2014

Quantitative, qualitative and in vitro evaluation of solid lipid nanoparticles containing 5-fluorouracil

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Thesis

entitled

Quantitative, Qualitative and In Vitro Evaluation of Solid Lipid Nanoparticles Containing 5-Fluorouracil

by

Mohamed Saleh Majrad

Submitted to the **Graduate Faculty** as partial fulfillment of the requirements for the

The Master of Science Degree in Pharmaceutical Sciences, Industrial Pharmacy Option

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August 2014

An Abstract of

Quantitative, Qualitative and In Vitro Evaluation of Solid Lipid Nanoparticles Containing 5-Fluorouracil

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The primary goal of this research work was to develop solid lipid nanoparticles (SLNs) containing 5-Flourouracil and to evaluate its effect on various cell lines. The solid lipid nanoparticles were prepared through a new temperature modulated solidification technique developed in our laboratory. Particle size analysis by dynamic light scattering (DLS) and morphology evaluation by transmission electron microscopy (TEM) demonstrated that the SLNs are nanoparticulates. Cytotoxic activity of SLN loaded 5-Fluorouracil showed a decrease in viability when compared to pure solution of 5-FU on PC-3 and Caco-2 cell line. Blank SLN showed no decrease in cell viability when the concentration increased. Biocompatibility studies of SLNs in human RBCs indicated that 5-FU SLN formulations are compatible. Bovine permeability study shows that apparent permeability for 5-FU SLN was 0.000348 cm/s and 1.339 cm/s for 5-FU solution. The preliminary results from various in vitro evaluations suggest that 5-FU loaded SLNs have the potential to be used as an anti-cancer drug delivery system.

Acknowledgements

I want to take this opportunity to express my deepest sincere thanks to my major advisor Dr. Nesamony for the unconfined assistance by all means whether it is related to research or outside research. Thank you for all your guidance and support; it's an honor to be part of your research group. I would like to thank my committee member Dr. Boddu for all the knowledge and guidance learned in his coursework. Special thanks to Dr. Liu for agreeing to be a part of my committee, I want to thank to Dr. Shah for being the faculty representative in my thesis defense. I am very grateful to Dr. Hoss for his assistance in providing me the financial assistance needed to complete the Industrial pharmacy degree. Thanks to Dr. Alexander for all the knowledge gained. Special thanks to Ali, Yangjie, Abulkareem, Nachiket, and Ameya for their support and encouragement that was viable in our two year program. I would like to express my never ending love to my beloved family. My wife is my best friend, soul mate, and her love gives me encouragement and support. Special thanks go to my three beautiful kids, Abdulmajeed, Wijdan and Saleh may Allah protect them and guide them to the right path. A million thanks to my great mother and father. Thanks to my brothers Waleed, Mousa, my sisters for their support and many thanks to my cousin Aisha Omar for the unlimited help she provided to my family. Thanks to all my cousins, nephews and friends for their support and encouragement during my studies, thank you Allah for making this come true.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

5-FU 5-Fluorouracil

GMS.....Glyceryl monostearate

Phe.....Phenylalanine

SLNs.....Solid Lipid Nanoparticles

Trp.....Tryptophan

Chapter 1

Introduction

1.1. Solid Lipid Nanoparticles

Solid Lipid Nanoparticles (SLN) were developed over the past decade as an alternative carrier system to emulsions, liposomes, and polymeric nanoparticles [1, 2]. SLNs offer several advantages over other colloidal carrier systems. They are composed of physiological lipids, which reduce the risk of acute and chronic toxicity [3, 4]. SLNs can be manufactured economically and in a large scale production using high pressure homogenization without the use of organic solvents [4]. SLNs are composed of lipophilic bioactives that is incorporated in a carrier lipid that remains solid at room and body temperature and are considered crystallized nanoemulsions [5]. The entrapment of the bioactive ingredients inside the solidified lipid matrix is considered one major advantage of SLNs [5, 6], this prevents the diffusion of the bioactive ingredients to the surface of the emulsion droplet and reduces the incidence of oxidative reactions [7, 8]. However, SLNs are also prone to instabilities because of their complex crystallization behavior due to occurrence of polymorphic transitions in solid lipid nanoparticles. Lipid polymorphisms can decide the location of drug entrapment with the drug either being

integrated in the matrix or attached to the surface of the submicron particle. According to the Noyes Whitney and Kelvin equation, the dissolution rate increases for poorly water-soluble drugs as the particle size decreases. These drugs when formulated as a nano-suspension show an increase in the dissolution rate and an increase in the absorption in the gastrointestinal tract. Nanoparticles can be designed to adsorb preferentially on organs or tissues depending on their relative hydrophobicity, particle charge, and surface properties [9]. The particle matrix in SLN is composed of solid lipid and contrasts with the lipid nature in emulsions and liposomes. And to achieve controlled drug release liquid lipid may be blended with solid lipids because in solid lipids drug mobility is considerably lower when compared to liquid lipids [7]. The incorporation of drugs in solid lipids instead of liquid lipids improves the stability of incorporated chemically sensitive lipophilic ingredients and has been shown to increase control over the release kinetics of encapsulated drugs. These beneficial effects are potentially attributed to a number of physicochemical phenomena that is associated with the physical state of the lipid phase. The rate of chemical degradation reactions may be reduced because the mobility of the reactive agents in a solid matrix is lower than in a liquid matrix. Also, the carrier lipid within individual liquid particles can be controlled and produce microphase separations of the active ingredients. This occurs by preventing the accumulation of active compounds at the surface of lipid particles where chemical degradation reactions frequently occur [10-14]. Bioactive compounds that are poorly absorbed into solid lipid nanoparticles have been shown to improve their biological absorption. It is shown that lipid digestion is slowed down by the use of solid matrix instead of liquid matrix leading to a sustained release of the encapsulated compound [15].

For a drug delivery system to be ideal, the system must have properties of sufficient drug loading, stable in environment conditions, controlled and targeted release, easy and inexpensive scale up procedure, selective for the site, biodegradable, non-toxic, and non-immunogenic.

SLNs are more stable formulations than liposomes and they confer prolonged drug release with no sterilization problems. Some SLN formulations are prone to gelation after certain period of storage time; usually this is avoided by changing the lipid composition and with the use of stabilizing surfactant mixtures. It is desirable to use a specific formulation in some cases because of toxicological considerations and stabilization of incorporated drugs against chemical degradation. Certain surfactant mixtures produced drug accumulation in the outer shell of the SLN particles [16]. The lipid gelation process is accelerated with increasing storage temperature and with increasing light exposure. The addition of a co-emulsifying surfactant with high mobility such as glyococholate can retard or prevent gelation [17].The SLN dispersion can be destabilized physically by external input of various forms of energy. It is also found that siliconization inhibited the lipid gelation process. The recrystallization index that is indicative of the degree of crystallization of lipids also has an effect on the long-term stability of the aqueous SLN. An increase in particle size growth is usually seen in dispersions that contain a highly recrystallized lipid phase. SLNs by the hot homogenization method are prepared through high-pressure homogenization of the melted lipid dispersed in an aqueous surfactant solution. The obtained nanoemulsion is cooled down and then recrystallizes to form the solid lipid nanoparticles. This usually depends on the nature of the lipid and can take place very quickly within minutes. On the other hand, it has been reported that

recrystallization of the lipid can be decreased for up to 3 weeks or even months. The general trend is that the crystallinity of the bulk material used for SLN production increases with increasing storage time and the recrystallization index of the SLN is below the crystallinity of the bulk material used [16]. Triglycerides will crystallize in the α modification, the α→ß transformation can be retarded by surfactants such as poloxamer [18, 19].

1.1.1. Influence of the shear forces and lipid concentration

Shear forces such as pushing through the needle of a syringe promote gelation in some suboptimal stabilized formulations of SLN. In some studies, Compritol SLN was stored in a shaking bath at 20° C at a frequency of 70 cycles per minute to induce and observe the process in a controlled manner. The degree of crystallinity and particle size were determined every day over a period of 2 weeks. In dispersions with a lipid concentration of 10 % particle aggregation could be detected after 3 days. The particle diameter increased from 0.77 ± 0.01µm up to 23.34 ± 0.19µm. The crystallinity increased with increasing time in the aqueous SLN dispersion and after solidification in the gel. The samples showed gelling within 5 days and the resulting gel became increasingly solid with storage time. The lipid concentration was reduced from 10 % to 5 % and 2 % to improve the stability of the SLN dispersion. The 10% stock SLN was diluted with de-ionized water and the ratio of lipid versus surfactant was the same. A reduction in aggregation was observed due to a lower probability of particle collision during the shaking process from a lower particle concentration [16].

The 5% SLN dispersion did not form a gel up to 2 weeks. The particles size increased after day 5 and solid particles were detected visually. The enthalpy increase was lower when compared with the 10% lipid concentration. The particle size of the 2 % dispersion remained the same (280.1 ± 5.0 nm) at day zero and 282.1 ± 3.9 nm after 14 days.

1.1.2. Influence of the surface of the packing material

To study the effect of contact between the particles and the surface of the packing material studies were done by varying the ratio of volume to contact surface in vials. Some glass vials were filled up to the top and some were filled to only one third capacity with a 10 % Compritol SLN dispersion and stored in a shaking bath at 20 °C and 70 cycles per minute. Gelation was accelerated and occurred 2 days quicker in the vials with a low volume to surface ratio. Aggregation and gelation of particles is not usually promoted by a particle-particle contact but rather by a particle to vial surface contact. Droplets in emulsions coalesce through adherence to the wall of the container and similarities exist in SLN and oil-in-water emulsions since the inner lipid phase is stabilized by surfactants.

1.1.3. Properties of the lipids: SLN versus bulk material

Energy input causes destabilization in Compritol SLN at higher kinetic energy leading to oscillation of lipid molecules promoting frequent collisions [16]. The number of particle contacts is further increased by shear forces that cause a partial ripping off and damage to the surfactant film on the surface of the particle promoting aggregation. Freitas at el. showed that fat fraction of Compritol SLN is not completely solidified as indicated from

the recrystallization index. The enthalpy of the physical mixture of the excipients were compared the recrystallization indices calculated from the enthalpy of the SLN by differential scanning calorimetry. During storage conditions, the Compritol dispersion is stable at 8 °C in the dark, recrystallization index increased slightly to about 87 %. The gelling system showed higher crystallinity of 130 %, this indicates that the whole fat fraction was solid. These changes are possibly because of the energetic changes occurring due to partial destruction of the surfactant film and with particle collision the lipid solidifies by bridging the particles.

1.1.4. Stability and storage conditions

SLNs and nanostructured lipid carriers (NLC) may perhaps contain additional colloidal structures, including mixed micelles, micelles, liposomes, and nanoemulsions [20]. SLN/NLCs contain additional features such as various polymorphic modifications, supercooled melts, non-spherical shapes and all these eventually affects the stability of the drug delivery system. During storage, there are several stability problems such as increase in particle size, gelation of the dispersion, and drug expulsion from the lipid matrix. Gelation occurs when networks and lipid bridges exist between the lipid particles [21] . In hot homogenization, the first product formed is super-cooled melt which usually has high drug loading capacity, but because the lipid transforms to lipid crystals there is a decrease in lipid drug loading capacity and eventually drug is expelled from lipid matrix. SLNs/NLCs physical stability is generally studied by evaluating measurements of particle size (photon correlation spectroscopy, laser diffraction), zeta potential (electrophoretic light scattering) and thermal analysis (differential scanning calorimetry). Many studies

have indicated that the physical stability of SLNs dispersion is more than a year [22-25]. The study reported by Frietas et al. assessed the effect of light and temperature on the physical stability of SLNs dispersions. The study shows that light and temperature enhanced particle growth and that gelation of the particles occurred within 7 days and 3 months storage in artificial light and day light, respectively. However, in the dark condition, the particle growth started after 4 months storage, zeta potentials decreased from -24.7 to below -18 mV when stored in light. They also found that the particle size was increased at elevated temperature that resulted in melting the lipids and modifying the lipid matrix. On the contrary, there was no significant change in particle size when stored in refrigerator for more than 3 months. Also, upon particle growth the zeta potential decreased from -24.7 to -15 mV. Developing and optimizing storage conditions can improve and enhance the physical stability of SLN formulations [16]. Storage temperature has been found to have a profound effect on the quality of SLNs and it was found that SLNs stored at 5 ± 3°C were stable beyond one year [22]. There particle size did not increase significantly after one year of storage and the entrapment efficiency decreased by 9 %. Total drug content was reduced by 3% suggesting that the SLNs retained their potency beyond the one-year study period. Long term stability for SLNs can be performed by storing the formulation in 3 different environments at 4 °C, room temperature, and at 40 °C. Approximately 10 mg SLN particle was placed in glass vial that is diluted with 10 ml water and after vortexing and sonication the samples were subjected to particle size analysis [26].

Stability of the SLN formulation is dependent on components added to formulate the product including the type of lipid used and the emulsifying agent [27]. Studies have

shown that SLNs stored at refrigerated temperature 4 ± 1 °C were more stable as this storage did not significantly change the particle size and entrapment efficiency when compared to particles stored in room temperature [28]. The lipid transformations in the SLN can be produced by input of kinetic energy such as temperature and light. This leads to changes of the melting point of the lipid and causes transformation of ß' and α to ß polymorph and this is usually accompanied by gel formation. However, formulations stored in refrigerator in dark conditions were found to withstand transformations [21]. Chemical investigation of the stability of the lipids used in SLNs was performed by gas chromatography along with transmethylation of the lipids at the methyl esters of the fatty acids. The idea here was to extract the lipid from the aqueous SLN dispersions [29]. In the study performed by Radomska-Soukharev the formulation incubated at 25 °C in which the lipids was made of triglycerides, Dynasan ® 118 showed the highest chemical stability [29].

