SYMMETRICAL 1,3-DIGLYCERIDES AS SOLID LIPID MICROPARTICLES
FOR CONTROLLED DRUG DELIVERY

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ABSTRACT

Solid lipid microparticles (SLM) made from triglycerides and waxes are promising colloidal systems for controlled drug delivery. In this study, new symmetrical 1,3-diglycerides comprised of dihydroxyacetone and lipids of varying chain length were synthesized and used to fabricate solid lipid microparticles via a modified solvent-emulsification evaporation method. Particles were physically characterized in terms of size, surface morphology, surface charge as a function of lipid chain length. Scanning electron micrograph images showed that lipid particles display a distinct surface morphology depending on lipid chain length, with morphology transitions from smooth to porous structures with increasing chain length. Results of zeta potential measurements showed that the spheres are negatively charged and are susceptible to Schiff base reaction or reductive amination reaction with primary and secondary amines such as linear-polyethylenimine on the surface. Hydrophobic (nile red) and hydrophilic (rhodamine-B) model drug compounds were incorporated into the microparticles to determine encapsulation efficiency and in vitro release kinetics. Release kinetics of the hydrophobic compound nile red showed increasing release kinetics with increasing chain length, while microparticles incorporating the hydrophilic compound rhodamine-B exhibited burst release characteristics in all cases. These results outline the initial characterization of dihydroxyacetone-based symmetrical 1,3-diglycerides as new materials for controlled drug delivery.
BIOGRAPHICAL SKETCH

Sara Yazdi was born on October 24th 1984 in Iran. She and her family immigrated to United States in October 1998 and settled in Needham, Massachusetts. She obtained her B.S. at the department of Chemical engineering at University of Massachusetts, Amherst in 2004. In addition, she participated in research experience for undergraduate (REU) program sponsored by Material Processing Center (MPC) at MIT in 2003, and completed National Nanotechnology Infrastructure Network (NNIN) REU program at Cornell University in 2002. Prior to her graduate studies, she also worked as a Formulation intern at Millennium Pharmaceuticals, Inc. in Cambridge, MA. She started her MS/PhD studies at Cornell University under supervision of Professor David Putnam in Fall 2004.
To my brother, for not ever giving up
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CHAPTER 1.

INTRODUCTION AND BACKGROUND

The discovery and use of polymeric and solid lipid particles as excipients for the delivery of drug molecules to targeted sites at a desired rate transformed the field of drug delivery, and caused an emergence of a new era in patient care. Controlled release technology allows delivery and release of a cargo therapeutic molecule to a physiological environment at a designated rate. Many drugs have a narrow therapeutic index, therefore requiring multiple injections resulting in poor patience compliance and increasing the incidence of infections and hemorrhages. Use of a controlled release system permits improving the availability of drugs with short \textit{in vivo} half life, and thus eliminating the need for multiple injections and reducing the possibility of side effects.

A diverse collection of natural and synthetic biodegradable polymers have been studied intensively for drug targeting and prolonged drug release. Natural biodegradable polymers such as human serum albumin, and collagen are limited in use because of high cost and their disputed purity, thus in last two decades, use of synthetic biodegradable polymers in drug delivery has increased considerably. Synthetic biodegradable polymers ranging from polyesters, polyanhydrides, poly(amides), poly(amino acids), poly(orthoesters), poly(urethanes) and poly(acrylamides) have been widely utilized to prepare drug loaded devices [1-3]. Among the most popular synthetic biodegradable polymers are thermoplastic aliphatic poly(esters) like poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and most importantly poly(lactic-co-glycolic acid) (PLGA). PLGA is approved by Food and Drug Administration (FDA) for use as implants (Zoladex®) and microparticles (Decapeptyl®, Enantone Depot®) for treatments of prostate cancer. Encapsulation of
therapeutic molecules inside solid matrices not only decreases the toxicity of free drug molecules in the blood stream, but also allows targeted delivery through surface modification of these particles via covalent or electrostatic attachment of certain moieties (sugars, amino acids, lipids, etc). Examples of surface functionalization for polymeric and liposomal particle systems range from the conjugation of PEI to the surface of PLGA microspheres for delivery of plasmid DNA, to surface functionalization of PLGA-b-PEG nanoparticles with RNA aptamers targeted toward treatment of prostate cancer cells [4].

Despite the considerable growth in this field, the costly large scale production and stringent FDA manufacturing regulations remains a substantial challenge [5]. In case of polymer based particles, despite their advantages of robust surface chemistry, and wealth of possible chemical modifications, the cytotoxicity associated with product resulting from subsequent degradation and scale up production continues to be an issue of pressing importance [6].

