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Santa Barbara

Accelerating Coagulation in Traumatic Injuries Using Inorganic Nanoparticles

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Chemistry

by

Damien Kudela

Committee in charge:

Professor Galen Stucky, Chair

Professor Craig Hawker

Professor Kevin Plaxco

Professor Linda Petzold

September 2015

The dissertation of Damien Kudela is approved.

Craig Hawker

Linda Petzold

Kevin Plaxco

Galen Stucky, Committee Chair

September 2015

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by

Damien Kudela

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VITA OF DAMIEN KUDELA
June 2015

EDUCATION

Bachelor of Arts in Chemistry, Cornell University, May 2008 (cum laude)
Bachelor of Arts in History, Cornell University, May 2008
Doctor of Philosophy in Chemistry, University of California, Santa Barbara, June 2015

PROFESSIONAL EMPLOYMENT

2006: Laboratory Assistant, Research and Development, Sanofi-Aventis, Bridgewater, NJ
2007-2008: Laboratory of Professor Jeffrey Varner, School of Chemical and Biological Engineering, Cornell University
2008-2009: GS-05 step 1, Office of the Science and Technology Adviser, US State Department, Washington, DC
2009-2011, 2015: Teaching Assistant, Department of Chemistry and Biochemistry, University of California, Santa Barbara
2009-2015: Laboratory of Professor Galen Stucky, Department of Chemistry and Biochemistry, University of California, Santa Barbara
2014-present: Founder and CEO, Cayuga Biotech

PUBLICATIONS

1. **D. Kudela**, A. May-Masnou, S. A. Smith, G. B. Braun, A. Pallaoro, T. T. Chuong, S. Hammond, M. J. Cohen, J. M. Galante, H. H. Rashidi, J. H. Morrissey, and G. D. Stucky. "Clotting Activity of Polyphosphate-Functionalized Silica Nanoparticles." *Angewandte Chemie International Edition*, 54 (2015), 4018-4022.
2. **D. Kudela**, A. May-Masnou, S. A. Smith, G. B. Braun, A. Pallaoro, T. T. Chuong, S. Hammond, M. J. Cohen, J. M. Galante, H. H. Rashidi, J. H. Morrissey, and G. D. Stucky. "Accelerating Clotting in the Presence of the Lethal Triad with Polyphosphate-Laden Silica Nanoparticles." *Nano Today*, (2015), submitted for publication.
3. **D. Kudela** and G. D. Stucky. "Inorganics and the Blood Coagulation System." *Journal of Trauma and Acute Care Surgery*, (2015), submitted for publication.
4. J. H. Yeon, N. Mainani, T. M. Schlappi, K. Y. T. Chan, J. R. Baylis, S. A. Smith, A. J. Donovan, **D. Kudela**, G. D. Stucky, Y. Liu, J. H. Morrissey, R. F. Ismagilov, and C. J. Kastrop. "Localizing polyphosphate enhances its ability to clot flowing blood plasma." *ACS Nano*, (2015), submitted for publication.
5. S.A. Smith, **D. Kudela**, A. May-Masnou, G. B. Braun, A. Pallaoro, T. T. Chuong, G. D. Stucky, and J. H. Morrissey. "Enhanced Factor XI-Dependent Thrombin Generation by Nanoparticle-Bound Polyphosphate." *Journal of Thrombosis and Haemostasis*, 11 (2013), 427.

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7. Y. Li, A. M. Sawvel, Y.-S. Jun, S. Nownes, M. Ni, **D. Kudela**, G. D. Stucky, and D. Zink. "Cytotoxicity and potency of mesocellular foam-26 in comparison to layered clays used as hemostatic agents." *Toxicology Research*, 2013; 2, 136-144.

AWARDS

Lindros Award for Graduate Students and Postdoctoral Scholars Translating Research to Medical Practice, 2014

UCSB New Venture Competition, 2nd place for Cayuga Biotech, 2014

UCSB Grad Slam, 2nd place, 2014

Nanotherapeutic drug selected by BioTechnology Acceleration Program (BioTAP) for licensable potential and development beyond early stage innovation, UCSB, 2013

Dobro Slovo Slavic National Honor Society, inducted 2012

Graduated BA, cum laude, in Chemistry from Cornell University, 2008

Honors Thesis, Professor R. Hoffmann, Chemistry, Cornell University, 2008

Dean's List Spring 2008, Fall 2007, Fall 2004, Cornell University

National Society of Collegiate Scholars, inducted Oct. 2005

Semi-Finalist, Presidential Scholars Award, 2004

FIELDS OF STUDY

Major Field: Accelerating Coagulation in Traumatic Injuries Using Inorganic Nanoparticles

The Effect of the Lethal Triad on Coagulation with Professor Galen Stucky

ABSTRACT

Accelerating Coagulation in Traumatic Injuries Using Inorganic Nanoparticles

by

Damien Kudela

Trauma remains the leading cause of mortality between the ages of 1 and 44 in the United States. Uncontrolled blood loss accounts for 50 % of all battlefield deaths and up to 25 % of civilian trauma deaths. This mortality is often the result of a severe clotting impairment known as acute traumatic coagulopathy. Therefore, hemorrhage control remains the *a priori* goal in the care of the critically injured patient. While great advances have been made in the resuscitation of the injured patient, attenuating bleeding and correction of coagulopathy remain vexing clinical problems. Current clotting treatments are plagued by concerns over excessive cost, poor stability, and safety issues.

In this defense, I present a silica nanoparticle (SNP) functionalized with polyphosphate (polyP) that mediates the body's natural clotting process. SNPs initiate the blood clotting system's contact pathway, while the endogenous short-chain polyP accelerates the common pathway via rapid formation of thrombin. This enhances the overall blood-clotting system, both by accelerating fibrin generation and by facilitating the regulatory anticoagulation mechanisms essential for hemostasis. Because of its low production cost, long-term stability at ambient conditions, and the potential to minimize side effects seen in

current treatments, the polyP-SNP therapeutic has the possibility to enable the body to re-establish hemostasis after traumatic injury, preventing massive blood loss and saving lives.

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I. Trauma

Midway upon the journey of our life
I found myself within a forest dark,
For the straightforward pathway had been lost.

Ah me! how hard a thing it is to say
What was this forest savage, rough, and stern,
Which in the very thought renews the fear.

So bitter is it, death is little more;
But of the good to treat, which there I found,
Speak will I of the other things I saw there.

I cannot well repeat how there I entered,
So full was I of slumber at the moment
In which I had abandoned the true way.
- Dante Alighieri, *Inferno*, Canto I, 1-12

A. Introduction

Hemorrhage control remains the *a priori* goal in the care of the critically injured patient. Uncontrolled blood loss accounts for up to 25 % of civilian trauma deaths and over 50 % of all battlefield deaths.(1-6) Within the first 48 hours after injury, hemorrhage causes the majority of trauma deaths (Figure 1).(7, 8) Current treatment calls for rapid transport to hospital for large volume resuscitation and surgical intervention to stem the loss of blood and continued resuscitation. Hemostatic resuscitation practices include aggressive blood product resuscitation rapid interventions, limited crystalloid infusion, surgical techniques to stem blood loss, and hemostatic adjuncts.(9, 10)

When a blood vessel is injured, the escaping blood comes into contact with tissue factor (TF) to initiate the extrinsic, or tissue factor, pathway of blood clotting (Figure 2). The extrinsic pathway is the biologically dominant mechanism for initiating coagulation in humans. F(actor) VII in the blood becomes activated (FVIIa) and binds with TF to form the

TF-FVIIa complex. Once complexed, TF-FVIIa accelerate activation of downstream FIXa and FXa. At the end of the tissue factor pathway, small concentrations of prothrombin are cleaved to produce thrombin.(11)

In addition to the extrinsic pathway, blood clotting can initiate via FXII and the intrinsic pathway through autoactivation or the introduction of negatively charged surfaces such as silica (Figure 2).(12-14) Once activated, FXIIa complexes with prekallikrein (PK) and high-molecular weight kininogen (HMWK) to produce larger quantities of FXIIa. In the presence of FXIIa, HMWK converts FXI into FXIa, which then converts FIX into FIXa.(15)

Whether initiated intrinsically or extrinsically, the activation of FIXa and FXa signal a shift from initiation to the common activation, or propagation pathway. The main goal of the common pathway is to accelerate coagulation and regulation of the blood clotting process through a thrombin burst, a mass production of thrombin (Figure 2).(16) This thrombin burst is produced by the prothrombinase (FXa-FVa complex) rapidly converting prothrombin into thrombin.(11) Aided by polyphosphate, thrombin autocatalyzes FXIa,(17) FVa,(18) FVIIIa,(19) to strengthen the mechanism and accelerate further thrombin production.(19) In the final stage of coagulation, thrombin converts fibrinogen into fibrin, activates FXIIIa,(20) and activates platelets(11) to assist in forming the physical clot that shuts injury and prevents further blood loss. FXIIIa cross-links fibrin monomers into polymers that form the backbone for strong clots.(16)

Because thrombin is so robustly procoagulant, it must be carefully regulated by the body. To prevent spread of coagulation away from the injury site, thrombin is captured by the surface-protein thrombomodulin (TM)(11) and inhibited by antithrombin-III (AT-III) (Figure 2).(21) This process turns thrombin into an anticoagulant. Upon capture by TM and AT-III,

thrombin promotes activation of protein C (aPC, Figure 2), which regulates the clotting cascade(11, 22-24) to keep the clotting localized. APC also accelerates fibrinolysis, the breakdown of the fibrin clot, which enables the vessel to heal after injury and return to hemostasis.(22-24) If overexpressed, thrombin's anticoagulant role can lead to severe clotting deficiencies in trauma patients.(23)

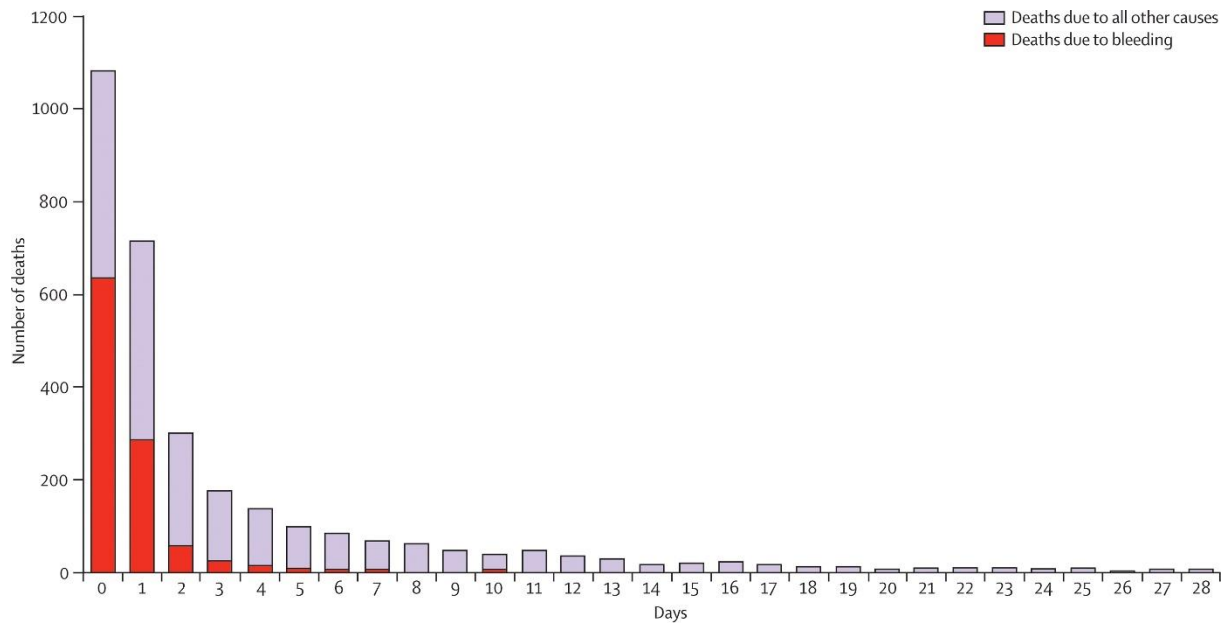


Figure 1. The majority of hemorrhage deaths occur within 24 hours of hospitalization. Reprinted from The Lancet, 376/6736, the CRASH-2 collaborators, Effects of tranexamic acid on death, vascular occlusive events, and blood transfusion in trauma patients with significant haemorrhage (CRASH-2): a randomised, placebo-controlled trial, 23-32, Copyright (2010), with permission from Elsevier.(8)

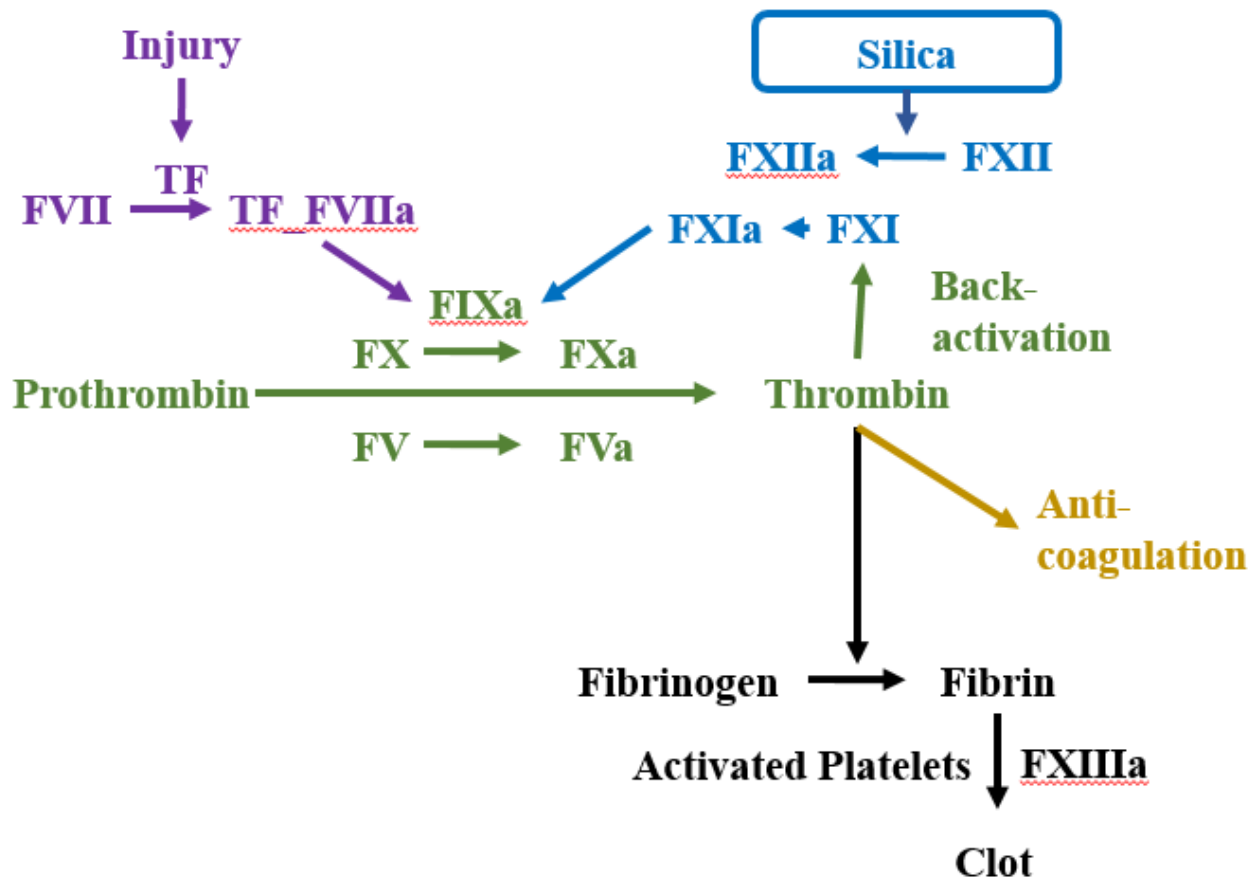


Figure 2. Simplified coagulation cascade. Coagulation can either be initiated by the exposure of blood to tissue factor (TF, extrinsic pathway) or to a negatively-charged surface such as silica that induces the activation of FXII through intrinsic (contact activation) pathway. Both initiation pathways meet at the common activation pathway, where FXa and FVa combine to produce large quantities of thrombin. In the final step, fibrinogen is converted into fibrin to form the physical clot.

