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PRINCIPAL INVESTIGATOR: Doctor, Allan

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

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Dr. Allan Doctor

E-Mail: adoctor @som.umaryland

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Center for Blood Oxygen
Transport and Hemostasis
(CBOTH)
670 W. Baltimore St.,8th Flr.
Baltimore, MD. 21201

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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 approved. CBOTH reopened in Y4 at 25% capacity, by Q4 we were fully operational; during that period, we completed biocompatibility, rheology, PK/biodistribution/elimination experiments and advanced modeling of PFC dosing requirements; we re-established our HS/R model (with new O2 delivery-consumption analyses), confirming EM V2 efficacy and initiated pilot work for our PFCRA survival model. In, we Y5Q1-3 reported on a further optimized EM shell (EM V2.1) with anticipated dosing interval for PFC of ~ 30h (major improvement) and presented calibration of HS severity in our PFC survival models to an ~ LD50 outcome for the negative control group (Lactated Ringers) and presented (near complete) 72h survival data for EM (non-inferior to WB, superior to crystalloid) in PFC Scenario A. In this FINAL REPORT for the project, we review all results, by MT and show final survival outcome and safety data for our PFC models; moreover, we summarize impact of the project and next steps (including awarded grants related to this project).

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.

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?

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 of high/low O_2 affinities *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. (completed, target 11/2022). PK for fully optimized EM shell (EM $V_{2.1}$): KC1003:DPPG:DPPC:DPPE(PEG2000):cholesterol (mol% = 5:7.5:54:3:30) indicates that a dosing interval of ~ 30h will be suitable for PFC (substantial improvement). (completed 03/2023)

Major Task 4: Develop EM PFC dosing.

Milestone #6: Confirm EM dosing strategy for rabbit PFC models; informed by MPS depletion model. (completed 03/2023)Also, see above.

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. (completed 09/2022).

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). (completed).

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 LR, blood and EM-based resuscitation) for HA administration. (completed, target 12/2022).

Major Task 10: Screen EM safety *in vivo*.

Milestone #14: Identify lab and histologic evidence of EM toxicity during resuscitation from PFC Scenarios A-C, in comparison to that observed in blood re-infusion and LR resuscitation groups. (completed 09/2022).

What was accomplished under these goals?

MT 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).

Table 1: Comparison of p50: RBCs, Hemoglobin (Hb), and ErythroMer (EM)

p50	7.2			7.4			7.6		
	Mean	±SEM	n	Mean	±SEM	n	Mean	±SEM	n
RBC	30.49	0.3665	6	24.76	0.4339	6	19.64	0.3941	6
Free Hb	14.32	0.6108	8	11.61	0.2928	15	10.69	0.525	8
Free Hb:DPG 1:1	13.23	0.3765	4	11.33	0.1141	4	9.66	0.2177	4
Free Hb:DPG 1:5	16.02	0.5166	4	12.82	0.4341	4	10.41	0.1156	4
Free Hb:RSR 1:1	17.82	0.31	2	15.93	1.315	2	14.52	1.555	2
Free Hb:RSR 1:5	32.11	1.015	2	27.94	1.14	2	24.47	1.355	2
EM Pure Hb	12.28	1.173	8	10.62	0.7003	12	10.48	1.101	7
EM Hb:DPG 1:1	15.07	0.8769	7	12.17	0.4874	8	11.66	0.6166	6
EM Hb:DPG 1:5	16.64	1.949	6	14.84	0.5243	6	12.42	0.4872	5
EM Hb:RSR 1:1	22.32	0	1	20.06	0	1	18.7	0	1
EM Hb:RSR 1:5	37.79	0.065	2	33.33	1.385	2	27.09	1.395	2

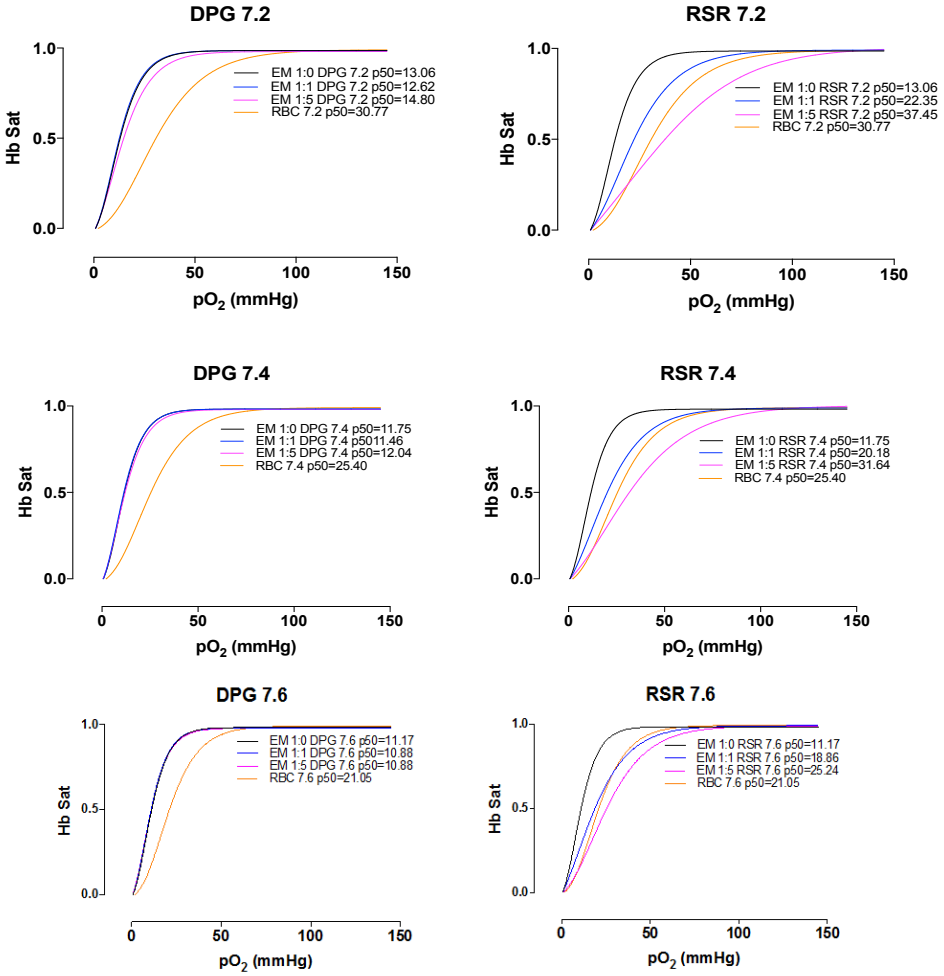
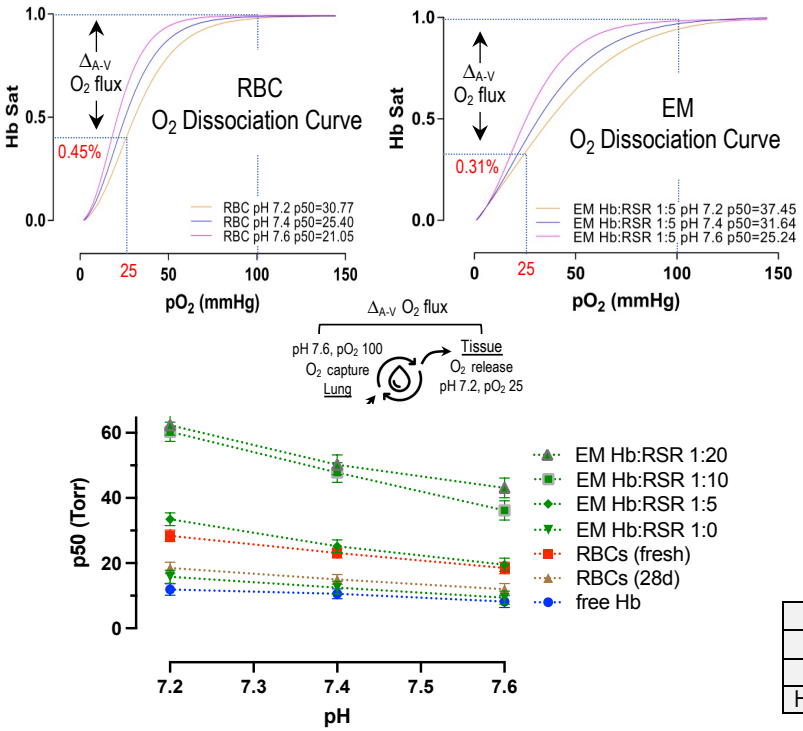
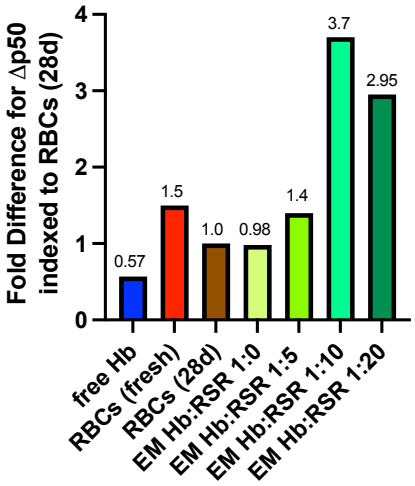


Fig. 1: Oxygen Dissociation Curve (ODC). By shifting the ratio in favor of the allosteric effector compared to Hb (5:1), the ODC is right shifted resulting in more efficient release of O_2 in response to reduced pO_2.

Figure 2. Arterio-venous O2 flux estimates for EM formulations with differing oxygen affinities (p50) for pH gradients commonly encountered during circulatory transit in the course of resuscitation for hemorrhagic shock (see cartoon/schematic depicting lung → tissue gradients). The relative potency (with regard to arterio-venous O2 flux per gram HbEM with the lowest oxygen affinity (Hb:RSR of 1:10) to that for stored RBCs (28d storage duration) is 3.7:1 (see fold difference plot); therefore an equipotent blood level (for Hb) of EM (relative to stored RBCs) is reduced by this difference in relative potency (see Table) and permits (a) conservative initial dosing and (b) extended dosing intervals – both enhancing EM suitability for PFC use.





	RBCs (fresh)	RBCs (stored)	EM
Circulating Hct	30	45	12
Circulating [Hb]	10	15	4
Hb per 300 mL unit	50	75	20

- Y2: Optimized design w/r/t biocompatibility, improved payload retention, and improved lyophilization/reconstitution, yielding EM-V2. This design is described in prior annual reports an in the following publication (full reprint in appendix):
  - Mittal N, Rogers S, Dougherty S, Wang Q, Moitra P, Brummet M, Cornett E, Kaye A, Shekoohi S, Buehler P, Spinella P, Pan S, and Doctor A. Erythromer (EM), a Nanoscale Bio-Synthetic Artificial Red Cell. In Blood Substitutes and Oxygen Biotherapeutics. Eds: Jahr J, Liu H, Kaye A, and Scher C. Springer Nature, SPi Global. 2021.
- Y3: Optimization of ErythroMer components and processes, including fabrication and assembly; cleanup; and lyophilization/reconstitution (details in prior annual reports).
  - Of note three complimentary proposals have been awarded to support additional optimization of the EM formulation:
    - NIH/NHLBI SBIR Phase I H193-004-0067 "Rapid Reconstitution of a Lyophilized, Bio-inspired, Artificial Red Blood Cell."
    - NIH/NHLBI PhIIb R44 HL135965-A1 "ErythroMer: Nanoscale BioSynthetic Red Cell Substitute"
    - DARPA HR001121S0027-FSHARP-FP-001 CONCERT: Consortium for Optimized Integration of Bio-artificial Blood Components for Adaptive Resuscitation and Therapy

This additional MT 1 activity is briefly summarized on the following page:

Optimization of EMv2 preparation (MT 1, Milestones 1 and 2). From the perspective of sample preparation and quality, formation of EM nanosuspensions were studied using various techniques such sonication, membrane hydration, ethanol injection and high pressure microfluidization. Interestingly some notable differences were still noticed (**Figure 3**). The generation of EM involved self-assembly of their constituent parts from a mixture of amphiphile in micellar state. The constituent parts are as follows: i) phospholipids (20 mole%); ii) mixture of hemoglobin and allosteric effector RSR13 and iii) lipid-amphiphile-precursor (80 mole%). EMv2 has been designed with a biocompatible lipid-oligomeric amphiphile chains across the surface, producing a net negative zeta potential, excellent payload retention and differential gas permeability. Moreover, unlike phospholipid bilayers in liposomal-based HBOCs, EM has a tunable membrane offering greater integrity due to counterionic Hb and precursor interaction and pH responsiveness. EM V2 can be classified as a hybrid-vesicles resulting from the combined self-assembly of both amphiphilic lipid-oligomer into an advanced vesicular structure. To afford such a design, the different parameters controlling both self-assembly and membrane structure must be tuned. Compared to EM V2, the cell biomimetic character of EM V1 is rather limited as polymeric amphiphile in EM V1 is entirely synthetic in nature, while lipid-oligomeric amphiphiles used in EM V2 are mostly natural components of the cell membrane. Optimization studies revealed that hybrid vesicular structures can be obtained according to the molar composition and thermodynamic phase of the phospholipids and precursor mixture. In the composition optimization experiment, the effect of precursor and cholesterol concentration on particle size was investigated by varying the concentration of precursor and cholesterol. All the samples were prepared by following the EMv2 procedure of probe sonication followed by purification using tangential field flow fractionation (TFF). DLS and zeta potential measurements were obtained in triplicate for each measurement. The average mean and their induvial std. dev. are calculated for each measurement and the results were compared to each other. Results indicated that increasing the cholesterol concentration will increase the stability and Hb encapsulation. (**Figure 4a**).

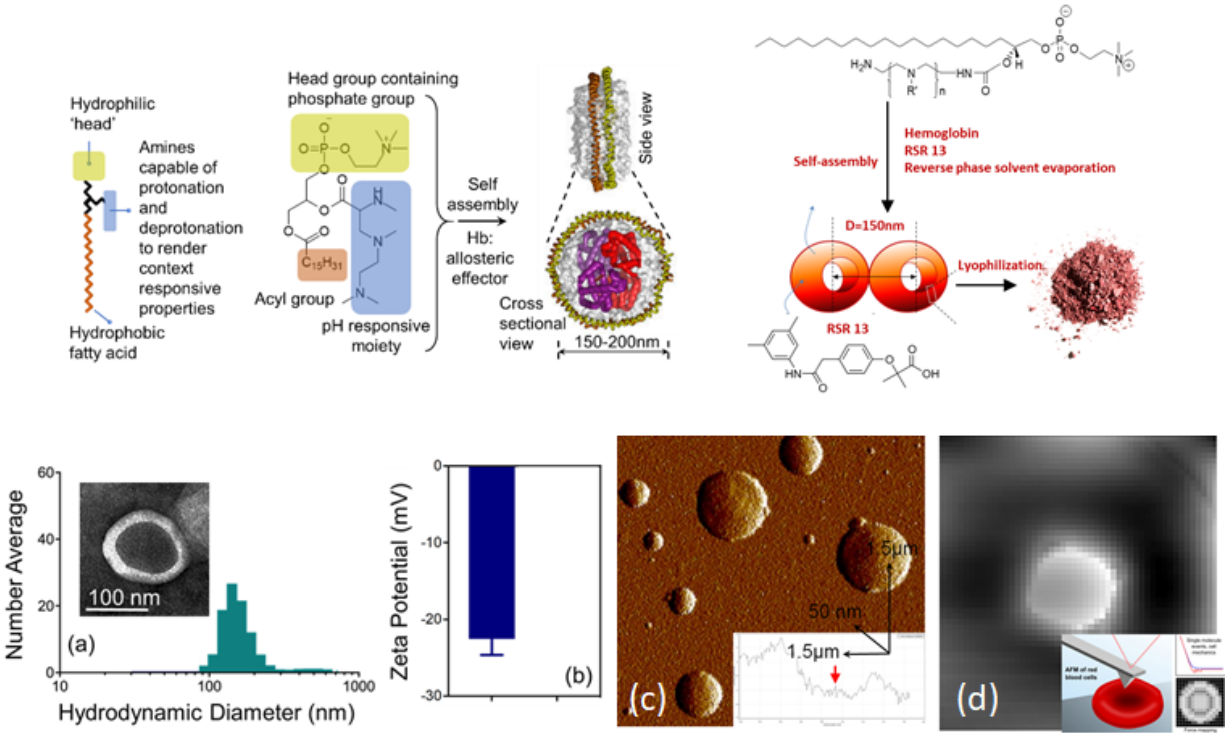


Figure 3. Schematic representation of the composition and self-assembly; chemical structure of the precursor and the process. (a) Hydrodynamic diameter and TEM image (inset) and (b) electrophoretic potential results. Tapping mode atomic force microscopic analysis of EMv2 and RBC (inset) from doi.org/10.1016/j.cis.2017.05.011 .

