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Trapped water of human erythrocytes and its application in cryopreservation

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Abstract

The novel differential scanning calorimetry method as a technique for determining human red cell volume during freezing process has been reexamined and has been shown to provide a final erythrocyte volume to be 53% of its isotonic value after freezing from 0 to −40 °C. A new type of electronic particle counter (Multisizer™ 3, Beckman Coulter Inc., USA) was used to measure cell volume changes in response to equilibration in anisotonic media, and which gave out an equilibrated volume to be 57% of cell isotonic value in solution of 3186 mOsm. Both of these results indicate that 34–40% of intracellular water is trapped and is unavailable for participation in osmotic shifts. These findings are consistent with the published data that at least 20–32% (v/v) of the isotonic cell water is retained within RBCs. Then the application of trapped water in both simulation of freezing models and freezing-drying control was pointed out.

Keywords: Differential scanning calorimetry/DSC; Electronic particle counter/EPC; Cell volumetric change; Red blood cell/RBC/erythrocyte

1. Introduction

Blood storage strategy plays a central role in blood banks. Currently, most of the blood is preserved in a freezer at 4 or −80 °C, and a few stored in a costly LN container at −196 °C. Capacity of this strategy depends on the amount of equipment, and a blood center is designed just for conventional clinical use. Although the blood bank can fulfill the ordinary need of blood product, it has been challenged recently by the emergency requirements for a vast amount of blood due to natural or man-made disasters and a worldwide research has been raised on a more efficient technology for long-term storage of erythrocytes. As a valuable alternative, freeze-drying technology has been studied extensively for its stability and large capacity in shelf life at room temperature and the convenience for clinical use [1–3]. During

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Table 1
Measured DSC heat releases (mean ± S.D., $n=3$)

DSC experiments	Cytocrit (α)	$\overline{q_f}$
1	0	285.377 ± 2.477
2	0.35	238.778 ± 4.879
3	0.45	229.416 ± 3.004
4	0.55	214.635 ± 3.534
5	0.62	205.996 ± 3.637

the freeze-drying process, some parameters are crucial for the quality of product, and one of them is the water content in an erythrocyte just prior to its following drying process, in other words, the water trapped in the cell after the freezing process (an unexacting call—unfreezable water). The solid water in the sample is sublimated (primary drying) firstly during the drying process, followed by a secondary drying process at a higher working temperature for the residual water (below its collapse temperature). To keep the viability of shelf cells, approximately 5–10% cell water must be withheld. It is very important to know the amount of trapped water prior to the secondary drying process in order to stop the costly freeze-drying process in good time. Although the trapped water could be observed by nuclear magnetic resonance (NMR) [4], it is more convenient to observe the water content in cells by differential scanning calorimeter (DSC), which has been applied widely in cryobiology and foods industry [5,6]. Recently, Devireddy and Bishof [6] have presented a novel DSC method for measurement of cell volumetric change during freezing process, and a theoretical conjecture was provided to explain this subzero phenomenon successfully. Following this, we developed a simplified analytical and experimental DSC method to determine the trapped water in the cell in the case of slow cooling [7]. This is the starting point to the hope that one would estimate the optimal cooling protocol in the freeze-drying process using DSC, and the trapped water in the sample could be measured consequently. Alternatively, a new type of electronic particle counter (Multisizer™ 3, Beckman Coulter Inc., USA) was used to determine the volumes of human RBCs in NaCl solutions of different grade osmolalities, in order to simulate cell volumetric change during the equilibrium freezing process. The EPC method was also used to determine the amount of trapped water in RBCs. These two methods agree well with each other. Both of the results may be of advantage to the design of the freeze-drying process.

