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Microscopy imaging of liposomes: From coverslips to environmental SEM

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ABSTRACT

A plethora of techniques for the imaging of liposomes and other bilayer vesicles are available. However, sample preparation and the technique chosen should be carefully considered in conjunction with the information required. For example, larger vesicles such as multilamellar and giant unilamellar vesicles can be viewed using light microscopy and whilst vesicle confirmation and size prior to additional physical characterisations or more detailed microscopy can be undertaken, the technique is limited in terms of resolution. To consider the options available for visualising liposome-based systems, a wide range of microscopy techniques are described and discussed here: these include light, fluorescence and confocal microscopy and various electron microscopy techniques such as transmission, cryo, freeze fracture and environmental scanning electron microscopy. Their application, advantages and disadvantages are reviewed with regard to their use in analysis of lipid vesicles.

1. Introduction

Much of the initial excitement in the discovery of liposomes (Bangham et al., 1965) was due to the fact that the spherical phospholipid bilayer offered a model for the cell membrane. Whilst it is a general requirement to visualise cells prior to application, the preliminary characterisation of liposomes often overlooks initial image analysis to confirm their structural integrity, with a potential over reliance on physico-chemical analysis methods such as particle sizing using laser diffraction techniques. In the application of such techniques there is an assumption that intact bilayer vesicles are present and no vesicle aggregation is occurring. Indeed most particle sizing methods struggle to differentiate between individual liposomes and aggregates, assuming the latter to be a single particle. However this can be verified simply and quickly using light microscopy and help confirm that fully formed vesicles have been produced.

When applying microscopic techniques for visualising liposomes, it is interesting to draw comparisons between how we work with these artificial cell membranes and cells; the use of light microscopy to determine cell growth and viability has been carried out for many decades as routine. From the initial seeding through to manipulation of cells, every stage is followed visually. One of the primary reasons that cells are viewed with relative ease is their size and their ability to adhere to surfaces (although the latter is not a pre-requisite). Many cells have an ability to attach to other cells, and to underlying substrates and materials through an array of cellular adhesion molecules. Indeed the substrate on which the cells grow can ultimately affect their biological function (Jokhdar et al., 2007).

Here the idea of phospholipid bilayer vesicles as a model for cells was revisited and reversed, forming the basis to enhance the techniques available in the laboratory for visualising liposomes and other bilayer constructs. Compared to cells, synthetic vesicles obviously have two major disadvantages; the first being the lack of adhesion proteins, and also their much reduced density in comparison to most cells (due to their lack of internal cellular moieties). However, an important factor to consider is that even initial interactions between cells and surfaces are dominated by electrostatic interactions, with ligand interactions not playing a role until they are within close enough proximity to form such interactions (Marshall et al., 1971). Therefore, it is generally these longer range electrostatic forces which are the controlling factors for cell absorption onto substrates (Dan, 2003), and such interactions could be similarly exploited with liposomes to enhance visualisation, especially as many liposomes studied are anionic or cationic in nature.

Unfortunately light microscopy is unable to provide comprehensive information about the lipid bilayer compared to the detail offered by other techniques, such as freeze fracture or cryo-electron microscopy. However, light microscopy does provide the advantage of obtaining an image of vesicles in a short period of time using

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standard laboratory equipment; information on the morphology of the vesicles, the heterogeneity of the sample and visualisation of the degree of vesicle aggregation over a defined time period can be determined with relatively little preparatory work. To help with the visualisation of liposomes by light microscopy there are various options including the use of hydrophilic dyes such as fluoroisothiocyanate (FITC) and carboxyfluorosceine (CF) (e.g. Bagatolli, 2007; Bouvrais et al., 2010) or alternatively lipophilic dyes such as Sudan Black B (Briz et al., 2000) and Oil Red O. Indeed using such fluorescent probes can support a range of physico-chemical studies of pharmaceutical systems: for example, micrographs of niosomes demonstrated enhanced dye retention of cholesterol-rich niosomes was a result of their spherical nature compared to the niosomes formulated in the absence of cholesterol which formed polyhedral structures (Arunothayanun et al., 2000).

1.1. Polarisation microscopy

In simplistic terms, image formation in polarisation microscopy is based upon the ability of polarised light to interact with polarisable bonds of ordered molecules in a direction sensitive manner (Murphy, 2001). Liquid crystals such as lamellar vesicles are anisotropic, i.e. their physical properties vary with the direction of measurement. For example plane-polarised light is rotated when travelling perpendicular to the layers in the lamellar phase. Because of this ability to rotate polarised light, liquid crystals are visible when placed between crossed polarisers, and this provides a useful means of detecting liquid crystalline state and thus lamellar vesicles. Images are formed due to diffraction, interference and existence of ordered molecular arrangements. The polarising microscope requires an analyser and polariser for monochrome images and the principles and microscope setup are shown (Fig. 1). For brightly coloured images, a lambda plate is also required which is made of a film of highly aligned linear organic polymers from mica and is birefringent. This introduces vivid interference colours to images of birefringent objects.

1.2. Electron microscopy

For greater magnification, electron microscopy can be employed. Its use has allowed an insight into the bilayer characteristics and made visualisation of small unilamellar vesicles possible. In the most basic terms, these systems work using an electron beam which is focussed by various lenses onto the sample surface. Electrons are then scattered in two ways, with some only their path will change, whilst others will collide and displace electrons around the nuclei of atoms in the sample. The electrons are then focused and magnified by a system of magnetic lenses to produce a projected image. The primary advantage of using this technique, over light microscopy, is the higher resolution which allows for the visualisation of smaller constructs. Light microscopes have a resolution of 200 nm, whilst the resolution of transmission electron microscopy is considered to be about 0.2 nm (Kane and Sternheim, 1978) thus information regarding structures of small unilamellar vesicles would be limited. Electron microscopy is also a relative quick method for the visualisation of vesicles under very high magnification.

In transmission electron microscopy (TEM), sample preparation involves a small amount of hydrated specimen being placed onto a grid. As vesicles are in direct contact with the grid surface this may affect their orientation or morphology before they are viewed. It is necessary to use a negative stain such as Uranyl Acetate or Osmium Tetroxide, so the vesicles can be viewed against the stained background. By using a negative stain, the background is stained rather than the liposomes; hence the outer region of a vesicle is black whilst the inner area is white. During the staining process, the specimen is blotted to remove excess stain. The use of such stains and preparatory techniques have been shown to cause changes to the original vesicular structure and the stains themselves can lead to the formation of dark and light fringes which could be misinterpreted as lamellar structures in the specimen being analysed (Talmon, 1982). Furthermore, once in the electron microscope chamber the system is placed under vacuum which causes further dehydration of the sample, which again may cause changes in the vesicle morphology such that the images can be poorly representative (Donald, 1998).

Early histology work using electron microscopy routinely involved chemical fixation, dehydration, cutting of thin sections and staining of cells prior to analysis. Such preparatory work has long been recognised to have significant effects on cell morphology (Bullivant and Ames, 1966). Thus methods which eliminated the need for exposing living cells to such physical and chemical effects experienced during chemical fixation and dehydration were developed. Cryo-electron microscopy can help circumvent some of the above issues: this is an adaptation of TEM in which thin vitrified aqueous films, kept in liquid nitrogen, are used. This technique allows for the visualisation of the architecture of vesicles in terms of the outer shape and the inner compartment (e.g. Perrie et al., 2001; Davidsen et al., 2005). The advantage of the rapid freezing of samples is that it limits ice crystal formation and allows small proteins and biological material in the inner compartments of vesicles to be preserved and details regarding the morphological characteristics, such as the number of bilayers, can be better distinguished. The lower limit for resolution with these systems is 4–5 nm, and the upper limit of ~500 nm is defined by the thickness of the film as scattering by water molecules becomes too great (Almgren et al., 2000). During the preparation of samples for use with this method, difficulties can be encountered in the production of uniform films due to variability in film thickness.

Cryo-preserved samples can also be visualised through the use of fracturing techniques as they offer a more detailed perspective of the arrangement of bilayers and the different phases that are present in lipid systems especially within multilamellar vesicles. It has been recognised that an absence of lipid specific fixatives and interference associated with negative stain techniques led to the more routine use of freeze fracture for imaging of hydrated lipid systems (Talmon, 1982). Early work using freeze fracture with liposomes (Hope et al., 1989) showed preparative procedures in conjunction with the properties of liposomes formed and their morphology upon freeze fracture. A sample composed of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (7:3 molar ratio) was shown (Hope et al., 1989) in its liquid crystalline state using ultra rapid cryofixation. A rippled effect can be exhibited on the fracture face with lipids exhibiting a more gel like state of organisation when cholesterol is absent (common with phosphatidylcholines and phosphatidylglycerols). However, when DMPC liposomes are taken below their Tc, a banded fracture plane is observed and conversely a smooth fracture is found when quenched above Tc. It is also observed that a large proportion of lipids adopt hexagonal phases upon fracturing, such as unsaturated phosphatidylethanolamine where a step like ordered pattern is observed (Hope et al., 1989). Thus the use of freeze fracture can not only reveal the local structure of the bilayer, but also give an insight into the lipid phase transitions that occur with the varying nature of lipids used for preparations. However freeze fracture can result in changes in the morphology of liposomes due to the mechanical stresses encountered during specimen preparation (Egelhaaf et al., 2003).