Lyophilization and resuspension (MT 1, milestones 1 and 2). Once lyophilized, the EM nanoparticles form easy-to-handle fluffy cakes (**Figure 1**) which require manual shaking and short resuspension times and maintain the size characteristics of the origin suspensions (**Figure 2b**). Indeed, it appears that most particles retained their initial size and surface properties during the lyophilization/resuspension process, and that aggregation is not an encumbering issue. The respective electrophoretic potential values (mV), was employed here as indicators of the stability of EM colloidal dispersions, also remained mostly unaffected by the lyophilization and resuspension procedure and may even improve marginally. Unlike earlier liquid preparations, these particles also incorporated RSR13 as allosteric effector. Results indicated that the presence of RSR13 helps with the stability of the particles (**Figure 2c**). For characterization, we have employed nanoparticle tracking analysis (NTA) to measure particle size by video tracking, simultaneously, many individual particles. This results in a particle size distribution of high resolution, particle concentration. Results indicated the presence of multiple population in the suspension which is very typical for a 'soft' particle. (**Figure 2d**). Overall, the results obtained as part of this study support the concept that suspensions of stabilized EM-nanoparticles can be lyophilized to yield ready-to-use powders which can be resuspended on demand without notable loss of particle quality (i.e., no significant aggregation).

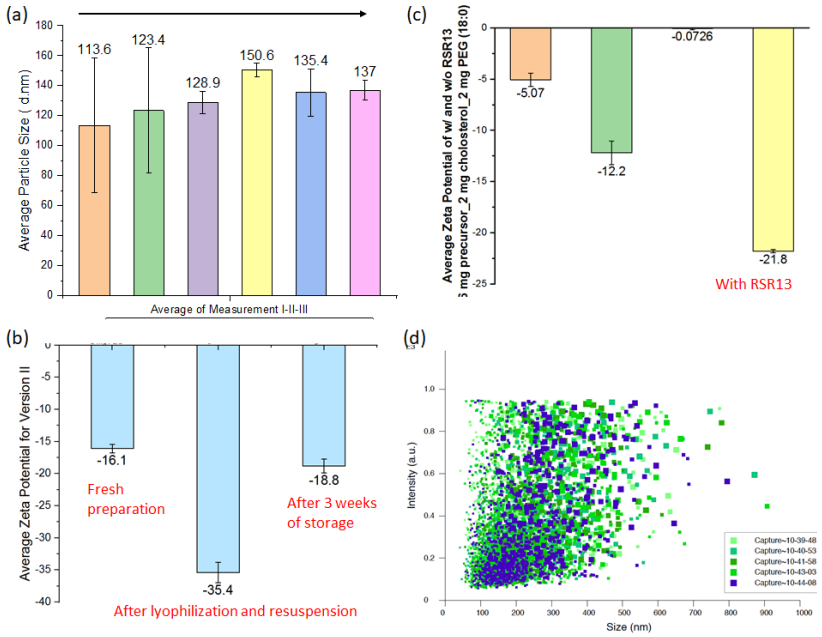


Figure 4. (a) Hydrodynamic diameter changes with the increasing concentration of cholesterol; (b) stability measurements immediately after preparation, after lyophilization and resuspension and after 3 weeks of shelf life study; (c) NTA study measures mean square displacement in two dimensions.

Sterility testing (MT 1, milestone 1 and 2). The EMv2 samples were subjected to membrane filtration 0.45µm cellulosic membrane filter. To investigate the presence of the microorganism in the EM sample, a bacterial culture test was done. LB agar plate was used as a nutrition media to culture the bacteria from EM unsterile sample. In a sterile environment, an aliquot of the EM sample was streaked on the plate and incubated at 37°C for 48h. Around 10 separate colonies were grown indicating the contamination of the sample with microorganisms (**Figure 5**). The number of colonies was found to be negligible indicating a very low concentration of microorganism's presence in the sample. The number of colonies in the region where the sample is diluted because of the streaking (low concentration region) showed no colonies which further confirmed the low bacterial concentration in the original sample (**Figure 5**).

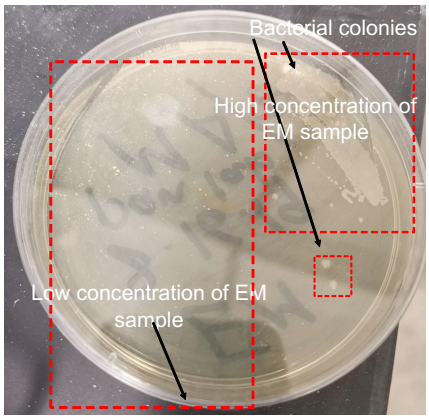


Figure 3. LB agar plate image after 48h incubation, 37°C after EM sample streaking. The lack of bacterial colonies is evident.

MT 2: Test efficacy of high/low O_2 affinities *in vivo.*

- Y2 Milestone #4: Establish O2 delivery benefit conferred by EM prototypes with high/low O2 affinities (defined as 20% improvement in tissue pO2 relative to current prototype). (completed 04/2019)

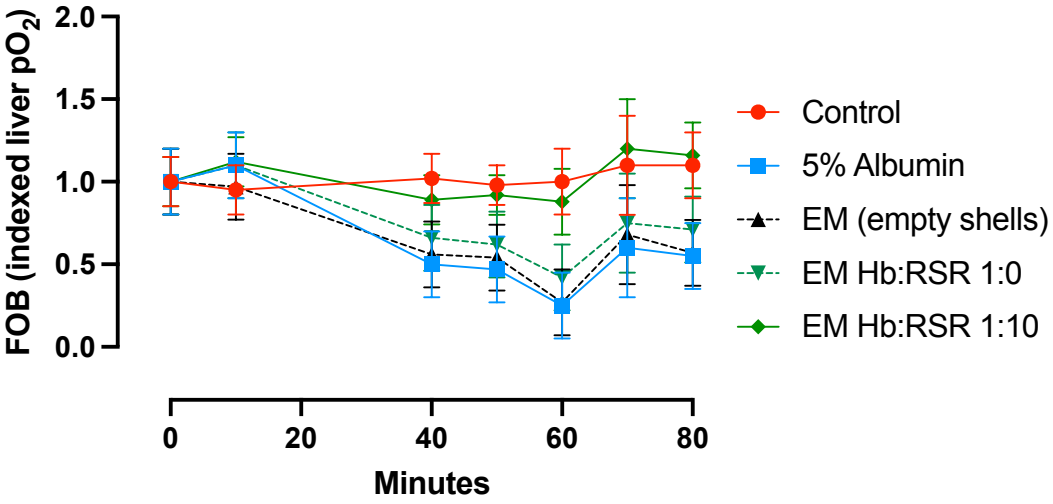


Figure 6. Liver pO2 for EM with high/low O2 affinities. The above plot represents relative differences in liver tissue pO2 (measured directly via fiberoptic phosphorimetry (OxyPhor G4) as described in prior annual reports and below, for MT 7-10. The model involved 40% blood volume replacement (via Exchange Transfusion, com pleted by 20m) and are reported for control (blood/blood exchange, 5% HAS, EM empty shells, and EM with low O2 affinity (Hb:RSR 1:10) and high O2 affinity (Hb:RSR 1:0) [also, see Figures 1 &2]. Low affinity EM shows non-inferiority to shed blood and clear (~ 40% improvement in liver pO2) superiority to high affinity EM (as expected). The low affinity formulation was used in the remainder of our *in vivo* experiments.

Major Task 3: Measure EM pharmacokinetics (PK).

Milestone #5: Calculate EM PK as a function of Blood Volume (BV)% replacement.

- 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) (Figure 7)
- 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 (Figure 8).
- 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 (Figure 9).
- Y5: We completed the PFC PK dose data acquisition (on EM V2) in Y4 and finalized PK modeling for fully optimized EM shell (EM V2.1): KC1003:DPPG:DPPC:DPPE(PEG2000):cholesterol (mol% = 5:7.5:54:3:30) in Y5, that indicated that a dosing interval of ~ 30h will be suitable for PFC (substantial improvement) – we completed the complex PK model below (Figure 10).

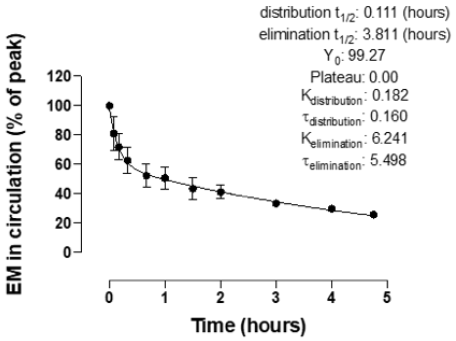


Figure 7: Initial analysis of top-loading experiments with EM-V2 in rabbits (n=6) demonstrated elimination $t_{1/2}$ ~ 4.5 hours. Top-loading volume was 10% of blood volume based on weight; the particle concentration of EM-V2 administered was 250 x 10^9 particles/mL in lactated ringers, which translates to a circulating concentration of 57.7 x 10^9 particles/mL.

Clodronate based depletion of the Mononuclear Phagocytic System (MPS) to simulate MPS saturation during massive transfusion (MT 4, milestone 6). Studies to date continue to support our hypothesis that EM clearance from the circulation is driven by the MPS, which may become saturated during massive blood volume replacement with EM. MPS saturation will result in a reduced EM clearance rate (extended circulation time). However, in our rabbit model of acute hemorrhagic shock/resuscitation, the ability to evaluate the extent to which this saturation will affect EM half-life is complicated by ischemia-reperfusion (which alters MPS saturation thresholds). To address this, we have recently employed selective macrophage depletion (and depletion of circulating monocytes/macrophage-precursors) with systemic injection of liposomal encapsulated clodronate (dichloromethylene bisphosphonate) prior to infusion with EM. This approach enabled us to fully appreciate the extent to which MPS saturation will increase the half-life/circulation time for EM. In order to evaluate the effect of tissue macrophages on EM circulation, we first used dose escalation studies with clodronate-liposome 24 hours prior to collection of tissues (liver and spleen). After determining dosing for maximal MPS depletion, we will employed fluorescently-labeled liposomes (empty/no clodronate but same composition as clodronate-liposomes) as an EM-surrogate (control), as these particles are known to be cleared by the MPS, to further validate our PK studies with EM. Testing the effect of MPS depletion 24 hours prior to administration of either EM or fluorescently-labeled liposomes will help us model the circulation time for EM when the MPS is severely or totally saturated or otherwise affected (ischemia-reperfusion injury).

Clodronate dose escalation studies (MT 4, milestone 6). The conditions for tissue macrophage identification in dissociated and collagenase-digested liver and spleen tissue had been previously established using mouse tissues (separate experiments), so these studies were optimized based on this prior work. To confirm the level of macrophage depletion, flow cytometry was used to evaluate the presence of tissue macrophages in the liver and spleen. We dissociated and collagenase-digested spleen, and liver tissue from the control rabbits (received empty liposomes) and the clodronate liposome-infused rabbits (0.75ml/kg, 1.5ml/kg, 3.0ml/kg, or 6.0ml/kg) for flow cytometry analysis at 24h post-infusion. However, during our preliminary testing, we found that interpretation of the spleen and liver data is complicated as clodronate treatment appears to cause an influx of neutrophils into the tissues. As this major neutrophil population made identification of macrophages more challenging, we optimized conditions with more specific macrophage-targeting antibodies, which have demonstrated promising results. Using the macrophage-specific antibodies, flow cytometry on the liver, spleen, and blood samples revealed a near-complete depletion of monocytes from the blood in the clodronate liposomes-treated rabbits as well as a near-complete depletion of tissue macrophages with the higher concentrations (3.0ml/kg, or 6.0ml/kg) of clodronate liposomes.

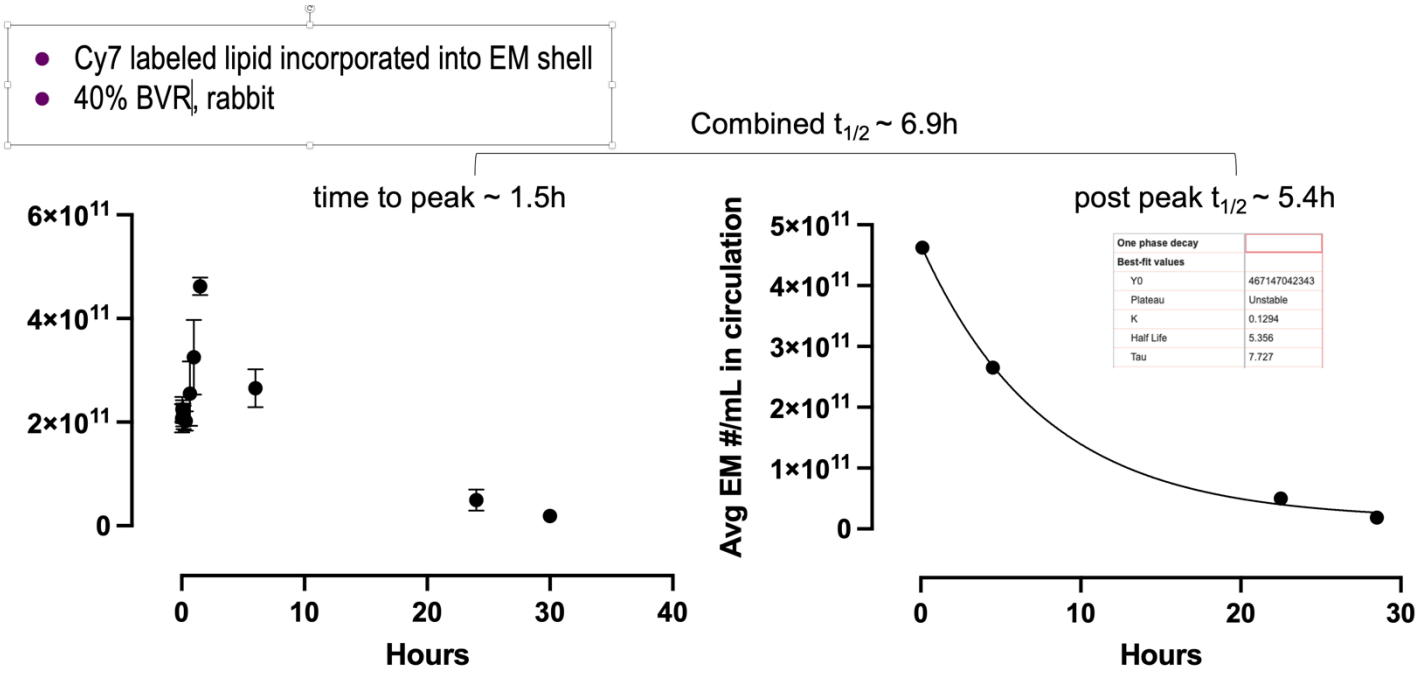
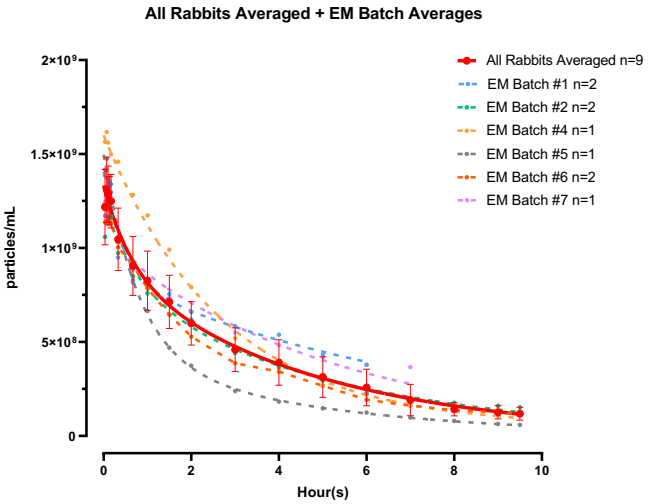


Figure 8: Blood volume replacement experiments with EM-V2 in MPS-depleted rabbits (n=6) demonstrated elimination $t_{1/2}$ ~ 6.9 hours (a 53% increase). Blood volume replacement was 40% of blood volume based on weight; the particle concentration of EM-V2 administered translated to a peak circulating concentration of 5.3 x 10^{11} particles/mL.

