INTRODUCTION

Lipids span a large and diverse class of naturally occurring organic compounds, which are non-polar in nature and hence, soluble in organic (less polar than water) solvents, while being insoluble/partially soluble in aqueous (polar) environments. They are majorly composed of chains of hydrocarbons in their highly reduced form. Most membrane lipids are amphipathic. The dual characteristic of these lipids is attributed to two different structural regions: a lipophilic (hydrophobic - uncharged) region consisting of chains of fatty acids (more precisely acyl groups) and a hydrophilic (charged) region containing polar head groups, which interacts differently with water than the hydrophobic region. The polar head groups project outside and get exposed to water, while the hydrophobic non-polar tails are shielded from water and stay inside. This rearrangement helps them in forming spontaneous molecular aggregates, and is relevant structural signatures of liposomes and membrane bilayers in an aqueous milieu. In biological systems, common forms of lipids include fats, waxes, sterols, phospholipids and triglycerides, *etc*., and are essential components of cell membranes, with functions such as energy storage, providing insulation, forming water repellent layers on leaves, providing building blocks for steroid hormones like testosterone and are involved in cell signalling. Lipids with saturated fatty acids have higher melting point, and the melting temperature commonly increases with increase in hydrocarbon chain length (uniform rod-like shapes of molecules) and decreases with the extent of unsaturation in the fatty acyl chains. Mammalian species use various biosynthetic pathways both to synthesize and break down lipids; however, some ‘essential’ lipids like α-linoleic acid and linoleic acid containing lipids cannot be synthesized by these pathways and therefore must be obtained from the diet.

1. Fatty acids

Fatty acids are long chain hydrocarbons containing a terminal carboxyl group, which may either be saturated or unsaturated, depending upon the number of hydrogen bonds. Most naturally occurring fatty acids have an unbranched chain of an even number of carbon atoms, from 4 to 28. Fatty acids are usually derived from triglycerides or phospholipids.

Types of fatty acids: Fatty acids having carbon–carbon double bonds (*i.e* less than the maximum number of hydrogen bonds) are known as unsaturated. A fatty acid having one double bond is called "monounsaturated", while fatty acids having multiple double bonds are known as "polyunsaturated". Comparably, fatty acids lacking any double bond (*i.e.* maximum number of hydrogen bonds) are known as saturated. Some common fatty acids are enlisted in Table 1.

Table 1. Commonly used saturated and unsaturated fatty acids.

Saturated fatty acid		**Unsaturated fatty acid**	
Common name	Chemical structure	Common name	Chemical structure
Caprylic acid	$CH_3(CH_2)6COOH$	Myristoleic acid	$CH_3(CH_2)3CH=CH(CH_2)7COOH$
Capric acid	$CH_3(CH_2)8COOH$	Palmitoleic acid	$CH_3(CH_2)5CH=CH(CH_2)7COOH$
Lauric acid	$CH_3(CH_2)10COOH$	Sapienic acid	$CH_3(CH_2)8CH=CH(CH_2)4COOH$
Myristic acid	$CH_3(CH_2)12COOH$	Oleic acid	$CH_3(CH_2)7CH=CH(CH_2)7COOH$
Palmitic acid	$CH_3(CH_2)14COOH$	Elaidic acid	$CH_3(CH_2)7CH=CH(CH_2)7COOH$
Stearic acid	$CH_3(CH_2)16COOH$	Vaccenic acid	$CH_3(CH_2)5CH=CH(CH_2)9COOH$
Arachidic acid	$CH_3(CH_2)18COOH$	Linoleic acid	$CH_3(CH_2)4CH=CHCH_2CH=CH(CH_2)7COOH$
Behenic acid	$CH_3(CH_2)20COOH$	Linoelaidic acid	$CH_3(CH_2)4CH=CHCH_2CH=CH(CH_2)7COOH$
Lignoceric acid	$CH_3(CH_2)22COOH$	α-Linolenic acid	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)7COOH$
Cerotic acid	$CH_3(CH_2)24COOH$	Arachidonic acid	$CH_3(CH_2)4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)3COOH$

Fatty acids can also be classified according to their chain lengths. These are as follows:

- Short-chain fatty acids (SCFA): Aliphatic tails of fewer than six carbons.
- Medium-chain fatty acids (MCFA): Aliphatic tails of 6–12 carbons.
- Long-chain fatty acids (LCFA): Aliphatic tails 13 to 21 carbons.
- Very long chain fatty acids (VLCFA): Aliphatic tails longer than 22 carbons.

2. General classification of lipid

The classification of lipids can generally be delineated as: simple lipids or homolipids (esters of fatty acids only with alcohols), compound lipids or heterolipids (esters of fatty acids with alcohols and other additional functional groups) and derived lipids (derived from simple and compound lipids by hydrolysis).

2.1. Simple lipid / Homolipid

Fats and oils	Waxes
Triglycerides	Sperm whale wax
Simple triglycerides	Beeswax
Mixed triglycerides	Carnauba wax

2.2. Compound lipids / Heterolipids

Phospholipis	Glycolipids
Phosphatids Phosphoglycerides Lecithin Cephalins Plasmalogens	Cerebrocides Kerasin Phrinosin Narvon Oxynorvon
Phosphoinositids Phsphotidylinositols	
Phosphosphingosids Sphingomyeline	

2.3. Derived lipids

Steroids	Terpenes	Carotenoids
Cholesterol	Monoterpenes	Lycopene
Coprostanol	Sesquiterpenes	Carotenes
Cholestanol	Diterpenes	Xanthophylls
Ergosterol	Triterpenes	
	Tetraterpenes	
	Polyterpenes	

2.1. Simple Lipids or Homolipids

Simple lipids are esters of fatty acid with various alcohols only.

- Fats and oils (mono, di and triglycerides).

These are tri-esters of fatty acid with glycerol. Fat is solid at room temperature, while oil is in liquid form.

2.1.1. Glycerides

Acylglycerols being the more accurate name for these, glycerides are esters formed from glycerol and fatty acids. Glycerol contains three hydroxyl functional groups, which can be esterified with one, two, or three fatty acids to form monoglycerides, diglycerides, and triglycerides, respectively.

2.1.2. Monoglyceride

Monoglycerides (also known by acylglycerols or monoacylglycerols) are a class of glycerides, which are composed of a molecule of glycerol linked to a fatty acid via an ester bond. As glycerol contains both primary and secondary alcoholic groups, two different types of monoglycerides may be formed: 1. monoacylglycerols, where the fatty acid is attached to a primary alcohol, or a 2. monoacylglycerols, where the fatty acid is attached to the secondary alcohol. Figure 1 shows general structures of monoglycerides.

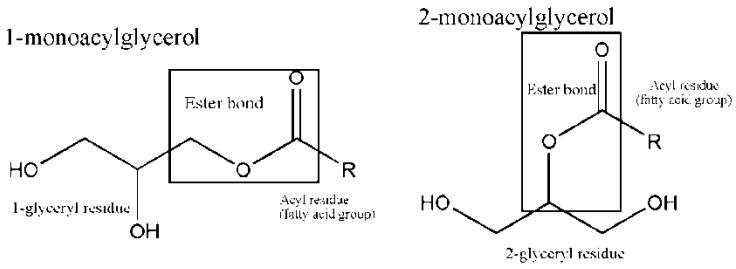

Figure 1. General structures of monoglycerides.

Examples of these include monolaurin, glycerol monostearate, glyceryl hydroxysterate, etc.

2.1.3. Diglyceride

A diglyceride, or diacylglycerol (DAG), is a glyceride consisting of two fatty acid chains covalently bonded to a glycerol molecule through ester linkages. Two possible forms of these are 1, 2-diacylglycerols and 1,3-diacylglycerols. DAGs can act as surfactants and are commonly used

as emulsifiers in processed foods. Figure 2 shows the common structures of diglycerides.

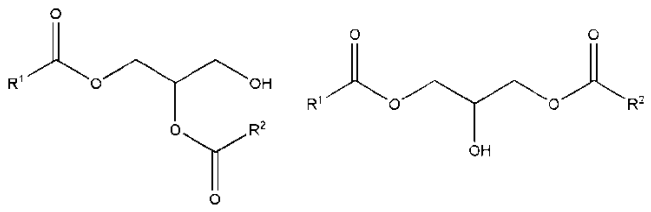

Figure 2. General structure of diglycerides and triglycerrides.

2.1.4. Triglyceride

A triglyceride (TG, triacylglycerol, TAG, or triacylglyceride) is formed by combination of one molecule of glycerol and three fatty acid molecules. Alcohols have a hydroxyl (–OH) group, organic acids have a carboxyl (–COOH) group. Alcohols and organic acids join to form esters. The three hydroxyl (-HO) groups in glycerol join with the carboxyl (–COOH) group of fatty acid, with removal of three molecules of water to form ester bonds as shown below:

$$HOCH_2CH\,(OH)\,CH_2OH + RCO_2H + R'CO_2H + R''CO_2H \rightarrow RCO_2CH_2CH\,(O_2CR')\,CH_2CO_2R'' + 3H_2O \qquad (1)$$

The three fatty acids (RCO_2H, $R'CO_2H$, $R''CO_2H$ in equation (1) above are usually structurally different. The chain lengths of different fatty acids in naturally occurring triglycerides vary, of which most contain about 16, 18, or 20 carbon atoms. Naturally occurring fatty acids found in plants and animals are typically composed of only even numbers of carbon atoms, reflecting the pathway for their biosynthesis from the two-carbon building-block acetyl CoA. Bacteria, however, possess the ability to synthesis odd- and branched-chain fatty acids. As a result, ruminant animal fat contains odd-numbered fatty acids, such as 15, due to the action of bacteria in the rumen. Many fatty acids are unsaturated, some are polyunsaturated (*e.g.*, those that are derived from linoleic acid).

Triglycerides are the main constituents of body fat in humans and animals, as well as vegetable fat. They are also present in the blood to enable the bi-directional transference of adipose fat and blood glucose from the liver, and

constitute a major component of oils. Triglycerides are further categorized as saturated and unsaturated. Saturated triglycerides, also known as solid lipids, have higher melting points and are more likely are solids at room temperature. On the other hand, unsaturated fatty acids (also known as fluid lipids), with double bond in the fatty acyl chains, have a lower melting point and are more likely to be liquid at room temperature.

Glycerides (mono, di and triglyceride) have been widely used to prepare different drug delivery systems, *viz*., liposomes, solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC) and other nanoformulations of topical, oral and parenteral drugs to increase solubility, improved bioavailability, reduced toxicity, and increased penetration. SLN and NLC are often composed of pure solid lipid or mixed with liquid lipid and stabilized by surfactants.

2.1.5. Waxes

Waxes are long alkyl chained esters having saturated and unsaturated fatty acid with monohydroxy alcohols, which have high molecular weight. They include higher alkanes and lipids, typically with melting points above about 40 °C, melting to give low viscosity liquids, and are malleable near ambient temperatures. Waxes are insoluble in water but soluble in organic, nonpolar solvents. Examples of these include cetyl palmitate, sorbitan tristerate, *etc*.

2.2. Compound Lipids or Heterolipids

Heterolipids are fatty acid esters with alcohol and additional functional groups.

2.2.1. Phospholipids (phosphatides)

Phospholipids are composed of fatty acid chains attached to a glycerol backbone. Phospholipids usually have two fatty acid chains and a third carbon of the glycerol backbone is occupied by a modified phosphate group as presented in Figure 3. Different phospholipids have different modifiers on the phosphate group, such as choline (a nitrogen-containing compound), serine (an amino acid), etc. Different modifiers give phospholipids different properties and roles in a cell. They are most abundant in cell membranes and serve as structural

components. They can form lipid bilayers because of their amphipathic characteristic.

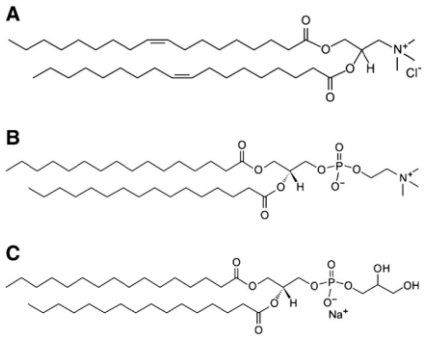

Figure 3. General structure of saturated and unsaturated phospholipids with different head groups:(A) 1,2-dipalmitoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) [DoTAP]; (B) 1,2-dipalmitoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) [DPPC] and (C) 1,2-dipalmitoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) [DPPG].

2.2.2. Glycolipids (cerebrosides)

Glycolipids are fatty acids with carbohydrates, which may contain nitrogen but without phosphoric acid, forming a glycosidic bond as shown in Figure 4. They also include some compounds like sulfolipids (sulfur containing functional group in the sugar moiety), gangliosides (containing negatively charged oligosaccharides with one or more sialic acid residues), and sulfatids (sulfate containing functional group in sugar moiety), which are structurally-related.

