STUDIES ON POLYHEDRAL NIOSOMES

A thesis presented by

Parinya Arunothayanun, B.Pharm.

in partial fulfilment of the requirements for the degree of

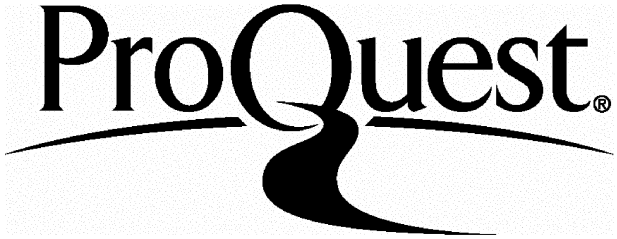
Doctor of Philosophy

of

the University of London

The School of Pharmacy
University of London
Brunswick Square
London, UK

October 1998



To Dad and Mum
Thank you for everything

แด่ อาเด อามา
ขอบคุณครับ สำหรับทุกอย่าง

The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!) but 'That's funny ...'

Isaac Asimov

ACKNOWLEDGEMENTS

The last three years in the School of Pharmacy have been one of the most fantastic times of my life. I would like to thank the following people who have been taking part in both my studies and contributing to my joyous experiences.

First and foremost, I would like to express my sincere gratitude to my supervisor, Professor Alexander T. Florence, for his superb guidance and encouragement during my time of study. I will always remember your excellent supervision (also with aspects of fun) which has been in the kindest, most open-minded and friendly way.

I wish to convey special thanks to Dr Ijeoma F. Uchegbu for invaluable techniques, consolation and friendship. Dr Duncan Q. M. Craig is gratefully acknowledged for his support and advice in thermal studies. I am deeply indebted to Dr John A. Turton for his superb supervision during the *in vivo* study. I would like to give my appreciation to Professor Gregory Gregoriadis for being kind to me during my stay in CDDR.

My thanks also go to Mr Dave McCarthy for his friendship and excellent microscopic work, Miss Clair Liu for her HSDSC introduction, Mr Thongchai Sooksawate for his help with micropipette techniques, Mr Douglas Banning for advice in shear flow studies, Ms Annie Cavanagh for immediate help with slides and posters, Mr Wilfred Baldeo for technical advice in HPLC, and Mr Steve Coppard for his help during *in vivo* studies. Miss Marie-Sophie Bernard, Mr Bipin Shah, Mr Sandeep Kiri, Miss Jayna Patel were also appreciated for fruitful collaborations.

The Government Pharmaceutical Organisation, Thailand is gratefully acknowledged for financial support.

For friendship, a big big thank go to Sasitorn, Jittima, Ruedeekorn, Sudax, Sakthi, Gill, Yvonne, Dejana, Ibrahim,Victoria, Roghieh, Dejana, Brenda, Steve, Kent, Malini, Begona, Mia, Jean Christophe, Kirsten and all my friends in CDDR. Affection from all Thai friends in London is greatly admired.

There were also times when I experienced down-hearted moments. It would never have been possible for me to be here and now without tremendous support from my family (and their long distance calls), and all my friends. I would like to thank Prapassorn, Porntip and, especially, Itsaraet for being such good friends of mine all the ways.

Finally, I would like to thank my parents, Dad Chookiat and Mum Kamnoi, for their love and prayers, (and also their Kao Mun Gai). I am so proud of you and glad to be your son.

ABSTRACT

Niosomes prepared from non-ionic surfactants were studied and characterised with regard to their physicochemical and biological properties. Hexadecyl diglycerol ether ($C_{16}G_2$) and a series of poly(oxyethylene) alkyl ethers form, with an equimolar amount of cholesterol, a mixture of largely spherical and tubular niosomes. In the absence of cholesterol, they form polyhedral structures below their phase transition temperature (Tm) and they transform into spheres on heating above Tm. Various properties, namely vesicle shape, size, encapsulation efficiency, membrane permeability, thermal behaviour, viscosity, and osmotic activity were investigated using in addition, as a comparator, sorbitan monostearate (Span 60) niosomes. The role of membrane composition was in particular an issue.

Rheological studies showed that viscosity of niosomes can be affected by a number of factors including vesicle shape. The values of intrinsic viscosity were used in attempt to predict the hydration and the volume fraction of the vesicle dispersions.

Polyhedral niosomes formed by $C_{16}G_2$ are less osmotically active, and more permeable than their spherical/ tubular counterparts, formed with cholesterol. The release profiles of 5(6)-carboxyfluorescein from both types of niosomes are also different and can be affected by the content of the hydrophilic surfactant poly(24) oxyethylene cholesteryl ether (Solulan C24) in the membranes.

The high encapsulation efficiency of luteinising hormone releasing hormone (LHRH) in $C_{16}G_2$ niosomes was achieved when these are prepared with the remote loading method (exploiting either pH or $(NH_4)_2SO_4$ gradients). *In vitro* studies showed that spherical/ tubular $C_{16}G_2$ niosomes are more stable than their polyhedral counterparts in rat plasma and muscle homogenate. Following intramuscular injection in rats, ^{125}I-LHRH solution was cleared from the site of injection within 2 h, whilst both polyhedral and spherical/ tubular $C_{16}G_2$ niosomes act as a depot and release ^{125}I-LHRH over 25 h and 49 h, respectively.

Extrusion of single niosomes (4-40 μm in diameter) from glass capillaries (5 μm in diameter) was studied as a biomimetic approach for pulsatile delivery. This was simply achieved but when vesicle diameter was larger than the capillary diameter, the vesicles can be extruded into a variety of simple or complex structures, depending on their initial ultrastructure, possibly a reflection of the differences in membrane elasticity of the vesicle formulations.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

LIST OF TABLES

ABBREVIATIONS

$C_{16}EO_2$	polyoxyethylene 2 cetyl ether
$C_{16}EO_5$	polyoxyethylene 5 cetyl ether
$C_{16}G_2$	hexadecyl diglycerol ether
$C_{16}G_3$	hexadecyl triglycerol ether
$C_{18}EO_2$	polyoxyethylene 2 stearyl ether
$C_{18}EO_5$	polyoxyethylene 5 stearyl ether
CF	5(6)-carboxyfluorescein
CPP	Critical packing parameter
DCP	Dicetyl phosphate
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
DRV	Dehydration-rehydration vesicles
DSC	Differential scanning calorimetry
DSPC	1,2-Distearoyl-sn-glycero-3-phosphocholine
HPLC	High performance liquid chromatography
HS	Hand-shaking
HSDSC	High sensitivity differential scanning calorimetry
I.D.	Inner diameter
LHRH	Luteinizing hormone releasing hormone
MW	Molecular weight
O.D.	Outer diameter
PBS	Phosphate buffered saline
PCS	Photon correlation spectroscopy
PEG	Polyethylene glycol
REV	Reverse-phase evaporation vesicles
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
TBS	Tris buffered saline
TEM	Transmission electron microscopy
TPGS	D-alpha tocopheryl polyethylene glycol 1000 succinate

CHAPTER 1

INTRODUCTION TO VESICULAR SYSTEMS

1.1. DEFINITIONS

Certain amphiphilic molecules self-assembly on dispersing in aqueous media to form sealed vesicles which are composed of bilayer membranes enclosing an aqueous core (Figure 1.1).

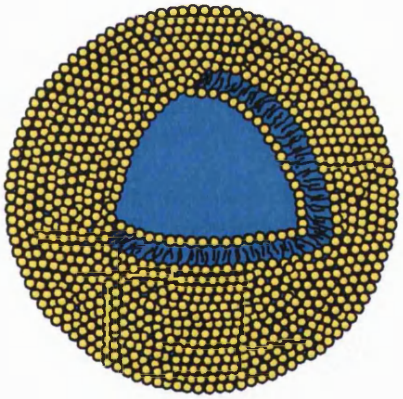


Figure 1.1: Schematic representation of a vesicle formed by a bilayer of amphiphilic molecules.

Vesicular dispersions formed by phospholipids, so called **"liposomes"**, have been used widely as cell models and drug delivery systems (Bangham *et al.*, 1965). The term **"niosome"** was initially used by L'Oreal, France (Vanlerberghe *et al.*, 1973), and refers to vesicles formed mainly by self-assembly of synthetic non-ionic surfactants with an optional addition of charged amphiphiles and cholesterol (Florence & Baillie, 1989). However, other names such as non-phospholipid liposomes or non-ionic liposomes have also been used since they are considered as analogues of liposomes.

Niosomes offer a versatile alternative to liposomes as their surfaces, which can subsequently elucidate the vesicle behaviour *in vivo*, can be modified by the choice of a wide range of surfactants with differing structure. Due to the fact that both liposomes and niosomes are vesicles comprising bilayers, their physicochemical properties have very much in common. This chapter reviews general knowledge on some aspects of vesicular systems relevant to the studies in this thesis.

1.2. FORMATION OF VESICLES

Amphiphilic molecules aggregate in aqueous solution to give a number of forms such as micelles, inverted micelles, spherical micelles, planar bilayers, and curved bilayers (vesicles). However, in non-polar organic media, "inverted" or "inverse" vesicles can also be formed (Kunieda & Rajagopalan, 1996). A critical packing parameter (CPP) of amphiphilic molecules, defined as

$$CPP = v/ l_c a_o \qquad (1.1)$$

has been derived to predict the structure of surfactant assemblies, where v = hydrocarbon chain volume, l_c = hydrocarbon critical chain length, a_0 = hydrophilic head group area (Israelachvili *et al.,* 1980; Israelachvili, 1991). A surfactant with a CPP value of 0.5-1 is predicted to form flexible bilayers which can aggregate into closed vesicles. However, the CPP calculated for some vesicle-forming amphiphiles do not fall in the suggested theoretical range, e.g. sorbitan monostearate has a CPP of 0.4 (Florence & Cable, 1993). It was argued that not only is the shape of an isolated molecule important but the combination also of the complex intermolecular interactions in the systems, e.g.

solvophobic forces, van der Waals forces, electrostatic interactions, entropy effects, and hydrogen bonds, that determine the shape of supramolecular assemblies (Gruner, 1987; Fuhrhop & Koning, 1994).

Apart from phospholipids, the various chemical structures of synthetic surfactants that form vesicles on hydration have been reviewed by Özer *et al.,* (1991), Florence (1993), and Uchegbu & Florence (1995). Formation of vesicles can also be induced by an interaction between positively and negatively charged amphiphiles e.g. in the mixture of sodium dodecyl sulfate and dodecyltrimethylammonium bromide (Kamenka *et al.,* 1992). It is noteworthy that vesicles can also be formed by monolayer membranes in the case of bolaform amphiphiles which carry two hydrophilic head groups, one on each end of the hydrophobic chain (Fuhrhop & Koning, 1994).

Various preparation methods to obtain vesicle dispersions with required optimum size and lamellarity have been reviewed (New, 1990), providing the concentration of amphiphiles exceeds the critical bilayer concentrations (CBC), the value depending on amphiphile; for example with distearoyl phosphatidylcholine, the CBC is on the order of 10^{-10} M (Hristova & Needham, 1995). Vesicles can be prepared simply by hydrating dried film of lipids or surfactants in round bottom flasks, the so called "hand-shaking method". Other techniques have also been developed to avoid the use of organic solvents e.g. formation of vesicles in gas streams (Talsma *et al.,* 1994), inducing micelle-vesicle transitions by removal of surfactants from lipids containing mixed micelles *via* dialysis (New, 1990), or by removing the water-interacting head group of surfactant *via* an enzymatic process (Chopineau *et al.,* 1994).

1.3. SOME PHYSICOCHEMICAL ASPECTS OF VESICLES

1.3.1. Thermal Behaviour of Vesicle Membranes

The physicochemical properties of the bilayer membranes can be diverse depending on the composition of the membranes which determines the polymorphic phase of the bilayers. Figure 1.2 shows some examples of bilayer geometries assumed by lipid-water systems. Bilayers undergo changes in physical state with temperature. Above the phase transition temperature (T_m) the bilayer exists as a two-dimensional liquid. Below this temperature the bilayer is a two-dimensional solid and the lipid molecules are tightly packed in a crystal lattice (Gruner, 1987).

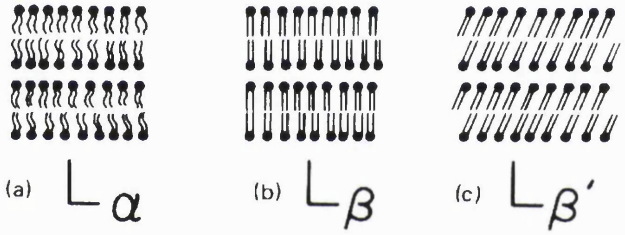


Figure 1.2: A schematic representation of lipid bilayer polymorphic phases. The L_β and $L_{\beta'}$ phases the chains are in a gel (frozen) state, whereas in the L_α phase is a liquid crystalline (melted chain) bilayer phase with fluid acyl chains.

The value of T_m of bilayers varies with the composition of membranes and reflects various properties. For example, the lipids with longer hydrophobic chains form membranes which have higher phase transition temperatures and are less permeable to entrapped solute compared to those formed by lipids with shorter chain lengths (Buckton *et al.*, 1992). The mechanochemical properties of membranes can be different depending on their polymorphic state (Evans & Kwok, 1982; Needham & Zhelev, 1996). Figure 1.3 shows an example of the difference in elasticity between gel state and liquid state membranes observed by manipulating a vesicle with a micropipette (Needham & Zhelev, 1996).

Addition of cholesterol into the gel state membranes has been found to increase the mobility of amphiphiles, this therefore increasing the overall membrane fluidity and decreasing the phase transition temperature and enthalpy (New, 1990; Taylor & Morris, 1995).

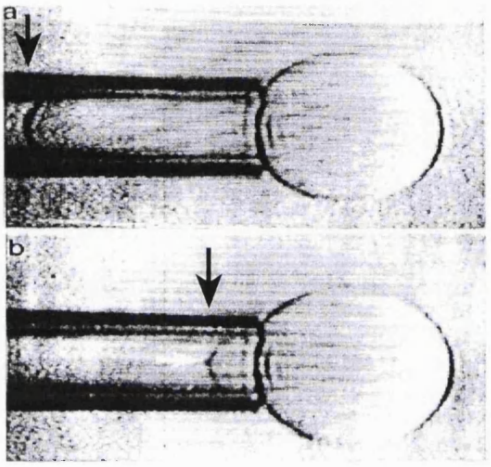


Figure 1.3: Videomicrographs of vesicles formed by stearoyloleoyl phosphatidyl choline and palmitoyloleoyl phosphatidyl ethanolamine (40:60) as the temperature is decreased: (a) liquid phase vesicle at 20°C; (b) gel phase vesicle at 13°C showing 20% reduction in vesicle membrane area when experimenting with equal suction. From Needham & Zhelev (1996).

1.3.2. Vesicle Shape Transformation

Vesicles can exhibit a variety of morphologies and shape transformations, as observed in biomembranes, since the molecules in the bilayers can move laterally along the membranes. Membrane fluctuations can be induced by various factors such as temperature, the bilayer asymmetry, or applied external forces (Lipowsky, 1995).

Cytomimetic behaviour has been widely observed. Fusion of vesicles (Figure 1.4) involves aggregation of vesicles with close apposition of bilayers following a destabilising process leading to merging of the bilayers and mixing of the contents of each vesicle. These processes can be induced by a number of factors including temperature (Allen *et al.*, 1990), pH (Hope *et al.*, 1983), osmotic stress (Döbereiner *et al.*,1993), and the presence of

fusogenic molecules such as polyethylene glycols (Yamazaki & Ito, 1990), cations or anions (Hope *et al.,* 1983; Allen *et al.,* 1990; Mosharraf *et al.,* 1995; Menger & Lee, 1995) which allow a fusion process through hydrophobic or charge interactions, depending on the nature and charge of the lipids (Israelachvili, 1991; Bernsdorff *et al.,*1996).

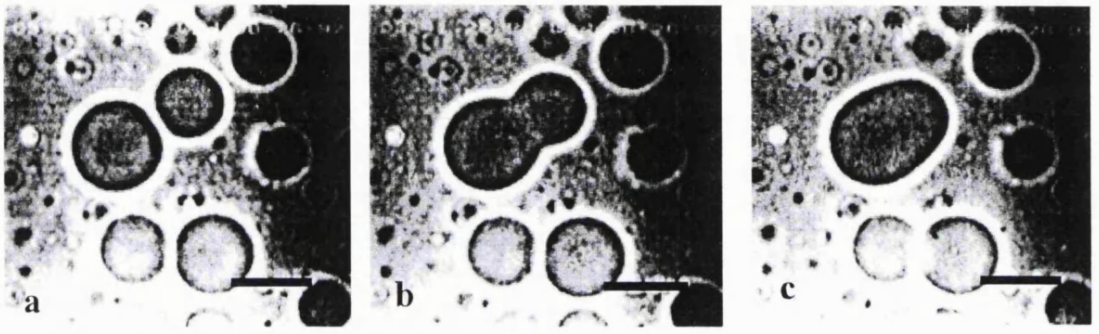


Figure 1.4: Fusion of two didodecyldimethylammonium bromide vesicles taking place over a few seconds after injecting sodium acetate in the vicinity of the vesicles (bar=25 μm). From Menger & Gabrielson (1995).

The healing process takes place if small defects are created due to the fluidity of the membranes; for example, following exposure to low concentrations of sodium cholate, didodecyldimethylammonium bromide vesicles were observed to break open and reseal into intact vesicles (Menger & Lee, 1995). However, it was found that membrane defects created by osmotic shrinkage can re-anneal in the liquid state, but not in the gel state (Disalvo *et al.,* 1996).

Membranes are sensitive to the change in temperature, especially near their phase transition temperature. Dimyristoylphosphatidylglycerol vesicles were reported to develop extended branches when heated to the transition range (Heimburg & Biltonen, 1994). Budding and fission of vesicles induced by heat, pH and, in many cases, osmotic gradients (Figure 1.5) reflect the processes of cell compartmentalisation (Döbereiner *et al.,*1993; Mathivet *et al.,*

1996). The events take place when the surface area-to-volume ratio of the vesicles is increased due to the increase in excess area and/or decrease in volume, as shown in figure 1.6.

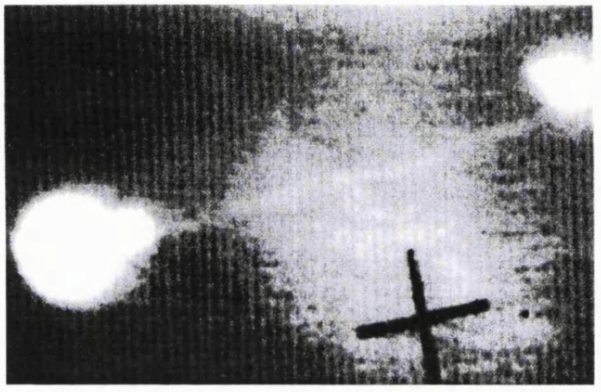


Figure 1.5: A single spherical egg phosphatidylcholine vesicle loaded with fluorescent dextran was transformed, by application of hyperosmotic glucose solution, into these three spheres connected by a narrow tether. Bar=10 μm. From Mathivet *et al.* (1996).

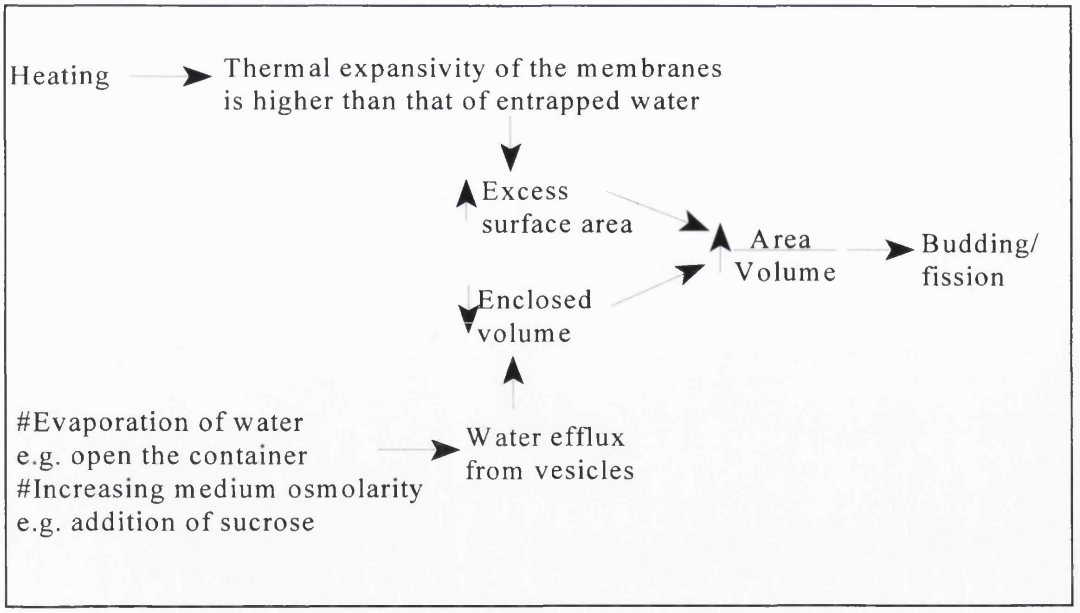


Figure 1.6: Schematic representing the mechanisms of budding/ fission processes, adapted from Döbereiner *et al.* (1993).

Conventional spherical vesicles can exhibit conformal diffusion, induced, for example , by temperature changes. The shapes of the vesicles change continuously and randomly, and finally reach an equilibrium non-spherical topology of which the elastic curvature energy of the fluid membranes is minimized under certain physical constraints; for example , genus 1 (vesicles with one hole) or genus 2 (vesicles with 2 holes) vesicles (Lipowsky, 1995; Michalet & Bensimon, 1995) (Figure 1.7).

Figure 1.7: Examples of conformal diffusion of a toroidal vesicle with holes: all shapes have the same bending energy, surface area, enclosed volume, and total mean curvature. From Lipowsky (1995).

Shape transformations have also been studied by exposing the vesicles to various physical agitations. For example, cylindrical vesicles which formed with dimyristoyl phosphatidyl choline can be split into a number of smaller vesicles when manipulated by optical tweezers (Lipowsky, 1995). Theoretical studies of the axisymmetric vesicles flowing down narrow capillaries showed that a vesicle experiences a shape transformation starting from a sphero-cylinder shape and ending in a bell shape on increasing of pressure gradient, as observed in red blood cells (Bruinsma, 1996). Effect of gravity was also studied theoretically which showed the variety of shapes a vesicle can exhibit (Kraus *et al.,* 1995). Application of a micropipette to manipulate cells (Artmann *et al.,* 1997) or vesicles allows one to observe their behaviour, individually, and gain more understanding of membrane behaviour under

physical stress (Evans & Kwok, 1982; Lasic & Needham, 1995; Needham & Zhelev, 1996). For example, Menger & Gabrielson (1995) found that an oligolamellar giant vesicle can be ruptured layer-by-layer, which results in a smaller vesicle using sharp micropipette.

1.3.3. Osmotic Behaviour

Cells posses semi-permeable membranes and respond to osmotic gradients (Yoneda, 1973). Their volume and/or turgor pressure are controlled by modulating cytoplasmic osmolality in response to changes in environmental osmolality by means of diffusion or transporter proteins. It is well known that lipid vesicles are osmotically sensitive (Bangham *et al.,* 1967). Osmotic stress can affect properties of vesicles in many aspects from inducing membrane undulation (Menger & Lee, 1995) to change in vesicle size (Sun *et al.,* 1986), shape (Berndl *et al.,* 1990) and membrane permeability (Iga *et al.,* 1989).

Under hypertonic gradients, osmotic shrinkage causes changes in the interlamellar distance in multilamellar vesicles (Viera *et al.,* 1996). Previous studies found that osmotic shrinkage can cause defects at the membrane surface, an event which is likely to occur when the bilayer is in the gel state (Disalvo *et al.,* 1996). Water outflow can also induce topological changes of vesicles, as discussed in section 1.3.2.

Changes in vesicle volume and area due to osmotic stress lead to changes in the elastic modulus of bilayers, their morphology, and to subsequent vesicle lysis (Senisterra *et al.,* 1988; Mui *et al.,* 1993). In hypotonic media, an excess of water influx beyond a critical point, depending on the system, produces an expansion of the bilayer resulting in changes in the area per molecule and membrane leakage (Sun *et al.,* 1986). For example, when

subjected to hypotonic solution, mast cell vesicles of mice can increase in diameter up to 73% (Brodwick *et al.,* 1992) compared to 152% found in inner medullary collecting duct cells of rats (Grunewald *et al.,* 1993). The latter was found to regulate the volume by releasing sorbitol, an intracellular osmolyte, to the external medium. The same response is also found in the secretion of bioactive substances, e.g. hormone is released from the intracellular vesicles of endocrine cells following cell swelling induced by medium hypo-osmolarity or permeant molecules (Inukai *et al.,* 1992).

1.3.4. Rheological properties

The rheological characteristics of vesicular suspensions have not been widely studied. A limited amount of work has been published on the rheology of dilute vesicle dispersions (Florence, 1993; Muzzalupo *et al.,* 1996). Some have concentrated on the viscoelastic nature of systems (Smeulders *et al.,* 1990; Hoffmann *et al.,* 1994 & 1995; Seki & Komura, 1995) or on their flow properties at high concentration (Kato *et al.,* 1983; Sakai *et al.,* 1997), both key topics.

Vesicles, particularly if multilamellar, might be compared, theoretically, with rigid sphere systems e.g. latex (Saunders, 1961; Wang, 1970), silica dispersions (Van der Werff *et al.,* 1989), or systems such as microemulsions (Matsumoto & Sherman, 1969; Attwood *et al.,* 1974; Baker *et al.,* 1984), or emulsions (Sherman, 1968; Kita *et al.,* 1977). Multilamellar vesicles have been considered as rigid spherical particles in previous studies by Hoffmann *et al.,* 1994 & 1995); unilamellar vesicles, which are not rigid spheres, have, however, also been treated as such (Smeulders *et al.,* 1990).

There are a number of rheological studies considering a vesicle dispersion as a model for an erythrocyte suspension (Bruinsma, 1996). One may consider red blood cells to be analogous to large uni- or pauci-lamellar niosomes in the blood; both are comprised of simple bilayers without any complex internal structures, although the high concentration of red blood cells in the vessels and their distinctive shape make it unlikely that their behaviour can be compared directly to that of dilute suspensions of spherical vesicles.

The effect of vesicle addition on the viscosity of topical gel preparations has been studied (Bonté *et al.*,1994). Rheological measurements are useful in monitoring the transformation of vesicles into micelles (Hassan *et al.*, 1996). The viscosity of vesicle dispersions is also important for their *in vivo* behaviour. One example is the work performed by Sakai *et al.* (1997) in which the surface of vesicles entrapping hemoglobin was modified with 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine-N-[poly(ethylene glycol)]. This was found to prevent the aggregation of vesicles, improve flow properties on mixing with blood and albumin, and to provide a better blood flow and O_2 supply profile *in vivo* compared to unmodified vesicles.

1.4. NON-SPHERICAL VESICLES

In living systems, although most cells appear to be spherical in shape, other structures, which include some liquid crystalline assemblies such as helical structures found in DNA, or in the visual systems, also exist. Many attempts have been put forward to build simpler models for such structures found in the living systems, using synthetic materials (Figure 1.8) which also lead to a large number of applications. Amphiphilic molecules can swell on hydration to form bilayers which, considered as a precursor, evolve to form spherical vesicles and a variety of self-assemblies.

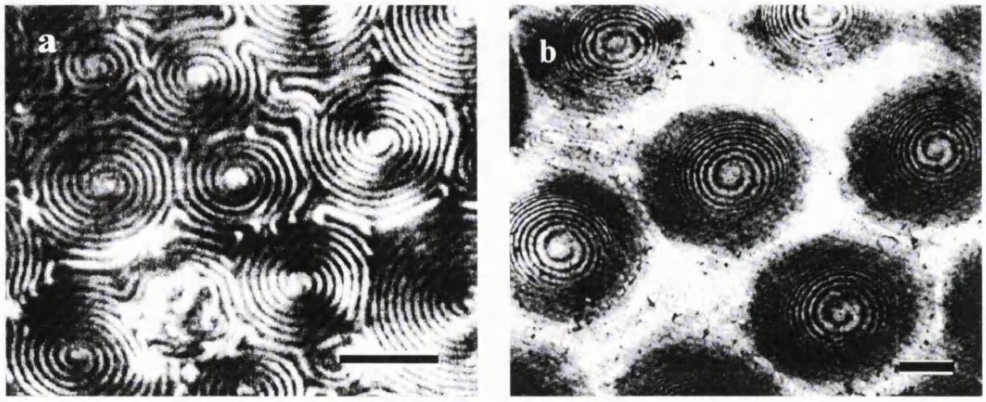


Figure 1.8: Helical structures of (a) the liquid crystals formed by p-Methoxybenzylidene-p-n-butylaniline and 10% cholesteryl nonanoate appear to be similar to those found in (b) corneal lens of beetles (bar=10 μm). From Brown & Wolken (1979).

Tubular or cylindrical vesicles are commonly found coexisting with spherical vesicles as a result from the swelling process of lecithin and synthetic analogues. They can also be formed by fusion of spherical vesicles; for example, addition of calcium chloride to phosphatidylserine vesicles leads to vesicle aggregation, fusion, and subsequent formation of multilayered cylinders (Fuhrhop & Helfrich, 1993). Spherical vesicles formed by didodecyldimethyl ammonium hydroxide transform into tubular vesicles when their hydrophilic groups are titrated with HBr. As a result from bilayer asymmetry, with hydroxide interior and bromide exterior, the smaller head group area of the bromide surfactant allows vesicles to swell to a point where they can no longer pack spherically and prefer tubular structures (Kachar *et al.,* 1984). Figure 1.9a shows plausible ultrastructures which can be formed following the scrolling of bilayers. Diacetylenic lecithins undergo an intermediate helical ribbon bilayers, due to the packing defects in closed spheres, to form hollow tubules so called "microcylinders" (Figure 1.9). It has also been shown that tubular vesicles can also be formed by hydrating the mixed surfactant systems of C14-maltoside: cholesterol: polysorbate 80 (40:40:20) (Uchegbu & Florence, 1995).

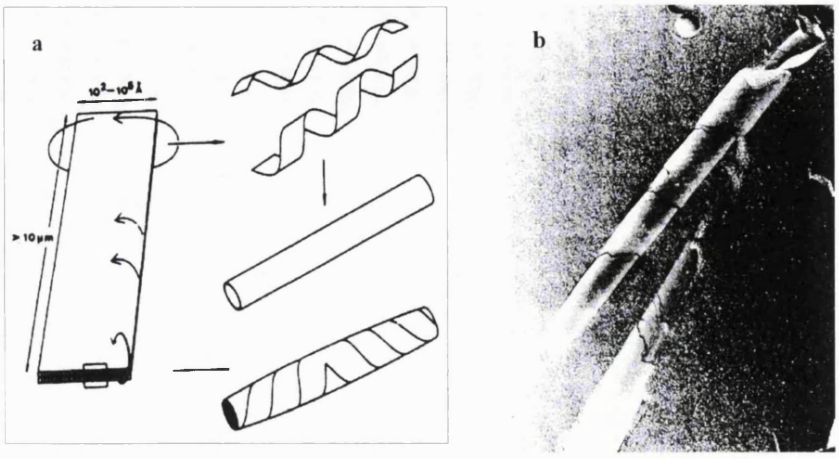


Figure 1.9: (a) Schematic representation of scroll formation from planar bilayers. From Fuhrhop & Helfrich (1993). (b) An example of hollow cylinder formed from a diacetylenic phospholipid. The tubules have a diameter of ca. 0.5 μm with the wall being made up of helically wrapped bilayers. From Schnur & Shashidhar (1994).

Disc-shaped structures, with sizes in the nanometre range, were observed with poly-15-oxyethylene glyceryl-α,α'-dioctadecylethers (Okahata *et al.,* 1981), and some glycolipids (Zariff *et al.,* 1994). Large disc-shaped vesicles of 15-100 μm, so called **discomes** (Figure 1.10a), can be obtained by cooling the mixed micellar liquid of $C_{16}G_2$ (hexadecyl diglycerol ether): cholesterol: Solulan C24 (poly-24-oxyethylene cholesteryl ether) (50:20:30) (Uchegbu & Florence, 1995). It was suggested that a heterogenous distribution of the different surfactants in the bilayer is responsible for the formation of such structures. This was evidenced by the formation of discomes induced by solubilisation of $C_{16}G_2$: cholesterol: dicetyl phosphate (69: 29: 2) and (47.5: 47.5: 5) vesicles with the soluble surfactant Solulan C24 (Uchegbu *et al.,* 1992) and octylglucoside (Seras *et al.,* 1992), respectively.

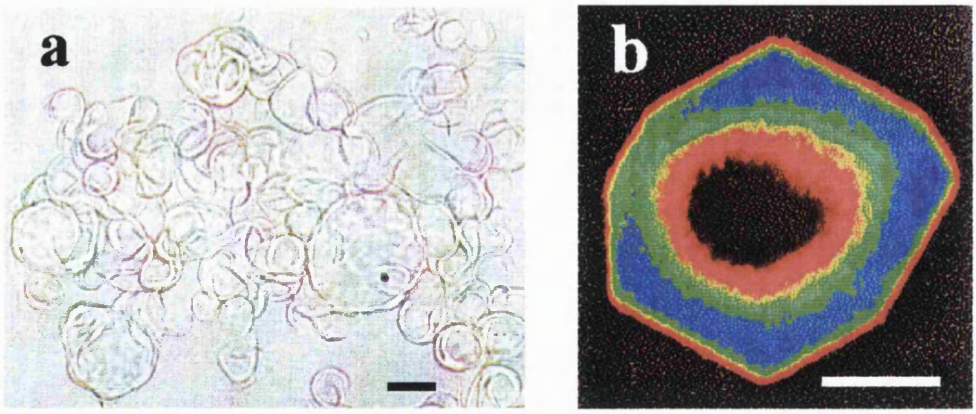


Figure 1.10: Some examples of non-spherical vesicles from the mixed surfactant system, $C_{16}G_2$:Solulan C24. (a) An optical micrograph of discomes obtained from the 50:50 mixture (bar=20 μm). (b) Confocal laser scanning micrograph in pseudocolours of a polyhedral vesicle obtained from the 91:9 mixture (bar=5 μm). From Uchegbu *et al.* (1996).

Polyhedral niosomes (Figure 1.10b) were found in the low-cholesterol region of the $C_{16}G_2$-cholesterol- Solulan C24 phase diagram (Uchegbu *et al.*, 1996). They possess straight edges and flattened contours with their membranes in the gel state (L_β) at room temperature (Uchegbu *et al.*, 1997). The release of encapsulated carboxyfluorescein and nicotinamide adenine dinucleotide has also been found to be thermo-responsive although the membrane are still in gel phase at studied temperature. Extruded and sonicated dipalmitoyl phosphatidyl choline vesicles (diameter <200 nm) were also observed to possess such faceted structures by Cryo-TEM when vitrified below the T_m, but appear to be smooth, spherical vesicles above the T_m (Andersson *et al.*, 1995). It was explained that in small vesicles with high membrane curvature the tilted bilayers $L_{\beta'}$ cannot be packed into close spheres when the bilayer changes from the L_α into the $L_{\beta'}$ phase. This packing problem leads to the formation of a number of flat bilayer areas, joined together by small highly curved edges.

More complicated structures can be obtained as a result of the association of each individual vesicle. Lipophilic polyamide dendrimers were found to form aggregates which

were, it was speculated, bundles of tubular vesicles associated into **vesicle helices** (Sakthivel *et al.*, 1998) (Figure 1.11a). Sternberg *et al.* (1995) reported the formation of multiple vesicle structures, so called **geodesic structures** (Figure 1.11b), which are aggregates of small spherical niosomes packed in a disordered face-centred cubic lattice and which appear to be similar to the structures found in certain bacterial membranes. Interestingly, the bola-amphiphiles $C_n[G_m]_2$ (n=10, m=2 and 3), dicarboxylic amides with two oligoglycine head groups, were found to form **vesicle-enclosing tubules** (Figure 1.11c) (Shimizu *et al.*, 1996). It was also reported that such structures do not form with $C_6[G_2]_2$ or by replacement of the glycine with proline or N-methylglycine. The multicompartment vesicles, so called **vesosomes** (Figure 1.11d), were constructed by encapsulating vesicle aggregates within large bilayer vesicles using a molecular-recognition process mediated by a biotin-streptavidin complex (Walker *et al.*, 1997).

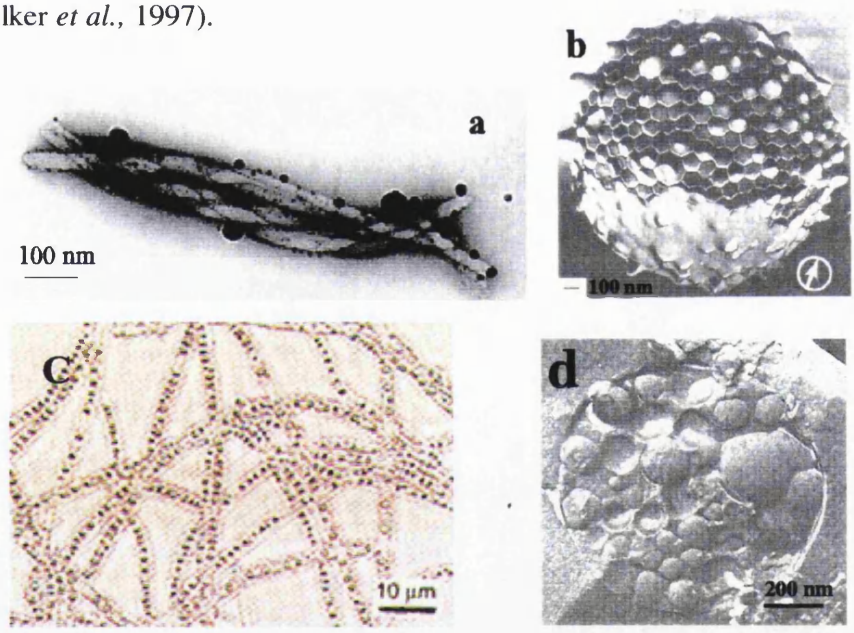


Figure 1.11: Some examples of complex vesicles. (a) A helical bundle of vesicles formed by a lipophilic polyamide dendrimers (Sakthivel *et al.*, 1998). (b) A geodesic structure formed by aggregation of sorbitan mono-oleate: cholesterol: dicetyl phosphate (19:19:2) niosomes (Sternberg *et al.*, 1995). (c) Uniform tubular structures which enclose a number of spherical vesicles formed by $C_{10}[G_3]_2$ (Shimizu *et al.*, 1996). (d) A vesosomes formed by wrapping the biotin-streptavidin-induced vesicle aggregates with a larger streptavidin-incorporated bilayer membrane (Walker *et al.*, 1997).

1.5. APPLICATIONS OF VESICULAR SYSTEMS

1.5.1. Medical Applications

Vesicular systems have been widely used as drug carriers which rely on the slow release of drugs from the systems to achieve sustained activity and reduced toxicity of drugs (Baillie, 1988; Florence & Baillie, 1989; Uchegbu *et al.,* 1995). They have also been used successfully as immunoadjuvants for a broad range of antigens from short peptides to particulates, and both humoral and cell-mediated immunity are induced (Hassan *et al.,* 1996). Use of radiopaque loading vesicles which accumulate in specific organs have been found useful in organ imaging (Erdogan *et al.,* 1996). Apart from parenteral delivery of vesicles, they have also been studied for other routes including transdermal (Schreier & Bouwstra, 1996) and ophthalmic routes (Durrani *et al.,* 1992). Vesicles have also been used orally to deliver peptide and protein drugs (Yoshida *et al.,* 1992), or as immunoadjuvants (Jackson *et al.,* 1990), since they can be uptaken by M-cells of Peyer's patches and translocated to the lymphatics (Childers *et al.,* 1990).

Vesicles have been so far considered as a biocompatible system although there are concerns over toxicity of amphiphiles used, a concern which depends on their chemical structures (Riess *et al.,* 1991; Hofland *et al.,* 1992; Mahe *et al.,* 1992; Lasic & Barenholz, 1996). Studies in cell culture showed that the toxicity of polyoxyethylene cholesteryl ethers are due to their free and micellar forms, being greatly reduced when the surfactant is incorporated as an integral part of vesicles (Dimitrijevic *et al.,* 1997). Vesicles formed by various phospholipids were found to show no myotoxicity, regardless of size, charge, and membrane fluidity (Al-Suwayeh *et al.,* 1996). Positively charged stearylamine-liposomes

exert cytolytic activity following intravenous administration in rabbits (Yoshida & Nakae, 1986). Non-ionic surfactants are generally biodegradable *in vivo* and show extremely low toxicity when administered by the subcutaneous and intramuscular routes to rats (Brewer, 1994).

There is a need to optimise the properties of vesicles e.g. size, membrane composition, surface characteristics, in order to obtain the required encapsulation efficiency and drug delivery pattern *in vivo*. Vesicles with larger size have higher volume to entrap hydrophilic drugs (Iga *et al.,* 1989). Addition of cholesterol into the vesicle membranes also increases entrapment efficiency due to packing effects on bilayers and the consequent reduction of free motion of the encapsulated drugs inside the vesicles (Taylor *et al.,* 1990). The presence of charged amphiphiles in the multilamellar vesicles increases the interlamellar distance which, in turn, enlarges the aqueous regions between closed bilayers, hence the higher entrapment (Panico *et al.,* 1997). Charged vesicles also enhance the incorporationof the drug which possesses opposite charge by the electrostatic interaction (Taylor *et al.,* 1990); for example, cationic lipids are used to increase the entrapment of DNA.

Whilst large vesicles are preferred when the drug loaded vesicles are administered intramuscularly, as they are not easily drained into the lymphatic systems, the use of small vesicles are required for intravenous injection. However, there is a need for an optimum size due to the size dependent affinity of complement in the recognition of vesicles which can promote the uptake into the reticuloendothelial systems. For example, Harashima *et al.* (1995) studied the intravenous fate of liposomes, with a size of 0.2-1 μm, and found higher degradation with the increased vesicle size, while vesicles with a diameter less than 70 nm

are rapidly removed from the circulation (Ghosh & Bachhawat, 1995).

Modification of the vesicle surfaces with hydrophilic polymers, such as derivatives of polyethylene glycol (PEG), glucuronide, or monosialoganglioside, provides "stealth" systems which prevent opsonisation and ensures the vesicle longevity in the blood stream (Oku & Namba, 1994). The presence of a fluorinated core in the membranes, as in vesicles formed with fluorinated phosphatidyl cholines, was found to enhance the retention of drug *in vivo*. This is due to their lipophobic character which reduces the adsorption and anchoring or lipophilic plasma proteins on the membranes, therefore inhibiting the recognition and uptake by the macrophages (Santaella *et al.,* 1993). Coated polymers can also provide a barrier to drug release which results in a prolonged release profile (Durrani *et al.,* 1992).

The use of vesicle for drug delivery purposes is not limited just to sustaining the release of drug from the vesicles but also to controlling the release of drug when required. Such release of drug can be triggered when subjected to certain stimuli. For example, near the phase transition temperatures, the vesicle membranes become highly leaky to water-soluble contents due to disorder at the boundaries between solid and fluid domains in the lipids. The concept of temperature-sensitive vesicles exploits such knowledge by choosing the proper combination of lipids, which provides vesicle membranes with the phase transition temperature near the temperature of tumors or the applied temperature in local hyperthermia (Iga *et al.,* 1989).

Attempts to control the delivery profile *in vivo* have been taken a step further in order to

target the drug loaded vesicles to specific sites or organs. One simple approach is an addition of antibodies recognizing antigens on tumour cell surfaces into the vesicle membranes which leads to the high concentration of drug at the tumour and thus reduces toxicity due to the presence of drug in other organs (Gabizon & Papahadjopoulos, 1988).

Vesicular dispersions have been formulated combined with other systems which can improve delivery profiles; for example, vesicle-in-water-in-oil (v/w/o) system (Yoshioka & Florence, 1994), drug and hydrogel co-entrapped in vesicles (Monshipouri & Rudolph, 1995), and vesicles loaded into polysulfone capillary fibers as an intraocular implant (Rahimy *et al.,* 1994).

1.5.2. Non-medical applications

Composed of bilayers, vesicles offer a particular valuable model for cells and cell membranes which serve the need to understand various behaviours of the living cells. They have been used to study the cytomimetic responses (see section 1.3), the spontaneous interbilayer transfer of lipids (Bittman, 1993; Suzuki *et al.,* 1995), the mechanisms of cell damage by toxic compounds e.g. asbestos (Erdogdu & Hasirci, 1994), or to reconstruct membrane proteins for pharmacological studies (Tank & Miller, 1983).

Vesicles have been used as catalytic substrates which offer suitable conditions for various chemical and biotransforming reaction, i.e. large surface area, hydrophilic/hydrophobic boundary, and their hydrophilic compartments (Lasic & Barenholz, 1996). They have been widely used in the food industries from immobilizing enzymes in cheese ripening processes to encapsulating food ingredients such as flavours, vitamins, and antioxidants (Reineccius,

1995). Cylindrical vesicles formed by diacetylenic lecithins (Figure 1.9) which can be polymerized and metal-coated also offer a number of applications such as microcathodes and anti-corrosive paints (Neitchev *et al.,* 1986; Schnur & Shashidhar, 1994).

OUTLINE OF WORK IN THESIS

Physicochemical properties of niosomes formed mainly by sorbitan monostearate (Span 60), hexadecyl diglycerol ether ($C_{16}G_2$), and a series of poly(oxyethylene) alkyl ethers are studied. In **Chapter 2**, the effect of vesicle membrane composition on their morphology, thermal behaviour, and the basic properties needed for the drug delivery such as mean size, entrapment efficiency, and membrane permeability are investigated. These are followed by studies to understand the flow properties of vesicles in **Chapter 3**, and their osmotic behaviour when challenged to a range of osmotic gradients in **Chapter 4**. *In vivo* studies in rats are presented in **Chapter 5**, to determine the ability of the niosomes as a depot at the site of injection for delivery of a model peptide drug, luteinizing hormone releasing hormone (LHRH). An attempt to achieve a pulsatile delivery pattern is shown in **Chapter 6**, where the application of micropipette technique is used as a model device for delivery of single niosomes. Such technique is also used for fabrication of microstructures from pre-formed niosomes. Conclusions and suggestions for future work are then presented in **Chapter 7**.

CHAPTER 2

THE IMPORTANCE OF MEMBRANE COMPOSITION TO THE PHYSICOCHEMICAL PROPERTIES OF VESICLES

2.1. INTRODUCTION

In order to formulate a vesicle dispersion, the lipid or surfactant which forms the vesicle membranes are considered as the main excipients. Optimum properties of vesicles such as size, shape, surface characteristics, and encapsulation efficacy, which lead to a required behaviour *in vivo*, can be obtained by using the proper compositions of such excipients along with suitable preparation methods.

Earlier studies in our laboratory showed that vesicles can form various structures on varying the amount of a hexadecyl diglycerol ether ($C_{16}G_2$) (Figure 2.1) , cholesterol and a poly (24) oxyethylene cholesteryl ether (Solulan C24) (Figure 2.2) (Uchegbu, 1996). In this chapter, we show the effect of membrane composition on the general properties of vesicles, including shape, size, entrapment efficacy and membrane permeability. Niosomes formed by $C_{16}G_2$ have been further investigated along with those formed by Span 60 (sorbitan monostearate) (Figure 2.1), a non-ionic surfactant commonly used to form vesicles. The morphology of niosomes was studied using various microscopic techniques including light

microscopy, transmission electron microscopy (TEM), and cryo-scanning electron microscopy (cryo-SEM), which reveal a variety of vesicle structures i.e. polyhedral, spherical, and tubular forms, depending on membrane composition, temperature, and preparation methods. The ability of a series of poly (oxyethylene) surfactants and some phospholipids (Figure 2.1) to form vesicles with various shapes in the same fashion as that found in $C_{16}G_2$ were also studied. The phase behaviour of niosomal membranes was investigated using high sensitivity differential scanning calorimetry. The interaction between bilayer membranes of polyhedral niosomes and spherical niosomes was also studied in order to exploit the polyhedral structures as a tool to determine transfer of membrane components. Laser diffraction and photon correlation spectroscopy were used for measuring mean vesicle diameter. The encapsulation efficiency of vesicles and their membrane permeability were studied using a fluorescence marker, 5(6)-carboxyfluorescein (CF). The ability of cholesterol to reduce membrane permeability was also exploited in an established multi-component system which contains a cocktail of vesicles with various cholesterol contents.

MATERIALS AND METHODS

2.2. MATERIALS

The materials used in the various experiments in this chapter are as given in Table 2.1. All reagents and chemicals were of analytical grade, unless otherwise stated. All materials were used as obtained from suppliers without further purification and the water source was from an ultra high quality reverse osmosis water purifier (Elgastat UHQPS - Elga, UK).

