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CONTRACTING ORGANIZATION: University of Maryland School of Medicine, Baltimore, MD

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14. ABSTRACT

The first ErythroMer prototype (EM-V1) was structurally stable and toroidal, with diameter ~ 1/50th that of RBCs and amenable to lyophilization and rapid reconstitution. In addition, EM p50 (with novel pseudo-Bohr effect), NO sequestration and vasoactivity were equivalent to RBCs – establishing POC for the bio-inspired design. In our novel rabbit hemorrhagic shock model, for both hemodynamic and O2 delivery outcomes, EMV1 was non-inferior to shed blood and superior to 5% Albumin. To further optimize biocompatibility, circulation time, and Hb payload density/retention, we developed EM-V2. In prior reports, we included data on V2 benchtop characterization, which recapitulates RBC physiology similarly to V1. Of note, just prior to the end of project Y2, Dr. Doctor was recruited from Washington University (WUSM) to the University of Maryland (UMB), to serve as founding director for the Center for Blood Oxygen Transport and Hemostasis (CBOTH). Our PFCRA team was reorganized and KaloCyte relocated from St Louis to Baltimore. Resulting from these changes, the timing of contract transition from WUSM to UMB, and from COVID19 related lab shutdowns, the only project work in Y3 was performed by KaloCyte. As such, two sequential NCEs have been requested and approved. CBOTH labs reopened in Y4 at 25% capacity; in Q1 our team completed new key biocompatibility experiments. In Q2, lab personnel density was liberalized to 50% and we completed new key PK experiments with advanced modeling of PFC dosing requirements, demonstrating suitability for PFC (dosing q 8-10h). In Q3, personnel density was liberalized to 75% capacity and we re-established our HS/R model, confirming EM V2 efficacy and initiated pilot work for our PFCRA survival model. In Q4 we completed analysis of EM impact upon blood rheology/viscosity, continued evaluation of EM V2 efficacy (with detailed O2 delivery consumption analyses) and completed calibration of HS severity to an LD50 model.

15. SUBJECT TERMS

ErythroMer (EM), Artificial Red Blood Cell (RBC), Prolonged-Field Care (PFC), PFC models, Resuscitation, Oxygenation, Hemorrhagic Shock, Pharmacokinetics, and Biocompatibility

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1. INTRODUCTION:

The overall goal of this project is to optimize a red blood cell substitute, ErythroMer (EM) for resuscitation of casualties with hemorrhagic shock. This will be accomplished by developing EM prototypes with optimal oxygen (O_2) binding affinity that allows for O_2 capture in the lungs and O_2 release in other tissues as well as optimizing formulation and dosing to achieve stable circulation suitable for PFC. EM will also be tested for compatibility with Thrombosomes and other hemostatic adjuncts to prevent dilutional coagulopathy via co-administration with EM. Finally, we will establish EM's efficacy and safety in resuscitation of a hemorrhagic shock model with/without Thrombosomes and/or hemostatic adjuncts.

2. KEYWORDS:

ErythroMer (EM), Artificial Red Blood Cell (RBC), Prolonged-Field Care (PFC), PFC models, Resuscitation, Oxygenation, Hemorrhagic Shock, Pharmacokinetics, and Biocompatibility

3. ACCOMPLISHMENTS:

What were the major goals of the project?

See Revised SOW for adjusted timeline, anticipated completion dates listed below.

Major Task 1: Select & Fabricate ErythroMer (EM) prototypes with high/low O_2 affinities.
Milestone #1: Select EM prototypes meeting high/low P50 targets (completed 04/2018).
Milestone #2: Fabricate selected EM prototypes for *in vivo* testing (completed 10/2018).

Major Task 2: Test efficacy *in vivo*.
Milestone #3: Obtain IACUC/ACURO approval (completed 01/2018 at WUSM; 10/2020 at UMB).
Milestone #4: Establish O_2 delivery benefit conferred by EM prototypes with high/low O_2 affinities (defined as 20% improvement in tissue pO_2 relative to current prototype). (completed 04/2019)

Major Task 3: Measure EM pharmacokinetics (PK).
Milestone #5: Calculate EM PK as a function of Blood Volume (BV)% replacement. (90% complete, target 10/2021). Added PK with MPS depletion (by clodronate), this simulates MPS saturation by massive transfusion, as expected in PFC (50% complete, target 02/2022)

Major Task 4: Develop EM PFC dosing.
Milestone #6: Confirm EM dosing strategy for rabbit PFC models; will be informed by MPS depletion model. (80% complete, target 02/2022).

Major Task 5: Determine EM:HA (hemostatic adjunct) compatibilities *ex vivo*.
Milestone #7: Obtain IRB/HRPO approval (completed 01/2018 at WUSM; 10/2020 at UMB).
Milestone #8: Confirm EM:HA *ex vivo* compatibility. (completed 01/2018).

Major Task 6: Develop goal-directed HA algorithm for EM-based dilutional coagulopathy (DC).
Milestone #9: Develop goal directed HA algorithm for EM-induced DC suitable for in vivo testing (rabbits). (completed 10/2018).

Major Task 7: Pilot PFC Scenarios.
Milestone #10: Obtain IRB/HRPO approval (completed 01/2018 at WUSM; 10/2020 at UMB).
Milestone #11: Pilot & Optimize PFC Scenarios (A, B, C) to achieve 50% 48h mortality for colloid resuscitation controls. (50% completed, target 02/2021).

NB. Major Tasks 8-10 run concurrently, efficiently using the same rabbits for multiple tasks.

Major Task 8: Establish EM efficacy *in vivo.*
Milestone #12: Establish EM efficacy in comparison to shed blood (O_2 delivery non-inferiority yes/no) and colloid resuscitation (mortality superiority yes/no). (25% completed, target 02/2022).

Major Task 9: Optimize PFC HA Algorithms *in vivo*.
Milestone #13: Optimize goal directed HA algorithm for DC and TIC during resuscitation in PFC Scenario B (uncontrolled hemorrhage, with dilutional coagulopathy) and PFC Scenario C (controlled hemorrhage + polytrauma, with TIC). Identify differences required (amongst colloid, blood and EM-based resuscitation) for HA administration. (25% completed, target 09/2022).

Major Task 10: Screen EM safety *in vivo*.
Milestone #14: Identify laboratory and histologic evidence of EM toxicity during resuscitation from PFC Scenarios A-C, in comparison to that observed in blood re-infusion and colloid resuscitation groups. (25% completed, target 09/2022).

What was accomplished under these goals?

What was accomplished under these goals?