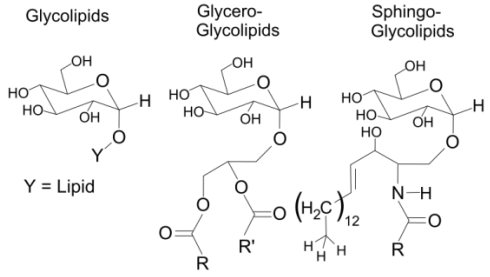

Figure 4. General structure of glycolipid derivatives.

These cerebrosides are important constituents of the brain and other tissues. They consist of at least one sugar unit, so they are also called glycosphingosides. They are like phospholipids, because they have a

hydrophobic region, with a polar region and two long hydrocarbon tails. Like phospholipids, glycolipids form lipid bilayers that are self-sealing and form the structure of cellular membranes.

2.3. Derived Lipids

These substances are derived by hydrolysis of compound and simple lipids. These fatty acids include alcohols, mono and diglycerides, carotenoids, steroids, and terpenes.

2.3.1. Steroids

Steroids are a class of lipid molecules which are identifiable by their structure of four fused rings. Although they do not resemble the other lipids structurally, steroids are included in lipid category because they are also hydrophobic and insoluble in water. All steroids have four linked carbon rings and several of them also have a short tail. Many steroids also have an -OH functional group attached at a particular site. Such steroids are also categorized as alcohols, and are thus known as sterols.

2.3.2. Cholesterol

Cholesterol is a well-studied lipid, because of the strong correlation of high blood cholesterol levels and the incidence of heart attack and stroke. It is a prominent member of a large class of lipids called isoprenoids, as they are formed by chemical condensation of a simple five carbon molecule, isoprene. It is an important component of cell membranes and plasma lipoproteins, and is an important precursor of many biologically important substances like bile acids and steroid hormones. It is abundant in nerve tissues and is associated with gallstones.

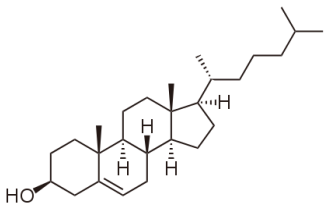

Figure 5. General structure of cholesterol.

General structure of cholesterol has been shown in Figure 5. Dietary cholesterol is found in saturated fats of animals (such as butter and lard), but vegetable oils do not contain cholesterol. Only a small portion of the body cholesterol comes from the diet. Most of it is produced in the body. Eating unsaturated fatty acids from vegetable oil helps lower blood cholesterol levels by reducing cholesterol synthesis in the body. However, eating saturated fats from animal fat elevates blood cholesterol and triglycerides and reduce the ratio of good to bad cholesterol.

3. Lung surfactant (LS)

The prevention of lung collapse is of vital importance in order to allow the lung to function properly, especially during deflation or expiration. To facilitate this, a lipid-protein material called lung surfactant (LS) lines the air-water interface (hypophase) of the lung alveoli. The alveoli are flaccid sacs in the lungs that carry out gaseous exchange. It is also within these alveoli that surfactant is produced. Surfactant is secreted by alveolar Type II cells as bilayer vesicles called lamellar bodies (LB), which transform to form tubular myelin (TM); TM are lipid-protein cross-hatched structures[1,2]. TM or the most surface active large aggregate fraction of LS becomes fully functional when it is readily adsorbed at the air-water interface of alveoli, to form a surface layer. Lung surfactant should be fluid enough to form adsorbed films but at the same time they should be rigid enough to lower surface tension (γ), when the film undergoes compression[3-6].

LS layers or films formed at the alveolar hypophase function to stabilize the lung by lowering γ close to near 1 mN/m values during compression. LS can adsorb rapidly to an air-water interface to reach equilibrium γ of 25 mN/m. Surface tension is produced when there exists a greater attractive force between water molecules in bulk than that of air at the interface. LS serve to lower γ by interacting with the intermolecular forces of attraction of water molecules in the bulk phase. It is this function of LS that shows that it is surface active and fully functional.

Various lung diseases and injuries may interfere with the proper functioning of LS. This is evident in case of diseases, such as acute respiratory disease syndrome (ARDS) and acute lung injury (ALI) where the normal

respiratory processes of the lung are compromised and LS is no longer able to function properly. Figure 6 compares a normal lung to a lung with ALI.

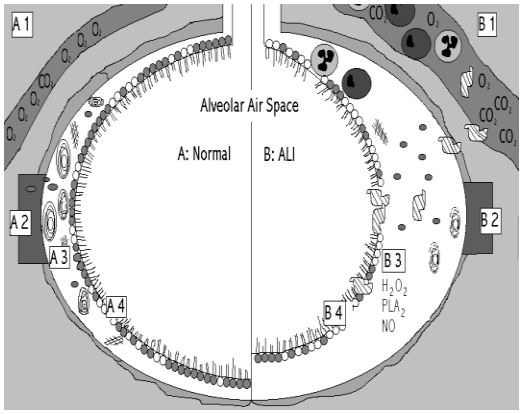

Figure 6. Schematic diagram of a normal alveolus compared with an alveolus suffering from acute lung injury (ALI). During ALI, serum proteins become inserted and DPPC is replaced by less surface-active phospholipids.

3.1. Composition of lung surfactant

In most mammalian species, LS is comprised of approximately 90% lipid and 10% protein by weight as demonstrated in Figure 7. The lipid portion contains various phospholipids as well as neutral lipids such as cholesterol. There are four surfactant proteins (SP) making up the protein portion of LS; they are surfactant proteins (SP-) A, B, C, and D. Earlier studies involved understanding the interaction of LS lipid and protein components.

Lipids in LS are approximately 80 - 90% phospholipids (PL) and may exist in different structural polymorphic forms. The most abundant PL is phosphatidylcholine (PC) which constitutes approximately 80% of most mammalian surfactant[7]. The major PC that is present in LS is dipalmitoylphosphatidylcholine (DPPC) and it is this PC that gives rise to the surface activity of LS, enabling surface tension to be greatly reduced. To allow this, this phospholipid becomes tightly packed in LS films to reduce γ to low values[2,6,8,9]. DPPC is also an unusual disaturated (16:0/16:0) acyl chain phospholipid not found in any other mammalian membranous system, suggesting it may have a special role in LS.

Neutral lipids, such as cholesterol, triglycerides, and free fatty acids are also present in LS. Cholesterol is present in significant amounts that are approximately 8-10% by weight of total lipids[6,7]. It was previously suggested that cholesterol increases fluidity and lowers the surface pressure, allowing the surfactant to cover more surface area and increasing the rate of adsorption of DPPC to the air-water interface[10].

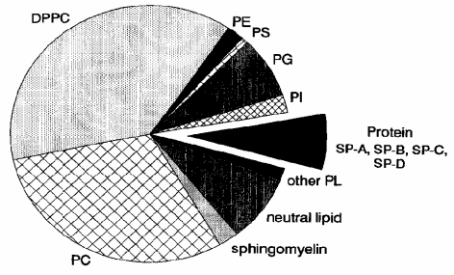

Figure 7. Pi diagram describing the composition of PS.

Phosphatidlyglycerol (PG) and phosphatidylinositol (PI) are two other phospholipids present in significant amounts in LS. Other PL includes phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM), lysophosphatidylcholine (LPC) and lyso-bis-phosphatidic acid (LBPA) which are present in minor amounts (1 - 3% by weight). Though cholesterol is responsible for fluidity, excess cholesterol may cause a problem because it prevents the tight packing of the PL in LS films upon compression and may limit the ability of LS to reduce γ to low values[11-13]. This is the reason why clinically useful surfactants, such as bovine lipid extract surfactant (BLES) used in this study, have the cholesterol component removed. This BLES preparation also allows us to sensitively alter the amount of cholesterol and thereby study the effect of cholesterol on surfactant.

3.2. Surfactant proteins and lung disease

Lung surfactant, as previously mentioned, contains four surfactant proteins, SP-A, SP-B, SP-C, and SP-D and their structure have been shown in Figure 8. SP-A and SP-D are hydrophilic glyco-proteins while SP-B and SP-C are hydrophobic smaller proteins which interact with the LS lipids. The hydrophobic proteins that have been associated to helping LS lipids to quickly be adsorbed at the air-water interface, increasing the adsorption rates of LS, and

promoting a more compressible monolayer film that is high in DPPC by film refinement[9]. Each of these proteins has possibly specific roles in surfactant function. SP-A and SP-D are members of a family of proteins called 'collectins', which are large multimeric glycoproteins[14]. These proteins can bind to various microbial ligands and can bind to pathogens to help keep the alveolar airspaces in a sterile condition, increasing the innate immunity[15]. Other studies are consistent with the proposed connection between immunity and collectins. A series of experiments involving knockout mice for SP-A and SP-D genes showed that the SP-A and SP-D deficient mice became more susceptible to infection due to the impaired ability of clearance of various microbes by alveolar microphages. SP-D -/- mice were affected by viral and not bacterial infections[16-18]. This may model why these pulmonary infections cause serious disease in humans, for example in ARDS patients, there is some deficiency of SP-A and SP-B in the lung lavage of human patients[19-21].

SP-B and SP-C are proteins that help enhance the surface activity of the phospholipid films of surfactant. Both are required for lung function and surfactant regulation. Each on their own (purified) or in combination enhances the rate of formation, of a surface-active phospholipid film, *in vitro*, at the air-water interface[22,23]. SP-B is a 17.2 KDa protein that is produced from a larger precursor found within type II cells of the alveoli[21,24]. SP-B was noted to increase adsorption rate of the LS lipids by accelerating the formation of a surface-active film of phospholipids at the air-water interface[25]. Studies by Cochrane and Revak, among others, have documented that SP-B improves surface activity, when added to a PL film, more so than SP-C[26-28].

A SP-B knockout study by Clark *et al*., 1995, using transgenic mice (SP-B -/-), showed that homozygous SP-B -/- mice died of respiratory failure just after birth. Even though lung development was normal in these mice, the lungs were unable to expand fully at birth due to lack of force induced by breathing[29]. It was concluded that lack of SP-B disturbed all aspects of surfactant homeostasis; including surfactant protein and phospholipid function, causing respiratory failure at birth[8,21,29-33]. Other studies mentioned that with a reduced amount of SP-B present in the neonatal mice, lung compliance was decreased[8]. Along with SP-B, SP-C also has possible unique functional roles in the making

of surface active surfactant film. Both SP-C and SP-B, have been known to regulate the processes that govern surfactant production and maintain the functioning of alveolar type II cells[34].

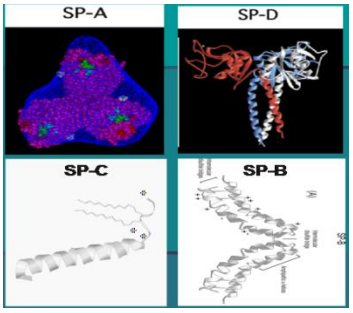

Figure 8. Structure of lung surfactant proteins.

Possmayer et al. suggested that SP-C appears to have a unique part in recycling the surfactant material that is removed from the film during cycling, back into the monolayer film. However, SP-B was more effective[9]. SP-C functions to improve surfactant's surface activity by increasing the rate of adsorption of the lipids to the air-water interface[9,25,34]. SP-C is the only surfactant protein solely synthesized in the alveolar type II cells and detected only in these alveolar cells of the mature adult lung[34-36].

A lack or deficiency of SP-C has been linked to respiratory dysfunction and interstitial lung disease seen in animal and human studies. SP-C deficiency has been reported in both infants and adults[30,34,37] in respiratory distress syndromes. SP-C knockout mice are unable to establish normal breathing[38]. To further examine the role SP-C played in lung function[35], used SP-C (SP-C -/-) knockout mice. SP-C gene was inactivated in nascent stem cells. Unlike the mice with a deficiency for SP-B, the SP-C-/- mice were however alive from birth and grew normally into adults without any signs of pulmonary abnormalities[35]. There was no SP-C mRNA or any mature SP-C found in these mice. What was detected however, included abnormalities in lung mechanics and instability of surfactant at low lung volumes, as tested using lung mechanics studies and a captive bubble surfactometer[35]. This instability may accompany respiratory distress syndrome seen in infants and adults[31,35]. Levels of surfactant associated proteins in lung lavage can serve as a biomarker in a disease such as ARDS[19]. In a more recent

study it was suggested that serum components may affect the ability of SP-B and SP-C to function properly, thereby inactivating LS[39].

