Characterization of cryobiological responses in TF-1 cells using interrupted freezing procedures

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Abstract

Cryopreservation currently is the only method for long-term preservation of cellular viability and function for uses in cellular therapies. Characterizing the cryobiological response of a cell type is essential in the approach to designing and optimizing cryopreservation protocols. For cells used in therapies, there is significant interest in designing cryopreservation protocols that do not rely on dimethyl sulfoxide (Me2SO) as a cryoprotectant, since this cryoprotectant has been shown to have adverse effects on hematopoietic stem cell (HSC) transplant patients. This study characterized the cryobiological responses of the human erythroleukemic stem cell line TF-1, as a model for HSC. We measured the osmotic parameters of TF-1 cells, including the osmotically inactive fraction, temperature-dependent membrane hydraulic conductivity and the membrane permeability to 1M Me2SO. A two-step freezing procedure (interrupted rapid cooling with hold time) and a graded freezing procedure (interrupted slow cooling without hold time) were used to characterize TF-1 cell recovery during various phases of the cooling process. One outcome of these experiments was high recovery of TF-1 cells cryopreserved in the absence of traditional cryoprotectants. The results of this study of the cryobiology of TF-1 cells will be critical for future understanding of the cryobiology of HSC, and to the design of cryopreservation protocols with specific design criteria for applications in cellular therapies.

keywords: TF-1, osmotic properties, hydraulic conductivity, osmotically-inactive fraction, solute permeability, cryopreservation, hematopoietic stem cells, dimethyl sulfoxide

Introduction

Cryobiology plays a key role in the long-term storage of native and engineered cells and tissues for research and applications in many disciplines. Cryopreservation, which involves storing cells and tissues at low subzero temperatures, is currently the only feasible method for the long-term maintenance of biological structure and function. Success in cellular therapies often depends critically on the number of functional cells transplanted, thus reviving interest in maximizing cell survival and function during collection, processing, and preservation. As new sources of cells are being explored, it is becoming clear that effective tools are required to understand the cryobiology of these cells.

The survival of cells and tissues after freezing and thawing depends on the ability of the cells to withstand a variety of stresses imposed by the cryopreservation protocol. As extracellular ice forms during cryopreservation, there is a resulting increase in extracellular solute concentration, which imposes osmotic stresses on the cell [41]. The osmotic response of the cell is largely dependent on the movement of water across the plasma membrane [40]. The movement of water across the membrane is the result of movement of water molecules by diffusion through the membrane or through water channels (aquaporins). The osmotic parameters governing the movement of water and cryoprotectants across the membrane are specific to each cell type; thus the osmotic responses to anisotonic conditions are different for different cell types. If a permeating cryoprotectant is present then both the net water and cryoprotectant movements depend on the osmotic parameters of the cell membrane. Therefore, measuring the osmotic parameters of the cell membrane is important to further understand the cryobiology of a cell.

Electronic particle counters have been used to measure cell volumes as a function of time

for cells exposed to anisotonic solutions [48]. In kinetic studies, sequential measurements of cell volumes allow determination of cell permeability characteristics by fitting the experimental data with theoretical models. Using this method, osmotic parameters have been determined for a variety of cell types including: human lymphocytes [21]; bovine chondrocytes [47]; pancreatic islet cells [31,61]; human corneal endothelial, stromal, and epithelial cells [9]; several African mammalian spermatozoa [17]; and HSC [16,22,60].

In addition to characterizing cellular osmotic responses, exploring how freezing results in cell damage, is also critical. Mazur *et al.*'s "two-factor hypothesis" of freezing injury [33,37] proposes that there are two independent mechanisms of damage during freezing: injury during slow cooling, where cell injury is caused by exposure to high solute concentrations as water in the extracellular solution is converted to ice; and injury during rapid cooling, where cell injury is related to the presence of intracellular ice [38,41]. Maximum survival is normally obtained by selecting a cooling rate that is sufficiently high to avoid the injury from exposure to the concentrated solutes, yet low enough that the cells can osmotically dehydrate to avoid intracellular ice formation. This approach almost always requires a cryoprotectant, such as Me_2SO. Like other permeating cryoprotectants, Me_2SO protects the cell primarily against slow cool injury by reducing the amount of ice formed at any temperature, hence reducing the concentrations of other solutes and injury related to exposure [67]. There are, however, drawbacks to using cryoprotectants in cryopreservation of clinical samples, which include cellular osmotic stress during addition and removal, toxicity to cells during processing and preservation, and transplant-related toxicity for the patient. A better understanding of the nature and kinetics of cellular responses to temperature-induced conditions would allow novel approaches to the cryopreservation of new cell types.

In most practical cryopreservation procedures, cells are typically cooled to a low temperature (e.g. -80°C); the process being empirically optimized for cell type by varying cooling rate and cryoprotectant type and concentration to minimize cryoinjury. However, it has long been known that there are critical subzero temperature ranges where interrupting the cooling process improves cell recovery [35]. There are two procedures specifically designed to explore cryoinjury by separating damage which occurs during the initial cooling to this critical temperature range, during time spent in this temperature range itself, and during subsequent cooling to storage temperatures. These two procedures are two-step freezing (interrupted rapid cooling with hold time) [14] and graded freezing (interrupted slow cooling without hold time) [44].

The two-step freezing procedure, used by Farrant et al. is a logical method to examine the effects of interrupted rapid cooling on cell recovery over a broad range of subzero temperatures and conditions. Using this procedure [14], human lymphocytes were cooled rapidly to various subzero temperatures and held for various periods of time before being either (a) thawed directly from that holding temperature or (b) rapidly cooled to -196°C (liquid nitrogen) before thawing (see schematic - Figure 1a). This two-step freezing procedure is uniquely different from previous procedures using interrupted cooling as it also includes a separate analysis of damage which occurs during the initial cooling to the hold temperature. In further studies, McGann and Farrant reported that subzero temperature and the length of hold time at that temperature were important factors affecting cell survival [45]. McGann also used two-step freezing to explore the differing actions of penetrating and non-penetrating cryoprotectants and for the cryopreservation of Chinese hamster fibroblasts with 20 % HES; with cell recovery comparable to 10 % Me_2SO [43]. It should be noted that the two-step procedure [14] is a modification of previously reported

procedures [2,54,57], which did not include the direct thawing step from the hold temperature.

A variation of the two-step freezing procedure, the graded freezing procedure, was used by McGann to investigate progressive cell injury during slow cooling [44]. In this procedure, samples were cooled at a low linear rate to various subzero temperatures before being either (a) thawed directly or (b) rapidly cooled to -196°C (liquid nitrogen) before thawing. The difference between the two-step and graded freezing procedures is that two-step freezing uses interrupted rapid cooling with a hold step at an intermediate temperature, while graded freezing uses interrupted slow cooling to an intermediate temperature, without a hold step (see schematic - Figure 1b). Both approaches allow separation of damage which occurs during the initial cooling phase from that which occurs during subsequent cooling to the storage temperature.

Although HSCs are routinely cryopreserved for clinical use using dimethyl sulphoxide (Me_2SO), this cryoprotectant is associated with morbidity and mortality in some HSC transplant patients [7,8,11,56,68] and contributes to osmotic stresses for stem cells [60]. As with other cells used in therapies, it would be beneficial to eliminate Me_2SO from the cryopreservation protocol. TF-1 cells have been used as a model cell type for hematopoietic stem cells (HSC). These cells express the CD34+ antigen and are able to differentiate into the various hematopoietic lineages [28,29,36], and as such have been used in various studies [4,5,30,36]. This study explores the cryobiology of TF-1 cells, a model for HSCs, by examining cryoinjury during interrupted rapid cooling and interrupted slow cooling procedures. Specifically, this study will use two-step freezing and graded freezing procedures to characterize the cryobiological responses of TF-1 cells.

Experimental materials and methods

TF-1 cell culture

TF-1 cells ((Lot #2056376) ATCC, Manassas, Virginia) were cultured at 37 °C in 5 % CO_2 in Modified RPMI 1640 Medium (ATCC) with 10 % fetal bovine serum (ATCC), and supplemented with 2 ng/mL recombinant human GM-CSF (Stemcell Technologies, Vancouver, Canada). Prior to experiments, cells were washed twice with serum-free RPMI media and incubated in serum-free RPMI overnight to accumulate the cells in the G_1/G_0 phase of the cell cycle [30], resulting in a more uniform cell size distribution. Cells were then centrifuged and re-suspended in serum-free RPMI (4×10^6 cells/mL) prior to experiments. Serum-free media was chosen for experiments in order to compare experimental results with and without different types of cryoprotectants.

Cell volume measurements

A Coulter Electronic Particle Counter (ZB1, Coulter Inc., Hialeah, Florida), fitted with a pulse-height analyzer (The Great Canadian Computer Company, Spruce Grove, AB, Canada) was used to monitor cell volume as a function of time as cells passed through the 100 μm aperture [18-20,48]. This system has been previously used to monitor changes in cell volume for a variety of cells in suspension [3,9,21,31,61], including HSC [16,59].

TF-1 cells (150-200 μL) were injected into well-mixed hypertonic experimental solutions (10 mL) at experimental temperatures that were maintained using a circulating water bath and a custom insulated jacket. Pulse heights, proportional to the cell volumes, were digitized and the time recorded as each cell traversed the aperture of the Coulter counter [48]. The upper and lower volume limits were 1750 ± 1 μm^3 and 218 ± 6 μm^3, respectively. There was approximately 2 % coincidence correction for the cell density used in the experiments [6]. Various concentrations of phosphate-buffered saline ((PBS) GIBCO Invitrogen Corp., Carlsbad, California) were prepared by diluting 10X PBS (GIBCO) with distilled water to final

osmolalities of 291 ± 6 (1X), 583 ± 25 (2X), 861 ± 22 (3X), 1150 ± 17 (4X) and 1434 ± 20 mOsm/kg (5X) respectively (mean ± sd of 16 replicates). Volume measurements to determine the permeability of Me_2SO used Me_2SO (Sigma, Missisauga, Canada) in 1X PBS for a final concentration of 1 M Me_2SO. Osmolalities were measured using a freezing-point depression Osmometer (Precision Systems Inc., Natick, Massachusetts), calibrated using 100, 300 and 500 mOsm/kg osmometry standards (Precision Systems Inc.).

For PBS solutions, the experimental temperatures were 4.8 ± 0.1, 12.9 ± 0.3, 23.3 ± 0.2, and 37.4 ± 0.1 °C (2-5X PBS data pooled mean ± sem; n=36) and 4.6 ± 0.2, 8.0 ± 0.2, 11.0 ± 0.2, 16.3 ± 0.2, 19.4 ± 0.3, and 28.8 ± 0.2 °C (3X PBS data mean ± sem; n=9). The temperatures were measured immediately following data acquisition while still in the insulated jacket using a Digi-Sense thermocouple thermometer (Cole Parmer, Anjou, Canada). For Me_2SO experimental solutions, 3 temperatures were investigated: 4.9 ± 0.3, 10.7 ± 0.4, and 23.4 ± 0.5 °C. For each experiment, 3 replicate measurements were performed for each solution at each temperature. The experiments were repeated a minimum of 3 times using cells from different passages. Latex beads (15 μm diameter; Beckman Coulter, Miami, Florida) were used as calibrators to convert pulse heights to actual volumes in 1X PBS and in the experimental solutions.

Cell permeability parameters

Osmotically-inactive fraction

Cellular osmotic parameters relevant to cryopreservation are the osmotically-inactive fraction, hydraulic conductivity, and solute permeability for permeating cryoprotectants and activation energies. The osmotically-inactive fraction (*b*) of the cell is the fraction of the cell volume not involved in the osmotic activities. The Boyle van't Hoff relationship [34] has been used to express equilibrium cell volume in solutions of impermeant solutes:

$$\frac{V}{V_o} = \frac{\pi^o}{\pi}(1-b)+b \qquad (1)$$

where V is the equilibrium cell volume (μm^3), V_o is the isotonic cell volume (μm^3), π^o is the isotonic osmolality (osmoles/kg water), π is the experimental osmolality (osmoles/kg water), and b is the osmotically-inactive fraction of the cell volume, a parameter found by fitting Eq. 1 to data.

