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Development and Characterization of Long-Circulating Emulsions to Target Solid Tumors

par

Joanna Rossi

Sciences pharmaceutiques

Faculté de pharmacie

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présenté par :

Joanna Rossi

a été évalué par un jury composé des personnes suivantes :

Dr. Emmanuelle Roux, président-rapporteur

Dr. Jean-Christophe Leroux, directeur de recherche

Dr. Christine Allen, co-directrice

Dr. François-Xavier Lacasse, membre du jury

Résumé

Des émulsions furtives ont été préparées à l'aide d'excipients approuvés pour une application pharmaceutique et ont été évaluées *in vivo* pour leur capacité à cibler des tissus néoplasiques. Les émulsions étaient composées d'une phase interne de triglycéride et émulsifiées avec un tensioactif synthétique (polysorbate 80) et un co-émulsifiant lipidique. Afin de produire des émulsions furtives, de la sphingomyéline d'œuf (ESM) a été choisie comme co-émulsifiant et différents dérivés de 1,2-distéaryl-*sn*-glycero-3-phosphatidyléthanolamine-poly(éthylène glycol) (DSPE-PEG) ont été greffés à l'interface de l'émulsion. Les dérivés sélectionnés de DSPE-PEG étaient la DSPE-PEG 2000, la DSPE-PEG 5000, et la DSPE-*N*-[pentaérythritol polyoxyéthylène glutaryl] (DSPE-4armPEG), (MM 2000). Les effets de l'ESM et de la DSPE-PEG (la concentration et la structure) sur la prolongation du temps de circulation et sur l'accroissement de l'accumulation dans les tissus néoplasiques ont été évalués sur des souris inoculées du mélanome B16 et de l'adénocarcinome du côlon C26 en sous-cutané.

Dans cette étude, nous rapportons que des émulsions furtives ont été obtenues en enrobant la surface des gouttelettes avec de la DSPE-PEG 2000 ou 5000. L'accroissement du temps de circulation n'a pu être atteint ni avec la DSPE-4-armPEG malgré le segment de PEG de masse molaire de 2000 ni avec l'ESM. Le temps de circulation accru des émulsions enrobées de PEG 2000 ou 5000 s'est traduit par une accumulation plus élevée dans les tumeurs C26, mais pas dans les tumeurs B16. Ces émulsions pouvaient améliorer la sélectivité d'agents anticancéreux

lipophiles pour certains tissus néoplasiques et aussi augmenter leur index thérapeutique.

Mots-clés : Émulsions furtives, poly(éthylène glycol), biodistribution, pharmacocinétique, vecteurs de médicaments

Abstract

Long-circulating emulsions were prepared using pharmaceutically acceptable excipients and evaluated *in vivo* for their ability to target neoplastic tissues. The emulsions were composed of a triglyceride internal phase and emulsified with a synthetic surfactant (polysorbate 80) and a lipid emulsifier. Attempts made to produce long-circulating emulsions included adding egg sphingomyelin (ESM) as a coemulsifier and grafting various 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-poly(ethylene glycol) (DSPE-PEG) derivatives into the emulsion interface. The DSPE-PEG derivatives selected were DSPE-PEG 2000, DSPE-PEG 5000 and DSPE-*N*-[pentaerythritol polyoxyethylene) glutaryl] (DSPE-4-armPEG), (MW 2000). The effect of ESM and DSPE-PEG concentration and structure in prolonging circulation time and enhancing accumulation into neoplastic tissues was assessed in mice bearing subcutaneously implanted B16 melanoma or C26 colon adenocarcinoma.

In this study, we report that long-circulating emulsions were obtained by coating the droplet surface with single chain DSPE-PEG 2000 or 5000. Circulation longevity could not be achieved with DSPE-4-armPEG despite the 2000 MW PEG segment nor with ESM. Enhanced circulation time of emulsions grafted with PEG 2000 or 5000 translated into higher accumulation into C26 tumors but not into B16. These emulsions can potentially enhance the specificity of lipophilic anticancer drugs towards neoplastic tissues and enhance the therapeutic index.

Keywords: Long-circulating emulsions, poly(ethylene glycol), biodistribution, pharmacokinetics, drug carriers

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Abbreviations

π	surface pressure
A	molecular area
A_π	molecular area at a particular surface pressure
Apo	apolipoprotein
AUC	area under the blood concentration-time curve
CHE	cholesteryl hexadecyl ether
Chol	cholesterol
CMC	critical micelle concentration
CO	cholesteryl oleate
C_s	modulus of compressibility
DLVO	Derjaguin, Landau, Vervey, and Overbeek theory of colloidal stability
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine
DOTAP	1,2-dioleoyl-*sn*-glycero-3-trimethylammonium propane
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine
DSPE	1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine

DSPE-4-armPEG	1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[(pentaerythritol polyoxyethylene) glutaryl]
DSPE-PEG	1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine-*N*-monomethoxy-[poly(ethylene glycol)]
EPR	enhanced permeation and retention
ESM	egg sphingomyelin
Gal	galactosylated
HDL	high density lipoprotein
HLB	hydrophile-lipophile balance
$HLB_{required}$	hydrophile-lipophile balance required
HPMA	hydroxypropylmethacrylamide
HSPC	hydrogenated soybean phosphatidylcholine
IDU-Ol_2	iododeoxyuridine-oleoyl
LCT	long-chain triglyceride
LL2	anti-B-cell lymphoma monoclonal antibody
LPL	lipoprotein lipase
LUV	large unilamellar vesicle
MCT	medium-chain triglyceride
MLV	multilamellar vesicle
MPS	mononuclear phagocyte system
MTD	maximum tolerated dose
ODN	oligonucleotide

O/W	oil-in-water emulsion
PA	phosphatic acid
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEG	poly(ethylene glycol)
PEG-*b*-PDLLA	poly(ethylene glycol)-*b*- poly(D,L-lactide)
PEG_{2000}-DPPE	poly(ethylene glycol)-modified 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine
PEG-DOPE	*N*-(monomethoxy-poly(ethylene glycol)-succinyl) phosphatidylethanolamine
PEG_{2000}-POPE	poly(ethylene glycol)-*N*- 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine
PEO	poly(ethylene oxide)
PEO-*b*-PPO-*b*-PEO	poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) (poloxamer)
Pgp	P-glycoprotein
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine
PS	phosphatidylserine
PS-80	poly(ethylene oxide) 20 sorbitan monooleate (polysorbate 80)

PSPC	1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphatidylcholine
PVA	polyvinylalcohol
PVP	polyvinylpyrrolidone
PVP-*b*-PDLLA	poly(*N*-vinylpyrrolidone)-*b*-poly(D,L-lactide)
RES	reticuloendothelial system
SCT	short-chain triglyceride
SM	sphingomyelin
SMANCS	conjugate of neocarzinostatin and poly(styrene-comaleic acid)
SUV	small unilamellar vesicle
T_c	transition temperature
TC	tricaprylin
TO	triolein
TPGS	D-α-tocopheryl polyethyleneglycol 1000 succinate
W/O	water-in-oil emulsion

For my loving husband

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CHAPTER 1: COLLOIDAL DRUG CARRIERS

1. Introduction

Conventional, low-molecular weight therapeutics often have the ability to traverse across various biological membranes and compartments, showing little or no selectivity for diseased tissues over healthy ones [1]. This poor specificity for the target site often leads to undesirable side-effects and low proportions of the administered dose reaching the intended site of action in the body. As a result, higher doses often need to be administered to achieve therapeutic concentrations at the target site. To circumvent this non-specific drug delivery, substantial efforts have been made to alter the pharmacokinetic and tissue distribution of drugs by incorporating or attaching them to colloidal systems such as liposomes, micelles, macromolecular prodrugs, polymeric nanoparticles, and emulsions. By including or linking them to colloidal carriers the distribution of the therapeutic agent no longer depends on the physicochemical properties of the drug molecule but instead is contingent on the features of the carrier.

One way in which colloids achieve selectivity is a result of their large size which restricts extravasation to locations in the body with permeable vasculature (see Figure 1). Solid tumors and sites of infection or inflammation often have porous blood capillaries, which allow for the passage of nano-sized colloidal drug carrier across the endothelium and into the extravascular space (passive targeting). Given that the majority of the vascular endothelium is continuous with tight junctions between neighboring endothelial cells, active compounds associated with colloidal carriers are prevented from reaching the extravascular space of most tissues in the body reducing many of the adverse side effects caused by drugs in the free form [2].

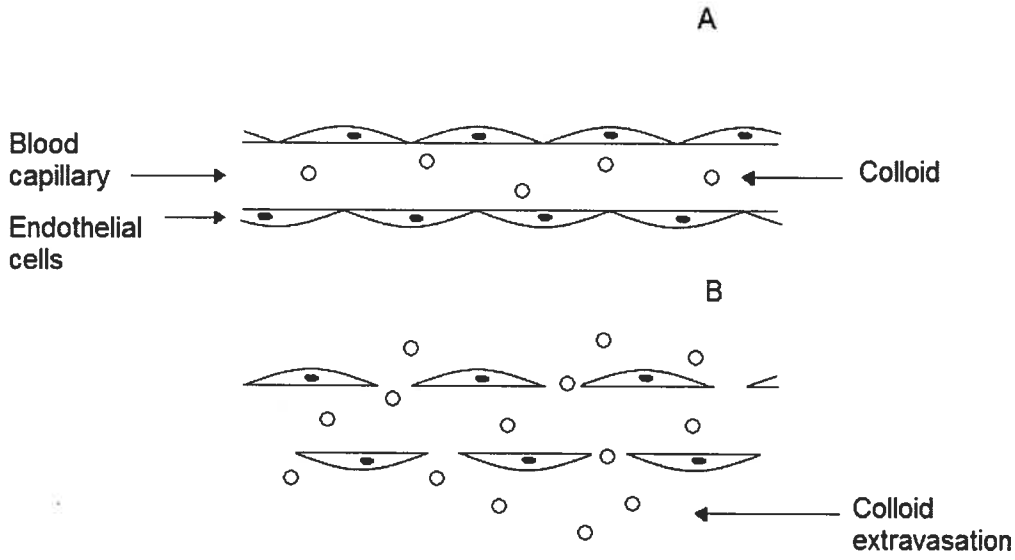

Figure 1: Capillary structure of normal tissues with a continuous endothelial lining (A) and tumors possessing enhanced vascular permeability (B).

In addition to the selectivity imparted by size, targeting moieties that are specific for determinants found primarily or in high amounts on the membrane of target cells can be attached to the surface of the carrier to enhance specificity (active targeting).

Another application of colloids is in the controlled release of therapeutics, whereby they act as reservoirs that release the encapsulated drug into the blood stream slowly. Such sustained release systems can maintain therapeutic drug levels in the blood, reducing the frequency of administration. Furthermore, colloidal drug delivery systems can also overcome efflux pumps such as P-glycoprotein (Pgp) by changing the pathway in which drugs enter the cell. Intracellular internalization of colloids by endocytosis locates the drug in an endosome/lysosome, which reduces interactions with Pgp compared to drugs in the free form that traverse across the cell membrane by diffusion [3]. Other advantages of colloidal drug delivery systems include protection of the encapsulated drug from premature degradation and enhanced intracellular delivery of certain therapeutic compounds. For instance, free or un-encapsulated genetic material requires a carrier to enter the cell because of the unfavorable electrostatic interactions between DNA and cell membranes.

Depending on their size and the physicochemical properties of the surface, colloids can be rapidly taken up by the cells of the mononuclear phagocyte system (MPS) and quickly removed from the systemic circulation. Such systems are ideal for macrophage targeting. On the other hand, colloids can exhibit long-circulating properties in blood and target sites in the body other than MPS tissues.

Indeed, colloidal drug delivery systems offer many benefits over drugs in the free form. These systems, however, vary in terms of physicochemical properties and

thus have different advantages and drawbacks. An overview of the most common colloidal drug delivery systems is briefly presented in the following sections.

2. Liposomes

Among the various colloidal drug delivery systems investigated, liposomes are the most widely studied. They can be formed from either synthetic lipids or lipids originating from biological membranes. Liposomes typically range in size from 50 – 10,000 nm and are classified as either small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs) or multilamellar vesicles (MLVs). LUVs have diameters usually between 100 to 500 nm and are bigger than SUVs [4]. They are composed of an aqueous inner core surrounded by a single lipid bilayer. In contrast, MLVs contain several concentric lipid bilayers and vary in size from 100 to 10,000 nm [4]. These unique structures permit the encapsulation of either hydrophilic or hydrophobic compounds. The behavior of these systems in the host depends largely on size, bilayer rigidity and surface charge [5, 6].

Based on composition and therapeutic application, liposomes are generally categorized into 4 major types which are conventional, 'stealth' or long-circulating, immunoliposomes (targeted), and cationic liposomes [7]. Conventional liposomes are usually quickly removed from the systemic circulation by the MPS and thus are more appropriate for macrophage targeting, local depot or antigen delivery (vaccination) [7]. Conversely, 'stealth' or long-circulating liposomes evade detection by the MPS and tend to extravasate into tissues with enhanced vascular permeability (i.e. solids tumors and sites of infection or inflammation) [8-10]. The most widely used method to produce liposomes with enhanced blood residence times is to coat the surface of

the carrier with poly(ethylene glycol) (PEG). This polymer provides long-circulating properties by reducing interactions with plasma proteins and cell surfaces because of its highly hydrated and flexible polymer chains [11, 12].

The specificity of encapsulated drugs towards the intended location can be enhanced by attaching ligands such as antibodies, antibody fragments, peptides, carbohydrates, vitamins or hormones onto the surface of the carrier that are specific for certain sites on the membrane of target cells [13]. If the encapsulated drug is to be delivered to non-MPS tissues, PEG can be grafted onto the surface of the liposome along with the targeting ligands to reduce uptake by macrophages [14]. Apart from being carriers for conventional drugs, cationic liposome complexes are being developed to protect genetic material (e.g. DNA and RNA) from degradation in the blood stream and enhance transfection into the cell [15].

As a result of the large effort in developing liposomes as drug delivery vehicles, several liposome formulations are presently on the market, such as Doxil®/Caelyx™, Myocet®, DepoCyt®, and AmBisome®, just to name a few [16]. Liposomal products currently on the market offer treatment for a wide range of illnesses including cancer, Kaposi's sarcoma, fungal infections, and meningitis.

Despite the many advantages of liposomal drug delivery systems there are several drawbacks, which include poor efficiency to load hydrophobic molecules as a result of the limited solubility in the lipid bilayer and poor stability during storage due to hydrolysis and/or oxidation of the lipids in the bilayer. Formulation stability can be improved by preparing the liposomes with saturated lipids and displacing the air with an inert gas such as argon.

3. Micelles

Micelles are core-shell structures formed from amphiphilic molecules such as low-molecular weight surfactants or block copolymers. These amphiphilic molecules self-assemble in aqueous solvents at concentrations above the critical micelle concentration (CMC). The hydrophobic core provides a cargo space for poorly-water soluble compounds, while the hydrophilic corona permits solubilization in aqueous media [17]. As a result of their structure, micelles can considerably enhance the solubility of hydrophobic molecules in water and possibly protect the sequestered drug from chemical and/or enzymatic degradation in the host.

Micelles can be classified as either low-molecular weight surfactant micelles or polymeric micelles depending on the molecular weight of the amphiphile. Low-molecular weight surfactant micelles typically have high CMC values and low core viscosity, resulting in poor stability upon dilution in solution and in the blood stream after intravenous (i.v.) administration [18]. In addition, many low-molecular weight surfactants cause adverse side reactions. For instance, Cremophor® EL has been associated with severe hypersensitivity reactions in many patients [19]. These side effects were also observed with polysorbate 80, but to a much lesser extent [20].

Compared to surfactant micelles, polymeric micelles have several advantages, such as reduced toxicity, higher drug loading capacity and greater stability upon dilution due to the lower CMC values. For example, poly(*N*-vinylpyrrolidone)-*b*-poly(D,L-lactide) (PVP-*b*-PDLLA) with 27 and 37% DLLA have CMCs of 10 and 6 mg/L, respectively [21]. These values are considerably lower than those of common

low molecular weight surfactants such as Cremophor® EL and polysorbate 80 with CMCs of 90 and 100 mg/L, respectively [22].

PEG is commonly used as the hydrophilic segment of the block copolymer to prolong systemic circulation time and target sites other than the cells of the MPS [23]. Attaching targeting ligands to the hydrophilic block of polymeric micelles can potentially improve drug delivery, although research on these systems is not well advanced yet [18]. Presently, a novel injectable polymeric micelle formulation of paclitaxel (Genexol®, PEG-*b*-PDLLA) is being evaluated in Phase II clinical trials in patients with advanced breast and non-small cell lung cancers [24]. This formulation was reported to have lower toxicity and enhanced efficacy in mice compared to the commercial low-molecular weight micelle formulation of paclitaxel (Taxol®) [25].

Micelles can also improve the delivery of genetic material by electrostatic complexation of polyanionic DNA with a cationic segment of a block copolymer, linked to a non-ionic, hydrophilic block. Neutralization of the oppositely-charged polyions produces a water-insoluble segment, which forms the core of the micelle in aqueous solvents [26]. The hydrophilic corona solubilizes the complex in aqueous media and enhances stability in biological fluids. Such systems are referred to as polyion complex micelles.

The main drawbacks of micelles as drug delivery systems include rapid drug leakage from the micelle and dissociation if diluted below the CMC. As a result, the kinetics of micelle dissociation and drug diffusion are important parameters to control.

4. Macromolecular prodrugs

Macromolecular prodrugs (also referred to as drug-polymer conjugates) are delivery systems in which therapeutic agents are covalently linked to a polymer. The polymer can be either naturally-occurring (e.g. starch amylase, pullulans, chitosan) or synthetic (e.g. PEG, poly-amino acids, hydroxypropylmethacrylamide (HPMA), polyvinylalcohol (PVA), polyvinylpyrrolidone (PVP)) [27]. These drug-polymer conjugates are water soluble and generally have high molecular weights (> 40 kDa) to overcome renal excretion and achieve extended plasma half-lives. These polymeric drugs can attain half-lives in the order of hours as opposed to a few minutes for drugs in the free form [28]. Due to their relatively large size, drug-polymer conjugates cannot diffuse through the cell membrane and thus are usually taken up by endocytosis.

For this system to be efficacious the drug must be released from the polymer at the target site, however, there are exceptions whereby the prodrug is active without cleavage. In general, release of the drug from the polymer can be either pH-triggered or enzymatic [27]. Prodrugs with pH-sensitive bonds can be cleaved in acidic conditions such as the extracellular space of solid tumors and/or the endosomal or lysosomal compartments after internalization by the cell [27]. In the case of enzyme-dependent release, the linker attaching the drug to the polymer is usually a peptide spacer such as Gly-Phe-Leu-Gly, which is susceptible to cleavage by intracellular enzymes [29]. Ideally, the macromolecular prodrug should remain stable in the blood circulation until it reaches the target site. Similar to other colloidal drug carriers, targeting moieties can be attached to macromolecular prodrugs to improve selectivity

for the target site [30]. Examples of macromolecular prodrugs presently on the market include PEG-interferon alpha, conjugate of neocarzinostatin and poly(styrene-comaleic acid) (SMANCS), and PEG-L-asparaginase [27].

Macromolecular prodrugs have two major disadvantages. First, for drugs that are inactive as a conjugate, cleavage from the macromolecule must occur sufficiently fast at the target site in order to achieve greater efficacy than the free drug. Second, non-biodegradable polymers larger than 40 kDa cannot be eliminated by renal filtration and thus will remain in the patient.

5. Polymeric nanoparticles

Polymeric nanoparticles consist of a dense polymer matrix, which can physically entrap hydrophobic compounds. These particulates range in diameter from 10 – 1,000 nm and can be prepared from either natural or synthetic polymers [28]. Nanoparticles are interesting drug delivery systems due to the dense polymer core, which can considerably sustain or control the release of physically entrapped molecules [31]. The release rate of the drug molecules from the nanoparticle is controlled by the diffusion of the drug through the polymer matrix and the erosion of the nanoparticle [32]. As a result of the polymeric matrix, nanoparticles are usually more stable than liposomes and micelles in the presence of biological fluids. In general, without proper surface modification nanoparticles are quickly removed from the systemic circulation by the MPS. Incorporating PEG at the surface greatly enhances the residence time of the nanoparticles in the blood stream.

The preparation of nanoparticles often requires organic solvents, which presents a disadvantage for the use of this type of carrier in the clinic. Another

potential problem is the biodegradability and toxicity of the degradation products. Consequently, the polymer must be carefully selected.

6. Emulsions

Emulsions are heterogeneous mixtures of two immiscible liquids (i.e. oil and water), whereby the one phase is dispersed as fine droplets in the other. The addition of an emulsifier or surfactant provides kinetic stability to the preparation by reducing the interfacial tension and increasing droplet-droplet repulsion through electrostatic and/or steric repulsive forces [33]. Similar to other drug delivery vectors, emulsions can protect the encapsulated drug against hydrolysis and enzymatic degradation in the blood compartment, lower the toxicity of cytotoxic compounds, and can also provide a certain level of selectivity towards target tissues, increasing the therapeutic index of many drugs [34]. The application of emulsions as intravenous drug delivery systems will be discussed in more detail in Chapter 2.

The goal of the present work was to develop long-circulating emulsions and characterize their accumulation into solid tumors. The performance of the emulsions was assessed *in vivo* in mice bearing either B16 melanoma or C26 colon adenocarcinoma. The experimental section and results of this study are provided in Chapter 3 in the form of a scientific article. A summary of these findings as well as additional data on formulation optimization and stability are presented and discussed in Chapter 4. Finally, the concluding remarks and perspectives are given in Chapter 5.

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CHAPTER 2: PRINCIPLES IN THE DEVELOPMENT OF INTRAVENOUS LIPID EMULSIONS

Joanna Rossi and Jean-Christophe Leroux*

Canada Research Chair in Drug Delivery, Faculty of Pharmacy, University of Montreal, P.O. Box 6128, Downtown Station, Montreal (PQ) CANADA H3C 3J7

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*To whom correspondence should be addressed

1. Introduction

Emulsions can be defined as heterogeneous mixtures of two immiscible liquids, in which one phase is dispersed as fine droplets in the other. Small oil droplets dispersed in a continuous water phase is termed an 'oil-in-water' (o/w) emulsion. The opposite of this system is a 'water-in-oil' (w/o) emulsion, whereby the water phase is dispersed in an oily external medium. Among these types, only o/w emulsions can be used for intravenous administration [1]. Emulsions are thermodynamically unstable systems that will eventually destabilize into two separate phases. A third component, the surfactant or emulsifier, is added to stabilize the preparation by reducing the interfacial tension and increasing droplet-droplet repulsion through electrostatic and/or steric repulsive forces [2]. The addition of an emulsifying agent however, only provides kinetic stability. Even though emulsions are unstable systems, surface active agents may provide stability for several years, making the system useful for practical application [2].