3.3. Inhibition of LS in disease

In certain diseases, LS can become dysfunctional and inactive. This is evident in various respiratory conditions, in adults, as acute respiratory distress syndromes (ARDS). The term ARDS was first coined by Ashbaugh *et al.* in 1967[40]. Unfortunately not all criteria of such conditions was defined. Hence, in 1993, a new definition for ARDS was created by the American-European Consensus Conference (AECC)[41]. This definition described the early onset of the syndrome where respiratory distress in adults was caused by lung collapse and inflammation[42]. As defined elsewhere by Learer and Evans[43], ARDS shows a difficulty in treating hypoxemia as well as the accumulation of exogenous substances in the lung which shows up on radiographs of patient's chest cavity.

ARDS can develop because of various underlying conditions, with the highest prevalence in patients with sepsis and septic shock[43]. ARDS is first suspected when one has difficulty breathing and shows signs of fluid retention (pulmonary oedema) in the lungs[43]. Inflammation of the alveoli occurs as well as fluid, rich in proteins, leaks into the lung due to increased permeability of the capillaries caused by epithelial damage[42,44]. Even in the early stages of ARDS, the alveoli become flooded with plasma proteins which can inactivate LS[45]. Lung surfactant of ARDS patients are shown to contain a decreased percentage of PC and PG, a decreased level of SP-A and such surfactant showed reduced surface activity[46].

Along with the above, most ARDS patients also show poor lung compliance, smaller lung volumes and poor gaseous exchange in the lung[46,47]. A study was conducted by Gregory *et al*., in 1991, in which they examined chemical composition and biophysical activity of surfactant in patients with ARDS[45]. They compared lung lavage of normal and at risk ARDS patients. They found abnormalities in LS composition in both at risk and ARDS patients which indicates that changes in LS components occurs early in the disease. Their results showed that minimal surface tension was found to increase compared to those

seen in normal patients which suggested that the ability of LS to lower surface tension was compromised[45].

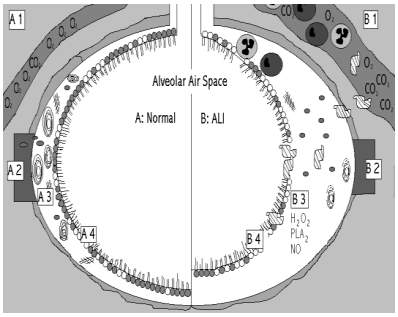

Figure 9. Schematicdiagram of a normal alveolus compared with an alveolus suffering from acute lung injury (ALI). During ALI, serum proteins become inserted and DPPC is replaced by less surface-active phospholipids.

The acute phase of ARDS is evident by a protein-rich fluid in the air spaces of the lungs, due to a more permeable alveolar-capillary barrier which is formed from microvascular endothelium and alveolar epithelium damage. The epithelium of the alveoli is composed of 90% (surface area) flat type I cells, which are easily injured and 10% (surface area) cubical type II cells (which produce LS) which are less susceptible to injury. Damage to the epithelium, mainly type I cells, can contribute to flooding of the alveoli, impaired removal of edema fluid from the alveolar spaces, and some injury to type II cells which may reduce the surfactant production and turnover[42,48,49]. Serum leakage into the airspace from more permeable capillaries is a characteristic of most ARDS patients. Studies have shown that serum proteins like albumin, haemoglobin and fibrinogen inhibit surfactant function by disrupting the ability of LS to lower surface tension upon addition of the proteins[47]. Not only serum proteins, but even cholesterol, lipoproteins, and ions can enter the lungs and cause surfactant dysfunction in injured lungs.

3.4. Cholesterol function in LS

Cholesterol has been suggested to play major functional roles in phospholipid cell membranes[50]. Cholesterol is mainly a rigid, hydrophobic molecule, with a -OH substituent, making it slightly amphipathic in character. The slight amphipathic nature enables anchoring of the molecule to the aqueous interface of the cell membrane. Here, cholesterol acts to make these fluid

membranes more rigid. Cholesterol also functions to give fluidity to certain membrane structures, to prevent the phospholipids from coagulating and crystallizing. The role of cholesterol in membranes is evident above and below a membrane's gel-liquid phase transition temperature. Cholesterol acts to fluidize the gel phase and it acts to rigidify the fluid phase[50,51]. Cholesterol has been shown to be a major constituent of lipid-rafts in biological membranes, where sphingomyelin-fluid lipids and cholesterol may accumulate for specific protein function[52]. Recently Serna *et al*., have also suggested that the SP-B and SP-C proteins may reside in similar lipid-raft structure for their function[53].

It has been previously shown[54] that cholesterol causes a reduction in the transition temperature of the lipid bilayer as well as lung surfactant[39]. Cholesterol also plays some role in LS by helping to improve the spreadability of the DPPC monolayers[55]. Studies suggest that the addition of cholesterol in amounts that mimic those found in normal lungs has no effect on functioning of exogenous surfactants. However, cholesterol removed from many clinical surfactants based on earlier studies (possibly due to experimental errors) has shown contradictory results[56]. Veldhuizen *et al*., suggested that cholesterol served in the processing and packaging of phospholipids into lamellar bodies and perhaps in their secretion, however it may not have any major role in γ reduction[13].

For cholesterol to have any role in the functioning of surfactant, it first must be incorporated in LS. The lung secretes up to 20% of its total cholesterol in the form of pulmonary surfactant[57]. The lung was also shown to be capable of endogenous cholesterol synthesis[58]. A study by Hass and Longmore, demonstrated that while the lung is more than capable of producing its own cholesterol, the main mechanism used in the production of surfactant cholesterol is the use of exogenous lipoprotein cholesterol[57]. In their earlier study, they reported that only 1% of surfactant cholesterol is of endogenous origin, synthesized in the lung. The rest, they mentioned, was supplied by serum low density (LDL) and high density lipoprotein (HDL) possibly entering through pulmonary arterial blood flow[57,59,60].

LDL is the major transporter of cholesterol to cells, including the lung, and is required for anabolic and energy purposes. LDL is spherical and has a cholesteryl ester core surrounded by a monolayer of free cholesterol,

phospholipids and an Apo-protein B (Apo-B) molecule. The Apo-B in the LDL is recognized by receptors in most cellular membranes. HDL delivers cholesterol to the liver *via* a process called reverse C transport as well as facilitates cholesterol removal from cells and tissues[61]. Hass and Longmore, also reported that receptors for LDL and HDL were present in the lung and suggested that cholesterol metabolism in the lung may be regulated by these lipoproteins[57,59].

Competitive binding studies concluded that the lung indeed had receptors for both LDL and HDL binding. Unlabeled LDL eagerly competed with labeled LDL for binding and addition of unlabeled HDL resulted in an increase in binding of labeled LDL. Unlabeled HDL was a strong competitor for the labeled HDL. Unlabeled LDL showed competition with the HDL only at high concentrations[57]. Furthermore, this study showed that deuterated [^{2}H] cholesterol uptake from both lipoprotein classes into surfactant occurs at least in part, by uptake by the lung, rather than an exchange since the lipoproteins inhibited endogenous synthesis. This result, coupled by lung surfactant cholesterol inhibition by LDL and HDL, showed that cholesterol metabolism in the lung was regulated similarly to that in other tissues[57,62].

Even though Hass and Longmore, found that the rate of uptake of the lipoproteins remained similar for both, the uptake rate for the protein portion of these lipoproteins was different, as LDL was found to have a higher rate. This suggested that LDL may be taken up as a whole particle, whereas the cholesterol in HDL may be transferred without definite uptake of the Apo-protein component. They further reported that majority of the surfactant cholesterol is derived from lamellar bodies, which are known to be storage sites of surfactant phospholipid components[57]. A later study by Davidson *et al*. in 1997, provided some evidence that the lung does endogenously produce surfactant cholesterol. There was no effect on cholesterol amount in any surfactant fraction, even with reduced serum cholesterol[59]. In 2001, Orgeig and Daniels infused a rat tail vein with [^{2}H] cholesterol and a large boost in cholesterol specific activity was seen in lamellar bodies (LBs) of surfactant within the first 30 minutes. After a two-day period, this increase was not seen in the alveolar surfactant. Further examination by studying the limiting membrane of the LBs showed a high concentration of LB cholesterol. They concluded that it appeared unlikely that LBs were the major

source or storage site of surfactant cholesterol, because the major portion of LB cholesterol was not released into the alveolar surfactant, as the limiting membrane of LB fused with the cell membrane upon exocytosis, keeping the cholesterol inside the cell (Orgeig and Daniels, 2001). Therefore, it is possible that the surfactant cholesterol synthesis occurred within the lung and was stored elsewhere from the phospholipid components. It seems from these studies as though there are possibly other sources of cholesterol in the type II cells[63].

A study by Voyno-Yasenenetskaya *et al*., in 1993 further examined lipoprotein-mediated (receptor mediated) signal transduction and exocytosis of surfactant from the alveolar type II cells of rat lung. They demonstrated that LDL and HDL stimulated signal transduction and surfactant secretion in type II cells. Next it was determined that alveolar type II cells themselves had receptors for LDL. Specific antiserum to the LDL receptor was immuneblotted to freshly isolated type II cells. It was concluded that membranes of the type II cells had LDL receptors, due to a band on the SDS-PAGE that migrated the same distance as the LDL band[64]. Similar observations were also observed by Hass and Longmore, where it was found that HDL and LDL delivered radio-labeled cholesterol first to lamellar bodies from type II cells and on to secreted surfactant[57,59,64]. Voyno-Yasenenetskaya *et al*., also suggested that HDL and LDL stimulation of surfactant secretion may only occur at a basal level, and the level increased due to hormones and other activators, through a signal transduction pathway. This showed that the lipoproteins not only supplied cholesterol to surfactant but affected the assembly and secretion of the surfactant from alveolar type II cells[64].

In all natural surfactants, as previously noted, cholesterol is the major neutral lipid present at about 8-10% (20 mol% of the PL) weight of phospholipids and thus a significant component. Previous studies have shown that cholesterol present in the LS films in physiological amounts has no effect on the ability of LS to reach low values[11]. The purpose of this amount of cholesterol is unclear, as it seems to have no effect on surface activity[12]. Keating *et al*., 2007, examined the effect of cholesterol on the biophysical as well as physiological properties of BLES. At normal LS levels, similar to those found in a normal lung, it was found that cholesterol had no significant effect on surface tension, similar

to systems with no cholesterol in LS. However, addition of cholesterol above 20% proved to prevent BLES from lowering γ. They also provided evidence that cholesterol added in excess, to the amount of DPPC, caused formation of a liquid disordered phase which affected surface activity[56]. These findings supported those of an earlier study by Gunasekara *et al*.,[11]. They also showed the inability of surfactant with high cholesterol content to lower the minimal surface tension and inability to provide structural stability to the lung. It was determined that cholesterol formed a complex with DPPC and the unbound cholesterol (that was not associated with saturated phospholipids) caused LS dysfunction[11].

All these studies may suggest that serum proteins may be the major cause of LS dysfunction as previously suggested[65]. Cholesterol either alone or in conjunction with serum proteins may be a major factor in LS inactivation in ARDS.

3.5. Serum protein role in ARDS

It is known that some serum proteins that leak from the capillaries into the fluid lining the lungs may render LS inactive. But exactly which protein is leaked and the mechanism to inactivate LS remain unclear. Previous studies on serum albumin, serum fibrinogen as well as C-reactive protein (CRP) have been conducted to show LS inactivation[46,65-67]. By using BLES with and without bovine serum albumin (BSA) in various weight percents (12.5-250%) and higher concentrations (2000-3000%), one study showed that a twenty-fold increase of protein can inhibit surfactant[68,69]. However, previous studies on serum proteins in lung lavages of dysfunctional surfactants have conclusively showed that the soluble protein fractions only increase by two to three-fold[9,67]. Thus, pathophysiological amounts of serum proteins may not be the main inactivator of LS.

Albumin is a major protein found in serum and is also a carrier of fatty acids. Various biophysical techniques such as Langmuir-blodgett balance, adsorption, and atomic force microscopy (AFM) were used to examine the inactivating effects of BSA. It was found that BSA, only in high amounts (20 fold) excess over what prevented surface tension from reaching low values. The hypothesis to rationalize the high amounts of BSA required was that BSA

interfered with lipid packing at the surface of the BLES monolayer film during compression. BSA adsorption to the interface was separate from BLES, as well as BSA attaching somehow to the polar lipid head groups of the lipids in BLES films[68].

Albumin is not the only serum protein known to inhibit LS function. Serum fibrinogen has been proven to inactivate LS[65]. A study by Devraj, 2005, also added evidence that serum proteins do in fact inactivate LS, however at high amounts. In this case, fibrinogen was the serum protein of choice. Fibrinogen (Fbg) was mixed with BLES in a range of solutions from 0.1:1 to 5:1, as well as 10:1 Fbg : BLES, wt/wt. These chosen concentrations mimicked that seen in lung disease. The high concentrations, 5:1 and 10:1 showed maximum inhibition. By using similar biophysical techniques that were used in the previous study on albumin, the inhibitory effects of fibrinogen were evident. It was seen that fibrinogen had prevented BLES from adsorbing quickly to the air-water interface[70]. Other studies on albumin, and other serum proteins, such as fibrinogen, using both extremely high and physiological concentrations of protein have yielded complementary results and showed inhibition in varying degrees[71-74]. The varying concentrations of proteins, various surfactant compositions, and different surface activity measuring techniques, make the studies difficult to compare[72].

