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**Perfluorooctyl Bromide Emulsions as *In Vivo* Carriers for Laser
Polarized ^{129}Xe for Magnetic Resonance Imaging:
The Effect of Phosphatidylcholine Chain-Length and Oxygenation
of the Emulsion on Longitudinal Relaxation Time (T_1) and Line-
Width.**

by

Daniel P. McPhee, B.Sc.

A thesis Submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of
Master of Science

Department of Biology

Carleton University
Ottawa, Ontario, Canada

September 2001

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Chair, Department of Biology

Thesis Supervisor

Carleton University
Date _____

Abstract

Hyperpolarized ^{129}Xe (HXe) can be dissolved in biologically compatible Perfluorooctylbromide (PFOB) emulsions while maintaining sufficient polarization for *in vivo* magnetic resonance imaging. Increasing the longitudinal (T_1) and the transverse (T_2^*) relaxation times of HXe dissolved in PFOB emulsions is critical if it is to be used for *in vivo* Magnetic Resonance Imaging. The effects of emulsion droplet size and oxygenation were studied to determine if these times might be increased. Emulsion droplet sizes were controlled by changing the amount of phosphatidylcholine (PC) used to stabilize the emulsion and by changing the chain-length of the PCs' hydrocarbon tail. It was found that the average droplet size could be decreased from 3.05 μm to 2.42 μm by using a PC with a 4 carbon tail (DBPC) rather than one with an 18 carbon tail (DOPC). With this decrease in size, line-width decreased significantly from 4.74 ppm to 2.42 ppm. There was no correlation between T_1 and droplet size. Oxygenation did not affect line-width but had a destructive effect on T_1 . It was possible to increase T_1 s by over 100 s through the rigorous elimination of oxygen.

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CC, CS, LB

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List of Abbreviations

DAPC	1,2-Diarachidoyl-sn-glycero-3-phosphatidylcholine
DBPC	1,2-Dibutyroyl-sn-glycero-3-phosphatidylcholine
DLPC	1,2-Dilauroyl-sn-glycero-3-phosphatidylcholine
DOcPC	1,2-Dioctanoyl-sn-glycero-3-phosphatidylcholine
DOPC	1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine
DOPE	1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholine
FWHM	Frequency width half max
HRTC	High resolution computed tomography
HXe	Hyperpolarized ^{129}Xe
MR	Magnetic resonance
MRI	Magnetic resonance imaging
NMR	Nuclear magnetic resonance
PA	Phosphatidic acid
PC	Phosphatidylcholine
PFC	Perfluorocarbon
PFOB	Perfluorooctyl bromide
PI	Phosphatidylinositol
PS	Phosphatidylserine
RBC	Red blood cell
RF	Radio frequency
SE	Scanning electron
SEM	Scanning electron microscopy
SNR	Signal-to-noise ratio
T_1	Longitudinal relaxation time
T_2	Transverse relaxation time
TR	Repetition time

Chapter 1

Introduction

Magnetic Resonance (MR) imaging is well known for its low patient risk. It does not use ionizing radiation and provides excellent soft tissue contrast (Hylton and Frankel, 1994). Conventional MR image contrast is provided largely by differences in proton longitudinal (T_1) and transverse (T_2) relaxation times. These times depend on tissue-specific factors such as tissue water content and biochemical properties.

Conventional MR imaging has, however, been unable to differentiate cancerous from benign breast lesions due to overlap in relaxation times (Santyr, 1994). It is also unable to image gas spaces (Hedlund *et al.*, 2000).

Non-reactive, noble gases such as helium (^3He) and xenon (^{129}Xe) can be used to image spaces, such as the lungs, where the proton concentration is insufficient to yield an image. The major obstacle to the use of these gases as a source of contrast is the low sensitivity and concentration ($\sim 10\text{mM}$) achievable in tissue (Albert, *et al.*, 1995; Patyal *et al.*, 1997), yielding a low signal to noise ratio (SNR). In order to increase SNR the nuclei must be laser polarized. With the laser polarization technique, it is possible to increase the sensitivity of ^{129}Xe detection by nearly five orders of magnitude (Albert *et al.*, 1995). In order to introduce hyperpolarized ^{129}Xe

(HXe) into tissue, including intravascularly, the gas must be dissolved in a non-toxic carrier. This carrier must also be able to protect the hyperpolarized gas from its environment in order to increase relaxation times, permitting imaging. The success of this carrier will depend a number of factors including

- (a) how quickly HXe depolarizes during transit (T_1 relaxation)
- (b) the rate of signal loss during image acquisition (T_1 and T_2^* relaxation)
- (c) the chemical shifts of the dissolved xenon

This thesis investigates the use of phosphatidylcholines with differing chain lengths to emulsify Perfluorooctyl bromide for the purpose of dissolved-phase ^{129}Xe MR imaging. It is expected that phosphatidylcholine chain length will affect:

- (i) emulsion stability
- (ii) droplet size
- (iii) T_1 relaxation time
- (iv) T_2^* relaxation time.

The effect of oxygenation of the emulsion was also measured in order to help determine the optimal conditions for *in vivo* injection of the HXe saturated emulsion.

1.1 Emulsions

Emulsions are a common way of bringing into solution substances that are otherwise insoluble in a given medium (Shinoda and Friberg, 1986). They are prevalent in many biological systems including mammalian milk, and have been made use of in foods such as ice cream and mayonnaise (El-Nokaly and Cornell, 1991). In the

pharmaceutical industry, emulsions are routinely used as a means of introducing hydrophobic compounds into the blood stream, an aqueous environment. Emulsions contain two or more immiscible liquids that are mixed through the addition of energy via shearing forces (Sjöblom, 1992). The liquid that is broken up into globules is termed the dispersed phase, while the surrounding medium is known as the continuous phase or the dispersing medium (Shinoda and Friberg, 1986).

An emulsion which has no surfactant is termed “unprotected” and is often unstable. Surfactants are also commonly referred to as stabilizers and emulsifiers. For the purposes of this thesis, stability will describe the actual separation of the emulsion. An emulsion that is unstable will separate such that a clear fraction can be seen on the bottom of the container. The term settling will be used to describe the actual settling of the droplets on the bottom of the container due to gravity. If the lower portion of the emulsion container is white and there is no visible clearing at the bottom, the emulsion has settled but is stable and may be shaken in order to resuspend the droplets. In this case it is possible that some of the droplets may have coalesced, increasing the size of the droplets. An oil-in-water emulsion will quickly separate into two phases as the unprotected oil droplets coalesce. However, the addition of a surfactant with a polar end and a hydrophobic end can serve as an interface between the two phases, increasing stability (Sjöblom, 1992).

1.1.1 Emulsifiers

In the case of oil-in-water emulsions, an emulsifier that is amphipathic is required. The emulsifier must form a stable interface between the water and the hydrophobic oil. There are only a few emulsifiers regarded as safe for parenteral administration. The most popular of these are Pluronic F68® (Poloxamer 188), Tween 80®, Polysorbate 80 and phosphatidylcholine (PC). Of these, PC is the only one that is biodegradable and can be metabolized, as it is an integral component of biological membranes. Non-biodegradable emulsifiers can only be excreted via the kidneys. Furthermore, Pluronic F68® has been shown to cause endogenous pyrogen release due to its impurities (Mueller and Heinmann, 1993). The USP23/NF18 (1995) describes lecithin as a complex mixture of acetone-insoluble phosphatides. It is well tolerated and has GRAS-status (Generally Recognized as Safe) as approved by the FDA. Kuksis (1985) and Prosise (1985) describe the isolation and purification of lecithins from different sources.

PC is a mixture of sn-glycerol-3-phosphatidylcholine backbones with different length acyl chains substitutions (figure 1.1). Fatty acids in Egg Yolk PC (EYP) are typically 16-20 carbons in length. The sn-1-chain is typically 16 C, whereas the sn-2-chain is usually 18 C (Gennis, 1989). Two common sources of PC are egg and soya. Depending on its source, lecithin contains other minor components such as phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylserine (PS) as well as lysophospholipids (Hansrani, 1980). There are reports of adverse reactions to the

parenteral application of soya lecithin-stabilized emulsions due to its higher PI content (Wretlind, 1976). As such, egg PC is most commonly used.

A PC molecule tends to orient itself at a slight angle at the interface. This is because the polar region of the head group extends as far as the second carbon atom of the sn-2 acyl chain. This, essentially, pulls the PC down (Gennis, 1989) (figure 1.1).

This thesis, in part, is concerned with the effect of chain length on emulsion droplet size and, consequently, the effect on HXe relaxation. As such, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) was used in place of egg PC. DOPC has twin 18 carbon acyl chains, each with one double bond. In this way, the variations in the extraction process and other trace phospholipids in EYP were controlled for. To study the effect of PC chain-length, 1,2-dibutyroyl-sn-glycero-3-phosphatidylcholine (DBPC) was used. It is a PC molecule with two four-carbon acyl chains.