1.2.1. Environmental scanning electron microscopy

Given the outlined disadvantages of fixing, staining or freezing of bilayer vesicle samples, imaging liposomes in their natu-

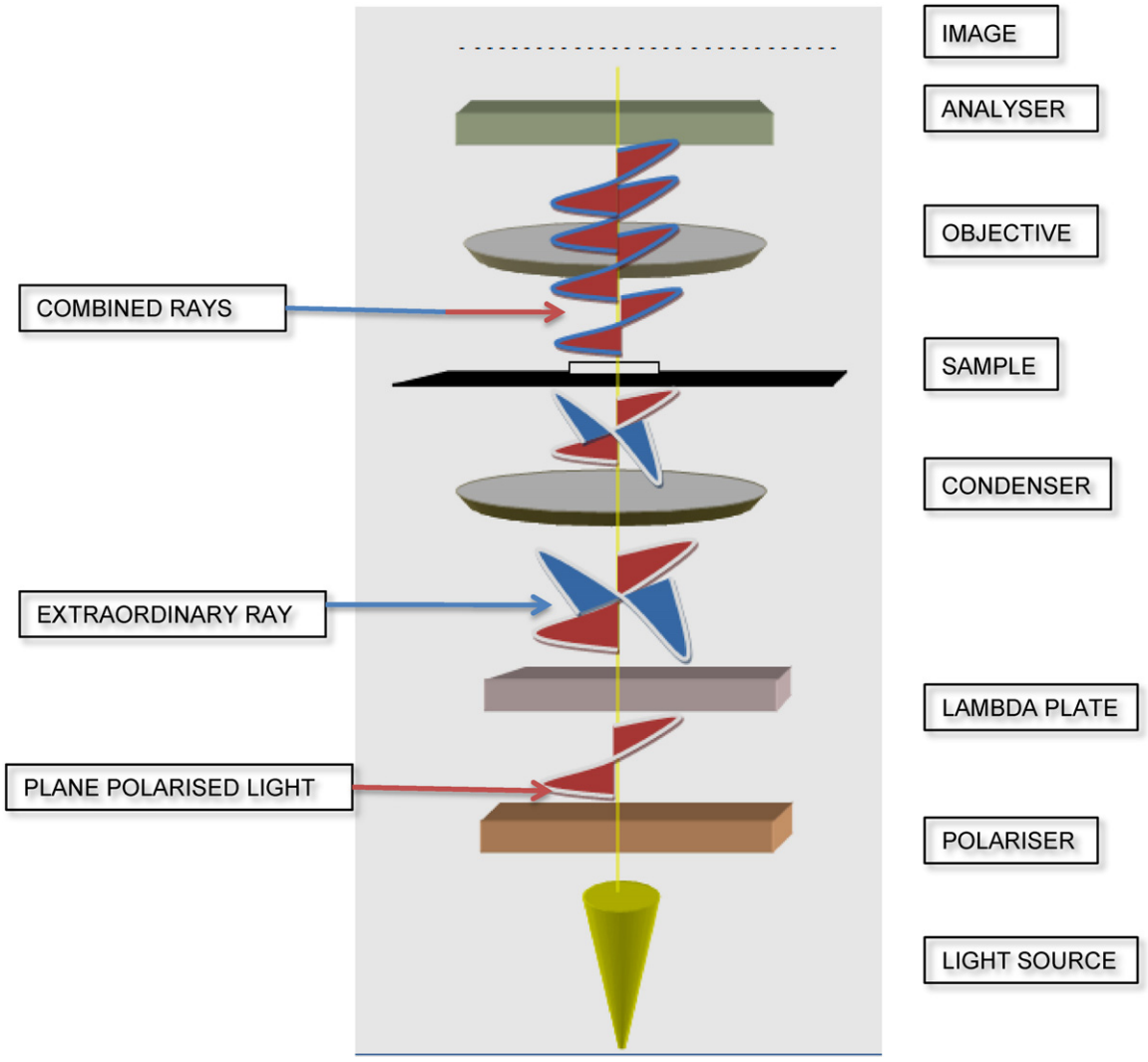


Fig. 1. Principle of polarisation microscope.

ral hydrated state is the ideal scenario for visualisation of these dynamic lipid structures. Environmental scanning electron microscopy (ESEM) is a novel imaging technique used within our group to study liposomes and niosomes. ESEM offers potential advantages to the above methods as the dynamic changes of wet systems, without prior sample preparation, can be viewed. The main distinguishing feature of ESEM compared to other techniques is the presence of vapour (usually water) in the sample chamber, made possible by a system of differential pumping zones which maintains the required high vacuum (10^{-6} Torr) for the electron gun whilst allowing for partial vacuum (10 Torr) in the sample chamber (McKinlay et al., 2004; Muscariello et al., 2005). The primary electron beam generates secondary electrons from the sample surface, which then encounter vapour molecules, which in turn become ionised and generate further secondary electrons, which in turn encounter adjacent vapour molecules, and so on, leading to a "cascade" amplification of the signal before reaching the detector (Fig. 2) (Manero et al., 2003; Muscariello et al., 2005; Robinson, 1975). Therefore due to this feature, ESEM avoids the need for prior preparation of samples and it can elucidate how different hydration environments and pressure changes can physically affect the vesicles in real time. This is highly relevant to formulation and stability studies. For example we have recently used ESEM as an alternative assay of liposome formulation and stability to dynamically follow the changes in structure of lipid films and liposome suspensions as water condenses on to or evaporates from the sample (Mohammed et al., 2004; Vangala et al., 2006). We were also able to study liposomes incorporating drugs within their bilayer and showed enhanced stability of liposomal bilayers containing 10% (mol/mol) ibuprofen compared to their drug free counterparts (Mohammed et al., 2004).

1.3. Fluorescent microscopy

Whilst commonly used for tracking of particulate delivery systems in biological environments, fluorescent microscopy can also provide valuable information regarding the structural attributes of various systems including bilayer vesicles. Fluorescence microscopy offers the potential to simultaneously add multiple probes within the vesicle formulation which are then distributed in the bilayer and provides a means for elucidating essential knowledge in regards to the dynamic structure of membranes (Bouvrais et al., 2010). Moreover, it allows assessment of more than one parameter, as probes could be placed in the internal aqueous compartment as well as the bilayer. For example, giant unilamellar vesicles (GUV) have been used for this type of study which range in size from 10 to 100 μm (Morales-Penningston et al., 2010). However, multilamellar vesicles can be large enough to also

