Order Number 9417469

Preparation and characterization of perfluorocarbon emulsions for biomedical applications

Habif, Stéphane Samuel, Ph.D.

City University of New York, 1994

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

PREPARATION AND CHARACTERIZATION OF PERFLUOROCARBON EMULSIONS FOR BIOMEDICAL APPLICATIONS

by

STEPHANE SAMUEL HABIF

A dissertation submitted to the Graduate Faculty in Chemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1994

This manuscript has been read and accepted for the Graduate Faculty in Chemistry in satisfaction of the dissertation requirements for the degree of Doctor of Philosophy.

07/13/93

Date

Chair of Examining Committee

8/26/93

Date

Executive Officer

Supervisory Committee

The City University of New York

Abstract

PREPARATION AND CHARACTERIZATION OF PERFLUOROCARBON EMULSIONS FOR BIOMEDICAL APPLICATIONS

by

Stéphane S. Habif

Adviser: Professor Henri L. Rosano

Considerable interest in perflubron (perfluorooctylbromide, PFOB) emulsions has arisen from their numerous potential biomedical applications, such as imaging solutions and oxygen-carrying solutions, i.e., red blood cell substitutes. The objective of the present research was to determine the factors involved in the stability of PFOB/saline emulsions with egg yolk phospholipids (EYP) as emulsifiers. Experimentation yielded an optimal emulsion preparation method based on high pressure homogenization (microfluidization), in which the phospholipids are first dispersed in the aqueous phase as vesicles at a temperature above their phase transition temperature. The vesicles are then broken down by high mechanical stress, resulting in the monomolecular adsorption of the emulsifier at the PFOB/saline interface. The emulsion obtained consists of both PFOB droplets (250 nm in diameter) encapsulated in a phospholipid monolayer, and PFOB-free phospholipid vesicles (80 nm in diameter). Since EYP has a complex and variable composition and is very sensitive to oxidation, either decaglyceroldioleate (10-2-O) or phospholipon 90H (PL), a hydrogenated phospholipid, was substituted for EYP in simpler model systems used in subsequent

investigations. Phospholipids and 10-2-O are virtually insoluble in both PFOB and saline, but if by the application of a large amount of mechanical work, for example through microfluidization, they can be made to adsorb monomolecularly at the o/w interface, they will reduce the interfacial free energy and provide a film capable of keeping its ordered structure under high stress and high temperature conditions. This in turn yields an emulsion capable of withstanding heat sterilization. But PL and 10-2-O provide no electrostatic charge at the droplet surface, and yield flocculated PFOB/saline emulsions but stable PFOB/saccharide solution emulsions. Furthermore, studies of PFOB/saline emulsions using a combination of PL and a charged surfactant showed that a negatively-charged interface results in a stable system while a positively-charged interface -- with the same zeta-potential amplitude -- results in flocculation. We may infer that EYP systems are probably stabilized against flocculation by a combination of both electrostatic and hydration forces due to the presence of minor components in the EYP mixture, which provide a negative charge and a strong hydration shell around the emulsion droplets.

Acknowledgements

My deepest thanks go to my friend and mentor at City College (CCNY), Dr. Henri L. Rosano, who taught me a lot about chemistry and, beyond chemistry, about Life itself. I will always remember fondly the years I spent under his guidance.

I want to thank the following people at Alliance Pharmaceutical Corp. (San Diego, CA) for the technological and financial support they provided throughout my research: Duane Roth (Chairman & CEO), Ron Hopkins, David Klein, Hank Graham, Tim Pelura, Ernest Schutt, Linda Smith, and -- in particular -- Ernie Green, who kindly agreed to serve on my thesis committee.

Thanks are also due to Kraft General Foods (Tarrytown, NY) for financial and technological support and for the use of the Tarrytown research facility. I would particularly like to thank the following: Charles Cante; Frank Del Valle; Fouad Saleeb; David Ikenberry, for his kind help and support; and John Cavallo, for his useful advice and for serving on my thesis committee.

I am grateful as well to Michael Aronson (Unilever Research U.S., Edgewater, NJ), another member of my thesis committee, for his interest in my research and his precious advice; to Jack Kearns (The Liposome Company, Princeton, NJ), for his help and advice; and to Larry Boni (Liposome), for running the TEM.

I thank Professors David Locke (Queens College, CUNY) and Horst Schultz (CCNY) for serving on my committee.

I owe a great debt to Christian Oleksiak, my very good friend and my fellow student at CCNY, for his penetrating criticism of my work,

and above all, for his continuous collaboration in my research efforts: "Working together, we made such a great team!"

I wish to thank Christian and the other scientists who contributed to the experimental work connected with my thesis.

Thanks are due to all my colleagues and friends at CCNY, who helped make my studies both enjoyable and rewarding.

I am grateful to Martha Browne for her friendship and her editorial assistance.

Finally, to my parents, Charlotte and Leon, my brother, Sebastien, and my wife, Louiza: this thesis is dedicated to you, with my undying gratitude for your unconditional love.

Table of Contents

Objective	1
Chapter One: Biomedical Applications of Perfluorochemicals	3
1.1 Red Blood Cells (RBC) Substitutes	3
1.1.1 "Red blood"	5
1.1.2 "White blood"	11
1.2 Other Biomedical Applications	14
1.2.1 Imaging applications	15
1.2.2 Cancer applications	16
1.2.3 Cardiovascular applications	17
1.2.4 Pulmonary applications	19
Chapter Two: Perfluorochemicals: Properties and Synthesis	21
2.1 Physical properties of PFCs	21
2.2 Synthesis of PFCs	23
Chapter Three: Phospholipids: Chemistry and Extraction	29
Chapter Four: Emulsion Formation and Stability	39
4.1 Emulsions	39
4.2 Emulsifiers	41
4.3 Creaming/sedimentation	44
4.4 Coalescence	47
4.5 Flocculation (aggregation)	48
Chapter Five Materials and Methods	51
5.1 Materials	51
5.2 Emulsion preparation	52
5.2.1 Sonication	52
5.2.2 Microfluidization	54

5.3 Sample preparation for TEM 57

5.4 Sedimentation field flow fractionation (SFFF) 57

5.5 Particle size measurements 58

5.6 Electrokinetic (Zeta) potential measurements 59

5.7 Surface and interfacial free energy measurements 60

5.8 Compression isotherms 61

Chapter Six: Results and Discussion 72

6.1 Preparation of PFC/saline emulsions using EYP as an emulsifier 72

6.2 PFOB/EYP/saline emulsions characterization 75

6.3 Properties of the phospholipid monolayer at the PFOB/saline interface 79

6.4 PFOB emulsions using 10-2-O as an emulsifier 83

6.5 Saturated PC as a replacement for EYP 84

6.6 Model for PFOB/EYP/saline emulsions 86

6.7 Role of the dispersed phase in emulsion stability 91

Conclusions and Perspectives 106

References 109

List of Illustrations

Chapter Two

Figure 2.1: Simons electrofluorination cell 27

Table 2.1: Structure and properties of several perfluoro-chemicals 28

Chapter Three

Figure 3.1 Structure of several biological phospholipids 34

Figure 3.2: Extraction of total lipids from egg yolk 35

Figure 3.3: Extraction of EYP from the total lipids 36

Figure 3.4: Alternative method of extraction of EYP from egg yolk 37

Figure 3.5: Purification of EYP 38

Chapter Five

Figure 5.1: Apparatus for sonication 64

Figure 5.2: Microfluidizer M-110T and attachments 65

Figure 5.3: Ultra-Turrax and microfluidizer with attachments 66

Figure 5.4: DSC curve of a 4% dispersion of PL in water prepared using the UT (13,500 rpm/10 min./65°C) 67

Figure 5.5: Apparatus for interfacial free energy measurement 68

Figure 5.6: Surface free energy of SDS solutions and PFOB/SDS solution interfacial free energy versus SDS concentration at 24°C 69

Figure 5.7: Apparatus for measuring surface pressure and surface potential (within a Faraday cage) 70

Table 5.1: Tyrode solution composition 71

Chapter Six

Figure 6.1: Transmission electron micrograph of a 52% v/v (100% w/v) PFOB/EYP/saline emulsion 94

Figure 6.2: Sedimentation field flow fractionation fractogram of a 52% v/v (100% w/v) PFOB/saline/EYP emulsion 95

Figure 6.3: (top) Surface pressure and surface potential isotherms and (bottom) surface pressure isotherms (compression - decompression) of (a) commercial EYP, (b) DPPC, and (c) PL on saline at pH 6.8 and 25°C 96

Figure 6.4: Structure of 10-2-O 97

Figure 6.5: (Top) Surface pressure and surface potential isotherms and (bottom) surface pressure isotherms (compression-decompression) of 10-2-O at 23°C 98

Figure 6.6: Particle size of PFOB/saline emulsions for uncharged and charged interfacial films 99

Figure 6.7: Viscosity versus shear rate for PFOB emulsions at 27°C 100

Figure 6.8: Transmission electron micrograph of a 45% v/v (90% w/v) PFOB/saline/PL dispersion 101

Figure 6.9: Light micrograph of a 45% v/v (90% w/v) PFOB/saline/PL dispersion 102

Table 6.1: Saline solution with a phosphate buffer 103

Table 6.2: Surface and Interfacial Free Energies (at 25°C) 104

Table 6.3: Interfacial Free Energy Measurements at 25°C 105

Objective

Perfluorochemical (PFC) emulsions have been developed for a variety of biomedical applications, including their use as oxygen-carrying solutions (i.e., red blood cell substitutes) in transfusions, as imaging solutions in medical diagnosis, and as adjuncts in cancer therapy. While a practical formulation -- made of perflubron (PFOB) dispersed in a saline solution by microfluidization using egg yolk phospholipids (EYP) as an emulsifier -- for these PFC emulsions had already been developed when I started my research in 1989, little was known about the factors involved in the stability of these emulsions.

My initial task was to prepare PFOB/EYP/saline emulsions given the constraints of injectability (non-toxicity, sterility, low viscosity) and efficiency (concentrated emulsion, i.e., high PFOB/saline ratio, and shelf life of at least one year at positive temperature). The structure and properties of the film at the PFOB/saline interface, which depend on the nature and composition of the emulsifier system, are the principal factors in emulsion stability. Thus most of my research has focused on the emulsifier and its surface properties.

After both emulsion formulation and method of preparation were optimized, the emulsions were characterized by photon correlation spectroscopy (PCS), sedimentation field flow fractionation (SFFF), and transmission electron microscopy (TEM).

Since EYP has a complex and variable composition and is sensitive to oxidation, a simpler model system for PFOB/EYP/saline emulsions was devised by replacing EYP with a simpler combination of

emulsifiers. The initial model system consisted of a synthetic emulsifier, decaglyceroldioleate (10-2-O), which was replaced in a subsequent model by a modified natural emulsifier that combined a hydrogenated phospholipid with some additives mimicking the role of the minor components in the EYP mixture.

Emulsion stability was assessed, essentially, by measuring the emulsion particle size by PCS under four conditions: prior to and after sterilization, and with and without dilution of the sample (either in water or in a solution with the same composition as the continuous phase -- in most cases, saline). When the four particle sizes are identical the emulsion is deemed stable.

The present work is composed of six main chapters and a concluding "conclusions and perspectives" section. The first chapter gives an overview of the biomedical applications of PFCs and a historical background of the development of PFC emulsions. The second chapter is devoted to the properties and synthesis of PFCs, the third to the chemistry and extraction of phospholipids. Chapter Four presents emulsion theory and principles and the main causes of emulsion instability. Chapter Five describes the materials and methods used in my experimental work. Chapter Six, the heart of my thesis, is devoted to the presentation and analysis of my results.

In a postscript I summarize my conclusions and suggest possible next steps in the investigation of emulsion stability.

Chapter One
Biomedical Applications of Perfluorochemicals

1.1 Red Blood Cells (RBC) Substitutes

The New Webster's Dictionary [1] defines it as "the red, viscid fluid which circulates in the body of men and animals." Behind this bland definition is a substance that has always captivated the human imagination: blood, the ultimate symbol of life. Nowadays, unfortunately, blood has also become a symbol of death, being one of the prime vectors of HIV, the virus causing AIDS. Fortunately, the development of new blood screening tests has reduced the chances of being infected through transfusions [2]. Since the introduction in 1985 of a blood test for HIV, the risk of acquiring HIV from a unit of transfused blood ranges in estimates from 1 in 1 million to 1 in 40,000 depending mostly on where the blood is collected. Widespread implementation in 1990 of a test for the hepatitis C virus reduced the estimated risk of contracting transfusion-transmitted hepatitis to values ranging from less than 1 in 1,000 to 1 in 200 per unit of blood, down 80 to 90 percent from estimated rates in the early 1960s [3]. But blood-screening technology is limited. First of all, all infectious diseases have a "window" period when infection cannot be detected. In the case of HIV screening, for example, the test consists of the detection of HIV antibodies; most people make antibodies 6 to 12 weeks after contracting the virus, but it can take as long as 36 months. Cases of HIV infection spread by blood transfusion after 1985 result probably from donors whose

blood was tested during the window period [3]. Moreover, not all blood-transmitted infectious diseases can currently be tested for; no test exists for malaria, for example. As Sandler and Popovsky put it [3], "the problem of blood-borne disease is like the mythological Hydra: eliminating one infectious agent is often only a short-lived victory, to be followed by another threat."

In order to avoid the risk of infection through the transfusion of donated blood, a surgical patient can be given his own, previously harvested blood (so-called autologous blood donation). Unfortunately, few people needing transfusions have the 42 days' advance notice necessary to donate their blood for future surgery, and autologous blood donation currently represents less than 5% of the blood collected in the United States [3].

The risk of contagion is not the only problem in using natural blood for transfusions. Blood is delicate and must be stored under refrigeration, it has a short "shelf life" of only about 3 weeks, and it requires a donor-recipient blood-type match. In addition, a worldwide shortage of blood makes the search for blood substitutes a major objective in biomedical research. At stake is a potentially lucrative share of the huge market in natural human blood. In 1988, between 12 million and 14 million units of blood (1 unit corresponds to 500 ml) were used in transfusions in the United States alone. With hospitals paying as much as $200 per unit, the domestic market approaches $2 billion annually. Estimates of the global market are as high as $10 billion a year [4, 5, 6].

Blood is a complex substance fulfilling numerous biological functions in the body such as transport of nutrients and metabolites,

transport of gases, protection against infection, and regulation of body temperature and pH. While no single substance is likely ever to replace blood in all its functions, the term "blood substitute" is widely used to designate a preparation intended primarily as a replacement for red blood cells (RBC) in the transport of oxygen and carbon dioxide. Such preparations, more appropriately termed "RBC substitutes," would mimic, not all of blood's functions, but its gas transport properties alone: already a very challenging goal to achieve. RBC substitutes, once perfected, would be used in transfusions on surgical patients, accident victims, and wounded soldiers to replace a large blood loss and keep up the flow of oxygen in the system, until the body can produce enough blood to replace the loss.

The search for RBC substitutes is currently proceeding on two fronts simultaneously as researchers develop both "red" and "white" bloods: modified, encapsulated, or synthetic hemoglobin solutions; and perfluorochemicals (PFCs) emulsions, respectively.

1.1.1 "Red blood"

Modified hemoglobin

Naunyn, in 1868, was the first to inject a hemoglobin (Hb) solution obtained from hemolyzed blood into a live animal [7]. The injection provoked adverse reactions resulting ultimately in cardiac arrest. The toxicity was later found to be associated with the presence of components of the erythrocyte membrane (proteins, neutral lipids, glycolipids, phospholipids, and lipoproteins) released upon membrane destruction [8]. To avoid stromal/lipid contamination,

several investigators developed methods of Hb isolation from RBCs that resulted in so-called "stroma-free" hemoglobin solutions (SFHS), virtually free of stromal contaminants [9, 10]. But even the purer preparations contain minute quantities of stromal lipids giving rise to such adverse effects as vasoconstrictor activity [9, 11], perturbations of lung mechanics and kidney functions, changes in coagulation activity, and increased antigenicity [8]. Moreover, the presence of endotoxin, even at non-pyrogenic levels, can be very harmful because of the powerful synergism between endotoxin and Hb [8].

Even if they were totally free of contaminants, SFHS could not be used as blood substitutes because

(1) they have too high an affinity for oxygen, and

(2) they have a very short circulation time.

To be available to the tissues the oxygen contained in the capillary blood must diffuse out of the capillary and into the mitochondria of the cells that need it. The driving force for this diffusion is the gradient in partial pressure of oxygen (PO_2) existing between the two sites. A high PO_2 must exist at the venous end of the capillary in order to ensure proper oxygen supply to the tissue [12]. The value of the PO_2 depends on the oxygen affinity of the hemoglobin, expressed as P_{50}, the PO_2 at which Hb is half saturated with oxygen at physiologic temperature (37°C) and pH (7.4). The higher the oxygen affinity the lower the P_{50}. In human erythrocytes the Hb oxygen affinity is modulated by 2,3-diphosphoglycerate (2,3 DPG), which maintains a high value of P_{50} [12], typically between 25 and 27 mmHg, providing for an efficient oxygen unloading. Naked hemoglobin, in the absence of 2,3 DPG, has a higher oxygen affinity

(P_{50} of 12-18 mmHg [8]), making the unloading of oxygen less efficient and resulting ultimately in a reduced oxygen supply. Free Hb also has a very short residence time in the plasma (half-life of 2 to 3 hours) [12]. Furthermore, Hb, which is composed of four protein chains, is highly unstable when removed from the erythrocyte and readily dissociates into dimers [13] that are rapidly filtered out of the blood, often resulting in long-term kidney damage [14]. In recent years, researchers have tried to overcome these problems by chemically modifying hemoglobin so it would be both stable and capable of releasing the oxygen more efficiently. The Hb molecule has been stabilized by intrachain crosslinking [13, 15, 16], by conjugation to dextran or PEG, or by polymerization (intermolecular crosslinking) [13, 15, 16, 17, 18]. The high oxygen affinity of SFHS has been reduced by covalent linkage of Hb to pyridoxal-5'-phosphate [12, 13, 17] or by carboxymethylation of Hb [18].

Hemoglobin is obtained from outdated stocks of donated human blood. Even if all outdated blood available were used to prepare modified hemoglobin solutions, however, only 5% of the need for blood transfusions could be satisfied, since the outdated blood amounts to only 600,000 units per year [19]. This has prompted the search for alternative sources of hemoglobin. Bovine hemoglobin solutions have been tested as potential RBC substitutes [5, 20]. Because the oxygen affinity of bovine Hb is regulated by chloride ions, abounding in human plasma, rather than 2,3-DPG, bovine Hb solutions have a low oxygen affinity (P_{50} of 28 mmHg) [8] and therefore the only modification necessary is the stabilization of the Hb tetramer by conjugation or polymerization.

Other researchers have obtained recombinant human hemoglobin, synthesized in hosts like yeasts [21], bacteria (E. coli [4, 6] or S. cerevisiae) or transgenic animals such as pigs [4, 14, 22, 23]. But these recombinant Hb must still be chemically modified to be usable. Another approach is the preparation of genetically engineered recombinant human hemoglobin [13, 15]. Researchers have succeeded in preparing a mutant form of human hemoglobin, with a reduced oxygen affinity and a stable tetramer, in the bacterium E. coli by modification of the gene that codes for the protein, but the potential presence of endotoxins has spurred criticism of this method [4, 14, 24, 25].

Many unanswered questions concerning the toxicity of these modified hemoglobins remain: presence of stromal/lipid components and endotoxins [8], potential toxicity of crosslinking agents such as glutaraldehyde [15], immunogenicity of non-human Hb [5, 15, 26], metabolic distribution and routes of excretion of modified Hb [15]. The most crucial point is that Hb might itself be toxic because of its high affinity for nitric oxide [22], the potential generation of damaging free radicals from its breakdown products [15], and/or its tendency to stimulate bacterial growth when removed from the erythrocyte [27]. Of the very few human trials of hemoglobin solutions conducted in recent years, half were halted because of adverse medical effects [6, 14, 26, 28]!

Encapsulated hemoglobin

An alternative approach to preparing an RBC substitute is the encapsulation of hemoglobin in liposomes. T.M.S. Chang was the first,

in 1957, to propose the use of encapsulated hemoglobin as an "artificial red cell" [29]. While Chang used synthetic polymers (nylons) to encapsulate hemoglobin, most groups are now employing a fairly standardized mixture of phospholipids and cholesterol consisting of roughly equal parts cholesterol and phosphatidylcholine, with 5-10% negatively charged lipids such as phosphatidic acid, phosphatidylglycerol, or dicetyl phosphate to prevent liposome aggregation and 1% α-tocopherol to reduce lipid peroxidation [30, 31, 32]. To maintain a low Hb oxygen affinity, researchers are either co-encapsulating hemoglobin with pyridoxal-5-phosphate or 2,3-DPG [33], or using bovine Hb [32].

The best method of preparation of liposome-encapsulated hemoglobin (LEH) seems to be high pressure homogenization in a Microfluidizer or a Gaulen homogenizer, since this yields multi-liter quantities of LEH, 0.2-0.5 μm in diameter (smaller than the smallest capillaries) while preserving the integrity of Hb [33].

Lyophilization seems to be the best method for treating LEH for long-term storage, since it greatly reduces the volume and mass of material for storage, eliminates the need for refrigeration, and prevents the oxidation of the hemoglobin. Provided a cryoprotectant such as the carbohydrate trehalose is added during LEH production, lyophilization also prevents the aggregation and coalescence of the liposomes.

A major problem with LEH, however, is the rapid rate of liposome removal from the circulatory system, which both undermines the efficiency of LEH as an RBC and has the potential to lead to

reticuloendothelial blockage, a serious problem for patients with a compromised immune system [19].

Research is now aiming at increasing the circulation time of LEH by modifying the size and surface charge of the liposomes, by altering the bilayer fluidity, and by incorporating glycolipids, glycoproteins, or gangliosides (sialic acid-containing glycolipids) into the phospholipid bilayer [32]. For example, Hunt and Burnette have incorporated synthetic glycolipids (non-antigenic) into the membrane of the liposomes, making them difficult for the phagocytes to recognize ("stealth" liposomes) and thus increasing their survival time [34]. In another study, a longer circulation half-life of 18 hours was documented for LEH made with hydrogenated (saturated) phospholipids composed of egg yolk phosphatidylcholine [33]. These results strongly suggest that the best way to extend the circulation lifetime is probably through modification of the liposome surface.

As a new way to encapsulate Hb, so-called multiple emulsions were developed: these consist of a concentrated Hb solution encapsulated in an oil phase, which in turn is dispersed in isotonic saline solution [35, 36]. However, these emulsions, which are not sterile, have a large particle size (several microns) and are not stable for more than a couple of days.

Synthetic hemoglobin-like chemicals

Tsuchida et al. have designed a totally synthetic oxygen carrier made of synthetic heme embedded into the phospholipid bilayer of liposomes (30 nm in diameter) [37, 38]. To prevent liposome aggregation and fusion they have polymerized (crosslinked) the

phospholipids of the bilayer. Although these model complexes are promising, they are not practical, since the low solubility of the heme into the lipid bilayer results in very low concentrations of "active" component in the final product; and both their efficacy (given their short circulation time) and their toxicity can be seriously questioned.

