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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 variability's. 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 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. 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 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.					
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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:

Year 1(Months 1-12)

As per the proposed SOW, the Year 1 Aims and Major Goals were:

Aim 1. Characterization of biodistribution, systemic risks and immune response of intravenously administered SynthoPlate™ in pigs.

Major Task 1: Characterize and Mitigate immune response (if any) to SynthoPlate dosing

Major Task 2: Characterize prothrombotic risks (if any) upon SynthoPlate administration

Major Task 3: Characterize biodistribution of SynthoPlate over time.

Major task 4: Establish a safe dosing protocol for SynthoPlate in pigs.

Majority of these tasks were completed in Year 1, except for histopathology of pig organs which were slightly delayed and extended into Year 2. This was because the 'histopathology kits' were on 'backorder'. Nonetheless, they were ultimately completed in early Year 2. Year 1 annual report was submitted in November 2018. Components of results stemming from Year 1 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 published in *Nature 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 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.

Aim 2. Evaluate hemostatic efficacy of pristine SynthoPlate™ and TXA-loaded SynthoPlate™ 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 and sterilization for shipment to UPitt
- Subtask 2: IACUC and ACURO approval at UPitt
- Subtask 3: Sham/Control studies in pig trauma model

SynthoPlate manufacture and sterilization was completed. IACUC and ACURO approval for pig model studies in Neal lab was approved 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. Sham/control studies in pig were initiated.

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
- CRADA was established with Dr. Chan's lab at ISR, accordingly.

Year 2 (Months 12-24)

As per the proposed SOW, the Year 2 Aim and Major Goals were:

Aim 2. Evaluate hemostatic efficacy of pristine SynthoPlate™ and TXA-loaded SynthoPlate™ 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 3: Continuation of studies with sham/control pig model
- Subtask 4: Evaluation of hemostatic effect and serum cytokines in SynthoPlate-dosed pig traumatic injury model

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

All pig model studies were conducted as per IACUC and ACURO approval 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 hemorrhage at the University of Pittsburgh. In our studies, 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 proceeded to investigate 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 (VBP, CBP and FMP) decorated on SynthoPlate and test for specific 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 CARPA as an etiology. The details of our studies and results are described in Year 2 Annual Report. From our 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*. As a result, we conjectured that the reaction we are seeing is most possibly a species-specific reaction reported for pigs and other cloven-hoofed animals that 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*). To further test this, we decided to carry out studies with the ex vivo analysis of pig lung macrophages. We also wanted to study the cardiopulmonary distress aspect in real time using ultrasound imaging in pigs. These approaches required us to modify the UPitt IACUC with amendments to allow these studies and then submit the amended

IACUC for further ACURO approval. Our thought process was if we confirm the species-specific PIM to be culprit regarding the cardiopulmonary reaction seen in pigs, then we will either 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. As for *Major Task 2 Subtask 1*, we successfully loaded TXA in SynthoPlate and characterized its encapsulation and release. The details of our studies have been incorporated in Annual Report of Year 2, and part of the TXA encapsulation work was included in a recent publication (*Girish et al., J Thromb Haemost, 2019; 17: 1632-1644*).

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 2: Shipment of SynthoPlate (SP) and Gentamicin-loaded SP 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

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 9cm² 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 may have instead spread throughout. Nonetheless, the SP and Genta-SP group showed some promising trend in healing, as described in detail in the Annual Report of Year 2. In the context of avoiding treatments running out of the wound area, we planned to revise the wound model set-up for the subsequent experiments in two ways: (1) Create only 16 wounds with more distance between each wound and (2) Sequestering the treatments to their appropriate wounds by platform wound devices used in other experiments by the Chan group. The plan was to follow the wounds out to 90 days as opposed to 28 to also examine re-epithelialization and scarring in the same animals. The new device set-up was described in Annual Report of Year 2, and IACUC amendments to reflect these studies were submitted for approval, with the hope of subsequent ACURO approval to carry out the studies in Year 3.

Year 3 (Months 24-36)

Aim 2. Evaluate hemostatic efficacy of pristine SynthoPlate™ and TXA-loaded SynthoPlate™ 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 4: Evaluation of hemostatic effect and serum cytokines in SynthoPlate-dosed pig traumatic injury model (after establishing the mechanism behind the transient cardiopulmonary reaction seen in injured pigs upon nanoparticle dosing)

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 2: Evaluate 'control particle + TXA' vs 'SynthoPlate + TXA' in pig trauma model

Major task 3: Identify effects of TXA additively mixed vs encapsulated in SP vesicle on organ injury and inflammation following trauma

Major task 4: Data analysis and report preparation on Sp Aim 2.

For Aim 2 swine studies, the model involves the use of male and female Yorkshire swine (30-35kg). As described in the Final Report of Year 2, 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. Therefore, we focused on studying the potential mechanism behind these observations. This required an amendment to our IACUC protocol, to enable the use of a bronchoalveolar lavage (BAL) to collect macrophages, Swan-Ganz catheterization, and ultrasound imaging of the pig, as well as addition of another personnel (Hernando Gomez) who could assist with these envisioned studies. The IACUC amendment request was submitted by Dr. Neal's (Co-I at UPitt) laboratory, and once the amendment was approved by UPitt IACUC, the revised IACUC was submitted for ACURO approval. No animal studies were performed under DM160354 award during this time while the IACUC protocol was under ACURO evaluation. The ACURO approval was obtained during February 2020. However, the studies could not be performed since all laboratories were closed due to COVID-19 related shutdowns and stay-at-home orders that started in March 2020.

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.

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

Major Task 3: Demonstrate SynthoPlate alone or with Gentamicin combination has a beneficial effect on re-epithelialization of a wound

Major Task 4: Final report generation

The animal use protocol associated for these studies, entitled, "A Validation Study of Scarring After Skin Loss Either as a Result of Trauma or Burns in a Porcine Animal Model - A Type Protocol," required amendments for procedural modifications as described in the Annual Report of Year 2. REASON FOR CHANGE(S): A-14-042-TS4 has always been intended to be an extension of the A-14-041-TS4 protocol. The A-14-042-TS4 protocol was written to be the large wound version of A-14-041-TS4. Although this treatment was initially applied to the wounds described in A-14-041-TS4, we found that the wounds were too small (3cm diameter) and close together and that the dressing applied was unable to keep the treatments contained to the wound they were applied to, likely because there was not enough space between the wounds for the tegaderm adhering the dressing to form an adequate seal. We proposed to move the treatment to the A-14-042-TS4 protocol so that we are able to make use of a larger wound diameter, which would result in less wounds per pig but more space in between wounds for the dressing to adhere. We also proposed to use the platform wound device (PWD) to keep the treatments contained to their respective wounds. The PWD has an adhesive rim that creates a strong seal around the wounds, thereby keeping the treatment in place. These modifications to the intramural IACUC was submitted and approved (A-14-042-TP) and was subsequently submitted to ACURO for further review and oversight. The expectation was that upon ACURO approval the studies would be resumed some time in February or March of 2020. However, the studies could not be performed since all laboratories were closed due to COVID-19 shutdowns and stay-at-home orders starting in March 2020.

For both **Aim 2** and **Aim 3** remaining studies, no research could be done as laboratories remained shut until September 2020. This created two issues: (1) the grant award DM160354 was ending in September 2020 and (2) The IACUC periods at both UPitt and ISR were expiring by that time. An additional issue was the anticipated challenge to do pig trauma hemorrhage model studies in the COVID-19 restricted room occupancy framework, because these studies require 5-6 people to be in close quarters within a room. To address these issues, we decided to : (1) Request a no cost extension to our remaining award funds for 12 months (to end in September 2021); (2) Resubmit IACUC and subsequent ACURO approval requests for the animal procedures; and (3) Submit request for a potential model change without changing the scope of the work for the non-rodent trauma hemorrhage model from pigs to rabbits which will still allow evaluating the SP and SP +/- TXA formulations in a DoD-relevant non-rodent trauma model. These three steps were taken accordingly. The 12-month NCE request was granted in Sep 2020. The IACUC and ACURO re-approvals for the pig models were obtained in Dec 2020. The rabbit model explanation and request were sent to the Scientific Officer for the grant and was allowed. Accordingly, IACUC was approved at UPitt for the rabbit trauma hemorrhage model and the resultant documents are currently in preparation for ACURO submission. Upon ACURO submission we anticipate doing the remaining Aim 2 studies in this new model between April-July 2021. In parallel we expect to complete the remaining Aim 3 studies in the pig burn model at Chan Lab (ISR) at the same time. This will allow us to carry out data analysis and report preparation in August and submit to DoD by September of 2021.

Year 4 (Months 36-48)

As described in Quarterly Reports and Final Report of Year 3, we requested a 'change of model' for Sp Aim 2 from swine to rabbit, and this was approved. Hence the **revised Sp Aim 2** is:

Sp Aim 2: Evaluate hemostatic efficacy of SynthoPlate doses in Rabbit Model of uncontrolled hemorrhage

Major Task 1: Evaluate post-injury SynthoPlate transfusion for reducing blood loss following trauma injury

Subtask 1: IACUC and ACURO approval for Rabbit Model

Subtask 2: SynthoPlate and Control particle manufacture and supply to UPitt for study in rabbit model

Subtask 3: Studies with Sham animals and animals injected with control (unmodified) particle

studies: rabbit model injected with sham or control particles and evaluation of hemorrhage outcome, and analysis serum cytokines for determination of systemic inflammation levels (baseline)

Subtask 4: Studies on animals injected with SynthoPlate doses and evaluation of hemostatic effect, survival and analysis serum cytokines for assessment of systemic inflammation and systemic thrombotic side effects (if any).

Regarding Subtask 1 under Major Task 1 of Revised Specific Aim 2, the Rabbit model IACUC was approved at UPitt (Neal Lab) in December, 2020. Accordingly, ACURO documents were prepared in January 2021 and submitted to DoD in February 2021. ACURO review of the UPitt IACUC led to a several questions and commentary for clarifications, and the IACUC documents were sent back to UPitt for review and amendment. In review of the IACUC, several amendments and corrections were required, which were carried out and re-submitted for IACUC approval. We are awaiting the amended IACUC approval, to then submit back to ACURO for approval. Due to anticipated lag-time regarding this amended IACUC approval at UPitt and subsequent review and approval by DoD ACURO, we communicated with the Scientific Officer and decided to submit a request for second NCE to be able to complete the Major Task 1 studies during Fall 2021 and Spring/Summer 2022. This second NCE request was submitted in September 2021 and was approved. The ACURO protocol for rabbit studies, DM160354.e005 entitled, "Exploring Dynamic Platelet Functions after Hemorrhagic Shock, Polytrauma, and Associated Coagulopathies in Rabbits," IACUC protocol number 20128298 from University of Pittsburgh, Protocol Principal Investigator Dr. Matthew Neal, was approved on 01/05/2022, and the rabbit studies were started in Spring of 2022 and completed in Summer of 2022.

The results of these studies were described in detail in our Y5Q3 report and has now been included in the Appendix section of this Final Report. The main findings were as follows:

- Treatment of the traumatically injured rabbits with SynthoPlate (SP) seems to preserve systolic BP and MAP better than control particles (CP). This is indicative of improved BP stabilization potentially due to improved hemostatic effect in SP-treated animals vs. CP-treated animals.
- SP does not affect native platelet count and native platelet aggregatory (ADP as agonist) capability. Interestingly, CP seems to have a deleterious effect on platelet count and aggregatory function. Thus, SP is safe towards native platelet status in rabbits.
- Analysis by Platelet Factor 4 (PF4) assay of rabbit plasma indicated that neither CP nor SP impacts the in vivo PF4 status in injected rabbits, further corroborating that SynthoPlate does not affect the native platelet profile in vivo.
- Analysis by catecholamine assay of rabbit plasma indicated that neither CP nor SP seems to impact the in vivo systemic catecholamine status in injected rabbits, suggesting that SP or CP administration does not cause systemic duress in the injected rabbits.
- The blood loss analysis from the traumatically injured rabbits were the most exciting results from the studies. Rabbits administered with SynthoPlate (SP) showed a significantly reduced blood loss compared to sham (saline buffer-treated) as well as compared to control particle (CP) treated rabbits. This indicates improved hemostatic effect in rabbits when treated with SP.

These results have been submitted (and now accepted) as a research presentation abstract at the EAST (Eastern Association for the Surgery of Trauma) annual meeting to be held in Jan 2023:

Platelet-inspired synthetic hemostatic nanoparticles improve hemostasis and hemodynamics in a rabbit model of intraperitoneal hemorrhage

Authors: *Srinivasan AJ*, Secunda ZA, Mota-Alvidrez RI, Luc NF, Disharoon D, Traylor B, Pawlowski CL, Bruckman MA, **Sen Gupta A**, **Neal MD**

Representative results from these studies are shown in the Appendix, including blood pressure data, platelet aggregometry data, PF4 analysis data, catecholamine assay data, histology data with Carstairs's stain to assess off target thrombotic risks, and blood loss data from trauma injury.

We are in the process of preparing a manuscript based on these results too, for submission in December 2022 for publication in early Spring 2023, and the DM160354 award will be acknowledged in both the EAST presentation as well as the manuscript.

Major Task 2: Major Task 2: Identify the effects of SynthoPlate $\pm$ TXA blood loss and hemodynamic changes following traumatic injury.

This specific major task component was canceled due to budgetary limitation. The limitation stemmed from the fact that during 2020 although no experimental work could be carried out regarding Aim 2 due to COVID-related lab shutdown, the research personnel salary components still needed to be covered. This resulted in a part reduction of 'residual budget' for Aim 2 in 2021, which necessitated partial reduction of proposed Aim tasks. Since the primary focus of the project is the evaluation of SynthoPlate, and the context of 'SynthoPlate + TXA' evaluation is secondary to it, we decided to cancel this secondary task to be able to cover the primary task with the residual budget. This aspect was communicated with the Scientific Officer.

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.

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

Major Task 3: Demonstrate SynthoPlate alone or with Gentamicin combination has a beneficial effect on re-epithelialization of a wound

Major Task 4: Final report generation

IACUC and ACURO approval for these studies were achieved during Spring/Summer 2021. Gentamicin-loaded SP was shipped to ISR in March 2021 and the swine burn model studies were resumed. The studies were carried for 90-day time span. Deep partial-thickness burns were induced by a thermocoupled 5cm diameter, brass burn device at 100°C for a duration of 15 seconds with a constant application force. The wounds were lightly debrided by cleaning with a wet cotton gauze and topical treatments were applied in a special device using a polyurethane device – Platform wound device (PWD). Assessments and reapplications of treatments occur on days 3, 7, 14, 21 and 28. The wounds are then followed with assessments and biopsies on days 60 and 90 which is the final endpoint. The wounds are likely re-epithelialized at day 28, so there were not any further treatment applications at that point. Scarring and epithelialization outcomes were assessed on a monthly schedule until day 90. At days 28, 60 and 90 biopsies were harvested for histology. In addition, on day 90 a large excisional strip biopsy was harvested postmortem for histological scar assessments. The overall study results showed slightly higher re-epithelialization for SynthoPlate-treated group but no statistically significant differences between the various treatment groups. Wounds treated with SynthoPlate were ~96% re-epithelialized whereas those treated with the standard-of-care (SOC, Silver sulfadiazine ointment) measured slightly less at ~92% ($p = 0.56$). Re-epithelialization in the Vehicle Control (Saline) group was ~97% and in the Gentamicin group was ~90%. In conclusion, re-epithelialization in wounds treated by SynthoPlate were slightly improved over the SOC, but this difference did not reach significance. Interestingly, when SynthoPlate was used in combination with Gentamicin, there was a trend showing that the mean re-epithelialization was decreased relative to both the SOC and Gentamicin alone. The percentage of wound contraction was monitored by applying

a tattoo to the borders of the area intended for burn creation on day 0. This tattooed area was then measured on each day of analysis and recorded. The mean area for each treatment group was calculated at day 90. The percentage of wound contraction was reported as a ratio of the size of the wound after 90 days of healing with its respective treatment relative to the initial wound size. A growth co-efficient was factored in, to account for porcine development and growth over the 90-day period in order to scale size changes not due to contraction. The results showed that no statistically significant differences were observed between the treatment groups. The wound contraction in the SOC group was the lowest with wounds measuring 93% of their initial size (Figure 2). Wound contraction in wounds treated with SynthoPlate was higher with wounds measuring 86% of their initial size. Relative to SynthoPlate alone, contraction improved in wounds treated in combination with Gentamicin at the wounds measured 91% of their initial size ($p = 0.67$). In the Vehicle Control (Saline) group, the wounds measured at 85% of their initial size and in the Gentamicin alone group the wound contraction percentage was 91% of initial wound size. In conclusion, the percentage of wound contraction in the treatment groups of SynthoPlate as well as SynthoPlate with Gentamicin did not improve outcomes relative to the SOC. Although mean percentage of wound contraction was improved in the combination treatment group of SynthoPlate with Gentamicin relative to SynthoPlate alone, this difference did not reach significance. Superficial blood flow was monitored through Laser Speckle analysis. Data was recorded at each assessment and wounds were monitored for their amount of superficial blood flow relative to normal, uninjured control skin to compare levels of blood flow. This analysis served as a surrogate for angiogenesis. At post-burn day 28, in the SynthoPlate treatment group, superficial blood flow was measured to be 168% of normal skin controls. Meanwhile in wounds treated with a combination of SPM with gentamicin, superficial blood flow was slightly lower at 160%. In wounds treated with the SOC, superficial blood flow decreased to 110% of normal skin controls ($p = 0.27$). In conclusion, superficial blood was elevated well above that of normal skin controls in the SynthoPlate treatment group as well as the SynthoPlate with Gentamicin group at post-burn day 28. These treatments were improved also in regard to the treatment with the SOC, but this observed difference did not reach significance. Wounds were monitored for the amount of bacterial load present in order to assess how well protected from infection each treatment group would be as they progressed through healing. Bacterial swabs were collected and cultured. The mean bacterial load in each group was assessed at post-burn day 3 through visual assessment with the MolecuLight Wound Imaging Device that allows visualization of bacteria on the culture dish. A scoring system was developed to stratify the amount of bacteria present in accordance with visual representation from 0-100 where higher the score greater the burden of bacteria. Notably, this consisted of the mean bacterial load being the lowest in wounds treated with gentamicin alone at 17/100. Meanwhile in wounds treated with the SOC, or for those in the SynthoPlate with gentamicin group, mean bacterial loads were significantly higher at 43/100 and 47/100, respectively ($p = 0.02$). Mean bacterial load of SynthoPlate alone was 53/100 and for wounds in the Vehicle Control (Saline) group was 57/100. In conclusion, Gentamicin decreased the amount of bacteria present on the wounds significantly. SynthoPlate did not decrease bacterial load relative to the SOC. However, when in combination with Gentamicin, the mean bacterial load was decreased although this did not reach significance. The vehicle control (saline) group had the highest

bacterial burden. These values served as representations of the antibacterial properties offered by each type of treatment to deep partial-thickness burns. The overall conclusion altogether from these studies is that SynthoPlate alone as well as Gentamicin-loaded SynthoPlate show a trend towards improved epithelization and blood flow (characteristics of improved healing), but at the current stage this observed trend has not reached a statistical significance. We envision that future studies, supported by subsequent contract awards from DoD will enable us to improve upon this by refining the SynthoPlate design to make it capable of augmenting fibrin and collagen formation (pro-healing matrix proteins), as well as delivering other therapeutic agents beyond Gentamicin.

Results from Aim 3 results was presented at the American Burn Association 2022 annual meeting under the following title:

Synthetic Platelet-Mimetics with Gentamicin: Outcomes in Deep Partial-Thickness Burns

Authors: Franklin Valdera, MD, Kristo Nuutila, MSc, PhD, Laura E. Cooper, MD, David E. Varon, BA, Jennifer Cooley, MD, Javier Chapa, BS, Rodney K. Chan, MD, Anders H. Carlsson, PhD

Department of Surgery, Brooke Army Medical Center, San Antonio, Texas; U.S. Army Institute of Surgical Research, San Antonio, Texas; Department of Surgery, University of Maryland Medical Center, San Antonio, Texas; University of Michigan Medical School, San Antonio, Texas; Department of Surgery, William Beaumont Army Medical Center, El Paso, Texas; Division of Combat Wound Repair, U.S. Army Institute of Surgical Research, San Antonio, Texas; US Army Institute of Surgical Research, San Antonio, Texas; The Metis Foundation, San Antonio, Texas

These results are also now being submitted as a manuscript in ***Journal of Burn Care and Research*** (please find PDF of manuscript attached at the end).

Opportunities for Training and Professional Development: During the limited but resumed activities of Year 4 in the midst of COVID vaccinations and somewhat restricted lab operations, the research has allowed the training of two PhD researchers (Aditya Girish and Norman Luc) as well as, several undergraduate researchers (Stephanie Huang, Stephanie Yang, Ankush Banerjee, Yvonne Ma, Kenji Miyazawa, Kelsey Swingle), 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 (Matthew Neal laboratory) and USAISR (Rodney Chan laboratory), to contribute to the reported studies. The researchers were also trained in writing technical reports. Similarly, at the University of Pittsburgh, under the guidance of Dr. Matthew Neal, several researchers were trained, including Shannon Halderman, Jurgis Alvikas, Adnan Hassoune and Roberto Mota to carry out various aspects of the Aim 2 studies. At ISR, under Dr. Rodney Chan's and Dr. Anders Carlsson's supervision, the team completed remaining Aim 3 studies in the pig burn model.

Results Dissemination: All PDFs are attached at the end

Aim 1 studies:

- *MHSRS Poster 2018*

Pre-Clinical Evaluation of SynthoPlate as an Intravenous Hemostatic Nanotechnology for Hemorrhage Control and Survival Benefit in Military-relevant Trauma Models

Authors: DaShawn A Hickman, Norman F Luc, Ankush Banerjee, Aditya Girish, Mitchell R. Dyer MD, Shannon Haldeman, Wei Li MD PhD, Ann Kim MD, Andrew Shevitz, Michael Bruckman PhD, Ujjal DS Sekhon, Christa L Pawlowski PhD, Vikram S Kashyap MD, Matthew D Neal MD, Anirban Sen Gupta, PhD.

Aim 2 studies:

- *Nature Scientific Reports Publication 2018, 8:3118 DOI:10.1038/s41598-018-21384-z*

Intravenous synthetic platelet (SynthoPlate) nanoconstructs reduce bleeding and improve ‘golden hour’ survival in a porcine model of traumatic arterial hemorrhage

Authors: DaShawn A. Hickman, Christa L. Pawlowski, Andrew Shevitz, Norman F. Luc, Ann Kim, Aditya Girish, Joyann Marks, Simi Ganjoo, Stephanie Huang, Edward Niedoba, Ujjal D. S. Sekhon, Michael Sun, Mitchell Dyer, Matthew D. Neal, Vikram S. Kashyap, Anirban Sen Gupta

- *EAST Trauma Conference Abstract (to be presented and published in 2023)*

Platelet-inspired synthetic hemostatic nanoparticles improve hemostasis and hemodynamics in a rabbit model of intraperitoneal hemorrhage

Srinivasan AJ, Secunda ZA, Mota-Alvidrez RI, Luc NF, Disharoon D, Traylor B, Pawlowski CL, Bruckman MA, Sen Gupta A, Neal MD.

Aim 3 Studies:

- *American Burn Association 2022 annual meeting conference presentation abstract :*

Synthetic Platelet-Mimetics with Gentamicin: Outcomes in Deep Partial-Thickness Burns

Authors: Franklin Valdera, MD, Kristo Nuutila, MSc, PhD, Laura E. Cooper, MD, David E. Varon, BA, Jennifer Cooley, MD, Javier Chapa, BS, Rodney K. Chan, MD, Anders H. Carlsson, PhD

- *Manuscript submitted to Journal of Burn Care and Research (JBCR-D-22-00309, publication anticipated in 2022/2023):*

Synthetic Platelets with Gentamicin: Outcomes in Deep Partial-Thickness Burns

Authors: Franklin Valdera, Kristo Nuutila, David E. Varon, Laura E. Cooper, Jennifer Cooley, Javier Chapa, Sean Christy, Norman Luc, Anirban Sen Gupta, Andrew Ditto, Michael Bruckman, Rodney K. Chan, Anders H. Carlsson.

