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Article

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Electrophysiology as a Real-time Biomarker for Human Red Blood Cell Monitoring in Extracorporeal Membrane Oxygenation

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ABSTRACT

Blood monitoring in extracorporeal membrane oxygenation (ECMO) is critical to managing the precarious balance between bleeding and thrombosis in ECMO support. Conventional monitoring including plasma free hemoglobin and activated clotting time are limited to once-daily measurements. Considering this limitation, we investigated surface zeta (ζ) potential, as a real-time biomarker for monitoring RBCs in ECMO. RBC ζ-potential is a negative electrical charge on the cell surface, providing repulsive force to prevent RBC aggregation in circulation. Human RBCs from 7, 14, 21, and 49 days stored blood were circulated through an ECMO circuit for 12 hours. Every 2 hours, ζ-potential, RBC concentration, intracellular Ca^{2+}, and sialic acid were measured.

The ζ-potential of RBCs decreased with blood circulation after only 8 hours of circulation. The decrease in ζ-potential was further amplified by blood storage from just 21 days. Ca^{2+} and sialic acid were also shown to increase with flow and storage duration. This suggests prolonged blood storage compounds the risk of RBC aggregation. Our results demonstrate ζ-potential can detect changes in RBCs in ECMO circuits *ex vivo*. The high temporal sensitivity of ζ-potential offers real-time detection of changes in RBC physiology to better predict cell aggregation and clotting risk, improving treatment outcomes for patients.

Keywords: surface zeta potential, red blood cell, ECMO, electrokinetics, blood storage

INTRODUCTION

Extracorporeal membrane oxygenation (ECMO) is a type of cardiopulmonary bypass system that provides temporary artificial heart and lung functionality to critically ill patients, also serving as a bridge to heart and lung transplantation[1]. ECMO systems consist of either a roller pump (typically used primarily for pediatric/neonatal patients) or centrifugal pump (used in both pediatric and adult patients) and an artificial lung (membrane oxygenator) which facilitates gas exchange and reoxygenation of the blood. These components are connected via heparin-coated tubing, forming a complete circuit. Unlike traditional cardiopulmonary bypass systems which are used on a short-term basis (up to 6-8 hours) during cardiac surgical procedures, ECMO systems frequently provide support for at least 12 hours or even days to weeks. Due to prolonged blood circulation, complications of bleeding and red blood cell (RBC) aggregation leading to thrombosis remain significant clinical challenges for ECMO patients[2,3]. In ECMO circuits, inflammatory and coagulation responses are exacerbated due to the increased interaction of cells with foreign surfaces and materials, resulting in blood clot formation.

In addition to these responses, other mechanisms of cell damage occur in ECMO circulation. One *in vivo* study showed a 60% loss of RBCs in ECMO systems due to bleeding and pump-induced hemolysis[4]. Other *ex vivo* studies have investigated the effects of flow rate for detecting thrombus formation, drug sequestration and pharmacokinetics, as well as differing pump and membrane oxygenator device models to inform ECMO circuit configuration and clinical treatment planning[5–8]. Over the past decade, blood storage and RBC aging have gained considerable attention due to the reported >96% of patients who require blood transfusions during in-hospital ECMO support[9]. Prolonged blood storage causes storage lesions which deleteriously affect cellular function and RBC membrane integrity, including cleavage of sialic acid and decreased cell membrane elasticity and deformability[10,11].

To our knowledge, no *ex vivo* studies to date have focused exclusively on monitoring RBCs in ECMO circuits despite the critical role of RBCs for oxygen delivery. Clinical laboratory techniques for monitoring RBCs during *in vivo* clinical ECMO support, which include plasma free hemoglobin (PfHb), a measure of cell damage and hemolysis, and red cell distribution width (RDW) for monitoring systemic inflammatory response, are limited to once-daily measurement to limit blood loss from frequent phlebotomy[12]. These measurements vary widely across clinical centers, yielding inconsistent results[13,14]. Anticoagulants, while often a first-line therapy for managing thrombosis in ECMO circuits, elevate the potential risk for cannula site bleeding and intracranial hemorrhage due to systemic anticoagulation effects[15]. Thus, a significant clinical need exists to monitor RBCs in ECMO systems, providing greater temporal measurement resolution and sensitivity, to better predict blood clot formation, blood transfusion and blood volume management in ECMO patients. Additionally, information on how prolonged storage may impact RBCs in ECMO will provide further insights into this management. We have previously demonstrated surface zeta potential as a means to provide high temporal resolution and measurement sensitivity for detecting changes in RBC electrophysiology under both normal cellular processes and for monitoring disease[16–18]. Surface zeta potential is an electrochemical property of cells in suspension and offers the ability to directly measure the RBC's negative surface charge and repulsive forces[19,20]. It has been shown to correspond with other biophysical RBC properties, including deformability and membrane potential[21].

Therefore, the present study sought to investigate the applicability of RBC surface zeta potential as a real-time biomarker for human RBCs. This is the first study to our knowledge focused on *ex vivo* monitoring of isolated RBCs in an ECMO circuit. Here, we evaluate the effects of blood circulation time, blood storage duration, and shear stress on the surface zeta potential response of RBCs circulating through a benchtop ECMO circuit.

METHODS

Human Subjects

Studies were conducted in accordance with 45 CFR 46 and the principles of the Declaration of Helsinki, with approval from the Institutional Review Board (Wake Forest University, Winston-Salem, NC). Participants in the study were screened for relevant self-reported health issues. All participants provided written, informed consent after having received a detailed explanation of the study procedures.

Blood Procurement and Preparation:

Units (~450 mL) of fresh human whole blood (type A and age matched, <55 years old) were drawn by venipuncture into blood collection bags containing sodium heparin anticoagulant and shipped overnight from a commercial blood bank (Zen-Bio, Inc., Durham, NC). Upon receipt, whole blood was stored at 4°C in refrigeration for up to 49 days. Following 7 days of blood storage, ~30 mL of whole blood was aliquoted, and the remaining whole blood was placed back in refrigeration for use in subsequent blood storage experiments (i.e., at 14, 21, 30, and 49 days post-draw). For experiments, RBCs were isolated from the whole blood by centrifugation, washing twice with room temperature normal saline (0.9% NaCl). Following isolation, packed RBCs were resuspended in 100 mL saline at 10% hematocrit in blood reservoir bags.

Benchtop ECMO Circuits:

Benchtop ECMO circuits (**Figure 1**) were constructed, each consisting of a roller pump (COBE Cardiovascular, Inc., Arvada, CO) and 100-mL blood bag connected by heparin-coated silicone tubing (ID: 1/4 in, OD: 3/8 in) and a volume-equivalent, 3D-printed reservoir to mimic the oxygenator, referred to as the Pump-only group. An Affinity Pixie™ Pediatric hollow-fiber membrane oxygenator (Medtronic Plc, Minneapolis, MN) was added to one circuit, referred to as the Pump+PIXIE group, to investigate the compounding effects of membrane oxygenators on the biophysical response of human RBCs. On the oxygenator circuits, pre-and post-oxygenator pressure monitoring was carried out using Model 6069 DELTRAN® disposable pressure transducers (Utah Medical, Inc, Salt Lake City, UT) connected to a two-channel PendoTech Pressure MAT data acquisition system (Mettler-Toledo International Inc, Columbus, OH). Flow rate was monitored in real-time using the digital display on the roller pump's front control panel. Before starting each experiment, the circuits were primed with normal saline to remove any air bubbles from the tubing and oxygenator[22,23].

