AWARD NUMBER: W81XWH-18-2-0061

TITLE: **Intravenously Infusible Nanoparticles to Stop Bleeding and Increase Survival Following Trauma**

PRINCIPAL INVESTIGATOR: Erin Lavik

CONTRACTING ORGANIZATION: Maryland, University of, Baltimore County

REPORT DATE: October 2019

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
Oct 2019	Annual	**09/30/2018 - 09/29/2019**

4. TITLE AND SUBTITLE

Intravenously Infusible Nanoparticles to Stop Bleeding and Increase Survival Following Trauma

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Erin Lavik

E-Mail: elavik@umbc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Maryland, University of, Baltimore County

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Uncontrolled bleeding is the leading cause of death in battlefield traumas. Following injury, hemostasis is established through a series of coagulatory events including platelet activation. However, with severe injuries, these processes are insufficient and result in uncontrolled bleeding. We need a therapy that that can be administered in the field to stop internal bleeding. While many agents have shown promise in small animal models, translating the work to large animal models has been exceptionally in great part because of complement activation to intravenously infused agents. We have been developing hemostatic nanoparticles for a number of years, and in the last year, we developed a formulation that does not trigger complement activation upon infusion but does lead to hemostasis in a porcine model. We propose a pilot study to test these hemostatic nanoparticles in a large animal pressure-targeted hemorrhagic shock polytrauma model that approximates injuries seen in a combat setting.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified		

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1. Introduction

Uncontrolled bleeding is the leading cause of death in battlefield traumas. Following injury, hemostasis is established through a series of coagulatory events including platelet activation. However, with severe injuries, these processes are insufficient and result in uncontrolled bleeding. Immediate intervention is one of the most effective means of minimizing mortality associated with severe traumas, and yet the only available treatments in the field are pressure dressings and absorbent materials which are effective for exposed wounds but cannot be used for internal injuries. What we need is a therapy that can be administered in the field by a medic to complement the pressure dressings and stop bleeding.

We have developed hemostatic nanoparticles based on functionalized nanoparticles that halve bleeding in a rat major femoral artery injury following intravenous administration. The hemostatic nanoparticles are safe and stable at room temperature. They halt bleeding significantly faster than other treatments including recombinant factor VIIa (NovoSeven ®) and improve survival in a number of rodent models of trauma including blast injury models. These hemostatic nanoparticles are administered intravenously meaning that they are injected and can reach internal injuries.

In this proposal, we are planning to test these hemostatic nanoparticles in a large animal model of trauma. We propose a pilot study to test these hemostatic nanoparticles in a large animal pressure-targeted hemorrhagic shock polytrauma model that approximates injuries seen in a combat setting. This model uses pigs because they are very similar cardiovascularly to humans and are considered a critical model in testing new hemostatic materials. This particular injury model is one that closely resembles the kinds of injuries sustained by military personnel in combat.

While many agents have shown promise in small animal models, translating the work to large animal models has been exceptionally challenging in great part because of complement activation. Complement is part of the innate immune system and is supposed to recognize foreign invaders. When the complement system is activated as it generally is in large animal models with nanoparticles, vasodilation happens which means that the vessels dilate and blood flow increases. Not only is this seen in pig models, but at least 10% of humans exhibit the same significant response during infusions of the nanoparticle formulation, DOXIL, as well as infusions of biologics. It has been challenging to design a hemostatic nanoparticle that does not trigger this system, but we have preliminary data that shows that our particles do not do trigger the complement system and vasodilation and, in fact, trigger hemostasis following trauma.

Based on this, we want to thoroughly characterize the complement response to these materials and test them in the large animal model of combat trauma in a pilot, small scale study to determine if we have made what may be the first successful hemostatic formulation to treat internal bleeding in the field.

All of this work will give us the critical insights we need to move to the clinic. Traumatic injury is the leading cause of death for both men and women between the ages of 5 and 44 worldwide, and blood loss is the primary cause of death at acute time points post injury in both civilian and battlefield traumas. The work we are proposing here will allow us to determine the safety and efficacy of the particles in large animal models of trauma as a critical step towards translation of the technology to the clinic. This has application not only in conflicts, but in car accidents and other traumas that can involve multiple injuries and internal bleeding.

