Dielectrophoretic Detection of Electrical Property Changes of Stored Human Red Blood Cells

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Abbreviations: crossover frequency (COF), glutaraldehyde (glt)

Abstract

The ability to transport and store a large human blood inventory for transfusions is an essential requirement for medical institutions. Thus, there is an important need for rapid and low-cost characterization tools for analyzing the properties of human red blood cells (RBCs) while in storage. In this study, we investigate the ability to use dielectrophoresis (DEP) for measuring the storage-induced changes in RBC electrical properties. Fresh human blood was collected, suspended in K2-EDTA anticoagulant and stored in a blood bank refrigerator for a period of 20 days. Cells were removed from storage at 5-day intervals and subjected to a glutaraldehyde crosslinking reaction to “freeze” cells at their ionic equilibrium at that point in time and prevent ion leakage during DEP analysis. The DEP behavior of RBCs was analyzed in a high permittivity DEP buffer using a three-dimensional DEP chip (3DEP) and also compared to measurements taken with a 2D quadrupole electrode array. The DEP analysis confirms that RBC electrical property changes occur during storage and are only discernable with the use of the cell crosslinking reaction above a glutaraldehyde fixation concentration of 1.0 w/v%. In particular, cytoplasm conductivity was observed to decrease by more than 75% while the RBC membrane conductance was observed to increase by more than 1000% over a period of 20 days. These results show that the presented combination of chemical crosslinking and DEP can be used as rapid characterization tool for monitoring electrical properties changes of human red blood cells while subjected to refrigeration in blood bank storage.

1 Introduction

Subcellular components such as the lipid bilayer membrane, cytoskeleton, and cytoplasm play essential roles in maintaining cellular function and cell health. Environmental influences such as disease, exposure to pathogens, and pharmacological interventions can impact the physiological characteristics and structural organization of these subcomponents, which can influence their biomechanical and bioelectrical properties. Electrical property changes have been correlated with many physiological processes, including the differentiation state of neural and embryonic stem cells [1,2]. During cell invasion and development, the malaria inducing parasite, Plasmodium falciparum induces electrical changes in human red blood cell (RBC) cytoplasm and membrane conductance [3]. Pharmacological interventions, such as cell exposure to ion channel blockers and anti-cancer treatments have been shown to induce changes in cancer cell electrical properties. Membrane potential has also shown to be an important indicator in biological function, including during the progression of cancer [4]. Similarly, recent studies demonstrate the significance of membrane conductance and cytoplasmic conductivity in contributing to the regulation of the circadian rhythm in human red blood cells [5] and in HL-60 cells undergoing apoptosis [6,7]. A significant and continuing growing body of work therefore demonstrates that cellular electrical properties have the potential to serve as label-free diagnostic markers in cell identification, disease detection, cell separation and for health monitoring [1,8,9].

One important diagnostic marker is the quality of stored human blood for transfusion [10]. Modern blood storage systems enable the donation and transfusion of blood to be dynamically separated in time and space. Large medical centers can use hundreds of units of RBCs a day, and the ability to transport and build large blood

storage inventory is an important requirement for medical institutions. However, such readily available blood storage often raises the question: how long can blood be stored before it is no longer safe and effective for blood transfusions?

RBCs show clear signs of degradation during storage, and there are many known physiological changes associated with RBC storage, including changes in cell morphology and loss of metabolism [11]. A number of RBC quality measures exist, including measuring hemoglobin concentration in RBC storage and measuring the potassium ion concentration in suspension solution, however, no such methods quantify the electrical properties of the RBCs themselves. One metric related to cell electrical properties is the diffusive loss of cytoplasmic cations, such as K^+, Na^+ and Ca^{2+} via the cell membrane while the blood is stored under refrigeration in a blood bank. In particular, the activity of the sodium-potassium ion pump is highly sensitive to temperature, and refrigerated RBCs have been shown to leak potassium into their suspending buffer during storage [12]. When reinfused, cells have the potential to reabsorb potassium which can alter the local extracellular potassium concentration. In cases with an infant or when a large volume of blood relative to the patient's circulating volume is required, such ion reabsorption can lead to death [12]. Development of low-cost and rapid methods to dynamically track the electrical properties of human red blood cells in storage could therefore play an important role in dynamically monitoring the health of stored blood.

One method to measure cell electrical properties is to use the electrokinetic technique, dielectrophoresis (DEP) [13]. Conventionally, DEP is performed using two-dimensional (2D) thin-film electrode arrays with quadrupole, interdigitated, comb-tooth or castellated structures that are typically fabricated atop a glass substrate and observed under an optical microscope. Cells suspended in a buffer of known electrical

conductivity and permittivity are deposited on the electrode array and subjected to a sweeping electric-field frequency. The electric field from the electrodes polarizes the cells and dielectrophoretically drives them to spatially assemble within the confines of the electrode array. The specific geometric location of the DEP cell assembly is based on cell and buffer electrical properties and the AC field frequency; cells will experience a different DEP force - both in direction and magnitude - depending on the frequency applied. The resulting frequency dependent DEP force curve, or DEP spectrum, will consist of finite field frequency ranges where cells are either attracted towards (positive DEP) or repelled away from (negative DEP) the high electric field regions as defined by the array's electrodes. The frequency in which this attraction/repulsion reverses and cell DEP motion ceases is defined as the DEP crossover frequency (COF). The frequency dependent DEP behavior can be measured optically and combined with an appropriate Maxwell-Wagner cell polarization model to empirically deduce cell electrical properties. In this manner, the entire cell suspension is utilized to measure the COF and therefore the resulting DEP method yields electrical properties reflective to the mean value of the entire cell suspension under observation.

While the 2D electrode array method is effective and responsible for many impactful contributions within the DEP community, it does suffer from several limitations. First, this method typically requires one to manually observe DEP-induced cell trajectories. This process requires a highly trained operator and can be tedious for large numbers of samples. Further this manual method is vulnerable to human error and bias. Second, very often this method is only useful in measuring cell COFs which represents single data points within the entire DEP spectrum. Therefore, significant experimental data and DEP behavior in the positive and negative DEP frequency ranges of the spectrum are often not taken into consideration. Such a constraint places

a limit on the ability to accurately correlate DEP behavior to DEP polarization theory and determine the cell electrical properties. In an effort to overcome the drawbacks of conventional 2D experiments and to realize DEP as a more formal analytical tool, Hughes et al. developed the 3DEP dielectrophoretic cytometer [14]. The 3DEP is capable of measuring a more complete DEP force spectrum and empirically calculate the cell suspension electrical properties.

