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ORIGINAL RESEARCH

TRANSFUSION

Rapid oxygen release from stored red blood cells can be preserved for longer with refined additive solutions

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Abstract

Background: Stored red blood cells (RBCs) progressively lose metabolic and structural features that are critical for efficient oxygen release, potentially reducing transfusion efficacy. Compared to the standard storage additive saline-adenine-glucose-mannitol (SAGM), successive refinements such as phosphate-adenine-glucose-guanosine-saline-mannitol (PAGGSM) and SOLX (AS-7) have demonstrated better preservation of metabolic and structural properties. Measuring RBC oxygen release permits further evaluation of such refinements within, and beyond, current storage durations.

Study Design and Methods: Leukoreduced whole blood (WB) units from six pools of three ABO/Rh-matched donors were processed into red cell concentrates and stored in SAGM, PAGGSM, or SOLX under standard blood bank conditions for 56 days. Periodic sampling during storage assessed hemolysis, adenosine 5′-triphosphate (ATP) content, and oxygen-unloading kinetics measured by single-cell oxygen saturation imaging or estimated by FlowScore recorded on a hematology analyzer.

Results: Hemolysis remained below the European regulatory threshold of 0.8% to day 49 in all units, with SOLX-stored units showing less variation and

Abbreviations: ANOVA, analysis of variance; AS, additive solution; ATP, adenosine triphosphate; BB, blood bank; C, celsius; $CaCl_2$, calcium chloride; CDL, Component Development Laboratory; CE, European Conformity; CI, confidence interval; CO_2, carbon dioxide; D42, day 42 of storage; DaDEHP, di(2-ethylhexyl) phthalate; FSC, forward scatter; Hb, hemogoblin; HCO_3^-, bicarbonate; Hct, hematocrit; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KCl, potassium chloride; kPa, kilopascals; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; $MgCl_2$, Magnesium chloride; ms, milliseconds; NaCl, sodium chloride; O_2, oxygen; PAGGSM, phosphate-adenine-glucose-guanosine-saline-mannitol solution; RBC, red blood cell; RCC, red cell concentrate; RET, reticulocyte; SAGM, saline-adenine-glucose-mannitol solution; SD, standard deviation; snHb, supernatant hemoglobin; SSC, side scatter; WB, whole blood.

more consistent late-stage compliance. SOLX-stored units preserved ATP for longer than SAGM and PAGGSM. Oxygen unloading from SOLX- and PAGGSM-stored RBCs was faster than with SAGM; a similar effect was reported for FlowScore, indicating the utility of this surrogate to benchmark additive solutions. Overall, storage in SOLX halved the rate of attrition in oxygen release kinetics, with a statistically significant benefit emerging by week 3 of storage.

Conclusion: Over 56 days, SOLX- and PAGGSM-stored RBCs demonstrated faster oxygen release kinetics than SAGM, as measured directly and by the flow-cytometric surrogate. Additionally, SOLX-stored RBCs maintained better ATP levels and hemolysis compliance.

KEYWORDS

additive solutionATPFlowScoreoxygen unloading, quality, RBC, red blood cell, storage lesion

1 | INTRODUCTION

Red blood cell (RBC) transfusions are a common and life-saving medical intervention. To ensure the continuity and consistency of supply, blood donations are processed and stored according to established methods and evaluated in terms of post-manufacture yields and post-expiry hemolysis probed in a representative subset of donations,[1,2] and from the increment in recipient hemoglobin (Hb).[3] Despite efforts to optimize storage, laboratory studies of stored red cell concentrates (RCCs) have described progressive biochemical and morphological alterations. This so-called storage lesion is characterized by ATP depletion, oxidative damage, membrane vesiculation and increased Hb-oxygen affinity[4–6] which may impair RBC oxygen handling[7–9] and compromise transfusion efficacy, particularly when the clinical imperative is to rapidly restore oxygen delivery (e.g. hemorrhagic shock, traumatic brain injury, or acute anemia).[9,10] Although multiple clinical trials have not resolved an effect of storage duration on outcomes, a large meta-analysis associated higher recipient mortality with transfusions of RCCs stored beyond 7–14 days[11] and has incentivized efforts to reduce the storage lesion. A suitable strategy is to implement changes to post-donation processing methods as these have measurable effects on RCC parameters[12–14] and may be readily implementable,[15–18] such as the use of additives.[19,20]

The saline-adenine-glucose-mannitol (SAGM) additive, in use for four decades,[21–24] is the current standard in Europe and Canada. SAGM sustains RBC viability through adenine-supported nucleotide recycling and mannitol-mediated membrane stabilization,[25] yet its hypertonicity and low pH (Table 1) accelerate in-bag metabolic exhaustion.[19,26] This has been associated with lower posttransfusion recovery, as compared to more recently developed additive solutions[6,31,32] such as the near-isotonic phosphate-adenine-glucose-guanosine-saline-mannitol (PAGGSM) solution[33] in which RBCs are better at maintaining ATP levels.[26,34] PAGGSM storage has now been adopted for di-(2-ethylhexyl)phthalate (DEHP)-free blood collection methods currently trialed in various jurisdictions to control higher post-expiry hemolysis observed without the traditional plasticizer.[35] A third storage solution approved in the United States and Europe is SOLX (originally EAS-81; AS-7), a hypotonic, chloride-free, high-bicarbonate formulation to maintain glycolytically favorable alkaline pH, preserve ATP and energy-dependent RBC features that are conducive for extended storage and transfusion viability,[28,36,37] including acceptable (>75%)[38] posttransfusion recoveries after 42–56 days of storage.[29,32]

Since the primary function of RBCs is to supply oxygen to tissues, the benefit of changing storage additive should consider metrics of oxygen handling, but these are not routinely measured.[18] To that end, we developed single-cell oxygen saturation imaging to track RBC oxygen unloading and investigate the impact of changes during storage.[39] Previously, we used this technology to demonstrate progressive attrition of oxygen release from stored RBCs,[40] which can be slowed under hypoxic storage[41] or restored at the storage end-point by biochemical rejuvenation which replenishes ATP and 2,3-diphosphoglycerate (2,3-DPG) levels.[40] To demonstrate meaningful impact on tissue oxygenation, we described how the storage-induced kinetic impairment correlated with reduced renal respiration in ex vivo perfused human kidneys, rescued by biochemical rejuvenation. These findings suggest a meaningful impact of the storage lesion on oxygen delivery to tissues and justify efforts to improve oxygen release kinetics prior

TABLE 1 Formulations of the additive solutions.