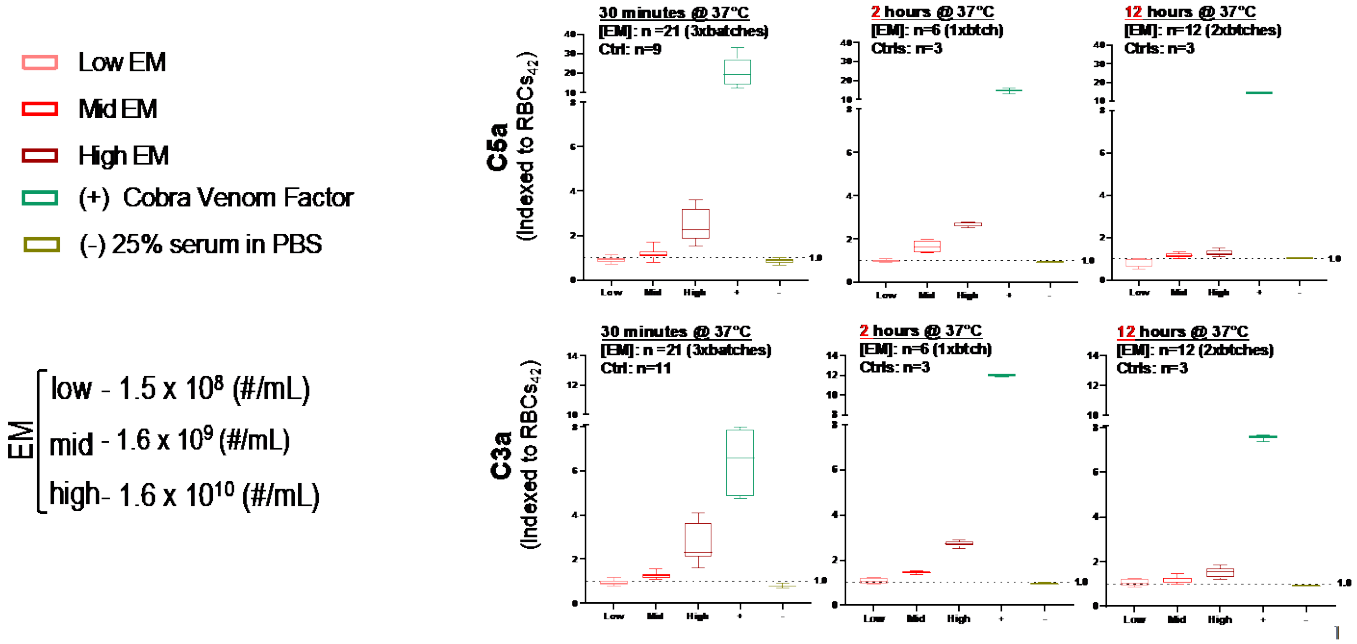
1. **Select & Fabricate ErythroMer (EM) prototypes with high/low O_2 affinities.**
   - Y1: Testing of the first EM prototype (EM-V1) completed
     - Identified EM-V1 with O_2 affinities that match RBCs or are >30% and <20% that of RBCs. (MT 1, Milestones 1 and 2).
     - The prototype with optimal O_2 affinity was tested *in vivo* in both PK and in oxygenation studies following controlled hemorrhage (MT 2; Milestones 3 and 4).
   - Y2: Optimized design w/r/t biocompatibility, improved payload retention, and improved lyophilization/reconstitution, yielding EM-V2.
     - Two complimentary proposals have been awarded to support additional optimization of the EM formulation:
       - DoD BA190035 (FOA USA-MRMC-BAA-2018-W81XWH18SBAA1) "Optimized Formulation, Delivery & Dosing for ErythroMer (Artificial Red Cell)"
       - NIH/NHLBI SBIR Phase I H193-004-0067 "Rapid Reconstitution of a Lyophilized, Bio-inspired, Artificial Red Blood Cell."
   - Y3: Optimization of ErythroMer components and processes, including fabrication and assembly; cleanup; and lyophilization/reconstitution (details in prior reports).
2. **Biocompatibility**
   Hemostasis
   - Y1-2 Biocompatibility was evaluated in *ex vivo* ROTEM analyses
     - EM-V1 and EM-V2 particles had no effect upon (*ex vivo*) hemostasis other than a correctable dilution effect, indicating need for co-administration of plasma and platelets when transfusion exceeds ~ 50% BV (e.g. massive transfusion) (see Y1 report). (MT 5, Milestone 7)
     - Generation of a goal-directed HA algorithm to optimize co-administration testing of hemostatic adjuncts to maintain hemostasis during resuscitation (see Y1 report). (MT 6, Milestone 8)
     - Extensive *ex vivo* analysis of EM biocompatibility with Thrombosomes (see Y2Q1 & Y2Q3 reports). (MT 5, Milestone 7)
       - EM-V2 (empty shells [focused study of surface biocompatibility] and Hb-loaded EM-V2) had no effect on coagulation studies (size distribution, aggregometry, surface marker expression, thrombin generation) with Thrombosomes (desired outcome).
     - Submission of two complimentary proposals to fund additional work exploring EM biocompatibility and co-administration with:
       - Freeze dried plasma: DoD PR190685 (FOA W81XWH-19-PRMRP-TTDA) "Freeze-Dried Hemostatic O_2 Carrier for Damage Control Resuscitation) (awarded, in partnership with Haima Therapeutics)
       - Synthetic platelets: DoD/DHA SBIR Phase I H193-004-0067 "Nanoformulated Dried Whole Blood Surrogate for Hemostatic Resuscitation". (not awarded)
   - Y3 – due to move from St Louis → Baltimore and pandemic lockdown, no work was performed on this task in Y3.

3. **Pharmacokinetics**
   - Y1: Top-loading (10% BV replacement) PK studies were completed with both EM-V1 and EM-V2. Analysis of EM-V2 in rabbits indicated a $t_{1/2}$ of ~4.5h. (MT 3, Milestone 5)
   - Y2: Confirmation of findings, in the context of 20 & 40% BV replacement (data in Y2Q3 report). We anticipate that PK in the setting of higher EM dosing (>40% BV replacement) may exhibit complex multi-phase elimination due to saturation of the mononuclear phagocytic system (MPS), the principal route of elimination for EM. We have designed experiments to test this hypothesis, employing an established liposomal clodronate model for MPS depletion (see Narrative below).
   - Y3: due to move (St Louis → Baltimore) and pandemic lockdown, no work was performed on this task.
   - Y4: We confirmed PK for the optimized EM shell and completed noncompartmental nonlinear modeling to determine a payload-based range of dosing intervals for PFC. We will also confirm anticipated $t_{1/2}$ extension (following MPS depletion) of labeled liposomes and then of EM in this model and with BV replacement > 40%. These results will influence dosing in our PFC models (below).
4. **Safety/Toxicity**
   - Y2: Key exploratory work evaluating rheologic impact of "Nanocrit" (BV comprised by EM particles) in relation to Hematocrit (BV comprised by RBCs).
     - Circulating concentrations of EM (50, 75, 125, 150, 300 x 10^9 particles/mL) were tested in both murine and rabbit models. Findings indicates a slight impairment of O_2 transport (liver pO_2, lactate) when 'NanoCrit' exceeded 150 x 10^9 particles/mL (data in Y2Q1, Q2 and Q3 reports).
     - This is important safety information and given [Hb]/particle, this issue will not limit our ability to provide adequate O_2 carrying capacity during resuscitation, since the circulating 'NanoCrit" can be maintained below this level and still achieve adequate circulating [Hb] to support O2 delivery.
     - Note: Given the differences in flow dynamics and vessel caliber, we anticipate that the therapeutic window (for elevated NanoCrit) will be broader in humans, allowing for greater concentrations/higher particle abundance to be tolerated.
   - Y3: due to move (St Louis → Baltimore) and pandemic lockdown, no work was performed on this task.
   - Y4: We continue to evaluate the effect of EM in the efficacy experiments planned in Y4 and report on the basic safety – toxicity screen embedded in our efficacy protocols (basic metabolic profile, liver enzymes, renal function panel). In Q1, we evaluated relative complement activation (to stored RBCs) for EM in human plasma. In Q3, we characterized cytokine responses to EM in human blood. These experiments were delayed by Luminex availability in the UMB core and will report these data below.
5. **Efficacy**
   - Y2: Rabbit hemorrhagic shock model pilot (MT 7, Milestone 10)
   - Initial shock studies demonstrated non-inferiority of EM resuscitation to shed blood and superiority of EM- to Colloid-based resuscitation (for MAP, lactate, tissue pO_2), (Data reported in Y2Q1-3).
   - Several features have been evaluated to optimize our shock model and establish appropriate conditions for further PK analysis with volume replacement.
     - Initial studies were performed using ventilation with 100% oxygen. However, to realistically simulate hemorrhagic shock under field conditions, we reduced FiO_2 to room air.
     - We have now completed initial pilot work to optimize our hemorrhagic shock + polytrauma model (with pseudofracture & quadricep crush injury and liver laceration).
   - Y3: Due to move from St Louis → Baltimore and pandemic lockdown, no work was performed on this task in Y3. "Dry lab" working included preparation for PFC resuscitation scenarios (acute bleed, uncontrolled hemorrhage, acute bleed with polytrauma) – this required design, purchase and installation of state of the art telemetry system (secured with Dr. Doctor seed fund) to enable 48h FPC model.
   - Y4: We expect to complete efficacy experiments remaining in the SOW in the upcoming NCE year.
     - Work stopped on this grant at WUSM on 6/7/2019.
     - Due to personnel turnovers at the sponsoring agency, grant transition was delayed.
     - As expected, CBOTH lab staffing advanced to 75% capacity in Y4Q3, at which point, we resumed rabbit efficacy experiments (MT 7-10). Below, we report that we have successfully re-established the acute hemorrhagic shock-resuscitation model and re-established the non-inferiority of EMV2 to blood and superiority of EM to 5%Albumin. We also initiated pilot work on our 72h PFC model and report initial survival data as we calibrate the model to an LD50 insult (complete for scenario A). Once the model is calibrated for all scenarios, we will begin efficacy studies of EM in PFC.