In this work, we demonstrate the use of DEP to quantify the storage-induced electrical property changes of human RBCs subjected to blood-bank refrigeration. In order to minimize ion leakage across the RBC membrane during DEP measurements, we utilize a glutaraldehyde crosslinking reaction prior to cell washing and suspension in a low conductivity-high permittivity zwitterion DEP buffer [15–17]. The glutaraldehyde crosslinks aminated protein groups and serves to inhibit ion loss through the membrane, while the zwitterion buffer reduces the second high frequency DEP COF to within the operating range of most standard function generators. We analyzed the influence of varying concentrations of glutaraldehyde on the DEP behavior of human RBCs over a period of 20 days and used the 3DEP to calculate the cell electrical properties. We also analyzed and compared the DEP spectrum obtained by the 3DEP with that of a 2D quadrupole electrode array in order to ensure these two methods do indeed provide comparable experimental COFs. Results suggest that the 3DEP method is capable of discerning storage-induced electrical property changes of human RBCs. With further development, we believe this glutaraldehyde-3DEP approach can potentially offer a rapid low-cost method for electrically quantifying the status of blood stored for transfusions and for detecting autologous blood transfusions in doping/cheating endurance athletes.

2 Theory

2.1 DEP-Shell Model

We utilize DEP to investigate the dynamic changes of RBC electrical properties when subjected to storage at 4 °C in a blood bank refrigerator over a period of 20 days. The DEP force and polarization equation can be derived for a homogeneous axisymmetric spherical particle and then modified for a RBC. The DEP force on a spherical particle suspended in an electrolyte media is given as [18,19]:

$$\vec{\mathrm{F}}_{\mathrm{DEP}} = 2\pi\varepsilon_o\varepsilon_{\mathrm{m}}\mathrm{r}^3\mathrm{Re}[\mathrm{K}(\omega)]\nabla\vec{\mathrm{E}}^2\,, \qquad (1)$$

where ε_o, ε_{m}, r and $\nabla\vec{\mathrm{E}}^2$, is the permittivity of free space, dielectric constant of the media, the radius of the particle and the gradient of the electric field squared, and $\mathrm{Re}[\cdot]$ represents the real part of a complex variable. $\mathrm{K}(\omega)$ is the Clausius-Mossotti factor (CMF),

$$K(\omega) = \left[\frac{\varepsilon_{\mathrm{p}}^* - \varepsilon_{\mathrm{m}}^*}{\varepsilon_{\mathrm{p}}^* + 2\varepsilon_{\mathrm{m}}^*}\right]. \qquad (2)$$

Physically, Eq. (2) describes the frequency dependent particle dipole moment, and is based on a complex permittivity of both the particle, $\varepsilon_{\mathrm{p}}^*$ and media, $\varepsilon_{\mathrm{m}}^*$. This factor describes the frequency dependent polarization, and ultimately the direction a particle will move in a field gradient under DEP. The DEP COF, for example occurs at a frequency such that $\mathrm{Re}[\mathrm{K}(\omega)] = 0$, while the particle exhibits positive DEP (pDEP) and negative DEP (nDEP) when $\mathrm{Re}[\mathrm{K}(\omega)] > 0$ and $\mathrm{Re}[\mathrm{K}(\omega)] < 0$, respectively.

Electrically, each domain – the particle and surrounding electrolyte - is modelled as a leaky dielectric which behaves as an equivalent resistor and capacitor in series. For a harmonic AC field with an angular frequency, ω this circuit exhibits a complex permittivity, ε^*as

$$\varepsilon^* = \varepsilon - i\,\frac{\sigma}{\omega}\,, \qquad (3)$$

which has a real part composed of the permittivity, ε and an imaginary part (i^2 = -1) that is a function of the angular field frequency, ω, and the electrical conductivity, σ.

Here, we consider the DEP force acting on a dilute suspension of human RBCs. The DEP particle force equation must be modified to account for the outer RBC membrane and inner cytoplasmic domain. Based on previous DEP work, we assume each individual human RBC is modelled as sphere of cytoplasm with an interior radius, r_1 surrounded by a shell of cell membrane with an outer radius, r_2 as determined by the cell membrane thickness. The polarization characteristics of both domains are captured with an effective complex permittivity term, ε_{eff}^* and can be shown to be [18,19]:

$$\varepsilon_{eff}^* = \varepsilon_{mem}^* \frac{\left(\frac{r_2}{r_1}\right)^3 + 2\left(\frac{\varepsilon_{cyto}^* - \varepsilon_{mem}^*}{\varepsilon_{cyto}^* + 2\varepsilon_{mem}^*}\right)}{\left(\frac{r_2}{r_1}\right)^3 - \frac{\varepsilon_{cyto}^* - \varepsilon_{mem}^*}{\varepsilon_{cyto}^* + 2\varepsilon_{mem}^*}}, \quad (4)$$

where ε_{eff}^* replaces ε_p^* equation (Eq. 2). This effective term is a function of the electrical properties of both cell domains with a complex permittivity of the cytoplasm, ε_{cyto}^* and the cell membrane, ε_{mem}^*.

It is worth briefly justifying our assumption to use a spherical polarization model as an approximation for capturing the DEP behavior of an inherently non-spherical biconcave shaped human RBC. An alternative single-shelled DEP model utilizes an oblate spheroid to capture the RBC disc shaped geometry. In some instances, this oblate model is necessary to properly fit DEP polarization data, as was shown previously using bovine RBCs [21]. Using the opensource DEP spectrum software, myDEP [20] one can show that a single-shelled spherical DEP model for this study largely captures the DEP spectrum – including both low and high COFs – produced from an oblate spheroidal polarization model to within ~90% accuracy. It should be

noted that the 3DEP commercial instrument software is not yet capable of performing regression analysis and data fitting with a non-spherical model. Therefore, while it is the only existing option within the instrument, it is still important to validate the use of a spherical assumption for any given cell system. Based on our DEP spectra comparisons, we believe a spherical assumption is a valid DEP approximation for human RBCs (experimentally measured mean radius, r_2 = 4.2 μm $\pm$ 0.4; membrane thickness = 7 nm) for our DEP experimental system.

3 Materials and methods

We now describe the fabrication and operation of the 2D and 3DEP devices, the high permittivity zwitterion DEP buffer and the RBC glutaraldehyde chemical crosslinking workflow.

3.1 Dielectrophoresis Devices

DEP experiments were performed using two different experimental methods. First, a more complete DEP spectrum (up to a field frequency of 45 MHz) was determined using the commercial 3DEP instrument (DEPtech, Heathfield, UK). Second, a 2D quadrupole electrode array was used to measure the cellular DEP COF and subsequentaly compared to that of the 3DEP spectrum.

