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14. ABSTRACT

Combat-associated traumatic hemorrhage remains a primary cause of 'preventable mortality' in the military. As evidenced by robust clinical studies and dictated by current Remote Damage Control Resuscitation (RDCR) principles, timely transfusion of blood components (platelets, RBC, plasma) to mitigate hemorrhagic shock strongly improves combat casualty survival. However, blood components, especially platelets, have very limited availability far forward and there is a severe lack of *field-deployable* platelet products to enable effective *in-field* Hemostatic Resuscitation (HR). Lyophilized, low-volume, portable, easily storable, saline-reconstitutable synthetic platelet surrogate technology can potentially address this significant challenge and improve survival outcomes. To this end, we have developed a liposome-templated synthetic platelet surrogate technology (**SynthoPlate (SP)**, ***US 9107845*** and ***US 93636383***, **TRL 4**) that has demonstrated systemic safety, targeted hemostatic efficacy and survival improvement in pilot studies in mouse, rat and pig hemorrhagic trauma models. Recently we have established the ability to lyophilize and reconstitute SynthoPlate particles, as well as the ability to sterilize them for long term storage (6-9 months), without compromising their stability and platelet-mimetic bioactivity. Building on these successful capabilities, we propose the translational development of the 'lyophilized SynthoPlate' (**Lyo-SP**) nanotechnology as an intravenous or intraosseous administrable platelet surrogate product for point-of-injury 'hemorrhage control' with a vision for RDCR application in combat-associated trauma.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION:

Combat-associated traumatic hemorrhage remains a primary cause of ‘preventable mortality’ in the military. As evidenced by robust clinical studies and dictated by current Remote Damage Control Resuscitation (RDCR) principles, timely transfusion of blood components (platelets, RBC, plasma) to mitigate hemorrhagic shock strongly improves combat casualty survival. However, blood components, especially platelets, have very limited availability far forward and there is a severe lack of <u>*field-deployable*</u> platelet products to enable effective <u>*in-field*</u> Hemostatic Resuscitation (HR). Lyophilized, low-volume, portable, easily storable, saline-reconstitutable synthetic platelet surrogate technology can potentially address this significant challenge and improve survival outcomes. To this end, we have developed a liposome-templated synthetic platelet surrogate technology (**SynthoPlate (SP)**, ***US 9107845*** and ***US 93636383***, **TRL 4**) that has demonstrated systemic safety, targeted hemostatic efficacy and survival improvement in pilot studies in mouse, rat and pig hemorrhagic trauma models. Recently we have established the ability to lyophilize and reconstitute SynthoPlate particles, as well as the ability to sterilize them for long term storage (6-9 months), without compromising their stability and platelet-mimetic bioactivity. Building on these successful capabilities, we propose the translational development of the ‘lyophilized SynthoPlate’ (**Lyo-SP**) nanotechnology as an intravenous or intraosseous administrable platelet surrogate product for point-of-injury ‘hemorrhage control’ with a vision for RDCR application in combat-associated trauma to achieve hemorrhage control and treat trauma-induced coagulopathies.

2. KEYWORDS:

Hemorrhage, Trauma, Coagulopathy, Platelets, Synthetic Platelets, Field-deployable, Lyophilizable, Intravenous, Intraosseous, Remote Damage Control Resuscitation

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The overall focus of the project is to evaluate, optimize and establish a nanoparticle-based lyophilized synthetic platelet surrogate for hemorrhage control and hemostatic resuscitation in far forward battlefield setting in the framework of Remote Damage Control Resuscitation (RDCR). The ‘synthetic platelet’ nanotechnology in focus here is called SynthoPlate (SP) and the lyophilized form is termed Lyo-SP.

The evaluation is to be carried out under the following Specific Aims:
Aim 1: In vitro evaluation of platelet-relevant bioactivity for Lyo-SP compared to freshly made non-lyophilized SP, utilizing microfluidics, aggregometry, flow cytometry and ROTEM, utilizing human (healthy donor and trauma patient) plasma and whole blood.

Aim 2: Evaluation of systemic safety, hemostatic efficacy and survival outcome for reconstituted Lyo-SP compared to non-lyophilized SP, varying dosage and time-of-administration in rat liver hemorrhage model.

Aim 3: Evaluation of systemic safety, method and time of delivery, hemostatic efficacy and survival outcome for reconstituted Lyo-SP compared to non-lyophilized SP, in pig intraperitoneal hemorrhage model.

What was accomplished under these goals?

In the period of 07.2020 – 07/2021, the proposed Major Tasks were conducted as follows:

Major Task 1: Process establishment for reproducible manufacturing, lyophilization, sterilization and rapid small volume aqueous reconstitution of Lyo-SP.

The subtasks under this category were as follows:

Subtask 1:

- Manufacture, lyophilization, sterilization and size/morphology/stability assessment of SP and Lyo-SP
- SP and Lyo-SP manufactured at Haima Therapeutics supervised by Dr. Mike Bruckman (Co-I) via subcontract agreement
- Fresh made SP and reconstituted Lyo-SP characterized for size and morphology (DLS, cryo-TEM) in Sen Gupta Lab
- SP and Lyo-SP shipped to LexaMed (Toledo, Ohio) for sterilization by E-Beam and shipped back to CWRU
- DLS and Cryo-TEM of sterilized SP suspension vs aqueous reconstituted sterilized Lyo-SP for size, morphology and stability characterization and comparison

Subtask 2: Manufacturing process documentation in scientific report format for future transfer to GMP manufacturing entity

Please note that even though the project was officially funded in July of 2020, all laboratory facilities at Case Western (including Sen Gupta's laboratories), as well as Haima Therapeutics (SP and Lyo-SP manufacturing company) were closed due to COVID-19 related shutdowns. 'Restricted operations' with very limited occupancy started from mid-September of 2020 on Case Western campus, but was only limited to crucial operations and not 'hands on' research. The purchasing department did not operate during Sep-Dec 2020 and thus no supplies could be procured. The purchasing and research operations could be technically initiated in January 2021.