Hydraulic conductivity

Jacobs and Stewart [24,25] used the following equation to describe the rate of water movement across the plasma membrane :

$$\frac{dV}{dt} = L_p ART(\pi_i - \pi_e)\rho \qquad (2)$$

where V is the volume of the cell (μm^3), t is the time (min), L_p is the hydraulic conductivity ($\mu m^3/\mu m^2$/min/atm), A is the cell surface area (μm^2), R is the universal gas constant ($\mu m^3 \cdot$atm/mol/K), T is the absolute temperature (K), π_e is the extracellular osmolality (osmoles/kg of water), π_i is the intracellular osmolality (osmoles/kg of water), and ρ is the density of water (assumed to be constant at 1.0×10^{-15} kg/μm^3). Experimental measurements of cell volume as a function of time in the presence of an impermeant solute were fit to Eq. 2, with the following initial conditions: i) the intracellular solution was at isotonic concentration and the extracellular solution was at the experimental concentration; ii) the cell volume was calculated from the Boyle-van't Hoff equation (Eq. 1). The differential equation Eq. 2 was integrated using Euler's method with sufficiently small discretization in EXCEL (e.g. time increment approximately 8×10^{-4} min). Experimental data were fit to the differential equation solutions using the least squares method (EXCEL Solver) to estimate a best fit value for L_p. The

assumptions used in these calculations are: i) intracellular and extracellular solutions are dilute, so the values of osmolality and concentration are the same; ii) the density of water is 1 g/mL; and iii) the cell surface area was calculated from the spherical cell volume.

Solute permeability

Jacobs and Stewart coupled Eq. 2 with the following equation to describe the volume changes and solute movement across a membrane as a function of time:

$$\frac{dS}{dt} = P_s A\left(C_s^e - C_s^i\right)\rho \qquad (3)$$

where S is the number of solute molecules (mole), P_s is the solute permeability ($\mu m^3/\mu m^2/min$), C_s is the solute concentration (molal), the superscript e denotes extracellular, and the superscript i denotes intracellular. In the presence of a permeant solute, experimental data were fit to Eqs. 2 and 3 using the following conditions: i) the initial conditions were that the intracellular solution was at isotonic concentration, containing no permeant solute; and ii) the experimental conditions were that the extracellular solution consisted of the permeant solute at the experimental concentration and an impermeant solute at the isotonic concentration. The differential equation Eq. 3 was integrated using Euler's method with sufficiently small discretization in EXCEL, and the experimental data were fit using the least squares method (EXCEL Solver) to estimate either P_s or both P_s and L_p.

Temperature dependencies of permeability parameters

Also relevant to cryopreservation are the temperature dependencies of L_p and P_s which are normally described with Arrhenius relationships [53]:

$$L_p = L_p^o \exp\left(-\frac{E_a^{Lp}}{R}\left(\frac{1}{T}-\frac{1}{T_o}\right)\right) \quad (4)$$

$$P_s = P_s^o \exp\left(-\frac{E_a^{Ps}}{R}\left(\frac{1}{T}-\frac{1}{T_o}\right)\right) \quad (5)$$

where L_p^o and P_s^o are the reference water and solute permeabilities, respectively, at the temperature T_o, E_a are the Arrhenius activation energies (kcal/mol), R is the universal gas constant (kcal/mol/K), and T is the absolute temperature (K). The Arrhenius activation energies for L_p or P_s, described by Eqs. 4 and 5 respectively, were determined by fits using linear regression of the natural logarithm of L_p or P_s as a function of the inverse absolute temperature.

Statistical analysis

Statistical comparisons used a standard two-way analysis of variance (ANOVA) (p=0.05 level of significance) for estimates of L_p and b respectively between the 4 groups of experimental temperatures and PBS concentrations, including evaluation of interactions between these parameters. A one-way ANOVA (p=0.05) was used to further evaluate statistical differences between temperatures for L_p. Due to small sample size, a Kruskal-Wallis non-parametric statistical test (p=0.05) was used to compare estimates of P_s when solved for separately from L_p, with estimates of P_s when solved for simultaneously with L_p. For all mean values reported, the standard error of the mean was also calculated and included in the result (i.e. mean ± sem).

Two-step freezing procedure (interrupted rapid cooling with hold step)

Samples of 0.2 mL TF-1 cell suspension in serum-free RPMI were transferred to glass tubes (6x50 mm; Fisher, Edmonton, Canada) and allowed to equilibrate at room temperature for 5 min. Control samples were either warmed in a 37 °C water bath or plunged directly into liquid nitrogen. Experimental samples were individually transferred into a stirred methanol bath

(Kinetics Thermal Systems, New York, USA) preset at -3, -6, -9, -12, -15, -20, -30, or -40 °C and allowed to equilibrate for 2 min at that temperature prior to ice nucleation with cold forceps. After nucleation, samples were held at the experimental temperature for 3 min before either thawing in a 37 °C water bath or plunging into liquid nitrogen. Plunge samples were stored in liquid nitrogen for a minimum of 1 hour prior to thawing in a 37 °C water bath. Sample temperatures were monitored throughout the experiments using a Type T thermocouple. Experiments were also performed using different hold times. Samples were cooled to various experimental hold temperatures (-5, -7, -9, -12, -25 °C), nucleated with cold forceps, and held for 0.5, 0.7, 1, 2, 3, 5, 7 or 10 min. Duplicate samples were used for each experimental measurement and each experiment was repeated in triplicate with cells from different passages. Samples cooled to hold temperatures of -20, -25, and -30°C spontaneously nucleated prior to reaching the hold temperature; however time zero for all experimental samples was calculated as time after nucleation.

Graded freezing procedure (interrupted slow cooling without hold step) no cryoprotectant

Samples of 0.2 mL cell suspension in serum-free RPMI were transferred to glass tubes and cooled in a 0 °C wet ice bath for 5 min. Control samples were removed and either warmed in a 37 °C water bath or plunged into liquid nitrogen. Experimental samples were transferred into a methanol bath (FTS Systems, Inc., Stone Ridge, New York) preset at -3 °C and allowed to equilibrate for 5 min prior to ice nucleation in each tube with cold forceps. The bath was then cooled at 0.9 °C/min, and the temperature monitored using a Type T thermocouple (Omega, Laval, Canada). At each experimental temperature (-3, -6, -9, -12, -15, -20, -30, and -40 °C), one set of duplicate samples was thawed directly in a 37 °C water bath and another set was plunged into liquid nitrogen. Plunge samples were kept in liquid nitrogen for a minimum of one hour

prior to being thawed in a 37 °C water bath. Duplicate samples were used for each experimental temperature. Other experiments used cooling rates of 0.2 and 0.5 °C/min. Each experiment was repeated in triplicate with cells from different passages.

Graded freezing procedure with Me_2SO

Samples of 0.2 mL cell suspension in 10 % Me_2SO in serum-free RPMI at room temperature were transferred to glass tubes and placed in a 0 °C ice bath for 5 min. Control samples were removed and either warmed in a 37 °C water bath or plunged into liquid nitrogen. Experimental samples were transferred into a methanol bath preset at -3 °C and allowed to equilibrate for 5 min prior to ice nucleation with cold forceps. Although this temperature is slightly above the predicted freezing point of this solution, the presence of ice crystals was verified by visual examination at the end of the hold time. After 5 min, the bath was cooled at 0.9 °C/min. At each experimental temperature (-3, -6, -9, -12, -15, -20, -30, and -40 °C), one set of duplicate samples was thawed directly in a 37 °C water bath and another set was plunged into liquid nitrogen. Plunge samples were kept in liquid nitrogen for a minimum of one hour prior to being thawed in a 37 °C water bath. Duplicate samples were used for each experimental temperature. Each experiment was repeated in triplicate with cells from different passages.

Assessment of cell recovery

Membrane integrity is widely used as an indicator of cryoinjury to cells, and there is evidence that the plasma membrane is a primary site of freezing-thawing injury [1,39]. Although not a rigorous test of cell function, the membrane integrity assay indicates the upper limit for cell viability. In these studies, the membrane integrity assay was performed by incubating cells with SYTO® 13 (Molecular Probes, Eugene, Oregon) and ethidium bromide (EB) (Sigma, Mississauga, Canada) [64]. SYTO® 13 (SYTO) permeates the cell membrane of all cells,

complexes with DNA, and fluoresces green under UV exposure. Ethidium bromide penetrates cells with damaged plasma membranes and also complexes with DNA fluorescing red under UV conditions.

The SYTO/EB stain was prepared daily from 1X phosphate-buffered saline (PBS) and stock solutions, which were stored at 4 °C (EB) and at -20 °C (SYTO). Final concentrations were 0.25 mM EB and 0.125 mM SYTO. Twenty μL of stain was added to 200 μL of cell suspension, mixed, and incubated for 2 min at room temperature. Fluorescent images were captured using a Leitz Dialux 22 fluorescence (440-480nm) microscope (Leitz, Wetzlar, Germany) fitted with a PIXERA Viewfinder Pro digital camera (Pixera Corporation, Los Gatos, CA, USA). A viability assessment program (The Great Canadian Computer Company, Spruce Grove, Canada) was used to quantify cell membrane integrity from digital images [26].

Results

Isotonic cell volume

Synchronizing TF-1 cells in the G_0/G_1 phases of the cell cycle resulted in a narrow log normal distribution [6,12], with a mean isotonic volume 916 ± 15 μm^3 (mean ± sem) from replicate samples. The width of the distribution was 916 ± 35 (± sd of the distribution),

Osmotic parameters (b, L_p, E_a^{Lp})

Figure 2 shows representative plots of mean cell volume as a function of time after exposure of TF-1 cells to 3X PBS at 4 different temperatures. Values for the osmotically-inactive fraction (b) and the hydraulic conductivity (L_p) were calculated by simultaneously fitting L_p and b to the data using Eq. 2, from cell volume measurements in different experimental solutions (2X-5X) and different temperatures (4.8 ± 0.1, 12.9 ± 0.3, 23.3 ± 0.2, and 37.4 ± 0.1 °C), to examine the concentration and temperature dependence of L_p and b.

Values of the osmotically-inactive fraction calculated for all the experimental conditions in Table 1 show that the osmotically-inactive fraction for TF-1 cells was not dependent on concentration ($p>0.05$), but was dependent on temperature ($p<0.05$). Further analysis showed that among temperatures, some were statistically significant, indicating possible slight temperature dependence. From an Arrhenius plot, the activation energy describing the temperature dependence for the osmotically-inactive fraction (E_a^b) in TF-1 cells was 0.80 ± 0.14 kcal/mol, which is very low. Furthermore, a two-way ANOVA analysis revealed that there was no statistically significant interaction effect between osmolality and temperature on b ($p>0.05$). Original data for all temperatures and osmolalities were pooled and the overall mean value for the fitted osmotically-inactive fraction was 0.355 ± 0.005.

A Boyle van't Hoff plot of equilibrium cell volume as a function of inverse osmolality for the pooled temperature data is shown in Figure 3. The y-intercept of the linear regression estimates the osmotically-inactive fraction to be 0.368 ± 0.004 (calculated with the LINEST function in EXCEL). There was no statistical difference between the osmotically-inactive fractions calculated by these two approaches (Mann-Whitney U test; $p>0.05$). The osmotically-inactive fraction calculated from the Boyle van't Hoff plot was chosen for use in further analysis for several reasons, including: it is the standard in cryobiology; and it is easier to interpret as it only uses equilibrium data, as opposed to kinetic data; which is evident in the smaller error.