1.1.2 Factors Affecting Emulsion Stability

Amphipathic surfactants, including PC, are hydrodynamically aligned around a droplet. They function as an interface by organizing the water around the droplet into an ordered shell while their hydrophobic tails interact with the dispersed oil. This shell of water is thermodynamically preferable over direct interactions between the polar water molecules and the hydrophobic hydrocarbons (El-Nokaly and Cornell, 1990). There are several factors that have strong effects on the stability of an

emulsion. Any decrease in the ordering of the water surrounding the droplet will result in a less stable emulsion. For this reason, using phospholipids with different head groups will have a strong effect. Increasing the temperature of the emulsion has a similar effect. In this case, the surrounding water shell is less ordered due to the addition of thermal energy (El-Nokaly and Cornell, 1990). Decreasing the order of the surrounding water shell increases the likelihood of droplets coming together and coalescing. Finally, emulsions made with homogeneous surfactants tend to be less stable than those emulsified using surfactants that are slightly heterogeneous or a mixture of surfactants (Shinoda and Friberg, 1986).

1.2 Perfluorocarbon Emulsions

1.2.1 Perfluorocarbon Emulsions as Gas Carriers

Perfluorocarbon-based emulsions have been used for over twenty years as carriers of various gases (Clark *et al.*, 1975). Perfluorooctylbromide (PFOB) is a colourless, odorless liquid that is insoluble in water. It has been shown to be safe when used in the body as an oral liquid or intravenous emulsion (Mattrey *et al.*, 1984). It is a chemically and biologically inert and stable compound, which resists decomposition by all but the strongest bases. No chemical transformations of PFOB have been observed in the body. It has high gas solubility and low viscosity (Flores-Aguilar *et al.*, 1995) and can dissolve up to 20 times more oxygen than water (Biro, 1993). It is immiscible in water and optically clear (Flores-Aguilar *et al.*, 1995).

Neat PFOB is used in various surgeries including vitreoretinal surgery (Meller *et al.*, 1998; Flores-Aguilar *et al.*, 1995). Fluosol®, a 'first generation' perfluorocarbon emulsion was approved by the FDA in 1985 and other products are undergoing clinical trials for use as a red blood cell (RBC) substitute (Biro, 1993; Alliance 2001; Medical 2001). Experimental studies in which PFC emulsions were used for the perfusion of isolated hearts (Siegel *et al.*, 1987; Kioka *et al.*, 1987; Silber *et al.*, 1991) indicate that perfusion with oxygenated PFC-containing media is capable of permitting the functional preservation of the heart for up to 24 hours.

Perfluorocarbon emulsions developed as RBC substitutes have the advantage of very small particle size (particles are one 30th to one 70th the size of RBCs) so they can reach areas where blockages impede blood flow (Medical Sciences Bulletin, 2001) and a relatively short retention time. PFOB, specifically, has a fairly short biological retention time, with a half-life of 3 days and their removal from the circulation is principally by phagocytic processes (Sartoris *et al.*, 1985), with a very small proportion being directly excreted by expiration (Biro, 1993). For an excellent review on perfluorocarbon based systems for oxygen delivery see Riess and Krafft (1997).

1.2.2 Perfluorooctylbromide Emulsions in Imaging

The presence of bromine in PFOB renders it radio-opaque, and it was initially used as a radiological contrast agent administered either orally to outline the gastrointestinal tract or as a bronchographic contrast agent (Mattrey and Long, 1988). In an

emulsion, after intravenous administration, it was used to enhance hepatosplenic computed tomographic (CT) images. In doses of 1 to 2 g/Kg body weight administered slowly (1 mL/min), the emulsion was tolerated well and provided superior images, permitting the identification of tumor metastases (Bruneton *et al.*, 1988). When administered as a blood pool contrast agent, its safety was further documented in experiments using dogs (Mattrey *et al.*, 1989) and pigs (Peck *et al.*, 1984); the hemodynamic effects were compared with those induced by standard iodinated ionic contrast agents. In both species, PFOB induced less hemodynamic disturbance than the known transient hypotension induced by ionic contrast material.

Subsequent investigations (Mattrey and Long, 1988) showed that intravenously administered PFOB emulsion also enhanced the ultrasonographic image of the liver and kidney, possibly by virtue of the high density and low acoustic velocity of the PFCs. In addition to enhancing radiographic and echographic images, PFOB was found to enhance diagnostic images obtained by MR (Mattrey and Long, 1988; Mattrey, 1989; Brown *et al.*, 1991). It can be ingested 'neat' to produce bowel images that are darkened due to the absence of proton signal.

The presence of the fluorine atoms in PFOB makes it a good MR imaging agent for blood pool applications. The ^{19}F nucleus, present in 100% natural abundance, is the second best to hydrogen for MR imaging applications (Mattrey, 1989). Its vascular retention makes it useful for showing the vascular pool in most solid organs.

1.3 Magnetic Resonance Imaging (MRI)

MRI is an extension of nuclear magnetic resonance (NMR). NMR was first observed by two groups, independently: Bloch, Hanson and Parkard (Bloch, 1946) at Stanford University detected a signal from the protons of water and Purcell, Torrey and Pound (1946) at Harvard University observed a signal from paraffin wax. The phenomenon of chemical shift was discovered in 1949 and 1950, when a number of investigators noted that nuclei of the same species absorbed energy at different frequencies (Abraham *et al.*, 1988). The theory laid out in this and following sections pertain to spin $\frac{1}{2}$ nuclei and is not, by any means, a complete overview of MRI. For a more complete review see Abraham *et al.* (1988) and Carrington and McLachlan (1967).

In conventional MRI, a large magnetic field is used to align or polarize the nuclei. This field is termed the B_0 field. Spatially, this is defined as the z-axis (figure 1.2a). A spin $\frac{1}{2}$ nuclei (such as ^{129}Xe) exists in one of two states, spin up or spin down. In the presence of the B_0 field the nuclei line up either with or against the field, depending on their spin. At thermal equilibrium there are slightly more spins in one direction over the other. This sets up a population ratio of the spin-up to spin-down nuclear states governed by the Boltzman distribution. It is the inequity between these two fractions that contribute to an MR signal, yielding a bulk magnetization, M .

Signal acquisition is made possible by ‘tipping’ the bulk magnetization into the transverse (xy) plane, perpendicular to the B_0 field (the field produced by the

magnet). Once in the xy-plane, the magnetization begins to precess about the z-axis like a gyroscope. The rate of this precession is proportional to the field strength. The frequency at which it precesses is detected by RF coils as a free induction decay (FID) (figure 1.3), with an intensity dependent on the xy-component of the bulk magnetization. A 90° pulse will yield the greatest signal as the entire magnetization is tipped into the xy-plane (figure 1.2b). As the bulk magnetization returns to the z-axis, the y component decreases, yielding less signal (figure 1.2c). Since the intensity of the signal picked up by the RF coils is the sum of the magnetization in the transverse plane, each successive pulse decreases the magnetization.

MRI uses magnetic field gradients to create a spatial frequency map that can be used to generate an image (Carrington *et al.*, 1967). Magnetic gradient coils are used to change the field strength along the z-axis. The gradient alters the resonant frequencies of the spins, since the frequency at which the nuclei precess is dependent upon the field strength. When Fourier transformed, this provides a spatial distribution of the spins.

The most wide spread medical use of MR imaging is in diagnostic radiology and it is the imaging method of choice for examining disorders of the central nervous system. It is, however, being increasingly used for imaging diseases in other organ systems and for tumour detection (Stark and Bradley, 1992; Edelman Warach, 1993).

1.4 Hyperpolarized Xenon for MR Imaging

All nuclei with odd number of nucleons, either protons or neutrons, possess the property of a magnetic moment (Carrington *et al.*, 1967) and can contribute to an MR signal. The magnetic moment of a nucleus is related to its gyromagnetic ratio. Since hydrogen nuclei (proton) has one of the highest gyromagnetic ratios (Abraham *et al.*, 1988) and 60-90% of biological tissues are composed of water (Campbell, 1993), proton imaging yields a large signal. For these reasons, current MRI relies mainly on the protons in tissue to provide the NMR signal (Albert *et al.*, 1994).

It is difficult to image nuclei other than protons due to their low density in tissue as well as their relatively low gyromagnetic ratios. Since no organic nuclei provide the necessary signal intensity, it is, therefore, desirable to introduce a biologically compatible species with the necessary signal intensity.

The technique of laser polarization or hyperpolarization of nuclei, first developed in 1950 (Kastler, 1950) can be used to hyperpolarize noble gases. ^3He and ^{129}Xe are the only nonradioactive nuclei suitable for NMR that can be laser polarized. ^{129}Xe is hyperpolarized through interactions with optically pumped rubidium vapour. The energy is transferred from the rubidium valence electrons to the nucleus of the ^{129}Xe via collisions. The polarization is typically 1-2% in our lab compared to $1 \times 10^{-6}\%$ normally. Albert *et al.* (1994) are able to achieve polarizations of up to 25% using a higher power laser and dynamically optimizing their oven temperature.

Initially, hyperpolarized noble gases were used to image animal lungs. Albert *et al.* (1994), Sakai (1996) and Wagsul *et al.* (1996) have obtained images of small animal lungs, both live and excised, using hyperpolarized ^{129}Xe . Hyperpolarized ^3He tends to be better suited to lung space imaging due to its low solubility in blood and tissue, while ^{129}Xe is promising for imaging the brain and other tissues (Patyal *et al.*, 1997). Albert *et al.* (1994) predicted the possibility of High Resolution Computed Tomography (HRTC)-like images of lung tissue density variation using ^{129}Xe gas. In addition, it may be possible to obtain images of the time-dependent dissolved ^{129}Xe distribution that reflects alveolar function since it dissolves readily in oils and lipid rich materials, including cellular membranes.

