INTERLEUKIN-2 IN AQUEOUS AND LIPID ENVIRONMENTS: UNDERSTANDING MOLECULAR INTERACTIONS THAT IMPACT PHARMACEUTICAL FORMULATION DEVELOPMENT

by

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ABSTRACT

REGINA MARIE VRIKKIS. Interleukin-2 in aqueous and lipid environments: understanding molecular interactions that impact pharmaceutical formulation. (Under the direction of DR. GLORIA ELLIOTT)

The continued development of pharmaceutical recombinant proteins as therapeutics necessitates a full understanding of the biomolecular interactions impacting structural and functional protein stability within different environments. Two such environments are investigated here for the therapeutic protein, interleukin-2 (IL-2). Aqueous surroundings are applicable in liquid formulations for storage and parenteral injection as well as some controlled release drug delivery strategies, while the pharmacokinetic targeting possibilities of a liposome nanoparticle drug delivery system for IL-2 dictates an understanding of molecular interactions within a lipid environment.

Proteins often require excipients to preserve structural and functional stability in aqueous environments, thus we investigated a novel organic salt, choline dihydrogen phosphate (CDHP), which has been shown to impart increases in thermal stability of some model proteins. Thermal stability is of particular interest for IL-2, which irreversibly aggregates upon thermal denaturation. A significant increase in the thermal midpoint transition temperature (12.5˚C) of IL-2 was seen in the presence of high concentrations of CDHP (680mM). Control formulations in sodium dihydrogen phosphate (NaDHP), choline chloride (ChCl), and sodium chloride (NaCl) revealed that the DHP anion is the primary contributor towards IL-2 thermal stability. Initial cytotoxicity studies indicated the need for lower concentrations (<40mM), suggesting the need for dilution prior to *in vivo* administration. Bioactivity of IL-2 in the presence of

30mM CDHP revealed that even small amounts of CDHP can affect the bioactivity of IL-2, demonstrating that increases in thermal stability do not necessarily lead to increases in bioactivity retention.

The second aim of this work was to investigate the interaction between IL-2 and lipid vesicles and the clinical implications of such an interaction, including release kinetics and bioactivity of released protein. Liposomes are of particular interest for IL-2 because of their potential as a lymphatic system targeting drug delivery strategy or as a means of decreasing the *in vivo* clearance rate of IL-2 in the body. Three phosphatidylcholine based lipids of various acyl chain lengths (DMPC, DPPC, and DSPC) which have a neutral charge at physiological pH and one phosphatidylglycerol based lipid (DSPG) which has a charge of (-1) at physiological pH were chosen for liposome formulations. The effect of liposome composition on IL-2 binding thermodynamics, IL-2 lipid saturation ratios, IL-2 release kinetics, lipid bilayer melting thermodynamics, and IL-2 bioactivity were studied. A positive correlation between acyl chain length and IL-2 binding and saturation ratios was found. Bioactivity of released IL-2 was not significantly altered as a result of formulation into the various lipid compositions. Aggregation phenomena of liposomes, which were altered upon IL-2 incorporation into each liposome formulation, impacted release kinetics. All phosphatidylcholine based liposome formulations disaggregated upon IL-2 addition, while formulations containing phosphatidylglycerol aggregated upon IL-2 incorporation. An IL-2 liposome interaction model is proposed to explain binding thermodynamics and aggregation behavior, which may facilitate the development of future IL-2 liposome formulations.

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CHAPTER 1: GENERAL OVERVIEW

1.1 Pharmaceutical Proteins and the Importance of Stability

The advent of recombinant DNA protein technology has facilitated the development of protein based drugs, which now comprise a sizable portion of drugs on the market and under clinical development. Such drugs are advantageous in that they are very specific in their action. Their unique, three dimensional structural conformations allow precise interactions with specific biomolecules to elicit exact biochemical reactions within the body. However, protein based drugs are often sensitive to the thermal, chemical, and physical stresses that occur during production, isolation, and bioformulation (Chang & Yeung, 2010). Unlike small molecule based pharmaceutical formulations, proteins are complex, three dimensional structures that are subject to unfolding, or denaturing, upon exposure to stresses such as heat, pH changes, mechanical stress, and exposure to interfaces. The road from the initial isolation of a protein from a cell or tissue to application is a long one, involving exposure to many of these stresses. Understanding the effect of these stresses, as well as how to mitigate them, is vital to producing, handling, and successfully storing a structurally and functionally intact protein product.

In order to understand the effect that certain types of stresses have on proteins, one must define a measure of protein stability. Protein stability is an extremely broad term, and can be explained in terms of structure and function, with the two concepts very closely related. Protein functional stability is a simple concept in that proteins have

specific jobs. An evaluation of the ability of a protein to carry out this task or subtasks can establish the functionality of the protein. The ability of a protein to function requires certain aspects of its primary, secondary, and tertiary (and for some proteins, quaternary) structure to remain intact and unmodified, and to have a certain amount of molecular mobility. These critical structural moieties are unique to an individual protein. Structural modifications as well as changes in molecular mobility that may occur during bioprocessing and formulation can affect the ultimate functionality of the final product. The effect of two bioformulation/bioprocessing conditions on the structural and functional stability of a pharmaceutical protein will be considered in this work: preparation within an aqueous formulation and integration within a nanoparticle drug delivery system.

1.2 Interleukin-2

Interleukin-2 is a therapeutic protein used to treat metastatic melanoma, the deadliest form of skin cancer. While the immune system naturally attempts to recognize and eliminate cancer cells (Burnet, 1967; Shankaran et al., 2001), some tumor cells continue to multiply after failing to provoke an immune response. Although localized tumor treatment primarily involves surgical resection, if the cancer has metastasized and spread to distal parts of the body, the preferred treatment options are chemotherapy or an immunotherapy, such as recombinant human IL-2 (rhIL-2). For simplicity, we will refer to clinical and laboratory grade rhIL-2 as IL-2 in this work.

Interleukin-2 is a protein that is naturally produced by the body during an immune response. It is a unique cancer treatment option in that it does not interact directly with the tumor cells (as in chemotherapy), but combats cancer by *enhancing* the immune

system response. Upon successful binding of IL-2 with its receptor (IL2-R) on activated immune cells called T-helper cells, IL-2 is responsible for a signaling pathway that results in the activation, production, differentiation, and stimulation of relevant cancer fighting immune cells (Smith, 1988). IL-2 therapy has been approved by the FDA for use as an immunotherapeutic agent to treat metastatic renal cancer since 1992 and metastatic melanoma since 1998. The current approved therapeutic regimen, a systemic approach necessitated by the widespread distribution of cancer at these late disease stages, involves repeated, high dose intravenous injections of IL-2 directly into the blood stream. Such a dosing schedule is required to maintain an effective circulating therapeutic level because of the high blood clearance rate of IL-2 (Barton-Burke, 2006 ; Konrad et al., 1990; Rosenberg et al., 1985). Unfortunately severe toxicity often requires cessation of treatment before a therapeutic benefit is attained (Rosenberg et al., 1987).

Despite the difficulties associated with the traditional administration method, IL2 therapy is for many patients their last hope for a cure. At these late cancer stages, complete remission of the cancer is an extremely unlikely scenario with most treatment options. Alleviation of patient pain or the extension of the patient's life by weeks or months may be considered a worthy goal. The currently approved therapeutic regimen for IL-2 has a complete response rate of 6% and partial response rate of 10% on metastatic melanoma (Corporation, 2008). A complete response results in permanent cancer remission, while a partial response results in diminished cancer growth. This is comparable to other immunotherapeutic and chemotherapeutic agents such as Ipilimumbab (complete plus partial response rate of 10.9% (Company, 2011; Hodi et al., 2010)) and Decarbazine (complete plus partial response rate of 10-20% (C. M.

Anderson, Buzaid, & Legha, 1995; Legha, 1989) but less than 2% complete response over a period of 5 years (Ahmann et al., 1989)). Because IL-2 is one of the extremely few metastatic melanoma treatment options that results in complete cancer remission in some patients, we have chosen it as the protein drug for this study. The IL-2 used clinically (Aldesluekin®) differs from native IL-2 in the following ways: (1) It is not glycosylated, (2) it has no N-terminal alanine, (3) it has serine substituted for cysteine at amino acid position 125, and (4) its aggregation tendencies are different. We have chosen this mutein for the current study.

1.3 Degradation Pathways of IL-2

Proteins denature via different pathways, thus it is important to be aware of what these typical paths are and what types of stresses may lead to this type of denaturation in order to formulate an appropriate strategy for dealing with them. Here we will review some of the most common degradation pathways of IL-2.

Chemical degradation of IL-2 occurs via oxidation and deamidation. Oxidation will be addressed first. While IL-2 containes several oxidation sensitive residues (methionine, tryptophan, histadine, and tyrosine), it is methionine residue 104 that is most readily oxidized (Kunitani et al., 1986). In liquid formulations, addition of L-methionine can serve as a competitive antioxidant to prevent IL-2 oxidation. Oxidation of the methionine residue comes into play during long term storage or in the presence of reducing agents such as potassium persulfate, an agent often used in making hydrogels (Cadee et al., 2001). Methionine oxidized IL-2 is separable by reverse phase high performance liquid chromatography (RP-HPLC), but it still retains full biological activity (M.Hora, 1990).

Deamidation is the deletion of an amide functional group from an organic compound. This results in damage to the protein side chains of amino acids asparagine and glutamine, with asparagine-88 being most susceptible to deamidation during long periods in aqueous storage. Deamidation is more likely to occur at higher temperatures, but does not appear to occur below 5˚C, illustrating the need for refrigeration of aqueous formulations of rhIL-2. The biological activity of IL-2 deamidated at asparagine-88 retains almost the same biological activity of native IL-2 (Sasaoki, Hiroshima, Kusumoto, & Nishi, 1992). While biological activity of IL-2 after oxidation or deamidation may be retained, these forms may illicit unwanted immune responses upon administration due to alterations in sequence or aggregation tendencies (FDA, 2013)

Native IL-2 has three cysteine residues at positions 58, 105, and 125, with a disulfide bridge forming between 58 and 105, leaving it vulnerable to mismatched cysteine pairing with 125. However, clinical grade IL-2 (Geigert, Solli, Woehlake, & Vemuri, 1993) as well as the laboratory grade IL-2 used in this study does not have a third cysteine residue at 125. It is replaced with a serine residue instead such that intramolecular disulfide scrambling is not possible. The disulfide bridge between residues 58 and 105 is required for full biological activity of IL-2 (Geigert et al., 1993). Substitution or deletion of either cysteine 58 or 105, which disrupts this bridge, results in 250 times or 8-10 times less IL-2 activity, respectively. While substitution of serine for cysteine at position 125 does not result in altered activity, deletion of this residue or residues in the vicinity of residue 125 results in a protein with negligible activity. This suggests that IL-2 structure around residues 58, 105 and 125 are imperative for biological function, with the areas around residues 58 and 125 being particularly important (Liang,

Thatcher, Liang, & Allet, 1986). Cleavage of this disulfide bond is promoted in the presence of high pH and copper ions (Geigert et al., 1993), thus pH control is extremely important.

Protein aggregation can occur through several mechanisms including reversible self association, aggregation of chemically altered or denaturated protein, nucleation governed aggregation (in which the addition of native monomers to a nucleus of oligomers becomes thermodynamically favorable upon the attainment of a critically sized nucleus), and aggregation induced by surface adsorption. Aggregation due to heat and shear stress results in conformationally altered protein (Philo & Arakawa, 2009). If this aggregation is reversible, this is not necessarily a biostability issue. For example, the ability of non-glycosylated IL-2, such as the laboratory grade IL-2 used in this study, to form heterodimers is relativity well documented, but dimerization does not appear to have a deleterious effect on protein functionality (Pellequer, Ollivon, & Barratt, 2004; Trinchieri & Clark, 1984; Welte, 1982). However, if aggregation of unfolded or partially unfolded protein is irreversible, protein bioactivity is permanantly lost. While many proteins are able to refold upon cooling after being thermally denatured, thermal denaturation of interleukin-2 results in irreversible aggregation of conformationally modified protein (K. D. Weaver et al., 2012). Thus, thermally denatured IL-2 results in permanent loss of biological activity.

Excipients of IL-2 are often chosen on the basis of their ability to prevent protein denaturation during lyophilization due to cold stress, dehydration stress, and interfacial exposure. The presence of stabilizers such as amino acids, a non-ionic surfactant (Tween 80), human serum albumin (HSA) are often included during lyophilization in order to

protect IL-2 from denaturation, but have a tendency to also induce aggregation and oxidation. Arginine and carnitine (a derivative of the amino acids lysine and methionine) have been included in lyophilized IL-2 formulations as amphiphilic amino acids that may presumably stabilize IL-2 by interacting with like regions on the similarly amphiphilic protein. However, such formulations are prone to aggregation upon mechanical perturbations after reconstitution within liquid formulations. Non-ionic surfactants stabilize IL-2 during lyophilization and during storage and prevent protein adsorption to glass and plastic surfaces by inhibiting hydrophobic interactions, but do lead to substantial oxidation of IL-2 (M.Hora, 1990). Human or bovine serum albumin is often added to IL-2 formulations in lyophilized, liquid, and liposomal formulations (P. M. Anderson et al., 1990; Bergers, Den Otter, Dullens, Kerkvliet, & Crommelin, 1993; Neville, Boni, Pflug, Popescu, & Robb, 2000) to prevent protein adsorption to glass or plastic surfaces (M.Hora, 1990), but may also form complexes during suboptimal storage conditions to affect immunogenicity of the final product (Braun & Alsenz, 1997).

The pH at which IL-2 is most stable at varies depending on which mutein is being examined. In general, proteins are most stable at their isoelectric point (pI) (Mosavi & Peng, 2003). The particular form of IL-2 we are using in this study, des-alanyl-1, serine-125 human interleukin-2, has a pI between 6.5 and 7.5 as determined by isoelectric focusing (IEF) by the manufacturer. Indeed IL-2 has been shown to be more prone to irreversible aggregation and protein loss at pH $\leq$ 6.5 (Gounili, 1999).

Clinical grade IL-2 (Aldesleukin©) is supplied as a lyophilized cake containing $18x10^6$ International Units (IU) (1.1mg protein) IL-2m 50mg mannitol, 0.18mg sodium docedyl sulfate (SDS), 0.17mg monobasic and 0.89mg dibasic sodium phosphate. The

mannitol serves as a bulking agent to provide a supportive matrix for the protein during lyophilization. SDS serves as a solubilizing agent after reconstitution within a liquid formulation (Corporation, 2008; Geigert et al., 1993; T. Arakawa, 1994). Monobasic and dibasic sodium phosphate serves to adjust the pH of the liquid formulation upon reconstitution with 1.2ml water to a pH of 7.2-7.8 (Corporation, 2008; Geigert et al., 1993).

1.4 Project Objectives

Given these IL-2 stability issues, innovations of therapeutic formulations that maintain protein functionality are clearly needed. Such formulations are not only needed for the storage and transport of IL-2, but also to support new directions in the administration of IL-2. Such a task necessitates a full understanding of the biomolecular interactions impacting structural and functional protein stability within different environments. Two such environments are investigated in this work: aqueous and lipid. Aqueous surroundings are applicable in liquid formulations for storage and parenteral injection as well as some controlled release drug delivery strategies (FIGURE 1), while the pharmacokinetic targeting possibilities of lipid based nanoparticle drug delivery system for interleukin-2 dictates an understanding of molecular interactions within a lipid environment.

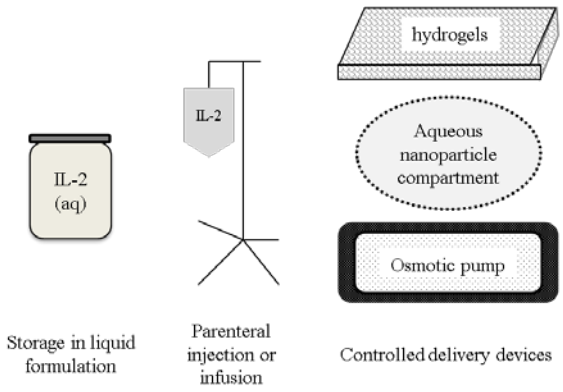


FIGURE 1: Exposure to aqueous environments within various drug storage and delivery strategies

1.4.1 Determination of IL-2 Stability in the Presence of a Novel Organic Excipient in an Aqueous Formulation

Aqueous formulations intended for injection are traditionally preferred for protein pharmaceuticals due to the bioavailability concerns of other routes of administration. Excipients are often required in order to preserve protein structural and functional stability in these aqueous environments. Because maximum shelf life is traditionally attained by lyophilizing protein pharmaceuticals, fragile protein therapeutics are often stored in a freeze dried formulation. As a result, most of the literature addressing IL-2 stability in the presence of stabilizing excipients is evaluated in the context of stabilization during lyophilization (M.Hora, 1990; Vemuri, 1992), which results in thermal, dehydration, and interfacial stresses (Carpenter, Pikal, Chang, & Randolph, 1997; Maa & Prestrelski, 2000; W. Wang, 2000). IL-2 stability in aqueous environments has been evaluated in the presence of anti-oxidants (Cadee et al., 2001), surfactants (Reyes et al., 2005; T. Arakawa, 1994), and as a function of external controls such as pH and temperature (Gounili, 1999). However, the lack of excipients for stabilization of IL-2 within an aqueous environment provided the motivation to explore new excipients in the first portion of this work.

The first aim of this work was to evaluate the impact of a novel organic salt, choline dihydrogen phosphate (CDHP), on IL-2 thermal and functional stability. CDHP has been shown to impart increases in thermal stability of some model proteins within an aqueous environment. Because irreversible denaturation and aggregation occurs after thermally induced unfolding (K. D. Weaver et al., 2012), thermal stability of IL-2 is extremely relevant. Furthermore, resistance to thermal stress in the presence of CDHP

was chosen as measure of stability because aggregation, oxidation, and deamidation (common degradation pathways for IL-2 (Cadee et al., 2001; Sasaoki et al., 1992; K. D. Weaver et al., 2012)) rates are expedited in the presence of thermal stress (Philo & Arakawa, 2009; Sasaoki et al., 1992), and thermal stress is a common factor in bioformulation and bioprocessing protocols (Gombotz & Pettit, 1995). Thermal stability is also often used as an indicator of shelf life (Weiss, Young, & Roberts, 2009). Individual ion contributions to thermal stability were probed by including control formulations of sodium dihydrogen phosphate (NaDHP), choline chloride (ChCl), and sodium chloride (NaCl) solutions. Because the biocompatibility of CDHP had not been established, cytotoxicity of CDHP was also evaluated. Bioactivity of IL-2 in the presence of CDHP was also measured by assessing the ability of IL-2 to promote survival and growth of an IL-2 dependent cell line.

1.4.2 Evaluation of IL2-Lipid Molecular Interactions that Influence the Nature of IL-2 Release Kinetics and Bioactivity, as Well as Liposome Aggregation Phenomena

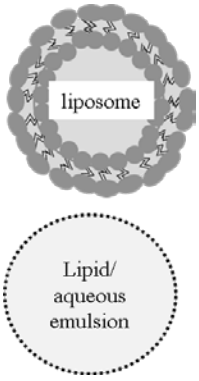


FIGURE 2: Lipid based drug delivery systems

In addition to stability within an aqueous environment, the second aim of this study evaluated IL-2 biomolecular interactions within a lipid environment. Lipid environments are relevant for drug delivery systems such as liposomes and lipid based two phase emulsions (Tamilvanan & Benita, 2004) (FIGURE 2). Liposomes are often used as drug delivery vehicles, and the targeting ability of these constructs may lead to improvements in IL-2 treatment. While the traditional systemic administration method for IL-2 is associated with superior or comparable metastatic melanoma remission rates compared to other immunotherapeutic

and chemotherapeutic agents (Ahmann et al., 1989; C. M. Anderson et al., 1995; Company, 2011; Corporation, 2008; Hodi et al., 2010; Legha, 1989), complete and partial remission rates remain at 6 and 10%, respectively. Furthermore, systemic administration of IL-2 is associated with dose limiting toxicities that often require patients to be removed from treatment for management of these adverse effects. Controlled release and targeting delivery systems, including hydrogels (Bos et al., 2004; Cadee, de Groot, Jiskoot, den Otter, & Hennink, 2002), liposomes (P. M. Anderson et al., 1990; Bergers et al., 1993; Kanaoka et al., 2001, 2003; Kanaoka et al., 2002; Kedar, Rutkowski, Braun, Emanuel, & Barenholz, 1994; Neville, Boni, et al., 2000), osmotic pumps (Nishimura, Uchiyama, Yagi, & Hashimoto, 1986), and polymer based release systems (Hsu, Lesniak, Tyler, & Brem, 2005; Rhines et al., 2003), have been developed to improve upon traditional IL-2 therapy. Therefore, the second aim of this work sought to evaluate the nature of the biomolecular interactions between IL-2 and a two phase lipid-water liposome nanoparticle system upon formulation and release, and the subsequent effect on structure and function.

IL-2 liposomes have been documented in literature (Bergers et al., 1993; Boni et al., 2001; Fleury, Ollivon, Dubois, Puisieux, & Barratt, 1995; Johnston, Reynolds, & Bystryn, 2006; Kanaoka et al., 2001, 2003; Kanaoka et al., 2002; Kedar et al., 2000; Kedar et al., 1994; F.J. Koppenhagen, 1997; F. J. Koppenhagen, Visser, Herron, Storm, & Crommelin, 1998; Kulkarni, Kasi, Tucker, & Pizzini, 1987; Neville, Boni, et al., 2000; Neville, Richau, et al., 2000; Pellequer et al., 2004), however there is little literature addressing fundamental liposomal design parameters affecting IL-2 interaction, release, and subsequent functionality. Because encapsulation of IL-2 within the interior of the

liposome requires exposing the protein to thermal and mechanical stress, we have chosen to adsorb the amphiphilic protein preformed liposomes (Kanaoka et al., 2001; F. J. Koppenhagen et al., 1998). Because protein interaction at interfaces may result in altered protein structure or function, and it was the goal of this work to probe the exact nature and effect of such an interaction as a function of liposome composition. The effect of liposome composition on IL-2 binding thermodynamics, IL-2 lipid saturation ratios, IL-2 release kinetics, lipid bilayer melting thermodynamics, and IL-2 bioactivity were assessed. Furthermore, because liposome vesicle size and aggregation tendencies can affect pharmacokinetics, the size distribution of liposomes was evaluated before and after IL-2 incorporation.

CHAPTER 2: GENERAL MATERIALS AND METHODOLOGIES

2.1 Materials

Lyophilized rhIL-2 (des-alanyl-1, serine-125 human interleukin-2) was purchased from GenScript USA Inc. (Piscataway, NJ). The IL-2 dependent CTLL-2 cell line was purchased from ATTC (Manassas, Va). RPMI 1640 culture medium and calcium/magnesium free phosphate buffer saline (PBS) were purchased from Mediatech (Manassas, VA). The IL-2 culture supplement from rat with concanavalin A (T-Stim) was purchased from BD Biosciences (San Jose, Ca). RPMI 1640 culture medium was purchased from Mediatech (Manassas, Va). Bovine serum albumin (FBS), MEM non-essential amino acid solution, 100mM sodium pyruvate solution, L-glutamine solution, HEPES, β-mercaptoethanol, penicillin, carboxy-fluorescein succinimidyl ester (CFSE), and streptomycin were purchased from Sigma Aldrich. Propidium iodide (PI) and trypan blue were purchased from Life Technologies (Rockville, MA)

2.2 Methods

2.2.1 IL-2 Aliquot Preparation and Storage

Lyophilized rhIL-2 from GenScript was reconstituted in sterile conditions with 0.1%BSA PBS at a concentration of 1mg/ml. The solution was equilibrated for 4 hours at 4˚C, divided into 10ul aliquots, and stored at -80˚C.

2.2.2 Protein Concentration Determination

2.2.2.1 Absorbance at 280nm

Protein concentration was determined by absorbance at A280nm. A standard curve was prepared from freshly thawed rhIL-2 aliquots.

2.2.2.2 Bicinchoninic Acid (BCA) Assay

The BCA Protein Assay (Thermo Fisher Scientific Inc., Rockford, IL) was used according to the manufacturer's instructions to quantify protein concentration for protein samples that were too dilute for characterization by absorbance at 280nm.

2.2.3 CTLL-2 Cell Culture

CTLL-2 cells were cultured in RPMI-1640 media supplemented with 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 1mM sodium pyruvate, 10mM HEPES, 50μM β-mercaptoethanol, penicillin (100U/ml), and streptomycin (50μg/ml), 10% FBS. The supplemented media will henceforth be referred to as complete media. Growth was maintained at 37˚C in a 5%/95% (CO_2/air) humidified chamber with the addition of 3% (v/v) T-stim. Cell density was maintained at 1-2 x 10^4 viable cells/ml and media renewed twice per week.

2.2.4 *In Vitro* rhIl-2 Activity Assay

The IL-2 bioactivity was determined using a cell based assay. CTLL-2 cells require biologically active IL-2 to survive and proliferate (Gillis & Smith, 1977), which can be quantified using a fluorescent cell viability marker, propidium iodide (PI), and proliferation marker, carboxyfluorescein succinimidyl ester (CFSE). CTLL-2 cells were recovered from culture suspensions, washed in RPMI-1640 culture medium (no FBS) twice, and re-suspended in PBS. An aliquot of 1x10^6 cells were stained by incubation

with 1µM CFSE for 8min at room temperature in a 50ml centrifuge tube covered with aluminum foil at room temperature. Staining was stopped by adding an equal volume of FBS, and incubating in the dark for 5 minutes. The cells were washed three times with complete RPMI medium. The cells were then plated at a concentration of at least $1.25x10^5$cells/well in a 96-well U-bottom culture plate with IL-2 under the appropriate experimental conditions. Control wells containing freshly prepared 0,1, and 10U IL-2 were also prepared. After 71 hours of incubation, PI was added at a final concentration of 0.5µg/ml, and incubation continued for another hour in the dark. At the end of 72 hours, the cells were recovered, washed twice, and re-suspended in PBS for flow cytometry analysis.