Table 2.1: Materials used

MATERIAL	SOURCE
5(6)-carboxyfluorescein (CF)	Sigma Chemical Company (UK)
$C_{16}EO_2$ (polyoxyethylene 2 cetyl ether, Brij 52)	Sigma Chemical Company (UK)
$C_{16}EO_5$ (polyoxyethylene 5 cetyl ether)	Sigma Chemical Company (UK)
$C_{16}G_2$ (hexadecyl diglycerol ether)	Donated by L'Oreal (France)
$C_{18}EO_2$ (polyoxyethylene 2 stearyl ether, Brij 72)	Sigma Chemical Company (UK)
$C_{18}EO_5$ (polyoxyethylene 5 stearyl ether)	Sigma Chemical Company (UK)
Chloroform (HPLC grade)	Rathburn Chemicals Ltd (UK)
Cholesterol	Sigma Chemical Company (UK)
1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)	Lipoid GmbH (Germany)
1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC)	Lipoid GmbH (Germany)
Disodium hydrogen phosphate	BDH Laboratory Supplies (UK)
Hydrochloric acid	BDH Laboratory Supplies (UK)
Isopropanol	BDH Laboratory Supplies (UK)
Phosphotungstic acid	TAAB Laboratories equipment (UK)
Potassium chloride	BDH Laboratory Supplies (UK)
Sodium dihydrogen phosphate	BDH Laboratory Supplies (UK)
Sodium chloride	BDH Laboratory Supplies (UK)
Sodium hydroxide	BDH Laboratory Supplies (UK)
Solulan C24 (poly-24-oxyethylene cholesteryl ether)	Donated by Ellis & Everald (UK)
Span 60 (sorbitan monostearate)	Sigma Chemical Company (UK)
Trizma HCl (tris(hydroxymethyl) aminomethane HCl)	Sigma Chemical Company (UK)
D-alpha tocopheryl polyethylene glycol 1000 succinate (TPGS)	Eastman Chemical Company (USA)

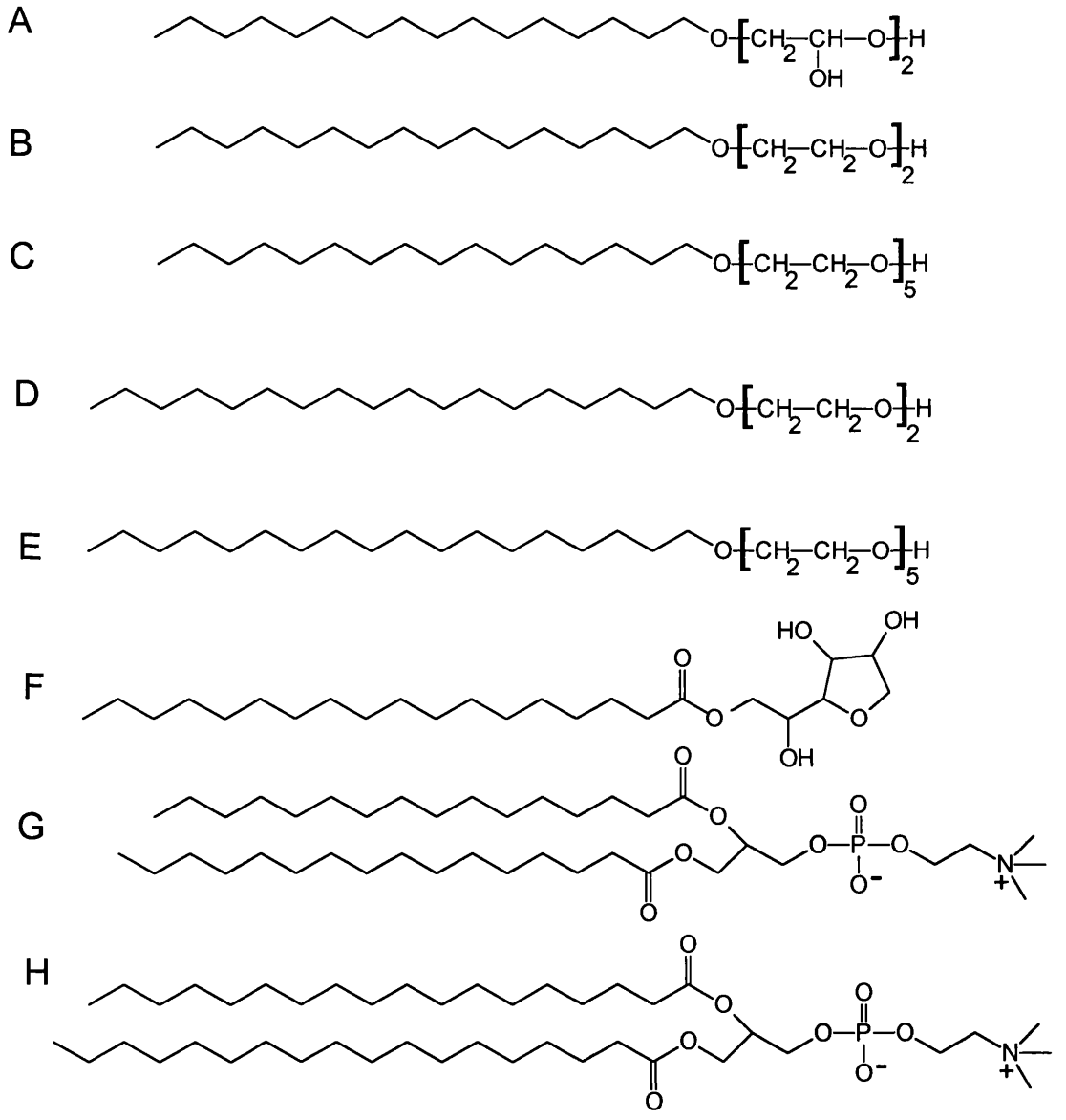


Figure 2.1: Molecular structures of vesicle forming surfactants and phospholipids used in the studies. A = $C_{16}G_2$, B = $C_{16}EO_2$, C = $C_{16}EO_5$, D = $C_{18}EO_2$, E = $C_{18}EO_5$, F = Span 60, G = DPPC, H = DSPC

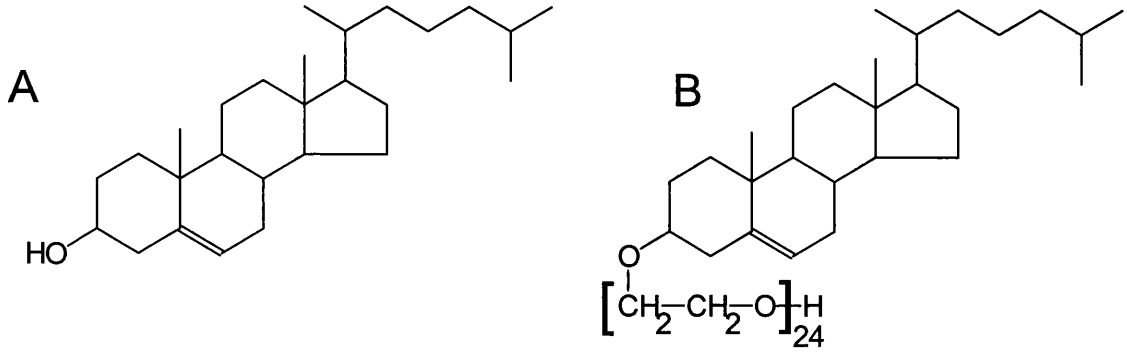


Figure 2.2: Molecular structures of (A) cholesterol and (B) Solulan C24

METHODS

2.3. GENERAL METHODS FOR PREPARATION OF NIOSOMES

Lipid/surfactant mixtures were dissolved in chloroform, and the solvent was removed under reduced pressure at 60°C, in a round bottom flask. Residual organic solvent was removed by drying the lipid film under a stream of nitrogen for 15 min. The obtained lipid/ surfactant film was then hydrated, with either water, 5mM CF in phosphate buffered saline (PBS; NaCl 140mM, Na_2HPO_4 0.18mM, $NaH_2PO_4.2H_20$ 3.2mM, KCl 2.7mM, pH adjusted with 1M NaOH), or in Tris buffered saline (TBS; Trizma HCl 10mM, NaCl 140mM, pH adjusted with 1M NaOH) (generally at pH 7.4, unless otherwise stated), at 60°C for 1 h with constant shaking on a mechanical shaker. The dispersions were then probe sonicated if small vesicles were required. Niosome dispersions, with a final lipid/surfactant concentration of 60 mM, were left to cool at room temperature and then kept overnight at 4°C before

experiments.

2.4. EFFECT OF MEMBRANE COMPOSITION ON VESICLE SHAPE

2.4.1. Cryo-scanning electron microscopy (Cryo-SEM)

Polyhedral and spherical/ tubular niosomes prepared from $C_{16}G_2$, Cholesterol, Solulan C24 in water were plunge frozen in Liquid Nitrogen Slush, transferred to the Philips XL20 scanning electron microscope and surface moisture removed by sublimation (at -40°C for 10 min). Samples were transferred back to a cryo-work station, gold coated at -180°C and photographed by SEM.

2.4.2. Temperature-induced vesicle shape transformation

Changes in morphology of niosomes were observed using a LINKAM system (BCS196) with temperature control fitted to a Nikon Microphot FXA light microscope.

2.4.3. An investigation into phase transition behaviour of polyhedral niosomes

The phase behaviour of niosomes with various membrane compositions were studied by using a high sensitivity differential scanning calorimeter (HSDSC) (MicroDSC, Setaram, UK). Approximately 300 mg of each niosome dispersion prepared in water, was introduced into the measurement vessel whilst an equivalent mass of water was introduced into the reference vessel. Samples were scanned at a rate of 1K/min from 10 to 70°C, followed by cooling to 10°C. The difference in heat flow between both vessels was measured as a function of temperature.

2.4.4. Transfer of cholesterol from spherical/ tubular niosomes to polyhedral niosomes

Polyhedral niosomes formed mainly by $C_{16}G_2$ or $C_{16}EO_5$ without cholesterol were mixed with equal amounts of their spherical/ tubular counterparts which contain equimolar quantities of cholesterol, sonicated or unsonicated. The mixtures were incubated at room temperature, or above their phase transition temperature (55°C) and left to cool at room temperature for 1 h prior to observation. The change in morphology of the polyhedral niosomes was observed using light microscopy.

2.5. EFFECT OF MEMBRANE COMPOSITION ON VESICLE SIZE AND ENCAPSULATION EFFICIENCY

2.5.1. Size of unsonicated niosomes

Niosomes prepared mainly from Span 60 and $C_{16}G_2$ with various amounts of cholesterol were sized on a Malvern MasterSizer X (UK) by diluting the dispersion with water. Results are expressed as mean volume diameters.

2.5.2. Size stability of sonicated $C_{16}G_2$ niosomes

Polyhedral and spherical/ tubular niosome dispersions formed by $C_{16}G_2$, Cholesterol, Solulan C24 were probe sonicated for 4 min, using an MSE PG100 150W probe sonicator with the instrument set at 15% of its maximum power output, and then left either at room temperature or 60°C. Size stability after sonication of submicron niosomes was followed by using the photon correlation spectroscopy (PCS) on a Malvern AutoSizer 2C, (UK) at 25°C by diluting the dispersion to 4 ml in filtered (0.22 μm pore size) water. Results are expressed as the z-average mean diameter.

2.5.3. Transmission electron microscopy (TEM)

Freshly sonicated and unsonicated polyhedral niosomes prepared from $C_{16}G_2$, Cholesterol, Solulan C24 in water were stained with 1% aqueous phosphotungstic acid prior to viewing. Photographs were taken at 100 kV using a Philips 201 transmission electron microscope.

2.5.4. Separation of unencapsulated material and determination of encapsulation efficiency

Niosomes were prepared loading CF as described in **2.3**. For Span 60 niosomes, the dispersions were probe sonicated for 4 min. 1 ml of the dispersion was washed by dilution with 7 ml of water or PBS (pH 7.4) and ultracentrifuged at 200,000 g (Sorvall Combi Plus, Sorvall Ltd., UK.) at 4°C for 45 min. The supernatant was discarded and the niosome pellets were resuspended with water or PBS (pH 7.4) before washing again. The washed pellets were then resuspended with 1 ml of water or PBS (pH 7.4). 0.1 ml of the dispersions was added into the 10 ml volumetric flask. To the flask was added 1 ml of isopropanol to disrupt the niosomes. The volume was then made up with water or PBS (pH 7.4). Further dilutions may be prepared in the case of the solution being too concentrated. A calibration plot was produced by diluting stock solutions of CF with water or PBS (pH 7.4) whilst ensuring that final dilutions contained 10%v/v isopropanol. The fluorescence of these solutions was measured on a Perkin Elmer LS-3 fluorescence spectrometer (excitation 486 nm, emission 514 nm). Encapsulation efficiency was calculated and expressed as a percentage of the available hydrating solute actually encapsulated.

2.6. MEMBRANE PERMEABILITY OF NIOSOMES

2.6.1. Release of CF from Span 60 niosomes

Span 60 niosomes were prepared entrapping CF, which were washed and the entrapment efficiencies estimated as previously described in sections 2.3 and 2.5.4. The release profiles of CF solution in PBS (pH 7.4), CF entrapped into niosomes formed by Span 60: cholesterol: TPGS (75: 15: 10), (60: 30: 10), (45: 45: 10); and also a mixture of empty niosomes (Span 60: cholesterol: TPGS, 45: 45: 10) and CF solution were studied by placing the dispersions (each sample contained 7.7 nmol of CF) into dialysis tubing (MW cut off = 12-14 kDa) suspended in 45 ml of PBS (pH 7.4) at 37°C. The dialysate was collected and analysed fluorimetrically over a 24 h period. Mixtures of CF prepared in PBS, CF entrapped in niosomes with 30% cholesterol, and in niosomes with 45% cholesterol (each portion contained 2.57 nmol of CF) were also studied in order to prepare multi-component systems with a given release profile.

2.6.2. Release of CF from $C_{16}G_2$ niosomes

Polyhedral and spherical/ tubular niosomes were formed by hydrating dry film of $C_{16}G_2$: cholesterol: Solulan C24 (91: 0: 9) and (45: 45: 10), respectively, with CF solution prepared in Tris buffered saline (TBS pH 7.4), washed and estimated the entrapment efficacy as previously described (section 2.3 and 2.5.4). The release of CF at room temperature was studied by placing 0.5 ml of niosome dispersions in dialysis tubing suspended in 40 ml of TBS. The dialysates were collected and analysed fluorimetrically over a 5 h period.

RESULTS AND DISCUSSIONS

2.7. EFFECT OF MEMBRANE COMPOSITION ON VESICLE SHAPE

It has been previously reported that polyhedral niosomes can be formed by $C_{16}G_2$ and $C_{16}G_3$ (a hexadecyl triglyceryl ether) in the absence or with very low amount of cholesterol (Cable & Florence, 1988; Uchegbu *et al.*, 1996). In addition, we also found that such polyhedral niosomes can be formed, in the absence of cholesterol, by a series of poly (oxyethylene) surfactants i.e. $C_{16}EO_2$, $C_{16}EO_5$, $C_{18}EO_2$, $C_{18}EO_5$, and a phospholipid DPPC but not with DSPC (Figure 2.1). Figure 2.3 showed some examples of polyhedral structures which, although possessing a crystal-like appearance, can encapsulate a hydrophilic solute CF as conventional spherical vesicles. It is noteworthy that these surfactants alone can form polyhedral niosomes, as the addition of low amounts of Solulan C24 (Figure 2.2) does not affect the shape of the vesicles, but provides a steric barrier on the vesicle surface which can prevent aggregation. As happens with $C_{16}G_2$ and $C_{16}G_3$ vesicles, these polyhedral structures no longer exist when the niosome dispersions were prepared with an equimolar of cholesterol and surfactants, and the spherical vesicles are formed (Figure 2.4). The morphology of such formulations was also observed using the cryo-SEM (Figure 2.5). Figure 2.5a showed that niosomes formed by $C_{16}G_2$: Solulan C24 (91:9), without cholesterol, are polyhedral in shape while those formed by $C_{16}G_2$: cholesterol: Solulan C24 (45:45:10) gave mixtures of spherical (Figure 2.5b) and tubular vesicles (Figure 2.5c&d).

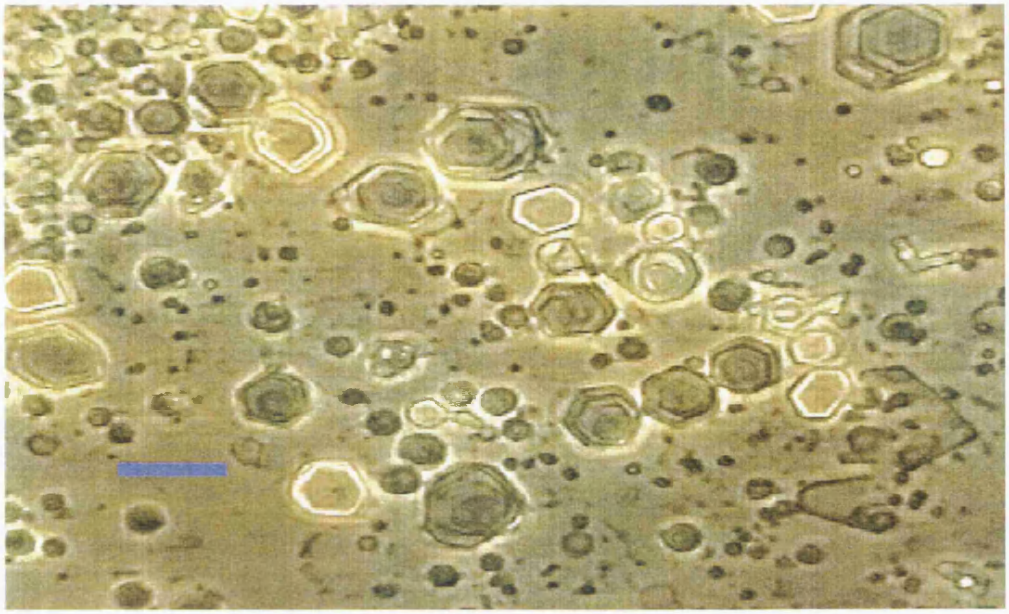


Figure 2.3a: Phase contrast videomicrograph of polyhedral niosomes formed by $C_{16}EO_5$ alone in PBS, pH 7.4. Bar = 20 μm.

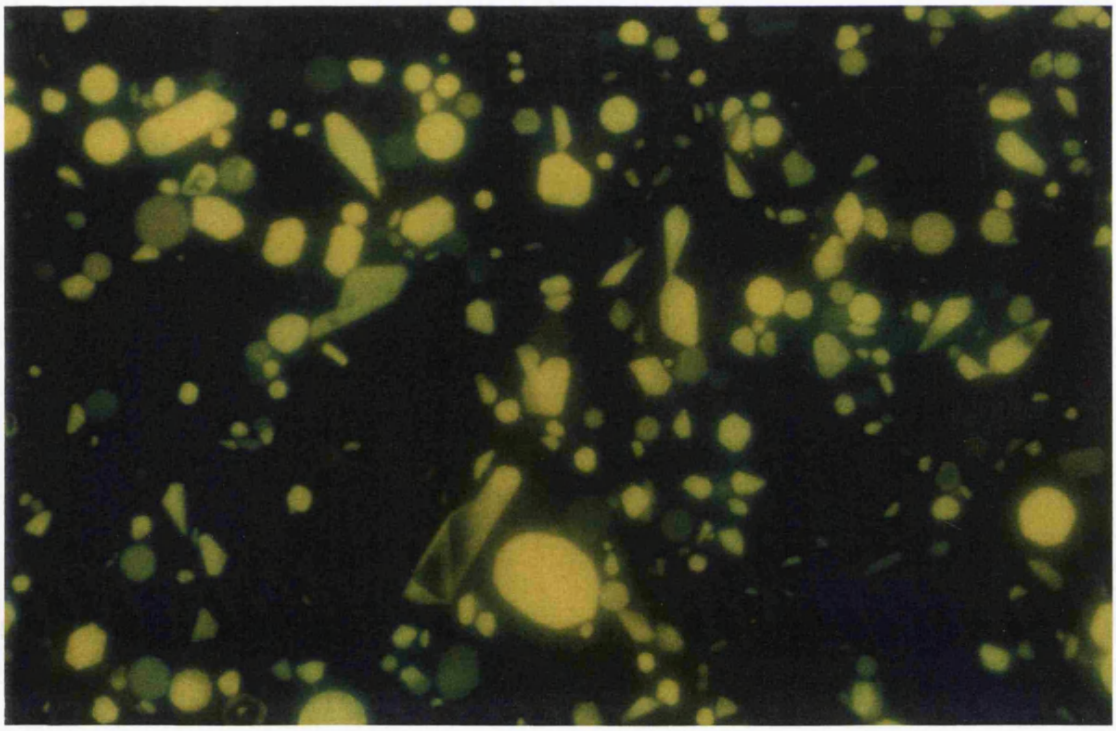


Figure 2.3b: Photomicrograph of CF loaded polyhedral niosomes formed by $C_{16}G_2$: Solulan C24 (91:9) in PBS, pH 7.4 observed under fluorescent light. 50 μm.

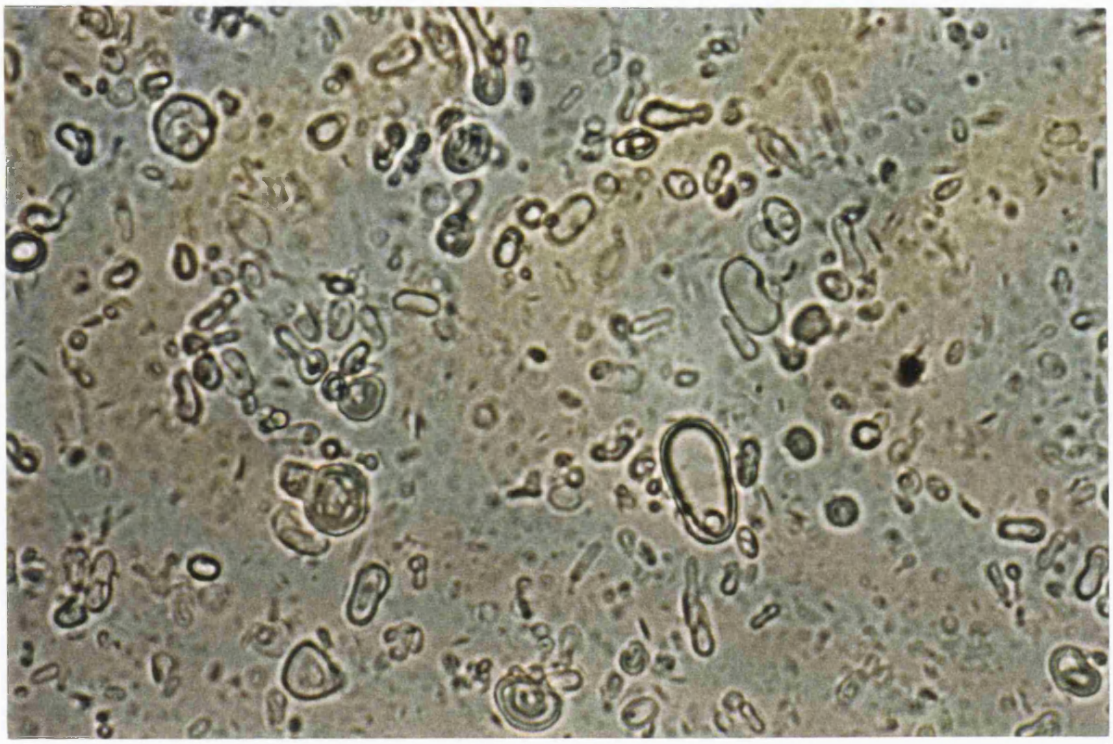


Figure 2.4: Photomicrograph of spherical niosomes formed by $C_{16}EO_5$: cholesterol: Solulan C24 (45:45:10). 20 μm.

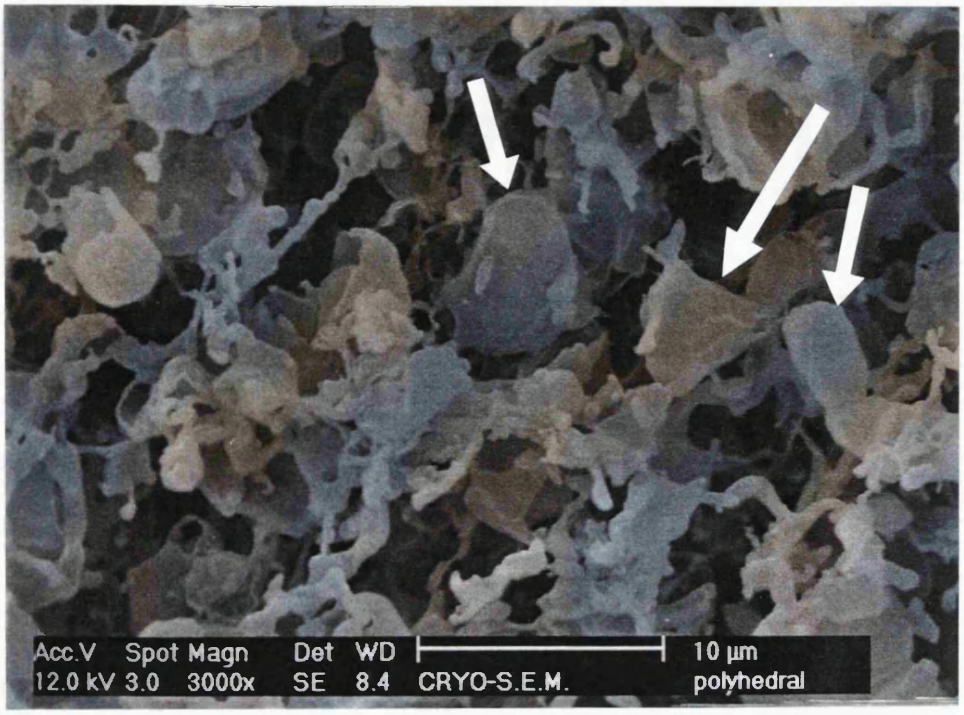


Figure 2.5a: Cryo-scanning electron micrograph of polyhedral niosomes formed by $C_{16}G_2$: Solulan C24 (91:9) in water.

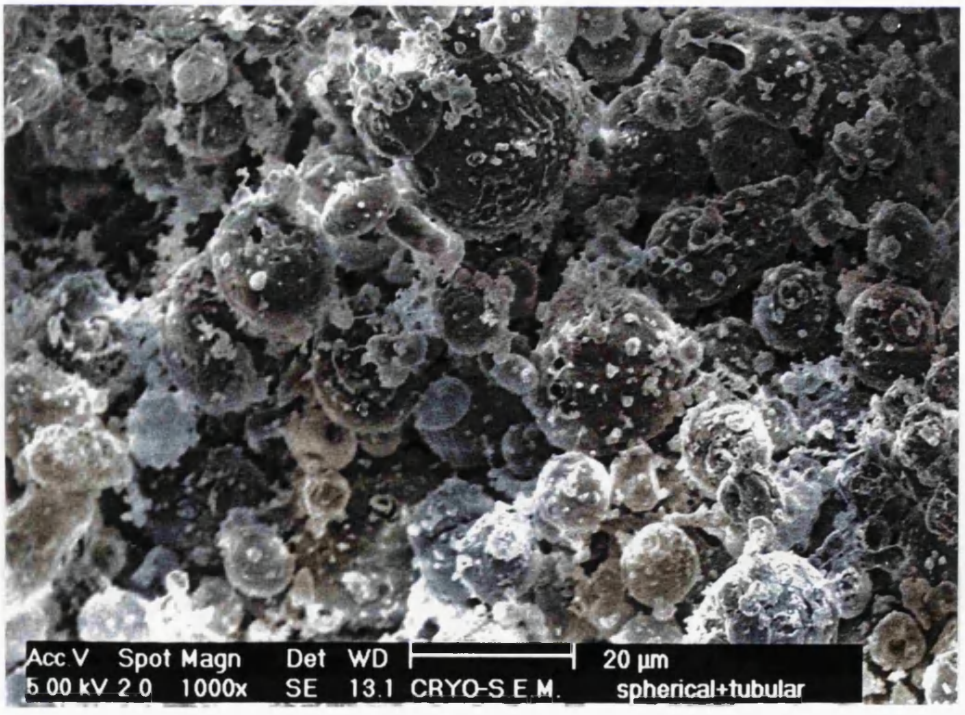


Figure 2.5b: Cryo-scanning electron micrograph of spherical niosomes formed by $C_{16}G_2$: cholesterol: Solulan C24 (45:45:10) in water.

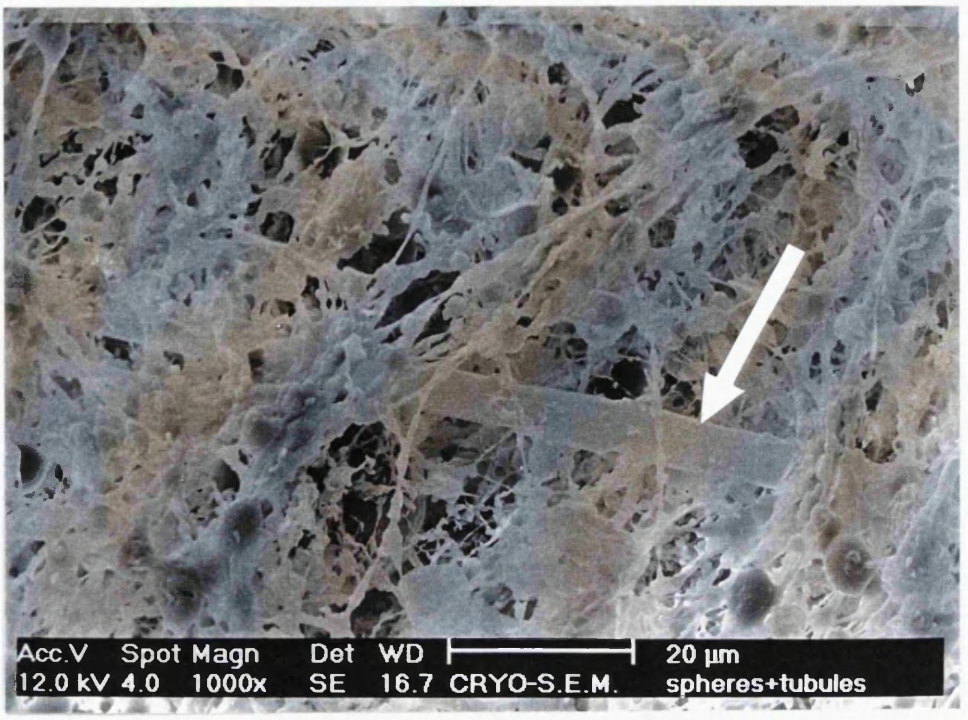


Figure 2.5c

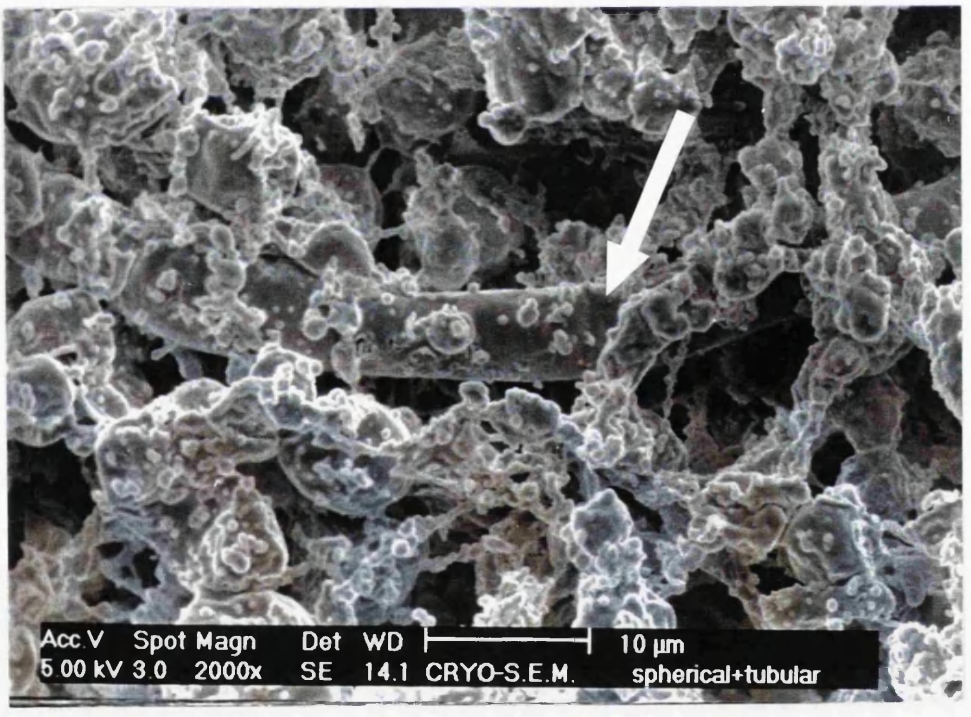


Figure 2.5d

Figure 2.5c & d: Cryo-scanning electron micrographs of tubular niosomes found in the dispersion of spherical niosomes formed by $C_{16}G_2$: cholesterol: Solulan C24 (45:45:10) in water.

2.7.1. Temperature-induced vesicle shape transformation

An observation of freshly prepared polyhedral niosomes revealed that these niosomes are first formed as spherical vesicles which, on cooling, transform into polyhedral structures. We therefore studied the morphological changes of these polyhedral structures using the hot stage microscope. On increasing the temperature, these polyhedral vesicles undergo a reversible shape transformation into spherical structures which were found to develop polyhedral structures on cooling down (Figure 2.6). The temperature in which the shape transformation occurs was found to depend on the type of surfactants (Table 2.2). Figure 2.7 showed a polyhedral vesicle formed by $C_{16}G_2$: Solulan C24 (91:9) which, on heating, transformed into a spherical vesicle, and on cooling, developed a group of small spherical vesicles before returning to polyhedral structures . For spherical/ tubular niosomes formed by $C_{16}G_2$:Cholesterol: Solulan C24 (45:45:10), fluorescence microscopy showed that vesicles remained morphologically intact even when the temperature was raised. However, on increasing the temperature to 80°C, vesicular "budding off" was seen, indicating a limitation in dimensions to keep the membranes in a stable conformation (Figure 2.8). This cytomimetic behaviour has previously been explained on the basis that an excess membrane area exists on heating which leads to an increase in the area-to-volume ratio and hence vesicular budding (Döbereiner *et al.*, 1993).

Figure 2.6a

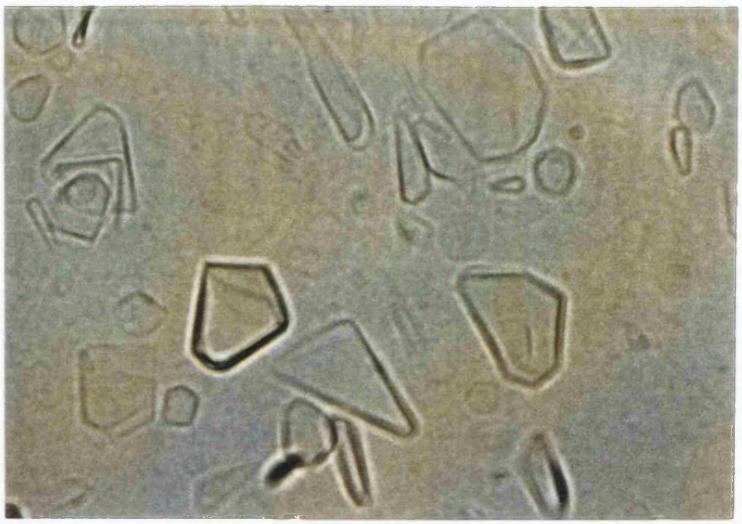


Figure 2.6b

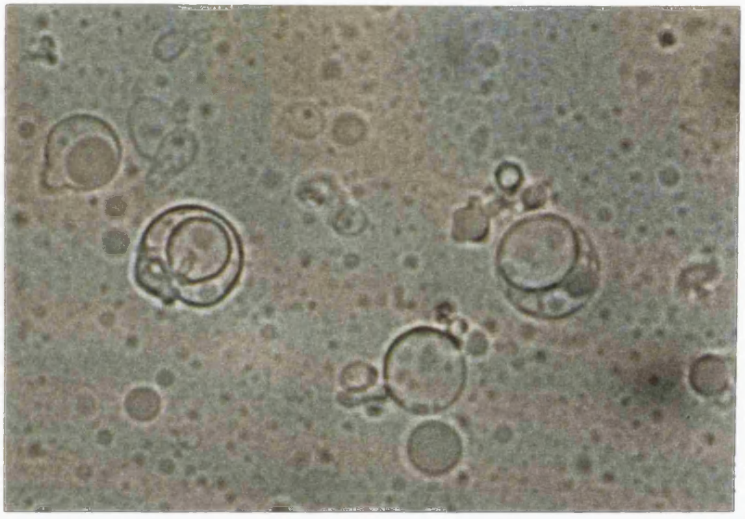


Figure 2.6c

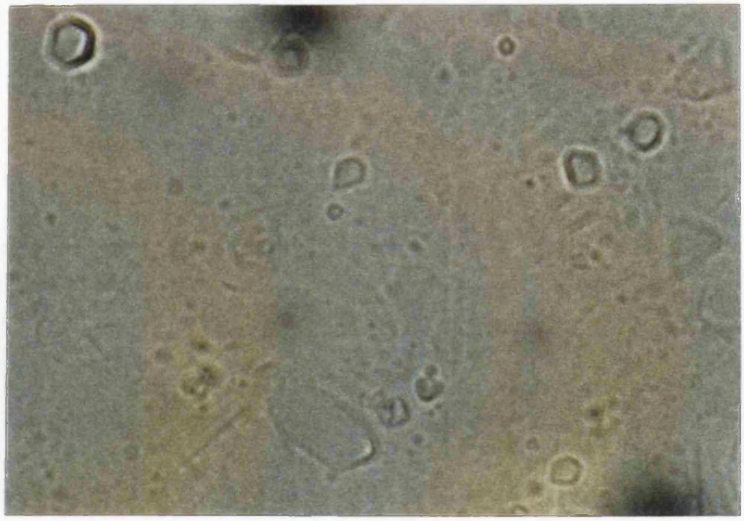


Figure 2.6: Photomicrographs of (a) polyhedral niosomes of $C_{16}EO_5$: Solulan C24 (91: 9) in water at room temperature which undergo a reversible shape transformation into (b) spherical niosomes on heating to 35°C, and then return to (c) polyhedral structures on cooling to 30°C. 20 μm

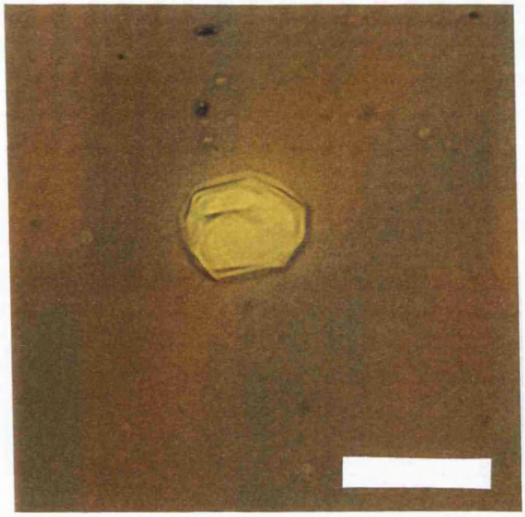


Figure 2.7a

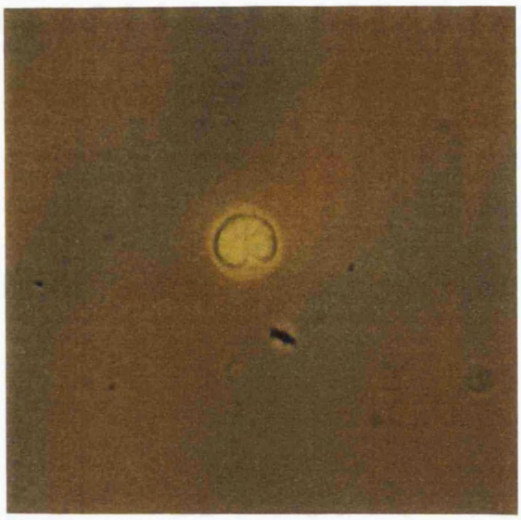


Figure 2.7b

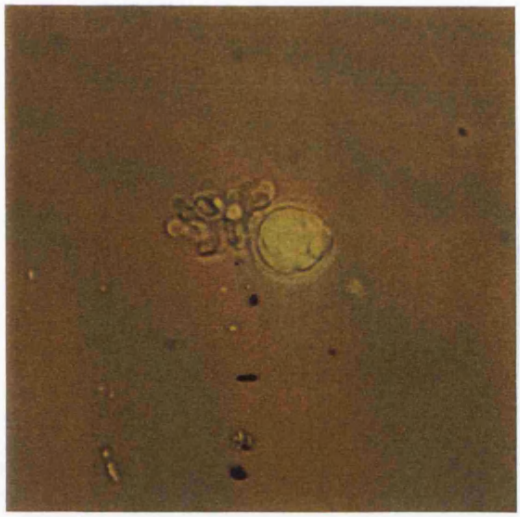


Figure 2.7c

Figure 2.7d

Figure 2.7: Fluorescence photomicrographs of a single CF loaded polyhedral vesicle formed by $C_{16}G_2$: Solulan C24 (91: 9) in PBS, pH 7.4 (a) at room temperature which was transformed into (b) a spherical vesicle on heating to 48°C. On cooling, the spherical vesicle splits into (c) a group of small spherical vesicles at 49°C and then again developed a faceted appearance at 35°C (d). 50 μm

Figure 2.8a

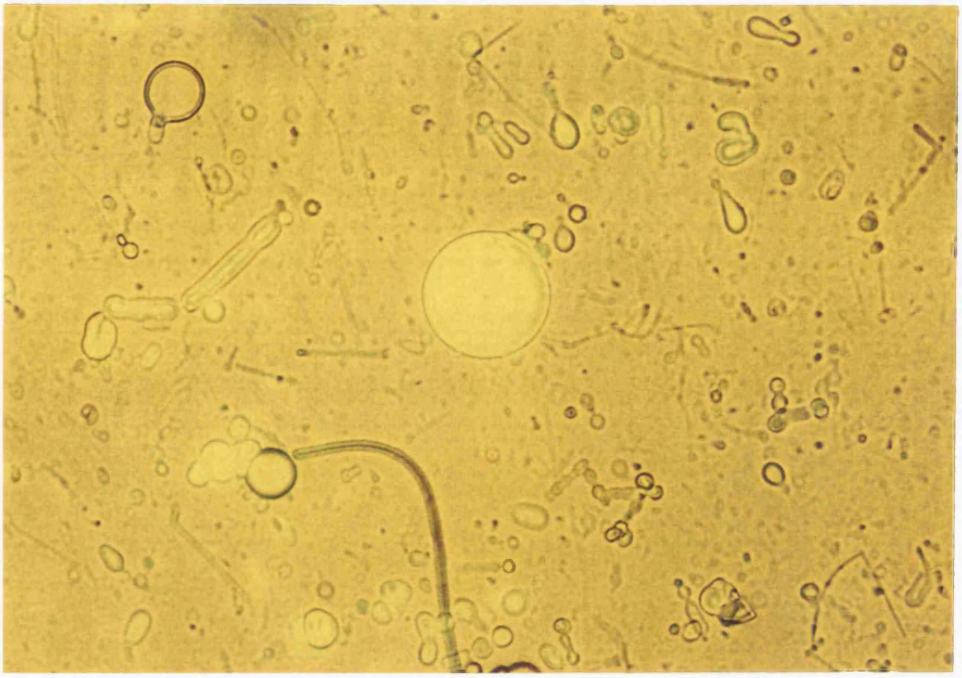


Figure 2.8b

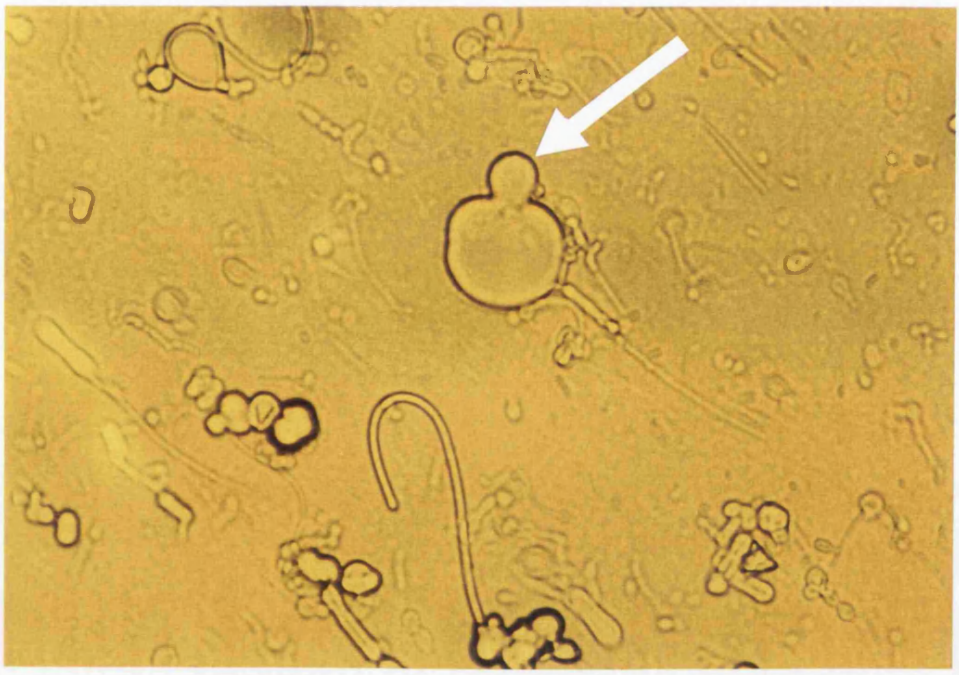


Figure 2.8: (a) Spherical/ tubular niosomes formed by $C_{16}G_2$: cholesterol: Solulan C24 (45:45:10) found to be intact on heating up to (b) 80°C where the vesicular "budding off" (→) was observed. 50μm

2.7.2. Phase transition behaviour of polyhedral and spherical/ tubular niosomes

The HSDSC studies were performed using niosomes formed by 91 mol% of $C_{16}G_2$, $C_{16}EO_2$, $C_{16}EO_5$, $C_{18}EO_2$, or $C_{18}EO_5$ with 9 mol% Solulan C24. The DSC traces of these polyhedral niosomes generally show a main endothermic transition on heating and a main exothermic transition on cooling (Figure 2.9). It was found that the temperature at which the vesicle membranes change from gel state to liquid state corresponds to the temperature at which the change of the polyhedral structure into the spherical shape occurs and this was also found to depend on the type of surfactants (Table 2.2). It is noteworthy that the DSC trace of polyhedral niosomes formed by $C_{16}G_2$:Solulan C24 (91:9) clearly showed a second transition after the main exothermic event (Figure 2.9). This, to some extent, may correspond to two events, the splitting of an adopted spherical vesicle into a group of small spheres and the re-formation of the polyhedral structures, observed on cooling down the heated vesicles (Figure 2.7). Membrane permeability of polyhedral niosomes was previously found to be dependant on temperature (Uchegbu *et al.*, 1997). Changing from the gel to the liquid phase by increasing the temperature above the phase transition temperature also changes their ultrastructures. The HSDSC therefore allows precise quantification of the temperature and energy associated with this transition. Considering the temperature at which the shape transformation of polyhedral niosomes formed by other surfactants take place (Table 2.2), it is noteworthy that the temperature detected agrees well with previous studies of poly (oxyethylene) alkyl ethers using HSDSC in that increasing of the alkyl chain length and decreasing of the poly (oxyethylene) chain length lead to an increase in transition temperature (Buckton *et al.*, 1992) hence the shape transformation temperature.

Although polyhedral vesicles formed by the surfactants used in this study are found below their phase transition temperature, it is not always the case that any gel state membranes will possess such faceted structures. For example, in the case of two phospholipids, DPPC (T_m = 41°C) and DSPC (T_m = 55°C) (Figure 2.1), we found that vesicles formed by DPPC, in the absence of cholesterol, also possess polyhedral structures, but those formed by DSPC appear to be spherical in shape at room temperature. This finding confirms that the ratio of molecular volume and surface area is an important factor which determines the arrangement of the amphiphiles in the membranes. Further investigations may be needed in order to predict the shape of vesicles formed by certain amphiphiles.

Table 2.2: Phase transition temperatures (T_m)† and the temperature at which polyhedral niosomes undergo a shape transformation into the spherical structures (T_{st})‡.

Polyhedral niosomes	T_m (°C)†	T_{st} (°C)‡
$C_{16}G_2$: Solulan C24 (91:9)	45.4	48
$C_{16}EO_5$: Solulan C24 (91:9)	29.0	35
$C_{16}EO_2$: Solulan C24 (91:9)	37.8, 47.2*	45
$C_{18}EO_5$: Solulan C24 (91:9)	48	60
$C_{18}EO_2$: Solulan C24 (91:9)	58.3	65

*$C_{16}EO_2$ used in the experiments contains antioxidants and shows 2 main transition peaks

† by HSDSC.

‡ by hot stage microscopy.

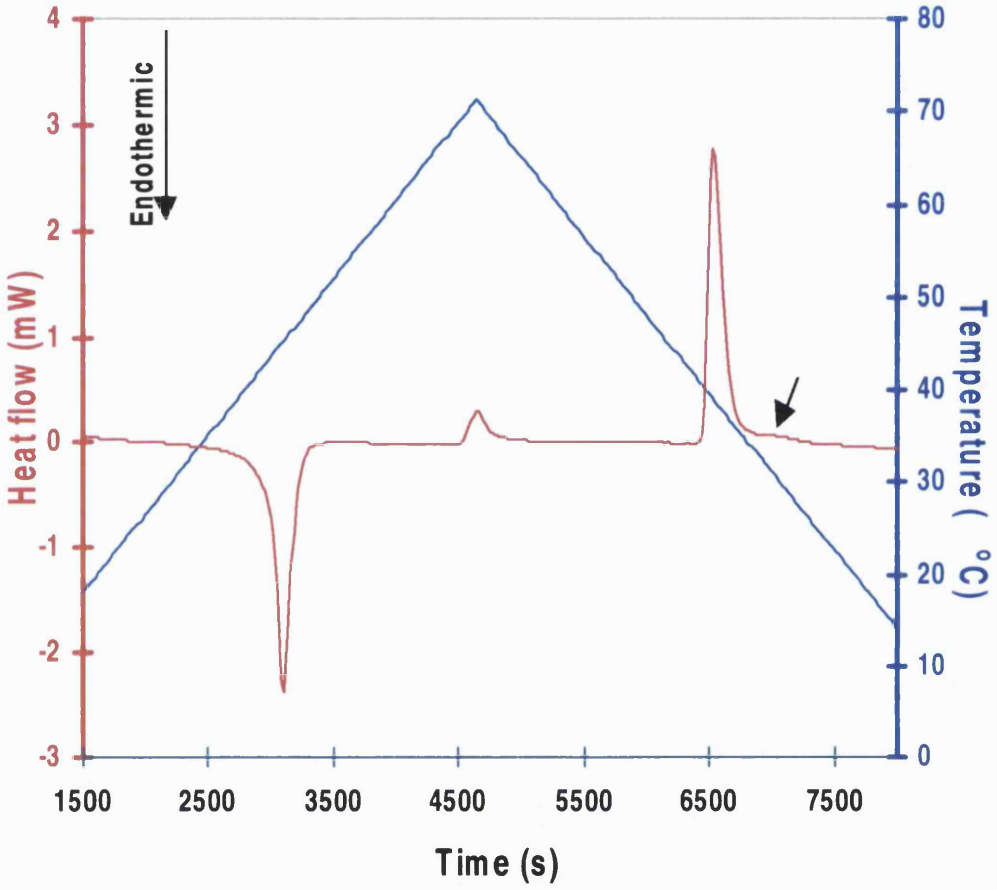


Figure 2.9: A DSC trace of polyhedral niosomes ($C_{16}G_2$: Solulan C24, 91:9) which shows a main endothermic transformation at 45°C on heating, with an exothermic transformation detected on cooling at 39°C. A second transition was also detected after the main exothermic peak (➔).