Lipid emulsions have traditionally been used for parenteral nutrition to deliver essential fatty acids to patients unable to acquire them in food. Due to the successful induction of lipid emulsions in parenteral nutrition, there has been increasing interest in developing emulsions as carriers for lipophilic drugs. Many intravenous lipid emulsion formulations are commercially available (Table 1) and a number of others are in clinical phase or in preclinical development (Table 2). Lipid emulsions are promising carriers for drug delivery due to their biocompatibility, reasonable stability, ability to solubilize high quantities of hydrophobic compounds and relative ease of manufacture at an industrial scale [3, 4]. In addition, emulsions can protect the

encapsulated drug against hydrolysis and enzymatic degradation in the blood compartment, reduce drug loss in infusion sets, lower the toxicity of cytotoxic compounds, and reduce the incidence of irritation and pain upon injection [1, 4]. They can also provide a certain level of selectivity towards target tissues, increasing the therapeutic index of many drugs [5]. However, after intravenous injection, lipid emulsions can acquire apolipoproteins and be metabolized as natural fats or be recognized as foreign bodies and taken up by the cells of the mononuclear phagocyte system (MPS; also known as the reticuloendothelial system (RES)) [6]. Evading the MPS or natural fat metabolism is necessary when the encapsulated drug is to be delivered to non-MPS organs or liver parenchymal cells, respectively. The *in vivo* fate of lipid emulsions can be controlled to a certain extent by altering the physicochemical properties of the carrier such as, droplet size, composition and surface properties. This chapter will discuss the main factors to consider when developing emulsions for intravenous injection.

2. Emulsion stability

Emulsions are thermodynamically unstable systems and will inevitably break apart into separate oil and water phases. Emulsion instability is caused by the increase in surface free energy (ΔG) as small droplets are formed as a result of the enhanced surface area (ΔA). Adding a surfactant to the mixture reduces the interfacial tension (γ_{ow}) at the oil-water interface facilitating globule rupture during emulsification and stabilizes the preparation (Eqn 1).

$$\Delta G = \gamma_{ow} \Delta A \tag{1}$$

It is important to state that surfactants only provide the emulsions with kinetic stability, which delays the destabilization process. Nevertheless, surface active agents can provide stability for several years, which is long enough for the system to be useful for practical purposes [2]. Emulsions that are thermodynamically stable are known as microemulsions. They are clear or translucent systems and do not require much energy input during emulsification. In contrast, emulsions are cloudy and require a greater amount of energy for emulsification [7]. The theory behind the formation of microemulsions is beyond the scope of this chapter.

2.1. Destabilization processes

Emulsion destabilization can be characterized by three separate processes: flocculation, coalescence and Ostwald ripening. Coalescence and Ostwald ripening are irreversible processes which lead to an increase in droplet size, requiring a large energy input to re-disperse the droplets. Flocculation, on the other hand, is reversible and occurs when droplets aggregate to form a clump of many individual droplets. The aggregated droplets move together as a cluster but each droplet still retains its separate identity. The interactions holding the droplets together are weak and can be broken by mild agitation. Even though floccules can be easily re-dispersed, they may eventually fuse together to form single, larger globules. The fusion of droplets is irreversible and is termed coalescence. Ostwald ripening, which also increases droplet size, occurs in polydisperse formulations wherein the smaller droplets are more soluble in the continuous phase than the larger ones. In this process, the oil from the smaller droplets dissolves in the aqueous phase and diffuses towards the larger droplets. This transfer of oil causes the big droplets to grow, while the smaller ones

decrease in size. As the small droplets continue to shrink, the Ostwald ripening effect is enhanced. The progressive increase in droplet size over time will eventually lead to complete phase separation. Adding too much surfactant may promote Ostwald ripening as the excess surfactant will form micelles which enhance the solubility of the oil in the aqueous phase. Ostwald ripening can be reduced by increasing the viscosity of the continuous phase, decreasing polydispersity, or adding a third component which has a lower solubility in the continuous phase than the oil [8, 9].

Depending on the density differences between the dispersed and continuous phases, individual droplets or floccules can cream or sediment. If the dispersed phase is lower in density than the continuous phase, the droplets or floccules will rise to the surface producing a highly concentrated layer of dispersed phase, which is known as a cream. In the case where the dispersed phase is higher in density than the continuous phase, a sediment will form at the bottom of the formulation. For o/w emulsions, creaming usually occurs since the oil phase is typically less dense than the aqueous phase. The rate of creaming or sedimentation can be linked to the size of the droplet by Stokes' equation (Eqn 2). According to this equation the limiting velocity of a falling sphere (υ) is:

$$\upsilon = \frac{2}{9}\frac{a^2 \Delta\rho}{\nu} g \qquad (2)$$

where a is the radius of the droplet, $\Delta\rho$ is the density difference between the dispersed and continuous phases, ν is the viscosity of the continuous phase and g is the acceleration due to gravity. Stokes' equation implies that droplets will rise or settle faster if the droplet size or the density difference between the dispersed and continuous phases is large, while an increase in continuous phase viscosity will slow

down the separation process. As a result, creaming or sedimentation can be delayed by reducing droplet size, decreasing the density differences between the two phases and increasing the viscosity of the continuous phase. Not much emphasis, however, is being placed on density adjustments to produce stable emulsions since there are a limited number of oils approved for intravenous administration and these oils have similar densities.

Submicron emulsions have colloidal properties and as a result are less susceptible than coarse emulsions to the gravitational forces in Stokes' equation [10]. Nanosized droplets are subjected to random Brownian motion and consequently are less inclined to cream or sediment. Brownian motion, however, does not provide complete protection against instability since droplets may aggregate or coalesce upon random collisions. Stability against these collisions depends on the attractive and repulsive forces acting on the droplets. Typically, emulsions are stabilized by either electrostatic or steric repulsive forces (or a combination of the two).

2.2. Electrostatic stabilization

The balance between attractive Van der Waals forces and electrostatic repulsive forces is described in the theory of colloidal stability, termed DLVO after its developers Derjaguin, Landau, Vervey, and Overbeek. If the net force is attractive, the droplets will either flocculate or coalesce. In contrast, if the net force is repulsive, the particles will repel each other and the system is stable. The attractive interaction between particles arises from Van der Waals forces and is experienced by all particles. Van der Waals forces dominate at short separation distances and the strength of this attractive force can be determined from the magnitude of the Hamaker

constant (A). Emulsions can overcome the attractive Van der Waals forces through electrostatic repulsion with charged emulsifying agents. Electrostatic repulsion is provided by the electric double-layer surrounding the droplet. The electric double-layer is characterized by an adsorbed layer of fixed counterions and a diffuse layer of ions that move freely with the fluid. Two approaching particles will experience a repulsive force as the electric double-layers overlap. The total potential of interaction between two droplets is the sum of the attractive van der Waals forces and the electrostatic repulsive forces (Eqn 3);

$$V_T = V_A + V_R \tag{3}$$

where V_T is the total interaction potential, V_A represents the attractive van der Waals forces and V_R signifies the electrostatic repulsive forces. The potential energy of interaction between two droplets as a function of separation distance is illustrated in Figure 2. The repulsive barrier generated by the electric double-layer corresponds to the maximum in the curve. The height of the energy barrier determines the stability of the emulsion and depends on the ionization of the surfactants.

For the system to be stable, the energy barrier must be high enough such that the droplets do not have enough kinetic energy to surpass it and reach the primary minimum. At the primary minimum (maximum attractive potential) droplet coalescence readily occurs. Flocculation takes place at the secondary minimum and contrary to coalescence, is reversible by providing a small amount of kinetic energy to overcome the weak attractive forces holding the droplets together. Flocculated droplets are prevented from coalescing as a result of this repulsive energy barrier. If the flocculated droplets have enough energy to surpass the energy barrier they will

easily reach the primary minimum and coalesce. The strength of the electrostatic forces can be quantified by measuring the zeta potential, which is the potential at the plane of hydrodynamic shear. Generally, emulsions are stabilized by electrostatic repulsive forces if the zeta potential is greater than ± 30 mV [2, 8, 11]. An emulsion stabilized by electric double-layer repulsion can be destabilized if the concentration of electrolytes is increased above a critical value. Adding electrolytes to an emulsion decreases the electric double-layer repulsion potential, while the van der Waals attractive potential remains unchanged. As electrolyte concentration increases, the repulsive forces stabilizing the colloid become weaker until the net force is attractive and stability is lost.

2.3. Steric stabilization

Emulsions can also be stabilized by steric repulsion by grafting long-chain polymers at the emulsion interface. Steric repulsion is a non-DLVO interaction that occurs due to the unfavorable overlap of the polymer chains as two particles approach each other [8, 12]. Steric stabilization occurs at short inter-droplet separation distances and can provide a strong barrier against coalescence [8]. Optimal steric repulsion can be achieved at high polymer surface density as desorption and chain rearrangement is minimized [8].

3. Elimination mechanisms for lipid emulsions

After intravenous injection, lipid emulsions may be metabolized in a manner similar to chylomicrons or might be recognized as foreign bodies and removed by the cells of the MPS. The mechanism of elimination from the body depends on the

physicochemical properties of the emulsion. Both mechanisms of elimination can occur for a given lipid emulsion, however, one process may be favored over another. This section describes the two primary pathways of lipid emulsion elimination from the body.

3.1. Lipid emulsions metabolized as endogenous chylomicrons

Depending on the composition and surface properties, lipid emulsions may be recognized as chylomicrons and be eliminated via the fat metabolism pathway. Chylomicrons are endogenous emulsions produced by the enterocytes of the small intestine after dietary lipids are ingested. They are rich in triglycerides and possess apolipoproteins A-I, A-IV and B-48 prior to entering the blood circulation (Figure 3) [13]. Chylomicrons are secreted into the lymph and enter the systemic circulation through the thoracic duct. After entering the blood, chylomicrons obtain Apo C-II and Apo E from the high-density lipoproteins (HDLs) and release Apo A-IV. In the capillaries of adipose tissues and muscle, lipoprotein lipase (LPL) located on endothelial cells adsorb onto the mature chylomicron and hydrolyze the triglycerides to fatty acids [14]. The fatty acids are then absorbed mainly by adipose tissues and muscle. During lipolysis, a substantial amount of phospholipid, Apo A and Apo C are transferred to the HDLs and the size of the chylomicron is reduced considerably. The remnant chylomicrons composed of mainly Apo B-48, Apo E and cholesterol are quickly removed from the blood by the liver. The uptake of remnant chylomicrons by the liver occurs via two Apo E-specific recognition sites on parenchymal cells, which are the low-density lipoprotein receptor (LDLr) and the remnant receptor [15-17].

Injectable lipid emulsions differ from chylomicrons in that they do not have apolipoproteins on the surface prior to entering the blood stream, although they may acquire them after systemic injection. Emulsions rich in triglycerides are known to acquire apolipoproteins (Apo C-I, C-II, C-III, E and possibly Apo A-IV), mainly from HDLs, soon after injection into the systemic circulation and are metabolized in a pathway comparable to that described for chylomicrons [15, 18, 19]. Among the apolipoproteins acquired Apo C-II and Apo E are essential for LPL activation and uptake of remnant emulsions by the liver, respectively [19].

Elimination of the lipid emulsion via the pathway of natural fat metabolism may be desirable when the liver parenchymal cells are the target site. On the other hand, if the target site is not the liver then apolipoprotein adsorption onto the emulsion should be avoided. The metabolism of lipid emulsions as natural fats is strongly dependent on the type of emulsifier [20, 21], the presence of cholesterol [22] and the chain length of the triglyceride oil [23].

3.2. Elimination by the mononuclear phagocyte system

If the body recognizes the lipid emulsions as foreign, they will be captured by the cells of the MPS, mainly the Kupffer cells of the liver and the macrophages of the spleen, and removed from the systemic circulation. The MPS takes up the emulsions via endocytosis and localizes them in the lysosomal compartment where they are degraded by enzymes [24]. The extent of emulsion clearance from the systemic circulation is enhanced by the adsorption of opsonins (proteins) onto the colloid surface. The bound proteins then interact with the receptors on monocytes and macrophages, facilitating endocytosis. Carriers that become bound to opsonins will be

rapidly cleared from the blood and prevented from reaching the target site(s) [24]. Immunoglobins and complement components such as C1q and C3 fragments (C3b, iC3b) are well known opsonins.

A major challenge in drug delivery using colloidal nano-carriers is to avoid clearance by the cells of the MPS when the target sites are non-MPS tissues. Overloading or saturating the MPS with large injections volumes has been shown to enhance the circulation time of lipid emulsions [25]. However, temporary impairment of the MPS may pose a health hazard to the patient [26]. Alternatively, the clearance rate of carriers from the blood can be altered by modifying the physicochemical properties of the emulsion, such as droplet size [27, 28] and surface characteristics [29]. This will be discussed in detail in section 4.

4. Biodistribution of lipid emulsions

The biodistribution of an emulsion after systemic injection is dependent primarily on the droplet size, composition and surface properties. A certain specificity towards the target site can be achieved by controlling the physicochemical properties of the emulsion. The principle factors that influence the biodistribution of emulsions has already been very thoroughly reviewed in a book chapter by Nishikawa [6]. This section provides a brief overview of these factors and has been updated with some recent work.

4.1. Effect of lipid emulsion size

It is well known that droplet size greatly influences the uptake of the emulsions by the MPS [27, 28]. In general, larger particles are more susceptible to

uptake by the MPS and are cleared more quickly from the systemic circulation. The influence of droplet size on the *in vivo* biodistribution of lipid emulsions was explored by Takino et al. [27]. The authors compared the biodistribution of large (250 nm) and small (100 nm) lipid emulsions composed of egg phosphatidylcholine (egg PC):soybean oil = 1:1. [^{14}C]Cholesteryl oleate ([^{14}C]CO), a highly lipophilic compound (log P = 18.3) that does not undergo lipolysis by LPL and remains associated with the emulsion, was incorporated into each emulsion to track the elimination of the whole droplet [30]. The large egg PC emulsion was rapidly eliminated from the blood with 60% of the injected emulsion recovered in the liver within 10 min. The small egg PC emulsion, however, remained in the blood for longer and accumulated less in the liver. Similarly, Lundberg et al. [28] reported that droplet size influenced emulsion clearance rate from plasma. They observed that the smallest emulsion (50 nm) survived the longest in plasma, whereas the larger emulsions (100 and 175 nm) were cleared more rapidly (Figure 4). The influence of emulsion-like lipid nanocapsule size (20, 50 and 100 nm) on the extent of complement activation and macrophage uptake was evaluated by Vonarbourg et al. [31]. Similar to emulsions, lipid nanocapsules are core-shell structures with an oily internal phase that is stabilized by a monolayer of emulsifiers. They differ from lipid emulsions in the physicochemical properties of the hydrophilic/hydrophobic interface. In lipid nanocapsules, the emulsifiers form a semi-rigid shell, while the interface is more fluid in emulsions. The authors observed that larger lipid nanocapsules were stronger activators of the complement and were taken up more by macrophages than the smaller ones.

The size of the lipid emulsion was also shown to influence lipolysis. Kurihara et al. [32] found that the rate of lipolysis was much faster for the small sized emulsions (~ 100 nm) *in vitro* compared to the larger ones (225-416 nm). However, after intravenous injection of these formulations in rats, they observed that the small sized emulsions remained in plasma longer than the larger ones, which is consistent with the studies of Takino [27] and Lundberg [28]. Consequently, even though small emulsions were better substrates for LPL, large emulsions were cleared from the blood faster, which suggests a greater uptake by the MPS.

Droplet size also determines the ability of the emulsion to escape the systemic circulation through the blood capillaries and reach the extravascular space. Capillary walls are composed of a single layer of endothelial cells surrounded by a basement membrane. They are classified into three types, continuous (intact), fenestrated or discontinuous (sinusoidal), based on their wall structure [33]. Both fenestrated and discontinuous capillaries have pores in the endothelium, while continuous ones have tight junctions between adjacent endothelial cells [34]. Continuous capillaries have an intact subendothelial basement membrane and can be found in most regions of the body such as the skin, connective tissue, skeletal and cardiac muscle, alveolar capillaries of the lung, and the brain [33]. In fenestrated capillaries, the pores (fenestrae) are approximately 40-80 nm in diameter and they can be either open (unobstructed) or covered by a thin diaphragm [33]. These capillaries have a continuous subendothelial basement membrane and are situated in the intestinal mucusa, pancreas, glomerulus, peritubular capillaries, endocrine glands, the choroid plexus of the brain and the ciliary body of the eye [33]. Discontinuous capillaries, on

the other hand, have large gaps between endothelial cells and are located in the liver, spleen and bone marrow [33]. The basal membrane is either absent, which is the case for the liver or discontinuous (spleen and bone marrow) [34]. The largest pore size in the capillary endothelium is believed to be approximately 100 nm [35]. Nanoscopic drug carriers are generally too large to diffuse across the capillaries of continuous endothelium. Their best opportunity to escape the systemic circulation is through the gaps between the endothelial cells of discontinuous capillaries. Consequently, colloidal drug carriers tend to accumulate in the liver, spleen and bone marrow.

Control over carrier size can impart some selectivity for the extravascular space of tumoral sites, reducing anticancer drug toxicity towards healthy tissues. This selectivity can be achieved by taking advantage of the difference in capillary structure between tumors and normal tissues. Tumor vasculature is often characterized as porous or "leaky" allowing enhanced permeation of colloidal particles across the endothelium and into the extravascular space. In addition, tumors have poor lymphatic drainage allowing colloids to be retained in the tissue for longer periods of time [36]. This increased permeation and retention of colloids is called the enhanced permeation and retention (EPR) effect [37]. The optimum size range for colloidal particle accumulation in tumors is generally accepted to be approximately 50-200 nm [38]. Particles in this size range can be convected from the blood vessel into the extravascular space through the porous vasculature of the tumor. Depending on the porosity of the tumor capillaries, particles above 200 nm may not pass through the pores and will be eliminated more quickly by the MPS. On the other hand, particles

less than 50 nm will easily extravasate through the discontinuous endothelium of the liver, spleen and bone marrow.

As a rule of thumb, for successful accumulation of drug in the tumor by the EPR effect, the concentration of colloidal carriers in the plasma must remain high for more than 6 hours [39]. The progressive extravasation of the carrier into the tumor tissue over several hours will result in increasing concentrations of anticancer drug in the vicinity of the cancer cells. Kurihara et al. [40] demonstrated that lipid emulsions under 230 nm in diameter could deliver more RS-1541, a highly lipophilic antitumor agent (13-O-palmitoyl-rhizoxin), to the tumor site (M5076 sarcoma cells) than larger droplets (Figure 5). The low concentrations of RS-1541 detected in the tumor for the larger emulsions is most likely due to the impermeability of the leaky tumor capillaries to large particles and their faster removal rates from blood. It was also observed that emulsions greater than 220 nm reduced the toxicity of RS-1541 as shown by the higher maximum tolerated dose (MTD) with increasing size (Table 3). All emulsions regardless of size (70 – 380 nm) suppressed tumor growth and improved survival at the MTD. The medium sized emulsions (220 nm), however, displayed the highest antitumor activity at the MTD due to the permeability of the tumor vasculature for the emulsions and reduced toxicity, permitting the injection of a higher dose. Hence, lipid emulsions can augment the delivery of cytotoxic compounds to tumoral sites and reduce systemic toxicity by suitable selection of the droplet size.

4.2. Effect of lipid emulsion composition and emulsifiers

Composition of the oil phase

The composition of the internal phase has also been shown to alter the biodistribution of lipid emulsions. Lutz et al. [41] observed that lipid emulsions composed of medium-chain triglycerides (MCTs) were cleared from plasma more quickly than those prepared with long-chain triglycerides (LCTs). This is probably due to the faster hydrolysis of MCTs by LPL and hepatic lipases compared to LCTs as a result of the greater solubility and mobility of shorter chain triglycerides at the oil/water emulsion interface [42].

Adding free cholesterol has also been shown to alter the metabolism of triglyceride emulsions. Maranhao et al. [22] observed that emulsions with low free cholesterol content (< 4, % w/w) were metabolized in a manner similar to chylomicrons, as shown by the faster removal rate of triglycerides from the blood than CO due to LPL mediated hydrolysis of the oil and greater uptake of CO than triglycerides by the liver. In contrast, emulsions with high free cholesterol (>16, % w/w) displayed similar triglyceride and CO removal rates from blood and equal uptake by the liver. The group also observed that emulsions containing high free cholesterol bound less Apo A-I, Apo A-IV and Apo C and more Apo E *in vitro*. Apo C-II is essential for LPL binding and activation and hinders liver uptake, while Apo E facilitates emulsion uptake by the liver. Hence, the presence of free cholesterol may modify the metabolism of the droplets by altering the binding of apolipoproteins onto the surface.

Phosphatidylcholine composition

The biodistribution of emulsions can also be altered by the phospholipid emulsifier. Lenzo et al. [43] demonstrated that the nature of the PC affected the metabolism of the emulsion in rats. Five lipid emulsions with different phospholipid emulsifiers were prepared. The phospholipids selected were egg PC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC). The average composition of each emulsion was similar and size was maintained at approximately 150 nm. The emulsions were radiolabeled with [^{14}C]triolein (TO) and [^{3}H]CO or dipalmitoylphosphatidyl[N-methyl-^{3}H]choline to monitor the hydrolysis of the triglyceride oil by LPL, the clearance of the entire colloid particle and the transfer of phospholipids to the HDLs, respectively.

The carriers emulsified with egg PC or POPC were metabolized in a manner similar to chylomicrons as shown by the rapid removal rate of [^{14}C]TO from plasma, consistent with hydrolysis by LPL and the efficient uptake of [^{3}H]CO (remnant emulsions) by the liver. DPPC-based emulsions remained in plasma the longest and the triglycerides associated with this emulsion disappeared very slowly, suggesting that the emulsion was less susceptible to hydrolysis by LPL. Moreover, the phospholipid radiolabel did not transfer to HDLs. A possible explanation for the above observations is the difference in chain unsaturation between the five phospholipid emulsifiers. The authors hypothesized that rapid hydrolysis of the

triglyceride oil by LPL and efficient transfer of phospholipids to HDLs requires a chain unsaturation at the glycerol 2-position.

4.3. Effect of surface charge

Lipid emulsions obtain their surface charge through the use of neutral, anionic or cationic emulsifiers. Most emulsions used in drug delivery are either neutral or negatively charged since cationic carriers are more prone to aggregate in the presence of plasma proteins. This susceptibility for aggregation in the bloodstream is a result of the electrostatic interactions with negatively charged plasma proteins. It is generally accepted that surface charge has an effect on the rate of particle uptake by the MPS, although the connection is far from straightforward. Other surface properties, such as the nature of the emulsifier may take precedence over the effects generated by surface charge. Davis et al. [44] found no clear correlation between zeta potential and the rate of emulsion uptake by mouse peritoneal macrophages, although emulsions with the weakest charge, prepared with the non-ionic surfactant poloxamer 338, had the slowest rate of uptake. Stossel et al. [45] found that emulsions with higher surface charge (positive or negative) were phagocytosed at a faster rate compared to neutral or weakly charged surfaces. Oku et al. [46] observed that uptake by the liver and spleen was greater for positively charged liposomes than neutral or anionic ones. The higher accumulation of cationic liposomes in the MPS organs may be due to both particle aggregation in the presence of serum and protein adsorption onto the colloid, which was observed to a lesser extent in neutral or anionic liposomes. Devine et al. [47] found that liposomes bearing a net positive or negative charge activated the complement in a dose-dependent manner, while no complement activation was

observed for neutral liposomes. Interestingly, long-circulating cationic lipid emulsions have been reported in the literature by careful selection of the emulsifier [48].

Over the past several years, there has been increasing interest in developing cationic lipid-DNA complexes for the improved delivery of genetic material [49, 50]. An advantage of using cationic carriers is the enhanced cellular uptake via endocytosis over neutral or negatively charged carriers [51]. This is due to the favorable electrostatic interactions of cationic particles with the negatively charged moieties on biological membranes. However, due to the tendency of cationic particles to aggregate in the presence of serum, the positive charge will need to be shielded, which will invariably reduce transfection efficiency in the absence of targeting ligands.