Figure 9 Pharmacokinetics (PK) and Biodistribution (Bio-D) 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. **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].

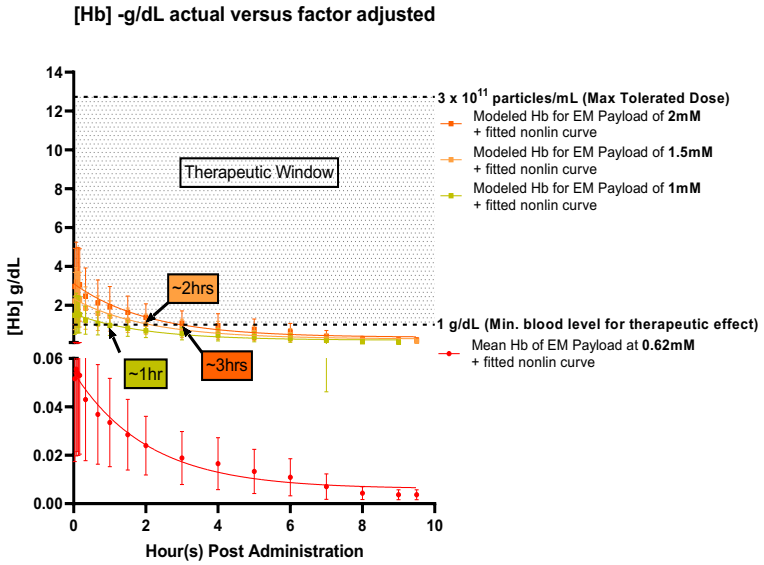
Hb NCA derived parameters:

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.	1.6×10^{11} ± 1.5×10^{10}	1.3×10^{9} ± 1.6×10^{8}	3.9×10^{9} ± 5.5×10^{8}	4.5×10^{9} ± 7.4×10^{8}	36.2 ± 11.5	2.1 ± 0.19	123.0 ± 16.9

Initial Modeling of NCA data to determine PFC dosing interval for EM V2, estimated to be 8h.



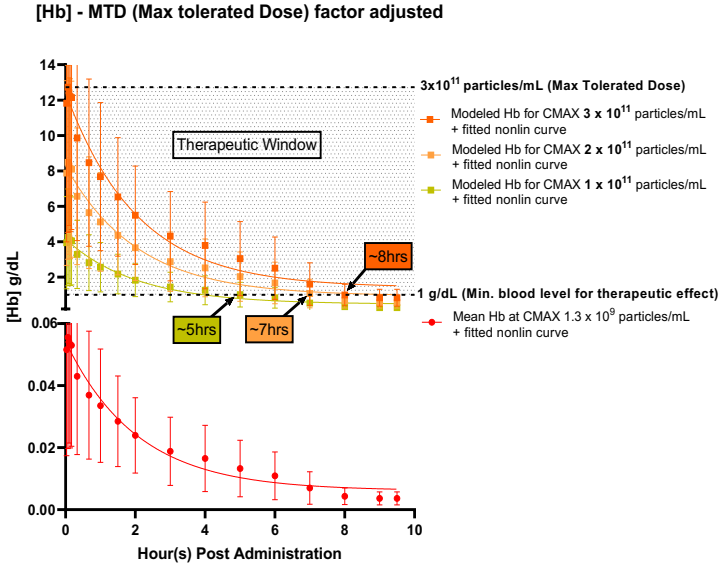
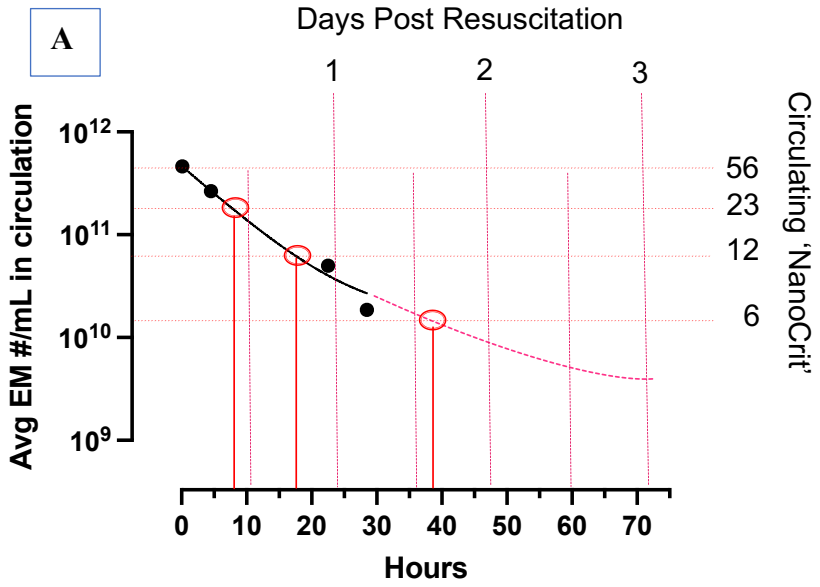


Figure 10 Finalized PK modeling for fully optimized EM shell (EM V2.1):
KC1003:DPPG:DPPC:DPPE(PEG2000):cholesterol (mol% = 5:7.5:54:3:30) in Y5, that indicated that a dosing interval of ~ 30h will be suitable for PFC (substantial improvement) – we completed the complex PK model below. (A) results from a 10% blood volume top loading experiment indicate that a dosing interval of ~ 15h would be required to maintain circulating EM-based 'nanocrit' equivalent to a (fresh RBC) hematocrit of ~ 30. (B) results from a 40% blood volume replacement in a model of full hemorrhaghic shock/resuscitation indicate that a dosing interval of ~ 30h would be sufficient to maintain an EM – based 'nanocrit' equivalent to a (fresh RBC) hematocrit of ~ 30. SEE Figure 2 (above) for description/justification of equipotent 'dose' analysis between EM (nanocrit) and RBCs (hematocrit) based upon quantified capacity to support oxygen flux/Hb from lung to tissue during circulatory transit.

A



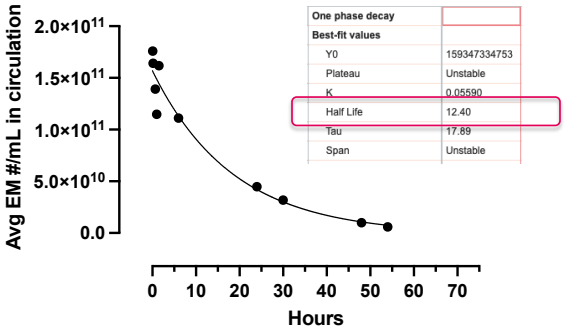
Estimated 'equipotent' Hct & Hb
For Δ A-V O_2 flux
Based upon Hb 'vehicle'
RBCs (fresh or stored) v EM

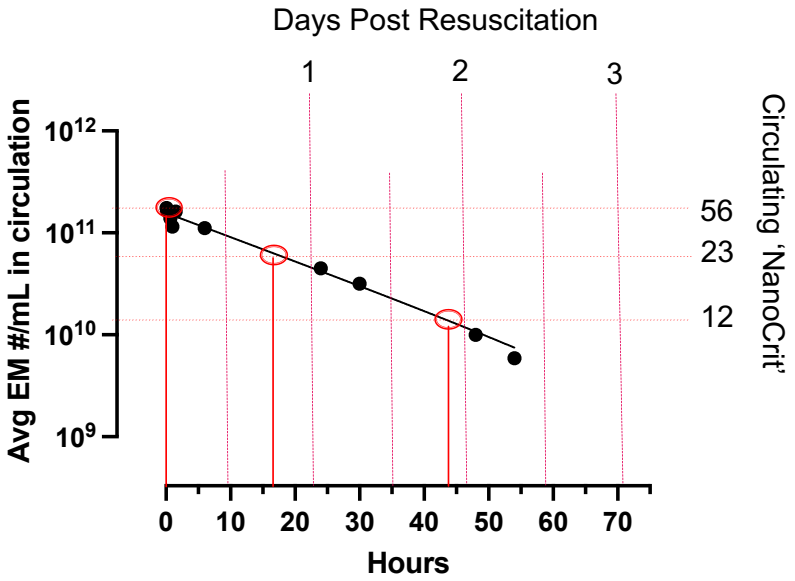
	RBCs (fresh)	RBCs (stored)	EM
Circulating Hct	30	45	12
Circulating [Hb]	10	15	4
Hb per 300 mL unit	50	75	20

- Cy7 labeled lipid incorporated into EM shell
- 10% BV 'top loading', rabbit

B

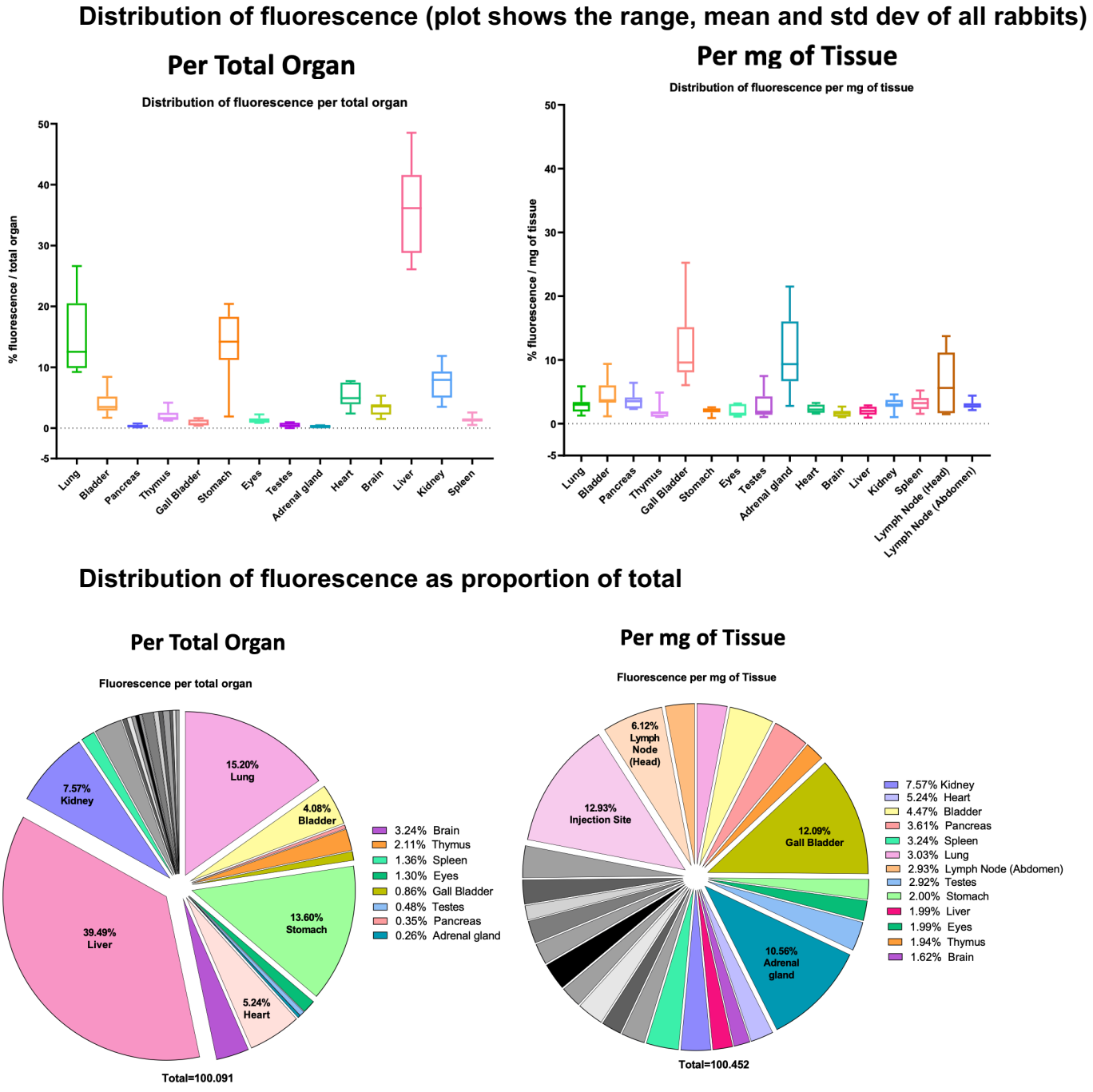
- Cy7 labeled lipid incorporated into EM shell
- 40% HS/R, 80min downtime
- 50% EM administered was labeled





	RBCs (fresh)	RBCs (stored)	EM
Circulating Hct	30	45	12
Circulating [Hb]	10	15	4
Hb per 300 mL unit	50	75	20

Figure 11. Analysis of EM route of elimination and biodistribution. Elimination Method All urine and bile was collected at the end of the (9h) experiment. **Elimination Result** 100% of eliminated EM-based fluorescence was found in bile; 0% was found in urine. **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). **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.