Trauma management has been influenced by combat since the dawn of humanity. As combat injuries tend to be more grievous and difficult to treat, advances in military medical care are often adapted in short order by civilian medical personnel. Hippocrates once stated, “He who would become a surgeon, let him join an army and follow it.” Evidence of bandages used to treat wounds and prevent further blood loss dates back to Homer’s Iliad. Modern military medicine begins with Napoleon’s chief surgeon, Dominique Jean Larrey. Larrey is credited with pioneering both the use of triage in treating injured soldiers as well as the advent of ambulances to retrieve soldiers from the battlefield and bring them to field hospitals.(25)

While 15-25% of trauma deaths occur as a result of external or internal hemorrhage,(3) this number rises to nearly 50 % on the battlefield.(3-6) Over the past century, the killed in action rates for US soldiers have remained relatively stable between 20 – 25 % of all casualties (Figure 3).(26) However, overall combat mortality has decreased because of a reduction in wounded soldiers who later died of their wounds (Figure 4).(5) This reduction in combat mortality is a direct result of better treatment protocols aimed at preventing injured soldiers from subsequently dying of their wounds. In World War II, 19 % of the wounded soldiers died. A slightly smaller fraction, 15 % of the wounded soldiers, died in Vietnam.(4, 5) In Iraq and Afghanistan, the US case fatality rate dropped below 9 %.(27) This significant decrease in case fatality rate occurred because of a revolution in trauma care that emphasized prehospital management to improve the condition of patients prior to arrival, thus leading to increased survival rates.

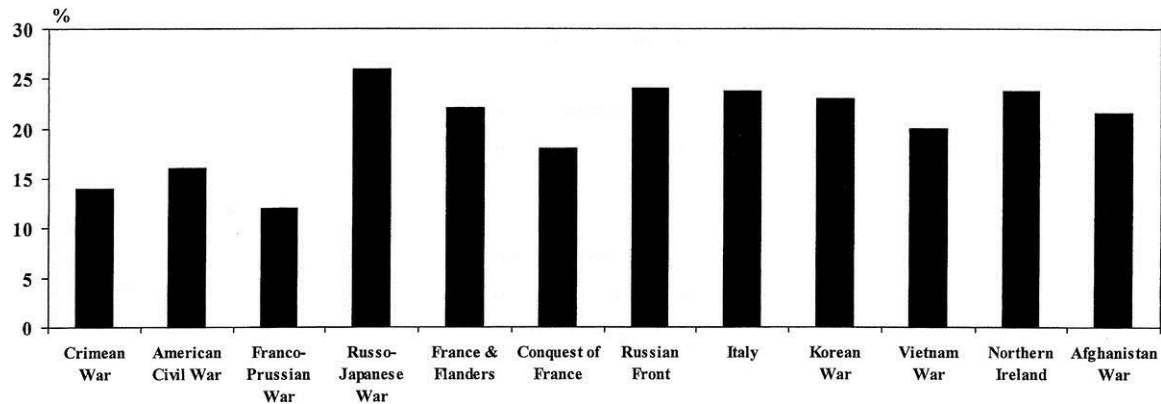


Figure 3. Percent of combat casualties killed in action from 1854-1989. Crimean War, 1854–55 (British battle casualties); American Civil War, 1861–65 (Union); Franco-Prussian War, 1870–71 (German); Russo-Japanese War, 1904–05 (Japanese); France and Flanders, 1914–18 (British); Conquest of France, 1940 (German); Russian Front, 1942 (German); Italy, 1944–45 (American); Korean War, 1950–53 (American); Vietnam War, 1964–73 (U.S. Marine Corps); Northern Ireland, 1970–84 (British); Afghanistan War, 1979–89 (Soviet). Reprinted from The Journal of Trauma and Acute Care Surgery, Vol. 54, Issue 5, HR Champion, RF Bellamy, P Roberts, A Leppaniemi, A profile of combat injury, S13-S19, Copyright (2003), with permission from Wolters Kluwer Health, Inc.(5)

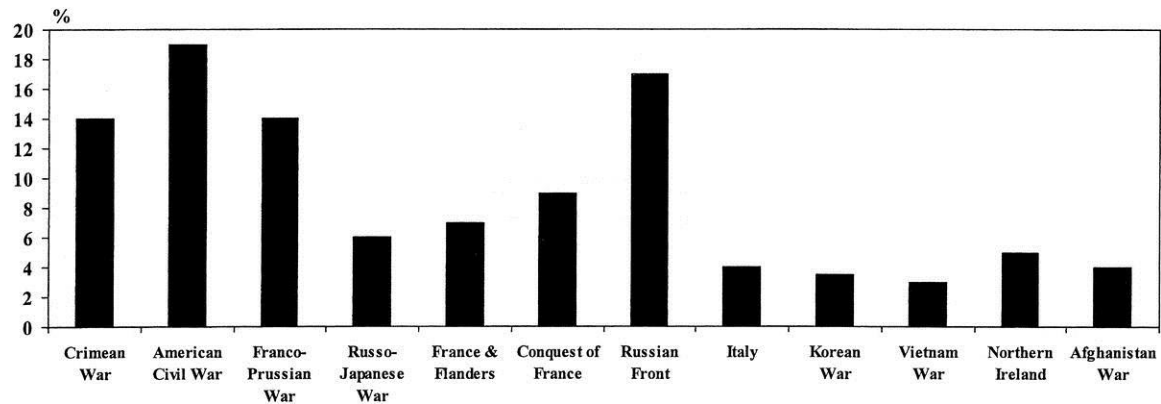


Figure 4. Percent of soldiers who died of their wounds after reaching a field hospital from 1854-1989. Crimean War, 1854–55 (British battle casualties); American Civil War, 1861–65 (Union); Franco-Prussian War, 1870–71 (German); Russo-Japanese War, 1904–05 (Japanese); France and Flanders, 1914–18 (British); Conquest of France, 1940 (German); Russian Front, 1942 (German); Italy, 1944–45 (American); Korean War, 1950–53 (American); Vietnam War, 1964–73 (U.S. Marine Corps); Northern Ireland, 1970–84 (British); Afghanistan War, 1979–89 (Soviet). Reprinted from The Journal of Trauma and Acute Care Surgery, Vol. 54, Issue 5, HR Champion, RF Bellamy, P Roberts, A Leppaniemi, A profile of combat injury, S13-S19, Copyright (2003), with permission from Wolters Kluwer Health, Inc.(5)

At the onset of Operation Enduring Freedom and Operation Iraqi Freedom, combat medics were unprepared for the type of casualties they would face. An unacceptable mortality rate continued to exist in the early years of the wars in Iraq and Afghanistan, when potentially survivable external wound deaths made up 15-28% of all combat fatalities.(5, 28) Of these potentially survivable deaths, 80 % occurred due to hemorrhage.(29)

One of the underlying reasons for increased mortality was a shift in the cause and location of injury. In Iraq and Afghanistan, explosions accounted for 72 % (65 % in Vietnam) of all injuries compared to 18 % (35 % in Vietnam) of wounds from gunshots.(30) The rise in injury due to non-penetrating or blunt injuries also led to an increase in internal injuries that are more difficult to treat due to their inaccessibility. Head and neck injuries made up 31 % of all casualties (16 % in Vietnam), truncal injuries rose to 27 %, (23 % in Vietnam), while extremity injuries fell significantly to 39 % (61 % in Vietnam).(30) As a result of inadequate treatment and protocols, the military needed to change trauma care protocols to adapt to new battlefield conditions. Spurred on by the US Special Operations Forces, by 2009, all six branches of the military had adopted the Tactical Combat Casualty Care (TCCC) guidelines for battlefield protocol in partnership with the Joint Trauma System (JTS) to systematically review combat casualties and their care.(28, 31) Improving prehospital care became the overarching focus due to the fact that 90 % of combat fatalities occur prior to arrival at a medical facility.(5) The TCCC and JTS have proven successful at training combat medical personnel to limit compressible, extremity bleeding mortality and have identified the first treatment protocol, using tranexamic acid, for patients suffering noncompressible hemorrhage on the battlefield.(28)

B. Acute Coagulopathy of Trauma

Clinicians have identified acute traumatic coagulopathy (ATC), an endogenous hypocoagulable state present in 25-40% of patients nearly immediately after injury.(32) ATC is a multifactorial process involving an endogenous hypocoagulable state occurring nearly immediately after injury complicated by issues such as the injury suffered, clotting abnormalities, cellular interaction, and inflammation (Figure 5).(33) Shock is suspected to instigate ATC after injury.(22)

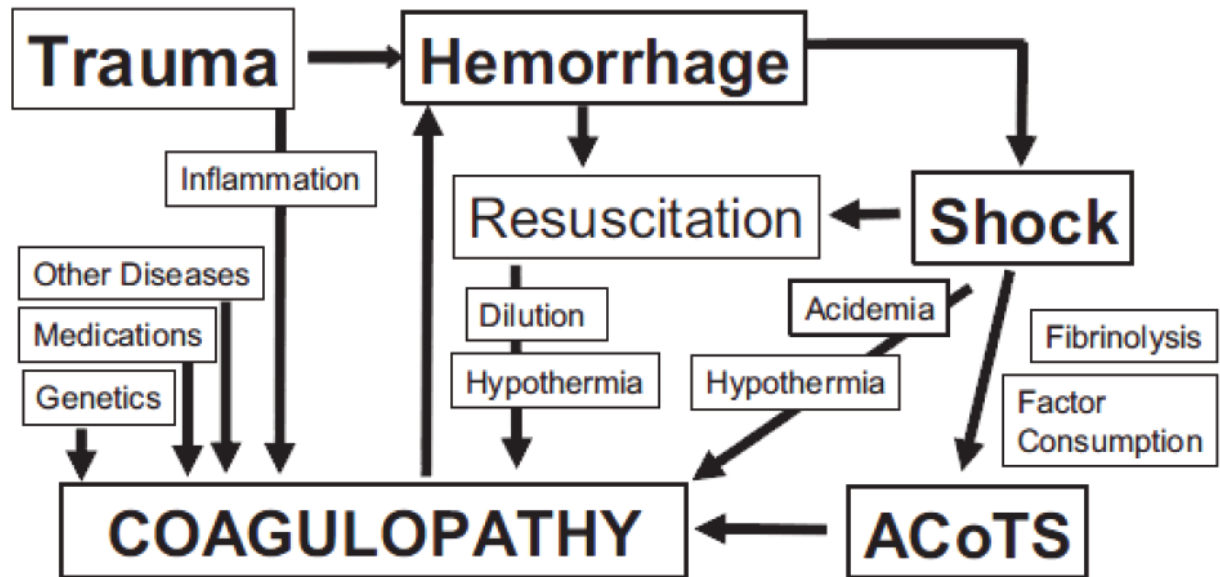


Figure 5. ATC develops endogenously as a response to multiple systemic factors including tissue injury and hemorrhage. Reprinted from Thrombosis Research, 129(5), D Frith, MJ Cohen, K Brohi, Animal models of trauma-induced coagulopathy, Pages No. 551-556, Copyright (2012), with permission from Elsevier.(34)

The onset of ATC is associated with worsened bleeding, increased need for transfusion, and a 4-fold increase in mortality.(33, 34) Within the first 24 hours after injury, coagulopathy results in an 8-fold increase in mortality.(35) This is due to a two-fold problem caused by coagulopathy. First, ATC leads to activation of protein C (aPC), which is the primary anticoagulant pathway. In the aPC pathway (Figures 6, 7), the thrombin that is produced at the wound site to accelerate clot formation is captured by thrombomodulin (TM) to form the thrombin-TM pathway.(22, 23) This converts thrombin from its main task as the key procoagulant in the clotting cascade(11) into a potent anticoagulant agent at the most critical time for the patient to clot.(22, 23)

Adding additional insult, the injury can dislodge tissue plasminogen activator (tPA) production from the endothelium into the bloodstream. In ATC, the elevated levels of aPC and tPA accelerate fibrinolysis (Figure 7), which is the breakdown of the physical fibrin clot into fibrin degradation products (FDPs). Thus, at the time the body has the greatest need to form clots to prevent further blood loss, the body's clotting mechanism becomes reversed. The protein needed to signal rapid clot formation instead initiates anti-clotting, while the clots that do form are quickly broken down and unable to stem the tide of blood loss.(22, 23) Medical treatment can exacerbate conditions and induce an iatrogenic coagulopathy through use of non-blood product resuscitative fluids that further deplete coagulation factors. The unfortunate sequelae of resuscitative efforts, iatrogenic coagulopathy, hypothermia, and acidosis form a 'lethal triad' that signal imminent mortality.(36) Therefore, it is no surprise that the appearance of ATC is a harbinger of mortality.

In the first 24 hours, doctors race against time to keep the patient from exsanguinating. Initially, coagulopathic patients receive massive transfusions of blood products to help

replenish procoagulant factors that have been lost.(22, 23, 33, 34, 37) If this proves insufficient, doctors move next to clotting agents such as the antifibrinolytic tranexamic acid(8) or recombinant clotting factors.(2, 38)

Even if the patient survives the critical first 24 hour period, grave risks other than hemorrhage complicate recovery. The risk for unwanted thrombotic events rises because the body is in a strongly hypercoagulant state to enable it to successfully clot in the presence of coagulopathy.(39, 40) The use of procoagulant agents to further induce clotting may raise the risk of thrombosis in trauma patients.(41) This results in a conundrum for doctors – how to treat bleeding and induce healthy clotting immediately after injury without putting the patient at-risk for life-threatening clots once the threat of exsanguination has passed?

In addition to major thrombotic events such as emboli,(39) the hypercoagulable state can also lead to disseminated intravascular coagulation (DIC).(42) In DIC, the hypercoagulable state results in procoagulant factor activation throughout the body despite the absence of injury. The resulting microthrombi can occlude healthy vessels (Figure 8).(42) However, only 5 – 10 % of all DIC cases are due to microthrombi.(43) Instead, DIC usually presents in patients as a secondary symptom of sepsis.(43) In this scenario, the whole-body hypercoagulable state exhausts the body's supply of clotting factors, leaving the patient again at risk for uncontrollable hemorrhage if sepsis or another condition leads to a later bleeding episode (Figure 8).(42) The only option for clinicians is to treat the underlying cause. Neither antifibrinolytics nor anticoagulants such as heparin are recommended for DIC patients as they can tip the precarious balancing act towards hemorrhage or thrombosis.(43)

In addition to thrombosis, patients who survive for 24 h after the onset of ATC face increased risks of organ failure, infection and inflammatory complications.(33) The

coagulation and inflammation pathways are interconnected as the body must first clot the wound after injury and then repair it via inflammation to return to homeostasis.(24, 44, 45) While necessary, this pathway can become perturbed. aPC is the integral anti-inflammatory protein.(24, 45) Because ATC leads to initial elevated levels of aPC, the hypercoagulable post-ATC state can lead to a crash in protein C levels.(46) In conjunction with DIC, unchecked inflammatory responses can damage organs,(42) increase the risk for infection,(44) and ultimately result in sepsis,(47) thus contributing to many of the non-hemorrhage trauma mortalities post-injury.