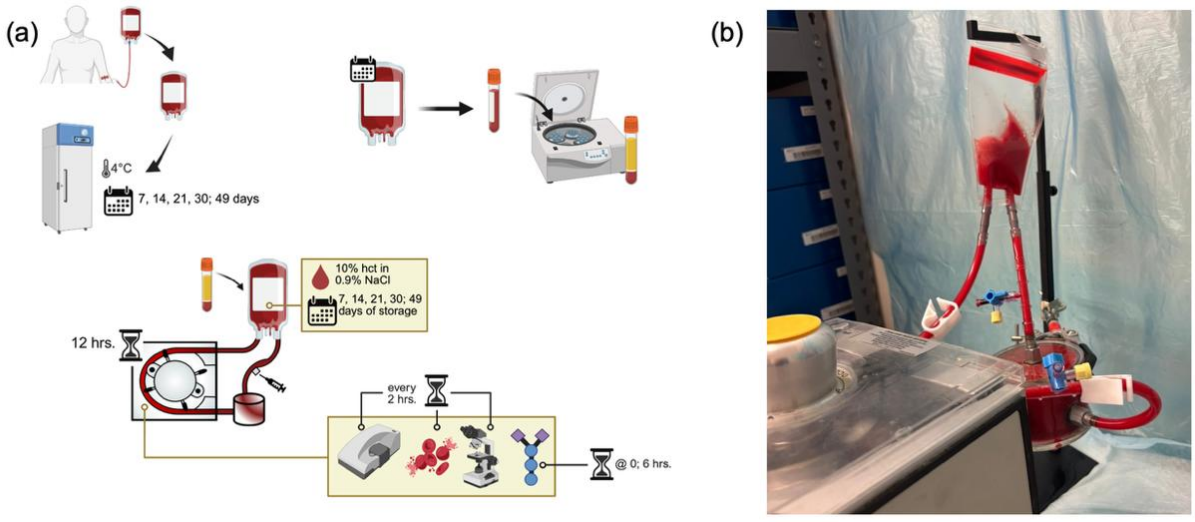

Figure 1: Workflow and set up for benchtop ECMO experiments. (a) Experiments were repeated on Day 7, 14, 21, 30, and 49 of blood storage for donors with blood type A, age matched (<55 y.o.). Surface zeta potential, percent hemolysis, and cell concentration and population % analysis were immediately performed for the "static" and "flow-exposed" samples every 2 hours, whereas sialic acid analysis was performed every 6 hours. (b). Benchtop ECMO circuit with volume-equivalent chamber to mimic the oxygenator (Pump-only group).

Washed RBCs were circulated through both ECMO circuits for 12 hours at a flow rate of 2 ± 0.2 (s.d.) L/min. A parallel "static" sample of 10% hematocrit was left sitting at room temperature in an Eppendorf tube. Every ~2 hours, 2 mL RBC suspension samples (1 mL of initial "waste" followed by a 1 mL sample for analysis) were drawn from each circuit. The analysis sample was pelleted by centrifugation and held at room temperature throughout the duration of experiments. Surface zeta potential, percent hemolysis, cell concentration and population percentage analysis was immediately performed for both the static and flow-exposed samples. Circuits were operated at room temperature (22 ± 2 °C). Oxygenator pressure drop and the pump flow rate were monitored and remained constant.

Measurement of Surface Zeta Potential in RBCs:

RBCs were pelleted by centrifugation and resuspended in an iso-osmotic, low conductivity (~430 μS/cm) buffer[24] at a final RBC concentration of ~2 x 10^6 cells/mL. All surface zeta potential measurements were made at 25 °C. Surface zeta potential response was carried out using a Zetasizer Pro Red Label instrument (Malvern Analytical, UK) which operates based on electrophoretic light scattering (ELS) technique[32,33]. ELS uses light scattering to quantify the movement of charged particles under an applied electric field (known as the electrophoretic mobility). Disposable cuvettes containing platinum-gold electrodes were loaded and an electric field was applied to induce cell movement and scattered light. The software converts the frequency of scattered laser light to electrophoretic mobility which is used to calculate surface zeta potential (ζ) using the using the Helmholtz-Smoluchowski expression[25], Equation (1):

$$\zeta = \frac{4\pi\eta}{\varepsilon} \cdot \mu_p f(\kappa\alpha) \quad (1)$$

where η and ε are the viscosity and dielectric constant of the suspending media respectively; f(κα) is the Henry Function (approximated at 1.5 for RBCs), which relates particle radius to electric double-layer thickness, and μ_p is the electrophoretic mobility derived from particle velocity and electric field strength.

Measurement of RBC Hemolysis:

To assess RBC damage in ECMO circuits, the percentage of hemolysis was quantified every 6 hours for both flow-exposed and static samples. For hemolysis measurements, following centrifugation, the supernatants of RBC samples were collected and resuspended in a 96-well plate at 10x dilution in normal saline (0.9% NaCl) at a volume of 200 μL per well. Percent hemolysis was determined at an optical density of 541 nm using a microplate reader (SynergyH1, BioTek Agilent Technologies, Durham, NC) calculated by Equation (2):

$$Hemoslysis = 100\left(\frac{A_s - A_b}{A_p - A_b}\right) \quad (2)$$

where A_S, A_p, and A_b are the absorbances of the supernatant, a 100% hemolysis reference sample, and the pure buffer solution, respectively[3].

Measurement of Sialic Acid Content:

Unbound, "free" sialic acid (SA_{free}) concentrations of static RBCs (measured at 0 and 6 hours) and flow-exposed RBCs (measured at 6 hours) was determined by the Warren Method using a commercial assay kit (QuantiChrom™ Sialic Acid, BioAssay Systems Inc., Hayward, CA) according to manufacturer protocols. SA_{free} was determined from the supernatant (~40 μL) of each RBC sample.

Measurement of Intracellular Calcium:

Packed RBCs were resuspended in Phosphate Buffered Saline (PBS) in 1 mL total volume at a final concentration of ~2 x 10^6 cells/mL. Fluo-4 AM (MedChem Express, Monmouth Junction, NJ) , a calcium-sensitive fluorescent dye, was added from stock at a final concentration of 1 μM and samples were incubated in the dark for 1 hour at 37°C. Following incubation, fluorescently labeled cells were pipetted into a black 96-well microplate. Intracellular calcium (Ca^{2+}) was determined by measuring fluorescent intensity at emission/excitation wavelengths of 485 nm/526 nm using a microplate reader (SynergyH1, BioTek Agilent Technologies, Durham, NC).

Measurement of Cell Concentration:

RBC concentration was determined from hemocytometer images at x20 magnification brightfield microscopy (ECHO Technologies, San Diego, CA). For each hemocytometer image, for each sample group (e.g., static and flow exposed RBCs in each ECMO circuit) cells on 0.2 mm x 0.2 mm grids were tagged green for "healthy" (exhibiting a normal concave RBC shape) or tagged red for echinocytes (e.g., cells with spikes formed on membrane surface), **Supplemental Figure 1**. The cell concentration was determined as the total number of cells within a 4 x 4 grid. The population % was determined as the ratio of each sub-population to the total cell concentration. ImageJ software (National Institute of Health) was used to perform all cell counting measurements.