Component	SAGM[a]	PAGGSM[b]	SOLX[c]
Glucose (mmol/L)	45	47	80
Adenine (mmol/L)	1.25	1.4	2
Mannitol (mmol/L)	30	55	55
Guanosine (mmol/L)	-	1.4	-
Sodium chloride (mmol/L)	150	72	-
Sodium gluconate (mmol/L)	-	-	-
Sodium citrate (mmol/L)	-	-	-
Sodium bicarbonate (mmol/L)	-	-	26
Citric acid (mmol/L)	-	-	-
NaH_2PO_4 (mmol/L)	-	8	-
Na_2HPO_4 (mmol/L)	-	16	12
Osmolality (mOsm/kg)	376	287	228–237
pH	5.7	5.7–6.0	8.5

Note: References: Formulation and pH (a) and (b) 13, 26, 27; (c) 26, 28, 29 Osmolality (a) and (b) Refs 26, 30; (c) Refs 20, 26, 28, 29.

to clinical trials.[42] However, single-cell oxygen saturation imaging is technically demanding and before it can be streamlined for routine measurements, we developed a flow-cytometric surrogate called FlowScore that leverages on the mechanistic relationship between RBC shape (i.e. diffusion path length) and the oxygen release time constant. FlowScore is accessible on hematology analyzers such as the widely disseminated Sysmex XN-10/−20 series, with the reticulocyte (RET) channel activated for obtaining side and forward scatter (FSC) parameters that interrogate RBC shape.[43] International validation studies have confirmed FlowScore's robustness across multiple blood manufacturers as a practical tool to address a gap in RBC quality monitoring.[43]

This study presents the first comparison of storage in SAGM, PAGGSM, and SOLX on oxygen release kinetics measured directly and estimated by surrogate. This comparison provides mechanistic insights into RBC functional preservation and confirms FlowScore as a useful quality marker for guiding optimization of RBC processes and evaluating storage solutions.

2 | STUDY DESIGN AND METHODS

2.1 | Processing, storage, and sampling

Whole blood (WB) donations (475 mL ± 10%) were collected into 66.5 mL of citrate phosphate dextrose anticoagulant using FQE614B collection packs (Macopharma, Tourcoing, France), following NHS Blood and Transplant procedures and donor consent protocols for these routine donations. These were held at 20–24°C and processed as follows within 24 h of venipuncture (median 18.87 hours; range 15.97–22.10 h, $n = 20$). After leucoreduction by inline filtration, six pools were made of 3–4 ABO/RhD-matched donations prior to three-way splitting. Each unit was centrifuged (3400 *g*, 15 min), its plasma removed by press, and resuspended in either 105 mL of Saline-Adenine-Glucose-Mannitol (SAGM; Macopharma), 110 mL of PAGGSM (Fresenius Kabi, Bad Homburg, Germany), or 110 mL of SOLX™ (prepared from SOLX A and SOLX B according to the manufacturer's instructions; Hemerus Medical, Minnesota, USA). Formulations are shown in Table 1. These final RCCs (6 per additive solution) were stored at 2–6°C throughout the study. On sampling days, units were gently mixed and 8–10 mL of blood was drawn into two sterile sampling pouches (VSE0000A; Macopharma) using a sterile tubing connection device (TSCD; Terumo). One pouch per unit was shipped at 2–6°C to Oxford for single-cell oxygen saturation imaging, and the second was stored at 2–6°C at NHSBT Cambridge for contemporaneous blood bank testing on storage days 2, 7, 21, 35, 42, 49, and 56. Figure 1 shows the study design.

2.2 | Measurements on stored blood

Samples of RCC were analyzed using an ABL90 FLEX blood gas analyzer (Radiometer, Crawley, UK) to measure pH, bicarbonate (HCO_3^-), oxygen (O_2), and carbon dioxide (CO_2). Hemoglobin concentration (total [Hb]; g/L), hematocrit (Hct) and mean cell volume (MCV) were performed on a Sysmex XN10 hematology analyzer (Sysmex Europe GmbH, Norderstedt, Germany). Hematocrit was also measured manually ('spun Hct'), where RCC samples within capillary tubes were centrifuged in a Hemata STAT-II (Separation Technology, Florida, USA) then placed on a slide scale to measure RBC pellet height as a percentage of the total volume height. A 'manual' estimate of MCV then calculated by dividing spun Hct volume by the RBC count. Supernatants were prepared by double centrifugation (2890 *g*, 10 min) for assays of supernatant hemoglobin (Sn[Hb]; g/L; the Harboe method),[44] potassium, glucose, and lactate concentrations using an Alinity C analyzer (Abbott, Illinois, USA). Hemolysis as a percentage of red cell mass was calculated as Sn[Hb]/total [Hb] × (100–Hct).[45] Two strategies were taken to calculate hemolysis; both used the same values for supernatant and total Hb but differed in how Hct was calculated, considering differences in storage solution osmolality. In the first, Hct was obtained from the

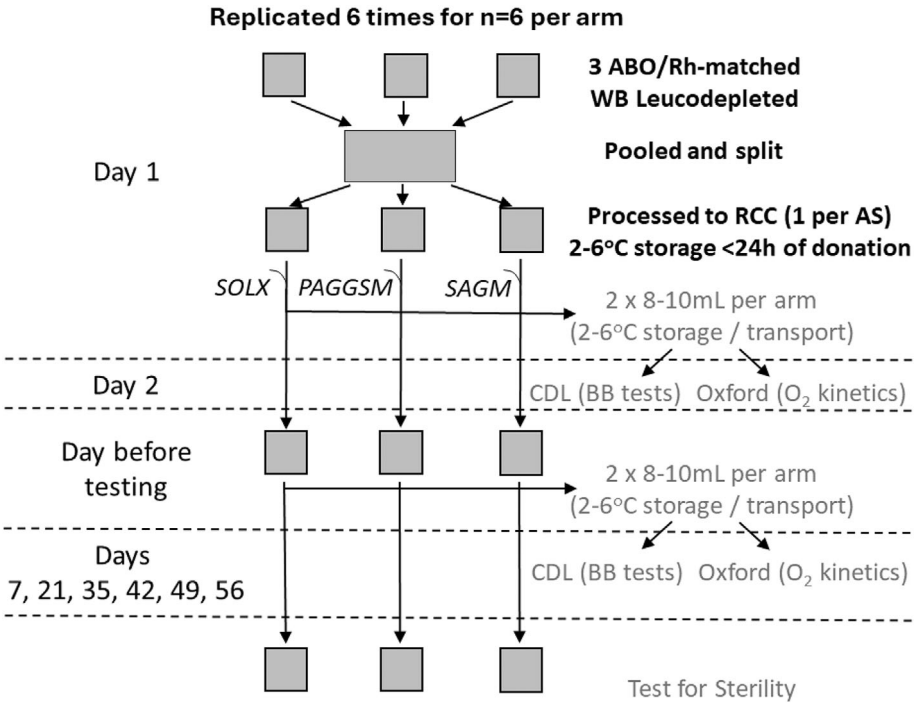

FIGURE 1 Storage study design. AS, additive solution; BB, blood bank; CDL, Component Development Laboratory; RCC, red cell concentrate; WB, whole blood.