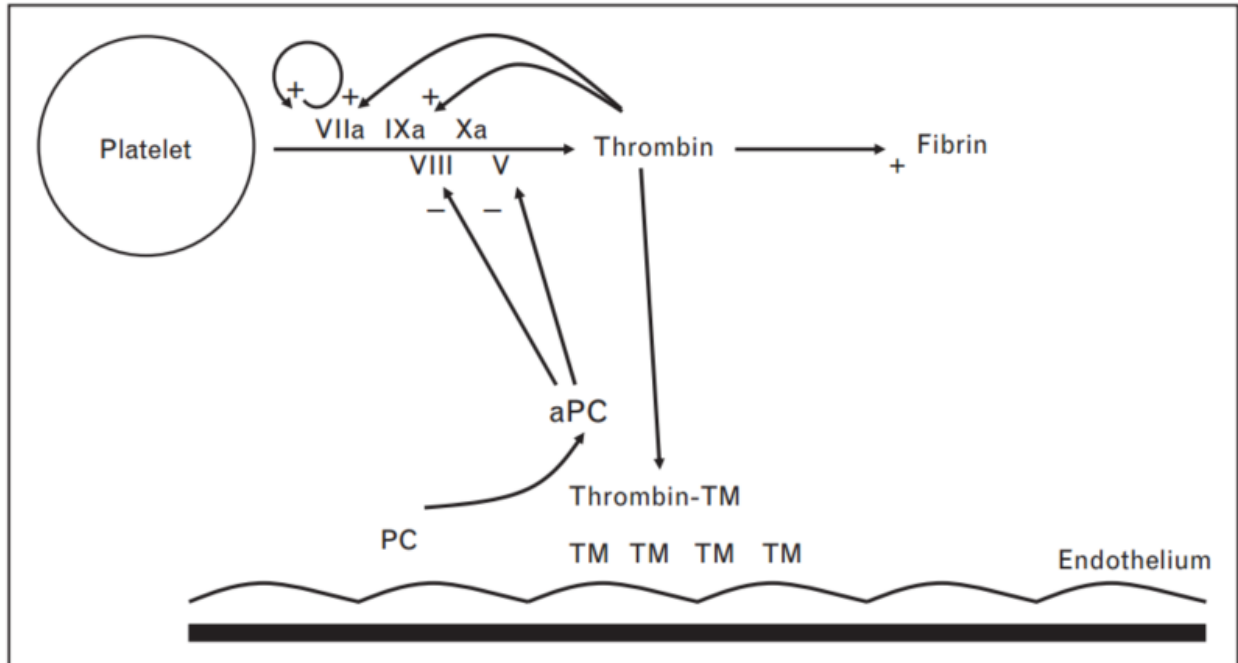


Figure 6. In ATC, thrombin binding to thrombomodulin (TM) accelerates. This increases activation of protein C (aPC) and leads to systemic dominance of the anticoagulant pathway. Reprinted from Current Opinion in Critical Care, 13(6), K Brohi MJ Cohen, R Davenport, Acute coagulopathy of trauma: mechanism, identification and effect, Pages No. 680-685, Copyright (2007), with permission from Wolters Kluwer Health, Inc.(22)

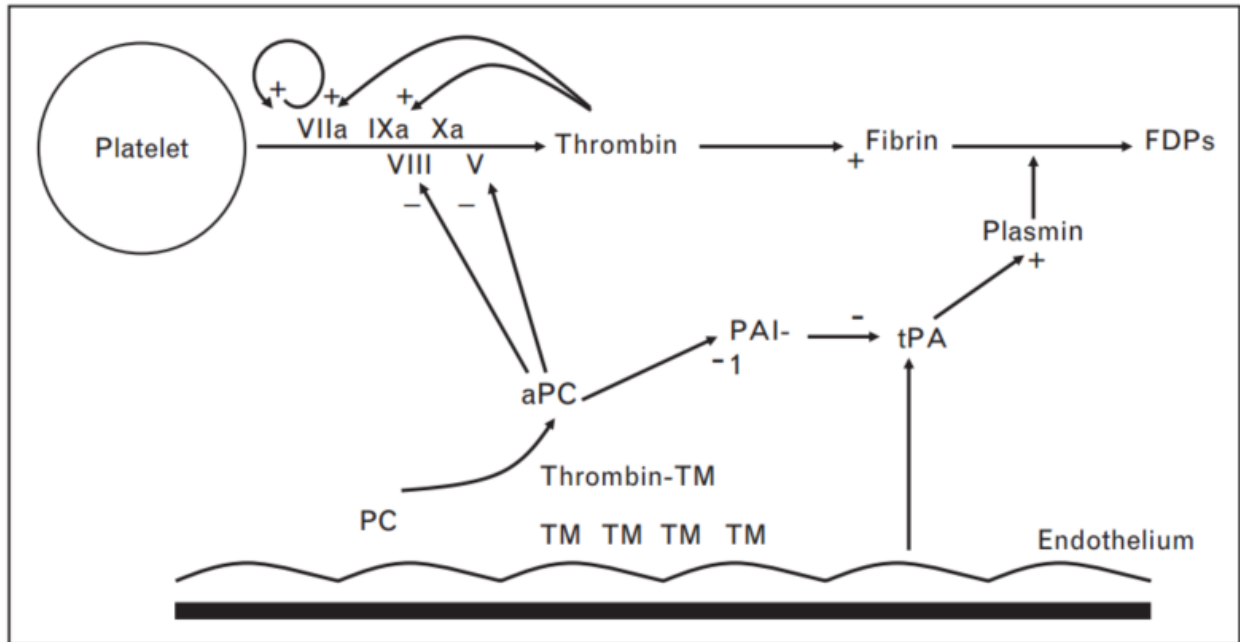


Figure 7. Injury leads to release of tissue plasminogen activator (tPA). Activated protein C (aPC) and tPA accelerate fibrinolysis – the breakdown of fibrin into fibrin degradation products (FDPs). Reprinted from Current Opinion in Critical Care, 13(6), K Brohi MJ Cohen, R Davenport, Acute coagulopathy of trauma: mechanism, identification and effect, Pages No. 680-685, Copyright (2007), with permission from Wolters Kluwer Health, Inc.(22)

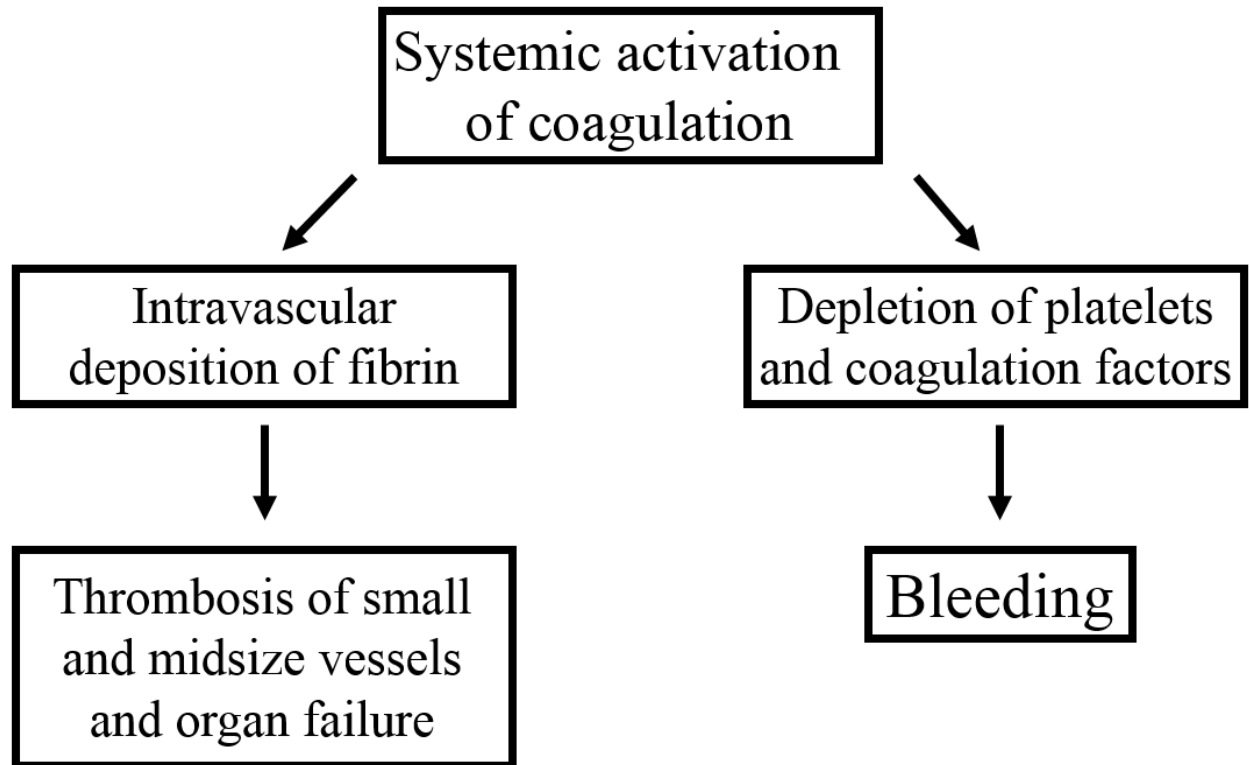


Figure 8. The hypercoagulable state that develops in response to ATC can lead to disseminated intravascular coagulation - a systemic activation of coagulation – that can cause two fatal conditions. Reproduced with permission from Levi M & ten Cate H (1999) Disseminated intravascular coagulation. *New England Journal of Medicine* 341(8):586-592, Copyright Massachusetts Medical Society.(42)

C. Blood Products

The use of blood transfusion to replace shed blood dates back to the First World War.(48) At the outset of the Second World War, US military medicine relied on freeze-dried plasma due to its ease of production, storage, and use.(48) Freeze-dried plasma was abandoned in favor of whole blood because it was ineffective in patients who went into shock.(49) Civilian blood banks were set up to answer this need, with 2,000 units of blood supplied per day to Europe and the Pacific in March 1945.(48) In Korea, guidelines dictated transfusing more whole blood than was lost as opposed to a 1:1 ratio in previous wars.(49) By the 1980s, whole blood transfusions began to be replaced by a combination of blood products - red blood cells (RBCs), fresh frozen plasma (FFP), and platelets.(48)

Severe trauma injuries often require massive transfusions – 10 or more RBC units in a 24 hour period – to replace blood that is shed after injury.(50-53) In combat hospitals, 5 % of patients receive massive transfusion.(50) The mortality rate is above 30 % for those receiving a massive transfusion of RBCs.(54) In civilian hospitals, 1 – 3 % of patients receive a massive transfusion,(53, 55, 56) 15 % of the most severe trauma patients receive a massive transfusion,(47) and the mortality rate for this population lies between 20 – 50 %.(7, 47, 51)

To determine the ideal transfusion protocol, varying transfusion ratios of blood products were tested (Figures 9, 10).(50) Researchers concluded that clinicians should aim for a ratio of

1:1:1 FFP:platelets:RBC to greatest survival rates and survival times (Figures 9, 10).(50, 57) In the subsequent Pragmatic, Randomized Optimal Platelet and Plasma Ratios (PROPPR) trial, the 1:1:1 ratio led to a slightly reduced rate of exsanguination deaths than 1:1:2.(58)

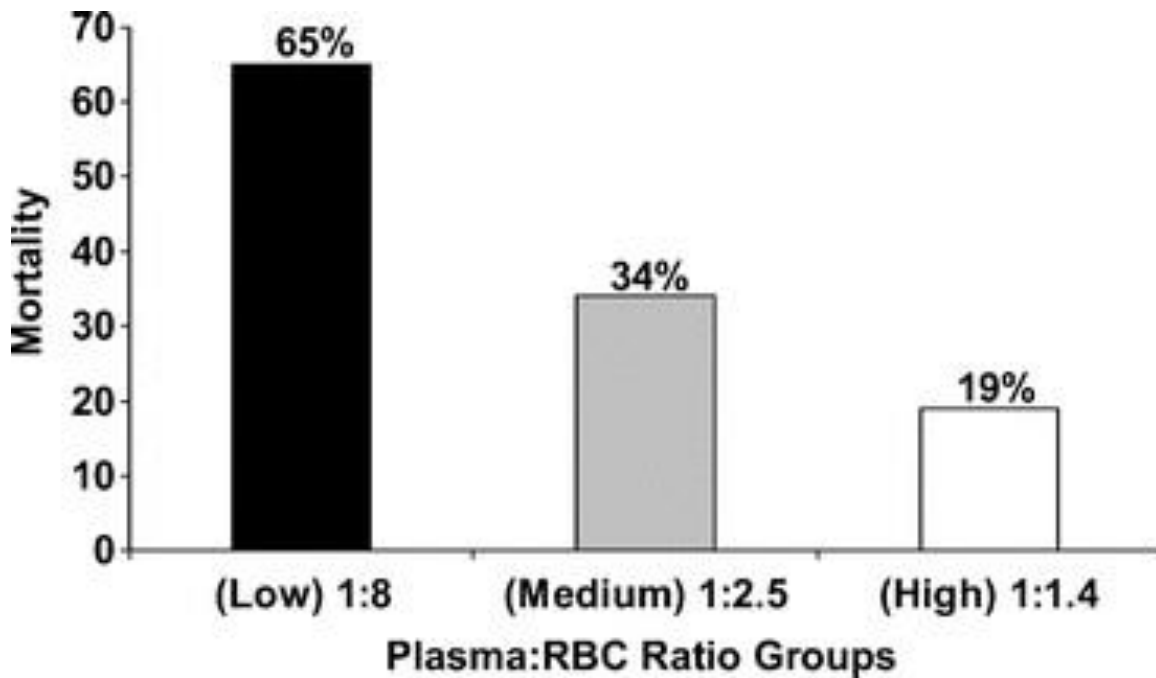
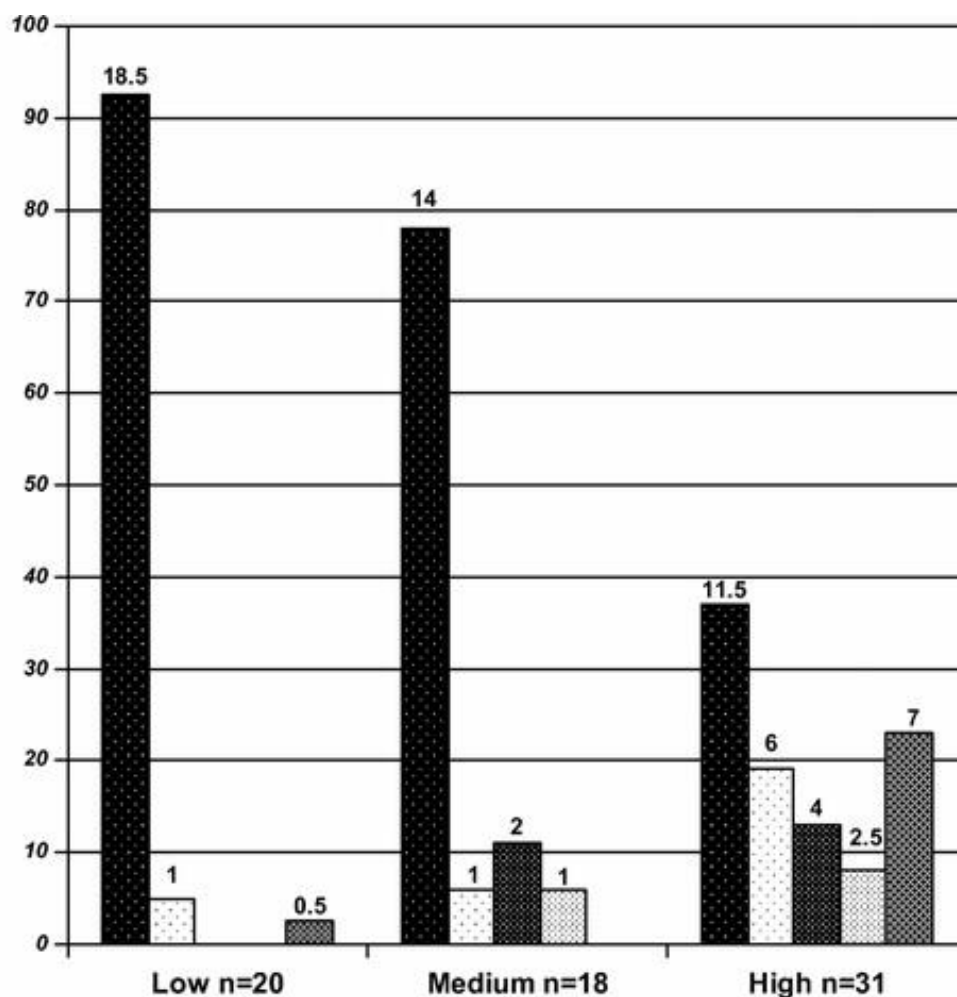