Xenon is extremely sensitive to its chemical environment, having a range in its resonance frequency or chemical shift of about 200 ppm (Albert *et al.*, 1994). This is a broad range compared with that of protons, which has a chemical shift range of about 10 ppm (Abraham *et al.*, 1988). For this reason, ^{129}Xe can be used for perfusion imaging of the brain, lung and other organs that are lipid rich. Groups including Swanson *et al.* (1997) and Duhamel *et al.* (2000) have produced dissolved-phase images of rat lung, heart, brain and kidneys. These results show the potential of dissolved phase ^{129}Xe for use in measuring cerebral blood flow, cardiac perfusion, kidney perfusion and lung function.

1.4.1 Longitudinal Relaxation Times (T_1)

Using HXe requires special considerations. Once hyperpolarized, the ^{129}Xe will return to thermal equilibrium over time. This ‘half-life’ is known as longitudinal relaxation

(T_1 relaxation). Keeping the hyperpolarized gas in a strong magnetic field and in a container that decreases interactions between the gas and the container help to maintain polarization. PFOB emulsions are also expected to increase T_1 relaxation times. In addition to its natural tendency to relax, the RF pulses supplied to the gas in order to tip the bulk magnetization into the xy-plane also decrease polarization.

Figure 1.4 shows the bulk magnetization being tipped by angle α , the remaining magnetization is the bulk magnetization multiplied by the cosine of the tip angle. For this reason, the tip angles must be small enough such that the HXe is not completely depolarized by the RF pulse sequence. On the other hand, the tip angle must be large enough for the xy-component to yield sufficient SNR. Any ferrous or paramagnetic material (including oxygen, steel and deoxyhaemoglobin) will also increase the rate at which the hyperpolarized nuclei relax. For this reason, care must be taken in the preparation and administration of the HXe in order to maintain a long T_1 .

1.4.2 Spectral Line-width [Transverse relaxation (T_2^*)]

In an NMR experiment, the signals are Fourier transformed to separate the complex superimposed frequencies that of which the FID is comprised. For each frequency the Fourier transform shows a series of Lorentzian-shaped peaks. The transverse relaxation time (T_2^*) describes the rate at which the xy-component of the magnetization dephases (figure 1.5). When the bulk magnetization is tipped into the xy-plane (figure 1.5a), it begins to precess. The width of the peak at half the

maximum signal is inversely proportional to the transverse relaxation time (T_2^*) (equation 1). During this precession the dipoles begin to dephase (figure 1.5b and c).

$$\text{FWHM} = (\pi T_2^*)^{-1} \quad \text{Eqn. 1}$$

Where FWHM is the width of the peak at half the height (frequency width half max).

Therefore, as T_2^* increases, line width decreases. Since the area under the peak is conserved, increasing T_2^* has the effect of increasing SNR.

1.4.3 Perfluorooctylbromide as a Carrier for Hyperpolarized ^{129}Xe

Further to the uses of PFOB emulsions described in the previous two sections, PFOB may be used as a carrier for HXe. Site directed injections of ^{129}Xe dissolved in a biologically compatible carrier would have the advantage of increasing the local concentration of ^{129}Xe relative to inhalation as a delivery method. When ^{129}Xe is inhaled it is dissolved in the blood stream and transported throughout the body.

Albert *et al.* (1995) have shown T_1 s of 4.5 s in red blood cells, 9.6 s in plasma, 26.3 s in distilled water. Their samples were prepared at atmospheric oxygen pressure.

These T_1 s are short but imaging with them is feasible as long as the HXe can get to the tissue of interest. The carrier would confer further advantages if the T_1 of dissolved HXe was longer than in blood. Groups including M.S. Albert at Harvard Medical School in Boston, US (Venkatesh *et al.*, 2000), and A. Bifone at The Clinical

Magnetic Resonance Group in Surrey, UK (Lavini *et al.*, 2000; Wolber *et al.*, 1999) have performed an intravenous injections using PFOB as a carrier for HXe.

The amount of HXe signal from a tissue is dependent on several factors including perfusion of the tissue, T_1 of HXe in the emulsion and blood and T_2^* relaxation times. T_1 is dependent on oxygenation and exchange time between the emulsion and its surroundings.

1.4.4 Spectral Line Broadening Due to Exchange of Xenon Between the PFOB Fraction and Water

Xenon experiences a very broad range of chemical shifts depending on its environment. Relative to the gas peak, the peak for xenon dissolved in water is found at 195 ppm, while the peak for xenon dissolved in pure PFOB is found at 104 ppm (figure 1.6). Since the PFOB must be emulsified for intravenous injections (as discussed in section 1.1), a third dissolved phase peak is seen at close to 110 ppm (figure 1.6). This peak tends to be broader than either of the pure peaks. This is a result of averaging between the water peak and the PFOB peak as the xenon nucleus finds itself jumping at random from one environment to another (Carrington and McLachlan, 1967).

There are two limiting cases, denoted fast exchange and slow exchange (Carrington and McLachlan, 1967). When the xenon nucleus finds itself in the slow exchange regime, all three dissolved phase peaks may be resolved, depending on the

variation in droplet sizes. This regime occurs when the emulsion droplets are sufficiently large and dilute, such that there is detectable portion of xenon nuclei experiencing only one environment. This is represented in figure 1.6 by arrow A, which remains in the water and arrow D, which remains in a droplet of PFOB. Arrows B and C represent xenon nuclei that are moving from one environment to another. These nuclei experience a chemical shift that is the average of the two frequencies (Carrington and McLachlan, 1967). The nuclei are jumping from one environment to the other so rapidly compared with the difference in resonance frequencies that they hardly lose phase from one time they are on a particular site to the next (Carrington and McLachlan, 1967).

Holding the water and PFOB fractions of the emulsions constant, the width of the line corresponding the exchanging nuclei is dependent on the size of the droplets, and as such, the dilution of the droplets. As the size of the droplets increases, the distance between droplets increases. Emulsions with smaller average droplet sizes can be considered more concentrated. They have more xenon nuclei in the fast exchange regime and few to none in the slow exchange regime. These nuclei have even less time to rephase between environments, resulting in a narrower line-width. Maximum line broadening occurs during an intermediate exchange regime. This occurs when the droplets are too small to be in the slow exchange regime but too large to be in fast exchange. For a more complete overview of the effects of exchange on line-width see Pople *et al.* (1959) and Carrington and McLachlan (1967).

1.5 Thesis Overview

The goal of this research was to determine the effects of PC chain-length on droplet size and in turn, the effects on the T_1 and T_2^* relaxation times for HXe in PC-stabilized PFOB emulsions. Phosphatidylcholines with twin 18 carbon tails (DOPC) and twin 4 carbon tails (DBPC) were used to stabilize PFOB emulsions. Their properties were compared with each other as well as published sizes and relaxation times for similar emulsions stabilized with Egg-Yolk-Phosphatidylcholine (EYP). This thesis was also concerned with the depolarizing effects of oxygen, which is paramagnetic. Protocols were developed to substantially reduce oxygen contamination.

It was found that DBPC produces smaller droplets and has a narrower line-width than emulsions prepared with DOPC. There was no significant difference between the T_1 s of the two emulsions; however, the DBPC stabilized emulsions had a significantly sharper line-width. It was also found that through rigorous elimination of oxygen from the emulsions, T_1 relaxation times increased from under one minute to almost three minutes.

Figure 1.1 Schematic of phosphatidylcholine showing its various regions as well as its orientation at the interface (adapted from Gennis, 1989).

Figure 1.2 A 3-space representation of (a) the bulk magnetization, M , along the z -axis, (b) a 90° pulse completely tipping the magnetization and (c) the projection of the magnetization (M_y) in the xy -plane as the bulk magnetization returns to the x -axis.

Figure 1.3 A free induction decay (FID) characterized by a sinusoidal offset frequency and an exponential decay constant (T_2^*)

Figure 1.4 Effect of the progressive saturation method on bulk magnetization.

Figure 1.5 Transverse relaxation of the bulk magnetization in the xy -plane. The magnetization dephases due to T_2^* as it precesses about the origin of the xy -plane.

Figure 1.6 The chemical shift of xenon due to its environment. The circles represent PFOB droplets of differing sizes, suspended in water. The lettered arrows (top) indicate the random movement of xenon in the emulsion and correspond to peaks on the spectrum (bottom). All chemical shifts are relative to the gas peak. This spectrum is not to scale.