3.2 3DEP Analysis

A detailed analysis of the operating theory and data analysis for the 3DEP has been described elsewhere [14]. The 3DEP system consists of a printed circuit board (PCB) chip which is inserted into a reader (Fig. 1A). The reader contains twenty independent DDS-signal generators each capable of delivering a 20 V_{pp} waveform source at frequencies up to 45 MHz. Each PCB 3DEP chip contains 20 small pinhole wells capable of initiating DEP. The chip is illuminated using a collimated light source

and monitored using an integrated CMOS camera. When an electric field is applied to each well, the resulting DEP force drives cell motion towards the well edges by pDEP, or into the well center by nDEP depending on the frequency applied. The resulting light intensity of each well is then used to compute a DEP force spectrum dataset (Fig. 1C). This process is repeated multiple times to increase the number of spectrum data points and improve experimental accuracy (Fig. 1D). The 3DEP makes it possible to measure the CMF of cells by monitoring the evolution of optical intensity passing through each chip well. Each resulting 3DEP spectrum is a relative DEP force spectrum and relates the CMF to the light intensity measured in each well through a mathematical factor that remains unchanged during the course of the DEP experiment [14]. The resulting spectrum is then not the complete DEP force, but instead is a relative DEP force, or CMF, which can be used to deduce RBC electrical properties by fitting these relative force spectra to the polarization model defined previously in Eqns. 2 – 4.

3.3 The Quadrupole Electrode Array

2D quadrupole electrodes were fabricated using vapor deposition and wet chemical etching techniques atop of thin glass coverslips (#1, 50 x 30 mm Fisher Scientific). Each slide was coated with 20 nm of chromium and 30 nm of gold using electron beam evaporation and subsequently patterned using a positive photoresist (Shipley 1318). The exposed metal was then etched using gold and chromium chemical etchant (Transene Company, Inc) to create a quadrupolar electrode array geometry with four individual pointed electrodes, each with a separation lengthscale of 50 μm and electrically accessible by a rectangular pad (Fig. 1B). A 20 Vpp voltage over a varying frequency range was then applied to every other electrode using a function generator (Rigol DG1022Z) while the other two non-active electrodes were

grounded. The voltage generated a well-defined high field region in the viscinity of the electrode edges and a low field region in the center of the array. The DEP COF was measured manually under brightfield microscopy by determining the field frequency in which cell assembly transitioned from pDEP to that of nDEP (Fig. 1B).

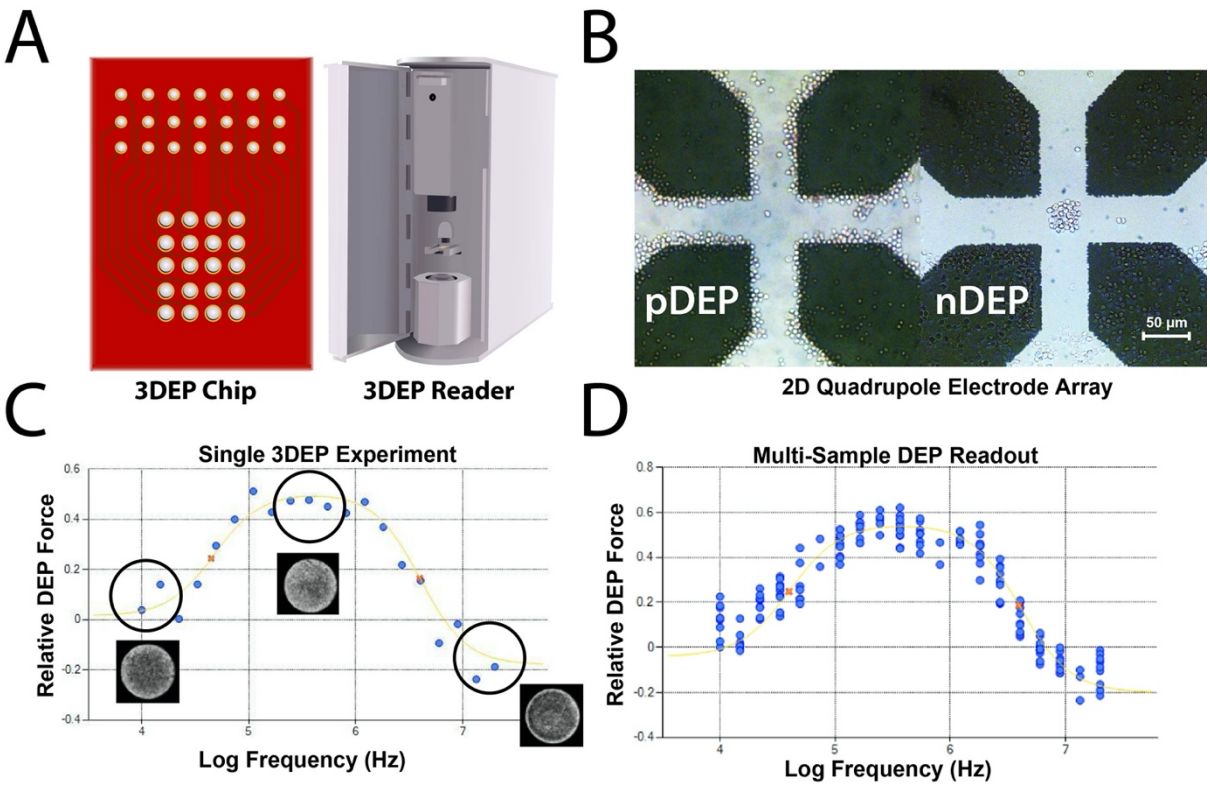


Figure 1. Dielectrophoresis Devices and Spectrum Analysis. (A) 3DEP chip and reader for measuring RBC DEP spectrum. (B) 2D quadrupolar array for DEP COF measurments. RBCs collect at the electrode edges by pDEP or in the center by nDEP depending on the AC frequency applied. (C) Relative DEP force spectrum from a single experiment is fit to a single-shell spherical model. The circled minima and maximum regions correspond to the DEP cell distributions within the pictured 3DEP pinholes. (D) Multiple 3DEP sample repeats (N = 7) superimposed and fit to the polarization model using regression analysis (R = 0.9487).

3.4 Overview of Buffer and RBC Electrokinetic Optimization

In this section we describe the preparation of our DEP buffer and the chemical treatment of human RBCs for DEP analysis. Our overall strategy is to minimize as much as feasible cytoplasmic leakage and interaction of a RBC interior with that

associated with resuspension in a high permittivity-low conductivity DEP buffer immediately prior to DEP analysis. Therefore, in order to effectively capture the storage related electrical property changes occuring in the RBCs we devised a chemical crosslinking scheme to diffusively “seal” the interior cell cytoplasmic ionic distribution during analysis in DEP buffer to more accurately elicidate RBC electrical property changes that occured while in refrigerated storage. Our previous work has demonstrated that chemical crosslinking by the fixation agent, glutaraldehyde is an effective approach to prevent cell leakage when cells are resuspended from physiological buffer into a low conductivity DEP buffer [15,21]. Without a crosslinking protocol, previous DEP studies with bovine RBCs showed that any storage induced electrical changes were not resolvable by DEP [15,21]. After glutaraldehyde treatment at the appropriate concentration, the chemically crosslinked cells are capable of handling DEP analysis in low conductive DEP buffers without cytoplasmic leakage or hemolysis. Therefore in this work, human RBCs were first treated with varying concentrations of glutaraldehyde in order to chemically crosslink the aminated proteins of the RBC and to determine the most effective concentration in which this effect is observed with human blood. While we could attempt to instead use an osmotically balanced DEP buffer as demonstrated in other DEP studies with human blood [5], previous work with storage related DEP analysis has shown that it is important to minimize the degree to which cells are capable of being osmotically influenced by a change in buffer composition. Mass transport across the membrane during DEP analysis has been shown to reduce DEP resolution and lead to dynnamically changing DEP behavior. Therefore, in this work we utilize chemical crosslinking to maximize DEP resolution for detecting age related electrical changes in human RBCs.