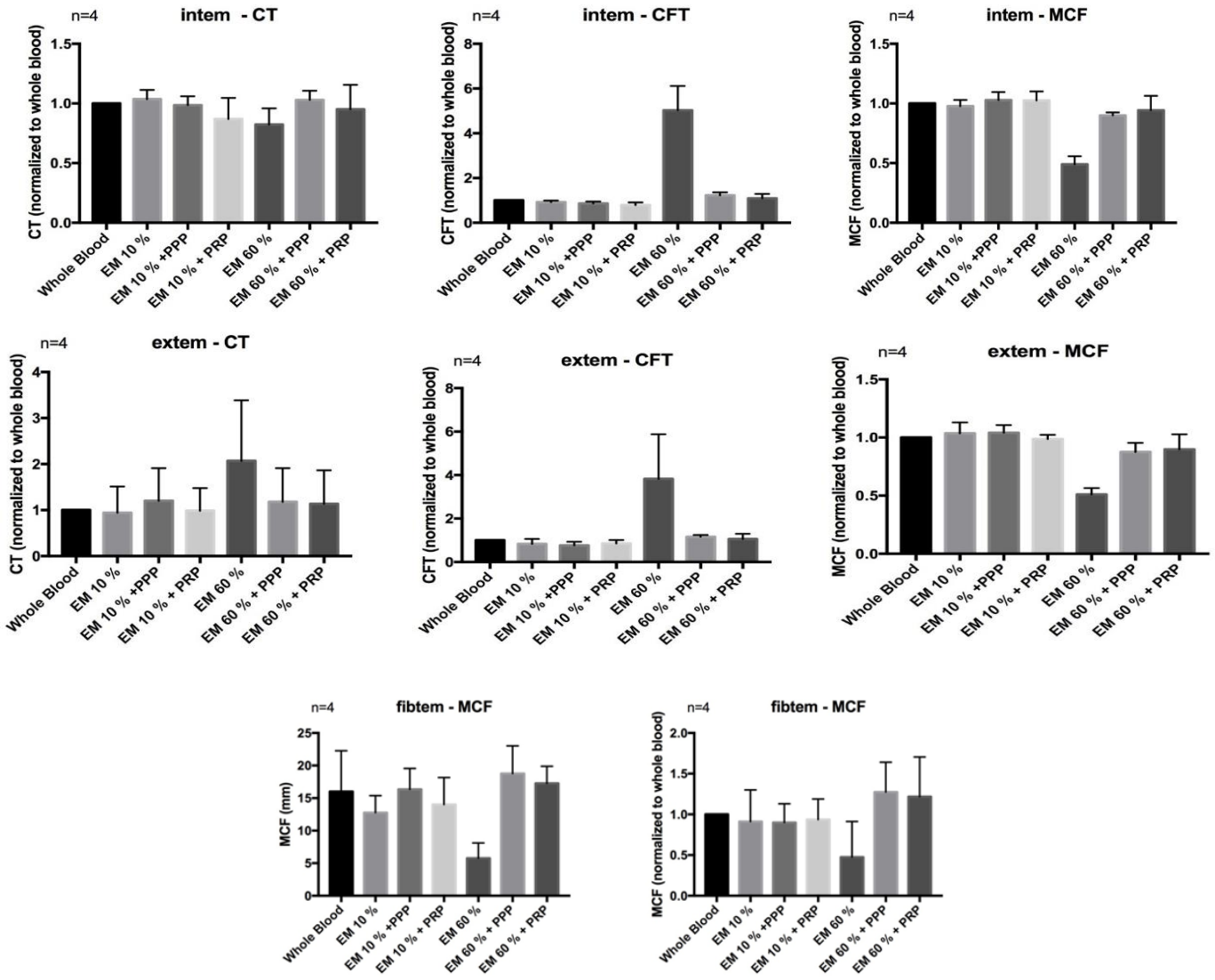
Major Task 4: Develop EM PFC dosing.

Milestone #6: Confirm EM dosing strategy for rabbit PFC models; informed by MPS depletion model. (completed 03/2023) Also, see above. While our initial PK modeling (from top loading and simple (non-shock) BVR experiments) suggested we would require EM dosing at ~ 8 hour intervals, as noted above (Figure 10) our final PK modeling (from actual hemorrhagic shock in our PFC Scenario A model) indicate we would require EM dosing at ~ 30h intervals. In the models with a single acute hemorrhage (Scenario A and C) all animals surviving beyond 24h demonstrated hemodyamic stability, resolution of lactic acidosis, and otherwise fully adequate resuscitation. Therefore, we performed serial dosing only in the Scenario B model (simulated uncontrolled hemorrage); the dosing schedule in Scenario B was driven by serial bleeding/resuscitation and is described in detail below in for MT's 7 & 8.

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). We first determined the impact of EM based resuscitation (blood volume replacement) upon hemostatic function to assess if the effect was limited to hemodilution or specific interference/interaction with hemostatic mechanisms. These experiments were performed both in vitro (human) and in vivo (rabbit) and are presented below (Figures 11 - 13).

Fig. 11: Coagulation study (ROTEM) of intrinsic pathway (INTEM, ellagic acid, phospholipid; EXTEM tissue factor activation). CT: Clotting Time; CFT: Clot Formation Time; MCF: Maximum Clot Firmness. *This experiment demonstrates dilutional coagulopathy becomes evident with dosing equivalent to 60% blood volume replacement by ErythroMer (buffer suspended) and that this coagulopathy is wholly 'rescued' by platelet poor plasma (PPP) replacement. Indicating that the EM particle (per se) has no effect upon (ex vivo) hemostasis.* **NB: Intem** – clot activation via the contact phase – assessment of factors XII, XI, IX, VIII, X, V, II, I, platelets and fibrinolysis; **Extem** clot activation by thromboplastin (tissue factor) – assessment of factors VII, X, V, II, I, platelets and fibrinolysis). **FIBTEM** – clot activation is in EXTEM, with addition of cytochalasin D, a platelet blocking agent – permitting specific assessment of fibrinogen levels and fibrin polymerization.



We also performed 'proof of concept' experiments to screen for: (1) volume-attributable EM-based dilutional coagulopathy, (2) EM-particle attributable impairment in clot kinetics/firmness, (3) *ability to correct* the above with commercially available, lyophilizable HA (PCC [Kcentra] & FC [RiaSTAP]). 50% dilution of whole blood (WB) altered clot kinetics/firmness; this impairment was largely 'rescued' by HA (PCC & FC) (Figures 7 & 8). ROTEM MCF recovery, however will likely require thrombosomes, in addition to HA (vide infra; also see Figure 8 - MCF primarily reflects platelet function).

Figure 12 Thromboelastometry during addition of EM & HA to WB (ex vivo): ROTEM metrics (see Fig. 8) following addition of buffer (BIS/TRIS, pH 7.2) or EM (in same buffer) to fresh WB (SD rat) [pH 7.2 simulates acidosis during shock]: (A) Clotting Time, (B) Clot Formation Time and (C) Maximum Clot Firmness. DC is similar for buffer & EM, excepting for CFT (less severe for EM). Next, 50% diluted (EM:WB, 1:1) samples were 'rescued' with PCC & FC (combined @ 1/20th-10th human dose); (D-F) ROTEM parameter recovery is shown (indexed to control WB values; * $p<0.05$). Recovery of all metrics was achieved, excepting MCF (likely requires thrombosomes).

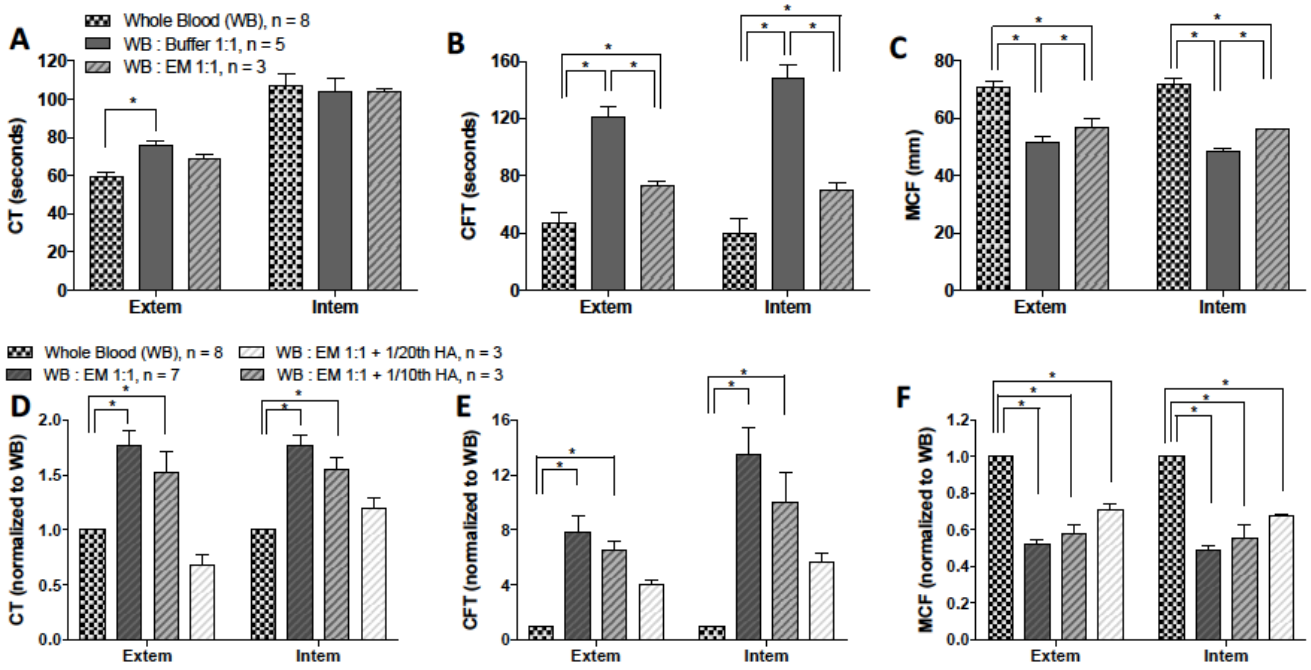
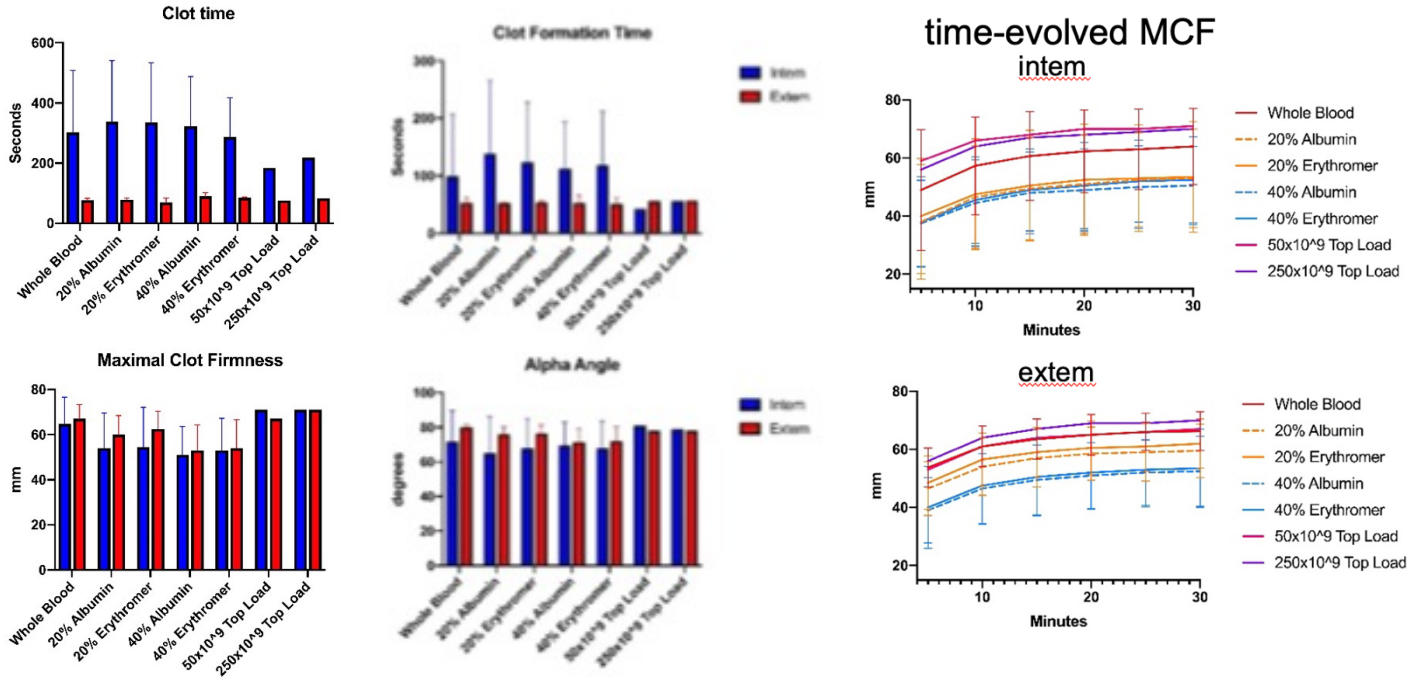


Figure 13. *In vivo* evaluation of EM-based dilutional coagulopathy. We confirmed the findings in Figure 11 (EM dilutional coagulopathy) in rabbits (simple dilutional coagulopathy, without additional interference in hemostatic function).



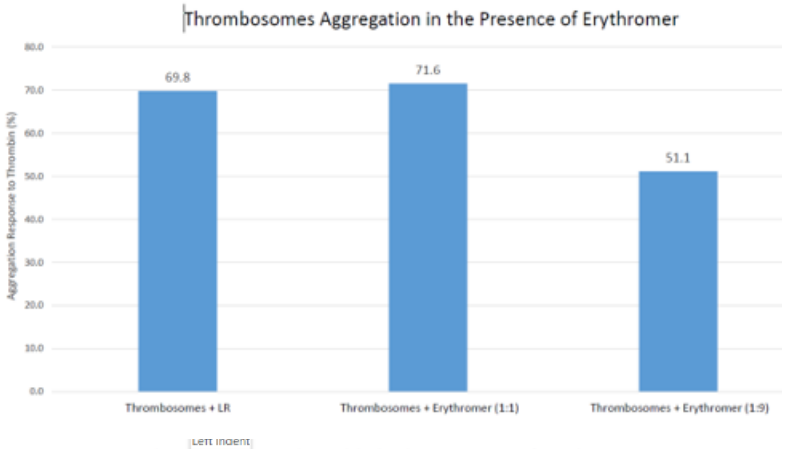
Finally, to complete MT5, we evaluated compatability amongst EM and thrombosomes (a freeze dried platelet derivative, developed by Cellphire, now in human PhII trials). We found that all biophysical and functional features of both thrombosomes and EM were preserved (>80%) in mixtures of both products likely to be encountered in the course of resuscitation for severe hemorrhagic shock.

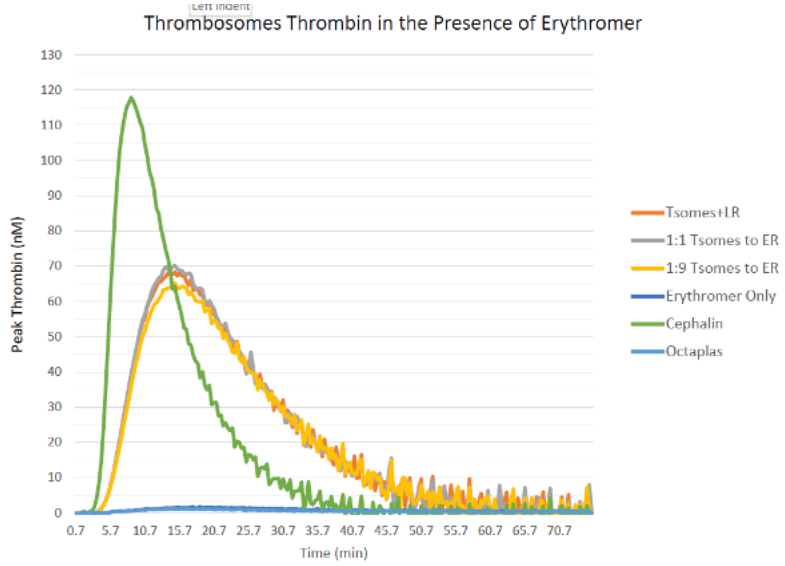
Figure 14. Functional assessment of Thrombosomes after mixture with EM EM and thrombosomes were mixed in ratios of either 1:1 or 1:9 (T:EM) and evaluated as shown below by flow cytometry (for size and activation as determined by surface marker), aggregation and ability to initiate thrombin generation.