Chapter 2

Materials and Methods

2.1 Preparation of Emulsions

Emulsions for all experiments, unless otherwise noted, were prepared in the following manner. The emulsion typically used was comprised of 4% w/v 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 90% w/v perfluorooctyl bromide (PFOB) and 53% w/v distilled water. Emulsions were typically made in 5.5 mL batches consisting of 2.6 mL PFOB, 2.9 mL ddH₂O and 220 mg DOPC. This emulsion will, hence forward, be termed the ‘standard emulsion.’ The mixtures were shaken and then sonicated using a Virsonic 60 probe sonicator at powers between 4 and 10, depending on the total volume of the mixture. Sonication time also depended on the volume. Larger volumes required longer sonication at a higher power in order to thoroughly emulsify. For volumes below 1 mL, sonication did not exceed 2 minutes. Volumes up to 6 mL were sonicated for 5 minutes. Smaller volumes have a tendency to incorporate air into them if the sonication power is too high. This causes the emulsion to foam. All lipids were purchased from Avanti Polar Lipids, Alabaster AL. Perfluorooctylbromide was purchased from Sigma-Aldrich, St. Louis MO.

2.1.1 Stability of the Emulsions

In order to determine the amount of 1,2-Dibutyroyl-sn-glycero-3-phosphatidylcholine (DBPC) required to stabilize an emulsion, quantities of DBPC were added which range from less than the molar equivalent of 4% w/v DOPC, to greater than 4% w/v DBPC (Table 3.1). DOPC-stabilized emulsions, due to their extended stability were simply observed over weeks and months for settling and separation. Attempts at stabilizing emulsions using 1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) were also made starting with 4% w/v DOPE and increasing to 12%.

2.2 Sizing of the Emulsion Droplets

It is important to know the size of the emulsion droplets if they are to be injected into animals. Droplets that are too large may plug capillaries and never reach the desired target. Another reason it is necessary to know the size of the droplets is for the exchange model, section 2.4. No single particle-sizing method was capable of describing the particle size distribution. It was, therefore, necessary to use several sizing methods. Each method was used to check results from the other methods.

2.2.1 Light Microscopy

A Reichert Nr. 337 639 (Austria) phase contrast microscope with a Wild 2302 (Heerbruff, Switzerland) camera was used to photograph the emulsion droplets. Kodak Tmax 400 film was used in the camera. Micrographs were taken of emulsions produced using phospholipids of varying chain lengths to determine if a relationship

existed between chain length and droplet size. This was also done to determine the size distribution of the standard 4% w/v DOPC emulsion. Table 2.1 lists the phospholipids with their abbreviations, chain lengths and quantities added. With the exception of the 50 mg DBPC and the 33 mg DOPC emulsions, the mass of each phospholipid to be added was calculated, keeping the molar ratios the same as in the 4% w/v DOPC emulsion.

The samples were contained and sonicated in 10 mL Pyrex tubes. Emulsions were sonicated for 5 minutes. The emulsions were far too concentrated to view clearly under the microscope. As such they were diluted 11 fold in distilled water. A drop of the diluted emulsion was placed on a microscope slide with paper shims on either side of the drop. A cover slip was placed on the shims to keep the cover slip from flattening out the droplets, thereby changing their apparent diameter.

Pictures were taken of each trial under the 40x objective. The oil immersion lens was attempted but there was too much movement on the slide, causing the pictures to blur. A micrograph of a micrometer was taken to measure the droplet sizes.

2.2.2 Scanning Electron Microscopy

Three emulsions were observed using a JEOL JSM 6400 scanning electron microscope (Japanese Electron Optic Limited, Japan). The standard 4% w/v DOPC emulsion as well as a 6% w/v DOPC emulsion and a 9.1% w/v DBPC emulsion

which was found to be most stable (section 3.1). The Coulter counter requires saline as an electrolyte to function properly. The effect of salination, both for Coulter counter data as well as *in vivo* injection, was uncertain. To detect any drastic changes in droplet size, a sample of the standard emulsion was also observed diluted in saline.

All samples were treated in the following fashion. One drop (about ½ mL) of sample was placed in an aluminum holder and flash frozen in liquid nitrogen. The drop was then planed using a cryo-microtome. The samples were etched at -90°C and coated with aluminum. The four samples were viewed at magnifications ranging between 50X and 20 000X.

2.2.3 Particle Sizer

Another attempt at determining the droplet sizes of emulsions with different chain length phospholipid emulsifiers was made using a particle sizer. The sizer used was a Submicron Particle Sizer, Autodilute model 370 (Santa Barbara, USA). This method uses light scattering to measure the particles. Emulsions were prepared in the same manner as for the light microscopy sizing, section 2.2.1. The particles are weighted in terms of both number and volume. For all measurements, the gaussian distribution was used unless the χ^2 value was above 3.84. This indicated more than one population of sizes. In these cases, the Nicomp® distribution was used. This sizer has an upper limit of 1 μm .

2.2.4 Coulter Counter

A Coulter counter (Particle Data Inc., Elmhurst IL) was used to size the emulsion droplets and describe the distribution. The coulter counter uses changes in resistance past an orifice to measure and count droplets as they are drawn through the orifice. The counter can be fitted with different sized orifices in order to change the range of sizes measurable. This counter was fitted with an orifice that had a lower limit of 2 μm .

Three emulsions were sized using this method: the standard 4% w/v DOPC-stabilized emulsion as well as a 6% DOPC emulsion and a 2% w/v DBPC-stabilized emulsion. The samples were diluted in saline, as the counter requires electrolytes to make its measurements. The samples were sized in triplicate and standardized since the values were concentration dependent. The mean size of each trial was used to calculate the mean particle size for each treatment. The variance between trials was used to determine significance. The standard deviation about the mean for each treatment was also calculated in order to describe droplet size variance. This was done by averaging the standardized data from the trials in each treatment before calculating the mean and standard deviation.

2.3 Dissolving Hyperpolarized ^{129}Xe in the Sample

There are many steps involved in dissolving HXe in a sample. Sections 2.3.1 through 2.3.4 outline the procedures involved in polarizing the gas and the actual mixing.

2.3.1 Gas Manifold

A gas manifold (figure 2.1) designed to move gas from one vessel to another via pressure gradients was designed by Pascale Sevigny (1999) and constructed by Steve Breeze (NRC, Ottawa Canada). High vacuum Teflon and glass components were used in the construction of the manifold. Table 2.2 lists the volume of each section of the manifold defined by the closed valves seen in figure 2.1.

To monitor pressure, the gas manifold had a Penning gauge (Edwards) to monitor high vacuum (10^{-7} to 10^{-2} mbar) and a vacuumeter (VM1500, Ebro) to monitor pressures from 0 to 1500 mbar. The vacuumeter was used for monitoring gas transfers. The manifold was maintained at pressures of about 8×10^{-6} Torr with a roughing pump and a diffusion pump (Alcatel).

2.3.2 Filling the Optical Pumping Cell

The optical pumping cell is a glass container with a volume of 60 cc. Its inner surface is coated with SurfaSil (Pierce Chemical Laboratories). The pumping cell contains 3 components: a small amount of rubidium metal, 800 mbar of natural abundance xenon (26.4%) ^{129}Xe , and 779 mbar of nitrogen. The rubidium is added to the cell upon construction. Rubidium is highly reactive with oxygen. For this reason, filling the cell must be done under high vacuum. To ensure this, the pumping cells were pumped on over night with high vacuum prior to filling. If the rubidium oxidizes the cell must be remade.

The optical pumping cell required refilling after about five transfers. The pumping cell was attached to the manifold as shown in figure 2.1. All valves were opened except valves 6,7,8 and 9. The manifold is pumped on until a vacuum of 8×10^{-6} mbar was reached. Valve 2 was then closed and the valve to the xenon reservoir (valve 8) was opened until the vacumeter read 284 mbar pressure in the manifold. The pumping cell was then cooled with liquid nitrogen in order to collect the xenon into the pumping cell. Valve 9 was opened until there was 200 mbar in the manifold. The pumping cell valve and valve 5 were then closed. The pumping cell was removed and allowed to thaw, while valve 2 was opened to pump the remaining nitrogen out of the manifold. The final pressure in the pumping cell was about 1.5 atm (800 mbar xenon and 779 mbar nitrogen).

2.3.3 Laser Polarization Setup

^{129}Xe was hyperpolarized using a 30 W Al-Ga-As diode array laser (Opto-Power, Tucson AZ) emitting at 794.8 nm. The beam was polarized with a $\frac{1}{4}$ wavelength circular polarizing filter and widened into a large parallel beam by a set of lenses. Figure 2.2 depicts the set-up used. The laser beam was set parallel (ie. along the z axis) to the magnetic field in the most homogeneous part of the fringe field of the magnet described in section 2.4. The field strength at the oven was approximately 100 Gauss, and was set about 1.5 m from the bore of the magnet (figure 2.2). The ^{129}Xe was irradiated for 20 minutes with the oven at 88°C.

2.3.4 Transfer of HXe from the Optical Pumping Cell to the Mixing Cell and Dissolving it into the Sample

The HXe was transferred from the optical pumping cell to a clean, evacuated glass mixing cell (figure 2.3). The transfer line was kept at room temperature to prevent rubidium from escaping the pumping cell. It was assumed little to no rubidium escaped the pumping cell and any that did escape, solidified in the transfer line. For *in vivo* studies it may be necessary to introduce a cold trap to the transfer line or cool the pumping cell after polarization in order to ensure there is no rubidium vapour carried with the HXe.

The mixing cell was evacuated to a pressure of about 8×10^{-6} using the manifold. Once evacuated, valve C (figure 2.3) was closed and the cell was removed from the manifold. The cell was then attached to the T-connector with the polarization cell and the aspirator. The polarization cell was placed in the oven as described in section 2.3.3. During polarization, valve B is open to evacuate the transfer line (ie. between valve A and C). After 20 minutes of polarization the transfer apparatus is removed from the oven and carried to the bore of the magnet. The aspirator valve (B) is first closed. Valve A is opened, releasing HXe to the line and the mixing cell is opened (valve C). Valves A and C are then closed to prevent air from entering the system or HXe from escaping.

The mixing cell is then removed from the T-connector (figure 2.4) and placed in the bore of the magnet to maintain polarization. The pumping cell, still connected

to the aspirator, was set aside. The resultant pressure in the mixing cell is in excess of 1 atm, decreasing with each transfer.

Holding the mixing cell close to the bore of the magnet, the samples were injected from a syringe through a rubber septum and vigorously shaken for 5 seconds. The mixing cell was then put in the 1 cm coil described in section 2.4.

2.4 NMR Spectroscopy

All NMR experiments were performed using a 1.89 T Magnex (Abingdon, England) 30 cm bore super-conducting magnet controlled by a MRRS (formerly SMIS) (Surrey, UK) MR5000 console. The mixing cell (described in section 2.3.4) was placed in a solenoid coil with an inside diameter of 1 cm, tuned to the xenon frequency (22.18 MHz). Relaxation measurements were obtained using a progressive saturation method adapted for HXe (Section 1.2.1).

The progressive saturation method was used to calculate T_1 using equation 2. The signal intensity (I_N) of a hyperpolarized sample after the Nth radio frequency (RF) pulse in a series of equally spaced pulses each with tip angle α is given by:

$$I_N = I_0 \cos^{N-1}(\alpha) \sin(\alpha) e^{-(N-1)T_R/T_{1\text{eff}}} \quad \text{Eqn. 2}$$

Where

$$T_{1\text{eff}} = T_1^{-1} - \ln(\cos \alpha) T_R^{-1}$$

Where I_0 is the intensity of the initial magnetization, T_R is the time interval between each pulse and T_1 is the longitudinal relaxation time. This is only true for hyperpolarized samples since initial magnetization is orders of magnitude greater than the thermal equilibrium value (Patyal *et al.*, 1997).

The T_1 data were processed and fitted with Eqn. 2 using MatLab (The Math Works Inc., Natick, MA). Chemical shift positions were referenced with respect to the xenon gas peak.

T_1 measurements were made using 32 pulses with a 120 second TR , unless otherwise noted. T_2^* measurements were calculated using the frequency width half max (FWHM) of the spectra peak corresponding to the chemical shift of ^{129}Xe in the emulsion according to Eqn. 1. The peak of the first pulse was used, as it had the greatest SNR. It also ensured that there was little or no settling or coalescence of the droplets, which may affect the line-width.

2.4.1 Tip Angle Calibration

There are a number of factors that affect the output power of the transmitter including the tuning of the RF coil to the resonance frequency and the loading of the RF coil. These factors affect how efficiently RF energy is transferred to the sample. As such, the tip angle must be calibrated experimentally. This was done using a pulse sequence with 6 pulses separated by a repetition time (TR) of 100 ms which is far

shorter than the T_1 , which is on the order of minutes. This sequence minimizes decay due to T_1 . The decay, in this case, is described by Eqn. 3 with $TR \ll T_1$:

$$I_{N+1}/I_N = \cos(\alpha) \quad \text{Eqn. 3}$$

That is, with the condition that the T_R is much smaller than T_1 , the decay is almost entirely due to the pulses.

2.4.2 Effect of Oxygenation on T_1

Samples of pure PFOB and distilled water were oxygenated by shaking them in a closed container with 1 atm of air. The pure samples were deoxygenated by pumping on the liquid with a roughing pump (0.01 Torr) in the mixing cell. The standard 4% w/v DOPC emulsion was used for these experiments. T_1 relaxation times of the emulsion samples were measured directly after preparation or after deoxygenation by slowly evacuating and agitating the container under a vacuum of 0.1 Torr to avoid excessive foaming. Care was taken to replenish any water removed by the evacuation. HXe was transferred to the mixing cell. In the case of the deoxygenated samples, the liquid was already in the mixing cell. For oxygenated samples, the liquid was injected into the mixing cell as shown in figure 2.3.

The tip angle was chosen to provide adequate SNR and recalibrated for each sample with values ranging from 7 to 20 degrees. Thirty-two RF pulses were used with a TR of 2-120 seconds.

2.4.3 Effect of Phospholipid Chain Length on Longitudinal Relaxation Time and Line-Width (T_2^*)

To determine the effect of chain length on the longitudinal (T_1) relaxation time, the relaxation times for the standard emulsion and the molar equivalent (2% w/v) DBPC emulsion were measured along with that of water and pure PFOB. All emulsions were made in a glove bag, saturated with argon, to minimize oxygen contamination. In order to remove as much oxygen as possible, it was necessary to do most of the preparation in an argon (BOC Gases) glove bag (Fisher Scientific). The glove bag contained all the necessary components and equipment, including the sonicator probe, reagents, a vacuum tube, a 1000 μ L pipetteman and tips and syringes for transport to the magnet (figure 2.5).

The lipid surfactant was weighed into a 13 mL graduated, snap cap tube (Falcon) in the atmosphere as there was no room for a scale in the glove bag. With all necessary materials in the glove bag, it was filled with argon and allowed to slowly deflate 5 times before the reagents were mixed and sonicated. All reagents were pumped on for ten minutes using a line running to a sink aspirator (Figure 2.5). The syringes were pulled apart and reassembled in order to remove any air from them. The emulsions were sonicated in the same manner as described in section 2.1.

The prepared samples were drawn into the syringe and any excess argon was ejected. The syringe cap was replaced and the syringe was removed from the glove bag through opening #4 (figure 2.5). At the magnet, the procedure was followed as outlined above (section 2.3.4).

Table 2.1 Composition of the emulsions photographed under the phase contrast microscope. The indicated amount of phospholipid was added to 290 μ L distilled water and 260 μ L PFOB

Phospholipid	Abbreviation	Chain length	Quantity	
			Mass (mg)	Weight per volume (%)
1,2-Dibutyroyl-sn-glycero-3-phosphocholine	DBPC	4	11.1	2.0
			50.0	9.1
1,2-Dioctanoyl-sn-glycero-3-phosphocholine	DOcPC	8	14.3	2.6
1,2-Dilauroyl-sn-glycero-3-phosphocholine	DLPC	12	17.4	3.1
1,2-Dipalmitoyl-sn-glycero-3-phosphocholine	DPPC	16	20.5	3.7
1,2-Dioleoyl-sn-glycero-3-phosphocholine	DOPC	18	22.0	4.0
			33.0	6.0
1,2-Diarachidoyl-sn-glycero-3-phosphocholine	DAPC	20	23.7	4.3

Table 2.2 Volumes of the manifold. The volumes describe sections of the gas manifold depicted in Figure 2.1. Volumes taken from Sevigny (1999).

Volume delimited by:	Volume (mL)
V#1 and V#2	145
V#2, V#3, V#4 and V#5	38.5
V#3, V#6, V#7 and the cold trap	15.5
V#5 and the outlet	4
V#4, V#8 and V#9	8

Figure 2.1 Gas manifold used for filling the optical pumping cell. The valves labeled 1-9 isolate the various sections of the manifold. The volumes of these sections can be found in table 2.2.

Figure 2.2 Schematic of the laser polarization apparatus

Figure 2.3 HXe transfer apparatus. The pumping cell is attached to a sink aspirator and the mixing cell via a T-connector and Swagelok® fittings.

Figure 2.4 Mixing HXe with the emulsion. The numbered arrows indicate the order of the steps involved.

Figure 2.5 Depiction of the argon glove bag used for deoxygenating emulsions. All equipment was placed in the bag through the front opening, #1. The coaxial cable from the probe was fed through an opening made in obstruction 2. The aspirator hose was fed through #3. Argon was fed into the system through #5 and syringes containing the deoxygenated emulsion were removed through #4.

Chapter 3

Results

3.1 Stability of the Emulsions

Emulsions stabilized with DBPC do not remain emulsified as long as those made with DOPC. Emulsions made using 4% w/v DOPC remain stable on the order of months. Settling did occur but simply shaking the emulsion tended to suspend the droplets for days to weeks. DBPC-stabilized emulsions are less opaque and have a slightly bluish hue compared with the stark white of the DOPC-stabilized emulsions. Five emulsions ranging in the quantity of DBPC were made and observed for separation (table 3.1). The 10 mg stabilized emulsion is 0.2% w/v less than the molar equivalent to the standard emulsion. It was not possible to stabilize the emulsion for an hour, as was the case with the 3.6% w/v stabilized emulsion. The 5.4% w/v stabilized emulsion stayed emulsified for approximately 2 hours, whereas, the 7.2% w/v stabilized emulsion separated after a day. The 9.2% w/v stabilized emulsion was stable after three days. Pure DOPE was not an effective stabilizer. It was unable to stably disperse the PFOB for any length of time.

3.2 Sizing the Emulsion Droplets

3.2.1 Light Microscopy

The micrographs of the emulsions clearly show a large range of sizes. Figure 3.1 is a micrograph of the standard emulsion. The droplets range from about 5 μm to droplets too small to resolve at this magnification. Going to the oil immersion lens did not prove fruitful as there were currents causing the droplets to move quite quickly at that power. The droplets were visible with the oil immersion lens but were not photographable as the exposure time of the camera was far too long, causing the droplets to appear as streaks in the micrograph. Many of the droplets in figure 3.1 are out of focus. These droplets were not considered for size as there was no way to measure their true diameter.

3.2.2 Scanning Electron Microscopy

The SEM micrographs (figures 3.2, 3.3 and 3.4) show that the majority of the droplets are submicron for the three emulsions observed. These figures show well, the density of the emulsions. There are few droplets that are greater than a micron. The arrow in micrograph B of figure 3.3 has a cluster of droplets, which may be one large droplet, covered in smaller droplets. This cluster is approximately 5 μm in diameter, including the smaller droplets around the perimeter. It is not clear if the droplets from any of the emulsions are substantially larger or smaller than the others.

The emulsion seen in micrograph B of figure 3.4 is diluted in saline. It is possible to see the solutes lining up due to the freezing, more so, than in the other micrographs. The droplets appear to line up with the solutes.

3.2.3 Particle Sizer

In all cases except the 0.85% w/v sample and the DAPC sample, there were two populations of sizes. Figure 3.5 displays the results of varying the chain lengths. For the large diameter populations of droplets for each chain length, the trend is the same whether the droplets are sized with respect to the number or the volume weighted. As chain length increased, the size of the droplets increased. Between the 18 carbon (DOPC) and the 20 carbon (DAPC) there was a plateau. By the number weighted curve, the size plateaued at about 0.825 μm . By the volume weighted curve the size plateaued at about 0.860 μm . It is important to note, however, that the upper limit of the particle sizer is 1 μm . The second, smaller population of sizes was under 0.1 μm except for the standard emulsion, which was 252.4 by volume and 236.9, by number. The smaller populations did not seem to follow any trend. The size of droplets was about 0.15 μm larger when measurements were weighted by volume.

The 0.85% w/v DOPC-stabilized emulsion did not produce droplets as large as the standard emulsion. They were approximately 180 nm smaller than the standard emulsion droplets.

3.2.4 Coulter Counter

Figure 3.6 is the plot of the size distribution for the standard emulsion, 6% w/v DOPC and 2% w/v DBPC emulsions output from the Coulter counter. The plot for each treatment is the result of standardizing and averaging three trials. It shows a long tail extending past 8 μm for all three emulsions. The upper limit of the Coulter counter was 36 μm but few droplets were seen past 8 μm . The plots are truncated at 1.5 μm due to the 2 μm lower limit of the orifice used. Figure 3.7 depicts the average droplet sizes and standard deviations between trials for the standard emulsion, 6% w/v DOPC stabilized emulsion and the 2% w/v DBPC stabilized emulsion. The error bars are very small and do not overlap indicating a significant difference in droplet size with differing treatment. The average size of droplets were found to be $3.05 \pm .01 \mu\text{m}$ for the standard emulsion, 2.80 μm for the 6% DOPC-stabilized emulsion and $2.42 \pm 0.01 \mu\text{m}$ for the DBPC-stabilized emulsion.

The variance in droplet size of each population of the differently stabilized emulsions can be seen in figure 3.6. While the mean size of droplets is significantly different between treatments (figure 3.7), there is a very large range in the size of the droplets in each treatment. As such, there is a great deal of overlap in droplet size between the treatments.

3.3 NMR Spectroscopy

All T_1 measurements were made by plotting the intensity of the emulsion peak as it decayed over time. A curve was then fitted to the data and using a value for the tip angle, calibrated for that trial, a value for the T_1 was calculated. Figure 3.8 is an example of a standard fit.

3.3.1 Effect of Oxygenation on T_1

Deoxygenated, neat PFOB has a very long T_1 relative to water and the emulsions (figure 3.9). However, oxygen has the greatest effect on the T_1 of pure PFOB.

Oxygenating the pure PFOB sample decreased its T_1 by more than 200 seconds, from 287.5 $\pm$ 83.9 s to 84 $\pm$ 23.7 s. Oxygenation had the least effect on the pure water sample. The T_1 of the oxygenated water is only 10 s shorter than that for the deoxygenated water. This is not a significant decrease due to sampling variance (figure 3.9).

The increase in T_1 of the standard emulsion due to deoxygenation was significant (figure 3.9). The T_1 of the oxygenated standard emulsion was 54.0 $\pm$ 22.7 s. Through rigorous deoxygenation, it was possible to increase the T_1 to 160.4 $\pm$ 23.4 s (figure 3.9).

3.3.2 Effect of Phospholipid Chain Length on Longitudinal Relaxation Time and Line-Width

3.3.2.1 T_1 Measurements

The T_1 measurements described here are those of the deoxygenated samples. The T_1 for the standard emulsion was found to be 160.38 s with a standard deviation of 23.44, N=5. The standard deviation is quite large due to one outlying data point. The average T_1 for the DBPC-stabilized emulsion was found to be 169.73 s with a standard deviation of 34.39, N=4. There is no detectable significant difference between these two averages. However, the sample size is very small and the data set for the DBPC-stabilized emulsion had one outlier that brought the average down. With the outliers removed the average T_1 for the DBPC-stabilized emulsion was 186.47 ± 9.6 s. The non-parametric Mann-Whitney test did not show a significant difference between the T_1 of the DBPC-stabilized and the standard emulsions.

3.3.2.2 Line-Width (T_2^*)

The line-widths measured for the emulsions were greater than those of the water and pure PFOB (figure 3.10). Both the water peak and the PFOB peak had line-widths of 0.36 ppm. The DOPC-stabilized emulsion had a line-width of 4.74 ± 0.54 ppm and the DBPC-stabilized emulsion had a line-width of 2.71 ± 1.54 ppm. The sampling standard deviations for the two emulsions do overlap (figure 3.10) but the Mann-Whitney test for independence shows there is a significant difference ($0.005 < P[U \geq 62] < 0.01$).

Table 3.1 Quantity of DBPC required to stably emulsify PFOB in water. The emulsions consisted of 0.26 mL PFOB, 0.29 mL ddH₂O and the indicated mass of DBPC.

Quantity of DBPC		Stability
Mass (mg)	Weight per volume (%)	
10	1.8	< 1 hour
20	3.6	< 1 hour
30	5.4	~ 2 hours
40	7.2	~ 1 day
50	9.1	> 3 days

Figure 3.1 Micrograph of the standard emulsion viewed through a phase contrast microscope.

Figure 3.2 Scanning electron micrographs of the standard emulsion.

Figure 3.3 Scanning electron micrographs of the 6% w/v DOPC emulsion. The arrow in B indicates a possible droplet that is in excess of 1 μm.

Figure 3.4 Scanning electron micrographs of the 2% w/v DBPC stabilized emulsion (A) and the standard emulsion diluted with saline (B).

Figure 3.5 Emulsion droplet sizes using a particle sizer. Five emulsions with different PC chain lengths were measured as well as one 0.85%w/v DOPC-stabilized emulsion. All other emulsions were stabilized with the molar equivalent of the 4% w/v DOPC-stabilized emulsion. The upper measurable limit of the Particle sizer is 1000 nm.

Figure 3.6 Distribution of the size of droplets in the standard emulsion (diamonds), 6% DOPC emulsion (squares) and the 2% DBPC emulsion (circles). The measurements were taken using a Coulter Counter. The dotted line at 2 μm indicates the lower accurate limit of the Coulter Counter.

Figure 3.7 Mean size and standard deviation between trials for the standard emulsion, 6% w/v stabilized DOPC emulsion and 2% w/v stabilized DBPC emulsion. The error bars represent sampling variance.

Figure 3.8 Plot of a typical T_1 fit to Eqn. 2.

Figure 3.9 Average T_1 s for oxygenated and deoxygenated 'Neat' PFOB, water and the standard emulsion as well as the deoxygenated DBPC-stabilized emulsion. The error bars represent sampling variance.

Figure 3.10 Line-widths (FWHM) for HXe dissolved in water, pure PFOB, the standard emulsion and the DBPC-stabilized emulsion. The error bars represent sampling variance.

Chapter 4

Discussion

This thesis investigated the effects of phosphatidylcholine chain-length on the size of Perfluorooctyl bromide emulsion droplets produced as well as the longitudinal relaxation time and line-widths of hyperpolarized xenon. It is known that oxygen plays a major role in depolarizing HXe (Hedlund *et al.*, 2000). For this reason, the effect of oxygen on T_1 was also quantified.

PFOB was used as the carrier for its ability to absorb gases and its potential for protecting the magnetization of HXe (see section 1.5). It is a biocompatible compound, capable of dissolving more than 15 times more xenon than water is capable (Pollack *et al.*, 1992). Another valuable trait that makes PFOB a good carrier is the narrow line-width exhibited by HXe when dissolved in PFOB. Other carriers, such as Fluosol® (20% w/v perfluorodecalin, Green Cross Corporation, Japan), experience severe dissolved-phase line broadening (Wolber *et al.*, 1999). In order to maximize SNR it is critical to minimize line-width since the area under the peak in a Fourier transform is conserved.

4.1 Stability of Emulsions

Emulsion stability is important for many reasons. These include consistency of the signal and data over time (ie. are the HXe relaxation properties changing over time?) as well as the need for a well-characterized emulsion if it is to be used for *in vivo* imaging. If the emulsion is to be used for intravascular injection it must remain stable since PFOB is an irritant. It is also important for the emulsion to remain stable since increasing the droplet size causes the emulsion to change from the fast exchange regime to an intermediate or slow exchange regime, as described in section 1.4.4. Maximum peak broadening is found in the intermediate exchange regime.

Emulsions stabilized with at least 4% w/v DOPC remain emulsified for months. Riess (1992) showed that 90% w/v PFOB emulsions containing less than 0.3% w/v EYP destabilize within a couple of minutes and that maximum stability using EYP is obtained at 4-6% w/v. Since the density of PFOB is 1.93 times that of water, over time, the fractions separate by weight and the droplets settle. When settled, the likelihood of the droplets coming together and coalescing increases. In the case of the standard emulsion it takes weeks to months for the emulsion to separate. Daily agitation may help to prolong stability by keeping the droplets apart. It is also possible that the settling rate may be used as an indication of droplet size. The stability of emulsions made using DOPC will be a little different due to impurities in EYP such as cholesterol but, nonetheless, are comparable. Undistilled materials, containing impurities such as trace amounts of other PCs and cholesterol,

tend to produce more stable emulsions (Shinoda and Friberg, 1986). Likewise, combinations of surfactants often help to further stabilize emulsions (Shinoda and Friberg, 1986).

In order to emulsify 90% w/v PFOB using DBPC, between 7% and 9% w/v was required. This is the molar equivalent of close to four times that of DOPC. 2% w/v DBPC-stabilized emulsions (the molar equivalent of the 4% w/v DOPC-stabilized emulsion) do not remain stable for more than an hour and will often not emulsify at all. This may be the lower limit using DBPC.

4.2 Droplet Sizes

It is clear that there was no one good way of sizing the droplets, however, the coulter counter proved to be the most useful of the sizing instruments used. The upper limit of the particle sizer was 1 μm while the lower limit of the coulter counter was 2 μm . Both instruments provide average particle sizes within their range although both are close to their respective limits. This suggests two populations of sizes. This idea is corroborated by Wolber *et al.* (1999), who measure the average droplet size, by number, of their 4% w/v EYP emulsion to be 0.4--0.5 μm . They found the size distribution by volume to be bimodal with the average size of the larger particles to be about 4 μm .

For all PCs used except DAPC (20 carbon chain), data from the particle sizer indicate a bimodal population among the smaller droplets. One population of

droplets in the submicron range is similar to Wolber's findings and the other population is between 50 and 250 nm (figure 3.5). Since the upper limit of the particle sizer is 1 μm , all droplets measured by the sizer are in the fast exchange regime (discussed further in section 4.4) and it is, therefore, not important to distinguish between them for any reason other than to describe the population. DAPC did not have any droplets in the smallest range described. Hence forward, all droplets described as large refer to droplets greater than 1 μm in diameter, small droplets are less than 1 μm .

It is likely that the particle sizer data does not give a true indication of the average droplet size. This is especially the case for emulsions stabilized with PCs of chain-lengths longer than 12 carbons. At this point, the sizes begin to plateau as they approach the upper limit of the machine. Measurements made with the Coulter counter show these emulsions to have some droplets as large as 30 μm (data not shown). Droplet size data acquired from the Coulter counter is also incomplete since the droplets are quite close to the 2 μm lower limit of the orifice used. The left tail of the otherwise normal distribution (figure 3.6) describing the population of large droplets is cropped at 1.5 μm for all sample measurements. However, this is not likely to alter the measured average size in a drastic manner. While these two methods are not adequate for describing the true population of droplets, they do cover the sizes Wolber *et al.* (1999) describe using 4% w/v EYP weighted by both number and volume. Further evidence that the size of the emulsion droplets are submicron

when weighted by number are the light micrographs (figure 3.1) and scanning electron micrographs (figures 3.2, 3.3, 3.4).

The majority of particles are submicron and there are few droplets larger than 1 μm per field. The phase contrast micrograph (figure 3.1) does not resolve the smaller droplets seen in the scanning electron micrographs (figures 3.2, 3.3, 3.4). The phase contrast micrograph does show several droplets that are greater than 1 μm amongst many more that are approximately 2—3 μm . There are also many droplets that appear to be smaller but are slightly out of focus. The level of focus of the micrograph is a limiting factor in the usefulness of the data. If a droplet has fuzzy edges, the plane of focus is not on the droplet's centre. Any measurements of the droplet sizes will, therefore, be an underestimation. For this reason, light microscopy is useful only as a means of visualizing the droplets and getting a general idea of their sizes, that is, most droplets are about a micrometer or smaller.

Phase contrast microscopy is able to resolve droplets measurable with the coulter counter. As mentioned in section 3.2.1, photographs taken using the oil immersion lens are of no use. This is due to currents on the slide. These cause far too much motion for the exposure time required, severely limiting the power of this technique. Chemical methods may be used to fix the samples, however, these fixing techniques may alter the droplets by interfering with the surfactants.

The scanning electron micrographs (figure 3.2, 3.3, 3.4) serve much the same purpose as the phase contrast micrographs, but are able to resolve droplets measurable by the particle sizer. This technique uses cryo-freezing with liquid nitrogen to fix the samples. Flash freezing the sample in such a way may best preserve the shape and size of the droplets. There are two problems with this technique. The first problem lies in the planing step whereby the frozen sample is planed using a cryo-microtome. This step is taken in order to produce a flat surface to view. While this helps to increase resolution by reducing the range of depths in a field, it may also serve to underestimate the size of droplets. If a droplet was planed near the bottom it would appear smaller than it actually was. The planing may also mash individual droplets that are grouped together into what appears to be one large droplet. This may be the case indicated by the arrow in figure 3.3 (B). The large droplet indicated is covered in smaller droplets and may in fact, be hundreds of smaller droplets with most of the definition between them obscured by the planing process. On the other hand, the definition that is observable in the mass may be due to the freezing process. The droplets freeze from the outside in. This tends to produce concentric rings as the droplet freezes. It may be these lines which are somewhat obscured by the planing process.

Where the phase contrast micrographs suggest most particles are submicron, the SE micrographs show this to be true. Figure 3.1, the phase contrast micrograph and the SE micrograph in figure 3.2 (A) is the same emulsion viewed under very close to the same magnification. The sample used for the phase contrast micrograph

was diluted in distilled water whereas the SE micrograph is undiluted. These micrographs show the effect of the planing and etching. It is difficult to distinguish between droplets in the SE micrograph, again, due to the difficulty in discerning large droplets from groups of droplets. None of the larger droplets visible in the phase contrast micrograph were observed in the SE micrograph and when viewed at a greater magnification (figure 3.2 B) no large droplets were seen.

It is desirable to dilute the emulsion in order to easily distinguish individual droplets, however, after etching, many of the droplets are no longer suspended in anything and fall away. This can be seen in the SE micrograph of the standard emulsion diluted in saline (figure 3.4 B). The micrograph shows more of a contoured surface rather than the coral-like structure seen in the other SE micrographs of the undiluted emulsions. During the freezing process, the droplets and solutes align in streaks (Ling, 2001). This aligning is advantageous in that it helps to suspend the droplets on the surface but contributes to the mashing effect previously described. While figure 3.4 (B) does not give a good indication of the size of the droplets, it does indicate that the droplets are stable when diluted in saline. This validates the use of the Coulter counter, which requires saline as an electrolyte.

Describing the droplets in terms of their larger population (measurable by the Coulter counter) is acceptable if the droplets are small such that the droplets are in fast exchange since the droplets of the smaller populations (measured by the particle sizer) are in fast exchange. This will minimize line broadening, increasing SNR (see

section 4.4). Signal broadening is discussed later in section 4.4. Of the methods used to measure droplet sizes, the Coulter counter yields the most useful data. This may be improved, however, by using an orifice that is capable of measuring a range from 0 to 20 μm . A dual wavelength particle sizer would also be a valuable tool since they are capable of measuring sizes in this range.

4.3 T_1 Relaxation Time

Prolonging T_1 relaxation time is the primary focus of this research. In order to generate *in vivo* images it is essential that the laser-polarized ^{129}Xe retain its hyperpolarization long enough to reach the tissue of interest with sufficient signal to image.

The greatest effect is seen in the pure PFOB sample while the smallest effect is seen in the pure water sample. There is little change in T_1 of the oxygenated samples between dilutions. However, the T_1 of oxygenated water is significantly longer than pure oxygenated PFOB (figure 3.9)

T_1 s of PFOB emulsions are extremely sensitive to oxygen due to its paramagnetic properties. T_1 s of up to several minutes are possible by the rigorous elimination of oxygen. As described previously (section 1.5.1), PFOB is an effective oxygen carrier. As such, elimination of oxygen is both difficult and more critical than when using carriers with less capacity to dissolve gases, such as water (figure 3.12). The presence of PFOB allows the effective oxygen content to exceed that which may

be present at 1 atm, increasing the amount of oxygen dissolved in the solution, further speeding T_1 relaxation. Figure 3.9 shows the effect of oxygenation on the T_1 of HXe in PFOB. Due to the amount of oxygen PFOB is capable of dissolving relative to water, oxygenation has the greatest effect in pure PFOB. It is for this reason that oxygenated water has a longer T_1 than oxygenated PFOB. As the PFOB is diluted (ie. emulsified in water), and the water fraction increases, the amount of gas the emulsion is capable of dissolving decreases. Observing the T_1 of HXe in the oxygenated samples as they are diluted from pure PFOB to pure water shows the importance of oxygen in the sample if the amount of PFOB in the emulsion is to be maximized. There is nearly a 50 second increase in T_1 between oxygenated PFOB and oxygenated water. This demonstrates the extent to which depolarization occurs due to oxygen dissolved in PFOB.

The difference in the average droplet size between DBPC and DOPC-stabilized emulsions was shown to be significant (figure 3.7). Contrary to expectations, there was no significant difference between the T_1 of the two emulsions (figure 3.9). However, the sample size was quite small. Further study to increase sample size and decrease sampling variance would be beneficial to determine if there is a significant effect on T_1 due to droplet size. If there is any effect at all, it is likely very small considering the average T_1 s of the two emulsions are less than 10 seconds apart. Although the samples underwent rigorous deoxygenation, it is extremely difficult, if not impossible to remove all of the oxygen. It is also extremely difficult to ensure the amount of oxygen dissolved in the sample is the same between samples.

These minute differences cause the sampling variance to be quite broad. As such, a large number of samples would be required in order to see a significant difference. PFOB is expensive (\$65 CDN for 2.6 mL) and as such, this goal may not be worth pursuing for a predictably small change in T_1 .

4.4 Line-Width (T_2^*)

As with T_1 relaxation time, increasing T_2^* is very important for *in vivo* imaging. As described in section 1.4.2, T_2^* is inversely proportional to line-width and as such, SNR. With increased SNR, resolution can be improved. It is much simpler to obtain good SNR *in vitro* since reactions with living tissue are far more complicated than reactions with the glass containers. However, SNR remains a critical factor for the routine that fits an exponential decay to the longitudinal relaxation data (figure 3.8), estimating T_1 . If the SNR is poor there is greater error associated with the fit, and consequently, the T_1 .

Wolber *et al.* (1999) show ^{129}Xe NMR spectral line-width to be droplet size dependent. They measured the line-width from an aged 0.47% w/v EYP-stabilized emulsion (several days old), 4% w/v EYP-stabilized and .85% w/v EYP-stabilized emulsions. They found the aged emulsion had a very narrow line-width (0.96 ppm), which they attribute to larger droplets. Line-widths from Wolber *et al.* (1999) described in this thesis have been corrected for the difference in field strengths. Compared with the 4% w/v EYP-stabilized emulsion at 19 ppm, this constitutes a very significant increase in SNR. Table 4.1 summarizes line-width results from both

the data in this thesis and data from Wolber *et al.* (1999). Experimental results using 4% w/v DOPC had a line width of 4.74 ± 0.54 ppm, substantially narrower than the same w/v EYP.

Wolber *et al.* (1999) demonstrated that line-width decreases as the size of the droplets increase, until droplets are approximately 5 μm . They speculate that when the droplets are greater than 5 μm they do not completely saturate with xenon. It is more likely that the droplets are far enough into the slow exchange regime such that they are approaching the line-width of the pure components (0.36 ppm).

The trend described by Wolber *et al.* (1999), at face, seems contradictory to the data obtained in this study. Droplets stabilized with DBPC were shown to be smaller than DOPC-stabilized droplets (figure 3.7) yet experienced a narrower line-width (table 4.1). Bearing in mind the exchange model described in section 1.4.4, they may not be conflicting. It is likely that the emulsions used in this study fall within the fast exchange regime, whereas the emulsions used by Wolber *et al.* (1999) are likely in the intermediate to slow exchange regimes. Figure 4.1 describes the relationship between their findings and those of this study. The line-width of the aged EYP-stabilized emulsion (0.96 ppm) is very close to the line-width of pure PFOB or distilled water. This is an indication that the droplets in their aged emulsion are very large, as was their intent. These droplets are probably in the slow exchange regime.

Their 4% EYP-stabilized emulsion experienced the greatest line broadening of all the emulsions (19 ppm). It is likely that this emulsion falls within the intermediate exchange regime. When Wolber *et al.* (1999) increased the size of the emulsion droplets by decreasing the amount of EYP from 4% to 0.85%, droplet size increased. This shifted the emulsion from the intermediate exchange regime into the slow exchange regime, narrowing the line-width (right half of the curve in figure 4.1).

The standard emulsion droplets, stabilized with 4% DOPC, were smaller [3.04 μm (figure 3.7)] than the 4% EYP-stabilized droplets [greater than 4 μm (Wolber *et al.*, 1999)] and had a narrower line-width (4.74 ppm as opposed to 19 ppm). The standard emulsion falls within the fast exchange regime (figure 4.1). The fact that the 2% DBPC-stabilized emulsion, which has even smaller droplets (2.4 μm), had an even narrower line-width (2.71 ppm) is further proof that droplets in this size range are in the fast exchange regime.

There is some possibility that the surfactant itself has some effect on line-width. There is a notable difference in line-width between 4% DOPC and the 4% EYP stabilized emulsions. However, it is clear from the range of line-widths described by Wolber *et al.* (1999) for the three EYP-stabilized emulsions that droplet size plays a major role. The direct effect of the surfactant is likely very small. It is more likely that the droplets are near the boundary between the intermediate and fast exchange regimes. In this case, small changes in average droplet size would manifest as drastic changes in line-width.

4.5 Conclusions and Future Work

It has been shown that using a shorter chain-length PC produces smaller droplets.

These droplets are less stable than those stabilized with longer chain-lengths.

Although it was shown that using DBPC to stabilize the emulsion produced smaller droplets than the standard DOPC-stabilized emulsion, the T_1 did not show any significant change, contrary to what was expected. However, the variance in T_1 s within each treatment was quite large. Further study to increase sample size is necessary. This would allow more powerful statistical testing to detect significance. The greatest cause of T_1 relaxation was oxygenation, which can reduce the T_1 of PFOB by up to 300 seconds.

Differences in line-width between the DOPC, DBPC and EYP-stabilized emulsions show that decreasing droplet size plays a significant role in decreasing line-width. Aside from increasing polarization, this may pose the greatest opportunity to increase SNR. There are several methods available that would produce smaller droplets with a narrower distribution, and possibly narrower line-widths. As seen in figure 3.6, there is a measurable number of droplets that are greater than 8 μm . Breaking these large droplets into droplets that are in the fast exchange regime would help to increase SNR. Since the volume of a droplet is dependent on the cube of its radius, the larger droplets have a very significant amount of the emulsified PFOB. A droplet with a diameter of 8 μm will have the approximate volume of 4025 droplets with a diameter of 0.5 μm . Forcing the

emulsion through a high pressure homogenization after sonication would both decrease droplet size and disperse the PFOB more uniformly. With additional surfactant to compensate for the increase in surface to volume ratio, homogenization would break these droplets in to smaller, more stable droplets. A high power bath sonicator may be able to accomplish this as well. Further study using other biocompatible surfactants deserves investigation to determine if the average droplet size can be decreased further. Decreasing the variation of droplet sizes in the emulsion may also help to decrease line-width.

One aspect of delivering HXe to a target tissue, which was not studied, was to ensure the xenon remained at the tissue long enough to image, rather than just rushing by in the blood stream. Targeting of HXe to specific regions of the body should increase the fraction of HXe going to the tissue of interest. This may be accomplished through immunotargeting with local injections. Much work has gone into liposome targeting as an aide to drug design (Allen, 1998; Langer, 1998; Gregoriadis and Florence, 1993; Gabizon, 1989). As important as targeting will be, the carrier itself must first be optimized. If the carrier cannot deliver a sufficient amount of HXe with a sufficiently long T_1 , the work will be halted before targeting methods need be developed.

Table 4.1 Experimental line-width results for DOPC and DBPC-stabilized emulsions as well as published results for EYP-stabilizing emulsions of differing lipid concentrations. All data for EYP-stabilized emulsions are from Wolber *et al.* (1999). It is important to note that the ratio of PFOB to lipid is very close to the same for the aged EYP sample and the 0.85% w/v EYP sample. All emulsions are 90% w/v except the aged EYP emulsion.

Lipid	Emulsion Lipid Component (w/v)	Line-width (ppm)
EYP	4%	19 [‡]
EYP	0.85%	5 [‡]
EYP (aged)	0.47%(50% w/v PFOB)	0.96 [‡]
DOPC	4%	4.74 ± 0.54
DBPC	2%	2.42 ± 1.46

[‡] Line-widths from Wolber *et al.* (1999) were multiplied by 1.59 in order to correct for the difference in field strengths.

Figure 4.1 Model relating droplet size to line-width using experimental data and EYP-stabilized emulsion data from Wolber *et al.* (1999). The scale across the top is an estimation of the boundaries of the fast, intermediate and slow exchange regimes.

Chapter 5

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