2. Major Activities:

For Major Task 1, scientist and research team at Haima Therapeutics, under the supervision of Dr. Michael Bruckman, has carried out studies on using various lyoprotectants to manufacture Lyo-SP and characterize the shape/size/morphology of the resultant nanoparticles to establish the optimized lyoprotectant formulation.

2.A. Lyoprotectant Optimization

The initial lyoprotectants that were evaluated were trehalose and dextrose, which in literature review have been shown to provide enhanced stability to liposomes (SynthoPlate is built on a liposomal platform). Trehalose and dextrose in HEPES were evaluated at three (3) concentrations 1%, 5%, and 10% (w/v) in HEPES and processed through the other lyophilization parameter groups as shown in Table 1 below. Test groups with trehalose and dextrose present within the liposome and outside the liposome was prepared by resuspending the lipid films with the various trehalose concentrations during SynthoPlate production per Haima's manufacturing protocol (MP004). A second lyoprotectant group with a higher concentration of trehalose (5% (w/v)) on the outside of the liposome and a lower concentration on the liposome inside (1% (w/v)) was prepared by mixing additional trehalose into the post-extruded SynthoPlate formulations.

Table 1: Lyoprotectants in Lyophilized SP Sample.

Sample ID	Rehydration Buffer Lyoprotectant	Lyoprotectant Location	Concentration % (w/v)
DD01	Dextrose in HEPES	Inside & Outside Liposome	1%
DD05	Dextrose in HEPES	Inside & Outside Liposome	5%
DD10	Dextrose in HEPES	Inside & Outside Liposome	10%
WD05	Dextrose in HEPES	Gradient Outside Liposome	5% Outside / 1% Inside
TT01	Trehalose in HEPES	Inside & Outside Liposome	1%
TT05	Trehalose in HEPES	Inside & Outside Liposome	5%
TT10	Trehalose in HEPES	Inside & Outside Liposome	10%
WT05	Trehalose in HEPES	Gradient Outside Liposome	5% Outside / 1% Inside
S	Saline (NaCl)	Inside & Outside Liposome	0.9%

2.B. SynthoPlate Freezing Optimization

Freezing optimization of the samples was accomplished by shell-freezing various concentrations and volumes of SP in 10% (w/v) Trehalose in HEPES as shown in Table 2 below. The shell-freezing is achieved by submerging the glass vial containing SP at approximately a 45° angle into liquid nitrogen at -196°C for 5 minutes while rotating at approximately 30 rpm. Each lyophilized freezing optimization group was resuspended in cell culture grade water, 0.45 µm filtered, and analyzed for size and charge to determine the optimal freezing conditions.

Table 2: Freezing optimization of lyophilized SP at various concentration and volumes.

Lyophilization Concentration (mg/mL)	SP Stock Concentration (mg/mL)	Final Lyophilization Volume (mL)	Volume of Stock Solution (mL)	Final Lyophilization Mass (mg)	Volume of Buffer (mL)
2.0	4.5	3.0	1.333	6	1.7
1.0	4.5	6.0	1.333	6	4.7
0.5	4.5	12.0	1.333	6	10.7
0.2	4.5	30.0	1.333	6	28.7

2.C. SynthoPlate Lyophilization Parameters Optimization
The initial two (2) secondary drying processes to be studied are:
1) Constant drying with sublimation at ambient temperature of 25°C and 0.2 mTorr for 24hr
2) A Primary drying phase with the lyophilizer shelf temperature precooled and increased at a rate of 1°C/min starting from -60°C to 0°C. This primary drying phase is followed by secondary drying for 1020 minutes with the lyophilizer shelf temperature increased at a rate of 1°C/min starting from -0°C to 25°C. This ramp temperature is intended to avoid stability issues with the phospholipid-peptide conjugates. Upon analysis of each secondary drying group, the goal is to determine if changes in secondary drying temperatures or ramp rates need to be adjusted, as well.
In addition, post-filtering the resuspended lyophilized SP with a 0.45 μm filter is to be utilized in the final step after resuspending the Lyo-SP.

2.D. Lyophilization Process Repeatability
The repeatability of the optimized lyophilization process including: shell freezing, primary and secondary drying, resuspension, and post-filtering was repeated across five (5) batches. These five batches were analyzed for charge and diameter measurements and the precision across batches was examined. Precision was determined by the coefficient of variation percentage (CV%) shown in equation 1 below.

$$\boxed{Coefficient\ of\ Variation = \frac{Standard\ Deviation}{Mean}\ x\ 100} \qquad \text{(Equation 1)}$$

2.E. Lyophilization Process Development Acceptance Criteria

-2.E.1. Lyoprotectant and Concentration Optimization Acceptance Criteria
The acceptance criteria of the lyoprotectant and concentration optimization is to maintain a hydrodynamic diameter within 25% of the pre-lyophilized particles, a negative charge of at least -5 mV, spherical and intact lamellar structural integrity, and the presence of binding functionality by BioFlux.

- 2.E.2. Lyophilization Process Repeatability Acceptance Criteria
The lyophilization process across 5 batches repeatability has an acceptance criteria of a CV% of less than or equal to 25% for its analysis endpoints. The lyophilization process analysis endpoints across 5 batches include: zeta potential, polydispersity index (PDI), mean number diameter, mean volume diameter, mean intensity diameter, maximum and minimum intensity diameter, and mean hydrodynamic diameter.

Lyophilizer Parameters
- Shell Freezing in Liquid Nitrogen (~196ºC) for 10 min, Held at ~196ºC for 15 min
- Primary Drying (0.700 mTorr and -45ºC for 240 min, Shelf Temp at 25ºC)
- Secondary Drying (0.200 mTorr and -45ºC for 960 min, Shelf Temp at 25ºC)

Lyoprotectant Groups (13 Groups)
- 1%, 5%, 10% Trehalose Inside and Outside Liposome
- 1%, 5%, 10% Dextrose Inside and Outside Liposome
- 1%, 5%, 10% Trehalose Outside Liposome Only
- 1%, 5%, 10% Dextrose Outside Liposome Only
- Saline

2.F. DSC Method Development and Optimization
DSC method optimization verified that the equipment is generating accurate results, optimized the test protocol to detect lyoprotectant incorporation, enabled identification of any product separation.