Figure 9. Transfusing plasma to RBC at a 1:1.4 ratio leads to low mortality rates. Reprinted from The Journal of Trauma and Acute Care Surgery, Vol. 63, Issue 4, MA Borgman, PC Spinella, JG Perkins, KW Grathwohl, T Repine, AC Beekley, J Sebesta, D Jenkins, CE Wade, JB Holcomb, The Ratio of Blood Products Transfused Affects Mortality in Patients Receiving Massive Transfusions at a Combat Support Hospital, 805-813, Copyright (2007), with permission from Wolters Kluwer Health, Inc.(50)








 Hemorrhage %†	92.5 ^a	78 ^a	37 ^b
 Sepsis %	5	6	19
 MOF %	0	11	13
 Airway/Breathing %	0	6	8
 CNS %	2.5	0	23
Time to death (hrs) ^{2*}	2 (1 – 4) ^a	4 (2-16) ^b	38 (4 – 155) ^c

Figure 10. Transfusing patients with a 1:1.4 plasma:RBC ratio decreases mortality due to hemorrhage and increases the survival time for a patient. Reprinted from The Journal of Trauma and Acute Care Surgery, Vol. 63, Issue 4, MA Borgman, PC Spinella, JG Perkins, KW Grathwohl, T Repine, AC Beekley, J Sebesta, D Jenkins, CE Wade, JB Holcomb, The Ratio of Blood Products Transfused Affects Mortality in Patients Receiving Massive Transfusions at a Combat Support Hospital, 805-813, Copyright (2007), with permission from Wolters Kluwer Health, Inc.(50)

D. Tourniquets

Though preventable given quick and effective treatment, patients suffering major arterial bleeds can exsanguinate in minutes.(59) Prior to 2003, tourniquet use was discouraged to prevent long-term tissue damage in limbs such as the need for amputation.(59) Even proponents of tourniquets followed the words of Larrey, who stated at the Battle of Borodino in 1812, “Better to lose the limb and save the life”. Quantitative tourniquet studies on the battlefield would not be undertaken until the turn of the 20th century with Lakstein’s retrospective study, which showed that the use of tourniquets in 91 Israeli Defense Force patients had a 100 % survival rate.(60) One of the most important results of the TCCC and JTS was the renewed emphasis of tourniquet use to treat major arterial bleed extremity injuries.(61)

Though extremity injuries fell to 39 % in Iraq and Afghanistan,(30) severe limb trauma accounted for up to 20 % of combat injuries.(60) As the rate of preventable deaths due to hemorrhage rose,(5, 28) TCCC guidelines adopted the use of tourniquets for prehospital control of extremity hemorrhage. In a study of patients at a combat hospital in Baghdad, Kragh et al. found that use of a tourniquet in the field increased survival by 13 %.(59) Survival rates skyrocketed to 80 % if the tourniquet was applied before the patient went into shock (Figure 11).(59) Over the 6 month period, Kragh found that tourniquet use saved 31 lives.(59) Overall, tourniquets have prevented mortality in as many as 2,000 US combat soldiers.(27)

Furthering the benefits of tourniquet use, Swan et al. tested conventional wisdom limiting tourniquet use if the injury is below the elbow or knee, if the patient reports pain as a result of tourniquet placement, or if manual pressure can be used to occlude the injured blood

vessel.(62) In all three conditions, conventional wisdom was invalid with a conclusion to use a tourniquet whenever possible to stop bleeding.(62) Along with the proliferation of hemostatic devices and tranexamic acid in trauma management (detailed below), tourniquet use was credited with preventing greater mortality after the Boston Marathon bombings.(27)

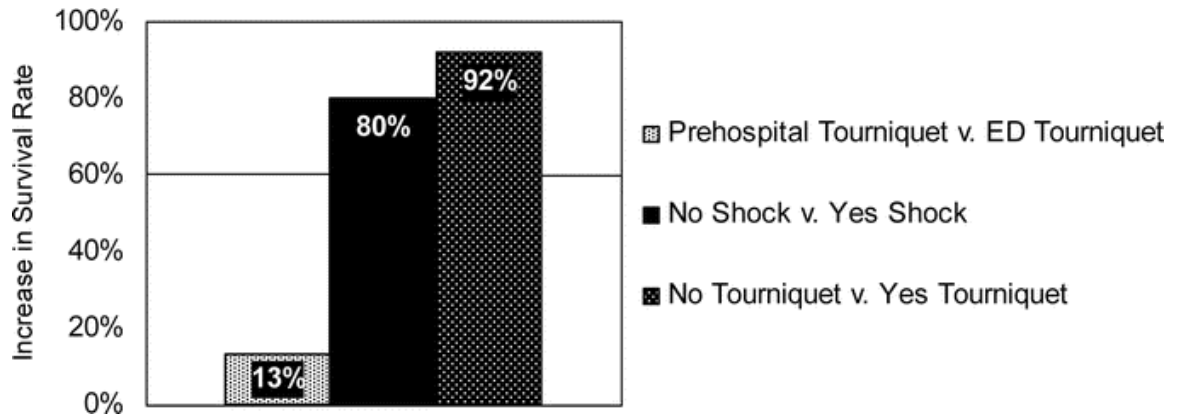


Figure 11. Prehospital tourniquet use in extremity injuries increased survival by 13 %. This rate increased to 80 % if the tourniquet was applied before the patient went into shock. Survival increased 92 % if a tourniquet was used at all. Reprinted from Annals of Surgery, Vol. 249, Issue 1, JF Kragh, TJ Walters, DG Baer, CJ Fox, CE Wade, J Salinas, JB Holcomb, Survival with emergency tourniquet use to stop bleeding in major limb trauma, 1-7, Copyright (2009), with permission from Wolters Kluwer Health, Inc.(59)

E. Topical Devices

For critical accessible injuries where tourniquets were ineffective, hemostatic application (delivery) agents such as impregnated gauzes were developed and rushed into service. In 2006, Pusateri, Alam, et al. postulated seven characteristics for an ideal hemostatic dressing. The ideal hemostatic dressing should (1) stop all arterial and venous bleeding within 2 minutes of application on the wound; (2) be ready to use immediately; (3) be simple to apply to the wound; (4) be lightweight and durable; (5) be stable and function at room temperature for at least 2 years and for several weeks at temperatures between -10°C and 55°C; (6) cause no adverse effects; (7) be inexpensive.(63)

Though many hemostatic agents were proposed and extensively tested, silica-based materials proved exceptional at improving clotting function for arterial bleeding. Silica-based materials clot quickly, are stable for years, are easily applied to wounds, and are inexpensive to produce, fulfilling Pusateri's characteristics of an ideal agent. While many variations of silica have been tested in preclinical and clinical trials, kaolin, the active ingredient in QuikClot Combat Gauze (Z-Medica, Wallingford, CT), was identified as a potential agent at UCSB,(64) found to meet all of the requirements put forth by Pusateri, Alam et al,(63, 65-69) and rushed to the battlefield.(70) As a result of this work, Professor Stucky was honored with the Department of Defense's Advanced Technology Applications for Combat Casualty Care Award in 2008. Kaolin-impregnated Combat Gauze remains the current device still recommended for all U.S. uniform service branches after seven years of field use under TCCC.(70) The identification and development of silica-based agents as clotting agents will be further explored in the second chapter.

Chitosan is a linear polysaccharide shellfish byproduct composed of (1–4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamide-2-deoxy-D-glucose (Nacetyl-D-glucosamine).(71) Sold commercially as the product Celox, chitosan is the preferred external wound agent used by the UK military medical command.(72) Upon contact with blood at the injury site, chitosan attaches to the damaged tissue and swells up to form a gel that seals the wound and prevents further blood loss.(73) Unlike dehydrated zeolite in the first generation of QuikClot,(63) chitosan does not release heat upon contact with blood.(73) Chitosan also acts outside of the coagulation milieu as it holds a positive surface-charge and acts by binding to anionic sites on red blood cells to initiate clotting.(71)

In addition to promoting clotting in open wounds, chitosan is also biocompatible and antimicrobial.(74) While no adverse effects have been reported with regard to shellfish allergies, the sole study on the safety of chitosan use in patients with shellfish allergies was conducted with only 10 participants.(75)

In conjunction with improved treatment protocols, the proliferation of silica-based hemostatic agents led to the reduction of case fatality rate in Iraq from nearly 20 % (76) at its peak in 2003 to 10.9 % during the troop surge in 2007-2008.(77) Adoption of TCCC guidelines, including kaolin silica hemostatic agents, by civilian trauma sectors were instrumental in preventing the loss of further life and limbs during the Boston Marathon Bombings.(27)

F. Recombinant Proteins

Recently, hemostatic compounds, such as recombinant human proteins, have been developed that are endogenous to the body. The most notable is recombinant factor VIIa (rFVIIa), the FDA's first approved recombinant protein treatment to be used in general

trauma patients despite its indication only for hemophilia patients. rFVIIa promotes hemostasis through its activation of the extrinsic coagulation pathway. rFVIIa binds to tissue factor, which is exposed at the injury site. As a secondary mechanism, rFVIIa also binds to platelets, increases thrombin burst, and increases clot strength.(38)

While recombinant proteins can be very effective, they also have serious limitations. Agents are designed for a specific subset of patients – e.g., rFVIIa for hemophiliacs. In general trauma patients, there are no FDA guidelines for dosage or administration and these drugs are often used as a last resort measure to save the patient.(78) In addition, recombinant proteins are extremely expensive. As such, drugs like rFVIIa are viewed as a panacea for late-stage bleeding when the reality is that these drugs are ineffective at this point of the trauma trajectory and could be far more effective if utilized before the patient's coagulation capabilities became irreparably impaired.(78) However, the excessive cost and safety questions in using these biological agents are major concerns that often limit use until late stage blood loss in trauma patients.(79)

Despite anecdotal successes, multiple studies showed that the use of rFVIIa does not significantly improve outcomes in bleeding patients and may result in thrombotic complications.(78, 80) O'Connell et al. found that use of rFVIIa in non-hemophiliac bleeding patients led to increased risk of thromboembolic event, particularly within rFVIIa's 2 hour half-life after rFVIIa administration cease.(41) In the larger CONTROL trial, rFVIIa was found to have no increase in thromboembolic risk compared to any other procoagulant treatment. More damning though, the CONTROL trial found that rFVIIa reduced blood product use but did not reduce mortality.(81) As a result, rFVIIa has fallen out of favor in the trauma community. Other agents, including prothrombin complex concentrates and

antifibrinolytics, are currently being evaluated but act within the coagulation milieu and are unlikely to provide improved coagulation and hemostasis in the setting of the severely impaired coagulation that is ATC.

In a study of 450,375 trauma patients, 1602 (0.36%) developed a venous thromboembolism after injury.(39, 40) Within the subset, roughly 600 suffered from a life-threatening pulmonary embolism. 18.7 % of patients with a pulmonary embolism died.(39) Risk factors for embolism include age, the type of injury suffered, presence of shock, and the need for major surgery.(39)

As with rFVIIa, prothrombin complex concentrates (PCCs) were developed for a specific subsection of hemorrhage patients.(82) PCC is composed of multiple proteins – prothrombin (FII), FVII, FIX, FX, and protein C – and is designed to quickly reverse the effect of the anticoagulant warfarin in patients who are hemorrhaging or require surgery.(83) As with rFVIIa, PCC is used off-label to treat trauma patients. Fries et al. found that in a dilutional coagulopathic model with a liver injury in swine, combining PCC with fibrinogen led to 100 % survival rate compared to less than 20 % with placebo.(84) In a similar swine liver injury model, Honickel et al. found that PCC and fibrinogen were also effective in minimizing blood loss and increasing survival rates under severely hypothermic conditions.(85)

PCC is now given with fibrinogen concentrate(86) to supplement the use of FFP. PCC and fibrinogen may improve coagulation profiles due to its ability to supplement the coagulopathic system with five unactivated factors as opposed to the single factor rFVIIa.(87) This increases the likelihood that coagulopathy cannot impair all five factor mechanisms. Aside from cost, the greatest concern with combined PCC and fibrinogen usage is the potential for thromboembolic events. In a study conducted at the University of Arizona,

7 % (3 of 45) of trauma patients receiving PCC and fibrinogen suffered unwanted thrombosis.(88) However, this risk of embolism was consistent with rates seen in patients receiving FFP without PCC.(87)

G. Tranexamic Acid

Tranexamic acid (TXA) is a synthetic version of lysine used in patients at risk for bleeding complications.(89) TXA was among a group of antifibrinolytics originally developed to minimize blood loss in patients at risk for postoperative complications.(90) Due to its success in reducing need for blood transfusion by one-third,(90) TXA use grew to include patients suffering traumatic injuries. In a Clinical Randomisation of an Antifibrinolytic in Significant Haemorrhage (CRASH-2) study, TXA lowered hemorrhage mortality significantly when compared to saline placebo ($p = 0.0077$, Table 1) in over 20,000 civilian patients.(8) As an antifibrinolytic, TXA is thought to reduce blood loss and overall mortality by binding to plasminogen, thereby inhibiting plasminogen's ability to break down the physical fibrin clots.(8) However, further studies on TXA and other antifibrinolytics posit that the mechanism for lowering mortality may be caused by TXA's ability to minimize the inflammatory effects of the protein plasmin.(91)

Table 1. Tranexamic acid lowered bleeding mortality without increasing non-bleeding mortality. Reprinted from The Lancet, 376/6736, the CRASH-2 collaborators, Effects of tranexamic acid on death, vascular occlusive events, and blood transfusion in trauma patients with significant haemorrhage (CRASH-2): a randomised, placebo-controlled trial, 23-32, Copyright (2010), with permission from Elsevier.(8)

	Tranexamic acid (n=10 060)	Placebo (n=10 067)	Relative Risk (95% CI)	p value (two-sided)
Any cause of death	1463 (14.5%)	1613 (16.0%)	0.91 (0.85–0.97)	0.0035
Bleeding	489 (4.9%)	574 (5.7%)	0.85 (0.76–0.96)	0.0077
Vascular occlusion	33 (0.3%)	48 (0.5%)	0.69 (0.44–1.07)	0.096
Multiorgan failure	209 (2.1%)	233 (2.3%)	0.90 (0.75–1.08)	0.25
Head injury	603 (6.0%)	621 (6.2%)	0.97 (0.87–1.08)	0.6
Other causes	129 (1.3%)	137 (1.4%)	0.94 (0.74–1.20)	0.63

Furthermore, the benefit of TXA is strictly time-dependent: TXA administration immediately after injury led to a much greater survival rate in patients.(92) Conversely, the use of TXA more than 3 hours after injury decreases survival rate (Figure 12), potentially due to the increased likelihood of disseminated intravascular coagulation in later-stage trauma patients.(92) The use of tranexamic acid within 1 hour lowered hemorrhage-related mortality from 7.7 % to 5.3 % compared to placebo; treatment between 1 – 3 hours lowered mortality from 6.1 % to 4.8 %.(92)

Based on the CRASH-2 study, the military adopted TXA as a key component of clinical hemorrhage care.(10) Army studies suggest that TXA remains stable in prehospital conditions for up to 12 weeks, though it is primarily used by surgeons in a hospital setting.(93) As in the CRASH-2 study, the Military Application of TXA in Traumatic Emergency and Resuscitative Surgery (MATTERs) found that TXA did lead to reduced mortality and need for massive transfusion for patients who survived for at least 24 hours. The greatest statistical data point for TXA was that it limited the need for massive transfusions in 1 out of 7 patients, whereas it produced a mortality benefit of only 1 in 67 patients.(10) However, the MATTERs study found no statistical difference between TXA and non-TXA use in preventing mortality within 24 hours.(10) The use of TXA in more severely injured patients is one of the many reasons that may account for this discrepancy. More concerning was the fact that TXA use in combat situations led to unwanted thrombotic events. However, the study size was too small to assess the risk for unwanted thrombosis as a direct result of TXA use.(10) While TXA is established as an excellent agent for reducing blood loss and restoring hemostasis, newer drugs are still needed to improve mortality in the 24 hour period after injury when the patient is at greatest risk for exsanguination.

