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14. ABSTRACT

Combat trauma-associated uncontrolled hemorrhage and coagulopathy remain the leading causes of morbidity and mortality in the military. Overwhelming evidence from military based resuscitation studies has indicated that platelet transfusion can significantly reduce these events in prolonged field care scenarios. However, platelet transfusion suffers from unique logistical and functional challenges in a far forward military setting, due to (i) limited availability and portability of platelet concentrates, (ii) special storage requirements to minimize platelet activation and granulation, (iii) high risk of bacterial contamination and (iv) very short shelf-life (3-5 days). Furthermore, blood type compatibility issues can limit early intervention. Other platelet-derived products, e.g., frozen (-80C), cold-stored (4C) or lyophilized platelets and platelet membrane-derived vesicle technologies (e.g. Infusible Platelet Membrane and Thrombosome) may suffer from similar limitations and performance variabilites. These challenges have led to robust research efforts for creating a shelf-stable, highly portable, readily deliverable 'platelet substitute' that can mimic platelet-mediated mechanisms of hemostasis, while avoiding systemic immunogenicity and off-target harmful effects. To this end, we have created a lipid-peptide conjugate based synthetic platelet technology (SynthoPlateTM, US patent 9107845, TRL 4), that mimics the inherent platelet-mediated mechanisms of primary and secondary hemostasis in a bleeding site-selective fashion, without presenting systemic risks.

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1. Introduction: Combat trauma-associated uncontrolled hemorrhage and coagulopathy remain the leading causes of morbidity and mortality in the military. Overwhelming evidence from military based resuscitation studies has indicated that platelet transfusion can significantly reduce these events in prolonged field care scenarios. However, platelet transfusion suffers from unique logistical and functional challenges in a far forward military setting, due to (i) limited availability and portability of platelet concentrates, (ii) special storage requirements to minimize platelet activation and granulation, (iii) high risk of bacterial contamination and (iv) very short shelf-life (3-5 days). Furthermore, blood type compatibility issues can limit early intervention. Other platelet-derived products, e.g., frozen (-80C), cold-stored (4C) or lyophilized platelets and platelet membrane-derived vesicle technologies (e.g. Infusible Platelet Membrane and Thrombosome) may suffer from similar limitations and performance variabilities. These challenges have led to robust research efforts for creating a shelf-stable, highly portable, readily deliverable 'platelet substitute' that can mimic platelet-mediated mechanisms of hemostasis, while avoiding systemic immunogenicity and off-target harmful effects. To this end, we have created a lipid-peptide conjugate based synthetic platelet technology (SynthoPlate™, US 9107845, TRL 4), that mimics the inherent platelet-mediated mechanisms of primary and secondary hemostasis in a bleeding site-selective fashion, without presenting potential systemic risks. In the current project, we seek to evaluate the point-of-care hemostatic efficacy and spatio-temporally targeted wound healing treatment applicability of the SynthoPlate™ nanotechnology in appropriate porcine models, with a vision to translate this technology for prolonged combat casualty care in a far forward setting. Our specific aims are:
Aim 1. Characterization of biodistribution, systemic risks and immune response of intravenously administered SynthoPlate™ in pigs.
Aim 2. Evaluation of hemostatic efficacy of pristine SynthoPlate™ and TXA-loaded SynthoPlate™ in a pig model of polytrauma.
Aim 3. Evaluate the efficacy of SynthoPlate™ alone or in combination with Gentamicin to provide wound protection and improve re-epithelialization in porcine wound models.
2. Keywords: Trauma, Hemorrhage, Burn, Wound, Transfusion, Platelets, Synthetic Platelets, TXA, Intravenous, Hemostasis, Pig Model

3. Accomplishments: As per the proposed SOW, the Year 1 major goals are:
Major Task 1. Characterize and mitigate immune response to SynthoPlate dosing in pigs
- Subtask 1: SynthoPlate manufacture and sterilization studies
- Subtask 2: IACUC and ACURO approvals
- Subtask: Complement and IgG analysis

Major Task 2. Characterize and mitigate systemic prothrombotic risks (if any) upon SynthoPlate administration.
- Subtask 1: SynthoPlate™ dose studies in pigs to monitor blood pressure, vitals, blood lab, aggregometry, ROTEM, D-dimer assays

Major Task 3. Characterize biodistribution of SynthoPlate over time
- Rhodamine-B fluorescence based UPLC analysis of SynthoPlate™ sequestration and biodistribution during 1 hr period

Major Task 4. Establish a safe dosing protocol for SynthoPlate in pigs
- Statistical analysis of all data generated during Mo 3-9, generation of progress report and safe dosage protocol documents to supply to collaborators

Specific Aim 1. Characterization of biodistribution, systemic risks and immune response of intravenously administered SynthoPlate™ in pigs. **(Months 1-12)**

<u>Major Tasks 1</u>

<u>Subtask 1:</u> SynthoPlate was manufactured in two different formulations Version 1 (SPv1) and Version 2 (SPv2) as shown in Figure 1. The major difference between the two formulations is the mole % of the platelet integrin GPIIb-IIIa binding fibrinogen mimetic peptide (FMP) to assess potential safety and efficacy outputs of these formulations in pigs. Both SPv1 and SPv2 were manufactured using a "film rehydration and extrusion" technique. The component lipids and lipid-peptide conjugates in the mole fractions shown in Figure 1 were dissolved in 1:1 chloroform:methanol. The solvent was removed under reduced pressure, and the thin lipid film was rehydrated with normal saline solution (0.9% NaCl) at a concentration of 1 x 10^5 moles of lipid per mL. This lipid suspension was was subjected to 10 freeze/thaw cycles and subsequent extrusion through 200 nm pore-diameter polycarbonate membranes using a pneumatic extruder (Northern Lipids, Burnaby, Canada) to create heteromultivalently decorated SynthoPlate vesicles. Dynamic light scattering (DLS) and electron microscopy characterization indicated fresh-made SynthoPlate vesicles were ~200 nm in diameter (Figure 2). The diameter does not seem to change over 6-month time period in storage (as saline suspension) at room temperature (Figure 2), which indicates significant promise of stability in storage. The SynthoPlate vesicles were subjected to two different sterilization methods: sterile filtration and E-beam (25 KGy and 40KGy). For this, first it was necessary to validate the sterilization methods by demonstrating that SynthoPlate itself does not inhibit the growth of contaminating organisms and hence does not interfere with the standard sterilization assays. For this, SynthoPlate™ samples were inoculated with 6 different challenge organisms, namely *Staphylococcus aureus, Kocuria rhizophila, Clostridium sporogenes, Bacillus subtilis spizizenii, Candida albicans* and *Aspergillus brasiliensis,* and allowed to grow in appropriate conditions for up to 5 days. Any differences in growth profiles between samples with or without SynthoPlate™ over the 5-