hematology analyzer (calculating Hct from RBC count and MCV measured in analyzer diluent), whilst the second measured Hct directly by the manual method.[28] For ATP quantification, RCC samples were kept on ice before deproteinized extracts were prepared[14] and stored at −70°C before testing by microplate assay using a coupled enzyme method.[46]

2.3 | Single-cell oxygen saturation imaging

The kinetics of oxygen release from RBCs were measured using a published method.[39,41] Briefly, RBCs were diluted 1000-fold in normal Tyrode's solution (130 mM NaCl, 4.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 20 mM HEPES; pH adjusted to 7.4 at room temperature by NaOH). Cells were incubated with 4 μM Calcein-AM and 16 μM CellTracker DeepRed for 10 min, then centrifuged and resuspended in dye-free solution. Cells were plated in a superfusion chamber with a borosilicate coverslip pretreated with 0.1% poly-L-lysine. A valve-operated, gravity-fed system exchanged solutions at the chamber between oxygenated normal Tyrode's solution (exposed to in air) and an anoxic reservoir bubbled with 100% N_2 gas and supplemented with 2 mM sodium dithionite. Fluorescence was excited at 640 and 488 nm, with emission signals recorded in the ranges 500–550 and 650–700 nm (OptoSplit, Cairns) and captured by an ORCA Hamamatsu camera. Integration times were set to 200 ms for green fluorescence and 30–95 ms for red fluorescence, adjusted to achieve a fluorescence ratio of ∼1. Ratiometric fluorescence was analyzed to determine the time constant of deoxygenation (τ) and oxygen-carrying capacity (κ), with values derived from the mean of three technical repeats, a minimum of 5 regions of interest, and at least two biological repeats, per sample. Unless stated, all reagents were of analytical grade from either Merck/Sigma-Aldrich or ThermoFisher.

2.4 | FlowScore

Blood samples were analyzed using a Sysmex XN10 hematology analyzer with the RET mode activated. Values from the RET-RBC-Y and RET-RBC-Z channels representing red cell FSC and side scatter (SSC) were used to calculate FlowScore.[43]

2.5 | Statistical analysis

Data were entered and analyzed using Prism 9.1.1 (GraphPad Software; San Diego, USA). A two-way repeated measures analysis of variance (ANOVA) was preferred to compare quality parameters over storage between the three arms, with a mixed effects model used where data points were missing by chance. Tukey's method was used to detect specific differences at each day of storage. Live cell recordings were collected by HCI software and analyzed using in-house macros.

3 | RESULTS

3.1 | Effect of alternative additive solutions on standard metrics of RCC quality

All RCC units met NHSBT specifications for volume, Hb content and Hct on day 2 of storage (Table 2). Unit volumes decreased over time because of repeated sampling but remained within UK specifications until day 35 (UK expiry),[47] falling nominally below specification in the final week of the study. Supernatant Hb concentration (sn[Hb]) increased in all additives (Figure 2A). Hct measured by the 'spun' method (i.e. in the additive solution) was greater than the estimate using the hematology analyzer, which dilutes samples into proprietary diluent. The discrepancy was greatest for the most hypotonic SOLX and lowest for most hypertonic SAGM (Figure 2B). Hemolysis as calculated using analyzer-estimated Hct showed a consistent rise over storage with no difference between additives (2-way ANOVA, 3-arm comparison $p = .660$; Figure 2C) and remained within UK regulatory limits (<0.8% in at least 75% of units) until day 42, at which point the values for mean (95% CI)($n \geq 0.8\%$) were: for SAGM 0.62% (0.26%–0.98%)(1); for PAGGSM 0.55% (0.1%–0.99%)[1]; for SOLX 0.63% (0.22%–1.04%).1 By day 49, values derived from analyzer Hct rose to: for SAGM 0.69% (0.34%–1.05%)(3); for PAGGSM 0.60% (0.22%–0.98%)(0); for SOLX 0.66% (0.38%–0.93%)(0). Hemolysis calculated using spun Hct also showed no difference between additives ($p = .095$) and remained within the limit until day 49, at which point values were: for SAGM 0.55% (0.31%–0.79%)(0); for PAGGSM 0.45% (0.18%–0.72%)(0); for SOLX 0.38% (0.25%–0.51%)(0). Applying this method of assessment, notably SOLX-stored RCCs produced less variation in hemolysis in later storage and consequently fewer above the limit. At day 56, hemolysis calculated from spun Hct was: for SAGM 1.04% (0.18%–1.90%)(5); for PAGGSM 0.76%(0.28%–1.24%)(3); for SOLX 0.60% (0.20%–1.00%)(1) (see Figure 2C, showing mean ± range). Consistent with hemolysis data, supernatant potassium concentrations increased comparably for the three additives, ranging from 17.2 to 19.2 mmol/L at day 7 and 55.0–56.2 mmol/L at day 42 (Table 3).

3.2 | Metabolic stability and energetics are preserved better under PAGGSM and SOLX storage

Glucose levels reflected the initial composition of the additive solutions, with SOLX maintaining consistently higher glucose concentrations throughout storage (Table 3; overall $p < .001$) and a higher rate of utilization over the whole storage period (0.4 mmol/day in SOLX; 0.3 mmol/day in PAGGSM, 0.28 mmol/day in SAGM). These results were proportional to lactate accumulation (Table 3; overall $p < .001$; 0.66, 0.52 and 0.49 mmol/day in SOLX, PAGGSM and SAGM, respectively). Despite higher lactate production, SOLX maintained pH over storage modestly more alkaline, with more units retaining measurable pH values at days 49 and 56 (Table 3) attributable to greater bicarbonate consumption from its initially higher concentration. This explains more CO_2 evolution from neutralization (Table 3). Cellular ATP levels initially increased in the first week (5–6 μmol/g Hb) before declining progressively. During this depletion phase, ATP was significantly higher under SOLX storage compared to SAGM from day 21 ($p < .01$; Figure 3A) and

TABLE 2 Component Parameters and Specification post-manufacture (Day 2) and later in storage (Days 35, 49).

