The synthesis and characterisation of novel ultra-flexible lipidic vesicles using propanol

Urwashi Sharad Naik, BSc

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Department of Pharmacy and Biomedical Sciences, University of Central Lancashire, Preston, UK

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DECLARATION

I declare that whilst registered as a candidate for the degree for which this submission is made, I have not been registered candidate for another award by any other awarding body. No material contained in this thesis has been used in any other submission for and academic award.

Signed

Abstract

There is a large population of microorganisms which reside deep within the skin and are not easily accessible or removed without damaging the skin layers. Therefore, it would be useful to design an effective drug delivery system that could deliver the antimicrobial agent deeper into the outmost layer of the skin, the stratum corneum.

The main objective of this study was to prepare a nanosized lipidic particulate drug delivery system that has an innate antimicrobial activity as well as the ability to carry an antimicrobial agent with the potential to deliver the drug through the skin. Similar to ethosomes and transfersomes, the use of edge activators such as sodium cholate hydrate, Span 80 and Tween 80, could make these lipidic nanoparticles more elastic and have the ability to be deformed allowing deeper penetration into the skin or applied topically.

In this study, thin film hydration technique was used to prepare the conventional formulations of liposomes, ethosomes and transfersomes by first dissolving DPPC, SPC, cholesterol and edge activators (Sodium cholate hydrate, Span 80, Tween 80) in either chloroform or chloroform: methanol mixture. The samples were then hydrated by a PBS (pH 7.4), doubled distilled water or distilled water-ethanol mixture following rotary evaporation for 60 minutes. The conventional formulations either with or without the model drug (chlorhexidine) were compared with the purposely-designed ultraflexible lipidic formulation, which is termed as propanosomes. Modifying the thin film hydration method, this study used propanol for the hydration step for the synthesis and optimisation of propanosomes. Propanol was selected over other solvents due to its more bactericidal property. All formulations were characterised using laser diffraction, zeta sizer and lipid extrusion techniques. The propanosomal formulations were visualised under TEM. The following size ranges were found with the following formulations: liposomes (1µm), ethosomes (6-8 µm), transferosomes (60-90 nm) and propanosomes (80-100 nm). These experiments also showed that ethosomes were more ultradeformable than liposomes and transfersomes as there was no change of the size upon extrusion. The propanosomal results showed that the mean particle size was inherited by transfersomes and deformability and charge were inherited by ethosomes concluding that the combining preparatory method of using transfersomes and ethosomes was successfully achieved. Antimicrobial studies on the formulations as disk diffusion testing was also conducted on the formulations. In conclusion, the results of this study have demonstrated that it is possible to synthesis and characterise novel lipidic particulate system and further optimisation on the nanolipidic system warrants potential application as drug delivery system for topical antimicrobial application.

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List of abbreviations

±	With or without
CHX	Chlorhexidine base
CHX salt	Chlorhexidine diacetate salt hydrate
D	Degree of deformability
DLE	Drug loaded ethosomes
DLL	Drug loaded liposomes
DLP	Drug loaded propanosomes
DPPC	Dipalmitoylphosphatidylcholine
DTF-SCH	Drug loaded transfersomes with Sodium cholate hydrate
DTF-SP	Drug loaded transfersomes with Span-80
DTF-TW	Drug loaded transfersomes with Tween80
E	Ethosomes
L	Liposomes
LUV	Large Unilamellar Vesicles
MLV	Multivesicluar Vesicles
P	Propanosomes
PBS	Phosphate Buffer Saline
PDI	Polydispersity index
PMFP	Polycarbonate membrane filter pore
SCH	Sodium cholate hydrate

Span 80	Sorbitan monooleate
SPC	Soybean Phosphatidylcholine
SUV	Small Unilamellar Vesicles
TEM	Transmission Electron Microscopy
TF-SCH	Transfersomes with sodium cholate hyrate
TF-SP	Transfersomes with Span-80
TF-TW	Transfersomes with Tween-80
Tween 80	Polysorbate 80

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1 Introduction

1.1 Skin model

Human skin is the main barrier for material permeation (Barry, 1983) and consists of inner skin region of 250 µm called the dermis and outer skin region of 50 µm known as the epidermis. The outer most layer of the epidermis is the stratum corneum (SC), often described as the horny layer of the skin and acts as a major biological barrier for absorption through the skin (Cevc, 2003).

There are three possible ways through which the penetrants can follow to pass through the skin: – the hair follicles with associated sebaceous glands, the sweat glands and the continuous appendages across SC (see figure 1.1) (Barry, 2001).

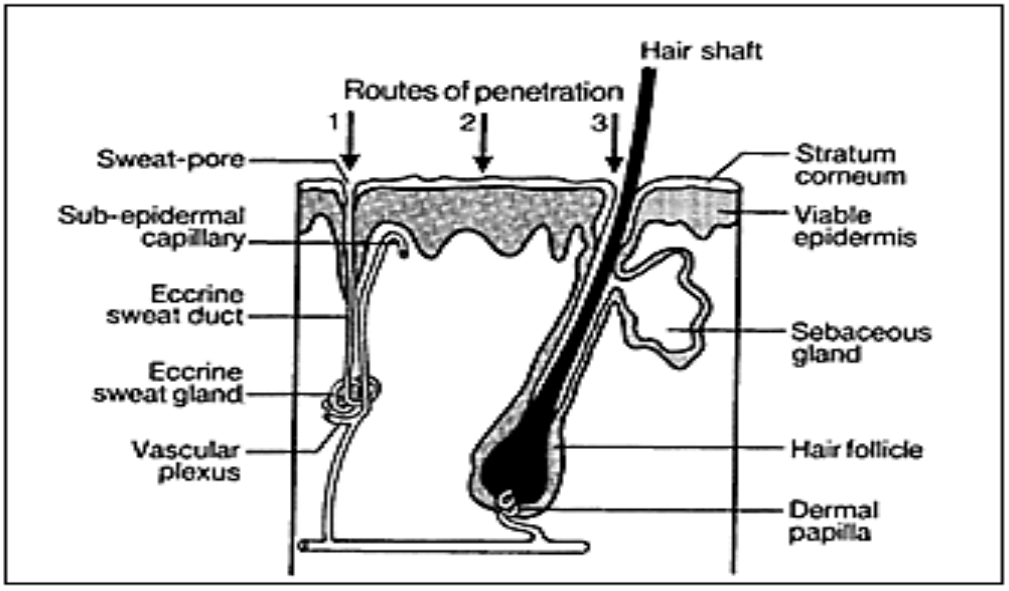

Figure 1.1: Simplified diagram of skin structure and micro routes of drug penetration: (1) via the sweat ducts; (2) across the continuous stratum corneum and (3) through the hair follicles with their associated sebaceous glands (Taken from Barry, 2001).

Only 0.1% of fractional appendageal are available for transportation (Barry, 2001) and SC provides the major barrier for drug transportation due to its 'brick and mortar' wall like structure (Barry & Williams, 1995). In this structure, the corneocytes of hydrated keratin consist of 'bricks' embedded in a 'motar' composed of multiple lipidic bilayers of ceramides, fatty acids, cholesterol and cholesterol esters. These bilayers form gel and liquid crystals domain regions. Most of the molecules pass

through this intercellular microroute and many techniques are emerging to disrupt this route (see in figure 1.2).

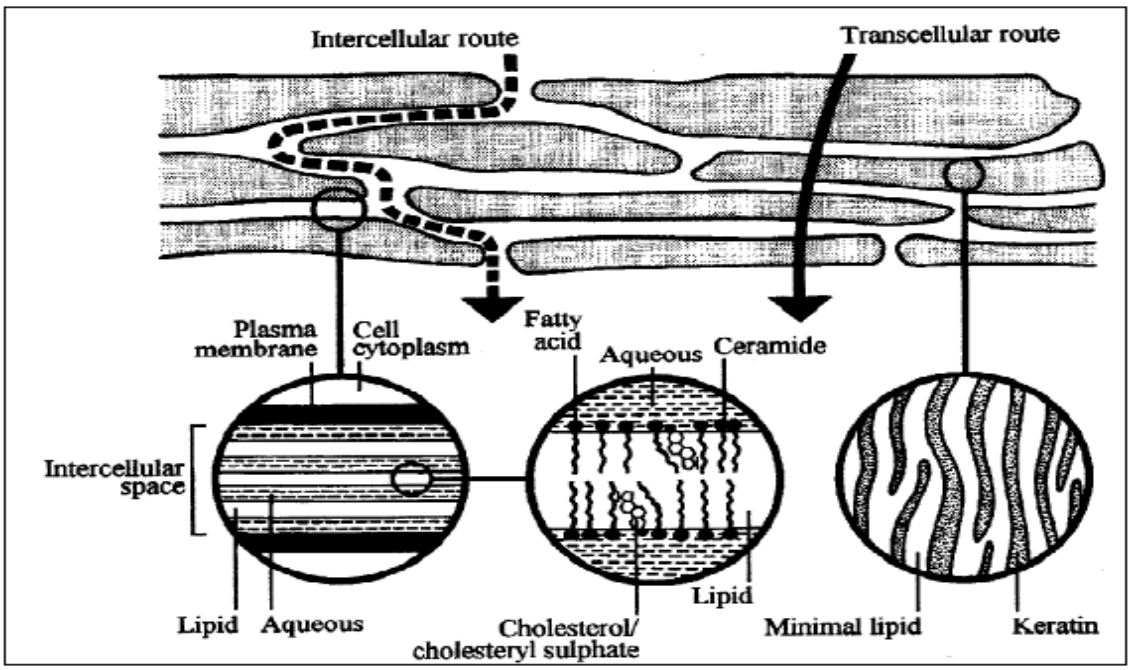

Figure 1.2: Diagram showing the stratum corneum and two microroutes of drug penetration (Taken from Barry, 2001).

1.2 Transdermal drug delivery

The transdermal route for drug administration has many advantages over other pathways such as avoiding the haepatic first-pass effect, the potential for controlled drug delivery, fewer side-effects and improved patient compliance (Barry, 1983). The major barrier for effective transdermal delivery is the low penetration ability of drugs through the skin mainly because of the SC layer which consists of coenocytes embedded in a lipid matrix. The lipid matrix composed of highly organized bilayer, containing free fatty acids, ceramides, cholesterol and cholesterol sulphate (Elias et al., 1977). Lipid matrix acts not only like"glue" but also seals the spaces between the cell and the skin (Trotta et al., 2002). Hence, the SC makes it very challenging for drug to pass through the skin (Wertz & Downing, 1989). Transdermal drug delivery approaches may depend upon fluidising or destroying this lipid bilayer (Barry, 1995).

1.3 Liposomes

Liposomes are small spherical nano-sized vesicles produced from phospholipids and other excipients such as cholesterol. Liposomes are vesicles made of concentric bilayers, which may entrap and deliver various molecules such as small drugs, genes and proteins (Barry, 2001; Samad et al., 2007). Liposomes were first discovered by Bangham et al. in 1964 when they were studying phospholipids as model biological membranes today and they have become important carriers in pharmaceutical sciences, biology, biochemistry and medicine. The word "liposome" is derived from the Greek words: 'lipos' meaning fat and 'soma' meaning body. Structurally, liposomes are concentric lipidic bilayers, which are made up of phospholipid molecules consisting of hydrophilic head group and hydrophobic tail group. The head group is water loving (i.e. hydrophilic) and the tail group is water repelling (i.e. hydrophobic) (Kimball's, 1981). In a cell, the head layer of phospholipid focuses outside of the cell is attracted towards the water in the environment and another layer of the head focus inside of the cell. The bilayer structure is formed by the combination of one hydrocarbon tail of one layer, which faces the other hydrocarbon tail of the other layer (Dua, et al., 2012). In the past 15 years, several liposomal drugs have been approved. Liposomal formulations are being used commercially for antifungal, anti-tumour and anti-bacterial agents (Samad et al., 2007).

1.3.1 Composition of liposomes

When phospholipids are exposed to water, they are self-assembled into double-layered aggregate bilayers shielding the hydrophobic portion (alkyl chains) from water, resulting in formation of liposomes. Phospholipids can be divided into synthetic phospholipids such as dipalmitoylphosphatidylcholine (DPPC) (Figure 1.3) and natural phospholipids such as soya phosphatidylcholines (SPC). There is no specific structure of soya phosphatidylcholine. It is a mixture of many molecules. The nature phospholipids can be found as different chain lengths and degree of unsaturation and plant-extracted phospholipid such as SPC contains high degree of polyunsaturation in the hydrocarbon chains (New, 1990a).

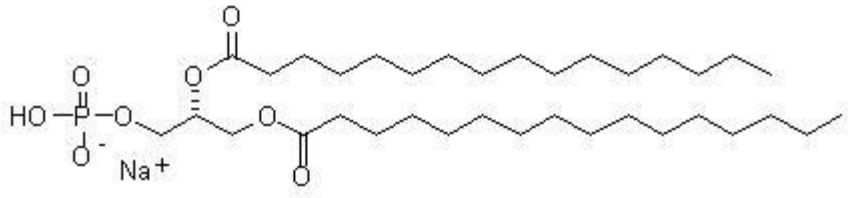

Figure 1.3: Chemical structure of Dipalmitoylphosphatidylcholine (DPPC)

1.3.2 Types of liposomes

Based on structural parameters such as size and number of phospholipids bilayers, liposomes are classified as small unilamellar vesicles, oligolamellar vesicles, multilamellar vesicles, large unilamellar vesicles, and multivesicular vesicles. Liposomes are found within a size range of about 50-500 nm, but can also be as large as 5µm (Arshady, 1999) (see Figure 1.4 and Table 1.1).

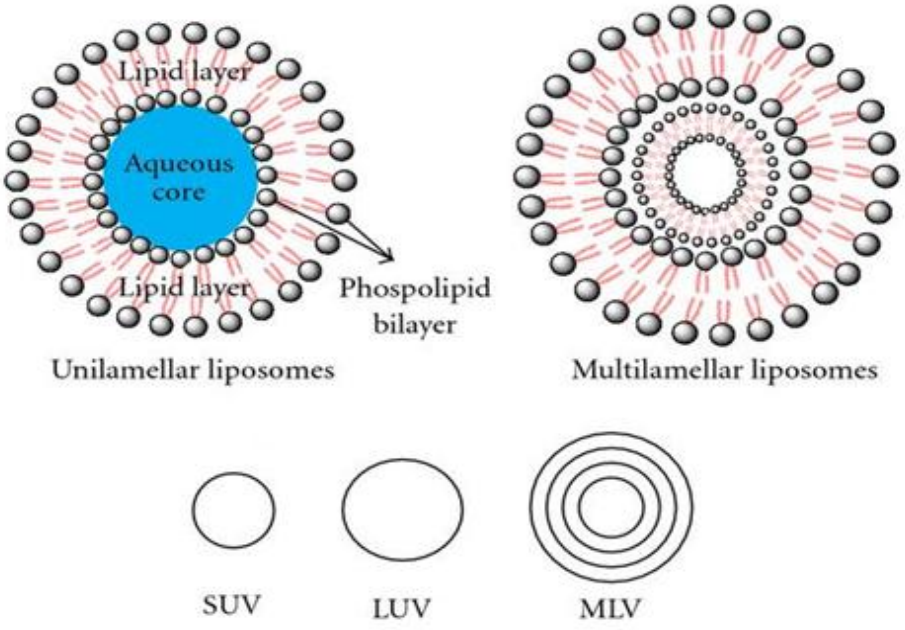

Figure 1.4: Schematic representation of basic structures and different types of liposomes. (Taken from http://www.hindawi.com/journals/jdd/2011/863734/fig1/)

Table 1.1: Types of liposome (Taken from: Dohert, 2004)

Types of vesicles	Abbreviation	Diameter	Number of lipid bilayers
Small unilamellar vesicles	SUVs	Around 100 nm	Single bilayers
Oligolamellar vesicles	OLVs	0.1 – 1 µm	Approximately 5 lipidic bilayers
Multilamellar vesicles	MLVs	500 – 5,000 nm	Concentric bilayers
Large unilamellar vesicles	LUVs	200 – 800 nm	Single bilayers
Multivesicular vesicles	MVVs	>1 µm	Multicompart-mental structure

1.3.3 General method of liposome preparation

There are many methods that can be used for preparation of liposomes. The most common and traditional method is the thin-film hydration method, also called hand shaking method or Bangham method (Szoka and Papahadjopoulos, 1980).

1.3.3.1 Thin film hydration for preparation of liposomes

Thin film hydration is the most common method for preparation of liposomes. For preparing liposomes, the lipids and cholesterol are first dissolved and mixed in an organic solvent to make a homogenous mixture. The solvent usually comprises either chloroform or chloroform: methanol (1:1 v/v). The major aim is to obtain a clear lipid solution for completely mixing the lipids. Once thoughly mixed, the organic solvent is removed using rotary evaporation to obtain a lipid film. Addition of aqueous phase with shaking generates a "milky" suspension of liposomes. This procedure has been described in many publications with minor modification in each depending on the properties needed for the liposomes following hydration. For instance, Elhissi and co-workers (2006) designed the vesicles to be ultradeformable by inclusion of surfactants in the lipid mixture prior to dissolution in the organic solvent. The prime limitation of the thin film hydration method is the low entrapment offered by the resultant vesicles and difficulty of generating liposomes on a large scale using this approach. However, at research level, this method is still widely used.

1.3.4 Advantages of liposomes

Liposomes may offer a range of advantages including enhancement of stability and manipulation of pharmacokinetics of the entrapped material. Drug degradation can be minimized by encapsulation of the drug in liposomes (Pinto-Alphandary et al., 2000). Previous research studies have demonstrated that liposomal formulations could enhance the targeting properties of the encapsulated drug and may also potentiate its therapeutic effect. Liposomes were first targeted for the treatment of intracellular pathogens (e.g. leishmaniasis and histoplasmosis) and life threatening systemic fungal infections (e.g. treatment of candidosis and aspergillosis by using amphotericin B-encapsulated liposomes (Ambisome®). Liposomal formulations may also be effective for antibacterial infections (Alder-Moore et al., 2002; Fielding et al., 1999). Intracellular pathogens such as *Salmonella*, *Mycobacterium*, and *Leishmania* located

in the phagocytic cells of reticuloendothelial system (RES) or outside the RES (e.g. lung tissue infected with *Klebsiella pneumonia*) show sensitivity towards entrapped antibiotics delivered by liposomes (Constant et al., 2006). In addition, liposomes have been extensively studied as carriers of anticancer drugs such as doxorubicin (Doxil®) opening a new horizon towards the role of nanotechnology in anticancer drug development (Huang et al, 2008).

1.3.5 Drawbacks of using liposomes

There is extensive research investigating the potential use of liposomes as drug carriers, and a range of liposomal formulations are used in clinical trials (Maurer et al., 2001; Immordino et al., 2006; Chen et al., 2010). However, one major disadvantage of liposomal formulations, which also contribute as major obstacles for the licensing of many liposome-based products are the instability of liposomes during storage. This is aggregated by the presence of aqueous phase which increases the rate of phospholipid hydrolysis (Kensil et al., 1981; Grit et al., 1989) and oxidation (Hunt et al., 1981). Liposomes can also be destabilized due to vesicle aggregation, leading to leakage of the entrapped material (Elhissi et al., 2010).

Another disadvantage of using liposomes is their fast elimination from the blood and the capture of liposomes by the cells of reticulo-endothelial system (Torchilin, 2005). Liposomes may be used for transdermal delivery of drug and could be employed in research as biological membrane models to predict how drugs and permeability enhancers interact with cells (El Magharby et al., 2008).

1.3.6 Applications of liposomes

Liposomes provide excellent advancement in dermal and/or transdermal bioavailability of drugs (Constant et al., 2006). As being a drug carrier, liposome may enhance drug permeability through biological membranes by facilitating the absorption of the drug through the percutaneous system (Constant et al., 2006).

Thus liposome-based formulations have been extensively studied for the following reasons:

1. Enhancing the uptake of antigens for topical immunization (e.g. tetanus toxin) (Gupta et al., 2010).

2. Oxygen species scavengers for the prevention of cutaneous photodamage (i.e. sodium ascorbyl phosphate) (Foco et al., 2005).

3. Enhancing the therapeutic effect of antihistaminic drug (i.e. cetirizine) via drug encapsulation (Elzainy et al., 2004).

4. For encapsulation of non-steroidal and steroidal anti-inflammatory substances (e.g. indometacin, hydrocortisone, dexamethasone) (Puglia et al., 2004; Cevc et al., 2004; Sinico et al., 2005).

5. For antitumoral and antiviral drugs (e.g. tamoxifen, methotrexate, idoxuridine, acyclovir) (Bhatia et al., 2004; Seth et al., 2004; Trotta et al., 2004; Seth et al., 2003).

6. For entrapment of immunosuppressive molecules (e.g. cyclosporin A), showing potential in the treatment of dermatological diseases characterized by an autoimmune T cell-mediated pathogenesis (Verma et al., 2004).

Contrasting with expert's previous research also shows that liposomes are not able to penetrate through deeper inside the skin (Mezei et al., 1980). As per rule, they are believed to be deformed and transformed as fragments (Touitou et al., 1994). Therefore, for chemical composition size, shape, and lamallarity are all relevant for formulation (Fresta et al., 1996). Phosphatidylcholine have multi-functioning properties for different applications. Formulations prepared from unsaturated phosphatidylcholines are preferred to support skin regeneration, prevent skin aging, prevent development of acene and enhance penetration of other active agents like vitamins and their derivatives into the skin. For skin protection against sunburns, nanoparticles and DMS (Derma Membrane Structure) are used but hydrogenated phosphatidylcholine may also be used (Fresta et al., 1996)

1.3.7 Lipids and Liposome Composition

An important parameter in liposomal properties is rigidity of the bilayers which is mainly influenced by lipid phase composition of the liposome, determining the liposomal properties including stability on storage and pharmacokinetic properties following *in vivo* administration (Chapman, 1975; Lee, 1975; Melchoir & Steim, 1976; Phillips, 1972). When vesicles are kept below the phase transition temperature (Tc) of the lipid phase they are considered to be 'solid' while when the preparation is stored above the phase transition temperature the vesicles are considered to be 'fluid' and exhibit higher permeability of the encapsulated drug. Phase transition temperature of a given phospholipid is a function of the acyl chain length and it increases with increasing the length of the acyl chain. The phospholipids which can carry the bulky side groups, unsaturated chains or branched chains e.g. cyclopropane ring exhibit considerable decrease in the Tc. The head groups of acidic phospholipids also have a considerable influence on the phase transition temperature. For example, phosphatidyserine, phosphatidyglycerol, and phosphatidic acid.

Another component to influence the bilayer fluidity and enhance liposome stability is cholesterol, which is a prominent component of eukaryotic biological membranes (Szoka & Papahadjopoulos, 1980). Cholesterol at high concentration may eliminate the phase transition temperature of the phospholipid, making drug release from the resultant liposomes less dependent on temperature (Tseng et al., 2007).

1.4 Transfersomes

In most of the published reports, it was observed that drugs applied on the skin tend to accumulate on the upper layer of the skin (Mezei and Gulasekharam, 1980; Mezei and Gulasekharam, 1982; Touitou et al., 1994; Fresta and Puglisi, 1996; Meidan et al., 1998a; Cheng and Chien, 1999). As reported by Mezei (1992), liposomes are not expected to penetrate through the stratum corneum of the skin. However, the vesicular transportation of the drug is still debatable (Barry, 2001). To overcome the difficulty of poor skin permeability, various approaches have been proposed including the use of transfersomes and ethosomes. Cevc's group in 1991 introduced 'transfersomes' which are the first generation of elastic vesicles (Figure 1.5).

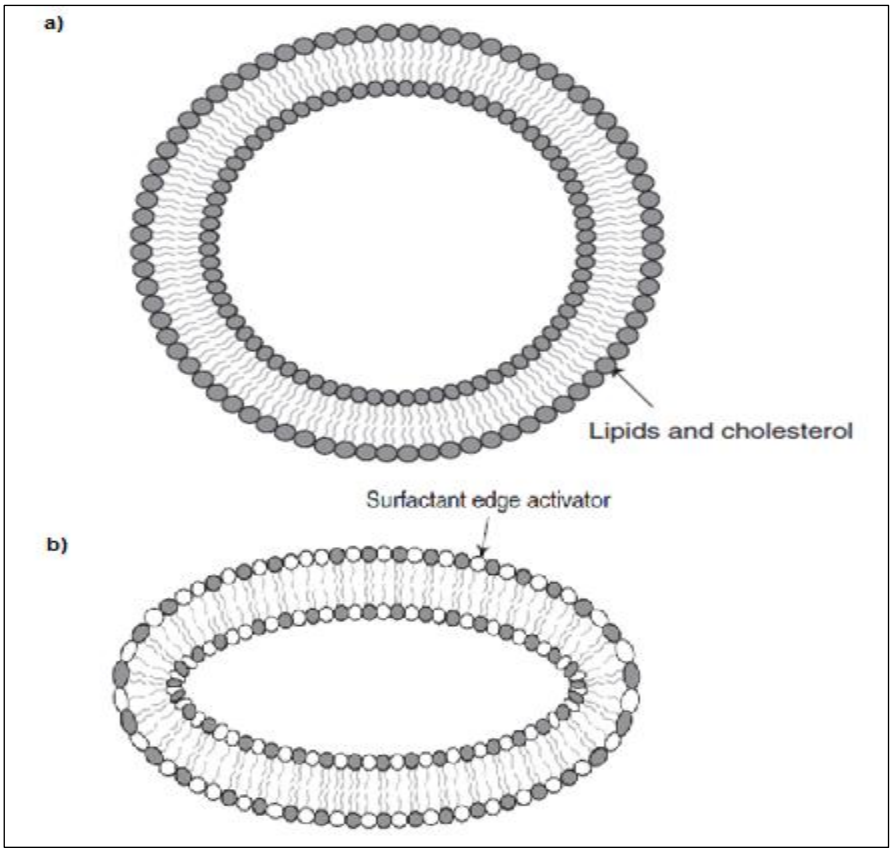

Figure 1.5: a) Rigid liposomes consisting of double chain lipids in the presence or absence of cholesterol and b) Transfersomes consisting of double chain lipids and an edge activator (Taken from Benson, 2006).

Transfersomes is a registered trademark by the German Company IDEA, AG. The name means the "carrying bodies", and is derived from the Latin word 'transferre', meaning 'to carry across', and the Greek word 'soma', for a 'body' (Prajapati et al., 2011). They are classified into phospholipid and detergent-based types (Choi & Maibach, 2005) and are prepared by phospholipids and edge activators. An edge activator is a single chain of surfactants with a high radius of curvature, which destabilises the lipidic bilayer of the vesicle and increases the deformability of the bilayer (Duangjit et al., 2011). It was claimed by the inventors of this type of vesicle that transfersomes are highly ultra-deformable and can squeeze through the stratum corneum and have less than one-tenth of the liposomal diameter. Thus, for sizes up to 200-300 nm they can penetrate through the intact skin (Fig 1.6) (Barry, 2001). When compared with subcutaneous administration, transfersomes improved *in-vitro* skin permeation of various drugs, penetrate the intact skin from *in-vivo* studies and

effectively transfer the therapeutic amount of drugs (Chang et al., 2007; Elsayed et al., 2006; Viriyaraj et al., 2009).