-2.F.1. SP Component Characterization by DSC
DSC characterization was used to verify that individual SP component thermal transitions match with vendor data and known melting temperatures (Tm). Test reagents include:
- DSPC Tm = 54.4°C
- Cholesterol Tm = 148°C
- DSPE-PEGs Tm = ~74°C
- Trehalose Tm = 203°C

2.G. DSC Method Development Acceptance Criteria

-2.G.1. Component Characterization Acceptance Criteria: The acceptance criteria for DSC results of SP components will be within +/- 10% of established values provided by the component manufacturers.

-2.G.2. Test Parameter Optimization Acceptance Criteria: The acceptance criteria for DSC results of test parameter optimization is an observable Tg and Tm with standard deviations of < 10°C.

-2.G.3. DSC Method Precision: DSC test method precision will be determined by the reproducibility of Tg and Tm amongst the five (5) runs. The CV% will be less than or equal to 25% per the ICH Q2B Validation of Analytical Procedures: Methodology Guidance.

3. Results pertaining to Major Task 1

3.1. Lyoprotectant and Concentration Optimization

3.1.1. Morphology and Size by Cryo-Transmission Electron Microscopy (Cryo-TEM)

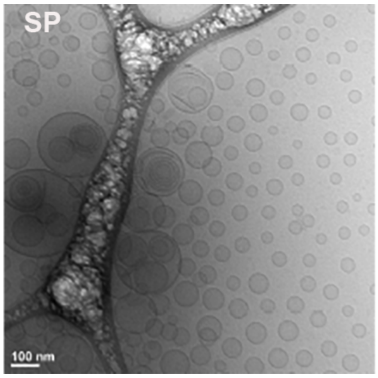
Cryo-TEM was utilized to obtain the morphology and size of lyophilized SP with rehydration buffers containing various base solutions including water, saline, and HEPES along with various lyoprotectants including trehalose. After lyophilization and reconstitution, circular morphologies were retained with all the rehydration buffers and lyoprotectants as shown with the representative Cryo-TEM of SP in 10% Trehalose in 4 mg/mL HEPES buffer in **Figure 3.1.1**. The Cryo-TEM images also revealed that multi-lamellarity decreased after lyophilization for all rehydration buffers and lyoprotectants and shown **Figure 3.1.1** and **Table 3.1.1**. In addition, the size measurements from the Cryo-TEM images showed that the size range in the diameter histograms remained constant after lyophilization for SP with rehydration buffers that contained lyoprotectants as represented by the Cryo-TEM image of lyophilized SP in 10% Trehalose in 4 mg/mL HEPES buffer.

3.1.2. Size and Charge of Lyophilized SP (Initial Screen in Rehydration Buffer)

Lyophilized SP was examined for size and charge by the Litesizer 500. The results of this initial screen revealed promising charge and size distributions for lyophilized SP in rehydration buffers containing 5% (w/v) lyoprotectants dextrose and trehalose as shown in Table 2.2.1. While the charges for the lyophilized SP with 5% (w/v) trehalose and 5% (w/v) dextrose remained more negative than -5 mV, the diameter size ranges increased for mean diameter intensity from 159 nm to 676 nm as shown in **Table 3.1.1** (see previous page). This increase in intensity diameter indicate that additional refinement for the lyoprotectant concentration will be needed (see next section).

3.1.3. Lyoprotectant Concentration Effect on Size and Charge

The concentration of the lyoprotectants dextrose and trehalose in HEPES-based rehydration buffer of SP and its effect on size and charge after lyophilization was examined at 1%, 5%, and 10% w/v using the Litesizer 500. The various concentrations of the lyoprotectants dextrose and trehalose demonstrated both an decrease in diameter size range with an increase of the lyoprotectant concentration as shown in **Table 3.1.2** (see next page). A gradient of increased lyoprotectant on the outside of the liposome (5% (w/w)) compared to the inside of the liposome (1% (w/v)) did not improve the increased diameter after lyophilization. Instead, the size range of these lyoprotectant-gradient SPs was the same size range as the SPs with 1% (w/v) lyoprotectant on both the inside and outside. Overall, 10%(w/v) trehalose resulted in intensity diameter size ranges the most similar before and after lyophilization with 62-382 nm and 99-282 nm, respectively. Now that 10% (w/v) trehalose had been shown to be optimal, this trehalose concentration effect was tested to be reproducible across multiple batches.



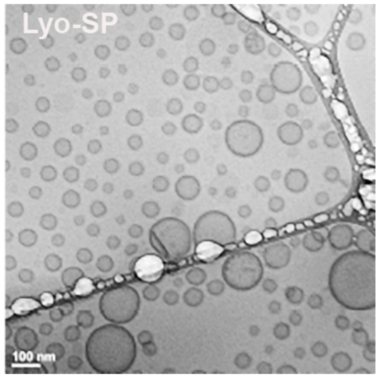


Table 3.1.1: Litesizer based charge and diameter data for SP vs Lyo-SP for various rehydration buffers

Buffer	Lyo	Zeta Potential Mean (mV)	Diameter Number Mean (nm)	Diameter Intensity Mean (nm)	Diameter Volume Mean (nm)	Litesizer PDI	Cryo-TEM Mean Diameter (nm)	Cryo-TEM PDI	Cryo-TEM Uni-lamelarity %
Saline	Pre-Lyo	-26 ± 4	124 ± 21	177 ± 49	150 ± 32	0.21 ± 0.03	115 ± 52	0.20	31%
	Post-Lyo	-32 ± 2	776 ± 598	855 ± 644	932 ± 742	0.40 ± 0.09	241 ± 103	0.18	10%
Water: 5% Trehalose	Pre-Lyo	-18 ± 10	96 ± 14	178 ± 7	127 ± 13	0.23 ± 0.03	160 ± 39	0.06	91%
	Post-Lyo	-20 ± 1	118 ± 15	888 ± 241	1004 ± 286	0.39 ± 0.07	N/A	N/A	N/A
HEPES	Pre-Lyo	-34 ± 5	108 ± 11	184 ± 44	140 ± 36	0.25 ± 0.03	127 ± 34	0.07	89%
	Post-Lyo	-32 ± 8	232 ± 254	544 ± 42	590 ± 467	0.45 ± 0.15	N/A	N/A	N/A
HEPES: 5% Dextrose	Pre-Lyo	-32 ± 4	83 ± 10	130 ± 14	100 ± 11	0.18 ± 0.03	108 ±38	0.13	91%
	Post-Lyo	-35 ± 3	129 ± 21	273 ± 65	3005 ± 4927	0.26 ± 0.02	131 ± 53	0.17	14%
HEPES: 5% Trehalose	Pre-Lyo	-20 ± 3	106 ± 9	159 ± 4	127 ± 8	0.21 ± 0.01	99 ± 53	0.29	85%
	Post-Lyo	-48 ± 5	153 ± 9	676 ± 310	1356 ± 764	0.41 ± 0.14	111 ± 43	0.15	22%