Results from monolayer balance studies provided further evidence that proved the inhibitory effects of fibrinogen (Fbg) on BLES. With addition of fibrinogen, BLES ability to lower surface tension upon compression was altered, allowing minimum attainable surface tension of 27 mN/m instead of 1 mN/m, characteristic of BLES films. This suggested that the protein interfered with the surfactant film.

BLES is a modified clinical surfactant extracted from bovine lungs and is composed of most LS lipids and proteins, SP-B and SP-C[75]. When spread into monolayer films, the gel lipids were oriented so that the hydrophobic fatty acid tails were pointing upward into the air, while the hydrophillic head groups were attracted to the water surface. By using techniques such as AFM, height differences between the fluid (liquid expanded) and gel (liquid ordered) phases were imaged and measured. Fluorescent microscopy, with the use of a

fluorescent probe, identified domains as dark (probe-free) regions throughout a bright fluid area[76-78]. When these films were compressed, a phase separation (transition) occurred and gel areas or domains, were formed in a "sea" of fluid lipids[79]. The domains, rich in DPPC lipids, were tightly packed together and tilted more perpendicular than the fluid phase lipids to the air-water interface. The proteins and other unsaturated or fluid phospholipids constituted the surrounding area. The domains changed shape and size and disappeared upon further compression, or when excess cholesterol or serum proteins were added[78].

Devraj (2005) conducted studies examining the structural appearance of BLES and BLES + Fbg films at various γ. The higher the concentration of fibrinogen added, the fewer BLES domains present; even at 100% fibrinogen, domains were non-existent. Using Raman spectrometry studies, it was seen that Fbg, somehow interacted with BLES and changed domain confirmation even in the gel phase, where the BLES lipids were packed tightly together[70].

Another serum protein used as a biomarker, indicative of ARDS, is C-reactive protein (CRP). CRP is an inflammatory associated protein that is known to increase in serum during the inflammatory process[80]. It is mentioned by Nag *et al*., 2004, that the ability of CRP to bind to PC molecules may become an important factor in surfactant inhibition *in situ*[66]. CRP can make its way into the lungs by means of serum leakage as well as lung macrophages may produce this protein. Drover, 2006, compared BLES and BLES + 10% CRP in several biophysical studies. Results indicated that when CRP was added to BLES, rate of adsorption to the air - water interface was decreased only at high amounts. Indicative from AFM and Raman studies, CRP agglutinated the gel domains and caused an increase in the gel phase domains and prevented the fluid stage transition[81]. Findings from Chang *et al*., indicated that perturbation of the monolayer or bilayer surface lipid-packing was necessary for CRP binding[82].

3.6. LS and interaction with whole fetal calf Serum

Previous studies[9,46,65], including some of the above, have established that serum proteins had a detrimental effect on surfactant function; however other factors of serum may affect LS at pathophysiological levels. Fetal calf serum (FCS) is very similar to that of human serum in composition. Not only does

human serum contain soluble proteins, like albumin, fibrinogen, CRP and other globulins, it also contains various ions, amino acids, sugars, (such as fructose and glucose) as well as serum lipids. These lipids may be in the form of LDL, HDL, triglycerides, fatty acids, cholesterol and cholesterol esters[83,84].

Whole fetal calf serum was first studied with LS by examining the structure and function of whole FCS and its constituent albumin and the effects on a clinical surfactant, BLES, as described below. Upper and lower airway models were studied to examine the effects of serum and albumin in the airways of the lung, by using a capillary surfactometer and a Langmuir balance, respectively. It was observed that a small pathophysiological amount of serum was needed to render BLES inactive in both airway models compared to 200-fold amount of a soluble protein[69].

Different amounts of FCS and albumin [0.1-2:1 BLES, dry (wt/wt)] on BLES dispersions were studied. As increasing amounts of albumin protein from 10% to 2000% by weight were added to BLES, a greater inhibitory effect took place. It was not until 500% wt or more albumin was added that maximal inhibition was seen. The same was seen for FCS with concentrations ranging from 5% to 1000% wt, but this time maximal inhibition was seen at even the lowest FCS/BLES concentration of only 5% wt. Further studies were conducted at concentrations ranging from 0.1% to 5% wt FCS. Results indicated that as little as 2% wt FCS was required to show the same inhibition of BLES as seen with 500% wt albumin[69,85].

This study provided compelling evidence that serum was 200 times more of a potent inhibitor than albumin, one of its major constituents, alone. From monolayer studies it was seen that serum perturbs lipid packing of the BLES lipids and prevented a low surface tension from being reached. Studies by Vidyasankar (2004) showed similar results with albumin but the BSA inhibitions were not as potent as with FCS described above. Earlier studies have shown that serum protein alone does not alter LS surface activity in a significant manner below high (10-20:1) serum protein: LS concentrations, suggesting physiological factors of serum may be responsible for LS inactivation[68].

Panda *et al*., published a study reporting that in hyperventilated injured rat lungs (ARDS model) the cholesterol content of dysfunctional surfactants increased two-fold compared to normal lungs with a concomitant threefold increase in soluble protein content[67]. Most studies[68,70,72,81,86,87] have found that only high levels (10-20:1) of serum protein: LS can cause LS inactivation. The study by Hillier (2005) showed evidence that other constituents of serum and not just single proteins may possibly be involved in LS inhibition. Whether it is the protein-protein or protein-lipid interactions is yet to be established[69].

To support the fore mentioned serum study, Parsons (2005), examined the functional and structural properties of a BLES monolayer with varying FCS concentrations (1:0.01-1:1, BLES: serum, dry wt/wt) as a preliminary investigation of how lung surfactant structures are altered and how whole serum components interacted to cause dysfunction. Serum inhibition of BLES was studied using a Langmuir surface balance, to see if surfactant's intrinsic ability to lower surface tension was compromised, and studied using both fluorescence and atomic force microscopy to examine film ultra-structure. From the monolayer balance studies, it was concluded that serum prevented surface activity of BLES or the ability of BLES to reach low surface tensions, caused incompressibility of surfactant films, and prevented 'squeeze-out' from occurring. The preliminary microscopy studies demonstrated that whole serum, at lower concentrations, disrupted larger gel-domain formation and caused smaller and more numerous domains to form[69,77,86]. At higher concentrations, serum had the ability to form its own film and prevented lipids from occupying any space in the film and thus likely inhibited surfactant lipid adsorption[77,86]. This study suggested that whole serum was indeed more potent in inhibiting BLES as was its protein constituents.

Although several reports are available in the literature describing the interaction studies between lipid monolayer and the disrupting agents, but till date the exact mechanism of film dysfunctionality remains unclear. The present work endeavours to address such an issue, whereby comprehensive physicochemical investigations have been undertaken in order to correlate the composition, film functionality and structure of lung surfactants in the presence of serum, cholesterol and LDL. The derived results are considered to shed light on the nature and cause of surfactant dysfunction.

4. The interest in new drug delivery systems

Treatments of acute or chronic illnesses have mostly been achieved by drug delivery systems such as tablets, capsules, suppositories, creams, ointments, liquids *etc.* since a long time. These systems have shown promises in facilitating rapid drug release. Drug efficiency is more often than not restricted by the drugs capability to reach their targeted therapeutic site of action. As a matter of fact, most of the administered dose of drugs is distributed to different parts of the body away from the intended site, which is also dependent upon the physicochemical and biomedical properties of drug, while a limited amount goes to the site of action. Identifiable caveats in the drug delivery systems such as short biological half-lives of newer drugs (e.g. peptide-based drugs), very potent drugs with strong side effects (e.g. tumour necrosis factor[88]), poor or sparse water solubility, inadequate bioavailability and their pricey development regime have paved way to increased interest in the possibility of delivering drugs to their desired site of action. Under such circumstances, the need for a novel drug-carrier system becomes imperative, in order to address the identifiable drawbacks. Ideally, this carrier system should be devoid of systemic toxicity (acute and/or chronic), stable in the physiological liquid, biodegradable, biocompatible, inert for the drug and the targeted tissue, having prolonged biological half-life, having a sufficient drug loading capacity and at the same time, protecting against systemic side effects by targeting of the drug to the desired tissue. The carrier-mediated release of the drug at the desired tissue should be in a precise manner to maintain a sustained and effectual drug level[89,90]. Also, the incorporated drug should include stable physicochemical properties. In addition, the feasibility of production scaling up with reasonable overall costs should be required.

One of the formal approaches to address this challenge is the effective use of colloidal drug carriers (Particles < 1 μm) in accordance with the different routes of drug administration. Particles of colloidal carriers usually range in size from 10 nm to 1000 nm consisting of materials in which the active ingredients (drugs or biologically active materials) are dissolved, entrapped, encapsulated, and/or to which the active ingredients adsorbed or attached[91]. Adjustment of the carrier properties such as particle size, particle rigidity and surface charge and surface hydrophobicities can potentially aid in the design of an apposite carrier systems, although a serious bottleneck in the use of colloidal carrier systems is its

rapid clearance from the blood-stream, in turn being assisted by the macrophages of the reticuloendothelial system (RES; mainly in the liver and spleen)[92,93]. The participation of macrophages in the pathogenesis of diseases such as human immunodeficiency virus (HIV) infection[94] is overwhelming and offers a fascinating prospect for the evaluation of cell-specific drug targeting *via* the use of colloidal carriers. Again, as mentioned earlier, modification of the properties of the carriers, such as particle size, surface charge and surface hydrophobicity can also help in reducing the RES clearance. On the other hand, monoclonal antibodies, such as those attached to the surface of the drug carriers, can aid in the specific targeting of drugs to the desired site of action[95]. Careful amendments of the carrier components may lead to drug release upon exposure to the specific macro-environment such as changes in pH[96], temperature changes[97] and the influence of a magnetic chamber[98]. As such, examples of site-specific and controlled drug delivery systems, developed as particulate carriers are: polymeric particles[99-101], liposomes[102,103], emulsions[104-108] and micelles[109]. Figure 10 gives an overview of the historical perspectives of the development of a typical colloidal carrier system.

The subsequent sections henceforth will deal with fundamental aspects, methods of preparation, characterization and applications of these systems.

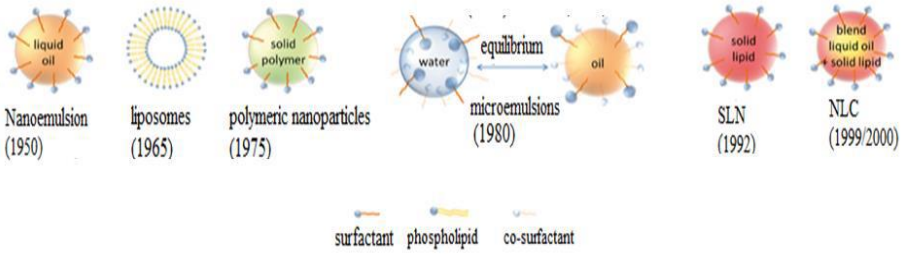

Figure 10. Schematic of historical development of colloidal carrier system.

5. Drug delivery systems

5.1. Nanocapsules and polymeric nanoparticles

Nanocapsules present themselves as a barrier made from polymers between the oily core and the aqueous surrounding environment. Methods such as solvent displacement[110,111] and interfacial polymerization[112,113] are common for nanocapsules preparation. Nanoparticle preparations make use of polymers, which include cellulose derivatives, poly-(alkylcyanoacrylates), poly-

(methylidene malonate), polyorthoesters, polyanhydrides and polyesters such as poly-(lactid acid), poly-(glycolic acid) and poly-(ε-caprolactone) and their co-polymers[114]. A number of techniques may be applied for production of polymeric nanoparticles, such as coacervation, solvent evaporation[115], solvent diffusion, interfacial polymerization, denaturation or desolvation of natural proteins or carbohydrates[116] and the degradation by high-shear forces, e.g. by high pressure homogenization[117] or by micro fluidization[118].