	Volume (mL) D2	Volume (mL) D35	Volume (mL) D49	Hb (g/unit) D2	% Hct (analyzer) D2	% Hct (spun) D2
UK specification[a]	220–340 (210–375)	N/A	N/A	>40 (>30)	50–70(40–70)	None
US specification[b]	300–400	N/A	N/A	50–80	55–65	None
SOLX[c]	304 (281–316)	249 (223–262)	215 (192–227)	62.1 (53.4–65.9)	63.6 (61.3–65.4)	82.8 (78.0–87.3)
PAGGSM[c]	302 (277–315)	246 (219–259)	210 (183–222)	63.0 (57.1–66.2)	65.6 (64.5–67.0)	75.9 (72.0–79.3)
SAGM[c]	308 (282–323)	250 (226–267)	216 (193–231)	62.6 (56.2–66.6)	63.6 (62.0–65.1)	70.0 (64.7–73.7)

[a]Values shown are standard specified range (range for release under clinical concession) (Ref 47).
[b]Values shown are routine/typical range (Ref 48).
[c]Measured values are shown as mean (range).

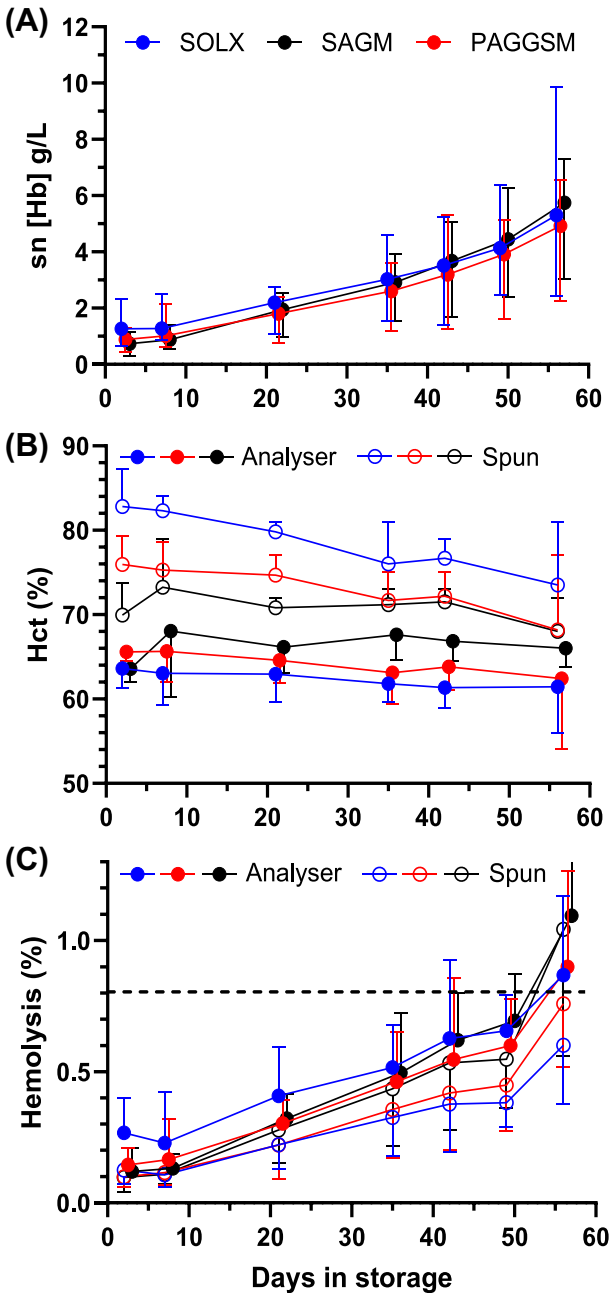

FIGURE 2 Measurements relating to hemolysis. Values are plotted (Mean ± range; $n = 6$ pools) for SOLX (blue), PAGGSM (red) and SAGM (black) arms of the study, over 56 days of storage. (A) Supernatant sn[Hb] as measured by Harboe method. (B) Hematocrit (Hct) as measured by 'spun' method (open symbols) and by Sysmex analyzer (closed symbols). (C) Hemolysis calculated from sn[Hb] and Hct from either spun (open symbols) or analyzer (closed symbols) methods. For clarity, bars extend in only one direction for plots B and C. The bar for hemolysis of SAGM at day 56 extended to 1.72%. Dotted line represents 0.8% hemolysis (UK limit; Ref 47). [Color figure can be viewed at wileyonlinelibrary.com]

higher under PAGGSM storage compared to SAGM at day 56 only ($p < .01$). Levels of ATP remained above the range 2.7–3 μmol/g Hb (drawn from previous proposals[6,49]; supported by other studies[31,32,50,51]) in all arms until day 35 (SOLX 4.4 ± 0.5, PAGGSM 3.9 ± 0.5, SAGM 3.3 ± 0.3 μmol/g Hb). By day 49, only SOLX-stored RCCs remained above this threshold (SOLX 3.6 ± 0.4, PAGGSM 3.0 ± 0.4, SAGM 2.4 ± 0.4 μmol/g Hb). By day 56, SOLX averaged above the threshold, with 4 out of 6 units exceeding 3 μmol/g Hb, compared to 2/6 in PAGGSM and none in SAGM. ATP levels recorded in SAGM-stored RCC at day 35 were not reached in PAGGSM until day 42 and in SOLX until day 56, indicating an advantage of ~1 week for PAGGSM and ~3 weeks for SOLX over SAGM.