	SynthoPlate v1 (SPv1)	SynthoPlate v2 (SPv2)
	Mole %	Mole %
DSPC	40.63%	46.50%
Cholesterol	39.32%	45.00%
DSPE-mPEG$_{1000}$	2.18%	2.50%
DHPE-Rhodamine	0.87%	1.00%
DSPE-PEG$_{2000}$-VBP	1.09%	1.25%
DSPE-PEG$_{2000}$-CBP	1.09%	1.25%
DSPE-PEG$_{2000}$-FMP	14.81%	2.50%

Figure 1

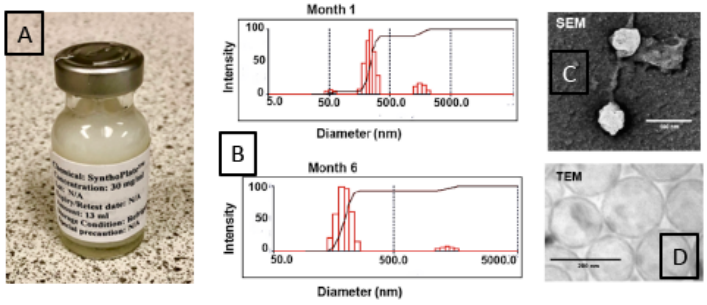

Figure 2. [A] Characteristic vial of manufactured SynthoPlate; [B] Representative DLS data for SynthoPlate at 1 month and 6 month upon storage; [C] and [D] are representative Scanning Electron Microscopy (SEM) and cryo-Transmission Electron Microscopy (cryo-TEM) imaged of SynthoPlate vesicles.

day period were recorded. Next, SynthoPlate™ samples were sterilized by filtration in 0.2 μm filter and by E-beam exposure at both 25 and 40 kGy doses. Sterility was assessed by observation of the samples for any visible growth of microorganisms over the course of 7 days. Furthermore, endotoxin burden in the formulation was evaluated using a standard chromogenic limulous ameobocyte lysate (LAL) assay. Briefly, SynthoPlate samples were incubated with 0.5% (v/v) of 1.5% $C_{12}E_{10}$ detergent for 20 minutes at 65°C followed by 1 hour at room temperature to solubilize the lipid components of SynthoPlate™ and release any masked endotoxin. Endotoxin burden in the detergent-treated SynthoPlate™ samples was quantified using the LAL assay.

25 kGy - LIQUID

Organism	Days of Incubation									
	1		2		3		4		5	
	T	C	T	C	T	C	T	C	T	C
B. subtilis	N	N	P	P	PE	PE	PE	PE	PE	PE
C. albicans	N	N	N	N	P	P	PE	PE	PE	PE
A. brasiliensis	N	N	N	N	N	N	P	P	PE	PE
S. aureus	N	N	P	P	PE	PE	PE	PE	PE	PE
K. rhizophila	N	N	N	N	P	P	PE	PE	PE	PE
C. sporogenes	P	P	PE	PE	PE	PE	PE	PE	PE	PE

40 kGy - LIQUID

Organism	Days of Incubation									
	1		2		3		4		5	
	T	C	T	C	T	C	T	C	T	C
B. subtilis	N	N	P	P	PE	PE	PE	PE	PE	PE
C. albicans	N	N	N	N	P	P	PE	PE	PE	PE
A. brasiliensis	N	N	N	N	N	N	P	P	PE	PE
S. aureus	N	N	P	P	PE	PE	PE	PE	PE	PE
K. rhizophila	N	N	N	N	P	P	PE	PE	PE	PE
C. sporogenes	P	P	PE	PE	PE	PE	PE	PE	PE	PE

Figure 3. Bacteriostatic and fungistatic analysis of SynthoPlate™ suspension exposed to E-beam sterilization and challenged with 6 different organisms, namely *Bacillus subtilis spizizenii, Candida albicans, Aspergillus brasiliensis, Staphylococcus aureus, Kocuria rhizophila,* and *Clostridium sporogenes,* which were allowed to grow in appropriate conditions for up to 5 days. T= test, C= control, N = No growth, P = positive growth and PE = positive growth established.

For all organisms tested, SynthoPlate™ itself did not have any bacteriostatic or fungistatic activity, and this data is shown in Figure 3. SynthoPlate™ solutions sterilized by either filtration or by E-beam exposure showed no visible growth of microorganisms, suggesting effective sterilization by all methods tested. The endotoxin bioburden of the concentrated SynthoPlate™ formulation (1 x 10^5 moles lipid per ml) was measured to be 3.5-5 EU/ml as per a standard chromogenic limulous ameobocyte lysate (LAL) assay (see Methods for details). When diluted in sterile saline to the in vivo dosing level of 5 x 10^4 moles lipid per ml, this equates to a dose of ~0.83 EU/kg, which is substantially less than the endotoxin safety threshold of ≤ 5 EU/kg for *in vivo* usage. This suggests that the SynthoPlate™ preparation can be safe for *in vivo* usage. To assess whether sterilization affects SynthoPlate™ particle stability, post-sterilization particle size distribution was measured by DLS and compared to fresh-made (unsterilized) particles. Furthermore, in order to measure whether sterilization affects the platelet-mimetic biofunctional abilities of SynthoPlate™, specific adhesion and aggregation assays were performed. To test whether sterilization affects the platelet-mimetic adhesive function of CBP and VBP moieties on SynthoPlate™ (to collagen and vWF, respectively), acid washed glass coverslips were coated with a solution of 1:1 vWF:collagen, and 200μl RhB-labeled SynthoPlate™ suspensions (0.4 mg/ml), un-sterilized or sterilized, were incubated on these coverslips in a 12-well plate for 30 minutes at 37°C on a shaker set at 100 rpm. Following this, SynthoPlate™ suspension was removed, and loosely bound particles were further washed away with PBS. The coverslips were then mounted onto microscope slides and imaged (using Zeiss AxioObserver inverted fluorescence microscope) for adhered SynthoPlate™ (Rhodamine B fluorescence, λ_{ex}=560 nm, λ_{em}=580 nm). To test whether sterilization affects the ability of FMP moieties to bind to active platelet integrin GPIIb-IIIa to amplify platelet aggregation, platelet lumi-aggregometry assays were performed using a PAP-8E (Bio/Data Corp, Horsham, PA, USA) instrument. For this,

