CONCENTRATION OF CRYOPROTECTANT IN WATER-IN-OIL MICRODROPLETS FOR SINGLE CELL VITRIFICATION

By

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B.S. in Mechanical Engineering, University of Missouri, 2006

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Abstract

Among the several challenges associated with vitrification of cells, a major roadblock is the requirement of high concentrations of cryoprotectant chemicals and the damages caused by exposure of cells to these high concentrations at physiological temperatures. It is thus desirable to minimize the time of exposure of cells with high concentrations of cryoprotectants to physiological temperatures. In addition, vitrification requires very rapid cooling rates. As cooling rates of a sample are limited by its size, it becomes ideal to use the minimum sizes of the sample to be preserved.

Certain organic oils, such as soybean oil, are made of triacylglycerols and are capable of dissolving small amounts of water due to the presence of ester groups, a property which enhances significantly with increasing temperature. This phenomenon was exploited to accomplish temperature controlled concentration of cryoprotectants in single water droplets with and without cells dispersed in the organic phase.

The organic phase used in the present work is soybean oil while glycerol is used as the cryoprotectant. Glycerol was found to be comparatively insoluble in soybean oil at 35 °C for up to 10 minutes. The present work employed heating on a temperature controlling stage and temperature increases of about 10K. Solutions of glycerol in DI Water were mixed with soybean oil and emulsions made by vigorous agitation. The water to oil concentration was kept at 0.1% v/v to simulate an infinite dissolution medium and to prevent different droplets from affecting each other. To prevent premature dissolution, the oil is saturated with water at room temperature by incubating for 48 hours. Micro-liter-sized droplets of the emulsion are placed on a heating/ cooling stage and droplets of 15-20 micron diameter are visually selected from polydisperse emulsion for observation under a microscope. Upon increasing temperature, water dissolves into the oil rendering the droplet highly concentrated with the oil-insoluble cryoprotectant. The experiment involved heating to 35 °C from room temperature, so that all water eventually dissolved into the oil. Droplets with an initial

concentration of 1M were found to be concentrated to about 3-4M in 90s while droplets starting at 2M were concentrated to 6M in about the same time. The entire process takes place over a time scale of about one minute, fast enough to minimize exposure times but slow enough to be precisely controllable.

This phenomenon is demonstrated to dynamically concentrate cryoprotectants within single cell-containing droplets. These droplets of sizes of about 30 micron diameter were concentrated to 3-4M from a starting concentration of 1M in about 300s. The cells are tolerant to this concentration process and do not die when subjected to it. The process may be used in practice to innocuously concentrate cell encapsulating droplets which may then be vitrified before they are exposed to high temperatures for fatally long time scales. With appropriate characterization, the controllability of the process will allow for choosing exact cryoprotectant concentration levels used for vitrification.

The demonstrated phenomenon has several other applications in cryobiology. Its controllability and speed may be used to dynamically modulate cryoprotectant concentrations in preservation protocols that require stepwise concentration or dilution. In addition, the process was found to be reversible and may thus be used for unloading cryoprotectants by controlled cooling as opposed to heating.

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I remain forever indebted to my parents, Dinesh and Neelu Bajpayee, and my uncle and aunt, Rakesh and Sunita Bajpai, for their constant encouragement and support in everything I do. Last but not the least I owe a debt of gratitude to my grandparents, Ravi Shanker and Dhanvanti Devi Bajpayee, and Shashi Shekhar and Shanti Mishra, who have always been sources of motivation and so important in my life.

This thesis is dedicated to Ambika. Many hours put into this work were taken away from her while she constantly wished for its success.

Contents

List of Figures

Nomenclature

A	Area
t	Time
M	Molecular Weight
D	Diffusion Coefficient
C	Concentration
ρ	Density
V	Volume
K	Constant
$\tilde{V}$	Dimensionless Volume
$\tilde{t}$	Dimensionless Time
C	Constant (Change in Solubility and Saturation Concentration of Water in Oil)
τ	Time Constant

Subscripts

w	Water
o	Oil
sat	Saturation
∞	At infinity
O	Initial

Chapter 1

Background and Introduction

1.1 Cryobiology and its Applications

Humans have been fascinated by the idea of suspending and preserving life for centuries. It was not until the last century however that research in this area gathered noticeable momentum [1]. Attempts have been made to preserve entire organisms, individual organs, as well as cells. Research beyond the cellular level has met with little success yet [2]. While cellular preservation is also an immature field with numerous unanswered questions, it has experienced greater development than organ or organism preservation. It is still an area of high interest within the medical community.

It is desirable to preserve cells for use in various applications that include organ transplants, regenerative surgery, reproductive medicine, revival of endangered species, and long term storage for research purposes. Liver transplant operations, for example, require a continuous supply of liver cells. Sperm and egg preservation is of prime importance to the reproductive medicine industry. Also, blood preservation for medicinal and military purposes is an area of constant interest. Lessons learnt at the cellular level are vital for successful transition to tissue and organ preservation.

1.2 Cryopreservation of Cells

Preservation of cellular life involves suspending molecular motion which translates to suspension of all chemical reactions. This prevents natural degradation and facilitates storage beyond normal life span. Traditionally this suspension is achieved by storage of the cells at very low temperatures, well below -100 °C. More recently, desiccated storage at higher temperatures has been explored and could potentially be

successful. This method however has not gained enough success to become mainstream [3]. The current work deals primarily with preservation by cooling, but may also be translated to the field of dry preservation.

To appreciate the challenges associated with cell preservation it is important to understand the mechanisms of cellular injury during the freezing process. Once the cells reach the state of cryogenic storage very little damage occurs. The only damages are due to free-radical formation caused by cosmic rays, which may take 2000-4000 years to significantly affect the cells [4]. It is the process of reaching that state where maximum injury is sustained. Formation of ice crystals in one form or the other leads to cell injury. Cells have been known to sustain injury under both slow and fast freezing conditions, although by seemingly different mechanisms [2]. When cells are subjected to slow cooling the ice starts forming outside the cells first. This results in an osmotic removal of water from the cell to its surroundings due to lower concentration of liquid water outside. As the result the cell dehydrates and eventually dies. On the other hand rapid cooling does not allow enough time for the water to escape from the cell and intra-cellular ice formation (IIF) occurs. This creates mechanically damaging ice crystals inside the cell that rupture the cell membranes and organelles.

Approaches to achieve cell preservation have involved the use of an optimum cooling rate that prevents IIF and too much dehydration, coupled with the use of cryoprotectant agents to inhibit ice formation. Cryoprotectant agents (CPAs) are glass forming liquids such as glycerol, dimethyl sulfoxide (DMSO), and ethylene glycol that inhibit the formation of ice crystals. Such methods are experimentally developed for individual varieties of cells for individual conditions, i.e. they are cell specific and

depending on a variety of factors including cell concentrations, storage temperature, freeze-thaw history and the cryoprotectant used [5]. Attempts have met with both successes and failures and are largely based on empirical methods.

The idea behind preservation is suspension of all motion. Motion can be suspended either by crystal formation or by vitrification. Whereas crystal formation is mechanically damaging, vitrification is considered to be mechanically harmless. Vitrification refers to the phenomenon of formation of glass upon cooling as opposed to formation of ice [6]. The viscosity of glassy state is high enough to consider it stationary over a time scale of centuries [4]. Glass is an amorphous state and no crystal formation or change in volume is observed during glass transition. Thus any mechanical damage is prevented while still ceasing all molecular motion.

The glass transition temperature of water is in the range of 136-165K [7,8]. Vitrification of water can be achieved by either cooling the sample at extremely fast rates of over10^6 K/ s, or at lower cooling rates by adding significant amounts of CPAs or mixtures thereof [2]. CPAs are used to reduce the cooling rates required for vitrification. The higher the CPA concentration, the lower the vitrification cooling rate will be. A pure CPA will form glass irrespective of the cooling rate [6]. Since vitrification rates for pure water are beyond practical limits it becomes essential to add enough CPA to achieve glass formation at practical cooling rates. The down side of this technique is that CPAs can themselves be biologically harmful, i.e. though they protect the cells from mechanical damage they might cause osmotic and toxic damage and must thus be used in moderation. It is therefore imperative to determine an acceptable combination of CPA concentration and achievable cooling rates.

According to Kresin and Korber [9], in order to achieve vitrification glycerol concentrations of more than 45% or about 6M controlled cooling rates of 100 K/s are required. Such high CPA concentrations would be lethal to the cell, if exposed to ambient temperatures for too long. If high concentrations must be used, it is desirable to minimize the exposure of the cells to ambient temperatures before cooling.

1.3 Emulsions & Micro-droplets in Chemistry and Biology

Ice formation is an aggregation phenomenon [10], whereby crystal formation begins at a nucleation site and then spreads through the sample and molecules interact with each other to arrange in defined crystal patterns. As the temperature of a sample is increased the probability of homogenous ice nucleation increases drastically. The probability of homogeneous ice nucleation also increases with sample size as there is a greater possibility of the occurrence of a nucleation site in a larger sample than a smaller one [11] [12]. Homogeneous ice nucleation refers to the spontaneous ice nucleation in a sample whereas heterogeneous ice nucleation refers to the phenomenon of artificially inducing ice nucleation by seeding [12]. Since micro-droplets of cell suspension would vitrify far more easily than bulk suspension, it is desirable to use those over bulk medium when attempting vitrification.

The current work combines the merits of using micro-sized samples and preventing exposure of highly CPA concentrated cells to ambient temperatures. A fast and precisely controllable, temperature stimulated method of concentrating cryoprotectant in micro-droplets in oil emulsion is proposed for single cell vitrification

Certain organic oils, such as soybean oil, are made of triacylglycerols and are capable of dissolving small amounts of water due to the presence of ester groups, a

property that enhances significantly with increasing temperature [13]. Previous work by He et al. has demonstrated shrinkage of dye and nanoparticle solution droplets in soybean and mineral oil emulsions by focused laser heating of about 0.1 K and the resulting concentration of the solutes in the droplets [13]. It was demonstrated that the solubility of water in soybean oil was far greater than that in other organic phase samples. Soybean oil contains free ester groups that attach with water and facilitate dissolution. At standard temperature and pressure soybean oil dissolves about 0.3% water of volume. This limit increases at higher temperatures. Upon dissolution of some water from an aqueous solution droplet into the oil phase the remaining droplet is rendered concentrated with the solute. He's technique was targeted primarily towards purposes of detecting molecules within droplets, especially when the number of molecules is limited.

More recently Jeffries et al [14] modulated the size of aqueous droplets in acetophenone and decanol emulsions and thus controllably increased and decreased chemical concentration in the droplets. Jeffries also used focused laser heating and employed a temperature increase of less than 1K.

In addition, Sgro et al [15] recently demonstrated freezing of micro-droplets containing single cells. By utilizing the vast difference in freezing points of the aqueous droplets and the surrounding organic phase Sgro showed that temperature control may be used to manipulate droplets for a variety of uses such as freezing, temperature cycling, sample archiving etc. Furthermore Sgro's experiment had suggestive evidence that freezing of cells trapped within micro-droplets did not significantly affect their viability.