6. **Team Transition to Baltimore**
   - Y2: Drs. Doctor, Pan and KaloCyte transitioned from WU, UIUC and the St. Louis Cortex District to join the Center for Blood Oxygen Transport and Hemostasis (CBOTH) at the University of Maryland, Baltimore – which Dr. Doctor directs. CBOTH is located in the new Health Sciences Facility (HSF) III and includes resources that will accelerate project task completion (10,000 sf labs, with six core labs: RBC and Hematology; Nanofabrication and Characterization; Imaging; Small Animal Surgery and Physiology; Analytical Chemistry; and Biospecimen Repository and Clinical Research.
     - Work stopped on this grant at WUSM on 6/7/2019.
     - Due to personnel turnovers at the sponsoring agency, transition of this grant was delayed.
   - Y3: The grant was successfully transferred from WUSM to UMB on 6/19/20, though funds were not activated for the Doctor Lab until 10/30/20. The grant was amended, effective date 20-Aug-2020, extending the period of performance to 29-Sept-2021.
   - Y4: After a complete pandemic-related shutdown, in Y4Q1, labs reopened, but at 25% capacity. In Y4Q2, lab capacity increased to 50% and our team completed new biocompatibility experiments demonstrating non-inferiority of EM (compared to stored RBCs) for complement activation in human plasma. In Y4Q3, lab staffing was increased to 75% and to 100% in Y4Q4.

Y4 Project Narrative: Here, we summarize work performed during Y4. As noted in prior reports, during the latter part of Y3, the UMB CBOTH laboratory underwent pandemic-related down. During Y4/Q1, CBOTH reopened with limited (25%) capacity. During this period, KaloCyte was able to continue use of temporary wet lab space in the UMB BioPark, made available to small commercial affiliates, and so was able to maintain operations during the pandemic shutdown. This work was limited to additional optimization and scaling of EM source materials and fabrication, to better prepare for an accelerated period of *ex vivo* testing that commenced in Y4/Q1 (biocompatibility) and continued in Q2 (pharmacokinetics/modeling). As noted above staffing progressively increased throughout Y4 and is now at 100%. Results from Y4 activity are presented below.

1. **Biocompatibility**
   a. **Complement activation in Human blood**
   i. **Method** 3 EM batches or stored RBCs (acquired from a commercial blood center, aged 42d) were incubated with freshly collected/prepared human plasma (healthy volunteers). RBCs and EM were adjusted to present the same surface area to blood (EM particle # was 50X stored RBC cell #). Samples were incubated for 30m, 1h, 12h and assayed for both C3a and C5a (Elisa).
   ii. **Results** EM, at particle concentrations likely to be used in massive transfusion, exhibit minimal complement activation relative to that elicited by stored RBCs.