2.7.2.a. Effect of cholesterol on niosome phase transition behaviour

Increasing the amount of cholesterol into gel phase membranes of the liposomes has been found to increase membrane fluidity to the extent where the phase transition is abolished (Taylor & Morris, 1995). In this study cholesterol was added to polyhedral niosomes at concentrations varying from 2.5% to 10% and also 45% which resulted in the formation of spherical/ tubular niosomes. The HSDSC traces of these dispersions showed that cholesterol decreased the temperature and energy associated with the phase transition (Figure 2.10) in a manner analogous to that seen for liposomal systems.

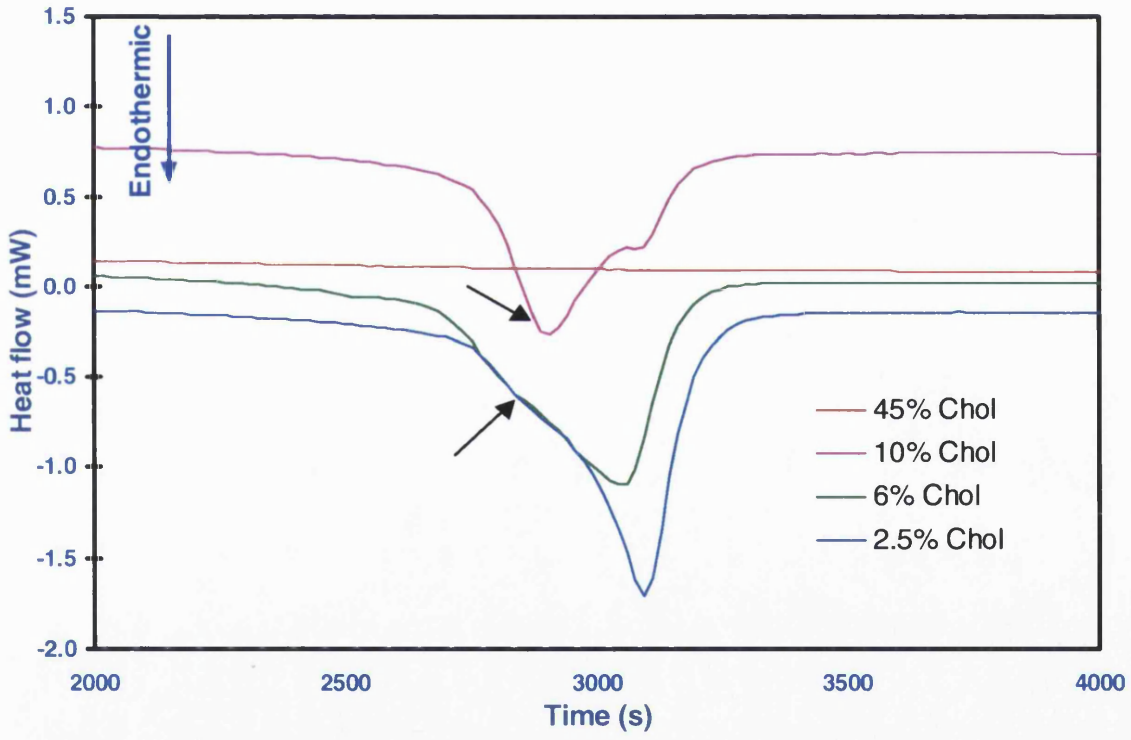


Figure 2.10: Endothermic events of niosomes formed by $C_{16}G_2$, cholesterol, and 10% Solulan C24. On increasing the cholesterol content in the membranes, the energy and temperature involving the transitions decrease and the pretransition (➔) was enhanced.

2.7.2.b. Effect of Solulan C24 on niosome phase transition behaviour

Polyhedral niosomes are formed by $C_{16}G_2$ with low levels of cholesterol and Solulan C24. The molecule of Solulan C24 has a cholesterol moiety, which penetrates into the hydrophobic core of vesicle membranes, covalently attached to a polyethyleneglycol (PEG) chain (Figure 2.2), which provide a steric barrier at the vesicle surface. In the absence of Solulan C24, $C_{16}G_2$ still forms such faceted structures although aggregation can be found. On increasing the level of Solulan C24, large disc-shape vesicles, so called "discomes", are formed (Uchegbu *et al.*, 1992 & 1996) (Figure 1.10a). Such slightly curved vesicles with some straight edges suggests that Solulan C24 allows faceted $C_{16}G_2$ to form membranes with higher curvature, perhaps in the same way as cholesterol although with less potential. Cholesterol was found to promote the pretransition peak of $C_{16}G_2$ systems (Figure 2.10). It is noteworthy that on increasing the amount of Solulan C24, a promotion of the pretransition on the endothermic peak and 2nd transition on the exothermic peak (Figure 2.11) was also observed. We also found a decrease in T_m and enthalpy in the main endothermic peak; however, in the smaller degree when comparing to an addition of cholesterol (Figure 2.12). Although the cholesterol moiety of Solulan C24, as a hydrophobic part of the molecule, is incorporated into the bilayer of $C_{16}G_2$ membranes, the ability to change the thermal behaviour of the membranes is somehow different from that of cholesterol. This finding indicates that the alteration in the structure of cholesterol results in the change in molecular orientation of other membrane components (Bernsdorff *et al.*, 1996), and sterol molecule itself, as observed with androstenol and cholesterol (McMullen *et al.*, 1994), in the bilayer membranes, hence the change in thermal behaviour of membranes.

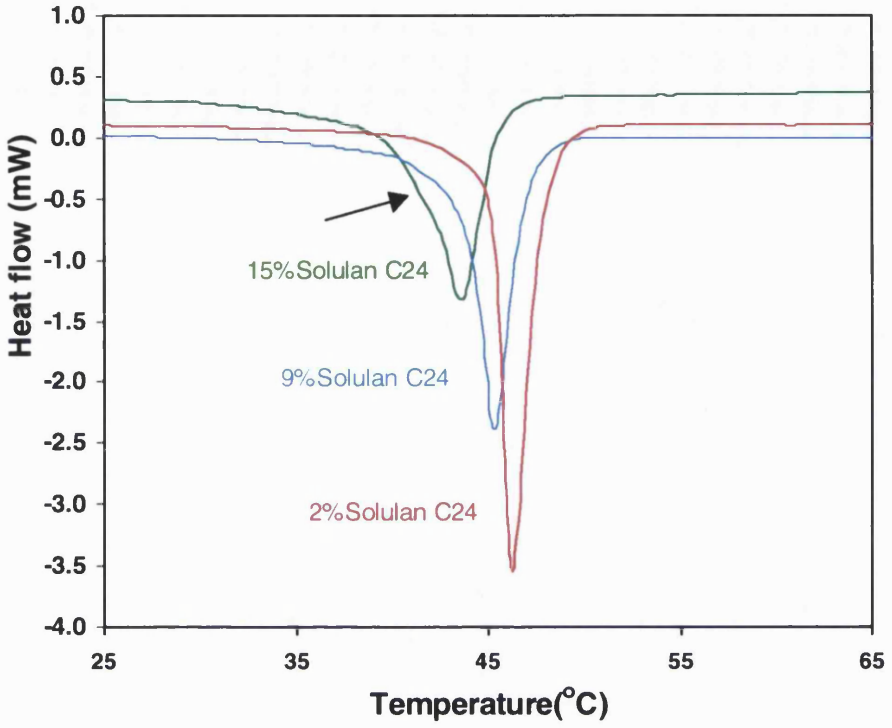


Figure 2.11a: Endothermic transformation of polyhedral niosomes formed by $C_{16}G_2$ in water on increasing the level of Solulan C24 to 15 mol%. The promotion of pretransition was observed on heating the sample up.

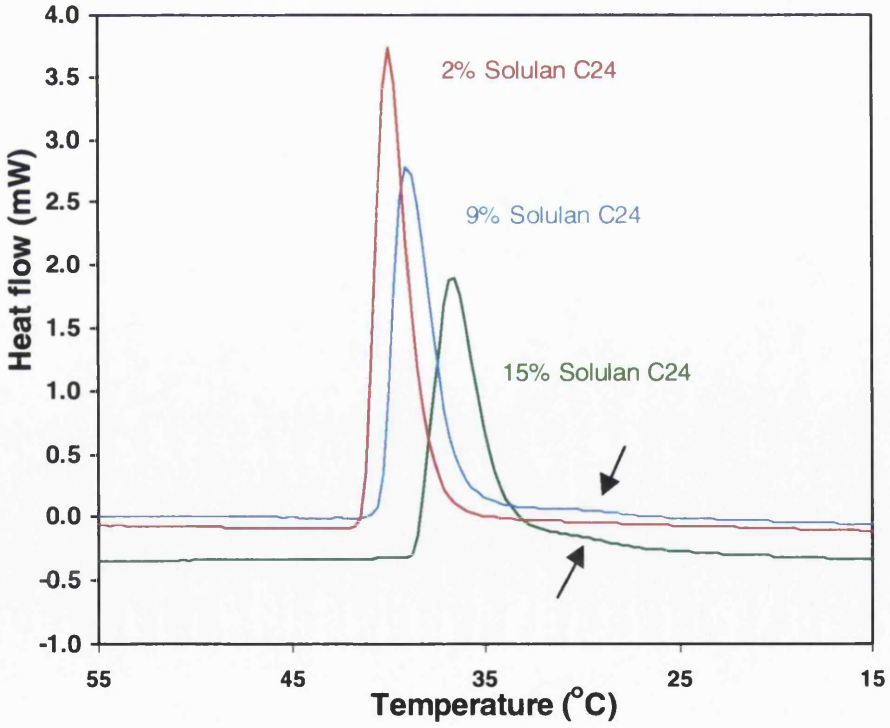


Figure 2.11b: Exothermic transformation of polyhedral niosomes formed by $C_{16}G_2$ in water on increasing the level of Solulan C24 to 15 mol%. The promotion of 2nd transition was observed on cooling.

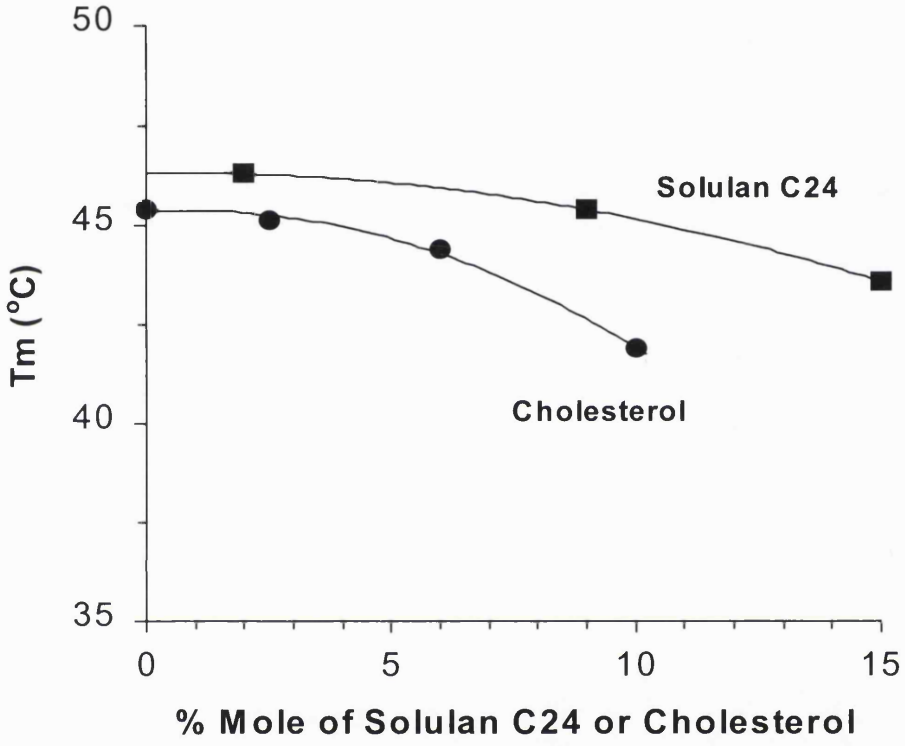


Figure 2.12: The T_m of polyhedral niosomes is reduced by increasing cholesterol in the systems formed by $C_{16}G_2$ and 10%Solulan C24 (●); and by increasing Solulan C24 in $C_{16}G_2$ systems without cholesterol (■).

2.7.3. Cholesterol transfer: observation through shape transformation

The interactions between lipid bilayers have been of great interest in recent years since such interactions play an important role in e.g. endocytosis, exocytosis, or infection processes of enveloped viruses. *In vivo*, liposomes have been found to undergo various membrane interactions with cells such as binding to cell surfaces, endocytosis and fusion, either at the cell surface or in prelysosomes. Liposomes have also been used for studying membrane interaction processes, for example, as a system to deliver cholesterol to cell membranes (Bittman, 1993; Yeagle, 1991). By application of fluorescent amphiphiles, the change of membrane components among vesicles can be quantified (Baraka *et al.*, 1996; Ott *et al.*, 1994; Ott & Wunderli-Allenspach, 1994). Here we show that polyhedral vesicles may be used qualitatively as a tool to provide evidence of the inter-bilayer transfer of cholesterol.

Mixtures of cholesterol-free polyhedral niosomes and their spherical counterparts which contain cholesterol in their membranes were incubated either at room temperature, or above their phase transition temperature in order to mimic the liquid phase membranes in nature (Bittman, 1993) which were then left to cool to room temperature for 1 h prior to light microscopy. Figure 2.13 shows that incubation of the mixtures of polyhedral niosomes of $C_{16}G_2$: Solulan C24 (91:9), and spherical niosomes of $C_{16}G_2$: cholesterol: Solulan C24 (45:45:10), above their phase transition temperature allows polyhedral niosomes to adopt curved membranes along some of their original faceted membranes. It is noteworthy that no significant differences were observed when sonicated or unsonicated spherical niosomes were used. As we have previously shown, increasing the amount of cholesterol in the systems allows $C_{16}G_2$ to form membranes with higher curvature (see section 2.7), this finding therefore suggests the presence of cholesterol in such curved regions. The membrane components can be transferred between bilayers by collision of the donor and acceptor

membranes which may be followed by a fusion process, and/ or through the aqueous phase without contact of the donor and acceptor membranes (Ott *et al.*, 1994; Yeagle, 1993). To specify the mechanisms by which the cholesterol transfer occurred would be complex, although the presence of large vesicles (Figure 2.13 b, e) may suggest that fusion processes may participate. In nature, the amount of cholesterol has been found to be different among different cell membranes; for example, the level of cholesterol in plasma membranes and secretory vesicles is higher than that found in the endoplasmic reticulum and mitochondria (Bittman, 1993). Our findings indicate that, even within a membrane, the distribution of cholesterol can be highly asymmetric. For the mixtures of polyhedral niosomes and spherical niosomes formed by $C_{16}EO_5$, we were not able to find polyhedral niosomes after incubation at 55°C for 1 h, indicating a total transformation into spherical vesicles (Figure 2.14). This finding suggests that the ability of membranes to permit interbilayer transfer depends highly on the type of membrane-forming lipids as observed in the case of $C_{16}G_2$ and $C_{16}EO_5$.

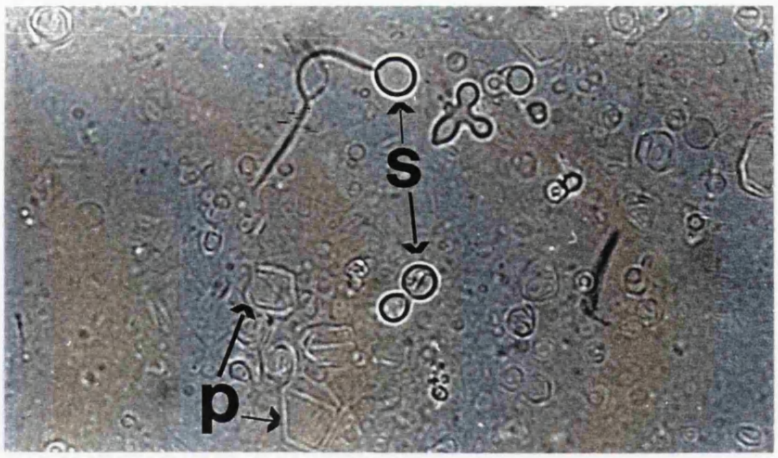


Figure 2.13a: Photomicrograph of a mixture of polyhedral niosomes of $C_{16}G_2$: Solulan C24, 91:9 (P→), and spherical niosomes of $C_{16}G_2$: cholesterol: Solulan C24, 45:45:10 (S→) before incubation at 55°C. 20 μm

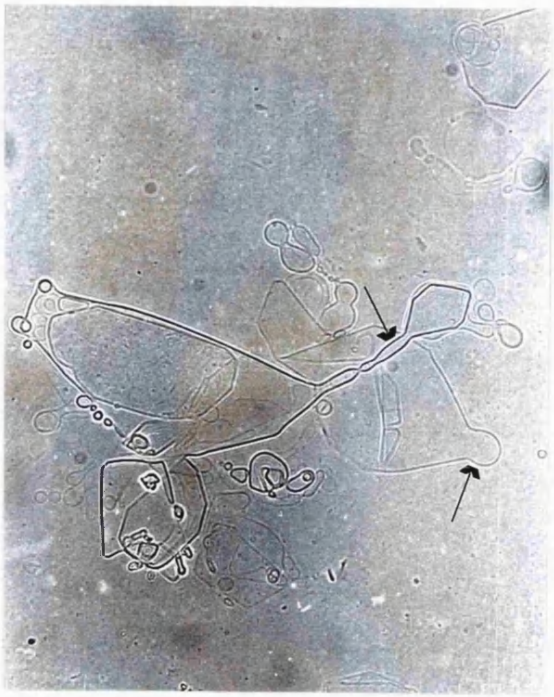


Figure 2.13b 40 μm

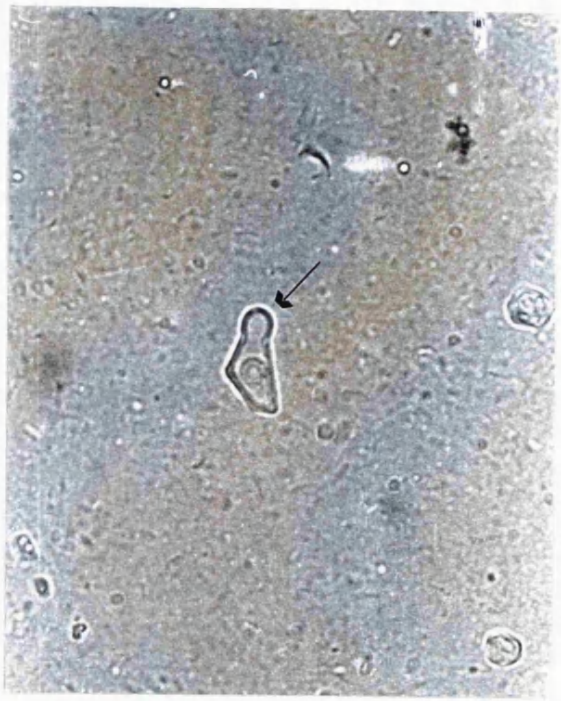


Figure 2.13c 20 μm

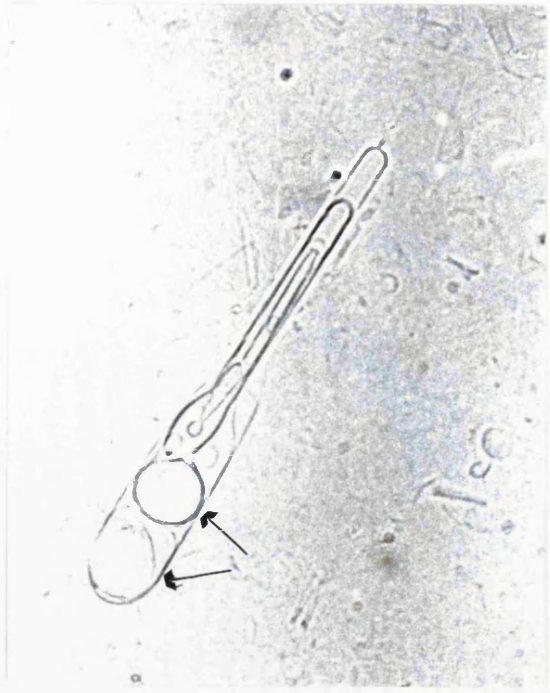


Figure 2.13d 20 μm

Figure 2.13e 20 μm

Figure 2.13b, c, d & e: Photomicrographs of polyhedral niosomes which possess curved regions (➜) observed at room temperature after 1 h incubation at 55°C of the polyhedral niosomes ($C_{16}G_2$: Solulan C24, 91:9); and unsonicated (b) or sonicated (c, d, e) spherical niosomes ($C_{16}G_2$: cholesterol: Solulan C24, 45:45:10).

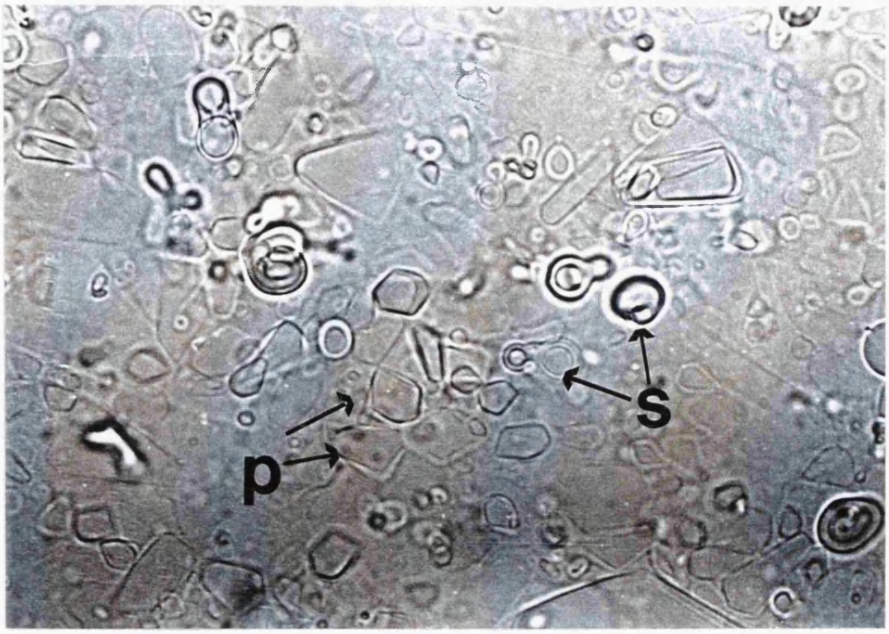


Figure 2.14a: Photomicrograph of a mixture of polyhedral niosomes (P→)and unsonicated spherical niosomes (S→) formed by $C_{16}EO_5$: cholesterol: Solulan C24 (91:0:9) and (45: 45: 10), respectively, before incubation. 20 μm

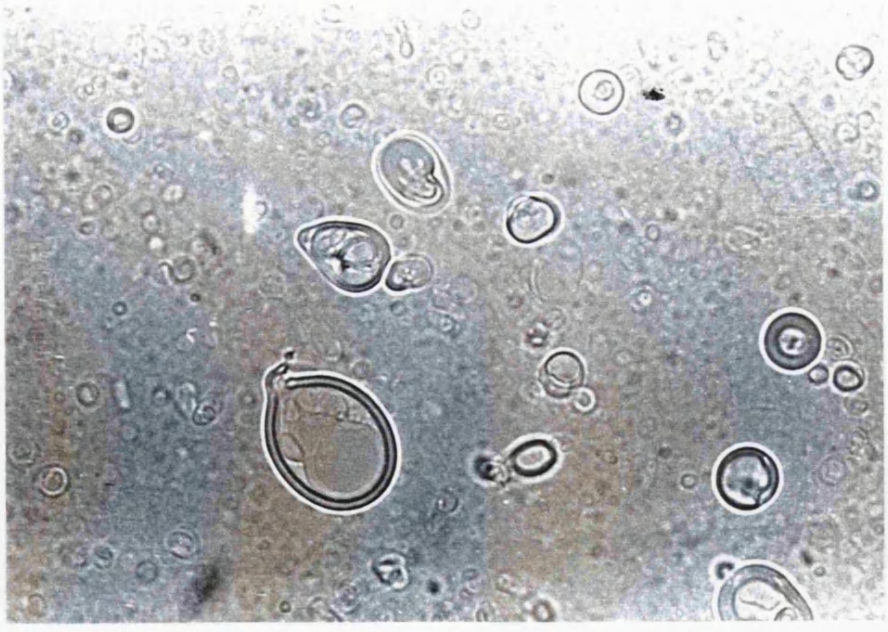


Figure 2.14b: Photomicrograph of a mixture of polyhedral niosomes and unsonicated spherical niosomes, formed by $C_{16}EO_5$: cholesterol: Solulan C24 (91:0:9) and (45: 45: 10) respectively, after incubation at 55°C for 1 h and leave to cool to room temperature for 1h. No polyhedral structure was observed. 20 μm

2.8. EFFECT OF MEMBRANE COMPOSITION ON VESICLE SIZE AND ENTRAPMENT EFFICACY

The ability of vesicles to entrap substances is highly dependent on vesicle composition, the method of preparation, and the properties of the substance to be entrapped (Blok *et al.*, 1976).

2.8.1. Span 60 niosomes

The amount of cholesterol in the membranes of Span 60 niosomes affects both size of sonicated niosomes and the entrapment efficacy (Figure 2.15). Cholesterol has been known to strengthen the membranes of vesicles hence resisting size reduction. The entrapment of CF in all sonicated niosomes is very low as sonicated vesicles have low volumes of entrapment (Vemuri *et al.*, 1990). The higher entrapment of niosomes with higher cholesterol content is therefore due to better solute retainability and larger vesicle size.

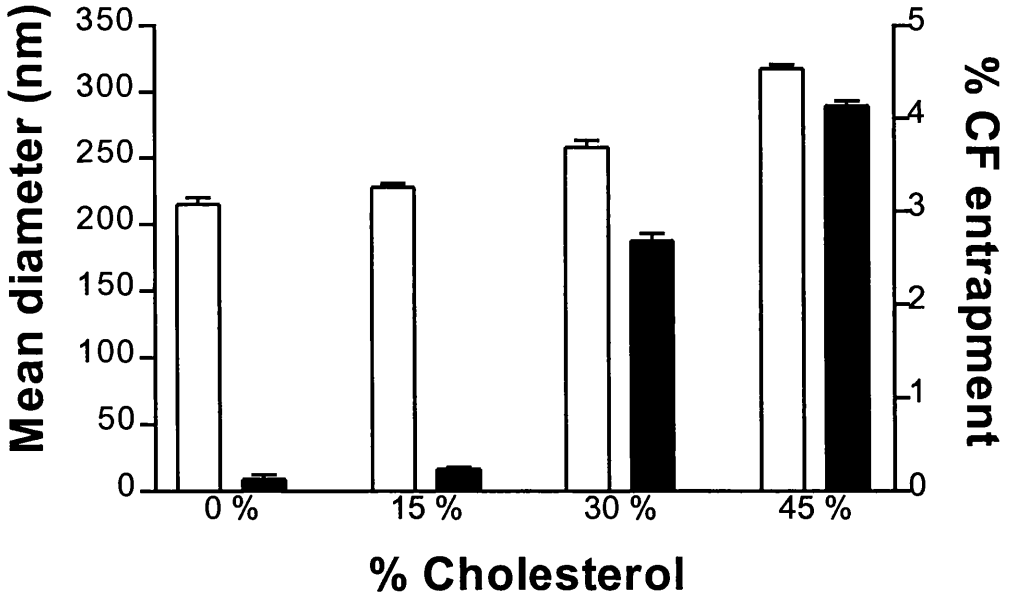


Figure 2.15: The mean diameter (nm) (white bars) and % entrapment (solid bars) of 4 minute-sonicated CF loaded Span 60 niosomes on varying concentration of cholesterol in the membranes

2.8.2. $C_{16}G_2$ niosomes

The mean size of unsonicated polyhedral and spherical/ tubular niosomes formed by $C_{16}G_2$: cholesterol: Solulan C24 (91:0:9) and (49:49:2) measured by using laser diffraction was 8 μm and 6.6 μm, respectively. The entrapment of CF inside the polyhedral niosomes was found to be higher than in spherical niosomes (Figure 2.16). It is likely that, in the case of polyhedral niosomes, increasing cholesterol into such faceted membranes allows them to form spherical vesicles with higher curvature and smaller size. An observation of CF loaded spherical/ tubular vesicles under fluorescent light microscope also suggested that very low amount of CF or none is entrapped inside the tubular vesicles compared to spherical vesicles. The lower entrapment of spherical/ tubular niosomes is therefore due to the smaller entrapped volume.

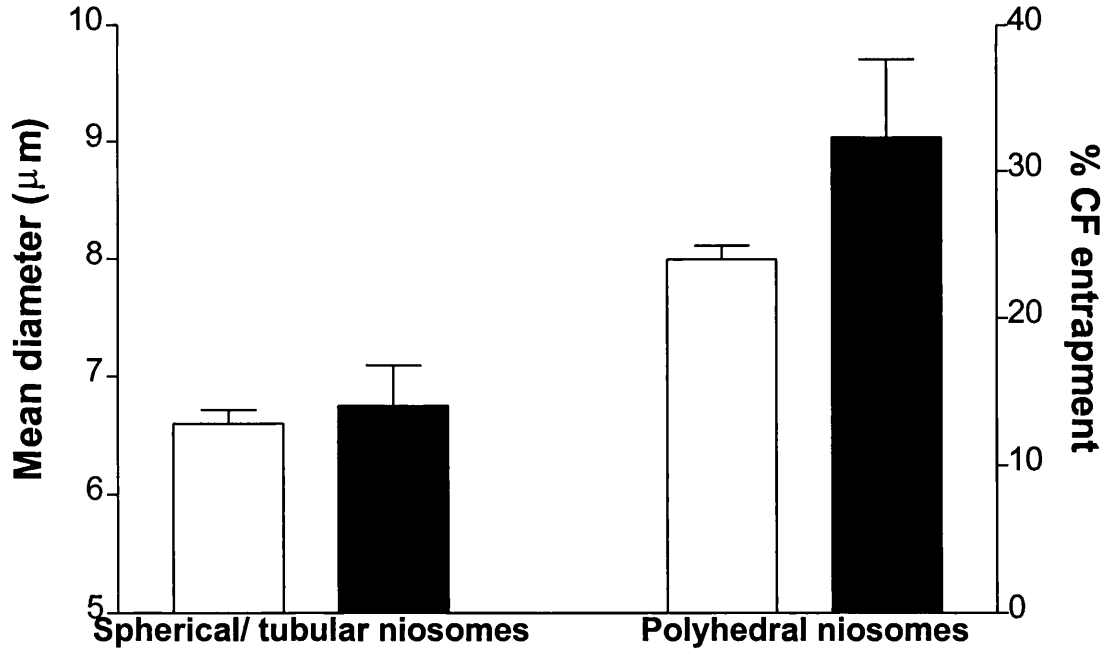


Figure 2.16: The mean diameter (μm) (white bars) and % entrapment (solid bars) of CF loaded unsonicated spherical/ tubular niosomes ($C_{16}G_2$: cholesterol: Solulan C24-49:49:2) and polyhedral niosomes ($C_{16}G_2$: Solulan C24-91:9)

2.8.2.a. Growth in size of sonicated polyhedral niosomes

When the size of $C_{16}G_2$ niosome dispersions was reduced by sonication and stored at room temperature, a difference in the size stability was observed between the spherical/ tubular and polyhedral formulations using PCS (Figure 2.17). Cholesterol rich-spherical/ tubular $C_{16}G_2$ niosomes possessed compositions that are stable after sonication. The size of the sonicated polyhedral niosomes was found to be stable when stored at high temperature (60°C) but not at room temperature. Transmission electron micrographs (TEM) of unsonicated polyhedral niosomes revealed that there are also some polyhedral vesicles in the nanometre size range (Figure 2.18a,b). In the freshly sonicated polyhedral niosomes, the large polyhedral vesicles could not be visualised using light microscope and only spherical vesicles were observed using TEM (Figure 2.18c & d). The large polyhedral vesicles could then be seen again by light microscope on leaving the sonicated polyhedral niosome dispersions at room temperature for 1 hour. This observation indicated the unstable spherical structures developed at the high energy state of $C_{16}G_2$ molecules in polyhedral niosome membranes once the membranes were excited by sonication.

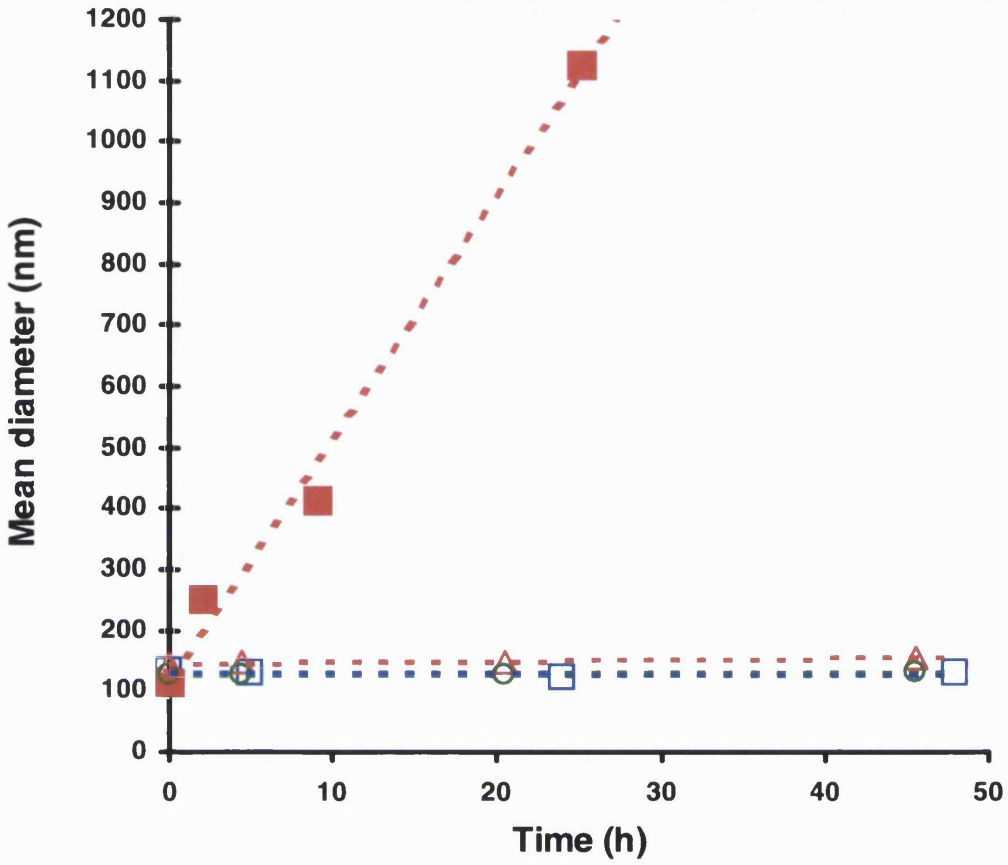


Figure 2.17: Size as a function of time and temperature of sonicated polyhedral niosomes ($C_{16}G_2$: Solulan C24, 91:9) stored at 25°C (■) and 60°C (❑); spherical/ tubular niosomes ($C_{16}G_2$: cholesterol: Solulan C24, 45:45:10) at 25°C (Δ); and spherical/ tubular niosomes ($C_{16}G_2$: cholesterol: Solulan C24, 49:49:2) at 25°C (○) (n=1)

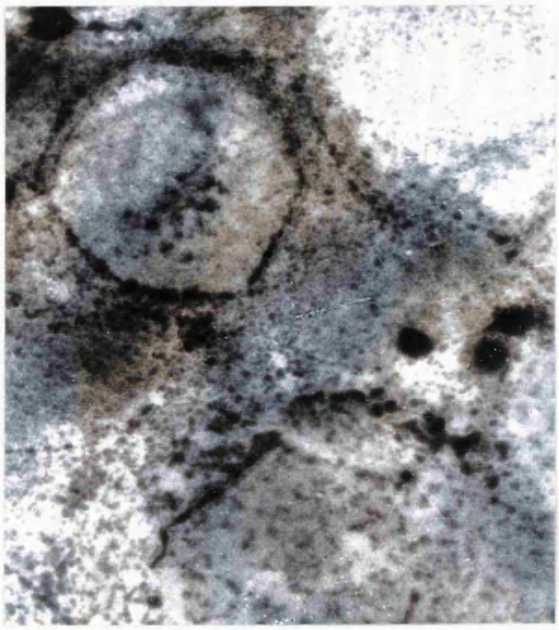


Figure 2.18a 1 μm

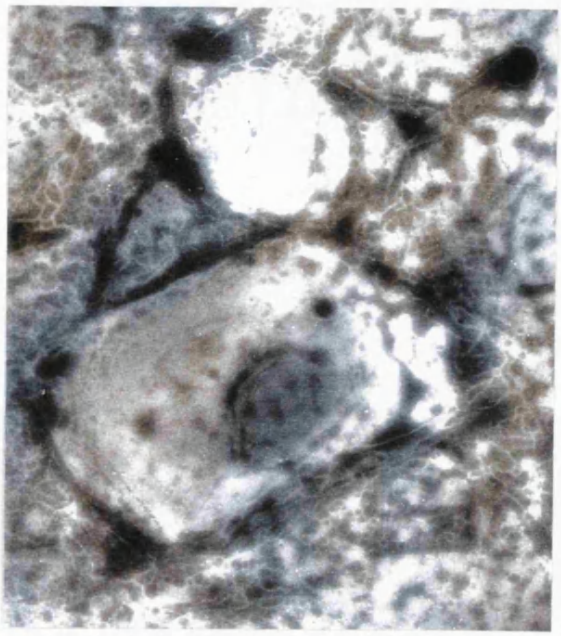


Figure 2.18b 1 μm

Figure 2.18a & b: Transmission electron micrographs of unsonicated polyhedral niosomes formed by $C_{16}G_2$: Solulan C24 (91: 9).

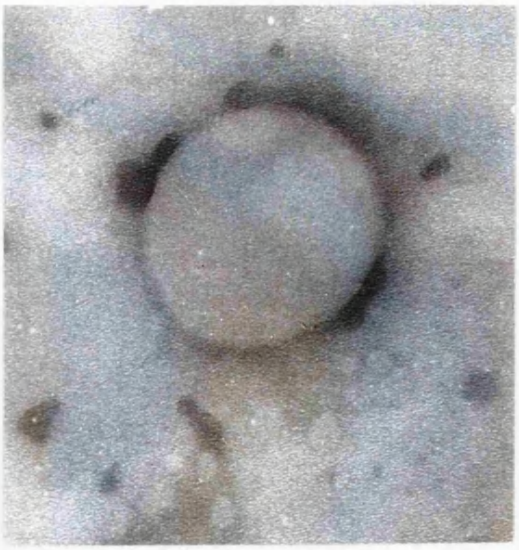


Figure 2.18c 0.2 μm

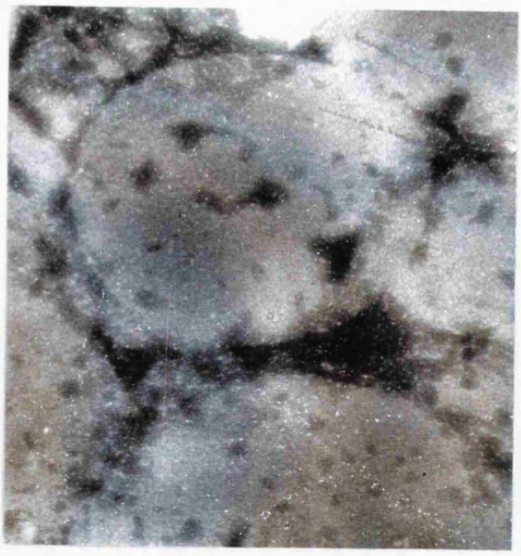


Figure 2.18d 0.5 μm

Figure 2.18c & d: Transmission electron micrographs of freshly sonicated polyhedral niosomes formed by $C_{16}G_2$: Solulan C24 (91: 9).

2.9. EFFECT OF MEMBRANE COMPOSITION ON MEMBRANE PERMEABILITY

2.9.1. Multi-component niosomes

Figure 2.19a shows that the ability to retain the solute inside the vesicles depends highly on the cholesterol content in the membranes. A slight reduction in the release rate of CF from the mixture of CF solution and empty niosomes was also found (Figure 2.19a). This finding suggests that a small amount of CF may be taken up into the vesicles by means of CF concentration gradient between the inside and outside of the vesicles.

As the release rate of a solute from niosomes can be modified by adjusting the cholesterol content in the membranes, we exploited such knowledge by establishing a multi-component system for sustained drug delivery in the same fashion as in microparticulate solid dosage forms which contains a mixture of dry pellets with various thickness of coated-polymer layer or mixtures of pellets with different release properties. Our system contains a portion of drug solution, and drug loaded in niosomes with 30% and 45% cholesterol. It was expected that, on delivery *in vivo*, the unentrapped drug will firstly give rise to the blood level following by the release of drug from 30% and 45% cholesterol- containing niosomes, respectively. Figure 2.19b showed the release profile of CF, as a model drug, from multi-component mixtures along with the predicted release profile calculated from the release of CF from each portion obtained from Figure 2.20a. This preliminary study showed that the release profile of the multi-component niosomes can be satisfactorily predicted and may be useful in the design of a system which provides a specified release pattern *in vivo*.

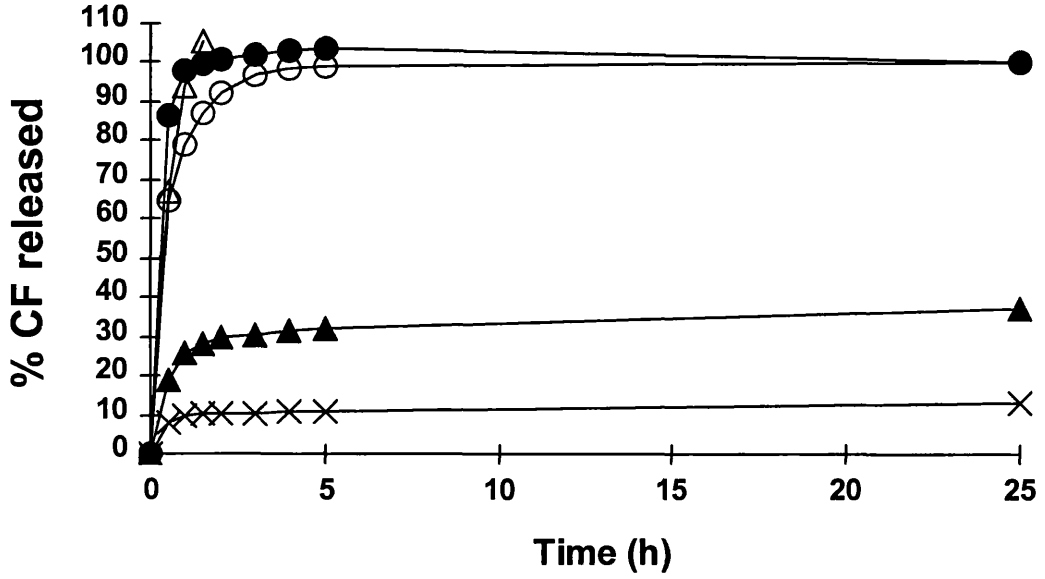


Figure 2.19a: Release profiles at 37°C of CF from (●) CF solution in PBS, pH 7.4; (○) a mixture of CF solution and empty niosomes of Span 60: cholesterol: TPGS (45:45:10); (Δ) CF loaded niosomes of Span 60: cholesterol: TPGS (75:15:10); (▲) CF loaded niosomes of Span 60: cholesterol: TPGS (60:30:10); and (×) CF loaded niosomes of Span 60: cholesterol: TPGS (45:45:10)(n=1)

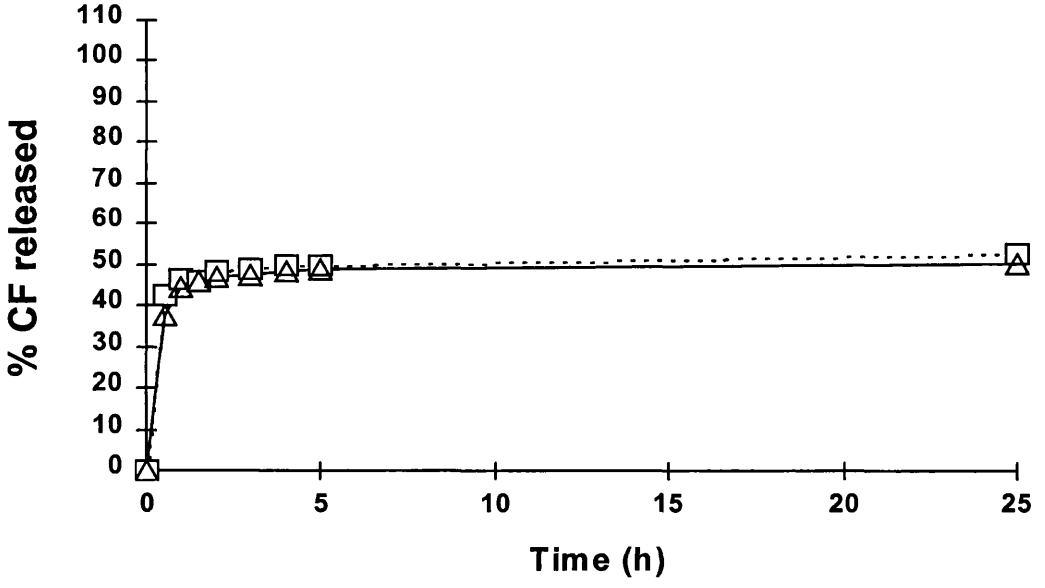


Figure 2.19b: Release profile at 37°C of CF from a multi-component dispersion containing equimolar amount of CF in CF solution, in Span 60 niosomes with 30% cholesterol and in Span 60 niosomes with 45% cholesterol (□); in comparison to the predicted release profile (Δ) calculated from the amount of CF released from each portion in Figure 2.19a (n=1)

2.9.2. Release of CF from $C_{16}G_2$ niosomes

It has been reported that polyhedral niosomes formed by $C_{16}G_2$ and Solulan C24 release more CF on increasing the temperature (Uchegbu *et al.*, 1997). On heating above the phase transition temperature, the vesicle membranes change from the gel to the liquid phase leading to higher membrane permeability (Iga *et al.*, 1989). The release study at room temperature showed that the membranes of polyhedral niosomes, although in gel state, are more permeable than those of spherical/ tubular niosomes (Figure 2.20). Gel phase membranes are known to be less permeable than liquid phase membranes; however, they were found to be permeable to water molecules (Blok *et al.*, 1976). It is the lack of cholesterol and perhaps the membrane defects formed due to irregular membrane structures which contribute to the higher permeability of polyhedral membranes compared to spherical/ tubular niosomes which, although in a fluid state, contain cholesterol, which reduces membrane permeability (Baillie *et al.*, 1985; De Gier *et al.*, 1968; Storm *et al.*, 1989), in the membranes.

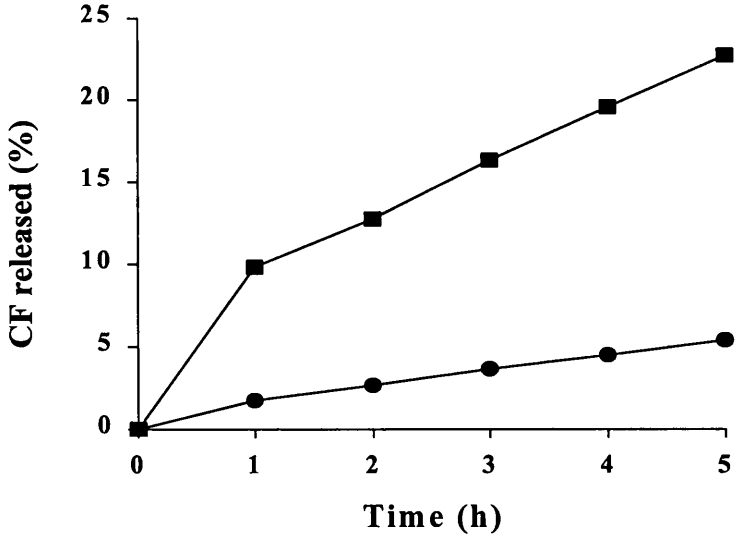


Figure 2.20: Release profile at room temperature of CF from polyhedral niosomes (■), and spherical niosomes (●) formed by $C_{16}G_2$: cholesterol: Solulan C24 (91:0:9) and (45:45:10), respectively

2.10. CONCLUSIONS

In conclusion, polyhedral niosomes can be formed by various amphiphiles in the complete absence of, or with very low amounts of cholesterol, and they are able to encapsulate and retain aqueous solutes, and might be of use in drug delivery systems. Transformation of polyhedral niosomes into the spherical shape occurs when they are heated above their phase transition temperature, or by introduction of cholesterol into their membranes following incubation with cholesterol-rich vesicles. The schematic (Figure 2.21) represents the molecular conformation of $C_{16}G_2$ niosomes, as in polyhedral form, and shows the assumed effect of adjusting several conditions on ultrastructure. The composition of membranes can be manipulated to obtain vesicles with required size, shape, and membrane permeability although further investigation may be needed to predict the shape of vesicles formed by certain surfactants.