4. Impact:

Impact on principal discipline. The studies conducted under the support of DM 160354 have provided valuable and exciting insight on the feasibility and translational potential of a platelet surrogate system (SynthoPlate) in hemorrhage control and burn wound care for battlefield-relevant traumatic injuries. The injury models used for the studies are in pigs (hemorrhage and burn) and rabbits (hemorrhage), which are important non-rodent models for translational evaluation, and in both models SynthoPlate showed promising therapeutic efficacy and reasonable safety. The studies also provided important insight that porcine models are very suitable for studying topically applied therapeutics, but in general may not be suitable for high throughput evaluation of intravenously dosed nanoparticle systems. This is due to the inherent hypercoagulability and cardio-pulmonary sensitivity of pigs to intravenously dosed nanoparticles, that then require additional administration management (slow infusion, pre- or post-treatment management with immune/inflammation modulators, rigorous modulation of nanoparticle surface charge and dose etc.) to allow execution of the studies. This aspect is also corroborated by several studies reported by other research groups studying intravenously delivered nanoparticle systems in pigs (and other cloven-hoofed animals). Thus, pigs (and other cloven-hoofed animals) are suitable for *topical* nanoparticle therapeutics evaluation but not that suitable for *intravenous* nanoparticle therapeutics evaluation. This cardio-pulmonary hypersensitivity of pigs (and cloven-hoofed animals in general) to intravenously dosed nanoparticles is rarely found in humans, and thus mitigating such issues for pigs may not have high translational relevance in humans. Rabbits on the other hand do not have such issues and is a highly suitable non-rodent model for studying intravenous nanoparticles. In our opinion, the most suitable translational path for in vivo evaluation of intravenous nanoparticle hemostat is Mouse/Rats → Rabbits → Dogs → NHP → Humans. Subsequent to the studies funded by DM 160354 (funded in 2017 and NCE till 2022), a separate DoD contract (PRMRP, PR 191632) was recently funded to us in 2020 with a focus on lyophilizing SynthoPlate using various carbohydrates as lyoprotectants for potential 'small carry volume' field-deployable technology for on-field hemorrhage control. Therefore the initial hemostatic efficacy studies of SynthoPlate supported by DM 160354 have now opened the doors on studying the lyophilizability and field-deployability of SynthoPlate, to potentially lead to an on-field applicable hemorrhage control and wound care technology. Although our studies are in the research and development phase, our data and design focused on a technological solution to reduce hemorrhage-associated preventable deaths of combat personnel in austere battlefield conditions, has high translational promise. With this in mind, SynthoPlate patents were

licensed to Haima Therapeutics, a biotech start-up that Anirban Sen Gupta (PI) has co-founded. Haima has now taken up the responsibility of scaled up manufacturing of SynthoPlate.

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 hemorrhage control and 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: One modest challenge that we faced during execution of our Aim 1 and Aim 2 tasks is the finding that pigs are hypersensitive and reactive to intravenously administered nanoparticle systems and show transient signs of cardiopulmonary distress, which ultimately resolve via modulation of dose infusion rate and nanoparticle surface charge, but nonetheless puts an operational obstacle in doing pig model studies. In our literature search on this matter, we found that this observation has been documented by several other researchers in studying other nanoparticle systems (e.g. hemoglobin-loaded nanoparticles as RBC surrogates, chemotherapy-loaded liposomal nanoparticles etc.) intravenously administered in pigs, and the current consensus is that this is attributed to a special type of macrophages (pulmonary intravascular macrophages, PIMs) that are present in pigs and other cloven-hoofed animals, but not in humans (and thus the human exhibition of such reactions to I.V.-administered nanoparticles is rare). We therefore rationalized that for our non-rodent studies we should choose a non-cloven hoofed animal, and based on our budget as well as reported DOD studies we selected rabbits as a suitable non-rodent model (based on robust documentation of blood product evaluation by DOD-funded investigators in rabbit trauma models). We requested this model change, which was approved. However, by the time the rabbit IACUC and ACURO were approved, operations were completely stalled due to COVID-19. Here we requested our first NCE which was approved. The biggest challenge of 2020 and 2021 was the stalling of research activities for almost 10 months, as well 'limited occupancy in labs' scenario, due to issues associated with COVID-19-related shutdown, supply chain issues and operational obstacles. Operations resumed during summer of 2021 (post-vaccination) and by that time some IACUCs had expired and required IACUC re-approval and ACURO re-approval. This also required a second NCE request which was approved. After these were obtained, we could resume our studies in rabbit models, and were able to complete our studies (revised SOW) with the two NCEs.

6. Products:

Aim 1 studies:

- *MHSRS Poster 2018*

Pre-Clinical Evaluation of SynthoPlate as an Intravenous Hemostatic Nanotechnology for Hemorrhage Control and Survival Benefit in Military-relevant Trauma Models

Authors: DaShawn A Hickman, Norman F Luc, Ankush Banerjee, Aditya Girish, Mitchell R. Dyer MD, Shannon Haldeman, Wei Li MD PhD, Ann Kim MD, Andrew Shevitz, Michael Bruckman PhD, Ujjal DS Sekhon, Christa L Pawlowski PhD, Vikram S Kashyap MD, Matthew D Neal MD, Anirban Sen Gupta, PhD.

Aim 2 studies:

- *Nature Scientific Reports Publication 2018, 8:3118 DOI:10.1038/s41598-018-21384-z*

Intravenous synthetic platelet (SynthoPlate) nanoconstructs reduce bleeding and improve 'golden hour' survival in a porcine model of traumatic arterial hemorrhage

Authors: DaShawn A. Hickman, Christa L. Pawlowski, Andrew Shevitz, Norman F. Luc, Ann Kim, Aditya Girish, Joyann Marks, Simi Ganjoo, Stephanie Huang, Edward Niedoba, Ujjal D. S. Sekhon, Michael Sun, Mitchell Dyer, Matthew D. Neal, Vikram S. Kashyap, Anirban Sen Gupta

- *EAST Trauma Conference Abstract (to be presented and published in 2023)*

Platelet-inspired synthetic hemostatic nanoparticles improve hemostasis and hemodynamics in a rabbit model of intraperitoneal hemorrhage

Srinivasan AJ, Secunda ZA, Mota-Alvidrez RI, Luc NF, Disharoon D, Traylor B, Pawlowski CL, Bruckman MA, Sen Gupta A, Neal MD.

Aim 3 Studies:

- *American Burn Association 2022 annual meeting conference presentation abstract :*

Synthetic Platelet-Mimetics with Gentamicin: Outcomes in Deep Partial-Thickness Burns

Authors: Franklin Valdera, MD, Kristo Nuutila, MSc, PhD, Laura E. Cooper, MD, David E. Varon, BA, Jennifer Cooley, MD, Javier Chapa, BS, Rodney K. Chan, MD, Anders H. Carlsson, PhD

- *Manuscript submitted to Journal of Burn Care and Research (JBCR-D-22-00309, publication anticipated in 2022/2023):*

Synthetic Platelets with Gentamicin: Outcomes in Deep Partial-Thickness Burns

Authors: Franklin Valdera, Kristo Nuutila, David E. Varon, Laura E. Cooper, Jennifer Cooley, Javier Chapa, Sean Christy, Norman Luc, Anirban Sen Gupta, Andrew Ditto, Michael Bruckman, Rodney K. Chan, Anders H. Carlsson.

Please note that Rabbit studies pertaining to Aim 2, that are being presented at EAST conference, will also be compiled into a manuscript and submitted early 2023. The DM 160354 award will be acknowledged in the manuscript.

7. Participants:

Name:	Anirban Sen Gupta
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	eRA Commons ID: ANIRBAN0426
Nearest person month worked:	2
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 R01 HL141080 (MPI), DM160354 (PI), PR 191632 (PI).

Name:	Norman Luc
Project Role:	PhD Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Norman Luc 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 manufacture and characterization of SynthoPlate for planned studies. He worked with two undergraduate researchers, in a team. He will continue to participate in remaining components of Aim 2 and Aim 3 including manufacture of SP, SP +/- TXA and Genta-SP to ship to collaborator labs for remaining studies.
Funding Support:	NIH R01 HL121212, DM 160354

Name:	Aditya Girish
Project Role:	PhD 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 and Aim 3 including manufacture of SP, SP +/- TXA and Genta-SP to ship to collaborator labs for studies.
Funding Support:	NIH R01 HL 129179, 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

Name: Jurgis Alvikas, MD

Project Role: General Surgery Research Resident

Research Identifier: Nearest person month worked: 4

Contribution to Project. Dr. Alvikas assists with the animal surgeries and analyzes the data, including blood and organ samples, of the trauma and hemorrhage models.

Name: Adnan Hassoune, MD

Project Role: Visiting Research Scholar

Research Identifier: Nearest person month worked: 4
Contribution to Project: Dr. Hassoune assists with the animal surgeries and organizes the data for studies on trauma and hemorrhage models.

Name: Rodney Chan, MD

Project Role: Subaward PI

Research Identifier: <https://orcid.org/0000-0002-5061-847X>

Nearest person month worked: 1.80 calendar months

Contribution to Project: Data Analysis, Animal Experiments

Name: Anders Carlsson, PhD

Project Role: Subaward Co-PI

Research Identifier: <https://orcid.org/0000-0002-4846-108X>

Nearest person month worked: 6.00 calendar months

Contribution to Project: Data Analysis, Animal Experiments

8. Quad Chart: FINAL Quad Chart attached.

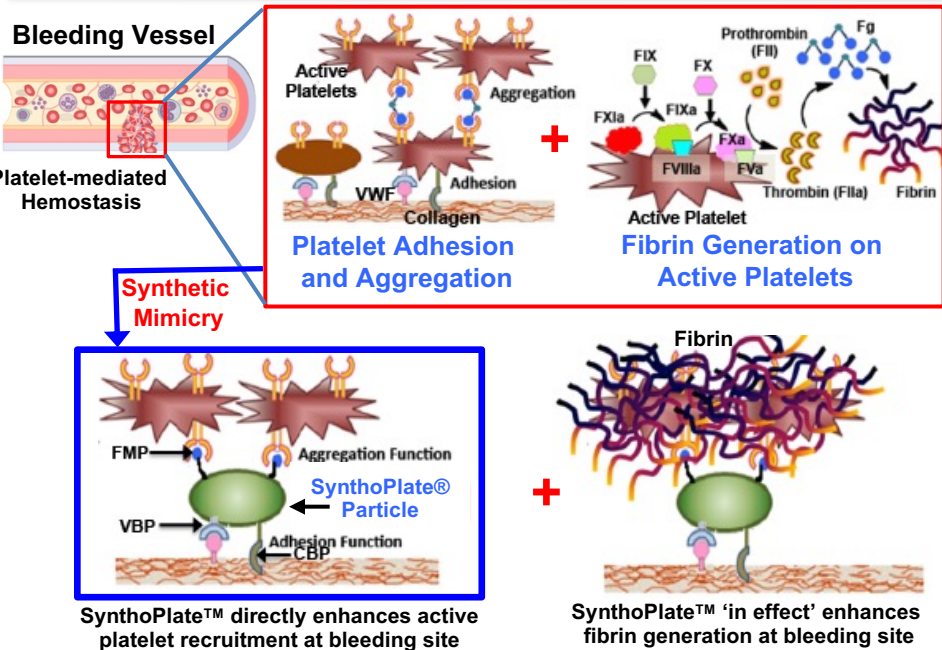
9. Appendices: All Manuscripts and Presentation Abstracts Attached.



SynthoPlate™ Nanotechnology For Intravenous Hemostasis and Wound Healing in Prolonged Field Care

Funding Opportunity Number: W81XWH-16-DMRDP-CCCRP-PFCRA

PI: Anirban Sen Gupta, PhD; Co-PI: Matthew D. Neal, MD; Co-I: Ronald Poropatich, MD, Co-I: Rodney K Chan, MD



Military Relevance in Prolonged Field Care

- In PFC and pDCR, transfusion of blood components, especially platelets, can render rapid hemostasis that significantly reduces combat-associated mortalities from trauma and exsanguination
- Platelet products, however, present issues of availability, portability, high bacterial contamination risk, and very short shelf-life that present significant logistical challenges for their efficient point-of-care (pre-MTF, RDCR) use in a far forward military setting
- **SynthoPlate™** is a lyophilized powder-form, sterile, shelf-stable, portable **synthetic platelet technology**, that can be reconstituted in saline at point-of-care, and administered I.V. to mimic, and amplify endogenous mechanisms of platelet-mediated hemostasis
- Beyond rendering efficient hemostasis to reduce morbidity and mortality risks, SynthoPlate™ particles can also act as targeted delivery platforms for spatio-temporally regulated delivery of bioactive agents to promote wound protection and healing

Technology: SynthoPlate™ (synthetic platelet nanotechnology)
Patent US 9107845; Technology Readiness Level: 4

Study Aims (with Milestone Components)

- **Aim 1: Characterize biodistribution, systemic risks and immune response of SynthoPlate™ in pigs**
 - Systemic safety, Maximum Tolerated Dose and Biodistribution
- **Revised Aim 2: Evaluate hemostatic efficacy of SynthoPlate™ in a rabbit model of trauma hemorrhage**
 - Hemostatic efficacy, Dose response, Dose optimization
- **Aim 3: Evaluate efficacy of SynthoPlate™ alone or in combination with Gentamicin for wound protection and healing in pigs**
 - Effect of SynthoPlate™ vs SynthoPlate™-Gentamicin combinations in wound protection and healing in burn and excision wounds

Timeline and Cost

Activities	Year 1	Year 2	Year 3-5
Systemic safety and biodistribution studies	Complete		
Hemostatic efficacy studies in vivo (pig model originally, revised rabbit model of trauma in Y 4,5)		Complete (No work possible in Y3 due to COVID; NCE and Revised SOW approved for Yrs 4 and 5)	
Wound protection and re-epithelialization studies		Complete	

TOTAL EXPENDED BUDGET = \$969,214

Pre-Clinical Evaluation of SynthoPlate™ as an Intravenous Hemostatic Nanotechnology for Hemorrhage Control and Survival Benefit in Military-relevant Trauma Models

¹DaShawn A Hickman, ⁵Norman F Luc, ⁵Ankush Banerjee, ⁵Aditya Girish, ²Mitchell R. Dyer MD, ²Shannon Haldeman, ³Wei Li MD PhD, ⁴Ann Kim MD, ⁴Andrew Shevitz, ⁵Michael Bruckman PhD, ⁵Ujjal DS Sekhon, ⁵Christa L Pawlowski PhD, ⁴Vikram S Kashyap MD, ²Matthew D Neal MD, ^{1,5}Anirban Sen Gupta PhD*



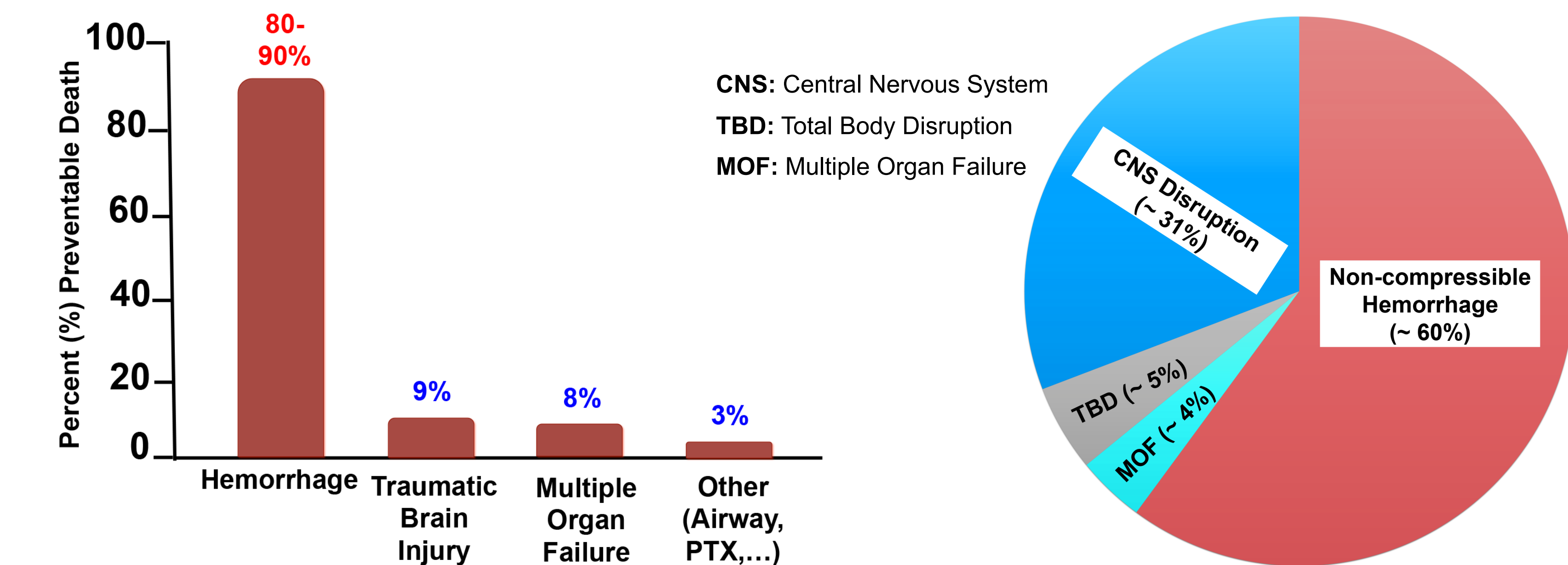
SEN GUPTA LAB
BIO-INSPIRED ENGINEERING for ADVANCED THERAPIES



¹Department of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH
²Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA
³Biomedical Sciences, Marshall University, School of Medicine, Huntington, WV
⁴Department of Vascular Surgery, University Hospitals Case Medical Center, Cleveland, OH
⁵Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH

Traumatic Hemorrhage in Combat Setting

Trauma-associated Non-compressible Hemorrhage is the Leading Cause of 'Preventable Death' in Pre-hospital Battlefield Conditions

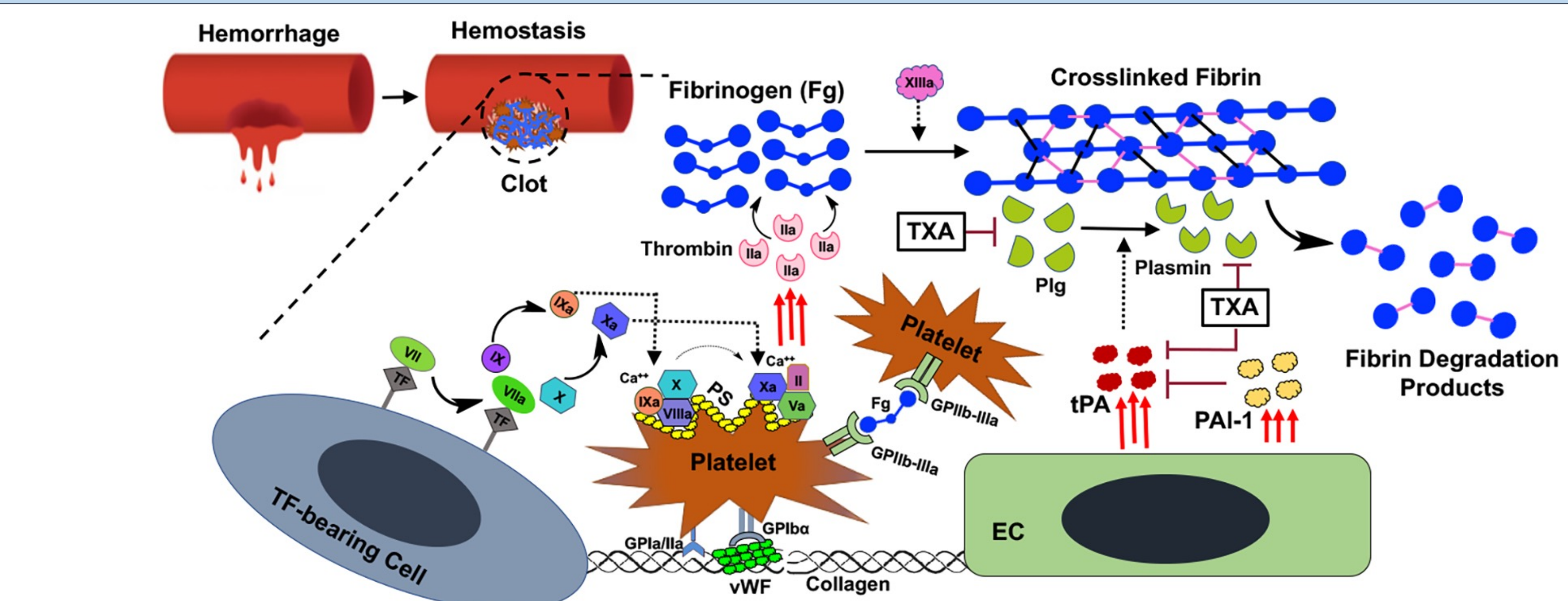


Adapted from [Eastridge et al, J Trauma, 2011](#)
and [J Trauma Acute Care Surg, 2012](#)

Adapted from [Morrison et al, J Trauma Acute Care Surg, 2013](#)

- Pre-hospital approaches to mitigate non-compressible hemorrhage and coagulopathy can significantly improve survival of military personnel suffering from combat injury.
- Rapid control of bleeding at or near the point of injury is critical to enable all subsequent resuscitative procedures.
- Early transfusion of whole blood (WB) or components (platelets, RBC, plasma) as part of Remote Damage Control Resuscitation (RDCR) can improve hemostasis and tissue oxygenation, and enhance survival.
- However, availability and accessibility of WB and components, especially platelets, are very limited in pre-hospital battlefield conditions due to difficult portability, high risk of bacterial contamination, and short shelf-life (3-5 days).

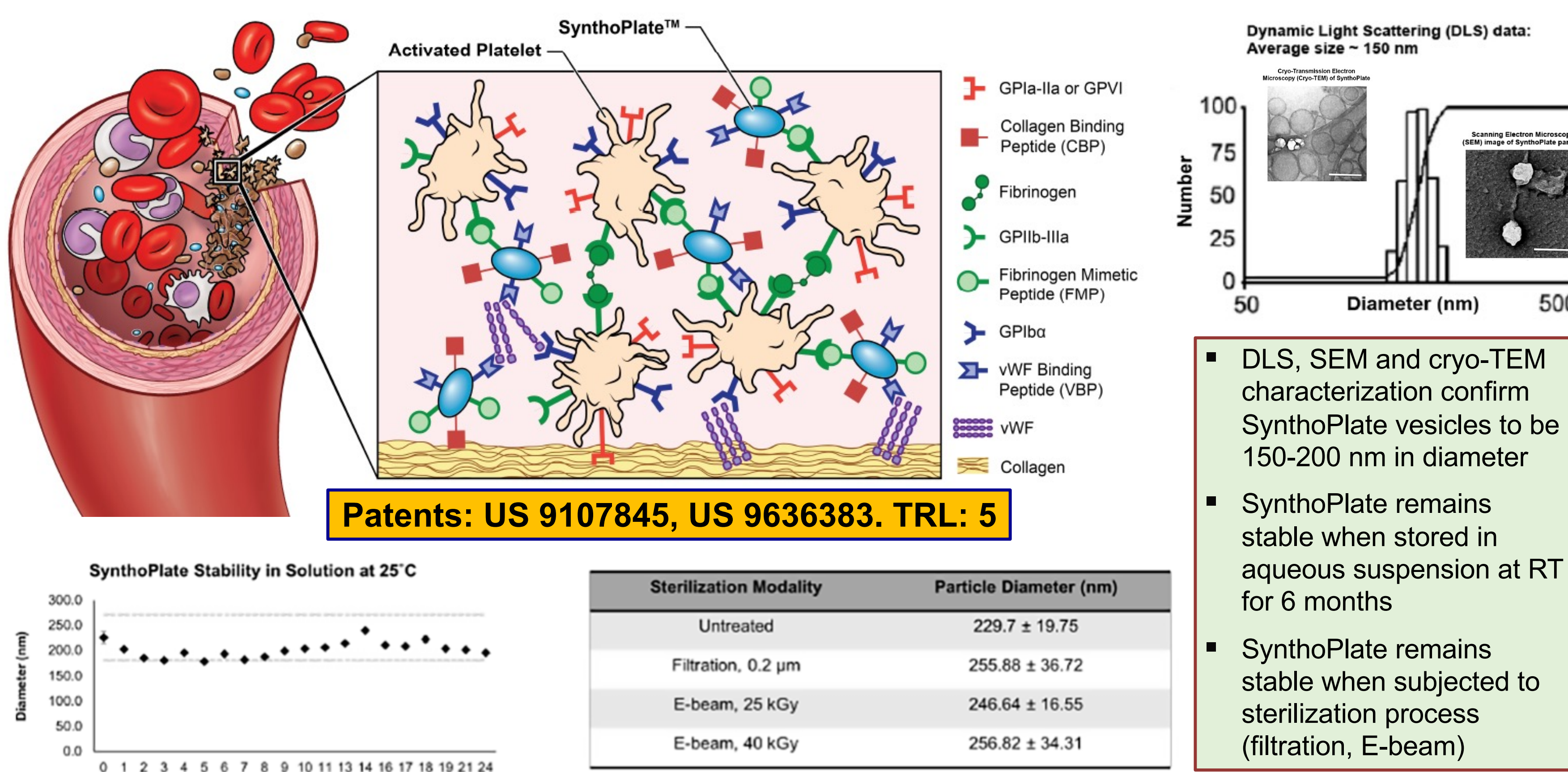
Platelets in Hemostasis



- Upon vascular damage, TF-bearing cells render TF-VIIa complex formation, leading to generation of initial small amount of thrombin (FIIa), that activates platelets and several key coagulation factors.
- Damaged vasculature also exposes sub-endothelial collagen and vWF, to which platelets can adhere and activate via distinct outside-in signaling pathways.
- Activated platelets can aggregate primarily via bridging of platelet surface integrin GPIIb-IIIa by the blood protein Fibrinogen (Fg) to form the 'platelet plug'.
- The surface of aggregated active platelets expose anionic phospholipids like phosphatidylserine (PS) that promotes co-localization of coagulation factors to form the tenase (FIXa + VIIIa + X) complex and the prothrombinase (FXa + FVa + FII) complex, to ultimately amplify the generation of thrombin (FIIa).
- Thrombin converts Fg to Fibrin which can self-assemble as well as undergo crosslinking via action of transglutaminase FXIIIa, to form a biopolymeric mesh that secures platelet plug as well as other blood components to form hemostatic clot.
- Crosslinked fibrin stability is closely regulated by Fibrin degrading action of Plasmin (generated from Plasminogen by action of EC-sourced tPA) as well as inhibition of tPA by agents like PAI-1.
- In severe trauma-associated hemorrhage, the above processes become highly dysregulated resulting in coagulopathy, hyperfibrinolysis and weakened clot structure, leading to impaired hemostasis.