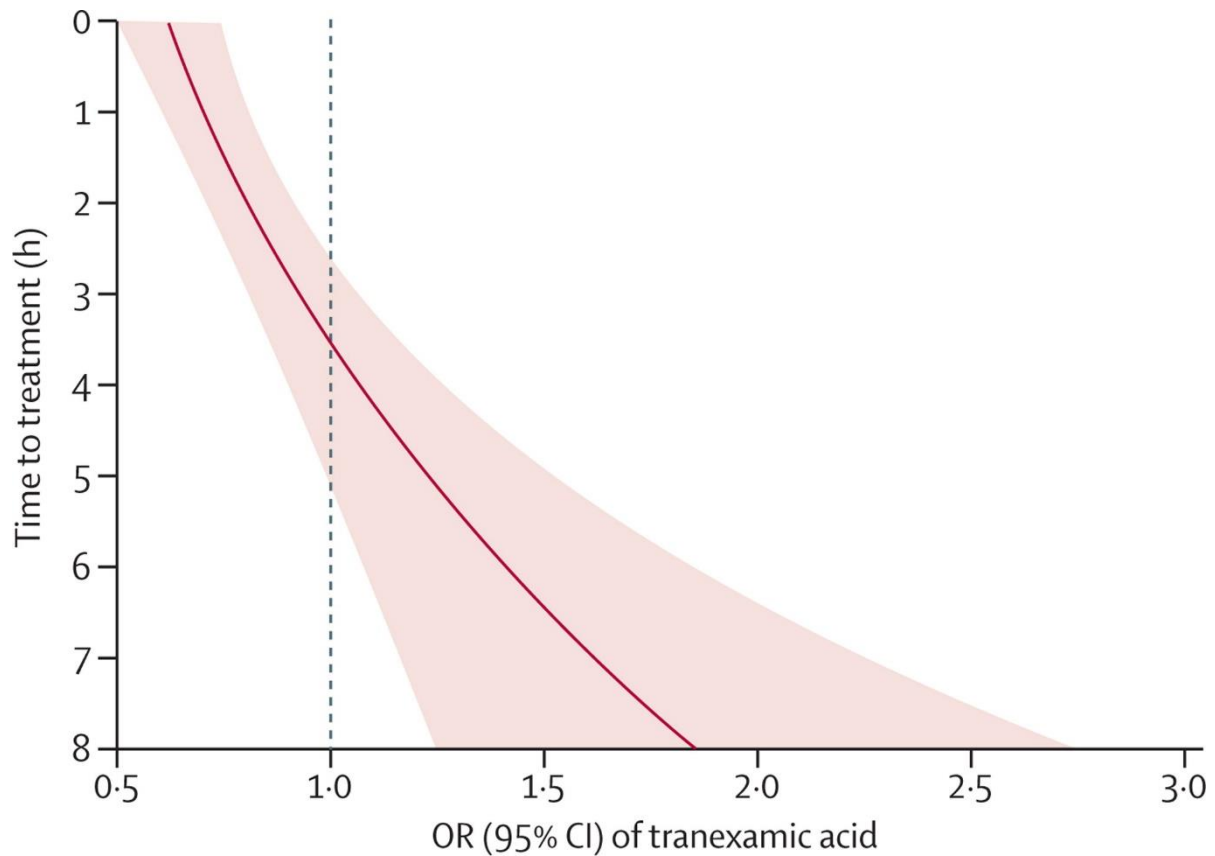


Figure 12. Survival odds ratio (OR) with a 95 % confidence interval (CI) for patients given TXA as opposed to placebo. OR is at 0.61 if TXA is given immediately upon injury and rises by a factor of 1.15 every hour until TXA. After 3 hours, patients given a placebo are more likely to survive. Reprinted from The Lancet, 377/9771, the CRASH-2 collaborators, The importance of early treatment with tranexamic acid in bleeding trauma patients: an exploratory analysis of the CRASH-2 randomised controlled trial, 1096-1101, Copyright (2011), with permission from Elsevier.(92)

H. Conclusion

The development of coagulopathy in the trauma patient is a key warning that intervention is required for survival. There are a myriad of agents that can aid the impaired clotting system to help replenish the blood that has been shed and prevent further loss of blood. At this time, prothrombin complex concentrates and tranexamic acid are the two most utilized clotting agents in trauma surgeries to restore clotting capabilities in patients. However, these agents are not without limitations. The extreme cost, storage requirements, and risk for unwanted thrombotic events if used contrary to indications must be weighed by the clinician before using these agents. One potential treatment that can mitigate these questions is the inorganic compound silica. Whether on its own or used to deliver a procoagulant payload, silica is used in a new class of agent that is cheaper, stable at ambient conditions, and functionalized to prevent unwanted coagulation. It is the preferred agent for point of care topical application as a hemostasis agent to stop major arterial bleeding that is unexpectedly induced either by combat or civilian trauma situations.

II. Silica Hemostatic Agents

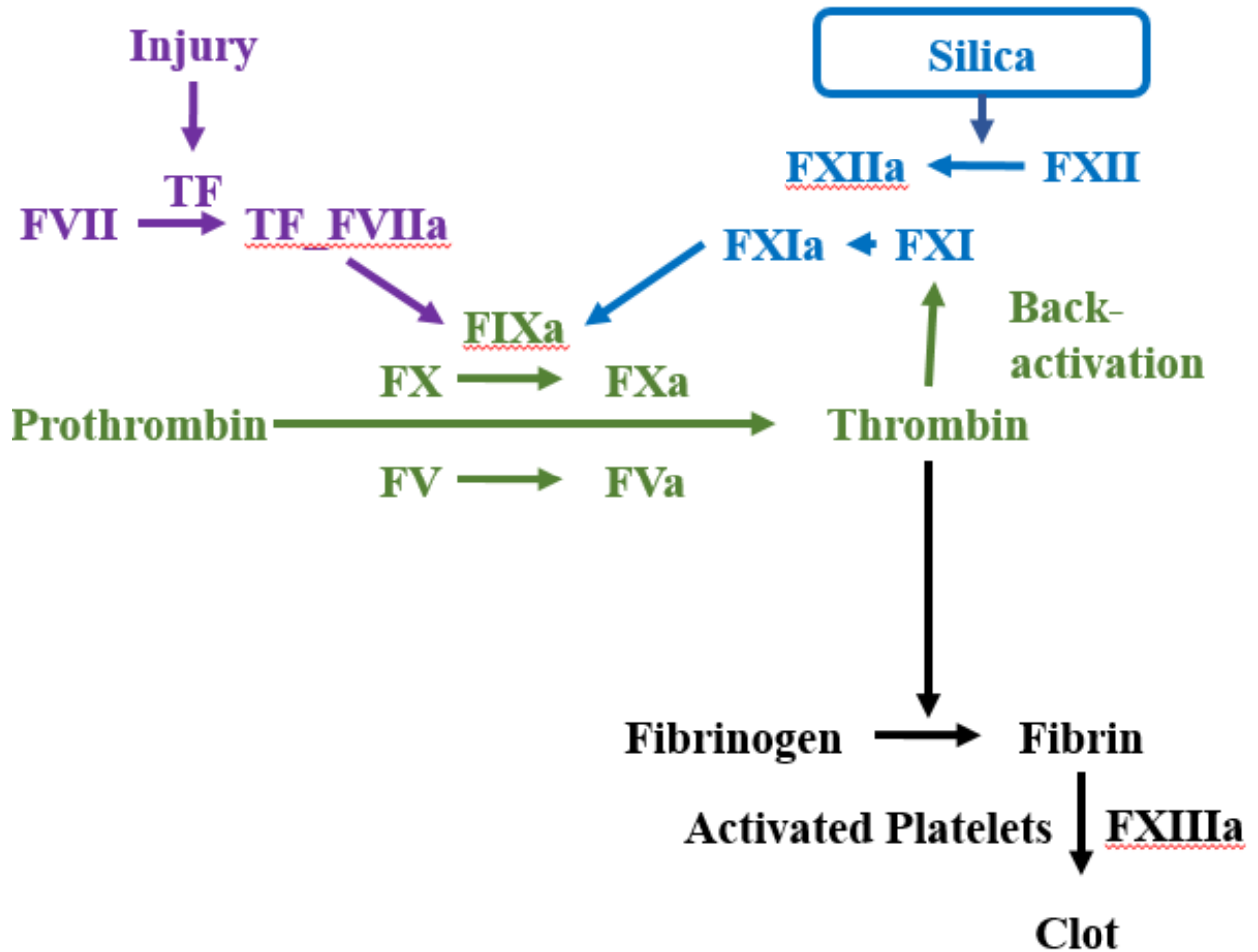
The fate of the wounded lays with those who apply the first dressing.

- Col. Nicholas Senn, US Army, 1844 - 1908

A. Introduction

Ratnoff first discovered Factor (F)XII, then known as the Hageman factor, in 1955 in diagnosing a patient who had prolonged clotting times for intrinsic pathway tests without abnormal clotting function during surgery.(94) By 1961, Margolis had discovered that FXII was activated by adsorption onto a silica nanoparticle surface, which initiated coagulation (Scheme 1). He further found that the clotting activity depended on the size of the silica particle, with peak clotting activity occurring at 30 nm.(95) While researchers used materials such as silica glass and kaolin for their clotting assays, they were not considered for hemostasis treatment.(96)

Scheme 1. Simplified coagulation cascade. In the presence of blood, silica induces the activation of FXII to initiate the intrinsic (contact activation) pathway.



B. Use of naturally occurring silicates to control bleeding.

Despite knowledge of its effect on FXII and the intrinsic pathway, the use of silica or silicates for hemorrhage treatment was not recognized until the 21st century. Out of many inorganic oxide compounds, a 3-D nanoporous aluminosilicate zeolite with a water accessible surface area of ~500 m²/g proved effective in lowering clot time while also accelerating clot growth (Figure 13).(13) This dehydrated calcium substituted zeolite (Na_{0.5}Ca_{5.75}(SiO₂)₁₂(AlO₂)₁₂·xH₂O), used commercially for nitrogen-oxygen separation from dry air, was serendipitously (97) found to be a good coagulating agent, and was initially applied in the same dehydrated powder particulate form as used for oxygen-nitrogen separation. This was the original aluminosilicate (QuikClot (QC)) that was used as a therapeutic for arterial bleeding in the field.

In 2004, Alam et al. showed that application of this zeolite-impregnated gauze reduced mortality to 0 % in swine with a severe groin injury (Figure 14).(98) Pusateri et al. found that impregnating the silicate zeolite on gauze for treating wounds reduced blood loss, reduced the need for resuscitative fluids, hastened the animal's return to hemostasis, and increased survival by 76 % on a lethal liver injury (Figures 14 and 15, Table 2).(63, 98) This marked a major improvement over what was then the state of the art for field use. However, there was intense heat released due to the exothermic adsorption of water onto the zeolite's dehydrated, high surface area (~500 m²/gm) 3-D nanostructure.

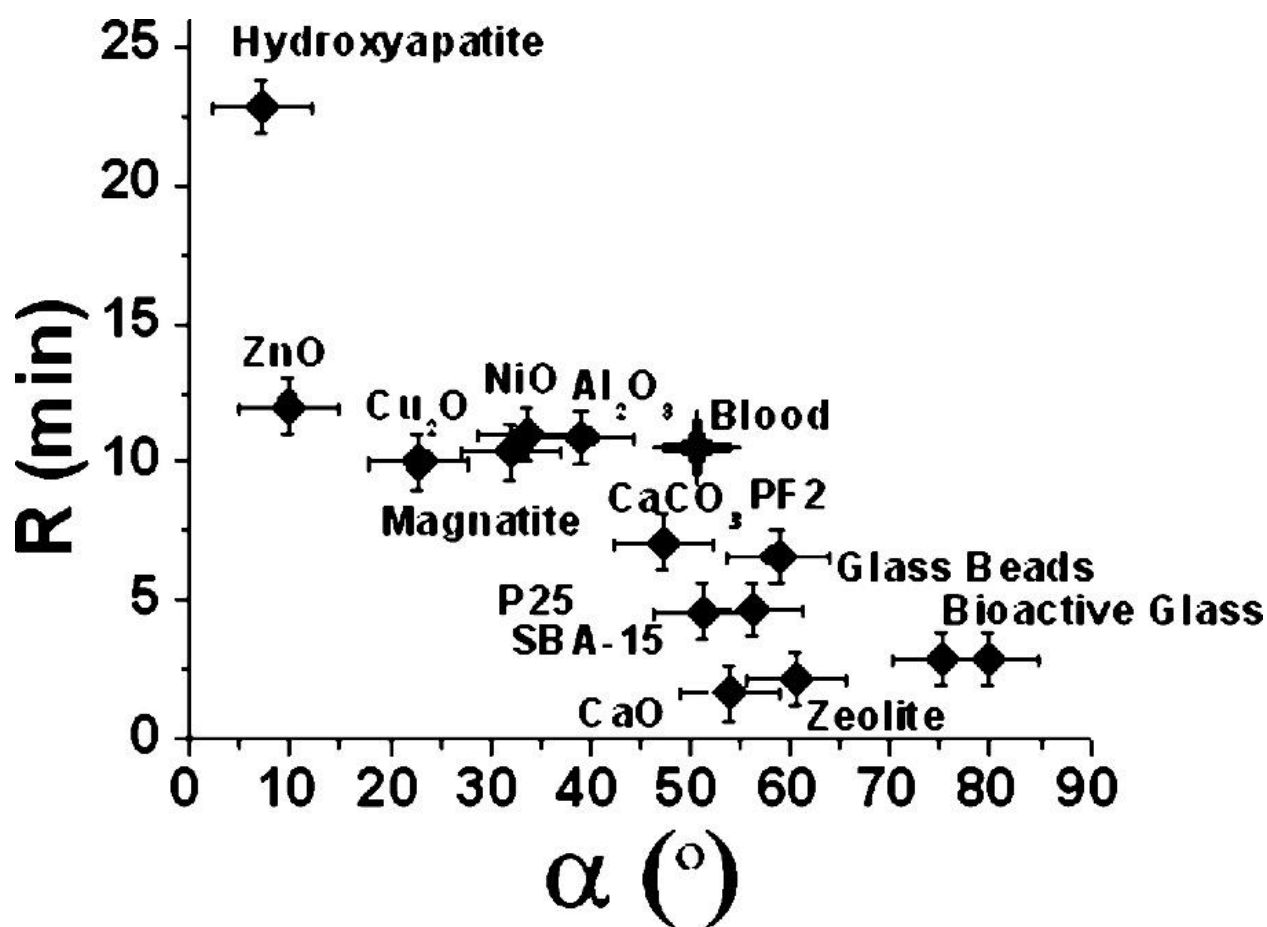


Figure 13. In vitro clotting time (R) and rate of clot growth (α) of various inorganic oxides. Zeolite is one of the strongest procoagulant oxides with a low clot time and large clot growth. Reprinted (adapted) with permission from Langmuir, Vol. 23, Issue 22, TA Ostomel, Q Shi, PK Stoimenov, GD Stucky, Metal oxide surface charge mediated Hemostasis, 11233-11238 , Copyright (2007) American Chemical Society.(13)