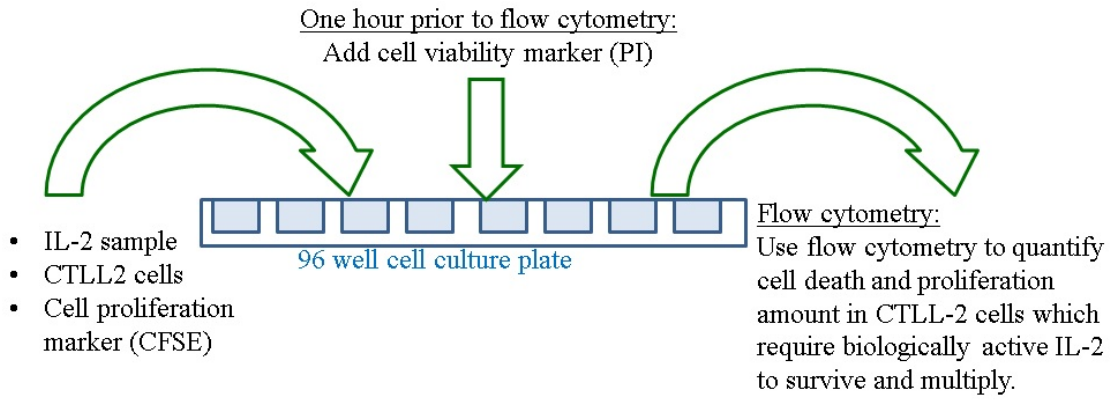


FIGURE 3: Assessment of IL-2 bioactivity by the ability of the protein to induce CTLL-2 cell proliferation and survival

CHAPTER 3: STABILITY OF INTERLEUKIN-2 IN AN AQUEOUS ENVIRONMENT: EFFECT OF TRADITIONAL AND NOVEL EXCIPIENTS

3.1 Introduction

Understanding the biomolecular interactions of pharmaceutical proteins in aqueous formulations that lead to stabilizing or destabilizing effects and the therapeutic implications of these interactions are of paramount importance to successful formulation design. Bioavailability challenges dictate that protein pharmaceuticals must typically be administered within a liquid formulation by injection. Protein drugs are quickly hydrolyzed and broken down within the gastrointestinal tract, resulting in oral bioavailability as low as <1% (Park, Kwon, & Park, 2011). Parenteral administration (injection), which has 100% bioavailability, is usually the preferred method for delivering protein therapeutics. Liquid formulation eliminates the complexity and cost of lyophilization (freeze-drying) in favor of a product that is much simpler for the end user to employ. Liquid formulations are also more convenient at the time of administration, eliminating the need for a reconstitution step of a lyophilized product. Furthermore the continued development of controlled release drug delivery systems requires some protein therapeutics to remain functional within an aqueous reservoir for an extended period prior to release *in vivo* (Fu, Klibanov, & Langer, 2000). Unfortunately, proteins in liquid formulation tend to degrade faster than in lyophilized formulations due to perturbations by surrounding water molecules. These water molecules tend to foster degradation of proteins by allowing more molecular flexibility and or via hydrolysis of the amino

linkages (Wei Wang, 1999) (Avis & Wu, 1996). If the tendency towards protein degradation in liquid formulation can be diminished, prolonged storage in the aqueous state can be achieved and the cost of protein therapeutics can be substantially reduced. Since the rate and extent of chemical and physical degradation pathways are impacted by the solvent environment, understanding how proteins denature and how to prevent it is critical to maintaining a stable and functional product.

Proteins are inherently unstable outside of their native environment; therefore stabilizing additives (excipients) are often added to improve shelf life. The range of stabilizing agents routinely employed as pharmaceutical excipients has, to date, been limited to a relatively small number of sugars, amino acids, polymers, salts, and detergents (Maclean, Qian, & Middaugh, 2002). Recently, various low melting point organic salts have been reported to stabilize proteins and DNA, decrease protein aggregation, and protect against thermal denaturation, raising the possibility of their use as additional excipients in pharmaceutical protein formulations (Byrne, Wang, Belieres, & Angell, 2007; K. Fujita, M. Forsyth, D. R. MacFarlane, R. W. Reid, & G. D. Elliott, 2006; Vijayaraghavan, Izgorodin, Ganesh, Surianarayanan, & MacFarlane; R. M. Vrikkis, Fraser, Fujita, Macfarlane, & Elliott, 2009). It has been reported that a combination of kosmotropic (water structure-making) anions and chaotropic (water structure-breaking) cations can stabilize proteins in aqueous solution, and this criteria can be employed to design organic salts that stabilize therapeutic proteins in liquid formulation (Constatinescu, Herrmann, & Weingartner; K. Fujita et al., 2007; Ru, Dordick, Reimer, & Clark, 1999). One such organic salt, choline dihydrogen phosphate (CDHP), has been reported to extend the shelf life of several model proteins (including

cytochrome C, lysozyme, and ribonuclease A) when formulated with 20% (*v/w*) water, supporting the retention of structure and activity of these proteins (K. Fujita et al., 2006; K. Fujita et al., 2007; R. M. Vrikkis et al., 2009). Stabilization effects have also been observed in model proteins when using lower concentrations (<40 wt %) of this salt (Constatinescu et al.; K. D. Weaver et al., 2011). A 3 year shelf life of cytochrome c at room temperature has been seen by Fujita et al in 80% w/w CDHP (Kyoko Fujita et al., 2007). Full functional activity of lysozyme has been maintained after room temperature storage of lysozyme in 80% w/w CDHP (R. Vrikkis, MacFarlane, & Elliott, 2009). A 20˚C increase in the thermal transition temperature of ribonuclease A in the presence of 4M CDHP (pH 7.0) has been reported, as well as an increase the reversibility of the reversibility of protein thermal denaturation from 5 to 80% in 0.1M CDHP (Constantinescu, Herrmann, & Weingärtner, 2010).

Protein structure is sensitive to many external, environmental conditions including temperature, pH, and concentration of denaturing or stabilizing solvents. Interleukin-2 is prone to several denaturation pathways including chemical, physical/mechanical, and thermal denaturation. Chemical denaturation pathways relevant for IL-2 include oxidation and deamidation. However, these pathways can be prevented with the use of antioxidants and organic solvents, respectively. Furthermore, the oxidized and deamidated forms of IL-2 have been shown to retain similar bioactivity and functionality as their native forms (Cadee et al., 2001; K. Sasaoki, 1989; Sasaoki et al., 1992; Wakankar & Borchardt, 2006). Physical denaturation pathways due to mechanical stresses such as agitation or exposure to an interface can occur via aggregation. If aggregation is reversible, and the protein refolds back into its native conformation, this is

not a stability issue. Aggregation of native IL-2, for example, can be prevented in the presence of sodium dodecyl sulfate (Arakawa, Philo, & Kenney, 1994). However, irreversible aggregation leads to irreversible losses in bioactivity. Thermal denaturation of many proteins is reversible upon cooling. However, IL-2 undergoes irreversible thermal denaturation and aggregation (Advant, Braswell, Kumar, & Kalonia, 1995; K. D. Weaver et al., 2012).

Therefore, thermal stability is an important parameter to consider for biologically active therapeutic IL-2 formulation, and is relevant for bio-preservation, protein storage, and bioformulation into some drug delivery systems (Maa & Prestrelski, 2000; Pellequer et al., 2004). Understanding this denaturation pathway and protective measures to prevent thermal denaturation may aid in a more rational process and formulation design. Furthermore, because certain degradation pathways (oxidation, deamidation, aggregation) may occur more quickly at higher temperatures (Cadee et al., 2001; Sasaoki et al., 1992; K. D. Weaver et al., 2012), exposure to thermal stress allows one to study protein resistance to such pathways in an accelerated manner.

Protein structure is stable up to a temperature termed the onset temperature (T_{onset}) at which the first protein unfolding event is detectable. The temperature at which native and unfolded protein populations exist at a 1:1 ratio occurs at a critical temperature designated as the thermal midpoint transition temperature (T_m). The T_m for a given protein can depend on environmental conditions (pH, ionic strength, concentration of denaturants/stabilizers). As thermal energy continues to be added to the system such that the activation energy required for denaturation is transferred to the protein and the system temperature exceeds past the T_m, it becomes thermodynamically favorable for the protein

to unfold or denature. The disruption of native protein structure(s) is accompanied by heat absorption, measurable calorimetrically as an enthalpy heat change (ΔH). It may or may not be reversible (or partially so), with aggregation of denatured protein typically accompanying irreversible unfolding. Thermal denaturation can be monitored by calorimetry, circular dichroism (CD), nuclear magnetic resonance (NMR), X-ray crystallography, and Fourier transform infrared spectroscopy (FTIR).

CDHP has been shown to extend the shelf life of model proteins when used at high concentrations as a solvent, and increase thermal stability of model proteins when used at lower concentrations. Further studies are needed to understand the mechanism of stabilization in different concentration ranges, to identify possible differences between the use of CDHP with relatively stable model proteins and less robust therapeutic proteins such as IL-2, and to extend biocompatibility studies to better anticipate possible complications of *in vivo* injection. The aim of the current study was to investigate effectiveness of CDHP as an excipient for use in IL-2 aqueous formulation. This is the first time a therapeutic protein such as IL-2 has been formulated with this novel solvent. We also determined the maximum concentrations that elicit no adverse cytotoxic effects *in vitro* following chronic exposure. In doing so we sought to define the effect of low concentrations of CDHP on IL-2 structure and function, as well as the biological activity of CDHP, using complementary *in vitro* models of splenocyte and melanoma cell cultures. Furthermore, we attempted to elucidate possible molecular interaction mechanisms responsible for any stabilizing or destabilizing effects on IL-2 thermal stability and bioactivity.

3.2 Materials and Methods

3.2.1 Materials

Lyophilized rhIL-2 was obtained, prepared, and stored as previously described in Chapter 2. CDHP was prepared in the MacFarlane laboratory, as previously reported (Fujita, MacFarlane, & Forsyth, 2005; Kyoko Fujita, Maria Forsyth, Douglas R. MacFarlane, Robert W. Reid, & Gloria D. Elliott, 2006; Kyoko Fujita et al., 2007). The B16-F10 mouse melanoma cell line was purchased from the National Cancer Institute Tumor Repository (Frederick, MD). Dulbecco's modified Eagle medium mixture F12 (DMEM), resazurin, sodium bicarbonate ($NaHCO_3$), choline chloride, and red blood cell lysis buffer were purchased from Sigma-Aldrich (St. Louis, MO). Sodium dihydrogen phosphate and sodium chloride were purchased from VWR International. 0.25% trypsin/EDTA was purchased from Life Technologies (Rockville, MA). A bicinchoninic acid (BCA) protein assay kit was purchased from Thermo-Fisher Scientific (Waltham,MA).

3.2.2 Solution properties

Solution osmolarities were measured using a vapor pressure osmometer (Wescor Vapro 5520, Logan, UT, USA).

3.2.3 IL-2 Resistance to Thermal Denaturation in 0-680mM CDHP

Resistance to thermal denaturation of IL-2 in aqueous solution was evaluated by heating IL-2 from a control temperature of 4˚C to a temperature between 45˚C-85˚. Samples were then cooled back to 4˚C to evaluate irreversible thermal denaturation. Aliquots of IL-2 (125 pg/mL) were diluted in PBS containing 0.1% (w/v) BSA and supplemented with CDHP pH 7.4 (0–680 mM). Control solutions of IL-2 in 680mM

NaH_2PO_4 (NaDHP), 680mM ChCl, and 680mM NaCl were prepared in a similar manner. All samples were heated to 45–85˚C in a dry bath incubator (Thermo Fisher Scientific). The temperature was monitored using a K-type thermocouple with a HH314A humidity temperature meter (Omega, Stamford, CT). Once the samples reached the prescribed temperature, they were immediately cooled to 4˚C prior to analysis by a sandwich ELISA.

3.2.4 Effect of Prolonged Exposure of CDHP to IL-2

To examine the effect of prolonged exposure to CDHP on IL-2, 125 pg/mL IL-2 in 0.1%(v/v) BSA PBS was supplemented with 0–60 mM CDHP with and without an appropriate amount of $NaHCO_3$ to maintain the pH 7.2. Samples were incubated in a water bath (IsoTemp 210, ThermoFisher Scientific) at 37˚C, removed at 24 or 48 hours, and stored at 4˚C prior to analysis by a sandwich ELISA.

3.2.5 rhIL-2 Binding Ability Retention by Sandwich ELISA

Structural integrity of IL-2 was inferred by evaluating its binding ability using a luminescent sandwich rhIL-2 enzyme-linked immunosorbent assay (ELISA) (Thermo-Fisher Scientific, Waltham, MA). This assay requires two structurally relevant conformational epitopes to remain intact in order for binding to occur (Byrne et al., 2007), as shown in FIGURE 4. While an ability of IL-2 to bind and lead to a luminescence signal does not necessarily indicate structural and functional integrity,

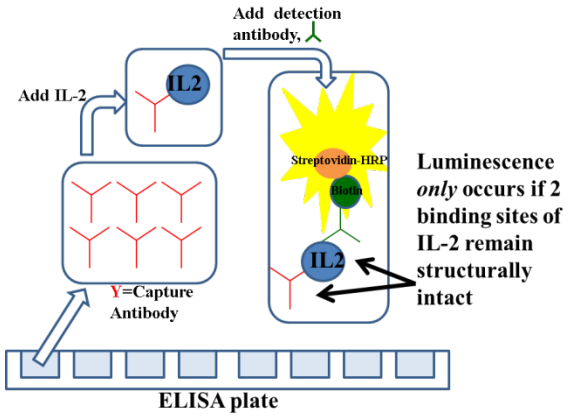


FIGURE 4: A sandwich ELISA requires the presence of two structurally intact binding sites on IL-2.

an inability to bind does indicate structural alteration to the protein. This method is a quick, efficient method to screen excipients. All samples were assayed in triplicate for each temperature point.

3.2.6 Cytotoxicity Assays

3.2.6.1 Rezasurin Reduction Assay

Cytotoxicity of CDHP was investigated as described in (O'Brien, Wilson, Orton, & Pognan, 2000) using a cell line (B16-F10 melanoma cells) and primary cells (splenocytes). Cells were plated (1×10^5 cells/well for splenocytes, 1×10^4 cells/well for B16-F10 cells) and cultured in 96-well plates in complete culture medium in the presence of 0-80mM CDHP. Because of the ability of CDHP to lower the pH of the media, the effect of buffering to pH≥7.2 with 0.15-0.33% (w/v) $NaHCO_3$ was also investigated. To maintain splenocyte growth, cultures were further supplemented with 500U/ml IL-2. After a prescribed length of time (4 hours for splenocytes, 18 hours for B16-F10 cells), rezasurin was added to a final concentration of 43.7μM. At 24 hours rezasurin reduction was measured colorimetrically (530/590 nm) (FIGURE 5).

3.2.6.2 Trypan Blue Exclusion Assay

A trypan blue exclusion assay was used to assess cytotoxicity effects CDHP. Cells were plated (5×10^5 cells/well for splenocytes, 2×10^5 cells/well for B16-F10 cells) and cultured as described in the resazurin reduction assay. At 24 hours cells were removed from the wells, stained with 0.04% (w/v) trypan blue, and total cell counts and viability were assessed using a Countess® automatic cell counter (Life Technologies, Rockville, MA) (FIGURE 5).

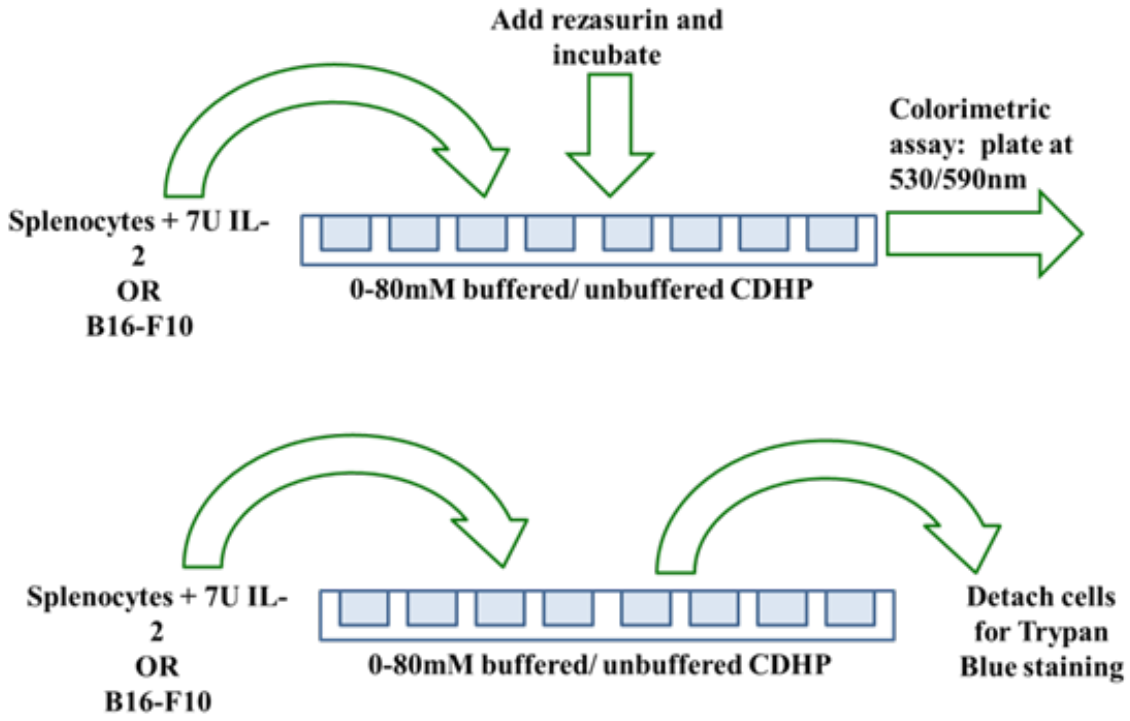


FIGURE 5: Cytotoxicity assays. Top: Rezasurin reduction assay. Bottom: Trypan blue assay.

3.2.7 CTLL-2 Activity Assay

IL-2 biological activity was measured by flow cytometry using a CTLL-2 reporter cell line. CTLL-2 cells were plated and cultured as previously described in the presence of 0,1, or 10 international units (IU) IL-2 with 0, 30, or 60mM CDHP buffered in $NaHCO_3$ (pH≥7.2). Cell viability and proliferation was quantified as defined in the previous chapter.

3.2.8 Statistical Analysis

All results are expressed as the means ± standard error of the mean (SEM) unless otherwise indicated. A one or two way ANOVA was used when appropriate to compare results from samples ±CDHP. Prism 5 software (Graphpad Software Inc., La Jolla, Ca.)

was used to perform statistical analysis. A $p<0.05$ was considered to be statistically significant.

3.3 Results

3.3.1 Effect of Aqueous Formulation Composition During Heating on Binding Ability Retention

Previous work in our lab evaluated the effect of CDHP on protein structure and thermal stability. The far-UV spectropolarimetry at 222nm was measured as IL-2 was thermally denatured and the unfolding temperature (T_m) was assessed, and it was found that the presence of CDHP increased the T_m of IL-2 (K. D. Weaver et al., 2012). This observation established a temperature range and CDHP concentration range to enable further investigation of IL-2 stability in clinically relevant formulations. The T_m values of IL-2 with and without CDHP present in solution are reported again here: (1) 30mM CDHP pH 7.4: Tm=60.2°C, (2) 185mM CDHP pH 7.4: Tm=63.0°C, (3) 680mM CDHP pH 7.4: Tm = 74.2°C, and (4) 30mM sodium dihydrogen phosphate (NaDHP): Tm =61.7°C pH 7.4. The experimental error in determining the T_m was 1˚C, so there was no statistical difference found between the T_m of IL-2 prepared in the control, 30mM NaDHP, and 30 or 185mM CDHP. However a significant increase in T_m was found when IL-2 was formulated in a 680mM CDHP solution. Interestingly, a comparison of IL-2 secondary structure in 30mM NaDHP and 30mM CDHP revealed an increase in ordered secondary structure content (α-helix and β-sheet content), suggesting an overall tightening of tertiary structure in the presence of CDHP as opposed to NaDHP which became more exaggerated at higher (up to 680mM) concentrations of CDHP (K. D. Weaver et al., 2012).

In the current work, the structure and function following heating IL-2 to temperatures below and above the T_m was evaluated in the presence of CDHP. A sandwich ELISA assay measures the ability of the protein to bind at two structurally relevant epitopes, providing an insight into conformational changes that may impact binding ability retention. It is important to note that the ELISA method does not provide a complete profile of structural integrity, and an ability to bind at these two epitopes does not necessarily indicate complete structural integrity. However an inability to bind does indicate significant structural alteration to the protein. Protein samples were heated from 45˚C to 85˚C, cooled immediately upon reaching the desired temperature, and evaluated using an ELISA. Several formulations were tested. We previously established 680mM as an appropriate CDHP concentration to impart a significant increase in the thermal stability of IL-2. In order to probe individual ion (choline and dihydrogen phosphate ions, specifically) contributions to protein thermal stability, a 680mM NaDHP and 680mM choline chloride formulation were also investigated as salt based excipients. Sodium chloride (NaCl), was included to enable comparison to a common inorganic salt. Each formulation was buffered to a pH of 7.4, and osmolality was also assessed. Solution osmolality measurements did not differ by more than 5% (CDHP = 1520 mmol/kg,NaDHP = 1530 mmol/kg,ChCl = 1501 mmol/kg, and NaCl = 1520 mol/kg).

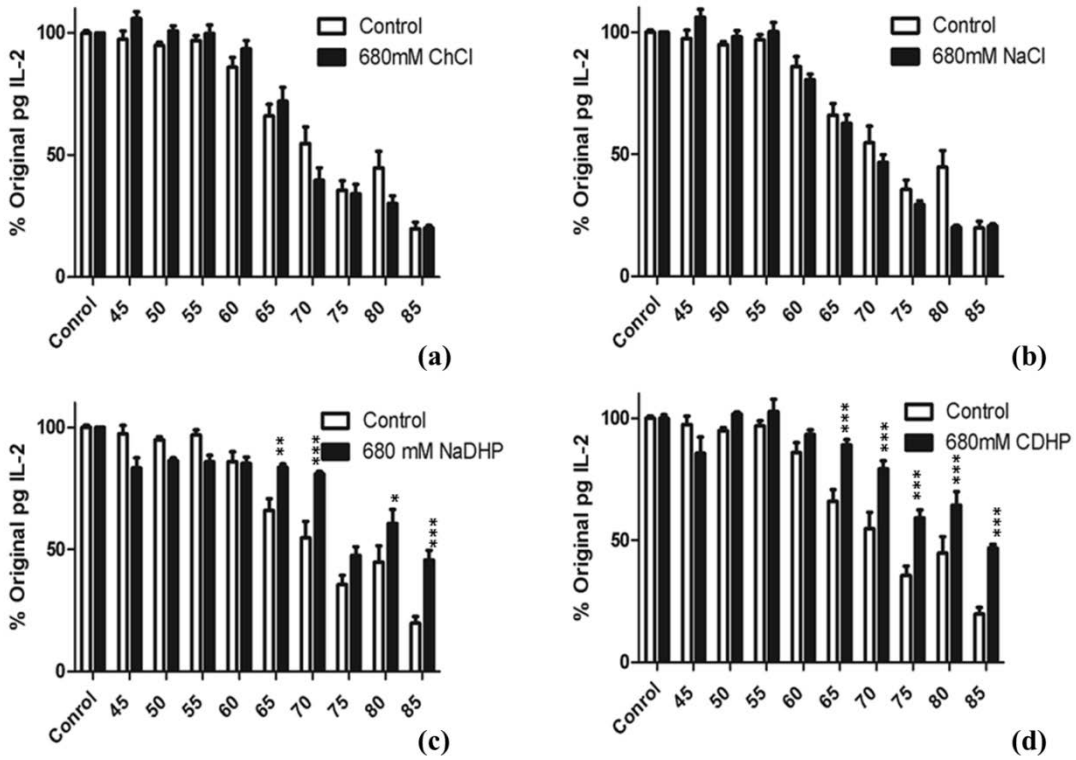


FIGURE 6: rhIL-2 formulated in 680mM pH7.4 CDHP and 680mM NaDHP remains structurally intact when heated. Sandwich ELISA measurements of rhIL-2 concentrations formulated in (a) 680mM ChCl pH7.4, (b) 680mM NaCl pH7.4, (c) 680mM NaDHP pH7.4, and (d) 680mM CDHP pH 7.4 versus no CDHP control (white bars), and heated at temperatures ranging from 45°C to 85°C. Data are expressed as mean relative rhIL-2 concentrations ±SEM, normalized to the initial amount (T0) of protein. n=9; *: $p<0.05$; **: $p<0.01$. Comparisons are made to the control formulation at each given temperature.