Spray drying is used in SLNs to increase or prolong stability especially for preparations intended for intravenous administration. Spray drying is investigated for SLNs as an alternative method to lyophilization to convert liquid dispersions into a dry powder. In order for spray drying to be effective, the melting point of the lipid matrix should be greater than 70 °C. Aqueous dispersions of SLNs can be converted by spray drying into a dry powder that can be stored for extended periods of time [30]. The spraying parameters and the chemical nature of the lipid phase, and the redispersion medium influenced the particle size of the final dried SLN powder [30].

Lyophilization also has been found to enhance the stability of SLNs. Cryoprotectants such sucrose, dextrose, trehalose, and mannitol were found to be beneficial to retain the

integrity of SLNs during and after lyophilization. SLNs that are lyophilized in the absence of cryoprotectants produced severe particle aggregation [31]. Trehalose is considered to be the most effective cryoprotectant in terms of inhibiting SLN particle growth [32]. A study done by Ghaffari et al, showed that SLNs of 150 nm size increased to 190 nm after lyophilization. However, at higher temperature the stored freeze dried particles did not have any significant particle size enlargement. Amikacin loaded solid lipid nanoparticles were designed to target the *Pseudomonas aeruginosa in* to reduce its dose or decrease the frequency of administration with the goal of reducing its side effects during long-term treatment. Amikacin loaded SLNs stored in aqueous dispersion form without freeze-drying showed rapid particle growth at increasing temperatures. Zeta potential of SLNs lyophilized was higher than those before lyophilization and the polydispersity index (PDI) after freeze-drying was less than 0.5. Activity of amikacin expressed no change after lyophilization and the release profile of the drug did not change even when particles were stored at various temperatures. There was a burst release in all the conditions stored [31]. Based on various stability results lyophilization is accepted as a suitable method to increase particle stability for long-term storage. Zeta potential of particles increased after freeze drying which may suggest that the risk of aggregation and enlargement of particles after redispersion is decreased. Release profile of amikacin SLN increased with increasing temperature partly because the lipid melts at higher temperature and causes changes in the crystalline structure of the lipid. The lipid content of the SLN dispersion should not exceed 5 % to prevent increase in particle size. Lyophilization can compromise the protective effect of the surfactant [33]. SLN aggregation can be decreased by the addition of cryoprotectors and to obtain a better

redispersion of the dry product. In the field of liposomes, the influences of the cryoprotectants have been widely investigated on the quality of the lyophilizates. Cryoprotectants favor the glassy state of the frozen sample and they decrease the osmotic activity of water and crystallization by preventing contact between discrete lipid nanoparticles [34-40]. They serve as a pseudo hydration shell by interacting with the polar head groups of the surfactants

Morphology studies performed by Ghaffari et al showed that particles lyophilized and redispersed, did not change their particle size significantly and verified that the shape and size of the SLNs are not influenced by the freeze drying method [41].

1.1.5. Gelling tendency: temperature versus shear forces

In Compritol SLN, the destabilization process and recrystallization behavior induced by temperature resulted in firm solid gels that are highly viscous by the effect of shear forces. DSC measurements over a period of 14 days on a 10% dispersion that was stored at 50 °C demonstrated an increase in melting point and crystal fraction. At higher temperature the shoulder caused by incorporated water was detected after 1 week. The melting point of 72.3 °C and the shape of the DSC curve were similar to that of the bulk lipid material after 2 weeks storage. However, Compritol samples containing 10 % stored at room temperature in a shaker demonstrated a different crystallization behavior [16]. After 5 days, the water shoulder disappeared and the enthalpy peaks at 50 °C storage did not narrow much. The melting point increased from 68.9°C at day 0 to 70.0°C at day 14. The 5% lipid dispersions behaved similar to the freshly prepared SLN from

day 0 until day 7 except for the missing water shoulder on the DSC curve. The curve is more round and blunt and the melting point increased to higher temperature after 14 days.

1.1.6. Differences in the modification of stable and unstable SLN

Changes that occur in the shape of the DSC heating curves during the gelation process indicate changes in the modification of the lipid matrix. Modification that are unstable are present in the α and sub α in freshly prepared SLN and these can be transformed into the more stable ß'. It was not possible to detect specifically the separated melting peaks for compritol because of its complex structure during the heating scans.

1.1.7. Active ingredient and lipid chemical stability

The active ingredient is included in nanoparticles not only to enhance skin delivery or control the rate of release of the drug, but rather to create a barrier to chemicals that negatively influence the stability of the active pharmaceutical ingredients. Lipid nanoparticles enhance the chemical stability of cosmetic actives such as vitamins and other compounds that are sensitive to light, oxidation and hydrolysis. Jenning and Gohla compared the chemical stability of all-trans retinol incorporated in SLNs with an emulsion. The chemical stability in retinol was more in the emulsion than in the expected SLN formulation [42]. The group then increased the solubility of retinol in SLNs by reducing the retinol loading and by increasing the oil to the lipid ratio creating a nanostructured lipid carrier. By doing so, they were able to obtain a more stable form of retinol than the microemulsion [42]. Another study performed by Teeranchaideekul et al. investigated the effect of formulation parameters on the chemical stability of ascorbyl

palmitate. In this study, different types of lipids, surfactant, antioxidants, and storage conditions including temperature and nitrogen gas flushing and the effect of drug loading capacity were evaluated [43]. The stability of ascorbyl palmitate was improved by the addition of antioxidants [44]. More than 90% of the active drug remained in the NLCs stored for 90 days at 4 °C after flushing with nitrogen gas and adding a combination of antioxidants which are butylate hydroxyanisol (BHA) and butylate hydroxytoluene (BHT) and DL-α-tocopherol (vitamin E) [43].

1.1.8. Lipid excipients

About 23 years ago, solid lipid nanoparticles (SLN) was invented and introduced as a novel drug delivery system for incorporating both hydrophobic and hydrophilic drugs. Extensive studies have been performed on lipid crystallization including polymorphic transitions; however little work has been done on the chemical stability of SLNs and the chemical stability of lipid matrix which is an important prerequisite for developing a formulation to be introduced to the clinic and the pharmaceutical market [45]. Radamska-Soukharev stored various formulations consisting of lipids of different percentages of mono-, di and trigylcerides and surfactants for 24 months [29]. The lipid content immediately dropped from 100% to 90-95% within 2 years after SLN production via hot homogenization. SLN formulation consisting triglycerides showed greater stability than those composed of mono-and diglycerides. SLNs made with triglycerides content of 97% demonstrated greatest chemical stability with retention of 96% lipids after 2 years storage. SLNs stability of mono-and diglyceride content of 95% was the lowest with a reduction in lipid content between 89-95% within 2 years of storage [29].

1.1.9. Chemical stability

1.2. Phospholipid stability

Membranes in liposomes are primarily constituted of phospholipids and these phospholipids consist of ester bonds which are sensitive to hydrolysis [46]. The organization of the lipid assembly can change from lamellar to a micellar system because of the chemical hydrolysis of the liposomal phospholipids [47]. Lysophosphatidylcholine and fatty acids are formed [48] and membrane permeability increased [49] when these transformations occur. The peroxidation of unsaturated acyl chains is generally accompanied by phospholipid degradation [49]. An increase in the permeability of the bilayer was increased by the lipid peroxidation. Degradation process resulted in a number of products with highly different chemical natures [46, 49]. As a result of these degradation processes phospholipid use in such formulations is very limited and is currently substituted with non-ionic surfactants to circumvent degradation problems [49].

1.2.1. Triglycerides stability

When triglycerides hydrolysis occurs, it degrades to mono-or di-glycerides with free fatty acids. They are less susceptible to hydrolysis than the external phospholipids because of their internal location in solid lipid nanoparticles.

1.2.2. Physical stability

1.2.3. Lipid modification

SLN suspensions have complex additional stability aspects compared to other lipid system because of their crystallization kinetics and the polymorphism of the dispersed lipid. Solid lipids show crystallinity and have a definite melting point as they move from solid to liquid state [50], an important aspect that should be considered when formulating SLN [7]. Matrix of SLNs is frequently composed of glycerides and the lipids that make SLNs are solid at room temperature [51]. Fusion of the ß form in long chain triglycerides (tristearin, tripalmitin) occurred at high temperature (68 °C to 60 °C, respectively) and for low chain triglycerides (trimyristin, trilaurin, 53 °C and 43°C, respectively). The size characteristics of the structure can alter the solidification phenomenon [6, 52]. Lipid nanocrystals melt at about 3 to 5 °C lower than the bulk material as a result of their small particle size [53].

Tryglycerides which are used in the preparation of nanoparticles are solid at room temperature did not crystallize upon cooling to common storage temperatures. Particles remained without crystallization in the liquid form for a number of months. Westesen and Bunjes experimented that particles colloidally dispersed in trimyristin and trilaurin remained in the liquid state at room temperature for several months. When the dispersion was cooled below the critical crystallization temperature no change occurred in particle crystallization. The particles can remain in a supercooled liquid state for a long time period of time and if this occurs, the emulsions of supercooled melts were formulated instead of SLNs [6]. The supercooled state of the droplets was not thermodynamically stable and upon long term storage gradual crystallization cannot be excluded due to the fact that the properties of the product will change. Such gelling or the expulsion of the incorporated drug results because of the crystallization process that leads to instabilities

in the SLN. When crystalline re-orientation occurs, this can result in changes of the charge on the particle surface and eventually on the measured zeta potential and the crystals can possess different charge densities. For glyceryl tribehenate SLN, it resulted an increase in zeta potential from -25 to -15 mV[21].

Polymorphism in SLN is the ability to reveal different unit cell structure in crystals, originating from molecular conformations and molecular packing. Polymorphism is an important physical stability processes which affects stability in solid dosage forms because various polymorphs have different thermodynamic properties such as melting points, X-ray diffraction, and solubility [50]. The main polymorphs in glycerides are the α, ß’ and ß forms, the α form can quickly transform to a form with better chain packing such as the ß' form. The transition of triglycerides of liquid melt from α to ß via the ß' was the pathway to the optimum packing form of the molecules. During storage at elevated temperatures, this unstable form gradually transforms toward the most stable form while losing the initial spherical surface structure [54]. Polymorphism influenced the nanoparticles content and the oil presence allowed for higher drug loads [55, 56]. Jenning showed that the in vitro results on skin showed that when water evaporates, it leads to solid modification changes of SLN dispersion causing drugs to be expelled from the lipids resulting in increase in penetration of drug into the skin [57]. The problem of lipid modification is not always solved with assignments to α, ß or ß' form. The complexity increases as a result of many subspecies and the interactions of the lipid with the emulsifiers. Westesen's group demonstrated that the decisive factor for the physical properties of SLN is the particle size [58].

Table 1.1
Lipids used for preparation of solid lipid nanoparticles.

Lipids	Matrix arrangement	Examples	Literature
Triglycerides	***Highly ordered***	Tricaprin	[59]
		Trilaurin	[6, 60, 61]
		Trimyristin	[6, 60, 62]
		Tripalmitin	[6, 17]
		Tristearin	[59, 63]
		Hydrogenated coco-glycerides	[64]
Hard fat types	*-*	Witepsol ® W 35	[63, 65, 66]
		Witepsol ® H 35	[63, 67, 68]
		Witepsol ® H 42	[68]
		Witepsol ® E 85	[64, 67]
Acylgylcerol mixtures	***Less ordered***	Glyceryl monostearate (Imwitor ® 900)	[62, 69-71]
		Glyceryl behenate (Compritol ® 888 ATO)	[70-72]
		Glyceryl palmitostearate (Precirol ® ATO 5)	[72]

Table 1.2
Surfactants used in the preparation of solid lipid nanoparticles.

Emulsifiers	**Literature**
Poloxamer 188	[65, 73, 74]
Poloxamer 182	[64]
Polysorbate 20	[75, 76]
Polysorbate 60	[77]
Polysorbate 80	[64]
Sodium cholate	[62, 64, 78, 79]
Sodium glycocholate	[17, 80]
Soybean lecithin	[60, 67, 80]
Soybean phosphatidylcholine	[78, 79]
Sorbitan tioleate	[81]

1.2.4. Ostwald ripening

Ostwald ripening a phenomenon observed in both solid dispersions and emulsions. Ostwald ripening originates from the dependence of particle solubility on their size. According to Ostwald-Freundlich equation, small particles have higher saturation solubility than larger ones [82], resulting in a drug concentration gradient between the small and large particles. As a result, molecules diffuse from higher concentration surrounding small particles to areas around larger particles with lower drug concentration. This phenomenon does not require physical contact of the colloidal

particles. Subsequently a supersaturated solution is generated around the large particles leading to drug crystallization onto the large particles. Ostwald ripening can be partially inhibited by narrowing particle size distribution which minimizes the saturation solubility difference and drug concentration gradients within the medium. Stabilizers can reduce the interfacial tension between the solid particles and liquid medium; therefore, preventing Ostwald ripening. Ostwald ripening can be mitigated by stabilizers as long as they do not enhance the drug solubility [83, 84]. It depends on the granulometry of particles in which species flux occurs from small to large droplets via the continuous phase. For SLN, particle size increase due to the dissolution of smaller crystals and deposition of the dissolved material on larger surfaces, resulting in the growth of large particles at the expense of smaller ones [85].