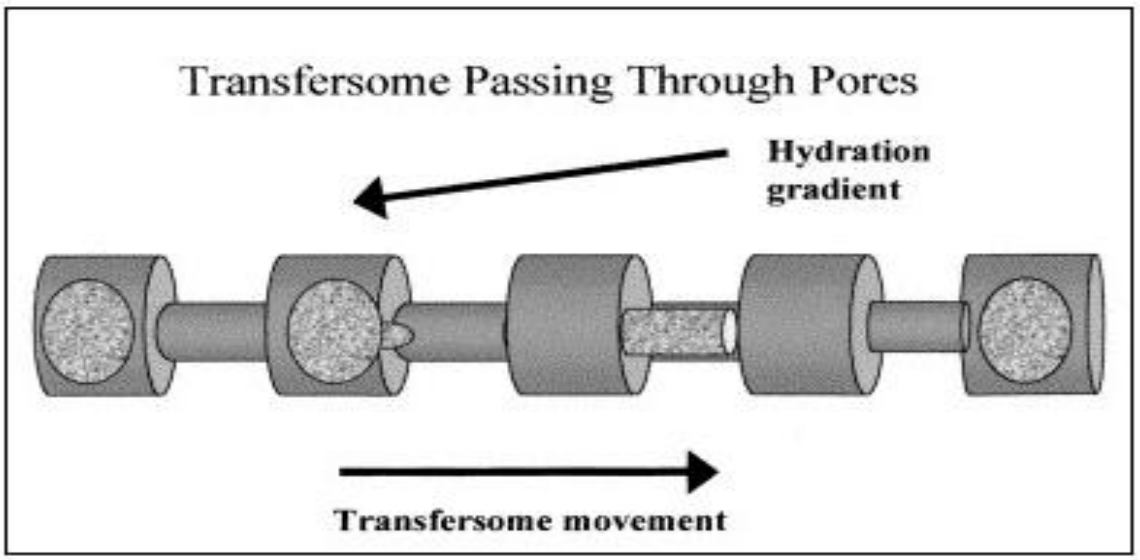

Figure 1.6: Ultra deformable transfersome squeezing through minute pores in the stratum corneum, driven by the water concentration gradient. The liposome with edge-activators thus penetrates from the horny layer surface (relatively dry) to the wet viable tissues (modified from Cevc et al., 1996) (Taken from Barry, 2001).

There are two important features to be considered. Firstly, in non-occlusive conditions, transfersomes require a hydration gradient to penetrate, driving the transfersomes through the horny layer in order to reach a depth of at least 30 μm (Aguiella et al., 1994). Secondly, transfersomes work best in *in-vivo* conditions, by virtue of their high and self-optimizing deformability (Choi & Maibach, 2005).

1.4.1 Functions of Transfersomes

Transfersomes can be described as lipid vesicles having deformability that facilitates their penetration through the pores of the skin even though the vesicle size is much larger than the pore size (Prajapati et al., 2011).

1. When transfersomes are applied to the skin, due to their deformability they exploit the hydrophilic pathways or ‘pores’ in-between the cells by widely opening the gaps in the SC layer and thus passing through together with its drug cargo without losing their integrity (Figure 1.7) (Prajapati et al., 2011).

2. Transferosomes are capable of transporting low or high molecular weight drugs, however, that is influenced by a range of formulation factors such as transferosomal composition, drug entrapment efficiency and the detailed application conditions such as pretreatment of vesicles, duration of skin exposure to the vesicles and the volume of formulation (Choi & Maibach, 2005).

3. In non-occlusive application, the flexibility of the transfersomes reduces the vesicular rupture in the skin and thus allows them to follow natural water gradient across the epidermis (Prajapati et al., 2011).

4. Transferosomes may protect the drug from degradation and provide an effective reservoir for sustained drug release (Cevc 1996).

5. Transferosomes can penetrate the SC layer either by intracellular or transcellular routes. Figure 1.7 shows the drug penetration across the human skin via these two routes (Schatzlein & Cevc, 1995; Panchagnula 1997).

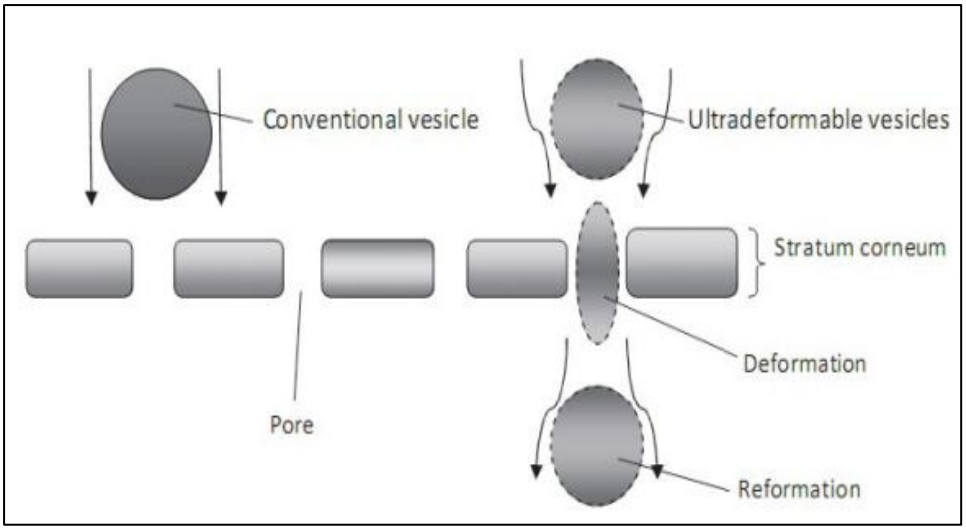

Figure 1.7: Schematic diagram showing the two microroutes of penetration in biological membranes (Taken from Prajapati et al., 2011).

1.4.2 Advantages of transfersomes

Transfersomes have advantages in the field of cosmetics and dermatology. It has been observed that transfersomes significantly improve the drug delivery via the transdermal route compared to conventional liposomes.

Transferosomes have been reported to be safe for application on skin and they may improve hydration of aged skin (Cevc et al., 2001).

1.4.3 Transfersomes versus other Carrier systems

Transfersomes can prolong the release and improve the pharmacological activity of drugs *in-vivo* (Jain et al., 2004). In this study, *in-vivo* performace of selected formulation was evaluated by using a carrageenan-induced rat paw edema model. It has been reported that transfersomes encapsulating dexamethasone can provide 82.3% inhibition of paw edema, compared to conventional liposomes ointments, which reduced the edema by around 38.3% and 25%, respectively. Similarly, triamcinolone acetonide, at a dose of 0.2 mg /cm^2 was able to suppress 75% of the edema in the ear of the rat over a period of 48 hours. In contrast, conventional formulation of this drug required almost a 10-fold increase in concentration to induce the same pharmacologic activity (Cevc & Blume, 2003). Other studies using transferosomes have reported similar results for Diclofenac where the therapeutic effect was prolonged and the concentration of the drug increased

almost by 10 times in tissues under the skin compared to commercial hydrogel formulations (Cevc & Blume, 2001).

In their studies, Paul et al (1998) showed that large molecules could be transferred through intact skin using transferosomes, resulting in successful immunization with a protein antigen. In that study, gap junction protein (GJP) were encapsulated in transfersomes and applied to the skin. The results showed that the specific antibody titers were higher than those elicited by subcutaneous injection of the protein using transfersomes, mixed micelles or conventional liposomes (Paul et al., 1998). Similarly, Cevc (2003) showed that the transdermal delivery of insulin by transfersomes through the skin was equivalent to insulin injected subcutaneously. In addition, Kim et al. (2004) investigated the effect of cationic transfersomes (containing DOTAP and Cholate) on transdermal delivery of DNA in mice. The results revealed that GFP (Green Fluorescent Protein) expression to be very high compared to that of free gene in PBS solution, and expression was detected in distant organs like liver and lungs. Thus, transfersomes may be developed further as non-invasive protein and gene delivery system (Cevc & Blume, 2003).

1.4.4 Mechanism of transfersome penetration through biological membranes

One mechanism of transferosomal penetration through the skin is by utilising its elastic nature, resulting in enhanced delivery of the encapsulated drug across the SC layer and subsequent drug release at the site of action (Honeywell-Nguyen & Bouwstra, 2003). Fang et al. (2001) investigated the mechanism of vesicular sytem across the skin with soybean PC liposomes containing enoxacin. Their results showed that after 12 hour of drug treatment, drug permeation across PC-treated skin was higher than that of nontreated skin. Similarly, Verma et al. (2004), using a model hydrophilic drug, demonstrated that PC liposomes carry both entrapped and unentrapped drug through the SC layer to the deeper layers of the skin. Transfersomes provided a penetration efficiency of 5-fluorouracil trasnfersomes through the SC of 13.5% when the drug entrapment efficiency was as low as 8.8% (El Maghraby et al., 2001).

However, it is agreed that higher drug entrapment in transferosomes is necessary to maximize the drug transportation through the SC (Honeywell-Nguyen et al.,

2003; Honeywell-Nguyen et al., 2003). The other proposed mechanism of enhanced penetration of transferosomes is attributed to the detergent-based elastic nature of the vesicles. Honeywell-Nguyen et al. (2003) proposed that detergent-based elastic vesicles can facililate drug transport by fast partitioning in the SC and do not penetrate into the deeper layers. There are four major steps in determining the effectiveness of the elastic vesicles. The first is about drug association with the vesicle bilayers, while the second is about the partitioning of vesicles into the SC. The third is the drug release from the vesicles once it is in the SC and finally, the fourth case is the portioning of the free drugs in the SC and subsequent delivery to the systemic circulation (Honeywell-Nguyen et al., 2003).

1.4.5 Preparation methods of transfersomes

The general method for preparation of transfersomes is the thin-film hydration technique described earlier. A thin film is prepared, hydrated and then the resultant vesicles are reduced to the desired size by sonication and homogenized via extrusion through polycarbonate membrane filters.

1.4.6 Application of Transfersomes

The following are the application of transfersomes for transdermal drug delivery.

1) **Diffusion of large molecules across the skin**

Due to higher molecular weight of some drugs (e.g. proteins), they are unable to cross through the skin and reside at the upper layer (SC) of the skin itself. In order to overcome this problem, transferosomes are non-invasive vehicles of large molecules for successful penetration across the skin. For example, transfersomes (transfersulin) encapsulating insulin can help to overcome the inconvenient subcutaneous administration of the drug (Planas et al., 1992).

Transfersomes are used as carriers for controlled release of interferon-α (INF-α) and interleukin-2 drug and for increasing the stability of drugs having immunomodulatory effects (Choi & Maibach, 2005). Transfersomes are widely used for carrying the proteins and peptides, as both of them are biogenic molecules due to which their transportation is difficult. When given via oral administration, the proteins and peptides are degraded in the gastointestinal GI tract whilst

transdermal penetration is not possible because of their large size. The bioavailablity obtained using transfersomes seems to be similar to that of subcutaneous injection of the same protein (Trotta et al, 2004; El Maghraby et al., 2000).

2) The topical delivery of vaccines for transdermal immunization

Due to their ultradeformable capability, transfersomes can be used to deliver vaccines transdermally following topical administration of vaccine in transferosome formulation. Trasnfersomes may entrap proteins such as integral membrane protein, human serum albumin and gap junction protein, hence, can be used for delivery of vaccines, offering avoidance of the painful injections. Moreover, higher IgA levels can be obtained with transferosomal formulations of vaccine, for instance, by transcutaneous delivery of hepatitis-B vaccine using transferosomes (Song & Kim, 2006).

When compared with subcutaneous injections, transfersome-based formulations of local anaesthetics showed similar permeation as subcutaneous injections demonstrated with studies employing lignocaine and tetracaine (Prajapati et al., 2011). In addition, the pain sensitivity is similar to subcutaneous bolus injections (80%) and the effect may last longer (Prajapati et al., 2011).

3) Transdermal deliveries for anti-cancer and herbal drug

Tansdermal delivery of the anti-cancer drug methotrexate via transfersomes showed ability to treat skin cancer. The presence of transfersomes within the skin structure may help for the provision of the nutrients and maintain the functions of the skin. Encapsulation of capsaicin provided better topical application compared to free capsaicin (Benson, 2006; Xiao-Ying et al., 2006).

1.4.7 Limitation of transferosomes

Transfersomes are chemically unstable because of their liability to oxidative degradation. Purity of natural phospholipids is another limitation against adoption of transfersomes as drug delivery vehicles. Also, transfersomal formulations are

expensive, owing to the high cost of some pure lipid ingredients used in formulation of these systems (Prajapati et al., 2011).

1.5 Ethosomes

Another specially designed vesicle able to allow transdermal delivery is ethosomes, which are non-invasive delivery carriers that allow the drug to reach the deeper skin layers and possibly the systemic circulation. They are a soft, malleable vesicles, which are actively, enhances the delivery of entrapped drugs. Ethosomes are composed mainly of phospholipids (e.g. phosphatidylcholine, phosphatidylserine, phosphatitic acid), high concentration of ethanol (20-45%) and water (Anitha et al., 2011). Touitou (2002) observed that ethosomes might enhance delivery of drug to the deep strata of the skin or to the systemic circulation (Anitha et al., 2011). Due to ethosomal high malleability, it may permeate through human skin and into the systemic blood circulation (Jain et al., 2004; Lopez-Pinto et al., 2005; Paolino et al., 2005; Touitou et al., 2000).

According to Perez-Cullell et al. (2000) fluid liposomes can delivered more fluorescence into stratum corneum than did rigid liposomes (Barry, 2001). Ethosomes are deformable liposomes containing higher amount of ethanol (up to 45%). It was proposed that alcohol fluidizes the ethosomal lipids and stratum corneum lipids, thus allowing the soft malleable ethosomes to penetrate (Ainbinder et al., 2005; Barry, 2002). The size range of ethosomes varies from tens of nanometers to micrometers (Anitha et al., 2011). The ethanol content may similar to surfactants as in case of transferosomes, conferring 10 times higher deformability of the vesicles (Godin et al., 2003; Jain et al., 2004).

1.5.1 Mechanism of permeation enhancement using ethosomes

As described previously by Touitou (2002), the exact mode of permeation enhancement using ethosomes is unclear. However, it is believed that the higher concentration of ethanol in ethosomes disrupts the lipid bilayer in the SC making it more permeable and thus giving the ethosomes a higher ability to squeeze through the small opening in the SC lipid (Touitou, 2001).

Substances that reversibly reduce the barrier resistance of the SC, such as ethanol, are commonly described as permeation enhancers (Anitha et al., 2011). Ethanol can interact with the polar head group of the lipid molecules, resulting in reduction of the melting point of the stratum corneum lipid, thereby increasing lipid fluidity and cell membrane permeability (Touitou et al., 1999). However, due to the interdigitation ethanol causes to the lipid bilayers, it was commonly believed that vesicles couldnot coexist with high concentrations of ethanol. Currently, ethanol might be used in relatively low concentrations in liposome formulations (Touitou, et al., 2000). Ethanol is a volatile solvent which can be easily evaporated at skin temperature (Anitha et al., 2011), leaving the supersaturated concentration of the drug in formulation. This may influence the drug flux across the membrane. Also, ethanol may alter the solubility of the SC, facilitating drug delivery (Verma, 2004).

In current studies, ethanol is found in low concentration in liposomal formulations; 7-10% for transfersomes. However, higher ethanol concentrations are used in proniosome formulations (30-50%) (Fang et al., 2001; Vora et al., 1998). Figure 1.8 shows how ethosomes may enhance penetration of drugs through lipid bilayers.

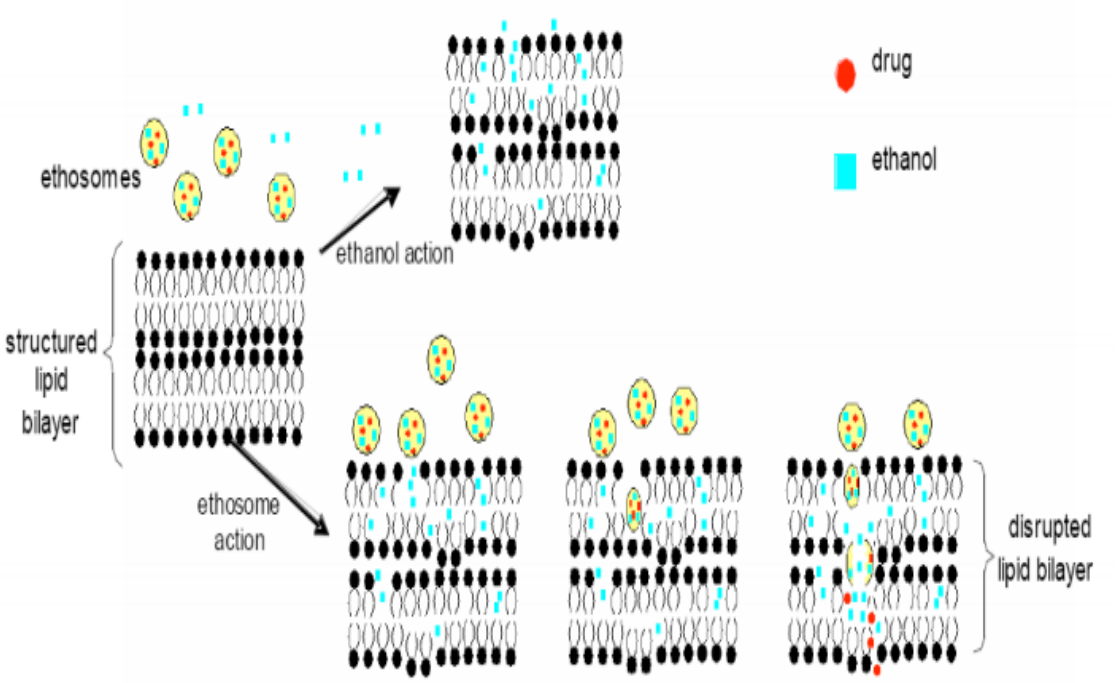

Figure 1.8: Diagrams showing the mode of drug penetration through lipid bilayers using ethosomes (Taken from Anitha et al., 2011).

1.5.2 Composition and preparation of ethosomes

Ethosomes are vesicular carriers composed of hydro-alcoholic or hydro/alcoholic/glycolic phospholipid in which the concentration of alcohol(s) is relatively very high (Friend, 1988).

Ethosomes can be prepared using the conventional thin film hydration technique (mentioned in chapter 2) or by addition of aqueous phase in a controlled manner to the alcoholic solution of phosphatidylcholine.

A. **Cold method** - In this method, phospholipids, drugs and other lipid materials are dissolved in ethanol in a covered vessel at room temperature by vigorous stirring with the use of mixer. Either Propylene glycol or other polyol are added while stirring (Jain, 2004). The mixture is heated to 30 $^{\circ}$C in a water bath. In a separate vessel the water is heated to 30 $^{\circ}$C and added to the mixture followed by stirring for 5 mins (Touitou, 2000a). The size of the ethosomal formuation can be decreased using either by sonication or extrusion and the formulation is then finally stored in the refrigerator (Manosroi, 2009).

B. Hot method – In this method, phospholipids are dispersed in water by heating in a water bath (40 °C) until a colloidal suspension is obtained (Jain, 2004). In a separate vessel, ethanol and propylene glycol are mixed and heated to 40 °C and once it reaches the desire temperature the organic phase is added to the aqueous one (Bhalaria, 2009). Depending on the drug hydrophilic/hydrophobic properties, it is either dissolved in water or ethanol solvent. The size of the ethosomal formulation can be decreased using probe sonication or extrusion (Touitou, 1996).

1.5.3 Advantages of ethosomes

In comparison to complicated methods such as iontophoresis and phonophoresis, ethosomes may be more capable of delivering large molecules such as peptides and protein molecules. It offers low risk and high patient compliance when it is administrated as semi-solid formulation (gel or cream). Ethosomes also have applications in pharmaceutical, veterinary, and cosmetic fields (Gangwar, 2010).

1.5.4 Ethosomes vs other carrier system

Ethosomes are similar in their structure to liposomes only difference in composition. Liposomes composed of phosphatidylcholine and cholesterol whereas ethosomes are made of high concentration of ethanol (Chourasia et al., 2011). The value of ethosomes lies in their capability of increasing the transdermal permeation of the entrapped material compared to liposomes or drug solutions of mixture containing ethanol and water (Dubey et al., 2007; Godin et al., 2003; Touitou et al., 2001).

The efficacy of ethosomes by increasing the entrapped drug for transdermal delivery is similar to that of transfersomes. In a study conducted by Elsayed et al. (2006), they reported that ethosomes have increased the transdermal permeability of the entrapped drug ketotifen when compared with trasnfersomes (Chourasia et al., 2011).

Godin et al. (2004) indicated differences in the data and they explained that existence of osmotic gradient across the skin was not important contrasting from

the data that was observed with elastic vesicles where permeation enhancement occurred only in non-occlusive conditions (Choi & Maibach, 2005).

Addition of ethanol in ethosomes can provide high flexibility of the vesicles to squeeze through the skin pores that are much smaller in diameter than the vesicles. Thus, ethosomes are much more efficient in delivery of the drug in terms of quantity and depth when compared to conventional liposomes that tend to accumulate at the upper layer of the skin due to the stratum corneum barrier (Touitou et al., 2001). In other research articles, it has been observed that due to the presence of ethanol, ethosomes provide good storage stability of the vesicles (Dubey et al., 2007).

1.5.5 Therapeutics application of ethosomes

Ethosomes are mainly used for transdermal route of drug delivery of various molecules. When tested, ethosomal preparations are used as replacement for liposomal preparation as they are much more effective permeation enhancers. Ethosomal carriers are used for delivery of hydrophilic and impermeable drugs. (Akiladevi & Basak, 2010).

1.6 Vesicle characterisation

The characterisation of the vesicular formulation was performed using the following techniques:-

1.6.1 Lipid extrusion of vesicles

Lipid extrusion is a technique in which multi-lamellar liposomes are forced through filters with defined pore sizes in order to optimise the size and size distribution of the vesicles. This technique is very popular for producing unilaminar vesicles (Hope et al., 1993). Extrusion of MLVs through polycarbonate membrane filters may lead to formation of a homogeneous population of LUVs (40-150 nm size range) (Hope et al., 1993).

1.6.2 Transmission Electron Microscope (TEM)

Transmission electron microscope (TEM) applies the same principle as light microscope, but uses electron in place of light. The wavelength of the light in light

microscope is the reason for poor resolution, whereas TEM uses “electrons” as a “light source” which has much less wavelength compared to light microscope and provides 1,000 times better resolution. Due to its high magnification effect, TEM can be used in various research fields such as in medical, biological and material science (Stadtländer, 2007). TEM can magnify the specimen within a range of 500-500,000X (Flegler et al., 1993).

In TEM the electron are projected through an ultrathin slice of the specimen and produces two-dimensional images.

1.6.3 Sonication for size reduction of lipid particles

Sonication is a commonly used technique for typically producing SUVs having size range of 15-50 nm. The most commonly used instruments are probe sonicator and bath sonicator for successfully producing SUVs (Dau, 2012). Sonication could be easier and less time consuming than extrusion for generation of small vesicles. Bath sonicators are used for homogenisation of the suspension which is done through the walls of the plastic/ glass tube where sample is present. Probe sonicators employ a tip (probe) that applies high ultrasound energy on the liposomal suspension, causing disruption of the lipidic bilayers (Dau 2012). The excessive heat generated during probe sonication may cause lipid degradation and contamination with the metal leaching from the probe (Hope et al., 1993). Due to these disadvantages bath sonicators might be preferred for SUV preparation, however, bath sonication is time-consuming (Dau, 2012).

1.6.4 Particle size analyser

The measurement procedure based on the fact that on modern laser diffraction device (LD) spatial distribution of scattered light is a function of the particle size of the analysed sample. The phenomena of light diffraction on particles can be vividly presented. As the stone hits the water surface, it forms various concentric rings of waves. The waves near the site of impact create highly intensive waves while the intensity decreases as moving away. Higher waves are created by larger stones and smaller waves are created by smaller stones. Similar phenomena take place for particles when illuminated. The LD measures the intensity of different rings and distance between the illuminating particles. The phenomena occurring

on illuminating particles are called light scattering wherein the scattering of the light is emitted in all directions. The spatial distribution of scattered light is commonly called the scattering pattern of particles. The scattering of the particles depend on the ratio of particle diameter (D) and the wave length of the incident light (λ). Based on D/ λ ratio the scattering pattern distinguished among the Fraunhofer, Mie and Rayleigh scattering (Stojanović & Marković, 2012).

When the particle size is 5-6 times larger than λ then it forms Fraunhofer scattering pattern. When the particle size is less than λ (e.g 10 times) then it forms Rayleigh scattering pattern and when the D/ λ ratio is around one it forms Mie's scattering (Stojanović & Marković, 2012).

In practice, the wavelength of the light source devices range from 633 to 900 nm (Keck et al., 2008) so that only particles larger than 4.5 µm can be analysed by Fraunhofer's approximation, and smaller by Mie's theory. Smaller particles scatters light of a lower intensity to larger angles, while the larger particles scattered light of relatively stronger intensity toward smaller angles. Since the intensity of scattered light to the front decreases with decreasing particle size, thereby sensitivity of the device is reduced and there is a limit of detection of particles below a certain size. However Mastersizer 2000 is limited for particle size detection from 0.02 - 2000 µm. The intensity of scattered light can be measured as laser beam passes through a dispersed particulate sample. The data is then analysed to calculate the size of the particles that created the scattering pattern (Mastersizer 2000 user manual, 2007). The machine analyse the particle size based on Fraunhofer and Mie's theories (Stojanović & Marković, 2012).

The dispersed sample particle passes through the focused beam of light and scattered the light at characteristic spatial angles. The Mastersizer 2000 uses two sources of light, one light source: HeNe laser which is a source of red light of wavelength of 633 nm placed in the axis of the instrument and the other light source is emitted by LED which is blue light of wavelength of 455 nm. In addition to the conventional detector for small angels in the form of concentric rings located in front of the measuring zone in the axis of the device, there are additional side detectors for the light scattered at angles smaller and greater than 90º

respectively. The Fourier lens is placed behind the measuring zone. There is also a filter separating the vertical and horizontal polarized light. After completion of the measurements the raw data of the software analysed using defined optical parameters. The standard data shows in LD measurements are marked as d (0.1), d (0.9) and d (0.5). D (v, 50) is the mass median diameter (marked by lowercase v) of the volume distribution. It is expressed in microns and indicated that 50% of the sample has a size smaller than that value, whereas 50% have a larger size. D (v, 0.1) indicates that 10% of the sample has a size smaller than that value, whereas D (v, 0.9) indicates that 10% are larger in size (Stojanović & Marković, 2012).

1.6.5 Zeta potential analyser

Zeta potential analyse the surface charge of the particle in a colloidal solutions. Nano particles have a surface charge which attracts the thin layer of ions of opposite charges to its surface. This double layer of particle travel with nano particle as it diffuses throughout the solutions (Zeta potential manual).

The particle surrounded by liquid exists as two parts: inner region which is called Stern layer and outer layer consisting of diffuse region were they bound less firmly. Within the diffuse layer consist of notional boundary wherein ions and particles form stable entity. When the particle moves the ions also moves within these boundaries and the ions beyond this boundary stay within the bulk dispersant medium. The potential at this boundary is called zeta potential. The magnitude of zeta potential gives stability to the colloidal system. If the particles in the suspensions possess large positive or negative charge, then all the particles will be moving away from each other as they tend to repel each other as their will be no tenderacy for the particles to come together. However particle possessing lower zeta potential then no force can prevent them to come close to each other. The general dividing line for knowing the particle stability and instability are either +30 mV or -30mV i.e. particles which are more positive than +30 mV and more negative than -30mV are considered to be more stable. However, particles possessing different densities compare to dispersant medium are more prone to get sediment (Malvern Tec Note).

