DOCTOR OF PHILOSOPHY

The formulation of artificial reference standards for use within the ELISPOT assay

Sagida Bibi

2013

Aston University

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Thesis summary

Whether to assess the functionality of equipment or as a determinate for the accuracy of assays, reference standards are essential for the purposes of standardisation and validation. The ELISPOT assay, developed over thirty years ago, has emerged as a leading immunological assay in the development of novel vaccines for the assessment of efficacy. However, with its widespread use, there is a growing demand for a greater level of standardisation across different laboratories. One of the major difficulties in achieving this goal has been the lack of definitive reference standards. This is partly due to the *ex vivo* nature of the assay, which relies on cells being placed directly into the wells. Thus, the aim of this thesis was to produce an artificial reference standard using liposomes, for use within the assay. Liposomes are spherical bilayer vesicles with an enclosed aqueous compartment and therefore are models for biological membranes.

Initial work examined pre-design considerations in order to produce an optimal formulation that would closely mimic the action of the cells ordinarily placed on the assay. Recognition of the structural differences between liposomes and cells led to the formulation of liposomes with increased density. This was achieved by using a synthesised cholesterol analogue. By incorporating this cholesterol analogue in liposomes, increased sedimentation rates were observed within the first few hours. The optimal liposome formulation from these studies was composed of 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol (Chol) and brominated cholesterol (Brchol) at a 16:4:12 μMol ratio, based on a significantly higher ($p<0.01$) sedimentation (as determined by a percentage transmission of 59 ± 5.9 % compared to the control formulation at 29 ± 12 % after four hours).

By considering a range of liposome formulations 'proof of principle' for using liposomes as ELISPOT reference standards was shown; recombinant IFNγ cytokine was successfully entrapped within vesicles of different lipid compositions, which were able to promote spot formation within the ELISPOT assay. Using optimised liposome formulations composed of phosphatidylcholine with or without cholesterol (16 μMol total lipid) further development was undertaken to produce an optimised, scalable protocol for the production of liposomes as reference standards. A linear increase in spot number by the manipulation of cytokine concentration and/or lipid concentrations was not possible, potentially due to the saturation that occurred within the base of wells. Investigations into storage of the formulations demonstrated the feasibility of freezing and lyophilisation with disaccharide cryoprotectants, but also highlighted the need for further protocol optimisation to achieve a robust reference standard upon storage. Finally, the transfer of small-scale production to a medium lab-scale batch (40 mL) demonstrated this was feasible within the laboratory using the optimised protocol.

Key words: Reference standard; ELISPOT assay; Liposomes; IFNγ; Immunological assays.

Acknowledgements

I would like to begin by thanking Professor Yvonne Perrie, my PhD supervisor for selecting me to carry out this project and for her support and advice throughout. I feel privileged to have had the opportunity to work with Yvonne on this project, a brilliant scientist whose work ethos was always inspiring. Her approachability and great sense of humour has really made these three years an enjoyable experience.

I would also like to acknowledge our collaborators throughout this project at NIBSC especially Dr Barry Walker, Dr Mei Mei and in particular Dr Donna Bryan for all her assistance with the automated reading. I would also like to thank my group at Aston University for making it a great three years and Jiteen Ahmed for his technical assistance throughout.

On a personal level, none of this would have been possible without the support of my family, in particular my husband Tahir Mahmood, and my children Aaliya and Haider for being patient and understanding. The support of my parents, Mazhar Hussain and Zabada Bagum has been instrumental throughout my studies, whose value for education has been instilled within me from a young age. Finally I would like to thank the rest of my family including my brothers, sister, sister-in laws whom are too numerous to name individually. They have all offered support whether it was practical or just an injection of humour, it really all helped to get this far.

Publications resulting from this and related work

Bibi, S, Lattmann, E, Mohammed, A. R. & Perrie, Y. 2012. Trigger release liposome systems: local and remote controlled delivery? *J Microencapsul,* 29**,** 262-76.

Bibi, S, Kaur, R, Henriksen-Lacey, M, Mcneil, S. E, Wilkhu, J, Lattmann, E, Christensen, D, Mohammed, A. R. & Perrie, Y. 2011. Microscopy imaging of liposomes: from coverslips to environmental SEM. *Int J Pharm,* 417**,** 138-50.

Related abstracts posters and presentations

Bibi, S, Bryan, D, Lattman, E, Walker, B, Perrie, Y (2011). Sedimentation characteristics of liposome formulations for use as reference standards in the ELISPOT assay. *38th Annual Meeting & Exposition of the Controlled Release Society, Maryland, USA Abstract #100184.*

Bibi, S, Bryan, D, Lattman, E, Walker, B, Perrie, Y (2011). Sedimentation characteristics of liposome formulations for use as reference standards in the ELISPOT Assay. *17th UKICRS Symposium,* Queens University, Belfast, UK.

Bibi, S, Bryan, D, Lattman, E,Walker, B, Perrie, Y (2011). Sedimentation characteristics of liposome formulations for use as reference standards in the ELISPOT Assay. *UKPHARM SCI East Midlands Conference Center, Nottingham.* Abstract #72.

Bibi, S, Bryan, D, Lattman, E,Walker, B, Perrie, Y (2011). The formulations of IFNγ liposomes for use as reference standards in the ELISPOT Assay. *ILS meeting, Liposome advances; progress in drug and vaccine delivery, School of Pharmacy, University of London.*

Bibi, S, Mohammed A, Perrie, Y (2012). The use of coated gold nanoparticles incorporated liposomes for a novel *in vitro* assay. *Liposome Research Days, Hangzhou China.*

Bibi, S, Bryan, D, Lattman, E,Walker, B, Perrie, Y (2012). Liposomes encapsulating IFNγ as reference standards in the ELISPOT Assay. *Postgraduate Research day,* Aston University.

Bibi, S, Bryan, D, Lattman, E,Walker, B, Perrie, Y (2012). Trigger release liposomes for use as reference standards in the ELISPOT assay. *12th European Symposium on Controlled Drug Delivery,* Egmond En Zee, Netherlands.

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Abbreviations

Brchol	Brominated cholesterol
BSA	Bovine serum albumin
BSA-FITC	Bovine serum albumin-Fluoroscein Isothiocyanate
Chol	Cholesterol
^{13}C NMR	Carbon 13 nuclear magnetic resonance spectroscopy
ConA	Concavalin A (ConA)
DPPC	2-dipalmitoyl-sn-glycero-3-phosphocholine
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DSC	Differential scanning calorimetry
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked Immunosorbent spot assay
FCS	Fetal calf serum
^{1}H NMR	Hydrogen nuclear magnetic resonance
IFN-γ	Interferon gamma
LUV	Large unilamellar vesicles
MLV	Multilamellar vesicles
PC	L-α Phosphatidylcholine
PBS	Phosphate buffered saline

PLC	Phospholipase C
PLA_2	Phospholipase A2
PMA	Phorbol 12-myristate 13-acetate
PVDF	Polyvinylidene Fluoride
rIFN-γ	Recombinant Interferon gamma
TLC	Thin layer chromatography
TGA	Thermogravimetric analysis
SUV	Small unilamellar vesicles

Chapter 1

General Introduction

Publications related to this Chapter:

BIBI, S, LATTMANN, E, MOHAMMED, A. R. & PERRIE, Y. 2012. Trigger release liposome systems: local and remote controlled delivery? *J Microencapsul,* 29**,** 262-76.

1.1 The ELISPOT assay

The ELISPOT assay is an immunological assay which was originally developed to measure antibody secreting B cells and is now commonly used for the detection of cytokine secreting T cells (Czerkinsky *et al.*, 1983). It is broadly based on the sandwich ELISA method, with the most significant difference being that live cells are used within the plate rather than antibody conjugates. The ELISPOT assay has become a useful tool as a prognostic immunological marker for predicting vaccine efficacy and disease monitoring especially in the quantification of $CD8^+$ T cell response (Cox *et al.*, 2006). The reason for its wide spread use is primarily due to the sensitivity of the assay as it can detect cytokine producing cells at the single cell level and thus the assay is able to provide a greater level of sensitivity than the Enzyme linked Immunosorbent assay (Cole, 2005). There are various types of ELISPOT assays available to measure many other cytokines responding to antigenic or mitogenic stimulation including IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17 and TNF-α; however, the Interferon-γ (IFNγ) ELISPOT assay is the most commonly used (Slota *et al.*, 2011). The level of sensitivity of this assay becomes particularly significant when studying responses to infection in which the T-cell responses can occur at low levels, such as with Mycobacterium *tuberculosis* (Lazarevic *et al.*, 2005). It has resulted in the development of a new test (T-SPOT.TB, Oxford Immunotech, UK) which is more specific than the tuberculin skin test as it measures the release of IFNγ in response to antigens present in Mycobacterium *tuberculosis* and not Bacilli Calmette- Guerin (BCG) (Lalvani and Pareek, 2010).

One of the more significant recent developments with the use of this technique has been the implementation of this assay in the field of vaccine development. In fact, the IFNγ ELISPOT is one of the two most used immunological assays for the assessment of emerging HIV vaccines (Cox *et al.*, 2006). In determining T-cell based responses, laboratories at the HIV Vaccine Trials Network (HVTN), the National Institute of Allergies and Infectious Disease (NIAID) and the National Institute of Health use it as their primary assay in vaccine trials (Moodie *et al.*, 2006). Other immunological assays such as lymphoproliferation assays and cytotoxic T lymphocyte

assays have limiting factors, which include the use of radioactivity, decreased sensitivity with cryopreserved samples and other technical complications of the assay, whereas the ELISPOT has virtually no lower detection limit as it is able to detect 1 responsive cell in 100,000 (Currier *et al.,* 2002). In addition, this eliminates the need for the use of radioisotopes and allows the use of cryopreserved samples, which becomes important for vaccine trials. Other developments in the field of ELISPOT assays include the dual spot assay, which enables the detection of more than one cytokine at the same time, and has been shown to differentiate between three subsets of cytokine secreting T cells (Okamoto and Nishida, 2005). In this assay, Th1 cells (producing IL-2 and IFN-γ) and Th2 (producing IL-4 and IL-5) could be detected. Th1 cells were developed with horseradish peroxidase and amino-ethyl-carbazole/H_20_2 for red spots and Th2 were developed with alkaline phosphatase and Vector blue. In addition, Th0 cells which have the ability to secrete both cytokines as they are a precursor for Th1 and Th2 cells were detected by chromogenic substrates visible by indigo spots. This shows that developments in this assay since its first emergence in the 1980s, has led to the assay becoming ever more complex.

The different stages of the ELISPOT assay shown in Figure 1.1 briefly comprise cytokine-specific antibodies being immobilised onto the ELISPOT plate to which cells (peripheral blood mononuclear cells isolated from whole blood are normally used) are added. These cells may also have undergone an additional pre-stimulation step prior to being placed onto the plate. At this stage the plates containing the immobilised antibodies and cells are left for between 18 – 48 hours, during this time memory T cells will produce and secrete cytokine such as IFNγ. The cells are then removed prior to the addition of a secondary antibody for detection, which is either biotinylated or enzyme conjugated. If it is biotinylated, then a streptavidin enzyme conjugate is subsequently added. Finally, a substrate will then produce a coloured spot where the cell has released cytokine (Figure 1.1).

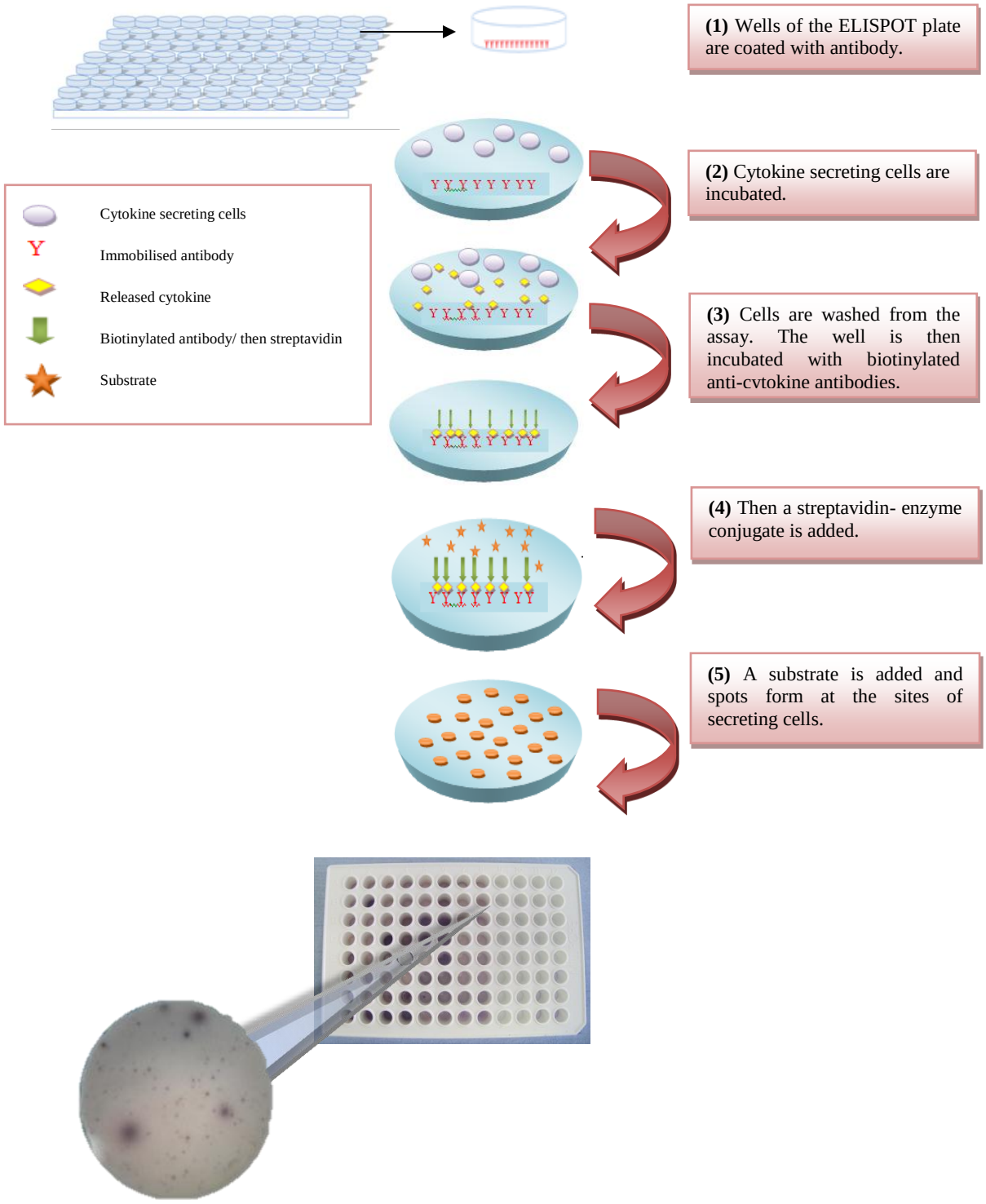


Figure 1.1 Illustrated principle of the ELISPOT assay with an example of a developed plate and an image of a representative well.

The plates used within the ELISPOT assay fall under two categories depending upon the polymer used for the membrane: nitrocellulose based plates or poly(vinylidene fluoride) (PVDF). The use of PVDF plates for ELISPOT assays has become more widespread due to the drawbacks of using nitrocellulose-based membranes. The nitrocellulose membranes were prone to cracking and breaking due the brittle nature of the material thus PVDF plates overcame many of these disadvantages (Weiss, 2005). The PVDF membrane is resistant to chemical degradation (other than when in contact with alkalis greater than pH 12), and has a greater degree of elasticity, hence are not prone to breaking. However, PVDF is a very hydrophobic material (with a surface energy of 21 dynes/cm) and requires some modification in order for it to be used with aqueous solutions (Weiss, 2005). Therefore, protocols generally require a pre-wetting step in alcohol and then an immediate exchange with water prior to antibody solutions being placed on the membrane. Other important properties of the PVDF membrane include the pore size, which is 0.45 µm, a porosity of 65-70 % and a membrane thickness of 135 µm. These membranes are broadly compatible with a wide range of solvents; however, detergents such as sodium dodecyl sulphate may interfere with any antibody binding within the well (Weiss, 2005, Weiss, 2012).

1.2 Standardisation issues with the ELISPOT

Recent literature (Smith *et al.,* 2009, Janetzki *et al.,* 2009) has shown that with the growing use of the assay has also emerged concern, in regards to standardisation and validation protocols across laboratories. The assay has an added complication when compared to the ELISA assays of the use of live cells; although in terms of physiology their integration into an '*in vitro'* assay (thus becoming *'ex-vivo'*) means that it is able to reveal more closely the true '*in vivo'* events, there is an added degree of variability. Different laboratory protocols for using ELISPOT assays exist and although there is some disagreement in the scientific community as to the true extent of this variability and its implications, there have been studies carried out in regards to this area. For example, a recent study showed that ELISPOT assays from multi-institutional laboratories

differed in their results from 20 to 100 fold (Janetzki *et al.*, 2009). The authors conclude that such variability compounds any attempts to compare the results between two laboratories and refer to it as 'a game of chance'. There are many factors which can influence the different results obtained by laboratories; for example a study by Smith *et al.*, (2009) examined factors such as the short *in vitro* stimulation step of cells prior to transference to the plate, differences in antibody coating concentrations, differences in cell incubation times and serum containing medium or serum free media. They found that in particular, the type of medium used, delays in processing of cells and the number of cells added all had a negative impact on the sensitivity of the assay. Even small differences can contribute to variation in results of the different laboratories and there are suggested procedures for standardisation. However, others have used such disparaging results to note that when assay procedure and data analysis is standardised, the detection of T-cells with precision was possible even with inexperienced operators (Zhang *et al.*, 2009). Yet in this case laboratories were using the same reagents, following the same protocol and using the same data analysis techniques and in reality such conditions would not occur, as laboratories optimise individual protocols, source their own reagents, and have individual data analysis platforms. Therefore, this does highlight the need for standardisation and validation of assays to compare results between laboratories and to validate in-house results. There are currently programmes in place to bring this about, one such being The Cancer Vaccine Consortium of the Sabin Vaccine Institute which aims to bring a level of standardisation through recommendations (Janetzki *et al.*, 2008). There are other consortiums which are also working to enhance the co-ordination across the many vaccine development research groups such as Transvac (European network of vaccine research and development), and through collaborations are working to develop reference standards which are essential for effective vaccine development (Transvac, 2013).

Variability in results obtained from ELISPOT assays are compounded by the sensitivity of the assay; many factors can cause variability to occur and these can include cell recovery

subsequent to thawing of peripheral blood mononuclear cells, operator to operator variability, reagents used, the protocol itself and methods for spot counting and analysis (Slota *et al.*, 2011).

These main variables are divided into three areas: biological, reagent and technical. The biological variables can include the actual cell numbers added to the wells of the assay, the incubation time for the assay and the antigens used. The reagent variables can include the antibodies used for coating; some laboratories use self-coated plates although pre-coated are now also available, the use of serum and even the substrates used for development. The technical variables can include parameters such as, the incubator, washing procedures, pipetting, counting procedures and the way in which cells are frozen/thawed (Jantezki and Britten, 2012). When the assay is being used in clinical trials there are rigorous validation criteria that need to be followed and the first and perhaps most important of these is to produce a validated 'standard operating procedure' (Cox *et al.*, 2006). If the assay procedure and the data analysis platform are standardised and validated then reproducible results are achievable. One method for assessing the variability of assays is using reference standards or controls.

1.3 Reference standards

Despite attempts to tackle the problem of the standardisation of immunological assays, it remains an on-going challenge, in particular for cytokines (Thorpe, 1998). Since the emergence of early crude 'natural' reference standards, significant progress has been made in this area and subsequently a large number have now been produced, overseen by the World Health Organisation that has a dedicated committee on biological standardisation. However, at present there are no commonly used standard references available for ELISPOT assays that could help standardise assay procedure and protocols thereby ensuring that the best vaccine candidates are put through to clinical trials (Janetzki *et al.*, 2009).

As part of assay standardisation and validation, reference controls have an essential role. According to the World Health Organisation (WHO, 2006) the purpose of the reference standard is:

"*…to facilitate standardised characterisation of biological samples, whatever type of measurement or method used. Their use enables the achievement of consistency in the measurement of key attributes of biologicals…and thus the development of internationally agreed criteria for acceptability*…"

The World Health Organisation is the body responsible for the standards for most biologically important proteins determined by immunoassay. These standards are validated by NIBSC (National Institute Biological Standards and Control), which are approved by the Expert Committee on Biological Standards (ECBS). There are many other organisations that work at a national and regional level, including the International Standard Organization (ISO), in projects concerning the standardisation of immunoassays (Stenman, 2001).

A reference standard for this particular assay is integral in allowing comparability of data from different laboratories, and to confirm the validity and accuracy of the individual results obtained (Thorpe, 1998). According to assay validation guidelines there should be *at least* one positive and one negative control. The definition of a positive is a known amount of purified analyte of interest added to the matrix of samples (Mata and Lohr, 2008). In fact, it is advisable to have a control that falls in the middle of a response range and one which is two or three times that of the assay cut off or negative control. The negative control in immune based assays is generally a pool of normal human serum. Subsequent to sourcing a control, stability studies need to take place to ensure their reliability under common storage systems. The same need exists for ELISPOT assays, established reference standards that can validate assays and can monitor the inter-assay variability. It is thought that the variability found within the ELISPOT assay can differ with the level of responses; therefore, for the purposes of validation should include assessments at a low, medium and high range of the responses (Slota *et al.*, 2011).

1.4 The current status of controls for the ELISPOT assay

Although the ELISPOT assay is an *ex vivo* assay there are currently controls which are used by various laboratories. For negative controls it is advised that six replicate wells are included which contain PBMCs without any stimulation and usually two types of positive control, namely a mitogen such as Phytohaemagglutin (PHA) and a pool of peptides (Cox *et al.,* 2006). Currently used controls are mitogens such as Phytohaemagglutin, Concavalin A (ConA) or Phorbol 12-myristate 13-acetate (PMA) used in conjunction with Ionomycin. The largest drawback of using these as positive controls within assays is that although they stimulate cells, they do not specifically target memory responses from T cells, and can produce responses in other subsets of cells. These non-specific responses can cause an overstimulation of the cells that can lead to inaccuracies within the assay (Janetzki *et al.,* 2004). Indeed, Janetzki *et al.,* (2004) confirmed the need for controls through the assertion that 'with assay-dependant controls for spot definition, the variability can be reduced to a minimum'.

There is not only recognition for the need for standardisation, but also attempts have been made to produce reagents that can be used for controls (Currier *et al.,* 2002). In this study a pool of peptides from Cytomegalovirus and Epstein Barr Virus were used, these are commonly encountered viruses for which T-cell responses have been elicited. The advantage of using such a pool is that it can provide information on the reliability of the PBMCs used in the assay whereas the use of other controls such as mitogens cannot. This is especially significant in vaccine trials where cryopreserved PBMCs are used. However, it is still very difficult to minimise the variability, even with a standard amount of this peptide pool, due to donor related differences from peripheral blood mononuclear cells added.

The need still exists for a standard that when placed into a well of an ELISPOT assay can provide a defined response, for example 200 spots and has no reliance on the PBMCs used within the plate to elicit a response upon stimulation. Such a control, could be used to assess the integrity of the plate coating, reliability of antigen from batch to batch and standardisation of

spot counting procedures albeit manual or automatic. In order to achieve an artificial reference standard for use within the ELISPOT assay, it will need to mimic the actions of the cell closely. The vesicle needs to encapsulate the cytokine and then be able to release gradually within the well of an ELISPOT assay to produce a spot. Such requirements can be fulfilled by liposomes.

1.5 Liposomes for use as artificial reference standards

Liposomes are small spherical vesicles that are composed of one or more lipid bilayers and formed by the dispersion of water insoluble phospholipids in water (Bangham *et al.*, 1965) (Figure 1.2). Initially it was their potential as artificial cells, more specifically in the study of biological membranes that was of interest. A significant development in the field of liposomes came with the realisation of their potential to encapsulate material and act as delivery systems for drugs, including proteins, enzymes, anti-tumour and anti-microbial agents (Gregoriadis and Ryman, 1971, Gregoriadis *et al.*, 1971, Gregoriadis, 1973).

Much of the research in liposomes has been directed towards their *in vivo* applications as drug delivery agents and decades of research have culminated in several liposome based products on the market for the delivery of drugs. As shown in Figure 1.3, liposomes have progressed from mere discovery in the late 1960s to the culmination of marketable formulations that are being used regularly for the treatment of diseases such as cancer presently. Liposomes not only have the ability to encapsulate material such as proteins, drugs and vaccines but can also be used for selective targeting of drugs; this can enhance the pharmacological effect and thus lead to a reduction in toxicity of the encapsulated drugs (Budai and Szogyi, 2001). The challenge has been to encapsulate the material of interest and also enhance the bioavailability (Allen and Cullis, 2012). The emergence of Visudyne® and very soon Thermodox® are the more sophisticated of liposomal delivery systems that have external trigger release mechanisms.

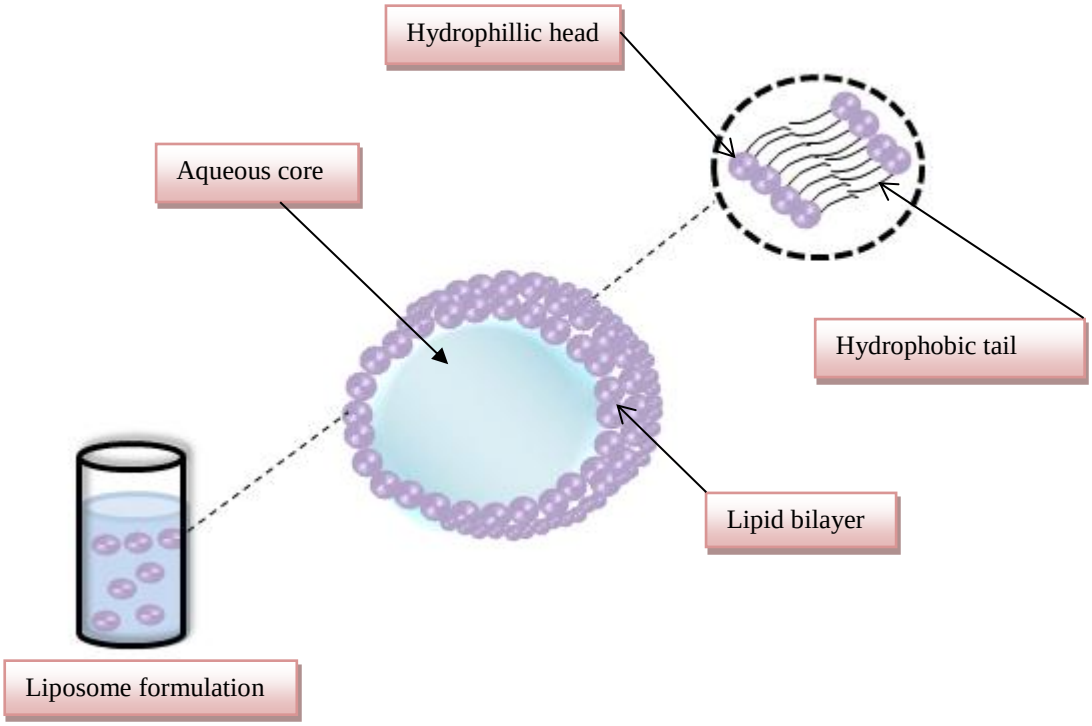


Figure 1.2 Schematic diagram of internal aspects of liposomes and the bilayer.

The nature of the composition of liposomes results in the entrapment of hydrophilic substances within the aqueous core and hydrophobic substances being attracted to/or incorporated within the bilayer itself. Liposomes vary in charge and size due to the lipids and manufacturing methods used, with the nomenclature based upon size and lamellarity; these are summarised below in Table 1 (Storm and Crommelin, 1998). By the nature of the lipids chosen and the manufacture technique, liposomes are tailored for specific requirements.

Liposomes can also be prepared from a variety of different methods depending upon the specific requirements of the formulation (Lasch *et al.*, 2003). To summarise briefly a few of these fundamental methods, the most basic and perhaps ubiquitously used method for the formulation of liposomes is commonly referred to as the 'dry film lipid hydration method'.

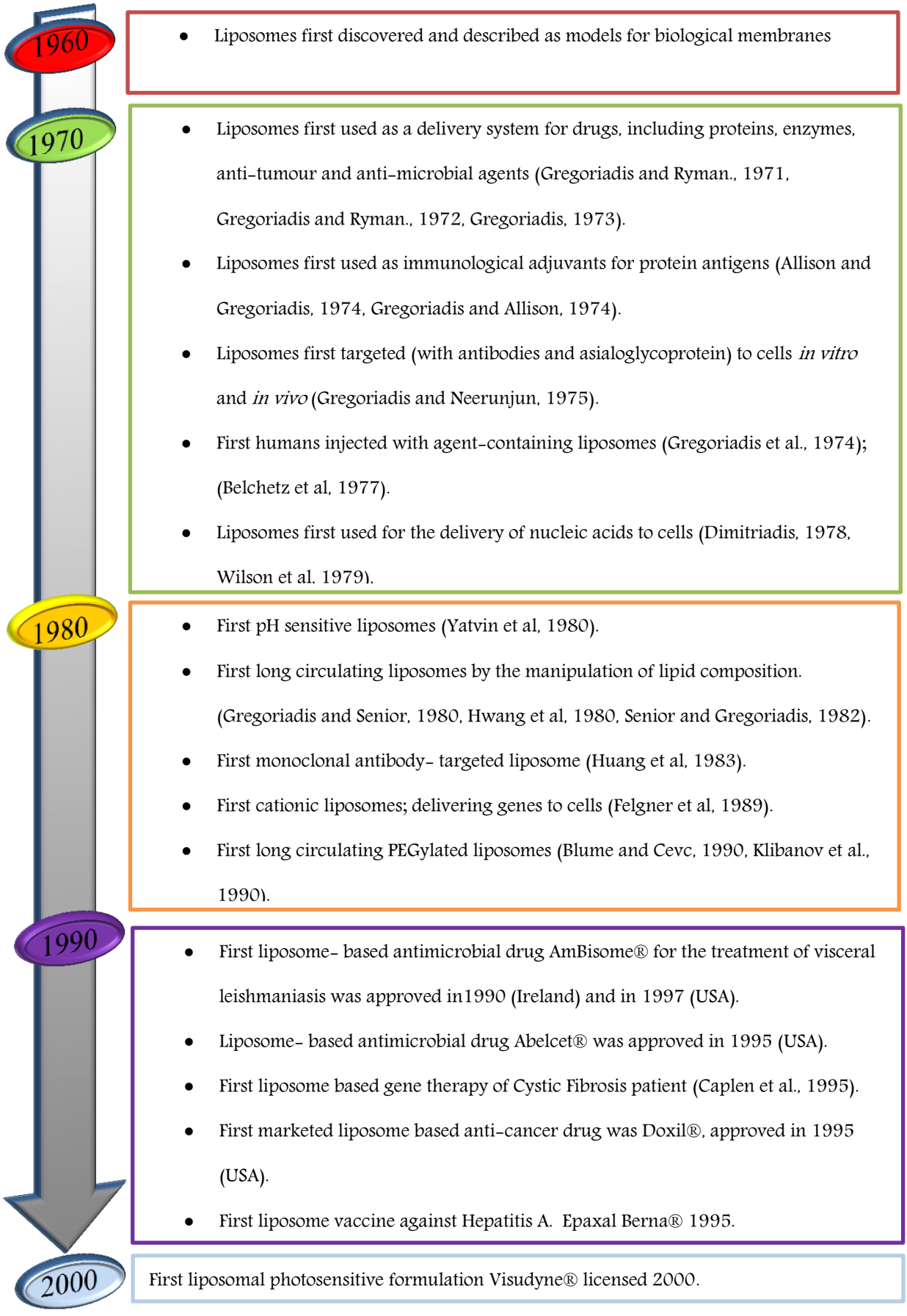


Figure 1.3 Timeline for the development of liposome systems.

Table 1.1 Liposome nomenclature with abbreviation and size ranges.

Abbreviation	Name	Size range
OLV	Oligolamellar vesicles	0.1-1 μm
ULV	Unilamellar Vesicles	All Size Ranges
SUV	Small Unilamellar Vesicles	20-100 nm
MLV	Multilamellar Large Vesicles	>0.5 μm
MUV	Medium Sized Unilamellar Vesicles	20-100 nm
LUV	Large Unilamellar vesicles	>100 nm
GUV	Giant Unilamellar vesicles	>1 μm
MVV	Multivescular vesicles	>1 μm

A dry lipid film is formed after the solvent in which the lipids are dissolved is evaporated on the rotary evaporator. The resultant film is subsequently hydrated with an aqueous solution that is pre-warmed above the phase transition temperature of the lipid. It is necessary for the suspension to be agitated for instance by vortexing and a heterogenous mixture of multilamellar vesicles (MLV) are produced. A further development resulted from the need to produce vesicles which were less than a micron in size; the process of sonication was introduced whereby pre-made multilamellar vesicles are sonicated either by placing the tip of a sonicator into the liposome dispersion or the sample is placed in the bath sonicator.

Another important development in the formulation of liposomes came with the advent of freeze-dried rehydration vesicles (Senior and Gregoriadis, 1989). Similarly, it is necessary to have pre-formed vesicles and the aim, through this method is to achieve higher entrapment efficiency. The technique involves taking sonicated liposomes, which are then frozen, lyophilised and rehydrated with the aqueous buffer. Extrusion of the final formulation reduces the size and produces a more homogenous formulation. The method of reverse-phase evaporation does not rely on the formulation of pre-formed vesicles instead; lipids are added to 3 mL of diethylether (with a small amount of chloroform or methanol to increase solubility if necessary) to which 1 mL of the aqueous phase is added. The resulting suspension is kept under inert gas and then sonicated until a clear or opalescent one-phase dispersion is obtained (Szoka and Papahadjopoulos, 1980). The remaining organic solvent is removed under rotary evaporation leading to the formation of a highly viscous gel which at a critical point will collapse and become an aqueous suspension, and as a final step dialysis is used to remove

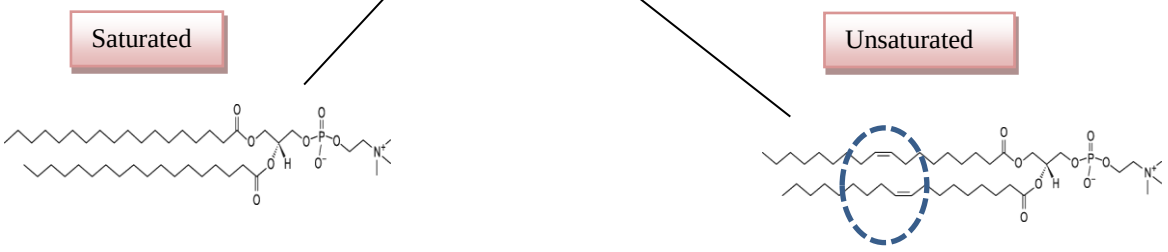
unentrapped material. These procedures are just a few examples of basic methods of which there are many adaptations used for manufacturing liposomes.

1.5.1. Liposomal attributes

Although lipids used for liposomal systems have a broadly similar structure in terms of a polar head group and two fatty acid chains, even slight variations of chemical structure can lead to a profound change in the properties and functionalities of liposomes produced from them. Differences can include the charge of polar head groups, the addition of polymers to the surface, the length and saturation of the fatty acid chains (Lian and Ho, 2001). For instance, lipid bilayers can exist in low temperature solid-ordered phase and above a certain temperature in a fluid disordered phase; this is called phase transition or T_m, it is at this temperature that the greatest liposome leakage is observed.

Table 1.2 Phospholipids and their transition temperatures (T_m).

Lipid	Abbreviation	No of Carbons	No of double bonds	Phase transition temperature T_m
Dimyristoylphosphatidylcholine	DMPC	14	0	23
Dipalmitoylphosphatidylcholine	DPPC	16	0	41
Distearoylphosphatidylcholine	DSPC	18	0	55
Dioleoylphosphatidylcholine	DOPC	18	1	-20



The T_m is tailored by lipid selection, as it is dependent upon the length of fatty acid chains and whether they are saturated or unsaturated. DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) is a saturated phospholipid which has 18 carbons in each hydrocarbon chain and has a T_m of 55 °C. DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) has a virtually identical chemical structure albeit the

presence of a single double bond in the chain yet it has a T_m of -20 °C (Table 1.2). The presence of this double bond results in decreased rigidity and an increase of the fluidity of the membrane and thus a decrease in the transition temperature results.

Increasing the chain length for a lipid will result in an increase of the T_m, for instance an 18-carbon chain for DSPC has a T_m of 55 °C whereas a 22 carbon chain has a T_m of 75 °C. There are wide varieties of lipids available with varying transition temperatures and the lipids selected for formulations are dependent upon the requirements. Sterols such as cholesterol are often used in combination with other lipids in liposome formulations to alter membrane properties dependent upon the concentration used (Gregoriadis, 1991). At low concentrations, the incorporation of cholesterol leads to enhanced membrane permeability whilst higher amounts are able to decrease membrane permeability and eliminate the phase transition. For liposomes tailored for *in vivo* drug delivery cholesterol is included to adjust rigidity in addition to reducing instability due to binding of serum proteins to the membrane (Lian and Ho, 2001).

1.5.2. Liposomes for the entrapment of rIFNγ

Liposomes are ideal as drug carriers; they are generally composed of non-toxic lipids and are therefore safe as delivery systems *in vivo* as they do not produce unwanted immune responses and yet are able to encapsulate internally, or bind, a variety of molecules to their surface (Lasic, 1998). The potential of liposomes as carriers has been exploited for a wide variety of different molecules including chemotherapeutic agents, antimicrobial drugs, imaging agents, antigens, peptides, hormones, and cytokines as well as genetic material to name a few (Gregoriadis, 1991). Although there are a variety of different types of ELISPOT assays available to enumerate cytokine secreting cells, the IFNγ ELISPOT assay is the most widely used in vaccine trials. For instance, it is routinely used for the quantification and enumeration of the human immunodeficiency virus (HIV)-specific CD8+ T cell responses (Streeck *et al.*, 2009). Therefore the aim of this study to encapsulate rIFNγ within liposomes and produce a reference standard that can mimic the action of T cell responses to facilitate assay standardisation.

Table 1.3 Properties of IFNγ adapted from (Farrar and Schreiber, 1993).

Property	IFNγ
Major Inducer	Antigens/ Mitogens
Molecular Weight (KDa)	17
Amino Acids	143
N-linked glycosylation	2 sites
Subunit composition	Non Covalent homodimer
pH stability	Labile
Cellular source	T cells and Natural Killer cells.

IFNγ is a dimeric protein, with an overall compact and globular shape. It is primarily helical with the absence of any β sheet in the structure. Thus, each dimer associates with another to form a non-covalent homodimer and each IFNγ dimer may be able to bind two IFNγ receptors (Farrar and Schreiber, 1993).

It should be noted the structure of all recombinant human interferon γ (rIFNγ) is different from the natural analogue (Table 1.3). There are structural differences in terms of the presence of additional amino acids not present in the natural form, most significantly all rIFNγ is not glycosylated and this is the reason that it is less stable in solution (Christova *et al.,* 2003). In addition to the reduced stability, other studies conducted have shown that the rIFNγ is also susceptible to physical stresses such as increased temperature and pressure (Goosens *et al.,* 2003; Tsanev et al 2007).

Liposomes have the ability to carry a wide variety of molecules, and have versatile structures for physical manipulation of characteristics and more importantly have low toxicity for therapeutic requirements (Storm and Crommelin, 1998). Liposomes have previously been used for the successful entrapment of cytokines (Goldbach *et al.,* 1995). More recently, liposomes encapsulated with cytokine have been used for *in vivo* purposes (Van Slooten *et al.,* 2001). The reason for incorporating rIFNγ in liposomes is due to its ability to act as an adjuvant; this immunomodulatory activity offers potential for the activation of systemic immune responses, for instance when administered simultaneously with a vaccine. In this instance, no adjuvant activity was seen with the addition of free IFNγ at the site of injection. This is most likely due to rapid clearance, yet liposome encapsulated rIFNγ proved to be more effective (Van Slooten *et al.,* 2001).

1.5.3 Liposomes and immunoassays

Liposomes have previously been used in immune based assays to provide signal amplification as either intact or lysed vesicles. These have involved encapsulation of various types of markers including fluorescent markers whereby response amplification is achieved upon liposome lysis through proteins like complement or mellitin, detergents such as Triton X and enzymatic lysis through the addition of Phospholipase C (Gomez-Hens and Fernandez-Romero, 2005, Edwards and Baeumner, 2006). The methods used in such assays involve basic modification of the Enzyme-linked Immunosorbant Assay (ELISA), with either the covalent binding of the liposome to the antigen or conversely the liposome is bound to an antibody or secondary molecules (Rongen *et al.*, 1997). Basic requirements for the use of liposomes in any kind of immune based assay would be to achieve high levels of encapsulation in order to meet the limits of detection of the assay. Therefore, the liposomes need to be very stable so that there is minimal unwanted leakage.

The applications of liposomes as reference standards within immunoassays have not been previously considered. The need to do this within ELISA based immune assays has not been necessary as reference standards can be produced from sera with the presence or absence of the required antibodies for positive and negative controls respectively. Whereas within the ELISPOT assay a defined amount of cytokine pipetted into a well would not result in discrete spot formation. Thus, although the use of liposomes to encapsulate cytokines has been carried out before, the use of liposome encapsulated cytokine within ELISPOT assays has not. Due to the nature of the ELISPOT assay, where each well is coated with antigen for IFNγ, a reference standard is required that could contain the rIFNγ and release the contents in a similar way to the cells in the rest of the assay. The release needs to be precise and definitive and as such may require a trigger release mechanism. Liposomes not only offer the potential to encapsulate cytokines but are also a delivery system that is similar in morphology to the cells normally placed upon the assay. The basic premise of this project has been to formulate liposomes that can be used as reference standards within the ELISPOT assay, as shown in Figure 1.4.

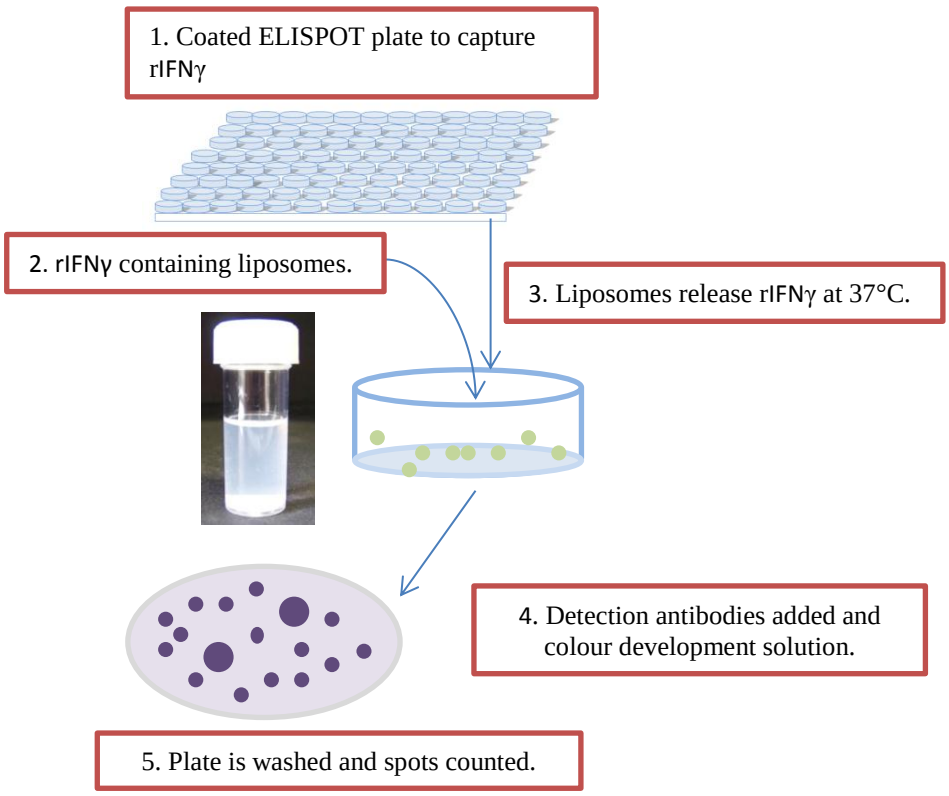


Figure 1.4 Schematic showing the methodology for liposome incorporation into the ELISPOT assay.

Unlike cells, which can be stimulated to release cytokines upon contact with an appropriate chemical or antigen, liposomes will only release in response to a trigger. It is important to consider these trigger release systems in the design of a standard for the ELISPOT assay (Figure 1.5). Although the encapsulation of proteins such as cytokines is well established, the dynamics of adding these vesicles to an existing immunoassay introduces a diverse set of challenges. For the liposomes themselves, it begins with the fundamentals of demonstrating the feasibility of encapsulation of rIFNγ in a sufficient concentration that will allow spot formation. In order to achieve this, the lipids chosen for the construction of such vesicles will need to allow release of the cytokine in a similar way to T-cells. Depending upon the lipids chosen, there will be other stresses on the cytokine to consider, such as heat, vortexing and centrifugation at high speeds.

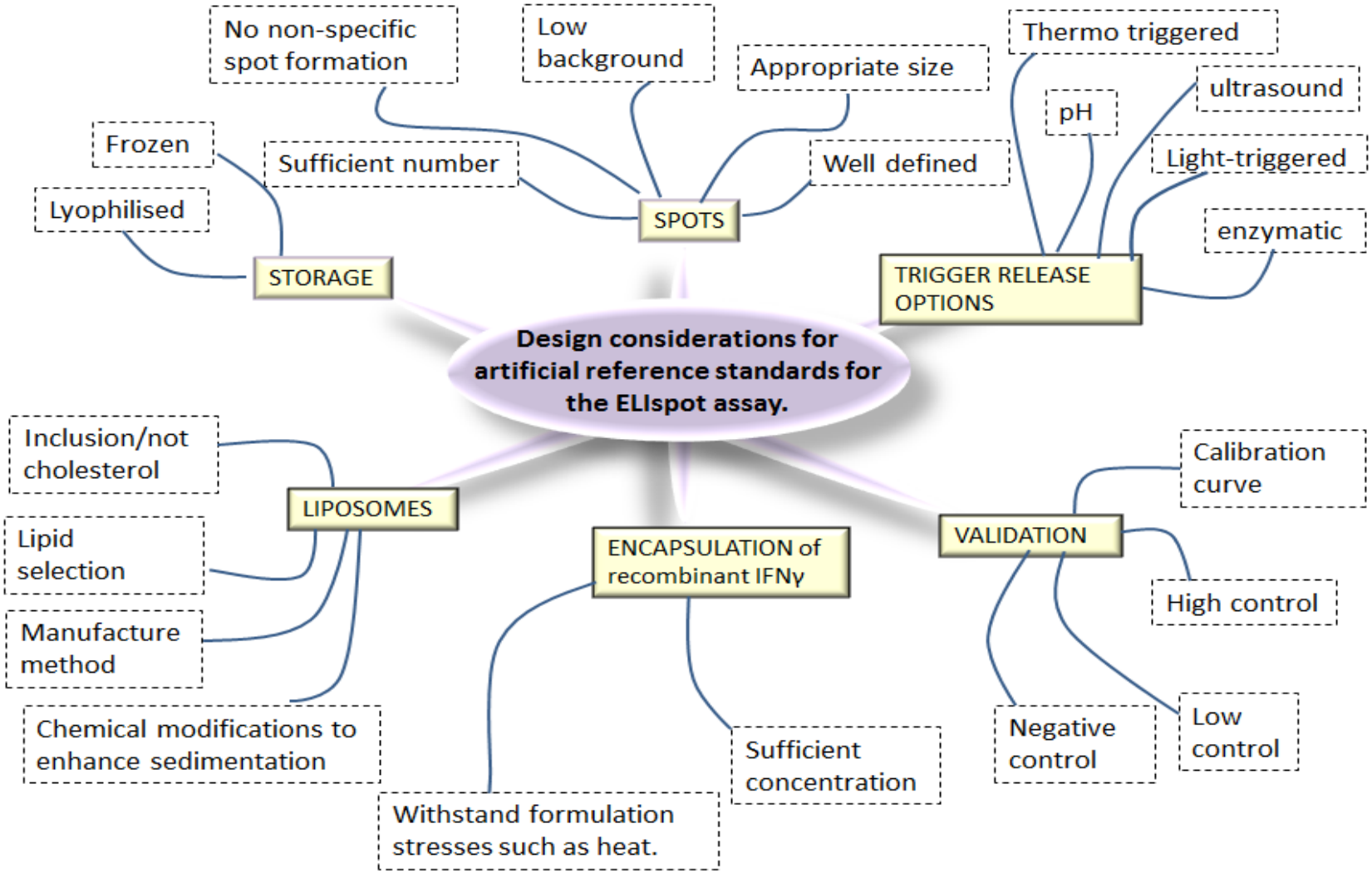


Figure 1.5 Design considerations for the formulation of a reference standard.

Other important factors that require consideration are the differences between the sedimentation rates of a liposome formulation and whether this can be enhanced to ensure minimal leakage of liposomal contents before contact with the membrane; thus chemical modifications to enhance sedimentation may aid this requirement. Any modifications will need to have a minimal effect on the overall structure of the formulation or release of the cytokine.

Once 'proof of principle' is established, there are still further challenges that need to be addressed such as the correct size, morphology, definition and number of spots. For the purposes of producing a final formulation, many other validation issues emerge such as producing a negative control and a formulation that can be stored either frozen or lyophilised. As shown in Figure 1.5, many pre-design parameters need to be taken into consideration prior to formulation of the artificial reference standards. Due to the novel nature of this work the dynamic between artificial vesicles and the ELISPOT assay will only become apparent upon practical application and thus further challenges may emerge in the process of optimisation to achieve a final product. The consideration of triggered release of liposomes is an important parameter as there is not an active secretion of cytokine as with cells.

1.6 Trigger Release Liposomes

T-cells release IFNγ upon exposure to a specific antigen such as the viral protein. However, liposomes require disruption of their bilayer to promote release of their entrapped moiety, and the rate of release can vary depending on the liposome formulation and the characteristics of the substance entrapped, thus possible trigger release systems need to be considered if these systems are to be exploited within an ELISPOT assay.

It is well established that liposome composition plays a significant role in liposome stability and many liposome formulations display limited stability. This is especially true within *in vivo* conditions; these include a fast clearance rate from the blood circulation due to the adherence of plasma proteins to the surface of the liposomes and a tendency to localise in the tissues of the mononuclear phagocyte system (MPS) particularly the liver and spleen (Allen, 1996). The *in vivo*

characteristics of such liposomes limits their effectiveness for the treatment of a wide array of diseases; however the ability of these systems to passively target the MPS has resulted in their use for the delivery of antimicrobial agents to infected macrophages as well as for antigen delivery (Allen *et al.,* 1995). Early work investigating the effects of liposome composition and its relationship to *in vivo* clearance demonstrated that the susceptibility of liposome formulations to opsonins and high density lipoproteins found within a biological environment varies with the phospholipids used and the inclusion of cholesterol (Gregoriadis, 1991, Kirby *et al.,* 1980). Such studies demonstrated that increasing the cholesterol content of liposomal formulations up to 50 molar % increased the retention of fluorescent dyes encapsulated within phosphatidylcholine liposomes due to restrictions in the mobility of the bilayer (Kirby *et al.,* 1980). Furthermore, it was shown that by using saturated lipids such as 1,2-distearoyl-sn-glycero-3-phosphocholine with transition temperatures greater than 37 °C and equimolar cholesterol, bilayer stability could be retained for over 48 hours in comparison to similar formulations using phosphatidylcholine (Gregoriadis, 1991).

The establishment of such fundamental principles in the early stages of liposome design demonstrated the enhancement of liposome stability through the tailoring of the phospholipids used and the inclusion of cholesterol to confer greater *in vivo* stability. Indeed increased circulation times *in vivo* was considered to be one of the key elements for enhanced drug targeting of liposomal based carriers, and a significant development in this area came with the coating of liposomes with a hydrophilic polymer particularly polyethylene glycol (PEG) (Blume and Cevc, 1990). The PEG coating was shown to enhance the steric stabilisation of formulations and reduced the binding of plasma proteins to the liposomal surface, resulting in longer circulation times (Lasic, 1998). The ability of such formulations to avoid rapid clearance by the MPS led to them being termed stealth liposomes. The enhanced *in vivo* characteristics of such formulations resulted in their use as delivery systems for a selection of different diseases due to a reduction in toxicity compared with drug alone (Maeda, 2001). This was found to be particularly effective within cancerous tissues where the leaky vasculature combined with damaged lymph drainage systems (resulting from angiogenesis) promotes the accumulation of liposomes at the tumour site, a

mechanism often referenced to as the enhanced permeability and retention (EPR) effect (Matsumara and Maeda, 1986, Maeda, 2001). Long circulating liposome formulations are now available for commercial use including; Ambisome® (for serious fungal infections encapsulating amphotericin B), Doxil® and DaunoXome® for the treatment of cancer (Storm and Crommelin, 1998). Even with such progress there remain further drug delivery hurdles to overcome. The increased permeability required for the penetration of the drug to within the tumour tissue varies within individuals and drugs such as doxorubicin have reduced therapeutic activity due to slow release and limited penetration deep into the tumour site, thus inhibiting effective drug distribution (Primeau *et al.,* 2005, Zasadzinski *et al.,* 2011). Not only can the effectiveness of the treatment be compromised, but in addition the cumulative effect of insufficient drug reaching the target site can increase drug resistance.

Given that the release of the drug from liposomes generally relies on liposomal degradation and breakdown, there is a continued need for more control and optimisation of drug release characteristics. To address this, there are a wide array of studies that have examined the possibilities of using trigger release liposomes for the delivery of encapsulated material. This includes photosensitive, thermosensitive, ultrasound triggered, enzymatic and pH triggered release (Figure 1.6) and as such these need to be examined as possible release mechanisms for liposomes within the ELISPOT assay (Bibi *et al.,* 2012). Although the application is very different, the requirements in terms of efficient encapsulation and release are much the same. Many of the studies for trigger release systems have focussed on the local environment of the tissue for target specific release. Although in the *in vitro* environment of the ELISPOT assay these conditions are absent, they can be designed to be a part of the assay, particularly for those wells containing the reference standards where the conditions required for ‘local release’ may include a change in pH or increased levels of specific enzymes.

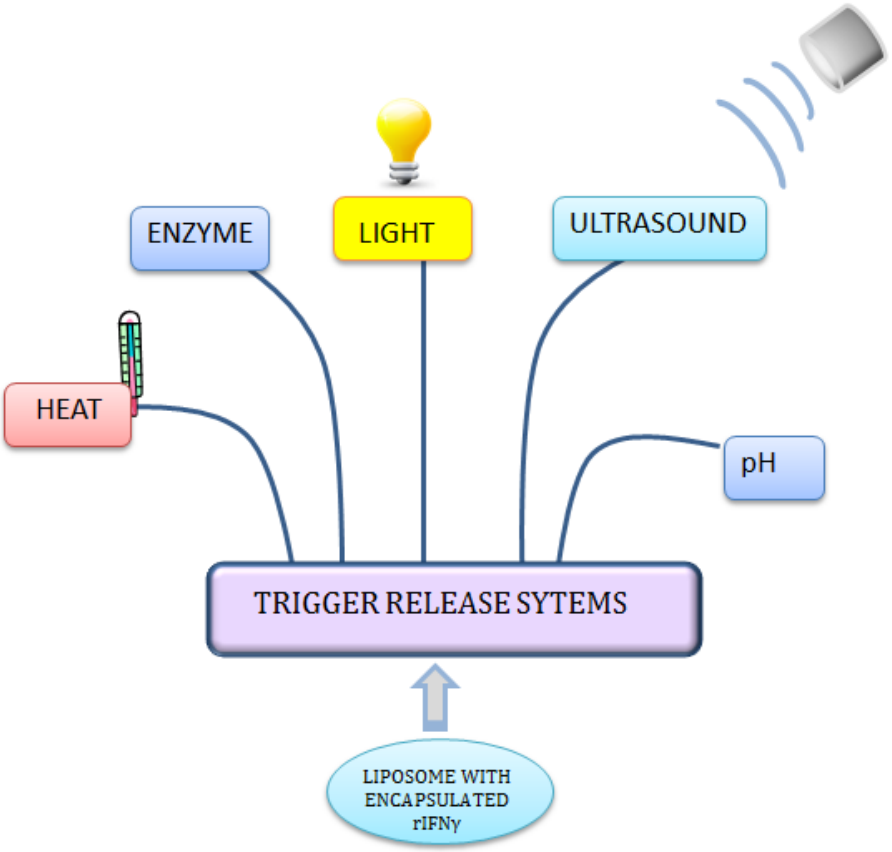


Figure 1.6 Trigger release systems for the release of encapsulated rIFNγ.

1.6.1 Enzyme triggered release from liposomes: Exploiting Phospholipase C

Given the structural attributes of liposomes, the main consideration for triggered release is the breakdown of the lipid bilayer structure. One of the primary design considerations for liposomes at the initial stages of development is maximal encapsulation of the drug and minimal leakage. This can be achieved by incorporating high transition temperature lipids and/or cholesterol or through bilayer manipulation (Senior and Gregoriadis, 1982, Gregoriadis and Senior, 1980, Lian and Ho, 2001, Zasadzinski *et al.*, 2011). The need for the stability of liposome vesicles to retain encapsulated material prior to reaching the target site and consequently effectively release all the liposomal contents upon reaching the site can be difficult to achieve. Thus, exploiting the local environment of the target site allows such a balance to be achieved as only the presence of the trigger, albeit elevated levels of enzymes or a more acidic pH, can cause release of the encapsulated material. This simultaneously deals with two parameters for improving formulations; the first being

reduced toxicity as release occurs only at diseased sites, and secondly an improved therapeutic index, as there is a trigger for release, as opposed to reliance on eventual liposomal breakdown.

However, in certain disease states the biological environment surrounding the affected tissue can become quite different to that of non-diseased areas. Exploiting the local environmental conditions for drug release is the premise for a range of prodrug therapies where compounds undergo biotransformation, either by chemical or enzymatic reaction, to release the active drug. The biotransformation is triggered through the exploitation of differences in the local environment at the site of action. For example, stilboestrol diphosphate is a phosphorylated synthetic oestrogen, which is cleaved to free stilboestrol through the action of phosphatase, which is found at higher concentrations in prostatic carcinomatous tissue. Similarly, in prostate, breast and pancreatic cancers there is a significantly higher expression of phospholipase A_2 (PLA_2), (Zhu *et al.*, 2011, Davidsen *et al.,* 2003). It is also well established that soluble extracellular enzymes are also found in inflammatory environments; for instance the synovial fluid from animals with arthritis contains enzymes such as lysozyme and cathepsins (Meers, 2001) and matrix metalloproteinases (MMPs), in particular MMP-2 and MMP-9 are widely thought to be associated with the development of human tumours (Sarkar *et al.,* 2005). Thus, it is these local environmental conditions that are exploited in the development of enzyme-triggered release systems (Figure 1.7) and for triggered release from liposomes, enzymes able to degrade lipids are of primary interest.

It is interesting to observe that even before the inadvertent official discovery of liposomes by Bangham *et al.,* (1965), that studies on the effects of enzymatic activity on aqueous dispersions of synthetic lipids were being carried out. Much of the early work was using Phospholipase C (PLC) due to the ease of availability and similarity to mammalian analogues (Macfarlane and Knight, 1941, Long and Maguire, 1954, van *et al.,* 1961). These groups of enzymes catalyse the hydrolysis of phospholipids producing diacylglycerol and phosphocholine (Figure 1.8) (Liao *et al.,* 2010).

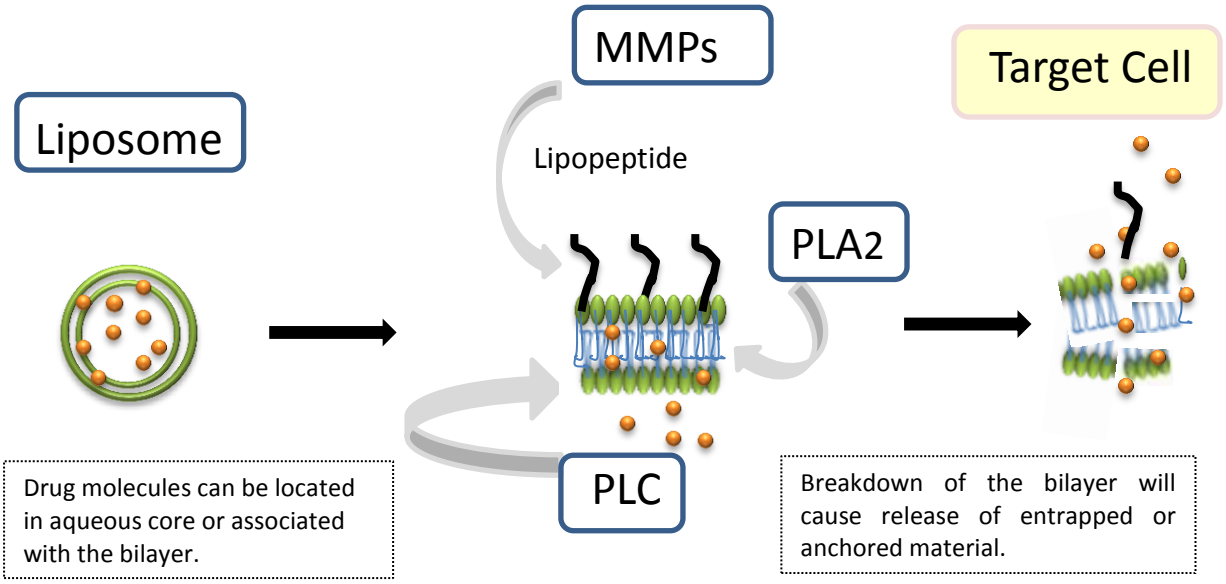


Figure 1.7 Schematic summary of various enzymes and location of action for degradation of the bilayer.

Whilst at this time not being termed liposomes, studies using PLC and liposomes were being carried out as early as the 1940s (Macfarlane and Knight, 1941, Long and Maguire, 1954) where it was found that saturated lecithins were resistant to the actions of the enzyme. However, these results were later contradicted in a study by Van et al (1961), where they used a similar methodology but concluded that it was not necessarily the difference between a saturated or unsaturated lipid that caused the differences in hydrolysis but rather the length of the lipid acyl chain. They found that PLC from *Clostridium perfringens (C. welchii*) was able to hydrolyse both saturated and unsaturated compounds; however, the rate of hydrolysis decreased significantly with increased acyl chain length. Whilst these early studies are not based on liposome vesicles but on aqueous dispersions, these findings have laid the foundations for the sophisticated enzymatic trigger release systems being designed today. Subsequent studies on the action of PLC on the lysis of lipid membranes (Hesketh *et al.*, 1972) found that liposomes prepared from lecithin with, or without, cholesterol and dicetylphosphate in molar ratios of 7:1:2 (using a 1:1 w/w molar ratio of PLC preparation to phospholipid) exhibited over 50 % loss of marker. However, no lytic action was observed when the membranes contained saturated lipids. Using thin layer chromatography, the authors demonstrated hydrolysis of the lecithin by PLC whereas

dipalmitoylphosphatidylcholine (DPPC) showed no conversion to diglyceride (Hesketh *et al.*, 1972).

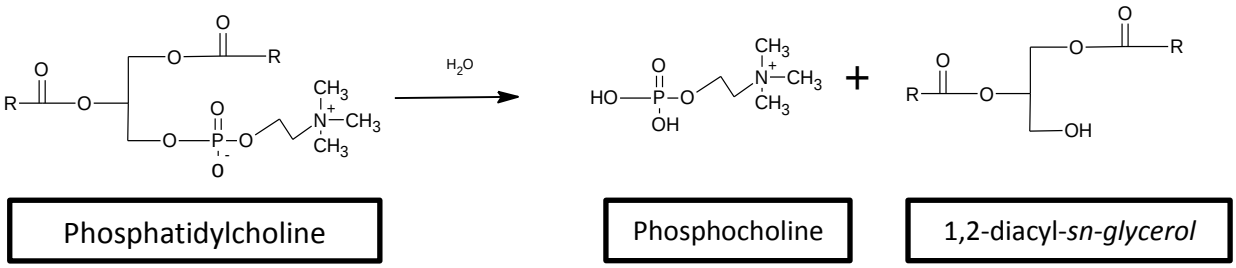


Figure 1.8 The hydrolysis of phosphatidylcholine by Phospholipase C (PLC).

Given the ability of PLC to digest lipid membranes, their potential to promote triggered release from liposomes was further exploited in the field of liposome-based immunoassays. In this type of assay, the enzyme detection element is replaced by a liposome encapsulating a marker molecule and lyses of antibody bound liposomes is caused by the presence of complement in serum or by mellitin, where inhibition of its lipid digesting action indicates the presence of the analyte (Rongen *et al.*, 1997). In a study assessing the actions of PLC in an *in vitro* assay, liposomes were prepared using the reverse phase evaporation method and composed of DPPC and cholesterol (9:1 molar ratio), with the entrapped fluorescent marker calcein. Thus, a simple and inexpensive assay for testing ultra-trace amounts of biological substances such as insulin was developed due to the inhibition of the lytic activity of the conjugates in the presence of insulin antiserum (Lim and Kim, 1997). Using an enzyme such as PLC within the ELISPOT assay to trigger release based upon these studies offers a feasible option as PLC woks at an optimal temperature of 37 °C.

1.6.2 Enzyme triggered release: Phospholipase A_2

Whilst the above studies considered the ability of PLC as a trigger to promote drug release from liposomes, for *in vivo* targeted release exploitation of local conditions of the tissue or surrounding fluid can present an appropriate environment to trigger the release of liposomal contents. This has been the basic premise of numerous recent studies (Zhu *et al.*, 2011, Davidsen *et al.*, 2003, Kaasgaard *et al.*, 2009). For example, PLA_2 enzymes found throughout nature in the venoms of bees and snakes as well as within mammalian pancreatic juices are also involved in phospholipid

metabolism and numerous cellular functions, and can either be located intracellularly or extracellularly (Arni and Ward, 1996). However, increased levels of this enzyme are also implicated in various diseases such as rheumatoid arthritis, ischemia, atherosclerosis, and particular cancers such as colorectal and human familial adenomatous polyps (Kudo and Murakami, 2002).

Thus, the presence of enhanced levels of this enzyme can offer a mechanism for the triggered release of liposomes that are stable under normal physiological conditions but release their contents at the above-mentioned sites. This was shown through a study in which elevated PLA_2 levels in inflamed tissue were exploited for potential triggered release of siRNA from liposomal systems (Foged *et al.*, 2007). Using the double emulsion technique, liposomes were formulated using DPPC, dipalmitoylphosphatidylglycerol (DPPG), and dipalmitoylphosphatidylethanolamine attached to polyetheylene gylcol (DPPE-PEG) to provide a hydrophilic stealth coating of the liposomes. Within these vesicles siRNA, which would target TNF-α silencing was entrapped. The cytokine TNF-α is over expressed by cells such as monocytes and macrophages in rheumatoid arthritis, and as such siRNA would be a highly specific way to silence the genes of interest (Foged *et al.*, 2007). The study carried out *in vitro* using HeLa cells found that there was increased uptake of siRNA into vesicular compartments that was concentration dependant and increased by the presence of PLA_2 suggesting the potential of such siRNA encapsulated liposomes in targeting and controlled release of siRNA in inflamed tissue (Foged *et al.*, 2007).

The idea of triggered release from liposomes does require appropriate stability and targeting of the liposomes to ensure accumulation of the liposomes at the site of action prior to their triggered release. PEGylated liposomes confer enhanced stability within the blood circulation, consequently various PEGylated nanoparticulate products are approved for clinical use (e.g. Doxil®/ Caelyx®). However their enhanced ability to accumulate at the site of the tumour is not matched by the rate of drug release, thus in a recent study Zhu *et al.*, (2011) the increased levels of PLA_2 associated with various malignant tumours was exploited to examine the effects on the rate of drug release from various lipid vesicles. Interestingly from this work, the authors noted that liposomal degradation and drug release is dependent upon the PLA_2 isoform expressed. In this study two groups were examined, namely IIA and III. From the formulations examined, it was found that the presence of

DSPE-PEG within the bilayer enhanced PLA_2 triggered release. This was attributed to the binding of PEG to DSPE; DSPE is zwitterionic under normal conditions yet when bound to PEG it becomes anionic, and this combined with the structural attributes of PEG may cause membrane restructuring which subsequently enhances the activity of the enzyme. In terms of the difference in isoforms used, there was a reduced level of release observed with group IIA when compared to group III (Zhu *et al.,* 2011). Such studies highlight how enzymes can be used with liposomes for bilayer degradation and how changes in the composition of the liposomes can alter release kinetics in the presence of PLA_2.

1.6.3 Enzyme triggered release: Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of highly homologous zinc dependent endopeptides that hydrolyse most of the constituents of the extracellular matrix and basement membrane components (Birkedal-Hansen *et al.,* 1993). They play a central role in a range of biological processes including embryogenesis, wound healing, angiogenesis and in diseases such as atheroma, arthritis, tissue ulceration and cancer (Visse and Nagase, 2003). Although these enzymes play an important role in the normal physiology of the body, they are also implicated in the development and metastasis of tumours. Whilst lipids are not a natural substrate for MMPs, which degrade components of the extracellular matrix, the presence of these enzymes in the local environment of tumours can be exploited for the purposes of liposome based trigger release. In order to achieve triggered liposomal release, peptides are incorporated into the liposomal bilayer that the enzymes can specifically target (Sarkar *et al.,* 2005, Elegbede *et al.,* 2008). For example, in a study carried out by Sarkar *et al.,* (2005) the development of a trigger release mechanism via MMP-9 using endopeptidases was shown. A triple helical collagen mimetic peptide conjugated to stearic acid and this lipopeptide was then incorporated with DSPC at 10:90 mol % respectively. These modified liposomes encapsulating carboxyfluorescein, when subjected to the MMP-9 enzyme digestion released 55 % of the marker after five hours. The authors also show specific release associated with this particular subgroup of enzymes because in the presence of a general proteolytic enzyme such as trypsin, there was no release (Sarkar *et al.,* 2005). The range of

enzymes used for release of the encapsulated material from liposomes can be carefully selected for the most effective release within the ELISPOT assay.

1.6.4 ADEPT - designing antibody-enzyme conjugate trigger release systems

Liposome based immuoassays have previously shown to use enzyme-analyte conjugates for *in vitro* purposes, similarly antibody-enzyme conjugates have been used for *in vivo* targeted therapy. Although PLC is not commonly found in areas of diseased tissue, the ability of such enzymes to covalently attach to biological molecules such as targeting antibodies offers an advantageous route for targeted release. It allows attached prodrugs to be activated specifically at the diseased site this type of treatment is termed Antibody-Directed Enzyme Prodrug Therapy (ADEPT) (Francis *et al.*, 2002).

ADEPT is a two-step targeted therapy with the antibody-enzyme conjugate targeting the tumour and then administration of a prodrug activated by the enzyme at the tumour site and is currently a growing area of research as a treatment option for advanced cancers. The emerging problems encountered during the developmental stages of this treatment have included toxicity issues related to the long half-life of the pro-drugs in circulation, subsequently leading to the additional administration of a clearing antibody (Mann, 2002).

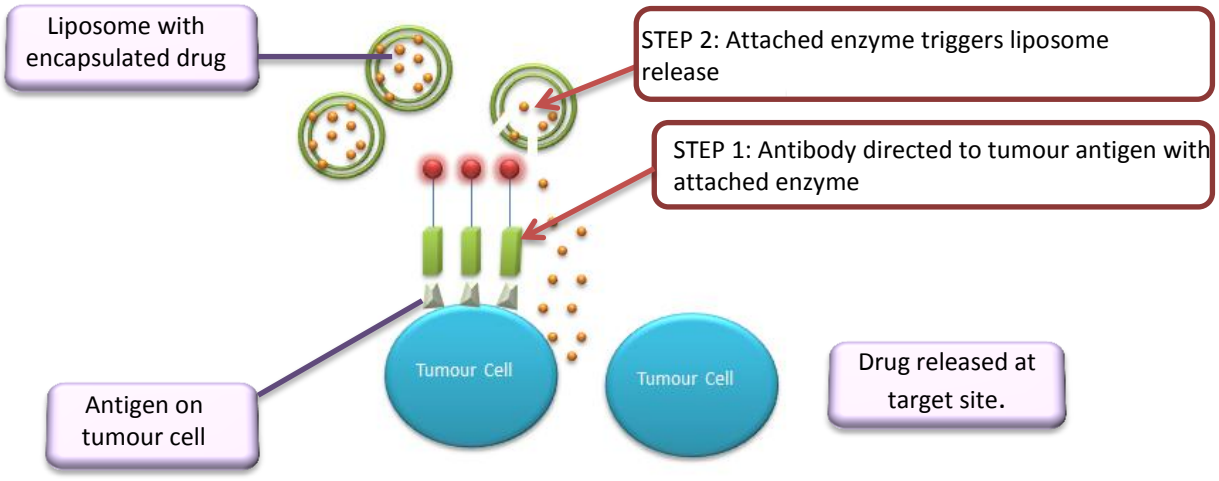


Figure 1.9 The potential use of ADEPT in conjunction with trigger release liposomes.

In terms of liposomal drug delivery, a similar mechanism could be employed to target an enzyme to a disease site using antibodies but rather than the enzyme-triggered conversion of a prodrug, the enzyme could trigger liposomal breakdown and release encapsulated material (Figure 1.9). An advantage of such a system would be controlled release of the drug at the target site and an increased amount of drug reaching the target site within the liposomal carrier system compared with the prodrug conjugates. Within the *in vitro* environment of the ELISPOT assay the enzyme could be attached to a liposomal vesicle which is only activated at 37 °C (the incubation temperature) or by an antibody which is attached to a separate liposomal vesicle. The design of such a system would offer a sophisticated trigger release system.

1.6.5 pH triggered release using fusogenic lipids

Triggered release mechanisms for drug release within cells have also improved targeted delivery. For example, endosomal drug release was achieved by designing fusogenic liposomes that are stable in the extracellular fluid environment, but when they are taken up within the endosomal compartment, the drop in pH results in the liposomes fusing with the endosomal membrane (Shi *et al.,* 2002). This can result in the delivery of the internal contents of the liposomes into the intracellular compartment either via endosomes or directly into the cytosol. Liposome triggered release in response to changes in pH can be achieved via a variety of methods including the use of fusogenic lipids (Connor and Huang, 1985, Simoes *et al.,* 2004) and has been commonly used in gene therapy systems.

pH triggered liposomes were originally designed to release encapsulated material in acidic environments especially areas surrounding tumours where the pH drops to around 5.0 (Lee *et al.,* 1996). The use of pH triggered liposomes is often designed for the acidic environment of endocytic vesicles. In this environment the pH is around 5 and sufficiently acidic to promote release (Drummond *et al.,* 2000). There are different mechanisms by which pH sensitive liposomes have been achieved but they are generally composed of phosphatidylethanolamine or a derivative and a compound, which is a weakly acidic amphiphile. One particular drawback of such formulations has been the loss of pH sensitivity in the presence of serum (Shi *et al.,* 2002, Simoes *et al.,* 2004).

Although a recent study has shown that liposomes formulated from egg phosphatidylcholine, dimethyldiooctadecylammonium bromide (DDAB), cholestryl hemisuccinate (CHEMS) and Tween-80 (25:25:49:1) showed rapid release and more stability than liposomes formulated with DOPE. This study also examined specific targeting of the folate receptor found in many tumours and found increased cytostolic release of entrapped markers (Shi *et al.,* 2002). For an *in vitro* environment such as the ELISPOT assay, the change in pH required for trigger release would have to be carefully controlled by the addition of a buffer in the wells with reference formulation. The main consideration with such pH-triggered release is the change in pH and the impact on the plate coated antigen and the encapsulated material.

1.6.6 pH triggered release and inducing fusion with peptides

Other types of pH-triggered liposomes include the use of synthetic fusogenic peptides within the liposomes vesicles. For example, in a study by Li et al (2004), a peptide containing the amino acid sequence (GALA) was chosen to confer pH sensitivity through a negatively charged side chain. Since the peptide is water soluble at a neutral pH and hydrophobic in an acidic pH, a drop in pH will promote a re-arrangement of the peptide within the liposomal vesicles with the peptide moving into the membrane, fusion of small unilamellar vesicles composed of unsaturated phospholipids will occur causing release of encapsulated material (Li *et al.,* 2004) .

1.6.7 pH triggered release and improving stability with polymers

The most current developments in this field of trigger release have come with the incorporation of pH titratable polymers. One of the problems of using phosphatidylethonolamine (PE) based formulations is rapid clearance and destabilisation by serum proteins; however, inclusion of polyethylene glycol has been shown to circumvent such issues with rapid elimination being observed when regular pH sensitive liposomes were tested *in vivo* but not for sterically stabilised liposomes (Simoes *et al.,* 2004). Polymers have been used extensively in the study of thermosensitive polymers including N-isopropylacrylamide (NIPAM). However, previous studies have shown that the incorporation of such copolymers in liposomal systems result in an increase of the lower critical solution temperature above 37 °C and the vesicles becoming pH sensitive. When

studied for the release from PC and PC/cholesterol liposomes, enhanced release of fluorescent markers was observed at 37 °C in acidic conditions similar to those found *in vivo*, with similar results observed with the incorporation of doxorubicin (Drummond *et al.,* 2000). Interestingly, it was also observed that the inclusion of PEG on the liposome surface did not affect the contents release in an acidic environment (Drummond *et al.,* 2000).

1.6.8 Remote triggered release

Although using the external environment of the target cell seems ideal to achieve site-specific delivery there are no guarantees that release will solely occur here. For instance, inflamed areas can have high levels of Phospholipase enzymes (Meers, 2001); therefore, treatment of tumours or cancers with patients with inflammation due to rheumatoid arthritis may interfere with the targeted release. Thus, an even higher level of sophistication is sought to achieve target specific release and the inevitable next step has been remote triggered release. Instead of relying on the biological environment of the target cell, release would be reliant on an independent external trigger. One of the strategies examined for this purpose has been the use of ultrasound; this is a desirable method for release because it offers area specific penetration into the body without being invasive. In terms of *in vitro* applications such as the ELISPOT assay there is potential for controlled release subsequent to the settling of the liposomes at the bottom of the well thus this type of release could offer reduced background.

1.6.9. Ultrasound triggered release

Ultrasound has been used for many years within the clinical setting for a wide variety of diagnostic imaging including obstetrics, gynaecology and cardiology (Abramowicz, 1997). However, in some instances the reflected signal intensity received by the detector from the tissue can become highly attenuated and therefore ambiguous for useful diagnosis (Cosgrove, 1997). In the late 1960s, it was found that small air bubbles caused within supravalvular injections of saline led to enhanced signal intensity. However, these dissolved rapidly within the blood and therefore more stability was required. Therefore, a combination of low diffusivity gases and materials to stabilise the outer shell such as lipids, polymers, and proteins were developed (Marxer *et al.,* 2011, Kaur *et al.,* 2009).

These are termed echo-enhancers or contrast agents and are effective at increasing signal intensity and reducing tissue attenuation, and there are several products on the market which are used for this purpose, of which Sonovue® uses phospholipids (Calliada *et al.,* 1998). The scope of microbubbles as contrast agents within more sophisticated imaging techniques such as magnetic resonance imaging (MRI), for the purposes of imaging fluid pressure has been examined for a stability assessment of two different gases; namely air filled gas (Vangala *et al.,* 2007) and nitrogen gas (Kaur *et al.,* 2009). For the stabilisation of these bubbles they were formulated with phospholipid 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) in the presence or absence of cholesterol and/or poly (ethylene glycol distearate) (PEG-distearate). The nitrogen gas microbubbles exhibited an enhanced level of stability in comparison to air-filled microbubbles (Kaur *et al.,* 2009, Morris *et al.,* 2008).

Liposomes designed for the purpose of ultrasound-triggered release are generally termed as echogenic liposomes; the basic requirement is an area of air to make them sensitive to ultrasound release. It is thought that the sound wave causes expansion of the contained air and thus of the vesicle itself, to a point where the increase in pressure can exceed the elastic limit of the bilayer and damage occurs to the bilayer thereby releasing the internal contents (Huang and MacDonald, 2004). Exposing liposomes to low frequency ultrasound can cause formation of transient pores due to the effect of ultrasound on the packing arrangement of the lipid chains within the membrane; this results in an increase in the permeability of the membrane and thus drug release (Schroeder *et al.,* 2009). The basic structure of liposomes used for ultrasound activation can be divided in three subtypes, the first would be a liposome which contains the entrapped drug with an internal compartment of air space bound by a monolayer (Huang, 2008). The second would comprise a more complex monolayer covered air bubbles within the aqueous compartment of the liposome prepared by reverse phase evaporation, and sterically stabilised with polyethyleneglycol, before being placed in vials of perfluoropropane gas, bath sonicated and the final subtype are liposomes which are conjugated to air bubbles through a biotin-avidin linkage (Huang, 2008).

It has also been suggested that lipid composition influences drug release from liposomes using ultrasound as a trigger (Evjen et al 2011). In this study, calcein was used as a model drug and

release was monitored by fluorescence self-quenching. Increased cavitation dose, resulted in a greater amount of release from DOPE based liposomes in comparison to formulations composed of DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine). At a cavitation dose of 2000 (1.13 MHz) the formulation of DSPE:DSPE-PEG 2000:cholesterol 68:8:30 mol % showed release of 34 % whereas with the formulation incorporating DOPE (DOPE:DSPC:DSPE-PEG 2000:cholesterol 58:11:5:26 mol %) 49 % release was observed. It is interesting to note that in this study cholesterol had a negative effect on sonosensitivity. The differences in the observations are thought to be due to the bilayer packing arrangements of the lipids which, when exposed to ultrasound can produce perturbations in the membrane (Evjen *et al.,* 2011).

Further investigations of liposome-microbubble complexes for drug delivery applications have been shown through constructs of microbubbles conjugated to the surface of gas-filled microbubbles used for the purpose of ultrasound-triggered release. It was found that short ultrasound treatment resulted in complete destruction of the bubble and release of a third of the entrapped dye and thrombin release was confirmed to be around 11 % (Klibanov *et al.,* 2010). There is an obvious potential in taking forward ultrasound triggered release particularly as it offers the potential for precise release at the target area and it is preferred alternative to more invasive techniques. In terms of the design, a formulation as a reference standard within an ELISPOT assay, microbubbles may not be able to sediment as effectively as the normal vesicles due to the gas that is used. This may influence effective spot formation.

1.6.10 Thermosensitive liposomes: exploiting phase transition temperatures of lipids.

In taking the literal meaning of thermosensitive, all lipids used in liposome formulations are thermosensitive because the nature of the bilayer will change in response to the surrounding temperature. The bilayer will undergo phase transitions from a more crystalline gel state to a more disordered fluid state (Figure 1.10) at increased temperatures and this affects the permeability of the membrane resulting in leakage of encapsulated material. The phase transition temperature of a liposome formulation is dependent upon the phospholipids used and formulations can therefore be designed for the application required (Vemuri and Rhodes, 1995).

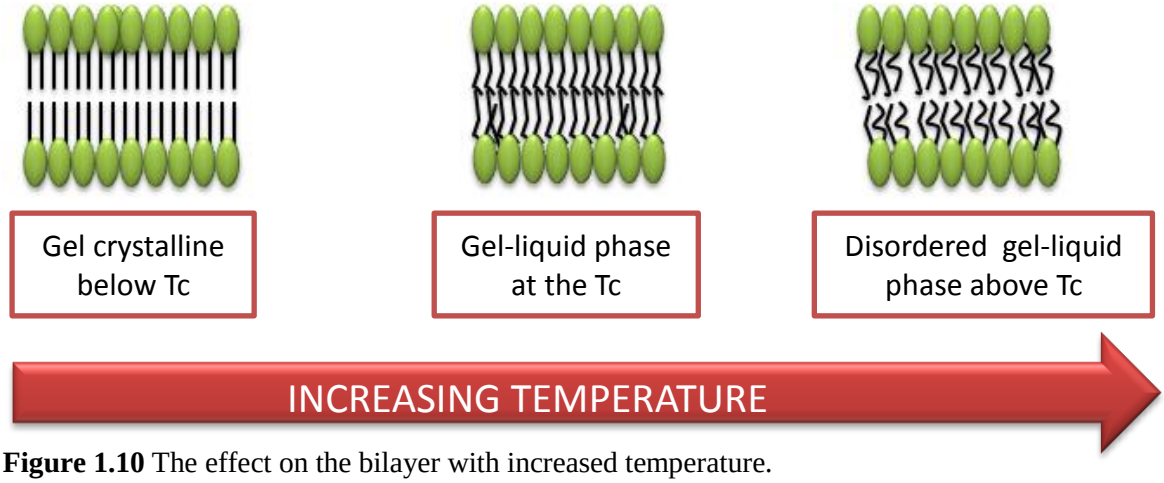


Figure 1.10 The effect on the bilayer with increased temperature.

Long circulating liposomes have the ability to evade destruction by the immune system prior to reaching the target site; however, the rational design of liposomes is now expanding to become even more specific with trigger release and target specific delivery. For instance, a recent study has examined the use of thermosensitive liposomes conjugated with a ligand attached to the liposome surface. These liposomes had a human epidermal growth factor 2 specific ligand which is used to target breast cancer cells. These liposomes were loaded with the cancer drug doxorubicin and the effects of hyperthermia were examined (Smith *et al.*, 2011). Formulations were made from DPPC and DSPE-PEG 2000 and cells treated with liposomes showed a 10-fold increase in binding to HER2+ cells in comparison to HER2- cells at 37 °C. The doxorubicin liposomes also exhibited a 2-3 fold higher accumulation in cells in comparison to unencapsulated liposomes. By using specialised ligands incorporated into the liposomes, termed affibody molecules, as alternatives to antibodies the high affinity ligand is able to bind with specificity to target proteins, which are upregulated in tumour tissues. They are composed of a three helix scaffold and are relatively small (6-9 kDa) yet they are extremely stable and can be readily expressed in bacteria or by peptide synthesis (Smith *et al.*, 2011).