1.2.5. Flocculation

In Brownian motion, colloidal particles will collide with neighboring particles. This depends on the relative magnitudes of the attractive and repulsive forces; some particles may adhere or repel one another. There is no effective interaction between the particles at long separation distances. However, as they move closer together, the van der Waals attraction dominates initially. These forces initially will give rise to the secondary minimum [86]. What determines if the system will flocculate or remain no-aggregated is the depth of the minimum. Particles will flocculate if the depth is larger compared to the thermal energy of the system and remain in a non-aggregated state if it is smaller [84]. Particles during flocculation cluster together without merging [87-89]

When attraction forces occur between the droplets, this causes them to cluster which results in the formation of a larger structure by bridging. Flocculation is a reversible phenomenon used to remove suspended matters from the water as the flocculate finally sediment. Considerable energy is required to form small liposomes and this created a thermodynamically unfavorable packing status. A mechanism to dissipate the excess surface energy originating from the distorted packing material is by aggregation and fusion of liposomes [90]. The aggregation of liposomes caused an increase to larger units and in principle this process is reversible by applying mild shear forces, by changing the temperature, or by binding metal ions that induced aggregation [49].

1.2.6. Zeta potential

Zeta potential (ZP) refers to the surface charge of the particles. ZP (±) indicates the degree of repulsion between close and similarly charged particles in the dispersion. This repulsion force prevents aggregation of the particles. Therefore, ZP is a useful parameter to predict the stability of the solid lipid nanoparticles dispersions.

According to the literature data [91], zeta potential values above 30 mV provide good stability and above 60 mV provide excellent physical stability. Short term physical stability represents a value of 20 mV; however, values in the range of -5 mV to +5 mV indicate fast aggregation. For low molecular weight surfactants and pure electrostatic stabilization, this is valid. But for high molecular weight stabilizers which act mainly by steric stabilization, this is not valid. In this case zeta potential of only 20 mV or much lower can provide sufficient physical stability [91, 92].

1.2.7. Creaming and sedimentation

Creaming is a reversible process describes how the emulsion droplets rise to the top of vial or to sink to the bottom as in sedimentation. Flocculates may either rise or sink depending on density differences between the dispersed and continuous phases. Oil droplets in the case of o/w emulsions, oil droplets typically have a lower density than the aqueous phase and will rise to the surface of the emulsion. In solid dispersions in w/o emulsions, because of the higher density of the dispersed solid than water, particles will sediment. The Stoke's equation provides an estimate of the rate of sedimentation or creaming [89, 93] which indicates the important role of particle size, medium viscosity and density difference between medium and dispersed phase in determining the sedimentation rate [94, 95]. Non-homogenous dispersion results from a competition between Brownian agitation and gravity. Robins reviewed the mechanisms of creaming and phase separation due to its commercial importance in food emulsions [96]. The most common strategy used to reduce particle settling is to reduce particle size. The approaches used to mitigate sedimentation problems are by matching drug particles density with medium viscosity [95, 97]. Particles settle independently in a deflocculated suspension as small size entities resulting in a slow sedimentation rate; however, caking occurs in densely packed sediment [98].

1.2.8. Coalescence

Coalescence is an irreversible rupture of the emulsion resulting in phase separation. SLN dispersions tend to cream or gel after particle contact. In contrast, rigid solid particles are expected to be stable against coalescence. Coalescence occurs when two or more droplets

merge to form a single larger droplet. This process leads to an irreversible breakdown, referred to as cracking of an emulsion [89]. In the absence of a primary maximum, rapid aggregation can occur leading to the formation of a strong, irreversible aggregated structure [86]. A network of three-dimensional aggregates with interconnections eventually fuses into a compact pack of particles, causing an irreversible caking of the dispersion [89].

1.2.9. Fusion

Corresponds to the membrane reorganization with relocation of individual lipid molecules between adjacent lipid layers in aggregates of liposomes. Fusion is an irreversible process and definitely the original structure of the liposome is lost [49]. One of the biological processes that is based on membrane fusion is intracellular trafficing inside macrophages. To increase the entrance of vesicles inside the cell, fusion process between biological membranes and vesicle is favored so that drugs can be carried inside the cytoplasm [99].

1.3. Stability of drug nanoparticles

1.3.1. Effect of dosage form on stability

Nanoparticlulate drugs have unique characterstics that have enabled them to be explored for extensive application in various dosage forms including oral, ocular, dermal, parenteral and other specialized delivery systems [100-104]. Some common stability issues are shared by different dosage forms such as sedimentation, particle agglomeration or crystal growth and their effects on drug products are quite different. In pulmonary drug

delivery, particle agglomeration could be a major issue since it affects deposition amount and drug efficacy. On the other hand, agglomeration in intravenous formulations can lead to blood capillary blockage and obstruct blood flow. The selection of stabilizers is strictly governed by FDA regulation and is closely related to dispersion medium and dosage form . Currently excipients allowed for inhalation are very limited where as for oral dosage form there is a wide variety of stabilizers approved [105].

The final drug products of nanoparticles exist in either dry powder or suspension form. Dry powder form include the dry powder inhaler, lyophilized powder for injection and oral tables or capusles. Stability profile of stored solid dosage forms are usually good, so to enhance the nanosuspension stability is to transform the suspension into solid form has become a common strategy to enhance stability profile [106, 107]. Nanosuspensions in which the drug nanoparticles are dispersed in a medium with or without stabilizers is where most of the reported stability concern arise. So the stability mechanisms of small and large biomolecule formulations are different because of their molecular structure differences. In protein peptide, maintaining the 3-dimensional molecular conformation such as the secondary and tertiary structure to keep their bioligical activities is one of the major stability issue [108, 109]

1.3.2. Agglomeration

Nanoparticles create high total surface energy from the large surface area and this is thermodynamically unfavorable. When particles agglomerate, they tend to minimize the surface energy and cause a variety of issues for nanosuspensions such as rapid settling and creaming, crystal growth and inconsistent dosing. Introducing stabilizers to

formulation is the common strategy to challenge this issue. Stabilizers are chosen based on their ability to provide a barrier to pevent nanoparticles from agglomeration and to contribute wetting to the surface of the particles[102, 106]. The classic Derjaguin-Landau-Verwey-Overbeek (DLVO) theory describes the statilization from electrostatic repulsion [110, 111]. Colloidal suspensions can be stabilized in both mechanisms aqueous and non-aqueous medium such as electrostatic repulsion and steric stabilization [94, 95, 101]. Adding ionic and non-ionic stabilizers into the medium respectively are two ways in which the mechanism mentioned above are achieved.

1.3.3. Stability of lipids

The term lipid in a broad sense includes triglycerides, partial glycerides, fatty acids, hard fats and waxes. The lipid matrix in SLN is made from physiological lipids which decreases the danger of acute and chronic toxicity which is a clear advantage of SLN [112]. The use of solid lipid instead of liquid lipid is beneficial for the release kinetics of encapsulated compounds and to improve the stability of incorporated chemically sensitive lipophilic ingredients. Because of the physiochemical characteristics associated with the physical state of the lipid phase, they are potentially effective. The rate of chemical degradation reactions may be retarded because the mobility of the reactive agents in a solid matrix is lower than in a liquid matrix [113-117]. In addition, separation of micro phase of the active ingredients and carrier lipid within individual liquid particles can be controlled, thus preventing the accumulation of active compounds where chemical degradion reactions occur at the surface of lipid particles. Also, it has been shown that the absorption of poorly absorbed bioactive compounds to be increased after

incorporation into solid lipid nanoparticles. It has been shown that lipid digestion can be slowed down by the use of solid lipid instead of a liquid matrix; therefore, allowing for a more sustained release of the encapsulated compound. The excipients of SLNs are surfactant of aqueous type that act as emulsifier to form o/w emulsion and the choice of stabilizers for SLNs dispersion mainly depends on the route of administration. Excipients are made of a solid hydrophobic core containing the drug either dissolved or dispersed in the core [118]. SLNs prepared by any technique whether it is by high pressure homegenization or miro emulsification are in dispersion form and upon long term storage results in instability primarily because of hydrolysis reactions. The stability of SLNs can be increased by converting into solid dry reconstituable powders through lyophilization or spray drying [119].

Lim at el. reported that upon irradiation of SLNs with 60 W incandescent bulb showed improvement in stability of all trans retinol encapsulated into SLN and upon incorporating a small amount of antioxidant in the SLN preparation there was 43 % improvement in stability [120]. The presense of surfactants have been found to inhibit flocculation as they produce electrostatic repulsion and steric hindrance between the particles [121, 122]. The liquid layer surrounding the particle is formed by two parts: an inner region called the stern layer in which the ions are strongly bound, and an outer diffuse region , in which the ions are less firmly attached. With in the diffuse layer, there is a notional boundary that forms between particles and ions and forms a stable entity. Ions within the boundary move when a particle move and any ions beyond the boundary do not travel with the particle. This boundary is called the surface of hydrodynamic shear and the potential that exists at this boundary is known as the zeta potential. Shahgaldian

et al. have investigated that ionic strength can have an influence on the stability of SLN, indicating the strongest destabilization occuring by sulphate ions [123]. Using surfactants for intravenous administration is still critically debated [124]. SLNs tested today proved to be non-toxic in vivo and in vitro systems. Muller et al. showed that animals didn't show any anaphylatic shock or related symptoms [125].

1.3.4. Drug release from SLNs

Investigations of the drug incorporation and release serves as an important tool in the design, development, and evaluation of potential drug carrier systems. The data on the release mechansims are still scarce; however, there are many studies concerning drug incorporation into SLN. A general issue observed with the release of drugs in SLNs is the burst release [126]. In general, the amount of drug in the outer shell and on particle surface is released in the form of a burst. On the other hand, drugs that are incorporated in particle core releases the drug in a prolonged manner. SLNs can be produced surfactant-free or with surfactants unable to solubilize the drug to avoid or minimize the burst release [127]. The extent of burst release can be controlled by controlling drug solubility in the aqueous phase during production and this can be controlled via the temperature and the surfactant concentration used. Olbrich and Muller showed that enzymatic degredation of the lipid matrix by lipase is caused by the lipid and the emulsifier used [128]. Appropriate balance between steric stabilizers and other surfactants should be optimized to modify drug release and particle degradation since lipases require a lipid interface for enzyme activation. For this reason, coating with hydrophilic over lipid nanoparticles is not easily recognized by these enzymes. Rapid

release of the drug is enhanced by factors that include large surface area, high diffusion coefficient due to small molecular size, low viscosity in the matrix and short diffusion distance for the drug e.g. the release from the outer nanoparticulate surface. There are a large number of drugs showing high lipophilicity and their structure have been studied with regard to their incorporation into SLN [127], examples include diazepam, oxazepam, cortison, betamethasone valerate, prednisolone, retinol, menadione, ubidecarenone[23], timolol [68, 129], pilocarbine [130], progesterone [69], doxorubicin [131], idarubicin [131], hydrocortisone [69], thymopentin [132], gadolinum (III) complexes [133], camptothecin [134, 135], acyclovir [136, 137], etomidate, and tetracaine [138]. Strong changes of the SLN characterstics such as particle size, zeta potential and lipid modification correlates to drug loading because drug incorporation implies the localization of the drug in the solid lipid matrix. There are several alternative incorporation sites (micelles, mixed micelles, liposomes, drug-nanosuspensions) beside the complex physicochemical status of the lipid (supercooled melt and other modifications). These precautionary remarks are very important and should be kept in consideration during the interpretation of published results. Characterization of SLN dispersion based on the particle size measurements are not sufficient. There is a large number of drugs including hydrophilic molecules that has been investigated to be incorporated into SLN. Few data exist on exactly where the drug localization site is present and the physical state of the drug molecule. In a study by Lukowski, he observed by electron diffraction that acyclovir is not molecularly dissolved in the lipid matrix [136].

1.3.5. Controlled release

An efficient delivery system to be well recognized must have the capability to transport the desired drug molecule without any potency loss before reaching the targeted tissues. Once the drug delivery reaches the destination, it has to release the drug molecule in a controlled manner. Release at non-targeted healthy tissues can lead to serious sife-effects for patients and this is particularly true for toxic anti-tumor drugs. To overcome the rapid removal of drug from the administration site associated with fat emulsions, and to enhance targeting, SLNs have been investigated as drug delivery vehicles [139]. Selective release at target sites can be achieved through modification of the SLN core via surface functionalization with pH-titratable peptides or polymers [140]. SLNs with longer-lasting produrg of butyric acid lipid matrix are biocompatible and have good drug-lipid electrostatic interaction. So to allow for a possible prolonged drug release, they are entrapped in intracellular compartments persistently and consistently and do not modify the specific effect of the active dug molecule [141]. Drugs can be targeted to tumors passively by conjugation to polymers and exploiting the enhanced permeability and retention (EPR) effect that occurs in tumors, areas of inflammation, and sites of infection. Solid tumors may contain leaky vasculature, most of the tumor vessels have an irregular diameter and the branching pattern is abnormal and they do not fit well into the usual calssification of arterioles, capillaries and venules. Due to the leaky vasculature of tumors, there is uncontrolled proliferation and angiogenesis and this allows the drug carriers below a specific size e.g. 200 nm to permeate through. Polymer conjugation approach which offers the possibility of incorporating two cancer drugs allows the release

of both drugs at the tumor site, and hence gives a synergestic effect enhancing the formulation efficacy. HPMA which is N-(2-hydroxypropyl)methacrylamide is a polymer used in conjugation and delivery of anticancer drugs [142]. Sometimes a peptide linker is attached to the polymer (HPMA) and the drug is cleaved from the polymer by the enzymes that are only present on the tumor antigens.