1.7 Chlorhexidine

As shown in Figure 1.9, chlorhexidine is an antimicrobial agent having a chemical formula of 1,1′- hexamethylene-bis-5-(4-chlorophenyl) biguanidide or 1:6 di (4-chlorophenyl-diguanido) hexane is a symmetric molecule with two ionizable guanidide moieties (Zeng et al., 2009).

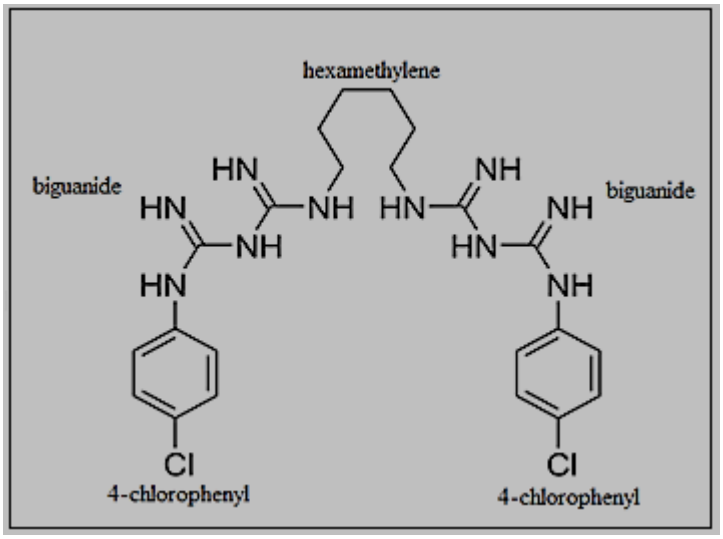

Figure 1.9: Chemical structure of Chlorhexidine [1:6 di (4-chlorophenyl-diguanido) hexane] (Taken from Lim and Kam, 2008)

Chlorhexidine (CHX) has a pKa value of 2.2 and 10.3 which makes it dicationic on a wide range of physiological pH values (Nerurkar et al., 1995). Its molecular weight is 505.45 g. It is an antimicrobial agent which is effective against both gram positive and gram negative bacteria, but with higher activity against gram negative bacteria owing to its negatively charged peptidoglycan layer cell wall to which the cationic granidium groups of CHX drug are believed to bind, acting as both bacteriostatic and bactericidal (Zeng et al., 2009).

Clinically, an amount of 2% of CHX in aqueous solution is used as antibacterial agent for the treatment of root canals (Zebnder, 2006). CHX is mostly used in its salt form such as CHX diacetate, dihydrochloride or digluconate as the preferred use of the salt form of the drug is attributed to its free base form being very insoluble in water. Saturated solutions of CHX diacetate (CHX-Ac_2) and dihydrochloride ($CHXCl_2$) have 2% and 0.2 % of CHX concentration respectively (Nerurkar, et al., 1995). The digluconate salt is clinically rised as diluted solution (2%) and available at much higher concentration of 20% (w/v) (Gjermo, 1989).

CHX is used as antimicrobial agent in endodontic therapy (Dolby, et al., 1972; Gjermo, 1989; Senior, 1976; Wang and Peng, 2006). CHX drug was developed as antiseptic agent by imperial Chemical industries, (Manchester, UK) in 1950s (Davies et al., 1954). In medical practices, it is widely use as antiseptic agent and an ingredient in personal hygiene formulations such as in mouthwashes and toothpastes (Denton, 2000).

1.7.1 Chemistry and physical characteristics of CHX

CHX solutions are colourless, odorless, and have an extremely bitter taste (Denton, 2000). The N- chlorinated derivatives of CHX when used topically, it covalently binds with the proteins of skin mucosa resulting in persisting anti-microbial effect with limited systemic absorption, even after oral ingestion (Boyce et al., 2002; Rushton, 1977; Cowen et al., 1979).

1.7.2 CHX mechanism of action

CHX disrupts the bacterial cell wall by adsorbing itself to the phosphate containing protein components. At lower concentration, it has bacteriostatic effect as it penetrates inside the bacterial cytoplasmic membranes causing leakage of cytoplasmic components. At higher concentration of CHX, it has bactericidal effect by forming irreversible precipitates with the intracellular adenosine triphosphate and nucleic acids after entering the cytoplasm via the damaged cytoplasmic membrane (Denton, 2000) (Figure 1.10).

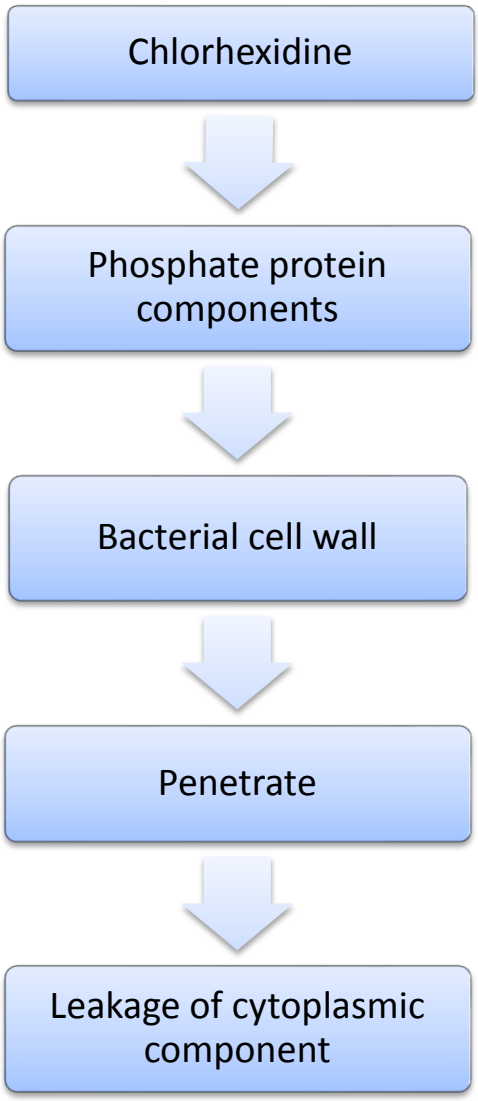

Figure 1.10: A flow diagram showing the action of CHX on bacterial cell wall at Bacteristatic condition.

CHX has bacteriostatic, bactericidal, fungicidal, fungisatic and antiviral properties (Davies et al., 1954; Denton, 2000). Longer exposure of CHX can increase the bactericidal effect against a wide range of bacteria (Figure 1.11). However, CHX has minimum effect against acid-fast bacilli or heat-resistant bacterial spores.

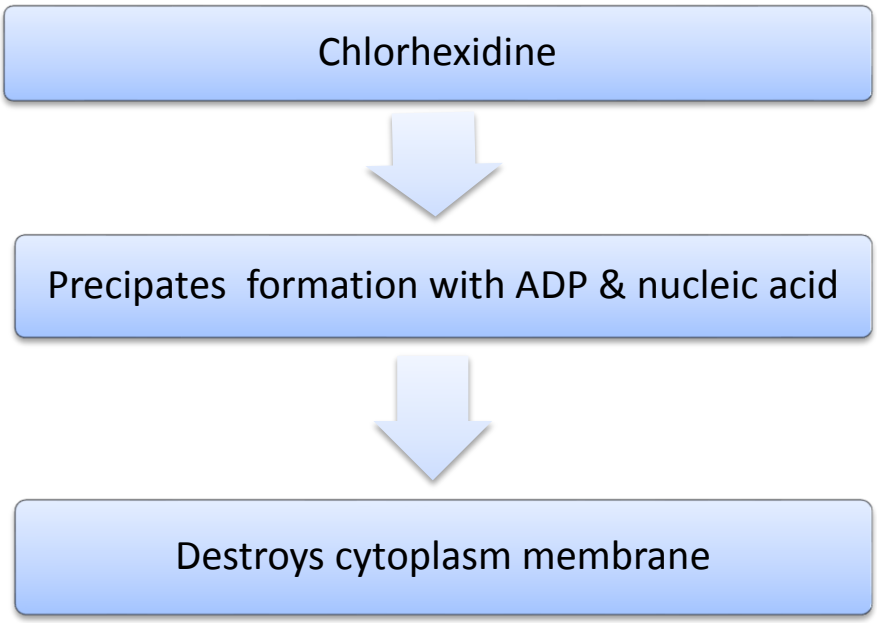

Figure 1.11: A flow diagram showing the action of CHX on bacterial cell wall at bactericidal condition.

1.7.3 Application of CHX drug

1) Oral antiseptic

CHX is widely used in mouthwash solutions, dental gels and toothpastes. It exerts bacteriostatic action when used in mouthwash solutions, were in it binds electrostatically to the oral mucosal surfaces, hence inhibiting dental plaque formation (Gjermo, 1989; Bral et al., 1988).

2) Hand disinfectant

The temporary habitation of hand flora (*S. aureus* and gram negative bacilli) on the superficial skin layers are associated with nosocomial infection and existence of resident flora (coagulase negative *S. epidermidis*) within the deeper skin layers are less likely to be pathogenic (Boyce et al., 2002).

CHX solutions are used as surgical hand disinfectants (Mulberry et al., 2001; Pereira et al., 1997; Larson et al., 2001).

The efficiency of skin antiseptic are measured in terms of log reduction of bacterial counts, where 1-log represents 10 fold decrease in bacterial counts (i.e. 90% bacterial elimination) and 20-fold decrease meaning 99% bacterial elimination. Although presence of total bacterial count does not consider the pathogenicity of bacterial remaining. However, the pathogenic bacteria (*S. aureus*) present onto the superficial skin of the hand are removed by hand washing. As suggested by US FDA, 1-log reduction of bacterial count at 1 min, 2-log reduction at 5 mins and 3-log reduction at 10 mins (Boyce et al., 2002).

The most effective antiseptic agents are alcohols followed by CHX and then povidone iodine. However, residual antimicrobial activity is fastest for CHX drug. Formulations containing both CHX and alcohol are most antimicrobial effective (Gjermo, 1989).

Newer handwash regimens contain CHX and alcohol hand rubs and they provide greater reduction in bacterial count compared to traditional methods of using surgical hand antiseptisis involving brush and sponge with either CHX gluconate (4%) or povidone iodine (10%) (Hibbard et al., 2002).

In a non-blinded trial observation, it was found that instead of using CHX (2%) and isopropyl alcohol (70%), the combined use of CHX and isopropyl alcohol achieved highest antibacterial count reduction and statically showed marked antimicrobial activity (Hibbard et al., 2002). Overall, the data show that CHX is a better antiseptic handwash and surgical skin preparation compared to iodine (Lim & Kam, 2008).

3) Venepuncture and vascular access

CHX mixture with alcohol provided superior antseptic for skin preparations prior to venepuncture and vascular access (Lim & Kam, 2008). Other benefits include shorter onset of action and long lasting antimicrobial activity in presence of pretentious material such as blood (Mimoz et al., 1999). A meta-analysis of 8 randomized trials, alcoholic CHX showed 50% risk reduction in catheter related bloodstream compared with povidone iodine. The risk reduction is due to the prolonged anti-microbial activity of CHX and its efficiency in the presence of

blood (Chaiyakunapruk et al., 2002). A 2% alcoholic CHX is used for skin antisepsis as per the guidelines for the preparation of intravascular catheter-related infectious disease (Baer, 2006).

4) Central venous catheters (CVCs)

CHX-silver sulfadiazine-treated CVCs may reduce the bacterial colonization. CHX disrupts the bacterial cell membrane which influx the silver ions into the bacterial cell where they bind to the DNA helix and impair bacterial replication (Mermel, 2001; Pearson et al., 1997; Logghe et al., 1997). The antimicrobial substances are dissolved in the surrounding tissues by leaching out the catheter and prohibiting the bacterial colonization and bacterial growth (Pearson & Abrutyn, 1997; Logghe et al., 1997; Tennenberg et al., 1997; Maki et al., 1997; Heard et al., 1998).

5) Placement of epidural catheters

In order to reduce the risk of epidural infections for skin, antiseptic solutions are used prior to the insertion of epidural catheters. It is suggested that bacterial colonization leading to epidural infection can be reduced along the epidural catheter. In a random controlled trial, the efficacy of chlorhexidine was tested against the controlled trial in which significant reduction of bacterial colonisation was observed along the catheter tips compared to control drug (Lim and Kam, 2008).

1.7.4 Adverse Effects of Chlorhexidine

In dental care products of chlorhexidine, adverse effects such as staining teeth, salivary calculus formation and transient dulling of taste sensation are rarely reported (Rushton, 1977; Gjermo, 1989).

1.8 Alcohol antiseptics

It has been observed that ethyl alcohol (ethanol) and isopropyl alcohol (propanol) (60-90% solution) have recently gained acceptance as hand antiseptics. Even though both of them have the germicidal characterstics, with better bactericidal

properties than bacteriostatic properties against vegetative forms of bacteria (Gram + and Gram -). These alcohols can also act as tuberculocidal, fungicidal, and virucidal against enveloped viruses but not effective against bacterial spores and nonenveloped viruses. The optimum bactericidal activity is in the range of 60-90% solution in water (v/v) and their bactericidal activity drops sharply when diluted to a concentration below 50% (Morton, 1950; Ali et al., 2001).

Previous studies have shown that at concentration of 60-80%, ethyl alcohol is a potent virucidal agent for lipophilic viruses (e.g. herpes, vaccinia, and influenza virus) and many hydrophilic viruses (e.g. adenovirus, enterovirus, rhinovirus, and rotaviruses but not hepatitis A virus (HAV) or poliovirus) (Tyler, et al. 1987, Kurtz et al., 1980). On the other hand, isopropyl alcohol is not active against non-lipid enteroviruses but fully active against lipid viruses. Moreover, isopropyl alcohol is slightly more bactericidal than ethyl alcohol for *E. coli* and *S. aureus* bacteria (Coultthard & Sykes, 1936). Isopropyl alcohol (20%) is effective in killing the cysts of *Acanthamoeba culbertsoni* (560) as chlorhexidine, hydrogen peroxide, and thimerosal (Turner et al., 1999).

1.9 Experimental plan

The experiments were performed by preparing control formulation (Liposomes, Ethosomes and Transfersomes) and ultraflexible nanosized lipidic formulation by thin film hydration either with or without the drug chlorhexidine. This was followed by performing antimicrobiological testing using disk diffusion tests on *S. aureus* grown agar plates in order to check the sensitivity of the bacteria using these formulations (as explained in Table 1.2).

Table 1.2: The table below shows the summarized experimental plan of the study present

Control formulation	**Drug (CHX)**		**Anti-microbiology Testing**
	With	**Without**	**Agar-Disk diffusion test**
Liposomes	+	-	*Staphylococcus aureus*
Ethosomes	+	-	
Transfersomes	+	-	
	+	-	
Special formulation			
Ultraflexible-nanosized lipidic formulation (Propanosomes)	+	-	

1.10 Agar-disk diffusion test

Agar disk diffusion test is one of the commonly used methods to check the bacterial susceptibility. Small filter paper disks (6mm) were impregnated with standard amount of antibiotic and placed on to the agar plate on which the bacteria have been swabbed. These plates were then incubated for overnight and the zone of inhibition was measured. The high susceptibility of the bacteria was indicated by larger zone of inhibition, while resistance was indicated by small or no zone of inhibition. Figure 1.12 shows a typical example of agar disk diffusion test.

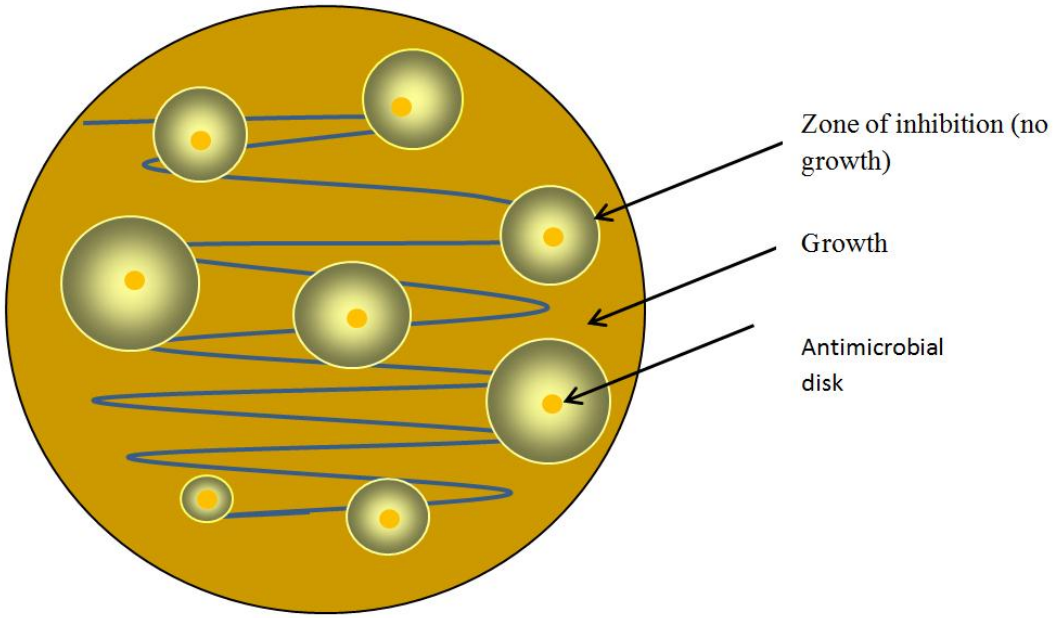

Figure 1.12: An example of plate showing disk diffusion study. The impregnated disk showing varying degrees of zone of inhibition. The results can be by measuring the diameter of each zone of inhibition.

1.11 Working Hypothesis

Anti-bacterial formulation with better skin penetration can be delivered as anti-bacterial drug to either act as bactericidal or bacteriostatic on deep routed *S. aureus* to treat skin infection. This project tests this hypothesis by preparing antibacterial formulations to stop the growth of the bacteria.

1.12 Main aim

The main aim of this project was to prepare anti-bacterial formulation propanosomes for the delivery of the drug to stop the growth of the bacteria.

1.13 Specific aims of Research

1. To prepare the control vesicle formulation: namely liposomes, ethosomes, transfersomes and optimize its preparatory protocol using thin-film hydration method.

2. To perform the characterization techniques including size analysis, zeta potential measurement and evaluation of the degree of deformability of the vesicles.
3. To design and optimize the novel vesicle formulations of propanosomes.
4. To perform the anti-bacterial testing using disk diffusion test.
5. To test the antibacterial property of the control vesicle formulation and propanosomes.
6. To investigate the antibacterial property of chlorhexidine for suppressing the growth of the bacteria using propanosome formulations.
7. To analyse the data by comparing and contrasting the antibacterial properties of the vesicles carrying with/without drug on bacteria.
8. To analyse the data and write up the MSc thesis.

1.14 Elements of originality

The novelity of the project is based mainly on the preparation of anti-bacterial ultradeformable lipidic vesicles. This is the new concept of the project as there is no published research for the use of antibacterial propanosomal formulations for the skin treatment. Moreover, this formulation not only provides the anti-bacterial activity but also deliver drug chlorhexidine permeating through the skin for killing *S. aureus*. This project has contributed towards the application of nanotechnology and it has broadened the scope of liposome applications for treatment of skin infections.

2 Materials and Methods

2.1 Materials

Soya phosphatidylcholine (SPC; Lipoid S-100) and 1, 2–Dipalmitoyl–sn–glycerol-3-phodphocholine (DPPC) were purchased from Lipoid GmbH, Switzerland. Cholestrol, Tween 80 solution, Span 80, Sodium cholate hydrate, Chlorhexidine base, Chlorhexidine diacetate salt hydrate, PBS (Phosphate buffered saline) tablets were supplied by Sigma Aldrich, UK. Chloroform (99+%) stabilised with 0.75% ethanol, Propan-2-ol (Iso-propyl alcohol), methanol (HPLC grade) and ethanol 99+% (GLC absolute grade) were all supplied by Fisher Scientific, UK.

Nutrient agar: 90 mm ready poured plate Oxoid from (Fisher Scientific, UK), autoclaved disks, autoclaved 20 microliter pipette tips, *Staphylococcus aureus* (NCIMB6571) culture medium from Microbiology laboratory UCLAN, Spreader [inoculating, L-shape, sterile polystyrene inner packs of 5 (80 bags)] were all obtained from Fisher Scientific Ltd, UK. The autoclaved distilled water for dissolving was obtained from UCLAN research laboratories, chlorhexidine diacetate salt was supplied by Sigma Aldrich, UK.

2.1.1 Equipments

Malvern Mastersizer 2000 and Malvern Zetasizer (Nano series (Nano 2S) were used for size analysis as described in Section 2.1.2 and 2.1.3. The technique of lipid extrusion and size reduction of the lipidic particles was also discussed, Büchi rotavapor from Büchi, Switzerland, bath sonicator (Model SC-20) from Fisher Scientific, UK, sterile cupboard (Uni Mat-BS class II Microbiological safety), vortex mixer from Stuart®, weighing balance, 2, 20, 200 and 1000 µl pipettes (gilson) and pipette tips, forceps, aluminium foil, 10 ml vials, 100 ml (round bottom short neck socket 24/29 100 ml) flasks, 5 ml volumetric flasks, sterile spatula, 10 ml and 5 ml beakers, pipette adaptors were all obtained from Fisher Scientific Ltd, UK and CM 120 Bio Twin transmission electron microscope (Philips Electron Optics BV, The Netherlands).

For the preparation of the lipidic particles, Lipofast Basic and stabiliser (Avestin,Inc), Nuclepore polycarbonate Track-Etch Membrane filters with a pore size of 12.0, 10.0 , 5.0, 1.0, 2.0, 0.8, 0.2, 0.4 µm were purchased from Fisher Scientific, UK.

2.1.2 Malvern Mastersizer 2000 particle size analyser

Malvern mastersizer 2000 (Model number: APA2000) was used to measure the particle size (0.02 - 2000 µm). This model analysed the samples based on two theories, firstly by Fraunhofer model (Mastersizer user Manual, 2007) stated that a scattering pattern was created when a solid, opaque disc of known size was passed through beam of light. This model did satisfy few particles but was not meant for all particles as very few particles were in disc shaped and others were transparent. Hence, the instrument also employed Mie theory which assumed that the amount of light passed through a particle was directly proportional to the light passed through or directly absorbed (Mastersizer 2000 user manual, 2007).

2.1.3 Zeta potential using Malvern Zetasizer Nano 2S

Malvern Zeta Nano (Nano series; Nano 2S) measured the particle zeta potential, size and molecular weight. The system also had the ability to perform autotitration measurements and trend measurements including the determination of protein melting point. Zeta potential was measured by determination of how fast the particles could move in liquid when an electrical field was applied i.e. velocity. This was relevant for the investigation when a certain range of particle size was desirable. (Zeta size user Mannual, 2007). The Zeta size analyser was used to check the zeta potential (mV) of the formulations by pipetting 800 µl of the sample into the zeta sizer cuvette which was then loaded into the machine. The system was allowed to calibrate for 2 minutes and the results were recorded in triplicates with 10 measurements for each experiment.

2.1.4 Preparation of thin lipid films using rotary evaporation

The rotary evaporator was used to remove the solvents from the mixture of solvents from the flask which was attached to the condenser under reduced pressure. The machine utilized the lower pressure than the atmospheric pressure which allowed the solvents to evaporate at a lower temperature. Furthermore, the rotation increased the surface area and therefore evaporation was facilitated.

2.1.5 Bath sonication

Bath sonicator depended on applying ultrasound energy on the sample, possibly resulting in size reduction of particles. The bath sonicator was operated as follows:-

The plan object was placed onto the secondary holding container. The cleaning solution was poured into the secondary container in order to completely submerge the object.

1) The secondary holding container was placed into the empty ultrasonic cleaner. The deionized water was poured into the ultrasonic cleaner until the water was half way up the side of the secondary container.
2) The desired time was set up on the ultrasonic bath.

2.1.6 Transmission Electron Microscopy (TEM)

The suspension sample was deposited on carbon-coated copper grids (400 mesh) and negatively stained with phosphotungstic acid (1% w/v). The sample was viewed using CM 120 Bio Twin transmission electron microscope (Philips Electron Optics BV, The netherlands). This method was adapted from that used by Elhissi and co-workers (2007).

2.1.7 Lipid Extrusion

Extrusion was used to reduce particle size and determine the flexibility of the formulations. This was performed repeatedly through the polycarbonate membrane filter pore. Depending on the formulations, 11 to 21 passes were sufficient to produce a uniform suspension formulation of the desire size, similar to the procedure previously employed for the preparation of large unilamellar vesicles (MacDonald, 1991) using a LiposoFast^{TM} mini-extruder.

2.1.8 Degree of deformability using lipid extruder

For the comparative measurements of elasticity of the bilayer of liposomes, ethosomes, transfersomes and propanosomes a lipid extruder was used to carry out the extrusion measurements (Bergh et al., 2001). At a constant pressure the vesicles were extruded through polycarbonate filter with defined pore size

(Nucleopore). The vesicular elasticity was expressed in terms of deformability index (D) which was proportional to j $(r_v/ r_p)^2$ where, 'j' is the weight of the suspension, which was extruded for a specific time through a polycarbonate filter of defined pore size, 'r_v' the size of the vesicle and 'r_p' the pore size of the vesicle membrane (Gupta et al., 2005).

2.2 Method and procedure used for vesicular preparation

The diagram below outlined the methods employed for producing a range of formulations such as liposomes, transferosomes, ethosomes and propanosomes (Figure 2.1). The thin film hydration method was used for the preparation of these formulations.

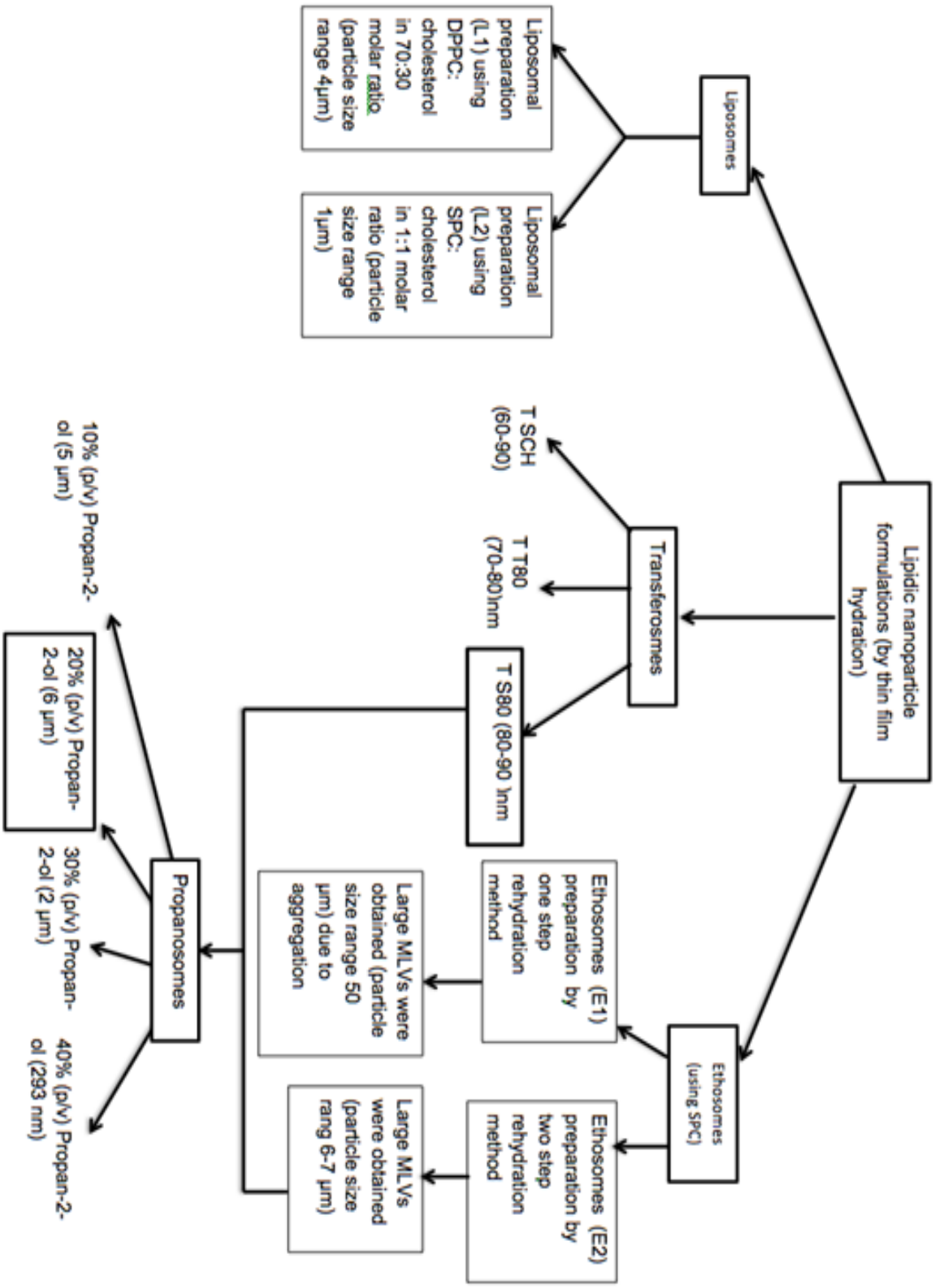

Figure 2.1: Flow diagram showing the different methods employed in the study to prepare a range of formulations.