3.3 | Oxygen release kinetics decay slower under PAGGSM and SOLX storage

As a proxy of the oxygen release time constant, FlowScore increased during storage from ~1.2 s at day 2 (Figure 3B). The rate of FlowScore attrition was highest in SAGM, reaching 1.71 ± 0.07 s by day 42 compared to 1.50 ± 0.04 and 1.43 ± 0.02 in PAGGSM and SOLX, respectively. By day 56, FlowScore in PAGGSM (1.66 ± 0.09) and SOLX (1.61 ± 0.06) approached levels observed at day 35 in SAGM (1.65 ± 0.07). After transport to the Oxford laboratories, single-cell oxygen saturation imaging interrogated oxygen-unloading kinetics in RBCs. Data are presented as histograms of oxygen-carrying capacity (κ) and oxygen release time constant (τ), shown in Figure 3C,D. κ remained stable over storage across all study arms, consistent with constant corpuscular hemoglobin content (MCH; Table 3). In contrast, τ increased progressively over storage (Figure 3D). This kinetic attrition was most pronounced in SAGM (119 ms/week), compared to PAGGSM (67 ms/week) and SOLX (70 ms/week) as calculated from lines of best fit. This was reflected in SAGM-stored RCC consistently producing higher τ values from day 21, compared to PAGGSM and SOLX. For instance, at day 42, SAGM averaged 1.66 ± 0.15 s whilst corresponding values were 1.35 ± 0.09 s ($p < .05$) for PAGGSM and 1.28 ± 0.08 s ($p < .01$) for SOLX. By day 56, τ in PAGGSM (1.50 ± 0.17 s) and SOLX (1.45 ± 0.15 s) approached those observed in SAGM at day 35 (1.55 ± 0.16 s), revealing that the refined additive solutions delayed kinetic attrition by 3 weeks (Figure 3D). This result is in broad agreement with FlowScore, which strongly correlated with τ ($p < .001$), further validating its utility as a surrogate for oxygen-handling kinetics. The relationship between FlowScore and τ fell on the same line with all storage conditions, indicating a common mechanism linking the direct measurement and its flow-cytometric proxy (Figure 4A). Similarly, the inverse relationship between FlowScore and

TABLE 3 Biochemical and cellular measures.

Days in storage		2	7	21	35	42	49	56
Potassium (mmol/L)	SOLX	6.0 ± 0.5***	19.2 ± 1.3*	39.4 ± 1.8	51.3 ± 2.0	55.3 ± 2.4	58.9 ± 2.5	61.7 ± 2.4
	PAGGSM	5.1 ± 0.4$^{\#}$	18.0 ± 1.1	39.4 ± 1.5	51.5 ± 1.7	56.2 ± 2.3	60.2 ± 1.9	63.3 ± 2.1
	SAGM	4.4 ± 0.2†	17.2 ± 1.3	38.0 ± 2.0	50.5 ± 2.1	55.0 ± 2.5	58.9 ± 2.2	61.9 ± 2.2
Glucose (mmol/L)	SOLX	41.4 ± 1.4***	37.8 ± 1.2***	31.5 ± 1.0***	26.7 ± 1.3***	24.8 ± 1.3***	22.9 ± 1.0***	21.0 ± 1.0***
	PAGGSM	32.1 ± 0.8$^{\#\#\#}$	29.3 ± 0.9$^{\#\#\#}$	24.2 ± 1.1$^{\#\#\#}$	20.5 ± 1.3$^{\#\#\#}$	18.9 ± 1.3$^{\#\#\#}$	17.3 ± 1.1$^{\#\#\#}$	16.0 ± 1.1$^{\#\#\#}$
	SAGM	29.9 ± 0.6†††	26.7 ± 0.8†††	21.8 ± 0.7††	18.5 ± 0.8†	17.1 ± 0.9	15.9 ± 0.9	14.7 ± 0.9
Lactate (mmol/L)	SOLX	5.3 ± 0.8*	11.6 ± 1.1	23.3 ± 1.2	32.0 ± 1.5**	35.3 ± 1.5***	38.6 ± 1.4***	41.1 ± 1.3***
	PAGGSM	5.8 ± 0.8	11.0 ± 1.2	20.2 ± 1.6$^{\#}$	26.8 ± 2.0$^{\#\#}$	29.5 ± 2.3$^{\#\#}$	32.2 ± 2.4$^{\#\#}$	34.1 ± 1.9$^{\#\#\#}$
	SAGM	7.0 ± 1.0	13.0 ± 1.1†	22.5 ± 1.3	28.3 ± 1.3	30.3 ± 1.5	32.4 ± 1.4	33.7 ± 1.0
[a]pH	SOLX	6.8 ± 0.02***	6.7 ± 0.02	6.6 ± 0.02*	6.5 ± 0.02**	6.4 ± 0.02*	6.4 ± 0.02	6.3 ± 0.01
	PAGGSM	6.7 ± 0.02$^{\#\#\#}$	6.6 ± 0.01$^{\#\#\#}$	6.5 ± 0.02$^{\#\#\#}$	6.4 ± 0.02$^{\#\#\#}$	6.3 ± 0.02$^{\#\#}$	Below limit	Below limit
	SAGM	6.9 ± 0.01†††	6.7 ± 0.02†††	6.5 ± 0.02††	6.4 ± 0.02	6.4 ± 0.01	6.3 ± 0.01	6.3 ± 0.01
HCO_3^- (mmol/L)	SOLX	16.0 ± 0.4***	16.1 ± 0.7***	12.6 ± 0.7***	9.8 ± 0.6***	8.5 ± 0.5*	7.2 ± 0.6***	5.6 ± 0.7**
	PAGGSM	9.6 ± 0.3$^{\#\#\#}$	9.5 ± 0.6$^{\#\#\#}$	7.7 ± 0.5$^{\#\#\#}$	6.0 ± 0.6$^{\#\#\#}$	5.3 ± 0.4$^{\#\#}$	4.6 ± 0.54$^{\#\#\#}$	3.5 ± 0.5$^{\#\#\#}$
	SAGM	11.9 ± 0.7†††	10.8 ± 0.8†	7.9 ± 0.5	6.3 ± 0.5	5.5 ± 0.8	5.0 ± 0.5	4.0 ± 0.5
CO_2 (kPa)	SOLX	14.0 ± 0.6***	16.4 ± 1.2***	18.7 ± 0.8***	18.9 ± 1.1***	18.0 ± 1.4*	17.9 ± 1.1***	14.9 ± 1.6**
	PAGGSM	11.0 ± 0.5$^{\#\#\#}$	12.7 ± 0.9$^{\#\#\#}$	14.2 ± 0.9$^{\#\#\#}$	13.7 ± 1.1$^{\#\#\#}$	13.3 ± 0.7$^{\#}$	13.5 ± 1.2$^{\#\#\#}$	10.8 ± 1.7$^{\#\#}$
	SAGM	9.2 ± 0.5†††	11.3 ± 1.1	13.0 ± 0.6†	13.5 ± 1.0	13.1 ± 1.6	13.5 ± 0.7	11.2 ± 0.9
MCH Sysmex (pg)	SOLX	30.6 ± 1.1	30.4 ± 0.9	30.5 ± 1.1	30.8 ± 1.1	30.7 ± 1.0	30.6 ± 1.0	30.7 ± 1.0
	PAGGSM	30.5 ± 1.0	30.5 ± 1.1	30.6 ± 1.1	30.8 ± 1.2	30.7 ± 1.1	30.8 ± 1.0	30.8 ± 1.0
	SAGM	30.5 ± 1.0	30.7 ± 1.0	30.6 ± 1.0	30.8 ± 1.0	30.9 ± 1.1	30.8 ± 1.1	30.7 ± 1.1
MCV (Sysmex) (fL)	SOLX	95.6 ± 2.6	93.4 ± 2.3	93.0 ± 2.2**	92.9 ± 2.2***	92.9 ± 2.3***	92.8 ± 2.0***	93.6 ± 2.4***
	PAGGSM	95.7 ± 2.3	95.2 ± 2.1	94.7 ± 2.5	94.7 ± 2.4	95.0 ± 2.3†††	95.3 ± 2.3	96.4 ± 2.6
	SAGM	95.4 ± 2.4	97.0 ± 2.4	99.9 ± 2.5†	101.6 ± 2.6††	102.7 ± 2.7††	103.4 ± 2.5††	104.8 ± 2.9††
MCV (Spun) (fL)	SOLX	124.5 ± 5.0***	122.0 ± 2.7***	118.0 ± 3.4***	114.2 ± 5.6	116.0 ± 2.8*	n.d.	112.0 ± 3.6
	PAGGSM	110.8 ± 5.1$^{\#\#}$	109.2 ± 3.3$^{\#\#\#}$	109.6 ± 4.6$^{\#}$	107.5 ± 2.8	107.4 ± 3.0$^{\#\#}$	n.d.	105.4 ± 4.6$^{\#}$
	SAGM	105.0 ± 6.0	104.5 ± 3.8	107.0 ± 3.6	107.1 ± 5.1	109.9 ± 3.6	n.d.	107.9 ± 4.6