In addition to chemically treating the RBCs, we also optimize our DEP buffer with a polarizable zwitterionic salt in order to increase the buffer permittivity. In doing so, the upper high frequency portion of the DEP spectrum associated with the second DEP COF is shifted into a lower frequency space and within the full working range of the 3DEP instrument. As such, a DEP spectrum which would normally require a > 60 MHz bandwidth to fully observe only requires an upper frequency of ~ 20 MHz. The following sections below describe the chemical cell crosslinking and DEP buffer preparation protocols.

3.5 High Permittivity DEP Buffer

The DEP buffer used in this work was a 0.8 M 6-aminohexanoic acid (AHA) (Millipore Sigma) dissolved in deionized water. The final buffer electrical conductivity was controlled by first polishing the 0.8 M AHA solution for 10 min in 5g/mL Dowex MR-3 ion exchange resin (Millipore Sigma) to remove trace salts, and to lower the solution's electrical conductivity to a baseline value. The buffer solution electrical conductivity was then adjusted to a final target value using 1X phosphate buffered saline (PBS). The final DEP buffer possessed an electrical conductivity of 100 μS/cm and a dielectric constant of 110, as verified in our previous electrokinetics work using fluidic dielectrophoresis (fDEP) [22,23].

3.6 Human Red Blood Cells

Single donor O- human blood (ZenBio, Inc) was drawn intravenously in 10 mL volumes and stored in DB vacutainer K2EDTA which contains di-potassium K2EDTA which blocks the coagulation cascade. All human donors passed required FDA screening and provided informed consent prior to blood collection. Collected blood was stored in a blood bank refrigerator (Jewett) at a temperature of 4^{o}C. Upon arrival, and prior to each use, blood hematocrit was measured using pocH-100i hematology

analyzer (Sysmex). A depiction of the RBC washing and crosslinking DEP workflow is shown in Fig. 2. For each DEP experiment, a 20μL volume of blood was removed from the vacutainer and suspended in 1X PBS. The cell suspension was then gently mixed by inverting the micro-centrifuge tube 15 times and rotating it gently for 30 seconds. The diluted whole blood suspension was then centrifuged at 2000 rcf for two minutes to pellet the RBCs and remove blood components. Then supernatant was discarded and replaced with 1X PBS and the sample was further washed for a total of three times. Cells were then either crosslinked in a dilute glutaraldehyde solution or used in DEP experiments. Prior any DEP experiment, cells were washed twice in the 0.8M AHA DEP buffer and centrifuged at 6000 rcf for two minutes to remove trace amounts of 1X PBS prior to dielectrophoretic characterization. The final washed RBCs were then immediately used in the DEP experiments.

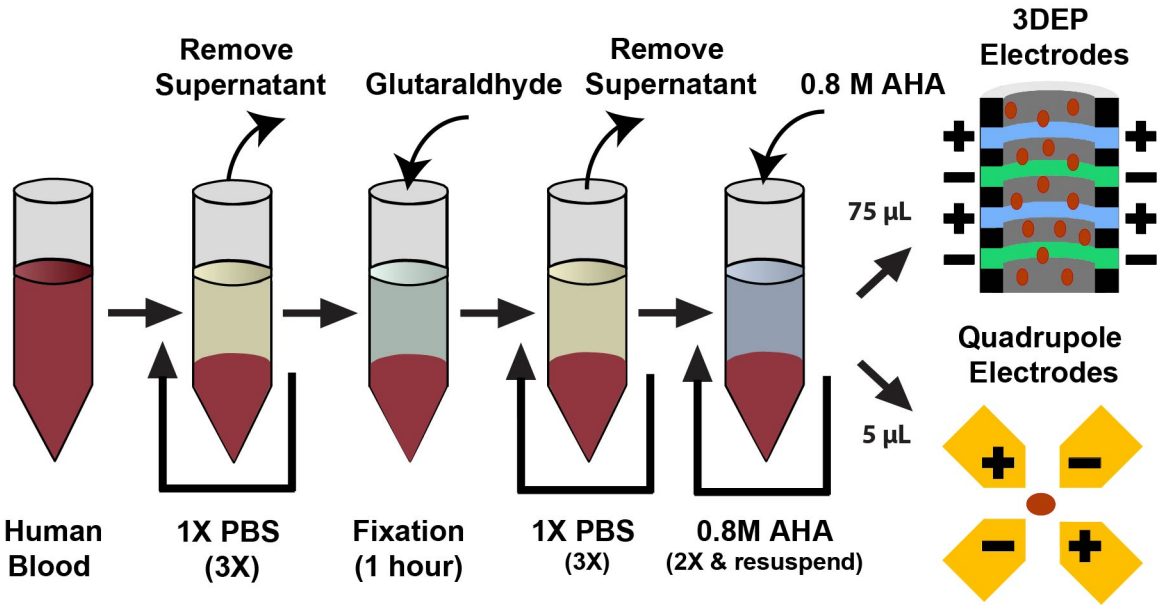


Figure 2. Human RBC glutaraldehyde crosslinking workflow. Whole human blood is washed three times in 1X PBS and subjected to chemical fixation for 1 hour. The cells are washed three times again in 1X PBS, washed twice in DEP buffer, and suspended in fresh DEP buffer for immediate DEP analysis.

3.7 Glutaraldehyde Crosslinking

Following the RBC wash in 1X PBS, cells were chemically crosslinked in glutaraldehyde in a similar manner as described previously [15,21]. As shown in the experimental workflow in Fig. 2, glutaraldehyde (EM Grade, 25%; Polysciences, Inc) was performed by first adjusting RBC concentration to 10^6 cells/ml in a 1X PBS solution. A 25 w/v% glutaraldehyde stock solution was used to produce varying RBC crosslinking concentrations (0, 0.05, 0.1, 0.5, 1.0, and 2.5 w/v %). Crosslinking reactions were performed atop a shaker (Standard Analog Shaker 3500, VWR) for 1 hour at 250 rpm. To quench the crosslinking reaction, samples were centrifuged at 2000 rcf for 2 minutes and the supernatant was replaced with 1X PBS. After three 1X PBS wash cycles, the samples were washed twice with 0.8M AHA DEP buffer and then suspended in fresh 0.8M AHA DEP buffer for immediate DEP analysis.