2.3 General procedure for preparation of formulations

All the formulations were prepared using thin film hydration technique. The phospholipid and cholesterol or phospholipid and surfactant were taken at a specific molar ratio or weight ratio, respectively in a round bottom flask. The desire mixture was dissolved in chloroform. For drug loaded formulations CHX base was added at this stage. The flask was then attached to rotary evaporator above the transition temperature of the lipid. The rotary evaporator was allowed to rotate at a speed of 280 rpm under a vacuum at 23 mbar for 60 minutes. A thin lipid film was obtained after 60 minutes. Then the vacuum pump was turned off to release the negative pressure and the flask was detached. The film was then hydrated either with distil water, PBS (7.4), water + ethanol mixture or 0.9% NaCl solution + propan2-ol mixture as to prepare the desire formulation. The resulting formulation was mixed manually by vigorous shaking and vortexing at 1800 rpm for 15 minutes. The flask was then kept for annealing for one hour or for rotation as per the desire formulation to be prepared. The annealing was performed to stabilise the bilayer membrane. The resultant formulation was then checked for further particle analysis using various techniques. The procedures of all the vesicular formulations are summerised in Table 2.1.

Table 2.1: A summery explaining the prepration of vesiclular formulations

Formulations	**Compositions of the ingredients**	**Condition of the rotatory evaporator and hydration of the formulation**
Liposomes	SPC: Chol (1:1) molar ratio DPPC : Chol (70:30) weight ratio	• Hydration with distilled water • 1 hour stable annealing
Ethosomes	SPC: Chol (1:1) molar ratio	• One step hydration • Two step hydration • Hydration with water + ethanol mixture • 1 hour stable annealing
Transfersomes	SPC: Surfactant (85: 15) (w/w)	• Hydration with PBS (7.4) solvent • 2 hours rotation in rotatory evaporator
Propanosomes	SPC: Surfactant (85: 15) (w/w)	• Hydration with 0.9% NaCl solution + propan-2-ol mixture • 1 hour stable annealing • 30 minutes bath sonication

2.2.1 Bacterial sensitivity test

Bacterial sensitivity towards the formulations was investigated using the agar disk diffusion test.

1) Agar Disk Diffusion Test

A culture of *Staphylococcus aureus* (NCIMB 6571) was prepared in a nutrient broth by aseptically diluting 0.1 ml of the culture into 9 ml of fresh nutrient broth followed by thorough mixing. The bacteria were spreaded in plates by using 0.1 ml of inoculum over the nutrient agar surface followed by incubation for one day to produce of a confluent lawn of bacteria on the agar medium. A swab was dipped into the culture medium and then it was dispersed onto the nutrient agar. This was passed three times over the same area to make sure it was fully covered. The forceps tip was sterilized by passing it three times on a Bunsen burner. Then, the sterile filter paper disk was picked up with the sterile forceps and was placed onto the disk of the agar medium. The lipid formulation (2 µl) was pipetted onto the disk area. The plate was covered with the lid and sealed with Paraflim. The plates were inverted and incubated at 37ºC for 18-24 hours.

The bacterial sensitivity was processed over three day's process involving:-

- Inoculation of the nutrient broth which was done on day 1,
- Disk diffusion test was performed on day 2 and
- Observation of the results on day 3

On day one, a drop of *S. aureus* (NCIMB 6571) was added to the broth of nutrients inside the incubator. A clean environment was essential so ethanol was used to clean the hood and areas where tests were performed. The inoculated tubes were placed into an orbital shaker at 36°C overnight using 1800 rpm setting. On day two, the inoculated tube was removed and cultured with 100 µl of *S. aureus* and then 100 µl cultured nutrient added to the agar plates. On day three, the cultured nutrients were spread and kept for 10 minutes and then autoclaved disks on each of the plates and lipid formulations added.

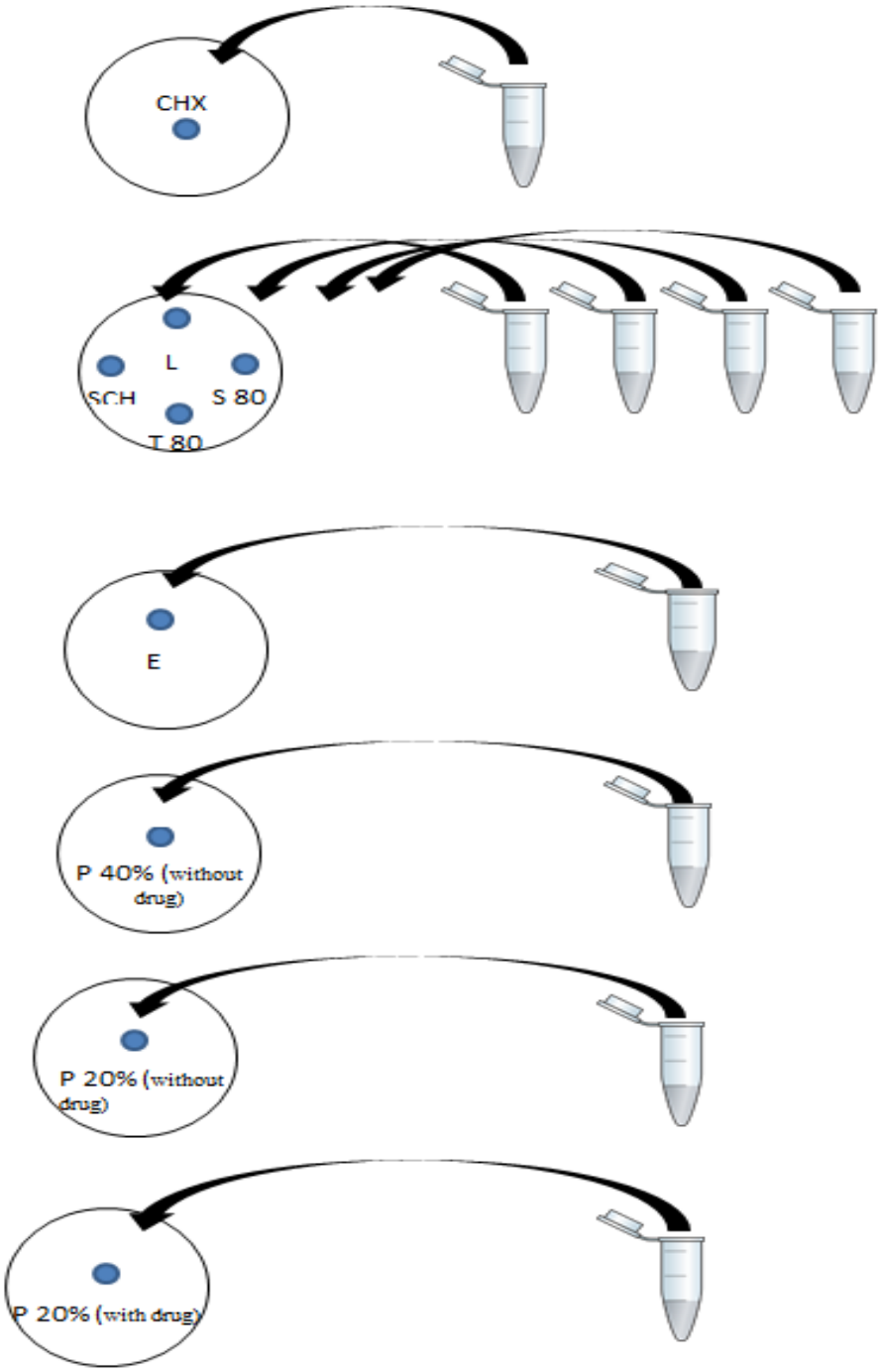

Figure 2.2: A flow diagram showing the disk diffusion testing procedure carried out for the lipidic samples.

2.2.2 Storage of the formulations

- All the above formulations were transferred into the vial covered with aluminium foil to protect it from light and this was kept in the refrigerator at 4 ºC subsequently.

2.2.3 Statistical analysis

The statistical analysis was performed by taking the means and SDs for all the samples. The optimized experiments were undertaken in duplicate. No student's t-test or similar statistical test was employed in the study due to time constain in doing serval experiments.

3 Results

3.1 Formulation optimising using lipid extruder

Figure 3.1 illustrates size analysis of the samples using a Malvern Mastersizer instrument to determine the span and size of the in various formulations.

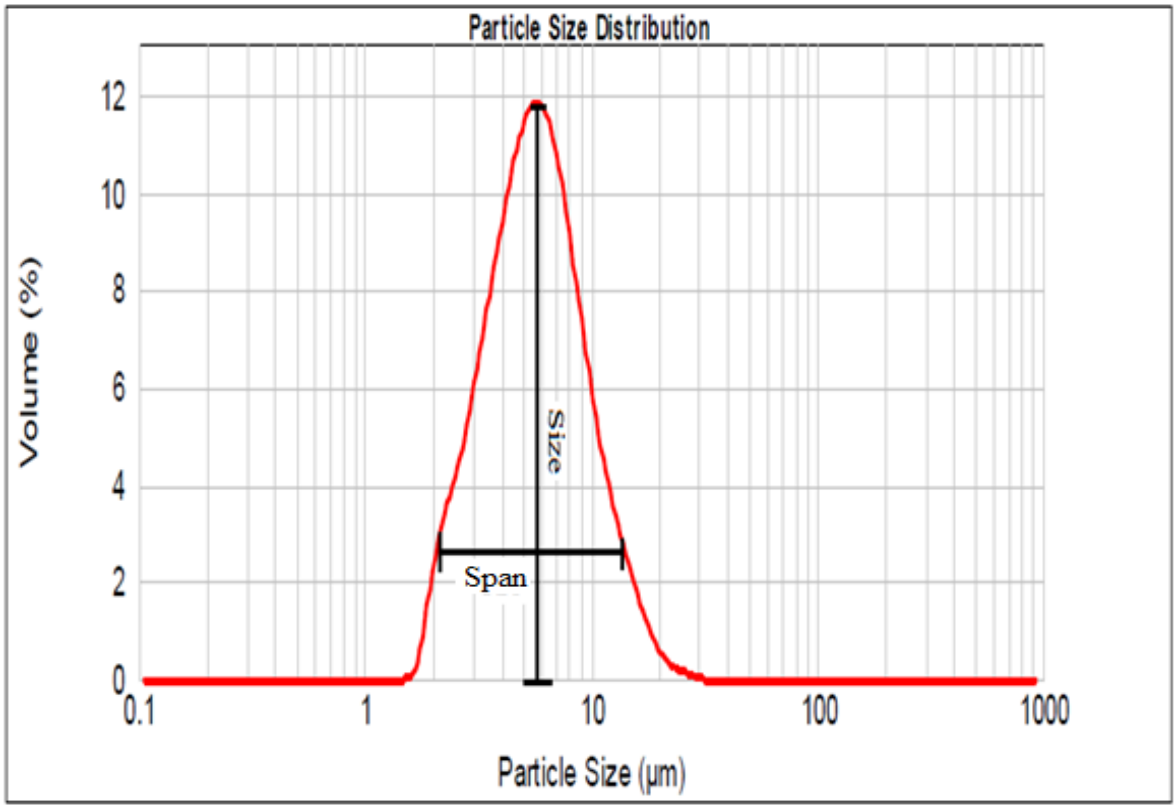

Figure 3.1: A typical bell shape for size distribution of particles in liposomal sample prior to extrusion.

3.2 Optimised vesicular formulations

In this section, the experiments performed in the developmental part (not shown) of the study were optimised to perform the experiments and the final conclusions were drawn based on these optimised results. It is also important to bear in mind, that laser diffraction (Malvern Mastersizer) was employed for measurement of particles having size of 1 µm or above. In this case, the term used to express the polydispersity was "Span". By contrast, for particles expected to have size below 1 µm, dynamic light scattering was employed (using Zetasizer Nanoseries) for most accurate measurements. Hence, it was not possible to compare the polydispersity of micro-sized particles with nano-sized particles.

Figure 3.2 shows the zeta potential of both L and DLL formulations. The results showed that the surface charge (i.e. zeta potential) values of empty liposomes were slightly negative and unaffected by extrusion. Inclusion of the drug has reversed the charge of vesicles in a way that was dependent on the size of the membrane pores (Figure 3.2).

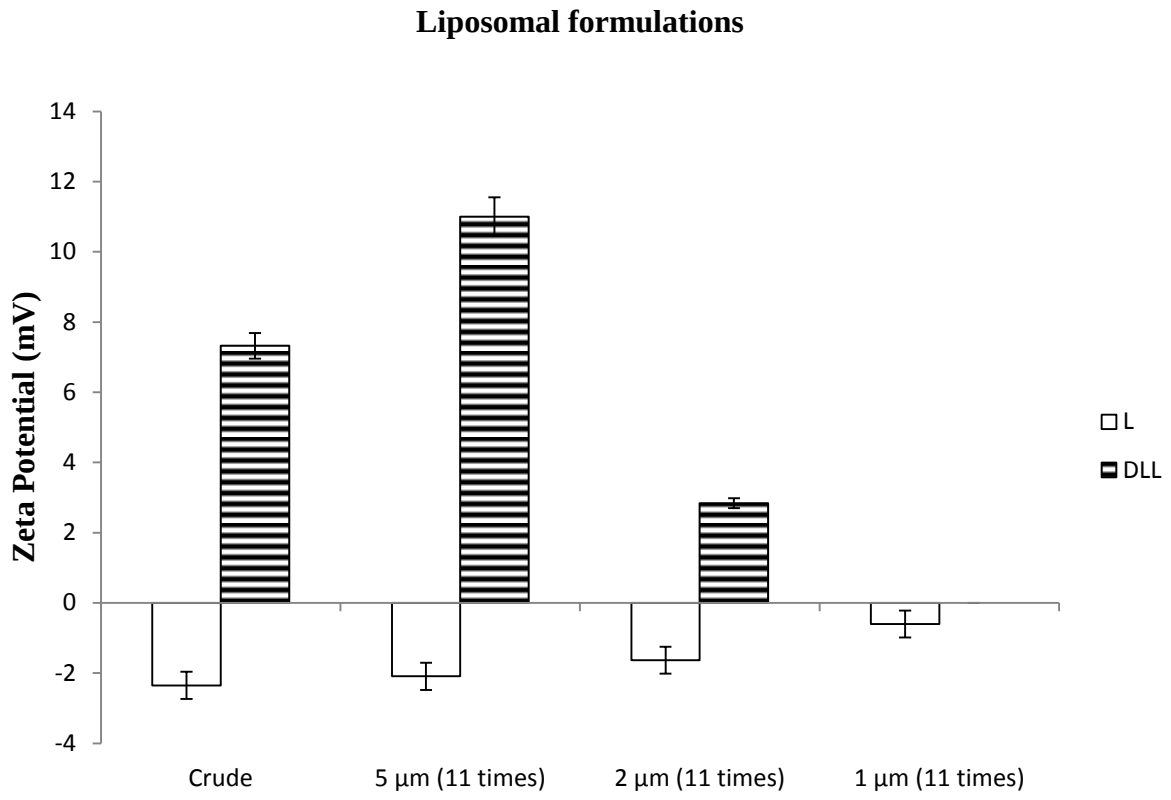

Figure 3.2: Bar chart showing the zeta potential of empty liposomes (L) and drug-loaded liposomes (DLL) following extrusion through membrane filters with different pore sizes (5, 2 and 1 µm). The Drug-loaded liposomes did not passed through the 1µm filters as the desire size was obtained in 2 µm polycarbonate membrane filters. The liposomes were prepared using SPC and cholesterol (1:1 molar ratio). The results represent the mean (±SEM) of 2 experiments (n=2).

Figure 3.3 clearly shows that there was a marked decrease in the mean size of liposomes with extrusion, regardless of drug inclusion. However, drug-loaded liposomes are always smaller than empty liposomes. Also, it was not possible to extrude drug-loaded vesicles through the 1 µm polycarbonate filters, suggesting interaction of the drug with the liposomal bilayers in a manner than affected vesicle size and bilayer rigidity.

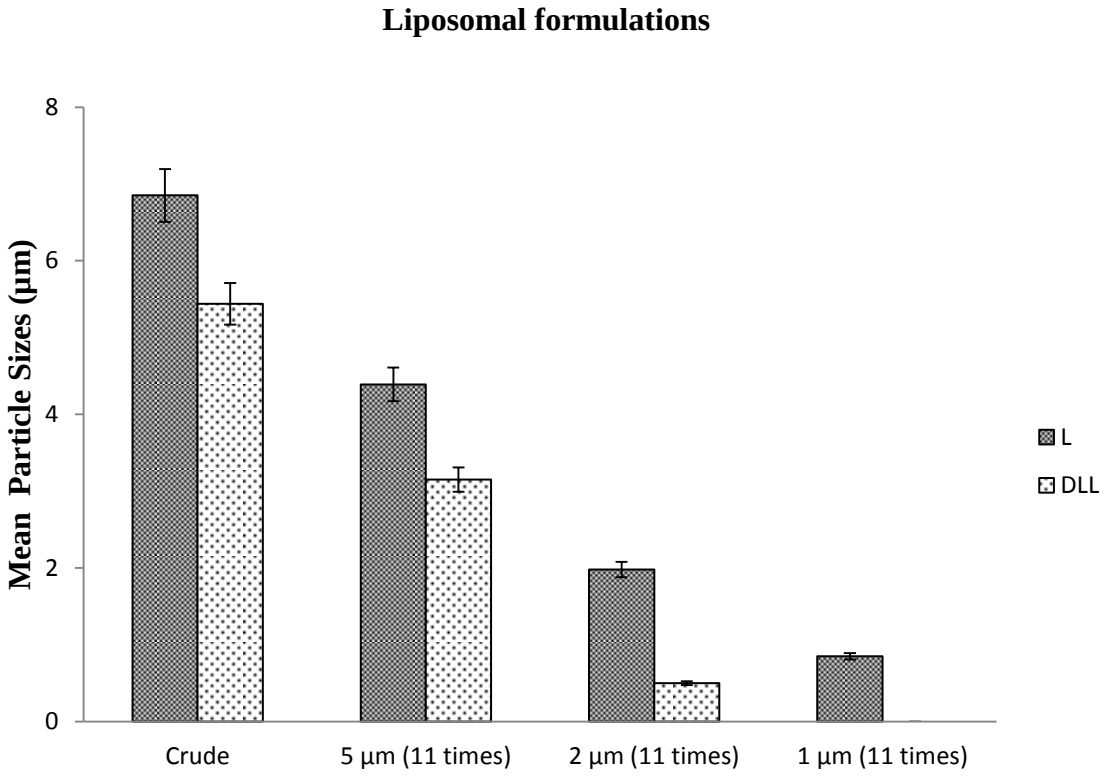

Figure 3.3: Bar chart showing the size of empty liposomes (L) and drug-loaded liposomes (DLL) following extrusion through membrane filters with different pore sizes (5, 2, 1 µm). The drug loaded liposomes did not pass through the 1 µm membrane filters as the desire size was obtained in 2 µm membrane filters. The liposomes were prepared using SPC and cholesterol (1:1 molar ratio). The results represent the mean (±SEM) of two experiments (n=2).

Figure 3.4 shows the relationship between Span and extrusion for L and DLL. Drug inclusion in the liposomal formulation caused a decrease in polydispersity of the extruded vesicles, suggesting the presence of chlorhexidine may enhance liposome dispersion properties by reducing the aggregation of the vesicles. This correlates well with the reduced vesicle size in formulations that included the drug (Figure 3.3).

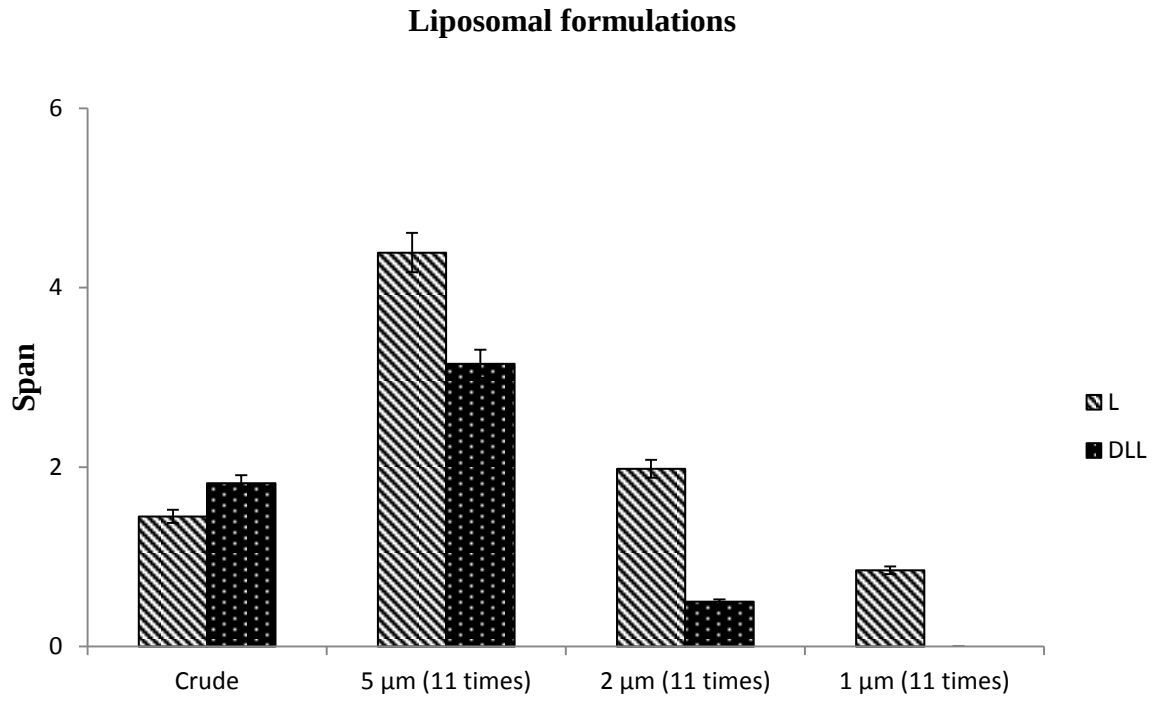

Figure 3.4: Bar charts showing the span for empty liposomes (L) and drug-loaded liposomes (DLL) following extrusion through membrane filters with different pore sizes (5, 2, 1 µm). The drug loaded liposomes did not pass through the 1 µm membrane filters as the desire size was obtained in 2 µm membrane filters. The liposomes were prepared using SPC and cholesterol (1:1 molar ratio). The results represent the mean (±SEM) of two experiments (n=2).

Figure 3.5 demonstrated that the zeta potential of both E and DLE were positive in ethosomes. The zeta potential values increased with drug incorporation into the formulations, again confirming that this drug changed the properties of lipid bilayers.

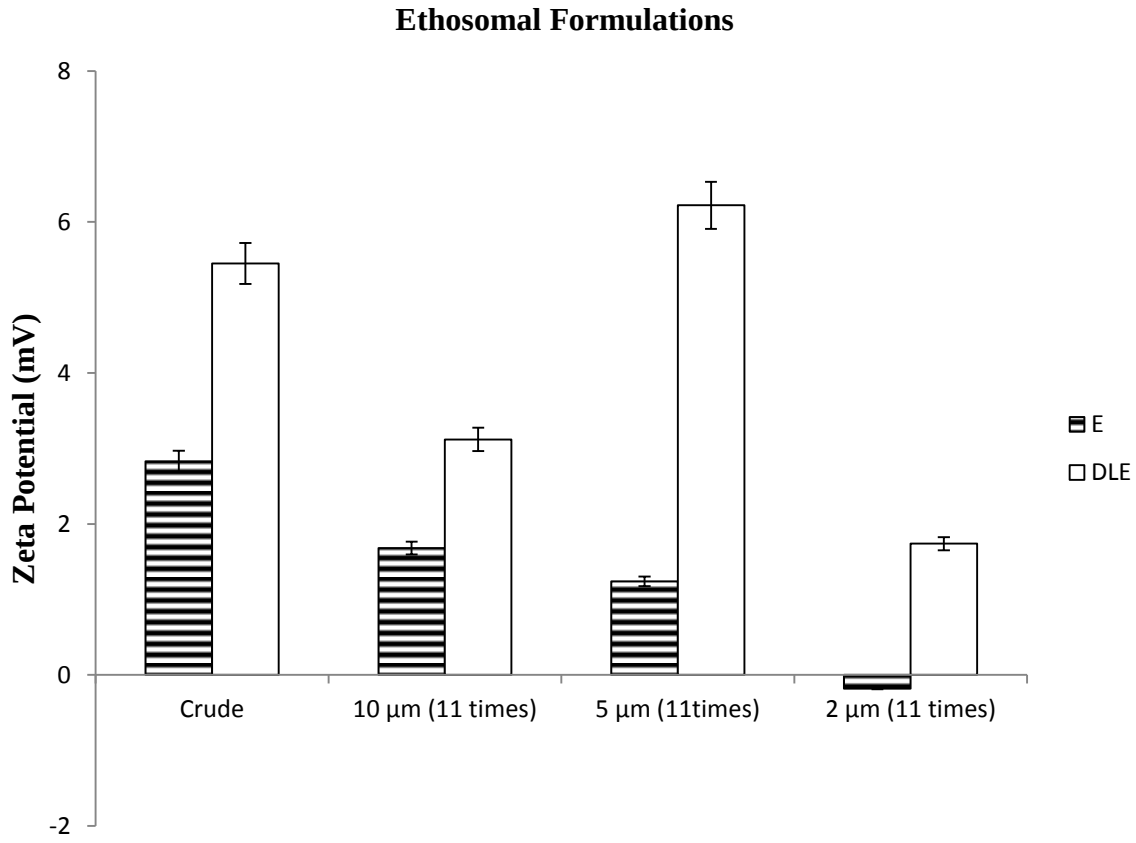

Figure 3.5: Bar charts showing zeta potential for empty ethosomes (E) and drug loaded ethosomes (DLL) following extrusion through membrane filters with different pore sizes (10, 5, 2 µm). The ethosomes were prepared using SPC and cholesterol (1:1 molar ratio).The results represent the mean (±SEM) of two experiments (n=2).