Note: All values are mean ± SD. Statistical significances by Tukey's post test; for SOLX v SAGM *($p < .05$), ** ($p < .01$), and *** ($p < .001$) and similarly for SOLX v PAGGSM (#) and PAGGSM v SAGM (†).
[a]pH statistics performed only up to day 42, after which values started to drop below the detection limit (on day 49 there were 5, 0, and 4 and on day 56 there were 4, 0, and 2 readable units out of 6 for SOLX, PAGGSM, and SAGM; respectively).

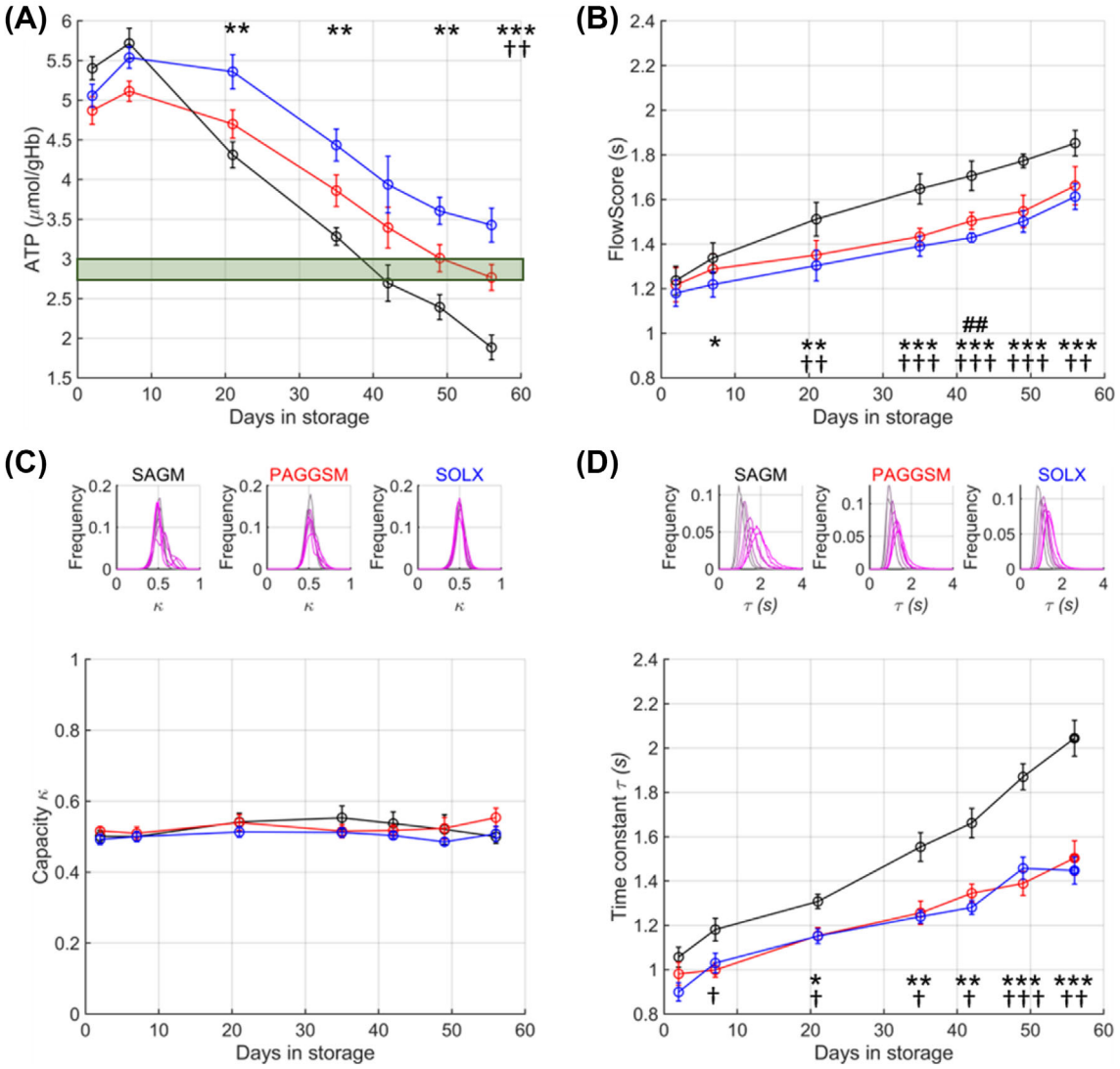

FIGURE 3 Cellular ATP, FlowScore, and oxygen unloading. Values are plotted (Mean ± SEM; $n = 6$ pools) for SOLX (blue), PAGGSM (red) and SAGM (black) arms of the study, over 56 days of storage. (A) Red cell ATP levels (green box indicates 2.7–3.0 μmol/gHb range of proposed quality thresholds; Ref 6, 49). (B) FlowScore calculated as described (Ref 43) from the Sysmex XN analyzer reticulocyte channel FSC and SSC parameters. (C) Upper panel: histograms of oxygen-carrying capacity (κ) from Day 2 (gray) to Day 56 (pink). Lower panel: averaged time course for the three storage solutions. (D) Upper panel: histograms of oxygen unloading time constant (τ) from Day 2 (gray) to Day 56 (pink). Lower panel: averaged time courses for the three storage solutions. Statistical significances by Tukey's post test; for SOLX v SAGM *($p < .05$,** ($p < .01$), *** ($p < .001$), and for PAGGSM v SAGM † ($p < .05$), †† ($p < .01$), ††† ($p < .001$) and SOLX v PAGGSM ## (p0.01). [Color figure can be viewed at wileyonlinelibrary.com]

ATP was overlapping for the three storage additives, indicating a common mechanism underpinning the relationship (Figure 4B).