SynthoPlate™ (synthetic platelet surrogate)

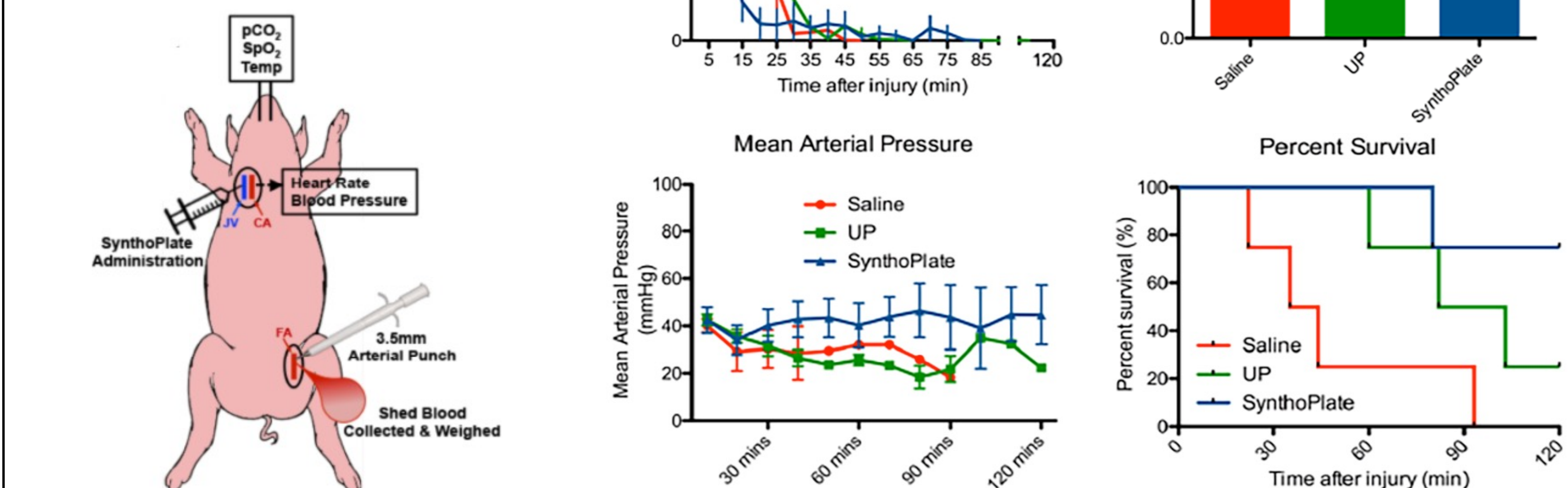
SynthoPlate Design: liposomal construct surface-decorated with three types of peptides



In Vivo Studies (Pig Artery Model)

Femoral artery trauma model

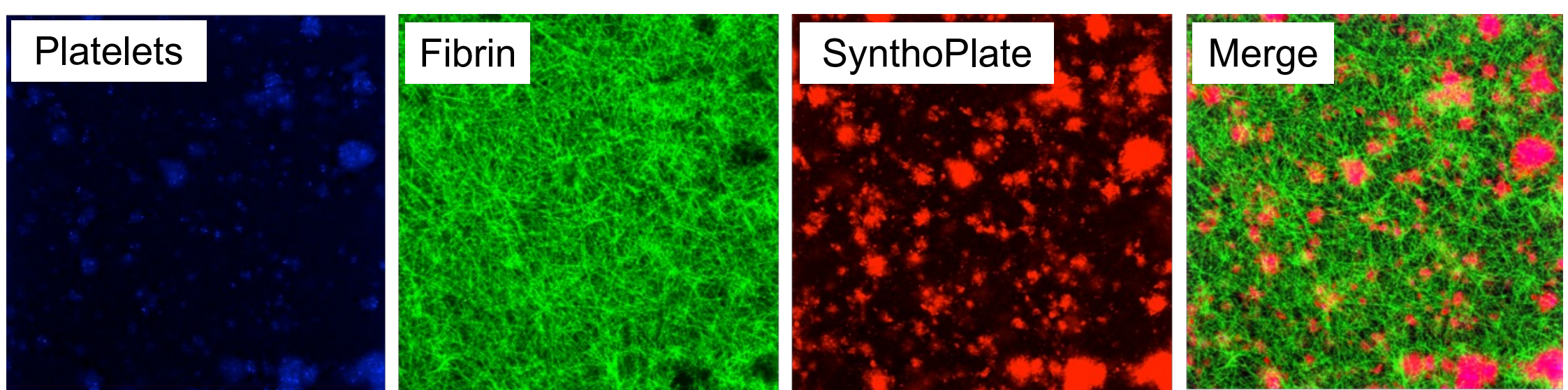
- Femoral artery injured with 3.5mm punch
- SynthoPlate administered I.V. post-injury
- Vitals and survival monitored upto 2 hrs
- Shed blood measured for blood loss



- SynthoPlate dose substantially reduced blood loss rate and total blood loss, and stabilized MAP over 2 hr window.
- SynthoPlate dose substantially enhanced survival in pigs following injury, compared to saline only or control particles.

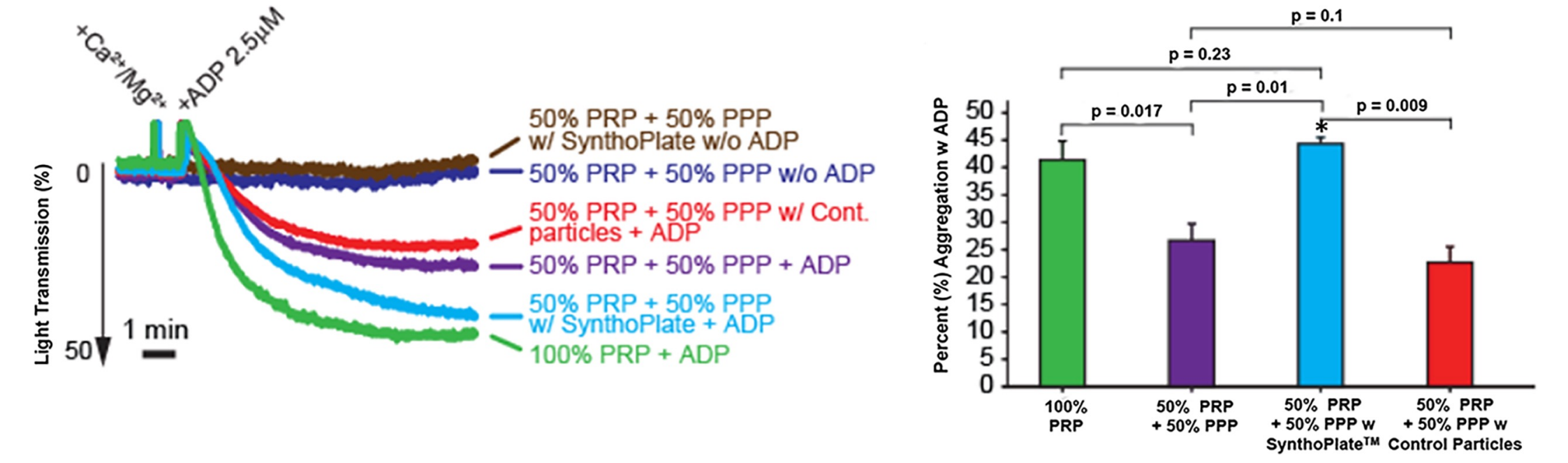
In Vitro Studies

Co-localization of SynthoPlate with Platelets in clots (confocal)

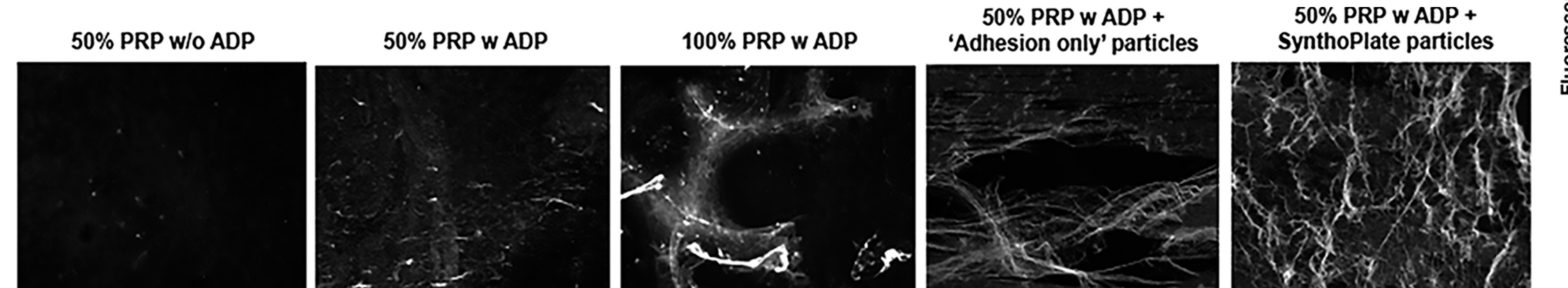


- SynthoPlate vesicles (red, Rhb-labeled) colocalize with active platelets (calcein-stained, pseudocolored blue) within green fibrin (AlexaFluor-647 stained, pseudocolored green) in PRP clots.
- SynthoPlate does not activate and aggregate resting platelets (i.e. no thrombotic risk towards circulating platelets), but can specifically enhance the aggregation of activated platelets.
- 'SynthoPlate + Platelets' significantly enhanced fibrin deposition in PRP clots over time, as evidenced by enhanced fibrin fluorescence by 15 min time point, and high D-dimer signal upon clot degradation, in microfluidic set-up.

Aggregometry

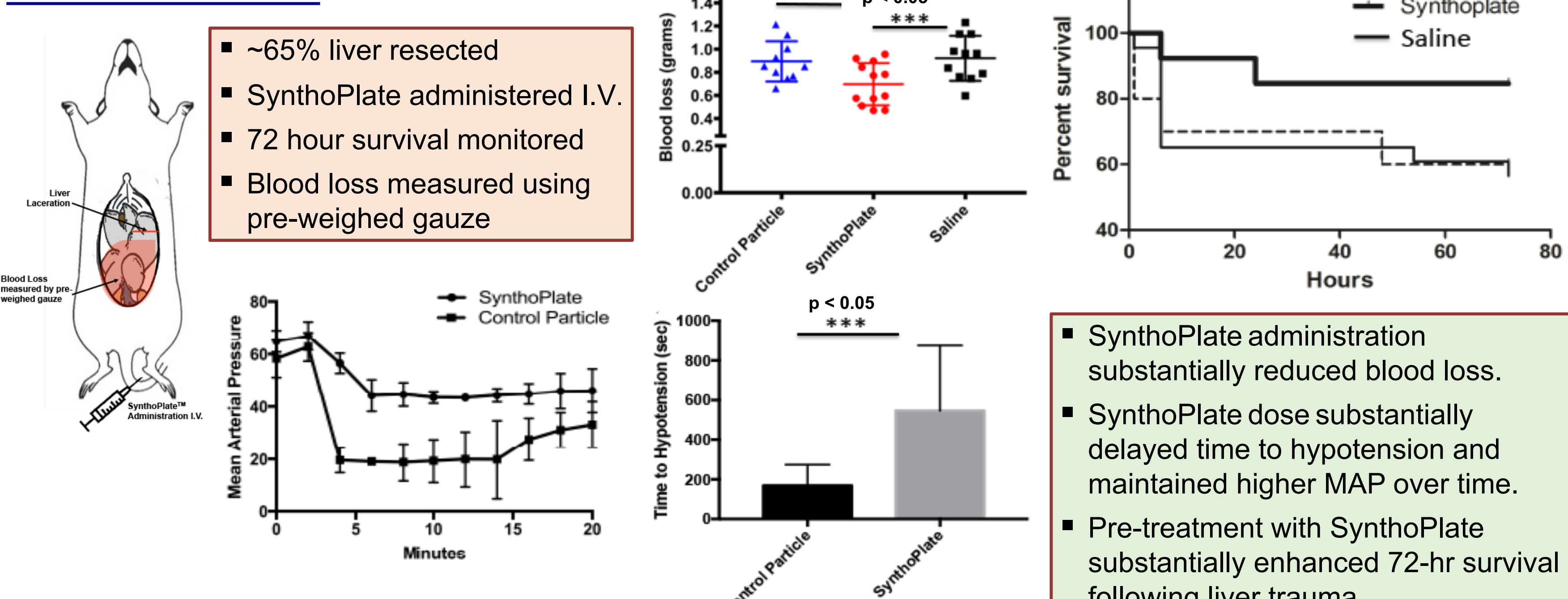


Site-directed fibrin generation by 'SynthoPlate + Platelet'

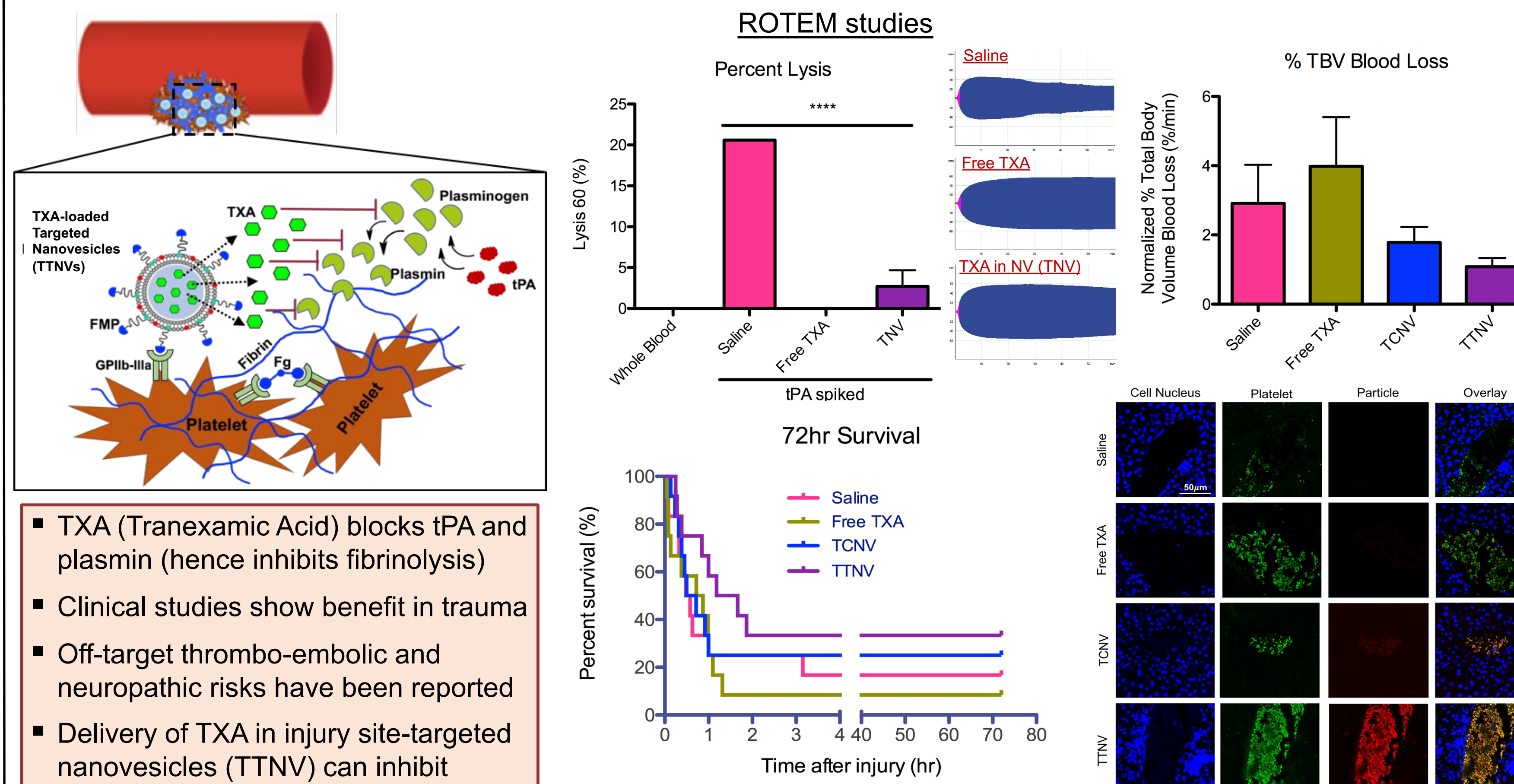


In Vivo Studies (Mouse Liver Model)

Liver trauma model



In Vivo Studies (Rat Liver Model with TXA)



- TXA (Tranexamic Acid) blocks tPA and plasmin (hence inhibits fibrinolysis)
- Clinical studies show benefit in trauma
- Off-target thrombo-embolic and neuropathic risks have been reported
- Delivery of TXA in injury site-targeted nanovesicles (TTNV) can inhibit hyperfibrinolysis site-selectively while avoiding off-target effects.
- Studies were conducted in a **rat liver hemorrhage injury model** where 30% of the rat liver was resected and the animals were administered with 0.5 ml injection volume of saline, free TXA, TCNVs or TTNVs

- TXA can be efficiently encapsulated in injury site-targeted nanovesicles (TTNV Encapsulation Efficiency ~ 64%).
- Ex vivo ROTEM analysis with tPA-spiked rat blood showed significant ability of TTNVs to stabilize clots and resist lysis at levels comparable to free TXA.
- Post-injury administration of TTNVs in rats substantially reduced blood loss, and increased 1-hr and 72-hr survival, compared to those treated with saline only or TXA-loaded undecorated control particles (TCNVs).

Summary and Future Studies

- SynthoPlate™** is a lipid-peptide based **Synthetic Platelet nanotechnology** that can mimic platelet-mediated mechanisms of hemostasis site-selectively.
- Pre-clinical studies in multiple trauma models indicate that SynthoPlate™ can provide significant benefit in hemorrhage control in pre-hospital battlefield scenarios where availability of natural platelet products may be highly limited.
- SynthoPlate™ can be further used as a platform vehicle for injury site-targeted delivery of therapeutic agents like TXA to augment hemostatic benefit in trauma.

References: Hickman DA, et al. *Sci. Rep.* 2018; 18 (1): 3118. doi: 10.1038/s41598-018-21384-z.
Dyer M, et al. *J. Trauma Acute Care Surg.* 2018; 84 (6): 917-923.

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Intravenous synthetic platelet (SynthoPlate) nanoconstructs reduce bleeding and improve 'golden hour' survival in a porcine model of traumatic arterial hemorrhage

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Traumatic non-compressible hemorrhage is a leading cause of civilian and military mortality and its treatment requires massive transfusion of blood components, especially platelets. However, in austere civilian and battlefield locations, access to platelets is highly challenging due to limited supply and portability, high risk of bacterial contamination and short shelf-life. To resolve this, we have developed an I.V.-administrable 'synthetic platelet' nanoconstruct (SynthoPlate), that can mimic and amplify body's natural hemostatic mechanisms specifically at the bleeding site while maintaining systemic safety. Previously we have reported the detailed biochemical and hemostatic characterization of SynthoPlate in a non-trauma tail-bleeding model in mice. Building on this, here we sought to evaluate the hemostatic ability of SynthoPlate in emergency administration within the 'golden hour' following traumatic hemorrhagic injury in the femoral artery, in a pig model. We first characterized the storage stability and post-sterilization biofunctionality of SynthoPlate *in vitro*. The nanoconstructs were then I.V.-administered to pigs and their systemic safety and biodistribution were characterized. Subsequently we demonstrated that, following femoral artery injury, bolus administration of SynthoPlate could reduce blood loss, stabilize blood pressure and significantly improve survival. Our results indicate substantial promise of SynthoPlate as a viable platelet surrogate for emergency management of traumatic bleeding.

In remote civilian locations and austere battlefield conditions, uncontrolled traumatic hemorrhage remains one of the leading causes of mortality^{1–7}. In such scenarios, transfusion of whole blood or blood components (RBCs, plasma and platelets) can significantly improve survival^{8–10}. Extensive trauma resuscitation studies have indicated substantial benefits of early platelet transfusion to treat hemorrhagic shock and enhance survival possibilities^{8–13}, however, the limited availability and portability, need for blood type matching, special storage requirements, high risks of bacterial contamination at room temperature and very short shelf-life (3–5 days at room temperature) of platelets, present severe logistical challenges for their applicability in pre-hospital scenarios^{14–24}. These issues have led to robust research efforts in improving the storage stability, portability and availability of platelets, e.g.

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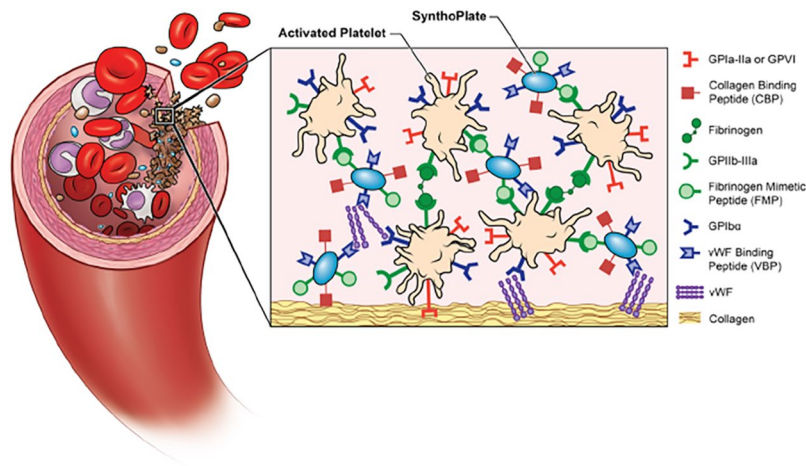


Figure 1. Schematic representation of SynthoPlate design and mechanism, showing nanoconstructs heteromultivalently decorated with VBP, CBP and FMP motifs to render platelet-mimetic interactions with vWF, collagen and active platelet integrin GPIIb-IIIa respectively, and thus amplify platelet-mediated primary hemostatic mechanisms at the injury site.

via lyophilization and cold-storage, as well as, developing pathogen reduction technologies to reduce contamination^{25–32}. However, these approaches have only marginally improved the pre-hospital applicability of platelets, and the research continues to find alternative platelet-based options, e.g. Isolated Platelet Membrane (IPM Cyplex) and Thrombosoma^{33–35}. These technologies involve isolation, purification, sterilization, temperature-stabilization and lyophilization of outdated platelets or platelet-derived membrane to utilize their residual hemostatic bioactivity. However, such processing can lead to significant ‘loss-in-function’ of hemostatically relevant glycoprotein on the platelet membrane surface^{36,37}, which can potentially lead to batch-to-batch functional inconsistencies. Due to such challenges with natural platelet based products, a parallel area of research has focused on the development of synthetic platelet surrogates^{38–40}. The design requirements for such platelet surrogates are: (i) large scale *in vitro* manufacturability, (ii) sterilizability without compromising biofunctional properties, (iii) long-term storage as suspension or lyophilized powder, (iv) easy portability, (v) intravenously administrable ‘on demand’ in pre-hospital scenarios, (vi) post-administration circulation safety without systemic risks, (vii) ability to mimic and amplify endogenous hemostatic mechanisms selectively at the bleeding site, and (viii) biodegradation and safe elimination from the body. Also, such synthetic platelet surrogates can potentially avoid the need for blood type matching and can be used more universally, since they would not bear blood antigens.