1.3.6. Influence of the emulsifier

The type of emulsifier and its concentration is of great importance in determining the quality of the SLN dispersion. The surface area of SLN increases in the presence of smaller size particles. An increase in the surface area leads to the Ostwald ripening phenomenon which results in phase separation resulting in thermodynamic instability. Emulsifiers are well accepted for human use and include poloxamers, lecithins, polyethoxylated monoglycerides, and polysorbates. The chemical stability of excipients used in the production of particles is required for successful formulation of drugs in SLN. In order to avoid the interaction of preservatives with lipids particles, SLNs are produced without preservatives and high pressure homogenization will sterilize the dispersion because of the destruction of bacteria cells under high pressure.

1.3.7. Influence of surfactant properties on physical stability of SLNs

The influence of surfactants alone or in combination with co-surfactants on particle size was evaluated during storage at 20 °C for 22 days on d32, d43 of tristearin SLNs. The lipid particles emulsified with low melting lecithin (PC75) alone or in combination with any of the co-surfactants showed aggregation or gelling after its preparation. The lipid

particles also aggregated and gelled after preparation when high melting lecithin (80H) alone or in combination when tween 80 was used. Tween 80 led to insufficient repulsive forces between the particles due to its non-ionic properties and thus forming flocculated particle and finally gelling. SLNs that were emulsified with 80H and Pluronic F68 resulted in formation of small droplets but extensive aggregation of particles was observed after 8 days of storage. However, SLNs emulsified with high-melting lecithin 80H used in combination with taurodeoxycholate resulted in formed SLNs with monomodal particle size distributions. SLNs were physically stable throughout 22 days of storage and this correlated with previous data from studies on SLN [143-145]. On the other hand, SLNs emulsified with 80H and phenylalnine (Phe) were stable for 22 days of storage and SLNs with 80H and combined with co-surfactants Tryptophan (Trp) or Tyrosine (Tyr) showed stability for 8 days and after 15 days they began to show some aggregation. Surfactants namely phospholipids have been shown to play a role in modulating the crystallization behavior of SLN in the pharmaceutical and cosmetics. Upon crystallization, phospholipids require a co-surfactant to stabilize the newly formed surface [146, 147]. Previous studies have shown that taurodeoxycholate is an effective co-surfactant, it is relatively expensive and has a bitter taste and is not food grade for this reason its replacement is important. Taurodeoxycholate is a superior co-surfactant for stabilizing SLNs interfaces emulsified with saturated phospholipids. The co-surfactants aromatic amino acids, phenylalnine (Phe) were effective in improving the polymorphic and physical stability of SLN [148].

1.3.8. Phase transition temperatures

SLNs emulsified with high melting lecithin 80H alone or in combination with a co-surfactant during heating from 20 ºC, there were two endothermic peaks detected at ~56 to 57 °C and 69 to 71 ºC. SLNs emulsified with low melting lecithin PC75 alone or in combination with a co-surfactant showed two endothermic peaks at ~ 56 to 57 °C and ~ 71 to 72 ºC upon melting. The presence of these two peaks corresponds to the melting of α- and ß-subcell crystals of tristearin [149, 150]. A small exothermic peak was observed at ~ 62 to 63 ºC when SLN was emulsified with 80H and amino acids (Phe, Trp, and Tyr) and this is due to the α→ß polymorphic transition [151]. Tristearin SLNs emulsified with 80H or PC75 as the main surfactant melted approximately 1 to 4 ºC lower than the corresponding bulk tristearin which is ~ 73 ºC [56, 144]. During crystallization, the most unstable and least ordered α-subcell crystals grow first because they require the least amount of molecular rearrangement to form according to the Ostwald's theory. Upon cooling, the α-subcell crystal structure will always form first. The activation energy in supercooled melts has to be overcome in order to form crystals [19]. If the activation energy is higher than the thermal energy of the system, formation of initial crystals will not occur. Nanodispersions consist of very small droplets and the presence of naturally occurring impurities such as dust or any other solid particles becomes less likely. This means that the lower the crystallization temperature smaller the droplets are because most of the lipid droplets will not contain any impurities to act as crystallization templates. There are small nuclei in the lipid matrix that are thermodynamically unstable and can re-dissolve, so in order to induce crystallization, these nuclei have to have a large enough size to be stable because the dispersion will persist in the supercooled state [56, 144]. Factors that initiate crystallization in the supercooled emulsions are impurities that act as

crystallization templates (heterogeneous nucleation) and cooling the materials below the crystallization temperature of the bulk lipid (homogenous crystallization) [152]. On the other hand, lipids that crystallize close or slightly below the onset crystallization temperature are prone to coalescence because during polymorphic transition from α-to ß-subcell crystals, the particles change in shape from spherical to plate-like resulting in an increase in the surface area of the particle [153]. The lipid particles will aggregate upon the attractive lipid-lipid interactions when the concentration or coverage velocity of the surfactant is too low to cover the newly formed hydrophobic surfaces [143, 153]. During heating to the melting point of the bulk lipid, particles with lipid-lipid interface will coalesce. The likelihood of particles containing impurities increases because the volume of the coalesced particle is higher resulting in higher crystallization temperature via heterogeneous nucleation. Supercooled melts are not unusual in SLNs because it describes the phenomenon that lipid crystallization may not occur below the melting point of the lipid even though the SLN is stored at room temperature [154]. The degree of crystallinity and lipid modification can be assessed using differential scanning calorimetry (DSC) and X-ray scattering to investigate the status of the lipid. DSC is based on the fact that different lipid modifications possess different melting points and melting enthalpies and X-ray scattering is used to assess the length of the long and short spacing of the lipid lattices.

1.3.9. Storage stability

There are remarkable similarities when comparing SLN and nanoemulsions with respect to their composition and production methods. SLNs cannot be regarded as colloidal lipid

dispersions with solidified droplets. However, SLNs have additional features such as different modifications, supercooled melt, non-spherical shapes and all these contribute to the stability of colloidal lipid suspension. Increase in particle size, gelation phenomena and drug expulsion from the lipid carrier are the major problems associated with storage stability. Supercooled melt is the first product formed after hot homogenization which represents a nanoemulsion. It is characterized by spherical lipid droplets and a high incorporation rate for drug molecules. When the transformation of the lipid melt to lipid crystals occur, it results in an increase in particle surface, the loading capacity of lipid is also decreased and as a result leads to increase in the stability problem. Therefore, as the stability of the lipid modification increases, the stability of the lipid dispersion decreases.

1.4. Toxicity aspects

SLNS are made from physiological compounds and one can predict that they are well tolerated in living systems because the metabolic pathway to degrade the lipids exists in the body. However, the toxicity of the emulsifiers has to be evaluated. For peroral or transdermal administration and i.m. or s.c. injection appropriate surfactants have to be used. Particle size is not that critical issue for these administration routes because the performance of SLN system might decrease because of low contents of microparticles, but will not cause toxic events. For parenteral administration, the absence of pyrogens must be assessed because SLNs may interfere with the pyrogen tests (limulus test) and cause gelation.

Originally SLNs were designed for controlled release of drugs after intravenous injection. But because of the change in the lipid droplet (nanoemulsions) to a solid core (SLN) this

should decrease drug delivery due to decreased drug diffusion coefficients. For i.v. injection, particle size distribution is a key issue due to the danger of capillary blockage; this could result in death due to fat embolism. The diameter of the capillaries is between 5 and 10 microns. For safety reasons, the particle size should be completely in the submicron range. On the other hand, nanoemulsions for parenteral nutrition in microparticles exceeding the size of the capillaries have been found in commercials [155, 156]. Larger amounts microdroplets are tolerated by human body but this should not be the case with SLN because a solid lipid is not deformable as oil in contrast to nanoemulsions. Blockage of capillary will occur if the particle size exceeds the size of the diameter of blood vessels. In the syringe needle, gelation of the low-viscosity SLN dispersion might occur and this forms a viscous suspension with unacceptable particle size. Serious hurdles for the development of SLN dispersion suitable for i.v. injection in clinical practices are solid state of the lipid and the danger of injection-induced gelation. Studies on the interaction of SLN with phagocytizing cells in vitro on human granulyctes have been performed [157, 158]. To compare SLNs with polymer particles and to assess the influence of the SLN composition on the phagocytosis rate, a luminol-based chemoluminescence was used. The phagocytosis rate of the poloxamer stabilized Compritol® and cetyl palmitate SLN was lower in comparison to polystyrene nanoparticles [158-160]. In order to distinguish the small differences in SLN phagocytosis, an indirect chemoluminescence assay was developed [161]. Poloxamine 908 prevented the uptake of Compritol® SLN more efficiently than poloxamer 407 [162].

1.4.1. In vivo fate

SLN particles for the in vivo fate will depend mainly on the following factors: the administration route, the SLN interaction with the biological surroundings that is adsorption of biological material on the particle surface and desorption of SLN components into the biological surrounding. Also, the lipid degradation by lipases and esterases via the enzymatic processes are very critical. Lipases are present in various organs and tissues and are the most important enzymes of SLN degradation. They split the ester linkage and form partial glycerides or glycerol and free fatty acids. The oil/water interface activates most lipases and the catalytic center is open [163-165]. Experiments done *in vitro* indicate that solid lipid nanoparticles show different degradation velocities by the lipolytic enzyme pancreatic lipase which serves as a function of their composition, the lipid matrix and the stabilizing surfactant [62, 166]. The length of the fatty acids chains in the triglycerides and the surfactants showed SLN degradation dependence. Longer fatty acid chains in the glycerides showed slow degradation. The surfactant effect on the degradation is to accelerate it such as cholic acid sodium salt and to hinder it because of steric stabilization such as poloxamer 407, poloxamer 188. Tween 80 which serves as a steric stabilizer and results show that the hindering effect on degradation process was less noticeable than that of poloxamer 407. This correlated with the number of ethylene glycol chains in the molecule that result in the degradation of the SLN through suppressing lipase/colipase complex.

1.4.2. Conclusion

Stability of drug nanoparticles remains a very challenging issue during pharmaceutical product development. SLN formulations have shown potential for oral delivery to improve GIT absorption and oral bioavailability of many drugs. These formulations are useful for sustained/prolonged release or targeted drug delivery. Stability is affected by various factors such as the dosage form, dispersion medium, delivery route, production technique, and the nature of drug molecule whether it is small or large biomolecules. Excipients used are of GRAS (Generally Recognized As Safe) status have been used in pharmaceutical and food products. SLNs must occupy a considerable place in the pharmaceutical market, it is essential that the pharmaceutical industries along with the academic research groups specialized in the development of new drug delivery systems engage in novel formulation technology and to enhance their scale up production and establish these formulations in the market. To understand the interaction of SLN with their biological surrounding such as adsorption/desorption, processes, enzymatic degradation, agglomeration, interaction with endogenous lipid carrier systems, further work needs to be done. Several administration routes are feasible for SLN administration and i.v. injection is the most challenging route because it requires absolute control of the particle size. The primary application of SLNs appears to be the dermal route of administration since results from such studies so far are encouraging and promising. To conclude, SLNs are complex systems with obvious advantages when compared to other colloidal carriers. More studies have to be done in terms of understanding the structure and dynamics of SLNs on a molecular level in *ex vivo* and *in vivo* conditions.

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Chapter 2

Significance of the Thesis Research

The significance of this research is to enhance the delivery of 5-Fluorouracil through the use of Solid Lipid Nanoparticles. Insufficient drug concentration because of poor absorption, rapid metabolism and elimination and drug distribution to other tissues along with high drug toxicity such as cancer drugs. High plasma fluctuations because of unpredictable bioavailability after peroral administration. Therefore, a promising strategy to overcome these problems involves the development of suitable drug carrier system. Solid lipid nanoparticles provide controlled and localized release of the active drug according to the specific needs of the therapy. The surface on the solid lipid nanoparticles can be modulated for specific tissue targeting using specific antibody to target tumor antigens. 5-FU was limited because of its high toxicity, short half-life, bas selectivity and low bioavailability. Goal of this research work was to develop solid lipid nanoparticles (SLNs) carrying 5-Flourouracil and to evaluate its effect on various cell lines. The solid lipid nanoparticles were prepared through a new temperature modulated solidification technique developed in our laboratory. Particle size analysis by dynamic light scattering (DLS) and morphology evaluation by transmission electron microscopy (TEM) demonstrated that the SLNs are nanoparticulates. Cytotoxic activity of SLN

loaded 5-Fluorouracil showed a decrease in viability when compared to pure solution of 5-FU on Caco-2 cell line. Blank SLN showed no decrease in cell viability when the concentration increased. Biocompatibility studies of SLNs in human RBCs indicated that 5-FU SLN formulations are compatible. Bovine permeability study shows that apparent permeability for 5-FU SLN was 0.000348 cm/s and 1.339 cm/s for 5-FU solution. The preliminary results from various in vitro evaluations suggest that 5-FU loaded SLNs have the potential to be used as an anti-cancer drug delivery system.