human whole blood was collected in citrated tubes from healthy consenting donors in accordance with protocols and guidelines approved by the Institutional Review Board (IRB) of University Hospitals Cleveland Medical Center (UHCMC, IRB Number 12-16-06).

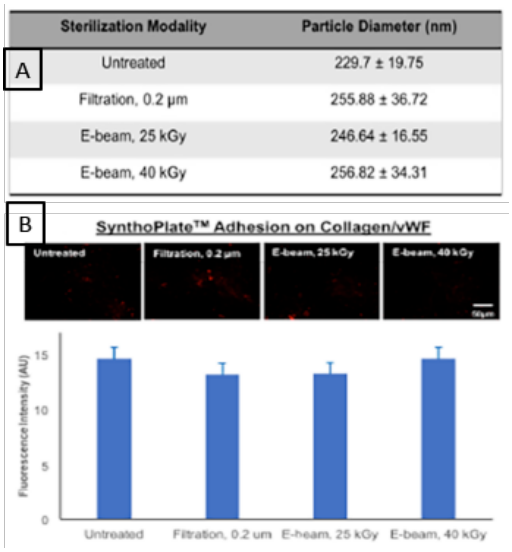

Figure 4. [A] DLS based size distribution analysis and [B] Microfluidics based collagen/vWF adhesion analysis show that sterilization by filtration or E-beam does not negatively affect the size, stability and biofunctionality of SynthoPlate vesicles.

It is important to note here that this IRB is not directly connected to the proposed aims (and corresponding Major Tasks) of the current PFCRA project but is an IRB approved in our laboratory to draw blood from healthy donor volunteers for isolating platelets and plasma, in the context of confirming SynthoPlate quality and activity towards healthy human platelets, before doing any other research studies. In several of our previous research projects we had attempted this quality assessment activity with commercially obtained bags of platelets (e.g. from Lampire) but found that there were 3-5 day delays in the commercial product getting to our lab, which affected the 'platelet-to-SynthoPlate activity' that we observed. Therefore we had decided to obtain an IRB to collect healthy human blood from volunteer donors and isolate fresh platelets to confirm SynthoPlate-to-platelet activity quality, every time we made SynthoPlate batches. The grant connected to this approved IRB is NIH R01 HL121212. This allows us to confirm SynthoPlate quality (via microfluidics and aggregometry) independent of the subsequent specific in vivo studies to be done with SynthoPlate pertaining to other project aims. This clarification is being provided to confirm that human subject use is not being performed using funds from the PFCRA award. Informed consent was obtained from all subjects as per this IRB-approved guideline. The whole blood was centrifuged at 150 x g for 15 minutes to obtain platelet rich plasma (PRP). Some of the PRP was further centrifuged at 2500 x g for 25 minutes to obtain platelet poor plasma (PPP). Aggregometry studies were carried out at 37°C with stirring at 1200 rpm. ADP-mediated aggregation was assessed with 225µl of 100% PRP or PRP diluted 50% (v/v) with PPP supplemented with fresh-made (un-sterilized) or sterilized (filtration and E-beam) SynthoPlate™ particles with a particle concentration of 5 x 10^{10}/mL, with or without agonist (2 x 10^{-5}M ADP). These adhesion and aggregation results of SynthoPlate™ post-sterilization were compared to that for fresh-made SynthoPlate™. After sterilization by either filtration or E-beam, SynthoPlate™ particles showed statistically insignificant variability in size compared to fresh-made (unsterilized) SynthoPlate™ (Figure 2), suggesting that the sterilization methods did not affect particle integrity, size and stability. For the microfluidics-based adhesion assays, representative images of Rhodamine B (red) fluorescent adhered SynthoPlate™, as well as, statistical data of fluorescence intensity analysis from multiple images per condition (5 images per condition), are shown in Figure 4. As evident from the results, sterilized SynthoPlate™

particles retained their capability to adhere to 'collagen + vWF'-coated surfaces at levels similar to freshly prepared SynthoPlate™ particles, indicating that sterilization by filtration or E-beam does not affect the platelet-mimetic adhesive activity of VBP and CBP moieties on SynthoPlate™. In the aggregometry studies, neither unsterilized nor sterilized SynthoPlate™ particles were found to induce any spontaneous platelet aggregation in the absence of ADP, but both were able to markedly enhance platelet aggregation in the presence of ADP, as shown in representative aggregometry data in Figure 5. Therefore, these results indicate that sterilization by filtration or E-beam does not affect the platelet-mimetic aggregatory activity of FMP moieties on SynthoPlate™. Hence, altogether these results establish that SynthoPlate™ can be effectively sterilized without affecting its platelet-mimetic bioactivity, which is important in the context of its translation through large-scale manufacturing and sterilization without compromising its hemostatic function.

Subtask 2: We obtained necessary IACUC and ACURO approvals (please see attached document) to carry out pig 'safety' studies upon SynthoPlate™ dosing.

