Published in final edited form as:

Anal Chem. 2010 February 15; 82(4): 1288–1291. doi:10.1021/ac9022742.

Concentration of Glycerol in Aqueous Microdroplets by Selective Removal of Water

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Abstract

Among the challenges associated with vitrification of cells, a major roadblock is the requirement of high concentrations of cryoprotectant (CPA) chemicals and the damages caused by prolonged exposure of cells to these high concentrations above the glass transition temperature. These effects are minimized with controlled CPA loading. Certain organic oils, such as soybean oil, are made of triacylglycerols and are capable of dissolving small amounts of water, a property which enhances significantly with temperature. This phenomenon was exploited here to accomplish temperature controlled concentration of glycerol in single water droplets dispersed in the organic phase. Emulsions of aqueous solutions of glycerol in soybean oil were made and subjected to temperature increase of 10 °C from room temperature. Upon increasing temperature, water dissolved into the oil rendering the 15–20 micron droplets concentrated an average of 3.6 times and 2.6 times for 1M and 2M starting concentrations respectively with the oil-insoluble glycerol in 90 – 110 seconds. This phenomenon could be used to dynamically concentrate CPAs within cell-containing droplets which may then be vitrified before being exposed to high temperatures for fatally long times.

INTRODUCTION

The idea behind cryopreservation is suspension of all motion, which may be achieved by crystal formation or vitrification. Whereas crystal formation is damaging, vitrification is considered to be harmless. Viscosity in the glassy state is high enough to be considered stationary over a time scale of centuries¹, preventing degradation during storage. Vitrification of pure water can be achieved by either cooling at extremely fast rates on the order of 10⁶ °C/s^{2, 3}, or at lower cooling rates by adding significant amounts of cryoprotectant agents (CPAs)^{2, 4}. Many pure CPAs will form glass at any cooling rate⁵. The down-side of adding large amounts of cryoprotectants is that CPA's might themselves be toxic at these high concentrations. As a result, it is imperative to minimize the time for which the cell experiences the high CPA concentration in order to maximize post-thaw cell survival. For example, in order to achieve vitrification at cooling rates below 100 °C/s, glycerol concentrations of more than 45% (6M) are required⁶. Such high concentrations could be lethal if held at ambient temperature for too long. Experiments on human granulocytes have suggested that less than half of the cells survive exposure to 2M glycerol for more than 5 minutes⁷.

Previous work has demonstrated concentration of solutes in water droplets surrounded by organic substances that dissolve small amounts of water, a quality that can be controlled accurately with temperature^{8, 9}. Dynamic concentration and modulation of microdroplets

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has been demonstrated using focused laser and thermoelectric heating with temperature differentials constrained to less than 1 °C^{10–12}. Upon dissolution of some water from an aqueous solution droplet into the oil phase the remaining droplet is rendered concentrated with dyes, salts, and nanoparticles^{9,10}. However, using this technique to concentrate cryoprotectant agents (CPA) has not previously considered. In addition, small temperature differentials used in these studies require long processing times (>10 min). There is a need to rapidly concentrate CPAs to vitrification levels (> 5M) to avoid toxic injury to cells.

Here we make use of bulk heating with large temperature differential ($\Delta T = 10\text{ }^{\circ}\text{C}$) to concentrate CPAs in aqueous micro-droplets dispersed within a soybean oil continuous phase, consequently achieving high CPA concentrations in relatively shorter time periods, over a time scale of less than two minutes. It is envisioned to encapsulate single cells in droplets, concentrate the CPA as heating pulls water out and cool for preservation immediately after reaching the desired CPA concentration. The increase in glycerol toxicity due to heating is negligible in the experimental time scale of less than two minutes¹³.

EXPERIMENTAL SECTION

Preparation of Soybean Oil, Polydisperse Emulsions, and Experimental Setup

Soybean oil at 25 °C dissolves a maximum of 0.3% water by volume⁹. To prevent dissolution before heating, the oil was saturated with DI water at 25 °C. DI water (1% v/v) was mixed in Soybean oil by vigorously agitating a test tube to create an emulsion. The test tube was agitated for 30 s until the contents appeared cloudy to the eye, and then allowed to stand vertically for 72 hours. The undissolved water gravitationally separated to the bottom leaving the saturated oil above which was decanted for experimental use. SPAN 80 (Sigma Aldrich, St. Louis, MO) was added (0.1 % v/v) to the saturated oil to enhance emulsion stability during experiments.

Polydisperse emulsions were generated using this saturated oil. To control the amount of oil available for dissolution, the dispersed phases were always mixed with the continuous phase of soybean oil in a 1:1000 ratio by volume. The oil and the dispersed phase were added together in a vial and vigorously agitated for about 30 seconds until the vial contents appeared slightly cloudy to the eye.

All droplet concentration experiments were performed on a Linkam® FDCS 196 thermal stage, mounted on an upright Nikon® Optiphot 2 microscope, to allow for microscopic observation of droplets at controlled temperature. Once polydisperse emulsions were prepared, a 2 μl droplet of these emulsions was pipetted on to a hydrophobic coverslip placed on the thermal stage maintained at 25 °C. Hydrophobic coverslips were used to ensure that the water droplets in the sample did not stick to the substrate and remained spherical throughout the experiments.

The samples were observed under the microscope and manually adjusted to bring droplets of between 13 and 18 μm diameter into the field of view. Once a droplet in the desired size range was clearly observable the stage was heated at a rate of 120 °C per minute to a final temperature of 35 °C. At this point video recording was started. Frames were recorded at 10X magnification using a PixeLINK A662 digital camera. A schematic of the experimental setup as well as a sample experimental run with shrinking droplets are shown in Figure 1.

Selection of cryoprotectant

Preliminary testing was done to explore the interaction of DMSO and glycerol dispersed in soybean oil phase. First, a polydisperse emulsion of DMSO in oil was made (1:1000 v/v) and a sample of that was heated from 25 °C to 35 °C, and observed under the microscope.

Droplets of DMSO were found to completely dissolve in soybean oil after 10 minutes. This discounted DMSO as a candidate cryoprotectant for the proposed concentration process. The same process was repeated with a polydisperse emulsion of glycerol in soybean oil (1:1000 v/v). The glycerol droplets however, did not dissolve in oil; glycerol could be concentrated and was thus selected as the cryoprotectant for these experiments.

Concentration of droplets

Solutions of glycerol in water were prepared for experiments. Glycerol concentrations of 0M (pure water), 1M, and 2M were studied. These solutions were also mixed with the prepared saturated soybean oil in a 1:1000 ratio by volume and polydisperse emulsions were generated. Samples of these emulsions were heated on the thermal stage from 25 °C to 35 °C; the object being to dissolve the water in the glycerol solution droplet into the soybean oil phase and render the remaining highly concentrated with glycerol. Droplets in the size range of 13 – 18 µm were observed over several runs. The time dependent diameters of the shrinking droplets were measured and since the droplets were essentially spherical the measured diameters were used to calculate the droplet volume.

THEORETICAL SECTION

A pseudo-steady state diffusion model is used under the assumptions that water is sparingly in soybean oil (0.3% v/v at 25 °C) and that the rate of diffusion of water through the soybean oil is much greater than the rate of shrinkage of the droplet. This pseudo-steady state assumption breaks down when the droplet is smaller than 0.5 µm. The diffusion coefficient of water in a 1M glycerol solution is about 950 µm²/s¹⁴, which is much greater than that of water in soybean oil estimated to be about 50 µm²/s⁹. Thus the resistance to mass diffusion within the glycerol solution droplet is neglected. This assumption may be considered valid at least until the glycerol concentration exceeds 6M, at which point the diffusion coefficient of water in glycerol solution reduces to about 400 µm²/s¹⁴. Thus the present model is assumed to be valid for droplet sizes of greater than 0.5 µm and glycerol concentrations of at least up to 6M, which covers the entire experimental regime.

For pseudo-steady state mass diffusion equation in radial symmetry, the rate of shrinkage of droplet area should be constant¹⁵. As confirmed experimentally for a drop of pure water in soybean oil by He et al⁹, the rate of change of area, A , is given by:

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

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 the saturation concentration of water in oil (assumed for the droplet surface) and the concentration far away from the droplet, respectively.

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

$$V = (Kt + V_o^{2/3})^{3/2}, \quad (2)$$

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

$$K = - \left(\frac{128}{9} \right)^{1/3} \pi^{2/3} \left(\frac{M_w D_{w,o} C_{sat}}{\rho_w} \right). \quad (3)$$

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

$$\tilde{V}^2 = (-Z \tilde{t} + 1)^3, \quad (4)$$

where $\tilde{V}$ is the dimensionless volume, $\frac{V}{V_0}$, $\tilde{t}$ is the dimensionless time, $\frac{t}{\tau}$, where τ is the time constant given by,

$$\tau = \frac{V_o^{2/3}}{|K|}. \quad (5)$$

Z is simply a multiple of K and describes the multiple by which the product $D_{w,o} (C_{sat} -$

$C_\infty)$ changes for a given temperature change, i.e. $Z = \frac{(D_{w,o} C_{sat})_{35^\circ C}}{(D_{w,o} C_{sat})_{25^\circ C}}.$

RESULTS & DISCUSSION

The aforementioned theory is initially validated using data for DI water, which is governed by the simple diffusion equation. In this case, the droplet volume eventually goes to zero assuming there is sufficient oil in which to dissolve the water. Emulsions of pure de-ionized water in soybean oil were observed after heating to 35 °C and as expected the droplets did completely dissolve and the time taken for dissolution varied between 50 and 90 s depending upon the initial droplet size (see Figure 2). Linear trend lines were fitted through each set of data according to Eq. (1). The close agreement with data validates the model for pure de-ionized water. In the case of aqueous glycerol droplets the squares of the dimensionless volume are plotted against dimensionless time and superimposed with theoretical curves in Figure 3. It is observed that the experimental data for all experimental runs follows the same trend and closely conforms to the theory for glycerol solutions as well.

For aqueous-glycerol droplets, the theory 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. Since the theory assumes that the dispersed are pure water, the experimental data was analyzed as a change in the free water volume with time. Free water volume is simply the total volume of the droplet at any given time less the volume of the glycerol and the volume of water that is bound to this glycerol. 2.63 water molecules bind to each glycerol molecule¹⁶. Consequently, for 1M glycerol concentration the glycerol and bound water comprise about 12% of the initial droplet volume, i.e. 88% volume of the initial volume the free water that can leave the

droplet and dissolve into the oil phase. For 2M concentration the glycerol and bound water comprise about 24% of the initial volume.

On average, the droplets stopped shrinking once they reached $28\% \pm 4.5\%$ and $38\% \pm 6.2\%$ of their original volume for 1M and 2M glycerol concentrations respectively. This is attributed in part to the binding of some water to the glycerol in solution. The apparent leftover free water in these drops may arise due to the drastic reduction in water transport due to increased glycerol concentration as well as due to the finite volume availability of oil per aqueous drop (1000:1 v/v).

Results for 1M and 2M droplets are summarized in Figures 3(b) and 3(c) respectively, in dimensionless form. The data conforms to theory until the end of the process where the slight discrepancies are due to saturation of oil with water in the droplet vicinity. It is observed that the value of the product $D_{w,o}C_{sat}$ increases 2.7 times on average in going through a temperature rise of 10 °C from 25 °C to 35 °C. The dimensionless plots are useful for predicting the shrinkage profile of a glycerol solution droplet in soybean oil at 35 °C if the initial droplet size is known. A simple manipulation gives the resulting glycerol molarity as,

$$M = M_i / \left[1 + \Phi_{fw} (\tilde{V} - 1) \right], \quad (6)$$

where M_i is the initial molarity and Φ_{fw} is the free water volume fraction which depends on the initial molarity. $\tilde{V}$ and the corresponding dimensionless time $\tilde{t}$ can be read from the model graph. For example, given a starting droplet diameter of 20 μm , a droplet of 1M initial concentration will be concentrated 3.05 times in 76 seconds (see Figure 3(b)). Note that the free water volume fraction for 1M concentration is 0.88, whereas a 1M 30 μm droplet would be concentrated 3.05 times in 170 seconds. On the other hand a droplet starting at 100 μm diameter and 1M concentration would take more than 29 minutes to concentrate to 2.3M, thus failing to achieve the quick concentration goal. This feature allows for estimation of the size ranges in which the proposed process would be feasible.

The concentration profiles of selected droplets are depicted in Figure 4. 1M droplets in the size range of 14.5–18 μm were concentrated to about 3M, while 2M droplets in the size range of 16–18.5 μm were concentrated to about 6M, all in 80 to 100 seconds; fast enough to minimize exposure times but slow enough to be controllable. All droplets, regardless of initial concentration and size, show similar general behavior.

It is important to note that we saturated the oil at 25 °C as the initial condition for our experiments. We then used a temperature jump of 10 °C to go to 35 °C to facilitate droplet shrinkage. This temperature increase lasts for less than two minutes and the potential increased toxicity of glycerol is negligible under these conditions¹³. Moreover, given that the glycerol concentration reached levels above 6M in less than two minutes, which are adequate for vitrification of biological samples, we did not study the effect of temperature beyond 35 °C. In addition, the cooling rates are extremely rapid for vitrification purposes (2500 – 30000 °C/min)^{17–19} and thus the time will be too short for the droplet to dilute back while cooling due to the reversible nature of the process. As a result our approach can ultimately be extended for use in biological cell preservation.

In summary, we demonstrate a temperature controlled method of concentrating cryoprotectant agents in aqueous micro-droplets suspended within a continuous phase of soybean oil. The analysis presented suggests that droplets follow a common pattern of shrinkage and concentration profile which may be predicted from theory under the given

conditions if the initial size and concentration are known. The use of bulk heating and significant temperature differential of 10 °C provides for a fast and simple process. The demonstrated technique could be used to dynamically concentrate cryoprotectants within cell-containing droplets which may then be vitrified. The process could allow for choosing exact cryoprotectant concentration levels used for vitrification. The method may also be used to modulate cryoprotectant concentration in preservation protocols that require stepwise addition or removal of cryoprotectant concentrations. In addition, this technique may prove to be a useful tool in various other chemical and biological applications.

Acknowledgments

The authors would like to thank Daniel T. Chiu for helpful discussions (with M.T.) and for critical reading of the manuscript. This study was partially funded by the National Institutes of Health Grants DK046270 and P41 EB002503.

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Figure 1. Schematic of experimental setup, and shrinking of 1M Glycerol Solution droplets in soybean oil

Figure 2.
Shrinkage of droplet surface area with time for DI water droplets in oil.

Figure 3. Dimensionless Volume Squared vs. Dimensionless Time for (a) DI Water Droplets in Oil, (b) Free water volume of 1M Glycerol Droplets in oil, (c) Free water volume of 2M glycerol droplets in oil.

Figure 4.
Molar Concentration of Glycerol vs. Time in droplets of different sizes and initial concentrations