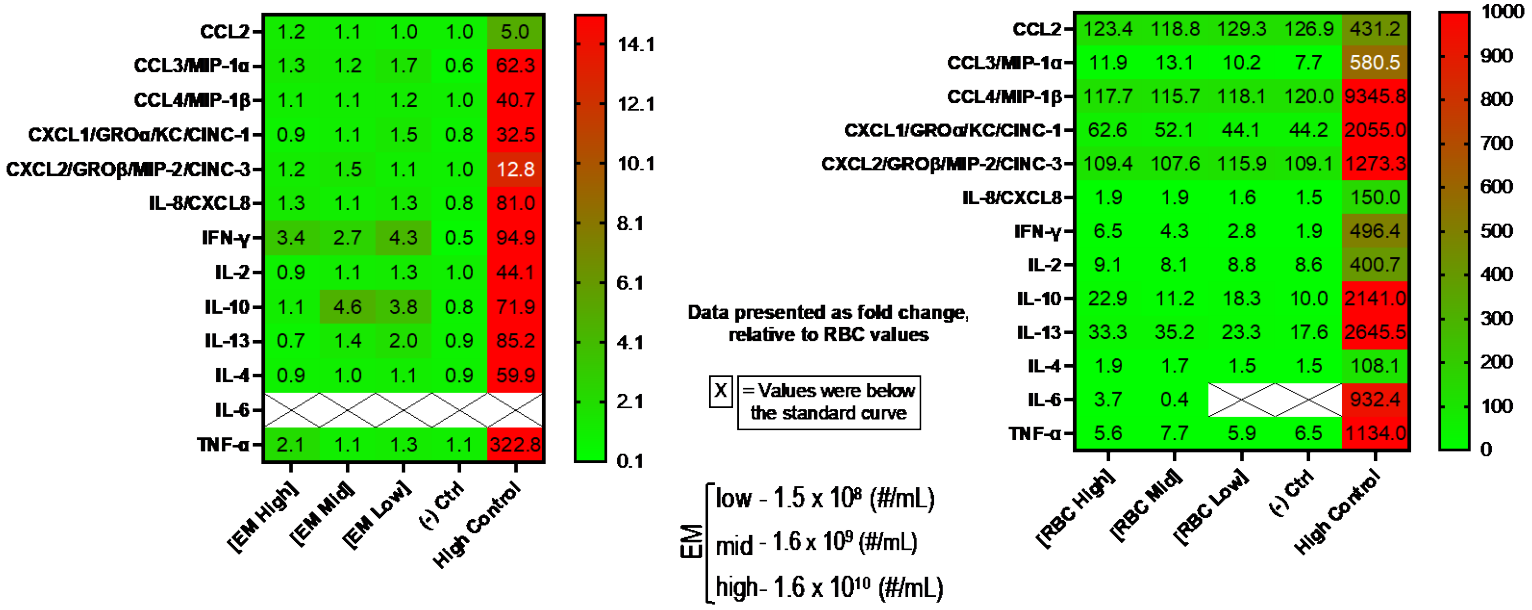


b. Cytokine response in Human Blood

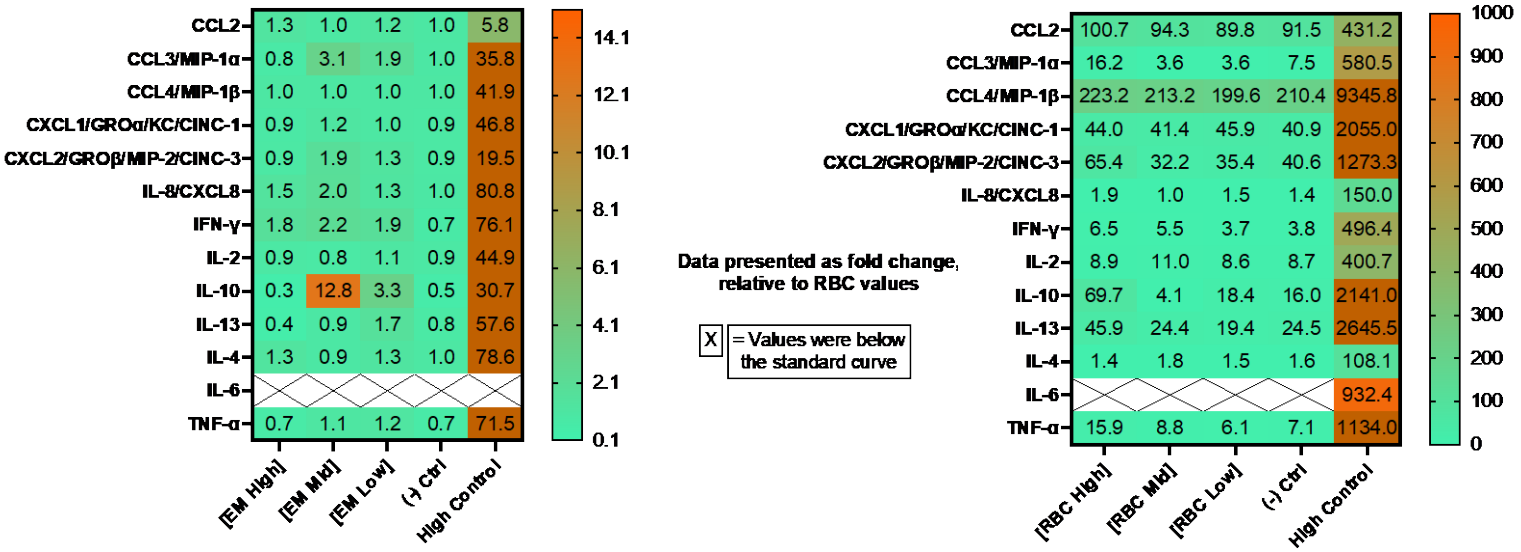
i. **Method** 2 EM batches or stored RBCs (acquired from a commercial blood center, aged 42d) were incubated with freshly collected/prepared human whole blood(healthy volunteers). RBCs and EM were adjusted to present the same surface area to blood (EM particle # was 50X stored RBC cell #). Samples were incubated for 30m or 120m and assayed for cytokine response (custom Luminex panel).

ii. **Results** EM, at particle concentrations likely to be used in massive transfusion, elicits minimal cytokine response relative to that by stored RBCs.

30 Minute Incubation in Whole Blood



120 Minute Incubation in Whole Blood

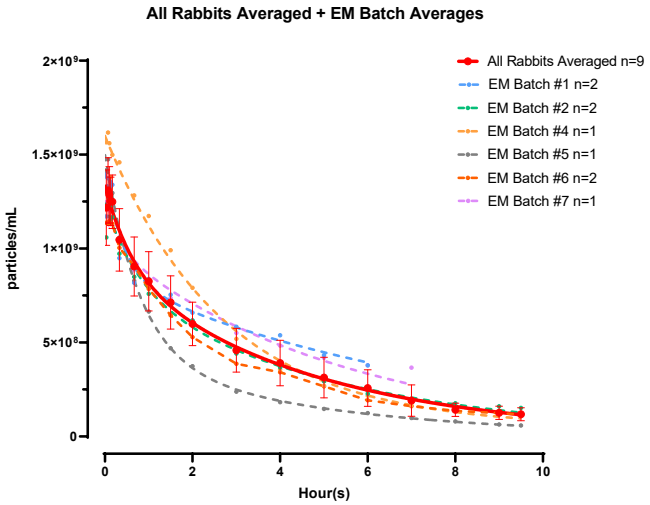


2. Pharmacokinetics (PK) and Biodistribution (Bio-D)

a. **PK Method** IRdye800-labeled EM to inject to achieve ~ 1.2 x 10^9 particles/mL, followed by serial blood sampling (9h), after which tissue, urine and bile was collected.

b. **PK Results** We found that EM, exhibited an elimination $t_{1/2}$ of ~ 4.5h. Non-compartmental modeling indicated that EM dosing every 7-8h would maintain [EM] within the anticipated therapeutic window.

Compartmental Analysis: Nonlinear Regression Two-Phase Decay Plot (constrained plateau to 0) demonstrated an elimination half life ($et_{1/2}$) of 4.6h.



Two phase decay	
Best-fit values	
Y0	Unstable
Plateau	= 0.000
PercentFast	32.05
KFast	1.591
KSlow	0.2168
Half Life (Slow)	3.197
Half Life (Fast)	0.4356
Tau (slow)	4.612
Tau (fast)	0.6285
Rate constant ratio	7.339

Noncompartmental Analysis: NCA metrics are presented below based upon EM particle # or [Hb].

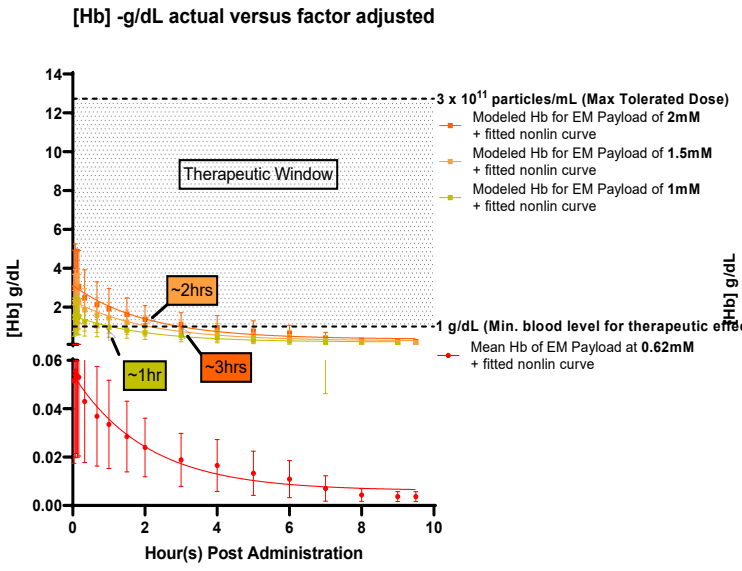
Hb NCA derived parameters:

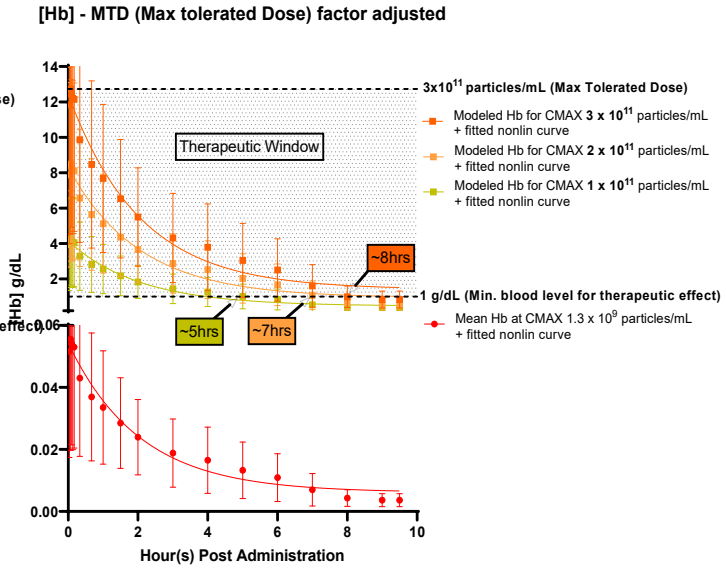
Rabbit #	Ave. [Hb] Dose (g)	Cmax (g/dL)	$AUC_{(0\text{-tlast})}$ (g/dL x hours)	$AUC_{(0\text{-}\infty)}$ (g/dL x hours)	Cl total (dL/hour)	$t_{1/2}$ (hours)	Vc (dL)
Ave. ± Std Dev.	0.067 ± 0.042	0.056 ± 0.036	0.15 ± 0.076	0.172 ± 0.09	0.397 ± 0.12	1.9 ± 0.19	1.219 ± 0.17

Particle NCA derived Parameters:

Rabbit #	Dose (particles)	Cmax (particles/mL)	$AUC_{(0\text{-tlast})}$ (particles/mL x hours)	$AUC_{(0\text{-}\infty)}$ (particles/ml x hours)	Cl total (mL/hour)	$t_{1/2}$ (hours)	Vc (mL)
Ave. ± Std Dev.	$1.6 \times 10^{11} \pm 1.5 \times 10^{10}$	$1.3 \times 10^{9} \pm 1.6 \times 10^{8}$	$3.9 \times 10^{9} \pm 5.5 \times 10^{8}$	$4.5 \times 10^{9} \pm 7.4 \times 10^{8}$	36.2 ± 11.5	2.1 ± 0.19	123.0 ± 16.9

Modeling of NCA data to determine PFC dosing interval

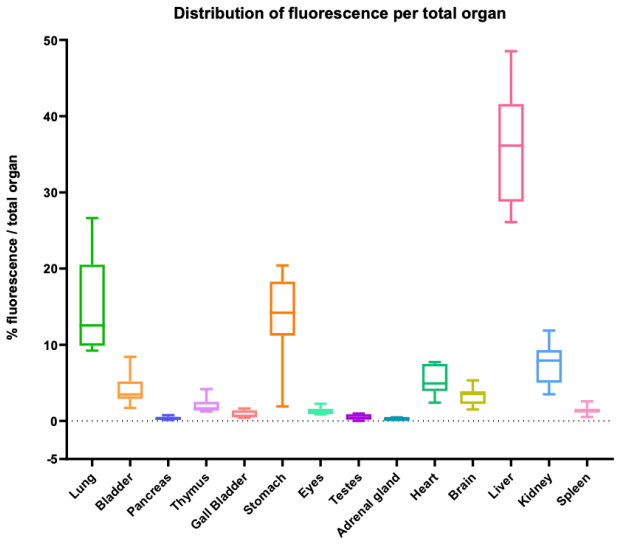




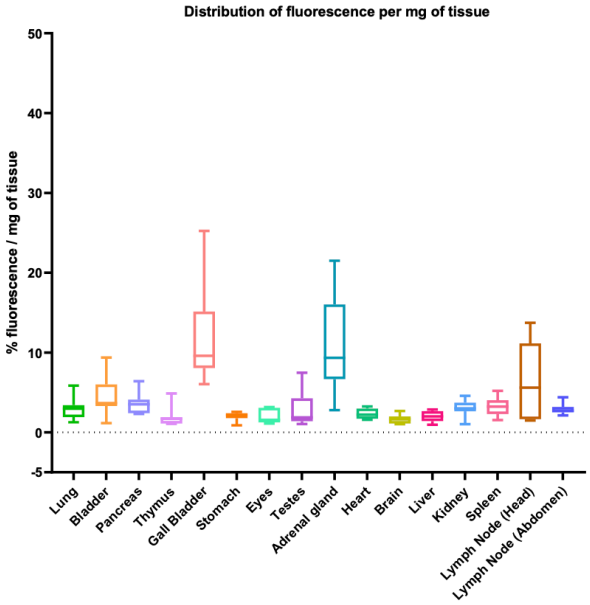
c. **Elimination Method** All urine and bile was collected at the end of the (9h) experiment.
d. **Elimination Result** 100% of eliminated EM-based fluorescence was found in bile; 0% was found in urine.
e. **Bio-D Method** Urine, bile and tissue from multiple sites (below) was collected, weighed, homogenized and assayed for EM abundance. Determine mean fluorescence/mg tissue for each organ & multiply by organ weight. calculate as proportion of total fluorescence (fluorescence/mg of all tissue).
f. **Bio-D Results** 100% of eliminated EM-based fluorescence was found in bile; 0% was found in urine. Also, As anticipated, EM partitions amongst organs in direct proportion to resident blood volume (organs were not flushed prior to analysis for fluorescence). Moreover, the principal route of elimination (as for other nanoparticles) is via the hepatic mononuclear phagocytic system, with biliary elimination.

Distribution of fluorescence (plot shows the range, mean and std dev of all rabbits)