2. Keywords

C5a, C3a, complement, alternative pathway, coagulation, hemostasis

3. Accomplishments

Specific Aim 1: Determine the hemostatic efficacy and complement response to hemostatic nanoparticles in vitro as a function of hemostatic nanoparticle concentration

Specific Aim 2: Perform a pilot study to test the efficacy and safety using intravenous (IV) administration of hemostatic nanoparticles in a large animal (swine) pressure-targeted hemorrhagic shock polytrauma model

Specific Aim 3: Confirm the mechanism of augmented hemostasis

Task 1: Confirm that hemostatic nanoparticles do not activate complement over a range of doses

Subtask 1: Synthesize and characterize hemostatic nanoparticles and controls including previous generation of complement-activating particles

Ideally, poly(lactic acid)-*b*-poly(ethylene glycol) block copolymer nanoparticles exhibit negative zeta potential of -11mV due to presence of carboxyl groups on the surface of the nanoparticles [1]. These nanoparticles were found to cause complement activation when administered in a large animal trauma model, and a correlation was observed between zeta potential and complement activation. It was found that highly negative or highly positive nanoparticles cause complement activation leading to vasodilation whereas neutral nanoparticles (i.e. particles with zeta potential between -3 and 3 mV zeta potential) did not cause complement activation. [2] Hence, current efforts are directed towards controlling zeta potential of hemostatic nanoparticles, and to this end, the role of cationic surfactants are investigated in this section

Using Cationic surfactant to modulate zeta-potential of nanoparticles

Cetyl trimethylammonium bromide (CTAB) is a cationic surfactant consisting of a 16-carbon chain hydrophobic end and a tertiary amine in the hydrophilic end (Fig 1). In previous report we had discussed the role of CTAB along with few other surfactants in controlling zeta potential of nanoparticles. For concentrations ranging from 60-120 mg of CTAB, the shift of zeta potential was negligible after a certain shift. Nanoparticles without CTAB with same polymer blend usually have a zeta-potential of -16 mV. While initially there is a shift, at higher concentrations, the zeta-potential shift does not change significantly. Hence, polymeric blend with terminal amine groups where used, and based on the results, a higher prominent shift in zeta potential was observed.

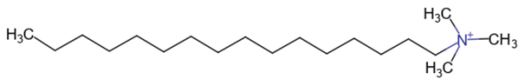

Figure 1: Cationic surfactant CTAB used to control zeta potential of hemostatic nanoparticles

The nanoparticles were prepared by dissolving the polymer in tetrahydrofuran and adding it drop wise to aqueous solution containing the cationic surfactant, i.e. hexadecyl methyl ammonium bromide (CTAB) dissolved in phosphate buffered saline. As stabilizer, 240mg of poloxamer was used in these nanoparticle batches. For the control nanoparticles, only poly(lactic acid)-b-poly(ethylene glycol) was used without any free amine side group or peptide conjugated polymer, with 120mg of poloxamer used as stabilizer. The nanoparticles were aliquoted for use and stored at -20 ℃. Lyophilization was done for 500ul of the nanoparticles, and the concentration of the nanoparticle in PBS solution was determined.

For polymeric blend consisting of PLLA-PEG-NH_2 and PLLA-PEG-GRGDS, as expected, increasing number of amines by increasing ratio of PLLA-PEG-NH_2 head to higher number of amine presence on nanoparticle surface, which resulted in positive shift in zeta-potential. Initially 45mg of PLLA-PEG-NH_2 and 45mg of PLLA-PEG-GRGDS were used. For further shift in positive direction, batches containing 60mg PLLA-PEG and 30mg of PLLA-PEG-GRGDS were prepared. The change in zeta-potential after a certain value is negligible for the nanoparticles with PLLA-PEG and increasing amount of CTAB (Fig 2). In case of using amine terminated and peptide terminated polymers, it is seen that at certain concentrations, the change is most significant in positive direction, while at concentrations of CTAB higher and lower than that, the change in zeta-potential is reversed (Fig 3). Using 120mg of CTAB, zeta potential of -3.44 mV was obtained while using concentrations of 100mg and 180mg resulted in zeta potential lower than the neutral range. This was further confirmed when PLLA-PEG-NH_2 and PLLA-PEG-GRGDS in 60mg and 30mg were used respectively. Using

130mg CTAB lead to lowest zeta potential of -0.223 mV while values higher and lower lead to zeta potential moving towards negative zeta potential (Fig 4).