The present work proposes similar concepts to concentrate cryoprotectants in droplets by raising their temperature. Unlike previous work, the heating is done in bulk as

opposed to focused heating, and the temperature increase employed is significantly greater, about 10 °C as compared to 0.1 °C as previously shown. The resulting effect is that water dissolves in larger quantities and a high concentration of solute is achieved in relatively short time periods. The entire process takes place over a time scale of about one minute, slow enough to be controllable and fast enough to minimize exposure to ambient temperatures. It is envisioned to cool the droplets for preservation as soon as the desired cryoprotectant concentration is achieved.

Chapter 2

Materials and Methods

2.1 Overview

It is proposed to generate cell encapsulating micro-droplets of water or freezing medium in a water-in-oil emulsion. Emulsions provide an easy method of obtaining multiple droplets without the fear of immediate coalescence. Droplets may also be stored or transported as emulsions. Polydisperse emulsions can be easily produced by mixing water and oil in desired proportions and agitating the mixture. If desired, monodispersed emulsions may also be produced using specially designed micro-fluidic devices [16]. The droplets in the generated emulsions are concentrated with a cryoprotectant of choice by bulk heating over a temperature range of 10 °C starting from room temperature. In addition, a simple mathematical model is developed to characterize droplet shrinkage and concentration. Finally the tolerance of HL-60 cells to the concentration process is explored.

2.2 Selection of Cryoprotectant

Experiments were conducted to determine the solubility of pure DI water, dimethyl sulfoxide (DMSO), and glycerol in soybean oil. Emulsions of each of the above were made in soybean oil by agitation and droplets in the size range of 14-20 μm were selected for observation. The samples were heated to 35 °C and held for 10 minutes. The heating setup and process are described in detail in the forthcoming text.

2.3 Generation of Polydisperse Emulsions

Depending on desired droplet composition, DI water, 1 M glycerol solutions, or 2 M glycerol solutions, were mixed with the continuous phase in a 1:1000 ratio. Such a low

concentration was used to simulate an infinite dissolution medium and prevent emulsion droplets from affecting each other. Soybean oil was used as continuous phase and incubated with DI water at room temperature for 72 hours. This was done to saturate the oil and prevent premature dissolution of the dispersed phase as that would interfere with the analysis as well as with the controllability of the process. Also, 0.1% by volume of SPAN 80® was added as surfactant to prevent the emulsion from coalescing.

1 ml of soybean oil was taken in a micro-vial and a 1 μl droplet of the dispersed phase was mixed into it at room temperature. The mixture was vigorously agitated for about 30 seconds until the vial contents appeared slightly cloudy to the eye. The created emulsion was used for experiments and found to be stable for periods of up to one hour.

2.4 Generation of Monodispersed Emulsions

Methods were developed to generate monodispersed emulsions of water in oil if so desired. This was done using specially designed microfluidic devices. Droplets are generated by introducing a stream of the dispersed phase into co-flowing streams of the continuous phase on a microfluidic device. Droplets detach from bulk when the shear force due to the flow of the continuous phase exceeds the interfacial tension within the dispersed phase. This method derives from demonstrations of similar techniques for generating oil-in-water emulsions [17].

Micro-devices used to generate droplets of water in oil phase were manufactured in PDMS using an SU-8 mold built on to a silicon wafer using established methods [16,18]. The insides of the channels were coated with water repellent, Aquapel® (PPG Industries, Pittsburgh, PA), to prevent sticking of water to the device. The device mask is shown in Figure 1.

Water is flown in channel A while oil comes in through channels B and C. Additional oil channels are added downstream to dilute the concentration of water in oil. According to Umbanhower et al [17], the size of the generated micro-droplets depends on the channel diameter, the surface tension of the dispersed phase, and the viscosity, density, and flow velocity of the continuous phase. The droplet diameter may thus be precisely controlled by varying the flow rate of the continuous phase. That is exactly why additional oil channels are added downstream; to achieve the desired dilution without affecting the required flow rate at the point of droplet generation.

Successful generation of monodispersed emulsion of DI water-in-oil was demonstrated using the manufactured devices. Figure 2 shows the equally spaced droplets downstream in the device. The monodispersed emulsion was not eventually used for droplet concentration experiments due to the complexity of the process. Polydisperse emulsions made by agitation were used instead, and droplets of the desired size were visually selected. In a completely automated process, however, it would be necessary to generate monodispersed emulsions on the micro-fluidic device.

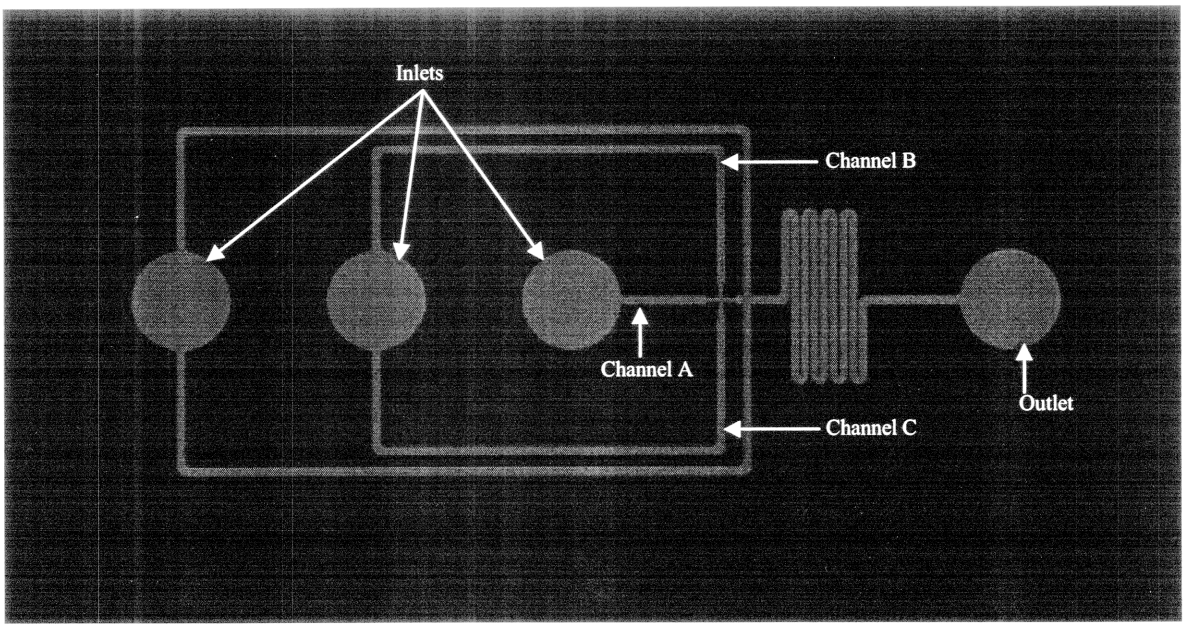

Figure 1: Mask of Microfluidic Device used to generate Monodispersed Emulsion

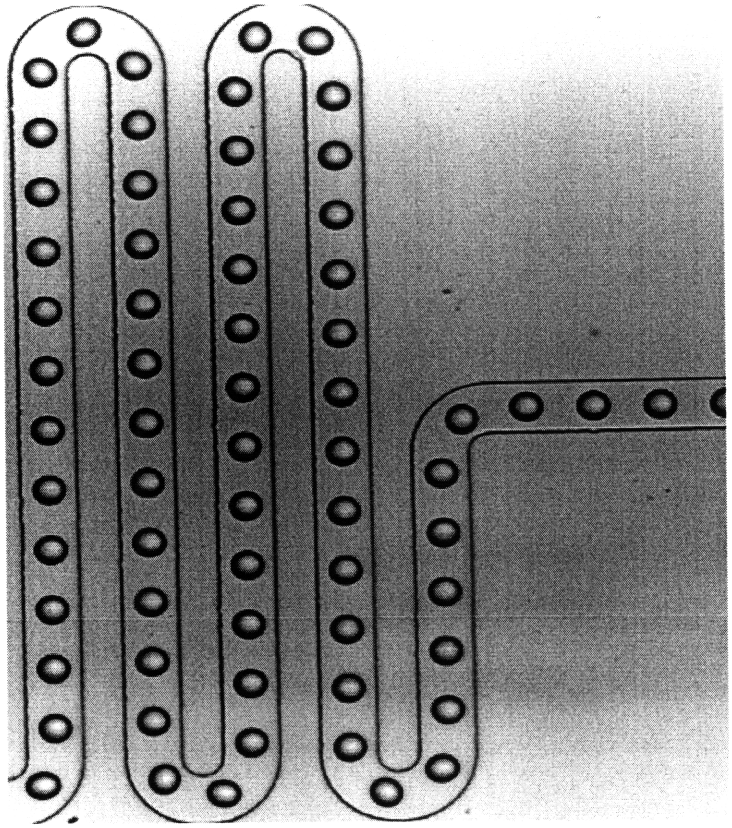

Figure 2: Equally Spaced Droplet Downstream in the Microfluidic device

2.5 Concentration of Droplets

Droplet concentration experiments were performed on a Linkam® FDCS 196 Thermal-stage, a microscope mounting automated device with precise temperature and environmental control. Photographs of experimental equipment are shown in Figure 3 and Figure 4. A schematic of the experimental setup is shown in Figure 5.

The heating/ cooling plate of the stage has an electrical resistance heater and liquid nitrogen cooling channels. The device is capable of providing a temperature from -196 °C to 130 °C and heating and cooling at rates of up to 120 °C per minute. A removable quartz crucible is placed on the thermal-stage plate. The crucible holds the experimental sample and is capable of being maneuvered in two directions on the horizontal plane while the experiment is in progress. Quartz is an ideal choice as it is a good conductor that facilitates heat transfer and its transparency allows for proper imaging. The stage chamber is also capable of being vacuumed. This vacuuming feature, however, was not used is the present work. Three stage controllers monitor the temperature, the flow of liquid nitrogen and the vacuum level respectively. The entire system is controlled by a PC with Linksys® 2.0 software as the user interface. The thermal-stage was mounted on an upright Nikon Optiphot® 2 microscope. Pictures were taken using a Pixellinx® A662 1.3 mega-pixel digital camera that is also connected to the controlling PC. Imaging was done at a magnification of 10X.