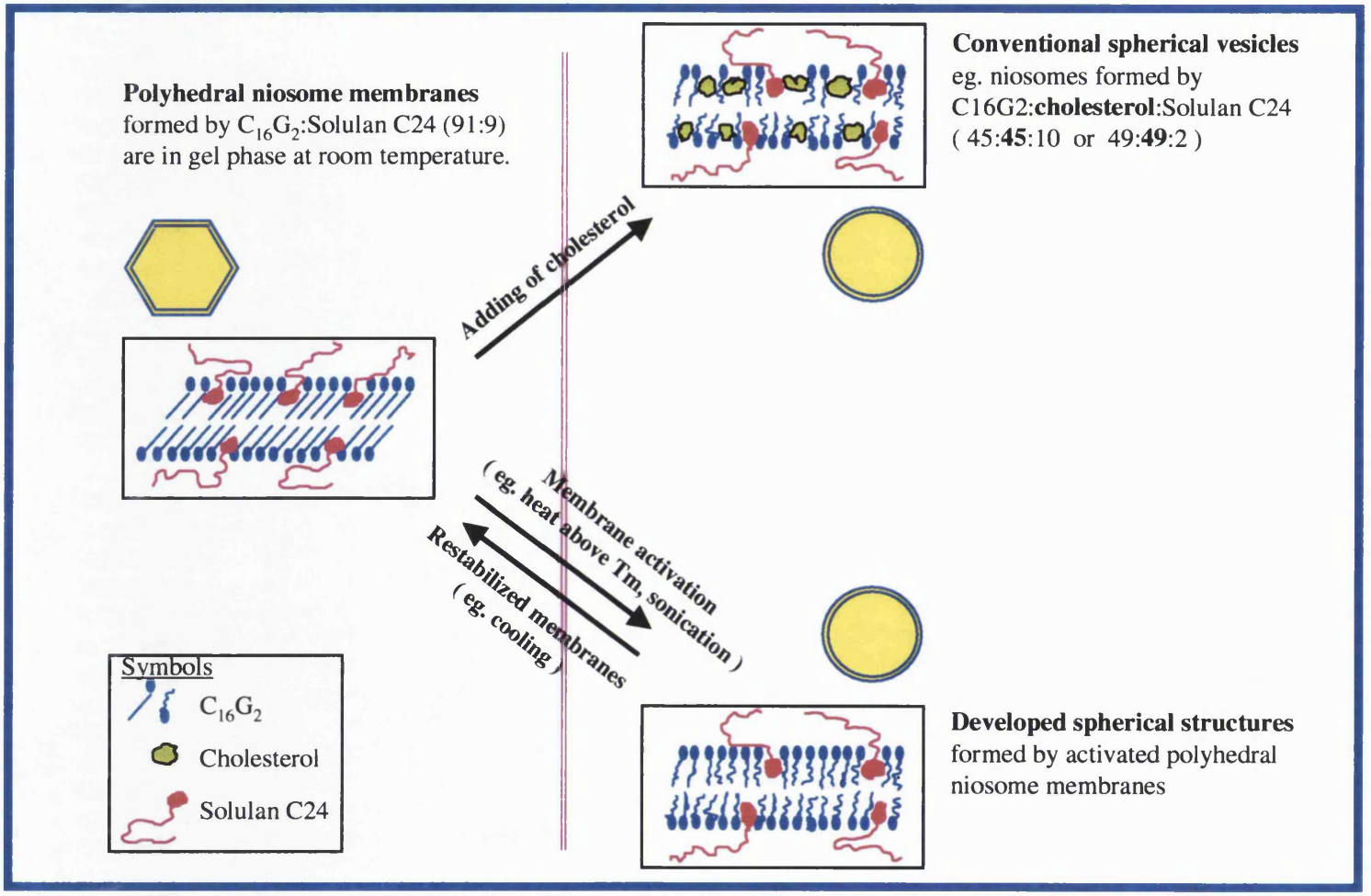


Figure 2.21: The schematic represents factors affecting shape and molecular conformation of polyhedral niosomes

CHAPTER 3

VISCOSITY OF NIOSOME DISPERSIONS

3.1. INTRODUCTION

The rheological properties of vesicle dispersions can be of great importance in a spectrum of instances from preparation and handling to delivery to targets in the body. Not only are flow properties of vesicle dispersions involved in such preparation steps as extrusion processes, where small vesicles are produced by extruding large vesicles through filter membranes under an applied pressure, but potentially also in filling processes. It could be argued, although there are no observations on the topic, that the viscosity of the vesicle dispersions is relevant to an understanding of the flow of vesicles from the syringe needle, perhaps even through small pores of skin, but certainly their movement in capillary blood vessels.

The rheological properties of colloidal dispersions such as suspensions, emulsions and microemulsions have been widely investigated, but there are surprisingly few rheological studies of vesicle dispersions. Vesicles can be considered to be a category of colloidal dispersion. However, although both emulsion and vesicle dispersions contain liquid

droplets which are dispersed in a continuous medium, the liquids which are entrapped inside the vesicles and which comprise the continuous medium are the same, i.e. water, or oil in the case of inverted vesicles, while emulsions are dispersions of water in oil, or oil in water. Moreover, emulsion droplets are coated with surfactant monolayer while vesicles can be composed of one to many *bilayers*. Whether or not existing theoretical models of rheological behaviour for colloidal dispersions, briefly reviewed below, can be applied directly to niosomes is open for debate.

3.1.1. Rheology of colloidal dispersions

The first hydrodynamic calculation of the effective viscosity of spherical particles was given by Einstein (1906, 1911). His equation (3.1) relates to the relative viscosity in steady shear flow, η_{rel}, of dilute dispersions of non-deformable solid spheres, to the volume fraction, φ, which they occupy in the system:

$$\eta_{rel} = 1 + a\varphi \qquad (3.1)$$

For this equation to be valid, $\varphi < 0.05$. The constant a equals 2.5 for non-deformable and non-interacting spherical particles. This constant may become less than 2.5 in non-rigid sphere dispersions, e.g. emulsions, as the flow lines may be partially transmitted through the suspended particles.

The intrinsic viscosity, $[\eta]$, which is defined by

$$[\eta] = \lim_{\varphi \to 0}\left(\frac{\eta_{rel} - 1}{\varphi}\right) = \lim_{\varphi \to 0}\left(\frac{\eta_{sp}}{\varphi}\right) \qquad (3.2)$$

is obtained by plotting $(\eta_{rel}-1)/\varphi$ (i.e. η_{sp}/φ) vs φ and extrapolating the data line to $\varphi = 0$. Deviation of the extrapolated value of $[\eta]$ from 2.5 can be ascribed to particle asymmetry and hydration. As any solvent layer tightly bound to the particle surfaces acts kinetically as an integral part of the particles, the intrinsic viscosity thus reflects the degree of solvation of particles. Hydration can be calculated from $[\eta]$ (see equation (3.10)). For non-hydrated particles asymmetry can be readily assessed also. If the particles are asymmetric, equation (3.1) is modified to

$$\lim_{\varphi \to 0} \frac{\eta_{sp}}{\varphi} = [\eta] = \nu \qquad (3.3)$$

where ν is related to the axial ratio of the particle when defined either as an oblate or prolate ellipsoid.

To deal with some non-rigid spherical particles, the Einstein equation (3.1) was extended by Taylor (1932) to apply to fluid droplets in which the fluid circulation within the globules was considered

$$\eta_{rel} = 1 + 2.5\varphi[(\eta' + \frac{2}{5}\eta_0)/(\eta' + \eta_0)] \qquad (3.4)$$

where η' is the viscosity of the liquid in the droplets and η_o is the viscosity of the medium. It is unlikely that there is any such circulation in most vesicle interiors because in emulsion particles the presence of an adsorbed stabilising layer effectively reduced the internal circulation (Sherman, 1968). Nonetheless in large vesicles some fluidity and extensibility might be anticipated and, indeed, can be observed in shear flow.

In the Einstein equation, the viscosity of a dispersion depends only on the total volume of disperse phase and is independent of their size because the theory does not consider the effects of other particles since only dilute non-interacting systems are considered. At higher volume fractions, interactions between particles are included, hence the higher-order terms in φ. There are a number of modified equations, mostly of the type

$$\eta=\eta_o(1+a\varphi+b\varphi^2+...)\,. \qquad (3.5)$$

One example is

$$\eta=\eta_o(1+2.5\varphi+6.2\varphi^2) \qquad (3.6)$$

in which the hydrodynamic interactions between particles with Brownian motion were considered (Batchelor, 1977).

Polydispersity of many colloids can complicate the interpretation of viscosity even though there is no size dependency in the Einstein equation (which, we recall, deals with unsolvated and non-interacting particles). When the crowding effect due to the polydispersity of particle diameter is considered, e.g. in a two-component system where spheres of size r_1 crowd spheres of size r_2, Mooney's equation (Mooney, 1951) is commonly applied

$$\eta_{rel}=\exp[a\varphi/(1-k\varphi)] \qquad (3.7)$$

where the symbols have the same meaning as before, *k* being the self-crowding factor implying a hydrodynamic interaction which increases as particle size decreases. This

equation had been modified and applied successfully in many colloidal systems including emulsions and microemulsions (Matsumoto & Sherman, 1969; Attwood *et al.*, 1974; Matsumoto & Kohda, 1980; Baker *et al.*, 1984), latex beads (Saunders, 1961; Wang, 1970), micelles (Muzzalupo *et al.*, 1996), and also erythrocytes (Navari & Gainer, 1973).

Particle asymmetry has a pronounced effect on the viscosity. Rods have a greater effect than discs in increasing viscosity (Barnes *et al.*, 1989):

$$[\eta]_{rods}=3(axial\ ratio)/10 \qquad (3.8)$$

$$[\eta]_{disc}=7[(axial\ ratio)^{5/3}]/100\ . \qquad (3.9)$$

These simple empirical equations show the relationship of intrinsic viscosity and the axial ratio of non-spherical particles.

3.1.2. Problems in the determination of volume fraction and hydration values of vesicle dispersions

Einstein's equation (3.1) shows that the relative viscosity, η_{rel}, of dilute colloidal dispersions depends on the volume fraction, φ, of the disperse phase. It is easy, in the case of emulsions, to access the value of φ as we know exactly the total volume of the disperse phase added to the dispersion. Similarly for micelles in aqueous media, provided that the density of the surfactant is known, φ is readily obtained.

Providing the mean radius of vesicles, the density of amphiphiles in the bilayers, bilayer

thickness, and lamellarity are known, the total number of vesicles in each dispersion can be calculated, and can allow the estimation of the volume fraction value. However, Smeulders *et al.*(1990) commented that such estimation, so called "dry weight method", leads to an estimation of φ with large error margins due to the uncertainties of those parameters. They therefore measured the steady-state viscosity of vesicles and determined φ by comparing this with the steady-state viscosity versus φ relationship of known real hard sphere models, assuming the vesicles behaved as hard spheres.

Hydration is of particular interest in pharmaceutical colloidal dispersions as hydrated surface layers prolong circulation in the blood after IV administration. When hydrated a particle occupies a larger effective volume fraction than its unhydrated counterpart, as Oncley equation (Alexander & Johnson, 1950; Elworthy & Macfarlane, 1965) defines:

$$[\eta]=\nu[\overline{V_2}+W_1V_1^0] \qquad (3.10)$$

where $\overline{V_2}$ is the partial specific volume of the particles; W_1 is the solvation expressed as g solvent/ g solute; and V_1^0 is the specific volume of the solvent; as before ν = 2.5 for spherical particles.

In most colloidal systems, e.g. emulsions, micelles, the hydration occurs at the surface of particles. Application of equation 3.10 therefore allows the calculation of the level of external hydration, if the shape is known. However, the lipids and surfactants in niosomes are hydrated and in the form of bilayers entrapping water or drug solution inside. This does not allow equation 3.10 to be directly applicable for the calculation of hydration, and it is

incorrect to use the volume of dry surfactant obtained from surfactant density to calculate the volume fraction of the niosomes. In addition to the fact that niosomes are highly hydrated, it is difficult to predict the volume fraction of niosomes which are formed either by the same concentration of lipids/surfactants of different types or even the same types but prepared by different methods. Vesicle dispersions can have various values of φ at any given concentration of lipid depending on their size, shape, and lamellarity. It is straightforward, however, to use the total *concentration* of lipids/surfactants in place of volume fraction to plot rheological behaviour (Florence, 1993; Hoffmann *et al.*, 1994; Hassan *et al.*, 1996; Muzzalupo *et al.*, 1996).

In this chapter, a number of variables which can influence the rheological properties of niosomes were investigated by using an Ostwald U-tube viscometer or a suspended level dilution viscometer. Such practical factors include vesicle concentration, vesicle size, vesicle shape, vesicle membrane composition, temperature, electrolytes, and preparation methods. The niosomes used as models were formed mainly by Span 60 or $C_{16}G_2$. Attempts to predict the volume fraction and the hydration value from the viscosity data were also made. The viscosity of micellar solutions of Solulan C24 were also measured to gauge the hydration of a micellar surface composed of Solulan C24 molecules.

MATERIALS AND METHODS

3.2. MATERIALS

The materials used in this chapter are as given in Table 2.1. Diethyl ether was supplied by

BDH Laboratory Supplies, UK. Dicetyl phosphate was obtained from Sigma Chemical, UK. The water source was an ultra high quality reverse osmosis water purifier (Elgastat UHQPS, Elga, UK).

METHODS

3.3. VISCOMETRIC STUDIES OF SOLULAN C24 MICELLAR SOLUTIONS

Solulan C24 was dissolved into water at 60°C and left to cool at room temperature. Viscosity measurements were performed in an Ostwald U-tube (size M2, Phillip & Harris, UK) contained in a thermostated water bath with the temperature set at 25, 35, and 45°C (± 0.1°C). Samples were diluted with water to the required concentrations and left to equilibrate for 1 h. Relative viscosity (η_{rel}) was calculated by comparing efflux time (n=3) with that of water.

3.4. VISCOMETRIC STUDIES OF SPAN 60 NIOSOME DISPERSIONS

3.4.1. Effect of vesicle concentration, size and membrane composition

Dried film mixtures of 300 µmol Span 60, cholesterol, Solulan C24 (45:45:10 and 49.5:49.5:1) were hydrated with 5ml of water at 60°C for 1 h. All dispersions were probe sonicated for a set period of time to obtain the required vesicle size. Particle sizing was performed using the photon correlation spectroscopy (PCS; AutoSizer 2C, Malvern, UK). Samples were also viewed by transmission electron microscopy (TEM) (Philips 201 microscope). Rheological studies were performed using a suspended level dilution viscometer (size 2, BDH, UK) at 25°C.

3.4.2. Effect of electrolytes

Dried film mixtures of 300 µmol Span 60, cholesterol, Solulan C24 (45: 45: 10) and Span 60, cholesterol, dicetyl phosphate (DCP) (45:45:10) were hydrated with 5 ml of water or 0.5 M NaCl solution at 60°C for 1 h. All dispersions were probe sonicated to obtain vesicle dispersions with the mean size of around 270 nm. The dispersions were diluted to the required concentrations with water or 0.5 M NaCl and the rheological studies were performed using a suspended level dilution viscometer (size 2, BDH, UK) at 25°C. Relative viscosity (η_{rel}) was calculated by comparing efflux time (n=3) with that of their hydrating solutions.

3.4.3. Effect of preparation methods and lamellarity

Niosomes were prepared from 300 µmol Span 60, cholesterol, Solulan C24 (45:45:10) by two methods, Method A and Method B.

Method A: Hand-shaking method (HS)- Dried film was hydrated with 5 ml of water at 60°C for 1 h.

Method B: Reversed phase evaporation method (REV)- Water (4 ml) was rapidly injected into the surfactants/ lipid solution prepared in 10ml of diethyl ether: chloroform (1:1) which then was bath sonicated for 3 min. The organic solvents were removed in a vacuum evaporator at 60°C followed with 15 min nitrogen flushing. The remaining dispersion was then adjusted to 5 ml with water.

Both dispersions were then probe sonicated for 5min twice and then characterized by TEM

and sized by PCS. Rheological studies were performed using a suspended level viscometer (size 2, BDH, UK) at 25°C.

3.5. VISCOMETRIC STUDIES OF $C_{16}G_2$ NIOSOME DISPERSIONS

3.5.1. Effect of vesicle shape and temperature

Polyhedral and spherical/ tubular niosomes were prepared by hydrating dry films of 300 μmol $C_{16}G_2$, cholesterol, Solulan C24 in the molar ratios 91:0:9 and 45:45:10, respectively, with 5 ml of water at 60°C for 1 h, storing the suspensions for 24 h before experiments. Viscosity measurements were performed in an Ostwald U-tube (size M2, Phillip & Harris, UK) contained in a thermostatted water bath with temperature set at 25, 35, 45, and 55°C.

RESULTS AND DISCUSSION

3.6. VISCOMETRIC STUDIES OF SOLULAN C24 MICELLAR SOLUTIONS

Solulan C24 itself forms micelles and the hydration of the 24 unit polyoxyethylene sheath can be calculated from the intrinsic viscosity by using the modified Oncley's equation (3.10). Assuming all densities are equal to unity, the intrinsic viscosity of micellar solutions of Solulan C24 at 25°C and 35°C of 12.2 ml g^{-1} and 8.2 ml g^{-1}, respectively (Fig. 3.1), provide the hydration value of 3.8 g g^{-1} surfactant at 25°C and 2.3 g g^{-1} surfactant at 35°C. Elworthy (1960) obtained a value of around 2 g g^{-1} hydration for cetomacrogol 1000, a polyoxyethylene monocetyl ether containing an average of 22 ethylene oxide units. These values are useful in estimating the external hydration of the niosomes.

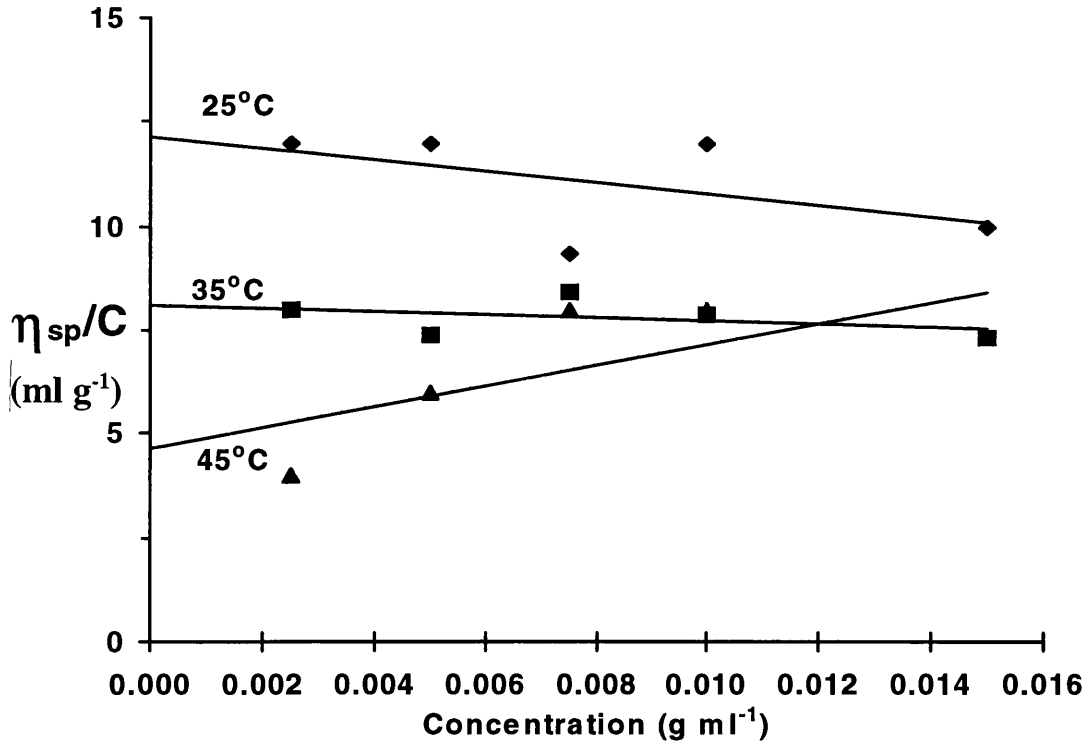


Figure 3.1: Reduced specific viscosity plots for Solulan C24 micellar solutions at different temperatures as a function of Solulan C24 concentration.

3.7. VISCOMETRIC STUDIES OF SPAN 60 NIOSOMES

3.7.1. Effect of vesicle concentration

Figures 3.2a&b show the effect of concentration on the relative viscosity of Span 60 niosomes of different mean diameters formed with 1% and 10% respectively of Solulan C24. Figure 3.3a&b show the plots of reduced specific viscosity (η_{sp}/C) versus concentration (C) of both systems. The values of intrinsic viscosity obtained from the intercept are shown in Table 3.1. Some examples of concentration-dependent rheological profiles are shown in many studies (Kato *et al.*, 1983; Hassan *et al.*, 1996; Muzzalupo *et al.*, 1996).

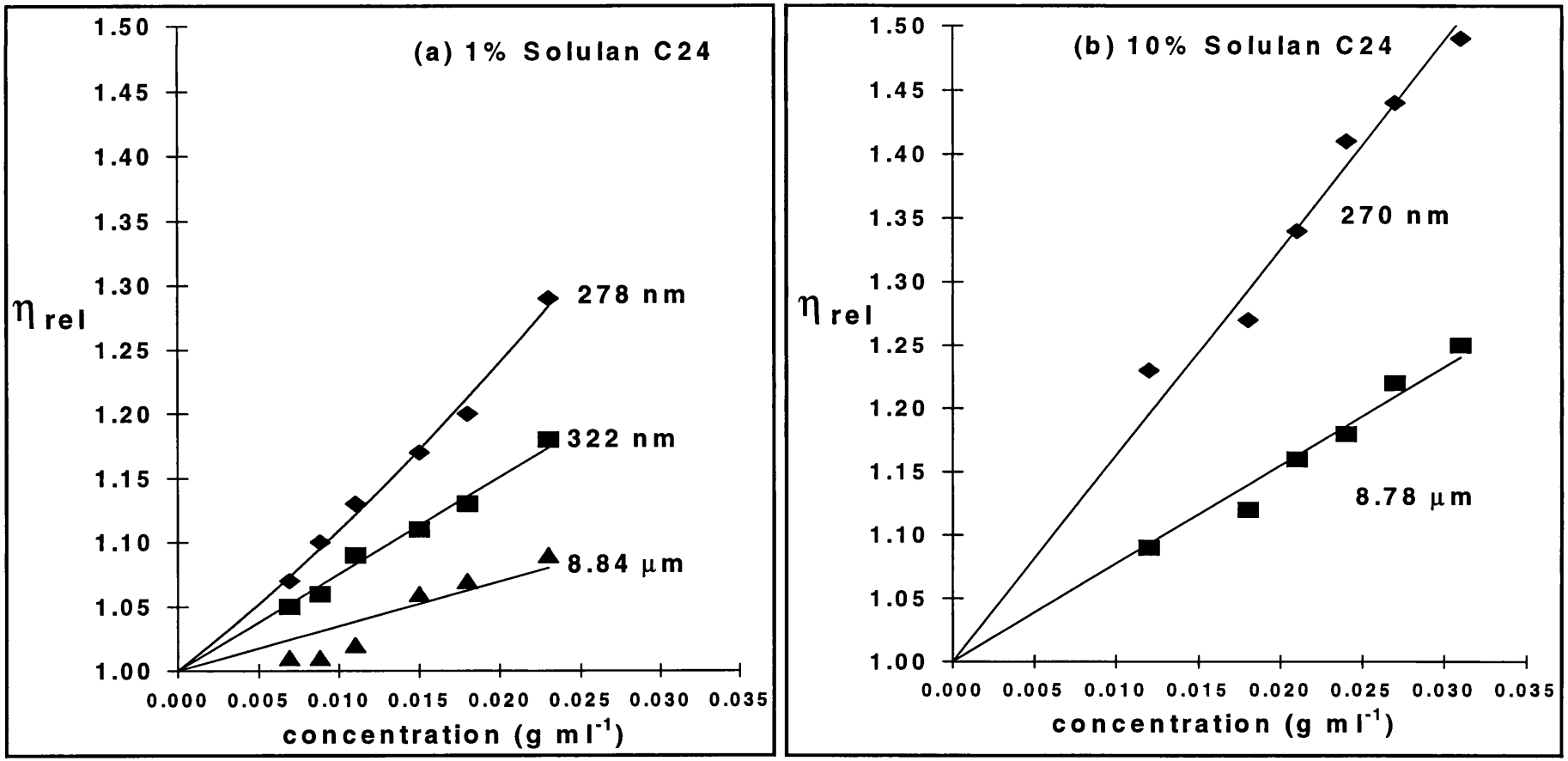


Figure 3.2: Relative viscosity of niosomes formed from Span 60: cholesterol: Solulan C24 (a) (49.5: 49.5: 1) and (b) (45: 45: 10) as a function of the concentration (C) of lipid/ surfactants in water at 25°C and of niosome diameter.

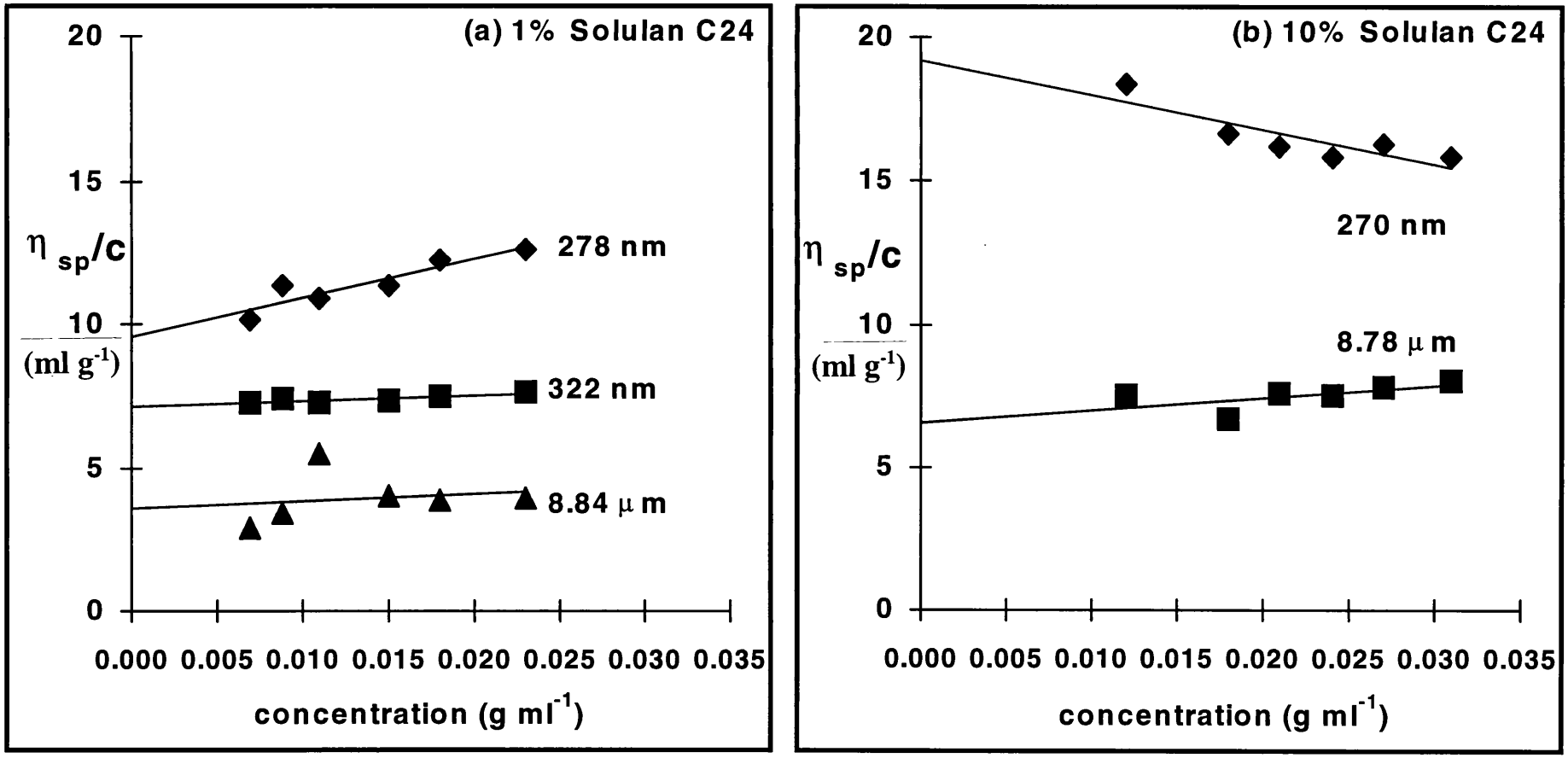


Figure 3.3: Reduced specific viscosity of niosomes formed from Span 60: cholesterol: Solulan C24 (a) (49.5: 49.5: 1) and (b) (45: 45: 10) as a function of the concentration (C) of lipid/ surfactants in water at 25°C and of niosome diameter.

Table 3.1: Intrinsic viscosity, [η], obtained from the intercepts of reduced specific viscosity (η_{sp}/C) against concentration (C) plots in water at 25°C.

Niosome Composition	Mean Size (μm)	[η] ($ml.g^{-1}$)
Span 60: Cholesterol: Solulan C24 (49.5:49.5:1)	0.278	9.6
	0.322	7.1
	8.84	3.5
Span 60: Cholesterol: Solulan C24 (45:45:10)	0.27	19.2
	8.78	6.5
Span 60: Cholesterol: Solulan C24 (45:45:10)		
-Sonicated from REV dispersion	0.154	19.9
-Sonicated from HS dispersion	0.16	12.9

3.7.1.a. Estimations of volume fraction and hydration

One of the main difficulties in the interpretation of data on the flow of dilute vesicle dispersions by way of estimating hydration lies in the calculation of volume fraction, φ, which comprises not only the surfactant and lipid content of the vesicles, φ_L, but also the internalised water, φ_w. We have no direct access to φ_w. The internal phase of unilamellar niosome dispersions must be considered to be at least partially aqueous (φ_w). The true volume fraction (φ) of the disperse phase may be estimated from

$$\varphi = \varphi_L + \varphi_W \qquad (3.11)$$

φ_L is obtained with some accuracy from the concentration of lipid/ surfactants (C) used to prepared the vesicles, but φ_w is not measurable directly although estimates may be made from a knowledge of surfactant hydration (Elworthy & Macfarlane, 1965).

As Oncley equation (3.10) shows, the hydrodynamic unit will, of course, include the water associated with the surface hydrophilic head groups hence for spherical non-deforming vesicles, on estimated this vital surface hydration can be made, as will be discussed below.

An attempt to predict φ was made to interpret the viscosity of systems formed by Span 60: cholesterol: Solulan C24 mixture as shown in Figure 3.2. If we assume that the Einstein equation (3.1) and the modified Oncley equation (3.10) apply to the systems under study, it should be possible to interpret [η] in terms of the hydration of the *exterior* of the vesicle, if φ is known. Figure 3.4 shows the model for a unilamellar vesicle with the rationale for the calculation of φ in both unilamellar and multilamellar systems. If the volume fraction of the vesicles is the volume fraction of the lipid/ surfactant mixture plus the volume fraction of entrapped water, assuming for the sake of calculation all densities are equal to unity, then we can convert reduced specific viscosity vs concentration (η_{sp}/C vs C) plots into η_{sp}/φ vs φ plots for a range of assumed relationships between φ and C, eg φ = 1.5C, φ = 2C, etc. For example, the calculation is simple: if the internal hydration of the vesicle is 1g/g surfactant, then φ = 2C. Figure 3.5 shows the data for 278 nm Span 60 niosomes containing 1% Solulan C24 obtained from Figure 3.2a plotted over the range φ = C to 3C. The values of [η] based on these assumptions are obtained as shown in Table 3.2 and the consequent values of hydration (i.e. surface hydration) estimated as W_1. If φ = 2C, the intrinsic viscosity is 4.79 ml g^{-1} which, from the modified Oncley's equation, implies a hydration value of 0.916 g g^{-1}, but when φ = 2C the intrinsic viscosity calculated for the 8.8 μm vesicles falls well below 2.5. If we assume φ = 1.5C (Fig 3.5), [η] = 6.39 ml g^{-1} which, from the modified Oncley equation, implies a hydration of 1.5 g g^{-1}.

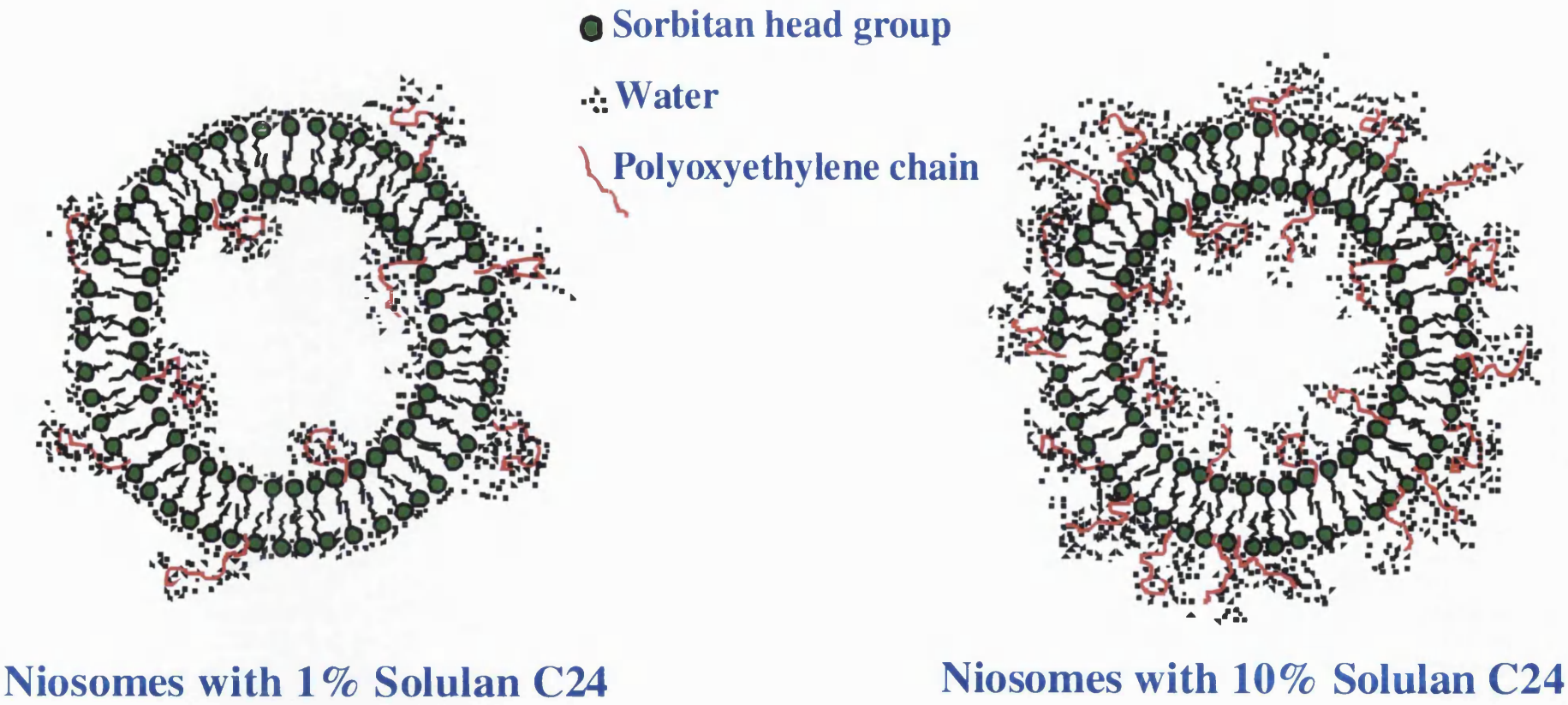


Figure 3.4: Diagrammatic representative of unilamellar niosomes containing low and high (1 and 10%) amounts of Solulan C24 with their hydration associated with the oxyethylene chains both internally and externally.

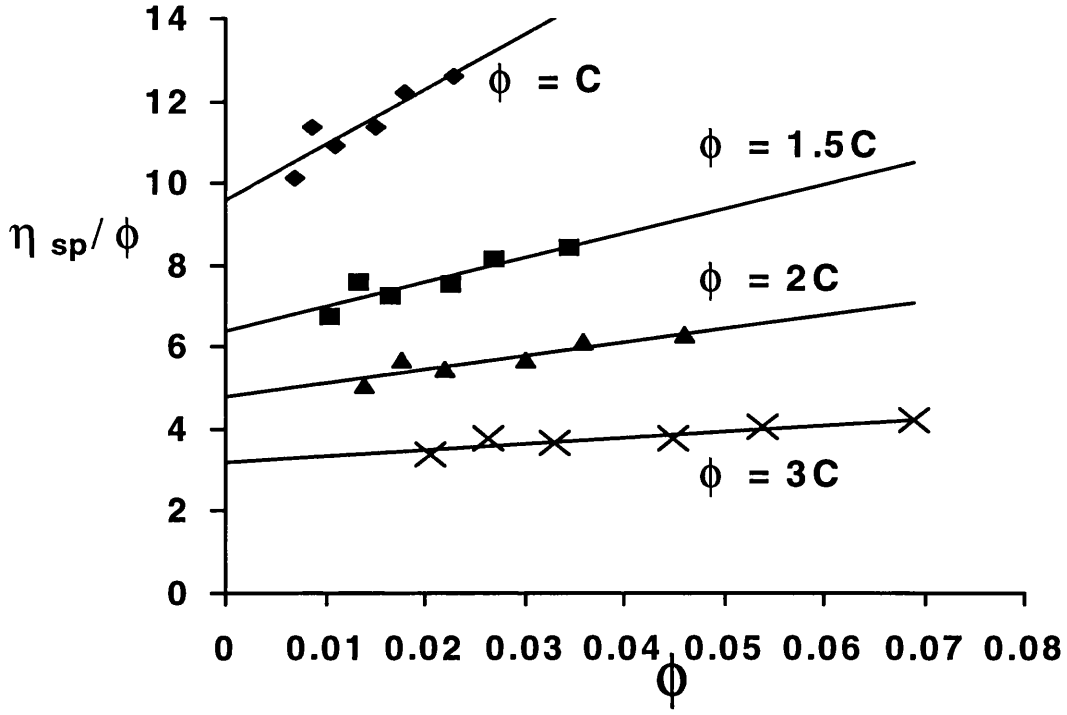


Figure 3.5: Plot of reduced specific viscosity, η_{sp}/φ versus φ, of niosomes prepared from Span 60: cholesterol: Solulan C24 (49.5: 49.5: 1) with mean diameter of 278nm using data from figure 3.2a, assuming φ = C, 1.5C, 2C, and 3C.

Table 3. 2: Intrinsic viscosity, [η], and hydration value (W_1) of 278 nm niosomes formed by Span 60: cholesterol: Solulan C24 (49.5: 49.5: 1) at various range of assumed volume fractions (φ).

φ	$[\eta]^{\dagger}$ ($ml.g^{-1}$)	$W_1^{\ddagger}$ ($g.g^{-1}$)
C	9.58	2.83
1.5C	6.39	1.55
2C	4.79	0.92
3C	3.19	0.28

$^{\dagger}[\eta]$ values are obtained from the intercepts of reduced specific viscosity (η_{sp}/φ) against estimated volume fraction (φ) plots in water at 25°C. $^{\ddagger}W_1$ is calculated from modified Oncley's equation $[\eta]=\nu[\overline{V_2}+W_1V_1^0]$: assuming ν = 2.5 for spherical particles; $\overline{V_2}$ (partial specific volume of the particles) and V_1^0(specific volume of water) are assumed to be equal and =1

If the niosome surface was composed entirely of Solulan C24, the maximum hydration that would be anticipated would be 3.8 g g^{-1} (assumed from the data on the micellar state) while the lowest value would be approximately 0.5 g g^{-1} the value for sorbitan, as deduced from data on analogous sugars. If for the system with 10% Solulan C24 it is assumed that the internal hydration is greater than that of the previous system because of the greater degree of water binding to the membranes, and it is assumed that $\varphi = 2C$, the value of $[\eta]$ obtained is 9.59 ml g^{-1} which suggests a surface hydration of 2.8 g g^{-1}. This hydration level falls well in the "theoretical"range of 0.5 to 3.8 g g^{-1} discussed above.

3.7.2. Effect of vesicle size

In the vesicular dispersions where the interaction between vesicle-vesicle and vesicle-solvent exist, one would expect their rheological properties to be affected by vesicle size. Figures 3.2a&b show that there is a clear effect of vesicle size on the relative viscosity of niosome suspensions. When vesicle size is reduced vesicles tend to form with lower degrees of lamellarity. A smaller vesicle size and greater number of vesicles mean an increase in the specific surface area of the disperse phase, hence a higher viscosity due to the increase in particle-solvent and particle-particle interactions. The size of vesicles is also a factor that controls the behaviour of, for example, carbomer gels containing vesicles, the viscosity of which was also found to decrease on increasing size (Bonté *et al.*, 1994).

3.7.3. Effect of vesicle membrane composition

Figure 3.2a & b show the relative viscosity of Span 60 niosomes containing 1% and 10% Solulan C24, respectively. The elevation of relative viscosity when increasing the level of Solulan C24 in the system can be clearly seen.

Modification of vesicle surfaces with polyethylene glycol (PEG) derivatives has been known to improve the vesicle circulation time in the body due to the hydration barrier and steric hindrance effects (Shimada *et al.*, 1995; Gabizon *et al.*, 1996). Increasing the surface hydration results in higher vesicle suspension viscosity. However, the addition of PEG onto the surface of vesicles can also improve the flow properties of niosomes if they otherwise tend to aggregate. Aggregation of particles results in the formation of particles with irregular shapes which then elevates the viscosity of dispersions. Modification of vesicle surfaces with long chain polymers can prevent the aggregation by steric hindrance thus reducing the viscosity of some dispersions (Sakai *et al.* 1997). The ability to reduce particle-particle interactions of grafted polymer sometimes can overcome the effect of size on rheological properties. Kato *et al.* (1983) found that modifying the surface of artificial red blood cells (ARBCs), sheep hemolysate-loaded vesicles, with a polysaccharide, carboxymethylchitin, leads to a reduction in vesicle aggregates and viscosity when compared to sheep red blood cells (SRBCs), despite the fact that ARBCs have a larger specific surface area than that of SRBCs.

3.7.3.a. Hydration

It is clear that the surface hydration of vesicles is an important determinant of biological fate through modifying vesicle-vesicle interactions and vesicle-cell interactions. Hydration also influences rheology and an examination of rheological behaviour can lead to an estimate of hydration. Thus [η] is determined by the degree of hydration of spherical vesicle surfaces.

Polyoxyethylene chains interact avidly with water (Antonsen & Hoffman, 1992), the number of bound water molecules depending on the number of oxyethylene units,

particularly when polyoxyethylated derivatives are in a micellar form (Elworthy & Macfarlane, 1962; Aldwinckle *et al.*, 1982; Miyajima *et al.*, 1989). An increased amount of Solulan C24 in the vesicle membranes will enhance the effective hydration of the surface of the vesicles, otherwise comprising sorbitan groups (Figure 3.4). The viscosity of the more hydrated form will be higher at any given value of concentration of lipids because of both the internal and the surface hydration.

In a unilamellar vesicle, the lipid/ surfactant bilayer occupies 20% of the total sphere volume hence $\varphi = 5C$, assuming all solute densities = 1 $g.ml^{-1}$. In a multilamellar system, $\varphi = C$ but only if there was no internal hydration, which is unlikely. However if we assume that the level of hydration of the sorbitan and polyoxyethylene groups are respectively 0.5 $g\ g^{-1}$ and 3.8 $g\ g^{-1}$, we can assume mean values of hydration of around 0.8 $g\ g^{-1}$ for systems containing 10% Solulan C24, assuming that the surface concentration of the Solulan is identical to the bulk concentration. It is reasonable then to assume values of $C < \varphi < 2C$ (see also Figure 3.5). The 1% Solulan system approximates more closely to $\varphi = 1.5C$.

3.7.4. Effect of electrolytes

Figure 3.6 shows the high viscosity of Span 60 niosomes containing dicetylphosphate (DCP) in water when compared to the dispersion of Span 60 niosomes without DCP but with a polyoxyethylated surfactant, Solulan C24. The viscosity of Span 60: cholesterol: DCP is dramatically decreased by the addition of NaCl. Addition of electrolytes to vesicle dispersions generally results in a decrease in the viscosity (Bonté *et al.*, 1994; Hoffmann *et al.*, 1994 & 1995; Muzzalupo *et al.*, 1996), an effect anticipated in charged dispersions and also found in latex bead systems (Wang, 1970).

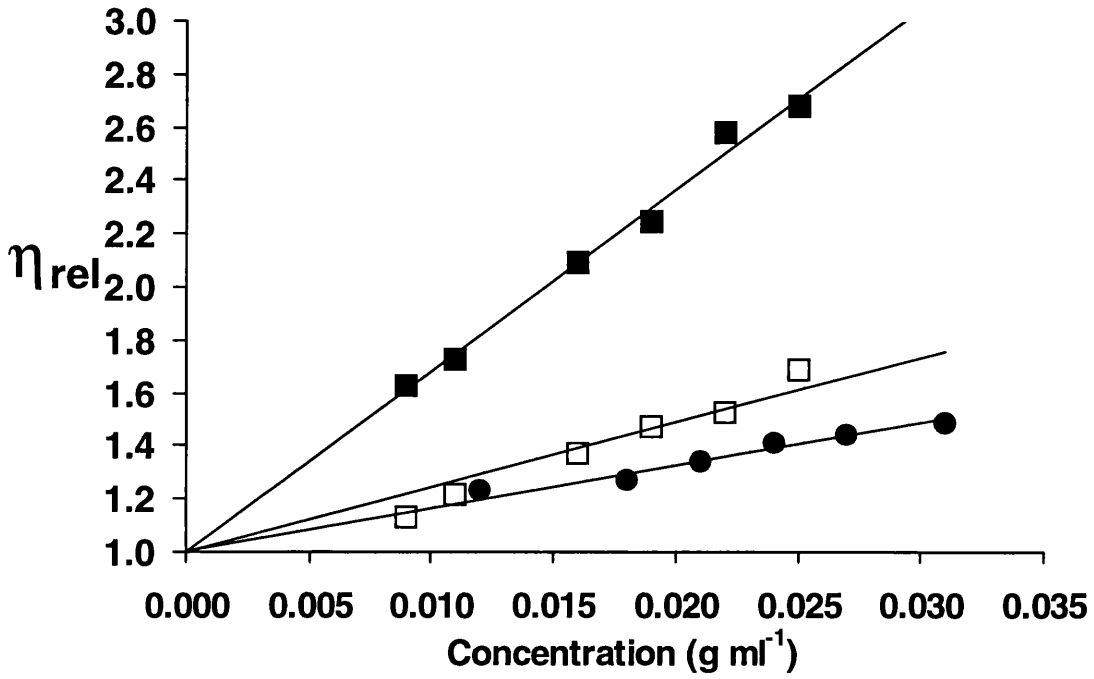


Figure 3.6: Relative viscosity of 270 nm-niosomes formed by (●) Span 60: cholesterol: Solulan C24 (45: 45: 10) in water; Span 60: cholesterol: dicetyl phosphate (DCP) (45:45:10) in (■) water and in (□) 0.5 M NaCl solution at 25°C.

Like other colloidal systems, vesicle surfaces, especially those containing charged lipid/ surfactants, possess an electric diffuse layer of ions (EDL). In this case the primary electroviscous effect, produced by the distortion from spherical symmetry of the counter-ion double layer around the charged particles due to the shear field should be considered. In the systems with higher concentrations of vesicles, there will also be concerns relating to the secondary electroviscous effect, which is the electrical repulsive force arising from the collision of charged particles. Van der Waarden (1954) explained that the viscosity of the dispersion of charged particles is highly dependant on an attraction of water molecules in the counter-ion double layer. Addition of electrolytes results in a reduction of double layer thickness around the suspension particles (Goodwin, 1974). The decrease in viscosity

of charged niosomes on adding NaCl was therefore explained in term of the reduction in viscous drag forces on the particles caused by fewer water molecules being adsorbed on the charge-neutralised vesicle surfaces.

3.7.5. Effect of preparation methods

Niosomes prepared by different methods may appear to vary in size and lamellarity. Multilamellar vesicles prepared from Span 60: cholesterol: Solulan C24 (45:45:10) by hand shaking method (HS) and reversed phase evaporation vesicles (REV) prepared from the same compositions were sonicated to a diameter of 160 nm. The viscosity profiles are shown in Table 3.1 and Figure 3.7 suggesting an additional complication of such preparation methods. Although the size distribution, measured by PCS, and the transmission electron micrographs of niosomes prepared by both methods were similar, multilamellar vesicles can be seen, and it is speculated that REV provides conditions more appropriate to the formation of unilamellar vesicles.

Considering the relationship between concentration and volume fraction, as discussed above, some clues are provided by the preparation of a largely unilamellar niosome formulation with a 10%mol of Solulan C24 and a mean diameter of *circa* 160 nm. In a unilamellar vesicle of this size the surfactant/ lipid would occupy (using the dimensions of Span 60 bilayer ~6nm) only 20% of the volume of the vesicle. If it was bilamellar, lipids occupy 38.5%. Thus φ ranges from 5C to 2.6C. Using the former value, the external hydration calculated (assuming a sphere) is ~0.6 g g^{-1} and the latter ~2 g g^{-1}, both in fact plausible figures as we have seen.

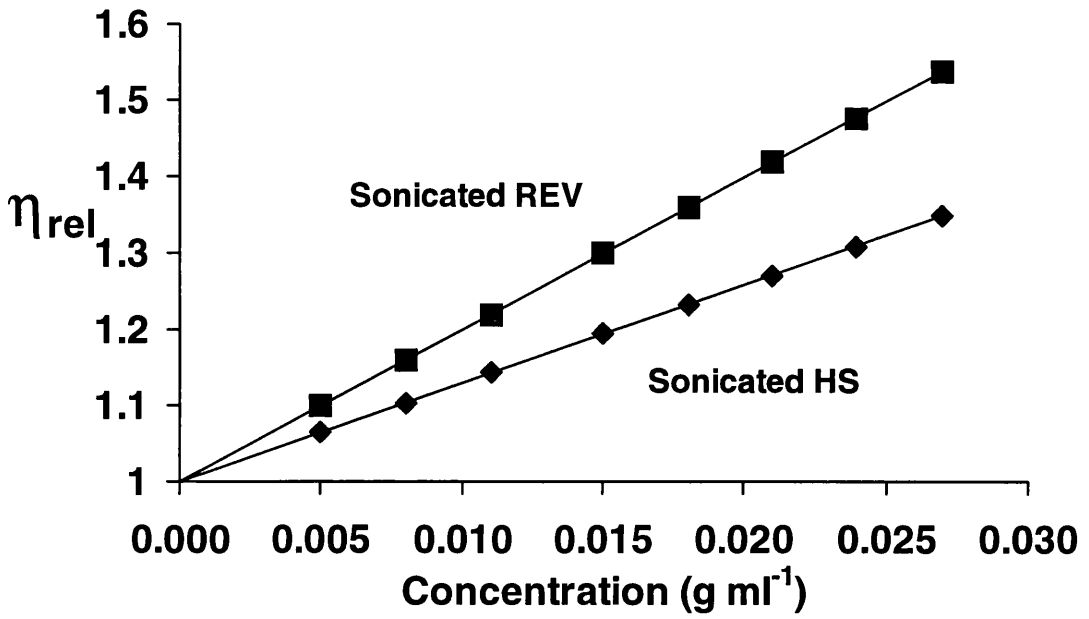


Figure 3.7: Relative viscosity of 160 nm niosomes formed by Span 60: cholesterol: Solulan C24 (45:45:10) prepared from sonication of reversed phase evaporation vesicles (REV) and hand shaken multilamellar vesicles (HS) in water at 25°C.