Microfluidic Shear Exposure Set-Up:

To induce the appropriate shear stress duration and magnitude, a linear Poiseuille shearing system was constructed comprised of a 38 mm segment of 400 μm I.D. polyethylene tubing and NE-1000 syringe pump (New Era Pump Systems, Inc). The pump was programmed for a 60 mL syringe (barrel ID: 28.90 mm) at a total infuse/withdraw volume of 121.3 μL, yielding a shear duration of 0.24 seconds per pass through the tubing. Infuse and withdrawal cycles were alternated for 25 cycles for a total shear duration of 6 s. Pump flow rate was set to 13.73 mL/min for the 61.25 Pa low exposure group and 33.45 mL/min for the 123 Pa high exposure group.

Oxidative Stress Change Exposure Set Up:

Blood suspensions were prepared by diluting stock 3% hydrogen peroxide (H_2O_2) in HBSS to obtain final treatment concentrations of 0.5, 1, 5, and 10mM, in a total working volume of 1mL at 10% hematocrit. To inhibit catalase, 40 μL of 100mM sodium azide was added to each sample prior to introducing H_2O_2 treatment. All samples were incubated in light-sensitive Eppendorf tubes (Eppendorf North America, Inc, Framingham, MA) in the dark at 37 °C for 2 hours. Following incubation, samples were washed twice by centrifugation at 700 x g for 5 minutes, and resuspended in our low conductive iso-osmotic media prior to surface zeta potential measurements.

Flow Cytometry Preparation:

RBC samples were tagged with DCFDA fluorophore (Abcam, Cambridge, UK) and incubated for 30 minutes at 37 °C according to manufacturer's protocols. 30,000 RBCs were analyzed using the BD Fortessa X20 flow cytometer system (BD Biosciences, San Jose, CA). The flow cytometry data was gated Forward Scatter Area (FSC-A) versus Forward Scatter Height (FSC-H) to isolate RBC populations and eliminate signal artifact.

RESULTS AND DISCUSSION

Surface Zeta Potential of RBCs in an ECMO Circuit:

In clinical practice, ECMO circuits are operated for 12 hours or longer and use whole blood stored for up to 42 days. We designed our experiments to mimic these conditions, however we isolated RBCs from whole blood, and operated ECMO circuits at 10% hematocrit. This allowed use of a single donor across the entire storage duration (removing donor to donor variability over storage times). Surface zeta potential measurements were performed for the "static" and "flow-exposed" samples every 2 hours. **Figure 2** shows the effects of blood storage duration and blood circulation on the surface zeta potential response of RBCs. We saw a significant decrease in surface zeta potential with blood storage duration (**Figure 2a**). We also saw a significant change in surface zeta potential with blood circulation time further compounded by blood storage duration (**Figure 2b-f**). At Storage Day 14 we saw a significant change in surface zeta potential due to flow effects after 10 hours. At Storage Days 21 and 30, surface zeta potential changes occur after 8 hours, and at just 4 hours in the oldest blood (Storage Day 49). These observations suggest that changes in the RBC physiology, that impact their ability to repel one another, are occurring within standard ECMO protocol timescales (circuit flow time and storage duration). Surface zeta potential may be an early indicator of these changes, which has clinical implications for early prediction of cell aggregation and clot formation in ECMO circuits.

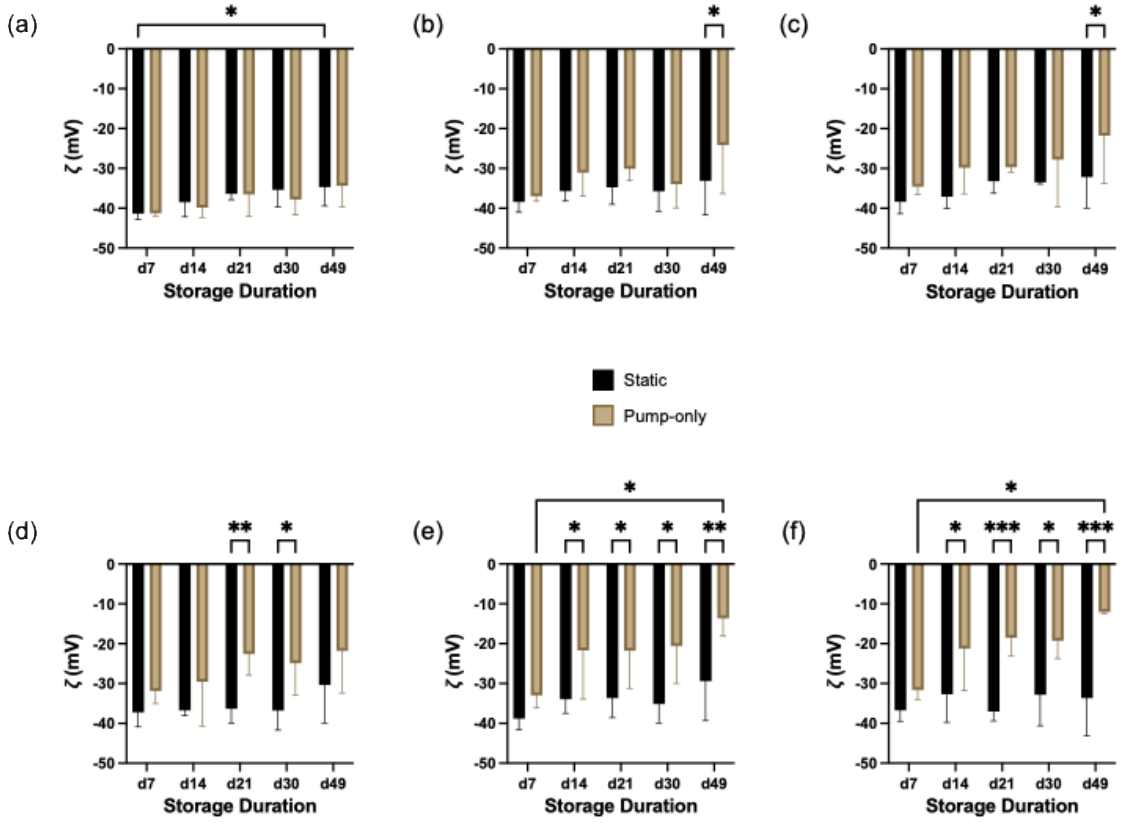

Figure 2: Changes in RBC surface zeta potential over increasing blood storage duration at (a). 2 hours, (b). 4 hours, (c). 6 hours, (d). 8 hours, (e). 10 hours, and (f). 12 hours of ECMO blood circulation, comparing flow and static samples. Data presented represents the mean ± (s.d.) for all donors (static: n=7; flow: n=3). Statistical comparisons between static and flow-exposed samples were made via Two-way ANOVA using Tukey's Multiple Comparison test; *p<0.05, **p<0.01. All values with P<0.05 were considered statistically significant.