Per Total Organ

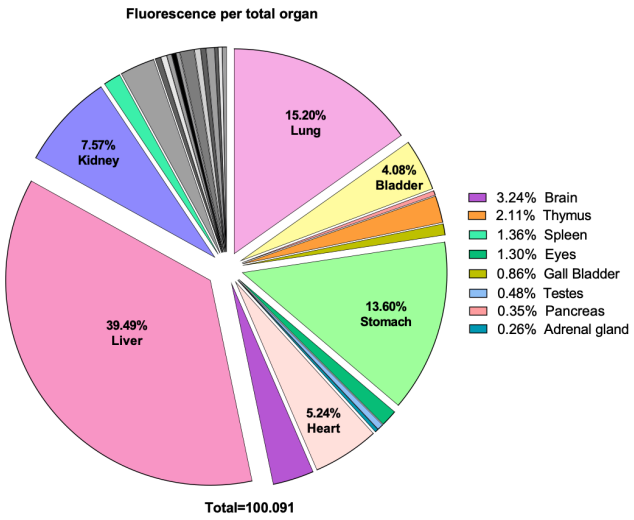


Per mg of Tissue

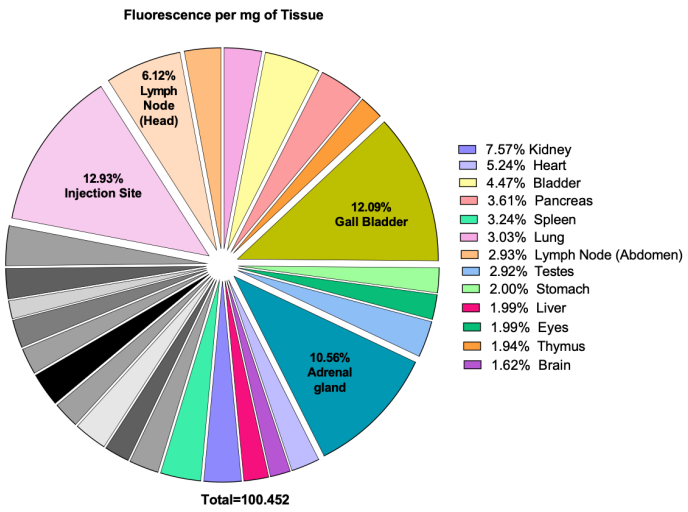


Distribution of fluorescence as proportion of total

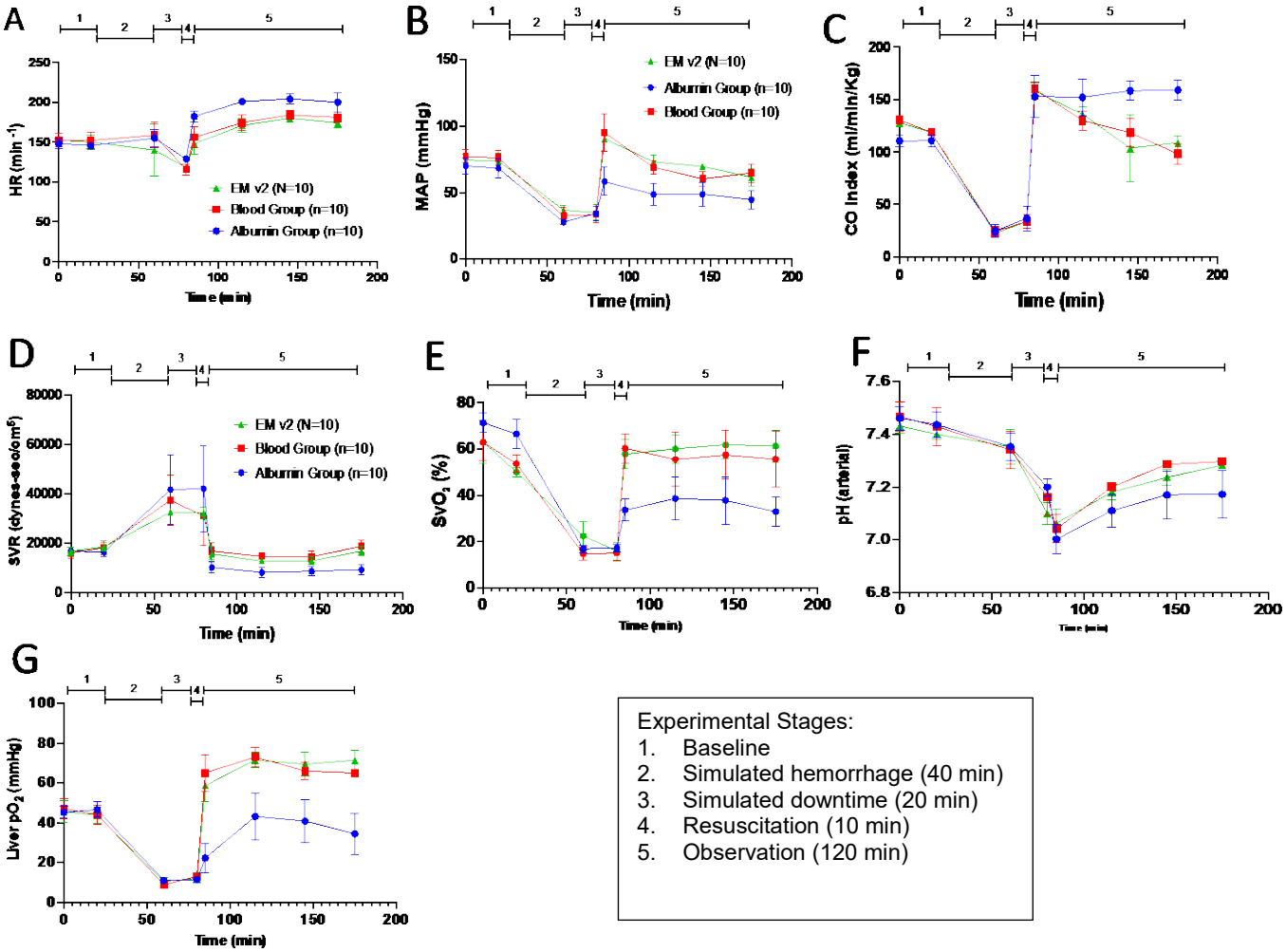
Per Total Organ



Per mg of Tissue



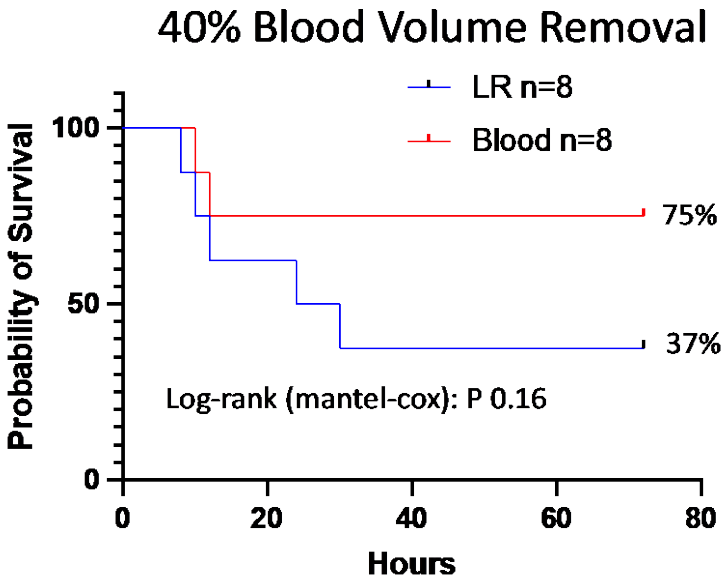
3. **Efficacy**
   a. **Acute Hemorrhagic Shock / Resuscitation Model (6h outcomes)**
      i. **Method** Rabbits (2.5kg) are instrumented, then hemorrhagic shock is induced by blood removal (40% BV, 40m according to a decelerating schedule (%ages are of the total BV to be removed: 50% over 1st 10m (~ 4 ml/h); then 25% over 2nd 10m (~ 2 mL/m); then 25% over next 20m (1 mL/m). Next 20 min downtime is simulated, followed by resuscitation (over 10m) by returning shed volume as actual shed blood, 5% albumin, or EM (in 5% albumin). Rabbits are then observed for 2h, then sacrificed
      ii. **Results** Data from our acute model affirms superiority of EM over 5% albumin and non-inferiority of EM to reinfusion of shed blood (where marked with *, RM-ANOVA, $p < 0.05$). These data are for our previously identified optimized post-resuscitation circulating concentration of EM (1×10^{11} particles/mL), which restores circulating [Hb] to ~ 70% of pre-resuscitation values. It is important to note the concordance between the direct measure of O_2 delivery (liver pO_2), indirect measures of O_2 delivery (SvO2, pH, lactate) and direct measures of cardiovascular compensation for inadequate O_2 content (HR, cardiac output, systemic vascular resistance) and overall restoration hemodynamic performance (mean arterial blood pressure). Our prior model (at Washington University in St Louis) did not include direct measurement of cardiac output and systemic vascular resistance; we have added that important functionality to this model, as one of the benefits in transitioning to the UMD Center for Blood Oxygen Transport and Hemostasis. It is also worth noting that systemic vascular resistance for the EM and blood groups are quite similar, affirming our safety data indicating similar NO trapping (by direct chemiluminescence measurement) and vasoactivity (by measurement in an ex vivo vascular ring array) between EM and RBCs.