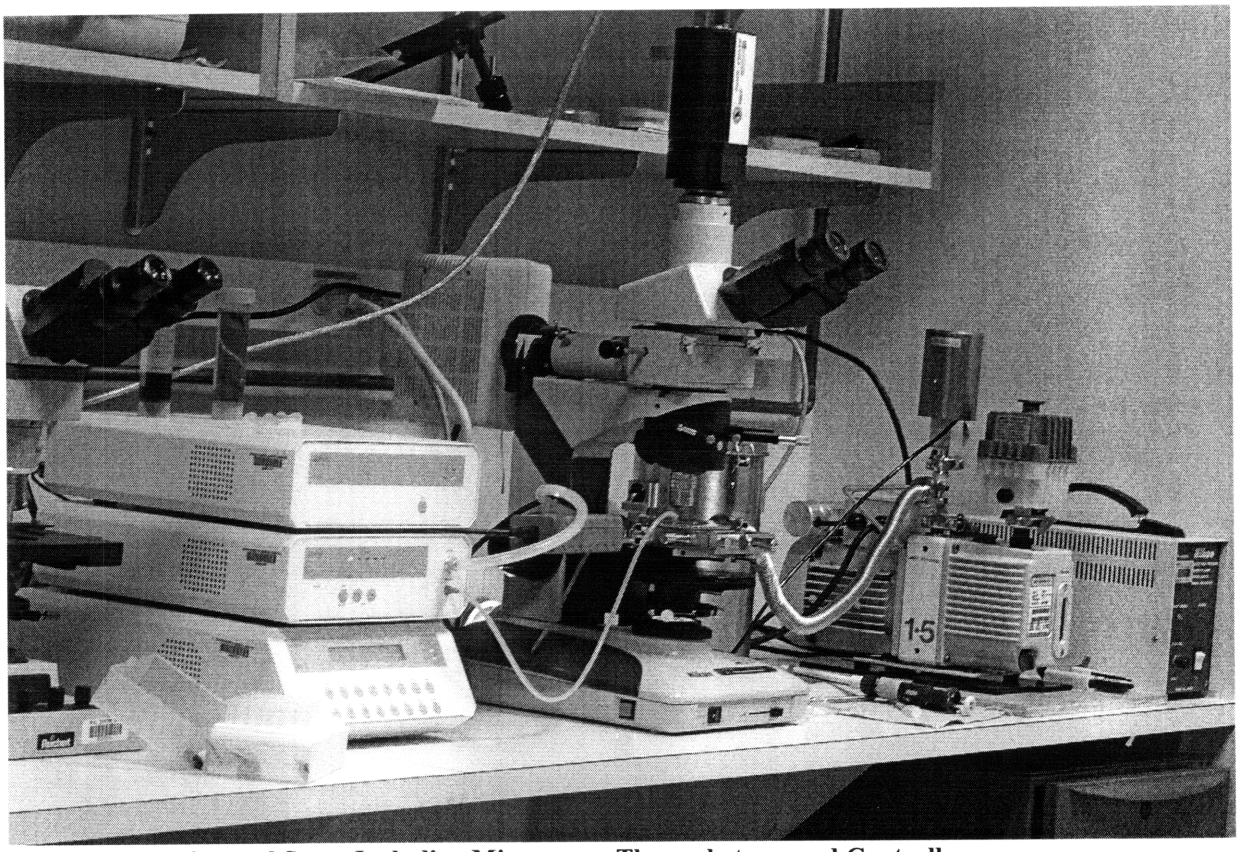

Figure 3: Experimental Setup Including Microscope, Thermal-stage, and Controllers

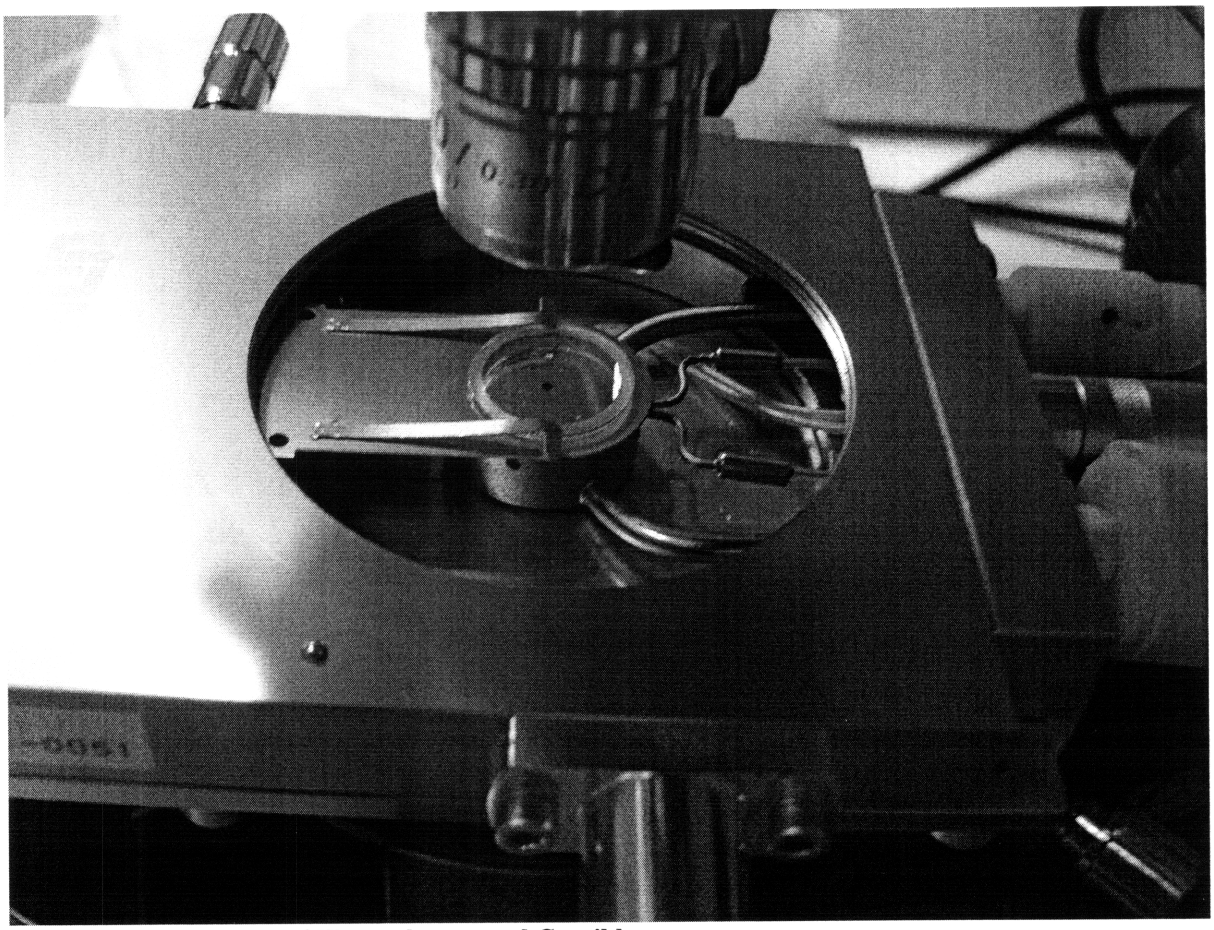

Figure 4: Close-up view of Thermal-stage and Crucible

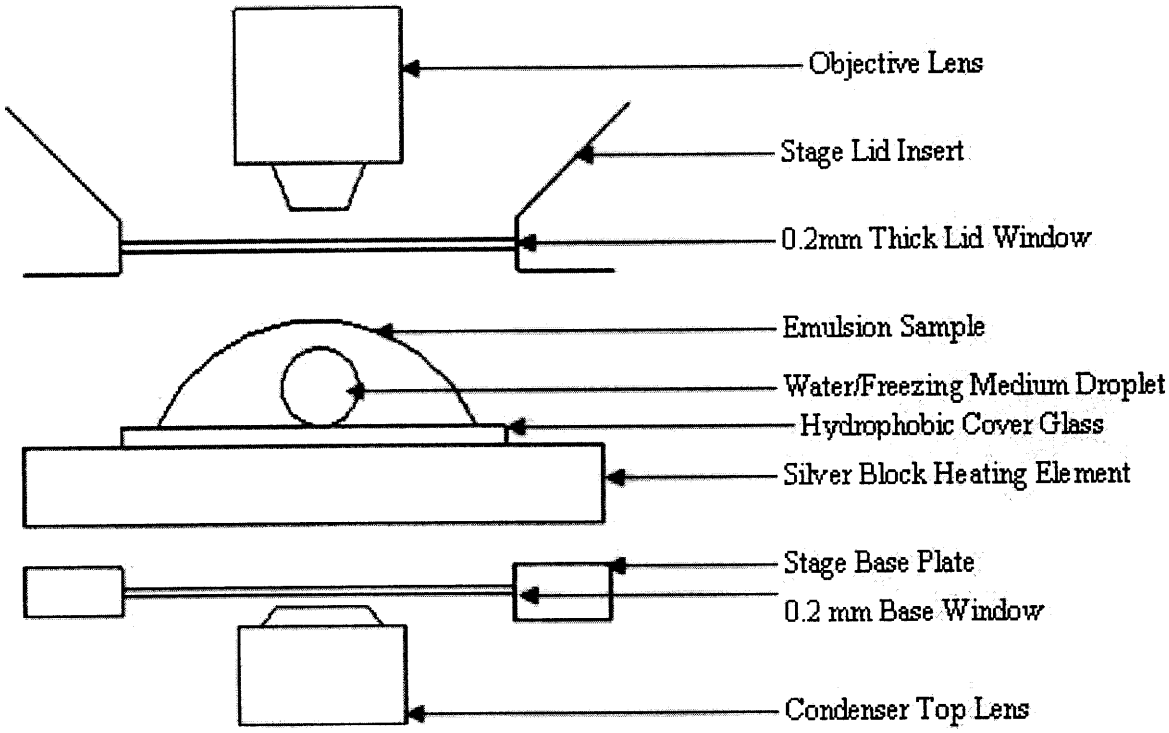

Figure 5: Schematic of the experimental setup

The thermal-stage silver plate was cleaned with isopropyl alcohol and preset to room temperature that varied between 23 and 25 °C. The quartz crucible was also cleaned with isopropyl alcohol and placed on the plate. A glass coverslip, made hydrophobic by coating with Aquapel®, was placed in the crucible. About 1-2 µl of the prepared emulsion sample was taken in a micro-pipette and placed on the hydrophobic cover slip. The samples were viewed under the microscope and moved around to visually select droplets of desired size which were then observed. In the present work, droplets in the size range of 13-18 µm diameters were used. Since the concentration of water in the emulsion was very low (1:1000) usually only a single droplet was observed in a field of view.

As soon as the samples were placed on the quartz crucible the stage lid was closed and the plate heated at a rate of 120 °C per minute to a final temperature of 35 °C. The fast rate of temperature increase simulates instantaneous heating. Video recording was started when the stage reached 35 °C. Videos were made at a rate 20 frames per minute. The experiments were repeated seven times.