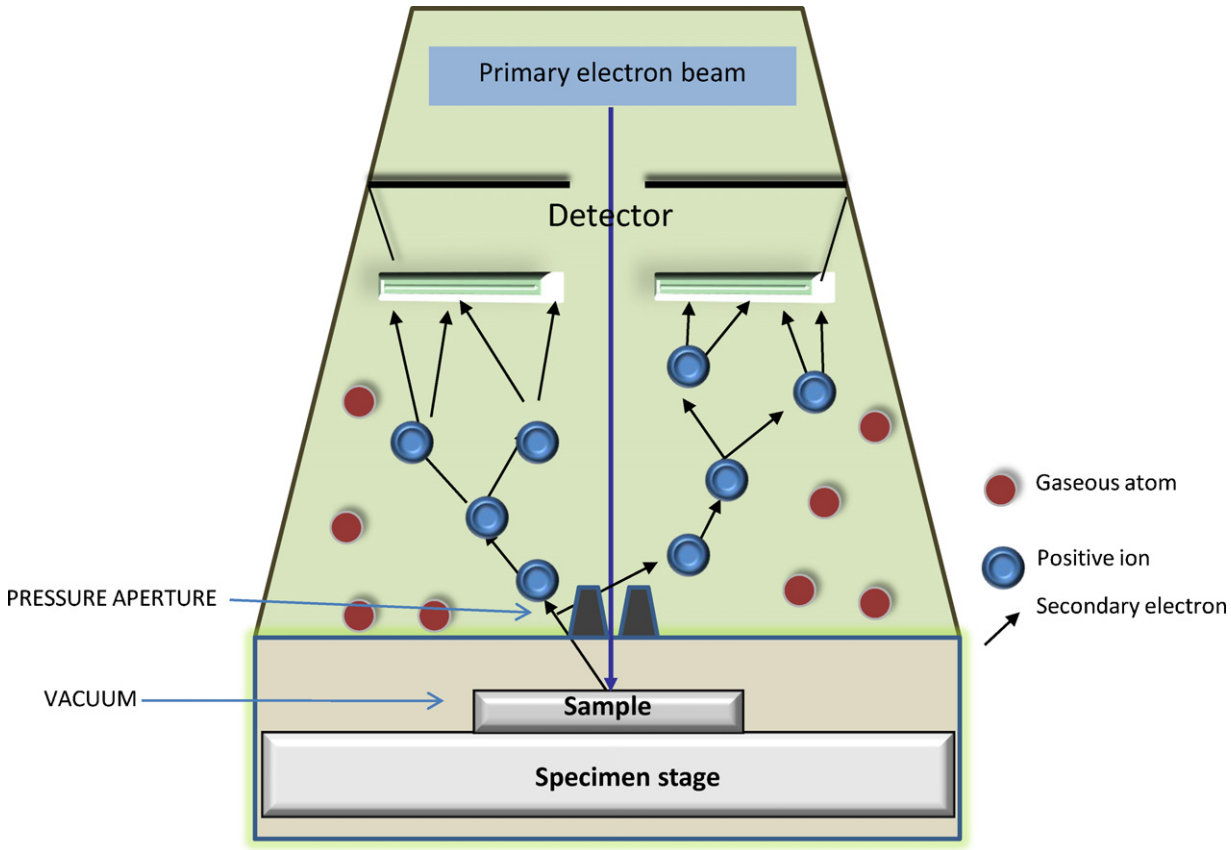


Fig. 2. Schematic representation of theory of environmental scannning electron microscopy and how an image is produced.

be considered using these techniques. Fluorescent microscopy has the same limitations in terms of magnification that conventional light microscopes have, however it offers the advantage of allowing areas of interest within the liposome constructs to become identified via the fluorescent label. The disadvantage is, of course, that the addition of probes to the membrane system may interfere with the properties of the system being investigated; however, recent studies have suggested that suitable concentrations can be used such that the effect of the probes can be negligible (Bouvrais et al., 2010). Another potential drawback of this method is that prolonged exposed to fluorescent light can result in bleaching and loss of fluorescence intensity.

The more recent advancement in this area has come with the development of the confocal scanning microscopy. This design has allowed much of the out of focus fluorescent light to be filtered out through the use of a pinhole, allowing images from a smaller depth of field to be taken. With confocal microscopy a z-stack of vesicles can be acquired. This means that a series of images is taken, beginning at the top of the vesicle(s), even slightly out of focus; then images are taken in defined intervals of focus. By taking numerous images 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. This has been shown recently (Vequi-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 (DOPG:Esm:Chol) were observed using 3D projections and the fluorescent dye $DilC_{18}$. The aim of their work was to locate regions of ordered and disordered phases. Through microscopy the formation of lipid rafts and how these are reduced when charged lipids are used was shown (Vequi-Suplicy et al., 2010). In contrast to freeze fracture, with confocal microscopy samples are viewed in a hydrated state albeit with the addition of fluorescent labels. Both fluorescent microscopy and confocal require the same preparation as for light microscope work, however greater care and speed needs to be considered during the actual viewing of the sample to avoid possible bleaching and more time is required for setting of the parameters and focussing the sample.

Overall given the choice of options available for imaging liposomes, the above advantages and disadvantages for all types of microscopy need to be carefully balanced against effects they may have upon the sample, as ultimately the final image obtained may have been affected through the process. Within this paper we outline a range of techniques available for quick validation purposes through to detailed morphology and stability studies.

2. Materials and methods

2.1. Lipids

Phosphatidylcholines used were: egg phosphatidylcholine (PC) (grade I), (Lipid Products, Epsom, Surrey, UK), dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) (all from Avanti lipids, Alabaster, AL, USA). Cationic lipids used were 3β-[N-(N′,N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) (Sigma–Aldrich, Poole, Dorset, UK) and dimethyldioctadecylammonium (DDA) (Avanti Polar Lipids, Alabaster, USA). 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE), and α,α′-trehalose 6,6′-dibehenate (TDB) and rhodamine-labelled 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lisamine rhodamine B Sulfonyl) (Rho-DPPE) were also from Avanti Lipids

(Alabaster, AL, USA). 1-Monopalmitoyl glycerol (C16:0) (MP) and cholesterol were from Sigma–Aldrich Company Ltd., Poole, UK.

2.1.1. Materials

Poly-L-lysine 0.1%, Oil Red O (ethylenedinitrilo)tetraacetic acid (EDTA), bovine serum albumin fluoroisothiocyanate (BSA-FITC) and ovalbumin fluoroisothiocyanate (OVA-FITC) were purchased from Sigma (Sigma–Aldrich Company Ltd., Poole, UK). Microslides 0.3 mm × 3 mm × 50 mm were from Cam Lab (Cambridge, UK) and frame seal chambers from Bio-Rad (Hertfordshire, UK). Microscope slides used were ground edges plain glass, coverslip no. 1.5 Borosilicate Glass 25 mm × 50 mm with thickness of 0.16–0.19 mm (Fischer Scientific Loughborough, UK).

2.2. Liposome and niosome preparation

The simplest way to produce liposomes remains the method based on that first outlined by Bangham et al. (1965). Briefly the lipid components are dissolved in a 9:1 solvent mixture of chloroform and methanol at the desired concentrations and placed in a round-bottom spherical Quick-fit flask. If a drug is to be incorporated within the bilayer, the required amount of drug is also added at this stage. The solvent is then evaporated on a rotary evaporator to obtain a dry film which is then flushed with oxygen-free nitrogen in order to ensure complete removal of all solvent traces. The film is hydrated by addition of 2 ml of ddH_2O or appropriate buffer and agitated vigorously until the thin lipid film is completely dissolved and transformed into a milky suspension. The hydration of the lipid film should be maintained above the gel-liquid crystal transition temperature (Tc) of the phospholipid (>Tc). For example, PC has a Tc of 0 °C and DMPC has a Tc of 23 °C, however, DSPC and DDA have a Tc above 55 °C and 47 °C, respectively, therefore the water added to hydrate the lipid film should be pre-warmed to a temperature above the Tc and the liposome solution should be maintained at this temperature during liposome formation. For the entrapment of hydrophilic drugs, a drug solution should be used to hydrate the dry film, however, this technique generally results in low hydrophilic drug entrapment. Where liposomes were prepared with Oil Red O, a 0.5% stock solution of Oil Red O was prepared using isopropanol and added to the round bottom flask with the appropriate lipids. This dry film was the rehydrated in 2 ml of ddH_2O.

2.3. Preparing samples for preliminary visualisation via light microscopy

An upright Zeiss Axiovert A1 microscope is used. All images were viewed using a X40 objective and Axiovision software was employed for image capture. A simple glass slide can be used and 20–30 μl of sample is sufficient. The coverslip is then placed on top of the sample.

2.3.1. Pre-coating microslides

A microslide holds a 50 μl volume. A small tube is attached to one side, to the other the pipette tip can be attached and the microslide can be placed in the sample preparation and then drawn up. At this point it is important to try and ensure that no air bubbles are present. To help visualise vesicles without the aqueous medium in which they are dispersed, coating of the microslide can be applied. However, consideration needs to be given to the type of coating in conjunction with nature of vesicles. For example, for a cationic coating then 0.1% poly-L-lysine can be used and this would allow a greater interaction with anionic vesicles; where it is more suitable to use an anionic coating, EDTA may be used. To coat the microslides, the appropriate coating solution is taken up into the microslide by attaching small, thin walled tubing to one end and then pipetted up avoiding air bubbles to ensure a smooth coating. This can then be ejected, and then the liposome preparation can be taken up. This should be allowed to remain in the microslide for at least 30 min to 1 h at room temperature. Then using a piece of tissue paper the liquid can be removed by inverting the microslide vertically and holding down onto the tissue paper until all the excess liquid is removed, whilst the liposomes remain on the microslide for microscopic analysis.

2.3.2. Frame seal chamber

Basically with these systems, each frame is sandwiched between two plastic liners. One side has a hole where there is a square, this is the upper side. The plastic liner at the bottom of the frame should be removed to allow the chamber to adhere onto the glass slide when pressed down firmly. The top cover can then be removed and an appropriate volume can be aliquoted into the chamber. The polyester cover can be placed across carefully then rubbed down if the effects of temperature are required for visualisation otherwise a glass coverslip can be placed across the top.

2.3.3. Polarisation

A Zeiss Axioscope A1 was fitted with a polarizer fixed with a rotatable lambda plate and with a removable analyser. The images are obtained using bright-field with either the monochrome or colour camera as appropriate. Images were taken using a X40 objective. Axiovision software was used for image capture.