1.1.2 "White blood"

Research in developing PFC-based RBC substitutes began in 1966 when Clark and Gollan [39] illustrated the high capacity of PFCs for dissolving oxygen by showing that mice submersed in oxygenated PFC could survive. Being insoluble in water, PFCs must be emulsified (i.e., dispersed as small droplets in a saline solution compatible with plasma), before being injected. The preparation of such emulsions requires the addition of emulsifiers (to stabilize the droplets) and salts (to adjust the osmotic pressure and pH), and an input of energy, usually by sonication or high-pressure homogenization (microfluidization). Sloviter and Kamimoto were the first, in 1967, to use a PFC emulsion to deliver oxygen to a perfused isolated rat brain [40]. Geyer reported in 1973 the successful (with definite survival) total exchange-perfusion of rats with PFC emulsions [41]. Fluosol-DA, the first potentially commercial PFC emulsion, was developed by scientists at Japan's Green Cross Corp. (Osaka, Japan) in 1978. Fluosol-DA consists of 20% (w/v) PFCs: perfluorodecalin (FDC, 14.0 g/dl), with a half-life in the body of ca. 7 days, and perfluorotripropylamine (FTPA, 6.0 g/dl), with a half-life of 65 days, emulsified by a combination of poloxamer 188 (Pluronic F68, 2.72 g/dl), egg yolk phospholipids (EYP, 0.40 g/dl), and potassium oleate

(0.032 g/dl) in a saline solution composed of glycerin (0.80 g/dl), sodium chloride (0.60 g/dl), sodium bicarbonate (0.21 g/dl), dextrose (0.18 g/dl), magnesium chloride (hexahydrate) (0.043 g/dl), calcium chloride (dihydrate) (0.036 g/dl), potassium chloride (0.034 g/dl) [42]. The first adult volunteers were given the PFC emulsion Fluosol-DA in 1979, and in 1982 the results of a large-scale (186-patient) Japanese clinical study of Fluosol-DA were reported: no untoward reactions were observed and measurements of oxygen delivery and consumption indicated a beneficial effect of the infusion [43]. After further human trials in Europe and in the United States, Fluosol-DA was approved by the U.S. Food and Drug Administration (FDA), in December 1989, for use in percutaneous transluminal coronary angioplasty (PTCA) or balloon angioplasty [44, 45]; this severely limited approval nevertheless paved the way for further and broader approvals of similar products. Unfortunately, these first-generation PFCs emulsions have several disadvantages: (1) due to low PFC concentrations (20% w/v), they require the concurrent administration of 60% to 100% oxygen with the associated potential oxygen toxicity; (2) due to their instability, Fluosol-DA-like emulsions must be stored frozen in three separate components that must be mixed prior to injection; (3) PFC emulsions have low circulation times (a few hours) and the reticuloendothelial system (RES), which is involved in PFC clearance, has been reported to be saturated, thus reducing the body's ability to clear other foreign substances (bacteria, viruses); (4) due to the high value of FTPA's half-life in the tissues, Fluosol-DA can be administered only in limited amounts; and

(5) PFCs have also been reported to accumulate in the liver and the spleen [19].

To achieve long-term storage stability and small particle size, while increasing circulation time and preventing blockage of the capillaries, Rosano and Gerbacia [46] first suggested, in 1973, the use of PFC microemulsions; these are thermodynamically stable, self-dispersible, transparent emulsions with particle size distributions around 100 Å. These microemulsions have low PFC concentration, however, and their biocompatibility has yet to be proven.

Over the past five years, the first-generation PFC emulsions have been superseded by improved "second-generation" formulations [47]. The principal developments have been: (1) the selection of PFCs with a higher purity and with lower half-lifetime in vivo; (2) the preparation of emulsions with greater PFC content and increased potential gas-carrying properties; and (3) the use of better emulsifiers, less toxic and capable of producing concentrated PFC emulsions stable for months when stored at room temperature.

The most promising second-generation PFC emulsion was developed by Long (San Diego, CA) in the mid-80s [48]. This emulsion, which is being developed by Alliance Pharmaceutical Corp. (San Diego, CA), consists of 90-100% (w/v) perflubron (PFOB, half-life of 4 days) emulsified by EYP in a saline solution isotonic to blood; it is stable for years when stored at 4°C and has been tested in clinical trials with no major side effects being reported. These EYP-based emulsions have considerable advantages over Pluronic F68-based emulsions: (1) because they are extracted from natural (animal) sources, phospholipids are well accepted by the body (they are the

major components of the cell membrane), and are commonly used in intravenous injectable emulsions for parenteral nutrition; (2) phospholipids have a much higher surface activity than Pluronic F68 (they yield very low surface and interfacial free energies); (3) EYP emulsions do not induce complement activation and do not interfere with circulation; and (4) Pluronic F68 has a cloud point at about 115°C, above which its effectiveness as a surfactant decreases sharply, rendering conventional sterilization at 121°C difficult [49].

PFCs emulsions have several advantages over blood or hemoglobin-based RBC substitutes: (1) since they contain no antigens, no typing or crossmatching between donor and recipient is necessary, and (2) PFCs are easily synthesized in large amounts from readily available materials and are free from infectious agents [19].

In addition to their role in the development of RBC substitutes, PFCs have extensive potential for other biomedical applications, which makes their study very compelling.

1.2 Other biomedical applications [50]

PFCs preparations are also being developed for imaging applications (contrast agents), and for oxygen-carrying applications other than as temporary RBC substitutes: these include cancer, cardiovascular, and pulmonary applications. PFOB emulsions are particularly rich in potential applications due to the presence of a bromine atom on the PFOB molecule.

1.2.1 Imaging applications

Long and Mattrey [48, 51] discovered PFOB to have properties making it useful as a contrast agent for imaging specific organs in the body. Pure PFOB can be used as an oral contrast agent to image the gastrointestinal (GI) tract with X rays or magnetic resonance (MR) scans. Because of the bromine atom attached to the molecule, PFOB creates a bright outline of the GI tract when viewed with X rays. MR imaging is based upon the resonance of hydrogen atoms found in human tissue; since PFOB contains no hydrogen, it creates a signal void during MR scanning that provides a dark outline marking the GI tract. PFOB emulsions can be used as intravenous agents for imaging the spleen or the liver, one of the key organs involved in determining the prognosis for cancer patients. It is important to confirm whether or not a primary cancer has spread to the liver and, if so, to determine the size of the tumor. When injected intravenously, PFOB particles accumulate in the normal tissue of the liver, resulting in excellent contrast between the normal tissue and tumors during computed tomography (CT) or ultrasound imaging. PFOB emulsions also have great potential for facilitating earlier diagnosis of the spread of cancer. Since cancer often spreads (metastasizes) from one part of the body to another through the lymphatic system, evaluation of the lymph nodes for metastatic cancer involvement typically follows diagnosis of a primary tumor. While there are no FDA-approved, effective, non-surgical methods currently available for determining the presence of cancer in lymph nodes, a very promising PFOB-based imaging agent is under development. This imaging technique is designed to exploit the well-

established tendency of PFOB droplets, once injected subcutaneously, to migrate into the nearby lymph nodes and opacify them on CT images. When perfected, the technique will provide a visual display of intranodal architecture that could enable radiologists to determine the presence or absence of malignancies within the nodes. By enabling safer and more accurate diagnosis of lymph node involvement at an earlier stage, which would in turn permit earlier treatment, such applications of PFOB emulsions thus offer hope for improved cancer survival rates. Due to the very low velocity of sound in PFCs, PFOB emulsions are also being developed for use with ultrasound to image blood flow within organs and to detect vascular defects (local constriction of blood vessels or blood clots).

1.2.2 Cancer applications

Over the past decade, the use of PFC emulsions has been explored in numerous preclinical studies as an adjunct to radiation and chemotherapy in the treatment of solid tumors [52, 53, 54]. Rapid and uncontrolled growth of malignant cells within solid tumors results in external pressure on capillaries, which causes them to constrict and collapse. As this process evolves, red blood cell passage and oxygen supply to the tumor become restricted, and as a result, most solid tumors have regions of cells that are poorly oxygenated, or hypoxic. Such regions tend to be more resistant to radiation and chemotherapy than cells that are well oxygenated. For example, it is estimated that three times more radiation is required to kill severely hypoxic cells than normally oxygenated cells. Higher doses and prolonged treatment with radiation and chemotherapy may be more

effective at killing hypoxic cancer cells; however, such intensified treatment also increases the extent of side effects, which can limit the benefit of therapy. PFOB emulsions have been shown in animal studies to increase the oxygen levels in hypoxic regions of cancerous tumors, making the tumor cells more responsive to both radiation and chemotherapy. Therefore, these dispersions may be useful as an adjuvant treatment by improving the effectiveness of lower-dose radiation and chemotherapy on cancer cells.

1.2.3 Cardiovascular applications

The heart needs large amounts of oxygen, which it normally extracts from the blood, and when deprived of oxygen, as when blood flow is restricted or blocked, the heart muscle deteriorates rapidly. PFOB emulsions offer a possible method of reducing such damage by providing an alternative oxygen-delivery system. Myocardial infarction (heart attack) occurs when an artery supplying the heart muscle becomes blocked, usually by a blood clot, with resultant reduction in the oxygen supply to portions of the heart: this can lead to permanent myocardial tissue damage. Animal studies simulating coronary artery blockage indicate that infarct damage may be reduced when a PFOB emulsion is administered. These results may be explained by the ability of small emulsion particles to traverse partially blocked capillaries, which are too narrow for the passage of red blood cells.

Heart disease is often detected before heart attacks occur and, when the problem is related to coronary artery obstruction, therapy can be selected either to open the artery or to graft a replacement

artery in the heart to "bypass" the obstruction. To open the blocked artery, a procedure known as percutaneous transluminal coronary angioplasty (PTCA) can be employed. In this procedure a small balloon, positioned on a catheter, is placed inside the obstructed vessel and then inflated to enlarge the vessel. When angioplasty procedures are employed, blood flow to a portion of the heart is temporarily blocked as a result of the balloon inflation. This reduction in blood flow carries a risk of causing oxygen deprivation, which may further complicate the patient's condition. Coronary angioplasty is occasionally used on an emergency basis to deliver thrombolytic drugs directly to coronary blood clots during the treatment of acute heart attacks. In such circumstances, PFOB emulsions may be used for intracoronary administration to supplement oxygen delivery to the blood-deprived region of the heart. When open-heart surgery is performed to reroute a coronary artery or to replace heart valves, the pumping action of the heart is temporarily stopped to allow intricate surgical intervention to take place. In this case, the patient's blood is oxygenated and circulated mechanically by a heart-lung machine, thereby bypassing the patient's own cardiopulmonary system. The machine's pump requires large volumes of priming fluid, preferably a standard saline solution. These water-based solutions are poor oxygen carriers, however, and may result in a diluted blood that fails to provide adequate oxygenation for some patients. When such a problem is anticipated, donor blood is often used as a priming agent. In effect, these patients are receiving a blood transfusion to compensate for the short-term oxygen need created by this procedure. Here again,

PFOB emulsions may be used as oxygen-carrying "priming" solutions to reduce patient exposure to the risks associated with donor blood.

1.2.4 Pulmonary applications

Respiratory distress syndrome (RDS), which affects both children and adults, can occur as a result of pulmonary infections, traumatic shock, severe burns, or inhalation of toxic substances. Patients with such conditions often accumulate fluid and debris in their lungs and suffer restricted flow of gases and impaired production of the surfactant required for normal lung operation. These conditions can lead in turn to the blockage of airways and the collapse of alveoli (air sacs), resulting in inadequate gas exchange. The standard therapy for RDS, mechanical gas ventilation (via respirator) administered under pressure, is inherently flawed: the patient's condition is often worsened by the high gas pressures required to inflate damaged and collapsed lung segments. Indeed, of approximately 200,000 cases of RDS reported annually in the United States, some 50 to 60 percent end in the death of the patient. If injected into the lungs, a PFOB-based combination surfactant/oxygen carrier would, because of its surface properties, reduce the surface free energy in the air sacs, allowing their inflation at significantly lower ventilation pressures; once inside the alveoli, it would facilitate oxygen and carbon dioxide exchange.

Many other potential biomedical applications exist for PFC-based preparations, e.g., organ preservation, drug delivery, biomedical research on bloodless animals, cerebral and intestinal ischemia [55], ocular applications, enhanced oxygenation to accelerate wound

healing [56], and transport of radio-labeled oxygen for imaging of ischemic tissues.

Chapter Two
Perfluorochemicals: Properties and Synthesis

Definition: The term perfluorochemical (PFC) will be used here to designate a fluorinated compound that may or may not contain a hetero atom such as nitrogen, oxygen, sulfur, or another halogen. PFCs are colorless, dense, inert, hydrophobic liquids with a low surface free energy and a high capacity to dissolve gases such as oxygen and carbon dioxide. Their unique physical properties make them good candidates for a variety of biomedical applications including medical imaging and RBC substitutes for temporary oxygen delivery. Typical PFCs are perfluorodecalin (FDC), perfluoro-tripropylamine (FTPA), bis-(F-butyl)-ethene (F-44E), and perflubron (PFOB or perfluorooctylbromide) (Table 2.1).

2.1 Physical properties of PFCs

PFCs are chemically inert due to the presence of very strong carbon-fluorine bonds (485 kJ/mole), the fluorine atoms offering steric protection to the carbon group [1]. The C-F bond, being unusually strong and short (1.36 Å), has a low polarizability resulting in very low interaction forces between the PFC molecules [2]. These weak intermolecular forces are responsible for the rather unique set of physical properties of PFCs, including low boiling points, high densities, very low surface free energies, low sound velocities, low dielectric constants, and a high capacity for dissolving gases.

PFCs' boiling points are lower than those of hydrocarbons with equal chain length and lower than those of other compounds with the same molecular weight, with the exception of noble gases [3].

Like their boiling points, the surface free energies of PFCs are much lower than those of the corresponding hydrocarbons. Because of their low surface free energies PFCs will wet all other materials [3].

The densities of PFCs are about twice those of saturated hydrocarbons. This explains why, although the absolute viscosities of the PFCs are much higher than those of corresponding hydrocarbons, their kinematic viscosities (the ratio of the absolute viscosity to density) are somewhat lower than those of hydrocarbons. Consequently, PFC liquids will pour like water [3].

PFCs have also extremely low refractive indices, low dielectric constants, and very low sound velocities. This last characteristic makes them suitable as diagnostic agents for ultrasound imaging.

PFCs are poor solvents and are either totally miscible or partially soluble in aliphatic hydrocarbons, depending on their molecular weight. As an example, perfluoro-n-pentane and n-pentane are completely miscible, in contrast to perfluoro-n-heptane and n-heptane, which coexist as two distinct phases [3].

PFC liquids have a very high solubility for gases (Table 2.1): oxygen solubility in PFCs is typically 40-50 vol%, whereas the solubility of carbon dioxide can be 3-4 times greater. This solubility depends upon the molecular volume of the dissolving gas and decreases in the order $CO_2 > O_2 > N_2$ [1]. In contrast with the characteristic sigmoid binding curve of oxygen to hemoglobin, oxygen

solubility in PFCs and their emulsions increases linearly with the partial pressure according to Henry's Law. The amount of gas dissolved depends upon the concentration of PFC and on its solubility coefficient for the gas, and is inversely related to temperature. The molecule structure and shape of PFCs are important determinants of their gas-dissolving properties, if, as is believed, gas molecules occupy "cavities" within the PFC liquid.

A crucial factor in the selection of a PFC for a biomedical application is the retention time of the PFC. Yokoyama has shown that retention increases exponentially with molecular weight. From Yokoyama's data Riess [4] has specified a range of molecular weights acceptable for blood substitutes from the standpoint of their excretion: its lower limit, below which the vapor pressure becomes too high and provokes lung emphysema, is set at about 460, corresponding to a vapor pressure less than 20 mm Hg, whereas the upper limit is about 520, corresponding to a half-retention time not exceeding two weeks. PFOB is a deviant point on Yokoyama's plot; it has a much lower retention time than expected from its molecular weight, which makes it a particularly attractive candidate for biomedical applications.

2.2 Synthesis of PFCs

There are two main approaches to PFC synthesis: substitution and oligomerization. In the substitution method, hydrogen atoms in a hydrocarbon analog of the desired compound are progressively replaced by fluorine. This can be achieved by direct fluorination

with elemental fluorine gas, cobalt trifluoride fluorination, or electrochemical fluorination (a process also known as electrofluorination).

Direct (vapor phase) fluorination

A stream of fluorine gas, diluted with an inert gas (nitrogen or helium), is reacted with the hydrocarbon in a reactor at 130-300°C. The reaction is highly exothermic and a mixture of perfluorinated, partially fluorinated, and fragmented products is formed [3]. The process has been improved by the use of low-temperature techniques allowing good control of the highly exothermic reaction.

Direct fluorination is essentially used for the synthesis of perfluorochemical compounds not accessible through other routes: sterically crowded PFCs, for example, because of the acid or kinetic instability of their hydrocarbon precursors or reaction intermediates.

Cobalt trifluoride fluorination

The vaporized hydrocarbon, often diluted with nitrogen, is fed into an agitated reactor containing cobalt trifluoride at 200-250°C. This process has the advantage over direct fluorination that elemental fluorine is not used directly, thus reducing the exothermicity of the reaction as well as the carbon-carbon bond cleavage. FDC is produced by the $CoCF_3$ fluorination method, but large amounts of partially fluorinated products are also obtained, due to incomplete fluorination, bond breaking, and isomerization [1, 3].

Electrofluorination

The electrochemical fluorination process, the one most commonly used, was discovered in 1950 by J.H. Simons [5] and has been developed since on a commercial scale in the production of a variety of fluorocarbons and fluorocarbons derivatives.

An electrochemical cell (Fig. 2.1) is charged with anhydrous hydrofluoric acid and an organic substrate in a ratio of approximately 10:1. The electrodes are attached to the lid as a pack of alternate anodes and cathodes but insulated from each other with inert spacers. A refrigeration unit capable of cooling the cell and a condenser to 0°C and below is necessary. When direct electric current at low voltage is passed through such a solution, hydrogen is evolved at the cathode and the organic material is fluorinated at the anode. Since highly fluorinated materials are not miscible with hydrogen fluoride, high boiling products collect as a separate bottom layer. A valve at the bottom of the cell allows the product to be drained off periodically [6].

Usually, functionalized hydrocarbons and aromatics are used as starting materials in electrofluorination processes, since hydrocarbons and hydrocarbon aromatics have low solubility in hydrofluoric acid and form non-conducting solutions. For example:

$$\underset{\text{n-octyl alcohol}}{CH_3(CH_2)_6-CH_2OH} \xrightarrow{EF} \underset{\text{perfluorooctane}}{CF_3(CF_2)_6-CF_3} + \text{other inerts}$$

The second approach to PFC synthesis is the oligomerization route, in which PFCs are synthesized by combining smaller, already fluorinated compounds. Oligomerization is advantageous for the synthesis of PFCs for in vivo use since pure compounds can be produced in a reproducible manner. PFOB can be synthesized by the oligomerization of tetrafluoroethylene [1].

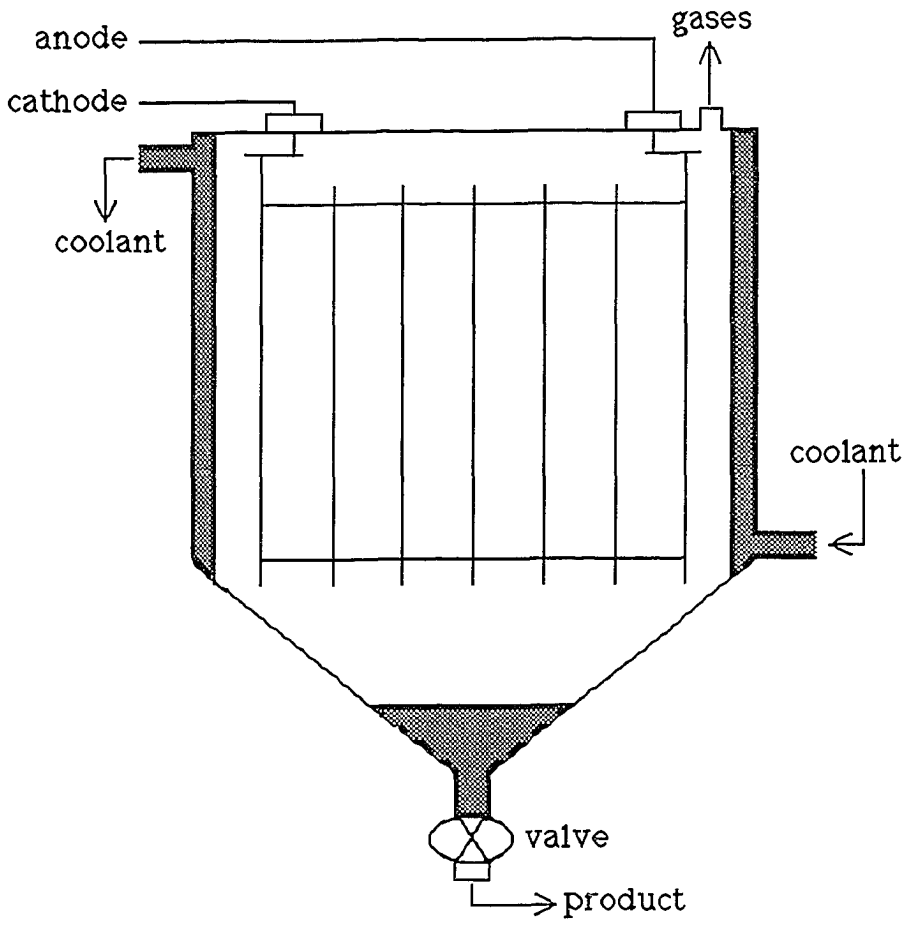
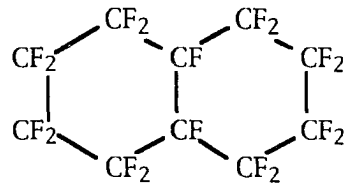


Figure 2.1: Simons electrofluorination cell (from [3])

Table 1: Structure and properties of several perfluorochemicals

Perfluorochemical (PFC)	MW	Density (g/ml@ 20°C)	BP (°C)	$t_{1/2}$ in organs (days)	vp (mmHg, 37°C)	O_2 solubility (vol%, 37°C)	CO_2 solubility (vol%, 37°C)
perfluorodecalin (FDC) 	462	1.91	142	7	12.5	40	140
bis-(F-butyl)-ethene (F-44E) $(n\text{-}C_4F_9)CH{=}CH(n\text{-}C_4F_9)$	462	≅2	129	7	12.6	50	210
perfluorooctylbromide perflubron (PFOB) $n\text{-}C_8F_{17}Br$	499	1.92	141	4	14.0	50	210
perfluorotripropylamine (FTPA) $N(n\text{-}C_3F_7)_3$	521	≅2	129	65	18.5	45	165

Chapter Three
Phospholipids: Chemistry and Extraction

Definition: Phospholipids are a class of phosphorus-containing chemicals usually found with fats. The major components of all biological membranes, they can be extracted from both animal and vegetable sources (e.g., egg yolk and soybeans). Phospholipids are divided into two subclasses: glycerophospholipids (the major phospholipids), derived from glycerol-3-phosphate, and sphingophospholipids (essentially sphingomyelin, SM), derived from ceramide (Fig. 3.1). The individual species of glycerophospholipids differ in the nature of the group (X) esterified to the phosphate: this group may be a hydrogen atom (phosphatidic acid, PA), choline (phosphatidylcholine, PC), ethanolamine (phosphatidylethanolamine, PE), serine (phosphatidylserine, PS), glycerol (phosphatidylglycerol, PG), or inositol (phosphatidylinositol, PI). They also vary in the chain length and degree of unsaturation of the acyl chain (X and Y) attached to the glycerol backbone. If one fatty acyl residue is hydrolyzed a lysophospholipid is formed. Phospholipids are amphiphilic molecules and have thus been widely used as emulsifiers.

Hydrolysis

Phospholipids are hydrolyzed by enzymes, called phospholipases, forming lysophospholipids (one acyl chain) and free fatty acids (FFA) (with phospholipase A and B); phosphatidic acid (with phospholipase D); or triglycerides (with phospholipase C).

Oxidation

Oxidation is the most important of all phospholipid degradation processes. It takes place on the acyl chain of unsaturated fatty acids and results in the formation of hydroperoxides, which are unstable and decompose, primarily to aldehydes. The oxidation stability of phospholipids depends on the following factors:

(1) presence of oxygen;

(2) presence of anti-oxidants (tocopherols (vitamin E));

(3) degree of unsaturation of the acyl chain (the more double bonds, the faster the oxidation: oxidation rates for oleic (18:1), linoleic (18:2), and linolenic (18:3) are approximately 1:10:25);

(4) heat (every 10°C rise in temperature doubles the rate of oxidation); and

(5) light (oxidation sensitivity increases with exposure to light).

Thermal Properties

Because of the hydrophobic hydrocarbon-water interaction between the phospholipid molecules and the aqueous phase, phospholipids form bilayers in dilute aqueous solution. At low temperature each phospholipid acyl chain is in the low energy all-trans configuration. If there were no intermolecular interactions, as the temperature is increased each chain would rotamerically disorder only very gradually. Lipid molecules that are packed into a bilayer are not free to disorder gradually, however: if one molecule attempts to rotate one of its acyl chains about a C-C bond, the terminal methyl group will bump into neighboring chains, which are

only about 4.8 Å distant. Thus, chain rotation is of necessity a cooperative event, one involving many molecules. This gives rise to a sharp anomaly called phase transition between an ordered gel state at low temperatures and a more disordered fluid (liquid crystalline) state at high temperatures [1].