The aim of the design process in such systems is to produce a vesicle that is not only target specific to the intended tissue but one that will also have the capability to release at the target area in order for the enhancement of drug delivery. Similarly, to this study the leaky character of liposome formulations during phase transitions can be exploited to design thermosensitive liposomes, especially as DPPC undergoes phase transition at 41°C (Needham and Dewhirst, 2001, Kono,

2001). However, instead of relying solely on the lipids themselves other techniques to modify the membrane characteristics are being used, such as the addition of lysolipids and polymers.

1.6.11 Thermosensitive liposomes and modifying phase transition with lysolipids

A recent study examined the formulation of liposomes using DPPC and monopalmitoylphosphatidylcholine (MPPC) for the delivery of arsenic trioxide (ATO;AS_2O_3) in thermo-triggered liposomes formulations. Similarly to cisplatin, this drug is an effective anti-cancer treatment but at higher doses there are toxicity problems. Liposomes are ideal carrier systems because they are generally composed of non-toxic lipids and able to encapsulate or bind a wide variety of molecules (Lasic, 1998). Liposome formulations in this study included a combination of DPPC and corresponding lysolipids, which have single chains (Winter *et al.,* 2011). In this study, a 10 % addition of lysolipid allowed release over 24 hours whereas formulations with a 5 % reduction of MPPC release reached a plateau after 10 hours. This elucidates the nature of tailoring phospholipids used and release kinetics as temperature affects the permeability of the membrane. Although release rates were comparable at 37 °C and 42 °C the presence of 10 % lysolipid resulted in a 15 % higher release rate when compared to formulations using 5 % lysolipid within the first hour (Winter *et al.,* 2011).

Similarly, the MPPC lysolipid incorporated into DPPC formulations with entrapped carboxyfluorescein enabled the liposome formulations to become more thermosensitive (Needham and Dewhirst, 2001). The presence of lysolipids in liposome formulations can reduce the stability of vesicles by partitioning in to the bilayer. It was found that the lysolipid containing liposomes showed a much more rapid release at 42 °C. These formulations were also tested *in vivo* in mice with tumours and it was found that that the growth delay for the tumours was extremely effective as none of the tumours showed regrowth for up to 60 days compared to non-thermosensitive stealth liposomes where tumours grew back 15 days after treatment. The hyperthermia, which is 42 °C for 1 hour, is thought to have produced enhanced permeability, thus long circulating stealth liposomes are able to accumulate at the site of the tumour because of increased vascular permeability (Needham and Dewhirst, 2001). Studies using lysolipids within formulations to enhance the

thermosensitive effects of liposome formulations appear to show promise. The rational design for a formulation to release at a specific temperature, slightly higher than the physiological temperature, would allow more target specific release, through heating of local areas where the drug has accumulated. This would offer many advantages, primarily reduced cytotoxicity as free drug will not be available in non-target areas as is the case with many anti-cancer drugs. This method allows greater control of release, which addresses one of the drawbacks of stealth liposomes, that although they can reach the target site the release from the vesicles is slow (Shum *et al.,* 2001).

1.6.12 Thermosensitive liposomes and modifying phase transition with polymers

Instead of relying solely on the melting point of individual lipids, thermosensitive polymers can be incorporated into the formulations (Kono *et al.,* 1999, Kono, 2001, Chandaroy *et al.,* 2001). There are a wide range of synthetic and natural polymers available that have the ability to be water insoluble below the lower critical solution temperature (LCST) and water soluble above the LCST. The change in the polymer chains from a hydrated or hydrophilic state to hydrophobic whereby the polymer chains contract and cause destabilisation of the membrane of the liposomes can be controlled by the exposure temperature (Figure 1.11), thus the liposomes become thermosensitive (Kono, 2001).

Association of the polymers is either through the aqueous incubation of polymers with liposome formulations (attached only to the outer liposomal membrane) or alternatively they can be a mixture of lipids and polymers used prior to formulation with polymer chains associated with both the internal and outer membrane. In a study by Kono *et al.,* (1999) where co-polymers were used, two different types of anchoring was investigated, one was in the middle of the chain and one at the end of the chain. These were combined with dioleoylphosphatidylethonolamine (DOPE) in calcein loaded liposomes. It was found that the polymers anchored at the terminal region exhibited significantly more release at 42 °C, with almost complete release of the contents within 5 minutes.

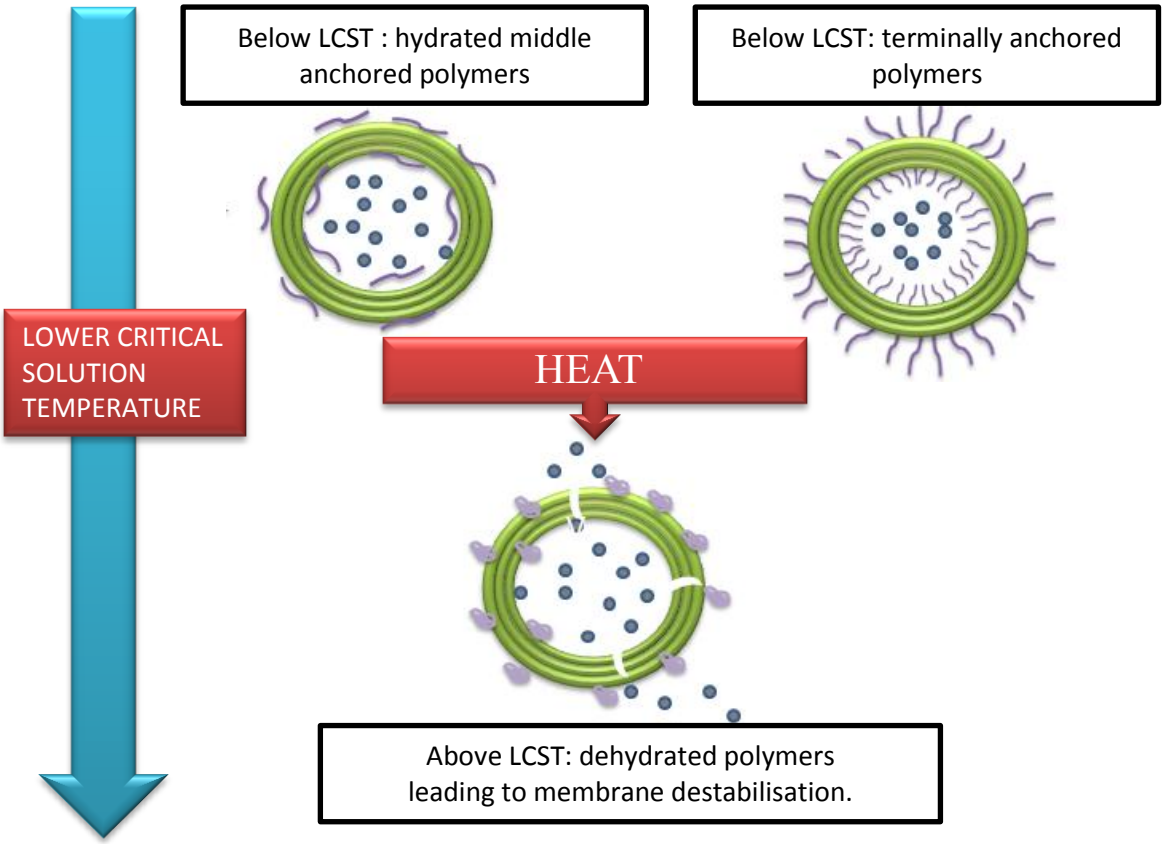


Figure 1.11 The effect of temperature on liposomes modified with polymers.

The polymer used is a poloxamer and these are tri-block polymers with a hydrophobic poly (propylene oxide) group in the middle of two polyethylene oxide hydrophilic groups (Chandaroy *et al.*, 2001). The results from this work showed that the presence of increasing percentages of cholesterol in the composition of the formulation resulted in a sharper transition for the release of contents, and that the temperature for the onset of release increased with decreasing pluronic concentration. Understanding the effects of release by modifying the composition of the formulations is a significant issue to consider in the rational design of liposome formulations. The use of di-stearyl poly (ethylene glycol 5000) was found to have no effect on the release characteristics of the formulations, which suggests that the presence of PEG does not hinder the pluronic molecules.

1.6.13 Light sensitive liposomes

Ultrasound as a technique is already widely employed within the medical field (Abramowicz, 1997) and therefore using liposomes to carry drugs to affected areas and triggering release via this method seems logical step forward. Light is not used to the same extent within the clinical setting,

which may explain the reduced number of research articles available in this area and the lack of success for *in vivo* applications of this particular trigger release system. Photo-triggered release requires an element within the liposome formulation to be light reactive; this can either be the lipid itself which may have been modified or a membrane anchored probe and contact of light of the appropriate wavelength to achieve release. In a study by Yavlovich *et al.,* (2011), in order to tailor the liposomes formulations for use within an *in vivo* environment, DPPC liposomes were formulated with diacetylene phospholipid (DC[8,9] PC) and DSPE-PEG200 with the encapsulated doxorubicin. Assessment of the released drug showed that laser treatment of cells with a 514 nm wavelength resulted in a 2-3 fold improvement in cells killed compared to untreated samples (Yavlovich *et al.,* 2011).

More novel uses of photo-reactive liposomes have been shown with the use of 3D tissue constructs (Smith *et al.,* 2007). This application is not aimed at drug delivery although theoretically can be applied to *in vivo* situations. In this instance photosensitive liposome formulations were made using a light sensitive lipid (1,2 –bis (4-(n-butyl) phenylazo-4'-phenylbutyroyl) Phosphatidylcholine) to be triggered by long wavelength ultra violet light. The entrapped calcium chloride upon release resulted in cross linking of the alginate solution and immobilisation of bone-derived cells for the construction of 3D cultures. By combining the lipid with a stable lipid, such as DPPC, trapped solutes are released upon with the appropriate trigger (Smith *et al.,* 2007). Light triggered release does not only have the option of a single parameter for causing release such as temperature as the formulation can be tailored for wavelength, intensity and duration of application. Currently, Visudyne® is one of the only approved liposome formulation which uses the principle of light triggered release and is used for a condition which occurs as a result of age related macular degeneration. Thus, theoretically light triggered release could be applied within an *in vitro* setting although this would require an additional step within the assay that is not a part of the normal protocol. The modification of the protocol itself to enable the use of a reference standard is not ideal as it may introduce further variability.

1.7 Triggered release systems and applications for the ELISPOT assay

The emergence of ThermoDox® for the treatment of a wide range of cancers highlights how trigger release systems are moving closer to becoming available for treatment (Allen and Cullis, 2012). This liposome formulation has been designed to release entrapped doxorubicin under conditions of mild hyperthermia and is now at the Phase III stage of clinical trials. Hence, the progression of trigger release systems is translating into relevant medical applications. Trigger release systems currently studied are broadly divided into two categories; those reliant on an external trigger such mild hyperthermia, ultrasound or light and those dependent on the local environment as in the case of enzymatic and pH triggered release.

The obvious advantage of an external trigger is that there is complete control of release if the formulations are completely stable; it is precisely this ability, which makes these systems interesting options for the release of encapsulated material in an ELISPOT assay. For instance, the success of enzymatic release relies on a successful interaction of the enzyme and the substrate and this may be governed by several factors as described in Table 1.4. Such factors include chain length and saturation of lipids chosen and although there have been no comprehensive studies examining these parameters the various studies in this field have shown that the ability of the enzyme to hydrolyse the substrate varies when these factors are changed. For instance, PLC was less effective with lipids of longer chain lengths (Van *et al.*, 1961) or saturated chains (Macfarlane and Knight, 1941, Long and Maguire, 1954). However, the impact of lipid choice may not be as significant for instance with the MMP based release systems as the target was not the bilayer itself but the endopeptidases attached to it. Thus, the actual composition of the bilayer is not going to play as significant a role as those enzymes such as PLA_2 that directly hydrolyse the lipid chains.

Although the aim of many, if not all, of these studies is to enhance the effectiveness of targeted delivery for *in vivo* purposes, for the purpose of producing an artificial reference standard the concept of triggered release systems needs to be applied to an *in vitro* environment. By carefully designing the liposomal system to be as stable as possible and incorporating a triggered release system this may lead to enhanced release, and possibly more effective spot formation.

Table 1.4 Liposome composition factors and their impact on enzymatic trigger release systems.

Parameters	Effect
Chain Length	Increased chain length leads to a more tightly packed bilayer. This may affect how the enzyme is able breakdown down the bilayer.
Saturation	Saturated phospholipids are able to pack more tightly; this can lead to more stable formulations for *in vivo* purposes, but conversely it has been shown to inhibit enzyme action.
Transition Temperature	Enzymes work at an optimal temperature range; *in vivo* this needs to be 37 °C thus the transition temperatures of the lipids must be chosen to achieve stable formulations, as it will vary as a direct result of composition of the overall phospholipid mixture.
Cholesterol	Increases the stability of the liposomes resulting in tighter packing therefore there may be a direct impact on the rate of release by the enzyme.
Polymers	The insertion of a polymer into the bilayer may also effect the dynamics of enzyme-lipid interaction as there maybe changes to the charge or the overall structure of the liposome
Charge	Lipids can be anionic, neutral or cationic and this may directly affect the interaction between the enzyme and substrate.

Any triggered release system considered for use in the ELISPOT assay needs to be compatible with the assay and the way in which it is used on a daily basis, therefore some of the triggered release systems may not be appropriate. Overall trigger release systems offer potential for enhanced release and if used in conjunction with the ELISPOT assay would require careful consideration for their integration within the assay. Therefore options such as light triggered release and ultrasound, although offering remote release in a controlled manner, would result in an additional step in the procedure of the assay which may affect other live cells present in the assay. However, options such as careful lipid selection for thermo-triggered release, enzymatic release and pH triggered release may offer more effective alternatives.

1.8 Aim and objectives

The aim of this project was to produce a liposomal system that could encapsulate recombinant interferon gamma (rIFNγ) for use as an artificial reference agent in an ELISPOT assay. The reference standard produced would aid in enhancing the reliability of the assay and would be

particularly useful in the assessment of *inter-* and *intra-* assay variability. In order to achieve this aim, the objectives for the study were;

- To show the feasibility of encapsulating the cytokine within a range of liposomal formulations including a formulation of increased density, and an assessment on the impact of the cytokine of the formulation process.
- To assess 'proof of principle' as to whether liposomes can produce spots within an ELISPOT assay and consequently which liposome compositions are most effective.
- To achieve defined spot formation of an appropriate size and morphology similar to those produced by peripheral blood mononuclear cells added to the assay. To produce a negative control with empty liposomes and a positive control with rIFNγ encapsulated cytokines.
- To consider trigger release systems as part of the design for more effective release of the liposomal contents for the production of increased spot numbers.
- To demonstrate low background levels with an optimised protocol for production that will allow clear visualisation of spots formed whether using manual or automated enumeration techniques.
- To assess the feasibility of the controls as reference standards through an external assessment with live cells.
- To assess product storage either as a frozen formulation or as a lyophilised standard with the inclusion of cryoprotectants and examine the feasibility of scale-up to produce a larger batch.

Chapter 2

Materials and Methods

2.1 Materials

Chemicals/Materials	Supplier
Ascorbic acid	Sigma-Aldrich, Poole, Dorset, UK
Alkaline phosphatase	Sigma-Aldrich, Poole, Dorset, UK
Biosieve filter	Fisher Scientific, Loughborough, UK
Bovine serum albumin	Sigma-Aldrich, Poole, Dorset, UK
Bovine serum albumin-Fluoroscein Isothiocyanate	Sigma-Aldrich, Poole, Dorset, UK
Bromine	Fisher Scientific, Loughborough, UK
Calcium chloride dihydrate	Sigma-Aldrich, Poole, Dorset, UK
Cholesterol	Sigma-Aldrich, Poole, Dorset ,UK
Chloroform	Fisher Scientific, Loughborough, UK
1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiIC),	Sigma-Aldrich, Poole, Dorset, UK
ELISA kits (Human interferon-γ)	Mabtech, Sweden.
ELISPOT plus (Human interferon-γ)	Mabtech, Sweden.
Ethylenediaminetetraacetic acid tetrasodium	Sigma-Aldrich, Poole, Dorset, UK
Frame seal chambers	Bio-Rad, Hertfordshire, UK
Glacial acetic acid	Fisher Scientific, Loughborough, UK
Interferon-γ Human	Sigma-Aldrich, Poole, Dorset, UK
Methanol	Sigma-Aldrich, Poole, Dorset, UK

Microplates 96 well black non-binding plates	Greiner, Stroutwater, UK
Microslides	Cam Lab, Cambridge, UK
Microscope slides	Fisher Scientific, Loughborough, UK
Molybdic acid	Sigma-Aldrich, Poole, Dorset, UK
Polycarbonate 1.2 μm and 3 μm filters	Millipore, Abingdon, UK
Phosphate buffered saline	Sigma-Aldrich, Poole, Dorset, UK
Phospholipase C from Clostridium Perfringens	Sigma-Aldrich, Poole, Dorset, UK
Phospholipase A2 from honey bee venom.	Sigma-Aldrich, Poole, Dorset, UK
Phosphorus standard solution	Sigma-Aldrich, Poole, Dorset, UK
Rhodamine –B	Sigma-Aldrich, Poole, Dorset, UK
RPMI without Glutamine	Biosera, Uckfield East Sussex, UK
Sodium phosphate dibasic	Sigma-Aldrich, Poole, Dorset, UK
Sucrose	Sigma-Aldrich, Poole, Dorset, UK
Trehalose Dihydrate	Sigma-Aldrich, Poole, Dorset, UK
Tris maleate buffer	Sigma-Aldrich, Poole, Dorset, UK

Lipids

2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)	Avanti Lipids, Alabama, USA
1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)	Avanti Lipids, Alabama, USA
1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)	Avanti Lipids, Alabama, USA
L-α Phosphatidylcholine (PC)	Sigma-Aldrich, Poole, Dorset, UK

2.2 Methods

2.2.1 Liposome Preparation

Liposomes were prepared using the dry film hydration method (Figure 2.1) (Bangham *et al.*, 1965). A range of lipids were used throughout the study including 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), phosphatidylcholine (PC) and cholesterol (Chol); which were dissolved in chloroform: methanol 9:1 at a concentration of 10 mg/mL.

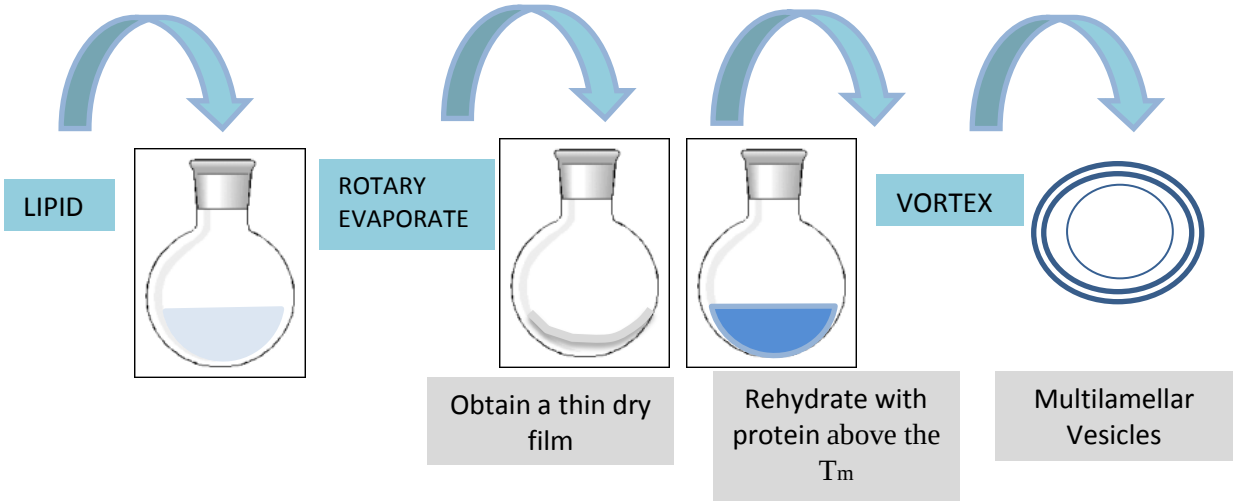


Figure 2.1 Method of (MLV) liposome preparation for protein encapsulation.

The majority of the preliminary work was carried out with either a total lipid amount of 32 µMol or 16 µMol with cholesterol containing formulations at a 1:1 equimolar ratio. The lipids were placed into a 50 mL round bottom flask and the solvent evaporated on a rotary evaporator to obtain a thin dry lipid film. The flask was further dried with nitrogen to remove any residual solvent. The hydration media with the moiety to be incorporated was warmed above the transition temperature for the lipids before being added to the flask. The suspension was allowed to stand for approximately 30 minutes in a water bath above the transition temperature with intermittent periods of vortexing, during which time multilamellar vesicles are formed and a milky suspension is obtained.

2.2.2 Liposome characterisation

2.2.2.1 Particle size characterisation

Size is an important characteristic to measure for liposomal formulations in the evaluation of the sample produced and laser diffraction techniques are commonly used. Advantages of this technique include a broad size range can be considered, small amounts of sample are used, robustness and precision of data (Tinke *et al.,* 2008). This is a technique based on the principle that particles present in the path of a laser beam will cause scattering of light and this scattering is related to the size of the particle. The scattering diffraction patterns occur because of all the particles present in the pathway of the beam and therefore information is provided in terms of the number of particles and size (Ma *et al.,* 2000). In the characterisation work carried out in this study all liposomes formulation underwent size characterisation using the Sympatec Helos (UK) particle sizer. For the measurements of size, 100 µL of a liposome formulation was pipetted into a glass quartz cuvette (a signal of at least 15 % was required for an adequate reading). Three measurements were taken for each formulation and an average result obtained for size and polydispersity. The Sympatec Helos (UK) uses a Helium Neon laser of 632.8 nm (Sympatec) with an R2 lens. Calculations for particle size distribution require a mathematical process to assess the scattering pattern using an optical model. There are various mathematical equations that can be used, the Sympatec uses the Phillips-Twoney method (Sympatec Limited, UK).

2.2.2.2 Measurement of zeta potential.

Zeta potential is an important characterisation aspect to consider in liposome characterisation, as electrostatic interfaces play a significant role in particle interactions and stability. The electrical charge on the surface of particle is related either to the ionisation of surface groups or to the adsorption of charged ions. The ions directly attracted to the surface form the stern layer and the ions, which surround this layer, form the diffuse or electrical double layer and contain both negative and positive ions (Florence and Attwood, 2011). The zeta potential is the measurable electrical potential at the shear plane (Hunter *et al.,* 1981).

Zeta potential measurements were carried out using the Zetasizer Nano (Malvern, UK). A 1:10 dilution was made of each liposome formulation in buffer and placed into the zetasizer cuvette, avoiding air bubbles. The Zetasizer Nano takes multiple readings for each formulation before providing an average with the standard deviation, this was repeated three times for each formulation with the average reading obtained for each formulation.

2.2.3 Morphological analysis of liposomes

2.2.3.1 Microscopy techniques and sample preparation

In conjunction with particle size characterisation, morphological assessment of formulations was carried out using microscopy. Light microscopy was used for the visualisation of prepared samples dispersed in an aqueous medium with different options available for the preparation of the sample as shown in Figure 2.2. The advantages and disadvantages of each method are also listed (Figure 2.2).

Figure 2.2 Summary of methods for sample preparation for visualisation of lipid vesicles. The advantages and disadvantages of each method with images taken on the light microscope using Zeiss Axioscope A1 under 40 X objective (Bibi *et al.*, 2011).

For the vast majority of liposomal images a standard glass slide/cover slip can give good visualisation of large vesicles such as multilamellar vesicles. This technique allows confirmation of the formulation heterogeneity and particle size data obtained using other methods. However microscopy can also substantiate other quantitative data for instance to show that encapsulation of a protein which is fluorescently labelled with a fluorophore, or to highlight the bilayer which can have the fluorescent marker incorporated inside (Szoka and Papahadjopoulos, 1980).

2.2.3.2 Light Microscopy

Light microscopy was used to consolidate data obtained from particle size characterisation and to assess the morphology of the vesicles. Generally, samples were viewed using standard glass slides with cover slips under a 40 X objective (Figure 2.2). The light microscope used was an upright Zeiss Axiovert A1 microscope with Axiovision software employed for image capture. On the glass slide 20–30 μL of sample was placed centrally, before a coverslip was placed on top. Images were viewed either in bright-field or phase contrast.

2.2.3.3 Confocal Microscopy

A 60 X oil objective was used with Helium and Argon lasers using a Leica confocal microscope. The fluorescent dye DilC (1,1'-dioctadecyl-3,3,3' ,3'-tetramethylindocarbocyanine perchlorate) was dissolved in chloroform at 0.2 mol% and incorporated in to the bilayer prior to rotary evaporation of the dissolved lipids. Once the thin lipid film was obtained the formulation was rehydrated with 4 mL of BSA-FITC at a concentration of 5 μg/mL. The unentrapped marker was removed by centrifugation at 29,771 *g* for 30 minutes at 4 °C. The formulation was then taken up into a microslide which is fixed onto a glass slide for viewing (Figure 2.2). This avoids immediate drying out of the sample if just a coverslip is used. Filters used were FITC for the BSA-FITC and TRITC for the DilC dye.

2.2.3.4 Freeze fracture microscopy

Electron freeze fracture is an important technique that can serve to highlight the structure and structural changes that have occurred in both biological and artificial vesicles, by assessing the fractured planes (Forge *et al.,* 1989). This technique was used for the analysis of liposomal membranes during the stability study assessment of liposomal formulations containing brominated cholesterol. A drop of each incubation mixture (approx. 5 µL) was placed on a ridged, gold specimen support or was sandwiched between two copper plates, for fracture in a double replica device. Samples were frozen by rapid plunging into a constantly stirred mixture of propane: isopentane (3:1) and cooled by liquid nitrogen. Fracture was subsequently performed on a Balzers BAF 400D apparatus at a temperature of – 110 ^{o}C. Replicas were floated free on distilled water, and cleaned in 40 % chromic acid. Images were viewed using a transmission microscope. The electron freeze fracture was carried out at the UCL, department of Audiology, UK by Professor Andrew Forge.

2.2.4 Centrifugation procedures used for the removal of unentrapped marker

To remove unentrapped protein the formulations were placed into 3.9 mL tubes, which were heat sealed and placed into an Ultramax centrifuge (Protocol 1; Table 2.1). The centrifuge cycle began once the vacuum was switched on and had reached < 50. Protocol 1 was used for the removal of unentrapped BSA-FITC. Subsequently for the removal of rIFNγ variations of the protocol were required for size selection and optimisation of the background in the ELISPOT wells (Protocol 2 to 5; Table 2.1). The ultra-centrifuge was used for speeds of 29, 771 g with the TLN-100 SN 32 rota or the standard universal centrifuge H232 for 10 mL tubes.

Table 2.1 Centrifugation protocols used for the removal of unentrapped protein. The ultra-centrifuge was used for speeds of 29, 771 g with the TLN-100 SN 32 rota or the standard universal centrifuge H232 for 10 mL tubes. Protocol 1 was used for the removal of unentrapped BSA-FITC and IFNγ. The protocol was further optimised to reduce background for the ELISPOT assay (protocol 2 – 5).

Protocol 1	Protocol 2	Protocol 3	Protocol 4	Final Protocol 5
1. Place in 3.9 mL centrifuge tubes make up to volume with PBS. Centrifuge at 29, 771 g for 30 mins.	**1.** Place in 3.9 mL centrifuge tubes make up to volume with PBS. Centrifuge at 29, 771 g for 30 mins.	**1**. Place in 3.9 mL centrifuge tubes make up to volume with PBS. Centrifuge at 29, 771 g for 30 mins.	**1.** Make up to 5 mL with PBS and centrifuge at 4°C for 15 minutes at 2660 g.	**1.** Make up to 5 mL with RPMI and centrifuge at 4°C for 15 minutes at 2660
2. Remove supernatant and resuspend in 2 mL of PBS.	**2**. Remove supernatant and resuspend in 2 mL of PBS.	**2.** Remove supernatant and resuspend in 2 mL of PBS.	**2.** Remove supernatant and resuspend in 2 mL of PBS.	**2.** Remove supernatant and make up to 5 mL with RPMI and centrifuge at 4°C for 15 minutes at 2660 g.
		3. Place in 3.9 mL centrifuge tubes make up to volume with PBS. Centrifuge at 29, 771 g for 30 mins.	**3**. Place in 10 mL centrifuge tube and make up to 5 mL with RPMI Centrifuge at 205 g at 4°C.	**3.** Remove supernatant, resuspend in 5 mL of RPMI, and add to 10 mL tube.
		4. Remove supernatant and resuspend in 2 mL of PBS	**4.** Resuspend in 1 mL in RPMI	**4.** Centrifuge at 205 g for 10 minutes and resuspend in 1 mL of RPMI medium.

2.2.5 Bromination of cholesterol

For the purpose of producing a reference standard in the ELISPOT assay it was thought that by producing an analogue of cholesterol the overall density of liposome formulations could be enhanced and would thus mimic more closely the density of live cells placed in the assay. By having an increased density, the aim was to enhance the sedimentation characteristics of such formulations.

2.2.5.1 Method for the synthesis of brominated cholesterol

A basic laboratory method for a bromination reaction for cholesterol was carried out (Kabara *et al.*, 1960). The reaction involves nucleophilic attack by the cholesterol C=C bond on bromine (Figure 2.3) with the formation of a tertiary carbocation. The formed cation undergoes secondary bromination via an attack from the back by the bromide ion to form the dibromocholesterol derivative, with the bromine atoms in the *trans* and diaxial configuration.

Figure 2.3 Schematic of the reaction steps for bromination of cholesterol (Fieser and Williamson, 1992).

The method for synthesis was as follows:

- In a 50 mL round bottom flask, 1 g of cholesterol was dissolved in 10 mL of ether.
- The solution was stirred and 5.0 mL of a 10 % solution of bromine in glacial acetic acid was added (0.10 g of bromine /1.0 mL).
- The mixture was left to stand at room temperature for 30 minutes so that the developing dibromocompound could precipitate from the solution. The solution was then cooled on an ice bath to complete the precipitation.
- The product was collected by suction filtration. This was then washed in the funnel, once with 5 mL of acetic acid and finally with water. After washing, the compound was left to dry under vacuum overnight.

2.2.5.2 Melting point determination of synthesised dibromocholesterol

The melting point was determined using basic Gallenkamp MP apparatus by visualisation after placing a small amount of pure cholesterol and the product into a glass capillary tube and into the apparatus. The temperature was increased gradually until a visible change occurred. This observed melting point was recorded and the process repeated three times. Clear defined melting point ranges can be used when compared to literature melting points to confirm the product required has been produced.

2.2.5.3 Differential Scanning Calorimetry for determination of melting point

Differential scanning calorimetry (DSC) is defined as the measurement of the change of difference in the heat flow rate to the sample and to a reference sample while they are subjected to a control temperature program (Höhne *et al.*, 2003). Although this analytical technique is primarily used to measure thermal phase transitions for this study, it was used for an accurate measurement of the melting point and for assessing the purity of the sample. 2 mg of each sample was placed into a T-0 aluminium pan and sealed. A sample and a reference pan were placed inside the instrument (DSC TA Instruments Q200,UK) Scans were run for the pure cholesterol (Sigma) and the product dibromocholesterol. The scan rate was 10 °C per minute commencing at 30 °C.

2.2.5.4 Thin Layer Chromatography

This method is used to differentiate between substances that differentially partition between two phases, the mobile phase and the stationary phase. Migration of the substance is a result of its varying affinity for the stationary and mobile phase (Sherma and Fried, 2003). The thin layer chromatography (TLC) method was used to assess the formation of the synthesised product by using cholesterol as the reference.

The prepared TLC plates had an adsorbent silica gel coating with aluminium backing. Small strips (2.5 cm x 6.5 cm) were cut from a 20 cm x 3 cm sheet of the solid support. A straight line was drawn horizontally 1 cm from the bottom of the strip. Three spots were applied in pencil equidistant apart as a guide for the application for spots for the product and pure cholesterol. Thus,

0.5 mg of the cholesterol and product were dissolved in 1 mL of chloroform. This was applied with a glass pipette to allow a small uniform spot to be formed. The solution for both the product and cholesterol were applied a few times in this way allowing the solvent to evaporate between additions. This was then checked under a short wavelength UV light to determine if enough had been applied. The chamber used for the development of the chromatogram was a large glass jar with a screw top lid. Solvent is poured into the jar to the depth of a few millimeters. The solvent was allowed to saturate the development chamber. The spotted plate was then placed into the container with the spotted end down with the solvent level below the spots. Various compositions were trialed including initially the literature method of hexane:ether:acetic acid 85:15:2 (Kabara and Mclaughlin, 1960). Visualisation was examined of this plate via both 10 % phosphomolybdic acid in ethanol and vannilin solution. The solvent used after optimisation was ether:acetic acid 98:2. Visualisation of the product via 10 % phosphomolybdic acid was carried out. When the solvent front had reached near to or exactly at the top of the plate it was removed from the devoloping chamber with clean forceps. The position of the solvent front was marked and allowed to evaporate. The plate was then immersed in 10 % phosphomolybdic acid. This was dried and then individual spots were marked.

2.2.5.5 Infrared Spectroscopy

The melting point data using the DSC was slightly different to the literature melting point and showed slight discrepancies between the two batches after the main melt, therefore for further characterisation an IR spectrum was carried out of the obtained product, as this technique can be used to ascertain identity and purity (Kalinkova, 1999). The IR spectrum produces sample peaks, which correspond to the frequency of vibrations of parts of the sample molecule thus information in regards to the structure in particular the presence of functional groups, can be ascertained (Stuart, 1997). The wavelengths were assigned using described absorption bands (Mendham *et al.*, 2000).

Infrared Spectroscopy is a technique that uses the vibrational frequencies of bonds between atoms in molecules. It is obtained by passing infrared radiation through the sample and the energy at

which the peaks appear in the absorption spectrum corresponds to the frequency of a vibration of a part of the sample molecule. The specific frequencies of IR radiation absorbed correspond to the functional groups present in the sample. It is widely used for the determination of structures and compounds (Mendham *et al.,* 2000). The Infrared spectroscopy machine used was Nicolet IR200, using potassium bromide (KBr) disks.

2.2.5.6 Preparation of KBr disks

The sample was prepared by weighing out 150 mg of potassium bromide (KBr) with 1 mg of the sample product. The KBR was of spectroscopic grade and was dry. The powders were then ground together with a mortar and pestle until the mixture was very fine. The disks and the die were cleaned with tissue to ensure they contained no residues. The disk was then placed into the die with the shiny side up and the slightly bezzled edge on the outer surface. A micro-spatula was used to add 150 mg of the powder into the tube and it was shaken in gently to ensure an even distribution. The die was then tapped in order to distribute the powder. The second disk was placed on top with the shiny side inwards towards the powder and the plunger was pushed down. The holder was screwed down when aligned with the die until it is firmly in place. The tube for the vacuum was then placed on the left hand side of the die and the dial on the right hand side turned until it was tight before the vacuum was switched on. The vacuum was left on for 2 minutes and then the pressure was increased to 8 tonnes and left for 10 minutes. The disk was then placed in a holder and put into the machine and the cover placed on top. Disks were prepared and analysed for both cholesterol and dibromocholesterol.

2.2.5.7 ^{1}H NMR and ^{13}C NMR

NMR is a spectroscopic analytical technique that uses the magnetic properties of nuclei. The instrument contains a strong magnet with a coil that supplies energy in the radio frequency range of the electromagnetic spectrum. Nuclei are able to absorb this energy and flip from a lower energy state to a higher energy state. There is another coil, which is able to sense these small energy changes. This information was analysed to provide information about the number, type and environment of the ^{1}H and ^{13}C atoms (Sanders and Hunter, 1993).

The carbon NMR spectrum allows not only the number of carbons to be established but also provides information in regards to how many hydrogens each carbon is bonded to, dependent on whether the peak is above or below the baseline (Malet-Martino and Holzgrabe, 2011). Approximately 2 mg of sample was placed into 0.5 mL of CDCL3 solvent, this particular solvent is used as it does not contain H atoms, which too would absorb energy in the same range as the other protons. This sample was placed in a thin glass tube that is placed into the NMR machine (Bruker 250MHz).

2.2.5.8 Calculation of percentage yield

The quantity of product produced during a chemical synthesis was determined by calculating the percentage yield. In order to this, the theoretical yield was calculated initially to determine the amount that would be obtained in perfect conditions. Then using the final weight of the product the actual percentage yield can be calculated (Equation 2.1). The following equation was used to do this:

Equation 2.1 Calculation for percentage yield.

$$\text{Percentage yield} = \begin{matrix}\text{Amount in moles} \\ \text{of limiting reagent}\end{matrix} \times \begin{matrix}\text{Ratio of limiting reagent} \\ \text{and Product}\end{matrix} \times \begin{matrix}\text{Molecular weight} \\ \text{of Product}\end{matrix}$$

The theoretical yield is the maximum amount, which can be obtained from a reaction. During a synthesis, the actual product obtained is normally less than this. This may be due to loss during synthesis or purification, or the reaction maybe incomplete and not converting all the reactants to product (Kotz *et al.*, 2009).

2.2.5.9 Second batch production of dibromocholesterol

Having synthesised and characterised the initial batch of dibromocholesterol a second batch was required within the project. In this instance, reproducibility of production was assessed alongside stability testing of the first batch. For comparative characterisation purposes TLC, DSC, ^{13}C NMR, ^{1}H NMR and TGA was carried out on both powders.

2.2.5.10 Stability assessment of dibromocholesterol and thermogravimetric analysis (TGA)

Subsequent to the synthesis and validation of the dibromocholesterol, it was important to assess its stability to ensure that it remained stable over the duration of the study. Thus after a six month period similar techniques were carried out including TLC, DSC, ^{13}C NMR, ^{1}H NMR and in addition thermogravimetric analysis. Thermogravimetric analysis (TGA) is an analytical method, which is used to provide an assessment of structural decomposition, oxidation and moisture gain or loss amongst other aspects. It measures the change in mass of the material as a function of temperature (Mansfield *et al.*, 2010). This was carried out by placing a small amount of the sample onto the TGA instrument using a Perkin Elmer Pyris 1 TGA. The samples were analysed between the temperature ranges of 50 °C and 140 °C at a scan rate of 10 °C/min. The values reported are the percentage loss in weight of the product.

2.2.6 Improving the sedimentation of liposomes

Sedimentation is defined as the migration of molecules or colloids in a centrifugal or gravitational field (Vesaratchanon *et al.*, 2007). Having carried out the chemical synthesis to modify the cholesterol it was necessary to assess the impact that the modification had on the ability of the liposomes to settle; it is interesting to note that in fact sedimentation of the liposome formulations is generally thought to be the indicator for an unstable system (Gregoriadis and Senior, 1984). However, the sedimentation requirements can vary, thus some uses will require a slower rate of sedimentation to aid stability; however, for the purpose of this study it was desirable to have an enhanced velocity of sedimentation.

2.2.6.1 Quantification of sedimentation

In order to assess liposomal sedimentation five different liposome formulations were examined (Table 2.2). All formulations were encapsulated with BSA-FITC to mimic the entrapment of cytokine, with the unentrapped material removed by centrifugation at 29, 771 *g* for 30 minutes at 4 °C.

Table 2.2 Formulations used for qualitative and quantitative sedimentation studies.

Lipid Composition	Morphology	Ratio of Lipids (μMol)
DPPC:Chol	MLV	16:16
DPPC:Chol	SUV	16:16
DPPC:Chol:Brchol	MLV	16:8:8
DPPC:Chol:Brchol	MLV	16:4:12
DPPC:Brchol	MLV	16:16

Percentage transmission was measured using a Jenway spectrophotometer; PBS was placed in a clear 5 mL cuvette and used as the reference blank. A wavelength of 592 nm was chosen from the visible electromagnetic spectrum to read the samples. Samples were measured at the start of the study and at 1, 2, 4, 12, and 18 hours.

2.2.6.2 Qualitative assessment of sedimentation

To substantiate the above quantitative data, a qualitative assessment was also carried out by placing the appropriate sample formulations in a 5 mL bijoux tube and taking photographs at 0, 1, 2, 4,12 and 18 hours. The camera used was a Kodak Easyshare Z86121S 8.1 Mega Pixels.

2.2.6.3 Viscosity Measurements

A comparison of viscosity was carried out to compare formulations of DPPC:Chol 16:16 μMol with the brominated cholesterol formulation chosen for continuing studies i.e. DPPC:Chol:BrChol 16:4:12 μMol. The viscosity measurements were carried out on an Anton Paar AMVn automated micro viscometer using a 1.6 mm bore tube with a ball diameter of 1.5 mm at 20.0 °C. In this viscometer a steel ball rolls down a glass tube containing the test liquid. The rate at which this ball falls of a known density and diameter is the inverse function of viscosity. In this way sedimentation can be related to Stokes Law (Equation 2.2.);

Equation 2.2 Stokes Law

$$V = \frac{d_{st}(p_s - p_o)\,g}{18\eta_o}$$

V = Rate of settling

d_{st} = The mean diameter of particles based on velocity of sedimentation

p_s = The density of the particles

p_o = The density of the dispersion medium

g = The acceleration due to gravity CONSTANT

η_o = The viscosity of the medium

2.2.7 Fluorescence assay optimisation.

It was essential to have a basic screening assay that would provide quantifiable release data for the formulations. Fluorescent assays are well established and have many advantages over other techniques including sensitivity, specificity, accuracy and flexibility (Vogel, 2008). The marker which was selected for entrapment for the initial studies was BSA-FITC; the advantage being that it was a protein with a conjugated fluorescent marker which allowed ease of detection. A similar assay format was used to measure temperature controlled contents release from liposomes containing Pluronic F127 (Chandaroy *et al.*, 2001). In this work, the excitation wavelength used was 492 nm and the emission wavelength was 519 nm.

2.2.7.1 Optimisation of wavelengths; spectral scans

An important aspect of validation was choosing the correct wavelengths for the fluorophore, as this needs to be optimised for the fluorometer used (Lakowicz, 2006). The best results are obtained when the excitation and emission wavelengths used for reading are not the same as the wavelengths of the excitation and emission of the fluorophore (Figure 2.4). If they are separated, a smaller

amount of excitation light will pass through the emission monochromator and onto the PMT (photomultiplier tube) resulting in a purer emission signal and more accurate data.

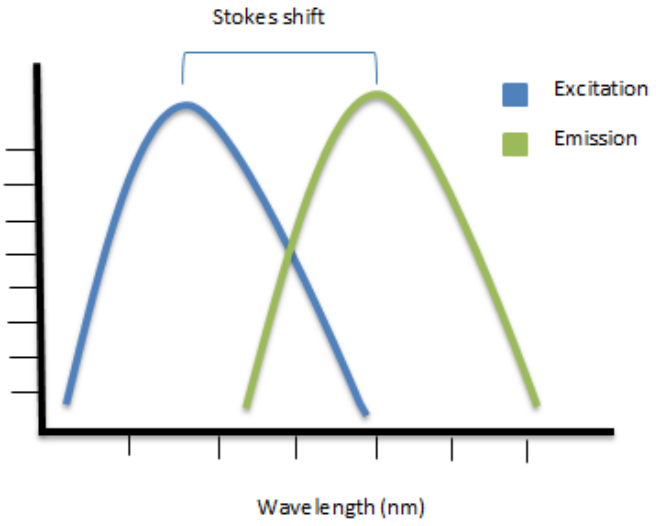


Figure 2.4 Excitation and emission peaks with Stokes shift.

Spectral scans were carried out at literature wavelengths, initially at 492 nm for excitation and 519 nm for the emission wavelength. However, to determine more precise wavelengths further spectral scans were carried out for excitation and emission, from 450 nm to 500 nm in increments of 10 nm. The assay was read on a microplate reader Spectramax Geminix (Molecular Devices Corporation). The plates used for all the fluorescence work were black non-binding 96 well plates. The excitation and emission wavelengths were optimised using spectral scans to obtain a purer emission signal.

2.2.7.2 Optimisation of the fluorescent assay with BSA-FITC

In establishing a fluorescence based release assay for the evaluation of the amount encapsulated within liposome formulations and their release characteristics it is essential to consider the validation of the assay. Validation characteristics, which should normally be taken into consideration, include accuracy, precision, repeatability, specificity, detection limit, quantification limit and range (ICH, 2006). These were considered a part of the protocol optimisation. Three calibration curves were prepared for BSA-FITC from a stock solution of 20 µg/mL. These were

assessed for linearity, accuracy and reproducibility using the calculation of linear regression and by the method of least squares as recommended by ICH guidelines (ICH, 2005).

2.2.7.3 Confirmation of speed, time, and frequency of centrifugation

In the establishment of the above method (2.2.6.2), the centrifugation procedure needs to be sufficient in terms of speed, time and frequency to ensure effective removal of unencapsulated BSA-FITC. Thus, the initial supernatant was measured for fluorescence and then the pellet was re-suspended and centrifuged again for 30 minutes. This was carried out for a further two steps to establish if all BSA-FITC was removed.

2.2.7.4 Effects of lysis chemicals on the BSA-FITC concentration curve

As the chemical used for lysis will still be present when the fluorescent marker would be analysed in the assay it was essential to assess how such components affect the final result as this impacts on the specificity of the assay. Three set of calibrators were made of BSA-FITC with concentration range up to 20 µg/mL in PBS. One set of calibrators were made from BSA-FITC 20 µg/mL, using 25% v/v of a 10% v/v Triton X solution, at each concentration the final volume was made up with PBS. The second set of calibrator dilutions were made using BSA-FITC 20 µg/mL with 25 % of isopropanol 1:1, the final volume is made up with PBS. Then 200 µL of each concentration was pipetted into the wells in triplicate. Thus, there were three calibration curves on a plate i.e. normal, Triton X and isopropanol. This was repeated in triplicate.

2.2.7.5 Determination of BSA-FITC release from liposomes

Having established and validated the various parameters for the release assay the method for measuring the amount of material entrapped within the pellet and total recovery was also carried out which is illustrated in Figure 2.5.

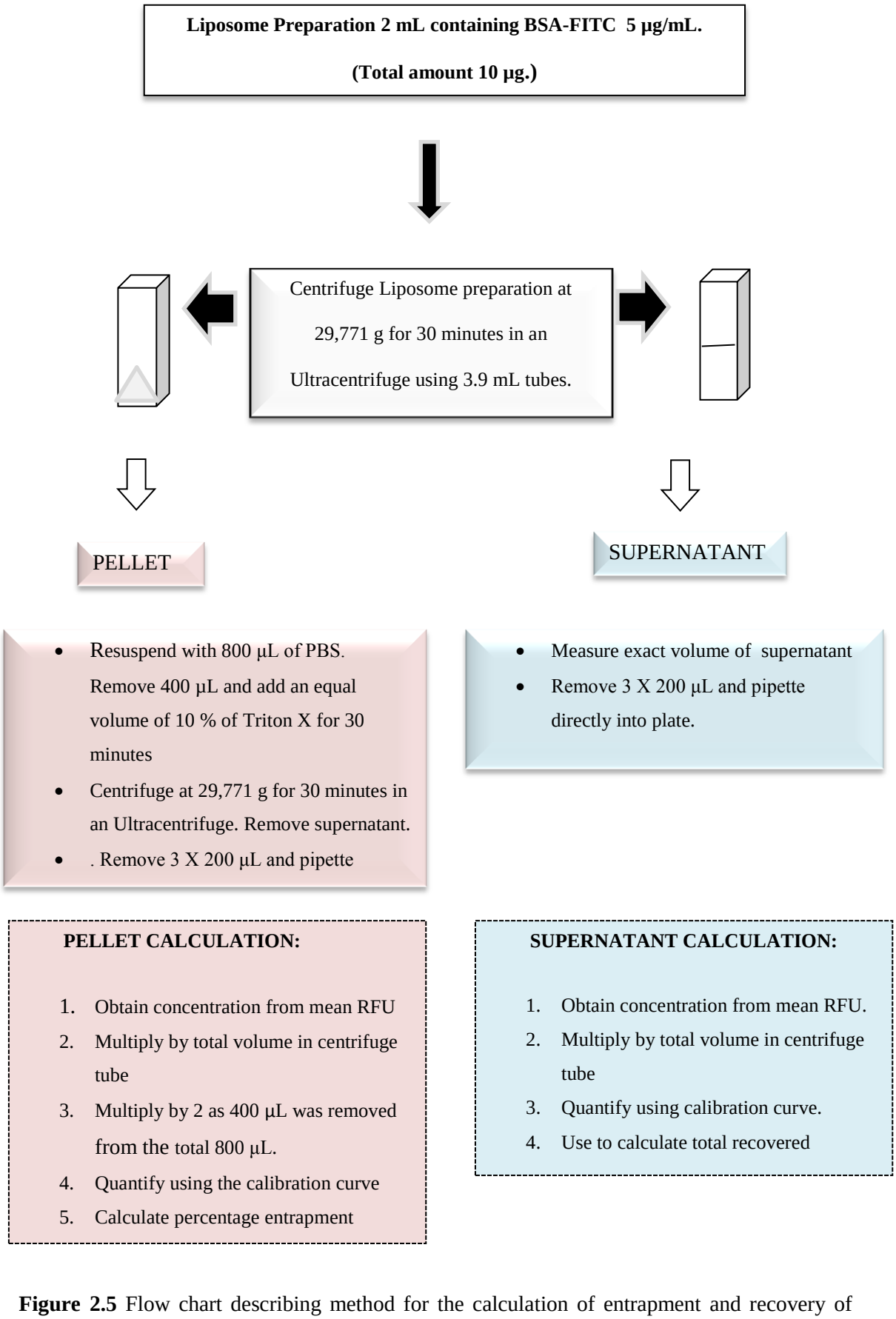


Figure 2.5 Flow chart describing method for the calculation of entrapment and recovery of BSA-FITC 5 µg/mL from the liposome formulations.

2.2.7.6 Limits of detection and quantification

According to ICH guidelines detection and quantitation limits of the background can be based on analysing an appropriate number of blank samples and calculating the standard deviation of these responses (ICH, 2006). The limit of the detection (LoD) is the lowest concentration which can be distinguished from replicates of a blank sample when they are tested without the presence of any analyte. The limit of quantification (LoQ) is used for the lowest level of analyte which can be precisely defined (Armbruster and Pry, 2008). The LoQ can be equal to the LoD but not lower; the limit of the detection was calculated from triplicate samples from three separate assays. The limit of quantification was defined as the lowest concentration with a coefficient of variation of < 20 % (Armbruster and Pry, 2008).

The following equations will be used to assess entrapment efficiency (Equation 2.3 and 2.4):

Equation 2.3 Calculation for percentage entrapped in liposomes.

$$\% \text{ Entrapped in liposomes} = \frac{\text{Amount in Pellet}}{\text{Amount added}} \times 100$$

Equation 2.4 Calculation for total recovery.

$$\% \text{ Total recovered} = \frac{\text{Amount in Pellet + Amount in Supernatant}}{\text{Amount added}} \times 100$$

2.2.8 Stability study of liposome formulations for reference standards

For the production of a liposome reference standard stability is an important aspect that needs to be considered, it is well established that liposomes are susceptible to a range of physical and chemical instabilities such as hydrolysis of lipids and oxidation (Mohammed *et al.,* 2006). Three batches of liposomes each for, 1) DPPC 32 μMol and 2) DPPC:Chol 16:16 μMol, 3)DPPC:Chol:BrChol 16:4:12 μMol were prepared by the dry film method. These formulations were characterised for particle size using the Sympatec and for zeta potential using the Malvern Nanosizer. One mL was

taken from each formulation and then placed at 4 °C, 25 °C and 40 °C stability cabinets. These were then characterised for particle size and zeta potential after formulation and then at specified time intervals; Day 1, 4, 7, 14 and 28. Microscope images were also taken of formulations to confirm stability using a Zeiss Axioscope A1 using a 40 X objective.

2.2.9 Size separation of liposomes

Centrifugation is arguably one of the simplest techniques for size separation and has been shown to be effective for nanoparticle separation of heterogenous batches (Gaumet *et al.,* 2007). In order to achieve separation using this technique two parameters were examined; speed and time of centrifugation. Low speeds and two centrifugation times were examined (five and ten minutes).

Density gradients have previously been used by Goormaghtigh and Scarborough *et al.,* (1986), for the removal of larger particles to obtain several size populations of small unilamellar vesicles. More recently liposome populations have been characterised using sucrose density gradients (Sanchez-Lopez *et al.,* 2009). By modifying sucrose concentrations, the viscosity of each solution is modified; thus the centrifugation process can be refined as the liposomes should distribute in different concentration bands of the sucrose (Goormaghtigh and Scarborough, 1986). Ultimately, when using this technique it is heavier particles that need to be retained. Where there were distinct bands both layers were removed and (subsequent to one wash to remove the sucrose) measured for particle size.

Filtration is a basic laboratory technique used for separation; and similar to centrifugation it is a ubiquitously used technique due to its ease of use and could also be applied to polydisperse systems to isolate specific size populations (Gaumet *et al.,* 2007). The aim was to find techniques, which were able to remove the smallest vesicles yet, retain the larger ones thus different filtration techniques were assessed. A biosieve was tested initially as this can be used for the separation of cellular debris and cells and thus was considered applicable for liposome vesicles. A basic buchner flask was set up and a weak vacuum was used. Other types of filters assessed were polycarbonate filters of 2 μm and 3 μm pore size. The formulations were assessed as previously by measuring

particle size of recovered liposomes from above the filter and the filtrate (formulation obtained once it has gone through the filter).

2.2.9.1 Centrifugation for the size separation of liposomes

Liposome formulations were centrifuged at 51 g, 115 g and 205 g (subsequent to centrifugation at 29,771 g for 30 mins to remove unencapsulated BSA-FITC). The pellet was re-suspended in 12 mL of PBS and 4 mL was subsequently placed into three tubes and centrifuged at the various speeds for five minutes the process was then repeated for ten minutes. This was carried out for three separate formulations, which were characterised for particle size and zeta potential. Two types of centrifuge were used in this study, the ultra-centrifuge and the TLN-100 SN 32 rota or the standard universal centrifuge H232.

2.2.9.2 Centrifugation with density gradients

Liposomes were prepared as described previously. 10 mL of density gradients were prepared of various strengths (w/v) using sucrose in distilled water. The following variables were examined;

10 %, 20 %, 40 %, 60 %, and 80 % sucrose

20 % and 80 % sucrose

5 %, 15 %, 25 %, and 35 % sucrose

} 2660 g for 30 min.

Initially 2 mL each of a wide range of density gradients were used for a preliminary screen, layered on top of each other and 2 mL of liposome formulation. To also consider a simpler option, as only the smaller particles were not required, a heavy and a low density gradient were chosen. Then as it became clear that separation was occurring around the lower bands, a wider range was chosen across the lower concentrations. The lower range was then repeated but at a greater speed of 354,000 g and a longer time point of 3 hours.

2.2.9.3 Filtration

Filtration was considered for size separation because of the relative ease of this technique. Ordinarily filtration is used to remove unwanted larger particles to retain the filtrate. In this instance, the liposomes of a smaller size would theoretically pass through the filters. Sample formulations of: DPPC:Chol:BrChol 16:4:12 µMol were prepared as described previously and re-suspended after centrifugation to remove unencapsulated BSA-FITC (29, 771 g for 30 minutes) and the pellet was re-suspended in 12 mL of PBS. A Buchner flask was set up with a weak vacuum (Figure 2.6) with a funnel (5 mm width). The filters were then placed inside, and the sample poured through.

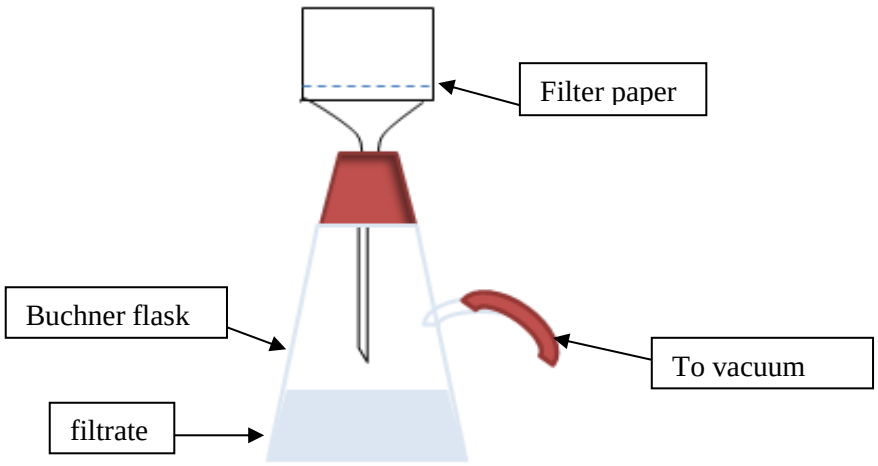


Figure 2.6 General set up for the assessment of filtration techniques.

The filtrate was collected and the filter was washed in 10 mL of PBS and then centrifuged at 2660 g for 30 minutes at 4 °C and then re-suspended in 1 mL of PBS. Three different filters were assessed; 5 µm biosieve filters, a 1.2 µm and 3 µm polycarbonate filters.

2.2.10 IFNγ ELISA assay for the measurement of entrapment in liposomes

The ELISA kit was provided with a cytokine standard which was used to construct the calibration curve. The standard is reconstituted to 1 µg/mL with the provided buffer, mixed thoroughly for five

minutes and aliquoted to be stored at -20 °C. A serial dilution was prepared 30 minutes prior to the start of the experiment with the stock standard and ELISA diluent to produce a calibration curve with a standard range of 3.16 – 1000 pg/mL. All sample dilutions were made with ELISA diluent. The following protocol was then carried out;

1. The strips were washed with 5x 300 µL/well of diluted wash buffer.
2. 100 µL/well of diluted cytokine standard and assay background control was added. Also the 'unknown samples' were added at this stage diluted in ELISA diluent at 1:1 ratio. The plate was covered with the adhesive cover and incubated at room temperature for 2 hours.
3. The wells were then washed with 5x 300 µL/well of diluted wash buffer.
4. The detection antibody was added which was also prepared in ELISA diluent to a final concentration of 1 µg/mL. The plate was then incubated at room temperature for a further 60 minutes.
5. The wells were then washed with 5 x 300 µL/well of diluted wash buffer. 100 µL/well of streptavidin-horseradish peroxidase conjugate was added which is diluted (1:1000) in the SA-HRP diluent.
6. Blank wells were left empty and the plate incubated at room temperature for 60 minutes. The wells were then washed with 5 x 300 µL/well of diluted wash buffer.
7. The wells were then developed with 3,3,5',5' Tetramethylbenzadine enzyme solution and incubated at room temperature in the dark for 15 minutes.
8. The colour development was then stopped using 100 µL/well of stop solution 1M H_2SO_4.

2.2.10.1 Reproducibility of the IFNγ assay

Three calibration curves of the kit associated calibrators were run in duplicate across three plate batches on three separate days. The standard deviation across the replicates was less than 1, therefore confirming the reproducibility of results across different plates and different days.

2.2.10.2 Preliminary titration for the estimation of cytokine concentration

In order to determine a suitable concentration to dilute the lyophilised rIFNγ (Sigma) cytokine; using the kit provided calibrators, a standard curve was run on the IFNγ ELISA assay (Mabtech). Three dilutions of the purchased cytokine at 1000, 500 and 250 pg/mL were assessed on the assay. To extrapolate unknown concentration, the four parameter logistic equations is used within Graph pad 4.0. These concentrations were diluted in a 1:1 ratio with ELISA diluents.

2.2.10.3 Titration optimisation for the estimation of cytokine concentration

In order to obtain an optical density which is near the optimal binding capability of the plate a repeat titration was carried out. The aim was to find the most suitable concentration of the recombinant IFNγ for use in the formulation of multilamellar vesicles to produce spots on the ELISPOT plate. The concentrations assessed were 5000 ng/mL, 2500 ng/mL, 1250 ng/mL, 625 ng/mL, 312.5 ng/mL, 156.25 ng/mL and 78.125 ng/mL subsequent to a 1:1 dilution in ELISA diluent.

2.2.10.4 Examining the effects of Triton X on the coating antibody

The coating antibody used for the ELISPOT plate was the same as the one used for the ELISA. Thus to prove that Triton X did not interfere with either the coating antibody or the rIFNγ to be used for encapsulation, the calibration curve was run alongside a calibration curve which included 50 % by volume of 10% v/v Triton X for each calibrator. This was run in triplicate.

2.2.10.5 Determining the effect of temperature on rIFNγ

As the formulation stages of producing liposomes involves the rehydration phase of the lipid film at a temperature above the T_m of the lipid, this will also result in exposing the cytokine to such higher temperatures. It was therefore important to ascertain what the effects of the temperature would be on the activity of the cytokine. The stock solution of $2.5x10^7$ U/mL diluted with ELISA diluent at a 1:1 ratio was placed at the various temperatures in a heat block for either 30 minutes or 1 hour time points. Once removed from the block they were allowed to cool to room temperature before adding to the ELISA plate with a 1:1 dilution with calibrator diluents.

2.2.10.7 Determining the effect of centrifugation on rIFNγ

1 mL of stock solution of IFNγ at $2.5x10^5$ U/mL was serially diluted to produce a calibration curve. The serial dilutions were divided into two portions. One set was centrifuged at 29,771 g for 30 minutes, then they were both ran on the IFNγ ELISA assay. This was repeated in triplicate to ascertain the effect of centrifugation on the cytokine.

2.2.10.8 Determining the effect of vortexing on rIFNγ

The stock solution of rIFNγ at $2.5x10^5$ U/mL was taken and aliquoted into 7 vials and then exposed to various times of vortexing to ascertain the effects on the cytokine. The time points assessed were 0, 0.5, 1, 2, 5, 10 and 15 minutes. The aliquoted solutions were tested for optical density on the IFNγ ELISA assay.

2.2.11 Calculation of entrapment efficiency of rIFNγ liposome formulations

For the purposes of quantifying the entrapped rIFNγ the liposomes were formulated as described in 2.2.9; however, additional steps were required to separate the pellet from the supernatant as both were assessed for the amount of rIFNγ present (Figure 2.7).

2.2.12 Formulation of rIFNγ liposomes as a positive and a negative control.

For the formulation of rIFNγ containing liposomes where the initial lipid concentrations were 16 μMol total these were rehydrated with 2 mL of 2.5 x 10^5 U/mL rIFNγ. After centrifugation (Table 2.1), the liposomes are re-suspended in 1 mL of RPMI. If 32 μMol total lipid amount was used then rehydration of the lipid film was with 4 mL of 2.5 x 10^5 U/mL rIFNγ. The liposome formulations were re-suspended in 3 mL of RPMI. The negative control consisted of liposomes rehydrated with PBS.

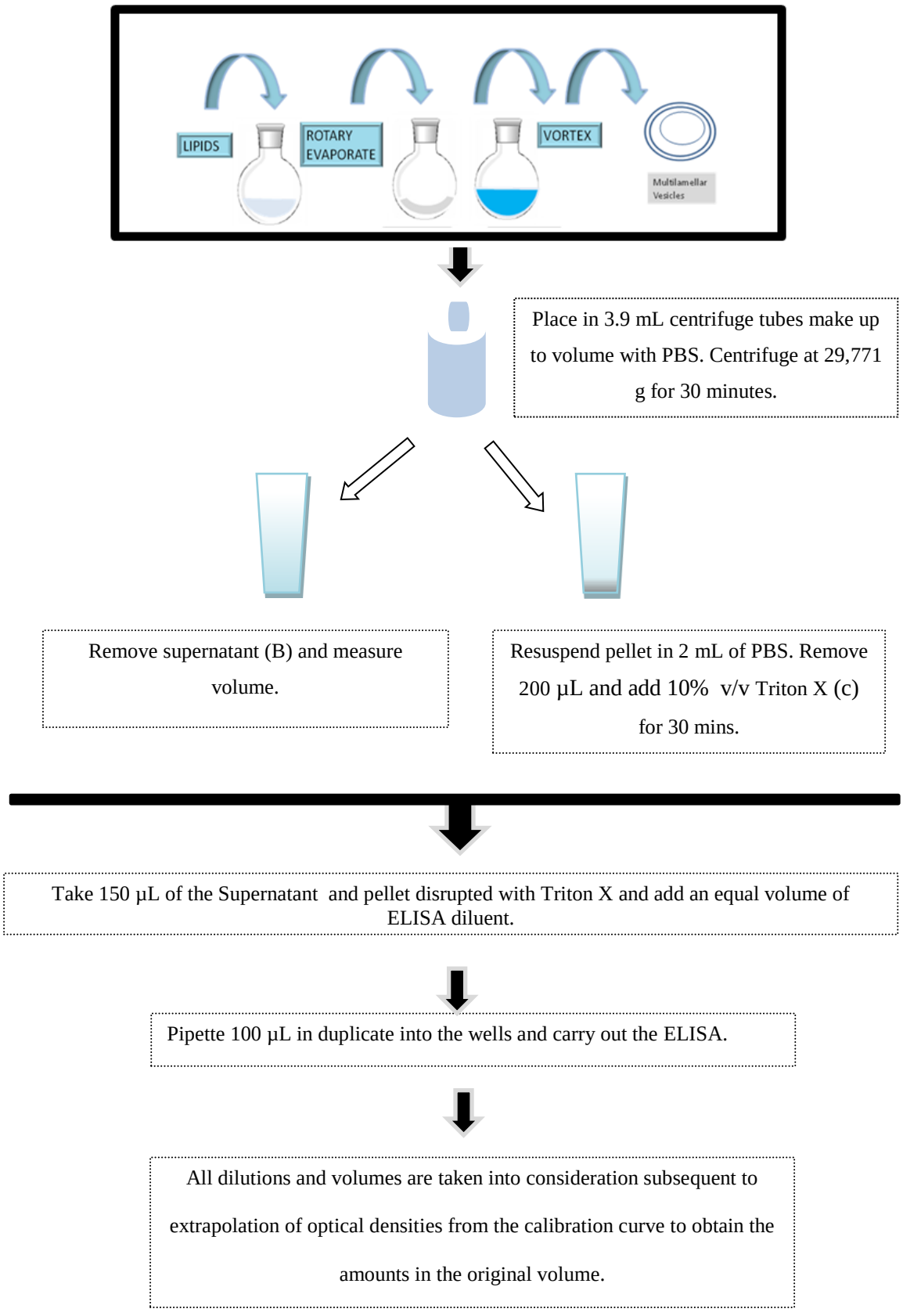


Figure 2.7 ELISPOT testing of liposome formulations; method for calculating entrapment efficiency.

2.2.13 ELISPOT protocol for the testing of liposome formulations

The ELISPOT kit contains plates, coating antibody, biotin for detection of the IFNγ and the secondary antibody streptavidin. The development reagent is BCIP/NBT-plus. The following protocol was followed as per the kit instructions:

1. The coating antibody was diluted to 15 µg/mL, in sterile PBS pH 7.4.

2. The ELISPOT plate was removed from the packaging and the membrane was pre-wet by adding 50 µL of 70 % ethanol for a maximum incubation time of 2 minutes.

3. The plate was washed 5 times with sterile water 200 µL/well.

4. 100 µL/well of coating antibody at 15 µg/mL was added and incubated overnight at 4 °C.

5. Excess antibody was removed by washing the plate in sterile water as above.

6. 200 µL of RPMI medium was then added to the wells containing 10 % FCS and incubated for 30 minutes at room temperature.

7. The plate was then washed with 5 times with sterile water 200 µL/well.

8. 100 µL/well of liposome formulations were pipetted into each well. The plate was then placed at 37 °C for 12 hours (minimum incubation time). Movement of the plate was avoided and wrapped in foil to avoid evaporation.

9. Addition of enzymes and Triton X; the enzymes were added at the same time as the liposome formulations. A volume of 100 µL/well was added per well. The Triton X was added upon removal of the plate subsequent to the liposome formulations being incubated overnight.

10. Then the plate was washed 5 times with sterile water 200 µL/well.

11. The detection antibody was added (100 µL/well) at a concentration of 1µg/mL in PBS containing 0.5 % FCS for 2 hours at room temperature.

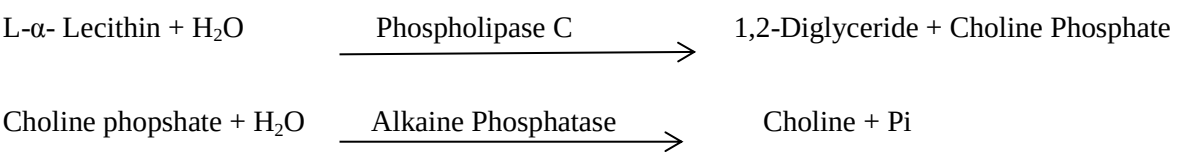
12. The plate was washed 5 times with sterile water 200 µL/well.

13. The substrate solution (BCIP/NBT-plus) was filtered through a 0.45 µm filter and 100 µL was added to each well until distinct spots emerged.

14. The plate was then washed extensively in tap water and left to dry.

2.2.14 Assessment of enzyme activity of PLC

Prior to carrying out work with the Phospholipase enzymes it was necessary to establish that the enzyme activity was acceptable, thus a basic Sigma quality control procedure was followed. The principle of the assay is based on the following:



2.2.14.1 A quality control PLC assay for confirmation of enzymatic activity

Briefly seven vials were labelled: test, test blank, standards 1-4 and a standard blank. 2 mL was added of 50 mM Tris Maleate Buffer pH 7.3 and 0.5 mL of 50 mM Calcium Chloride solution to each vial. Subsequently 1.5 mL was added of 2.0% w/v *L – α*- phosphatidylcholine to all vials. Then 0.9 mL of the enzyme dilution solution (1.0% w/v) bovine serum albumin (BSA) prepared with 50 mM tris maleate buffer) was added to the test and the test blank. To prepare standards 1- 4: 0.25 mL, 0.50 mL, 0.75 mL and 1.0 mL were added of the prepared phosphorus standard stock solution (20 µg/mL, 0.645 µ/mole) to each vial with 0.75 mL, 0.50 mL and 0.25 mL of deionised water added to standards 1-3 consecutively. 1.0 mL of deionised water was added to the standard blank.

The vials were then mixed by swirling and allowed to equilibrate at 37 °C and 0.1 mL was added of prepared PLC solution containing 0.1 – 1.0 U/mL in cold 1.0% w/v BSA (using 50mM Tris

maleate buffer pH 7.3 at 37 °C). All vials were incubated at 37 °C to allow the PLC solution to hydrolyse the lecithin for an incubation time of 15 minutes. Then 0.9 mL of a 270 mM ethylenediaminetetraacetic acid (EDTA) solution (pH 7.3) was added to all vials to stop the reaction of the PLC. In order to quantify the choline phosphate 0.1 mL of alkaline phosphate was added to all vials and 0.1 mL of PLC to the 'Test' blank. The vials were mixed by swirling before incubated at 37° C for 120 minutes. During the incubation time and 30 minutes before use, the AMES colour reagent was prepared by adding 10 mL of 10% w/v ascorbic acid Solution and 6 mL molybdic acid solution and 54 mL of deionised water. Vials were mixed by swirling and stored in the dark at room temperature.

From the vials 1 mL was transferred from the 'test', 'test blank', standards 1 - 4 and 'standard blank' into fresh vials 0.5 mL is added of 20% w/v sodium lauryl sulfate solution (SDS) and 3.0 mL of the AMES colour reagent. All vials were mixed and then incubated at 37 °C for 60 minutes. These were then transferred to cuvettes and the absorbance was read on the spectrophotometer at 660 nm. Background was taken into account; all the standard blank readings were subtracted from all the readings of the standards and the test blank from the test readings. A standard curve was then plotted for absorbance at 660 nm versus micromoles of phosphorus in 5 mL. The activity of the enzyme was confirmed with that stated on the purchased vial of PLC. One unit will liberate 1 micromole of water soluble organic phosphorus from α- phosphatidylcholine at pH 7.3 at 37 °C, this was carried out using the below equation (Equation 2.5) once the micromoles of phosphorus is extrapolated from the curve.

Equation 2.5 Calculation for the activity of the enzyme.

$$\text{Units/mL enzyme} = \frac{(\text{Micromoles of phosphorus})\ (\text{df})}{(15)\ (0.1)}$$

2.2.14.2 Microscopy assessment of enzyme activity

For confirmation of enzyme activity using microscopy two liposome formulations of PC and PC:Chol were made from 16 μMol and 8:8 μMol without IFNγ as the size of the cytokine is too

small to be visualised by means of light microscopy. 100 µL of liposome formulation was added to 100 µL of the following enzymes at the specified concentrations (Table 2.2.):

Table 2.3 PC:Chol liposome formulations tested with various enzyme concentrations with phospholipase A_2 and phospholipase C using bright field microscopy.

Microslide number	Liposome formulation	PLA_2 added U/mL	Microslide number	Liposome formulation	PLC added U/mL
1.	PC	0	**1.**	PC	0
2.	PC	1	**2.**	PC	1
3.	PC	5	**3.**	PC	5
4.	PC	10	**4.**	PC	10
5.	PC:Chol	0	**5.**	PC:Chol	0
6.	PC:Chol	1	**6.**	PC:Chol	1
7.	PC:Chol	5	**7.**	PC:Chol	5
8.	PC:Chol	10	**8.**	PC:Chol	10

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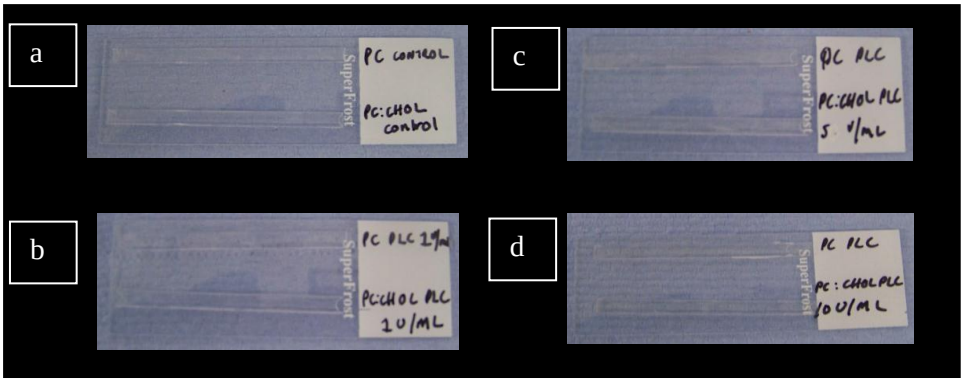

Figure 2.8 Enzymatic degradation (using PLC and PLA_2) of liposome formulations in microslides of **(a)** Control formulations of PC 16 µMol and PC:Chol 8:8 µMol and **(b)** 1 U/mL,**(c)** 5 U/mL and **(d)** 10 U/mL of PLC.

Axioscope A1 light microscope. The microscopy pictures were taken at the start , after 1 hour and after an overnight incubation at 37 °C. The mixtures were prepared at room temperature and immediately placed into the microslides as shown (Figure 2.8).

2.2.15 Evaluation of the pre-wetting stage of the ELISPOT assay

Evaluation of the pre-wetting stage was carried out at the ethanol incubation time of one minute prior to antibody coating. As a control there were wells with no ethanol and then 15 µL, 25 µL and 50 µL of 70% v/v alcohol for PC:Chol formulations. Subsequent to testing various amounts of

ethanol for the pre-wetting stage, for further confirmation the effects of adding 25 µL and 50 µL was assessed across liposome formulations of various compositions.

2.2.16 Trigger release assessment; enzymatic and detergent triggered release

A microscopy study was carried out, to confirm the optimal buffer and concentration for enzymatic release within the ELISPOT assay using PC:Chol formulations. The enzymatic concentrations were assessed in two two buffers 1) 10 mM Tris, 150 mM NaCl and 10 mM calcium chloride and 2) 10 mM PBS. Subsequent to the selection of the Tris based buffer increased concentrations of the enzyme were assessed for both PLC (1 U/mL, 4 U/mL and 16 U/mL) and PLA_2 (1 U/mL, 5 U/mL and 10 U/mL) respectively. 100 µL of the enzyme dilutions were added to each well. For detergent triggered release, Triton X 10% v/v was pipetted into the wells at the end of the overnight incubation step. It was left for 15 minutes prior to washing at room temperature.

2.2.17 Manual counting of spots formed on the ELISPOT plates

The spots were counted manually by taking an enlarged image of the well using a stereomicroscope (Figure 2.9).

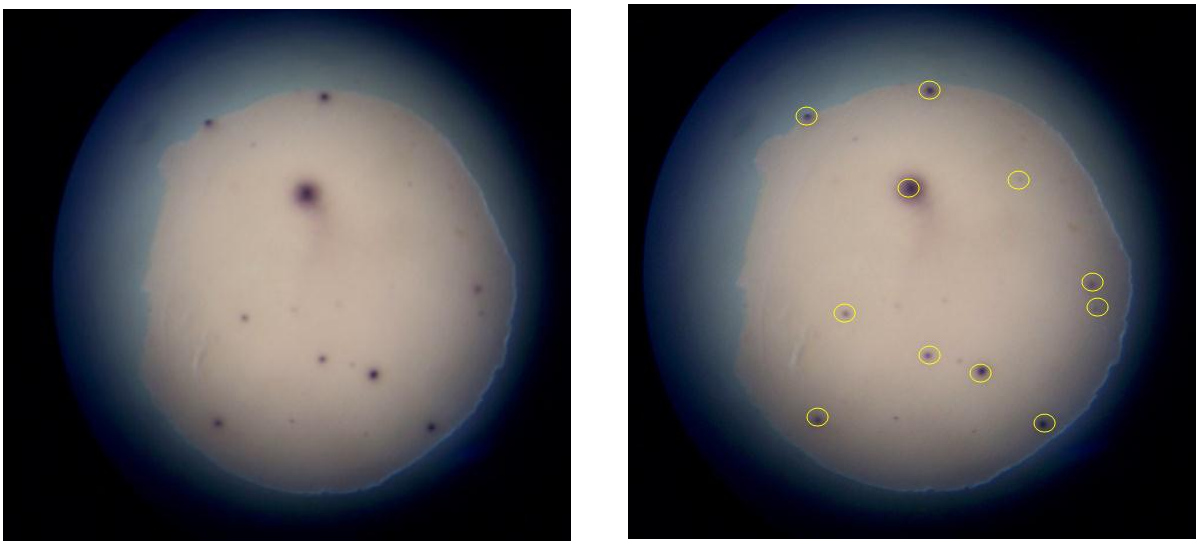

Figure 2.9 Image captured of an ELISPOT well and highlighted spots to illustrate counting procedure.
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An ELISPOT puncher was used to remove individual wells from the plate, each well was pushed out on to a foam pad and then placed onto a glass slide and secured in place by placing a coverslip on top. This was due to the difficulty of visualisation from the plate. A stereomicroscope was used for the magnification of the wells and a camera was used to take a picture through the eyepiece. The spots were then counted from uploaded images (Figure 2.9) as these could be enlarged for clarity. Each formulation was run in triplicate.

2.2.18 Automated Counting

Plates for automated counting were sent to NIBSC after colour development. These were read using the Jenner (University of Oxford settings).

2.2.19 Dynamic viscosity measurements

Dynamic viscosity measurements were carried out to compare the viscosity of the different liposome formulation in PBS, RPMI and RPMI with 10 % FCS. These were carried out using an Anton Par AMVn automated micro viscometer. Approximately 1 mL of liposome formulation was filled into the glass capillary tube and the measurements recorded at an angle tilt of 50° and -50° and carried out at 20 °C.

2.2.20 Establishing a calibration curve in the ELISPOT assay

In order to test the feasibility of a calibration curve the resuspending volume was changed from 2 mL to 1 mL. From this stock, a serial dilution was made in RPMI at a 1:1 ratio (Figure 2.10). There were 6 calibrators or dilutions in total. ‘Empty’ liposome formulations can serve as the negative control.

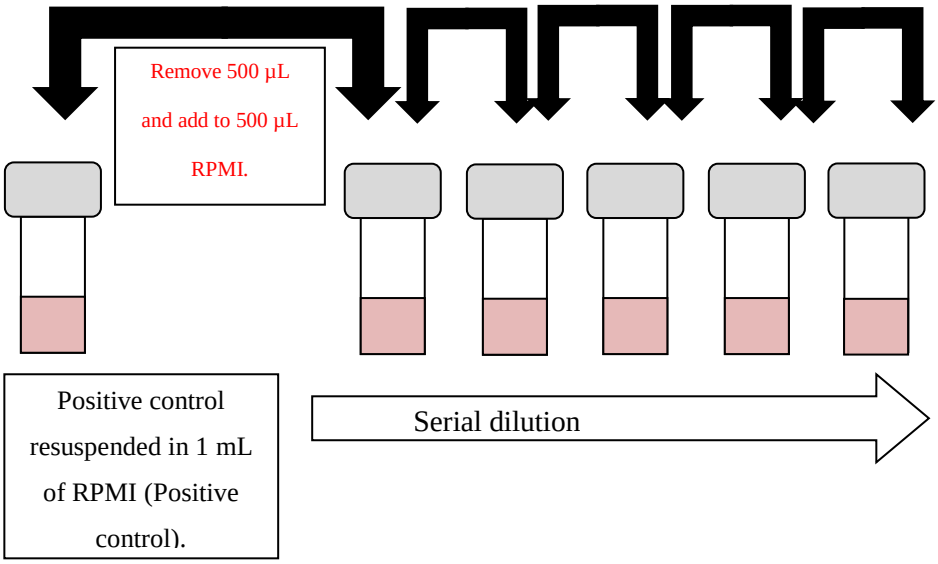


Figure 2.10 Serial dilution for the positive control in 1:1 RPMI.

2.2.21 Freeze dried liposomes with rIFNγ

The formulations were frozen at -70 °C for at least 30 minutes. The freeze drier was pre-cooled to -50 °C which takes 50 minutes. Primary drying was initiated at -40 °C (50mTorr) for 36 hours and then secondary drying at 20 °C (50mTorr) for 6 hours. The freeze drier used was the Virtis Advantage Bio- Pharma process Systems UK.

After the formulation of liposomes they were re-suspended in 500 µL of buffer and then 500 µL of cryo-protectant solution to make a final amount equivalent to the amount required prior to freeze drying. The lyoprotectants assessed were the disaccharides trehalose and sucrose at concentrations ranging from 50 mM to 200 mM for PC formulations and at 50 mM and 100 mM for PC:Chol formulations. Upon completion of the freeze-drying cycle, the liposome formulations were re-suspended with 1 mL of RPMI 1640 medium.

2.2.22 Freeze- thaw experiment of liposomes with rIFNγ

Liposomes formulations of PC 16 µMol and PC:Chol 8:8 µMol were made and then re-suspended in 1 mL of 100 mM trehalose cryoprotectants. These were split into two 500 µL volumes with one

vial for each placed at -70 °C for 1 hour. After the formulation had thawed completely both were placed onto the assay in triplicate and incubated at 37 °C.

2.2.23 Preparation of liposomes and characterisation for scale up

The small 4 mL batch was prepared as described previously using a 50 mL round bottom flask, for the larger batch it was necessary to use a 500 mL flask (Figure 2.9). An assessment of the entrapped BSA-FITC was carried out using the fluorescence assay as described previously (2.2.7). The calibration curve constructed from the serial dilution used for quantification is shown in figure 2.10. Size characterisation was carried out using the Sympatec Particle sizer. Ordinarily 100 µL of liposomes formulation is pipetted into a glass quartz cuvette (A signal of at least 15 % is required for an adequate reading). Three sample readings were taken for each formulation and an average result was obtained for size and polydispersity.

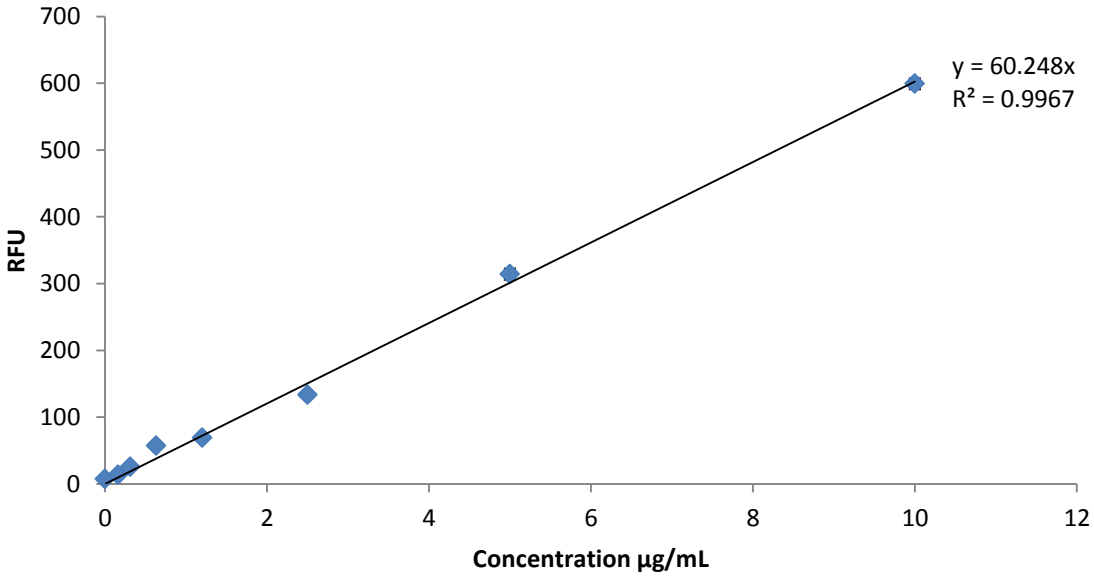


Figure 2.11 Calibration graph for BSA-FITC 5 µg/mL from the stock solution for the calculation for the amount of protein entrapped.

The time on the rotary evaporator was increased from 20 minutes to 45 minutes to ensure the evaporation of solvents for the larger batch. The flask was purged with nitrogen for four minutes, to remove any residual solvent. 1 mL of the small and large batch was removed for the assessment of

recovery and entrapment efficiency. The rest of the large batch was divided into triplicate aliquots to allow the assessment of the effect of freeze-drying with three different lyoprotectant concentrations namely 50 mM, 100 mM and 200 mM of trehalose in H_2O, 20 mM Tris buffer, PBS and three samples were left without any cryoprotectant. Subsequently the size of both the liposome formulations was measured and for the larger batch size characterisation was repeated again once all the freeze-dried vials were re-suspended. An assessment of the entrapped BSA-FITC was carried out using the fluorescence assay as described previously (2.2.7). The calibration curve constructed from the serial dilution used for quantification is shown in figure 2.10. Size characterisation was carried out using the Sympatec Particle sizer. Ordinarily 100 µL of liposomes formulation is pipetted into a glass quartz cuvette (a signal of at least 15 % is required for an adequate reading). Three sample readings were taken for each formulation and an average result was obtained for size and polydispersity.