As opposed to the emulsions and liposomes, nanocapsules and polymeric nanoparticles in contrast, can effectively provide enhanced shielding to the incorporated sensitive drug molecules. This augmented protective effect is due mainly to the polymeric barrier (for nanocapsules) and the solid polymeric matrix (for polymeric nanoparticles), respectively. In turn, controlled drug release from these carrier systems is also achievable[119-122]. However, polymer-based nanoparticles have a very many recognizable shortcomings, *e.g.* the residues of the organic solvents used in the production process, the toxicity from the polymer itself and the difficulty of the large-scale production[123,124]. Also, polymer erosions, drug diffusions through the matrix or desorptions from the surface are some of the other factors impeding their efficient application. In addition to this, the concentration of prepared polymeric nanoparticle suspensions is low, as much as less than 2%. Nowadays there are many products in the market for therapeutic use based on polymeric nanoparticles, a few instances being decapeptyl, gonapeptyl depot, and enantone depot.

5.2. Liposomes

In aqueous solution, dispersion of neutral PLs lead to the formation of structures with closed vesicle, which resemble cells morphologically. These closed vesicles are named "liposomes" (fat bodies) and consist of hydrated bilayers as presented in Figure 11. The characteristics of liposomes is that not only they can mimic cell membrane structures, but also can potentiate either encapsulation of hydrophilic materials in the inner liposome-water phase or can lead to association of the lipophilic materials within the lipid bilayer. Studies on the fate of liposomes and entrapped agents were initiated as early as1970s[125]. Liposomes may be of divergent nature based on sizes, numbers, positions of lamellae (multilamellar vesicles: MLV versus multivesicular vesicles: MVV),

charges, and bilayer rigidities (liquid crystalline versus gel state), and depending on the selection of lipids, the preparation techniques and conditions. The comportment of liposomes both *in vitro* and *in vivo* are heavily influenced by the above parameters[126,127]. The opsonization process, leakage profiles, disposition in the body, and shelf life, *etc*., depend on the type of liposome involved.

Hence, it becomes imperative to carefully choose relevant liposomal constituents and accurate preparation technique, in order to facilitate efficient characterization of the produced liposomes. In spite of the fact that liposomes generally display decent biocompatibility, biodegradability and, in certain cases, low cytotoxicity and immunogenicity[128,129], one major deficiency is their structural volatility *in vitro* and *in vivo*[126,127].

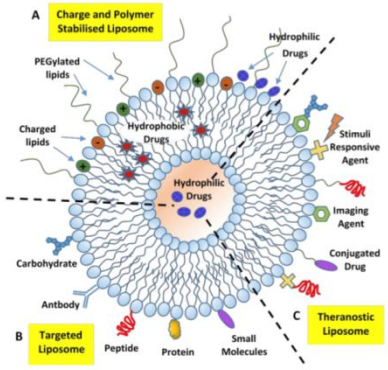

Figure 11. Schematic diagram of three different types of drug delivery systems based on liposomes: (A) charge and polymer stabilized liposome, (B) targeted liposome, and (C) theranostic liposome.

Drug leakage or particle growth is a resultant defect associated with the physico-chemical instability of liposomes. Incorporation of lipophilic drugs in low concentrations in PL bilayers[130] also limits the use of liposomes. There is no surprise that akin toother colloidal drug delivery systems, liposomes are also easily entrapped by the macrophages of the RES and subsequently cleared rapidly[131]. Weighing the pros and cons, it is but indicative that liposomes do not optimally present their application as a pharmaceutical carrier system. To overcome the aforementioned caveats, 'niosomes' were introduced. "Niosomal" structural/chemical stability is enhanced by their saturated hydrocarbon chains and intramolecular ethereal bindings[132,133].

5.3. Microemulsions and nanoemulsions

Microemulsions are transparent or translucent, optically isotropic single-phase liquid solutions with low viscosity. They are stable thermodynamically and are bicontinuous in nature, typically composed of water, oil, surfactant and co-surfactant[134-136]. The solubilizing power of microemulsions spans both hydrophilic and lipophilic drugs, and they are far more than solubilizing efficiency of micellar solutions. The high surface-active agent concentrations in microemulsions limit their use to dermal and oral applications[137,138]. In 1950s saw the introduction of nanoemulsions to cater to the need of parenteral nutrition[139].

Nanoemulsions have been used since a fairly good amount of time as drug carriers for lipophilic activities. Several pharmaceutical products such as etomidat lipuro, diazepam lipuro, disoprivan, stesolidand lipotalon[139-141] are based on nanoemulsion system and have long been introduced into the market.

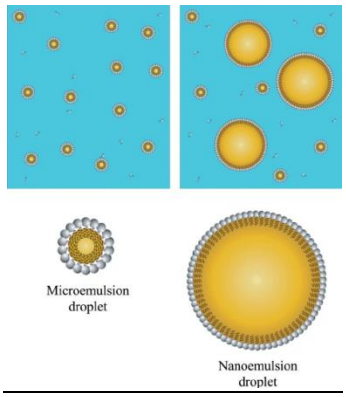

Figure 12. Schematic diagram of microemulsion and nanoemulsion.

Composed of a heterogeneous system of two immiscible liquids (one liquid is dispersed as droplets in the other one)[142-144], nanoemulsions require an input of source of energy, and also the obtained liquid-in-liquid dispersion is thermodynamically unstable[145]. Reduction of the local and systemic side effects, *e.g.*, reducing pain during injection and haemolytic activity caused by the high emulsifying agent concentration in the solubilization-based formulation[107] are some of the advantages of nanoemulsions over solubilization-based formulations (microemulsions) when it comes to drug delivery. Unfortunately, the lipophilic loaded drug can lead to stability issues as they partition between the oil droplets and the aqueous medium[146]. In addition, the desirable property of sustained release of drugs from nanoemulsions is not meet due to high mobility of the

loaded drug, dissolved in the oily phase. As such, a rapid release of the drug from its carrier system was reported in several studies[147]. To address this issue, attempts made expend towards newer drug delivery systems where the advantages of fat emulsions and retention of the incorporated drug for a longer period are simultaneously met. To increase the drug retention, some groups have replaced the liquid oil phase of the fat emulsions with a solid lipid to produce solid lipid nanoparticles (SLN).

6. Solid lipid nanoparticles

6.1. Definitions and physical structure of solid lipid nanoparticles

The sections above delineate several positive traits of lipid emulsions. It stands to reason that lipid emulsions have been used for many years as parenteral nutrition and as colloidal drug carrier system for delivering substances having poor aqueous solubility. However, prolonged drug release cannot be achieved in relation to the liquid state of the oil droplets, and is recognized as one of the major shortcomings of this delivery system. Liposomes, on the other hand is the other interesting parenteral carrier systems, which was described initially during the 1960s by Bangham et al. and was first introduced as a drug delivery vehicle in the 1970s[148]. The main idea behind development of this carrier system is to effectively lessen the toxicity of the incorporated drugs and enhance the efficacy of the treatment regime. Bottlenecks in the formulation and development of liposomes were that they were limited by physical stability, drug leakage and difficulties in up scaling.

The 1990s saw solid lipid nanoparticles (SLN or liposphere or nanosphere), which were developed as analogous means for colloidal carrier system for emulsions, and for liposomes in the sustained release drug delivery system. SLN are usually prepared by the substitution of the liquid phase of lipid (oil) of lipid emulsion by a solid phase of lipid, which means that the lipid particle matrices are not only solid at room and body temperature, but also stabilized by surfactants. The examples of these lipids include complex triglyceride mixtures, highly purified triglycerides, or even waxes[149]. SLN formulations *via* several routes of applications, such as parenteral, pulmonary, oral, ocular, rectal *etc*, have been developed and their exclusive characterization has been done *in vitro* and *in vivo*[149].

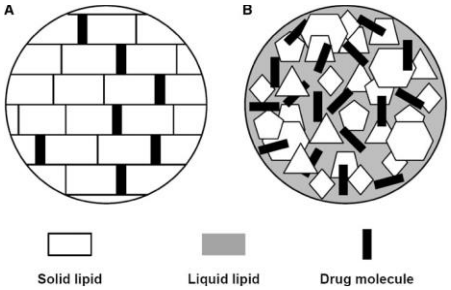

Figure 13. Schematic representation of a perfect lipid crystal in SLN (A) and a crystal lattice with many imperfections in NLC (B).

6.2. Models for incorporation of active compounds into SLN

Three fundamentally different models for incorporating active ingredients into SNL exist as presented in Figure14. Relative proportions of lipid, drug, and surfactant used in formulations and the use of hot or cold homogenization as the production conditions guide the acquisition of structures.

6.2.1. Homogeneous model

When the drug is homogeneously dispersed as molecules or amorphous clusters within the lipid matrix, a homogeneous matrix model is seen, which is also referred to as a solid solution model (Figure 14A). Lipid nanoparticles prepared by cold homogenization technique or a hot homogenization technique employing highly lipophilic drugs without the use of surfactants or drug-solubilizing molecules especially fall under this category. Likewise, a cold homogenization technique makes sure that the solubilized drug is dispersed homogeneously within the bulk of lipid. Subject to high pressure homogenization, lipid nanoparticles with a homogeneous matrix are formed due to mechanical agitation. Similarly, lipid droplets are inclined to crystallize with no phase separation between drug and lipid, when the end product of a hot homogenization is cooled rapidly. Drugs exhibiting extended release from particles go hand in hand with these models[150], example including prednisolone-loaded SLN system that presents slow release of prednisolone, usually from day 1 to week 6[151,152].

6.2.2. Drug-enriched shell model

A schematic of the drug-enriched shell model is depicted in Figure14B. When a lipid core is enclosed by a drug-enriched outer shell, it is known as a drug enriched shell model. Hot liquid droplets with hastened cooling form lipid nanoparticles with phase separation and gives rise to such structures. During production, the lipid precipitation mechanism and repartitioning on cooling can aptly explain the drug-enriched shell morphology. Hot homogenization leads to droplets presenting themselves as a blend of melted lipid and drug particles, which upon accelerated lowering of temperature hastens the process of lipid precipitation at the core with a contemporaneous increase in drug concentration in the outer liquid lipid. Complete cooling leads to precipitation of a drug-enriched shell. This structural model is suitable for facilitating drug release in bursts. Dermatological SLN formulations that necessitate enhanced drug penetration make use of such rapid release techniques, in addition to the occlusive effect of the SLN[150]. Studies have shown that the sustained release of clotrimazole from a topical SLN formulation was due to its drug-enriched shell structure (Souto et al. 2004). At elevated temperatures, the solubility of the drug in the surfactant-water mixture is another reason that can encourage precipitation of drug in the shell. The increase of solubility in the surfactant solution promotes partial movement of drug out of the lipid core, during the hot homogenization process. In contrast, a decrease of solubility of the drug in the surfactant solution is seen with the cooling effect of the dispersion. This facilitates enrichment of drugs in the shell, in scenarios under which lipid core solidification has already been initiated[150].

6.2.3. Drug-enriched core model

When the process of recrystallization is opposite to that of the drug-enriched shell model, a drug-enriched core model is acquired. Figure 14C shows a schematic of a drug-enriched core model, the morphology of which depicts that the drug tends to crystallize prior to the lipid. The solubilisation of the drug in the lipid melt is carried out close to its saturation solubility. Super-saturation of the drug in the lipid melt is caused by subsequent cooling of the lipid emulsion; this promotes drug recrystallizing prior to lipid recrystallization. Further cooling recrystallizes the lipid, which forms a membrane around the previously

crystallized drug-enriched core. In accordance with Fick's law of diffusion, this morphology is suitable for drugs that require extended release over a certain period[153].

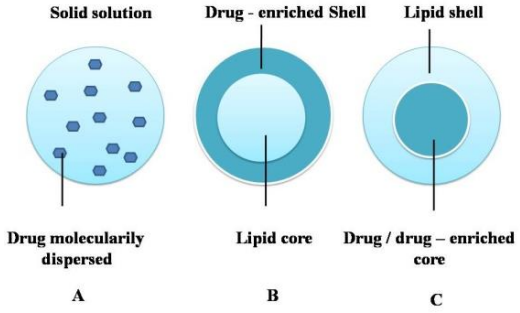

Figure 14. Models of incorporation of active compounds into SLN (a) homogeneous matrix; (b) drug-enriched shell model; (c) drug-enriched core model.

7. Nanostructure lipid carriers

7.1. Definitions and physical structure of nanostructured lipid carriers

The end of 1990s saw the introduction of nanostructure lipid carriers (NLC), which are further modifications on SLN. Evolving as an SLN of the new generation, NLCs contained particles with solid-lipid matrix with a nanometer-scale average diameter. While addressing the limitations counteracted in conventional SLN systems, NLCs strive to increase the payload and avoid expulsion of drugs[154]. Three different types of NLCs were proposed, which also included the oil droplets dispersed in a solid lipid matrix. This should in turn combine high drug loading caused by the liquid-lipid and controlled release caused by the solid-lipid (Figure 13B).

7.2. Structure of Nanostructured Lipid Carriers

Analogous to SLN, three distinct morphologies are recommended for NLCs as demonstrated in Figure 15. These are a function of the location of incorporation of drug molecules (Jenning et al. 2000 a, b and c)[151].