Differential scanning calorimetry (DSC) study of this characteristic gel-to-liquid crystal transition has shown that it depends essentially on the nature of the polar phospholipid head groups and on the length and degree of unsaturation of the acyl chains. For a given degree of unsaturation, the longer the chains, the higher the phase transition temperature (PTT). For a given chain length, the more unsaturated the chains, the lower the PTT. In biological lipids, the double bond on unsaturated acyl chains will be in cis position, causing a kink in the acyl chain. This kink introduces some perturbation in the interfacial film, resulting in a less efficient packing (smaller size of the cooperative unit) than with saturated acyl chains. The poorer the packing -- i.e., the less ordered the film -- the lower the PTT. For example, distearoylPC (DSPC), a saturated phospholipid with 18 carbons on the acyl chains, has a PTT of 55°C, compared to -17°C for dioleylPC (DOPC), with acyl chains having 18 carbons and one double bond in cis position, and to 41°C for dipalmitoylPC (DPPC), with 16 carbons and no double bonds.

Solubility

Soluble in organic solvents such as hexane, benzene, or chloroform, phospholipids are insoluble in acetone and thus easy to separate from other lipids by precipitation with acetone. At

temperatures above their PTT, phospholipids are dispersible in water, forming a lamellar phase. The bilayers can be resolved into vesicles (or liposomes) by mechanical agitation, the size of the vesicles and the number of bilayers in their membrane depending on the mechanical work provided and on the phospholipid concentration.

Hydrogenation

Under extreme conditions, phospholipids can be hydrogenated to yield products that are resistant to oxidation and have a higher PTT.

Extraction of phospholipids from egg yolk

Several authors have proposed methods to extract and purify egg yolk phospholipids [2, 3]; mine was inspired by the Wells & Hanahan extraction method [4]. After total lipids were extracted from egg yolk using a chloroform/methanol (2:1) solution (Fig. 3.2), phospholipids were precipitated in cold acetone (Fig. 3.3). The precipitate was washed and the solvent removed, yielding EYP. Oxidation had to be avoided to prevent phospholipids degradation. The resultant EYP did not yield stable emulsions. (I also used the method of Ramesh et al. [5], consisting of the denaturation of lipoprotein complexes by methanol, extraction of phospholipids with chloroform, and chloroform fraction wash with saline (Fig. 3.4), with similar results.) During phospholipid precipitation some non-polar compounds were entrapped within the crystal structure. I found that the best way to purify my EYP was to redissolve it in chloroform (with a minimum amount of methanol) and to add Silicagel (silicic

acid) (Fig. 3.5). The slurry was thoroughly stirred. The polar compounds were adsorbed on Silicagel, while the non-polar compounds remained in the solvent. The slurry was then filtered out and rinsed several times with chloroform. The polar compounds were eluted using methanol, which was finally roto-evaporated, allowing recovery of purified EYP (PEYP). See below (6.1) for more extensive discussion of EYP extraction and purification techniques.

Composition of EYP

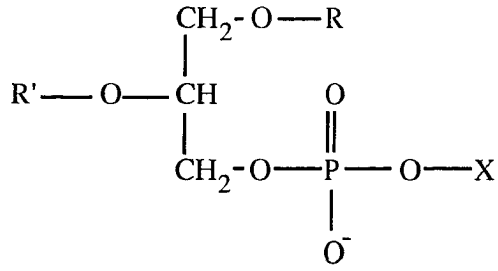
Normalized composition (w/w%) obtained by averaging the composition of EYP from five different suppliers:

PC	70 ± 1.5
PE	18 ± 1.0
LysoPC	6 ± 2.1
SM	3 ± 0.2
Cholesterol	3 ± 0.1

Additional minor components included phospholipids (PI, PS, PG) and pigments, among others.

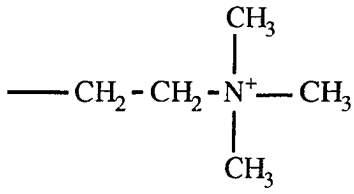
The composition of EYP depends essentially on the method of extraction and purification.

Glycerophospholipids

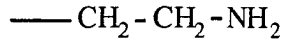


R, R'= fatty acid residues

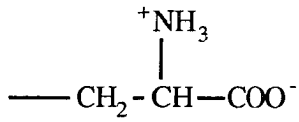
X= —H Phosphatidic acid (PA)



Phosphatidylcholine (PC)

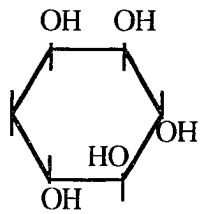


Phosphatidylethanolamine (PE)



Phosphatidylserine (PS)

Phosphatidylglycerol (PG)



Phosphatidylinositol (PI)

Sphingomyelin

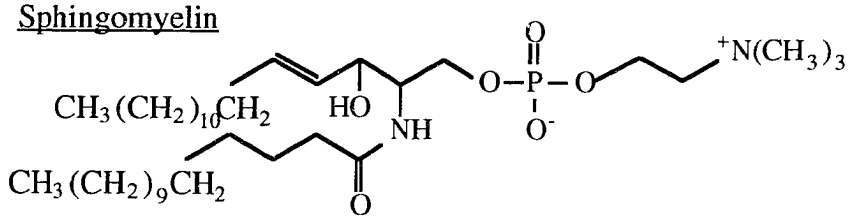
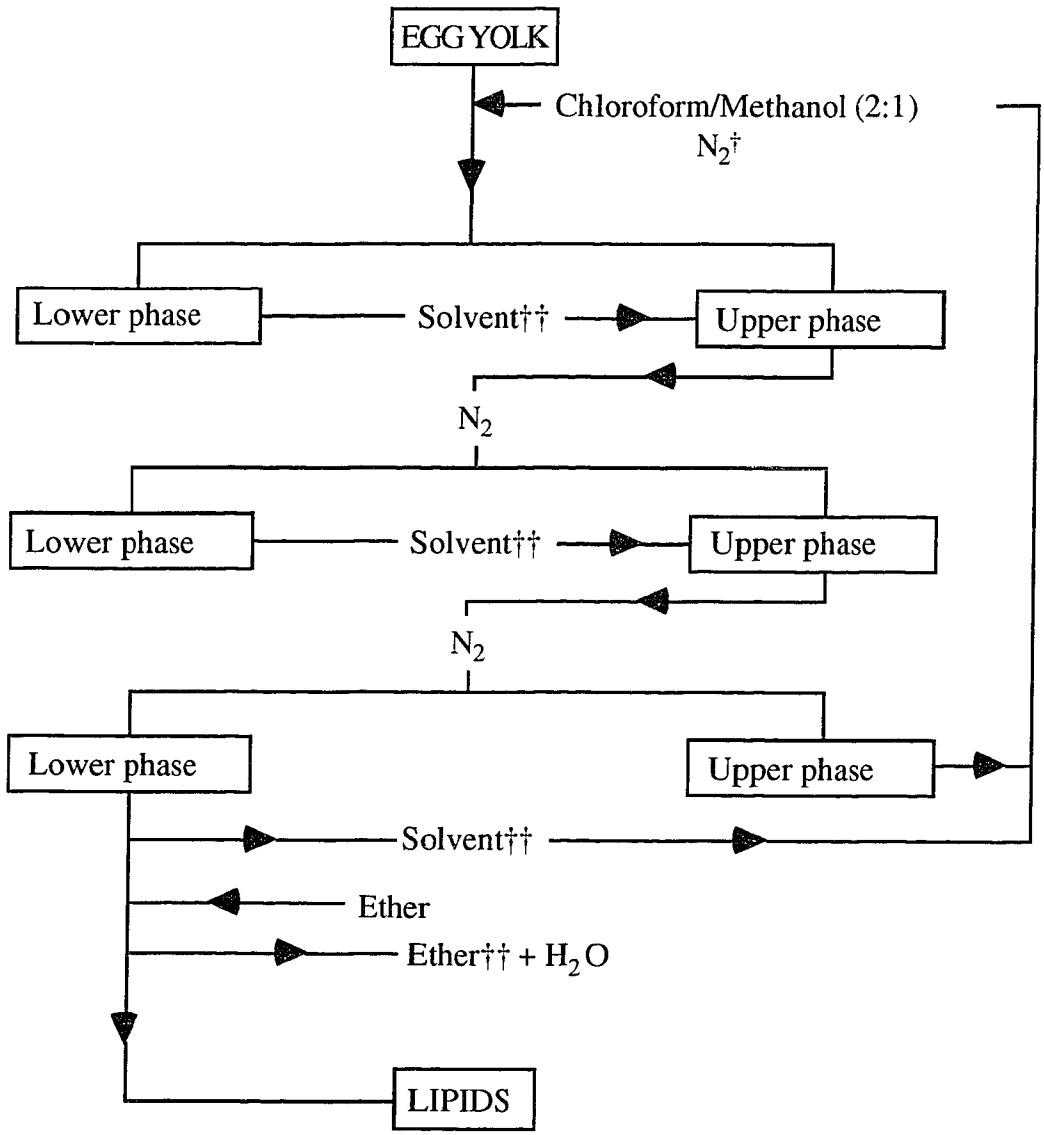
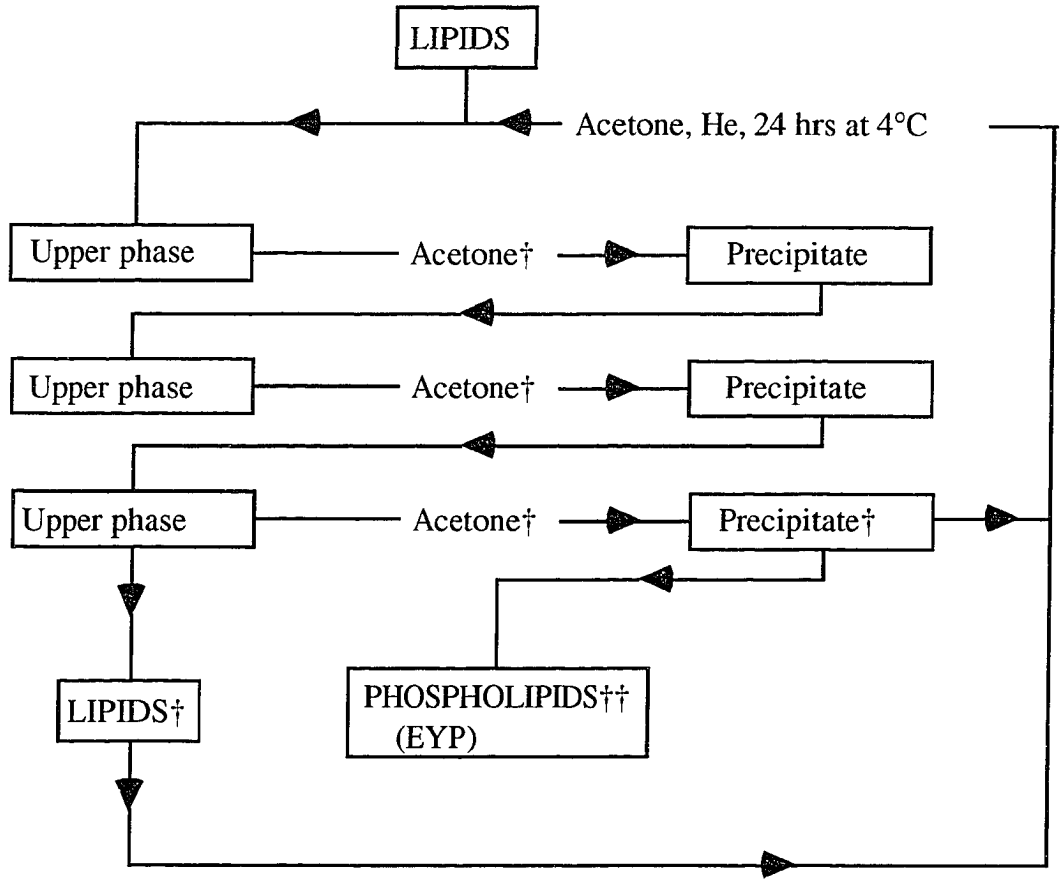


Figure 3.1 Structure of several biological phospholipids



†Separating Funnel; N_2 Bubbling
††Rotary evaporator

Figure 3.2: Extraction of total lipids from egg yolk



†Rotary evaporator
††Phospholipids in acetone + Helium stored at 4°C in the dark

Figure 3.3: Extraction of EYP from the total lipids

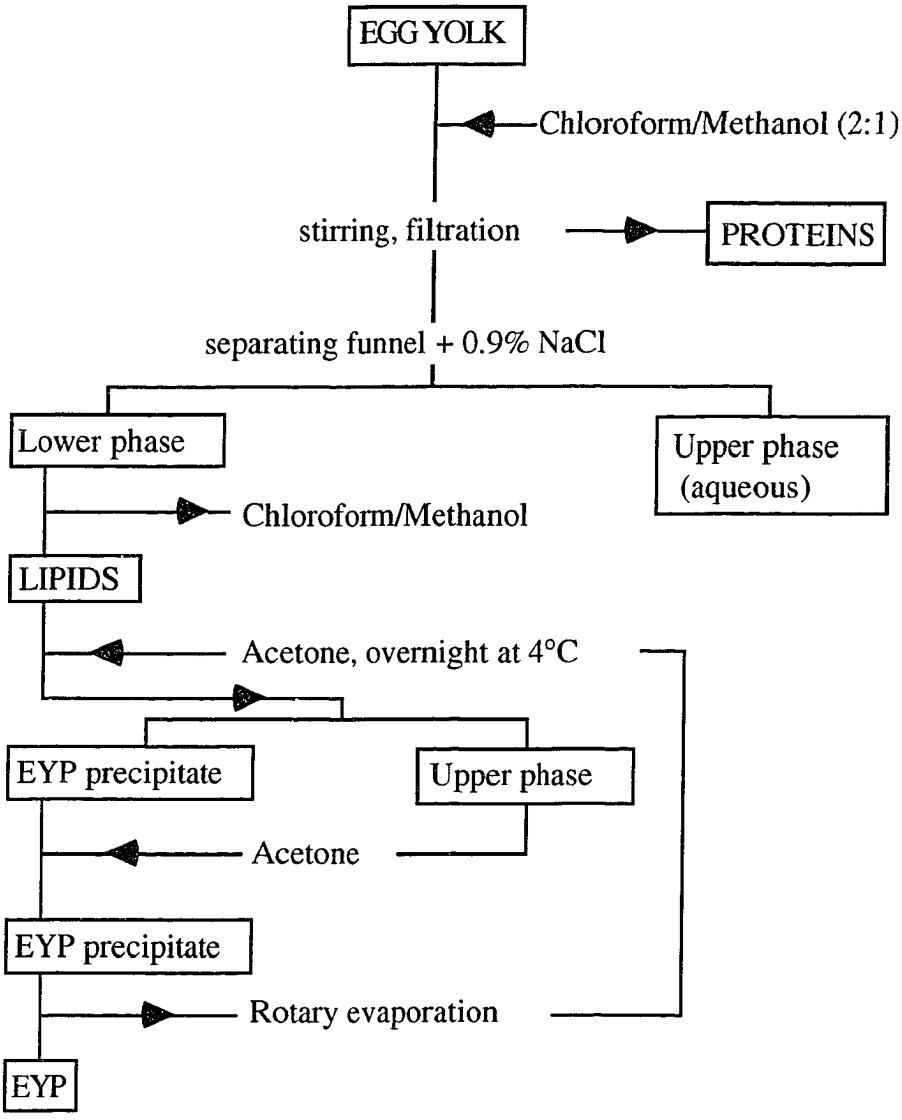


Figure 3.4: Alternative method of extraction of EYP from egg yolk

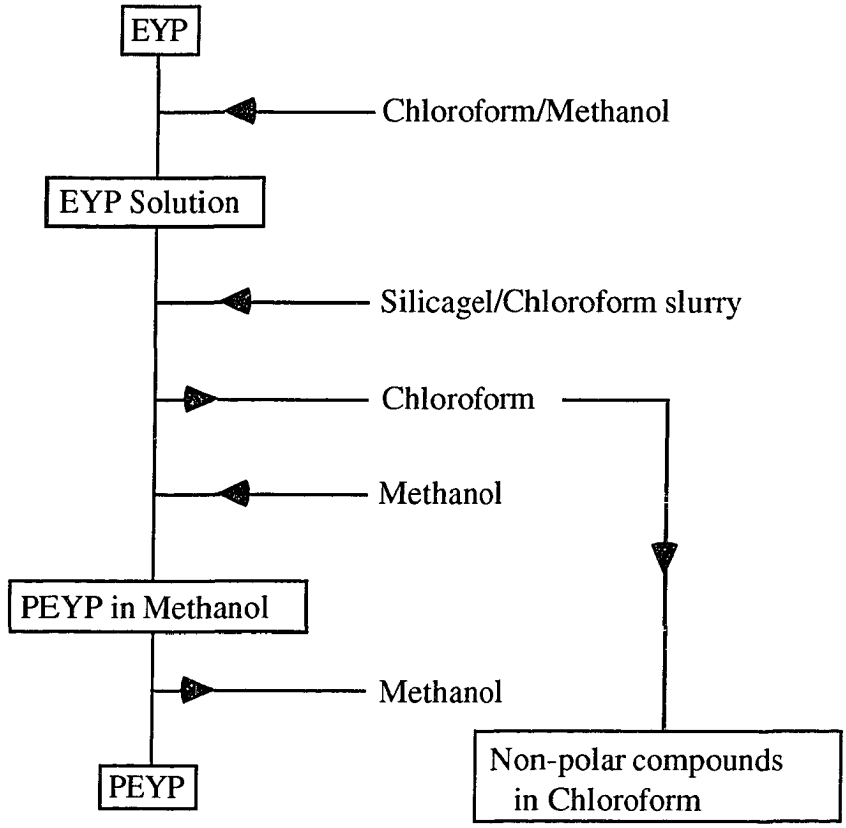


Figure 3.5: Purification of EYP

Chapter Four
Emulsion Formation and Stability

4.1 Emulsions

An emulsion is a dispersion of two immiscible (or partially miscible) liquids. Usually, one of the phases is aqueous -- the so-called "water" phase -- and the other is an "oil" in the wide sense, i.e., a hydrophobic substance such as an aliphatic or aromatic hydrocarbon (e.g., a triglyceride, benzene) or a PFC (PFOB, FDC). When the oil is the dispersed phase the emulsion is called an oil-in-water (o/w) emulsion, and when the aqueous medium is the dispersed phase it is called a water-in-oil (w/o) emulsion. The visual appearance of an emulsion results from the scattering of light by the droplets of the dispersed phase and depends on the droplet size. An emulsion with a particle size larger than 0.3 μm is milky-white-opaque. A particle size between 0.1 and 0.3 μm will give a gray-translucent emulsion. Dispersions with a particle size between 0.01 and 0.1 μm are transparent and are called microemulsions. One of the most interesting physical properties of an emulsion is its stability. Emulsion *instability* is promoted by three distinct phenomena: creaming (or sedimentation); flocculation (droplet aggregation); and droplet coalescence, resulting ultimately in phase separation.

Creaming, or sedimentation (the two terms essentially describe the same phenomena from opposite points of view), arises from a difference in density between the two phases and results in an

increased concentration of the dispersed phase at the emulsion surface (or bottom). Creaming (or upward sedimentation) will occur if the density of the dispersed phase is lower than that of the continuous phase (the usual case for hydrocarbon oil emulsions). An opaque layer of "cream" will be observed at the surface while the bottom will be a translucent liquid poor in dispersed oil droplets. Conversely, sedimentation (or downward creaming) will occur if the density of the oil phase is higher than that of the continuous phase as is the case for emulsions of PFCs. By nature creaming/sedimentation is a process readily reversible by a slight mechanical agitation. Although creaming facilitates flocculation by bringing emulsion droplets close together, it is not necessarily accompanied by this phenomenon.

Flocculation corresponds to the aggregation of emulsion droplets resulting in the apparition of flocs (clusters of aggregated particles) of increasing size that will migrate -- depending on their density -- either to the surface or to the bottom of the vial, where they will form a tridimensional gel-like network. A flocculated emulsion will eventually have the same appearance as a creamed emulsion, with a translucent layer resulting from the exclusion of the excess continuous phase from the oil-droplet network. Flocculation usually happens more quickly than creaming and, if it is not irreversible by physical means, is reversible only through the application of a much greater amount of mechanical work than in the case of creaming; this is due to the existence of strong interdroplet attractive forces in flocculated systems.

Flocculation is usually followed by coalescence of droplets resulting ultimately in phase separation. It is difficult if not impossible to suppress totally these causes of instability (i.e., creaming, flocculation, coalescence) but they can be diminished or slowed down to yield a system stable during a given period dictated by experimental or industrial requirements. Commercial emulsions must typically be stable over a period of several months or years.

4.2 Emulsifiers

Despite the definition presented in 4.1 of an emulsion as a binary, two-liquid dispersion, in practice, any emulsion used in a laboratory or commercial context is a *tricomponent* system consisting of a dispersed phase, a continuous phase, and an emulsifier -- the last element an agent facilitating emulsification and promoting emulsion stability. Emulsifiers are usually divided into four categories according to the electrostatic charge they bear in solution: (a) non-ionic (no charge), (b) cationic (positive charge), (c) anionic (negative charge), and (d) amphoteric (zwitterionic, no net charge). A more useful classification of emulsifiers involves their ability to form preferentially o/w or w/o emulsions. Indeed, the type of emulsion formed depends essentially on (a) the relative volumes of the two phases and (b) the nature of the emulsifier. The higher its phase volume, the more likely a liquid will be the continuous phase. (Emulsions have nevertheless been prepared in which the dispersed phase occupies more than 90% of the total volume [1].) The relative affinity of the emulsifier for the aqueous phase and the oil phase is

instrumental in determining the type of emulsion formed: Bancroft's rule states that the phase in which the emulsifier is the more soluble tends to be the dispersion medium. The amphiphilic nature of many emulsifiers, nonionic surfactants in particular, can be expressed in terms of an empirical scale of HLB (hydrophile-lipophile balance) numbers, based on a concept enunciated by Griffin in 1946 [2]. The HLB reflects the ratio of the hydrophilic character over the lipophilic character of a given emulsifier; the most hydrophilic emulsifiers will accordingly have the highest HLB values. A surfactant with a high HLB value (8-15) will preferentially yield an o/w emulsion, while one with a low HLB value (3-6) will give a w/o emulsion. HLB numbers can be used as a first approximation in emulsion formulation, but are inevitably followed by much trial-and-error testing. A major disadvantage of the HLB concept is that it measures the overall affinity of the emulsifier for the oil or the aqueous phase by numerically computing the contributions of the polar and the non-polar part of the surfactant molecule without taking into account the geometry or the spatial conformation of the molecule. Indeed, emulsifiers with totally different structures and emulsifying properties can have the same HLB number. Similarly, both a mixture of two (or more) emulsifiers with distinct individual HLB values and a pure emulsifier can have the same overall HLB number, while yielding emulsions with different stabilities; it is empirically well established that a mixture of surfactants usually yields more stable emulsions than a pure surfactant. Another disadvantage of the HLB concept is that it fails to take temperature effects into account. For example, an o/w emulsion stabilized by a polyoxyethylene

nonylphenylether [3] can be inverted to a w/o emulsion simply by heating it above a particular temperature called its phase inversion temperature (PIT). With increasing temperature the hydration of lyophilic groups like polyoxyethylene decreases and the emulsifier becomes less hydrophilic (its HLB decreases), until the emulsion inverts from o/w to w/o. Since temperature is an important factor in emulsion formation and stability, nonionic emulsifiers are more effectively selected for a given formulation by using the HLB-temperature (or PIT) system developed by Shinoda et al. [4]. When selecting an emulsifier of whatever category, of course, one must also take into account the interactions of the emulsifier with the other components of the system (e.g., dispersed phase, salts, proteins, polymers) and the environment (e.g., pH, temperature, presence or absence of oxygen).

By forming an adsorbed interfacial film around the dispersed droplets, emulsifiers both facilitate the emulsification process and foster stability by acting to prevent flocculation and coalescence; this stabilizing effect occurs via a complex and variable mechanism. Some aspects of the stabilization process will be discussed below when the major factors involved in the stability of PFC/aqueous emulsions with phospholipids as emulsifiers are examined in detail; see Chapter Six.