3.8. VISCOMETRIC STUDIES OF $C_{16}G_2$ NIOSOMES

3.8.1. Effect of vesicle shape and temperature

Viscometric studies of polyhedral and spherical niosomes clearly demonstrate the influence of average vesicle shape. Figure 3.8a & b show the relative viscosity of polyhedral niosomes formed by $C_{16}G_2$: Solulan C24 (91:9) and that of spherical niosomes formed by $C_{16}G_2$: cholesterol: Solulan C24 (45:45:10). At 25°C and 35°C, the viscosity of polyhedral niosomes is much higher than that of spherical/ tubular niosomes due to their faceted structures which resist to flow.

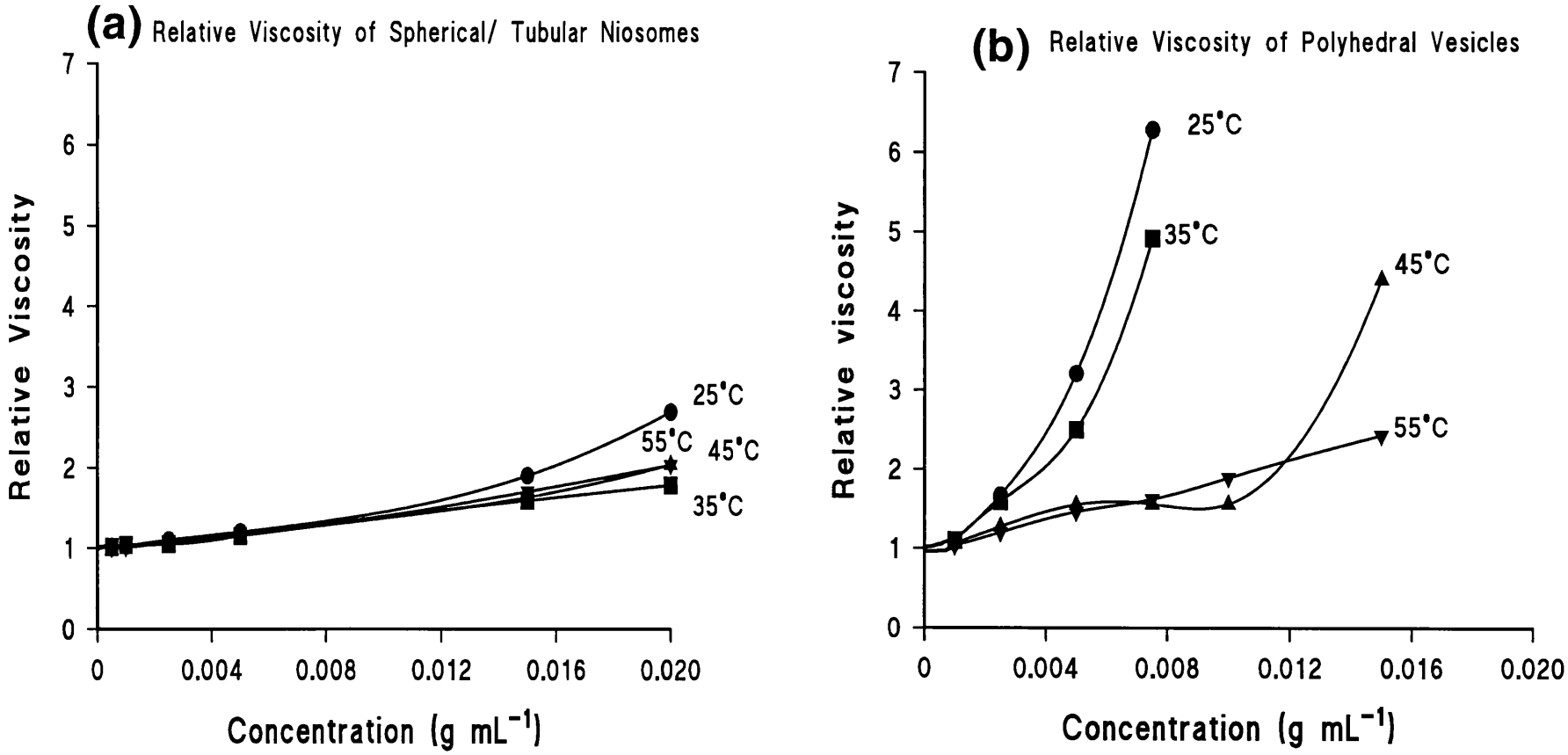


Figure 3.8: Relative viscosity of (a) spherical/tubular niosomes and (b) polyhedral niosomes of $C_{16}G_2$: cholesterol: Solulan C24 in the ratios 45:45:10 and 91:0:9, respectively at different temperature.

As shown in chapter 2 (section 2.7), the polyhedral niosomes have faceted structures at room temperature and at 35°C. On increasing the temperature above the transition temperature (T_m) of 45°C polyhedral niosomes undergo transformation into spherical structures, while cholesterol-rich spherical niosomes remain intact. There are thus different temperature effects on the flow pattern of both systems (Figure 3.8). On increasing the temperature, the viscosity of both niosomes decreases due to reduced particle-solvent interactions. However, the decrease in viscosity of spherical niosomes (Figure 3.8a) is less dramatic than that of polyhedral niosomes (Figure 3.8b), highlighting the overriding importance of shape change.

It is not necessarily always true that increasing temperature will result in decreased viscosity. Heimburg & Biltonen (1994) found an unusual increase in η_{rel} of dimyristoyl phosphatidylglycerol vesicles over the range of main transition temperature (20-28°C) while η_{rel} below and above this range was low. This effect was due to the domination of the non-spherical vesicles with extended arms existing in the transition range. Hassan *et al.* (1996) found the increase in viscosity of cetyltrimethylammonium hydroxynaphthalene carboxylate (CTAHNC) vesicles at temperatures greater than 50°C to be due to the transformation of vesicles into elongated wormlike micelles.

3.9. CONCLUSIONS

Niosomes serve as an interesting model system in rheological studies as their behaviour can be modified by varying either internal factors such as vesicle concentration, size, shape, membrane composition, or external factors such as temperature and salinity. It is recognised that further work needs to be done to allow the intrinsic viscosity of niosome dispersions to be used directly as an indicator of hydration. Insights gained from these studies should be useful not only for basic research but, *per se* in situations where the flow of suspensions are important as in manufacturing processes and the rheological behaviour *in vivo* which seem to have been a largely neglected topic.

Chapter 4

OSMOTIC BEHAVIOUR OF POLYHEDRAL NIOSOMES

4.1. INTRODUCTION

Cells are made of selectively permeable membranes which allow an unequal diffusion of water across their membranes when the osmotic pressure of the solutions separated by the membranes is different. To examine the behaviour of cells and the permeability of lipid membranes when subjected to solute gradients, many studies have been performed using lipid vesicle models. This chapter comprises a further characterisation of polyhedral niosomes formed mainly by $C_{16}G_2$ in comparison to their spherical counterparts formed with an equimolar amount of cholesterol. Changes in vesicle size were examined as a function of niosome structure and composition by challenging the vesicles to a range of osmotic gradients in which both internal and external salt gradients were established.

In this study, osmotic behaviour of niosomes was investigated by following the change in

size due to osmotic gradients using low angle laser light scattering (LALLS), the so called laser diffraction method which also provides an equivalent-sphere size distribution for non-spherical particles (ISO/DIS 13320). It is difficult to describe the size of a polyhedral structure as only a spherical particle can be described by one number which is its diameter (Allen, 1992; Bernhardt, 1994). However, by means of the spherical equivalence principle, we can obtain a diameter of an equivalent sphere model which has the same measured property as our non-spherical particles, depending on the technique used, e.g. sedimentation velocity, surface area, or volume in this case. The fact that every particle scatters light, the measured intensity distribution data can then be transformed to the volume of the particle by means of mathematical models. Therefore, the change in light diffraction pattern due to swelling or shrinkage of polyhedral vesicles allows the change in size of their equivalent spheres to be followed.

In addition to the change in vesicle size, membrane permeability was studied by following the release of a water soluble marker, 5(6)-carboxyfluorescein (CF), from both polyhedral and spherical/ tubular niosomes when subjected to osmotic gradients. The effect of pH on the release profiles of CF was also studied.

MATERIALS AND METHODS

4.2. MATERIALS

All materials used in this chapter were from the same source stated in Table 2.1.

METHODS

4.3. OSMOTIC ACTIVITY OF POLYHEDRAL NIOSOMES

Polyhedral vesicles, prepared by hydrating a film of $C_{16}G_2$: Solulan C24 (91:9) with water at 60°C and storing for 24 h, were mixed with glucose or sodium chloride solutions to give an external phase of 0.93 M glucose or 0.154, 1.0, or 2.0 M NaCl, respectively, with water as a control. Alterations in particle size in response to osmotic gradients were recorded by low angle laser light scattering (MasterSizer X, Malvern, UK). Polyhedral vesicles were also prepared containing 2.0 M NaCl using a hydrating solution of 2.0 M NaCl instead of water. 2.0 M NaCl loaded niosomes were diluted 10 times in either 2.0 M NaCl solution or water and the vesicles then sized. Niosomes prepared from $C_{16}G_2$: Cholesterol: Solulan C24 (49:49:2) and (45:45:10) which form mixtures of spherical and tubular vesicles served as control dispersions.

4.4. THE RELEASE OF CF FROM POLYHEDRAL NIOSOMES

4.4.1. The influence of osmotic gradient on CF release

The effect of the "external" and "internal" hypertonic solution on CF release was studied. The preparation of niosomes with an external hypertonic solution involved hydrating appropriate surfactant/ lipid mixtures, namely $C_{16}G_2$: Solulan C24 (91:9), (98:2), and $C_{16}G_2$: Cholesterol: Solulan C24 (49:49:2), with CF (5mM) prepared in water at 60°C for 1 h. This was followed 24 h later by the removal of unentrapped material by exhaustive dialysis at 4°C (Visking tubing - MW cut off = 12-14 kDa). Entrapped CF was estimated by the disruption of the niosomes with isopropanol followed by the dilution of the resulting solution with water and measuring the fluorescence (Perkin-Elmer LS-3 fluorescence

spectrometer; excitation wavelength 486nm, emission wavelength 514nm). Washed niosomes were then placed in dialysis tubing suspended in 0.154M, or 2.0 M NaCl solution followed by periodic fluorimetric analysis of the dialysate. The temperature was maintained at 25°C and sampling was perfomed over a 5h period.

Niosomes containing a hypertonic salt solution were prepared in the same way as described above except that surfactant/ lipid mixtures were hydrated with a solution of CF (5mM) in 2.0 M NaCl solution. This niosome dispersion was also kept for 24h. Exhaustive dialysis against 2.0 M NaCl at 4°C was then performed to remove unentrapped solute. The release of CF was then measured by placing these suspensions in a dialysis bag suspended in 2.0 M NaCl or water, as before.

4.4.1.a. Effect of NaCl on the phase transition behaviour

Studies were conducted using a high sensitivity differential scanning calorimeter (HSDSC) (MicroDSC, Setaram, UK). Approximately 300 mg of each polyhedral niosome dispersion (60mM) prepared in water or 2.0 M NaCl was introduced into the measurement vessel whilst an equivalent mass of water or 2.0 M NaCl was introduced in the reference vessel. The difference in heat flow between the vessels was measured as a function of temperature. Samples were scanned at a rate of 1K/min from 10 to 70°C, followed by cooling to 10°C.

4.4.2. The influence of pH on CF release

Polyhedral niosomes ($C_{16}G_2$: Solulan C24 - 91: 9) and spherical/ tubular niosomes ($C_{16}G_2$: Cholesterol: Solulan C24 - 49: 49: 2) were prepared by hydrating surfactant/ lipid films with CF (5mM) in Tris buffered saline (TBS) 1/10 strength at pH 5 or 8. Dispersions were

stored for 24 h and subsequently exhaustively dialysed against TBS 1/10 strength at the same pH at 4°C to remove unentrapped CF. Entrapped CF was estimated by the disruption of the niosomes with isopropanol followed by the dilution of the resulting solution with TBS at the same pH and measuring the fluorescence (excitation 486nm, emission 514nm). The release of CF was measured by placing these suspensions in a dialysis bag suspended in pH 5 and pH 8.

RESULTS AND DISCUSSION

4.5. OSMOTIC ACTIVITY OF POLYHEDRAL NIOSOMES

As anticipated, the dispersion of various niosome formulations in environments of varying osmotic strength caused changes in vesicle diameter. The size distribution obtained by laser diffraction equipment correlates well with optical microscopy. The degree of size change was dependent on the actual nature of the vesicles and the concentration of salt or glucose solution (Figure 4.1& 4.2) in the continuous phase. Generally polyhedral vesicles showed less response to a salt osmotic gradient compared to spherical/ tubular formulations (Figure 4.1 & Table 4.1). On being dispersed in glucose solution the mean size of all niosomes also decreased (Figure 4.2) although the degree of shrinkage was less for the polyhedral niosomes. Previous authors reported that large unilamellar vesicles of dipalmitoyl phosphatidylcholine can decrease in volume up to 80% when subjected to 0.20-0.25 M NaCl gradient (Disalvo *et al.*, 1996). A real life example is the change in size of sea urchin eggs by the tonicity of the media which showed that the surface of eggs can shrink by 50% without wrinkling (Yoneda, 1973). The ability of vesicles to increase in size shown in Table

4.1 does not seem to be excessive when compared to the 200-300% increase in volume of giant unilamellar vesicles formed by didodecyl ammonium bromide (DDAB) and cholesterol following a direct injection of water into the vesicles (Menger & Lee, 1995). The mast cell vesicles of the beige mouse are other examples found to increase in diameter up to 73% when subjected to hypotonic solution (Brodwick *et al.*, 1992). Although it has been previously shown that the degree of size change following osmotic gradients depends on the initial diameter of vesicles (Sun *et al.*, 1986), the results suggest that the composition of the vesicle membranes appeared to be a more important factor in determining their osmotic response. With spherical/ tubular niosomes the sensitivity to osmotic stress was not dependent on the level of Solulan C24 in the vesicles. Although the vesicle dispersions are polydisperse, changes in the mean diameter of the vesicle population were used as there was a shift of the overall size distribution to a lower diameter range. On the other hand when the osmotic stress across these spherical/ tubular niosome membranes arose from internal salt concentration to the niosomes, low levels of Solulan C24 (2%) increased the reaction of these niosomes to osmotic stress. A 60% increase in size was observed for niosomes containing NaCl but dispersed in water (Table 4.1) compared to a 25% decrease in size after 5h for niosomes containing water dispersed in 2.0 M NaCl (Figure 4.1). This observation compares favourably with earlier studies on erythrocytes which have a greater ability to swell than to shrink (House, 1974). In addition, their response to osmotic stress was fairly rapid, as can be deduced from the calculated water flux across the membrane (Figure 4.3).

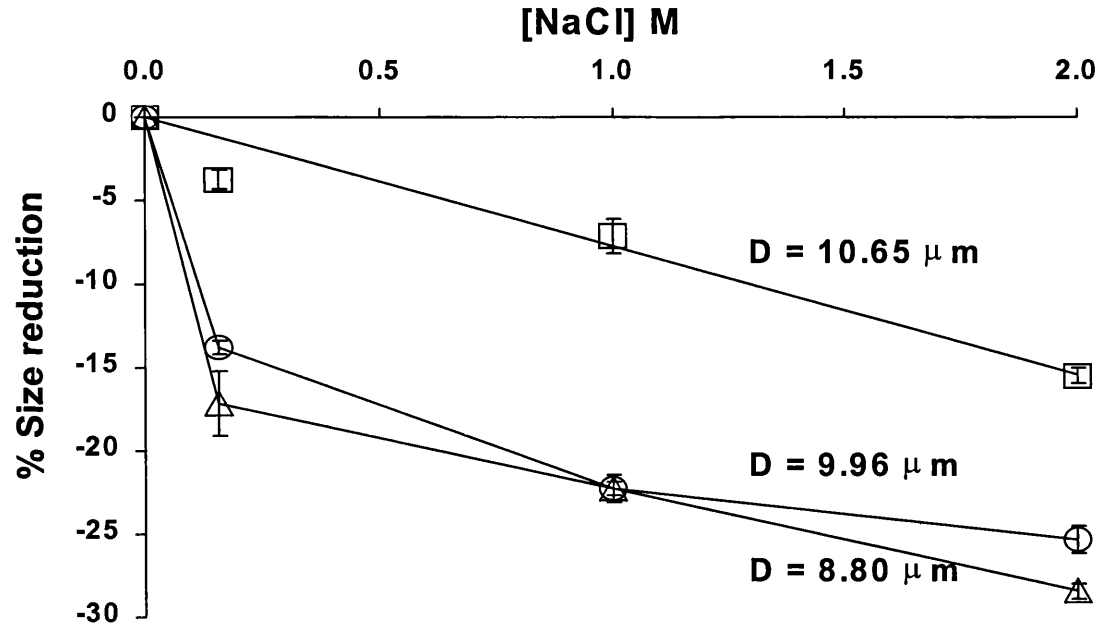


Figure 4.1: The reduction in niosome mean diameter (niosomes encapsulating water) 5h after dispersion in NaCl solutions, (□) polyhedral niosomes - $C_{16}G_2$, Solulan C24 (91: 9), (O) spherical/ tubular niosomes - $C_{16}G_2$: cholesterol: Solulan C24 (49: 49: 2), (Δ) spherical/ tubular niosomes - $C_{16}G_2$: cholesterol: Solulan C24 (45: 45: 10). The initial diameters of niosomes (D) are shown.

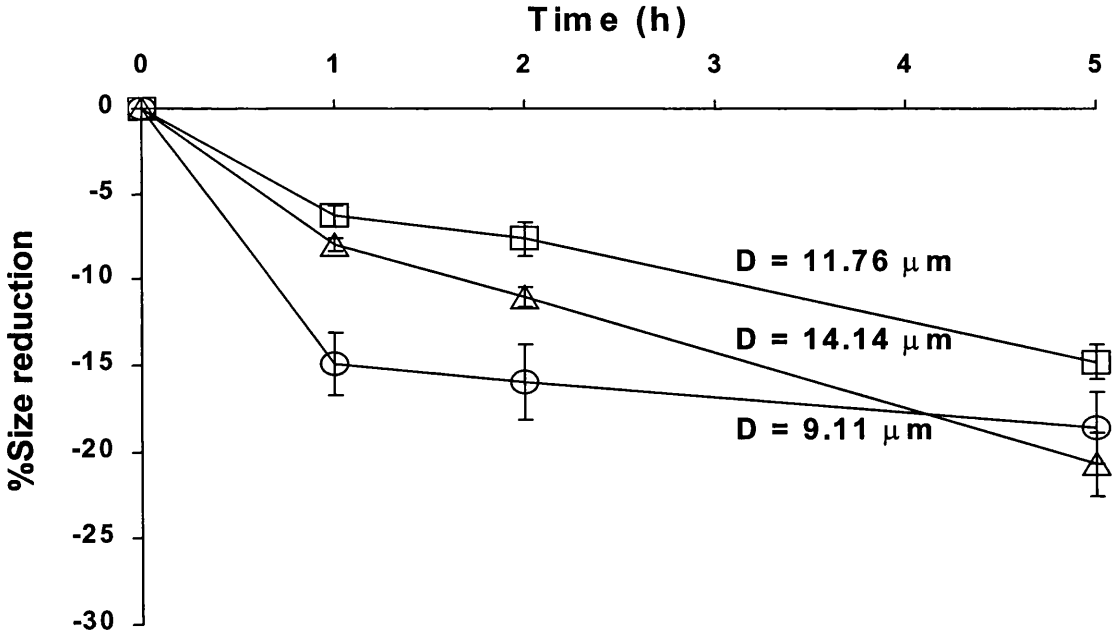


Figure 4.2: The reduction in mean niosome diameter (niosomes encapsulating water) as a function of time after dispersion in glucose solution-symbols as in Figure 4.1.

Table 4.1 :The increase in niosome mean diameter (niosomes encapsulating 2 M NaCl) after being dispersed in water for 5 h.

Composition of niosomes	% Size increase
Polyhedral (C16G2, Solulan C24 - 91:9)	10
Spherical/ tubular (C16G2, Cholesterol, Solulan C24 - 45:45:10)	23
Spherical/ tubular (C16G2, Cholesterol, Solulan C24 - 49:49:2)	60

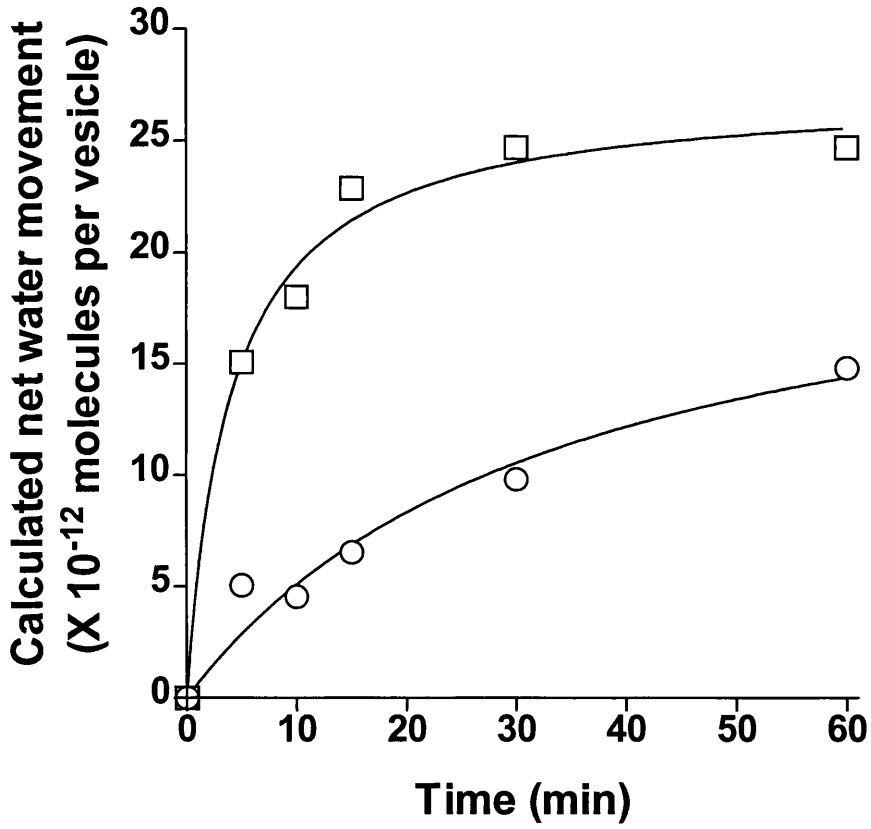


Figure 4.3: The calculated number of water molecules diffusing from spherical/ tubular ($C_{16}G_2$, cholesterol, Solulan C24, 49:49:2) vesicles as a function of time. Water flux is obtained by assuming the reduction in volume is caused by water efflux from the vesicles in (O) 0.15 M NaCl; and (□) 2 M NaCl. The estimated initial net water flux for niosomes dispersed in 0.15 M and 2 M NaCl are $1.2x10^7$ molecules mm^{-2}. sec^{-1} (or 0.12 $ml.cm^{-2}.h^{-1}$) and $4.6x10^7$ molecules. mm^{-2}. sec^{-1} (or 0.49 $ml.cm^{-2}.h^{-1}$) respectively, compared to those in animal epithelial tissues which are in range of 0.4-75.0 $ml.cm^{-2}.h^{-1}$ (House, 1974).

Selective permeability is the key to an osmotic stress reaction and osmotic gradients will be rapidly abolished if the membrane is permeable to the osmotic solute. The observation that polyhedral niosome membranes are less likely to show size changes than those of vesicles formed with the inclusion of cholesterol, agrees well with previous observations (Baillie *et al.*, 1985) in which vesicles formed from 100% of $C_{16}G_3$ (hexadecyl triglycerol ether) showed reduced size changes when compared to vesicles prepared containing cholesterol. The incorporation of cholesterol into liposomal membranes decreases the membrane permeability to solutes (De Gier *et al.*, 1968; Baillie *et al.*, 1985). This decreased solute permeability will allow for the selective membrane permeability to water and, in turn, produces the type of measurable reaction to osmotic stress observed here.

4.6. THE RELEASE OF CF FROM POLYHEDRAL NIOSOMES

4.6.1. The influence of osmotic gradient on CF release

It is possible that there are a number of factors in operation in determining the osmotic activity of niosomes. Figure 4.4a&b show the release of CF from the polyhedral and spherical/ tubular niosomes when dispersed in NaCl solutions. The permeability of polyhedral niosomes to entrapped CF was greater than that of spherical/ tubular niosomes, as compared in figure 4.5, and we believe that this higher permeability is in part responsible for the minimal change in size in response to osmotic stress. When glucose solutions were used to produce osmotic gradients the osmotic stress reaction of these polyhedral niosomes was increased (Figure 4.2) in comparison to the reaction to salt gradients (Figure 4.1). This may be due to a decreased permeability of the polyhedral niosomes to glucose when compared to the electrolytes.

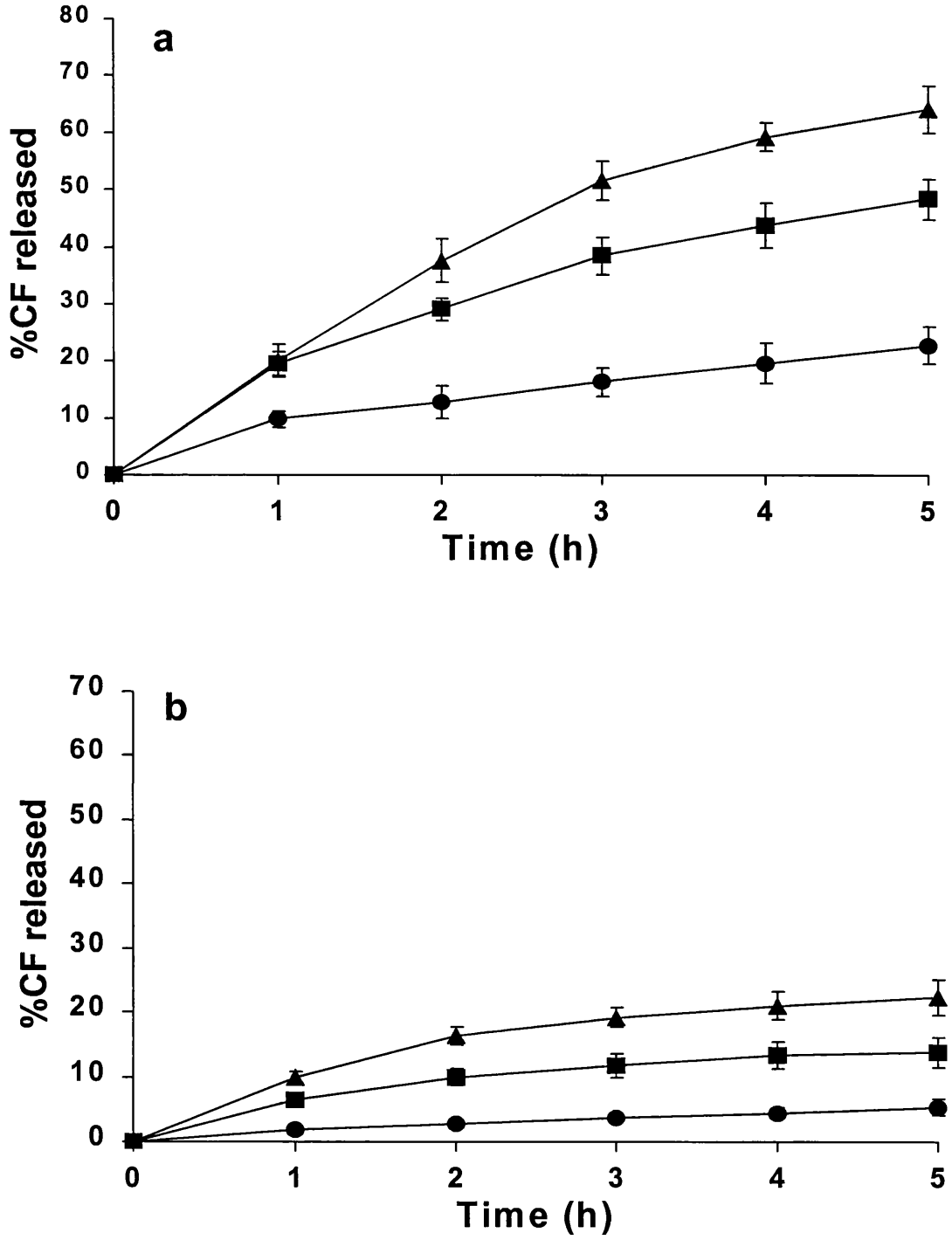


Figure 4.4: The cumulative CF released from (a) polyhedral and (b) spherical/ tubular niosomes (niosomes encapsulating CF alone) after dispersion in (●) water, (■) 0.154M NaCl, and (▲) 2M NaCl over a 5 h period.

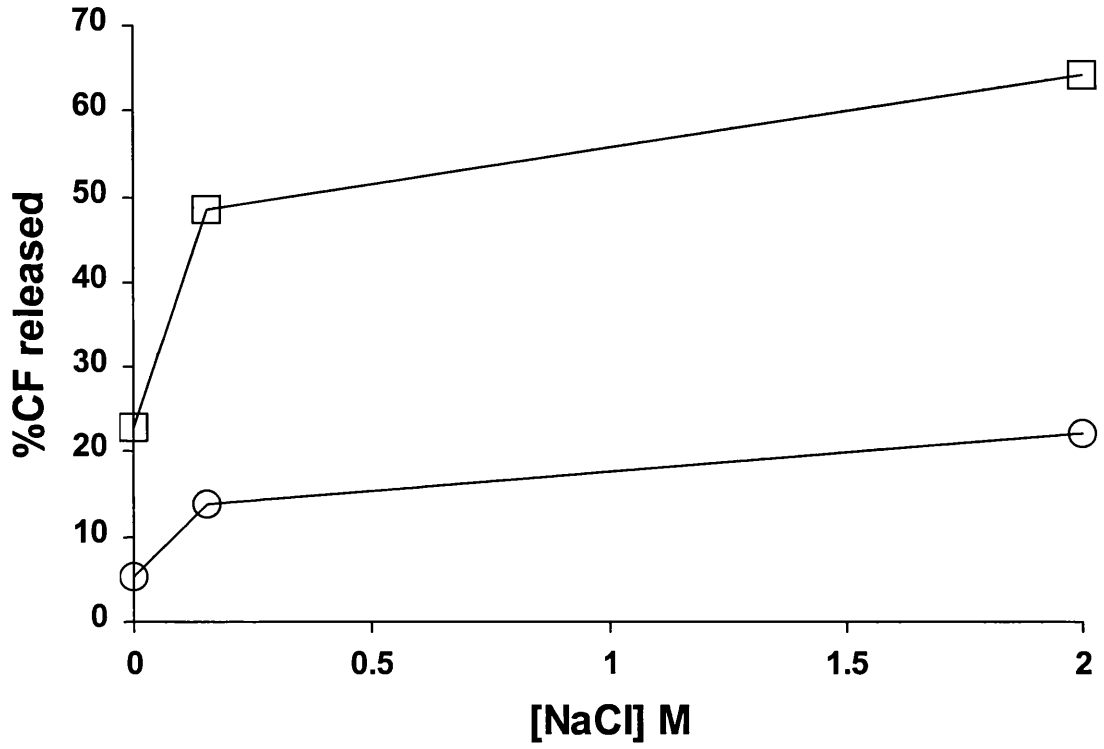


Figure 4.5: The cumulative percentage of CF released from polyhedral (□) or spherical/ tubular (O) niosomes (niosomes encapsulating CF alone) 5 h after dispersion in NaCl solutions.

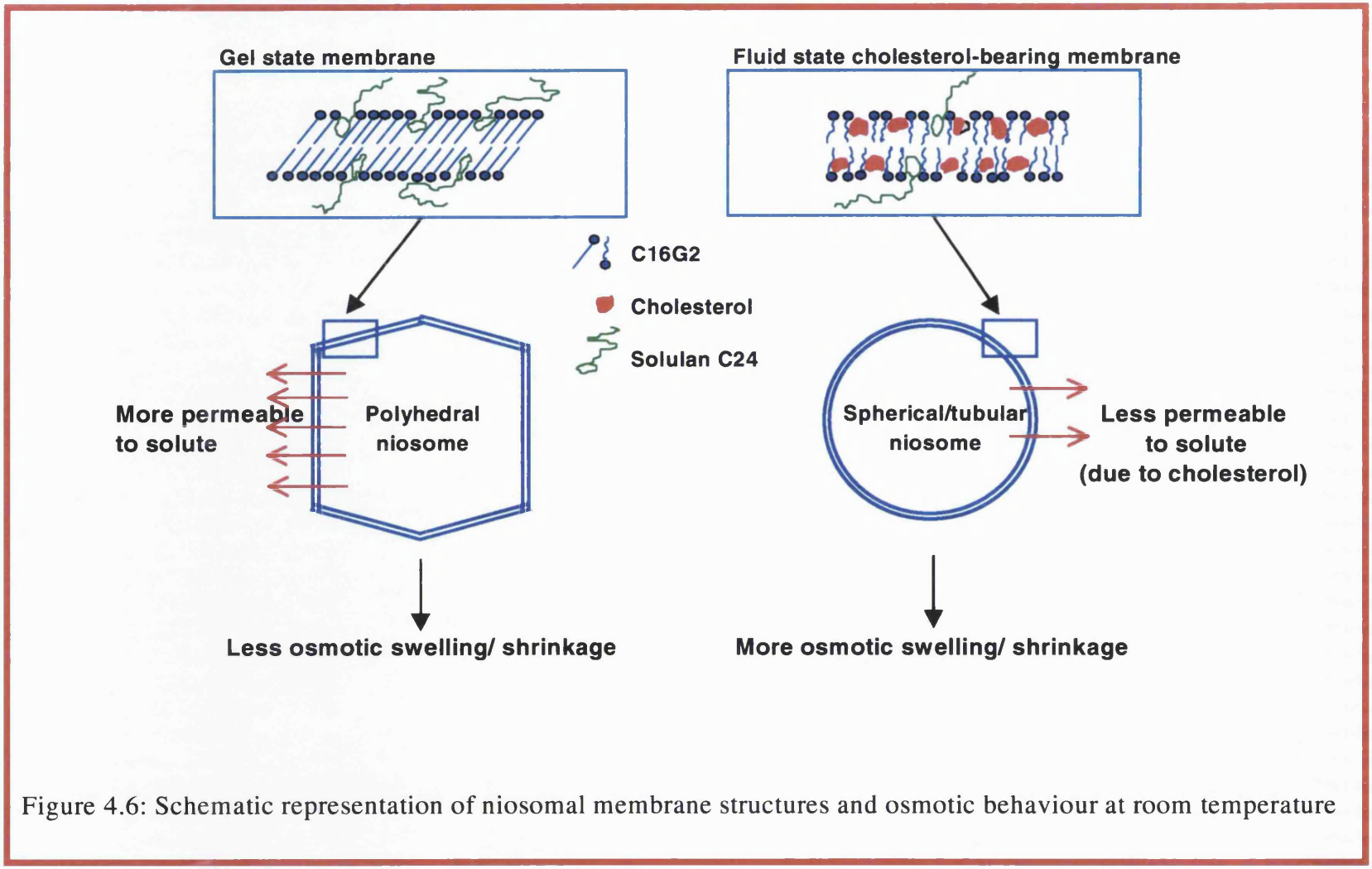


Figure 4.6: Schematic representation of niosomal membrane structures and osmotic behaviour at room temperature

There is some evidence from these studies of coupled flux of water and CF. It might have been expected that the release of CF into NaCl or glucose solutions would be enhanced as water exited the vesicles (Disalvo *et al.*, 1996). The release of CF from both polyhedral and spherical/ tubular niosomes into external salt solutions (0.15 & 2.0 M NaCl) was, however, only slightly greater than the release of CF from these niosomes into water (Figure 4.5). The degree to which osmotic gradient affects both types of niosomes appears similar, although the polyhedral niosomes are intrinsically more permeable to CF (Figure 4.5). This suggests that the lower sensitivity of the polyhedral niosomes in response to an osmotic stress is, in addition to being a function of the increased permeability of the membrane, also a function of the decreased elasticity of the membranes which are less likely to be able to accommodate changes in vesicle size (Sun *et al.*, 1986). The diagram describing the osmotic properties of polyhedral and spherical/ tubular niosomes is shown in Figure 4.6. Polyhedral niosome membranes are in the gel phase at room temperature, have a faceted appearance and are thus, presumably, more rigid than niosome membranes in which the gel to liquid phase transition is abolished as with cholesterol containing membranes. The spherical/ tubular nisomes are therefore able to change in size following osmotic gradients and less permeable due to the presence of cholesterol.

4.6.1.a. The effect of co-entrapped NaCl on CF release

As a test of the coupled flux, the influence of water flux into the vesicles on CF release was studied. The co-entrapment of CF with 2.0 M NaCl changed the release profile of CF from both polyhedral and spherical/ tubular niosomes (Figure 4.7). Spherical/ tubular niosomes showed a higher rate of release into a hypotonic medium (water) than into an isotonic (2.0 M NaCl) medium. A similar response has been recorded for liposomes (Yoshikawa *et al.*,

1983; Iga *et al.*, 1989). Polyhedral niosomes which were found to possess more leaky membranes, however, release very low amounts of CF irrespective of osmotic conditions (hypotonic or 2.0 M NaCl) (Figure 4.7). Figure 4.7b shows that polyhedral niosomes containing 9% Solulan C24, showed no increase in release in a hypotonic medium and release rates remained at the same level as when dispersed in 2.0 M NaCl (Figure 4.7a).

When the amount of Solulan C24 in polyhedral niosomes was reduced from 9% to 2%, the release of CF in hypotonic medium increased (Figure 4.8), as happens with conventional vesicles (Figure 4.7b). Figure 4.9 shows the CF loaded polyhedral niosomes of $C_{16}G_2$: Solulan C24 (91:9) prepared in NaCl and left to dialyse in water for 3 days at room temperature had become swollen (Figure 4.9b) when compared to those dialysed in the isotonic NaCl solution (Figure 4.9a). The same event was also observed for polyhedral niosomes with 2% Solulan C24 (Figure 4.10). It is noteworthy that niosomes formed with 9% Solulan C24 still contain high amounts of CF (Figure 4.9b) compared to those formed with 2% Solulan C24 (Figure 4.10b). The photomicrographs appeared to correlate well with the release studies. NaCl which can salt out polyoxyethylene chains (Graham, 1992) may cause membranes containing high levels of polyoxyethylated compounds (Solulan C24) to become more hydrophobic, leading to a lower permeability to CF. Small changes in the hydration level of membrane-forming polyoxyethylated surfactants are crucial to membrane ultrastructure as the complete formation of membrane depends on the amounts of water (Usselmann and Müller-Goymann, 1984).

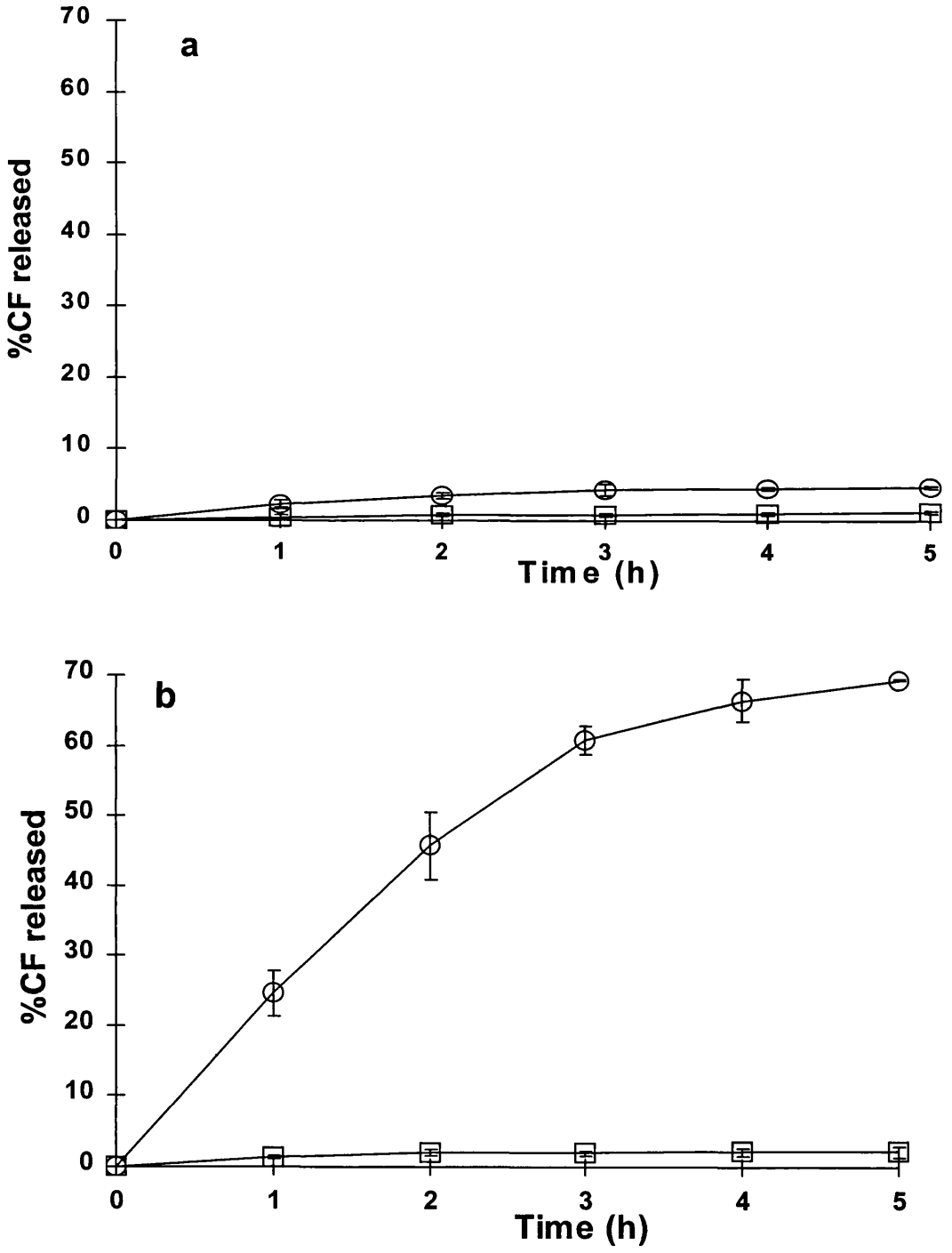


Figure 4.7: The release of CF from (□) polyhedral niosomes - $C_{16}G_2$: Solulan C24 (91: 9) and (O) spherical/ tubular niosomes - $C_{16}G_2$: cholesterol: Solulan C24 (49: 49: 2) (encapsulating CF + 2.0 M NaCl) (a) into isotonic media (2.0 M NaCl) and (b) into hypotonic media (water).

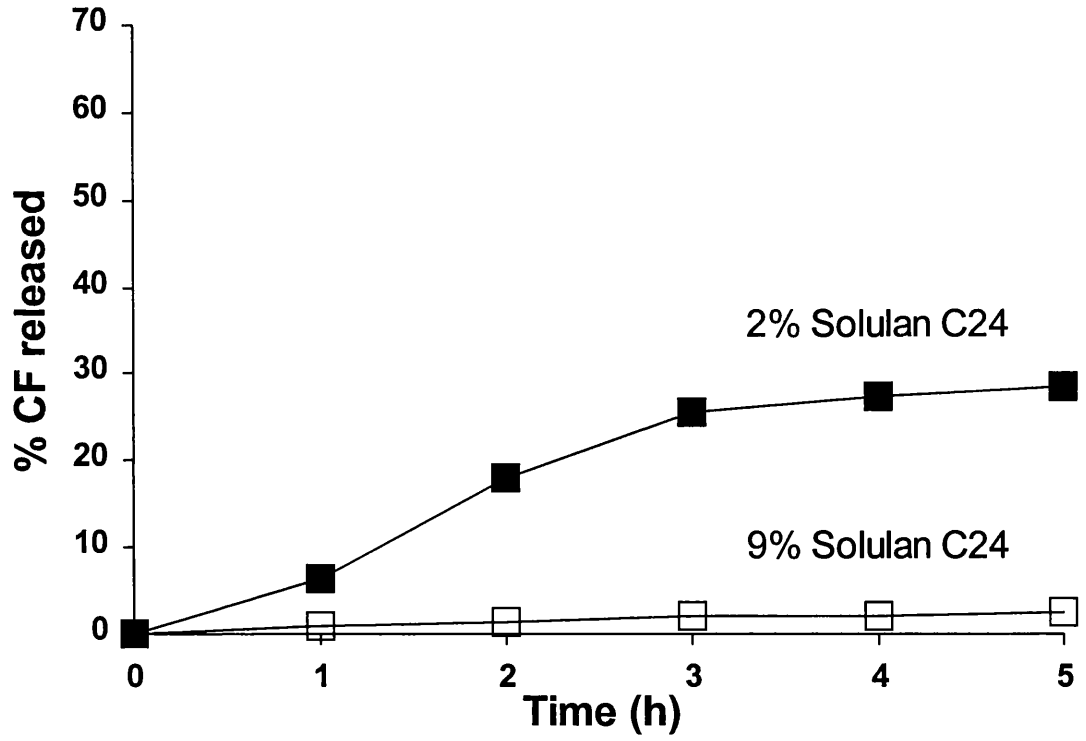


Figure 4.8: The release of CF from polyhedral niosomes (niosomes encapsulating CF + 2.0 M NaCl) into hypotonic media (water), (□) polyhedral niosomes - $C_{16}G_2$: Solulan C24 (91: 9), (■) polyhedral niosomes - $C_{16}G_2$: Solulan C24 (98: 2).

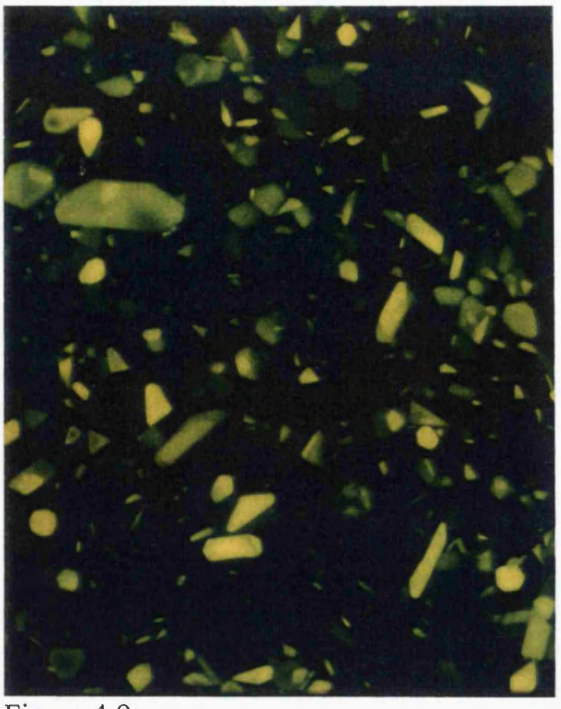


Figure 4.9a

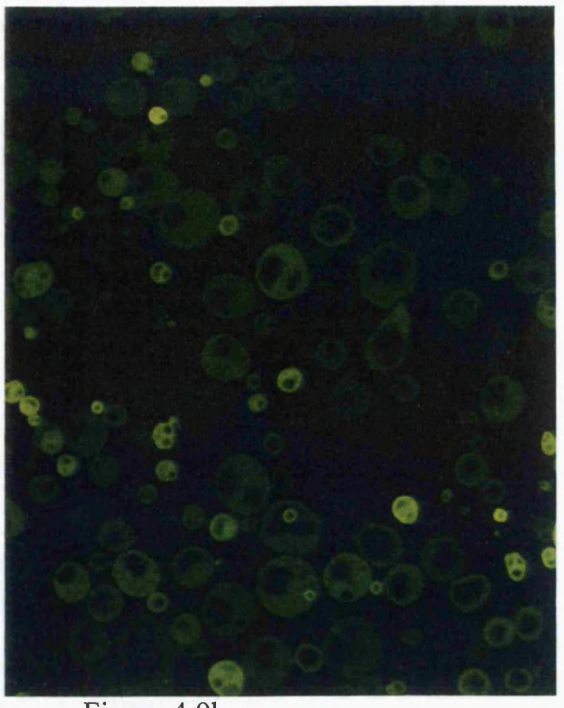


Figure 4.9b

Figure 4.9: Photomicrographs of polyhedral niosomes of $C_{16}G_2$: Solulan C24 (91:9) encapsulating CF prepared in 2.0 M NaCl and then dialysed against (a) 2.0 M NaCl and (b) water. 25 μm

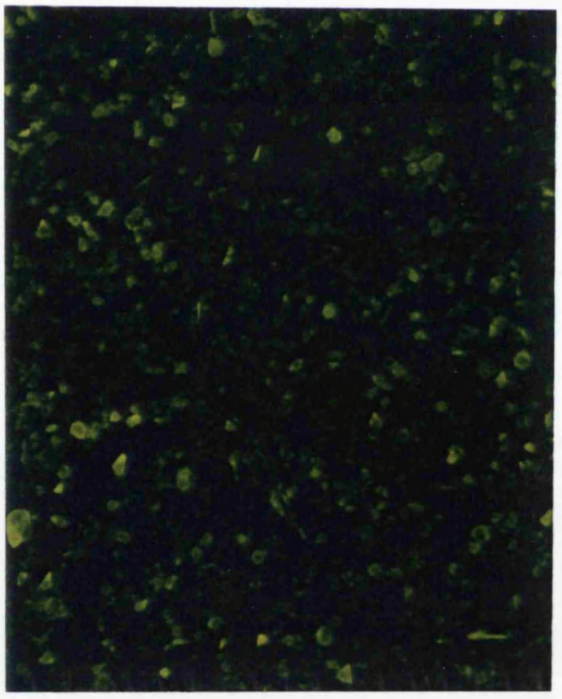


Figure 4.10a

Figure 4.10b

Figure 4.10: Photomicrographs of polyhedral niosomes of $C_{16}G_2$: Solulan C24 (98:2) encapsulating CF prepared in 2.0 M NaCl and then dialysed against (a) 2.0 M NaCl and (b) water. 25 μm

4.6.1.b. Effect of NaCl on the phase transition behaviours of polyhedral niosomes

The HSDSC was used to investigate the effect of NaCl on the permeability of different membrane compositions as previously used to examine the interactions between proteins and liposome membranes (Lo & Rahman, 1995). The presence of NaCl was found to have no effect on the phase transition of niosomes which contained 2% Solulan C24 (Fig. 4.11). The HSDSC traces showed an influence of NaCl on the phase transition of polyhedral niosomes composed of 9% Solulan C24 (Fig. 4.12) suggesting that NaCl alters the niosome membrane structure. The promotion of separated endotherm and exotherm in the presence of NaCl is also clearly shown when increasing the level of Solulan C24 to 15% (Fig. 4.13). These observations showed that the level of Solulan C24 in the niosome membranes is responsible for the change in thermal behaviour and release characteristics, caused by NaCl. DSC was earlier used to investigate the reduced transition temperature in the micelles formation of poloxamers (block copolymers of ethylene oxide and propylene oxide) on addition of NaCl (Hecht *et al.*, 1995). The effect was explained in terms of water desorption from poloxamer molecules by NaCl which, in turn, increases the hydrophobicity of the molecules and thus induces micellization at lower temperatures. Unlike the poloxamer micelles, we have not observed a reduction in transition temperature in our vesicle dispersions but the enhancing effect of the pretransition shoulder of endothermic peaks and 2nd transition of the exothermic peak to the level of 2 separated peaks. The interaction of NaCl and Solulan C24 seem clearly responsible for the change in thermotropic behaviour of $C_{16}G_2$ niosomes. It is possibly the desolubilisation of the ethylene oxide chains by NaCl that enhances hydrophobicity in the Solulan C24 molecules which may consequently induce the change in molecular orientation, phase behaviour, and solute retainability of vesicles.