2.6 Dimensionless Analysis

In order to generalize the experimental results, a dimensionless analysis was performed. Assuming perfect mixing in the continuous phase the rate of shrinkage of droplet area should be the same, i.e. the area should change linearly with time regardless of the droplet size. As suggested by He [13], the rate of change of area is given by,

$$\frac{dA}{dt} = -\frac{8\pi M_w D_{w,o}(C_{sat} - C_\infty)}{\rho_w} \qquad (1)$$

where, M_w is the molecular weight of water, $D_{w,o}$ is the diffusion coefficient of water in soybean oil, ρ_w is the density of water, and C_{sat} and C_∞ are concentrations of water in oil at the droplet surface and far away from the droplet respectively. In the present scenario where the dilution of water in oil is 1:1000, C_∞ is assumed to be zero.

Equation (1) may be modified and integrated to depict to describe volume as a function of time according to,

$$V = (Kt + V_O^{2/3})^{3/2} \tag{2}$$

where t is time, V_o is the initial volume, and K is a constant given by,

$$K = -5.197\left(\frac{M_w D_{w,o} C_{sat}}{\rho_w}\right) \tag{3}$$

Upon non-dimensionalization equation (2) may be written as,

$$\tilde{V}^2 = (C\tilde{t} + 1)^3 \tag{4}$$

where C is a dimensionless constant, $\tilde{V}$ is the dimensionless volume, $\frac{V}{V_O}$ and $\tilde{t}$ is the dimensionless time, $\frac{t}{\tau}$, where τ is the time constant given by,

$$\tau = \frac{V_O^{2/3}}{K} \tag{5}$$

Note that C is an artifact of the change in the value of K with temperature. K contains the diffusion coefficient and saturation concentration of water in oil, both of which are temperature dependent. C is simply a multiple of K and describes how many times the product $D_{w,o}C_{sat}$ changes due to temperature increase from 25 °C to 35 °C.

The experimental data was non-dimensionalized and compared to theoretical predictions for pure water. Thus, the theory is expected to accurately predict the data for DI water which is governed by the simple diffusion equation and eventually the droplet volume goes to zero. For glycerol droplets however, it is expected to accurately predict the data in the initial part of the curve but diverge from it as time increases and the droplet volume reaches a steady non-zero value. In addition to the insoluble glycerol left behind some water will bind to the glycerol in solution and not dissolve into the oil. To match the theory as closely as possible the data was analyzed as change in the free water volume with time.

2.7 Cell Encapsulating Droplets

Following investigations on DIW and glycerol solution droplets emulsions of cell encapsulating droplets were also experimented upon and some preliminary results obtained.

HL-60 cell line was used as the sample cells in the current work. The HL-60 cells were derived from an acute myeloid leukemia patient, and are a desirable model for studying normal as well as leukemia cells in humans [19]. Their average diameter is about 12.4 μm [20], which makes them conveniently sized for encapsulation in the droplet sizes under investigation. HL 60 cells are suspension cells and do not attach to in culture. This property makes them further suitable for the current work. The cells that were used ranged from seven to ten generations in culture. Figure 6 shows the HL 60 cells used at 40X magnification.

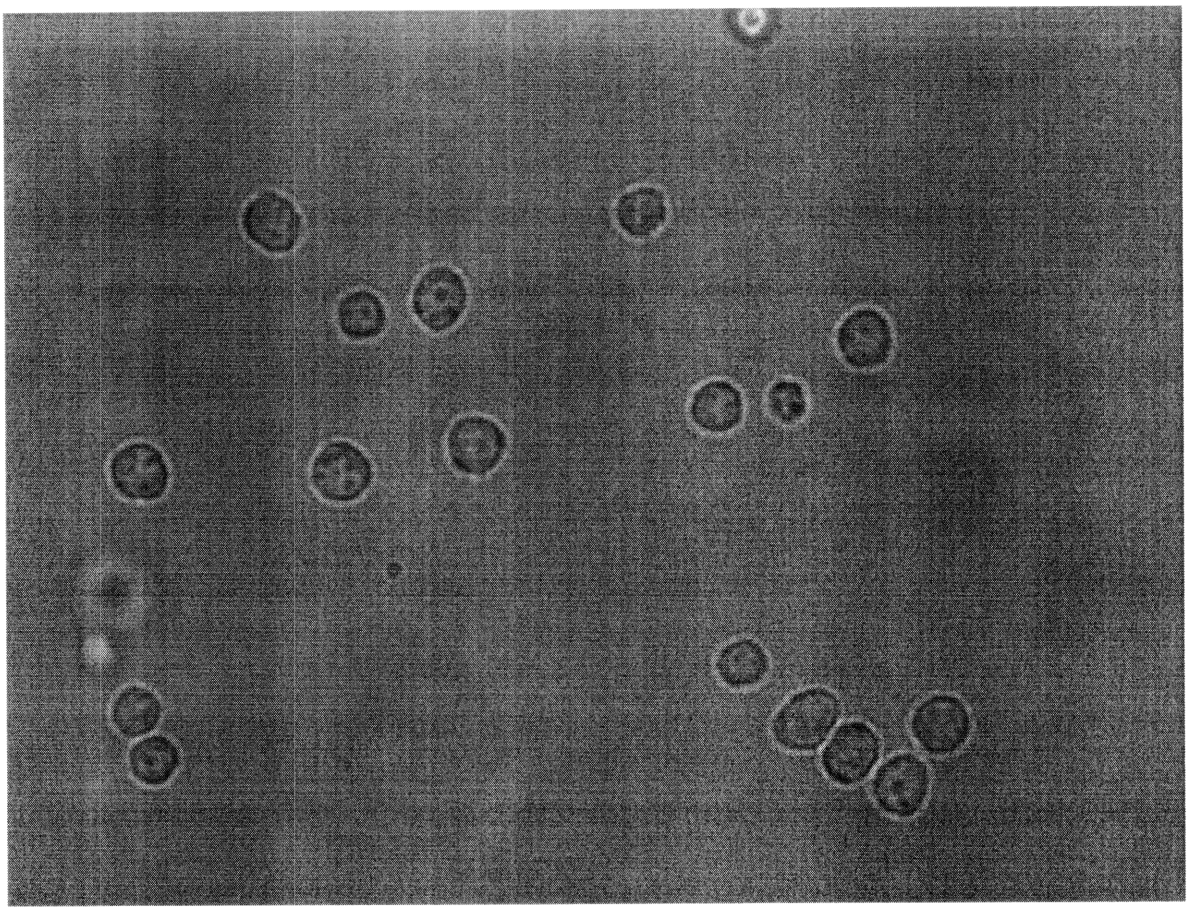

Figure 6: HL 60 Cells at 40X Magnification

The cells were cultured in RPMI Medium (GIBCO®, Carlsbad, CA) added to 1% Penicillin Streptomycin and 10% Fetal Bovine Serum. To prepare the freezing suspension, cells were taken out of culture and suspending in Hank's Buffer Salt Solution (HBSS) at 25 °C. Cells were counted using a Coulter Counter and a concentration of 25 million cells/ ml of suspension was created. This concentration results in the probability of finding a cell in one out of ten emulsion droplets. Finally, desired amounts of glycerol were added to produce 1 M glycerol solutions.

To a fraction of the samples was added the cell staining dye, 0.02% Calcien AM to enable visualization under fluorescence and allow for testing the survivability of the cells to the concentration process. In addition, fluorescent nanoparticles (Duke Sci. Corp., Palo Alto, CA) of size 51 nm diameter were added to make the droplet-oil interface visible. The nanoparticles were diluted to a concentration of 2.8×10^{13} particles/ ml. When viewed under the green fluorescent channel, live cells would appear as bright spots while the droplets would appear lighter but still clearly differentiable from the surrounding oil.

Droplet concentration experiments were conducted on an Indium Titanium Oxide (ITO) heater micro-fabricated in the lab. The ITO heater provided a heating platform that was also transparent, thus allowing for proper visualization under the microscope. These samples were visualized at a magnification of 20X and photographed using a SPOT® RT 1.4 megapixel digital camera. The samples were moved around to visually select droplets that contained single cells which were then observed and analyzed. The sample droplets used ranged from 18 μm to 31 μm in diameter. As in earlier experiments the samples

were placed on hydrophobic coverslips to ensure that the droplets did not become sessile and remained almost spherical.

The ITO heater operated at heating rates lower than that of the thermal-stage and the heating rates were difficult to control. The samples prepared at room temperature were placed on the heater which heated up slowly to 35 °C. Videos were made at 1-2 frames per minute. The experiments were repeated 5 times which included 3 runs under fluorescence.

Chapter 3

Results & Discussion

3.1 Heating of Droplets to Select Cryoprotectant

In an attempt to identify the most suitable cryoprotectant for the current scenario the dissolution of pure Water, DMSO and Glycerol in soybean oil was observed. It was found that water and DMSO were completely dissolved in soybean oil after holding for 10 minutes at. On the other hand glycerol did not dissolve at all into the oil. The results are portrayed in Figure 7. The bar chart shows change in normalized volume, V/V_o, where V_o is the initial volume. Glycerol was thus selected as the cryoprotectant of choice for the current work. Free water in the droplets would dissolve but not the glycerol, and as a result render the remaining droplets concentrated with CPA.

3.2 Concentration of DI Water Droplets in Soybean Oil Emulsion

The shrinkage of DI Water Droplets in Soybean Oil was monitored to characterize the behavior of droplets without cryoprotectant and obtain sound experimental employment of the theory developed for shrinkage of pure water droplets.

Emulsions of pure de-ionized water in soybean oil were observed after heating to 35 °C. As expected the droplets completely dissolved in oil. The time taken for the droplets to completely dissolve in oil varied between 50 and 90 s depending upon the droplet size. A sample series of photographs taken is depicted in Figure 8.

The shrinkage of droplets was monitored and the data collected is plotted in Figure 9. Linear trend lines were fitted through each set of data. It is noticed that the area-time profile is approximately linear and the slopes of different lines are similar in value. This accurately represents the theory.