But in order to establish a conceptual framework in which to discuss the experimental work itself and to evaluate the results, we must begin with a more theoretical consideration of the three essential phenomena involved in emulsion instability -- or destabilization: creaming, coalescence, and flocculation.

4.3 Creaming/sedimentation

In the absence of external forces, particles in suspension are in constant thermal motion, continually changing direction as a result of random collisions. This random motion, generally referred to as Brownian motion, results in a continual fluctuation of concentration on a molecular scale. Molecules or particles tend to migrate from a region of high concentration to a region of lower concentration, with a flow rate (F_d) given by Fick's first law of diffusion:

$$F_d = \frac{dm}{dt} = -DA\frac{dc}{dx} \tag{4.1}$$

where:

$\frac{dm}{dt}$ is the mass of substance diffusing in the x direction in a time across an area A,

D is the diffusion coefficient, and

$\frac{dc}{dx}$ is the concentration gradient.

The diffusion coefficient of a particle in suspension is related to the frictional coefficient (f) of the particles by Einstein's law of diffusion:

$$Df = kT \tag{4.2}$$

For spherical particles the frictional coefficient is given by Stokes' law:

$$f = 6\Pi\eta a \tag{4.3}$$

where:

η is the viscosity of the continuous phase, and

a is the radius of the particle.

Combining equations (4.2) and (4.3), we have

$$D = \frac{kT}{6\Pi\eta a} = \frac{RT}{6\Pi\eta a N_A} \tag{4.4}$$

where:

R is the gas constant, and

N_A is Avogadro's constant.

Therefore, combining equations (4.1) and (4.4), we have

$$F_d = -A\frac{RT}{6\Pi\eta a N_A}\frac{dc}{dx} \tag{4.5}$$

In Brownian motion, accordingly, the flow of particles across an area A increases when the concentration gradient and the temperature increase and decreases when the viscosity of the medium and the size of the droplets increase.

Emulsion particles are also moving under the influence of gravity (creaming or sedimentation) with a flow rate (F_s) given by:

$$F_s = \frac{dm}{dt} = cA\frac{dx}{dt} \tag{4.6}$$

The velocity, $\frac{dx}{dt}$, of the sedimenting particles is obtained by equating the driving force on the particle and the resistance of the liquid: this is a good approximation when particles are moving slowly, as it is the case for emulsion droplets:

$$f\frac{dx}{dt} = m(1-\nu\rho)g \tag{4.7}$$

where:

m is the mass of the particle,

ν is the specific volume of the particle, and

ρ is the density of the continuous phase.

N.B.: the factor $(1-v\rho)$ allows for the buoyancy of the liquid.

For a spherical particle:

$$m = \frac{4}{3}\Pi a^3 \rho_2 \tag{4.8}$$

where:

ρ_2 is the density of the spherical particle $\left(\rho_2 = \frac{1}{v}\right)$

Combining equations (4.3), (4.7), and (4.8), we have

$$\frac{dx}{dt} = \frac{2}{9}\frac{a^2(\rho_2 - \rho)g}{\eta} \tag{4.9}$$

Combining equations (4.6) and (4.9), we have

$$F_s = \frac{2}{9}A\frac{a^2(\rho_2 - \rho)g}{\eta}c \tag{4.10}$$

This is valid if we assume that:

(1) the motion of the spherical particle is slow;

(2) the continuous phase extends an infinite distance from the particle (i.e., the emulsion is extremely dilute); and

(3) the liquid medium is continuous compared with the dimensions of the particle, which is the case for emulsions in which the droplets are much larger than the molecules constituting the continuous phase.

Thus, the flow of particles subject to a gravitational field across an area A increases with increasing particle size, difference in density between the particle and the continuous phase, and concentration of

dispersed phase; it decreases with increasing viscosity of the continuous phase.

If we consider only the diffusion and sedimentation processes for an extremely dilute ideal emulsion of slowly moving uncharged spherical particles with a monodispersed particle size distribution, the higher the diffusion rate and the lower the sedimentation rate, the more stable the system.

Comparison of equations (4.5) and (4.10) shows that the following factors favor stability:

(1) a high temperature, since it favors diffusion;

(2) a low difference in density between the dispersed particles and the continuous phase; and

(3) a small particle size, since it increases diffusion and decreases sedimentation.

4.4 Coalescence

Coalescence is a natural process in emulsion systems since the lowest energy state is reached upon total phase separation (minimum interfacial area). Despite extensive research the molecular mechanisms involved in membrane fusion remain largely obscure [5, 6]. Obviously, a necessary condition for fusion is the establishment of mutual contact between the film of the particles involved [5]. In order to avoid coalescence, therefore, one must avoid flocculation, a better-understood phenomenon.

4.5 Flocculation (aggregation) [7-22]

Frequent encounters between particles in emulsions occur due to Brownian motion, to gravity (creaming or sedimentation), and to convection. Whether such encounters result in permanent contact or whether the particles rebound and remain free is determined by the forces between them. We may distinguish three types of forces:

1. Van der Waals forces: these are always attractive between particles of the same nature.

2. Electrostatic forces: due to the interaction of the electrical double layer surrounding the particles, these always lead to repulsion between particles having surface charges and surface potentials of the same sign and magnitude. More precisely, this repulsion is due to the interaction of the ions adsorbed at the surfaces, tempered by the space charges in the double layers.

Whereas van der Waals forces fall off as an inverse power of the separation between the particles and have a range comparable to the particle size, the electrostatic repulsion falls off as an exponential function of the distance and has a range of the order of the thickness of the electrical double layer.

There is always some uncertainty about the absolute value of the electrostatic forces, since the value of the surface potential is always an estimate, usually based on the value of the zeta (electrokinetic) potential, which corresponds to the potential at the plane of shear radius; but this in turn depends on the technique used in the measurement (electrophoresis, electroosmosis, streaming potential, sedimentation potential).

3. Hydration (steric) forces: when two particles, each carrying an adsorbed layer of highly hydrated compounds, approach one another closely, two effects lead to repulsion. (1) The hydrated chain, if its extended length is larger than the distance between the surfaces, loses some conformation; its contribution to the free energy of the system is thus increased, resulting in a repulsive force (the "volume restriction effect"); and (2) when the chains belonging to two particles overlap, the concentration increases locally, with, again, an increase in the free energy (the "osmotic effect") [7].

From my investigations into PFOB emulsions, I believe that in order to avoid flocculation in these systems the emulsion must be designed to allow a combination of electrostatic and steric stabilization. The strong temperature dependence of sterically stabilized dispersions does not allow for stability in the wide range of temperatures (4°C-121°C) and pressures experienced by these emulsion systems. Conversely, electrostatically stabilized dispersions are insensitive to changes in temperature, but the presence of large concentrations of ions (with their screening effect on the surface potential) means that, in practice, PFOB emulsions cannot be stabilized by electrostatic forces alone. An optimum combination will consist of (a) a major emulsifier providing for stability against coalescence (zwitterionic phospholipids); and (b) one (or more) minor components. All minor components will have (i) an anchor group insoluble in saline and compatible with the phospholipid film structure *and* (ii) either a charged polar head group or a highly hydrated head chain well soluble in the solvent, both being large

enough to produce an effect but not so large as to disrupt the film packing (hemolysis). On the basis of my latest experimental results, a combination of cholesteryl hemisuccinate and ethoxylated cholesterol is a promising candidate for the optimal stabilizing additive for phospholipid-based emulsion systems.

Chapter Five
Materials and Methods

5.1 Materials

Phospholipon 90H (PL) was purchased from American Lecithin Company (Danbury, CT) and was used without further purification. PL is composed of 93 ± 3% PC with 85% of the acyl chains being stearic acid residues (18:0) and 15% of the acyl chains being palmitic acid residues (16:0), and of 4% (max.) lysoPC. Perflubron (PFOB) was generously supplied by Alliance Pharmaceutical Corp. (San Diego, CA) and was redistilled twice before use. Perfluorodecalin (FDC), also supplied by Alliance, was used as supplied. Captex 300, a medium chain triglyceride (MCT) was purchased from Karlshamns (Columbus, OH). Caprol 10G-2-O: decaglyceroldioleate (10-2-O) was graciously supplied by Karlshamns. EDTA was purchased from Harleco (Philadelphia, PA). Dipalmitoyl phosphatidylcholine (DPPC), stearylamine (99%) (SA), dicetyl phosphate (DCP), and cholesteryl hemisuccinate (tris salt) (CHS) were purchased from Sigma Chemical Company (St. Louis, MO). The sodium dodecyl sulfate (SDS) used in surface free energy experiments was the generous gift of Unilever Research USA, Inc. (Edgewater, NJ); unlike commercial samples, it did not exhibit a minimum in surface free energy (due to the presence of dodecyl alcohol) prior to the critical micellar concentration (CMC). Silicagel: Merck, grade 60, 60 Å.

5.2 Emulsion preparation

5.2.1 Sonication

Principle

Sonication is based on the interaction of sound with matter through the process of cavitation; this generates a very high energy on a molecular scale, energy that is quickly dissipated by heat. During sonication, ultrasound waves are generated that consist of cycles of compression and expansion, alternately pushing the molecules of the liquid together and pulling them apart. During the expansion cycle, a sound wave of sufficient intensity can generate cavities that will grow and contract during subsequent compression and expansion cycles. Once a cavity can no longer absorb energy from the sound wave it will implode, generating intense heat that will raise the temperature of the liquid in its immediate surroundings and thus create a "hot spot." When sonication is used to emulsify liquids, ultrasonic compression and expansion stress liquid surfaces, overcoming the cohesive forces that hold a large droplet together; the droplet bursts into smaller ones, and eventually the liquids are emulsified [1].

Equipment

Sonicator ultrasonic processor model W-385, Heat Systems, Ultrasonics Inc. (Farmingdale, NY) equipped with a standard tapered titanium microtip (catalogue # 419). Rosette cell enclosed in a temperature-controlled jacket (Fig. 5.1).

Procedure: Sloviter's method

PFC emulsions are prepared according to a procedure described in example 1 of Henry A. Sloviter's U.S. patent [2]. To 7 ml of freshly prepared cold Tyrode solution (see Table 5.1 for composition) (pH=7.4) in a 15 ml Rosette cell in ice is added 960 mg of EYP. The mixture is sonicated at 110 watts for 15 sec. Sonication is repeated once after an interval of 1 min. To this dispersed lecithin at 0°C is added 4 ml of perfluorocarbon oil (FDC and PFOB). The mixture is sonicated as before for eight 15-sec. periods with an interval of 1 min. after each sonication. The resultant milky white emulsion is centrifuged at 4°C for 60 min. at 100 g. The emulsion is then transferred into a 20 ml vial, which is sealed and sterilized by conventional hospital autoclave procedures at 121°C/140 kPa(20 psi) for 15 min.

Modified procedure

Fourteen ml of Tyrode and 1.92 g of EYP are placed into a 100 ml beaker with a magnetic stirrer. The system is stirred 15 to 30 min. until all the EYP is visually dispersed. Then, 8 ml of PFC is added while stirring for 10 more minutes, and 12 ml of the resulting mixture is transferred into a Rosette cell for sonication using either Sloviter's procedure or another method. This enables us to assess the effect of mechanical work on emulsification and emulsion stability. As expected, a minimum amount of mechanical work is required in order to produce an emulsion. The optimum sonication parameters seem to be 5 times 2 min./10 sec. intervals/100-120 watts using a Rosette cell in cold water. The emulsion is centrifuged and sterilized as before.

Observations

1. It is difficult to standardize the mechanical work because the energy imparted to the system depends on how deeply the tip of the probe is plunged into the liquid and how long it stays there. During the emulsification process, then, the probe should be lowered to several different positions in the liquid. The use of a Rosette cell helps create turbulence, ensuring that all the liquid receives a similar amount of mechanical work (no stationary phase).

2. Since the metal probe erodes during sonication, a centrifugation step is necessary in order to remove the fragments of titanium.

3. Sonication can be used for processing small volumes (15-20 ml) only; the process does not scale up well.

5.2.2 Microfluidization

Principle

During processing, the feed stream is pumped into a specially designed interaction chamber in which fluid sheets interact at very high velocities and pressures, up to 450 m/s and 117 MPa (17,000 psi), respectively. The fixed microchannels within the chamber provide an extremely focused interaction zone of intense turbulence in which the liquid is submitted to a combination of high shear forces, impact, and cavitation, and the emulsion droplets are reduced into smaller ones. Since the interaction chamber has a small volume, the entire stream is subjected to a constant high mechanical work (uniform energy input), with the yield a population of particles having small size and low polydispersity (uniform product). Particle

size may be adjusted by controlling the interaction pressure. Modifications to the original design enable us to work under a nitrogen atmosphere and to recycle the liquid, thus obtaining even greater homogeneity.

Equipment

Ultra-Turrax T25 (UT) (IKA Works, Inc., Cincinnati, OH) fitted with a dispersing tool S25 KG-25 F. The UT is a high-speed dispersing and emulsifying apparatus for processing flowable liquid media in batch operation. The medium to be processed is sucked in from below by the rotor, emerging at the sides through the stator slots. The stator to a large extent prevents the rotation of the medium, and it allows the introduction of large mechanical energies in a very small space. The vortex formation commonly found with stirring is greatly reduced. In the shearing gap between rotor and stator about 1,000 times more energy is introduced into the medium than in stirring. A special attachment enabled processing in the absence of oxygen (under nitrogen).

Microfluidizer (MF) M110-T (Microfluidics Corp., Newton, MA), plus attachments custom made for the experiment (Fig. 5.2 & 5.3).

Procedure

The emulsifier is first dispersed in the aqueous phase by magnetic stirring at 65-70°C until a milky dispersion is obtained. Then the mixture is submitted to mechanical work (3 min./13,500 rpm/65°C) in a UT. The oil is added and the system is mixed (5 min./13,500 rpm/65°C) until it yields a homogeneous preemulsion; this preemulsion is fed into a MF (69 MPa(10,000 psi)/5-7 passes) and the resulting emulsion, contained in glass vials (15 and 50 ml) with

rubber stoppers and crimped aluminum seals, is sterilized in a static autoclave (AMSCO) 121°C/140 kPa(20 psi)/15 min. The total volume of each emulsion sample is 300 ml.

Observations

1. Since unsaturated phospholipids are very sensitive to oxidation, the emulsification process was carried out under a nitrogen atmosphere.

2. Since phospholipids should be in their liquid crystalline phase (above their PTT) to ensure their dispersibility in aqueous solution, the emulsion preparation was performed at a temperature greater than the phospholipid PTT. EYP, with both saturated and unsaturated fatty acid chains, and a PTT around -10°C (variable depending on EYP composition), is dispersible at room temperature; emulsions made with EYP were prepared at 15-20°C. PL, a hydrogenated phospholipid containing only saturated acyl chains, has been found (by differential scanning calorimetry (DSC) measurement, Fig. 5.4) to have a PTT of 52°C, so the emulsion preparation was performed at 60-65°C.

3. Since the interaction chamber of the MF has a small volume, the emulsion components should be preemulsified to yield a homogeneous (coarse) emulsion. This is achieved by homogenization in the UT prior to microfluidization.

4. Experiments showed that emulsions with a constant composition prepared with the MF at 10,000 psi/10-14 passes or 5,000 psi/5-7 passes had the same particle size. Thus, for a given emulsion composition, the MF will give similar-sized particles provided sufficient mechanical work is applied to the system; the

actual size seems to be determined by the geometry of the interaction chamber.

5. Sterilization is a crucial step in the process and must not be overlooked. Leaving the emulsion at high temperatures (above 50-60°C) for extended periods of time appears likely to result in emulsion instability (coalescence), even in phase separation. The emulsion should accordingly be sterilized, and subsequently cooled down, as fast as possible. A cold bath is recommended. The cooling rate is limited by the vials' resistance to breakage upon sudden temperature variations. The sterilization procedure should be precisely designed to yield sterile emulsions; the Parenteral Drug Association (PDA) suggests an overkill minimum lethality factor of F_0=12 [3], corresponding to an exposure of 12 min. at 121°C in the center of the vial. A sterilization time of 15 min. seems to be the minimum acceptable for the vials used (15-50 ml).

5.3 Sample preparation for transmission electron microscopy (TEM)

After rapid freezing in liquid propane, each sample was fractured and coated (Pt replica at 45° angle) for TEM.

5.4 Sedimentation field flow fractionation (SFFF)

In SFFF, emulsion particles are separated according to their size and density by a Colloid/Particle Fractionator, model S101 SedFFF (FFFractionation Inc., Salt Lake City, UT), attached to a Spectra-Physics Isochrom LC Pump (Spectra-Physics, San Jose, CA). A Linear UVis 200 Spectrophotometer (Linear Instrument Corp., Reno, NV),

was used to generate these data. In this technique, a stream of carrier liquid is introduced into a ribbonlike channel of 0.4 to 1 meter in length and 0.02 cm in diameter, fitted inside a centrifuge basket. The largest particles in the sample cloud, since they are driven by the strongest sedimentation forces, are compressed closest to the wall of the channel. The sample particles are then displaced downstream by the carrier liquid flow. But since the flow between the walls is parabolic in form, fast in the center and slow near the walls, particles relatively far from the wall are carried downstream more rapidly than those closer to the wall. The separated sample particles, entrained by the carrier liquid, are eventually eluted from the instrument for detection [4]. Caldwell et al. [5] reported the use of the combined SFFF-photon correlation spectroscopy (PCS) methods in emulsion characterization.

5.5 Particle size measurements

Photon correlation spectroscopy (PCS) -- also known as quasi elastic light scattering (QELS) -- was used to evaluate the size of the emulsion particles.

Principle

Colloids are in constant thermal (Brownian) motion. This motion causes the intensity of light scattered from the particles to vary with time. Since large particles move more slowly than small particles, the rate of fluctuation of the light scattered from them is also slower. PCS uses the rate of change of these light fluctuations to determine the size distribution of the light-scattering particles. The particle size

was measured for samples prior to and after sterilization. To avoid multiple scattering, emulsions should be diluted, and I diluted each sample in two ways: in a solution with the same composition as the continuous phase, and in distilled water. If a sample yields a much smaller particle size value when diluted in water than when diluted in the continuous phase, the larger size is the size of an aggregate of particles that breaks down into single droplets when diluted in water. If dilutions in the two solvents lead to the same observed particle size, no flocculation has occurred and the "real" size of an individual droplet is measured in both solvents. An increase in measured "real" particle size after sterilization reflects coalescence.

Procedure

Measurements were carried out on a ZetaSizer 3 (ZS3) Particle Electrophoresis and Multi Angle Particle Size Analyzer (Malvern) equipped with a 5mW He-Ne laser of 633nm wavelength. The ZS3 can measure the size of particles with diameters in the 5 - 5,000 nm range. All the emulsion samples were diluted 500- to 2000-fold prior to being measured. Measurements were carried out in the AZ4 cell (4 mm diameter) at 25°C at a fixed angle of 90°.

5.6 Electrokinetic (Zeta) potential measurements

Principle

Microelectrophoresis is the measurement of the movement of colloidal particles when they are placed in an electric field. The measurement can be used to determine the sign of charges on the particles and also their electrophoretic mobility, which is related to

the surface charge and zeta potential. The ZS3 measures the velocities of particles during electrophoresis experiments. As the velocity must be measured in the stationary layer, a small volume is defined by the crossing point of two laser beams. Where the two beams cross they produce interference fringes. Particles inside the scattering volume interact with these fringes to produce scattered light, which oscillates over time in a way depending on the speed of the particles. The light scattered by the particles is collected by the photomultiplier.

Procedure

The zeta potential of emulsion particles was measured by microelectrophoresis using the ZS3 operating in cross-beam mode at 25°C with samples diluted 500- to 2000-fold and contained in the AZ4 cell.

5.7 Surface and interfacial free energy measurements

Wilhemy blade method

Surface free energy was measured using a sandblasted platinum blade suspended to a microforce transducer-amplifier (model 311A, Sanborn Co., Waltham, MA). The amplified signal was sent to a recorder (model SRG, Sargent Welch, Springfield, NJ). A sandblasted Teflon blade (wetted by PFCs) was used to determine interfacial free energies. All glassware was cleaned with potassium dichromate/sulfuric acid solution. Distilled water was used in all preparations.

Drop volume method

For purposes of calibration, the surface free energy of aqueous solutions of increasing concentration of SDS and the interfacial free energy between PFOB and SDS solutions were measured using a sandblasted platinum blade and a sandblasted Teflon blade respectively, suspended from a tensiometer.

Next, a microsyringe was used to form a drop of PFOB near the interface between pure PFOB and SDS solutions (Fig. 5.5). The volume of the drop (average of 10 measurements of 10 drops) and its lifetime (i.e., the time before it coalesced with the bulk of PFOB) were recorded. The interfacial free energy was calculated from the volume of the PFOB drop and the calibration curve obtained with the Teflon blade method (Fig. 5.6).

5.8 Compression isotherms [6]

The experimental apparatus used for measuring surface pressures (Π) and potentials (ΔV) is shown in Figure 5.7. All the experiments were conducted within a Faraday box. The surfactant solutions in n-hexane-ethanol (95% - 5% respectively) were deposited onto the aqueous surface with an "Alga" micrometer syringe (Burroughs Wellcome and Co., London). The substrate and the film were retained in a fused silica trough and Teflon tape (10.7 x 2.5 x 31 cm) of 1.3 liter capacity. The surface was cleaned by dusting with calcinated talcum powder, which was removed with the aid of a glass tip connected to an aspirator.

Surface pressures (Π) were determined from surface free energy measurements, which were made by suspending a sandblasted platinum blade from a transducer-amplifier (Model 311A, the Sanborn Co., Waltham, MA). The transducer output was recorded continuously on a recorder (model SRG, Sargent Welch, Springfield, NJ). The surface free energies were reproducible within $\pm$ 0.2 dyne.

Surface potentials (ΔV) were measured with an air-ionizing electrode (a Radium-226 source, U.S. Radium Corp., Morristown, NJ) placed 1 to 2 mm above the surface of the liquid substrate and connected to a precision potentiometer, a high-input resistance electrometer (Model 610A, Keithley Instruments, Inc., Cleveland, OH), and a trough electrode (Ag/AgCl) dipped into the bulk of the aqueous substrate. The radioactive electrode was connected to the input terminal of the electrometer with Amphenol low-noise graphitized shielded cable and connectors. The entire circuit was grounded. The e.m.f. of the cell composed of the radioactive electrode, trough electrode, potentiometer, and electrometer, all connected in series, was measured immediately after cleaning the surface of the aqueous substrate (Vo), and compared with the e.m.f. obtained after spreading a film on the surface (V). The difference between the two e.m.f.'s (V - Vo) is the surface potential (ΔV). The potentiometer opposed an appropriate fraction of the cell e.m.f., and the electrometer output was recorded continuously. Sensitivity of the surface potential measurements was about $\pm$ 1 mV, and data reproducibility was within about $\pm$ 5 mV.

An automatic barrier drive with variable speed control permitted the determination of an optimum compression rate and reproducible

Π vs. σ (σ is the surface area per surfactant molecule) and ΔV vs. σ isotherms. A compression rate of 0.15 to 0.3 percent decrease in area per second was found to be optimum for this study.

The surfactant solutions were prepared by dissolving about 12.5 mg of the surfactant per 25 ml of hexane 99+%, with 5% of ethanol absolute 200 proof added when necessary.

All glassware was cleaned with freshly prepared sulfuric acid/potassium dichromate (sulfochromic) mixture and rinsed thoroughly with distilled water. The substrates were cleaned by foaming with nitrogen (the foam was removed several times by sweeping the surface). The substrates gave adequately stable baseline readings for both the surface pressure and the surface potential measurements [4].