2.2.24 Statistical analysis

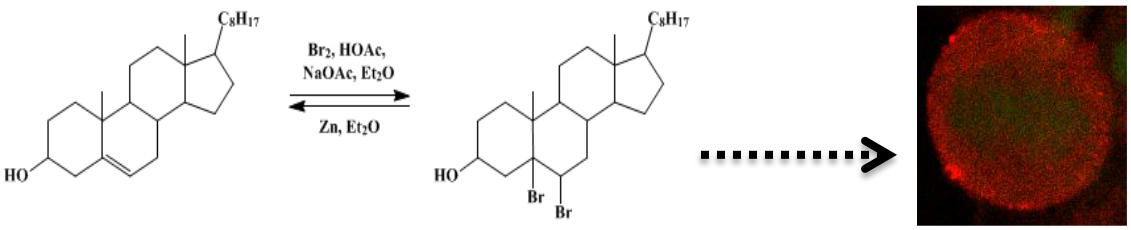
Where appropriate, the data has been examined for statistical significance using one way analysis of variance (ANOVA) and a multiple comparison post-test Tukey to compare all data sets. Graph pad software was used. Significance is set at $P<0.05$; for an extremely significant result $p<0.01$. ANOVA Two way analysis and the Bonferroni post-test has also been used where appropriate Significance is set at $P<0.05$ and for an extremely significant result $p<0.01$. In addition, the similarity factor F2 has been used to determine the similarity of the curves this is a logarithmic transformation of the sum-squared error of differences between the test T_2 and the reference values between 50 – 100 are acceptable according to the FDA regulations (Yuksel *et al.*, 2000).

Chapter 3

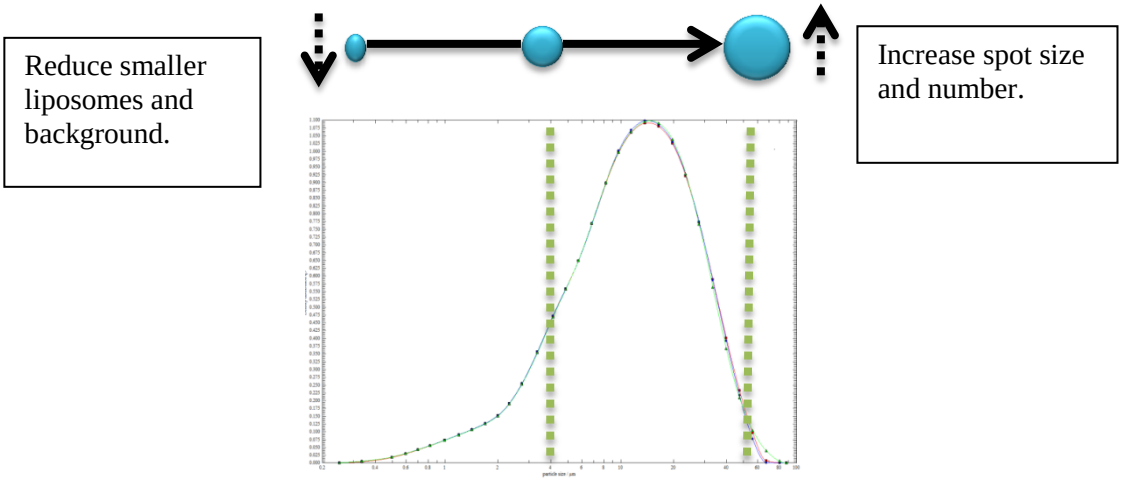
Design considerations for the formulation of liposome reference standards for the ELISPOT assay

Graphical abstract

a. Bromination of cholesterol to increase sedimentation rates;



b. Size selection of heterogenous liposome formulations;



3.1 Introduction

There are many advantages in using liposomes for the encapsulation of cytokines over other colloidal systems, with the most significant being their versatility thus, structural and physicochemical characteristics such as size, lipid composition, charge and fluidity of the phospholipid bilayers can be modified (Storm and Crommelin, 1998). Liposomes have been used for the entrapment of a variety of different molecules, ranging from small molecules and drugs through to therapeutic proteins however, their application within an ELISPOT assay was a novel concept (Gregoriadis and Ryman, 1971, Gregoriadis *et al.,* 1974). The novel application of liposomes within an immunoassay setting requires careful consideration of the design aspects for the formulations to produce an effective reference standard to replace stimulated cells normally used for positive controls.

3.1.1 Liposomes as artificial cells

The basic premise of this study was to produce liposomes that can mimic the actions of cells by the steady release of entrapped cytokine onto a pre-coated membrane. The surface area of the membrane for an ELISPOT well is approximately 0.3 cm^2. The T-cells placed into the well are spherical with a diameter of 10-15 µm and thus a cell count of 150,000 cells would constitute a monolayer (Weiss, 2012). The cells placed into the well have the ability to sediment and once in contact with the membrane they release cytokine to produce a spot. T-cells are lymphocytes and such cells have a minimal cytoplasm with small mitochondria and small amounts of polyribosomes, rough endoplasmic reticulum, and a small golgi apparatus (Turgeon, 2004). They have a predominant nucleus, which is approximately 7 µm (Henrikson and Mazurkiewicz, 1997). In attempting to produce an artificial vesicle that is able to mimic the actions of a lymphocyte the aforementioned physical attributes of the liposomes must also be taken into consideration such as the morphology, size of the vesicle and their ability to sediment in a manner similar to cells. Initially the discovery of liposomes was considered important as a potential model for the cell membrane and as an important tool for the study of membrane biophysics (Bangham *et al.,* 1965,

Gregoriadis, 1991). Although liposomes are structurally similar to cells in terms of their bilayer, they also have many notable differences, which may affect their behaviour as artificial reference standards when designed to mimic the action of cells. There have been many studies in which it has been necessary to describe cells as spherical colloidal particles however such descriptions over simplify the complex nature of the membrane (Dan, 2003). It is precisely such differences between cells and liposomes that are important pre-design considerations for this study; however, in their simplest form liposomes lack any other physical structures within the internal compartment and encapsulate only aqueous media. Furthermore, the liposomes formed are often variable in the size, lamerallity and size distribution (Sanchez-Lopez *et al.,* 2009).

3.1.2 Manipulating liposome formulations for the ELISPOT assay

The structural differences between liposomes and cells may affect the ability of the liposome vesicles to sediment to the bottom of the wells in a similar way to the cells as according to Stokes' law; the size of the vesicles and their density affects the sedimentation rate. The dominant initial interactions between cells and surfaces are electrostatic interactions, with ligand interactions becoming more significant when cells are within close enough proximity to surfaces to form such interactions (Marshall *et al.,* 1971 and Dan, 2003). Thus, to tackle such fundamental differences the method chosen to enhance sedimentation characteristics of the liposome vesicles was a basic modification to increase the molecular weight of either the phospholipid component or the sterol component. After consideration, the method chosen was the bromination of the cholesterol component of the liposome preparation as it is a relatively straightforward laboratory synthesis and achieved in a short period of time (Grant and Latimer, 2003). By modifying cholesterol the majority of the formulation remains the same, thus the aim was to retain the overall physicochemical characteristics of the liposomes other than the density of the vesicles.

Unlike T-cells, which are generally between 10-15 µm in size, when a liposome formulation is produced it is a heterogenous mixture of vesicle sizes (Sanchez-Lopez *et al.,* 2009). The mean diameter of a formulation could be for instance, 10 µm; however, within each preparation there will be liposomes both larger and smaller than the desired size range. Given that, the particle size is also

a controlling factor in sedimentation; a more homogeneous particle size range is desirable to reduce variation in sedimentation rates. Previous studies have shown the use of filtration with liposomes to produce a more homogenous population; using polycarbonate filters and extrusion methods multilamellar liposomes are forced through the filters reducing the size from several microns to less than a micron (Olson *et al.,* 1979, Vemuri and Rhodes, 1995). However to achieve homogeneity size has been reduced, other strategies which can be adopted to select the desired size and are routinely used with cells include centrifugation at different speeds and combining centrifugation with the use of sucrose density gradients (Alberts *et al.,* 2008, Weissig *et al.,* 1991). Such techniques can be adopted to select a given size range from a heterogenous liposome batch (Weissig *et al.,* 1991).

3.2 Aims and Objectives

In order to produce an artificial reference standard for the ELISPOT assay that can release cytokine in a way similar to T-cells, the main aim of this project was to design the formulation so that it closely mimicked the action of cells. The sedimentation velocity of the particles was an important aspect of the pre-formulation considerations. Therefore to enhance sedimentation rates, synthesis of brominated cholesterol and subsequent incorporation within the formulations was a means to increase mass of each individual vesicle. A second factor was to produce a vesicle suspension of large vesicles with a low particle size range. To achieve this it was necessary to investigate some basic methods for size separation, thus the objectives of the study were:

- To synthesise and validate brominated cholesterol.
- To incorporate brominated cholesterol, at various ratios, within liposome formulations and assessment of the effect on the rate of sedimentation.
- To measure and validate a fluorescence assay for the measurement of the entrapment efficiency of various liposome formulations with bovine serum albumin conjugated with fluorescein isothiocyanate (BSA-FITC).

- To investigate various basic size separation techniques to assess their effectiveness to produce a more homogeneous size population.
- To integrate the most effective techniques for size selection into a method for the production of liposome-entrapped cytokine formulations as artificial reference standards.

3.3 Results and Discussion

3.3.1 Synthesis and characterisation of dibromocholesterol

A basic laboratory method for the bromination reaction of cholesterol was identified (Kabara *et al.*, 1960, Fieser *et al.*, 1992).

Figure 3.1 The organic synthesis reaction for the formation of dibromocholesterol from cholesterol.

The reaction itself involves the breaking of a double bond and the addition of two bromine atoms in the specified positions (Figure 3.1). For the purposes of this study, two batches of dibromocholesterol were synthesised (batch 1 and 2 respectively). In order to validate the initial batch of dibromocholesterol the techniques used were; thin layer chromatography, melting point determination, and infrared spectroscopy. Due to the results obtained, further validation was necessary, including a more precise melting point determination using differential scanning calorimetry, ^{1}H-NMR and ^{13}C-NMR. For the second batch, similar characterisation techniques

(other than infrared spectroscopy were carried out due to the close comparability of the results to the first batch) and where possible this data was combined for both batches.

3.3.1.1 Thin Layer Chromatography (TLC)

The dibromocholesterol product was assessed using TLC; the solvent used after optimisation was ether:acetic acid 98:2. This solvent provided clear spots for the product which had a different migration rate to the well defined spot for cholesterol (Figure 3.2).

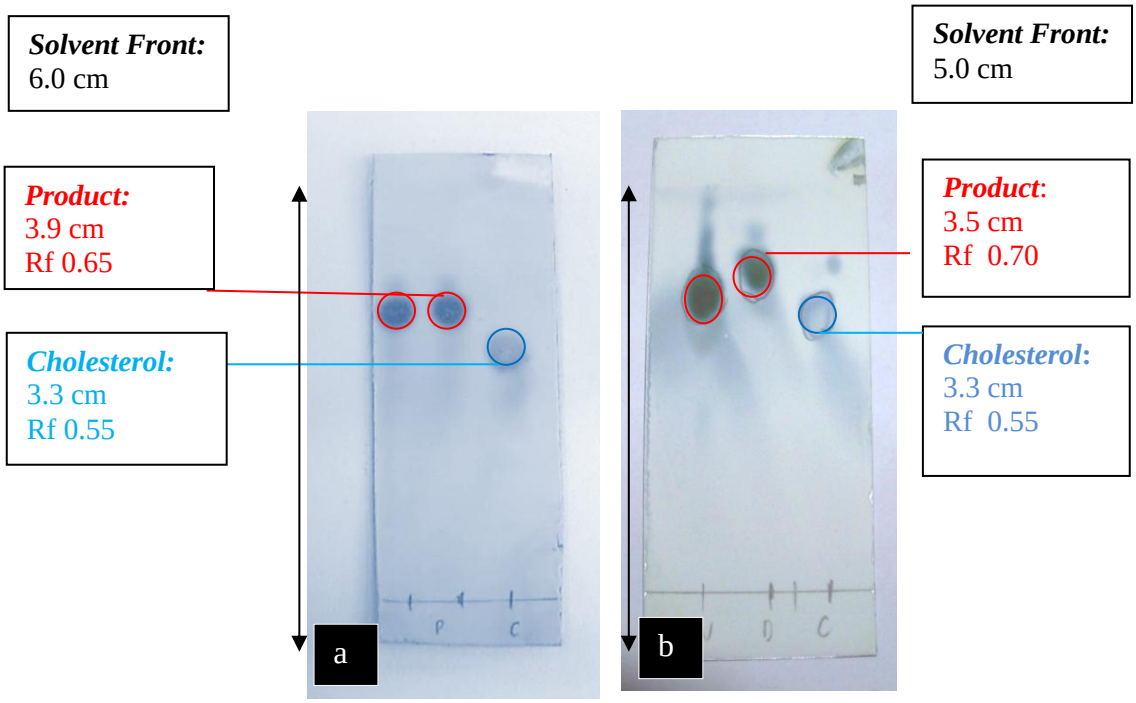


Figure 3.2 TLC plates for **(a)** dibromomocholesterol compared to **(b)** cholesterol.

There was also a good correlation between the Rf values for both of the batches which confirmed the reproducibility of the method for synthesis. The reference substance used was cholesterol and the values of 0.55 for the Rf values across the two chromatograms (Figure 3.2 a and Figure 3.2 b) indicated that the experimental parameters were consistent (Kar, 2005). The actual Rf values cannot be compared directly to other literature values as they are not definitively constant. They vary with many experimental factors such as temperature, humidity, the nature of the solvent and even the size of the container, the values indicate that a new compound has been formed for both

batches and there were no distinct spots above the product to indicate impurities (Krupadanam *et al.*, 2001; Sharma, 2005).

3.3.1.2 Melting point determination

Using the Gallenkamp melting point apparatus a small amount of pure cholesterol and the product were placed into a glass capillary tube and into the apparatus for assessment. For reference, pure cholesterol was used and this was in the range of the literature values expected of 149-150 ^{o}C (Table 3.1; Barton and Miller, 1950). However, the melting point of the product was found to be higher (119-121 ^{o}C; Table 3.1) than previously reported in the literature (112-114 ^{o}C; Barton and Miller, 1950).

Table 3.1 Visualised melting points of cholesterol and obtained product with the related literature values (Barton and Miller 1950).

Melting point determination is dependent upon several factors including the quantity of sample used, rate of heating and the purity and chemical characteristics of the substance, thus observed melting point values can differ from *true* values due to a lack of uniformity in the distribution of heat during the process of melting (Gilbert *et al.*, 2011). For further confirmation, the technique of differential scanning calorimetry (DSC) was used (Figure 3.3) for a more precise melting point determination (Palermo and Chiu, 1976). DSC can accurately determine the melting point and differentiate between different crystal forms (Storey *et al.*, 2011). In addition to the previously discussed TLC, DSC can further support chromatographic methods (Giron *et al.*, 1997).

The DSC thermogram showed the onset for the melting of cholesterol was 148.29 °C and the main peak at 149.38 °C (Figure 3.3); this was within the literature melting value range. The melting point for the dibromocholesterol using the DSC was not within the literature stated value of 112-

114 ° C (Barton and Miller, 1950): the onset of melting was 111.20 °C (Figure 3.3) and the main melt was at 117 °C. The scans show that the peak for cholesterol is sharp; however, the peak for the dibromocholesterol is broader with an additional secondary peak after the main melt. The reference peak of cholesterol was similar to that obtained in a previous study by Cui *et al.*, (2010) with a sharp melt observed at 149.8 °C. The broader peak and the presence of the secondary peak for dibromocholesterol suggested there may be an impurity (Palermo and Chiu, 1976). Although generally the nature of a DSC experiment results in some degree of broadening associated with phase changes which occur during melting (Storey and Ymen, 2011).

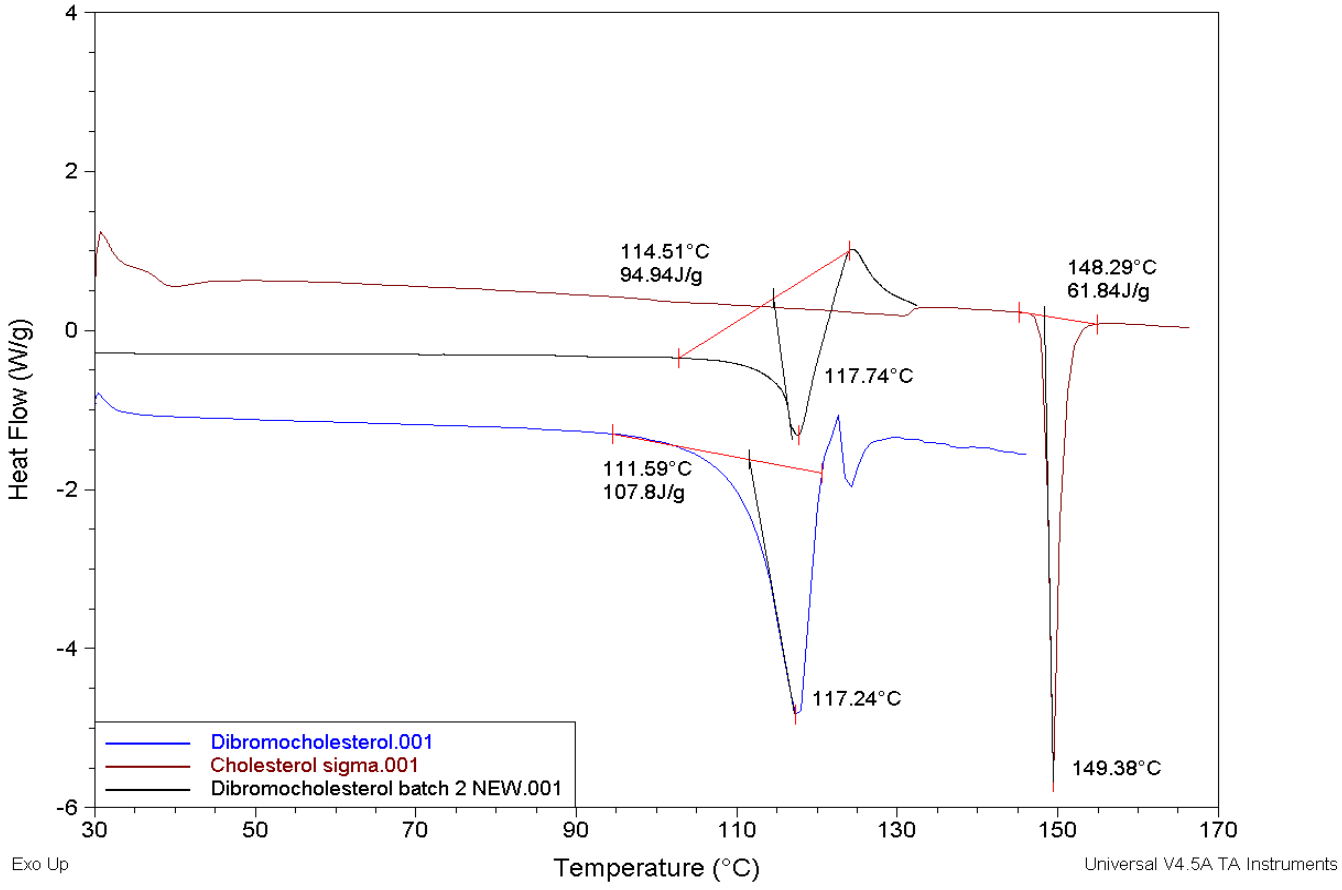


Figure 3.3 DSC thermograms for melting point determination of cholesterol and dibromocholesterol; **(a)** Cholesterol, red, top thermoscan, **(b)** dibromocholesterol batch 1, blue thermoscan, **(c)** dibromocholesterol batch 2, black thermoscan.

Any differences between the scan after the main melt could be due to impurities or residual components from the synthesis, as even small amounts of impurities can broaden the melting range (Palermo and Chiu, 1976). For assessment and determination of the presence of such impurities,

spectroscopic techniques are generally used, such as infrared spectroscopy to determine structural analysis through determination of functional groups (Ahuja, 2007).

3.3.1.3 Infrared spectroscopy

Cholesterol and the dibromocholesterol were both analysed by IR spectroscopy (Figure 3.4). One of the main functional groups present in dibromocholesterol and cholesterol is the –OH attached to the carbon on position 3 of the first ring.

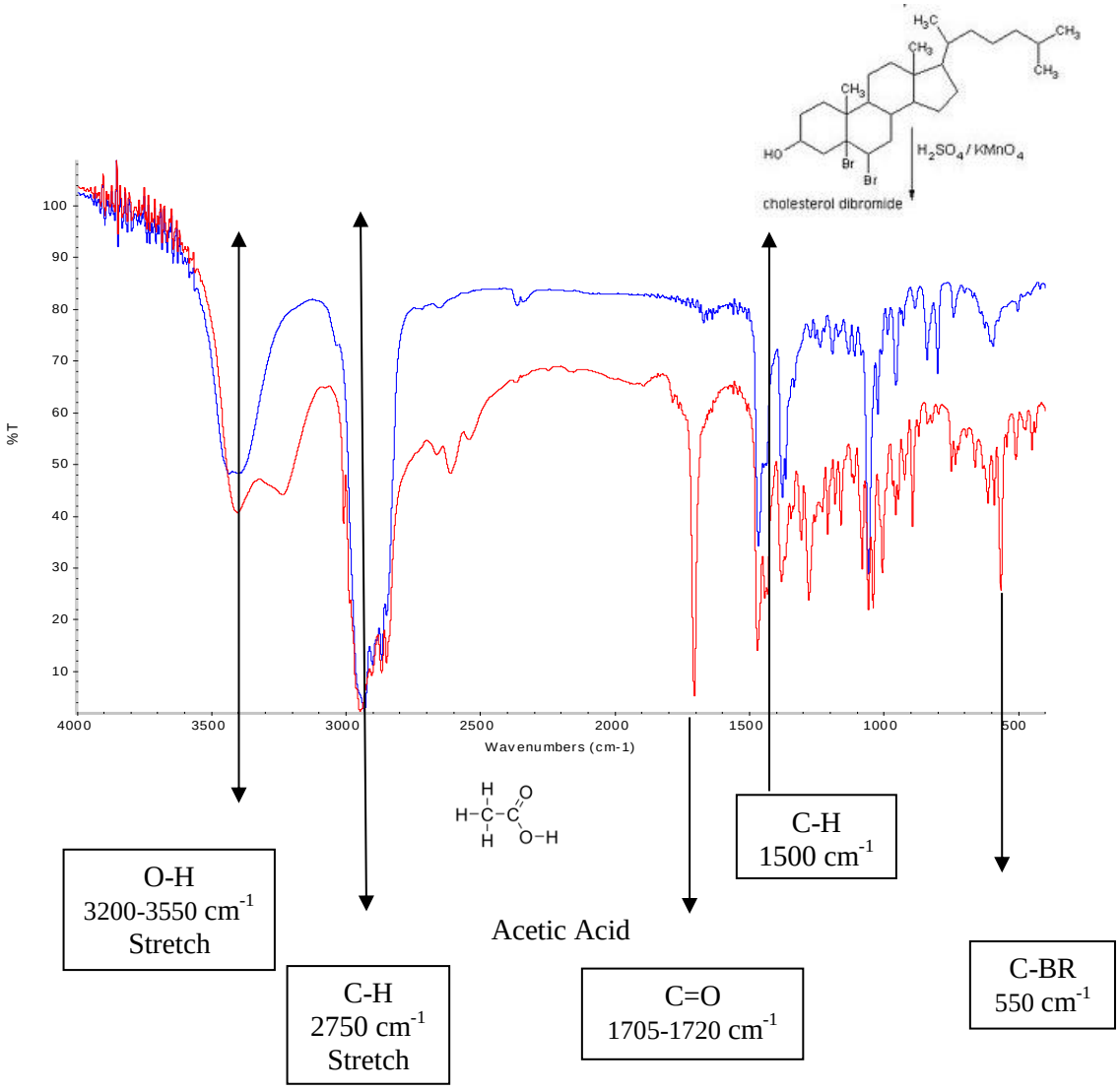


Figure 3.4 Infrared Spectrum for cholesterol (Blue) and product (Red).

The spectrum shows that it was present on both the starting material and the synthesised product. Other similar peaks include C-H bends and stretch at 1500 cm^{-1} and 2750 cm^{-1} respectively. However, there were two peaks present in the synthesised dibromocholesterol, which were not

present in the cholesterol. There is a peak at 550 cm^{-1} which corresponds to a C-Br bond and in addition a C=O peak at around 1700 cm^{-1} (Figure 3.4). Both the DSC scans (Figure 3.3) and the presence of the above C=O peak at 1705 – 1720 cm-1 (Figure 3.4) suggested an impurity may be present. In the reaction mechanism proposed by Fieser and Williamson, (1992), they state that 'when material prepared is dried to constant weight at room temperature, it is obtained as the 1:1 dibromide/acetic acid complex'. Through the process of chemical synthesis, chemical impurities can result and their origin could be from raw materials, solvents, intermediates and by products (Ahuja, 2007). Given the DSC scans show the melting point to be slightly different from the literature, combined with the presence of the second melt and the C=O in the infrared spectrum, suggested that this could be the case. Also of note is that ordinarily this reaction is for purification of crude cholesterol; whereas the starting material for this study is of 99 % pure (Grant *et al.*, 2003, Fieser and Williamson, 1992). The infrared spectrum obtained for pure cholesterol was similar to that observed in other studies and the direct overlay of the pure and synthesised material served to highlight the strong presence of the additional C-Br band at 550 cm^{-1} (Laugel *et al.*, 2005). It is interesting to observe that acetic acid for the purposes of drug synthesis has a low toxic potential, and its presence within defined low quantities is acceptable according to ICH guidelines (ICH, 2006). A further technique that is commonly used for analysis of synthesised products is nuclear magnetic resonance (NMR). This technique is used to study the behaviour of nuceli when subjected to an externally applied field and thus for this study ^{1}H and ^{13}C nuclei were examined for the purposes of structural characterisation of any present molecules (Lohr *et al.*, 2006).

3.3.1.4 Hydrogen NMR.

Cholesterol was analysed (Figure 3.5), as well as the synthesised batches of dibromocholesterol shown by a representative scan (Figure 3.6) to assess how the environment of bonded hydrogens changed subsequent to chemical synthesis. Highlighted in Figure 3.5, are the two hydrogen atoms most affected by the attachment of the strongly electronegative bromine atoms during the synthesis in the cholesterol NMR.

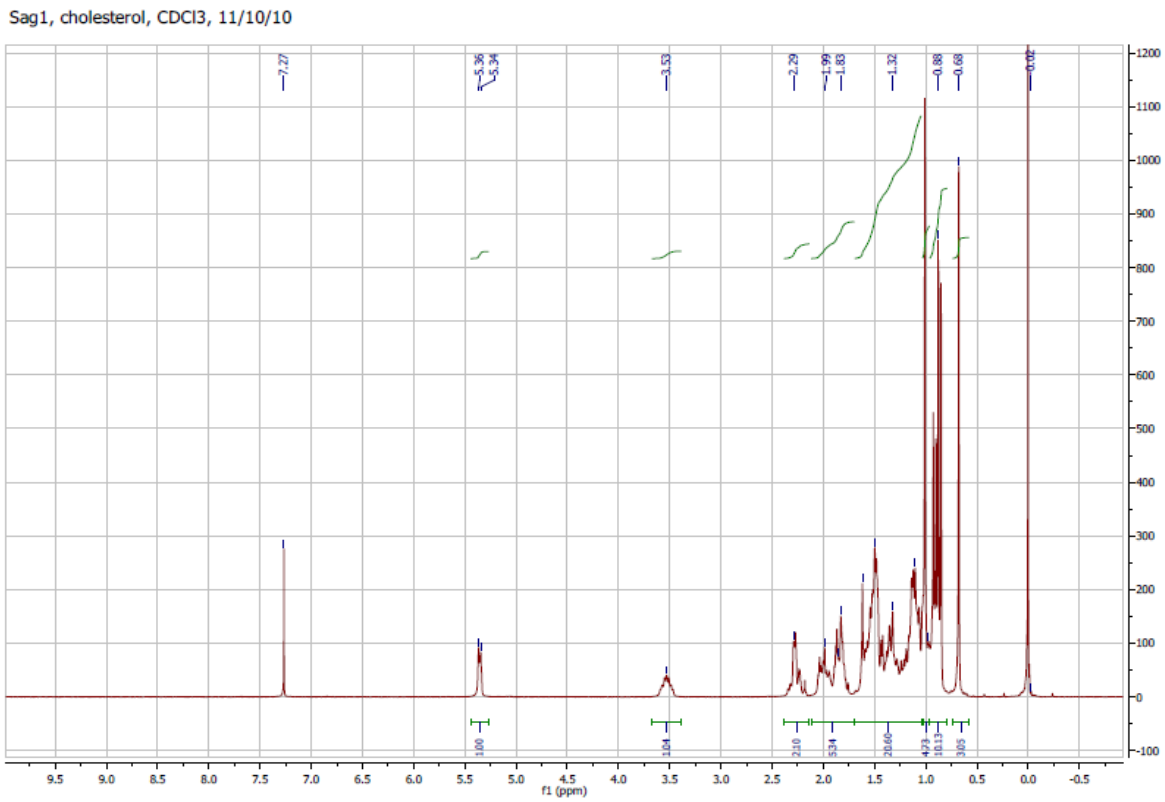


Figure 3.5 ^{1}H NMR of cholesterol.

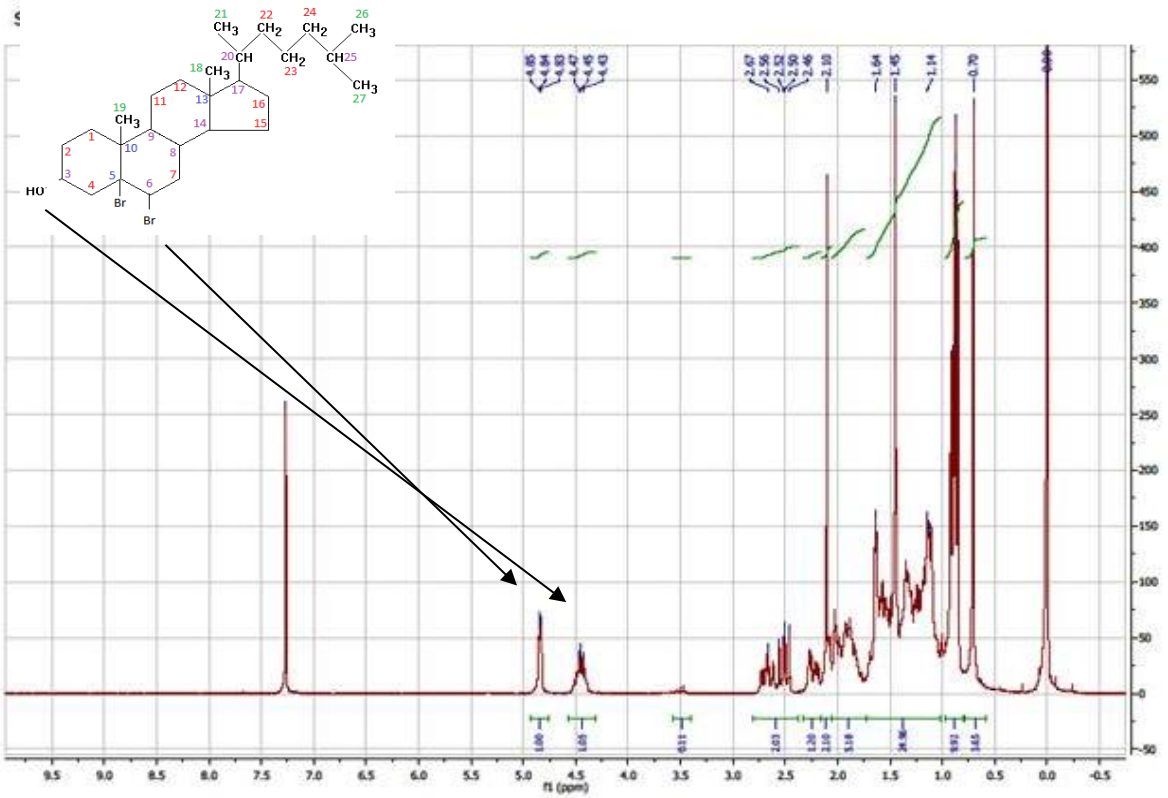


Figure 3.6 ^{1}H NMR of dibromocholesterol.

There were two downfield peaks which were multiplets (Figure 3.5); there was a peak at 3.5 (ppm) which can be attributed to the hydrogen attached to the oxygen and a second peak which was from the hydrogen attached at around 5.5 (ppm) carbon 6 associated with the double bond. It can be seen that with the synthesised product (Figure 3.6) both these peaks have shifted; the 3.5 (ppm) shifts to 4.5 (ppm) and the peak attributed to the hydrogen attached to the OH group moves from 5.5 (ppm) to 4.75 (ppm). There was also no evidence of a second compound within the spectrum. Hydrogen NMR can also provide information about the number, type and environment of the ^{1}H atoms (Malet-Martino and Holzgrabe, 2011).

If the double bond from the cholesterol is broken as predicted by the reaction mechanism (Figure 2.3), and bromine atoms attached, there should be a specific change visible in the ^{1}H NMR spectrums. The ^{1}H-NMR data (Figure 3.6) suggests that the proposed synthesis has taken place and there are no obvious indications of impurities or complexes being present.

3.3.1.5 Carbon NMR

Further characterisation was carried out using ^{13}C NMR. Figure 3.7 shows the structure of dibromocholesterol with all the carbons labelled in colours corresponding to the number of hydrogens attached. In Figure 3.8, quaternary and C-H carbons were shown to be above the base line and CH_2 and CH_3 were shown to be below. The red represented the CH_2, the green CH_3, the lilac CH and the blue were quaternary carbons with no attached hydrogens. The Carbon NMR (Figure 3.8) confirmed the presence of 27 atoms (Figure 3.7), as well as the bonding environment of each. Carbon NMR was used to determine the presence or absence of the suggested dibromide/acetic acid complex (Fieser and Williamson, 1992).

If such a complex was present, there should have been more than the 27 carbons in the NMR spectrum not accounted for by the structure of dibromocholesterol; this technique is sensitive enough to detect low molecular weight compounds in solution provided they contain the nuclei of interest (Malet-Martino and Holzgrabe, 2011).

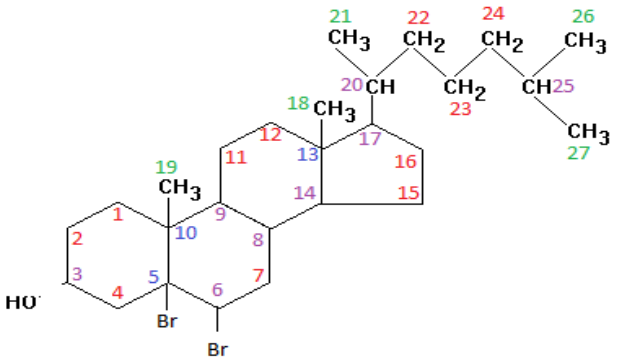


Figure 3.7 Structure of dibromocholesterol with all carbons numbered.

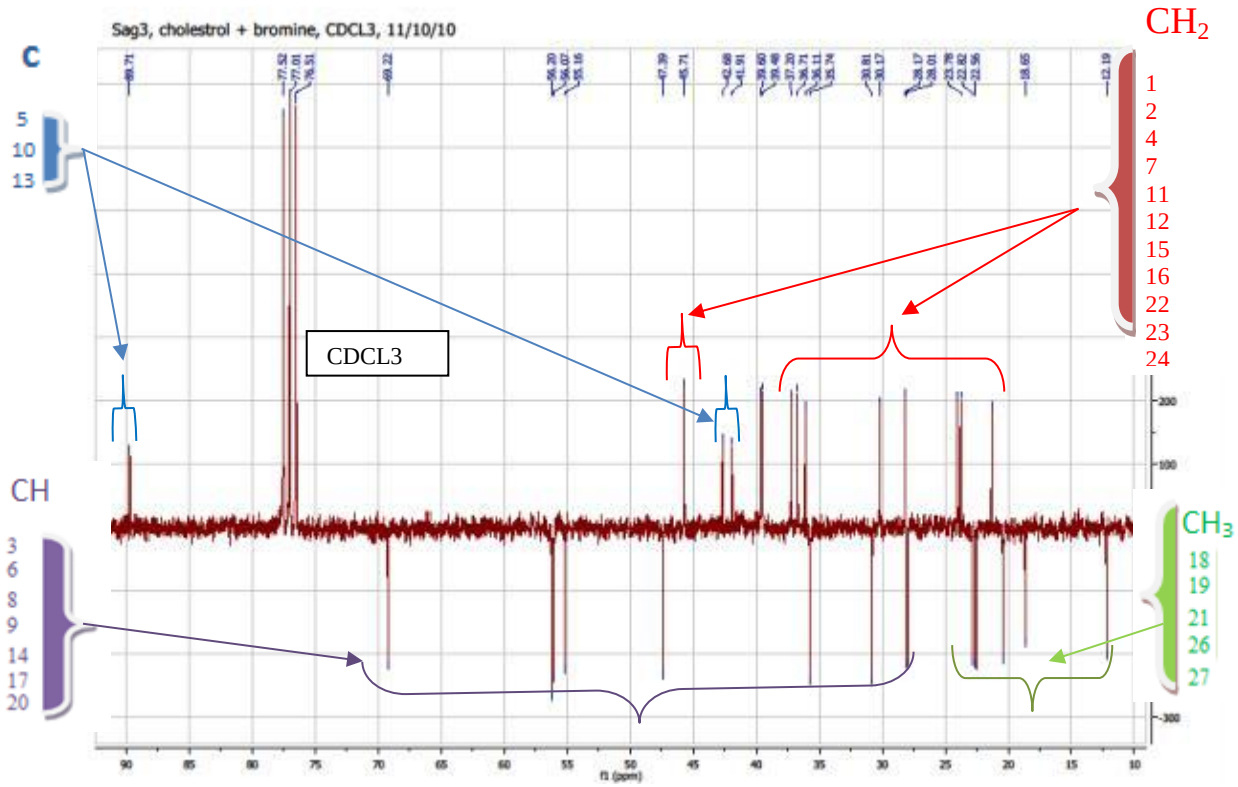


Figure 3.8 [13]Carbon NMR of dibromocholesterol.

The most significant aspect of the NMR results is that there was no such evidence of a dibromide complex as initially indicated by the IR spectrum. Confirmation was due to the absence of an additional two carbon atoms. Overall, the validation data suggests that dibromocholesterol is the synthesised product and slight discrepancies between literature and obtained melting points could

be due to differences in the purity of the starting material. The NMR data suggests that the reaction has taken place as expected and resulted in the formation of dibromocholesterol.

3.3.1.6 Percentage yield

It was necessary to calculate the yield obtained from the synthesis of the product to assess the efficiency of the reaction, if the yield was substantially low it would suggest further manipulation of the method chosen for synthesis was required (Smart, 2002).

Table 3.2 The percentage yields of the two batches of synthesised dibromocholesterol.

Batch Number	Initial Weight of cholesterol (g)	Theoretical Yield (g)	Final weight of Product (g)	Actual Yield (%)
1	1.0	1.632	1.0151	62
2	2.0	2.720	2.0100	74
		Average		68

The overall average yield obtained from the two synthesised batches was 68 % (Table 3.2). The *theoretical* yield is the mass of the product, which should be formed in the chemical reaction according to the stoichiometry of the balanced equation whereas the *actual* yield is the mass of the product formed in the chemical reaction (Bettelheim *et al.,* 2004). It is difficult to conclude exactly where the loss may have occurred, this may be due to transference loss or an incomplete reaction. If the percentage yield is too low, then consequently a small amount of final product would be formed from even with a large amount of starting material and the process maybe too inefficient to be useful; however, an average yield of 68 % was considered suitable for the required amounts of lipid needed for subsequent studies.

3.3.1.7 Six month stability assessment of dibromocholesterol

Having synthesised dibromocholesterol the stability of the synthesised product, when stored as a powder, was an important aspect to consider. The product needs to show stability upon storage and subsequently its incorporation into the liposome formulations needs to fulfil minimum stability requirements. The dibromocholesterol powder was stored at -20 °C for 6 months and was validated

as previously outlined with characterisation techniques including; TLC, DSC, ^{1}H NMR and ^{13}C NMR. In addition, moisture gain was assessed using thermogravimetric analysis. The data obtained was compared either directly to a newly synthesised batch (TLC, TGA) or to the original data (^{1}H NMR and ^{13}C NMR).

3.3.1.8 TLC of dibromocholesterol (6 months vs. newly synthesised)

Thin layer chromatography was used to compare the newly synthesised batch to the 6 month old dibromocholesterol to assess any change in the product. In this chromatogram (Figure 3.9), the original product was the dibromocholesterol, which had been stored for 6 months.

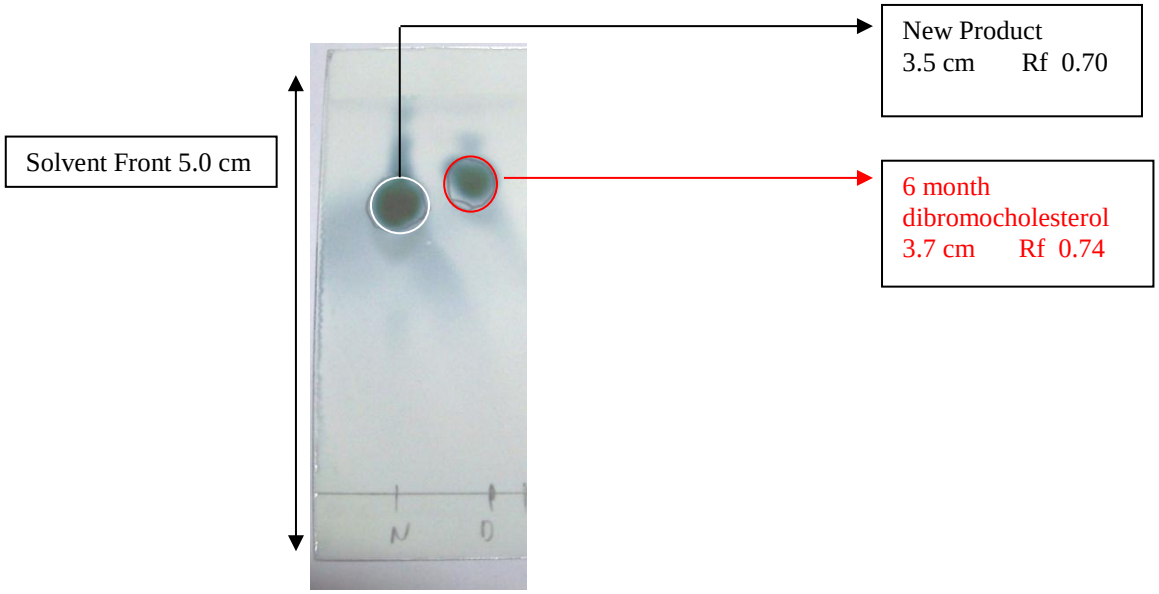


Figure 3.9 TLC plate showing a newly synthesised batch and dibromocholesterol stored for 6 months.

The Rf value was slightly different to the value originally obtained which was 0.65; however, the value obtained of 0.74 was directly comparable to the newly synthesised batch of 0.7. Moreover, there were no definitive additional spots to indicate impurities from degradation. Having knowledge of the basic stability characteristics of synthesised products is essential for practical aspects of the study; the loss of stability could lead to decomposition or the formation of new substances (Voronin, 2000).

3.3.1.9 DSC for melting point confirmation of stored dibromocholesterol

DSC was used previously for the determination of an accurate melting point and was therefore repeated to ascertain if any changes had occurred upon storage of the dibromocholesterol. The sample of the 6 month dibromocholesterol was tested and this scan was compared to the original to determine stability. The overlaid scans (Figure 3.10) showed that the main melt for the newly synthesised product and a batch stored for 6 months were very similar (117 °C and 119 °C respectively). This slight difference in melting point may be a result of the storage of dibromocholesterol at -20 °C (in the absence of a desiccator).

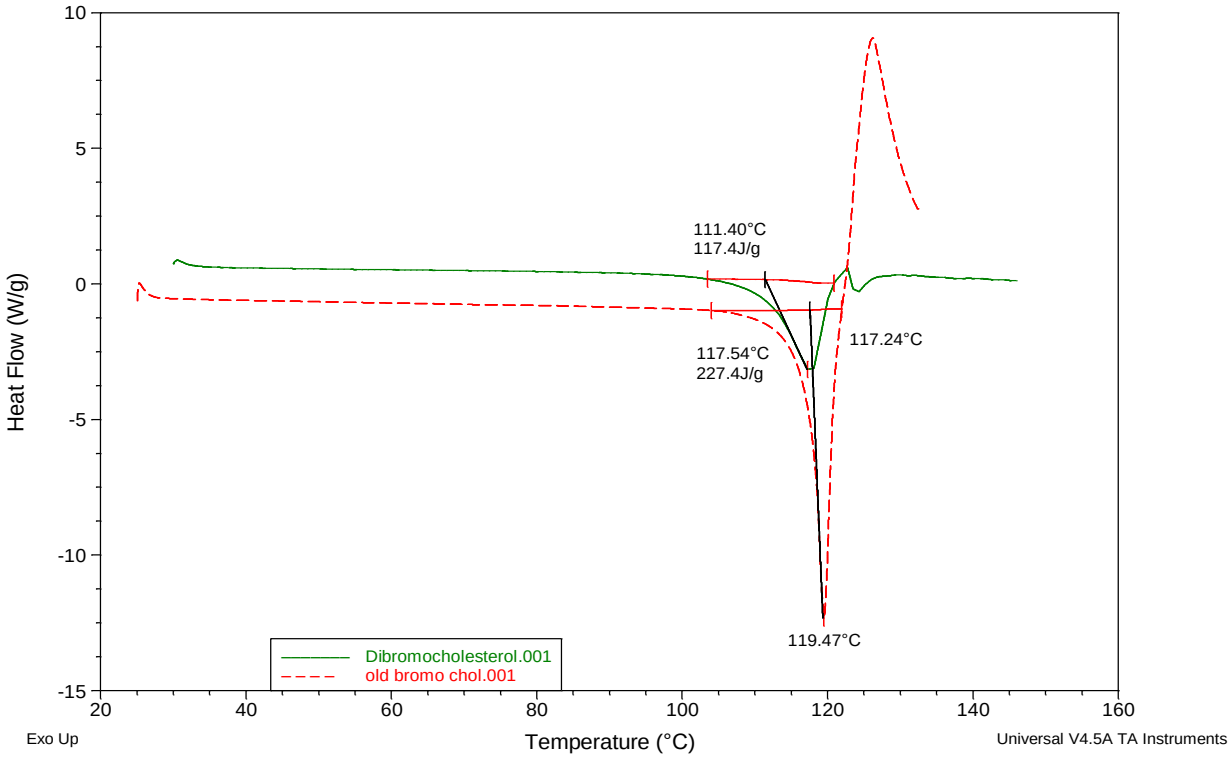


Figure 3.10 DSC thermogram of initial batch overlaid with dibromocholesterol after 6 months storage.

It is well established that the presence of water is a destabilising factor and that a higher water content is associated with a higher rate of chemical degradation (Ohtake and Shalaev, 2013). Although the vial was stored in a sealed container the dried powder may have absorbed water which could affect the melting point; this can be confirmed through thermogravimetric analysis as

this is a quantitative measurement which can determine the moisture content related to weight loss for stability studies (Yu, 2001) (see section 3.3.2.4).

3.3.1.10 ^{13}C NMR and ^{1}H NMR of stored dibromocholesterol

Stability testing of the stored dibromocholesterol involved repeating ^{1}H NMR and ^{13}C NMR studies. This would elucidate any structural changes that may have occurred over time. The ^{1}H NMR scans obtained were directly comparable to the initial scan, and neither scan showed any structural differences in terms of the signals obtained (Figure 3.11 vs. Figure 3.6). Similarly there were no visible difference in the carbon NMR scans, with the signals being highly reproducible (Figure 3.12 vs. Figure 3.8), suggesting no measurable structural degradation had taken place.

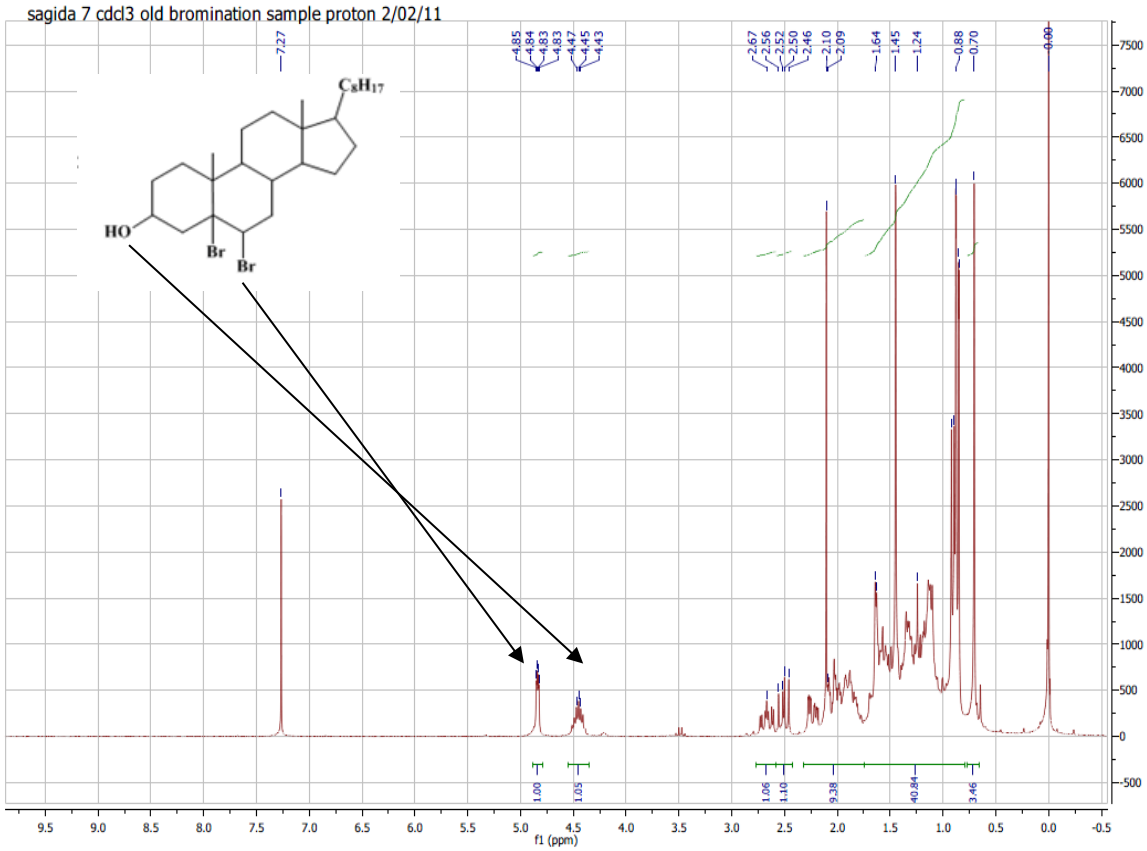


Figure 3.11 ^{1}H NMR scans of dibromocholesterol retested after 6 months.

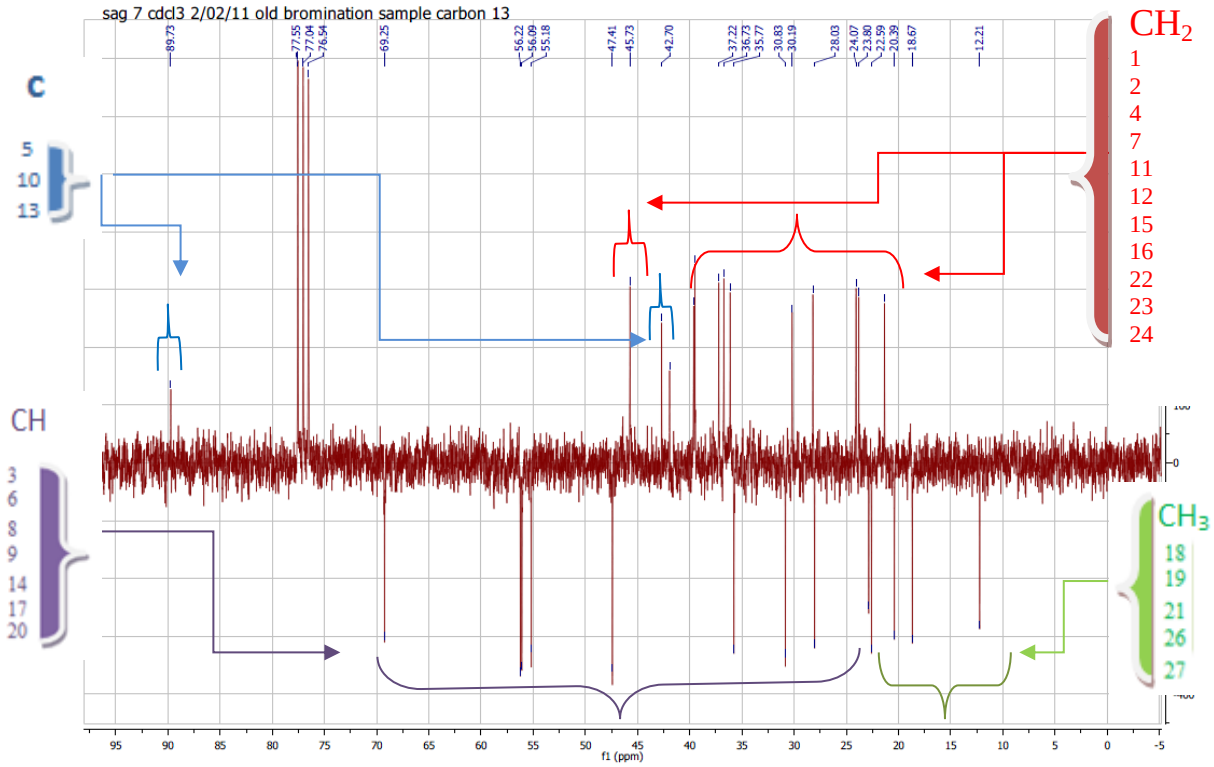


Figure 3.12 ^{13}C NMR scans of newly synthesised batch of dibromocholesterol (6 month stability).

3.3.1.11 TGA analysis of newly synthesised and stored dibromocholesterol

To provide an assessment of structural decomposition, oxidation and moisture gain or loss, thermogravimetric analysis (TGA) was carried out on both batches of dibromocholesterol in triplicate as shown in Figure 3.13. The TGA scans showed the older batch of dibromocholesterol had a mean moisture loss at 5.14 ± 0.54 % (Figure 3.14 b) compared to the newly synthesised batch which had a loss of 4.40 ± 0.21 % (Figure 3.13). Statistical analysis of the data showed no significant difference between the moisture loss from either the freshly prepared or the 6 month stored samples. This suggests that moisture gain over storage was not significant and that the samples were not hygroscopic in nature. Other studies assessing the hygroscopicity of samples consider an increase in mass of material between 0.2 % to 2 % to be slightly hygroscopic; as defined by the European Pharmacopoeia (Murikipudi *et al.*, 2013). However, given the starting material had a moisture content of around 4 % this may still be sufficient to enhance chemical degradation over longer term (May, 2013). FDA guidance for industry for pharmaceutical

development recommends a moisture content of less than 2 % and between 1 % to 3 % for freeze-dried products (ICH, 2009, May, 2013).

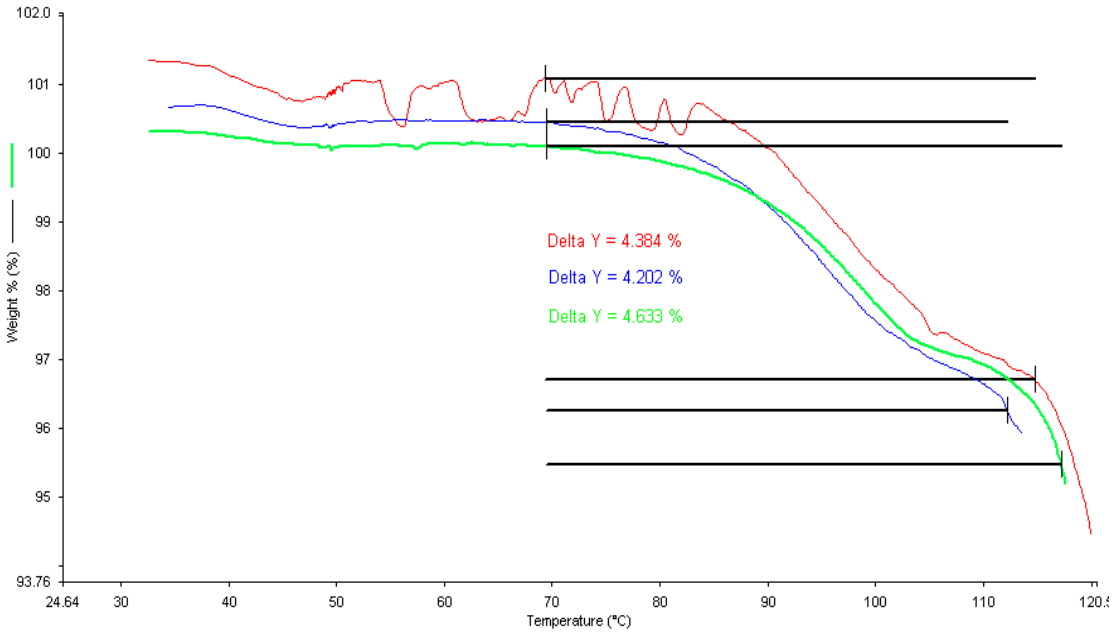


Figure 3.13 TGA scans of newly synthesised batch of dibromocholesterol .

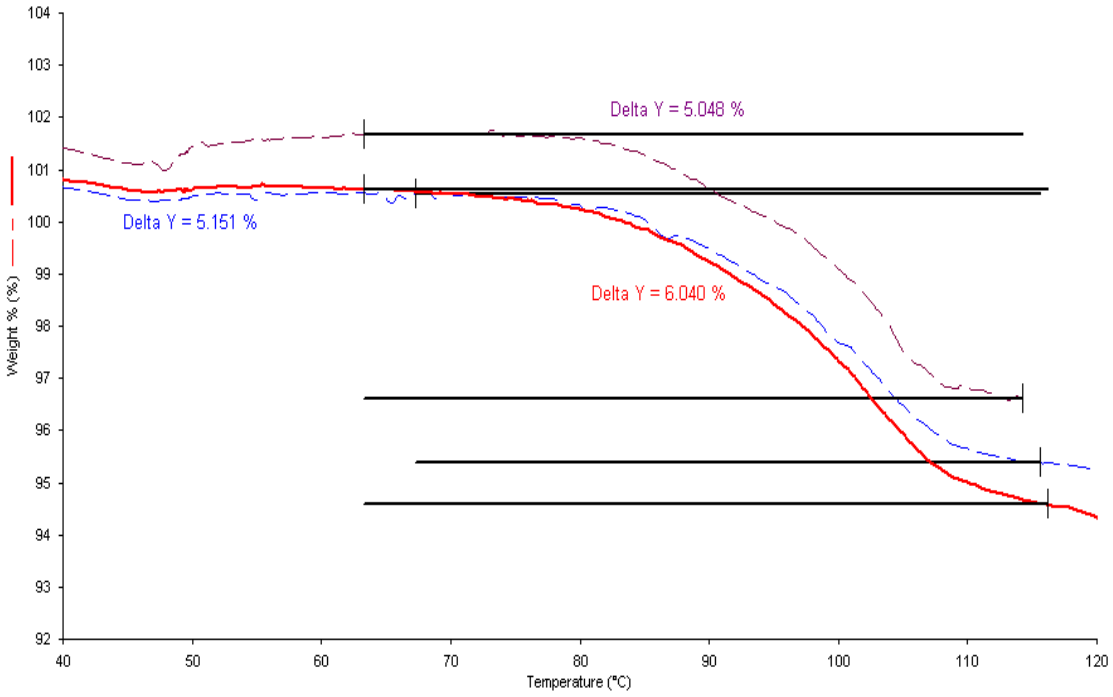


Figure 3.14 TGA scans of previous batch of dibromocholesterol.

3.3.2 Sedimentation studies of liposomes and physicochemical characterisation

Subsequent to the synthesis and characterisation of the dibromocholesterol, it was necessary to assess the impact of its incorporation within liposomal formulations. An assessment of the comparative effect on the sedimentation efficiency of liposome formulations of DPPC:Chol as multilamellar vesicles (MLV) and small unilamellar vesicles (SUV) with those containing dibromocholesterol in differing ratios was made (Table 3.3). For all the formulations included in the study physicochemical characterisation of the particle size and zeta potential were carried out. The results (Figure 3.15) from the particle size data showed there was no significant difference between the brominated MLV liposomes of DPPC:Chol:Brchol and the non-brominated DPPC:Chol MLV formulations. The particle sizes for the brominated vesicles of DPPC:Chol:BrChol 16:8:8 μMol, DPPC:Chol:Brchol 16:4:12 μMol and DPPC:Brchol 16:16 μMol were 10.7 ± 1.3 μm, 11.1 ± 2.5 μm and 8.5 ± 0.7 μm respectively (Figure 3.15). The DPPC:Chol 16:16 μMol MLV formulations had an average particle size of 10.18 ± 2.7 μm (Figure 3.15).

However, there was a significant difference ($p<0.001$) between the zeta potential for the non-brominated MLV formulations and the brominated formulations. The non-brominated formulations of DPPC:Chol 16:16 μMol had an average zeta potential of -6.92 ± 1.17 mV (Figure 3.14). The brominated formulations of DPPC:Chol:Brchol 16:8:8 μMol, DPPC:Chol:Brchol 16:4:12 μMol and DPPC:Brchol 16:16 μMol had average zeta potentials of -33.87 ± 3.87 mV, -37.70 ± 4.65 mV and -43.47 ± 5.42 mV respectively (Figure 3.15). There was no significant difference observed between the formulations containing increasing amounts of brominated cholesterol.

The size distribution (span) for each of the formulations showed that although similar values were obtained across the MLV formulations, the span is significantly decreased ($p<0.001$) for the SUV formulations. For instance for DPPC:Chol MLV formulations and DPPC:Chol SUV formulations this decreased from 2.3 ± 0.01 to 0.42 ± 0.05 respectively. The process of sonication is a widely used to technique for the production of SUV vesicles which are normally less than a micron and the resultant decrease in span is thus expected (Lapinski *et al.*, 2007).

It was interesting to observe that there was no significant difference in size across the formulations as the aim in using a synthesised derivative of cholesterol was to retain the physiochemical characteristics of normal liposome formulations. The lack of differences observed in the particle size when comparing the formulations suggested that the cholesterol and dibromocholesterol have inserted into the bilayer in a similar way. Cholesterol fills into the spaces within the acyl chains of the lipid bilayer as it orients itself so that the hydroxyl group is towards the aqueous phase and the hydrophobic steroid ring is parallel to and buried within the chains of the phospholipids (Ohvo-Rekila *et al.*, 2002).

Table 3.3 The five Liposome formulations used in the sedimentation study.

Lipid Composition	Morphology	Ratio of Lipids (µMol)
DPPC:Chol	MLV	16:16
DPPC:Chol	SUV	16:16
DPPC:Chol: BrChol	MLV	16:8:8
DPPC:Chol: BrChol	MLV	16:4:12
DPPC: BrChol	MLV	16:16

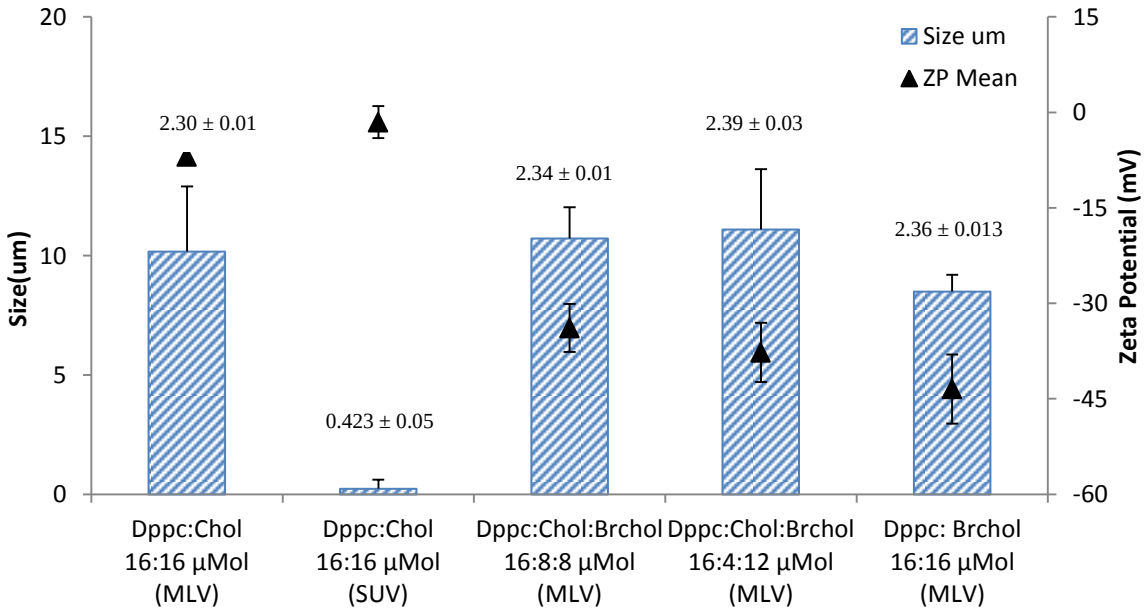


Figure 3.15 The size (µm), zeta potential (mV) and span of MLV and SUV formulations of DPPC:Chol with and without dibrominated cholesterol. Formulations of DPPC:Chol 16:16 µMol MLV and SUV with formulations containing brominated cholesterol of DPPC:Chol:BrChol 16:8:8 µMol, DPPC:Chol:Brchol 16:4:12 µMol and DPPC:Brchol 16:16 µMol used within the sedimentation studies. Results represent mean ± SD of n =3 batches.

The difference observed in the zeta potential was more notable in the liposome formulations incorporating dibromocholesterol; the electrical potential at the membrane surface becomes more anionic thus changing an important physiochemical characteristic of the formulation (Figure 3.15). The nature and extent of the charge on the surface of liposomes impacts upon stability, kinetics and within the *in vivo* environment even the biodistribution of the vesicles (Lian *et al.,* 2001).

However, negatively charged liposomes can have longer stability due to reduced aggregation and fusion (Lasic and Papahadjopoulos, 1998). Thus subsequent to characterisation sedimentation was assessed qualitatively (through images taken of formulations at specified time points) and quantitatively (by measuring percentage transmission using a spectrophotometer). Microscope images of the MLV formulations were used to assess the formation of liposomes further to the physicochemical characterisation.

3.3.2.1 Consideration of liposome morphology using bright field microscopy

The images obtained (Figure 3.16) for the MLV liposome formulations of (a) DPPC:Chol 16:16 μMol showed that the formulation produced spherical vesicles in general and were heterogenous in nature. The images for the formulations containing brominated cholesterol (Figure 3.16 b – d) confirmed that the liposomes were spherical in nature and were similar to the DPPC:Chol liposome formulations. As was suggested by the more negative zeta potential of the brominated-liposomes (Figure 3.15) there appeared to be a decreased level of aggregation of liposomes with the inclusion of the brominated cholesterol in the vesicles (Figure 3.16 b to d respectively). The higher negative surface potential could account for the vesicle repulsion (Lian *et al.,* 2001).

In many previous studies light microscopy techniques have been used in addition to particle size characterisation for the confirmation of size distribution and the morphological nature of MLV preparations, although this cannot be a complete picture due to the limits of the microscope (Szoka and Papahadjopoulos, 1980). However, this technique highlights clearly the heterogenous nature of the MLV vesicles within the samples, which may become more important during the developmental stages of liposomal reference standards.

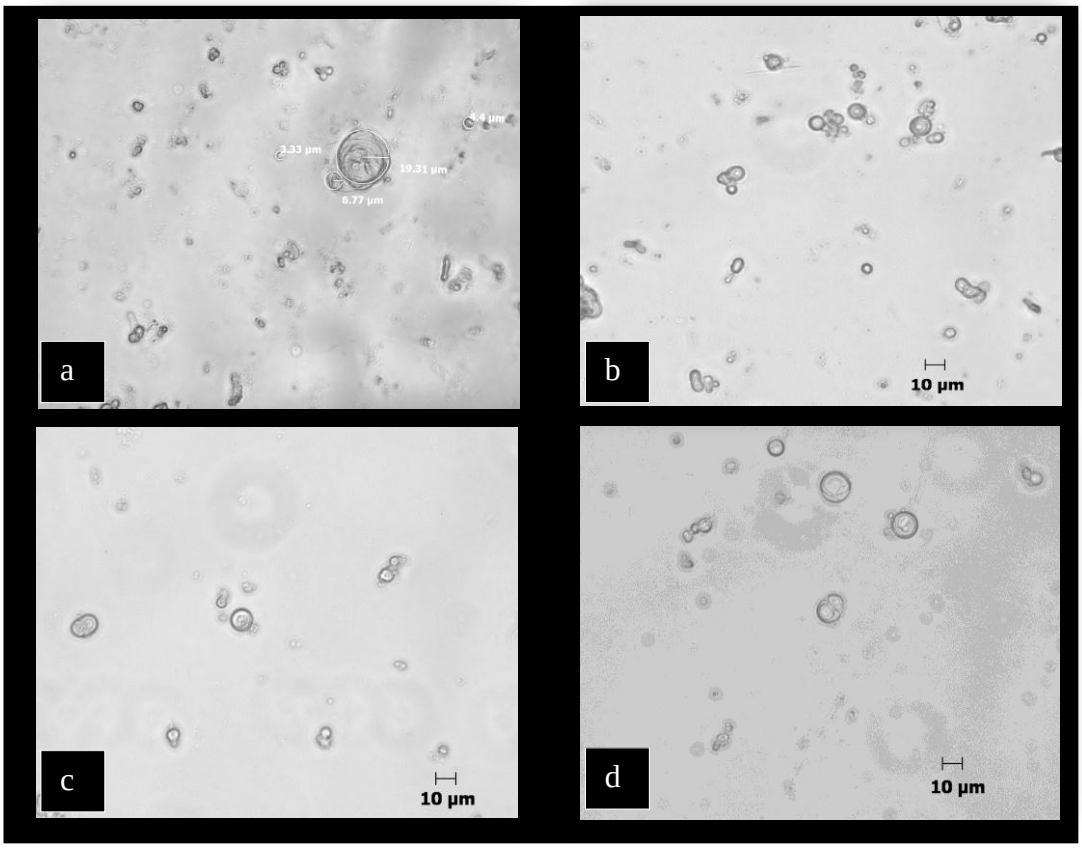


Figure 3.16 Light microscope images viewed under a 40X objective of formulations of DPPC:Chol with and without dibrominated cholesterol; **(a)** DPPC:Chol 16:16 µMol MLV**, (b)** DPPC:Chol:Brchol 16:8:8 µMol, **(c)** DPPC:Chol:BrChol 16:4:12 µMol and **(d)** DPPC: Brchol 16:16 µMol.

3.3.2.2 Sedimentation rates for liposome formulations; a quantitative and qualitative assessment

Liposome formulations with increasing dibromocholesterol (Table 3.3) were quantitatively assessed for their 'settling time' using a spectrophotometer (Figure 3.17). As the liposomes settle to the bottom of the cuvette, more light is able to pass through. Liposomes formulations were also placed into bijoux tubes for qualitative assessments of sedimentation and images were taken at the beginning of the study then at defined time points of 1, 2, 3, 4, 12 and 18 hours (Figure 3.18).

The results from the quantitative assessment (Figure 3.17) showed that that over the period of 18 hours the ability of the liposome formulations to sediment was significantly different ($P<0.001$) for the SUV formulations (DPPC:Chol 16:16 µMol) compared to all other MLV formulations. Even by the first hour of the study there was a low percentage transmission for the formulations of DPPC:Chol SUV, which was significantly different ($P<0.001$) when compared to the formulations

of DPCC:BrChol 16:16 μMol (1.3 ± 0.12 % and 19 ± 8.8 % respectively). At this time point there was a significant difference between the control formulations of DPPC:Chol 16:16 μMol (MLV) and the formulations of DPPC:BrChol 16:16 μMol ($P<0.01$) showing percentage transmissions of 0.13 ± 0.06 % and 19 ± 2.75 % respectively, with the formulation containing the brominated cholesterol at a 1:1 mole ratio showing effective sedimentation.

The observed trend was not consistent for the remaining time period of the study and after 4 hours the DPPC:Chol:BrChol, 16:4:12 μMol formulations had a significantly higher ($p<0.01$) percentage transmission of 59 ± 5.9 % compared to the control formulation at 29 ± 12 %. This was not significantly different from the formulations containing the highest dibromocholesterol content (DPPC:BrChol 16:16 μMol 46 ± 7.0 % (Figure 3.17).

By the 12 hour time point the percentage transmission was significantly ($p<0.001$) lower for the DPPC:Chol 16:16 μMol (SUV) formulations compared to those formulations incorporating brominated cholesterol and for the control DPPC:Chol 16:16 μMol (MLV) formulation ($p<0.001$). However, there was no significant difference between the control MLV formulations of DPPC:Chol 16:16 μMol and the brominated formulations at this time point or at the final time point of 18 hours. The qualitative results (Figure 3.17) confirmed that DPPC:Chol 16:16 μMol MLV formulations required over 4 hours to show physical evidence of settling, and the SUV formulations showed the slowest rate of sedimentation with some physical evidence of settling; however the suspension remained turbid, even after 18 hours. Figure 3.18 also confirmed that the formulations containing brominated cholesterol were clearer over the first few hours compared to the control formulations of DPPC:Chol 16:16 μMol (MLV).

In terms of application, the smaller vesicles are not practically applicable for the ELISPOT assay, however they do elucidate the behaviour of the smaller particles within formulations. Smaller vesicles are less dense and as outlined by Stokes' Law have a slower sedimentation rate (Versaratchanon *et al.,* 2007). In addition, aggregation of liposomes is ordinarily associated with larger liposomes due to the greater flatness of the membranes that allows greater contact between areas of membranes (Casals *et al.*, 2003).

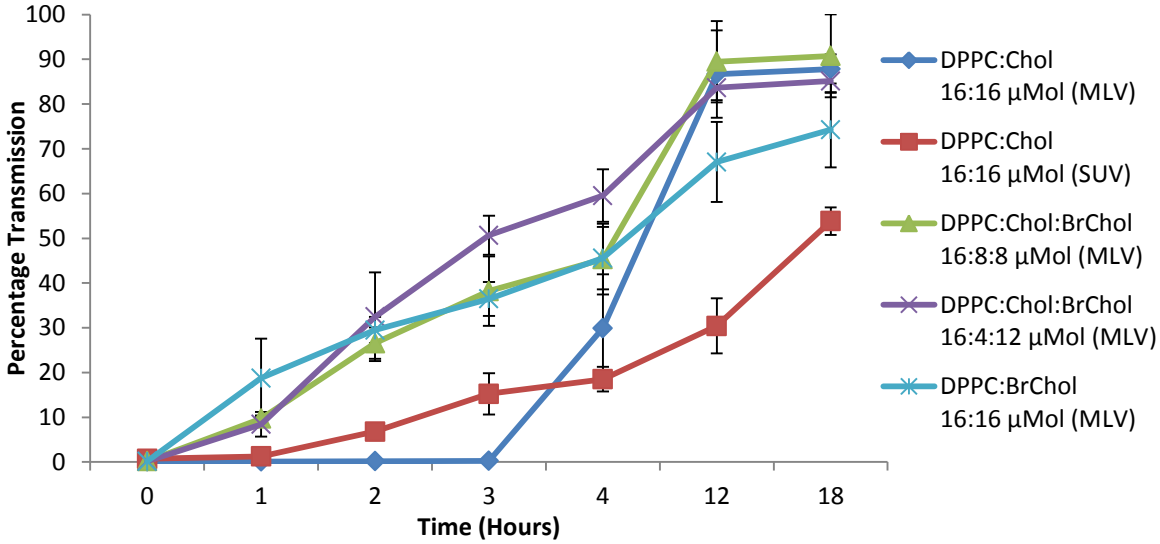


Figure 3.17 Percentage transmission over an 18 hour period of liposome formulations containing brominated and non-brominated cholesterol. (1) DPPC:Chol (MLV) (2) DPPC:Chol (SUV) (3) Dppc:Chol:Brchol 16:8:8 µMol, (4) Dppc:Chol:Brchol 16:4:12 µMol and (5) DPPC:Brchol 16:16 µMol. Results represent mean ± SD, n =3 batches.

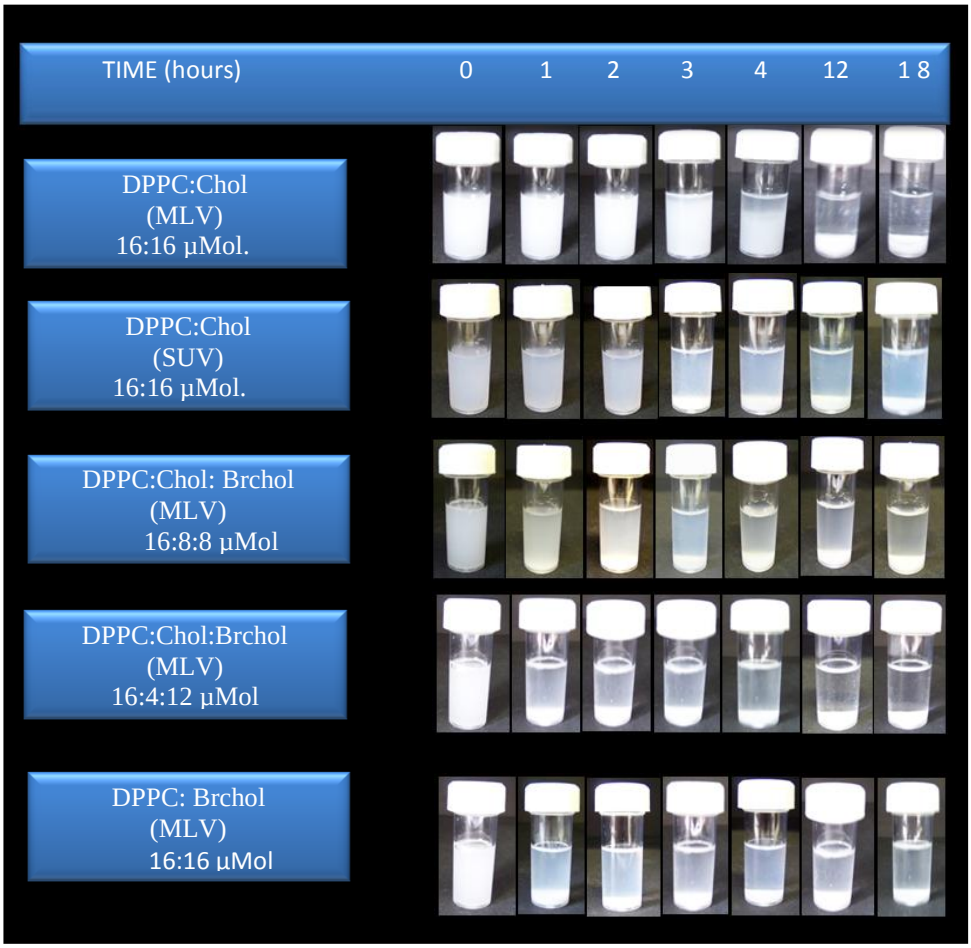


Figure 3.18 Photographic images over an 18 hour period of liposome formulations containing brominated and non-brominated cholesterol; (1) MLV and (2) SUV formulations of DPPC:Chol 16:16 µMol and MLV formulations containing brominated cholesterol of (3) DPPC:Chol:Brchol 16:8:8 µMol, (4) DPPC:Chol:Brchol 16:4:12 µMol and (5) DPPC:Brchol 16:16 µMol.

Thus, it can be anticipated from the observed results that smaller vesicles, which have a slower rate of sedimentation would be detrimental for an ELISPOT assay as they may not settle effectively at the bottom of the individual wells. These results show that by four hours, formulations with brominated cholesterol show enhanced settling ability compared to the non-brominated MLV formulations (Figure 3.18). This initial enhanced sedimentation rate suggests the increased density has a greater impact over this period, whilst this effect does not remain over longer periods (12 or 18 hours) which are normal endpoints for ELISPOT assays although they can even be as long as 24 hours (Smith *et al.,* 2009). By the final time point the majority of vesicles have settled. Stokes' law dictates that observed increase in sedimentation rates occur due to an increase in various factors including the size and density of the particle (Banker and Rhodes, 2002). However in the case of the liposome formulations containing brominated cholesterol a linear increase was not observed in the sedimentation velocity across all of the time points, perhaps due to the observed differences in surface charge between the formulations. The presence of charge (as observed with the formulations containing dibromocholesterol) is normally associated with increased stability (Casals *et al.*, 2003).

The negative charge also leads to greater repulsion between the vesicles; this would hinder caking and lead to a more diffuse and less compact sediment layer (Martin, 2003). The increase in sedimentation rate of the brominated vesicles in the first few hours could be of particular significance once applied to the ELISPOT assay to minimise leakage, until the liposome vesicles have reached the bottom of the well. For further studies the formulation of DPPC:Chol:BrChol at 16:4:12 μMol (which were most effective by the four hour time point) were considered to be a promising formulation for further assessment alongside other neutral formulations. The enhanced sedimentation efficiency in the early stages of the assay may prove to be important.

3.3.2.3 Dynamic viscosity data for the assessment of liposomal formulations

The synthesis of the dibromocholesterol and its incorporation into liposome vesicles was considered to increase the density of the vesicles, however another factor controlling sedimentation according to Stokes' law is viscosity (Banker and Rhodes, 2002). It was necessary to confirm

whether there were any differences in viscosity for both the formulations with and without brominated cholesterol (Table 3.4). The results confirmed that there was no significant difference in viscosity of MLV formulations not containing brominated cholesterol and a brominated formulations of (DPPC:Chol:BrChol 16:4:12 μMol), both of which were similar to the viscosity of water which is 1.0019 (Martin, 2003).

Table 3.4 Viscosity of DPPC:Chol:BrChol 16:4:12 μMol and DPPC:CHOL 16:16 μMol liposome suspensions. Results represent mean ± SD of n =3 batches.

Lipid Composition	Ratio of Lipids (μMol)	Dynamic Viscosity (mPas)	Kinetic Viscosity (mm^2/s)
DPPC:Chol	16:16	1.03 ± 0.03	0.94 ± 0.04
DPPC:Chol :Brchol	16:4:12	1.07 ± 0.04	0.97 ± 0.05

As no significant difference were previously observed in the vesicle size between the non-brominated and brominated samples (Figure 3.14), it can be concluded that the initial increase in sedimentation observed, according to Stokes' law (Equation 3.3), can only be accounted for by the increased density of the liposomes.

3.3.3 BSA-FITC assay optimisation; spectral scans to establish optimal wavelengths

Initially scans were carried out at the literature stated wavelengths of 492 nm for excitation as shown in Figure 3.19 (a) and 519 nm for the emission wavelength as shown in Figure 3.19 (b) (Chandaroy, 2001). The results showed a small wavelength difference between the two scans and by using a wavelength of 492 nm for excitation there would be a large overlap between excitation and emission. Therefore, to determine a more appropriate excitation wavelength to use in order to obtain a purer signal, a more precise spectral scan was carried out (Figure 3.20 a) from 450 nm to 500 nm in increments of 5 nm.