In figure 3.6, the results shows that the mean particle size of ethosomes considering extrusion and drug incorporation the data reveal that extrusion did not affect the size of ethosomes and the drug increased the size of vesicles independently of extrusion. This indicates the deformability of ethosomes; hence extrusion did not reduce the size of vesicles. Also, the drug did not affect the vesicle deformability and flexibility. This so far shows that shear-responsiveness of ethosomes is different from that of liposomes.

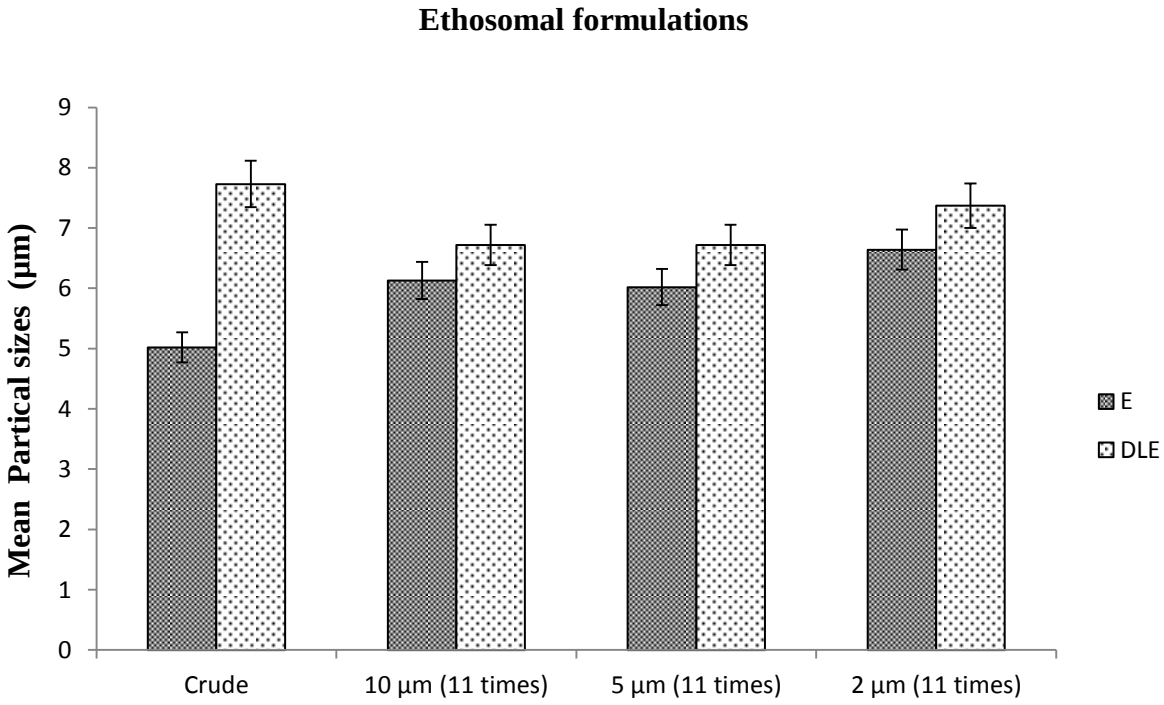

Figure 3.6: Bar charts showing mean particle sizes for empty ethosomes (E) and drug loaded ethosomes (DLL) following extrusion through membrane filters with different pore sizes (10, 5, 2 µm). The ethosomes were prepared using SPC and cholesterol (1:1 molar ratio). The results represent the mean (±SEM) of two experiments (n=2).

The polydispersity of ethosomes, as a result of drug inclusion and extrusion, is very difficult to interpret (Figure 3.7). For empty ethosomes, the effect of extrusion on polydispersity was mild. By contrast, for drug-included ethosomes, the polydispersity was high prior to extrusion, decreased upon extrusion through 10 µm and 5 µm filters and partially regained its high polydispersity when extruded through the 2µm filters (Figure 3.7). These findings indicate aggregation of drug containing ethosomes only when high shear was applied such as that provided using the small (2 µm) polycarbonate membrane filters. Further investigations are needed to understand the manners of interaction between chlorhexidine and ethosomal bilayers.

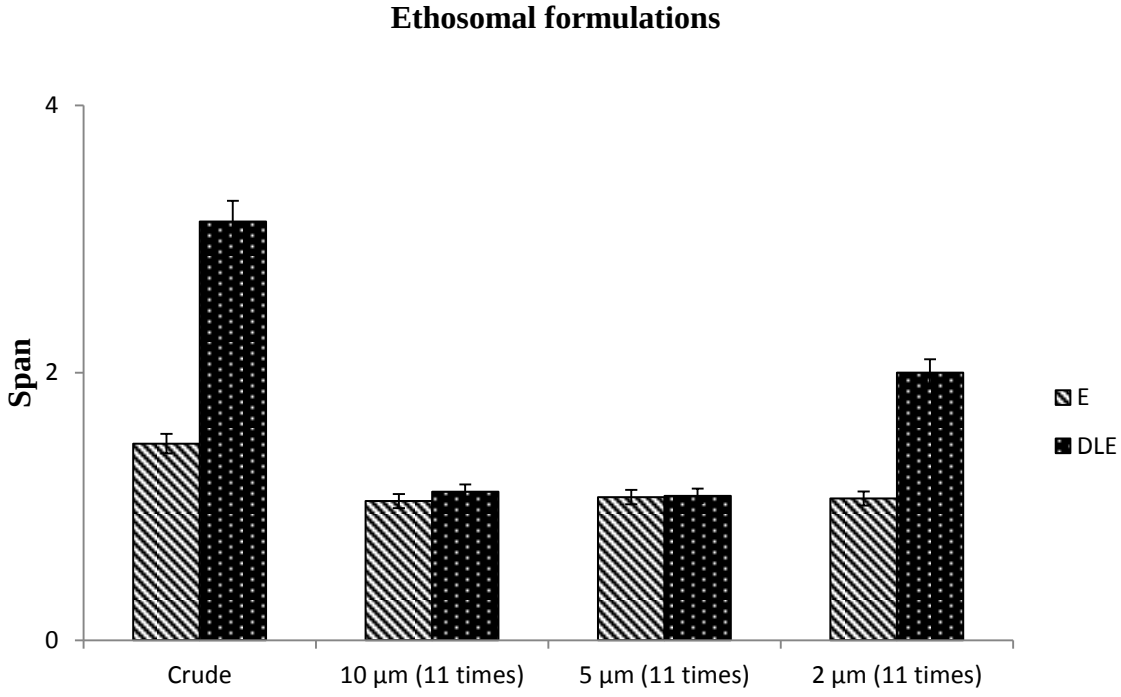

Figure 3.7: Bar charts showing span for empty ethosomes (E) and drug loaded ethosomes (DLL) following extrusion through membrane filters with different pore sizes (10, 5, 2 µm). The ethosomes were prepared using SPC and cholesterol (1:1 molar ratio). The results represent the mean (±SEM) of two experiments (n=2).

For transferosmal formulations, the zeta potential ranged from slightly negative to slightly positive when no drug was included (Figure 3.8). However, upon incorporation of the drug, all formulations had positive zeta potential measurements, suggesting the interaction of the drug with the transferosomal bilayers. The effect of extrusion on the zeta potential of transferosomes was small.

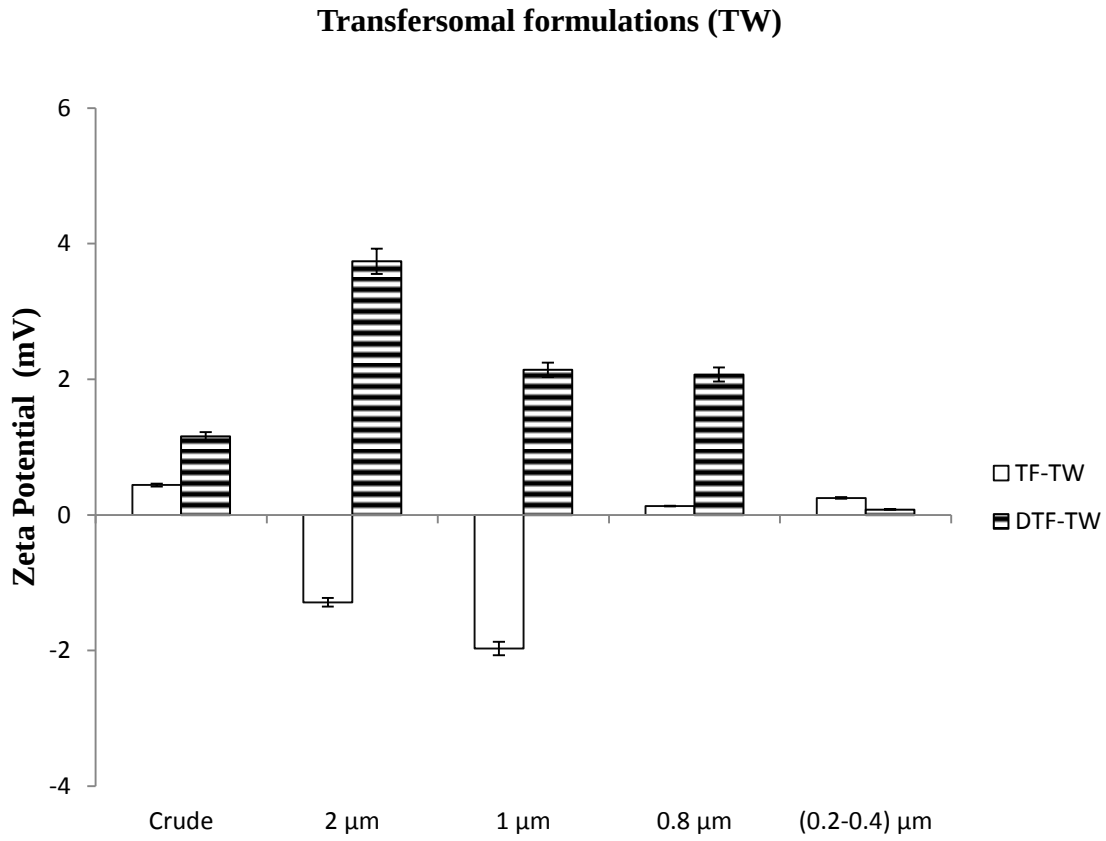

Figure 3.8: Bar charts showing zeta potential for empty transfersomes using Tween 80 (TF-TW) and drug loaded trasnfersomes using Tween 80 (DTF-TW) following extrusion through membrane filters with different pore sizes (2, 1, 0.8 and sandwich between 0.2-0.4 µm). The transfersomes were prepared using SPC and Tween 80 surfactant (85: 15) % (w/w) weight ratio. The results represent the mean (±SEM) of two experiments (n=2).

Unlike ethosomes which showed high ultradeformability with no major change in size upon extrusion (Figure 3.6), transferosomes underwent size reduction upon extrusion and tended to have the size of the filter pores through which they were extruded (Figure 3.9). Also, in general, drug did not affect the size of transferosomes (Figure 3.9) although it increased the zeta potential (Figure 3.8).

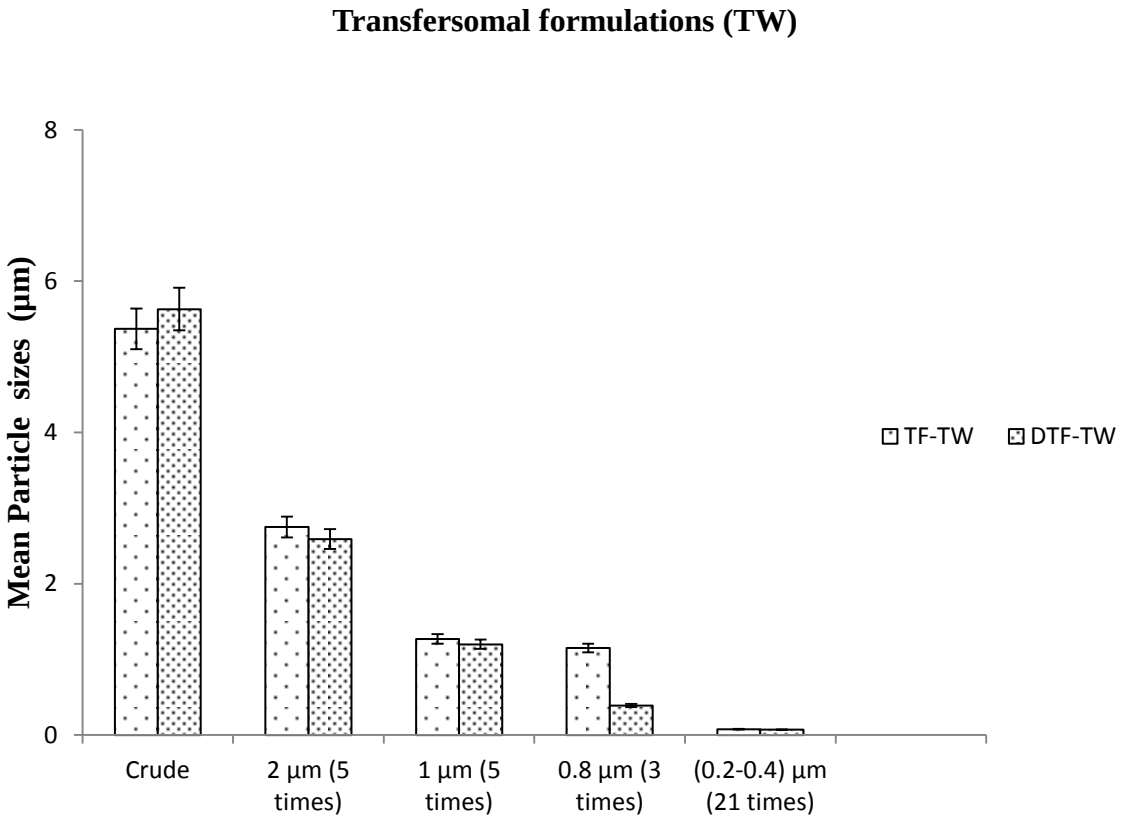

Figure 3.9: Bar charts showing mean particle sizes for empty transfersomes using Tween 80 (TF-TW) and drug loaded trasnfersomes using Tween 80 (DTF-TW) following extrusion through membrane filters with different pore sizes (2, 1, 0.8 and sandwich between 0.2-0.4 µm). The transfersomes were prepared using SPC and Tween 80 surfactant (85: 15) % (w/w) weight ratio. The results represent the mean (±SEM) of two experiments (n=2).

Figure 3.10 exhibits the polydispersity of both TF-TW and DTF-TW of crude and vesicles extruded through 2, 1, 0.8 and (0.2-0.4) µm filters. The results showed that the polydispersity markedly decreased by extrusion of the crude vesicles through 2 µm and 1 µm membrane filters, regardless of the presence of chlorhexidine. The same observation was seen when the 0.8 µm extruded vesicles were further pushed through the 0.2-0.4 µm filters, indicating the fragmentation of vesicles upon shearing. Both the TF-TW and DTF-TW were similar in polydispersity, indicating that addition of drug was not responsible for size distribution changes.

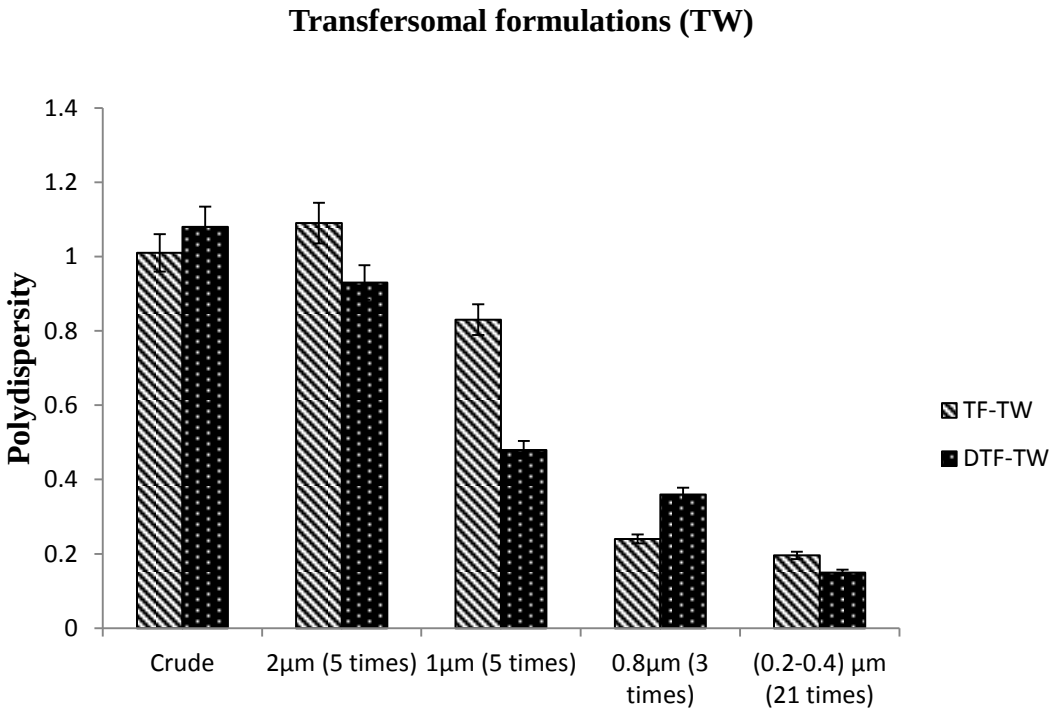

Figure 3.10: Bar charts showing polydispersity for empty transfersomes using Tween 80 (TF-TW) and drug loaded trasnfersomes using Tween 80 (DTF-TW) following extrusion through membrane filters with different pore sizes (2, 1, 0.8 and sandwich between 0.2-0.4 µm). The transfersomes were prepared using SPC and Tween 80 surfactant (85: 15) % (w/w) weight ratio. The results represent the mean (±SEM) of two experiments (n=2).

Figure 3.11 shows the zeta potential of both TF-SP and DTF-SP of crude and vesicles extruded through 2 µm, 1 µm, 0.8 µm and 0.2-0.4 µm membrane filters. The empty trasnfersomes had slightly negative zeta potential, whilst drug-loaded transferosomes had slightly positive zeta potential values, indicating that surface charge of the vesicles was reversed upon incorporation of the drug, again confirming the interaction of this drug with the lipid bilayers of vesicles.

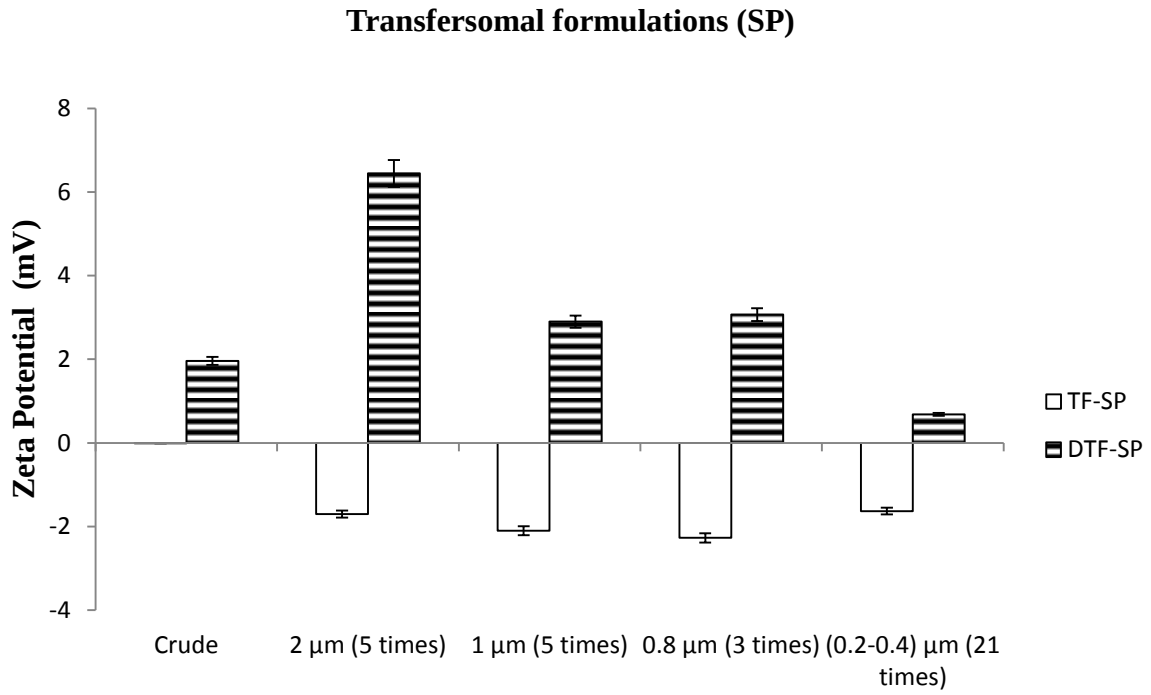

Figure 3.11: Bar charts showing zeta potential for empty transfersomes using Span 80 (TF-SP) and drug loaded trasnfersomes using Span 80 (DTF-SP) following extrusion through membrane filters with different pore sizes (2, 1, 0.8 and sandwich between 0.2-0.4 µm). The transfersomes were prepared using SPC and Span 80 surfactant (85: 15) % (w/w) weight ratio. The results represent the mean (±SEM) of two experiments (n=2).

The Figure 3.12 demonstrates that extrusion resulted in size reduction of the vesicles. Also, incorporation of the drug had no effect on the size of extruded or unextruded transferosomes. Size reduction of transferosomes upon extrusion was consistent with the findings seen earlier with liposomes (Figure 3.3), however for liposomes the drug tended to reduce vesicle size whilst with transferosomes no effect of the drug on particle size was seen (Figure 3.12).

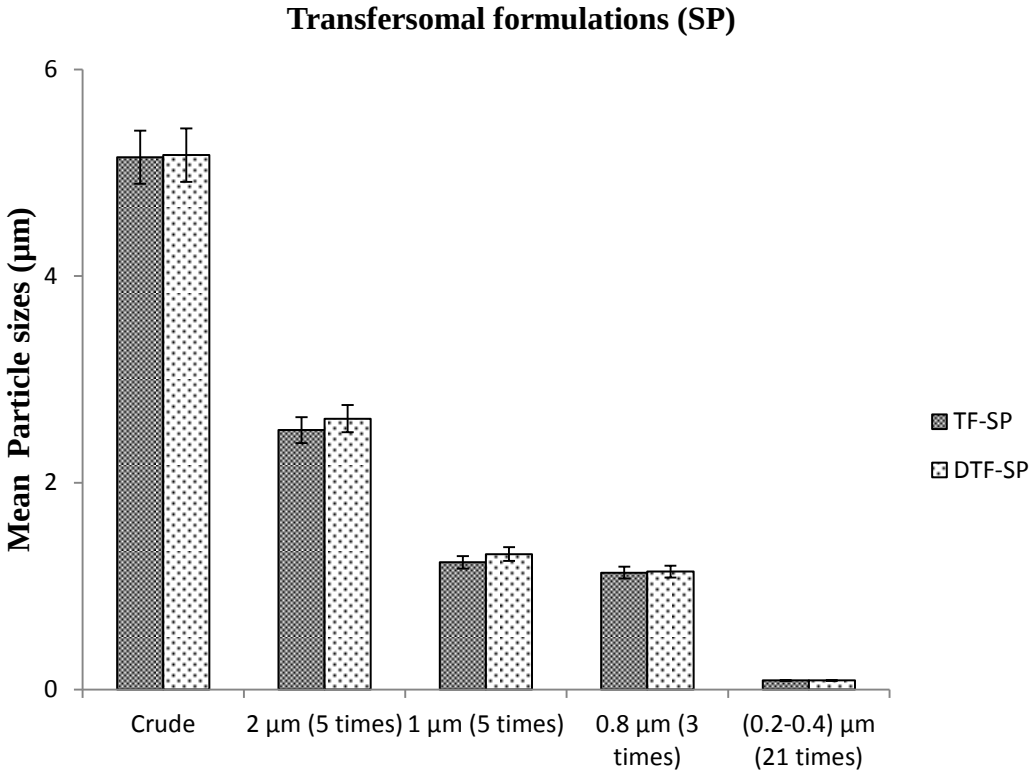

Figure 3.12: Bar charts showing mean particle sizes for empty transfersomes using Span 80 (TF-SP) and drug loaded trasnfersomes using Span 80 (DTF-SP) following extrusion through membrane filters with different pore sizes (2, 1, 0.8 and sandwich between 0.2-0.4 µm). The transfersomes were prepared using SPC and Span 80 surfactant (85: 15) % (w/w) weight ratio. The results represent the mean (±SEM) of two experiments (n=2).

Figure 3.13 shows that the polydispersity of both TF-SP and DTF-SP of crude and extruded vesicles 2, 1, 0.8, (0.2-0.4) µm PMFP. The results show that the polydispersity markedly decreased upon extrusion in both TF-SP and DTF-SP. However, polydispersity of the particles in DTF-SP was observed to be higher than that of TF-SP. These results clearly indicate that the addition of drug is responsible for the change in polydispersity of the vesicles. It is worth mentioning that whilst median size was unaffected by drug inclusion (Figure 3.12), the drug increased the presence of relatively small and large vesicles in the sample, hence broadening the size distribution (i.e. increasing polydispersity).

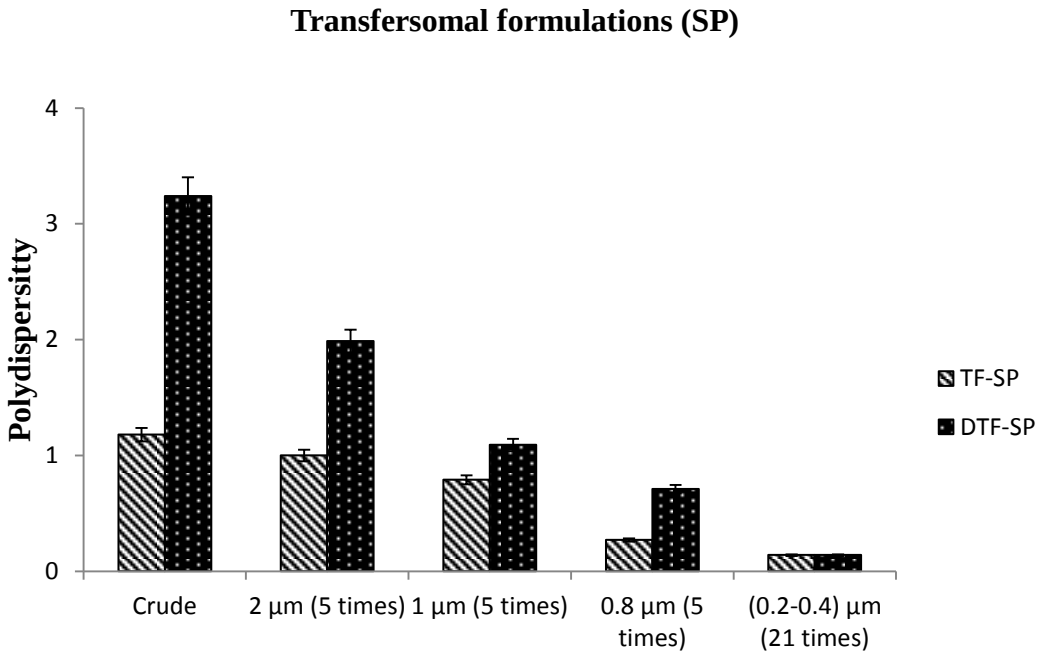

Figure 3.13: Bar charts showing polydispersity for empty transfersomes using Span 80 (TF-SP) and drug loaded trasnfersomes using Span 80 (DTF-SP) following extrusion through membrane filters with different pore sizes (2, 1, 0.8 and sandwich between 0.2-0.4 µm). The transfersomes were prepared using SPC and Span 80 surfactant (85: 15) % (w/w) weight ratio. The results represent the mean (±SEM) of two experiments (n=2).