Table 3.1.2: Litesizer data for charge and diameter of SP in a concentration range of 1%, 5%, and 10% (w/v) of the lyoprotectants Trehalose and Dextrose in HEPES

Description	Lyo	Zeta Potential Mean (mV)	Hydrodynamic Diameter Mean (nm)	Mean Diameter Number (nm)	Mean Diameter Intensity (nm)	Mean Diameter Volume (nm)	PDI	Minimum Intensity Diameter (nm)	Maximum Intensity Diameter (nm)
1% Trehalose in HEPES	Pre	-30	182	77	166	104	0.22	58	392
	Post	0	16203	32	0.66	45	0.39	0.48	2271
5% Trehalose in HEPES	Pre	-22	133	77	117	93	0.19	38	211
	Post	-28	68087	108	144	131	0.40	74	884
10% Trehalose in HEPES	Pre	-16	216	83	171	112	0.22	62	382
	Post	-6	98697	117	147	130	0.45	99	282
1% Dextrose in HEPES	Pre	-38	178	107	145	141	0.22	82	291
	Post	-7	79187	257	300	306	1.37	179	1364
5% Dextrose in HEPES	Pre	-35	152	87	141	107	0.23	66	271
	Post	-29	85347	170	292	212	0.38	127	519
10% Dextrose in HEPES	Pre	-30	140	82	130	100	0.23	62	240
	Post	-16	5651	313	426	0	0.37	194	1406
1% Dextrose Inside & 5% Dext Outside	Pre	-37	158	60	151	112	0.24	69	295
	Post	0	47797	1012	1126	1288	0.50	571	2597
1% Trehalose Inside & 5% Trehalose Outside	Pre	-34	149	85	138	105	0.21	64	262
	Post	-6	59966	57	58	114	0.48	54	1367
Saline	Pre	-26	157	88	152	110	0.24	66	316
	Post	-6	27529	79	155	120	0.29	112	2332

3.2. Lyophilization Process Parameter Optimization

Based on Lyo-SP particles with the smallest diameters, the lyophilization process was determined to include shell-freezing, primary and secondary drying, and resuspension followed by 0.45 μm filtering. Post-filtering after suspension demonstrated a decrease in all batches of Lyo-SP hydrodynamic diameter (< 646 nm), number diameter (< 103 nm), volume diameter (<159), intensity diameter (< 286 nm), maximum intensity diameter (< 1034), and PDI (< 0.30) (**Table 3.2.1**). Meanwhile, the charge was maintained at levels pre-lyophilization (**Table 3.2.1**).

3.2.1. Repeatability of Lyophilization Process with 10% (w/v) Trehalose and Filtering

The size and charge of lyophilized SP with 10% (w/v) trehalose in HEPES was evaluated for reproducibility across five (5) batches. Across the five (5) repeat batches, the mean intensity diameter ranged from 159 nm to 286 nm with a CV% of 22%, which was an acceptable variability compared to the acceptance criteria of ≤ 25% (**Table 3.2.1**). Similarly, the zeta potential, mean number diameter, mean volume diameter, PDI, minimum intensity diameter, and maximum intensity diameter all had CV% ≤ 25% (**Table 3.2.1**). Only the calculated hydrodynamic diameter had CV% of 44%, which was due to its range between 228 nm – 646 nm. Aside from the hydrodynamic diameter calculation, the five (5) repeat batches of lyophilized SP with 10% (w/v) trehalose was highly repeatable with CV% for its calculated measurements. Notably, the zeta potential of the lyophilized and filtered SP was maintained at a mean of -28 mV across 5 batches with a CV% of 15%, which indicated the presence of intact peptide conjugates on the surface of the SP particles.

Please see **Table 3.2.1.** on next page.

Table 3.2.1: Litesizer charge and diameter data for five (5) repeat lyophilization batches of SP with 10% (w/v) Trehalose and prechilled shelves at each process stage: pre-lyophilization, post-lyophilization, and post-filtration.

Pre-Process	Sample Description	Lyo	Zeta Potential Mean (mV)	Hydrodynamic Diameter Mean (nm)	Mean Diameter Number (nm)	Mean Diameter Intensity (nm)	Mean Diameter Volume (nm)	PDI	Minimum Intensity Diameter (nm)	Maximum Intensity Diameter (nm)
No Pre-Filter	Run #1 SP4.0 with 10% Trehalose	Pre-Lyo	-27	128	78	124	95	0.19	61	221
		Post-Lyo	-32	328	95	215	138	0.31	71	1976
		Post-Filter	-21	228	87	226	113	0.28	66	541
	Run #2 SP4.0 with 10% Trehalose	Pre-Lyo	-27	128	78	124	95	0.19	61	221
		Post-Lyo	-32	1232	78	156	101	0.21	61	2964
		Post-Filter	-32	241	85	215	110	0.23	66	748
	Run #3 SP4.0 with 10% Trehalose	Pre-Lyo	-30	138	86	132	104	0.19	66	241
		Post-Lyo	-29	10000	129	159	142	0.42	107	2324
		Post-Filter	-29	646	56	159	159	0.20	52	1034
Pre-Lyo Filter	Run #1 Pre-Filtered SP4.0 with 10% Trehalose	Pre-Lyo	-30	138	86	132	104	0.19	66	241
		Post-Lyo	-32	2742	100	194	138	0.23	77	4818
		Post-Filter	-30	394	103	286	127	0.30	77	953
	Run #2 Prefilterd SP4.0 with 10% Trehalose	Pre-Lyo	-30	138	86	132	104	0.19	66	241
		Post-Lyo	-35	10000	99	270	121	0.35	77	10000
		Post-Filter	-30	494	102	187	126	0.27	71	953
Inter-Batch Mean		**Post-Filter**	**-28**	**401**	**87**	**215**	**127**	**0**	**66**	**846**
Inter-Batch STDEV			**4**	**176**	**19**	**48**	**19**	**0**	**9**	**201**
Inter-Batch CV%			**-15%**	**44%**	**22%**	**22%**	**15%**	**16%**	**14%**	**24%**

Table 3.2.2. below depicts DLS and Zetasizer data from characterization of Lyo-SP manufactured using the protocol metrics described in Section 2.1 and resuspended in PBS.