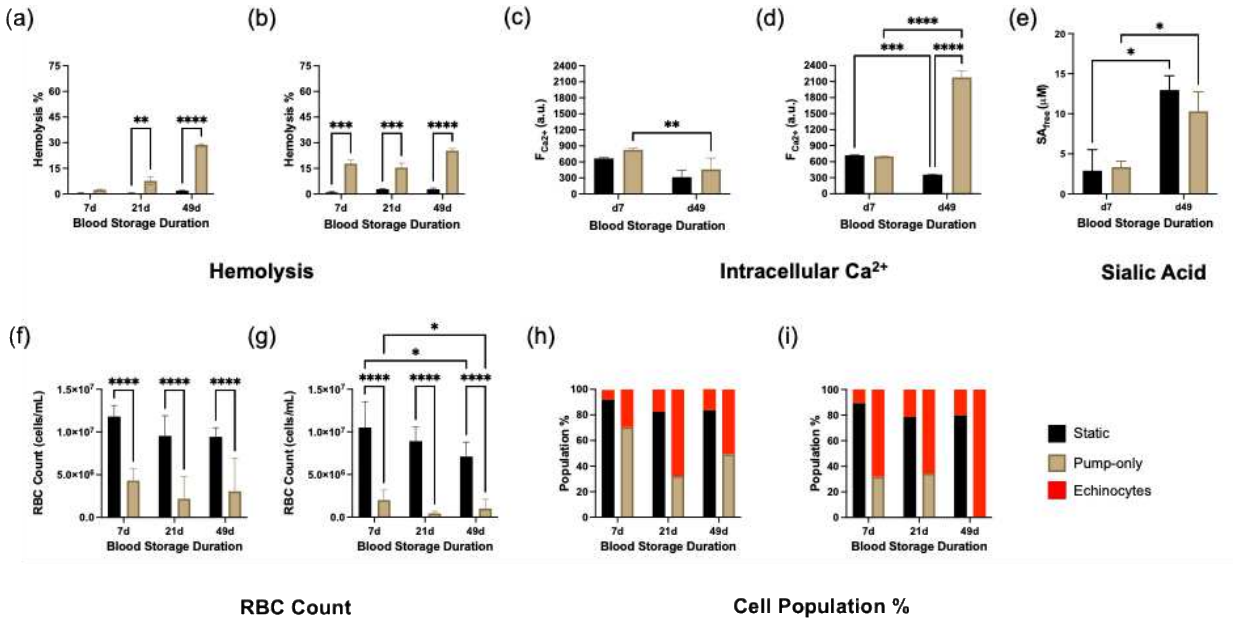

Figure 3: Change in RBC Hemolysis over increasing blood storage duration from 7 to 49 days following (a). 6 hours and (b). 12 hours of ECMO blood circulation. Change in Intracellular Ca^{2+} over increasing blood storage duration following (c). 6 hours and (d). 12 hours. (e). Change in free sialic acid (SA_{free}) concentration following 6 hours of ECMO blood circulation in Pump-only circuit. Change in cell concentration (f, g) and population % (h, i) over increasing blood storage duration following 6 (f, h) and 12 (g, i) hours of ECMO blood circulation. Data presented for all panels represents the mean ± (s.d.) for a single (n=1) donor. Statistical comparisons between static and flow-exposed Pump-only samples were made via Two- ANOVA using Tukey's Multiple Comparison test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. All values with p<0.05 were considered statistically significant.

Hemolysis in flow-exposed RBCs increased compared to the static samples after 6 hours (**Figure 3a**). Following 12 hours of blood circulation, an even more pronounced increase in hemolysis was observed for flow-exposed RBCs compared with static samples (**Figure 3b**). Longer storage time exacerbated hemolysis, with the most prevalent occurring at 49 days storage for both 6 and 12-hour blood circulation timepoints.

Both intracellular Ca^{2+} and sialic acid are important biophysical parameters used to characterize blood damage in human RBCs. Intracellular Ca^{2+} regulates cell volume and deformability, while bound sialic acid contributes up to 70% of the RBC's negative surface charge[26]. Here, the Ca^{2+} fluorescent intensity did not change between static or flow-exposed groups after 6 hours (**Figure 3c**). However, after 12 hours, a significant increase ($p<0.001$) in intracellular Ca^{2+} intensity was observed for flow-exposed RBCs following 49 days of blood storage (**Figure 3d**). The concentration of SA_{free} also increased with blood storage duration following 6 hours in ECMO circulation (**Figure 3e**).

Total RBC count decreased (**Figure 3f**) following 6 hours of blood circulation, with a similar but more pronounced trend following 12 hours of blood circulation (**Figure 3g**). Conversely, the percentage of echinocytes in the bulk cell population increased (**Figure 3h**) for flow-exposed RBCs compared to static RBCs following 6 hours of blood circulation, with a more pronounced trend following 12 hours of blood circulation (**Figure 3i**). We suspect both results are due to flow exposure in our ECMO circuit. Taken together with the observed increase

in hemolysis, this suggests sequestering of lysed RBCs from circulation, as well as the presence of RBC oxidative damage as the drivers for the changes we see in surface zeta potential. Again, storage time also was a significant factor when comparing static samples and flow-exposed samples to one another.

We anticipated that the decreasing surface zeta potential magnitude with ECMO circulation is due to exposure of circulating RBCs to flow-induced mechanical shear stresses—corroborated by an increase in both SA_{free}, and intracellular Ca^{2+}concentrations (suggesting mechano-activation of the Gardos channel)[27]. From the broader perspective of RBC biophysical properties, several authors have shown decreases in surface zeta potential to be associated with blood storage[28] and RBC aging[29], as well as increasing fibrinogen-RBC binding and subsequent blood clotting risk[30]. Collectively, these studies support a hypothesis that blood storage compounds shear exposure effects to the surface zeta potential response in RBCs, and has the potential to elevate blood clotting risk in ECMO circuits due to the decrease in repulsive forces.

To further probe this hypothesis, we added a membrane oxygenator to our ECMO circuits. Membrane oxygenators are a critical component of the ECMO system providing artificial lung functionality by reoxygenating RBCs. However, as RBCs pass through the oxygenator they are repeatedly subjected to periodic squeezing between membrane fibers. This squeezing mechanism as demonstrated by Pan *et al*, results in a multi-stage progression of cell membrane fatigue, characterized by initial elastic deformation and progressing to complete hemolysis[31]. Considering the increased shear exposure, we expected to see a more pronounced change in the surface zeta potential response in RBCs. Interestingly, we observed the converse surface zeta potential response where the addition of the Affinity Pixie™ oxygenator caused an increase in surface zeta potential (**Figure 4**).

We did see a significant increase ($p>0.0001$) in hemolysis for the Pump+PIXIE flow group (**Figure 5 a-b**), and significant increase in intracellular Ca^{2+} between static and Pump+PIXIE groups in the freshest blood (Day 7) after 12 hours of circulation and in the oldest blood (Day 49) at just 6 hours of circulation (**Figure 5 c-d**). Although SA_{free} concentration did increase with blood storage duration in both static and flow-exposed RBCs (**Figure 5e)**, surface zeta potential did not decrease as expected and as shown in prior studies[32,33].Thus, we suspect that the observed increased sialic acid is a byproduct of the blood storage, and not directly a result of blood circulation or shear stress exposure in our Affinity Pixie™ Oxygenator circuit. No change in cell concentrations and population % were observed with blood storage or circulation time due to the addition of the oxygenator (**Figure 5 f-i**).