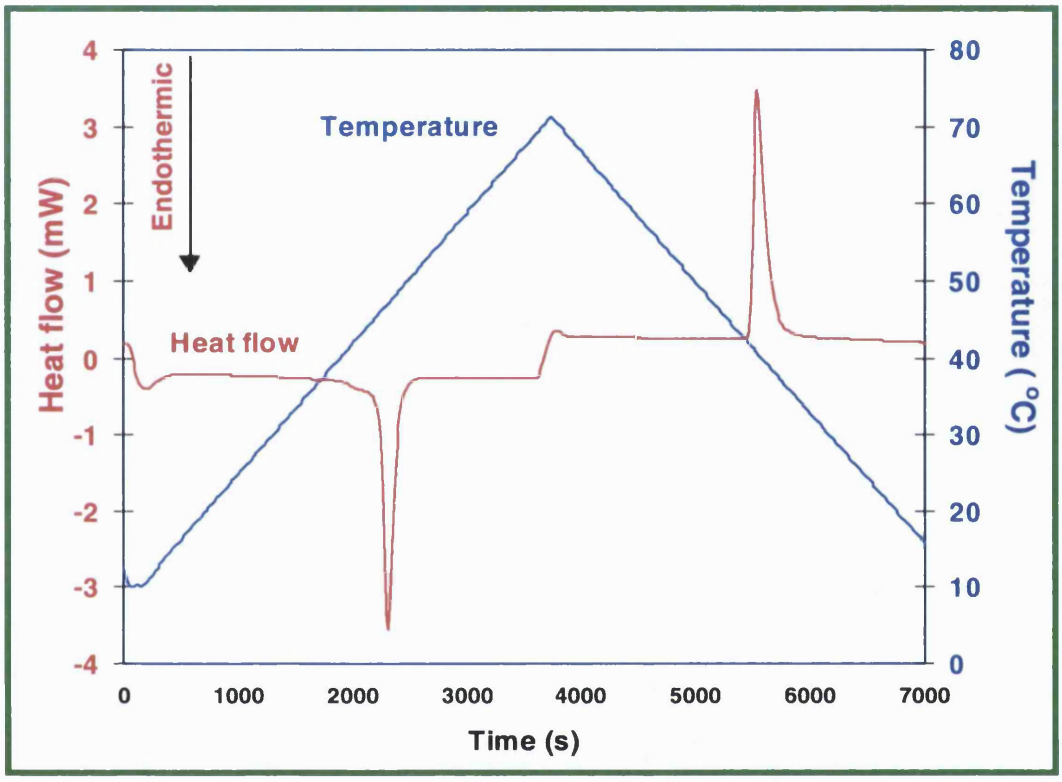


Figure 4.11: The HSDSC traces of the 2nd repeated heating/ cooling cycle of niosomes composed of 2% Solulan C24 in the presence of 2.0 M NaCl. None of the repeated cycles was found to differ from the first cycle.

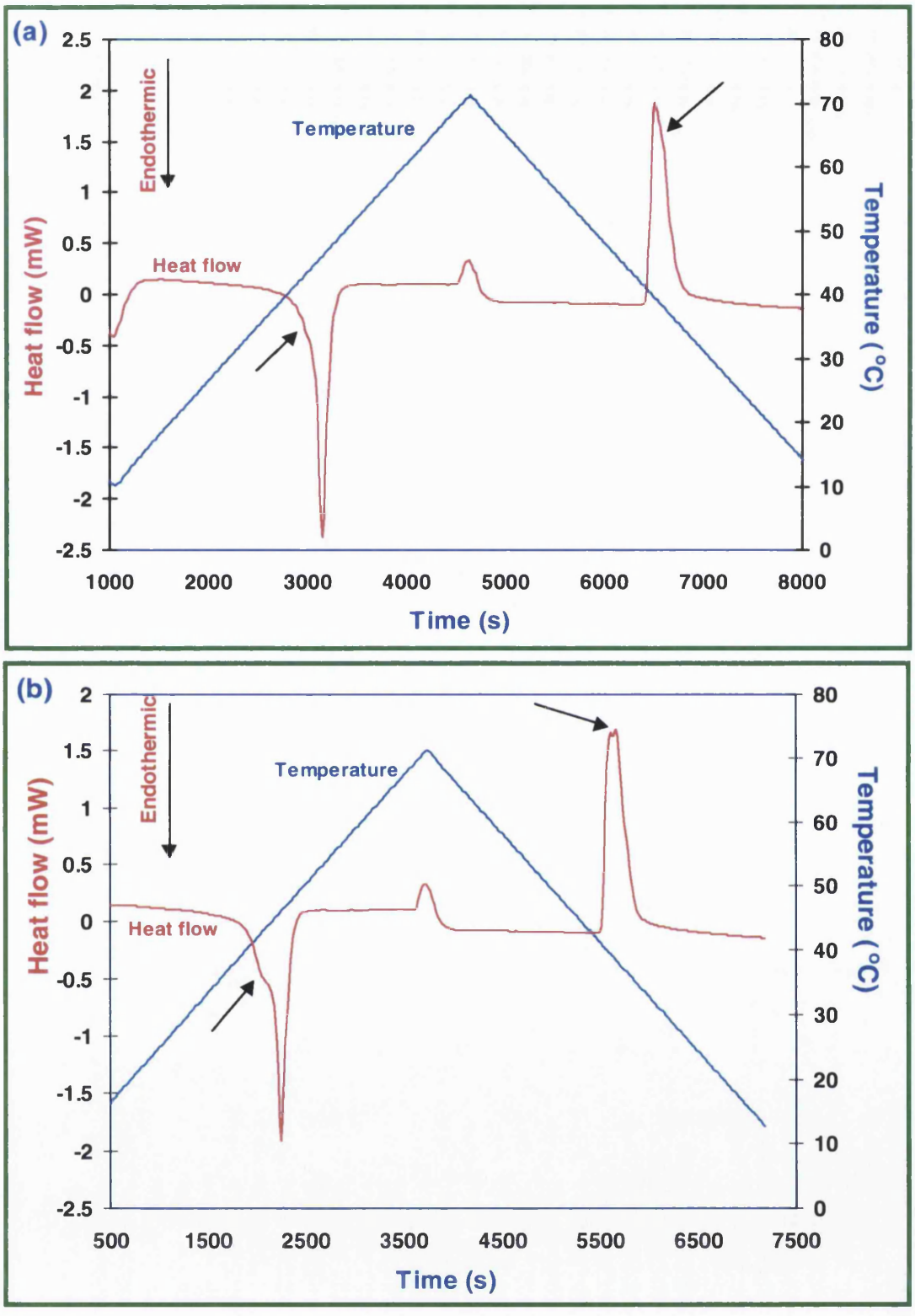


Figure 4.12: (a) 9% Solulan C24-Polyhedral niosomes heated and cooled in the presence of 2.0 M NaCl showed a shoulder (➜) on the main transition. (b) Repeated cycling showed further enhancement of the shoulder and separated peaks.

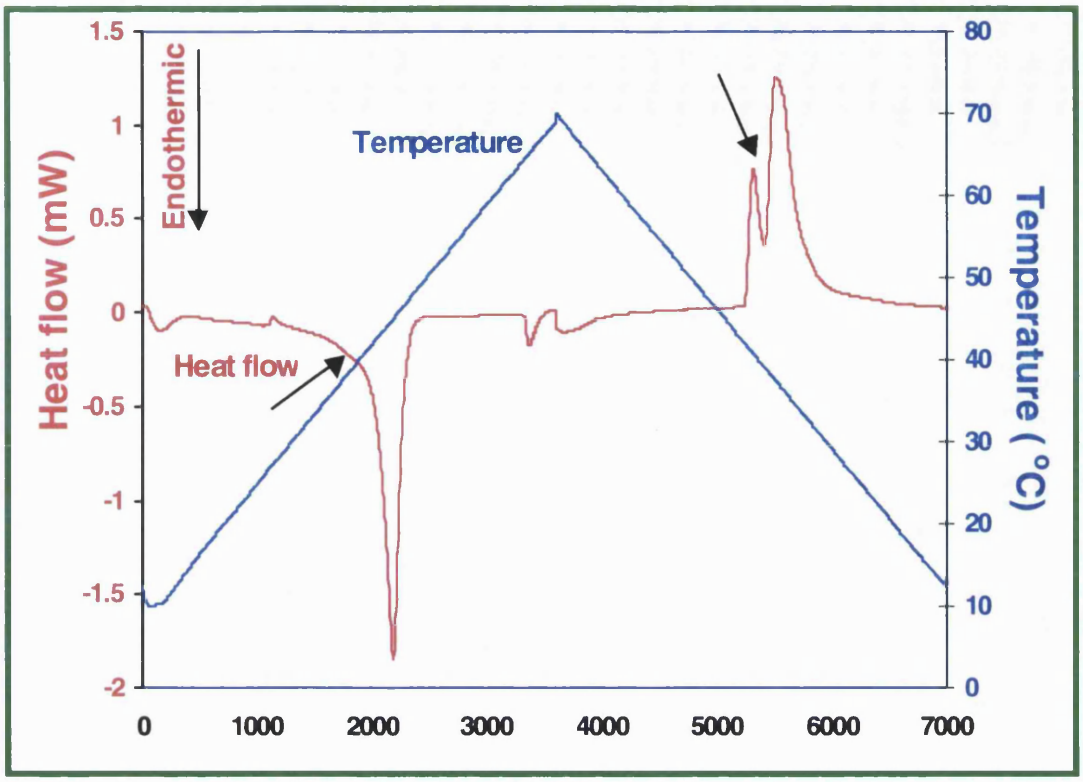


Figure 4.13: 15% Solulan C24-Polyhedral niosomes heated and cooled in the presence of 2M NaCl showed a shoulder (➜) on the main endothermic transition and separated exothermic transition peaks.

4.6.2. The effect of pH on CF release

As carboxyfluorescein (Figure 4.14) is an organic carboxylic acid with a pKa of 6.4, at pH 5 it is undissociated while fully ionised at pH 8. Figure 4.15 shows that the CF release was greatest from polyhedral niosomes at both pH values. At pH 8, when CF molecules are predominantly dissociated, the release rates were generally higher than at pH 5 (Figure 4.15). Formation of the stable form of drugs inside the vesicles can exert a retaining effect on drugs (Lasic *et al.*, 1995; Vemuri and Rhodes, 1994). At pH 5, CF (5.0 mM) precipitates from solution overnight. From these studies it is concluded that these two different types

of niosomes were able to differentiate between both the ionised and unionised species, preferring to retain the more lipid soluble species, which might have precipitated within the vesicle interior.

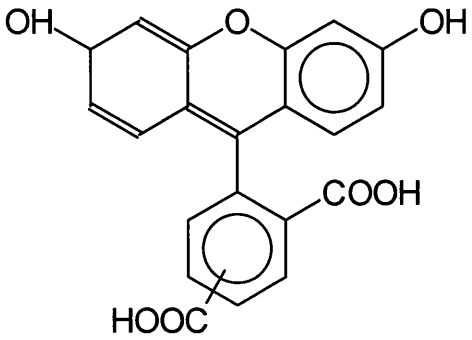


Figure 4.14: Molecular structure of 5(6)-carboxyfluorescein

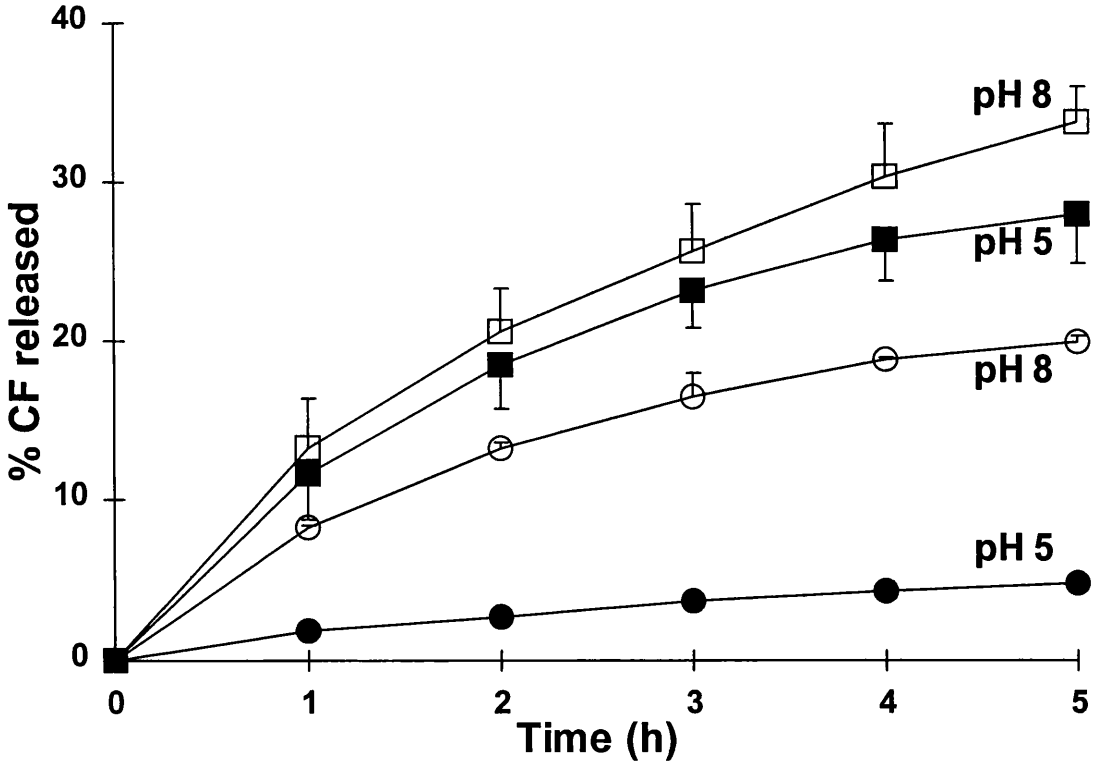


Figure 4.15: The release of CF from (□) polyhedral niosomes - $C_{16}G_2$, Solulan C24 (91: 9), (O) spherical/ tubular niosomes - $C_{16}G_2$: cholesterol: Solulan C24 (49: 49: 2) at pH 5 (filled symbols) and pH 8 (open symbols).

4.7. CONCLUSION

Physical characterisation of polyhedral niosomes found in cholesterol poor areas of the $C_{16}G_2$ - cholesterol - Solulan C24 ternary phase diagram revealed a decreased tendency to swell and shrink when subjected to osmotic gradients compared with spherical niosomes. The latter are found in areas where $C_{16}G_2$ and cholesterol are in a 1:1 molar ratio. This is due to a combination of a high membrane permeability to solutes and a more rigid and less deformable membrane structure. High levels (9%) of the polyoxyethylene cholesteryl ether compound Solulan C24 in polyhedral niosomes reduced the release rate of CF when co-entrapped with NaCl. This is suggested that this is the result of salting out of the hydrophilic polyoxyethylene chains by NaCl which subsequently reduces the permeability of the membrane to the hydrophilic CF. These findings may have a potential use in terms of drug delivery. A study of CF release at pH 5 and pH 8 revealed that the more soluble form of the aqueous volume marker CF was released to a greater extent than the insoluble moiety in both polyhedral niosomes and spherical/ tubular niosomes.

CHAPTER 5

IN VITRO/ IN VIVO EVALUATION OF LUTEINISING HORMONE RELEASING HORMONE (LHRH) LOADED NIOSOMES

5.1. INTRODUCTION

5.1.1. LHRH

Luteinizing hormone-releasing hormone (LHRH), also called gonadotrophin-releasing hormone (GnRH), is a decapeptide hormone (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, Figure 5.1) produced and secreted in a pulsatile manner (every 60-90 minutes) from the hypothalamus. It regulates the pituitary gland to synthesise and secrete luteinising hormone (LH) and follicle stimulating hormone (FSH) which control testicular and ovarian functions (Conn & Crowley 1991).

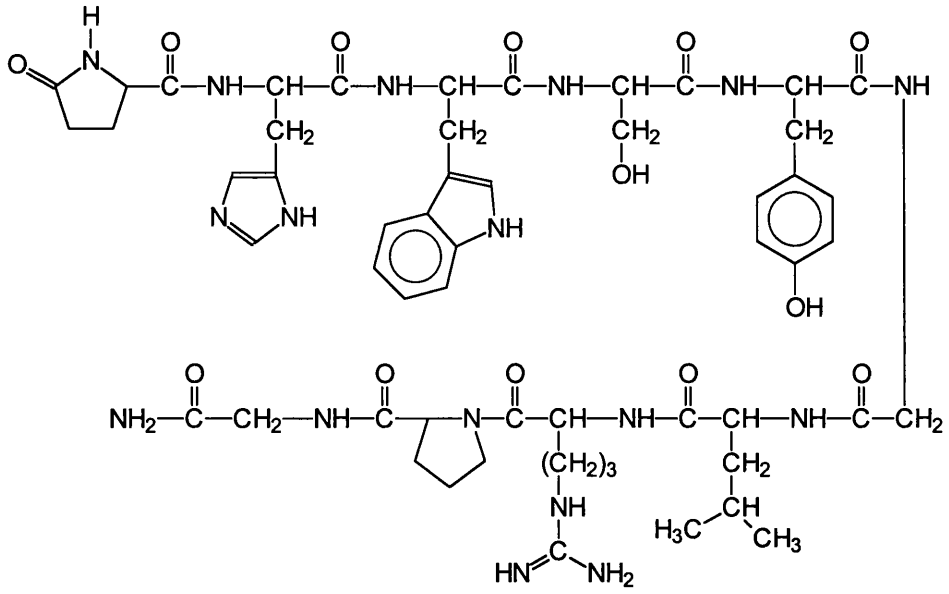


Figure 5.1: Molecular structure of LHRH

LHRH has been found to be stable and have a long shelf life over a wide range of pH and temperatures (Shi *et al.*, 1984; Helm & Muller 1990; Motto *et al.*, 1991; Powell *et al.*, 1991). However, like most therapeutic peptides, it is highly susceptible to various enzyme degradations hence has a very short plasma half life. Degradation is mediated by endopeptidases at the Tyr^5-Gly^6 bond, in combination with enzymes that hydrolyse the $pyroGlu^1$ or cleave the peptide on the carboxyl side of Pro (Sandow *et al.*, 1981). Substitution of the amino acids in position 6, 10, and others, therefore, results in longer-acting analogues which are also more potent than native LHRH (Conn & Crowley 1991).

LHRH is used for the treatment of amenorrhoea and infertility associated with hypogonadotrophic hypogonadism and multifollicular ovaries. There is the need for LHRH to be administered in a pulsatile manner for restoring the pituitary-gonadal function. Continuous exposure of the gonadotrophs to LHRH results in an inhibition of pituitary-

gonadal axis through a process of LHRH receptor down-regulation, desensitisation of the pituitary gonadotrophs, and a suppression of circulation levels of LH and sex steroids. These findings led to the second clinical application where the reversible suppression of the pituitary-gonadal axis is needed, for example, in the treatment of hormone-dependent malignant neoplasms, typified by prostate and breast cancer, and endometrial carcinoma. However, it is more convenient, clinically, to use other synthetic LHRH agonists which are more potent and possess prolonged activity.

5.1.2. Approaches to the delivery of LHRH

In order to induce fertility, there is interest in mimicking the natural release profile of LHRH in nature, i.e. in a pulsatile manner. The use of transdermal iontophoresis has been studied as a means to obtain such a release pattern (Heit *et al.,* 1993; Bommannan *et al.,* 1994). The short plasma half life (2-4 min) (Conn & Crowley 1991; Dollery 1991; Lee *et al.,* 1991), the hydrophilicity (partition coefficient 0.0451) (Banks & Cession, 1985) and the size of the molecule (MW=1,182 Da) are main obstacles for the continuous delivery of LHRH. Apart from using its synthetic analogues, many strategies have been employed to improve the absorption and its plasma half life. One approach is by modifying the chemical structure of the hormone. An example is the conjugation of a lipidic moiety to LHRH (Hillery *et al.,* 1996a&b). Such conjugates form microparticles which do not only protect the hormone from enzymes but also increase its hydrophobicity, both useful when the drug is delivered orally. Another approach is by incorporating the hormone, or its analogues, into various pharmaceutical preparations. These mainly include colloidal carriers e.g. emulsions (Morel *et al.,* 1994), liposomes (Schäfer *et al.,* 1987a&b), and microcapsules (Ogawa *et al.,* 1989). The release of LHRH is also prolonged when incorporated into viscous polymeric

solutions (Lorenz *et al.,* 1985) or matrices (Lee & Chien 1995; Han *et al.,* 1995). Addition of absorption enhancers (Chiou *et al.,* 1992) and/ or peptidase inhibitors (Han *et al.,* 1995) also improve the activity of the hormone *in vivo.*

5.1.3. *In vitro/ in vivo* evaluation of LHRH loaded $C_{16}G_2$ niosomes

The differences in basic properties of polyhedral, and spherical/ tubular niosomes, formed by $C_{16}G_2$: cholesterol: Solulan C24 with molar ratios of 91:0:9 and 49:49:2, respectively, have been compared previously. In chapter 4, polyhedral niosomes, possessing non-cholesterol membranes, were found to be more permeable to carboxyfluorescein (CF) than their spherical counterparts. However, the co-entrapment of sodium chloride with CF in polyhedral niosomes results in a very low CF release rate, regardless of the osmotic conditions. The irregular faceted shape of polyhedral niosomes results in a dispersion with a higher viscosity than that formed by their spherical/ tubular counterparts as shown in chapter 3. Such rheological properties may be relevant in allowing polyhedral niosomes to act as a good depot system which will remain longer at the site of injection than their spherical counterparts following an intramuscular injection and will not be easily drained into lymphatic systems.

LHRH was selected as a model peptide, as discussed above, to investigate the possibility of using polyhedral and spherical/ tubular niosomes, and polyhedral niosomes prepared in the presence of NaCl, as sustained drug delivery systems. A variety of preparation techniques were studied in an attempt to obtain a high level of LHRH entrapment in the vesicles. The release of the hormone from niosomes *in vitro* was performed in 5% rat skeletal muscle homogenate and in rat plasma. *In vivo* experiments were performed by following the clearance of LHRH from the intramuscular injection site in the rats.

MATERIALS AND METHODS

5.2. MATERIALS

The materials used in this chapter are as given in Table 2.1. All reagents and chemicals were of analytical grade, unless otherwise stated. All materials were used as obtained from suppliers without further purification and the water source was from an ultra high quality reverse osmosis water purifier (Elgastat UHQPS - Elga, UK). Luteinizing hormone releasing hormone (LHRH) acetate salt and radiolabelled ^{125}I-LHRH, were obtained from Sigma Chemicals, UK. Pentobarbitone sodium (Sagatal) was obtained from Rhône Mérieux Ltd., Harlow, Essex, UK.

METHODS

5.3. PREPARATION OF LHRH LOADED POLYHEDRAL NIOSOMES

Six methods of preparation of LHRH loaded polyhedral niosomes, formed from $C_{16}G_2$: Solulan C24 (91: 9) (60 μmoles) and LHRH acetate (2 mg/ml), were investigated (n=3). These are listed below.

Method A: Direct hydration (hand-shaking) at pH 7.4 -Dried surfactant film was hydrated with LHRH acetate prepared in phosphate buffered saline (PBS) pH 7.4 by mechanical shaking at 55°C for 30 min.

Method B: Direct hydration at pH 3.0 -Dried surfactant film was hydrated with LHRH acetate prepared in PBS pH 3.0 by mechanical shaking at 55°C for 30 min.

Method C: Dehydration-rehydration vesicles (DRV) -Dried surfactant film was hydrated with 5ml of water by mechanical shaking at 55°C for 30 min before adding 1ml of LHRH acetate in water. The niosome dispersion was then freeze-dried overnight and rehydrated with 1ml of PBS pH 7.4.

Method D: Reverse-phase evaporation vesicles (REV) -Surfactants were dissolved in 10ml $CHCl_3$: diethyl ether (1:1). 1ml of LHRH acetate in PBS pH 7.4 was injected into the dispersion which was then bath sonicated for 2 min and the organic solvents vacuum evaporated.

Method E: Remote loading by $(NH_4)_2SO_4$ gradient -Dried surfactant film was hydrated with 1ml of 120mM $(NH_4)_2SO_4$ by mechanical shaking at 55°C for 15min. The dispersion was ultracentrifuged at 4°C, the pellets redispersed with LHRH acetate in PBS pH 7.4 and mechanical shaken gently at 60°C for 15min.

Method F: Remote loading by pH gradient -Dried surfactant film was hydrated with 1ml PBS pH 3.0 by mechanical shaking at 55°C for 30min. The dispersion was ultracentrifuged at 4°C, the pellets redispersed with LHRH acetate in PBS pH 7.4 and mechanical shaken gently at 60°C for 15min.

The dispersions were washed twice with PBS pH 7.4 (or at pH 3.0) by ultracentrifugation

at 4°C. The entrapped LHRH acetate was analysed using reverse phase HPLC (μBondapak C18, 3.9X150mm, Mobile phase: 0.03M CH_3COONH_4 :ACN 70:30, flow rate 0.6 ml/min, detection wavelength 279 nm).

For the *in vitro/ in vivo* evaluation, polyhedral and spherical/tubular niosomes prepared from $C_{16}G_2$: cholesterol: Solulan C24 (91:0:9) and (49:49:2), respectively, were loaded with ^{125}I-LHRH (1.67 μCi/ ml in PBS pH 7.4) using the pH gradient method described in **Method F**. To study the effect of NaCl, phosphate buffer at pH 7.4 prepared in 2 M NaCl was used instead of PBS pH 7.4, except when the washed niosomes were redispersed prior to injection. Niosomes were sized using a laser diffraction method (MasterSizer X, Malvern Instruments Ltd., Worcestershire, UK).

5.4. ANIMAL HUSBANDRY

200 g male Wistar rats (Bantin and Kingman Universal Ltd., Aldbrough, Hull, UK), were housed in groups of 3 to 6, bedded on sawdust and given diet (Rat and Mouse No. 1, SDS Ltd, Witham, Essex, UK) and drinking water *ad libitum*. A temperature of 20-22°C was maintained with a relative humidity of 45 to 65% and a 12: 12 h light: dark cycle (lights on at 07.00 h). Animals were acclimatised for at least 5 days before each experiment and were observed daily for clinical signs of illness.

5.5. *IN VITRO* EVALUATION

5.5.1. Preparation of plasma

Rats were killed by intra-peritoneal injection of pentobarbitone sodium. Blood was

removed from the abdominal aorta, anticoagulated with lithium heparin, the plasma removed after centrifugation and stored at -30°C.

5.5.2. Preparation of 5% muscle homogenate

Rats were killed as above. Skeletal muscles were removed from the posterior and anterior thighs, weighed and homogenised with 19 parts PBS, and aliquots stored at -30°C.

5.5.3. Evaluation of niosomes *in vitro*

A 0.1 ml sample of each niosome dispersion was mixed with 0.9 ml of rat plasma, or 0.9 ml of 5% muscle homogenate, in a glass vial which was then incubated at 37°C in a thermostated water bath (Grant Instruments [Cambridge] Ltd., Cambridge, UK). 0.1 ml aliquots were collected at 30 min, and 2, 5 and 9 h and mixed with 1.9 ml PBS before ultracentrifugation. Radioactivity in 1ml of supernatant was counted (1275 Minigamma Gamma Counter, LKB Wallac, Turku, Finland), and the count/min in 2 ml was calculated as % LHRH released by comparison with the initial count/min of 0.01 ml of niosome dispersion.

5.6. *IN VIVO* EVALUATION

Male Wistar rats, 190 to 230 g, were injected intramuscularly (25 GA 5/8 needle; right posterior thigh) with 0.1ml of ^{125}I-LHRH prepared in PBS pH 7.4, or spherical/ tubular niosomes, polyhedral niosomes, or polyhedral niosomes prepared with a high concentration of NaCl. At 10 min, and 2, 7, 25, 32 and 49 h after injection, animals (n=2) were weighed and killed by IP injection of pentobarbitone sodium and blood collected into lithium heparin. The radioactivity remaining at the injection site was determined by removing the

injected leg and detaching the thigh muscles. The radioactivity in whole blood (2 ml) and muscle was counted using the gamma counter. The ^{125}I-LHRH in the total blood volume at each time point was calculated, based on the formula of total blood volume being 6 ml/100 g body weight in the mature male rat (Joint working group on refinement 1993), and the count of radioactivity in 0.1 ml of ^{125}I-LHRH solution or niosome dispersion.

RESULTS AND DISCUSSION

5.7. EFFECTS OF PREPARATION METHODS ON ENTRAPMENT EFFICACY

Figure 5.2 shows that only a small amount of LHRH acetate could be entrapped into polyhedral niosomes using passive loading methods, i.e. direct hydration at pH 7.4 and 3.0, and DRV and REV, in comparison with higher levels of entrapment obtained by active loading methods, i.e. $(NH_4)_2SO_4$ gradient and pH gradient. Calculated on the basis of available LHRH (2mg), the percentages of LHRH entrapment obtained by passive loading methods were in the range of 14%-24%. The $(NH_4)_2SO_4$ gradient and pH gradient methods led to entrapment of 44% and 51%, respectively. These figures correspond to ratios of LHRH to surfactants of 0.0124 and 0.0145 μmol entrapped LHRH/ μmol of surfactants, respectively.

Bangham *et al.* (1965) described the preparation of drug loaded vesicles using a hand-shaking method, but the amount of compound entrapped with this technique tends to be low. Many preparation methods aimed at improving the level of entrapment have been described (see, e.g., New 1990). For example, the binding of peptide to lipids can be

enhanced by modifying the electrical charge or the hydrophobicity of the peptide (Surewicz & Epand 1985). However, LHRH is very hydrophilic and becomes a univalent cation at physiological pH. An increase in lipid-peptide interaction *via* electrostatic binding was described by Schäfer *et al.* (1987b) by the addition of phosphatidic acid, an anionic lipid, into the membranes of phosphatidyl choline vesicles. In our present non-ionic systems, we obtained very low entrapment of LHRH by passive loading methods (Figure 5.2); probably due to the lack of hydrophobic and electrostatic interactions. The highest entrapment was achieved by the remote loading method (so called active loading), which makes use of either the difference in pH, or in the amount of $(NH_4)_2SO_4$, between the inside and the outside of the vesicle. It is also of interest that LHRH (a decapeptide with a MW of 1,182 Da) can be introduced through the vesicle membrane using a pH gradient (Mayer *et al.*, 1989 & 1992; Vemuri & Rhodes 1994; Boman *et al.*, 1995), or an $(NH_4)_2SO_4$ gradient (Lasic *et al.*, 1992 & 1995), as has been demonstrated for other small molecules such as doxorubicin (MW of 543.5 Da). In our studies, the highest amount of drug to be entrapped using the pH gradient method was 0.0145 μmol LHRH/ μmole surfactants. This level appears low in comparison with values achieved for other drugs. For example, 0.202 μmol doxorubicin/ μmol lipid was reported by Madden *et al.* (1990). We used a low concentration of LHRH (1 mM surfactants: 0.028 mM LHRH), in comparison with Madden *et al.* (1990) (1 mM lipid: 0.2 mM drugs), and the amount of LHRH encapsulated was up to 51% of the available drug.

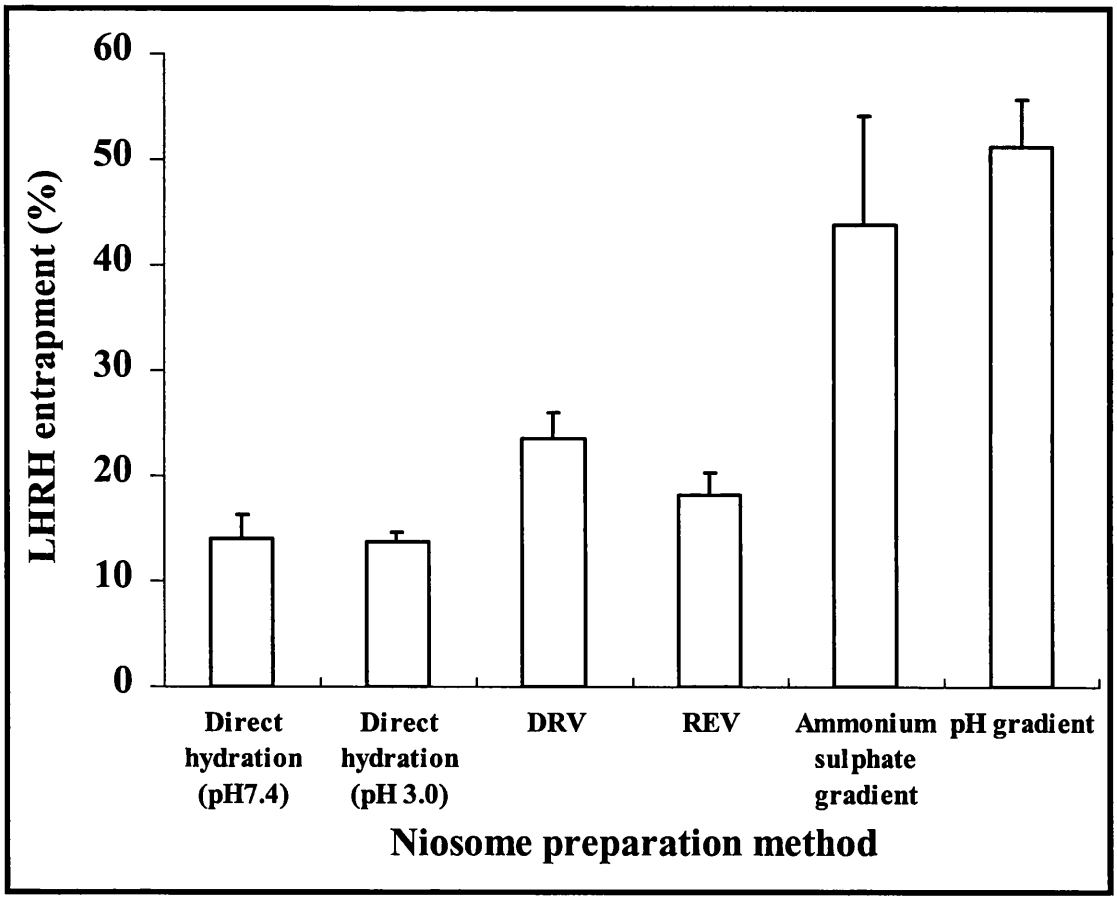


Figure 5.2: Entrapment of LHRH acetate (as %) into polyhedral niosomes by 6 different preparation methods. Data are expressed as mean and sd.

5.8. *IN VITRO* STABILITY STUDY

When incubated with plasma, all the entrapped ^{125}I-LHRH was released from polyhedral niosomes within 30 min (Figure 5.3). However, with polyhedral niosomes in 5% muscle homogenate, about 30% of the entrapped ^{125}I-LHRH was released in 30 min, and this level then remained constant until the end of the experiment at 9 h. When incubated in plasma, spherical/ tubular niosomes released approximately 80% of ^{125}I-LHRH by 30 min and the release profile then remained constant until 9 h. However, spherical/ tubular niosomes in muscle homogenate released only about 9% by 30 min, the amount then gradually rising to 24% at 9 h.

The stability of parenteral drug delivery systems can be evaluated by measuring drug release during incubation in serum or plasma (Guzmán *et al.,* 1989; Storm *et al.,* 1989; Kumar *et al.,* 1993; McCormack & Gregoriadis 1994), and muscle homogenates (Arrowsmith *et al.,* 1983). It is seen (Figure 5.3), that both polyhedral and spherical vesicles are more stable in a 5% muscle homogenate than in plasma. This finds a parallel with the findings of Zuidema *et al.* (1994) who showed that plasma had a higher potential than muscle components to induce solute leakage from liposomes. Figure 5.3 also demonstrates that spherical/ tubular niosomes released less ^{125}I-LHRH than polyhedral niosomes and these results compare with earlier findings using CF as a marker.

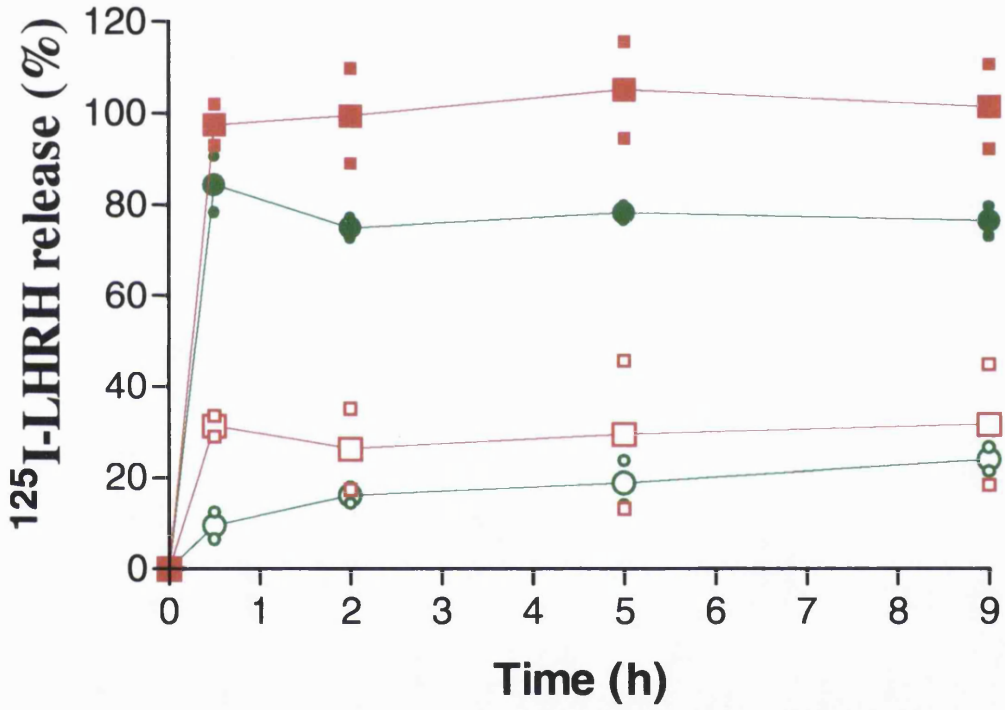


Figure 5.3: Release profile of ^{125}I-LHRH from polyhedral niosomes over a 9 h period in plasma (■) and in 5% muscle homogenate (□), and from spherical/tubular niosomes in plasma (●) and in 5% muscle homogenate (○). Individual data (small symbols) at each time point are shown along with their means (large symbols with line).

5.9. *IN VIVO* CLEARANCE STUDY

All niosomes used *in vivo* were unsonicated and the mean sizes were in the range 6 to 9 μm. Figure 5.4a illustrates the clearance of ^{125}I-LHRH, from the injection site in the muscle of the rat hind limb, in both polyhedral and spherical/ tubular niosomes in comparison with ^{125}I-LHRH prepared in PBS. It is seen that 99% ^{125}I-LHRH prepared in PBS was cleared from the site of injection in the first 2 h, while 14% and 70% of injected ^{125}I-LHRH prepared in polyhedral and spherical/ tubular niosomes, respectively, was still present at the injection site at this time. All the ^{125}I-LHRH in polyhedral niosomes was cleared from the injection site by 25 h, while 8.5% of the radioactivity in spherical/ tubular niosomes could still be detected.

Figure 5.5a shows the level of ^{125}I-LHRH in the blood over the 49 h period after dosing. The results are represented as the estimated amount of radioactivity in the total blood volume, expressed as a percentage of the dose of radioactivity administered. ^{125}I-LHRH prepared in PBS was released from the injection site into the blood very rapidly, giving the highest detected level at 12 min post dosing. The level then decreased with time. In comparison, niosome preparations tended to sustain the release of drug into the blood and prolong the blood level of the drug. Polyhedral niosomes gave a maximum but lower blood level at 2 h. Spherical/ tubular niosomes served as a better sustained release system as they slowly released drug into the blood with the maximum level at 7 h post dosing, the level then gradually decreasing with time. It is seen that these results (Figure 5.5a) closely parallel the clearance data (Figure 5.4a).

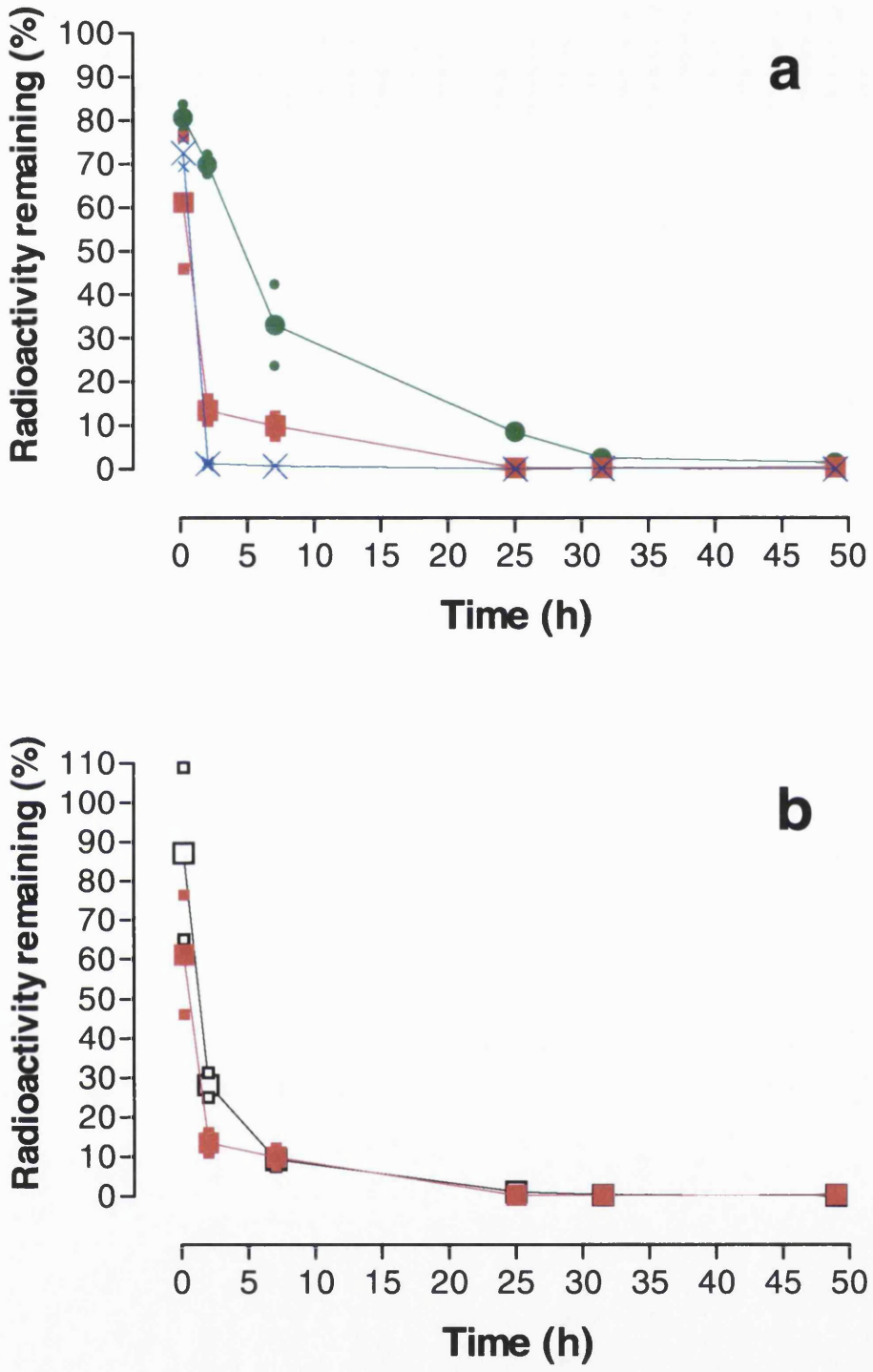


Figure 5.4: ^{125}I-LHRH as % remaining at the intramuscular injection site. **(a)** ^{125}I-LHRH prepared in PBS at pH 7.4 (X), in polyhedral niosomes (■), and in spherical/tubular niosomes (●). **(b)** ^{125}I-LHRH prepared in polyhedral niosomes (■) (as presented in Figure 5.4a), and in polyhedral niosomes with NaCl (□). Individual data (small symbols) at each time point are shown along with their means (large symbols with line).

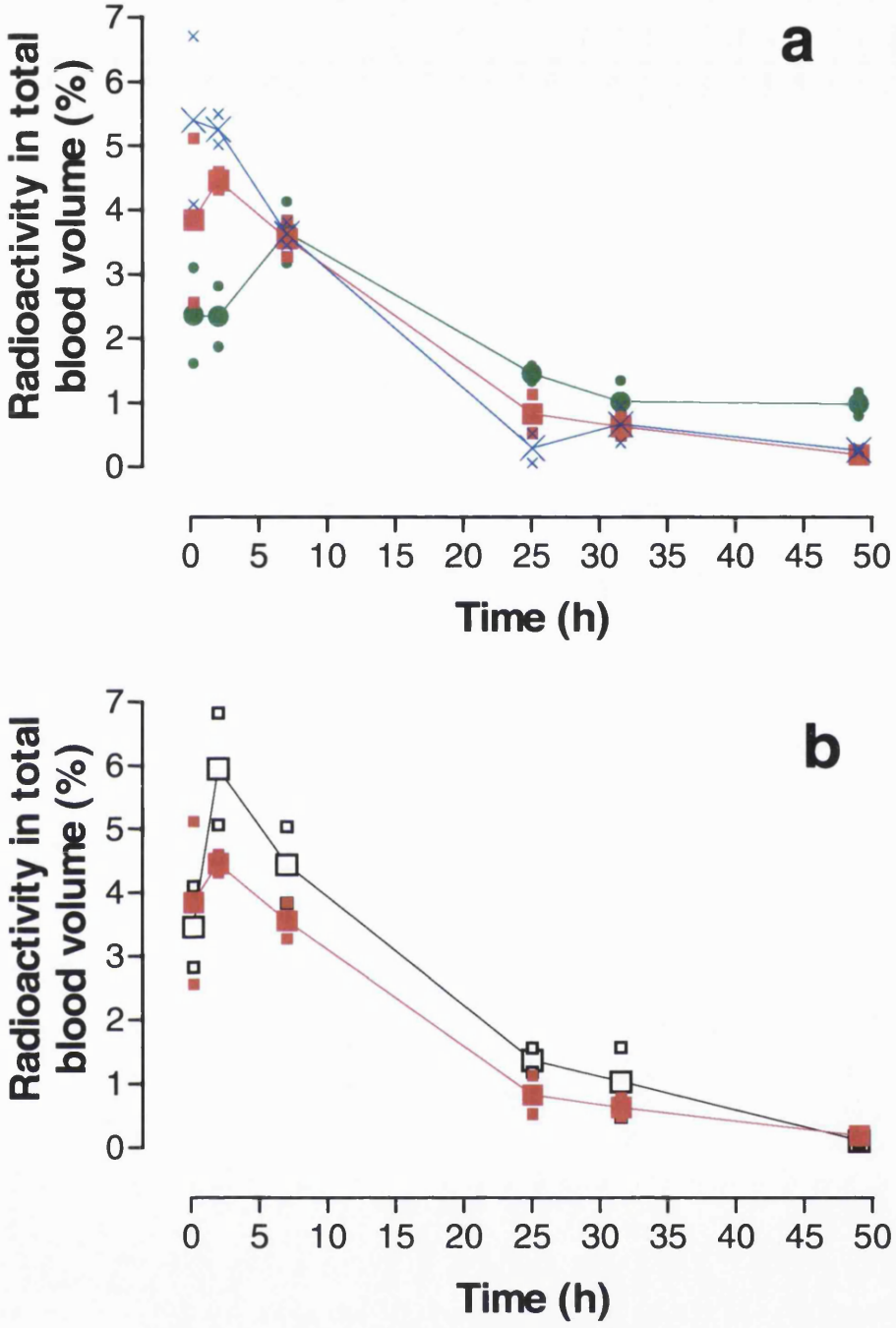


Figure 5.5: ^{125}I-LHRH as % detected in blood after intramuscular injection. The amount of radioactivity in the total blood volume was estimated and expressed as a percentage of the dose of radioactivity administered. **(a)** ^{125}I-LHRH prepared in PBS at pH 7.4 (X), in polyhedral niosomes (■), and in spherical/tubular niosomes (●). **(b)** ^{125}I-LHRH prepared in polyhedral niosomes (■) (as presented in Figure 5.5a), and in polyhedral niosomes with NaCl (□). Individual data (small symbols) at each time point are shown along with their means (large symbols with line).

Liposomes, on intramuscular injection, provide a depot effect, with the gradual release of drug from the site of injection (Zierenberg & Betzing 1979; Ohsawa *et al.,* 1985; Schäfer *et al.,* 1987a; Richards *et al.,* 1995). In the present work it was found that niosomes formed mainly from $C_{16}G_2$ are able to protect LHRH from being cleared immediately from the injection site.