As comprehensively reviewed elsewhere^{39–41} and summarily discussed later in this article, several ‘synthetic platelet’ designs have been reported in the past that primarily mimic platelet’s aggregatory capability only via surface-decoration of polymeric, lipidic or albumin particles with fibrinogen (Fg) or Fg-relevant peptides. While these designs have shown some promise as intravenous hemostats, the majority of them have only been reportedly evaluated in small animal bleeding models and only in a pre-treatment framework, i.e. particle administration before bleeding injury. No synthetic platelet design has yet been reported regarding evaluation in a large animal model, that is also in an emergency/rescue framework (i.e. particle administration after bleeding injury) in traumatic bleeding. We have developed a synthetic platelet surrogate, SynthoPlate, by leveraging a clinically relevant biocompatible liposomal platform that is heteromultivalently surface-decorated with three types of peptides: von Willebrand Factor (vWF)-binding peptide (VBP) and collagen-binding peptide (CBP) to mimic platelet’s bleeding site-selective *adhesion* mechanisms and active platelet integrin GPIIb-IIIa-binding fibrinogen-mimetic peptide (FMP) to mimic activated platelet *aggregation* mechanism (Fig. 1)^{42–44}. This integrative biomimetic approach to combine platelets’ *adhesion* and *aggregation* functions on a single particle platform is highly unique and can also be adapted to other particle platforms beyond the SynthoPlate’s liposomal platform⁴⁵. The chemical components and manufacturing details for SynthoPlate are provided in the Methods section and the relevant chemical synthesis schemes are shown in Supplementary Figure S.1. We have previously reported the detailed biofunctional and mechanistic characterization of SynthoPlate nanoconstructs⁴⁶ and have also demonstrated that the heteromultivalent integration of platelet-inspired *adhesion* and *aggregation* mechanisms on the same particle platform results in higher hemostatic capability compared to designs bearing ‘*adhesion* only’ or ‘*aggregation* only’ functions^{43,45}. We further demonstrated that prophylactic administration of SynthoPlate in normal as well as thrombocytopenic mice could augment hemostatic capability in a non-trauma bleeding model (bleeding time reduction in murine tail transection)^{43,45}. Building on these studies, here we sought to investigate the translational promise and hemostatic capability of SynthoPlate in emergency management of traumatic exsanguinating hemorrhage in a pilot scale study in large animal (pig) model. For this, we first characterized the effect of long-term storage (in saline) on SynthoPlate stability, as well as, the effect of sterilization on SynthoPlate stability and biofunction *in vitro*. Next, for *in vivo* studies, we adapted a femoral artery uncontrolled hemorrhage model in pigs, that has been utilized for evaluation of hemostatic dressings^{47,48}. In traumatic exsanguinating hemorrhage, significant number of patients succumb within the first 1–2 hours, and hemostatic intervention within

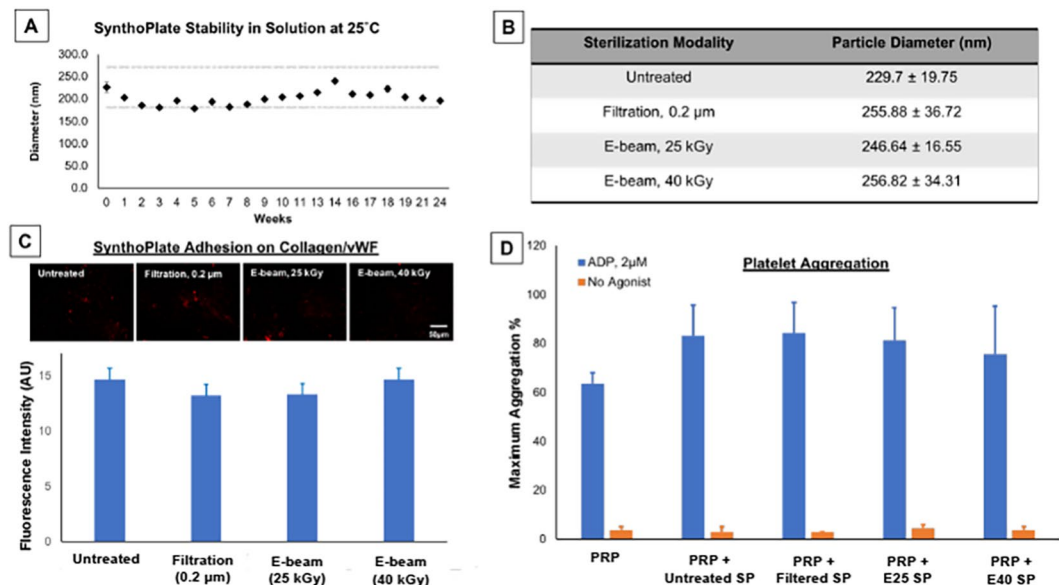


Figure 2. [A] SynthoPlate diameter (size distribution) analysis over a 6-month period demonstrated that the particles retained their size within 20% of their starting diameter when stored in a 0.9% NaCl solution at 25 °C, indicating long term stability; [B] After sterilization with filtration or E-beam, SynthoPlate showed minimal alteration in size (diameter) compared to fresh-made (unsterilized) samples, indicating that the sterilization did not affect particle stability; [C] Representative fluorescent images and quantitative analysis of surface-averaged fluorescence intensity of adhered SynthoPlate showing that sterilized SynthoPlate (red) maintained its ability to adhere to 'collagen + vWF'-coated surface at levels similar to fresh-made (unsterilized) SynthoPlate, indicating that sterilization did not affect the biofunctional properties of VBP and CBP; [D] Aggregometry analysis showed that neither fresh-made (unsterilized) or sterilized SynthoPlate induced spontaneous aggregation of platelets without agonist (w/o ADP), but both unsterilized and sterilized SynthoPlate markedly increased platelet aggregation in the presence of ADP, indicating that sterilization did not affect the biofunctional property of FMP on SynthoPlate and also SynthoPlate did not have any thrombotic risk towards resting platelets.

this window (known as the 'golden hour') can significantly improve survival^{10,49,50}. Our model results in the loss of ~1 liter of blood within 15–20 minutes, resulting in 75% mortality within first 45 min. Therefore, we utilized this model to test the SynthoPlate technology I.V.-administered immediately following injury, and the animals were observed for up to 2 hours to monitor blood loss, blood pressure, heart rate and survival. The systemic safety and biodistribution of SynthoPlate was also characterized. Administrations of saline or of unmodified (control) particles were used as comparison groups.

Results

SynthoPlate stability in storage. Liposomal nanoconstruct instability is reflected by large alterations in size (diameter) stemming from particle disassembly, aggregation and clustering. Hence Dynamic Light Scattering (DLS) analysis was used to monitor SynthoPlate stability in saline suspension over a 6-month period. Fresh-made liposome batches show a size (diameter) variability of $\pm 15\%$ in laboratory scale manufacture. As shown in Fig. 2A, our analysis showed that when stored as a suspension in 0.9% NaCl at room temperature, SynthoPlate particles showed additional size variability over time of about $\pm 5\%$, thus maintaining their size within an overall variability of $\pm 20\%$ of their starting diameter for up to 6 months. Representative DLS data at Month 1, Month 3 and Month 6 are shown in Supplementary Figure S.2. Also, by visual inspection, there were no signs of particle aggregation or settlement out of solution. This suggests that SynthoPlate is amenable to long-term storage under these conditions and indicates their potential for pre-hospital availability as a small volume suspension without compromising stability.

SynthoPlate sterilization and its effects. SynthoPlate sterilization studies by filtration (0.2 µm filter) as well as E-beam exposure (25 and 40 kGy) were carried out with 6 different challenge organisms, namely *Staphylococcus aureus*, *Kocuria rhizophila*, *Clostridium sporogenes*, *Bacillus subtilis spizizenii*, *Candida albicans* and *Aspergillus brasiliensis*, as described in the Methods section. For all organisms tested, SynthoPlate itself did not have any bacteriostatic or fungistatic activity, and this data is shown in Supplementary Figure S.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 (1×10^5 moles lipid per ml) was measured to be 3.5–5 EU/ml as per a standard chromogenic limulus amoebocyte lysate (LAL) assay (see Methods for details). When diluted in sterile saline to the *in vivo* dosing level of 5×10^4 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* usage⁵¹. Therefore, our SynthoPlate preparation can be considered safe

for *in vivo* usage. Furthermore, after sterilization by either filtration or E-beam, SynthoPlate particles showed statistically insignificant variability in size compared to fresh-made (unsterilized) SynthoPlate (Fig. 2B), suggesting that the sterilization methods did not affect particle integrity, size and stability.

To test whether sterilization affects the platelet-mimetic adhesive function of CBP and VBP moieties on SynthoPlate, Rhodamine B-labeled (red fluorescent) SynthoPlate particles in saline suspension, unsterilized or sterilized, were incubated on 'vWF + collagen' coated glass coverslips in a 12-well plate with stirring at 120 rpm, following which the coverslips were washed and imaged (experimental details provided in Methods section). 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 Fig. 2C. 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. To test whether sterilization affects the ability of FMP moieties on SynthoPlate to interact with active platelet integrin GPIIb-IIIa for amplifying platelet aggregation, lumi-aggregometry assays were performed using platelet rich plasma (PRP) isolated from citrated human whole blood. These assays were performed with 100% PRP or PRP diluted 50% (v/v) with platelet poor plasma (PPP) supplemented with fresh made (unsterilized) or sterilized (filtration and E-beam) SynthoPlate particles, with or without addition of platelet agonist ADP (experimental details in Methods section). In these 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 Fig. 2D. Representative raw data from aggregometry studies are shown in Supplementary Figure S.4. 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 future translation through large-scale manufacturing and sterilization without compromising its hemostatic function.

SynthoPlate cytotoxicity analysis. SynthoPlate was incubated with 3T3 human fibroblasts (see Methods for culture conditions and viability analysis) at doses relevant to the pig dose used in the current studies (0.5 nM) and double the dose (1 nM). Cell viability was analyzed by standard MTT based metabolic assay and compared to cell cultures without SynthoPlate incubation (i.e. culture media only). As shown in Supplementary Figure S.5, the cell metabolic activity was not affected by SynthoPlate incubation (i.e. no statistical difference), indicating that there is no cytotoxicity associated with SynthoPlate at the dose (as well as double the dose) used *in vivo* in the current studies.

***In vivo* Safety and Biodistribution Studies in Pigs.** The systemic safety of SynthoPlate in pigs within the 'golden hour' (60 min) time-frame, was tested by administering the SynthoPlate dose (dosage calculation shown in Supplementary Section S.6) in un-injured pigs intravenously through the jugular vein. Administrations of saline or unmodified (control) particle dose were used as comparison. Two animals per treatment group (i.e. 6 total) were used for these studies. The effects of the treatments on vitals, blood chemistry, complement (C3 to C3a) activation, platelet function (aggregation) and blood clot viscoelastic properties (using ROTEM) were examined for the 60-minute post-administration period (experimental details in Methods section). Sixty minutes post treatment administration, pigs were euthanized and major organs (lungs, heart, liver, kidney, spleen) were harvested for histological (using H&E stain) and biodistribution (using UPLC on tissue homogenate) analysis. As shown in Fig. 3A, following administration of unmodified particles or SynthoPlate in uninjured pigs, none of the animals showed any substantial alteration in their vitals compared to saline. *Ex vivo* analysis of arterial blood collected from the pigs post treatment administration showed no significant changes in the blood analysis parameters between saline and the two particle treatment groups (Fig. 3B), indicating that the SynthoPlate dose did not affect physiological parameters of blood *in vivo*. Also, as shown in Fig. 3C, no significant alterations in the C3:C3a plasma concentration ratios were found in animals administered with unmodified particles or SynthoPlate. It is important to note here that complement ratio alterations of 4-fold or greater is reportedly considered to be a risk indicator for drastic activation of complement system and deleterious reactions like complement activation-related pseudoallergy (CARPA)^{52–54}. SynthoPlate (as well as control particles) did not show any such effect compared to saline. Therefore, altogether these results suggest that within our experimental dose and animal observation window, administration of SynthoPlate did not cause any alarming systemic risks. The biodistribution analysis of SynthoPlate (as well as unmodified particles) in uninjured pigs is shown in Fig. 3D, demonstrating that both types of particles have similar organ distribution profile during the 1-hour circulation period. Within this window, most particles of either type were found to be cleared to the liver, possibly by the reticulo-endothelial system (RES), as this mechanism is known for clearance of PEG-ylated liposomes⁵⁵ (base particle for the design of SynthoPlate nanoconstructs). Interestingly ~30% of the injected dose of particles was found to still remain in the systemic circulation (blood), suggesting that this amount can be potentially still available after the 1-hour period to keep localizing at the injury site for hemostatic action. H&E staining-based histopathological analysis of harvested organs from SynthoPlate-injected un-injured animals showed no signs of off-target thrombi in the tissue samples (Fig. 4). Combining these findings with our previous report that SynthoPlate does not activate (and aggregate) resting circulating platelets and also does not spontaneously trigger thrombin (and fibrin) generation in plasma^{43,45}, we rationalize that our SynthoPlate dose was safe in pigs, with minimal systemic pro-thrombotic, pro-coagulant and complement activation risks.

Hemostatic Efficacy Evaluation in Femoral Artery Bleeding Model. The pig femoral artery hemorrhage model is described in detail in the Methods section and an experimental schematic is shown in Fig. 5.

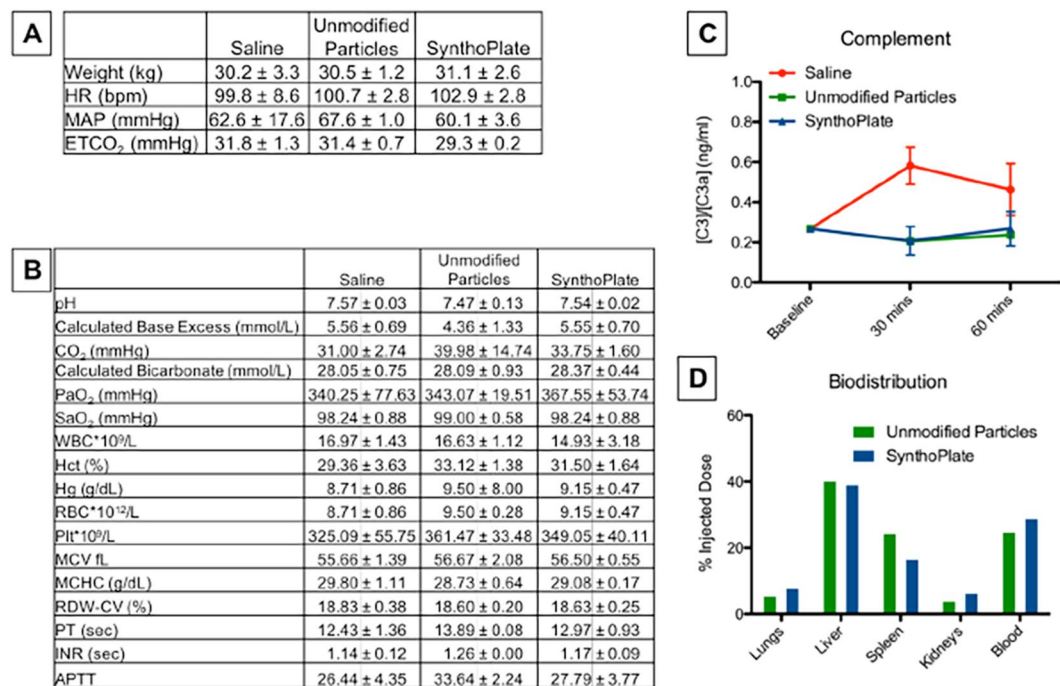


Figure 3. [A] Average vitals over the course of the experiment of uninjured pigs and [B] average blood lab values over the course of the experiment of uninjured pigs upon administration of unmodified particles or SynthoPlate, showed that there are no statistically significant differences in values compared to saline administration, indicating that SynthoPlate administration has no detrimental physiological effect; [C] Analysis of C3:C3a plasma concentration ratios in blood drawn from pigs administered with unmodified particles or SynthoPlate showed no statistically significant alterations of this ratio compared to saline administration groups, indicating that in the dose and administration protocol used, SynthoPlate did not have complement activation (and CARPA) risk; [D] Biodistribution analysis from harvested organs indicated a similar distribution profile for unmodified particles and SynthoPlate, with a majority of particles cleared through the liver.

Briefly, an acute hemorrhagic injury (near transection) was caused in the femoral artery with a 3.5 mm arterial punch and 1 minute following injury, saline, unmodified (control) particles or SynthoPlate particles were administered as a bolus dose through the jugular vein. Four animals per treatment group (i.e. 12 total) were used for these studies. Vitals, blood loss, hemodynamic parameters and survival were monitored in real time for 60 minutes (golden hour) and an additional 60 minutes (i.e. total observation for 120 min). During these observations, blood was also drawn through a carotid angi catheter to run platelet aggregometry and ROTEM analysis *ex vivo*. At the end of experiments, pigs were euthanized (unless they had already succumbed earlier from their injury), and organs and clot were harvested for histological and fluorescence-based distribution analysis (experimental details in Methods section). As shown in Fig. 6A, bolus administration of SynthoPlate immediately following femoral artery injury, resulted in substantial reduction of the blood loss rate over time. It is important to note here that the condition of the saline-treated pigs rapidly declined after injury as indicated by their mean arterial pressure (Fig. 6C) and 75% of them died before 60 minutes (Fig. 6D); therefore, blood loss rate for this group appears to approach zero after this time point (Fig. 6A). Meanwhile, pigs injected with unmodified (control) particle dose continued to undergo low rates of bleeding due to a delay in destabilization of mean arterial pressure (MAP) (Fig. 6B), but ultimately one of them succumbed while actively bleeding around 60 minutes and two other died around 100 minutes (Fig. 6D), though bleeding stopped in both of them at around 60 minutes possibly due to hemorrhage-induced hypotension (Fig. 6A and C). SynthoPlate-treated pigs exhibited significantly lower blood loss rates than comparison groups during the first 30 min and achieved full hemostasis (at 15 min, 18 min, 30 min and 80 min for the 4 pigs respectively) such that the blood loss rate for this group also became zero by the 80-minute time point (Fig. 6A). Furthermore, these animals exhibited stabilized MAP (Fig. 6C) throughout the 120-minute period. Total blood loss for SynthoPlate-treated pigs (normalized to body weight and time survived) was also found to be significantly lower to comparison treatment (Fig. 6B). As shown in Fig. 6D, SynthoPlate-treated animals showed a significantly improved survival, with 100% surviving the golden hour (first 60 minutes) and 75% maintaining survival at the end of 120 minutes. In comparison, 75% of the saline-treated animals died within the first 60 min and 75% of the unmodified (control) particle-treated animals progressively died during the 60–120 min period. Immunohistochemistry and fluorescence imaging analysis of harvested clots from the hemostasized femoral artery of SynthoPlate-treated animals revealed significant incorporation of SynthoPlate in the platelet-rich clot at the site of injury (yellow overlay of green platelets and red SynthoPlate), but no such red fluorescent particle presence was found at the arterial injury site for unmodified (control) particle-treated pigs (representative fluorescence images for the three treatments shown in Fig. 7). Also,

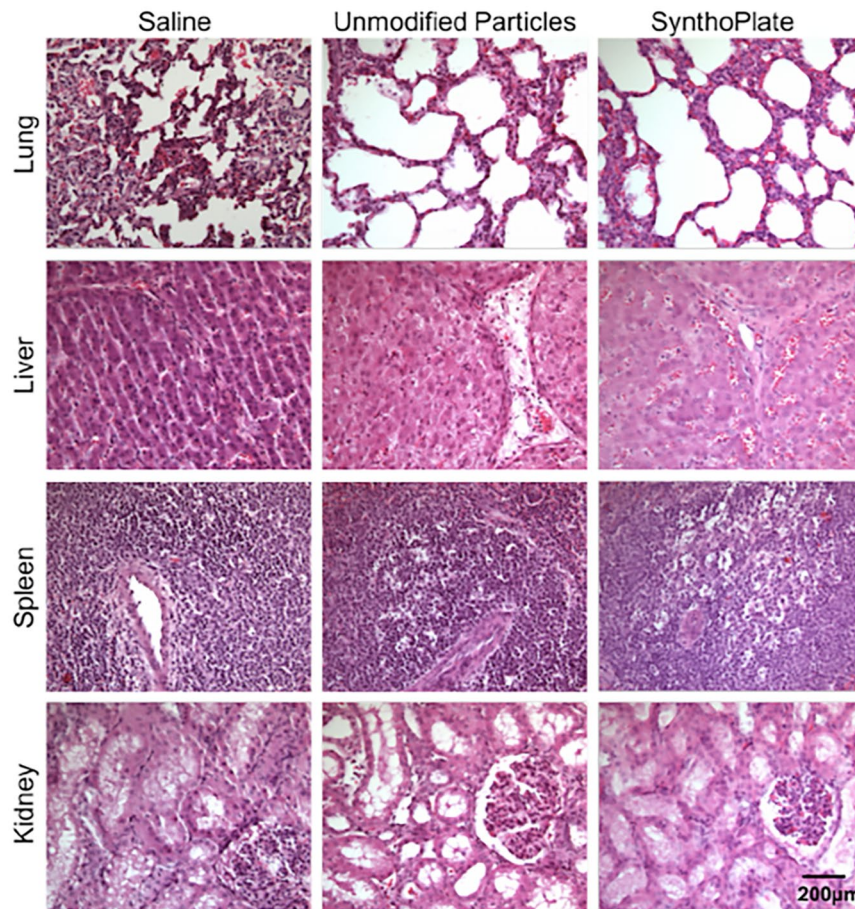


Figure 4. Representative hematoxylin and eosin (H&E) stained histology images (32 $\times$ magnification) of organ samples from pigs treated with saline, unmodified particles or SynthoPlate particles, show that SynthoPlate (as well as unmodified particles) does not cause any thrombotic risks in organs; scale bar of 200 μ m shown in bottom row kidney image is applicable to all histology images.

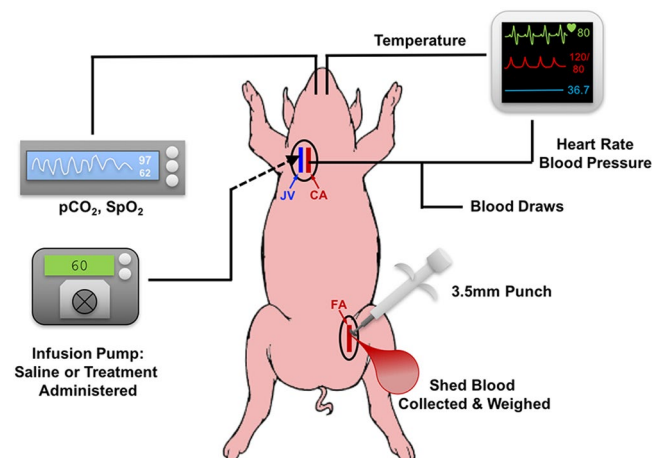


Figure 5. Schematic representation of pig femoral artery hemorrhage model setup. A CO₂ sensor was placed at the end of the endotracheal tube and mechanical ventilation was provided, EKG electrodes were placed on the pig's limbs, a pulse-oximeter probe was placed on the pig's mouth, an esophageal temperature probe was placed to measure core temperature, an angiocatheter was placed in the carotid artery to acquire invasive blood pressure and also withdraw blood samples for *ex vivo* analysis, an angiocatheter was placed in the internal jugular vein to deliver saline (or nanoparticle treatments) via an infusion pump.