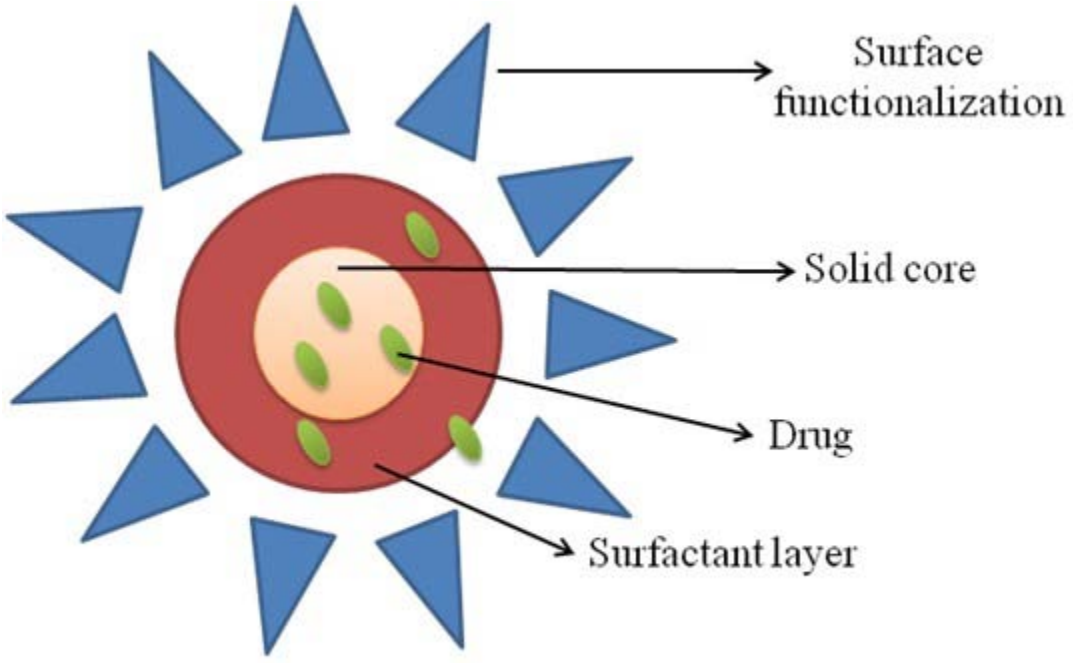

Figure 2-1: Schematic diagram of Solid Lipid Nanoparticles

Chapter 3

Chemicals used (partial list)

Glyceryl monostearate

An aliphatic carboxylic acid ester with HLB value of 3.8. a nonionic emulsifier, emollient, stabilizer and plasticizer in food products, cosmetics as well as in many pharmaceutical formulations. It is a glycerol ester of stearic acid. It occurs naturally in the body as a by-product of the breakdown of fats, and is also found in fatty foods. GMS is a food additive used as a thickening, emulsifying, anti-caking and preservative agent; an emulsifying agent for oils, waxes, and solvents; a protective coating for hygroscopic powders; a solidifier and control release agent in pharmaceuticals; and a resin lubricant. It is also used in cosmetics and hair care products. GMS is in the FDA inactive ingredient guide.

5-Fluorouracil

One of the oldest chemotherapeutic drugs commonly used against colon, stomach, and pancreatic cancers. It's a fluorinated analog of pyrimidine base uracil, which is metabolized intracellularly to its active form, fluorodeoxyuridine monophosphate. It is an

s-phase active anticancer agent and has no activity on cells when they are in G_0 or G_1; it induces cell cycle arrest and apoptosis by inhibiting the cell's ability to synthesize DNA.

Tween 80

A hydrophilic nonionic surfactant used as an excipient to stabilize suspensions and emulsions. It is also used as a solubilizing and wetting agents in creams, ointments, and medical preparations such as vitamins, oils, oral and parenteral suspensions and anticancer agents. Used as an emulsifier often used in foods and cosmetics. This synthetic compound is a viscous, water-soluble yellow liquid. Tween 80 is also used as a surfactant in soaps and cosmetics, or a solubilizer such as in a mouthwash. The cosmetic grade of tween 80 may have more impurities than the food grade. Tween 80 is an excipient that is used to stabilize aqueous formulations of medications for parenteral administration.

Chapter 4

Quantitative, Qualitative and In vitro Evaluation of Solid Lipid Nanoparticles Containing 5-Fluorouracil

4.1. Introduction

Targeting an anticancer drug to the disease location is a distinctive feature desirable in cancer therapeutics. The aim is to convey sufficient amounts of the drug to a tumor without affecting healthy tissues. Alternative drug delivery systems for the use as carriers of anticancer drugs that are currently under investigation include polymeric microspheres, macromolecule conjugates, and liposomes. These systems were found in laboratory settings to offer several advantages when compared to conventional dosage forms in that they improve efficacy and reduce toxicity [1]. Solid lipid nanoparticles (SLN) have been particularly found to be useful as drug carriers for both water soluble and poorly water soluble drugs [2]. SLNs as a particulate drug carrier system can improve therapeutic effectiveness and increase safety of chemotherapeutic agents such as doxorubicin, trastuzumab, capecitabine, cis-platin, and 5-fluorouracil (5-FU) with a particle size that is distributed in submicron (50-1000 nm) range [3]. SLNs are generally prepared using biocompatible lipid components that remain in the solid state at room and body temperatures. They can be prepared using a variety of lipids including fatty acids;

mono, di, or triglycerides; glycerides mixtures, or waxes and the formulation can be stabilized by using biocompatible surfactants. [4]

SLNs have gained growing interest in the field of cancer chemotherapy because of their passive ability to accumulate in tumor tissues and the possibility for site-specific drug release [4]. 5-Fluorouracil (5-FU) is one of the oldest chemotherapeutic drugs that have been used commonly against colon, stomach, breast, and pancreatic cancer. It is a fluorinated analog of the pyrimidine base uracil, which is metabolized intracellularly to its active form fluorodeoxyuridine monophosphate. DNA synthesis is inhibited by the active form and this is done by inhibiting the normal production of thymidine. 5-FU is inactive when cells are in G0 or G1 phases, but shows anticancer activity in the S-phase of the cell cycle. It is soluble in water [5], and upon intravenous administration, it causes severe toxic effects on the gastrointestinal tract, hematological, neural, cardiac and dermatological reactions [6]. The bioavailability of 5-FU is limited because of the rapid breakdown in the blood, liver and other organs, bad selectivity and high toxicity [7]. It has a half life in blood of 8 to 20 min after IV injection in humans [8]. For this reason, there is a need to effectively delivery 5-FU and SLNs have potential utility in this application. SLNs as a colloidal carrier system possess the advantages of polymeric nanoparticles, liposomes and fat emulsions and the ability to avoid disadvantages such as acute and chronic toxicity [9].

SLNs have been developed for the delivery of 5-Fluourouracil using several methods one of which is the hot homogenization method. Mao et al. investigated the properties of 5-FU loaded SLNs prepared by hot homogenization method and the factors that influence the formulation [10]. 5-FU was found to effectively reduce MDA-MB-468 tumor growth

when prepared using phospholipids [11] and also effective in lung cancer as an inhalation therapy [12]. The drug was deficient and limited in its use in clinical application when prepared as 5-FU liposomes, 5-FU magnetic fibroin/chitosan microspheres, microemulsions [13].

In the present study, 5-Fluourouracil incorporated SLNs were prepared by a temperature modulated solidification technique. This procedure is simple, reproducible and does not require the use of organic solvents and is feasible for large scale up. In this method, the environmental temperature is carefully modulated to solidify and form the lipid nanoparticles. In comparison to other SLN preparations such as high shear homogenization, multiple emulsion-ultrasonication method, ultra sound and solvent evaporation, the novel temperature modulated solidification technique consist of compositions that are biodegradable and non-toxic. The objective of the present study was to prepare 5-Fluorouracil loaded solid lipid nanoparticles via temperature modulated solidification and evaluate their effectiveness as an anticancer drug delivery system.

4.2. Materials and methods

4.2.1. Materials

Glyceryl Monostearate (GMS) and D- Trehalose, Anhydrous were purchased from Fisher Scientific (Pittsburg, PA); Tween 80 (Polyoxyethylene (20) Sorbitan Monooleate) and 5-Fluorouracil (5-FU) were purchased from Spectrum Chemical Manufacturing Corporation (NewBrunswick, NJ); and Amicon Ultra-15 Centrifugal Filter Units were purchased from EMD Millipore (Billerica, MA). Deionized water was obtained from the

central deionized water line in our laboratory. Methanol (HPLC grade), triethylamine (HPLC grade), sterilized phosphate buffer solution (PBS), fetal bovine serum (FBS) and human red blood cells (RBCs) were purchased from Fisher Scientific (Fair Lawn, NJ). Eagle's Minimum Essential Media (EMEM) from Meditech Inc., (Manassas, VA). The human intestinal epithelial colon adenocarcinoma cell lines (Caco-2 ATCC HTB-37) were obtained from ATCC, Manassas, VA.

4.2.2. Preparation of 5-Fluourouracil loaded solid lipid nanoparticles

An accurately weighed quantity of 5-FU was added to 1 g of glyceryl monostearate (GMS) that was equilibrated for 48 hours at 75º C, 1500 RPM after placing in a thermostated shaker (Multi-therm shaker H5000-H, Benchmark Scientific Inc., NJ, USA). 2 g of Tween 80 was added to the drug-GMS mixture and vortexed at 2900 rpm for 20 s every 5 min for one hour to obtain a homogenous dispersion. The Deionized water was heated to (80 ºC) which is slightly above the temperature of the molten lipid phase. The molten lipid phase was then dispersed into the heated deionized water, placed in an ice bath, and stirred at 4000 rpm for 45 min by a high shear mixer (model L5M-A, Silverson, USA). The resulting SLN dispersion was used for further characterization. To isolate the SLNs, an aliquot of the aqueous SLN dispersion usually 12 ml was placed in a 100 kDa Amicon Ultra-15 centrifugal filter unit and centrifuged at 7830 RPM for 30 min and temperature maintained at 15°C in a centrifuge 5430R (Eppendorf AG, Hamburg, Germany). The SLNs retained by the filter were rinsed, collected and redispersed in deionized water before lyophilization. Anhydrous D-trehalose, was used as a cryoprotectant in an amount that was equivalent to the amount of glyceryl monostearate.

After that the mixture was frozen quickly at -80 °C and lyophilized for 72 hours in the lyophilizer (FreeZone 2.5 liter benchtop freeze dry system, Labconco, MO, USA) at -49 °C and vacuum maintained at 0.140 mBar. SLNs free of drug were prepared by an identical procedure apart from the drug addition step. The freeze dried blank and 5-FU loaded SLN were collected and samples were and stored in a dessicator at room temperature (~23 ºC) for further characterization.

4.2.3. Particle size and zeta potential measurements

Dynamic light scattering (Nicomp 380 ZLS, CA, USA) was used to determine particle size and the zeta potential of 5-FU loaded SLNs (lyophilized and non-lyophilized, respectively). DLS is equipped with a 100 mW He-Ne laser of wavelength 658 nm and a photodiode array detector. For lyophilized samples, usually 1 mg was redispersed in 10 ml of deionized water, vortexed and sonicated until no particles can be seen (Fisher Scientific model FS60D, Pittsburgh, PA) for 30 min prior to measurement. Non-lyophilized samples were diluted (10X) with deionized water and vortexed until homogenous prior to measurement. For particle size determination, about 0.8 ml of all sample were transferred to disposable Durex® borosilicate glass culture tubes (Kimble Chase, Vineland, NJ) and measured at 23 °C at a scattering angle of 90°. The particle size was expressed as a volume-weighted diameter. For zeta potential measurements, all samples were transferred to standard plastic cuvettes and measured at 23 °C and at a scattering angle of 14.06° in the DLS instrument operated under electrophoretic light scattering mode (ELS). Nicomp software was used for data acquisition and analysis.

4.2.4. Imaging by transmission electron microscopy (TEM)

Approximately 5 mg of lyophilized SLNs were redispersed in 10 ml of deionized water. The sample is vortexed and sonicated for 30 minutes and non-lyophilized SLNs were diluted (10X) with deionized water and vortexed until homogenous. From the prepared sample, one drop was pipetted onto a Formvar/Carbon 400 mesh copper grid (Ted Pella, CA). The excess sample was removed after 10 minutes using a lint-free wipe; the sample was air-dried overnight at room temperature prior to imaging using a scanning transmission electron microscope (Hitachi HD-2300A, Hitachi High Technologies America, IL, USA) operating at an acceleration voltage of 200 kV.

4.2.5. Stability studies

5-Fluorouracil loaded solid lipid nanoparticles were stored for 3 months at 4 °C, ~23 °C and 40 °C. The samples were characterized for particle size, polydispersity index, zeta potential and encapsulation efficiency upon storage. The results were expressed as mean ± SD. The student t-test was applied to examine the significant difference between characteristics of SLN at 1st day and 90th day.

4.2.6. Cell viability Studies

PC-3 cells (ATCC CRL-1435) were grown in a sterile, polystyrene 24-well plate (Costar®, USA) ATCC-formulated F-12K supplemented with 10 % (v/v) fetal bovine serum (FBS) and penicillin/streptomycin (100 units/ml) and incubated in 5% CO_2 atmosphere at 37^{o} C for 72 h. The cells were then exposed to 50 µg/ml, 100 µg/ml, 150 µg/ml and 200 µg/ml of reconstituted blank and 5-FU loaded SLNs in EMEM. In the

control wells, similar amounts of EMEM were added. After 2 h of exposure, each well was washed with 100 µl of PBS. The cells were detached by adding 50 µl trypsin solution containing 0.05% trypsin and 0.02% EDTA. After 2-5 min, 200 µl EMEM was added; the contents of each well were transferred to microcentrifuge tubes and centrifuged at 1000 x g for 5 min. The pellets were resuspended in 300 µl EMEM and the supernatants were removed. After that, 10 µl of each dispersions were mixed with 10 µl 0.4% trypan blue, and 10 µl of this mixture was transferred to a counting slide and observed under Luna automated cell counter (Logos Biosystems, Korea). The percent cell viability was (compared to the controls) calculated by the Luna automated cell counter.