2. Theoretical background

2.1. Theory of DSC method

It was assumed that: (a) intracellular ice formation does not occur at slow cooling rates; (b) intracellular water diffusing out of cells will freeze extracellularly as soon as it mixes into extracellular media during the freezing process; and (c) all components in the cell suspension (including solution and cells) have the same density. Under these assumptions, total latent release from the start of the freezing to any final temperature during the freezing process can be described as follows [6,7]:

$$q_f = K_f \cdot \alpha + B_f \tag{1}$$

$$K_f = \left[\frac{V_0 - V_f}{V_0}\right] \cdot \Delta H_f - q_{ec,f} \tag{2}$$

$$B_f = q_{ec,f} \tag{3}$$

From Eqs. (1)–(3), one can obtain Eq. (4) to calculate cell volume at any ending temperature:

$$V_f = V_0\left(1 - \frac{K_f + B_f}{\Delta H_f}\right) \tag{4}$$

where q_f is the total latent heat release per unit mass of the cell suspension at any ending temperature during the freezing process, caused by the freezing of extracellular water and the water diffused out of cells; $q_{ec,f}$ is the total heat release resulting from the freezing of water which is originally in extracellular media, not including water diffused out of cells during the freezing process; α is the cytocrit of cell suspension; V_f is the final cell volume; V_0 is the originally isotonic cell volume; ΔH_f is the fusion heat of unit mass of pure ice.

From Eq. (1), we can see that q_f is a linear function of α. If q_f of cell suspensions with different α was experimentally measured, the slope K_f and intercept B_f in Eq. (1) can be determined by linearly fitting Eq. (1) with experimental data. Then substitution of the two values into Eq. (4); the ending volume V_f can be determined.

2.2. Principle of EPC method

A new type of EPC (Electronic Particle Counter, Multisizer™ 3, Beckman Coulter Inc., USA) was used to measure the mean volumes of human RBCs in NaCl solutions of different osmolalities. The EPC principle is an electrical sensing zone technique, which is based on measuring changes in electrical resistant between two electrodes that are produced when particles pass through the sensing zone. The particles pass through the zone by being drawn through an aperture under vacuum. Each particle displaces its own volume of electrolyte. The volume displaced is measured as a voltage pulse. The height of each pulse is directly proportional to the volume of the particle, while it has nothing to do with the shape of the particle. The software (Multisizer 3 V3.51) together with EPC can give the percentage distribution of particle amount, volume and surface area (Multisizer 3 Operator's Manual, PN 8321681 Rev. B).

RBC volume changes with the osmotic pressure of the solution suspends in it, which can be well described by Boyle–van't Hoff relationship, that is $V=M_0/M\times(V_0-V_b)+V_b$. Where V_0 is the isotonic cell volume at isotonic osmotic pressure (M_0), V is the cell volume at osmotic pressure M and V_b is the osmotically inactive cell volume [8]. In this study, M was controlled by adjusting the concentration of NaCl solution (or more directly described, controlled by modified osmolality of the solution), then the RBC volume was measured using EPC. From Boyle–van't Hoff relationship, one can see, if M is increased to be infinite, then the cell volume tends to be V_b.

3. Materials and methods

3.1. Preparation of RBCs

Human RBCs were obtained from a local blood bank (Red Cross and Blood Center of Hefei, Anhui Province, China), and used in DSC experiments within 12 h. The plasma was removed and the cells were re-suspended in filtrated (PALL-Gelman Laboratory, Super-200, 0.2 μm filtrate membrane) 0.9% NaCl solution. Cell washing processes: RBC suspensions were centrifuged for 2 min at 390 *g* using a centrifuger (Hettich Zentrifugen, MIKRO 22), then the upper solution was removed, and the RBCs were re-suspended in filtrated 0.9 NaCl solution. This process was repeated two or three times. One sample of the cell suspensions had a final cell concentration of approximately 3×10^8/ml, which was prepared for EPC. The other samples were prepared to have five different cytocrits of 0, 0.35, 0.45, 0.55, 0.62 (after being centrifuged, different amount of 0.9% NaCl solution was added, and a pick of each sample was centrifuged for 30 min at 585 *g* in a cytocrit-tube to measure the cytocrit), which were prepared for DSC.

3.2. DSC experimental procedures

i. Calibration of DSC. The temperature scale of DSC (Pyris-1, Perkin-Elmer Corporation, Norwalk, CT) was calibrated by the melting point of *n*-decane ($C_{10}H_{22}$, 243.45 K or −29.7 °C for 99 purity) and indium (156.7 °C for 99.9 purity). The transition enthalpies were based on the heat of fusion of indium (28.45 J/g). The instrument was carefully calibrated two–three times. In order to minimize the thermal lag, a low and evenly distributed sample mass (12 μl, usually 9–12 mg) and a low scan rate (5 °C/min) were used as described in Ref. [7].