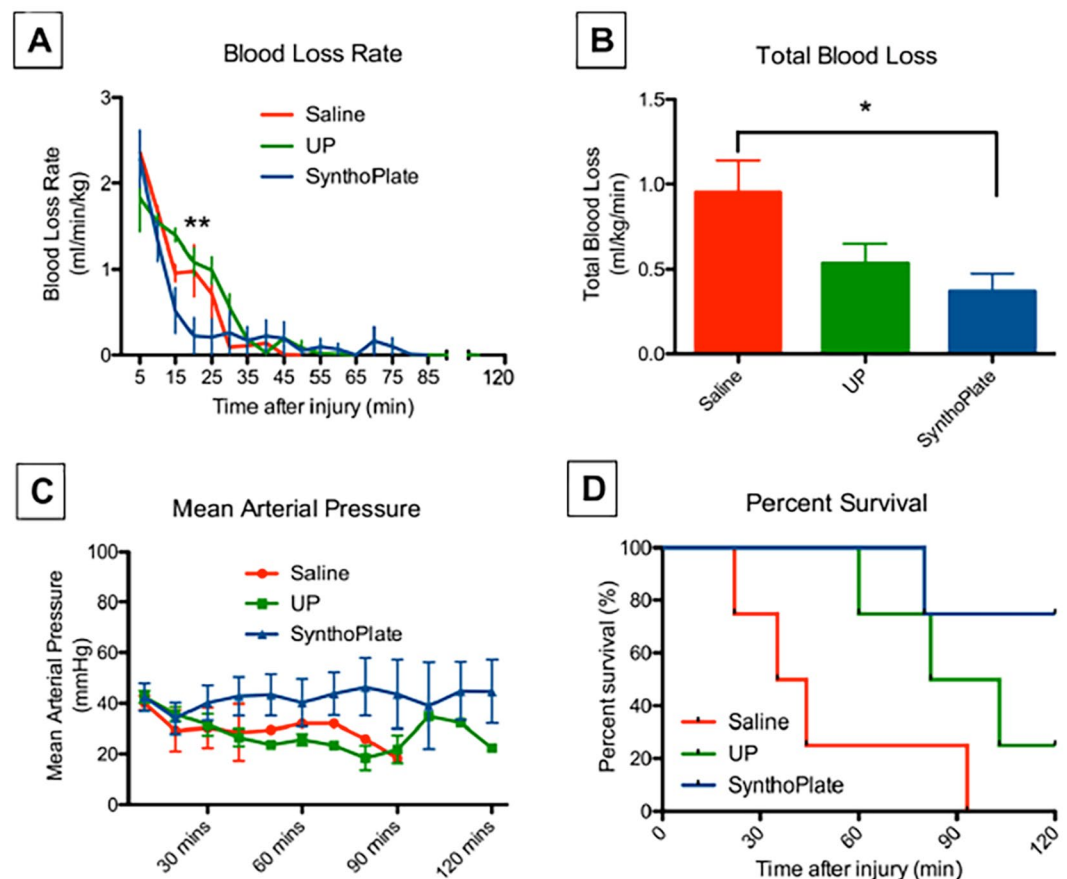


Figure 6. Hemostatic efficacy analysis in injured pigs shows that [A] pigs administered with SynthoPlate had a reduced blood loss rate (blue) compared to those treated with saline (red) and unmodified particles (UP, green), especially within first 30 min post treatment administration, where blood loss rate in SynthoPlate treated pig was significantly lower than those treated with unmodified (control) particles and saline (** $p < 0.01$); [B] SynthoPlate administration in pigs also resulted in significantly lower total blood loss compared to saline administration (* $p < 0.05$); [C] SynthoPlate administration in pigs resulted in maintenance and stabilization of a higher average mean arterial blood pressure (MAP) over time (data points shown for every 10 min); [D] SynthoPlate administration in pigs resulted in a significant enhancement of survival, with 100% surviving the first 60 min (golden hour) and 75% surviving the additional 60 min ($p < 0.05$), compared to administration of saline (25% survival by 60 min and 0% survival by ~90 min) or unmodified particles (50% survival by 90 min, 25% survival at 120 min).

similar to the safety experiments in uninjured pigs, during the golden hour (60 min) there were no drastic changes in the C3a:C3a plasma concentration for SynthoPlate-treated or unmodified particle-treated animals compared to saline-treated animals (Supplemental Figure S.7.A). Furthermore, the biodistribution of the unmodified particles and SynthoPlate (analyzed at the point of pig death or euthanasia) were similar (Supplemental Figure S.7.B). Aggregometry studies showed that pigs treated with SynthoPlate had a stable platelet aggregation profile while pigs treated with unmodified (control) particle demonstrated a trend for decreasing platelet aggregation and pigs treated with saline showed a trend for platelet hyper-aggregation (Supplemental Figure S.8.A). ROTEM analysis of blood revealed no presence of coagulopathy in SynthoPlate or saline treated animals, as indicated by clotting time (CT), maximum clot firmness (MCF), and amplitude 10 minutes after CT (A10) values from EXTEM and FIBTEM assays that all fall within 'normal' reference range, based on criteria established in previous studies (Supplemental Figure S.8.B)⁵⁶. Unmodified particle treated animals did begin to show signs of coagulopathy at the 120-minute time point as indicated by MCF and A10 EXTEM and FIBTEM values that are below the reference range (Supplemental Figure S.8.B). Altogether, the data suggest that SynthoPlate has the ability to reduce blood loss rate as well as total blood loss (Fig. 6A and B), stabilize MAP (Fig. 6C) and increase overall survival (Fig. 6D) after traumatic hemorrhage injury, due to the ability of SynthoPlate to enhance active platelet recruitment and aggregation to form hemostatic clot at the injury site and prevent coagulopathy.

Discussion

Traumatic exsanguinating hemorrhage continues to be a major cause of military and civilian fatalities in pre-hospital settings, where blood components have limited availability^{15,24}. In such scenarios, volume resuscitation with saline or plasma expanders shows only limited benefit⁵⁷. Currently, studies are being directed at

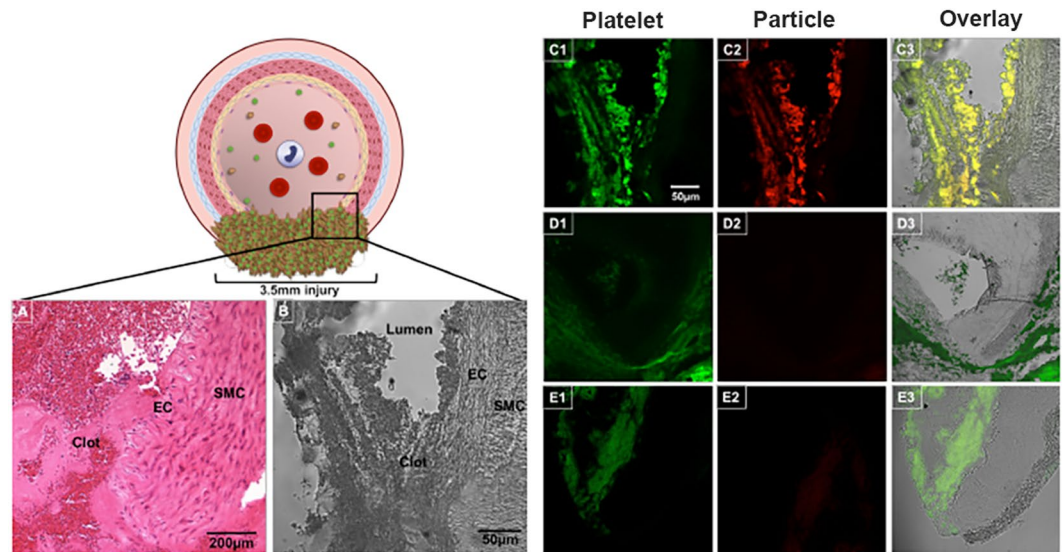


Figure 7. Schematic representation and representative images of hemostasized injury site in the femoral artery of pigs treated with SynthoPlate or saline or control particles: [A] Representative hematoxylin and eosin (H&E) stained histology image (32× magnification) and [B] representative bright field image (10× magnification) of the site of injury (transected artery) with the injured vessel components (EC: endothelial cell, SMC: smooth muscle cell) and hemostatic clot in view for injured pig treated with SynthoPlate; [C1] representative fluorescence image (10× magnification) of FITC-anti-CD42b (green fluorescence) stained platelets, [C2] Rhodamine B-labeled (red fluorescence) SynthoPlate particles and [C3] overlay of C1 and C2, in the same field of view as the brightfield image B demonstrating that red fluorescent SynthoPlate is co-localized with green fluorescent platelets at the site of injury within the hemostatic clot; [D1, D2 and D3] are similar representative images for injured pig treated with saline, and [E1, E2 and E3] are similar representative images for injured pig treated with unmodified (control) particles showing that some platelets indeed localize at the injury site to promote hemostasis but presence of control particle is minimal at the site and therefore control particles do not have the ability to augment hemostasis; scale bar of 50 µm shown in C1 is applicable to all fluorescent images.

improving the survivability in such conditions by anti-fibrinolytic agents like tranexamic acid (TXA) as well as by improving blood component storage, portability and availability through cold-storage and lyophilization^{27,58–63}. Time is critical in mitigation of exsanguinating hemorrhage since significant mortalities occur within 1–2 hours of injury, and hemorrhage control within this time can significantly improve survival^{49,50}. Therefore, intravenously transfusable synthetic RBC and platelet surrogates that can be administered early at point-of-injury can have tremendous benefit in emergency mitigation of hemorrhagic shock^{39–41}.

In the area of synthetic platelet surrogates, past approaches have focused mostly on mimicking only the *aggregatory* mechanism of platelets by decorating micro- and nanoparticles with fibrinogen or fibrinogen-relevant peptides, essentially creating a ‘super-fibrinogen’ construct^{39,41}. However, platelet’s native hemostatic action is a co-operative concert of injury site-selective *adhesion* and *aggregation* mechanisms⁶⁴. Hence in our design, we hypothesized that integrating these two mechanisms on a single particle platform will allow a superior mimicry of platelet’s hemostatic mechanisms selectively at the bleeding site while minimizing off-target effects. We previously tested this hypothesis via heteromultivalent decoration of liposomal as well as albumin-based nanoparticles with VBP, CBP and FMP motifs and successfully validated the superior hemostatic efficacy of this integrative design compared to ‘*adhesion* only’ and ‘*aggregation* only’ designs^{43,45}. SynthoPlate is a refined liposome-based version stemming from this integrative design⁴⁴, and we have recently demonstrated its hemostatic efficacy in a non-trauma bleeding model in severely thrombocytopenic mice⁴⁶. Building on this, here we investigated the translational promise of the SynthoPlate technology *in vitro* (sterilization effects and storage stability) and *in vivo* (large animal hemorrhage model). Our studies demonstrate that sterilization of SynthoPlate by either filtration or E-beam does not affect the product’s stability and platelet-mimetic biofunctionalities. Our studies also indicate that long-term storage in suspension does not affect SynthoPlate stability. Altogether, these data suggest that sterile suspensions of SynthoPlate can potentially allow long-term storage and availability for hemorrhage mitigation in pre-hospital settings.

Our *in vitro* and *in vivo* studies clearly demonstrate that the SynthoPlate dose did not have any cytotoxicity and systemic risks in the experimental conditions tested. Future studies will be directed at assessing dose escalation based pharmacology and toxicology analysis, as well as, evaluating the potential of multiple doses. Following arterial injury and bolus administration of SynthoPlate (or control) particles, our results show that compared to saline (0% survival), SynthoPlate treatment renders 100% survival during the ‘golden hour’. Interestingly, unmodified (control) particles showed ~50% survival during this first hour, however, survival for this treatment group fell to 25% during the second hour of the experimental window. This can be attributed to the fact that the unmodified particles seemed to transiently improve MAP, possibly due the colloidal nature of injected liposome suspension transiently increasing blood colloidal osmotic pressure (BCOP). However, since unmodified particles

were incapable of biofunctionally enhancing hemostasis, these animals continued to bleed at a higher rate and succumbed. In contrast, a combination of hemodynamic stabilization and hemostatic enhancement significantly improved the survival of the SynthoPlate-treated pigs within and beyond the ‘golden hour’. It is to be noted that, because of MAP stabilization and longer survival time, the blood loss rate data (Fig. 6A) apparently suggests that the SynthoPlate-treated animals continued to bleed (albeit at a very low rate) up to and beyond the ‘golden hour’. However, this is a ‘survival bias’, since majority of injured animals treated with saline or unmodified particles essentially could not recover from being rapidly hypotensive, which resulted in ‘apparent reduction’ of blood loss rate and ultimately death. Interestingly, several ‘externally applicable’ hemostatic dressings were recently evaluated in a pig femoral artery hemorrhage model, that showed overall survival to be 50–60% in the 1-hour period⁴⁷. Considering that in our studies, administering only a single bolus intravenous dose of SynthoPlate, with no additional dressings, resulted in 100% survival of the pigs for 60 minutes (and 75% for 120 min), we rationalize that the superior hemostatic capability of SynthoPlate can be potentially combined with externally applied hemostatic dressings, to further improve the hemostatic outcomes in pre-hospital scenarios. We also note that in the current studies the treatments were administered within 1 minute after injury and we have not yet evaluated the ‘administration time window’ for SynthoPlate following injury to determine at what point of delayed administration its hemostatic benefit may fail to increase survival. Our future studies will further evaluate these possibilities in porcine trauma models. Our current findings therefore strongly demonstrate the potential of SynthoPlate as a viable synthetic platelet surrogate for point-of-injury management of traumatic hemorrhagic.

Materials and Methods

Materials. The lipid components, namely, 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-1000] (DSPE-mPEG₁₀₀₀), 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG₂₀₀₀-Mal) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[azido(polyethylene glycol)-2000] (DSPE-PEG₂₀₀₀-Azide) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Rhodamine B-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DHPE-RhB) was purchased from Invitrogen (Carlsbad, CA, USA). The peptides CTRYLRHPQSWVHQI (VBP), C[GPO]₇ (CBP) and cyclo-{Pra}CNPRGD{Tyr(OEt)} RC (FMP) were custom-synthesized by Genscript (Piscataway, NJ, USA). VBP and CBP were conjugated to DSPE-PEG₂₀₀₀-Mal via thiol-maleimide coupling⁶⁵ and FMP was conjugated to DSPE-PEG₂₀₀₀-azide via copper-catalyzed alkyne-azide cycloaddition (CuAAC)⁶⁶ (schematics shown in Supplementary Figure S.1), conjugates were purified by dialysis and characterized by MALDI-TOF mass spectrometry. Sterile normal saline solution (0.9% NaCl) was purchased from Baxter (Deerfield, IL, USA). Cholesterol, rat tail type I collagen, copper(II) sulfate (CuSO₄), Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), sodium ascorbate, decaethylene glycol monododecyl (C₁₂E₁₀) and the Mammalian Cell Lysis Kit were from Sigma-Aldrich (Saint Louis, MO, USA). Ethylenediamine Tetraacetic Acid (EDTA), cellulose dialysis tubing (MWCO 2k and 3.5k), phosphate buffered saline (PBS), chloroform, methanol, LAL Chromogenic Endotoxin Quantification kit, Penicillin-Streptomycin (PS), DMEM without phenol red and Vybrant MTT Cell Proliferation Assay Kit were purchased from Fisher Scientific (Pittsburgh, PA, USA). Citrated human whole blood was drawn from healthy, aspirin-refraining donors via venipuncture according to IRB approved protocols. Adenosine di-phosphate (ADP) and soluble calf skin type I collagen were from Bio/Data Corporation (Horsham, PA, USA), and human vWF (FVIII-free) was from Hematologic Technologies (Essex Junction, VT, USA). For rotational thromboelastometry (ROTEM) analysis, all reagents were purchased from ROTEM (Munich, Germany). For *in vivo* studies on pigs, Yorkshire Farm Pigs were purchased from Shoup Investments Ltd. Telazol, isoflurane and pentobarbital were obtained from Patterson Veterinary (Greely, CO, USA). The Beadbug Microtube Homogenizer was purchased from Benchmark Scientific (Edison, NJ, USA). The Guinea Pig Complement C3 ELISA kit and Human Complement C3a des Arg ELISA kit were from Abcam (Cambridge, MA, USA). Mouse-derived embryonic fibroblast cell line 3T3 was provided graciously by the laboratory of Eben Alsberg, Case Western Reserve University. High glucose Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were from American Type Culture Collection (Manassas, VA, USA).

Manufacture of SynthoPlate. SynthoPlate was manufactured using ‘film rehydration and extrusion’ technique as described previously^{42,44}. Briefly, DSPC, cholesterol, DSPE-PEG₂₀₀₀-VBP, DSPE-PEG₂₀₀₀-CBP, DSPE-PEG₂₀₀₀-FMP and DSPE-mPEG₁₀₀₀ were homogeneously mixed at 0.475, 0.45, 0.0125, 0.0125, 0.025 and 0.025 mole fractions, respectively, in 1:1 chloroform:methanol. Solvent was removed via rotary evaporation, and the thin lipid film was rehydrated with normal saline solution (0.9% NaCl) at a concentration of 1×10^5 moles lipid per mL. This lipid suspension was subjected to 10 freeze/thaw cycles and subsequent extrusion through 200 nm pore diameter polycarbonate membrane 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 vesicles were ~200 nm in diameter.

Long term Storage-in-Suspension Studies. In order to evaluate the stability of SynthoPlate under storage, particles were manufactured as described above, packaged into polypropylene tubes, purged with nitrogen gas and stored for up to 6 months at room temperature (~25 °C). Particle size distribution was measured weekly via Dynamic Light Scattering (DLS). This data was considered as reflective of particle stability over time, since large variations in size would be indicative of particle instability.

Studies on SynthoPlate sterilization and its biofunctional effects. SynthoPlate samples were evaluated at Lexamed (Toledo, OH, USA) for sterilization via filtration and E-beam exposure. First, it was necessary to validate the sterilization methods by demonstrating that SynthoPlate itself does not inhibit the growth of

common 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-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 limulus amoebocyte lysate (LAL) assay. Briefly, nanoparticle samples were incubated with 0.5% (v/v) of 1.5% C₁₂E₁₀ 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 endotoxin⁶⁷. Endotoxin burden in the detergent-treated SynthoPlate samples was quantified using the LAL assay.

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 as adapted from our previous reports^{43,46}. 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 200 µl 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, $\lambda_{\text{ex}} = 560 \text{ nm}$, $\lambda_{\text{em}} = 580 \text{ nm}$). 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 (UHMC, IRB Number 12–16–06). Informed consent was obtained from all subjects as per this IRB-approved guideline. The whole blood was centrifuged at $150 \times g$ for 15 minutes to obtain platelet rich plasma (PRP). Some of the PRP was further centrifuged at $2500 \times g$ 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 225 µl 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 $5 \times 10^{10}/\text{mL}$, with or without adding agonist ($2 \times 10^{-5} \text{ M}$ ADP). These adhesion and aggregation results of SynthoPlate post-sterilization were compared to that for fresh-made (unsterilized) SynthoPlate.

In vitro cytotoxicity assay with SynthoPlate. 3T3 cells were grown in DMEM supplemented with 10% FBS and 1% PS. Cultures were maintained in a humidified atmosphere of 5% CO₂ at 37 °C. 3T3 cells were seeded into 96 well plates at 5000 cells/well. Within 24 hours, media was removed and seeded wells were treated with media containing SynthoPlate particles versus media without the particles. SynthoPlate particles were applied at 0.5 nM and 1 nM concentrations, which correspond to the dose administered to pigs and double the dose administered to the pigs respectively. All treatments were suspended in phenol red free DMEM and $n = 8$ was maintained for all test groups. The treated cells were incubated at normal culture conditions for 24 hours. After 24 hours, a standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess metabolic viability of the cells. In this assay, metabolic activity (i.e. cell viability) reduces the MTT dye to insoluble formazan which has a purple color, and the intensity of this can be assessed by colorimetric (i.e. UV-Visible spectrometry) technique to reflect cell viability. For this, post incubation with or without SynthoPlate, media was removed and fresh phenol red free media and MTT stock solution were applied to all wells. The cells were incubated at normal culture conditions for 4 hours. After 4 hours, a solution of SDS-HCl was mixed into all wells, and incubated for a final period of 4 hours. Finally, the formazan intensity (i.e. cell metabolic viability) was quantified with UV-visible spectrometry, measuring absorbance at 570 nm.

In vivo Safety and Biodistribution Studies in Pigs. 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 (IACUC, Protocol Number 2015-0135). 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 CO₂ sensor was placed at the end of the endotracheal tube. Mechanical ventilation was provided to keep end-tidal CO₂ 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 angi catheter was placed in the carotid artery to acquire invasive blood pressure and withdraw blood samples for *ex vivo* testing. Another angi catheter was placed in the internal jugular vein to deliver saline or particle treatments. The 50 ml dose of SynthoPlate (or control particles) to be administered in the pigs was 1×10^{11} particles/ml (per calculation shown in Supplementary Section S.4) followed by a 450 ml saline infusion. Invasive arterial blood pressure, CO₂, SpO₂, 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 angi catheter at baseline, 30 minutes, and 60 minutes. At the end of experiments, pigs were euthanized with an I.V. overdose of pentobarbital (0.22 ml/kg).

In order to evaluate the systemic safety of SynthoPlate in pigs within the ‘golden hour’ time-frame, without any confounding effects from injury, treatments were administered (at the bolus dose stated previously) in a pilot group of non-injured animals (total $n = 6$; saline: $n = 2$, unmodified particles: $n = 2$, SynthoPlate: $n = 2$), and the animals were observed for 1 hour. The surgeons were blinded to the administered treatments, and blinding was ensured by a researcher 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. It has been reported that nanoparticles (including liposomes) can sometimes lead to a hypersensitivity reaction and complement activation (known as complement activation-related pseudoallergy or CARPA)⁵². Therefore, the risk for such reactions was assessed *ex vivo*. 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 of saline, unmodified particles or SynthoPlate. 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 $2500 \times g$ and measurements of C3 and C3a were carried out using Guinea Pig Complement C3 ELISA kit and Human Complement C3a des Arg ELISA kit respectively (Abcam, Cambridge, MA, USA). Pig whole blood samples were also analyzed for pH, calculated base excess, 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), and, activated partial thromboplastin time (APTT) compared between blood of saline-treated, unmodified particle-treated and SynthoPlate-treated pigs. Furthermore, platelet aggregometry was conducted on PRP isolated from pig blood samples, using methods described previously. 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. Three random samples 200–350 mg were removed from each pig organ and placed into pre-weighed homogenizing tubes, and the wet weight of each organ sample was recorded. The samples were then vacuum dried and their dry weights recorded. The dry organs were homogenized with a BeadBug Microtube Homogenizer using a Mammalian Cell Lysis kit and then centrifuged (20 minutes, $12,000 \times g$) to separate the organ tissue from the supernatant. The supernatant was added to 1:1 methanol:chloroform to dis-assemble the Rhodamine B labeled lipids of SynthoPlate in the samples. These samples were analyzed with Ultra Performance Liquid Chromatography (UPLC) using a fluorescence detector ($\lambda_{\text{ex}} = 560 \text{ nm}$, $\lambda_{\text{em}} = 580 \text{ nm}$) to assess Rhodamine B fluorescence. Biodistribution of the SynthoPlate particles was determined by calculating the nanogram (ng) of particles per milliliter (ml) of supernatant utilizing an appropriate calibration curve that correlates RhB fluorescence intensity to particle concentration. Samples from each organ were also processed for histological analysis. For this, samples were embedded in paraffin and sectioned and stained with hematoxylin and eosin (H & E stain). The resultant slides were imaged on a Zeiss Axiophot microscope and images were obtained with Zeiss Axiovision camera and software.