4.4. Long-circulating lipid emulsions

After intravenous administration, colloidal drug carriers are rapidly taken up by circulating monocytes and macrophages in the liver, spleen and bone marrow. Avoiding the MPS is crucial when the emulsions are to be delivered to non-MPS cells. Prolonged circulation of the drug carrier is also necessary to achieve passive targeting of tumoral tissues via the EPR effect. Modifying the colloidal surface such that the carriers are invisible or "stealth" to opsonins and macrophages is an approach investigated to increase the circulation time of submicrometer emulsions in blood.

Sphingomyelin

The presence of sphingomyelin (SM) at the oil/water interface has been shown to reduce the uptake of the emulsions by the MPS. Takino et al. [27]

demonstrated that adding SM to an egg PC and soybean oil formulation increased the circulation time of the submicrometer emulsions in blood and decreased liver and spleen uptake. The emulsions were composed of egg PC:soybean oil = 1:1 and egg PC:SM:soybean oil = 0.7:0.3:1 with [^{14}C]CO incorporated as a radiolabeled tracer. The AUC of the SM emulsion was 1.6 times larger than the one emulsified with egg PC only. Similarly, Redgrave et al. [21] observed that increasing the amount of SM enhanced the circulation time of the carrier in plasma (Figure 6) and reduced uptake by the liver (Figure 7). Even though SM and PC share a common phosphorylcholine polar head group, there are structural discrepancies between the two molecules that reflect their different physical properties in colloidal systems. SM has a high content of saturated acyl chains relative to naturally occurring PCs and has a stronger hydrogen bonding capacity, which may alter monolayer rigidity and interactions with blood components [52].

Poly(ethylene glycol)-lipids

A very widely used and effective method to avoid clearance by the MPS is to incorporate poly(ethylene glycol) (PEG) (also known as poly(ethylene oxide) (PEO)) at the colloid surface using a lipid derivative. PEG, a hydrophilic and flexible polymer, creates a zone of steric hindrance around the carrier which decreases the rate and extent of opsonin binding [53]. PEG is widely accepted for intravenous administration because it is a biocompatible, non-toxic and non-immunogenic polymer. Moreover, PEG-lipid derivatives are amphiphilic and as a result can be used as a coemulsifier as well. Liu et al. [29] observed the influence of PEG molecular weight on the biodistribution of lipid emulsions composed of castor oil and egg PC.

The PEG-lipid derivatives investigated were dioleoyl N-(monomethoxy PEG succinyl)phosphatidylethanolamine (PEG-DOPE) (MW 1000, 2000, and 5000) and poly(ethylene oxide) 20 sorbitan monooleate (polysorbate 80). Emulsion droplet size was maintained at approximately 200 nm so that the circulation behavior was only dependent on the surface properties of the emulsions. It was observed that emulsion circulation time in blood depended on the length of the PEG chain. PEG_{2000}-DOPE and PEG_{5000}-DOPE kept the emulsions in the blood the longest. Approximately 60-70% of the injected dose remained in the blood after 30 min. PEG_{1000}-DOPE and polysorbate 80 emulsions demonstrated comparable behavior *in vivo* with 47% of the injected dose remaining in the blood after 30 minutes. The high emulsion concentration observed in the blood for PEG_{2000}-DOPE and PEG_{5000}-DOPE translated into lower accumulation in the liver. Consequently, coating an emulsion surface with PEG of sufficient chain length can confer long circulating properties to submicrometer emulsions.

Hoarau et al. [38] evaluated two different processes to incorporate 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-monomethoxy-[PEG] (PEG-DSPE) into lipid nanocapsules. The conventional method was the first investigated and involved the addition of PEG-DSPE with the other surfactants during the emulsification of the oil. The second method evaluated was post-insertion, wherein an aqueous micelle solution of PEG-DSPE was added to the preformed lipid nanocapsules and then incubated for 90 min at 60°C. The authors observed that the post-insertion method enhanced the amount of PEG-DSPE that could be incorporated into the nanocapsule compared to the conventional process. For the conventional

method, the amount of PEG_{2000}-DSPE and PEG_{5000}-DSPE could not exceed 3.4 and 1.5 mol% of the total surfactants, respectively as physical instability would occur. In contrast, PEG-DSPE could be incorporated into the lipid nanocapsules at higher quantities (6-10 mol%) using the post-insertion method, regardless of the PEG chain length. Consequently, the PEGylated lipid nanocapsules prepared by the post-insertion technique circulated longer in blood due to the greater PEG density at the surface (Figure 8). For example, the AUC increased 5-fold as the proportion of PEG_{2000}-DSPE increased from 1.7 to 10 mol%.

Other methods to enhance circulation time

Surfactants containing PEO chains such as, PEO-*b*-poly(propylene oxide)-*b*-PEO (PEO-*b*-PPO-*b*-PEO, poloxamers) and PEO-hydrogenated castor oil (cremophors) have also been investigated to enhance the hydrophilicity of emulsion surfaces to reduce opsonin binding and uptake by the MPS. Lee et al. [54] demonstrated that emulsions coated with poloxamer 338 reduced the amount of ibuprofen octyl ester delivered to the MPS organs. Ueda et al. [55] investigated the influence of ethylene oxide number in PEO-hydrogenated castor oil surfactants on menatetrenone clearance rate from plasma and distribution to MPS organs. They observed that a minimum of 20 ethylene oxide units (MW = 880) is required to prolong menatetrenone circulation time in plasma. The prolonged circulation of emulsions containing greater than 20 ethylene oxide units translated into a lower accumulation of menatetrenone in the liver. Menatetrenone incorporated into emulsions with 10 ethylene oxide units were rapidly removed from plasma and were taken up to a greater extent by the liver.

4.5. Active targeting of selected cells

Drug delivery systems utilizing ligands that specifically recognize determinants on the surface of target cells has been extensively investigated in liposomes [56-58] and macromolecular prodrugs [59]. However, few studies have been done with emulsions. Incorporating ligands onto the emulsion interface is a promising method to enhance specificity towards the target site(s). For this method to be successful, lipid emulsions must have the appropriate ligand(s) anchored onto the surface, be able to reach the target cells, bind to the receptors and either enter the cell or empty the contents in the vicinity of the cell.

Lipid emulsions associated with apolipoprotein E

Apo E has an affinity for both the remnant and LDLr receptors on hepatocytes and is an important mediator in the uptake of emulsions and lipoproteins by the liver. Incorporating Apo E on lipid emulsions provides an opportunity to target hepatocytes. Rensen et al. [60] investigated the possibility of using lipid emulsions associated with Apo E as drug carriers for a model antiviral prodrug, iododeoxyuridine-oleoyl (IDU-Ol_2), to selectively target hepatocytes for improved therapy of hepatitis B viral infection. The emulsions were prepared using natural lipids (egg PC, lysophosphatidylcholine, cholesterol, TO and CO) and had a mean size of approximately 80 nm to mimic natural chylomicrons. The lipid emulsions were radiolabeled with [^{14}C]CO and [^{3}H]IDU-Ol_2 to track the *in vivo* distribution of the whole droplet and prodrug, respectively. After intravenous injection, lipid emulsions pre-loaded with Apo E were removed faster from serum and were taken up more by the liver than the control emulsions (lipid emulsions not pre-loaded with Apo E)

(Figure 9). The uptake of the carrier by the liver reached approximately 70% of the injected dose for the Apo E pre-loaded emulsion compared to only 30% for the control. The prodrug exhibited similar removal rates from serum and uptake by the liver as the carrier. The authors also showed that the carrier and prodrug were mainly taken up by parenchymal cells with little accumulation in the endothelial or Kupffer cells. Introducing lactoferrin, a glycoprotein that blocks Apo E mediated uptake of lipoproteins by parenchymal cells, prior to injecting the lipid emulsions resulted in a considerable reduction in emulsion uptake by the liver.

Sugar-coated emulsions to target hepatocytes

Incorporating Apo E onto the lipid emulsion is a complex process which may cause reproducibility and stability issues [61]. Another method to enhance selectivity for hepatocytes is to incorporate sugars such as galactose on the surface of the lipid emulsion to target the carbohydrate receptors on hepatocytes. Ishida et al. [61] investigated the biodistribution of galactosylated (Gal) and non-galactosylated emulsions after intravenous injection in mice. The results demonstrated that the Gal-emulsion was more quickly removed from the blood compared to the bare-emulsion, whereby the AUC values for the Gal-emulsion and bare-emulsions were 1.9 and 3.7 (% of dose x h/mL), respectively. In addition, the uptake of the Gal-emulsion by the liver was 3.2-times greater than the bare-emulsion. Moreover, Gal-emulsions were taken up 7.4-times more by parenchymal cells than non-parenchymal compared to only 4.3-times for the bare-emulsions. These findings suggest that introducing galactose on the surface of lipid emulsions is a promising method for delivering drugs to hepatocytes.

Antibody/peptide conjugation onto long circulating lipid emulsions

Cancer cells often over-express certain antigens or receptors, which provides another possible method to enhance the selectivity of anticancer drugs towards tumor tissues [58]. Antibodies, antibody fragments, or synthetic peptides can be incorporated onto the carrier surface to specifically recognize the antigen/receptors on cancer cells and offers a possible solution to the non-specific and slow uptake of colloidal carriers by cancer cells. Linking antibodies to liposomes has been widely studied, however, much more progress is required for its successful application [58]. In theory, the techniques applied to liposomes can be carried over to lipid emulsions. Ideally, the tumor targeting ability of antibodies can be coupled with the long circulating properties of PEGylated lipids. To avoid the interference between the PEG chain of PEGylated lipid emulsions and the antibodies incorporated into the emulsion surface, antibodies linked to PEG chains have been developed in recent years. Lundberg et al. [62] successfully conjugated an anti-B-cell lymphoma monoclonal antibody (LL2) onto a lipid emulsion by coupling LL2 to PEG-DSPE. The immunoreactivity of the LL2 conjugated emulsion was tested by determining their binding affinity to WN, the anti-idiotype antibody to LL2. The results showed that increasing the density of LL2 at the surface enhanced the binding of the emulsion to WN up to 40 antibodies per droplet.

4.6. Drug leakage from emulsions

Drug retention within the droplet after intravenous administration is another important factor to consider when designing lipid emulsion not only for dissolution purposes but as carriers for lipophilic drugs. Controlling the biodistribution of the

entire droplet will not enhance the therapeutic effect if the drug is released from the carrier before it reaches the target site. Takino et al. [27] suggested that the drug must have adequate lipophilicity (log P greater than 9) to remain sufficiently incorporated in the emulsion in the blood circulation. The influence of lipophilicity on drug leakage was observed by Kurihara et al. [63]. The group evaluated the pharmacokinetics of two lipophilic anticancer agents, rhizoxin and RS-1541, incorporated into lipid emulsions after intravenous injection in rats. The lipophilicities of rhizoxin and RS-1541 were very different with log P values of 1.9 and 13.8, respectively. After intravenous injection, rhizoxin was removed much more quickly from plasma than RS-1541 and distributed more to the liver, lung and intestine. The different pharmacokinetic profiles can be attributed to the lower retention of rhizoxin within the lipid emulsion after injection. Similarly, Sakaeda et al. [64, 65] found that sudan II with a log P of 5.4 was rapidly released from the lipid emulsion in plasma. Consequently, the lipid emulsion did not alter the pharmacokinetics of sudan II. The lipophilicity of a drug can be increased by chemically modifying the drug. A drawback of this approach, however, is that chemical modifications may reduce efficiency or completely inactivate the drug.

5. Preparation of emulsions for intravenous administration

To be used for intravenous administration, emulsions must be biocompatible, biodegradable, non-toxic, sterile, isotonic, physically and chemically stable, and non-immunogenic [1]. Moreover, droplet size must be small enough to avoid forming pulmonary emboli. To achieve these requirements, the excipients, additives and manufacturing conditions must be carefully selected. In addition, a complete

physicochemical characterization of the emulsions is necessary, followed by a long-term stability testing schedule on all promising formulation candidates. The following section describes the basic factors that need to be considered when developing emulsions for intravenous injection.

5.1. Excipient and formulation considerations

Internal phase (oils)

To comply with the essential requirement of biocompatibility, research on injectable emulsions has been primarily focused on the use of vegetable oils (triglycerides) as the oil phase [66]. Triglyceride oils can be characterized as long, medium or short chain depending on the number of carbon atoms per hydrocarbon chain. LCTs contain 14, 16, 18, 20 or 22 carbons in a fatty acid chain, which may or may not have unsaturations [24]. MCTs are derived from coconut oil and contain saturated fatty acids with chains of 6, 8, 10 or 12 carbons [1, 24, 67]. Lastly, short-chain triglycerides (SCTs), such as triacetin and tributyrin have chain lengths of only 2 and 4 carbons, respectively. LCTs and MCTs should be considered in the initial stages of formulation as many of these oils are approved for injection and are found in a number of FDA (Food and Drug Administration) approved products [67] (Table 4). The choice of oil is usually dependent on the solubility and stability of the drug. MCTs have 100-fold greater water solubility than LCTs and consequently are typically better solubilizers for drugs since most hydrophobic drugs have some polarity [24, 67]. Kan et al. [68] reported that triglycerides with short fatty acid chains were better solubilizers for paclitaxel, a lipophilic anticancer drug. They reported that paclitaxel solubility increased as the number of carbons per hydrocarbon chain

decreased. For example, tributyrin (C4) and tricaproin (C6) provided greater paclitaxel solubility than tricaprylin (C8) and other plants oils with a mixture of 6-22 carbons per hydrocarbon chain. Triacetin (C2) gave the highest paclitaxel solubility at a value of 75 mg/mL. Triacetin, however, is not approved for injection and might be difficult to emulsify due to its relatively high water solubility.

Vitamin E (DL-α-tocopherol) has been investigated as an alternative biocompatible oil to triglycerides to solubilize highly lipophilic drugs [69-71]. Constantinides et al. [70-72] have formulated a submicrometer emulsion of paclitaxel with high drug loading (8-10 mg/mL) using vitamin E as the internal phase and d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) and poloxamer 407 as the emulsifiers (TOCOSOL®-paclitaxel). This formulation is less toxic and has greater antitumor activity in mice bearing B16-melanoma tumors than the commercial formulation for paclitaxel (Taxol®). At the MTD for Taxol® (20 mg/kg), TOCOSOL®-paclitaxel showed greater tumor regression than Taxol® on a q3dx5 dosing schedule. Moreover, tumor growth was suppressed further at higher doses of this formulation (40 mg/kg and 60 mg/kg). TOCOSOL®-paclitaxel is currently in Phase III clinical trials [72]. In addition to being a solubilizer for poorly soluble drugs, vitamin E may also provide some therapeutic value. Bartels et al. [73] examined the influence of vitamin E, administered intravenously in an emulsion before surgery, on ischemia and reperfusion (I/R) injury in a double-blinded study on 68 patients. I/R injury is usually an outcome of liver surgery, which causes oxidative stress and cell damage. The results of the study indicated that administering vitamin E prior to surgery may reduce the impact of I/R injury in liver surgery. The antioxidant

activity of vitamin E has also been shown to protect against doxorubicin-induced cardiotoxicity in animal studies [74, 75]. Moreover, vitamin E was found to enhance the anticancer activity of doxorubicin on human prostatic carcinoma cells *in vitro* [76].

Another possible internal phase for intravenous emulsions is perfluorocarbons. Emulsions containing perfluorochemicals have been investigated as contrast agents for diagnostic tissue imaging or as carriers for the transport of oxygen offering an alternative to blood transfusions [77, 78]. Perfluorochemicals are chemically inert, synthetic molecules containing carbon and fluorine atoms, and are capable of dissolving considerable amounts of oxygen [78]. They are hydrophobic and as a result require emulsification for dispersion in aqueous media. Several types of perfluorochemicals have been investigated such as, perfluorooctyl bromide ($C_8F_{17}Br$), perfluorodecyl bromide ($C_{10}F_{21}Br$) and perfluorodichlorooctane ($C_8F_{16}C_{12}$). Imavist® (formally known as Imagent®) and Oxygent® are perfluorocarbon emulsions presently undergoing clinical trials as an ultrasound contrast agent and artificial blood substitute, respectively.

Emulsifiers

The purpose of surfactants is to emulsify the oil phase and to provide physical stability against flocculation and coalescence during storage, which may be for extended periods of time. Surfactants provide physical stability by reducing the oil-water interfacial tension and promoting droplet-droplet repulsion. Injectable emulsions are frequently emulsified with natural lecithins obtained from either egg yolk or soybeans. These lipids are biocompatible, biodegradable and have relatively

good emulsifying properties [79]. Lecithins differentiate in the nature of the headgroup and in the length and degree of saturation of the acyl chains. The headgroup can be either phosphatidic acid (PA), ethanolamine (PE), serine (PS) or PC and determines the surface charge of the emulsion. At pH 7, PE and PC headgroups are uncharged, while PA and PS are anionic. Surface charge can promote long-term emulsion stability by electrostatic repulsion and can influence its biodistribution *in vivo*.

The length and degree of saturation of the acyl chains greatly influences the gel-liquid phase transition temperature (T_c) and the surface properties of lipid bilayers (liposomes) and monolayers (emulsions). The T_c refers to the temperature at which the lipids shift from a highly ordered gel state to a less ordered fluid. Saturated lipids generally have phase transitions above room temperature (e.g. T_c of 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC; C18:0) is 58°C) [12, 79]. Introducing unsaturations or reducing the length of the acyl chains decreases the T_c substantially (e.g. T_c of DOPC (C18:1) is -22°C and T_c of DMPC (C14:0) is 23°C) [12, 79]. Most natural phosphatides have chain lengths of 16 to 18 carbons, however, chains with as little as 4 carbons also exist. Nii et al. [79] observed that both PC acyl chain length and degree of chain unsaturation influenced the ability of the lipid to emulsify tricaprylin (glyceryl trioctanoate). PCs having shorter and saturated acyl hydrocarbon chains were more effective emulsifiers as they produced emulsions with smaller mean globule size with less change in appearance and droplet size over time.

Lipid emulsions are often co-emulsified with a biocompatible synthetic surfactant to enhance emulsification properties. An example of an oil requiring co-

emulsification is vitamin E. Previous studies have shown that stable tocopherol emulsions cannot be prepared with lecithin as the sole emulsifier [72]. A possible explanation for this observation is the greater polarity of tocopherol compared to vegetable oils due to the presence of a hydroxyl group on the aromatic ring. The enhanced polarity of tocopherol may solubilize more lecithin in the emulsion core resulting in less emulsifier at the interface to stabilize the system. Consequently, a more hydrophilic co-emulsifier is required to emulsify tocopherol. The synthetic surfactants approved for intravenous injection are few and include polysorbates, cremophors and poloxamers.

To aid in the initial selection of emulsifiers, the hydrophile-lipophile balance (HLB) method is widely used. HLB is a system which classifies surfactants on an arbitrary scale based on the relative proportions of the hydrophilic and hydrophobic parts on the molecule. Each surfactant is given a number usually between 0 and 20. If the HLB value is high, the surfactant has a relatively large number of hydrophilic groups and is more soluble in water. In contrast, surfactants with low HLB are more hydrophobic and will consequently be more easily dispersed in organic phases. In general, stable w/o emulsions are formed from surfactants with low HLB, whereas those with high HLB are typically used to make stable o/w emulsions. HLB values for several synthetic emulsifiers that are approved for intravenous injection are listed in Table 5. The HLB method also classifies the oil, but in terms of HLB "required" ($HLB_{required}$). The $HLB_{required}$ specifies the HLB of the emulsifier that will produce the most stable emulsion. Oils are usually given two $HLB_{required}$ values, one to produce a stable o/w emulsion and the other for a stable w/o emulsion. This method allows the

formulator to match the HLB of the emulsifiers with the $HLB_{required}$ of the oil to produce a stable emulsion. The $HLB_{required}$ values to produce a stable o/w emulsion for cottonseed oil, safflower oil and soybean oil are 7.85, 7.72 and 7.66, respectively (Crodamol catalogue).

The HLB concept is advantageous in the initial screening stage of emulsion development as it reduces the number of emulsifiers to consider for a given type of oil. Although, the formulator should be aware that the HLB method has serious limitations, which arise from the fact that only the molecular structure of the individual surfactant is considered and the emulsion as a whole is ignored [8]. For instance, the HLB method does not take into account pertinent factors such as the conformation of the surfactant, the salinity of the aqueous phase or temperature [80]. Consequently, even if HLB and $HLB_{required}$ are correctly matched the emulsion produced may not be stable.

Aqueous phase

The isotonicity of an injectable emulsion is important in order to avoid disturbing the state of cells in contact with the formulation. The final osmolarity should be between 200 and 300 mOsm kg^{-1} and can be achieved by adding isotonizing agents such as glycerol, sorbitol and xylitol to the aqueous phase. Glycerol is more commonly used and can be found in most parenteral emulsions including Intralipid®, Lipofundin N®, Liposyn® and Soyacal®. Ionic agents such sodium chloride can also adjust osmolarity, however, they should be avoided as the ions can destabilize emulsions (see earlier). The pH of the final emulsion may need to be adjusted and this can be done by adding small amounts of HCl or NaOH. The desired pH is usually

between 7 and 8 in order to maintain physiological compatibility and minimize hydrolysis of the oil and phospholipids [81].

Antioxidants are often added to the emulsion to eliminate or reduce oxidation of the drug, oil and emulsifier [8, 67]. Common antioxidants used for injectable formulations include α-tocopherol, deferoxamine mesylate and ascorbic acid [81]. The formulation may also require the use of preservatives to resist against microbial growth. Microorganisms may change the physicochemical properties of the emulsion, such as color, odor, pH and physical stability, and may present a health hazard [8]. Common preservatives used in injectable preparations include phenol, cresol and methyl, ethyl or propyl esters of para-hydroxybenzoic acid [82].

5.2. Emulsion preparation

The most common process to manufacture emulsions is to incorporate the drug during the emulsification of the oil [66]. Another method to incorporate the active ingredient is to add a sterilized solution of the drug dissolved in a solvent to a pre-formed and sterilized emulsion. This method is not often done due to stability issues that may be encountered such as drug precipitation in the aqueous phase and emulsion cracking [1, 4]. The first step in emulsion preparation is usually to dissolve the water-soluble components (isotonizing agent and preservatives) in the aqueous phase and the lipophilic compounds (drug and perhaps the antioxidant) in the oil phase. The emulsifier can be dispersed in either phase. Both phases are typically heated and agitated to facilitate the dispersion of the various components [67]. The oil phase is then added to the aqueous phase. As the oil is added, the mixture is agitated with the aid of a medium-shear mixer and is usually heated. The rate of addition

should be optimized because adding the oil phase two quickly can lead to incomplete dispersion of the oil in the aqueous phase [67]. The temperature and duration of heating in the premix stage depends largely on the thermosensitivity of the drug, oil and emulsifier(s). This premix stage produces a coarse emulsion and can have a substantial impact on the final product [66]. A premix that is uniform with a droplet size under 20 μm, generally produces a final emulsion that is more unimodal and physically stable [66]. Following the premix stage, droplet size must be decreased to less than 5 μm in diameter and preferably below 1 μm, to avoid blocking the capillaries of the lungs. To produce emulsions with small droplet size microfluidization or high-pressure homogenization are usually used. Microfluidization is a process whereby a liquid mixture is forced by high pressure through an interaction chamber, which splits the stream in two and then recombines them at ultrahigh velocities [83]. The product can then be recycled to reduce droplet size further. The combination of high shear, turbulence and cavitation generated by this apparatus can produce submicron emulsions with a narrow size distribution [84]. In high-pressure homogenization, fluid is forced at high pressure by means of a plunger pump through a very narrow channel. Depending on the type of homogenizer the fluid may then collide head-on with another high velocity stream or hit a hard-impact ring. Droplet size is reduced by cavitation, high-shear forces and high-speed collisions with other droplets [85]. Pressure, temperature and number of passes are parameters that can be controlled and influence the efficiency of droplet size reduction.