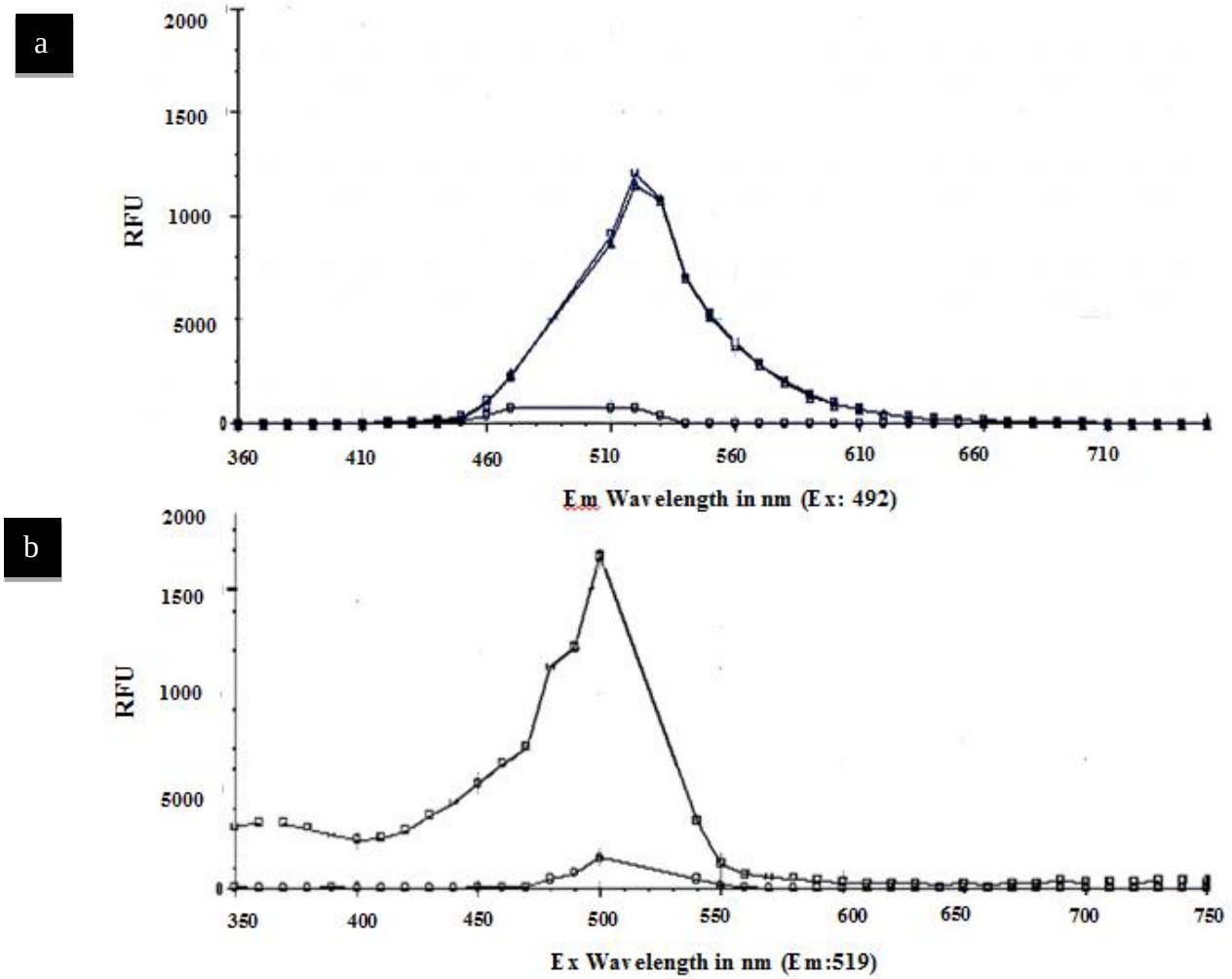
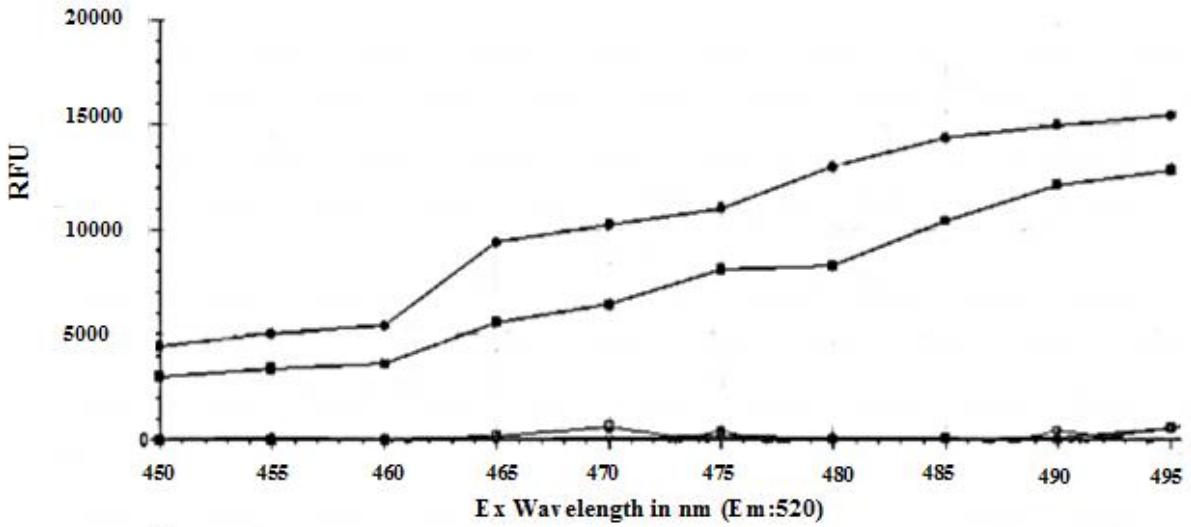


Figure 3.19 Spectral scans for excitation and emission wavelengths for BSA-FITC; **(a)** The excitation wavelength spectra at 520nm **(b)** The emission wavelength spectra at 490nm.

a



b

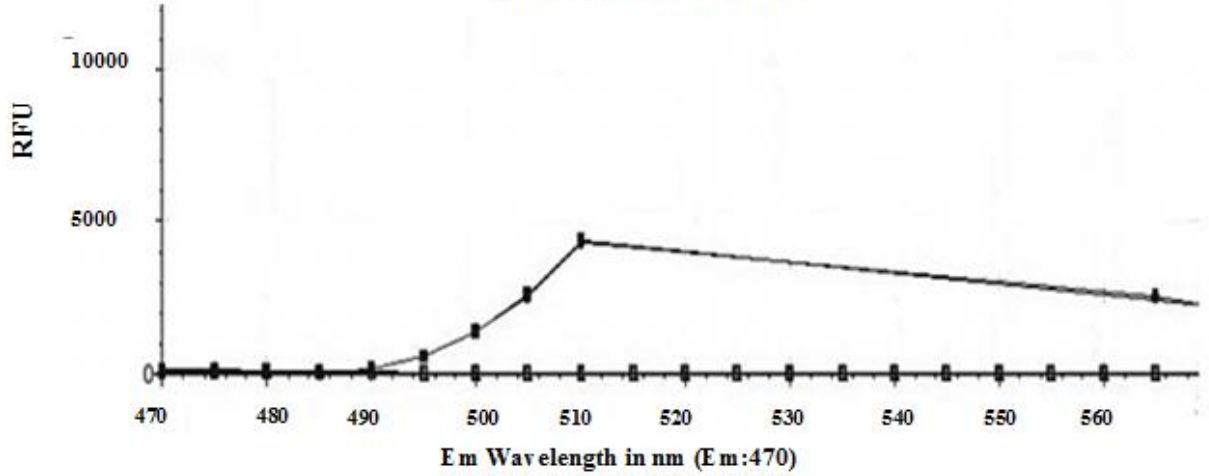


Figure 3.20 Spectral scans for **(a)** excitation wavelengths from 470nm to 570nm and the **(b)** emission wavelength scanned from 470nm to 570nm, the lower line depicted is of PBS.

A wavelength was required which was lower than 492 nm, yet still high enough to be able to provide an appropriate signal for the assay (emission was fixed at 520 nm). Based on these scans, the selected excitation wavelength was 470 nm and confirmation was obtained of a clear emission signal through the final spectral scan (Figure 3.20 b).There are a number of factors which can affect the accuracy of fluorescent measurements and these can include instrumental variations (Birdsall *et al.,* 1983).

The difference between the excitation and emission wavelength is referred to as Stokes' shift, if the shift is large there is less interference in the assay from incident light and the assay is more sensitive (Wild, 2001). Optimisation of the spectral scans is thus an essential aspect of the development of an assay and the spectral analysis has resulted in an appropriate excitation and emission wavelength (480 nm and 520 nm) respectively.

3.3.3.1 Endpoint fluorescence assay to confirm chosen wavelength

The concentration of the BSA-FITC to be used within the liposome preparations for entrapment was prepared at 5 µg/mL. A calibration curve was constructed from a serial dilution of the stock solution, the data points were plotted as a linear regression curve, and a trend line added (Figure 3.21). The R^2 value indicated the validity of the concentration curve. The calibration curve (Figure 3.21) represents the average of three separate calibration graphs. The results show the demonstration of linearity by dilution of a standard stock solution. Visual inspection of the graph confirms this to be a linear relationship as a function of analyte concentration (Figure 3.21) and was further evaluated by calculation of a regression line and by the method of least squares as recommended by ICH guidelines (ICH, 2005).

According to ICH guidelines (ICH, 2005) on the validation of analytical procedures demonstration of linearity, accuracy, precision, and reproducibility are required. Accuracy is defined as the closeness of agreement between the values, which is accepted either as a true value or from a reference value; the precision is the closeness of agreement between a series of obtained measurements. For a calibration curve the test results should be directly proportional to the concentration of an analyte. The guidelines specify that for the establishment of linearity a

minimum of 5 concentrations is recommended. For this study, 8 concentrations have been used (Figure 3.21). An appropriate range was used for the concentration curve as the maximum amount used of BSA-FITC per liposome formulation was 10 µg. The value of 1 for R^2 indicates a perfectly linear relationship thus the obtained value of 0.99 indicates a good statistical fit (Freund *et al.*, 2006). Repeatability of the assay has also been shown as a minimum of 9 determinations covering the specified range; three replicates were run for the three assays. According to ICH guidelines (ICH, 2006) other aspects of the assay that should be validated include the limits of detection/quantification and the specificity of the assay.

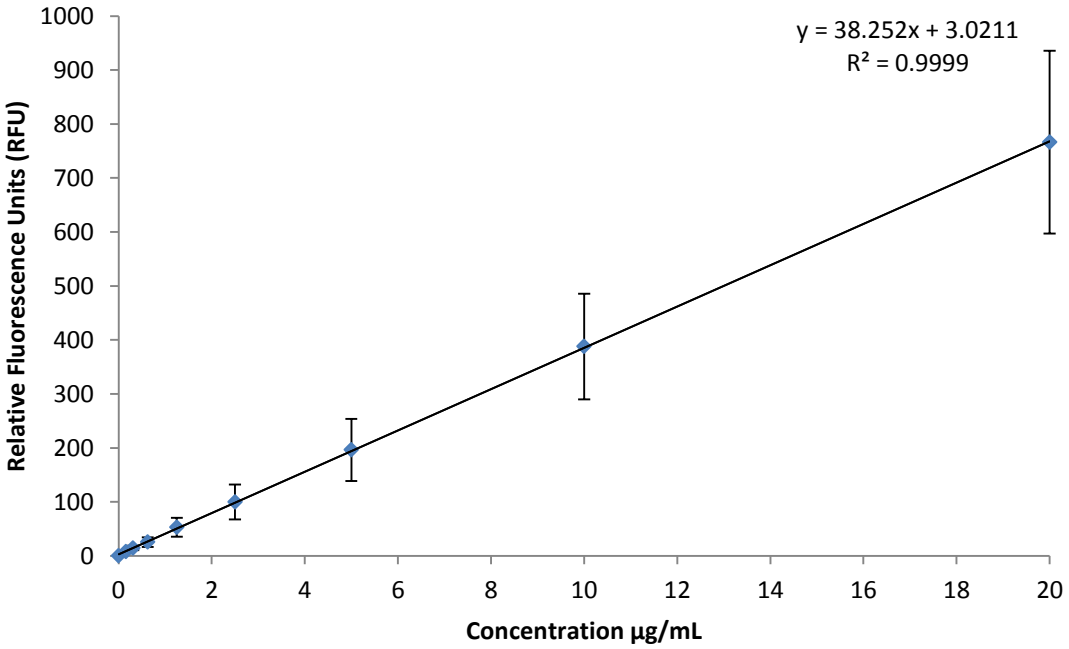


Figure 3.21 Calibration curve for BSA-FITC. Results represent mean ± SD of triplicate assays.

3.3.3.2 Limits of detection and quantification

The limit of detection is the lowest amount of analyte detected but not quantified and the limit of quantification is the lowest amount of analyte, which can be quantitatively determined. The limit of the detection calculated, from triplicate samples from three separate assays was 0.929 ± 0.05 µg/mL and the limit of quantification was 2.015 ± 0.06 µg/mL.

3.3.3.3 Speed, time and frequency of centrifugation

Subsequent to the production of the liposome formulations, it was essential for the precision of the assay to remove all unencapsulated material. The results obtained (Figure 3.22) confirmed that one centrifugation step at 29,771 g was sufficient to remove the fluorescent molecules. The remaining concentration after one centrifugation step was 0.74 ± 0.21 µg/mL, which was below the limits of quantification, specified previously (section 3.3.4.2). Subsequent centrifugation showed no further decrease in the levels of fluorescence, with no statistical difference between the levels of fluorescence after 30, 60 or 90 minutes of centrifugation (Figure 3.22).

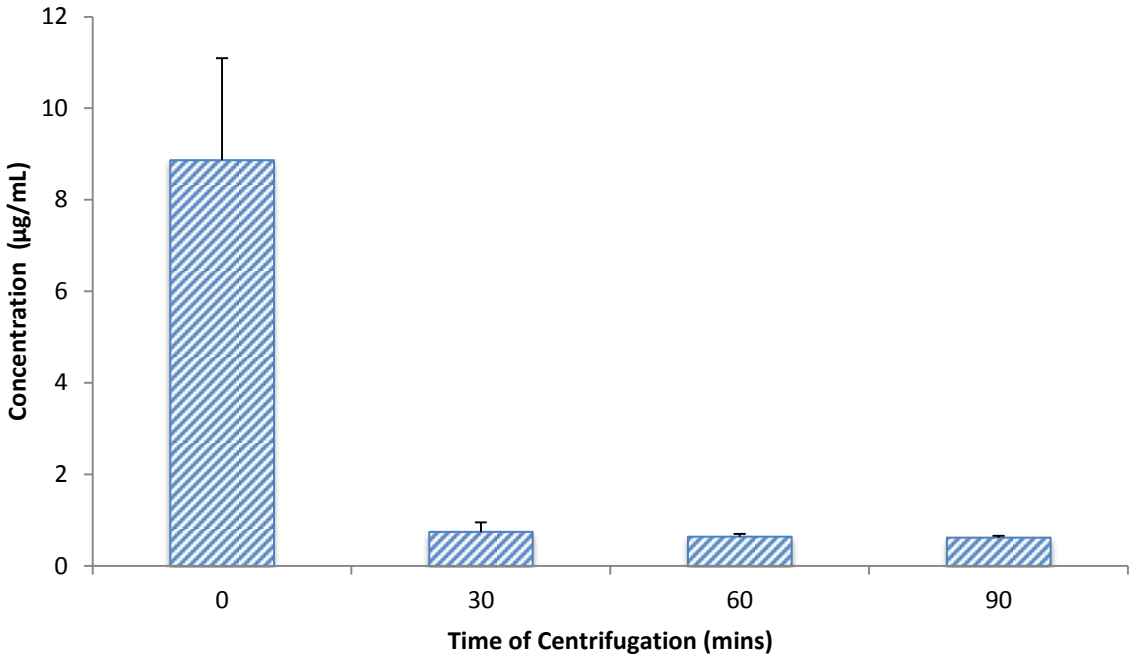


Figure 3.22 Concentration of BSA-FITC remaining in the supernatant subsequent to three steps of 30 minutes of centrifugation for up to 90 minutes. Results represent mean ± SD of n =3 assays.

The removal of unencapsulated material especially in the formulation of multilamellar vesicles was an essential part of establishing a methodology for analysis of encapsulated material. Previously used methods include separation by a gel filtration column as shown by Daleke *et al.*, (1990) or dialysis and ultracentrifugation (Szoka *et al.*, 1980). The results in Figure 3.22 show that effective removal of unencapsulated BSA-FITC was feasible.

3.3.3.4 Effects of lysis chemicals on the BSA-FITC concentration curve

A lysis agent was required for the disruption of the liposomal vesicles in order to measure the amount of entrapped material and thus it was necessary to establish how the presence of the agent would interfere with any readings from the assay. Of the two lysis agents tested (isopropanol and Triton X; Figure 3.23), isopropanol was shown to significantly increase the measured fluorescence activity ($p<0.05$) whilst the presence of Triton X made no significant difference even at the higher concentrations of 5 µg/mL and 10 µg/mL. A good linearity of the assay was shown even in the presence of isopropanol and Triton X with R^2 values similar to the normal calibration (0.9886, 0.9991, and 0.9998 respectively).

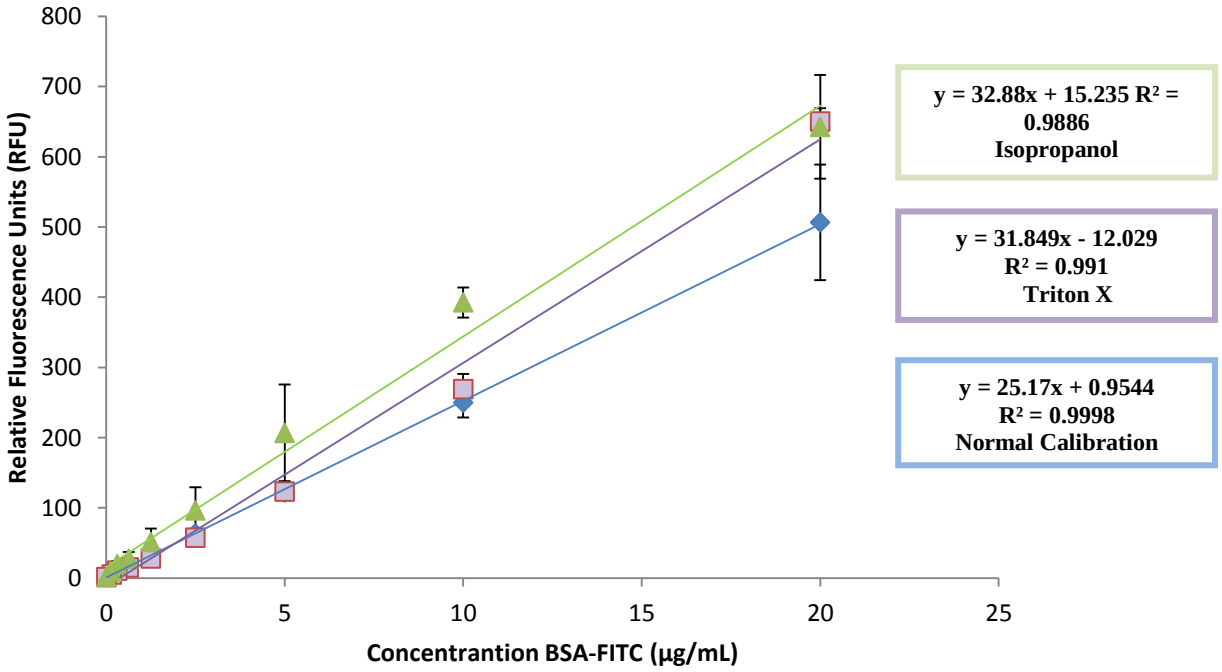


Figure 3.23 The effect of isopropanol and Triton X on the BSA-FITC fluorescence assay. Results represent the mean ± SD of triplicate assays.

Based on the results obtained Triton X was chosen as a disruption agent for liposomes as it interfered the least with the assay as according to ICH guidelines the specificity is defined as the ability to assess unequivocally the analyte in the presence of components which maybe present upon analysis with Triton X this is possible (ICH, 2006). In addition, this non-ionic surfactant has

been used extensively for the purposes of liposome disruption (Urbaneja *et al.*, 1988, Ruderman and Grigera, 1986, de la Maza and Parra, 1996).

3.3.3.5 Determination of BSA-FITC release from liposomes

Having optimised the various parameters of the assay, it was necessary to establish if BSA-FITC could be encapsulated within the liposomal formulations of DPPC:Chol 16:16 µMol and DPPC:Chol:BrChol 16:4:12 µMol and thus quantify the amount entrapped.

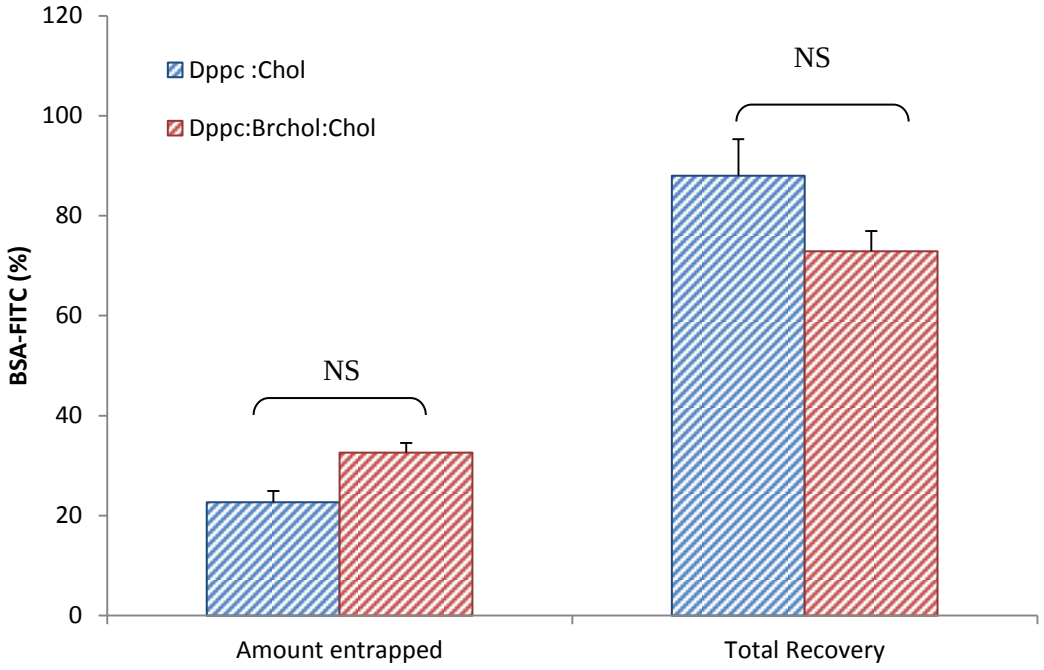


Figure 3.24 The total recovery and entrapment of BSA-FITC (%) of DPPC:Chol 16:16 µMol and DPPC:Chol:Brchol 16:4:12 µMol preparations. Mean particle size of liposome formulations of DPPC:Chol 13.4 ± 5.26 µm and DPPC:Chol:Brchol 11.8 ± 3.1 µm. Results represent mean ± SD of n =3 batches.

Protein entrapment for DPPC:Chol and DPPC:Chol:BrChol formulations were not significantly different (22.6 ± 2.25 % and 32.64 ± 1.91 % respectively; Figure 3.24) with total recovery of the protein being above 70 % in both cases. Studies by McWilliam and Stewart, (1989) using neutral liposomes (PC) for the encapsulation of protein have shown entrapment levels of up to 29 %, similar to levels observed in the results in Figure 3.24. However in the studies conducted by McAllister *et al.*, (1999) the dehydration-rehydration method was used, which involves freeze drying empty liposomes and resuspending them with the protein in buffer, entrapment levels of up

to 45 % were observed. This method promotes higher entrapment due to morphological changes, which occur during the process of freezing and thawing of the liposomes and can result in increased aqueous volumes (Mayer *et al.,* 1996). There is a wide array of methods available for the formulation of liposomes; the two aforementioned techniques are the dry film lipid hydration method and the dehydration-rehydration vesicles method. Other techniques include the reverse phase evaporation method in which liposomes are formed from water-in-oil emulsions using buffers and excess organic phase this is carried out under reduced pressure and have been found to have 4 times higher entrapment efficiencies as large unilamellar vesicles are formed (Szoka and Papahadjopoulos, 1978). The methods used for the formation of liposomes can impact upon the entrapment efficencies as generally MLV have low entrapment efficiencies which is thought to be due to the bilayers reducing the internal aqueous space (Szoka and Papahadjopoulos, 1978).

In terms of the specific entrapment of rIFNγ there are also variations observed in literature in regards to entrapment effciencies. In a study by Slooten *et al.*, (2001) neutral liposomes of PC prepared by the dry film hydration method and upon assessment for the entrapment of a cytokine showed very low entrapment efficiency at 5 %. Within these studies, the authors also observed that using liposomes with a more negative surface potential dramatically increased the entrapment efficiency. For instance for DPPC:DPPG which had a surface potential of -36 ± 6 mV showed entrapment efficiencies of 89 ± 1 %. In addition the higher observed entrapment by Slooten *et al.,* (2001) of negatively charged liposomes was not observed in the results shown in this study (Figure 3.24); even though the negative surface potential for the DPPC:Chol:BrChol was similar. The higher entrapment noted by Slooten *et al.,* (2001) in their anionic liposomes may be a result of electrostatic interactions, if the protein was slightly cationic in nature. In addition to electrostatic interactions, higher entrapment efficiencies have been attributed to the inclusion of cholesterol within liposome formulations. For example, Panico *et al.,* (1997) noted that tighter packing effects, promoted by cholesterol induced high (30-65 % depending on the protein) immunomodulatory protein entrapment in MLV composed of 10 mg lipid for DPPC and DPPC:Chol at 9:1 mg. In the results shown in this, study (Figure 3.24) there was no significant difference in the amount entrapped even with the inclusion of cholesterol. In another study using liposomes for the

entrapment of rIFNγ with the dry film hydration method, observed entrapment levels were 11 % but using a freeze-thaw method the IFNγ encapsulation increased to 29 % (Goldbach *et al.*, 1999.)

3.3.3.6 Confocal Microscopy

An important aspect of this study was to use light microscopy in order to confirm developments in vesicle formation, size and morphology. The inclusion of a fluorescent probe in the bilayer can aid visual observation of vesicles and highlight the upper limits of the size distribution (Szoka and Papahadjopoulos, 1980). It was evident from the images (Figure 3.25 a) that the liposome vesicles from a formulation of DPPC:Cholesterol 16:16 μMol were well-formed vesicles with generally, a spherical morphology (Figure 3.25 a). Furthermore, BSA-FITC can be seen entrapped within the internal aqueous core of the vesicles (Figure 3.25 b and c), with rhodamine being used as a bilayer marker. Liposome formulations of DPPC:Chol:BrChol at 16:4:12 μMol were made using BSA-FITC at 5 μg/mL.

To visualise the internal aqueous compartment and the bilayer simultaneously, an additional fluorescent marker (dilC at 0.2 mol % at probe:lipid molar ratio) was added to the lipids prior to rotary evaporation and observed on a Leica multiphoton confocal microscope using an 63 X oil objective (Fig 3.25 d - i). The images show BSA-FITC entrapped within well formed vesicles and the aqueous compartment.The use of fluorescence microscopy further enhances the information which was obtained from the more basic light microscopy images. Using mulitiple probes within the vesicles allows them to be distributed in the bilayer and, in this case BSA-FITC was shown to be within the aqueous phase apartment as would be expected and previously reported (Bouvrais *et al.*, 2010).

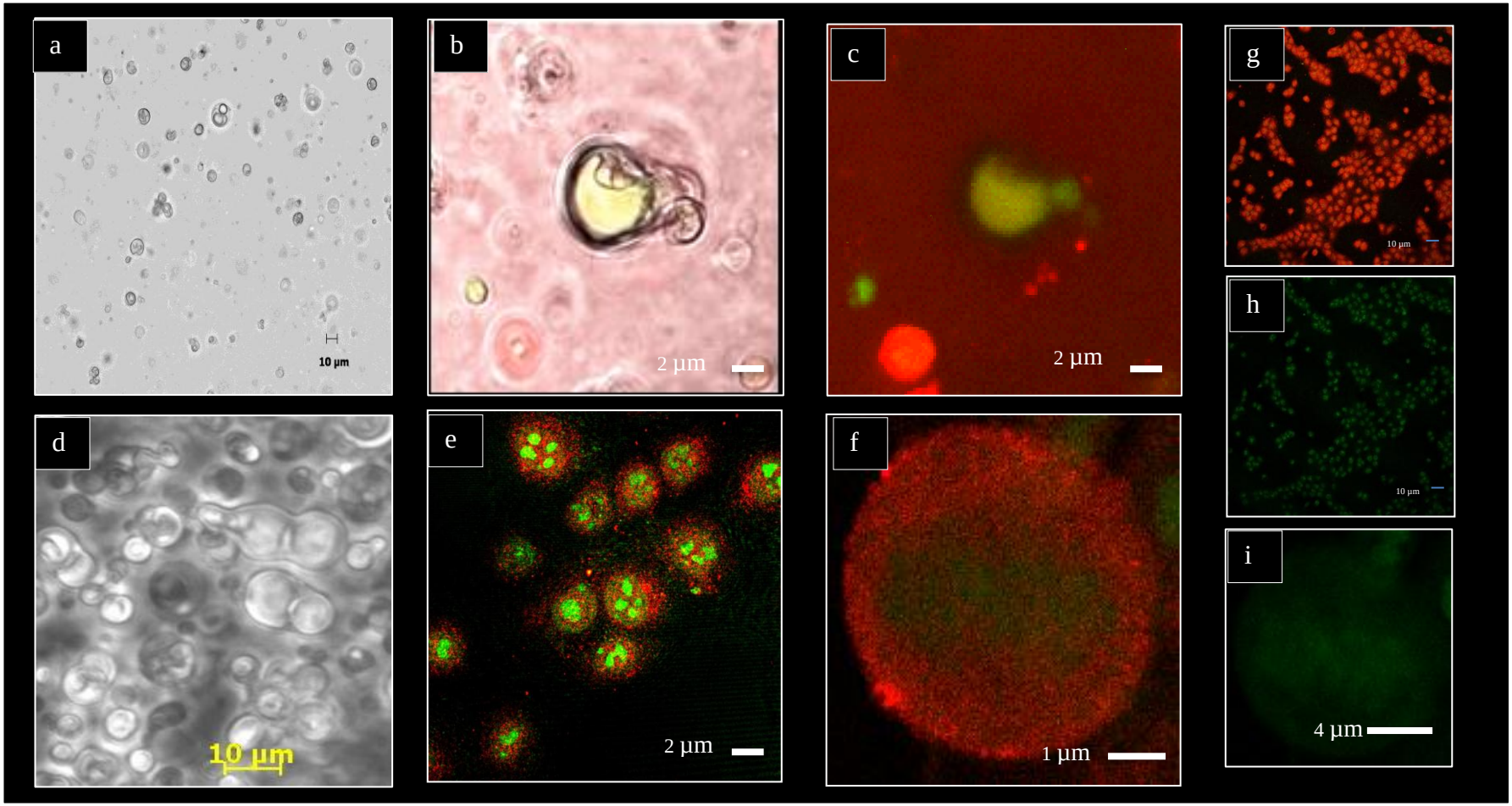


Figure 3.25 Confocal microscopy pictures of DPPC:Chol:BrChol at 16:4:12 µMol liposomes **(a-i)** taken with a multiphoton Confocal Microscope using a 40 X objective. The lipid bilayer is shown by the red colour, and the internal aqueous compartment by the green fluorescent marker. A Zeiss Axiovert A1 and a 40 X objective were used for image **(a)** and a Leica confocal microscope for subsequent images. The green fluorescent marker shown is BSA-FITC and the red marker is the dye dilC for all images except **(c)** where Rhodamine was used.

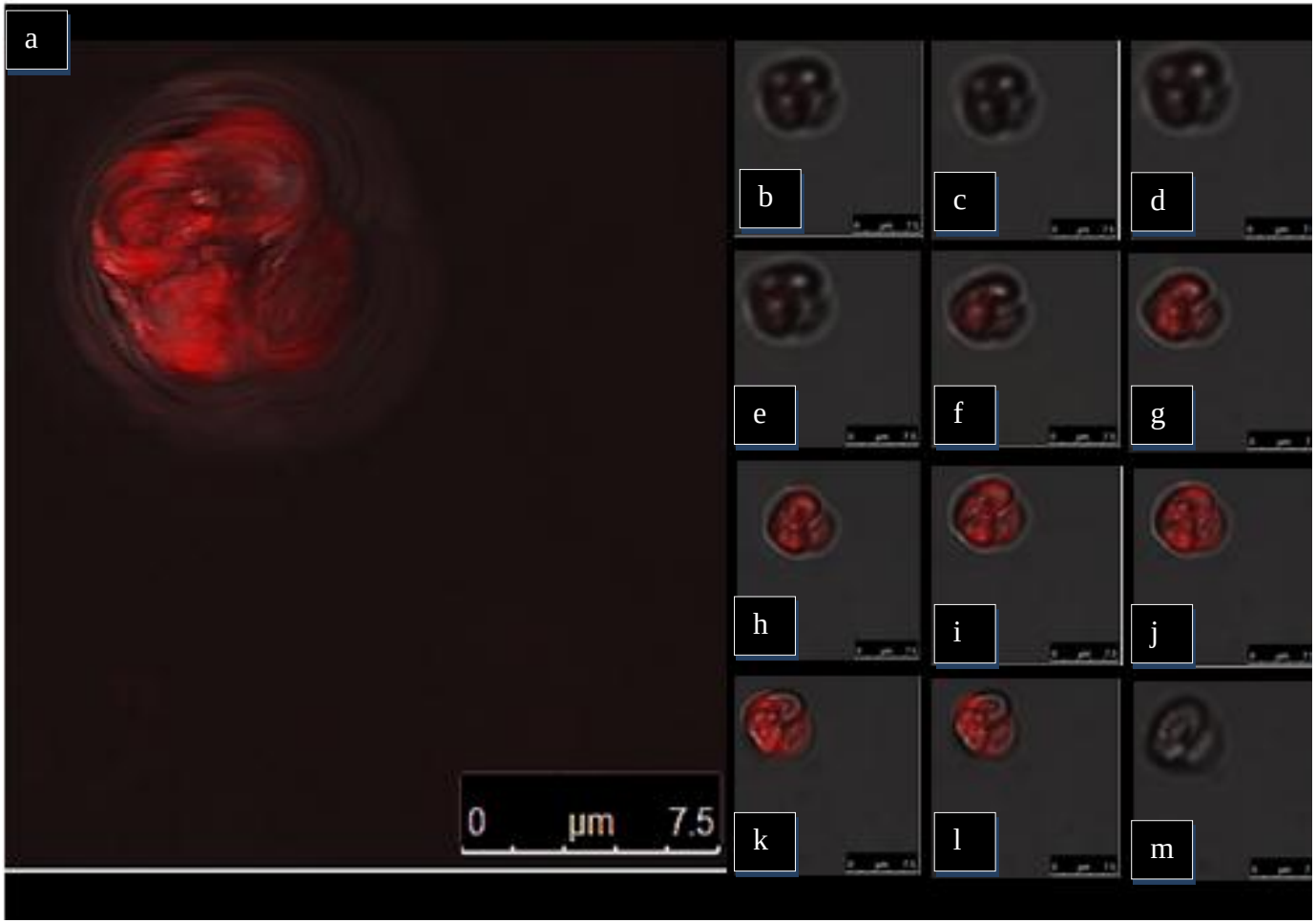


Figure 3.26 (a)The construction of a 3D visualisation of a liposome using confocal microscopy of DPPC:Chol liposomes with the fluorescent marker Dilc added at 0.2 mol %. Representative slices used for the construction of the 3D image are also shown (b – m). Aleica confocal microscope was used under a 40 X objective.

Figure 3.25 e and f show the full vesicle structure; this was carried out using confocal microscopy by taking numerous images; thus, a three-dimensional image can be built up of the vesicle. Image software can show each 'slice' from the vesicle and allow the rendered 3D projection to be visualised at 360 °C angles. An example of how this was achieved is shown in Figure 3.26, the 3D construction (Figure 3.26 a) of DPPC:Chol (16:16 μMol) liposomes was made using a number of z-stacks taken of the liposome vesicles (Figure 3.26 b – m). This has been shown recently (Vequil-Suplicy *et al.,* 2010) where giant unilamellar vesicles made of 1,2-dioleoyl-s-3-phosphn-glycero-[1-rac-glycerol] sodium salt, egg sphingomyelin and cholesterol were observed using 3D projections and the fluorescent dye DilC_{18}. Through microscopy the formation of lipid rafts was shown and how these are reduced when charged lipids are used. The images for the purposes of this study demonstrate the defined morphology and the successful encapsulation of protein marker within the liposomes for both neutral liposomes and the liposomes containing synthesised dibromocholesterol.

3.3.4. Stability assessment of liposome formulations

Subsequent to the synthesis of dibromocholesterol it was important to assess the stability of the liposome formulations thus a stability study was carried out assessing formulations of DPPC 32 μMol, DPPC:Chol 16:16 μMol and DPPC:Chol:BrChol 16:4:12 μMol. In this way the effects of placing the aqueous dispersions at 4 °C, 25 °C and 40 °C was observed by assessing the three physicochemical parameters of size, zeta potential and the size distribution of the vesicles (span).

3.3.4.1 Stability of saturated lipids in liposome formulations

In order to assess the impact of the presence of the dibromocholesterol formulations made up of the individual components were assessed thus in this instance DPPC formulations of 32 μMol. The study of stability at the three temperatures of 4 °C, 25 °C and 40 °C was carried out over 28 days.

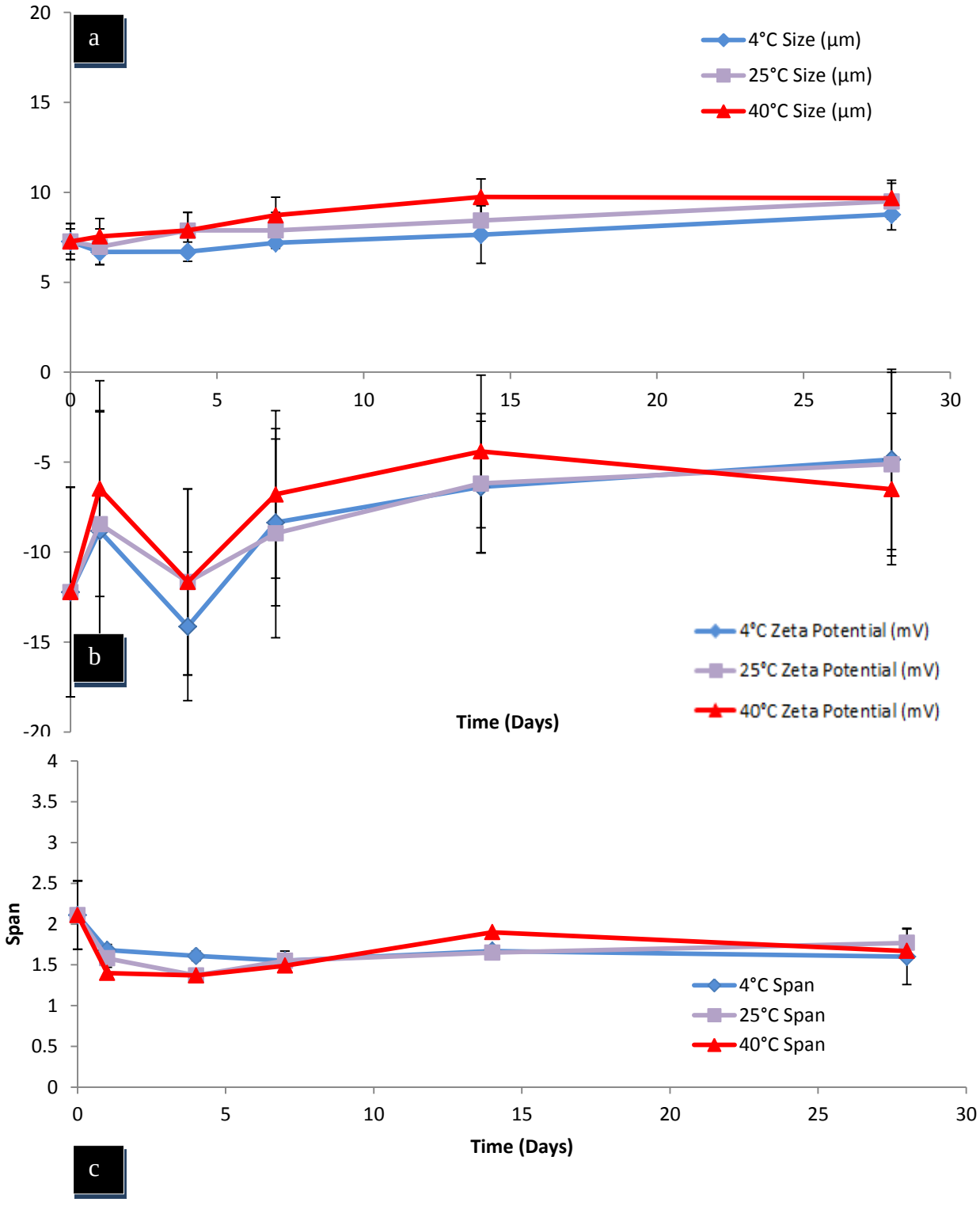


Figure 3.27 Stability assessment of saturated phospholipids (DPPC 32 µMol) over 28 days at 4 °C, 25 °C and 40 °C. Measurements were taken of (a) Size (µm) and (b) zeta potential (mV) and (c) span measurements at Day 0, 1, 4, 7, 14 and 28. Results represent mean ± SD of n =3 batches.

In this study, DPPC 32 μMol formulations were assessed for variations in size, zeta potential and span (Figure 3.27 a-c), there was no statistical significance across the assessed parameters over the 28 days even at 40^{o}C. DPPC is a commonly used phospholipid and is known to exhibit greater stability and less leakage over a range of temperatures due to the relatively higher transition temperature (Tc) of the synthetic phospholipid of 41°C (Chen *et al.,* 2010). DPPC is a saturated phospholipid and is thus more stable than the unsaturated counterparts, which are more susceptible to oxidation (which take place via a free radical chain mechanism) (Grit and Crommelin, 1993).

As the storage conditions assessed were not above the transition temperature of the lipid, the liposomes were stable over the 28 day study although neutral liposomes normally have a tendency to aggregate this was not observed in the results obtained (Casals *et al.,* 2003). The results observed (Figure 3.27) highlight that the single lipid component of DPPC 32 μMol forms stable liposomes and even in conditions of accelerated stability testing at 40 °C were able to remain stable as indicated by the physicochemical characterisation results.

3.3.4.2 Stability of saturated lipids with the inclusion of cholesterol

Stability assessments of aqueous liposome formulations of DPPC were previously assessed and shown to be stable, however the inclusion of cholesterol for the stability of the vesicles was necessary. Similarly, assessments were made of size, surface potential and size distribution over 28 days at defined time points. DPPC:Chol (16:16 μMol) formulations exhibited stability in regards to size and zeta potential over the 28 day period of the study (Figure 3.28 a and Figure 3.28 b) and there was no significant difference observed at any stage. In terms of the size distribution, the span was lower at day 7 for 40 °C and thus when compared to the other time points had a significant difference ($p<0.01$) although when comparing the span from day 0 to 28 there was no significant difference for any of the temperatures assessed in the study (Figure 3.28 c). The observed stability assessment (Figure 3.28) that combined an unsaturated lipid (DPPC) with cholesterol in equimolar ratio exhibited stability over the 28 day study.

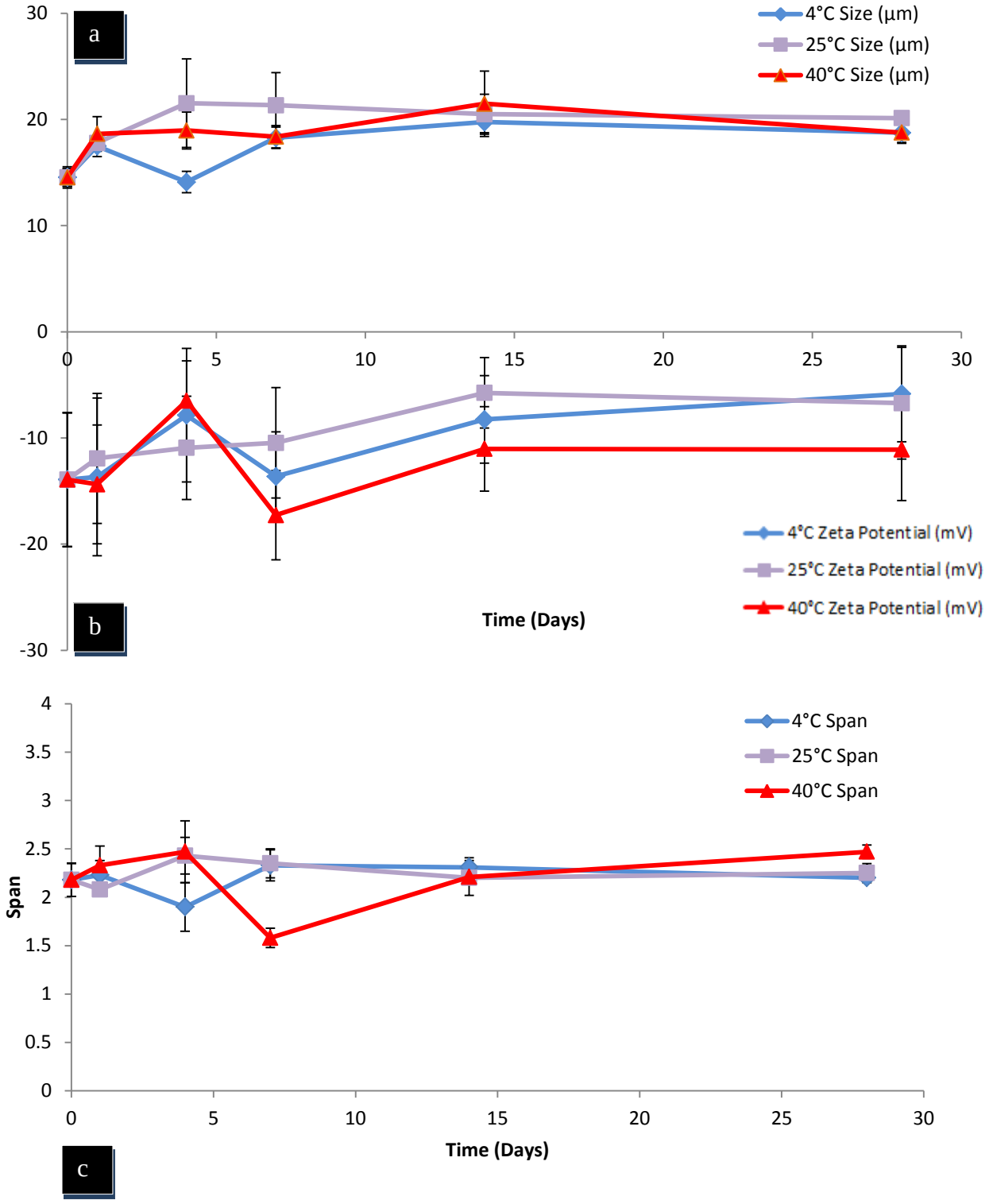


Figure 3.28 Stability assessment of saturated phospholipids with cholesterol (DPPC:Chol 16:16 µMol) over 28 days at 4 °C, 25 °C and 40 °C. Measurements were taken of (a) Size (µm) and (b) zeta potential (mV) and (c) span measurements at Day 0, 1, 4, 7, 14 and 28. Results represent mean ± SD of n =3 batches.

This data concurs with the findings found within literature on the stability of liposomal membranes with the inclusion of cholesterol; an amount of 33 % mole ratio is sufficient to eliminate the transition temperature (Szoka and Papahadjopoulos, 1980). Cholesterol alters the fluidic nature of the membrane and its behaviour in the liposome membrane is dependent upon the temperature of the surrounding environment (Chen *et al.,* 2010). Below the T_m it is thought to contribute to the disorder of the gel state however above the T_m it is able to enhance the stability of the liquid state and thus reduce membrane permeability (Chen *et al.,* 2010). Other studies have shown that phosphatidylcholine liposomes containing high concentrations of cholesterol were stable at 4 °C for several days and even by 53 days their stability had only declined moderately (Kirby *et al.,* 1980). Sulkowski *et al.,* (2005) found using NMR ^{31}P analysis that when cholesterol is present the structural changes in the bilayer decrease with increasing temperature whereas higher membrane fluidity is observed for formulations of DPPC alone. Although in the observed results in this study liposome formulations of DPPC were as stable as those containing cholesterol. In relation to producing stable reference standards in can be seen that the inclusion of cholesterol and lipids with transition temperatures above 40 °C allow enhanced stability of liposomal formulations; however the challenge lies in balancing the need for effective release and stability upon storage.

3.3.4.3 Stability of formulations containing the synthesised brominated cholesterol

Subsequent to the synthesis of brominated cholesterol and inclusion within liposomal bilayer many parameters were assessed including: physicochemical characterisation of size and zeta potential, vesicle morphology through microscopy techniques, sedimentation efficiency and the ability to encapsulate a protein marker. The studies thus far had shown that DPPC formulations with lipid alone and those formulations containing cholesterol were stable at 4 °C, 25 °C and 40 °C. It was important to assess the impact of the inclusion of dibromocholesterol under the same conditions. Therfore liposome formulations of DPPC:Chol:BrChol 16:4:12 μMol were placed at 4 °C, 25 °C and 40 °C in stability cabinets over 28 days.

In terms of vesicle size (Figure 3.29 a), at 4 °C and 25 °C there was no significant difference in size when comparing the formulations from day 0 to day 28 although there were some fluctuations

over the time points, the general trend suggests liposomes were stable over the 28 day period (Figure 3.29 a). However, at 40 °C there was a significant ($p<0.05$) increase in the size of the formulation after only 1 day with vesicle size increasing from (10.95 ± 0.64 µm) to (13.3 ± 0.93 µm) respectively. At this elevated temperature, on storage the vesicle size continued to increase by day 7 to 18.37 ± 1.06 µm (Figure 3.29 a). This instability of vesicles at higher temperature storage was also noted by light microscopy (Figure 3.29). By day 7 that there were instances of complete vesicle disintegration and fusion, thus any stability data would have been inaccurate and the study at this temperature was terminated at this point for size, span and zeta potential although light microscopy images were taken until the termination point at day 28 of the study.

In terms of zeta potential (Figure 3.29 b) by the first day there was no significant difference in surface charge under any of the storage conditions. The surface potential was significantly different ($p<0.05$) by day 4 and 7 when compared to day 0 at 40 °C changing from -51.27 ± 7.14 mV to -76.37 ± 1.06 mV. At 4 °C and 28 °C there were no significant differences observed over the 28 days. There were also no significant changes to the span over the 28 day study. The light microscopy images confirmed the pattern in stability which was observed from the particle size data (Figure 3.30) with vesicles at 4°C retaining shape and morphology and at 25 °C some morphological changes became apparent (at the day 14 timepoint). At 40 °C evidence of degradation was visible (Figure 3.30).

Electron microscopy images of the formulations were also shown comparing the formulation at day 0 to the formulations at 4°C, 25°C and 40°C at day 18 (Figure 3.31). The multilamellar nature of the bilayer was clearly evident from the formulation at day 0 and the fractured planes clearly show the individual bilayers (Figure 3.31 a - c). However an assessment of the formulations at day 18 indicates that bilayer fusion at 25 °C and 40 °C, although the vesicle itself seems to have maintained shape in some instances (Figure 3.31 h), there seems to be a lack of an internal aqueous compartments suggesting collapse of the bilayers (Figure 3.31 j - l).

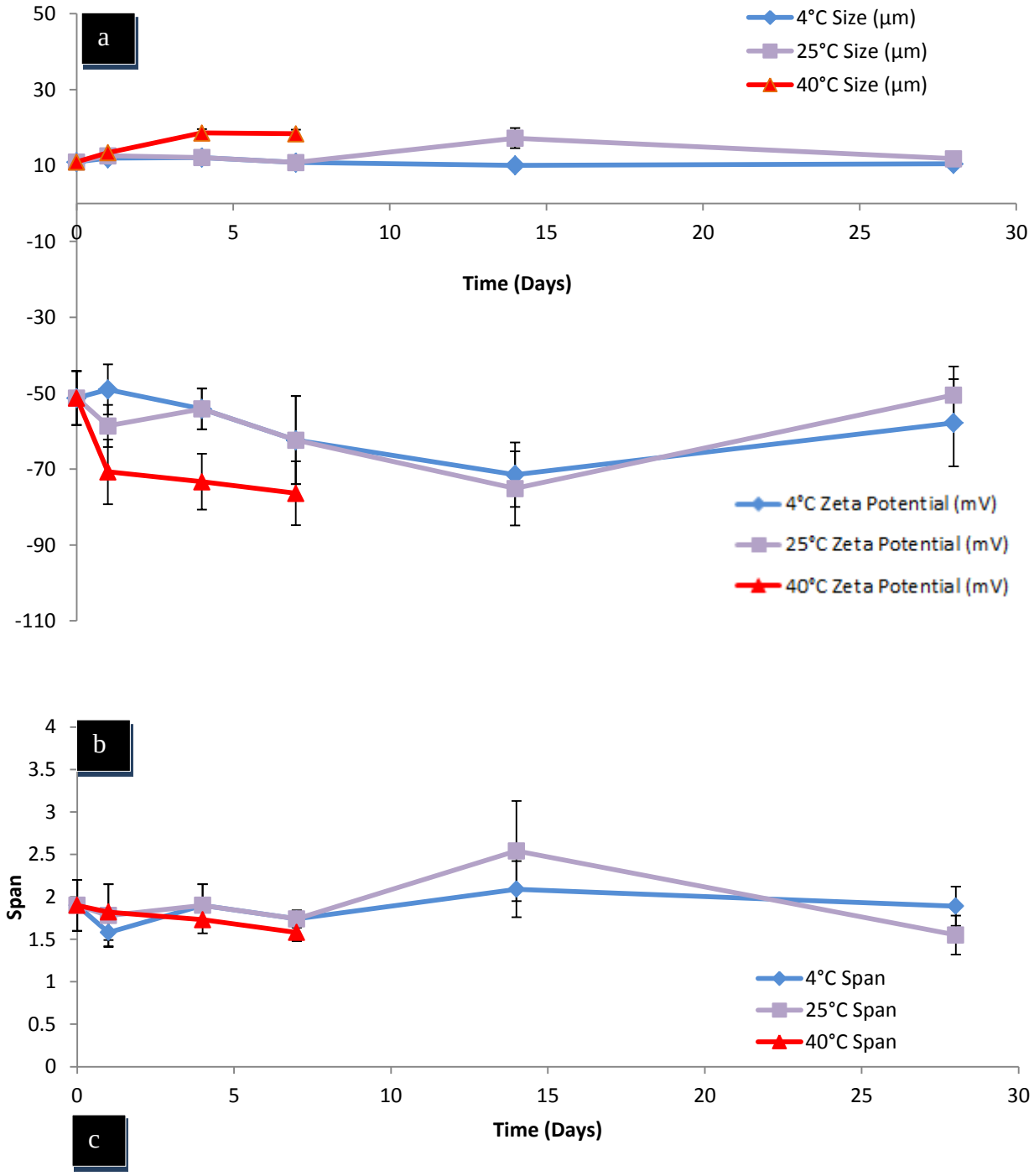


Figure 3.29 Stability assessment of liposome formulations containing brominated cholesterol (DPPC:Chol:BrChol 16:4:12 µMol) over 28 days at 4 °C, 25 °C and 40 °C. Measurements were taken of **(a)** Size (µm) and **(b)** zeta potential (mV) and **(c)** span measurements at Day 0, 1, 4, 7, 14 and 28. Results represent mean ± SD of n =3 batches.

Particle size variation, chemical stability, and leakage have proven to present problems during the long-term storage of liposomes (Crommelin and Brommel, 1984). The formulations tested of DPPC:Chol:BrChol 16:4:12 µMol had a strong negative zeta potential, and this is normally associated with increased stability of colloidal particles as they can remain dispersed. The zeta

potential of the formulations was -50 mV at day 0 and became more negative over the duration of the study, thus by this definition is considered to depict good stability (Plessis *et al.*, 1996). The variations observed in size of the aqueous formulations compares to those observed in literature wherein increases in the mean volume diameter have been observed at 4 °C and 25 °C of neutral and charged liposomes, and similarly instability is enhanced at higher storage temperatures (Plessis *et al.*, 1996). These observations are confirmed by Pahadjopoulos and Szoka, (1980), who comment that the retention of encapsulated material in terms of the storage of liposomes was optimal at 4 °C, and then 25 °C and 40 °C.

Such observations compare well to the results obtained in this study, which show that at 4 °C the changes to the physicochemical parameters assessed were reduced, and showed that storage was possible at 4 °C for a limited time (Figure 3.27, 3.28 and 3.29). At the higher temperatures, there was qualitative evidence of the impact of increased temperature on the changing morphology of the vesicles from light microscopy. Such effects can be attributed to the acyl lipid chains as they are susceptible to oxidation and more so in the presence of other factors (such as trace amounts of transition metal ions or radiation) and can lead to changes in the bilayer and also hydrolysis of the phospholipids catalysed by acids and bases (Mohammed *et al.*, 2006). The formulations containing DPPC alone or DPPC with equimolar cholesterol were stable although the inclusion of the dibromocholesterol did affect the stability of the liposomes at the higher temperature of 40 °C (Figure 3.29).

This was particularly evident through both the light and electron microscopy images (Figure 3.30 and Figure 3.31). The electron freeze-fracture technique can elucidate the effects that are occurring at a bilayer level and have shown the fusion of the bilayers for the vesicles at the higher storage temperatures (Meyer, 2001). The stability study was terminated at day 7 for those formulations at 40°C, as the physicochemical characterisation data of size and zeta potential in conjunction with the microscopy showed that aggregation and collapse of the vesicles had occurred.

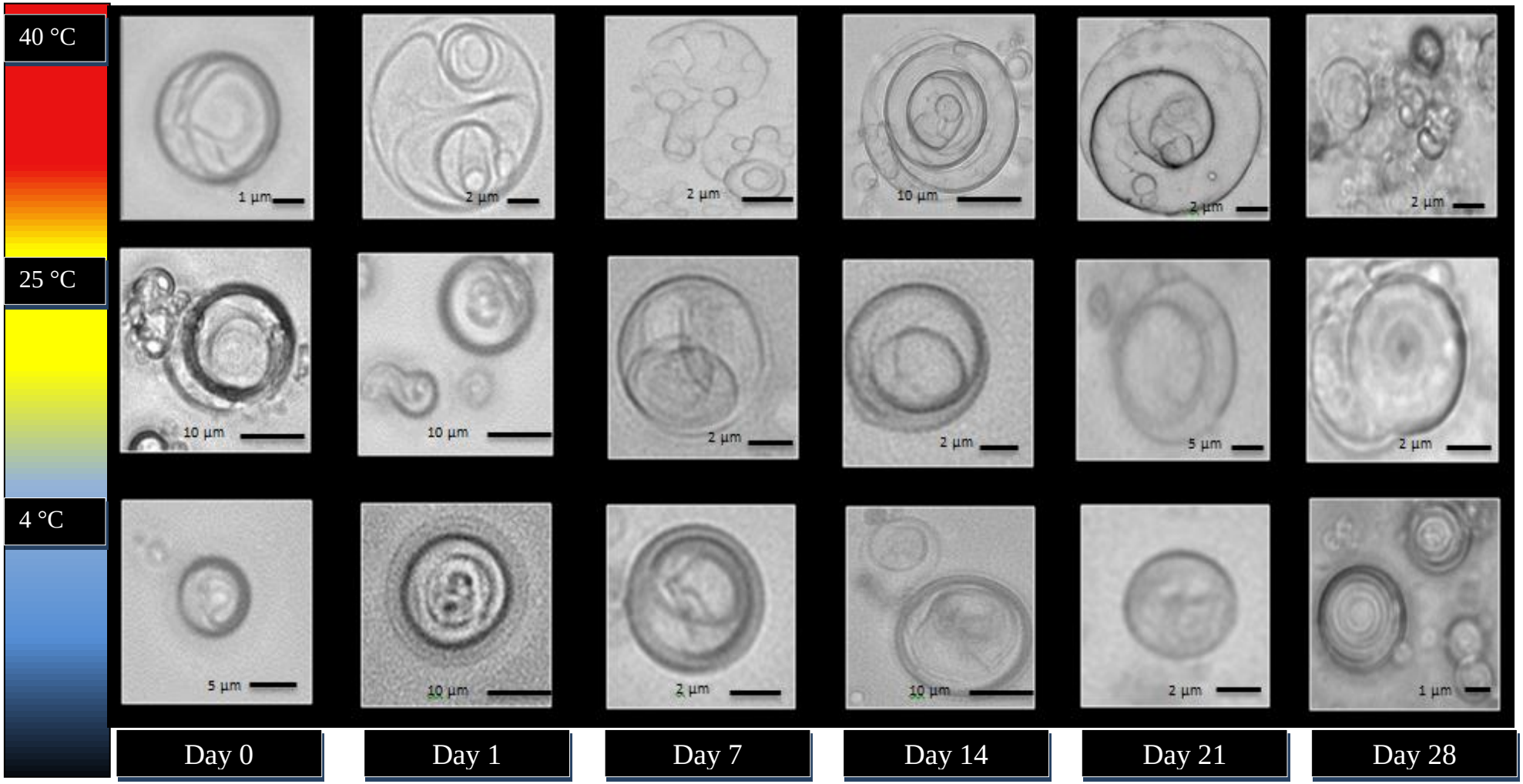


Figure 3.30 Representative light microscopy images taken throughout the duration of the stability study from formulations containing brominated cholesterol (DPPC:Chol:BrChol 16:4:12 µMol). Images were taken of formulations at 4 °C, 25 °C and 40 °C at Day 0, 1, 4, 7, 14 and 28.

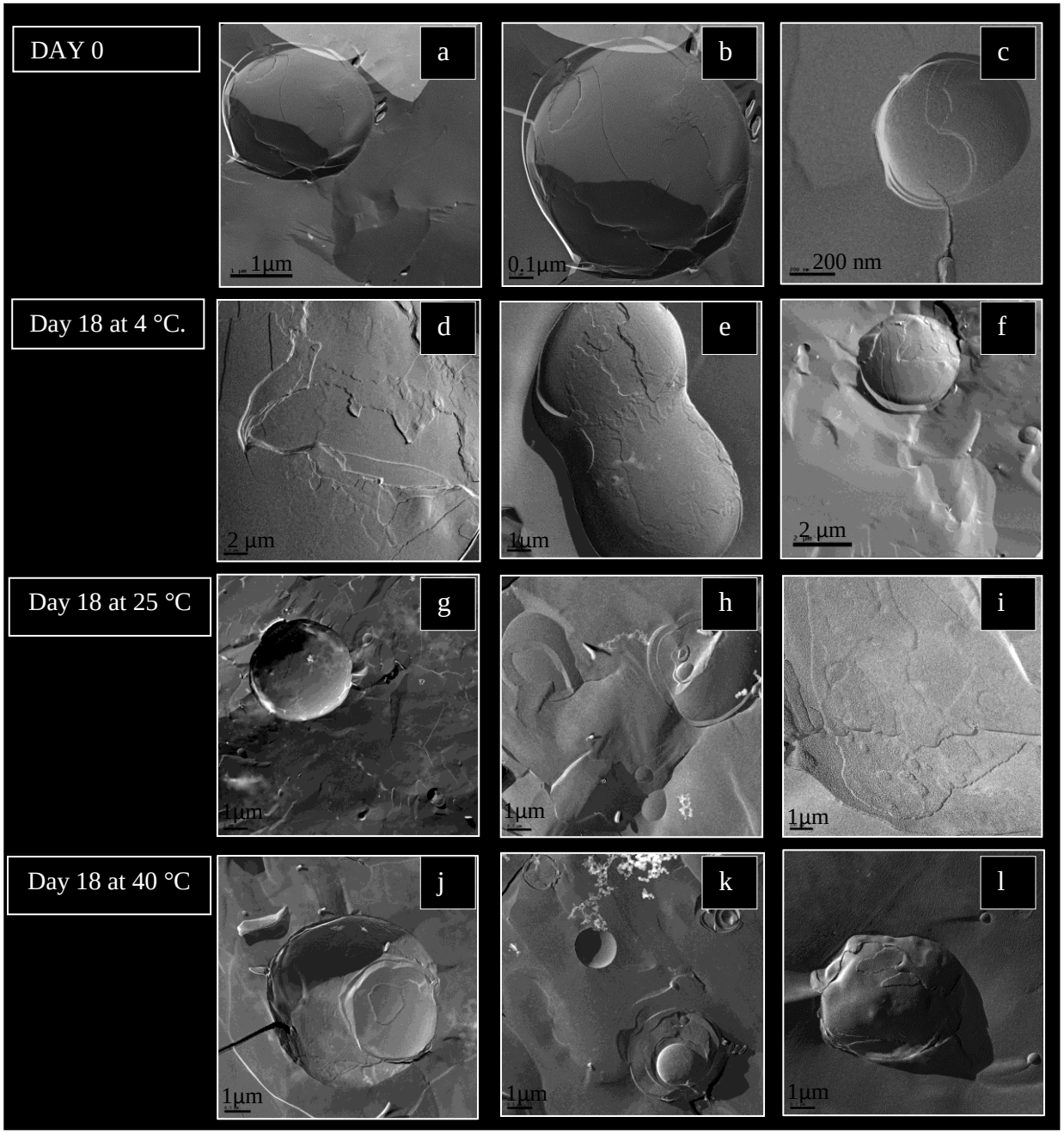


Figure 3.31 Freeze fracture electron micrographs of DPPC:Chol:BrChol 16:4:12 µMol at day 0 with formulations from day 18 at 4 °C at 25 °C and 40 °C.

Thus, overall the results have confirmed that liposomes as a carrier are stable with neutral lipids such as DPPC and with the inclusion of cholesterol, although the formulations of DPPC:Chol:Brchol were not as stable at the higher temperature of 40 °C. How stability impacts on the effectiveness of these formulations within the ELISPOT assay is not necessarily evident from this data, as a formulation is required that will rapidly sediment then release the cytokine entrapped, thus whilst it may seem a negative characteristic for the formulations to be unstable this may not be the case for this application. Protein retention was not assessed as a part of this study as

a wide range of liposomal compositions need to be tested within the ELISPOT assay to confirm which formulations are best suited in terms of release characteristics. For instance DPPC:Chol liposomes may have the ability to retain the protein well due to their stable nature; however this may not translate into effective spot formation, as the kinetics of release is an important factor within the assay (Lehmann, 2005). It is also of note that the formulations are placed directly on the ELISPOT assay overnight once hydrated, thus long-term stability of the aqueous formulations may not be necessary. The long-term storage of liposomes would require lyophilisation (freeze-drying) and the presence of cryoprotectants (Mohammed *et al.*, 2006).

3.3.5 Size Separation of liposome formulations using centrifugation, density gradients, and filtration

Previous work carried out has included the assessment of the sedimentation of liposome formulations subsequent to the incorporation of synthesised dibromocholesterol to increase the density of vesicles. However, the thin-film hydration method (Bangham *et al.,* 1969) used in the protocol because it one of the simplest methods, produces a heterogenous population of multilamellar vesicles whose size range can vary widely (Sharma *et al.,* 1997). Thus, various methods including; centrifugation, density gradients, and filtration techniques were investigated in order to remove some of the smaller vesicles that may lead to increased background with an ELISPOT assay if they are unable to settle to the bottom of the well and produce spots.

3.3.5.1 Variation of centrifugation speed and time

For the purposes of the removal of smaller vesicles, the basic technique of centrifugation was investigated. Formulations of DPPC:Chol:BrChol 16:4:12 μMol were subjected to five and ten minutes of centrifugation at speeds of 51 g, 115 g and 205 g (Figure 3.32 and Figure 3.33). After removal of the supernatant, the pellets were re-suspended in PBS. Formulations were characterised for size and span prior to and subsequent to centrifugation. The cumulative size distribution curves were also assessed to elucidate any shift of the curve subsequent to centrifugation (Figure 3.32 b, c, d and Figure 3.33 b, c, d).

At the centrifugation speed of 51 g, there was a significant ($p<0.001$) increase in the size of the formulation after centrifugation for both five (Figure 3.31) and ten minutes (Figure 3.33). The initial size of the formulations prior to centrifugation at five and ten minutes were 10.64 ± 0.30 μm and 7.91 ± 0.41 μm respectively and subsequent to 5 and 10 minutes centrifugation at 51 g this increased to 16.14 ± 0.51 μm and 11.44 ± 0.65 μm respectively (Figure 3.32 a and Figure 3.33. At the higher speeds of 115 g, again after five minutes there was a significant ($p<0.05$) increase in size when compared to the formulation prior to centrifugation (from 10.64 ± 0.30 μm to 12.78 ± 0.67; Figure 3.32). However, this was not the case for centrifugation after 10 minutes at 51 g, when comparing the size of the formulation prior to centrifugation no significant difference was observed. At 205 g there were no significant changes in the size observed at either five of ten minutes of centrifugation, similarly there were no other significant changes to the span (Figure 3.32 c and Figure 3.33 c).

The heterogeneity of the liposomal formulation significantly ($p<0.01$) decreased at the slowest centrifugation speed of 51 g at 5 minutes with an observed change in span value of the formulation prior to centrifugation at 1.74 ± 0.04, which decreased to 1.44 ± 0.04. This change is also visible from the overlaid cumulative distribution curves shown in Figure 3.32 b. At the higher centrifugation speed of 115 g and 205 g there was no significant difference in the span from the formulation prior to centrifugation. At ten minutes of centrifugation at 51 g, 115 g and 205 g there was no significant changes in the span data. The results indicate that the use of slower speeds does result in the pelleting of larger vesicles hence the observed increase in size however as the centrifugal pressure is increase on the formulation more of the smaller vesicles also become pelleted. The data obtained for centrifugation at five minutes (Figure 3.32) supports this in terms of particle size and span data however when centrifuging for the slightly longer time of ten minutes a significant increase in size was observed at the slower speed although this was not evident from the span data.

There have been various techniques previously investigated for the very purpose of size separation of liposomes, one such technique was size exclusion chromatography in particular the use of sepharose chromatography for the separation of MLV from SUV liposomes (Lundahl *et al.,* 1999).

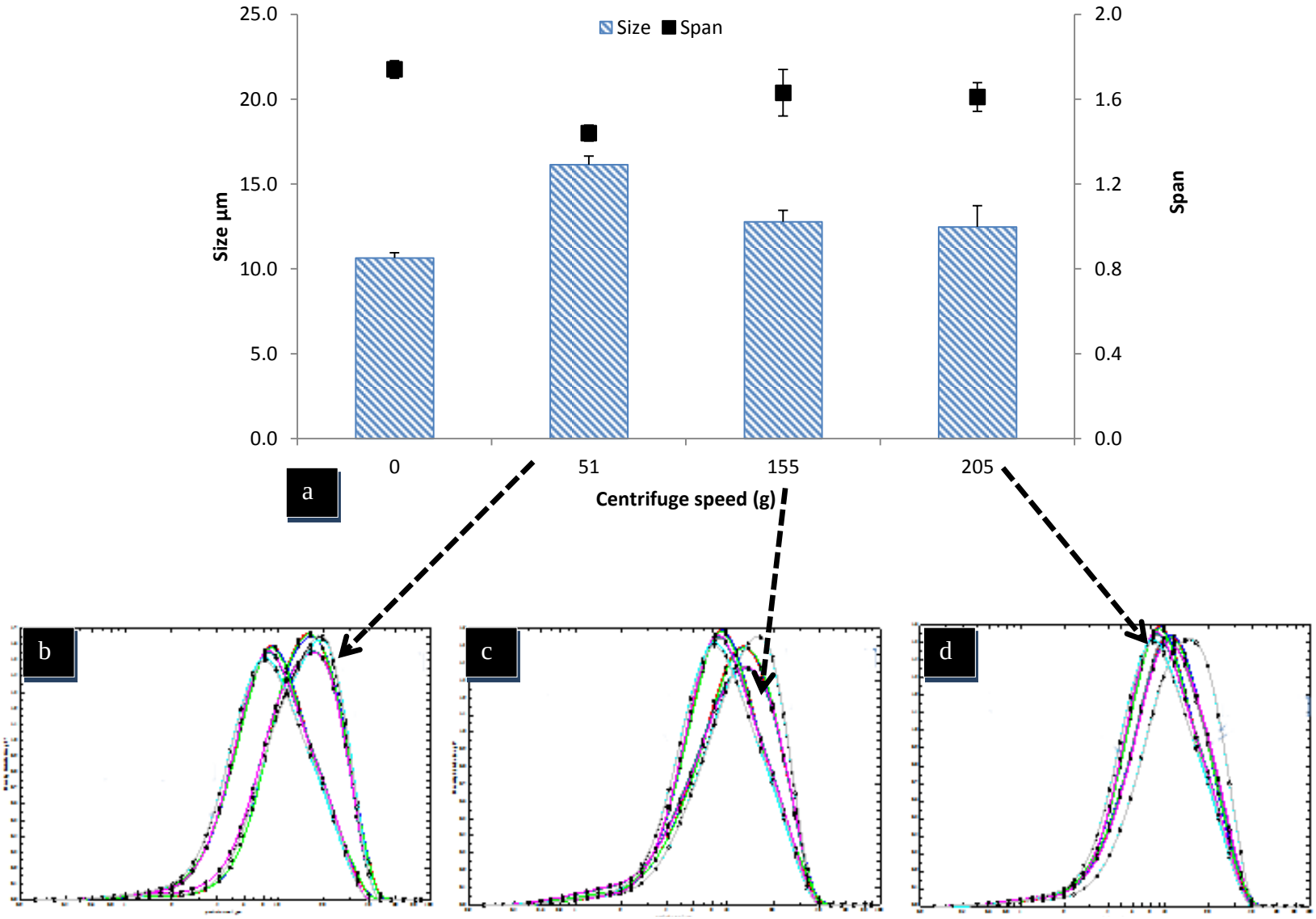


Figure 3.32 (a) Size and span data for formulations for formulations of DPPC:Chol:BrChol (16:4:12 µMol) after five minutes of centrifugation at with cumulative size distribution curves for n=3 for the corresponding speeds **(b)** 51 g, **(c)** 155 g and **(d)** 205 g overlaid with formulations prior to centrifugation. Results represent mean ± SD of n =3 batches.

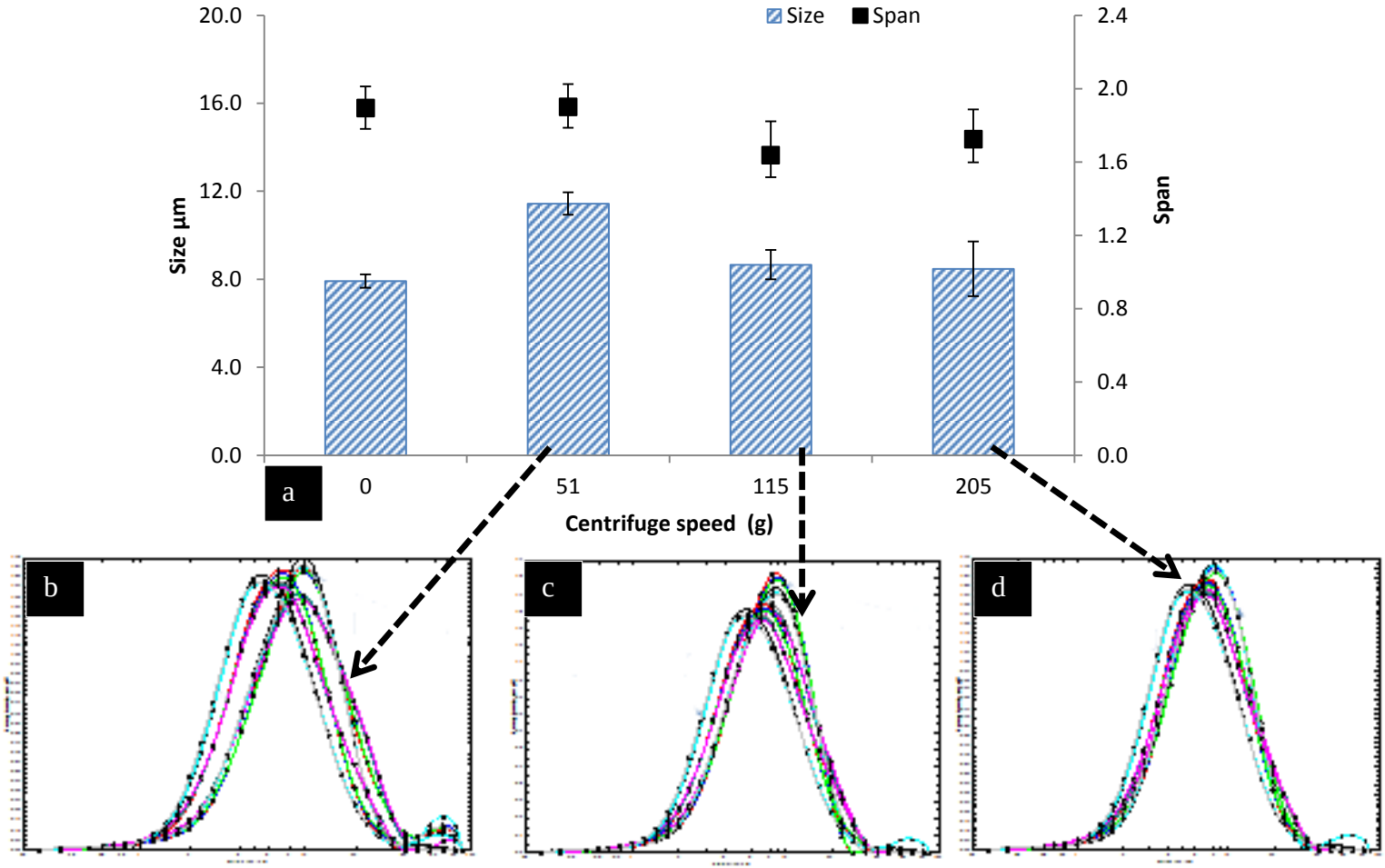


Figure 3.33 (a) Size and span for formulations for formulations of DPPC:Chol:BrChol (16:4:12 µMol) after ten minutes of centrifugation with cumulative size distribution curves for the corresponding speeds **(b)** 51 g, **(c)** 155 g and **(d)** 205 g overlaid with formulations prior to centrifugation. Results represent mean ± SD of n =3 batches.

However, one of the major disadvantages observed with such chromatographic techniques was the loss of formulation through the process itself, which in some studies has been as high as 40 %; with losses attributed to adsorption of liposomes to the gel matrices and physical blocking of the gel pores (Huang, 1969, Szoka and Papahadjopoulos, 1980). For the purposes of this project, the liposomes are essentially artificial cells and thus techniques focused upon were those ordinarily applicable for cell-based work, such as centrifugation (Alberts *et al.,* 2008). The use of centrifugation for the isolation of a size population from a polydisperse batch is a technique that can be carried out with relative ease (Gaumet *et al.*, 2007). In addition, the separation of MLV from SUV liposomes has been previously demonstrated using centrifugation at speeds of 100,000 g and 159,000 g as discussed by Szoka and Papahadjopoulos, (1980). If such high speeds are able to remove smaller vesicles then the protocol in place for the removal of unentrapped BSA-FITC used previously (section 3.3.4.3) would to some extent already be removing some of the smaller particles. The results from this centrifugation study indicated that it was possible to further enhance the removal of smaller vesicles and utilise centrifugation for the separation of smaller particles in the formulation; however, this required a short centrifugation time and a slow speed for the effect to be evident in particle size data.

3.3.5.2 Sucrose density gradients in conjunction with centrifugation

Subsequent to the use of centrifugation as a means to separate liposome formulations, an assessment was made of the use of sucrose density gradients in conjunction with centrifugation. Thus, initially a range of sucrose density gradients were assessed 10% w/v, 20% w/v, 40% w/v, 60% w/v an 80% w/v at 2660 g for 30 minutes (Figure 3.34). Then the combination of a heavy and light sucrose density gradient made up from 20% w/v and 80% w/v were also assessed at the centrifugation speed of 2660 g for 30 minutes (Figure 3.35). Three liposome formulations of DPPC:Chol:Brchol were produced 16:4:12 μMol and were initially characterised for size (μm) and zeta potential (mV). The average size of the formulations (n=3) was 9.1 ± 0.32 μm and the average zeta potential was -37.5 ± 3.9 mV. For the initial investigations a wide range of concentrations of sucrose were used at 2660 g in order to fractionate them into bands of various sizes; however, it was found that only two distinct bands formed.

Removed liposomes from each band underwent analysis for particle size and size distribution (Figure 3.34). There was no significant difference in the mean particle size of the two populations observed. There was however, a significant difference ($p<0.05$) in the span when comparing the formulations prior to centrifugation with those obtained in the upper and lower layer (this was 2.19 ± 0.17 to 1.71 ± 0.20 and 1.69 ± 0.08 respectively). Thus, there was an impact upon the heterogeneity of the formulation but not upon the particle size (Figure 3.34). In the separation of individual cell types from whole blood density gradients are ubiquitously used in particular two density gradients (Histopaque 1077 and Histopaque 1119); when the whole blood is layered on to the top of the two density gradients, and subjected to centrifugation cells will separate into different bands (Slifkin and Cumbie, 1992). As this work inherently involves viewing the liposomes as cells the same principle was applied to the liposome formulations and a 20% w/v and 80% w/v solution of sucrose was used (Figure 3.34). However, upon assessment of particle size and span, there was no significant difference in the liposomes obtained in the two obtained bands (Figure 3.35).

The investigative work on density gradients was not successful for the size separation of liposomal vesicles. One of the reasons for this difficulty was the lack of density difference between the liposome vesicles. For density gradients to be successful, the particles must separate due to the differences in sedimentation rate, determined by size, shape, and density (Sanchez-Lopez *et al.*, 2009). The fractionation of small unilamellar vesicles has been shown previously using glycerol density gradients at 19% v/v and 22% v/v by centrifugation at 45,000 rpm for 14 hours (Goormaghtigh and Scarborough, 1986). It was not practical to have a method, which would involve this duration of centrifugation as this could affect the stability of the entrapped cytokine. In addition, such a precise level of separation of very small vesicles was not required. The use of reduced viscosities of sucrose may aid the fractionation process (Goormaghtigh and Scarborough, 1986). According to Stokes law sedimentation rate is affected by viscosity, settling is reduced by the increase in viscosity (Troy, 2006). Thus in the subsequent investigations reduced concentrations of sucrose were used and assessed at a short and longer time points.

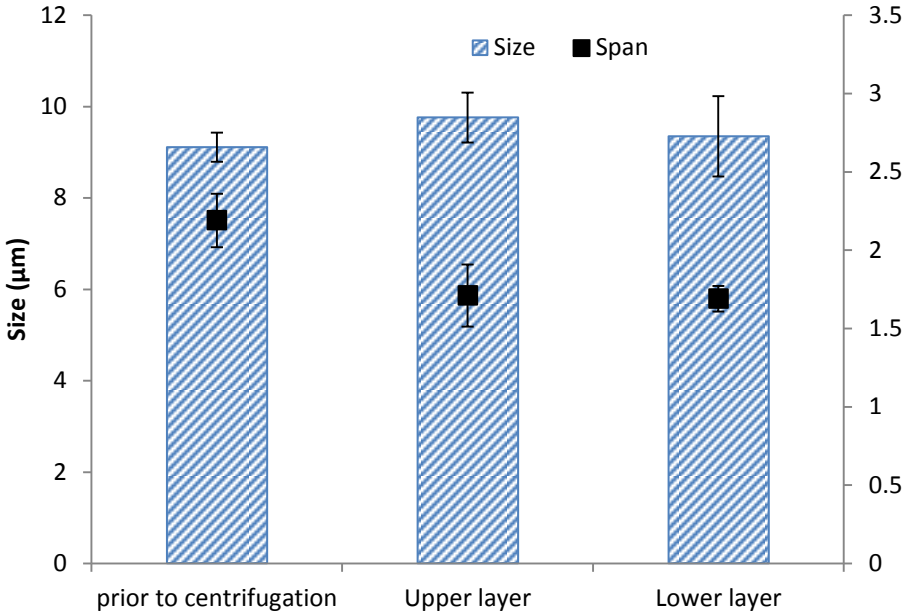


Figure 3.34 Size (µm) and span data for liposome formulations fractionated using sucrose density gradients at 10, 20, 40, 60 and 80% (w/v) sucrose. Results represent mean ± SD of n =3 batches.

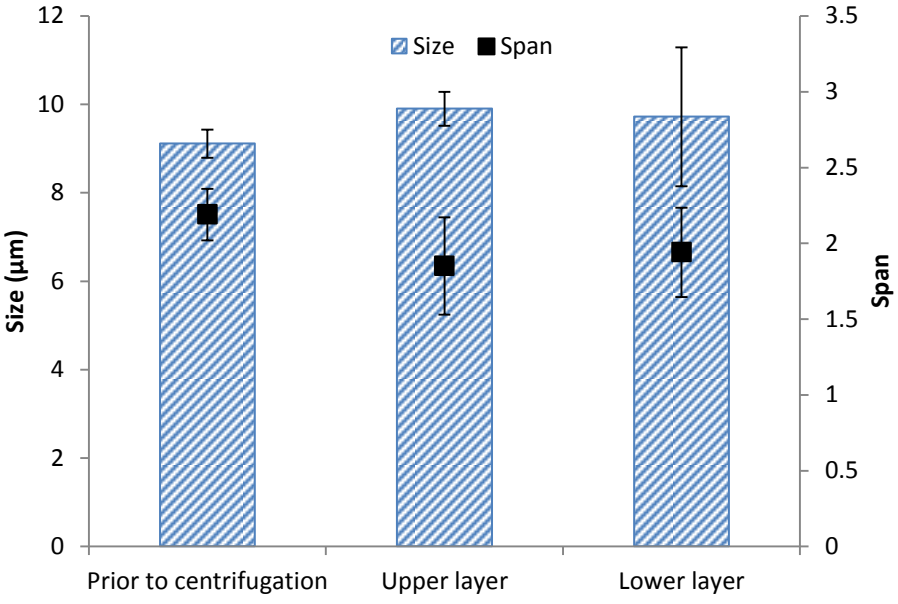


Figure 3.35 Size (µm) and span data for liposome formulations fractionated using sucrose density gradients at 20 % and 80% (w/v) sucrose. Results represent mean ± SD of n =3 batches.

3.3.5.3 The use of low viscosity, sucrose density gradients for the size separation of liposomes

To assess the effect of lower viscosity density gradients for the size separation of liposomal formulations with centrifugation, density gradients were prepared at 5, 15, 25 and 35% w/v with sucrose and centrifuged at 1) 2660 g at 30 minutes and 2) 434,902 g for 3 hours.

The formulations of DPPC:Chol:Brchol were produced 16:4:12 μMol were characterised for size and size distribution. When the lower viscosity density gradients were centrifuged with the liposomal formulations at 2660 g for thirty minutes (Figure 3.36), there was a significant difference in the span ($p<0.05$) and a significant difference in the size ($p<0.01$). When comparing the formulations prior to centrifugation to the upper layer separated band after centrifugation; the size increased from 9.20 ± 0.24 μm to 12.91 ± 0.29 μm and the span from 2.10 ± 0.14 to 1.65 ± 0.13 (Figure 3.36). There was no significant difference in size observed when the original formulation was compared to the particles in the lower band. The shift in the cumulative size distribution curves elucidates the change in size and span qualitatively (Figure 3.36 b). The cumulative size distribution curves of the lower band showed very little difference compared to the formulation prior to centrifugation (Figure 3.36 c). The effect of increased centrifugation time to 3 hours using the same density gradients (Figure 3.37) resulted in no significant difference in size by increasing the speed to 434, 902 g. In fact the band separation was not as clear.

These results (Figure 3.36) indicate that it is possible to significantly change the particle size and size distribution of a heterogeneous formulation by the removal of smaller vesicles using density gradient centrifugation through the optimisation of the speed, time and selection of density gradients. Although the upper band had the larger particle size, this was contradictory to that which is normally expected. An explanation could be due to the complexity of sedimentation of charged colloidal particles in viscous solutions. Chiu *et al.*, (2012) comments that the effect of the electrical double layer surrounding each charged particle can distort the fluid flow relative to the particle and this causes the induction of a sedimentation/migration potential. Thus, the anionic nature of the vesicles (Figure 3.14) may have affected the sedimentation velocity of the liposome vesicles.