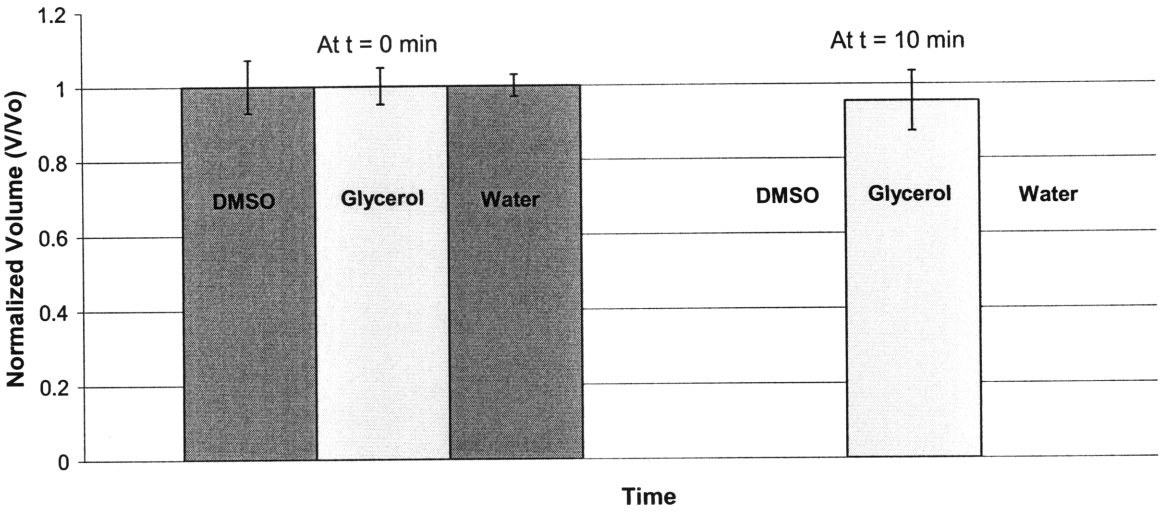

Figure 7: Initial and Final Volume Comparison after holding 10 minutes at 35 C

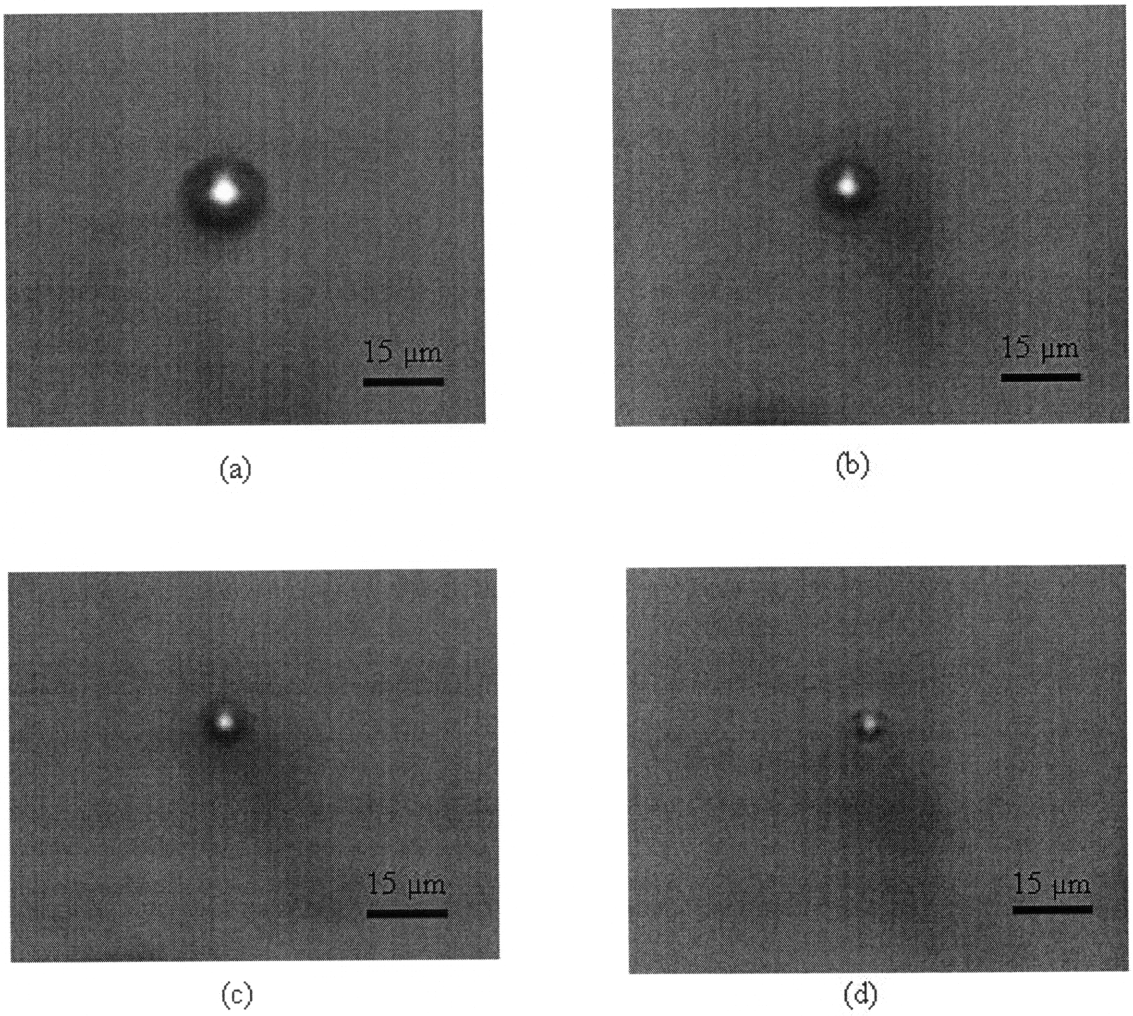

Figure 8: DI Water droplets in Soybean Oil at 35 °C at (a) t = 0s (b) t = 18s (c) t = 31s and (d) t = 46 s

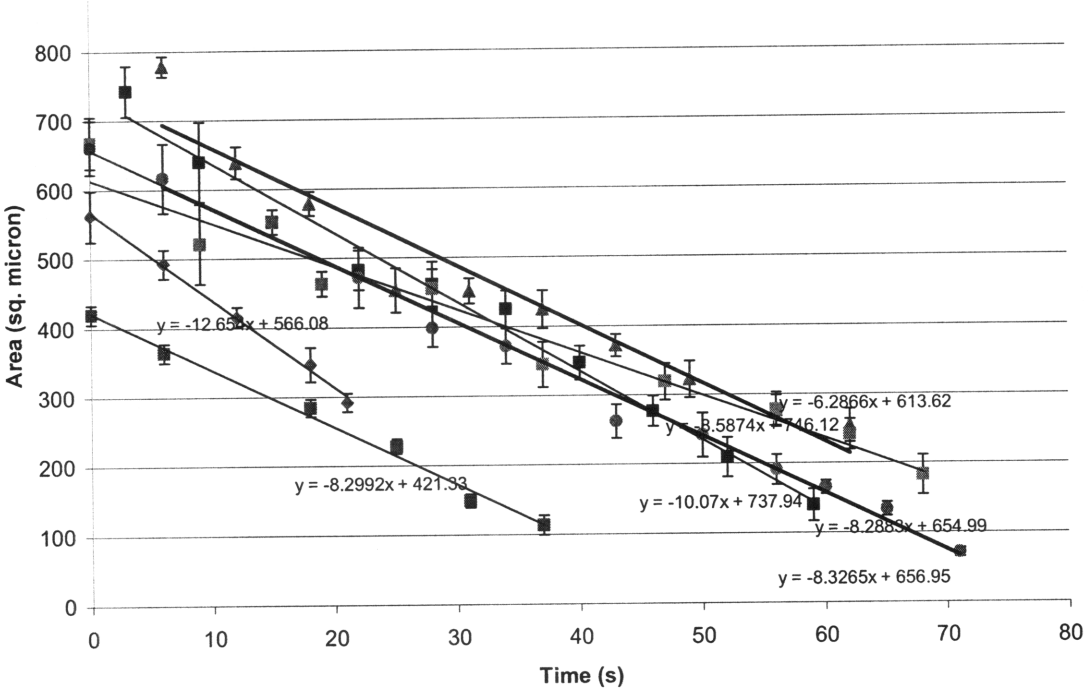

Figure 9: Shrinkage of droplet surface area with time for DI Water droplets in oil (Different Patterns correspond to different runs and droplet sizes)

Deriving from the dimensionless analysis, the squares of the dimensionless volume are plotted against dimensionless time and superimposed with theoretical curves in Figure 10. It is observed that the experimental data for all experimental runs follows the same trend and closely replicates theory.

3.3 Concentration of Glycerol Solution Droplets

Observations on pure water droplets were followed by investigation of the experiments with glycerol solution droplets.

Unlike droplets of pure DI water, the glycerol solution droplets did not dissolve completely into the oil. Figure 11 shows a sample series of photographs of 1M glycerol solution droplets. The droplets stopped shrinking once they reached about a third of their original volume. This is attributed in part to the presence of glycerol in the droplets as well to some water binding to the glycerol in solution. At the given concentration the hydration number of glycerol is 2.63, i.e. that many water molecules bind to each glycerol molecule [21]. It was calculated that for 1M glycerol droplets the final volume should be about 12% of the initial volume while for 2M glycerol droplets it should be about 24% of the initial volume. This includes the volume of the remaining glycerol. The droplet volume data collected is plotted in Figure 12 for 1M and 2M glycerol solution droplets in the form of curves of normalized volume against time. For comparison, one data set each of pure water and pure glycerol droplets is also depicted.

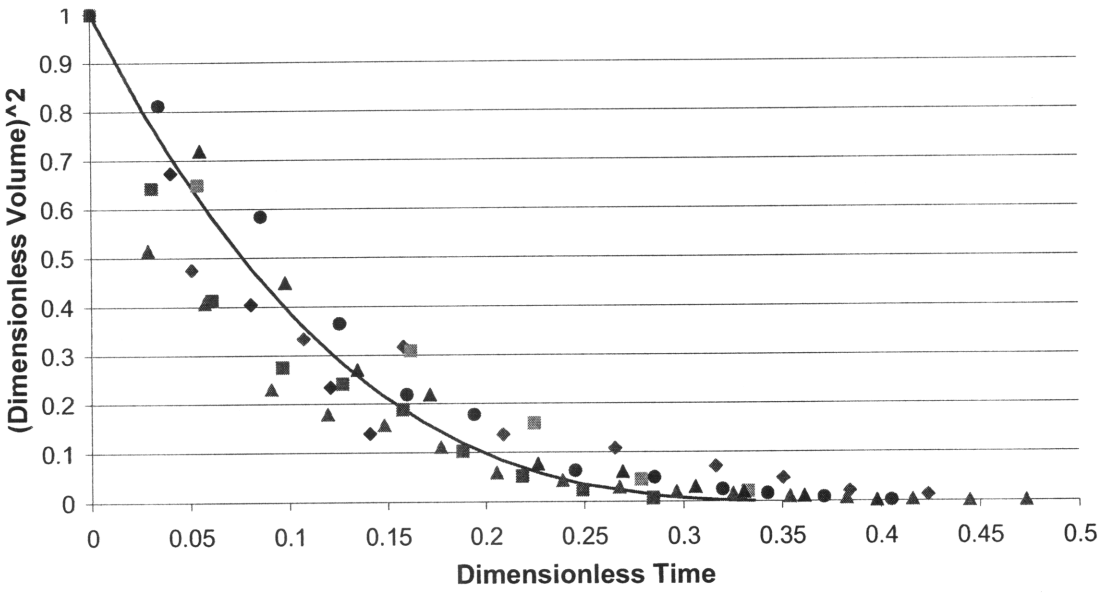

Figure 10: Dimensionless Volume Squared vs. Dimensionless Time for DI Water Droplets in Oil (Different Patterns correspond to different runs and droplet sizes)