Experimental Groups					
Sample	Volume Thrombosome	Volume of Lactated Ringers	Volume of ErythroMer	Thrombosomes Concentration	ErythroMer Concentration
Thrombosomes + LR	3 mL	2.1 mL	0 mL	1 x 10^6/µL	0 x 10^6/µL
Thrombosomes + ErythroMer (1:1)	3 mL	0 mL	2.1 mL*	1 x 10^6/µL	1 x 10^6/µL
Thrombosomes + ErythroMer (1:9)	3 mL	0 mL	2.1 mL*	1 x 10^6/µL	9 x 10^6/µL
ErythroMer only	0 mL	4.2 mL	0.9 mL	0 x 10^6/µL	9 x 10^6/µL

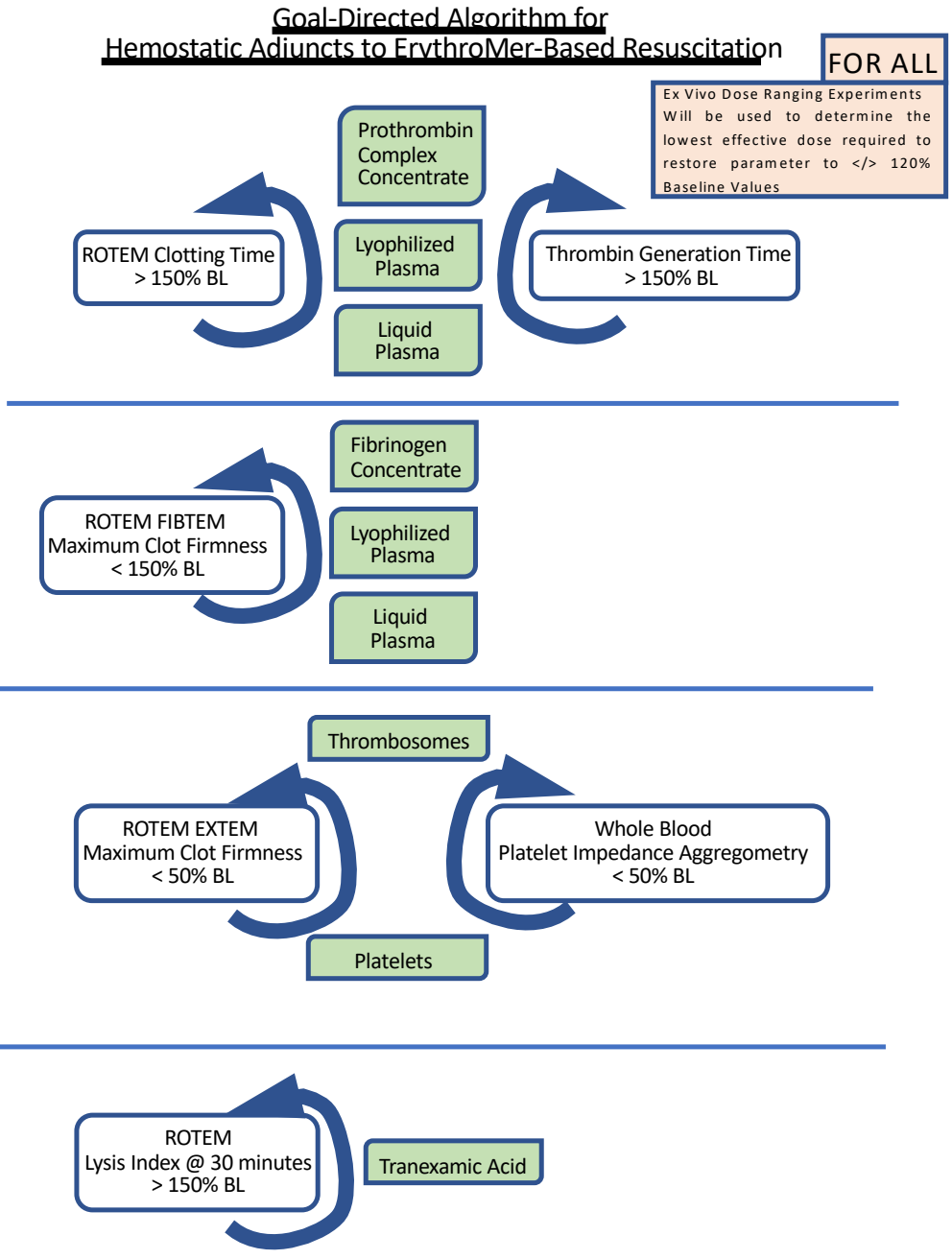
Flow Size Distribution			
Sample	Thrombosomes + LR	Thrombosomes + Erythromer (1:1)	Thrombosomes + Erythromer (1:9)
<0.5 µm (%)	0.1	0.1	0.1
0.5 to 0.9 µm (%)	89.1	90.2	91.0
0.9 to 2.5 µm (%)	10.0	9.1	8.3
>2.5 µm (%)	0.7	0.6	0.6

Flow Surface Markers			
Sample	Thrombosomes + LR	Thrombosomes + Erythromer (1:1)	Thrombosomes + Erythromer (1:9)
CD41+	83.3	83.3	79.7
CD62+ 0.5µm-2.5µM	90.2	91.7	91.7
AV+ 0.5µm-2.5µM	99.3	98.7	98.5



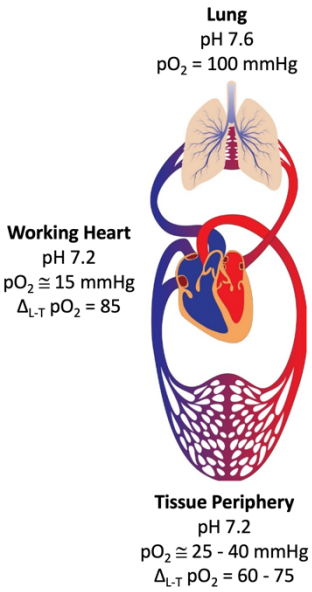


Major Task 6 & 9: Develop goal-directed HA algorithm for EM-based dilutional coagulopathy (DC). Milestone #9: Develop goal directed HA algorithm for EM-induced DC suitable for in vivo testing (rabbits). (completed 10/2018). Based upon the data acquired in MT 5 (Figures 11-14) we designed the goal directed HA algorithm **(Figure 15, below)** for potential use in PFC scenarios, as shown below. This algorithm was initially intented to be empirically evaluated in our PFC model of Trauma Induced Coagulopathy (Scenario C, described in detail for MTs 7 & 8, below). However, despite a ~ 50% mortality in our control group (Lactated Ringers resuscitation), and evidence of severe shock and coagulopathy, post resuscitation values in the model did not meet the relatively conservative thresholds below for administration of hemostatic adjuncts – any attempt to create a more severe coagulopathy resulted in mortality > 75 – 80% in our controls, suggesting we may require a different species or polytrauma model to test combined administration of EM and hemostatic adjuncts. Of note, we will be doing so in two complimentary projects: (1) DoD CRRP RDTRA W81XWH-20-C-0144 Nanotechnology Enabled Dried Whole Blood Surrogate (WBS) for Hemostatic Resuscitation (2) DARPA HR001121S0027-FSHARP-FP-001 CONCERT: Consortium for Optimized Integration of Bio-artificial Blood Components for Adaptive Resuscitation and Therapy [both projects will evaluate combined administration of EM, freeze dried plasma and synthoplate (nanoparticle based platelet mimetic) in multiple models of polytrauma/coagulopathy).

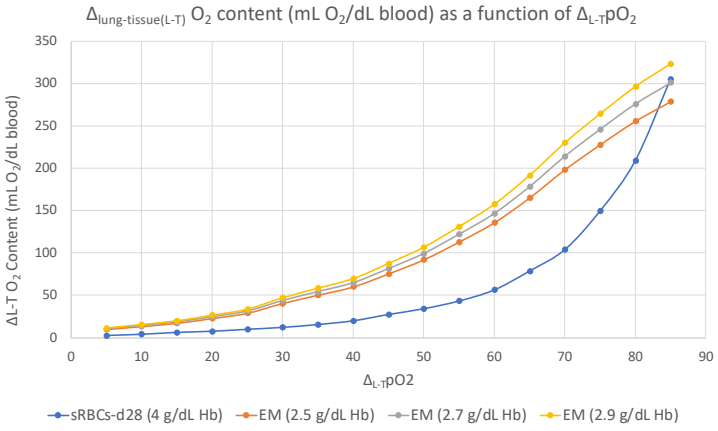


Major Task 7: Pilot PFC Scenarios. Milestone #10: Obtain IRB/HRPO approval (completed 01/2018 at WUSM; 10/2020 at UMB). Milestone #11: Pilot & Optimize PFC Scenarios (A, B, C) to achieve 50% 48h mortality for colloid resuscitation controls. (completed 09/2022). Pilot experiments optimizing severity of bleed/hemorrhage, polytrauma insult and simulated uncontrolled bleeding have been presented in detail in prior reports. Here, we will focus on presenting efficacy data in the fully optimized models (for both acute physiologic outcomes and for 72h survival), below. Additionally, we present the empiric/modeling basis for EM dosing in the model, based upon the relative potency experimental data from MT 1, Figures 1-2 (above). As for Figure 2, the following metrics were determined for Hb potency, treated as formulated either in stored RBCs (28 d storage duration) or in EM.

- **O_2 Transport Capacitance:** $\Delta_{\text{lung-tissue (L-T)}}$ O_2 content (mL O_2/dL blood)
- **O_2 Content** = % O_2 Saturation x [Hb] (g/dL) x 1.34 mL O_2/gm Hb
- **$\Delta_{\text{lung-tissue (L-T)}}$ O_2 content:** Lung O_2 Content (pH 7.6, pO_2 100) – Tissue O_2 Content (pH 7.2, pO_2 15 - 95)
- These data were then plotted against pO_2 gradient ($\Delta_{L\text{-}T}$ pO_2) to determine equipotent [Hb] as formulated in stored RBCs or in EM.



A



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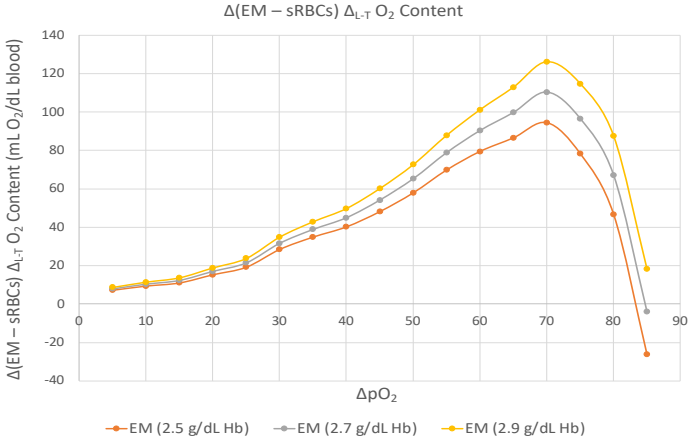


First (A), we plotted O_2 Transport Capacitance: $\Delta_{\text{lung-tissue (L-T)}}$ against the anticipated range in gradients in pO_2 between lung (set at 100 mmHg) and tissue (range from 95 to 15 mmHg). Note the increase in O_2 released by Hb as the pO_2 gradient between lung and tissue increases from L → R along the X axis. Also note that EM demonstrates superiority in potency for O_2 release for any given gradient until tissue pO_2 falls to 15 mmHg. **Then (B),** The absolute superiority in O_2 release by EM was plotted against the same anticipated range in pO_2 gradients – which defines the circulating blood [Hb] at which EM and stored RBCs are equipotent for O_2 delivery at extreme tissue hypoxia (pO_2 15 mmHg in working heart). ***Therefore our target post resuscitation blood [Hb] (as EM) for our PFC scenarios was set at 2.7 g/dL.*** Finally, as a table-top exercise (unrelated to our PFC models), we estimated various resuscitation volumes for in field management of a 70 kg trauma victim (Table, left).

		Vol./Unit (mL)	[Hb] in blood unit (mM; g/dL)	Resuscitation Volume (L)	Hb dose (gm)	Circulating $[Hb]_{dose}$ (g/dL)
sWB + Crystalloid	sWB	500	2.4; 15.0	1.5	225	4
	Crystalloid			1.25		
EM + Crystalloid	EM	500	1.62; 10.1	1.5	152	2.7
	Crystalloid			1.25		
EM only	EM-Conc.1	917	0.82; 5.1	2.75	141	2.5
	EM-Conc.2	917	0.88; 5.5	2.75	152	2.7
	EM-Conc.3	917	0.95; 5.9	2.75	163	2.9

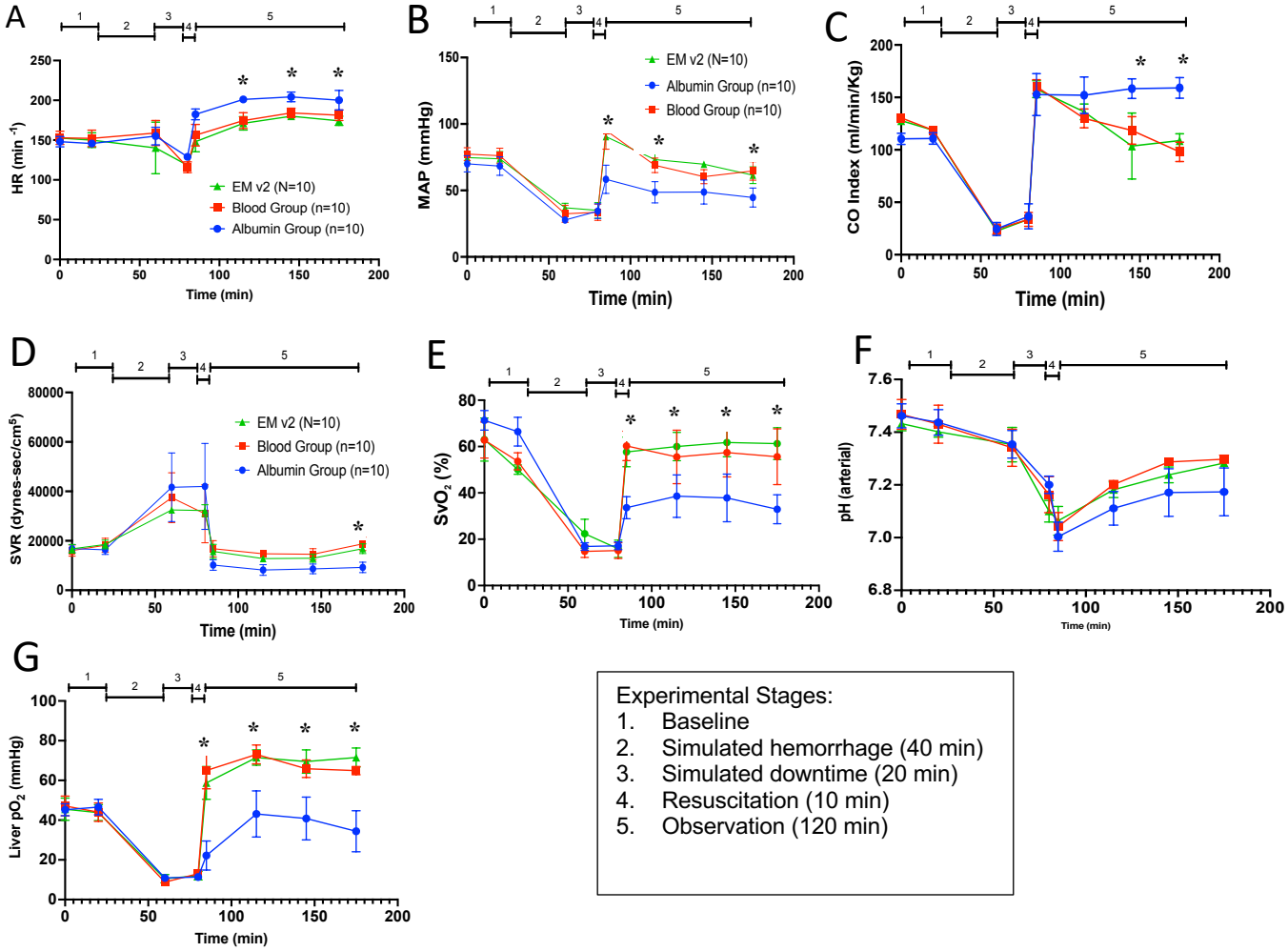
***Above values assume flow characteristics are the same**

Variables that affect flow:

- Pressure gradient (same for blood and EM)
- Impedance:
  - Shear-dependent viscosity
  - Yield point
  - Vessel diameter (Vasoactivity) – need comparison to stored RBCs

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). (completed).