The niosome formulations used in the present experiments were not sonicated, as it has previously been shown that the size of polyhedral niosomes can increase following sonication (see chapter 2.8.2.a.). The mean size of both the polyhedral and the spherical/ tubular niosomes were within the range 6 to 9 μm. Zuidema *et al.* (1994) suggested that small liposomes (< 200 nm) can be cleared from the injection site by the lymphatic system, while larger vesicles (4.9 μm) have been found to have a longer retention time at the site of injection (Jackson 1980). Figure 5.4a illustrates that ^{125}I-LHRH in PBS was cleared within 2 h from the site of injection. However, the clearance of both polyhedral and spherical/ tubular niosomes was more gradual. It was conjectured that polyhedral niosomes, having a higher viscosity than spherical/ tubular niosomes (as shown in chapter 3.8), might remain at the site of injection for a longer period. However, it is seen (Figure 5.5a) that the polyhedral niosomes released more ^{125}I-LHRH into the blood at early time points, in comparison with the spherical/ tubular niosomes. The latter vesicles, therefore, were able to prolong the blood level of ^{125}I-LHRH, probably due to their lower membrane permeability. These results compare, therefore, with the earlier *in vitro* stability study data.

LHRH is fairly stable in aqueous solution over a wide range of pH and temperature (Shi *et al.,* 1984; Helm & Muller 1990; Motto *et al.,* 1991; Powell *et al.,* 1991). Following intravenous injection in man, LHRH is eliminated mainly by metabolic breakdown and hence has a very short half life (2 to 4 min) (Conn & Crowley 1991; Dollery 1991; Lee *et al.,* 1991). The levels of ^{125}I-LHRH detected in the blood (Figure 5.5a) were very low, indicating that the peptide rapidly becomes accessible to protease enzymes on release from the niosomes.

In chapter 4 (section 4.6.1.a.), we reported that the co-entrapment of NaCl with CF reduces the membrane permeability of polyhedral niosomes. However, only a slight improvement in the release profile of LHRH was observed *in vivo*. Co-entrapment of NaCl with ^{125}I-LHRH in polyhedral niosomes was found not to change the release pattern of the drug from the injection site to a significant extent (Figure 5.4b), nor the level of ^{125}I-LHRH in the blood (Figure 5.5b), although the amount of radioactivity released in both instances was slightly higher in the case of polyhedral niosomes prepared with NaCl.

Liposomes may remain at the site of injection for more than 1 week (Richards *et al.,* 1995); however, their ability to retain solute is an important factor in their use as a sustained release system. Such an ability is related to the composition of the membranes, their lamellarity (Schäfer *et al.,* 1987a), and the presence of cholesterol in the membranes which reduces their permeability (De Gier *et al.,* 1968; Baillie *et al.,* 1985; Storm *et al.,* 1989). Polyhedral niosomes formed without cholesterol, although being in the gel phase, are therefore more leaky than their spherical/ tubular counterparts.

5.10. CONCLUSIONS

The method of preparing LHRH acetate loaded niosomes affects the amount of peptide entrapped. A high LHRH acetate loading can be achieved using active loading methods following either pH or $(NH_4)_2SO_4$ gradients. Niosomes can be used as sustained drug delivery systems which stay at the injection site and gradually release drug into the blood. The shape of the vesicle, which gives a high viscosity to suspensions of polyhedral niosomes, is not as important as the composition of the vesicle membranes, which governs membrane permeability. Spherical/ tubular niosomes possess more stable membranes than polyhedral niosomes due to the presence of cholesterol.

CHAPTER 6

MANIPULATION OF NIOSOMES: APPROACHES TO BIOMIMETIC PULSED DELIVERY AND FABRICATION OF MICROSTRUCTURES

6.1. INTRODUCTION

In this chapter, glass capillaries with defined diameters were used to manipulate niosomes with various sizes and membrane compositions in order to establish two new applications of niosomes. The first part of this chapter demonstrates an attempt to deliver single vesicles or groups of vesicles, from glass micropipettes, in a pulsatile manner. This delivery process is considered as a biomimetic approach which may be useful in designing drug delivery devices. The latter part of this chapter presents an attempt to fabricate various microstructures by extrusion or suction of pre-formed niosomes with glass micropipettes.

6.2. A BIOMIMETIC APPROACH TO PULSATILE DELIVERY

Nature provides superb models for the pulsed delivery of active agents. Release of bioactive agents, such as neurotransmitters in synaptic vesicles (Figure 6.1) and neurohormones, is triggered by a variety of stimuli, to give quantal pulses of active molecule (Figure 6.2a). There is a need in some cases for pulsatile drug delivery systems. Figure 6.2b shows a possible model for a pulsed delivery device in which a group of vesicles, containing an active agent (drug, toxin, peptide, etc.) are injected from a capillary, a process which can be regarded as "biomimetic". Another model of a pulsed delivery device might include the ejection of single vesicle from a capillary as illustrated in figure 6.3. While it is possible to obtain pulsed release pattern of solutions from capillaries e.g. by using programmable infusion pumps, there may be merit in delivering drug at intervals entrapped within vesicles. We investigated here the delivery of single vesicles, and groups of vesicles, from glass capillaries with defined diameters as a model of a pulsatile delivery device. LHRH, a neuroendocrinological hormone secreted from hypothalamic neurons in discrete pulses, was chosen as a model for the quantitative release study.

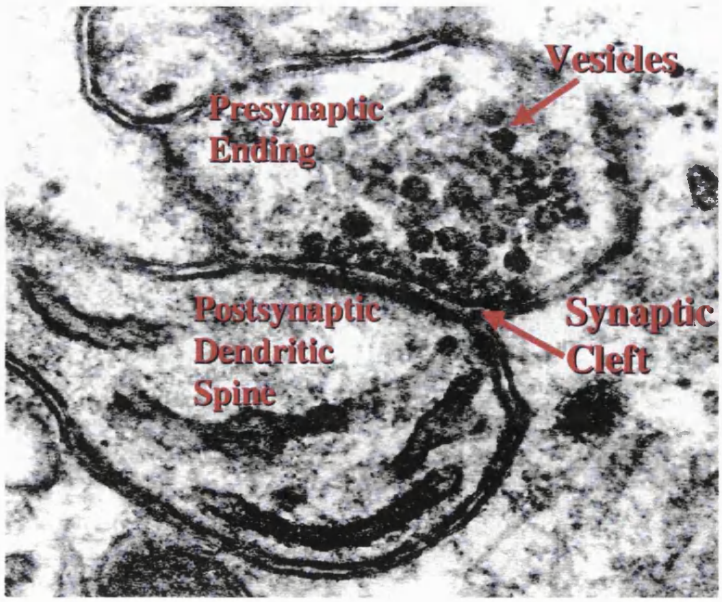


Figure 6.1: An electron micrograph of a synapse onto the spine of a Purkinje cell dendrite. Reproduced from Levitan & Kaczmarek (1991).

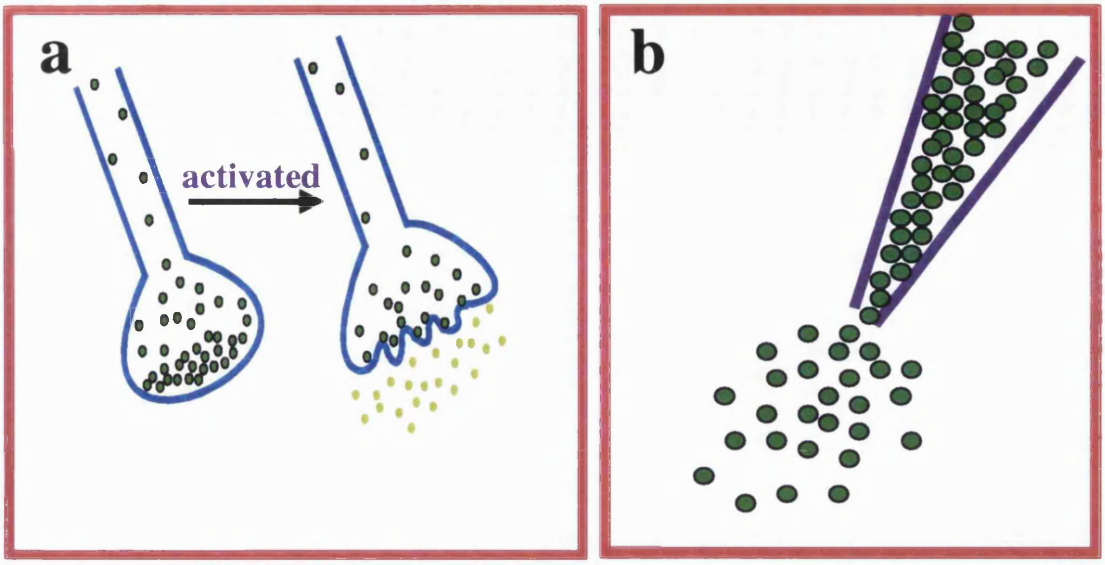


Figure 6.2: (a) Schematic representing the releasing of active agents from the end terminal of a neuron. Vesicles are accumulated in the axon terminal and, on stimulation, the vesicular content (e.g. neurotransmitters) is released to the extracellular space by exocytosis. (b) Schematic representing a suggested biomimetic delivery device. A group of vesicles which contain active agents is released in a pulsatile manner from the reservoir, i.e. the vesicle-containing capillary.

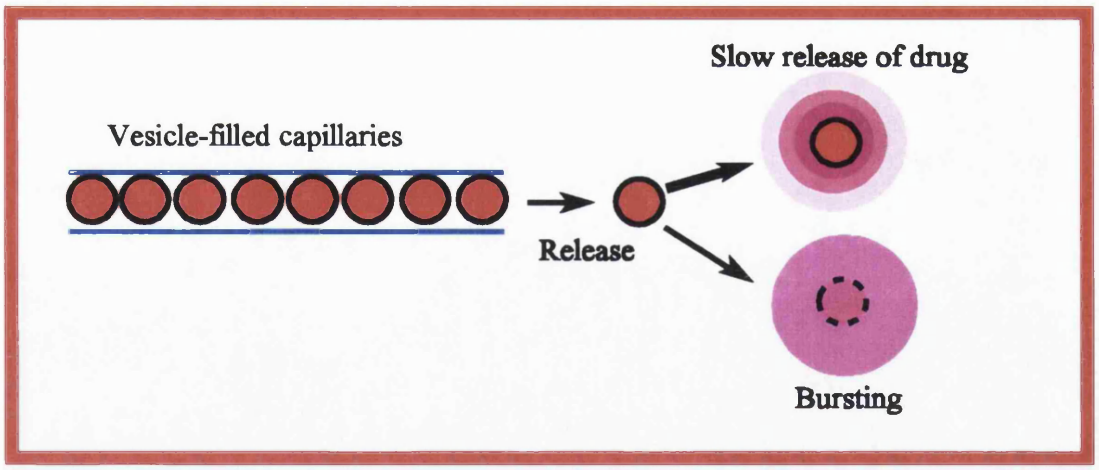


Figure 6.3: Schematic representing the delivery of single vesicles from a capillary; the vesicles can be pressure or electrical driven from the capillary which then release the encapsulated drugs either by gradual diffusion or membrane rupture.

MATERIALS AND METHODS

6.2.1. Materials

The materials used in this chapter are as given in Table 2.1. Sorbitan monooleate (Span 80), luteinizing hormone releasing hormone (LHRH) acetate, and ^{125}I-LHRH were purchased from Sigma Chemical, UK. Tetradecyl-β-D-maltoside (C_{14}-maltoside) was obtained from Calbiochem Corporation, USA. Sodium dodecyl sulphate (SDS) was purchased from Aldrich, UK. Polystyrene microspheres (co-polymerized with 2% divinyl benzene/ 10% methyl methacrylate/ 10% methacrylic acid), with a mean diameter of 4.4 μm were purchased from Bangs Laboratories, USA. All materials were used as obtained from suppliers without further purification. Borosilicate glass capillaries, with an inner diameter (I.D.) of 1.17 mm and outer diameter (O.D.) of 1.5 mm, were obtained from Clark Electromedical Instruments, UK. Polyamide coated fused silica capillary tubing, with an I.D. of 5 μm and an O.D. of 0.285 mm, were obtained from SGE Ltd., UK.

METHODS

6.2.2. Preparation of niosome filled micropipettes

Niosomes (60 mM lipid/ surfactant) were prepared by hand-shaking method in water or PBS pH 7.4, as described in section 2.3. The resultant dispersions were centrifuged to obtain fractions of various vesicle sizes. The fractions were collected and sized by laser diffraction (MasterSizer X, Malvern, UK), in some cases, diluted with the medium, and then filled into the micropipettes with defined exit diameters (5-10 μm). These

micropipettes were obtained by heating and mechanical pulling borosilicate glass capillaries, with an I.D. of 1.17 mm, with a vertical pipette puller (KOPF 720, David KOPF Instruments, USA).

6.2.3. Preparation of LHRH loaded niosomes

LHRH loaded niosomes were prepared by hydrating dried films of C_{14}-maltoside: cholesterol: Solulan C24 (45:45:10) (60 μmol) with LHRH acetate (1mg/ml) in water, using ^{125}I-LHRH (1.67 μCi/ ml) as a tracer. The dispersions were washed twice with water by ultracentrifugation at 4°C and the pellets were redispersed with 1 ml of water. The radioactivity of unwashed and washed niosomes were counted (1275 Minigamma Gamma Counter, LKB Wallac, Turku, Finland) and the amount of entrapped LHRH was calculated.

6.2.4. Extrusion of microspheres and niosomes from micropipettes

The micropipettes loaded with niosomes or microspheres were connected to an electronic pump which controlled the frequency and duration of nitrogen (pressure ~0.5-5 Psi) used to drive the vesicles or microspheres from the micropipettes into a Petri dish filled with PBS. The events were followed using a video camera attached to an inverted microscope (Diavert, Leitz, Germany).

In a quantitative release study, LHRH loaded niosomes, with ^{125}I-LHRH as a tracer, were ejected from the micropipette (I.D. of 10 μm, N_2 pressure of 5 Psi) every 2 min for a duration of 5 s into a Petri dish filled with 4 ml of 2% SDS solution which disrupts the vesicles and allows the release of the entrapped LHRH. 0.5 ml of the medium was collected, and refilled with 0.5 ml of 2% SDS, every minute. The radioactivity of the

samples was counted and the amount of released LHRH was calculated by comparison with the count/min of washed niosomes.

6.2.5. Preparation of niosome filled capillaries

Niosomes were also loaded into silica capillary tubing, 5 cm in length, an I.D. of 5 μm, and an O.D. of 0.285 mm, by heating on one end of the capillary followed by immersing the other end into the niosome dispersion. The outer surface of the capillary was cleaned with isopropanol. The capillaries were broken, plunge frozen in Liquid Nitrogen Slush, and then observed by cryo-scanning electron microscopy (cryo-SEM) as described in chapter 2 (section 2.4.1).

6.2.6. RESULTS AND DISCUSSION

Figure 6.4 demonstrates the successful extrusion of a group of niosomes from the micropipettes with an exit diameter of 5 μm. The release profiles of LHRH obtained after pulsatile extrusion of a group of LHRH loaded niosomes from the micropipettes is shown in figure 6.5. This, potentially, would provide the basis for a prototype programmed release device showing pulsed release patterns.

The extrusion of niosomes is affected by their concentration and polydispersity. At high concentration (60 mM lipid/surfactants), niosomes can aggregate and obstruct expulsion if the nitrogen pressure used is low, e.g. 0.5 Psi. If the vesicle size is larger than the exit diameter (e.g. >2 times), obstruction of the micropipettes can also be observed. However, these problems can be overcome by using micropipettes with exit diameters larger than the vesicle size, or by increasing the pressure of nitrogen following aggregation (e.g. 5-7 Psi).

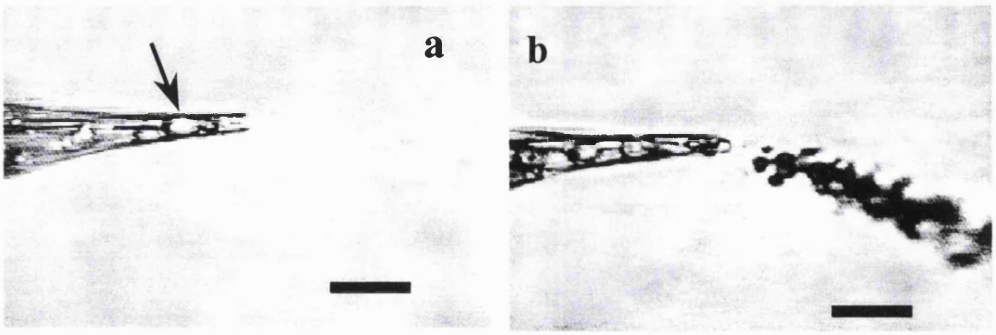


Figure 6.4: Extrusion of niosomes formed from C_{14}-maltoside: cholesterol: Solulan C24 (45:45:10) through the micropipette with an exit diameter of ~5 μm by nitrogen pressure at 0.5 Psi. (a) Large niosomes (arrow) can obstruct the exit at low pressures. (b) On increasing the pressure of nitrogen to 5 Psi, the obstructed vesicle is ejected, followed by a group of vesicles. Bar = 20 μm.

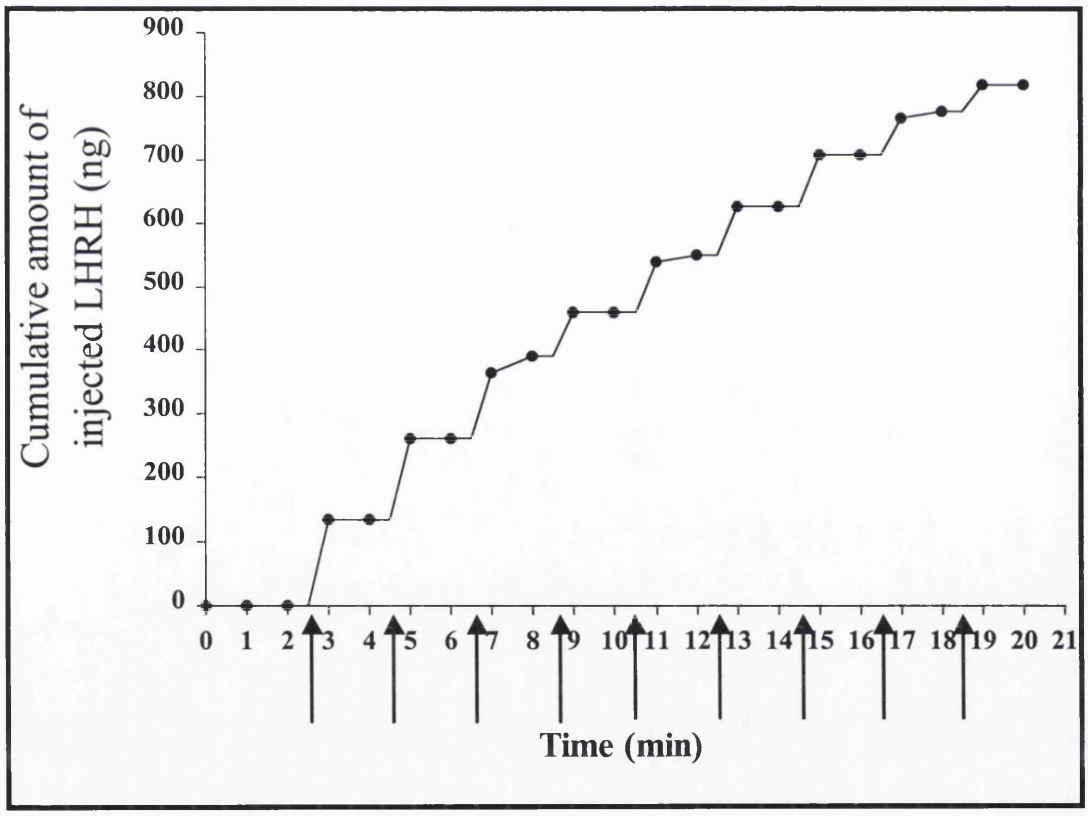


Figure 6.5: Release profile of LHRH acetate obtained by pulsatile expulsion of 3.5 μm LHRH acetate loaded niosomes (C_{14}-maltoside: cholesterol: Solulan C24, 45:45:10) from a micropipette with an I.D. of 10 μm. N_2 pressure (5 Psi) used to drive the vesicles from the micropipette was applied (see arrow) for a duration of 5 s every 2 min.

For sustained release, vesicles have been studied loaded into polysulfone capillaries which gradually release their content following implantation (Rahimy *et al.*, 1994). Recently, Kiser *et al.* (1998) applied the micropipette technique to study the release of drug from individual hydrogel microspheres following an applied electrical poration. The systems were regarded as analogues of secretory granules.

Here we show an attempt to deliver single vesicles in a pulsatile manner using the micropipettes as model devices. Figure 6.6 a & b demonstrate the successful expulsion of single microspheres and niosomes, respectively, from the micropipettes with an exit diameter of 5 μm. Both microspheres and niosomes used were diluted 10 times before filling into the capillaries to prevent their aggregation at the exit of micropipettes. The expulsion of individual microspheres can be achieved with low N_2 pressure as the diameter of microspheres used is smaller than the exit diameter of micropipettes (Figure 6.6a), whilst pulsatile delivery of single vesicles of which the diameter is smaller or slightly larger than that of the exit was found to be possible at low pressure (Figure 6.6b).

An attempt to load niosomes into a capillary tubing as the model shown in figure 6.3 was made. Figure 6.7 shows a cross section of silica capillary tubing, with an I.D. of 5 μm and O.D. of 0.285 mm, which was filled with vesicles and then broken to observe the vesicles loaded inside the capillary by a cryo-SEM. The presence of vesicles (Figure 6.7b) suggests the possibility of delivering single vesicles in a manner similar to the model shown in figure 6.3 although there may be a need for designing of delivery devices more applicable *in vivo*. Further work is also needed for studying the delivery of drug loaded vesicles as the release profile of bioactive substances from the ejected vesicles can be complicated and different from those delivered as solutions.

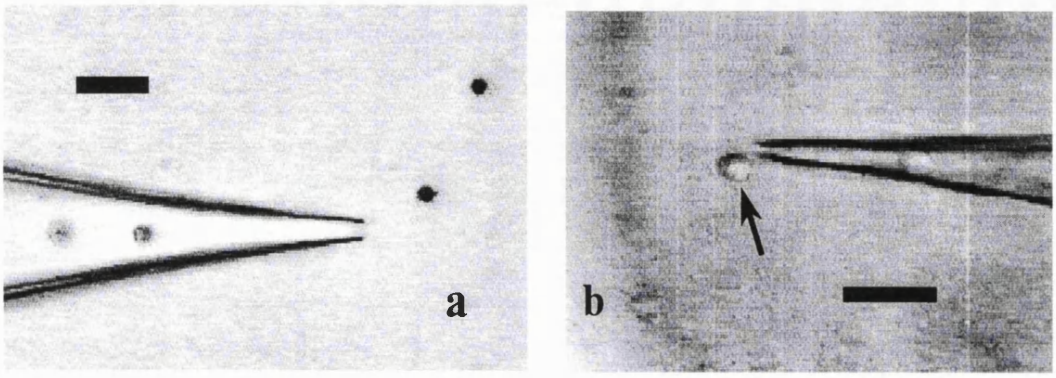


Figure 6.6: Extrusion of single (a) polystyrene microspheres (diameter 4.4 μm) and (b) niosomes formed by $C_{16}EO_2$: cholesterol: Solulan C24 (45:45:10). The diameter of the micropipette exit is ~5 μm and that of the vesicle (arrow) is slightly larger. Bar = 20 μm.

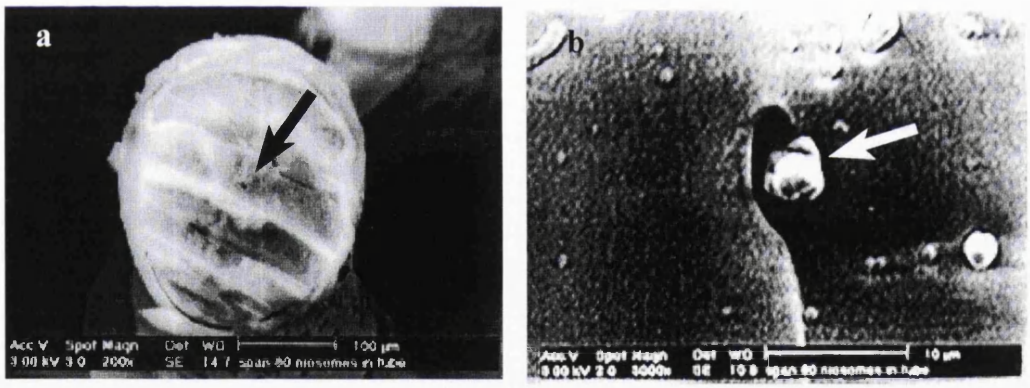


Figure 6.7: (a) A Cryo-scanning electron micrograph of a cross section of a silica capillary tube, with an I.D. of 5 μm (arrow) and an O.D. of 0.285 mm, which was loaded with niosomes (Span 80: cholesterol: Solulan C24, 45:45:10). The outer surface of capillary was cleaned with isopropanol, and the capillary was then broken before observation. The arrow indicates the inner hole in which the niosomes were loaded. (b) At higher magnification, a vesicle (arrow) can be observed located in the inner hole of capillary. Note that some vesicles are shown at the surface of the capillary cross section as a result of the breaking of the capillary prior to the microscopy.

6.3. MICROFABRICATION OF SURFACTANT STRUCTURES

Formation of pre-designed microstructures is important for the construction of many mechanical devices and have been well studied using a number of materials including semiconductors, metals, and polymers (Kim *et al.*, 1995; Moreau, 1988). However, the use of self-assembling amphiphilic molecules is relatively rare (Schnur & Shashidhar, 1994) and the appearance of the structures which can be formed relies greatly on the properties of amphiphilic molecules themselves. In this part of the work we attempt to create a variety of membrane-based microstructures by manipulating individual pre-formed vesicles. The process employs simple extrusion or suction of polyhedral or spherical niosomes formed by polyoxyethylated surfactants, in the size range of 4-40 μm, by micropipettes with an exit diameter of 5 μm. This technique aims to establish an approach to microfabrication.

MATERIALS AND METHODS

6.3.1. Materials

The materials used in this chapter are as given in Table 2.1 and section 6.2.1.

METHODS

6.3.2. Preparation of niosomes and micropipettes

Polyhedral niosomes were made of $C_{16}EO_5$: Solulan C24 (91:9) while spherical niosomes were made of $C_{16}EO_2$: cholesterol: Solulan C24 (45:45:10). Micropipettes with an exit diameter of 5 μm were prepared as described in 6.2.2.

6.3.3. Extrusion and suction of single niosomes with micropipettes

In the extrusion process, the micropipettes were filled with niosomes and connected to an electronic pump which controlled the frequency and duration of nitrogen (pressure was ~0.5-3.5 Psi) used to drive the vesicles from the micropipettes into a Petri dish filled with PBS. In the suction processes where negative pressure was needed, the capillaries were connected to a syringe and the suction of niosomes, which were dispersed in a Petri dish, was manually controlled. The events were followed using a video camera attached to an inverted microscope.

6.3.4. RESULTS AND DISCUSSION

Fabrication of well-defined microstructures relies on either the capability to manipulate molecular building blocks or on the ability of the molecules themselves to self-assemble (Drexler, 1992; Crandall, 1996). Here we describe a simple procedure-capillary manipulation-to prepare a variety of microstructures from the pre-formed vesicles.

Manipulating vesicles with micropipettes has been used to measure the elasticity of vesicle membranes to gain information of their mechanochemical properties (Evans & Kwok, 1982; Needham & Zhelev, 1996), and we have exploited the fact that vesicle membranes are deformed under shear stress. While small spherical vesicles with the diameters equal or slightly larger than the exit diameter of micropipettes can be released intact from the micropipettes, as shown above in section 6.2, those with diameters larger than the exit of capillaries can be sheared, following the extrusion or the suction, to an extent that the membranes cannot retain their integrity. Permanent new structures can, therefore, be developed from the deforming vesicles. While the diameter of micropipette exits are 5 μm

throughout the experiments, the structures can be varied depending on the diameter (4-40 μm), the bilayer composition, and the initial ultrastructure of the vesicles.

Figure 6.8 shows the extrusion of a single spherical vesicle with a diameter of ~30 μm. The vesicle is split into a clutch of smaller spherical vesicles (Figure 6.8c). These daughter vesicles are sometimes connected by a thin tubule, or tether, also the result of the vesicle extrusion (Figure 6.8c). This tether is sufficiently elastic to allow the daughter vesicles to oscillate (Figure 6.8c&d).

Large polyhedral niosomes which have bilayer membrane low in cholesterol and with a diameter of ~30 μm can be formed on extrusion into elongated curved-vesicular rods as shown in figure 6.9. Figure 6.10 shows the consecutive extrusion of 3 separated polyhedral niosomes, each with a diameter of ~20-30 μm. Each vesicle was extruded to form an elongated structure which then fuses with the previously extruded vesicle (Figure 6.10). This event shows that it may also be possible to join the membranes of separated vesicles by an applied shear stress. On extruding smaller polyhedral niosomes, generally of the order ~10 μm diameter, straight rod-like structures were obtained as shown in figure 6.11. It is clear that the structural appearance of extruded polyhedral niosomes depends greatly on the initial diameter of the vesicles.

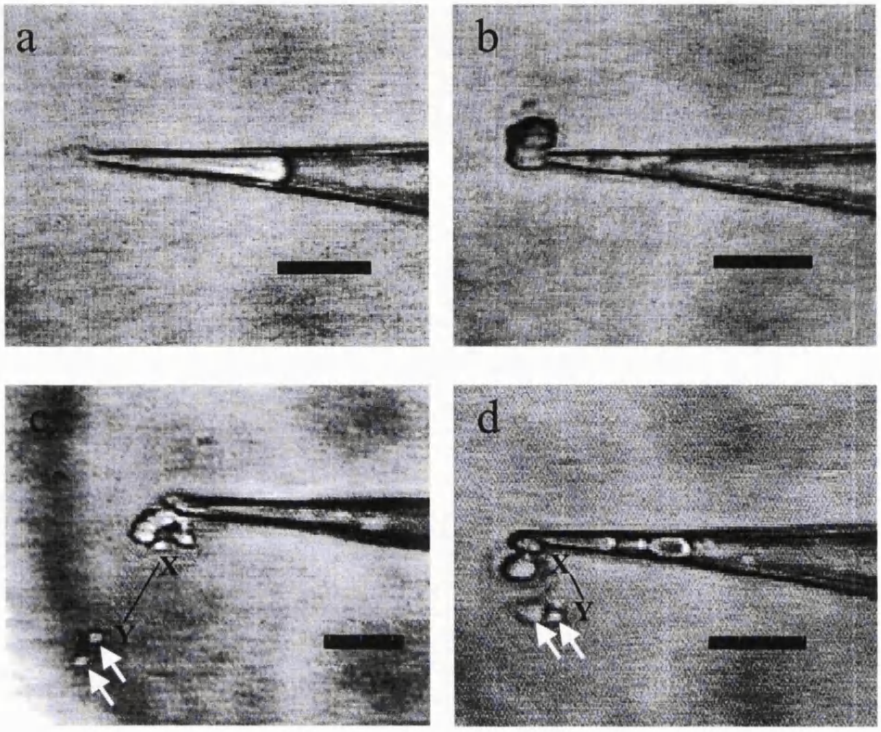


Figure 6.8: (a) Extrusion of a 30 μm-spherical vesicle, formed from a mixture of $C_{16}EO_2$: cholesterol: Solulan C24 (45:45:10), from a micropipette with an exit diameter of 5 μm. (b) The vesicle reforms, once extruded, into a spherical shape with a size larger than the exit. (c&d) The vesicle splits into a group of smaller daughter spherical vesicles (see arrow) which sometimes are linked together by a thin microtubule, which length is represented by the line X-Y. Such a tether is flexible as the distance between two groups of daughter vesicles (X-Y) changes on agitating the medium as in c&d. Bar = 20 μm.

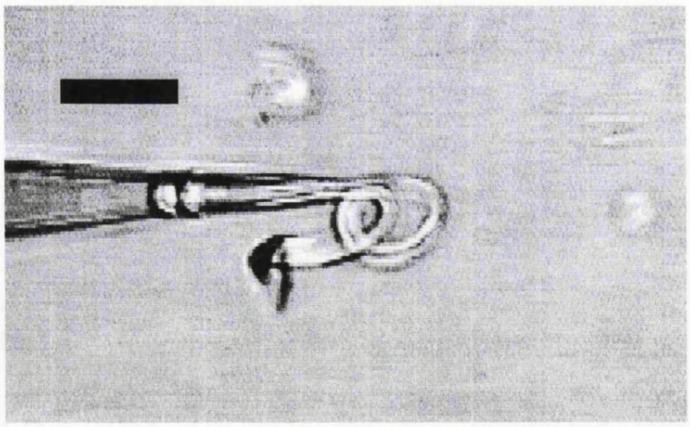


Figure 6.9: Extrusion of a 30 μm-polyhedral vesicle, formed from a mixture of $C_{16}EO_5$: Solulan C24 (91:9), from a micropipette with an exit diameter of 5 μm. The vesicle does not reform after extrusion and was sheared to form an elongated curved structure. Bar = 20 μm.

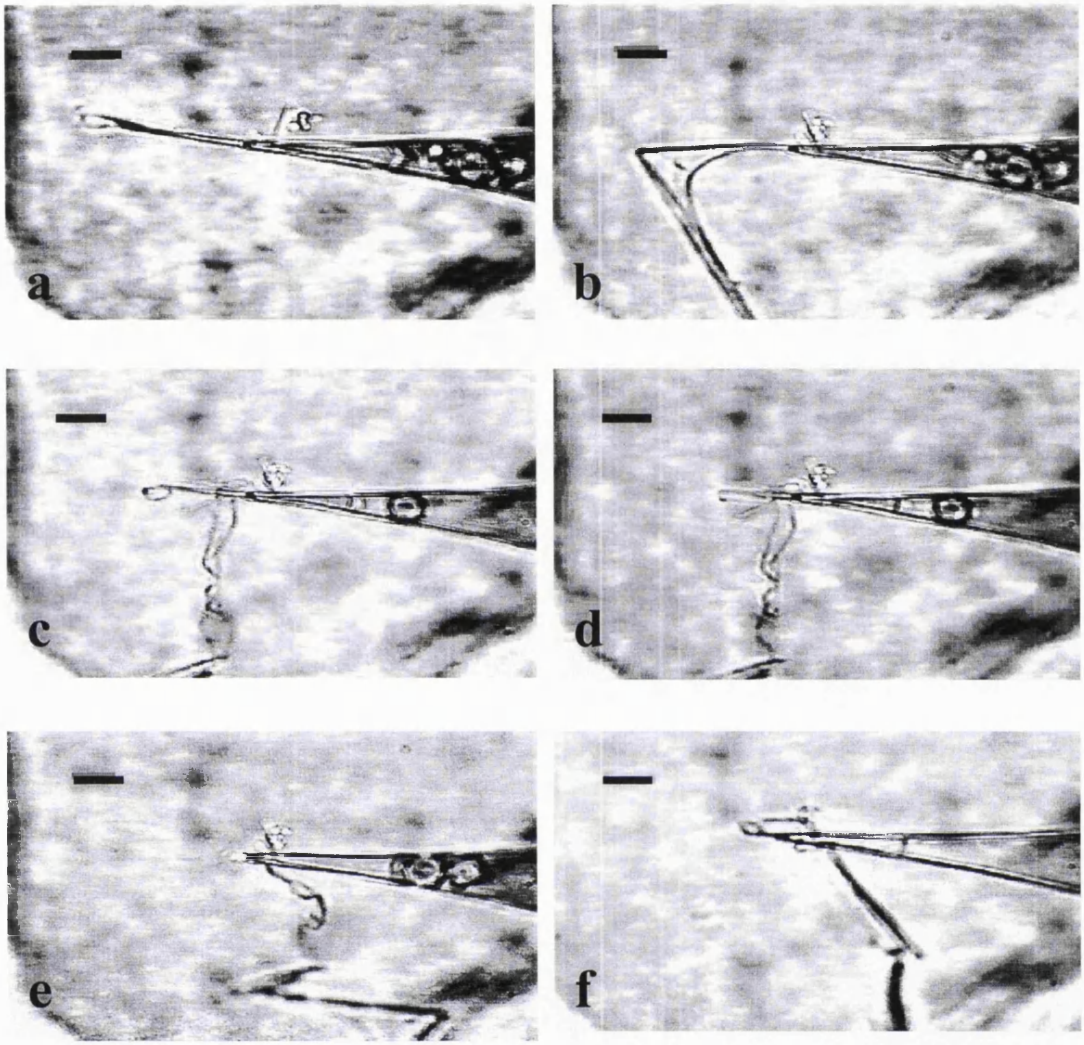


Figure 6.10: Extrusion of three consecutive, separate polyhedral niosomes, formed from a mixture of $C_{16}EO_5$: Solulan C24 (91:9) with diameters ~20-30 μm, from a micropipette with an exit diameter of 5 μm. The vesicles were extruded into elongated structures. Bar = 20 μm.

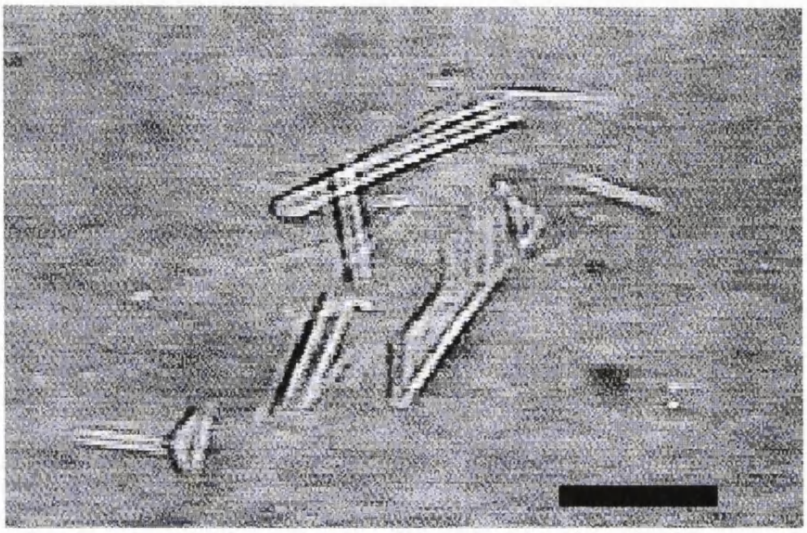


Figure 6.11: Straight rods obtained by extruding polyhedral niosomes, formed from a mixture of $C_{16}EO_5$: Solulan C24 (91:9) with a diameter ~10 μm, from a micropipette with an exit diameter of 5 μm. Bar = 20 μm.

Cholesterol is the key factor which liquifies the gel state membranes into liquid crystalline state increasing the mobility of the amphiphiles in the membranes (Demel & De Kruyff, 1976), hence the difference in flexibility of the polyhedral (cholesterol-poor) and spherical (cholesterol-rich) niosomes. Although spherical niosomes cannot retain their integrity on extrusion, they can reform into spherical vesicles. Polyhedral niosomes possess a faceted structure with their membranes in the gel state at room temperature. The phase transition temperature of polyhedral niosomes formed from a mixture of $C_{16}EO_5$ and Solulan C24 is 29°C measured by high sensitivity differential scanning calorimetry (Table 2.2). This prevents the vesicles from reforming into vesicles once extruded. However, in figure 6.10 the elongated structures appeared to be wider than the exit exhibiting some structural elasticity.

Changes in shape of niosomes following the extrusion prompt questions on the behaviour of vesicular systems on being delivered *in vivo*. It has been known that vesicles with sizes > 5 μm can remain at the intramuscular site of injection for a longer period than do small spherical vesicles (Zuidema *et al.*, 1994) However, whether the vesicles can retain their integrity when they are sheared in the muscles following intramuscular injection should be investigated, as this may reduce the ability of the vesicles to retain the drug at the site of injection. Vesicle dispersions with the size larger than the diameter of blood capillaries may be safely injected intravenously if the vesicles can be squeezed as observed here by glass micropipettes.

Intact niosomes can also be manipulated by drawing them into micropipettes by careful suction. When only parts of the vesicle, and not the whole vesicle, are drawn into the micropipettes, and then released, the membranes of spherical niosomes tend to be removed into the micropipettes, while the remaining membranes anneal. This results in the formation of smaller spherical niosomes. Figure 6.12 shows the removal of bilayers of an oligolamellar spherical vesicle which finally resulted in the smaller spherical vesicles (Figure 6.12c). Similar events have previously been observed by rupturing vesicle membrane layers with a sharp micropipette (Menger & Gabrielson, 1995). Here parts of the vesicle membrane, and not the whole vesicle as in the extrusion process, were drawn into the micropipette. Ejection of the drawn vesicles revealed that the peeled membranes form a group of smaller spherical vesicles, linked together in a chain (Figure 6.12d).

The membranes of polyhedral niosomes are not sufficiently flexible to heal themselves following suction (Figure 6.13). This allows them to be shaped as required. Figure 6.13c

shows an apparent hole in the membranes of a polyhedral niosome after a part of the membrane was removed by a rapid suction. The lack of membrane elasticity of polyhedral niosomes is highlighted when vesicles were carefully drawn into the micropipettes to form polyhedral vesicles with elongated "arms" (Figure 6.13d&f).

We have so far succeeded in the formation of vesicles with a hole or extended branches, straight and curved vesicular rods, and vesicular chains. While the mechanisms of the processes are not yet clearly understood, this technique may be considered as one approach to microfabrication.

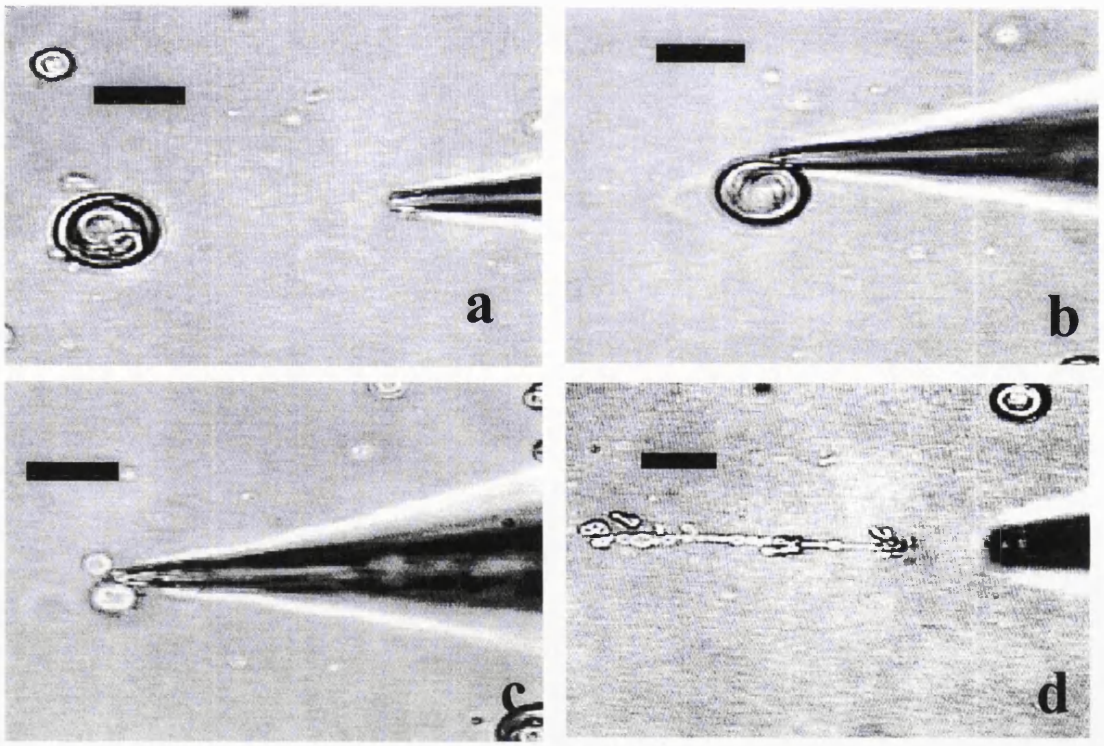


Figure 6.12: Suction of an oligolamellar spherical vesicle, formed from $C_{16}EO_2$: cholesterol: Solulan C24 (45:45:10), into a micropipette with a 5 μm diameter opening. (a, b&c) The membrane layers were peeled and the resultant vesicle becomes smaller in size. (d) Following suction, extrusion of the vesicle revealed the chain of spherical vesicles shown. Bar = 20 μm.

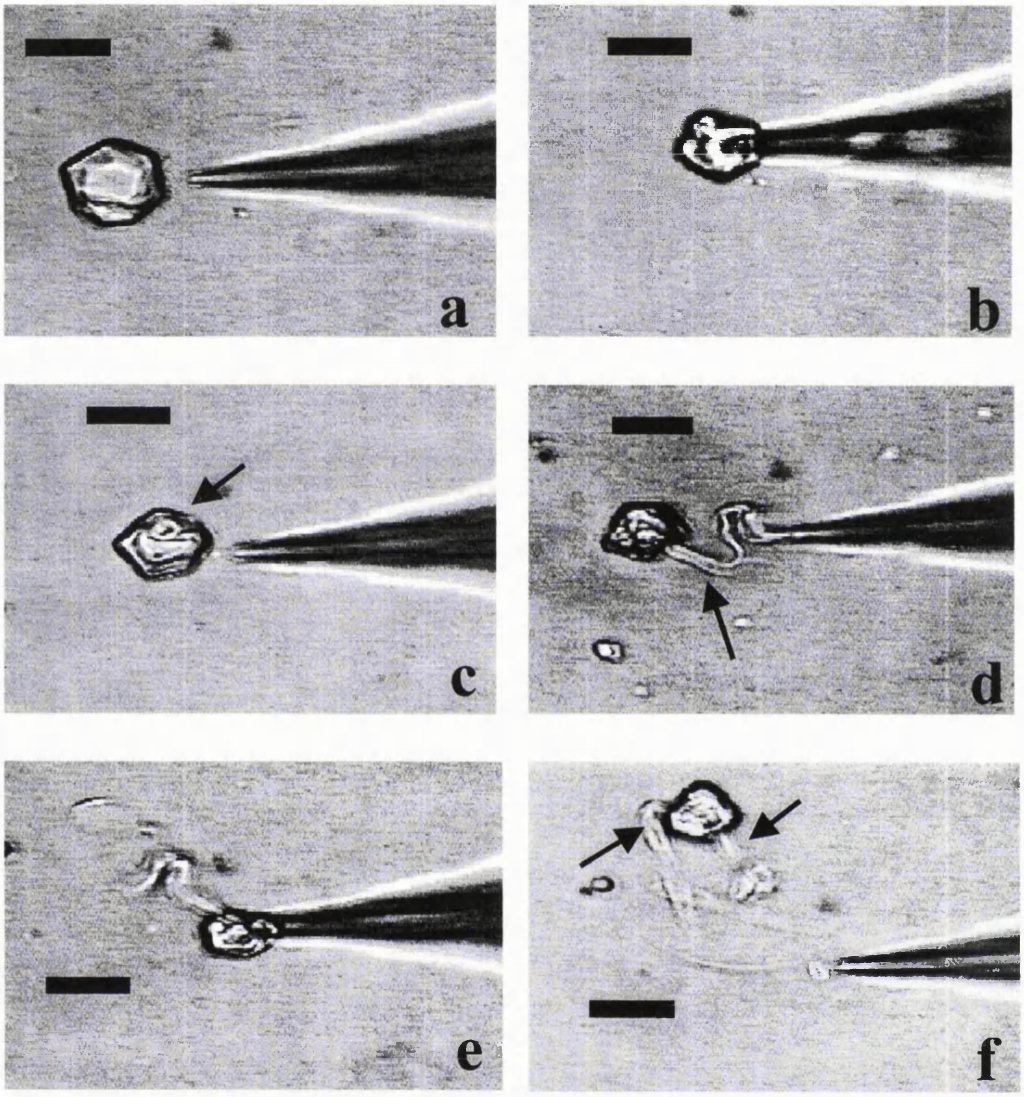


Figure 6.13: Suction of a polyhedral vesicle, formed by poly (5) oxyethylene cetyl ether: poly (24) oxyethylene cholesteryl ether (91:9), into a micropipette with an orifice diameter of 5 μm. The vesicle (a) was drawn toward the capillary following by rapid suction to remove a part of the vesicle membrane (b). The resultant vesicle (c) showed what appears to be an un-annealed hole after the part of the membrane was completely drawn into the capillary tubing (see arrow). By careful suction and release, the membranes of polyhedral vesicle were stretched resulting in the formation of vesicle with appendages (d, e & f) (see arrow). Bar = 20 μm.

6.4. CONCLUSIONS

We have used micropipettes as a model device for delivery of single niosomes and as a tool for fabrication of microstructures. Both studies were performed in a similar manner by manipulating niosomes with various sizes, membrane compositions, and ultrastructures. Successful extrusion of niosomes, and microspheres, with sizes smaller than the exit diameters of micropipettes suggests a possibility for potential programmed release device. Insights gained from the studies of niosomes with diameters larger than the exit of micropipettes should be useful in microfabrication of lipid/surfactant structures which themselves can act as templates for manufacture of permanent structures. The technique offers a three-dimensional process which allows construction of the microstructures from self-assembling systems. It is recognised that further work needs to be done to allow there to be greater control over the form and nature of the final structure, but the simplicity of the technique has its own significance. The study of the influence of formulation additives and the effect of relative diameter of vesicles and micropipette exits, form the basis of continuing studies.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

7.1. CONCLUSIONS

We have studied niosomes with regard to their physicochemical and biological behaviour. The vesicle size, shape, entrapment efficiency, and membrane permeability can be controlled by varying both preparation methods and the composition of the membranes. These parameters were found to affect various properties including the viscosity, osmotic activity, in vivo behaviour of the prepared niosomes.

Studies of polyhedral vesicles showed that their faceted membranes can be constructed by certain amphiphiles but that this ability is affected by the presence of cholesterol in the membranes. Polyhedral niosomes transform into spherical structures upon increasing the

temperature above their phase transition temperature, due to increased membrane fluidity. The inhomogeneous distribution of cholesterol in the membranes, as observed on incubating with the cholesterol-rich spherical niosomes, also locally increases membrane fluidity, resulting in the formation of diverse structures.

Studies of dilute vesicle viscosity provide important information which may be applicable in formulation programs or relevant to their behaviour *in vivo*. The unique structure of vesicular systems offers a challenging model for rheological studies, although viscosity is generally affected by similar factors to those found in other colloidal systems. The hydration and the volume fraction of the vesicles, both directly inaccessible parameters, were estimated using the intrinsic viscosity as a tool. The values were found to be affected by a number of factors both internal, i.e. vesicle size, shape, lamellarity, membrane composition, and external such as temperature or electrolytes. Although further investigation may be needed, this prediction so far provides reasonable estimated values of these critical parameters.