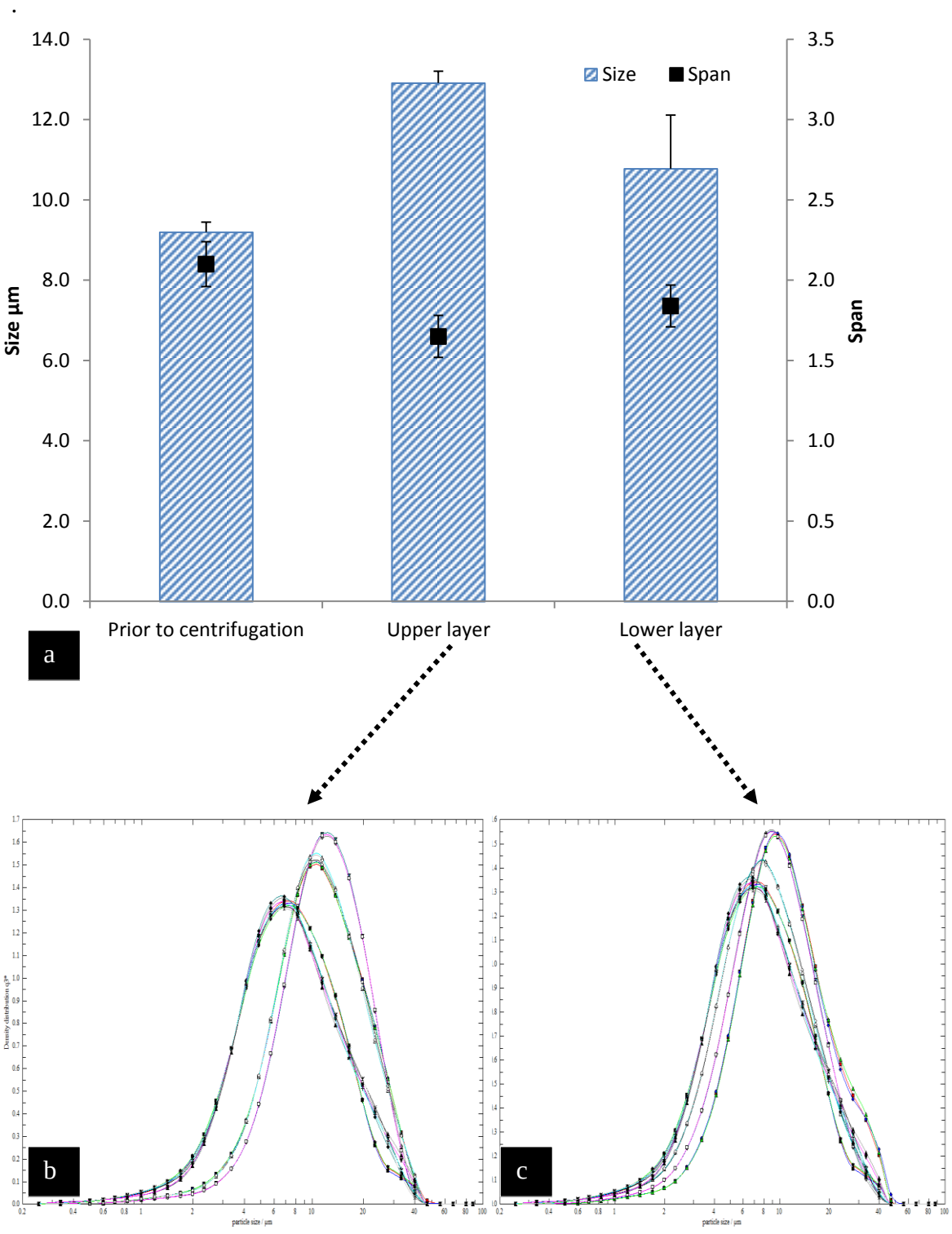


Figure 3.36 The characterisation of liposome formulations for size from obtained fractions subsequent to centrifugation (2660 g for 30 minutes) with density gradients at 5, 15, 25 and 35% sucrose (w/v). Results are shown for **(a)** mean particle size (µm) and span (**b)** Cumulative size distribution curves for the upper and **(c)** lower band. Results represent mean ± SD of n =3 batches.

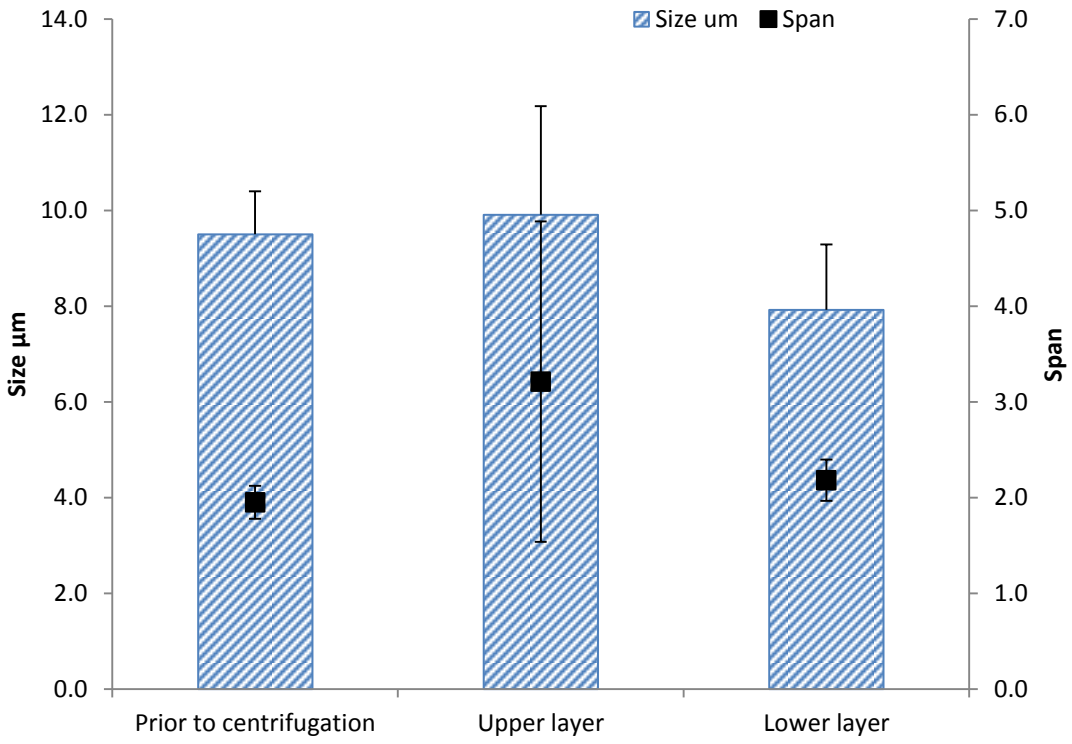


Figure 3.37 Size measurements for obtained fractions of liposome formulations subsequent to centrifugation (2660 g for 3 hours) with density gradients at 5, 15, 25 and 35% w/v. Results are shown for (a) mean particle size (µm) and span (b) Cumulative size distribution curves for the upper and (c) lower band. Results represent mean ± SD of n =3 batches.

By using sucrose density gradients in conjunction with centrifugation similarly to chromatographic techniques of separation there is contact of the vesicles with the matrix which was sucrose in this case, Stokes' law of sedimentation does not take into consideration any potential interactions or affinity of the vesicles with the dispersion medium (Banker and Rhodes, 2002). Thus the anionic nature of the vesicles with the presence of the sucrose brings additional parameters that effect the sedimentation rate. An additional aspect not considered within this study was actual recovery. In future work consideration of quantification of recovered formulations would be required. The recovery would affect the overall effectiveness of such techniques for the production of reference standards on an industrial scale. In addition, unlike centrifugation without density gradients, there are additional preparative steps that need to be carried out prior to and subsequent to centrifugation.

3.3.5.4. Use of a biosieve filter for the size separation of liposomes

Subsequent to investigation of centrifugation and density gradients, another technique assessed was filtration and whether it could be used for basic size separation of liposomes. Three batches of DPPC:Chol:BrChol were produced (16:4:12 μMol) of an average size of 10.6 ± 0.3 μm and the average zeta potential was -28.8 ± 2.1 mV (data not shown). The formulations were placed through the top of a filtration system using a mild vacuum and were subsequently collected from both above and below the filter. The change in size from the original liposome formulation (Figure 3.38) to those liposomes recovered from the top of the filter was significant ($p < 0.05$) changing from 10.94 ± 0.75 μm to 14.17 ± 1.55 μm and the decrease in the span from 1.94 ± 0.66 to 1.27 ± 0.14 was highly significant ($p < 0.001$).

The microscopy pictures (Figure 3.38 a-c) of the formulations collected above and below the filter show the presence of a few vesicles in the upper filtrate this suggests that the pore size was maybe slightly too large. However, if this was the case more would have been expected in the lower filtrate this suggested that the recovery from the filter is ineffective.

Filtration techniques have been previously used with aim of the removal of larger particles from liposomal formulations or for extrusion purposes to produce a more homogenous population of smaller vesicles (Szoka and Papahadjopoulos, 1980, Vemuri and Rhodes, 1995). Such techniques have also been used within the field of cellular work, although perhaps not as ubiquitously as centrifugation for the removal of cellular debris (Allegraza, 2008). Membrane microfiltration is used within the biological processing industry for the separation of particles in the size range of 0.1–10μm (Cláudia Sousa *et al.*, 2002). Thus, the application of filtration systems were considered for heterogenous liposome formulations and although it proved promising in the ability to produce a significant chance in the particle size; the inefficiency to actually keep all particles above this size on top of the filter is a significant drawback. The significant increase in size and the decrease in span although promising would need to be offset with a good recovery otherwise would not be cost effective especially for industry-based production of a reference standard.

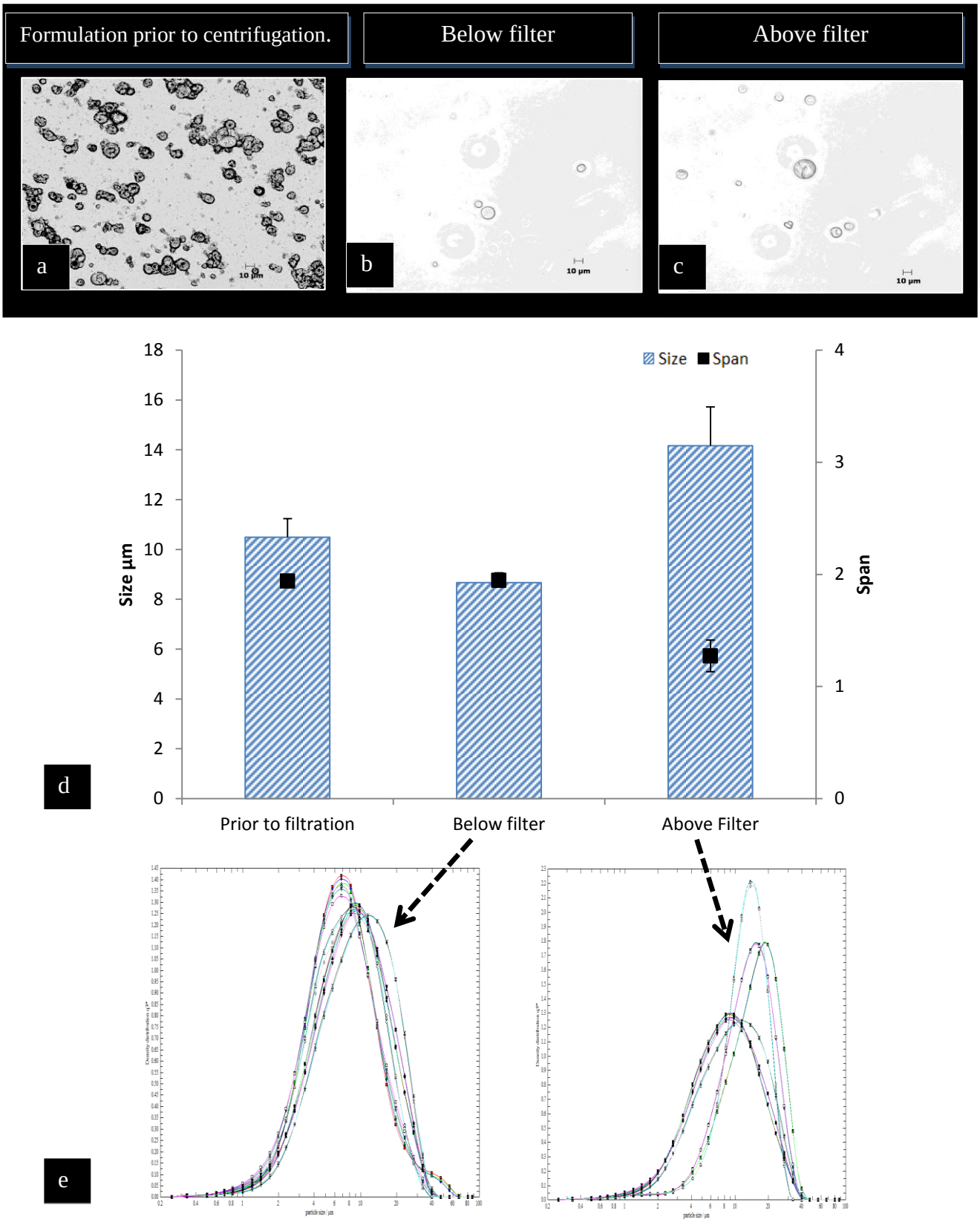


Figure 3.38 The effects on morphology and particle size of filtration on liposomes using a biosieve. Microscope pictures of original formulations (a) and upper (b) and bottom filtrate (c), with span and size data for formulations prior to and subsequent to filtration with a biosieve and **(e)** Cumulative size distribution curves. Results represent mean ± SD n=3 batches.

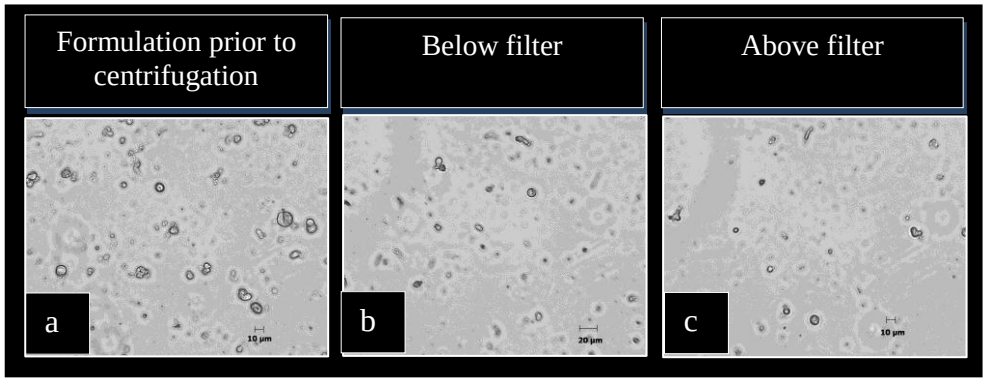
The problem of adsorption of vesicles to the membranes of the filters themselves has also been previously observed (Szoka and Papahadjopoulos, 1980). Such results highlight the importance of recovery and how this was highlighted through light microscopy, thus there would need to be careful consideration of the effectiveness and efficiency of techniques.

3.3.5.5 Filtration using polycarbonate filters of 3μm and 1.2 μm pore size for the size separation of liposomal formulations

As previously three liposome batches of DPPC:Chol:BrChol were produced 16:4:12 μMol these were initially characterised for size (μm) and zeta potential (mV). The average size of the formulations (n=3) was 8.25 ± 0.5 μm and the average zeta potential was -32.0 ± 7.8 mV (results not shown). Although the light microscope images suggested that the liposomal formulations filtered through the polycarbonate filters of 3 μm and 1.2 μm had become smaller (Figure 3.39 a – c and Figure 3.40 a –c) after the filtration process there was no statistically significant difference either in the particle size data nor the size distribution (Figure 3.39 d and Figure 3.40 d).

Polycarbonate filters are generally used for the process of producing a homogenous population of smaller vesicles when they are pushed through such filters under pressure using an extruder (Endruschat and Henschke, 2000; Vemuri and Rhodes, 1995); the results suggest that the aim of retaining the larger particles above the filter was not achieved. One of the reasons why size separation was not achieved is that liposomes are not solid particles but are made from flexible lipid membranes that can be easily deformed upon passing through narrow pores or can be excluded from larger ones (Lundahl *et al.,* 1999). The results suggest that polycarbonate filters would perhaps be more suitable for obtaining smaller size populations as shown through the previous studies in which they have been used.

Overall, the techniques assessed for the size separation of liposomes have highlighted how such simple vesicles in terms of structure are in fact quite difficult to separate in terms of size. This is due to the many factors that can impact on the effectiveness of such techniques especially as liposomal interaction with membranes or matrix can affect recovery, thus perhaps the use of centrifugation is the most practical technique for the purposes of this work.



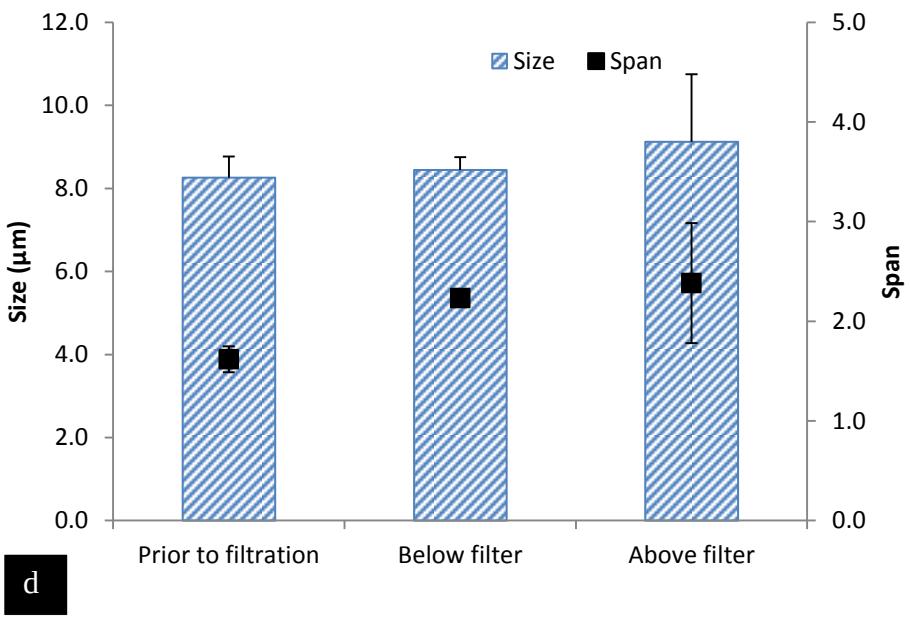
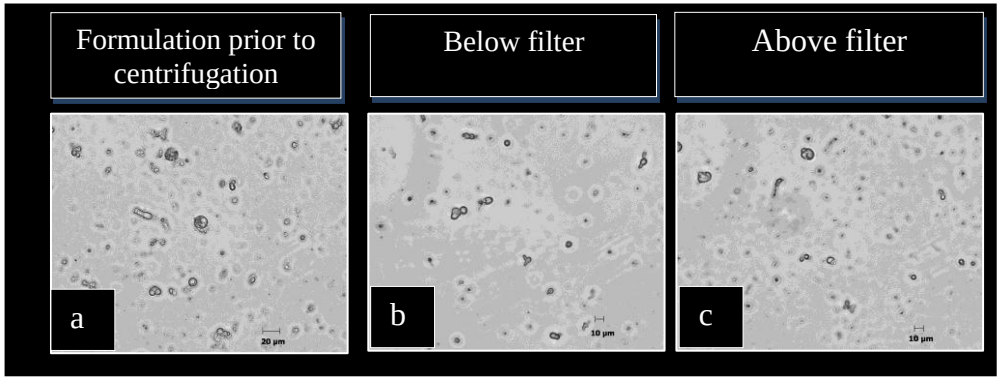


Figure 3.39 The effects on morphology and particle size of filtration using a 3 µm polycarbonate filter on DPPC:Chol:Brchol formulations. Bright field microscopy images **(a)** prior to centrifugation **(b)** liposomes below the filter and **(c)** above the filter**.** Size (µm) and Span **(d)** data prior to and subsequent to filtration. Results represent mean ± SD of n =3. batches.



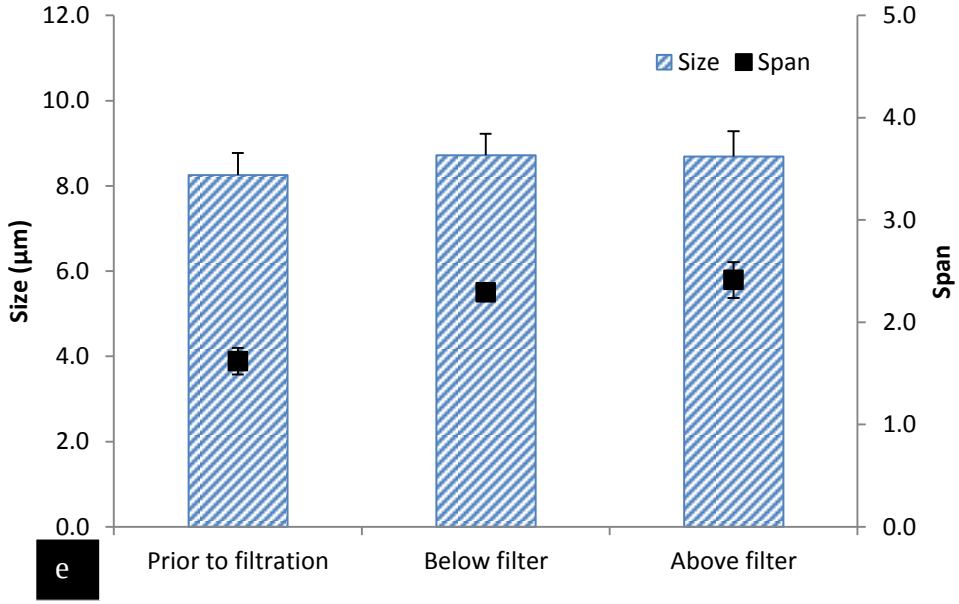


Figure 3.40 The effects on morphology and particle size of filtration using a 1.2 µm polycarbonate filter on DPPC:Chol:Brchol formulations . Bright field microscopy images **(a)** prior to centrifugation **(b)** liposomes below the filter and **(c)** above the filter. Size (µm) and Span data **(d)** prior to and subsequent to filtration. Results represent mean ± SD of n =3

3.4 Conclusion

Although fluorescence assays are well established it was important to validate the various aspects of the assay and method to be used for the measurement of entrapment and release from liposomes. Thus, the protocol developed offers a good basic screening assay for liposome formulations developed to measure release characteristics. Brominated cholesterol was synthesised and characterised thoroughly to reveal the modification had taken place, this was carried out through a wide variety of techniques including; TLC, ^{13}C NMR, ^{1}H NMR and IR spectroscopy. Stability testing on brominated cholesterol stored for six months established that it remained stable and there was no degradation. The sedimentation studies have elucidated the behaviour of liposomes in terms of their ability to settle; the results have shown that the presence of small vesicles in an SUV formulation tend not to settle. Thus, the size of the vesicles was shown to be related to their density and attempts made to increase the overall density by the addition of brominated cholesterol were complicated by the negative zeta potential of the formulations. Therefore the formulation containing a lower ratio of brominated cholesterol (DPPC:Chol:BrChol 16:4:12 μMol) of the total cholesterol content may provide more optimal sedimentation characteristics for future work. However, the microscopy images have elucidated that vesicles with or without the modified cholesterol are successfully able to encapsulate a marker and should therefore theoretically entrap the cytokine.

The results for size separation have clearly elucidated the difficulties in the separation of liposomes of different sizes. A mixture of cells such as whole blood can be quite effectively separated using centrifugation and density gradients because of the difference in density of the individual components. However, although the liposome formulations have a wide range in size from less than 1μm to greater than 10 μm the actual difference in density between each vesicle is much smaller. The results from the filtration in general suggest that if any of the techniques have shown promise for further optimisation it is filtration with the biosieve; however, the size of the pore is 5 μm and this is the minimum attainable for this type of filter. At this stage the inefficiency of keeping all the particles above this size on top of the filter is another drawback. This initial

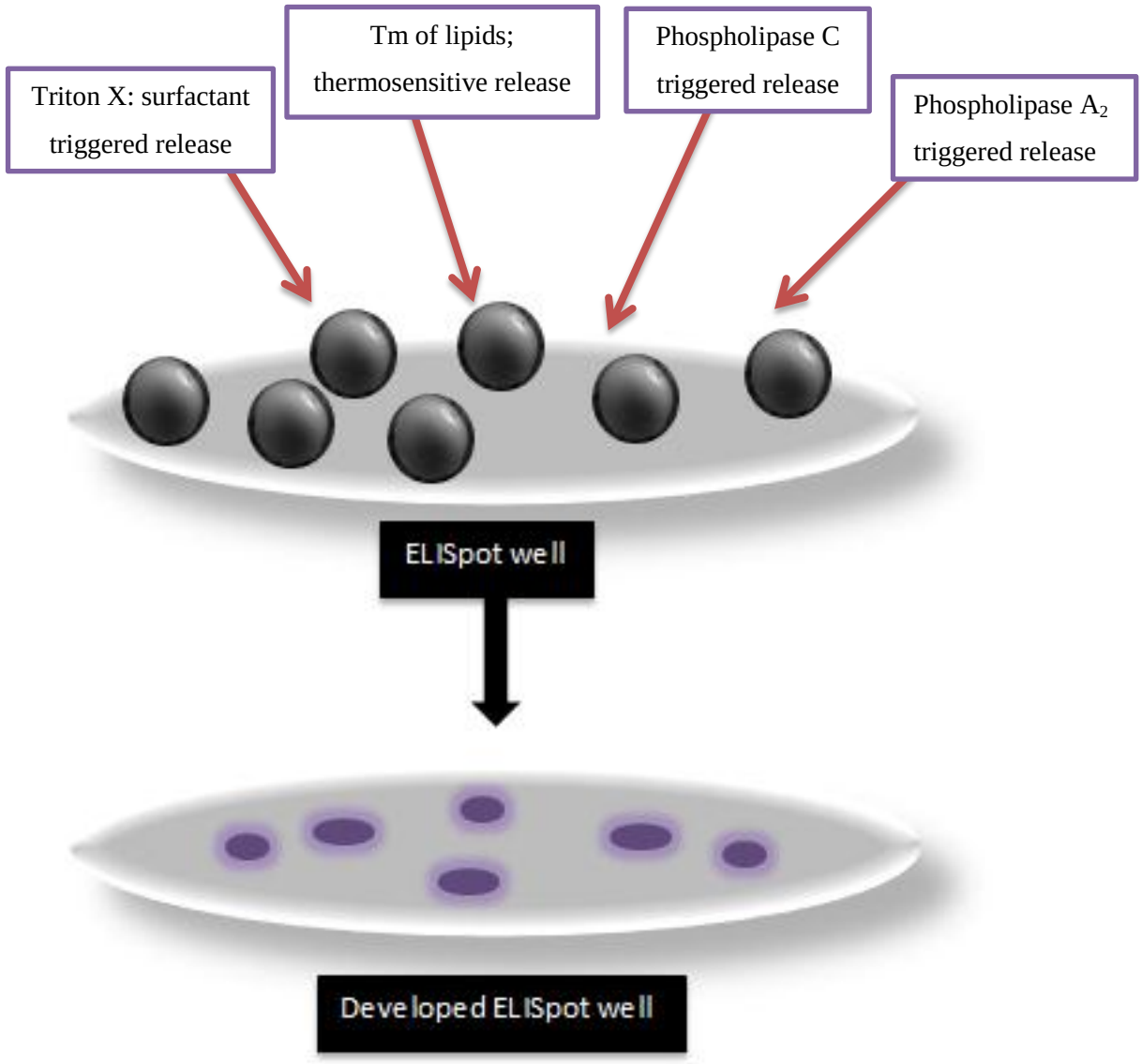
screening is useful because at this stage it may be sufficient to use centrifugation for short time periods and low speeds; as a means of removing many of the smaller vesicles. The use of density gradients had some promising results with lower viscosities of sucrose. The density gradients at 5, 15, 25 and 35 % w/v sucrose show a greater level of separation of the small and larger particles than the sucrose density gradients of higher viscosities. It remains a relatively simple technique with minimal effect on the vesicles thus there is further scope for optimisation.

The overall aim of such preliminary investigations was to circumvent issues, which may arise as a result of the structural differences between liposomes and cells; as the ideal scenario for the reference standards is for them to rest at the bottom of the plate and then release. The size of the spots are on average between 30 µm to 150 µm (Janetzki *et al.,* 2004) and yet the liposome vesicles as MLVs will be on average approximately 10 µm however they will solely contain the cytokine. The real challenge of this project will be fine tuning the kinetics of release, if the release is too rapid it can produce 'large, fuzzy spots' and if it is too slow and steady it will result in 'smaller, denser spots' (Lehman, 2005).

Chapter 4

Interferon γ containing liposomes in conjunction with trigger release mechanisms for use within an ELISPOT assay

Graphical abstract



4.1 Introduction

Interferon γ is a cytokine that displays immunomodulatory, antiviral, and antiproliferative effects (Goldbach *et al.,* 1995). It plays an important role in the destruction of invading microorganisms and has thus in many previous studies thought to be an effective therapeutic agent which could be encapsulated within liposomes to protect it from *in vivo* degradation (Christian and Hunter, 2012).

4.1.1 The challenges of using Interferon γ in liposomes

The challenge of this study was not only incorporating the cytokine within the liposomes, but also in retaining sufficient activity to produce a robust reference standard for use within an *in vitro* immunoassay. In previous studies, the rIFNγ used within liposomes was in the recombinant form (Anderson *et al.*, 1981). There are important differences in the IFNγ in its natural form and that of the recombinant form which is isolated from *E.Coli* cells (Curling *et al.,* 1990). Human interferon γ (Figure 4.1) contains only one tryptophan amino acid, consisting of 143 amino acids in total including two n-glycosylation sites and 28 lysine and arginine residues whereas the *E.Coli* derived rIFNγ is not glycosylated and contains an extra methionine residue (Farrar and Schreiber, 1993, Döbeli *et al.,* 1988).

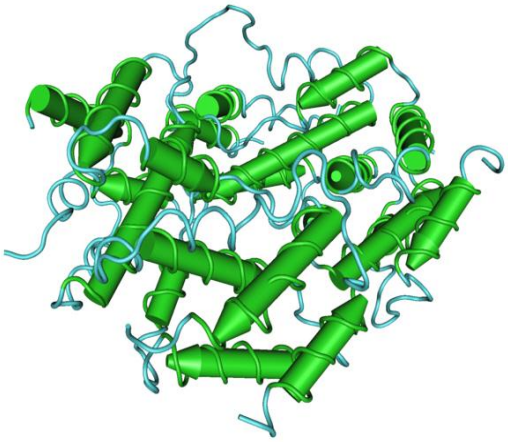


Figure 4.1 Computer model of human IFN γ.

The recombinant interferon is less stable in solution and has the tendency to aggregate (Christova *et al.,* 2003). rIFNγ is an unstable protein demonstrated by previous studies in which inactivation occurred through the formulation process of the liposomes (Ishihara *et al.,* 1990). The absorption of

the protein can also occur at liquid-solid interfaces and thus there are many challenges to ensure the effective encapsulation of the cytokine into the liposomes (Goldbach *et al.*, 1995). However, successful formulations of liposomes containing rIFNγ, even with single lipid components such as DMPC for *in vitro* or *in vivo* purposes have previously been demonstrated (Anderson *et al.*, 1994). Thus establishing the effects of the formulation process on the cytokine is an important aspect in the assessment of the feasibility of using the cytokine within liposomes in the ELISPOT assay.

4.1.2 Controlling the release from liposomal formulations

It is well established that liposomes can release their contents in a gradual and sustained manner, (Saravolac *et al.*, 1996). The challenge of this study lies with optimising a release pattern so that it is similar to that of the cells and the way in which they secrete cytokines. The stability of the liposomal membrane in terms of the mechanical strength and its ability to act as a permeable barrier relies upon the constituent lipids and sterols used in the formulation. Such liposomal membranes exhibit well-ordered gel phases below the lipid phase transition (T_m) and disordered liquid phase transitions above this temperature; as the T_m varies on the length of the lipid chain and the level of saturation it is a means by which membrane permeability can be controlled (Maurer *et al.*, 2001). The presence of cholesterol can have a significant impact too, as lower concentrations can increase permeability and higher concentrations (> 30 mol %) can eliminate the transition temperature and dramatically increase the stability (Szoka and Papahadjopoulos, 1980). Thus a wide variety of formulations of varying transition temperatures, with and without the presence of cholesterol require investigation as the correct release pattern cannot be predicted for the novel application of the liposomes within the ELISPOT assay.

4.1.3 Triggered release from liposomal formulations

To produce a liposomal reference standard, which can release cytokine in a similar way to cells various mechanisms, need to be investigated including thermosensitive release of liposomal contents through the manipulation of liposomal composition; however, this alone may not achieve

the desired release characteristics and thus an external trigger may be required. The use of trigger release mechanisms such as enzymatic, photo-triggered, pH and ultrasound for the release of liposomal contents has been widely exploited for *in vivo* purposes (Elegbede *et al.*, 2008, Sarkar *et al.*, 2008, Connor and Huang, 1985, Burke *et al.*, 2011, Shum *et al.*, 2001). However, some trigger release options are not feasible within the ELISPOT assay, including pH-triggered release and light triggered release as the protein itself is susceptible at a pH below 4.8 or above 9.5. Therefore, there is a risk that designing a pH dependant system brings an additional element of possible structural degradation of the cytokine (Christova, 2003). Use of light triggered mechanisms would require changing the basic ELISPOT protocol, as an additional step for the application of the light would be required. Movement of the plate once the cells have been placed in the wells is detrimental to the quality of the spots formed and should be avoided (Weiss, 2005). Suitable triggered release options include the use of a surfactant such as Triton X to cause the breakdown of the liposomal bilayer; such techniques are ubiquitously used to lyse liposomes and if rapid release is required would be very effective (Urbaneja *et al.*, 1988). Enzymatically triggered release also seems a favourable option to investigate as both the enzymes PLC and PLA_2 work at an optimal temperature of 37 °C (Op den Kamp *et al.*, 1972) which is the incubation temperature for the assay.

4.2 Aims and Objectives

The overall aim of this work was to establish the 'proof of principle' as to whether liposomes can encapsulate rIFNγ and are subsequently able to produce spots within an ELISPOT assay. Furthermore whether spot formation can occur with thermosensitive release or whether additional triggered release elements such as enzymes or detergents are necessary for enhanced spot formation.

The objectives for this study were:

- The characterisation of liposomes formulated from lipids of varying transition temperatures and the inclusion or absence of cholesterol to be used within the study as potential reference standards.

- To use an IFNγ ELISA assay for the optimisation of the concentration of the recombinant cytokine which would be sufficient to produce spots on the ELISPOT assay.
- To use an IFNγ ELISA to show encapsulation and recovery of the cytokine subsequent to the formulaton process for a range of liposome compositions.
- To carry out and optimise an ELISPOT assay and assess 'proof of principle' and whether the formation of spots from encapsulated recombinant cytokine is feasible.
- To assess the physical effects of the formulation process on the integrity of the rIFNγ with assessment of parameters such as temperature, centrifugation and vortexing.
- To carry out ELISPOT assays for the quantitative assessment of thermosensitive, enzymatic and detergent triggered release mechanisms in terms of spot number and qualitative assessments of the background for the wells.
- To optimise enzyme concentrations and buffers for the dilution of PLA_2 and PLC.
- In addition examine the effects of different pre-wetting volumes of ethanol across different formulations in order to establish iptimal parameters for the ELISPOT assay.

4.3 Results and discussion

4.3.1 Optimisation of cytokine concentration, validation and assessment of entrapped cytokine using an IFNγ ELISA assay

The ELISA assay and the ELISPOT assay are two similar immune-enzymatic assays based generally on the same principles however there are some fundamental differences. The ELISA assay is for quantification purposes; exactly 'how much' is secreted whereas the ELISPOT assay enumerates the *number* of cytokine secreting cells (Kalyuzhny, 2005). It was an important initial step to optimise the amount of cytokine, which would be sufficient to allow spot formation to occur from the artificial reference standards, thus the ELISA and ELISPOT kits that have been chosen are coated using the same antibody at the same concentration (Mabtech). There were also other important factors which needed to be assessed including the various steps of the formulation process on the integrity of the cytokine.

4.3.1.1 Validation of the ELISA assay for rIFNγ measurement

In order to establish the protocol for an IFNγ ELISA assay and subsequently demonstrate the reproducibility of the plate coating and the protocol, three calibration curves were made using the kit standards and ran on three different days on three plates. The overlay from the three curves was highly reproducible, with low standard deviations for each calibrator (<1) and the R^2 value of 0.99 and therefore confirmed the accuracy, precision and robustness of this assay for quantitative analysis of the recombinant Interferon γ used for encapsulation (Figure 4.2). The limits of detection were 1 pg/mL which was the lowest concentration which could be detected but not necessarily quantified. The limit of quantification was 3.16 pg/ mL and the standard range was 3.16 pg/mL to 1000 pg/mL. The *inter* and *intra* assay variation measured by the coefficient of variation was 4.2 % and 2.7 % respectively.

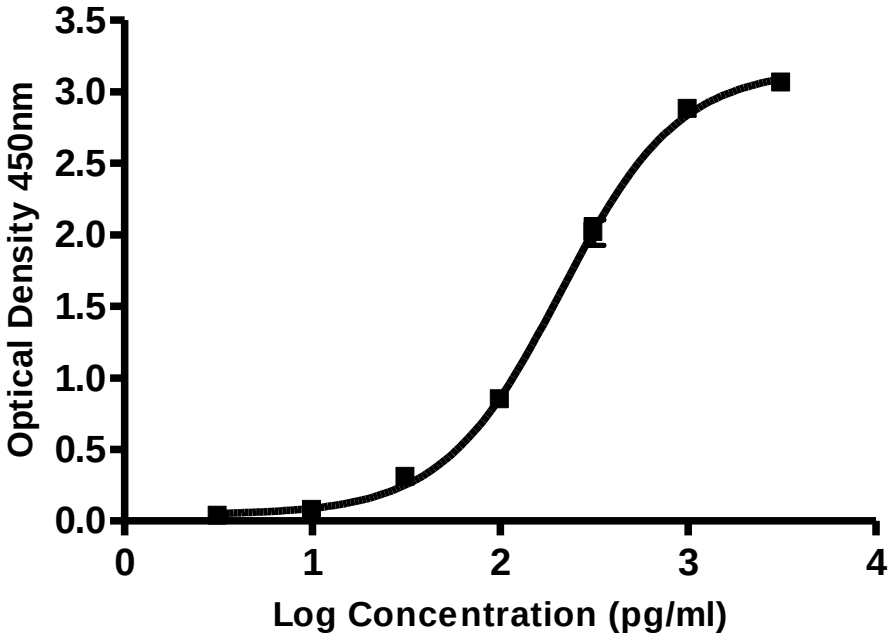


Figure 4.2 Sigmoidal dose-response curves for IFNγ calibration curves repeated in triplicate using standards with ELISA kit. The bottom and the top of the curve defined as 0.04 and 3.16 OD, Log EC50 2.323, Hill slope 1.387. The R^2 value calculated was 0.99. Results shown are for the mean ± SD triplicate assays.

As the assay components were purchased as a complete kit (Mabtech, Sweden), it was in fact validated, comprehensively QC tested and complied with European Regulations ISO 13485 and 90001 (ISO, 2003) and (ISO, 2008) respectively. Therefore, it was not necessary to validate the kit itself but it was appropriate to confirm that reproducibility, precision and accuracy were achievable through the operator. According to ICH guidelines (ICH, 2005) in the validation of analytical procedures linearity, accuracy, precision, and reproducibility need to be shown. Accuracy is defined as the closeness of agreement between the value, which is accepted either as a true value or from a reference value; the precision is the closeness of agreement between a series of obtained measurements. For a calibration curve, the test results should be directly proportional to the concentration of an analyte. The guidelines specify that for the establishment of linearity a minimum of 5 concentrations are recommended. For this study, 7 concentrations were provided (Figure 4.2). The value of 1 for R^2 indicates a perfectly linear relationship thus the obtained value of 0.99 indicates a good statistical fit (Freund *et al.*, 2006).

4.3.1.2 Titration of the recombinant cytokine

A titration of the human recombinant interferon γ purchased (Sigma) was necessary in order to obtain an approximate concentration strong enough to produce spots on the ELISPOT assay thus a high optical density on the ELISA assay was required. Using the kit standard curve (Figure 4.3) to extrapolate concentrations, dilutions of recombinant IFNγ cytokine (Sigma) of 1000 pg/mL, 500 pg/ mL and 100 pg/mL were tested to examine their suitability for encapsulation purposes. The lyophilised cytokine (1 mg in amount) was diluted in phosphate buffered saline with 0.5% bovine serum albumin. Three titres initially tested were in duplicate and the optical densities obtained shown in Table 4.1.

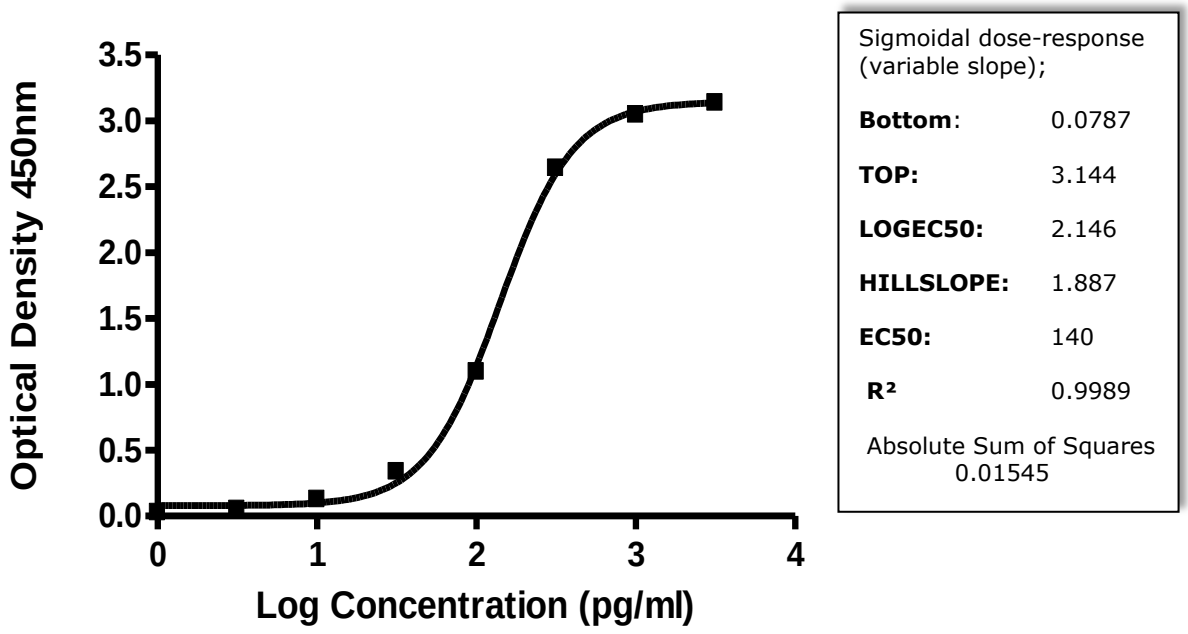


Figure 4.3 Dose-response curves for IFNγ with the ELISA kit standards for the extrapolation of dilutions of stock recombinant Interferon γ.

Table 4.1 Optical density of tested dilutions of rIFNγ and extrapolated concentrations carried out in duplicate.

Concentration (pg/mL)	Optical Density	Extrapolated value (pg/mL)
1000	0.0325	1.078
500	0.0205	1.048
100	0.0555	1.136

The concentration in the first column of Table 4.1 derived from the dilution of the purchased recombinant cytokine was used within the ELISA assay to determine their *actual* concentration. The coating antibody used in the ELISA assay is the same as the one used for the ELISPOT assay and therefore an indicator for the necessary concentration of the purchased IFNγ required for the liposome formulations. As observed in Table 4.1 the concentration of the purchased cytokine and the extrapolated concentrations were very different; for instance at 1000 pg/mL of the purchased cytokine on the ELISA assay provided an extrapolated concentration of 1.078 pg/mL.

The values obtained for the optical density were within the limits of detection (1.0 pg/mL); however, they were well below the limits of quantification (3.75 pg/mL) for the assay (Table 4.1). The results suggest that the concentration in mass defined by the kit standards was not directly

comparable to the stock rIFNγ for encapsulation within the liposomes. Thus, the titration was repeated at stronger concentrations with the aim of obtaining a concentration as close to the upper end of the optical density of the assay as possible (Figure 4.4).

The result from the repeat titration confirmed that a much stronger concentration was required to obtain higher optical densities than predicted (Figure 4.4 and Table 4.2). Even with the highest concentration tested at 5000 ng/mL of the recombinant cytokine, the optical density was 2.697 (Table 4.2) whereas it was evident from the curve that saturation was at an optical density of 3.1 before the curve begins to plateau (Figure 4.4). This was used as a basis to make the stock solution at a much stronger concentration, which was 10,000 ng/mL (10 µg/mL). Due to the lack of comparability between the kit standards and the stock solution of cytokine, it was necessary in further work to use the stock solution to construct a calibration curve to allow for the calculation of entrapment.

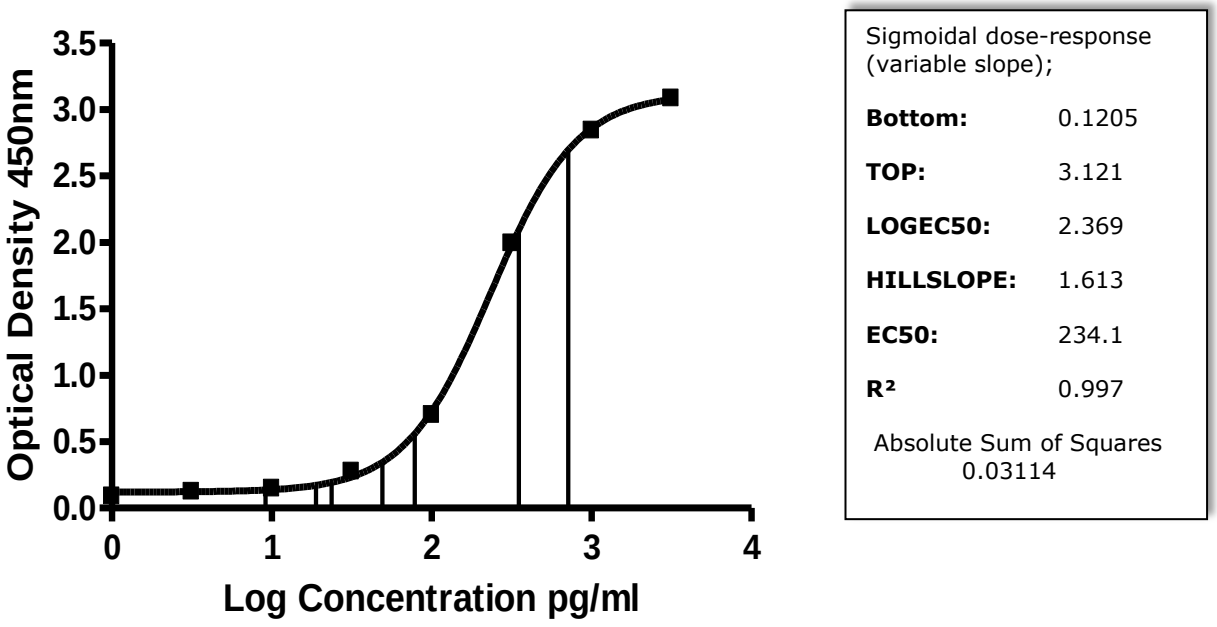


Figure 4.4 Extrapolation of dilutions of titrated concentrations of rIFNγ, for encapsulation within liposomes. Results represent mean ± SD of triplicate assays.

Table 4.2 Results for the mean optical density of extrapolated concentrations of specified dilutions of rIFNγ.

Concentration (ng/mL)	Optical Density	Extrapolated value (pg/mL)
5000	2.69	716.9
2500	2.093	350.7
1250	0.558	78.3
625	0.345	49.2
312.5	0.193	23.6
156.25	0.171	18.9
78.125	0.137	9.1

In the preliminary titration, there was an assumption that the concentration of the standard curve and the rIFNγ dilutions tested were directly comparable, however the results indicated that this was not the case. An explanation for this substantial difference in the activity as explained within literature is due to the variability observed during the production of recombinant interferon gamma, especially in glycosylation of the polypeptide chains; as this variability can result in reduced activity (Curling *et al.,* 1990). Various methods can be used to produce recombinant cytokines including *E. Coli*, yeast and Chinese hamster ovary cells (CHO) however, production of cytokine even using the same method can lead to different forms (Mire-Sluis *et al.*, 1999). The rIFNγ used within this study was *E.Coli* derived and produced in bacterial plasmids thus the variability found in the expression levels using this method is common and serves as an on-going challenge in the biotechnology industry (Sharma *et al.*, 2011). Therefore, it was not unexpected for there to be such discrepancy between the kit interferon (Mabtech, Sweden) and the purchased rIFNγ (Sigma, UK). In fact according to Mire-Sluis *et al.*, (1999) the biological potency of one amount from a manufacturer such as 1 mg of can have a very different activity to that from another.

However, the purpose of this study was to obtain a concentration for the cytokine that would be suitable for the formulation of the liposomes for the production of spots within an ELISPOT assay. The concentration of 10 µg/mL also equates to 2.5 x 10^5 U/mL in terms of the specific activity of the cytokine. Although it was simpler to initially work with the stock concentration in terms of amount, for the purposes of calculating encapsulation of cytokine within liposomes. In terms of the validation work for the ELISPOT assay subsequently, it was necessary to work in specific activity because to quantify the biological activity of different cytokine preparations biological activity has

to be expressed and not mass units. This is calculated from a bioassay and allows for more comparability between different laboratories (Mire-Sluis *et al.*, 1999).

4.3.1.3 Examining the effects of Triton X on the coating antibody and rIFN gamma

As the kit standards were no longer to be used, it was necessary to confirm that a reproducible curve could be obtained with a serial dilution of the rIFNγ stock solution at 10 µg/mL. It was also important to establish that liposome lysis with 10% v/v Triton X for the measurement of encapsulated protein would not affect the optical density of the calibration curve by interference with the assay.

The results showed (Figure 4.5) that there was no significant difference between the two curves when various parameters of the graph including the bottom, top, Log EC50 and hill slope were compared. The R^2 value for the normal calibration vs. Triton X was 0.996 and 0.995 respectively. The results from the overlay show the calibration curve produced from the stock 10 µg/mL solution of recombinant cytokine produced a highly reproducible curve. Furthermore, the Triton X did not affect reproducibility. This was thus a suitable lysis agent for both the ELISA assay for the determination of percentage encapsulation and within the ELISPOT assay as a means to show complete release.

If Triton X caused interference within the assay then it would not be an acceptable lysis agent, as the specificity of the assay would be affected; according to ICH guidelines, the specificity is the ability to assess explicitly the analyte in the presence of other components that maybe present (ICH, 2006). However, the results have shown that there was no interference with the use of Triton X and this was a suitable non-ionic surfactant for the lysis of liposomal membranes. Its suitability further confirmed through literature, showed its extensive use for the purposes of liposome disruption, and was therefore used for subsequent studies. (Urbaneja *et al.*, 1988, Ruderman and Grigera, 1986, De la Maza and Parra, 1996).

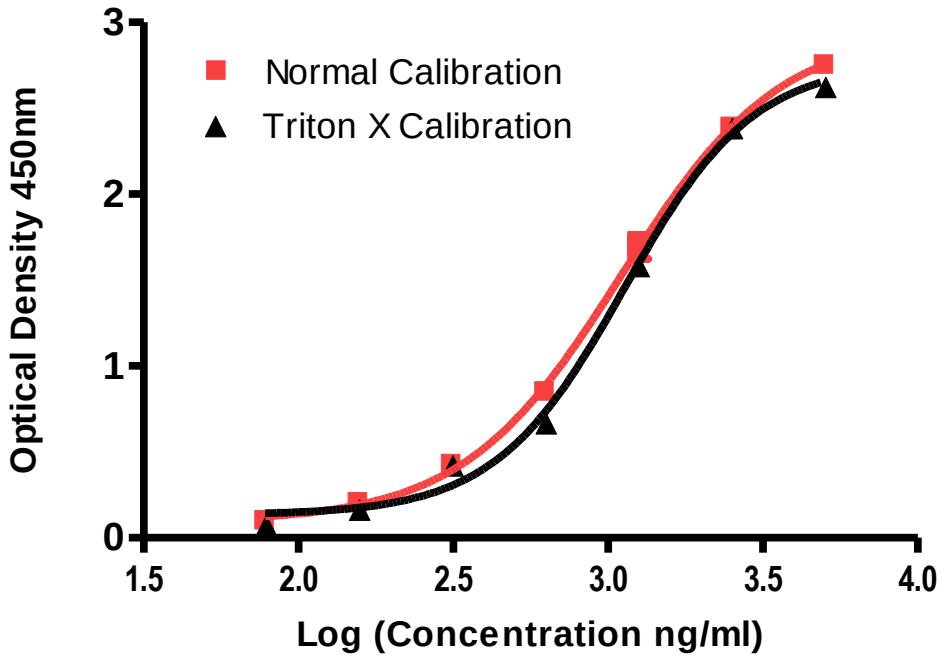


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Figure 4.5 The effect of Triton X on the rIFNγ calibration curve. Results represent mean ± SD of n =3 assays.

4.3.2 Determining the effects of the physical stresses of the formulation process

An assessment of the effects of the formulation process on the cytokine was necessary to establish the impact on activity of the protein. It was important to determine the effects of temperature, centrifugation and/or vortexing on the rIFNγ and consequently on the ability of the protein to bind to the coating antibody. During the formulation process, the temperature was required to be above the transition temperature for the lipids for liposomes to form, with encapsulated rIFNγ (Anderson and Omri, 2004). Thus dependent upon the lipid used the temperature can range from room temperature to 60 °C.

4.3.2.1 Determining the effect of temperature on rIFNγ

To assess which formulations were suitable for the encapsulation; 500 µL of the rIFNγ (10 µg/mL) was placed in a dry heat block for either 30 minutes or 1 hour (Figure 4.6). It was found that as the temperature increased there was a significant ($p<0.05$) change in the optical density from 40 °C to 50 °C, from 2.89 at 40 °C the optical density decreased at 50 °C for 30 minutes and 1 hour to 2.53 and 2.47 respectively. Thereafter the decrease for both incubation times remained highly significant

($p<0.001$) compared to the optical density at 40 °C. When comparing the difference between the two incubation times at each temperature there was no significant difference observed at 40 °C or 50 °C; however, subsequently at 60°C and 70 °C the difference in optical density was highly significant ($p<0.001$). At 60 °C, the optical density at 30 minutes incubation and 1 hour incubation was 2.73 ± 0.14 and 1.71 ± 0.21 respectively. At 80 °C the difference in optical density was not significant and at 90 °C was significant ($p<0.05$) again, by which point the optical density was at 0.61 ± 0.12 and 0.22 ± 0.02 (Figure 4.6). These results show that even small changes in temperature can have a significant impact on the activity of the rIFNγ, thereby limiting working temperatures that could be used for the manufacture and application of the liposome systems.

In previous studies, liposome encapsulation of rIFNγ has been successful using the dry film hydration method. Various formulations including lipids such as PC, EPG (1,2-diacyl-sn-glycero-3-[phospho-rac-(1-glycerol)] , DPPC and DPPG (1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol) which have a range of transition temperatures (<20°C to 41°C) above which the formulation process occurs (Van Slooten *et al.,* 2001). Furthermore, assessment of the bioactivity of the rIFNγ through a TNFα bioassay found that the activity was unaffected by the formulation process (Van Slooten *et al.,* 2001). In fact other studies have shown that at at 50 °C the infrared spectrum of the rIFNγ exhibited minor changes; however, above this temperature it was observed that with increasing exposure to higher temperatures there were irreversible changes to the secondary structure (Goossens *et al.,* 2003). Such results agree with the observations from this study which suggest that although there was an observed decrease in the optical density using the rIFNγ ELISA assay at 50 °C the actual level of activity remaining would be acceptable for the formulation of liposomes using the dry film lipid hydration method. However, formulations above this temperature may result in irreversible damage to the cytokine.

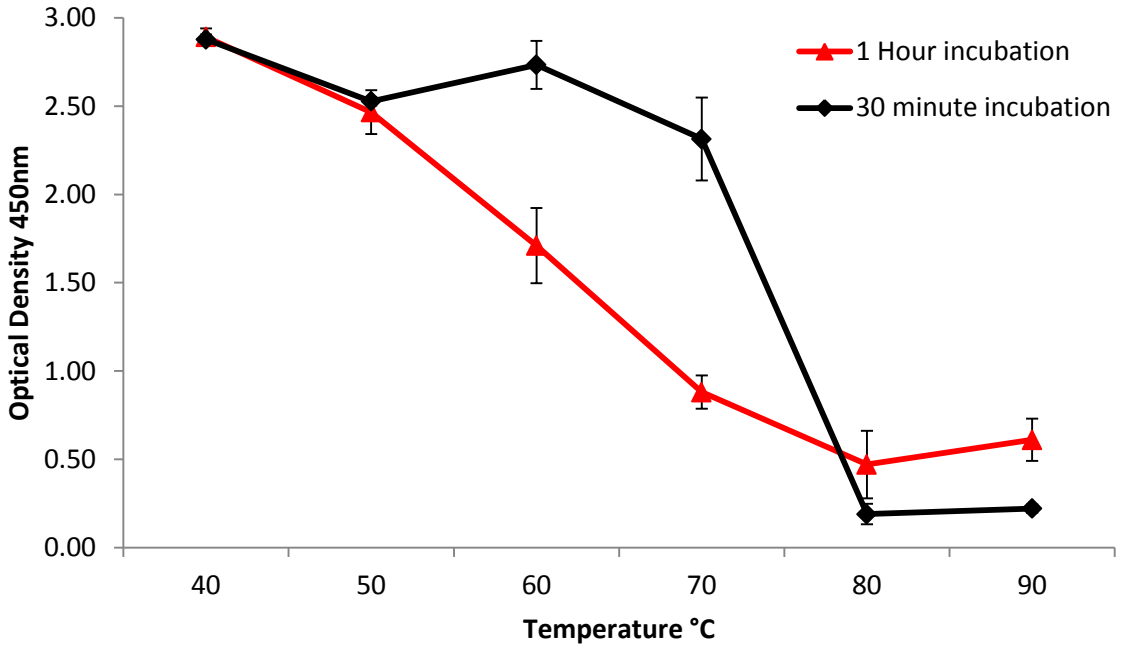


Figure 4.6 Effect of increased temperature on 10 µg/mL recombinant IFNγ for 30 minutes and 1 hour. Results are the mean of triplicate samples tested ± SD.

4.3.2.2 Determining the effect of centrifugation on rIFNγ

Three serial dilutions were made and divided into two; one set was centrifuged at 29, 771 g for 30 minutes and the other was not. These were then tested on the ELISA IFNγ assay (Figure 4.7). From the results (Figure 4.7) it can be seen that the curves do not overlay precisely; however a statistical analysis for the two curves using the similarity factor F2 test which is a logarithmic transformation of the sum-squared error of differences at all the calibrator dilutions was calculated to be 52. This represents the closeness of the two curves as acceptable values are 50 – 100 according to FDA guidelines for dissolution curves (Yuksel *et al.*, 2009). This suggests that although centrifugation has some effect on the optical densities although overall the curves were simlar and thus centrifugation was a suitable technique within the formulation process which was compatible with the rIFNγ.

The differences observed in the curves maybe due to the calibrators for the centrifuged curve being transferred to plastic tubes sealed and then centrifuged, some protein may have been lost due to it being adsorbed at interfaces such as the tube. This is a result of electrostatic interactions between a

charged adsorbent material and oppositely charged amino acid chains on the protein (Hlady *et al.*, 1999).

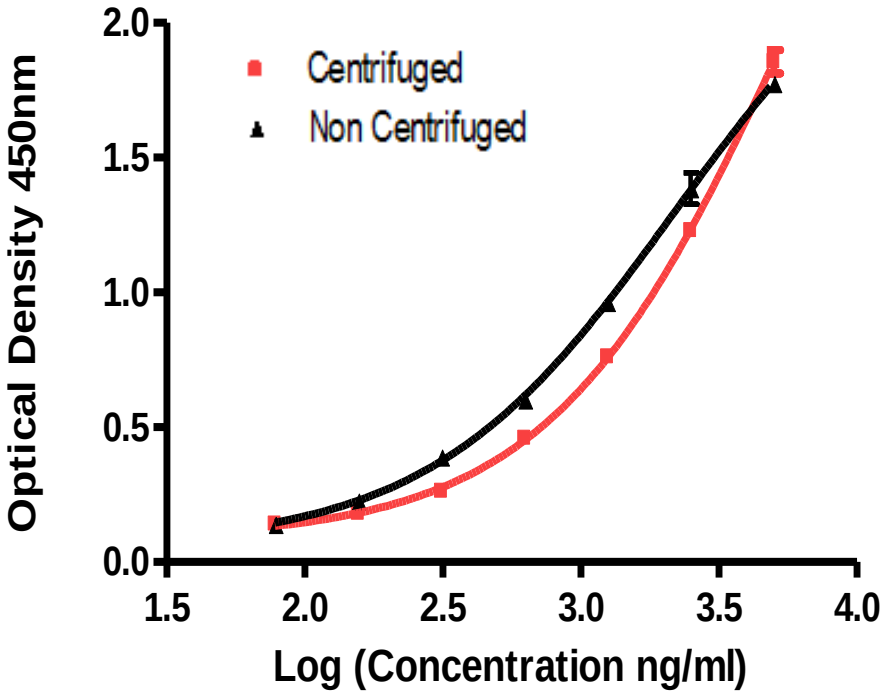


Figure 4.7 The effects of centrifugation at 29, 771 g for 30 minutes on the optical density of the calibration curve on an ELISA IFNγ assay vs. a normal calibration. Results are the mean of triplicate samples tested ± SD.

Furthermore, the adsorption process can result in protein denaturation. Other studies have shown that recombinant IFNγ can be susceptible to a loss of activity when subjected to mechanical stresses, such as shearing forces and increased surface tension (Tsanev *et al.*, 2007). In terms of the formulation process, the centrifugation of the protein is for the purposes of removing the free IFNγ so any loss observed should not affect the encapsulated rIFNγ.

4.3.3.3 Determining the effect of vortexing on rIFNγ

Vortexing the dry lipid film is an important step subsequent to hydration of the film, thus the stock solution was vortexed for various time points ranging from 30 seconds to 15 minutes and analysed for biological activity on the ELISA assay.

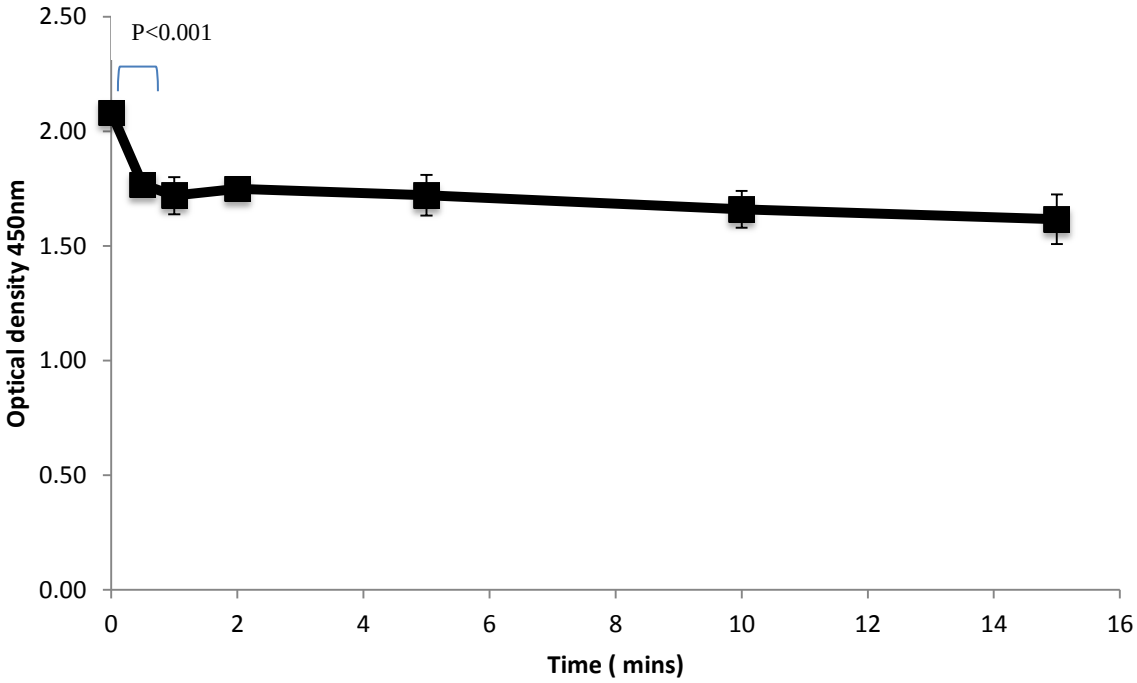


Figure 4.8 The effect of vortexing on the recombinant interferon cytokine. Results show are the mean ± SD for triplicate assays.

The results in Figure 4.8 show that rIFNγ was sensitive to vortexing, after only 30 seconds a significant ($p<0.05$) reduction in protein activity was noted from 2.0 ± 0.02 to 1.77 ± 0.04 (optical density). However, continued vortexing for up to 15 minutes did not have any further significant effects on the optical density (Figure 4.8). Mechanical stresses such as increased pressure, temperature, and shearing has been shown to affect the biological activity of rIFNγ (Goossens *et al.*, 2003). During the encapsulation process, the exposure of proteins to mechanical stress can lead to the denaturation of sensitive proteins (Hwang *et al.*, 2000). The formulation process for this study would not require such prolonged periods of vortexing and would be limited. In addition, previous studies have shown that it has been possible to formulate liposomes with rIFNγ and thus confirm that although the protein can show instability it is still feasible to form liposomes (Van Slooten *et al.*, 2001, Anderson *et al.*, 1981, Eppstein and Stewart, 1982).

4.3.4 Physicochemical characterisation and entrapment of rIFNγ with varying liposome compositions

Having established the feasibility of using an ELISA IFNγ assay to establish encapsulation efficiency (section 4.3.1), prior to liposome formulations being placed in an ELISPOT assay it was important to assess a wide range of lipids with varying transition temperatures. Once liposomes were formulated they were characterised for size and zeta potentials and subsequently establish ecapsulation efficiency.

4.3.4.1 Characterisation of liposome formulations

The purpose of this study was to assess a wide range of liposome formulations which were composed of either 16 μMol for formulations without cholesterol and 8:8 μMol for those with cholesterol (Table 4.3). 'Empty' and rIFNγ encapsulated formulations were made for each corresponding composition (Figure 4.9). For the formulations containing rIFNγ there was no notable differences or trends in the various liposome formulations. The PC 16 μMol 'empty' liposome formulation was significantly smaller ($P<0.05$) when compared to other formulations such as PC:Chol , DPPC:Chol, DMPC, DMPC:Chol and DOPC, although there was no significant difference between empty PC formulations and the size of PC rIFNγ encapsulated formulations. In terms of zeta potential, the formulations exhibited no signicant difference when compared between formulations other than DPPC:Chol:BrChol 8:4:4 μMol which showed either a signicant difference ($p<0.05$) or a highly significant difference ($p<0.001$) with remaining formulations. This is due to the anionic nature of the formulations which have a zeta potential -21.9 ± 6.9 mV whereas the other formulations are more neutral, for instance PC has a surface potential of -6.8 ± 3.9 mV.

Table 4.3 Liposomal formulations for empty and rIFNγ containing liposomes for characterisation and the entrapment of rIFNγ.

Formulation	Lipid composition (µMol)	Formulation	Lipid composition (µMol)
1. PC empty	16	**10.** DPPC:Chol:BrChol IFNγ	8: 2: 6
2. PC IFNγ	16	**11**. DMPC empty	16
3. PC:Chol empty	8: 8	**12.** DMPC IFNγ	16
4. PC:Chol IFNγ	8: 8	**13.** DMPC:Chol empty	8: 8
5. DPPC empty	16	**14**. DMPC:Chol IFNγ	8: 8
6. DPPC IFNγ	16	**15**. DOPC empty	16
7. DPPC:Chol empty	8: 8	**16.** DOPC IFNγ	16
8. DPPC:Chol IFNγ	8: 8	**17**. DOPC:Chol empty	8: 8
9. DPPC:Chol:BrChol empty	8: 2: 6	**18**. DOPC:Chol IFNγ	8: 8

For the purposes of producing a reference standard for the ELISPOT assay, it was difficult to predict which formulation would provide optimal characteristics for the effective entrapment of rIFNγ and consequently effective spot formation within the ELISPOT assay. Thus, it was necessary to characterise a wide range of formulations. A range of lipids were chosen and in addition cholesterol was added at an equimolar ratio, the inclusion of cholesterol can decrease the permeability of the membrane (Kirby and Gregoriadis, 1983, Kirby *et al.*, 1980). The lipids chosen were PC, DMPC, DPPC, and DOPC and these have transition temperatures of <20 °C, 23 °C, 41 °C, -22 °C respectively (Vemuri and Rhodes, 1995). The characterisation of the liposomes generally showed no significant difference across the formulations. Some variability was due to producing the formulations on three separate occasions, the dry film hydration method produces a heterogenous mixture of liposomes and this is where the variation is introduced not only in terms of size but also lamerallity and is one of the major drawbacks for this method (Wagner and Vorauer-Uhl, 2011, Szoka and Papahadjopoulos, 1980). The statistical significance observed in size between the empty PC formulations when compared to other formulations was not deemed to be important for the purposes of assessment of formulations on the assay as no spots would be

expected. The effect of any variation would become more significant subsequent to an assessment of encapsulation.

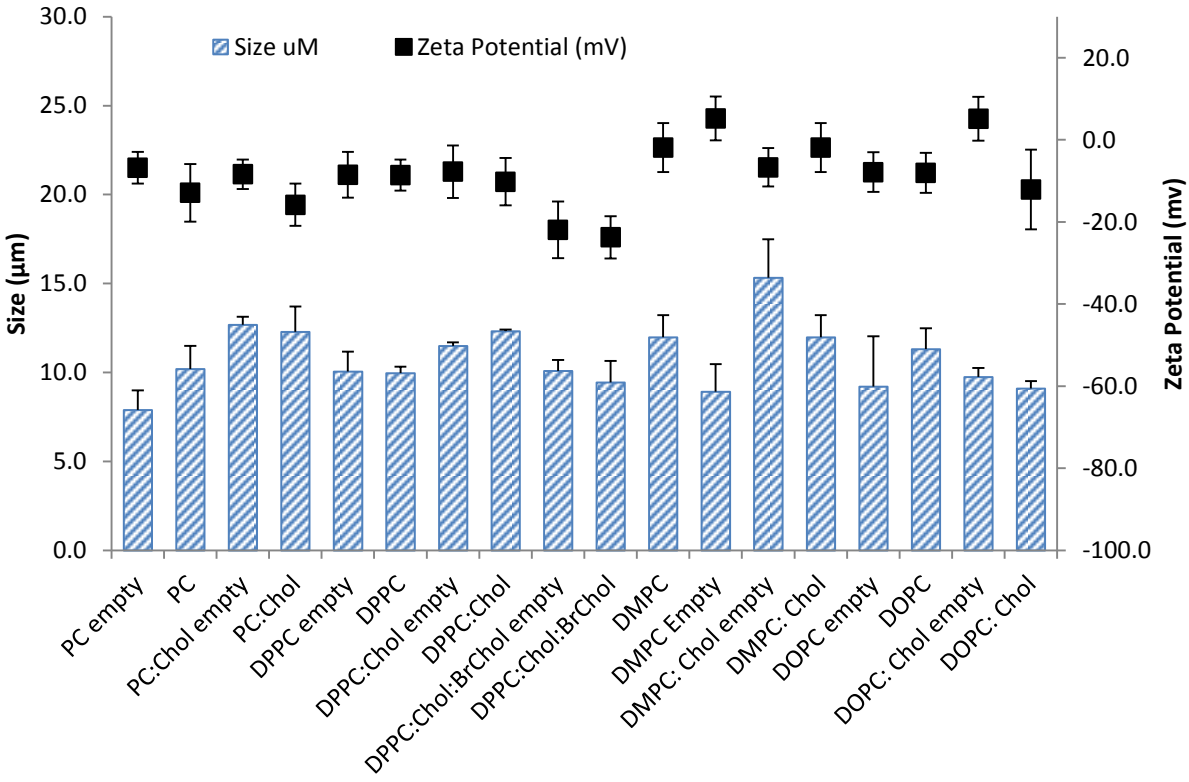


Figure 4.9 Physicochemical characterisation of size (µm) and zeta potential (mV) for both empty and rIFNγ containing liposmes with and without cholesterol and brominated cholesterol. Liposome formulations were made using 16 µMol total lipid amount. Results represent mean ± SD for n=3 batches.

4.3.4.2 Encapsulation of rIFNγ in varying liposome compositions

The results from the ELISA assays (Figure 4.9) showed that encapsulation of the rIFNγ was feasible, with the percentage encapsulation ranging from 13 ± 9% for DOPC to 25 ± 4.4 % for DPPC:Chol, with no statistical significance between the encapsulation efficiency for the formulations. Results obtained for the total recovery of protein were more variable (Figure 4.10); Statistical analysis revealed the p value across the groups was significant ($p<0.05$). The recovery ranged from 57 ± 7.6 % for DPPC:Chol: to 83 ± 8 % for DMPC. All formulations were significantly different from DPPC:Chol:Brchol ($p<0.05$) in terms of recovered protein and there

was also a significant difference ($p<0.05$) between DPPC vs. DMPC:Chol 40 ± 6.5 % and 91 ± 4.7 % (Figure 4.10).

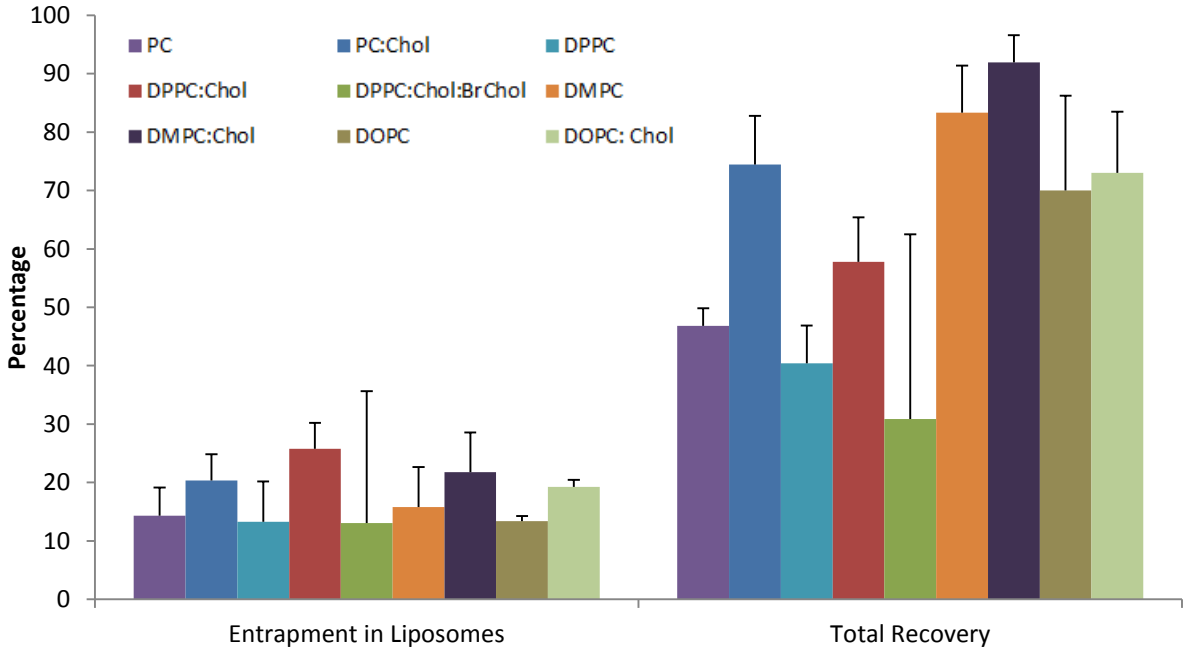


Figure 4.10 Encapsulation and recovery (%) for rIFNγ containing liposmes using ELISA assays. Liposome formulations were made using 16 µMol total lipid with cholesterol containing liposomes at 8:8 µMol. Results represent mean ± SD for n=3 batches.

Previously reported entrapment efficiencies for rIFNγ containing liposomes for topical delivery include 10-20 % using the dehydration rehydration method (Weiner *et al.*, 1989). Other studies have shown the successful entrapment of cytokine in positive and negative liposomes with the inclusion or absence of cholesterol, with entrapment levels for REVs at 10-20 % and 4-5 % for MLVs (Eppstein and Stewart, 1982). Furthermore, the authors observe that the inclusion of cholesterol in the phosphatidylserine containing liposomes was necessary for stable association of the rIFNγ for more than 2 days at 4 °C and for 24 hours at 37 °C (Eppstein and Stewart, 1982). This was an interesting observation, as although there was no statistically significant difference between the formulations in terms of entrapment in this study (Figure 4.10), the mean entrapment suggest a trend of higher entrapment for the formulations containing cholesterol (with the exception of liposomes containing dibromocholesterol). When PC and PC:Chol formulations were compared, for instance, the entrapment values were 14 ± 4 % and 20 ± 4.4 % respectively. The

trends found in the data (Figure 4.10) agree with Eppstein *et al.*, (1982). The studies mentioned from literature do not include values for the assessment of total protein recovery however as previously discussed the variability in the amount recovered could be due to the mechanical stress of the formulation process as the free interferon is exposed (Hwang *et al.*, 2000).

4.3.5 Determining the activity of phospholipase C and phospholipase A_2

Given that a range of formulations were shown to encapsulate rIFNγ, the next stage was to consider release mechanisms for the system. Release from liposome formulations can be enhanced using various methods of trigger release for the purposes of controlled release (Allen and Cullis, 2013). The purpose of the study was to assess the effectiveness of enzymatic triggered release particularly of PLC and PLA_2 activity of purchased enzymes for their ultimate use within the ELISPOT assay to assess their impact on spot number. Prior to the incorporation of enzymes within the assay an activity assessment was carried out both qualitatively and quantitatively to show that the enzyme was active and able to degrade the liposomes. Three aqueous dispersions of pure lipids were tested; PC, DOPC and DPPC and the breakdown of the lipids measured using absorbance values, which, were converted to enzyme activity using a phosphate standard curve (Figure 4.11).

The assay was a quality control check to ensure that there was activity of the enzyme, prior to the assessment of triggered release work within the ELISPOT assay. The observed results suggested that the observed activity of the enzyme varied dependent upon the lipid. Phosphatidylcholine seemed to show the greatest level of enzymatic activity and was significantly higher ($P<0.001$) than DOPC and DPPC with measured activity at 0.97 U/mL, 0.63 U/mL and 0.24 U/mL respectively. Previously PLC has been used within an immune based assay for the release of entrapped fluorescent marker (Kim and Park, 1994). The purpose of this study was to incorporate the enzyme for more effective release from liposomal formulations within the ELISPOT assay. Initially it was necessary to confirm the activity of the enzyme itself. The quality control assay (Figure 4.11) showed that there was enzyme activity and that this varied with the different lipid compositions assessed. Both PLA_2 and PLC work by catalysing the hydrolysis of the phospholipids to produce phosphocholine and diacylglycerol (Liao *et al.*, 2010).

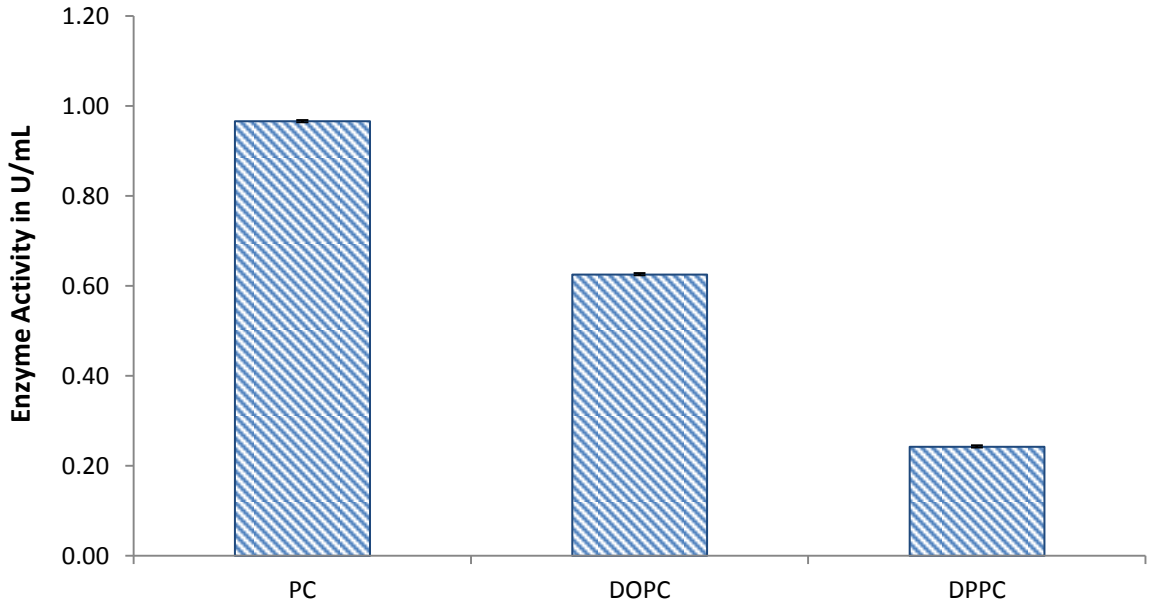


Figure 4.11 Quality control asssesment of the activity of PLC carried out with aqueous dispersions of PC, DOPC and DPPC. Results shown are mean ± SD for n=3 preparations.

The results observed concurred with findings by Van *et al.,* (1961), where differences in the hydrolysis of lipids by PLC were thought to be due to the variation in the length of the acyl chain with longer chains leading to a decrease in the rate of hydrolysis. Although other research has pointed to the level of saturation of the chain as an important determinate for the rate of hydrolysis; with saturated chains being more resistant (Long and Maguire, 1954). The results confirm that the enzyme activity was greatest with the unsaturated PC and then for DOPC which has an 18 carbon chain length and 1 double bond. Least activity was exhibited for DPPC, which has a 16 carbon chain length however, has no double bonds (Chen *et al.*, 2010). For further confirmation of enzyme activity using microscopy, PC and PC:Chol (16 μMol and 8:8 μMol respectively) without rIFNγ were placed into microslides with specified concentrations of PLA_2 and PLC (Table 4.4). Microscope images were obtained on the Zeiss Axioscope A1 light microscope at the time points of 0, 1 hour and after an overnight incubation at 37 °C; subsequently the microscope slides were assessed for changes in morphology of the vesicles.

Table 4.4 Lipids and enzyme concentrations assessed for PLA_2 and PLC activity using PC 16 μMol and PC:Chol 8:8 μMol (of mean particle size 8.68 ± 0.02 μm and 11.09 ± 0.03 μm respectively) for the light microscopy study.

Microslide number	Liposome formulation	PLA_2 added U/ mL	Microslide number	Liposome formulation	PLC added U/ mL
1.	PC	0	**1.**	PC	0
2.	PC	1	**2.**	PC	1
3.	PC	5	**3.**	PC	5
4.	PC	10	**4.**	PC	10
5.	PC:Chol	0	**5.**	PC:Chol	0
6.	PC:Chol	1	**6.**	PC:Chol	1
7.	PC:Chol	5	**7.**	PC:Chol	5
8.	PC:Chol	10	**8.**	PC:Chol	10

The microscopy results show that there is enzymatic action and this is most visible with overnight incubations, at concentrations between 1 U/mL and 5 U/mL (Figure 4.12 - 4.13). From the qualitative assessments of the images, the effect of the PLC was evident for both the formulations of PC (Figure 4.12 a) and PC:Chol (Figure 4.12 b). There was visible evidence of the breakdown of liposomes but larger liposomes showed some level of aggregation. Similar results were observed for the PLA_2 enzyme (Figure 4.13 a and b) with breakdown of liposomes into smaller particles and aggregation of larger liposomes. Previous studies have shown similar effects were observed when PLC was added to PC liposomes and examined using cryo-electron microscopy, which allows much further insight into the changes in the bilayer (Basanez *et al.*, 1997). In a study by Basanez *et al.*, (1997), the action of the enzyme induced vesicle fusion by initially entering an intermediate stage where the vesicles became aggregated and closely packed before then fusing into larger vesicles. There was no evidence observed of larger vesicles formed although there was observed aggregation. It was not possible to conclude conclusively from the images, the optimal concentration of the enzyme for the assay and thus a variety of enzyme concentrations would need assessment within the ELISPOT assay.