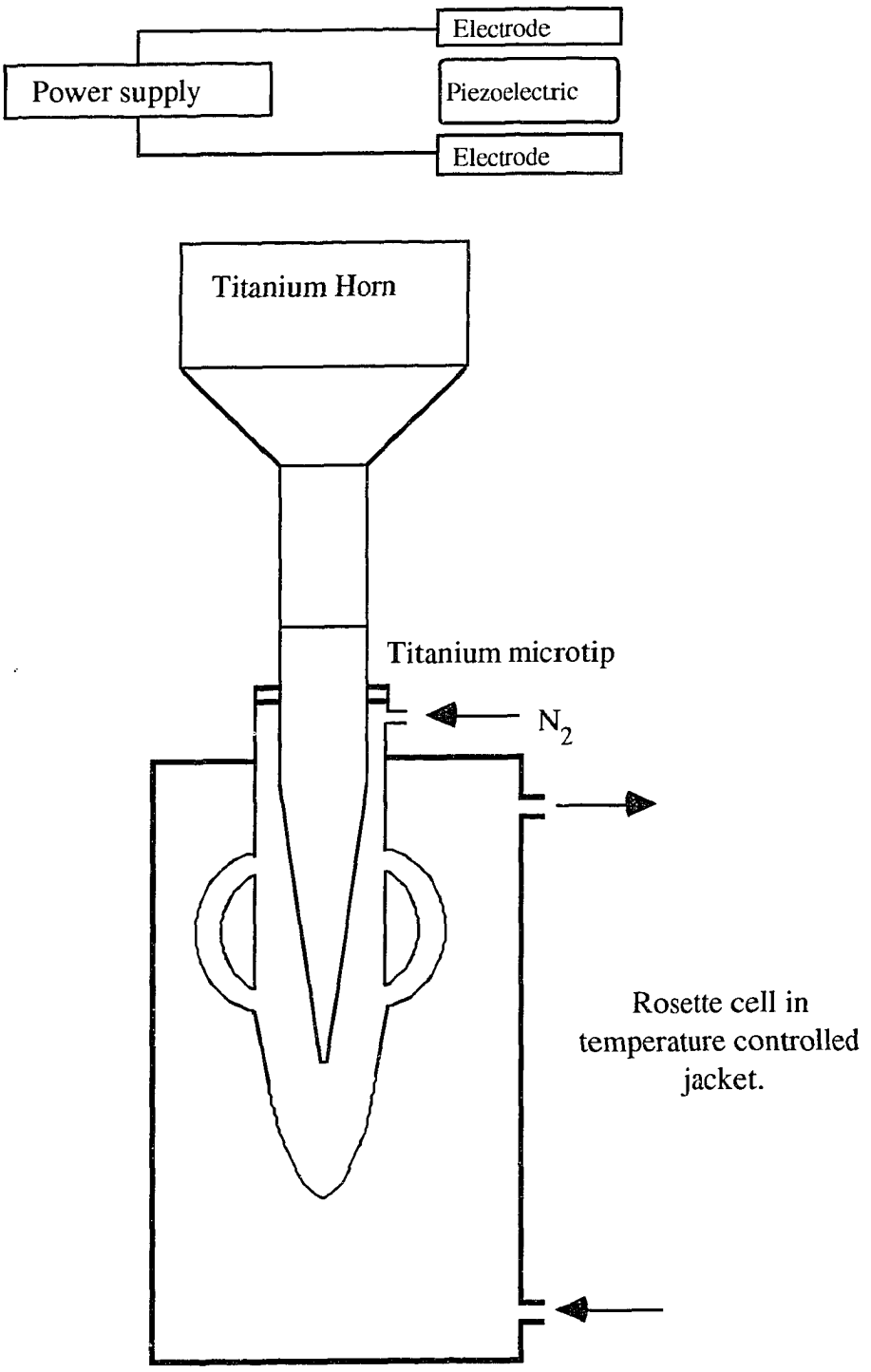


Figure 5.1: Apparatus for sonication

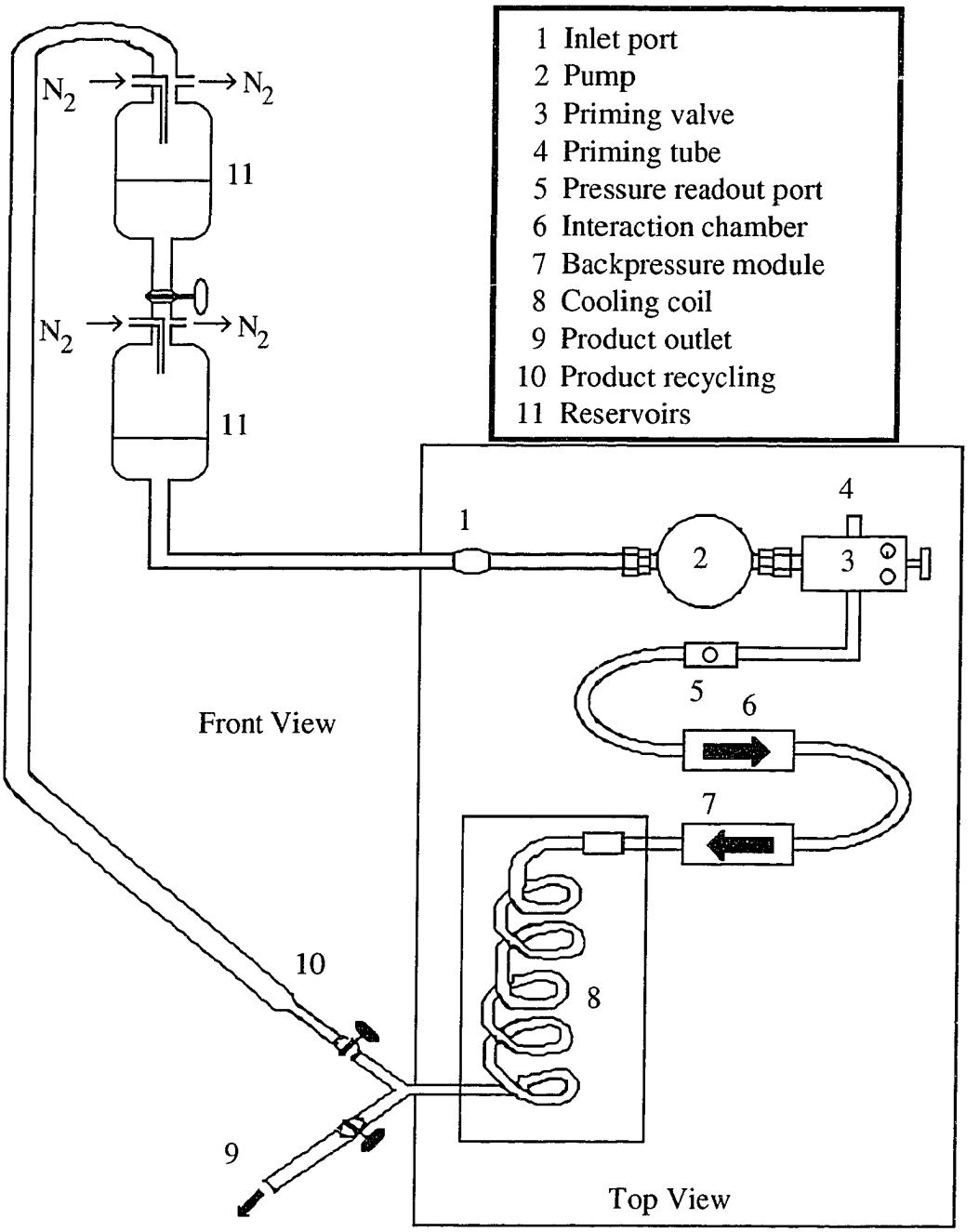


Figure 5.2: Microfluidizer M-110T and attachments

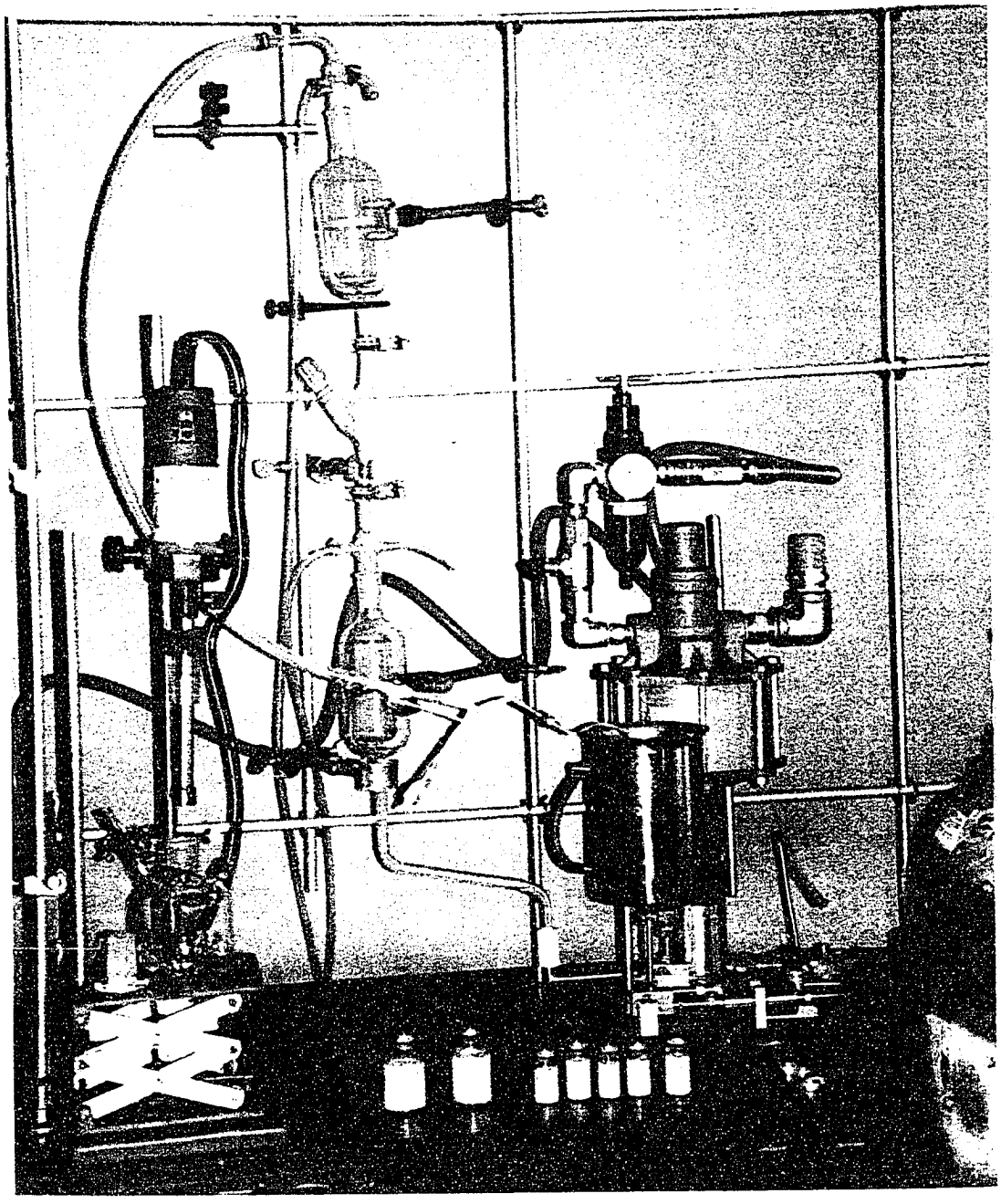


Figure 5.3: Ultra-Turrax and microfluidizer with attachments

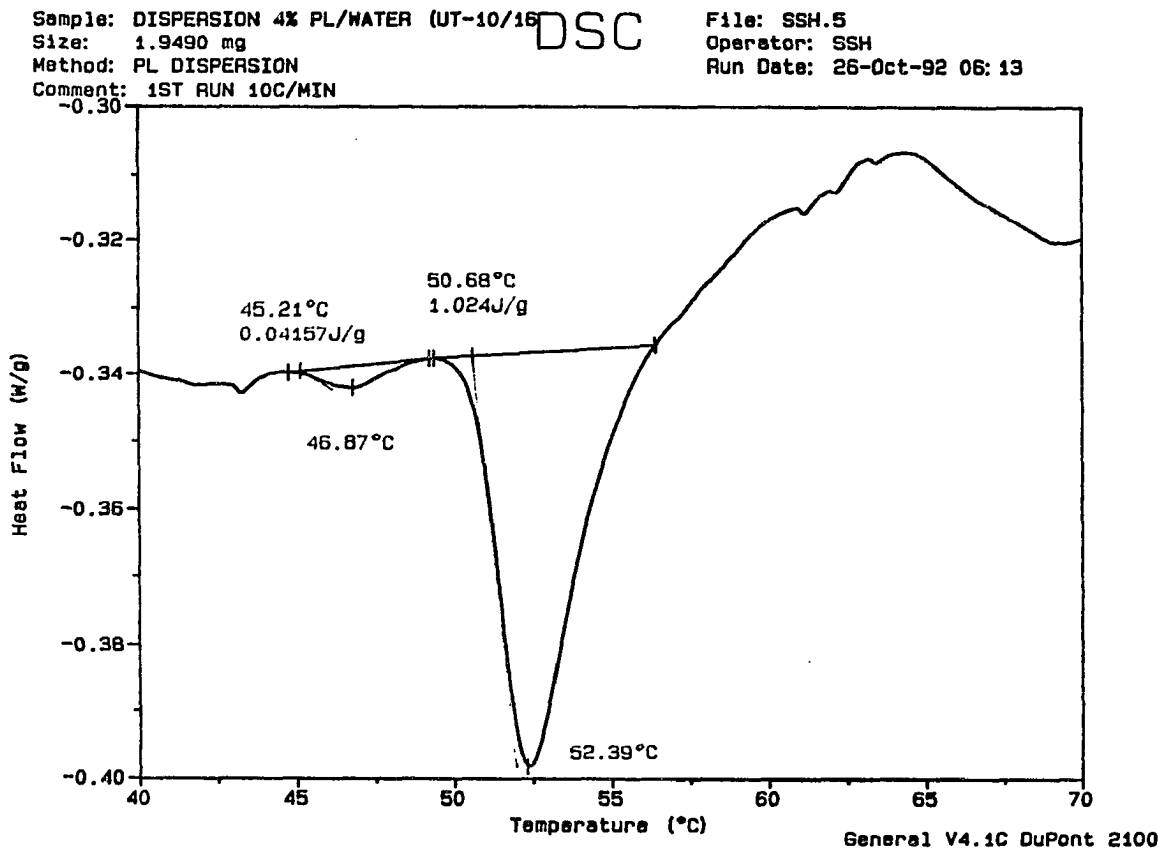


Figure 5.4: DSC curve of a 4% dispersion of PL in water prepared using the UT (13,500 rpm/10 min./65°C)

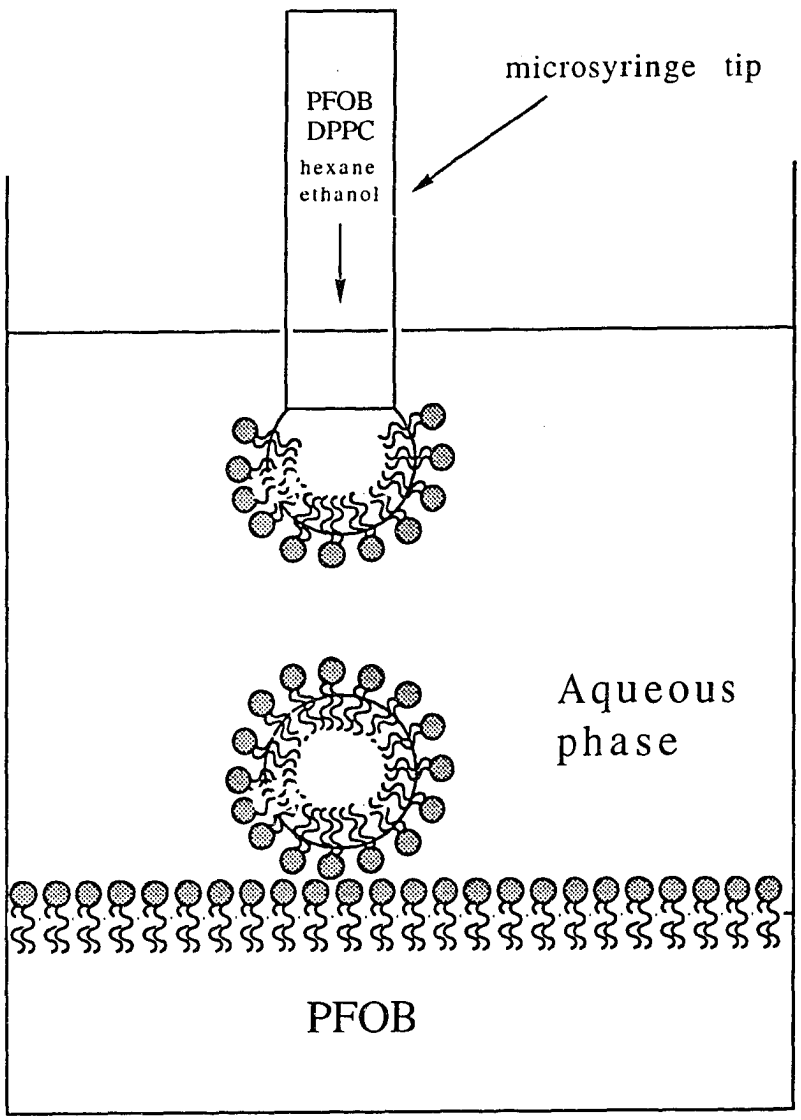


Figure 5.5: Apparatus for interfacial free energy measurement

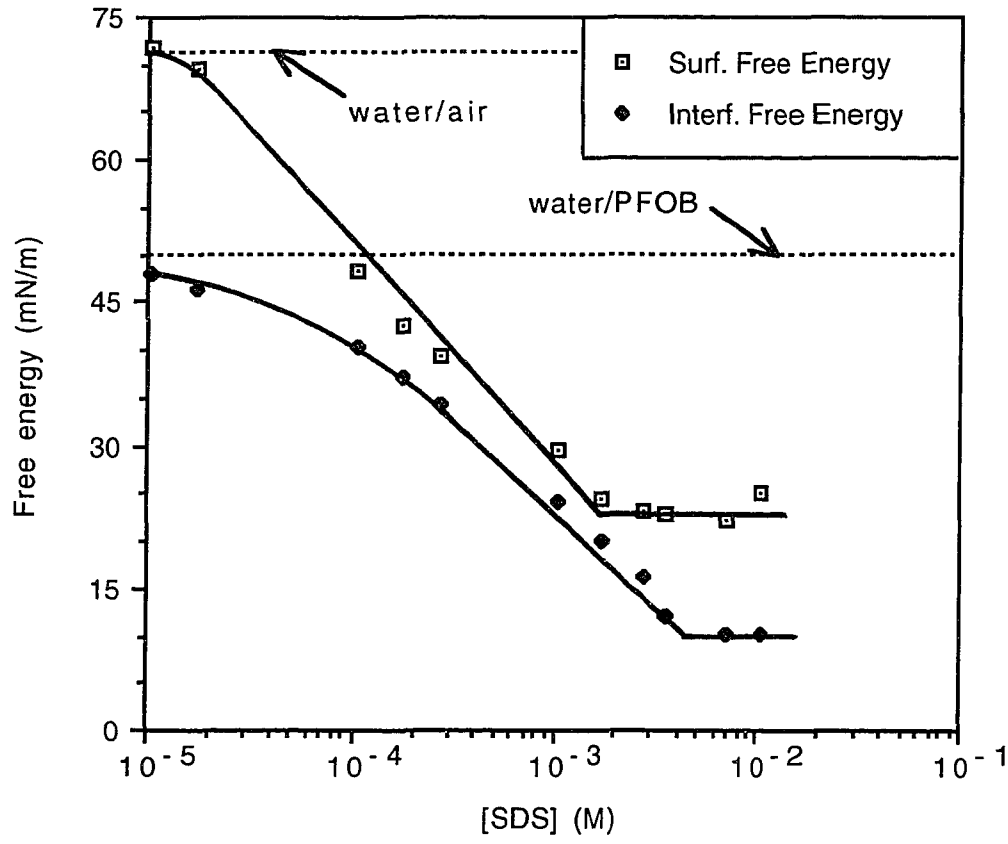


Figure 5.6: Surface free energy of SDS solutions and PFOB/SDS solution interfacial free energy versus SDS concentration at 24°C

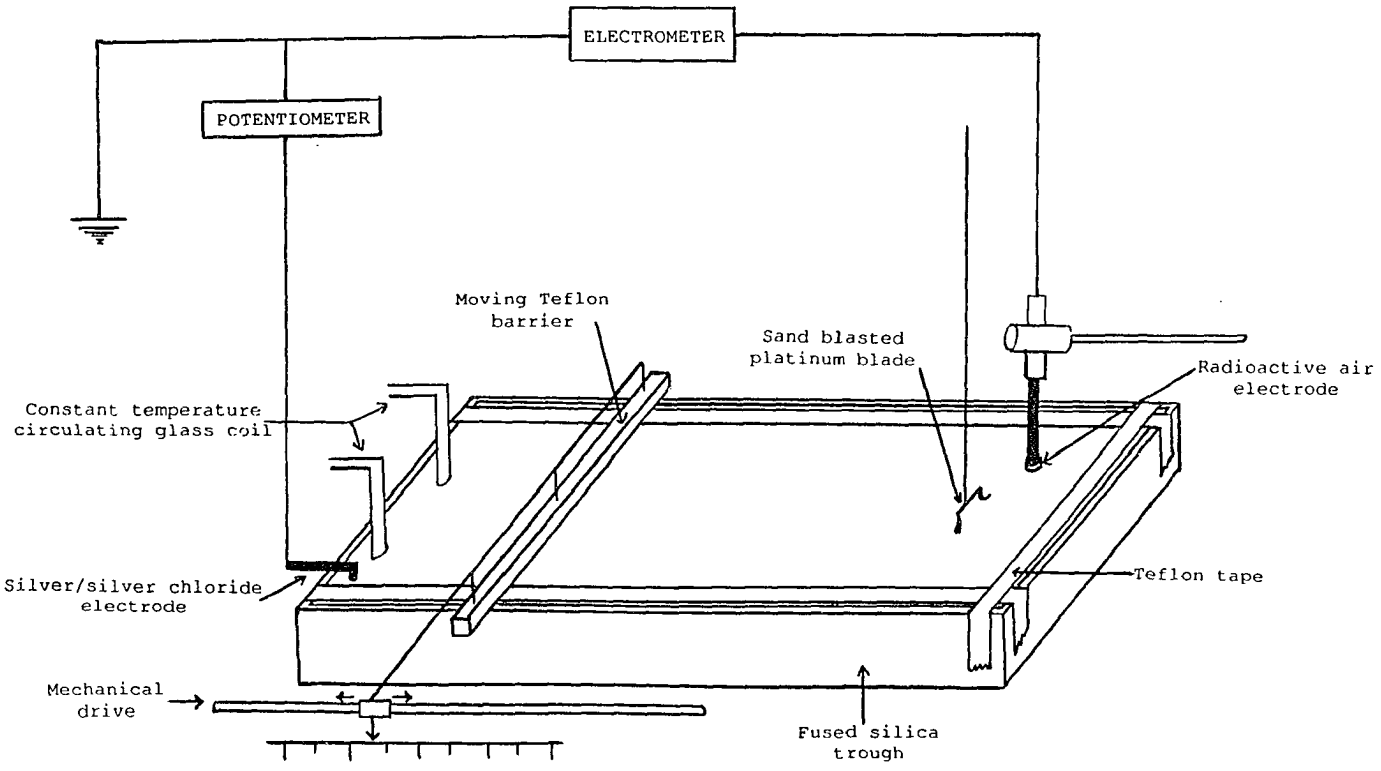


Figure 5.7: Apparatus for measuring surface pressure and surface potential (within a Faraday cage)

Table 5.1: Tyrode solution composition

	Concentration (g/L)
NaCl	7.5
KCl	0.15
$CaCl_2$	0.2
$MgCl_2$	0.07
NaH_2PO_4	0.4
$NaHPO_3$	0.2
Glucose	0.2

Chapter Six
Results and Discussion

6.1 Preparation of PFC/saline emulsions using EYP as an emulsifier

As stated in Chapter Five, Materials and Methods, I began my current research program in 1989 by attempting to prepare stable PFC/saline emulsions using a method patented by Henry A. Sloviter [1]. Several lots of commercial EYP were tested with either FDC or PFOB, but no system produced a visibly stable emulsion, and it was evident that Sloviter's method did not allow complete dispersion of EYP in Tyrode solution. Although a modification of the procedure yielded an improved EYP dispersion in Tyrode solution, all systems showed some phase separation after 24 hours' storage. I drew several important conclusions that largely shaped the direction of my subsequent investigations:

(1) The preparation of PFC emulsions requires a large amount of mechanical work, certainly larger than suggested by Sloviter in his patent.