7.2.1. NLC type I or "Imperfect crystal" type

NLC type I or imperfect crystal types exhibit imperfections in structured solid matrix. Glycerides composed of various fatty acids can lead to augmentation of such imperfections. Increasing the number of imperfections can

also give a boost to drug accommodation. Imperfect crystal NLCs are prepared by mixing spatially different lipids, as opposed to using only solid lipids. This gives them the leverage to obtain "maximum imperfections". Hence, accommodation of more drug molecules is facilitated, either in molecular form or as amorphous clusters. Using variable lengths of fatty acid chains further leads to formation of a solid matrix with flexible distances. In addition, drug-loading is enhanced by inclusion of a small amount of liquid lipid[153].

7.2.2. NLC type II or "Multiple" type

NLC type II or oil-in-lipid-in-water type is the second kind of NLC, also known as the multiple type. The oil solubility of lipophilic drugs is higher than that of its solid lipid solubility. This is the principle used in formation of multiple type NLC. In this kind, higher amounts of oil are blended in solid lipids. Oil molecules are easy to disperse into the lipid matrix at low concentrations. Addition of oils more than that of its solubility promotes phase separation leading to production of tiny oily nano-compartments surrounded by the solid lipid matrix. These models facilitate precise drug release with the lipid matrix preventing leakage of drug[151]. Lipophilic drugs are solubilized in oils and multiple types of NLCs are formed during the cooling process of a hot homogenization.

7.2.3. NLC type III or "Amorphous" type NLC

NLC type III or amorphous type NLC is the third kind of NLC. Drug expulsion is promoted by crystallization. To curb this effect, preparation of NLCs involves judicious mixing of solid lipids with special types of lipids such as hydroxy octacosanyl hydroxystearate, isopropyl palmitate or medium chain triglyceride (MCT). Non-crystalline (amorphous), solid, lipid nanoparticles are formed thus. The core of the lipids solidifies to an amorphous state. The maintenance of the polymorphic nature of the lipid matrix minimizes drug expulsion in this case.

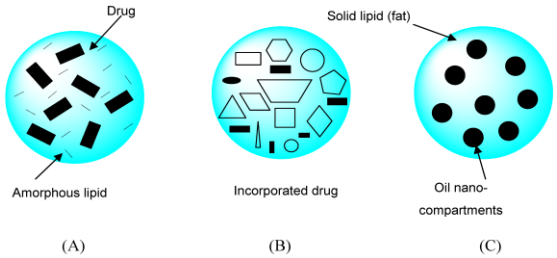

Figure 15. Drug incorporation models of nanostructured lipid carriers (A) the amorphous type, (B) imperfect type, and (C) multiple type.

8. Preparation techniques for lipid nanoparticles:

Different approaches are used to produce finely dispersed lipid nanoparticle dispersions. The various procedures are precisely described in this section:

8.1. High Pressure Homogenization (HPH)

High pressure homogenization (HPH) can be performed at high temperatures (hot HPH technique) and is a suitable method for the preparation of NLC or at or below room temperature (cold HPH technique)[155-159]. The hot HPH is prepared by melting the lipid and the drug together at precisely 5 °C above the melting point of the lipid. This is further mixed with an aqueous surfactant solution at the same temperature. A rapid stirring leads to formation of a hot pre-emulsion. The hot pre-emulsion is then processed at a temperature-controlled high pressure homogeniser, generally a maximum of three cycles of 500 bars are sufficient. Recrystallization of the obtained nanoemulsion occurs once it has been cooled down to room temperature forming the relevant NLC. An apposite technique for handling hydrophilic or temperature labile drugs is the cold HPH. Lipids and drugs are melted together and then quickly pulverised under liquid nitrogen forming solid lipid microparticles. Brisk stirring of the particles in a cold aqueous surfactant solution leads to formation of a pre-suspension. Homogenization of this pre-suspension at or below room temperature delivers formation of NLC, the conditions for homogenization generally being five cycles at 500 bars. Extensive studies of the influence of homogeneous type, applied pressure, homogenization cycles and temperature on particle size distribution have been carried out in the recent past[158,160-162]. Both HPH techniques are

suitable for processing lipid concentrations of up to 40%, which usually yield very narrow particle size distributions (polydispersity index < 0.2)[163,164].

8.2. Production of SLN *via* microemulsions

A suitable method for the formulation of SLN has been developed and optimized by the Gasco group through microemulsions and adapted by different labs[165-169]. Preparation of a warm microemulsion is done by stirring, then dispersing with further stirring in excess cold water (typical ratio 1:50), using an especially industrialized thermostatic syringe. This warm microemulsion typically contain 10% molten solid lipid, 15% surfactant and up to 10% co-surfactant. The excessive water produced thus is discarded either by ultra-filtration or by lyophilisation. This is done in order to facilitate enhancement of particle concentration. Extensive studies on factors such as microemulsion composition, dispersing device, temperature and lyophilisation on the size and structure of the obtained SLN have previously been reported. It is to be noted that the process of removing excess water from the formulated SLN dispersion is a challenging aspect when it comes to consideration of particle size. In addition, concentrations of surfactants and co-surfactants (e.g. butanol) in increased amount are necessary for formulation purposes, however less desirable with respect to regulatory purposes and application.

8.3. Preparation by solvent emulsification-evaporation or - diffusion

Until now, attempts have been made by several research groups to produce SLN by precipitation. In the solvent emulsification-evaporation method[170-172], water-immiscible organic solvent (e.g. toluene, chloroform) is used to dissolve the lipid which subsequently undergoes an aqueous phase emulsification before evaporation of the solvent under reduced pressure. Once the solvent is evaporated, SLN is formed by precipitation of lipids. A major benefit of this method is that it is also applicable for thermolabile drugs, as heat is not applied during its preparation. However, issues might be related to solvent residues in the final dispersion. The limited solubility of lipid in the organic material also makes these dispersions quite diluting. Lipid concentrations in the final SLN dispersion are characteristically found to be around 0.1 g/l, making it imperative to boost the particle concentration by means of methods such as ultra-

filtration or evaporation. Solvents which are partially water-miscible are used (*e.g.* benzyl alcohol, ethyl formate) in the solvent-diffusion method[173,174]. The first step is to mutually saturate them with water in order to warrant the thermodynamic equilibrium of both liquids. The next step is to dissolve the lipid in water-saturated solvent and consequently emulsify with solvent-saturated aqueous surfactant under pre-eminent temperatures. After addition of superfluous water (typical ratio: 1:5 – 1:10), the diffusion of the organic solvent from the emulsion droplets to the continuous phase causes the SNL to precipitate. Moderately diluting dispersion, which requires concentration by means of ultra-filtration or lyophilisation is produced very much like the production of SLN *via* microemulsions. Very narrow to average particle size (around 100 nm) distributions are reached at by the two solvent evaporation methods.

8.4. Preparation by W/O/W double emulsion method

A unique method for the preparation of SLN loaded with hydrophilic drugs based on the solvent emulsification – evaporation technique was introduced lately[155]. In this method, the hydrophilic drug is encapsulated along with a stabilizer to preclude drug partitioning into the external water phase during solvent evaporation in the internal water phase of a W/O/W double emulsion. This technique is used for preparing sodium cromoglycate-containing SLN. Nevertheless, the average size is in the range of micrometers. Hence, the term ‘‘lipospheres’’is a misnomer for these particles.

8.5. Preparation by high speed stirring and/or ultrasonication

These types of SLN were prepared by spray congealing followed by lipid nanopellets produced by high speed stirring or sonication from lipid microparticles[175,176]. Major improvements with this technique rely on the facts that it is easily produced and the equipment is commonly available in every laboratory. The issue with high speed stirring originates from broader particle size distribution in the micrometer range. The result of this is physical instability, an example being particle growth upon storage. Using higher surfactant concentrations could possibly bring improvements to this technique, which could potentially be associated with toxicological problems after parenteral administration. Another possible caveat is the metal contamination due to ultra-

sonication. Hence, previous studies have relied upon methods to improve the stability of the obtained SLN dispersions. High speed stirring is combined with ultra-sonication nowadays at elevated temperatures for some time[177,178]. This aids in obtaining reasonably narrow and physically stable distributions, although the concentration of lipid is relatively low (< 1%) when compared to the surfactant concentration, which is high.

9. Characterization of nanostructured lipid carrier

Characterization of lipid nanoparticle formulations should be carried out carefully as a requirement in order to facilitate the formation of dispersions having the relevant properties for the desired application. The complex structural features coupled with the size of the colloidal lipid nanoparticle dispersions accounts for a challenging task of their characterization. The size of the colloidal particles promotes modification of physical features such as increasing solubility and the propensity to lead to formation of super-cooled melts. On dispersion of the bulk materials into nanoparticles, their properties such as polymorphism and crystallinity might significantly alter. Attributes, which further complicate the scenario is the potential co-existence of additional colloidal structures such as micelles, vesicles and emulsions in the dispersions. A comprehensive scheme for characterization is necessary in order to investigate the structural behaviour of these complex colloidal carriers. Characterization of drug carriers can nonetheless be done by several physicochemical methods. Few advances in analytical methods and equipment such as the ones which use laser light scattering techniques for particle size analysis, zeta potential determination, differential scanning calorimetry, and a range of electron-microscopic techniques can potentially be used. In general, the characterization of colloidal particles have undergone several improvements, including their physical state and stability, which have been carefully monitored over the past decade or so.

9.1. Dynamic light scattering

Dynamic Light Scattering (DLS) may also be referred as photon correlation spectroscopy (PCS) or Quasi-Elastic Light Scattering and is a technique, which aids in estimation of the mean particle size and the width of the particle size distribution expressed as polydispersity index (PDI) in the sub-

micron range as shown in Figure 16. The particle size is measured as the diameter of the sphere, which diffuses with the same velocity as the particle being measured. DLS measurements are based on the tenets of the light scattering phenomena, in which the statistical intensities of fluctuations of the scattered light from the particles is measured. These fluctuations are a result of random movement of the particles in the dispersion medium, more popularly known as the Brownian motion.

The structure of a DLS device usually involves a laser light to illuminate a tiny volume of the sample in question, primarily composed of a dilute suspension of particles. A photomultiplier is used to measure the intensity of the scattered light from the particles after it has been collected by a lens at a certain angle (usually 90º or 173º). At identifiable fluid viscosity and temperature, the diffusion rate of the particles depends on their size. Hence, the rate of fluctuation of the scattered light intensity guides the calculation of the size of these particles. Small suspended particles diffuse relatively faster than the larger ones, causing rapid fluctuations in the scattered light. A correlator is used to estimate the detected intensity signals by using the auto-correlation function, $G(\tau)$. Correlators compare between two different signals or a signal with itself at varying intervals of time. Then, the diffusion coefficient (D) of the particles is calculated from the decay of correlation function. Equation (2) below shows that the hydrodynamic diameter (d_h) of the particle is inversely proportional to D, measured by the instrument. The d_h of the particles can be calculated by the Stokes-Einstein equation.

$$d_h = \frac{kT}{3\pi \eta D} \qquad (2)$$

where, d_h is the hydrodynamic diameter, D is the translational diffusion coefficient, which measures the velocity of the Brownian motion, k is the Boltzmann's constant, T is the absolute temperature and η is the viscosity of the solution.

As indicated above, the diffusion of small particles is faster than that of the larger ones leading to a strong fluctuation in the scattering signal with a much rapid decaying given by the auto-correlation function, $G(\tau)$. In case of a

monodisperse system, *i.e.* one which is composed of molecules of the same mass/size, G(τ) is given by a single exponential, but where the system is polydisperse (if more than one size/mass of particles is present), the G(τ) is represented as poly-exponential. Polydispersity Index (PDI) is calculated by making use of the deviation from a single exponential, which is in turn a measure of the width of size distribution. The standard PI of 0.0 is recorded when the particle population is essentially monodispersed. A relatively narrow distribution is indicated at PDI values in the range of 0.10-0.20, while values ≥ 0.5 indicate very broad distributions.

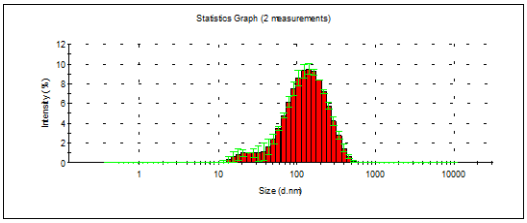

Figure 16. Particle size distribution of NLC (PEG-25-SA-NLC).