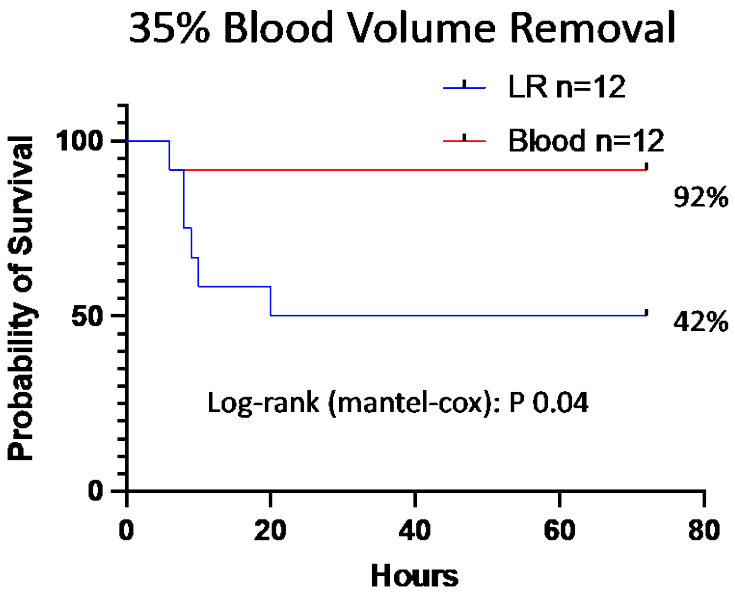


b. PFC Hemorrhagic Shock / Resuscitation (72h outcomes)

i. **Method** This complex protocol commences with a procedure to implant telemetry (carotid arterial pressure catheter) for monitoring HR, MAP and to implant tunneled internal jugular CVL, followed by 2d recovery. Hemorrhagic shock is induced as in the acute model (40% BV removal via the same decelerating blood loss schedule). Likewise, the downtime and resuscitation are identical to the acute model (LR is substituted for 5. Rabbits are then observed for 30m, then recovered. After which – HR, RR, and BP are monitored continuously via telemetry; if HR < 40 BPM or BP < 40 mmHg for > 30m, then euthanize.

ii. **Results** In Y4Q3, we piloted our 72h PFC model; our goal is to calibrate the severity of modeled hemorrhage (combination of %BV removal and downtime) to an LD50 model, using crystalloid-based resuscitation (since 5% albumin is not available for actual PFC). Although the #subjects are not sufficient to demonstrate mortality differences, our initial data do suggest that while 40% BV removal and 20m downtime is tolerated after resuscitation with 5% albumin; this level of hemorrhage is too severe for our PFC model, following LR-based resuscitation. In Y4Q4, we continued to pilot this model, calibrating the %BV removed to achieve LD50. We found that 35% blood volume removal resulted in 42% survival in the LR group, with excellent separation between blood/LR (with N=12 per group). In Y5, we will begin comparisons of blood and LR to EM based resuscitation.





What opportunities for training and professional development has the project provided?

In Y4Q1, multiple team members attended and presented at the American Association of Blood Banks (AABB); while we did not present results from this project, Dr. Doctor gave a lecture on transfusion decision making and anemia tolerance, our team also attended many presentations related to support of patients with hemorrhagic shock and resuscitation. In Y4Q2 Dr. Doctor delivered grant rounds at St. Jude Research Institute (discussing transfusion decision making). Multiple team members are at various stages in their research training; this project has greatly enhanced their education.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

As outlined above, our work has been paced by UMB lab reopening schedule. In the next reporting period, we expect to: complete our extended clodronate-based PK studies and initate evaluation of EM in our 72h PFC model. An adjusted milestone schedule was presented in our modified SOW (appendix to the Y4Q3 report) which described a schedule to complete our planned PK and oxygenation/acute shock studies in our PFC (72h survival models). These PFC models will be exploited to further our understanding of dosing (PK), biocompatibility, safety and efficacy. We will use this set of experiments to optimize our hemostasis/oxygenation algorithm in concert with Cellphire's platelet derived product, to determine compatibility of these products in *in vivo*.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

1. We have developed a unique rabbit model for precise evaluation of acute hemorrhagic shock and simulation of pragmatic in-field resuscitation; this model (uniquely) includes specific readouts for tissue oxygen tension as well as ability to independently evaluate impact of dilutional and trauma-induced coagulopathy.

2. We have identified a new parameter that helps determine maximum tolerated dosing for artificial RBCs or any encapsulated hemoglobin based oxygen carrier (HBOC) – we term this parameter the 'NanoCrit' and is the nanoparticle (encapsulated HBOC) correlate for the Hematocrit (which represents the %age of blood volume occupied by red blood cells. The combination of the NanoCrit and Hematocrit determine blood viscosity, which if increased beyond tolerance, may impair blood flow and oxygen delivery.

What was the impact on other disciplines?

1. As noted above, our hemorrhagic shock and resuscitation models enable independent evaluation of the two major causes for coagulopathy encountered in resuscitation of combat casualties (dilutional and trauma-induced coagulopathy); we have used these models in related projects to evaluate efficacy of hemostatic resuscitation (RDCR) – with plasma and platelets.

What was the impact on technology transfer?

This project involves significant partnership with KaloCyte, Inc. – a startup created to commercialize ErythroMer. During this project period, KaloCyte has made significant progress in advancing EM to a commercial product:

1. Raised additional significant private funding.
2. Relocated from the St Louis Cortex District to the UMB Biopark; the KaloCyte space (offices and lab) is embedded in the UM School of Medicine Center for Blood Oxygen Transport and Hemostasis.
3. KaloCyte has filed two additional provisional patents related to EM development.
4. KaloCyte has completed a strategic plan for EM development, with specific targets set for preIND (Q1, 2022), IND (Q3, 2022) and FIM testing (Q4, 2022).
5. **KaloCyte has now filed a pre-IND package with the FDA Submission Tracking Number (STN):PS007073; proposed dates for the pre-IND meeting are Proposed dates: Nov 29, 30, Dec 1-3. We anticpate reporting the results of that meeting, with written feedback from FDA in our next report.**

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

There have been no changes to the defined Aims, Major Goals or Tasks with regard to content. However, just prior to the end of project Y2, Dr. Doctor was recruited from Washington University (WUSM) to the University of Maryland (UMB), to serve as founding director for the Center for Blood Oxygen Transport and Hemostasis (CBOTH), a major resource that will accelerate EM development. Related to this transition, there were changes with other key personnel, which have been detailed in prior reports, including transition of KaloCyte from the St Louis Cortex District to the Baltimore Biopark (KC lab is now embedded in CBOTH, facilitating collaboration). Resulting from these changes, the timing of contract transition from WUSM to UMB, and from COVID19 related lab shutdowns, the only project work in Y3, was performed by KaloCyte; information is provided in detail below. As such, an NCE was requested and approved, enabling the team to resume progress (NCE-Y4) towards completing project goals; a time-adjusted SOW is included in this report. Additionally, given the extent of the lab shutdown and pace of reopening, In Y4Q3, we requested an additional NCE Year, with a a modified budget, justification and SOW.