4 | DISCUSSION

This study is the first to benchmark three additive solutions in terms of their effect on oxygen handling in stored RBCs. The results demonstrate a benefit of advances in storage solution design with PAGGSM and SOLX, over the standard formulation of SAGM. Combined with recent findings linking faster oxygen release with better tissue oxygenation, the study outcomes indicate a potential for refined additives to improve transfusion outcomes. A collateral outcome is the demonstration that FlowScore can accurately resolve the benefit of storage in PAGGSM or SOLX over SAGM, paving the way for using this accessible surrogate to guide further improvements in blood storage methods.

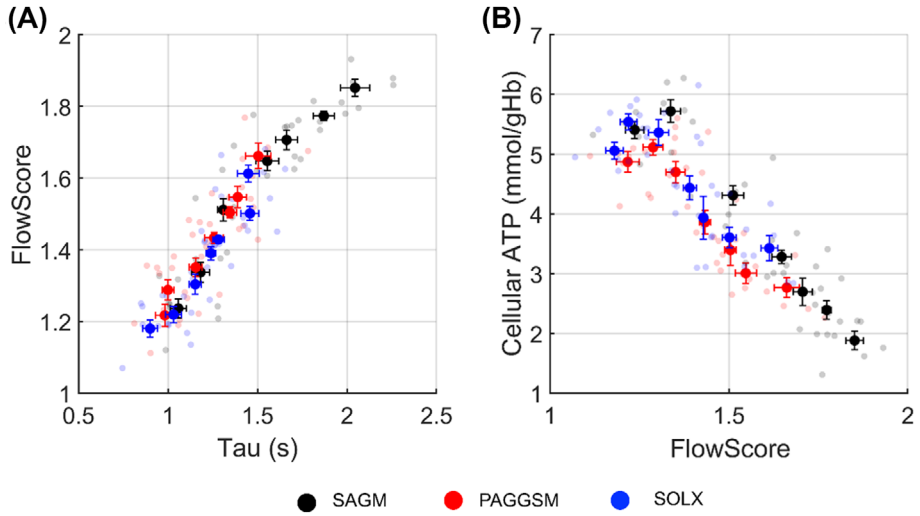

FIGURE 4 Consistent relationship between FlowScore and oxygen-release time constant or ATP across additive solutions. (A) Relationship between τ, the time constant of RBC oxygen unloading, and corresponding FlowScore calculated from analyzer over storage and (B) between FlowScore and ATP. The relationships are comparable and overlapping between the three different additives (SOLX: blue, PAGGSM: red, SAGM: black). Values are plotted as mean ± SEM. [Color figure can be viewed at wileyonlinelibrary.com]

4.1 | Hemolysis measurement and additive solution osmolality

Quantifying hemolysis at expiry is a mandatory quality-control measure implemented across blood agencies, although methodology varies, prompting efforts to harmonize practice.[52] As this study compared additive solutions of differing chemical formulations, the influence of osmolality on hemolysis calculations became apparent. In hyperosmotic/hypertonic SAGM, RBCs occupy less volume,[30] shrinking the Hct and resulting in a larger supernatant fraction that dilutes the cell-free sn[Hb]. Conversely, swollen RBCs in hypotonic SOLX occupy more volume, thus concentrating sn[Hb]. Using sn [Hb] and spun Hct measurements obtained under the same osmotic conditions would accurately report hemolysis. However, hematology analyzers resuspend RBCs in standardized diluent prior to determining cell volume from which Hct values are calculated. SAGM-stored cells had the smallest discrepancy in Hct between spun and analyzer methods, whereas SOLX-stored cells had the greatest difference. This inversion of cell volumes between analyzer and solution osmolalities was observed previously,[26,27] and leads to hemolysis overestimation in PAGGSM and SOLX versus SAGM— thereby providing a rationale to recalibrate analyzer-derived Hct when transitioning from SAGM to alternative storage solutions. Utilizing spun Hct and sn[Hb], all study arms remained below the 0.8% hemolysis threshold until day 49. This would also be true for SOLX but not SAGM, calculating with lower Hct values measured previously (SOLX∼70%; SAGM∼60%).[28] The hemolysis values were consistent with observations from units processed to SOLX after 'overnight hold'.[32] The lower variation in SOLX and PAGGSM versus SAGM, observed late in storage, indicates a restraining effect on hemolysis by the newer additives that, if replicated at blood bank production scales, would enhance RBC quality-control sensitivity.

4.2 | Glycolytic flux and ATP preservation

SOLX-stored RBCs had higher glycolytic flux than in SAGM or PAGGSM, fueled by more glucose and accelerated by the higher pH.[34] Consequently, ATP levels in SOLX were better maintained over storage, being significantly higher than in SAGM at most timepoints and higher than PAGGSM at day 56, consistent with previous studies.[26,28,34] Using this performance metric, the improvement over SAGM storage was equivalent to ∼1 week with PAGGSM storage and ∼3 weeks in SOLX storage. ATP is required for cytoskeletal turnover, stabilizing its membrane interactions which maintain deformability and the biconcave shape,[53] critical for rapid oxygen diffusion, to minimize microvesiculation, and for a sufficient pool for release as a vasodilator.[54] These factors may contribute to the modest correlation between posttransfusion recovery and ATP that underlies the 2.7 or 3 μmol/gHb thresholds[6] and signifies levels of other glycolytic intermediates also diminishing later in storage and associated with poorer recoveries.[55] A limitation here is that levels of 2,3-DPG were not measured, the study being performed since termination of the commercial

reference method. However, previous studies have shown more in SOLX than in PAGGSM and SAGM up to mid-storage day 21.[26,34]

4.3 | Metrics of oxygen release from RBCs

Oxygen release kinetics were assessed directly by single-cell oxygen saturation imaging. In contrast to the oxygen-carrying capacity, which was constant over storage, the oxygen-release time constant became slower under all three storage conditions, with SAGM exhibiting a faster decline than PAGGSM or SOLX. On day 2, time constants for all arms were approximately 1 s, similar to values for freshly drawn blood.[43] By day 35, SAGM-stored cells had exceeded 1.5 s, proceeding to 2 s by day 56. In contrast, PAGGSM- and SOLX-stored cells maintained faster oxygen release, reaching 1.2 s at day 35 and 1.5 s at day 56. Slower oxygen release aligns with ATP depletion, which transitions RBC to a lower-energy spherical state that expands oxygen diffusion path lengths[39] and is followed by a depletion of 2,3-DPG, which strengthens buffering by Hb and has the effect of slowing oxygen egress from cells.