9.2. Particle charge and zeta potential

Predictions of the storage stability of colloidal dispersions are measured by the zeta (ξ) potential (ZP). There is less probability for particle aggregation with high ZP, *i.e.* with charged particles because of electrostatic repulsion. The fact that reduction of ZP agrees well with the reduction in physical stability is well-studied now. As a general rule, the gradation of the electrostatic stabilization (and thus, physical stability) can be delineated as being excellent for ZP >-60 mV and good for ZP >-30 mV[179,180]. However, for systems having steric stabilizers (non-ionic macromolecules), this rule cannot be applied since adsorption of steric stabilizers will decrease the ZP by causing shift in the shear plane of the particle. In his article, Müller has discussed the relationship between ZP and surfactants in great details[157].

The ZP is inversely proportional to energy inputs, majorly light and temperature. As the higher temperatures and increases in light intensity cause an elevation in the kinetic energy of the system, SLN aggregation and gelation occurs with a reduced ZP. Reorientations in the crystalline structure of the lipids are brought in about by this energy input[179,181]. As a result of this change, changes in the particle surface charge (Nernst potential) and consequently, the

measured ZP is affected. In addition, different sides of a crystal may possess different charge densities (*e.g.* aluminium silicates like Bentone™). One-dimensional crystal growth (*i.e.* formation of long b crystals[182] brings about changes in the surface ratio of differently charged crystal sides and accordingly, changes in the measured ZP. Aggregation of lipid crystals to building up a network is facilitated by the reduction in ZP (and electrostatic repulsions)[179].

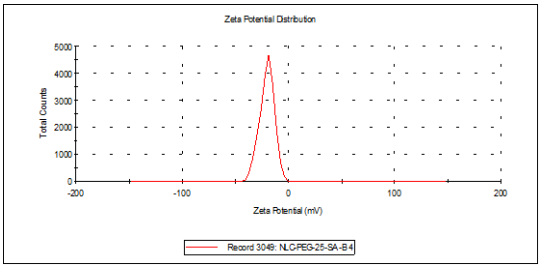

Figure 17. Zeta potential distribution of NLC (PEG-25-SA-NLC).

ZP of SLN dispersions have previously been shown to decrease as a result of autoclaving, especially for nanoparticles comprised of fatty acids[183]. The zeta (ξ) potential can directly be calculated from electrophoretic mobility based on the Smoluchowski equation (equation 3):

$$v = \left(\frac{\varepsilon \cdot E}{\eta}\right)\xi \qquad (3)$$

Where v is the measured electrophoretic velocity, η is the viscosity, ε is the electrical permittivity of the electrolytic solution, and E is the electric field.

9.3. Morphology

Primary information with regards to size distribution, morphology, surface topography and internal structure of lipid nanoparticles can be offered by the frequently used advanced microscopic techniques, *e.g.* scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM). Two and three dimensional morphological information are given by TEM and SEM, respectively. The large depth of field of SEM comes off as a foremost advantage, which translates to images of relatively large structures being all in-focus[184]. However, SEM does not provide internal details[185], because of its low resolving power (~3 - 4 nm), unlike TEM[186], which has a resolving power of 0.4nm[184], and can provide information on internal structure of lipid

nanoparticles[187]. Even hydrated specimens can be analysed by Environmental SEMs (ESEMs). Although their working pressures are not as low as SEM and coating before measurement is not required[188], they have low resolution rendering them insufficient to obtain detailed structural information of nanoscale materials, such as those giving the surface properties and architecture[187]. Cryogenic TEM (cryo-TEM), cryogenic SEM (cryo-SEM) and freeze fracture TEM are few other microscopic techniques that have been effectively made use off or visualizing lipid nanoparticles[189-193] that allow the sample to be observed near its natural state. Klang et al, provides further details about advanced microscopic techniques[187].

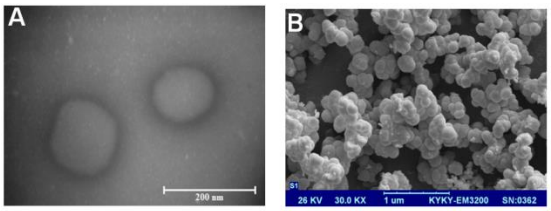

Figure 18. TEM (A) and SEM (B) images of PEG - 100 - SA coated SLNs.

On the other hand, use of techniques as Atomic Force Microscopy (AFM) have resulted in delivery of relevant information on the size, shape and surface morphological information of nanoparticles because of its high resolution (up to 0.01nm) [194]. AFM uses piezo-electric transducers to provide control over spatial positions of the probing tip relative to the sample surface with great accuracy and reproducibility. This, as a result is able to map the surface topology on an atomic or nanometre scale. Usually, surface profiles of uncoated non-conductive samples may be evaluated by AFM under atmospheric conditions and/or liquid/wet environment[188,194]. However, submicron particles exhibit rapid movements due mainly to the Brownian motions, rendering them imperative to be fixated/dehydrated for assessing shape parameters via the tiny tip of AFM[194]. The methods in the scale of 1-1000 nm include the SEM and the freeze-fracture electron microscopy. These techniques have been used inherently to classify the non-spherical shapes of SLN systems. However, these methods are not able to generate 3D-pictures and are also limited to measurements in vacuum, *i.e.* in aqueous-free environments. Hence, the influence of external agents on the time evolution of targets is not seen. High resolution AFM could potentially address these shortcomings.

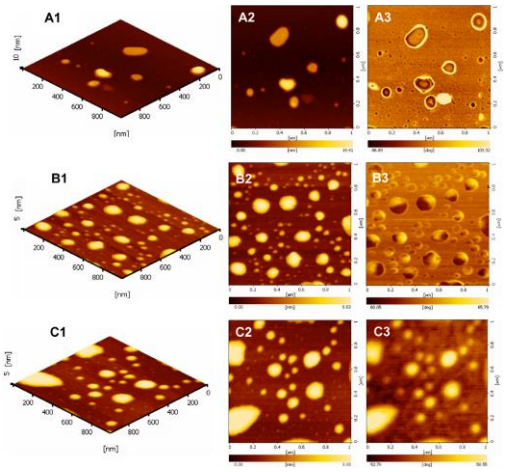

Figure 19. AFM image of γ -oryzanol loaded lipid nanoparticles (LN) at 0% liquid lipid (A), 5% liquid lipid (B) and 10% liquid lipid (C): 3D images (1), topographic images (2) and phase images (3). All images were scanned over a 1 × 1 μm^2 area.

9.4. Crystallization

Two of the significant constraints for lipids are polymorphic transition and temperature for crystallization. Characteristics such as lipid crystallinity degree and modification of the lipid beg special attention with regards to quality of the product. Solid nanoparticles involve polymorphic transitions. Rearrangement of the lipid molecules coupled with increase in lattice intensity promotes transition to a more stable lipid polymorph[195]. The arrangement of the lipid within the lipid nanoparticle is supposed to be less ordered than the bulk materials[177]. The crystallization behaviour, the degree of crystallinity and the crystal modifications of the matrix constituents compared to the bulk materials are all influenced by the production method, the presence of surfactant, melting point of the lipid, lipid concentration, drug incorporation and the high dispersity coupled with the small particle size of the resulting systems[196]. The internal structure of the particles along with the colloidal state of the dispersion are affected by the stabilizers, when it comes to particle size and stability. This consideration is imperative for the development of drug carriers based on lipid nanosuspensions[197]. Recrystallization at room temperature is generally hampered by depression of the melting and recrystallization points brought by crystalline structures alteration. As a result of this, liquid, amorphous or partially crystallized metastable systems are formed[181,196,198]. In addition, propensity to

recrystallize is thoroughly disturbed by the melting temperature of the lipid. Crystal behaviour and lipid modifications have a strong correlation to drug incorporation and rates of release[199]. The loading capacity of nanoparticles is further influenced by the lipid crystal order and variances in the supramolecular structures of the polymorphs. A distorted organization of the lipid crystals tends to prefer enhancement of drug loading capacity[177,195]. Changes in dispersion stability and drug loading are significant contributions from the sorts of crystal polymorph and the kinetics of transitions[195].

The lipid polymorphs may be told apart by X-ray diffraction method, which allows clear identification of these different polymorphic forms by their spacing[196,200] evaluation of the lengths of long and short spacing of the lipid lattice[107]. While X-ray diffraction facilitates differentiation amongst crystalline and amorphous materials, on the other hand, differential scanning calorimetry (DSC) can distinguish among amorphous solids and liquids. In a way, X-ray diffraction measurements endorse the polymorphism exhibited by DSC measurements.

^{1}H NMR spectroscopy is particularly useful for characterizing liquid lipid domains inside the SLN. With the incorporation of the liquid lipids in the lipophilic phase of SLN, which is thought to improve the loading capacity of the particles, including solid lipid with high crystallinity, the resulting particles are solid, but the oil inside the particle may remain in a liquid state. ^{1}H NMR observables give information regarding the mobility, arrangement and environment of the oil molecules[151].

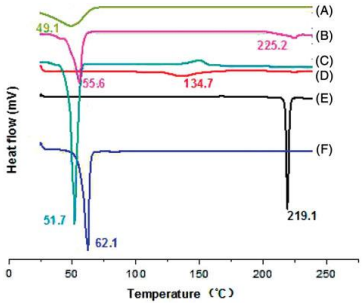

Figure 20. DSC image of (A) BCA–PEG–NLC and its ingredients, (B) physical mixture, (C) PEG–SA, (D) LEC, (E) BCA, and (F) GMS.

It also aids in accounting for the drug distribution within compositions and provide relevant evidence on the mobility of the drug molecules included in the lipid matrix. This is of utmost importance given the fact that sustained drug release from the lipid particles is a challenging task, despite high drug loading being easily attained with high drug mobility[201].

9.5. Drug incorporation into SLN and NLC

Considerations to be taken into account when dealing with the loading capacity of the drugs in the lipid are[107] drug solubility in the melted lipid, tendency of drug melt to be miscible with the lipid melt, chemical and physical texture of solid matrix lipid, polymorphic state of the lipid material.

Models of SLN drug incorporation as depicted in Figure 14 are the solid solution model, core-shell model; drug-enriched shell, drug-enriched core. A solid solution model disperses the drug in the lipid matrix with production of the particles by the cold homogenization process without the use of surfactant or drug-solubilizing surfactant. The melting behaviour of the lipid matrix assesses the strength of drug interactions with the lipid[202]. X-ray diffraction can be used as a standard tool to validate the molecular dispersion of the drug in the particles. The extended release of drug for as long as a number of weeks can be attained with solid lipid particles[203]. On the other hand, the drug-enriched shell model of drug incorporation partitions the drug molecules from the liquid oil phase to the water phase *via* hot homogenization. The liquid state of the particles accelerates the segregation into the external phase. The extent to which the drug partitions is directly proportional to the drug solubility in the aqueous phase and the temperature. As a result, elevated temperature leads to higher saturation solubility of the drug in the water phase. When the O/W nanoemulsion produced is cooled, the drug solubility in water drops down with temperature drops. This leads to re-partitioning of the drug into the lipid phase. When the recrystallization temperature of the lipid is reached, a solid lipid core is formed. The reduced solubility in aqueous media leads to enhancements of the pressure on the drug to promote re-partitioning, when the temperature of the system is decreased. As a result of the core being inaccessible to the drug due to crystallization, the drug concentrates in the still liquid outer shell of the SLN and/or on the particle surfaces[204,205]. The drug-enriched core model of drug incorporation gives a drug-

enriched core when a drug which is dissolved in the lipid melts at or close to its saturation solubility. Following this, when the nanoemulsion is cooled, the drug becomes supersaturated in the melted lipid leading to drug precipitation earlier than lipid recrystallization. Subsequent lowering of temperature promotes to the recrystallization of the lipid surrounding the drug as a membrane. This lipid membrane houses the drug at a concentration consistent with the saturation solubility of the drug at the recrystallization temperature of the lipid[107]. This entrapment model shows that the nature and amount of surfactant surrounding the SLN core determines the quantity of SLN associated drug. Heiati et al., have previously shown that drugs such as dexamethasone, dexamethasone palmitate and a zidothymidine palmitate, which are amphiphilic in nature, was not seemingly integrated within the triglyceride core of SLN, an example being that of trilaurin (drug-enriched shell model). Also, drug incorporation was affected by the increase in phospholipid strength, and could possibly be tuned by changing the concentration of phospholipids[206].

9.6. Controlled drug delivery

The drug release from lipid nanoparticles could be appreciated well by the general attributes outlined below (Venkateswarlu and Manjunath 2004)[199]:

- The drug release and drug partition coefficient are inversely related.
- Smaller particle size and hence larger surface area elevates the amount of drug released.
- A homogeneous dispersion of the drug in the lipid matrix encourages gradual release of drug.
- If the lipid carrier is less crystalline and drug is highly mobile, they facilitate expedited drug release.