2.4. Transmission electron microscopy

Morphological analysis was carried out by TEM using a JEOL 1200EX TEM fitted with a LaB6 filament, with an operating voltage from 40 to 120 kV. A small drop of sample was placed on a polymer filmed copper grid and allowed to stand for 2 min. The excess sample was removed using filter paper, followed by the addition of 10 μl of uranyl acetate. The grid was then allowed to stand for another 2 min, washed in distilled water and air dried, forming a thin film, which was viewed at 70 kV. Non-ionic based vesicles containing monopalmityol glycerol, cholesterol and dicetylphosphate were used.

2.5. Cryo-electron microscopy

Cryo-electron microscopy involves forming a thin aqueous film on a bare specimen grid (3–4 mm thick, with a fine 700 mesh honeycomb pattern of bars) by dipping the grid into the liposome suspension. After blotting the suspension-coated grid on filter paper, the thin film produced was rapidly (1 s) vitrified by plunging the grid into ethane and cooled to its melting point with liquid nitrogen. Preparation and blotting of thin films was carried out in a controlled environment using a fully automated system (PC-controlled, up to vitrification). The vitrified film was mounted in a cryo-holder (Gatan 626) and observed at 170 °C in a transmission microscope (Philips CM12) operating at 120 kV. Micrographs were taken using low-dose conditions.

2.6. Freeze–fracture microscopy

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) cooled by liquid nitrogen. Fracture was performed on a Balzers BAF 400D apparatus at a temperature of −110 °C. Replicas were floated free on distilled water and cleaned in 40% chromic acid. Images were viewed using a transmission microscope.

2.7. Environmental SEM imaging of liposomes

The ESEM sample holder was loaded with liposome formulation and examined under saturated water vapour conditions. Gradual reduction of pressure from (5.00 Torr to ~1 Torr) in the sample chamber resulted in controlled dehydration of the sample environment. The effect of the hydration medium on the stability of drug-free liposomes under controlled dehydration conditions was investigated using distilled water. In our studies ESEM analysis was performed using a Philips XL30 ESEM-FEG (Philips Electron Optics (FEI), Eindhoven).

2.8. Fluorescence microscopy

Liposomes composed of dimethyldioctadecyl ammonium (DDA) and trehalose dibehenate (TDB) (5:1, w/w) were produced using the lipid-film hydration method with the inclusion of a tracer amount (1 mol%) of rhodamine-labelled 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lisamine rhodamine B Sulfonyl) (Rho-DPPE). The lipid-film was hydrated with Tris buffer (10 mM, pH 7.4), followed by addition of FITC-labelled ovalbumin (OVA). Liposomes with OVA were viewed on glass coverslips at 100× magnification using phase-contrast and fluorescence filters.

2.9. Confocal microscopy

Two liposome preparations were made of 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol (8:8 μmol) using the lipid film hydration method. For one preparation the dry film was rehydrated with 2 ml of a 5 μg/ml of BSA-FITC solution. The sample was prewarmed above the Tc of DPPC (41 °C) before it was added. It was then vortexed and kept above the Tc for 30 min to allow the formation of multilamellar vesicles. The second sample was rehydrated with a solution of rhodamine (5 μg/ml) and prepared similarly. Once the vesicles were formed the sample preparations were centrifuged to remove unencapsulated material and resuspended in 2 ml of PBS. 100 μl of each preparation was taken and vortexed together and approximately 25 μl was placed into a frame seal chamber and sealed with a coverslip. Samples were viewed under a X63 oil objective using a Zeiss multiphoton confocal scanning microscope. A laser source for FITC at 470 nm and for Rhodamine 510 nm was used.

3. Results and discussion

3.1. Primary screen of liposomes

The simplest and quickest way to visualise prepared samples which are dispersed in an aqueous medium in the laboratory is to use basic light microscopy, however there are a range of options that can be used to initially screen bilayer vesicles (Fig. 3). For example, using a standard glass slide/cover slip can give good visualisation of large vesicles such as gas-filled liposomes (Fig. 3a) and mulitlamellar vesicles (Fig. 3b). The heterogeneous size range of both formulations can be easily demonstrated using this method, and this can help conform particle size analysis data obtained using other methods.

Coating of the microslides to promote liposome 'capture' can also be undertaken. For example, coating the microslide with poly-L-lysine helps to increase the electrostatic interactions with anionic liposomes which come into contact with the bottom of the slide and when the liquid is gently removed a sparse, hydrated layer of liposomes attached to the surface can be obtained. The image shown in (Fig. 3c) is a liposome preparation of phosphatidylcholine and cholesterol 8:8 μmol that has been taken up and left for between 30 min to 1 h within a poly-L-lysine coated microslide. By adding a dye such as Oil Red O, the density of liposomes can be increased and thus when the liquid is removed an even layer of liposomes remains (Fig. 3d). Anionic coatings, such as EDTA, can also be applied which can be used for cationic preparations such as DDA liposomes (Fig. 3e) where similarly once the liquid is removed, transiently attached liposomes remain (Fig. 3f). This method can be further applied to investigate how cells and liposome might interact. For example, immune cells such as lymphocytes or neutrophils can be added to visualise how they would interact with the adhered liposomes. Alternatively, the microslide offers an ideal surface to grow certain cells such as endothelial cells (Cook et al., 1993), and subsequently a liposome preparation could be added allowing visualisation of cell–liposome interactions.

With frame seal chambers, a defined volume can be placed into a sealed area, offering an improved method for visualisation than using just a coverslip. They come with a provided polystyrene cover (Fig. 3g) although, if preferred, a coverslip can be placed on for greater clarity (Fig. 3h). Using this set up, the potential for visualising liposomes as they are heated to observe changes in morphology is perhaps the most interesting, especially as heated stages are available to enable this. Therefore, in general, whilst detailed morphological information from ordinary light microscopy may be limited, there are a range of simple and cost-effective methods which can be adopted to give preliminary screening of formulations to assess their general morphology, size range and heterogeneity.

3.2. Morphological observations made using polarized light

The light microscope can easily be upgraded to offer polarisation images; either as monochrome or in colour (Fig. 4a and b, respectively). This offers an alternative method for visualising liposomes to confirm the formation of vesicles. Theoretically lipid vesicles are anisotropic due to the ordered molecular arrangement of the bilayers and when polarised light is forced to interact with the layers in the lamellar phase between cross polarisers, the maltese cross should be visible (Fig. 4a and c). When further interference is introduced to the polarised light with the introduction of the rotatable lambda plate, due to its birefringent nature, vivid colours are created as seen (Fig. 4b). In a larger heterogenous sample of niosomes (Fig. 4c), not all of the vesicles exhibit the maltese cross; on closer analysis it can be seen that it is the larger vesicles which do not, this maybe because they have single unilamellar bilayers. However it is difficult to make conclusive observations regarding the bilayer characteristics solely from polarisation images.

3.3. Studies using transmission electron microscopy

For enhanced detail and morphological investigations higher resolution imaging is required. In such instances electron microscopy can be used. Examples of TEM images of bilayer vesicles are shown (Fig. 5). Again a heterogeneous population of vesicles can be seen with sizes ranging from less than 100 nm to over 1 μm (Fig. 5a and b). In these studies, TEM was used to visualise the effects of freeze-drying on vesicles, with image analysis of vesicles prior to freeze-drying (Fig. 5a) and those subjected to freeze-drying and rehydration (Fig. 5b). The images can be used to support the effectiveness of the freeze drying protocol, as they show that upon rehydration morphologically intact vesicles have formed similar to those prior to freeze-drying, but obviously additional techniques are required to confirm other characteristics such as drug retention, etc. Due to the heterogeneity of the sample used, the resolution of the light microscope would not be sufficient to see the size range of such vesicles with this level of clarity.

The final image taken using TEM (Fig. 5c) has been included to demonstrate the artefacts of staining. In agreement with previous

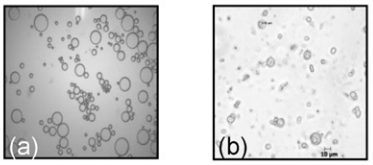
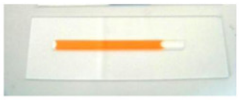
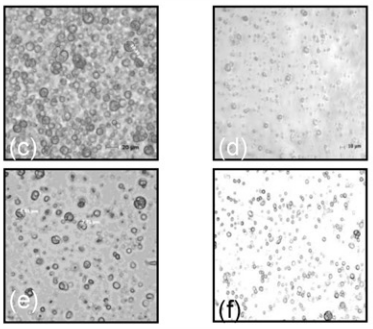
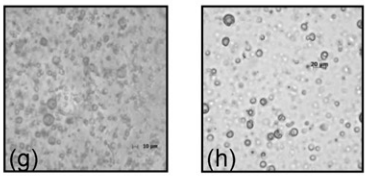
Method	Advantages	Disadvantages	Examples
Standard glass slide with cover slip 	Simple and quick to prepare. Inexpensive. Immediate visualisation. Good resolution as sample is visualised through a coverslip depth of 0.16 mm to 0.19 mm.	Minimal depth and sample spreading. Reducing number of liposomes per field of view. Drying out of sample relatively quickly	
Micro-slide 	Depth of wet sample allows visualisation of heterogeneity of sample. Drying out of sample reduced as edges can be sealed. Possible to pre-coat microslide E.g. with Poly-l-Lysine or EDTA. Opportunity to grow cells on the microslides.	O.3 mm depth thus resolution may not be as sharp as with a cover slip. At least 50 μl of sample required to fill micro-slide. Slightly more time consuming if micro-slide is coated. Slightly more expensive.	
Frame Seal Chamber 	A defined volume (e.g. 25 μl) is placed into the frame. A cover slip can be placed over the top of the frame for enhanced resolution. The sample can be sealed reducing dehydration of sample. Ease of preparation. Frame seal chamber can withstand heat up to 100 °C.	Most expensive although still reasonable. Frame seal polystyrene cover provided does not offer the same resolution as a cover slip.	