After the desired droplet size is achieved, the formulation is filtered to remove large droplets or debris and sterilized. Sterilization can be achieved by autoclaving or by filtration through a 0.22 μm cartridge filter. The heat generated by autoclaving can cause the oil and lecithin to hydrolyze liberating free fatty acids, which will reduce the pH of the formulation. The conditions for sterilization by autoclaving will need to be selected carefully to minimize the degradation of heat sensitive products. Filter sterilization on the other hand greatly reduces the heat burden on the emulsion [67]. Not all emulsions can be sterilized by filtration, as a mean droplet size less than 200 nm is an essential requirement. Large droplets may clog the 0.22 μm cartridge filter and prevent sterile filtration [86]. The main manufacturing steps involved in the production of intravenous emulsions are outlined in Figure 10.

5.3. Emulsion characterization

Injectable emulsions are often characterized for mean droplet diameter, size distribution, surface charge and phase inversion temperature. The aforementioned properties are useful in predicting emulsion stability, biocompatibility and *in vivo* biodistribution. Control over droplet size and size distribution can impart some specificity towards target tissues and are also important predictors of biocompatibility as droplets greater than 5 μm can potentially form pulmonary emboli. The maximum allowable droplet size for intravenous administration, however, is unclear. Emulsions containing few droplets above than 5 μm might not necessarily cause any adverse reaction since capillary blockage may be reversible by droplet degradation and large droplets may pass through small capillaries by deforming [87]. Burnham et al. [88] found that fat droplets greater than 7.5 μm in diameter could deform and pass through

pulmonary vasculature without difficulty. There are a number of techniques available to measure mean droplet size and size distribution of emulsions. Dynamic light scattering (also known as photon correlation spectroscopy or quasielastic light scattering), atomic force microscopy, static light scattering (or intensity light scattering) and electron microscopy are frequently used to determine the size and size distribution for droplets below 1 μm [38, 89]. However, apart from atomic force microscopy and electron microscopy, the upper limit of detection on these instruments prevents the evaluation of the droplet size distribution above 5 μm. For detection of droplets greater than 5 μm, light obscuration, electrical-sensing zone (Coulter Counter) and optical microscopy are appropriate methods [90-92].

Surface charge measurements are also useful indicators of emulsion biocompatibility. Surfaces with a net positive charge are more likely to aggregate in the blood stream in the presence of plasma proteins than negatively-charged or neutral droplets. Charged surfaces can also impart physical stability to the emulsion by prevented/reducing coalescence upon random collisions through electrostatic repulsion (see earlier). The surface charge of an emulsion droplet can be obtained through zeta potential measurements using laser Doppler anemometry [93]. Lastly, phase inversion temperature, the temperature at which the emulsion changes from o/w to w/o or vice versa, can be a useful predictor of emulsion stability during temperature altering processes such as heating, emulsification and sterilization [94].

5.4. Stability measurement

The stability of an emulsion formulation is vital for its use in clinical applications. The formulation must display physical, chemical and microbial stability

for at least one year, if not more. The difficulty in emulsion formulation is that the system must be stable in aqueous solution, as opposed to polymeric micelles [95] or nanoparticles [96] which can be easily stored as a lyophilized powder whereby they have less opportunity to destabilize. Creaming and a visible layer of oil are classic signs of a physically unstable emulsion, while formulation discoloration is a typical indication of chemical instability. A long-term stability testing schedule should be performed on all promising formulation candidates, whereby each emulsion is stored at various temperatures ranging from 4 to 40 °C. The emulsions should be monitored for changes in size, pH, drug content, zeta potential, viscosity, electrical conductivity and chemical composition [81].

Physical stability

The long-term stability of an emulsion is difficult to estimate and only time can actually tell you whether the formulation is stable. Waiting for extended periods of time to find out whether a number of formulation efforts are stable is very impractical and there are methods available to accelerate stability testing. Most accelerated tests induce physical instability by increasing the number of collisions between globules. Accelerated tests based on sample heating, however, are not reliable as they do not reflect the environment of samples kept under storage conditions. Heating the sample not only enhances collisions but also diminishes the protective action of adsorbed surfactants, increases the solubility of all components, promotes the degradation of heat sensitive products, alters the electric double-layer and reduces surfactant adsorption at the emulsion interface, which can cause a potentially stable emulsion to destabilize, leading to erroneous results [2, 81].

Steam sterilization is considered an acceptable temperature raising accelerated test as it approximates the environment that an emulsion would experience during autoclaving. Excessive shaking and freezing and thawing cycles are other commonly used accelerated stability testing processes as these techniques predict the conditions that a formulation will be subjected to during transportation and storage [81]. Another good method to estimate emulsion stability is to make size measurements frequently several weeks after the formulations are prepared. Emulsions that increase in size over time during the first few weeks in storage will invariably destabilize [2]. If there is no change in size, the formulator can have some hope that the emulsions will be physically stable long term [2].

Chemical stability

Injectable emulsions can undergo chemical changes by oxidation and hydrolysis of the oil and/or emulsifier [67]. Chemical instability can be detected by formulation discoloration and by changes in pH due to the increase in free fatty-acids. Chemical instability can be reduced by storing the emulsions refrigerated, protected from light and in sealed containers with a layer of an inert gas, typically argon. These precautionary methods will reduce hydrolysis of the oil and emulsifier(s) [67]. Degradation of the encapsulated drug can also occur during storage. Consequently, the integrity of the encapsulated drug over time must also be determined [67].

Some groups have investigated storing lipid emulsions as a lyophilized powder to overcome some of the stabilized issues encountered when stored in solution [97, 98]. Freeze-drying emulsions, however, is difficult as the droplets may crack during the lyophilization process and is rarely done. Bensouda et al. [99]

evaluated the influence of a number of cryoprotective agents (glucose, mannitol, sorbitol, maltose, lactose, glycin and dextran) on the success of the freeze-drying process. Glucose, maltose, sorbitol and lactose provided protection against changes in particle size, while mannitol, dextran and glycin offered no protective action.

6. Lipid emulsions for the delivery of nucleic acid-based drugs

Gene therapy is the science in which nonfunctional genes are substituted, altered or supplemented for the treatment of genetic or acquired diseases. The difficulty in the successful application of gene therapy is the complexity of delivering functional genetic material such as plasmid DNA, antisense oligonucleotides (ODN) or small interfering RNA into the cell. This is a result of their rapid degradation in plasma and their inability to cross cell membranes due to their hydrophilic and polyanionic nature, and relatively large size. Viral vectors have been extensively investigated for gene delivery [100]. However, concerns over host inflammatory and immune responses have created a demand for non-viral vectors [101-103]. As such, cationic liposomes are commonly investigated because they enhance transfection of DNA into the cell [104, 105]. Nonetheless, a major shortcoming of this technology is the formation of large aggregates with time and the reduced transfection of the liposome/DNA complex in the presence of serum [106].

Cationic lipid emulsions have been considered as alternative non-viral gene delivery vectors to liposomes. Complexation occurs through electrostatic interactions between the nucleic acids and the cationic lipid emulsifiers. The lipid emulsion/DNA complex can be prevented from forming large aggregates in the presence of serum by

co-emulsification with an appropriate non-ionic surfactant [107]. Yi et al. [108] prepared a cationic lipid emulsion/DNA complex that retained greater than 60% transfection efficiency in the presence of 90% v/v serum. This formulation was composed of soybean oil, 1,2,-dioleoyl-*sn*-glycero-3-trimethylammonium propane (DOTAP) as the cationic surfactant and co-emulsified with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-N-[PEG 2000] (PEG_{2000}POPE). The relatively high transfection efficiency of the lipid emulsion/plasmid DNA complex can be attributed to its ability to resist aggregation in the presence of serum, which may be due to steric stabilization by the PEO chains of PEG_{2000}POPE. Indeed, Kim et al. [109] observed that adding a co-emulsifier containing a PEO chain such as polysorbate 80 or the Brij series (PEO 4, 7, 10 or 23 lauryl ether) produced DNA-complexed emulsions that could resist changes in size in the presence of serum. In contrast, surfactants without a PEO group such as sorbitan monooleate (Span 80), mannide oleate (Montanide 80) and oleyl alcohol aggregated in the presence of serum and during DNA complexation. The authors also observed that the presence of PEO in both polysorbate 80 and Brij interfered with the electrostatic interactions between DNA and the emulsion interface. DNA and cationic lipid interactions were reduced when the polysorbate 80 content or PEO chain length in the Brij series increased. Despite the progress, much still needs to be done for the successful application of cationic emulsions to deliver genetic material *in vivo*. Indeed, cationic lipid emulsions will most likely face the same problems as other non-viral gene delivery systems.

7. Conclusions

Lipid emulsions are promising carriers for highly lipophilic drugs due to their biocompatibility, reasonable shelf-life, aptness for large scale manufacture and ability to solubilize large quantities of drug in their oily core. Lipid emulsions can also alter the biodistribution of incorporated drugs and enhance specificity towards target tissues by passive and active methods. Evading the MPS or natural fat metabolism is necessary when the encapsulated drug is to be delivered to non-MPS organs or liver parenchymal cells, respectively. Long-circulating lipid emulsions can be obtained by reducing droplet size and by grafting long-chain hydrophilic polymers, such as PEO, to the emulsion interface. Moreover, active targeting using ligands that recognize specific determinants on cells can enhance specificity and uptake by target cells. It should be kept in mind that control over droplet biodistribution alone will not enhance the therapeutic effect as the drug may leak out of the carrier before reaching the target site. In general, drugs with higher lipophilicities (log $P > 9$) are retained better within the emulsion after intravenous administration. In addition to being carriers for lipophilic drugs, lipid emulsions have also been adapted to deliver genetic material and are an alternative non-viral vector to liposomes.

Several therapeutic lipid emulsions are commercially available for clinical use and other formulations are presently undergoing clinical trials. The use of intravenous lipid emulsions in the clinic will expand as new and less toxic formulations are discovered.

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9. References

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Tables

Table 1: Several commercially available emulsions for intravenous injection

Product	Drug	Manufacturer	Indications
Diazemuls®	Diazepam	Pfizer and Pharmacia	Anti-convulsive, sedative, muscle relaxant
Diazepam-®Lipuro	Diazepam	B. Braun	General anesthesia
Diprivan® *	Propofol	AstraZeneca	General anesthesia
Etomidate-®Lipuro	Etomidate	B. Braun	General anesthesia
Limethason® *	Dexamethasone pamitate	Mitsubishi Pharmaceutical	Rheumatoid arthritis
Liple® (Lipo-PGE_1)	Prostaglandin E_1 (PGE_1)	Mitsubishi Pharmaceutical	Vasodilator, platelet inhibitor
Propofol-Lipuro®	Propofol	B. Braun	General anesthesia
Lipo-NSAID® - Ropion® *	Flurbiprofen axetil	Kaken Pharmaceuticals	Pain reliever
Vitalipid® *	Vitamins A, D_2, E, K_1	Fresenius Kabi	Parenteral nutrition

* Based on the formulation of Intralipid® (10 or 20% soybean oil; 1.2% egg lecithin; 2.5% glycerol)

Table 2: Some intravenously injectable emulsions in development and in clinical trials

Drug	Product Name	Indications	Clinical*	Ref.
Aclacinomycin A	-	Cancer chemotherapy	-	[110]
Amphotericin B	-	Treatment of fungal infections	-	[97, 98]
Beta-elemene	SDP-111	Cancer chemotherapy	-	[111]
Cyclosporine A	-	Immunosuppressant	-	[112]
Docetaxel	SDP-014	Cancer chemotherapy	-	[111]
Perfluorooctyl bromide	Imavist®	Ultrasound contrast agent	Phase III	[113]
Paclitaxel	TOCOSOL/S-8184	Cancer chemotherapy	Phase III	[72]
Paclitaxel	SDP-013	Cancer chemotherapy	-	[111]
Perfluorooctyl bromide	Oxygent®	Artificial blood substitute	Phase III	[78]
Propofol	Ampofol®	General anesthesia Sedation	Phase III	[114]
Propofol	IDD-D propofol	General anesthesia Sedation	Phase III	[115]
Alpha-tocopherol succinate	SDP-112	Cancer chemotherapy	-	[111]
Vincristine	-	Cancer chemotherapy	-	[116]

* Status as of March 2006

Table 3: Antitumor activity of RS-1541 emulsion formulations against M5076 sarcoma at the MTD

Mean Diameter (nm)	Dose (MTD)[a] (mg/kg)	Tumor Diameter[b] (%)	Tumor Growth Delay[c] (day)	I.L.S.[d] (%)	Cure (on day 120)
Surfactant solution	6.0	213	17	62	0/6
70	4.5	166	24	66	0/6
100	4.5	113	29	69	0/6
220	15.0	13	>61	>224	4/6
380	40.0	18	56	>195	3/6

[a] RS-1541 was given in each formulation to M5076 bearing BDF1 mice via a single i.v. injection at MTDs on day 13 after inoculation (6 mice were used for each group).
[b] Tumor diameter on day 44 divided by that on treatment day.
[c] Days required for the tumors to reach again the diameter on treatment day following a therapy.
[d] Increase in life span: ratio (%) of median survival days in a treatment group of mice to that in the control group (37 days).
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Table 4: List of oils used in commercial emulsions for parenteral nutrition

Oils	Commercial Product name	Manufacturer
LCTs		
Cottonseed oil	Lipofundin®	B. Braun
Safflower oil	Liposyn®	Abbott Laboratories
Soybean oil	Intralipid®	Kabi-Pharmacia
	Soyacal®	Alpha Therapeutics
	Travamulsion®	Travenol Laboratories
	Liposyn III®	Abbott Laboratories
	Lipofundin S®	B. Braun
	Trivé 1000®	Egic
Safflower oil:Soybean oil	Liposyn II®	Abbott Laboratories
LCTs + MCTs		
Soybean oil:MCTs, (1:1)	Lipofundin® MCT/LCT	B. Braun

Table 5: Several non-phospholipid surfactants approved for intravenous administration in at least one country or under clinical investigation

Chemical Name	Common Names	Properties	MW	Ref.	HLB	Ref.
Poly(ethylene oxide) 35 castor oil	Cremophor EL	Non-ionic	2515	[117]	13.5	[118]
Poly(ethylene oxide) 40 castor oil	Cremophor RH 40	Non-ionic	N/A	-	14-16	[119]
Poly(ethylene oxide) 60 castor oil	Cremophor RH 60	Non-ionic	N/A	-	15-17	[119]
Poly(ethylene oxide) 20 sorbitan monolaurate	Polysorbate 20	Non-ionic	1225	[120]	16.9	[120]
Poly(ethylene oxide) 20 sorbitan monopalmitate	Polysorbate 40	Non-ionic	1282	[120]	15.6	[120]
Poly(ethylene oxide) 20 sorbitan monooleate	Polysorbate 80	Non-ionic	1310	[121]	15.0	[120]
PEO_{80}-*b*-PPO_{27}-*b*-PEO_{80} [a]	Poloxamer 188	Non-ionic	8400	[122]	29	[118]
PEO_{101}-*b*-PPO_{56}-*b*-PEO_{101} [a, b]	Poloxamer 407	Non-ionic	12600	[123]	22	[124]
d-α-tocopheryl polyethylene glycol 1000 succinate [b]	TPGS	Non-ionic	1513	[121]	13	[72]
Poly(ethylene oxide)-15-hydroxystearate	Solutol HS-15	Non-ionic	958	-	14-16	-
Deoxycholic acid	-	Anionic	392	-	24	[72]
Glycocholic acid	-	Anionic	465	-	N/A	-

[a] Poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide)
[b] In Phase III clinical trials

Figures

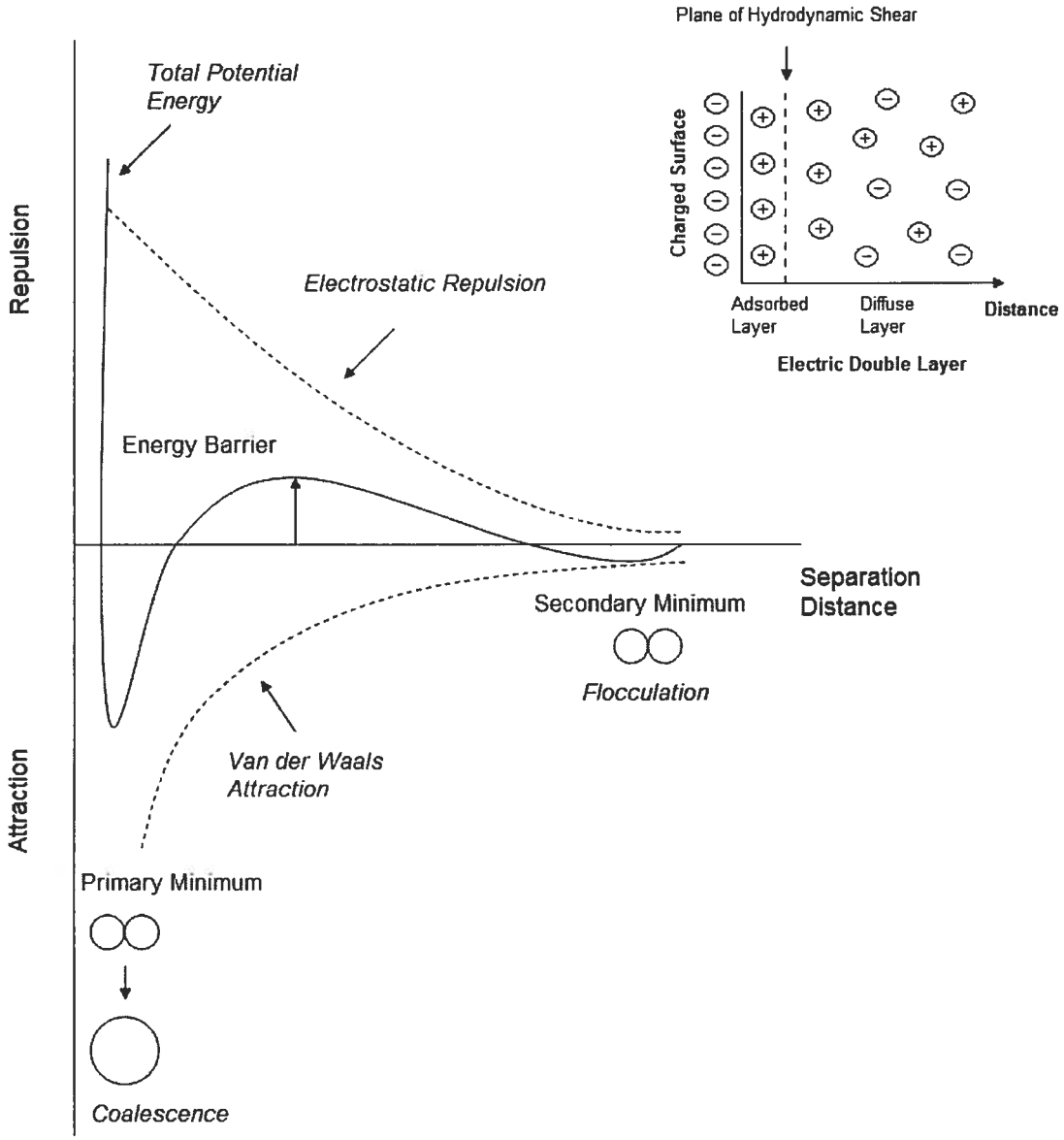

Figure 2 : The total potential energy of interaction between two droplets as a function of separation distance (electrical double layer repulsion and van der Waals attraction).

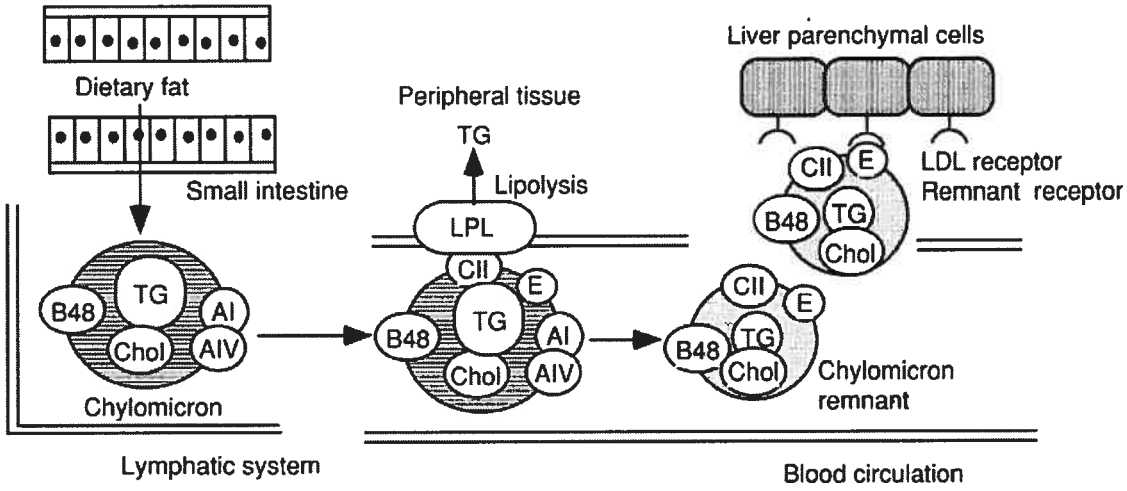

Figure 3 : Absorption and metabolism of dietary fat. Dietary fats are metabolized and incorporated into chylomicrons in the small intestine. Then, chylomicrons enter the blood circulation via the thoracic duct. During circulation, the triglycerides of chylomicrons are rapidly hydrolyzed via lipoprotein lipase (LPL) on endothelial surfaces, then chylomicron remnants are produced. Finally, chylomicron remnants are cleared by the liver by the LDL or remnant receptors. TG, triglyceride; Chol, cholesterol; AI, apolipoprotein AI; AIV, apolipoprotein AIV; B48, apolipoprotein B48; CII, apolipoprotein CII; E, apolipoprtoein E. Reprinted with permission from Elsevier Ref. [13] Copyright 2000.