In an effort to overcome the above difficulties, researchers have aimed to design a particulate system that is cost effective for large scale production and at the same time meets the FDA regulations. This carrier system needs to be physically stable whilst miscible with a wide variety of drugs and is easy to produce and cost effective. With that framework in mind, in the past decade, physiological lipids (i.e. triglycerides, cholesterol) have drawn significant attention as alternative materials to polymeric and liposomal systems, due to their potential advantages especially in terms of low toxicity and biocompatibility [7].

The term lipid applies a broad family of triglycerides, partial glycerids, fatty acids, steroids, and waxes. There is a vast variety of literature and reviews on use of lipids as drug delivery carriers; however, there are not that many studies focused on potentials of diglycerides in specific compared to rest of the lipids family
(i.e. triglycerides). Solid lipid microparticles (SLM) are reportedly used to encapsulate various drugs such as clobetasol (anti itch, for treatment of skin inflammation) and GnRH antagonist (hormones) as well as used to encapsulate hepatitis B surface antigen for mucosal delivery against hepatitis-B [6, 8-11].

Solid lipid particles are derived from lipids that are solid at room temperature as well as at body temperature and are stabilized by surfactants. The lipids include highly purified triglycerides, complex glyceride mixtures and waxes [12]. While lipid particles offer excellent physical stability, protection of the incorporated drug from degradation, and controlled drug release in addition to good tolerability, they suffer from drawbacks such as low drug encapsulation and drug expulsion after polymorphic transition during storage [13].

In this study we report synthesis of symmetrical 1,3-diglycerides based upon dihydroxyacetone (DHA). DHA is a constituent of glycolysis pathway, and is FDA approved for oral and topical administration, making it an attractive starting material for the synthesis of new biomaterials and facilitating their translation into clinical practice [14-16]. The advantages of using DHA-derived biomolecules lies in the fact that once degraded the resulting molecules are eliminated via its metabolic pathway (Creb cycle). This study intends to describe initial experimental results of synthesis and use of symmetrical 1,3-diglycerides and their associated features, applied production methods, drug incorporation and drug release mechanism as well as surface modification.

Modifying a synthetic route originally established by Bentley and McCrae[17], a set of six lipids with varying chain length were synthesized and used to produce particles in micron range. In this paper, in vitro control release kinetics, zeta potential, as well as particle size and morphology were investigated as a function of lipid chain length. The presence of adjustable lipid chain length thus changeable degradation rate
makes room for tailor made drug delivery carriers that can be modified depending on the nature of the encapsulating drug, taking us one step closer to individualized medicine.
CHAPTER 2.

SPECIFIC AIMS

The objective of this study is to understand how the rate of release of an encapsulated model drug correlates as a function of the lipid chain length, our working hypothesis is that rate of release will increase with the decrease in lipid chain length. The following specific aims are defined to evaluate our hypothesis:

1. To synthesize DHA-derived symmetrical 1,3-diglycerides. Using a synthetic route modified from Bentley and McCare [17], an array of symmetrical 1,3-diglycerides will be synthesized in high yields, and their characterization will entail $^1$H NMR spectroscopy and elemental analysis.

2. To fabricate solid lipid particles. Particles will be made using solvent-emulsification/evaporation method, and will be characterized in terms of size, charge and morphology.

3. To characterize the rate of release of model drug as a function of lipid chain length. The in vitro controlled release behavior will be studied through encapsulation of a range of model drugs with varying range of hydrophobicity and hydrophilicity. Our working hypothesis is to find a correlation between encapsulation efficiency and the nature of model drug as well as lipid chain length.

The investigation of these specific aims will provide us with a detailed general background knowledge of symmetrical 1,3-diglycerides as solid lipid particles in preparation for more advanced encapsulation and in vitro release study.
CHAPTER 3.

METHODS OF LIPID PARTICLE PRODUCTION

There are various methods to make lipid particles and the choice of the technique is contingent upon the nature of the lipid, the encapsulating drug, as well as the intended use.

3.1.1 Preparation by solvent emulsification-evaporation

In the solvent emulsification technique, lipid is dissolved in a water-immiscible solvent (i.e. chloroform, toluene), emulsified in an aqueous surfactant solution, followed by evaporation of the organic solvent yields lipid particles (Figure 1). An advantage of this technique is the absence of high temperature, making it a suitable method for heat sensitive drugs; however, the drawback of residual organic solvents still remains. In the solvent diffusion technique, the lipid is dissolved in a partially water-miscible organic solvent and then emulsified in an aqueous surfactant solution. The diffusion rate of the water-miscible solvent to aqueous phase is very rapid, thus causing turbulence at the interface between organic solvent and aqueous phase. The turbulence, referred to as Marangoni effect [18], leads to assembly of PVA molecules around emulsion droplets, setting off spontaneous droplet formation. The reduced solubility of lipid drug complex at the emulsion interface causes immediate precipitation of lipid and accompanying drug thus encapsulating drug inside the lipid matrix with organic solvent diffusing out toward the continuous phase. The dispersions are fairly dilute and particles can be collected via filtration or centrifugation [12, 19]. This method is widely utilized for encapsulation of hydrophobic drugs, however depending on the nature of the drug, low encapsulation efficiency maybe a problem at which point the process is modified/changed increase encapsulation efficiency.
3.1.2. High pressure homogenization (HPH)

There are two methods of high pressure homogenization, hot and cold. In hot HPH, lipid and drug are melted together at approximately 5 °C above the lipid melting point; the melt is mixed with aqueous surfactant solution (i.e. poly (vinyl alcohol)) at the same temperature. The hot pre-emulsion solution is stirred at high speeds and then processed with a temperature controlled homogenizer. The process is followed by cooling to room temperature and crystallization of lipid particles. In cold HPH, the melt containing molten lipid and drug is ground under a stream of liquid N₂, leading to formation of lipid microparticles. The presuspension is stirred in a cold aqueous surfactant solution followed by homogenization. The advantages of HPH technique are the narrow particle size distribution as well as absence of any organic solvents [12]. The advantage of this method is that there are no organic solvents involved, however this method is limited to temperature insensitive drugs.
3.1.3. Preparation by Water-in-oil-in-water (w/o/w) double emulsion method

In w/o/w double emulsion technique is a modification of solvent emulsification-diffusion method specifically aimed at encapsulating hydrophilic drugs. In this method, the drug is solubilized in an internal aqueous phase of a w/o/w double emulsion, along with a stabilizer to retard drug loss to the external aqueous phase during solvent evaporation [20]. High speed stirring and/or ultrasonication can also be utilized to yield lipid particles. Particles can be collected through ultra filtration as well as centrifugation.

3.1.4. Preparation using other methods

Other preparation methods such as microchannel emulsification technique [21], spray congealing, and cryogenic micronisation are also utilized. Particle prepared using microchannel emulsification are prepared through forced flow of that dispersed phase through a continuous surfactant phase, the resulting particles are reportedly monodisperse and the size of the droplet is dependent on the design of the microchannel [13].

3.2. Methods of analytical characterization of lipid particles

Extensive characterization of lipid particle properties made through various methods provides us with the opportunity to select a method depending on the costs, desired particle size distribution, and recovery efficiency.

3.2.1 Size measurements

Size is one of the deciding factors for pharmaceutical applications and characteristics of well-formulated system will include a narrow size distribution. When it comes to delivery of drugs via intravenous injections, particles larger than 5-μm can cause embolism [22]. In addition, the size of the particles can trigger the
capture mechanism though phagocytosis, a process in which large and insoluble particles are enveloped by the plasma membrane and internalized [23], subsequently influencing the bioavailability of the drug encapsulated particles. Particle size measurements are usually done using dynamic light scattering (also known as photon correlation spectroscopy (PCS)) and laser diffraction (LD) for particle size spanning nanometers to 5-6 μm, for particles larger than 6 μm, Coulter counter method [24] in which the electrical resistance produced by particles suspended in buffer solution, while passing through an aperture, is usually utilized. The resulting displaced volume of buffer solution, which is proportional to the particle size will create a voltage pulse that is collected to create a particle size distribution [25].

3.2.2 Charge measurements

Zeta potential $\zeta$ is an assessment of the surface charge of colloidal dispersion and often the key to understanding dispersion and aggregation in particle population. The greater the zeta potential ($|\zeta|$) the less likely the suspension is to become unstable, because charged particles repel one another and therefore overcome the tendency to aggregate [22]. Surface charge is measured using a zeta sizer which measures the electrophoretic mobility across the diffusive layer of the particles.

3.2.3 Morphology

Particle morphology, studied using scanning electron microscopy (SEM), is an indicative of particle composition and origin and it also serves to better the understanding of in vitro controlled release in case of drug encapsulated particles. Particles with higher surface:volume ratio allow more buffer access and deeper penetration thus a faster degradation rate. Particle morphology also has an impact on the recognition process by the cells, thus becoming an important characteristic [26].
3.2.4 Encapsulation efficiency

Encapsulation efficiency (EE) is defined as percentage of drug experimentally encapsulated over the amount the drug originally fed into the system whilst it being a function of preparation method it is also a function of the hydrophilic/hydrophobic nature of the drug, for example O/W emulsion technique is often used for encapsulation of hydrophobic drugs while W/O emulsion is utilized to encapsulate hydrophilic drug [13]. In a study done by Cortesi et al. encapsulation of hydrophobic drug in the matrix, using the melt dispersion method was as low as 2% compared to 70% encapsulation efficiency in case of hydrophobic drug prepared using the same technique. In an effort to increase encapsulation efficiency, the preparation method of the lipid particle was changed to w/o/w double emulsion and the subsequent efficiency was raised to 50% [20].

3.3. Drug release from lipid particles

Kinetics of drug release is an important aspect of the particle characterization. In vitro release gives an insight into the drug distribution inside the matrix as well as information about the release mechanism. It has been shown that the release profile is primarily influenced by modification on the lipid matrix, surfactant concentration and fabrication parameters [27, 28]. Wissing et al. [12] proposed the following structural models for drug encapsulated lipid particles,
Depending on the nature of the encapsulated drug and method of preparation, the three primary outcomes are: a drug enriched core particle, a drug enriched shell or a particle with drug homogenously dispersed throughout the matrix. Those particles with drug enriched shell often display a burst release behavior rather than sustained release over an extended period of time. Also, depending on the particle size, those enriched on the surface often display rather low encapsulation efficiency.

### 3.4. Surface Modification of particles

Surface modification of drug encapsulated particles for targeted delivery to specific cell types would greatly enhance the effectiveness of drug delivery by these vesicles [4, 29, 30]. In the case of polymeric particles, some examples of surface modification include PEGylated particles that have shown great potential as long circulating systems for intravenous injections and polyethylenimine (PEI)-conjugated PLGA microspheres [29] which are strong contenders in field of gene delivery to cells. Some other examples of surface modified particles are, PLGA-\(b\)-PEG nanoparticles functionalized with aptamers [4] utilizing EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride)/NHS chemistry. These surface modified particles were then recognized by antigens expressed on the surface of
prostate cancer cells. Similarly Kasturi et al. [30] employed a modified EDC/NHS chemistry to conjugate PEI to the surface of PLGA microspheres, yielding cationic microparticles useful for delivery of plasmid DNA.

However, examples of surface modified lipid particles are scarce especially in the case of mono- and diglycerides, and their ability as alternative surface modified carriers for drug delivery remains in question. But nevertheless, it has been shown that lipid particles combine the virtues of liposomes and polymeric particles in a sense that they are biodegradable, can incorporate hydrophobic/hydrophilic drugs, and have an enhanced stability of the incorporated drugs, and are deemed more feasible for scale-up [6].
CHAPTER 4.

MATERIALS AND METHODS

4.1. Materials

1,3-Dihydroxyacetone dimer (DHA), palmitoyl chloride, myristoyl chloride, and lauroyl chloride, as well as anhydrous pyridine, sodium cyanoborohydride and chloroform were all obtained from Sigma-Aldrich and used without further purification. Poly(vinyl alcohol) (PVA, MW~25000, 88 mole% hydrolyzed) and polyethylenimine-linear (PEI, MW~25000) were purchased from Polysciences Inc. Dichloromethane (DCM), acetone, tetrahydrofuran (THF) and ether were purchased from J.T.Baker. Rhodamine-B was purchased from Fluka.

4.2. Synthesis of symmetrical 1,3-diglycerides

Symmetrical 1,3-diglycerides were synthesized through modification of a previously reported method by Bently and McCrae [17]. The general procedure is as it follows: dihydroxyacetone (DHA) is stirred in chloroform under flow of N₂ at room temperature, and acyl chloride and anhydrous pyridine are added drop wise in that order to the heterogeneous mixture, respectively. The mixture is stirred for 3 hrs followed by extraction of chloroform layer with water portions. Chloroform is evaporated using rotary evaporation and the remaining solid is re-crystallized using methylene chloride-ether combination. ¹H-NMR spectra were recorded at room temperature with a Brucker AF-300 spectrometer operating at 300.13 MHz.
4.3. Fabrication of Solid Lipid Microparticles (SLM) using spontaneous emulsification-solvent evaporation method

Lipid (0.1 gm) is dissolved in a 3:2 ratio of DCM:acetone by vigorous vortexing for approximately 10 seconds. The lipid solution was poured into 450 ml of 2.5% PVA solution stirring at 800 rpm and left for 3 hrs to allow full evaporation of all organic solvents. Particles were separated by centrifugation followed by multiple washes (3×) with water. The particles were resuspended in de-ionized water and lyophilized for a minimum of 12 h and were stored at -20°C. The results were fine powder particles with approximate yield of 75% (relative to original weight of lipid). The fabrication variables that were examined are provided in Table 1.
Table 1: Summary of modified solvent emulsification-evaporation method for concentration $C_A$ and $C_B$ lipid solution

<table>
<thead>
<tr>
<th>Method</th>
<th>Disperse Phase</th>
<th>Dispersion Medium</th>
<th>Stirring speed</th>
<th>Process duration</th>
<th>Particle isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsification-solvent evaporation $C_A$</td>
<td>Dissolved Lipids (0.1gm) in 5 ml of 3:2 ratio of DCM:Acetone $C_A= [0.02] \text{ gm·ml}^{-1}$</td>
<td>2.5 and 5 wt% poly(vinyl alcohol)</td>
<td>800 rpm at 25°C</td>
<td>1.5 hrs</td>
<td>Centrifugation followed by washing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emulsification-solvent evaporation $C_B$</td>
<td>Dissolved Lipids (0.1gm) in 10 ml of 3:2 ratio of DCM:Acetone $C_B= [0.01] \text{ gm·ml}^{-1}$</td>
<td>2.5 and 5 wt% poly(vinyl alcohol)</td>
<td>800 rpm at 25°C</td>
<td>3 hrs</td>
<td>Centrifugation followed by washing</td>
</tr>
</tbody>
</table>

4.4. Particle characterization

Particle morphology as well as size was studied at low voltage (5 kV) using scanning electron microscopy imaging (LEICA 440) after coating with palladium. The samples were suspended in 1:10 diluted Phosphate buffer saline and surface charge was investigated using a Malvern Zetasizer-Nano ZS (Malvern, UK).

4.5 Surface Modification using Linear-PEI

PEI (31mg: 581μmol) and (30 μmol) of lipid microspheres were incubated in 5 ml of sodium borate buffer (pH 8.5). After addition of sodium cyanoborohydride (5 equiv/lipid), the reaction was incubated for 4 hrs at 40 °C. At the end of the reaction duration, particles were washed 4× in excess 1M NaCl solution [30] to remove physically adsorbed PEI. Afterward, particles were resuspended in 1:10 dilution of PBS buffer and zeta potentials was measured in triplicates.
4.6 Encapsulation efficiency of SLM

The encapsulation efficiency of lipid microparticles was determined by complete drug recovery from melted microparticles, followed by comparison with the theoretical maximum drug loading. For microparticles containing rhodamine-B, encapsulated drug was recovered from 5.0 mg of lipid particles by melting in 1-ml of phosphate buffered saline (PBS) at five degrees above the corresponding lipid melting point. The resulting emulsion was cooled to room temperature and any re-solidified particulate matter was removed by centrifugal filtration (Ultrafree-MC, Millipore). The concentration of rhodamine-B in the supernatant was quantified in a microplate spectrofluorometer (Spectramax GeminiEM; Molecular Devices, Sunnyvale, CA) using 96-well black assay plates (Corning, Inc). The excitation and emission wavelengths were 540 nm and 625 nm, respectively. Complete recovery of nile-red from particles was performed in a similar fashion, with the exception that particles were melted in ethanol due to the insolubility of nile red in PBS. The excitation and emission wavelengths of nile red were 550 nm and 650 nm, respectively.

4.7 In vitro drug release

*In vitro* drug release was determined by suspending 5.0 mg of microspheres in 1-ml of PBS in amber microcentrifuge tubes (Eppendorf) and incubating at 37 °C with
rotation (60 rpm). For rhodamine-B, samples were filtered, and the fluorescence was quantified in a microplate spectrofluorometer at each given time interval. Rhodamine-B loaded samples were discarded after each time interval reading, thus each data point represents three dedicated samples. In the case of nile red, samples were first centrifuged (16,000 RCF) to pellet solids. The supernatants were then removed for fluorescence measurements, and pellets were resuspended in fresh buffer and allowed to rotate until the next time interval.

4.8 Statistical Analysis

The statistical significance of experimental results was examined using the two sample Students’ t-test with p<0.05. The calculated errors were set to standard mean error for all experimental results.
CHAPTER 5.

RESULTS AND DISCUSSION

5.1. Lipid characterization

Symmetrical 1,3-diglycerides were successfully synthesized following the McCrae[17] synthetic route with a yield in excess of 70%. Acyl chlorides were chosen such that the final product was a physiological lipid, for example palmitoyl chloride derived from palmitic acid, a fatty acid freely present in various metabolic pathways, was selected to synthesize C_{16} lipid and so on (Table 2). In addition, to derivation from physiological molecules, all of the synthesized lipids have melting points that are above room temperature making them ideal candidates for use as powder or particulates in drug delivery.

![Table 2: Lipids and their corresponding acyl chloride](image)

Elemental analysis results for C_{12}-C_{18} lipids showed results within acceptance range, (Table 3). Proton nuclear magnetic resonance (\(^1\)H NMR) was also utilized to further characterize the products. Chemical shifts (δ) were measured in ppm using deuterated chloroform, CDCl\(_3\) as solvent.
Table 3: Lipids elemental analysis results and corresponding melting points

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Lipid</th>
<th>Melting Point (°C)</th>
<th>E. analysis - theoretical</th>
<th>E. analysis - experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>%C</td>
<td>%H</td>
</tr>
<tr>
<td>C₁₆</td>
<td>C₃₅H₆₆O₅</td>
<td>76-78</td>
<td>74.13</td>
<td>11.75</td>
</tr>
<tr>
<td>C₁₄</td>
<td>C₃₁H₅₆O₅</td>
<td>73-76</td>
<td>72.87</td>
<td>11.46</td>
</tr>
<tr>
<td>C₁₂</td>
<td>C₂₇H₅₆O₅</td>
<td>69-70</td>
<td>71.32</td>
<td>11.08</td>
</tr>
<tr>
<td>C₁₀</td>
<td>C₂₃H₄₂O₅</td>
<td>64-65</td>
<td>69.31</td>
<td>10.62</td>
</tr>
<tr>
<td>C₈</td>
<td>C₁₈H₃₄O₅</td>
<td>56-58</td>
<td>66.63</td>
<td>10.01</td>
</tr>
</tbody>
</table>

Figure 4: ¹H-NMR spectra of C₁₂ lipid (dodecanoic acid 3-dodecanoyloxy-2-oxo-propyl ester) C₁₂ lipid’s spectra serves as a representative of the remaining lipids.

5.2 SLM characterization

5.2.1 Size

Particle sizes were measured using scanning electron microscopy (SEM) images that were obtained at low voltage (5 kV). Sizing results collected from multiple image analyses using Image J software showed polydisperse particle distributions in the micron size range. The effect of lipid concentration and surfactant
concentration on the final particle size with constant stirring rate and temperature was determined by serial variation of both parameters. The results showed that increasing the lipid concentration led to an increase in mean diameter (Figures 5 and 6), whereas increasing the surfactant concentration did not significantly change particle size (Figures 7 and 8). Statistical significance of particle size as a function of lipid chain length was inconclusive because of the polydispersity of the particle size distribution.

Figure 5: Effect of pre-emulsion lipid solution concentration on particle size for a constant 2.5% PVA concentration. Error bars represent ±SEM.
Figure 6: Effect of pre-emulsion lipid solution concentration on particle size for a constant 5% PVA concentration. The mean particle diameter for high concentration C_{12} lipid, as well as C_{10} and C_{8} is statistically significant from its low concentration counterpart (n=3, p<0.05, ±SEM), indicated by asterisks (*).
Figure 7: Effect of varying PVA concentration with constant high pre-emulsion lipid solution concentration. At high concentration (0.02 g/ml), the mean particle diameter of C12 lipid sample decreases with increasing surfactant concentration, and is statistically significant (n=3, p<0.05, ±SEM), denoted by an asterisk (*).
Figure 8: Effect of varying surfactant concentration with constant low pre-emulsion lipid solution concentration. At low concentration (0.01 g/ml), mean particle diameter for C8 lipid sample and C10 lipid sample as well as C14 and C16 diameters decreases with increasing surfactant concentration, and is statistically significant (n=3, p<0.05, ±SEM) as indicated by asterisks (*).

5.2.2 Morphology

The particle surfaces were irregular with unique patterns depending on the lipid chain length. In spontaneous emulsification, the solvent must diffuse out into the external aqueous phase first prior to evaporation. The irregular surface morphology of the lipid particles may therefore be a result of rapid solvent removal, leading to local precipitation or crystallization of the lipids [1]. Further inspection of the particle surfaces revealed that the morphology is distinct for each lipid chain length. The smaller lipids (C8 and C10) show a relatively smooth surface compared to C12 through C16. Additionally, the surface structures become more prominent as the lipid chain length increases (Figure 9).
Figure 9: Clockwise from top-left, (A) C₈ particle morphology showing a smooth surface followed by (B) C₁₀, (C) C₁₂, (D) C₁₄, and (E) C₁₆. Note the increasing porosity with increasing lipid chain length.
5.2.3 Charge

Zeta potential measurements showed that the SLMs are negatively charged in a 1:10 dilution of PBS in water. The results indicate the presence of hydrolyzed ester groups on the surface. Along with hydroxyl PVA side chains, the ester groups of the diglycerides likely rearrange in such manner to associate with the aqueous phase. Upon removal of the organic solvent, these ester groups remain exposed on the surface of the newly formed lipid particles, leading to their hydrolysis and exposure of negatively charged carboxyl groups (Figure 10).

![Figure 10: Zeta potential of lipid microparticles in 1:10 dilutions of PBS:Water. All lipid particle surfaces retain a negative charge.](image)

5.3. Surface modification of SLMs

Particle surface modification provides the framework for targeted delivery of drugs. A myriad selection of literature discusses successful conjugation of functional groups to the surface of particles, for example Zanta et al. [31] successfully
conjugated galactose to branched PEI via imine formation with lactose and subsequent condensation of PEI-gal with plasmid DNA forming neutral galactose-bearing complexes. The PEI-gal complexes were further tested for \textit{in vitro} gene delivery to liver (hepatocytes). Following the footsteps of Zanta \textit{et al.}, the modified synthetic route was used to conjugate linear-PEI onto the surface of lipid microparticles, (Figure 3 and Scheme 2).

Initial zeta potential measurements showed that lipid particle are negatively charged, the negative charge is attributed to the presence of hydrolyzed carbonyl groups on the surface. Zeta potential measurements acquired after the reaction with linear-PEI showed a positive surface charge. The first positive control experiment consisted of incubation of lipid in PBS medium for reaction duration, and the second positive control experiment consisted of incubation of lipid with linear-PEI in the absence of NaCNBH$_4$, followed by zeta potential measurements. In the negative control experiment PLGA particles replaced their lipid counter parts and underwent the reaction with identical conditions. The positive control experiments suggest that linear-PEI interacts with the exposed carbonyl groups both covalently as well as electrostatically. Surface zeta potential was slightly less positive for particles that underwent 4× wash with 1M NaCl compared to the ones washed with DI-H$_2$O, the reduction in zeta potential is due to removal of electrostatically attached linear-PEI onto the surface. The negative control experiment with PLGA particles showed that linear-PEI does not interact with carboxyl groups present on the surface covalently, however the electrostatic interaction between linear-PEI and the negative PLGA surface is quite strong. The results of the Students’ t-test analysis showed that resulting surface charge is statistically significant between different chain lengths, the zeta potential becomes more positive as the lipid chain length increases. Another interesting observation was the results of the reaction between lipid particles and
linear-PEI in the absence of NaCNBH₄; however more rigorous experiments are required to explain the observed behavior, (Figures 11 and 12).

Recent drug delivery approaches have employed various synthetic routes for covalent attachment of moieties with positive charge. The increased interest in cationic particles stems firstly from the large population of therapeutics molecules such as DNA, oligonucleotides, and proteins are negatively charged, thus the electrostatic interaction between the two could stabilize the complex and lead to uptake by target cells. Secondly, a positively charged complex will maintain contact with the negatively charged outer cell membrane and thus will enable efficient internalization of the particle [32]. A vast variety of literature have reported successful functionalization of polymeric particles with linear and branched PEI, likewise our initial attempts to functionalize the surface of lipid particle have been successful however further characterization of the cationic lipid particle still remains.

Scheme 2: Reductive amination reaction pathway
Figure 11: Results of reductive amination reaction of linear-PEI with C₁₂ lipid in presence of NaCNBH₄ and corresponding control experiments. Columns marked by * are statistically significant from each other, (n=3, p<0.05). Negative control experiment with PLGA particles shows statistical significance in zeta potential with NaCl wash versus water wash, NaCl (1M) solution washes remove electrostatically attached linear-PEI onto the surface and indicates the lack of covalent bonding between linear-PEI and carboxylic acid groups present on the surface of PLGA particles. Positive control experiment results shows zeta potential intensity of C₁₂ lipid particles are statistically significant compared to those that underwent the reductive amination reaction with linear-PEI. The resulting zeta potential intensity of particles that underwent C₁₂-PEI reaction in the absence of NaCNBH₄ is also significant from those that underwent the reaction in presence of NaCNBH₄, the difference can be contributed to changes in pH of the reaction environment, (n=3, p<0.05, ±SEM)
Figure 12: Results of reductive amination reaction for C_{12}-C_{16} particles. Columns marked by * are statistically significant. The zeta potential intensity increases as the lipid chain length increases, the difference between the lipids’ zeta potential intensity is apparent after the reaction with linear-PEI, (n=3, p<0.05, ±SEM).

5.4. Encapsulation Efficiency Measurements

Rhodamine-B (hydrophilic model drug) and nile red (hydrophobic model drug) were successfully encapsulated in lipid particles, although with very different efficiencies. For rhodamine-B loaded particles, the encapsulation efficiency was less than 10 percent, whereas for nile red the efficiency exceeds 70 percent (Figures 13 and 14). Factors that impact encapsulation efficiency range from the method of preparation to the nature of interaction between the model drug and the encapsulating matrix. Low encapsulation efficiency in the case of rhodamine-B can be attributed to the
hydrophobicity of the lipid matrix, the porous nature of the lipid microparticles, as well as the solubility of rhodamine-B in the aqueous PVA solution. Amongst all lipids, C8 with the shortest lipid chain displayed the highest encapsulation efficiency of rhodamine-B.

For nile red the results are very different. All lipids encapsulated significant amounts of nile red (>70%). Additionally the Students’ t-test analysis of encapsulation efficiency for nile red loaded lipid particles showed that the difference between the lipids is not statistically significant (p>0.05). The high encapsulation efficiency in the case of nile red is attributed to a more favorable interaction between the hydrophobic model drug and the encapsulating lipid matrix.