3.8 DEP Experiments

Blood aliquots were collected after fixed storage time points (1, 5, 10, 15 and 20 days) after the blood draw and crosslinked. DEP analysis with the 3DEP and 2D electrodes were performed immediately after PBS and AHA washing. For 3DEP experiments, a gel loading pipette tip was used to load 75 µL of a RBC suspension into a 3DEP 806 chip (DEPtech, Heathfield, UK). The top surface of the chip was then covered with a 20x20 mm glass coverslip and loaded into the 3DEP reader. The pin connections between the 3DEP reader and the DEP chip then delivered an AC potential to each of the 20 chip pinholes for 30 seconds. This experiment resulted in a point-wise dataset for a relative DEP force spectrum (Fig. 1C). To improve accuracy the experiment was repeated, for example N = 7 times, and the combined dataset was regressively fit to the single-shell polarization model and used to deduce the RBC

electrical properties (Fig. 1D). This process was then repeated across varying RBC storage time and glutaraldehyde crosslinking concentrations.

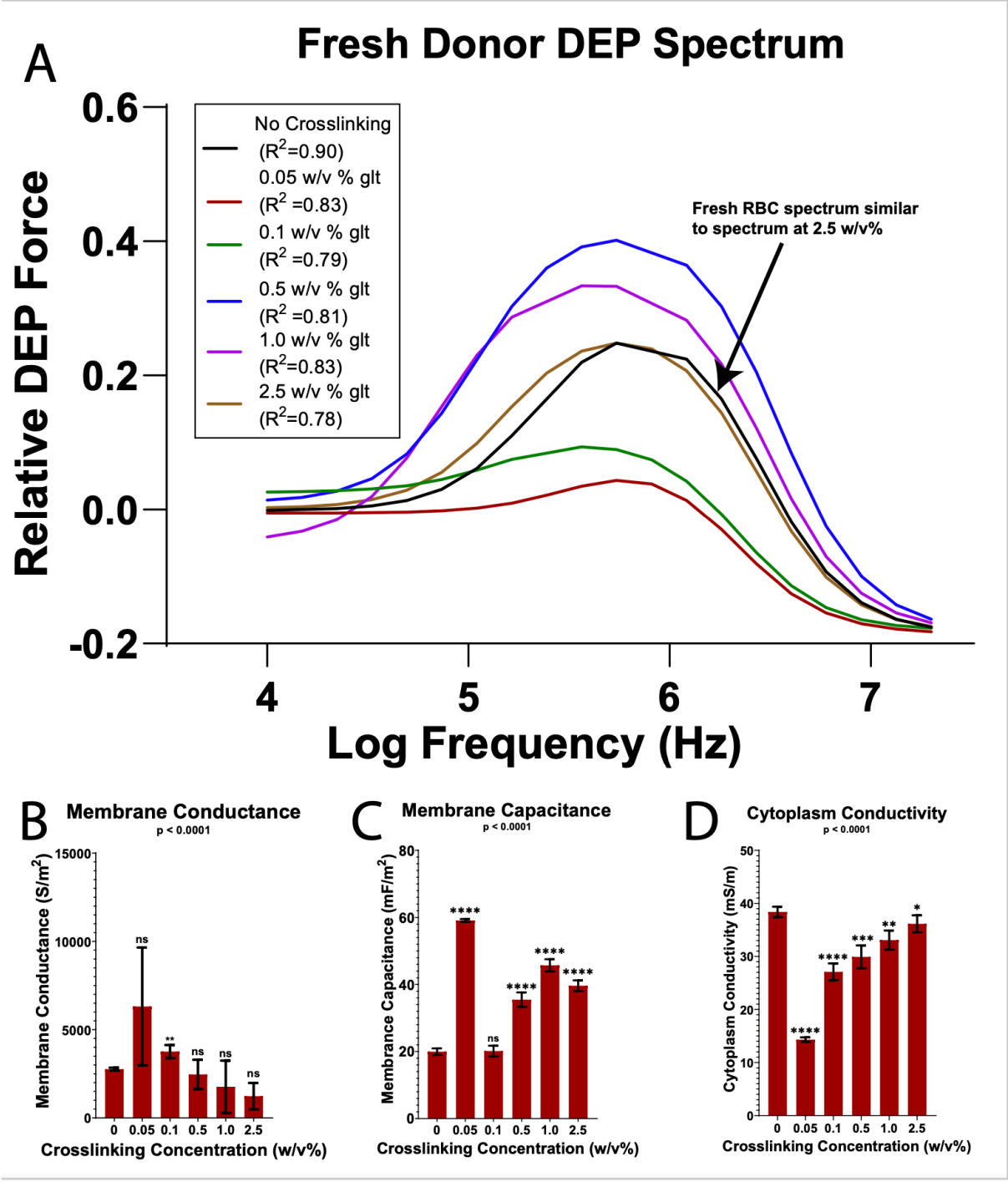


Figure 3. DEP Spectrum and electrical properties of fresh donor RBCs. (A) 3DEP model spectra of RBCs for varying glutaraldehyde concentrations, n=5 for crosslinked, and n=10 for no crosslinked RBCs (B) RBC membrane conductance, (C) RBC membrane capacitance, and (D) RBC cytoplasm conductivity (b-d represent mean ± SEM, n=5 for cross-linked, n=10 for no crosslinking, and p<0.0001 by one-way ANOVA and compared to w/v% control

using an unpaired t test with Welch's correction (**, ***,**, and * and correspond to p values < 0.0001, 0.001, 0.01, 0.05 and ns denotes p > 0.5).**