Figure 3.14, shows that zeta potential using TF-SCH and DTF-SCH formulations was independent of extrusion and drug inclusion; using SCH in transferosomes showed positive zeta potential values which were also unaffected by incoprpration of the drug (Figure 3.14). Hence, zeta potential was dependent on lipid phase composition such as the presence/absence of chlorhexidine and certain surfactants and deformability enhancers (e.g. edge activators).

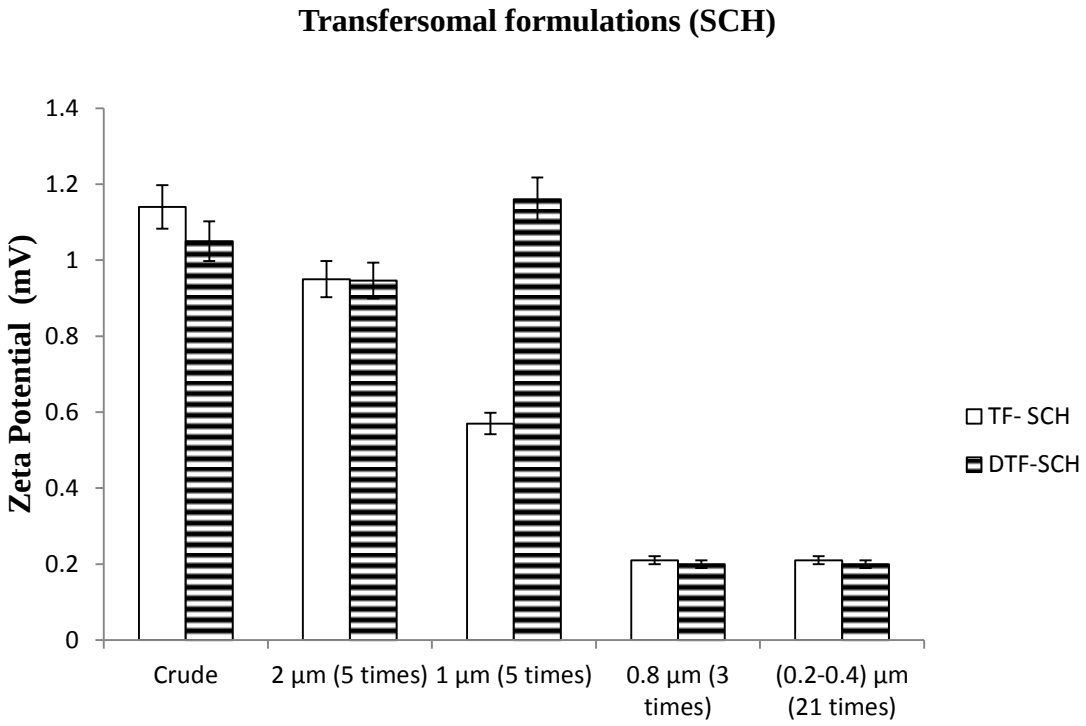

Figure 3.14: Bar charts showing zeta potential for empty transfersomes using sodium cholate hydrate (TF-SCH) and drug loaded trasnfersomes using sodium cholate hyrate (DTF-SCH) following extrusion through membrane filters with different pore sizes (2, 1, 0.8 and sandwich between 0.2-0.4 µm). The transfersomes were prepared using SPC and Sodium cholate hydrate surfactant (85: 15) % (w/w) weight ratio. The results represent the mean (±SEM) of two experiments (n=2).

Although the effect of drug on zeta potential of SCH transferosomes was mild (Figure 3.14), the drug tended to generate larger transferosomes compared to the corresponding drug-free formulations (Figure 3.15). For both drug-free and drug-containing formulations, the transferosome size was reduced by extrusion. The particle size measurements were larger in DTF-SCH compared to TF-SCH. This shows that addition of the drug was responsible for the increase in particle size of the SCH transferosomes (Figure 3.15).

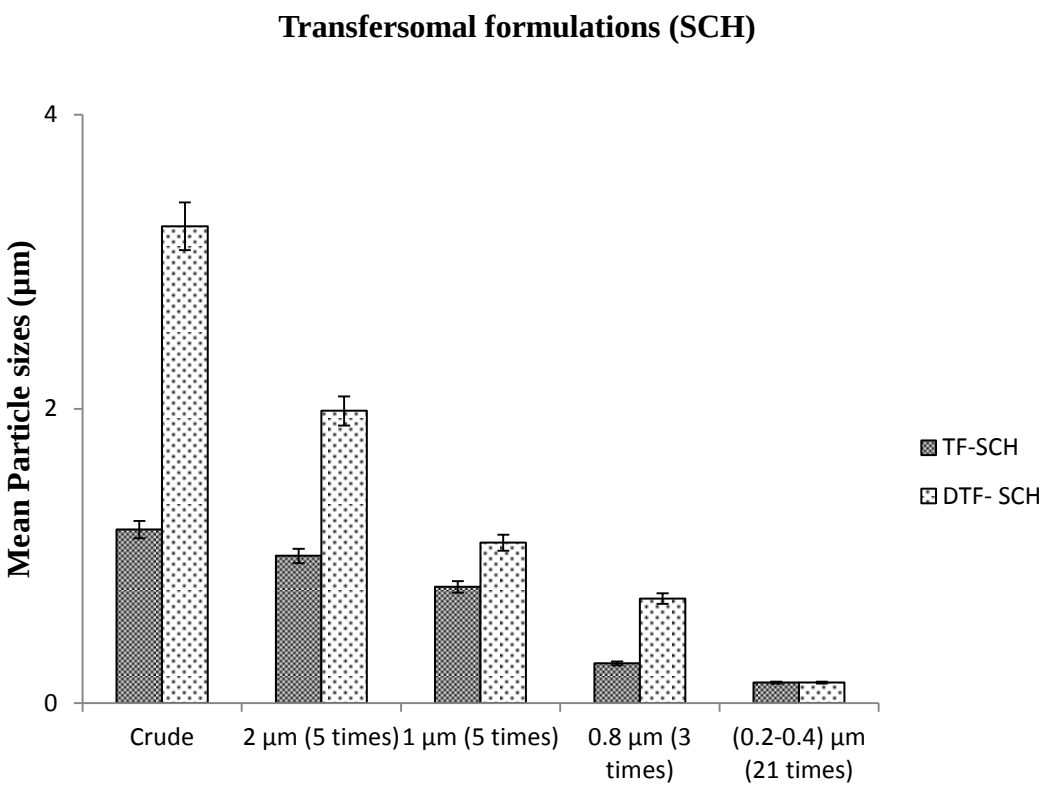

Figure 3.15: Bar charts showing mean particle sizes for empty transfersomes using SCH (TF-SCH) and drug loaded trasnfersomes using SCH (DTF-SCH) following extrusion through membrane filters with different pore sizes (2, 1, 0.8 and sandwich between 0.2-0.4 µm). The transfersomes were prepared using SPC and Sodium cholate hydrate surfactant (85: 15) % (w/w) weight ratio. The results represent the mean (±SEM) of two experiments (n=2).

Figure 3.16 indicates that the polydispersity of both TF-SCH and DTF-SCH of crude and 2 µm, 1 µm, 0.8 µm, 0.2-0.4 µm PMFP was progressively reduced by extrusion, and there was no effect of the drug on the vesicle polydispersity, except for the 1 µm extruded vesicles which demonstrated an increase in vesicle polydispersity as a result of drug incorporation. Further studies are needed to understand the reason behind the different behaviour of this particular formulation.

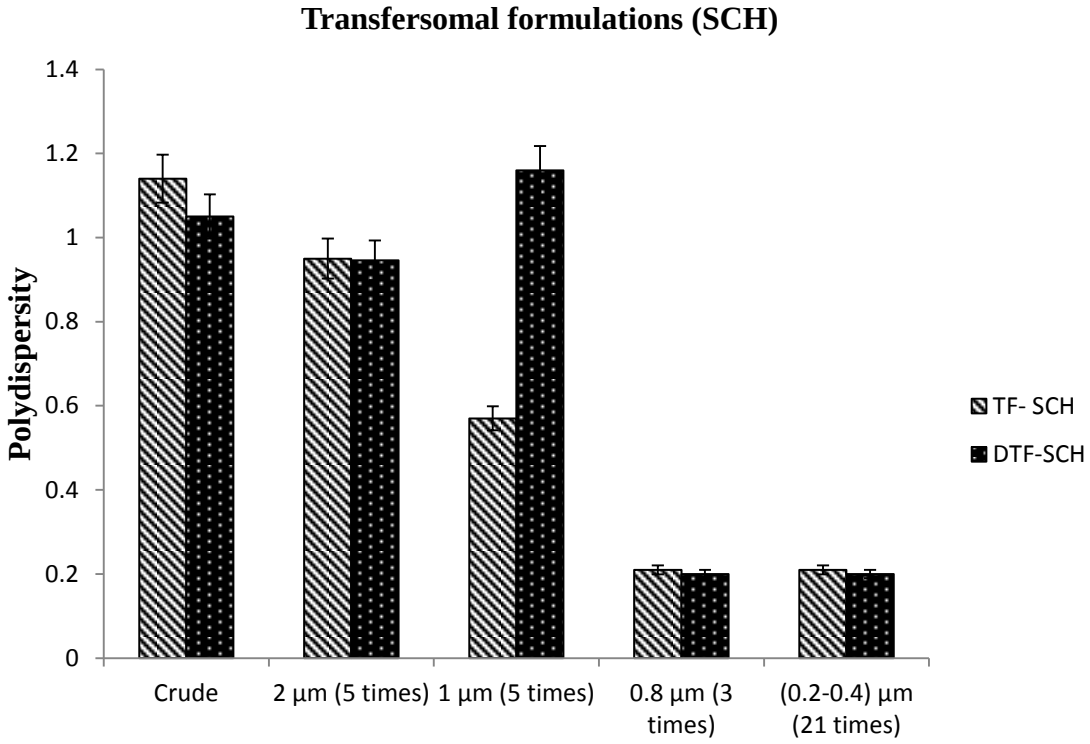

Figure 3.16: Bar charts showing polydispersity for empty transfersomes using SCH (TF-SCH) and drug loaded trasnfersomes using SCH (DTF-SCH) following extrusion through membrane filters with different pore sizes (2, 1, 0.8 and sandwich between 0.2-0.4 µm). The transfersomes were prepared using SPC and Sodium cholate hydrate surfactant (85: 15) % (w/w) weight ratio. The results represent the mean (±SEM) of two experiments (n=2).

The Figure 3.17 demonstrates the zeta potential measurements of propoanosomes unextruded and those extruded through successive polycarbonate membrane filters (2, 1, 0.8, 0.2-0.4 µm). For empty formulations, the zeta potential was slightly negative. The inclusion of drug has demonstrated capability to make the zeta potential positive, suggesting an interaction between chlorhexidine and propanosomal bilayers. Interestingly, in the drug-containing formulations also shown a trend of an increase in zeta potential was observed when the vesicles were extruded through the smaller pores. This might be attributed to the enhanced interaction between the drug and the bilayers owing to the increased surface area of the lipid following extrusion.

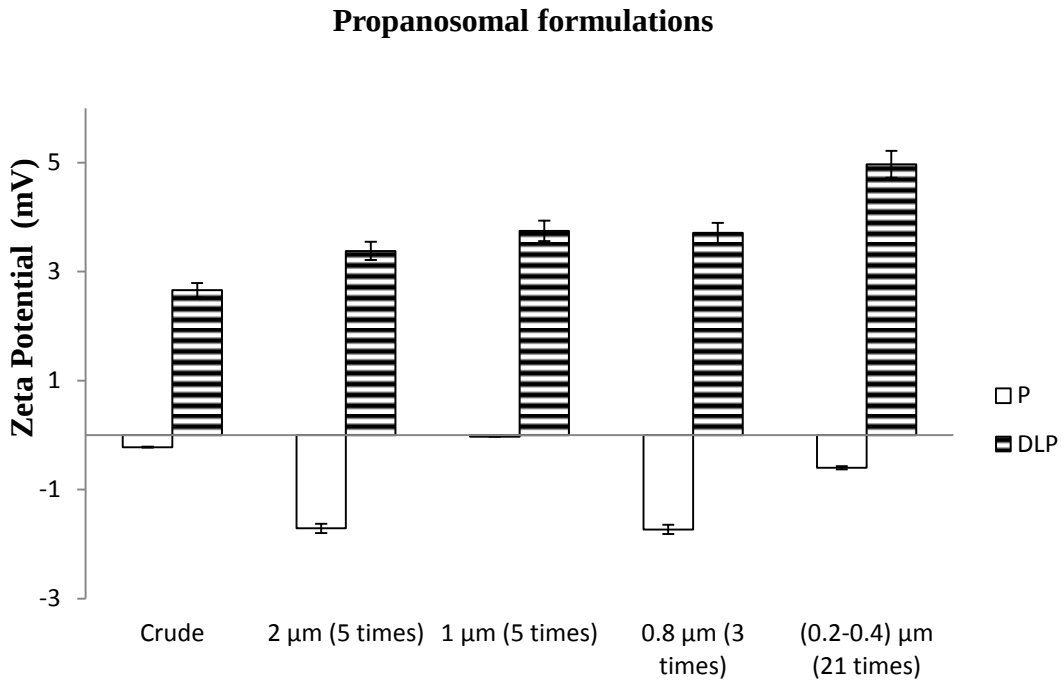

Figure 3.17: Bar charts showing zeta potential for empty propansomes using (P) and drug loaded propansomes (DLP) following extrusion through membrane filters with different pore sizes (2, 1, 0.8 and sandwich between 0.2-0.4 µm). The transfersomes were prepared using SPC and Span 80 (85: 15) % (w/w) weight ratio. The results represent the mean (±SEM) of two experiments (n=2).

Figure 3.18 shows the particle size of both P and DLP of crude and vesicles extruded through 2, 1, 0.8, 0.2-0.4 µm PMFP. The result shows that the size markedly decreased by extrusion, and there was no effect of the drug on the size of propanosomes.

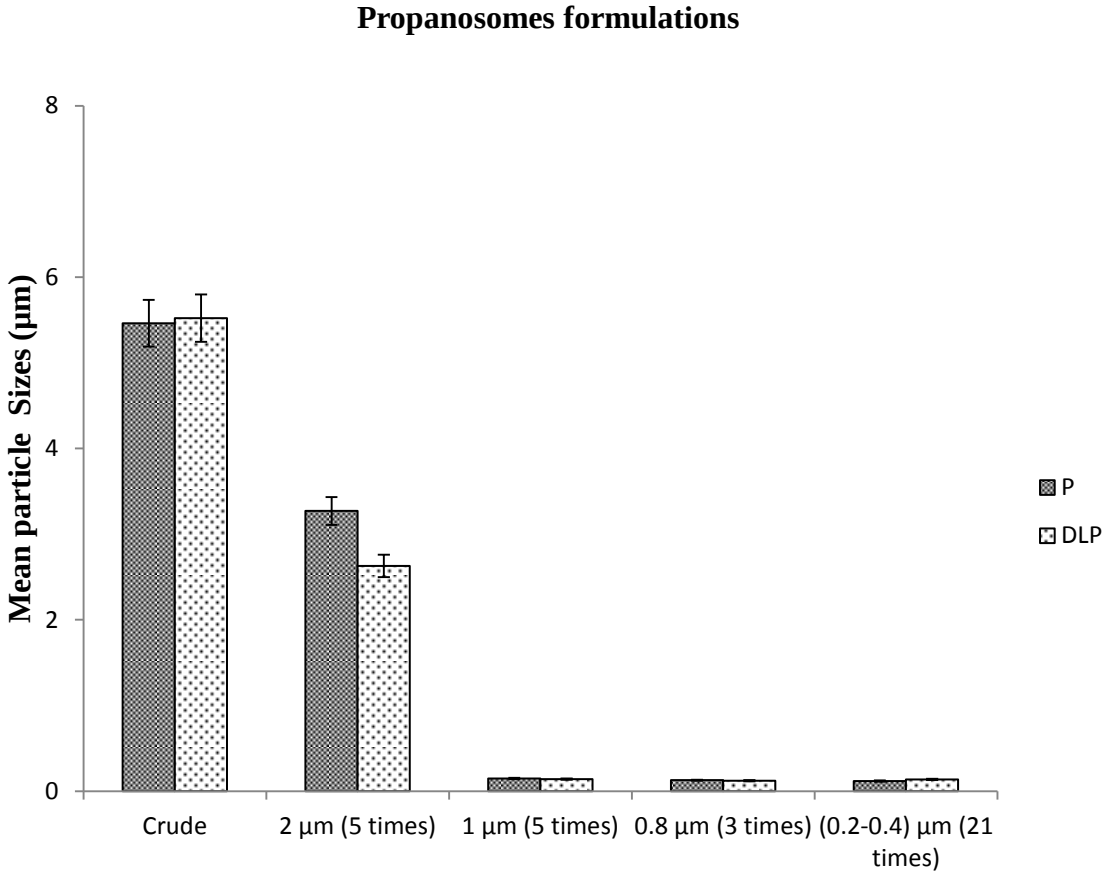

Figure 3.18: Bar charts showing mean particle size for empty propansomes using (P) and drug loaded propansomes (DLP) following extrusion through membrane filters with different pore sizes (2, 1, 0.8 and sandwich between 0.2-0.4 µm). The transfersomes were prepared using SPC and Span 80 surfactant (85: 15) % (w/w) weight ratio. The results represent the mean (±SEM) of two experiments (n=2).

Figure 3.19 shows the polydispersity of propanosomes to be minimally dependent on formulation, considering the Span measurements for the crude vesicles and vesicles extruded through 2 µm and 1 µm filters, and the PDI values for the formulations extruded through 0.8 µm, and 0.2-0.4 µm filters.

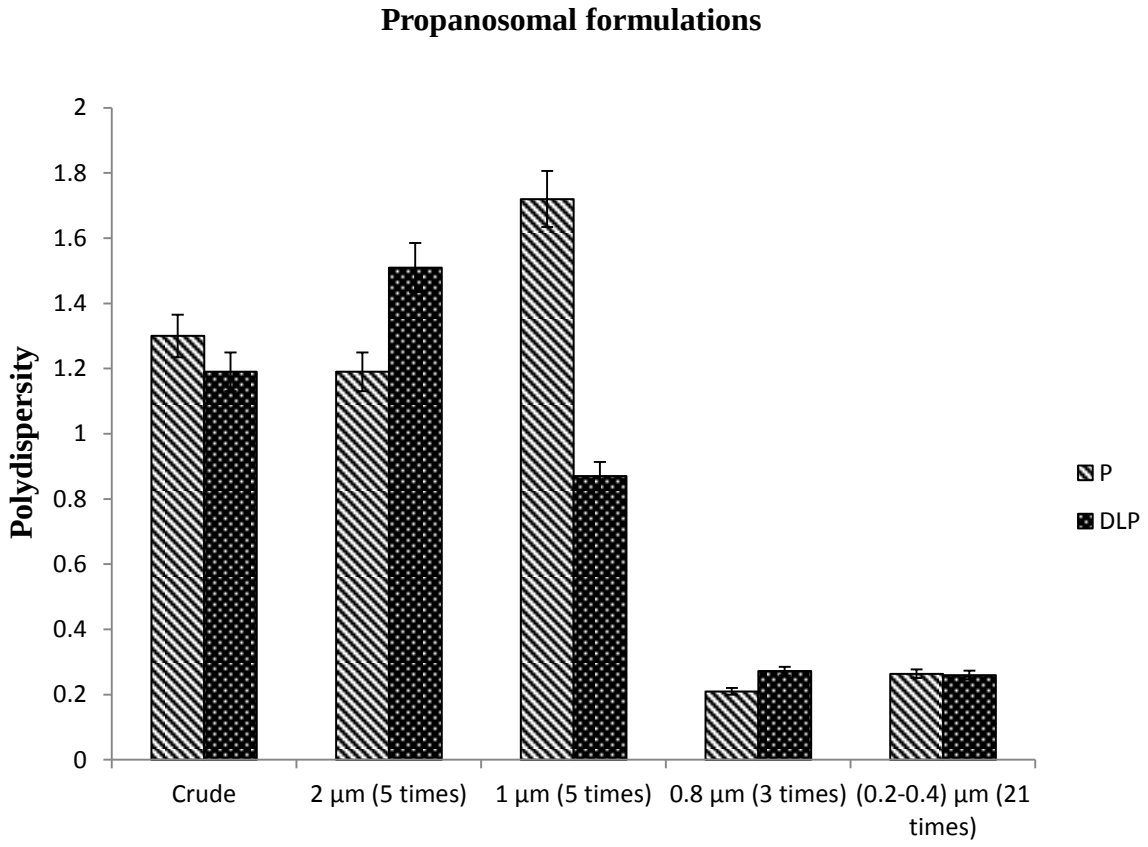

Figure 3.19: Bar charts showing polydispersity for empty propansomes using (P) and drug loaded propansomes (DLP) following extrusion through membrane filters with different pore sizes (2, 1, 0.8 and sandwich between 0.2-0.4 µm). The transfersomes were prepared using SPC and Span 80 surfactant (85: 15) % (w/w) weight ratio. The results represent the mean (±SEM) of two experiments (n=2).

3.2.1 Degree of Deformability (D) testing Results

In all the below results presented below, the mean of (0.2- 0.4) µm PMFP was taken as 0.3 µm for calculation of D for trasnfersomal and propanosomal formulations.

In Figure 3.20, while passing the liposomes from crude to 5 µm PMFP, a lot of pressure was required to pass them through the membrane as the size of the liposomes in the crude stage was large as 7 µm (results not shown). For DLL the formulation was not allowed to pass through 1 µm PMFP as the desire size of the vesicles were obtained at 2 µm PMFP stage. The degree of deformability for empty liposomes shows inconsistency through progressive extrusion. However, for drug-loaded liposomes the D is decreasing through extrusion.

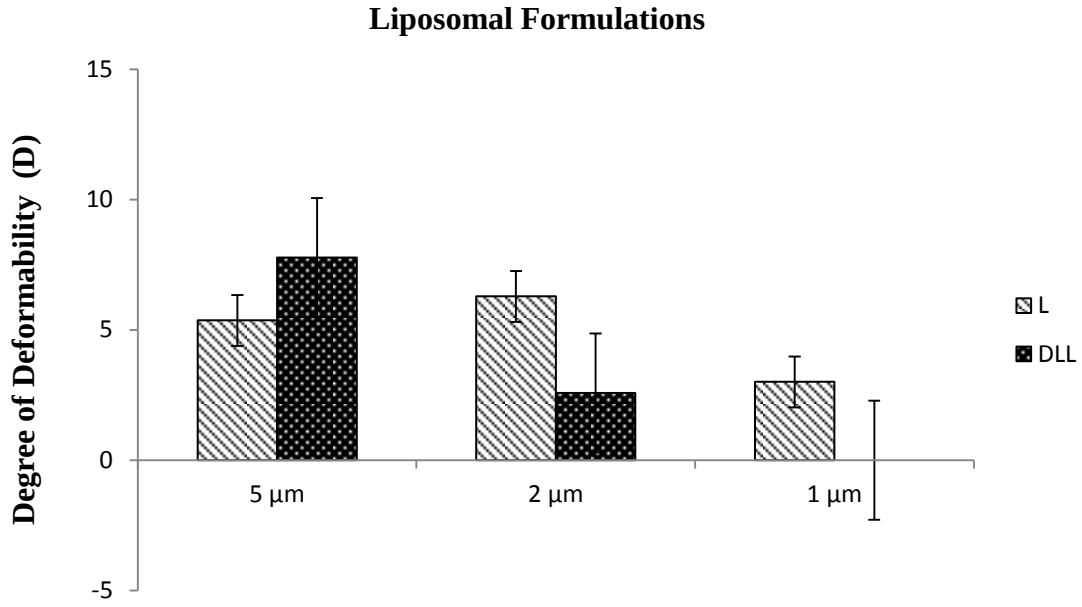

Figure 3.20: Bar charts showing degree of deformability of empty liposomes (L) and drug-loaded liposomes (DLL) following extrusion through membrane filters with different pore sizes 5µm, 2 µm and 1 µm. The results represent the mean (±SEM) of two experiments (n= 2).

As shown in Figure 3.21, throughout the whole procedure of extrusion, ethosomes were hard to extrude due to their large particle size while passing from crude to 5 µm PMFP. The degree of deformability for E and DLE are the same through progressive extrusion. However, for DLE there is marked increase in D due to aggregation of the particles.

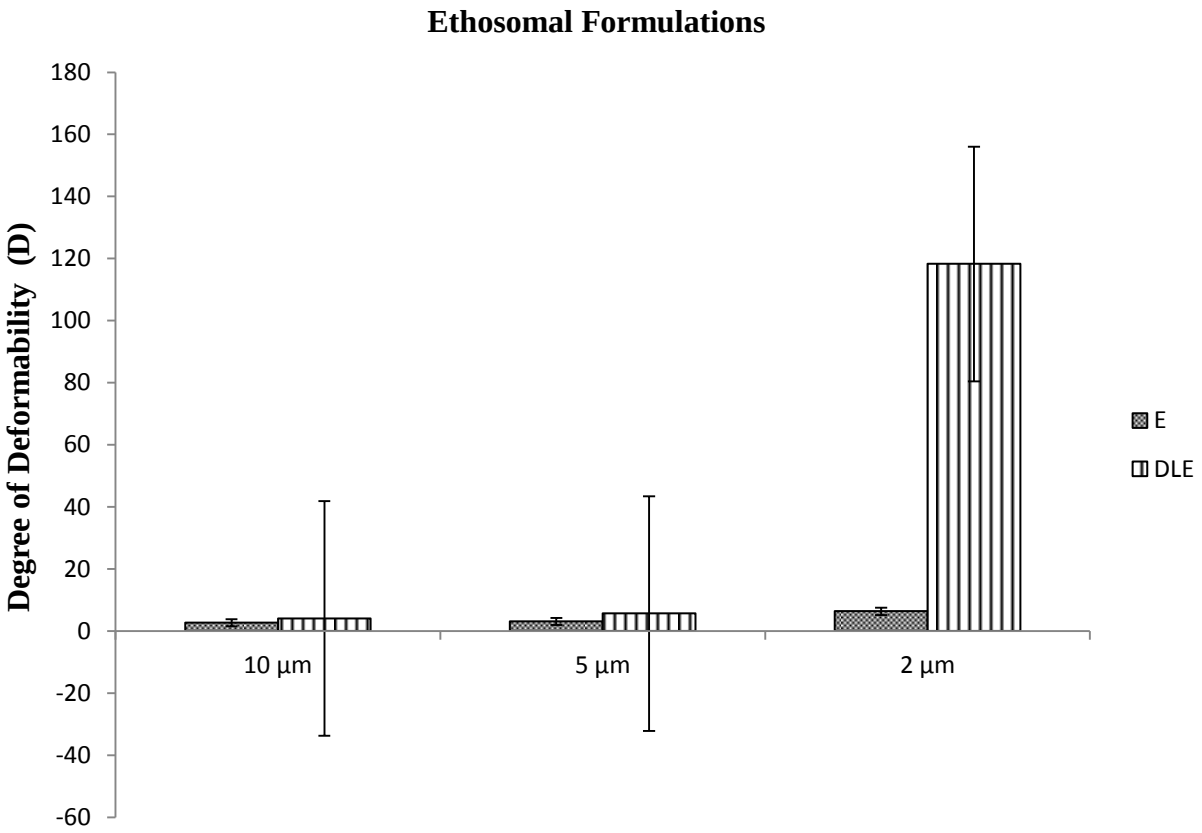

Figure 3.21: Bar charts showing degree of deformability for empty ethosomes (E) and drug-loaded ethosomes (DLE) following extrusion through membrane filters with different pore sizes 10 µm, 5 µm and 2 µm. The results represent the mean (±SEM) of two experiments (n= 2).