Femoral Artery Bleeding Model in Pigs. Figure 5 shows a representative experimental set-up of the model for these studies. Pigs (total $n = 12$; Saline: $n = 4$, unmodified particles: $n = 4$, SynthoPlate: $n = 4$) were prepared as described previously. The skin at the inguinal area of the thigh was incised $\sim 10 \text{ cm}$, the femoral artery was exposed and injured (near transection) using a 3.5 mm aortic punch (Scanlan International, St Paul, Minnesota, USA). Saline or particle treatments were administered 1 minute after injury via the jugular at a rate of 50 ml/min. Blood loss from femoral artery injury was measured by carefully suctioning the shed blood without disturbing the injury site, into a container that was weighed every 30 seconds for the first 10 minutes, every minute for the next 20 minutes, and every 5 minutes thereafter until bleeding stopped due to death or hemostasis. Blood mass was correlated to blood volume by approximating blood density to be 1000 kg/m^3 . With saline administration, which is a standard volume resuscitation strategy for treating hemorrhage in pre-hospital scenarios, this injury resulted in an average of 31.5 mL/kg blood loss in 24 minutes, and showed only 25% survival within the first 60 min (i.e. within the ‘golden hour’ period). At the end of experiments, pigs were euthanized with an I.V. overdose of pentobarbital (0.22 ml/kg).

Hemostatic Efficacy Evaluation in Femoral Artery Bleeding Model. The femoral artery bleeding model was performed as described above. Induction of the injury was designated as time-point zero (0). Saline or particle (unmodified control or SynthoPlate) treatments were administered 1 minute after injury via the jugular, (50 ml particle concentration 1×10^{11} particles/ml followed by 450 ml saline) at 50 ml per minute. Blood loss was measured as described previously. Arterial blood was drawn via the carotid artery angiocatheter at baseline, and 15, 30, 60 and 120 minutes, to run *ex vivo* analysis. At the end of experiments, pigs were euthanized, major organs (lungs, heart, liver, kidney, spleen) and clot were harvested, weighed and fixed in formalin. Organ samples were prepared and analyzed for SynthoPlate (or control unmodified particle) biodistribution at time of death as described previously for uninjured animals. Sections of the femoral artery injury site from SynthoPlate-treated animals were deparaffinized and rehydrated by washing with xylene 2 times for 2 minutes, 1:1 xylene: ethanol for 3 minutes, ethanol 2 times for 3 minutes, 95% ethanol for 3 minutes, 70% ethanol for 3 minutes and finally 50% ethanol for 3 minutes. Antigen retrieval was performed by incubating in Tris-EDTA buffer in a water bath at 60°C overnight. Slides were washed, blocked in 10% serum with 1%BSA in TBS for 2 hours, incubated with the FITC-labeled (green fluorescent) CD42b antibody (for staining platelet glycoprotein GPIIb/IIIa), washed, protected

with a cover-slip and then imaged (using a Zeiss AxioObserver inverted fluorescence microscope). Brightfield, SynthoPlate fluorescence (red, Rhodamine B) and platelet fluorescence (green, FITC) were captured for the same field of view.

Statistical Analysis. Statistical analysis of aggregometry, biodistribution, blood loss data, and complement data was done using two-way ANOVA with a Bonferroni post-test. Vitals data, blood analysis data and ROTEM data were analyzed using one-way ANOVA with a Tukey post-test. Survival data was analyzed using a Log-rank test. In all analyses, significance was considered to be $p < 0.05$.

Data Availability. Majority of data generated and analyzed during this study are included in this article (and its Supplementary Information files). Additional raw data (e.g. raw data of size distribution analysis by DLS, H&E staining data of harvested organ sections from all animals, raw data from ROTEM and aggregometry analysis etc.) will be available from the corresponding author on reasonable request.

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Author Contributions

A. Sen Gupta, V. Kashyap and M. Neal directed research. E. Niedoba, C. L. Pawłowski and D. A. Hickman prepared all particles, and dynamic light scattering characterizations. D. A. Hickman, C. L. Pawłowski, N. F. Luc, U. D. S. Sekhon, J. Marks and M. Sun carried out *ex vivo* aggregometry and ROTEM studies. A. Girish carried out *in vitro* cytotoxicity studies with SynthoPlate. S. Ganjoo and S. Huang carried out *in vivo* complement studies. D. A. Hickman and C. L. Pawłowski carried out histology and immunohistochemistry studies. D. A. Hickman, C. L. Pawłowski, A. Kim, A. Shevitz, S. Ganjoo, S. Huang, E. Niedoba, N. F. Luc, U. D. S. Sekhon, J. Marks and M. Sun carried out *in vivo* pig studies. D.A. Hickman, C.L. Pawłowski, M. Dyer, M. Neal and A. Sen Gupta carried out data analysis, interpretation and wrote the paper.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-018-21384-z>.

Competing Interests: A. Sen Gupta is a co-inventor on patents related to SynthoPlate technology: US 9107845 and US 9,636,383. The SynthoPlate trademark is currently recorded with USPTO (U.S. Serial Number: 86–829,160). The other authors state that they have no conflict of interest.

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809 **An Allogeneic Fat-First Approach to Burn Reconstruction Mitigates Adhesion and Soft Tissue Deficit.**

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Introduction: Adipose and adipose-derived stem cell therapies have met success as adjunctive treatment during burn reconstruction with well described benefit in the delayed-treatment of soft-tissue deficits. While the use of allogeneic skin is well-described, adipose tissues have typically remained autologous. Allogeneic fat is not commonly used in burn care, however, in large, complex burns where autologous tissue is limited adipose may not be readily available for harvest or use. Understanding the efficacy of allogeneic tissues in this setting is critical to expand our reconstructive options. Here we describe a protocol utilizing allogeneic fat as well as examine the efficacy of this approach on burn-wound contractures, adhesions, and soft-tissue deficits.

Methods: Female, Yorkshire swine received 16, 4x4 cm full-thickness burns. After 48 hours, eschar was removed to fascia. Wounds were stratified to receive either A) No Reconstruction, B) Skin-Only, C) Fat-Only, D) Immediate-Skin, Delayed-Fat, or E) Immediate-Fat, Delayed-Skin. All fat utilized was allogeneic sourced from vendor-matched swine. At 8-weeks post-engraftment animals were sacrificed and all wounds were collected for photography, ultrasound, histology and serum studies.

Results: Use of allogeneic fat significantly improved terminal soft-tissue thickness under both immediate and delayed administration ($p < 0.05$). Immediate use of allogeneic fat significantly improved tissue mobility vs. untreated and skin graft controls ($p < 0.05$). Contracture was most significantly affected by timing of skin graft placement, however, could be further mitigated under standard delayed-fat approached with allogeneic tissue.

Conclusions: Here we demonstrate use of allogeneic fat in both traditional-delayed and a fat-first approach with significant mitigation of adhesion when applied as an initial basal layer. Both immediate and delayed allogeneic fat were sufficient to improve on soft tissue deficits.

810 **Synthetic Platelet-Mimetics with Gentamicin: Outcomes in Deep Partial-Thickness Burns**

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Introduction: Infection and prolonged inflammation in deep partial-thickness burns can lead to inadequate healing. In addition to their role in hemostasis, activated platelets also contain granules with anti-inflammatory properties which may impact wound healing. However, portability and storage of platelets remain a challenge. Synthetic platelet-mimetics (SPM) avoid these difficulties, but like natural platelets, SPM have vesicles that can transport bioactive agents, such as antibiotics. We sought to evaluate wound healing outcomes in deep partial-thickness burns treated topically with SPM.

Methods: A total of 30 circular, deep partial-thickness burns were created on the dorsum of 2 porcine models. Each wound measured 5cm in diameter and was standardized using a thermocoupled burn device. Sets of six wounds were randomized into five groups: SPM, gentamicin alone, SPM with gentamicin, a vehicle control (saline), or dry gauze (the standard of care, SOC). Additionally, two separate 5cm diameter circular areas were demarcated to serve as normal skin controls. Wounds were assessed at post-burn days 3, 7, 14, 21, 28, 60 and 90 with a variety of non-invasive imaging and punch biopsies. Treatments were re-applied at each assessment. The primary outcome was the amount of wound re-epithelialization, measured histologically, at post-burn day 28. Secondary outcomes consisted of the percentage of wound contraction, amount of superficial blood flow, and mean bacterial load.

Results: The amount of wound re-epithelialization in wounds treated with SPM was 96% whereas those treated with the SOC measured only slightly less at 92% ($p = 0.56$). The percentage of wound contraction was 39% in the SPM with gentamicin group, while in the SOC group contraction was higher at 45% ($p = 0.20$). Moreover, superficial blood flow in the SPM group was measured to be 168% of normal skin controls, while wounds in the SPM with gentamicin group were slightly lower at 160%, and in wounds treated with the SOC blood flow was 110% of normal skin controls ($p = 0.27$). The mean bacterial load in each group was measured at post-burn day 3 and notably consisted of bacterial load being the lowest in wounds treated with gentamicin alone at 17/100, meanwhile in wounds treated with the SOC or those in the SPM with gentamicin group, mean bacterial loads were significantly higher at 43/100 and 47/100, respectively ($p = 0.02$).

Conclusions: SPM applied to deep partial-thickness burns did not significantly improve measured outcomes over the

SOC. Although numerical improvements were shown in all measured outcomes, no significant differences were noted. The theoretical and observed potential for SPM to improve wound healing deserves additional evaluation in larger pre-clinical studies.

811 Statistical Analyses of Multiple Burn Wounds within an Animal

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Introduction: Porcine animal models are frequently used to study the effects of various burn wound treatments and conditions. In these studies, multiple wounds are created on the dorsum of an anesthetized pig. Wounds within an animal are correlated, and the analysis of such a design should account for this dependence. Two recent studies are used as examples to illustrate the use of random effects for animal to account for the dependence of wound measurements within each pig.

Methods: In both studies, treatments were randomized to wound location, and neither contained repeated measurements within a wound.

Study #1 investigated the effectiveness of enzymatic debridement. Forty 3-cm diameter partial-thickness burn wounds were created on the dorsum of 5 anesthetized pigs, arranged in 4 rows and 10 columns. Dorsal quadrants with 10 wounds each were created by combinations of two conditions: wounds that were wet or dry and wounds that were treated for either 72 or 96 hours. Debridement Efficiency Scores were calculated for each wound and evaluated for differences by inferential statistical comparisons between experimental treatments and conditions. The multilevel statistical model contained Treatment Group (5 levels), Treatment Time (2 levels: 72 hour and 96 hour), and Wet/Dry Condition (2 levels) as fixed effects, which were crossed to create a 3-way block, resulting in 2 wounds per animal for each Treatment, Wet/Dry, and Time combination. Random effects of animal and treatment block within animal were included in the model.

Study #2 investigated the effectiveness of an experimental treatment applied at 3 different post-burn injury time points versus a standard of care (SOC). Up to 12 5-cm² deep partial thickness wounds were created in 2 rows and 6 columns on each dorsum of 6 anesthetized pigs. Each treated wound was compared with a SOC wound located along the same cranio-caudal axis. Paired differences for wound closure % were calculated and analyzed with time as a fixed effect with a random effect for animal.

Results: For Study #1, there were 200 total wounds, and for Study #2 there were 72 total wounds. These experimental units are not independent, as required by statistical testing. For both studies, there was graphical evidence that the burn wound measurements differed both within and between pigs. The statistical tests accounted for this variability by incorporating the effects due to pigs.

Conclusions: Matching the statistical model to the experimental design is critical for correct interpretation of the results. Failure to use the correct model by assuming wounds within a pig are independent (and thereby ignoring any effect due to the pig) will result in erroneous statistical test results and less powerful tests of treatment differences.



Anirban Sen Gupta <axs262@case.edu>

Fwd: FW: Authorship Modification: Journal of Burn Care & Research, Volume 43

Anders Carlsson <anders.h.carlsson.swe@gmail.com>

Tue, Mar 29, 2022 at 11:51 PM

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Dear Anirban,

Franklin successfully updated the authors on the abstract. Please see the forwarded email chain.

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The author's corrections appear below:

My name is Franklin. I am reaching out in regard to the publication:

810 Synthetic Platelet-Mimetics with Gentamicin: Outcomes in Deep Partial-Thickness Burns, *Journal of Burn Care & Research*, Volume 43, Issue Supplement_1, April 2022, Pages S210–S211, <https://doi.org/10.1093/jbcr/irac012.358>**Published:**

23 March 2022

There has been an update from our colleagues regarding authorship. Can we please update the author list with the following names and affiliations?

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Journal of Burn Care & Research

Synthetic Platelets with Gentamicin: Outcomes in Deep Partial-Thickness Burns --Manuscript Draft--

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Abstract:	Background: Prolonged inflammation and infection in burns may cause inadequate healing. Platelet granules contain anti-inflammatory mediators that impact wound healing . Synthetic platelets (SP) avoid portability and storage difficulties of natural platelets and can be loaded with bioactive agents. We evaluated wound healing outcomes in deep partial-thickness (DPT) burns treated topically with SP loaded with antibiotics. Methods: Thirty DPT burns were created on the dorsum of 2 Red Duroc hybrid pigs. Six wounds were randomized into five groups: SP alone, SP loaded with gentamicin vesicles, SP with gentamicin mixture, vehicle control (saline), or dry gauze as the standard of care (SOC). Wounds were assessed from post-burn days 3-90. Primary outcome was re-epithelialization percentage at post-burn day 28. Secondary outcomes included wound contraction percentage, superficial blood flow relative to normal skin controls, and bacterial load score. Results: Re-epithelialization with the SOC was 93%, SP alone measured 97%, SP loaded with gentamicin vesicles was 100%, and SP with gentamicin mixture was 89%. Wound contraction was 7% in the SOC and was 10% in both the SP loaded with

	gentamicin vesicles and SP with gentamicin mixture groups. Superficial blood flow in the SOC was 103%, SP alone was 170%, and the SP and gentamicin mixture was 155%. Bacterial load score in the SOC was 2.2/5.0 and was significantly less at 0.8/5.0 in SP loaded with gentamicin vesicles ($p>0.05$). SP and gentamicin mixture scored 2.3/5.0. Conclusion: Topical SP treatment did not significantly improve outcomes. However, SP loaded with gentamicin infused vesicles decreased bacterial load.
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August 25th 2022

David N. Herndon, MD
Editor-in-Chief, Journal of Burn Care and Research

Dear Dr. Herndon,

Attached please find our revised manuscript, entitled “Synthetic Platelets with Gentamicin: Outcomes in Deep Partial-Thickness Burns” to be considered for publication in the Journal of Burn Care and Research.

Prolonged inflammation and infection in burns may cause inadequate healing. Platelets are vital in stopping ongoing blood loss resulting from wounds sustained during trauma—particularly in burns when they reach partial and full thickness depths or are associated with other severe injuries. It is well known that platelets initiate hemorrhage control by coalescing at the site of injury and promote the activation of the clotting cascade. However, in addition to their known role in hemostasis, platelet also contain inflammatory modulators within their granules.

Synthetic platelets avoid portability and storage difficulties of natural platelets and can be loaded with bioactive agents. Therefore, we evaluated wound healing outcomes in deep partial-thickness burns treated topically with synthetic platelets loaded with antibiotics.

We want to declare that the data in the manuscript is original. The paper is not under consideration elsewhere and none of its contents have been previously published. All authors have read and approved all versions of the manuscript and its content.

Sincerely,
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Synthetic Platelets with Gentamicin: Outcomes in Deep Partial-Thickness Burns

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AD and MAB are employed by Haima Therapeutics LLC that provided the synthetic platelets for the study. All other authors declare no conflicts of interest.

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The views expressed in this article are those of the author(s) and do not reflect the official policy or position of the U.S. Army Medical Department, Department of the Army, DOD, or the U.S. Government. Research was conducted in compliance with Animal Welfare Act, the implementing Animal Welfare regulations, and the principles of the Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee approved all research conducted in this study (Protocol #A-21-001). The facility where this research was conducted is fully accredited by the AAALAC.

Synthetic Platelets with Gentamicin: Outcomes in Deep Partial-Thickness Burns

ABSTRACT

Background: Prolonged inflammation and infection in burns may cause inadequate healing. Platelet granules contain anti-inflammatory mediators that impact wound healing. Synthetic platelets (SP) avoid portability and storage difficulties of natural platelets and can be loaded with bioactive agents. We evaluated wound healing outcomes in deep partial-thickness (DPT) burns treated topically with SP loaded with antibiotics.

Methods: Thirty DPT burns were created on the dorsum of 2 Red Duroc hybrid pigs. Six wounds were randomized into five groups: SP alone, SP loaded with gentamicin vesicles, SP with gentamicin mixture, vehicle control (saline), or dry gauze as the standard of care (SOC). Wounds were assessed from post-burn days 3-90. Primary outcome was re-epithelialization percentage at post-burn day 28. Secondary outcomes included wound contraction percentage, superficial blood flow relative to normal skin controls, and bacterial load score.

Results: Re-epithelialization with the SOC was 93%, SP alone measured 97%, SP loaded with gentamicin vesicles was 100%, and SP with gentamicin mixture was 89%. Wound contraction was 7% in the SOC and was 10% in both the SP loaded with gentamicin vesicles and SP with gentamicin mixture groups. Superficial blood flow in the SOC was 103%, SP alone was 170%, and the SP and gentamicin mixture was 155%. Bacterial load score in the SOC was 2.2/5.0 and was significantly less at 0.8/5.0 in SP loaded with gentamicin vesicles ($p>0.05$). SP and gentamicin mixture scored 2.3/5.0.

Conclusion: Topical SP treatment did not significantly improve outcomes. However, SP loaded with gentamicin infused vesicles decreased bacterial load.

KEY WORDS: Synthetic platelets, burn wound healing, re-epithelialization, burn infection

INTRODUCTION

Burns are very common injuries with over four-hundred thousand visits to the Emergency Department annually. [1] Although over 90% of burns are sent home without requiring hospitalization, [2] they may still inflict serious harm with debilitating outcomes which are variable and can increase the risk of factors such as infection and chronic inflammation.

As burn depth and extent worsens, there is a decrease in the protective barrier of skin and therefore the likelihood of infection increases, which can lead to poor outcomes. [3] As a consequence, infection can present both local and systemic effects, highlighting the importance of optimizing a clean wound microenvironment to maximize prevention. [4] Infection may also cause poor healing and cosmetic outcomes when persistent. [5]

Additionally, inflammation plays a vital role in burns and wound healing in general. However, prolonged inflammation can delay healing and lead to excessive fibrosis and hypertrophic scar formation following burn injury. [6] When occurring near joints, an orifice, or on hands, hypertrophic scarring and fibrosis from ongoing inflammation may also lead to functional deficits. [7] Given these potential consequences secondary to burns and their relationship with inflammation and infection, timely and appropriate treatment of these phenomena is paramount.

Platelets are vital in stopping ongoing blood loss resulting from wounds sustained during trauma—particularly in burns when they reach partial and full thickness depths or are associated with other severe injuries. It is well known that platelets initiate hemorrhage control by coalescing at the site of injury and promote the activation of the clotting cascade. [8] However, in addition to their known role in hemostasis, platelet also contain inflammatory modulators within their granules. [9] These contents may have the ability to impact burn wound healing as the use of

platelet rich plasma (PRP) has been shown to improve healing outcomes through recruitment of other cells and providing anti-inflammatory effects in other studies involving soft and hard tissues. [10,11]

Contrasting the several benefits that platelets provide through hemostasis, cell recruitment, and immunomodulation, their use also poses a number of drawbacks and challenges to overcome. Given the only source of platelets is to obtain them through phlebotomy, acquisition is restricted since less than 10% of the U.S. population donating blood products annually. [12] Storage is limited by the shelf-life of platelets which can be as short as 5 days along with their requirement to be kept at room temperature which complicates their portability in austere locations or remote locations such as military deployments. [13,14] Acquisition cost of platelets is also expensive, estimated to be over \$500 per unit. [15] Given the several factors involved with natural platelets, studies have turned to manufacturing alternatives with similar properties that overcome the barriers of natural platelets.

Synthetic platelets have been manufactured and studied to address the limitations posed to natural platelets by donor-limited availability, storage, risk of bacterial contamination, and portability. [16] The barriers of natural platelets are overcome whilst also creating an artificial platelet technology that mimics the ability of natural platelet adhesion during primary hemostasis and aggregation in secondary hemostasis. [17]. Synthetic platelets offer similar benefits of natural platelets to treat these common injuries such as burns, while avoiding the same barriers of use. Given this, we performed a study to explore the use of synthetic platelets alone and in combination with gentamicin as a treatment of deep partial-thickness porcine burns. We sought to evaluate wound healing outcomes and antimicrobial properties in comparison to the standard of care treatment.

Synthetic platelets will be utilized two ways, the first is loaded with gentamicin into their vesicles and the second with synthetic platelets grossly mixed with gentamicin and not loaded into vesicles as this will secondarily evaluate the spatio-temporal release of a bioactive agent within the vesicles, analogous to platelet granules. The primary outcome will be the percentage of wound re-epithelialization at post-burn day (PBD) 28 to evaluate the proportion of healing. Secondary outcomes will include parameters measuring the quality of healing: percentage of wound contraction, amount of superficial blood flow, and bacterial load score.

METHODS

Animal, Anesthesia, and Analgesia

Research was conducted in compliance with Animal Welfare Act, the implementing Animal Welfare regulations, and the principles of the Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee approved all research conducted in this study (Protocol #A-21-001). The facility where this research was conducted is fully accredited by the AAALAC. The study design was a pre-clinical porcine model with two, red Duroc hybrids (Midwest Research, Glencoe, MN, USA). Each were female, adolescent at the initiation of the study, weighed between 55-70 kg, and housed individually. The animals were fasted at least 8 hours prior to planned procedures. Anesthesia and analgesia were used for all procedures. Intramuscular injections (IM) of glycopyrrolate (0.01 mg/kg) or atropine (0.02 mg/kg) were used as needed to reduce salivary secretion and block vagally mediated bradycardia during wound creation. Anesthesia consisted of IM tiletamine-zolazepam (Telazol, 4-6 mg/kg) or Ketamine (10-25mg/kg) and 3-5% isoflurane and oxygen via a snout mask. Pigs were intubated with endotracheal tubes and placed on mechanical ventilation and anesthesia was maintained with 1-4% isoflurane.

Heart rate, peripheral capillary oxygen saturation, core body temperature, muscular relaxation, and urine output were monitored throughout each procedure. Additional analgesia was given subcutaneously (SC) as needed prior to the procedure with buprenorphine SR-LAB (0.1-0.24 mg/kg) or hydromorphone (0.2-0.6 mg/kg). The animals were transferred to their respective holding rooms at the completion of each procedure. Observation occurred twice every 24 hours for the first 72 hours following each procedure. Additional analgesia was administered as needed based on post-surgical observations. Animals were kept for a total of 90 days to examine wound healing progression and were euthanized under surgical anesthesia according to institutional IACUC policies using a commercial euthanasia solution at the study conclusion.

Porcine Burn Model

The timeline for the study from burn creation, treatment application, assessments, and conclusion are outlined in the study design (Figure 1). Two anesthetized pigs were placed in prone positioning, dorsum and flanks were trimmed and sanitized. Along each dorsum, up to 15 deep partial-thickness burns (DPT) (5cm in diameter) were made using a thermocouple burn device with a 5cm circular brass plate. Burns were separated by at least 3cm to minimize site-to-site influence. The burn device was heated to 100°C and applied to the skin at a constant pressure for 15 seconds to create each DPT burn. Temperature probes and infrared cameras were used to confirm consistent temperatures between burn creations. Burn sites were tattooed (Superior Tattoo, Phoenix, AZ, USA) along their borders to monitor wound contraction. Two additional 5cm diameter circular areas were demarcated on the dorsum, serving as normal skin growth controls.

The number, size, and placement of burns were determined based on prior studies and the dimension of a representative 55 kg pig with less than 10% TBSA burned. Thirty DPT burns, 15

per pig, were randomized into one of five groups: 1) SP with gentamicin loaded into vesicles (SP Loaded) (n=6), 2) SP grossly mixed with gentamicin (SP Mixed) (n=6), 3) SP alone (n=6), 4) dry gauze as the standard of care (SOC) (n=6), or 5) vehicle control with saline (VC) (n=6). Excluding the SOC treatment group, all wounds were covered with a platform wound device (PWD) (Applied Tissue Technologies LLC, Hingham, MA) to ensure treatments remained on over the wound topically without interference. Briefly, PWDs are occlusive dressings applied to a wound that can be instilled with topical compounds through an infusion port. This allows treatments to remain in the chamber and prevents the external environment from coming into contact with the wound as the PWD is adhered circumferentially to adjacent, normal skin with an adhesive. [18,19] Topical compounds were instilled into each PWD using a 10cc syringe. A total of 5mL was applied to each wound.