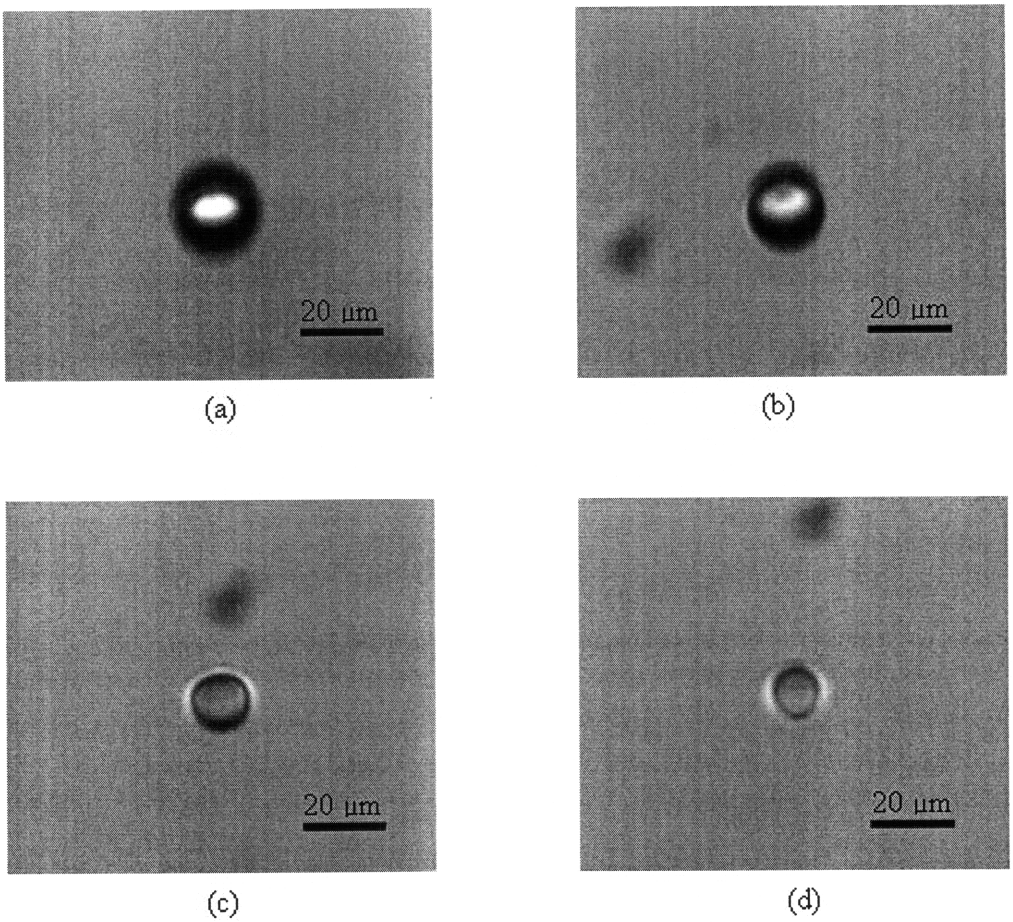

Figure 11:1M-Glycerol droplets in Soybean Oil at 35 °C at (a) t = 0s (b) t = 30s (c) t = 60s (d) t = 120 s

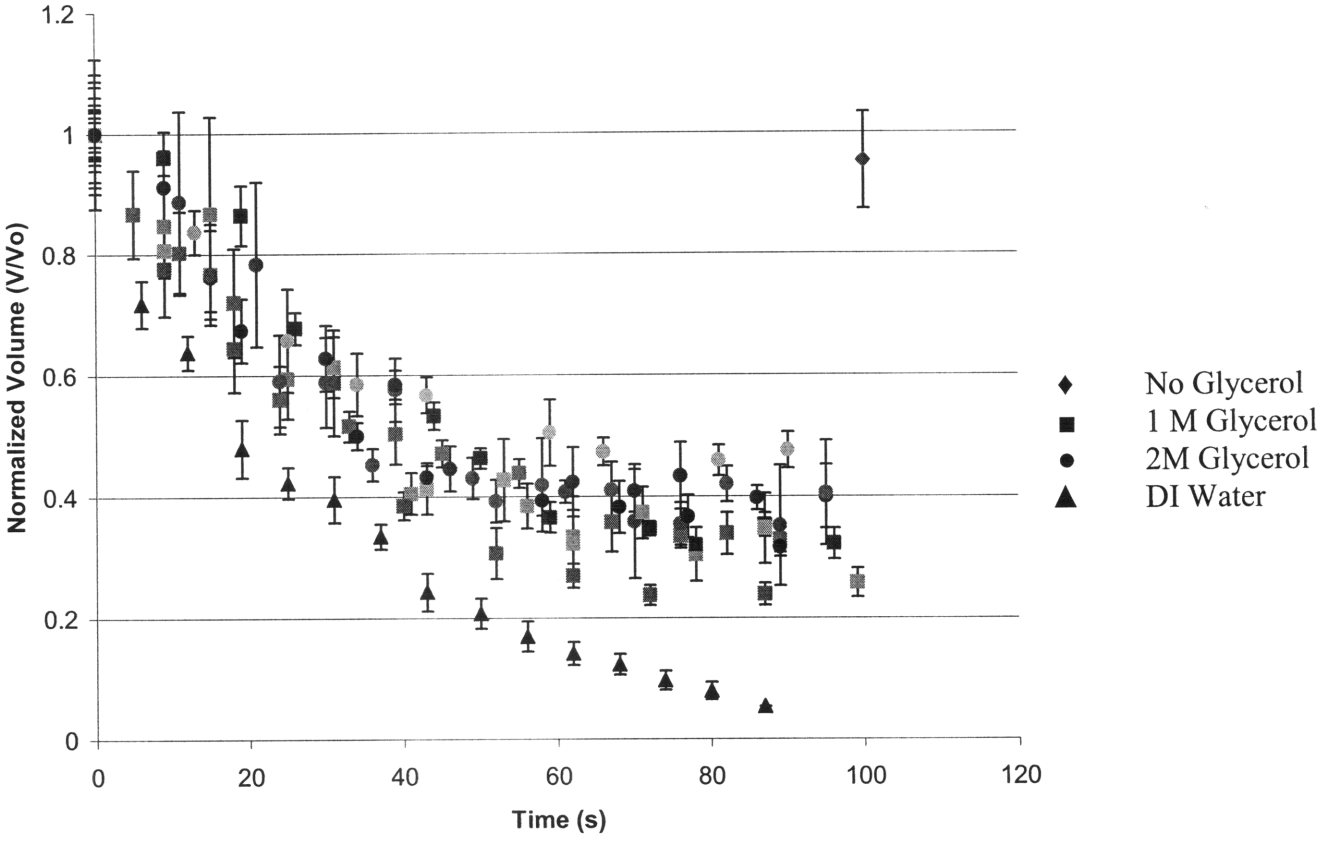

Figure 12: Change in Normalized Volume with Time for 1M and 2M Glycerol Droplets in Oil (DIW and Pure Glycerol shown for comparison)

The experimental steady state for 1M solution droplets was found to be between 23-34% of their original volume, while it was slightly higher for 2M solution droplets, in the range of 32-45%. The discrepancy from the expected values may be attributed to non-ideal mixing and the resulting saturation of oil in the vicinity of the sample droplets. Deriving from the dimensionless analysis, the squares of the dimensionless free water volume are plotted against dimensionless time in Figure 13 and Figure 14 for 1M and 2M glycerol solution droplets respectively. The plots are comprehensive and contain data from experiments on all glycerol solution droplets in oil emulsions. It is observed from the dimensionless plot that all glycerol droplets, regardless of size and conditions follow the same general trend. The data is superimposed with theoretical curves of shrinking of pure water droplets. Again the data closely replicated theory until the end of the process where the slight discrepancies are due to saturation of oil in the droplet vicinity.

The dimensionless plots are very useful in the sense that they allow for prediction of the shrinking profile of a glycerol solution droplet in soybean oil at 35 °C if the initial droplet size is known. Upon fitting a third degree polynomial line through the data it is observed that the value of the product $D_{w,o}C_{sat}$ increases 2.88 times on average in going through a temperature rise of 10 °C from 25 °C to 35 °C.

Based on the above analysis of glycerol solution droplets in oil, it is inferred that under conditions of the current experiment, the droplets can only be concentrated about 3-4 times their original concentration. Figure 15 depicts the concentration values over time of different glycerol solution droplets under experiment.

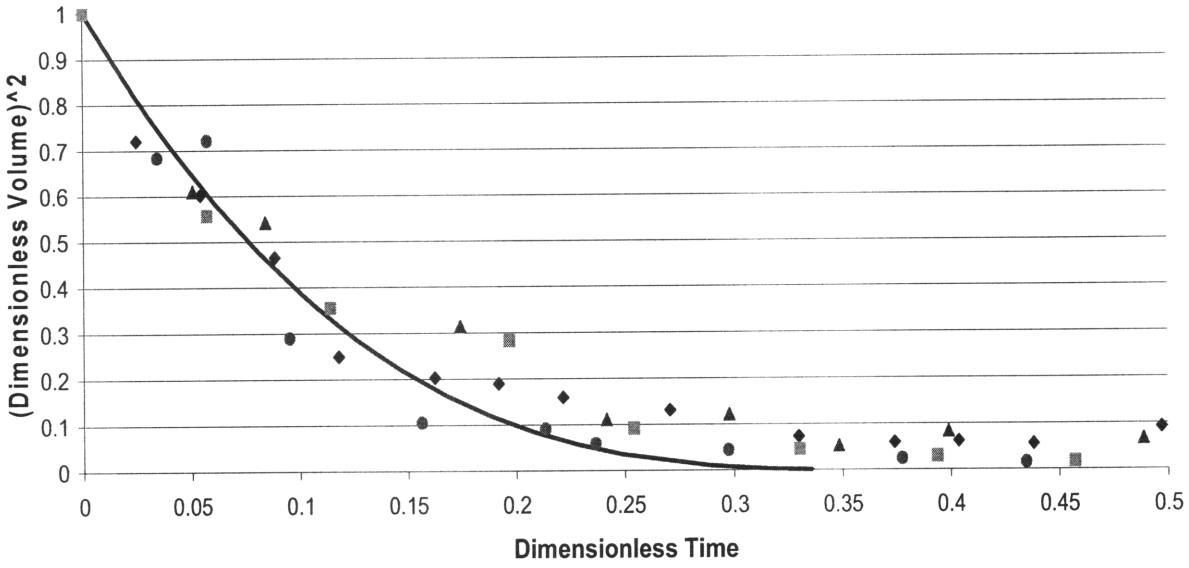

Figure 13: Dimensionless Free Water Volume Squared vs. Time for 1M Glycerol Droplets in Oil (Different Patterns correspond to different runs and droplet sizes)