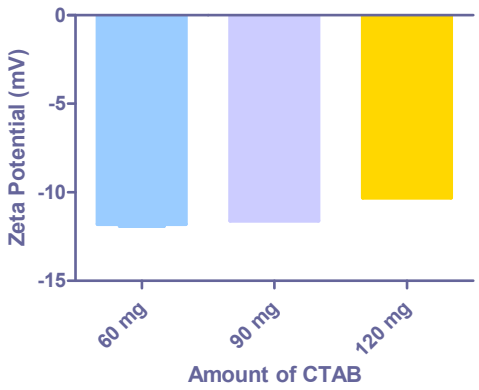

Figure 1: Change in Zeta-potential for nanoparticles with 90mg PLLA-PEG-COOH and 30mg PDLA

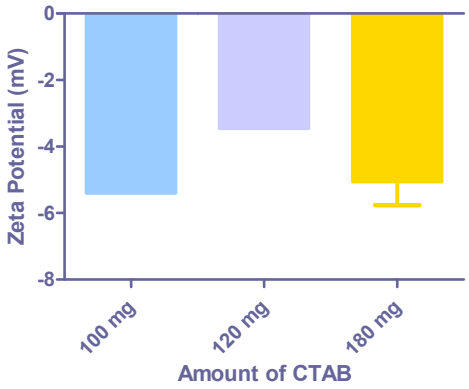

Figure 2: Change in Zeta-potential for nanoparticles with 45mg PLLA-PEG-NH2, 45mg PLLA-PEG-GRGDS and 30mg PDLA

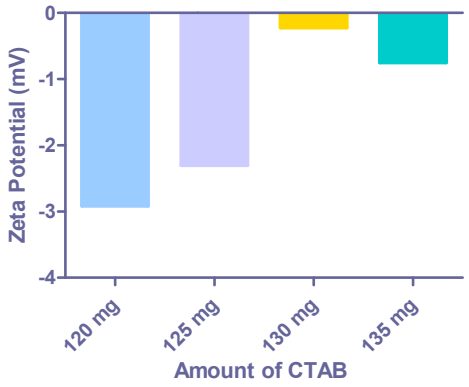

Figure 3: Change in Zeta-potential for nanoparticles with 60g PLLA-PEG-NH2, 30mg PLLA-PEG-GRGDS and 30mg PDLA

Based on the trend observed on effect of amount of CTAB and PLLA-PEG-NH2 on controlling zeta potential, nanoparticles were prepared by changing the ratios and amounts of the two to prepare nanoparticles of different charges and zeta potentials. These nanoparticles were then used in ELISA assays to quantify the complement protein levels and these were recharacterized during the assays as well. The results for the complement assays will be discussed in the next section. A major observation was that the zeta potential of the nanoparticles was drifting in the negative direction. The results are summarized below:

Table 1: The PLLA-PEG-NH_2+PLLA-PEG-GRGDS nanoparticles used in the complement assay [1]

Modification	Mean Zeta potential (mV)	Z-Average (nm)	Zeta Potential during assay
125 mg CTAB	-2.3±0.197	412.4±6.093	-4.94
130mg CTAB	-4.99±0.289	392±1.464	-6.41
130mg CTAB	-1.26±0.319	329±6.174	-4.29
130mg CTAB	-1.7±0.119	507.3±28.81	-3.29
130mg CTAB	-1.91±0.216	445.1±3.844	-5.51
Control	-16.3±0.556	329.2±1.762	-16.0

A stability study was then run for nanoparticles, were the suspensions were diluted in PBS and zeta potential was noted. It was observed that with higher amounts of dilution of the nanoparticles, the zeta potential drifted towards the negative, suggesting CTAB was dissolving out of the nanoparticles and moving into the aqueous phase (Fig 5).