ii. Measurement of the total latent heat release. Human RBCs suspension initially equilibrated at 4 °C was cooled at 5 °C/min until the extracellular ice nucleated in the solution, observed as a sharp negative peak on the DSC thermogram (typically approx. −12 °C). The sample was then re-equilibrated at a certain subzero temperature −0.53 °C (T_{ph}, phase change temperature of 0.9% NaCl solution) for 4 min. The sample was then cooled from T_{ph} to T_f (−0.53 to −40 °C) at 5 °C/min. The latent heat release from the freezing sample was calculated using the Pyris-1 DSC software, the sigmoidal baseline was drawn between the

phase change temperature and −25 °C. Each sample was measured three times.

3.3. EPC experimental procedures

i. Preparation of NaCl solutions of different osmolalities. NaCl of different mass was dissolved in deionized water produced by KFLOW-R050ACB water clean system (KFLOW water system CO., Ltd), filtrated using 0.2 μm filtrate membrane (PALL-Gelman Laboratory, Super-200). Then NaCl solutions of different osmolalities (118, 185, 260, 302, 670, 863, 1349, 1583, 1741, 2252, 2682, 2800, 3186 mOsm) were produced. The osmolality values were measured using a freezing point depression osmometer (Osmomat 030 cryoscopic osmometer, ±5 mOsm, German), before measurement it was calibrated with deionized water (0 mOsm) and standard NaCl solution (300 mOsm).
ii. Measurement of RBC volume under different osmolalities using EPC. The above prepared RBCs were added into different beakers (capacity of 50 ml) full of NaCl solutions (50 ml) of different osmolalities, numbered 1–13, equilibrated for 15 min under room temperature. EPC was validated using standard particles (CC Size Standard L10) before each sample was measured. Then the cell suspensions were measured one by one using EPC. The software was set to count 30 000 cells each run, every sample was measured five times (70-μm aperture tube was selected in this measurement). The software then gave out the mean volume of the approximately 30 000 RBCs in the sample using its statistical module. The mean volumes of five runs were averaged to give the last value, which is the equilibrated volume of RBCs under this osmolality.

4. Results

4.1. DSC method

The measured latent heat releases from RBC samples were list in Table 1, and they were also plotted as a function of cytocrit as shown in Fig. 1. Through the linear fitting of the data, it was found that $B_f = 285.08$ (J/g), and $K_f = -127.52$ (J/g).

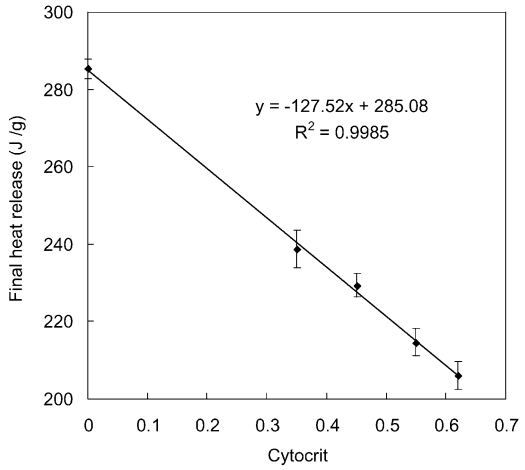

Fig. 1. Total latent release (mean ± S.D., $n = 3$) from RBC suspensions as a linear function of cytocrit during the freezing process from −0.53 to −40 °C.

Substituting the determined B_f and K_f into Eq. (4), assuming the latent heat of pure ice (ΔH_f) is approximately 333.9 J/g [6]. V_f was determined to be 53% of the cell isotonic volume (Table 2).

4.2. EPC method

Fig. 2 is the relationship between equilibrated volume of RBCs and the osmolality of the suspension. According to the EPC results, when the osmolality is more than 2500 mOsm, RBC volume has no further shrinkage, which is in agreement with Ref. [9]. The final volume of RBC is approximately 57% of its isotonic volume, which can be compared with the result of the DSC method. From the above results, it can be concluded that the water transport concentrates on the osmolality scale of 118–1000 mOsm.