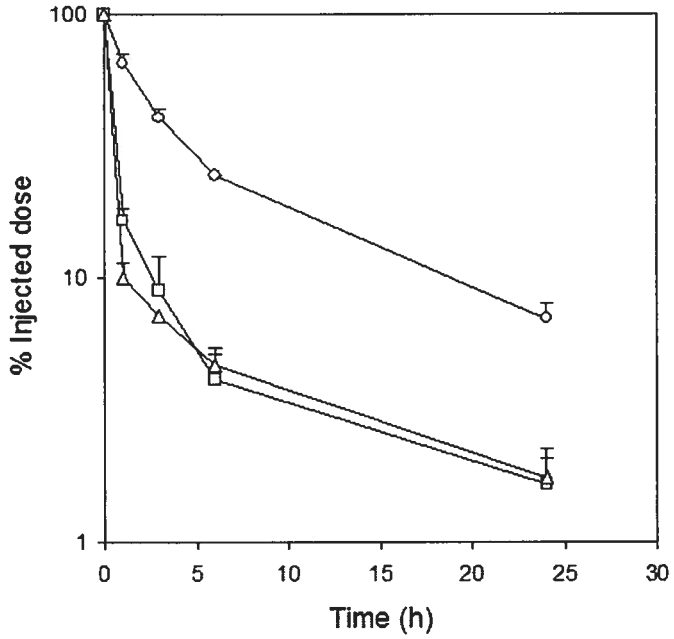

Figure 4 : Effect of particle size on the clearance of cholesteryl oleate (CO) label from plasma as a function of time after intravenous administration into mice. The emulsions were composed of triolein (TO): 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC):polysorbate 80: polyethylene glycol modified 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (PEG_{2000}-DPPE) (2:1:0.4:0.1, w/w) . The droplet sizes of the emulsions injected were 50 (○), 100 (□) and 175 nm (Δ). Adapted with permission from Elsevier Ref. [28] Copyright 1996.

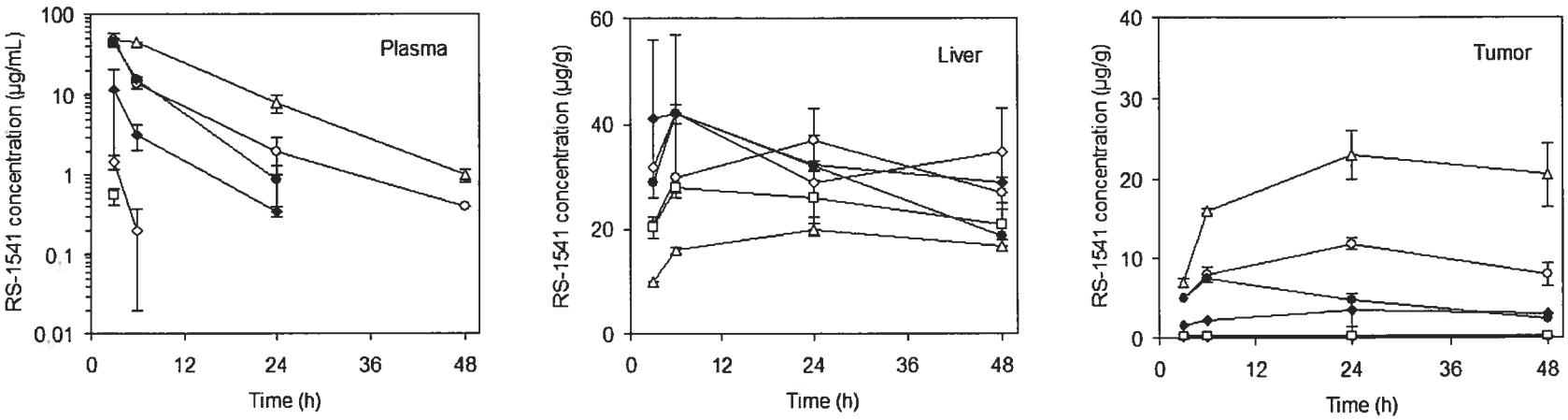

Figure 5 : Concentrations of 13-O-palmitoyl-rhizoxin (RS-1541) in the plasma, liver, and tumor after a single intravenous administration of various sizes of emulsion formulations and a surfactant solution of RS-1541 to mice bearing M5076 sarcoma at a dose of 5 mg/kg. The emulsion droplet sizes were 110 (Δ), 230 (○), 350 (♦), 410 (◊), 630 nm (□) and the surfactant solution (●). Each value represents the mean ± S.E. of three mice. Adapted with permission from Springer Science and Business Media Ref. [40] Copyright 1996.

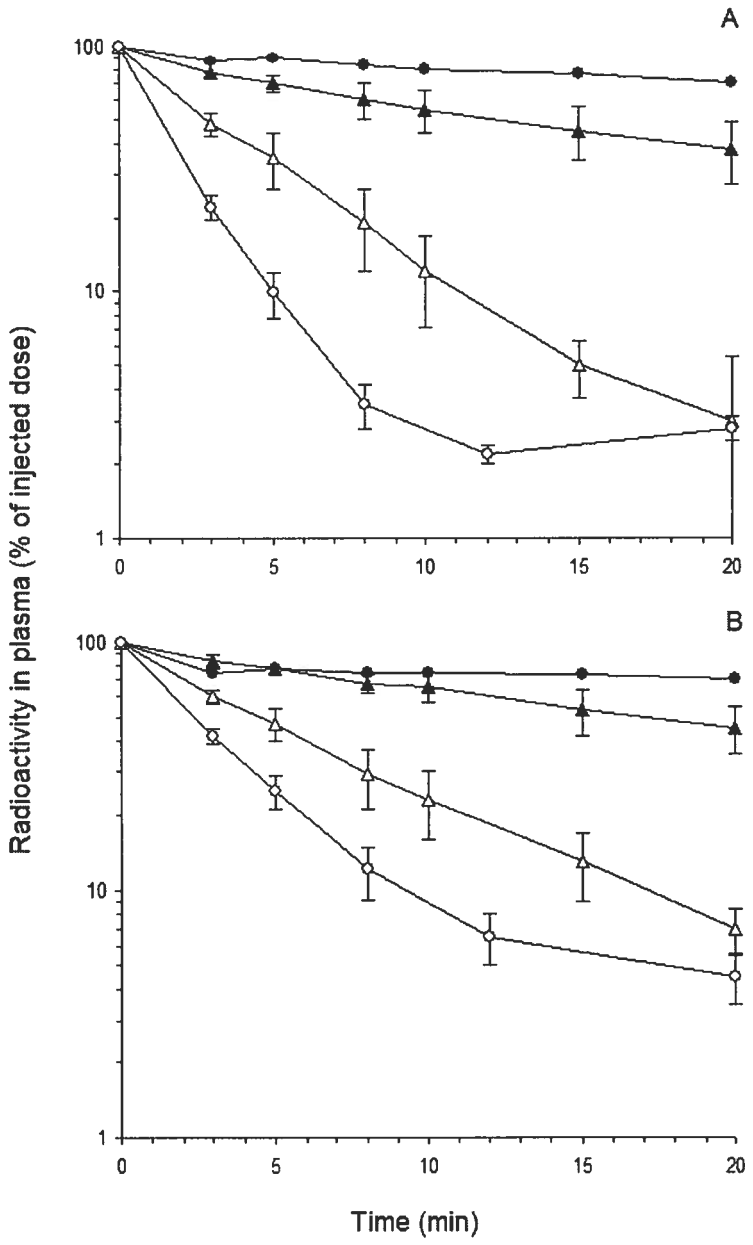

Figure 6 : Radioactivity in plasma of triolein (TO) and cholesteryl oleate (CO) labels after injection of emulsions stabilized by mixtures of sphingomyelin (SM) with egg phosphatidylcholine (egg PC). TO-CO-cholesterol emulsions stabilized with mixtures of SM and egg PC were injected intravenously in conscious rats. Plotted are the data for labeled TO (A) and CO (B) incorporated in the emulsions remaining in the plasma at 3, 5, 8, 12 and 20 min after injection. Results are means ± S.E. of at least four experiments for each observation. SM 100% (●), SM/egg PC 50/50 (▲), SM/egg PC 25/75 (Δ), egg PC 100% (○). Adapted with permission from Elsevier Ref. [21] Copyright 1992.

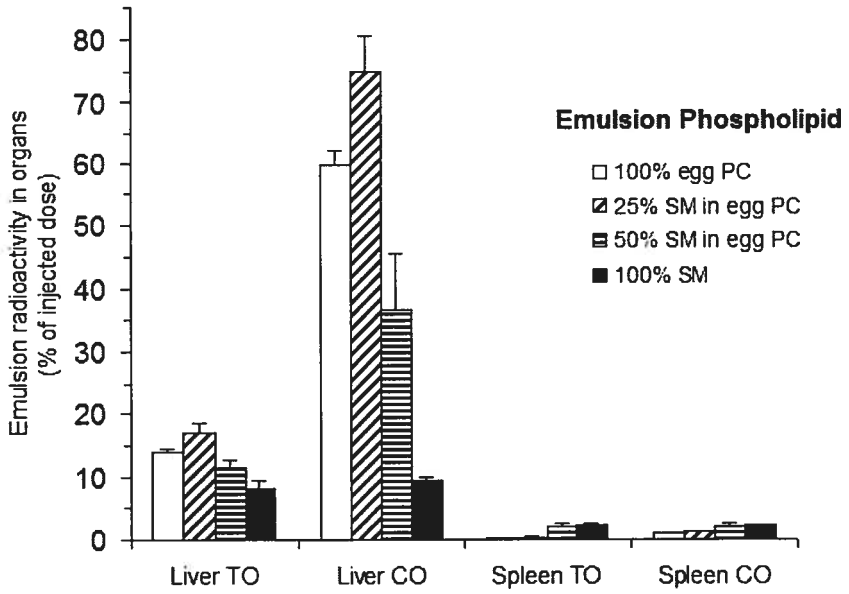

Figure 7 : Radioactivity in the liver and spleen of triolein (TO) and cholesteryl oleate (CO) labels after injection of emulsions stabilized by mixtures of sphingomyelin (SM) with egg phosphatidylcholine (egg PC). TO-CO-cholesterol emulsions stabilized with mixtures of SM and egg PC were injected intravenously in conscious rats. Organ uptakes of radioactive TO and CO labels in the emulsions were measured 20 min after injection. Results are means ± S.E. of at least four experiments for each observation. By analysis of variance the differences between groups were statistically significant with $P < 0.01$ for liver TO, $P < 0.001$ for liver CO, $P < 0.01$ for spleen TO and $P < 0.025$ for the spleen CO. Adapted with permission from Elsevier Ref. [21] Copyright 1992.

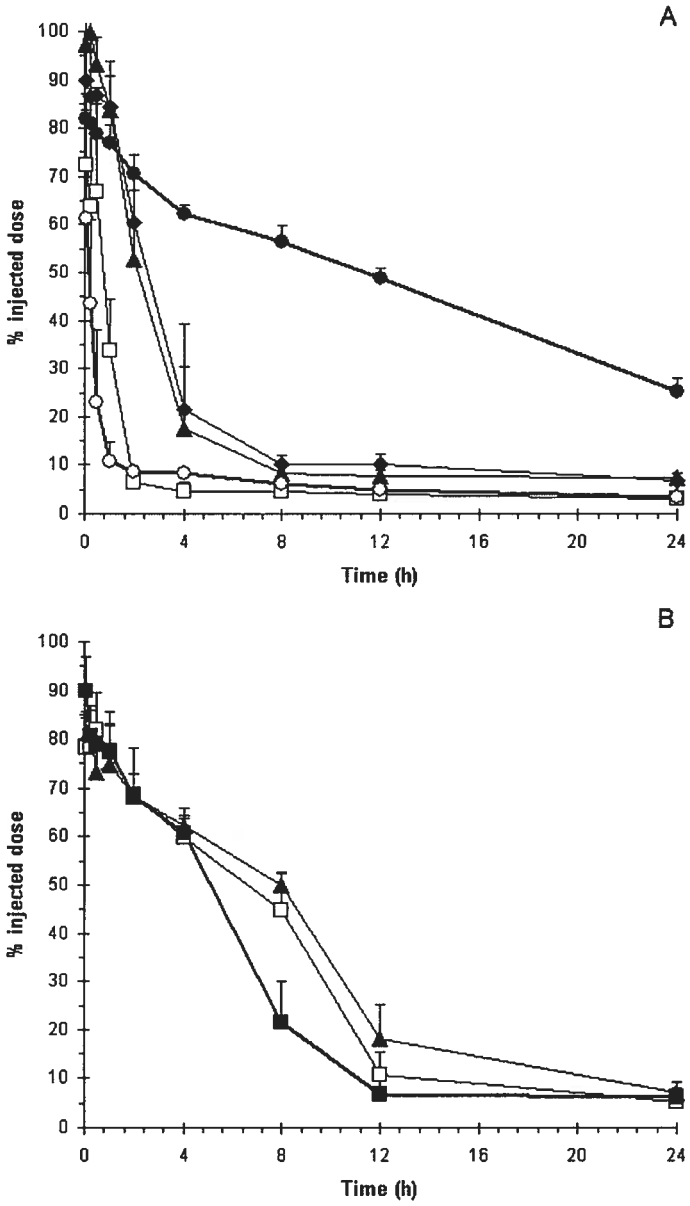

Figure 8 : Blood concentration-time profile of stealth liposomes and different lipid nanocapsule formulations prepared by the conventional (A) or post-insertion method (B). Mean ± SD (n =3 to 5). *A:* PEGylated liposomes (●), plain lipid nanocapsules (○), PEGylated lipid nanocapsules with 1.7 mol% 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-monomethoxy-[polyethylene glycol] (PEG_{2000}-DSPE) (□), PEGylated lipid nanocapsules with 1.4 mol% PEG_{5000}-DSPE (▲), and PEGylated lipid nanocapsules with 3.4 mol% PEG_{2000}-DSPE (♦). *B:* PEGylated lipid nanocapsules with 6 mol% PEG_{2000}-DSPE (■), PEGylated lipid nanocapsules with 6 mol% PEG_{5000}-DSPE (□), and PEGylated lipid nanocapsules with 10 mol% PEG_{2000}-DSPE (▲). Formulations were injected intravenously at a dose of 2 mg lipids/rat. Adapted with permission from Springer Science and Business Media Ref. [38] Copyright 2004.

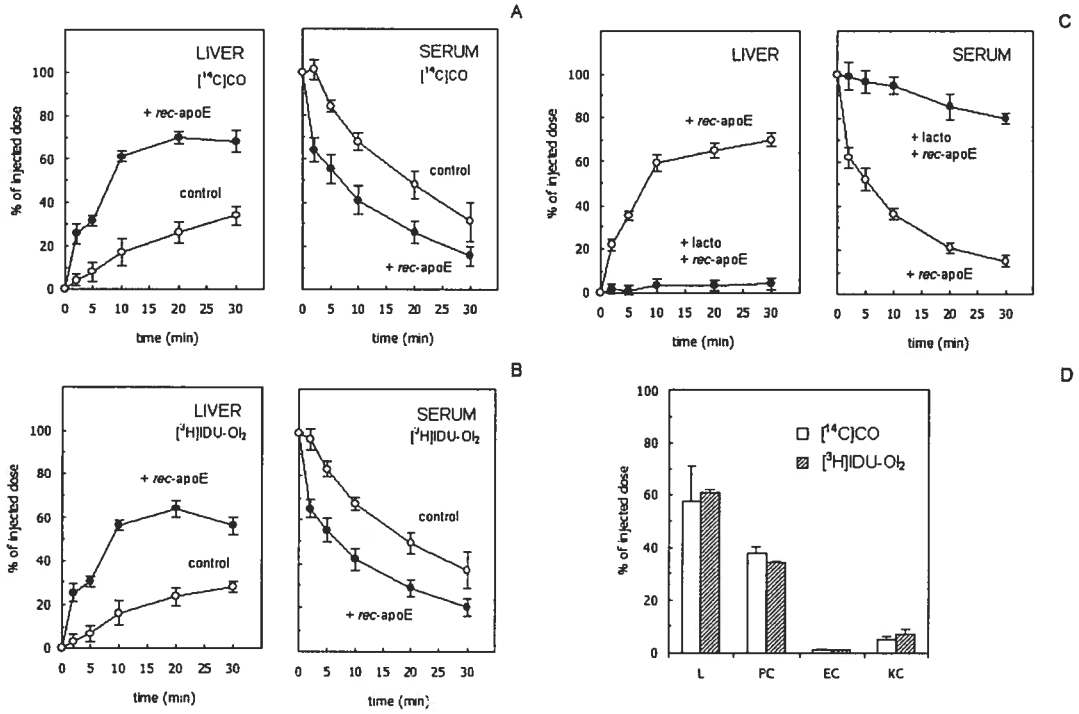

Figure 9 : Liver uptake and serum decay of the control and human recombinant (*rec*) apo E-enriched emulsion-iododeoxyuridine-oleoyl ([^{3}H]IDU-Ol$_2$) in rats, in the absence or presence of lactoferrin. Control and *rec*-apo E-enriched emulsions, double-labelled with [1-^{14}C]cholesteryl oleate ([^{14}C]CO) and [^{3}H]IDU-Ol$_2$ were injected into fasted anaesthetized rats. *A, B, C:* At the indicated times, the liver uptake and serum decay of [^{14}C]CO (A) and [^{3}H]IDU-Ol$_2$ (B) were determined. The liver uptake and serum decay of *rec*-apo E-enriched emulsions were also determined after preinjection of lactoferrin (C). *D:* At 30 min after injection of emulsion-*rec*-apo E-IDU-Ol$_2$, the liver was perfused. Total liver (L) association was determined and parenchymal (PC), endothelial (EC), and Kupffer (KC) cells were subsequently isolated. Values are means ± s.d. of three experiments. Adapted with permission from Macmillan Publishers Ltd. Ref. [60] Copyright 1995.

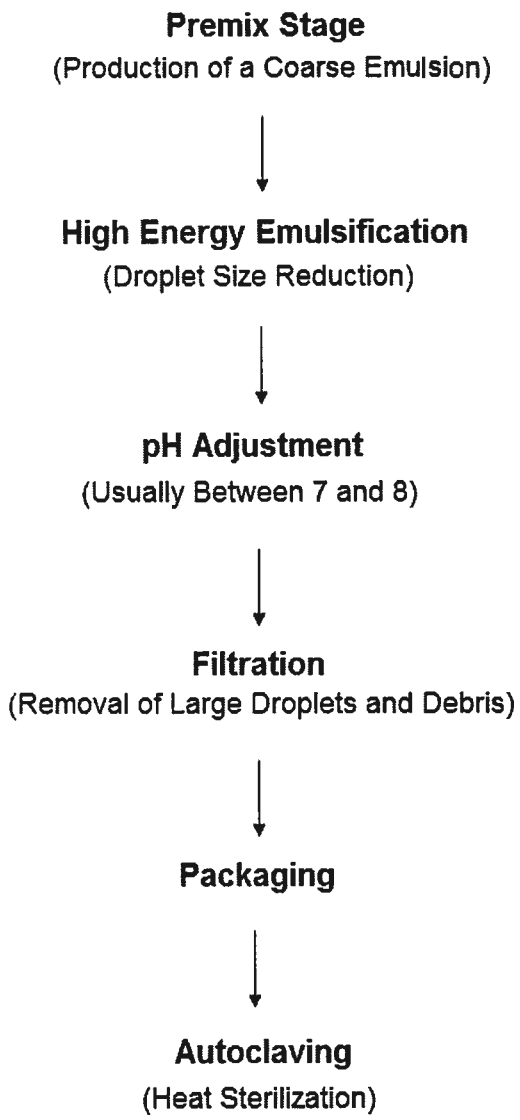

Figure 10 : The main manufacturing steps involved in the production of intravenous emulsions.

CHAPTER 3: PRESENTATION OF THE ARTICLE

Long-Circulating Poly(Ethylene Glycol)-Coated Emulsions to Target Solid Tumors

Joanna Rossi[1], Suzanne Giasson[1,2], Mohamed Nabil Khalid[1], Pascal Delmas[3], Christine Allen[4], and Jean-Christophe Leroux[1,*] (in preparation)

[1]Canada Research Chair in Drug Delivery, Faculty of Pharmacy and [2]Department of Chemistry University of Montreal, C.P. 6128 Succ. Centre-ville, Montréal, Québec, Canada H3C 3J7
[3]Bioxel Pharma Inc., 3075, ch. des Quatre-Bourgeois, Suite 440, Sainte-Foy, Québec, Canada G1W 4Y5
[4] Leslie Dan Faculty of Pharmacy, University of Toronto, 144 College Street, Toronto, Ontario, Canada M5S 3M2

*To whom correspondence should be addressed

1. Abstract

Purpose. The purpose of this study was to develop long-circulating emulsions and evaluate their ability to target neoplastic tissues. ***Methods.*** Nano-sized emulsions (100 – 120 nm) composed of tricaprylin (TC)/polysorbate 80 (PS-80)/hydrogenated soybean phosphatidylcholine (HSPC) and TC/PS-80/egg sphingomyelin (ESM) (5:3:1, *w/w*) were prepared by probe sonication. They were incubated with various poly(ethylene glycol)-distearoylphosphatidylethanolamine derivatives (DSPE-PEG) including DSPE-PEG 2000, DSPE-PEG 5000 and DSPE-N-[(pentaerythritol polyoxyethylene) glutaryl] (DSPE-4-armPEG) (PEG MW 2000). The effect of ESM, DSPE-PEG 2000 concentration and various DSPE-PEG derivatives in prolonging circulation time and enhancing accumulation into neoplastic tissues was assessed *in vivo* in mice bearing subcutaneously implanted B16 melanoma or C26 colon adenocarcinoma. ***Results.*** No significant differences were detected between TC/PS-80/HSPC and TC/PS-80/ESM emulsions *in vivo*. Only emulsions coated with either PEG 2000 or 5000 had prolonged circulation time in blood and were able to accumulate significantly into solid tumors. Emulsions grafted with DSPE-4-armPEG were removed quickly from blood despite the 2000 MW PEG segment. The ability of the long-circulating emulsions to extravasate at the tumor site depended on the tumor model, whereby the C26 tumors were more permeable to the emulsions than B16. ***Conclusion.*** Incorporating single-chain PEG 2000 or PEG 5000 at the interface significantly prolonged the residence time of emulsions in blood and enhanced their ability to target solid tumors. Such nano-sized emulsions could potentially improve the delivery of incorporated anticancer drugs to neoplastic tissues.

2. Introduction

One of the major difficulties in cancer therapy is to achieve good specificity of antineoplastic agents for their target site(s). As a result of their toxicity towards healthy tissues, many anticancer drugs are often administered at doses that are subtherapeutic. Over the past three decades, a great deal of research has been devoted to altering the pharmacokinetics and biodistribution profiles of these drugs by encapsulating them in colloidal drug carriers such as liposomes [1], nanoparticles [2] and polymeric micelles [3]. These nanoscopic systems can enhance drug accumulation at the tumor site and reduce distribution to healthy tissues, provided that the encapsulated cargo remains associated with the carrier after intravenous (i.v.) injection [4, 5]. One method in which drug carriers achieve this selectivity is by the enhanced permeation and retention (EPR) effect, which exploits the difference in capillary structure between healthy and cancerous tissues [6]. Tumor vasculature is generally porous or "leaky" allowing enhanced permeation of colloidal particles across the endothelium and into the extravascular space. Moreover, tumors have poor lymphatic drainage allowing colloids to be retained in the tissue for prolonged periods of time [7, 8]. For successful accumulation into the tumor by the EPR effect, the concentration of drug carrier in the blood must remain high for more than 6 hours [7]. However, after intravenous administration, colloidal drug carriers are usually recognized as foreign bodies and are rapidly taken up by circulating monocytes and macrophages in the liver, spleen and bone marrow. The ability of colloidal particles to evade the mononuclear phagocyte system (MPS) and exhibit long residence times

in blood depends largely on carrier size and the physicochemical properties of the surface.