4 Results and discussion

4.1 3DEP Spectra of Fresh Donor Cells

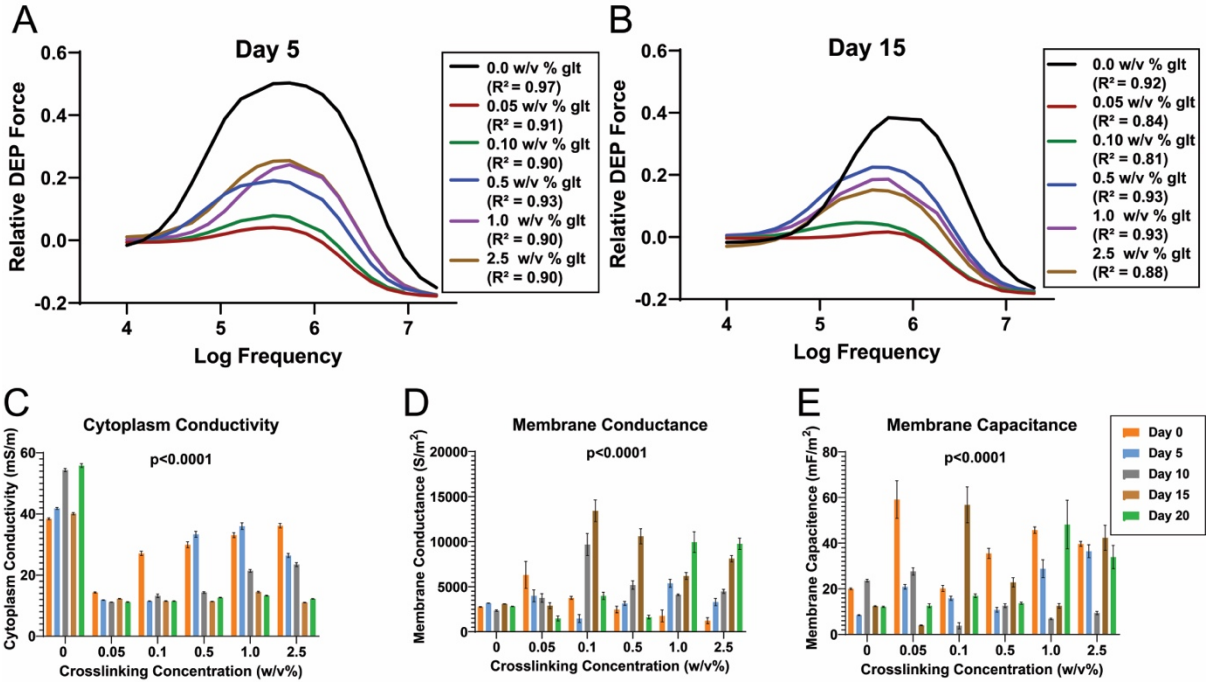
The 3DEP spectra of fresh single donor human RBCs was first measured at different glutaraldehyde crosslinking concentrations. Shown in Fig. 3A., the DEP spectrum of fresh donor cells is plotted for varying glutaraldehyde concentrations. For fresh RBCs, the DEP spectrum of non-crosslinked cells closely mimics the spectrum measured for fresh cells crosslinked at 2.5 w/v%, as indicated by the two overlapping spectra. Each fresh RBC spectrum then was used to calculate the cell membrane conductance (Fig. 3B), membrane capacitance (Fig. 3C), and cytoplasm conductivity (Fig. 3D). For each 3DEP spectrum fitting experiment, the mean value of the cell radius was measured using the sample population. Shown in Fig. 3B, the cell membrane conductance initially increased and then decreased with continued increases in glutaraldehyde concentrations, and finally plateaued above a glutaraldehyde concentration greater than 0.5 w/v%. We observe an inverse trend with membrane capacitance above concentrations of 0.05 w/v%, as capacitance steadily increased with glutaraldehyde concentration from 19 mF/m^2 to 41 mF/m^2 between 0.1 w/v% and 2.5 w/v% and was observed to stabilize at concentrations above 0.5 w/v% (Fig. 3C). Finally, we observed an initial drop in cytoplasm conductivity at a low 0.05 w/v% crosslinking concentration followed by a steady increase with increasing crosslinking concentrations (Fig. 3D). As observed from the measured spectra, the use of the high permittivity DEP buffer enabled both the low and high frequency COFs and a significant portion of the high frequency DEP spectrum to be measured within the 45 MHz bandwidth of the 3DEP. These experiments demonstrate that a glutaraldehyde

crosslinking concentration of 2.5 w/v% is likely to fully crosslink the RBC, as indicated by the observation that cell membrane electrical properties stabilize and plateau with crosslinking concentrations between 1.0 and 2.5 w/v%. These results also suggest these higher concentrations serves to prevent membrane ion leakage during DEP analysis as indicated by the cytoplasm conductivity at 2.5 w/v% approaching to within 5% of the original non-crosslinked RBC values. It is therefore anticipated that a crosslinking concentration of 2.5 w/v% provides the most optimum crosslinking concentration for our DEP analysis.

4.2 DEP Analysis of Stored RBCs

RBCs were stored and subsequently washed and crosslinked according to the workflow presented in Fig. 2. The DEP spectrum was measured after 5, 10, 15 and 20 days of refrigeration. Select spectra obtained for storage times of 5 and 15 days across five glutaraldehyde concentrations are plotted in Fig. 4A and 4B, respectively, to illustrate the 3DEP data collection workflow. As shown, the spectra are dependent on both the storage time and the glutaraldehyde concentration. In order to validate the 3DEP method with that of the DEP quadrupole method that was used in our previous bovine RBC work, we measured the high frequency COF for both fresh and stored RBCs at storage time points of 5, 10 and 15 days for all crosslinking concentrations. DEP motion within the quadrupole was observed under brightfield microscopy. The AC field frequency for when the first RBC “crossed over” from the quadrupole high field region to the low field region, and later the frequency where the last cell crossed over, was optically determined and measured. The average of these two values was plotted as the high frequency COF and compared with the COF measured by the 3DEP. As shown in Fig. 5 for fresh RBCs (day 0), and cells stored for 5 and 15 days,

412 the 3DEP COFs agree well with the values obtained by the 2D quadrupole across all
413 measured glutaraldehyde concentrations. As such, we believe the 3DEP instrument is
414 a more complete DEP measurement method when compared to point-wise COF
415 measurements obtained with 2D electrode arrays.



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417 **Figure 4. 3DEP spectra and electrical properties of stored human RBCs. (A)**
418 **3DEP spectrum for RBCs stored 5 days. (B) 3DEP spectrum for RBCs stored 15**
419 **days. (C) RBC cytoplasm conductivity, (D) RBC membrane conductance and (E)**
420 **RBC membrane capacitance showed statistical significance from a ordinary**
421 **two-way ANOVA analysis with p < 0.0001 for both the row and column**
422 **interactions; average +/- SEM, and n=10 for 0 w/v% otherwise n=5.**
423

424 The 3DEP was then utilized to deduce the RBC cytoplasm conductivity,
425 membrane conductance and membrane capacitance across the varying
426 glutaraldehyde concentrations and storage times. As shown in Fig. 4C-D, and as
427 observed in previous work with bovine RBCs, no discernable different in RBC electrical
428 properties was observed with human RBC storage age at a 0 w/v% glutaraldehyde
429 concentration. However, the discernment between storage-induced RBC cytoplasm
430 conductivity and membrane conductance increased with glutaraldehyde concentration

(Fig. 4C-D). As observed, the optimum crosslinking concentration for human RBC DEP resolution occurs at a glutaraldehyde concentration between 1.0 and 2.5 w/v% and is very similar to what was observed in previous crosslinking DEP experiments with bovine RBCs [15,21].