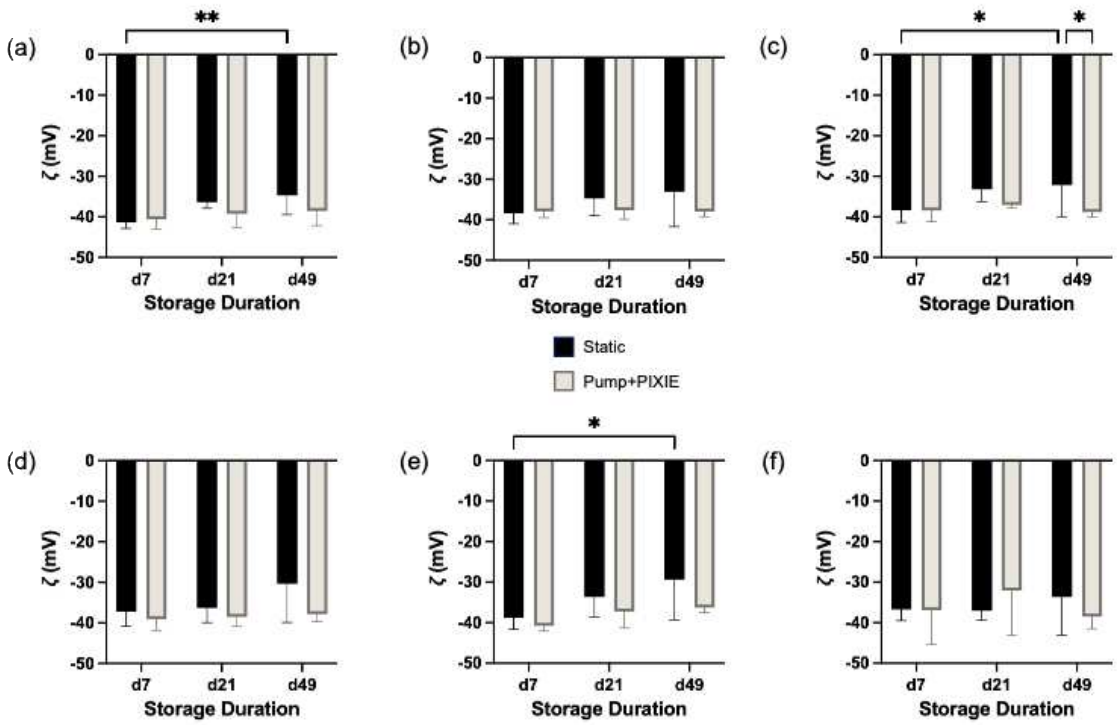

Figure 4: Changes in RBC surface zeta potential through the Affinity Pixie™ Oxygenator circuit over increasing blood storage duration at (a). 2 hours, (b). 4 hours, (c). 6 hours, (d). 8 hours, (e). 10 hours, and (f). 12 hours of ECMO blood circulation. Data presented represents the mean ± (s.d.) for all donors (static: n=7; flow: n=4). Statistical comparisons between static and flow-exposed Pump+PIXIE samples were made via Two-way ANOVA using Tukey's Multiple Comparison test. All values with P<0.05 were considered statistically significant.

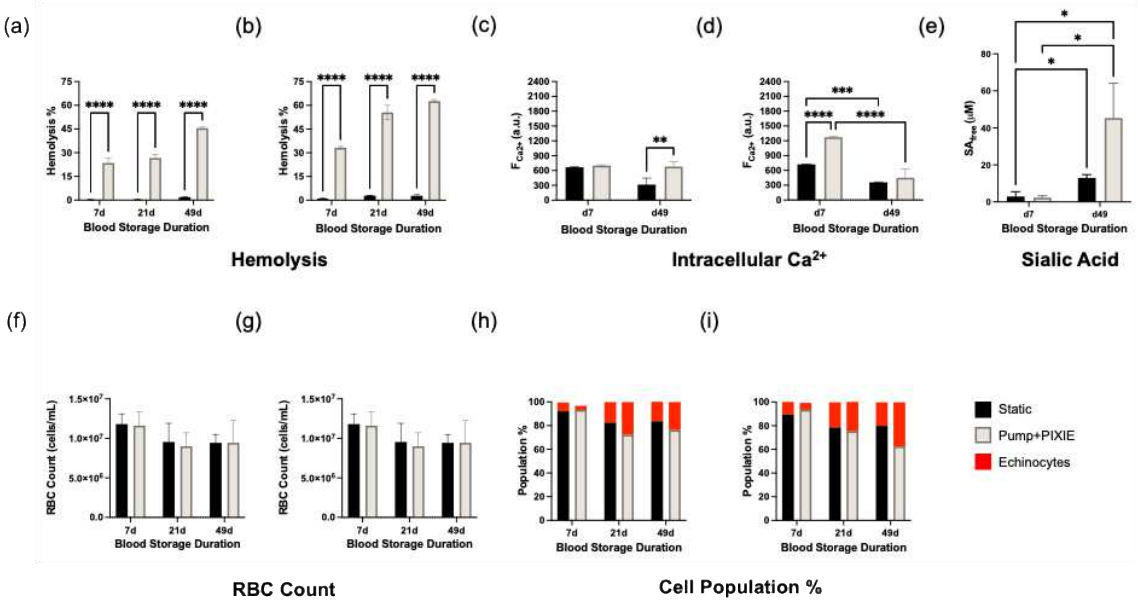

Figure 5: Change in RBC Hemolysis over increasing blood storage duration from 7 to 49 days following (a). 6 hours and (b). 12 hours of ECMO blood circulation. Change in Intracellular Ca^{2+} over increasing blood storage duration following (c). 6 hours and (d). 12 hours. (e). Change in free sialic acid (SA_{free}) concentration

following 6 hours of ECMO blood circulation in Affinity Pixie™ Oxygenator circuit. Data presented for all panels represents the mean ± (s.d.) for a single (n=1) donor. Statistical comparisons between static and flow (Pump+PIXIE) samples were made via Two-way ANOVA using Tukey's Multiple Comparison test; *p<0.05, **p<0.01, ****p<0.0001. All values with p<0.05 were considered statistically significant.

Potential Considerations in Observed Surface Zeta Potential Changes:

Our original hypothesis was that our observed surface zeta potential measurements were a result of shear exposure on the RBCs in ECMO flow. With the addition of the oxygenator, we expected to see those effects increase the observed changes in surface zeta potential (with a reduction in overall surface potential). Interestingly, we did not observe that, leading us to question to what extent is zeta potential influenced by shear exposure? Mulholland *et al* showed that in a roller pump ECMO circuit, a peak shear stress of 150-400 Pa is experienced by approximately 1.49 x 10^{-7} dL of blood volume due to the flow mechanics (occurring when both rollers simultaneously occlude the tubing) [24]. Based on this calculation and our experimental parameters (e.g., circuit volume and pump speed) we estimated that ~3% of total RBCs in our circuits are exposed to that maximum shear stress. While it is unlikely the low percentage number of sheared RBCs is responsible for our observed decrease in the bulk surface zeta potential responses, we were interested to know what level of shear stress (and percentage of total cells exposed) would alter surface zeta potential to a detectable level. To accomplish this, a shearing system adapted from McNamee *et al*[34] was constructed to expose 100% of RBCs in suspension to a known level of mechanical shear stress (**Figure 6a**). Even at maximum shear stress magnitude and exposure time (i.e., 123 Pa for 6 seconds) no surface zeta potential or cell concentration changes (**Figure 6a-b**) were observed, despite increased intracellular Ca^{2+} compared to static samples (**Figure 6c**).