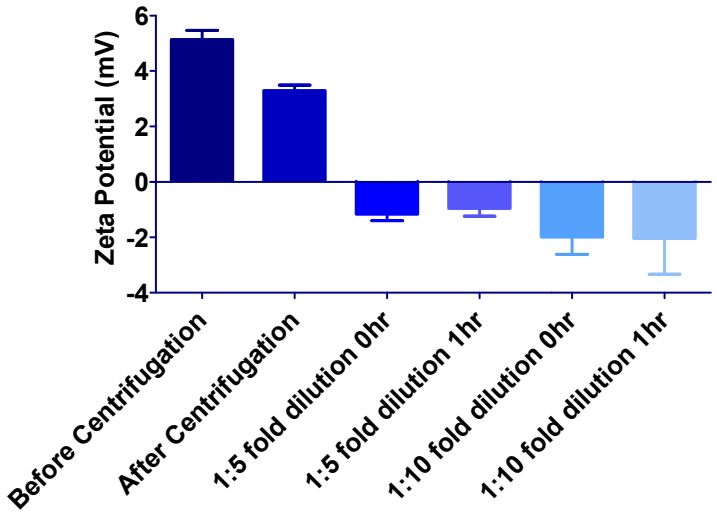

Figure 5: Change in zeta potential with increasing incubation time and dilution of nanoparticles measured in 10mM KCl solution

Currently, we are investigating few other approaches for stable formulations of the nanoparticles and investigating the impact of changing parameters in reaction for conjugating peptides. The goal is to reduce the amount of carboxyl groups on the surface which is the source of the neutral nanoparticles. Moreover, the role of dextran coating is also being investigated currently, were nanoparticles are being coated with dextran and its impact on complement activation will be investigated.

Subtask 2: Optimizing complement Assays for human and porcine whole blood with polymeric nanoparticles

The aim of these experiments was to determine the effect of zeta potential on complement protein levels in human and porcine whole blood. The nanoparticles with polymeric core and cationic surfactants were used in these experiments. The methods and results are summarized in this section.

[1] Batches mentioned include: NM-256, NM,SD-257, SD-001, NM,SD-259, NM-260, NM-261

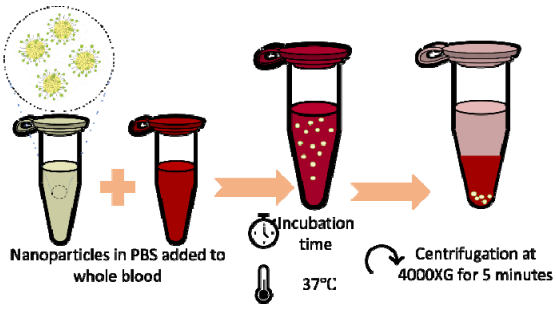

Figure 6: Heparinized whole blood is incubated with nanoparticles at dosage of 0.25mg/ml and 1mg/ml and incubated for 45 minutes (for human blood), and for 15 minutes, 30minutes and 45 minutes (for porcine blood) and plasma separated was used for complement assays

The fold change in C5a is represented in the figure below (Fig 7). Reducing zeta potential decreased the complement activation as seen for reduced amount of C5a. . The positive control zymosan showed the expected change in C5a levels. The change in complement level with changing zeta-potential was also observed, however, the fold change was less than 2-fold for all samples, crossing 2-fold for only the nanoparticles having zeta potential of -16.3 mV and the positive control zymosan.

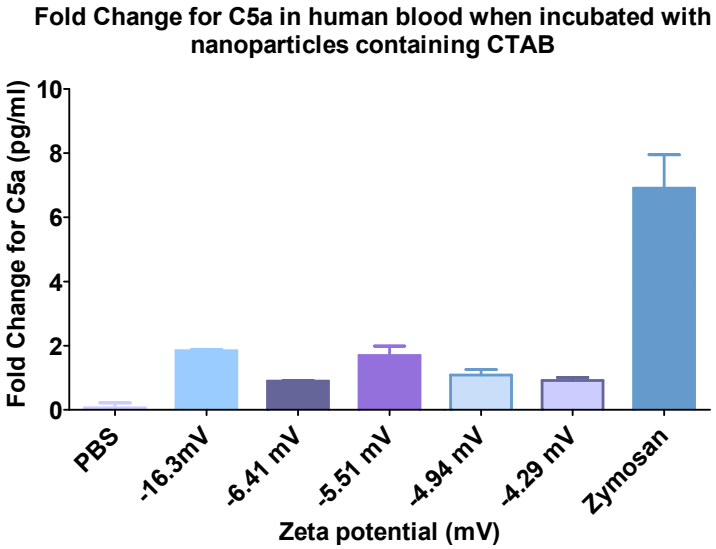

Figure 7: The fold change was determined in the human plasma samples with the 100-fold dilution of samples