Fig. 3. Summary of methods used in the laboratory for visualisation of lipid vesicles and the advantages and disadvantages of each with images taken on the light microscope using Zeiss Axioscope A1 under X40 objective. All liposomes have Oil Red O encorporated. A glass slide with coverslip is used, images are shown of: (a) gas microbubbles, (b) liposomes of PC:CHOL 8:8 μmol. A microslide is used and placed on a glass slide images are shown of: (c) a poly-l-lysine coated microslide: PC:CHOL 8:8 μmol liposome preparation has been taken up and left for approximately 30 min. (d) The liquid is removed and a layer of liposomes remains. (e) Anionic coated microslide with 0.1% EDTA coated for approximately 30 min: cationic DDA liposomes used. (f) The liquid is removed and transiently attached liposomes remain. Frame seal chambers are used on a glass slide, images are shown of: (g) DPPC:CHOL 8:8 μmol viewed under a polystyrene cover (h) DPPC:CHOL 8:8 μmol viewed under a glass coverslip.

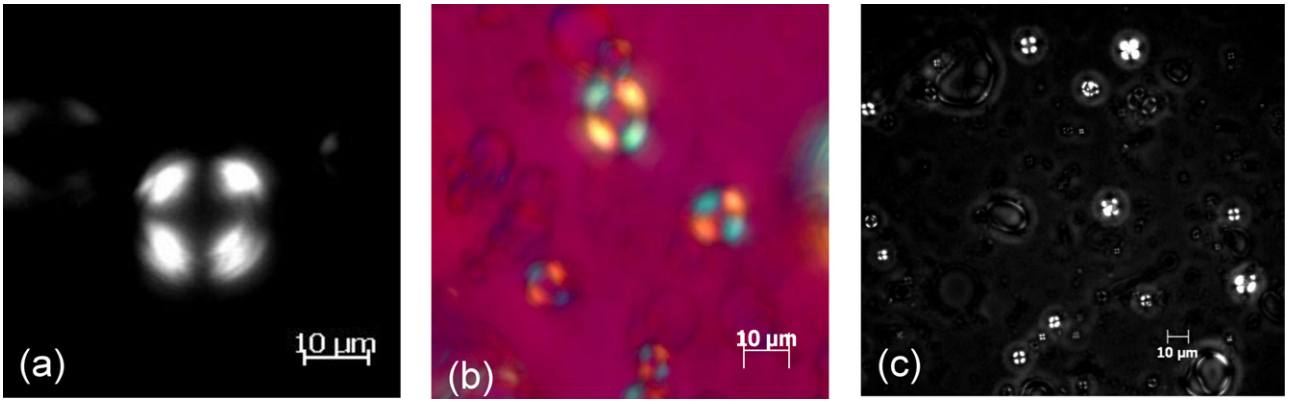


Fig. 4. Polarized light microscopy. These are images obtained using a Zeiss Axioscope A1 under a X40 objective using brightfield with a polarizer. (a) The image is of DSPC:Chol liposomes 8:8 μmol viewed under a frame seal chamber with coverslip. A monochrome camera was used. (b) The lambda plate is used to obtain this image of DPPC:CHOL 8:8 μmol viewed in a microslide and a colour camera used. (c) Heterogenous sample of multilamellar niosomes viewed using a coverslip image taken with a monochrome camera. Axiovision 4.3 Software is used.

studies (Talmon, 1982), dark and light shades can be seen as a result of remnants of the dye used. The vesicle in terms of morphology seems dehydrated in comparison to (Fig. 5a and b). It is advisable that two separate techniques should be used in order to obtain a more reliable picture (Talmon, 1982). This highlights the need for the user to critically assess the quality of images obtained and subsequently use complementary microscopic techniques to consolidate information about the vesicles. It also clearly highlights the damage that can be caused to samples simply through the process.

3.4. Getting inside vesicles

The above described methods can all be used to visualise vesicles, however they do not allow us to look inside the vesicle and

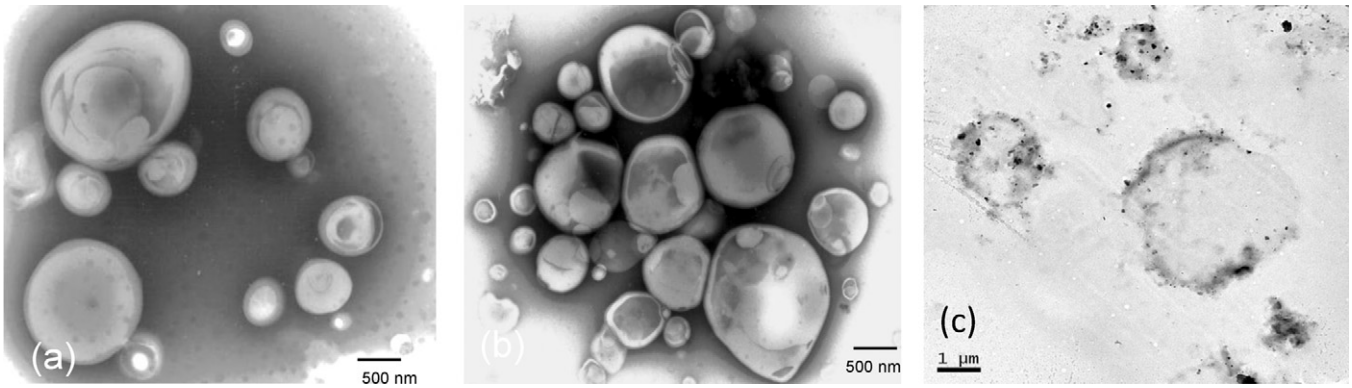


Fig. 5. Transmission electron microscope images of niosomes (a) pre-freeze drying, (b) post-freeze drying, (c) vesicles which have been damaged during TEM exhibiting artefacts of negative stain.

investigate the lamellae in terms of their construction and organisational detail for this we must look to (or with) other methods.

3.4.1. Cryo-electron microscopy

Similarly to TEM, cryo-electron microscopy allows direct visualisation of the sample using electron microscopy. The main difference is that the liquid samples are frozen rapidly into the form of a thin vitrified film. Fig. 6a–c shows vesicles visualised using cryo-EM. Vesicles are shown with multiple bilayers (Fig. 6a) that are in close contact with each other images. Fig. 6b and c shows mixed populations of largely unilamellar vesicles from which, the number of bilayers as well as the size of vesicles and other morphological characteristics can be easily determined.

The use of cryo-electron microscopy has been reported to be especially beneficial where proteins or DNA have been encapsulated and Alfredsson (2005) reviews the use of cryo-TEM for studies involving DNA and lipid structures (lipoplexes): examples are given of LUVs containing a cationic lipid and a poly(ethylene)glycol coating are used to entrap antisense oligonucleotides. The cryo-electron micrographs show that in the absence of oligonucleotide, unilamellar liposomes are formed yet when oligonucleotides are present the membranes are forced into close contact. By increasing the ratio of lipids and oligonucleotides, the amount of multilamellar liposomes is also increased, which is elucidated from the micrographs (Alfredsson, 2005).

Studies on lipoplexes utilising cryo-electron microscopy in conjunction with small angle X-ray scattering has been carried out (Lasic et al., 1997). Lipoplexes were prepared by rapid mixing of DNA with liposome solutions. From the micrographs, particles in the size range of 0.2–0.5 μm were observed and shapes varied from stacks of flat bilayers to concentric and amorphous aggregates. Using the two techniques of cryo-TEM and X-ray scattering, it was shown that the structure of these complexes can be estimated and DNA is adsorbed as single parallel helices (Lasic et al., 1997). Similar studies carried out by Huebener et al. (1999) have also shown micrograph images of DNA-coated unilamellar vesicles which are adsorbed to each other. These vesicles were deformed at the regions of contact and the thickness of the entire band was calculated at 13.5 nm, which is sufficient for two bilayers at 5.1 nm with a monolayer of DNA at 2 nm. Thus cryo-electron microscopy has been used as a fundamental technique in the understanding of the nature of liposome and DNA complexes, and size and morphological characterisation can be elucidated as well as bilayer thickness, even estimations of the nature of the material adsorbed between them.