Subtask 3: All *in vivo* porcine studies were carried out in accordance with relevant guidelines and regulations approved by Case Western Reserve University Institutional Animal Care and Use Committee and ACURO (**IACUC, Protocol Number 2017-0105, ACURO approval May 7, 2018**). Yorkshire Farm Pigs 25–36 kg were acclimated to the laboratory space for 48 hours. Pigs were fasted for 12 hours before experiments but had free access to water. Pigs were sedated with Telazol (6–8 mg/kg) injected intramuscularly, intubated with an endotracheal tube and anesthetized with isoflurane (1–5% to effect). A CO_2 sensor was placed at the end of the endotracheal tube. Mechanical ventilation was provided to keep end-tidal CO_2 and respiration rate initially at normal values. EKG electrodes were placed on the pig's limbs. A pulse-oximeter probe was placed on the pig's mouth (cheek or tongue). An esophageal temperature probe was placed to measure core temperature, which was maintained between 36 °C–38 °C with a water-filled warming blanket. An angiocatheter was placed in the carotid artery to acquire invasive blood pressure and withdraw blood samples for *ex vivo* testing. Another angiocatheter was placed in the internal jugular vein to deliver particle treatments. The 50 ml dose of SynthoPlate administered in the pigs was calculated to deliver a dose of 3, 1.5, 0.75, and 0.5 mg/kg followed by a 450 ml saline infusion. For an infusion treatment of SynthoPlate, the 50 mL dose of SynthoPlate was mixed into 450 mL saline prior to infusion. The basis of choosing these doses was from our preliminary data obtained in 2017 where we observed safety in a pilot group of pigs with 0.75 mg/kg of SynthoPlate. For current studies we therefore started at 4 times higher dose and decided to do escalating or de-escalating doses based upon effect on the pig (adaptive dosing protocol). Invasive arterial blood

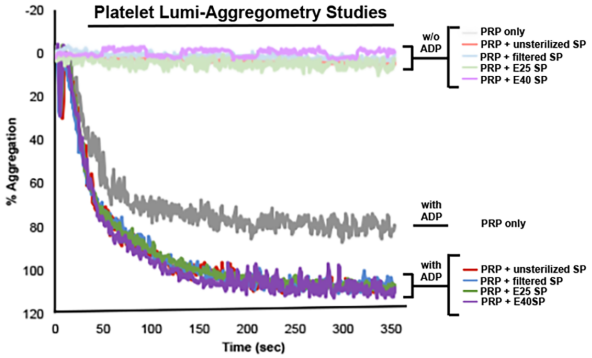

Figure 5. Representative aggregometry data for SynthoPlate interaction with resting and ADP-activated platelets

pressure, CO_2, SpO_2, temperature, and heart rate were recorded every 30 seconds for the first 10 minutes, every minute for the next 20 minutes, and every 5 minutes thereafter for a total of 60 minutes. Arterial blood was drawn via the carotid artery angiocatheter at baseline, 15 minutes, 30 minutes, and 60 minutes. At the end of experiments, pigs were euthanized with an I.V. overdose of pentobarbital (0.22 ml/kg). SynthoPlate concentration to dose in pigs in pigs were calculated as follows: A typical unit of platelets for human transfusion contains $\geq 3 \times 10^{11}$ platelets. Since actual platelets are ~10 times larger (in diameter) than SynthoPlate™, we decided to scale the dose concentration of SynthoPlate™ by 1 order of magnitude, to 5×10^{12} particles per dose in a 50 ml volume, resulting in $\sim 10^{11}$ Particles per ml.

In order to evaluate the systemic safety of SynthoPlate in pigs within a 1-hour post-administration time-frame without any confounding effects from injury (as proposed in grant application), SynthoPlate v2 (vs saline control) were administered in a group of non-injured animals (total n=20; 3 mg/kg n=1; 1.5 mg/kg n=5, 0.75 mg/kg n=5, 0.5 mg/kg n=5, saline n=4), and the animals were observed for 1 hour. To evaluate the effect of administration of SynthoPlate v1, treatments were administered either at a bolus or infusion dose of 1.5 mg/kg in a group of non-injured animals (total n=5; bolus n=2; infusion n=3), and the animals were observed for 1 hour. The research personnel were blinded to the administered treatments, and blinding was ensured by the PI who assigned a randomly generated code to each treatment. The effects of the administered treatments on vitals, blood chemistry, platelet function (aggregation), and blood clot viscoelastic properties were examined ex vivo. The risk for complement activation was assessed ex vivo with the blood sampling. For this, specific complement activation marker (C3 to C3a) was measured in the blood drawn from the pig at baseline as well as 30 and 60 minutes post administration treatment. In complement activation, C3 is cleaved into C3a and C3b leading to a decrease in plasma C3 levels and an increase in plasma C3a levels, and thus the C3:C3a ratio can indicate such risks. For these studies, platelet poor plasma was isolated from pig blood samples by centrifuging for 25 minutes at 2500 x g and measurements of C3 and C3a were carried out using C3 and C3a des ELISA kit respectively (Abcam, Cambridge, MA, USA). As shown in representative data in Figure 6, following administration of SynthoPlate™ in pigs, none of the animals showed any substantial alteration in in the C3:C3a plasma concentration ratios in comparison to saline control, that can be considered as drastic complement activation. We speculate that the situation may be different in 'injured' pigs that we will study during our Year 2 plan.

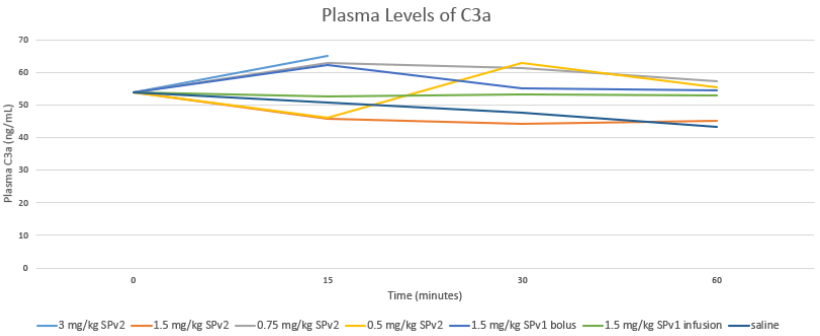

Figure 6. Complement C3a analysis on pig blood post SynthoPlate dose.

Major Task 2

Subtask 1: Pig whole blood samples were also analyzed for pH, calculated base excess,

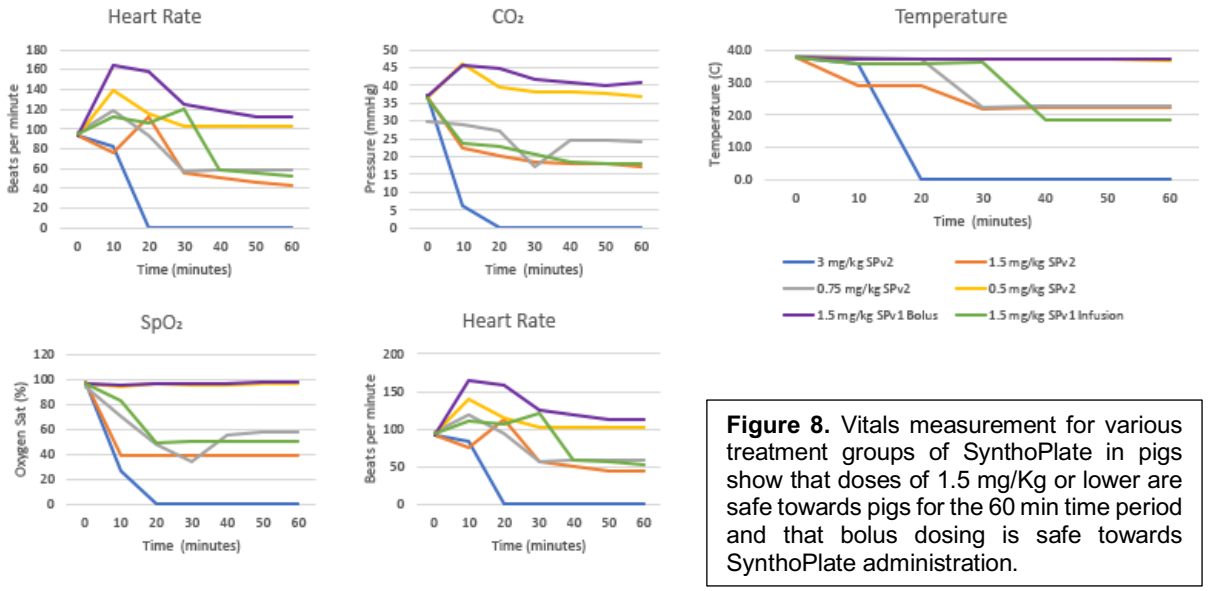

Figure 8. Vitals measurement for various treatment groups of SynthoPlate in pigs show that doses of 1.5 mg/Kg or lower are safe towards pigs for the 60 min time period and that bolus dosing is safe towards SynthoPlate administration.

carbon dioxide (CO_2), calculated bicarbonate, partial pressure of oxygen (PaO_2), oxygen saturation (SaO_2), white blood cell count (WBC), hematocrit (Hct), hemoglobin (Hg), red blood cell count (RBC), platelet count (Plt), mean corpuscular volume in femtoliters (MCV fL), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width- coefficient of variation (RDW-CV), prothrombin time (PT), international normalized ratio (INR), activated partial thromboplastin time (APTT), D-dimer, glucose, sodium, potassium, chloride, bicarbonate, anion gap, urea nitrogen, creatinine, glomerular filtration rate (GFR), calcium, albumin, total bilirubin, direct bilirubin, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total protein. These parameters were compared between dose levels and administration methods. Furthermore, platelet aggregometry was conducted on platelet-rich plasma (PRP) isolated from pig blood samples, at 37°C with stirring at 1200 rpm and aggregation mediated by ADP as the agonist. In addition, effect of the various treatment groups on clot viscoelastic properties was assessed by Rotational Thromboelastometry (ROTEM) via analysis of drawn blood samples. At the 1-hour time point pigs were euthanized and major organs (lungs, heart, liver, kidney, spleen) were harvested, weighed and fixed in formalin for use in biodistribution and histopathological analysis. Figure 7 shows the survival of the pigs after administration of SynthoPlate v2. There was at least one death in each de-escalating SynthoPlate group

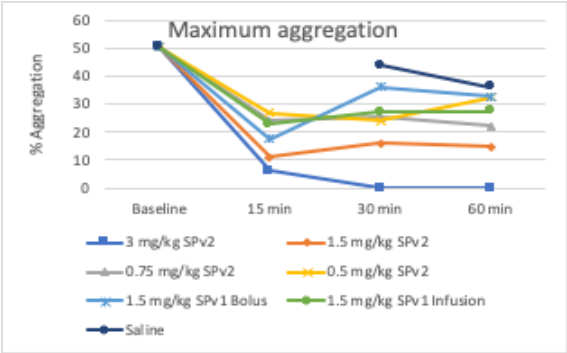

Figure 9. Ex vivo lumi-aggregometry data of ADP-activated platelet aggregation for the various doses of SynthoPlate.

until the whole 0.5 mg/kg group (all 5 pigs) survived for the 60 min period. The acute response that we observed with the highest dose of SynthoPlate V2 (3 mg/Kg) in pigs, prompted us to investigate in a whether such SynthoPlate response can be mitigated by dosing speed (e.g. bolus vs infusion) or modifying the surface chemistry (increasing FMP and hence PEGylation density since FMP is conjugated to lipid by PEG spacer). Thus we investigated the potential safety of SynthoPlate v1 via bolus and infusion. Figure 7 shows that with a 3 times higher dose than SynthoPlate V2 (1.5 mg/Kg of SP V1 vs 0.5 mg/Kg of SP V2), 2 out of 2 pigs survived in bolus group and 2 out of 3 pigs survived in the infusion group. Figure 8 shows vitals of the various treatment groups and the doses tested. It is apparent from these observations that 0.5 mg/Kg dose of SynthoPlate (SP) Version 2 and 1.5 mg/Kg dose of SynthoPlate (SP) Version 1 are safe towards pig vitals, and also both SynthoPlate Version 1 and 2 are safer as a bolus (which can be considered favorable in terms of on-field administration in a far forward setting).

Figure 9 shows lumi-aggregometry based ex vivo studies on pig platelet aggregation response to the various SynthoPlate doses in platelet-rich plasma isolated from pig blood samples acquired at various time points post SynthoPlate administration. Here, platelet agonist ADP was used to initiate platelet aggregation. Interestingly, at higher doses of SynthoPlate (1.5g mg/Kg and 3 mg/Kg), the ability of ADP-activated platelets to aggregate appear to be lower, compared to that in presence of lower doses of SynthoPlate (0.5 mg/Kg and 0.75 mg/Kg). Comparison controls are shown for saline (no SynthoPlate dose). This indicates that there may be an optimum platelet: SynthoPlate ratio above which the SynthoPlate particles will potentially have an inhibitory effect on platelet aggregation

CT (secs)

Test	Treatment	Time point				Reference Range
		Baseline	15 min	30 min	60 min	
EXTEM	3.0 mg/kg SPv2	53.607	48.352			41-59
	1.5 mg/kg SPv2		49.317 ± 2.799	48.684 ± 0.774	27.898 ± 20.887	
	0.75 mg/kg SPv2		47.926 ± 9.149	29.878 ± 3.233		
	0.5 mg/kg SPv2		55.830 ± 7.757	50.958 ± 8.670	47.152 ± 6.962	
	1.5 mg/kg SPv1 bolus		53.762 ± 14.298	46.715	57.379 ± 17.248	
	1.5 mg/kg SPv1 infusion		46.824 ± 9.306	48.358 ± 5.959	45.357 ± 9.181	
	Saline			53.428 ± 13.325	41.592 ± 20.943	
FIBTEM	3.0 mg/kg SPv2	53.630	70.047			42-57
	1.5 mg/kg SPv2		54.441 ± 3.618	53.630 ± 0.000	53.630	
	0.75 mg/kg SPv2		46.509 ± 5.777	45.353 ± 18.859		
	0.5 mg/kg SPv2		56.214 ± 6.734	46.556 ± 7.088	48.558 ± 10.933	
	1.5 mg/kg SPv1 bolus		49.747 ± 0.963	55.231	62.767 ± 1.602	
	1.5 mg/kg SPv1 infusion		43.083 ± 10.867	45.468 ± 3.892	43.304 ± 10.679	
	Saline			59.963 ± 13.123	41.911 ± 24.854	

A10 (mm)

Test	Treatment	Time point				Reference Range
		Baseline	15 min	30 min	60 min	
EXTEM	3.0 mg/kg SPv2	66.357	40.164			69-78
	1.5 mg/kg SPv2		62.653 ± 3.739	66.812 ± 0.643	70.902 ± 6.428	
	0.75 mg/kg SPv2		55.556 ± 2.479	56.683 ± 4.804		
	0.5 mg/kg SPv2		61.318 ± 1.850	62.915 ± 3.814	64.615 ± 3.726	
	1.5 mg/kg SPv1 bolus		60.154 ± 0.612	61.549	63.493 ± 0.029	
	1.5 mg/kg SPv1 infusion		50.155 ± 19.288	59.732 ± 5.090	62.671 ± 1.303	
	Saline			52.495 ± 0.505	43.693 ± 14.020	
FIBTEM	3.0 mg/kg SPv2	33.407	6.681			32-49
	1.5 mg/kg SPv2		26.464 ± 6.091	29.050 ± 6.162	33.407	
	0.75 mg/kg SPv2		16.217 ± 4.498	22.591 ± 1.468		
	0.5 mg/kg SPv2		25.574 ± 6.113	30.123 ± 4.671	27.395 ± 4.297	
	1.5 mg/kg SPv1 bolus		25.155 ± 1.828	27.144	27.840 ± 0.000	
	1.5 mg/kg SPv1 infusion		19.341 ± 9.494	23.564 ± 6.413	33.647 ± 4.634	
	Saline			53.241 ± 8.098	50.927	

MCF (mm)

Test	Treatment	Time point				Reference Range
		Baseline	15 min	30 min	60 min	
EXTEM	3.0 mg/kg SPv2	69.446	44.193			71-79
	1.5 mg/kg SPv2		65.984 ± 3.851	69.909 ± 0.655	74.076 ± 6.547	
	0.75 mg/kg SPv2		59.932 ± 3.345	61.599 ± 3.345		
	0.5 mg/kg SPv2		65.293 ± 1.850	66.273 ± 2.934	67.826 ± 3.372	
	1.5 mg/kg SPv1 bolus		63.672 ± 1.251	66.512	68.006 ± 0.654	
	1.5 mg/kg SPv1 infusion		55.382 ± 17.917	64.045 ± 4.511	67.113 ± 0.681	
	Saline			52.878 ± 0.007	46.001 ± 10.757	
FIBTEM	3.0 mg/kg SPv2	34.593	7.461			33-50
	1.5 mg/kg SPv2		27.654 ± 6.167	30.833 ± 5.318	34.593	
	0.75 mg/kg SPv2		16.165 ± 4.657	23.016 ± 0.444		
	0.5 mg/kg SPv2		27.274 ± 6.690	31.092 ± 5.546	26.737 ± 7.341	
	1.5 mg/kg SPv1 bolus		26.652 ± 2.423	29.750	29.701 ± 1.047	
	1.5 mg/kg SPv1 infusion		20.389 ± 8.501	24.039 ± 6.354	34.451 ± 3.963	
	Saline			53.132	50.992	

Figure 10. Ex vivo ROTEM analysis of pig blood post SynthoPlate administration

possibly due to steric hindrance via volume exclusion between platelets. Figure 10 shows the clotting time (CT), maximum clot firmness (MCF), and amplitude 10 minutes after CT (A10) parameters obtained via ROTEM analysis (EXTEM and FIBTEM assays) of porcine blood samples (obtained post SynthoPlate administration). As indicated by the data, the CT, A10 and MCF outputs for SynthoPlate dose of 3mg/Kg seem to be drastically out of range, compared to that for all other lower SynthoPlate doses (1.5, 0.75 or 0.5 mg/Kg). This has led us to postulate that a bolus dose of SynthoPlate V1 at 1.5 mg/Kg is the maximum safe 'threshold' to subsequently test in the injured pig model as proposed for Year 2 of the grant proposal. Figure 11 shows representative biodistribution profile of SynthoPlate (and unmodified control liposome particles as comparison) at the end of the 60 min time period post administration. As evident from the data, in the absence of injury SynthoPlate (as well as control unmodified particles) get cleared from circulation predominantly in the liver and spleen, with minimum accumulation in the lungs and kidneys. Approximately 65-75% of the injected dose are cleared in this fashion by 60 min time point, while 25-35% still remain in circulation. These observations are in agreement with reported biodistribution profiles for many liposomal formulations. For subsequent studies in the injury model in pigs (Year 2), we anticipate that this biodistribution profile will change since a significant portion of the SynthoPlate is expected to localize at the injury site.

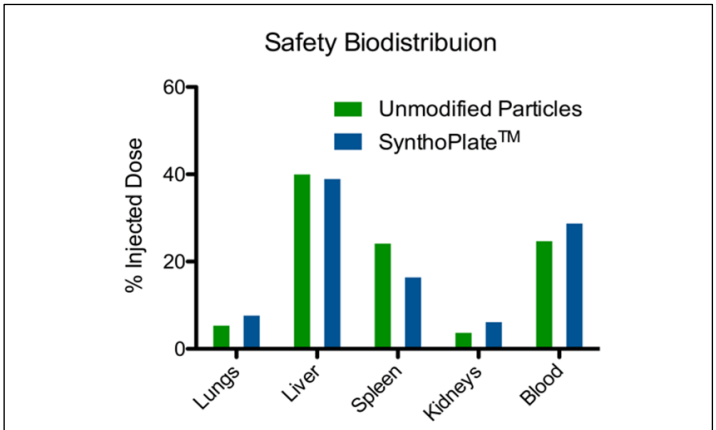

Figure 11. Representative biodistribution of SynthoPlate in pigs during 60 min post I.V.-administration.

Incomplete aspects of Specific Aim 1: There was a delay in our ACURO approval due to our mistake regarding submission of some necessary documents. As a result, the pig studies were slightly delayed in their execution. Furthermore, there was some delay in September regarding obtaining some of the histopathology staining kits from companies which designated them as 'backordered'. As a result, a few analyses aspects currently remain incomplete (studies are ongoing), regarding histopathologic analysis of systemic thrombotic incidents (if any) in various organs that were excised from the pigs upon euthanasia (D-dimer analysis and platelet thrombi analysis as part of Major Task 2). Nonetheless, based upon our currently analyzed results as described above, we are manufacturing SynthoPlate V2 and supplying to Dr. Matthew Neal's laboratory at University of Pittsburgh for initiating studies proposed in Specific Aim 2 to be carried out during months 6-36.

Specific Aim 2. Evaluation of hemostatic efficacy of pristine SynthoPlate™ and TXA-loaded SynthoPlate™ in a pig model of polytrauma. **(Months 6-36)**

Major Task 1

Subtask 1: Initial batches of SynthoPlate V2 and control particles have been manufactured and shipped to Dr. Neal's laboratory at University of Pittsburgh to begin evaluation in injured pigs. The studies in Dr. Neal's laboratory are currently ongoing.
Subtask 2: Dr. Neal's laboratory at University of Pittsburgh has obtained necessary IACUC and ACURO approvals (please see attached).

Specific Aim 3. Evaluate the efficacy of SynthoPlate™ alone or in combination with Gentamicin to provide wound protection and improve re-epithelialization in porcine wound models. **(Months 6-36)**
Subtask 1: A CRADA is being established with the help of METIS Foundation.
Subtask 2: SynthoPlate has been manufactured for shipment to Dr. Chan's lab upon complete execution of CRADA.

Opportunities for Training and Professional Development: During Year 1, the research has allowed the training of an MD PhD researcher (DaShawn Hickman), a PhD researcher (Aditya Girish), two post-doctoral researchers (Dr. Christa Pawlowski and Dr. Michael Bruckman), a Masters student researcher (Norman Luc) as well as, several undergraduate researchers (Simi Ganjoo, Stephanie Huang, Ankush Banerjee, Yvonne Ma, Kenji Miyazawa), in various aspects of in vitro, in vivo and ex vivo studies focused on SynthoPlate™. These researchers have worked under my mentorship, along with regular consultation with veterinary specialists at the Animal Research Center (ARC) at Case Western, as well as, with our collaborators at University of Pittsburgh and USAISR, to carry out the reported studies. The researchers were also trained in writing technical reports and have contributed heavily towards preparing a manuscript where some of the studies were included as part (now published in Nature Scientific Reports).

Results Dissemination: Components of results stemming from the above-described studies were included in a manuscript titled "***Intravenous synthetic platelet (SynthoPlate™) nanoconstructs reduce bleeding and improve 'golden hour' survival in a porcine model of traumatic arterial hemorrhage*****"**, that was recently published in Nature Scientific Reports (*Scientific Reports,* Vol 8, Article Number: 3118, 2018). Some of the results were also part of a recent presentation on the SynthoPlate™ nanotechnology that was given by Dr. Anirban Sen Gupta (PI) to researchers at NAMRU San Antonio as part of the ORISE program. Some of the results were also exhibited as part of a poster presented in the Hemorrhage Control program at MHSRS 2018.

Plans for next reporting period: During the next reporting period we will:
- Continue studies of control particle and SynthoPlate™ in the laboratory of Dr. Neal (Co-I) at University of Pittsburgh, for the pig polytrauma studies.
- Establish CRADA with Dr. Chan's (Co-I) lab at USAISR to be able to ship SynthoPlate™ to the lab for initiation of evaluation studies in pig burn wounds.