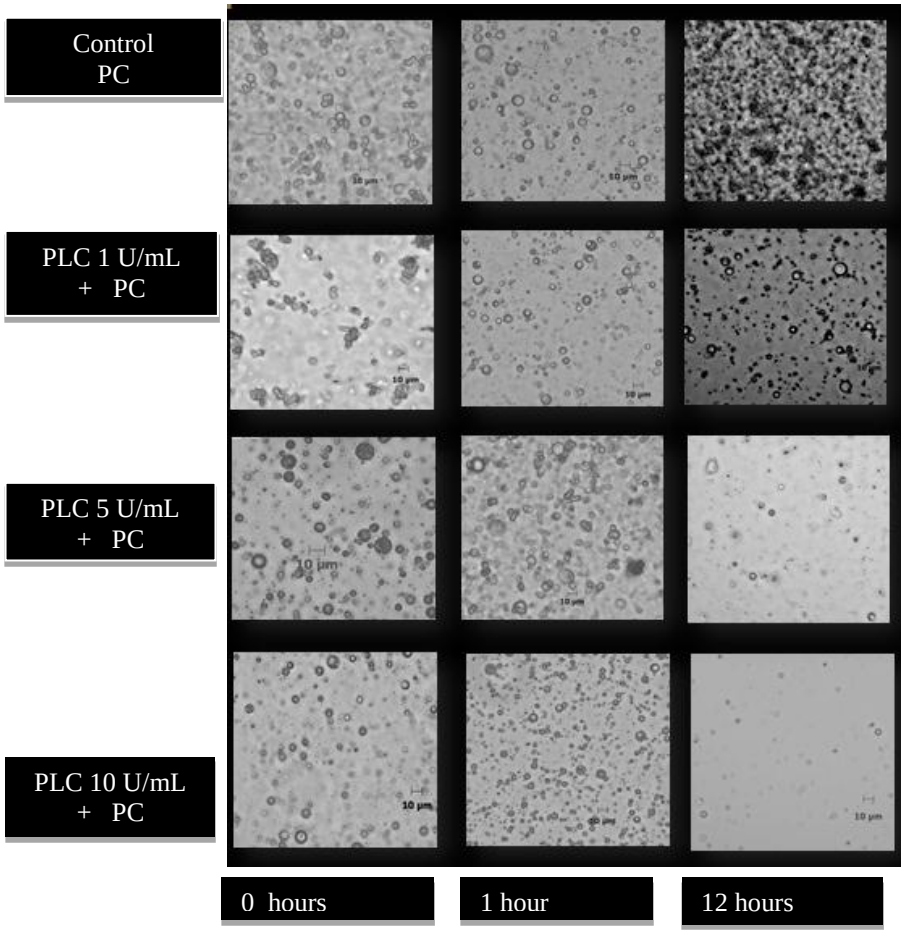


Figure 4.12 a; Light microscope images of a PC 16 µMol formulation (8.6 ± 0.02 µm mean particle size) incubated with or without various concentrations of PLC enzymes.

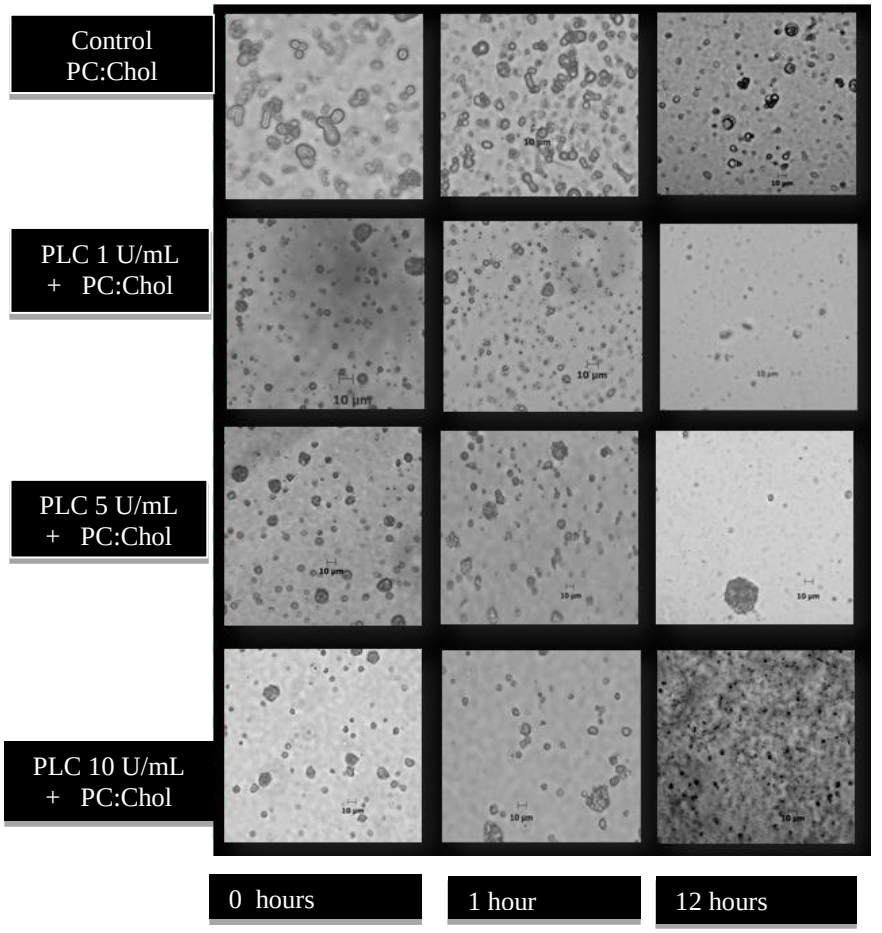


Figure 4.12 b; Light microscope images of a PC:Chol 8:8 µMol formulation (11.09 ± 0.03 µm mean particle size), incubated with or without various concentrations of PLC enzymes.

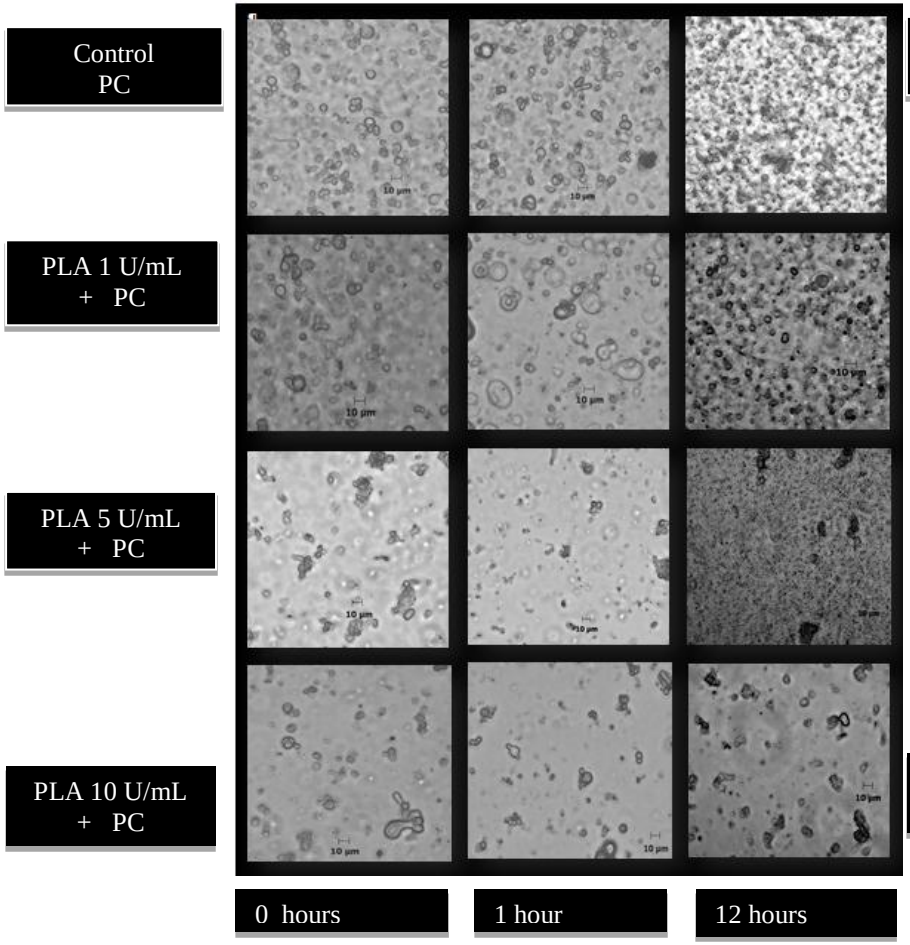


Figure 4.13 a Light microscope images taken of a PC 16 µMol formulation (8.6 ± 0.02 µm mean particle size) incubated with or without various concentrations of PLA_2 enzymes.

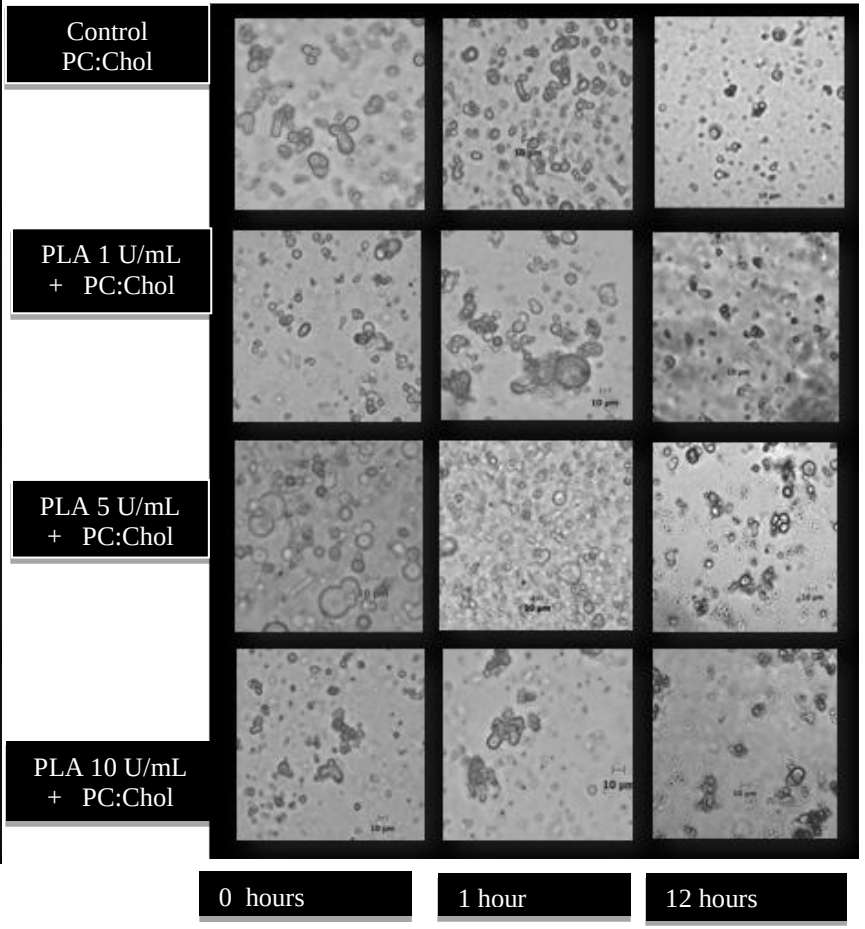


Figure 4.13 b Light microscope images of a PC:Chol formulation 8:8 µMol (11.09 ± 0.03 µm mean particle size) incubated with or without various concentrations of PLA_2 enzymes.

4.3.6 An assessment of pre-wetting volume for the optimisation of the ELISPOT protocol for the use of liposomal reference standards

The ELISPOT protocol ordinarily has a pre-wetting step with 70% v/v ethanol and is a recommended aspect of the protocol due to the hydrophobic nature of the membrane; however, this can vary from protocol to protocol in terms of the amounts used and the length of time in the well (Janetzki *et al.*, 2005). As this is a step left to the discretion of the user and as the application of liposomes in the assay was a novel concept, it was important to assess whether variation of the volume of ethanol used for this pre-wetting step would have any impact on the liposomes prior to establishing the coating procedure.

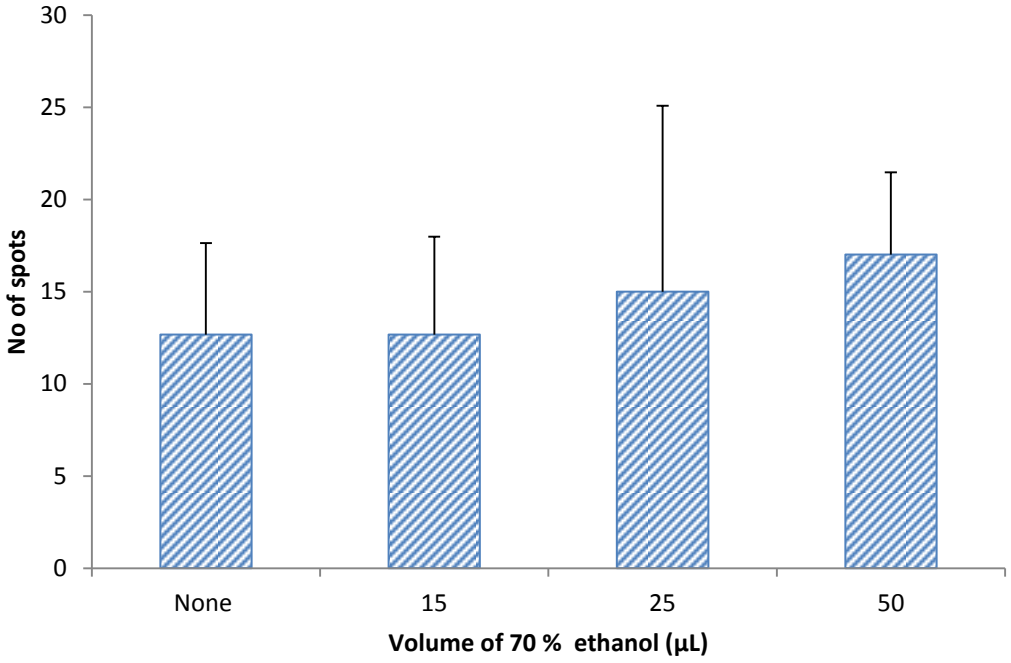


Figure 4.14 The effect of variation of the pre-wetting volume of 70 % ethanol on spot numbers generated for PC:Chol (8:8 µMol) formulations with an average size of 12.28 ± 2.4 µm. Results represent mean ± SD of n=3 batches.

The results observed (Figure 4.14) indicate that there was no significant difference in the spot number generated upon variation of the pre-wetting volume. The assay also served as 'proof of principle' that in fact liposomes were able to produce spots on the ELISPOT assay (Figure 4.15). The spots were visible under the stereomicroscope and showed that the encapsulation, release, and binding of the recombinant IFNγ to the well membrane was achieved.

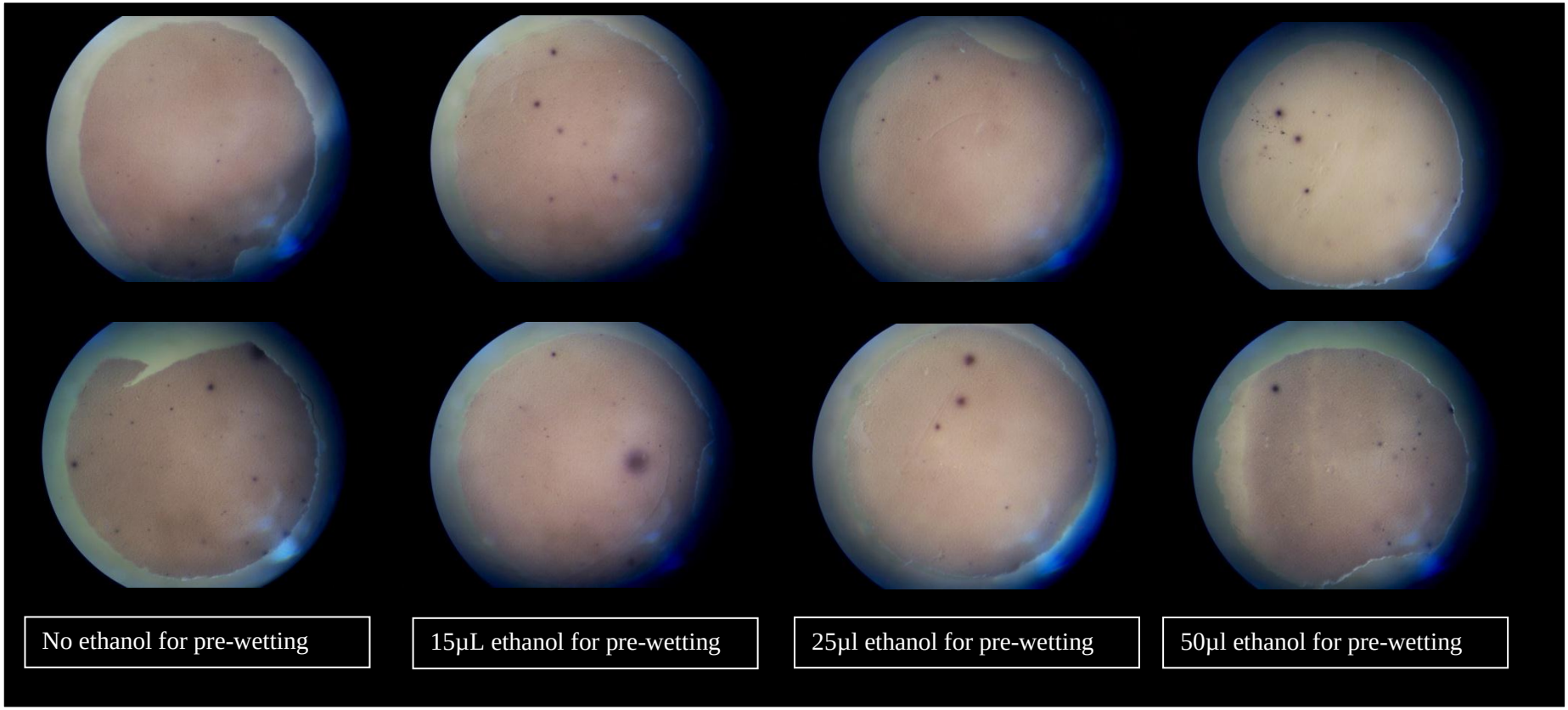


Figure 4.15 Photographic Images obtained with 2.8 X magnification using the Leica stereomicroscope with spots counted with various pre-wetting volume of 70% Ethanol. Images shown are representative and are of the n=1 PC:Chol formulation and were obtained using a Kodak camera over the eyepiece.

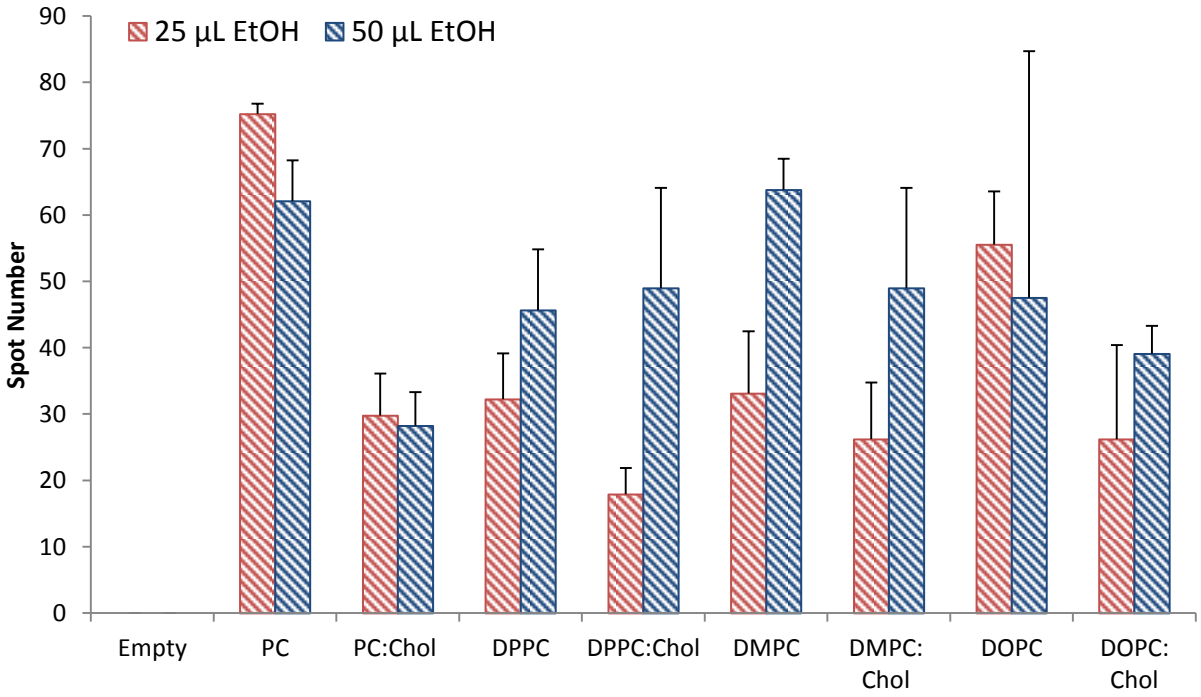


Figure 4.16 The effect of changing the pre-wetting volume on empty PC liposomes and rIFNγ containing liposomes of PC, PC:Chol, DPPC, DPPC:Chol, DMPC, DMPC; Chol, DOPC and DOPC:Chol. Total lipid amounts were 16 µMol. Results represent mean ± SD of n =3 batches.

An assessment was made of the pre-wetting volumes of 25 µL and 50 µL across liposomal formulations made from various lipids with and without equimolar cholesterol (Figure 4.16). When comparing the effects of using 25 µL and 50 µL of ethanol there was no significant difference shown for PC, PC:Chol, DPPC, DMPC, DOPC or DOPC:Chol liposome formulations. However, there was a significant ($p<0.001$) difference for the formulations of DPPC:Chol and DMPC. It was interesting to observe that the formulations of PC 16 µMol gave a significantly higher ($p<0.001$) spot number compared to all the other formulations other than DOPC at 25 µL pre-wetting volume. These results show no significant variation in spot number across the formulations at 50 µL pre-wetting volume and the formulation that gave the highest spot number was for the formulation of PC 16 µMol. The results from this assessment for the pre-wetting volume with all the formulations served to confirm that 50 µL of 70 % ethanol would be used for subsequent work.

Previous studies have shown with cells that the absence of a pre-wetting step prior to antibody coating of the wells can result in reduced spot number and can affect background, with results showing up to a 30 % reduction in spot number (Weiss, 2005). From the results observed it was also evident that different liposomal compositions are affecting spot number; it has become well established that different liposomal compositions will have very different properties in terms of release characteristics (Maurer *et al.*, 2001). This is based on whether lipids are saturated or unsaturated, the length of the alkyl chain and the inclusion of cholesterol (Vemuri and Rhodes, 1995). Higher transition temperature lipids such as DPPC were less leaky at 37 °C and seven at the 50 µL pre-wetting volume had the least mean spot number compared to other lipids in the absence of cholesterol (46 ± 9 spots). The effect of using cholesterol within the formulations was most prominent when comparing the PC 16 µMol formulations to the formulation containing cholesterol 8:8 µMol where the mean spot number was observed to be 62 ± 6 and 28 ± 5 respectively. Although DOPC has the lowest transition temperature it did not have the highest spot number, this may be due to the increased levels of background observed. The formulation process retained the activity of the encapsulated rIFNγ for all of the formulations assessed; thus, the 'proof of principle' was established for a range of liposomal formulations.

4.3.7 Establishing the buffer to be used for enzyme dilutions and a preliminary trigger release assessment

The four different variables that were to be examined for each formulation were;

(a) thermo-triggered release ie the effects of placing the liposome formulations in the ELISPOT well and placing them at 37 °C which is an essential step of the ELISPOT assay (it is the'incubation period' for the cells ordinarily used for this assay).

(b) Enzymatic triggered release, i.e. this will initially be assessed using phospholipase C to promote the release of cytokine through the breakdown of the bilayer.

(c) Triggered release using a detergent, in this case Triton X at 10% v/v concentration. Prior to examining the trigger release options on a range of formulations it was important to ensure the

conditions for enzyme were optimal, this included examining the buffer and the concentration of the enzyme to be used.

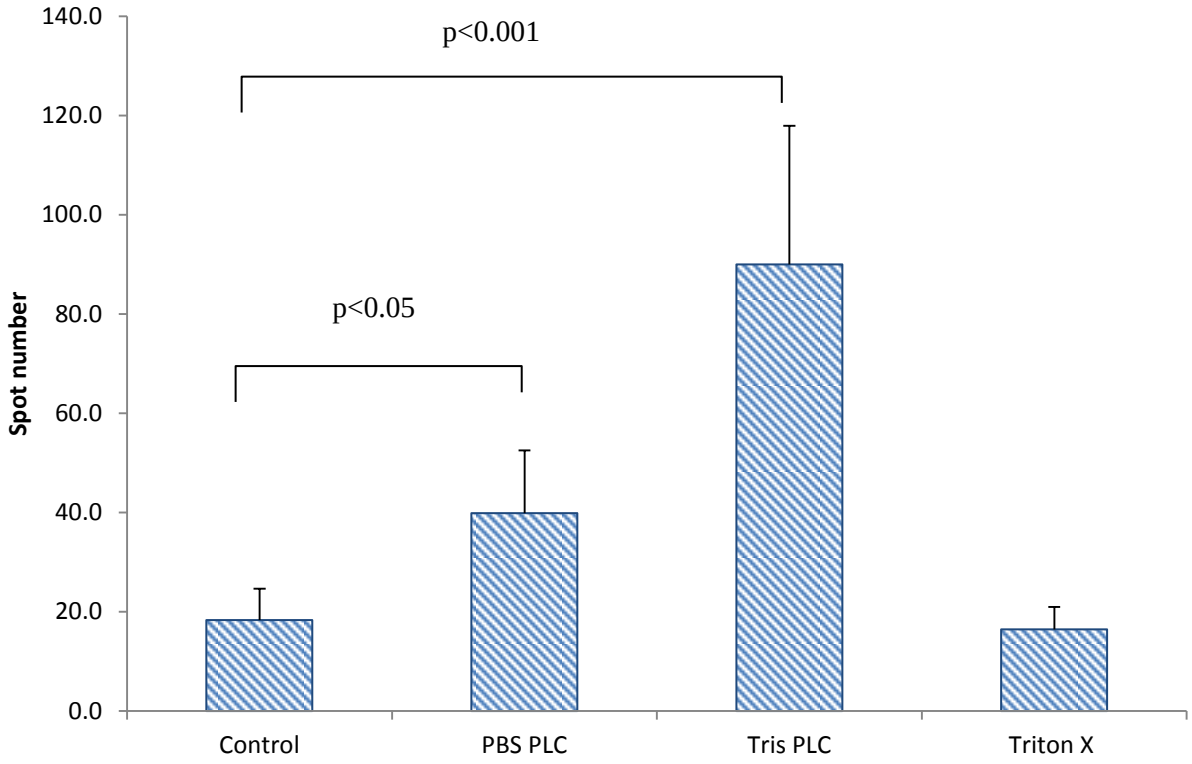


Figure 4.17; ELISPOT spot number obtained for the assessment of PLC triggered release mechanisms in PBS and Tris and Triton X release from PC:Chol formulations with an average size of 12.11 ± 0.9 µm and the zeta potential of -10.23 ± 5.31 mV (data not shown). Results represent mean ± SD of n =3 batches.

Statistical analysis of the results (Figure 4.17) has shown that when comparing the control to the PLC in PBS the results are significantly different ($p<0.05$). However, there was a greater statistical significance between the control and the PLC in Tris buffer ($p<0.001$) with 18 ± 6 spots and 90 ± 28 spots respectively. There was no statistical significance between the results of the control formulation and Triton X triggered release. The results showed that the most suitable buffer for the dilution of PLC, which maintained the activity of the enzyme was the Tris based buffer, (Tris 10Mm, 150mM NaCl and 10mM Calcium Chloride). Triton X was also added to the liposome formulation and had a significantly decreased ($p<0.001$) spot number compared to the trigger release mechanism (PC:Chol + PLC in Tris) 16.4 ± 5 and 90 ± 28 spots respectively.

It was important to ensure that prior to making assessment of release of liposomal formulations in the presence of external triggers that smaller assays were carried out to optimise parameters; ensuring the correct buffer was used for the enzymes was an essential pre-requisite for the assay to establish optimal conditions. The results (Figure 4.17) indicate that the Tris based buffer-containing $CaCl_2$ allowed the enzyme to work more effectively in the hydrolysis of the liposomal membranes. These results (Figure 4.17) concur with previous studies which have highlighted the catalytic properties of PLC enzymes was dependant on calcium as a cofactor and increases with rising calcium concentration (Ellis *et al.,* 1998). Other studies that have isolated PLC from *Bacillus Cereus,* used a Tris based buffer with the addition of 2 μMol $CaCl_2$ in the assessment of activity (Kleiman and Lands, 1969). Even with the PBS there was an increase in activity observed compared to the control formulation with the presence of the PLC. This agrees with previous studies (Holopainen *et al.,* 2002) that have shown that PLC successfully hydrolysed giant unilamellar liposomes and the authors comment that the vesicle size and curvature plays significant role in the outcome of the enzymatic reactions taking place on the lipid membrane.

Triton X is a well-established non-ionic surfactant used for the lysis and degradation of liposomal membranes and was therefore assessed for the purposes of method optimisation (Urbaneja *et al.,* 1988). The lack of an increase in spot number was thus, not expected (Figure 4.17). In the qualitative assessment of the well it was observed that the control formulation in the absence of any triggers (Figure 4.18 a), showed defined spots with reduced background whereas the wells in which enzymatic triggers are used (Figure 4.18 b and d). These spots seemed less defined, possibly smaller and yet greater in number. In Figure 4.18 c, in which the Triton X was added to the settled liposomes, the background was high and spot number reduced. This can be attributed to the highly viscous nature of the surfactant, which is perhaps disturbing the layer of liposomes on the wells and causing release, whilst reducing contact of the vesicles with the membrane (Spicer *et al.,* 1986). Alternatively, the kinetics of release was too abrupt as this is thought to reflect upon spot morphology, density and general shape (Lehmann, 2005).

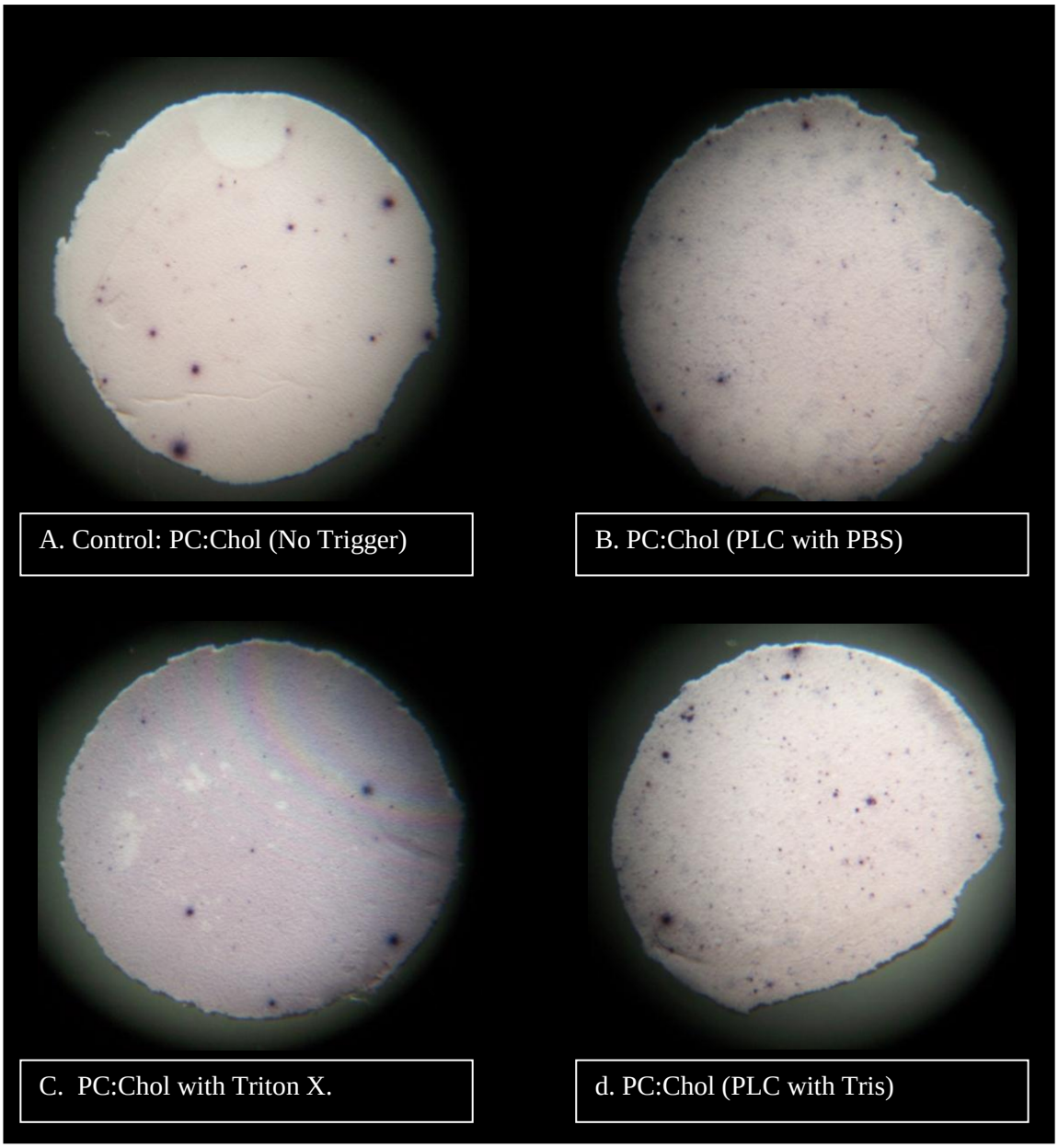


Figure 4.18 Selected images of ELISPOT wells for the effects of triggered release; **(a)** PC:Chol with no trigger **(b)** PC:Chol with PLC in PBS buffer, **(c)** PC:Chol with Triton X **(d)** PC:Chol with Tris images viewed under a stereomicroscope.

4.3.8 Establishing the effect of varying enzymatic concentration on spot number

Further to establishing that Tris was the optimal buffer for the assay, the next stage was to assess the effect of increased enzymatic concentration on spot number within the ELISPOT assay.

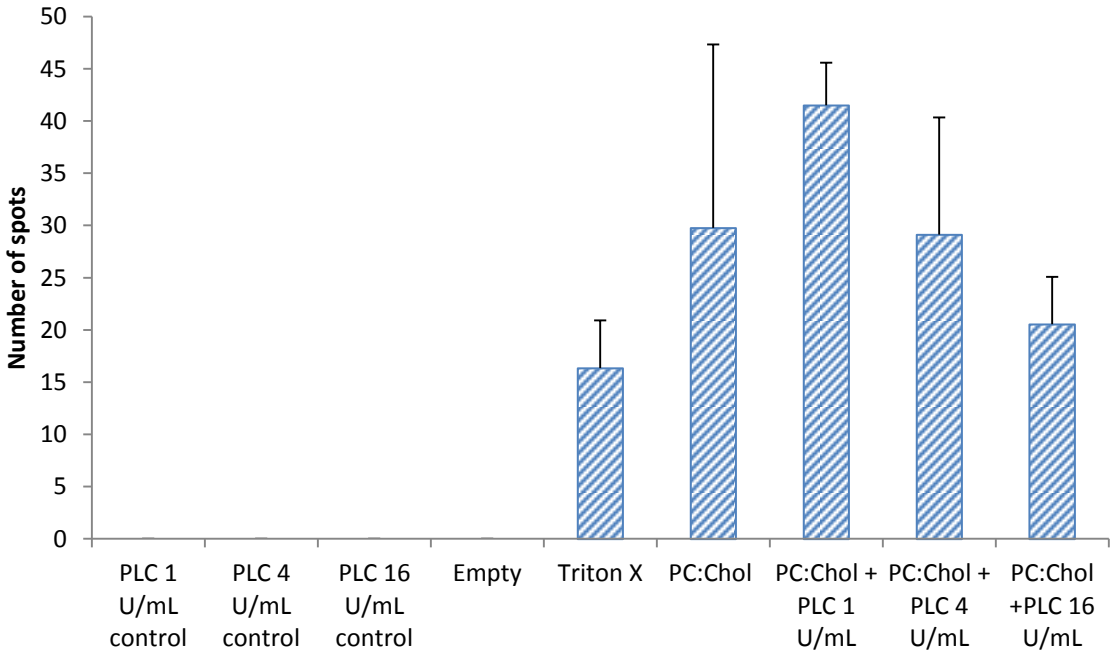


Figure 4.19 ELISPOT numbers generated for PC:Chol (8:8 µMol) formulations with PLC concentrations of 1, 4 and 16 U/mL and Triton X 10% v/v. Controls of 'empty' PC:Chol formulations and neat enzyme solutions were also assessed. Results represent mean ± SD of n =3 batches.

The PC:Chol formulations were measured for size and empty formulations were 10.3 ± 0.6 µm and IFNγ containing PC:Chol formulations had a mean particle size of 11.2 ± 1.3 µm (data not shown). The results show (Figure 4.19) that there was no significant difference between each of the variables when comparing the release to the control PC:Chol. As previously observed, the mean spot number for the detergent triggered release produced the least number of mean spots 16 ± 5 and the highest mean spot number was 41.5 ± 4.1 produced, when the liposomes were in the presence of PLC at 1 U/mL. Based on the observed results, even at the lowest concentration, activity of the PLC was evident. The enzyme solutions, which were ran as controls showed no spots thus confirming that the enzyme concentrations did not interfere with the membrane. Empty liposome formulations also did not promote spot formation. The ELISPOT images from all three assays in Figure 4.19, similarly confirmed high background levels for the Triton X, and enhanced levels for those wells where enzymatic triggers were used.

Figure 4.20 ELISPOT images for establishing the effective concentration of PLC with PC:Chol formulations. PLC concentrations assessed were 1 U/mL, 4 U/mL and 16 U/mL and Triton X 10% (v/v) were added to PC:Chol formulations.

There are two types of staining problems previously associated with the use of cells and these include specific and non-specific background; the first of which arises because of the secreted molecule diffusing away from the cell and the latter due to the adsorption of detecting components of the assay to the membrane (Kalyuzhny, 2005). The images suggest that with triggered release mechanisms and the Triton X the combination of enhanced release is resulting in such non-specific background staining although the presence of the liposomal membranes are not interfering with assays as the empty liposomes shown very clear wells.

As further confirmation, three different concentrations (1 U/mL, 5 U/mL and 10 U/mL) of PLA_2 were assessed. In Figure 4.21 results for the control and PLA_2 triggered release are shown.

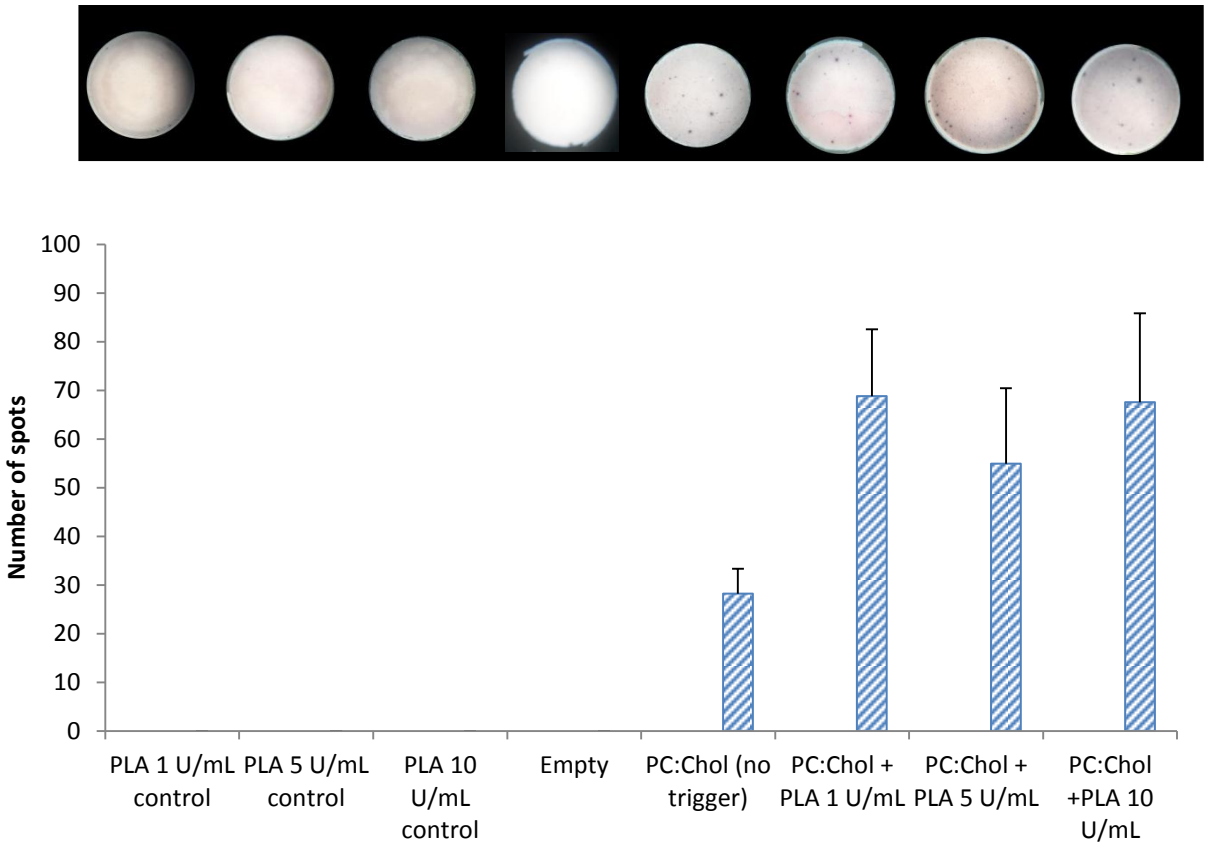


Figure 4.21 ELISPOT results to determine the phospholipase A_2 concentration for the triggered release of liposome formulations. Results represent mean ± SD of n =3 batches.

Size characterisation for PC:Chol formulations was carried out; empty formulations had a mean particle size 10.12 ± 0.04 µm and rIFNγ formulations had a mean particle size of 8.16 ± 0.33 µm (data not shown). It was necessary to confirm a suitable concentration of PLA_2, which for use in further studies. Concentrations assessed were 1 U/mL, 5 U/mL and 10 U/mL. The results (Figure 4.21) show there was a significant increase ($p<0.05$) in the number of spots when comparing the control to the concentration of PLA_2 at 1 U/mL (28 ± 5 vs. 68.8 ±13.7 respectively). There was also a significant difference ($p<0.05$) between the control and the PLA_2 at 10 U/mL (28 ± 5 vs. 67.6 ± 18.3 respectively). There was however no significant difference between the three concentrations. The results (Figure 4.21) confirm that a final concentration of 1 U/mL was sufficient for the triggered release of liposomes. It was interesting to observe that increasing concentrations of PLA_2

did not result in a linear increase in spot number. This pattern was also observed previously with PLC and suggests that manipulating the release to have the correct kinetics is perhaps more important than complete disintegration of liposomal membranes. As liposomes have not been used within the ELISPOT assay previously, the increased speed of release can perhaps be compared to the presence of having too many cells within a well. Zhang *et al.,* (2009) observed that increasing the number of cells did show a linear increase in spot number, until a critical point after which elevated levels of background and spot confluence resulted in a loss of this linear relationship. Whilst higher concentrations may actually be more effective in liposomal breakdown, 'overload' of the well with free cytokine is also a concern; thus 1 U/mL was deemed a suitable concentration for further studies.

4.3.9 ELISPOT results for thermo-triggered, PLC and Triton X triggered release

Having optimised and established activity of the enzyme and confirmed the actual methods for addition of trigger release vehicles a range of formulations were assessed to establish the optimal formulations to further progress the study and these were;

- PC and PC:Chol
- DMPC and DMPC:Chol
- DPPC and DPPC:Chol
- DPPC:Chol:BrChol
- DOPC and DOPC:Chol.

As previously particle size characterisation was carried out for all the formulations in this study; PC (9.8 ± 1.3 µm), PC: Chol (11.4 ± 2.1 µm), DPPC (10.3 ± 1.1 µm), DPPC: Chol (12.1 ± 1.4 µm), DMPC (8.94 ± 0.9 µm), DMPC: Chol (11.7 ± 2.1 µm), DOPC (7.8 ± 1.1 µm), DOPC: Chol (11.2 ± 0.8 µm) and DPPC:Chol:BrChol (8.5 ± 2.1 µm) and there were no significant differences observed (data not shown).

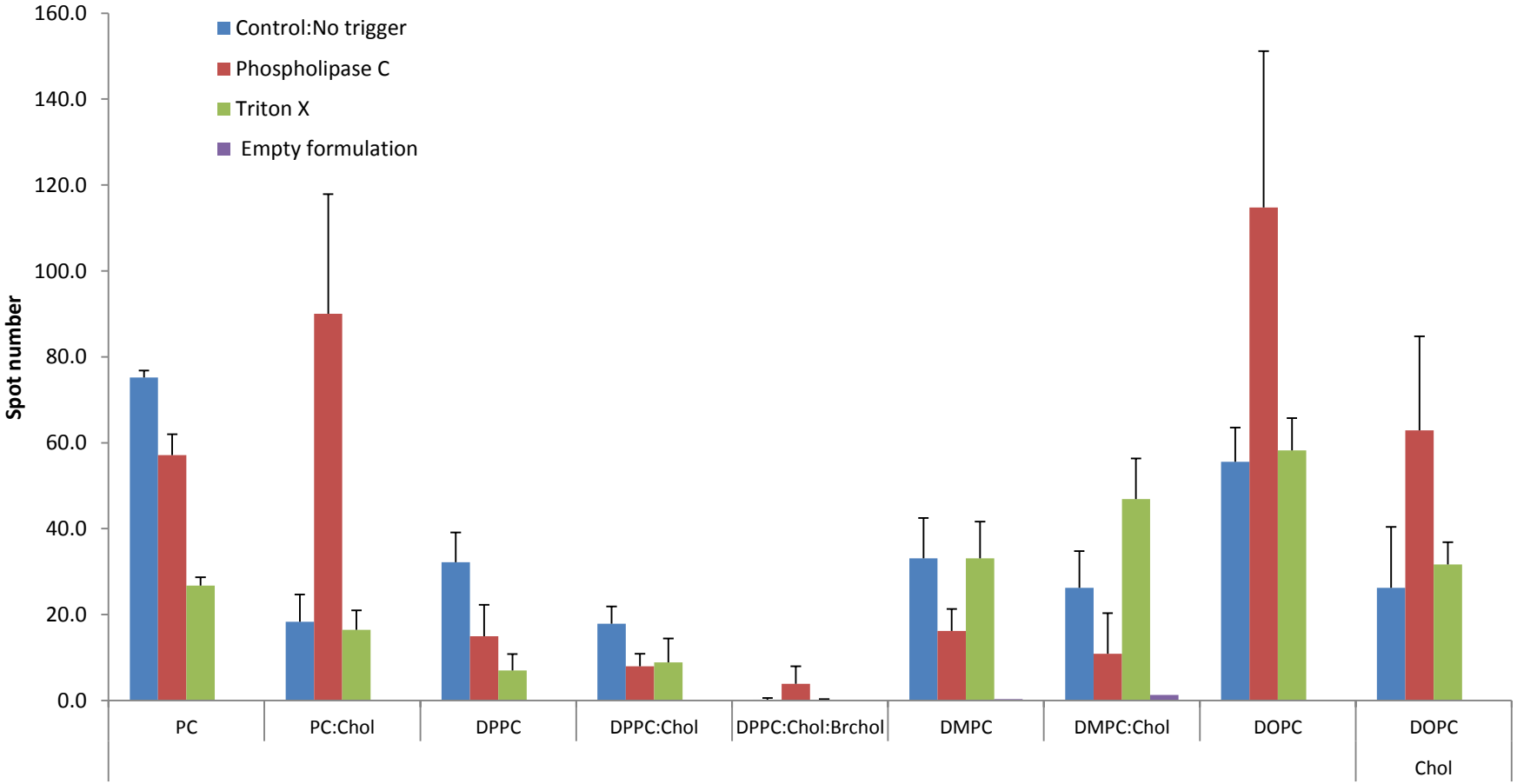


Figure 4.22 Assessment of enzymatic (PLC) and Triton X trigger release mechanisms using the ELISPOT assay for liposome formulations of PC, PC:Chol, DPPC, DPPC:Chol, DMPC, DMPC:Chol, DOPC, DOPC:Chol (16 µMol without cholesterol and 8:8 µMol with cholesterol) and DPPC:Chol:Brchol (16:4:12 µMol). Results represent mean ± SD of n =3 batches.

The formulations were assessed (Figure 4.22) for thermo-triggered release, promoted due to the assay being placed in the incubator at 37 °C overnight, Triton X for surfactant-based release, and enzymatic release with PLA_2 and PLC at a concentration of 1 U/mL with Tris based buffer containing calcium. Results were analysed for each aspect of release across the different liposomal formulations. From Figure 4.22 it can be seen that for thermo-triggered release, which relies on no external trigger, the optimal formulation in terms of spot number was PC which was significantly higher ($p<0.001$) than all formulations other than DOPC with 75 ± 1.6 spots and 56 ± 8.0 spots respectively. Although the background, levels observed were higher for DOPC formulations. The least spot number was observed for formulations containing the synthesised dibromocholesterol with few spots present. Even with additional trigger release mechanism present, such as PLC and Triton X, there was no further significant increase in spot number. In the analysis of enzymatic triggered release using PLC it was observed that a significant increase in spot number upon addition of the enzyme when compared to the control, only occurred for three formulations; PC:Chol ($p<0.001$), DOPC ($p<0.001$), and DOPC:Chol ($p<0.01$) with spot numbers counted to be 90 ± 28, 114 ± 36 and 62.9 ± 21.8 respectively. There was no significant difference in the formulations for Triton X triggered release when compared to thermo-triggered release other than for PC ($P<0.001$) where spot number In fact decreased upon addition of the detergent.

Enzymatic triggered release of PLA_2 was similarly assessed (Figure 4.23).These results show that only two formulations (PC:Chol and DMPC) demonstrated a significant ($p<0.001$) increase in spot number compared to the formulations in the absence of PLA_2. The spot numbers for PC:Chol formulations increased 2-fold from 28 ± 5 to 69 ± 16 with the presence of the PLA_2 similarly for DMPC there was over a 2-fold increase from 64 ± 4 spots for the control to 146 ± 7.8 for PLA_2 triggered release, thus the greatest increase in spot number was for DMPC. Although the enzymatic triggered release from PLA_2 gives an increased spot number, this simultaneously increases overall background level and an increased level of very small spots that are extremely difficult to discern from background on some occasions (Figure 4.24). Overall, from Figure 4.23 and 4.24 it can be seen

that the PC:Chol control formulations have consistently given well-defined spots and a reduced level of background without the need of a trigger.

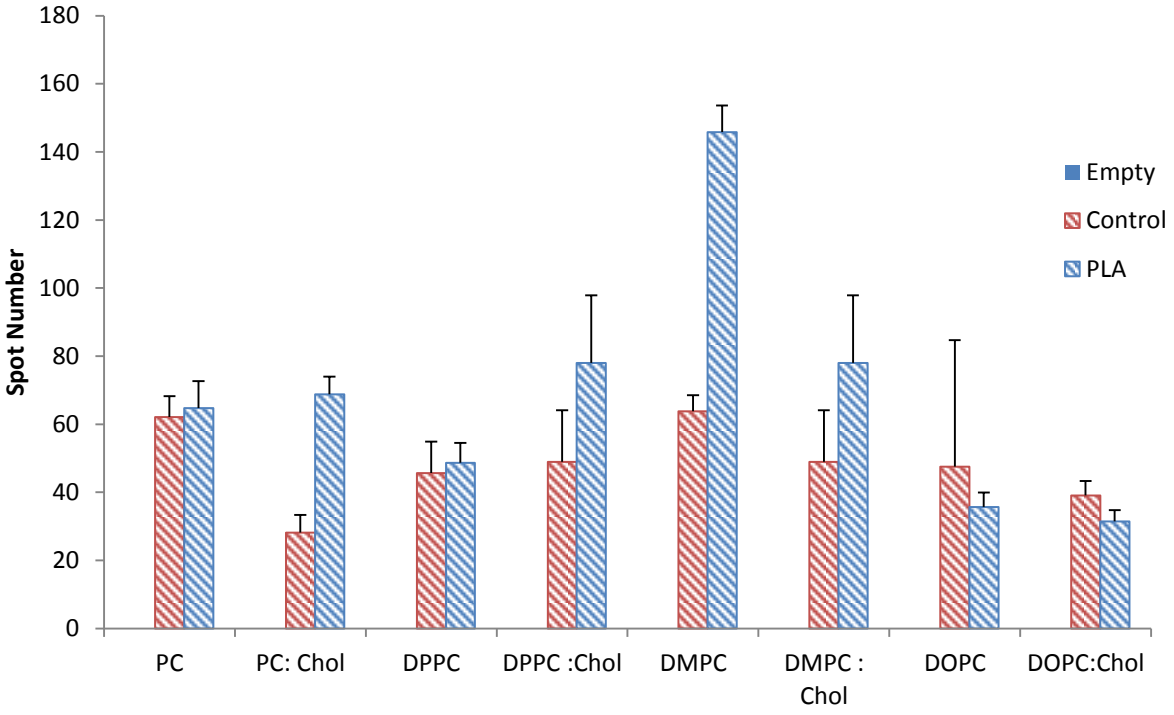


Figure 4.23 The effects of PLA_2 on spot number for liposome formulations of varying lipid compositions including PC, PC:Chol, DPPC, DPPC:Chol, DMPC, DMPC:Chol, DOPC and DOPC:Chol placed in the ELISPOT assay. Results represent mean ± SD of n =3 batches.

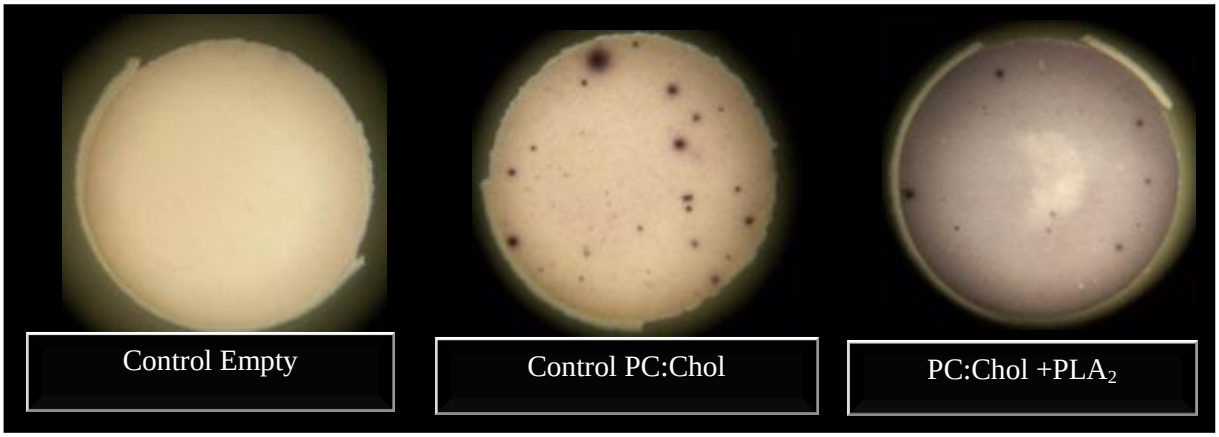


Figure 4.24 Representative ELISPOT wells of PC:Chol 8:8 µMol enlarged to compare the effects of trigger release mechanisms.

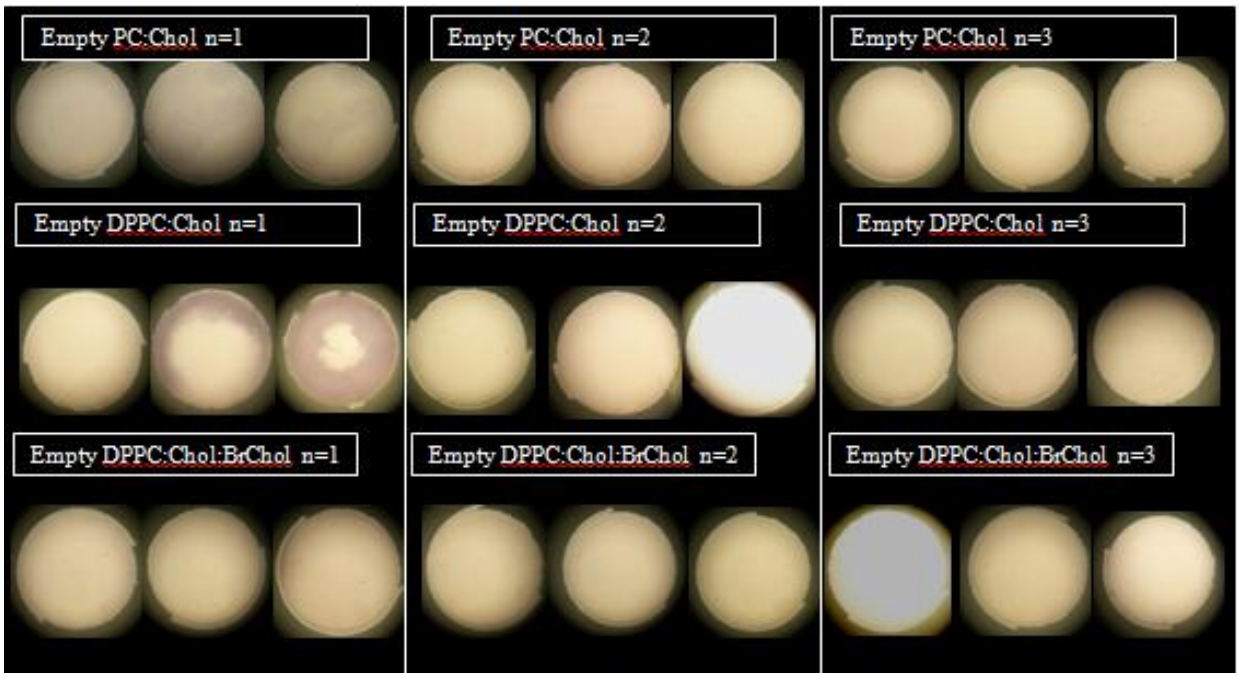


Figure 4.25 ELISPOT wells of control formulations of liposomes without rIFNγ.

The results observed for thermo-triggered release can be explained in terms of two factors; the transition temperature of the lipid and the inclusion of cholesterol. Figure 4.20 showed that with the formulations containing solely lipid, enhanced release was observed generally in accordance to the T_m of the lipid thus PC and DOPC had the greatest spot numbers and they have a T_m of -15 °C and -20 °C (Szoka and Papahadjopoulos, 1980). Spot numbers for thermo-triggered release were the least for DPPC, which is a saturated phospholipid with a T_m of 41 °C, thus the bilayers remained in the ordered gel state at the incubation temperature of 37 °C. Therefore, DPPC formulations retained the encapsulated rIFNγ more effectively, than for instance DMPC, which has a transition temperature of 23 °C (Maurer *et al.,* 2001). Leakage is thought to be maximal at the T_m of the lipid due to the existence of the two phases; the gel state and the liquid crystalline state, which causes bilayer defects (Lasic, 1998). However, with the inclusion of cholesterol as is well established within literature there was an altered the pattern of release observed. The presence of cholesterol at 1:1 equimolar ratio for all of the lipid formulations (other than DPPC:Chol:Brchol) resulted in a decrease in spot number. The presence of cholesterol changes the nature of the bilayer through improved stability of the bilayer

by reducing the fluidity of the bilayer and thus the movement of molecules through the membrane (Kirby *et al.*, 1980, Gregoriadis and Davis, 1979).

Triton X is an effective detergent used for lysis of cells and liposomes; however, it is known that its presence can lead to interference in some immunoassays (Lepage *et al.*, 1993). The Triton X wells (Figure 4.19) have increased background and reduced spot number. This may be due to the complete release of the rIFNγ from the liposomes or some level of interaction with the well itself or a combination of both. The results indicate the use of Triton X to enhance release for this study was ineffective. However, the results observed for PLC triggered release was promising for selected formulations such as PC:Chol. Increased spot numbers were also observed for PLA_2 with PC:Chol and DMPC; however, enzymatic release did also increase the level of background. For the purposes of in *in vivo* targeted delivery, areas of elevated enzymatic levels in disease state are often sought for rapid and effective release (Davidsen *et al.*, 2001, Kaasgaard *et al.*, 2009). However the suggestion from the data and images obtained was that use of triggered mechanisms alters the kinetics of rIFNγ release from liposomes and the resultant increase in release may be too rapid, as even when increased release rate occurs with cells on the ELISPOT plate the spots are larger and less defined (Lehmann, 2005). Furthermore elevated background levels with cells used in an ELISPOT assay can be as a direct result of increased secretory activity (Lehmann, 2005)

The use of the liposomal formulations containing the synthesised dibromocholesterol proved ineffective for spot formation within the ELISPOT assay, perhaps due to the high negative surface charge of the formulation, which may be detrimental for settling of the liposomes onto the PVDF membrane. The process of sedimentation of colloidal particles is somewhat complicated, there is literature that supports theories of how sedimentation velocity can be influenced by the size of the container, much remains unknown about the fundamental properties of the long-range nature of hydrodynamic interactions between particles (Guazzelli, 2006). Even though the initial aim was to produce vesicles, which were higher in density, how the synthesised dibromocholesterol inserted into the liposomal bilayer and consequently its interaction with the encapsulating material and the surrounding hydrodynamic environment was uncertain. However, its application within the ELISPOT

assay proved unfeasible and was not suitable for further development as a reference standard within the ELISPOT assay. The formulations of PC, PC:Chol and DMPC have shown effective spot formation without any elevated levels of background and promising formulations for further development and optimisation.

4.4 Conclusion

Establishing an ELISA assay initially was essential to demonstrate quantitatively the level of encapsulation with liposomes as well as the effect on activity of the cytokine that other factors such as temperature or the presence of Triton X may have. The assay was both reproducible and accurate for the purposes of this study. Encapsulation of the recombinant IFNγ was been achieved with a wide variety of formulations of varying lipid and/or cholesterol composition. The results from the ELISA assay suggest that the cytokine was able to withstand a variety of temperatures up to 55 °C for up to 30 minutes, which are sufficient for formulation purposes using a range of lipids. This allows us to investigate lipids with a range of transition temperatures for both thermo-triggered release and enzymatic release. A protocol for carrying out the ELISPOT assay was established, with an assessment of the pre-coating stage by using different volumes of 70 % ethanol. There seems to be no significant difference between spot numbers between the wells on variation of this parameter but the pre-wetting volumes of ethanol has shown that it can affect spot number and will thus be an integrated part of the protocol.

More significantly, there are clear defined spots on the ELISPOT plate, which demonstrate that, the principle that liposome vesicles can release rIFNγ encapsulated onto the PVDF membrane of the plate to produce spots in a similar way to cells. Although the 'proof of principle' has been established; it has been found that the optimised formulation containing brominated cholesterol was not effective at producing spots on the wells and thus can be eliminated as a formulation to be optimised. Triton X triggered release has also shown to be ineffective; as there are high levels of background associated with its use in the wells. Such levels of background would be detrimental to producing a robust

reference standard furthermore the viscosity of the Triton X solution also meant that upon being pipetted into the well sedimented liposomes were moved back upwards, this disturbance of the settled liposomes would result in release occurring but not whilst the vesicles were in direct contact with the PVDF membrane. This study proves that it has no benefit as a trigger release mechanism in an ELISPOT assay and there was not used for further studies. From the control formulation where there are no external trigger release systems other than the heat at 37 °C from incubation there is reduced background and a better level of defined spots. Such formulations are less susceptible to changes in the pre-wetting volume although the issue of very small spots still emerges but this is less so than having additional trigger release mechanism present. The formulations of PC and PC with cholesterol (1:1) have consistently showed low levels of background, well-defined spots and good reproducibility even when the pre-wetting volume was changed. From the work thus far completed with no attempt at optimisation of background or size of vesicles, they seem like promising formulations candidates to take forward for thermo-triggered release.

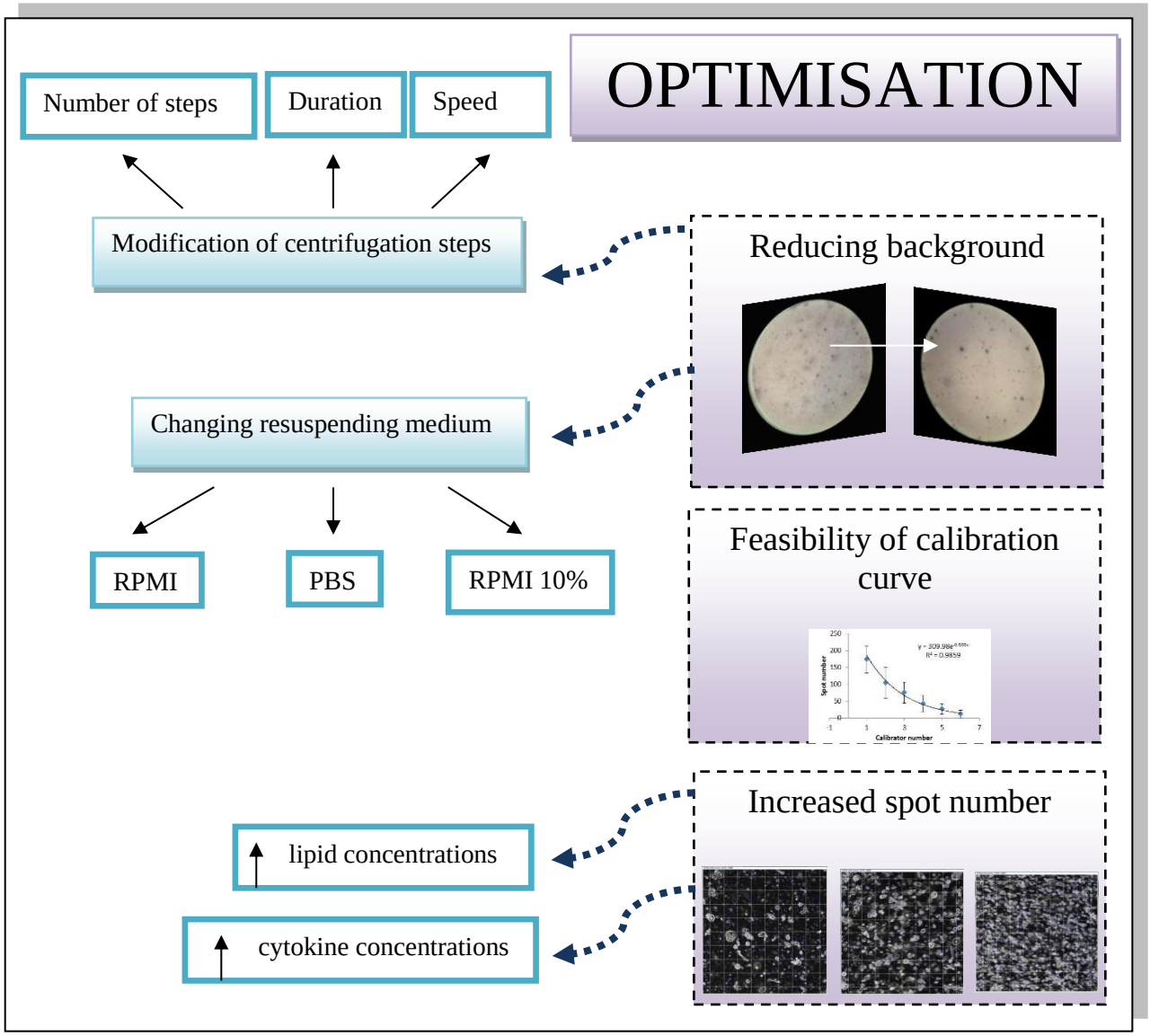
The enzymatic release studies so far indicate that the enzyme is working to degrade the vesicles for selective formulations such as PC:Chol and DMPC, however, for the majority of formulations at present this release is not translating in to an increased number of well-defined spots. Background levels when using the enzymes in the wells tends to be high, including diffuse spots and an increased number of very small spots. From the ELISPOT results, it is difficult to make decisive conclusions about the effectiveness of enzymes with formulations because they may be very effective at degradation and release of contents such as the Triton X, but this effectiveness may not be translating directly into an increase into spot number. The increase in number of smaller spots could also be counteractive when attempting to produce robust reference standards. Thus although ‘proof of principle’ has been established, further optimisation of the reference standards was required to ensure that they are robust and background levels are low and as such all parameters which could further enhance the reliability of the artificial reference standards was further investigated.

Chapter 5

The optimisation of liposome formulations

as artificial reference standards for the ELISPOT assay

Graphical abstract



5.1 Introduction

The need for reference standards for immunological assays has become more apparent over the past decade, particularly for the ELISPOT assay, as concerns regarding variability of assays have grown (Smith *et al.,* 2009, Janetzki *et al.,* 2009). It has been found that immunoassays have been particularly problematic with regard to achieving the aim of standardisation (Wadhwa and Thorpe, 1998). The proof of concept for a novel artificial reference standard using liposomes within the ELISPOT assay was previously shown (chapter 4). However, further parameters required investigation, including optimisation of the protocol and establishing how such a novel reference can fulfil the criteria required of established reference standards.

5.1.1 Reducing background levels when using liposomes in the ELISPOT assay

The ELISPOT assay is established for the use of cells, and even for this purpose, the importance of reducing or eliminating background staining is an important consideration (Grant *et al.,* 2005, Janetzki *et al.,* 2005, Kalyuzhny, 2005). The assay is able to detect low-level cytokine secreting cells and this has resulted in its widespread use within vaccine clinical trials (Moodie *et al.,* 2006). However, non-specific staining can give high background levels, leading to challenges in spot detection and quantification (Grant *et al.,* 2005).

In the previous work (chapter 4), liposomes were formulated in PBS. However, many ELISPOT assay protocols use cells placed in either media, or media supplemented with 10-20 % fetal bovine serum (Janetzki *et al.,* 2005). More recently, the cancer vaccine consortium (CVC-CRI) has identified the choice of serum in ELISPOT assays as one of the leading causes for variability within assays and found that serum free medium performs as well as qualified medium/serum combinations (Janetzki *et al.,* 2010). Therefore, it was necessary to assess the feasibility of using alternative resuspending medium to PBS that was previously used; especially as cell medium RPMI with or without 10 % FCS may help to reduce the inconsistencies observed with background variability.

Spot enumeration is normally conducted using a stereomicroscope, there are also automated processes available, but in both cases background levels need to be low to ensure accuracy (Janetzki *et al.*, 2004). In addition, there are difficulties associated with the use of manual counting techniques such as the difficulty in counting high spot numbers, limited resolution making it difficult to recognise and exclude artefacts, and the counting of small and faint spots (Janetzki *et al.*, 2004). Thus, the importance of introducing an artificial standard with low levels of background and high levels of spot clarity are essential.

5.1.2 Investigating the feasibility of calibration curves and enhancing spot number

Generally, for the production of reference standards there has been a rule of 'like versus like' where the standard should mimic as closely as possible the test sample. However, there are an increasing number of exceptions to this rule, and according to the World Health Organisation (WHO) guidelines, no longer considered essential with some reference standards having qualitative rather than quantitave purposes (WHO, 2006). Reference materials are generally for the purpose of assay calibration, and invariably used in the form of a dose response curve, although they can also be used comparatively for comparing the performance of various assays and their ability to detect similar analytes (Thorpe, 1998). Based on this, it was important to investigate the feasibility to produce calibration curves from a serial dilution of the highest control.

Other parameters, which also required investigation, were the possibility of increasing spot number to produce a control. Optimisation of the control, which assesses whether the optimal lipid or cytokine concentration have been achieved are important parameters that require assessment. Examining the impact of changing these parameters on spot number will determine the limits for the reference standard in terms of maximum spot number achievable.

5.2 Aims and Objectives

Therefore, the aim of the work was to optimise the formulation process so as to reducing background and enhancing spot number. Based on this, the objectives were as follows:

- To assess the effect of resuspending liposome formulations with cell medium, in particular RPMI or RPMI with 10 % FCS and their compatibility within the assay.
- To assess the feasibility of a calibration curve for the assay, using a serial dilution from the main reference standard.
- To examine a range of lipid concentrations and subsequently assess the impact upon spot numbers.
- To assess whether increased cytokine concentration can improve spot number and/or clarity of the spots obtained in the assay.
- To compare spots produced from liposomes and those from live cells and automated reading of the ELISPOT assay and manual counting.

5.3 Results and Discussion

5.3.1 Assessment of optimised liposome formulations for triggered release

Three liposomes formulations 1) PC, 2) PC:Chol and 3) DMPC were further investigated for triggered release within the ELISPOT assay, based on results from Chapter 4. There was no significant difference in the size or zeta potential across the formulations (Table 5.1). In the assessment of trigger release using either thermo-triggered or enzymatic-triggered release with PLA_2 and PLC, it was found that the background levels for the assays were high from qualitative assessments (Figure 5.1). The representative wells of the thermo-triggered release control formulations shown with individual spots marked (Figure 5.1); this indicates the challenge of discerning the spots from the image alone (although when viewing directly under the stereomicroscope there was slightly greater clarity).

The overall results (Figure 5.2) indicate that in terms of thermo-triggered release there was no significant difference in observed spot numbers between the three liposome formulations (PC, PC:Chol and DMPC; Figure 5.2). Generally, the use of the enzymatic triggers PLA_2 or PLC did not increase in spot numbers (Figure 5.2). However, only very low levels (1.3 ± 1) were detectable for the PC:Chol formulation in the presence PLC and spot numbers produced by the PC formulation in the presence of PLC were also significantly lower (two-fold; $p<0.05$) than those produced by the DMPC formulation.

Table 5.1 Characterisation data for the formulations of PC, PC:Chol and DMPC used within the ELISPOT assay. Results represent mean ± SD for n=3 batches.

Formulation	Lipid composition (µMol)	Size (µm)	Zeta Potential (mV)
PC empty	16	7.98 ± 0.09	-9.72 ± 6.80
PC:Chol empty	8:8	10.99 ± 0.20	-5.70 ± 5.70
DMPC empty	16	8.11 ± 0.23	-8.88 ± 9.36
PC rIFNγ	16	8.39 ± 0.06	-6.08 ± 6.32
PC:Chol rIFNγ	8:8	9.08 ± 0.01	-6.30 ± 7.80
DMPC rIFNγ	16	10.06 ± 0.05	-5.17 ± 7.85

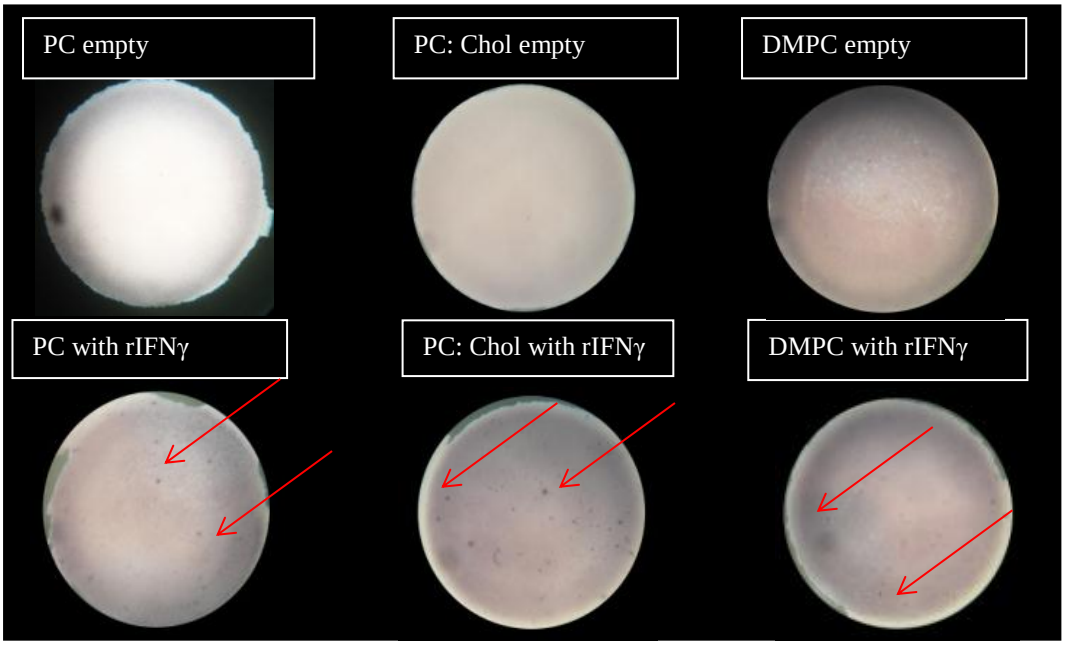


Figure 5.1 Representative ELISPOT wells for wells containing (a) 'empty' liposomes for PC, PC:Chol and DMPC and (b) control formulations containing rIFNγ without external triggers.

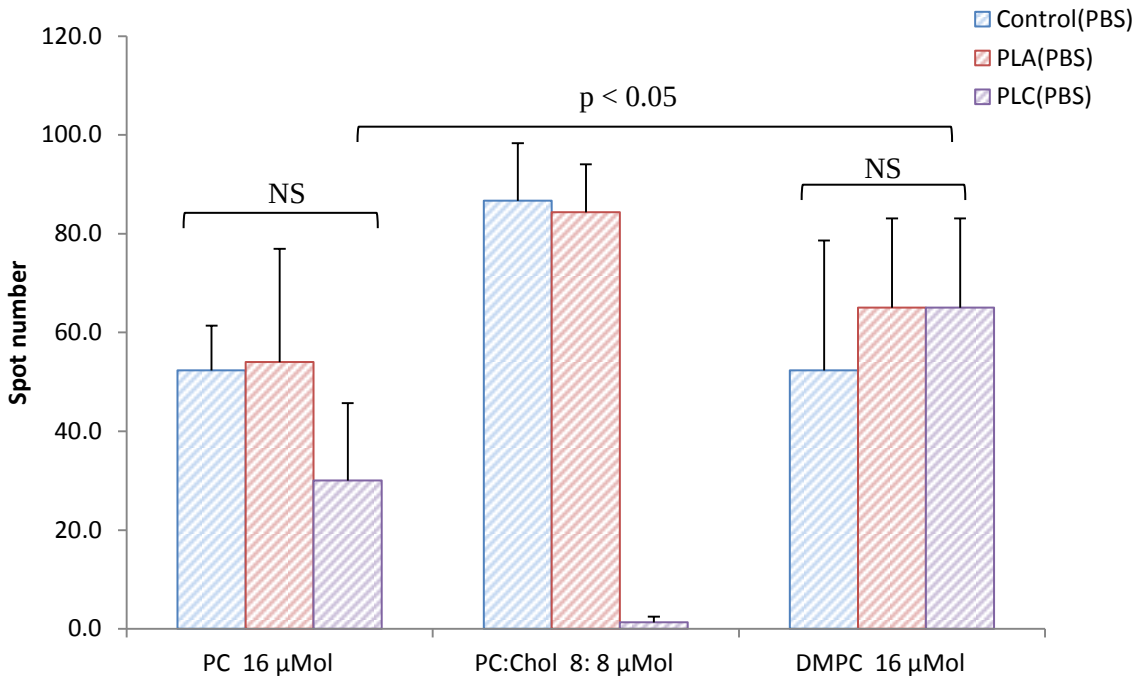


Figure 5.2 ELISPOT numbers for PC 16 µMol, PC:Chol 8:8 µMol and DMPC 16 µMol liposome formulations with thermo-triggered release (control) and enzymatic triggered release using PLA_2 and PLC. Results represent mean ± SD for n=3 batches.

Overall, these results show that using PLA_2 and PLC as enzymatic triggers for release offers no benefit in terms of spot number (Figure 5.2). Previous results have confirmed that the enzymes can disrupt liposomes and promote triggered release (chapter 3), and there are numerous examples (e.g. Meers, 2001) where such enzymes, in particular PLA_2, have effectively promoted release of liposomal contents. Thus the degradation of the liposomal membranes and subsequent protein release are most likely occurring; however, it maybe that the kinetics of release was not beneficial for spot formation. Indeed degradation of the liposomes may be too rapid and occurring prior to sedimentation of the liposomes and increase background levels. When cells are used within these assays to promote spot formation, the relationship between spot size is based on various parameters including cytokine secretion rate, net amount produced, binding and lateral diffusion of the cytokine (Karulin and Lehmann, 2012). A rapid secretion is thought to produce large spots which are not well defined (Lehmann, 2005).

In terms of thermo-triggered release, the lack of discernible differences between the three formulations of PC, PC:Chol and DMPC, these control formulations had no external trigger release mechanisms; the release of the encapsulated rIFNγ was a result of the permeability changes to the membrane occurring at the incubation temperature of 37 °C (Szoka and Papahadjopoulos, 1980). In a study by Anderson and Omri, (2010) the effect of different liposomal compositions were assessed for *in vitro* stability at 37 °C using DSPC, DPPC and DMPC liposomes prepared by sonication. It was found that DSPC formulations (which had the highest transition temperature of 55 °C) gave the highest retention (85 ± 10 %) of radiolabelled inulin at 37 °C and the lowest retention was observed for DMPC liposomes at 53 ± 5.3%. Whilst this may suggest that PC liposomes would give the highest spot number when used in the ELISPOT assay, the accuracy of this assay is dependent upon a good contrast between the background and spot formation. In the presence of high background levels spots become masked, compromising quantification (Kalyuzhny and Stark, 2001) thus enhanced release rates of rIFNγ within the assay may not translate into localised release of the protein when liposomes sediment in the well of the ELISPOT assay, which is required for effective spot formation with low background levels.

5.3.2 Identifying the appropriate suspension media for liposomal reference standards for greater compatibility within the ELISPOT assay

To further enhance the compatibility of the artificial liposome reference standards (PC 16 μMol and PC:Chol 8:8 μMol) within the ELISPOT assay, the liposomes were re-suspended in either 1) PBS, 2) RPMI or 3) RPMI with 10 % FCS. When comparing the choice of suspension buffer (Figure 5.3), there was no significant difference in the spot numbers produced by either PC and PC:Chol liposome formulations in PBS or RPMI. However, when suspended in RPMI with 10 % FCS spot numbers were significantly reduced ($p<0.05$ and $p<0.01$ for PC and PC:Chol formulations respectively; Figure 5.3). Indeed there was a two-fold reduction in the generation of spot number (Figure 5.3) for PC (from 97.11 ± 21 in PBS to 45 ± 17 in RPMI with 10% FCS) and with the PC:Chol formulations over a five-fold reduction in spot number (from 85 ± 21in PBS to 15 ± 7.7 in RPMI with 10 % FCS). As

previously noted there was no significant difference between the formulations in the same buffers (Figure 5.3).

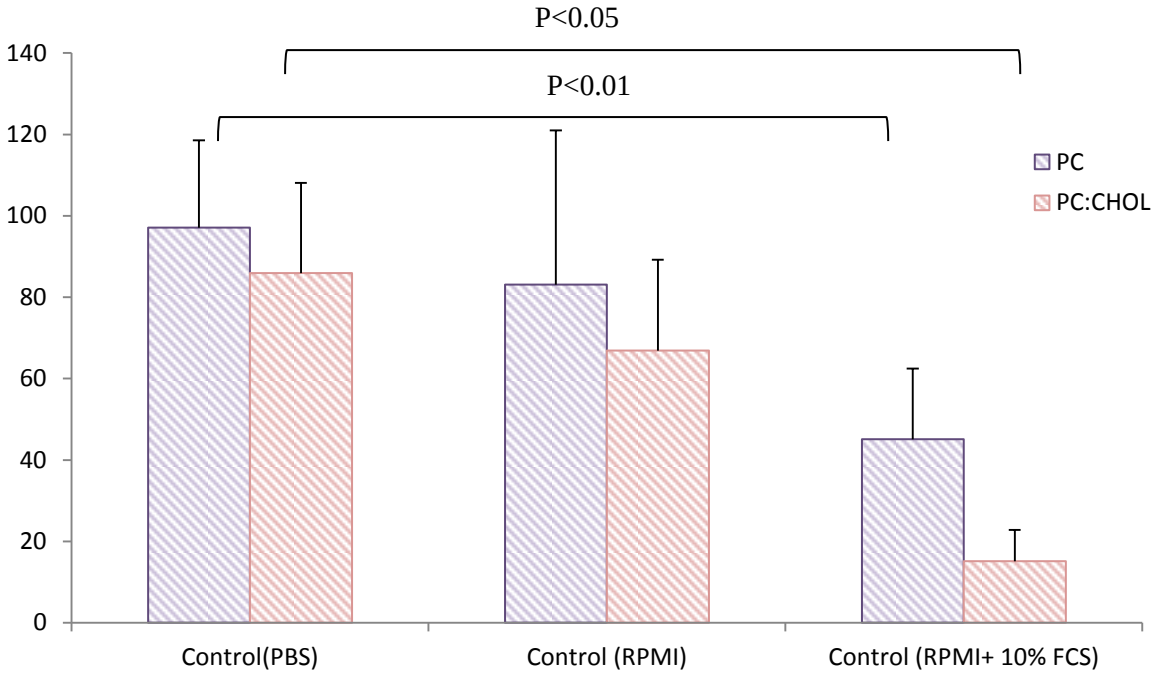


Figure 5.3 Spot numbers generated for formulations of PC 16 µMol and PC:Chol 8:8 µMol subsequent to two centrifugation steps and the effects of different resuspending medium. (Size: 11.92 ± 0.24 µm and 12.01 ± 2.4 µm respectively, data not shown). Results represent mean ± SD for n=3 batches.

The results showed that RPMI can be used as the re-suspending buffer instead of PBS allowing the reference standards to be in the same media as the rest of the samples placed within the assay thus making it more compatible (Hempel, 2004). Cells used in the ELISPOT assay are ordinarily in RPMI as it contains all the necessary carbohydrates, amino acids, vitamins and inorganic salts required for the cells and it is often necessary to add animal or human derived serum (Ng and Schantz, 2010). However, the additional presence of serum in the re-suspension media resulted in the reduction in spot number, and this may be due to serum induced liposome instability. A recent study (Hossann *et al.*, 2012) investigating the effect of serum on thermosensitive release of liposomes found the liposomes were influenced by serum components and the constitutive proteins can adsorb to liposomes and affect the integrity of the membrane bilayer through partial penetration promoting leakage of the entrapped material. However, previous studies by Gregoriadis and Senior (1980) have shown that

through the inclusion of 50 mole % of cholesterol in liposomes prepared from PC, DPPC or DMPC had enhanced stability, and *in vivo* permeability was significantly reduced through a reduction in opsonisation. For instance, the leakage of carboxyfluorescein from liposomes prepared from DSPC was reduced from 86.5 % to 32.4 %, and for PC liposomes retention increased from 0 to 86.3 %.