The effects of sonication and freeze-thawing on aggregate size on aqueous DPPC dispersions has also been shown using Cryo-TEM (Kim et al., 2007). Their images demonstrated that after a freeze thaw cycle there were aggregates with an approximate size range of between 30 and 300 nm. Morphologically it is observed that two types of vesicles were visible; the more 'frozen' looking vesicles whilst others are 'curled' around themselves. Flat membranes were also observed most likely from broken vesicles. After a second cycle of sonication, the samples were again visualised using cryo-TEM whereby vesicle shape seems to be restored. Here, cryo-TEM was used to determine the effectiveness of a process used regularly in liposome preparation.

Whilst there are many advantages of this method, cryo-TEM is not without drawbacks, the most significant of these is that a two dimensional image is obtained of three dimensional objects. The low electron doses used in cryo-TEM also means the images are not as clear as with other methods. Other limitations include the formation of amorphous ice which could result in constraints on the object as well as the use of blotting which too could result in artefacts similarly to TEM.

3.4.2. Freeze–fracture microscopy

To address the above problems regarding obtaining information on the 3D nature of vesicles, freeze–fracture can be used. In this method samples are cryo-fixed however vesicles are then fractured; this is normally preferentially across the hydrophobic domains thus surface structure of the membrane interior is exposed.

Although one of the earliest techniques for visualisation of vesicle structure, freeze–fracture remains a key tool for investigations of bilayer organisation. For example, investigations of a multilamellar liposomes using freeze–fracture are able to show in detail the multilamellar construction of the systems in terms of the tight bilayer packing (e.g. Fig. 6d and e). We are also able to investigate the nature of lipid bilayers using this technique: in Fig. 6f, smaller unilamellar constructs can be seen with distinct 'rippling' on the lamellar surface. The rippled effect is the most common bilayer deformation (Meyer and Richter, 2001). It reveals some disorder in the transitions of the acyl chains prior to freezing. One interpretation for this could be that ripples are formed where the molecules have a different tilt direction to the way in which the rest are packed. Incubation of the vesicle between pre-transition and actual transition temperature can also induce ripples. In contrast the liposomes in Fig. 6d and e show a crystalline arrangement of the numerous bilayers.

In their studies to assess the effects of sonication and freeze thawing on aggregate size of DPPC dispersions, Kim et al. (2007) also used freeze–fracture microscopy, as well as cryo-TEM, to look at DPPC liposomes quenched above and below their TC. A smooth fracture with some symmetrical perturbations is seen with the liposomes in the gel phase, however, the fluid rippled effect is seen

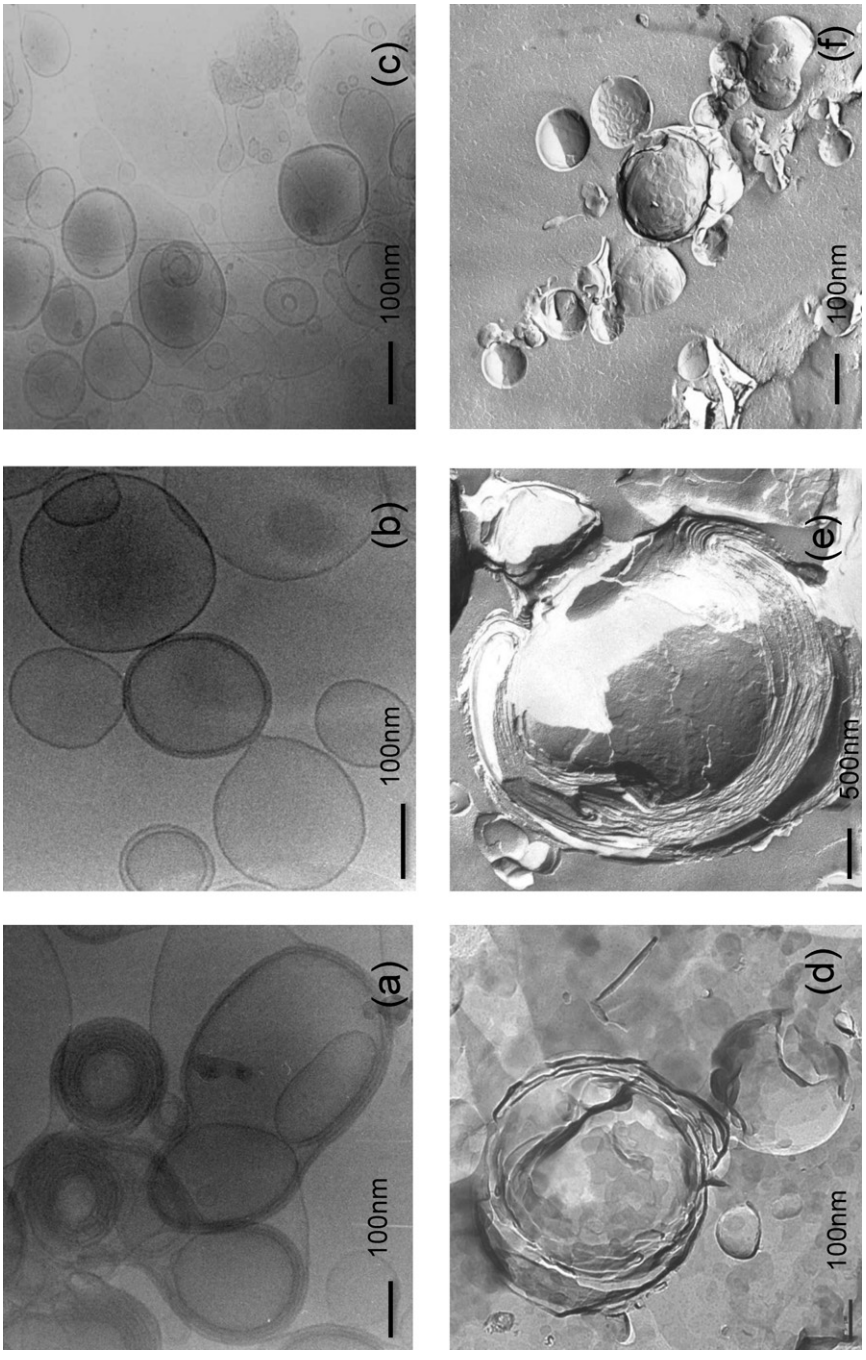


Fig. 6. Cryo-electron microscope images of (a) multilamellar and (b and c) unilamellar liposomes. Freeze fracture image of (d) multilamellar niosome, (e) mulitlamellar liposomes and (f) SUV liposomes.