(2) Sonication might not be the emulsification method of choice, given that (a) it is difficult to standardize the mechanical work, (b) the metal probe erodes during sonication (fragments of titanium must be removed), (c) only small volumes can be processed, and (d) the process does not lend itself well to large-scale processing.

(3) EYP is very sensitive to oxidation and the experiment must be carried out under an inert atmosphere (nitrogen or helium).

(4) Since different lots of EYP processed under identical experimental conditions yielded emulsions with different degrees of stability, the nature of the EYP used in the formulation must play a major role in emulsion stability.

In order to investigate the role played by the components of EYP on emulsion stability, I decided to prepare my own EYP and study its behavior in emulsions.

During the preparation of EYP, some triglycerides, free fatty acids (FFA), cholesterol, and other materials remain entrapped within the phospholipid crystals. When the EYP is separated from the total lipid fraction by precipitation in cold acetone, the crystals have a yellow color due primarily to the presence of xanthophylls. The EYP was purified before use; in practice, the best results in terms of emulsion stability were obtained with the most highly purified EYP, which was also visually the whitest. The ability of EYP to form stable emulsions, then, seems to be affected dramatically by the presence or absence of certain compounds in the EYP mixture. After the method of extraction and purification of EYP that gave the most stable emulsions was identified, the emulsion formulation was optimized. Several emulsions were prepared using different PFOB/saline ratio in order to find the optimum concentration of the dispersed phase (PFOB) in the emulsion: 85-100% w/v (44-52% v/v). The optimum EYP/PFOB ratio was found to be 9% (w/v) corresponding to EYP concentrations of 4% and 4.5% (w/v) for 90% and 100% (w/v) PFOB emulsions respectively. For a given amount of the dispersed phase, the more surfactant in the emulsion, the smaller the particle size, until a limiting lower value is reached that corresponds to the

maximum curvature of the film; this value depends on the structure and spatial configuration of the surfactant molecules at the interface. Ishii et al. [2] found a similar optimum EYP/oil ratio in their preparation of fat emulsions for parenteral nutrition. When more surfactant is used in the formulation (EYP/PFOB ratio is larger than 9% w/v) the excess EYP will be present as PFOB-free phospholipid vesicles. This excess surfactant has been deemed detrimental to the stability of the emulsion by other researchers [3]. I found no evidence that the excess EYP was either detrimental or beneficial to emulsion stability, but lower amounts of emulsifier are certainly desirable a priori from both biomedical and cost-efficiency standpoints [3]. And specific evidence that the excess phospholipid (as vesicles) has detrimental effects on cholesterol metabolism in the case of fat emulsions used in parenteral nutrition has been presented by Hajri et al. and Untracht [4, 5].

Questions of EYP apart, the Tyrode solution specified in Sloviter's patent seemed unlikely to produce really stable emulsions. The polyvalent ions (Ca^{2+}, Mg^{2+}) it contains are known to adsorb to the negatively charged emulsion droplet; in so doing they reduce its surface charge, thus reducing the repulsive (electrostatic) force between the droplets until it equals the attractive van der Waals force [6]. This point is called the critical flocculation concentration (CFC): the higher the charge of the cation, the lower the CFC. So polyvalent ions must be avoided as detrimental to emulsion stability. Moreover, Tyrode itself is unstable, and a freshly prepared batch is essential in order to avoid pH change and precipitate formation (calcium carbonate). As an alternate to Tyrode I first used a saline

solution (isotonic to blood), containing NaCl as a sole salt, THAM (Tris) to buffer the solution at pH 7.4 (similar to that of blood), and EDTA to form a complex with polyvalent ions. During the emulsification and the sterilization processes the emulsion is subjected to great temperature variations, however, and the dissociation of the Tris buffer between acidic and basic forms varies noticeably with temperature; hence the pH of the emulsion will vary during processing. In order to avoid such pH fluctuations I replaced THAM with a phosphate buffer less sensitive to temperature variations (Table 6.1). The saline solution is composed of a salt (NaCl), a buffer (NaH_2PO_4, Na_2HPO_4), and 1.5 mM EDTA added to complex polyvalent ions. The pH and osmolality are 7.4 and 291 mOsm/kg water respectively, identical to the plasma values. The second solution at pH = 6.8 corresponds to a pH where the phospholipid hydrolysis is minimized.

Finally, I used high-pressure homogenization (microfluidization) instead of sonication in the emulsification process because it permits the preparation of stable PFOB/EYP/saline emulsions with a low polydispersity in particle size. The advantage of microfluidization over sonication was recently confirmed by another research group [3].

6.2 PFOB/EYP/saline emulsions characterization

A typical 100% (w/v) PFOB/saline emulsion prepared by microfluidization using 4.5% (w/v) EYP as an emulsifier was found to be stable over four years at 5°C [7]. The emulsion exhibits a minimal

settling, readily reversible with light shaking (by hand). This settling at the bottom of the vial is simply the result of the sedimentation of the emulsion droplets due to the high density of PFOB (d_{20} = 1.92 g/cm^3).

An electron micrograph of this system (Fig. 6.1) reveals a polydispersed collection of semi-spheres averaging 250 nm diameter, as confirmed by PCS measurements (245 ± 33 nm).

A SFFF fractogram of the same system shows particles separated according to their size and density (Fig. 6.2). It exhibits two peaks. The smaller peak, which corresponds to one third of the total EYP fraction by mass [8], represents PFOB-free phospholipid vesicles of density similar to that of saline. The larger peak (two thirds of the total EYP fraction by mass [8]) represents PFOB droplets with a density about twice that of saline.

The small particles (80 nm) visible on the electron micrograph are probably PFOB-free vesicles.

We may calculate the theoretical amount of EYP required for the monomolecular interfacial coverage of 100 ml of a 100% w/v (52% v/v) PFOB/saline emulsion, assuming that only 67% by mass of the total EYP is adsorbed at the interface, the rest being present as PFOB-free phospholipid vesicles.

The total surface area (A) of the emulsion droplets is given by:

$$A = 4\Pi a^2 \times N \qquad (6.1)$$

where:

a is the radius (Å), and

N is the number of emulsion droplets.

A is also given by:

$$A = n\,\sigma \tag{6.2}$$

where:

n is the total number of EYP molecules at the PFOB/saline interface, and

σ is the surface area per EYP molecules (Å^2/molecule).

Combining equations (6.1) and (6.2), we have

$$N = \frac{n\,\sigma}{4\Pi a^2} \tag{6.3}$$

The number of emulsion droplets (N) is also given by the ratio of the total volume of dispersed phase (V) and the volume of an individual emulsion droplet:

$$N = \frac{3V}{4\Pi a^3} \tag{6.4}$$

Combining equations (6.3) and (6.4), we have

$$n = \frac{3V}{a\,\sigma} \tag{6.5}$$

Given that:

$V = 52 \times 10^{24}$ Å^2

a =1250 Å as determined by PCS

σ=60 Å^2/molecule [9]

Then:

$n = 2.1 \times 10^{21}$ molecules

The mass (m) corresponding to n molecules of EYP is given by:

$$m = \frac{n\,M}{N_A} \tag{6.6}$$

where:

M is the molecular weight of EYP, and

N_A is Avogadro's constant.

Given that: M=780 g/mol

$m = \underline{2.7}$ g

Since only 67% by mass of the total EYP is adsorbed at the PFOB/saline interface, the total (theoretical) amount of EYP (m_T) necessary to prepare such an emulsion will be:

$m_T = \underline{4.0}$ g

My system contains 4.5 g EYP, and there is thus a monolayer of EYP adsorbed at the PFOB/saline interface.

The above calculations suggest that the optimum EYP/PFOB ratio would be around 5% (w/v), corresponding to an EYP concentration of 2.6% (w/v) in the emulsion, where all the EYP molecules would be adsorbed at the o/w interface. In practice, however, for such a ratio the particles have a much higher radius than expected, due to the presence of PFOB-free vesicles. This discrepancy is due to the higher affinity of phospholipid molecules for other phospholipid molecules than for the oil phase (see below, section 6.3), which results in a low bilayer-to-monolayer conversion (BMC) yield during the emulsification process. In this process, the phospholipids are first dispersed as vesicles with bilayered membranes in saline solution; then the oil is added and a large amount of mechanical work is applied in order to break the vesicles and bring the phospholipid molecules to the o/w interface. The BMC is not total, however, and a sizable amount of phospholipids remains in solution as vesicles or liposomes. In order to increase the efficiency of the BMC, a small amount of mechanical work should be provided during the dispersion of the phospholipids in the aqueous phase; it is well

established that the higher the mechanical work, the smaller and the less lamellar the vesicles, and that small unilamellar vesicles (SUV) are much more difficult to break than large multilamellar vesicles.

The dispersion is thus composed of (a) PFOB droplets stabilized by a phospholipid monolayer adsorbed at the interface and (b) small PFOB-free phospholipid vesicles.

6.3 Properties of the phospholipid monolayer at the PFOB/saline interface

The surface free energy (γ) of PFOB, FDC, saline, and several alkanes and various interfacial free energies (γ_i) were determined using the Wilhemy blade method. The work of cohesion (W_c) and the work of adhesion (W_a) were calculated using the following equations:

$$W_c = 2\gamma \tag{6.7}$$

$$W_a = \gamma_{o/air} + \gamma_{aq/air} - \gamma_{o/aq} \tag{6.8}$$

The results are given in Table 6.2. PFCs have low surface free energies (18.1 and 19.7 mN/m for PFOB and FDC, respectively) due to a low work of cohesion (36.2 and 39.4 mN/m for PFOB and FDC, respectively), which in turn results in a high capacity for dissolving gases such as oxygen (50% (v/v) and 40.3% (v/v) per atmosphere at 37°C for PFOB and FDC, respectively [10]).

In contrast, the PFC/saline interfacial free energy is high (50 and 56.2 mN/m for PFOB and FDC, respectively), making it difficult to disperse PFCs in the aqueous phase.

We may use n-hexadecane as a model for the phospholipid acyl chains. The work of cohesion of water (144 mN/m) is high compared

to the work of cohesion of n-hexadecane (56 mN/m), which explains the rejection of phospholipids from the structure of water, resulting in their vesicle-formation ability. The work of adhesion PFC/n-hexadecane (43.5 and 41.8 mN/m for PFOB and FDC, respectively) is slightly higher than the work of adhesion PFC/saline (40.1 and 35.5 for PFOB and FDC, respectively), which explains the higher affinity of PFCs for hydrocarbon chains than for saline solution. In fact, the interfacial free energy between PFCs and hydrocarbons is very low (2.5 and 5.8 mN/m for PFOB and FDC, respectively), and PFOB is actually soluble in n-octane at room temperature. But the work of cohesion of n-hexadecane (56 mN/m) is larger than the work of adhesion PFC/n-hexadecane (43.5 and 41.8 mN/m for PFOB and FDC, respectively), which explains why so much mechanical work is needed to make PFC/saline emulsions using phospholipids as emulsifiers. A high energy is needed to break the vesicles, that is to take the bilayers apart so that the phospholipid acyl chains can adsorb, quasi-irreversibly, at the PFOB/saline interface.

Phospholipids are basically insoluble in both PFOB and saline, but I predicted that, if brought to the interface, they would strongly adsorb, reducing the interfacial free energy and providing the structured film necessary for emulsion stability.

To verify this hypothesis, the PFOB/saline interfacial free energy was measured in the presence of an interfacial film of dipalmitoyl phosphatidylcholine (DPPC, a pure phospholipid) using the drop volume method. A drop of a combination of PFOB, DPPC, hexane, and ethanol was formed near the PFOB/saline interface. The volume of the drop and its lifetime at the interface (i.e., the time before it

coalesced with the bulk of PFOB) were recorded. The interfacial free energy drops from 50 mN/m between pure PFOB and saline down to about 1 mN/m in the presence of an interfacial film of DPPC (Table 6.3). Furthermore, while the "naked" droplet coalesces right away with the bulk of PFOB, it remains intact overnight at the interface when encapsulated in a phospholipid monolayer, showing the existence of a structured interfacial film. In contrast, while the water-soluble surfactant SDS reduces the PFOB/saline interfacial free energy to about 10 mN/m (Fig. 5.6), thus facilitating the emulsification, it does not form a structured film strongly adsorbed at the interface. PFOB/saline emulsions prepared with SDS are unstable, starting to break (phase separation) as soon as they are made.

Further evidence for the existence of a structured interfacial film of phospholipid at the PFOB/saline interface was provided by compression isotherms of monolayers of EYP, PL, and DPPC (Figs. 6.3 a, b, and c, respectively). They show that, in all cases, the film has a high collapse pressure (45 to 65 mN/m) and a high surface potential (+400 to +550 mV for a molecular surface area of 50 $Å^2$) and that the compression/decompression curves have no, or very little, hysteresis.

A high value of the collapse pressure proves that the phospholipid film is highly structured and regularly packed. Too many irregularities in the film structure would result in a weaker film unable to withstand high pressure (i.e., a film with low collapse pressure). A film of pure DPPC or PL (phospholipids with saturated lipid chains) has a collapse pressure of 65 mN/m, evidence of a well-

packed film. A lower value (45 mN/m) was found for EYP; the lesser degree of organization of the EYP film is due, first, to a kink in the unsaturated lipid chains resulting from double bonds in cis position and, second, to the presence of impurities (minor components of EYP such as cholesterol or FFA) capable of disturbing the packing.

A high value of the surface potential is further evidence of a highly organized interfacial film. The surface potential represents the potential across the interface and is directly proportional to the vector sum of the dipole moment of each phospholipid molecule under the surface of the air-ionizing electrode (about 10^{14} phospholipid molecules). A well-organized film results in aligned dipole moments, which in turn yield a maximum value for the surface potential.

The compression/decompression curves show that the system does not lose its integrity while compressed. Hence, phospholipids yield emulsion droplets with a structured film that can take stress and return to its equilibrium state with no physical change [11].

Traditionally, in studies of emulsion formation surfactants soluble in water or in oil have been the center of attention while surfactants soluble in neither were ignored. But low surfactant solubility in both the continuous and the dispersed phases will insure a very high surfactant adsorption at the interface and a very low interfacial free energy, yielding a stable system. This can be achieved only by providing a large amount of mechanical work during the emulsification process in order to bring the phospholipid molecules to the o/w interface, the surfactant having a higher affinity for itself than for the saline solution or the PFOB.

6.4 PFOB emulsions using 10-2-O as an emulsifier

EYP is a variable and complex combination of phospholipids and minor components and is very sensitive to oxidation. Accordingly, I decided to use an alternative emulsifier as a simpler model for my system.

In a first series of experiments decaglycerodioleate (10-2-O) was used as a replacement for EYP. A polyglycerol ester prepared from a direct alkaline polymerization of glycerol followed by esterification with oleic acid [12, 13], 10-2-O is available commercially as a combination of various isomers differing by the size and the degree of esterification of the polyglycerol, the length and the degree of unsaturation of the fatty acyl chains, and positional isomerism of the fatty acids on the polyglycerol. 10-2-O is a polyglycerol with an average of ten molecules of glycerol esterified by two molecules of oleate (Fig. 6.4).

Commercial samples of 10-2-O yielded stable sterile 100% w/v PFOB emulsions when a 5% dextrose solution was used as a continuous phase. With a saline solution the system was unstable, due to the presence of free fatty acids (FFA, 2.8% as oleic, determined by titration method: A.O.C.S. Official method Ca 5a-40) in commercial 10-2-O. In the presence of ions, FFA form soaps detrimental to emulsion stability. Using another sample of 10-2-O with almost no FFA (0.04% as oleic) yielded flocculated systems.

Investigation of the surface properties of 10-2-O yielded conclusions similar to those for EYP. 10-2-O, being insoluble in both

phases, will strongly adsorb at the PFOB/saline interface, where it will reduce the interfacial free energy and provide a structured film, as evidenced by interfacial free energy (drop volume method) and compression isotherm measurements. The interfacial free energy drops from 50 mN/m between pure PFOB and saline to 6 mN/m in the presence of a film of 10-2-O, and, as before, the droplets are stable overnight. A monolayer of 10-2-O has a high collapse pressure (40 mN/m) and a high surface potential (+310 mV for a molecular surface area of 100 $Å^2$), and the compression/de-compression curve shows no hysteresis (Fig. 6.5).

6.5 Saturated PC as a replacement for EYP

In a second series of experiments, phospholipon 90H (PL), a hydrogenated phospholipid with simpler and more standard composition than EYP, was used as a sole emulsifier to prepare emulsions of PFOB.

Like EYP, pure phospholipids (DPPC, PL) dramatically reduce the PFOB/saline interfacial free energy, with resultant lowering of the energy required in the emulsification process, and provide a structured interfacial film. Nevertheless, they do not produce stable PFOB/saline emulsions. Direct observation of the emulsions shows a rapid (within 48 hours after sterilization) settling to the bottom of the vial of a gel-like phase with a high (thixotropic) resistance to the flow (supernatant/infranatant ratio is 50:50 v/v). Such a quick and massive settling of a viscous, gel-like phase is characteristic of a flocculated system. Furthermore, the particle size measured by PCS

goes from 650 nm before sterilization to about 1 μm after sterilization when the emulsion is diluted in saline solution, while with dilution in distilled water the particle size, about 300 nm, is similar before and after sterilization, again indicating a flocculated system (Fig. 6.6). The flocculation phenomenon was confirmed by viscoelastic measurements [11]. This system exhibits high specific viscosity values (100 to 2,000 cP) that make it unsuitable for intravenous injection (Fig. 6.7). A transmission electron micrograph (TEM) (Fig. 6.8) obtained for the same system after 2-month storage at room temperature shows a multitude of small droplets (150-200 nm in diameter) clustered around the surface of very large droplets (5 to 10 μm in diameter), evidence that PFOB/PL/saline systems flocculate and eventually coalesce. The coalescence was confirmed by a light micrograph clearly showing large PFOB droplets (10 μm in diameter, Fig. 6.10). The creaming is controlled by the time required for the aggregates to sediment to the bottom of the vial, which is governed by Stokes' law [6]. Thus the size of the flocs can be estimated from equation (4.9). Given that it takes them 48 hours to settle to the bottom of the vial (average distance of sedimentation: 5 cm) in a medium with a viscosity of 2,000 cP, the flocs are approximately 35 μm in size. Their time of formation is only a few minutes, however.

So why does EYP yield stable PFOB/saline emulsions while 10-2-O and PL produce flocculated systems?

The lowering of the interfacial free energy and the formation of a structured interfacial film are necessary conditions for emulsion formation and stability, but they are not sufficient. A single isolated

droplet would be stabilized by a structured film, but when considering the stability of an emulsion one must evaluate the role of interdroplet forces in preventing droplet aggregation (flocculation).

10-2-O is a nonionic emulsifier yielding an uncharged interfacial film. PC is a zwitterion at pHs higher than 3. The phosphocholine headgroup is oriented approximately parallel (within 30°) to the surface of the PC interfacial film [14, 15] yielding a zero net charge at the surface. This was confirmed by measuring the zeta potential of PFOB/PL/saline emulsions; the value found -- 3 ± 7 mV -- is almost zero and is similar to the value (-1 mV) obtained by Washington et al. [16] for soya oil/DPPC/water emulsions.

These findings -- that an uncharged interface yields flocculation and that the presence of saccharides in the continuous phase (a dextrose solution for 10-2-O systems) can prevent flocculation -- prompted me to study the role of electrostatic and hydration forces in preventing flocculation. This effort bore fruit: an examination of the effects of charging the interface and of using a saccharide solution as a continuous phase enabled me to design a model for PFOB/EYP/saline emulsions.

6.6 Model for PFOB/EYP/saline emulsions

Previous work on the stability of fat emulsions for parenteral nutrition [16] showed that flocculation could be prevented by the addition of negatively charged surfactants to the pure phospholipids. An increase in the amplitude of the zeta potential was correlated with a lesser tendency to flocculate [6, 17-20], and vice versa [21].

Stalidis et al. [22] reached similar conclusions for o/w emulsions of hydrocarbons prepared with a nonionic surfactant (Tween 80) as a primary emulsifier and stabilized against flocculation by addition of an anionic surfactant (SDS): "the greater the zeta potential the greater is the stability."

Investigation of PFOB in saline emulsions using a binary mixture of PL and a positively or negatively charged surfactant, stearylamine (SA) and cholesteryl hemisuccinate (CHS), respectively, showed that electrostatic forces alone could not explain the flocculation phenomenon. An emulsion (PL/SA 9:1 mol/mol) with a positive zeta potential (17 ± 7 mV) flocculates. A viscous phase settles at the bottom of the vial (supernatant/infranatant ratio is 27:73 v/v) and the particle size is high (around 600 nm) both before and after sterilization when the emulsion is diluted in saline, while dilution in water yields a system with a 330 nm particle size both before and after sterilization (Fig. 6.6). Flocculation was confirmed by viscoelastic measurements [11].

In contrast, an emulsion (PL/CHS 9:1 mol/mol) with a negative zeta potential (-14 ± 6 mV) is stable. After 4-month storage at room temperature, the emulsion shows very little settling (supernatant/infranatant ratio is 4:96, v/v), the particle size is around 300 nm before and after sterilization both in saline and water (Fig. 6.6), and viscoelastic measurements confirm the absence of flocculation with a low specific viscosity (10 cP) constant with increasing shear rate (Fig. 6.7) [11].

One could argue that the increase in stability could be due to the insertion of cholesterol moieties (in the form of CHS) into the

interfacial film, which would decrease the rigidity of the film of PL, thus stabilizing the droplets, since it is known that cholesterol decreases the rigidity of phospholipid films at temperatures below the PTT and increases the rigidity above the PTT. The difference in stability between EYP and PL emulsions would then be explained solely on the grounds of the mechanical (rheological) properties of the interfacial film. Because of its the unsaturated phospholipid acyl chains, a film of EYP will be much more fluid and elastic than a film of PL, with saturated acyl chains well packed at the interface (as evidenced by a high PTT), resulting in a hard-sphere behavior of the emulsion droplets. But while this could explain a better stability against coalescence, it could not account for the absence of flocculation. Also, emulsions made with a combination of PL and cholesterol (PL/chol. 19:1, 9:1, and 8:2 mol/mol) flocculate, showing no improved stability over an emulsion made with PL as sole emulsifier. Finally, charging the interface negatively by using DCP instead of CHS resulted in a similar stabilization (no flocculation).

Two systems with zeta potentials of comparable magnitude but with opposite signs (PL/SA and PL/CHS) yield emulsions with different degrees of stability: a negatively charged interface prevents flocculation more effectively than a positively charged interface. So flocculation cannot be explained solely by electrostatic force considerations. Washington [23] reached the same conclusions in his work on fat emulsions, stating that the hydration forces "should receive at least as much attention as the electrostatic forces in the consideration of emulsion stability."

The zeta potential is the potential at the surface of a sphere having a radius equal to the shear radius; its value is smaller than the value of the potential at the emulsifier head group surface because of the distance and the screening effect of the counterions. This effect has been studied for EYP-stabilized fat emulsions by Hall et al. [24], who found a linear relationship between the square of the zeta potential and the inverse of the ionic strength for sodium chloride solutions (ζ^2 vs. $[NaCl]^{-1}$). A negatively charged interface will be screened by cations, a positively charged interface by anions. The difference in stability between the two systems studied (PL/SA and PL/CHS) probably arises from the fact that the counterions have different degrees of hydration. The cations, being more hydrated than the anions, are more effective in preventing flocculation because of their higher "effective" (hydrated) radius, the emulsion being stabilized by steric forces as well as electrostatic forces. Studying o/w emulsions of hydrocarbons prepared with a nonionic surfactant (Tween 80) and stabilized against flocculation by the addition of cetyldimethylbenzylammonium chloride (CDBACl), Avranas and Stalidis [25] found that the adsorption of the cationic surfactant occurs primarily by ion exchange between the $CDBA^+$ and Na^+ ions in the absence of any chemical bond, the $CDBA^+$ lying flat on the surface of the film according to interfacial free energy results. They concluded that "the existence of electrostatic forces seems to be of less importance in the stability of these emulsions which are mainly stabilized with steric stabilization factors due to the high concentration of the nonionic surfactant."