Another experimental parameter we considered could be responsible for our observed changes in surface zeta potential in our Pump-only group was chemical exposure to the custom 3D printed chamber we used to volume-match the oxygenator in our original experiment. Printed plastic components, such as that used in our Pump-only group, have been shown to cause oxidative stress (OS) and cytotoxicity to RBCs[36], thus, we also tested blood circulating without

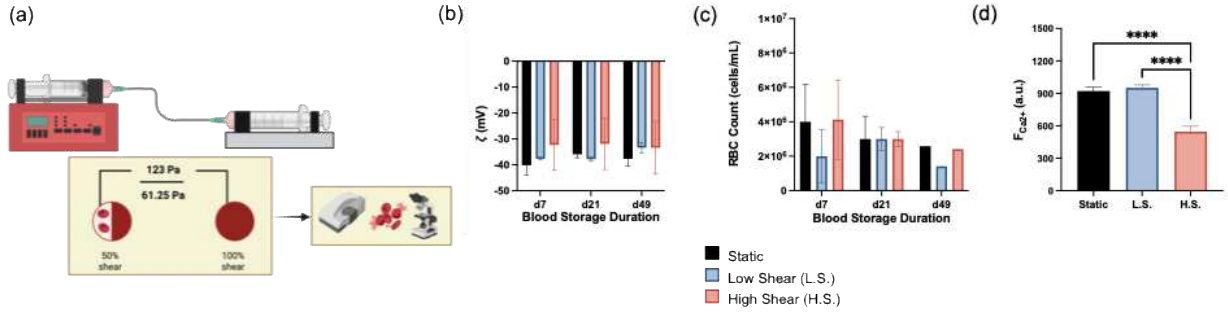

Figure 6: A shearing system designed to expose 100% of RBCs to maximum shear stress levels in ECMO. (a).Schematic of benchtop shearing system adapted from McNamee *et al.* (b). No changes observed in RBC surface zeta potential and (b). RBC concentration over increasing blood storage duration following exposure to low (L.S. = 61.25 Pa) and high (H.S.=123 Pa) mechanical shear stress. Data presented represents the mean ± (s.d.) for a (n=3) donors (c). Change observed in intracellular Ca^{2+} over increasing blood storage duration following exposure to low (61.25 Pa) and high (123 Pa) mechanical shear stress. Data presented represents the mean ± (s.d.) for a single (n=1) donor. Statistical comparisons between static, low shear stress, and high shear stress magnitude groups were made via One-way and Two-way ANOVA using Tukey's Multiple Comparison test; ****p<0.0001. All values with p<0.05 were considered statistically significant.

the reservoir (silicone tubing only). We only saw minimal surface zeta potential changes in this group (Tubing-only) compared to the flow group with the 3D-printed reservoir (**Figure 7a**). Further, we visually observed a progression of blood color from bright red to dark brown over 12 hours of circulation in both of circuits (**Figure 7b-e**). This observation was accelerated by blood storage, suggesting that storage time increases susceptibility of RBCs to OS[37], an important consideration when monitoring inflammatory and coagulation response in ECMO patients.

Although higher percent hemolysis was observed in the Pump+PIXIE group, indicating increased shear stress due to RBCs squeezing through membrane fibers, we suspect that the membrane oxygenator may provide chemical protection against OS, based on no changes in blood discoloration **(Figure 7f)** or surface zeta potential **(Figure 4)** compared to Pump-only flow samples. To probe the impacts of OS on surface zeta potential, we treated RBCs with increasing concentrations of hydrogen peroxide (H_2O_2) and observed that the surface zeta potential decreased with chemical OS damage (**Figure 8a**), confirmed by flow cytometry analysis (**Figure 8b**).

This further confirmed our hypothesis that OS is a likely mechanism to explain the changes in flow samples we observed in our Pump-only group. Many membrane oxygenators are coated with synthetic polymers to enhance biocompatibility and mitigate the formation of blood clots. In fact, the Affinity Pixie™ oxygenator used in our study is coated with Balance™ Biosurface, a heparin-free coating comprised of a negatively charged hydrophilic layer with bonded (negative) sulphonated polymer groups, which mimic the native endothelium[38]. Armstong *et al*[39] demonstrated that sulphonated polymer groups (also referred to as polyethylene glycol, PEG) lower the viscosity of RBCs in plasma via binding to blood group-specific antigens on the membrane surface, inhibiting cell agglutination (clustering) and enhancing RBC survival in circulation. Jovtchev *et al* also showed that binding of PEG polymer particles to the RBC membrane can elevate the steric (negative) charge of RBCs increasing repulsive force between cells[40]. This phenomenon might explain the increase in surface zeta potential that we observe in our Affinity Pixie™ oxygenator circuit, compared to the Pump-only flow samples.

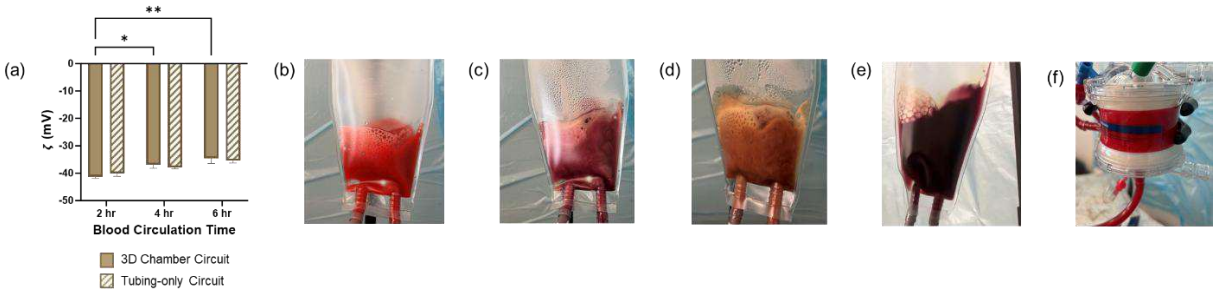

Figure 7: Comparison of circuits with and without a 3D printed reservoir. (a). Changes in RBC surface zeta potential between Pump-only circuit configurations at 7 days blood storage. Data presented represents the mean ± (s.d.) for a single (n=3 technical replicates) donor. Statistical comparisons between 3D Chamber and Tubing-only circuit groups, and over 6 hours of blood circulation time, were made via Two-way ANOVA using Tukey's Multiple Comparison test; ****p<0.0001. Comparison of blood color changes following (b). 2 hours, (c). 6 hours and (d). 12 hours of blood circulation for Pump-only ECMO circuits.