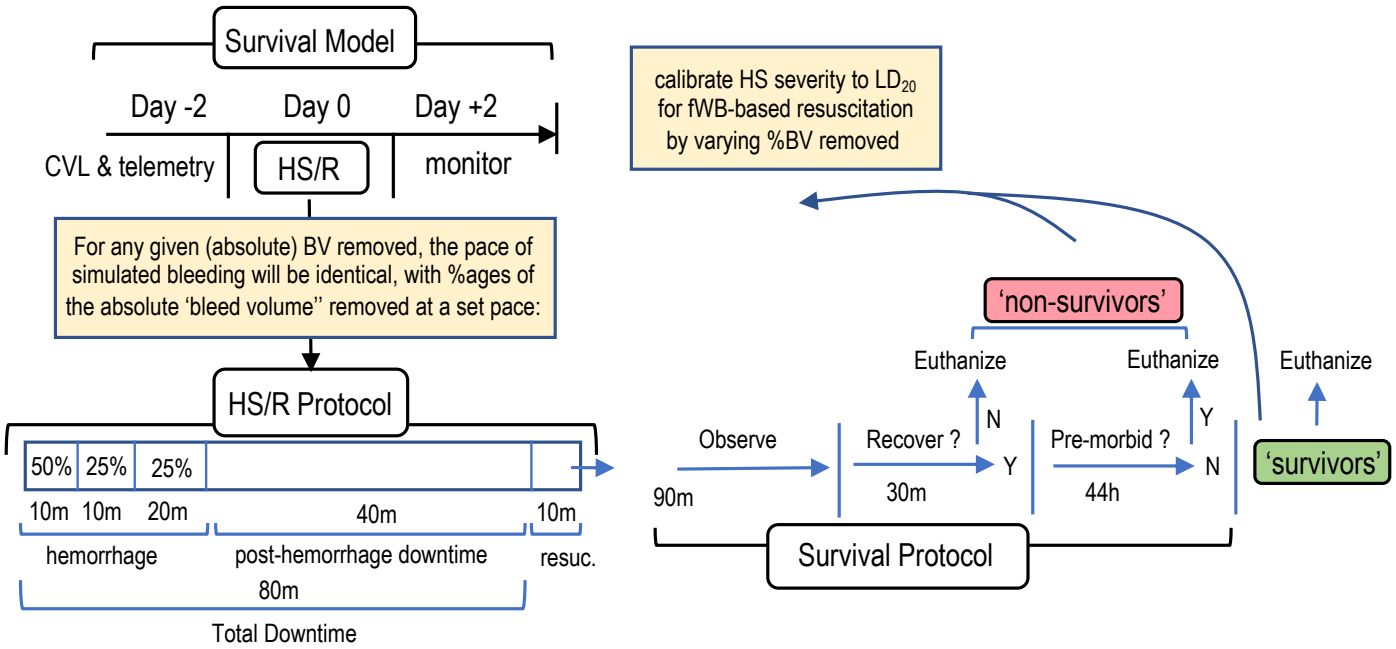
Acute Hemorrhagic Shock / Resuscitation Model (2h outcomes) Method Rabbits (2.5kg) are instrumented, then hemorrhagic shock is induced by blood removal (40% BV, 40m according to a decelerating schedule (%ages are of the total BV to be removed: 50% over 1st 10m (~ 4 ml/h); then 25% over 2nd 10m (~ 2 mL/m); 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**. Results (Figure 16, below)** 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



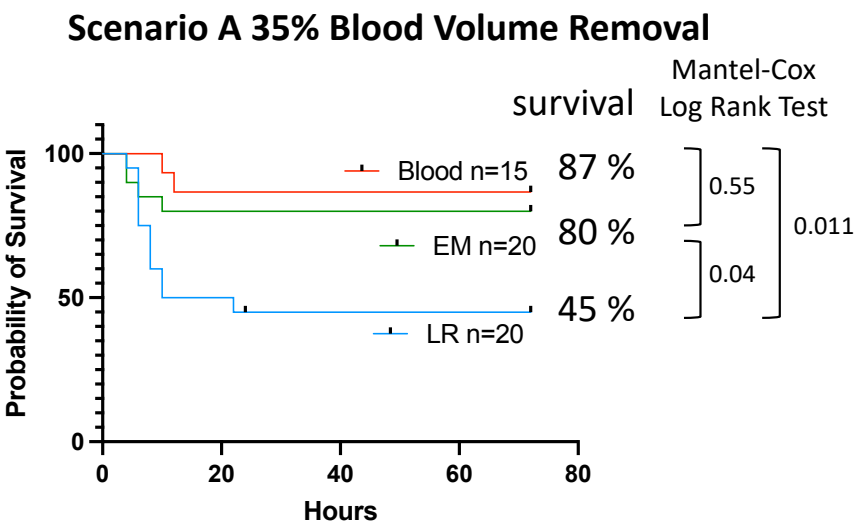
RBCs.

PFC Hemorrhagic Shock / Resuscitation (72h outcomes)

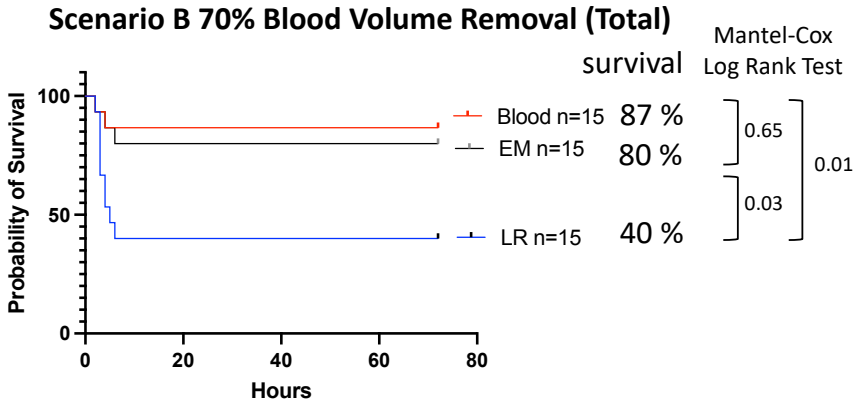
Scenario A – 'simple' controlled hemorrhagic shock without polytrauma Method This complex protocol **(Figure 17, below)** commences with a procedure to implant telemetry (carotid arterial pressure catheter) for monitoring HR, MAP and to implant tunneled internal jugular CVL, followed by 2d recovery. Hemorrhagic shock is induced as in the acute model (initially we performed 40% BV removal via a decelerating blood loss schedule. Here, to fully optimize outcome difference between resuscitation with fresh (shed) whole blood and crystalloid (LR) we extended downtime to 40m (from 20m), resuscitation is otherwise identical to the acute model (LR is substituted for 5% albumin). The decelerating bleeding schedule, total downtime (from injury to resuscitation) and the resuscitation fluid realistically model in-field resuscitation under austere conditions. Rabbits are then observed for 90m, then recovered. After which – HR, RR, and BP are monitored continuously via telemetry; if HR < 40 BPM or BP < 40 mmHg for > 30m, then rabbits are assessed as 'pre-morbid' – classified as 'non-survivors' for analysis and euthanized. Though stated elsewhere, all resuscitation is isovolemic to the shed blood volume (for each individual rabbit). The EM resuscitation 'fluid' is comprised of particle count ~ 1x1013 EM particles / mL, with p50 at pH 7.4 of ~ 55 Torr (dynamic range across pH 7.2 to 7.6 ~ 15 Torr) and [Hb] ~ 750 uM (these data are averages across the multiple EM batches tested in the model, CV < 10% across batches). As noted in prior reports, EM offers greater 'potency' than RBCs for O2 flux across the pH and pO2 gradients typically seen in massive transfusion resuscitation (lung – tissue gradients of pH: 7.6 – 7.2 & pO2 100 – 25, respectively (typical fresh RBC p50 is ~ 28, with dynamic range across pH 7.2 – 7.6 ~ 10 Torr); therefore, EM 'potency' for trans-circulatory (lung to tissue) O2 flux for the above metrics is ~ 80% that of whole blood.



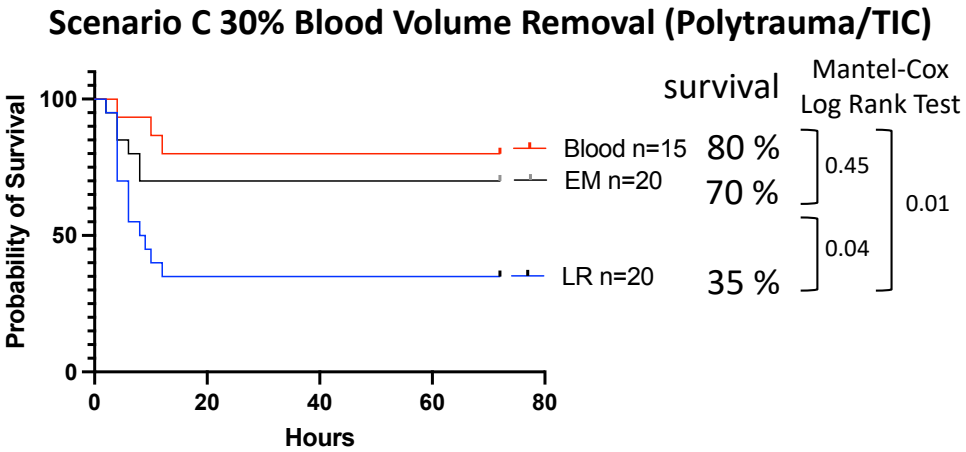
Results In multiple prior reports, Y4Q3 – Y5Q3, we presented model pilot/optimization data for our 72h PFC model; our goal has been to calibrate the severity of modeled hemorrhage (combination of %BV removal and downtime) to an LD50 model, using crystalloid-based resuscitation (since 5% albumin is not available for actual PFC). We have now fully optimized the model (35% blood volume removal) to an ~ LD50 model for LR based resuscitation. Below, we present survival outcomes for the three groups tested in Scenario A: shed blood, LR, EM (we have now completed 15 rabbits in each group; the Cox model continues to demonstrate non-inferiority to blood and is approaching superiority to crystalloid (LR) (EM v LR p-value fell from 0.21 to 0.08 and EM v Blood p-value rose from 0.47 to 0.57, as n in EM group increased from 8 to 15) – see figure below.



Scenario B – 'uncontrolled' hemorrhagic shock without polytrauma The intent of this model is to explore the survival impact of need for ongoing, serial resuscitation in the setting of 'uncontrolled' (e.g. non-compressible) bleeding, but without full-blown trauma induced coagulopathy. **Method** Hemorrhage was induced with removal of ~ 60% of the BV used to initiate shock in Scenario A (20% (absolute) BV, with identical resuscitation sequence/timing), followed by removal of an additional 10% (absolute) BV every ½ hr X 5 (total 70% BV); after each BV removal, resuscitation (with either shed blood, LR, or EM) was administered ½ hr following serial BV removal (total bleeding/resuscitation period is ~ 6 ½ hours). The specific BV removal volume/schedule was piloted to target ~ 50% 72h mortality (LR group). **Results** Below we present survival outcomes for the calibrated 'uncontrolled' hemorrhagic shock model (without polytrauma) after substantial pilot work, we settled on the bleeding & resuscitation schedule above to simulate a period of extended bleeding/resuscitation. This model achieved our goal of ~ LD50 and with good separation of controls.



Scenario C – Controlled hemorrhagic shock with polytrauma-associated trauma induced coagulopathy (TIC) The intent of this model is to explore the survival impact of polytrauma and trauma induced coagulopathy in the setting of resuscitation for hemorrhagic shock arising from an acute, controlled bleed (as in Scenario A, we did not propose to evaluate polytrauma/TIC in the setting of uncontrolled bleeding) **Method** Following telemetry and vascular access placement (as for Scenario A), the model commenced the following day with reinduction of anestheisa and modeled severe polytrauma involved bilateral lower extremity pseudofracture and partial liver laceration (detail for each, below), followed by a planned BV removal schedule and resuscitation sequence identical to Scenario A for which – volumes removed were piloted to target 50% 48h mortality (LR group). After this procedure, animals were allowed to stabilize for 1h, then recovered and monitored for 72h (analgesia per protocol) **Pseudofracture:** Femurs and tibias from a donor rabbits were isolated and morselized (mortar and pestle). Subject rabbits first underwent bilateral lower- extremity crush injury (clamp musculature adjacent to the femur for 40s), into which 150 mL morselized bone was injected**. Partial liver laceration:** Following pseudofracture creation, a laparotomy was performed, exposing the left lateral lobe of the liver. One-third of the lobe was sharply divided, left in the abdomen and the wound closed. In prior work with this model, we found the transected liver remnant to be nonviable; the liver injury did not result in significant hemorrhage. These model features (hemorrhagic shock with severe tissue injury/exposure) are expected to promote TIC evolution. **Results** We quantified acute effects of this model w/r/t oxygen delivery and TIC initiation and reported those data in our Y2 Annual Report. Our goal has been to calibrate the severity of modeled hemorrhage + polytrauma to an ~ LD50 model, using crystalloid-based resuscitation (since 5% albumin is not available for actual PFC). Our initial data suggested that while 35% BV removal and 40m downtime was workable for Scenario A, this level of hemorrhage is too severe (30% survival) for our Scenario C model. Our preliminary results indicated that 30% blood volume removal resulted in 60% survival in the LR group, (with N=8 per group). In Y5Q2, we evaluated 30% BV removal for extended downtime (40m, as for Scenario A), which resulted in an LD45 outcome. We have now completed these experiments with excellent separation of the positive (blood) and negative (LR, with some worsening of mortality) control groups and completed EM testing in Q3-4 of Y5.