Even when cells are used in assays, there is a general increase in the use of cell free medium (Ng and Schantz, 2010). The addition of the serum is thought to interfere with cell culture and subsequent analysis and disadvantages include lot–to-lot variation and the presence of inhibitors or toxins, which cause unwanted reactions or interference (Ng and Schantz, 2010). Within ELISPOT assays, the pre-testing of serum batches is recommended to ensure low background reactivity and optimal antigen specific spot reduction. Furthermore, it has been reported by the Assay Working Group of the Cancer Vaccine Consortium (CVC-CRI) that the choice of serum to be the leading cause for variability and suboptimal performance in large international ELISPOT proficiency panels (Mander *et al.*, 2010, Janetzki *et al.,* 2010, Smith *et al.,* 2009). To overcome the variability in the effect of batches received, and changes in serum properties over time, a large-scale harmonisation trial reported by Mander *et al.*, (2010) (organised through The Cancer Immunotherapy Immunoguiding program) showed a direct comparison of serum and serum free medium on the ELISPOT assay. The recommendation was put forward that serum supplemented media could be eliminated, as detection rates and background were similar. A similar large-scale trial further confirms this recommendation, which has shown that one type of serum free medium showed increased spot number (Janetzki *et al.*, 2010).

Whilst it was most likely that the use of serum in this study resulted in decreased spot number with liposomes possibly due to a destabilising effect, changes in the viscosity of the resuspending buffer was also tested (as according to Stokes' law, viscosity difference can affect sedimentation behaviour (Banker and Rhodes, 2002)). The results in Table 5.3 confirmed that there was no significant difference in the viscosities between the formulations.

Table 5.2 PC (16 μMol) formulations assessed for differences in viscosity between 1) PBS, 2) RPMI and 3) RPMI with 10 % FCS. Results represent mean ± SD for n=3 batches.

	Dynamic Viscosity[mPa.s]	Kinetic Viscosity[mm2/s]
PBS	1.07 ± 0.11	1.07 ± 0.11
RPMI	0.98 ± 0.00	0.98 ± 0.11
RPMI with 10% FCS	0.97 ± 0.00	0.97 ± 0.00

5.3.3 Reducing background in ELISPOT wells by modification of centrifugation steps for the removal of non-incorporated rIFNγ

The basic protocol established for the removal of unentrapped protein subsequent to formulation was one centrifugation step at 29,771 g for 30 minutes at 4°C (chapter 4). However, the results observed (section 5.3.1) have shown that background levels are varying between assays. The effects of modifying the protocol, to include an additional centrifugation step of 29,771 g for 30 minutes at 4 °C was assessed with liposome formulations composed of 1) PC, 2) PC:Chol or 3) DMPC (total 16 μMol) were assessed. The results for the physicochemical characterisation (Table 5.3) show that there was no significant difference in the physicochemical attributes of the formulations in terms of their vesicle size or zeta potential, as previously noted.

Table 5.3 Characterisation data for liposomes composed of PC, PC:Chol or DMPC. Results represent mean ± SD for n=3 batches.

Formulation	Lipid composition (μMol)	Size (μm)	Zeta Potential (mV)
PC	16	10.4 ± 1.27	-13.0 ± 7.94
PC: Chol	8: 8	8.99 ± 1.09	-5.96 ± 6.82
DMPC	16	7.83 ± 0.81	-6.88 ± 6.95

In terms of their efficacy as ELISPOT standards (Figure 5.4), there was a significant ($p<0.001$) increase in detectable spot number from the pre-centrifuged liposome formulations to the spots counted after the first centrifugation step. For liposomes prepared with PC and DMPC there was over a three-fold increase in mean spot number, and for PC:Chol liposomes there was over a five- fold

increase in spot number (Figure 5.4). However, the addition of a second centrifugation step, made no significant difference in spot number for any of the formulations and there was no significant difference between the three formulations for spot number at each stage (Figure 5.4). In terms of a qualitative assessment of the wells the impact of an additional centrifugation step did not make a notable difference to the background of the wells, although the intensity of the individual spots was slightly improved from the first centrifugation step (Figure 5.4 b) and subsequent to the second centrifugation step (Figure 5.4 c).

The increased spot number noted in the assay after removal of unentrapped rIFNγ from the liposome preparations, can be attributed to the presence of the free cytokine diffusing across the well prior to centrifugation, leading to high background levels and lower spot definition (Chambers *et al.,* 2010). This excess cytokine is detrimental to the specificity and sensitivity of the assay and can be related to other immunological assays such as ELISA assays. High protein binding plates are used to enhance detection in ELISA assays, but can result in high background levels and samples are either titrated to reduce the concentration plated or lower binding plates are used (Chiswick *et al.*, 2012).

Given that there was no significant difference in results after one or two centrifugation steps, this suggests that one centrifugation step can sufficiently remove free rIFNγ to an appropriate level. A two-step centrifugation protocol has previously been used for the effective removal of unentrapped protein, for vaccine containing formulations (Gregoriadis *et al.,* 1999). In this study, the liposome formulations were centrifuged at 40,000 g for 60 minutes at 4 °C and then the process was repeated again to ensure the effective removal of free vaccine. Thus, although an additional centrifugation step may be beneficial to ensure the removal of residual protein in this instance this did not translate into increased spot numbers, although background levels were improved slightly.

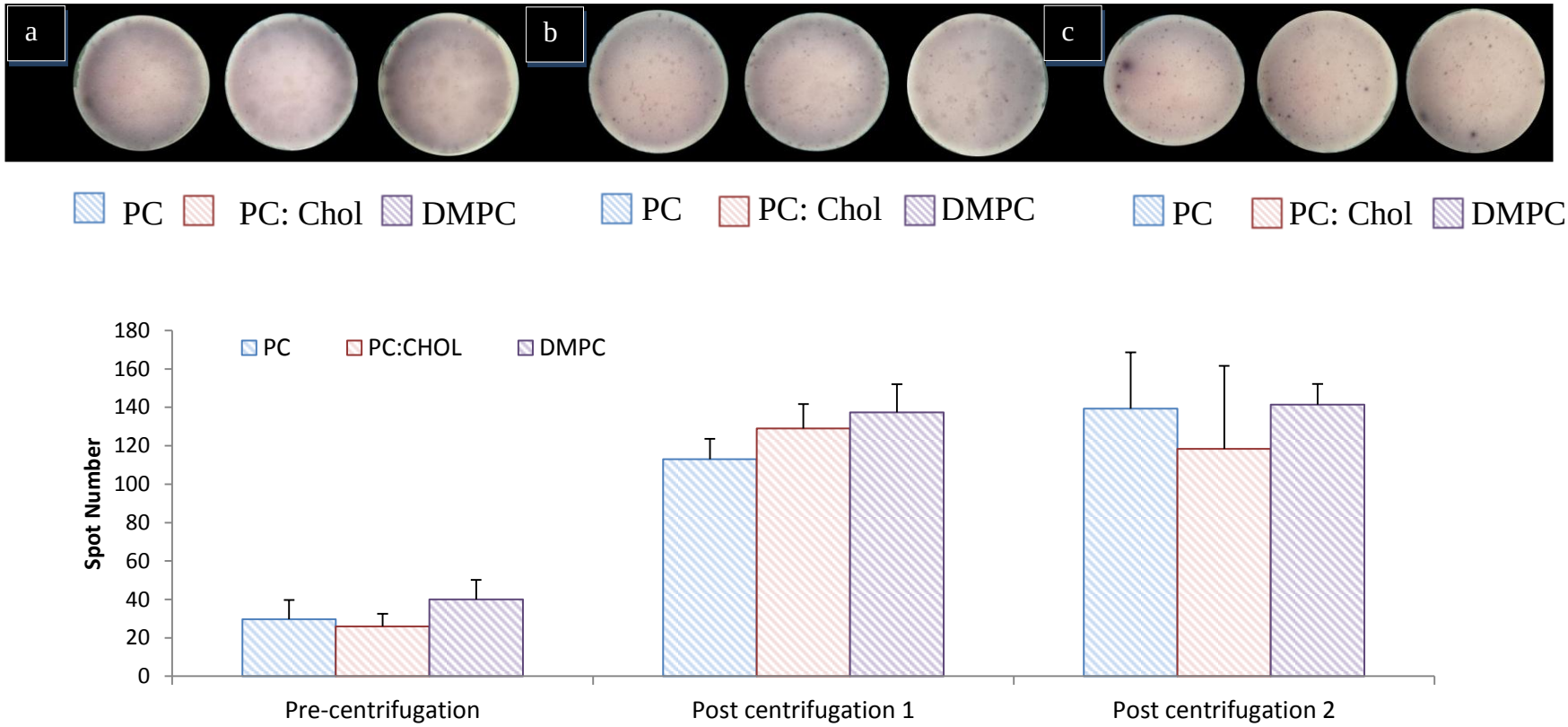


Figure 5.4 The spot numbers generated for PC, PC:Chol and DMPC liposome formulations (16 µMol total for each), shown pre-centrifugation and post centrifugation at 29, 771 g for 30 minutes and after the second centrifugation at 29, 771 g for 30 minutes. Representative ELISPOT images are also shown (a) pre-centrifugation (b) post- centrifugation 1 and (c) post -centrifugation 2 for each liposome formulation. Results represent mean ± SD for n=3 batches.

5.3.3.1 Reducing background of ELISPOT wells by removal of smaller liposomal vesicles

Previous results have shown that the addition of a slower centrifugation step for a short duration can aid the retention of larger liposomes and the removal of smaller liposomes (chapter 3). The inclusion of a slow speed and short duration step was included within the current protocol for the assessment on spot numbers. The protocol for the formulation and removal of unencapsulated rIFNγ included the following stages:

1. Centrifuge the formulations at 29, 771 g for 30 minutes at 4 °C.
2. Remove supernatant and re-suspend pellet in 3.9 mL of PBS.
3. Centrifuge the formulations at 29, 771 g for 30 minutes at 4 °C.
4. Remove the supernatant and re-suspend the pellet in 5 mL of PBS.
5. Centrifuge the formulations at 205 g for 10 minutes at 4 °C.
6. Resuspend formulations in 2 mL of RPMI.

The results in Figure 5.5 show that PC and PC:Chol formulations (16 μMol total) assessed using the optimised protocol showed no significant increase in spot numbers counted. However, there were improvements in background levels and spot clarity (Figure 5.6) especially when compared to the background of wells from the previous protocols such as Figure 5.4. The aim of adding the additional centrifugation step (205 g for 10 minutes at 4 °C) was to assess the effects of removing some of the smaller vesicles. The MLV formulations produced are heterogenous in nature (Figure 5.7) and thus there will be particles less than a micron in size, for such particles to pellet centrifugation speeds of 100,000 *g* are required (Gregoriadis and Senior, 1980). Differential centrifugation has been used extensively within the cellular field as an established method for the speration of cellular components and depends upon the fact that such components differ in size and density and will therefore sediment at different rates (Suckling and Suckling, 1980). Larger and denser particles will centrifuge more rapidly, so in terms of cell work small proteins and free lipid will remain in the supernatant (Suckling and Suckling, 1980). Based on such principles, and the previously observed effects of reducing the

number of smaller vesicles in the formulation (Chapter 2), such a step should form a part of an optimised protocol.

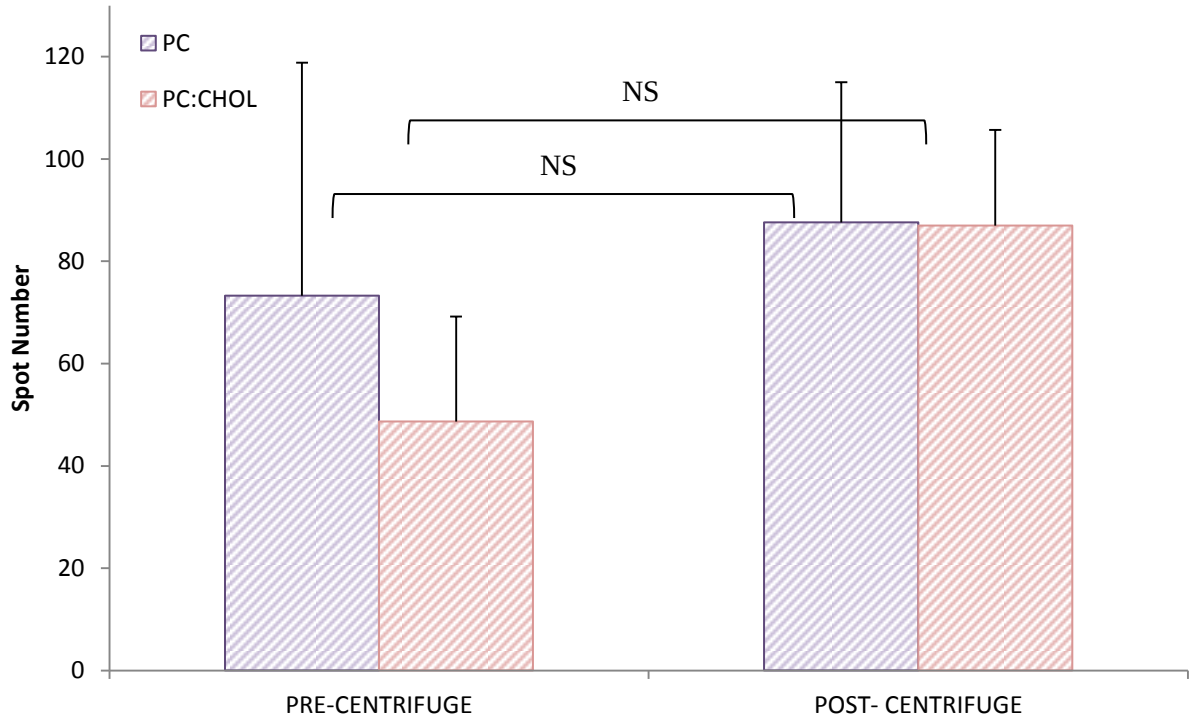


Figure 5.5 ELISPOT spot numbers to show liposome formulations of PC and PC:Chol (16 µMol total lipid) pre and post centrifugation (size 8.2µm ±0.24 and 9.5 µm ±1.19 respectively). Results represent mean ± SD for n=3 batches.

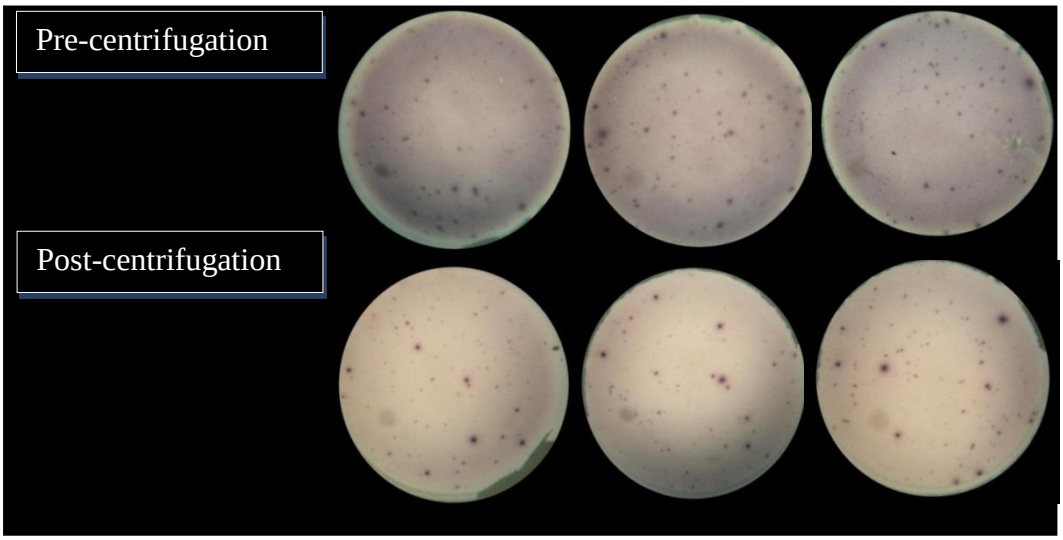


Figure 5.6 Representative images from ELISPOT wells of PC (16 µMol) wells pre and post removal of SUV liposomes.

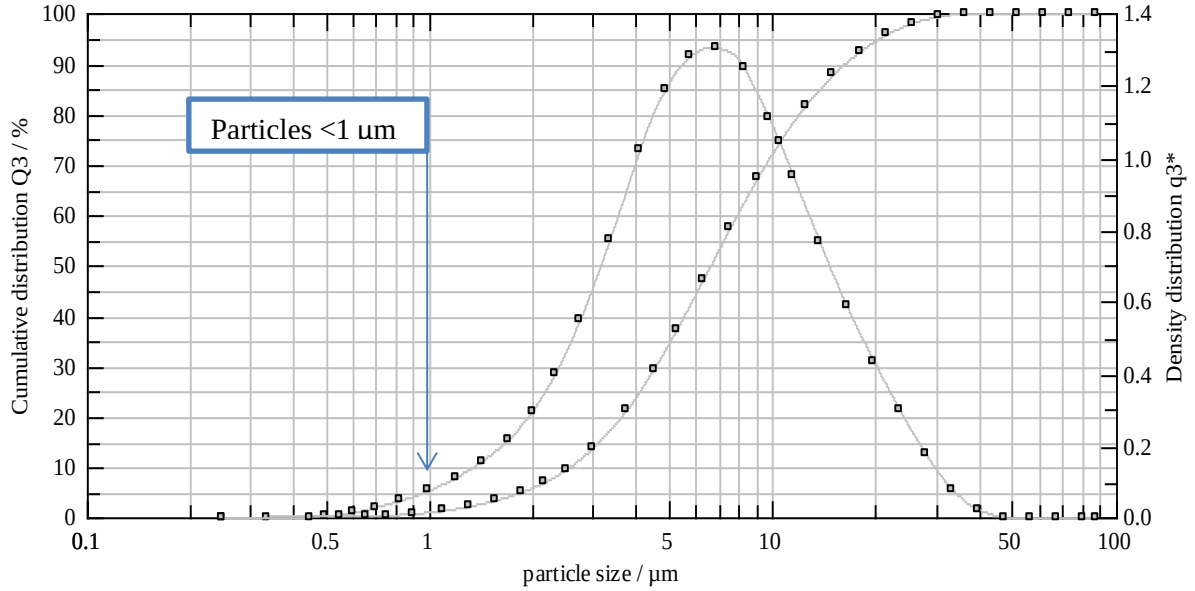


Figure 5.7 Cumulative size distribution curve shown for a PC:Chol liposome formulation with a mean particle diameter of 8.29 ± 0.06 µm.

5.3.3.2 Adaptation of the protocol to meet the needs of scale-up requirements

Although a protocol with low levels of background and high levels of spot clarity had been achieved (section 5.3.4), it was time consuming both in terms of the overall process time for the formulation procedure and in terms of the different preparative steps for the operator. In terms of biological material, it is important that the manufacturing process in place can be scaled up from the small amounts required in the laboratory, to large commercial needs (Evans, 2007). In terms of producing a reference standard, at a certain stage in development a larger batch size that can be used for routine testing, calibration and validation will be required (Raut and Hubbard, 2010). At this stage, the protocol consisted of two 30-minute centrifugation steps using the ultra-centrifuge, which involved pipetting into specialised 3.9 mL tubes without an air bubble and then heat sealing prior to each centrifugation. The centrifuge then required time for the vacuum to be at the correct level, which adds another ten minute for each run. Subsequent to each centrifugation step, it was necessary to re-disperse the pelleted liposomes.

Thus, the manufacturing process was quite long even for a small batch. This was not only a logistical issue but can also effect the protein as discussed in Chapter 4; the process of centrifugation and

vortexing could impact on the protein integrity, thus a protracted formulation process should be avoided (Christova *et al.*, 2003). Therefore the protocol for preparation of the liposome formulations was further modified (outlined below), with the ultra-centrifuge steps removed. Instead, there was an additional slower centrifugation step incorporated into the protocol using a lab centrifuge for the reduction of the background by simultaneously removing free rIFNγ and smaller liposomal vesicles. The modified protocol consisted of:

1. The 2 mL formulations made up to 5 mL with PBS in a 10 mL tube.
2. Centrifuge for 15 minutes at 2660 g at 4 °C.
3. Re-suspend liposomal pellet in 5 mL of RPMI.
4. Centrifuge at 206 g for 10 minutes at 4 °C.
5. Re-suspend liposomal pellet in 1 mL in RPMI.

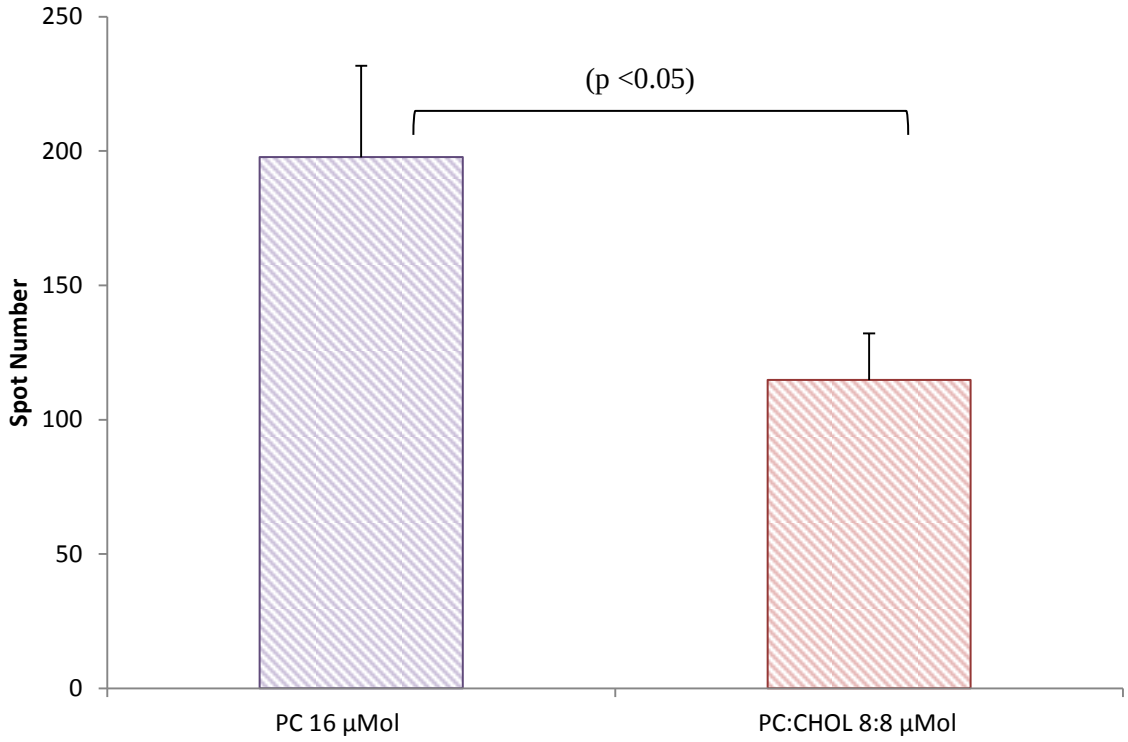


Figure 5.8 ELISPOT spot numbers for the formulations of PC 16 µMol and PC:Chol 8:8 µMol for a modified protocol to meet scale-up requirements (Size of formulations: 9.5 ± 1.24 µm and 11.5 ± 1.25 µm respectively). Results represent mean ± SD for n=3 batches.

Through the re-suspension of the final pellet in 1 mL as opposed to the normal 2 mL, there was an observed increase in spot number compared to previous assays (Figure 5.8 vs. Figure 5.5). In terms of spot number, there was a significant difference ($p<0.05$) in the mean between liposomal formulations for PC and PC:Chol with spot numbers of 198 ± 34 and 115 ± 17 respectively (Figure 5.8). Qualitative assessment of the wells showed that the background was low and the spots counted without any difficulty, as they were distinctive from the background (Figure 5.9). In terms of production of formulations, this was a more user friendly and quicker protocol. For this study, the initial concentrations of the formulations were 9 mg/mL but during centrifugation, the increase in volume results in a concentration of 1.8 mg/mL. Thus, larger volumes of RPMI could be used to ensure effective removal of unentrapped cytokine. It is perhaps this reduction in the observed background levels (Figure 5.9), the removal of smaller vesicles, and changing the re-suspending volume, which has allowed effective spot formation.

Figure 5.9 ELISPOT wells for formulations of PC a 16 μMol and PC:Chol 8:8 μMol formulations from a modified protocol to meet scale-up requirements. Total spot numbers evaluated by manual counting for the liposomal formulations of PC and PC:Chol were 198 ± 34 and 115 ± 17 respectively.

In numerous protocols where centrifugation has been used as a means to remove unentrapped material, centrifugation speeds used are generally high (Szoka and Papahadjopoulos, 1980). This is often necessary to enhance recovery of the liposomes particularly with small unilamellar vesicles (Gregoriadis and Senior, 1980). However, in a study by Dey-Hazra *et al.,* (2010) examining the detection of circulating platelet derived micro-particles, different centrifugation protocols were investigated for their extraction from plasma. It was found that at slower centrifugation speeds at 1500 *g*, the amount of microparticles were 10 to 15 fold higher compared with protocols using centrifugation speeds of 5000 *g* ($p<0.001$). This confirms that the careful selection of centrifugation protocols can result in the separation of smaller particles.

5.3.4 Testing the feasibility of a calibration curve and inter batch variation

Previous work has shown that liposome encapsulated rIFNγ can be used within ELISPOT assays to produce an artificial reference standard. It was important to assess the feasibility of using this control and carrying out a serial dilution to produce a calibration curve. Liposomal formulations of PC and PC:Chol (16 μMol total) were characterised for size and zeta potential (Table 5.4). There was no significant difference observed in the size or zeta potential of the formulations. The serial dilution of the neat standard controls were carried out in RPMI at a 1:1 ratio with the initial 1 mL control produced from the formulation protocol.

The results observed for both PC and PC:Chol liposome reference standards (Figure 5.10 and 5.11 respectively) indicated that the calibration curves were not linear. However, these fit an exponential trend line (or for logarithmic conversion the power trend line). The R^2 value for the PC liposome formulation was 0.998 and the R^2 value for PC:Chol formulation was 0.986. The images show that spots were well defined and the background decreases by the dilution of the formulations in RPMI (Figure 5.12).

Table 5.4 Characterisation data for PC:Chol and PC liposome formulations (16 μMol total). Results represent mean ± SD for n=3 batches.

Formulation	Lipid composition (μMol)	Size (μm)	Zeta Potential (mV)
PC rIFNγ	16	7.21 ± 0.33	-6.56 ± 9.22
PC:Chol rIFNγ	8: 8	10.22 ± 3.37	-13.2 ± 10.1

ICH guidelines recognise that immunoassays in particular, do not always demonstrate a linear relationship, as observed from the results of this study. Therefore, the analytical response was described by an appropriate function of the concentration (ICH, 2006). Although the R^2 values indicated a good fit, there was a high standard deviation for each dilution. The coefficient of variation is a statistical term, which can be used to express the variability of a set of data and is defined as the ratio of the standard deviation to the mean (Jones, 2002). It is typically used within immune assays to show the variance, a CV % of 20 is normally used to define the limits (Shah *et al.*, 1992). The coefficient of variation across the three formulations for the PC liposome formulation at each calibration dilution ranged from 38.5 % to 61 %, and for PC:Chol liposomes from 22.8 % to 94 %.

Other ELISPOT studies have shown coefficient of variations for *inter-* and *intra-* assay to be 21.9 % and 24.7 % respectively (Kumar *et al.*, 2001). Furthermore, a study examining IFNγ release from PBMCs in cancer patients have shown the *inter-* assay variation to be 15 % (Asai *et al.*, 2000), suggesting that the variability from using the liposomes as standards remains higher than required. However, in terms of a reference standard this is acceptable, because for production purposes there is production of a single, validated batch. For instance, when cytokine standards are produced for the WHO ordinarily a batch of 3500 ampoules maybe produced and validated; thus the variability within the batch would be expected to fall within defined limits (R. Mire-Sluis *et al.*, 1998).

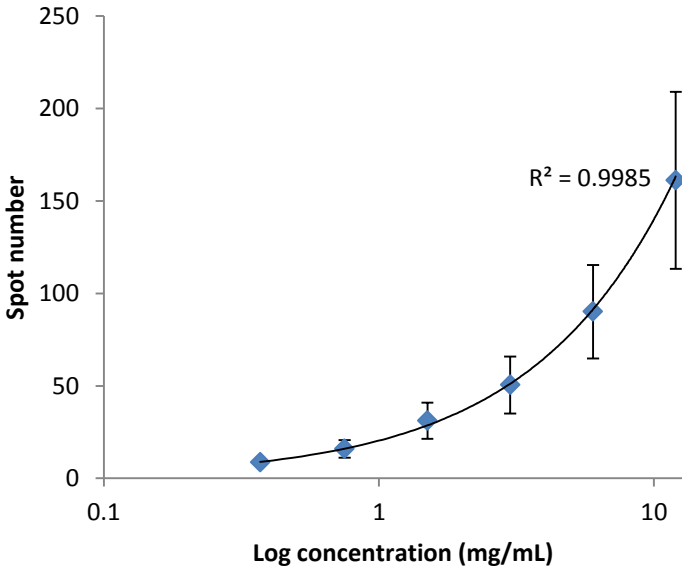


Figure 5.10 Serial dilutions of PC liposomes (16 µMol) with rIFNγ. Results are shown with an exponential trend line. Results are mean ± SD for n=3 assays.

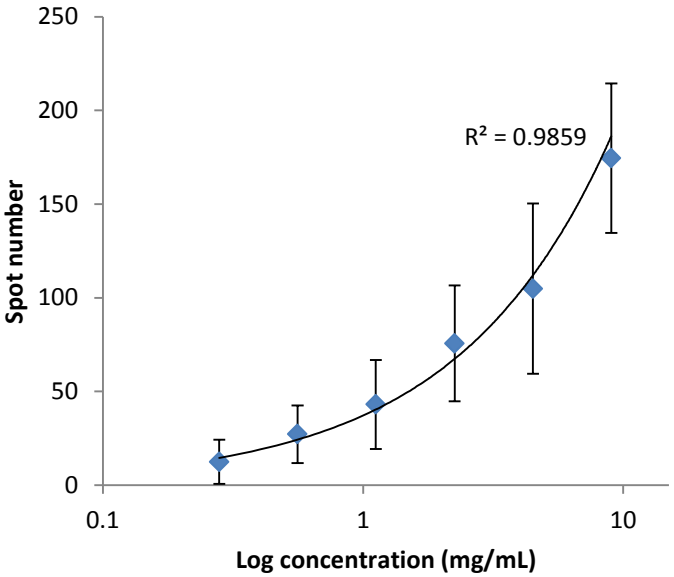


Figure 5.11 Serial dilutions of PC:Chol (8:8 µMol) liposomes with rIFNγ. Results are shown with an exponential trend line. Results are mean ± SD for n=3 assays.

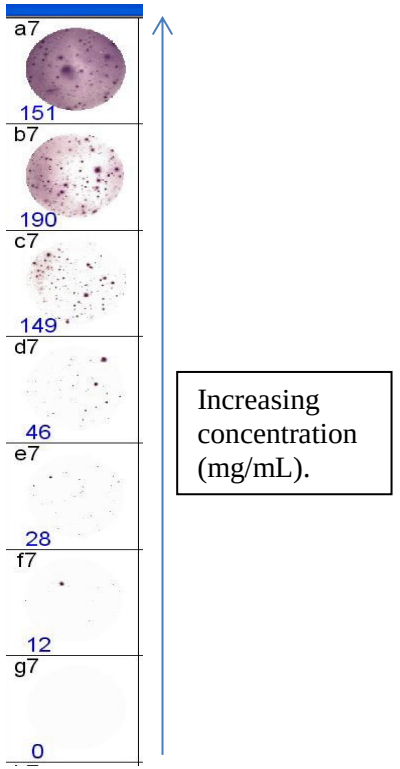


Figure 5.12 A representative assay for PC 16 µMol formulations with single wells shown for increasing concentrations.

5.3.4.1 Reproducibility assessment of a single batch of reference standard to assess intra batch variation

Subsequent to the formulation of a single batch of PC 16 μMol liposomes with rIFNγ (particle size 9.79 ± 0.3 μm; results not shown), assessment of batch reproducibility was required. From the stock preparation, a 1:1 serial dilution in RPMI was carried out three times and ran across three plates and the results for the individual replicates are shown in Figure 5.13, with the average shown in Figure 5.14. The results for each serial dilution, per well, are also shown (Figure 5.15). The three calibration curves similarly followed an exponential trend line and demonstrated a good fit as shown by the R^2 values of 0.995, 0.99 and 0.99 for n=3 assays (Figure 5.13). Similarly, for the average of the three-combined curves (Figure 5.14), the R^2 value was 0.99 and the points for each dilution followed the exponential trend line closely. The standard deviations on each dilution show a reduced variation compared to those in Figure 5.8 and Figure 5.9. The results for the values for each well (Figure 5.15) further highlight the reproducibility across the three assays.

The coefficient of variation was < 15 % for calibrator 1, 2, 3 which were the three strongest dilutions then for the lower part of the curve it was <30 %. Thus, the average coefficient of variation for the serial dilution was 23.6 %, which shows an acceptable level of reproducibility at this stage of optimisation. In some studies using the ELISPOT assay, an acceptable coefficient of variation was defined as ≤ 20 %, although in the study of responses from ten participants that observed variation was actually 25 – 36 % (Comin-Anduix *et al.*, 2006).

The results for the assessment of reproducibility were promising because although the batch-to-batch variability was high in previous results, this study has shown that one batch can produce good reproducible results. Within immunoassays variability between different batches can occur with many showing large assay errors (Deshpande, 1996). The overall shape of the curve also followed a non-linear pattern, but most immunoassays exhibit a non-linear relationship between the response and the analyte (Deshpande, 1996).

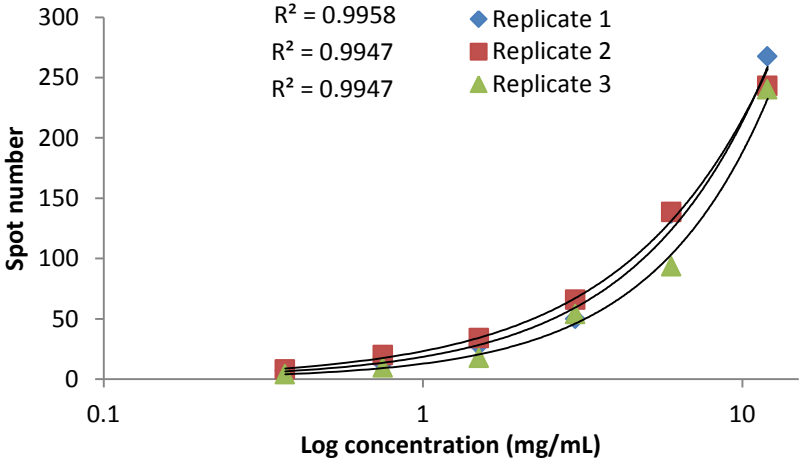


Figure 5.13 Three replicate curves from one batch of PC 16 µMol formulation on three separate assays with R^2 values.

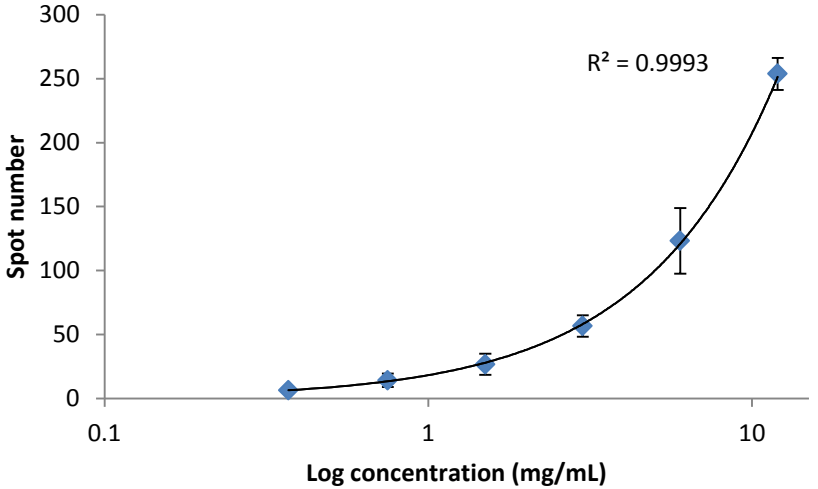


Figure 5.14 The mean of three replicate curves from one liposomal batch of PC 16 µMol with R^2 values. Results represent mean ± SD, n =3.

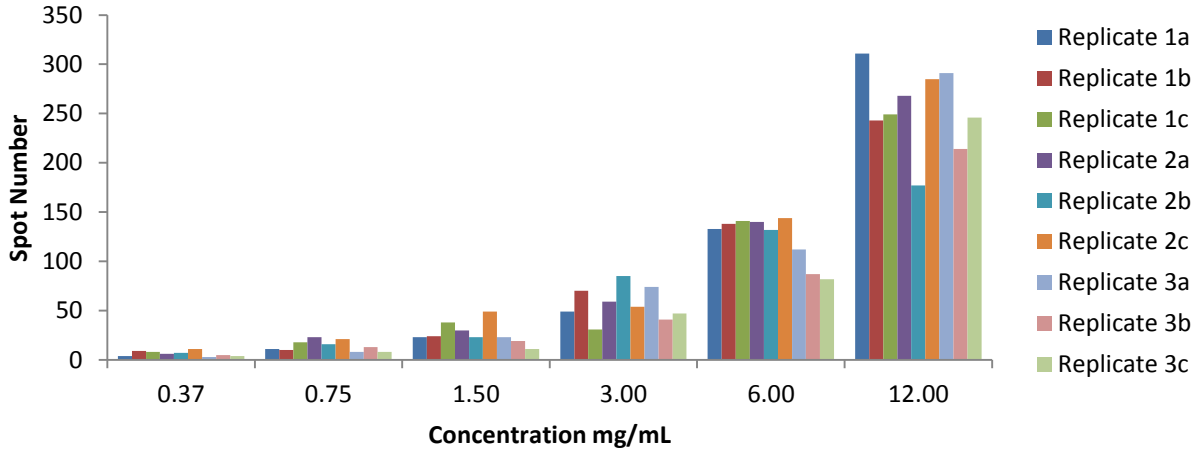


Figure 5.15 Individual results for spot numbers for each replicate for the triplicate assays of PC 16 µMol.

In a study by Janetzki *et al.,* (2006) 200,000 cells per well were plated with subsequent dilutions at 100,000, 50,000 and 25,000 cells per wells and the number of spots were counted with and without stimulation from a peptide pool. Similarly, triplicate wells were tested for each condition. The shape of the curves obtained are comparable with R^2 values in the experiment for stimulated versus non-stimulated at >0.9 and >0.84 respectively. Within the scope of ELISPOT literature, a calibration curve has not previously shown for a reference standard. Ordinarily reference standards for the ELISPOT assay rely on stimulating cells to produce high levels of IFNγ. There are three main types of existing controls mitogens, ionophores and peptide pools (Currier *et al.,* 2002). Mitogens which include; Phytohaemagglutin (PHA), Concavalin A (ConA), Lipopolyscaccharide (LPS) induce proliferation in a variety of cell types particularly T-lymphocytes and ionophores such as phorbol myristate acetate (PMA) mimic early signal transduction pathways and activate human T-lymphocytes to secrete cytokines (Moreland, 2004, Chopra *et al.*, 1989). The disadvantages of such controls such as PHA is that patches can occur in the wells, spots can vary widely in size, intensity and shape and loss of activity during storage (Currier *et al.,* 2002). In this study an artificial reference standards has been shown to produce a reproducible calibration curve, thus the 'proof of concept' has been extended and this study shows that artificial vesicle with encapsulated cytokine are able to mimic the actions of the cell on the ELISPOT assay.

5.3.5 Quantitative comparison of automated and manual reading.

Although much of the work for this study had, thus far used manual ELISPOT counting, normally automated procedures are used to reduce reader bias (Cox *et al.,* 2006). Therefore, it was important to assess how the automated reading of a calibration curve with the reference standards would perform when compared to the manual reading using a stereomicroscope. With three batches of PC 16 μMol liposome formulations containing rIFNγ, serial dilutions using 1:1 RPMI were carried out with each dilution placed on two separate plates. Results were analysed by manual counting using a stereomicroscope and automated counting at NIBSC.

The observed results (Figure 5.16 a) confirm that previous assays using the manual counting method were comparable to the assays read using automated reading. In addition, it showed that the spots produced by the reference standards were of a suitable size, morphology and counted effectively through the automated procedure (Figure 5.16 b and c). The curve used was the fit spline curve and statistical analysis of the curves using F2 similarity factor resulted in an obtained value of 50.5 values between 50 – 100 are acceptable according to the FDA regulations (Yuksel *et al.,* 2000). Thus, the two curves are considered similar and results obtained thus confirm that for the purposes of this study, spot-counting using a manual method was comparable to the automated method.

The use of automated reading is recommended as a means for standardisation of protocols and it is thought to reduce the variability observed by manual counting (Janetzki *et al.,* 2005). In a study by Almeida *et al.,* (2009), automated and manual protocols for spot enumeration were compared for PBMCs from six healthy individuals (fresh) and with cryopreserved cells. It was found that similar cells counts were obtained for both methods; with the median manual count at 66.18 spots and a range from 22 to 128.21 spots generated compared with automated at a median cell count of 69 and a range of 22 to 128.1 spots generated for fresh cells (Almeida *et al.,* 2009). Similar comparable results were observed with cryopreserved cells, and indicate that automated and manual spot enumeration techniques can provide similar results.

Manual counting procedures are carried out using a stereomicroscope, which typically consists of a 4 X objective lens and further 10 X magnification through the eyepiece (Kaluzhny, 2005). This technique relies solely on the operator to assess the spot number. Although time consuming and tedious, it is thought to have a higher sensitivity in identifying faint spots of smaller sizes although no significant differences were observed at the weaker concentrations in this study, the mean spot numbers counted were higher for the manual count (Hagen, 2012). The use of automated procedures within the field of ELISPOT is advised on many levels including harmonisation of results between different laboratories, automated counting offers many advantages including the reading of multiple plates, reduction in bias and increased accuracy (Cox *et al.,* 2006).

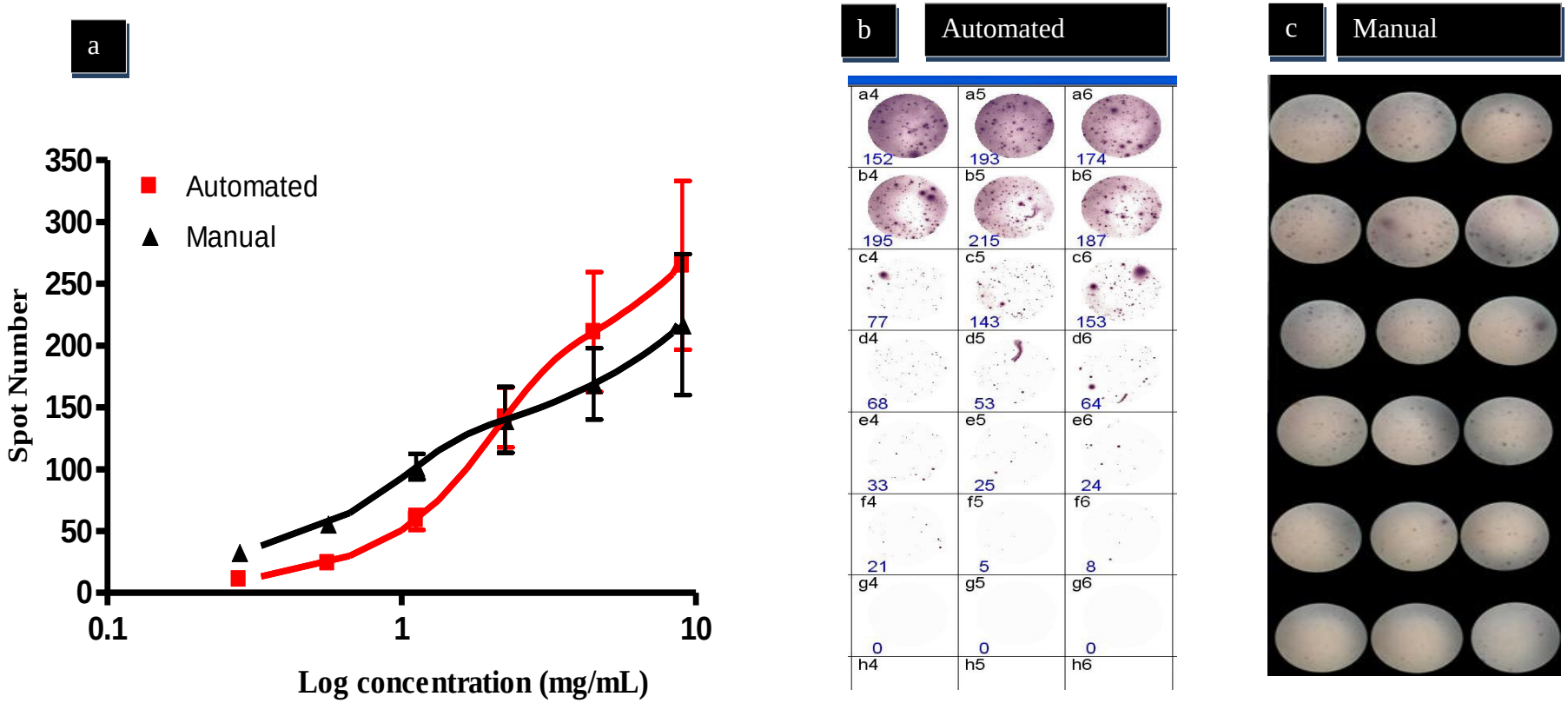


Figure 5.16 A quantitative and qualitative comparison of manual and automated reading procedures for the ELISPOT assay.(**a)** A fit spline curve comparison between automated and manual counting of ELISPOT wells of three batches of PC 16 µMol with an average size of 7.96 µm ± 1.41 and average zeta potential of -7.51 ± 4.65 mV. Results shown are for n=3 assays ± SD. (**b)** Images for ELISPOT wells using automated reading **(c)** Images for spots counted manually using a stereomicroscope.

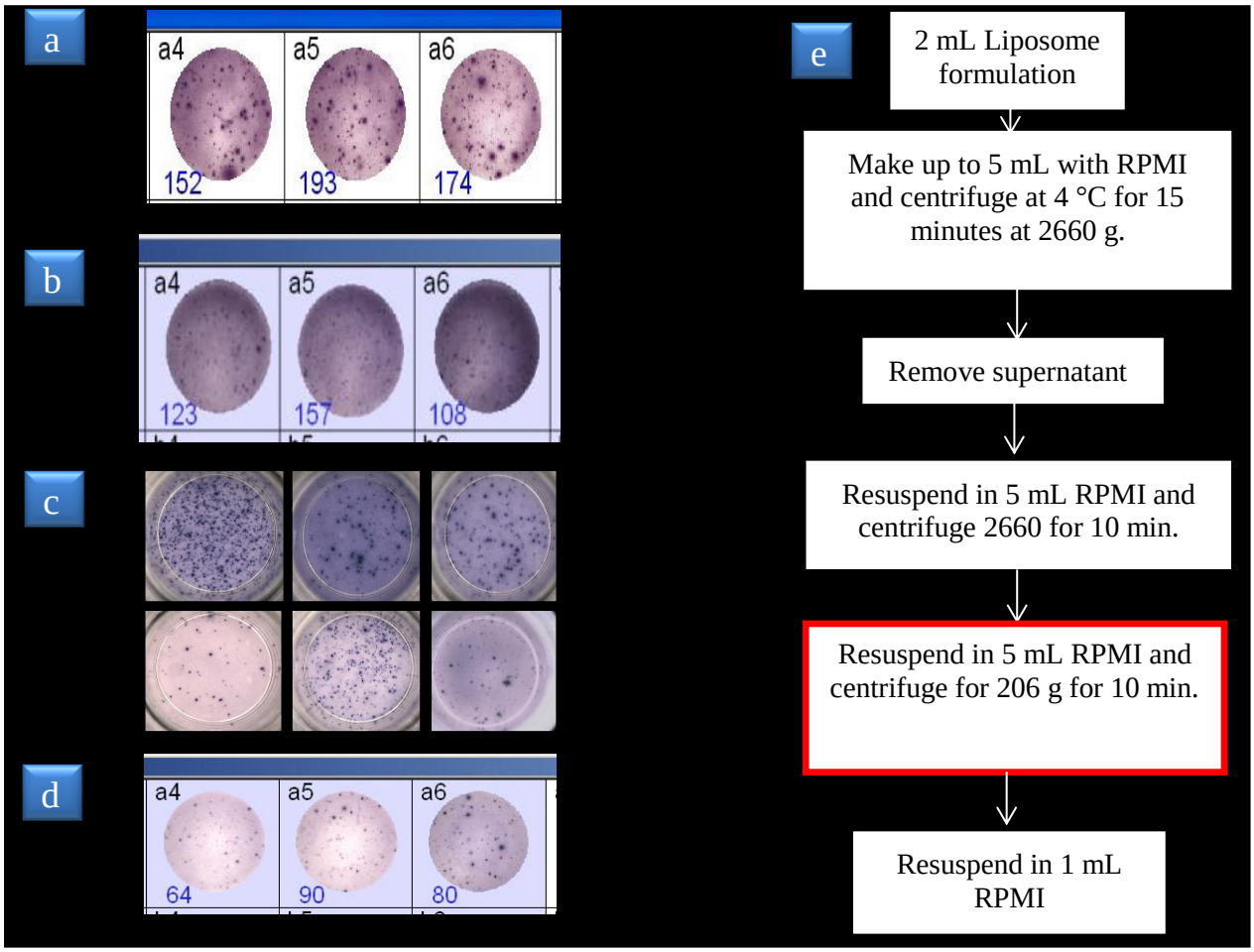


Figure 5.17 ELISPOT images for automated reading and the final protocol. Artificial reference standards are shown for two assays with automated reading **(a)** and **(b)** highlighting high levels of background in wells in comparison to **(c)** live cells. The final protocol when applied to automated reading is shown **(d).** The protocol **(e)** for the removal of free rIFNγ used for the assay (Table 2.4; protocol 4).

The final protocol should be adaptable for all situations as required by the user; this is a part of having a robust reference standard (ICH, 2006). However when assays were assessed using automated counting procedures, qualitative assessments revealed higher background levels (Figure 5.16 b and 5.17 a and b). This was particularly notable when compared to the cells normally plated in the assay (Figure 5.17 c). To address this, an additional step was brought back into the protocol of 2660 *g* for 15 minutes at 4 °C. This resulted in more acceptable levels of background (Figure 5.17 d). Thus, the final protocol (Figure 5.17 e) meets end user requirements in that it offers potential for scale up to larger batches and robust enough to achieve low levels of background was established (Figure 5.17 e). Unless the formulation procedure for the formulation of reference standards was robust then this will

limit the potential of transfer from smaller batch production to larger scale requirements (Wagner, 2011).

5.3.6 A qualitative assessment of live cells vs. artificial reference standards

It was necessary to make a qualitative assessment of how spots produced from live cells compared to those produced from the formulated artificial liposome reference standards. This was an important issue as the reference standard needed to mimic the actions of live cells and produce spots. In addition, the wells needed to have comparable background levels. Thus, live cells were used to produce spots (Figure 5.18) and images taken using a stereomicroscope to compare the wells directly to the wells that have liposome reference standards. The images of the live cells (Figure 5.18) highlight the spot definition against the low levels of background observed.

Figure 5.19 a, shows a representative image for the artificial liposomal reference standard and Figure 5.19 b shows an ELISPOT well produced from cells. From qualitative observations the liposome-induced spots do not diffuse out the way the cell forming spots do; the live cells seem to produce larger spots. The T-cells ordinarily placed into the wells are spherical with a diameter of 10-15 μm and are thus physically a similar average size to the liposomes used (Weiss, 2012).

Spot sizes within cells can vary due to different cell types within the mixed populations that are placed onto the wells. For instance, macrophage derived spots for IL-10 cytokine are much smaller than T-cell antigen induced spots (Lehmann, 2005). It is also thought that reduced cytokine activity from a cell results in the formation of smaller spots (Lehmann, 2005). This suggests that the levels of entrapped cytokine are not equivalent to the amount of cytokine released by the live cells. Although the images (Figure 5.19) have shown productions of spots from an artificial reference standard are not exactly the same, the similarity is close enough to make it difficult (without prior knowledge) to discern the two.

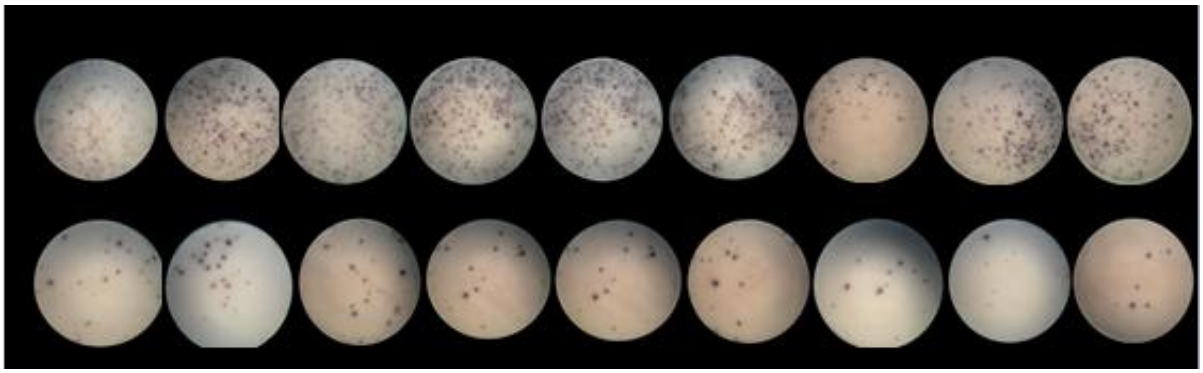


Figure 5.18 ELISPOT plate sent from NIBSC of peripheral blood mononuclear cells stimulated with phorbol 12-myristate 13-acetate (PMA), and purified protein derivative (PPD). PMA stimulated cells are shown in the top row and PPD stimulated cells shown in the lower row.

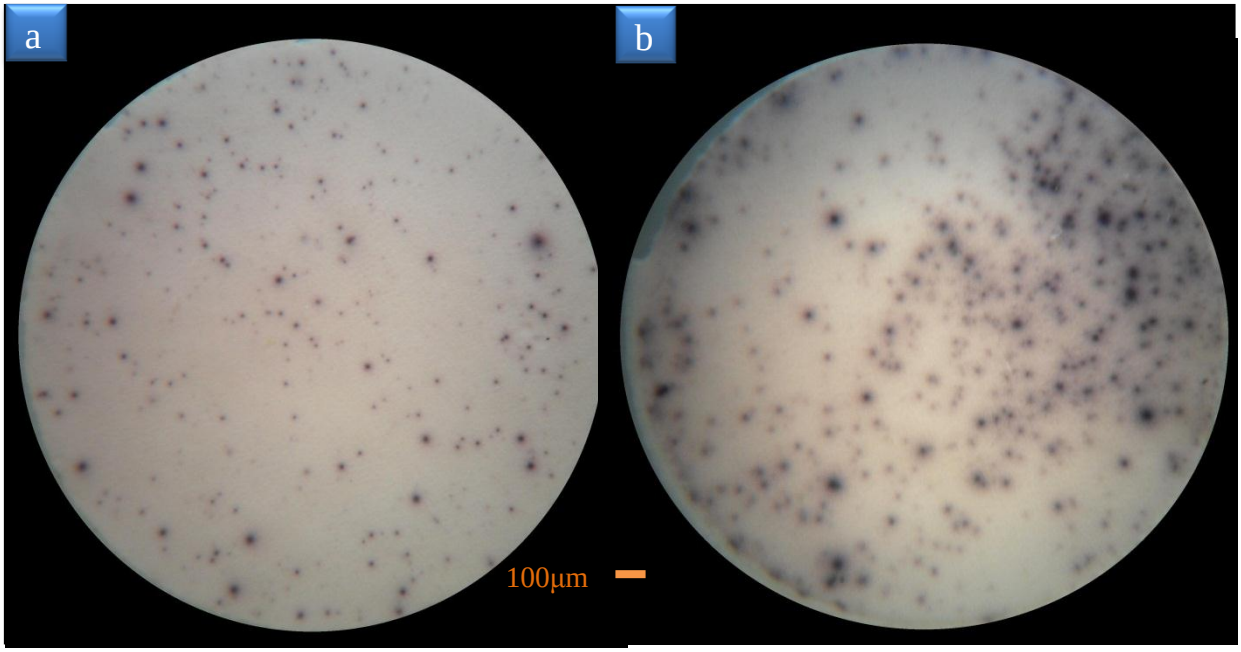


Figure 5.19 A direct comparison of a well from an ELISPOT assay of cells and an artificial reference standard. (a) A well from an ELISPOT plate of a PC (16 µMol) liposome formulation with rIFNγ and (b) A well from an ELISPOT of PBMCs stimulated with PMA (phorbol 12-myristate 13-acetate).

Immunoassays' can be particularly problematic from a standardisation view-point because of their ability to bind selectively to different molecular species of an analyte (Thorpe, 1998). In terms of the ELISPOT assay, the challenge for finding a robust reference standard is due to the nature of the assay, which is *ex vivo* and cells (generally peripheral blood mononuclear cells (PBMCs) or other subsets from the blood) are used directly on the assay. There are currently no reference standards used consistently from laboratory to laboratory and in conjunction with variations in protocol this has

greatly affected standardisation (Janetzki and Britten, 2012). Liposomes have not been used within the assay previously, but as shown in Figure 5.19 they can be directly comparable. It is not necessary for reference standard production to be the same as the components of the assay and according to WHO guidelines; reference standards may differ from the formulation or matrices of the examined substances (WHO, 2006).

5.3.7 Assessing the effects of spot number on increasing lipid concentration

For the purposes of producing a reference standard, a high positive control maybe needed, thus with the aim of increasing the spot number, the effect of increased lipid concentration was investigated. The formulations assessed were for PC at 16, 32 or 64 μMol and PC:Chol (1:1 ratio) at the same total molar concentrations (Table 5.5). Also assessed were pooled formulations consisting of two smaller concentrations, such as 16 μMol and 16 μMol, to make up the larger concentration of 32 μMol of total lipid. The formulations were characterised for size (Table 5.5). There was no significant difference in size observed for the formulations including the pooled batches, thus any affects seen in the ELISPOT data are a direct result of increased lipid concentration.

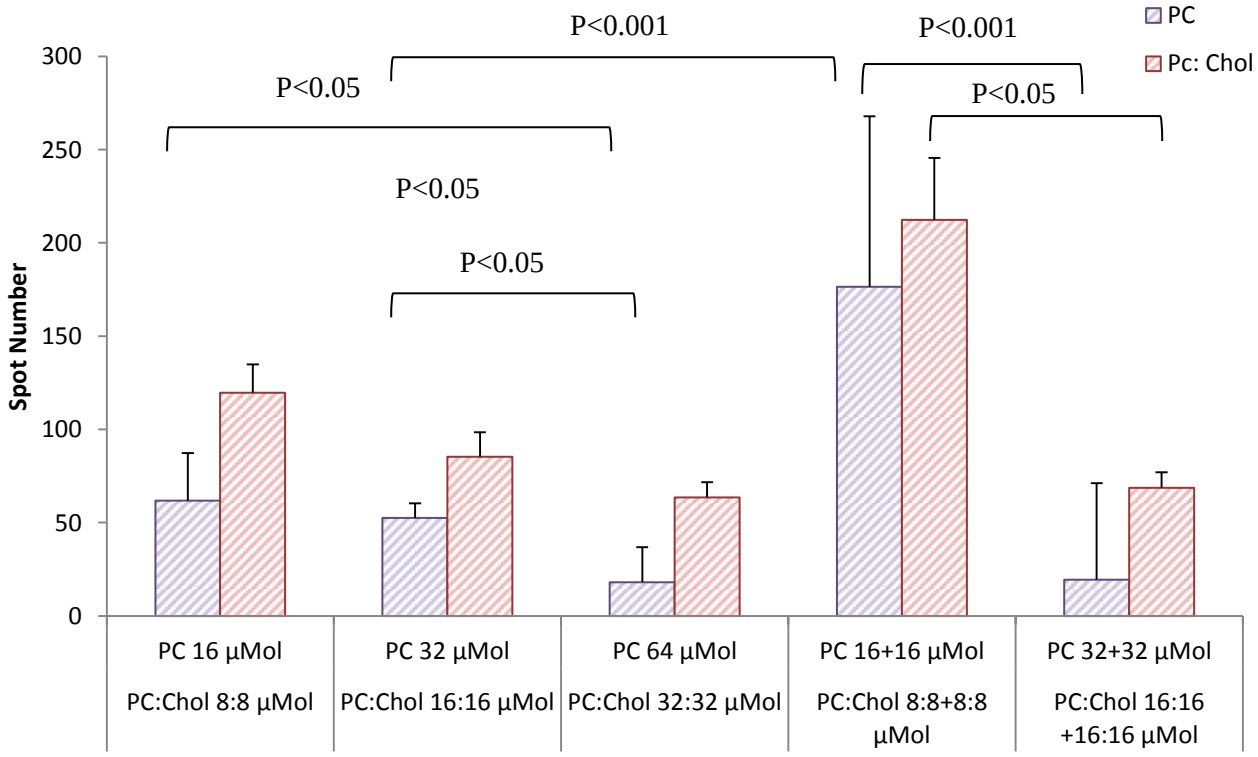
The results observed (Figure 5.20) indicated that as the lipid concentration increased there was no increase in spot number. In fact for liposome formulations of PC 16, 32 or 64 μMol, there is a trend of decreasing mean spot number with significant differences ($p<0.05$) observed for PC formulations of 16 μMol and 32 μMol, when compared with 64 μMol formulations (Figure 5.20). Spot numbers reduced from 62 ± 15 spots to 18 ± 8 (PC 16 μMol vs. PC 64 μMol) and 53 ± 13 spots generated to 18 ± 8 (PC 32 μMol vs. PC 64 μMol) respectively. Similar results (Figure 5.20) for the PC:Chol formulations are shown; as the lipid concentrations increased there was a trend of decreasing spot number as the lipid concentrations increased from 8:8 μMol, 16:16 μMol and 32: 32 μMol (from 120 ± 25 to 85 ± 8 to 64 ± 19 respectively; Figure 5.20). There was a significant decrease ($p<0.005$) of nearly two-fold for PC:Chol formulations of 8:8 μMol when compared to PC:Chol formulations at 32: 32 μMol lipid concentration.

There was an increase in spot number for the pooled batch of 16 μMol + 16 μMol for the PC liposome formulations, which was significantly higher than the other lipid concentrations of 16, 32 and 64 μMol ($p<0.001$; Figure 5.20). For the PC:Chol pooled liposomes batches of 8:8 μMol + 8:8 μMol there was over a 3-fold increase in mean spot number and it was statistically significant when compared to 32:32 μMol ($P<0.001$). Whilst the pooled batches for PC and PC:Chol formulations showed statistically significant increases in spot number at 8:8 + 8:8 μMol and 16+16 μMol compared to non-pooled batches; this trend did not continue when higher concentrations of the pooled batches were used. Thus PC:Chol 16:16+16:16 μMol formulations and PC 32+32 μMol formulations generated significantly lower spots numbers ($p<0.05$ and $p<0.001$ respectively) with almost a 3 fold and 9 fold decrease respectively, when compared to the lower concentration pooled batches (Figure 5.20).

The results observed for the effect of increasing lipid concentration were interesting as there was no linear increase in spot number observed with the non-pooled formulations. In a study by Zhang *et al.*, (2009) a wide range of PBMC cell concentrations were tested to assess the relationship between ELISPOT results and increasing cell number. Thus serial dilutions used ranged from 1.6×10^6 to 5.0×10^5 PBMC per well (stimulated with CMV peptide PP65). A linear relationship was observed between cell number and spots generated between 5.0×10^4 and 4.0×10^5 cells per well. However, in Figure 5.21 a similar conclusion cannot be applied, there was no evidence of the confluence of spots furthermore background levels appeared to be acceptable (Figure 5.21). In the study by Zhang *et al.*, (2009) spot numbers of up to 753 were observed for some of the higher cell concentrations, yet however the liposomal reference standards for the non-pooled formulations generated 120 spots (Figure 5.20). Further investigative work was carried out by similarly using a serial dilution at the three different concentrations of PC:Chol liposome formulations (Table 5.6).

Table 5.5 Size characterisation for 5 formulations of PC and PC:Chol with increasing concentrations of lipids or lipid and cholesterol in a 1:1 ratio. Results represent mean ± SD, n =3.

Formulation	Lipid concentration (µMol)	Size (µm)
PC:Chol	8:8	8.72 ± 0.98
PC:Chol	16:16	8.49 ± 1.06
PC:Chol	32:32	9.06 ± 0.54
PC:Chol (pooled 16:16)	8:8 + 8:8	8.91 ± 1.43
PC:Chol (pooled 32:32)	16:16 + 16: 16	9.53 ± 0.60
PC	16	7.13 ± 1.26
PC	32	6.94 ± 1.27
PC	64	7.19 ± 0.89
PC (pooled 32)	16 + 16	7.91 ± 1.49
PC (pooled 64)	32 + 32	8.02 ± 1.29



Fi**gure 5.20** The effect of increasing lipid concentrations for PC and PC:Chol liposome formulations. on spot numbers generated. Results represent mean ± SD of n =3 batches.

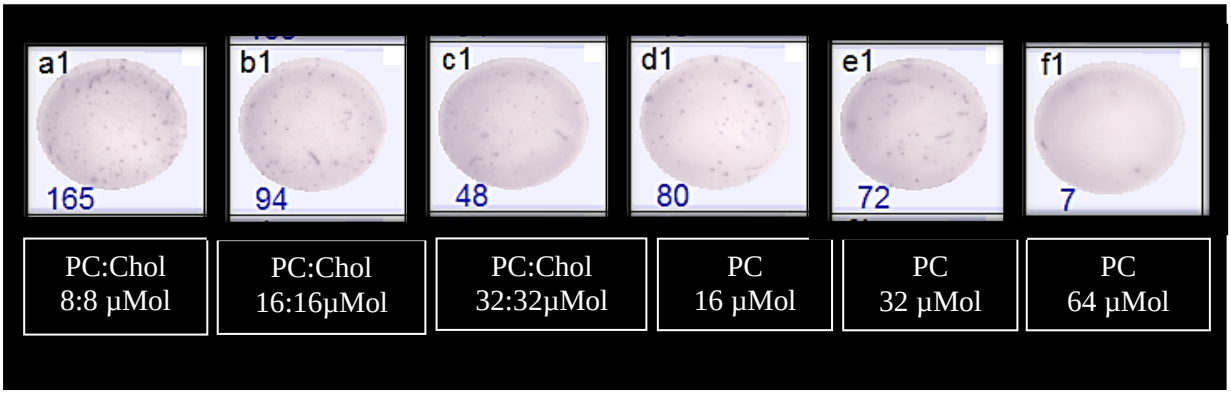


Figure 5.21 Representative ELISPOT wells for increasing lipid concentration for liposome formulations of PC and PC:Chol.

The results shown in Figure 5.22 were shown to have high standard deviations across the three different lipid amounts used for the liposomal formulations. However, there are notable differences in the trends between the three concentrations of the PC:Chol formulations. For the lower concentration (8:8 µMol; Figure 5.20), the spot number reduces from approximately 100 spots to below 20 spots per well with increasing concentration of liposomes as would be expected (Figure 5.22). In contrast, at higher concentrations (16:16 µMol and 32:32 µMol), initial spot counts are lower (approximately 80 and 40 spots per well respectively; Figure 5.22). On dilution of these two concentrations, spot numbers increase to maximum spot counts of approximately 100 spots per well for the 16:16 µMol concentration and approximately 60 spots per well for the higher concentration (Figure 5.22). Subsequent further dilution of these two liposome concentrations results reduces spot number to similar levels as the 8:8 µMol liposome concentration (Figure 5.22).

Thus, the results indicate that at the lower concentrations the relationship between spot number and liposome concentrations is very similar to that observed when PBMCs were plated out using serial dilutions (Zhang *et al.*, 2009, Janetzki *et al.*, 2006). However, the relationship between spot numbers at higher liposome concentrations was not observed with cells. This suggests that there is a hindrance to spot formation by excess liposomes being present. Although when live cells are used within the assay cell number optimisation is necessary, the critical parameter for spot formation to occur is the effective contact of the vesicles and the membrane (Dittrich and Lehmann, 2012).

Table 5.6 Characterisation data for the increasing lipid concentration of PC:Chol formulations of 8:8 µMol, 16:16 µMol and 32:32 µMol . Results represent the mean ± SD of n=3 batches.

Formulation	Lipid composition (µMol)	Size (µm)	Zeta Potential (mV)
PC:Chol	8:8	11.67 ± 0.80	-8.97 ± 7.44
PC:Chol	16:16	10.91 ± 0.60	-10.5 ± 8.70
PC:Chol	32:32	10.40 ± 1.16	-10.5 ± 5.77

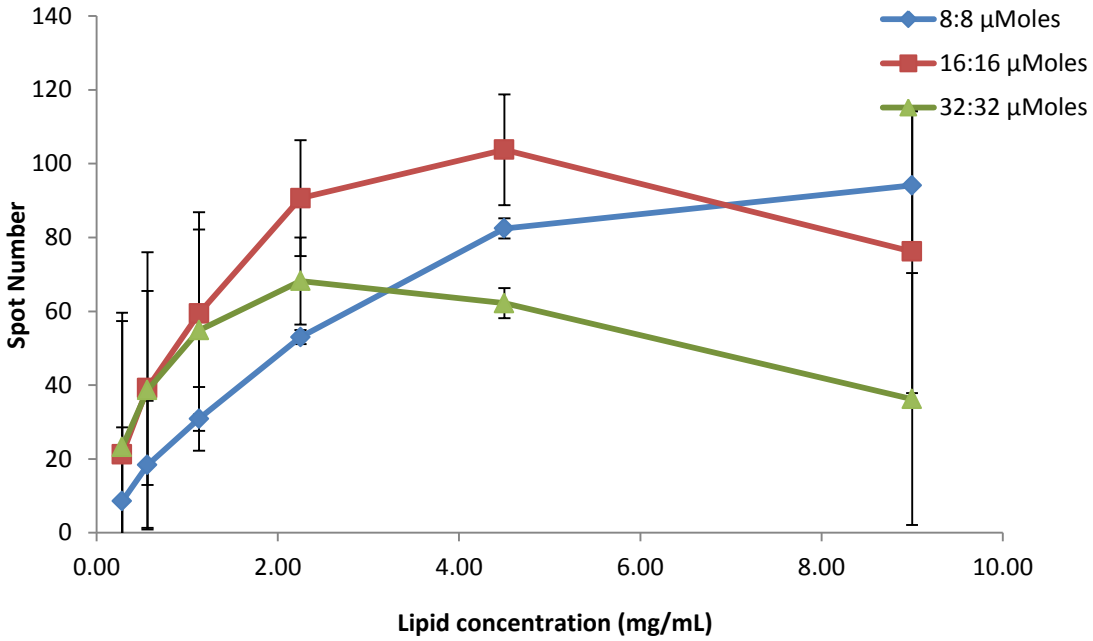


Figure 5.22 Liposomal formulations of PC:Chol of increased lipid concentrations 8:8 µMol, 16:16 µMol, and 32:32 µMol as serial dilutions for n=3 batches ran across three separate assays.

Once this occurs then can the secreted molecule can be captured onto the antibody-coated membrane, this is true for cells and thus can equally be applied to liposomes. In an assessment of the quality of staining for live cells, it has been shown that the presence of 'dead' cells can cause high background staining or a lack of spot formation; where PBMCs have been plated out with a high numbers of dead cells (30 -50 %) this resulted in high background staining (Kalyuzhny, 2005). Although high levels of background were not observed in the liposome studies, perhaps excess liposomes cause a lack of spot formation due to an oversaturation of the membrane itself. Thus to further investigate the effect of increasing lipid concentration, fluorescent microscopy was used with entrapped model protein BSA-FITC.

5.3.8 Fluorescent microscopy study of increasing lipid concentration

To investigate the relationship between the encapsulated biological material and the liposomes themselves, a confocal microscopy study was conducted with BSA-FITC as the model protein. Liposome formulations of 8:8 μMol, 16:16 μMol and 32:32 μMol were prepared with 5 μg/mL BSA-FITC. Image J software was used to convert the obtained images to 3D surface plot separate the non-fluorescent aspects of the image and the fluorescent liposomes. A qualitative assessment of the images (Figure 5.23) showed that as the lipid concentration increased, there was an increase in the total number of liposomes, but not a similar trend in liposomes containing detectable levels of fluorescently labelled protein. This would suggest that increasing the lipid concentration in the formulation, whilst increasing the number of liposomes did not increase protein-loaded liposomes, and this would result in reduced spot formation. The presence of the increased number of liposomes without sufficient protein incorporated may create a spatial hindrance to 'spot forming' liposomes in the well.

In Figure 5.24, manual counting was carried out for the total number of liposomes visible per image and the number of fluorescent liposomes. Whilst acknowledging this is not an exact count as there may be liposomes there which are not visible, this method is suitable for measuring the number of liposomes in the size range required for the assay (Szoka and Papahadjopoulos, 1980). The microscopy images confirmed there was a saturation effect and although there were hundreds of liposomes visible in an image (Figure 5.23 and Figure 5.24), only a small proportion of these had enough fluorescence to be visible in the fluorescent images. From the surface plots, this was particularly evident (Figure 5.23), thus as the concentration of lipid increases the number of visible liposomes increases.

The quantification of the results (Figure 5.25) show that for increasing concentrations of PC:Chol liposomes from 8:8 μMol through to 32:32 μMol, the number of BSA-FITC containing liposomes increased from 67 to 245 respectively (Figure 5.25). There was also a high proportion of non-fluorescent liposomes; at the 8:8 μMol concentration there were 173 liposomes with no visible fluorescence, at 16:16 μMol 149 liposomes and at 32:32 μMol this had further increased to 293

(Figure 5.25). This work can be related back to the ELISPOT assay; if such numbers of liposomes are present in the well that do not have enough cytokine to form a spot, then they will hinder spot formation of those liposomes which do contain enough entrapped cytokine.

A recent study carried out by Sunami *et al.,* 2006 aimed to assess the entrapment volumes for individual liposomes prepared using the freeze-thaw method with the purpose of selecting out those liposomes with higher volume entrapments. A plasmid DNA library encoding a gene of interest was encapsulated into liposomes (composed of 1-Palmitoyl-2-oleoyl-sn-phosphatidylcholine (POPC), 1-palmitoyl-2-linoleoyl-sn-phosphatidylcholine (PLPC), 1-stearoyl-2-oleoyl-sn-phosphatidylcholine (SOPC), and 1-stearoyl-2-linoleoyl-sn-phosphatidylcholine (SLPC), distearoyl phosphatidyl ethanolamine–polyethyleneglycol 5000 (DSPE–PEG5000) and cholesterol at 129:67:48:24:18:14) with green fluorescent protein. In the study, fluorescent activated cell sorting was applied to liposomes to assess the intensity of fluorescence and those exhibiting higher levels of fluorescence were then separated from those with lower fluorescent intensity levels (Sunami *et al.,* 2006). It was interesting to observe that the differences in intensity could be as high as 10 fold between such liposomes. Overall, the study showed the feasibility of selectively removing liposomes with lower levels of entrapment to enhance protein reactions that could occur within liposomes.

Such results confirm that in heterogenous liposomal formulations although overall entrapment is quantified, the individual liposomes within a population can vary in terms of the entrapped volumes. Thus in terms of the ELISPOT assay, if the entrapped volume of cytokine is too low, even though overall liposomal numbers may be higher, then it could be postulated that spot formation is hindered as these liposomes will still take up space on the membrane surface. In terms of liposome formulations, previous studies have shown that high concentrations of lipids were required to increase the passive entrapment of DNA into liposomes and furthermore this resulted in an increase in the number of ‘empty liposomes’, although entrapment did not exceed 20 % of DNA (Lee and Huang, 1996). Ideally, a ‘high’ control would be able to reach higher spot numbers; however, the image analysis indicates that this may not be achievable if the proportion of ‘empty liposomes’ is high. To investigate this further, the effect of increased cytokine concentration was considered.

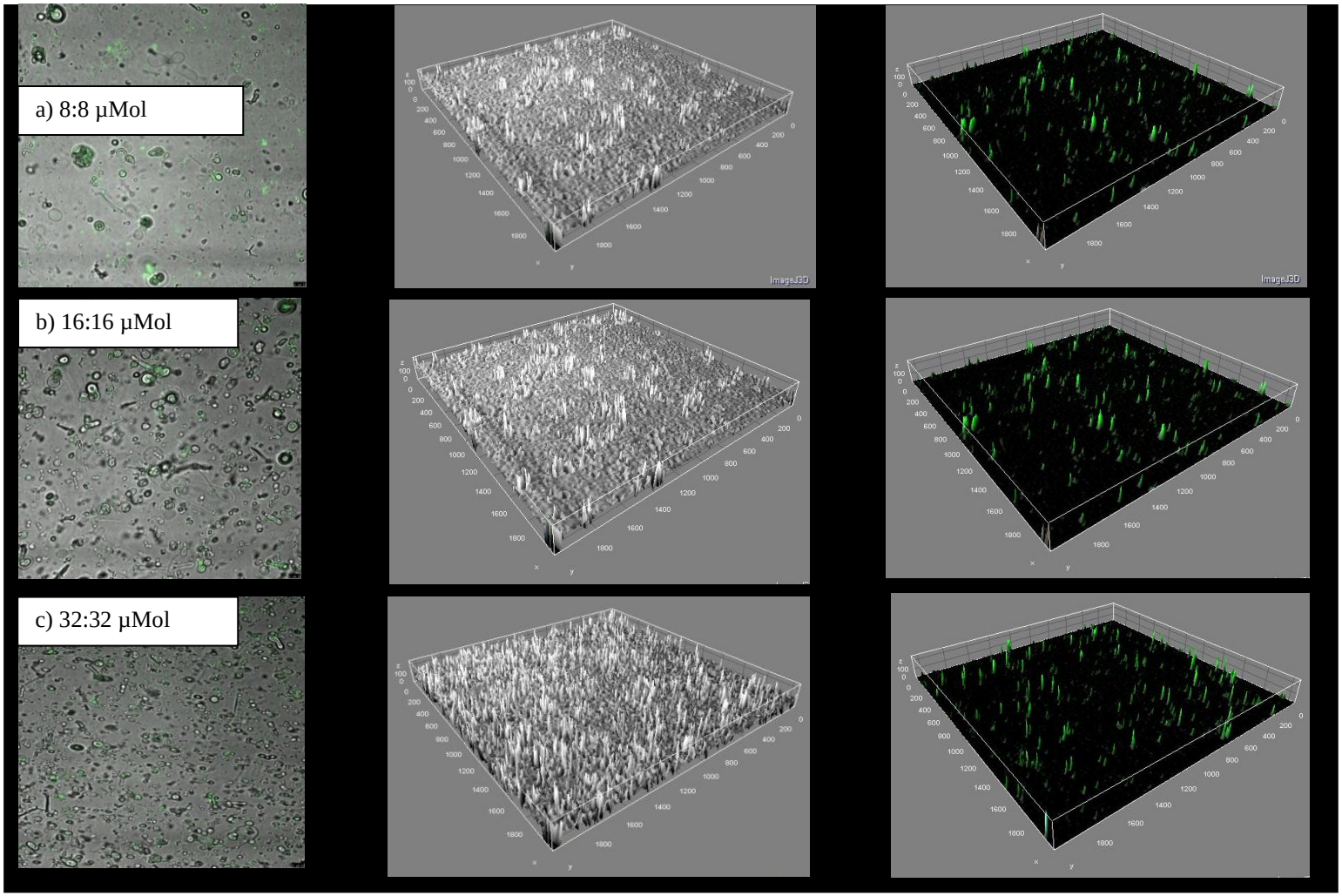


Figure 5.23 Confocal images of three liposomal formulations of PC:Chol with surface plots at **(a)** 8:8 µMol, **(b)** 16:16 µMol and **(c)** 32:32 µMol encapsulated with BSA-FITC 5 µg/mL.

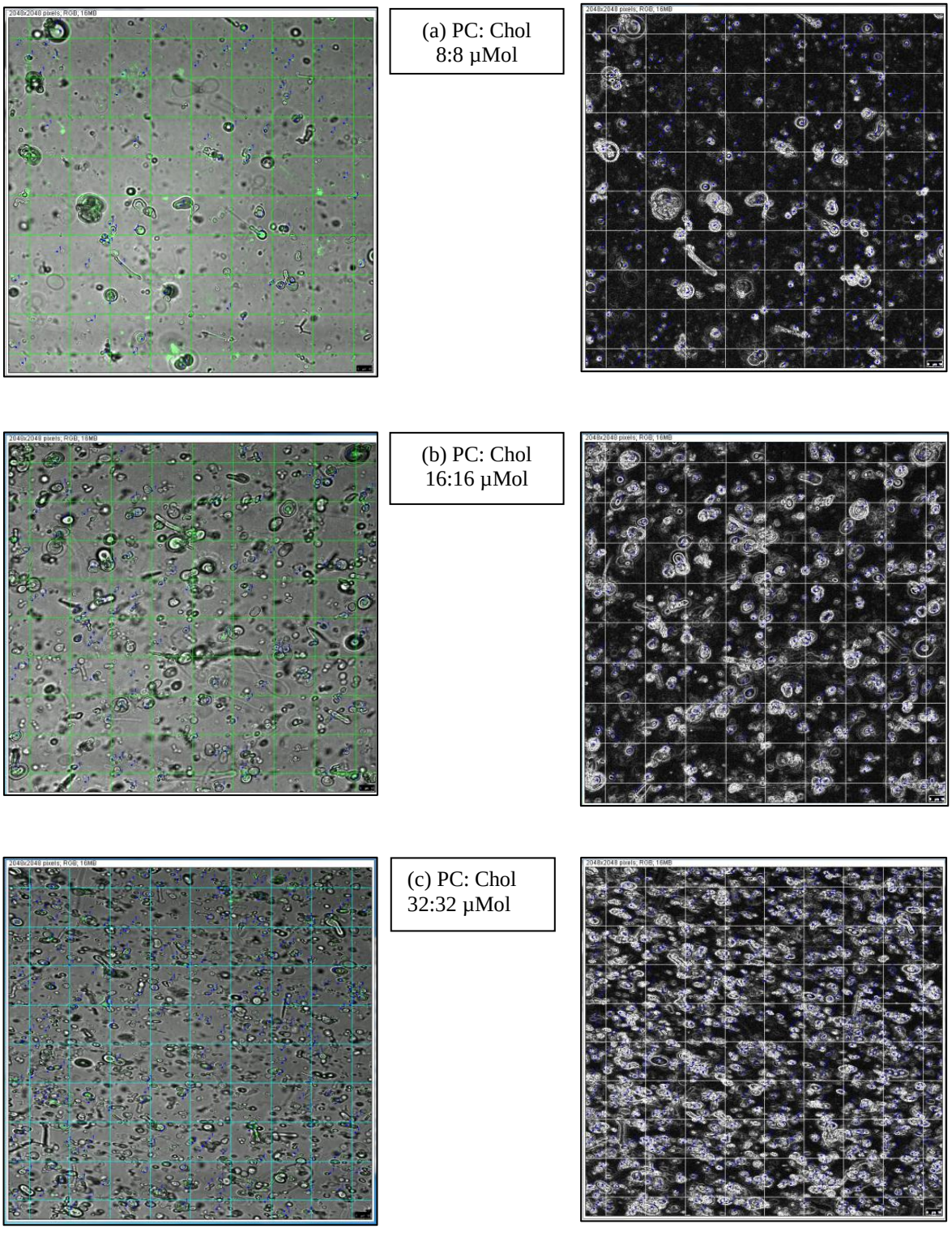


Figure 5.24 Quantifying from fluorescent confocal images the visibly fluorescent and non-fluorescent liposomal formulations of **(a)** PC:Chol at 8:8 µMol, **(b)** 16:16 µMol and **(c)** 32:32 µMol encapsulated with BSA-FITC 5 µg/mL with gridlines added using image J software.

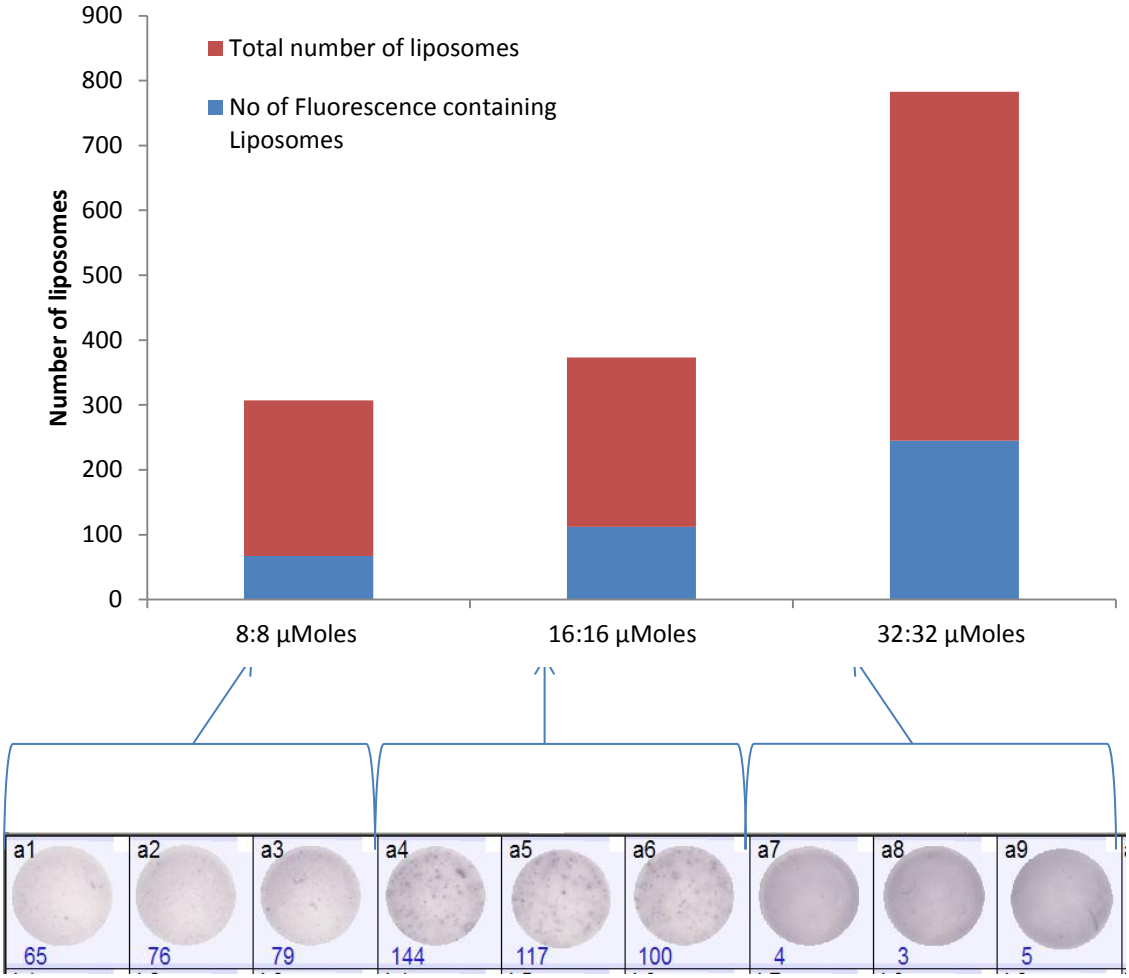


Figure 5.25 Quantification of the images for the formulations of (a) PC:Chol at 8:8 µMol, (b) 16:16 µMol and (c) 32:32 µMol encapsulated with BSA-FITC 5 µg/mL by counting the total number of liposomes and visibly fluorescent liposomes. Below the histogram are representative ELISPOT wells for the highest lipid concentrations for each calibration curve.