A detailed summary of the major changes (scientific team and administrative activity) was provided as an appendix to our Y3 Annual Report.

An NCE request and modified SOW was provided as an appendix to our Y4Q3 quarterly report.

Actual or anticipated problems or delays and actions or plans to resolve them

In the project Y3, major delays resulted from: (1) team transition from St Louis to Baltimore and (2) administrative delays in grant transfer from Washington University in St Louis to the University of Maryland and (3) the COVID-19 pandemic and response. These issues and our mitigation plans were described in detail in the Y3 annual report; at which time, an NCE was requested and approved, enabling the team to resume progress (NCE-Y4) towards completing project goals. Our labs reopened in Y4, but at 25% capacity; as described above, in Y4Q1 our team completed new key biocompatibility experiments demonstrating non-inferiority of EM (compared to stored RBCs) for complement activation in human plasma.

In our Y3 annual report we stated the following: "We have discussed this situation in detail with DoD program staff and considering the carefully measured pace at which the University of Maryland is re-introducing laboratory activity (100% capacity may not occur for another 6-8m, as a function of pandemic activity and policy evolution), we do anticipate having sufficient information (regarding lab activity) to submit another NCE and revised SOW in our Y4Q2 report that will include a comprehensive mitigation plan to resume aggressive pursuit of experimental goals for this PFCRA."

As indicated in our Y4Q2/3 quarterly reports we now have an adjusted schedule that will enable us to complete the project aims, and we are now operating under an approved NCE with a modified budget, justification and SOW as recently submitted.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Multiple manuscripts are in preparation/submission, as indicated above.

Books or other non-periodical, one-time publications.

A new book is now in press with a chapter will be devoted to ErythroMer: Title: Blood Substitutes and Oxygen Therapeutics, Ed: Jonathan Jahr, Chapter 22: ErythroMer. Allan Doctor

Other publications, conference papers and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

KaloCyte, Inc maintains a website that provided updated information on company activity and press releases: https://www.kalocyte.com/ The UMB Center for Blood Oxygen Transport and Hemostasis has opened it's website, which includes information related to EM development: https://www.medschool.umaryland.edu/CBOTH/

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

1. During Y1, an exclusive license was obtained by KaloCyte from Washington University for all relevant patent rights for EM-V1: Pan D, **Doctor A**, Spinella PC and Lanza GM. Blood Substitute Composition and Method of Use. U.S. Patents # 9,486,508; 9,655,952 & 9,750,241. 2016. Also filed in: Europe, Australia, Canada, Japan and South Africa.

2. A provisional patent application has been filed by KaloCyte for its development of the novel composition (EM-V2). Pan D, Spinella PC and **Doctor A.** Self-Assembling Oxygen Carrier Compositions. US Patent (Provisional) # 63,014,665. 2020. *This patent is now published: October 28, 2021 as Publication number US- 2021-0330754-A1.*

3. A provisional patent application has been filed jointly by KaloCyte and UMB for a novel system to prevent interference by ErythroMer of laboratory instrumentation, in blood of trauma victims who received EM. Pan D, Mittal N and **Doctor A**. Compositions and Methods for Removing Bio-Synthetic Nanoparticles from Bodily Fluids. US Patent

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

The table below indicates the various changes in institutional participation and roles with transfer of this award from Washington University in St Louis to the University of Maryland, Baltimore (UMB). Dr. Doctor (PI) transitioned from WUSM to UMB and also recruited Dr. Pan (UI) to join him at the new Center for Blood Oxygen Transport and Hemostasis. The collaboration with Dr. Bochicchio (WUSM), a trauma surgeon who supported our animal models was transitioned to Dr. Wang (UMB) a small animal surgeon and Director of the CBOTH Small Animal Surgery and Physiology Core who will support our animal models. Dr. Spinella (WUSM), who advised regarding our hemostasis assays, remained at WUSM, without change in effort. Dr. Gill moved to American University; his role is unchanged. Dr. Zuppa (CHOP) who supported our pharmacokinetic analyses will be replaced by Dr. Buehler (UMB), a pharmacologist specializing in HBOC evaluation recruited to CBOTH from FDA CBER. KaloCyte – also moved from St. Louis to Baltimore and with this move Dr. Richards was replaced with Dr. Mittal and Dr. Wang was replaced with Dr. Yildiz. There are no changes with regard to Cellphire.

Investigator Changes

Institution	Original Award		After Transfer of Award	
	Investigator	Role	Investigator	Continued Role or Change
Washington University (WUSM)	Doctor	PI		Moved to UMB
	Bochicchio	CoI – surg model		Replaced by Wang (UMB)
	Spinella	CoI - hemostasis		CoI – hemostasis (remains at WUSM)
	Gill	CoI - biostats		Moved to AU
University of Maryland, Baltimore			Doctor	PI, Moved from WUSM
			Pan	CoI, Moved from UIUC
			Buehler	CoI, replaces Zuppa (CHOP)
			Wang	CoI, new – surgical model

Institution	Original Award		After Transfer of Award	
				Replaces Bochicchio
			Rogers	CoI, moved from WUSM EM benchmarking
University of Illinois (UI)	Pan	CoI – bioengineer		Moved to UMB
Children's Hosp Philadelphia (CHOP)	Zuppa	CoI – pharmacology		Replaced by Buehler (UMB)
American University			Gill	CoI - biostats Moved from WUSM
KaloCyte, Inc	Richards	CoI – ErythroMer	Mittal	replaces Richards
	Wang	CoI – ErythroMer	Yildiz	replaces Wang
Cellphire, Inc	Fitzpatrick	CoI – Thrombsomes	Fitzpatrick	No change

Changes to Personnel (Lab Staff)

Institution	Original Award		After Transfer of Award	
	Personnel	**Role**	**Personnel**	**Continued Role or Change**
Washington University (WUSM)	Xue Lin	Doctor Lab staff		No longer on project
	Jose Aldana	Bochicchio Lab Staff		No longer on project
	Rohit Rasane	Bochicchio Lab Staff		No longer on project
	Sarbani Ghosh	Bochicchio Lab Staff		No longer on project
	Anja Fuchs	Bochicchio Lab Staff		No longer on project
University of Maryland, Baltimore (UMB)			Mary Brummet	Doctor Lab Manager
			Alex Lander	Doctor Lab Technician
			Tori Boyer	Doctor Lab Research Coordinator
			Parikshit Moitra	Pan Lab Postdoctoral Fellow
University of Illinois (UI)	Dinabandhu Sar			No longer on project
	Maha Alafeef			No longer on project
Children's Hosp Philadelphia (CHOP)				
American University				
KaloCyte, Inc	Richards	CoI – ErythroMer	Nivesh Mittal	CoI (replaces Richards)
	Wang	CoI – ErythroMer	Tugba Yildiz	Formulation Scientist (replaces Wang
			Shannon Dougherty	KaloCyte Lab Manager
			Darci Bartlett	KaloCyte Program Manager
Cellphire, Inc				No changes

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

none

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

Updated Quad Chart