Lyoprotectant in HEPES Group	Lyoprotectant Conc. (%, w/v)	Lyo	Zeta Potential Mean (mV)	Hydrodynamic Diameter Mean (nm)	Mean Diameter Number (nm)	Mean Diameter Intensity (nm)	Mean Diameter Volume (nm)	PDI	Minimum Intensity Diameter (nm)	Maximum Intensity Diameter (nm)
Trehalose	1%	Pre	-30	182	77	166	104	0.22	58	392
		Post	0	16203	32	0.66	45	0.39	0.48	2271
	5%	Pre	-22	133	77	117	93	0.19	38	211
		Post	-28	68087	108	144	131	0.40	74	884
	10%	Pre	-16	216	83	171	112	0.22	62	382
		Post	-6	119591	62	89	74	0.47	49	282
Dextrose	1%	Pre	-38	178	107	145	141	0.22	82	291
		Post	-7	79187	257	300	306	1.37	179	1364
	5%	Pre	-35	152	87	141	107	0.23	66	271
		Post	-29	85347	170	292	212	0.38	127	519
	10%	Pre	-30	140	82	130	100	0.23	62	240
		Post	-16	5651	313	426	0	0.37	194	1406
1% Dextrose Inside & 5% Dext Outside		Pre	-37	158	60	151	112	0.24	69	295
		Post	0	47797	1012	1126	1288	0.50	571	2597
1% Trehalose Inside & 5% Trehalose Outside		Pre	-34	149	85	138	105	0.21	64	262
		Post	-6	59966	57	58	114	0.48	54	1367
Saline		Pre	-26	157	88	152	110	0.24	66	316
		Post	-6	27529	79	155	120	0.29	112	2332

Table 3.2.2.: Characterization of Lyo-SP formulations using DLS (size) and Zetasizer (charge)

As evident from the above data, substantial size increase and charge heterogeneity were found when comparing fresh-made SP with aqueous reconstituted Lyo-SP. We hypothesized that this heterogeneity is potentially due to conglomeration of a certain percentage of SP upon lyophilization and resuspension. We also postulated that the resuspension medium being PBS potentially influences the charge heterogeneity. Additionally, the heterogeneity was demonstrably higher for Dextrose compared to Trehalose as a lyoprotectant. Therefore, the following revised strategies were implemented:

- Precooled Shelf in Lyophilizer protocol (-80C)
- Pre-Filter SP prior to Freezing and Lyophilization to remove large size fraction and Filter resuspended Lyo-SP through 0.45 micron sterile filter
- Alternatively, vortex fresh SP and resuspended Lyo-SP for 10 seconds to study whether this eliminates large aggregate fraction

Table 3.2.3 below depicts DLS and Zetasizer data from characterization of Lyo-SP manufactured using the revised protocol metrics described above.

Lyoprotectant in HEPES Group	Lyoprotectant Conc. (%, w/v)	0.4 um Filter	Zeta Potential Mean (mV)	Hydrodynamic Diameter Mean (nm)	Mean Diameter Number (nm)	Mean Diameter Intensity (nm)	Mean Diameter Volume (nm)	PDI	Minimum Intensity Diameter (nm)	Maximum Intensity Diameter (nm)
Trehalose	10%	Vortexed	-16	13525	555	726	853	0.39	282	1318
		Filtered	-5	588		151		0.28	107	498
Dextrose	10%	Vortexed	-10	75690	490	552	595	0.5	306	879
		Filtered	-3	1035	268	358	380	0.33	174	636

Table 3.2.3: Characterization of Lyo-SP formulations processed with filtration and vortexing

3.3. Chemical Stability by DSC

The chemical stability of Lyophilized SP and its components was determined by DSC analysis. Initially, the individual components of SP: DSPC, DSPE-PEG(1K), cholesterol, and trehalose were analyzed by DSC to determine their measured melting temperature and how it compared to theoretical values (**Figure 3.3.1** and **Table 3.3.1**). The measured melting temperature of the SP components was within 1% of the theoretical values and repeatable across replicates with %CV lower than 25%. Thus, the DSC method was shown to be accurate and repeatable and therefore a validated assay per the ICH Q2B Validation of Analytical Procedures: Methodology. Next steps will be evaluation of fully assembled SP.

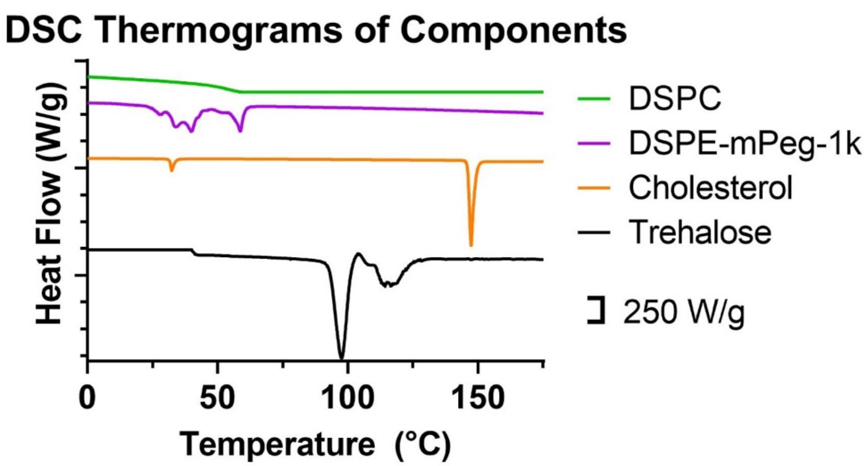


Figure 3.3.1. DSC analysis of individual molecular components of SP.