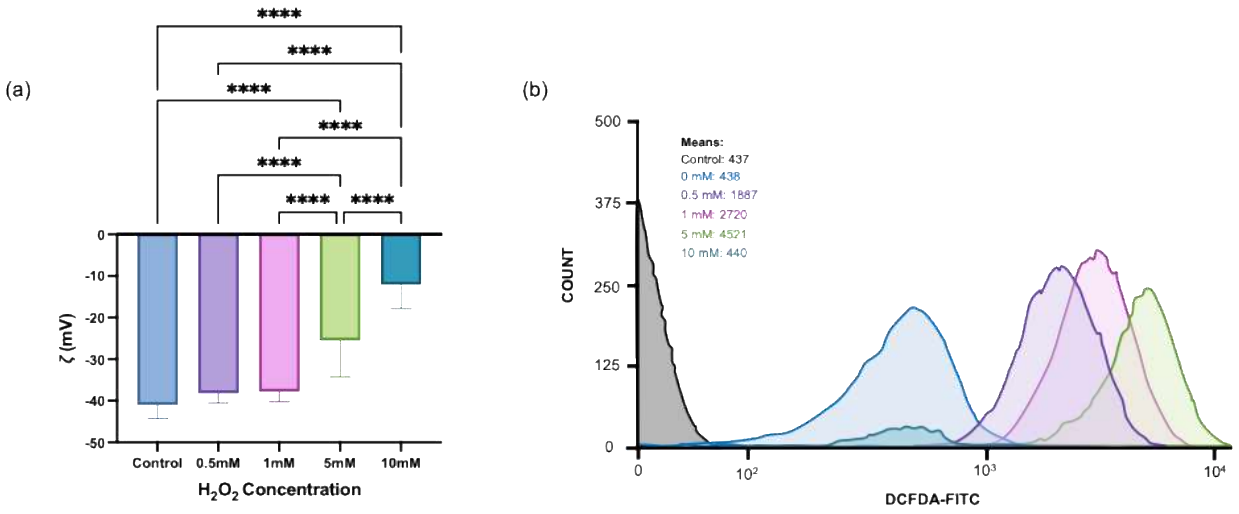

Figure 8: Changes in RBC surface zeta potential with increasing chemical oxidative stress. (a). OS induced by hydrogen peroxide treatment (H_2O_2). Data presented represents the mean ± (s.d.) for (n=3) donors. Statistical comparisons between H_2O_2 treatment groups of were made via One-way ANOVA using Tukey's Multiple Comparison test; ****p<0.0001. (b). Flow cytometry histogram negative control (black) positive control, 0 mM (dark blue) and, 0.5 mM (purple), 1 mM (pink), 5 mM (green), and 10 mM (teal blue) H_2O_2 treatment and a negative control using DCFDA fluorophore stain to detect presence of reactive oxygen species (ROS).

This study has several limitations and potential for expansion. First, surface zeta potential was evaluated in isolated RBCs in the absence of interactions with other blood components found in whole blood (e.g., fibrinogen, platelets and leukocytes). Considering that decreases in surface zeta potential magnitude have previously been associated with altered RBC-fibrinogen interactions[30], further whole blood experiments would be needed before translating the current findings to a clinical environment. Furthermore, the hematocrit used in this study (10%) is lower than observed in ECMO systems, which is ~40% found in whole blood. The isolation of RBCs and lower hematocrit were necessary to minimize inter-donor variability, allowing for analysis at multiple blood storage timepoints across a single donor. We showed a consistent response across multiple donors in this study, thus future work can more confidently use whole blood from various donors to better mimic clinical settings. Finally, while the RBCs were circulated mechanically through an oxygenator, no gas exchange took place, limiting our hypothesis about the contributions of the oxygenator to the chemical surface exposure of the RBCs. Future studies will be conducted using a more clinically translatable hematocrit range (between ~40%) as well as with whole blood using a multi-donor experimental time study.

CONCLUSIONS:

In the present study we investigated the applicability of surface zeta potential as a biomarker for real-time monitoring of human RBCs in *ex vivo* ECMO circuits. We demonstrated that surface zeta potential can detect flow-induced changes in RBCs due to oxidative stress, however, it is limited (at least from the observations in this study) in detecting hemolysis and shear stress changes. Further, the progressive decrease in surface zeta potential from blood storage Day 7 to Day 49, and the surface zeta potential decrease as early as 8 hours of blood circulation (on Day 21 of storage) show the impact of storage duration on RBC electrophysiology in ECMO. This highlights the use of surface zeta potential as a biomarker to help refine blood banking guidelines

to improve clinical outcomes. Lastly, the increase in surface zeta potential in Affinity Pixie™ oxygenator flow samples suggests that membrane oxygenators protect RBCs from OS (despite increased hemolysis) which may have benefits for blood preservation. Future work will investigate expanding the size of our Affinity Pixie™ oxygenator circuit to determine if less time through the oxygenator produces the same protective effects. Additionally, introducing oxygen into the circuit would allow for closer clinical correlation, and investigate the relationship between oxygen binding and RBC surface charge.

These findings not only provide a scientific basis for more refined blood banking protocols, but the high temporal sensitivity of surface zeta potential measurements (compared once-daily ACT and PfHb measurements) is clinically important because it offers the ability to detect real-time changes in RBC physiology and predict cell aggregation and clotting risk before it occurs, which can improve treatment outcomes for patients requiring ECMO life support.

Statistical Analysis:

All statistical analysis was carried out using Prism 11 (GraphPad Software, La Jolla, CA). Comparisons between groups were performed with either One or Two-way analysis of variance (ANOVA) with Tukey's Multiple Comparison test. All statistical analysis was performed at a significance level (α) >0.05. All p-values >0.05 were deemed statistically significant.

DATA AVAILABILITY STATEMENT

Data will be made available upon reasonable request to the Corresponding Author and with approval by the authors**.**

REFERENCES

1. Bartlett, R. *et al.* The history of extracorporeal membrane oxygenation and the development of extracorporeal membrane oxygenation anticoagulation. in vol. 50 081–090 (Thieme Medical Publishers, Inc., 2024).
2. Annich, G. Extracorporeal life support: the precarious balance of hemostasis. *Journal of Thrombosis and Haemostasis* **13**, S336–S342 (2015).
3. Sniderman, J., Monagle, P., Annich, G. M. & MacLaren, G. Hematologic concerns in extracorporeal membrane oxygenation. *Research and Practice in Thrombosis and Haemostasis* **4**, 455–468 (2020).
4. Bilodeau, K. S., Saifee, N. H. & Chandler, W. L. Causes of red blood cell loss during extracorporeal membrane oxygenation. *Transfusion* **63**, 933–941 (2023).
5. Bertin, S. *et al.* Effects of Extracorporeal Membrane Oxygenation Circuits on Drug Sequestration: A Review of Ex Vivo Experiments. *Journal of Clinical Medicine* **14**, 8060 (2025).
6. Heith, C. S. *et al.* Effects of an ex vivo pediatric extracorporeal membrane oxygenation circuit on the sequestration of mycophenolate mofetil, tacrolimus, hydromorphone, and fentanyl. *The Journal of Pediatric Pharmacology and Therapeutics* **24**, 290–295 (2019).
7. Meyer, A. D. *et al.* Effect of blood flow on platelets, leukocytes, and extracellular vesicles in thrombosis of simulated neonatal extracorporeal circulation. *Journal of Thrombosis and Haemostasis* **18**, 399–410 (2020).
8. Sarathy, S. *et al.* Flow monitoring of ECMO circuit for detecting oxygenator obstructions. *Annals of biomedical engineering* **49**, 3636–3646 (2021).