Using the ELISA method, IL-2 binding ability retention was not significantly impacted below the T_m (45˚C-60˚C) in any formulation (FIGURE 6a-d). Similar results were seen for IL-2 formulated in a control formulation of 0.1%BSA. At temperatures exceeding 60˚C, a significant decrease in structurally intact IL-2 concentration was seen in the 680mM ChCl, 680mM NaCl, and control formulations (FIGURE 6a-b), with only 72 ± 5.6, 62.5 ± 3.6%, 66 ± 4.7% structurally intact protein left at 65˚C, respectively. This effect was further exaggerated at increasing temperatures where nearly 20% further loss was seen with every 5˚C increment. Significant decrease in binding ability retention occurred at 65˚C in control IL-2 solutions formulated in 0.1% BSA PBS (66±4.7%).

At temperatures ≥65˚C, IL-2 formulated with 680mM NaDHP and 680mM CDHP retained a significantly larger portion of intact protein as compared to the control solutions (83.6±1.3% and 89.1±2.1%), losing only approximately 10% with every 5˚C increment (FIGURE 6c-d). Overall, IL-2 formulated in 680mM NaDHP and CDHP retained a larger amount of intact protein up to 23.3˚C above the T_m as compared to the control (19.7 ± 2.8% IL-2 incubated alone vs. 45.7 ± 3.9% in NaDHP and 46.8 ± 1.6% in CDHP formulation at 85˚C).

3.3.2 Effect of Aqueous Formulation Composition During Prolonged Exposure to CDHP on Binding Ability Retention

To determine the effect of prolonged exposure of CDHP on IL-2 structural integrity, IL-2 was incubated with 0, 30, and 60mM CDHP (± $NaHCO_3$ to buffer to pH≥7.2) at 37˚C in sterile conditions for 24 and 48 hours. A significant decrease in binding ability after 24 hours was seen in samples incubated with CDHP, with no further decrease seen when incubation length was extended to 48hours (FIGURE 7a). This effect was

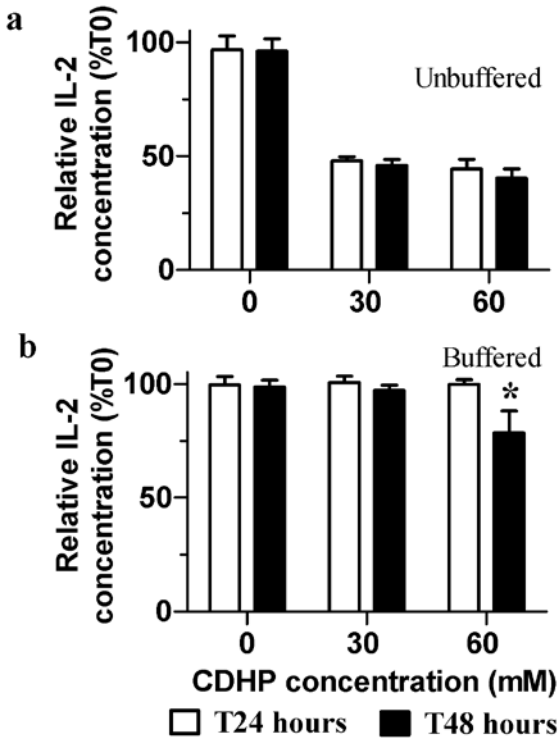


FIGURE 7: rhIL-2 remains structurally intact after a lasting exposure to near-isosmotic buffered CDHP. Sandwich ELISA measurements of rhIL-2 concentrations incubated 24 h (white bars) and 48 h (black bars) in 30–60 mM CDHP (upper panel), or CDHP in NaHCO3 (pH ≥ 7.2) (lower panel). Data are expressed as mean relative rhIL-2 concentrations ± SEM, normalized to the initial amount (T0) of protein. n = 6; *: $p<0.05$.

nullified when pH was maintained ≥7.2, with a slight decrease in intact protein occurring between 24 and 48 hours incubation in the presence of buffered 60mM CDHP (FIGURE 7b).

3.3.3 Cytotoxicity of CDHP Formulations

Depending on the site and mode of in vivo delivery of a CDHP containing formulation, chronic cellular exposure levels to CDHP could vary considerably. While it was not the goal of this work to evaluate the biocompatibility of CDHP it is of interest to

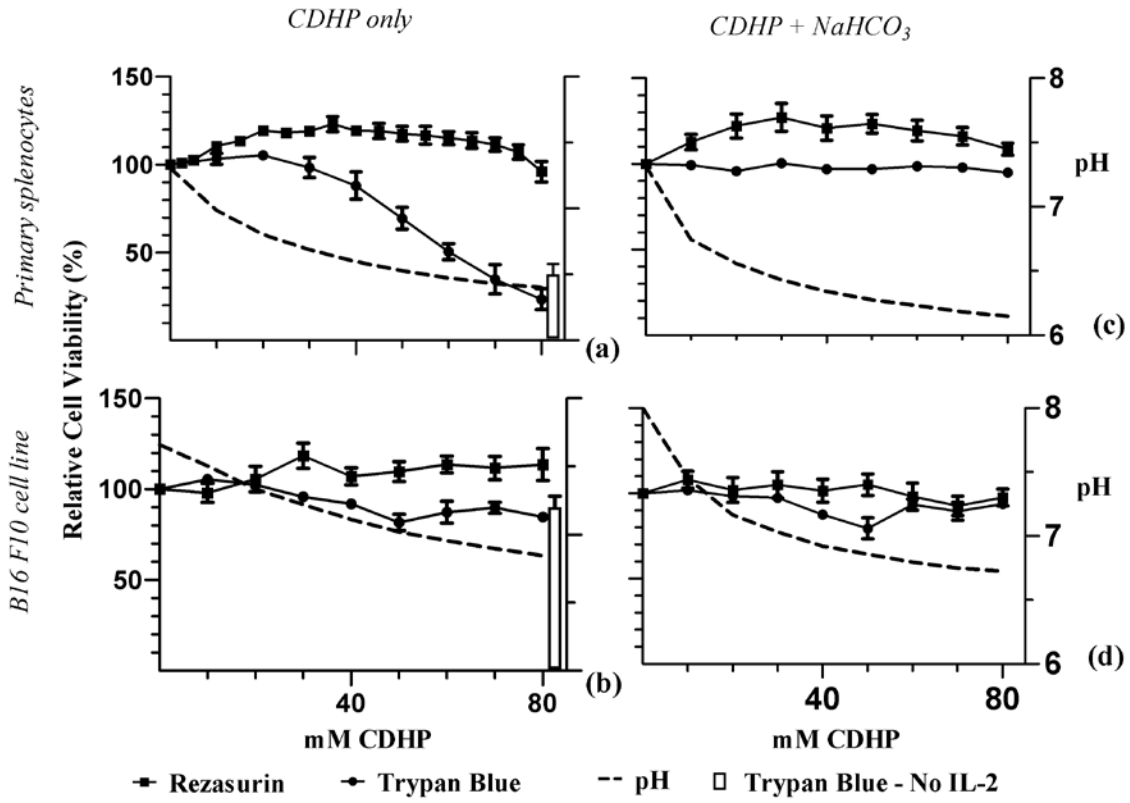


FIGURE 8: Near-isosmotic CDHP is not cytotoxic when buffered in vitro. Primary splenocyte culture (a) and B16F10 cell line (b) viability, after 24 hours of chronic exposure to 0-80mM CDHP (left panel), or CDHP in NaHCO3 (pH≥7.2) (right panel), as measured by trypan blue exclusion (circle) and resazurin reduction (square) assays. The white bars indicate splenocyte viability culture in IL-2-free media containing 80mM CDHP. Data are expressed as mean relative cell viability, normalized to no CDHP control samples ±SEM, and compared to culture media pH (dashed line). n=6 for splenocytes and n=9 for B16F10 cells.

determine whether or not CDHP must be eliminated as a possible pharmaceutical excipient on the basis of cytotoxicity. Such a biocompatibility issue is extremely relevant to the development of a therapeutic IL-2 formulation. Here we investigated the effect of CDHP on primary splenocyte cultures exposed to 0–80mM CDHP concentrations in complete RPMI culture medium.

At fairly dilute concentrations of CDHP (10mM), complete RPMI culture media pH decreased from 7.3 to 7.0, ultimately decreasing to pH 6.7 above 30mM CDHP. These conditions were found to be cytotoxic to splenocytes according to the trypan blue assay (FIGURE 8). Splenocyte culture viability decreased significantly at 40mM CDHP (88±7.8%, $p<0.05$). At 80mM CDHP, viability decreased further to 37.7±8.2. Because splenocyte viability decreased to 50% after 24 hours of incubation in the absence of a 100IU IL-2/well supplement (data not shown), IL-2 was present in all culture wells. To determine if the ultimate cytotoxic effects seen by unbuffered CDHP was primarily due to deleterious effects on IL-2 or on the splenocytes themselves, a control well was added containing 80mM CDHP (no $NaHCO_3$) and no IL-2. This lead to no statistical difference in cell viability (37.7±8.2% with IL-2 versus 23.4±5.9%) suggesting cytotoxic effects seen were not primarily due to denaturation of IL-2.

To discern whether cytotoxicity was due to pH changes or to the CDHP concentration itself, the solution was buffered to pH ≥7.2 by adding an appropriate amount of $NaHCO_3$ to 0-80mM CDHP solutions. This lead to approximately 100% viability of splenocytes retained upon incubation with buffered 80mM CDHP for 24 hours. From this data, we can conclude that any cytotoxic effects induced by CDHP on splenocytes can be negated by buffering to pH ≥7.2.

Interestingly the rezasurin reduction assay did not mirror the results obtained by the trypan blue exclusion assay. Viability appears to increase upon the addition of CDHP (no $NaHCO_3$). However, this apparent viability increase can actually be interpreted in terms of an increase in metabolic cell stress, because the resazurin reduction assay is a cell metabolism based assay (Rampersad, 2012). At concentrations 10-40mM CDHP, there was an apparent hormetic effect in which an increase in relative resazurin reduction was observed before a return to baseline rezasurin reduction was observed at concentrations of 50-80mM CDHP. Such an effect was negated when samples were buffered with $NaHCO_3$ (FIGURE 8).

3.3.4 Effect of Formulation Composition During Heating on Ability to Support IL-2 Dependent Cell Line

Structural integrity is often a prerequisite for IL-2 biological activity (Shu-Mei Liang, 1986), but is not the only requirement. Biological activity of IL-2 was assessed using the CTLL-2 cell line, a murine cytotoxic T cell line that requires IL-2 to survive and proliferate (Gillis & Smith, 1977). A CDHP concentration of 30mM was chosen based on the result of the initial cytotoxicity studies that indicated a cytotoxic effect >30mM CDHP (FIGURE 8a). This concentration also avoided any osmotic stress effects upon CTLL-2 cells, which could obscure data representing interaction site between IL-2 and IL-2R displaying CTLL-2 cells. Furthermore, dilution *in vivo* upon administration will likely result in a lower CDHP concentration present at the interaction of IL-2 and IL-2R positive cells. In the absence of IL-2, there were no significant differences in cell death between control samples (no CDHP) or samples incubated in 30 mM CDHP with NaHCO3 ($pH \geq 7.2$) (FIGURE 9a, 18.4 ± 2.5 and 15.5 ± 1.5% cell death, respectively).

Furthermore, in samples formulated in the absence of IL-2, no significant differences were observed in cell proliferation between samples incubated with and without CDHP (FIGURE 9b).

As expected, increasing the amount of rhIL-2 in cell culture medium significantly reduced CTLL-2 cell death while increasing the proportion of cells undergoing proliferation in a dose dependent manner FIGURE 9a-b. Cell viability was not significantly decreased in samples containing 1 and 10IU IL-2 in the presence of 30mM CDHP (FIGURE 9a). However, in samples containing 1 and 10IU IL-2 and 30mM CDHP, the percentage of proliferating cells was diminished (from 43±5.7% to 28±5.1% in samples containing 10IU IL2, $p<0.01$). Collectively these results suggest that even at concentrations as low as 30mM, CDHP does have an adverse effect on IL-2 bioactivity as evidenced by a reduction in the ability of CTLL-2 cells to proliferate.

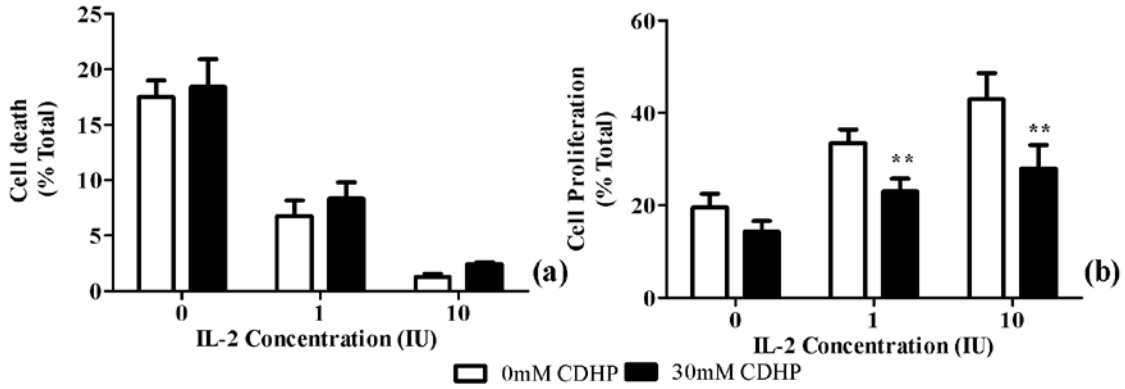


FIGURE 9: rhIL-2 activity decreases when formulated in buffered 30mM CDHP. Flow cytometry analysis of CTLL-2 cell viability (a) and proliferation (b), respectively measured by propidium iodide and carboxyfluorescein succinimidyl ester stainings, after 24 hours stimulation with 1-10IU rhIL-2 in presence (*black bars*) or absence (*white bars*) of 30mM CDHP in $NaHCO_3$ (pH≥7.2). Data are expressed as mean percentile of dead or proliferating cells ±SEM, among total cells in each sample. n=9 for both assays.

3.4 Discussion

In this work, the stability of IL-2 in an aqueous environment in the presence of a novel excipient, CDHP, was investigated. Resistance to thermal stress was chosen to measure the stabilizing/destabilizing effects upon IL-2. CDHP was chosen for study as a possible excipient for IL-2 based upon its ability to increase the T_m of model proteins such as lysozyme and cytochrome c in previous work (Kyoko Fujita et al., 2006; Kyoko Fujita et al., 2007; R. Vrikkis et al., 2009). The T_m, which is the temperature at which the folded and unfolded proteins exist in a 1:1 ratio, is often used as a measure of thermal stability. A higher T_m indicates a more thermally stable protein (Cooper, Johnson, Lakey, & Nollmann, 2001). However the concentrations studied in these investigations were extremely high (≥80% w/w). While this range may be appropriate for storage applications, such a high concentration is not easily injectable into the body without dilution. Therefore we chose to investigate the use of CDHP to thermally stabilize IL-2 at concentrations which might be appropriate for injection or continuous release devices.

We have previously published evidence that the presence of 680mM CDHP (pH 7.4) resulted in an increase in the T_m of IL-2 by 12.5˚C (K. D. Weaver et al., 2012). While an increase in thermal stability was seen, the thermal denaturation pathway for IL-2 remained irreversible in both 185 and 680mM CDHP formulations as evidenced by precipitation of aggregated protein upon cooling. Irreversible aggregation upon IL-2 unfolding has been observed elsewhere in the presence of 5% w/v glucose and 10mM sodium acetate, pH 4.0 (Arakawa, Philo, & Kita, 2001). Because IL-2, unlike lysozyme and cytochrome c (K. D. Weaver et al., 2012), does not refold upon cooling, an increase in the T_m is extremely relevant.

It is known that protein stability is greatly influenced not only by its interaction with water, but by how any other co-solvent molecules present interact with the water molecules around the protein (Zhao et al., 2006). Salts containing a mixture of chaotropic cations (ions that preferentially interact with the protein itself) and kosmotropic anions (ions which preferentially interact with surrounding water molecules) have been shown to optimally stabilize proteins (Kyoko Fujita et al., 2007; Zhao et al., 2006). In this work, a similar increase in thermal stability was seen for IL-2 in both 680mM CDHP and 680 NaDHP formulations (pH ≥7.2), indicating that the kosmotropic anion, dihydrogen phosphate, is the primary contributor to thermal stability. Protection against protein thermal denaturation in the presence high concentrations of kosmotropic anion containing salts via non-specific interactions has been observed elsewhere in literature (Komsa-Penkova, Koynova, Kostov, & Tenchov, 1996; Tadeo, Pons, & Millet, 2007; Vonhippel & Wong, 1964; Yamasaki, Yano, & Aoki, 1991). Because kosmotropic anions preferentially interact with water molecules surrounding the protein, they are able to stabilize proteins via a salting-out effect (Arakawa, Bhat, & Timasheff, 1990; Zhao et al., 2006) essentially shielding the protein from potential denaturing interactions with the surrounding water molecules.

The stabilizing ability of specific ions has been ranked on the well-known Hoffmeister series. While dihydrogen phosphate ranks as a stabilizing kosmotropic ion, the chloride ion is ranked nearly in the middle. This suggests that chloride has a relatively impartial role in protein stability and solubility (Zhang & Cremer, 2006), hence the salts containing the strongly kosmotropic dihydrogen phosphate anion are more likely to

prevent thermal denaturation than those containing a neutrally poised anion, such as sodium or choline (Zhang & Cremer, 2006).

Surface tension effects may also play a role in the observed increase in thermal stability of IL-2 by CDHP. Organic salts have been observed to increase the surface tension of solvents in direct proportion to their concentration in solution. Thermal stability, as measured by an increase in T_m, has been shown to directly correlate with the surface tension of organic salt solutions for a range of proteins, with variations between proteins attributed to differences in their physico-chemical properties (Kaushik & Bhat, 1999). The increased solvent surface tension preferentially stabilizes the folded protein conformation over the denatured one, which has a much higher surface area than the folded state (Tadeo, Lopez-Mendez, Castano, Trigueros, & Millet, 2009). We have observed a concentration dependent effect on IL-2 thermal stability (K. D. Weaver et al., 2012), suggesting that the increased surface tension as CDHP concentration increases may also be playing a role in the stabilizing effect of CDHP on IL-2.

It should be noted that because the phosphate containing salts require the addition of some sodium hydroxide to increase the pH, the ionic strength of these solutions are likely marginally higher than the chloride containing solutions, but the similarity in the osmolality of these solutions would suggest that this difference would be modest. Ionic strength can certainly play a part in protein stability (Yamasaki et al., 1991), but in the absence of detailed studies on the speciation of ions in solution it is difficult to uncouple ionic strength as a stabilization effect in the current study. It is likely that the charge-shielding effects of high ionic strength formulations (Miki, Kakuyama, & Soda, 1997), together with stabilizing increases in surface tension are both contributing to some degree

to increases in thermal stability. However, the dramatic difference in the thermal stability observed between solutions containing different anions would suggest that the anion kosmotropicity is the dominant stabilizing effect.

Unlike previous studies using model proteins (Kyoko Fujita et al., 2007; R. Vrikkis et al., 2009), data from these studies provide evidence of protein activity changes at CDHP concentrations as low as 30mM, which led to decreased IL-2-induced CTLL-2 cell proliferation in vitro. As opposed to lysozyme, IL-2 does not exert its biological activity by catalyzing enzymatic reactions. Instead IL-2 binds to a high-affinity receptor consisting of three subunits: IL-2Rα (p55), IL-2Rβ (p75) and γc(p64). These receptors are expressed by specific immune cell subsets, which mediate IL-2 activity (Minami, Kono, Miyazaki, & Taniguchi, 1993). To survive and proliferate, CTLL-2 cells, which display the IL-2R complex, require IL-2 to interact with the IL-2 receptor. Therefore, CTLL-2 cells are commonly used to assess IL-2 activity/stability (Gillis & Smith, 1977). The reduced ability of IL-2 formulated in 30mM CDHP to trigger CTLL-2 cell proliferation without affecting survival may be due to modifications in the tertiary structure of the protein. Interestingly, ELISA data demonstrated that rhIL-2 formulated in buffered CDHP in NaHCO3 (pH ≥7.2) shows no signs of structural integrity disruption. To be detected by a sandwich ELISA, two conformational motifs have to be recognized by two separated monoclonal antibodies, thus providing indirect evidence of tertiary protein structure integrity (at least in the regions involved in this binding interaction). In addition, previous CD scan analysis performed on rhIL-2 formulated in CDHP (680 mM, pH 7.4) demonstrated no evidence of disruption of IL-2 tertiary structure. It did, however,

suggest some tightening of secondary structure as evidenced by an increase in α-helix and β-sheet content (K. D. Weaver et al., 2012).

The loss of rhIL-2 functionality, when formulated with CDHP, could be explained in the context of an increase in protein thermal stability. While the parameters that define thermal stability (improved intramolecular hydrogen bonding, hydrophobic packing, Van der Waals interactions) may interact to create a more conformationally robust protein, they can also restrict conformational mobility to limit protein functionality (Vogt & Woell, 1997). To explore this further in terms of thermodynamic equilibrium, we can look at a protein folding and unfolding pathway as shown by EQUATION 1.

$$N^{\downarrow mobility} \leftrightarrow N \leftrightarrow I \leftrightarrow U \rightarrow U^{irrev.}$$

$$I \leftrightarrow A \rightarrow A^{irrev.}$$

EQUATION 1: Protein folding-unfolding pathways

The native protein (N) is the correctly folded protein with full biological activity. A typical unfolding pathway proceeds to the right in which the protein may exist in an intermediate (I) or partially unfolded conformations, which can fully unfold (U) or aggregate (A). These unfolding and aggregation pathways may be reversible or irreversible ($U^{irrev.}$, $A^{irrev.}$). An increase in the thermal stability may push the thermodynamic equilibrium so far to the left that the native protein transitions to a more compact, less mobile conformation ($N^{\downarrow mobility}$). Such a conformation may result in altered biological activity due to decreased conformational mobility (Kendrick et al., 1997).

IL-2 binds to the IL-2R complex through a sequence of events. The cytokine first interacts with IL-2Rα through a large hydrophobic binding surface surrounded by a polar border, resulting in a relatively weak interaction (Kd 10^8 M) (Malek & Castro, 2010). The

IL-2Rα-IL-2 binary complex leads to a necessary conformational change in IL-2 to encourage association with IL-2Rβ via polar interactions, forming a ternary structure with intermediate stability (Kd 10^9 M). Finally, the complex IL-2Rα-IL-2Rβ-IL-2 complex then interacts with the final IL-2R subunit, γc, through a weak interaction with IL-2 and a stronger interaction with IL-2Rβ to produce a stable quaternary IL-2-IL-2R complex (Kd 10^{11} M) (Gillis & Smith, 1977). This orchestrated series of events obviously requires a significant amount of IL-2 conformational mobility. It is possible that the interaction between IL-2, surrounding water molecules, and CDHP decreases the kinetic freedom of the protein, thus impacting the ability of IL-2 to interact with IL-2R displayed on the CTLL-2 cells, and decreasing the ultimate proliferation of these IL-2 dependent cells. The increase in IL-2 secondary structure content in the presence of CDHP that has been observed elsewhere (K. D. Weaver et al., 2012) supports such a hypothesis. If CDHP shifts the equilibrium towards the more compact N^* state (EQUATION 1), full biological activity, which requires the fully flexible native (N) conformation, will be compromised. Furthermore, while our study focused on structural integrity of IL-2, we cannot rule out that CDHP also interferes in the assembly of the high affinity, three-unit IL-2R, impacting IL-2 ability to interact with CTLL-2 cells chronically exposed to CDHP.

These data are of particular interest given that IL-2 is a relatively unstable protein compared to lysozyme (an antibacterial protein secreted in saliva and mucus) or cytochrome C (heme protein component of the mitochondrial electron transport chain), which are robust proteins reported to retain functionality when exposed to acidic conditions and reactive oxygen species (Olteanu et al., 2003; Proctor & Cunningham, 1988). IL-2, on the other hand, is a cytokine that is physiologically secreted into the

buffered and thermally stable conditions of the circulation. These fundamental differences in the physiological roles of these three proteins may explain the higher functional sensitivity of IL-2 compared to lysozyme and cytochrome c despite similarities in molecular weight [14.7 kDa (cytochrome C), 12 kDa (lysozyme), and 15.5 kDa (IL-2)]. Maintaining the pH at near physiologic levels was important for maintaining the structural integrity of IL-2. IL-2 activity losses have been observed elsewhere in acidic conditions due to the disruption of disulfide bond integrity (Shu-Mei Liang, 1986). However when IL-2-dependent cells were treated with pH controlled CDHP based formulations, activity levels were still reduced.