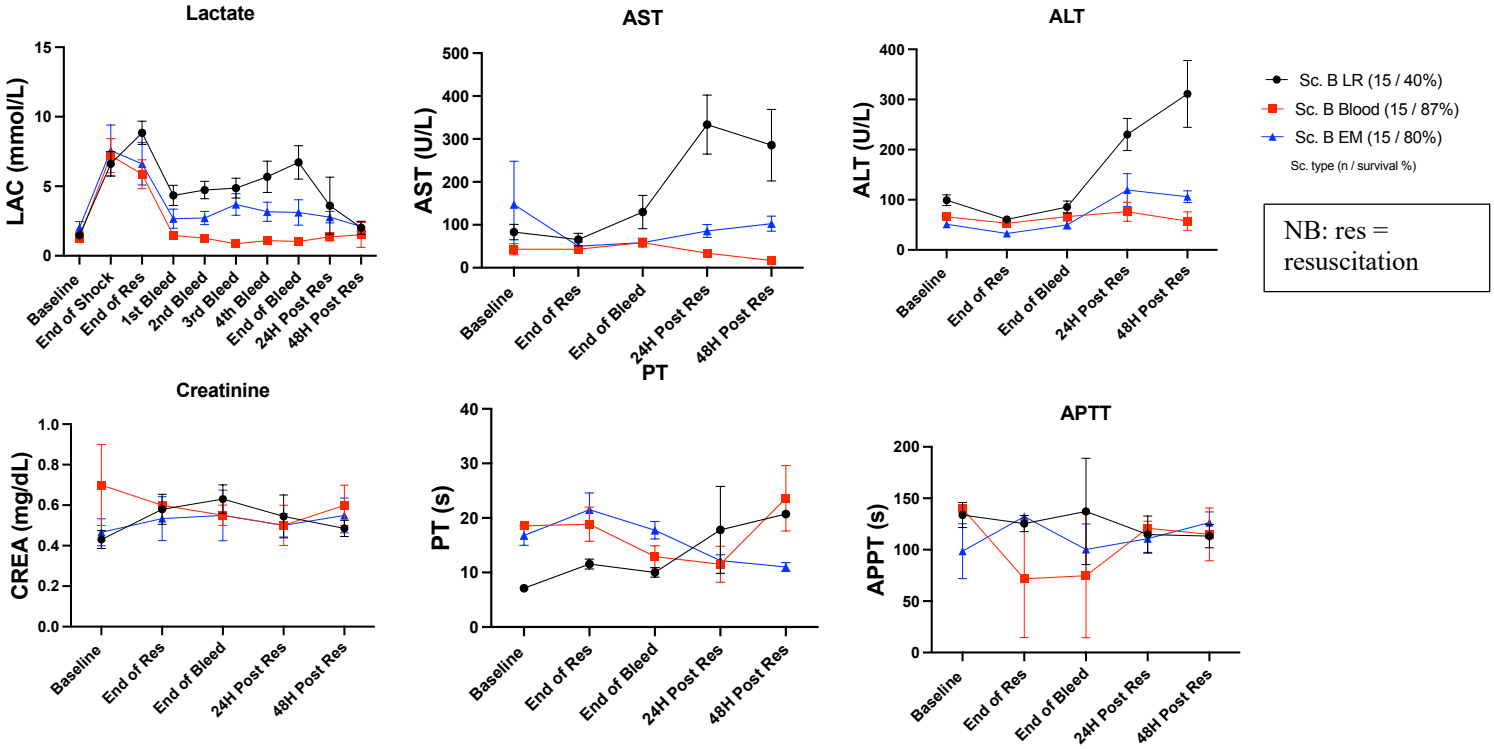


Conclusion: In all our 72h survival PFC models, which modeled three complementary clinical scenarios: A: acute controlled bleed, B: uncontrolled bleed, C: acute controlled bleed with polytrauma/TIC – EM demonstrated non-inferiority to positive control (blood) and superiority to negative control (LR, crystalloid).

Major Task 10: Screen EM safety *in vivo*.

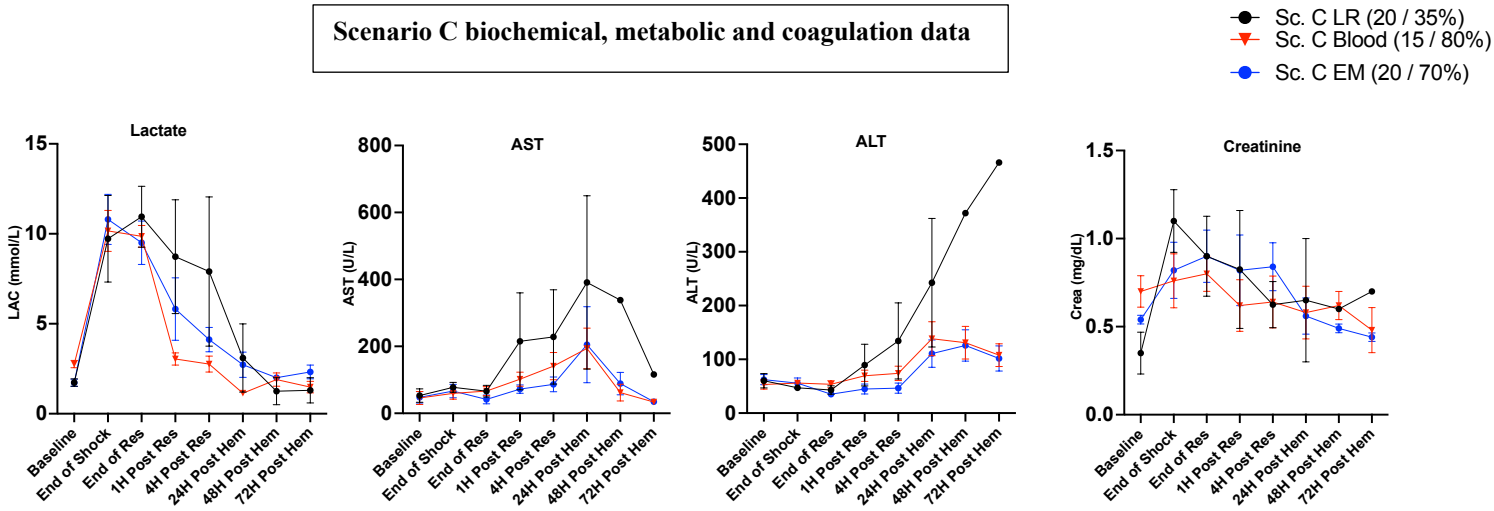
Milestone #14: Identify lab and histologic evidence of EM toxicity during resuscitation from PFC Scenarios A-C, in comparison to that observed in blood re-infusion and LR resuscitation groups. (completed 09/2022). We presented Scenario A basic metabolic data above; her we present detailed metabolic and hemostasis profile data from Scenario B and C here. **In Scenario B**, we found relative superiority for EM > LR based resuscitation with regard to lactate and hepatic enzymes, but no evidence of injury in any group with regard to creatinine or hemostasis. Of note, none of our histopathologic evaluation (simple H&E staining of kidney, liver, heart) demonstrated any injury in any group.

Scenario B biochemical, metabolic and coagulation data



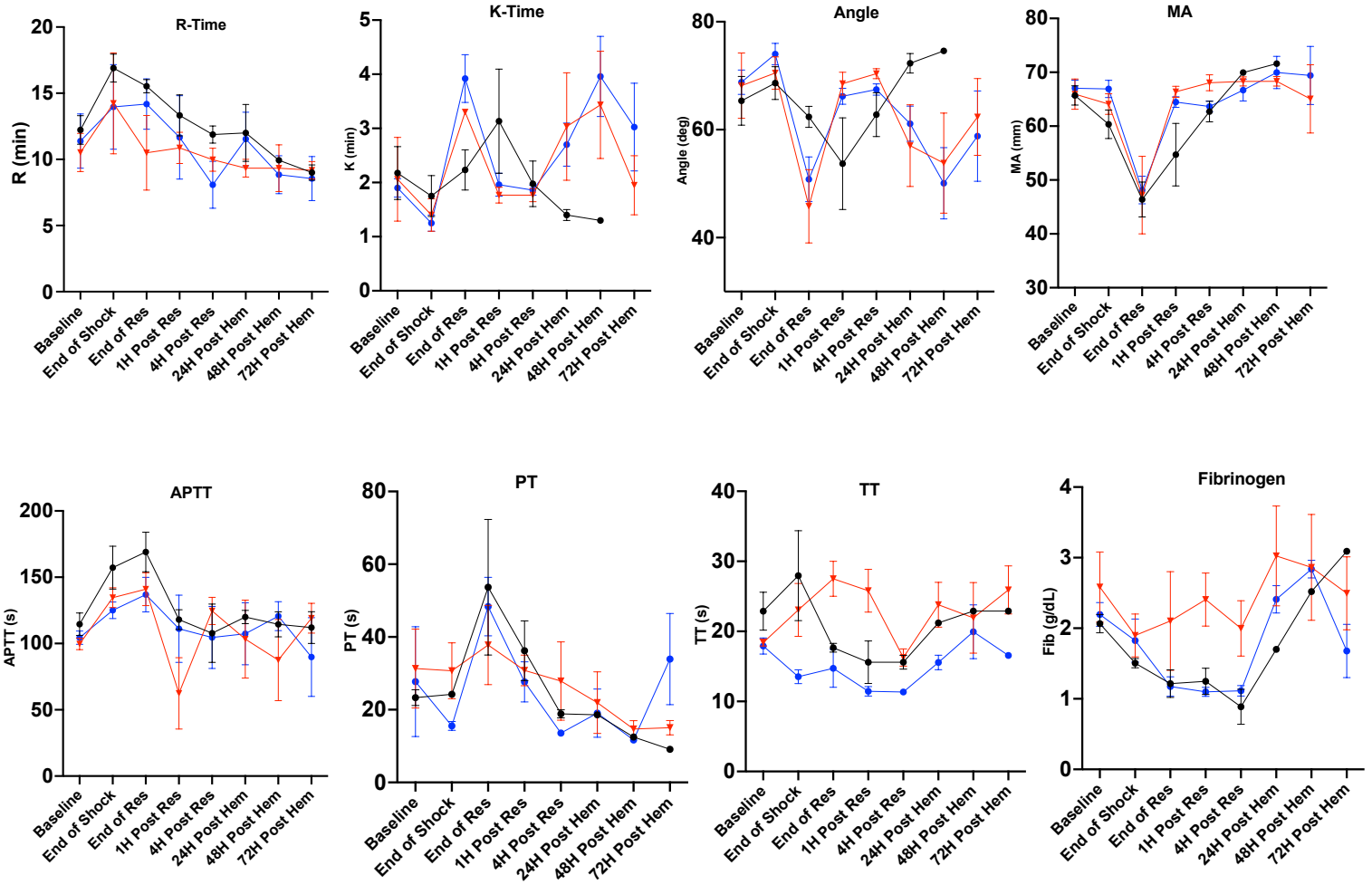
NB: res = resuscitation

Scenario C biochemical, metabolic and coagulation data



In **Scenario C**, similarly, we found relative superiority for EM > LR based resuscitation with regard to lactate and hepatic enzymes, but no evidence of injury in any group with regard to creatinine. Of note, none of our histopathologic evaluation (simple H&E staining of kidney, liver, heart) demonstrated any injury in any group. Analysis of hemostatic function in Scenario C involved functional testing (TEG6S) ; those data are shown below.

With regard to functional hemostasis in **Scenario C**, similarly, we found slight relative superiority for EM > LR based resuscitation with regard to a single TEG parameter (R-time), but no consistent evidence of benefit that exceeded that conferred by LR. We did observe a slight benefit to blood based resuscitation, particularly for R-time and fibrinogen. Although the model was quite severe (note control mortality > 50% and peak lactate > 10 mmol) a benefit to whole blood based resuscitation was not as apparent as expected. We anticipate additional model development may be required to further elucidate this issue.



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

Throughout the project, multiple team members attended and presented at the following meetings: American Association of Blood Banks (AABB), American Society for Hematology (ASH), Military Health Sciences Research Symposium (MHSRS), Trauma and Hemostasis Research Network (THOR); at each of these meetings our team attended many presentations and workshops related to support of patients with hemorrhagic shock and resuscitation.

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?

Title of Project: Nanotechnology Enabled Dried Whole Blood Surrogate (WBS) for Hemostatic Resuscitation
Source of Funds: DoD CRRP RDTRA W81XWH-20-C-0144
Project Period and Amount: 08/01/2020-07/31/2022
Role: coI (PI: Bruckman (Haima Therapeutics))
Major Goals: The goals of this project are: (1) to establish WBS co-formulation process and physico-chemical compatibility of EythroMer with synthetic platelets & freeze-dried plasma, (2) to optimize WBS hemostatic and oxygenation capabilities, and (3) establish WBS efficacy in multi-scenario rabbit models of hemorrhagic shock/resuscitation.

Title of Project: CONCERT: Consortium for Optimized Integration of Bio-artificial Blood Components for Adaptive Resuscitation and Therapy
Source of Funds: DARPA HR001121S0027-FSHARP-FP-001
Project Period and Amount: 01/23/2023-01/22/2027 (Phase I & II)

01/23/2027-01/22/2028 (Phase III)
Role: PI
Major Goals: The goal is to advance pre-clinical development of a whole blood analogue (WBA) from bio-artificial component prototypes through successful IND submission, (1) with features that recapitulate performance of whole blood in models of complex polytrauma, (2) is produced in a shelf-stable and field-deployable form that (3) can be cost-effectively scaled to meet DoD needs.

Title of Project: ErythroMer: Nanoscale BioSynthetic Red Cell Substitute
Source of Funds: NIH/NHLBI PhIIb R44 HL135965-A1
Project Period and Amount: 03/01/2023-02/29/2026
Role: coI (Alp, KaloCyte)
Major Goals: The goals of are to conduct IND enabling studies of EM, comprising: detailed non-GLP pharmacokinetic, biodistribution metabolism and excretion studies with exploratory toxicology, GLP hemocompatibility, GLP-safety pharmacology and GLP expanded acute dose toxicology studies to determine starting doses of EM in an initial clinical trial.
*Impact Score 13, R44 zone of consideration = 10-40, awaiting council review (10/2022)

How does your candidate product aid the Warfighter, Veteran, Beneficiary, and/or General Population?

~ 20% of military casualties are in hemorrhagic shock on arrival to field hospitals and an additional 5% require urgent transfusion. Hemorrhage is estimated to account for ~ 90% of survivable battlefield deaths: lives that could be saved with better hemostasis and field-ready blood, blood components, or blood substitutes. In fact, it is estimated that if *in-field* transfusion had been available, ~1,000 (24%) combat fatalities (2001-10) would have been survivable; recent military data also indicates that pre-hospital red blood cell (RBC) transfusion improves survival. A new (MHSRS, 2016) analysis of transfusion efficacy (air medevac, Afghanistan, 2102-5) indicates a striking 6-fold (30 day) survival benefit for in-transit transfusion; notably this benefit was time-sensitive [benefit was either 20-fold or 0, if transfusion occurred within- or after the 1st 13 minutes after lift-off]. As such, field-deployed EM could transform trauma care in military settings, and is particularly suited to address the challenges inherent in PFC settings.