Caco-2 cells (ATCC HTB-37) were grown in a sterile, polystyrene 24-well plate (Costar®, USA) in Eagle's Minimum Essential Medium (EMEM) supplemented with 20 % (v/v) fetal bovine serum (FBS) and 2 mmol/1 L-glutamine and penicillin/streptomycin (100 units/ml) and incubated in 5% CO_2 atmosphere at 37^{o} C for 72 h. The cells were then exposed to 50 µg/ml, 100 µg/ml, 150 µg/ml and 200 µg/ml of reconstituted blank and 5-FU loaded SLNs in EMEM. In the control wells, similar amounts of EMEM were added. After 2 h of exposure, each well was washed with 100 µl of PBS. The cells were detached by adding 50 µl trypsin solution containing 0.05% trypsin and 0.02% EDTA. EDTA is a Calcium chelator which will mop up the remaining divalent cations and if trypsin is allowed to stay in contact with the cells for too long, cell viability will reduce. After 2-5 min, 200 µl EMEM was added; the contents of each well were transferred to microcentrifuge tubes and centrifuged at 1000 x g for 5 min. The pellets were resuspended in 300 µl EMEM and the supernatants were removed. After that, 10 µl of each dispersions were mixed with 10 µl 0.4% trypan blue, and 10 µl of this mixture was

transferred to a counting slide and observed under Luna automated cell counter (Logos Biosystems, Korea). The percent cell viability was (compared to the controls) calculated by the Luna automated cell counter.

4.2.7. Hemolysis assay

Human red blood cells (RBCs) were washed to remove the anticoagulant and serum proteins, they were washed three times with normal saline solution. An erythrocyte stock dispersion (ESD) was prepared by adding 100 µl of washed RBCs to 900 µl normal saline solution. From the ESD, 200 µl was added to 1000 µl of 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml of both reconstituted blank and 5-FU loaded SLNs that were dissolved in normal saline solution. The samples were mixed until homogenous and then incubated for an hour at 37 °C. Samples were then centrifuged at 750 x g for 3 min in order to remove intact erythrocytes and other debris. From the supernatants, 150 µl was added to a mixture of 3000 µl of absolute alcohol and hydrochloric acid (40:1 v/v) and centrifuged again at 1000 x g for 3 min. UV spectrophotometer (Agilent 8453 UV-Vis spectrophotometer) was used to measure the absorbance of the collected supernatants) at 398 nm against blank samples containing absolute alcohol and hydrochloric acid mixture. The results were correlated with control samples of 0 % lysis which consist of (normal saline) and 100% lysis which consist of (distilled water) [14, 15]

4.2.8. Permeability studies

Bovine colon permeation studies were conducted using static glass Franz diffusion cells, placed in a temperature controlled water bath at 37 ± 0.5 °C. The thickness of the bovine

membrane was approximately 0.3mm. The membranes were placed on top of the receptor chamber of the Franz diffusion cell with the luminal side was facing the donor chamber. It was then capped by the donor chamber so that when the drug formulation was placed in the donor chamber it would penetrate the luminal side first. Phosphate buffer 0.2 M was used as the receptor solution (pH 7.4). The receptor solution was degassed by high speed stirring under vacuum in Nuova II stirrer connected to a vacuum pump for approximately 30 min prior to use in the permeation studies. The Franz cells were assembled using vacuum grease and a metallic clamp to create a leak proof seal between donor and receptor compartments. Known volume of the receptor solution was added to Franz cell (~15 ml) and the colon was allowed to equilibrate with the receptor solution for at least 1 h before starting the experiment. Colon barrier integrity was assessed prior to the beginning of the experiment by assuring there was no leakage from the neck of the Franz diffusion cell and no bubbles are present. Sink conditions were maintained throughout the duration of the experiment and sample was taken at zero min, 15 min, 30 min, 45min, 1h, 1.5h, 2h , 2.5h, 3h, 4h , 6h, 8h , 10h and 12 h and 300 µl was removed and replaced with equal amount of volume of fresh PBS.

4.3. Results and Discussion

4.3.1. Preparation of solid lipid nanoparticles

In this study, the preparation of SLNs was done using glyceryl monostearate as the lipid and tween 80 as the surfactant via a temperature modulated solidification method. The preparation was based on compositions optimized by uniformly changing various process parameters. We changed the ratio of drug to lipids, the concentration of surfactant and

emulsifying time and evaluated how these parameters affected SLN formation process. The preparation method involves regulating the temperature of the surrounding environment and merging two steps; the formation and concurrent solidification of lipid nanodispersion. To test the influence of various preparation parameters on the particle size, the SLN dispersions were evaluated by DLS. Shear forces are applied by magnetic stirring combined with a sudden cooling of the dispersion medium to form the SLN. When the amount of the lipids increased in the formulation, an increase in the size of particle was observed which indicated a concentration dependent increase in particle size after the lipid concentration exceeded a threshold value. Surfactant added to the formulation reduces the interfacial tension between the lipid and water producing a nanosuspension which subsequently forms the SLNs during the cooling phase [16]. Our goal was to use the least amount of surfactant to limit potential toxicities that could be related to the presence of surfactant especially when testing in cell cultures, human RBC, and animal tissues. The optimized formulation contained a lipid:surfactant ratio of 1:2, 150 ml of deionized water was used as the dispersion medium, and stirring time was 45 min.

Three different methods of purifications were reported to recover and purify the SLNs to enhance their stability after solidifying the lipid in an SLN suspension. These methods are ultracentrifugation, dialysis and ultrafiltration [17]. In our preparation of SLNs, we used the ultrafiltration process to remove excess surfactant from the nanosuspension before it is lyophilized. Lyophilization is a recognized technique to improve the physical and chemical stability of lipid based preparations in aqueous media. It leads to changes in osmolarity and pH followed by evaporation of the water under vacuum thus averting

Ostwald ripening and hydrolysis reaction [18]. Cryoprotectants are usually added to minimize particle aggregation; it has been considered an excellent technique during freeze-drying improving the long-term stability of various pharmaceutical products. Cryoprotectants also reduce stresses that destabilize the lipid nanosuspension, decrease aggregation, and prevent irreversible fusion of nanoparticles that destabilize the lipid nanoparticulates [ ]. The most commonly used cryoprotectants include the sugars such as trehalose, sucrose, and mannitol. Cryoprotectants act by immobilizing the nanoparticles within their glassy matrix and protect them against destabilization by aggregation [20]. The choice of crytoprotectant and the ratio was selected after methodically changing using trehalose, sucrose, and mannitol compositions during SLN preparation and evaluating the particle size of the freeze-dried SLNs. The freeze-dried SLNs were visually inspected for quality of the solid powder as well. Trehalose was found to form uniformly free flowing SLNs upon lyophilization of the lipid nanosuspension and hence was used in our formulation. Trehalose was used to protect the nanoparticles from aggregation in a ratio of 1:1 of lipids to trehalose that produced the SLN after lyophilization.

4.3.2. Particle size

Particle size is a critical parameter used to evaluate the quality of SLN formulation. The size of particles before freeze-drying were 18 ± 4 nm and after freeze-drying the particle size increased to 525 ± 6 nm. The particle size increased for lyophilized particles compared to non-lyophilized but remained in the nanometer size range. Lyophilization is used to prolong the stability of 5-FU loaded SLN by limiting the risk of particle

aggregation through the use of cryoprotectant. Following lyophilization, a moderate increase in particle size of SLNs has been reported by many research groups [21]. A moderate increase in particle size was observed when 5-FU was encapsulated in the SLNs. In the case of SLNs, the drug molecules associate with aliphatic chains of the fatty acids resulting in an increased in particle size. Cryoprotectants have been shown to improve long-term stability and dispersibility of SLNs but at the same time contribute to increase in particle size during lyophilization. Additionally trehalose may solidify around the surface of the SLNs during freeze drying and this contributes to the particle size increase seen in SLNs. The lyophilized SLNs possessed particles below 600 nm size range and were found to easily disperse in water when reconstituted after freeze-drying.

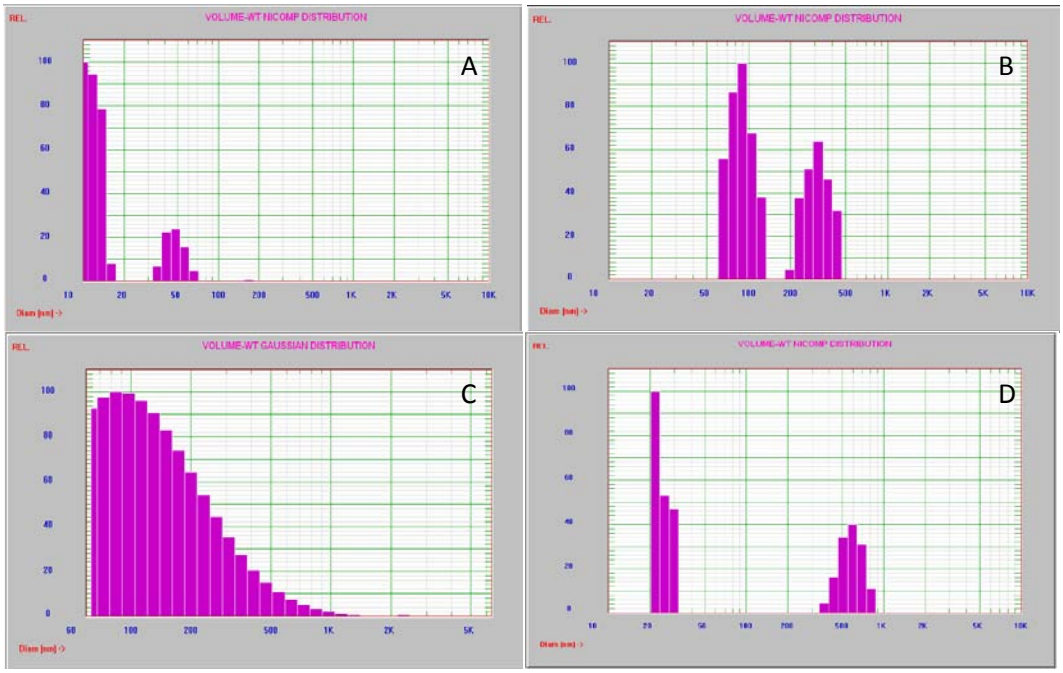

Figure 4-1: A) Particle size distribution of blank SLN before lyophilization; B) Particle size distribution of blank SLN 1 day after lyophilization;C) Particle size distribution of 5-FU loaded SLN after lyophilization at day 1;D) Particle size distribution of 5-FU loaded SLN after 60 days lyophilization

4.3.3. Zeta potential

The zeta potential was measured for 5-FU drug loaded aqueous SLN dispersion and lyophilized redispersed SLNs. Zeta potential for particles before lyophilization was approximately -17.3 mV and for particles after lyophilization was -18.5 mV. Zeta potential of the SLNs was negative on all formulations confirming glyceryl monostearate which is a fatty acid ester imparting a negative surface charge on the lipid particles. It indicates the overall charge a particle acquires in a specific medium and contributes to the degree of repulsion between close and similarly charged particles in the dispersion. High zeta potential (negative or positive) prevents aggregation of the particles due to electric repulsion and electrically stabilizes the nanoparticle dispersion. On the other hand, low zeta potential leads to when inter-particle attraction and the dispersion coagulates or flocculates. 5-FU is an unionized metabolite with a low molecular weight and is quite soluble in water and since cell membranes overall charge is negative, cell associations of negatively charged nanoparticles have been reported to be mediated by proteins and cell internalizations via the process of receptor-mediated endocytosis. Cellular interaction with charged ions or molecules shows that negatively charged ions or molecules will decrease the surface interactions and positively charged particles will increase the surface interactions [22]. Changes in zeta potential can occur as a result of the cellular interaction and internalization process. This is possibly due to vesicular transport based cell endocytosis engulfing portions of the surface plasma membrane to form vesicles around the substance inside the cells [23].

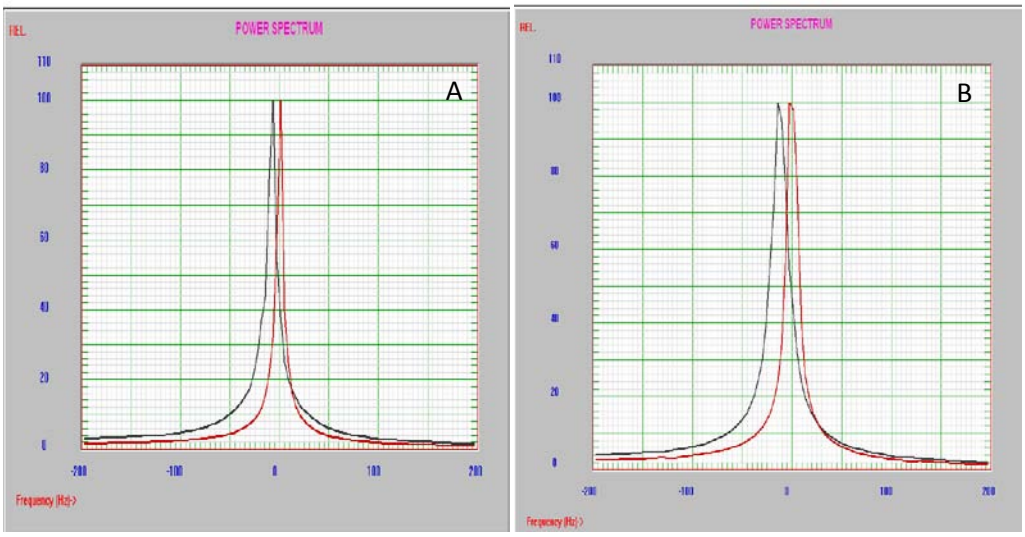

Figure 4-2: A) Representative zeta potential of 5-FU loaded SLN after 1 day lyophilization;B) Representative zeta potential of 5-FU loaded SLN after 60 days lyophilization

4.3.4. TEM

Transmission electron microscopy is a technique used to determine the shape and morphology of lipid nanoparticles. This technique can also help to evaluate the particle size. Particle shape is an important factor, since particles are preferentially required to be spherical. It provides additional information about the internal structure of the nanoparticles [24]. It utilizes electron transmission through the sample and provides direct information on the particle shape and size. The shape and surface morphology of blank and 5-FU loaded SLN dispersion and redispersed lyophilized SLNs were studied using TEM. The TEM images (figure 3) revealed the spherical shape of the lipid nanoparticles and confirmed the size of the particles to be in the nanometer range corroborating DLS data. Figures 3(a) and 3 (b) show SLN in aqueous dispersion before lyophilization and the particles are of diameter below 300 nm. TEM images of lyophilized SLN showed increased particle size (Figs. 3 (c) and 3 (d). TEM images of

lyophilized drug loaded SLNs suggest a core-shell model with a drug-enriched core. The dark spherical structures are the SLNs and the ultra thin carbon film on the foley carbon support film coating present in the 400 mesh copper TEM grid is the fibrous material. The blank lyophilized redispersed SLN show a well-defined shell. In drug loaded lyophilized redispersed SLN uniform density was observed throughout the structure indicating the homogeneity of the solid lipid matrix.