Table 3.3.1: DSC measured melting point temperatures compared to theoretical values for SP components: DSPC, cholesterol, trehalose, and DSPE-PEG(1k).

Melting points extracted from DSC thermograms				
Results in Degrees Celsius	**Cholesterol**	**DSPC**	**Trehalose**	**DSPE-PEG1k**
Expected	148	54.4	97	
Measured Mean	147.5	54.0	97.8	28.1 - 58.7
Standard Deviation	0.71	2.12	0.38	
Percent Difference from Expected	0.34%	0.77%	0.85%	
Coefficient of Variation	0.48	3.92	0.39	
Sample Replicates	2	3	2	1

Conclusion and Next Steps: The optimal lyoprotectants and concentration appears to be trehalose at 10% (w/v) in HEPES rehydration buffer, which has shown to protect the charge and size of the liposomes after lyophilization compared to other lyoprotectant parameters. The shell freezing, lyophilization with primary and secondary drying, and post-filtering has demonstrated to be a repeatable (CV% < 25%) across five batches.

4. <u>Major Task 2:</u> Establishment of particle stability, morphology, and platelet mimetic bioactivity of Lyo-SP utilizing microfluidics, aggregometry, flow cytometry, ROTEM and TEG analysis with healthy human donor blood.

Subtask 1:

- Manufacture SP and Lyo-SP as per above-established process protocol for bioactivity evaluation
- Manufacture of SP and Lyo-SP at Haima, shipment to LexaMed for sterilization and shipped back to CWRU

BioFlux Microfluidics system was acquired from Fluxion Biosciences and installed in the Sen Gupta laboratory (Wickenden 5th floor) at Case Western. The system set-up is shown below in Figure 4.1:

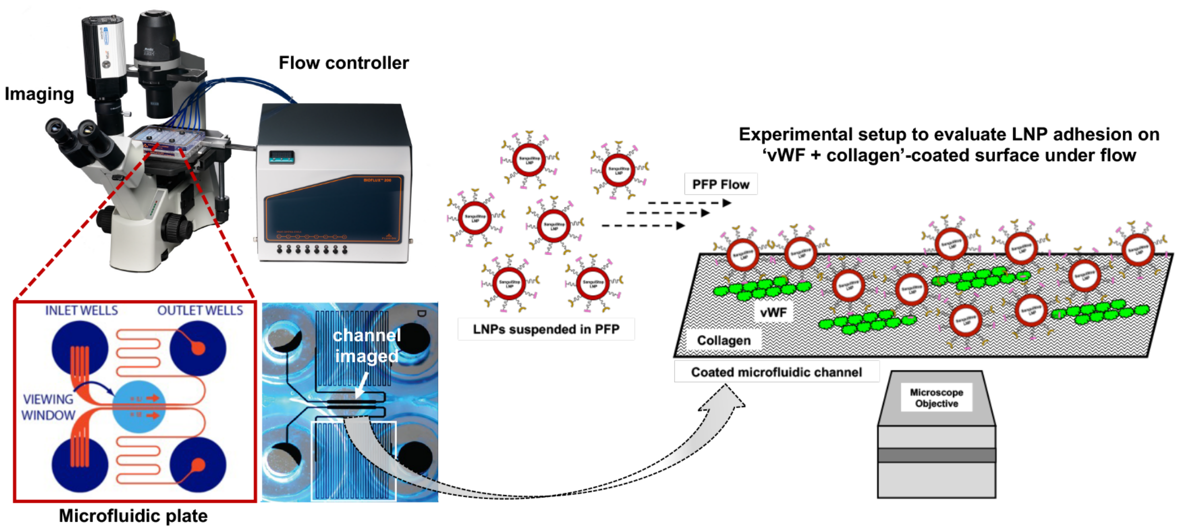


Figure 4.1. BioFlux microfluidic system set-up where channels can be coated with relevant proteins (e.g. collagen, vWF etc.), and fluorescently labeled lipid nanoparticles (LNPs), e.g. SP or Lyo-SP, can be flowed over the coated channels at controlled shear rate and imaged in real time using inverted fluorescence microscope. Platelets in plasma (e.g. PRP, PPP) or whole blood can also be introduced in the channel for analysis.

This system allows custom-made microfluidic well-plates to be observed/imaged under an inverted fluorescence microscope. The microfluidic channels are etched in between the wells of the plates and the flow velocity (hence shear stress) is regulated pneumatically using a 'flow controller' module. The microfluidic channels can be coated with hemostasis-relevant proteins (e.g. collagen, vWF etc.), and SP or Lyo-SP suspension in PBS, platelet suspension in plasma, as well as whole blood can be flowed through the channel at controlled shear rate. By virtue of pre-labeling SP (and Lyo-SP) with Rhodamine B and platelets with Calcein, it becomes possible to image the SP (and Lyo-SP) as well as platelet behavior on the coated microchannels, and also analyze SP (or Lyo-SP)-to-platelet interactions.

Initial studies in the BioFlux system were done by coating the surface with rat tail collagen, and equine collagen, and flowing platelet-rich plasma (PRP) on it where the platelets in the plasma were pre-stained with Calcein (green fluorescence). The purpose of these studies was to optimize the collagen coating protocol (both the source and the concentration of collagen) to enable significant platelet adhesion and activation from PRP under high shear flow (30-60 dyn/cm^2), since this will be the ‘positive control’ condition for efficient hemostatic performance. Representative results at 60 dyn/cm^2 are shown in **Figure 4.2**:

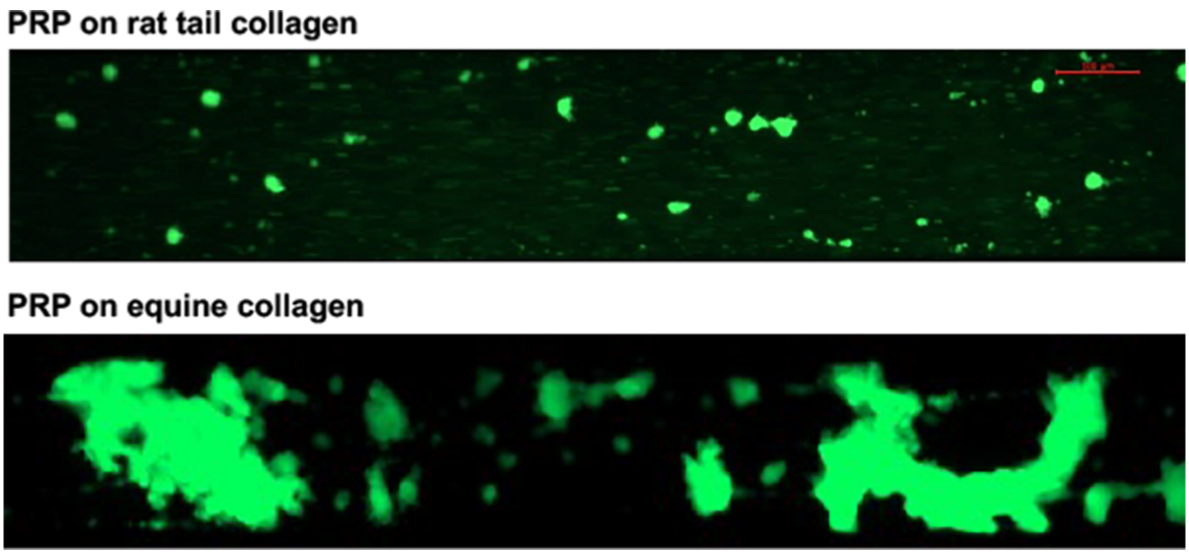


Figure 4.2: Representative fluorescence microscopy images of calcein-stained platelets (green fluorescent) binding to and forming aggregates on collagen in BioFlux microfluidic channel under flow at 60 dyn/cm^2 shear.

As evident from the results, platelets were more responsive to equine collagen compared to rat tail collagen, in terms of forming hemostatically relevant activation and aggregate formation. This is potentially due to the presence of a higher amount of endogenous vWF complexed with the equine collagen compared to rat collagen (information provided by the collagen supplier company Chronolog). Therefore, for all subsequent studies the equine collagen was used coat the BioFlux microchannels.

Next set of studies involved flowing Rhodamine-B (RhB) labeled SP versus control nanoparticle (no peptide decoration), on collagen-coated microchannels (coated with 40µg/ml equine collagen type-I) in presence of 10µg/ml vWF exogenously added in the flow volume. The rationale for these studies was to establish/confirm the baseline surface-coverage of SP versus control nanoparticles on the hemostatically relevant protein (vWF + collagen) coated surface, as an important parameter to subsequently evaluate SP versus Lyo-SP in terms of surface-coverage. RhB fluorescence (orange red) was used for these evaluations and fluorescence video microscopy was carried out for 10 min per experiment. The binding kinetics as well as surface coverage (as a % of total surface) were quantified as results reflective of SP adhesion criteria to ‘vWF + collagen’-coated surface. Representative images are shown below in **Figure 4.3**:

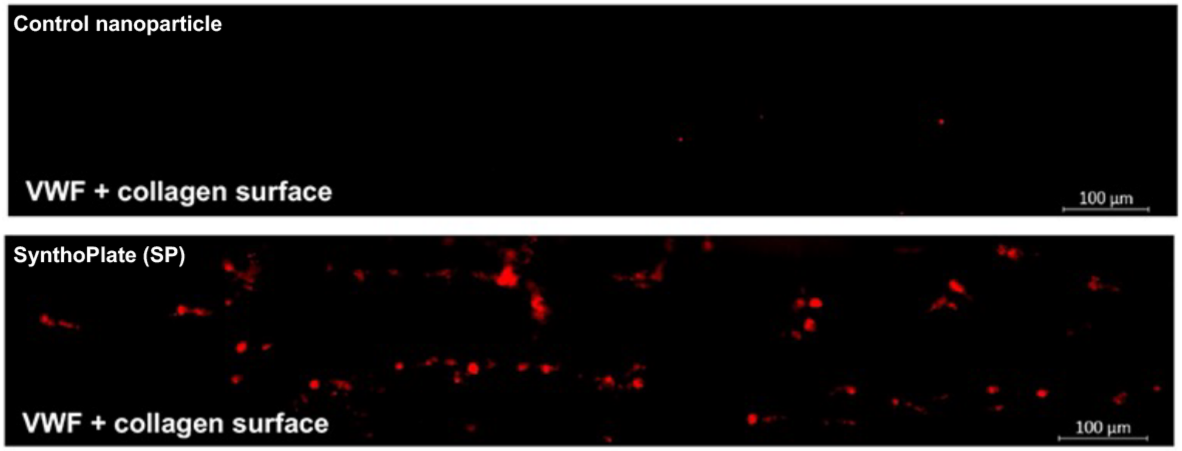


Figure 3: Representative fluorescence video microscopy images of RhB-labeled (orange red fluorescence) control nanoparticle (liposome without any peptide decoration) versus SynthoPlate (SP liposome bearing VBP + CBP + FMP peptides) adhesion to 'vWF + collagen'-coated microfluidic surface at 60 dyn/cm^2 shear flow.

As evident from the above Figure, control nanoparticles are unable to bind to the 'vWF + collagen'-coated microfluidic channel, whereas SP is able to substantially bind to this channel under high shear flow. Therefore, the platelet-mimetic hemostatically relevant adhesive capability of SP is confirmed by these early BioFlux studies.

Further studies will be continued in Year 2 to establish the hemostatically relevant bioactivity efficiency of aqueous-reconstituted Lyo-SP compared to fresh-made aqueous suspension of SP, in the context of rescuing hemostatic effect in 'simulated platelet dysfunction' settings in BioFlux.