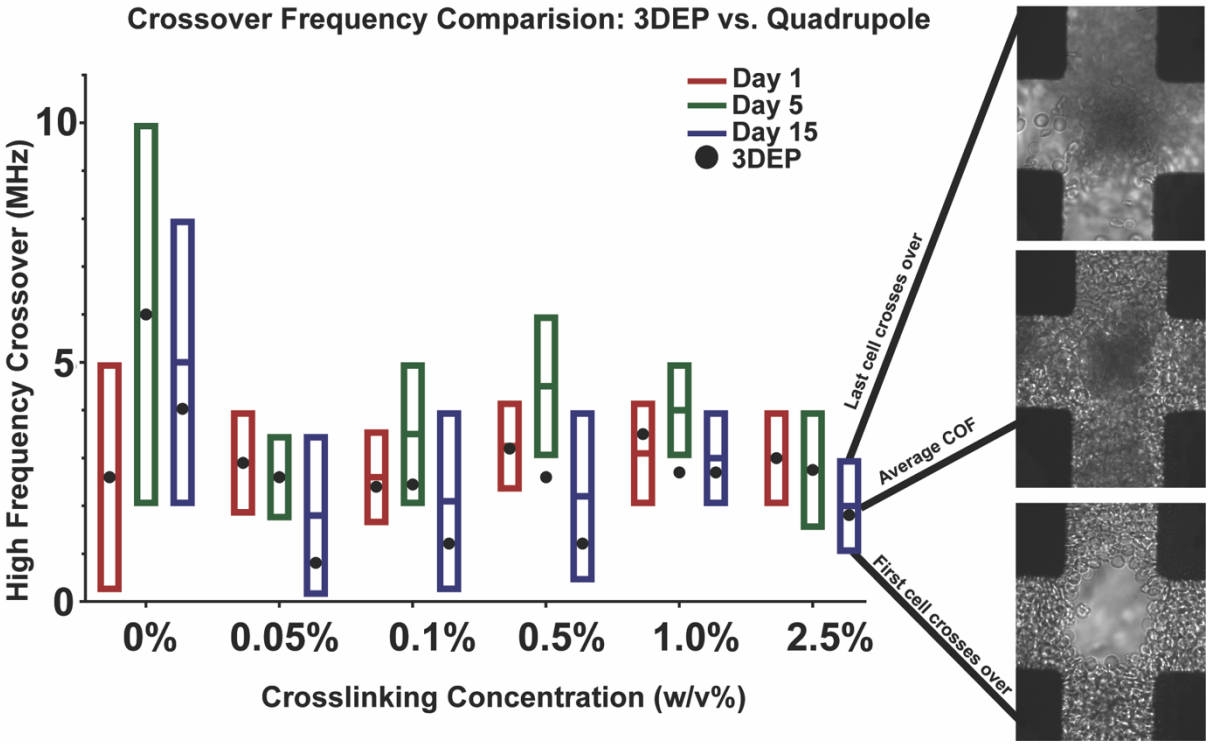

Figure 5. Human RBC Crossover comparison between 3DEP spectrums and 2D quadrupole array for RBCs at day 1, 5 and 15 of storage.

The RBC cytoplasm conductivity and membrane conductance exhibited observable trends with increasing storage time at a glutaraldehyde concentration of 2.5 w/v% (Fig. 4C-E). Here, the cytoplasm conductivity was observed to steadily decrease from an initial value of 0.036 S/m to 0.026, 0.023, 0.011, and 0.012 S/m with increasing storage times of 5, 10, 15 and 20 days, respectively. Conversely, the membrane conductance was observed to increase with increasing storage time, increasing from an initial value of 500 S/m^2 to 3000, 5000, 10,000, 12,000 S/m^2, at storage times of 5, 10, 15, and 20 days, respectively. Interestingly, the membrane

conductance appears to have an inverse relationship with the cytoplasm conductivity, as shown in Fig 6. While this trend presented is from a single human donor, this inverse relationship between cytoplasm conductivity and membrane conductance was been consistently observed with 9 total blood donors that were studied using this DEP method. However, a more detailed investigation into the DEP variability in electrical properties and COF across the human donors will be the subject of a future study. The inverse correlation between decreasing conductivity and increasing conductance has also been observed in other DEP work. In Henslee et al. for example, the authors showed an anti-phase relationship between these two electrical parameters [5]. We speculate here that this inverse trend is driven by the single-shelled relationship between the cell cytoplasm conductivity and the surrounding cell membrane. In the absence of cell hemolysis, a main driver for ion transport and a decrease in RBC cytoplasm conductivity during refrigerated storage is diffusive ion flux from the cytoplasm and across the cell membrane through the physical migration of ions across this domain. Ionic concentration within the cell membrane would therefore increase during this process and subsequently lead to an increase in membrane conductance. Further storage experiments using both dynamic DEP measurements and inductively coupled plasma mass spectrometry (ICP-MS) are needed to better understand the relationship between RBC DEP and ionic composition overtime and will be the subject of a future study. While a clear trend was observed between cytoplasm conductivity and membrane conductance with increasing storage time, no storage time dependence was observed with membrane capacitance for any experimental glutaraldehyde crosslinking concentration. It is worth noting that our 3DEP measured cytoplasm conductivity values (~10 – 40 mS/cm) are lower than values reported elsewhere (~100 mS) for human RBCs [5]. While our results demonstrate that

glutaraldehyde is capable of minimizing dynamic ion leakage across RBCs, the cell handling conditions may also contribute to the electrical state of the RBCs. These conditions were held constant when RBCs were washed and crosslinked for this work, however, an understanding of how varying centrifuge conditions and incubation temperatures ultimately influence RBC electrical properties is likely warranted to better understand this observed electrical. Previous reported values, for example, were acquired after RBCs had been incubated at 35 – 37 ^{o}C for 48 h [5], while this DEP work maintained cell samples at a continuous refrigerated temperature of 4 ^{o}C. We therefore believe that cell handling and incubation conditions can strongly influence the RBCs electrical state and can give rise to measured differences such as what is observed here.