Modifying the colloid surface with a hydrophilic and flexible polymer such as poly(ethylene glycol) (PEG) is widely used to prolong circulation time [9-11]. The longevity of PEGylated colloids is attributed to the highly hydrated and flexible PEG chains, which reduces interactions with plasma proteins and cell surfaces [12, 13]. Incorporating sphingomyelin (SM) at the interface is another approach that has been shown to enhance the systemic circulation time of emulsions and liposomes [14, 15]. For example, Takino et al. [15] reported that adding SM to an egg phosphatidylcholine (PC) emulsion prolonged circulation time and decreased uptake by the MPS organs. Similarly, Redgrave et al. [14] demonstrated that increasing the proportion of SM to PC further enhanced circulation time and resulted in a corresponding decrease in liver uptake. Moreover, liposomes composed of SM/cholesterol (Chol) (55/45, molar ratio) were shown to prolong the half-life of encapsulated vincristine, ciprofloxacin and vinorelbine [16-19]. In fact, this SM/Chol formulation with encapsulated vincristine was evaluated in Phase III clinical trials [20]. SM confers circulation longevity to emulsions and liposomes by enhancing membrane rigidity, resulting in lower membrane permeability and greater stability *in vivo* [21-24]. This membrane rigidifying effect of SM is a result of its high content of saturated acyl chains relative to naturally occurring PCs and its ability to form intermolecular hydrogen bonds [21, 25].

Among the various drug delivery systems for cancer therapy, emulsions are promising carriers due to their biocompatibility, reasonable stability, ability to

solubilize large quantities of lipophilic compounds and relative ease of manufacture at an industrial scale [26, 27]. Despite the vast amount of literature available on emulsions as carriers for anticancer drugs, not much work has been devoted to characterizing the accumulation of the droplet itself into solid tumors. The purpose of this study was to develop long-circulating emulsions using pharmaceutically acceptable excipients and evaluate their ability to target neoplastic tissues. The influence of egg SM (ESM) and distearoylphosphatidylethanolamine (DSPE)-PEG concentration and structure in prolonging circulation time and enhancing accumulation into solid tumors was assessed in mice bearing subcutaneously implanted B16 melanoma or C26 colon adenocarcinoma.

3. Materials and methods

3.1. Materials

Hydrogenated soybean phosphatidylcholine (HSPC, >99% PC), egg sphingomyelin (ESM) and 1,2-distearoyl-*sn*-glycero-3-phosphatodylethanolamine-*N*-monomethoxy-[PEG 2000] (DSPE-PEG 2000) were purchased from Northern Lipids Inc. (Vancouver, BC, Canada). Tricaprylin (TC) and poly(ethylene oxide) 20 sorbitan monooleate (polysorbate 80, PS-80) were obtained from Sigma-Aldrich (Oakville, ON, Canada). DSPE-PEG 5000 was bought from Avanti Polar Lipids Inc. (Alabaster, AL) and DSPE-N-[(pentaerythritol polyoxyethylene) glutaryl] (DSPE-4-armPEG) (PEG MW 2000) was from the NOF Corporation (Tokyo, Japan). [^{3}H]-Cholesteryl hexadecyl ether ([^{3}H]-CHE, 51.0 Ci/mmol), Hionic-Fluor®, Soluene 350® and Solvable® were obtained from Perkin Elmer (Woodbridge, ON, Canada). Sodium chloride (0.9% *w/v*) injection USP was purchased from B. Braun Medical Inc. (Irvine,

CA). All products mentioned above were used without further purification. Chloroform and hexane (purification grade), purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada), were further purified by filtration through drying columns on a PureSolv system (Innovative Technology Inc. system, Boston, MA) and distilled prior to use. Ethanol was obtained from Commercial Alcohols Inc. (Brampton, ON, Canada) and distilled. Water was deionized with a MilliQ® purification system (Millipore, Bedford, MA).

3.2. Compression isotherms

Surface pressure - molecular area (π-A) isotherms of HSPC, ESM, PS-80, HSPC/PS-80 and ESM/PS-80 were measured with a Langmuir-Blodgett trough (300 x 200 x 5 mm) from Nima Technology (Coventry, England). Surface pressure was determined by means of a Wilhelmy plate attached to a microbalance. The subphase was a phosphate buffered saline (PBS, pH 7.4) composed of 75 mM sodium chloride, 53 mM sodium phosphate dibasic, and 13 mM sodium phosphate monobasic, filtered through a 0.22-µm membrane. The temperature of the subphase was maintained at 25°C with a thermostated, circulating water bath. Solutions of HSPC and ESM were prepared at a concentration of 1 mg/mL in chloroform and hexane:ethanol (95:5, *v/v*), respectively. Fifty microliters of solution were deposited dropwise at the air/water interface using a microsyringe. For PS-80, dissolved in chloroform (0.25 mg/mL), 20 µL of solution were spread on the subphase. In the case of the mixed monolayer isotherms (0.25 mg/mL total amphiphiles), 20 µL of HSPC/PS-80 (1:3, *w/w*) dissolved in chloroform and ESM/PS-80 (1:3, *w/w*) prepared in hexane:ethanol (95:5, *v/v*) were deposited. The films were compressed at a constant barrier speed of 8

cm^2/min after initial delay periods of 30 min and 1 h for lipids spread in chloroform and hexane:ethanol, respectively. To reduce contamination from the atmosphere, the entire apparatus was located in a closed Plexiglas cabinet.

From the π-A isotherms, monolayer compressibility at a given surface pressure (π) was calculated using equation (1):

$$C_s = (-1/A_\pi)(dA/d\pi)_\pi \quad (1)$$

where C_s is the modulus of compressibility and A_π represents the molecular area at a particular surface pressure.

3.3. Preparation and characterization of emulsions

The emulsions were prepared by probe sonication using a Sonic Dismembrator (model 550, Fisher Scientific, Pittsburgh, PA). Prior to sonication, TC, PS-80 and either ESM or HSPC were mixed together under magnetic stirring above the phase transition temperature of ESM and HSPC (ca. 55°C) for 10 min. The dispersing phase (0.9% NaCl in water) was then added to the premix and the formulation was heated/mixed at 55°C for an additional 20 min. The volume was adjusted with 0.9% NaCl in water until the lipid phase represented 9% (*w/v*) of the emulsion. After the premix step, the formulation was sonicated at medium intensity (80 W) for 40 s on pulse mode (pulse every 2 s for 0.2 s). The composition of oil and emulsifier was TC/PS-80/HSPC or ESM (5:3:1, *w/w*). PEGylated emulsions were prepared by incubating an aqueous micelle solution of DSPE-PEG with a preformed ESM emulsion for 1.5 h at 60°C. The amount of DSPE-PEG added to the emulsions corresponded to either 10 or 15 mol% of the total surface components (excluding

TC). [^{3}H]-CHE, a non-exchangeable lipid derivative, was incorporated into each emulsion during emulsification to track the distribution of the entire droplet *in vivo*.

The mean hydrodynamic diameter and size distribution of the emulsions were determined by dynamic light scattering with a Malvern Autosizer (Malvern Instruments Ltd, Malvern, UK) at 25°C and a fixed angle of 90°. Measurements were performed in triplicate after dilution of the emulsion in water.

3.4. Biodistribution studies

The *in vivo* experiments were performed on either C57BL/6 mice (female, 18-21 g) bearing B16-F10 melanoma or Balb/C mice (female, 18-21 g) with C26 colon adenocarcinoma. Animal care and handling were approved by the Animal Welfare and Ethics Committee of the University of Montreal in accordance with the Canadian Council on Animal Care guidelines. The hair on the back and hind legs of the mice were removed by shaving. The B16 and C26 cells were harvested by trypsinization, resuspended in growth medium and injected subcutaneously in three separate locations on the back of each mouse. For the B16 cells, approximately $1x10^7$ cells in 50 µL of growth medium were delivered per implantation site, while $2x10^6$ of C26 cells in 50 µL were injected. The formulations were administered when the tumor volume reached approximately 20 mm^3. The volume (V) of each tumor was calculated from equation (2):

$$V = 1/2(4\pi/3)(L/2)(W/2)H \quad (2)$$

where L is the length, W is the width and H is the height.

The emulsions were prepared as described above and diluted with NaCl (0.9%) for injection. The tumor-bearing mice were anesthetized with isofluorane and

administered 5.4 mg of lipids/mouse (excluding DSPE-PEG) in a 100-μL injection volume via the subclavian vein. Each mouse received approximately 0.7 μCi [^{3}H]-CHE.

The mice (n=4-5 per group) were sacrificed at 2, 6 and 12 h post injection. At each time point, the blood, tumor and organs of interest (liver, spleen, heart, lungs, kidneys, and a piece of muscle) were extracted and assayed for [^{3}H]-CHE content. Blood was sampled by cardiac puncture and placed into pre-weighed scintillation vials. Excess blood was removed from the organs by passing a continuous flow of 0.9% saline through the systemic circulation via the heart. The tumors and organs of interest were then harvested, wiped and weighed. The blood was digested in a mixture of isopropanol/Soluene® (1:1 to 1:3 *v/v*) or isopropanol/Solvable® (1:1 to 1:3 *v/v*) at 60°C until complete solubilization. The samples were allowed to cool down to room temperature and were then discoloured with hydrogen peroxide (H_2O_2, 30% *v/v*) in aliquots of 100 μL. The organs and tumor were dissolved in Soluene® or Solvable® tissue solubilizers at 60°C until total digestion. Hionic-Fluor scintillation cocktail (10 mL) was added to the solubilized tissues and the samples were stored overnight at 4°C in the dark prior to counting. The amount of [^{3}H]-CHE radioactivity in blood, organs and tumor was assayed using a Beckman liquid scintillation counter (model LS 6500, Beckman, Fullerton, CA). The quenching of radioactivity due to the digested tissues was corrected with a quench curve. The percent injected dose (ID) of emulsions in blood was determined by assuming that the total mass of blood represents 7.2% of the mouse body weight [28]. Formulations were compared at each

time point with a one-way analysis of variance followed by Fisher's post-hoc test. The level of significance was a p value < 0.05.

4. Results and discussion

4.1. Compression isotherms

The compressibility and molecular conformations of two high phase transition lipids, HSPC and ESM, were characterized at the air/water interface using the Langmuir balance technique. Isotherms of pure HSPC and ESM monolayers in PBS buffer (pH 7.4, 25°C) are presented in Figure 11A. For clarity, only one isotherm for each compound was selected from a series of reproducible profiles. The ESM monolayer displayed a two-dimensional phase transition from liquid-expanded (chain disordered) to liquid-condensed (chain ordered). This transition region can be seen more clearly in Figure 11B by the change in slope of the π-A isotherm, expressed as the modulus of compressibility (see equation 1). The two-dimensional phase transition is consistent with previous reports, however, the sharpness of the transition region has been shown to vary depending on several factors including subphase temperature, chain length and heterogeneity in acyl chain composition [29, 30]. Naturally occurring SMs such as bovine brain SM and ESM tend to broaden the transition region compared to pure 18:0 SM and 16:0 SM, which are the main lipids in bovine brain SM and ESM, respectively [29]. The broader transition is most likely due to the heterogeneity in acyl chain composition, which would affect the alignment/ordering of the chains. The HSPC film, on the other hand, did not undergo an order-disorder transition like ESM. Instead, HSPC shifted from gas directly into a highly ordered liquid-condensed phase and then to a collapsed regime (Figure 11A).

HSPC formed a less compressible film at the air/water interface as indicated in Figure 11B by the lower the modulus of compressibility compared to ESM at all surface pressures. Another important difference between the molecules is that ESM could form a more densely packed film at higher surface pressures. Indeed, above about 25 mN/m, the area occupied per molecule was smaller for ESM than HSPC (Figure 11A).

The differences between HSPC and ESM molecules at the air/water interface can be rationalized by their structural discrepancies. Both ESM and HSPC share a common zwitterionic phosphorylcholine headgroup, however, they differ in the degree of chain unsaturation and in their ability to form hydrogen bonds. HSPC, like other PCs, contains two acyl chains that are esterified to a glycerol backbone. These acyl chains are approximately the same length, however, unlike most naturally occurring PCs, HSPC contains no unsaturations (major component is 16:0, 18:0). For ESM, a saturated acyl chain is linked to a sphingosine through an amide bond. The greater compressibility of ESM may be due to the unsaturation present between carbons 4 and 5 of the sphingosine, which could produce a more disordered and fluid monolayer than HSPC. The HSPC molecules are more ordered in the hydrophobic region due to the long and completely saturated acyl chains and thus are less compressible in the liquid phase. These results are consistent with the findings of Smaby et al. [29], whereby ESM was more compressible than a saturated PC with long acyl chains, such as distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC) (18:0, 18:0), at all surface pressures. In addition, ESM was more compressible than 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphatidylcholine (PSPC) (16:0, 18:0) and 1,2-

dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) (16:0, 16:0) at surface pressures above 5 and 10 mN/m, respectively [29]. Below these surface pressures, PSPC and DPPC, were in a chain-disordered regime and were slightly more compressible than ESM [29].

In addition to the differences in chain unsaturation, ESM has a much greater hydrogen bonding capacity than HSPC due to the amide and hydroxyl groups, which are not present in phospholipids. The intra- and intermolecular hydrogen bonding within and between ESM molecules may facilitate a more condensed organization of the head group region reducing the degree of hydration in the polar region and allowing for denser packing of the molecules upon compression compared to HSPC. These results are consistent with the findings of other group which report a lower degree of head group hydration in SM containing monolayers and bilayers compared with PCs [31-33].

The influence of PS-80, a major component in the emulsion, on the phase behavior of HSPC and ESM is illustrated in Figure 12A. For all isotherms, the collapse pressure was not achieved. Consequently, the monolayers were compressed until the minimum area of the trough was reached. In the presence of PS-80, HSPC and ESM displayed gaseous and liquid-phase behavior over the range of molecular areas investigated. These mixed monolayers were more fluid and compressible than pure HSPC and ESM films. The larger, more hydrated head group of PS-80 (poly(ethylene oxide) 20 sorbitan) would take up more space than phosphorylcholine at the air/water interface, preventing close intermolecular contact. All three monolayers exhibited similar compressibility (Figure 12B), however, ESM/PS-80

formed a more densely packed film as indicated by the smaller surface areas occupied by this mixture. A hypothesis proposed to explain this phenomenon is that ESM may reduce head group hydration by intra- and intermolecular hydrogen bonding within ESM molecules and between neighboring ESM molecules or with PS-80. As a result, the reduction in hydration of the head group region could permit denser packing of the molecules. In the context of injectable emulsions for drug delivery, incorporating ESM into a formulation emulsified with PS-80 may potentially enhance circulation time by the tighter molecular packing at the emulsion interface. Owing to the interesting properties of ESM/PS-80 monolayers, we then investigated *in vivo* whether ESM could enhance the circulation longevity of an emulsion coemulsified with PS-80.

4.2. Biodistribution studies

Oil-in-water emulsions with mean diameters ranging from 100 to 120 nm were prepared by probe sonication (Table 6). The internal phase consisted of TC and was emulsified with a combination of PS-80 and either HSPC or ESM. The weight ratio of oil and emulsifier was kept constant at 5:3:1 (TC/PS-80/HSPC or ESM). TC was chosen as the internal phase because medium chain triglycerides (MCTs) are generally better solubilizers for drugs than long chain triglycerides (LCTs) and thus these emulsions could potentially be used to encapsulate drugs [34-36]. PS-80 was the main emulsifier since it is well tolerated for i.v. application and the high density of short PEG segments at the emulsion interface may extend systemic circulation [37-39]. Only emulsions prepared with ESM were incubated with various DSPE-PEG derivatives. The final concentration of DSPE-PEG in the formulation represented 10

to 15 mol% of the total surface components (excluding TC). The presence of DSPE-PEG did not considerably alter the mean size of the emulsion (Table 6).

The influence of ESM and DSPE-PEG 2000 in prolonging the circulation time of emulsions was first assessed in C57BL/6 mice inoculated with B16 melanoma. To track the distribution of the droplets *in vivo*, the emulsions were labeled with [^{3}H]-CHE. This marker has been commonly used for biodistribution studies since it is highly lipophilic and non-exchangeable [40-42]. In this first experiment, three different formulations were administered: TC/PS-80/HSPC, TC/PS-80/ESM and TC/PS-80/ESM/(10 mol%)DSPE-PEG 2000. As shown in Figure 13A, the non-PEGylated HSPC and ESM emulsions were quickly removed from the systemic circulation with less than 10 % of the ID remaining in blood after 2 h. Emulsions containing ESM circulated slightly longer in blood than HSPC, however, statistical significance could not be demonstrated ($p > 0.05$). The slightly higher residence time for the ESM emulsion may be attributed to a tighter monolayer at the interface, which could potentially enhance the stability of the emulsion in blood. This tighter monolayer, however, was insufficient to provide significant enhancement in circulation longevity. As anticipated, Figure 13A shows that PEGylation of the TC/PS-80/ESM emulsion with 10 mol% PEG 2000 significantly prolonged circulation time ($p < 0.05$). Two hours after administration, approximately 35% ID was still present in blood. Surprisingly, despite the long-circulating properties of the PEGylated emulsion, all formulations showed similar accumulation into B16 melanoma tissues (Figure 13B). After 12 h, uptake into the tumor was between 1.2 and 2.2% ID/g of tissue. The comparable accumulation into the tumor for short and

long-circulating emulsions may be a result of low pore cut-off size in the tumor vasculature, restricting droplet extravasation across the vessel wall. Depending on the cell line, tumors can have varied pore cutoff sizes, ranging from 200 nm to 1.2 μm [43]. Despite the limited distribution into B16 melanoma, all emulsions accumulated more in the tumor than in the muscle (Figure 13B and C). Our results for vector distribution into the tumor are slightly lower compared to long-circulating liposomes (~ 100 nm in diameter) whereby accumulation into B16 melanoma (inoculated intramuscularly or in the hind footpad) was between 4.5 to 13.2% ID/g 24 h after injection [44]. The lower uptake of our PEGylated emulsions into B16 melanoma may be due to differences in the site of tumor inoculation, the size of the tumor at the time of injection and the time point at which the tumor was harvested. It was found that tumor uptake increased 1.7-fold, when liposome diameter was reduced from 138 to 97 nm without any significant change in blood residence time, demonstrating the importance of size on colloid extravasation into B16 melanoma [44].

The effect of DSPE-PEG 2000 content (10 and 15 mol%) and different DSPE-PEG derivatives in prolonging circulation time was then explored in Balb/C mice inoculated with C26 colon adenocarcinoma. In this study, five different formulations were injected: TC/PS-80/ESM, TC/PS-80/ESM/(10mol%)DSPE-PEG 2000, TC/PS-80/ESM/(15 mol%)DSPE-PEG 2000, TC/PS-80/ESM/(10 mol%)DSPE-PEG 5000, and TC/PS-80/ESM/(10 mol%)DSPE-4-armPEG. Elimination profiles of the emulsions from blood are shown in Figure 14A. Comparable to the study in mice bearing B16 tumors, the non-PEGylated ESM emulsion was quickly removed from blood with less than 20% of the ID remaining in the circulation 2 h post injection.

Also similar to the mice inoculated with B16 melanoma, the emulsions containing 10 mol% PEG 2000 prolonged circulation time with about 40% of the ID still present in the blood after 2 h. Increasing the concentration of PEG 2000 to 15 mol% or enhancing the length of the PEG chain to 5000 g/mol did not further prolong blood residence time. It is possible that at 10 mol% PEG 2000, the colloid reached its optimum protection with PEG and any additional increase in concentration and chain length would not enhance circulation time further. Previous studies by our group have shown that at low PEG concentrations (less than 4 mol%), small increases in PEG concentration or increasing chain length from 2000 to 5000 g/mol enhanced the circulation time of lipid nanocapsules [40]. However, at 6 mol% and above, almost no difference was observed with increasing PEG 2000 concentration [40, 45] or PEG chain length (2000 to 5000 g/mol) [40]. Similarly, Liu et al. [37] observed that increasing the concentration of PEG 2000 at the interface prolonged circulation time until a plateau in blood concentration was reached after approximately 5 mol%. Although the amount of DSPE-PEG anchored to the droplet surface was not quantified in our study, the lack of detection of micelles in the region of 3-10 nm by dynamic light scattering suggests that most of the DSPE-PEG was indeed bound to the droplets (data not shown).

Even though grafting density was high (10 mol%), droplets coated with DSPE-4-armPEG did not circulate as long as the other single chain DSPE-PEG derivatives (Figure 14A). The PEG chain lengths of each arm were probably too short to provide sufficient protection against opsonization. Indeed, the number of repeating ethylene oxide units was only 11 per arm for 4-armPEG (equivalent to about 500

g/mol per arm) compared to 45 and 113 for PEG 2000 and PEG 5000, respectively. Previous groups have shown that the circulation time of the carrier is strongly dependent on PEG chain length [46, 47]. For example, Allen et al. [47] observed that liposomes grafted with PEG 1900 and PEG 5000 prolonged the blood residence time of the carrier, while liposomes coated with shorter chain PEG-lipid derivatives (i.e. PEG 750 and PEG 120) were removed more quickly from the systemic circulation.

Enhanced circulation time of PEGylated emulsions in blood translated into higher accumulation into C26 tumors (Figure 14B). The distribution of emulsions grafted with PEG 2000 or PEG 5000 into the tumor was between 7.4 and 10.6% ID/g after 12 h, while only 2.6 and 3.9% ID/g was detected in the tumor for plain and 4-armPEG coated emulsions, respectively. Even though plain and 4-armPEG coated emulsions extravasated less into C26 tumors, all emulsions displayed selectivity for the tumor over the muscle. Indeed, no more than 1.1% ID/g was distributed to the muscle (Figure 14B and C). Our results are in general agreement with those obtained with stealth liposomes, whereby their accumulation into subcutaneously implanted C26 colon adenocarcinoma after 1 and 16 h post i.v. injection was approximately 7 and 18% ID/g, respectively [48]. These liposomes (~ 90 nm in diameter) reached a maximum accumulation of about 20% ID/g at the 24 h time point [48].

Regardless of the similar circulation times of emulsions with 10 mol% PEG 2000, accumulation was greater in C26 tumors compared to B16 at all time points. The greatest difference was detected at 12 h post injection, whereby the emulsion exhibited a 5-fold greater accumulation into C26 tumors than B16 (Figure 13B and Figure 14B). This observation may be explained by lower vascular permeability of

the B16 tumor compared to C26. In addition to leaky vasculature, other factors such as differences in blood vessel density, blow flow rate, and interstitial pressure in different tumor types can affect particulate accumulation [8]. Our finding is contrary to the results of Ishida et al. [49] who reported that B16 and C26 tumors showed similar permeability to liposomes with mean diameters ranging from 60 to 400 nm. The discrepancy between the two studies may be attributed to differences in vascular permeability and tumor volume at the time of injection.

The uptake of the emulsions by different organs after 2, 6 and 12 h post i.v. injection is presented in Figure 15. As expected, the emulsions distributed mainly to the MPS organs, with the majority of the formulation accumulating in the liver. For all emulsions, accumulation into the lungs, heart and kidneys was low. The enhanced blood levels of emulsions with PEG 2000 (10 and 15 mol%) and PEG 5000 translated into lower uptake by the liver. In contrast, the plain and 4-armPEG-coated emulsions which were cleared from blood faster accumulated more in the liver.