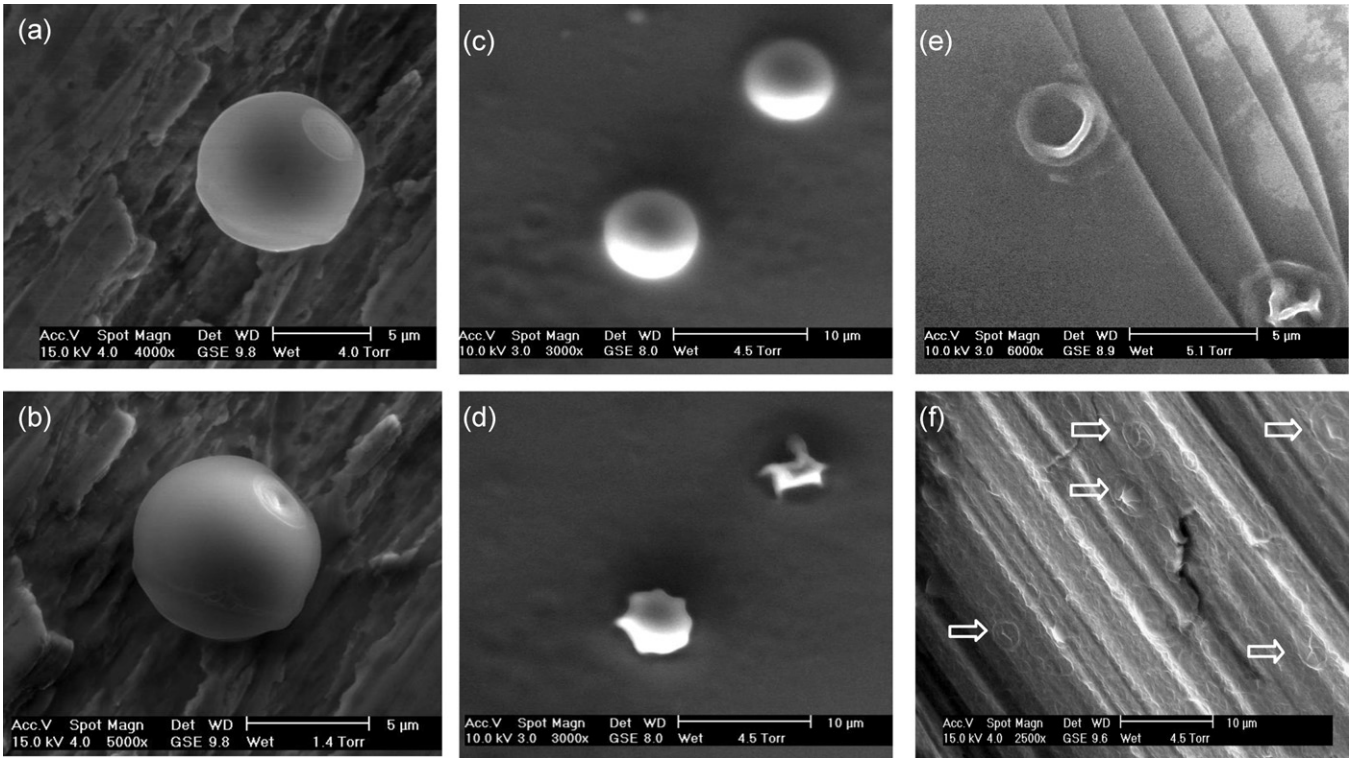


Fig. 7. Environmental scanning electron microscope images. (a and b) Propofol-loaded liposomes formulated from POPC and cholesterol (89:11, mol%). (c and d) Cationic liposomes composed of DDA:TDB (89:11, mol%). (e and f) Liposomes formulated using 1,2-distearoyl-3-trimethylammonium propane and cholesterol (89:11, mol%).

with the liposomes quenched above their Tc (at 55 °C). The ripples observed are like 'parallel stripes' as described by Meyer and Richter (2001). The presence of different lipids with different phase transitions are more likely to form separate domains and these can be best shown through the appearance of ripples, changes in curvature and tilting (Meyer and Richter, 2001).

Freeze–fracture investigations have also been used to examine structures formed in the interaction of cationic liposomes with plasmid DNA (Sternberg et al., 1994). In this study structural modifications were observed as a result of varying DNA concentration and incubation time. The freeze fracture micrographs of lipoplexes with a short incubation time or low DNA concentration show a few semi fused liposomes whilst those incubated for longer periods showed 'spaghetti like' structures thought to be DNA with a lipid coating.

In general, freeze fracture offers a greater resolution in image, as higher doses of electrons can be used compared to other methods. In addition bilayer characteristics can be investigated in detail, with the effects of temperature on phase transitions being shown using this method (Meyer and Richter, 2001; Hope et al., 1989). Similarly to cryo-TEM the sample is frozen, however it is a replica of the fractured surface that is examined, as the fracture splits the membrane along the bilayer to reveal the inner aspects of the separate leaflets of the bilayer in face view. Unlike cryo-TEM, the image is not 2D and more of the different aspects of the vesicle structure can be visualised.

3.5. Environmental scanning electron microscopy

Environmental scanning Electron Microscopy allows the visualisation of dynamic changes that occur to vesicles as physical environmental parameters are changed. For example, images are shown in Fig. 7 which reveal the different effects pressure reduction may have on liposomal systems. Fig. 7a and b shows propofol-loaded liposomes formulated from POPC and cholesterol (89:11, mol%). At a pressure of 4 Torr a defect in the outer membrane of these multilamellar vesicles can be seen and as we reduce the pressure to 1.4 Torr, the external lipid bilayers appear to be removed to gradually reveal the inner bilayers of the vesicles (Fig. 7b). In the case of Fig. 7c and d, these are cationic liposomes composed of DDA:TDB (89:11, mol%) and unlike the POPC:Chol liposomes, these vesicles, although initially maintaining a spherical shape (Fig. 7c), begin to shrink even when maintained under the sample pressures (Fig. 7d). With some formulations, the vesicles are not stable enough to be effectives visualised using this method as can be seen in Fig. 8e and f. These liposomes were formulated using the cationic lipid 1,2-distearoyl-3-trimethylammonium propane and cholesterol (89:11, mol%), and when examined using ESEM were shown to burst or collapse instantly. These studies show, in real time, the large differences in bilayer stability various liposome formulations offer and the dynamic nature of these images cannot be achieved with techniques where the preparations are frozen.

3.6. Fluorescent and confocal microscopy

Fluorescence microscopy allows fluorescent probes to be directly attached to regions of interest, albeit the internal protein or the bilayer and subsequently allows the visualisation of these specific molecules that fluoresce in the presence of excitatory light. It is widely used in biophysics to reveal important information about the structure and dynamics of membranes. Especially with systems such as giant unilamellar vesicles, where information can be obtained regarding membrane shape, size, fluidity and the presence of domains (Bouvrais et al., 2010).

A typical example of the application of this method is shown (Fig. 8a–c) where liposomes have been dual-labelled, so that both liposomal bilayers and surface adsorbed protein can be visualised simultaneously. The rhodamine-labelled lipid DPPE (rho-DPPE) was incorporated into the lipid bilayer thereby allowing for the

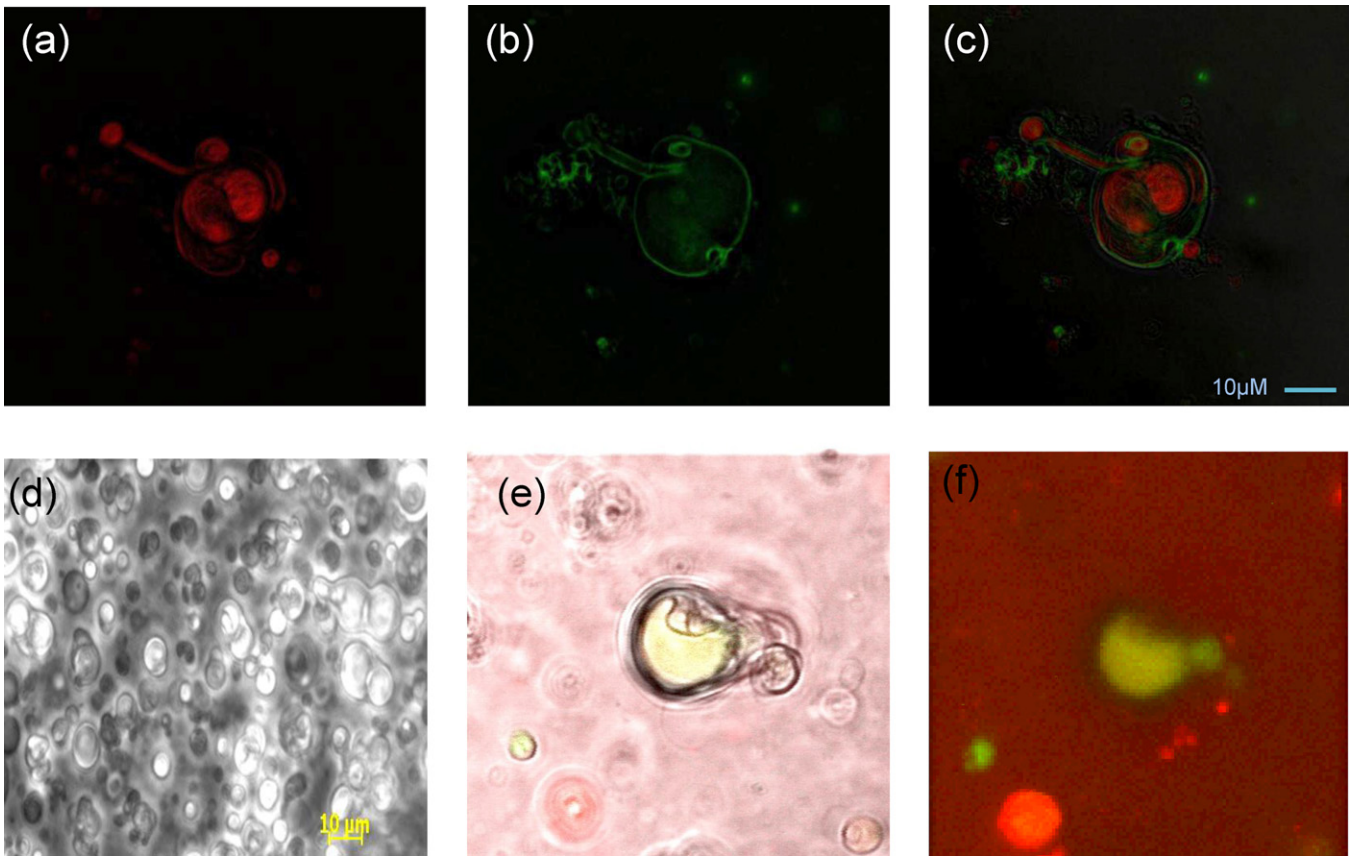


Fig. 8. Fluorescent images showing the multilamellar structure of a large cationic liposome composed of dimethyldioctadecyl ammonium (DDA) and trehalose dibehenate (TDB), with the inclusion of a tracer amount of rhodamine-labelled DPPE incorporated into the lipid bilayer (a). FITC-labelled ovalbumin was added to the liposomes post-formation resulting in surface adsorption mediated by electrostatic interactions (b). A composite image of the liposome and ovalbumin (c) and (d) are DPPC:CHOL liposomes viewed under a X40 objective under phase contrast using Zeiss Axioscope A1. (e and f) Obtained using a Muliphoton Confocal Microscope. Zeiss Axiovert. DPPC:CHOL liposomes were viewed under a X63 Oil objective using a Multiphoton Laser Scanning Microscope.

