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A method to measure permeability of red blood cell membrane to water and solutes using intrinsic fluorescence

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ABSTRACT

Background: Designing effective cryopreservation procedures for cells requires knowledge of permeability of cell membrane to water and solutes. To determine cell membrane permeability, one needs to measure the rate of cell volume changes in anisotonic environment. Red blood cells (RBCs) respond very quickly to changes in extracellular solutes concentration, which complicates the use of traditional methods. Preservation of RBCs from umbilical cord blood for neonatal transfusions is currently broadly discussed in the literature, but data on osmotic permeability of cord RBCs is controversial. Therefore, alternative methods to determine osmotic membrane permeability of these cells are warranted. We describe a technique to measure rapid changes in RBC volume through changes in the intensity of RBC autofluorescence.
Methods: To induce osmotically-driven changes in RBC volume, we rapidly mixed human RBCs with anisotonic solutions in a stopped-flow spectroscopy system and the intensity of intrinsic RBC fluorescence was measured.
Results: We found that change in RBC volume cause a proportional change in the intensity of RBC autofluorescence. This phenomenon occurs due to the self-quenching of RBC hemoglobin autofluorescence at high intracellular concentrations.
Conclusions: This novel method to determine osmotic permeability of RBCs overcomes the limitations of traditional techniques and has numerous clinical applications.

1. Introduction

Cryopreservation – preservation of cells and tissue at subzero temperatures below which biochemical reactions do not occur at appreciable rates – is an effective means to preserve the quality and functionality of biological material for clinical transplantation and research purposes [1]. To prevent damage to cells during exposure to such low temperatures, special chemical compounds termed cryoprotectants are used. Glycerol is a common cryoprotectant used in the cryopreservation of red blood cells [2]. The cell membrane plays a very important role in cryopreservation, governing the transport of water and solutes between the cytoplasm and the extracellular milieu and regulating osmotic changes of the cell during freezing [3]. To design effective cryopreservation procedures for cells and tissues and avoid freezing injury, it is critical to know cell osmotic parameters, such as the permeability of cell membrane to water and solutes [4–7]. To determine cell membrane permeability, one needs to measure the rate of cell volume change when placed in an anisotonic environment [8]. Red blood cells (RBCs) respond very quickly to changes in extracellular solute concentrations [9]. Therefore, it is difficult to measure the rate of RBC volume change with traditional methods, such as using an electronic particle counter [10].

Stopped-flow spectroscopy can quantify rapid changes of RBC volume and is a common method used to measure RBC osmotic permeability. Two stopped-flow approaches are used in this regard. The first measures RBC volume based on changes in the intensity of light scattered by RBCs [9,11–13]. However, the limitation of this method is that several factors besides cell volume can influence the intensity of scattered light. Among these factors are the dependence of cell refractive index on the intracellular permeable solute concentration [14], discoid shape of RBCs [13], or membrane aggregation [15]. The second approach monitors changes in cell volume based on the self-quenching of a fluorescent dye, such as carboxyfluorescein diacetate (CFDA), entrapped inside the cell. This method was first introduced by Chen et al. [15], and was later used by others for RBCs [16,17]. However, a significant portion of light emitted by fluorescent dye is absorbed by hemoglobin [18], which complicates this technique.

Evidence suggests that RBCs obtained from umbilical cord blood (cord RBCs) contain the same type of hemoglobin as blood of newborns [19] and may be a superior alternative to adult RBCs presently transfused

Abbreviations: CFDA, carboxyfluorescein diacetate; hemoglobin A, adult hemoglobin; hemoglobin F, fetal hemoglobin; CPD, citrate–phosphate–dextrose; g, gravitational acceleration constant; SAGM, saline–adenine–glucose–mannitol; V/V_0, relative volume; fluorescence/$fluorescence_0$, relative autofluorescence intensity; RFU, relative fluorescence unit.

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to neonates with anemia [20–22]. Although cord RBCs are usually discarded during hematopoietic stem cells isolation from cord blood, a number of studies have demonstrated that transfusions of autologous cord RBCs are both safe and effective in the treatment of anemic neonates [23–29]. Unlike RBCs from adult blood, cord RBCs deteriorate very quickly during traditional storage at 1–6 °C [25,30] and would benefit from low temperature preservation. Despite many studies having documented the successful cryopreservation of adult RBCs, no protocol for cryopreservation of cord RBCs has been developed. Knowledge of osmotic permeability of cord RBCs to water and solutes is required to design effective cryopreservation methods for these cells. However, literature data on cord RBC permeability are both limited and controversial [9,31]. Therefore, development of the improved methods to determine cord RBC permeability is warranted.

Literature reports indicate that RBC hemoglobin has autofluorescent properties [32–35]. Hirsch et al. showed that adult hemoglobin (hemoglobin A) and fetal hemoglobin (hemoglobin F), when excited at 280 nm, emit light with a maximum intensity at ~325 nm [32]. Alpert et al. demonstrated maximum emission of human adult hemoglobin at 334 nm [33]. We propose that RBC permeability to water and solutes can be measured using intrinsic fluorescence (autofluorescence) of intracellular hemoglobin. Rapid changes in RBC water volume in response to exposure of cells to an anisotonic environment can be captured by measuring changes in intrinsic hemoglobin fluorescence intensity, since measured fluorescence is reduced at increased hemoglobin concentrations resulting from reduced cell volume.

2. Materials and methods

2.1. Experimental samples

We used 2 sources of RBCs for this study: RBCs from peripheral blood of adult donors (adult RBCs), and RBCs from umbilical cord blood (cord RBCs). The Canadian Blood Services Network Center for Applied Development in Vancouver provided adult RBCs for our experiments. RBCs were stored in CPD (citrate–phosphate–dextrose) anticoagulant and SAGM (saline–adenine–glucose–mannitol) preservative at 1–6 °C and were used in experiments within 10 days of collection. The Alberta Cord Blood Bank supplied cord RBCs, as a waste product, after stem cell isolation from umbilical cord blood. Cord blood collected from a placenta was stored at room temperature for up to 67 h prior to stem cell isolation [36]. Cord RBCs were stored in our laboratory at 1–6 °C and used in experiments within 24 h of isolation from cord blood. Prior to experimentation, cord RBCs were washed with saline 1–3 times by centrifugation at 2200 ×*g*, 4 °C for 5 min. The hematocrit of cord RBCs was then adjusted to approximately 60% by the addition of saline to the RBC pellet. Ethics approval for the study was obtained from the University of Alberta Health Research Ethics Board (Biomedical Panel) and Canadian Blood Services Research Ethics Board.

2.2. Experimental solutions

RBC autofluorescence was monitored in the presence of various concentrations of NaCl, prepared by diluting 12% (w/v) stock solution (Baxter) with distilled water to yield final concentrations (% w/v) of 0.46, 0.68, 0.8, 0.9, 1.25, 1.6, 2.3, 2.55, 3.5, and 6.1. Glycerol (3.5% w/v)–NaCl (0.9% w/v) solution was prepared by diluting 35 g glycerol (99.5 +%, Sigma Aldrich, Inc.) and 9 g NaCl (≥99.0%, Sigma Aldrich) with distilled water to 1 l.

2.3. Stopped-flow system

A SpectraMax Gemini EM dual-scanning fluorescence microplate reader (Molecular Devices) was used to measure equilibrium autofluorescence of RBCs and hemoglobin in various osmolalities. Data were acquired and analyzed using SOFTmax PRO software (ver 5.3, Molecular Devices). All measurements were made at ambient temperature (mean = 24.6 °C, range 22.1–27.8 °C).

An SX20 stopped-flow reaction analyzer (Applied Photophysics, Ltd.) was used to measure rapid kinetic changes of RBC autofluorescence upon mixing with solutions of various osmolalities. Excitation and emission slit widths were set to 3 mm (equivalent to a wavelength bandwidth of 13.95 nm). The 20 μl optical cell had a 10 mm pathlength and 1 ms dead time (during which mixing occurred). Data were acquired using ProData SX software (Applied Photophysics). All measurements were made at ambient temperature (mean = 20.2 °C, range 19.3–21.2 °C).

2.4. Integrity of RBCs

RBC hemolysis (membrane damage) was measured in all experimental NaCl solutions (NaCl concentration ranging from 0.68% (w/v) to 3.5% (w/v)). Fifty microliters of RBCs were pipetted into 1 ml NaCl solution and were allowed to equilibrate at room temperature for approximately 5 min. RBC hemolysis was determined by spectrophotometric measurement of total and supernatant cyanmethemoglobin according to Drabkin's method [37] with a correction for hematocrit. Controls for total hemoglobin were prepared from Stanbio Tri-Level Hemoglobin controls (Stanbio Laboratory). The hematocrit of the RBC sample was measured, using a microhematocrit centrifuge (Hettich), as the ratio of the volume occupied by packed RBCs to the volume of a whole RBC sample.

2.5. Preparation of lysed RBCs (negative control)

Adult RBCs were lysed by three consecutive freeze–thaw cycles; cells were immersed in liquid nitrogen, and were then thawed at 37 °C. Lysed RBCs were run in parallel with intact RBCs in equilibrium and kinetics experiments. Specifically, control samples were prepared by adding 5 μl (in equilibrium experiments) or 20 μl (in kinetics experiments) lysed RBCs into 1 ml NaCl solution (NaCl concentration ranging from 0.68% (w/v) to 3.5% (w/v)).

2.6. Fluorescence spectrum of adult and cord RBCs

We chose 280 nm as an excitation wavelength for both cord and adult RBCs based on literature values [32]. The maximum emission wavelength was determined in 4 NaCl solutions; 0.68%, 0.9%, 1.6%, and 3.5% (w/v). Emission spectra of adult and cord RBCs were scanned in a fluorescence microplate reader (range 330–750 nm, 325 nm cut-off filter, step 10 nm) and in a stopped-flow analyzer (range 280–650 nm, step 2 nm).

2.7. Measurement of RBC autofluorescence

2.7.1. Equilibrium

We measured autofluorescence of RBCs equilibrated in NaCl solutions at concentrations of 0.68%, 0.8%, 0.9%, 1.25%, 1.6%, 2.55%, and 3.5% (w/v) NaCl. Five microliters of RBCs were pipetted into 1 ml NaCl solution and equilibrated at room temperature for approximately 5 min to allow the RBC volume to stabilize. Two hundred microliters of each sample were pipetted in triplicate into 96-well Microfluor 1 black flat bottom microtiter plates (ThermoScientific). After mixing, samples were excited at 280 nm, and emission was measured at the optimized wavelength in top-read mode. Background autofluorescence of NaCl solutions (without RBCs) was also measured in triplicate and later subtracted from autofluorescence of RBC samples. Lysed RBCs served as a control. Fluorescence was expressed in relative fluorescence units (RFUs).