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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^{TM}, 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^{TM} 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^{TM} in pigs.
Aim 2. Evaluation of hemostatic efficacy of pristine SynthoPlate^{TM} and TXA-loaded SynthoPlate^{TM} in a pig model of polytrauma.
Aim 3. Evaluate the efficacy of SynthoPlate^{TM} 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 2 Aim and Major Goals were:

Aim 2. Evaluate hemostatic efficacy of pristine SynthoPlate^{TM} and TXA-loaded SynthoPlate^{TM} in porcine model of polytrauma.

Major Task 1. Demonstrate that post-injury SynthoPlate transfusion results in a significant reduction in blood loss following polytrauma

- Subtask 1: SynthoPlate manufacture, sterilization, supply to UPitt: (platelet-mimetic nanoparticle manufacture from lipid-peptide conjugates using reverse phase evaporation and extrusion; shipment to UPitt).
- Subtask 2: Continued IACUC and ACURO approvals (with amendments).
- Subtask 3: Studies with Sham animals and animals injected with control (unmodified) particle studies: pig polytrauma model injected with *sham* or *control* particles and

evaluation of hemorrhage outcome, and analysis serum cytokines for determination of systemic inflammation levels.

- Subtask 4: Studies on polytrauma injury pigs injected with *SynthoPlate doses* and evaluation of hemostatic effect and analysis serum cytokines for mitigation benefit of systemic inflammation.
- Subtask 5: Statistical analysis, report generation, manuscript preparation on Major Task 1 of Sp Aim 2.

Activities under Major Task 1

Subtask 1: SynthoPlate was manufactured according to the formulation shown with the following composition: Distearylphosphatidylcholine (DSPC) at 46.5 mol%, Cholesterol at 45 mol%, Polyethylene glycol (1000 Da molecular weight)-terminated Distearylphosphatidylethanolamine (DSPE-PEG_{1000}) at 2.5 mol%, Peptide-modified polyethylene glycol (2000 Da molecular weight)-terminated Distearylphosphatidylethanolamine (DSPE-PEG_{2000}) with DSPE-PEG_{2000}-VBP at 1.25 mol%, DSPE-PEG_{2000}-CBP at 1.25 mol% and DSPE-PEG_{2000}-FMP at 2.5 mol%. These various components at the above-described mole fractions 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) characterization indicated fresh-made SynthoPlate vesicles were ~200 nm in diameter. Figure 1 shows a schematic of the SynthoPlate design. Figure 1 also shows the temperature stability of this suspension-phase SynthoPlate. As evident from Figure 1, SynthoPlate is stable (minimal change in diameter size distribution) at 4°C as well as room temperature. Hence, for shipment to Neal lab at University of Pittsburgh, particles as prepared were shipped overnight and upon receipt, the Neal lab stored the particles in the fridge at 4°C until use in the in vivo pig model.

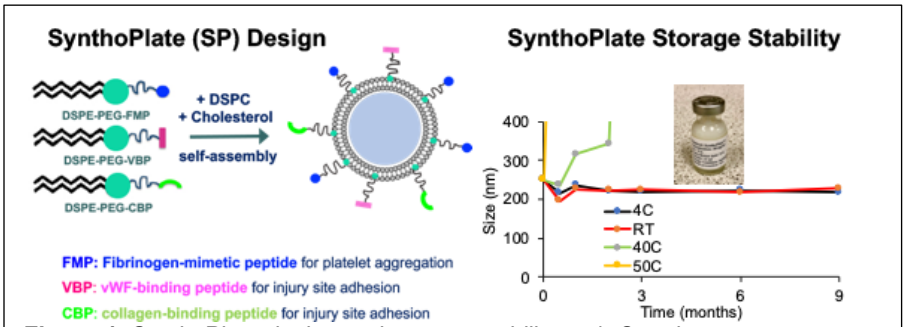


Figure 1. SynthoPlate design and storage stability at 4oC and room temperature

Subtask 2: All pig model studies were conducted as per IACUC and ACURO approval for for Neal lab studies at UPitt: DM160354.04 entitled, "Exploring Dynamic Platelet Functions after Hemorrhagic Shock, Polytrauma, and Associated Coagulopathies in Swine," IACUC protocol number 17110765, Protocol Principal Investigator Matthew Neal. New personnel (a new animal surgeon) was added to Neal Lab IACUC during Quarter 1 of Year 2. The actual study method and assessment criteria were not altered at all, and

hence we did not anticipate that this would be considered 'non-compliance' in ACURO framework. We received a letter of noncompliance for a protocol violation during the Quarter 2. When advised of the noncompliance, the new individual suspended participation until official approval was available, and we delayed additional studies until the protocol was fully reviewed and approval granted, effective 07-MAR-2019. Upon approval of ACURO, we resumed swine studies of uncontrolled intraperitoneal hemorrhage at the University of Pittsburgh.

Subtask 3 and 4: The swine model involves the use of male and female Yorkshire swine (30-35kg). First, a sham pig experiment was conducted with induction of general anesthesia, establishment of central venous access, placement of pulmonary arterial catheter, and femoral cutdown for arterial catheter placement. Serial arterial blood gas (ABG) measurements were utilized to standardize mechanical ventilation settings and monitor pH. Following this, subsequent experiments were performed to standardize the liver laceration. A guide affixed to a sterilized razor was constructed to create a reproducible 6cm long and 2cm deep laceration to the left lateral segment of the liver. Two of these animals received "control" particles which were the undecorated liposomal particles. Blood loss was quantified by measurement of shed intraperitoneal blood. Thromboelastography was performed. Results including blood loss, base excess, TEG, whole blood platelet aggregometry, and mortality were collected. End organs including liver, lung, brain, spleen, and heart were harvested and histopathology analysis was carried out. Plasma was collected for cytokine analysis at baseline and serial timepoints and results were generated from pooled analysis of multiple animal experiments.

For the model study, the pigs were sedated using TKX (Telazol, Ketamine, Xylazine) at 1 ml/50lb intramuscular injection in the thigh and transported to the surgery suite. The pigs receive oxygen and isoflurane 2-4% and undergo endotracheal intubation. Swine are ventilated on volume control mode, with tidal volume of 6-8ml/kg, inspired fraction of O_2 of 0.4 and are sustained under anesthesia with isofluorane 2-2.5%. The animals are monitored with EKG monitor and pulse-oximeter in the tail, and maintenance fluids (5% dextrose and normal saline) are started through a peripheral ear vein at 1ml/kg/hr. After cleaning the surgical areas with iodopovidone, the swine are instrumented with a right internal jugular pulmonary artery catheter through an 8 French introducer, a right femoral vein 1- French introducer and a right femoral artery triple lumen catheter. Animals are then allowed to stabilize for 30 minutes before starting hemorrhage. At this point, a laparotomy is performed and the liver laceration initiates uncontrolled intraperitoneal hemorrhage. The resuscitation is then undertaken per IACUC/ACURO protocol, and shed blood is collected and quantified from laparotomy pads placed in the abdomen. A total of 5 animals were utilized for model validation. These animals involved varying the severity of liver laceration including orientation (cruciate vs linear laceration) and the depth of laceration. Blood loss was highly variable, ranging from 54-2026gm of shed intraperitoneal blood. We have standardized the laceration utilizing a depth guard placed on a razor to regulate the degree of penetration, and a standard position on the left lateral segment of the liver was chosen for a laceration. This model (see Figure 2) yields a more reproducible volume of blood loss ranging from 106-656gm and was associated with 100% survival past the initial time of injury and resuscitation, whereas mortality in the

initial cohort of 5 animals was 40%. Data points collected from each run included: blood loss, hemodynamics, respiratory rate, CVP, oxygen saturation, arterial blood gas, Na, K, Cl, Ca, CO_2, Gluc, BUN, Cr, Hgb, HCT, anion gap, whole blood platelet aggregometry (collagen agonist), thromboelastography (TEG).

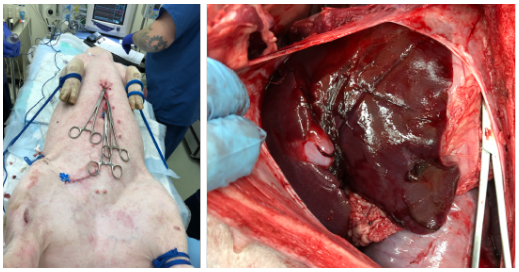


Figure 2. Set-up of pig liver laceration model and closer view at the liver laceration injury to induce intraperitoneal hemorrhage.

The following groups were first tested in the model: no treatment (i.e. no particles) and control particles. No major effects were observed in terms of heart rate, hemodynamics and histology in the animals injected with control particles. Histology of organs excised post-euthanasia are shown below in Figure 3:

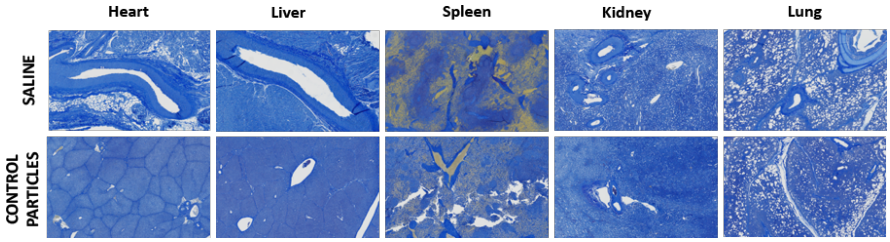


Figure 3: Histology of sham animals and control particle injected animals

Following this, studies were undertaken for comparing the effect of control particles versus SynthoPlate dosing in the swine hemorrhage model. The following groups were tested in the model: no treatment, control particles, 3-peptide decorated (VBP, CBP and FMP) SynthoPlate particles (0.64-1mg/kg), 2-peptide decorated combinations (CBP-VBP, CBP-FMP, VBP-FMP) and FMP-decorated particles alone. In our Year 1 studies in non-injured pigs, the intravenous administration of SynthoPlate did not cause any acute response (reported in Year 1 final report document, as well as published in our paper in *Nature Scientific Report*). Additionally, in trauma models of mice and rats, post-injury intravenous administration of SynthoPlate did not seem to cause any acute systemic effects (published previously in *J Trauma* and *J Thromb Haemost*). Interestingly however, the post-injury administration of SynthoPlate in the pig trauma model showed transient hemodynamic changes (tachycardia) and oxygen desaturations following the dose administration, along with transient coloration of the skin. We currently do not have a definitive conclusion regarding whether this is a result of the particles themselves, or whether this is related to a species (pig)-specific pulmonary macrophagic or innate immune response (e.g. see Skotland, T. *Theranostics 2017; 7(19): 4877–4878.*) or complement-mediated pseudo-allergic response (CARPA) that have been reported regarding some nanoparticle formulations in pigs previously by other researchers (e.g. see *Szebeni J. Mol Immunol 2014; 61: 163-173, PMID: 25124145*). Our observations led us to investigate the combinations of various peptide components decorated on SynthoPlate and test for peptide interactions as a source of the cardiopulmonary

changes. To this end, we collected plasma for a planned analysis of cytokine expression by Luminex. We also measured complement activation levels before and immediately following injury utilizing ELISA kits for C3a and C5a to assess complement activation-related pseudoallergy (CARPA) as an etiology.

To determine the effect of SynthoPlate (SP) and control particle (CP) administration on the porcine cytokine profile, we performed a Luminex assay measuring Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), interferon-γ (IFNγ), IL-1α, IL-1ß, IL-1RA, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18 and TNFα. We determined the cytokine profiles of plasma obtained from pigs treated with SP or CP as well as from untreated pigs as a control. We also tested the effects of 3 types of dual decorated particles – collagen-binding peptide and von Willebrand factor-binding peptide (CBP-VBP), collagen-binding peptide and fibrinogen mimetic peptide (CBP-FMP), von Willebrand factor-binding peptide and fibrinogen mimetic peptide (VBP-FMP). Additionally, we tested a single decorated particle, fibrinogen mimetic peptide only (FMP-only). Both EDTA and citrate were used as the anticoagulant, as indicated. The results are shown in Tables 1, 2 and 3. All values are in nanograms/milliliter (ng/ml). Baseline timepoint corresponds to blood draw prior to laparotomy, post-laceration timepoints corresponds to blood draw 15 minutes after liver laceration and final timepoint corresponds to timepoint 30minutes after particle injection. Control particle (CP) vs SynthoPlate (SP) results are below:

	EDTA Plasma								
	SP (1mg/kg)			**CP (1mg/kg)**			**Untreated**		
Analyte	Baseline	Post-Laceration	Final	Baseline	Post-Laceration	Final	Baseline	Post-Laceration	Final
GM-CSF	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
IFNγ	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
IL-1α	0.009	0.008	0.008	0.008	0.008	0.007	0.002	0.003	0.003
IL-1ß	0.062	0.043	0.032	0.005	0.011	0.009	0.040	0.039	0.058
IL-1RA	0.088	0.133	0.197	0.054	0.05	0.061	0.113	0.198	1.771
IL-2	0.028	0.020	0.020	0.051	0.055	0.049	0.024	0.029	0.033
IL-4	0.065	0.059	0.043	0.063	0.067	0.066	0.036	0.034	0.042
IL-6	0.029	0.027	0.037	0.002	0.008	0.029	0.014	0.057	0.205
IL-8	N/A	N/A	N/A	N/A	N/A	N/A	0.010	0.02	0.026
IL-10	0.041	0.037	0.032	0.046	0.05	0.047	0.024	0.028	0.039
IL-12	1.114	0.962	1.055	2.214	2.21	2.086	0.616	0.543	0.505
IL-18	0.338	0.354	0.456	0.262	0.297	0.307	0.26	0.359	0.592
TNFα	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 1. Cytokine Profile of EDTA plasma obtained from SP or CP treated pigs vs. untreated pig. N/A – indicates analyte concentration is below the range of the assay.

Results from pigs treated with dual peptide modified particles vs SP shown below:

	Citrate Plasma										
	SP (1mg/kg)		**SP (0.64mg/kg)**			**CBP-VBP (1mg/kg)**		**VBP-FMP (1mg/kg)**			
Analyte	Baseline	Final	Baseline	Post-Injection	Final	Baseline	Final	Baseline	Post-Laceration	Post-Injection	Final
GM-CSF	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
IFNγ	13.246	14.486	14.364	13.873	13.119	4.735	2.384	23.508	21.507	24.626	24.418
IL-1α	0.015	0.011	0.022	0.022	0.02	0.016	0.016	0.041	0.04	0.043	0.04
IL-1ß	0.088	0.051	0.103	0.100	0.087	0.076	0.056	0.244	0.198	0.218	0.199
IL-1RA	0.13	0.125	0.135	0.979	2.98	0.236	0.204	0.295	0.293	0.34	0.318
IL-2	0.211	0.164	0.164	0.166	0.15	0.083	0.08	0.281	0.247	0.276	0.306
IL-4	0.954	0.619	0.234	0.247	0.234	0.192	0.191	0.715	0.629	0.765	0.619
IL-6	0.037	0.065	0.058	0.064	0.07	0.041	0.05	0.097	0.092	0.106	0.094
IL-8	N/A	0.043	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
IL-10	0.121	0.073	0.139	0.112	0.106	0.077	0.069	2.504	2.712	2.83	2.646
IL-12	0.507	0.374	1.349	1.084	1.071	1.177	0.974	0.593	0.53	0.531	0.52
IL-18	0.553	0.433	0.597	0.656	0.877	0.338	0.397	1.318	1.395	1.353	1.318
TNFα	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 2. Cytokine Profile of citrate plasma obtained from SP, CBP-VBP and VBP-FMP treated pigs. N/A – indicates that the analyte concentration is below the range of the assay.

	Citrate Plasma			
	CBP-FMP (1mg/kg)	**FMP-only (1mg/kg)**	**CP (1mg/kg)**	**Untreated**

Analyte	Baseline	Post-Laceration	Post-Injection	Final	Baseline	Post-Laceration	Post-Injection	Final	Baseline	Final	Baseline	Final
GM-CSF	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.055	0.041	N/A	N/A
IFNγ	11.877	12.539	6.394	9.786	7.964	5.728	7.201	8.371	29.826	12.473	N/A	N/A
IL-1α	0.009	0.007	0.004	0.006	0.005	0.005	0.005	0.006	0.143	0.086	0.01	0.01
IL-1ß	0.035	0.026	0.005	0.016	N/A	N/A	N/A	N/A	0.827	0.529	0.033	0.042
IL-1RA	0.413	1.062	1.476	3.588	0.109	0.103	0.12	0.141	0.482	0.411	0.037	0.066
IL-2	0.053	0.038	0.032	0.035	0.015	0.007	0.007	0.016	0.961	0.603	0.099	0.099
IL-4	0.165	0.137	0.119	0.121	0.099	0.086	0.089	0.089	2.525	1.413	0.054	0.061
IL-6	0.026	0.034	0.034	0.042	N/A	0.013	0.028	0.044	0.409	0.257	0.009	0.04
IL-8	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.042	0.036	N/A	N/A
IL-10	0.101	0.064	0.54	0.066	0.019	0.010	0.012	0.019	1.237	0.607	0.073	0.084
IL-12	0.844	0.648	0.571	0.597	1.069	0.9	0.959	0.989	0.759	0.484	0.599	0.637
IL-18	0.523	0.575	0.523	0.553	0.222	0.23	0.262	0.325	3.413	2.328	0.262	0.317
TNFα	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 3. Cytokine Profile of citrate plasma obtained from CBP-FMP, FMP-only, CP treated pigs or from an untreated pig. N/A –indicates that the analyte concentration is below the range of the assay.

Complement (C3 and C5) analysis results are shown below:

		C3a (pg/ml)	C5a (ng/ml)
SP (1mg/kg)	Baseline	802.0355	0.911
	Post-Laceration	1482.939	N/A
	Final	1073.458	N/A
CP (1mg/kg)	Baseline	534.6305	0.480
	Post-Laceration	227.704	0.572
	Final	318.17	N/A
Untreated	Baseline	Above the range of the assay (>2098.438pg/ml)	N/A
	Post-Laceration	1343.082	N/A
	Final	921.795	N/A

Table 4. Complement C3a and C5a levels in EDTA pig plasma. N/A – indicates that the analyte concentration is below the range of the assay.

From our current studies it seems that systemic CARPA is not an issue in the pigs, with our control or SynthoPlate particles. It is important to note here that we also saw that complement activation was not an issue in our uninjured pigs and pilot studies of femoral artery bleeding model in pigs that we carried out prior to the current PFCRA-funded studies and published in *Nature Scientific Reports*. We are currently continuing our studies with the ex vivo analysis of lung macrophages to see whether our findings coincide with the reports from others that pigs and other *cloven-hoofed animals* have a special type of macrophages called Pulmonary Intravascular Macrophages (PIMs) that are sensitive to any nanoparticle (*Skotland, T. Theranostics 2017; 7(19): 4877–4878*). Apparently, this PIM phenotype is rare in humans such that the cardiopulmonary distress effects from similar nanoparticles are not expected to happen in humans. If we confirm this to be the case with our continued studies, so as to establish that the *transient tachycardic effects* are related to PIM sensitivity and not necessarily a limitation of our nanoparticle formulation, we will request an evidence-based amendment to our IACUC or ACURO to allow pre-treatment based depletion of these PIMs in pigs (e.g. by pre-treatment dosing of indomethacin or clodronate-loaded liposomes), or if needed, then request a change of species in our model (most possibly to rabbits), for subsequent studies in the future.

Subtask 5: We are currently in the process of compiling our data into manuscript format and anticipate to submit to peer-reviewed journals, as well as present at MHSRS conference in 2020.

Major Task 2. Evaluate the potential synergistic effect of SynthoPlate and site-specific delivery of TXA on blood loss and hemodynamic changes after injury.

Subtask 1: Manufacture and characterization of SynthoPlate loaded with TXA (TXA loading and release kinetics characterization from SynthoPlate particles) and shipment of TXA-loaded nanoparticles and control nanoparticles to UPitt.

In vitro loading and release studies of TXA in SynthoPlate particles have been carried out. For these studies TXA was labeled with a fluorescent molecule (Fluorescein, green fluorescence) and the labeled TXA was dissolved in PBS. This labeled TXA solution was loaded within the vesicles during the lipid hydration step of the manufacturing process. The TXA dose was chosen as 93mg/kg in relevance to clinical doses. Resultant TXA-loaded vesicles were characterized for size by dynamic light scattering (DLS). The encapsulation efficiency (EE) of TXA was measured by destabilizing the vesicles with Triton-X, exhaustively releasing the TXA and estimating this released TXA with ascorbic acid (AA)-based colorimetric assay, with absorbance spectrometry at 390 nm. Following this, similarly TXA-loaded vesicles were added to PBS (pH 7.4) in a 96-well plate ($7.5x10^7$ particles/µl) and diffusive release of TXA from the vesicles was recorded using a BioTek plate reader (excitation: 494nm /emission: 518 nm) at every 10 min for first 2 hours and then every hour for additional 10 hours, at 37°C. Figure 4 shows representative results of encapsulation efficiency (EE) of TXA in various batches of vesicles, demonstrating a consistent EE of ~65%. As shown in Figure 4, the diffusive release of TXA from such vesicles in physiological buffer (pH 7.4) at 37°C followed first order kinetics, with minimal release (10-20%) over first two hours and ~80% release over the next 10 hours.

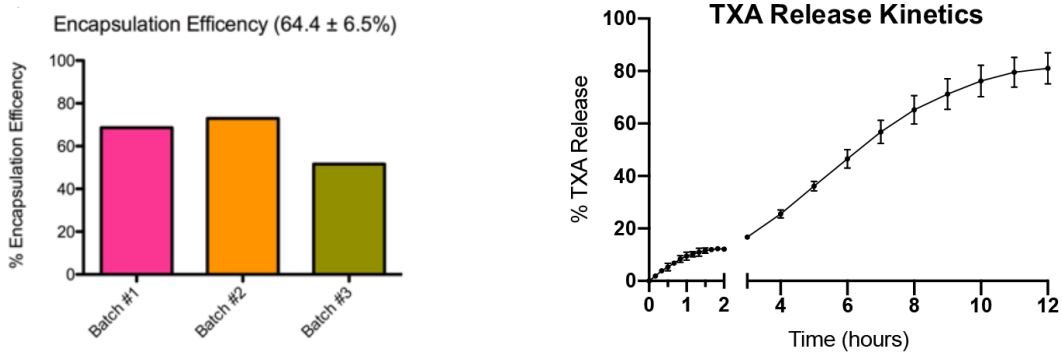


Figure 4. Encapsulation efficiency of TXA in vesicles and release kinetics of TXA from vesicles.

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.

Major Task 1: Demonstrate that SynthoPlate alone has beneficial effect on wound protection and inflammation.

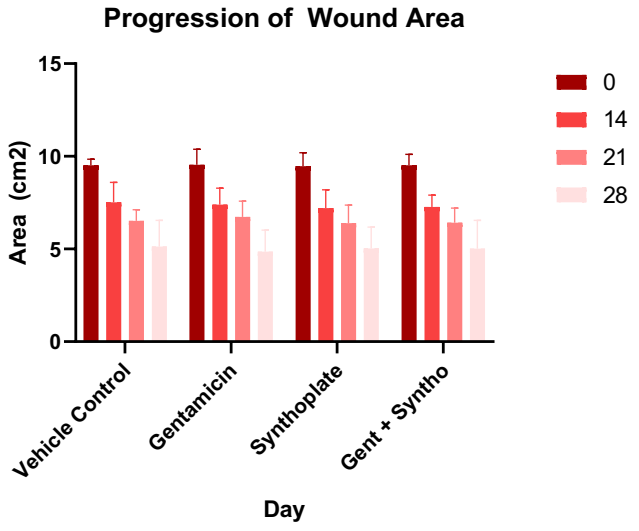
Subtask 1: CRADA establishment with Dr. Chan's lab, USAISR
Subtask 2: Shipment of SynthoPlate and Gentamicin-loaded SynthoPlate to Chan lab

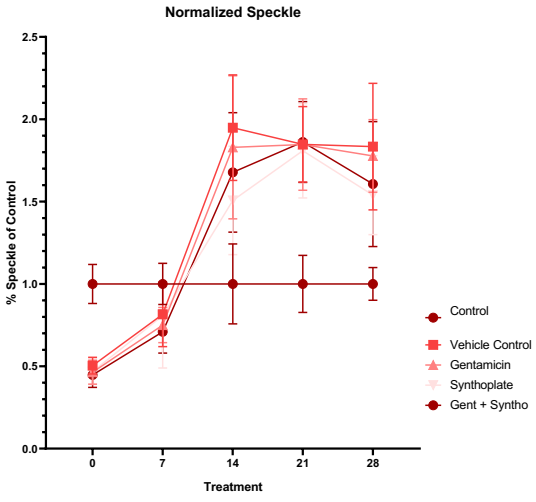
Subtask 3: Studies with localized application of SynthoPlate on burn wound in pigs for healing efficacy
Subtask 4: Report development on Major Task 1

Major Task 2: Demonstrate that SynthoPlate-Gentamicin combinations provide wound protection and decrease inflammation by decreasing the bacterial burden and local inflammatory markers.

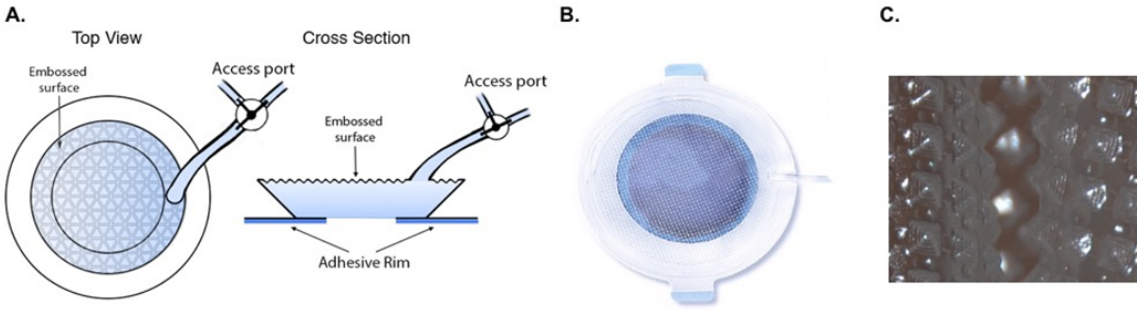
Subtask 1: Manufacture and characterization of SynthoPlate + Gentamicin formulations (loading + release), supply to Dr. Chan.
Subtask 2: Studies with SynthoPlate + Gentamicin in pig burn wounds

In Major Task 1 of this Aim, the subtask 1 was completed in Year 1. Subtask 2-4 of Major Task 1 and Subtask 1 and 2 in Major Task 2 were initiated in Year 2 of the project and is currently being continued. For this, unloaded SynthoPlate (SP) and Gentamicin-loaded SynthoPlate (Genta-SP) were adequately characterized in Sen Gupta lab and shipped to Chan lab for pig burn model studies. For these studies, 32 9cm^2 deep partial thickness burns were made on the dorsum of a pig. In an effort to keep each treatment localized to the appropriate wound, a 2-layered piece of gauze cut to the size of the wound was secured to each site with tegaderm. The appropriate treatment – normal saline, gentamicin, SynthoPlate, or Genta-SynthoPlate was applied to each wound. A larger tegaderm was then used over all the wounds to further secure the treatments, and finally loban was used to cover the entire back. Unfortunately, despite efforts to sequester each of the treatments, upon return to the operating room one week later, it was clear that the treatments had not remained on their individual wounds but instead spread throughout. We plan to analyze the treatments by histology, examining the presence of rhodamine in tissue biopsies. If the treatments did in fact spread between wounds, the rhodamine will be found in wounds that were not intended to be treated with SynthoPlate. The SP and Genta-SP group showed some promising trend in healing:





In the context of avoiding treatments running out of the wound area, we plan to revise our wound model set-up. The group has hypothesized that one of the issues with the prior model is that the wounds were too close together, resulting in an inability to form a strong seal around each wound. For the next experiment, we plan to create only 16 wounds with more distance between each wound. The treatments will be sequestered to their appropriate wounds by platform wound devices used in other experiments by our group. We also plan to follow the wounds out to 90 days as opposed to 28 to also examine re-epithelialization and scarring in the same animals. The set-up is shown below:



A. Cross-section of the platform wound device composed of polyurethane with an adhesive flexible ring and access port to allow injection of active treatment in liquid or gel form and withdrawal of fluid from the wound. **B.** Photograph of the platform wound device **C.** Pattern of small pyramids on the inner surface of the platform wound device promotes even distribution of liquid or hydrogel.

Opportunities for Training and Professional Development: During Year 2, the research has allowed the training of an MD PhD researcher (DaShawn Hickman), a PhD researcher (Aditya Girish), a Masters student researcher (Norman Luc) as well as, several undergraduate researchers (Stephanie Huang, Stephanie Yang, Ankush Banerjee, Yvonne Ma, Kenji Miyazawa), in various aspects of in vitro, in vivo and ex vivo studies focused on SynthoPlate formulations. 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 manuscripts, scientific reports and posters. Similarly, at the University of Pittsburgh, under the guidance of Dr. Matthew Neal, several researchers were trained, including Shannon Halderman, Jurgis Alvikas and Adnan Hassoune, and these researchers took part in carrying out the in vivo studies. At ISR, under Dr. Rodney Chan's supervision, Dr. Anders Carlsson carried out the pig burn model studies with his team.

Results Dissemination: Components of results stemming from the above-described studies were part of a recent poster presentation on SynthoPlate nanotechnology and TXA delivery with it, that was given by Dr. Anirban Sen Gupta (PI) in the Hemorrhage Control program at MHSRS 2018. The studies initiated in 2019 are planned to be continued in 2020 (Aim 2 and Aim 3 studies span Years 2 and 3), and the final data will be analyzed and presented in 2020.

Plans for next reporting period: During the next reporting period we will:
- Continue studies in the laboratory of Dr. Neal (Co-I) at University of Pittsburgh, to determine the cellular and molecular basis of transient cardiopulmonary distress observed in the pig model upon administration of SnthoPlate.
- Continue pig burn model studies in Dr. Chan's (Co-I) lab at USAISR to evaluate SP and Genta-SP in pig burn wounds.
- Prepare technical report on these activities and prepare appropriate manuscripts and presentations.

4. Impact:

Impact on principal discipline. The biggest impact from the Year 2 studies as described above is the finding that SynthoPlate can be manufactured reproducibly in large batches and stored at 4^{o}C as well as room temperature, without affecting stability and bioactivity. The other important finding from Year 2 is that intravenous SynthoPlate administration in pig intraperitoneal hemorrhage model causes transient cardiopulmonary distress that passes after 10-15 min. This finding has led us to extensively study literature on nanoparticle testing in pig models and the two salient findings from the past reports are that, the hemodynamic instability and cardiopulmonary distress are either due to systemic complement activation (CARPA) or organ-specific macrophage activation (pulmonary intravascular macrophages or PIMs) in cloven-hoofed animals. Based upon our studies we do not think that CARPA is an issue in our model, but PIM activation may be a potential issue. We are investigating this aspect further through our continued studies so as to enable us to make an informed decision about either deactivating the PIMs with therapeutic pre-treatment or to transition the model to a different species. This cardiopulmonary distress is not an issue in the pig burn model studies, as it is not intravenous but a topical administration framework. Although our studies are in the development phase, our data and design are driven towards a technological solution to reduce hemorrhage-associated preventable deaths of combat personnel in austere battlefield conditions.

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

Impact of technology transfer. The studies have added data to our existing patent on SynthoPlate, to 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 adding new personnel to Dr. Neal's approved IACUC (no procedural changes) but not getting the

subsequent ACURO approval for this IACUC change. Once we were notified of this as a non-compliance, the new person suspended his participation in the studies and we waited until the ACURO was approved in May 2019. The studies were continued following that and are still being continued as per the approved IACUC and ACURO protocol.

6. Products: Nothing to report.

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 (PI), NIH R01 HL129179 (PI), NIH R35 GM119526 (Co-I), DM160354 (PI).

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 vitro and in vivo models. He was responsible for carrying out majority of the research in Year 1 (Aim 1 studies) and in Aim 2 studies during the first six months of Year 2. He worked with two undergraduate researchers, in a team. He graduated in May 2019 with his PhD.
Funding Support:	NIH R01 HL121212, AHA Fellowship Grant 178CPRE33670016

Name:	Aditya Girish
Project Role:	Masters Student at Case Western

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Aditya Girish is a graduate researcher in the Sen Gupta laboratory, who actively contributes to SynthoPlate™ manufacture, characterization, shipment to collaborator labs, data analysis and report. He is responsible for carrying out the TXA formulation in the clot-targeted particles and its evaluation in vitro using ROTEM. He will continue to participate in remaining components of Aim 2 including manufacture of SynthoPlate to ship to Neal Lab at UPitt during the next reporting period.
Funding Support:	NIH HL 129179, DM160354

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 research assistant in the Sen Gupta laboratory, and is responsible for manufacture of SynthoPlate and Gentamicin-loaded SynthoPlate, characterization of these formulations using aggregometry, microfluidics and ROTEM, and shipment to collaborator laboratories at UPitt and ISR. He will participate in the remaining components of Aim 2 and Aim3.
Funding Support:	DM160354

Name: Matthew D. Neal, MD
Project Role: co-PI
Research Identifier: Nearest person month worked: 1
Contribution to Project: Dr. Neal leads the experimental design and analysis for all studies proposed under Specific Aim 2. He meets regularly with Dr. Sen Gupta and his team via phone as well as in person, for planning and execution of pig hemorrhage studies as described in Aim 2.

Name: Danielle Reiser
Project Role: animal technician
Research Identifier: Nearest person month worked: 1
Contribution to Project: Ms. Reiser prepared IACUC and ACURO documents for UPitt and coordinated administrative efforts and planning for swine studies.

Name: Shannon Haldeman
Project Role: Research Specialist
Research Identifier: Nearest person month worked: 4
Contribution to Project: Ms. Haldeman is the lead animal surgeon and coordinates all aspects of the swine trauma and hemorrhage model.

Name: Brian S. Zuckerbraun, MD
Project Role: co-I
Research Identifier: Nearest person month worked: 1
Contribution to Project: Dr. Zuckerbraun is an expert in hemorrhagic shock and resuscitation and serves as a consultant for the swine shock models

8. Quad Chart: Year 2 Quad Chart attached.

9. Appendices: IACUC and ACURO approvals for Rodney Chan (ISR), attached.