Table 2. In vitro clotting parameters for various ion-exchanged zeolites. Swine survival rate shows that only unexchanged zeolite led to 100 % swine survival. Springer and the Journal of Thrombosis and Thrombolysis, Vol. 22, 2006, 55-67, Host-guest composites for induced hemostasis and therapeutic healing in traumatic injuries, TA Ostomel, PK Stoimenov, PA Holden, HB Alam, GD Stucky, Table 3, original copyright notice) is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media.(99)

Agent	R (min)	α (°)	MA (mm)	Survival (%)
Ag-Exchanged Zeolite	0.9 ± 0.7	52.0 ± 7.2	75.6 ± 8	75 (6/8)
Ba-Exchanged Zeolite	1.8 ± 0.2	64.6 ± 1.2	76.9 ± 1.2	75 (6/8)
Na-Exchanged Zeolite	2.1 ± 0.2	62.0 ± 0.6	$73.3 \pm$	57 (4/7)
K-Exchanged Zeolite	2.2 ± 0.6	59.6 ± 2.9	77.5 ± 0.9	0 (0/2)
Sr-Exchanged Zeolite	2.1 ± 0.2	60.8 ± 1.9	73.7 ± 2.6	0 (0/2)
Citrated sheep blood	10.9 ± 1.3	50.2 ± 11.3	77.4 ± 2.5	NA
Zeolite	1.8 ± 0.1	67.8 ± 1.3	79.6 ± 1.1	100

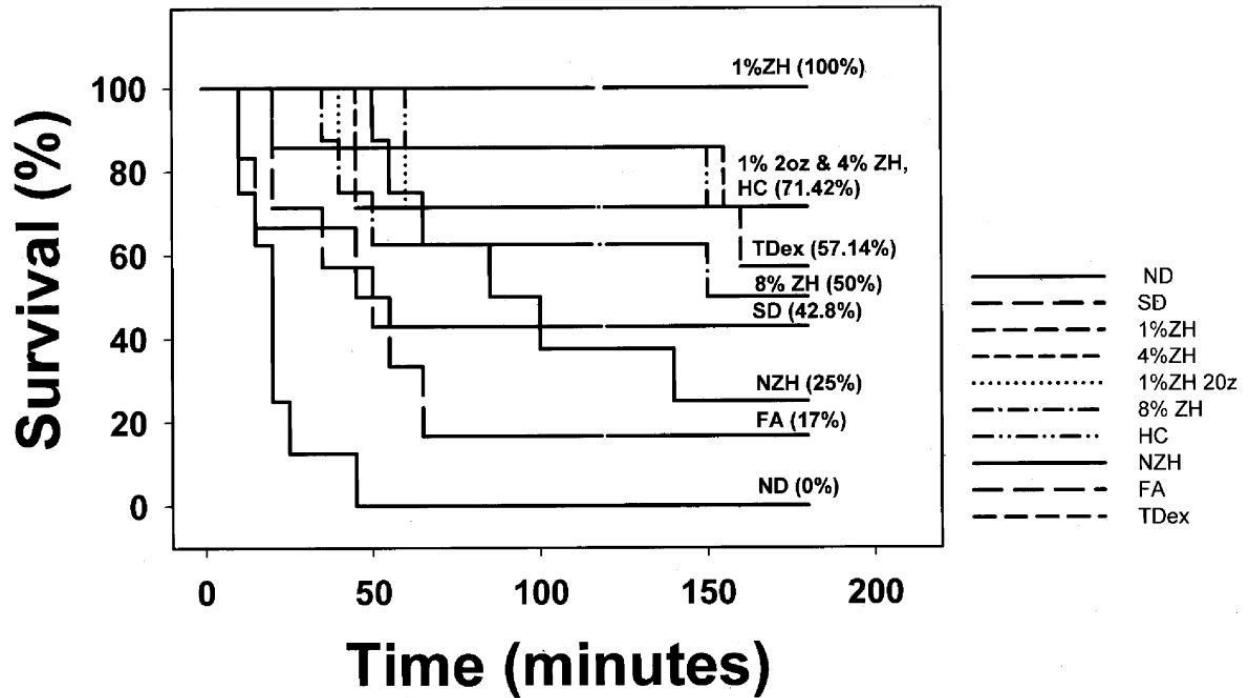


Figure 14. Survival rate over time for a complex swine groin injury for various treatments. ND, no dressing; SD, standard dressing alone; 1% ZH, SD + 3.5 oz of zeolite hemostat with 1% residual moisture; 4% ZH, SD + 3.5 oz of zeolite hemostat with 4% residual moisture; 1% ZH 2oz, SD + 2 oz of zeolite hemostat with 1% residual moisture; 8% ZH, SD + 3.5 oz of zeolite hemostat with 8% residual moisture; HC, SD + HemCon hemostatic dressing; NZH, SD + 3.5 oz of nonzeolite hemostat; FA, SD + Fast Act bovine hemostatic dressing; TDex, SD +30 g of TDex hemostatic powder. Reprinted from The Journal of Trauma and Acute Care Surgery, Vol. 56, Issue 5, HB Alam, Z Chen, A Jaskille, RILC Querol, E Koustova, R Inocencio, R Conran, A Seufert, N Ariaban, K Toruno, P Rhee, Application of a zeolite hemostatic agent achieves 100% survival in a lethal model of complex groin injury in swine, 974-983, Copyright (2004), with permission from Wolters Kluwer Health, Inc.(98)

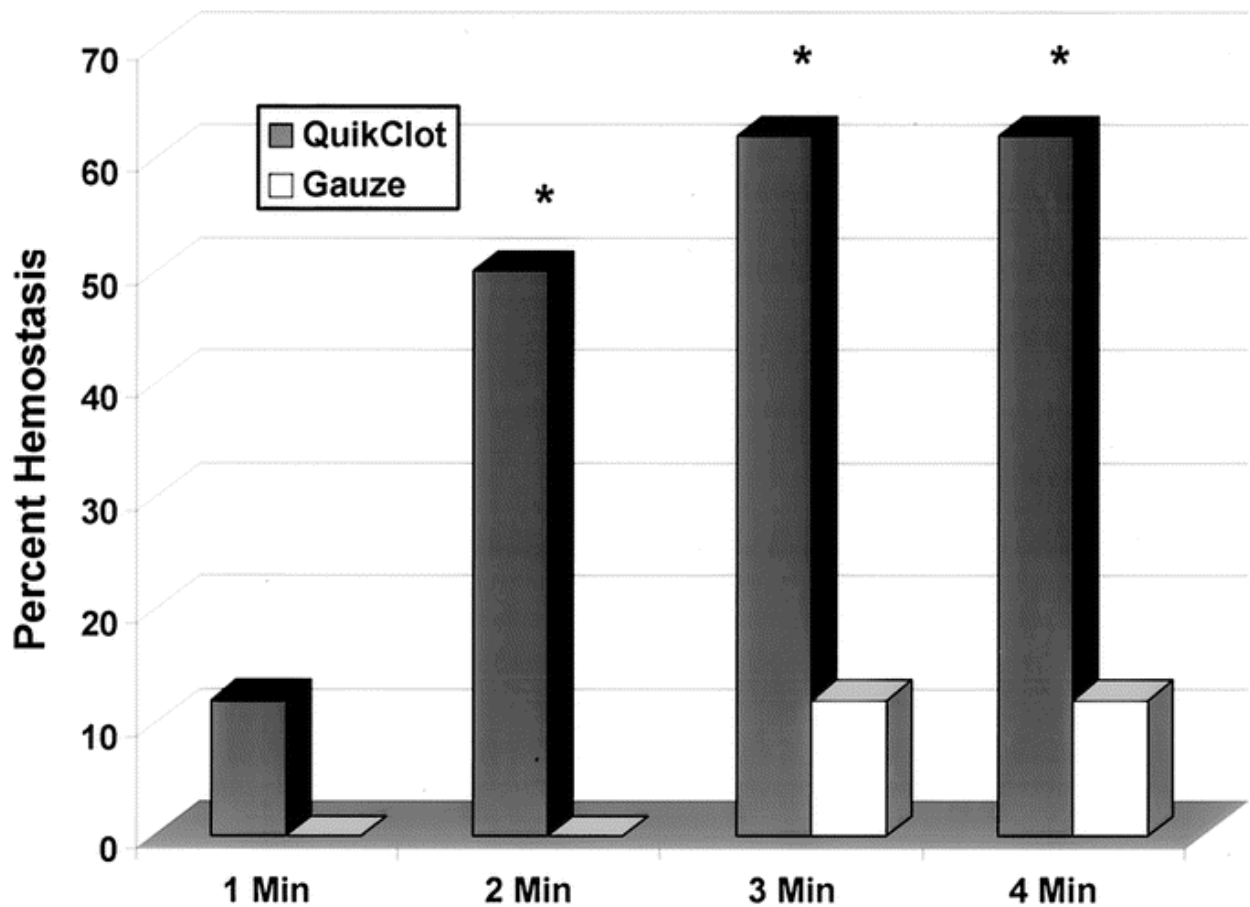


Figure 15. QC Zeolite achieves hemostasis after liver injury in swine faster than standard gauze. Reprinted from The Journal of Trauma and Acute Care Surgery, Vol. 57, Issue 3, HB AE Pusateri, AV Delgado, EJ Dick, Jr., RS Martinez, JB Holcomb, KL Ryan, Application of a Granular Mineral-Based Hemostatic Agent (QuikClot) to Reduce Blood Loss After Grade V Liver Injury in Swine, 555-562, Copyright (2004), with permission from Wolters Kluwer Health, Inc.(63)

Eventually the use of this dehydrated zeolite was abandoned due to the huge release of heat upon contact with aqueous media, including blood, that led to serious burns when applied (Table 2, Figure 4).(63, 64) Pusateri et al. found that application of QC zeolite raised the temperature at the wound site to 93°C (Table 2). Adding 10g of QC zeolite to 10 ml of stirred blood resulted in a 140.4 °C peak temperature (Figure 16).(63, 64) Titration of the zeolite with small amounts of water before packaging to eliminate the extreme heat generated by the dry zeolite gave a large improvement, over untreated wound hemostasis, in reducing the time necessary to initiate arterial clotting, increase the rate of clot formation and enhance the strength of the resulting clot.(99) Unfortunately, the *in vivo* and in-the-field effectiveness of this slightly hydrated zeolite to produce a high survivability for external major arterial bleeding was not adequate.(99)

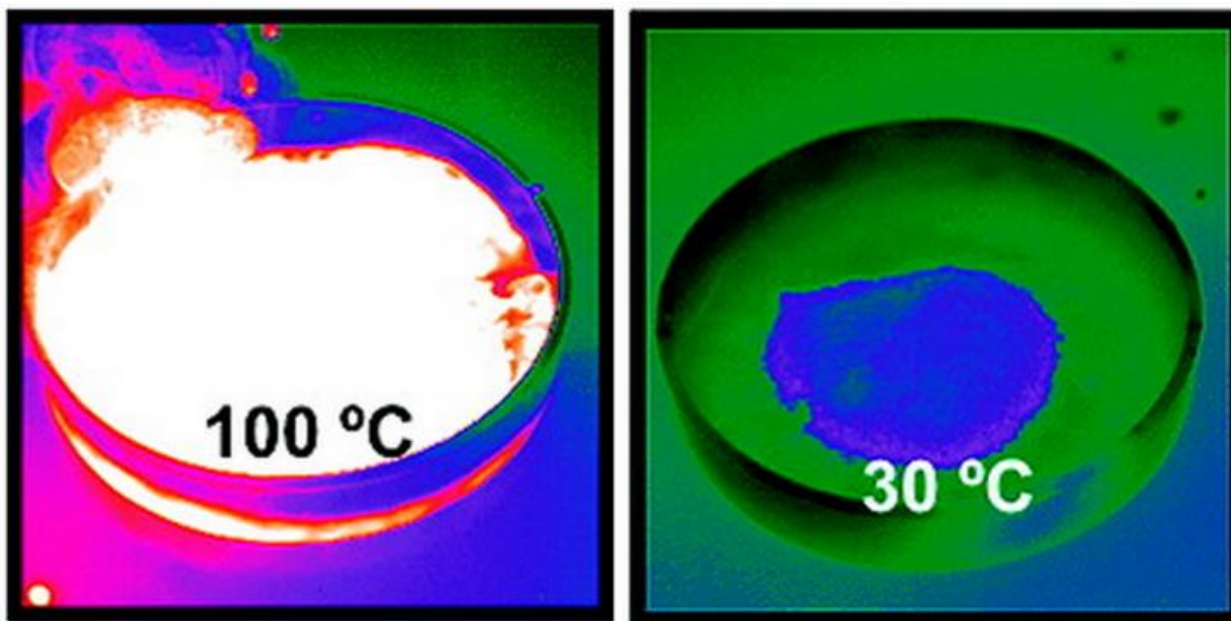


Figure 16. Thermal images show that QuikClot zeolite releases significant heat upon addition to water. Kaolin does not. Reprinted from Chemistry of Materials, Vol. 19, Issue 18, SE Baker, AM Sawvel, N Zheng, GD Stucky, Controlling bioprocesses with inorganic surfaces: Layered clay hemostatic agents., 4390-4392, Copyright (2007) American Chemical Society.(64)

Table 3. Post-injury effects of standard gauze and QC zeolite on lethal liver injury in swine. Reprinted from The Journal of Trauma and Acute Care Surgery, Vol. 57, Issue 3, HB AE Pusateri, AV Delgado, EJ Dick, Jr., RS Martinez, JB Holcomb, KL Ryan, Application of a Granular Mineral-Based Hemostatic Agent (QuikClot) to Reduce Blood Loss After Grade V Liver Injury in Swine, 555-562, Copyright (2004), with permission from Wolters Kluwer Health, Inc.(63)

Variable	Standard Gauze	QC Zeolite	<i>p</i> Value of Difference
Post-treatment blood loss (ml)	5338 +/- 806	1397 +/- 806	<0.01
Resuscitation fluid used (ml)	9686 +/- 1260	5574 +/- 1260	0.04
Peak temperature at tissue interface (°C)	37.5 +/- 0.01	93.3 +/- 10.5	<0.01
Survival to 1h	1/8 (12%)	7/8 (88%)	<0.01
Survival time (min)	44.5 +/- 3.6	58.3 +/- 1.8	<0.01