Porcine whole blood was also used to determine complement activation due to the nanoparticles. Previously heparinized porcine plasma was used to determine the change in complement protein levels. Heparinized porcine whole blood was used to determine the changes in this study, as for plasma the change in complement protein levels for zymosan, a known complement activator, caused decreased C5a level with longer incubation time and higher concentration of zymosan. C5a level should not decrease of there is no complement activation or increase if activation takes place based on the pathway. This raised question, whether porcine plasma was an appropriate matrix , hence whole blood is used in these consequent experiments. The role of incubation time and dosage was investigated and as complement activation in porcine animals is faster, lower incubation times of 15 minutes and dosage lower than 0.25 mg/ml would help in generating signal that represents expected change in the porcine complement protein levels with changing zeta potential (Fig 8). Further optimization will be done in the porcine blood matrices for detecting the complement protein levels.

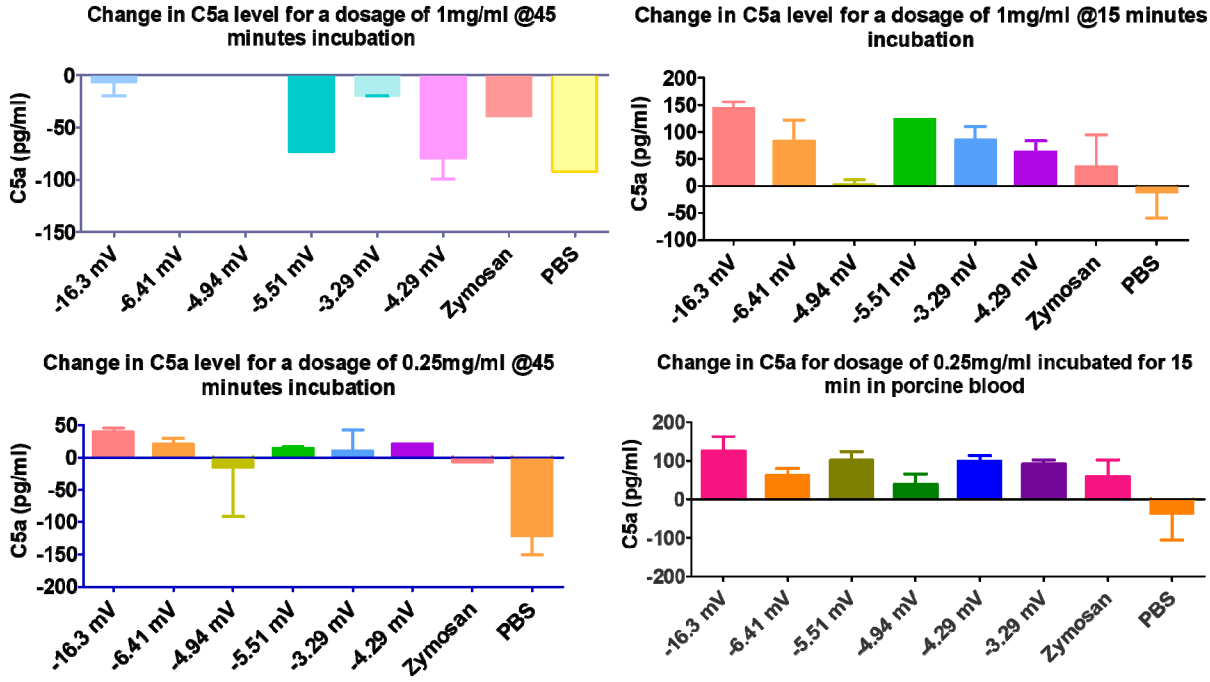

Figure 8: Impact of incubation time and nanoparticle dosage in complement activation in heparinized porcine whole blood