Table 2 shows the calculated hydraulic conductivity for TF-1 cells at different temperatures and extracellular osmolalities. Two-way ANOVA statistical tests indicated that L_p changed significantly with temperature ($p<0.001$), but did not change significantly with extracellular osmolality ($p>0.05$). This test also revealed statistically significant interaction between the effects of osmolality and temperature on L_p ($p<0.05$), indicating evidence that the

effect of temperature is dependent on concentration in predicting L_p. Due to this interaction effect, statistical significance should be interpreted with caution. Values at different osmolalities were pooled for each experimental temperature. In order to calculate the activation energy of L_p, Eq. 4 was linearized by taking the natural logarithm of each side of the equation and the data were plotted as $\ln L_p$ as a function of inverse absolute temperature. The pooled mean values in Table 2 were fitted to this equation using linear regression, giving an activation energy for L_p of 14.2 ± 1.1 kcal/mol. An Arrhenius plot of the experimental data and the fitted line is shown in Figure 4. The data for 3X PBS and pooled data for 2-5X PBS are plotted separately as experimental techniques were slightly different (i.e. n=9 and n=36, respectively).

Me_2SO permeability properties

Figure 5 shows the typical volume responses when TF-1 cells are exposed to a solution containing 1 M Me_2SO at 3 different temperatures. Using EXCEL Solver, P_s was calculated using two different approaches: 1) using values for L_p that were calculated from E_a^{Lp}, and fitting data to Eqs. 1, 2 and 3 to get values for P_s;, and 2) fitting data to Eqs. 1, 2 and 3 to get values for P_s and L_p. The values for P_s and L_p in Table 3 show that for pair-wise comparison of the two parameter estimation methods at each temperature give the values within experimental error ($p>0.05$), with the exception of L_p at 23.4 °C ($p<0.001$). A Kruskal-Wallis statistical test indicated that temperature is a significant predictor of both P_s and L_p, regardless of the calculation approach used to determine these parameters ($p<0.001$). The mean values in Table 3 were fitted to the linearized Arrhenius equation, Eq. 4, (using linear regression), giving an activation energy for P_s of 13.8 ± 0.1 kcal/mol and 16.3 ± 1.1 kcal/mol, respectively for the two different methods of calculation. For the second method, the activation energy for L_p was also calculated (17.5 ± 0.9 kcal/mol).

Two-step freezing experiments

Figure 6 shows experimental results for TF-1 cells, suspended in serum-free RPMI without cryoprotectant, cooled rapidly to various experimental temperatures and held for 3 min, prior to direct thaw or plunge into liquid nitrogen. Data were normalized with control TF-1 samples (i.e. TF-1 samples at room temperature; 94.4 ± 0.2 %). Samples cooled to hold temperatures of -20 °C and below, spontaneously nucleated prior to reaching the hold temperature. The direct thaw samples showed little loss of membrane integrity at hold temperatures down to -9 °C, but membrane integrity declined sharply between -9 and -20 °C, indicating that damage incurred during cooling to, or at the hold temperature. For samples plunged into liquid nitrogen, some protection against injury was conferred by holding for 3 min at -3 to -15 °C. Maximum membrane integrity after plunge and thaw was 62.2 ± 2.1 % and 61.8 ± 6.7 % at hold temperatures -12 °C and -15 °C, respectively. Membrane integrity for TF-1 cells that were plunged directly from room temperature was 6.7 ± 1.3 % and demonstrates that the absence of ice nucleation at high subzero temperatures and holding before plunging results in lower membrane integrity after thawing.

Effect of hold times in two-step freezing

Figure 7 shows the membrane integrity of TF-1 cells as a function of hold time at -5 or -25 °C. Data were normalized with control TF-1 samples (i.e. TF-1 samples at room temperature; 91.6 ± 2.7 % and 91.3 ± 2.2 %, respectively).For direct thaw samples at -5 °C, membrane integrity remained high for up to at least 10 min. Cells plunged into liquid nitrogen from -5 °C showed maximum membrane integrity with a 2 min hold time (65.4 ± 0.5 %). Comparable results for membrane integrity were obtained for hold temperatures between -7 °C and -9 °C (data not shown). Cell damage was evident during cooling to a hold temperature of

-25 °C, which limited the membrane integrity of cells plunged into liquid nitrogen. Samples cooled to -25 °C spontaneously nucleated prior to reaching the hold temperature. Figure 8 shows contour plots of membrane integrity for TF-1 cells subjected to two-step freezing over a range of hold temperatures and hold times. Figure 8a shows that cells directly thawed after being held for various times at the hold temperatures show progressive decrease in membrane integrity as the hold temperature was reduced below -9 °C and to a lesser extent, as hold time was increased. Figure 8b shows that cells held for 1-3 min at temperatures between -5 °C and -7 °C prior to plunging into liquid nitrogen, resulted in the highest percentage of membrane integrity (60 %). A hold time of greater than 5 min resulted in a marked decrease in cell survival. This data indicates that there is a zone of subzero hold temperatures (-5 °C to -12 °C) which, when held for 1-6 min, confers protection against cryoinjury associated with plunging to liquid nitrogen temperatures.

Graded freezing without cryoprotectant

Membrane integrity as a function of experimental temperature for TF-1 cells cooled at 0.9 °C/min, prior to direct thaw or plunge into liquid nitrogen is shown in Figure 9. Data were normalized with control TF-1 samples (i.e. TF-1 samples at 0 °C; membrane integrity 92.5 ± 2.9 %). Samples cooled to hold temperatures of -20 °C and below, spontaneously nucleated prior to reaching the hold temperature. Similar results were found for cooling rates of 0.2 °C/min and 0.5 °C/min (data not shown). Data from all 3 cooling rates demonstrated a progressive decline in membrane integrity for cells thawed directly from the experimental temperature, with a 50% loss at -12 °C, indicating injury related to exposure to the concentrated extracellular solution. Cells plunged into liquid nitrogen from the experimental temperature incurred additional injury. The zone of subzero experimental temperatures between -3 °C and

-9 °C confers some protection against injury during plunge into liquid nitrogen (membrane integrity 30.1 ± 0.7 % at -9 °C). Membrane integrity for TF-1 cells that were plunged directly from 0 °C was 11.8 ± 2.7 %.

Graded freezing with Me_2SO

Figure 10 shows the recovery of TF-1 cells cooled at 0.9 °C/min in 10 % Me_2SO to various subzero temperatures ranging from -3 to -40 °C, and either thawed directly or plunged into liquid nitrogen. Data were normalized with control TF-1 samples (i.e. TF-1 samples at 0 °C; membrane integrity 91.4 ± 3.2 %). Maximum membrane integrity after plunge and thaw was 71.5 ± 4.1 % at a plunge temperature of -15 °C. Note, for comparison, that the membrane integrity of TF-1 cells following a conventional HSC cryopreservation protocol (cooling 0.9 °C/min to -40 °C and then plunging into liquid nitrogen) was 63.0 ± 6.4 %. Membrane integrity for TF-1 cells in 10 % Me_2SO that were plunged directly from 0 °C was 30.9 ± 3.1 %. It also demonstrates that when comparing the results to having no Me_2SO present, Me_2SO confers protection even without ice nucleation at high subzero temperatures.

Discussion

Cell recovery following cooling to low temperatures depends in part on cellular osmotic responses which are described by a set of osmotic parameters. The average size of TF-1 cells (916 ± 15 μm^3) was higher than that previously reported for both human bone marrow $CD34^+$ cells of 345 μm^3 [16] and umbilical cord blood $CD34^+$ cells of 274 ± 13 μm^3 [22].

In the range of osmolalities studied, TF-1 cells follow the Boyle van't Hoff relationship yielding an osmotically-inactive fraction of 0.37. A previously reported value for human bone

marrow hematopoietic $CD34^+$ cells was 0.205 [16], and for human umbilical cord blood $CD34^+$ cells was 0.32 [60] and 0.27 ± 0.01 [22]. The value of osmotically-inactive fraction reported here is also within the range for a variety of mammalian cell types (0.2-0.41) [9,16,17,21,31,46,52].

Permeability parameters were calculated from cell volume measurements between 4 and 37 °C, showing that osmotically-inactive fraction was largely independent of temperature but that hydraulic conductivity was strongly temperature-dependent. The value for L_p was 0.339 μm/min/atm at 20 °C. The value reported here for L_p is higher than the L_p previously reported for human cord blood $CD34^+$ cells of 0.168 ± 0.03 μm/atm/min at 20 °C [22,60], indicating that the rate of water movement in TF-1 cells is larger than that in other HSC. The L_p value is also within the range reported for many nucleated mammalian cells, such as rat megakaryocytoporietic cells, Chinese hamster lung fibroblast cells, bovine immature oocytes, chondrocytes, corneal endothelial, epithelial and stromal cells [52].

The Arrhenius activation energy of L_p of 14.2 kcal/mol reported here, is within the range of some other mammalian cells (9-16 kcal/mol) [9,21,31,51]. Based on L_p values reported by Hunt et al. at 2 temperatures [22], we calculated the E_a^{Lp} in cord blood $CD34^+$ cells to be 18.8 kcal/mol. It has been reported that an E_a^{Lp} of <6 kcal/mol may be indicative of channel-mediated water transport [13], and an E_a^{Lp} >10 kcal/mol may indicate transport water across the plasma membrane primarily by solubility-diffusion [13].

The conventional method for cryopreserving HSC, utilizes Me_2SO as a cryoprotectant [65]. The calculated permeability of Me_2SO in TF-1 cells, 14.3 μm/min at 22 °C, is higher than reported for human cord blood $CD34^+$ cells (9.4 μm/min (22 °C) [60]; 4.4 μm/min (20 °C) [22]), but within the range of reported values found for a variety of mammalian cell types [51],

including human corneal endothelial, keratocyte and epithelial cells [10], hamster islet cells [3], human granulocytes [58], canine red blood cells [32], and neonatal porcine islet cells [15].

In the absence of cryoprotectants, this study showed that using two-step freezing (interrupted rapid cooling with hold time), TF-1 cells sustained less injury at lower subzero hold temperatures than using graded freezing (interrupted slow cooling without hold time). However, using two-step freezing, there was also damage associated with cooling down to and exposure at the hold temperatures. Cells thawed directly from the hold temperatures showed a decline in membrane integrity with decreasing temperature, reaching 50% at -17 °C, indicating that a major portion of cells were damaged prior to being plunged into liquid nitrogen. This damage is associated with cooling down to the hold temperature and exposure at the hold temperature, with intracellular ice formation as a likely cause. These results also demonstrate that for hold temperatures below -15 °C, no additional damage is incurred by cooling to liquid nitrogen temperatures, implying that the cells remaining intact at the end of the hold step have lost a sufficient amount of intracellular water and that there is no additional damage due to intracellular ice formation during the plunge. The highest recovery (membrane integrity) for samples thawed from liquid nitrogen were 62.2 ± 2.1 % at a hold temperature of -12°C and 61.8 ± 6.7 % at a hold temperature of -15 °C, both with a hold time of 3 min. Avoidance of intracellular ice formation during the plunge step requires osmotic dehydration during the hold step. The hydraulic conductivity and its activation energy are different for different cell types, so the optimal hold temperature and time will be cell-type dependent.

Using the graded freezing procedure, this study demonstrated the progressive damage incurred by slow cooling TF-1 cells to subzero experimental temperatures, indicating the deleterious effect of exposure to concentrated extracellular solutions. There was a marked

difference between the membrane integrity for cells directly thawed and those plunged into liquid nitrogen, which has also been shown for other cell types [49,50,63,65,66]. The maximum recovery for plunge samples using graded freezing without Me_2SO was 27.8 ± 0.8 %, while results from the literature for other HSCs were 32 % for nucleated white cells and 17 % for $CD34^+$ cells [65].

Table 4 compares maximum recovery of TF-1 cells cooled in liquid nitrogen (plunge thaw) using different freezing protocols. The maximum two-step freezing recovery (membrane integrity 62.2 ± 2.1 %) was higher than recovery using graded freezing profiles without Me_2SO (membrane integrity 30.1 ± 0.7 %). Furthermore, the two-step freezing membrane integrity (without cryoprotectant) were comparable with membrane integrity of TF-1 cells cooled using a conventional cryopreservation protocol (cooling at 0.9 °C/min in 10 % Me_2SO to -40 °C and plunging into liquid nitrogen) of 63.0 ± 6.4 %. Although there is no true standard cryopreservation method for HSCs, there is an overall conventional approach of slow cooling in 5-10 % Me_2SO solution. Based on the HSC cryopreservation literature, this approach leads to recoveries of 79 ± 5 % (membrane integrity; 1 °C/min with plunge at -50 °C using 10 % Me_2SO) [23], 67.4 ± 2.0 % (1 °C/min with plunge at -60 °C using 10 % Me_2SO) [62], and 85.8 ± 6.8 % (4 °C/min with plunge at -44 °C using 5 % Me_2SO) [65]. While the exact cooling protocols and Me_2SO solutions may differ to varying degrees from the present study, the influence of Me_2SO on increasing cell survival at lower temperatures during slow cooling is a commonality. The trends using graded freezing with Me_2SO, are comparable to those seen using graded freezing with nucleated white blood cells, [65], CD34+ UCB stem cells [65], and chinese hamster fibroblasts [42]. The maximum recovery obtained using graded freezing with Me_2SO was 71.5 ± 4.1 %; however, this was using a relative high plunge temperature of -15 °C (Table

4).

The data in this study are also comparable to previously published results by Karlsson et al. [27] using slow cooling and Me_2SO with mouse oocytes. They showed low cell recovery at high plunge temperatures, which increased at lower plunge temperatures. They also suggested that the observed cell damage at high subzero plunge temperatures was the result of intracellular ice formation, based on predictions of a mechanistic model [27], which would also explain the plunge results in this study.

The use of Me_2SO as a cryoprotectant has been identified as a problem for HSC transplant patients due to its clinical toxicity [7,11,56,68]. The experimental results reported here for TF-1 cells without cryoprotectants indicate that higher cell recovery is possible by manipulating the cooling profile. This data indicates that there is a zone of subzero hold temperatures (-5 to -12 °C) and hold times (1-6 min), that confers cryoprotection comparable to the conventional 10 % Me_2SO solution with slow cooling down to-40 °C in TF-1 cells. The pattern of recovery as a function of hold time and temperature was similar to that previously shown for fibroblasts [45].

By comparing the post-thaw recovery of TF-1 cells, following slow cooling, with water permeability results, it is evident that cells with a relatively low hydraulic conductivity are damaged during slow cooling without a cryoprotectant (membrane integrity 27.8 ± 0.8 %). However, human red blood cells (RBC), which have a much higher L_p (12 μm/min/atm at 22-24 °C) [51], sustain even greater injury during slow cooling (hemolysis >95 %) [55].

Practical cryopreservation procedures have largely been limited to the use of constant cooling rates. Interrupted freezing procedures, such as two-step and graded freezing, provide useful insight into the mechanisms of damage occurring at various temperatures throughout the

cooling profile [44]. These procedures allow manipulation of different variables of the cryopreservation protocol, including cooling profiles, plunge temperatures, cryoprotectants, and storage temperatures. In this study, both procedures allowed additional characterization of the cryobiology of cells, and the ability to cryopreserve TF-1 cells (a model for HSC) without the need for Me_2SO and to improve recoveries when using Me_2SO. Ultimately, this approach will enable to systematic exploration of alternatives to constant cooling rates.

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Figure 1. Schematics of (a) two-step freezing, including initial rapid non-linear cooling to hold temperature, hold time, and either directly thawing or plunging and then thawing following storage time, and of (b) graded freezing, including initial slow linear cooling to experimental temperatures and either direct thawing or plunging and then thawing.

Figure 2. Representative cell volume kinetics of TF-1 cells exposed to 3X PBS at 4 different temperatures. The symbols show the experimental volume measurements averaged over 300 msec time intervals and the solid lines represent Eqs. 1 and 2 fitted to the data using the least squares method, yielding a value of L_p and b at each temperature.

Figure 3. The Boyle van't Hoff plot for TF-1 cells. The symbols represent the experimental relative equilibrium cell volumes in hypertonic solutions and the solid line represents the linear regression fit of Eq. 1 to the data. The y-intercept gives the osmotically-inactive fraction, b.

Figure 4. Arrhenius plot of the natural logarithm for L_p (μm/min/atm) of TF-1 cells (± sem) as a function of inverse temperature (K^{-1}) (± sem). The open diamond symbols represent all experiments done in 2-5X PBS, with different concentration data pooled (n = 36). The closed symbols represent all experiments done in 3X PBS (n = 9). The solid line represents the linear regression of the data for calculation of the activation energy (E_a) from the slope (R^2=0.93).

Figure 5. Representative plots of cell volume kinetics of TF-1 cells exposed to 1M DMSO at (a) 4°C, (b) 10°C, and (c) 22°C. The symbols show the experimental volume measurements averaged over 300 msec time intervals and the solid lines represent Eqs. 1, 2, and 3 fitted to the data using the least squares method, yielding a value of P_s at each temperature.

Figure 6. Two-step freezing. Membrane integrity for TF-1 cells (± sem; normalized) in serum-free RPMI media cooled to various subzero plunge temperatures from room temperature, held 3 minutes, and then either thawed directly (dashed) or plunged into liquid nitrogen (solid) before

1 being thawed.

2 Figure 7. The membrane integrity of TF-1 cells (± sem; normalized) in serum-free RPMI media
3 as a function of hold time for cells cooled rapidly to -5 °C (black) or -25 °C (grey), held for a
4 period of time before either thawed directly (dashed) or plunged into liquid nitrogen (solid)
5 before being thawed.

6 Figure 8. Contours of membrane integrity of TF-1 cells (normalized) in serum-free RPMI media
7 after being cooled rapidly to various subzero hold temperatures and held for a duration ranging
8 from 0.5 to 10 minutes before being either (a) thawed directly or (b) plunged into liquid nitrogen
9 prior to thawing.

10 Figure 9. Graded freezing without cryoprotectant. Membrane integrity of TF-1 cells (± sem;
11 normalized) in serum-free RPMI media after being cooled at 0.9 °C/min to various subzero
12 experimental temperatures and either thawed directly (dashed) or plunged into liquid nitrogen
13 (solid) prior to thawing.

14 Figure 10. Graded freezing with Me_2SO. Membrane integrity of TF-1 cells (± sem; normalized)
15 in 10 % Me_2SO in RPMI media after being cooled at 0.9 °C/min to various subzero experimental
16 temperatures and either thawed directly (dashed) or plunged into liquid nitrogen (solid) prior to
17 thawing.

18

TABLE 1. Osmotically-inactive volume, *b*, values for TF-1 cells (mean ± sem; n = 9)

Osmolality (osm/kg)	**Temperature**			
	4.8 ± 0.1 °C	12.9 ± 0.3 °C	23.3 ± 0.2 °C	37.4 ± 0.1 °C
583 ± 7	0.399 ± 0.006	0.336 ± 0.021	0.334 ± 0.008	0.294 ± 0.022
861 ± 6	0.347 ± 0.024	0.374 ± 0.014	0.338 ± 0.006	0.339 ± 0.016
1150 ± 4	0.387 ± 0.027	0.385 ± 0.019	0.355 ± 0.007	0.344 ± 0.019
1434 ± 5	0.368 ± 0.009	0.400 ± 0.012	0.371 ± 0.008	0.312 ± 0.012
pooled mean	0.375 ± 0.010[a]	0.374 ± 0.010[a]	0.349 ± 0.004[a,b]	0.325 ± 0.009[b]

Different superscripts indicate statistical difference ($p<0.05$)

TABLE 2. Hydraulic conductivity, L_p, values for TF-1 cells (mean ± sem; n = 9)

Osmolality (osm/kg)	Temperature			
	4.8 ± 0.1 °C	12.9 ± 0.3 °C	23.3 ± 0.2 °C	37.4 ± 0.1 °C
583 ± 7	0.075 ± 0.003	0.119 ± 0.005	0.355 ± 0.012	1.348 ± 0.093
861 ± 6	0.070 ± 0.003	0.123 ± 0.005	0.395 ± 0.013	1.413 ± 0.074
1150 ± 4	0.075 ± 0.004	0.119 ± 0.004	0.407 ± 0.010	1.447 ± 0.106
1434 ± 5	0.074 ± 0.004	0.112 ± 0.002	0.386 ± 0.013	1.512 ± 0.061
pooled mean	0.074 ± 0.002^{a}	0.118 ± 0.002^{a}	0.386 ± 0.006^{b}	1.425 ± 0.044^{c}

Different superscripts indicate statistical difference ($p<0.05$)

TABLE 3. Me_2SO Permeability properties for TF-1 cells (mean ± sem; n = 9)

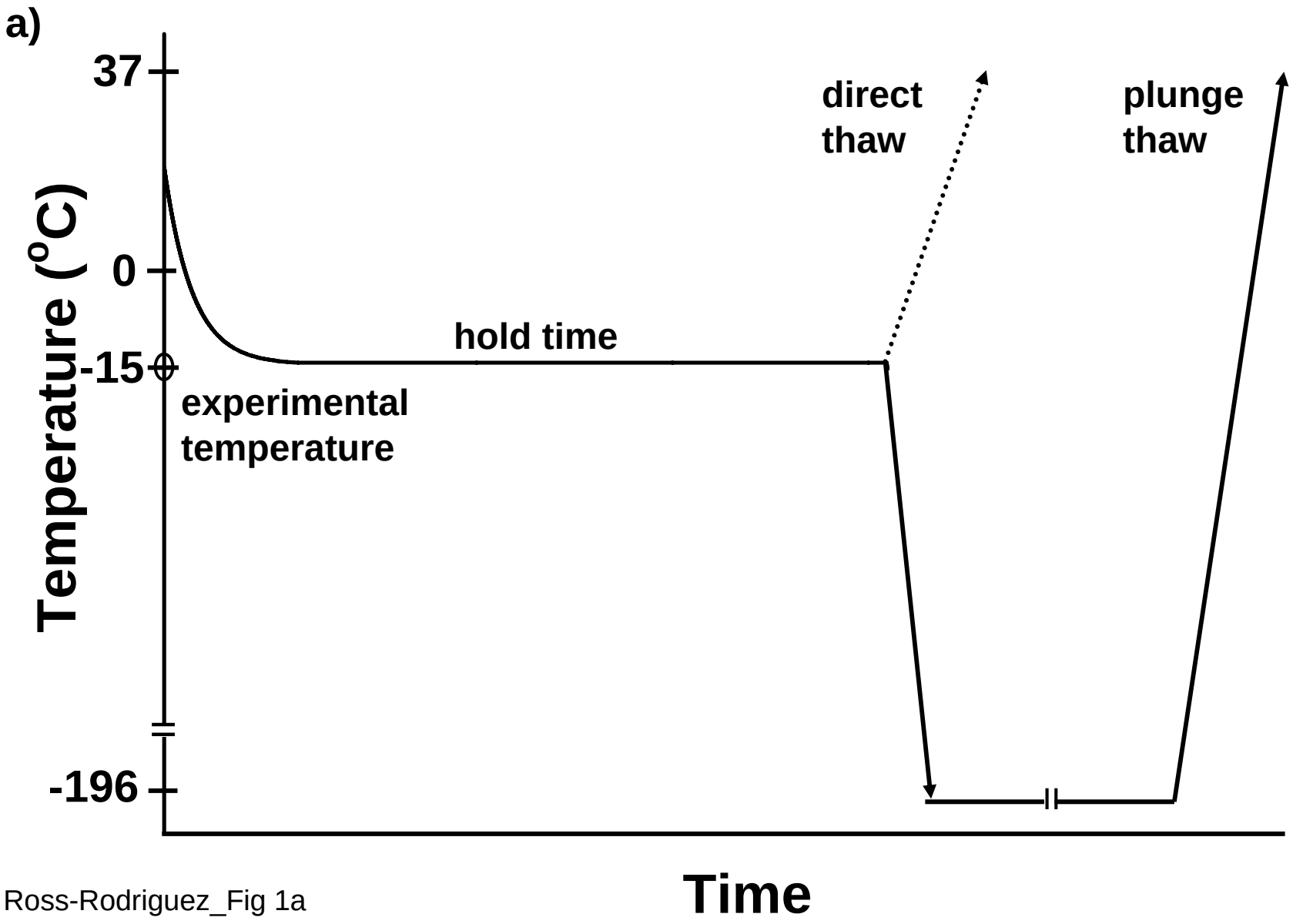
1M Me_2SO	**Using calculated L_p from E_a^{Lp}**		**Solving for L_p and P_s simultaneously**	
Temperature (°C)	**L_p (µm/min/atm)**	**P_s (µm/min)**	**L_p (µm/min/atm)**	**P_s (µm/min)**
4.9 ± 0.3	0.09	3.36 ± 0.40	0.08 ± 0.00	2.92 ± 0.22
10.7 ± 0.4	0.15	5.67 ± 0.51	0.17 ± 0.01	6.00 ± 0.76
23.4 ± 0.5	0.45	15.95 ± 1.08	0.61 ± 0.02*	18.8 ± 1.66
E_a (kcal/mol)	14.2	13.8 ± 0.1	17.5 ± 0.9	16.3 ± 1.1

* Signifies statistically significant difference from the P_s value estimated using the method of calculating L_p from E_a^{Lp} ($p<0.001$)

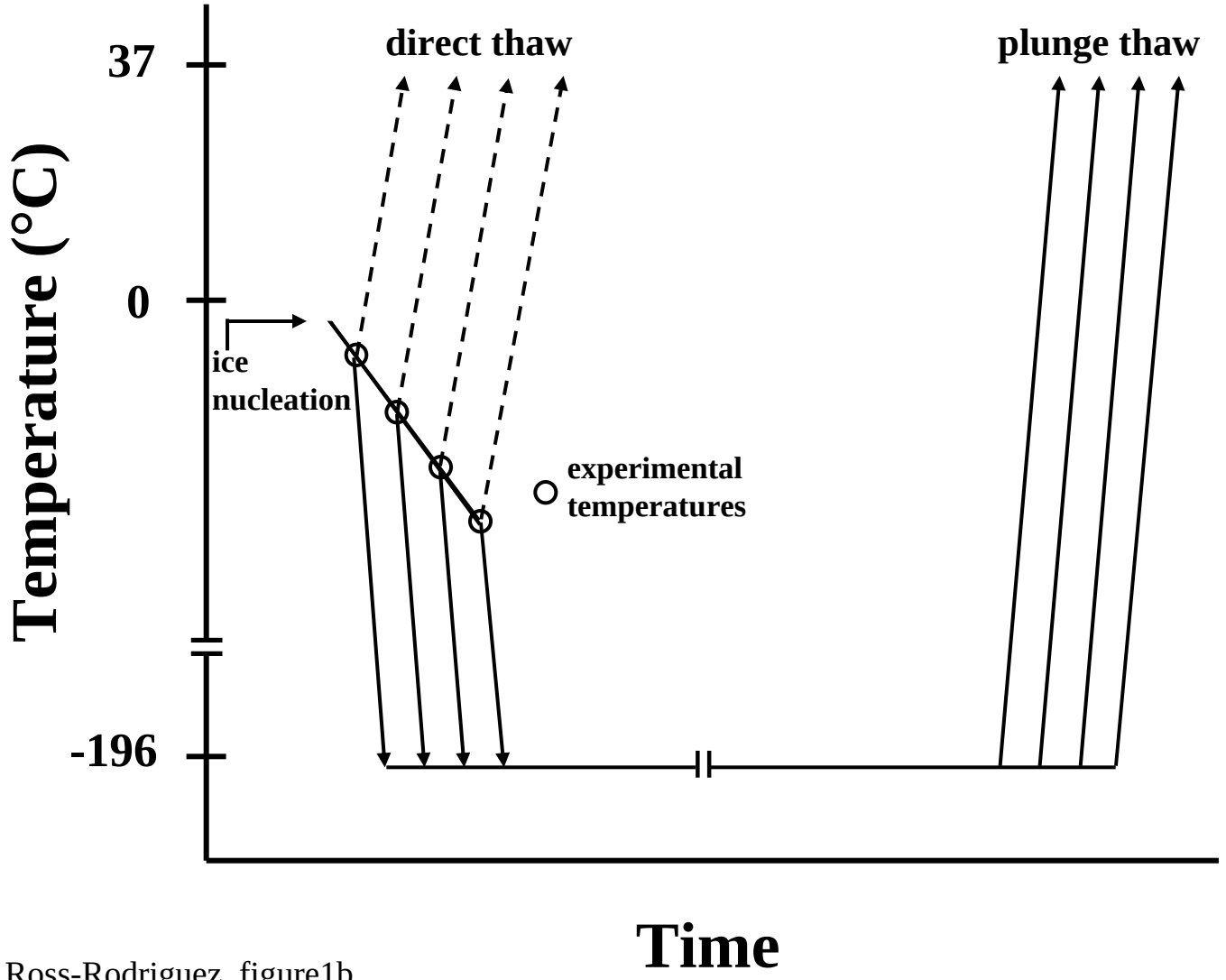
TABLE 4. Maximum recovery of TF-1 cells (plunge thaw), as assessed by membrane integrity (mean ± sem; normalized).

Protocol	**Maximum recovery after storing in liquid nitrogen**
Conventional Freezing protocol[1]	63 ± 6.4 %
Graded Freezing (without 10% Me_2SO)[2]	30.1 ± 0.7 %
Two-step Freezing (without Me_2SO)[3]	62.2 ± 2.1 %
Graded Freezing (with 10% Me_2SO)[4]	71.5 ± 4.1 %

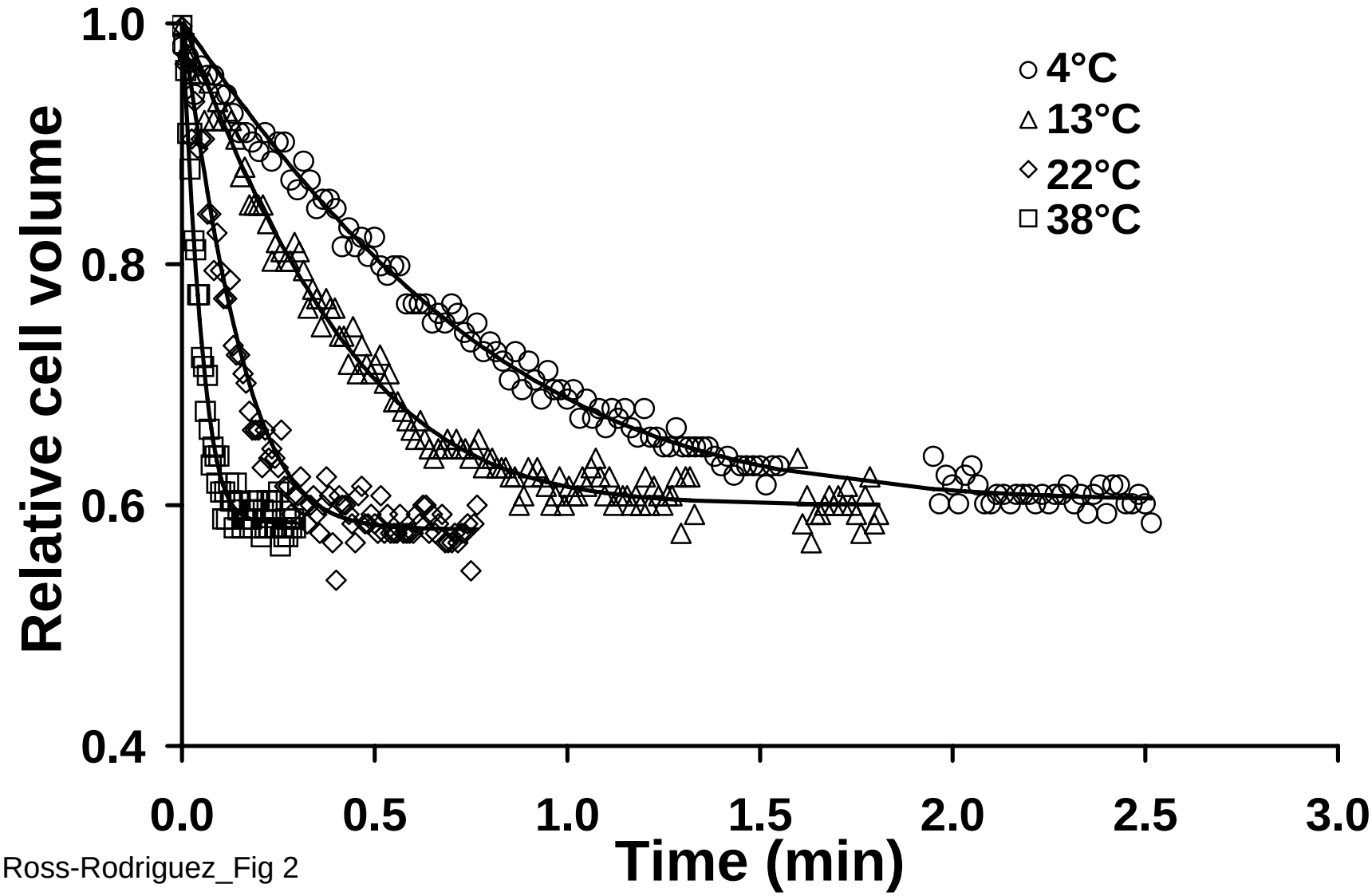
[1]0.9 °C/min to -40 °C and plunge
[2]0.9 °C/min to -9 °C and plunge
[3]non-linear cooling with 3 minute hold at -12 °C followed by plunge
[4]0.9 °C/min to -15 °C and plunge



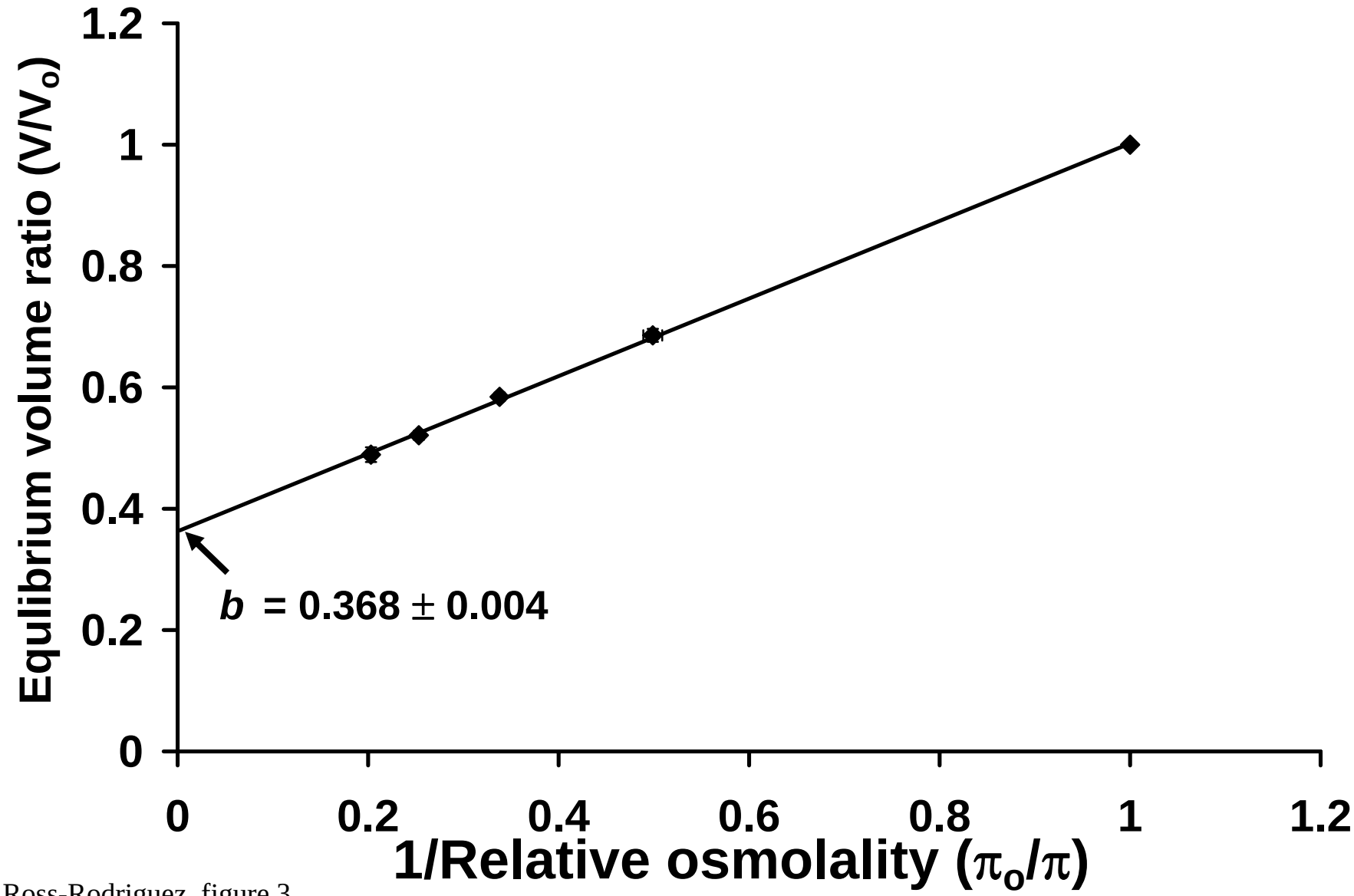
Ross-Rodriguez_Fig 1a



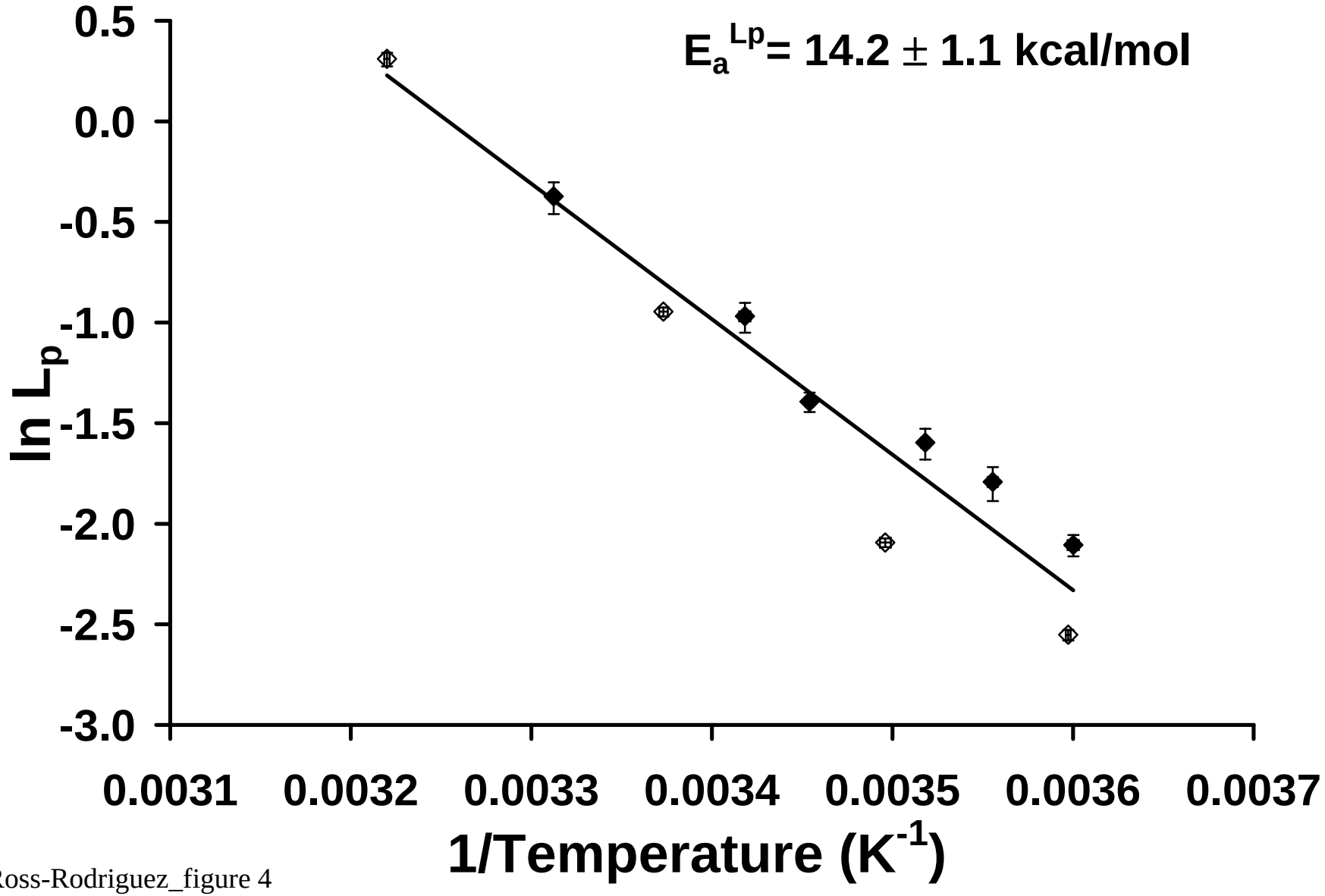
Ross-Rodriguez_figure1b



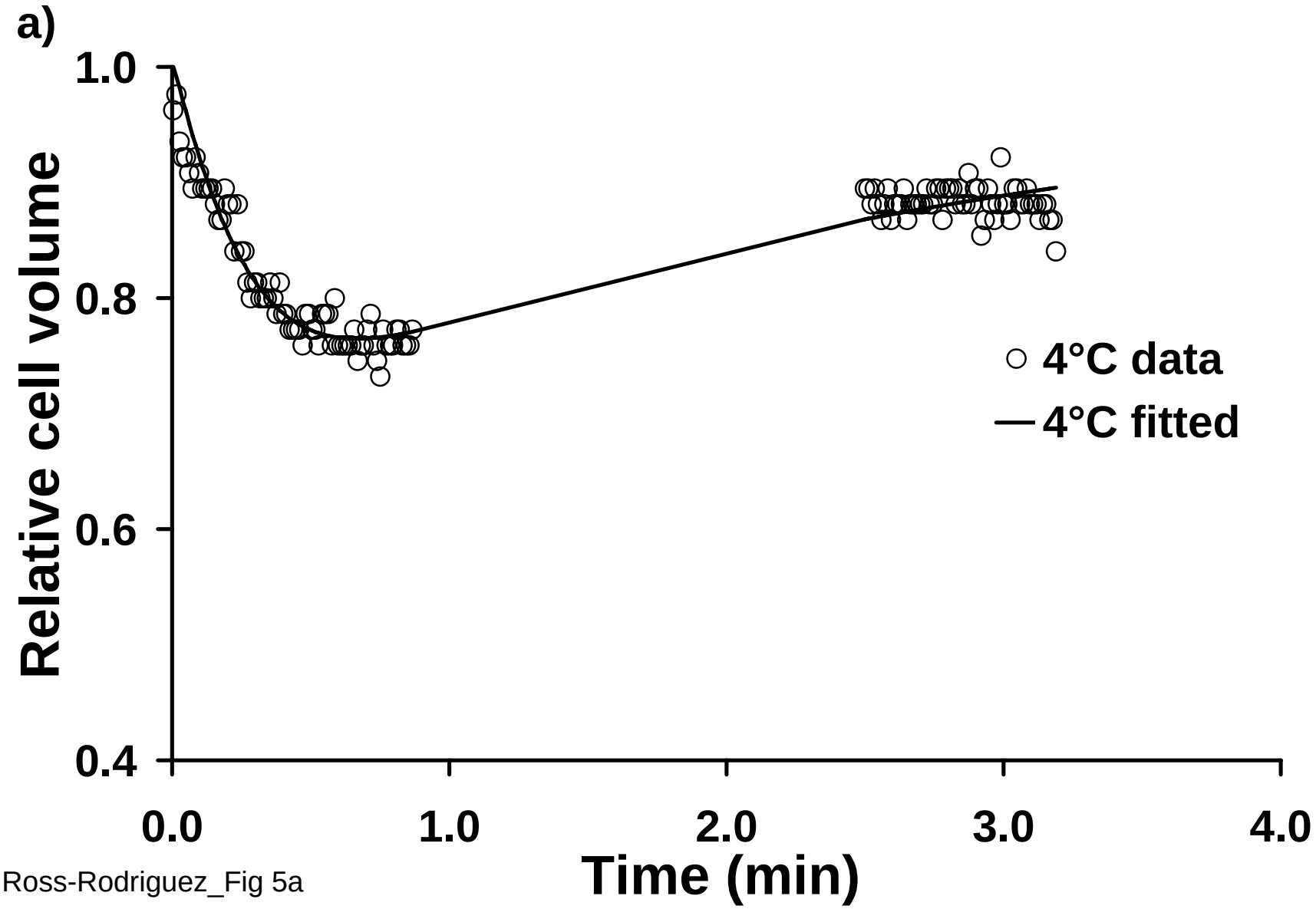
Ross-Rodriguez_Fig 2



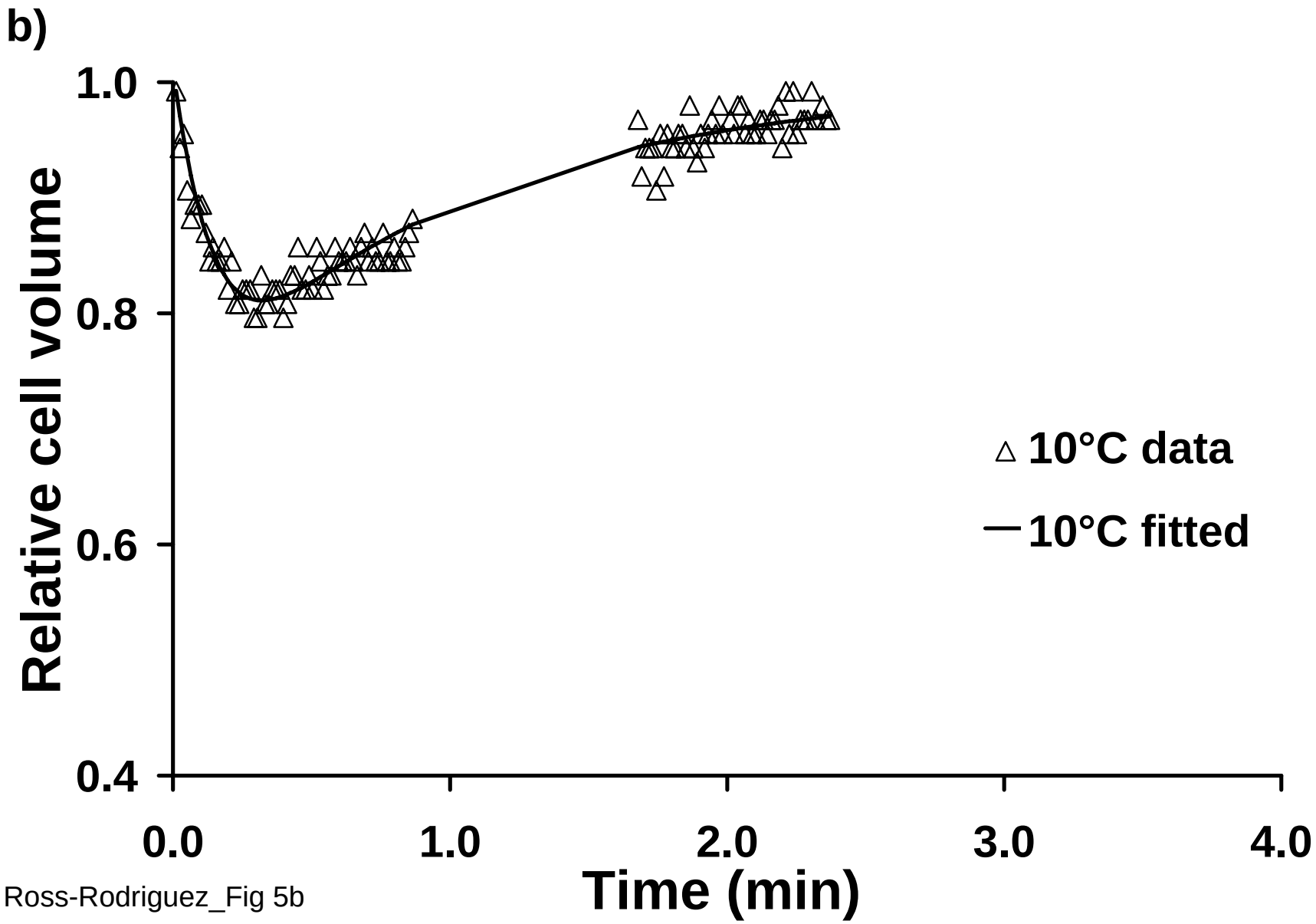