Research then shifted to focusing on what properties of the zeolite system made the dehydrated zeolite so effective as an arterial hemostasis agent when the zeolite system was interfaced with the blood clotting cascade system.(63, 64, 99) Calcium delivery to the blood system from the high surface area calcium zeolite ($\text{Na}_{0.5}\text{Ca}_{5.75}(\text{SiO}_2)_{12}(\text{AlO}_2)_{12} \cdot x\text{H}_2\text{O}$), local dehydration of the blood at the point of injury, the increase of temperature at the wound site, and the inorganic surface-blood factor interface interactions and chemistry were considered as possible clotting agent control variables for enhanced clotting efficiency.(99)

While modestly higher temperatures ($> 37^\circ\text{C}$) and the release of calcium give positive responses for more efficient clotting, the most important variable identified from studies of a variety of possible inorganic agents was the inorganic particle surface charge as determined by measurements of the zeta potential in simulated body fluid (Figure 17).(64) In fact, it was found that the appropriate choice of zeta potential, which varies over a wide range for inorganic agents, could be used to selectively initiate and amplify both clotting and its antithesis, anticlotting in plasma and whole blood. With this as a guide, the most effective candidate was determined to be the non-swellable silicate clay kaolin, which does not exfoliate blood transportable low molecular weight aluminosilicate species,(66) has no exothermic reaction with water or blood, but as a hemostasis agent is as effective as calcium dehydrated zeolite.(14)

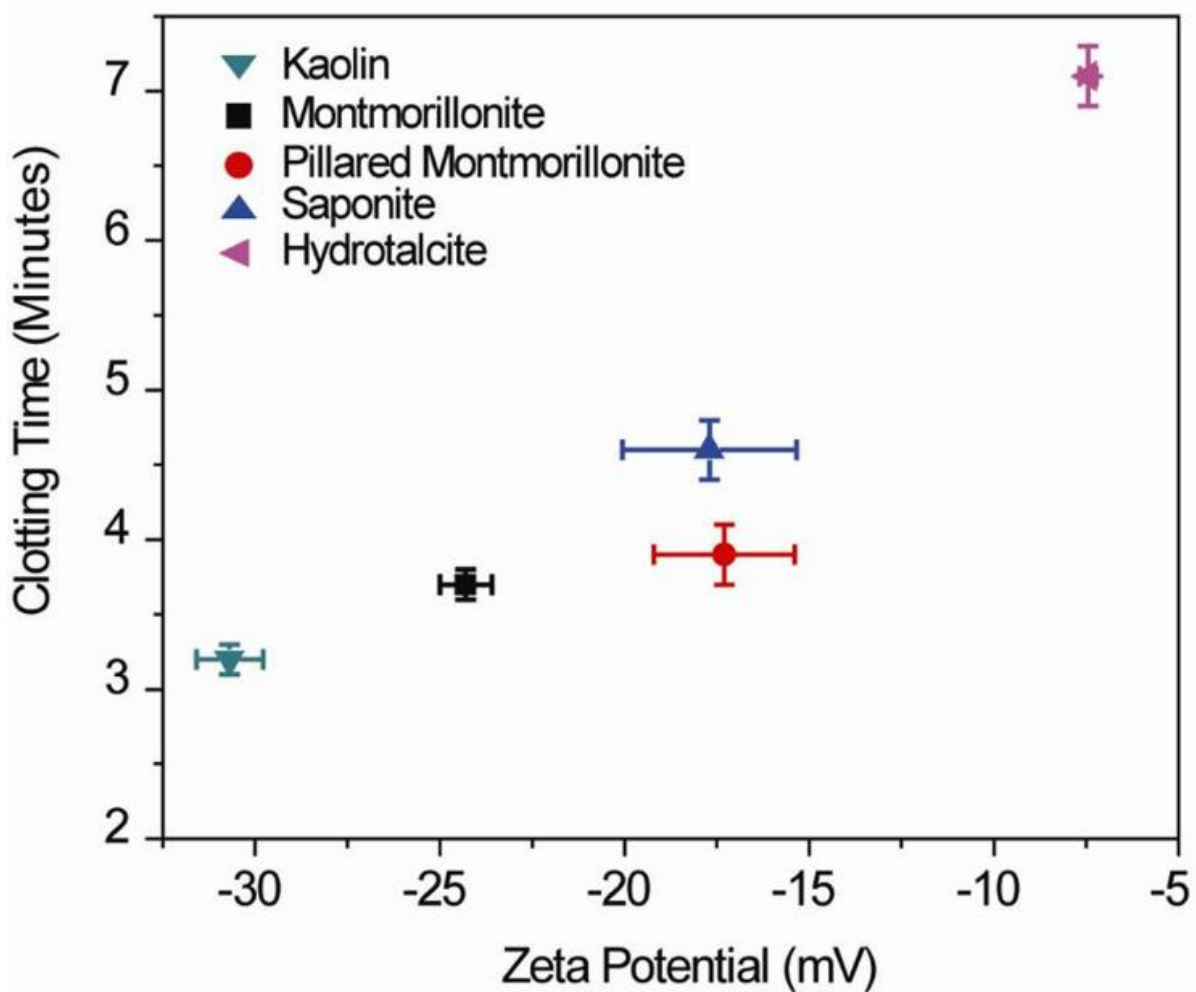


Figure 17. Decreasing surface zeta potential leads to decreasing clot time in silicate clays. Reprinted from Chemistry of Materials, Vol. 19, Issue 18, SE Baker, AM Sawvel, N Zheng, GD Stucky, Controlling bioprocesses with inorganic surfaces: Layered clay hemostatic agents., 4390-4392, Copyright (2007) American Chemical Society.(64)

Several other hydrated clay groups were tested. In addition to activating FXII, aluminosilicate smectite clays such as montmorillonite swell upon contact with blood, restricting blood flow while initiating coagulation at the point of injury. Studies conducted with porcine blood showed that Na-montmorillonite reduced clotting time by nearly one-third (Figure 18).(14) Further refinement led to the development of the powder WoundStat (WS), a topical agent that combined smectite clay with a superabsorbent polymer to minimize blood loss.

However, smectite clays also had the most serious side effects. *In vivo* studies with members of the swellable aluminosilicate layered clay family show significant *in vivo* cytotoxicity, probably due to their propensity for exfoliation.(100-102) As a result, WS was quickly removed shortly after FDA approval due to safety concerns over its cytotoxicity (Figure 19), the long-term effect of WS on treated vessels, and the occlusion of WS treated vessels by thrombin.(100, 102, 103)

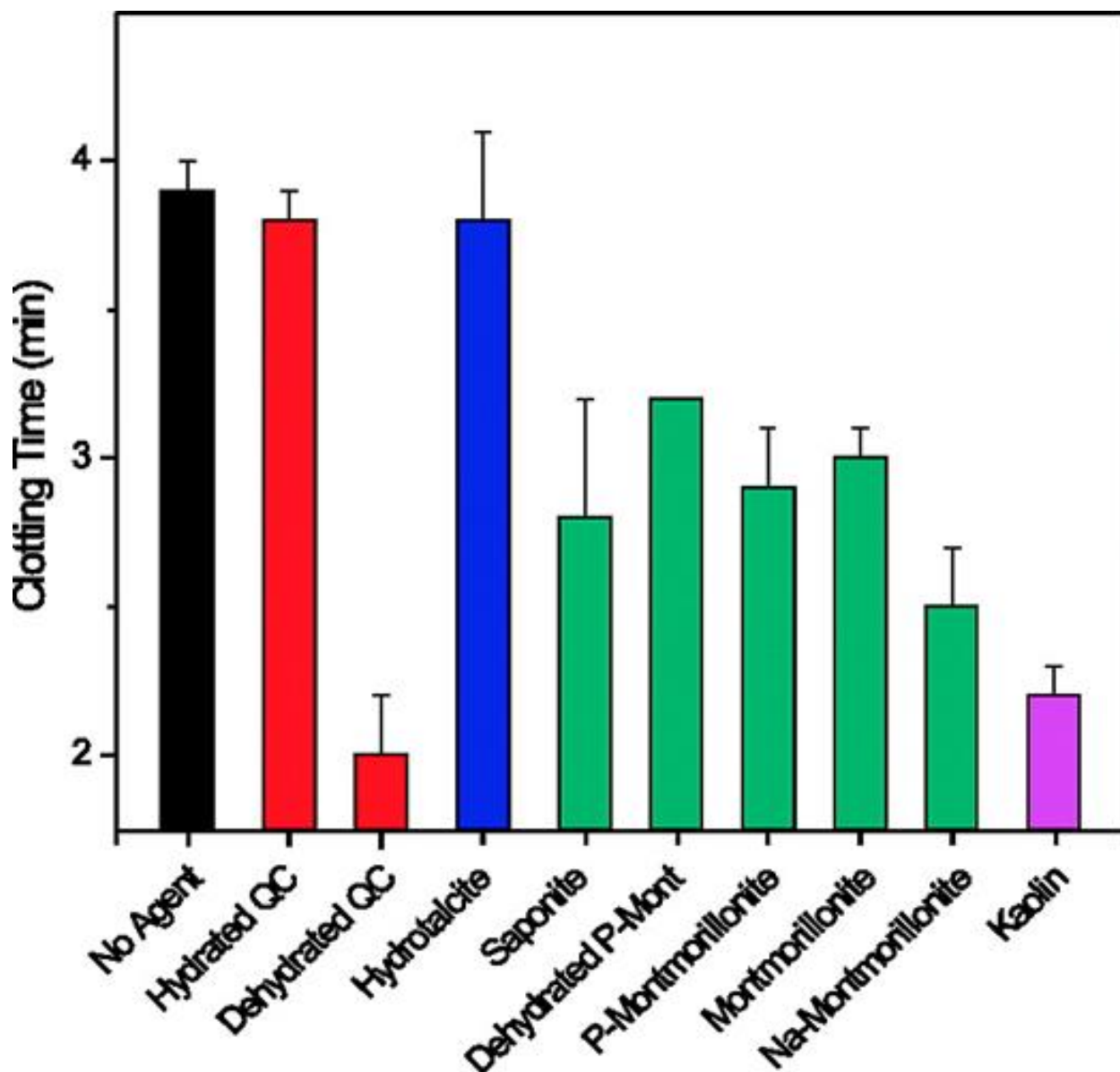


Figure 18. Clotting times of various silicates in porcine blood. Zeolites are in red. Non-silicate hydrotalcite is in blue. Smectites are in green. Kaolin in magenta. Reprinted from Chemistry of Materials, Vol. 19, Issue 18, SE Baker, AM Sawvel, N Zheng, GD Stucky, Controlling bioprocesses with inorganic surfaces: Layered clay hemostatic agents., 4390-4392, Copyright (2007) American Chemical Society.(64)

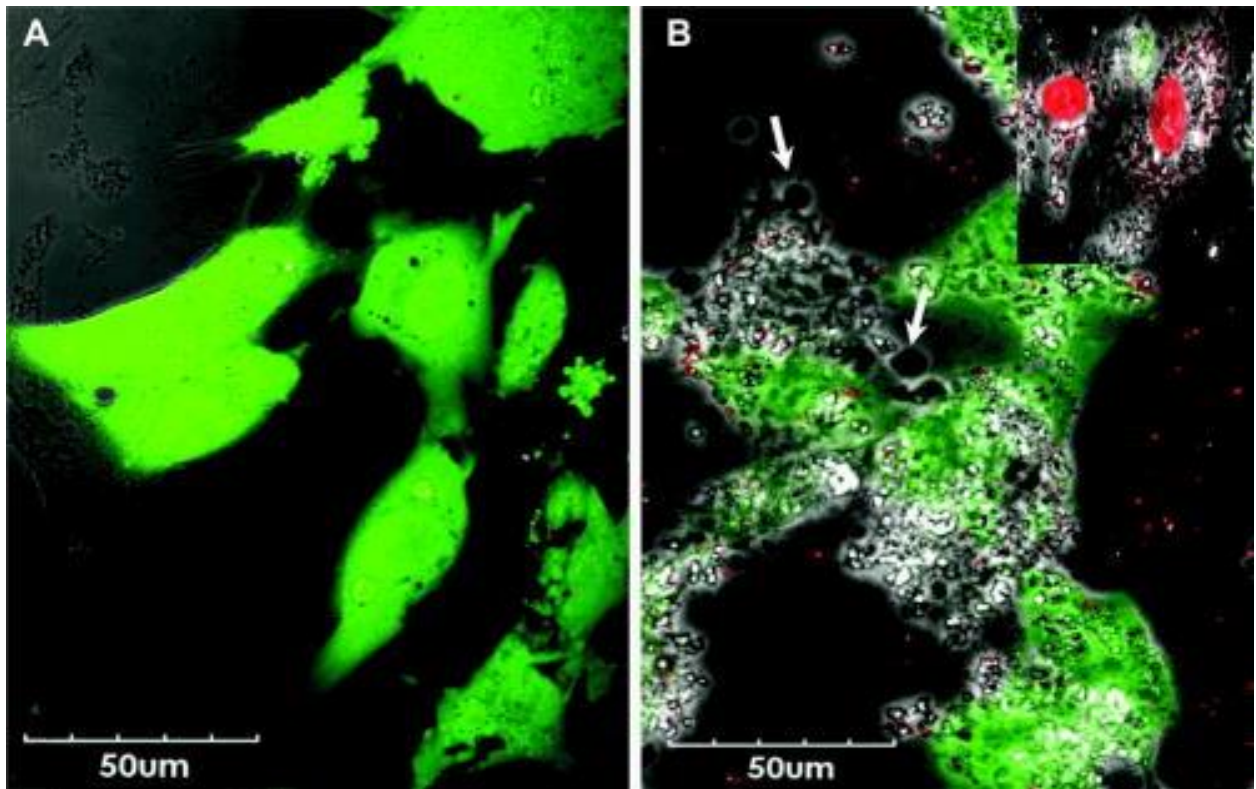


Figure 19. (A) Untreated HUVEC cells and (B) HUVEC cells after treatment with WoundStat. Untreated HUVEC cells are healthy, while those treated with WoundStat show early stages of cell death. Reprinted from The Journal of Trauma and Acute Care Surgery, Vol. 71, Issue 3, PD Bowman, X Wang, MA Meledeo, MA Dubick, BS Kheirabadi, Toxicity of Aluminum Silicates Used in Hemostatic Dressings Toward Human Umbilical Veins Endothelial Cells, HeLa Cells, and RAW267.4 Mouse Macrophages, 727-732, Copyright (2011), with permission from Wolters Kluwer Health, Inc.(100)

Of all naturally-occurring clays, independent extensive porcine *in vivo* blood clotting investigations by both Navy and Army medical research laboratories showed that kaolin ($\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4 \cdot 2 \text{H}_2\text{O}$) was highly effective as a blood clotting agent for major arterial bleeding from open wounds, yet, unlike zeolite, kaolin does not release heat upon contact with blood .(64) Extensive studies on the *in vivo* biocompatibility of kaolin were also made.(64)

The use of kaolin in clotting assays dates back to the 1950s, but surprisingly was not considered as a hemostasis agent.(96) In the more recent studies referenced above with porcine blood, kaolin was found to induce clots with nearly the same strength (Figure 18) as dehydrated zeolite without the significant exothermic release (Figure 16).(64) By 2011, the percentage of combat casualties who died of their wounds fell below 5 % thanks significantly to the proliferation of topical hemostatic agents such as kaolin-based Combat Gauze.(29)