Discussion

Various methods have been investigated so far to control zeta-potential of nanoparticles and so far, using PLLA-PEG-NH_2 has resulted instable change in zeta-potential. However, as discussed in previous reports, the addition of PLLA-PEG-GRGDS leads to nanoparticles that are not entirely neutral (between -6 and -7 mV). Hence, we had investigated the role of cationic surfactants which have resulted in neutral nanoparticles, however their stability over time has needs to be further assessed. Whether longer carbon chain would lead to better anchoring of cationic surfactants into the nanoparticles would be a possible route of investigation. Currently, we are working on a reversed NHS/EDC conjugation of GRGDS peptide to PLLA-PEG-NH2 where carboxyl of the peptide will conjugate with the NH2 end of the polymer. This may help in reducing the exposed carboxyl groups on the surface and control zeta-potential. Traditionally, the amine end of the peptide binds to the carboxyl group of the PLLA-PEG through NHS/EDC conjugation. The role of dextran coating are also being investigated, where nanoparticles coated with dextran are prepared, characterized, and presence of dextran on nanoparticle surface will be confirmed through NMR. The prepared nanoparticles will be used in complement assays to investigate complement activation in blood matrices due to their presence. For initial assessment of these nanoparticles in complement activation, we have been working on optimizing complement assays. The role of serum is being investigated currently to compare with plasma and whole blood to determine suitable blood matrix for these assays. While C5a for human blood matrices have shown expected outcomes, assay more suitable for porcine samples is being investigated (C3 and C3a assay) and role of dosages and incubation times are being optimized.

Citations

1. Lashof-Sullivan, M., et al., *Hemostatic Nanoparticles Improve Survival Following Blunt Trauma Even after 1 Week Incubation at 50 (°)C.* ACS biomaterials science & engineering, 2016. **2**(3): p. 385-392.
2. Onwukwe, C., et al., *Engineering Intravenously Administered Nanoparticles to Reduce Infusion Reaction and Stop Bleeding in a Large Animal Model of Trauma.* Bioconjugate chemistry, 2018. **29**(7): p. 2436-2447.

4. Impact

Because we have had to delay the in vivo work until January, we have spent our time refining both the complement assays and the hemostatic nanoparticle synthesis. The result is that we have developed assays that are significantly more sensitive than previous assays for quantifying complement activation. This work is essential for developing infusibles that are safe for use in a range of applications, but it particularly important for developing particles that are suitable for application in trauma applications since trauma leads to complement activation in the absence of interventions.

The delay had another benefit which is that we used the extra time to refine the chemistry of the hemostatic particles so that, even in the absence of a complement inhibitor, they appear to not activate complement even at high concentrations. We will confirm this in vivo, but the in vitro data is quite promising.

5. Changes/Problems

Right now, our major concern is making sure the pig experiments can be carried out efficiently in January. We are continuing to work with companies to obtain the appropriate complement inhibitors.

6. Products

We are in the process of writing up the optimized assays for assessing complement activation in vitro in both porcine and human blood. We are also writing up a paper on the development of novel, molecularly controlled hemostatic nanoparticles with steal properties. We anticipate submitting these for publication during the first quarter of 2020.

7. Participants & Other Collaborating Organizations

The team at the Naval Medical Research Unit, San Antonio that is heading up the pig study is:

Commander Jacob Glaser, MD
Deputy Department Head
Naval Medical Research Unit-San Antonio (NAMRU-SA)

8. Special Reporting Requirements

PROTOCOL (_1 of _1 total):

Protocol NAVY-19-05: Local approval Feb 21, 2019, ACURO approval March 19, 2019

Title: Intravenously Infusible Nanoparticles to Decrease Bleeding and Increase Survival Following Polytrauma in a Swine Model (Sus scrofa domesticus)

Target required for statistical significance: 58

Target approved for statistical significance: 70 (Included 6 animals for Technical Refinement and 6 possible cull animals)

SUBMITTED TO AND APPROVED BY:

APPROVED BY 711TH HUMAN PERFORMANCE WING AT THE TRI-SERVICES RESEARCH LABORATORY 21 FEBRUARY 2019. APPROVED BY ACURO 19 MARCH 2019

STATUS: APPROVED

TOTAL PROTOCOL(S): 1

9. Appendices

A quad chart is attached.