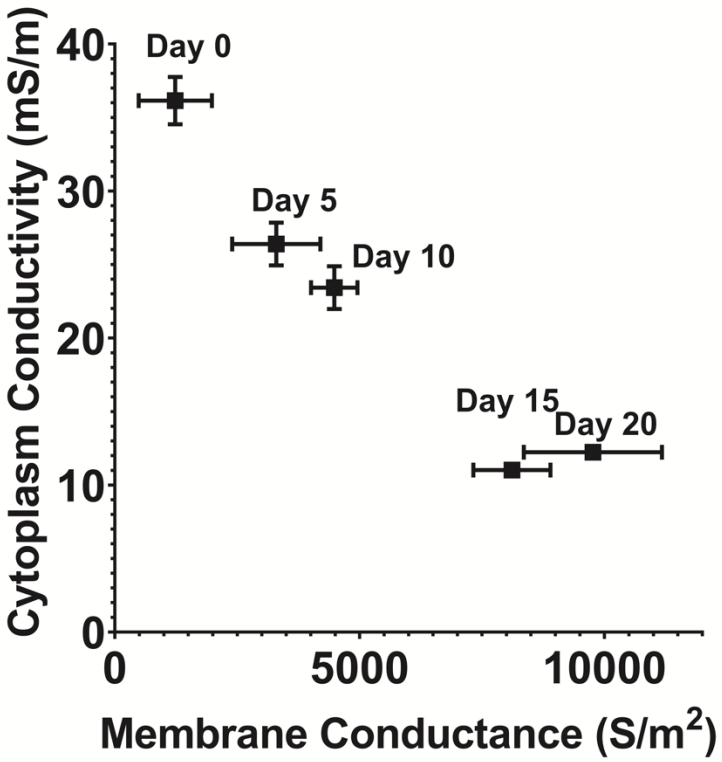

Figure 6. RBC cytoplasm conductivity decreases with increasing membrane conductance with increasing storage time.

The 3DEP was able to resolve the electrical property changes in single-donor human RBCs with increasing storage time using a chemical crosslinking method. The cell crosslinking reaction served to prevent ionic leakage across the cell membrane during DEP analysis and therefore the cellular ionic composition more accurately represented that which existed in the original storage buffer prior to crosslinking. It is well known that RBC ion channels are sensitive to changes in temperature and human red blood cells leak potassium ions during storage [12]. Ion leakage such as this would likely lead to a reduction in the cytoplasm conductivity and an increase in ionic species within the cell membrane. If, however, glutaraldehyde was not utilized in this work, the ion depleted cytoplasm and ion accumulated membrane would dynamically respond to a resuspension in the low conductivity DEP buffer and dynamically reach a new ionic equilibrium with their surroundings. Such communication with the surrounding cell buffer would ultimately lead to an internal cytoplasm conductivity that is not representative of the leakage that occurred during the storage process.

Glutaraldehyde is a well understood cell fixation agent which crosslinks the aminated protein groups of the RBC [24]. These results suggest that the polymerization of the cell membrane significantly reduces the rate of ion leakage across the cell membrane during DEP analysis if a large enough crosslinking concentration is utilized. In this work, DEP resolution occurred at crosslinking concentrations above 1.0 w/v%. Below this concentration a trend emerged dynamically and became discernable in a manner that was dependent of the age of the RBC sample. In particular, the initial observable trend for the cytoplasm conductivity was strongly influenced by RBC storage time. No age-related trend was observable in RBCs without crosslinking, but a measurable difference in cytoplasm conductivity was first observed with the youngest RBC populations. Shown in Fig. 4C,

a crosslinking concentration of 0 and 0.05 w/v% showed no observable change in conductivity with storage time. However, at a concentration of 0.1 w/v% a difference in cytoplasm conductivity was observable for a single sample: fresh day 1 cells. Interestingly, the sample storage age in which a measurable difference was first observed increased with increasing crosslinking concentration. At a concentration of 0.5 w/v%, for example, day 0 and day 5 RBCs exhibited measurable differences in cytoplasm conductivity, while the remaining cell sample storage times showed no discernable electrical differences at this crosslinking concentration. Further increases in crosslinking concentration (>1.0 w/v%) then produced observable differences in the cytoplasm conductivity across the entire experimental time course. Given that the RBCs shed membrane proteins during storage, fewer available crosslinking sites are available as storage age increases. We observe here that a smaller crosslinking concentration is required to influence ionic leakage in “fresh” cells than what is required for cells stored at the longer 5-, 10- and 20-day timepoints. This interplay between storage age and minimum crosslinking concentration for preventing cell leakage is therefore likely due to the age-dependent availability of RBC aminated crosslinking sites. While clear statistically significant differences in RBC cytoplasm conductivity and membrane conductance are observable over a refrigeration period of 20 days at crosslinking concentrations > 1.0 w/v%, no such difference was detected in the membrane capacitance over this period for any concentration. As such, it is likely refrigerated RBC storage over a period of 20 days did not significantly alter the membrane permittivity, thickness or cell surface area to the extent to which a change in membrane capacitance could be detected by the 3DEP.

5 Concluding remarks

In this work, we have presented a glutaraldehyde chemical crosslinking method for the detection of human RBC electrical properties by DEP. Single donor type O- human RBCs were stored in a blood bank refrigerator over a period of 20 days. Blood samples were withdrawn from refrigerated storage at varying time points 0, 5, 10, 15, and 20 days and chemically crosslinked in varying concentrations of glutaraldehyde ranging from 0 w/v% - 2.5 w/v%. Crosslinked cells were then suspended in a high permittivity 0.8M AHA buffer in order to increase the buffer permittivity and subsequently reduce the frequency bandwidth required to obtain a full DEP spectrum. Using this chemical crosslinking high permittivity buffer system, we performed human RBC DEP analysis using a commercial 3DEP instrument. The high frequency COF obtained using the 3DEP method was compared and shown to agree well with the COF values obtained using a conventional 2D quadrupolar electrode array. DEP spectra from 3DEP experiments were measured and fitted using nonlinear regression to a spherical single-shell polarization model in order to determine the RBC cytoplasm conductivity, membrane conductance and membrane capacitance over a period of 20 days of refrigerated storage. We show that at chemical crosslinking concentrations above 1.0 w/v%, 3DEP analysis yielded discernable differences in cell electrical properties with increasing storage time. In particular, the RBC cytoplasm conductivity was observed to increase while the membrane conductance decreased with refrigerated storage time. No trend was observed in RBC membrane capacitance with increasing storage time. Further, no RBC electrical differences were resolvable by DEP without glutaraldehyde crosslinking. The chemical crosslinking approach serves to eliminate ionic diffusion across the cell membrane when cells are resuspended in DEP buffer for analysis. Therefore, this chemical crosslinking method enables the

discernment of electrical property changes during storage and prevents cells from reestablishing ionic equilibrium with the low conductivity DEP buffer. This assay can successfully detect differences in blood storage, or starvation age, which is currently challenging to perform using patch clamp or single cell electrophysiology techniques. Interestingly, an inverse relationship between cytoplasm conductivity and membrane conductance is observed. This work offers a robust method for characterizing the quality of blood electrically and has applications in blood transfusion storage logistics and for detecting age related electrical property changes in blood samples from endurance athletes which have undergone illegal autologous blood transfusions. Future efforts will focus on better understanding the species involved with ionic leakage and understanding how these measurements vary across human donors.

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Conflict of interest

The authors declare no financial interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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