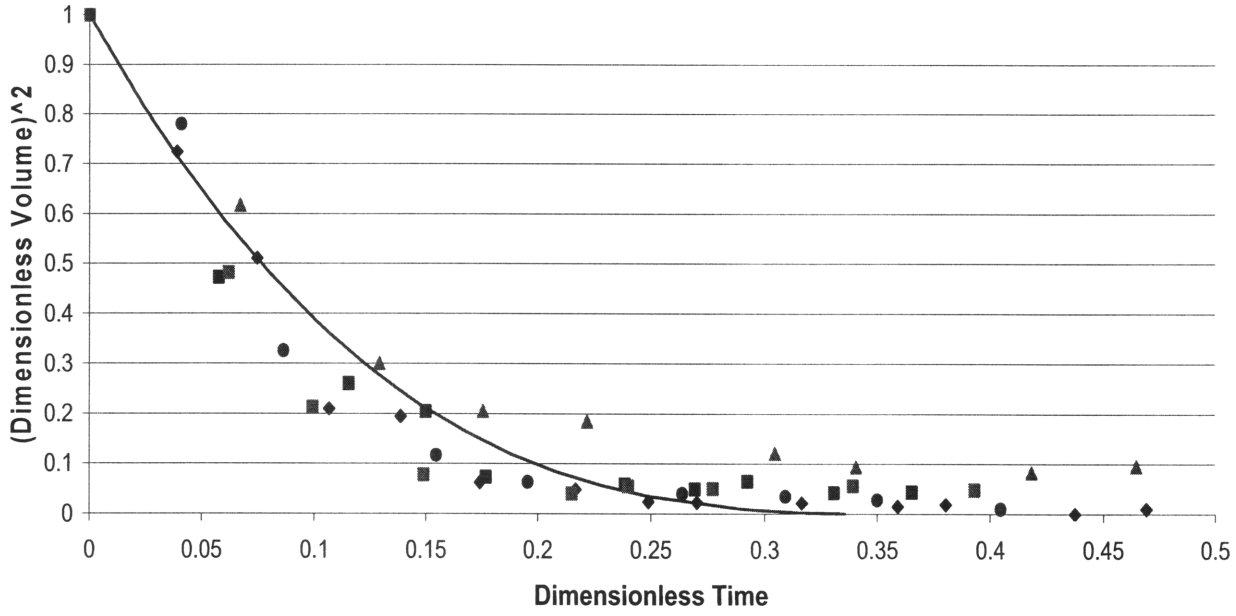

Figure 14: Dimensionless Free Water Volume Squared vs. Time for 2M Glycerol Droplets in Oil (Different Patterns correspond to different runs and droplet sizes)

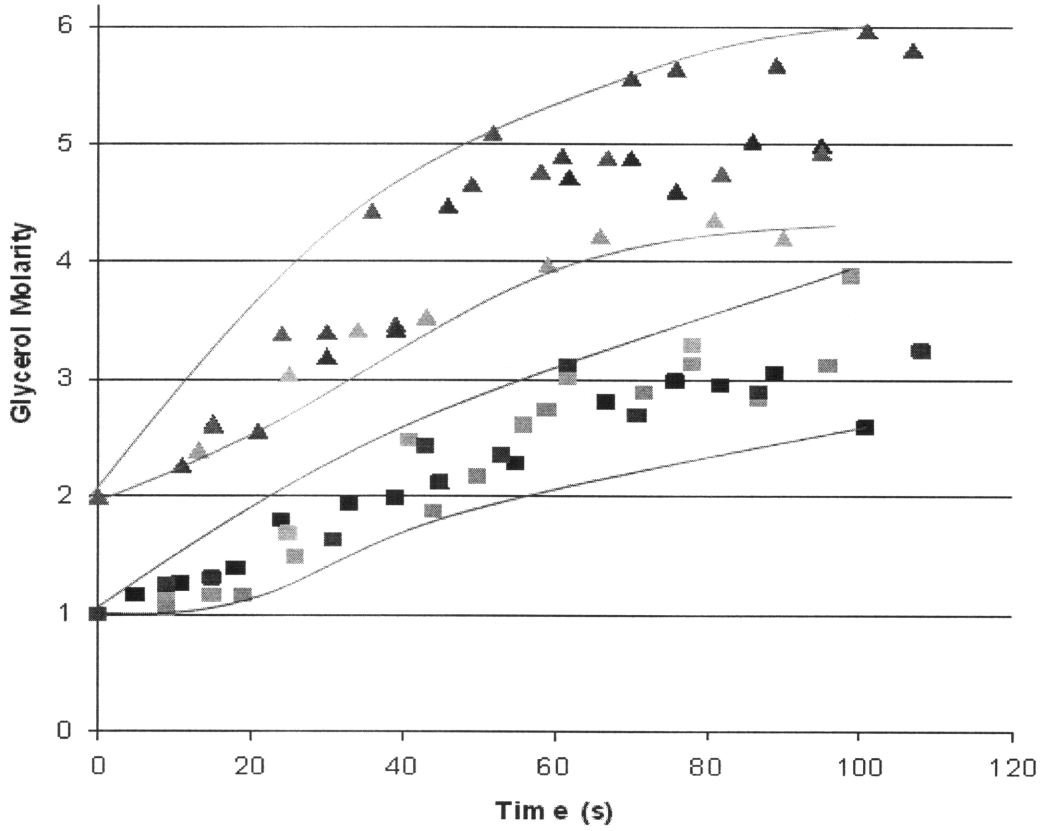

Figure 15: Increase in Glycerol Concentration with Time for Droplets of 1M and 2M initial concentration

3.4 Concentration of Cell Encapsulating Droplets

1M Glycerol Solution droplets in oil containing single cells exhibited similar behavior to droplets without cells. Figure 16 depicts a sample series of 1M glycerol droplets with an encapsulated cell. When visualized under fluorescence in the green channel, the cells appeared as bright stops while the encapsulating droplets appeared lighter but were clearly differentiable from the surrounding oil as shown in Figure 17. The shrinkage profile of cell encapsulating droplets is depicted in Figure 18. The time taken for the process to reach steady state is longer than that for earlier experiments due to the greater size of the droplets. However, as seen from the dimensionless plot in Figure 19, the time taken is actually less than that expected for the size of the droplets. This is due to overheating of the samples beyond 35 °C since the ITO heater was not precisely controlled. The overheating is also reflected in the fact the droplets shrink more than those in earlier experiments with 1M droplets shrinking to between 19-30% of their original size. The droplets were concentrated 3-5 times their original concentration.
In order to develop the pure water shrinkage model for this case, experiments on DI Water droplets in oil were run on the ITO heater and the slopes of area shrinkage monitored. The dimensionless data is superimposed against that computed for pure water droplets and is observed to closely replicate theory. The product $D_{w,o}C_{sat}$ increased 5.39 times its value at 25 °C. This large increase, especially as compared to the 2.88 times increase upon heating to exactly 35 °C suggests that the product is very sensitive to temperature.

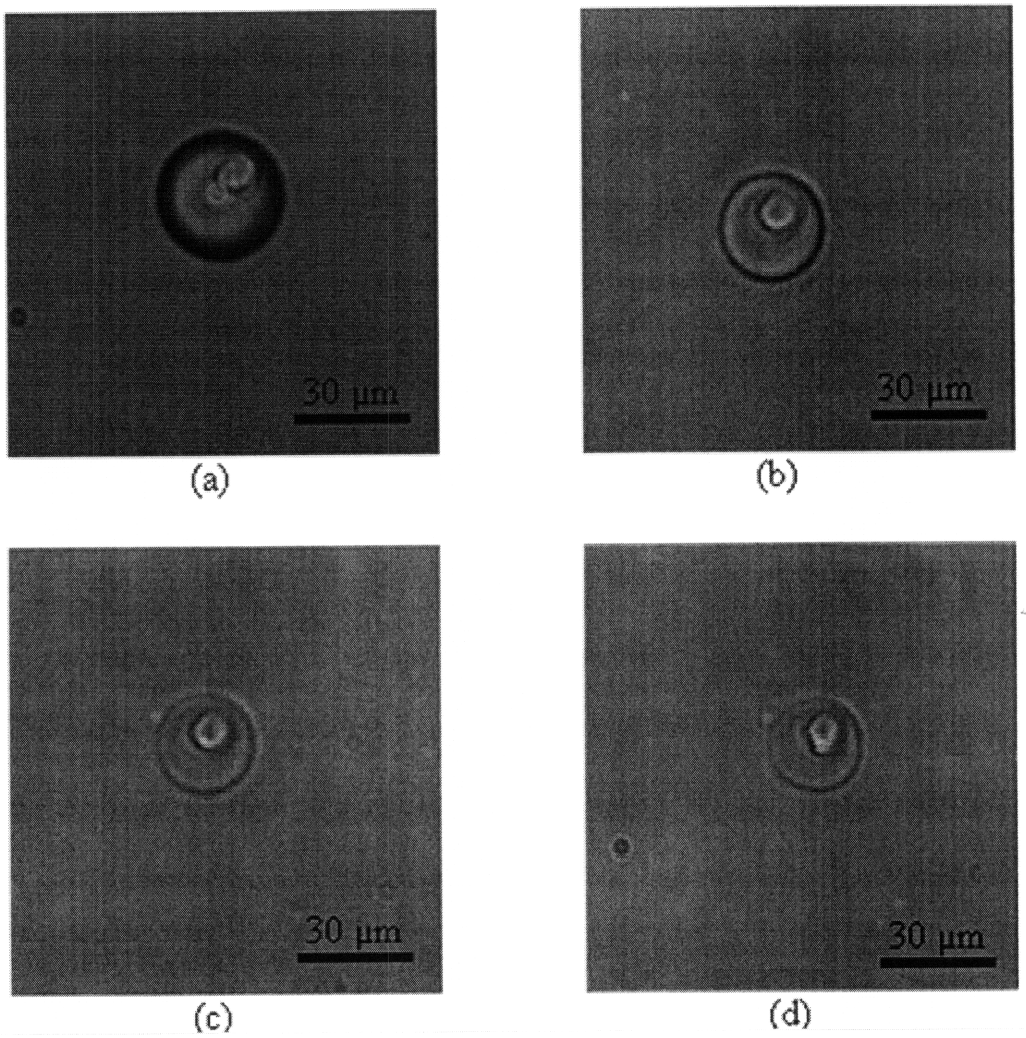

Figure 16: 1M Glycerol Cell Encapsulating Droplet in Soybean Oil at 35 ºC at (a) t = 0s (b) t = 60s (c) t = 120s and (d) t = 240 s