Ross-Rodriguez_figure 3



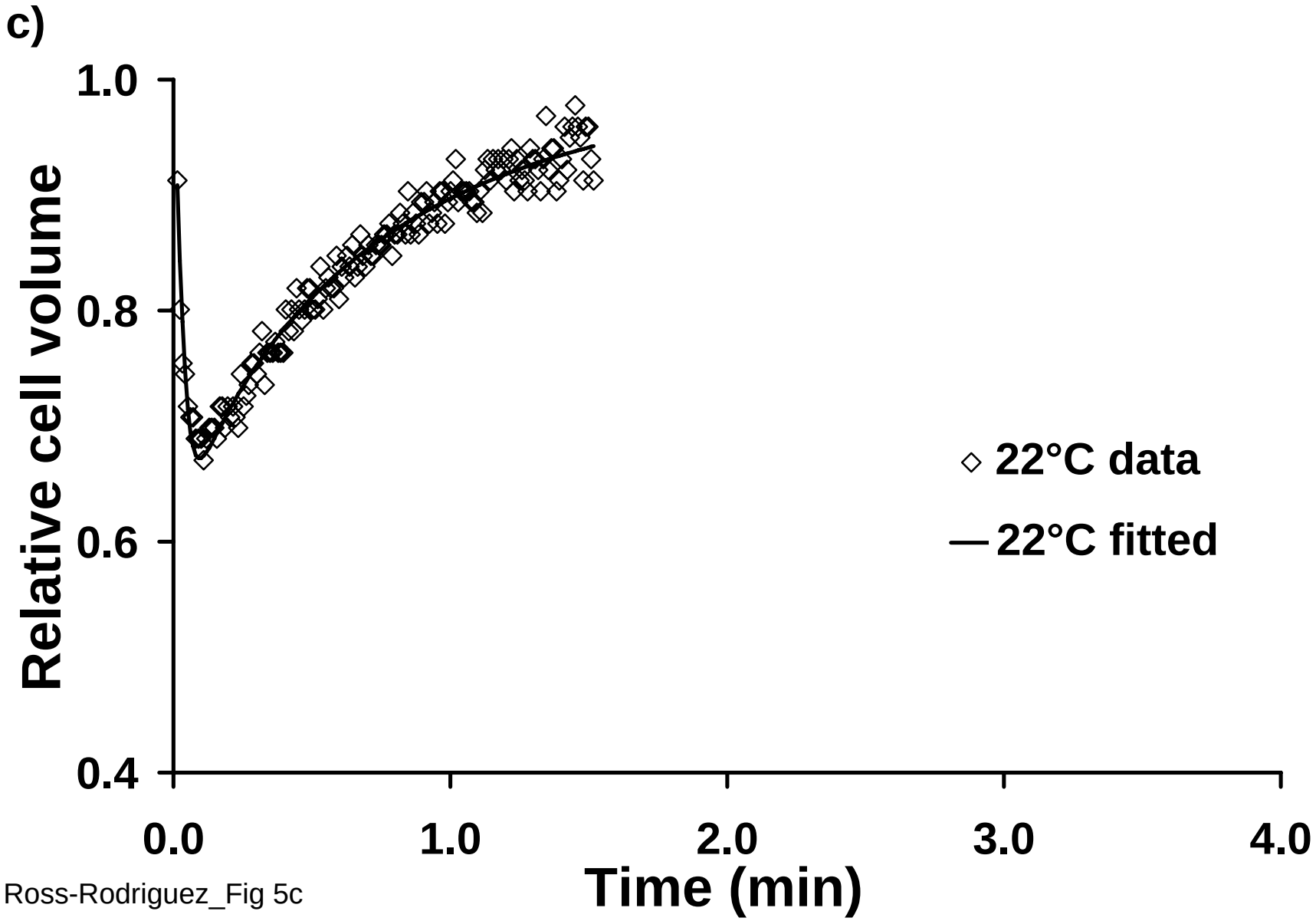
Ross-Rodriguez_figure 4



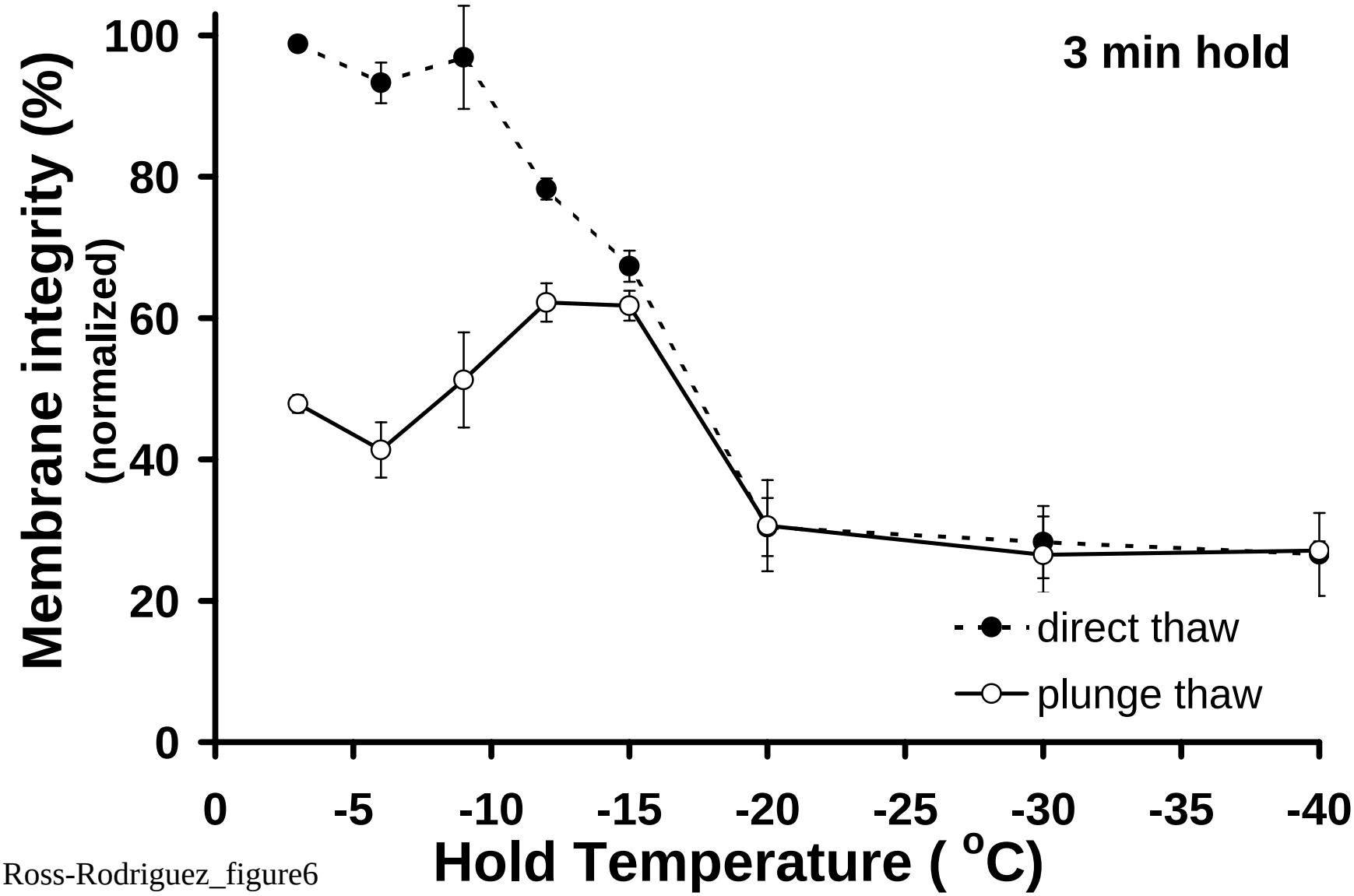
Ross-Rodriguez_Fig 5a



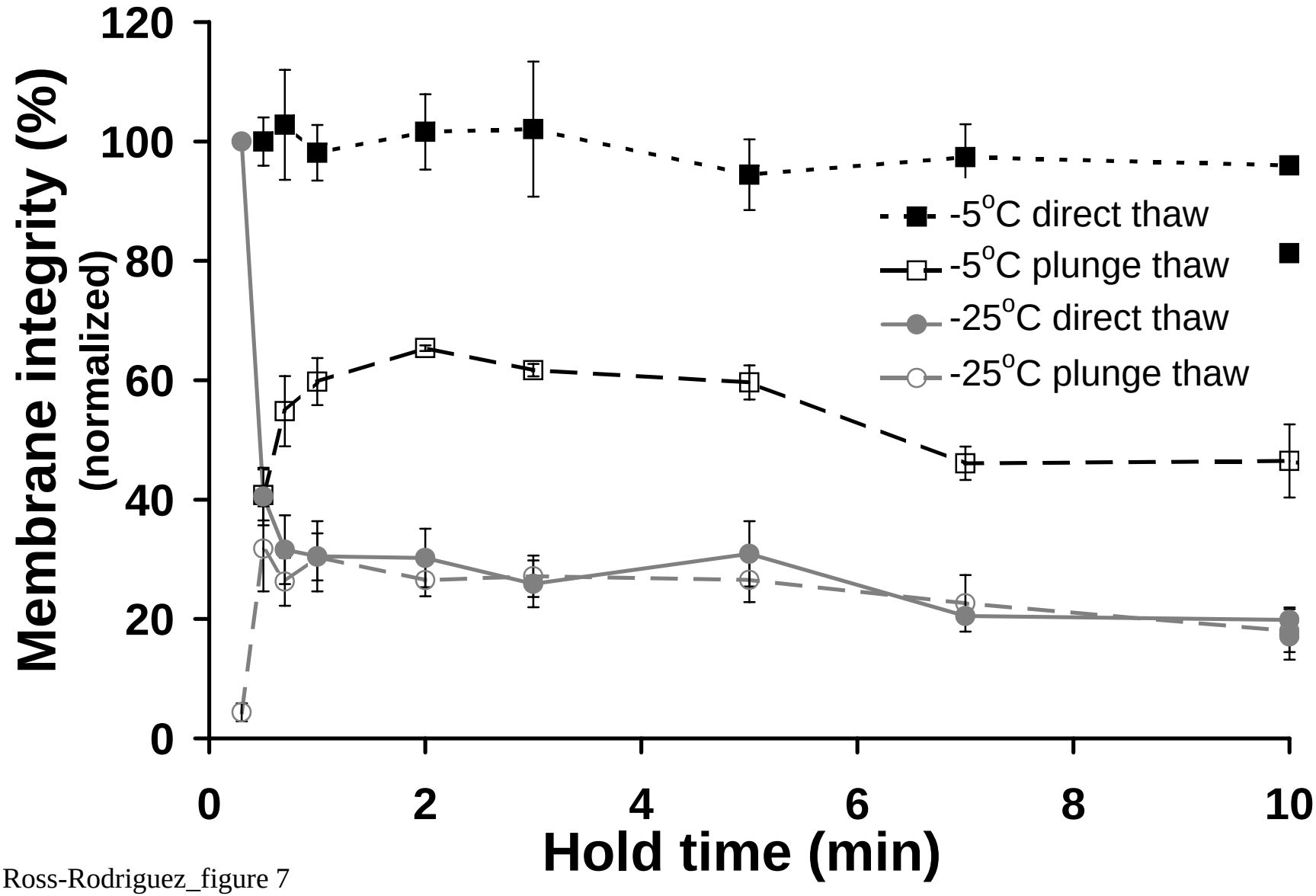
Ross-Rodriguez_Fig 5b



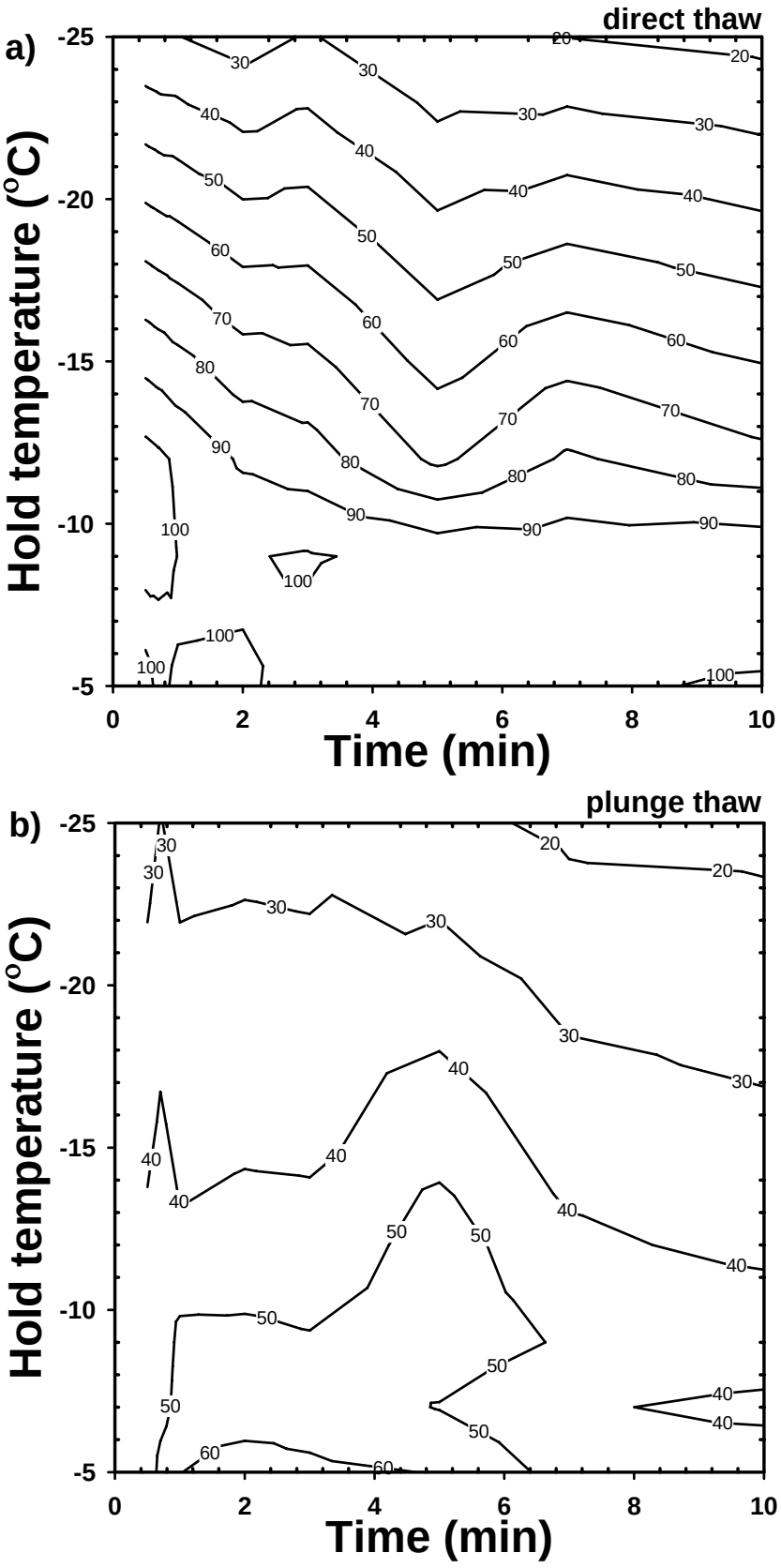