9. Shin, Y. *et al.* Association between blood transfusion and early mortality in patient undergoing extracorporeal membrane oxygenation. *Scientific reports* **15**, 27145 (2025).
10. Godin, C. & Caprani, A. Effect of blood storage on erythrocyte/wall interactions: implications for surface charge and rigidity. *European biophysics journal* **26**, 175–182 (1997).
11. Wagner, S. J., Glynn, S. A. & Welniak, L. A. Research opportunities in optimizing storage of red blood cell products. *Transfusion* **54**, 483 (2013).
12. Ghaleb, S. *et al.* Correlation among hemolysis biomarkers in pediatric patients undergoing extracorporeal membrane oxygenation. *The Journal of ExtraCorporeal Technology* **53**, 125–129 (2021).
13. Baghaei, R. *et al.* Red Cell Distribution Width as a Predictor of Outcome in Cyanotic Congenital Cardiac Surgery. (2022).
14. Shin, D. A., Lee, J. C., Shin, H., Cho, Y.-J. & Kim, H. C. Point-of-care testing of plasma free hemoglobin and hematocrit for mechanical circulatory support. *Scientific Reports* **11**, 3788 (2021).
15. Nellis, M. E., Dalton, H. & Karam, O. Quantifiable bleeding in children supported by extracorporeal membrane oxygenation and outcome. *Critical care medicine* **47**, e886–e892 (2019).
16. Henslee, E. A. *et al.* Rhythmic potassium transport regulates the circadian clock in human red blood cells. *Nature communications* **8**, 1978 (2017).
17. Hughes, M. P. *et al.* V m-related extracellular potentials observed in red blood cells. *Scientific reports* **11**, 19446 (2021).

18. Lavi, E. D., Crivellari, F. & Gagnon, Z. Dielectrophoretic detection of electrical property changes of stored human red blood cells. *Electrophoresis* **43**, 1297–1308 (2022).
19. DEMİR, A. The Importance Of Zeta Potential In Biological Cells. *Prof. Dr. Hasan Akgül Prof. Dr. Engin Şahna Prof. Dr. Zeliha Selamoğlu* 19 (2024).
20. Kalandadze, L. & Tsintsadze, M. The influence of zeta potential on the microrheological properties of erythrocytes. in (2022).
21. Hughes, M. P. The cellular zeta potential: cell electrophysiology beyond the membrane. *Integrative Biology* **16**, zyae003 (2024).
22. Schmidt, B. S. *et al.* Timing of adding blood to prime affects inflammatory response to neonatal cardiopulmonary bypass. *Cardiology in the Young* **27**, 480–487 (2017).
23. Alipour, E., Jordan, J. E., Poudel, L., Files, D. C. & Kim-Shapiro, D. B. In Vitro Reduction of Device Thrombosis Using a Combined Nitrite and Red Light Treatment. *bioRxiv* 2023–06 (2023).
24. Hoettges, K. F. *et al.* Ten–Second Electrophysiology: Evaluation of the 3DEP Platform for high-speed, high-accuracy cell analysis. *Scientific reports* **9**, 19153 (2019).
25. Sze, A., Erickson, D., Ren, L. & Li, D. Zeta-potential measurement using the Smoluchowski equation and the slope of the current–time relationship in electroosmotic flow. *Journal of colloid and interface science* **261**, 402–410 (2003).
26. Seaman, G., Knox, R., Nordt, F. & Regan, D. Red cell aging. I. Surface charge density and sialic acid content of density-fractionated human erythrocytes. *Blood* **50**, 1001–1011 (1977).
27. Lew, V. L. The calcium homeostasis of human red blood cells in health and disease: interactions of PIEZO1, the plasma membrane calcium pump, and gardos channels. *Annual Review of Physiology* **87**, 257–277 (2025).

28. Silva, D. C. *et al.* Optical tweezers as a new biomedical tool to measure zeta potential of stored red blood cells. *PloS one* **7**, e31778 (2012).
29. Huang, Y. *et al.* Human red blood cell aging: correlative changes in surface charge and cell properties. *Journal of cellular and molecular medicine* **15**, 2634–2642 (2011).
30. Carvalho, F. A., de Oliveira, S., Freitas, T., Gonçalves, S. & Santos, N. C. Variations on fibrinogen-erythrocyte interactions during cell aging. *PLoS One* **6**, e18167 (2011).
31. Pan, Y., Li, Y., Li, Y., Li, J. & Chen, H. Fatigue of red blood cells under periodic squeezes in ECMO. *Proceedings of the National Academy of Sciences* **119**, e2210819119 (2022).
32. Seaman, G., Knox, R., Nordt, F. & Regan, D. Red cell aging. I. Surface charge density and sialic acid content of density-fractionated human erythrocytes. *Blood* **50**, 1001–1011 (1977).
33. Hadengue, A. L., Del-Pino, M., Simon, A. & Levenson, J. Erythrocyte disaggregation shear stress, sialic acid, and cell aging in humans. *Hypertension* **32**, 324–330 (1998).
34. McNamee, A. P., Tansley, G. D. & Simmonds, M. J. Sublethal mechanical trauma alters the electrochemical properties and increases aggregation of erythrocytes. *Microvascular research* **120**, 1–7 (2018).
35. McNamee, A. Physical alterations to erythrocytes following sublethal mechanical stresses. (2020).
36. Luximon, A. & Luximon, Y. New technologies—3D scanning, 3D design, and 3D printing. in *Handbook of Footwear Design and Manufacture* 477–503 (Elsevier, 2021).
37. Kassa, T., Jana, S., Baek, J. H. & Alayash, A. I. Impact of cold storage on the oxygenation and oxidation reactions of red blood cells. *Frontiers in Physiology* **15**, 1427094 (2024).

38. Zhang, M. *et al.* Anti-thrombogenic surface coatings for extracorporeal membrane oxygenation: a narrative review. *ACS biomaterials science & engineering* **7**, 4402–4419 (2021).
39. Armstrong, J. K., Meiselman, H. J. & Fisher, T. C. Covalent binding of poly (ethylene glycol)(PEG) to the surface of red blood cells inhibits aggregation and reduces low shear blood viscosity. *American journal of hematology* **56**, 26–28 (1997).
40. Jovtchev, S., Djenev, I., Stoeff, S. & Stoylov, S. Role of electrical and mechanical properties of red blood cells for their aggregation. *Colloids and surfaces A: physicochemical and engineering aspects* **164**, 95–104 (2000).

AUTHOR CONTRIBUTIONS STATEMENT

B.K.E., E.A.H. and J.E.J.: Designed all experiments. **B.K.E.** Carried out experiments, performed statistical and data analysis and drafted the initial manuscript; **J.N.S.** assisted with execution of experiments, data analysis, and manuscript editing. **E.A.H. and J.E.J.:** Provided advice on data analysis, technical feedback and revisions prior to final manuscript submission.

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