To distinguish the interaction between CDHP and cells as opposed to IL-2 and to further probe the biocompatibility of CDHP, cytotoxicity of CDHP on two cell systems was evaluated in this work. Our lab has previously conducted CDHP cytotoxicity assays upon J774 mouse macrophages to rank choline salts in order of toxicity. These studies established an EC50 value (effective concentration that causes cell death in 50% of the population after 48 hours of exposure) of 20 mM for CDHP (Katherine D. Weaver, Kim, Sun, MacFarlane, & Elliott, 2010). Here we also examined relative toxicity to other cell types (primary splenocytes and B16-F10 cells) to understand these toxic effects in greater detail. By maintaining a near physiological pH, cells tolerated chronic exposure (24–48 h) to CDHP at levels ≤80 mM level, a concentration resulting in a moderately hypertonic solution. While these results are preliminary assessments of biocompatibility and cannot be directly extrapolated to predict *in vivo* response, the results suggest that CDHP appears to be as benign as other dissociating physiological salts under typical culture conditions. Extending toxicity testing to higher levels of chronic exposure would have resulted in

excessive osmotic stress to cells, and would not have revealed any further direct toxicity information about CDHP. These data suggest that if appropriately defined solution osmolarities can be achieved, there is no immediate cellular toxicity. Ideally an intravenously administered medication should have an osmolality near physiological levels (300 mOsm/L) and no more than 600 mOsm/L to prevent inflammation (Hicks & Becker, 2006), although there are certainly exceptions to this. Peripheral parenteral nutrition is often administered at levels up to 750mOsm/L, while central parenteral nutrition is administered up to 1400mOsm/L. The rate of infusion must also be considered. For example in an *in vivo* experiment by Kuwahara et al. (Kuwahara, Asanami, & Kubo, 1998), venous epithelial cells tolerated osmolarities of 820 mOsm/kg for 8 h, 690 mOsm/kg for 12 h, and 550 mOsm/kg for 24 h of a nutritional solution. Similarly, an intramuscularly administered volume of 0.5 mL of 300–1100 mOsm/kg solution has been reported to be well tolerated (Nony, Girard, Chabaud , Hessel, & Thébault, 2001). Assuming complete dissociation of the salts used in this study, the osmolarities of the buffered 680mM solutions would be expected to be approximately 3000mOsm/kg (too high to be administered parenterally without dilution or CDHP removal prior to administration). This is calculated based upon the measured osmolality of a 680mM CDHP (buffered to pH 7.4 with $NaHCO_3$) of 1520mmol/kg, given in section 3.3.1. By extension, a 30 and 80mM CDHP (pH $\geq$7.2) solution would be approximately 130 and 350mOsm/kg. Thus the concentrations that studied in this work reveal the potential for achieving a beneficial protein stabilization effect at concentrations that are hypothetically injectable, or which could be employed in controlled releases devices. Depending on the osmolarity/ osmolality limitations of the chosen administration route,

possible *in vivo* administrations schemes for IL-2 formulated with CDHP are illustrated in FIGURE 10a-d. At CDHP concentration requiring no dilution/ removal prior to administration, formulations could be given according to FIGURE 10a and c. If the CDHP concentration results in an osmolality that is deemed not safe for the chosen administration route, the IL-2 CDHP formulation can be diluted prior to injection according to FIGURE 10c. If such a preparation is formulated within a controlled release device, the device may have to be engineered so as to release IL-2 and CDHP at different release rates so that cell and tissue exposure to CDHP maintained within appropriate osmolality guidelines (FIGURE 10d).

Utilization of different cells enabled a more comprehensive understanding of the specific effects of CDHP on biological systems. Robust cell lines such as the B16-F10 melanoma or J774 macrophages (R. Vrikkis et al., 2009) are transformed, immortalized cells capable of surviving relatively adverse conditions such as acidic pHs, limited nutrient availability, and pro-apoptotic signals. It is therefore not surprising that we found a lack of cytotoxicity for CDHP (0–80mM) on the B16-F10 cell line despite decreases in culture medium pH (pH<7.0). To further explore CDHP biocompatibility, we also performed cytotoxicity assays using primary cell cultures of freshly isolated splenocytes. This model presents a more physiologically relevant system (compared to immortalized tumor cells). Splenocytes are a mixed population of motile, spleen leucocytes that propagate in culture without attaching to solid surfaces. Using this splenocyte model system, significant cytotoxic activity was detected at levels of CDHP as low as 40mM when pH was not tightly regulated. However this effect could be countered by increasing the buffer capacity of the media with $NaHCO_3$ supplementation, thus maintaining pH

near physiologic levels. Interestingly, cytotoxicity was only detectable in cultured splenocytes by performing direct cell counts (trypan blue exclusion assays), as data from metabolism-based assays (resazurin reduction) did not mirror the cell count data. Indeed, the metabolic resazurin reduction assays actually demonstrated what, at first analysis, appeared to be increased relative cell viability in the presence of CDHP (10–40mM). These measurements highlight a fundamental feature of metabolism-based methods of measuring cell viability (e.g., resazurin, MTT); early cell death events can be masked by increased cellular metabolism. This feature can lead to an overestimation of cell survival, but it also provides the opportunity to quantify cell stress via changes in metabolic activity following chronic exposure to non-cytotoxic (as assessed by trypan blue exclusion) levels of CDHP (Hashimoto et al., 2004; Takeda et al., 2002). Collectively these data demonstrate that at non-cytotoxic CDHP levels (0–40mM), CDHP significantly increased cell stress, but this can be effectively countered by increasing the buffer capacity with $NaHCO_3$ to maintain a physiologic pH in the solutions.

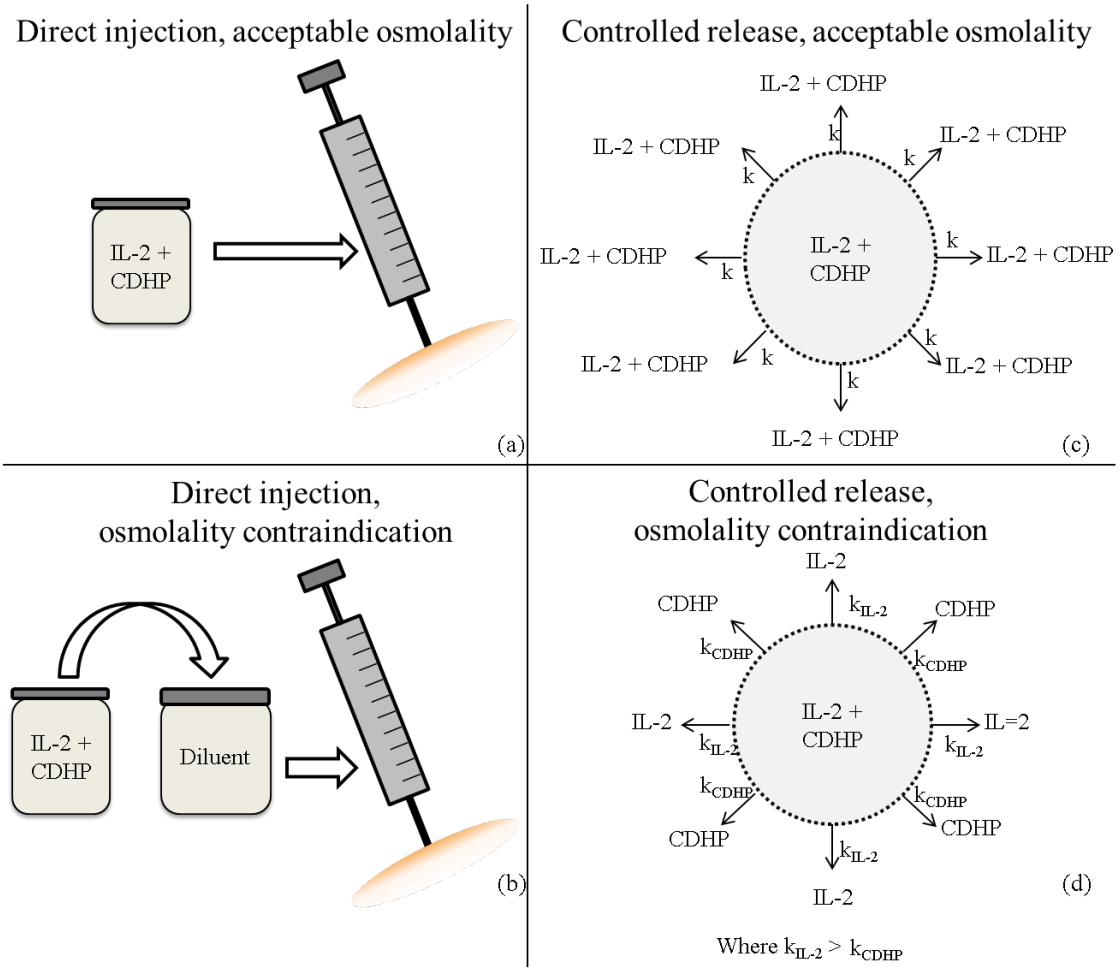


FIGURE 10: Possible in vivo administration strategies for IL-2 CDHP formulations

3.5 Conclusions

We had previously reported that CDHP increases the thermal stability of the therapeutic protein IL-2, and in this study we further investigated the applicability of CDHP as a stabilizing excipient for aqueous IL-2 formulations. The use of CDHP (pH≤7.2) allowed a better retention of IL-2 structural integrity at high temperature with no apparent cytotoxicity toward splenocytes and B16-F10 melanoma cells up to 80mM. Formulating IL-2 with CDHP (pH≤7.2) reduced its biological activity *in vitro* but the protein retained the ability to bind at two structurally relevant sites as measured by an

ELISA. This suggests that a trade-off might be necessary between thermal stability and biological activity if residual CDHP is expected to be present when IL-2 binds to its receptor. This trade-off may be acceptable when formulations are expected to experience thermal excursions, such as might occur in the creation of new IL-2 delivery devices such as controlled release liposomes and nanoparticles. In the current study it was found that the kosmotropic anion, dihydrogen phosphate, provided the dominant stabilizing effect while the anion choice had a relatively neutral effect. This suggests further opportunity to optimize the cation choice in order to design solution properties that are most desirable for IL-2 formulation.

CHAPTER 4: STABILITY OF INTERLEUKIN-2 IN A LIPID NANOPARTICLE ENVIRONMENT: EFFECT OF LIPID COMPOSITION

4.1 Introduction

Many therapeutic proteins, including interleukins, interferons, hormones, and enzymes are rapidly cleared or degraded within the body. As a small protein (<50,000kDa), IL-2 is cleared from the body via kidney filtration and extraction (Gibbons, Luo, Hannon, Braeckman, & Young, 1995) or via a receptor mediated mechanism (Piecilelli et al., 1996). The high blood clearance rate of IL-2 (Barton-Burke, 2006 ; Konrad et al., 1990; Rosenberg et al., 1985) necessitates a high and frequent dosing schedule to maintain an effective circulating therapeutic level. This dosing size and frequency can induce severe toxicity which often requires cessation of treatment (Rosenberg et al., 1987). To overcome the fast clearance rates and toxicity associated with systemically administered IL-2, nanoparticle carriers have been examined as a way to target tumor tissue and/or increase circulation time and thus improve IL-2 therapeutic outcome. Liposomal nanoparticles are flexible carriers in that they have the ability to incorporate both hydrophilic (within the aqueous interior) and hydrophobic molecules (within the hydrophobic bilayer), are easily manufactured, are biodegradable, and can be formulated with a variety of non-toxic lipids.

One strategy to improve IL-2 therapy is to increase the circulation time of IL-2 using liposomes. IL-2 liposome removal from the general circulation by macrophages can be minimized or avoided by liposomal surface modification with hydrophilic

polymers. Coating with hydrophilic polymers such as PEG has been shown to decrease binding of serum proteins to liposomes. Binding of these serum proteins, called opsonins, is the first event in the removal of liposomes from circulation by macrophages (Immordino, Dosio, & Cattel, 2006). Such 'stealth' liposomes have been shown to increase the half-life of the circulation time for IL-2 administered intravenously by 10-30 times compared to free IL-2 (Kedar et al., 2000).

Another strategy to improve IL-2 therapy using liposomes is to target these liposome carriers to specific cells, tissues, or systems where they ultimately release their payload. Targeting of drugs not only maximizes the efficiency of the drug dose, but decreases the adverse effects associated with drug interaction with non-targeted cells and tissues. It is the 'holy grail' of many cancer studies today seeking to target local tumor cells while avoiding healthy tissue. Indeed, localized administration of IL-2 within the tumor vicinity has been shown in several studies to be more effective and less toxic than systemic administration. Peritumeral IL-2 injections were shown to inhibit the growth of experimentally induced fibrosarcoma tumors in mice by Burbenik et al, while Jacobs et al observed a higher survival rate in patients receiving localized administration of free IL-2 to treat nasopharyngeal carcinoma (Bubeník, 1990; Jacobs, Sparendam, & Den Otter, 2005).

In addition to targeting tumor tissue, lymphatic targeting using liposomes may improve IL-2 immunotherapy. Such a strategy has not been studied for IL-2. The adverse effects of this immunotherapeutic are associated with IL-2 interaction with certain entities within the blood stream (namely natural killer cells and neutrophils), while the beneficial effects are due to the interaction of the protein with the IL-2 receptor (IL-2R)

expressing activated CD4+ T-cells, which reside largely in the lymphatic system (Caligiuri, 1993; Stites, 1994; van Haelst Pisani et al., 1991). Furthermore, because the lymphatic route is one of the primary routes for tumor metastasis, lymphatic targeting of IL-2 is an avenue of enormous potential.

The targeting ability and pharmacokinetics of liposomes are largely a factor of liposomal carrier size, administration route, and surface characteristics (charge, modification with hydrophilic polymers) (Gabizon, 2001; Hawley, Davis, & Illum, 1995; Christien Oussoren & Storm, 2001). One route in particular that has been evaluated for

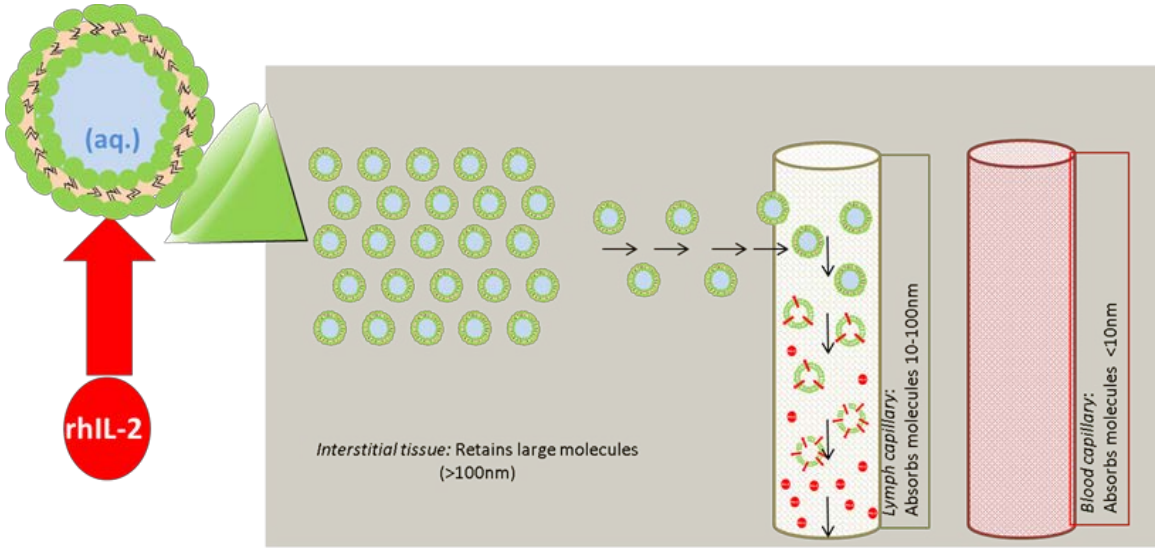


FIGURE 11: Lymphatic targeting of IL-2 using optimally sized liposomes

lymphatic targeting by liposome carriers is the subcutaneous route (C. Oussoren & Storm, 1999; Christien Oussoren & Storm, 2001; C. Oussoren et al., 1998; C. Oussoren, Zuidema, Crommelin, & Storm, 1997; Zuidema, Kadir, Titulaer, & Oussoren, 1994). Based on molecular weight, one could expect a little less than 50% of the administered dose of IL-2 (MW=15.4kDa) to accumulate within the lymph. The rest of the dose could be expected to accumulate within the blood plasma (Andreas Supersaxo, Hein, & Steffen,

1990). Indeed, after subcutaneous administration of IL-2 to pigs, about half the injected dose was recovered in the lymph, while the remainder was recovered in the plasma (Chen et al., 2000). Particles between 10-100nm are optimally absorbed into the lymphatic capillaries from the subcutaneous or interstitial space, with lymphatic uptake reaching approximately 70% of the injected dose (Christien Oussoren & Storm, 2001). Smaller particles are preferentially absorbed by the blood capillaries (Andreas Supersaxo et al., 1990; Swartz, 2001). Larger particles remain within the tissue, which can be used as to create a depot for a continuously released drug (Chung, Yang, & Tsai, 2006; Meyenburg, Lilie, Panzner, & Rudolph, 2000; S. S. Wang, Yang, & Chung, 2008). Therefore, liposomal carriers offer the possibility of increasing the amount of IL-2 absorption into the lymphatic system (FIGURE 11).

IL-2 liposomes have been documented in literature (Bergers et al., 1993; Boni et al., 2001; Fleury et al., 1995; Johnston et al., 2006; Kanaoka et al., 2001, 2003; Kanaoka et al., 2002; Kedar et al., 2000; Kedar et al., 1994; F.J. Koppenhagen, 1997; F. J. Koppenhagen et al., 1998; Kulkarni et al., 1987; Neville, Boni, et al., 2000; Neville, Richau, et al., 2000; Pellequer et al., 2004). Most studies have attempted to increase IL-2 encapsulation using potentially denaturing methodologies (Bergers et al., 1993; Fleury et al., 1995; Johnston et al., 2006; Kulkarni et al., 1987; Neville, Boni, et al., 2000). Encapsulation of drugs within the interior of the liposome typically requires breaking and reforming the vesicles through sonication, multiple freeze/thaw cycle, or extrusion of the lipid-drug mixture, exposing the drug molecule to mechanical stress and elevated temperatures (at least 10˚C above the phase transition temperature of the lipid). These strategies are less of a concern for small molecule drugs with no secondary structure, but

protein drugs are sensitive to thermal and mechanical denaturation. The ability to mitigate or avoid these stresses is of paramount importance for fragile and often expensive protein drugs, such as IL-2. Adsorption of IL-2 to preformed liposomes allows one to bypassing thermal stress and mechanical stress. Because IL-2 undergoes irreversible thermal denaturation (Foureau et al., 2012; K. D. Weaver et al., 2012), avoidance of thermal stress during the formulation of an IL-2 liposome carrier is desirable. (Foureau et al., 2012; Pellequer et al., 2004; K. D. Weaver et al., 2012).

Several authors have noted IL-2 association with the lipid bilayer, even when traditional encapsulation methods are employed (Bergers et al., 1993; F. J. Koppenhagen et al., 1998; Neville, Boni, et al., 2000). Neville et al observed bulges within DPPC liposome lamellae in IL-2 containing liposomes indicating intercalation of the protein using electron microscopy. IL-2 was also observed on the surface as indicated by the ability of IL-2 to bind to an anti-IL-2 antibody (Neville, Boni, et al., 2000). Bergers et al was able to modulate IL-2 incorporation efficiency into PC:PG (9:1) liposomes by modulating pH and ionic strength in order to maximize electrostatic interaction between the liposome surface and IL-2. They concluded that since this result could not be explained by differences within the aqueous interior space, IL-2 must associate with the bilayer, although the extent of hydrophobic as compared to electrostatic interaction was not investigated (Bergers et al., 1993). Koppenhagen et al also found evidence for IL-2 lipid bilayer association. When IL-2 was added to preformed DPPC liposomes, the steady state fluorescence of IL-2 increased, indicating changes in the microenvironment of IL-2's tryptophan residue (F. J. Koppenhagen et al., 1998). Only one study has probed the effect of modulating lipid acyl chain length on IL-2 interaction with lipid bilayers.

Kanaoka et al adsorbed IL-2 to preformed DMPC (12 carbons in the acyl chain), DPPC (14 carbons), DSPC (16 carbons) liposomes, and found the highest incorporation efficiency for DSPC. All these liposomes are neutrally charged, and the effect of including a negatively charged lipid was investigated by incorporating IL-2 into DSPC:DSPG (10:1 molar ratio) liposomes. The incorporation efficiency for DSPC:DSPG (10:1) liposomes was similar to that of DSPC liposomes. These incorporation efficiencies were evaluated by comparing the amount of free IL-2 versus bound IL-2 after separation of these two populations via gel filtration chromoatography. (Kanaoka et al., 2001). While the similar IL-2 incorporation ratios seen for DSPC and DSPC:DSPG liposomes suggested a hydrophobic interaction rather than electrostatic association, the exact nature of this interaction was explored.

Although IL-2 association with lipid bilayers has been observed and assumptions on the nature of this interaction (whether the interaction is governed by hydrophobic versus electrostatic) have been made on the basis of incorporation efficiencies upon modulation of pH, ionic strength (Bergers et al., 1993), and liposome lipid composition (Kanaoka et al., 2001), the thermodynamics of this interaction have not been specifically characterized. It is apparent that lipid choice affects IL-2 interaction with the liposome carrier (Kanaoka et al., 2001). However, no study has been performed comparing IL-2 release kinetics from its liposomal carrier and bioactivity the released protein as a function of liposome composition. Thus the relationship between liposome composition, binding and release thermodynamics or kinetics, and the subsequent functionality cannot be established given current literature. Because design of controlled release drug delivery

constructs requires the optimization of release kinetic behavior as well as the release of a functional product, these relationships are of clinical concern.

Another parameter that has been largely ignored in the formulation of IL-2 adsorbed to liposomes is the potential for change in the size distribution upon IL-2 incorporation. Because of its proclivity to associate with lipid bilayers, a size increase would be expected. Size, which affects diffusion and absorption of liposomes between cells, tissues, and systems *in vivo*, is therapeutically relevant for targeting purposes (C. Oussoren et al., 1997).

To avoid the thermal and mechanical stresses accompanying traditional encapsulation methods, this study will adsorb IL-2 to preformed liposomes. The simplicity of such a preparation may translate to cost effective scale up production. Adsorption of IL-2 to preformed liposomes is possible due to the amphiphilicity of IL-2, which contains regions of hydrophobicity and hydrophilicity. The majority of the protein contains regions of hydrophobicity (Christoph Alexander Bergmann, 1991), which may have a proclivity to associate with the hydrophobic lipid bilayer. In addition to hydrophobic interaction between IL-2 and the lipid bilayer, electrostatic interactions may be involved in adsorption of IL-2 to the bilayer. Electrostatic interaction may serve as the primary interaction between the protein and liposome, or may also be a precursor to further adsorption into the lipid acyl chain region for proteins with more hydrophobic regions (Norde, 1996).

We hypothesize that predominantly hydrophobic proteins will likely associate with the hydrophobic acyl chains of a lipid bilayer. Therefore the choice of lipids with longer acyl chains will exploit this interaction. Here we investigate three saturated

phosphatidylcholine based lipids with acyl chain lengths of 14, 16, and 18 carbons. These lipids are 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), respectively. These lipids carry an overall neutral charge at pH 7.4. Phosphatidylcholine is a major part of biological cell membranes, is non-toxic, and is one of the most common lipid types used in pharmaceutical liposome formulations.

The size of IL-2 makes it likely that the protein will not fit completely within this acyl chain region (Cornell & Separovic, 1983; Lewis & Engelman, 1983; Rickert, Wang, Boulanger, Goriatcheva, & Garcia, 2005), and at least some portion of the protein will be in close proximity to the phospholipid head groups of the lipid. Hence it is foreseeable that IL-2 association with lipid bilayers can be modulated by increasing the acyl chain length and head group interaction. Several studies have employed this strategy, and successfully adsorbed IL-2 onto preformed liposomes containing PC and PG head groups, which results in a lipid bilayer with a negative charge (-1) at physiological pH (Kanaoka et al., 2001, 2003; Kanaoka et al., 2002; F. J. Koppenhagen et al., 1998). The predominant mechanism of interaction was not directly probed in these studies, and the advantage of including a charged lipid head group, phosphatidylglycerol (PG), in the formulation rather than using only neutrally charge lipids was not readily apparent. Presumably the choice was made, not on the basis of IL-2 liposome interaction, but interaction between liposomes themselves. Negatively charged lipids such as PG are traditionally included in formulations to discourage liposome aggregation, and thus increase colloidal stability, through electrostatic repulsion. While cationic liposomes resist aggregation as well, they are typically used to neutralize and carry negatively

charged DNA (Escobar-Chávez, 2010), and are limited in their use due to instability, rapid clearance, and toxicity (Immordino et al., 2006). In this work, we have included 1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DSPG) to assess the effect of a negative charge on IL-2 lipid interaction as well as liposome aggregation phenomena.

In this work we provide a fundamental understanding of IL-2 lipid bilayer interaction by systematically evaluating the effect of lipid acyl chain length and lipid bilayer charge on the strength and nature of IL-2 liposome interaction, the release kinetics of IL-2 from the vesicle, and the implications on protein stability. Because protein stability is of utmost concern to maintain a therapeutically effective drug, the thermal and mechanical stresses associated with encapsulation methods have been avoided, and adsorption to preformed liposomes employed. Understanding the nature of IL-2 liposome interaction and the pharmaceutical implications (such as the release rate of IL-2 from the carrier and its subsequent bioactivity) will improve the rational design of IL-2 liposome drug delivery constructs.

4.2 Materials and Methods

4.2.1 Materials

4.2.2 Liposome Preparation

Liposomes composed of DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), and DSPC (1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol)) were prepared from lipid powder or chloroform suspensions. Chloroform was removed from lipid suspensions by evaporation under argon gas. The resultant lipid powder was dissolved in water, heated to at least 10˚C above the phase transition temperature of the

lipid, and allowed to solubilize for at least an hour. The suspensions were vortexed with 2-3 glass beads of 1mm diameter for several seconds to create a heterogeneous suspension of multilamelar liposomes (MLVs). The heated liposome suspension was passed through a polycarbonate filter with 100nm sized pores at least 21 times using a MiniExtruder from Avanti Polar Lipids, Inc (Alabaster, AL). The resultant 100nm unilamellar liposome suspension was stored at 4˚C overnight before use. All liposome concentrations are given as lipid concentrations.