Ross-Rodriguez_Fig 5c



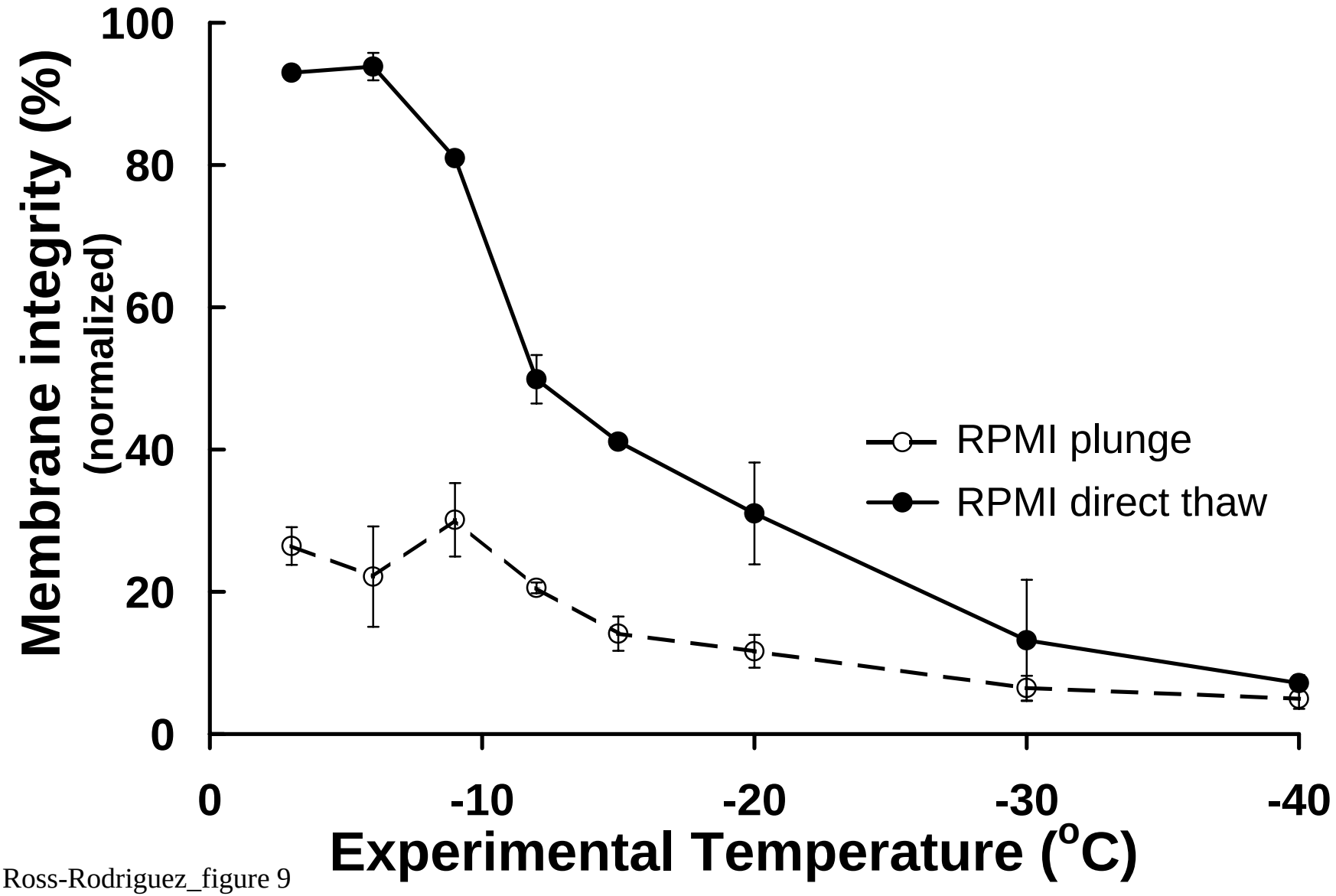
Ross-Rodriguez_figure6



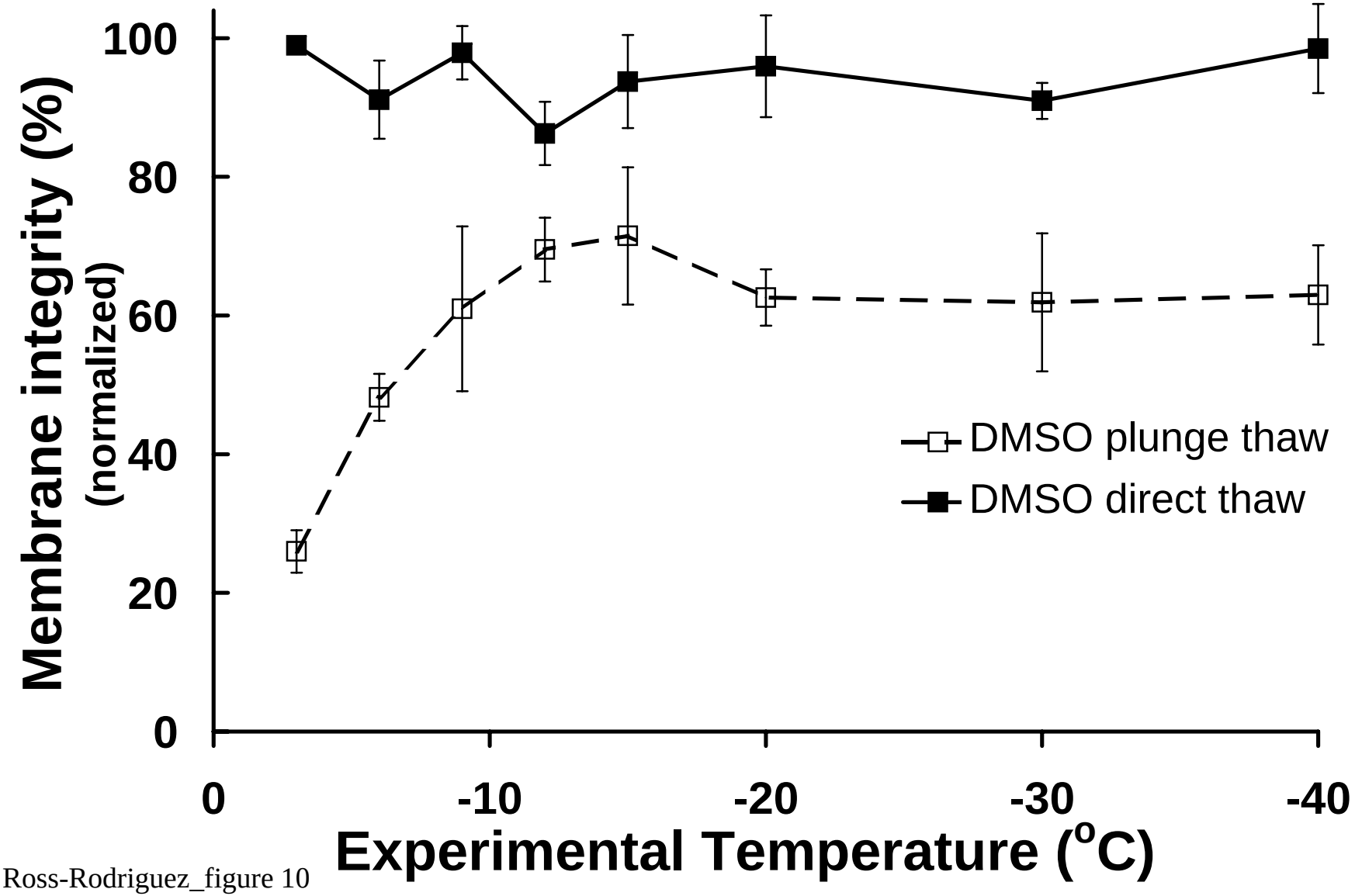
Ross-Rodriguez_figure 7



Ross-Rodriguez_figure 8



Ross-Rodriguez_figure 9



Ross-Rodriguez_figure 10