Histologic Analyses

The primary outcome of the study was the percentage of re-epithelialization at PBD 28 which was measured via histologic analysis. Each wound was sampled with a 6mm punch biopsy. Tissues were fixed in 10% neutral-buffered formalin for at least 48-hours, dehydrated, and embedded in paraffin for analysis. A microtome (Leica Biosystems, Buffalo Grove, IL, USA) was utilized to create 5-7 μ m sections which were stained with hematoxylin and eosin (H&E) and Mason's trichrome. Histologic sections were scanned using a DS-Fi1 camera (Nikon Corporation, Japan). The analyses were carried out using ImageJ software (NIH, New York, NY).

To evaluate other parameters associated with the quality of healing, both dermal-epidermal thickness as well as the quantity of rete ridges within a biopsy specimen were analyzed at PBD 90. Dermal-epidermal thickness was quantified by measuring three different areas on along each

biopsy wound edge and taking the mean of the measurements. Rete ridges were quantified by measuring the number of them within a 1000 um linear distance along the dermal-epidermal junction of each biopsy similarly at three different areas and subsequently calculating the mean. These values were then compared with the SOC.

Macroscopic Wound Closure

This outcome was monitored and analyzed on PBD 28 using the Silhouette Star wound imaging device and software (ARANZ Medical Ltd, Christchurch, New Zealand). Wounds were surveyed and images were recorded. The percentage of area that was healed grossly was calculated through objective measurement. For each treatment group, the mean was obtained, and groups were compared.

Wound Contraction

Percentage of wound contraction was measured at PBD 90 via the Silhouette Star (Aranz Medical Ltd., New Zealand) imaging device and software. To account for the adolescence of the porcine models and their maturity through the duration of the study, a growth coefficient of 0.74 was incorporated to scale size changes not due to contraction. Wound contraction was reported as a ratio of the size of the wound after 90 days of healing with its respective treatment relative to the initial wound size.

Bacterial Load

Wounds were evaluated on PBD 3 collectively to detect the presence and amount of bacteria. They were not deliberately inoculated. Samples were collected in sterile cryovials with 1

mL of sterile phosphate buffered saline. Serial ten-fold dilutions were made from each sample and 100 μ L of each dilution was plated onto a tryptic agar plate. Plates were incubated overnight at 37°C for 3-5 days. Bacterial load was quantified by the amount of colonization detected in each plate. The amount of growth was documented and converted to a numerical scoring system. Wounds were assigned a score from 0 – 5 and the mean of each treatment group was quantified. A score of 0 signaled no detected growth and 1 represented the presence of isolated colonies only. Score of 2 meant light growth, in that there were more than sparse colonies which were becoming a lawn of colonies but not quite. Score of 3 signified that a complete lawn of colonies was present. Score of 4 represented moderate growth with a denser lawn and a score of 5 was consistent with a heavy growth of a colony lawn throughout.

Wound Bed Microcirculation

High resolution Laser Contrast Speckle Imaging (LSCI) was utilized to assess the amount of superficial blood flow present in each wound. This was done with the use of the PeriCam PSI HR (Perimed AB, Järfälla, Sweden) being placed 20-30 cm above the wound. Image size was set to correspond to a 25 cm² area of skin and the acquisition rate was set to 21 images per second with a spatial resolution of 0.2 mm/pixel. The amount of perfusion was calculated based on each pixel of the digitized image. Mean perfusion levels for each wound were analyzed and calculated using PIMSoft 1.3 software (Perimed AB). The amount of blood flow in each wound was evaluated relative to normal skin controls at PBD 28.

Statistical Analysis

Statistical comparisons were performed using GraphPad Prism 9.3.1 (Graph Pad software, Inc. La Jolla, CA). Data is presented as mean $\pm$ standard error of mean (SEM). Comparison of treatments was performed using unpaired one-way ANOVA with Tukey's multiple comparison test and p-values < 0.05 were considered statistically significant. N number for each treatment group was 6.

RESULTS

Wound Healing

Re-epithelialization

The percentage of wound re-epithelialization at PBD 28 was the primary outcome of this study. No significant differences were found on analysis of treatment groups relative to the SOC (Figure 2a). Wounds treated with the SOC had a PBD 28 re-epithelialization of 92.7% $\pm$ 5.1. In wounds treated with SP alone, re-epithelialization was 97.3% $\pm$ 2.7, $p = 0.50$. Re-epithelialization at PBD 28 in wounds treated with SP loaded with gentamicin was 100% $\pm$ 0.0 and in those treated with SP mixed with gentamicin was 88.7% $\pm$ 11.3, $p = 0.21$ and $p = 0.78$, respectively. Re-epithelialization at PBD 28 in the VC group was 97.3% $\pm$ 2.7, $p = 0.45$.

Macroscopic Wound Closure

The percentage of the wound noted to have grossly healed at PBD 28 was analyzed as an adjunct to the histologic progression of re-epithelialization to discern whether or not a correlation was present. There were no significant differences in the treatment groups relative to the SOC (Figure 2b) nor did the findings here correlate with the amount of re-epithelialization found histologically. Wounds treated with the SOC had a macroscopic closure of 92.2% $\pm$ 0.06.

Whereas macroscopic wound closure in wounds treated with SP alone was 95.9% $\pm$ 0.02, $p = 0.56$. Macroscopic closure in wounds treated with SP with gentamicin loaded vesicles was 97.3% $\pm$ 0.03, and in those treated with SP mixed with gentamicin macroscopic closure measured 89.7% $\pm$ 0.05, $p = 0.48$ and $p = 0.79$, respectively. Macroscopic closure at PBD 28 in the VC group was 96.5% $\pm$ 0.03, $p = 0.58$.

Wound Bed Microcirculation

Superficial blood flow was monitored at wound surveillance intervals and analyzed on PBD 28, serving as a surrogate for wound angiogenesis as absolute values were obtained relative to uninjured skin controls with subsequent comparison to the SOC. Superficial blood flow was elevated well above that of normal skin controls in all treatment groups at post-burn day 28, however no significant differences were noted on analysis (Figure 2c). All reported percentages are relative to normal skin controls. In wounds treated with the SOC, superficial blood flow was 103% $\pm$ 22.5. In the SP alone treatment group, superficial blood flow was 170% $\pm$ 10, $p = 0.29$. Meanwhile in wounds treated with SP mixed with gentamicin and those with SP with gentamicin loaded vesicles, superficial blood flow was 155% $\pm$ 0 and 163% $\pm$ 2.5, $p = 0.20$. Superficial blood flow was 162% $\pm$ 13.5 in the VC group, $p = 0.35$.

Quality of Healing

Wound Contraction

The percentage of wound contraction was reported as a ratio of the size of the wound after 90 days of healing with its respective treatment relative to the initial wound size at PBD 0. After analyzing treatment groups relative to the SOC, no significant differences were noted (Figure 3a).

The percentage of wound contraction in wounds treated with the SOC was 7% $\pm$ 0.04. In wounds treated with SP alone, wound contraction was 14% $\pm$ 3.7, $p = 0.23$. Meanwhile, wound contraction in those treated with SP mixed with gentamicin was 10% $\pm$ 2.5, $p = 0.72$. Similarly wound contraction was 10% $\pm$ 4.6 in wounds treated with SP with gentamicin loaded vesicles, $p = 0.67$. In the VC treatment group, wound contraction reached 15%, $p = 0.33$.

Dermal-Epidermal Thickness

Measured histologically at PBD 90, SP grossly mixed with gentamicin significantly improved dermal-epidermal thickness over the SOC, 2412 μ m $\pm$ 426 vs 929 μ m $\pm$ 263, respectively, $p = 0.005$. The remainder of treatment groups did not significantly improve dermal-epidermal thickness relative to the SOC (Figure 3b). In wounds treated with SP alone, thickness measured 1264 μ m $\pm$ 487, $p = 0.51$. Meanwhile in wounds treated with SP gentamicin loaded vesicles, thickness measured 1919 μ m $\pm$ 531, $p = 0.16$. The VC group measured 1320 μ m $\pm$ 385, $p = 0.24$.

Rete Ridges

The mean quantity of rete ridges present was measured histologically at PBD 90 and analyzed. There were not any significant increases in the quantity of rete ridges relative to the SOC (Figure 3c). All quantities are expressed as the number of rete ridges per 1000 μ m. In wounds treated with the SOC, there were 5.3 $\pm$ 1.5 rete ridges present. SP alone treated wounds measured with 4.8 ridges $\pm$ 1.6, $p = 0.67$. In those treated with SP with gentamicin loaded vesicles and SP grossly mixed with gentamicin the quantity of rete ridges present was 5.8 $\pm$ 1.7 and 6.0 ridges $\pm$ 1.2, $p = 0.29$ and 0.72, respectively. Wounds with the VC had 5.5 $\pm$ 1.3 ridges, $p = 0.91$.

Bacterial Load

All wounds were monitored for bacterial load present and analysis was conducted on PBD 3 (Figure 4). Wounds treated with the SP, SP mixed, SP loaded, VC and SOC scored 2.67 ± 0.33 , 2.33 ± 0.33 , 0.83 ± 0.48 , 2.83 ± 0.17 and 2.17 ± 0.17 respectively. The difference between SP and SP loaded, VC and SP loaded showed a significance of $p > 0.01$. The difference between SP mixed and SP loaded, SOC and SP loaded showed a significance of $p > 0.05$.

DISCUSSION

In the setting of prolonged field care, treatment of wounds and burns are important for protecting soldiers from infection, optimizing wound healing outcomes, and stabilizing injuries in the event of an impending transfer to a higher level of care. Use of topical synthetic platelet application in far forward scenarios mitigates the limitations of natural platelets such as portability, special storage requirements and contamination risks of blood products e.g., platelet concentrates, and a very short shelf-life (3-5 days) at room temperature due to high risk of bacterial contamination. Briefly, synthetic platelets are designed by utilizing a polyethylene glycol (PEG)-modified lipid vesicle platform, refined by heteromultivalent ligand decoration with VWF-binding (VBP), collagen-binding (CBP), and active GPIIb-IIIa-binding Fg-mimetic peptides (FMP) to synthesize each vesicle. [20] Furthermore, these vesicles are amenable to degradation by bleeding site-relevant enzymes, phospholipases or thrombin. This provides a way to trigger enzyme release of encapsulated bioactive agents from the particles to treat wound sites, such as antimicrobials. Through these properties, synthetic platelets could have a large impact in the treatment of wounded soldiers in far forward scenarios.

Deep partial-thickness burns were chosen for this study as they are common among burn types and replicate injuries sustained in realistic injury depths sustained by civilians and soldiers alike. Furthermore, there is risk of hypertrophic scarring from ongoing inflammation as well as risk of infection from bacterial colonization of forming eschar on the wound. Gentamicin is often used to treat burns for its gram-negative coverage and even more so for its ability to treat pseudomonas, an infection that is common amongst burns.

Many numerical improvements were observed regarding wound healing capability as well as the quality of healing in the treatment of DPT burns through a porcine pre-clinical model. However, the application of SP alone, with a gentamicin mixture, or with gentamicin loaded vesicles, did not meet the primary outcome, improving DPT burn re-epithelization at PBD 28 relative to the SOC. Dermal-epidermal thickness was significantly increased relative to the SOC when DPT wounds were treated with SP mixed with gentamicin at PBD 90. Collagen deposition is paramount for multiple wound healing processes including modulation of inflammation, encouraging angiogenesis, re-epithelialization, and contributing to tensile strength to the newly formed scar. [21-24] Indeed a lower density of collagen fibers, in addition to other factors, is associated with the aging process where it decreases with time. Consequently, a lesser density of collagen has a predisposition to inferior wound healing. [25,26] It can be extrapolated that a higher density of collagen within the confines of a healing wound is therefore favorable to an extent—assuming ongoing re-organization, degradation and breakdown. Given the contrast of collagen deposition, the increased thickness of collagen and skin with the application of SP mixed with gentamicin may provide an ideal environment that is favorable for ideal wound healing outcomes.

SP loaded with gentamicin in vesicles significantly decreased the mean bacterial load of DPT wounds on PBD 3 relative to the SOC in this porcine model. Given the spatio-temporal

release of gentamicin from within SP vesicles, the steady release of gentamicin from vesicles provided an extended antimicrobial effect. This protection allowed for a significantly decreased presence of bacterial flora in the wound bed. With regard to an ongoing effect and presence, the formulation of SP is another important point to address. Creating SP as a hydrogel instead of a liquid formulation is ideal to provide wounds with a protective layer that will remain in place, not leak from the area, and can be utilized in scenarios of prolonged field care. This is important as a proper formulation can remain over the site of interest and provide a targeted treatment with a spatio-temporal release of antimicrobial properties in addition to the aforementioned wound healing benefits of SP.

Platelet-rich plasma (PRP) has been shown in multiple clinical studies to improve recovery and wound healing. [27-30] PRP has also been validated in other preclinical studies for burn wound healing. Venter et. al. explored the use of PRP in partial and full thickness injuries using a murine model. Their results contrast our study in that they show a significant improvement of macroscopic wound closure. [31] However, interpretation of findings from rodent studies is difficult. They are loose-skinned animals whose major wound healing mechanism is contraction while in pigs and humans, wounds heal mainly from granulation tissue formation and re-epithelialization. [31] Still their results are encouraging in that platelet application aided wound healing. Singer et. al. performed a study closer to our own comparing application of topical PRP vs topical antibiotics in DPT burns with a porcine model. Similarly to our results, they did not find significant differences between treatment groups regarding re-epithelialization at PBD 28. [32] Antibiotics used were a triple therapy of Neosporin, polysporin, bacitracin. While animals were more identical in this study, their wounds were also smaller at 2.5cm in diameter relative to our 5cm diameter burns.

Importantly, PRP did not perform inferiorly to topical antibiotic therapy thus warranting further platelet studies such as ours.

Despite the benefits found in SP application, as with all studies there were several limitations in this study. Small sample sizes to compare groups necessitates larger differences to show a significant change and likely had an impact on the parameters that did not reach significance. Larger trials with these formulations could address this. The animals were juvenile and went through adolescence over the course of the 90-day study. During this time, they nearly doubled in body weight, effecting the scale by which wound contraction was measured and requiring a correlation coefficient to eliminate their growth as a confounder. All forms of photographic and histologic analyses were standardized as much as possible but ultimately were subject to interpretation by research personnel.

To conclude, application of SP alone, when loaded with gentamicin in vesicles, or when mixed with gentamicin did not significantly improve wound healing capability outcomes relative to the standard of care, dry gauze. However, SP mixed with gentamicin grossly did significantly improve dermal-epidermal thickness and may be associated with improved quality of healing. Additionally, SP loaded with gentamicin in vesicles was significantly associated with a decreased bacterial load on post-burn day 3, which may provide antimicrobial benefits through the spatio-temporal release of bioactive agents from SP vesicles. The theoretical and observed potential for SP to improve wound healing capability deserves additional evaluation in larger preclinical studies.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

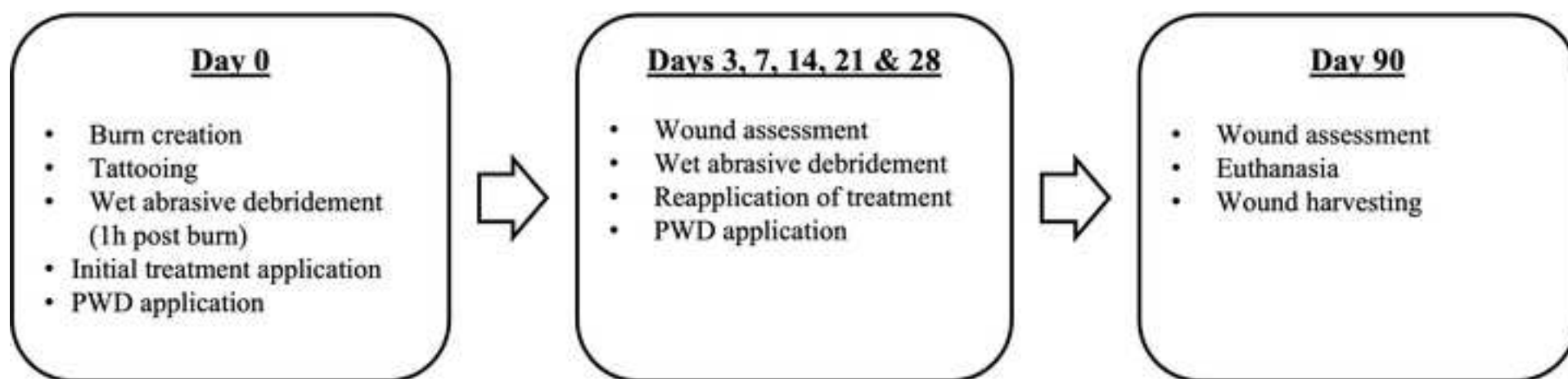
Figure 1. Study Design. Consort diagram which shows the layout of the study from initiation to conclusion with the key time points of treatment application and wound assessments.

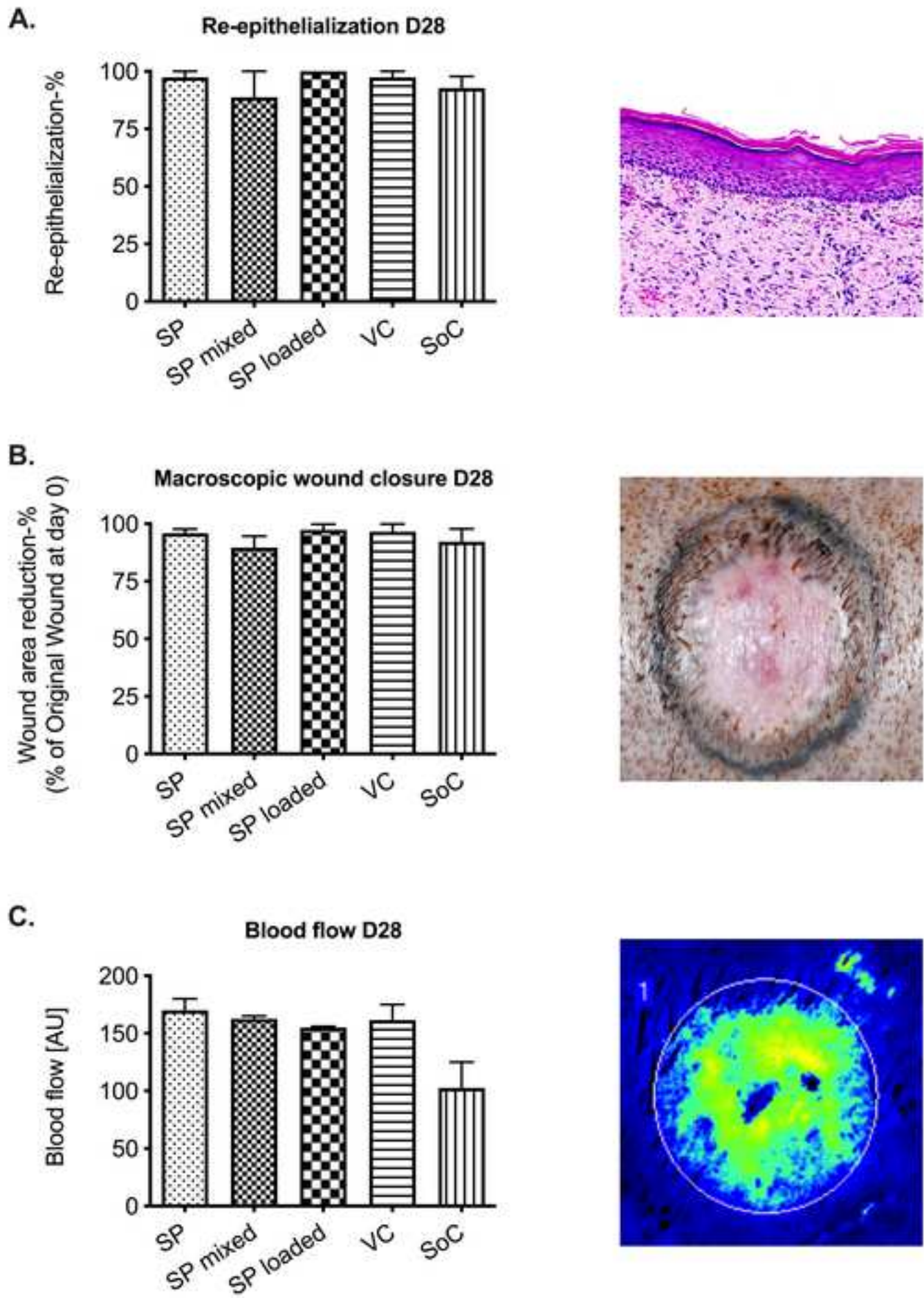
Figure 2. Wound Healing. **A.** Re-epithelization percentages from histologic evaluation at PBD 28 comparing the SOC, vehicle control (VC) with saline, SP alone, SP mixed with gentamicin, and SP with gentamicin loaded vesicles. With the graph is a representative slide of a wound from the SP loaded treatment group. **B.** Macroscopic wound closure percentages are displayed after evaluation at post-burn day 28 comparing the SOC, vehicle control (VC) with saline, SP alone, SP mixed with gentamicin, and SP with gentamicin loaded vesicles. A representative image of the SP mixed group is shown at PBD 28. **C.** The percentages of superficial blood flow are depicted as a surrogate of angiogenesis at PBD 28 comparing the SOC, vehicle control (VC) with saline, SP alone, SP mixed with gentamicin, and SP with gentamicin loaded vesicles. These values represent the comparison to normal, intact skin controls.