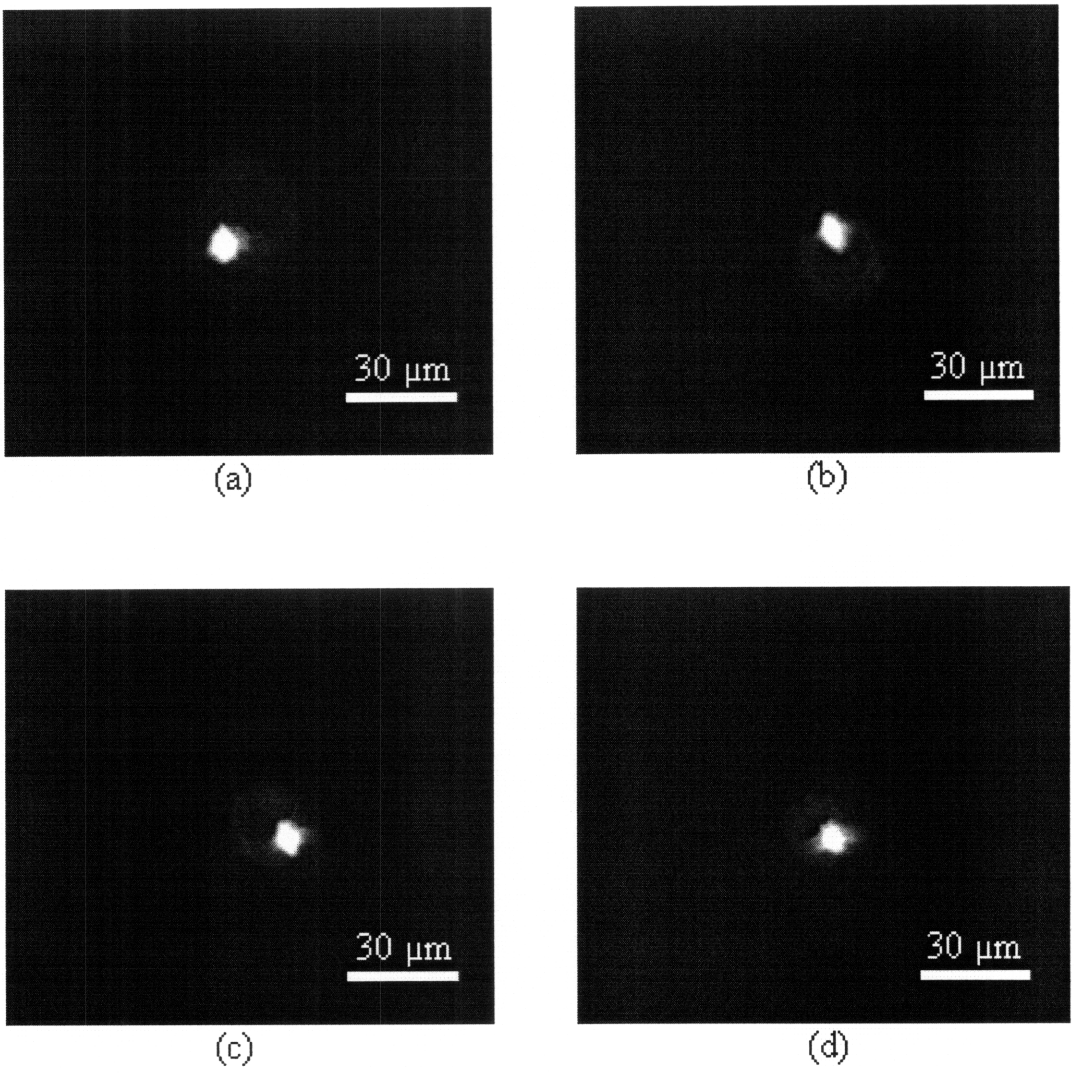

Figure 17: 1M Glycerol Cell Encapsulating Droplet in Soybean Oil under Fluorescence at 35 °C at (a) t = 0s (b) t = 120s (c) t = 240s and (d) t = 300 s

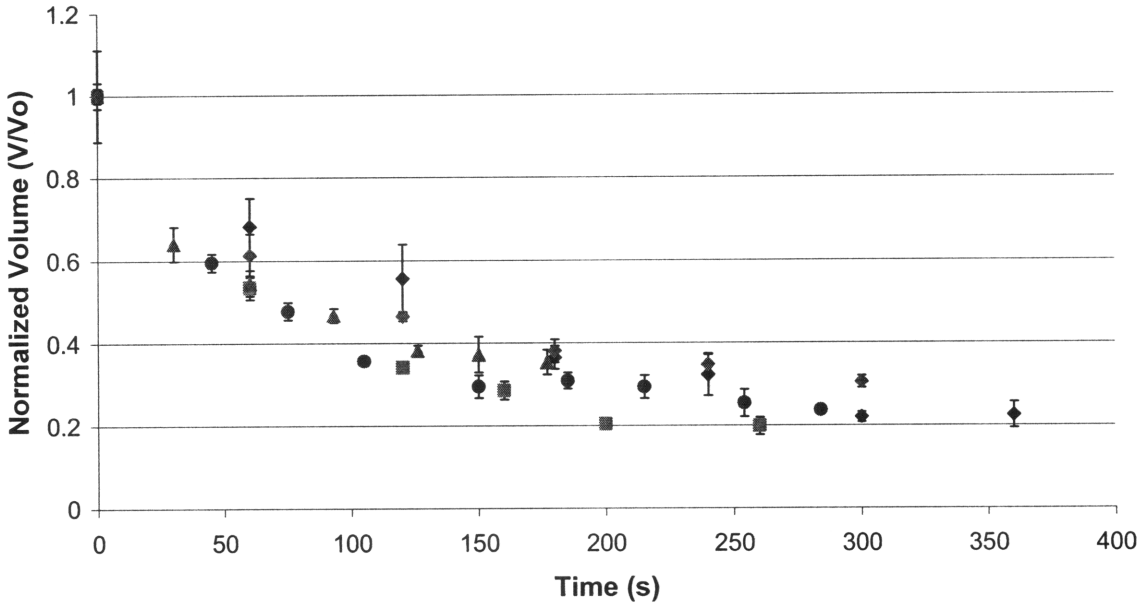

Figure 18: Change in Normalized Volume with Time for 1M Glycerol Cell Encapsulating Droplets in Oil (Different Patterns correspond to different runs and droplet sizes)

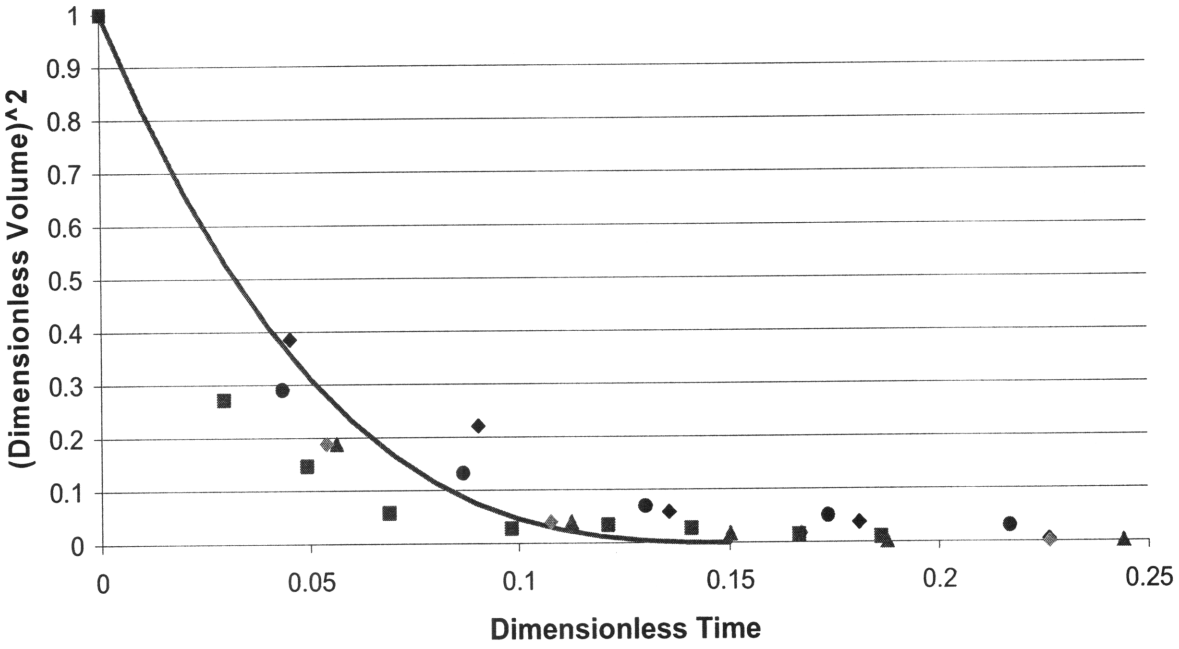

Figure 19: Dimensionless Free Water Volume Squared vs. Time for 1M Glycerol Cell Encapsulating Droplets in Oil (Different Patterns correspond to different runs and droplet sizes)

Chapter 4

Conclusion and Outlook

A novel method of concentrating cryoprotectant in water-in-oil micro-droplets is demonstrated. It is observed that droplets may be concentrated based on a temperature change and with good control over concentration rate and final concentration. The dimensionless analysis presented suggests that droplets follow a common pattern of shrinkage and that shrinkage and concentration profile of a droplet may be theoretically predicted under the given conditions if the initial size and concentration is known.

Droplets in the size range of 15-20 μm were shown to be concentrated with glycerol three times their initial concentration in about 90 seconds, while those in the size range of 30 μm concentrated three to five times in 300 seconds. These time scales fulfill the goal of developing a concentration mechanism that is slow enough to be controllable while yet fast enough that cells at high concentration are not exposed to ambient temperatures for fatally long periods. The experimental results on cells suggest that the concentration dynamics exhibited by cell encapsulating droplets are similar to those of droplets without cells. Tolerance of HL 60 cells to the concentration process is confirmed by the fact that the cells remained alive within the droplets throughout the experiments.

The demonstrated technique could be used to dynamically concentrate cryoprotectants within cell-containing droplets which may then be vitrified before they are exposed to high temperatures for fatally long time scales. With appropriate characterization, the controllability of the process will allow for choosing exact cryoprotectant concentration levels used for vitrification. Although not part of the current work, the demonstrated method may be used to modulate cryoprotectant concentration in

preservation protocols that require stepwise concentrations. In addition, since the process is reversible it may be used for post-thaw unloading of cryoprotectant from cells.

Further work required to put the cryoprotectant concentration technique in practice is to investigate the vitrification/ freezing processes of concentrated cells in droplets.

In addition, a completely automated system is visualized wherein emulsions would be generated on a microfluidic device and single cells would be encapsulated in droplets. Once concentrated by flowing over a heated stage the droplets would be plunged into liquid nitrogen by means of a valve or another gadget. Depending on factors such as type of cell, type of medium, goal of procedure, and the desired end point, one should be able to dial in a pre-plunging CPA concentration by adjusting the rate of flow over the thermal-stage.

The insight gained from the present effort combined with that from furthering of the idea could provide complete control over single cell preservation and increase its rate of success. Besides developing a useful and exciting application this controlled investigation method would provide deeper understanding of the science of cellular preservation. Lessons learned here move cryobiology another step closer to its founding goal and add to the hope of suspending life and bringing it back to verve in the exact same form as it was preserved eons ago.

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