As shown in Figure 3.22, the transfersomal formulations were smoothly extruded through all the PMFP. However, while passing through 1 µm to 0.8 µm PMFP slightly back pressure of the sample was observed. A marked decreased in D for both TF-TW and DTF-TW could be observed thorugh progressive extrusion.

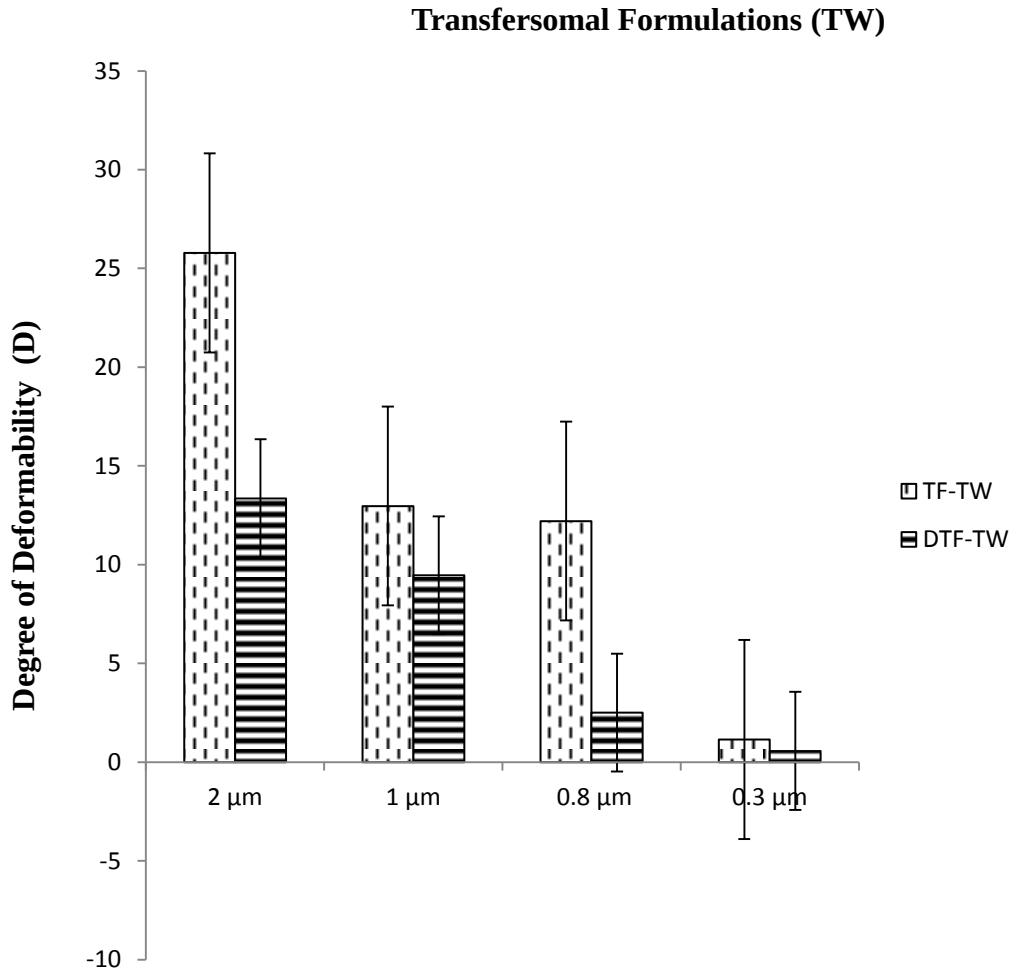

Figure 3.22: Bar charts showing degree of deformability for empty trasnfersomes using Tween 80 (TW) and drug-loaded transfersomes using Tween 80(DTF-TW) following extrusion through membrane filters with different pore sizes 2 µm, 1 µm, 0.8 µm and 0.3 µm. The results represent the mean (±SEM) of two experiments (n= 2).

In Figure 3.23, the results show that the transfersomal formulations were smoothly extruded through all the PMFP. A marked decreased in degree of deformability for both TF-SP and DTF-SP could be observed through progressive extrusion.

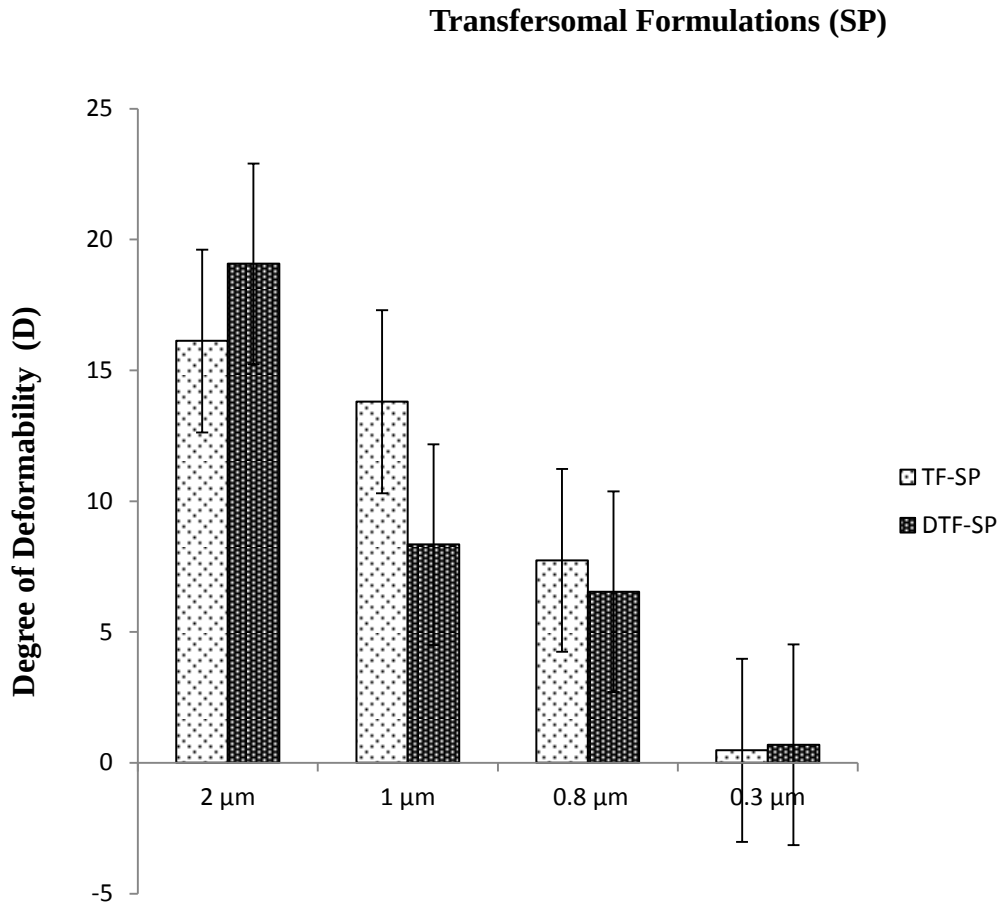

Figure 3.23: Bar charts showing degree of deformability of empty trasnfersomes using Span 80 (SP) and drug-loaded transfersomes using Span 80(DTF-SP) following extrusion through membrane filters with different pore sizes 2 µm, 1 µm, 0.8 µm and 0.3 µm. The results represent the mean (±SEM) of two experiments (n= 2).

As illustrated in Figure 3.24, the transfersomal formulations were smoothly extruded through all the PMFP. However, while passing through 2 µm to 1 µm PMFP slightly back-pressure of the sample was observed. A marked decreased in D for both TF-SCH and DTF-SCH could be observed thorugh progressive extrusion.

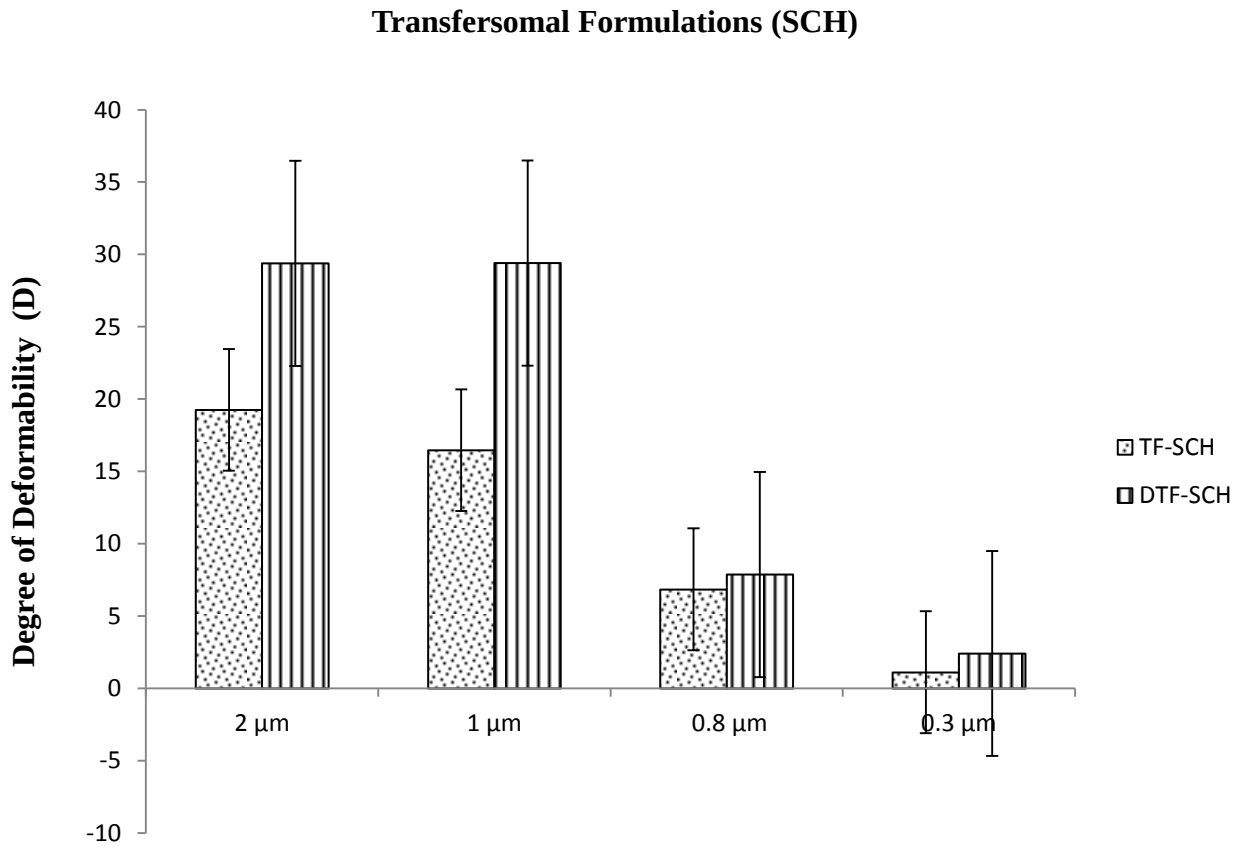

Figure 3.24: Bar charts showing degree of deformability of empty trasnfersomes using Sodium cholate hydrate (SCH) and drug-loaded transfersomes using Sodium cholate hydrate (DTF-SCH) following extrusion through membrane filters with different pore sizes 2 µm, 1 µm, 0.8 µm and 0.3 µm. The results represent the mean (±SEM) of two experiments (n= 2).

As revealed in Figure 3.25, the propanosomal formulations were smoothly extruded through all the PMFP. However, while passing through 2 µm to 1 µm PMFP, the sample look more diluted. A marked decreased in D for both P and DLP could be observed through progressive extrusion.

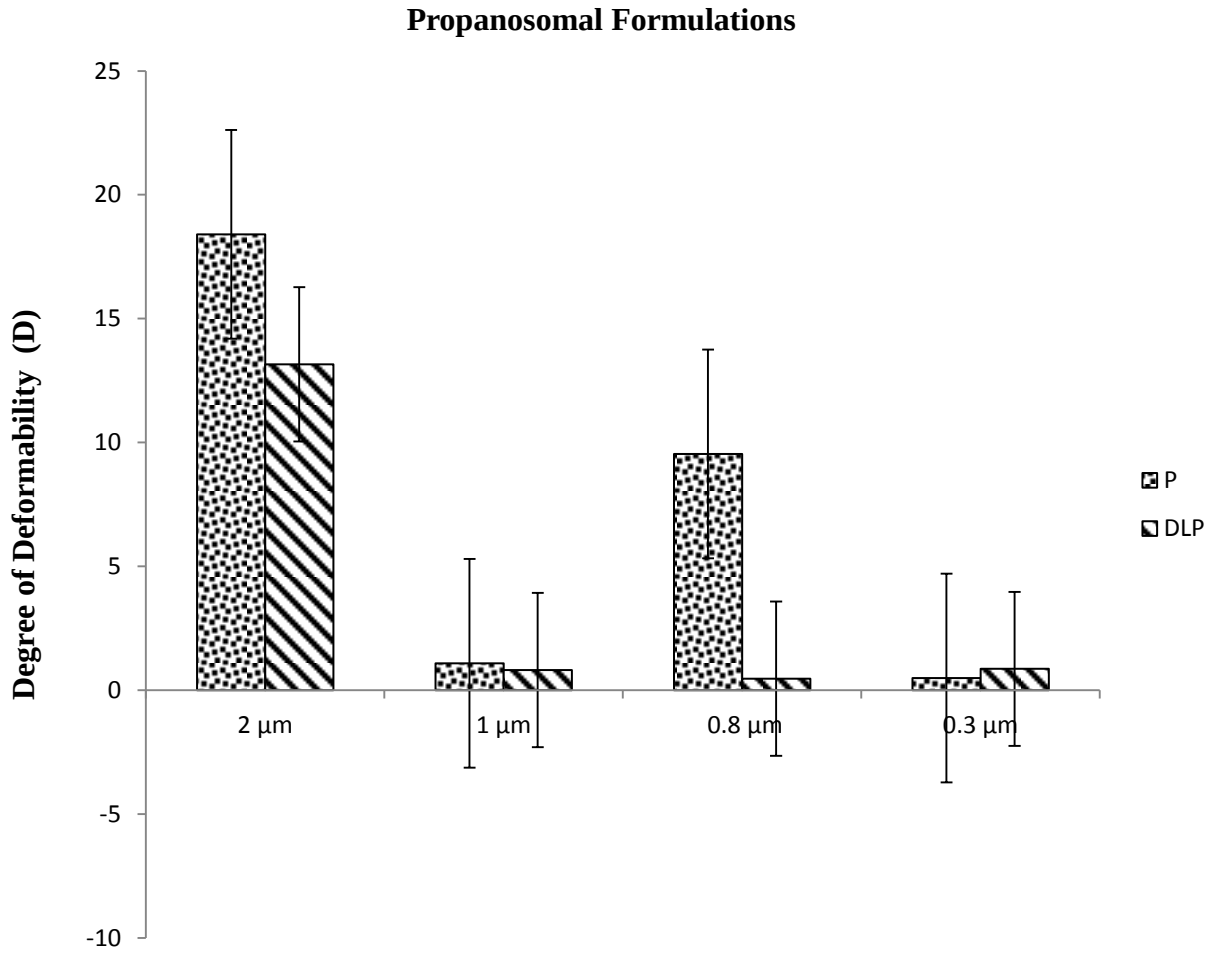

Figure 3.25: Bar charts showing degree of deformability of empty propansomes and drug-loaded propanosomes (DLP) following extrusion through membrane filters with different pore sizes 2 µm, 1 µm, 0.8 µm and 0.3 µm. The results represent the mean (±SEM) of two experiments (n= 2).

3.2.2 Disk diffusion testing

Agar disk diffusion testing was performed by addition of 2 µl of the formulations within the respective disks. The formulation treated disks were then impregnanted into the agar plates containing confluence of bacteria. The analyses of the disks were performed by overnight incubation at 37 ˚C for 18-24 hours in the incubator. The susceptibility of the bacteria towards the formulations was checked by measuring the zone of inhibition. A typical example showing the sample, which was added on the impregnated disk, contains 2 µl of chlorhexidine diacetate salt hydrates (15 mg/ml) testing, which is exhibiting the zone of inhibition (see Figure 3.26).

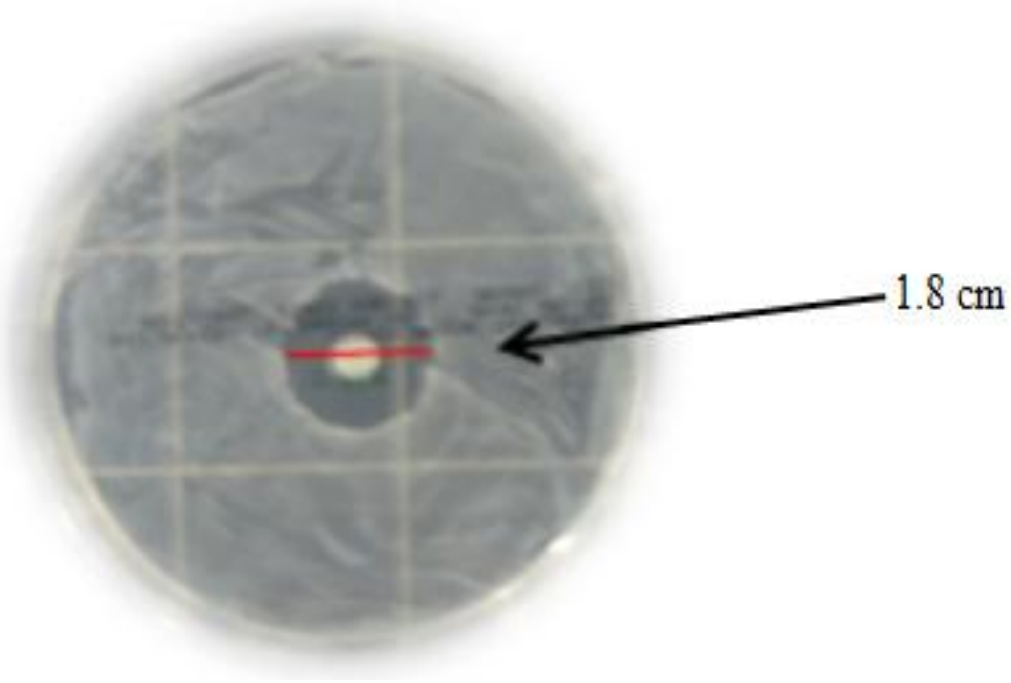

Figure 3.26: A photograph showing the plate exhibiting the measuring zone of inhibition (1.8 cm). The light grey shading represents a conflue of bacteria and the dark grey region represents no growth of the test bacteria (n=1).

Table 3.1 illustrates that none of the fomulations exhibited any zone of inhibition indicating that the bacteria showing no sensitivity in response to formulations. The only disk shown the zone of inhibition was the disk impregnanted with the solvent containing CHX diacetate salt hydrate. These results atleast revealed that the drug can be used against the bacteria.

Table 3.1: Table showing responses towards agar disk diffusion tests performed using various formulations and solvents.

Sr. No.	Formulations (Abbrevations)	Zone of inhibition (cm)	Total number of experiment performed (n)
01	L	-	2
02	TF-SCH	-	2
03	DTF-SCH	-	2
04	TF-S80	-	2
05	DTF-S80	-	2
06	TF-TW	-	2
07	DTF-TW	-	2
08	E	-	2
09	DLE	-	2
10	P (20%) v/v	-	2
11	DLP (20%) v/v	-	2
12	P (40 %) v/v	-	2
13	DLP (40%) v/v	-	2
14	Solvent containing 40% ethanol-water mixture	-	2
15	Solvent containing 70% ethanol-water mixture	-	2
16	Solvent containing 20% propanol+ 0.9% NaCl mixture	-	2
17	Solvent containing 40% propanol+ 0.9% NaCl mixture	-	2
18	Solvent containing 70% propanol+ 0.9% NaCl mixture	-	2
19	CHX diacetate salt hydrate (15 mg/ml)	1.4	1
		1.8	1

3.2.3 TEM Results

Figure 3.27 illustrates the structure of propanosomes prepared using SPC asn Span 80 surfactant. The picture indicted the unilamellar shape of vesicles.

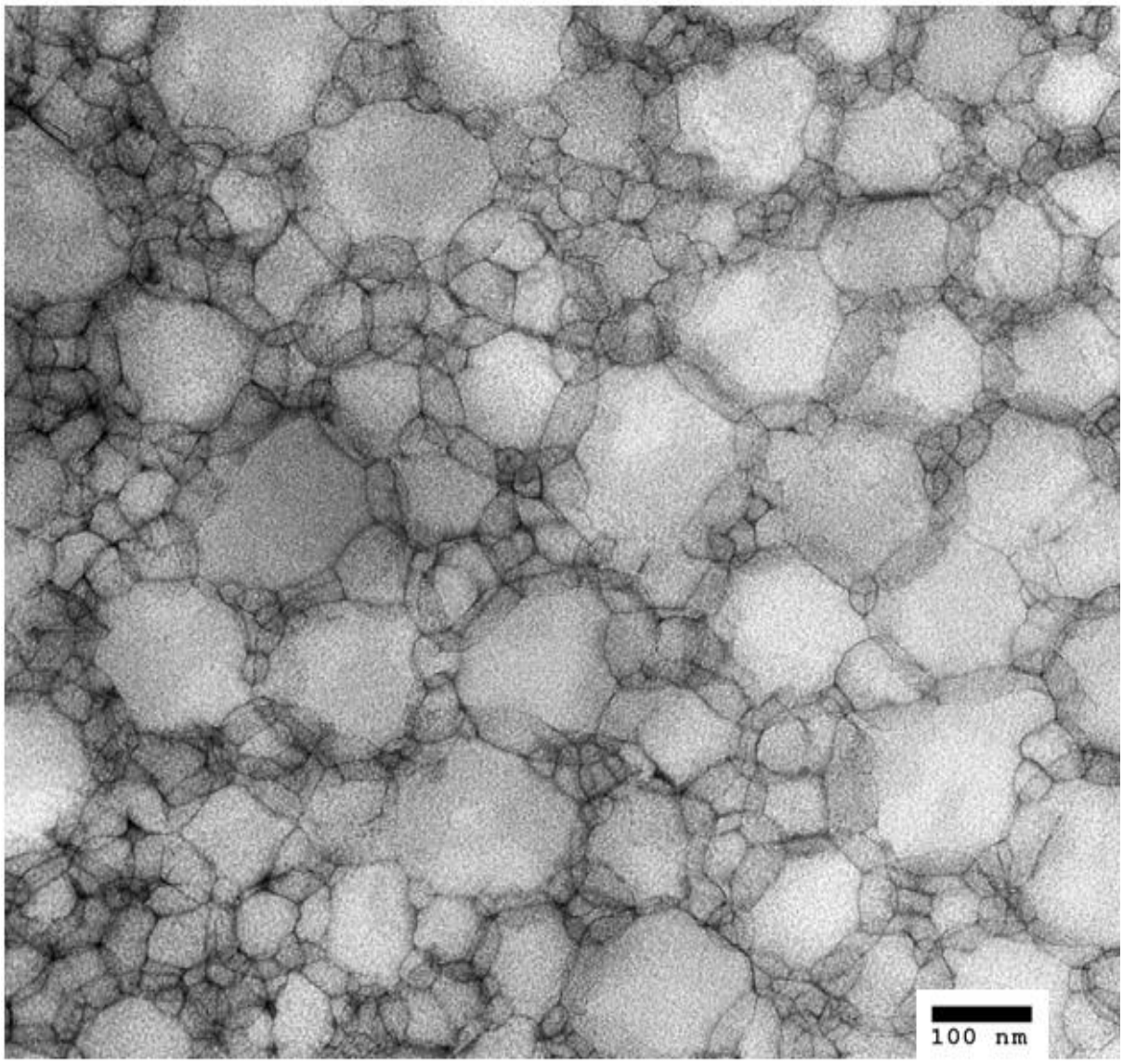

Figure 3.27: Transmission electron micrograph of propanosomes prepared using 20% propanol. This micrograph is typical of 2-3 such different experiments.

Figure 3.28 illustrates the structure of drug-loaded propanosomes. It indicates the disruption in the bilayer of popanosomes. It showed that after adding the drug the membrane got disrupted although the concentration of the drug remained low in the formulation.

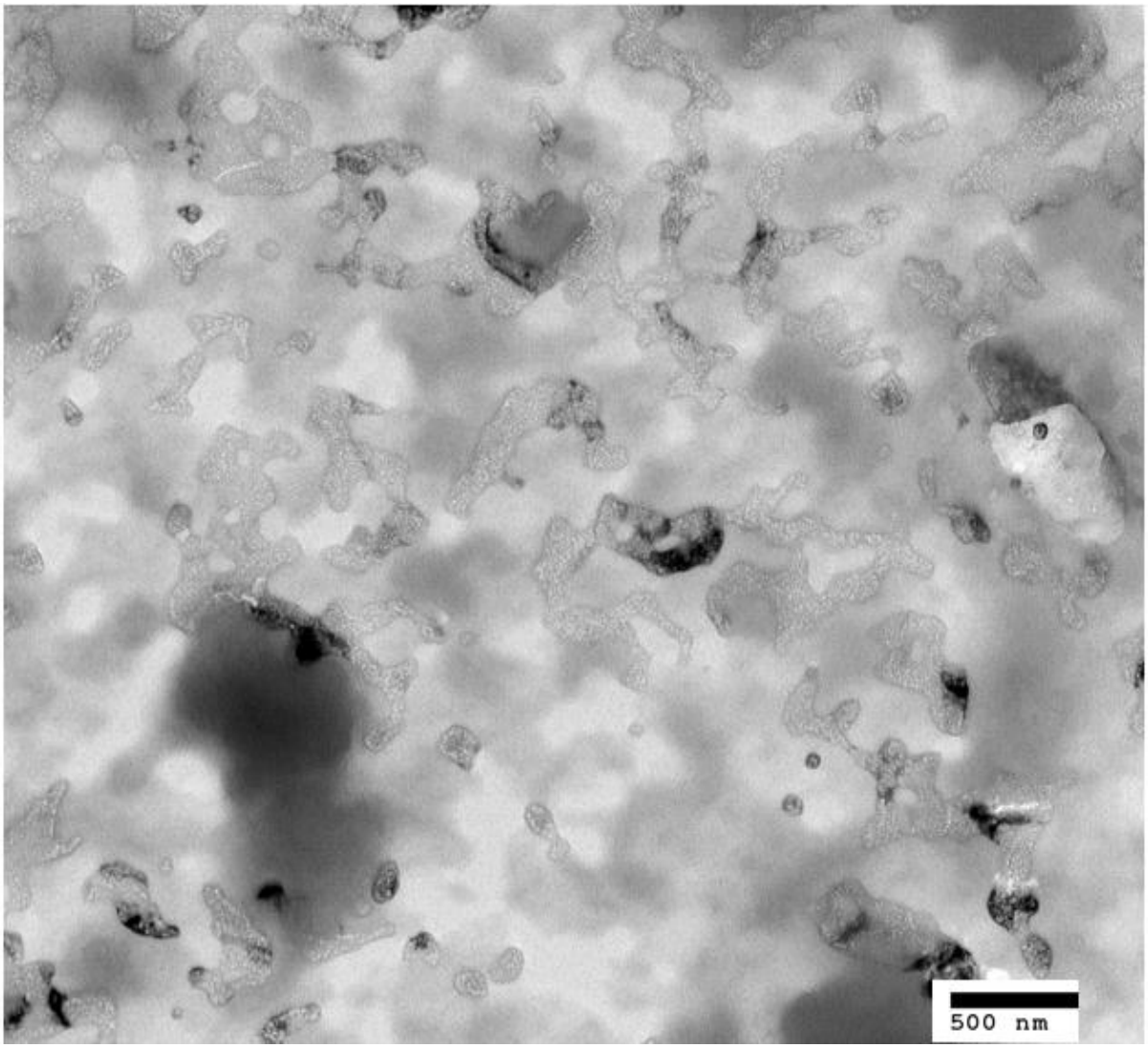

Figure 3.28: Transmission electron micrograph of drug loaded propanosomes prepared using 20% propanol. This micrograph is typical of 2-3 such different experiments.