- Prepare technical report on these activities and prepare appropriate manuscripts.

4. Impact:

Impact on principal discipline. The biggest impact from the Year 1 studies as described above is the feasibility demonstration of SynthoPlate™ as a viable platelet surrogate for potential pre-hospital hemorrhage control treatment, demonstrated in a large animal model. The studies are indeed in their early phases, but the data is highly promising to indicate a technological solution to reduce hemorrhage-associated preventable deaths of combat personnel in austere battlefield conditions. The studies also provided vary valuable insight regarding sterilization potential of SynthoPlate™, which will be a critical component in strategic planning of the GMP manufacture of this product in future IND phases of its development. The fact that the filtration or E-beam sterilization did not show any drastic detrimental effect on SynthoPlate™ stability and platelet-mimetic bioactivity, is highly encouraging towards its translation. Additionally, the fact that an intravenous bolus dose of SynthoPlate™ did not indicate major immune response or systemic thrombotic complications in pigs, is highly exciting regarding its in vivo safety, especially considering the fact that past 'synthetic platelet' designs have shown serious issues with in vivo safety. If successfully translated, the potential impact of this technology on military medicine is tremendous.

Impact on other disciplines. The studies further strengthened the evidence and of 'heteromultivalent surface-decoration' approach for biointeractive nanomedicine. Nanomedicine is a highly significant field of biomedical engineering. These studies expanded the potential of these fields in hemorrhage control.

Impact of technology transfer. The studies have added data to our existing patent on SynthoPlate™, that can potentially expand its use (CIP) in prolonged field care in trauma.

Impact on society beyond science and technology. The research allowed broader discussions of the potential of military medicine in civilian trauma scenarios with a variety of audience both within and outside the university.

5. Changes/Problems: There was an issue during Quarter 1 and 2 regarding IACUC and ACURO approval to carry out the pig safety studies pertaining to Aim 1. The IACUC for the model studies in pigs was approved at Case Western on CWRU on August 24, 2017 (IACUC Protocol 2017-0105). However, the PI made an error in timely submission of the approved IACUC document to additional ACURO evaluation and approval and had started the pig studies which was reported during Quarter 1 and 2. This resulted in a 'non-compliance' and the error was quickly realized. The studies were immediately stopped and all necessary documents were submitted for ACURO approval. The ACURO approval letter was obtained in May, 2018 (DM160354.03 entitled, "SynthoPlate Nanotechnology For Intravenous Hemostasis and Wound Healing in Prolonged Field Care", May 7, 2018) and the studies were resumed. This event had resulted in a slight delay to carry on and

conduct subsequent studies, but has not hindered the overall progress towards meeting the Year 1 milestones. The approved total 25 pigs have been studied during Year 1.

Also, there was some delay in September regarding obtaining some of the histopathology staining kits from companies which designated them as 'backordered'. As a result, a few analyses aspects were started a few weeks later than anticipated (e.g. histopathologic analysis of systemic thrombotic incidents (if any) in various organs that were excised from the pigs upon euthanasia (D-dimer analysis and platelet thrombi analysis as components of Major Task 2)). Nonetheless, the progress towards proposed aims have not been affected drastically and the SynthoPlate batches are being manufactured to be sent to Dr. Neal's laboratory for Year 2 studies.

6. Products: 1 published manuscript (Nature Sci Rep) and 1 poster (MHSRS 2018).

7. Participants:

Name:	Anirban Sen Gupta
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	eRA Commons ID: ANIRBAN0426
Nearest person month worked:	4
Contribution to Project:	Dr. Sen Gupta is the overall director and trainer for the current (and proposed) studies, and mentored all researchers involved.
Funding Support:	NIH R01 HL121212, NIH R01 HL129179, NIH R35 GM119526, AHA Grant-in-Aid 17GRNT33661005, DM160354

Name:	Michael Bruckman
Project Role:	Senior Research Associate
Researcher Identifier (e.g. ORCID ID):	eRA Commons ID: Mbruckman
Nearest person month worked:	2
Contribution to Project:	Michael Bruckman joined the Sen Gupta laboratory in November 2017 to carry out synthetic hemostat research. He will be responsible for the manufacture of

	SynthoPlate™ for Year 2 and completing the histopathology analysis of pig organs, along with a graduate and two undergraduate students, in a team.
Funding Support:	DM160354

Name:	DaShawn Hickman
Project Role:	MD PhD Graduate Student (MSTP program at Case Western)
Researcher Identifier (e.g. ORCID ID):	eRA Commons ID: DHICKMAN
Nearest person month worked:	4
Contribution to Project:	DaShawn Hickman is a graduate researcher in the Sen Gupta laboratory, focusing on evaluating SynthoPlate™ in several small and large animal bleeding models. He was responsible for carrying out majority of the research described in the current progress report. He will carry out the other major tasks of Aim 1 during the next reporting period. He is also the first author of the manuscript published in Nature Scientific Reports. He works with a post-doctoral and two undergraduate researchers, in a team.
Funding Support:	NIH R01 HL121212, AHA Fellowship Grant 178CPRE33670016

Name:	Norman Luc
Project Role:	Masters Student at Case Western
Researcher Identifier (e.g. ORCID ID):	ORCID ID: 0000-0003-0020-5850
Nearest person month worked:	4
Contribution to Project:	Norman Luc is a graduate researcher in the Sen Gupta laboratory, focusing on SynthoPlate™ manufacture and

	its evaluation in several small and large animal bleeding models. He was responsible for carrying out making of SynthoPlate, as well as ex vivo studies on pig blood samples described in the current progress report. He will carry out remaining components of Aim 1 (histopathology) as well as manufacture SynthoPlate to ship to Neal Lab at UPitt during the next reporting period. He is also a co-author of the manuscript published in Nature Scientific Reports.
Funding Support:	DM160354

Other organizations, namely University of Pittsburgh and USAISR, that are co-investigators in the current grant award, are to start their contributions from Year 2 onwards.

8. Quad Chart: Please see below.

9. Appendices: IACUC and ACURO approvals, please see below.