5. Discussion

From the published data, the osmotically inactive volume of RBC is approximately 28.3% of its isotonic volume, $V_b = 28.3\% V_0$, measured using both the dry weight method and the isotope meth-

od, which has been described in detail elsewhere [10]. Together with our experimental results, $V_f = 53–57\%V_0$, one can obtain the trapped water of RBC to be $V_{t,w} = 24.72–28.7\%V_0$ or $V_{t,w} = 34.4\% \sim 40\%V_{0,w}$, where $V_{0,w}$ is the initially isotonic water volume in RBC. That is, only 60–65.7% of intracellular water was apparently osmotically active (i.e. Ponder's R was approx. 60%).

Human erythrocytes responded as osmometers to increases in the external concentration of non-permeating solute, but the volume of intracellular water varied less with osmolality than would have been predicted for an ideal osmometer. Ponder's R was obtained directly by subtracting V_b from V_f, and was found to be 0.60–0.66.

In 1963, Peter Mazur developed a mathematical model based on thermodynamics principle, which describes cell volume change during freezing process [11]. During freezing of cell suspension, ice forms first in the extracellular solution that suspended the cells due to the block effect of cell membrane to the growth of ice towards the inside of the cells [12]. The presence of extracellular ice induces a chemical potential difference across the cell membrane which then causes intracellular water to move towards the extracellular solution, the consequent reduction in cellular volume was modelled as follows [11,12]:

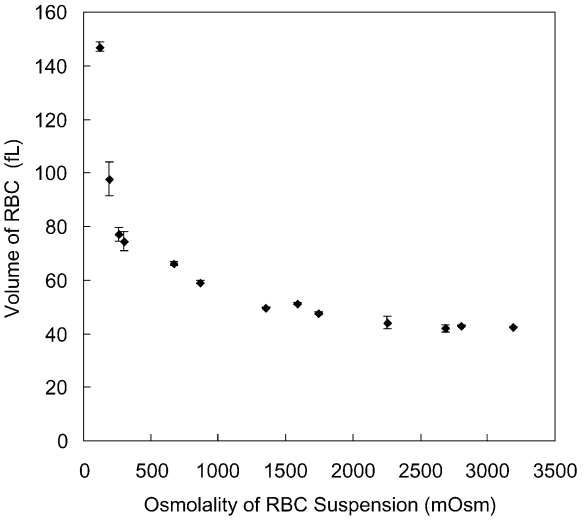

Fig. 2. RBC volumes under different osmolalities from 118 to 3186 mOsm (mean ± S.D., $n = 5$).

$$\frac{dV}{dT} = \frac{L_p A_c RT}{B v_w}\left[\ln\frac{V - V_b}{V - V_b + \phi_s n_s v_w} - \frac{\Delta H_f}{R} \times\left(\frac{1}{T_{m0}} - \frac{1}{T}\right)\right] \quad (5)$$

$$L_p = L_{pg}\exp\left[-\frac{E_{L_p}}{R}\left(\frac{1}{T} - \frac{1}{T_R}\right)\right] \quad (6)$$

Table 2
Measured RBC volumes under different osmolalities

Osmolality (mOsm)	Volume (*fL*)					Mean volume (mean ± S.D., *fL*)
	1	2	3	4	5	
118	149	148.4	147.1	146.3	144.7	147.1 ± 1.710
185	107.2	100.6	95.82	93.71	90.98	97.662 ± 6.389
260	80.85	78.66	75.93	76	74.39	77.166 ± 2.569
302	79.93	76.18	72.61	70.86	72.52	74.42 ± 3.640
670	67.14	64.86	66.36	66.78	66.28	66.284 ± 0.869
863	58.47	59.64	59.67	59.02	59.29	59.218 ± 0.497
1349	49.58	49.92	49.83	49.68	49.7	49.742 ± 0.133
1583	52	51.48	51.37	51.18	50.94	51.394 ± 0.396
1741	48.04	48.17	47.44	47.17	47.16	47.596 ± 0.480
2252	43.85	46.58	46.72	41.87	42.1	44.224 ± 2.344
2682	41.14	41.14	41.15	43.59	43.58	42.12 ± 1.337
2800	43.19	43.13	43.13	43.02	43.06	43.106 ± 0.067
3186	42.51	42.62	42.52	42.65	42.62	42.584 ± 0.064

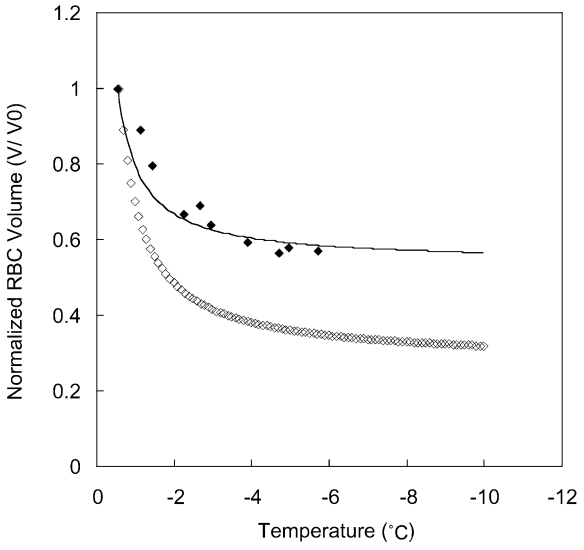

Fig. 3. RBC volumetric change during equilibrium freezing process.

where V is the cell volume, T is the absolute temperature, L_{pg} is the permeability of the membrane to water at a reference temperature T_R, E_{Lp} is the apparent activation energy for the permeability process, R is the gas constant, B is the cooling rate, A_c is the effective membrane surface area for water transport, v_w is the partial molar volume of water, n_s is the number of moles of solutes in the cell, V_b is the osmotically inactive cell volume, $\varphi_s = 2$ is the disassociation constant for salt in water, ΔH_f is the heat of fusion of pure ice (in order to agree with Ref. [7], its value is assumed to be 333.9 mJ/mg in the temperature range of interest from 0 to -20 °C), T_{m0} is the melting point of pure ice.

In Fig. 3, the solid rhombic points were obtained from EPC experiments, which simulated freezing conditions by suspending RBCs in hypertonic solutions of different osmolalities and using EPC to determine the average magnitude of cell volume change. The solid line is the results of solving Eq. (5) using V_f instead of V_b. From Fig. 3 one can see the results have a good accordance with the EPC experimental data, while the hollow rhombic points has a poor accordance with it. This suggests that, for RBCs, the osmotically inactive volume V_b, cannot be directly used in the conventional freezing model of the cell, it must be replaced by V_f. Levin et al. had developed a model describing the effects of hydration on the water content of human erythrocytes [13], and using it they predict at least 16.65% of the isotonic cell water content will be retained with RBCs placed in hypertonic solutions. Our results firmly support the predicted ones.

Fig. 4 is the typical result for the influence of the cooling rate on RBC volume change during the freezing process. This can be used in the optimization of freezing or freeze-drying procedure, and the simulation of the intracellular ice volume fraction of RBC during deep freezing.

From this study, a substantial fraction of the water within erythrocytes will not take part in the osmotic action of RBCs. We call this part of the cell water 'trapped water', as it is kept in the cell during the freezing process and cannot flow out with the dramatically increasing extracellular osmolality after ice forms outside the cell. The trapped water is mainly composed of 'bound water' (the water within the erythrocytes 'bounded' to solutes, mostly proteins) and perhaps a fraction of free water (the water that can take part in osmotic behavior, but still trapped in the cell

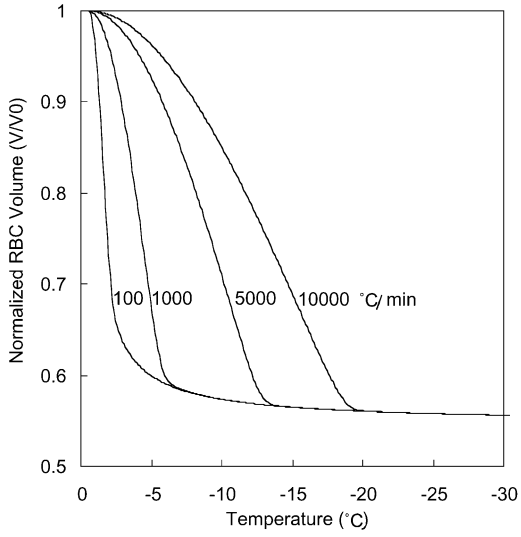

Fig. 4. RBC volumetric change at different cooling rate.

under subzero temperatures). Successful design for RBCs cryopreservation, especially freezing-drying preservation must fully consider the influence of trapped water.

Acknowledgments

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