Several studies have reported the ability of emulsions to enhance the accumulation of anticancer agents into solid tumors compared to the free drug [5, 50, 51]. While other reports have shown a greater reduction in tumor volume over time when drugs are encapsulated into long-circulating emulsions versus free drug [52-55]. However, despite the vast amount of literature on emulsions as drug carriers in cancer therapy not much work has been devoted to characterizing the accumulation of the droplet itself into solid tumors. In this study, we clearly demonstrated that nanosized PEGylated emulsions prepared with commonly used pharmaceutical excipients can passively target neoplastic tissues. The degree of emulsion accumulation into the

tumor was dependent on the PEG coating and tumor type, whereby C26 colon adenocarcinoma was more permeable to the emulsions than B16 melanoma. These emulsions can potentially enhance the selectivity of lipophilic anticancer drugs towards tumor tissues and increase their therapeutic index.

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Table 6: Properties of the lipid emulsions administered to mice bearing either B16-F10 melanoma or C26 colon adenocarcinoma

Formulation[a]	**PEG content (mol%)**[b]	**Mean Diameter (nm)**	**PDI**	**Tumor**
TC/PS-80/HSPC	-	113	0.27	B16-F10
TC/PS-80/ESM	-	103	0.32	B16-F10
TC/PS-80/ESM/DSPE-PEG 2000	10	107	0.25	B16-F10
TC/PS-80/ESM	-	110	0.29	C26
TC/PS-80/ESM/DSPE-PEG 2000	10	114	0.26	C26
TC/PS-80/ESM/DSPE-PEG 2000	15	103	0.23	C26
TC/PS-80/ESM/DSPE-PEG 5000	10	121	0.21	C26
TC/PS-80/ESM/DSPE-4-armPEG	10	110	0.24	C26

[a] The weight ratio of lipids except for DSPE-PEG was kept constant (TC/PS-80/X = 5:3:1, *w/w*, where X is either HSPC or ESM).

[b] Expressed as mol% of total surface components (excluding TC).

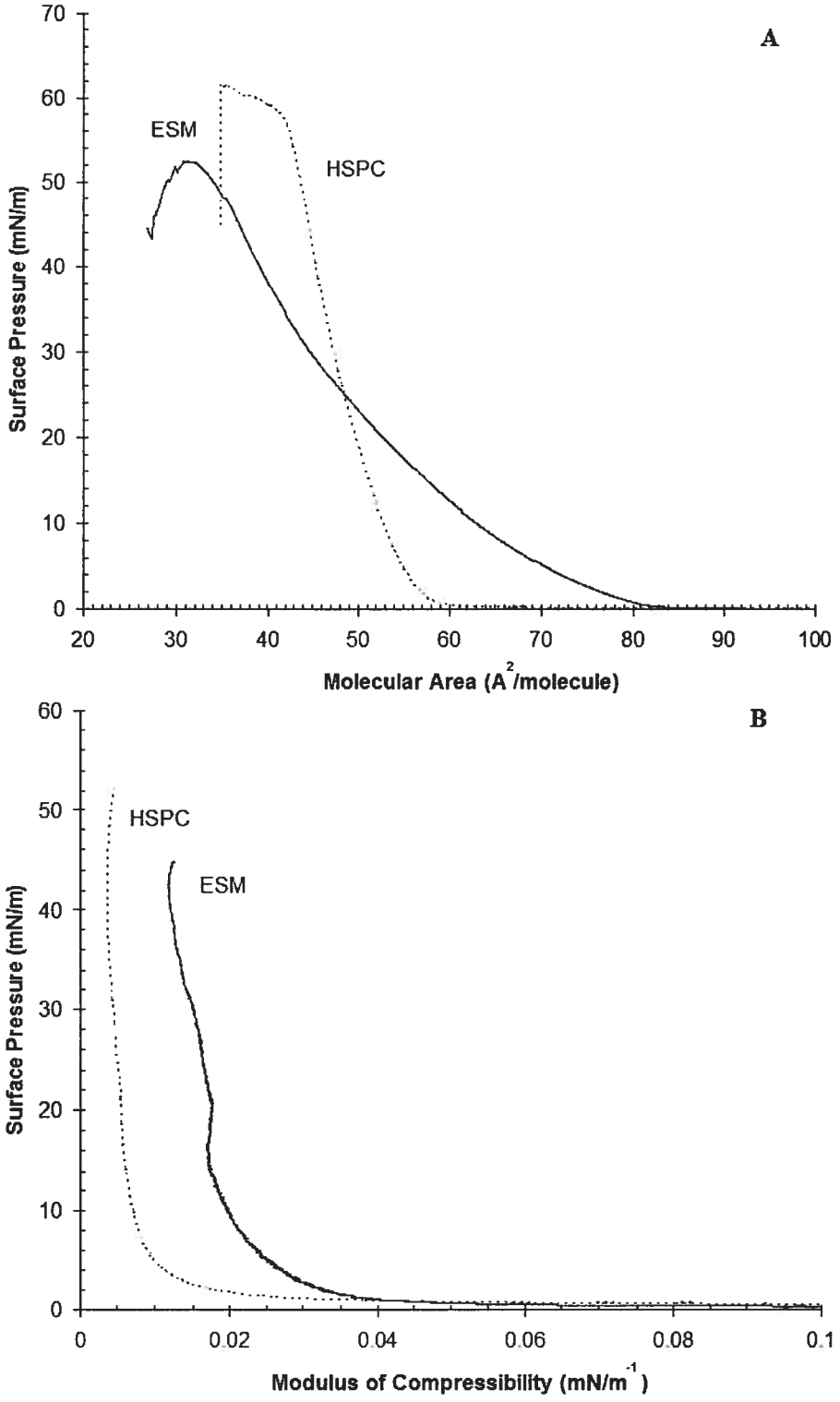

Figure 11: Surface pressure versus molecular area (A) and surface pressure versus modulus of compressibility (B) plots of HSPC and ESM at the air/water interface. Subphase conditions: PBS pH 7.4, 25°C.

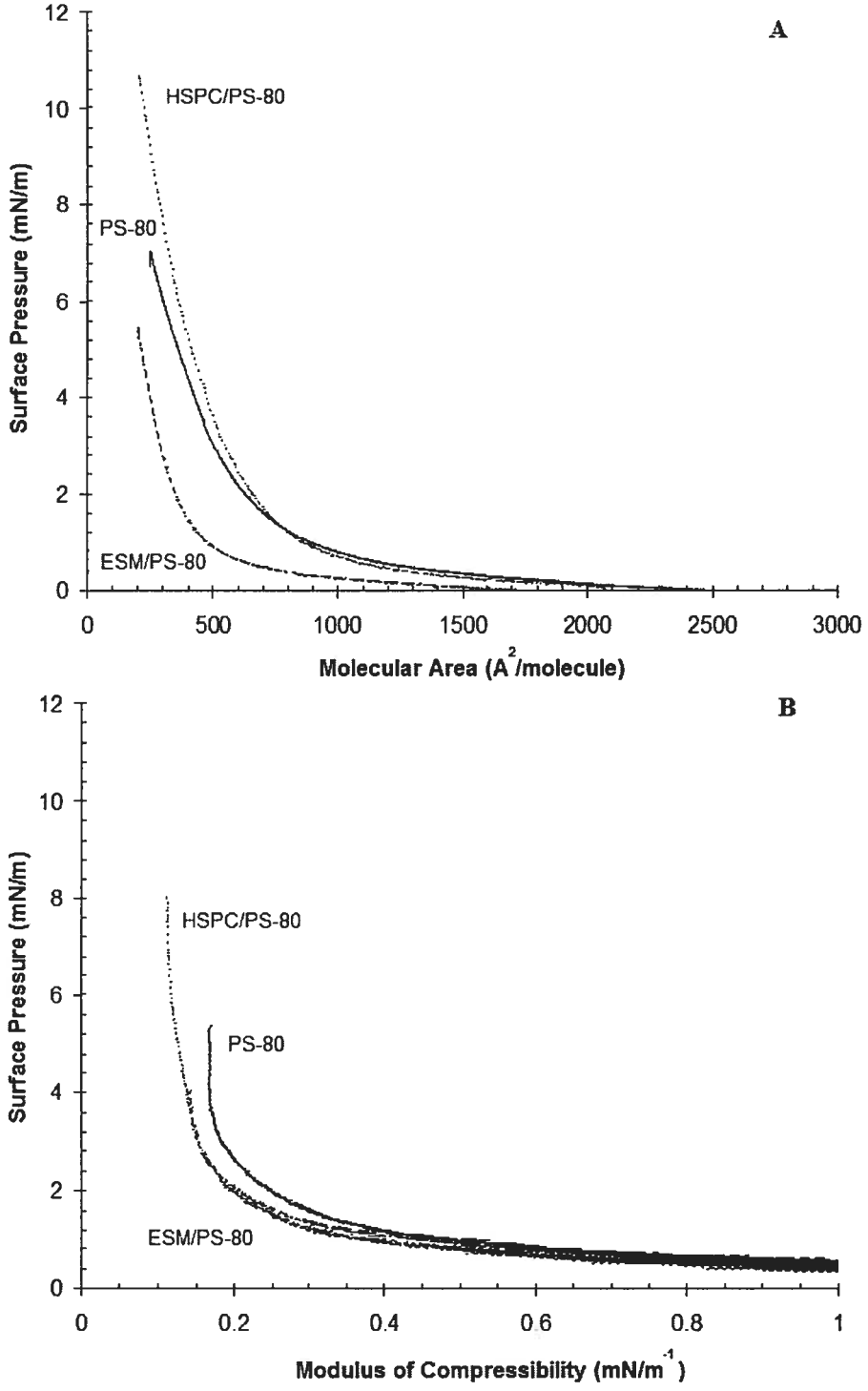

Figure 12: Surface pressure versus molecular area (A) and surface pressure versus modulus of compressibility (B) plots of PS-80, HSPC/PS-80 (1:3, *w/w*) and ESM/PS-80 (1:3, *w/w*) at the air/water interface. Subphase conditions: PBS pH 7.4, 25°C.

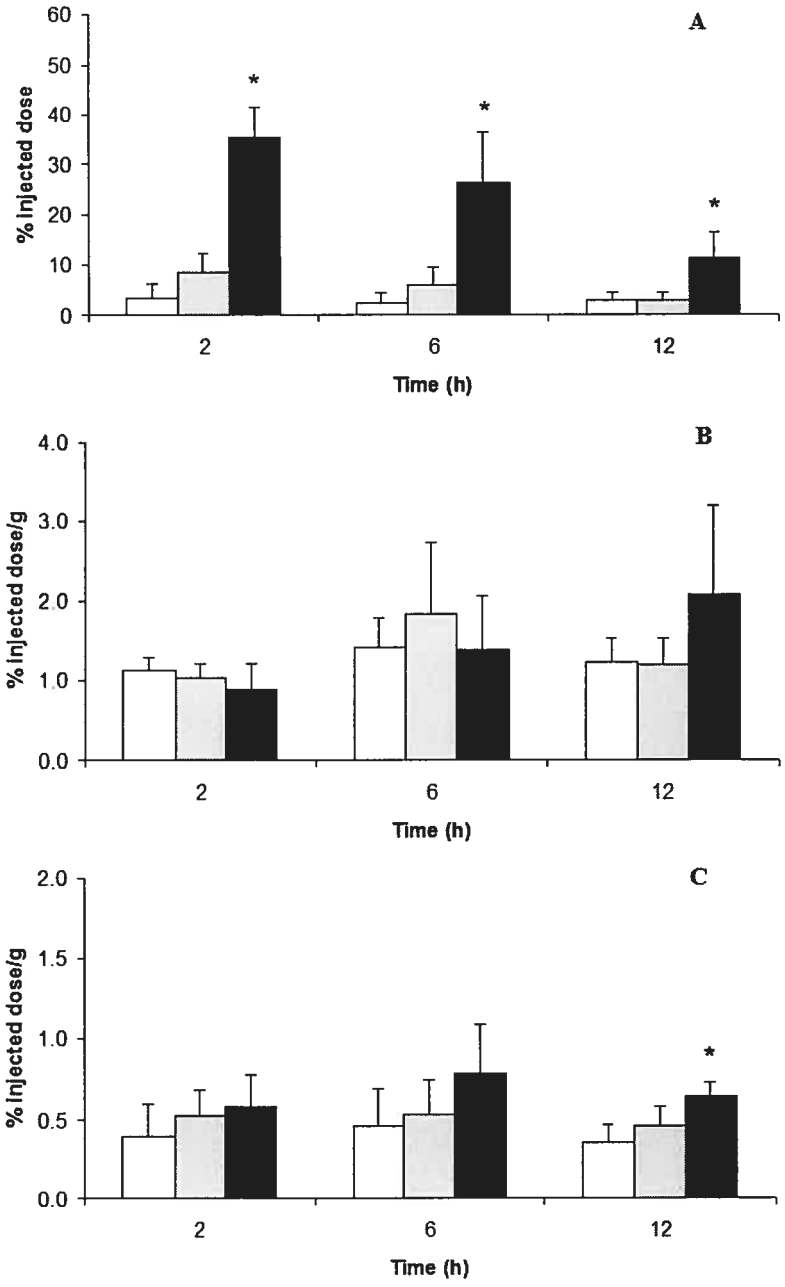

Figure 13: Elimination profile of emulsions from blood (A) and distribution to B16-F10 melanoma (B) and muscle (C) after i.v. injection in C57BL/6 mice. Each mouse was administered 5.4 mg of lipids (excluding DSPE-PEG) in a 100-µL injection volume. Mean + SD (n = 4-5 mice/group). TC/PS-80/HSPC (□), TC/PS-80/ESM (▫), and TC/PS-80/ESM/(10 mol%)DSPE-PEG 2000 (■). Statistically significant differences between plain and PEGylated emulsions are indicated. * $p < 0.05$.

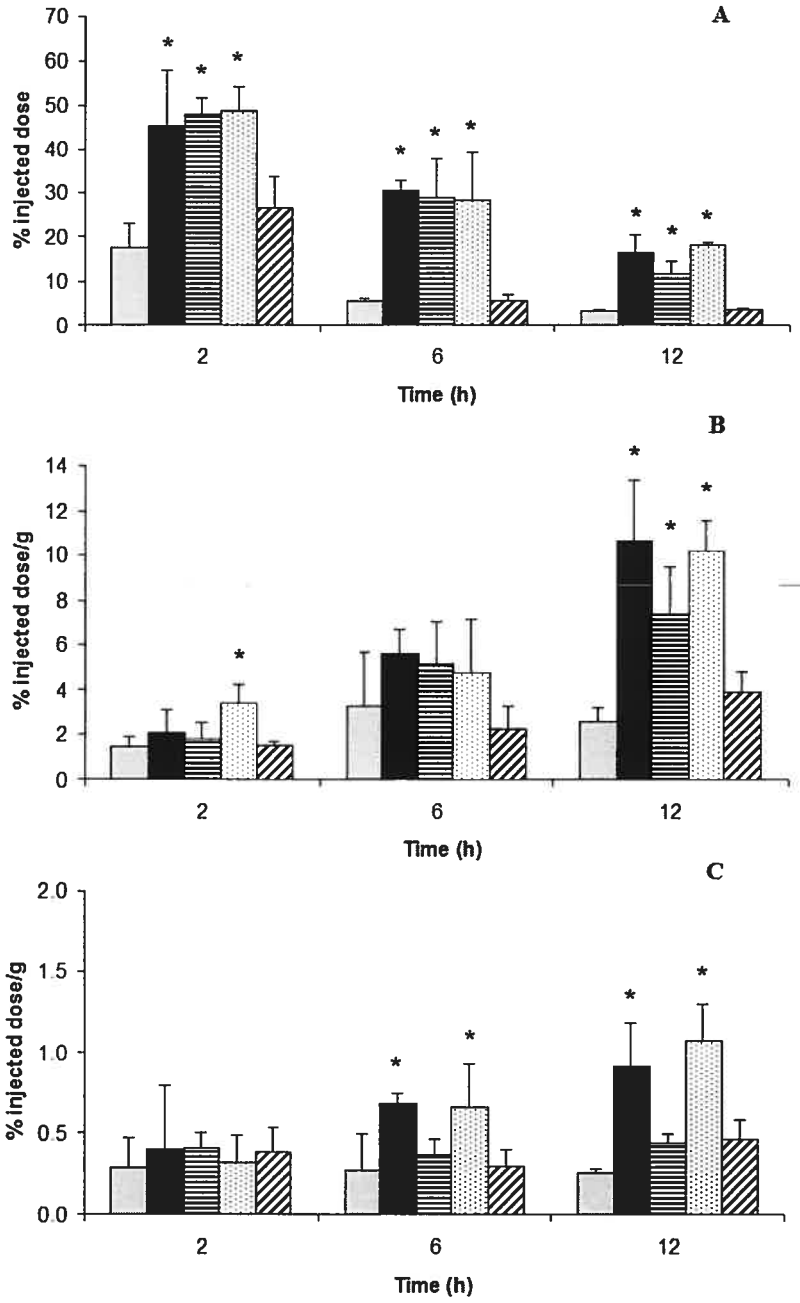

Figure 14: Elimination profile of emulsions from blood (A) and distribution to C26 colon adenocarcinoma (B) and muscle (C) after i.v. injection in Balb/C mice. Each mouse was administered 5.4 mg of lipids (excluding DSPE-PEG) in a 100-µL injection volume. Mean + SD (n = 4-5 mice/group). TC/PS-80/ESM (▫) and TC/PS-80/ESM/(10 mol%)DSPE-PEG 2000 (■), TC/PS-80/ESM/(15 mol%)DSPE-PEG 2000 (▤), TC/PS-80/ESM/(10 mol%) DSPE-PEG 5000 (▩), TC/PS-80/ESM/(10 mol%)DSPE-4-armPEG (▨). Statistically significant differences between plain and PEGylated emulsions are indicated. * $p < 0.05$.

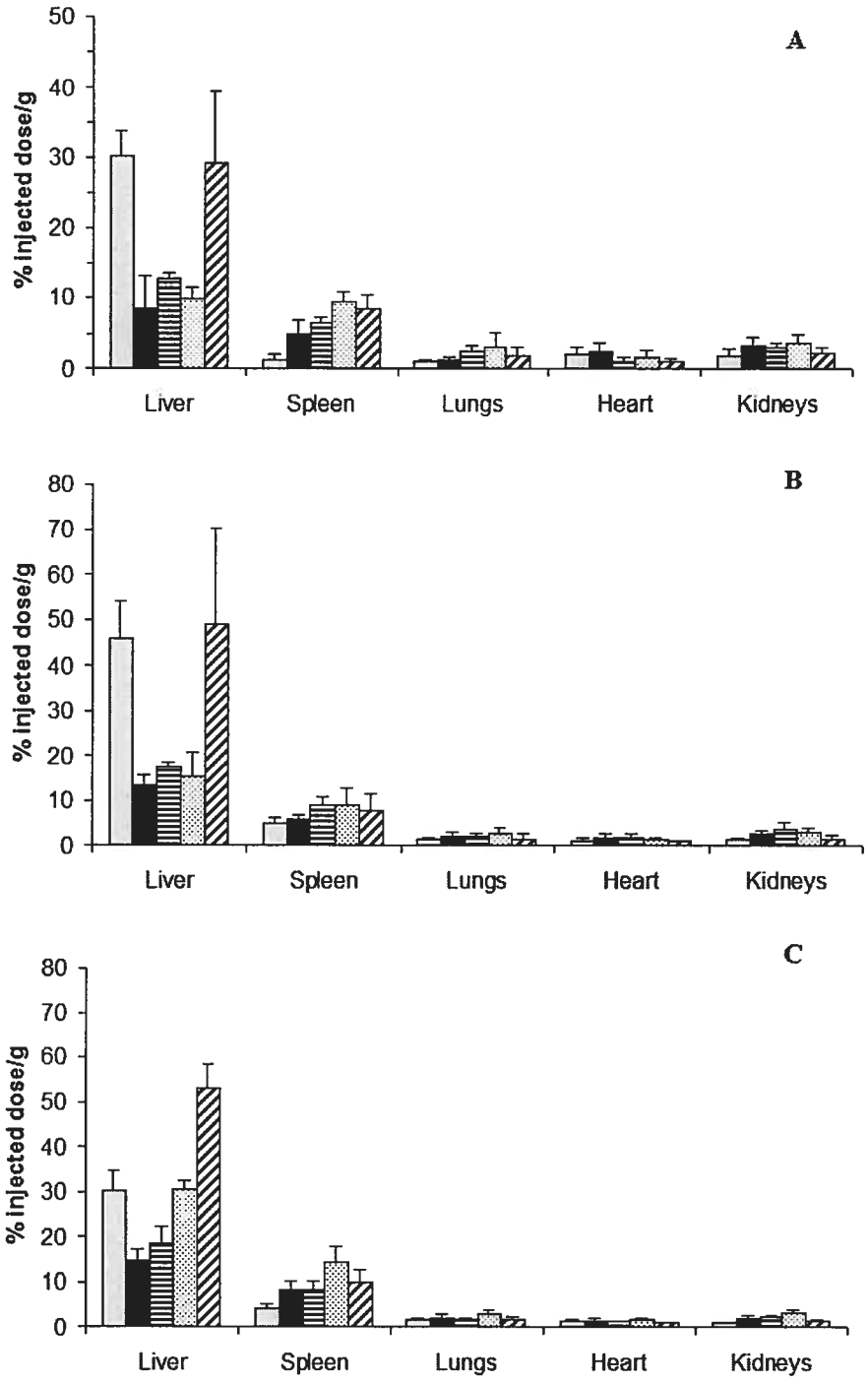

Figure 15: Effect of DSPE-PEG derivatives on the tissue distribution of the emulsions in Balb/C mice inoculated with C26 colon adenocarcinoma after 2 h (A), 6 h (B), and 12 h (C) post i.v. injection. Mean + SD (n = 4-5 mice/group). TC/PS-80/ESM (▫) and TC/PS-80/ESM/(10 mol%)DSPE-PEG 2000 (■), TC/PS-80/ESM/(15 mol%)DSPE-PEG 2000 (▤), TC/PS-80/ESM/(10 mol%) DSPE-PEG 5000 (▩), TC/PS-80/ESM/(10 mol%)DSPE-4-armPEG (▨).

CHAPTER 4: DISCUSSION

1. Formulation optimization

For emulsions to accumulate into neoplastic tissues by the EPR effect, the droplets must be smaller than the pores of the tumor blood capillaries [1] and must possess sufficiently long residence times in blood to extravasate progressively at the tumor site over time [2]. The ability of emulsions to evade the MPS and circulate long after intravenous (i.v.) injection depends largely on the hydrodynamic diameter of the dispersed droplets and the physicochemical properties of the surface [3-5].

Our strategy to achieve long residence times in blood and accumulation into solid tumors was to develop an emulsion with a unimodal size distribution centered on 100 nm that was co-emulsified with egg sphingomyelin (ESM). This lipid emulsifier was selected since several previous studies have shown that ESM prolonged the systemic circulation time of emulsions and liposomes [6-8]. The desired droplet size was realized by varying emulsifying parameters such as formulation composition (surfactant type, oil/surfactant ratio) and the energy input for droplet size reduction (sonication duration and intensity). This section describes the development of a nano-sized emulsion formulation that would later be coated with various 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-poly(ethylene glycol) derivatives (DSPE-PEG) including DSPE-PEG 2000, DSPE-PEG 5000, and DSPE-4-armPEG, and evaluated *in vivo* for their ability to prolong circulation time and accumulate into solid tumors.