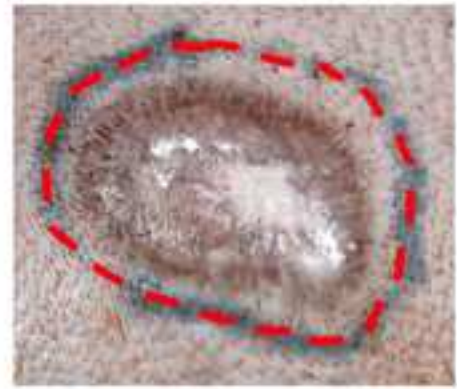
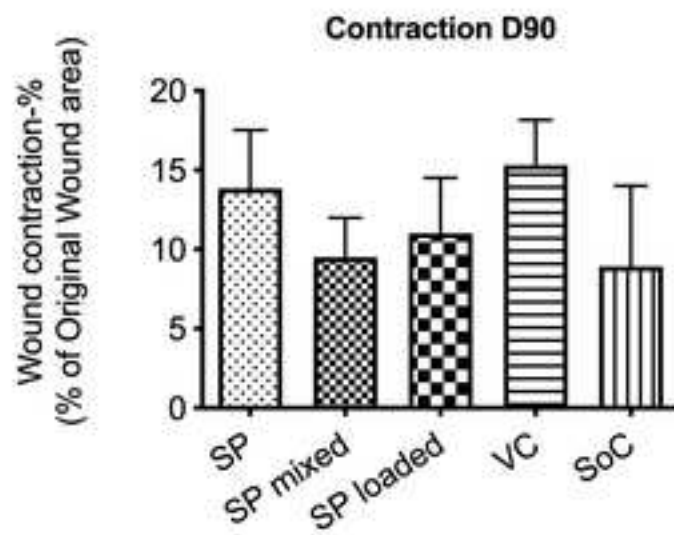
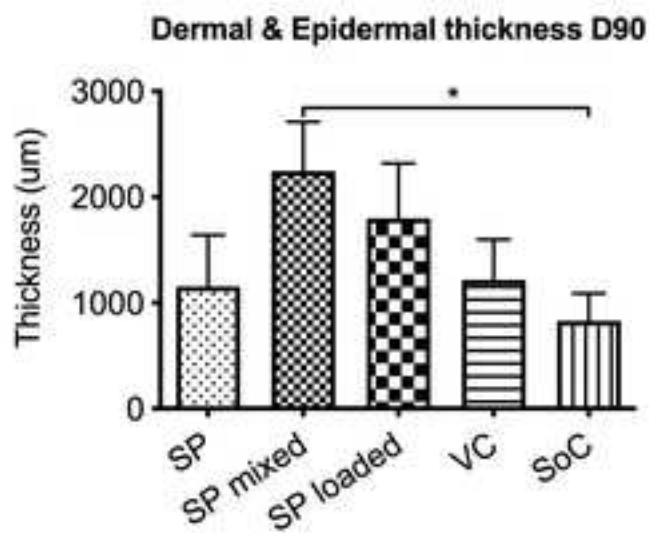
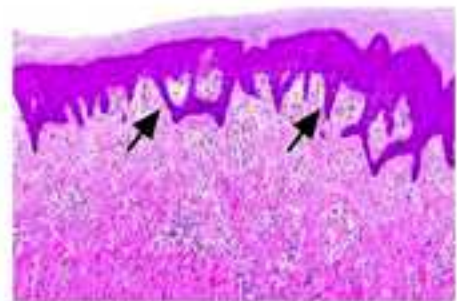
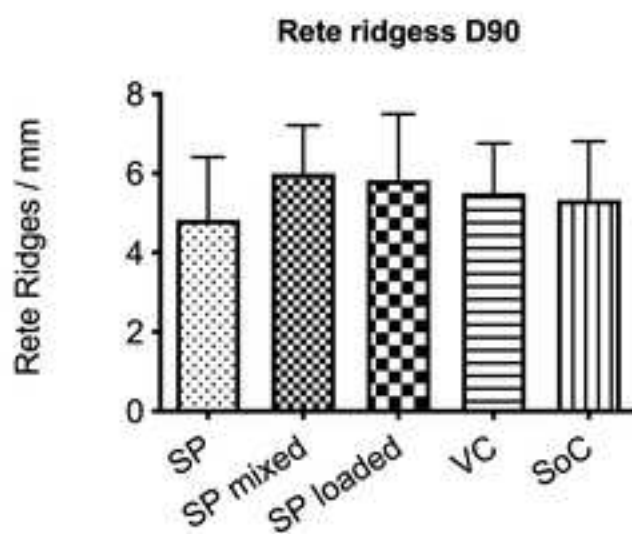
Figure 3. Quality of Healing. **A.** Wound contraction percentages at PBD 90 are depicted graphically to compare the SOC, vehicle control (VC) with saline, SP alone, SP mixed with gentamicin, and SP with gentamicin loaded vesicles. An image at PBD 90 of the SP alone treatment group is shown with the tattooed border representing the amount of contraction. **B.** Varying dermal-epidermal thicknesses were noted at PBD 90 in each treatment group and are depicted comparing the SOC, vehicle control (VC) with saline, SP alone, SP mixed with gentamicin, and SP with gentamicin loaded vesicles. Significant differences are marked with an asterisk. With this is a histologic example of the SP mixed treatment group. **C.** The mean quantity of rete ridges in

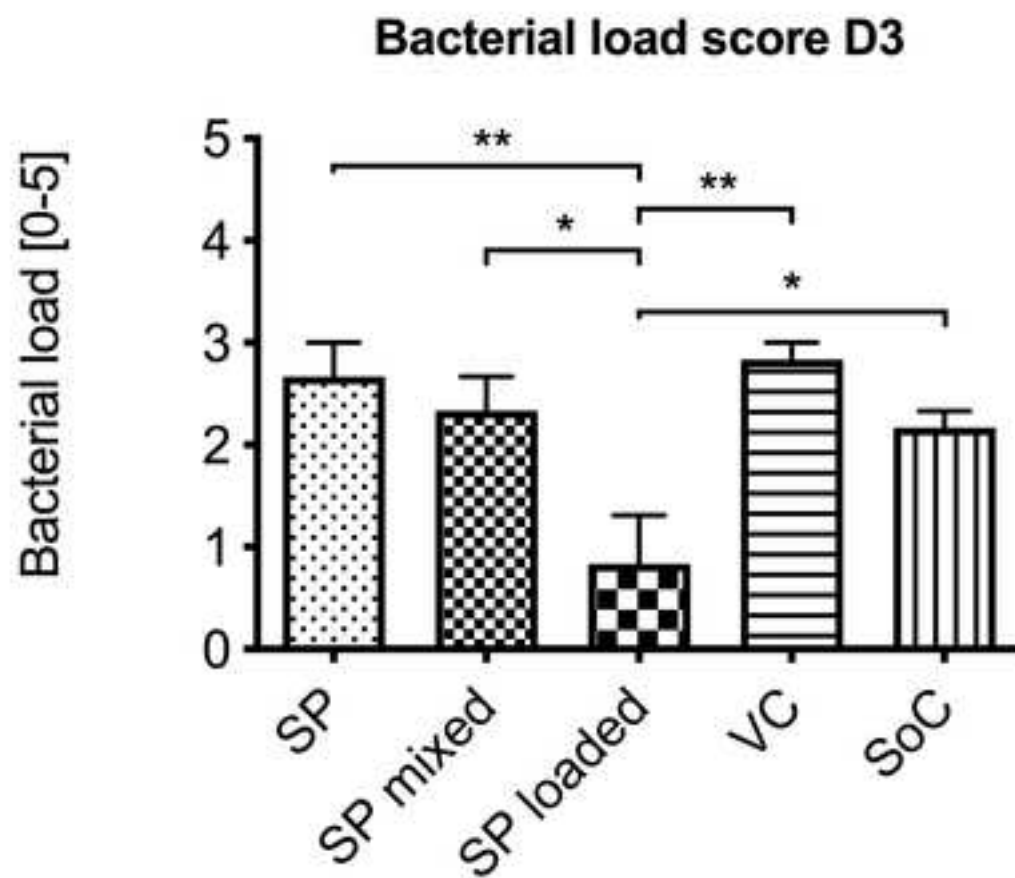
each biopsy wound edge at the dermal-epidermal junctions were determined at PBD 90 in each treatment group and are depicted comparing the SOC, vehicle control (VC) with saline, SP alone, SP mixed with gentamicin, and SP with gentamicin loaded vesicles. With this is a histologic example from the SP alone treatment group showing multiple rete ridges along the dermal-epidermal junction.

Figure 4. Bacterial load. Serial ten-fold dilutions were made from each culture swab and 100 μ L of each dilution was plated onto a tryptic agar plate. Plates were incubated overnight at 37°C for 3-5 days. Bacterial load was quantified by the amount of colonization detected in each plate (Representative photo on right) . The mean bacterial load was quantified and analyzed at PBD 30 in each treatment group and are depicted comparing the SOC, vehicle control (VC) with saline, SP alone, SP mixed with gentamicin, and SP with gentamicin loaded vesicles. Significant differences are denoted with an asterisk.





A.**B.****C.**



ICMJE DISCLOSURE FORM

Date: 8/26/2022

Your Name: Anders Carlsson, Kristo Nuutila, David Varon, Franklin Valdera, Jennifer Cooley, Javier Chapa, Sean Christy, Rodney Chan, Laura Cooper

Manuscript Title: Synthetic Platelets with Gentamicin: Outcomes in Deep Partial-Thickness Burns

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Platelet-inspired synthetic hemostatic nanoparticles improve hemostasis and hemodynamics in a rabbit model of intraperitoneal hemorrhage

Srinivasan AJ, Secunda ZA, Mota-Alvidrez RI, Luc NF, Disharoon D, Traylor B, Pawlowski CL, Bruckman MA, Sen Gupta A, Neal MD

Objectives:

Early platelet transfusion is associated with decreased mortality in patients with traumatic hemorrhage. However, donor supply constraints and short shelf-life severely limit potential platelet usage. SynthoPlate (SP) is a platelet-inspired synthetic nanoparticle designed by surface-decorating liposomes with peptides that mimic injury-site platelet adhesion to vWF and collagen, as well as fibrinogen-mediated platelet aggregation. SP has showed hemostatic benefit in murine, rodent and porcine hemorrhage models. We sought to evaluate hemostasis and hemodynamic effects of SP in a rabbit model of abdominal hemorrhage.

Methods:

24 adult male New Zealand white rabbits (2.5-3.5kg) were pretreated with either buffer, control particles (CP), or SP. Under general anesthesia with invasive monitoring, rabbits underwent laparotomy with standardized splenic and hepatic injury. Hemodynamics were monitored for 30 minutes and blood loss was quantified. Complete blood counts, aggregometry, and plasma catecholamine assays were performed at pre- and post-intervention, and post-trauma timepoints. Analysis used ANOVA and post-hoc Tukey testing with $\alpha=0.05$.

Results:

Rabbits in the SP group had significantly lower weight-normalized blood loss compared to both buffer and CP animals (21.1 vs 33.2 vs 40.4 g/kg, $p<0.001$). Areas under the curve (AUC) for mean arterial and systolic pressure were calculated to evaluate hemodynamics over time. SP animals had higher systolic AUC compared to buffer and CP animals (1589 vs 1353 vs 1172mmHg*min, $p=0.01$), though post-hoc differences were only significant for the SP:CP comparison ($p=0.01$). Platelet counts, catecholamine levels, and aggregometry were similar between groups.

Conclusion:

SP pretreatment reduced blood loss and improved hemodynamic outcomes in a rabbit model of abdominal hemorrhage. SP has potential as a platelet surrogate with donor-independent availability and scalable manufacture, for intravenous hemostatic management of traumatic bleeding.

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Significant Results:

A. Rabbit Polytrauma Model

Anesthesia is induced in New Zealand white rabbits weighing 2-4kg using 30 mg/kg ketamine and 3 mg/kg xylazine intramuscular injection. The animal is then endotracheally intubated and anesthesia is maintained using 1-4% isoflurane. Peripheral intravenous access is placed in the ear vein, maintenance fluids are started, and an arterial line is placed in the ear artery. For the remainder of the experiment the animal is continuously monitored using electrocardiographic tracings, arterial line pressures, SpO₂ (oxygen saturation via tongue probe), and end-tidal carbon dioxide levels. Following fur removal and povidone iodine prep, a cutdown is performed in the right neck and the internal jugular vein is isolated, controlled, and cannulated with plastic tubing; continuous central venous pressure monitoring is established at this time. A pre-treatment blood sample (3mL) is obtained. The animal is then treated with the intervention (buffer, control nanoparticles, or SynthoPlate nanoparticles) via peripheral injection over 1 minute. After 5 minutes, a post-treatment blood sample is obtained. The traumatic injury is then performed. A generous midline laparotomy is made and 3 pieces of a pre-weighed (20g) collection of gauze are inserted in bilateral flanks of the peritoneal space (2 on the left, 1 on the right), abutting the retroperitoneum. Another piece of the pre-weighed gauze is folded once and placed in the gastrohepatic space. The spleen is identified and medialized, and the medial 1cm is amputated with scissors before replacing the spleen in the left upper quadrant of the abdomen. The left lateral and right lobes of the liver are identified, and 5 lacerations are made in each of these lobes with a #10 blade; the lacerations are each 1cm in diameter and 1 cm deep. The laparotomy is temporarily closed with clamps and the animal is monitored continuously with recording of hemodynamic data at 2 minute intervals for a timeframe of 30 minutes. After 30 minutes, a final blood sample is taken. The gauze is removed from the abdomen and the remaining pre-weighed gauze is used to remove the residual hemoperitoneum and clots. The total weight of the blood-saturated gauze is compared to the previously known weight of the dry gauze to quantify blood loss. The animal is sacrificed with potassium chloride and tissue samples are taken from the liver, spleen, kidney, lung, and heart. These tissue samples are snap-frozen in liquid nitrogen before storing at -80C.

B. Blood Sample Processing

Whole blood samples are collected at the specified timepoints in blood collection tubes containing 3.2% sodium citrate in a 1:9 ratio and inverted gently to mix. Complete blood counts were obtained in duplicate using a Coulter Ac-T diff2 hematology analyzer, results are reported as the average of the two technical replicates. Remaining blood samples were centrifuged twice sequentially at 2,500g for 15 minutes at room temperature to separate platelet poor plasma (PPP). The PPP was aliquoted, snap-frozen in liquid nitrogen, and stored at -80C until use in assays.

C. Whole Blood Impedance Aggregometry

Samples were collected pre-treatment, post-treatment and final (30min post polytrauma) for each rabbit per treatment group (n: 5-8 per group). Platelet count was determined as per the method section above. We then determined the correct dilution for each sample for a total volume of 1mL of each sample to be ran. Whole blood was diluted with 0.9% saline solution at a 1:2.5 ratio. Optimal platelet count per sample was determined at a working range between 150×10^3 - 250×10^3 platelets per μ L per our knowledge from previous studies.

Whole blood was subjected to ADP-induced platelet aggregation by stimulating platelet aggregation with ADP (20uM) (Bio/Data™ 101312) as well as tested a positive control for each run using stimulation with 0.1UI of Thrombin (CHRONO-LOG 386). Samples were recalcified using Calcium Chloride (100 mM stock), samples were incubated with stimulant for 5-10min before each run. Data was collected using CHRONO-LOG® Model 700 Aggregometer by measuring impedance and was analyzed using AGGRO/LINK®8 software. Each sample was run twice for accuracy.

D. Platelet Factor 4 (PF4) and Catecholamine Quantification

PF4 was quantified in stored PPP using a commercial enzyme-linked immunosorbent assay kit (Rabbit PF4 ELISA, MyBioSource) according to manufacturer instructions. Total plasma catecholamines were quantified in stored PPP using commercially-available ELISA (Rabbit Catecholamine, CA ELISA Kit, Biotang Inc) designed to quantify epinephrine, norepinephrine, and dopamine concentrations. This second assay was also performed according to manufacturer instructions.

E. Statistical Analysis

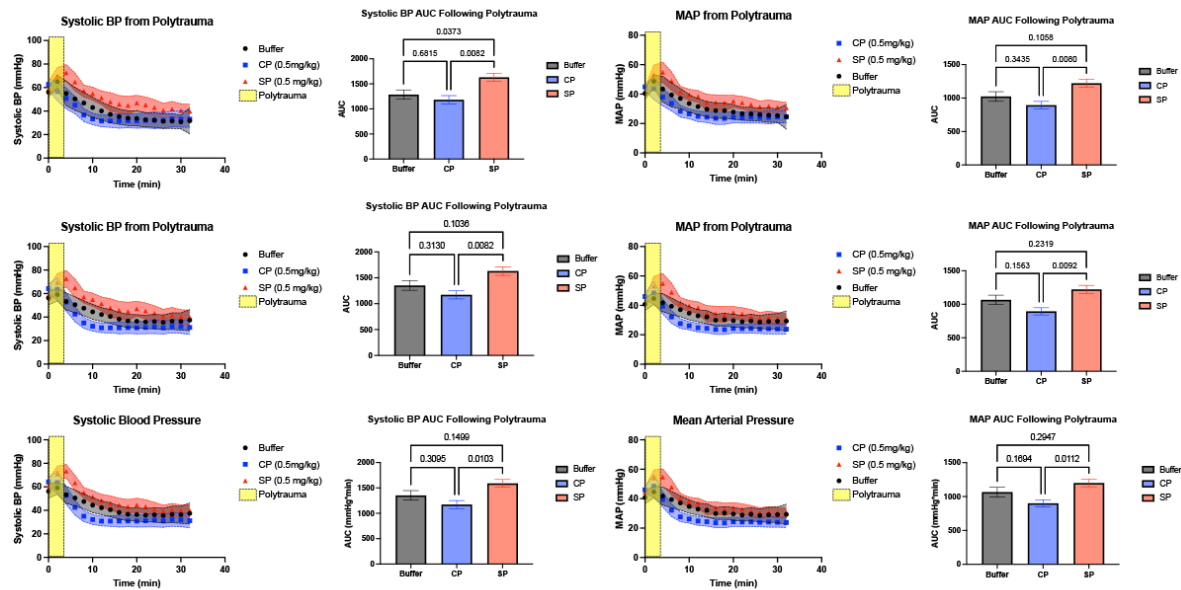
A sample size of 8 animals per treatment group was chosen based on prior experience with small-animal polytrauma models in our laboratory as well as preliminary rabbit polytrauma model data. A total sample size of 24 animals gave an 80% power to detect a 10g/kg change in blood loss from a baseline mean of 30g/kg and standard deviation 8g/kg. All statistical analysis was performed using GraphPad Prism. Continuous data are represented as mean +/- SEM and were compared among treatment groups using one- and two-way ANOVA with post-hoc Tukey tests for between-group comparisons. Areas under the curve (AUC) were calculated using identical technique for the blood pressure vs time curves of individual animals, and these AUC values were compared among the three groups using one-way ANOVA and post-hoc Tukey tests. Significance was declared at $p < 0.05$.

F. Results from particle-injected rabbit studies

In our last quarterly report we had presented our initial data on the rabbit studies, including systolic and diastolic BP, mean arterial pressure (MAP), heart rate (HR), platelet count and weight-normalized blood loss. We continued these studies for the remaining number of rabbits allowed by the current project funds (total 22 rabbits allowed), measuring the above parameters, as well as measuring some additional blood component parameters ex vivo from the sampled blood volume (e.g. PF4, catecholamine, aggregometry) to gain additional insight regarding the 'effect' of the nanoparticle treatment.

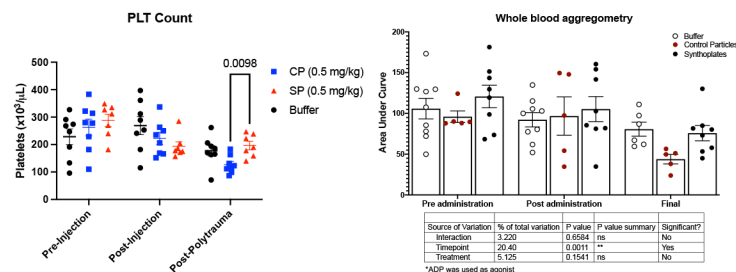
The following are the results from these studies:

F.1. Representative Systolic BP and MAP results:



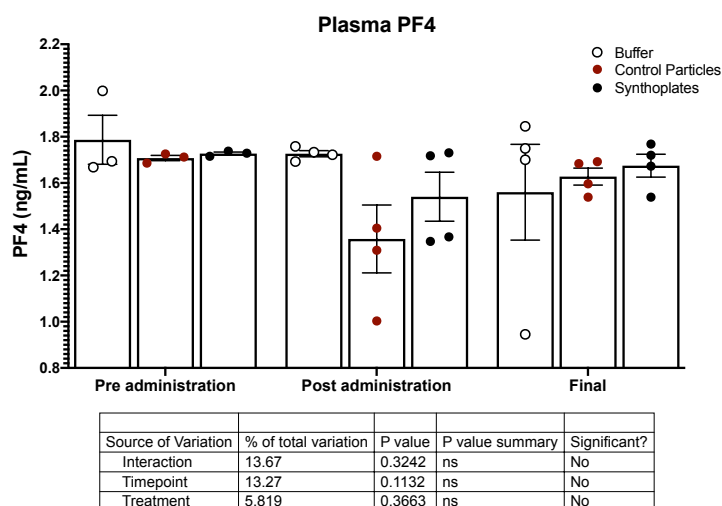
As evident from the representative data above, treatment of the traumatically injured rabbits with SynthoPlate (SP) seems to preserve systolic BP and MAP better than control particles (CP). This is indicative of a degree of BP stabilization potentially due to improved hemostatic effect in the SP-treated animals compared to the CP-treated animals.

F.2. Representative Platelet Count and aggregometry results:



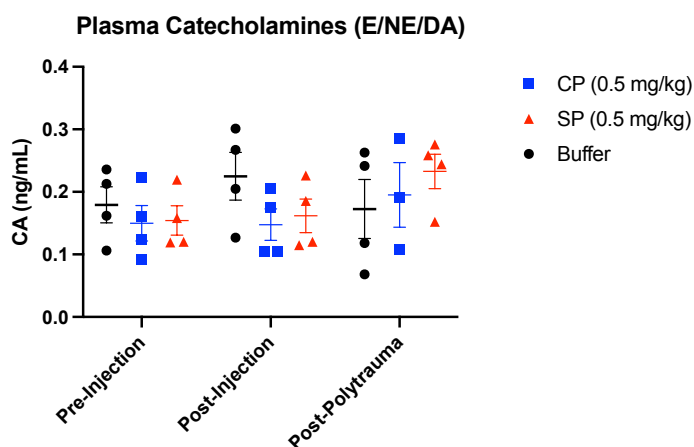
As evident from the data, SP does not affect native platelet count and native platelet aggregatory (ADP as agonist) capability. Interestingly, CP seems to have a deleterious effect on platelet count and aggregatory function. Thus, SP is safe towards native platelet status in rabbits.

F.3. Representative results from PF4 analysis:



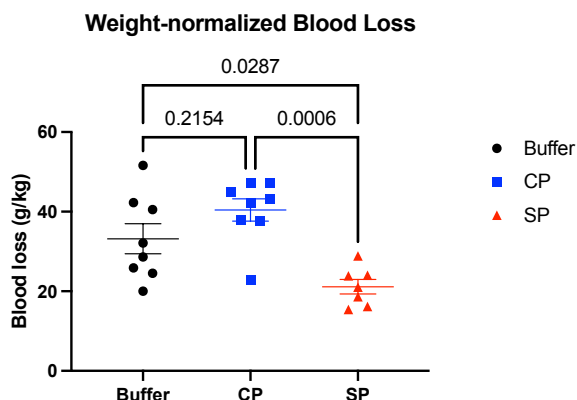
As evident from the PF4 analysis data, neither CP nor SP seems to impact the in vivo PF4 status in injected rabbits. This further corroborates that SynthoPlate does not affect the native platelet status in vivo.

F.4. Representative results from Catecholamine analysis:



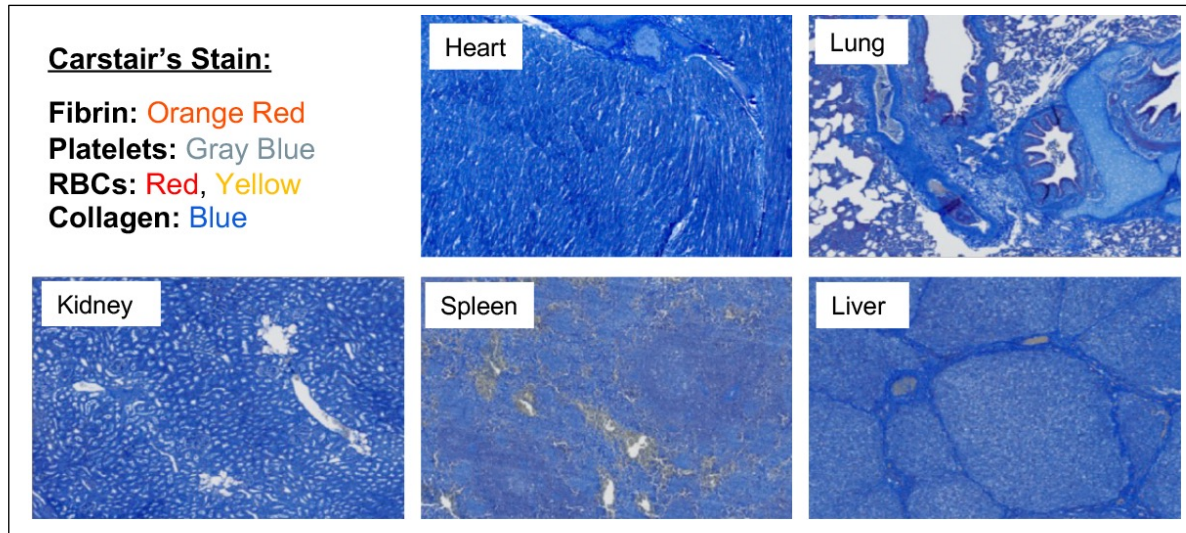
As evident from the catecholamine analysis data, neither CP nor SP seems to impact the in vivo systemic catecholamine status in injected rabbits. This suggests that SP or CP administration does not cause systemic duress in the injected rabbits.

F.5. Representative blood loss data in rabbits:



The blood loss analysis from the traumatically injured rabbits were the most exciting results from the studies. Rabbits administered with SynthoPlate (SP) showed a significantly reduced blood loss compared to sham (saline buffer-treated) as well as compared to control particle (CP) treated rabbits. This indicates improved hemostatic effect in rabbits when treated with SP.

F.6. Histology analysis of clearance organs in SP-treated rabbits with Carstair's Stain:



Carstair's staining was carried out in harvested clearance organs from SP-treated rabbits post euthanasia. The goal here was to determine whether SP dose poses systemic thrombotic risks in off-target organ/tissue beds. The above figure states the various staining colors for Carstair's, and shows representative histology data for the major clearance organs. As evident from the data, no major presence of orange-red fibrin or gray-blue platelets were observed in the any of the clearance organs. The presence of some platelets and RBCs in spleen and liver are expected as these are major storage and clearance organs for these blood cells, but the absence of fibrin indicates that there were on off-target thrombotic risks upon SP dosing in the rabbits. This corroborates the in vivo safety profile of SP.