4.2.3 Determination of Binding Constants, Stoichiometric Ratios, and Enthalpic and Entropic Contributions to Binding

The binding constants of IL-2 interaction with the liposomal bilayer were monitored with NanoITC and analyzed with NanoAnalyze software (TA Instruments, Lindon, UT). In addition to the binding constants, the data from these experiments were also used to determine the optimal stoichiometric ratios of IL-2 to lipid as a function of lipid composition within liposomes. These protein:lipid ratios were later used to optimize the concentration of IL-2 to adsorb IL-2 to preformed liposomes before evaluation of release kinetics, bioactivity, and liposome size and melting transition thermodynamics.

The Nano ITC used in this study employs a differential power compensation, which measures the change in power needed (μJ/s) required to maintain the system at a constant temperature. In all experiments, an IL-2 solution was contained within calorimeter cell and the lipid vesicles were injected at discrete intervals via a titration syringe. Shown in FIGURE 12a and b are (a) typical raw data and (b) integrated titration curves fitted to a multiple independent binding site model during an isothermal titration

calorimetry experiment.

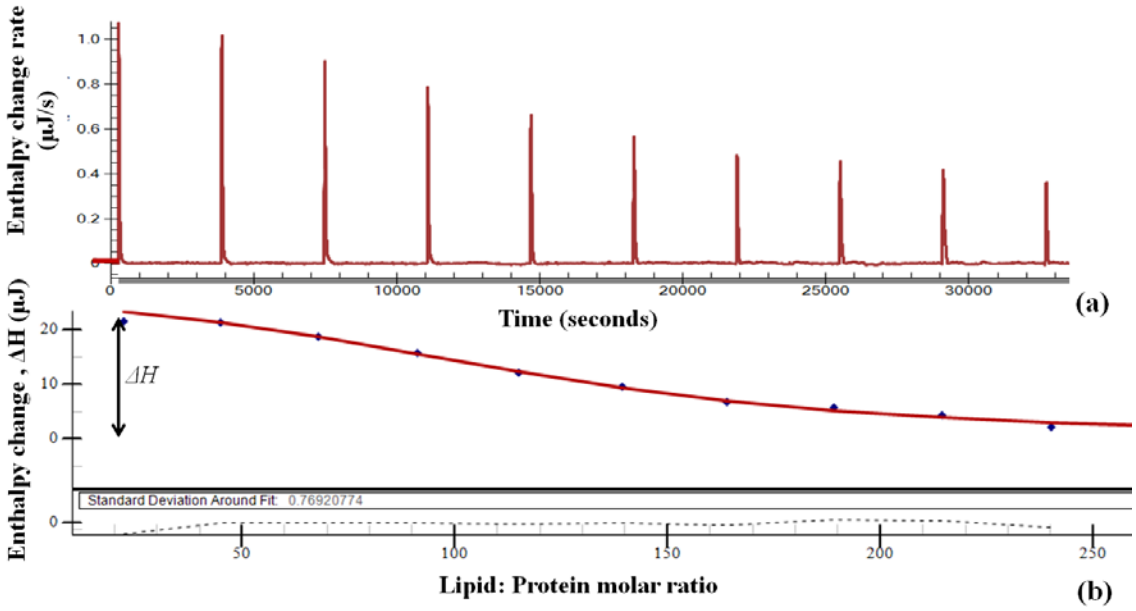


FIGURE 12: Typical isothermal calorimetric titration of liposome into IL-2 solution. Here a 20mM DPPC is injected in 5µl intervals into 300µL 15µM IL-2 at 37˚C (a) Raw data showing power compensation required by ITC to maintain constant temperature as each titration results in a binding event (NOTE: exothermic event shown here with peaks pointing up) (b) Integration of raw data, subtraction of the appropriate blank experiment (liposomes into water only to account for heat due to dilution) , and fitting with a non-linear regression technique allows determination of enthalpy change and stoichiometry of binding reaction.

During the initial titration, all of the injected liposomes bind to the protein. As the lipid titrations continue, free IL-2 becomes bound to lipid molecules and is removed from the bulk solution, leaving less protein available for binding. This is evidenced by a decrease in binding reaction enthalpy as lipid titrations continue (FIGURE 12a). At the end of the titration sequence, all binding sites are saturated and no heat other than the heat of dilution is observed. A blank experiment is run with an identical liposome into water scan to measure the heat of dilution (not shown). The area under these peaks, which is equal to the enthalpy change, is calculated and plotted as a function of the molar ratio of lipid to protein. Shown is a typical binding reaction fit with an independent binding site model, which assumes all binding reactions can be described by the same

binding constant and each binding event is independent of one another (FIGURE 12b). The overall enthalpy change, ΔH, of the event is given on the y-axis and the lipid to protein ratio is given on the x-axis.

The sigmoidal shape of the titration curve is consistent with an independent binding site model, which assumes that each individual binding site on the macromolecule (IL-2, in this case) is independent of all other binding sites. The equations used to fit this integrated titration data are obtained from algebraic manipulations of fundamental chemical thermodynamic relationships.

It was assumed that free IL-2 (IL_2) and lipids within an empty, preformed liposome ($Lipid$) bind to form an IL-2 lipid (liposome) complex ($IL2\_Lipid$) according to EQUATION 2.

$$IL2 + Lipid \underset{K_{dissociation}}{\overset{K_{association}}{\rightleftharpoons}} IL2\_Lipid$$

EQUATION 2: Binding equilibrium reaction

The binding constant, $K_{association}$, is defined as in EQUATION 3, where the brackets indicate molar concentrations. The binding constant is the inverse of the dissociation constant, $K_{dissociation}$. It stands to note that this binding constant (between IL-2 and lipid within a liposome) can be expected to be different for IL-2 bound to free lipid, because lipids within the inner bilayer leaflet of the liposome may not be available for binding.

$$K_{association} = \frac{[IL2\_Lipid]}{[IL2][Lipid]} = (K_{dissociation})^{-1}$$

EQUATION 3: Binding and dissociation constant

As the titrations continue, the concentrations of the IL-2 lipid complex, free IL-2, and free lipid constantly change, but the ratio of $K_{association}$ does not. Now let us define two constants in terms of these changing concentrations.

$$C_{Lipid} = [Lipid] + n[IL2\_Lipid]$$

EQUATION 4

$$C_{IL\_2} = [IL\_2] + [IL2\_Lipid]$$

EQUATION 5

The two variables, C_{Lipid} and C_{IL_2}, are constants, known from the initial concentrations of the protein and empty liposomes at time zero. The stoichiometry, n, is the number of binding sites on the IL-2 molecule that bind with each lipid molecule. In other words, for a single IL-2 molecule, there are 'n' number of lipids involved in the binding reaction. If EQUATION 4 and EQUATION 5 into EQUATION 3 are combined, we can solve for [IL2_Lipid], which takes the form of an algebraic quadratic equation in which b=$\left(nC_{Lipid}K_{association} + C_{IL\_2}K_{association} + 1\right)$.

$$[IL2\_Lipid] = \frac{b - \sqrt{(b)^2 - 4nK_{association}^2 C_{Lipid} C_{IL\_2}}}{2nK_{association}}$$

EQUATION 6

Now we turn to the parameter, n. The heat or energy of the reaction, Δq, can be defined in terms of the total change in enthalpy, ΔH.

$$\frac{\Delta q}{(n\Delta[IL2\_Lipid]\Delta V_{cell})} = \Delta H$$

EQUATION 7

In EQUATION 7, the quantity $n\Delta[IL2\_Lipid]$ represents the concentration of the complex in units of moles per volume, while the term ΔV_{cell} represents the volume

change within the cell as a result of the titrations and binding reaction. This equation is combined with EQUATION 6 and fit to minimize the error of $K_{association}$, n, and ΔH.

Once $K_{association}$, n, and ΔH have been determined, one can easily solve for the Gibbs free energy change and the entropic contribution to binding. R and T represent the gas constant and temperature, respectively.

$$\Delta G = -RTlnK_{association} = \Delta H - T\Delta S$$

EQUATION 8: Gibbs free energy equation of binding reaction

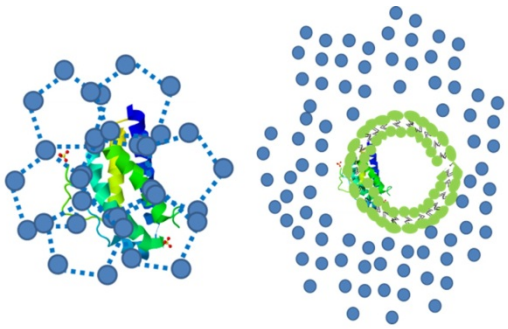


FIGURE 13: Entropic contribution to IL-2 liposome interaction. Left: Because IL-2 is a hydrophobic molecule, water molecules preferentially interact with one another than IL-2. Water molecules surround free IL-2 in an organized clathrate formation. This corresponds to a lower system entropy. Right: As IL-2 is incorporated into the liposome and removed from the aqueous environment, these organized clathrate cages are broken, corresponding to a higher system entropy.

The enthalpy (ΔH) and entropy (ΔS) contributions to binding can be used here as an indication of the nature of IL-2 lipid interaction. The insertion of a protein into a lipid bilayer may be enthalpy (ΔH) driven, and influenced by electrostatic interactions, H-bonds, or van der Waals interactions (short, attractive forces between neighboring acyl chains). Interaction between IL-2 and the liposome may also be driven by hydrophobic interactions between the protein and water molecules, an entropy (ΔS) driven phenomenon. An increase in the system entropy (as defined by the boundaries of the calorimeter cell in which the reaction is taking place) arises due to the elimination of orderly, clathrate water structures around a hydrophobic protein as a hydrophobic protein penetrates into the lipid bilayer (Tanford, 1980) (FIGURE 13).

All binding constants and reaction enthalpies were measured by lipid into peptide titrations. Liposome solutions (0-200mM lipid solution) were degassed by vacuum and injected in aliquots of 2-10ul into the sample cell, where 300ul of a degassed 6.54-15uM solution of IL2 was present. Protein and liposome solution concentrations and injection volumes were optimized in order to obtain the entire binding curve. All titrations were performed in triplicate.

4.2.4 Preparation of IL-2 Liposomes

4.2.4.1 Adsorption of IL-2 to Preformed Liposomes

IL-2 was adsorbed onto preformed liposomes in the following manner. The protein was added to the liposome suspension at an IL-2:lipid molar ratio, n, as defined and determined within the isothermal calorimetry experiments. These suspensions were gently mixed and incubated at 4˚C for 30 minutes. It has been shown in literature that incubation time has a minimal effect on the amount of IL-2 incorporated in liposome, and that 30minutes was a sufficient amount of time for IL-2 to adsorb into lipid bilayers (F. J. Koppenhagen et al., 1998) .

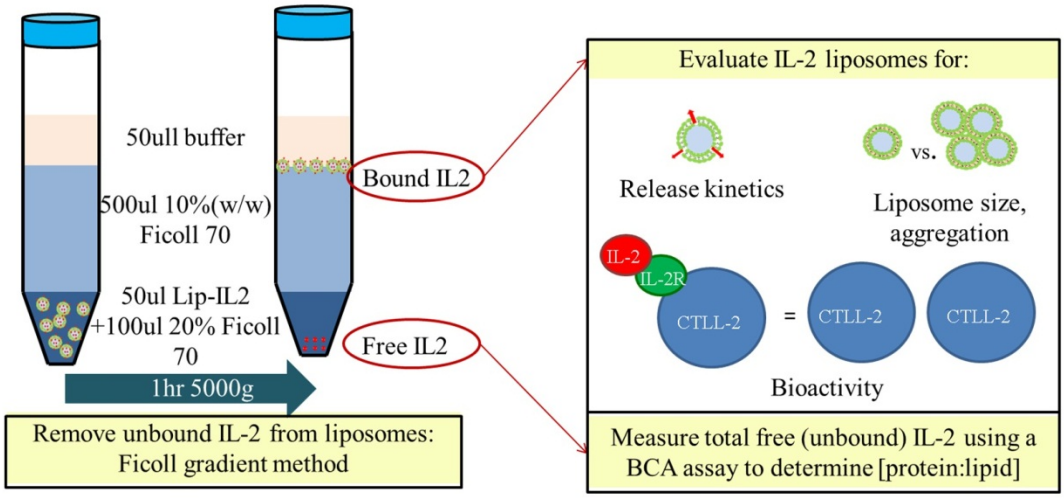


FIGURE 14: Separation of free and bound IL-2 from liposomes prior to analysis

4.2.4.2 Separation of Free and Bound IL-2

To ensure all free IL-2 not adsorbed to preformed liposomes after incubation was removed prior to evaluation of *in vitro* IL-2 release kinetics, IL-2 liposome size distribution and melting transition thermodynamics, or bioactivity evaluation of released IL-2, free and liposome bound IL-2 were separated by a Ficoll gradient method as in (Weissig, 1993). A comparison of the various separation methods available to separate free and unbound drug from liposome particles in a study by Dipali et al found that this method was a cost effective, rapid separation method with optimal sample recovery (Dipali, Kulkarni, & Betageri, 1996). The method protocol was as follows. A layered suspension of a 100ul IL-2 liposome suspension, 200ul 20% w/w Ficoll in water, 1ml 10% w/w Ficoll in water, and 100ul water was prepared in a 2ml centrifuge tube. The layered suspension was spun at 5000F for 1hr on a Thermo Scientific IEC Micromax FR Centrifuge. The IL-2 bound to liposomes partitioned in the top 100ul, and was carefully removed for further analysis. To confirm molar ratios of protein to lipid obtained by isothermal titration calorimetry, the bottom 100ul (where unbound IL-2 partitioned after centrifugation) was recovered. Protein concentration was quantified by a BCA assay and A280nm.

4.2.5 Protein Localization in Liposome by Evaluation of Alteration of Thermotropic Melting Transition of Liposomes Upon IL-2 Incorporation

Differential scanning calorimetry is one method used to detect changes to the melting phase transition behavior of lipid bilayers (Demetzos, 2008) as a function of protein and small solute molecule incorporation (Lo & Rahman, 1995). At lower temperatures, lipids exist in an ordered gel state in which all the rigid, extended acyl

chains are in an all trans-configuration. As the temperature is raised to the main transition temperature of the lipids (T_m), the lipids enter a disordered liquid crystalline phase in which more gauche conformations are present and more intra and intermolecular motions occur. The approach to T_m is marked by an increase in the specific heat capacity (C_p) of the lipid. The integrated area under the peak defined by $C_p(T)$ within the range of the onset and completion temperatures defines the calorimetric enthalpy change, ΔH_{cal}. The onset and completion temperatures were chosen at the intersection of the tangents to the baseline and the rising portion of the peak. The width of the peak at the half height, $\Delta T_{1/2}$, indicates the sharpness or cooperativity of the peak. Perturbations in these parameters upon protein interaction may indicate insertion or incorporation of the protein into the bilayer. The electrostatic or hydrophobic interactions within some protein-lipid systems can cause alterations in this phase transition. Papahadjopoulos et al have classified proteins in three categories based upon their effects on this melting phase transition of phospholipids. Category 1 proteins are hydrophilic and adsorb into the bilayer surface through electrostatic interaction. Category 2 proteins are also adsorbed onto the surface through electrostatic interaction, but are partially imbedded into the bilayer through hydrophobic interaction. Category 3 proteins penetrate into the core of lipid bilayers through predominately hydrophobic interaction (Papahadjopoulos et al., 1975). Characterization of how this interaction alters the thermodynamic parameters of melting (enthalpy change, T_m, or the $\Delta T_{1/2}$) can provide insight into where a protein is localized in a lipid bilayer in some cases (Demetzos, 2008). Observations observed in literature are shown in TABLE 1. The nature and extent of these perturbations upon IL-2 incorporation are probed here.

Category	Model protein	Neutral Lipid (DPPC)			Anionic Lipid (DPPG* or DPPS**)		
		Tm	ΔT1/2	ΔH	Tm	ΔT1/2	ΔH
1	Rnase A*	↔	↔	↔	↑	↑	↓
2	Cytochrome c*	↔	↔	↔	↕	↕	↕
3	Cyclosporin A*	↔ or ↓	↑	↕	n.e.	n.e.	n.e.
	Lipophilin**	n.e.	n.e.	n.e.	↓	n.e.	n.e.

TABLE 1: Alteration of thermodynamic parameters of the lipid melting phase transition upon protein interaction. ↔ indicates no change, ↕ indicates an decrease at low protein:lipid ratios and an increase at higher ratios, ↓ indicates an increase, ↑indicates a decrease, and n.e. indicates that this parameter was not evaluated. *Data from (Demetzos, 2008; Papahadjopoulos, Moscarello, Eylar, & Isac, 1975), **Data from (Boggs, Wood, Moscarello, & Papahadjopoulos, 1977)

Differential scanning calorimetry (DSC) was performed using a MicroCal VP-DSC system (GE Healthcare). Data was analyzed using Origin software. All samples were degassed prior to use. The scanning rate was set to 1˚C/minute. The enthalpy change, T_m, or the $\Delta T_{1/2}$ of the melting phase transition of liposome suspensions (0.3mM-1mM) prior to and after IL-2 adsorption were determined. Free IL-2 was removed from the IL-2 liposome suspension prior to analysis using a Ficoll separation gradient method.

4.2.5.2 Determination of Total Phosphorous

The accuracy of the enthalpy change measured by DSC is dependent on the accuracy of lipid concentration. Inaccurate estimation of liposome (lipid) concentrations can result from pipetting error, weighing error, or lipid loss during the extrusion process. To confirm liposome concentrations prior to DSC, the total phosphorous concentration was analyzed using the Bartlett assay (Chen, Toribara, & Warner, 1956; Fiske & Subbarow, 1925). All reagent solutions were prepared using deionized water. Briefly, ~0.1μmoles of each sample (empty liposomes and IL-2 liposomes) was placed on the bottom of a borosilicate glass tube, and all solvent removed under an argon stream. A standard curve of 0-0.228μmoles (0-350μl) was prepared from a 0.65mM phosphorous

standard (Sigma-Aldrich, St. Louis, MO). Several boiling stones were added to each tube, a glass marble placed on the top to the tubes, and solutions were heated with 450µl 8.9N H2SO4 (Thermo Fisher Scientific, Rockford, IL) in a fume hood to 200-215˚C for 20-25minutes in a dry bath incubator (Thermo Fisher Scientific). Tubes were cooled to ambient temperature for 5 minutes, 150ul H2O2 added, and heated for 30minutes at 200-215˚C . Tubes were removed from heat and cooled for 5 minutes. A volume of 3.9ml distilled water and 0.5ml 2.5% (wt/vol) ammonium molybdate (VI) tetrahydrate (Sigma Aldrich) solution were added to each tube and mixed thoroughly. Next, 0.5ml 10% (wt/vol) were then added and thoroughly mixed. Tubes were covered with a marble, heated to 100˚C for seven minutes, and cooled. Absorbance of samples was read at 820nm.

4.2.6 Evaluation of Liposome Swelling and Aggregation Upon IL-2 Adsorption by Dynamic Light Scattering

Because size is an important parameter governing pharmacokinetics of nanoparticle drug carriers, alteration in the particle size due to swelling or aggregation was evaluated. Dynamic light scattering (DLS) is a method that can be used to determine the size distribution of particles in suspension. As laser light hits these particles, a time dependent fluctuation in Rayleigh scattering can be observed. These fluctuations occur because the distance between particles, which are in Brownian motion, are changing. A second order autocorrelation function ($C(\tau)$) is generated to quantify these fluctuations.

$$C(\tau) = Ae^{-2\Gamma\tau} + B$$

EQUATION 9

$$\Gamma = q^2 D$$

EQUATION 10

Here A and B are constants, τ is the delay time, and Γ is a function of the diffusion coefficient, D, and the scattering vector, q, which is dependent on the scattering angle, refractive index, and laser wavelength. A non-linear fitting algorithm can be used to fit this measured function, which can then be used to obtain a diffusion constant, D. The diffusion constant is related to the radius of a diffusing sphere as described by the Stokes Einstein equation, which defines the diffusion constant as a function of the Boltzmann's constant (k_B), temperature (T), solvent viscosity (η), and particle diameter (d).

$$D = \frac{k_B T}{3\pi\eta d}$$

EQUATION 11

Particle size before and after IL-2 incorporation was studied using dynamic light scattering using a 90Plus Particle Size Analyzer (Brookhaven Instruments Corporation, Holtsville, NY) at a fixed scattering angle of 90˚ and a temperature 25˚C. The particle size distribution was obtained using ZetaPlus Particle Sizing software provided by Brookhaven Instruments. Free IL-2 was removed from liposome suspensions immediately prior to analysis by the Ficoll separation method.

4.2.7 *In Vitro* IL-2 Release Kinetics From Liposome

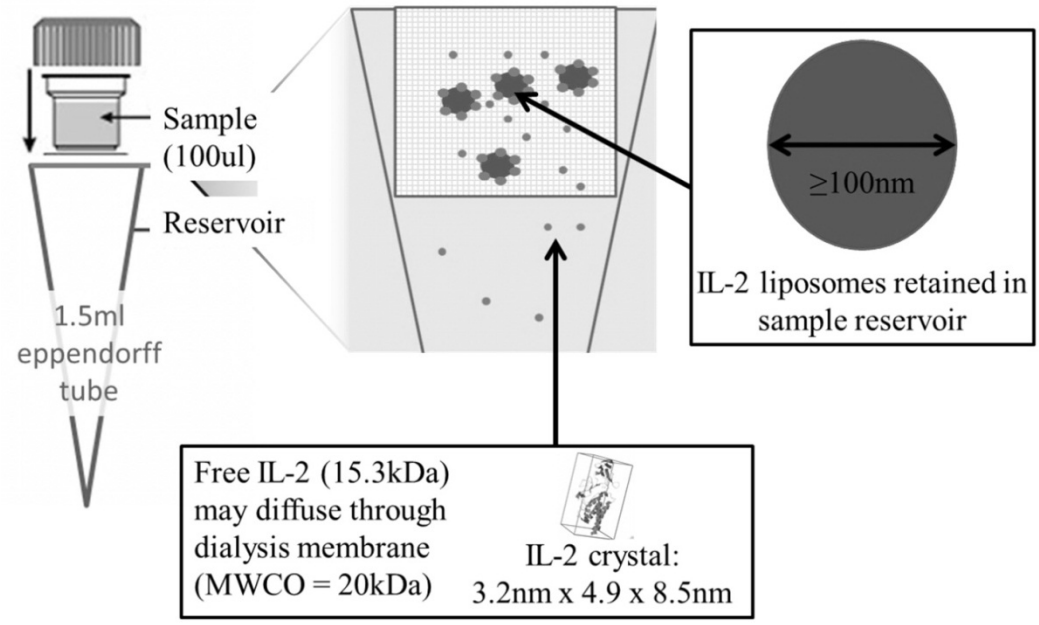


FIGURE 15: Evaluation of IL-2 release from liposomes *in vitro*

Release kinetics of bound IL-2 from the liposome were monitored by a sample and replace dialysis technique, as recommended in (D'Souza & DeLuca, 2006). Release kinetics of IL-2 from liposomes were monitored as shown in FIGURE 15. Prior to evaluation of IL-2 release from liposomes, free IL-2 was removed with a Ficoll separation gradient method. A volume of 100ul of a sample IL-2 liposome solution was placed in a presoaked Slide-A-Lyzer MINI dialysis device with a MWCO of 20kDa (Thermo Fisher Scientific Inc, Rockford, IL) which was then placed in 1.5ml centrifuge tube (reservoir) with approximately 1000ul water. All release kinetics were performed at 37˚C to mimic temperature conditions in the body. The molecular weight cutoff (MWCO) was chosen such that liposomes and protein bound to liposomes would remain in the dialysis unit, but free, released protein could pass through the dialysis filter into the reservoir. A stir bar, rotated a medium-low speed, was placed in the bottom of the

centrifuge tube (the reservoir) to provide continuous agitation. All water was removed from the reservoir at hourly intervals for testing, and an equal volume of fresh water was replaced in the reservoir at each testing interval. This sample and replace dialysis method was continued for 6 hours. During the next 18 hours, the dialysis release kinetic experiment was continued without removing water from the reservoir. At the end of 18 hours (24 hours total), free protein was removed from the reservoir and protein concentration quantified.

4.2.10 Bioactivity of Released IL-2

To assess whether released IL-2 was still biologically active after its release from liposomes, IL-2 liposomes were prepared as detailed in section 4.2.4. Free IL-2 was removed as previously described. IL-2 was released from liposomes and separated by a dialysis technique by a similar method as described in section 4.2.7. 100ul of the sample IL-2 liposome solution was placed in a presoaked Slide-A-Lyzer MINI dialysis device with a MWCO of 20kDa (Thermo Fisher Scientific Inc, Rockford, IL) which was then placed in 1.5ml centrifuge tube (reservoir) with approximately 1000ul water. Dialysis was performed at 4˚C to preserve maximum protein bioactivity during the release. IL-2 was allowed to dialyze for approximately 24 hours. A stir bar, rotated a medium-low speed, was placed in the bottom of the centrifuge tube (the reservoir) to provide continuous agitation. A control sample of IL-2 only was placed in an identical reservoir tube with a stir bar during the dialysis to account for any bioactivity losses due to incubation or stirring.

The total amount of IL-2 released from liposomes after this 24 hour dialysis period was quantified according to section 2.2.2. All samples were diluted to 120μg/μl. This total

(folded and unfolded) IL-2 concentration was chosen as it represents the required concentration of control IL-2 (after 24 hours at 4˚C under constant agitation by a rotating stir bar) to induce optimal growth and viability of the IL-2 dependent cell line, CTLL-2. The ability of IL-2 to promote CTLL-2 survival and proliferation, used as an indicator of IL-2 bioactivity retention, was evaluated using flow cytometry as previously described (see section 2.2.4).

4.3 Results

4.3.1 Determination of Binding Constants, Stoichiometric Ratios, and Enthalpic and Entropic Contributions to Binding

Isothermal titration calorimetry (ITC) was used to determine the thermodynamic parameters of IL-2 binding to neutrally charged lipid membranes of various acyl chain lengths (14, 16, and 18 carbon chains corresponding to DMPC, DPPC, and DSPC, respectively). The effect of lipid charge was investigated by evaluating liposomes composed of DSPC:DSPG (10:1 molar ratio). This formulation was composed of lipids (DSPC and DSPG) with an 18 carbon acyl chain, and had an overall negative charge of (-1) due to the inclusion of DSPG. ITC experiments were set up so that liposome solutions were injected into an IL-2 peptide solution, resulting in an exothermic binding reaction. Concentrations and injection volumes were chosen so as to observe the entire isothermal biding reaction curve.

All binding isotherms in these experiments follow a sigmoidal shape when the lipid :IL-2 molar ratio is plotted on the x-axis and the enthalpy of binding is plotted on the y-axis as shown in FIGURE 12. Such a shape is consistent when binding receptor

sites are equivalent and independent of one another, thus supporting a multiple independent binding site model.

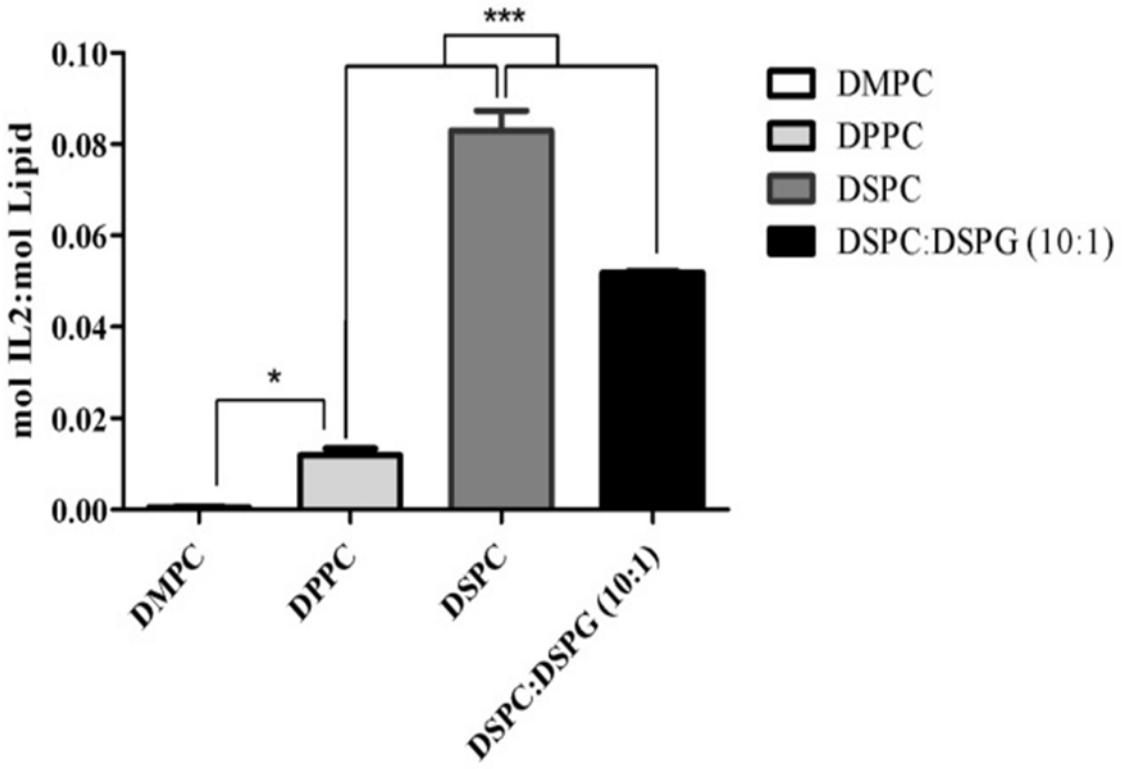


FIGURE 16: Molar ratio of IL-2 to lipids in liposome composed of DMPC, DPPC, DSPC, and DSPC:DSPG (10:1 molar ratio). Reported values are given as the average ± SEM. n=3. *:p<0.05; **:p<0.01, ***:p<0.001. Statistical differences between lipid compositions are reported with connecting lines.

The molar ratios of IL-2 to lipid within liposomes of different lipid compositions were determined. For a liposome into protein titration (as performed here), the lipid: protein molar ratio is traditionally obtained as in FIGURE 12b. We have chosen to report protein: lipid ratios for ease of comparison with other IL-2 liposomes in literature, which typically report protein: lipid molar ratios. The molar ratio of IL-2 to protein molecules increased with lipid acyl chain length (0.0005:1±0.000048 for DMPC, 0.012:1±0.002 for DPPC, and 0.083:1±0.004 for DSPC) as seen in FIGURE 16. A statistical difference was

seen between DMPC and DPPC ($P<0.05$), DMPC and DSPC ($P<0.001$), and DPPC and DSPC ($P<0.001$). The inclusion of a negatively charged lipid head group with an identical acyl chain length in the DSPC:DSPG (10:1) formulation decreased the amount of IL-2 absorbed into the bilayer from 0.083 moles IL-2 per mole lipid to 0.052±0.00033 moles IL-2 per mole lipid ($P<0.001$). Thus, both lipid acyl chain length and charge impacted the amount of IL-2 incorporated into liposome formulations.

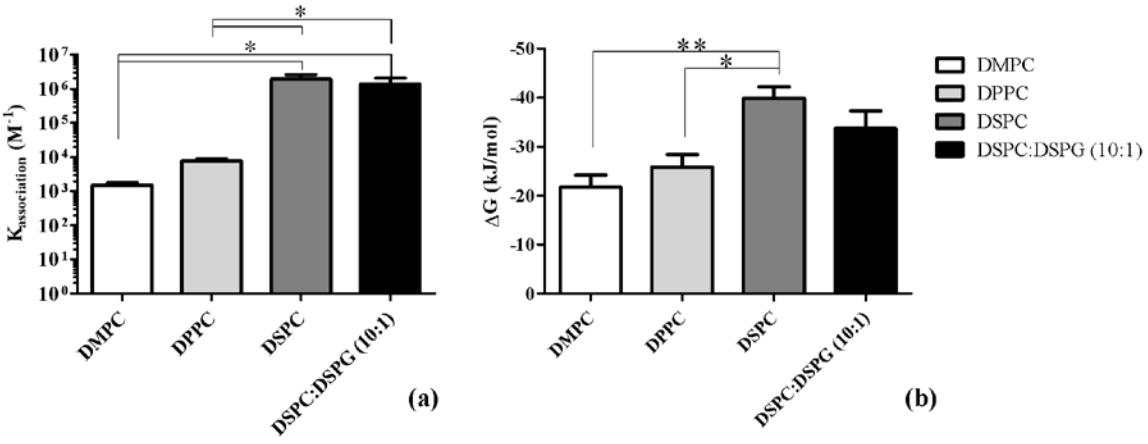


FIGURE 17: (a) Binding constants and (b) Gibbs free energy change upon binding of IL-2 to liposomes composed of DMPC, DPPC, DSPC, and DSPC:DSPG (10:1 molar ratio). Reported values are given as the average ± SEM. n=3, *: $p<0.05$; **:$p<0.01$. . Statistical differences between lipid compositions are reported with connecting lines.

Binding constant between lipids within liposomes and IL-2 were also assessed. FIGURE 17a illustrates the dependence of the binding or association constant ($K_{association,}$ M^{-1}) of IL-2 to lipid membranes on the acyl chain length of the lipids employed. This binding constant is defined by EQUATION 3, and represents the probability of the formation of a bound IL-2 lipid complex within a liposome. There is no statistical difference between the binding constant of IL-2 to DMPC and DPPC. However, there is a significant difference ($P<0.05$) between the binding constant of IL-2 to DMPC and DSPC (K_a=1.5E 3± 3E2 M^{-1} and 1.9E6 ±6.9E5 M^{-1}, respectively) and between DPPC and DSPC (K_a=7.7E 3±1.3E3 M^{-1} and 1.9E6±6.9E5 M^{-1}, respectively). Incorporation of a

negatively charged lipid does not lead to a statistically significant difference in the binding constant (DSPC:DSPG K_b=1.4E6 ± 6.9E5 M^{-1}) when compared to DSPC alone. These data show that the binding constant of IL-2 to liposomes is primarily dependent on lipid acyl chain length as compared to the inclusion of a negatively charged lipid.

To further illustrate the dependence of IL-2 binding to lipids within a liposome on lipid acyl chain length, the favorability of the binding reaction, as given by the Gibbs free energy change (ΔG), was also obtained. FIGURE 17b shows the increase in ΔG at 37˚C upon binding of IL-2 to DMPC (ΔG= -21.713±2.523 kJ/mol), DPPC (ΔG= -25.837±2.538 kJ/mol), and DSPC (ΔG= -39.842±2.33 kJ/mol). A more negative ΔG value indicates a more favorable binding reaction. A statistical difference between thermodynamic favorability of binding at 37˚C is seen between DMPC and DSPC ($P<0.01$) and DPPC and DSPC ($P<0.05$). No significant difference is seen between DSPC and DSPC:DSPG (DSPC:DSPG ΔG= -33.692±3.63 kJ/mol).

Finally, the contribution of electrostatic versus hydrophobic forces to the binding reaction was probed and calculated according to EQUATION 8. FIGURE 18 shows the entropy (ΔS) and enthalpy (ΔH) contributions to binding between IL-2 and the lipid membrane. The entropic driving force behind this binding event increases as the acyl chain length increases in the number of carbons (TΔS is equal to 21.7±2.52 kJ/mol for DMPC, 25.59±2.576 kJ/mol for DPPC, and 33.4942.281 J/mol for DSPC). A statistically significant difference is seen between DMPC and DSPC ($P<0.01$) and DPPC and DSPC ($P<0.05$). No significant difference with regards to entropic contribution to binding is seen upon the incorporation of a negatively charged lipid head group (TΔS is equal to 33.494±3.544 kJ/mol for DSPC:DSPG (10:1)). Enthalpy change upon IL-2 incorporation

into lipid membranes increases with acyl chain length, although this change is only statistically significant between DMPC (ΔH=-0.012±0.003 kJ/mol) and DSPC (ΔH=-0.348±0.111 kJ/mol) ($P<0.05$). No statistical significance is seen upon incorporation of a negatively charged PG head group (ΔH = -0.197±0.087 kJ/mol).These data reflect that the driving force behind IL-2 liposome binding interaction is hydrophobic in nature for all lipid compositions tested.

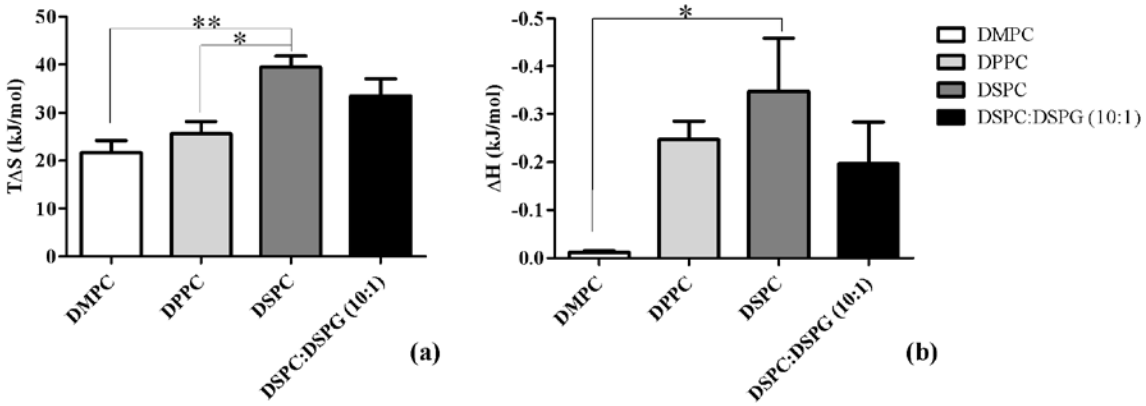


FIGURE 18: (a) Entropy (TΔS) and (b) enthalpy (ΔH) changes of upon IL-2 binding to liposomes composed of DMPC, DPPC, DSPC, and DSPC:DSPG (10:1 molar ratio). Experiments were performed at 37˚C. Values are reported as an average ±SEM. n=3, *: $p<0.05$; **:$p<0.01$. . Statistical differences between lipid compositions are reported with connecting lines.

4.3.3 Protein Localization in Liposome by Evaluation of Alteration of Thermotropic Melting Transition of Liposomes Upon IL-2 Incorporation: Effect of Liposome Composition

Name	Acyl chain length (# carbons)	Liposome Only			IL-2 Liposome		
Nuetral lipids		Tm (°C)	ΔT1/2	ΔH (kcal/mol)	Tm	ΔT1/2	ΔH (kcal/mol)
DMPC	14	24.26±0.20	1.38±0.04	5.70±0.05	24.28±0.10	1.35±0.02	5.21±0.17
DPPC	16	41.33±0.05	1.58±0.01	6.97±0.05	41.52±0.18	1.50±0.24	6.8±0.41
DSPC	18	54.29±0.17	1.59±0.01	15.76±0.61	54.25±0.03	1.42±0.09	15.98±0.44
Inclusion of a negatively charged lipid (DSPG)							
DSPC: DSPG (10:1)	18	55.56±0.23	2.43±0.30	19.18±0.35	55.41±0.09	1.8±0.20**	24.90±2.31*

TABLE 2: Thermodynamic parameters of liposome melting phase transition prior to and after IL-2 incorporation. Data are expressed as averages ± SEM. n=3. Statistical differences are indicated between 'Liposome Only' and 'IL-2 Liposome' of the same formulation. *:$p<0.05$, **:$p<0.01$.

The transition temperatures and enthalpies of the IL-2 free liposomes studied here fall within 10-30% enthalpy change values for DMPC, DPPC, and DSPCseen in literature (Mabrey & Sturtevant, 1976). As expected, increased acyl chain length leads to a higher unfolding temperature and an increased enthalpy change during the gel-liquid phase transition due to the importance of van der Waals interactions in liposome bilayer stability. The inclusion of a negatively charged lipid, DSPG, with DSPC did not result in a change in T_m, as expected, because each of these lipids has an equivalent T_m. The enthalpy and cooperativity of the phase transition of IL-2 free liposomes including a

negatively charged lipid is altered due to the electrostatic interactions between head groups as expected.

No significant change in the T_m, $\Delta T_{1/2}$, or ΔH of the lipid melting phase transition upon IL-2 incorporation was observed for DMPC, DPPC, and DSPC. The inclusion of a negatively charged lipid resulted in an alteration in the melting transition behavior upon IL-2 incorporation behavior as shown by the DSC trace in FIGURE 19. Specific changes to the melting transition thermodynamic parameters for DMPC, DPPC, DSPC, and DSPC:DSPG liposomes upon IL-2 incorporation can be seen in TABLE 2. Because the thermal denaturation of IL-2 occurs over the same temperature range as the DPPC, DSPC, and DSPG lipids, the enthalpy values for the lipid phase transition were corrected for IL-2 unfolding enthalpy contributions. Incorporation of IL-2 into the lipid membrane of DSPC:DSPG liposomes leads to a decrease in the $T_{1/2}$ from 2.42±0.3˚C to 1.795±0.199˚C ($P<0.01$). The broadening peak upon IL-2 incorporation into these liposomes coincides with an increase in enthalpy from 19.1844±351.4 kcal/mol to 25.4±2.31225 kcal/mol ($P<0.05$).

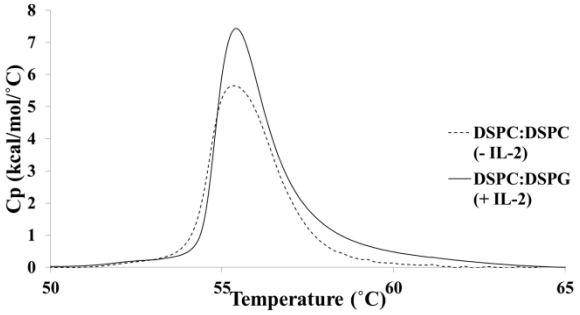


FIGURE 19: Thermodynamic traces of DSPC:DSPG (10:1) liposomes +/- IL-2 using DSC.

4.3.3 Evaluation of Liposome Swelling and Aggregation Upon IL-2 Adsorption by Dynamic Light Scattering

The liposomal particle size was measured by dynamic light scattering. Two phenomena can affect the particle size, and they are addressed separately here. The first item is the expected size increase due to IL-2 incorporation into the lipid bilayer. Let us first consider an empty liposome that has been extruded to a diameter of $d_{LIPOSOME\_ONLY}$=100nm. The surface area of this liposome (outer and inner leaflets), $SA_{LIPOSOME}$ can be defined as a function of the diameter. The total surface of the liposome, $SA_{LIPOSOME}$ can then be used to approximate how many lipid molecules are within the vesicle based upon the surface area of a single lipid head group, SA_{LIPID}, the thickness of the bilayer, t, and the total number of lipids within the vesicle, N_{LIPIDS}. The surface are of a phosphatidylcholine lipid head group within a vesicle has been approximated to be 0.61-0.74nm^2 (Huang & Mason, 1978; Murzyn, Rog, & Pasenkiewicz-Gierula, 2005), and a phosphatidylglycerol lipid head group has been estimated to be only 0.007nm^2 smaller (Murzyn et al., 2005). We will therefore approximate the surface area of a single lipid (phosphatidylcholine or phosphatidylglycerol) as SA_{LIPID}=0.675nm^2. The thickness of the bilayer in DMPC, DPPC, and DSPC liposomes, t, is 4.6, 5.2, and 5.9nm (Cornell & Separovic, 1983; Lewis & Engelman, 1983).

$$SA_{LIPOSOME} = 4\pi\left(\left(\frac{d_{LIPOSOME}}{2}\right)^2 + \left(\left(\frac{d_{LIPOSOME}}{2}\right) - t\right)^2\right)$$

EQUATION 12

$$N_{LIPIDS} = \frac{SA_{LIPID}}{SA_{LIPOSOME}}$$

EQUATION 13

Based upon this calculation we can approximate that there are N_{LIPID}=84871, 83864, and 82706 lipid molecules within a 100nm DMPC, DPPC, and DSPC liposome, respectively. Assuming a similar acyl chain length as DSPC liposomes, N_{LIPID} in a 100nm DSPC:DSPG (10:1) liposome is approximately 83573 lipid molecules.

Now let us consider the case where IL-2 is incorporated into the liposomes. To calculate the new vesicle diameter increase upon IL-2 incorporation ($d_{IL\text{-}2\_LIPOSOME}$) , we must know how many IL-2 molecules we expect to incorporate ($N_{IL\text{-}2}$) and the total surface area increase due to the incorporated IL-2 molecules ($SA_{IL\text{-}2}$). The number of IL-2 molecules we can expect to incorporate, $N_{IL\text{-}2}$, can be calculated as a function of the molar ratio of protein to lipid ($R_{IL\text{-}2:Lipid}$). The molar ratios as determined in section 4.3.2 were used in this calculation.

$$N_{IL-2} = N_{LIPIDS} R_{IL-2:Lipid}$$

EQUATION 14

The total increase in surface area due only to incorporated IL-2 molecules, $SA_{IL\text{-}2}$, can be calculated as a product of the number of IL-2 molecules, $N_{IL\text{-}2}$, and the surface area increased due to a single IL-2 molecule, $SA_{SINGLE\_IL\text{-}2}$.

$$SA_{IL-2} = N_{IL-2} SA_{SINGLE\_IL-2}$$

EQUATION 15

An approximation of the contribution of a single IL-2 molecule can be made on the basis of its crystal packing structure, which has an orthorhombic crystal lattice structure with dimensions of 3.171, 4.97, and 8.468nm (Arkin et al., 2003). Consider two

cases of IL-2 incorporation FIGURE 20. In case (a), each IL-2 molecule results in an increased surface area of $SA_{SINGLE\_IL\text{-}2}$=3.171nm x 4.97nm. This will result in the smallest possible vesicle size increase. In case (b) each IL-2 molecule results in an increased surface area of $SA_{SINGLE\_IL\text{-}2}$=8.468nm x 4.97nm. This will result in the largest possible vesicle size increase. We will consider these two extremes in our theoretical

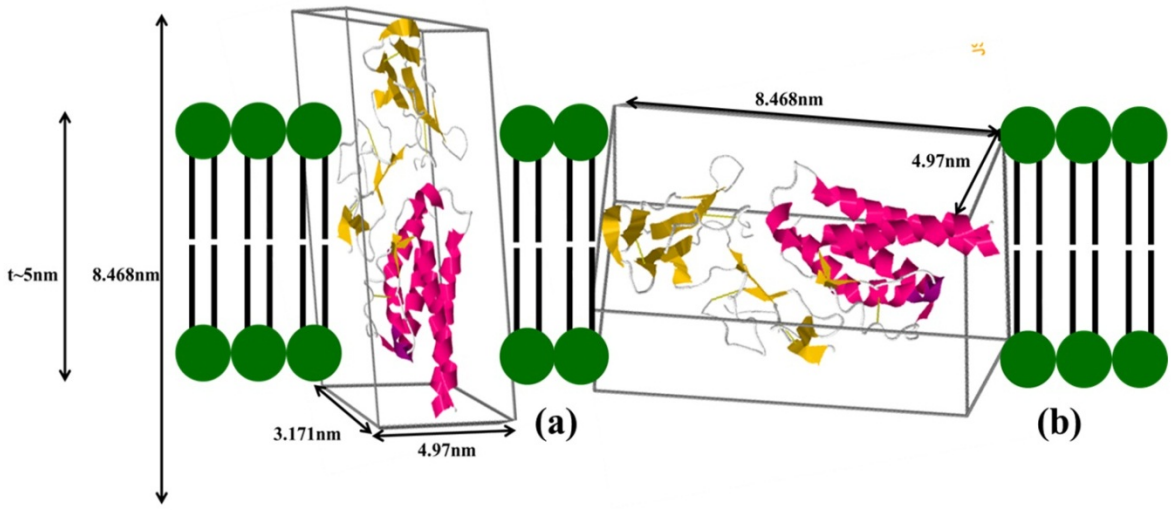


FIGURE 20: Two possible orientations of IL-2 in lipid bilayer. Protein crystal structure image obtained from Protein Data Bank (PDB Identifier 1M47)

calculations.

Finally, we determine the new diameter of the liposome with incorporated IL-2, $d_{IL\text{-}2\_LIPOSOME}$, by the following relationship.

$$SA_{IL-2\ LIPOSOME} = SA_{LIPOSOME} + SA_{IL-2}$$

EQUATION 16

The diameter of the new IL-2 containing vesicle, $d_{IL\text{-}2\ LIPOSOME}$, is calculated according to the same strategy as the empty liposomal vesicle.

$$SA_{IL-2IPOSOME} = 4\pi\left(\left(\frac{d_{IL-2\ LIPOSOME}}{2}\right)^2 + \left(\left(\frac{d_{IL-2\ LIPOSOME}}{2}\right) - t\right)^2\right)$$

EQUATION 17

The diameter of this theoretical liposome, $d_{IL\text{-}2Liposome}$, due to swelling upon IL-2 incorporation was calculated and compared to experimentally determined liposome diameters obtained by DLS (FIGURE 21). Theoretical minimum increase in surface area assumes the longest portion of IL-2 is oriented parallel to lipid acyl chains, while theoretical maximum increase in surface area assumes the longest portion of IL-2 is oriented perpendicularly to the lipid acyl chains. The error bars for the theoretical values are indicative of minimum and maximum theoretical diameter increases as a result of the orientations in FIGURE 20.

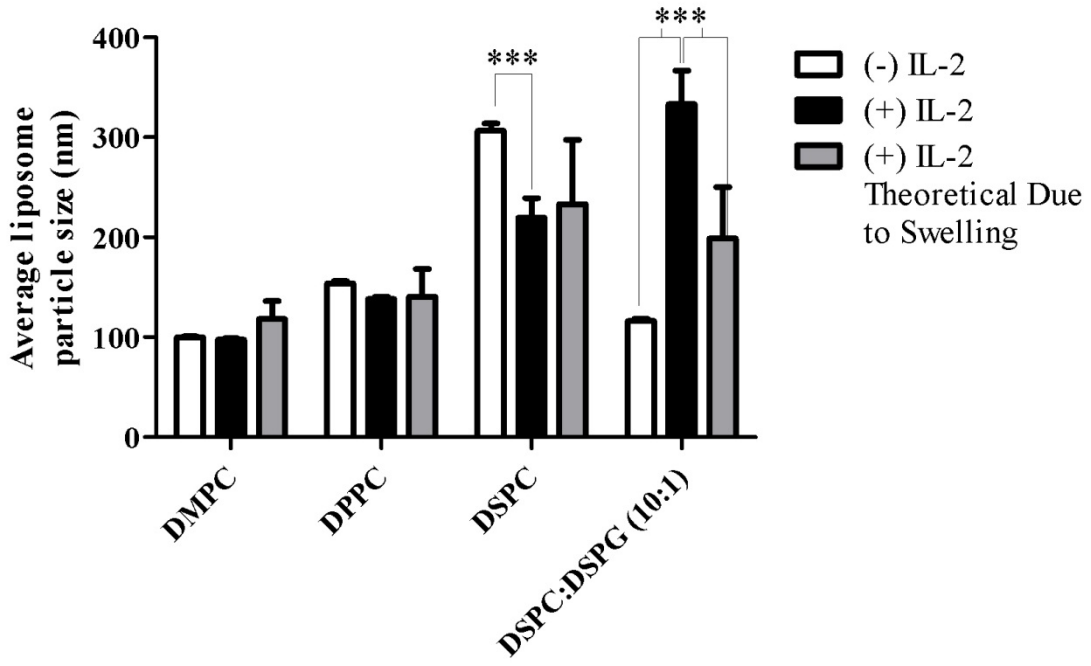


FIGURE 21: Average liposome particle size prior to and after IL-2 incorporation as measured with DLS and the theoretical size increase of liposomes upon IL-2 incorporation due to swelling. Data are presented as average±SD. For theoretical calculations, SD bars are based on the maximum and minimum liposome diameters upon IL-2 incorporation due to swelling. n=3. ***$p<0.001$. Statistical differences are indicated between the same lipid compositions.

FIGURE 21 shows that for empty, neutrally charged liposomes, as the lipid acyl chain length of neutrally charged liposomes increases, the average particle size in the population increases. However the incorporation of IL-2 into these neutrally charged liposomes leads to a significant decrease in diameter. The diameter of the IL-2 liposome increases by approximately 40 and 100% for DPPC and DSPC liposomes, respectively, as compared 100nm diameter the liposomes were initially extruded at. However, the theoretical diameters of IL-2 liposomes composed of neutrally charged lipids based upon swelling do not significantly differ from these experimentally determined values. This suggests that the increase in diameter of the neutrally charged liposomes can be explained by swelling upon IL-2 incorporation into the bilayer.

The opposite event occurs upon IL-2 incorporation into a negatively charged liposome (DSPC:DSPG). DSPC:DSPG liposomes with no IL-2 have an average

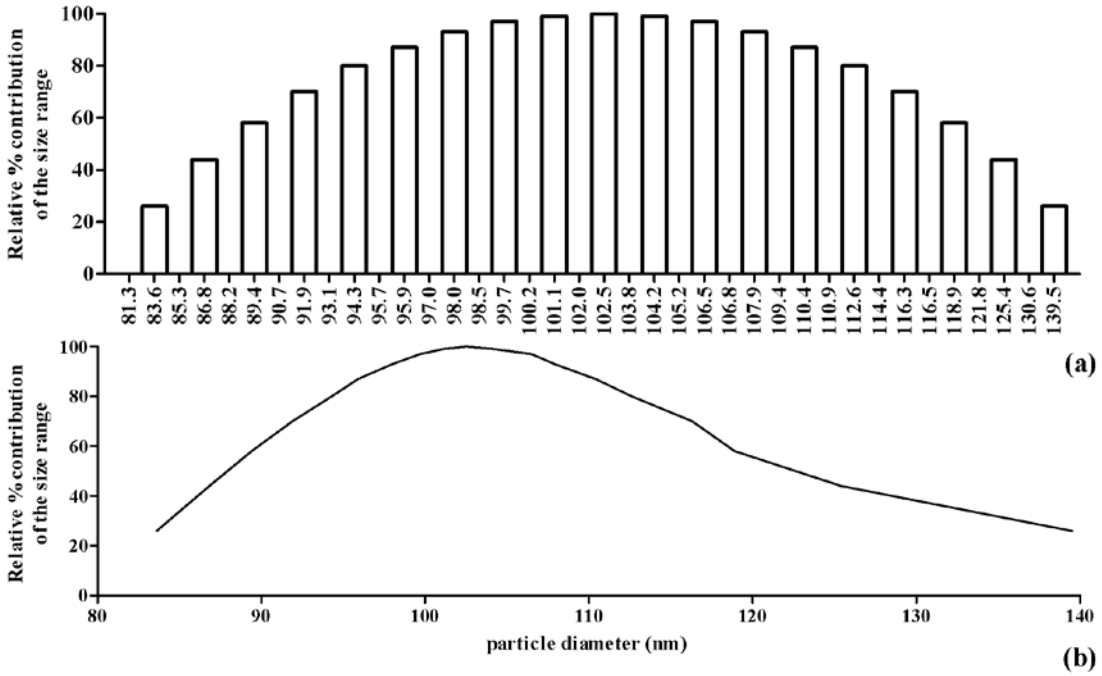


FIGURE 22:(a) Histogram of DMPC liposome particle sizes prior to IL-2 incorporation as measured by DLS. (b) Line graph adapted from histogram data to allow for easier visual comparison between samples.

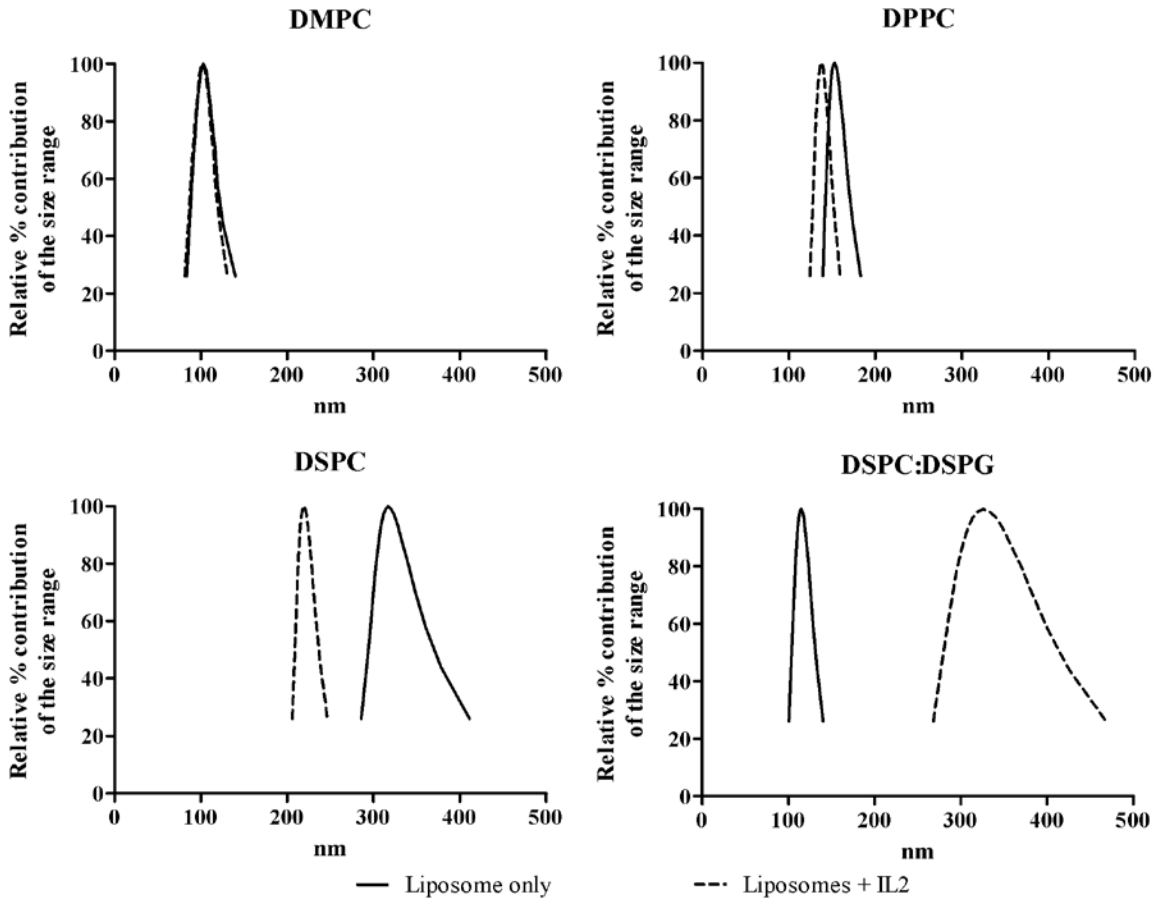


FIGURE 23: Liposome diameter distribution prior to and after IL-2 incorporation in DMPC, DPPC, DSPC, and DSPC:DSPG liposomes.

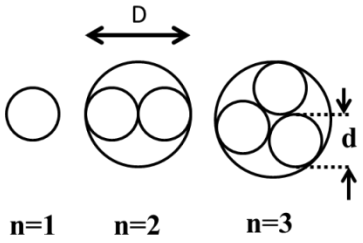
diameter of 116.15nm, which is very close to the 100nm diameter at which they were initially extruded at. However, the particle diameter increases 3 fold upon IL-2 incorporation. Because this increased IL-2 liposome diameter is significantly different than the theoretically calculated diameter upon IL-2 incorporation ($p<0.001$), swelling due to protein incorporation does not appear to be the only cause.

While swelling is certainly one factor that may affect the average particle diameter determined by DLS, aggregation may also lead to this result. To further investigate whether aggregation has occurred, we analyzed the size distribution of

liposomes in solution prior to and after IL-2 incorporation. From the DLS data, we obtain a distribution of all measured particle sizes within a liposome population. A histogram of these particle sizes within a DMPC liposome population prior to IL-2 incorporation is shown in FIGURE 22a. To allow for visual comparison of any shifts or widening of the population distribution, FIGURE 22a can also be visualized with a line graph as in FIGURE 22b. We have chosen to display the size distribution in this manner for the rest of the data.

FIGURE 23 shows how the size distribution of liposomes prior to and after incorporation of IL-2 is affected. Aggregation is typically manifested as a widening and skewing of the size distribution (Volodkin, Ball, Schaaf, Voegel, & Mohwald, 2007). The size distribution shape of DMPC and DPPC liposomes does not change prior to and after IL-2 incorporation. This suggests that IL-2 incorporation does not induce DMPC or DPPC liposome aggregation. The similarly shaped distribution peak of IL-2 DSPC liposomes indicates that these IL-2 liposomes do not aggregate. While no aggregation is seen for neutrally charged liposomes composed of DMPC (+/- IL-2), DPPC (+/-IL-2), or DSPC (+IL-2), empty DSPC liposomes do appear to aggregate as evidenced by a widening of the size distribution peak. FIGURE 23 also shows that negatively charged DSPC:DSPG liposomes with no IL-2 do not appear to aggregate, but do appear to aggregate upon IL-2 incorporation as evidenced by a widening and skewing of the peak.

Because aggregation is suggested by DLS data in DSPC liposomes without IL-2 and DSPC:DSPG liposomes containing IL-2, we will expound upon how many liposomes appear to be aggregating together in each formulation. To do this, we can make a comparison of the size distribution range of each of these formulations with theoretically calculated liposome aggregate sizes. Consider n=1,2,3…vesicles with diameter, d, aggregating together. The diameter of the sphere, D, that these aggregates fit within, as shown in FIGURE 24, are measured as the particle size by DLS. Assuming a spherical vesicle shape and assuming these spheres do not overlap, the diameter of the sphere containing the vesicle aggregates, D, can be calculated geometrically. These values have been calculated by Kravitz (Kravitz, 1967). We have calculated the theoretical size of empty DSPC liposomes based upon a liposome diameter size of 100nm. Calculations for DSPC:DSPG liposomes with IL-2 are based on a 'swollen' liposome size of 150.878nm (FIGURE 21). Based on the aggregate sizes calculated by FIGURE 24 and the size distributions given by FIGURE 23, empty DSPC liposomes tend to aggregate in groups of approximately 6-7, while IL-2 DSPC:DSPG liposomes tend to aggregate in groups of 4.



n	D
1	d
2	2d
3	2.15d
4	2.41d
5	3d
6	3d
7	3.6d

FIGURE 24: Diameter of smallest sphere, D, containing n number of smaller spheres of identical diameter, d. Values of D based upon d calculated by Kravitz (Kravitz, 1967).

4.3.4 *In Vitro* IL-2 Release Kinetics From Liposome: Effect of Liposome Composition

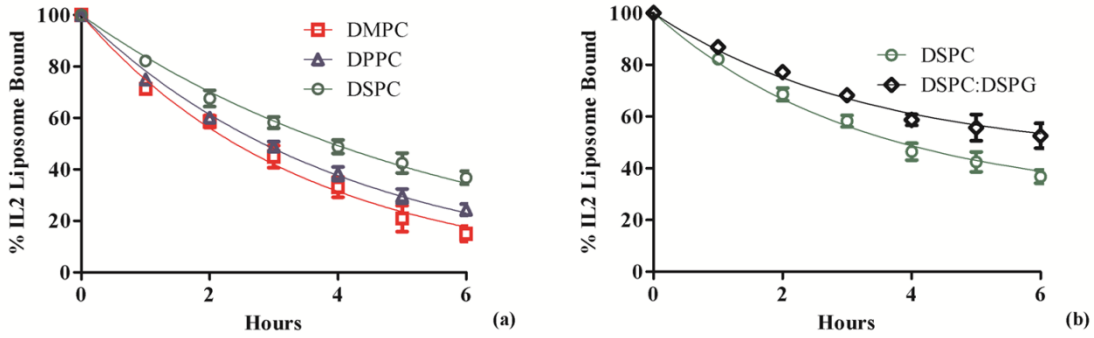


FIGURE 25: Release kinetics of IL-2 from (a) DMPC, DPPC, and DSPC liposomes and (b) DSPC and DSPC:DSPG (10:1) liposomes

FIGURE 25 shows the *in vitro* release kinetics of IL-2 from neutral and charged liposomes, evaluated using a dialysis sample and replace method. Non-incorporated, free IL-2 was removed from the liposome suspension prior to release kinetics evaluation, and it was assumed that all IL-2 was incorporated at time zero (i.e. 100% of the IL-2 was liposome bound). Release kinetics were evaluated over the first 6 hours. After each hourly sampling period, the released IL-2 was removed and fresh water was added to the reservoir to continue to drive diffusion of released IL-2 from the sample dialysis chamber (see section 4.2.7). FIGURE 25a depicts the effects of acyl chain length in neutrally charged liposomes, while FIGURE 25b shows the effects of the incorporation of a negatively charged head group. Each release profile follows an exponential release kinetic model, which has been observed in the literature of IL-2 liposomes (Neville, Boni, et al., 2000).

$$IL2_{bound}(t) = 100[e^{-kt}]$$

EQUATION 18: Exponential kinetic release model, where $IL2_{bound}(t)$ is the percentage of IL-2 remaining bound to the liposome at time, t (measured in hours), and K is the rate of IL-2 released, expressed in hr^{-1}.

	DMPC	DPPC	DSPC	DSPC:DSPG (10:1)
K (1/hr)	0.286±0.007937	0.2442±0.00564	0.1769±0.00537	0.1204±0.005027
R squared value of fit	0.9820	0.9847	0.9669	0.9641

TABLE 3: Exponential kinetic release model parameters for IL-2-liposomes. All release rates are significantly different ($p<0.001$); n=3.

The rate of IL-2 released, k (hr^{-1}), decreases as the acyl chain length increases in neutrally charged liposomes DMPC, DPPC, and DSPC ($p<0.001$). As a comparison, an exponential fit from data extrapolated from Nevile et al and fit with EQUATION 18 revealed an *in vitro* release rate of IL-2 from 177nm DPPC liposomes of approximately $k=0.2126hr^{-1}$, which differs from our value by ~13%. Furthermore, the inclusion of a negatively charged lipid with DSPC (DSPC:DSPG) decreases the rate of IL-2 released *in vitro* by nearly 30%.

After the first 6 hours, the remaining IL-2 liposome constructs were incubated overnight. While hourly sampling and replacing of release media in the reservoir does drive diffusion and thus ultimately affects the release kinetics observed, this still allowed us to make comparisons between formulations of total released IL-2 after 24hours. After 24 hours, there was 5.853±0.847, 8.320±1.827, 28.898±5.203, and 45.305±3.387% IL-2 remaining bound to liposomes. Because the experimental parameters were not identical, no direct comparison between the exponential model used for IL-2 release during the first 6 hours and the amount of IL-2 released after the remaining 18 hours can be made. Rather, the second portion of the release kinetic experiment reflects the nature of the chemical thermodynamic equilibrium state of binding and association. As shown by the results obtained from ITC, the binding constant increases as acyl chain increases. The

binding constant is a function of the concentrations of free and bound components, and reflects the likelihood of a bound complex. IL-2 binds to DSPC liposomes with a higher binding constant than DMPC, thus there is a higher probability of IL-2 binding the DSPC liposomes than DMPC liposomes at any given time.

4.3.5 Bioactivity of Released IL-2: Effect of Liposome Composition

Bioactivity of IL-2 was assessed by its ability to prevent CTLL-2 cell death and lead to cell proliferation (See section 2.2.4). No decrease in bioactivity was seen in any samples, indicating IL-2 released from the carrier did not result in structural changes that affected bioactivity.

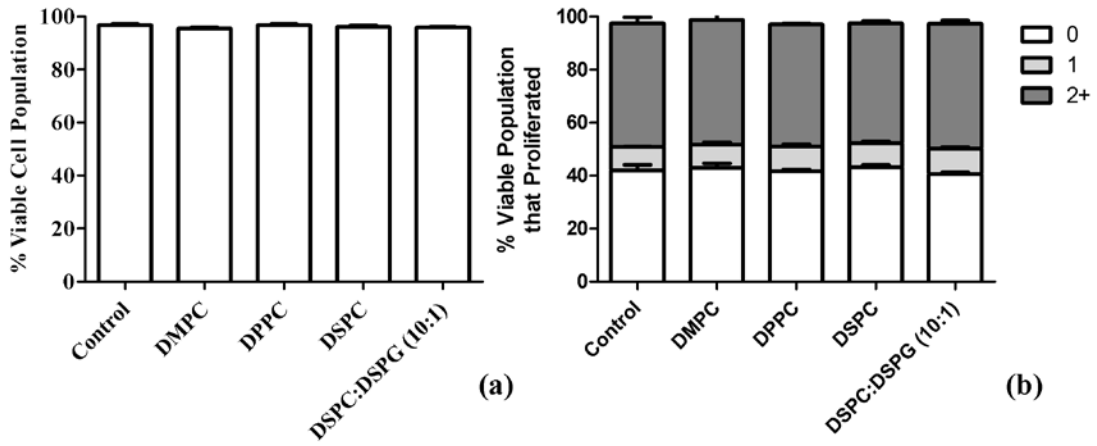


FIGURE 26: Bioactivity of released IL-2 from liposomes. Bioactivity of IL-2 is a function of its ability to lead to CTLL-2 viability (a) and number of cell proliferation cycles among cells that remained viable (b)

4.4 Discussion

In this study, we were interested in evaluating the effect of liposome lipid composition on IL-2 liposome interaction as evidenced by binding thermodynamics, membrane perturbations, IL-2 release kinetics, and functional retention of IL-2. Because To avoid thermal and mechanical stresses associated with encapsulation of the protein

within the interior of the liposome, we chose to absorb IL-2 onto preformed liposome bilayer membranes. Lipid acyl chain effects were examined by formulating with neutrally charged lipids of acyl chain lengths 14, 16, and 18 carbons (DMPC, DPPC, and DSPC, respectively). The effect of a negative charge inclusion was probed by formulating a DSPC:DSPG (10:1) liposome, which is composed of lipids with 18 carbon acyl chains. The choice to include a phosphatidylglycerol (PG) based lipid, which has a net charge of (-1) at pH 7.4, was based upon its inclusion within several IL-2 liposome formulations in the literature (P. M. Anderson et al., 1990; Bergers et al., 1993; Boni et al., 2001; Kanaoka et al., 2001, 2003; Kanaoka et al., 2002; F. J. Koppenhagen et al., 1998). PG has been used in literature to promote IL-2 – liposome association by electrostatic interaction (Bergers et al., 1993) and to prevent liposome aggregation. Electrostatic interaction is often thought to precede adsorption at solid/liquid interfaces (Adamczyk & Warszyński, 1996).

In this study, we have demonstrated evidence that IL-2 associates with neutral and negatively charged liposomes through a predominantly hydrophobic interaction. Predominantly hydrophobic interaction between IL-2 and the lipid acyl chains as opposed to electrostatic interaction is demonstrated by alterations in the thermodynamic binding parameters (obtained by ITC) as the acyl chain length is changed within neutral liposome formulations, but no modification in the thermodynamic parameters when a negatively charged lipid (DSPG) is included within the formulation. While ITC has been used to quantify protein-lipid interactions in the literature (Schote, Ganz, Fahr, & Seelig, 2002; Swamy & Sankhala, 2013), this is the first time to our knowledge that the thermodynamic parameters of binding (enthalpy and entropy contributions, binding constants) for an IL-2

liposome system has been quantified. Our ITC results showed that the enthalpy contributions to binding (ΔH) are approximately two orders of magnitude smaller than the entropic contribution (TΔS) in all liposome formulations, regardless of lipid composition. This entropic contribution increases as the acyl chain length (and thus the area available for hydrophobic interaction with IL-2) increases. However, because no statistical difference in the entropic contributions is seen between IL-2 binding to neutral (DSPC) or charged (DSPC:DSPG) liposomes, electrostatic contributions to binding are likely to be minimal (FIGURE 18). These large entropic changes are indicative of a disruption of the orderly clathrate water structures surrounding free IL-2 in solution (a system with a very low entropy) as the protein enters the acyl chain region of the lipid bilayer and the solvent water molecules are free to assume a wide variety of arrangements (a system with much higher entropy). The minimal enthalpy contributions observed are due to electrostatic interaction, van der Waals interactions, and H-bonds. (Swamy & Sankhala, 2013; Tanford, 1980).

The importance of hydrophobic interaction between IL-2 and the acyl chains of the lipid bilayer within a liposome system is further supported by the protein: lipid molar ratios and binding constants obtained for each system by ITC. As the acyl chain length of neutrally charged liposomes (DMPC, DPPC, and DSPC) is increased, IL-2:lipid molar ratio (FIGURE 16) and binding constant between IL-2 and the lipid bilayers (FIGURE 17) are increased. However, there is no difference in the strength of the binding interaction between IL-2 and neutral (DSPC) or charged liposomes (DSPC:DSPG) (FIGURE 17). One possible reason for an increased IL-2 incorporation into liposomes with longer acyl chains is that the thickness of the bilayer, and thus the hydrophobic

surface area of the bilayer with which IL-2 can interact, is larger. The lengths of DMPC, DPPC, and DSPC acyl chains have been calculated elsewhere to be 2.3, 2.6, and 2.95 ±0.01nm, respectively (Lewis & Engelman, 1983). We will approximate the acyl chain region of these lipid bilayers to be twice these values, which means that the thickness of DMPC, DPPC, and DSPC bilayers would be expected to differ by 0.6-1.3±0.02nm. This difference in length is comparable to the dimensions of a single IL-2 crystal molecule, which has an orthorhombic crystal lattice structure with dimensions of 3.171, 4.97, and 8.468nm (Arkin et al., 2003). A second reason for the difference in stoichiometry between IL-2 and DMPC, DPPC, and DSPC liposomes may be the difference in bilayer acyl chain packing. This is the predominant structural change that occurs as acyl chain increases (Cornell & Separovic, 1983). Increased packing may increase the ability of IL-2 to interact with lipid bilayers due to the proximity between the protein and the bilayer hydrophobic chains.

In addition to the ITC evidence for a predominantly hydrophobic interaction between IL-2 and the hydrophobic acyl chain region, this hydrophobic interaction also appears to impact the rate at which IL-2 is released as shown in FIGURE 25a. While there is not a direct mathematical relationship between the binding strength constant, $K_{association}$ (M^{-1}), and the release rate constant, k (hr^{-1}), given in EQUATION 18, there is a correlation in this work. The larger binding constant for IL-2 to neutral lipids with longer hydrophobic acyl chains results in a slower release rate of IL-2 *in vitro*.

DSC was used to further probe the interaction of IL-2 and liposomes to infer the location of IL-2 within the bilayer. The lamellar gel to liquid-crystalline phase changes (i.e. melting) of bilayer lipids have been studied as a means of probing solute and protein

interaction with lipid membranes. During this cooperative transition hydrophobic acyl chains in a rigid, extended, all-trans conformation increase in intra- and intermolecular motion and many chains change to a gauche conformation (Ruthven & Ronald, 2011). Alterations to the thermodynamic parameters of this lipid phase transition upon protein incorporation or adsorption have been categorized according to the manner in which proteins interact with the bilayer as detailed in 4.2.5. Category 1 proteins associate with the lipid head group through predominantly electrostatic interaction, category 2 proteins are incorporated within both the lipid head group and acyl chain regions, and category 3 proteins associate with only with the lipid acyl chains (Lo & Rahman, 1995). DSC revealed no change in thermodynamic parameters for DMPC, DPPC, and DSPC liposomes. Indeed, IL-2 adsorption onto DPPC liposomes has been shown in literature to result in no significant difference in thermodynamic parameters of lipid melting (F. J. Koppenhagen et al., 1998). This is indicative of a category 1 or category 2 proteins as given in TABLE 1. Given the evidence of hydrophobic interaction between IL-2 and neutral or anionic liposomes, we suspect that IL-2 behaves more like a category 2 protein.

Further support for IL-2 as a category 2 protein is given by the alteration of the anionic (DSPC:DSPG) lipid bilayer melting phase transition parameters. IL-2 incorporation into anionic DSPC:DSPG liposomes results in an increase in ΔH in a similar manner as cytochrome c, a category 2 protein (when formulated above a 4% molar protein to lipid ratio) (Demetzos, 2008). However, while IL-2 did alter the $\Delta T_{1/2}$ upon incorporation into anionic liposomes (DSPC:DSPG) like cytochrome c upon incorporation to DSPG liposomes, the alteration was not identical (TABLE 2). Cytochrome c results in an increase in $\Delta T_{1/2}$ of DSPG lipid bilayers (when formulated

above a 11.5% molar protein to lipid ratio) while IL-2 resulted in a decreased $\Delta T_{1/2}$ upon incorporation to DSPC:DSPG lipid bilayers. The difference between IL-2 and cytochrome c may be due to the lower incorporation of IL-2 as compared to cytochrome c in the DSC studies. IL-2 was able to be formulated at no higher than 3.4% molar protein: lipid ratios, while cytochrome c was formulated at concentrations above 11.5% in DSPG lipid bilayers (Lo & Rahman, 1995). It is also possible that the thermodynamic parameter changes to the lipid bilayer melting phase transition observed for IL-2 DSPC:DSPG liposomes as compared to (IL-2 free) DSPC:DSPG liposomes is a function of liposomal aggregation upon IL-2 incorporation. Aggregation of lipid bilayers may increase the stability of the liposome bilayer structure, ultimately increasing the enthalpic contribution (increased ΔH) required to disrupt this new, more complex lipid structure. By the same logic, the cooperativity of this new, more integrated, aggregated liposomal structure could be expected to be higher (decreased $\Delta T_{1/2}$). Both of these phenomena are seen in our work.

In addition to the observance of a chiefly hydrophobic IL-2 liposome interaction, we have also seen evidence for liposome vesicle aggregation and de-aggregation phenomena upon IL-2 incorporation. DLS was used to determine the average size of the liposomes before and after extrusion. While neutrally charged, empty DMPC liposomes show no aggregation, there was a three-fold increase in the average liposome particle size as the acyl chain length of the lipids increases from 14 carbons (DMPC) to 18 carbons (DSPC) in neutrally changed liposomes. Some aggregation is expected in liposomes with composed of neutrally charged lipids due to a lack of electrostatic surface repulsion, but we observe aggregation phenomena dependent on chain length. This may be attributed to

localized surface charge distribution. While phosphatidylcholine based liposomes are composed of lipids with an overall neutral charge, they do exhibit a very small negative surface charge at low ionic strengths (Makino et al., 1991), with this charge being strongest for DMPC and weakest for DSPC. The reason for this is due to the orientation of the head group. Phosphatidylcholine lipids have a negatively charged phosphate group and a positively charged choline group. At low ionic strengths, DMPC, DPPC, and DSPC the positively charged choline group is buried within the bilayer, while the phosphate portion in oriented such that it is closer to the surface of the bilayer. The angle between the lipid head group and acyl chain increases as the acyl chain length increases due to lipid molecule packing constraints, leading to more equal surface exposure of the negative and positive charges on the lipid head group (Makino et al., 1991) (FIGURE 27). This would be expected to result in a decreased electrostatic repulsion between vesicles, and thus aggregation. Indeed, such a trend is reflected in our data for the phosphatidylcholine lipids before IL-2 incorporation for DSPC liposomes (FIGURE 21). Not surprisingly, empty DSPC:DSPG liposomes, which have an overall negative charge, electrostatically repel one another and resist aggregation. As shown in FIGURE 21, empty DSPC:DSPG liposomes exhibit good colloidal stability and retain an average particle size of 116nm.

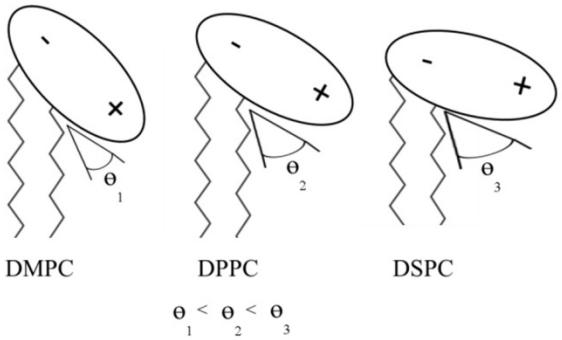


FIGURE 27: Nuetral lipid head group orientation as a function of acyl chain length

While empty DSPC liposomes aggregated and empty DSPC:DSPG liposomes did not, aggregation phenomena of neutrally and negatively charged liposomes composed of

18 carbon acyl chains were altered upon IL-2 incorporation. IL-2 incorporation to DSPC liposomes causes a marked narrowing of the particle size distribution (FIGURE 23) and an average particle size that is significantly smaller than the empty liposome (FIGURE 21). Furthermore, the average size of the IL-2 liposomes is not statistically different than the theoretically calculated value. Thus the aggregation phenomena observed in the empty DSPC liposomes is reversed after IL-2 incorporation suggesting that vesicle fusion is not responsible for larger vesicle size observed in empty DSPC liposomes.

Upon incorporation of IL-2, negatively charged DSPC:DSPG liposome particle size increases by a factor of 3. Aggregation of negatively charged liposomes has been observed in the literature upon protein incorporation into the bilayer (Kiyota, Lee, & Sugihara, 1996; Persson, Thorén, & Nordén, 2001). Kiyota et al evaluated the incorporation of small, amphiphilic, α-helical model peptides composed of hydrophobic leucine and positively charged lysine amino acids into neutral and charged liposomes. Incorporation into neutrally charged liposomes did not cause vesicle aggregation, but incorporation of some amphiphilic peptides into anionic liposomes did, as evidenced by turbidity measurements. The aggregation of anionic liposomes occurred when the positively charged residues were exposed on the liposome surface, allowing for interaction with neighboring anionic vesicles (Kiyota

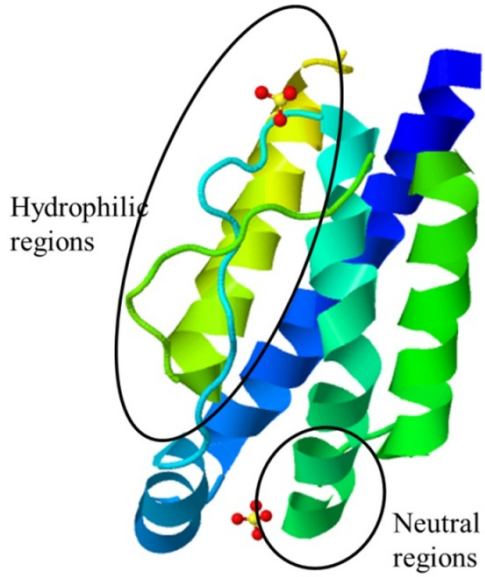


FIGURE 28: Hydrophilic and neutral regions of IL-2 (image from Protein Data Bank viewer, PDB Identifier 1M47)

et al., 1996). While IL-2 is a relatively hydrophobic cytokine, it does have both hydrophilic and hydrophobic regions (C. A. Bergmann, Landmeier, & Kaplan, 1991). These hydrophilic, hydrophobic, and neutral subunits of IL-2 have been evaluated by Bergman by the generation of a hydrophilicity plot using the Hopp Woods method. Hydrophilic subunits were found to lie between amino acid residues 47-56 and 95-133 (Christoph Alexander Bergmann, 1991). These sections contain four positively charged amino acids at Arg 120, Lys 48, Lys 49, and Lys 54. The hydrophilic and neutral regions of IL-2 have been circled in FIGURE 28.

Assuming that amphiphilic IL-2 interacts with neutral and anionic liposomes in a similar manner as described for small amphiphilic helical peptides by Kiyota et al (Kiyota et al., 1996), we have proposed a theoretical interaction for IL-2 to DMPC, DPPC, DSPC, and DSPC:DSPG liposomes, shown in FIGURE 29. Because the hydrophilic and hydrophobic residues are clustered together (FIGURE 28), the proposed model shown may not necessarily require major protein conformational structure alterations upon IL-2 incorporation. The hydrophobic portion of the protein embeds within the lipid acyl chain region, while the more hydrophilic regions are excluded from this hydrophobic region (Groves, 2006). The more hydrophilic region of the protein extends into the aqueous environment. We

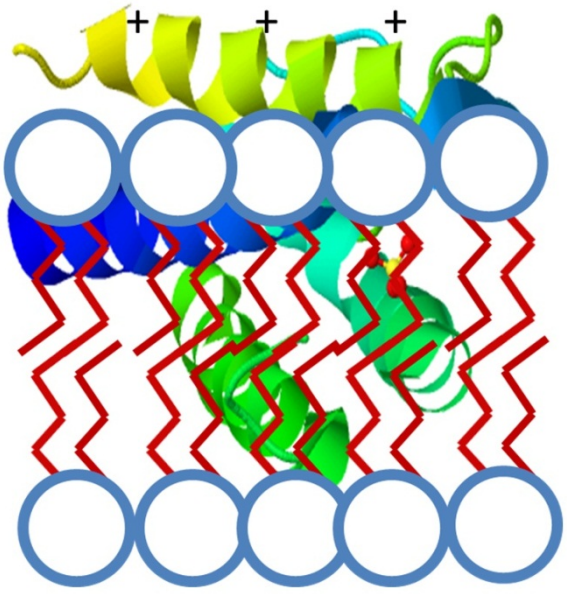


FIGURE 29: Proposed interaction model for IL-2 interaction with DMPC, DPPC, DSPC, and DSPC:DSPG liposomes.

will assume that this region includes Lys 48, Lys 49, Lys 54 and Arg 120 (Christoph Alexander Bergmann, 1991), which are all positively charged. Because the hydrophobic regions of the protein outnumber the hydrophilic regions, the bulk of the protein is assumed to be embedded within the hydrophobic acyl chains. This would lead to a larger hydrophobic involvement in binding, which is supported by the larger entropic contribution observed by our ITC experiments. Evidence for a mainly hydrophobic interaction is also corroborated by the observed dependence of stoichiometry and the binding constant on acyl chain length, as previously discussed. Our ITC data also suggests a small amount of electrostatic interaction between IL-2 and neutral as well as anionic liposomes (FIGURE 18b), which also supports our proposed model (FIGURE 29). Furthermore, there is evidence in the literature supporting such a model. Neville et al observed disturbances in the acyl chain region of DMPC liposomes upon IL-2 incorporation, indicating IL-2 at least partially partitioned within the hydrophobic bilayer. Part of the IL-2 molecule was also displayed on the surface of the liposome as evidenced by the ability of an anti-IL-2 antibody to bind to the IL-2 liposome (Neville, Boni, et al., 2000). Koppenhagen et al observed no change to secondary structure upon IL-2 incorporation into DPPC liposomes, but did see a change in the steady state fluorescence of the single tryptophan (Trp) residue in IL-2. This residue, Trp121, is next to Arg 120, which is one of the cationic amino acids that we propose to be extended outside the hydrophobic acyl chains (F. J. Koppenhagen et al., 1998).

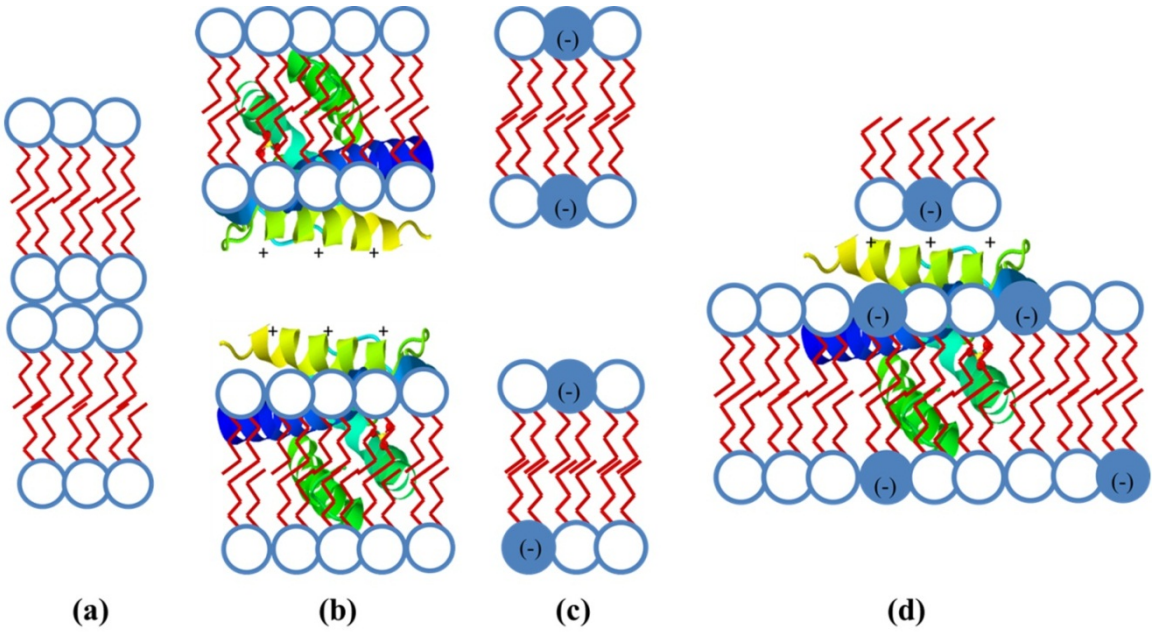


FIGURE 30: Aggregation and de-aggregation due to IL-2 incorporation to neutral and anionic liposomes. (a) Aggregation of neutrally charged empty DSPC liposomes. (b) Exposure of positively charged residues on hydrophilic regions of IL-2 on the surface of the DSPC liposome increases electrostatic repulsion between the surfaces of IL-2 DSPC liposomes. (c) Colloidal stability (no aggregation) of empty anionic DSPC:DSPG (10:1) liposomes. (d) Exposure of positively charged residues on hydrophilic regions of IL-2 on the surface of the DSPC:DSPG liposome results in electrostatic attraction with negatively charged PG head groups of neighboring vesicles.

This proposed theoretical model of IL-2 liposome interaction also explains the liposome aggregation and de-aggregation phenomena observed upon incorporation IL-2 incorporation into DSPC and DSPC:DSPG liposomes as shown in FIGURE 30. Empty DSPC liposomes (FIGURE 30a) tend to aggregate due to a lack of electrostatic repulsion, resulting in the increased aggregation observed in our work. Based on the theoretical model of IL-2 lipid interaction, exposed hydrophilic regions of the protein containing Lys or Arg (positively charged) would lead to electrostatic repulsion of IL-2 DSPC liposomes (FIGURE 30b). Thus IL-2 incorporation into DSPC liposomes serves to increase the colloidal stability of the liposome vesicle in a similar manner as other hydrophilic polymer surface coatings used in liposome formulations (Mufamadi et al.,

2011). Empty DSPC:DSPG liposomes (FIGURE 30c), do not tend to aggregate due to the electrostatic repulsion between the negatively charged head groups. Incorporation of IL-2 into DSPC:DSPG liposomes (FIGURE 30d) may lead to aggregation due to electrostatic attraction between the exposed positive charges on the liposome surface and the negatively charged PG head groups of neighboring IL-2 DSPC:DSPG liposomes.

This proposed theoretical model of interaction and its role in aggregation also clarifies some of the observations not entirely explained by hydrophobic interactions. For example, the release rates of IL-2 from neutrally charged liposomes correlate with the length of the hydrophobic acyl chain. By this logic, the IL-2 release rate from DSPC:DSPG liposomes should be similar that to that of DSPC liposomes. However we observe IL-2 releases more slowly from DSPC:DSPG liposomes than DSPC liposomes (FIGURE 25). Furthermore, we observed that upon further release over 24hours, a larger amount of IL-2 was retained by DSPC:DSPG liposomes as opposed to DSPC (28.898±5.203% IL2 remaining bound to DSPC liposomes versus 45.305±3.387% for DSPC:DSPG liposomes. Aggregation of DSPC:DSPG liposomes containing IL-2 could result in IL-2 that is trapped between vesicles, ultimately slowing release during the 6 hour sample and replace dialysis release kinetic experiment (FIGURE 25) and increasing the amount of IL-2 not released during the next 18 hours of dialysis (see section 4.3.4), as compared to neutrally charged DSPC IL-2 liposomes.

The aggregation behavior of IL-2 DSPC:DSPG liposomes may also explain why the ratio of protein to lipid is lower for DSPC:DSPG versus DSPC (0.083±0.004 for DSPC versus 0.052±3.3E-4, $p<0.001$ for DSPC:DSPG, FIGURE 16) even though there is no statistical difference between these two formulations with regards to binding strength

($K_{association}$) or enthalpy and entropy contributions to binding. The lower amount of protein incorporated into DSPC:DSPG liposomes may be a reflection of decreased available liposome surface area available for binding due to aggregation upon IL-2 incorporation to these anionic liposomes.

Finally, bioactivity of released protein was not altered as evidenced by FIGURE 26. This is a very promising result and may be a reflection of the how IL-2 is incorporated within the lipid bilayer. Our model assumes that the distinct regions of hydrophobicity and hydrophilicity shown in FIGURE 28 remain grouped together within the lipid bilayer (FIGURE 29). Such a model may not require major protein conformational changes from the native structure. Indeed, no major structural alterations upon IL-2 incorporation to DPPC liposomes were observed by Koppenhagen et al as evaluated by circular dichroism (F. J. Koppenhagen et al., 1998).

4.5 Conclusions

In this work we sought to evaluate the biomolecular interactions between IL-2 and liposomes composed of phosphatidylcholine based lipids and a mixture of phosphatidylcholine and phosphatidylglycerol lipids in order to aid in the rational design of IL-2 liposome drug delivery constructs. The effect of acyl chain length and lipid charge on protein-lipid interaction and the subsequent implications for pharmaceutical formulation were considered. We found that hydrophobic interaction of IL-2 with the lipid acyl chains was the primary driving force behind IL-2 and liposome incorporation in each case, and that an increase in acyl chain length led to a more efficient and tightly bound IL-2 – liposome compound. Bioactivity of released protein was not significantly altered. However, incorporation of IL-2 into these liposomes significantly changed the

size and aggregation behavior of the vesicles, and ultimately the kinetic release IL-2, elements that ultimately influence the pharmaceutical and pharmacokinetic behavior of drug loaded liposomes *in vivo*.

The effect of IL-2 incorporation into lipid vesicles is extremely important to consider in rational drug delivery system design. Our data indicates that higher IL-2 incorporation ratios and slower release kinetics may be obtained for IL-2 liposomes may be obtained by formulating with lipids with longer acyl chains. While negatively charged lipids are often included in liposomal formulations to prevent aggregation, anionic liposomes tend to aggregate when formulated with IL-2 within the bilayer. Neutrally charged liposomes, which are often assumed to aggregate due to a lack of electrostatic interaction, have excellent colloidal stability (i.e. do not aggregate) when formulated with IL-2 within the bilayer. Aggregation of IL-2 loaded anionic liposomes slowed the release rate, but also decreased the protein to lipid molar ratio. Depending on the therapeutic goal, one formulation may be preferred to the other. While aggregation of nanoparticles intended for circulation is not desired, it may be desirable in a depot controlled release construct.

CHAPTER 5: CONCLUSIONS, IMPLICATIONS, AND FUTURE DIRECTIONS

In this work we have evaluated specific stability issues of IL-2, a pharmaceutical protein, within aqueous and lipid environments. We chose the evaluate IL-2 thermal stability within an aqueous environment. The ability to withstand thermal stress is particularly relevant for IL-2 because its thermal denaturation pathway is irreversible. Thermal stress also speeds some of the denaturation pathway rates specific to IL-2, such as oxidation, deamidation, and aggregation (Cadee et al., 2001; Philo & Arakawa, 2009; Sasaoki et al., 1992; K. D. Weaver et al., 2012). Thermal stress is often a factor in liposome formulation as well; to achieve encapsulation of a protein drug within the aqueous compartment of a liposome, the protein and liposome is traditionally heated together to at least 10˚C above the thermal transition temperature of the lipids before size homogenization via extrusion or sonication. Therefore lipid choice is often limited to low transition temperature lipids, and it was of interest whether mitigation of such thermal stresses with the excipients examined in this work could allow a wider selection range of lipids for liposome formulation.

We chose a promising organic salt, CDHP, as a possible thermal stabilizer of IL-2 on the basis of previous studies by our lab and others which found CDHP increased the thermal midpoint transition temperature (T_m) of model proteins formulated with CDHP. We examined specific ion effects and found that the conformational stability imparted to IL-2 was primarily due to the presence of the kosmotropic ion, dihydrogen phosphate.

While high concentrations of CDHP were able to increase the T_m of IL-2 and impart conformational stability to the protein at temperatures above this temperature, formulating IL-2 with CDHP does come at a bioactivity cost. This decrease in bioactivity was proposed to originate from the same factors responsible for the increase in thermal and conformational stability. CDHP contains a kosmotropic ion, which tends to preferentially interact with the water molecules surrounding the protein, thereby kinetically protecting IL-2 from perturbations of bulk water molecules. However this kinetic stability may result in a decrease in molecular mobility required for full IL-2 bioactivity. If thermal stress is unavoidable, CDHP may be used to thermally stabilize the protein; however protein concentrations must be adjusted to account for the change in bioactivity of the final product. In this work we also explored formulation options that avoided thermal stress by IL-2 encapsulation into liposomes. IL-2 was successfully adsorbed onto the bilayer of liposomes, with a clear relationship between acyl chain length and the IL-2: lipid ratios obtained. The lipid showing the highest strength interaction with IL-2, DSPC, would have been restricted from use if traditional encapsulation methodologies had been chosen because the protein would have to be heated above its T_m. By avoiding thermal and mechanical stress and adsorbing IL-2 to preformed liposomes, the bioactivity of the subsequently released IL-2 was maintained.

The interaction between IL-2 and the lipids chosen in this study was driven primarily by hydrophobic mechanisms. It was also found that because the incorporation of IL-2 into the lipid bilayer presumably changed the surface charge distribution, aggregation phenomena of the final product, not just the IL-2 free liposome, is extremely important to consider. Aggregation and de-aggregation phenomena occur after IL-2

incorporation to neutral and charged liposomes based upon the deviation of liposome size from the extruded size upon IL-2 incorporation are particularly relevant from a therapeutic standpoint. Liposome carrier size is an extremely important variable in targeting of specific tissues, organs, or systems. For example, although it has not been attempted before, targeting of IL-2 to the lymphatic system using a liposomal carrier has promise due to the unique action of this drug. It has been proposed in literature that the adverse effects of this immunotherapeutic are associated with IL-2 interaction with certain entities within the blood stream (namely natural killer cells and neutrophils), while the beneficial effects are due to the interaction of the protein with IL2R expressing activated CD4+ T-cells, which reside largely in the lymphatic system (Caligiuri, 1993; Stites, 1994; van Haelst Pisani et al., 1991). By this logic, targeting of the lymphatic system may enhance the therapeutic benefits of IL2 therapy, while limiting toxicity. While several routes to target the lymphatic system exist including, intradermal (i.d) (A. Supersaxo, Hein, Gallati, & Steffen, 1988), intraperitoneal (i.p.) (Maincent et al., 1992), intragastric (Potluri & Betageri, 2006), and pulmonary routes, it has been found that 10-100nm sized liposomes are efficiently transported from subcutaneous tissue into the lymphatic system (Christien Oussoren & Storm, 2001; C. Oussoren et al., 1998; C. Oussoren et al., 1997; Zuidema et al., 1994). Because IL-2 incorporation into neutral liposomes increased vesicle diameter by up to 3 fold (from the extruded diameter) in DSPC formulations, liposomes intended for subcutaneously administered lymphatic targeting must be extruded at smaller diameters. Further study is needed to optimize the size upon extrusion and protein lipid molar ratios.

Because nanoparticles are commonly injected into the body with the expectation that they will circulate to some desired region in the body, aggregation of nanoparticles is not typically desired. However aggregation may hold promise as a liposomal depot/reservoir. For example, subcutaneous or intramuscularly administered liposomes that aggregate will not be able to diffuse into blood or lymphatic circulation, but may release their cargo locally. Maintaining low levels of locally administered IL-2 has been shown to result in better therapeutic outcomes rather than high doses of systemic IL-2 (Bubeník, 1990; Bubenik & Indrova, 1987; Den Otter et al., 2008).

The results from these studies have revealed the sensitivity of IL-2 to thermal stress, and revealed the potential tradeoffs between thermal stability and bioactivity. Given this data, we have formulated several liposomal carriers without exposure to thermal stress that were able to release bioactive IL-2. We have characterized the nature of the interaction between these carriers and IL-2 and have evaluated any aggregation effects. These results may aid in the further development of targeting liposomal systems or depot drug delivery designs for IL-2. Formulating liposomes composed of the longest acyl chains (DSPC and DSPC:DSPG) resulted in the highest protein incorporation ratios and the slowest release of IL-2 *in vitro*. While IL-2 liposomes composed of neutrally charged lipids showed little to no aggregation, anionic IL-2 liposomes (DSPC:DSPG) showed significant aggregation tendency. Aggregation not only has an impact on the pharmacokinetics of nanoparticle carriers, but it also has an impact on the interaction between IL-2 and the lipid membrane. Aggregated DSPC:DSPG IL-2 liposomes resulted in a slower release of IL-2. Furthermore, aggregation of anionic liposomes upon IL-2 incorporation may have decreased the protein to lipid molar ratios obtained. Depending

on the pharmacokinetic, release profile, and dosing needs, these data suggest that lipid acyl chain length and lipid charge can be optimized to control these relevant pharmaceutical parameters.

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