multilamellar or 'onion-skin' structure of DDA:TDB liposomes to be viewed (Fig. 8a). Addition of FITC-labelled OVA (Fig. 8b) to DDA:TDB:rho-DPPE liposomes (post formation) resulted in surface adsorption of the OVA, due to opposing electrostatic interactions. Fig. 8c shows a composite image of liposomes (red) and protein (green), therefore giving an indication as to the relationship between both components (For interpretation of the references to color in this sentence, the reader is referred to the web version of the article.).

To accommodate fluorescence imaging, light microscopes can be upgraded; however confocal laser scanning microscopy has become more attractive due to the greater clarity of the images obtained. Resolution of a fluorescent image can be enhanced using confocal microscopy as a lot of the out of focus fluorescent light can be reduced. An example of image analysis of liposomes using confocal microscopy is shown in Fig. 8d–f. Multilamellar vesicle of DPPC:Chol were initially prepared entrapping BSA-FITC. A phase contrast image of the vesicles, shows the formation of a heterogeneous suspension of multilamellar vesicles is shown in Fig. 8d. During confocal scanning microscopy a Z-stack was carried out, a 2D X-Z image is shown in Fig. 8e. The image shows a large MLV predominately encapsulating the fluorescent marker but there are also smaller vesicles present. All of the images from the z-stack are then reconfigured to provide a three dimensional image (Fig. 8f). As can be seen, the inner compartment of the vesicle is not a perfect sphere, the upper area of the image shows how the lipid bilayers have taken up some of the area of the internal compartment and there is a small area which could be another fused smaller liposomes or even a part of the same structure on the right hand side. The three dimensional image shows the distribution of the marker within the vesicle.

Interactions between cells and liposomes can also be effectively investigated using confocal microscopy. For example, in a study to assess the efficiency of gene delivery of a novel cationic liposome into cultured human cells, both fluorescence and confocal microscopy were used (Serikawa et al., 2000). DNA plasmids expressing green fluorescent protein and delivered by the cationic liposomes (composed of DC-6Î14, O,O P-ditetradecanoyl-N-(K-trimethylammonioacetyl) diethanolamine chloride, dioleoylphosphatidylethanolamine and cholesterol). Thus, after incubation cells were visualised under a fluorescence microscope without fixation and green coloured cells were enumerated in culture. In this instance the transfection efficiency was visualised by the presence of the GFP protein within the cells. Confocal laser scanning microscopy was also used by labelling the plasmid DNA with ethidium monozide. Transfected cells were fixed immediately after transfection and also at subsequent fixed time points. It was shown that the percentage of cells containing the labelled plasmid increased for both non tumourigenic and tumourigenic cells, reaching 80% after 8–12 h transfection. The presence of fluorescent probes within cells can be visualised using this microscopy technique and then data can be validated using flow cytometry, as it was done in this study. This offers a unique opportunity to assess effectiveness of cationic liposomes delivery systems, especially those used for transfection purposes. Various similar studies which have utilised these techniques have been reviewed by Torchilin (2005), which highlight their ability to provide information in regards to interaction between nanoparticulate delivery systems and cells.

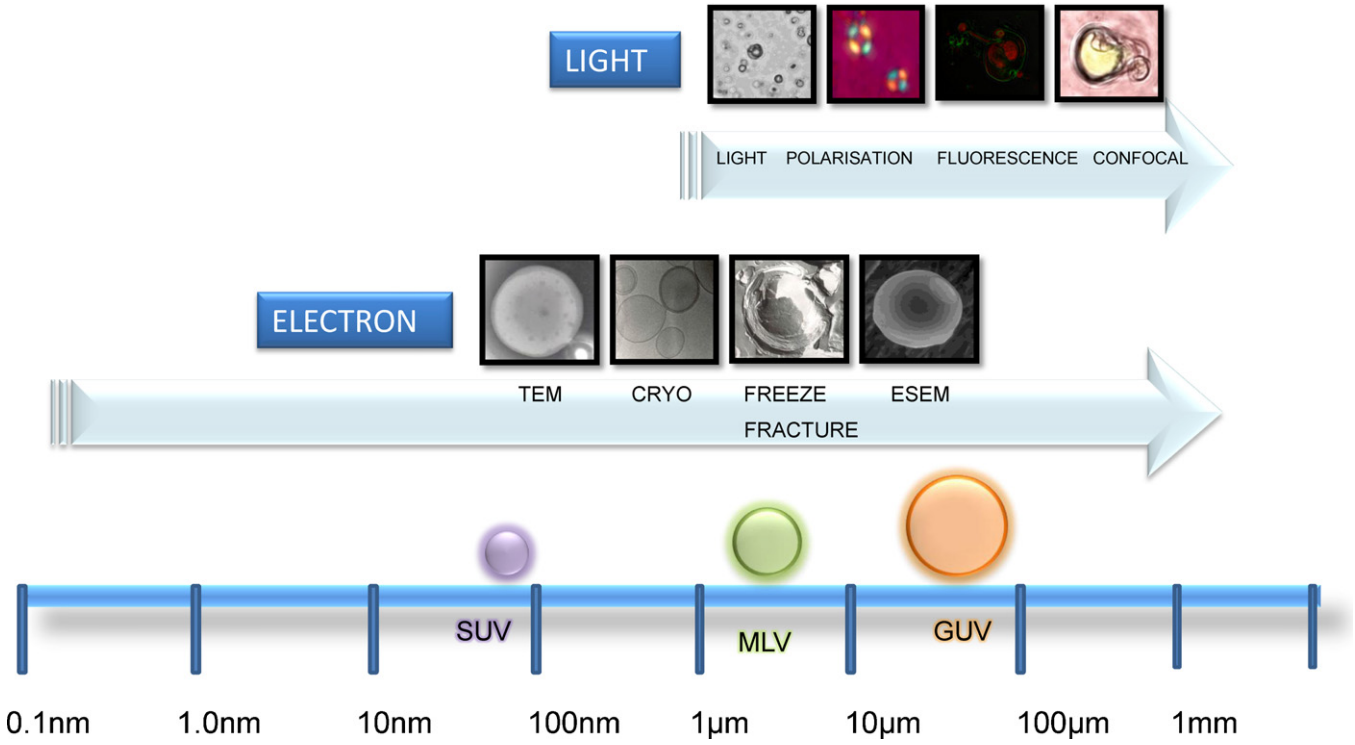


Fig. 9. Summary of microscopic techniques which can be used for the visualisation of liposomes, niosomes and other bilayer vesicles.

4. Conclusion

Imaging of liposomes and other vesicles can be achieved from a basic light microscope through to more advanced techniques such as ESEM and confocol, with the primary considerations being the vesicles size and bilayer characteristics being investigated, as summarised in Fig. 9. For example, when using wet samples other methods of sample preparation can to be considered compared to simply adding a coverslip on the top of a small drop of sample. In laboratories where multilamellar vesicles are produced routinely, size and vesicle morphology should be confirmed on the microscope as an essential aspect of the preliminary characterisation work. These simple methods could help reduce problems that may occur due to poor vesicle formation or aggregation prior to other methods being undertaken and polarisation microscopy offers a quick way to identify the presence of vesicles through the presence of the maltese cross. With such a diverse variety of microscopic techniques available for researchers, it is essential that the limitations and advantages of each technique are considered in relation to the work being carried out. Vesicles are in an aqueous environment when they are made, and exposure to varying environmental conditions such as for cryo-electron microscopy, TEM or freeze fracture will result in the sample being affected and ESEM should be considered as a novel method for visualisation of liposomes in a hydrated state. Using ESEM the effects of changing pressure on the vesicle and the way in which it collapses can also be monitored. However other techniques, such as confocal scanning microscopy, can also be employed to track liposomes in biological conditions and to monitor structural attributes with the advantage that it offers greater resolution of vesicles than standard fluorescent microscopy.

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