1.1. Emulsion composition

Formulation optimization began by screening through various synthetic surfactants to identify the ones which were most suitable to emulsify tricaprylin (TC) with ESM. This initial screening study was performed at various sonication intensities

and durations, while the amount of ESM and TC was kept constant. The influence of synthetic surfactant type was evaluated in a system composed of TC/ESM/X (5:1:3, *w/w*), where X was either poloxamer 188, span 40, sodium deoxycholate, sodium cholate, sodium glycocholate or polysorbate 80 (PS-80). The emulsions were prepared and characterized following the protocol described in Appendix I. From this study, PS-80 had the best emulsifying properties since these emulsions had superior physical stability under certain sonication conditions. Consequently, PS-80 was used in future optimization studies.

Following these experiments, the oil/surfactant ratio and PS-80/ESM ratio were varied while maintaining constant sonication conditions (duration: 25 s, intensity: medium (72-84 W)). Refer to Appendix II for the procedure used to prepare the emulsions. The influence of emulsion composition on droplet diameter and PDI is presented in Figure 16. As expected, smaller droplets were produced at lower oil/surfactant ratio due to increased lowering of the interfacial free energy with higher emulsifier content (Figure 16A) [9]. On the other hand, at low oil/surfactant ratio PDI values were larger, which is probably due to the formulation of small micelles as a result of the relatively large quantity of surfactant (Figure 16B). As oil/surfactant ratio increased, PDI values diminished while droplet diameter became larger. The formulation that provided the best compromise between mean diameter and PDI was with an oil/surfactant ratio and PS-80/ESM ratio of 1.25 and 3, respectively. With this formulation (TC/PS-80/ESM = 5:3:1, *w/w*), droplet size and PDI was 145 nm and 0.29, respectively (Figure 16A and B).

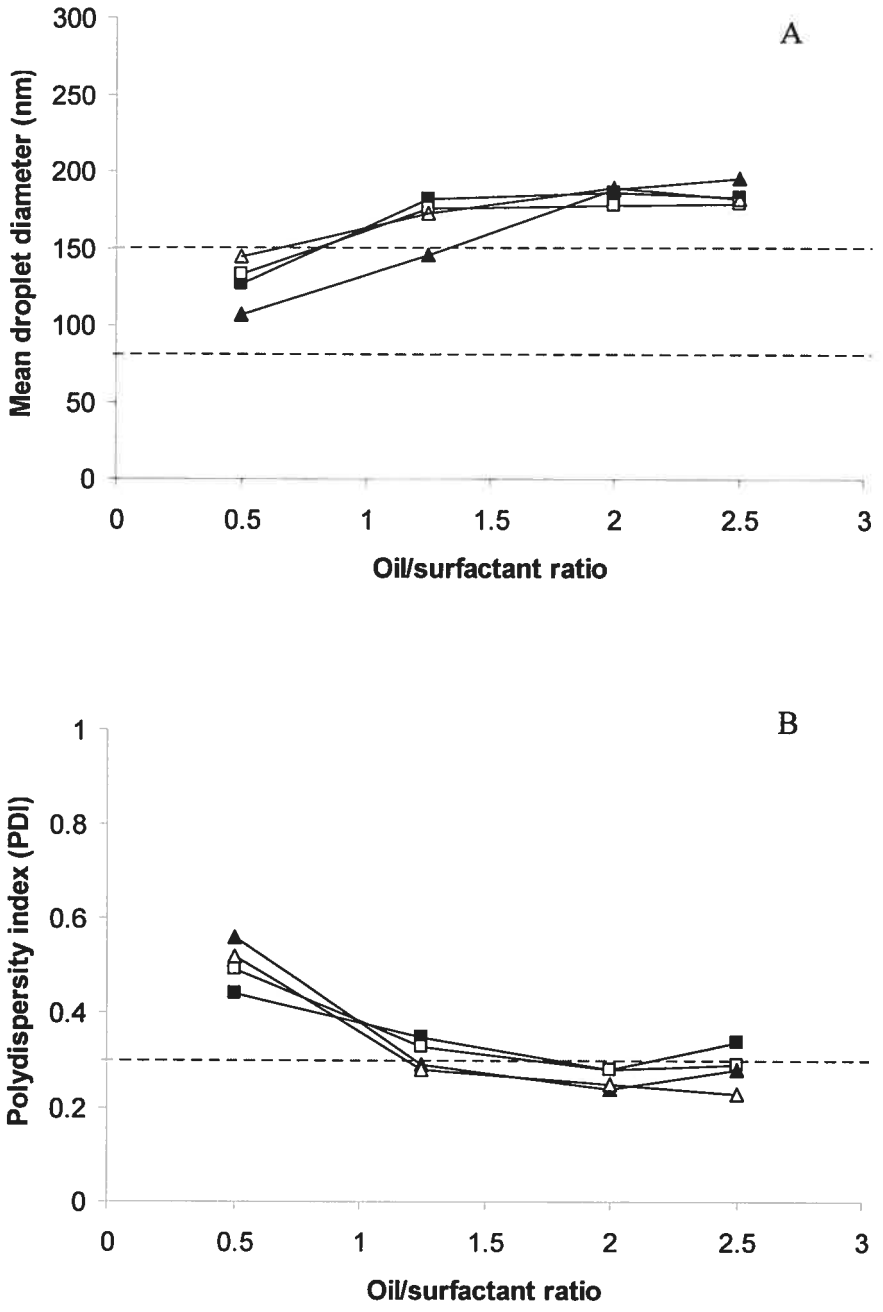

Figure 16: Influence of the proportions of TC, PS-80 and ESM on mean droplet diameter (A) and size distribution (B) of the emulsion. Sonication conditions were kept constant at medium intensity (72-84 W) for 25 s. The weight ratio of PS-80/ESM was either 0.6 (■), 1 (□), 3 (▲) or 7 (Δ). The external phase was citrate buffer at pH 5. (- - -) indicates the range of acceptable diameter and PDI.

1.2. Sonication parameters

Sonication is a process which reduces globule size by inducing cavitations through the mechanical vibrations generated by the sonicator probe. The collapse of these vapor bubbles produces high energy shockwaves throughout the liquid mixture which breaks up the droplets [10]. The influence of sonication parameters, such as intensity and duration on droplet diameter and PDI was investigated, while the proportion of TC/PS-80/ESM (5:3:1, *w/w*) was maintained constant. Refer to Appendix II for the procedure used to prepare the emulsions. As anticipated, Figure 17 shows that increasing sonication time and intensity reduced emulsion droplet size and PDI. This can be explained by the stronger mechanical vibrations induced by increasing the power input, producing more powerful shock waves that could break up the initial globules into smaller droplets [10].

Sonication at medium (72-84 W) or medium-high (92-102 W) intensity produced emulsions with comparable mean diameters. At these power inputs, droplet size leveled-off at approximately 100 nm (Figure 17A). PDI, on the other hand, could be decreased further by sonicating at medium-high intensity for longer durations (60 and 120 s) (Figure 17B). Eventually these emulsions may be interesting carriers for hydrophobic anticancer drugs. To avoid possibly overheating the formulation with high sonication intensity and duration, which could potentially degrade heat sensitive drugs, the parameters chosen for the *in vivo* studies were 40 s at medium intensity. These conditions were sufficient to produce an emulsion with a diameter of about 100 nm and acceptable polydispersity (< 0.3).

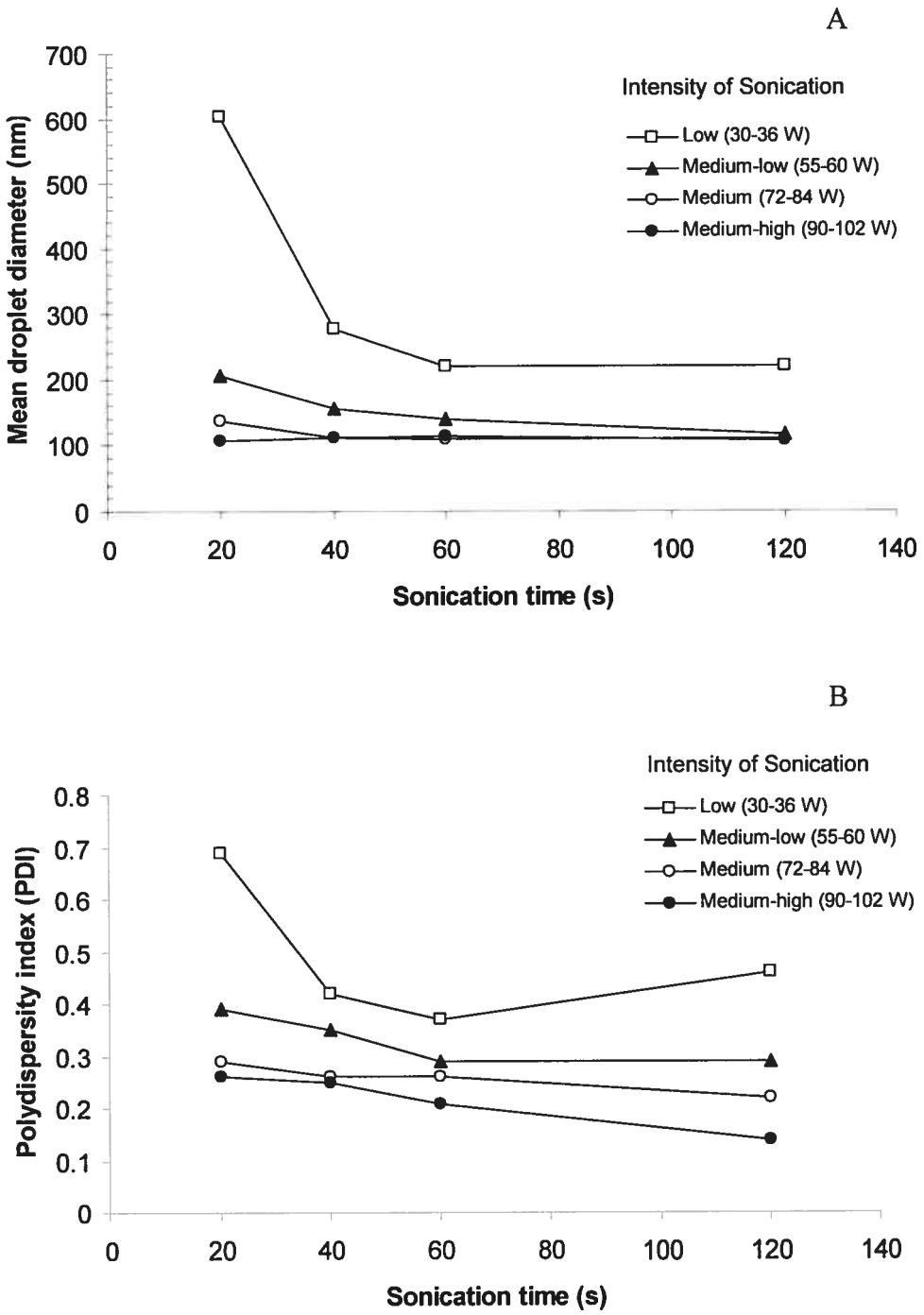

Figure 17: Influence of sonication intensity and time on mean droplet size (A) and size distribution (B). The proportion of TC, PS-80 and ESM was kept constant (5:3:1, *w/w*). The external phase was 0.9% *w/v* NaCl in water.

2. Emulsion stability

Several promising formulations were stored at ambient conditions and checked for physical stability over time. The emulsions were examined visually for signs of phase separation such as creaming, sedimentation, and oil droplets. In addition, droplet diameter was monitored over time by dynamic light scattering. The stability of several non-PEGylated emulsions is presented in Table 7. For all emulsions, size increased over time while PDI decreased. Several hypotheses are proposed to explain the change in emulsion size over time. One hypothesis is that the emulsions are physically unstable and are showing signs of destabilization. The increase in droplet size may be an indication of coalescence (fusion of separate droplets) due to insufficient electrostatic or steric forces to overcome the attractive Van der Waals forces as two globules approach each other [11]. Electrostatic stabilization was minimal since the emulsions were prepared with non-ionic emulsifiers. Alternatively, the polydispersity in droplet size may have promoted Ostwald ripening [12]. This destabilization process occurs when the small droplets transfer oil to the larger ones causing the big ones to grow while the small ones shrink. Another possibility is that the increase in size may be partially due to microbial growth since the emulsions were not sterilized prior to storage and no preservatives were added to the formulation.

Visually the emulsions did not show any indications of physical instability over two months. Signs of phase separation were observed after 3 months and obvious phase separation was observed after 4 months.

Table 7: Stability of several non-PEGylated emulsions over time

TC (mg)	ESM (mg)	PS-80 (mg)	Sonication time (s)	Day 0		1 Month		2 Months	
				Diameter (nm)	PDI	Diameter (nm)	PDI	Diameter (nm)	PDI
100	20	60	25	139.1	0.37	191.4	0.11	220.9	0.08
100	20	60	35	128.6	0.28	172.1	0.10	212.0	0.06
145	20	60	25	177.9	0.26	263.3	0.25	216.8	0.07
145	20	60	35	165.4	0.26	206.9	0.19	215.8	0.11
145	60	60	25	173.1	0.29	238.6	0.19	246.5	0.17
145	60	60	35	147.8	0.26	200.3	0.14	225.1	0.14

The formulations were sonicated at medium intensity (72-84 W)
Subphase: 0.9% (*w/v*) NaCl in water

Emulsions composed of TC/PS-80/ESM (5:3:1, *w/w*) were then incubated with DSPE-PEG 2000 at various concentrations (0, 6, 10 and 15 mol%). The physical stability of the emulsions was evaluated by monitoring changes in droplet size over time (Table 8). Surprisingly, emulsions coated with PEG 2000 increased in size over time similar to the non-PEGylated formulations (Table 7 and Table 8). It was expected that the polymer chains grafted to the surface of the emulsion droplets would provide physical stability through steric hindrance at high grafting densities. A possible explanation for this observation is that the appropriate combination of surfactants to provide an efficient film at the interface was not found. For instance, the considerable difference in alkyl chain length between the surfactants (PS-80 and ESM) and those of the TC core may be an issue. In addition or alternatively, the increase in droplet size may be a result of microbial growth which could destabilize a formulation prematurely.

Table 8: Stability of the PEGylated emulsions over time

DSPE-PEG 2000 content (mol%)	Day 0		Day 8		Day 16		Day 28	
	Diameter (nm)	PDI	Diameter (nm)	PDI	Diameter (nm)	PDI	Diameter (nm)	PDI
0	144.8	0.34	155.2	0.17	164.7	0.11	178.0	0.10
6	141.6	0.32	169.1	0.18	162.4	0.15	194.7	0.11
10	149.5	0.33	164.0	0.23	183.9	0.15	178.6	0.15
15	144.1	0.32	179.2	0.22	188.8	0.15	199.2	0.15

The weight ratio of lipids except DSPE-PEG 2000 was kept constant (TC/PS-80/ESM = 5:3:1)
Sonication conditions: 40 s, medium intensity (72-84 W)
Subphase: Millipore water

3. Compression isotherms

Following the initial optimization study, ESM monolayers were characterized at the air/water interface in PBS buffer (pH 7.4) and compared to a high phase transition phospholipid (hydrogenated soybean phosphatidylcholine, HSPC) using the Langmuir balance technique (refer to Figure 11A and B in Chapter 3). These lipids were characterized alone and in the presence of PS-80 for monolayer compressibility, molecular conformation and fluidity. For the pure lipids, two important differences were identified between the molecules. First, ESM formed a more compressible film than HSPC as indicated by the more gradual increase in surface pressure as molecular area decreased. The disparity in monolayer compressibility between the two molecules may be attributed to differences in chain unsaturation. The acyl chains of HSPC are completely saturated, while a double bond is present in ESM between carbons 4 and 5 of the sphingosine backbone. The unsaturation in ESM might produce a more fluid and disordered monolayer than HSPC. The second difference observed between the molecules is that ESM could form a more densely packed film above about 25 mN/m (see Figure 11A in Chapter 3). The ability of ESM to form a

tighter monolayer can be explained by its greater hydrogen bonding capacity compared to phosphatidylcholines (PCs) due to the presence of an amide and hydroxyl group in the molecular structure. The intra- and intermolecular hydrogen bonding within and between ESM molecules may decrease head group hydration, which could permit the molecules to pack more tightly. Previous studies have reported that ESM head groups are less hydrated in monolayers and bilayers compared to PCs, such as DPPC and egg PC [13, 14].

The fluidity and compressibility of both HSPC and ESM monolayers increased considerably when mixed with PS-80 (see Figure 12A and B in Chapter 3). Over the range of molecular areas investigated, both HSPC/PS-80 (1:3, *w/w*) and ESM/PS-80 (1:3, *w/w*) displayed almost identical compressibilities. However, compared to pure PS-80 and HSPC/PS-80, ESM/PS-80 formed a more densely packed film at the air/water interface (the mixture occupied the smallest area). A possible explanation for this observation is a reduction in hydration of the polar head group due to the formation of intra- and intermolecular hydrogen bonds with ESM and maybe also with PS-80. The lower head group hydration could permit denser packing of the molecules. The tighter molecular packing of ESM/PS-80 monolayers may potentially enhance monolayer stability in plasma and prolong the systemic circulation time of an emulsion.

4. Biodistribution studies

Based on the results of the monolayer experiments, we compared ESM versus HSPC emulsions *in vivo*. The emulsions were prepared with the same proportions of oil and surfactant (TC/PS-80/HSPC or ESM = 5:3:1, *w/w*) and sonicated at the same

conditions (medium intensity, 40 s). This basic formulation was chosen for the biodistribution experiments based on the optimization studies, whereby emulsions of ~ 100 nm with acceptable PDI values (< 0.3) were obtained under these conditions. Substituting HSPC for ESM did not considerably alter the size of the emulsion (refer to Table 6 in Chapter 3). These emulsions were administered intravenously via the subclavian vein to mice inoculated with B16 melanoma. After i.v. injection no significant difference was observed between ESM and HSPC emulsions ($p > 0.05$) (Figure 13A, Chapter 3). Although a statistical significance could not be demonstrated, the % ID at the 2 h time point was slightly higher for the emulsion containing ESM, which may be attributed to a tighter monolayer at the interface.

Following these experiments, the influence of PEG concentration, chain length, and structure in prolonging systemic circulation time was investigated. This was done by coating emulsions composed of TC/PS-80/ESM (5:3:1, *w/w*) with various DSPE-PEG derivatives including DSPE-PEG 2000, DSPE-PEG 5000 and DSPE-N-[pentaerythritol polyoxyethylene) glutaryl] (DSPE-4-armPEG) (PEG MW 2000). Droplet diameter was maintained constant between 100 – 120 nm such that blood residence time was dependent only on the physicochemical properties of the carrier (refer to Table 6 in Chapter 3). As expected, inserting DSPE-PEG 2000 or PEG 5000 into the emulsion interface at high grafting densities (10 or 15 mol%) significantly prolonged circulation time ($p < 0.05$) (Figure 14A, Chapter 3). Despite the 2000 MW PEG segment and high grafting density (10 mol%), emulsions coated with DSPE-4-armPEG did not exhibit higher residence times in blood compared to plain, non-PEGylated emulsions. The inability of DSPE-4armPEG to extent

circulation time is probably due to the lower molecular weight PEG segment per arm (500 g/mol) compared to single-chain PEG 2000 and PEG 5000. Previous groups have shown that the circulation time of the carrier was lower when coated with shorter PEG chains (i.e. PEG 750 and PEG 120) compared to PEG 1900, 2000 and 5000 [15, 16].

Enhanced circulation time of emulsions grafted with PEG 2000 or 5000 translated into higher accumulation into C26 tumors but not into B16 (Figure 13B and Figure 14B, Chapter 3). The lower uptake of emulsions by B16 melanoma may be due to several factors including lower vascular permeability and differences in blood vessel density, blow flow rate, and interstitial pressure in different tumor types [17].

The uptake of the emulsions by the organs at different time points is presented in Figure 15 in Chapter 3. The emulsions were taken up mainly by the liver followed by the spleen. As anticipated, the emulsions which had higher blood residence times accumulated less in the liver.

5. References

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CHAPTER 5: CONCLUSION

Oil-in-water submicrometer emulsions are promising vehicles for the delivery of highly lipophilic anticancer drugs to neoplastic tissues. The main advantages of emulsions over other colloidal drug delivery systems include the potential to solubilize high quantities of lipophilic compounds, relative ease of manufacture at an industrial scale, and reasonable stability under storage conditions. In this study, we clearly demonstrated that nanosized PEGylated emulsions prepared with commonly used pharmaceutical excipients can passively target solid tumors. The efficacy of emulsion uptake into the tumor was dependent on the physicochemical properties of the surface and the tumor model. These emulsions can potentially enhance the selectivity of lipophilic anticancer drugs towards tumor tissues, resulting in reduced systemic toxicities and increased therapeutic index, provided that the encapsulated cargo remains associated with the vector after i.v. injection. These results will undoubtedly improve the understanding of emulsion accumulation into solid tumors.

Future work will involve enhancing the physical stability of the emulsions in storage. Ideally, the formulation should be physically and chemically stable for at least one year. Moreover, the ability of the emulsions to incorporate hydrophobic anticancer drugs ought to be investigated, followed by an assessment of drug retention *in vitro* and *in vivo*. In general, molecules with high lipophilicities (log P > 9) are retained better within the emulsion in the presence of biological fluids [1, 2]. Thus, in order to achieve high drug levels at the tumor site it is recommended to load these emulsions with highly lipophilic compounds (log P > 9).

The ongoing advancement in delivery systems for anticancer drugs will eventually lead to safer and more tolerated therapies. Hopefully, this study will enhance interest in developing emulsions as carriers for antineoplastic agents.

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APPENDIX I

The emulsions were prepared by first mixing all components (oil, lipid surfactant, and synthetic surfactant) under gentle agitation (stirring bar) above the phase transition temperature of ESM for 5-10 min. The dispersing phase (0.9% *w/v* NaCl in water) was then added to the premix and the formulation was heated/mixed at 55°C for an additional 20 to 60 min. After the addition of external phase the dispersed phase represented 9% (*w/v*) of the emulsion. The formulations were then sonicated in batches of 1 mL in a 1.5 mL eppendorf at various intensities and durations on pulse mode (pulse every 2 s for 0.2 s). After sonication, a sample of formulation was taken and measured for mean hydrodynamic diameter by dynamic light scattering using a Malvern Autosizer (Malvern Instruments Ltd, Malvern, UK) at 25°C and a fixed angle of 90°. Each formulation was then extruded several times (~ 11 times) through a 100 - 200 nm filter using a 1 mL hand-held extruder. Following extrusion a second size measurement was made for each formulation. The emulsions were stored at ambient conditions and monitored visually for signs of phase separation such as creaming, sedimentation and oil droplets.

APPENDIX II

The emulsions were prepared by first mixing all components (TC, PS-80, and ESM) under gentle agitation (stirring bar) above the phase transition temperature of ESM for 5-10 min. The dispersing phase (0.9% *w/v* NaCl in water, citrate buffer at pH 5 or Millipore water) was then added to the premix and the formulation was heated/mixed at 55°C for an additional 20 to 45 min. After the addition of external phase the dispersed phase represented between 6 to 14% (*w/v*) of the emulsion. The formulations were removed from the heat and vortexed for ~ 2 min. The emulsions were then sonicated in batches of 1 mL in a 1.5 mL eppendorf at medium intensity for 25 to 40 s on pulse mode (pulse every 2 s for 0.2 s). Size measurements were made by dynamic light scattering using a Malvern Autosizer (Malvern Instruments Ltd, Malvern, UK) at 25°C and a fixed angle of 90°.