It is well known that vesicles behave like osmometers. The kinetics and the amplitude of swelling and shrinkage provide information on the elastic properties and permeability of the bilayers. We have shown that the initial ultrastructure of the vesicles and the membrane composition are also important factors in controlling the response to the imposition of osmotic stress. In isotonic media, the leakage of solutes from the vesicle lumen depends greatly on the concentration gradients of the solute between inside and outside of the vesicles. This flux can be reduced with an addition of cholesterol to the membranes. Low cholesterol levels make polyhedral niosomes generally more permeable when compared to

their spherical counterparts. The leakage of entrapped solutes from the vesicle lumen is enhanced in the presence of an osmotic gradients. In hypertonic media, the leakage of solutes is increased as a result of the coupled out of flux of solutes and water, whereas in the hypotonic media it is caused mainly (and speculatively) by the reduced crowding of lipids in the vesicle membranes following the swelling process.

The sustained release of LHRH from niosomes administered intramuscularly was clearly shown by both the slow removal of ^{125}I-LHRH from the injection site and the maintainance of blood level over periods of several hours. Low blood levels of ^{125}I-LHRH indicate the degradation of the labile peptide once incorporated into the circulation as free hormone. This suggests that higher doses or repeated doses may be needed to give a biological response. By comparing polyhedral and cholesterol-rich spherical niosomes, it was clearly demonstrated that the effectiveness of the niosomes as an IM depot relies greatly on their low membrane permeability, the factor which overrides the shape and the viscosity of niosome dispersions.

The future of pulsatile delivery by releasing single vesicles seems promising as demonstrated using glass capillaries as model devices. Distortion following the extrusion of the vesicles of which the diameter is larger than the exit of the capillaries was observed. Although the findings have raised concerns as to whether the same events may occur *in vivo*, these have led to the new application of the systems in microfabrication which exploits the difference in membrane elasticity of vesicles with different compositions.

7.2. FUTURE WORK

Further investigations following on the results obtained in this thesis are suggested below.

❋ Geometrical dimensions, e.g. molecular surface area and volume, of amphiphiles used in this thesis which form polyhedral niosomes may be examined. This may provide more information on how such faceted structures are formed and allow prediction of the vesicle ultrastructures formed with certain amphiphiles.

❋ The use of multicomponent niosomes dispersions, in which mixtures of niosomes with various membrane permeabilities, was shown in chapter 2 to provide a predetermined accumulated release profile *in vitro*. Further work should be performed *in vivo* to provide more information on the pharmacokinetics of these systems. This may lead to a new concept in niosomal delivery such as previously found successful with multiparticulate solid dosage forms.

❋ As shown in chapter 4, the effect of Solulan C24 and sodium chloride on the membrane permeability of polyhedral niosomes may be examined. This should provide knowledge on the development of vesicular systems which are osmotically non-responsive and which, therefore, prolong the release of drugs.

❋ The remote loading methods which use pH or $(NH_4)_2SO_4$ gradients allow drugs to be loaded into the vesicles prior to the treatment, which can avoid the leakage problems on storing. As shown in chapter 5, these methods were found to allow the entrapment of the decapeptide, LHRH. It will be interesting to investigate the encapsulation limit of drugs,

correlated with molecular weight and solubility, particularly of large molecules such as peptides and proteins.

* In chapter 6, pulsatile delivery of single vesicles was proved to be possible. This may be followed with the design of delivery devices which can be used clinically. Distortion of vesicles on shearing may be examined on delivery of large vesicles in complex milieu such as muscle or blood capillaries.

* Fabrication of microstructures from the preformed vesicles may be investigated regarding the relationship of vesicle and capillary diameter, membrane composition, applied pressure, and the resultant structures.

* Rheological studies of niosomes may be elaborated which may include the viscosity of vesicles themselves at high concentration and the effect of added vesicles on the flow of blood.

BIBLIOGRAPHY

Al-Suwayeh, S.A., Tebbett, I.R., Wielbo, D. and Brazeau, G.A. (1996) *In vitro- in vivo* myotoxicity of intramuscular liposomal formulations. *Pharm.Res.* **13,** 1384-1388.

Aldwinckle, T.J., Ahkong, Q.F., Bangham, A.D., Fisher, D. and Lucy, J.A. (1982) *Biochim.Biophys.Acta* **689,** 548-560.

Alexander, A.E. and Johnson, P. (1950) *Colloid Science,* Oxford University Press, Oxford, pp. 345-379.

Allen, T. (1992) *Particle Size Measurement Vol.1- Powder Sampling and Particle Size measurement,* 4th edn., Chapman & Hall, London.

Allen, T.M., Hong, K. and Papahadjopoulos, D. (1990) Membrane contact, fusion, and hexagonal (H_{II}) transitions in phosphatidylethanolamine liposomes. *Biochemistry* **29,** 2976-2985.

Andersson, M., Hammarström, L. and Edwards, K. (1995) Effect of bilayer phase transitions on vesicle structure and its influence on the kinetics of viologen reduction. *J.Phys.Chem.* **99,** 14531-14538.

Antonsen, K.P. and Hoffman, A.S. (1992) In: Harris, J.M. (ed.) *Poly (Ethylene Glycol): Chemistry-Biotechnical and Biomedical Applications,* Plenum Press, New York, pp. 15-28.

Arrowsmith, M., Hadgraft, J. and Kellaway, I.W. (1983) The stability of a prodrug, liposome and a prodrug-liposome complex in simulated biological conditions. *Int.J.Pharm.* **17,** 91-98.

Artmann, G.M., Sung, K.L.P., Horn, T., Whittemore, D., Norwich, G., Shu, C. (1997) Micropipette aspiration of human erythrocytes induces echinocytes *via* membrane phospholipid translocation. *Biophys.J.* **72,** 1434-1441.

Attwood, D., Currie, L.R.J. and Elworthy, P.H. (1974) Studies of solubilized micellar solutions. III. The viscosity of solutions formed with nonionic surfactants. *J.Colloid Interface Sci.* **46,** 261-265.

Baillie, A.J. (1988) Niosomes: a putative drug carrier system. In: Gregoriadis, G. and Poste, G. (eds.) *Targeting of Drugs,* Plenum, New York, pp. 143-151.

Baillie, A.J., Florence, A.T., Hume, L.R., Muirhead, G.T. and Rogerson, A. (1985) The preparation and properties of niosomes- non-ionic surfactant vesicles. *J.Pharm.Pharmacol.* **37,** 863-868.

Baker, R.C., Florence, A.T., Ottewill, R.H. and Tadros, Th.F. (1984) Investigations into the formation and characterization of microemulsions. II. Light scattering conductivity and viscosity studies of microemulsions. *J.Colloid Interface Sci.* **100,** 332-348.

Bangham, A.D., De Gier, J. and Greville, G.D. (1967) Osmotic properties and water permeability of phospholipid liquid crystals. *Chem.Phys.Lipids* **1,** 225-246.

Bangham, A.D., Standish, M.M. and Watkins, J.C. (1965) Diffusion of univalent ions across the lamellae of swollen phospholipids. *J.Mol.Biol.* **13,** 238-252.

Banks, W.A. and Cession, A.J. (1985) Peptides and the blood-brain barrier: lipophilicity as a predictor of permeability. *Brain Res.Bull.* **15,** 287

Baraka, M.E., Pécheur, E.I., Wallach, D.F.H. and Philippot, J.R. (1996) Non-phospholipid fusogenic liposomes. *Biochim.Biophys.Acta* **1280,** 107-114.

Barnes, H.A., Hutton, J.F. and Walters, K. (1989) Rheology of suspensions. In: Barnes, H.A., Hutton, J.F., Walters, K. (eds.) *An Introduction to Rheology,* Elsevier, Oxford, pp. 115-139.

Batchelor, G.K. (1977) The effect of Brownian motion on the bulk stress in a suspension of spherical particles. *J.Fluid Mech.* **83,** 97-117.

Berndl, K.J., Lipowsky, R., Sackmann, E. and Seifert, U. (1990) Shape transformations of giant vesicles: extremes sensitivity to bilayer asymmetry. *Europhys.Lett.* **13,** 659-664.

Bernhardt, C. (1994) *Particle Size Analysis- Classification and Sedimentation Methods,* Chapman & Hall, London.

Bernsdorff, C., Wolf, A. and Winter, R. (1996) The effect of temperature and pressure on structural and dynamic properties of phospholipid/ sterol mixtures - a steady-state and time-resolved fluorescence anisotropy study. *Z.Phys.Chem.***193,** 151-173.

Bittman, R. (1993) A review of the kinetics of cholesterol movement between donor and acceptor bilayer membranes. In: Finegold, L. (ed.) *Cholesterol in Membrane Models,* CRC Press, Boca Raton, pp. 45-65.

Blok, M.C., Van Deenen, L.L.M. and De Gier, J. (1976) Effect of the gel to liquid crystalline phase transition on the osmotic behaviour of phosphatidylcholine liposomes. *Biochim.Biophys.Acta* **433,** 1-12.

Boman, N.L., Cullis, P.R., Bally, M.B. and Mayer, L.D. (1995) Preclinical and clinical activity of liposomal doxorubicin. In: Shek, P.N. (ed.) *Liposomes in Biomedical Applications,* Harwood Academic, Chur, pp. 85-103.

Bommannan, D.B., Tamada, J., Leung, L. and Potts, R.O. (1994) Effect of electroporation on transdermal iontophoretic delivery of hormone (LHRH) *in vitro. Pharm.Res.* **11,** 1809-1814.

Bonté, F., Chevalier, J.M. and Meybeck, A. (1994) Determination of retinoic acid - liposomal association level in a topical formulation. *Drug Dev.Ind.Pharm.* **20,** 2527-2534.

Brewer, J.M. (1994) Non-ionic surfactant vesicles as delivery systems. In: *Proceedings of*

the Second Conference on Industrial Immunology, Chameleon Press, London, pp. 34-36.

Brodwick, M.S., Curran, M. and Edwards, C. (1992) Effects of osmotic stress on mast cell vesicles of the beige mouse. *J.Membrane Biol.* **126,** 159-169.

Brown, G.H. and Wolken, J.J. (1979) Visual systems: The optical and photoreceptor structures. In: *Liquid Crystals and Biological Structures,* Academic Press, London, pp. 119-144.

Bruinsma, R. (1996) Rheology and shape transition of vesicles under capillary flow. *Physica A* **234,** 249-270.

Buckton, G., Chowdhry, B.Z., Armstrong, J.K., Leharne, S.A., Bouwstra, J.A. and Hofland, H.E.J. (1992) The use of high-sensitivity differential scanning calorimetry to characterise dilute aqueous dispersions of surfactants. *Int.J.Pharm.* **83,** 115-121.

Cable, C. and Florence, A.T. (1988) Mixed poly(glycerol)-poly(oxyethylene) ether niosomes. *J.Pharm.Pharmacol.* **40,** 30P

Childers, N.K., Denys, F.R., McGee, N.F. and Michalek, S.M. (1990) *Regional Immunology* **3,** 8-16.

Chiou, G.C.Y., Shen, Z.F. and Li, B.H.P. (1992) Effects of permeation enhancers BL-9 and Brij-78 on absorption of 4 peptide eyedrops in rabbits. *Acta Pharm. Sin.* **13,** 201-205.

Chopineau, J., Lesieur, S. and Ollivon, M. (1994) Vesicle Formation by Enzymatic Processes. *J.Am.Chem.Soc.* **116,** 11582-11583.

Conn, P.M. and Crowley, W.F. (1991) Gonadotropin-releasing hormone and its analogues. *New Engl.J.Med.* **324,** 93-103.

Crandall, B.C. (1996) Molecular engineering. In: Crandall, B.C. (ed.) *Nanotechnology. Molecular Speculations on Global Abundance,* MIT Press, London, pp. 1-46.

De Gier, J., Mandersloot, J.G. and Van Deenen, L.L.M. (1968) Lipid composition and permeability of liposomes. *Biochim.Biophys.Acta* **150,** 666-675.

Demel, R.A. and De Kruyff, B. (1976) The function of sterols in membranes. *Biochim.Biophys.Acta* **457,** 109-132.

Dimitrijevic, D., Lamandin, C., Uchegbu, I.F., Shaw, A.J. and Florence, A.T. (1997) The effect of monomers and of micellar and vesicular forms of non-ionic surfactants (Solulan C24 and Solulan 16) on Caco-2 cell monolayers. *J.Pharm.Pharmacol.* **49,** 611-616.

Disalvo, E.A., Campos, A.M., Abuin, E. and Lissi, E.A. (1996) Surface changes induced by osmotic shrinkage on large unilamellar vesicles. *Chem.Phys.Lipids* **84,** 35-45.

Dollery, C. (1991) Dollery, C. (ed.) *Therapeutic Drugs,* Churchill Livingstone, Edinburgh, pp. G54-G58

Döbereiner, H.-G., Käs, J., Noppl, D., Sprenger, I. and Sackmann, E. (1993) Budding and fission of vesicles. *Biophys.J.* **65,** 1396-1403.

Drexler, K.E. (1992) Stratergies for molecular systems engineering. In: Crandall, B.C. and Lewis, J. (eds.) *Nanotechnology. Research and Perspectives,* MIT Press, London, pp. 115-146.

Durrani, A.M., Davies, N.M., Thomas, M. and Kellaway, I.W. (1992) Pilocarpine bioavailability from a mucoadhesive liposomal ophthalmic drug delivery system. *Int.J.Pharm.* **88,** 409-415.

Einstein, A. (1906) Eine neue Bestimmung der Molekuldimension. *Ann.Physik* **19,** 289-306.

Einstein, A. (1911) Berichtigung zu meiner Arbeit: Eine neue Bestimmung der Molekuldimension. *Ann.Physik* **34,** 591-592.

Elworthy, P.H. (1960) The size, shape and hydration of cetomacrogol 1000 micelles. *J.Pharm.Pharmacol.* **12,** 260T-266T.

Elworthy, P.H. and Macfarlane, C.B. (1962) Surface activity of a series of synthetic non-ionic detergents. *J.Pharm.Pharmacol.* **14,** 100T-102T.

Elworthy, P.H. and Macfarlane, C.B. (1965) The physical chemistry of some non-ionic detergents. *J.Pharm.Pharmacol.* **17,** 65-82.

Erdogan, S., Ozer, A.Y., Ercan, M.T., Erylmaz, M. and Hincal, A.A. (1996) In vivo studies on iopromide radiopaque niosomes. *S.T.P.Pharma Sci.* **6,** 87-93.

Erdogdu, G. and Hasirci, V.N. (1994) The use of liposomes in the investigation of mechanisms of asbestos damage. *Experientia* **50,** 785-787.

Evans, E. and Kwok, R. (1982) Mechanical calorimetry of large dimyristoyl phosphatidylcholine vesicles in the phase transition region. *Biochem.* **21,** 4874-4879.

Florence, A.T. (1993) New Drug Delivery Systems. *Chem.Ind.* 1000-1004.

Florence, A.T. and Baillie, A.J. (1989) Non-ionic surfactant vesicles-alternatives to liposomes in drug delivery? In: Prescott, L.F. and Nimmo, W.S. (eds.) *Novel Drug Delivery and Its Therapeutic Applications,* John Wiley, New York, pp. 281-296.

Florence, A.T. and Cable, C. (1993) Non-ionic surfactant vesicles (niosomes) as vehicles for doxorubicin delivery. In: Gregoriadis, G., Florence, A.T., Patel, H.M. (eds.) *Liposomes in Drug Delivery,* Harwood Academic, Chur, pp. 239-253.

Fuhrhop, J.H. and Helfrich, W. (1993) Fluid and solid fibers made of lipid molecular bilayers. *Chem.Rev.* **93,** 1565-1582.

Fuhrhop, J.H. and Köning, J. (1994) *Membranes and Molecular Assemblies: The Synkinetic*

Approach, The Royal Society of Chemistry, Cambridge, pp. 20-97.

Gabizon, A., Chemla, M., Tzemach, D., Horowitz, A.T. and Goren, D. (1996) Liposome longevity and stability in circulation: effects on the in vivo delivery to tumors and therapeutic efficacy of encapsulated anthracyclines. *J.Drug Targeting* **3,** 391-398.

Gabizon, A. and Papahadjopoulos, D. (1988) Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc.Natl.Acad.Sci.,*USA **85,** 6949-6953.

Ghosh, P.C. and Bachhawat, B.K. (1995) Effect of surface modification with glycolipids and polysaccharides on *in vivo* fate of liposomes. In: Lasic, D. and Martin, F. (eds.) *Stealth Liposomes,* CRC Press, Boca Raton, pp. 13-24.

Goodwin, J.W. (1974) The Rheology of Dispersions. In: Everett, D.H. (ed.) *Colloid Science Vol. 2. Specialist Periodical Reports,* Chemical Society, Margate, pp. 246-293.

Graham, N.B. (1992) Poly (ethylene glycol) gels and drug delivery. In: Harris, J.M. (ed.) *Poly (Ethylene Glycol) Chemistry-Biotechnical and Biomedical Applications,* Plenum Press, New York, pp. 268

Gruner, S.M. (1987) Materials Properties of Liposomal Bilayers. In: Ostro, M.J. (ed.) *Liposomes. From Bilayers to Therapeutics,* Marcel Dekker, New York, pp. 1-38.

Grunewald, J.M., Grunewald, R.W. and Kinne, R.K.H. (1993) Ion content and cell volume in isolated collecting duct cells: effect of hypotonicity. *Kidney Int.* **44,** 509-517.

Guzmán, M., Selles, E. and Abérturas, M.C. (1989) Effect of human plasma on the stability of large multilamellar liposomes with digitoxin. *Drug Dev.Ind.Pharm.* **15,** 387-399.

Han, K., Park, J.S., Chung, Y.B., Jeong, N.J., Park, H.B. and Robinson, J.R. (1995) Development of luteinizing-hormone-releasing hormone (LHRH) delivery systems for vaginal mucosal route. *Archives of Pharmacal Research* **18,** 325-331.

Harashima, H., Hiraiwa, T., Ochi, Y. and Kiwada, H. (1995) Size dependent liposome degradation in blood: *in vivo/ in vitro* correlation by kinetic modeling. *J.Drug Targeting* **3,** 253-261.

Hassan, P.A., Valaulikar, B.S., Manohar, C., Kern, F., Bourdieu, L. and Candau, S.J. (1996) Vesicle to micelle transition: rheological investigations. *Langmuir* **12,** 4350-4357.

Hassan, Y., Brewer, J.M., Alexander, J. and Jennings, R. (1996) Immune responses in mice induced by HSV-1 glycoproteins presented with ISCOMS or NISV delivery systems. *Vaccine* 1581-1589.

Hecht, E., Mortensen, K. and Hoffmann, H. (1995) L_3 phase in a binary block copolymer/ water system. *Macromolecules* **28,** 5465-5476.

Heimburg, T. and Biltonen, R.L. (1994) Thermotropic behavior of dimyristoyl

phosphatidylglycerol and it's interaction with cytochrome c. *Biochemistry* **33,** 9477-9488.

Heit, M.C., Williams, P.L., Jayes, F.L., Chang, S.K. and Riviere, J.E. (1993) Transdermal iontophoretic peptide delivery: *in vitro* and *in vivo* studies with luteinizing hormone releasing hormone. *J.Pharm.Sci.* **82,** 240-243.

Helm, V.J. and Muller, B.W. (1990) Stability of gonadorelin and triptorelin in aqueous solution. *Pharm.Res.* **7,** 1253-1256.

Hillery, A.M., Toth, I. and Florence, A.T. (1996a) Co-polymerised peptide particles II: oral uptake of a novel co-polymeric nanoparticulate delivery system for peptides. *J.Control.Rel.* **42,** 65-73.

Hillery, A.M., Toth, I., Shaw, A.J. and Florence, A.T. (1996b) Co-polymerised peptide particles (CPP) I: synthesis, characterisation and in vitro studies on a novel oral nanoparticulate delivery system. *J.Control.Rel.* **41,** 271-281.

Hoffmann, H., Thunig, C., Schmiedel, P. and Munkert, U. (1994) Surfactant systems with charged multilamellar vesicles and their rheological properties. *Langmuir* **10,** 3972-3981.

Hoffmann, H., Thunig, C., Schmiedel, P. and Munkert, U. (1995) Gels from surfactant solutions with densely packed multilamellar vesicles. *Discuss.Faraday Soc.* **101,** 319-333.

Hofland, H.J., Bouwstra, J.A., Verhoef, J.C., Buckton, G., Chowdry, B.Z., Ponec, M. and Junginger, H.E. (1992) Safety aspects of nonionic surfactant vesicles-a toxicity study related to the physicochemical characteristics of nonionic surfactants. *J.Pharm.Pharmacol.* **44,** 287-294.

Hope, M.J., Walker, D.C. and Cullis, P.R. (1983) Ca^{2+} and pH induced fusion of small unilamellar vesicles consisting of phosphatidylethanolamine and negatively charged phospholipids. A freeze fracture study. *Biochim.Biophys.Res.Commun.* **110,** 15-22.

House, C.R. (1974) *Water Transport in Cells and Tissues,* Edward Arnold, London, pp. 240-428.

Hristova, K. and Needham, D. (1995) Physical properties of polymer-grafted bilayers. In: Lasic, D. and Martin, F. (eds.) *Stealth Liposomes,* CRC Press, Boca Raton, pp. 35-49.

Iga, K., Hamaguchi, N., Igari, Y., Ogawa, Y., Toguchi, H. and Shimamoto, T. (1989) Heat-specific drug release of large unilameelar vesicle as hyperthermia-mediated targeting delivery. *Int.J.Pharm.* **57,** 241-251.

International standard ISO/DIS 13320. Particle size analysis guide to laser diffraction methods. By Technical Committee ISO/TC24.

Inukai, T., Wang, X., Greer, S.E. and Greer, M.A. (1992) Cell swelling induced by medium hyposmolarity or isosmolar urea stimulates gonadotrophin-releasing hormone secretion from perifused rat median eminence. *Brain Res.* **599,** 161-164.

Israelachvili, J.N. (1991) *Intermolecular and Surface Forces,* 2nd edn, Academic Press, London, pp 366-394.

Israelachvili, J.N., Marcelja, S. and Horn, R.G. (1980) Physical properties of membrane organisation. *Quart.Rev.Biophys.* **13,** 121-200.

Jackson, A.J. (1980) The effect of route of administration on the disposition of inulin encapsulated in multilamellar vesicles of defined particle size. *Res.Comm.Chem.Path.Pharm.* **27,** 293-304.

Jackson, S., Mestecky, J., Childers, N.K. and Michalek, S.M. (1990) Liposomes containing antidiotypic antibodies. An oral vaccine to induce protective secretory immune-response specific for pathogens of mucosal surfaces. *Infect.Immun.* **58,** 1932-1936.

Joint working group on refinement (1993) Removal of blood from laboratory mammals and birds. *Lab.Anim.* **27,** 1-22.

Kachar, B., Evans, D.F. and Ninham, B.W. (1984) Video enhanced differential interference contrast microscopy: a new tool for the study of associated colloids and prebiotic assemblies. *J.Colloid Interface Sci.* **100,** 287-301.

Kamenka, N., Chorro, M., Talmon, Y. and Zana, R. (1992) Study of mixed aggregates in aqueous solutions of sodium dodecyl sulfate and dodecyltrimethylammonium bromide. *Coll.Surfaces* **67,** 213-222.

Kato, A., Arakawa, M. and Kondo, T. (1983) Flow properties of hemolysate-loaded liposome suspensions. *Biorheology* **20,** 593-601.

Kim, E., Xia, Y., Whitesides,G.M. (1995) Polymer microstructures formed by moulding in capillaries. *Nature* **376,** 581-584.

Kiser, P.F., Wilson, G. and Needham, D. (1998) A synthetic mimic of the secretory granule for drug delivery. *Nature* **394,** 459-462.

Kita, Y., Matsumoto, S. and Yonezawa, D. (1977) Viscometric method for estimating the stability of w/o/w-type multiple phase emulsions. *J.Colloid Interface Sci.* **62,** 87-94.

Kraus, M., Seifert, U. and Lipowsky, R. (1995) Gravity-induced shape transformations of vesicles. *Europhys.Lett.* **32,** 431-436.

Kumar, S., Singh, T., Khar, R.K., Sharma, S.N. and Chauhan, U.P.S. (1993) Evaluation of sterically stabilized liposomes as a vehicle for targeting technetium-99m labelled radiopharmaceuticals. *Pharmazie* **48,** 613-616.

Kunieda, H. and Rajagopalan, V. (1996) Formation and structure of reverse vesicles. In: Rosoff, M. (ed.) *Vesicles,* Mercel Dekker, New York, pp. 79-103.

Lasic, D.D. and Barenholz, Y. (1996) Handbook of nonmedical applications of liposomes-from design to microreactors. Vol III, CRC Press, Boca Raton, 237.

Lasic, D.D., Ceh, B., Stuart, M.C.A., Guo, L., Frederik, P.M. and Barenholz, Y. (1995) Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery. *Biochim.Biophys.Acta* **1239,** 145-156.

Lasic, D.D., Frederik, P.M., Stuart, M.C.A., Barenholz, Y. and McIntosh, T.J. (1992) Gelation of liposome interior. A novel method for drug encapsulation. *FEBS Lett.* **312,** 255-258.

Lasic, D.D. and Needham, D. (1995) The "stealth" liposome: a prototypical biomaterial. *Chem.Rev.* **95,** 2601-2628.

Lee, V.H.L., Traver and Taub (1991) Enzymatic barrier to drug delivery. In: Lee, V.H.L. (ed.) *Peptide and Protein Drug Delivery,* Marcel Dekker, New York, pp. 324-326.

Lee, Y. and Chien, Y.W. (1995) Oral mucosa controlled delivery of LHRH by bilayer mucoadhesive polymer systems. *J.Control.Rel.* **37,** 251-261.

Levitan, I.B. and Kaczmarek, L.K. (1991) *The Neuron. Cell and Molecular Biology,* Oxford University Press, Oxford, pp. 3-31.

Lipowsky, R. (1995) The morphology of lipid membranes. *Curr.Opin.Struct.Biol.* **5,** 531-540.

Lo, Y.L. and Rahman, Y.E. (1995) Protein location in liposomes, A drug carrier: A prediction by differential scanning calorimetry. *J.Pharm.Sci.* **84,** 805-814.

Lorenz, D., Fechner, K. and Mehlis, B. (1985) Studies on the influence of viscosity-increasing pharmaceutical additives on the liberation of the peptide gonadotropin releasing hormone (GnRH) in solution. *Pharmazie* **40,** 631-637.

Madden, T.D., Harrigan, P.R., Tai, L.C.L., Bally, M.B., Mayer, L.D., Redelmeier, T.E., Loughrey, H.C., Tilcock, C.P.S., Reinish, L.W. and Cullis, P.R. (1990) The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey. *Chem.Phys.Lipids* **53,** 37-46.

Mahe, A.M., Manoux, J., Valla, A., Follana, R., Zarif, L., Greiner, J., Vierling, P. and Riess, J.G. (1992) Perfluoroalkylated surfactants-relationships between structure and acute toxicity in mice. *Biomaterials artificial cells and immobilization biotechnology* **20,** 1025-1027.

Mathivet, L., Cribier, S. and Devaux, P.F. (1996) Shape change and physical properties of giant phospholipid vesicles prepared in the presence of an AC electric field. *Biophys.J.* **70,** 1112-1121.

Matsumoto, S. and Kohda, M. (1980) The viscosity of w/o/w emulsions: An attempt to estimate the water permeation coefficient of the oil layer from the viscosity changes in diluted systems on aging under osmotic pressure gradients. *J.Colloid Interface Sci.* **73,** 13-20.

Matsumoto, S. and Sherman, P. (1969) The viscosity of microemulsions. *J.Colloid Interface Sci.* **30,** 525-536.

Mayer, L.D., Madden, T.D., Bally, M.B. and Cullis, P.R. (1992) pH Gradient-Mediated Drug Entrapment in Liposomes. In: Gregoriadis, G. (ed.) *Liposome Technology: Entrapment of Drugs and Other Materials,* 2nd edn. CRC Press, Boca Raton, pp. 27-44.

Mayer, L.D., Tai, L.C.L., Ko, D.S.C., Masin, D., Ginsberg, R.S., Cullis, P.R. and Bally, M.B. (1989) Influence of vesicle size, lipid composition, and drug- to- lipid ratio on the biological activity of liposomeal doxorubicin in mice. *Cancer Res.* **49,** 5922-5930.

McCormack, B. and Gregoriadis, G. (1994) Drugs-in-cyclodextrins-in liposomes: a novel concept in drug delivery. *Int.J.Pharm.* **112,** 249-258.

McMullen, T.P.W., Lewis, R.N.A.H. and McElhaney, R.N. (1994) Comparative differential scanning calorimetric and FTIR and ^{31} P-NMR spectroscopic studies of the effects of cholesterol and androstenol on the thermotropic phase behavior and organization of phosphatidylcholine bilayers. *Biophys.J.* **66,** 741-752.

Menger, F.M. and Gabrielson, K.D. (1995) Cytomimetic organic chemistry: early developments. *Angew.Chem.,Int.Ed.* **34,** 2091-2106.

Menger, F.M. and Lee, S.J. (1995) Induced morphological changes in synthetic giant vesicles: growth, fusion, undulation, excretion, wounding, and healing. *Langmuir* **11,** 3685-3689.

Michalet, X. and Bensimon, D. (1995) Observation of stable shapes and conformal diffusion in genus 2 vesicles. *Science* **269,** 665-668.

Miyajima, K., Baba, T. and Nakagaki, M. (1989) Interaction of polyoxyethylene cholesteryl ethers with liposomal membranes. *Colloid Polym.Sci.* **267,** 201-208.

Monshipouri, M. and Rudolph, A.S. (1995) Liposome-encapsulated alginate. Conrolled hydrogel particle formation and release. *J.Microencapsulation* **12,** 117-127.

Mooney, M. (1951) The viscosity of a concentrated suspension of spherical particles. *J.Colloid.Sci.* **9,** 162-170.

Moreau, W.M. (1988) *Semiconductor Lithography: Principles and Materials*, Plenum, New York.

Morel, S., Gasco, M.R. and Cavalli, R. (1994) Incorporation in lipospheres of [D-Trp-6] LHRH. *Int.J.Pharm.* **105,** R1-R3

Mosharraf, M., Taylor, K.M.G. and Craig, D.Q.M. (1995) Effect of calcium ions on the surface charge and aggregation of phosphatidylcholine liposomes. *J.Drug Targeting* **2,** 541-545.

Motto, M.G., Hamburg, F.P., Graden, D.A., Shaw, C.J. and Cotter, M.L. (1991)

Characterizationof the Degradation Products of Luteinizing Hormone Releasing Hormone. *J.Pharm.Sci.* **80,** 419-423.

Mui, B.L.S., Cullis, P.R., Evans, E.A. and Madden, T.D. (1993) Osmotic properties of large unilamellar vesicles prepared by extrusion. *Biophys.J.* **64,** 443-453.

Muzzalupo, R., Ranieri, G.A. and La Mesa, C. (1996) Translational diffusion and other physicochemical properties of a bolaform surfactant in solution. *Langmuir* **12,** 3157-3161.

Navari, R.M. and Gainer, J.L. (1973) In: Gabelnick, H.L. and Litt, M. (eds.) *Rheology of Biological Systems,* Charles C Thomas, Illinois, pp. 49-69.

Needham, D. and Zhelev, D.V. (1996) The mechanochemistry of lipid vesicles examined by micropipet manipulation techniques. In: Rosoff, M. (ed.) *Vesicles,* Mercel Dekker, New York, pp. 373-444.

Neitchev, V.Z., Kostadinov, A.P. and Bideaud, F.A. (1986) Glycoprotein effect on the osmotic permeability of liposomes. *Int.J.Biochem.* **18,** 459-464.

New, R.R.C. (1990) *Liposomes: a practical approach,* Oxford University Press, Oxford, pp 19-91.

Ogawa, Y., Okada, H., Heya, T. and Shimamoto, T. (1989) Controlled release of LHRH agonist, leuprolide acetate, from microcapsules: serum drug level profiles and pharmacological effects in animals. *J.Pharm.Pharmacol.* **41,** 439-444.

Ohsawa, T., Matsukawa, Y., Takakura, Y., Hashida, M. and Sezaki, H. (1985) Fate of lipid and encapsulated drug after intramuscular administration of liposomes prepared by the freeze- thawing method in rats. *Chem.Pharm.Bull.* **33,** 5013-5022.

Okahata, Y., Tanamachi, S., Nagai, M. and Kunitake, T. (1981) Synthetic Bilayer Membrane Prepared from Dialkyl Amphiphiles with Nonionic and Zwitterionic Head Groups. *J.Colloid Interface Sci.* **82,** 401-417.

Oku, N. and Namba, Y. (1994) Long-circulating liposomes. *Critical Reviews in Therapeutic Drug Carrier Systems* **11,** 231-270.

Ott, S., Schurtenberger, P. and Wunderli-Allenspach, H. (1994) Liposomes and influenza viruses as an in vitro model for membrane interactions. II. Influence of vesicle size and preparation methods. *Eur.J.Pharm.Sci.* **1,** 333-341.

Ott, S. and Wunderli-Allenspach, H. (1994) Liposomes and influenza viruses as an *in vitro* model for membrane interactions. I. Kinetics of membrane fusion and lipid transfer. *Eur.J.Pharm.Sci.* **1,** 323-332.

Özer, A.Y., Hincal, A.A. and Bouwstra, J.A. (1991) A novel drug delivery system: non ionic surfactant vesicles. *Eur.J.Pharm.Biopharm.* **37,** 75-79.

Panico, A., Pignatello, R., Cardile, V. and Puglisi, G. (1997) Preparation of liposome

formulations containing immunomodulatory peptides. *Pharm.Acta Helv.* **72,** 1-10.

Powell, M.F., Sanders, L.M., Rogerson, A. and Si, V. (1991) Parenteral peptide formulations: chemical and physical properties of native luteinizing hormone-releasing hormone (LHRH) and hydrophobic analogues in aqueous solutions. *Pharm.Res.* **8,** 1258-1263.

Rahimy, M.H., Peyman, G.A., Chin, S.Y., Golshani, R., Aras, C., Borhani, H. and Thompson, H. (1994) Polysulfone capillary fiber for intraocular drug delivery: *in vitro* and *in vivo* evaluations. *J.Drug Targeting* **2,** 289-298.

Reineccius, G.A. (1995) Liposomes for controlled-release in the food-industry. *ACS Symposium Series* **590,** 113-131.

Richards, R.L., Hailey, J.R., Egan, J.E., Gordon, D.M., Johnson, A.J., Alving, C.R. and Wassef, N.M. (1995) Intramuscular fate and local effects of liposomes as carriers for drugs and vaccines. In: Shek, P.N. (ed.) *Liposomes in Biomedical Applications,* Harwood Academic, Chur, pp. 167-178.

Riess, J.G., Pace, S. and Zarif, L. (1991) Highly effective surfactants with low hemolytic-activity. *Advanced Materials* **3,** 249-251.

Sakai, H., Takeoka, S., Park, S.I., Kose, T., Nishide, H., Izumi, Y., Yoshizu, A., Kobayashi, K. and Tsuchida, E. (1997) Surface modification of hemoglobin vesicles with poly(ethylene glycol) and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. *Bioconjugate Chem.* **8,** 23-30.

Sakthivel, T., Toth, I. and Florence, A.T. (1998) Synthesis and physicochemical properties of lipophilic polyamide dendrimers. *Pharm.Res.* **15,** 776-782.

Sandow, J., Clayton, R.N. and Kuhl, H. (1981) Endocrinology of Human Infertility: New Aspects. In: Crosignani, P.G. and Rubin, B.L. (eds.) *Proceedings of the Serono Clinical Colloquia on Reproduction No. 2,* Academic Press, London, pp. 21-21.

Santaella, C., Frezard, F., Vierling, P. and Riess, J.G. (1993) Extended in vivo blood circulation time of fluorinated liposomes. *FEBS Lett.* **336,** 481-484.

Saunders, F.L. (1961) Rheological properties of monodisperse latex systems. I. Concentration dependence of relative viscosity. *J.Colloid Sci.* **16,** 13-22.

Schäfer, H., Schmidt, W., Berger, H. and Bergfeld, J. (1987a) Pharmacokinetics of gonadotropin-releasing hormone and stimulation of luteinizing hormone secretion after single dose administration of GnRH incorporated into liposomes. *Pharmazie* **42,** 689-693.

Schäfer, H., Schmidt, W. and Lachmann, U. (1987b) Preparation and properties of GnRH-loaded multilamellar liposomes. *Pharmazie* **42,** 674-677.

Schnur, J.M. and Shashidhar, R. (1994) Self-Assembling Phospholipid Tubules. *Advanced Materials* **6,** 971-974.

Schreier, H. and Bouwstra, J. (1996) Liposomes and niosomes as topical drug carriers-dermal and transdermal drug delivery. *J.Control.Rel.* **30,** 1-15.

Seki, K. and Komura, S. (1995) Viscoelasticity of vesicle dispersions. *Physica A* **219,** 253-289.

Senisterra, G.A., Gagliardino, J.J. and Disalvo, E.A. (1988) Osmotic dependence of lysophosphatidylcholine lytic action on liposomes in the gel state. *Biochim.Biophys.Acta* **94,** 264-270.

Seras, M., Handjani-Vila, R.-M., Ollivon, M. and Lesieur, S. (1992) Kinetic aspects of the solubilization of nonionic monoalkyl amphiphile-cholesterol vesicles by octylglucoside. *Chem.Phys.Lipids* **63,** 1-14.

Sherman, P. (1968) Rheology of Emulsions. In: Sherman, P. (ed.) *Emulsion Science,* Academic Press, London, pp. 284-351.

Shi, Y.F., Sherins, R.J., Brightwell, D., Gallelli, J.F. and Chatterji, D.C. (1984) Long-Term Stability of Aqueous Solutions of Luteinizing Hormone- Releasing Hormone Assessed by an *In Vitro* Biossay and Liquid Chromatography. *J.Pharm.Sci.* **73,** 819-821.

Shimada, K., Miyagishima, A., Sadzuka, Y., Nozawa, Y., Mochizuki, Y., Ohshima, H. and Hirota, S. (1995) Determination of the thickness of the fixed aqueous layer around polyethyleneglycol-coated liposomes. *J.Drug Targeting* **3,** 283-289.

Shimizu, T., Kogiso, M. and Masuda, M. (1996) Vesicle assembly in microtubes. *Nature* **383,** 487-488.

Smeulders, J.B.A.F., Blom, C. and Mellema, J. (1990) Linear viscoelastic study of lipid vesicle dispersions: Hard-sphere behavior and bilayer surface dynamics. *Phys.Rev.A* **42,** 3483-3498.

Sternberg, B., Moody, M.F., Yoshioka, T. and Florence, A.T. (1995) Geodesic surfactant structures. *Nature* **378,** 21.

Storm, G., Van Bloois, L., Steerenberg, P.A., Van Etten, E., De Groot, G. and Crommelin, D.J.A. (1989) Liposome encapsulation of doxorubicin: pharmaceutical and therapeutic aspects. *J.Control.Rel.* **9,** 215-229.

Sun, S.-T., Milon, A., Tanaka, T., Ourisson, G. and Nakatani, Y. (1986) Osmotic swelling of unilamellar vesicles by the stopped-flow light scattering method. Elastic properties of vesicles. *Biochim.Biophys.Acta* **860,** 525-530.

Surewicz, W.K. and Epand, R.M. (1985) Role of peptide structure in lipid peptide interactions-high-sensitivity differential scanning calorimetry and electron-spin resonance studies of the structural-properties of dimyristoylphosphatidylcholine membranes interacting with pentagastrin-related pentapeptides. *Biochemistry* **24,** 3135-3144.

Suzuki, K., Okumura, Y., Sato, T. and Sunamoto, J. (1995) Membrane protein transfer

from human erythrocyte ghosts to liposomes containing an artificial boundary lipid. *Proc.Japan Acad.* **71,** 93-97.

Talsma, H., Van Steenbergen, M.J., Borchert, J.C.H. and Crommelin, D.J.A. (1994) A novel technique for the one-step preparation of liposomes and nonionic surfactant vesicles without the use of organic solvents. Liposome formation in a continuous gas stream: the 'bubble' method. *J.Pharm.Sci.* **83,** 276-280.

Tank, D.W. and Miller, C. (1983) Patch-clamped liposomes. Recording reconstituted ion channels. In: Sakmann, B. and Neher, E. (eds.) *Single-Channel Recording,* Plenum Press, London, pp. 91-105.

Taylor, G.I. (1932) The viscosity of a fluid containing small drops of another fluid. *Proc.Soc.London A* **138,** 41-48.

Taylor, K.M.G. and Morris, R.M. (1995) Thermal analysis of phase transition behaviour in liposomes. *Thermochim.Acta* **248,** 289-301.

Taylor, K.M.G., Taylor, G., Kellaway, I.W. and Stevens, J. (1990) Drug entrapment and release from multilamellar and reverse-phase evaporation liposomes. *Int.J.Pharm.* **58,** 49-55.

Uchegbu, I.F., Bouwstra, J.A. and Florence, A.T. (1992) Large disk-shaped structures (discomes) in non-ionic surfactant vesicle to micelle transitions. *J.Phys.Chem.* **96,** 10548-10553.

Uchegbu, I.F., Double, J.A., Turton, J.A. and Florence, A.T. (1995) The biodistribution and tumoricidal activity of doxorubicin sorbitan monostearate (Span 60) niosomes. *Pharm.Res.* **12,** 1019-1024.

Uchegbu, I.F. and Florence, A.T. (1995) Non-ionic surfactant vesicles (Niosomes): physical and pharmaceutical chemistry. *Adv.Colloid.Interface Sci.* **58,** 1-55.

Uchegbu, I.F., McCarthy, D., Schätzlein, A. and Florence, A.T. (1996) Phase transitions in aqueous dispersions of the hexadecyl diglycerol ether ($C_{16}G_2$) non-ionic surfactant, cholesterol and cholesteryl poly-24-oxyethylene ether: vesicles, tubules, discomes and micelles. *S.T.P.Pharma Sci.* **6,** 33-43.

Uchegbu, I.F., Schätzlein, A., Vanlerberghe, G., Morgatini, N. and Florence, A.T. (1997) Polyhedral non-ionic surfactant vesicles. *J.Pharm.Pharmacol.* **49,** 606-610.

Usselmann, B. and Müller-Goymann, C.C. (1984) Struktureller aufbau von cholesterol-polyoxyäthylenfettalkoholäther-wasser-mischungen. *Progr.Colloid Polymer.Sci.* **69,** 56-63.

Van der Waarden, M. (1954) Viscosity and electroviscous effect of emulsions. *J.Colloid.Sci.* **9,** 215-222.

Van der Werff, J.C., De Kruif, C.G., Blom, C. and Mellema, J. (1989) Linear viscoelastic behavior of dense hard-sphere dispersion. *Phys.Rev.A* **39,** 795-807.

Vanlerberghe, G., Handjani-Vila, R.M., Berthelot, C. and Sebag, H. (1973) Synthese et activité de surface compar'ee d'une serie de nouveaux derivés non-ioniques. *Proceedings of the 6th International Congress on Surface Active Agents, Zurich, 11-15 Sept, 1972* 139-155.

Vemuri, S. and Rhodes, C.T. (1994) Development and characterization of a liposome preparation by a pH- gradient method. *J.Pharm.Pharmacol.* **46,** 778-783.

Vemuri, S., Yu, C.-D., Wangsatorntanakun, V. and Roosdorp, N. (1990) Large scale production of liposomes by a microfluidizer. *Drug Dev.Ind.Pharm.* **16,** 2243-2256.

Viera, L.I., Senisterra, G.A. and Disalvo, E.A. (1996) Changes in the optical properties of liposome dispersions in relation to the interlamellar distance and solute interaction. *Chem.Phys.Lipids* **81,** 45-54.

Walker, S.A., Kennedy, M.T. and Zasadzinski, J.A. (1997) Encapsulation of bilayer vesicles by self-assembly. *Nature* **387,** 61-64.

Wang, Y.L. (1970) Electroviscous effects of concentrated polystyrene latexes. *J.Colloid Interface Sci.* **32,** 633-641.

Yamazaki, M. and Ito, T. (1990) Deformation and instability in membrane structure of phospholipid vesicles caused by osmophobic association: mechanical stress model for the mechanism of poly(ethylene glycol)-induced membrane fusion. *Biochemistry* **29,** 1309-1314.

Yeagle, P.L. (1991) Modulation of membrane function by cholesterol. *Biochimie* **73,** 1303-1310.

Yeagle, P.L. (1993) The biophysics and cell biology of cholesterol: an hypothesis for the essential role of cholesterol in mammalian cells. In: Finegold, L. (ed.) *Cholesterol in membrane models,* CRC Press, Boca Raton, pp. 1-12.

Yoneda, M. (1973) Tension at the surface of sea urchin eggs on the basis of liquid-drop concept. *Adv.Biophys.* **4,** 153-190.

Yoshida, E. and Nakae, T. (1986) Cytolytic activity of liposomes containing stearylamine. *Biochim.Biophys.Acta* **854,** 93-101.

Yoshida, H., Lehr, C.M., Kok, W., Junginger, H.E., Verhoef, J.C. and Bouwstra, J.A. (1992) Niosomes for oral delivery of peptide drugs. *J.Control.Rel.***21,** 145-153.

Yoshikawa, W., Akutsu, H. and Kyogoku, Y. (1983) Light-scattering properties of osmotically active liposomes. *Biochim.Biophys.Acta* **735,** 397-406.

Yoshioka, T. and Florence, A.T. (1994) Vesicle (niosome)-in-water-in-oil (v/w/o)emulsions: an *in vitro* study. *Int.J.Pharm.* **108,** 117-123.

Zariff, L., Gulik-Krzywicki, T., Reiss, J.G., Pucci, B., Guedj, C. and Pavia, A. (1994) Alkyl

and perfluoroalkyl glycolipid-based supramolecular assemblies. *Coll. Surfaces A* **84,** 107-112.

Zierenberg, O. and Betzing, H. (1979) Pharmacokinetics and metabolism of i.m. injected polyenylphosphatidylcholine liposomes. *Arzneim.Forsch.Drug Res.* **29,** 494-498.

Zuidema, J., Kadir, F., Titulaer, H.A.C. and Oussoren, C. (1994) Release and absorption rates of intramuscularly and subcutaneously injected pharmaceuticals (II). *Int.J.Pharm.* **105,** 189-207.

LIST OF PUBLICATIONS

Papers:

1. Arunothayanun, P., Turton, J.A., Uchegbu, I.F. and Florence, A.T. Preparation and in vitro/ in vivo evaluation of luteinizing hormone releasing hormone (LHRH) loaded polyhedral and spherical/ tubular niosomes. *J.Pharm.Sci.,* in press.

2. Arunothayanun, P., Uchegbu, I. F., Craig, D. Q. M., Turton, J.A. and Florence, A. T. *In vitro/ in vivo* characterisation of polyhedral niosomes. *Int.J.Pharm.,* in press.

3. Florence, A.T., Arunothayanun, P., Kiri, S., Bernard, M.-S. and Uchegbu, I.F. Some rheological properties of nonionic surfactant vesicles and the determination of surface hydration. *J.Phys.Chem.B*, in press.

4. Arunothayanun, P., Bernard, M.-S., Craig, D. Q. M., Uchegbu, I. F. and Florence, A.T. The effect of processing variables on the physical characteristics of non-ionic surfactant vesicles (niosomes) formed from a hexadecyl diglycerol ether. *Submitted to Int.J.Pharm.*

5. Arunothayanun, P., Uchegbu, I.F. and Florence, A.T. Osmotic behaviour of polyhedral non-ionic surfactant vesicles (niosomes). *Submitted to J.Pharm.Pharmacol.*

6. Arunothayanun, P., Sooksawate, T., Florence, A.T. Microfabrication of surfactant structures by manipulating a single vesicle. in preparation.

Book chapter:

1. Arunothayanun, P. and Florence, A.T. Rheology of niosome dispersions. In: Uchegbu, I.F. (ed.) *Synthetic surfactant vesicles. An introduction to niosomes and other non-phospholipid systems,* Harwood Academic, in press.

Abstracts:

1. Arunothayanun, P., Uchegbu, I.F. and Florence, A.T. (1996) Properties of polyhedral niosomes. *Pharm.Res.* **13 (suppl.),** s-159.

2. Florence, A.T., Uchegbu, I.F. and Arunothayanun, P. (1996) The morphological diversity of niosomes: some physical and biological consequences. *Liposome advances: progress in drug and vaccine delivery,* December, 16th-20th, 1996. The School of Pharmacy, University of London, UK.

3. Arunothayanun, P., Bernard, M.-S., McCarthy, D., Uchegbu, I. F., and Florence, A. T. Morphological studies of $C_{16}G_2$ niosomes using microscopy. *3rd UKCRS symposium on controlled drug delivery,* June 1st, 1997. University of Manchester, UK.

4. Bernard, M.-S., Arunothayanun, P., Uchegbu, I. F., and Florence, A. T. Rheological and morphological study of polyhedral and spherical/ tubular niosomes. *3rd UKCRS symposium on controlled drug delivery*, June 1st, 1997. University of Manchester, UK.

5. Arunothayanun, P., Craig, D. Q. M., Uchegbu, I. F., and Florence, A. T. (1997) An investigation into the phase behaviour of polyhedral niosomes and high sensitivity differential scanning calorimetry. In: *Proceed. 24th Int'l. Symp. Control. Rel. Bioact. Mater.* The Controlled Release Society, USA, **24**(1), 977-978.

6. Arunothayanun, P., Turton, J.A., Uchegbu, I.F. and Florence, A.T. (1997) Preparation

and *in vitro/ in vivo* evaluation of luteinizing hormone releasing hormone (LHRH) loaded niosomes. *Pharm.Res.* **14(suppl.),** s-286.

7. Arunothayanun, P., Uchegbu, I. F., Craig, D. Q. M., Turton, J.A. and Florence, A. T. *In vitro/ in vivo* characterisation of polyhedral niosomes. *2nd European Workshop on Particulate Systems,* May 22nd-23rd, 1998, Paris, France.

8. Arunothayanun, Uchegbu, I.F., Craig, D.Q.M., Turton, A.J., Florence, A.T. (1998) Investigations into the *in vitro/ in vivo* behaviour of polyhedral niosomes. *J.Pharm.Pharmacol.* **50(suppl.),** 169.

9. Arunothayanun, P., Kiri, S., Florence, A.T. (1998) Viscosity of niosome dispersions. *Eur.J.Pharm.Sci.* **6(suppl.),** s4.

10. Arunothayanun, P., Florence, A.T., Sooksawate, T. Approaches to a biomimetic delivery device: release of single niosomes from capillaries. *Eur.J.Pharm.Sci.* **6(suppl.),** s61.