![Figure 13: Encapsulation efficiency for rhodamine-B loaded lipid particles. Columns marked by asterisk (*) are statistically significant. Encapsulation efficiency of lipids C_{10}-C_{16} show statistical significance for encapsulation of rhodamine-B model drug, (n=9, p<0.05, ±SEM). No particular trend is observed between encapsulation efficiency and lipid chain length. Lipids show distinct crystallization behavior and](image-url)
smooth morphology in case of shorter chain length lipids (C₈-C₁₀), a possible explanation for high encapsulation efficiency of C₈ compared to longer chain length lipids.

Figure 14: Encapsulation efficiency for red nile loaded lipid particles. Encapsulation efficiency results for lipids C₈-C₁₆ for nile red (hydrophobic model drug) show high values of more than 70%.

5.5. In vitro controlled release behavior

The in vitro release curves were created using measurements conducted at 37°C for rhodamine-B as well as for nile red over a period of 24 hours. Results for rhodamine-B containing lipid microspheres show a burst release showing an expulsion of approximately 5-50% of encapsulated rhodamine-B over the initial hours [33]. This immediate release of the drug is attributed partly to the drug molecules associated with particle surface, or those entrapped in an outer thin shell of particle and partly due to
porous morphology of lipid particles. In contrast, the in vitro release curves for red nile samples showed slower release of 5-15% of the encapsulated nile red over the initial hours, in addition, longer chain length lipids (i.e. C\textsubscript{14}-C\textsubscript{16}) showed faster release compared to shorter chain lipids (i.e. C\textsubscript{8}-C\textsubscript{12}), (Figures 15 and 16). There are two possible explanations for the observed release behavior, firstly the release behavior is an approximate indication of nile red’s distribution in the lipid matrix, with nile red’s presence deeper in the core of the particle in case of smaller lipids, thus displaying slower release. In contrast, in case of larger lipids nile red is distributed closer to the surface and subsequently shows faster release. Secondly, slower release of smaller lipids maybe due to their smaller particle size, however particles prepared using solvent emulsification evaporation method are polydispersed, thus making it difficult to evaluate the effect of particle size on release profile confidently. A key determinant of drug release and degradation is the particle size [34], larger particle size leads to smaller surface:volume ratio thus leading to decreasing release rates. In addition during the fabrication process, smaller particles harden faster thus impacting the drug distribution within the lipid matrix. Noting the strong influence of the above factors, it becomes more difficult to obtain conclusive results regarding the existing interactions between the model drug and encapsulating matrix when varying surface:volume ratio amongst the particles themselves and from one lipid sample to another introduces a large and undesirable variability. Thus it becomes more essential to explore alternative means of particle fabrication that produce monodisperse particles in advance of in vitro release experiments.
Figure 15: Release of rhodamine-B from lipid microparticles. All lipids display burst release characteristics in the initial few hours of experimental time course, (n=3, ±SEM). The observed trends between rhodamine-B release and lipid chain length are unclear.
Figure 16: Nile red release from lipid microparticles. All lipids display slow release for the duration of 24 hours ($n=3$, ±SEM). Release rates increase with increasing lipid chain length.
CHAPTER 6.

CONCLUSION AND FUTURE DIRECTIONS

A family of DHA derived symmetrical 1,3-diglycerides with various hydrocarbon chain length were successfully synthesized and used to produce solid lipid microspheres via solvent emulsification-evaporation. Zeta potential measurements showed that the spheres are negatively charged and are susceptible to Schiff base reaction or reductive amination reaction with primary and secondary amines on the surface. Size measurements showed that particle distribution have a high polydispersity index, thus making it difficult to effectively measure encapsulation efficiency as well as \textit{in vitro} controlled release profile.

In an effort to resolve the aforementioned difficulties, alternative fabrication methods will be pursued to lower the polydispersity index of size distribution, thus allowing confident measurements of encapsulation efficiency. Studies have attributed the low drug encapsulation to formation of a perfect crystal lattice with few imperfections, thus leaving little or no room to accommodate drug molecules. A possible solution to this problem is to mix different lipids or make use of more complex lipid with different chain length thus inhibiting the formation of a perfect crystal lattice.

In an attempt to determine drug distribution inside the lipid matrix, Confocal microscopy will be utilized to provide us with a visual evidence of model drug residence inside the lipid matrix. In addition, differential scanning calorimetry (DSC) of drug encapsulated lipids will clarify whether drug and the lipid matrix are phase immiscible leading to low drug encapsulation efficiency. In addition to the above experiments, a modified hot homogenization method will be utilized to assess the rate
of release of a model drug, as well as size and morphology as a function of preparation method.

In conclusion, more studies are needed to evaluate the potentials of DHA-derived solid lipid particles as excipient for controlled drug delivery, however initial experimental show great promise as functional biomaterials compared to polymeric particles and liposomes.
REFERENCES