Figure 3.29 illustrates the structure of drug-free propanosomes prepared using 40 % (p/v) propan-2-ol which imparted a membrane disrupting effect in propanosomes.

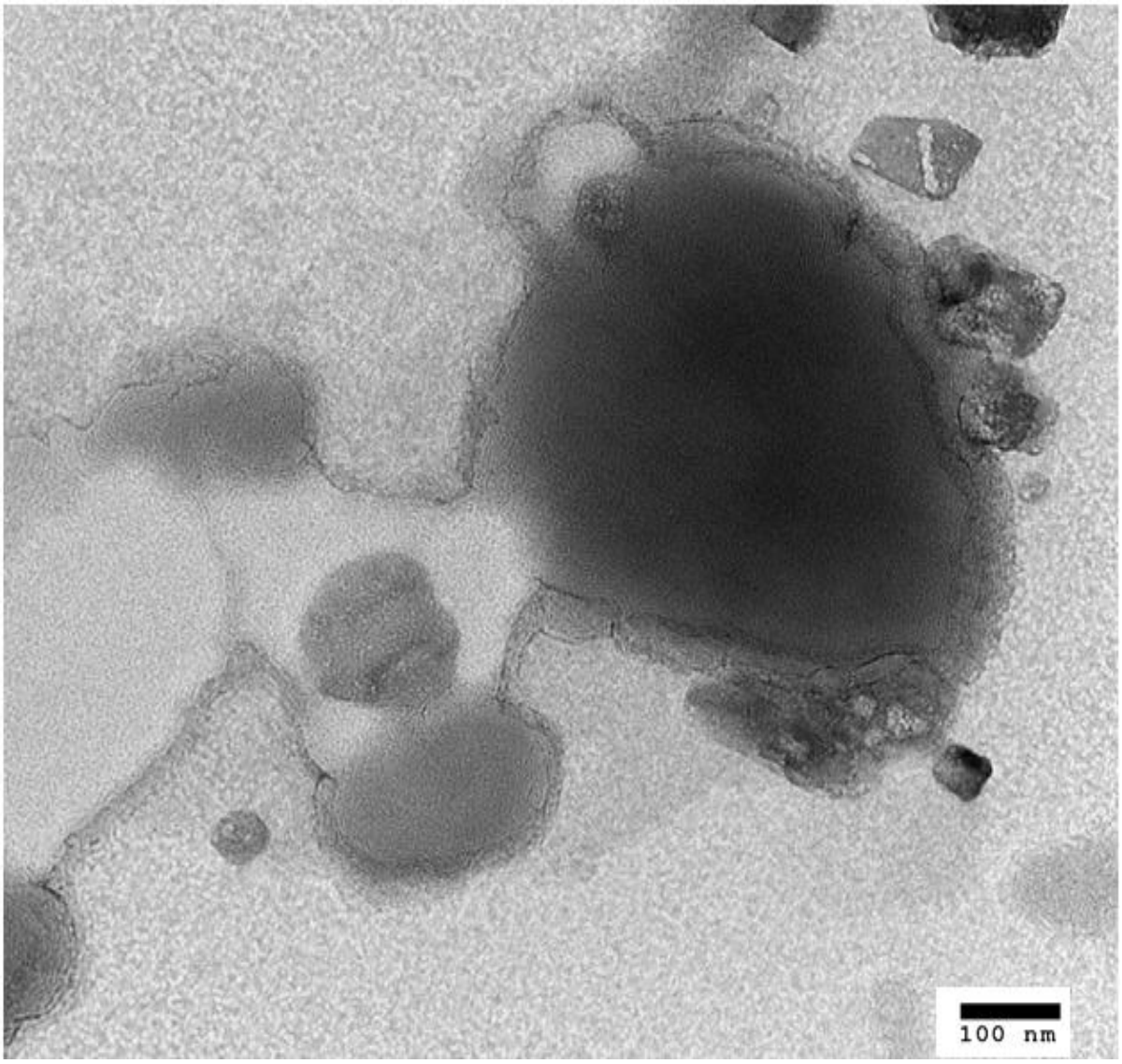

Figure 3.29: Transmission electron micrograph of empty propanosomes using 40 % (p/v) propan-2-ol. The picture was taken at 100 nm magnification. This micrograph is typical of 2-3 such different experiments.

4 Discussion

4.1 Reasoning for optimised results

During the course of experiments all formulations were prepared using thin-film hydration method. This method yields heterogeneous sized population of MLVs over 1 µm in diameter. Further extrusion technique was employed to achieve a homogenous population of formulation of the desired size. The size and zeta potential of the formulations were then analyzed (Doherty, 2004).

The optimised results consisted of data obtained from particle size analysis which includes, size and size distribution (Span or PDI) and from zeta potential measurements. While laser diffraction was used to measure the size that is expected to be 1 µm or larger and dynamic light scattering was employed to measure the size of particles in the sub-micron range. As reported by McConnell (1991), liposomes made from unsaturated phospholipids (such as SPC) have high fluidity when compared to saturated phospholipids and they spread more rapidly at the air/ liquid interface due to their low phase transition, and is expected to be adsorbed on the W/O interface rapidly (Wang et al., 2011).

The crude heterogeneous populations of formulations were then allowed for sizing for homogeneous distribution within a certain size range. The sizings of the liposomes were performed by sequential extrusion at relative low pressure using polycarbonate membrane filter pores. It is necessary to seal the membrane holder tight to avoid any possible leakage during extrusion of liposomes (Olson, et al., 1979). In the drug-loaded formulations chlorohexidine base was added in the solvent of the lipid phase, as it is a lipophilic in nature, hence was able to easily dissolve with the lipids.

Chlorhexidine base, 1,6-di-(4-chlorophenyldiguanido) hexane is a white crystaline solid (m.p. 132 ºC, mw 505.5) (Figure 1.13). CHX base and its salts are widely used as antiseptics and disinfectants in pharmaceutical and cosmetic formulations (Wade et al., 1994). Previous studies have shown that encapsulation of CHX base proven to be more effective against several bacteria when released into the target site. Furthermore, studies have shown that sustain release of base enhances drug delivery by mediating a more direct and prolonged contact between the carrier and the bacteria, skin surface and skin follicles are shown to present a prolonged *ex-*

vivo topical antimicrobial activity against *Staphylococcus epidermidis*. The *in-vivo* studies may confirm these findings (Lboutounne et al., 2002).

For optimised results SPC was used over DPPC for the preparation of the formulations. The reason for SPC to be chosen over DPPC for performing the further experiments was due to its lower transition temperature. Hence, hydration and extrusion were performed at room temperature which was highly convenient. As DPPC having Tc as 41º C (Szoka & Papahadjopoules, 1980) and SPC having Tc of minus 15º C (O'Neill and Leopold, 1982), the lower transition temperature of SPC offers advantages over higher transition temperature of DPPC as the bioactive drugs will be release slowly in DPPC due to its higher transition temperature. Also, liposomes made from DPPC were more time consuming to prepare and extrude. It has been proven that liposomes with high phase transition temperature may release the drug more slowly than liposomes made from lower phase transition temperatures (Betz et al., 2005; Mozafari & Mortazavi, 2005). As mentioned by El Maghraby et al. (2006), DPPC are rigid in structure due to which it would be difficult for them to pass through small PMFP for extrusion. Moreover, it was preferred to keep the same phospholipid for all the formulations as SPC to offer higher ultra-deformable properties.

Figure 3.2 revealed that zeta potential values of empty liposomes were negative due to the presence of SPC mixture of lipids; this came in agreement with other reports (O'Neill and Leopold, 1982). Using drug loaded liposomes the zeta potential drastically increases to positive values, due to the presence of CHX base which is symmetrical molecule with two *p*-chlorophenyl substituted biguanide groups, which provides four sites for protonation (Nishihata et al., 1994). There are two pK_a values observed for CHX (pKa = 10.3 and pKa = 2.2) and between pH 4 and 8, the CHX dication is the major species in solution (Hugo et al., 1964). Therefore, the CHX dication localized at the nanocapsule-solvent interface would confer a positive charge on the colloidal carrier (Lboutounne et al., 2002).

Interaction of lecithin and cholesterol without non-ionic surfactant exhibited negative charge on empty liposomes (Muthprasanna et al., 2010). Addition of CHX base within the formulation made the net overall charges of the formulation

positive (Lboutounne, et al., 2002). This clearly support the agreement of other findings with the findings reported in this project.

Figure 3.3 showed that progressive extrusion has reduced the size of liposomes effectively, regardless of drug inclusion in the formulations. However, the drug loaded liposomes were similar or not much different from empty liposomes as the added CHX base was of very low concentration (0.02% w/w). The other related compound, poly-hexamethylene biguanide (PHMB) shows no interaction with neutral PC, but a strong preference for anionic phosphatidylglycerol headgroup (Ikeda et al., 1983), which was not used, in the present report. This indicates that if concentration within the SPC based formulations was increased, it would expect that the liposomal formulation would not be able to encapsulate drug at higher concentration. Optimisation of entrapment of the drug should be carried out for further experimental analysis.

When empty liposomes (L) were compared with the drug-loaded liposomes (DLL), the span was smaller for DLL (Figure 3.4). This indicates that DLL formulations were less polydispersed. The decrease in polydispersity of DLL might be attributed to higher zeta potential of these formulations compared to empty liposomes resulting in individual repulsion of the vesicles and decrease in vesicle aggregation.

Figure 3.5 showed the positive zeta potential values for ethosome formulations. The reason of the positive zeta potential is not clear and needs furture research.

Figure 3.6 demonstrated that mean size of the ethosomes which was unaffected by drug inclusion in the formulations. In the previous studies, it has been shown that short chain alcohols may have an effect on the main transition temperature of the phospholipid (Rowe, 1983). It had also shown that this interdegetated phase have been performed on MLVs preparation. This phase may be present in SUVs and extrusion technique plays an important role in determining interdegetation (Komatsu et al., 1993). Ethanol seems to change the packing of the bilayer, making the lipid bilayers interdegetated and may displace the drug to the outer layer of the ethosomes. The small particle size of DLE compared to E may be due to the presence of CHX base in the interdegetated bilayers. The similarity in size is due to high flexibility of the vesicles because of addition of ethanol as permeation

enhancer to promote elasticity of the vesicles. The particle sizes of ethosomes are comparatively higher than other formulations due to the presence of ethanol which is responsible for large sized vesicles. It also makes the ethosomes deformable (Muthprasanna et al., 2010).

Figure 3.7 showed that for empty crude ethosomal preparation, the polydispersity was similar to that of the drug loaded ethosomes. The polydispersity of the formulations remained consistent due to the elasticity of the vesicles (seen in extrusion through 10 and 5 µm filters) and for 2 µm PMFP, the empty ethosomal vesicles were destabilized, possibly resulting in translocation of the drug due to excessive extrusion pressure.

Tween 80 was added in the formulation as edge activator in order to improve the elasticity of the vesicles. It is a nonionic surfactant with a large head group (containing about 20 polyoxyethylene units) and an HLB value of 15, making it miscible in water. Thus, Tween 80 is expected to partition between lipid bilayers and aqueous phase (El Maghraby et al., 2004).

Tween 80 contains the ethylene oxide and a long hydrocarbon chain imparting both lipophilic and hydrophilic characteristics to the vesicular formulation. It has been reported that Tween 80 may interact with the polar head groups of the lipids and the modification of H-bonding and ionic forces may occur (Nokhodchi et al., 2003).

The DTF-TW formulations had positive zeta potential values due to presence of CHX base whilst TF-TW had negative zeta potential measurements when using 2 and 1 µm polycarbonate membrane filters (Figure 3.8). The TF-TW formulations crude and those extruded through 0.8 µm and 0.2-0.4 µm filters had positive zeta potential values (Figure 3.8) which may be due to the presence of Tween 80. The present results disagree with Lee et al. (2005) who reported that Tween 80 transfersomes exhibited a more intense negative zeta potential compared to the transferosomes manufactured in this study. However, zeta potential may lead to greater repulsion between the bilayers, thus an increase in the size of Tween 80 containing vesicles could be seen (El Zaafarany et al., 2010). Figure 3.9 demonstrated a negligible difference in particle size between TF-TW and DTF-TW. Previous reports have shown homogeneously sized transfersomes following

extrusion through polycarbonate membranes (Ita et al., 2007). Due to higher HLB value of Tween 80, it may form larger particle size transfersomes compared to other surfactants (El Zaafarany et al., 2010).

In other studies, it has been observed that Tween 80 can lower the affinity for lipids so they do not favour lipid vesicle formulation as shown by Muthuprasanna et al. (2010). The present results disagree with these represented by Muthuprasanna et al. (2010), as the transfersomal formulations using Tween 80 were successfully produced and moreover, they were reduced in size upon extrusion. Difference in findings between different studies might be attributed to different formulation conditions.

Figure 3.10 demonstrated that the transfersomes for both TF-TW and DTF-TW were monodispersed, indicating the formulation protocol used in this study offered high control over particle size distribution of these vesicles. Span 80 has the shortest hydrophobic hydrocarbon chain and is a nonionic surfactant (Ita et al., 2007; Wei et al., 2007). It has relatively small head group compared with Tween 80, as it lacks the polyoxyethylene units. It has higher affinity towards lipids as it has lower HLB value of 4.3. It is lipophilic and immiscible with water and thus its distribution in lipid is higher than water (El Maghraby et al., 2004).

Figure 3.11 showed transfersomes formulation attributing overall positive zeta potential. The reasons are not clear. The experiment needs future research.

In a recent study conducted by Muthprasanna et al., (2010), the surfactants during formulation acted as an efficient emulsifing agent resulting in decreasing the particle size and promoting vesicle flexibility and penetration ability. This is in agreement with the results shown in Figure 3.12 using TF-SP with a particle size range of 80-90 nm. This could be due to the influence of Span 80 (oil and water emulsifier). Hence, Span 80 is suitable for producing small sized vesicles that are capable to pass through PMFP allowing higher drug proportions to be delivered (Hobbs et al., 1998; Unezaki et al., 1996). Also, Span 80 has high hydrophobicity, which also adds to its advantages.

The polydispersity was higher in DTF-SP and TF-SP (Figure 3.13). This is due to instability of the formulation, which was accompanied by foam formation. As

Span 80 is anti-foaming agent, it reduces the surface tension but here addition of the drug has caused formation of foam. Foam formation should always be avoided because it reduces the uniformity of the formulation (Jin et al., 2008).

Sodium cholate hydrate is a solubilising agent, which may increase the solubility of poorly soluble materials. Based on HLB values, SCH has a value of 16.7, revealing its low affinity towards lipids (El Maghraby et al., 2004). Studies have shown that anionic surfactants cause greater tissue damage than non-ionic surfactants (Som et al., 2012). Hence, SCH was not preferred for further analysis of novel lipidic formulations.

SCH is an anionic surfactant because its hydrophilic moiety bears a negative charge (Som et al., 2012). Unexpectedly, Figure 3.14 showed that zeta potential values were positive for all formulations including TF-SCH and DTF-SCH. This might be attributed to the high hydrophilic properties of SCH; hence it was not heavily associated with the lipid bilayers.

Figure 3.15 demonstrated an interaction of SCH and CHX base might be responsible for the larger particle size in the formulation for DTF-SCH that may contribute to drug leakage or instability of the bilayer upon inclusion of the drug in the formulation. Figure 3.16 showed that the polydispersity was similar for both the TF-SCH and DTF-SCH formulations. Previous findings have shown the presence of SCH to be responsible for making the particle size distribution more uniform (Ping and Jiao, 2000). However, the increase in polydispersity for DTF-SCH may indicate instability of the formulation, which could be due to excessive pressure applied upon extrusion through 1 µm PMFP.

Propanosomes were prepared by combining the ethosomal and trasnfersomal protocol. For enhancing the penetration property, propanol was added for improving the flexibility of the membrane bilayers, surfactant Span 80 was added in order to make ultra-deformable lipidic formulations. The formulations are supposed to serve as anti-bacterial without the drug loaded in the formulation by exploiting the antibacterial properties of propanol. The antibacterial susceptibity of the formulation was discussed in section 4.3.

Figure 3.17 demonstrated a positive surface charge for drug-loaded propanosomes. As expected, the drug-loaded formulation contains the CHX base which is contributing overall to the yield of a positive charge. The empty propanosomes had negative zeta potential measurement, confirming the responsibility of the drug for the positive zeta potential. This also clearly reveals the association of the drug with the bilayers of the propanosomes.

Figure 3.18 showed no difference in particle size when empty propanosomes and drug-loaded propansomes were compared. The propanosomes had smaller particle size as expected for transfersomal formulation, indicating that the formulation was flexible enough to pass through PMFP. However, drastic decrease from 1 µm PMFP to 0.2-0.4 µm PMFP showed that the formulation possibly became instable upon pressure application (e.g. upon extrusion).

Figure 3.19 showed monodispersed distribution of the particles, however, the drug-loaded propanosomes may exhibit instabilities, as the polydispersity was inconsistent through progressive extrusion. It is possible that extrusion has caused leakage of the drug from the bilayers in the form of crystals. This may contribute to the broad size distribution by having propanosomes particles in the nano range and drug crystals in the micro range within the same preparation. This agrees with light microscopy observation using other hydrophobic drugs (e.g. beclometasone dipropionate) in liposome formulations (Batavia et al., 2001).

4.2 Degree of deformability

Figure 3.20 represents the degree of deformability (D) study for L and DLL showing higher D value compare to other formulations (ethosomes, trasnferosomes and propansomes). This is due to higher particle size of liposomes compared to other formulations, as the liposomes were more rigid in structure due to the presence of cholesterol. However, the passage for liposomes through the filter pores was more difficult through the small pores, hence higher pressure was required.

Moreover, ethosomes had extremely low D (Figure 3.21) which could be due to the presence of ethanol as a penetration enhancer. The D for ethosomes are same

and low for 10, 5 and 2 µm PMFP passage for E and DLE with an exception for increased D using drug loaded ethosomes. This is possibly due to aggregation of the particles, which underwent extensive stress due to extrusion. The deformability in the transfersomes is influenced by the presence of edge activators that could be correlated with the difference in chemical structure of these molecules.

In previous studies, it has been shown that Tween 80 may confer very high deformability on vesicles. This could be due to the higher flexible and non-bulky hydrocarbon chain of Tween 80. By studying the findings in Figures 3.22-3.24, the same phenomenon could be observed where in the D is decreasing in size in both TF-TW and DTF-TW. However, it has been observed that TF-TW had higher D in comparison with DTF-TW. This could be due the presence of drug in DTF-TW. Also studies showed that Span 80 has lowest deformability, due to their hydrophobicity which reduces the formation of transient hydrophilic holes in the membrane, hence minimizing the amphiphilic property of the bilayers and reducing membrane fluidity (El Zaafarany et al., 2010). The results for the experiments in this project (Figures 3.22-3.24) agree with the study conducted by El Zaafarany and co-workers (2010). Finally, sodium cholate hydrate containing trasnfersomes showed least D compared to Tween 80 and Span 80 containing transfersomal formulations. Sodium cholate hydrate has steroid-like-structure which are bulkier than the other hydrocarbon chain containing surfactants (El Zaafarany et al., 2010).

The significance of drug release could be explained by variations in molecular ordering and deformability caused by edge activator (i.e. the edge activator possessing transfersomes shows highest deformability with highest drug release and vice-versa (El Zaafarany et al., 2010).

Figure 3.25 showed inconsistency of the D values of the propanosomal formulation regardless of the presence of drug. Further investigations of the propanosomal formulation through extrusion are needed for the introduction of a convincing justification.

4.3 Agar disk diffusion testing

The number of methicillin-resistant (MR) *Staphylococcus aureus* and MR coagulase negative *staphylococci* (CoNS) strains has been increasing (Jain et al., 2008). Methicillin-resistant *Staphylococcus aureus* (MRSA) strains have also emerged as a frequent cause of community acquired infections (Naimi et al., 2003). It is also becoming one of the major causes for bacterial infections underlying soft tissues of the skin among the patients who are visitors in clinics and office-based practices (Nathwani et al., 1998; Tice et al. 1995; Dykhuizen et al., 1994). To determine the susceptibility of these bacteria, there are several methods such as disk diffusion test that helps in determining the MIC (Minimum Inhibitory Concentration). The experiments conducted in this study, MIC testing was performed to determine the antimicrobial activity of the formulations against the *S. aureus* bacteria. CHX diacetate salt hydrate is an effective antibacterial drug against these bacteria as shown in Figure 3.38.

A test for control formulations, i.e. liposomes, transfersomes with sodium cholate hydrate, transfersomes with sodium cholate and transfersomes with Tween 80 were individually added on the impregnated disks. It could be seen that the disk result shows that these control formulations did not show any zone of inhibition around the disk. The results were as expected as they included no drug. However, all formulations containing the drug were expected to show zones of inhibition but the results showed no zones of inhibition (Table 3.3). This was probably due to the contaminents within the formulations which may be acting as a source of nutrient for bacterial growth.

It was expected to get a zone of inhibition upon using ethosomal formulations as ethosomes contain 40% ethanol even though ethanol is used as antiseptic. Throughout this series of experiment, it has been proved that ethanol has less antibacterial activity as stated by Macdonnell and Rusell (1999) in which they have clearly mentioned that ethanol is effective against viruses. A reason behind the unexpected finding for ethosomes is possibly due to the added phospholipid and cholesterol that may act as bacterial growth promoter.

Similarly, Table 3.3 showed propanosomes based on the concentration of propanol within the formulation which caused no zone of inhibition, indicating they have no

killing effect of bacteria. This was also the case when CHX was included within propanosome formulations. In these experiments, propanosomes were supposed to show a zone of inhibition as propanol is considered slightly more effective against bacteria compared to ethanol (Coulthard & Skyes, 1936). The possible reason could be the added phospholipids and surfactants that were utilised by the bacteria as a source of nutrition for bacterial growth.

Table 3.1 (sr. no 1-13) showed a selection of formulations chosen for investigation as to check whether they cause zone of inhibition as in previous experiment the formulations were not showing any zone of inhibition. Table 3.1 (sr. no. 14 and 15) further demonstrated an investigation aiming to determine the specific concentration of ethanol (i.e. whether 20 and 40%) that would be effective against bacterial growth. The results showed no zone of inhibition, which indicates that even 20 or 40% of ethanol was not effective against bacteria. The possible reason could be the low amount of solution used for analysis.

Similarly, Table 3.1 (sr no. 16-18) was presented to investigate the concentration of propanol required to inhibit the bacterial growth. The concentrations investigated were 20, 40 and 70% propanol in 0.9 % NaCl solution. As for ethanol, none of these solutions showed a zone of inhibition, possibly also because of the low amount used. Perhaps the clinical studies have been demonstrated that 70 % (p/v) propan-2-ol have been demonstrated as significantly a better skin disinfectant in patients receiving pre-operative skin antisepsis on abdominal sites (shown n = 106) (Hibbard et al., 2000).

4.4 TEM results

Transmission electron microscopy indicates the general images of propanosomal vesicular formation. At 100 nm magnification, the particles seemed to be unilamellar vesicles. The image indicated that the propanosomal formulation was composed of small unilamellar vesicles (Figure 3.27).

When drug loaded propanosomes were observed at 500 nm magnifications under TEM, the image shown that the lipid bilayers were disrupted which might be due

to addition of the drug. However, no drug particles were observed, as the concentration of added drug was too low; also it seems that no clear propanosomes were formed (Figure 3.28). The Figure also showed the disrupted structure of the vesicles probably due to higher concentration (40% propanol) included in the formulation. The image has clearly indicated membrane disruption and open structures (Figure 3.29).

5 Conclusion and Scope of Future studies

5.1 Conclusion

Novel lipidic formulations of propanosomes were successfully prepared using the thin-film hydration technique. They were ultra-deformable and reduced in size 80-100 nm upon passing through PMFP via lipid extrusion. The propanosomes also acted as carriers for CHX base for potential skin treatment application.

The reported results showed that the mean size of propansomes was inherited from transferosomes and the deformability and charge characteristics of ethosomes. The plan of combining the preparatory method for trasnsfersomes and ethosomes were successfully achieved in the form of propanosomes. However, the agar disk diffusion antimicrobial testing showed no antimicrobial property of propanosomes with or without the drug, CHX. However, it was acknowledged that the drug content may have been too low for any significant antimicrobial effect to be observed. The use of surfactant Span 80 as the edge activator for the preparations of propanosomes provided better results so it can be optimised for further testing.

TEM images have indicated SUVs of propanosomes, but the particle sizes were all disrupted. The reason is unknown, but this probably may have been due to the preparatory ingredients such as the inclusion of propanol, an alcohol similar to ethanol that is known to induce structural differences in lipidic membranes. The results also show the interesting property of the propanosomes such as smaller size, positive surface charge, ultradeformability which can be investigated further for skin treatment.

5.2 Scope for future studies

1) The concentration of added drug should be increased followed by investigating the antimicrobial efficiency.

2) Different forms of CHX such as base and salts should be tested individually by incorporating them in the formulation with the aim of enhancing the antimicrobial effects.

3) DSC studies can be performed to intensify and expand the knowledge of drug interaction with the bilayers of lipid-based vesicles. DSC could also provide information on the role of excipients used in bilayer fluidity and permeability.

4) Stability testing can be performed for the formulations by keeping them for six months and checking their size and zeta potential and their ultradeformability.

5) Propanosomes with 20% propanol added showed no anti-microbial effect so intermediate conentrations (25, 30, 20% (v/v) should be tested for further investigastion.

6) Propanosomes and other formulations deformability studies can be performed on a skin model.

7) Transfersomes made from combinations of Span 80 and Tween 80 may produce better MLVs which are more stable compared to Tween 80. Further studies can be performed based on their combitional preparation (Muthprasanna, 2012).

8) The concentration of Span 80 in the formulation should be optimized further for testing the entrapment efficiency of the drug.

9) The agar disk diffusion test may not be the best test to check the susceptibility of the bacteria. Hence using an alternative bacterial sensitivity test is required.

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