While study of ideal composition for resuscitative fluids is ongoing, it is evident that for those in hemorrhagic shock, volume replenishment alone (without O_2 carrying capacity) is insufficient. Moreover, with massive blood loss or with ongoing bleeding from non-compressible injuries, resuscitation with an O_2 carrier alone may be complicated by endogenous or acquired coagulopathy (dilutional), *particularly in prolonged field care (PFC) scenarios*. A field-deployable O_2 carrier that is compatible with lyophilized humoral hemostatic components and platelets will enable balanced on-scene resuscitation that treats both shock and coagulopathy during the critical 'golden-hour' after injury. We recognize that modern, pragmatic in-field trauma resuscitation (e.g. remote damage control resuscitation, RDCR) for hemorrhagic shock calls for proactive (rather than reactive) 'balanced' ratio administration of blood components (1:1:1 ratio of RBC:plasma:platelets), or use of whole blood. We envision that EM, delivered with lyophilized HAs, could be proactively administered in PFC conditions.

At the conclusion of this project, we have acquired empiric data to inform proportions, composition and timing for such a 'balanced ratio' approach. With these data, obtained via a robust *ex vivo* analysis platform and 3-scenario *in vivo* PFC model to dissect relative contributions of shock, hemodilution and tissue trauma in the setting of EM-based resuscitation, we hope to inform a PFC-suitable 'balanced ratio' algorithm for administration of a lyophilized, field-deployable 'blood components': e.g. O_2 carrier (EM), in combination with HA. In this fashion, combined use of EM/HA during PFC, even for casualties with severe polytrauma, can bridge to natural blood replacement prior to and during transport to central facilities.

Describe the steps and relevant strategies required to move the candidate product (knowledge or tangible) to the next phase of development and/or commercialization. Address any issues with intellectual property. }}

We have now about to lock design on our lead candidate prototype for EM and are well positioned to continue aggressive accrual of IND enabling data after formally transitioning fabrication/scaling to an identified CDMO (Southwest Research Institute) through projects that will be funded through the awards indicated above. We have already had a successful pre-IND meeting with FDA (report and formal response already submitted as ancillary to prior annual reports). We expect EM will be regulated by the Center for Biologic Research Evaluation (CBER), Office of Blood Research and Review (OBRR), in the Division of Hematology. During this project, we will initiate a Type B Pre-IND meeting with CBER to review plans for IND submission - which will include a comprehensive plan for safety studies and our first-in-man (FIM) Phase 1 protocol. Thereafter, the IND will be submitted and we plan to commence the FIM study following the FDA 30-day IND review process. Following Phase 2 clinical study, we would request another Type B End of Phase 2 Meeting with FDA to discuss our plan for biologics license application (BLA) submission, with a formal request for Priority Review. We will then work to initiate clinical trials of EM for hemorrhagic shock under the CBER authority. Following a successful Phase III clinical trial, we would seek FDA approval of a BLA for EM as a therapeutic product.

{{Final report, cont'd:

How would you classify your **lead candidate product**? *Please choose the best option or add explanation for multiple selections.*

(a)Therapeutic (Small Molecule, Biologic, Cell/Gene Therapy):

4. IMPACT:

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

1. **Short-Term Impact** At the conclusion of this project, we have optimized the ErythroMer (EM) prototype O2 affinities (Aim 1); crafted a dosing strategy suitable for PFC (Aim 2); determined compatibility amongst EM and lyophilized hemostatic adjuncts (HA) (e.g. prothrombin complex concentrates, fibrinogen concentrates and tranexamic acid) and have developed a goal-directed algorithm for HA correction of EM-based dilutional coagulopathy (Aim 3); and have established EM efficacy and safety in PFC models of hemorrhagic shock resuscitation (Aim 4). In achieving these goals, we have prepared EM for next stage of preclinical evaluation and development and, specifically, have readied EM for: 1) continued scale-up, as well as formal GLP/GMP documentation/certification and 2) pre-IND Type B meeting with the FDA. More generally, the precisely calibrated, novel hemorrhagic shock and polytrauma models developed in Aim 4 will uniquely augment resources for the resuscitation research community.

2. **Long-Term Impact** There is a commonly appreciated, critical unmet need for a safe, effective, and practical O2 transport agent to serve as an alternative to stored human RBCs. A successful artificial O2 carrier must demonstrate context-variable O2 binding, without NO sequestration. We have addressed this challenge by designing the first HBOC with dynamic context-responsive properties that enable effective O2 transport during physiologic stress. As an Hb-encapsulating, toroidal-shaped nanoparticle formulated by self-assembly of amphiphilic polymer, EM will constitute a new class of formally engineered bio-synthetic hybrid 'artificial cells' (e.g., 'cell-mers'). As elaborated in our Research Plan – the RBC-emulating physiologic performance of EM is an emergent property of biochemically-encoded 'wetware'; this (integrated shell and payload) design strategy has the potential to disrupt and fundamentally alter our approach to numerous complex therapeutic challenges.

3. Additionally, 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.

4. Finally, 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?

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 and UMB have filed several patents related to EM development (though not specifically funded by this project) Those patents are listed below.

   Invention Title ”Blood Substitute Composition and Method of Use”. The applicant for all three patents 9,486,508; 9,655,952 & 9,750,241 is the WASHINGTON UNIVERSITY , St . Louis , MO (US). This invention was made with government support under HL094470 and NS073457 grants.

   Invention Title ”Self-Assembling Oxygen Carrier Compositions”. PCT WO 2021/217010 is the publication number. The application number for this is PCT/US2021/028854. The applicant is KALOCYTE, INC.

   Invention Title ”Compositions and Methods for Removing Bio-Synthetic Nanoparticles from Bodily Fluids”. The application number 17/692,289. The applicants are UMB, UMBC and KALOCYTE, INC.

4. KaloCyte has now filed a pre-IND package with the FDA Submission Tracking Number (STN):PS007073. We submitted that document and reported the results of that meeting, with written feedback from FDA in prior annual reports and our live IPR with CCRP.

What was the impact on society beyond science and technology?

Public Benefit Pre-hospital care for civilian trauma remains a challenge, even in developed countries. 47 million Americans live more than one hour from a trauma center and most ambulances do not carry blood. Notably, while ~ 20% of the US population is rural, this group experiences 60% of trauma deaths. Trauma victims in hemorrhagic shock who do not receive blood until hospital arrival suffer mortality of 17 – 54% and mortality increases with time and distance to definitive care. The National Academy of Sciences has estimated that 30,000 US civilian trauma deaths/year occur are preventable, and that approximately 20,000 deaths that are preventable occur due to hemorrhage in the prehospital phase of resuscitation. Additionally, in an urban civilian disaster, delays in blood product readiness arise despite medical center proximity, further increasing mortality risk from hemorrhagic shock. As for military PFC settings, a field-deployable O_2 carrier that is compatible with lyophilized plasma and platelets will enable composition of a balanced resuscitation fluid that treats both shock and coagulopathy - and push advanced transfusion therapy for life-threatening civilian trauma to the field setting.

A successful blood substitute, with easy in-field portability and administration may fundamentally change Resuscitation and Transfusion Medicine. Rather than replace/compete with human RBC transfusion, initial EM utilization is anticipated to be in pre-hospital, military and other austere environments, where conventional transfusion is not possible. In addition, EM properties (lack of vasoconstriction and immune reactivity) offer advantage over RBCs in certain hospital settings: (1) priming bypass circuits for cardiac surgery and (2) emergency use in bleeding, acutely decompensating patients, for whom cross-matching would delay resuscitation. We also anticipate interest in stockpiling EM in large-scale civilian emergency depots and developing world blood banks. See Table 1 for a more complete listing of envisioned opportunities of use.

As noted above, an easily reconstituted blood substitute, amenable to extended ambient storage would have significant impact upon pre-hospital care for trauma victims in hemorrhagic shock, as well as enable creation of easily deployable 'strategic reserves' for blood bank support of disaster management. In addition, maintenance of a robust blood supply presents challenges in the developing world; specifically, creation and maintenance of processing and storage facilities for a decentralized blood bank system is not fiscally feasible. Moreover, in many countries, a pathogen-free donor pool is difficult to identify and maintain, and civil unrest leads to unpredictable surges in blood product requirements. For these reasons, a pathogen-free, lyophilized blood substitute would fulfill an unmet need in these areas as well.

Table 1	Opportunities for ErythroMer Use	
	Application	**Location / Setting**
General Transfusion	**When stored RBCs are undesirable** Low-volume resuscitation Limit alloimmunization Limit transfusion immunomodulation Limit risk of infectious transmission	Pre-hospital, ED, OR, ICU, military environments
Hemorrhagic shock	**When stored RBCs are unavailable**	Pre-hospital, civilian disasters, military environments, undeveloped countries, complex cross-matches, use in austere or remote locations
Perioperative Maintenance of O_2 delivery	**When stored RBCs can be avoided** Brief periods of controlled blood loss	OR, ICU
Improve Regional O_2 delivery	**Beyond conventional efficacy of stored RBCs** Target O_2 affinity to context Link pharmaceuticals to particle shell Ex vivo organ perfusion Nanoparticle perfusion through vascular obstruction (e.g. MI, stroke, PE, etc.)	Pre-hospital, ED, OR, ICU

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 implemented an adjusted schedule that enabled us to complete the project aims, and we completed the project successfully operating under an approved NCE with a modified budget, justification and SOW as submitted in our Y3/4 reports.

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

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**
  Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Ghaffarian R, Rogers S, Dougherty S, Wang Q, Moitra P, Brummet M, Buehler P, Spinella P, McGhee W, Alp E, McGovern E, Pan S, and **Doctor A**. Erythromer (EM), Bio-Synthetic Artificial Red Cell: Design, Biophysical Characterization and *in vivo* pre clinical results. **Science Translational Medicine.** *Anticipated submission March, 2023*.

Wang Q, Oghelvbie J, Lang E, Brummet M, Buehler P, Spinella P, Ghaffarian R, McGovern E, Pan S, and **Doctor A**. Multi Scenario Survival Models of Complex Hemorrhagic Shock for Prolonged Field Care. **Journal of Trauma.** *Anticipated submission March, 2023*.

Ghaffarian R, Rogers S, Dougherty S, Wang Q, Moitra P, Brummet M, Buehler P, Spinella P, McGhee W, Alp E, McGovern E, Pan S, and **Doctor A**. Multi-compartment pharmacokinetic modeling, bio distribution and elimination for ErythroMer – a nanoscale biosynthetic artificial RBC. **Transfusion.** *Anticipated submission March, 2023*.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Mittal N, Rogers S, Dougherty S, Wang Q, Moitra P, Brummet M, Cornett E, Kaye A, Shekoohi S, Buehler P, Spinella P, Pan S, and **Doctor A**. Erythromer (EM), a Nanoscale Bio-Synthetic Artificial Red Cell. In **Blood Substitutes and Oxygen Biotherapeutics.** Eds: Jahr J, Liu H, Kaye A, and Scher C. Springer Nature, SPi Global. 2021.

Other publications, conference papers and presentations.

Synthetic Biology Meets Bio-Inspired Engineering Potential Path to a Whole Blood Analogue
Trauma Hemostasis and Oxygenation Research (THOR) Network, Bergen, Norway, 2022

"Bio-Inspired Artificial Red Blood Cell: Design, Pre-Clinical Results and Novel Indications" BloodNet BiAnnual Research Seminar. San Diego, CA. 2022.

- **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**

This project is focused upon developing Erythromer, which is a bio-synthetic artificial RBC technology for in-field transfusion support of severe hemorrhagic shock.

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

Patents related to this project – but not directly funded by this project are listed below.
Invention Title "Blood Substitute Composition and Method of Use". The applicant for all three patents 9,486,508; 9,655,952 & 9,750,241 is the WASHINGTON UNIVERSITY , St . Louis , MO (US). This invention was made with government support under HL094470 and NS073457 grants.
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Invention Title "Compositions and Methods for Removing Bio-Synthetic Nanoparticles from Bodily Fluids". The application number 17/692,289. The applicants are UMB, UMBC and KALOCYTE, INC.

- **Other Products**

Nothing to report.

7. Participants & Other Collaborating Organizations

Name:	**Allan Doctor**
Project Role:	**PI**
Research Identifier (e.g. ORCID ID):	**N/A**
Nearest person month worked:	**3**
Contribution to Project:	**As PI, Dr. Doctor has directed all aspects of the project.**

Name:	**Paul Buehler**
Project Role:	
Research Identifier (e.g. ORCID ID):	**N/A**
Nearest person month worked:	**1**
Contribution to Project:	Dr. Buehler assisted with pharmacokinetics/pharmacodynamics calculations and modeling.

Name:	**Dipanjan Pan**
Project Role:	**CoI – bioengineer**
Research Identifier (e.g. ORCID ID):	**N/A**
Nearest person month worked:	**3**
Contribution to Project:	Dr. Pan supervised ErythroMer oxygen affinity optimization, shell labeling and in vitro biophysical and functional characterization.

Name:	**Mary Brummet**
Project Role:	**Doctor Lab Manager**
Research Identifier (e.g. ORCID ID):	**N/A**
Nearest person month worked:	**6**
Contribution to Project:	Ms Brummet coordinated lab experimental schedules and resources and personally performed multiple in vitro assays.

Name:	**Stephen Rogers**
Project Role:	**CoI, EM benchmarking**
Research Identifier (e.g. ORCID ID):	**N/A**
Nearest person month worked:	**5**
Contribution to Project:	Dr. Rogers assisted in animal physiology experiments and was responsible for bench top characterization of ErythroMer.

Name:	**Tobi Rowden**
Project Role:	**Doctor Lab Research Coordinator**
Research Identifier (e.g. ORCID ID):	**N/A**
Nearest person month worked:	**1**
Contribution to Project:	Ms Rowden is a clinical research coordinator; she assisted in securing fresh human blood samples for early controls (in vitro testing).

Name:	**Qihong Wang**
Project Role:	**CoI, new – surgical model**
Research Identifier (e.g. ORCID ID):	**N/A**
Nearest person month worked:	**9**
Contribution to Project:	Dr. Wang is the Director of the CBOTH Small Animal Surgery and Physiology Core. He performed all animal surgeries (vascular access and telemetry) and supervised animal physiology experiments.

Name:	**Phillip Spinella** **Washington University**
Project Role:	**CoI – hemostasis**
Research Identifier (e.g. ORCID ID):	**N/A**
Nearest person month worked:	**1**
Contribution to Project:	Dr. Spinella supported all hemostasis and coagulation analyses.

Name:	**Nivesh Mittal** **KaloCyte, Inc.**
Project Role:	**CoI – ErythroMer**
Research Identifier (e.g. ORCID ID):	**N/A**
Nearest person month worked:	**2**
Contribution to Project:	Dr. Mittal is responsible for fabrication of ErythroMer and evaluating bench top characterization of ErythroMer.

Name:	**Jennifer Richards** **KaloCyte, Inc.**
Project Role:	**CoI – ErythroMer**
Research Identifier (e.g. ORCID ID):	**N/A**
Nearest person month worked:	**2**
Contribution to Project:	Dr. Richards was responsible for supervision, implementation, and refinement of thedevelopment program for ErythroMer.

Name:	**Jefferson Gill** **American University**
Project Role:	**CoI - biostats**
Research Identifier (e.g. ORCID ID):	**N/A**
Nearest person month worked:	**1**
Contribution to Project:	Dr. Gill provided biostatistical support.

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:

Attached Separately

9. APPENDICES:

Attached Separatey.