These general guidelines above outline the significance of the production parameters in controlled drug delivery. With the employment of cold homogenization technique, the lipid phase loaded with drug rests mainly in the solid state during the production step (solid solution model). This leads to decreased mobility of the drug and entrapment of an increased amount of drug related to the hot homogenization technique, *i.e.* least partitioning to the water phase. In addition, drug release is much more sustained in contrast to the hot homogenization technique. The so-called ‘adeptness’ of entrapment alters with

the surfactant concentration and the production temperature used during hot homogenization. The SLN release profile *via* the hot homogenization technique reads as an initial burst effect in the first five minutes: a very prominent fast drug release followed by a much slow and sustained release of drug for the drug-enriched shell model (*i.e.* 100% within <5 min). Müller and co-workers have focused on probing the drug release mechanisms and their governing dynamics. With this in mind, newer SLNs came into picture, which incorporated several model drugs with diverse physicochemical properties. Studies on tetracain and etomidate bases as lipophilic drugs, and iotrolan (Schering AG, Berlin) as a hydrophilic drug were conducted under many of triglyceride SLN formulations. Etomidate and tetracain loaded nanoparticles have been reported to show almost comprehensive drug release initially over few minutes, which does not necessitate relying on the technique of production[207].

Studies on the release pattern of microparticles showed that the initial release in bursts is because of the site of drug in the SLN and drug surface area[202]. Thus, a possible solution to this effect was to increase the particle size to attain prolonged release, as in case of lipid microparticles. The feature of burst release of the drug was due to the fact that the drug was entrapped in SLN according to the drug enriched shell model leading to a comparatively short distance of diffusion. Another important consideration to be taken into account is the selection of a feasible surfactant that would interact with the outer shell and influence its structure. A surfactant in its low concentration gives rise to a minimal burst and sustained drug release. The significance of the homogenization was shown in the same study. The solid solution model accounts for the extended release by the molecular distribution of the drug in the lipid, although the employment of hot or cold homogenization method and the nature of the lipid change the profile for the drug release. For example, cold homogenization leads to sustained drug release with no distinct burst. Such trends may be recognized as due to the homogenous molecular distribution of the drug in the solid lipid matrix, which furthers the formation of a solid dispersion before homogenization and the process of cold homogenization leading to particle formation, which is retained as a solid dispersion. Due to this effect, there is enhanced release of prednisolone over a period of up to six weeks. The release profiles of the drugs incorporated could be modified more effectively by

the solid state of SLN. As a matter of fact, the placement of the drugs in the correct region is of primary importance to account for their release behaviour[208]. Solid state of particles at room temperature enforces controlled drug release, where there is decrease in mobility of the drugs, whereas the crystalline nature of the particles controls the drug release behaviour.

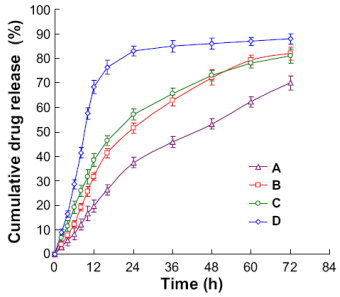

Figure 21. *In vitro* drug release profile of different NLCs: (**A**) Tf_{10k}-PTX-DNA-NLC, (**B**) Tf_{5k}-PTX-DNA-NLC, (**C**) PTX-DNA-NLC, and (**D**) Taxol®.

Another previous study has shown in favour of these results[205]. Fick's law of diffusion governs the membrane controlled release by the drug enriched core model of drug incorporation[152]. High drug loading capacity coupled with sustained release characteristics is common with NLC formulations, where the oil content of the particles solubilizes the drug.

The amorphous and imperfect type of NLCs facilitates improved flexibility to attain the preferred extended release[153]. In case of a highly lipophilic drug such as clotrimazole, a rapid drug release is favoured by NLC in comparison to SLN. Properties as the entrapment efficiency and drug release profiles depend on the drug and lipid concentrations, also promoted by higher propensity to crystallize along with higher lipid concentrations of SLN[209]. A recent study reports that ascorbyl palmitate (amphiphilic structure) was entrapped in SLN and NLC. With SLN and NLC, the rate of drug penetration across human skin from was statistically identical. Drug entrapment model of SLN (drug-enriched shell model) and the crystallization behaviour of NLC are responsible for this similarity in trends (Fig. 14)[210].

10. Applications

When compared to other different drug delivery systems, nanostructured lipid carriers (NLCs) display better stability and ease of upgradability to

production, an attribute imperative to several modes of targeting. Colloidal drug delivery systems are based largely on NLCs, as they are biodegradable and last at least for a year. Drug delivery can be facilitated both *in vivo* and *in vitro* to the liver and to actively phagocytic cells, respectively. A few of the number of potential applications of NLCs are enlisted below:

10.1. NLCs in brain delivery

NLCs have come off as a novel drug delivery system in the brain[211]. Several recent studies report NLCs carrying drugs to be delivered in specific regions of human brain[212,213]. Previous reports have also depicted that the novel nanometric chitosan coated NLCs are actually safe and non-toxic for intranasal (i.n.) administration and have successfully delivered drugs to the brain in a single dose *via* i.n. route[214]. This has opened up novel prospects to less invasive administration routes to approach the brain by passing the limiting step of the blood brain barrier (BBB). In addition, surface charge and particle size have been shown to affect drug delivery to the brain. Alam reported that epilepsy can effectively be treated by successful targeting of the potent lamotrigine[215]. Similarly, i.n. administration of NLCs of valproic acid (VPA) has offered superior protection against MES seizure[216]. Tsai has suggested a novel approach for explicit brain targeting of drugs *via* the intravenous (i.v.) route[213]. Hence, it has been heavily indicated that NLCs may expand the drug's capability to penetrate pass the BBB and has come off age as a promising drug targeting system for the treatment of CNS disorders.

10.2. NLCs for topical delivery

NLCs have been incorporated with several drugs for topical application. These include tropolide[217], imidazole antifungals[218], anticancers[219], vitamin A[220], isotretinoin[221], ketoconazole[222], DNA[223], flurbiprofen[224] and glucocorticoids[225]. The superb ability of NLCs to increase drug accumulation, drug retention, sustained release, localized effect, and maximal therapeutic antifungal efficacy are some of the plausible mechanisms for the improvement and hence they provide an advanced cutaneous drug accumulation[226-228]. Strong points of delivering the drug directly to the site of action is facilitated by formulations of acitretin–NLC gels in treating psoriasis patients[226]. Also, it is now well

documented that a couple of drugs with strikingly varied polarities may potentially be used in combinations of NLCs for augmented drug penetration and restricted skin irritation[229]. It has also been shown that NLCs display prominent effect compared to SLN for topical delivery.

10.3. NLCs as cosmeceuticals

Nowadays, added potential applications of NLCs lie in the preparation of sunscreens and as an active carrier agent for molecular sunscreens and UV blockers[225]. One *in vivo* study reported that there was enhanced skin hydration by almost 31% after 4 weeks by addition of just 4% SLN to aconventional cream[217]. Also, sustained release occlusive topical applications have been few of the formulations involving SLN and NLCs[230]. Superior localization is obtained on vitamin A in skin upper layers with glyceryl behenate SLNs in comparison to conventional formulations[220]. The effects of topical α-tocopherol was explored by Lopez-Torres *et al.*,[231] on the application on epidermal and dermal tissues and its ability to inhibit UV-induced oxidative damage. A superior capability of NLCs to increase the antioxidant *in vitro* penetration into rat skin was demonstrated by a comparison between SLN and NLC containing resveratrol[232].

10.4. NLCs as a targeted carrier for cancer

There are reports which suggest that NLCs could be used as an effective drug carrier to treat neoplasms[233]. In order to design drug release enhancers, tamoxifen, an anticancer drug have been incorporated in NLC after i.v. administration in breast cancer, which also augment the permeability and retention effect[234]. Similarly, targeting tumourous cells have been attained with NLCs loaded with drugs like methotrexate[235] and camptothecin[236]. Local SNL injections formulations loaded with the drug, mitoxantrone decreases the toxicity and recover the safety and bioavailability of the drug[237]. On the other hand, doxorubicin (Dox) efficacy was enhanced by including them in SLNs[238]. The method that was followed to formulate this included that Dox was complexed with soybean-oil-based anionic polymer and dispersed together with a lipid in water to lead to formation of Dox-loaded solid lipid nanoparticles. Such system was shown to have a superior efficacy and brought down the number of breast cancer cells.

10.5. Oral NLCs in anti-tubercular chemotherapy

Rifampicin, isonizide, pyrazinamide-loaded SLN and NLC systems are such formulated anti-tubercular drugs, which could improve patient compliance in addition to reducing the dosing frequency of the drug[239]. The nebulization in animal was reported to be improved by incorporating the above drug in SLN with enriched bioavailability[240]. The usefulness of lipid nanoparticles to evade adverse drug – drug interactions can be seen for the combinations of drugs like isoniazid (INH) and rifampicin (RIF), which are to be used with caution[241]. Rifampicin-loaded NLC drugs coated with mannose could be accurately targeted both *in vivo* and *in vitro* displaying minimal cytotoxicity and rendered safe for systemic administrations[242].

10.6. NLCs for potential applications in agriculture

When incorporated in SNL, essential oil extracted from *Artemisia arboreseens*L showed reduced rapid evaporation, when compared to emulsions and hence the systems have found effective use in agriculture as a relevant carrier of ecologically safe pesticides[243]. The preparation of SLN was done hereby the use of compritol 888 ATO as lipid and poloxamer 188 or Miranol Ultra C32 as surfactant.

Several different forms of monoglycerides, diglycerides, triglycerides, waxes, phospholipids and fatty acids have been exclusively used to develop NLCs. However, sorbitantristearate (Span 65) have not been commonly used for preparation of NLC in previous literature, as one of the lipidic component, despite its known biocompatibility. That imperfection/void spaces in the NLC matrices would exist when a combination of soy lecithin and stearic acid is used is a known fact. Hydrocarbon chain of the stabilizers also has significant contributions on generating imperfections. The stabilizers' effect on the behaviour of the solution phase and thermal properties of NLC such as temperature of maximum heat flow (T_m), peak width at half maxima ($\Delta T_{1/2}$), change in enthalpy (ΔH), change in heat capacity (ΔC_p) and crystallinity index (C.I.) have not been extensively studied. The mismatch of hydrocarbon chains is expected to make the formulation a novel carrier for lipophilic, hydrophilic as well as amphiphilic drug molecules. Drug localizations in NLCs could be potentially estimated by spectroscopic as well as thermal investigations. In

addition to this, the effect of hydrocarbon chain length on the entrapment efficiency, loading content and release kinetics are not very well characterized for this type of small molecules loaded in NLC. Studies carried out by several research groups indicate that the severe side effects could drastically be reversed, when loaded with suitable drug delivery system. Our goal is to validate this rationale for NLC formulation using Lidocaine (LIDO) and procaine hydrochloride (PRO.HCl), which are commonly used as local anaesthetics. To reduce dose frequency, relieve skin irritation (caused by high dose of anaesthetics) as well as to sustain the anaesthetic effect, such formulations are considered worthy to be investigated, and are extremely relevant.

In addition, NLCs composed of a mixture of saturated (solid) and unsaturated (liquid) triglyceride, phospholipid and fatty acid may be a very fresh and interesting alternative in place of the established fatty acid and triglyceride combinations. This is because such blends are known to be polycrystalline in nature, can boost the physical stability, efficiency to encapsulate, release behaviour, therapeutic efficiency, *etc*. Despite there being a number of instances previously found in literature on NLCs involving a mixture of saturated (solid) and unsaturated (fluid/liquid) lipids, comparative studies designating the effects of unsaturated lipids and saturated lipids on the physicochemistry of NLCs are sparse. Hence, a handsome scope of research in the field of NLCs is the employment of varied combination of saturated and unsaturated lipids. Contemporary studies have indicated that UA has latent anti-tumor effects and shows cytotoxic activity against various types of cancer cell lines[244-247]. Notwithstanding the above potentials, clinically UA has been limited because of its modest solubility in water, which leads to its crippled bioavailability and insignificant *in vivo* pharmacokinetics. This study revolves around evaluation and comparison of the saturated and unsaturated lipid comprising NLCs, in order to determine if differences in composition can alter the performance of these systems.

Moreover, drug delivery systems in the form of PEG coated nanostructured lipid carriers have been studied for delivery of several cancer therapeutic molecules by oral route. Nonetheless, it is not common in literature to find combination of tristearine and oleic acid as one of the lipidic component

and PEG - 25 / 55 - stearate as coating agent in preparing NLCs. Thus, in the present study, we aim to prepare conventional NLC as well as PEG-coated orcinol glucoside-loaded nanostructured lipid carriers for the purpose of targeting gastrointestinal tract cancer with enhanced anticancer activity for oral delivery of orcinol glucoside (OG). The current study proposes to explore the aforementioned systems through extensive physicochemical characterization.

References

References are given in BIBLIOGRAPHY under Introduction (pp. 162 - 173).