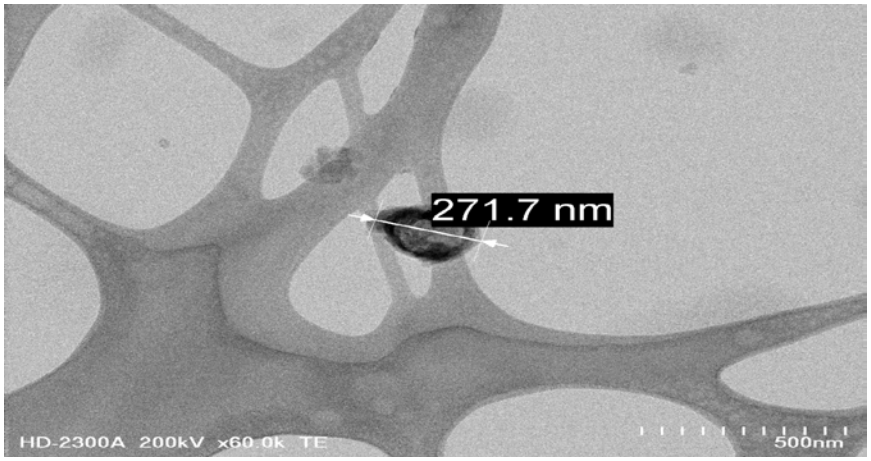

Figure 4-3A: TEM image of lyophilized blank SLN

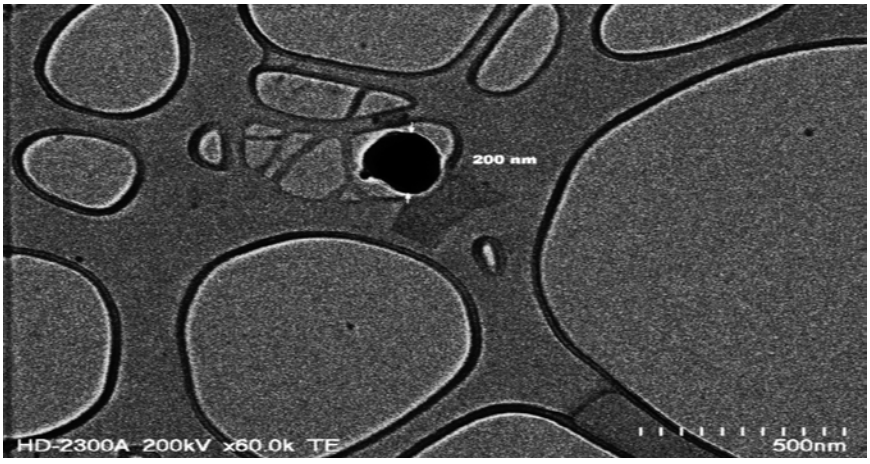

Figure 4-3B: TEM image of lyophilized 5-FU SLN

4.3.5. In vitro stability of SLNs

Stability of the SLN formulation is a critical factor that determines its success as a drug delivery system. SLNs principal features include their suitability to optimize drug loading and release profile, protection of incorporated labile drugs, long shelf life and low chronic toxicity, site specific targeting and excellent physical stability [25, 26]. However, one common instability problem with SLN dispersion is modifications in the lipid and colloidal dispersions mostly affecting the physical stability of SLNs such as lipid crystallization, polymorphic transitions, and gelling phenomena. For this reason, SLN dispersions were lyophilized to minimize potential instabilities that may occur during the preparation process. The physicochemical stability of lyophilized 5-FU loaded SLNs was monitored by measuring particle size and the zeta potential for the SLNs stored at 4 °C, room temperature (~ 23 °C) and 40 °C. There was no significant difference in the zeta potential of the 5-FU loaded SLNs in refrigerated room temperature and at 40 °C over 90 days of storage period. This indicates that in lyophilized SLNs, the surface charged remained stable regardless of the storage condition. On the other hand, particle size of 5-FU loaded SLNs increased in all three storage conditions demonstrating some variability with time. The SLN particle size after 60 days of storage at room temperature was 525 nm. Obviously the particle size is increasing over time and currently various strategies are being investigated to address this problem. There were no differences in Zeta potential in the three storage conditions with respect to time.

4.3.6. Evaluation of cytotoxic activity

PC-3 cells were used to study the cytotoxity of the SLNs using a Trypan blue exclusion assay. The automated cell counter used in this study is equipped with a 5 MP digital camera that captured images that were analyzed by the proprietary software available with the cell counter. In cell suspension, the trypan blue stain the dead cells but does not stain viable and living cells since these do not absorb the dye. The principle behind this is that a live cell possesses an intact membrane that excludes trypan blue dye; however, dead cells can take up the dye because the cell membrane integrity is lost [27]. The data obtained after 2 hrs of SLN exposure, is presented as percent cell viability shown in figure 4 (a). Cells treated with various concentrations of blank SLNs showed no significant difference ($p < 0.05$) in cell viability when compared to control. Blank SLN formulation data reveal that cells tolerated the exposure and that the SLN dispersion was compatible with the cells. Other researchers have reported biocompatibility of glyceryl behenate SLNs in Caco-2 cells [28]. For the cells treated with 5-FU loaded SLN, there was a concentration dependent decrease in cell viability. The PC-3 cells exposed to 5 µL and 10 µL drug loaded SLN dispersions showed approximately 98 % viability. Treatment with 15 µL viability decreased the cell viability to 95 %. Viability of cells treated with 20 µL further decreased to 89 %. One-Way ANOVA Tukey's test was used to compare means; 5-FU loaded SLN viability data on cells shows a significant difference ($p < 0.05$) when compared to cells exposed to nutrient medium and blank SLNs. There was also a significant difference between cells treated with 10µl and 20µl 5-FU SLN. The concentration dependent increase in cytotoxicity observed with 5-FU loaded SLNs is a notable achievement in cell culture especially when the blank SLNs were biocompatible and did not produce any biological effect.

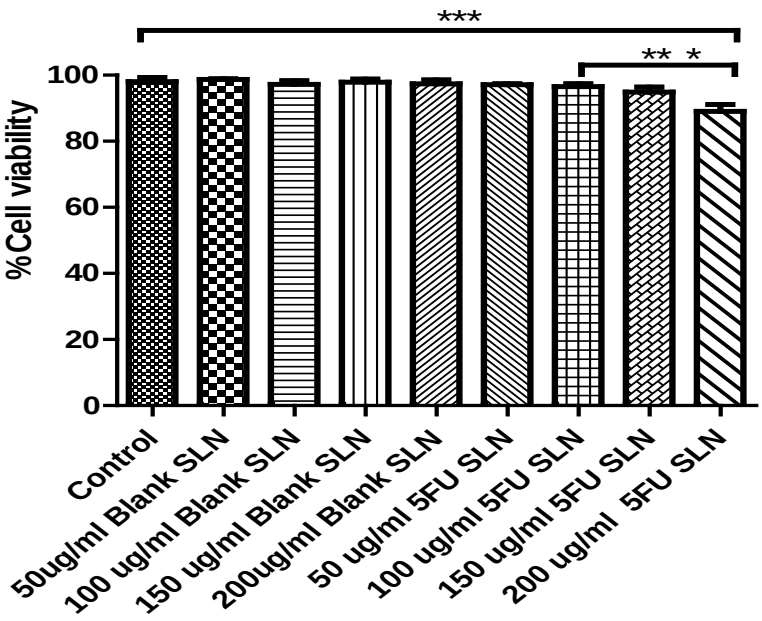

Figure 4-4A: Cell viability of PC-3 cells treated with blank and drug loaded 5-FU SLN

The aim of the present study was to evaluate the cytotoxic effect of the drug incorporated, versus free drug, on the human epithelial colorectal adenocarcinoma cell line, Caco-2. Cells were incubated for 2 hours with 5-FU solution and 5-FU loaded SLNs containing identical 5-FU concentrations. Viability (%) of cells treated with 5-FU solution was 87 % and for cells treated with 5-FU loaded SLNs was 79 %. 5-FU loaded SLN viability data on cells show a significant difference ($p < 0.05$) when compared to control. 5-FU loaded SLNs showed higher toxicity to Caco-2 cells when compared to 5-FU solution. 5-FU SLNs decreased the cell viability and cell proliferation in a concentration dependent manner which was more evident after 2 hours of exposure. Serpe et al demonstrated that HT-29 cells were more sensitive which resulted in low viability to cholesteryl butyrate SLN than when it's combined with doxorubicin concentrations. [29]. 5-FU loaded SLNs were further tested on Caco-2 cells for 4 hours. Cells were incubated with 5-FU solution

and 5-FU loaded SLN both formulations containing equivalent amounts of for 4 hours. Viability (%) of cells treated with 5-FU solution was 85 % and for cells treated with 5-FU loaded SLN 82 %. The incorporation of 5-FU SLN resulted in less viability when compared to 5-FU solution indicating drug release and permeation of 5-FU from the drug loaded SLNs. The increased cytotoxicity may be attributed to elevated drug concentration within cells.

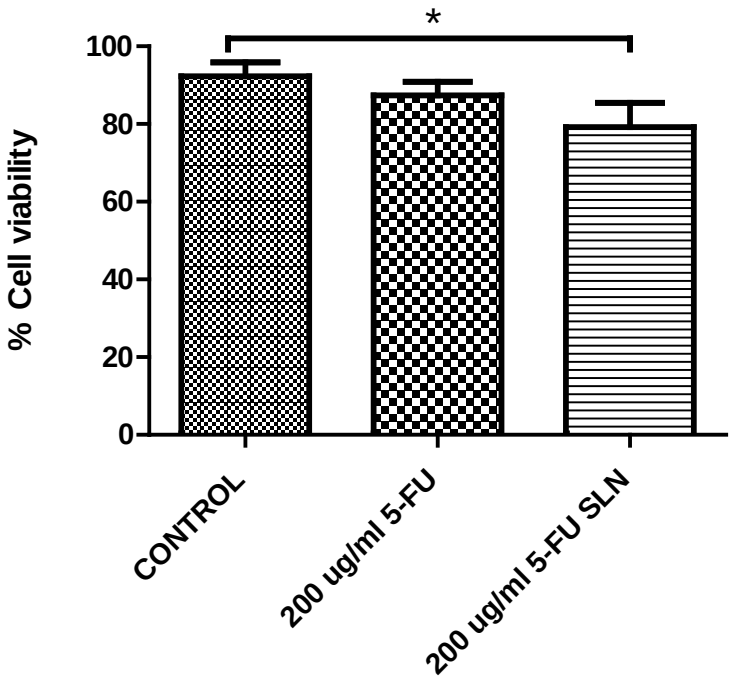

Figure 4-4B: Cell viability of Caco-2 cells treated with 5-FU solution and 5-FU loaded SLN for 2 h exposure

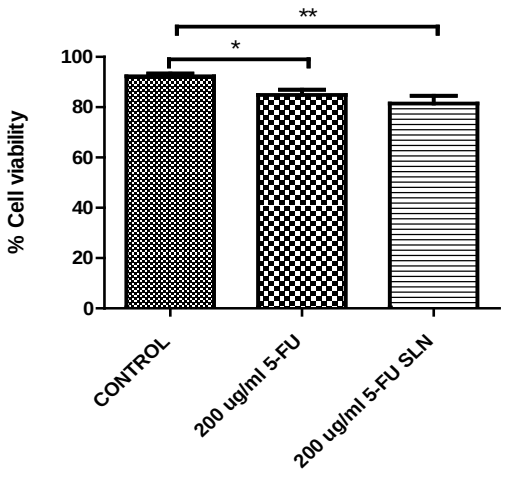

Figure 4-4C: Cell viability of Caco-2 cells treated with 5-FU solution and 5-FU loaded SLN for 4 h exposure

4.3.7. Evaluation of biocompatibility in human RBC's

Hemolysis is a potentially life-threatening condition that results in destruction of red blood cells *in vivo* that can lead to anemia, jaundice, and other pathological conditions. Therefore the hemolytic potential of all intravenously administered pharmaceutical formulations must be evaluated. One potential route of administration of 5-FU loaded SLNs is parenteral administration. For this reason the biocompatibility of the SLN formulation in RBC's is an important indicator of its in vivo tolerance in biological systems [30]. Red blood cells (RBCs) were collected by centrifugation and resuspended in normal saline. Human RBCs were incubated with various concentrations of lyophilized redispersed 5-FU loaded SLNs. For the positive control the RBC suspension was mixed

with distilled water which produces 100% lysis, and for the negative control RBC's were treated with normal saline producing no hemolysis [31] [32]. The RBCs were washed and centrifuged several times to remove debris. After washing an erythrocyte stock dispersion was formed and diluted with saline. From the stock dispersion, 200 µl was diluted with saline and treated with appropriate volume of stock SLN formulation to form 1 ml. After 1 h of incubation at 37 ºC, then centrifuge at 750 g for 3 min. 150 ml of supernatant was added to 3 ml of ethanol:HCl (40:1 v/v) solution and centrifuged. The absorbance was measured using a spectrophotometer at an absorbance wavelength of 398. The concentrations used to assess hemolysis were (0.025 mg/ml, 0.05 mg/ml and 0.1 mg/ml) and identical concentrations were used for blank SLNs and drug loaded SLNs. The results of hemolysis study are presented in figure 5(a). The percent hemolysis showed an increase when the concentration of 5-FU loaded SLN increased from 0.025 to 0.1 mg/ml. However, the drug loaded and blank SLNs produced RBC hemolysis that is under the critical limit of 5% hemolysis. This limit is reported as the upper limit for compatibility of materials with blood [33] . In a study performed by Jain et al., 2010, they showed that mannosylated SLNs containing doxorubicin showed 6.1 ± 0.2% hemolysis. In figure 5 (b) shows the effect of SLNs on RBC's when higher concentrations (0.125 mg/ml, 0.25 mg/ml and 0.5 mg/ml) of blank and 5-FU loaded SLNs were used in the RBS hemolysis study. The data indicates that blank SLNs and 5-FU loaded SLN formulation were tolerated by the RNC's and the overall hemolysis was below the critical limit of 5%.