PFOB emulsions made with pure PL in a saccharide solution show that hydration forces alone can significantly limit flocculation: PFOB/PL/9.5%(w/v) sucrose solution emulsions show some settling (supernatant/infranatant is 25:75 v/v) after 4-month storage at room temperature. The particle size is somewhat larger when the emulsion is diluted in the continuous phase (sucrose solution) than in water (450 nm and 300 nm, respectively), both before and after sterilization. The specific viscosity is low (15 to 10 cP) (Fig. 6.7). PFOB/PL/5%(w/v) dextrose solution emulsions show less flocculation: very little settling was observed after 4-month storage at room temperature (supernatant/infranatant ratio is 10:90 v/v). The particle size is 360 nm when the emulsion is diluted in the 5% dextrose solution and 300 nm when it is diluted in water, both before and after sterilization. The specific viscosity is significantly smaller than that of the PFOB/PL/saline emulsion (40 to 11 cP against 2,000 to 90 cP) (Fig. 6.7).

One might speculate that it is not the presence of saccharides that stabilizes the emulsion but the absence of salts, but this possibility is easily eliminated by considering a PFOB/PL/water emulsion: with no salts present the emulsion flocculates very quickly, like a PFOB/PL/saline emulsion.

Studying the effect of glucose on the flocculation of fat emulsions by monovalent and divalent cations, Washington et al. [26] found that "glucose produces a considerable stabilizing effect, which does not appear to be due to electrokinetic phenomena (no change in zeta potential), and can only be understood in terms of the van der Waals or solvation components of the interaction potentials between the

droplets." Known to be protein structure stabilizing agents [27], saccharides have recently been shown to stabilize PC vesicles against aggregation and fusion during freeze-thawing and freeze-drying with a mechanism of stabilization involving the interaction of the sugar with the phospholipid polar group. The sugar is probably hydrogen-bonded to the phosphodiester group of the phospholipid, thereby replacing the water of hydration [28, 29].

I believe that the stabilization of emulsion particles by saccharides is similar to the stabilization of colloidal suspensions by polymers through a mechanism known as steric stabilization [30]. The emulsion droplets are fully coated with sugars adsorbed at the surface in place of the water of hydration and are themselves highly hydrated (due to their numerous hydroxyl groups), so the sugar layers on different particles repel one another, yielding a quasi-stable system.

6.7 Role of the dispersed phase in emulsion stability

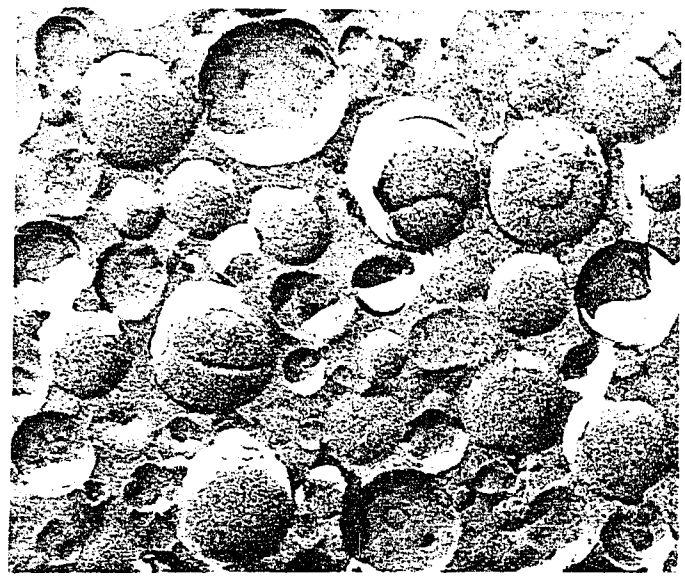
A series of emulsions were prepared using a medium chain triglyceride (MCT), Captex 300, as a dispersed phase with either 10-2-O or phospholipids. The results were similar to those obtained with PFOB emulsions in terms of stability and particle size. Similar results were also obtained when other PFCs (FDC, F-44E) were used as the dispersed phase, with the exception of perfluorophenanthrene, which gave a smaller particle size (0.13 μm in diameter). Emulsions of n-alkanes (C10, C12, C14, and C16) prepared with PL/CHS (9:1 mol/mol) in a saline solution all broke during sterilization (phase

separation). Studying compression isotherms of monolayer of PL in combination with an oil (PFC, MCT, n-alkane), Oleksiak [31] found no penetration of the PFCs or MCT in the phospholipid monolayer but did find a penetration of n-alkanes in the film. His results for MCT systems were confirmed by a study of phospholipid monolayers at the triolein-saline interface that showed that PC forms a highly condensed monolayer with a large lateral attractive interaction and very little penetration of triolein molecules [32]. This could explain the peculiar stability, even at elevated temperatures, of PFC or MCT emulsions made with phospholipids as emulsifiers. Since the usual method of breaking an emulsion down to its components is by the application of heat, it may be surprising to realize that emulsions of phospholipids can be heat-sterilized without breakage [33].

In emulsions of n-alkanes the phospholipid chain penetrates into the oil phase. This penetration increases as the temperature is raised until a surfactant phase (sometimes called "middle phase") appears: the packing of the film is loosened by the interdigitation of hydrocarbon molecules between the phospholipid molecules, and the interfacial free energy between the oil and water is minimal [34]. This phenomenon results in the loss of the film cohesion and in a redistribution of the surfactant molecules towards the formation of bilayers, and ultimately in emulsion breakage.

This was checked by subjecting the emulsions to various temperatures instead of sterilizing them. At 65°C all the systems broke after 30 minutes to 1 hour, while emulsions were stable (no phase separation) for months at 4°C. The difference could not be explained by kinetic considerations alone. In the case of PFC or MCT

emulsions there is insufficient affinity of the oil for the surfactant to yield a surfactant phase (during sterilization), and it is because phospholipids have such a low affinity for both the water and the oil phases that, if they can be made to adsorb at the o/w interface, they will yield emulsions capable of withstanding high temperature treatments (sterilization).



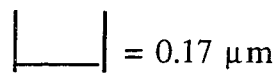


Figure 6.1: Transmission electron micrograph of a 52% v/v (100% w/v) PFOB/EYP/saline emulsion

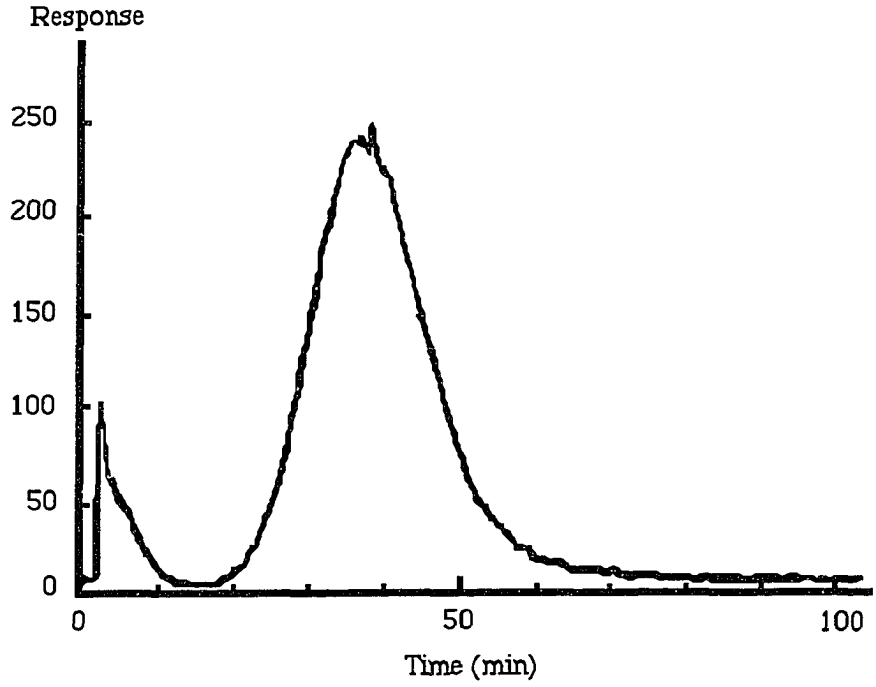


Figure 6.2: Sedimentation field flow fractionation fractogram of a 52% v/v (100% w/v) PFOB/saline/EYP emulsion

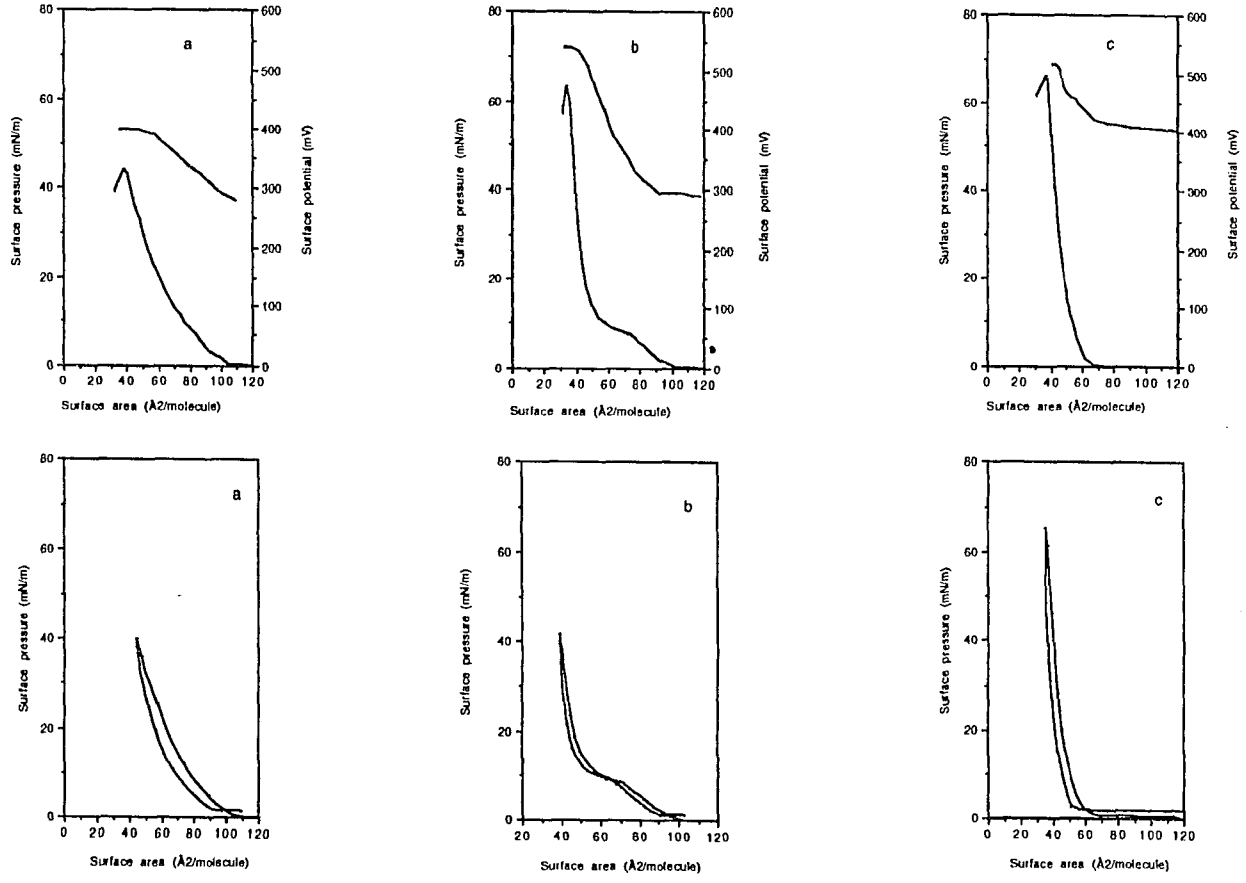
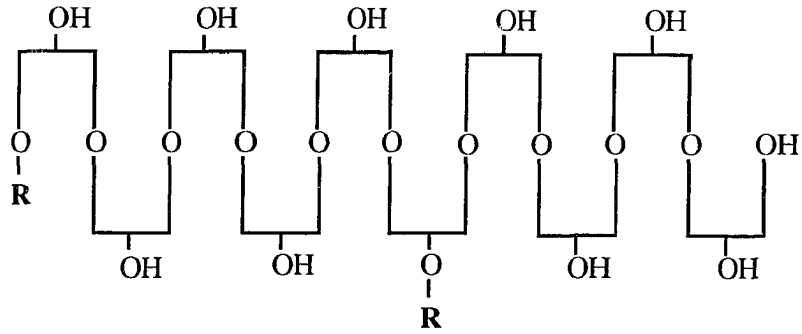


Figure 6.3: (top) Surface pressure and surface potential isotherms and (bottom) surface pressure isotherms (compression - decompression) of (a) commercial EYP, (b) DPPC, and (c) PL on saline at pH 6.8 and 25°C

Decaglycerol Dioleate (10-2-O)



R = Oleic acid residue

Figure 6.4: Structure of 10-2-O

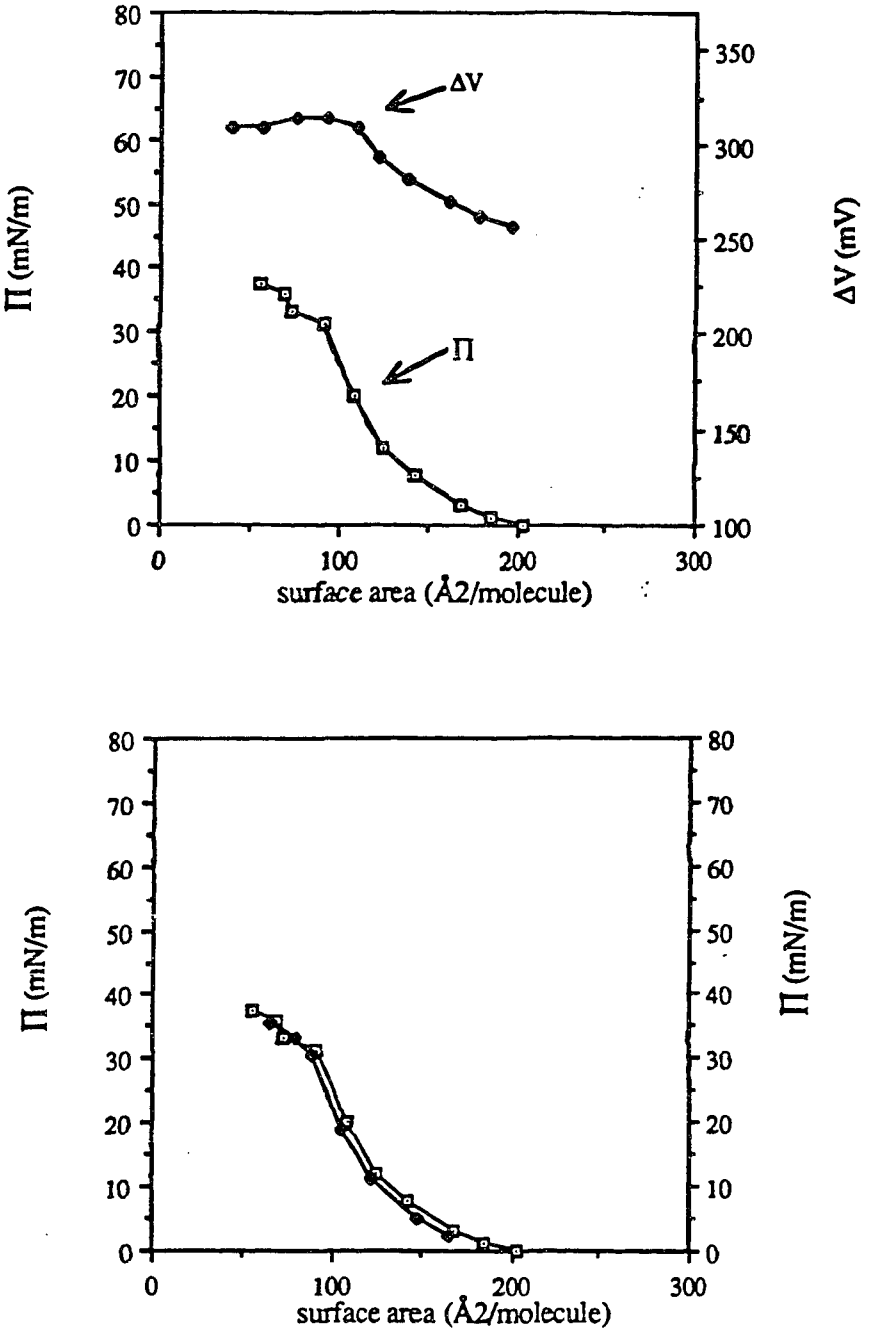


Figure 6.5: (Top) Surface pressure and surface potential isotherms and (bottom) surface pressure isotherms (compression-decompression) of 10-2-O at 23°C

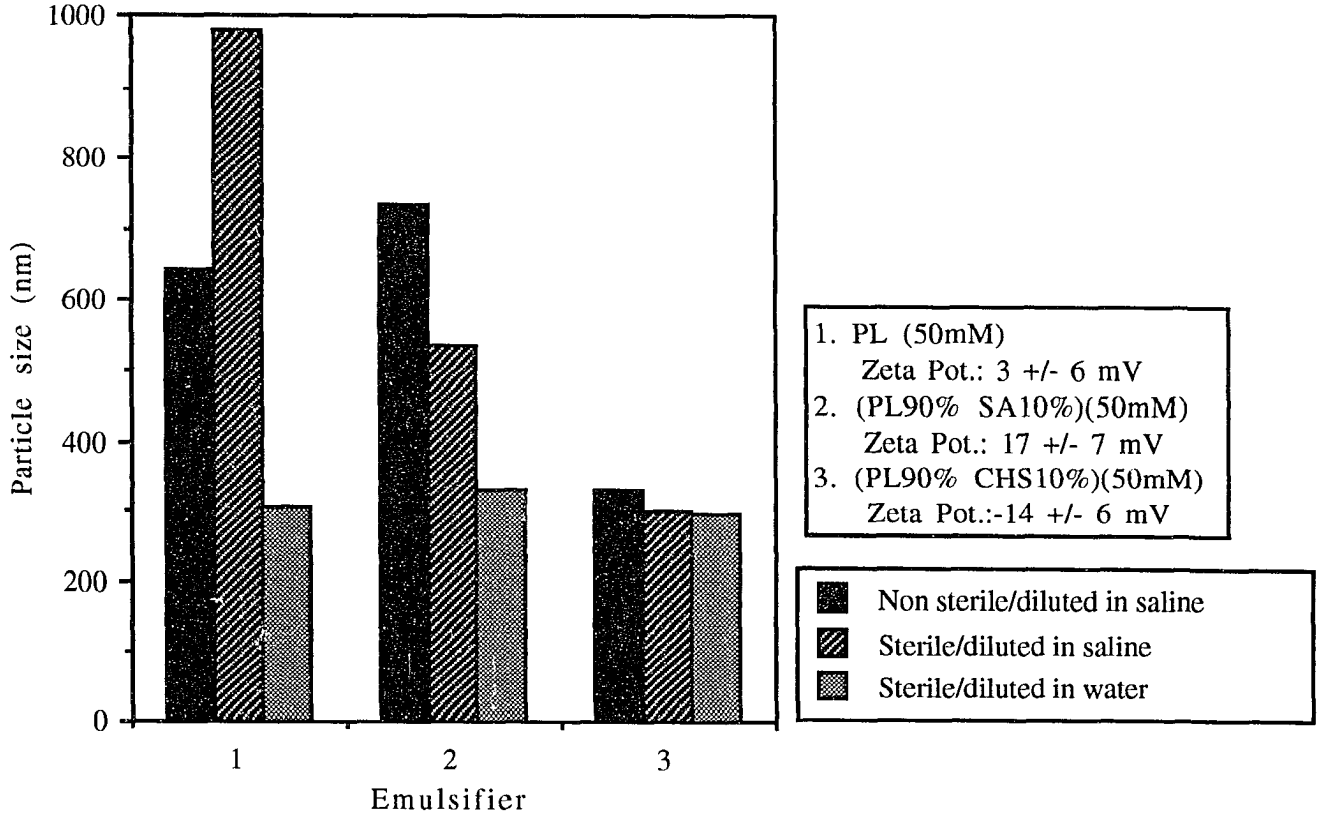


Figure 6.6: Particle size of PFOB/saline emulsions for uncharged and charged interfacial films

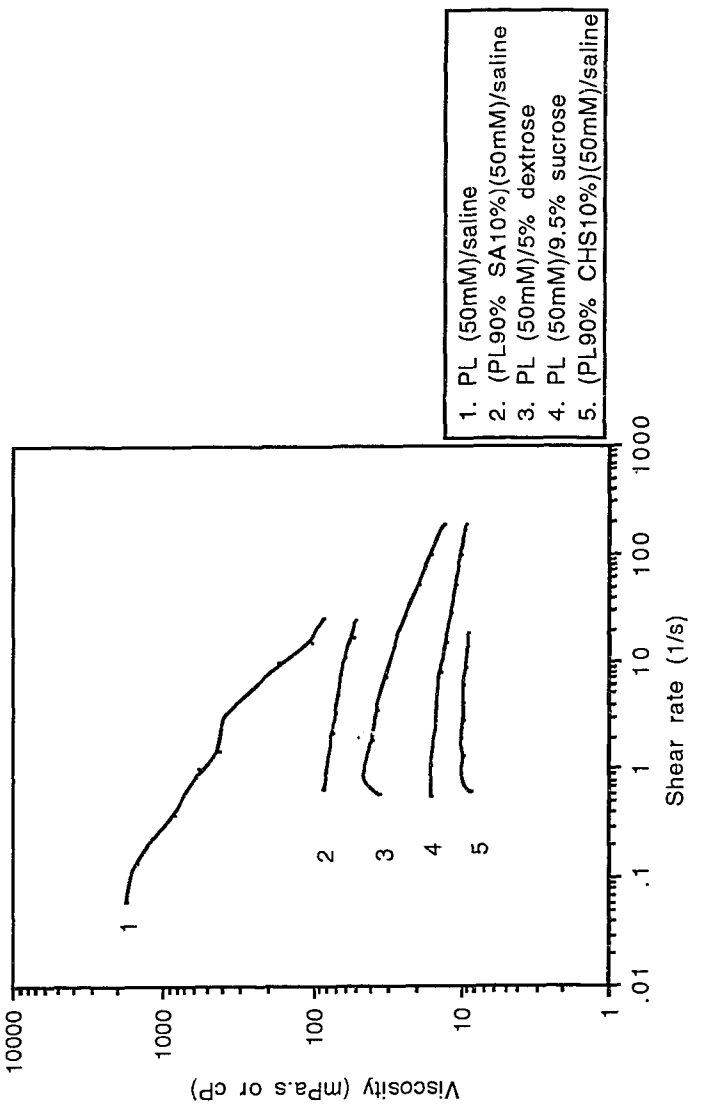


Figure 6.7: Viscosity versus shear rate for PFOB emulsions at 27°C

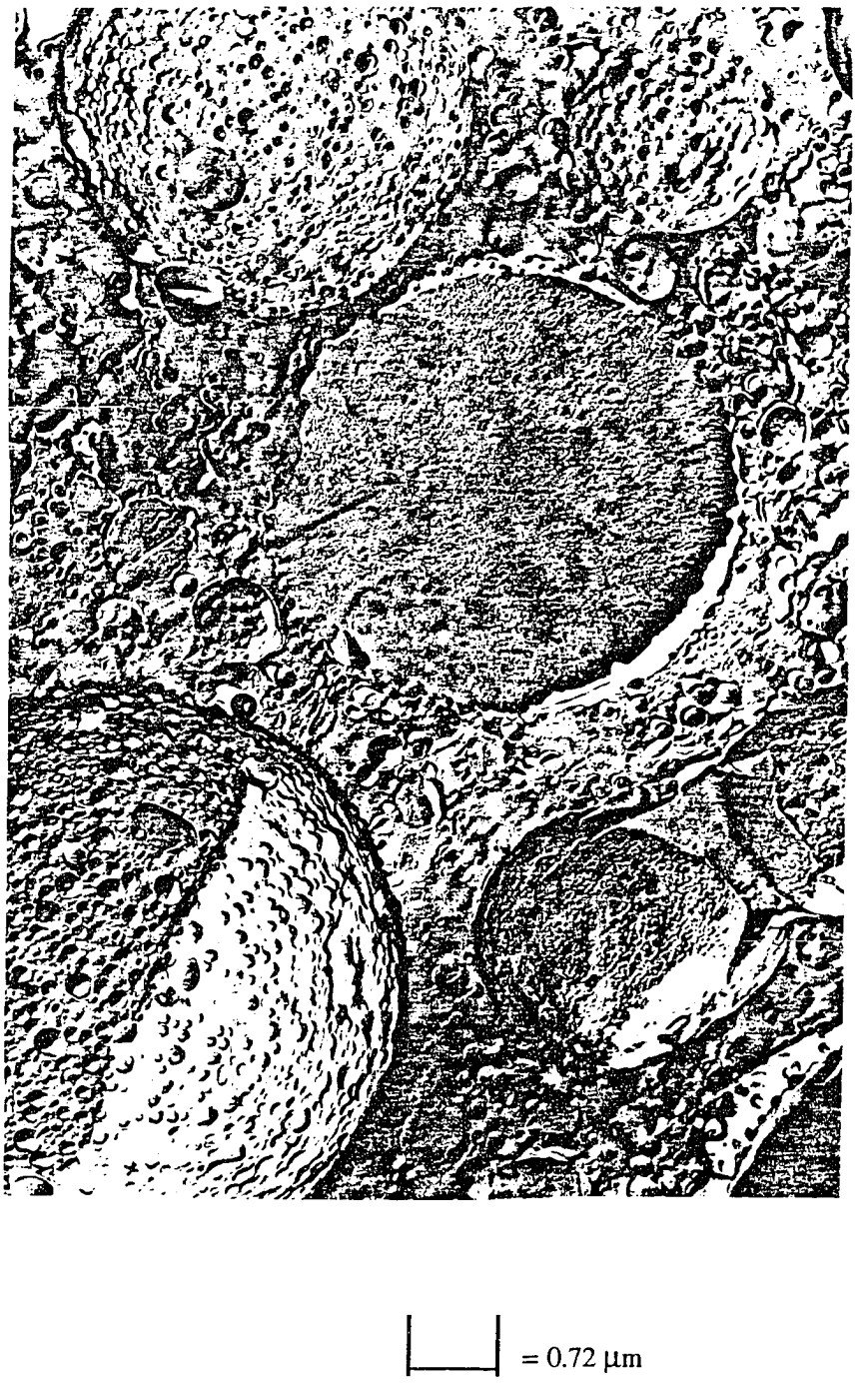
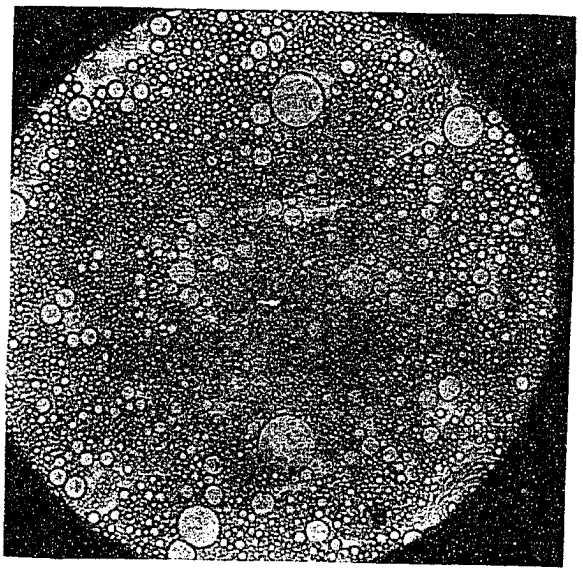