5.3.9 Assessment of increasing cytokine concentration to achieve higher spot numbers

Subsequent to assessing the effect of increasing lipid concentration on spot number, it was also important to determine the effect of increasing the concentration of the encapsulated cytokine on spot number within the ELISPOT assay. From Figure 5.26 a trend of increased spot number for the first three concentrations is notable, thereafter there is a sharp decrease is spot number. The change in increasing concentration only had a statistical difference between 2 x 10^5 U/mL and 4 x 10^5 U/mL ($p<0.05$), where there was a seven fold decrease in spot number (Figure 5.26). The reduced spot number at high concentrations may be due to the high levels of background observed. The results show that the highest spot number is achieved at the concentration that has been used throughout the

study, which is 2 x 10^5 U/mL. At higher concentrations, a change in protocol maybe necessary to ensure the removal of the higher levels of unentrapped rIFNγ. Even with cells, the presence of too much secreted cytokine can result in high levels of background and thus requires optimisation of the cell number (Chambers, 2010). Whether this would actually influence spot numbers is difficult to predict as the limitation could be the formulation method itself. Traditional methods for the preparation of MLV experience low aqueous trapped volumes and entrapment efficiencies which are improved by using other methods such as freeze-thaw techniques and dehydration-rehydration vesicles (Mayer *et al.*, 1986).

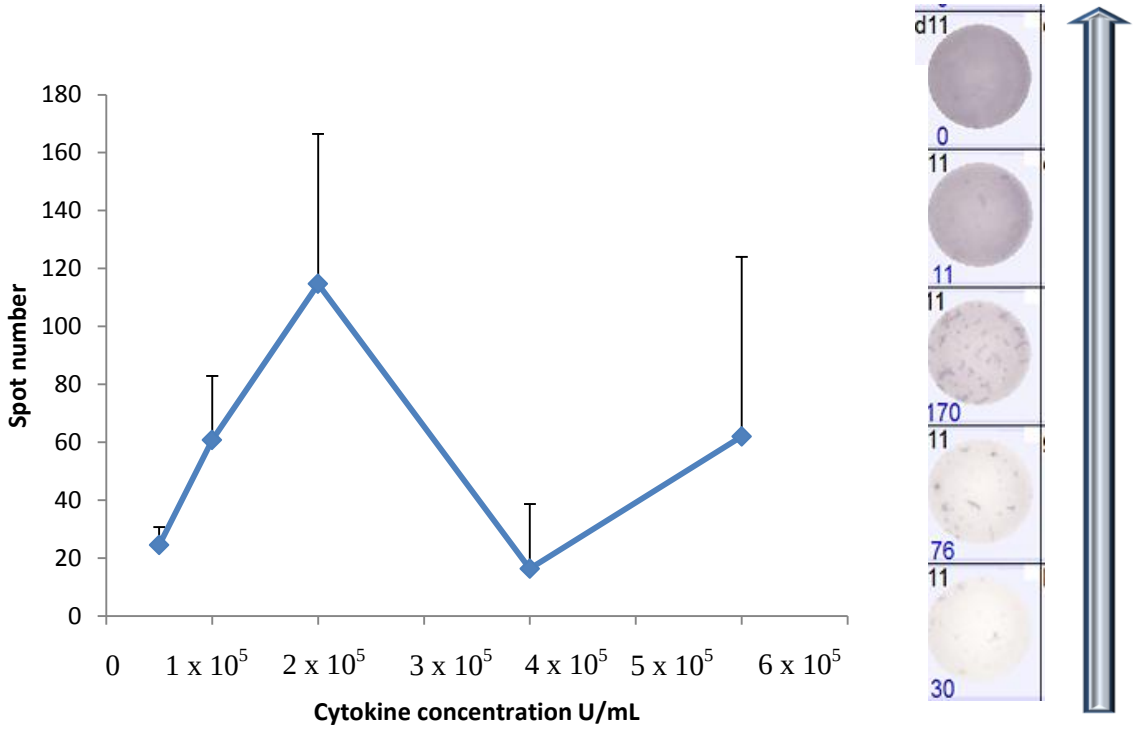


Figure 5.26 Spot numbers generated for increasing cytokine concentration, representative wells shown from PC:Chol 8:8 µMol particle size 7.8 ±0.49 µm. Concentration of rIFNγ tested was 5 x 10^4, 1 x 10^5, 2 x 10^5, 4 x 10^5 and 6 x 10^5 U/mL. Results represent mean ± SD, n =3.

5.4 Conclusion

Having established 'proof of principle' as shown in the previous chapter, the work in this chapter focused initially on optimisation of the procedure to produce high quality reference standards that would be user friendly. One of the principle conclusions derived was that background levels of unentrapped interferon can vary the quality of the ELISPOT results. However, this can be controlled through optimisation of the centrifugation steps. The addition of a centrifugation step made a significant difference to background levels. Using the ultra-centrifuge at 29,771 g RPM for 30 minutes for two runs and an additional slow step at 205 g for 10 minutes was perhaps the optimum protocol in terms of just background levels; however, logistically it was very time consuming. Modification of the protocol ensured that background levels were acceptable using a bench centrifuge normally used for cell work. Using RPMI for the resuspending buffer and for the final formulation had a notable impact on reducing overall background, thus the final protocol at this stage was one centrifugation step at 2660 g for 15 minutes followed by 205 g for 10 minutes. The premise of the second slower step was to remove smaller, less dense particles that may find it difficult to sediment but would probably add to background levels of interferon. The protocol though optimised required an additional change when the variability of automated reading for counting spot number was introduced. Modification of the protocol by the re- introduction of an additional centrifugation step of 2660 g for 15 minutes at 4 ° C was sufficient to rectify this issue.

From establishing 'proof of principle' that liposomes can encapsulate rIFNγ, the studies have shown the feasibility that a calibration curve can be produced from the formulation, which shows an exponential trend line with high R^2 values consistently obtained. There is a good level of reproducibility when running the same formulation batch as a calibration curve across different plates; however, the reproducibility of different batches can vary. The results from the calibration curves are promising, showing that a serial dilution can produce a standard curve.

In terms of trigger release, relying on the phase behaviour of the lipids at 37 ℃ to release the cytokine without an additional trigger such as an enzyme, seemed to work successfully. It was also evident that

using liposomes as reference standards is about getting exactly the right amount of release. The addition of an external trigger release mechanism such as an enzyme caused background levels to become elevated. It can be speculated that release was increased however, it became too great for effective spots to form on the well, thus thermosensitive release was more successful at this stage. The kinetics of release and its importance is apparent even for cells Lehman, (2005), comments that a rapid secretion rate will produce larger, less defined spots whereas a slower, steadier release of cytokine results in smaller and denser spots. It terms of producing a reference standard, by relying on the phase behaviour of the liposomes at 37 °C the actual standard is simpler to produce and use for the operator. The formulations of PC and PC:Chol have shown the 'right kinetics' and it has become apparent that perhaps these are the formulations that should be taken forward for further studies.

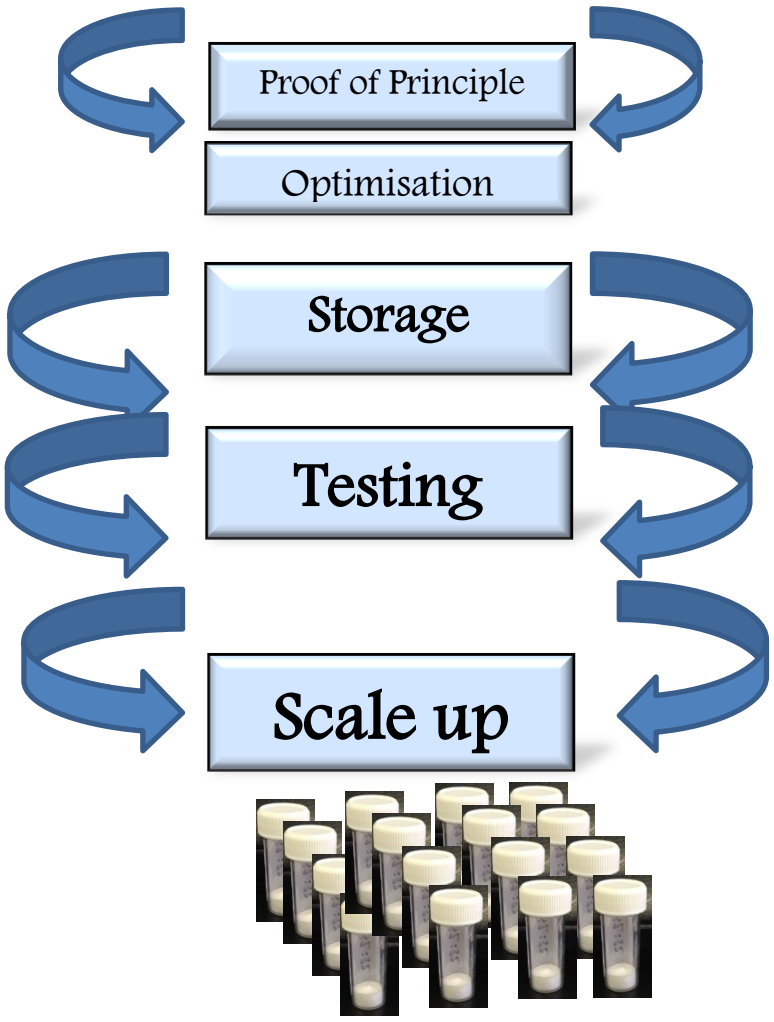
The assessment of achieving a 'high' positive control by increasing spot numbers however has proved more elusive. The relationship between increased lipid concentration and increased cytokine concentration is complex as there was no immediate increase in spot number observed. The microscopy work has aided in explaining why this is the case for increased lipid concentration as it was observed that many liposomes do not have enough encapsulated material to observe *any* fluorescence suggesting that similarly liposomes without enough cytokine would not form spots. Increased concentrations of cytokine did not affect spot number either but this may be due to the requirement of further refinements in the protocols depending upon the concentrations used.

Overall, the spot number and morphology of the spots formed from the artificial reference standards appear comparable although not perfectly similar. PC and PC:Chol are promising formulations for use within the reference standards without additional trigger release mechanisms.

Chapter 6

Feasibility of scaling up and lyophilisation of reference standards

Graphical abstract



6.1 Introduction

6.1.1 Enhancing stability of liposome reference standards and consideration of production scale-up

Stability and long-term storage are important pre-requisites to the development of a reference standard and lyophilisation has enabled many reference standards to fulfil these criteria (Matejtschuk *et al.*, 2005). Liposomes are susceptible to chemical instabilities, such as oxidation or hydrolysis, particularly with lipids containing unsaturated chains (Vemuri and Rhodes, 1995; Mohammed *et al.*, 2006). Even when liposomes are frozen or freeze-dried, they may undergo membrane rupturing which can lead to the loss of encapsulated material. One way to avoid this is for the liposomes to be freeze-dried in the presence of the lyoprotectant such as sucrose or trehalose (Bakás, 2000); however, the choice of cryo/lyoprotectants can play an important part (Christensen *et al.*, 2008, van Winden and Crommelin, 1999).

One theory proposed is that the sugars interact directly with the proteins or the head groups of the phospholipids of the bilayer membrane. The sugars replace the water molecules on the surface membrane, causing suppression in the bilayer transition temperature (T_m), which can cause leakage from the liposomes. The second theory relates to the formation of a glassy matrix around the liposomes upon dehydration. This matrix prevents the interaction between adjacent bilayers reducing mechanical stress and therefore enhancing bilayer integrity. A third theory for the protective effect of sugars relates to their kosmotropic effect. The charged ions present stabilise the structure of bulk water and reduce the amount of water at the membrane-water interface. This prevents damage by water during freezing and drying.

As liposome cryo/lyoprotectants, disaccharides such as sucrose and trehalose, which are naturally found in plants and animals protecting them from extremely cold environments, are particularly useful and form an established part of the freezing protocols for many pharmaceuticals within the biopharmaceutical industry (Christensen *et al.*, 2008). The advantage of using disaccharides is that they are small enough to interact with the phospholipid head groups whilst having a high Tg; sucrose and trehalose have Tg of -30 °C and -29 °C respectively (Chen *et al.*, 2010). Other cryoprotectants

found to be particularly effective are the oligosaccharides inulin and dextran, with inulin being more effective for PEGylated liposomes (Hinrichs *et al.*, 2005).

Given the basic 'proof of principle' and further optimisation of liposomes as potential ELISPOT reference standards has resulted in a practical and effective formulation process (chapter 5). The subsequent stage involved further testing of the reference standard as a part of the initial validation procedure (Cox, 2004). The testing of the reference standard forms an essential part of the validation procedure and is a necessary pre-requisite before the introduction of the reference standard into widespread use (Gold *et al.*, 2010). Many variables can affect the outcomes of an assay; the operator technique, equipment used, whether procedures are manual or automated thus, where possible evaluation of the material should be carried out under varying conditions (Janetzki *et al.*, 2008).

There are many available liposomal products that have achieved commercial success, including biological vaccines as well as drugs (Allen and Cullis, 2012). Therefore, scalable liposome techniques for the production and purification of liposomes on a large scale have been successful with many companies developing efficient procedures (Wagner *et al.*, 2002). In terms of this project, establishing the feasibility of scale-up was important for the project.

6.2 Aims and Objectives

Therefore, the aim of the work in this chapter was to optimise procedures for storage of the reference standards and to consider operator variability and the feasibility of scale-up of the production method. In order to achieve this aim, the objectives of the study were as follows:

- To investigate the appropriate format to facilitate storage and shipment of liposomes.
- To assess the robustness of the reference standards, through external testing with other operators.
- To assess the feasibility of making increased batch sizes that may be required for further validation processes.

6.3 Results and Discussion

6.3.1 Assessing storage options for liposome reference standards

An assessment was made of liposome formulations (PC, 32 μMol and PC:Chol, 16:16 μMol) for spot numbers generated pre-frozen and frozen at -70 °C (Figure 6.1). Although there was no significant difference observed across the formulations for PC:Chol rIFNγ reference standards pre and post freezing (with an average spot number generated of 120 ± 12 and 96 ± 27 respectively) there was a significant drop ($p<0.001$) in spot number for PC liposomes stored at -70 °C. There was over a two-fold decrease in spot number (from 135 ± 6 prior to freezing to 62 ± 6 spots subsequently; Figure 6.1). When comparing spot numbers generated for PC and PC:Chol formulations after freezing and thawing there was a significant difference ($p<0.05$) in spot number with just over 1.5 fold higher spot numbers generated for PC:Chol liposome formulations.

These results suggest that using 100 mM trehalose at -70 °C as a cryoprotectant would allow the liposome formulations to be stored and upon thawing, the cytokine retained enough activity for spot generation. As a general guideline, it is recommended to have 2 g cryoprotectant per g of phospholipid; within this study, the lipid to cryoprotectant ratio used was at 1:3 for the formulations (Mohammed *et al.,* 2006). In this study, liposome formulations using soy bean phosphatidylcholine (PC) and Dicetylphosphate at a 10:1 molar ratio were assessed for leakage of CF and it was found that storage at -30 °C was optimal for the freezing of liposomes (85 % retention) even without cryoprotectant through careful manipulation of the freezing rate. Although the absence of cryoprotectant did result in some levels of liposome aggregation and fusion. This was stabilised by the addition of cryoprotectants such as glycerol 10% v/v and mannitol 10 % m/v the authors attribute this to cryoprotectant interaction with the bilayer, reduction of ice crystallisation and a lower eutectic temperature in the presence of cryoprotecant (Fransen *et al.,* 1986). Previous studies have also shown that formulations of SUV containing soybean phospahtidylcholine and dicetylphosphate prepared at a 1:10 molar concentration in the presence of trehalose at 5% v/v had over 95 % CF retention at either -50 °C and -75 °C, with no significant variation in liposome size (Talsma *et al.,* 1991).

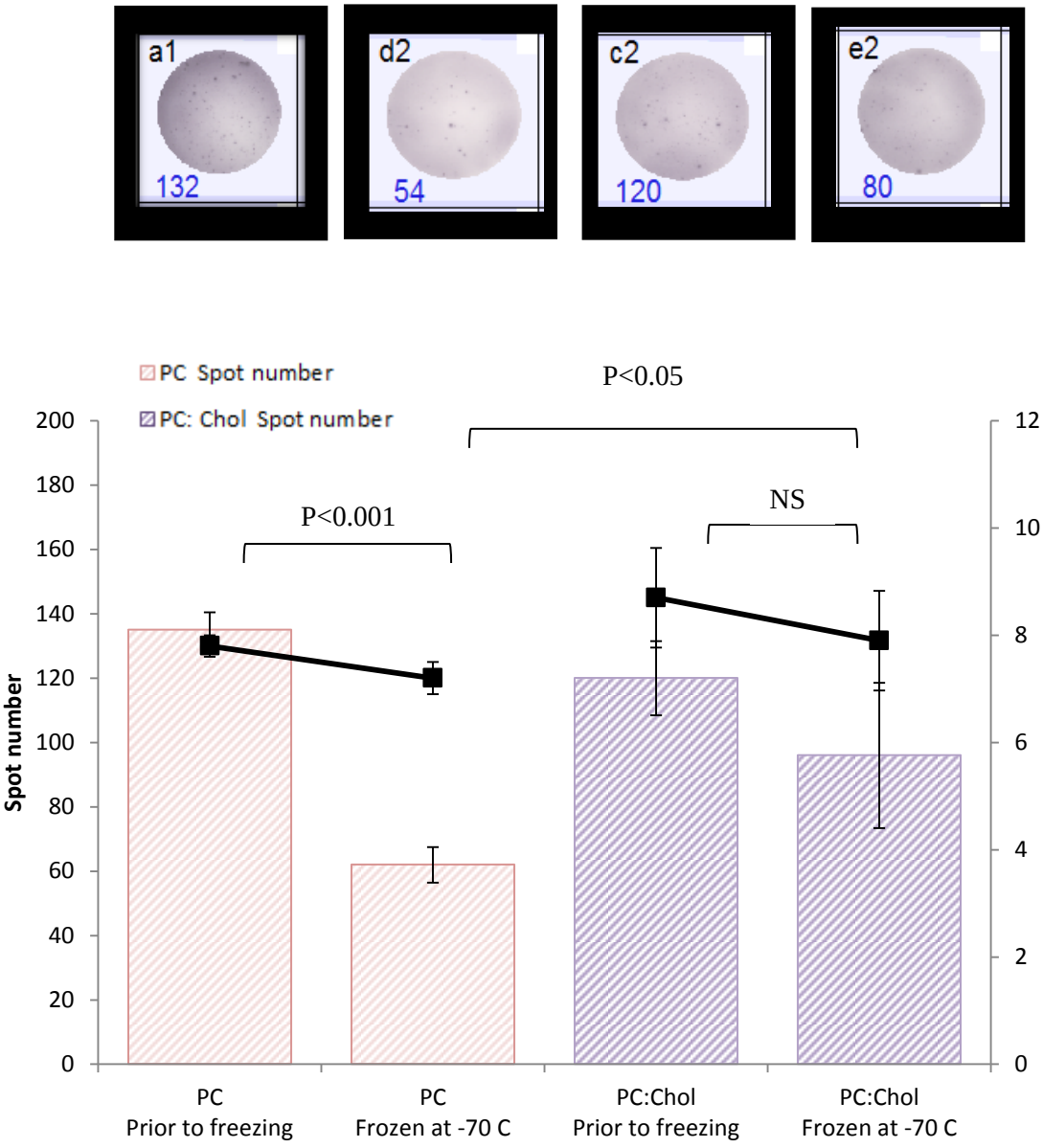


Figure 6.1 Assessment of PC and PC:Chol liposomes (32 µMol total lipid amount) for size (µm) and ELISPOT number for formulations prior to and subsequent to freezing at -70 °C. Results represent mean ± SD for n=3 batches.

6.3.2 Lyophilisation of liposome formulations encapsulating protein

Although it was shown in Figure 6.3.1 that MLV liposomes could potentially be frozen as reference standards, similarly to reference standard sera used as controls for ELISA assays, the ideal format for a reference standard is lyophilisation (WHO, 1990). Thus, using BSA-FITC as a model protein, PC:Chol liposome formulations with a mean particle size of 7.7 ± 0.1 µm (data not shown) were used to assess the effect of lyophilisation on protein loading and liposome size. To consider the role of

lyoprotectants, the lipid to trehalose ratios assessed were (1) 1:2 (w/w), (2) 1:4 (w/w), and (3) 1:8 (w/w) in conjunction with four aqueous media: 1) sodium bicarbonate, 2) Tris, 3) PBS and H_2O (Figure 6.2).

In terms of protein loading (Figure 6.2) the absence of cryoprotectant resulted in a significantly lower ($p<0.001$) protein loading (%) when compared to the pre-lyophilised formulations (4.22 ± 0.18 % vs. 8.34 ± 0.15 % respectively). The presence of trehalose at 50 mM, 100 mM and 200 mM did not prevent the loss of entrapped protein due to freeze-drying, with significant ($p<0.05$) decreases in protein loading compared to pre-lyophilised formulations. However, the loading values were significantly higher ($p<0.05$) for lyoprotectants in sodium bicarbonate buffer and Tris buffer when compared to formulations with no cryoprotectant. When assessing the impact of changing lyoprotectant concentration with the four different types of aqueous media for the post-lyophilised formulations, no significant differences were observed (Figure 6.2).

The impact of lyophilisation on the mean size of the liposome formulations was also investigated; results (Figure 6.2) showed that in the presence of the cryoprotectants (even at 50 mM) there was no significant difference in size when comparing pre-lyophilised formulations to lyophilised formulations. However, in the absence of any cryoprotectant the size was significantly higher ($p<0.001$) when compared to the pre-lyophilised formulations, increasing from 7 ± 2.16 μm to 17.68 ± 3.31 μm. This suggested that aggregation had occurred and was further confirmed through confocal microscopy (Figure 6.3).

During freeze-drying the protein is placed under a variety of stresses including low temperature stress, freezing stresses, the formation of ice crystals, increased ionic strength, changes in pH and drying stresses (Wang *et al.,* 2000). Many studies indicate that the correct choice of buffer for the lyophilisation procedure is critical due to the inherent pH changes which can cause crystallisation of the buffering species, especially sodium phosphate buffer (Wang *et al.*, 2000). From the results, the impact of the lyophilisation process was evident through the decrease in the entrapped protein and although the presence of the lyoprotectants reduced this loss, it was not prevented completely.

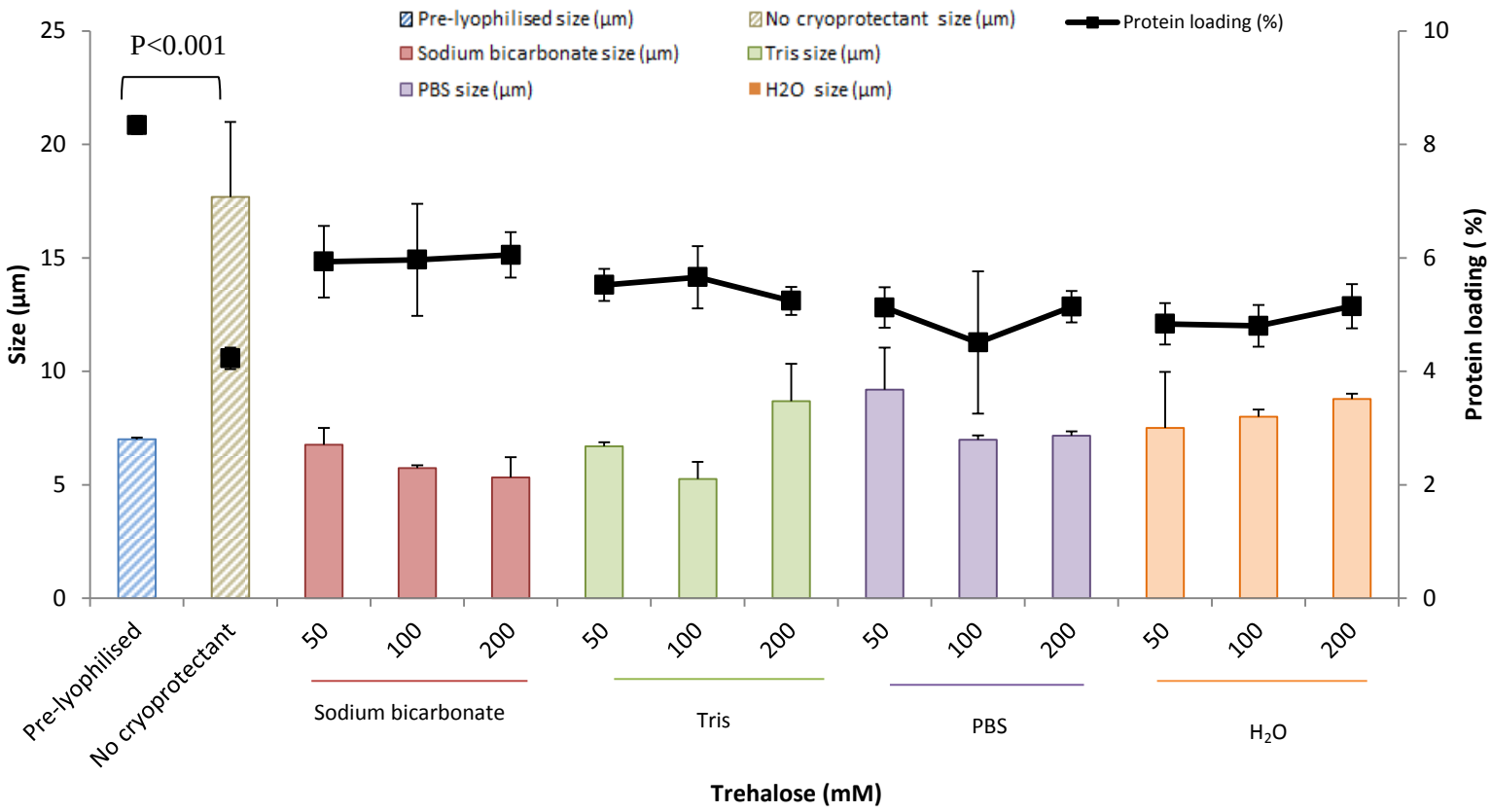


Figure 6.2 Protein loading (%) and size (µm) of PC:Chol liposomes entrapped with BSA-FITC (5 µg/mL) pre and post lyophilisation with trehalose at 50 mM, 100 mM and 200 mM in Sodium bicarbonate, Tris, PBS and H_20. Results represent mean ± SD for n=3 batches.

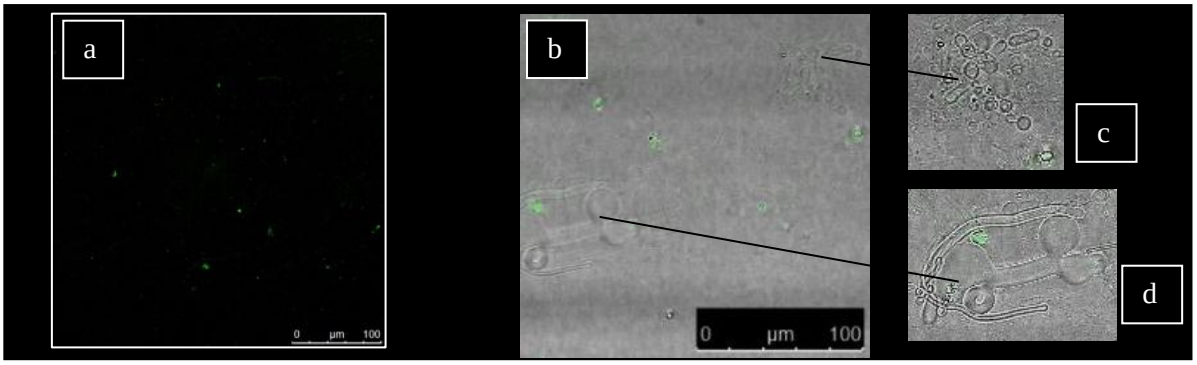


Figure 6.3 Confocal microscopy images of **(a)** and **(b)** lyophilised PC: Chol liposomes and enlarged areas **(c)** and **(d)** showing aggregation and fusion of the vesicles.

Studies by Van Winden et al (1997), examining the effect of freeze-drying of larger MLV vesicles of DPPC:DPPG (10:1 molar ratio) in the presence of sucrose (10% w/v), found that freeze-dried liposomes showed a higher ($p<0.01$) permeability for CF into the vesicles when compared to non-freeze dried liposomes. Thus, the studies confirm that freeze-drying affects the bilayer properties of the membrane and can explain the loss of protein observed during lyophilisation. Other studies confirm that, despite the presence of lyoprotectants, that 'repacking' of the bilayer components takes place during and after rehydration (Zhang *et al.,* 1997). The observed increase in size upon lyophilisation in the absence of cryoprotectant was shown to have occurred due to the aggregation and breakdown of the liposomal bilayers (Figure 6.3); such increases in size in the absence of cryoprotectant have also been observed in studies by Stark *et al.,* (2010). In this study the size of unprotected liposomes composed of DSPE-PEG 2000, 1-stearoyl-2-hydroxy-sn-glycero-3-[phospho-rac-1-glycerol],1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) or DSPC (5:28:57 molar ratio) significantly increased ($p<0.05$) whereas those liposomes in which glucose, trehalose or lactose was added at a 1:10 ratio showed no significant difference in size compared to freshly prepared samples. Although our results indicate that even at cryoprotectant concentrations as low as 1:2 (w/w) lipid to carbohydrate ratio the trehalose was able to stabilise the size of the vesicles. Overall, the results indicate that the Tris buffer is suitable for the trehalose dilution and the presence of cryoprotectant is important for the retention of protein and maintaining the integrity of the liposomes.

6.3.2.1 Optimisation of lyoprotectant concentration for the liposome reference standards

Although a general assessment of the impact of lyophilisation had been carried out (Section 6.2.2), the effect of lyophilisation on the rIFNγ incorporating liposomes was also required and the direct impact upon spot numbers to determine an optimised cryoprotectant concentration as a part of the protocol. Thus, formulations of PC 32 μMol were lyophilised at a range of lipid to lyoprotectant ratios, including 1:1.5 (w/w), 1:3 (w/w), 1:6 (w/w), and 1:9 (w/w) for both sucrose and trehalose. Subsequently formulations of PC:Chol (16:16 μMol) were also assessed at 1:1.5 (w/w) and 1:3 (w/w) lipid to lyoprotectant ratios with sucrose and trehalose.

As shown in Figures 6.4 a and 6.4 b there was a significant decrease ($p<0.001$) in spot number observed upon lyophilisation in the absence of cryoprotectant with, for example over a 7.5 fold reduction in generated spot numbers produced by PC liposomes freeze-dried compared to freshly prepared formulations. Generally, the process of freeze-drying and rehydration in the absence of a lyoprotectant also resulted in an increase in vesicle size. In terms of identifying an optimum lyoprotectant concentration, whilst there was no discernible trend for the PC liposomes (Figure 6.4 a and b respectively); at 100 mM of sucrose (188 ± 31 spots; 6.62 ± 1.83 μm mean particle size) or 100 mM trehalose (147 ± 5 spots; 8.32 ± 1.2 μm mean particle size) they gave comparable spot numbers and liposome size profiles to the freshly prepared PC liposomes (161 ± 62; 7.85 ± 1.26).

Based on the results for PC formulations, PC:Chol liposomes were freeze-dried using both sucrose and trehalose over a concentration range of 0 to 100 mM. Similar trends were observed with the PC:Chol liposomes to those of PC liposomes; in the absence of cryoprotectant there was significant decreases in spot numbers ($p<0.001$) compared to freshly prepared liposome formulations (Figure 6.5). The addition of 50 mM lyoprotectant also resulted in significantly lower spot numbers and overall 100 mM lyoprotectant giving more the comparable spot number and liposome size (Figure 6.5).

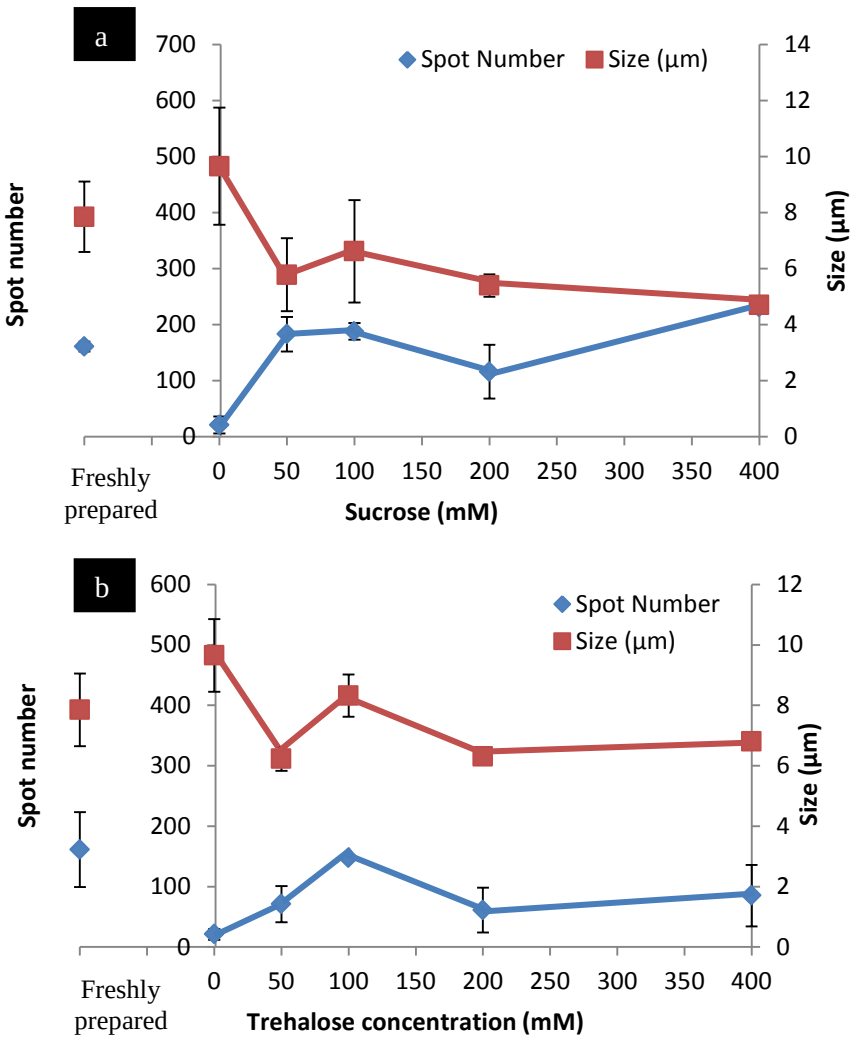


Figure 6.4 The effect of increasing **(a)** sucrose and **(b)** trehalose concentration on the size (µm) and ELISPOT number of PC 32 µMol liposome formulations. Results represent mean ± SD for n=3 batches.

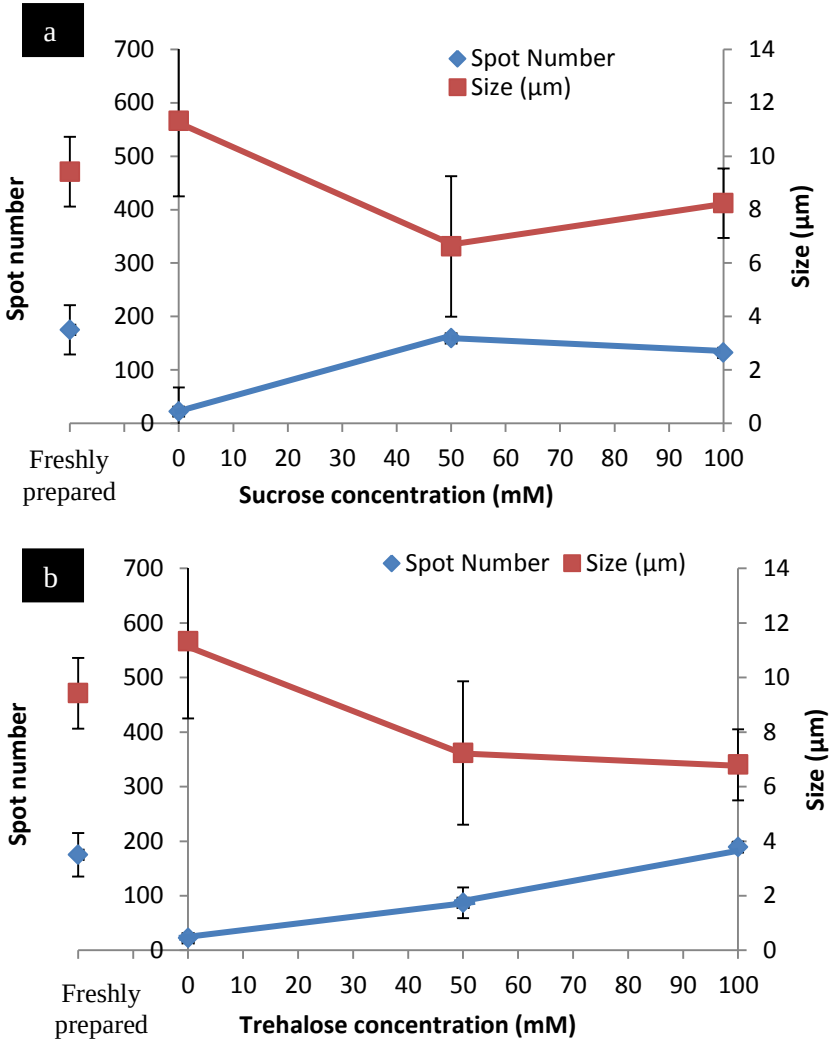


Figure 6.5 The effect of increasing **(a)** sucrose and **(b)** trehalose concentration on the size (µm) and ELISPOT number of PC:Chol 16:16 µMol liposome formulations. Results represent mean ± SD for n=3 batches.

Other studies assessing the impact of lyophilisation on proteins such as siRNA was carried out with liposomes composed of 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) chloride and dioleoylphosphatidylethanolamine (DOPE) at a 2:1 molar ratio with a final lipid amount of 5 mg (Yadava *et al.,* 2008). It was found that there was no significant differences between freshly prepared formulations and those lyophilised in the presence of glucose (p=0.2), sucrose (p=0.46), trehalose (p=0.74) and lactose (0.86) whereas lipoplexes in the absence of cryoprotectant exhibit an 88 % loss of activity and furthermore become amorphous and larger in size (Yadava *et al.,* 2008). In terms of the ELISPOT similar results were observed; in the absence of cryoprotectant with a significant reduction in spot number. The stability of liposomes with trehalose has previously been reported to have allowed the retention of 100 % of the encapsulated material subsequent to lyophilisation (Crowe *et al.,* 1987). In this study large unilamellar vesicles (LUV) liposomes composed of Palmitoyloleoyl-phosphatidylcholine (POPC) and phosphatidylserine (PS) at 90:10 mol % were used with trehalose and increasing retention of isocitrate was observed with increasing trehalose concentrations which was optimal at 1.8 g trehalose/g of phospholipid. This stability was attributed to the depression of the transition temperature of the lipids in the dry phase in the presence of trehalose.

Although these formulations showed a good level of reproducibility across the three batches (Figure 6.4 and 6.5), the issue of elevated background highlighted that the presence of the cryoprotectants was an issue. When the sucrose at 200 mM concentration was placed in wells without the reference standards, there was no observed background or spots (Figure 6.6 a) and there were instances of batch failures (e.g. Figure 6.6 b and c). Thus producing liposomes in a lyophilised format requires further optimisation, as even at lower lyoprotectant concentrations the observed background levels were high (Figure 6.6 d). Thus for further work, frozen formulations were used with 100 mM trehalose as the final concentration.

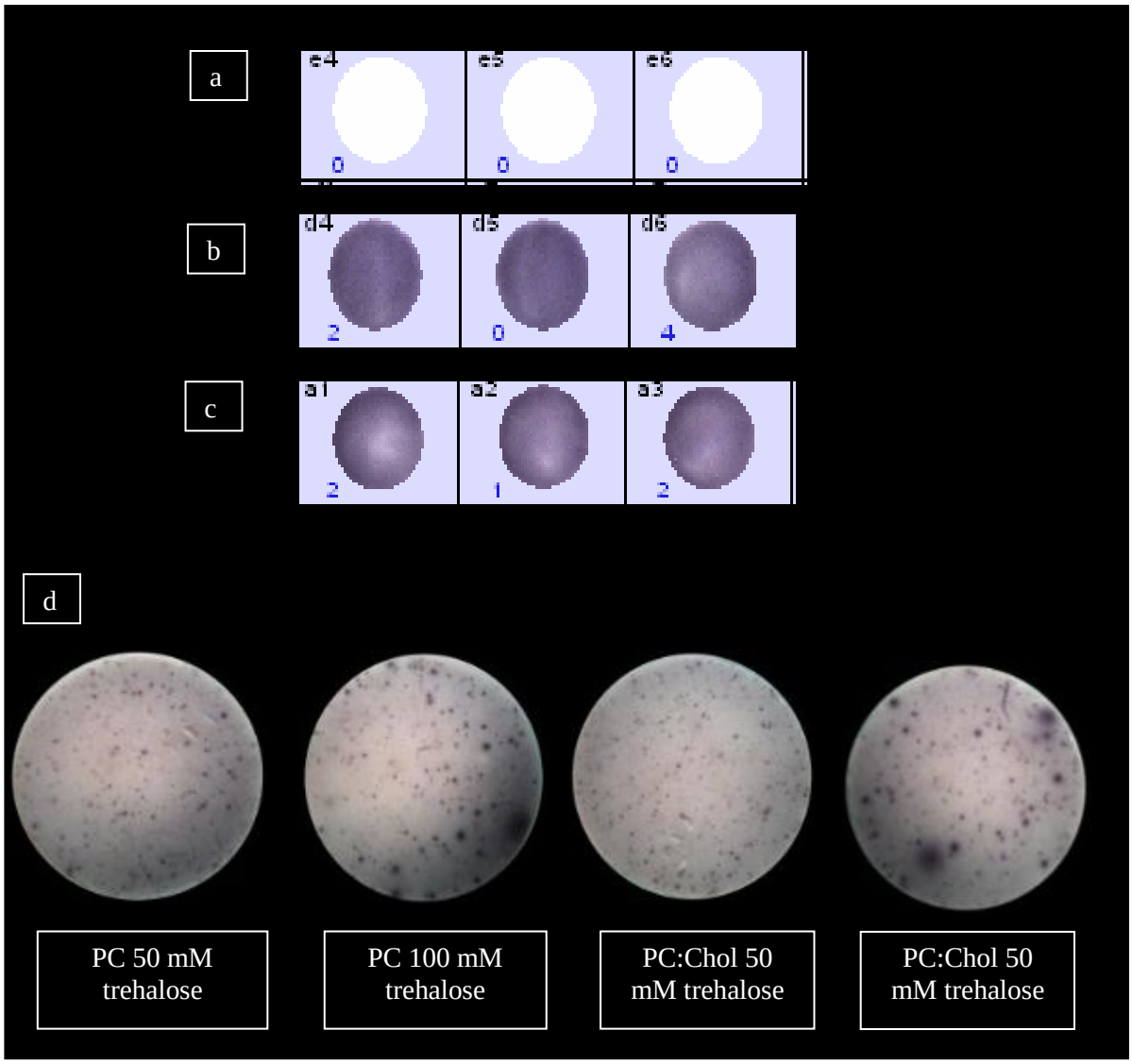


Figure 6.6 Representative ELISPOT results for assays post-lyophilisation **(a)** control sucrose concentration at 200 mM **(b and c)** results for no observed spots and elevated background levels attributed to batch failure **(d)** PC and PC:Chol wells for trehalose concentrations of 50 mM and 100 mM (1:3 w/w lipid to lyoprotectant ratio).

6.3.3 External assessment of formulations

To enable a direct comparison of the liposomal standards with the commonly used peripheral blood mononuclear cells, pre-formulated controls (PC 32 μMol and PC: Chol 16:16 μMol) were sent to NIBSC. For the study, liposome formulations that offered both high and low spot numbers that differed by a 1 in 3 dilution were used. Liposomes were prepared in RPMI and trehalose 100 mM, frozen and sent on dry ice to the operator. The assay plan details the layout of the artificial liposomes and the cell based controls (Figure 6.7 a).

Briefly, the artificial reference standards (Figure 6.7 a) sent from Aston were in rows A-C (column 1-6) and cells added to the assay included stimulated cells as high positive controls. In Figure 6.7 b in rows F 1-3 (cell based positive control) there were too many cells for the automated reader to detect, F 4-6 labelled as high positive 1 were cells stimulated with PMA and G 1-3 were also stimulated cells for a positive control. Cells without any additional stimuli were included as the negative control for cells and shown in H 1-6 (figure 6.7 b). From the results of the assay, the background levels were appropriate (Figure 6.7 b) across both the cell based controls and the artificial reference standards. There were low levels of spots observed for the negative controls included in the assay for both the artificial reference standards and unstimulated cells. RPMI medium alone also showed no spots again confirming that there was no contamination or non-specific spot formation (Figure 6.7 b). In terms of spot number for the controls sent to NIBSC (Figure 6.8), there was a significantly ($p < 0.001$) higher spots formed with the PC 'high' formulations which were 2.5 fold higher than PC:Chol preparations (82 ± 4 and 32 ± 5 spot numbers generated respectively; Figure 6.8). The spot numbers generated for the PC:Chol 'low controls' were 31 ± 7 spots and not significantly different to the PC:Chol formulation at 3 fold higher concentration (PC:Chol Low control; Figure 6.8).

Liposomes are able to encapsulate a wide variety of biological molecules although generally tailored for *in vivo* release (Gregoriadis and Allison, 1974, Gregoriadis *et al.*, 1971).The results in Figure 6.7 b and Figure 6.8 have shown that it is feasible to use artificial reference standards within an *ex-vivo* ELISPOT assay. For current controls within ELISPOT assays, cell preparations have been frozen and stored in liquid nitrogen, without significantly affecting their morphology or their maturation signal (Kalyuzhny, 2005) suggesting that using frozen rather then freeze-dried formulations is an option. In a recent study, it was shown that the viability of blood cells (PBMCs) collected from 285 patients was assessed for effects following cryopreservation and shipment (Olson *et al.*, 2011). A significant reduction in cell recovery was observed ($p<0.001$) and decreased cell viability and the authors attributed this to damage occurring during shipment and would not be evident prior to cryo-preservation or even immediately after.

a

	1	2	3	4	5	6	7	8	9	10	11	12
A	Negative control (1)			Negative control (1)			Negative control (1)			Neg control med (1)		
B	High Pos PC: Chol (1)			High Pos PC: Chol (2)			High Pos PC: Chol (3)			High Pos med (1)		
C	Low Pos PC: Chol (1)			Low Pos PC: Chol (2)			Low Pos PC: Chol (3)			Low Pos med (3)		
D	High Pos PC (1)			High Pos PC (1)			High Pos PC (1)			High Pos med (3)		
E	RPMI											
F	High Pos Cell (1)			PMA/PHA Pos (1)								
G	Low Pos Cell (1)			Control (1)								
H	No stimulation control (1)											

b

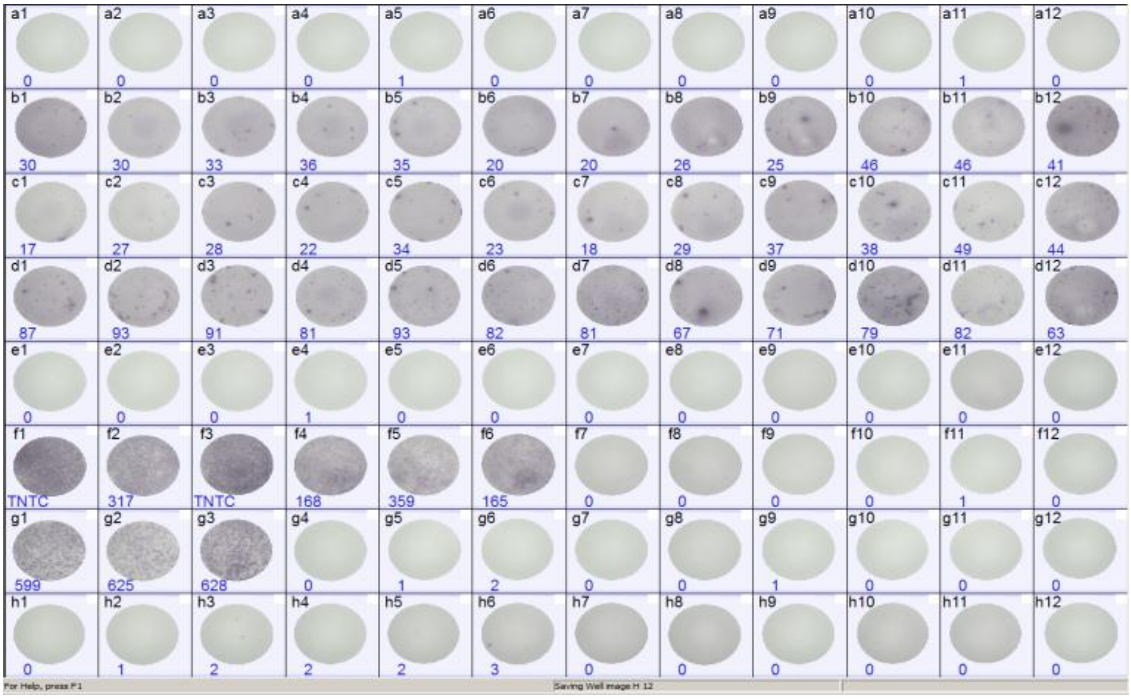


Figure 6.7 (a) Plate layout of the ELISPOT detailing where the artificial and live controls were ran within the assay **(b)** ELISPOT results for the assay of controls.

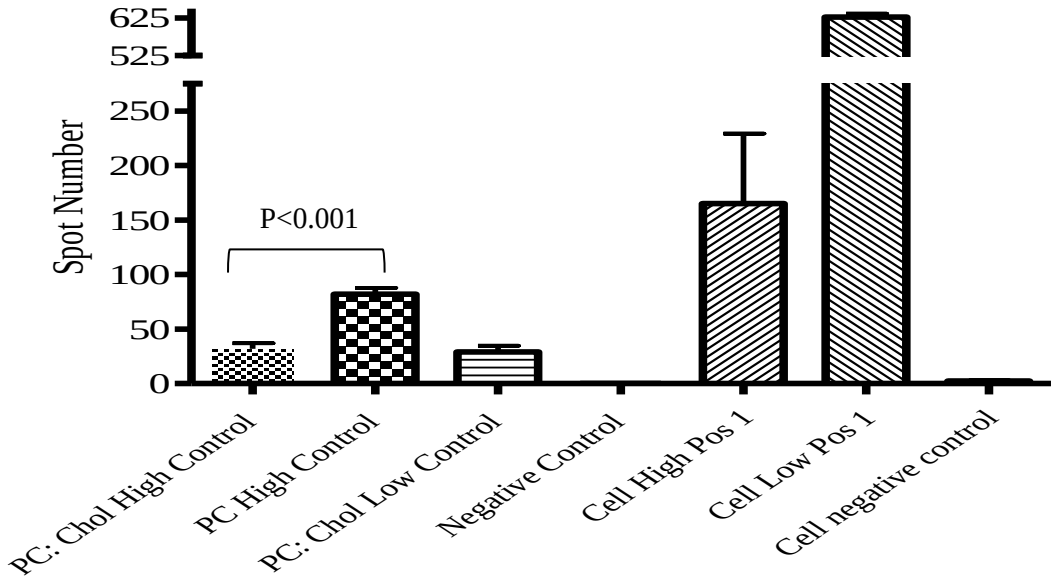


Figure 6.8 ELISPOT results for spot numbers observed with artificial liposomal and live cell reference standards of PC: Chol 'high' controls, PC 'high' controls, PC: Chol 'low', liposomal negative control Results represent mean ± SD for n=3 vials. Cell based positive high control 1, cell based positive control 2 and unstimulated cells as a negative control. The cell based controls are n=1 in triplicate wells.

6.3.4 The feasibility of scale up of liposomal reference standards

To assess the feasibility of scaling up the production of the ELISPOT reference standards, BSA-FITC (5 μg/mL) was used as a model protein to investigate producing a larger batch of liposome reference standards. The process used detailed in Figure 6.9, shows the procedure for production. A larger batch was made of PC:Chol 160:160 μMol to a total volume of 40 mL and the small batch of 16:16 μMol PC:Chol of a 4 mL volume. Assessments were made of particle size, entrapment efficiency and the morphology of the vesicles.

In terms of the scale up procedure, some adaptations to the basic protocol were necessary to account for the increased volumes. Due to the larger volume of lipids, it was necessary to use a 500 mL flask size and the time on the rotary evaporator increased to 45 minutes with further additional time for the flushing of N_2 for the removal of any residual solvents. Lipid hydration was for 30 minutes as modifications at this stage may affect the integrity of the protein.

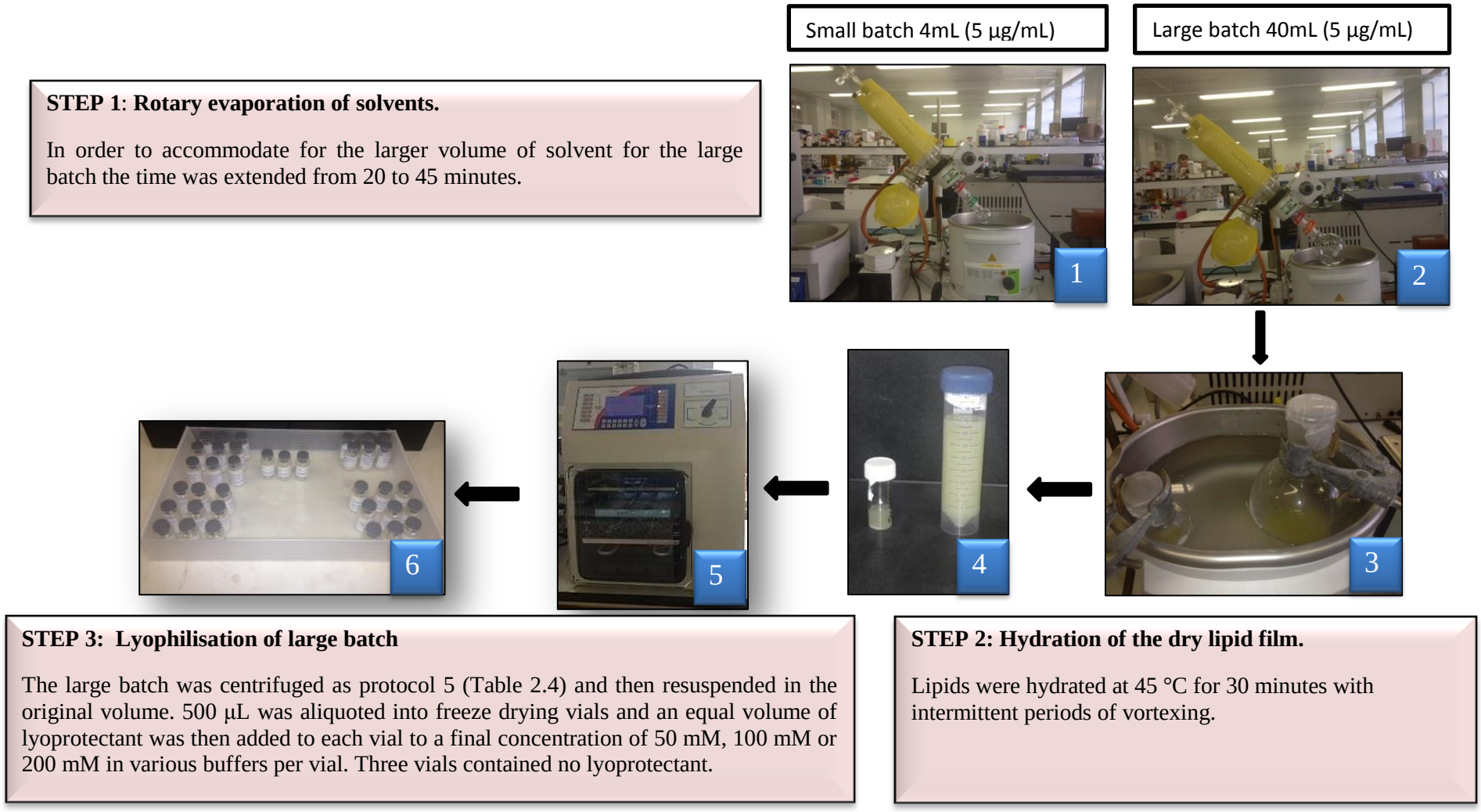


Figure 6.9 Scale up procedure and lyophilisation of large-scale batch of 40 mL PC: Chol 160:160 µMol with encapsulated BSA-FITC 5 µg/mL and production of a smaller batch of PC:Chol 16:16 µMol (4 mL).

Table 6.1 Particle size data for the batch of PC:Chol 16:16 µMol and PC:Chol 160:160 µMol.

Batch Size	Lipid Composition (µMol)	Volume (mL)	Size (µm)
Large	160:160	40	7.00 ± 0.1
Small	16:16	4	8.04 ± 0.2

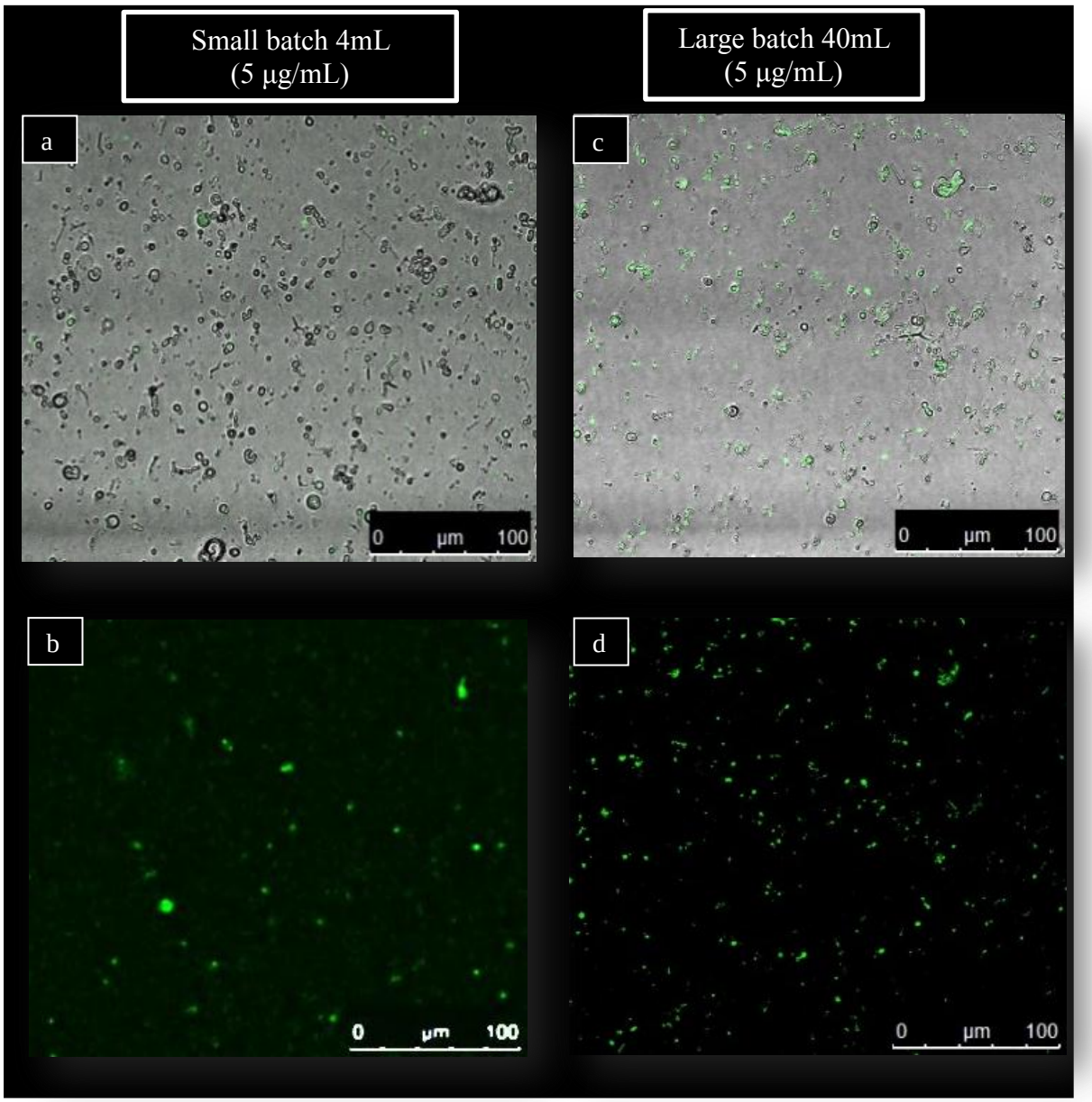


Figure 6.10 Confocal images using the 40X objective and the Leica Confocal microscope of the (a and b) small batch PC:Chol 16:16 µMol and (c and d) large batch PC: Chol 160:160 µMol with entrapped BSA-FITC 5 µg/mL.

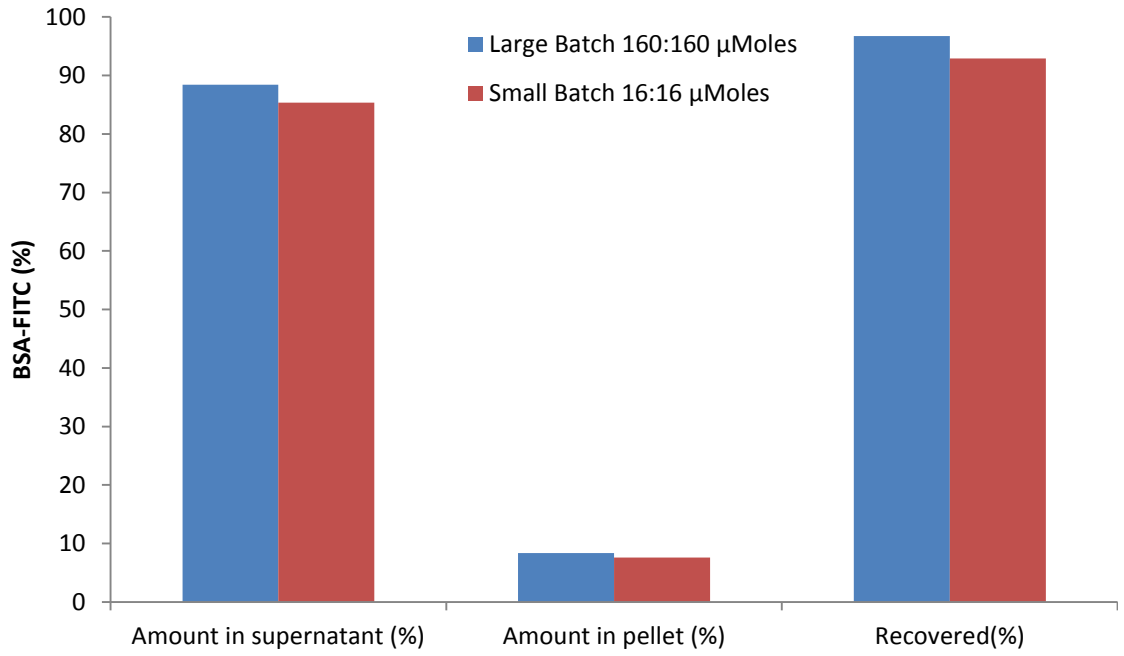


Figure 6.11 Entrapment and recovery of BSA-FITC 5 µg/mL for the large scale-up batch of PC: Chol 160:160 µMol and the small batch 16:16 µMol.

The particle sizes for the small and large batch (Table 6.1) were similar at 8.04 ± 0.02 µm and 7.00 ± 0.1 µm. The liposomes morphology and entrapment of the fluorescent marker was confirmed by confocal microscopy which also further confirmed that the vesicles from the small batch (Figure 6.10 a) were a similar size to the vesicles of the large batch (Figure 6.10 c). The fluorescent images clearly confirm that both the small and large batch were able to entrap fluorescent marker (Figure 6.10 b and d respectively).

Further to the confocal microscopy pictures, which confirmed the presence of the protein, quantification of the entrapped fluorescently labelled protein also confirmed that the entrapment of the marker had occurred using the previously validated assay detailed in chapter 3. For the purposes of quantification a calibration curve was constructed. The extrapolated values for the amount entrapped shown in Figure 6.11 for the small and large batch were 7.58 % and 8.34 % respectively. The total amount of protein recovered was also similar for the small and large batch of 93 % and 97 % respectively. Thus, the results have confirmed that scale-up with the lipid hydration method is achievable with minimal differences in the characterisation or entrapment values obtained.

In this study the feasibility of producing a larger batch of PC:Chol liposomes was assessed. These were produced via the lipid hydration method and although it is a relatively simple manufacturing process for the formulation of liposomes, it would not normally be considered for large scale production due to the low encapsulation efficiencies and the presence of multilamellar vesicles which are of limited clinical use (Lasic,1998). However, for the purposes of producing rIFNγ liposomes for use in the ELISPOT assay, MLV liposomes have been shown to be effective thus the lipid hydration method can be adopted. A study by Justo and Moraes, (2011) has shown that scale up of laboratory based methods was achievable, the method used was the ethanol injection method which produces small unilamellar vesicles (SUV) through ethanol mixing of dissolved lipids (PC and cholesterol at a molar ratio of 60:40), with an aqueous solution by fast injection. Smaller batches produced were 10 mL and scale-up was carried out by using 4:76 volume ratio of organic to aqueous solution and it was shown that liposomes of the larger batch were a comparable size to the small batch 132-146 nm and 122 nm respectively.

6.4 Conclusion

Overall, the results observed suggest that storage of the artificial reference standards produced from liposomes can be either in a frozen or a lyophilised format. However, there were drawbacks for both methods, as reductions in spot numbers were observed both upon freezing and with lyophilisation. Investigations into the optimisation of the type of lyoprotectant, the lipid to lyoprotectant ratio and the buffers used revealed that 100 mM concentration of either trehalose or sucrose were appropriate with no advantages of using higher concentrations in terms of spot numbers generated. Trehalose at 100 mM concentration was chosen when an assessment was made across both PC and PC:Chol liposome formulations.

As an assessment for the feasibility of sending controls externally for testing, liposomal formulations were frozen using 100 mM trehalose and sent to NIBSC. These artificial reference standards were ran alongside the ‘live cell’ controls and although spot numbers were lower than anticipated particularly for the PC:Chol ‘high’ control although with PC formulations spot numbers of just over 80 were

achieved. Thus, the results are promising in terms of feasibility for the production of reference standards that can be tested at different laboratories. The studies highlighted the further need for optimisation to enhance the activity of the 'recovered' liposomes subsequent to freezing and freeze-drying. Another important aspect of achieving a batch of controls that could be assessed externally was carried out by scaling up the process from 4 mL to 40 mL using a model protein BSA-FITC and was shown to be feasible both in terms of protein loading and maintaining the size of the liposomes.

There are many liposomal products on the market today successfully produced on an industrial scale including Doxil (Sequus), Ambisome (Nexstar), Visudyne (QLT) and Marqibo (Talon) (Allen, 2012) In 2001, the FDA published guidelines particularly referring to liposomal products including defining lipid components, excipients, characterisation and manufacture (Wagner and Vorauer-Uhl, 2011). Thus, the transition from the lab bench to commercial applications is viable with liposomal reference standards. Although in terms of the present study optimisation of storage protocols would be required.

Chapter 7

General discussion

The focus of this study has been on the ELISPOT assay, which is now one of the leading immunological assays used in the assessment of immune responses for vaccine clinical trials (Moodie *et al.,* 2006). Based broadly on the sandwich ELISA principle, it is an assay that was first described in 1983 and has become one of the most widely used methods to measure antigen-specific T-cell responses (Slota *et al.,* 2011). The assay plays an important role in the concerted drive to find an effective vaccine for the treatment of HIV amongst other diseases. The advantages of the assay include its capability for rapid screening, its cost effectiveness and the ability to carry out large scale screening for vaccine candidates (Streek *et al.,* 2009). It is a quantitative assay able to assess the cellular responses to vaccine candidates at a single cell level (Janetzki *et al.,* 2005). In the last few decades there has been hundreds of promising vaccine candidates found within laboratories with over a dozen entering into clinical trials. Although there have been successful developments of novel vaccines (Keith *et al.,* 2013) there have been some high profile failures of HIV vaccine candidates such as at the phase II stage for Merck (Harris, 2009). Such failures are costly on both a social and a political level, with estimated financial losses running into several hundred million dollars for Merck, and last year alone 34 million people worldwide were living with HIV (Harris, 2009). In the search to find answers for such large scale failures questions have been raised in regards to the future role of the ELISPOT IFNγ assay within the field of vaccine trials (Streek *et al.,* 2009). This is primarily because the *ex vivo* assay was unable to serve as a successful correlate for the protective immune response of the vaccine (Streek *et al.,* 2009).

The transition of this assay within thirty years from a newly developed technique into an integrated part of vaccine clinical trials has led to more demands of the assay in terms of performance, repeatability, and reliability that naturally lead to a focus on validation and standardisation of the assay (Janetzki *et al.,* 2005). Due to the nature of the ELISPOT assay, the results are dependent upon a number of technical variables that can influence the precision of the assay if not highly standardised (Almeida *et al.,* 2009). The large-scale studies in which the assay is used are particularly challenging in ensuring this, especially due the number of steps involved, and if there is a lack of automated procedures in place (Almeida *et al.,* 2009). This lack of standardisation across laboratories for the

ELISPOT assays have highlighted through harmonisation studies the wide range of variability observed (Janetzki and Britten, 2012). The issues highlighted through the studies have included the lack of a commonly defined consensus to determine the level of the immune response observed, variability in the reagents used and other protocol procedures (Moodie *et al.*, 2006, Smith *et al.*, 2009). The overall results of such differences across laboratories inevitably lead to a lack of harmonisation between laboratory data, which highlights the need for greater standardisation. Standardisation is the process for ensuring that all the methods for determining a particular concentration of analyte are able to give the same results and to achieve agreement between different methods particular reference standards are required (Wild, 2001).

The concept of producing a novel reference standard that would be feasible within the ELISPOT assay was the challenge for this study. The vehicle selected to achieve this aim had to be as similar to the cells as possible to be successful. A possible option to achieve this was with liposomes, considered ideal as models for biological membranes (Johnson and Bangham, 1969). The hydration of a dry lipid film formed enclosed vesicles with lipid bilayers and thus resembled cellular membranes (Lian and Ho, 2001). In terms of applications, a significant development came with the realisation of their capability to encapsulate biological materials such as drugs, protein and enzymes (Gregoriadis and Ryman, 1971). The aim was to design a reference standard, which could use these existing liposomal carriers through the entrapment of rIFNγ and they would therefore mimic the actions of cells.

Ordinarily in the development stages of a product the 'proof of concept' can be one of the first aims of a project; however, in this case the developmental stages began with pre-design considerations (Figure 7.1). The flow diagram shows the stages of development for the reference standard and this began with pre-design considerations. This stage encompassed considering how such a novel application for the liposomes would be successful. Although the liposomes offered a model biological membrane, they were not cells and had inherent structural differences.

From the outset, it seemed unlikely that placing the liposomes into ELISPOT wells and simply expecting them to reach the well membrane in similar time to the cells was unlikely, and thus careful consideration of the sedimentation characteristics of the liposomes was necessary. Therefore, the work in chapter 1 focused on assessing the sedimentation behaviour of liposomes. The results confirmed that they took a few hours to show complete settling thus in order to increase the efficiency of sedimentation a cholesterol analogue was synthesised. The basic premise was to sustain the inherent characteristics of cholesterol whilst modifying the liposomes to have a slightly increased density compared to more traditional liposomal formulations that incorporated cholesterol. Subsequent to the successful synthesis and characterisation of the brominated cholesterol formulations results showed there were improvements to the sedimentation efficiency of liposomes at specific ratios of the dibromocholesterol. In these initial stages, it was important to show that indeed the method for liposome formulation chosen could encapsulate proteins especially with the formulation containing synthesised dibromocholesterol. The entrapment of the protein was feasible as shown through a validated fluorescent assay.

Another parameter taken into consideration as a pre-design assessment (Figure 7.1) was the size of the liposomes. The method chosen for the formulation of the liposomes was the lipid film, hydration method (Bangham *et al.*, 1965). This method produces multilamellar vesicles which can range in size from 0.1 - 15 µm, have varying size distributions and lamerallity (Gomez-Hens and Fernandez-Romero, 2005). The aim was assessing the impact of various techniques in order to remove the smaller vesicles and retain the larger vesicles. A variety of techniques were assessed which would be applicable for cells such as differential centrifugation, centrifugation with sucrose gradients and filtration.

The aim was not to produce a homogenous population of smaller vesicles, which is invariably the case for *in vivo* applications of liposomes thus extrusion, homogenisation and sonication were avoided (Szoka and Papahadjopoulos, 1980). The techniques assessed were those generally used for cellular applications, so were more compatible considering that the liposomes were artificial cells.

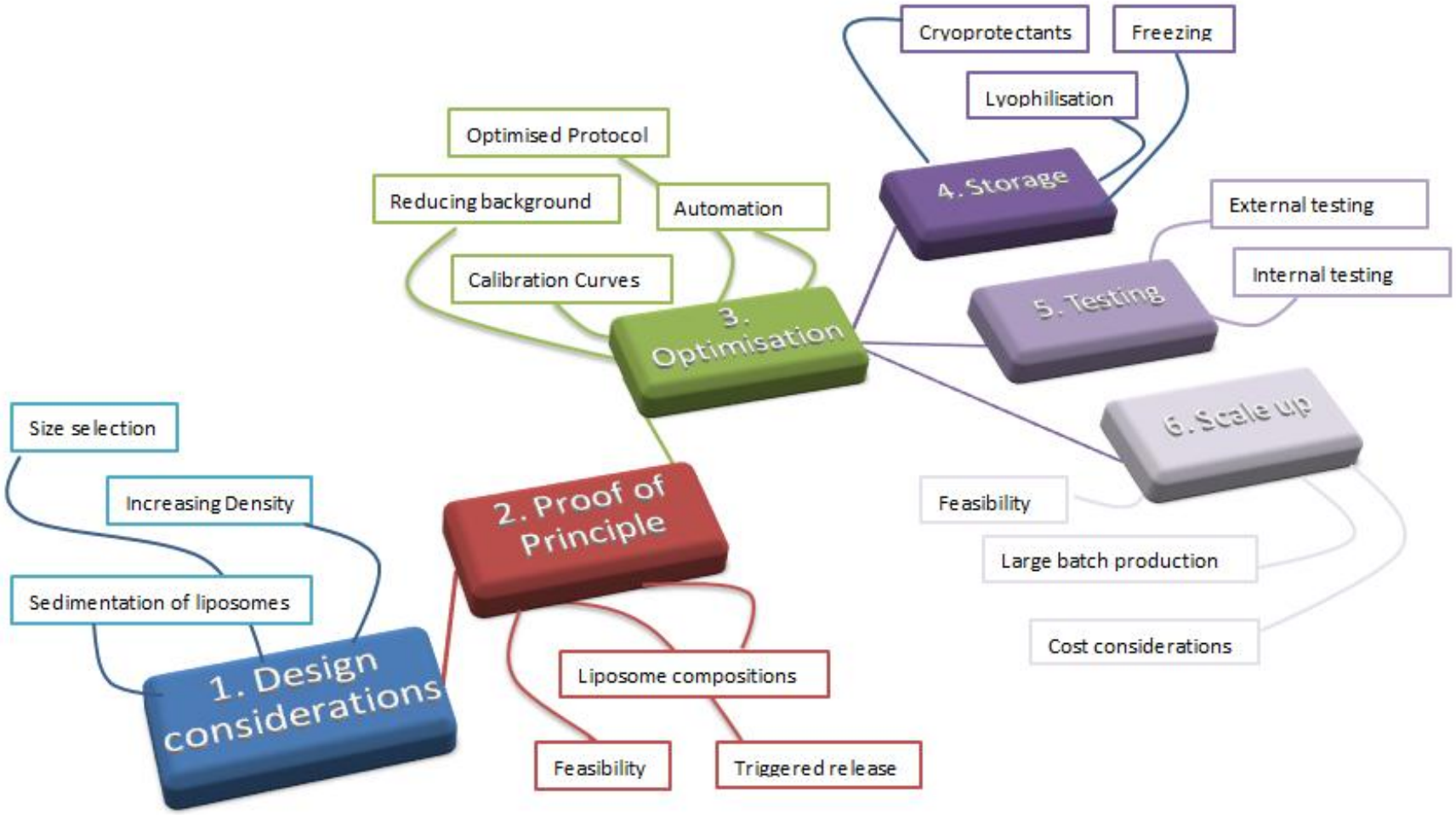


Figure 7.1 Flow diagram summarising the stages for the development of a novel artificial reference standard.

The conclusions from this work indicated that size separation was achievable using all the techniques to some extent; however, in terms of recovery of the liposomes and ease of procedure the most effective was slow speed centrifugation for short periods. The pre-design considerations were an important stage for examining the potential effect of the differences between liposome formulations and cells.

The next stage of development involved establishing the 'proof of principle' (Figure 7.1) and whether it was feasible that liposomes could encapsulate rIFNγ, whether the recombinant cytokine could retain its activity subsequent to the formulation process and the assessment of a wide range of liposomal compositions in conjunction with triggered release mechanisms. Similarly, to other studies, which had used interferon gamma within liposomes, entrapment was successful, and in agreement with the studies after the formulation process sufficient levels of cytokine activity were sustained (Van Slooten *et al.,* 2001, Anderson *et al.,* 1981). From the assessments of a wide range of liposome formulations of varying composition for thermo-triggered release, enzymatic release using PLA_2 and PLC and detergent triggered release, using Triton-X it became apparent that differences in the liposomal composition did affect spot formation on the ELISPOT assay. The utilisation of mechanisms other than thermo-triggered release was not effective overall. In terms of thermo-triggered release, all formulations tested showed some spot formation other than the formulations with the synthesised dibromocholesterol. These results were disappointing as the successful synthesis and incorporation of the dibromocholesterol within liposomes were achieved yet in its application within the ELISPOT assay the formation of spots had been unsuccessful. However, the 'proof of principle' was established, the incorporation of liposomes encapsulated with rIFNγ was not only feasible but their utilisation within an *ex vivo* ELISPOT assay was shown. At these stages liposome formulations of PC and PC:Chol were promising candidates for further optimisation. In terms of the other triggered release mechanisms it was observed that Triton-X was detrimental to spot formation and that the presence of enzymes only caused a significant increase in spot formation for two of the formulations of PC: Chol and DMPC. The results were an early indication that it wasn't simply about releasing all of the liposomal contents immediately but that

the kinetics of release was important. Thus, triggered release mechanisms for the liposome formulations were not successful within the assay.

Thus the introduction of a novel reference standard within an ELISPOT assay was shown, however these preliminary assays although promising were not part of an optimised protocol thus the subsequent stages of development (Figure 7.1) was to work on producing a protocol that was robust, offered reduced background levels and was feasible enough for scale-up procedures at a later stage. Through the introduction of the novel reference standard within the ELISPOT assay the membrane came into direct contact with 'foreign' material, which was not ordinarily used within the assay. It became evident that variability introduced such as automated reading increased background levels. The optimised protocol that could still produce reference standards that were similar to the spots produced from live cells was feasible. One of the limitations of the artificial reference standard highlighted at this point, was the difficulty in achieving increased numbers for a positive control. The spot numbers for positive controls using PBMCs can reach up to 500 spots (Zhang *et al.*, 2009). However, alterations in the lipid or the cytokine concentration did not result in increased spot numbers in this region. The general spot number observed for liposome formulations has been in the region of up to 100 spots. This is due to the numbers of liposomes, which do not have enough entrapped protein to form spots but are perhaps impeding the spot formation of those vesicles that do, by taking up space on the membrane well. However, the spot numbers obtained were sufficient for some studies in which the general cut-off for positive response was 55 spots (Hendrik *et al.*, 2009).

The difficulties for having a reference standard for the ELISPOT assay lies in the nature of the assay itself. The assay method involves adding cells directly into the wells and these cells will secrete appropriate levels of cytokine dependent upon the responses to antigens (Almeida *et al.*, 2009). Thus normally a standard which is used within an assay follows the broadly accepted rule of 'like versus like' where the material acting like the reference is as similar to the material being tested. Thus, the lack of such a reference standard within the ELISPOT assay explains the problem tackled within this study.

Previous attempts to find robust reference standards for the assay have included T-Cell lines, clones or other PBMCs but these are limited due to both their wider applicability and lack of definitive spot numbers for the actual number of cells (Janetzki and Britten, 2012). Currently many assays use positive controls as their cells stimulated with mitogens such as PHA, ConA or PMA/Ionomycin, as they can induce secretion of cytokine from cells however they can result in overstimulation and thus too many spots within a well (Janetzki and Britten, 2012). The problem of not knowing how many spots are produced from the positive controls remains the issue with all available reference standards for the ELISPOT assay. More recently studies have focused on the production of a pool of viral peptides made from a panel of epitopes from the Influenza virus (Flu), Cytomegalovirus (CMV) and Epstein Bar Virus (EBV) that were used to elicit IFNγ release from T-cells (Currier *et al.,* 2002). The results were promising as the size, shape and appearance of the spots could be used for establishing acceptance criteria; however, again they are unable to provide the answer for placing a reference standard on the assay which can give a definitive response say of 100 spots which can then be compared between laboratories. The aim of this study was to produce a reference standard that from assay to assay could give a defined response, so that even inexperienced ELISPOT users would be confident from the control values that they have followed the ELISPOT protocol correctly. By the optimisation stage of the protocol, the idea of an artificial reference standard within the ELISPOT assay had moved from a concept to a reality.

In Figure 7.1, the stages of the project that followed optimisation of the protocol were about application. Proving that liposomes could encapsulate rIFNγ and be used within the ELISPOT assay was not sufficient if they were used as part of a validation process, it would also be important to establish a storage format for the reference standards. Many reference standards arrive lyophilised, and for the liposomal rIFNγ formulations, this option was also possible in the presence of lyoprotectants such as sucrose and trehalose. Although background levels were elevated once more and consistency from one freeze-drying cycle to the next was not shown, lyophilisation was achievable. However, the studies highlighted that extensive further work was required in order to optimise such a process. Due to the unreliability observed with the freeze-drying cycles the reference standards sent externally were frozen and sent on dry ice. The external operator ran the

controls alongside live cells and demonstrated that spot formation occurred and that the spots were of a sufficient size and morphology for detection by automated reading procedures. This again highlights the feasibility of the reference standards as controls for the ELISPOT assay. The feasibility of scaling-up production for larger batches with a model protein with similar physicochemical characteristics and entrapment levels to the smaller batch was demonstrated although the final stages of storage and testing require further investigative work.

The 'proof of principle' that an artificial reference standard that can entrap rIFNγ and release the cytokine within the confines of an ELISPOT well to produce a spot on a membrane was demonstrated. This brings a promising and novel reference standard that could aid standardisation within the field of immune response assessment. The studies have highlighted the necessity for extensive further investigations in particular with the storage requirements for the assay; once optimised then further testing can be carried out. There is still much scope for investigating the lyophilisation process by examining optimised cycles, assessing freezing protocols, and possibly looking at a wider range of cryoprotectants and buffers. The possibility of using cytokine within the formulation process that has not previously been freeze-dried may also substantially help to retain the activity of the protein upon lyophilisation within liposome vesicles. Another area that requires investigation is the possibility of freezing the liposomes in a similar way to cells. Currently such processes have ensured the integrity of the cells and may prove to be more beneficial for liposomes encapsulating rIFNγ. Once such optimisation has taken place, the production of larger batches of liposomal reference standards with rIFNγ is feasible and thus more extensive testing with carefully designed validation studies to assess the robustness of the controls. The requirements of reference standards are stringent and even more so for the ELISPOT assay where there has been a wide variability in results observed. Although the incorporation of the synthesised dibromocholesterol within the formulations was not successful in this instance (or necessary due to the successful release of cytokine promoted by temperature) perhaps, other options for increasing the density of the liposomes without affecting the integrity of the cytokine or the release kinetics could further enhance the performance of the artificial reference standards. Assigning a fixed range for the controls would remain an ultimate objective but would only be feasible once effective storage of

the reference standards has been achieved This may ultimately lead to the elusive ‘true gold reference standard for the ELISPOT’.

In summary, the objectives of the research were to show that liposomes could encapsulate cytokine and then release the encapsulated material within the confines of the ELISPOT assay to produce defined spots of an appropriate morphology. The results from this study have shown that this was feasible across a wide range of formulations with liposomes composed of phosphatidylcholine and phosphatidylcholine with cholesterol being particularly effective. Thus, ‘proof of principle’ was established however, challenges in terms of high background levels were observed. These were overcome through the optimisation of the protocol for the production of the reference standards, which allowed clear visualisation of spots both by automated and manual reading procedures. Furthermore, external assessment of the liposomes with ‘live cells’ showed they could be incorporated within the assay as an integrated part of the normal protocol for the operator. The final objectives of considering product storage were shown as a frozen and lyophilised format although optimisation is still required in this area, in addition the feasibility of scale-up procedures to produce a larger batch were demonstrated. Overall, the main objective to produce artificial reference standards for use within the ELISPOT assay has been shown.

Chapter 8

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