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

The project enabled training and professional development opportunity for multiple scientists and researchers at Haima Therapeutics (Michael Bruckman, Christa Pawlowski, Andrew Ditto, Shrijal Desai, Sana Sayed, Ujjal Sekhon) and at Sen Gupta laboratory at Case Western (Anirban Sen Gupta, Nathan Rohner, Norman Luc). Under the supervision of Michael Bruckman (Haima) and Anirban Sen Gupta (Case Western), the researchers were able to undergo training in operational aspects of nanoparticle manufacture, lyophilization, physical property (size, charge) characterization and bioactive property (adhesion, aggregation) characterization. They executed all studies, acquired data, analyzed data and prepared scientific documents that enabled preparation of this annual report.

How were the results disseminated to communities of interest?

Presenting the study results at MHSRS 2021 was not possible because of conference cancellation due to COVID-19 restrictions. It is anticipated that data will be presented at MHSRS 2022. Results are being disseminated currently through the progress reports and annual report submitted via eBRAP that are open to unrestricted public disclosure.

What do you plan to do during the next reporting period to accomplish the goals and objectives.

During the next reporting period the three following aspects are anticipated to be executed:

- Complete SOPs and GMP-transferable documentation for manufacture of Lyo-SP
- Major in vitro evaluation of hemostatic activity conservation of Lyo-SP compared to SP
- Major in vitro evaluation of the effect of Lyo-SP versus SP in trauma patient blood sample
- Initiation of safety studies of SP versus Lyo-SP in intravenous dosing in rats

4. IMPACT:

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

The main Impact of Year 1 studies is the establishment of the potential of effectively lyophilizing a synthetic platelet surrogate (SynthoPlate, SP to Lyophilized SynthoPlate, Lyo-SP) with reproducible quality and characteristics, demonstrating conservation of hemostatic bioactivity. If successfully executed and translated, the Lyo-SP product can provide significant benefit as a field-deployable, easiy portable, on-demand aqueous reconstitutable intravenous hemostatic agent for hemorrhage control in RDCR settings.

What was the impact on other disciplines?

The findings may influence the methodology and process development of other therapeutic products based on lipidic nanoparticles (e.g. chemotherapies, vaccine LNPs etc.) to achieve lyophilized versions of these products that are easily portable in pre-hospital settings without the need of special containers and cold chain, to help management of various pathological conditions.

What was the impact on technology transfer?

The lyophilization method development for Lyo-SP is anticipated to result in new Intellectual Property related to lyophilization formulations and process conditions. Haima Therapeutics, the manufacturing entity for Lyo-SP, is in the process of legal consultation for such potential IP.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

The predominant problem in executing the project tasks in their early phase was the complete shutdown of experimental operations during whole of 2020 and initial few months of 2021 due to COVID-19 related restructions. Operations were resumed in early 2021 and significant efforts were made to accelerate the pace of the task execution to 'catch up' to the proposed SoW as much as possible. Although significant success was achieved to this end, there is still an existent lag regarding some sub-tasks (e.g. sterilization studies, trauma patient blood studies etc.). We anticipate catching up to this lag as we operate through 2022.

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

The delays that were imparted due to COVID-19 related restrictions during the first 6-8 months of the Project beginning (July-December 2020, January 2021) has resulted in a lag period regarding execution of Tasks and Sub-tasks as described in SoW, and this lag is anticipated to carry over as the project operations progress. With this anticipation, the PI (Sen Gupta) is planning to request a 12-month No Cost Extension (NCE) to complete all the proposed tasks of the project (anticipated end date to be July 2024 instead of July 2023).

Changes that had a significant impact on expenditures

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

Significant changes in use of biohazards and/or select agents

6. **PRODUCTS:**

" **Nothing to Report.**

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

Journal publications.

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

Other publications, conference papers and presentations.

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

- **Technologies or techniques**

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

- **Other Products**

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Anirban Sen Gupta, PhD
Project Role: PD/PI
Research Identifier: https://orcid.org/0000-0002-5773-0667
Nearest person month worked: 3
Contribution to Project: Dr. Sen Gupta provided overall direction and guidance, for experimental design, execution and analysis for studies proposed under the Specific Aims of the project. Dr. Sen Gupta also prepared and submitted quarterly progress report. In addition, Dr. Sen Gupta carried out communication and planning with collaborators at Haima and UPitt for subsequent phases of the project that will be carried out at current location.

Norman Luc
Project Role: Researcher at CWRU in Sen Gupta lab
Research Identifier:
Nearest person month worked: 12
Contribution to Project: Mr. Luc contributed to assisting in assisting with DSC and Litesizer analysis of SP and Lyo-SP.

Nathan Rohner, PhD
Project Role: Senior Research Associate at CWRU in Sen Gupta lab
Research Identifier:
Nearest person month worked: 8
Contribution to Project: Dr. Rohner oversaw procurement, installation and training of DSC and Litesizer in Sen Gupta lab, and assisted with SP and Lyo-SP analysis on these instruments.

Name: **Michael Bruckman**
Project Role: PI (CEO at Haima)
Nearest person month worked: 3
Contribution to Project: Michael managed the project operations, updated the budget documents, and assisted in any protocol development and results evaluation.

Name: **Christa Pawlowski**
Project Role: Key Personnel (COO at Haima)
Nearest person month worked: 2
Contribution to Project: Christa assisted with managing the project operations and assisted in any protocol development and results evaluation. Dr. Pawlowski led the subaward and subcontract document preparation. Finally, she assisted in developing the document control system and template documents for Haima.

Name: **Andrew Ditto**
Project Role: Key Personnel (Senior Scientist at Haima)
Nearest person month worked: 3
Contribution to Project: Andrew assisted with project management with Michael and Christa. He led the Cryo-TEM experiments. In the past quarter, he also helped get the clean room installed. Andrew is also leading the lyophilization process method development studies.

Name: **Emily Gahagan**
Project Role: Other Personnel (Research Scientist II at Haima)
Nearest person month worked: 5
Contribution to Project: Emily executed the SynthoPlate physicochemical and functional characterization experiments.

Name: **Shrijal Desai**
Project Role: Other Personnel (Research Scientist I at Haima)
Nearest person month worked: 3
Contribution to Project: Shrijal executed any required SynthoPlate manufacturing and also assists in any physicochemical and functional characterization.

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?

Lead organization: Case Western
Collaborating organizations: Haima Therapeutics, University of Pittsburgh

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: