Published in final edited form as:

Transfusion. 2009 July ; 49(7): 1435–1442. doi:10.1111/j.1537-2995.2009.02133.x.

Changes in Band 3 oligomeric state precede cell membrane phospholipid loss during blood bank storage of red blood cells

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Abstract

BACKGROUND—Lipid loss in the form of vesicles contributes to the red blood cell (RBC) storage lesion, and this loss of lipid is correlated with changes in membrane protein function. Sensitive spectroscopic techniques were used to measure changes in Band 3 oligomeric state during storage of RBCs, compared to metabolic changes and phospholipid loss. The aim of the study was to determine whether changes in the macromolecular organization of membrane proteins occur before, coincident with, or after lipid loss during RBC storage.

STUDY DESIGN AND METHODS—Five RBC units were collected from normal volunteers and stored under standard blood bank conditions, and both metabolic changes and lipid loss were measured by multiple assays. Band 3 oligomeric state was assessed by time-resolved phosphorescence anisotropy and fluorescence resonance energy transfer of eosin-5-maleimide-labeled RBC ghosts.

RESULTS—Extracellular pH decreased and extracellular potassium increased rapidly during cold storage of blood. Band 3 on the RBC membrane exhibited a shift from small to large oligomers early in the storage period and before detectable loss of phospholipid from the RBC membrane. The immobilized fraction of Band 3, that which is tethered to the cytoskeletal network via spectrin and ankyrin, did not change during cold storage.

CONCLUSION—Our results demonstrate that changes in the macromolecular organization of membrane proteins on the RBC occur early in storage, and these changes may induce phospholipid loss, irreversible morphologic changes, and loss of function during RBC storage.

During blood bank cold storage, red blood cells (RBCs) progressively lose deformability and elasticity due to irreversible changes in the membrane.¹ Morphologically this is observed first as a reversible shift toward discocyte formation and later as progressive irreversible formation of spherocytocytes.² These irreversible changes in deformability, elasticity, and morphology underlie the poor in vivo survival of transfused RBCs.¹ Recent studies have demonstrated that there may be a correlation between number of days of cold storage of RBCs and adverse outcomes including mortality among transfused patients,^{3,4} presumably due to structural or functional changes in red blood cells (RBC) occurring during storage. This phenomenon has been referred to as the RBC storage lesion.^{1,4}

The underlying mechanism behind RBC membrane changes during storage is largely unknown, but theories proposed include changes in spectrin–protein 4.1–actin interaction,^{1,5}

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CONFLICT OF INTEREST

No conflicts of interest to disclose.

spectrin oxidation,^{1,6} lipid peroxidation,⁴ spontaneous lipid loss in the form of vesicles,¹ or phospholipid asymmetry due to ATP depletion or ineffective ATP utilization at low pH.⁷ Several studies have identified mechanisms to reduce loss of membrane lipid in the form of vesicles during cold storage.^{8–10} Preservative solutions that maintain ATP levels have been developed and are effective in reducing lipid loss⁸ and hemolysis^{8,11} and preventing phospholipid asymmetry;¹² however, they do not prevent morphologic changes in the RBC or decreased deformability during storage.¹¹ Until the primary mechanism or mechanisms of RBC membrane structural changes are understood, it will be difficult to identify storage conditions that will prevent irreversible membrane changes leading to loss of function of transfused RBCs. An experimental approach that allows sensitive detection of changes in the organization of cytoskeletal membrane proteins on intact RBCs during storage would help elucidate the mechanism of the RBC storage lesion.

The goal of our study is to determine whether changes in the organization of cytoskeletal membrane proteins can be detected during cold storage of RBCs and to determine whether these changes occur before, coincident with, or after lipid loss from RBCs during storage. We measured metabolic changes occurring during RBC storage and loss of lipid from the RBC membrane using established^{6,8–10} and novel assays for 5 RBC units collected and stored under standard blood bank conditions. We also labeled Band 3 on the RBC membrane with eosin-5-maleimide (EMA) to perform time-resolved phosphorescence anisotropy (TPA) and fluorescence resonance energy transfer (FRET) experiments on the same 5 RBC units on Days 0, 12, 20, 29, 42, and 49 of cold storage. TPA has previously been shown to resolve distinct populations of Band 3 oligomeric complexes in the RBC membrane and is thus a powerful spectroscopic tool to determine the effect of RBC storage on the organization of membrane proteins.^{13,14} Fluorescence resonance energy homotransfer is an independent (compared to TPA) technique that also detects large changes in the organization of Band 3 in the RBC membrane.¹⁵ The results allow us to compare the time scale of changes in RBC metabolic parameters, lipid loss, and macromolecular organization of membrane proteins during cold storage of RBCs.

MATERIALS AND METHODS

Preparation of labeled RBCs

Five normal volunteers were solicited to donate 1 standard unit of whole blood collected in a 500-mL collection bag set containing 70 mL CP2D (Leukotrap WB, Pall Corp., East Hills NY). Within 8 hours the whole blood was passed through an in-line leukoreduction filter and centrifuged at 4000 r.p.m. for 5 minutes in a centrifuge (Sorvall RC3BP, Thermo Fisher Scientific, Waltham, MA) equipped with a HBB-6 rotor. Plasma was expressed after centrifugation and AS-3 solution (Nutricel, Haemonetics, Union, SC) containing citrate, phosphate, dextrose, adenine, and sodium chloride added to produce leukoreduced RBC units of 300 to 395 mL having hematocrit levels of between 45 and 55% according to institutional blood bank procedures. RBC units were stored in a monitored blood bank refrigerator at 4 to 6°C for the duration of the experiment.

On Days 0, 12, 20, 28, 42, and 49 of PRBC storage two 7-mL aliquots and two 3-mL aliquots (total, 20 mL) were removed using a sterile connecting device from the RBC units for TPA, metabolic, and lipid loss experiments. Experiments were initially planned for Days 0, 12, 20, 28, and 42 of storage, but due to a problem with the laser system TPA experiments could not be performed on Day 42. For this reason experiments were extended to Day 49 to determine an endpoint for changes in Band 3 oligomeric state. RBCs were labeled with EMA (Invitrogen, Carlsbad, CA) under conditions previously demonstrated to result in specific (>80%) labeling of Band 3 in the RBC membrane.¹⁶ Under these conditions EMA covalently labels Band 3 at one extracellular exposed lysine residue, lysine-430.¹⁷ Labeled

RBC ghosts were then prepared by hypotonic lysis in 20 vol of 5 mmol/L sodium phosphate, pH 7.4 (phosphate buffer). RBC ghosts were washed in phosphate buffer by centrifugation at 4°C at $5820 \times g$ for 15 minutes, followed by resuspension in phosphate buffer. This was repeated three times to remove hemoglobin (Hb). During and after ghost preparation RBCs were handled on ice and in the absence of direct overhead fluorescent lighting, and all TPA experiments were performed within 24 hours of ghost preparation. The study design was approved by the Mayo Clinic Institutional Review Board.

Metabolic experiments with RBC aliquots

After thorough mixing RBC aliquots were injected into a blood gas analyzer (ABL 725, Radiometer Medical A/S, Bronshøj, Denmark) for all pH and initial potassium measurements. For potassium values of greater than 20 mmol/L (due to linearity limits on the Radiometer), 7-mL aliquots of RBCs were centrifuged at $4000 \times g$ for 1 minute, and supernatant was analyzed on a chemistry analyzer (Cobas Integra 400, Roche Diagnostics, Indianapolis IN) for potassium.

Lipid and Hb measurement

Seven-milliliter aliquots of RBCs were centrifuged at $1865 \times g$ for 20 minutes at 4°C to separate RBC pellet from supernatant. Aliquots of RBC supernatant and RBC pellet were frozen at -70°C until later analysis. RBC acetylcholinesterase (AChE) activity has been used as a marker for lipid loss from the RBC membrane because AChE activity is found only in RBC membranes and in lipid vesicles lost from the membrane, and loss of AChE activity has been shown to be linearly correlated with loss of phospholipid in the form of vesicles during cold storage.^{6,10} AChE activity of RBC pellet and supernatant after centrifugation at $1865 \times g$ as described above was determined by a modification of the technique described by Ellman and colleagues.¹⁸ The ratio of esterified to unesterified cholesterol in supernatant was determined by measuring supernatant esterified and unesterified cholesterol on a chemistry analyzer (Hitachi 911, Roche Diagnostics, Indianapolis, IN) using reagents provided by Wako Chemicals (Richmond, VA). Hb concentration in the RBC supernatant was measured using a plasma/low Hb photometer (HemoCue AB, Ängelholm, Sweden).

TPA and homotransfer experiments

The optical laser system and data collection for TPA have been described previously.^{19,20} Anisotropy is similar to light polarization and allows detection and differentiation of membrane protein oligomeric state based upon relative mobility of various oligomers, aggregates and complexes.^{13,14,19} The TPA decay, $r(t)$, is given by

$$r(t) = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH}),$$

where I_{VV} and I_{VH} are obtained by signal-averaging the time-dependent decay in anisotropy after 300 vertically polarized laser pulses, with a single detector and emission polarizer that alternates between the vertical (I_{VV}) and horizontal (I_{VH}) positions every 300 pulses. The laser repetition rate was 70 Hz and data was collected to 5 milliseconds, so a typical $r(t)$ acquisition required 10 to 15 minutes to collect 100 loops, or cycles, of 600 pulses (300 in each orientation). G is an instrumental correction factor.^{19,20} For TPA experiments, labeled RBC pellet was diluted into phosphate buffer to less than 0.1 absorbance unit (AU) at 523 nm.²¹ An enzymatic oxygen removal system²⁰ was used and data collection was performed at 37°C after 10 minutes' incubation to allow oxygen removal and temperature equilibration.

TPA data was fit to a sum of two exponentials plus a constant¹³ and normalized to the initial (r_0) anisotropy such that

$$r(t)/r_0 = A_1 \exp(-t/\phi_1) + A_2 \exp(-t/\phi_2) + A_\infty, \quad (1)$$

where amplitude 1 (A_1) and ϕ_1 have previously been shown to represent the relative fraction and rotational correlation time of small Band 3 oligomers, amplitude 2 (A_2) and ϕ_2 the relative fraction and rotational correlation time of large Band 3 oligomers and aggregates, and A_∞ (r_∞/r_0) the relative fraction of Band 3 immobilized (tethered) to the membrane via ankyrin and spectrin.¹³ The rapid (15–20 μ sec) decay constant detected in previous experiments^{13,14} was not detectable in our system due to fluorescence “blinding” of the detection system for the first 5 to 15 microseconds of data collection. Previous studies have established that approximately 25% of Band 3 exists as dimers and small Band 3 oligomers; while approximately 50% of Band 3 is composed of large oligomers and aggregates of Band 3 that are not motionally restricted by the cytoskeletal network. The remainder of the Band 3 in the membrane is immobile on the time scale of phosphorescence anisotropy and represents Band 3 attached to the cytoskeletal network via ankyrin and spectrin. Thus, similar to previous TPA experiments performed on EMA-labeled RBC ghosts, we were able to resolve distinct populations of Band 3 oligomers on the intact RBC membrane and detect changes in Band 3 oligomeric state in response to membrane perturbants^{13,14} (in this case cold storage).

FRET (homotransfer) experiments were performed on a fluorometer (Eclipse, Varian, Inc., Palo Alto, CA) as described previously¹⁵ by diluting labeled RBC pellet into phosphate buffer to less than 0.02 AU at 600 nm. For excitation wavelengths greater than 550 nm, additional RBC pellet was added to increase signal intensity as described previously.¹⁵ Steady-state anisotropy was measured at room temperature by measuring fluorescence in each of the four (I_{VV} , I_{VH} , I_{HV} , I_{HH}) excitation and emission polarizer positions for 10 seconds each, with steady state fluorescence anisotropy defined as

$$r = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH}),$$

where G is measured as $G = I_{HV}/I_{HH}$. This was repeated varying excitation wavelength from 475 to 555 nm and measuring emission at 600 nm. FRET (homotransfer), and thus Band 3 aggregation, was observed as a decrease in the anisotropy r as reported previously.¹⁵

Compared to FRET, TPA is more effective at detecting changes in the relative fraction of mobile Band 3 oligomers because the time-dependent decay in anisotropy always detection of distinct populations of oligomers separately during the experiment.^{13,14} However, changes in residual anisotropy in TPA experiments are partially dependent on the orientation of the spectroscopic probe (EMA),^{13,15} potentially confounding interpretation of these changes. FRET experiments detect the mean oligomeric state of Band 3 and thus are less sensitive to changes in the relative fraction of mobile Band 3 oligomers.¹⁵ However, FRET can be used as an independent (compared to TPA) means to assess large changes in the fraction of aggregated Band 3¹⁵ and thus FRET experiments were included in the experimental design for this purpose.

RESULTS

Metabolic changes and lipid loss during cold storage

Extracellular pH decreased and potassium increased rapidly with cold storage of RBCs (Table 1), as observed previously.⁸ Changes in pH and potassium were significant on Day 12 (the first time point measured) compared to Day 0. It has been observed previously that these metabolic changes occur during the first week of blood storage⁸ and more rapidly than either depletion of ATP⁸ or irreversible changes in RBC morphology.² Supernatant free Hb increased gradually with significant increases in free Hb evident on Day 42 (Table 1). Other investigators have found that the percentage of hemolyzed RBCs increases slowly over several weeks of cold storage.^{8,12}

RBC AChE activity has been used as a marker of lipid loss and vesiculation during PRBC storage.^{6,10} AChE activity of the RBC pellet and/or percent release of AChE activity into supernatant after centrifugation have been found to correlate well with the extent of lipid loss from the RBC membrane in the form of vesicles.^{6,10} AChE activity of the RBC pellet decreased slightly after 12 or 20 days of cold storage, but significant changes in AChE activity were not detected until Day 28 (Table 1). When AChE activity of the supernatant was measured and used to calculate percentage of AChE release from the RBC membrane (supernatant activity/supernatant activity + RBC pellet activity) as done previously,⁶ percentage of AChE activity released from the membrane increased significantly on Days 42 and 49 coincident with the decrease in RBC pellet activity (data not shown).

Because cholesterol in the RBC membrane will be unesterified cholesterol,²² while cholesterol derived from residual volunteer plasma will be largely esterified,²³ we reasoned that measuring the ratio of unesterified to esterified cholesterol in the supernatant would be another means to assess the degree of membrane vesiculation during cold storage. The percentage of unesterified cholesterol present in the RBC supernatant increased gradually and became significant on Day 28, coincident with significant decreases in RBC pellet AChE activity and trends toward increased supernatant free Hb. Together the measurements demonstrate that significant lipid loss from the RBC membrane occurs later in the RBC storage period. At least one other study has observed that significant loss of phospholipid occurs only after 20 days of cold storage.⁵

Changes in Band 3 oligomeric complexes during cold storage

TPA and FRET experiments of EMA-labeled RBC ghosts were performed on Days 0, 12, 20, 28, 42, and 49 coincident with metabolic and lipid loss experiments; with the exception that TPA was not performed on Day 42 due to a problem with the laser system. TPA decays were fit to a sum of two exponentials plus a constant (Equation 1); representing the relative fraction of dimers and small oligomers (A_1), large (A_2) Band 3 aggregates, and immobile (A_∞) Band 3 oligomers tethered to the cytoskeletal network via spectrin and ankyrin.

Anisotropy decays for 1 RBC unit taken on Days 0, 12, 20, and 49 are shown in Fig. 1. Decays for Days 12, 20, and 49 were normalized to the initial anisotropy (r_0) on Day 0 for ease of comparison. When normalized, the initial anisotropy (r_0) and residual anisotropy (r_∞) are nearly identical over 49 days of storage, indicating that the fraction of immobile Band 3 tethered to the membrane cytoskeleton (A_∞ in Table 2) does not change. The amplitude of the most rapid motion (dimers and small oligomers, A_1 in Equation 1) decreased by Day 12 of cold storage, which can be seen in Fig. 1 as less decay in anisotropy over the first approximately 1500 microseconds. There was a further decrease in the initial (approx. 1500 μ sec) anisotropy decay by Day 49, although most of the effect of cold storage on small Band 3 oligomers occurred in the first 12 days (Fig. 1).

Fitting of the individual TPA decays as described previously²⁰ demonstrates a significant increase in the rotational correlation time assigned to the smallest Band 3 oligomers (ϕ_1 in Equation 1), which reached statistical significance as early as Day 12 (Table 2). The amplitude (relative fraction, A_1) assigned to these small oligomers shows a trend toward decreasing over storage time, which did not reach significance. Because fitting of the TPA decays was not constrained (i.e., amplitudes and correlation times were allowed to vary freely to find the best fit), a shift from small oligomers toward large aggregates could result in either decreased amplitude (relative fraction, A_1) or increased rotational correlation time (ϕ_1) during fitting of TPA decays. The amplitude (relative fraction, A_2) of the very large oligomers increased over this time period (Table 2) consistent with an increase in the relative fraction of Band 3 present in large aggregates. The normalized residual anisotropy (A_∞ in Equation 1) did not change over the 49 days of cold storage (Table 2), demonstrating that the relative fraction of Band 3 immobilized on the time scale of the experiment did not change. Together the results suggest that the primary effect of cold storage was to move the equilibrium of small and large Band 3 oligomers toward large aggregates without impacting the population of immobile Band 3 tethered to the RBC membrane via spectrin and ankyrin.

FRET (homotransfer) experiments were also carried out to allow an independent means to evaluate the oligomeric state of Band 3 during cold storage. FRET has been shown to be sensitive to large changes in Band 3 oligomeric state induced by such agents as melittin or zinc chloride (inducing complete aggregation of Band 3).¹⁵ Complete aggregation of Band 3 by zinc chloride or melittin was shown to decrease fluorescence anisotropy (increase FRET) at emission wavelengths near the excitation maximum, while at wavelengths of greater than 550 nm anisotropy approached the theoretical limit of 0.40 due to progressive loss of FRET at low-energy excitation.¹⁵ We observed trends toward decreased anisotropy (increased FRET) at wavelengths near the excitation maximum, which reached significance only on Day 49 (Fig. 2). This is consistent with the increased amplitude of large aggregates (Amplitude 2) by TPA, which also becomes significant on Day 49 (Table 2). Thus, FRET experiments are consistent with TPA in that a shift toward large Band 3 oligomers is detected, although FRET is less sensitive to these changes than is TPA. This is not unexpected given that complete aggregation of Band 3 by melittin induced a change in anisotropy of only 0.05 compared to native RBCs.¹⁵

DISCUSSION

During cold storage, RBCs undergo metabolic changes, loss of lipid, and decreases in elasticity and deformability leading to decreased survival after transfusion.¹ Decreasing ATP levels or ineffective utilization of ATP may account for some lipid loss and hemolysis during storage,^{8,12} although morphologic changes still occur during cold storage even when preservatives that maintain ATP levels^{8,12} and prevent phosphatidylserine redistribution to the outer membrane leaflet¹² are used.^{1,11}

Rapid changes in extracellular pH and lactate, as well as intracellular 2,3-diphosphoglycerate acid, are seen in the first 1 to 2 weeks of cold storage,^{8,12} although whether these metabolic changes are associated with morphologic changes is not known. Current theories of the mechanism of the storage lesion suggest that changes in spectrin–protein 4.1–actin interaction,^{1,5} spectrin oxidation,^{1,6} lipid peroxidation,⁴ or spontaneous lipid loss in the form of vesicles¹ may contribute to irreversible structural changes in the RBC membrane that decrease posttransfusion survival of the RBC and are presumably implicated in adverse outcomes associated with transfusion of “older” blood.

Band 3, ankyrin, and spectrin are the principle proteins in the membrane cytoskeletal network responsible for maintaining RBC deformability and elasticity.¹ Little information is

available on the organization of membrane cytoskeletal proteins during cold storage. One study demonstrated some Band 3 aggregation in stored RBCs by indirect immunofluorescence, although only 15% to 20% of stored RBCs showed this effect after 30 days of storage.²⁴ In contrast another investigator demonstrated Band 3 aggregates by sodium dodecyl sulfate–polyacrylamide gel electrophoresis during the first 4 days of cold storage,²⁵ although it is unclear how these denatured protein aggregates relate to the oligomeric state of Band 3 in the intact cell membrane. It has also been reported that Band 3 content decreases in RBC membranes and increases in vesicles lost from the RBC membrane, over the storage period.^{25,26}

We evaluated Band 3 oligomeric state in intact RBC membranes using sensitive spectroscopic probes and directly compared the time scale of membrane protein aggregation to metabolic changes and lipid loss that occur during blood bank storage of RBCs. The early changes in Band 3 oligomeric state detected by the more sensitive spectroscopic technique (TPA) represent a novel finding that has not been reported previously using methods that detect Band 3 organization in intact membranes. These changes may precede and be directly responsible for loss of Band 3 from the membrane and appearance of aggregated Band 3 in vesicles that occurs later during storage.^{24,27}

It is possible that the later changes we detected (decreased FRET on Day 49) represent a continuation of this membrane protein aggregation and reflect changes reported using techniques such as indirect immunofluorescence.²³ Alternatively the early shift from dimers toward larger oligomers, and the later appearance of very large Band 3 aggregates, could be driven by unrelated mechanisms. Our study indicates that during cold storage changes in the oligomeric organization of the freely mobile fraction of Band 3 occur within the first 2 weeks of storage and before lipid loss (presumably in the form of vesicles) from the RBC membrane.

Both spectrin oxidation and defects in spectrin–protein 4.1–actin complex formation have been shown to correlate with phospholipid loss from the RBC during cold storage.^{5,6} Band 3 clustering due to Hb denaturation is postulated to be a primary mechanism of targeting RBCs for removal from circulation,^{28,29} and previous investigators have found that antioxidants are effective in reducing IGG antibody binding to Band 3 (an indirect measure of Band 3 aggregation) during cold storage.³⁰ Thus oxidation of Band 3, spectrin, and/or other proteins may be responsible for changes in membrane protein organization and loss of RBC function during cold storage. Lipid peroxidation may also be the primary mechanism of both membrane protein reorganization and phospholipid loss during cold storage, as antioxidants that prevent lipid peroxidation during cold storage are effective in preventing both lipid loss and morphologic changes of the RBC.⁴

The changes in Band 3 oligomeric state we observed are probably not due to disruption of the cytoskeletal interactions between Band 3, spectrin, and ankyrin, because hereditary hemolytic disorders that affect the membrane cytoskeleton have been shown to dissociate immobile Band 3 complexes into smaller and/or more mobile Band 3 oligomers.^{21,31} Rather, our observations are consistent with an early effect of protein oxidation, lipid peroxidation, or some other process on Band 3 oligomeric structure, which may lead to vesiculation and loss of phospholipid from the membrane.

Our study was limited in that the earliest time point studied was after 12 days of cold storage and no measure of RBC morphology was included. In addition we did not measure Hb or Band 3 content within vesicles as has been done previously.^{26,27} We also used supernatant AChE activity as a measure of lipid loss rather than measuring vesicle lipid content directly, although these two parameters have been shown to be closely related.^{6,10} Future studies will

include 1) more RBC units and earlier time points to further differentiate changes in Band 3 oligomeric state from either phospholipid loss or metabolic changes and 2) a measure of RBC morphology to determine whether membrane protein reorganization or lipid loss better correlates with irreversible changes in RBC morphology underlying decreased survival of transfused RBCs.

In conclusion, we studied metabolic changes, lipid loss, and Band 3 oligomeric state during cold storage of RBCs under standard blood bank conditions. Metabolic changes (decreased pH, increased potassium) occur early in the storage period, but it is currently unclear how or whether these may relate to subsequent phospholipid vesicle loss and morphologic changes of the RBC. The freely mobile fraction of Band 3 undergoes a transition toward large oligomers early in the storage period of the RBCs, and these changes appear to precede phospholipid loss from the RBC membrane.

Acknowledgments

Supported by Grant GM27906 and AG26160 to DDT.