Figure 6.8: Transmission electron micrograph of a 45% v/v (90% w/v) PFOB/saline/PL dispersion



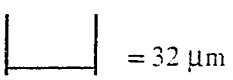


Figure 6.9: Light micrograph of a 45% v/v (90% w/v) PFOB/saline/PL dispersion

Table 6.1: Saline solution with a phosphate buffer

pH = 7.4

Compound	Concentration g/L	Molarity mmol/L soln	Molality mmol/kg H_2O	Osmolality mOsm/kg H_2O
NaCl	5.1	87.1	88.1	176
EDTA (diNa)	0.6	1.5	1.5	4.5
NaH_2PO_4	0.5	4.2	4.2	12.6
Na_2HPO_4	4.6	32.4	32.8	98.4
	10.8			291

H_2O = 989.2 g/L

pH = 6.8

Compound	Concentration g/L	Molarity mmol/L soln	Molality mmol/kg H_2O	Osmolality mOsm/kg H_2O
NaCl	5.1	87.1	88.1	176
EDTA (diNa)	0.6	1.5	1.5	4.5
NaH_2PO_4	1.8	15.1	15.3	45.9
Na_2HPO_4	3.2	22.5	22.7	68.1
	10.8			294

H_2O = 989.2 g/L

Table 6.2: Surface and Interfacial Free Energies (at 25°C)

	γ (mN/m)	W_c		γ_i (mN/m)	W_a
PFOB	18.1	36.2			
FDC	19.7	39.4			
Saline	72.0	144.0	/PFOB /FDC	50.0 56.2	40.1 35.5
n-hexadecane	27.9	55.8	/PFOB /FDC	2.5 5.8	43.5 41.8
n-dodecane	26.0	52.0	/PFOB /FDC	1.5 4.4	42.6 41.3
n-decane	23.3	46.6	/PFOB	0.4	41.0
n-octane	21.9	43.8	/PFOB /FDC	miscible 1.5	- 40.1

$$W_c = 2\gamma$$

$$W_a = \gamma_{o/air} + \gamma_{aq/air} - \gamma_{o/aq}$$

Table 6.3: Interfacial Free Energy Measurements at 25°C

sample	interfacial free energy
PFOB/Hexane/Ethanol, 71/25/4 w/w	50 mN/m
DPPC/PFOB/Hexane/Ethanol, 1/71/24/4 w/w	<1mN/m
10-2-O/PFOB/Hexane/Ethanol, 1/71/24/4 w/w	6 mN/m

Conclusions and Perspectives

During the emulsification process phospholipids are first dispersed in the aqueous phase as vesicles (i.e., spherical particles with bilayered membranes). Then, because of the higher affinity of the phospholipid molecules for each other than for the aqueous or the oil phase, a large amount of mechanical work is needed to break down these vesicles and bring the phospholipid molecules to the PFOB/saline interface, where (being virtually insoluble in both PFOB and saline) they will adsorb monomolecularly and quasi-irreversibly, reducing the interfacial free energy and providing a film capable of keeping its highly ordered structure under high stress and high temperature conditions. This in turn yields an emulsion capable of withstanding heat sterilization. The emulsion droplets must be protected against aggregation to avoid flocculation and subsequent coalescence. My results show that they are probably stabilized against flocculation by a combination of electrostatic (coulombic) and hydration (steric) repulsive forces.

Hence the key to emulsion stability is the existence of a highly hydrated, negatively charged interfacial film with a low affinity for both the oil and the aqueous phase; minimal penetration of the surfactant chains within the oil bulk ensures the preservation of the film integrity at elevated temperatures, while a low solubility of the surfactant in the aqueous phase ensures its strong, quasi-irreversible adsorption at the interface.

Thus, EYP's tendency to yield stable emulsions is most probably due to its naturally balanced composition: it is essentially composed

of major zwitterionic phospholipids (e.g., PC and PE), which provide a structured interfacial film, and of minor components that reduce the interfacial free energy to very low values and provide the interfacial film with a negative charge and a strong hydration shell (e.g., PA, PS, PG, PI).

Although my research has shed some light on PFC emulsion stability, much work remains to be done. For example, the rheological properties of the interfacial film must be examined and related to emulsion characteristics (e.g., particle size), to emulsion functionality (in particular, to residence time in circulation), and to emulsion stability. Also, while PFC emulsions of the second generation (e.g., PFOB emulsions) exhibit a markedly improved shelf-stability over PFC emulsions of the first generation (Fluosol-DA), which were so unstable that they had to be kept frozen as three separate components to be co-emulsified prior to use, they display no improvement in their intravascular persistence, being cleared from the circulation in five to six hours. An increase in residence time is essential if these emulsions are to serve as effective RBC substitutes; one possible avenue of approach is through heightening the emulsion particles' "stealthiness" to make them less recognizable by the macrophages. I hope to achieve this goal by increasing the hydrophilicity at the surface of the emulsion particle by the addition of a highly hydrated compound that would adsorb at the interface without destabilizing the interfacial film: i.e., a surfactant with an anchor group that would fit neatly between the phospholipid acyl chains and with a polar group highly hydrated but compatible with the phosphocholine group of the phospholipid molecules.

Experimentation with ethoxylated cholesterol compounds is in progress, and the first results are promising. Once the optimum size for the ethyleneoxide moiety and the optimum concentration have been identified, I hope to be able to increase both the intravascular persistence and the shelf-stability of the emulsion. Indeed, the highly hydrated ethyleneoxide molecules seem to form an efficient (steric) barrier against flocculation.

References

Chapter One

1 *New Webster's Dictionary and Thesaurus*, Ottenheimer Publishers, New York, NY, 1991.

2 [n.a.] *American Family Physician* 45:2787 (1992).

3 Sandler, S.G.; and Popovsky, M.A. *Technol. Rev.* 93:22 (1990).

4 Fisher, L.M. *The New York Times* Jul. 8, 1992.

5 Berreby, D. *Discover* 12:28 (1991).

6 Andrews, E.L. *The New York Times* Feb. 18, 1990.

7 Naunyn, B. *Arch. Anat. Physiol. Wiss. Med.* 4 (1868).

8 Sheffield, L.C.; and DeLoach, J.R. *Biotechnol. Appl. Biochem.* 14(3):249 (1991).

9 Lang, M.E.; Korecky, B.; Anderson, P.J.; and Biro, G.P. in Piiper et al. (Eds.) *Oxygen transport to tissue XII*, Plenum Press, New York, NY, 1990, p. 225.

10 Rabiner, S.F. *Fed. Proc.* 34(6):1454 (1975).

11 Biro, G.P. *Biomat., Art. Cells, Art. Org.* 16(1-3):595 (1988).

12 Biro, G.P. *Can. Med. Assoc. J.* 129:237 (1983).

13 Snyder, S.R.; and Walder, J.A. *Biotechnol. Ser.* 19 (Biotechnol. Blood):101 (1991).

14 Moffat, A.S. *Science* 253:32 (1991).

15 Ogden, J.E. *Trends in Biotechnology* 10(3):91 (1992).

16 Mok, W.; Chen, D-E; and Mazur, A. *Fed. Proc.* 34(6):1458 (1975).

17 Sehgal, L.R.; Rosen, A.L.; Gould, S.S.; Sehgal, H.L.; and Moss, G.S. *Chemtech* 21:116 (1991).

18 Manning, L.R.; Morgan, S.; Beavis, R.C.; Chait, B.T.; Manning, J.M.; Hess, J.K.; Cross, M.; Curell, D.L.; Marini, M.A.; and Winslow, R.M. *Proc. Natl. Acad. Sci. USA.* 88:3329 (1991).

19 Kahn, R.A.; Allen, R.W.; and Baldassare, J. *Blood* 66(1):1 (1985).

20 Beard, J. *New Scientist* [n.v.]:36, Jan. 20, 1990.

21 Coghlan, A. *New Scientist* 129:26 (1991).

22 Hilts, P.J. *The New York Times*, Jun. 16, 1991.

23 Marwick, C. *The World & I* [n.v.]:230, Mar., 1992.

24 Looker, D.; Abbott-Brown, D.; Cozart, P.; Durfee, S.; Hoffman, S.; Matthews, A.J.; Miller-Roehrich, J.; Shoemaker, S.; Trimbles, S.; Fermi, G.; Komiyama, N.H.; Nagai, K.; and Stetler, G.L. *Nature* 356:258 (1992).

25 Erickson, D. *Scientific American* 266:106 (1992).

26 Pool, R. *Science* 250:1655 (1990).

27 Otto, B.R.; Verweij-van Vught, A.M.J.J.; and MacLaren, D.M. *Nature* 358:23 (1992).

28 Mitchell, J. *Wall Street Journal*, Apr. 11, 1991.

29 Dayton, L. *New Scientist* [n.v.]:42, Jun. 3, 1989.

30 Kossovsky, N.; and Millet, D. *MRS Bulletin* 16(9):78 (1991).

31 Hunt, C.A.; Burnette, R.R.; MacGregor, R.D.; Strubbe, A.E.; Lau, D.T.; Taylor, N.; and Kawada, H. *Science* 230:1165 (1985).

32 Mobed, M.; Nishiya, T.; and Chang, T.M.S. *Biomat., Art. Cells & Immob. Biotech.* 20(1):53 (1992).

33 Goins, B.; Rudolph, A.S.; and Ligler, F.S. *Biotechnol. Ser.* 19(Biotechnol. Blood):117 (1991).

34 Bollands, A. *New Scientist* 115:65 (1987).

35 Zheng, S.; Beissinger, R.L.; and Wasan, D.T. *J. Colloid Interface Sci.* 144(1):72 (1991).

36 Borwanker, C.M.; Pfeiffer, S.B.; Zheng, S.; Beissinger, R.L.; Wasan, D.T.; Sehgal, L.R.; and Rosen, A.L. *Biotechnol. Prog.* 4(4):210 (1988).

37 Tsuchida, E.; and Hasegawa, E. *Artif. Organs Today* 1(1):23 (1991).

38 [n.a.] *C&E News* [n.v.]:42, Jan. 14, 1985.

39 Clark, L.C.; and Gollan, F. *Science* 152:1755 (1966).

40 Sloviter, H.A.; and Kamomito, T. *Nature* 216:458 (1967).

41 Geyer, R.P. *N. Engl. J. Med.* 289:1077 (1973).

42 Yamanouchi, K.; and Heldebrant, C. *Chemtech* [n.v.]:354, June 1992.

43 Mitsuno, T.; Ohyanagi, H.; and Naito, R. *Ann. Surg.* 195:60 (1982).

44 *FDC Report*, 8th January 1990.

45 Garrelts, J.C. *DCIP, The Annals of Pharmacotherapy* 24:1105 (1990).

46 Rosano, H.L.; and Gerbacia, W.E. U.S. Patent 3,778,381; Dec. 11, 1973.

47 Lowe, K.C. *Adv. Mater.* 3(2):87 (1991).

48 Long, D.M.; Long, D.C.; Mattrey, R.F.; Long, R.A.; Burgan, A.R.; Herrick, W.C.; and Shellhamer, D.F. in Chang, T.M.S.; and Geyer, R.P. (Eds.) *Blood Substitutes*, Marcel Dekker, New York, NY, 1989, p. 441.

49 Riess, J.G. *Artif. Organs* 15(5):408 (1991).

50 Largely drawn from Otisville BioPharm Annual Report 1988 (Otisville, NY) and Alliance Pharmaceutical Corp. Annual Report 1992 (San Diego, CA).

51 Mattrey, R.F. *Am. J. Radiol.* 152:247 (1988).

52 Biro, G.P.; and Blais, P. *CRC Crit. Rev. Oncol. Haem.* 6:311 (1987).

53 Teicher, B.A.; and Rose, C.M. *Cancer Res.* 44:4285 (1984).

54 Teicher B.A.; and Rose, C.M. *Science* 223:934 (1984).

55 Voynikov, T.I.; Nikolova, I.N.; Suzuki, A.; and Higashino, H. *Neurosciences* 16:591 (1990).

56 Kaufman, R.J. *Biotechnol. Ser.* 19(Biotechnol. blood):127 (1991).

Chapter Two

1 Lowe, K.C. *Adv. Mater.* 3(2):87 (1991).

2 Meinert, H.; Fackler, R.; Knoblich, A.; Mader, J.; Reuter, P.; and Rohlke, W. *Biomat., Art. Cells & Immob. Biotech.* 20(1):95 (1992).

3 Sargent, J.W.; and Seffl, R.J. *Fed. Proc.* 29(5):1699 (1970).

4 Riess, J.G. *Artif. Organs* 8(1):44 (1984).

5 Simons, J.H. U.S. Patent 2,519,983; 1950.

6 Dixon, D.D.; and Holland, D.G. *Fed. Proc.* 34:1444 (1975).

Chapter Three

1 Nagle, J.F. *Ann. Rev. Phys. Chem.* 31:157 (1980).

2 Folch, J.; Lees, M.; and Sloane Stanley, G.H. *J. Biol. Chem.* 226:497 (1957).

3 Ramesh, B.; Prabhudesai, A.V.; and Viswanathan, C.V. *JAOCS* 55:501 (1978).

4 Wells, M.A.; and Hanahan, D.J. in *Methods in Enzymology*, Academic Press, New York, NY, Vol. 14, 1969, p. 179.

5 Ramesh, B.; Adkar, S.S.; Prabhudesai, A.V.; and Viswanathan, C.V. *JAOCS* 56:585 (1979).

Chapter Four

1 Princen, H.M.; Aronson, M.P.; and Moser, J.C. *J. Colloid Interface Sci.* 75(1):246 (1980).

2 Griffin, W.C. *Proc. Sci. Sect. Toilet. Goods Assoc. No.* 6(6):43 (1946).

3 Shinoda, K.; and Saito, H. *J. Colloid Interface Sci.* 30(2):2258 (1969).

4 Shinoda, K.; and Sagitani, H. *J. Colloid Interface Sci.* 64(1):68 (1978).

5 Wilshut, J.; Scholma, J.; and Stegman, T. in Gaber, B.P.; et al. (Eds.) *Biotechnological applications of lipid microstructures, based on the proceedings of the workshop on technological applications of phospholipid bilayers, vesicles, and thin films (1986, Tenerife, Canary Islands)*, Plenum Press, New York, NY, 1988, p.105.

6 Bailey, S.M.; Chiruvolu, S.; Israelachvili, J.N.; and Zasadzinski, J.A. *Langmuir* 6(7):1326 (1990).

7 Overbeek, J.Th.G. *J. Colloid Interface Sci.* 58(2):408 (1977).

8 Friberg, S.E.; and Solans, C. *Langmuir* 2(2):121 (1986).

9 Friberg, S.; Jansson, P.O.; and Cederberg, E. *J. Colloid Interface Sci.* 55(3):614 (1976).

10 Aronson, M.P. *Langmuir* 5(2):494 (1989).

11 Fairhurst, D.; Aronson, M.P.; Gum, M.L.; and Goddard, E.D. *Colloids and Surfaces* 7:153 (1983).

12 Bibette, J. *J. Colloid Interface Sci.* 147(2):474 (1991).

13 Dickinson, E. *J. Colloid Interface Sci.* 132(1):274 (1989).

14 Vincent, B.; Luckham, P.F.; and Waite, F.A. *J. Colloid Interface Sci.* 73(2):508 (1980).

15 Dickinson, E.; and Eriksson, L. *Adv. Colloid Interface Sci.* 34:1 (1991).

16 Feigin, R.I.; and Napper, D.H. *J. Colloid Interface Sci.* 74(2):567 (1980).

17 Heyes, D.M.; McKenzie, D.J.; and Buscall, R. *J. Colloid Interface Sci.* 142(2):303 (1991).

18 Dolan, A.K.; and Edwards, S.F. *Proc. R. Soc. Lond. A.* 337:509 (1974).

19 Lafuma, F.; Wong, K.; and Cabane, B. *J. Colloid Interface Sci.* 143(1):9 (1991).

20 Melik, D.H.; and Fogler, H.S. in Shah, D.O. (Ed.) *Macro- and Microemulsions Theory and Applications*, American Chemical Society, Washington, D.C., 1985, p. 461.

21 Napper, D.H. *J. Colloid Interface Sci.* 58(2):390 (1977).

22 Ottewill, R.H. *J. Colloid Interface Sci.* 58(2):357 (1977).

Chapter Five

1 Suslick, K.S. *Scientific American* [n.v.]:80, Feb., 1989.

2 Sloviter, H.A. U.S. Patent 4,497,829; Feb. 5, 1985.

3 Joslyn, L.J. in Block, S.S. (Ed.) *Disinfection, Sterilization, and Preservation 3rd Ed..*, Lea & Febiger, Philadelphia, PA, 1983, p36.

4 Rosano, H.L.; Habif, S.S.; Oleksiak, C.B.; Pelura, T.J.; and Cavallo, J.L. in Mittal, K.L.; and Shah, D.O. (Eds.) *Surfactants in Solution*, Vol. 11, Plenum Press, New York, NY, 1991, p.431.

5 Caldwell, K.D.; and Li, J. *J. Colloid Interface Sci.* 132(1):256 (1989).

6 Experiments performed by Christian B. Oleksiak.

Chapter Six

1 Sloviter, H.A. U.S. Patent 4,497,829; Feb. 5, 1985.

2 Ishii, F.; Sasaki, I.; and Ogata, H. *J. Pharm. Pharmacol.* 42:513 (1990).

3 Krafft, M.-P.; Rolland, J.-P.; and Riess, J.G. *J. Phys. Chem.* 95:5673 (1991).

4 Hajri, T.; Ferezou, J.; and Lutton, C. *Biochim. Biophys. Acta* 1047:121 (1990).

5 Untracht, S.H. *Biochim. Biophys. Acta* 711:176 (1982).

6 Washington, C. *Int. J. Pharm.* 66:1 (1990).

7 Riess, J.G.; Dalfors, J.L.; Hanna, G.K.; Klein, D.H.; Krafft, M.-P.; Pelura, T.J.; and Schutt, E.G. *Biomat., Art. Cells and Immob. Biotech.* 20:839 (1992).

8 Pelura, T.J. Private communication.

9 Huang, C.-H. *Biochemistry* 8(1):344 (1969).

10 Lowe, K.C. *Adv. Mater.* 3(2):87 (1991).

11 Oleksiak, C.B.; Habif, S.S.; and Rosano, H.L. "Flocculation of perfluorocarbon emulsion using phospholipids as emulsifiers." To be published in *Colloids and Surfaces* (1994).

12 Garti, N.; and Aserin, A. *JAOCS* 59(7):317 (1982).

13 McIntyre, R.T. *JAOCS* 56:835A (1979).

14 Seelig, J.; MacDonald, P.M.; and Scherer, P.G. *Biochemistry* 26(24):7535 (1987).

15 Scherer, P.G.; and Seelig, J. *Biochemistry* 28:7720 (1989).

16 Washington, C.; Chawla, A.; Christy, N.; and Davis, S.S. *Int. J. Pharm.* 54:191 (1989).

17 Washington, C.; and Davis, S.S. *Int. J. Pharm.* 39:33 (1987).

18 Rubino, J.T. *J. Parenter. Sci. Technol.* 44(4):210 (1990).

19 Stampa, B.; Lucks, J.-S.; Müller, B.W.; and Müller, R.H. *J. Colloid Interface Sci.* 143(1):188 (1991).

20 Davis, S.S. in Hanin, I.; and Pepeu, G. (Eds.) *Phospholipids: Biochem., Pharm., Anal. Consid., [Proc. Int. Colloq. Lecithin], 5th*, Plenum Press, New York, NY, 1990, p. 69.

21 Johnson, O.L.; Washington, C.; Davis, S.S.; and Schaupp, K. *Int. J. Pharm.* 53:237 (1989).

22 Stalidis, G.; Avranas, A.; and Jannakoudakis, D. *J. Colloid Interface Sci.* 135(2):313 (1990).

23 Washington, C. *Int. J. Pharm.* 64:67 (1990).

24 Hall, S.B.; Gaskin, P.W.; Duffield, J.R.; and Williams, D.R. *Int. J. Pharm.* 70:251 (1991).

25 Avranas, A.; and Stalidis, G. *J. Colloid Interface Sci.* 143(1):180 (1991).

26 Washington, C.; Athersuch, A.; and Kynoch, D.J. *Int. J. Pharm.* 64:217 (1990).

27 Arakawa, T.; and Timasheff, S.N. *Biochemistry* 21:6536 (1982).

28 Strauss, G.; Schurtenberger, P.; and Hauser, H. *Biochim. Biophys. Acta* 858:169 (1986).

29 Hauser, H.; and Strauss, G. in Gaber, B.P.; et al. (Eds.) *Biotechnological applications of lipid microstructures, based on the proceedings of the workshop on technological applications of phospholipid bilayers, vesicles, and thin films (1986, Tenerife, Canary Islands)*, Plenum Press, New York, NY, 1988, p.71.

30 Dickinson, E.; and Eriksson, L. *Adv. Colloid Interface Sci.* 34:1 (1991).

31 Oleksiak, C.B. Unpublished results.

32 Handa, T.; Saito, H.; and Miyajima, K. *Biochemistry* 29:2884 (1990).

33 Hansrani, P.K.; Davis, S.S.; and Groves, M.J. *J. Parenter. Sci. Technol.* 37(4):145 (1983).

34 Shinoda, K. *Progr. Colloid & Polymer Sci.* 68:1 (1983).