4.4 | FlowScore as a surrogate for oxygen release kinetics

Hematology analyzers, including Sysmex XN-series, are highly standardized flow cytometers, attractive for developing sensitive and rapidly translatable new parameters to correlate with functional metrics from labor-intensive, localized methods. From the Sysmex RET channel, parameters of FSC (RET-RBC-Y) and SSC (RET-RBC-Z)[40] were used to calculate FlowScore.[43] FlowScore increased (slowed) over storage in all arms, with SAGM showing a more pronounced rise than PAGGSM and SOLX. The FlowScore trajectory in SAGM (∼1.2 at day 2, ∼1.7 at day 42) matched values reported in blood agency datasets[43] Notably, FlowScore in SAGM at day 35 was not reached in PAGGSM and SOLX until approximately day 56, matching trends in direct oxygen-unloading measurements and similar to ATP depletion rates. Despite differences in storage solution osmolality that affect cell volume and dependent measures (Hct, hemolysis), FlowScore robustly tracked the oxygen-unloading time constants. Independent of storage duration, this strong relationship of FlowScore with τ, reflected in its relation to [ATP], spans multiple additive solution formulations (Figure 4), making FlowScore a powerful tool for evaluating new storage solutions, whilst its simplicity promises utility in blood banking, where rapid and cost-effective metrics are needed.[6,15,17,18]

4.5 | Regulatory context, in vivo recovery, and future directions for improved RBC storage

Current regulatory approvals for RBC additive solutions reflect evolving strategies to extend storage while maintaining quality, according to accepted measures. SAGM is CE marked and widely used across Europe and in Canada for up to 42 days, though restricted to 35 days in some jurisdictions, such as the United Kingdom and Netherlands, when WB is held at room temperature for up to 24 hours prior to processing. The less widely disseminated PAGGSM was first licensed in Germany for storage of RBCs up to 49 days in the early 1990s.[56] A single in vivo study with a sample size of eleven units reported mean 24-h radiolabeled in vivo RBC recoveries of only 74.5% or 74.6% at 49 days,[27,30] falling just below the current US/EU regulatory threshold of ≥75%,[57] indicating only modest improvement over conventional additives, like SAGM. In contrast, RBCs stored in SOLX

TABLE 4 24-h Cr^{51} radiolabeled in vivo recoveries (%) based on hold conditions of WB (hours) prior to processing and red cell storage duration (days).

WB hold: Storage period	SAGM	PAGGSM	SOLX
<8 h: 35 days	83.5 ± 5.3% [$n = 4$][22]		
<8 h: 42 days	77.4 ± 4.7 [$n = 6$][22] 73.3 ± 6.6 [$n = 10$][23] 81 ± 5 ($n = 20$)[24]		88 ± 5 [$n = 27$][29]
<8 h: 49 days		74 .5 ± 4.4 [$n = 11$][56]	
<8 h: 56 days			82 ± 3 [$n = 27$][29]
>20 and <24 h at RT: 42 days (RT = Room Temperature)			86.2 ± 4.9 [$n = 28$][32] 86.2 ± 5.5 [$n = 25$][58]

(AS-7) have demonstrated more robust ATP preservation and reduced hemolysis variability, which now also includes better preservation of oxygen release kinetics. SOLX is currently approved in the United States for 42-day RBC storage explicitly including use with WB held at room temperature for up to 24 h prior to processing, a regulatory advantage that enhances operational flexibility. It received CE marking in Europe for storage up to 56 days, with efforts underway to secure similar 56-day approval in the United States.

While this study highlights the importance of directly assessing oxygen release kinetics under extended storage, it is critical to recognize that this metric represents one component of ensuring effective tissue oxygenation. The regulatory gold standard remains the 24-h radiolabeled in vivo recovery metric, which reports the proportion of transfused RBCs that remain in circulation after one day; a prerequisite for these transfused cells to load and unload oxygen at tissues. Thus, both high-quality in vitro functional markers and satisfactory in vivo recoveries are essential to ensure extended storage translates into meaningful clinical benefit. Table 4 summarizes select published 24-hour radiolabeled in vivo recovery data under different WB hold and storage durations, emphasizing the stronger dataset available for SOLX relative to older solutions.

The evolving regulatory landscape, including global moves to eliminate ortho-phthalate plasticizer (e.g., DEHP) from blood collection systems, underscores the need for advanced additive solutions like SOLX to show clear benefits in both in vitro and in vivo quality, particularly in non-DEHP collection and storage containers. Early feasibility studies suggest SOLX RBCs stored in non-DEHP containers maintained or improved hemolysis, ATP, and morphology compared to conventional AS-1 RBCs stored in DEHP plasticized PVC containers.[58] Future investigations with direct assessments of splenic clearance or patient oxygen delivery will be crucial to confirm these extended in vitro profiles truly translate into superior clinical outcomes.

5 | CONCLUSION

In summary, this study demonstrates that both SOLX and PAGGSM significantly improve RBC quality and function over storage compared to the current standard, SAGM, with SOLX demonstrating the most sustained preservation of ATP, hemolysis compliance, and oxygen-handling metrics through day 56 of storage. These findings span both conventional (e.g., ATP, hemolysis) and functional (e.g., FlowScore, oxygen release kinetics) indicators of RBC quality. The strong correlation between oxygen-unloading time constants and FlowScore, irrespective of storage regime, further confirms it as a valuable tool for assessing RBC quality. These findings have important implications for transfusion medicine; for efforts to improve the efficacy of RBC transfusions within current expiries, whilst seeking to extend RBC storage duration.

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CONFLICT OF INTEREST STATEMENT

MZ is an employee of Hemerus Medical, LLC. JRH is an inventor of SOLX (AS-7) additive solution and a consultant for Hemerus Medical, LLC. JR, RC, PS and PAS are named on a patent application for FlowScore.

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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