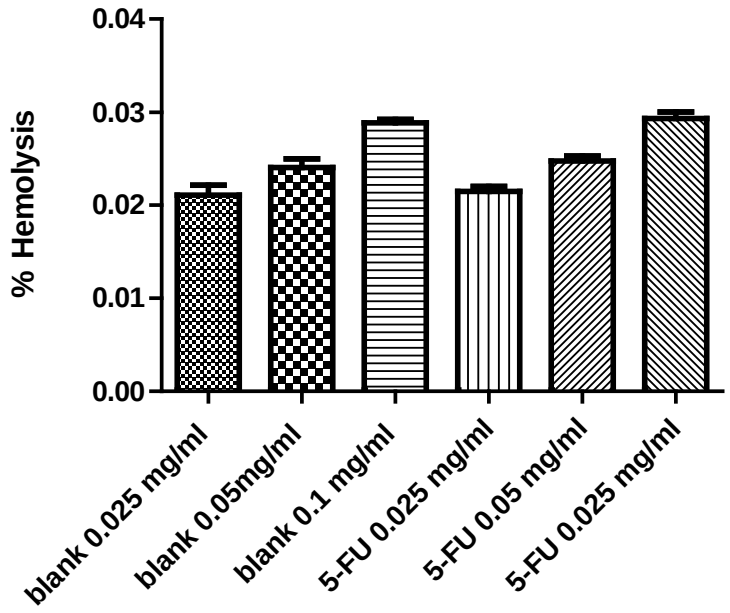

Figure 4-5A: Hemolysis assay of blank and 5-FU loaded SLN on the biocompatibility of RBCs, The data represent the mean values (n =3) ± S.D.

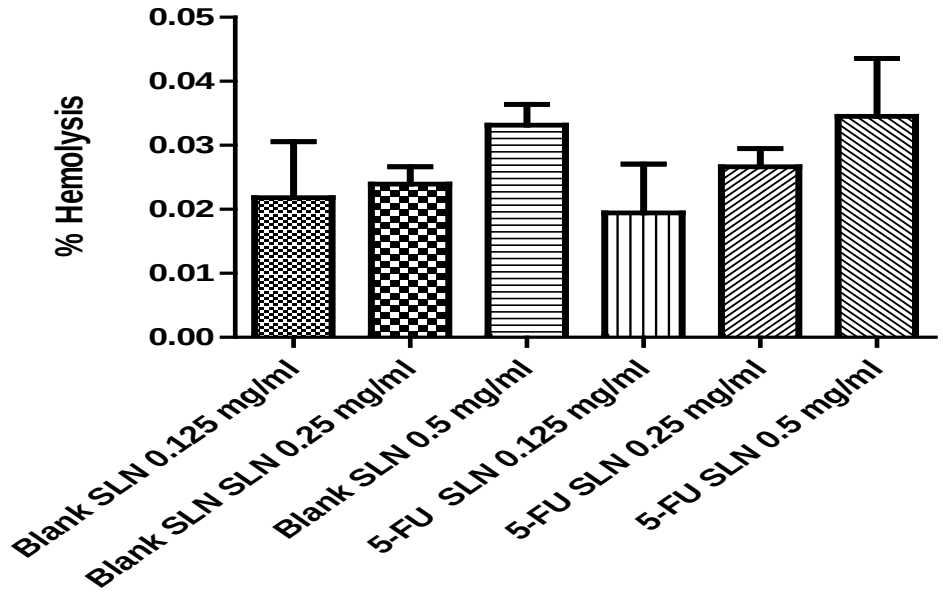

Figure 4-5B: Hemolysis assay of blank and 5-FU loaded SLN on the biocompatibility of RBCs, The data represent the mean values (n =3) ± S.D.

4.3.8. Permeability study

Bovine colon tissues were obtained from a local slaughter house and used for the determination of the in vitro colon permeability of 5-FU loaded SLNs. This study was performed to assess the utility of the SLN formulation to be used for oral administration. A side-by-side diffusion apparatus set-up was used in this study performed using Franz diffusion cells. The membranes were kept in phosphate buffer, pH 7.4 and washed for about 30 min before placing them between the donor and the receptor chambers. The receptor chamber contained approximately 15 ml of phosphate buffer in all cells [34], and was kept under magnetic stirring. The temperature was maintained at 37 ± 0.5 ˚C in the outer jacket of the Franz Diffusion cells. 5-FU solution was prepared in phosphate buffered saline (pH 7.4). 800 mg of 5-FU SLN was mixed in 4 ml of PBS and vortexed until solution became homogeneous and 1ml of the nanosuspension was placed in the donor compartment. 300 µl of the buffer was withdrawn from the receptor chamber and an equal amount of fresh PBS replaced to maintain equilibrium conditions. Samples were analyzed using reversed –phase high performance liquid chromatography (HPLC). The amount of 5-FU in the buffer was then determined using the chromatogram area via fitting to the line equation obtained from a 5-FU calibration curve. Subsequently the bovine membrane permeability was determined by normalizing the FLUX to the donor concentration, C*d* from the equation.

$$\text{Permeability } (P_{app}) = \frac{\text{FLUX}}{C_d}$$

C*d* is the drug in the donor chamber and FLUX = (dM/dt)/A, M is the cumulative amount of drug transported in time t and A is the bovine membrane surface area that is exposed

to the permeant [35]. All the experiments were performed in triplicate and results are expressed as mean ± standard deviation. The apparent permeability =FLUX=SLOPE= 1.319/ C*d = 3786*µg/ml =0.000348 cm/s and for 5-FU solution it was 1.339 cm/s.

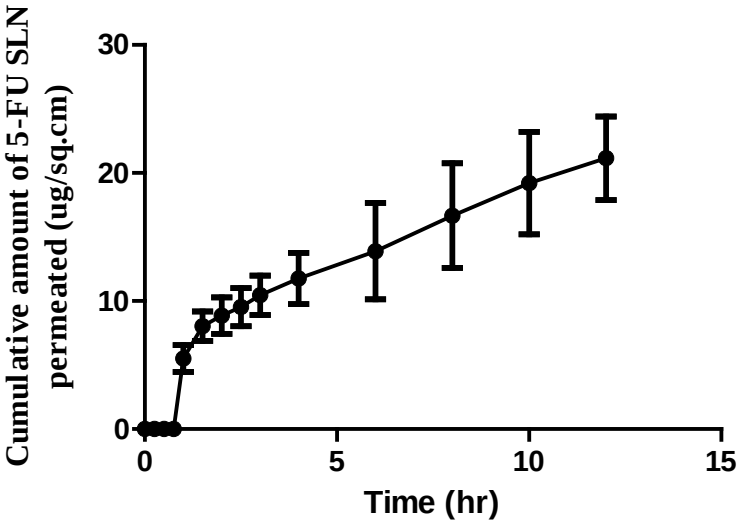

Figure 4-6A: Permeability of 5-FU SLN in bovine colon, the data represents the mean values (n =3) ± S.D.

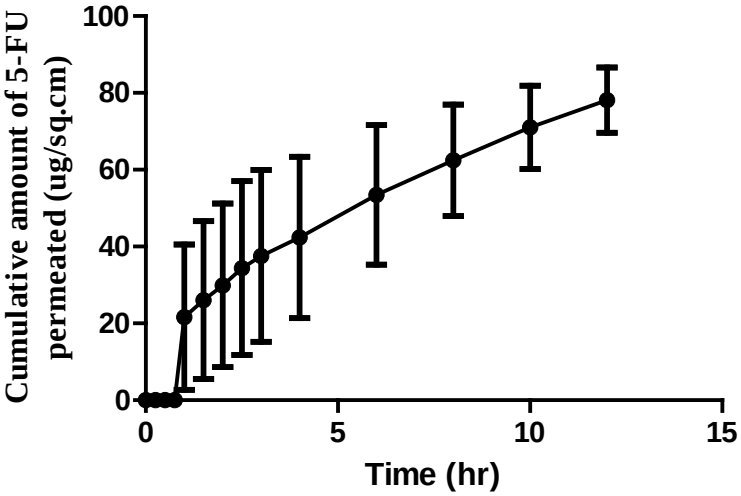

Figure 4-6B: Permeability of 5-FU pure solution in bovine colon, the data represents the mean values (n =3) ± S.D.

4.3.9. *5-FU Calibration curve and HPLC method development*

A stock of 1 mg/ml of 5-Fluorouracil was further diluted in water to obtain the above concentrations. 20µl of each concentration was injected and ran in triplicates directly into and analyzed by reverse-phase high performance liquid chromatography (HPLC) using Waters HPLC e2695 separation module equipped with a photodiode array detector (Waters 2998). A C18 column (Waters symmetry column, 3.5µm, 4.6 x 75 mm) was used as the stationary phase. The mobile phase composition consisted of pH 7.0 phosphate buffer, USP/acetonitrile/ethanol (60:20:20 v/v) and had an isocratic flow rate of 0.4 ml/min at 25 °C. 5-FU was detected at 264 nm and Empower 3.0 software was used for data analysis. All experiments were performed in triplicate, and results were reported as mean ±standard deviation.

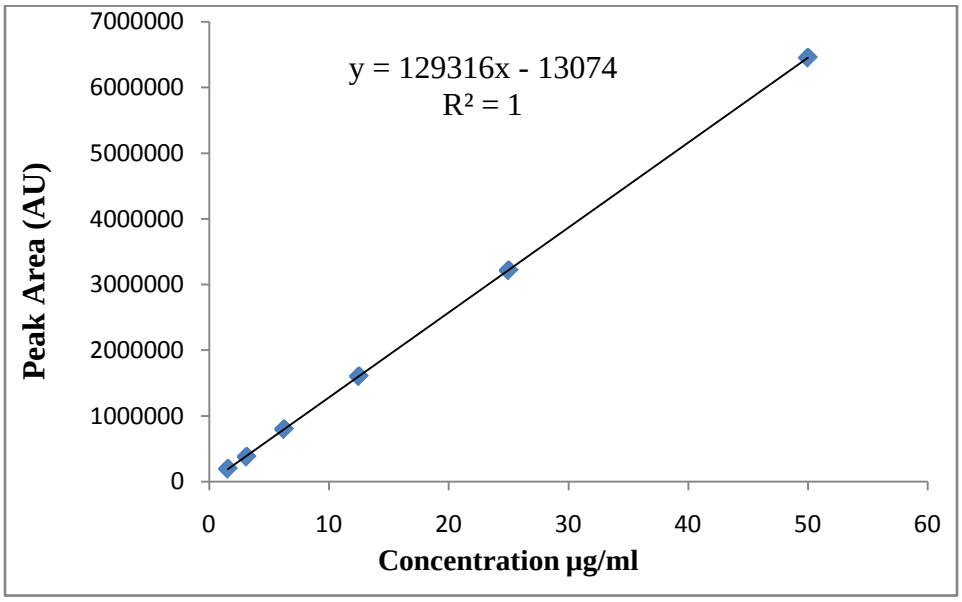

Figure 4-7: Calibration curve of 5-Fluorouracil

4.4. Conclusion:

In this present study, 5-Fluorouracil incorporated SLNs were successfully prepared via a temperature-modulated solidification method developed in our research laboratory. The particle size analysis and TEM imaging confirmed the formation of non-aggregated, spherical particles in the nanometer size range. Particle size of the SLN dispersion was approximately 18 nm and for redispersed lyophilized solid lipid nanoparticles was below 600 nm. Results from the three months stability testing indicates that lyophilization is an important method for improving the stability of SLNs. This allowed the SLN formulation to be stored in room temperature as indicated by the near constant particle size and zeta potential. Cell toxicity studies in PC-3 and Caco-2 cells demonstrated concentration dependent increase in cytotoxicity. Blank and 5-FU loaded SLNs formulations were found to be biocompatible when human RBCs were exposed to the concentration ranges tested. Data from our permeability study indicates that the apparent permeability is high and is likely to be bioequivalent to immediate release. Finally, our developed technique is simple and reproducible, and has the potential for scale-up for SLN formulation containing anti-cancer agents without the need of high energy and free of organic solvents.

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