We thank Igor Negrashov and Ewa Prochniewicz for technical assistance with TPA and FRET experiments in the Biophysical Spectroscopy Facility at the University of Minnesota; John O'Brien and Joe McConnell for help with AChE and cholesterol measurement; Richard Emery for help in preparation of RBC units and aliquots; and the technologists in the Metabolic Hematology laboratory at Mayo Clinic for EMA labeling of RBCs.

ABBREVIATIONS

AChE	acetylcholinesterase
EMA	eosin-5-maleimide
FRET	fluorescence resonance energy transfer
TPA	time-resolved phosphorescence anisotropy

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Fig. 1.

TPA decays of EMA-labeled RBCs from 1 RBC unit sampled on Days 0, 12, 20, and 49 of cold storage. For sake of comparison, the decays on Days 12, 20, and 49 are normalized to the initial anisotropy (r_0) on Day 0. There is no change in normalized residual anisotropy (r_{∞}/r_0) over 49 days of cold storage. However, there is less anisotropy decay in the first approximately 1500 microseconds on Days 12, 20, and 49 compared to Day 0.

Fig. 2.

FRET (homotransfer) of EMA-labeled RBCs, detected as a decrease in anisotropy at wavelengths near the excitation maximum, on Days 0 and 49 of cold storage. Each point represents the mean ($\pm$ SD) of 5 RBC units sampled on Days 0 and 49. Differences in anisotropy between Days 0 and 49 were significant ($p < 0.05$) at all wavelengths measured.

TABLE 1

Metabolic changes and lipid loss during RBC cold storage for 5 RBC units on Days 0, 12, 20, 28, 42, and 49 of cold storage (n = 5, all data mean ± SD)

Day of storage	pH	Potassium (mmol/L)	Free Hb (mg/dL)	Unesterified cholesterol (%)	RBC AChE (U/mL)
0	6.83 ± 0.02	1.9 ± 0.2	62 ± 29	28 ± 2	11.6 ± 1.1
12	6.63 ± 0.01 *	22.2 ± 0.8 *	86 ± 44	32 ± 4	11.5 ± 1.1
20	6.54 ± 0.01 *	30.3 ± 1.1 *	82 ± 44	33 ± 4	10.4 ± 1.4
28	6.48 ± 0.01 *	36.5 ± 1.3 *	100 ± 37	35 ± 4 *	8.5 ± 1.0 *
42	6.37 ± 0.02 *	43.3 ± 0.9 *	148 ± 35 *	36 ± 3 *	5.0 ± 0.7 *
49	6.33 ± 0.02 *	50.1 ± 1.6 *	182 ± 64 *	39 ± 4 *	6.2 ± 1.5 *

* Significant difference from baseline (Day 0) value (p < 0.05).

Amplitudes and correlation times from fitting Equation 1 to EMA-labeled TPA decays of 5 RBC units on Days 0, 12, 20, 28, and 49 of cold storage (n = 5, all data mean ± SD)

TABLE 2

Day of storage	A ₁	φ ₁ (μsec)	A ₂	φ ₂ (μsec)	A _∞ (r _∞ /r ₀)
0	0.22 ± 0.01	191 ± 7	0.17 ± 0.02	5005 ± 1	0.53 ± 0.01
12	0.23 ± 0.02	236 ± 7 *	0.19 ± 0.02	5014 ± 1	0.55 ± 0.04
20	0.20 ± 0.02	243 ± 10 *	0.20 ± 0.01	5014 ± 1	0.55 ± 0.01
28	0.22 ± 0.02	241 ± 44 *	0.21 ± 0.04	5013 ± 2	0.55 ± 0.01
49	0.19 ± 0.04	316 ± 24 *	0.24 ± 0.04 *	5010 ± 10	0.54 ± 0.06

* Significant difference from baseline (Day 0) value. A₁ and A₂ reflect the relative fraction of small (A₁) and large (A₂) Band 3 oligomers present, while A_∞ reflects the relative fraction of immobile (tethered to cytoskeleton) Band 3 complexes. φ₁ and φ₂ represent the rotational correlation times of small and large oligomers determined from fitting TPA decays as described under Materials and Methods.