The important conclusion from the studies is that there is tremendous variability in the efficacy and safety of different silica and silicate systems. Studies on the use and application of silica to activate the intrinsic pathway have taken advantage of naturally occurring silicates such as zeolites(98), smectites(64), and kaolin.(64), which are plentiful, produced cheaply, and have long shelf lives so that they are cost-effective in ton quantities. However these nanostructure materials are in fact themselves complex systems that can affect and direct the body and blood clotting systems in markedly different ways, with the most effective, from a biocompatibility and hemostasis view, being pure nanostructured silica (nanoparticles or mesostructured) and kaolin.(104)

Table 4. Kaolin-based Combat Gauze greatly improves outcomes in treating groin arterial hemorrhage in swine. Reprinted from The Journal of Trauma and Acute Care Surgery, Vol. 67, Issue 3, BS Kheirabadi, MR Scherer, JS Estep MA Dubick, JB Holcomb, Determination of Efficacy of New Hemostatic Dressings in a Model of Extremity Arterial Hemorrhage in Swine, 450-460, Copyright (2009), with permission from Wolters Kluwer Health, Inc.(105)

Outcome	HemCon	Celox-D	Trauma-Stat	Placebo	Combat Gauze
Initial Hemostasis achieved (# applications)	0/6 (12)	0/6 (12)	1/10 (20)	1/6 (12)	3/10 (17)
Total time bleeding stopped (min)	3.0 ± 2.2	0.5 ± 0.04	35.7 ± 22.2	57.9 ± 36.2	134.6 ± 22.2
Pretreatment blood loss (ml/kg)	19.2 ± 1	21.8 ± 1.8	19.3 ± 1.2	19.3 ± 1.6	18.2 ± 0.7
Posttreatment blood loss (ml/kg)	108.2 ± 7.5	113.8 ± 8.2	79.8 ± 13.8	75.5 ± 23.8	37.4 ± 17.3
Total resuscitation fluid (ml/kg)	175.3 ± 24.8	189.1 ± 16.2	160.3 ± 14.4	186.2 ± 41.9	123.9 ± 27.2
Survival rate	0/6	0/6	2/10	0/6	8/10
Survival time (min)	74.3 ± 10.5	74.2 ± 5.8	90.0 ± 15.3	121 +/- 19.3	167.3 ± 5.9
Peak wound temperature (°C)	36.8 ± 0.2	36.6 ± 0.2	36.6 ± 0.3	37.0 ± 0.1	35.7 ± 0.3

Despite improvements in clotting function, concern remains over the toxicity of aluminosilicates in the human body.(106) In addition, human cell cytotoxicity studies of kaolin as well as swellable clays show substantial HUVEC cell degradation for all clays,(107) but not for mesoporous silica.(104) The cytotoxicity of aluminosilicates for HUVEC cells, which are also used as models for studying blood-brain barrier processes, suggests that they should not be used for direct applications to open head injury wounds. Concern remains that a buildup of aluminosilicate species throughout the body might impair brain and kidney function. Pure silica species, however are much less HUVEC cell cytotoxic, and mesoporous silica with 10 kDa polyethylenimine improves cell viability without negative side effects.(108) Recent *in vitro* studies also suggest that magnesium ions in laponite ($\text{Na}_{0.7}\text{Si}_8\text{Mg}_{5.5}\text{Li}_{0.3}\text{O}_{20}(\text{OH})_4$) improve cell growth.(109, 110)

Table 5. Kaolin greatly increases outcome compared to unimpregnated gauze.
Reprinted from Journal of Surgical Research, Vol. 183, Issue 2, MJ Sena, G Douglas, T Gerlach, JK Grayson, KO Pichakron, D Zierold, A pilot study of the use of kaolin-impregnated gauze (Combat Gauze) for packing high-grade hepatic injuries in a hypothermic coagulopathic swine model, 704-709, Copyright (2013), with permission from Elsevier.(65)

Value	Kaolin Gauze	Plain Gauze	P value
Injury area (cm ²)	37 (6)	36 (8)	0.46
Fluid administered (ml/kg)	7 (29)	44 (45)	0.06
Blood loss (ml/kg)	25 (26)	58 (56)	0.05
Survival after 2 h (yes: no)	7:1	4:4	0.11

C. Inorganics to treat non-compressible, blunt trauma wounds

In Iraq and Afghanistan, wounds due to blunt and non-penetrating objects greatly increased the number of injuries in the head, neck, and trunk.(30) These injuries are non-compressible, which limits the effectiveness of tourniquets and topical agents. There is presently little that can be done to stem internal bleeding prior to arrival at a surgical care center. The main concern in all instances of non-compressible hemorrhage is that patients exhibiting severe blood loss will become coagulopathic and die before surgery can restrict blood loss.(9) Acute traumatic coagulopathy (ATC) can develop as a result of tissue injury and hemorrhagic shock and is further complicated by other multifactorial, systemic factors.(34)

Improving on the ideal topical hemostatic agent, the ideal agent to control non-compressible hemorrhage in the prehospital setting would have the following properties: [1] long shelf-life and stability in weather extremes, [2] reasonable cost, [3] capability to promote key functions of the blood clotting system at the injury site including coagulation, regulatory anti-coagulation and fibrin formation, [4] lack of off-target adverse effects. Such an agent must at the same time enable treatment during the transport phase when the patient is at the greatest risk for exsanguination.

D. Use of functionalized silica as a drug delivery agent

Pure silica is generally considered to be a non-toxic material, and often used in drug delivery studies.(111-117) Due to its negative surface charge, silica is also a potent contact activator in the bloodstream.(64, 95) , which can be mediated with covalently attached surface capping agents such as polyethylene glycol (PEG).(114) The particle's intrinsic negative surface charge at body pH allows FXII to bind to the silica surface and become

activated so that it is inherently a procoagulant.(95) Mesoporous nanoparticles can be gated to release therapeutic agents at targeted sites, which has been well demonstrated for cancer treatment.(113) Unlike pure silica, current materials used for treating external hemorrhage generally contain particles in the micrometer range, which are too large to easily traverse capillaries and unsuitable for use as intravenous therapeutics.(118) Consequently, the ideal construction of an intravenous hemostat with a silica nanoparticle (SNP) core would shield the SNP surface from exposure to the systemic circulation, while targeting exposure of the SNP carrier surface and the attached drug at the site of injury. Cytotoxicity tests are one of the main challenge thresholds needed to validate that the use of the SNPs intravenously is safe.

Porous SNPs and p-doped silicon nanoparticles with a thin coating of silica are attractive for use in drug delivery and imaging due to key tunable and readily accessible properties such as small size, pore volume, large surface area, uniformity in synthesis, and processability.(115) Sailor et al. has shown that silicon nanoparticles can be used to image tumors and internal organs in mice without significant side effects (Figure 20).(119) Most important to drug delivery schematics, mesoporous silica can be loaded with proteins or drugs for targeting and delivery to systems and trauma sites in the body.(104, 120) For example, Slowing et al. have successfully synthesized mesoporous silica nanoparticles (MSNs) with an average diameter of 100 nm, 900 m²/g surface area, 2 nm pore size, and 0.9 cm³/g pore volume (Figure 21).(115) Silica has also been proven to be biodegradable at a pH of 7.4, though the degradation process takes several hours or longer depending on the silica wall or particle thickness.(121) Lu et al. found that injecting mice with 50 mg/kg MSNs

showed no effects of toxicity over a two month study. Furthermore, the silica that did not degrade in the body was removed from the body through liver and kidney excretion.(122)

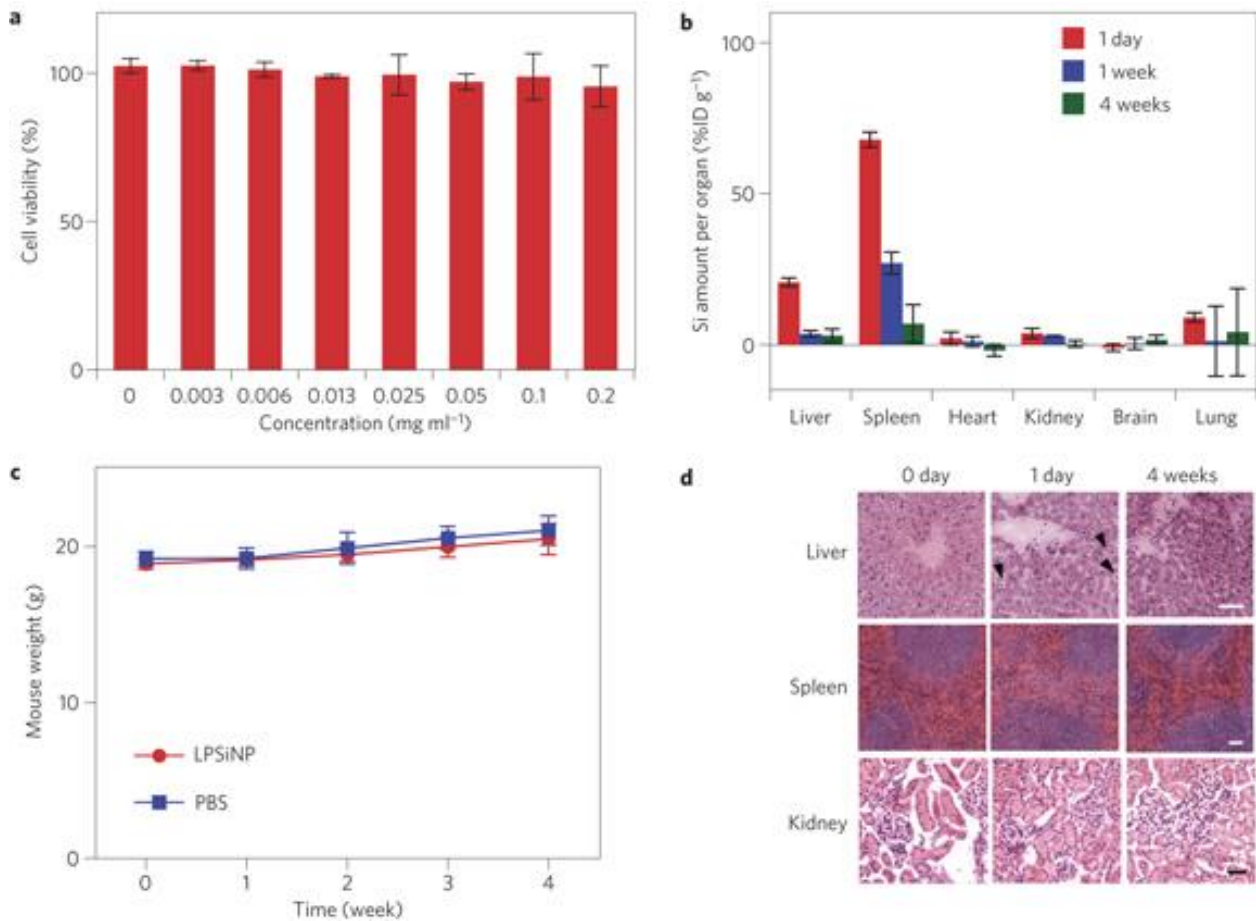


Figure 20. (a) Sailor's silicon nanoparticles were viable in in vitro HeLa cell assays. (b) In vivo biodistribution of silicon nanoparticles after 4 weeks in a mouse. (c) The mouse weight did not change whether the mouse was injected with particles or PBS buffer. (d) Liver, spleen, and kidney histology at various time points. Reprinted by permission from Macmillan Publishers Ltd: Nature Materials, Vol. 8, Issue 4, J-H Park, L Gu, G von Maltzahn, E Ruoslahti, SN Bhatia, MJ Sailor, Biodegradable luminescent porous silicon nanoparticles for : in vivo: applications, copyright (2009).(119)

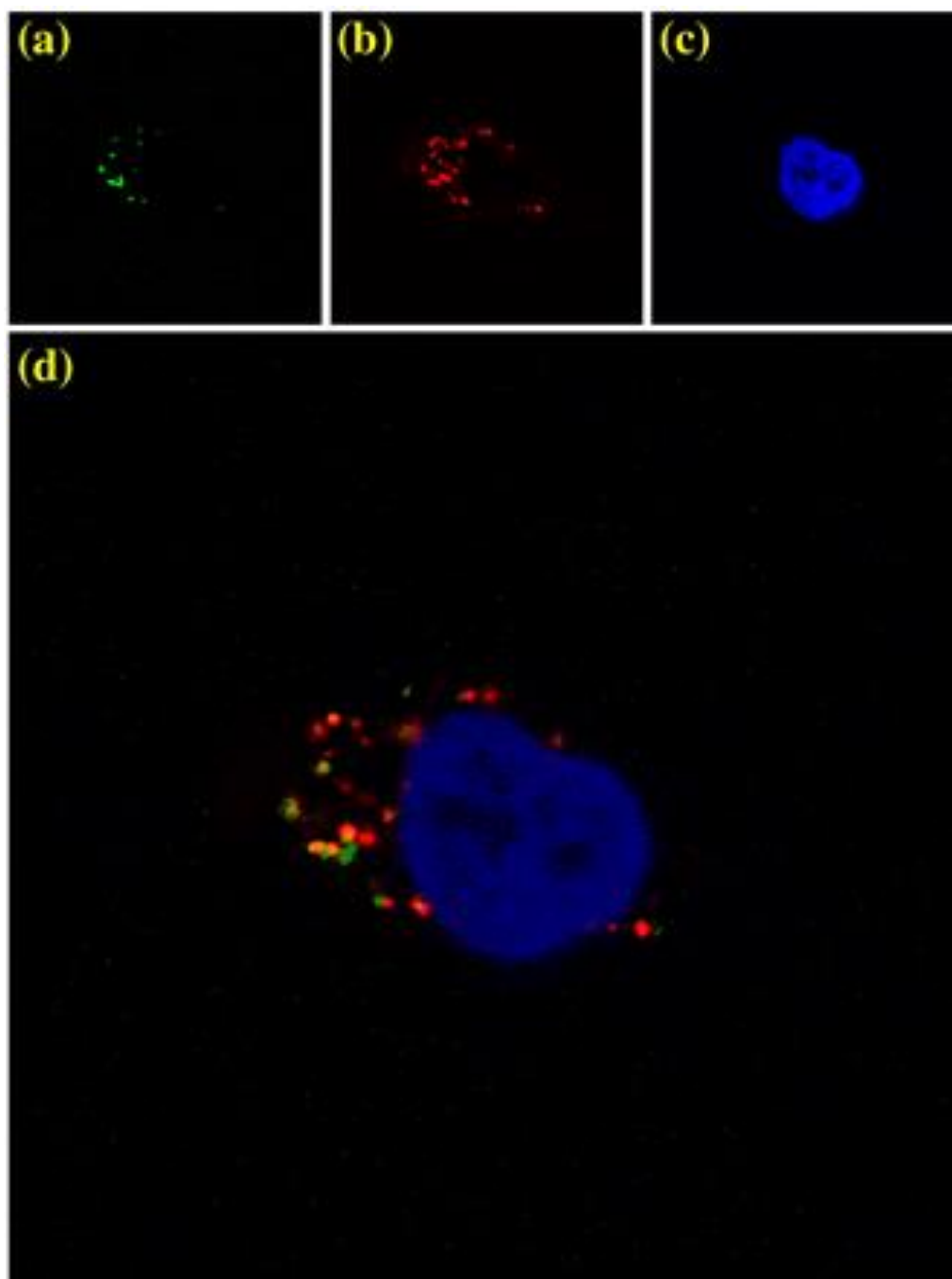


Figure 21. Confocal fluorescence images of fluorescein labeled MSN (green, panel a; red, panel b) endocytosed by HeLa cells (blue) show that MSNs with high surface charge escape cellular uptake; d) shows panels a), b), and c) merged together. Reprinted from *Advanced Drug Delivery Reviews*, Vol. 60, Issue 11, II Slowing, JL Vivero-Escoto, C-W Wu, S-Y Lin, Mesoporous silica nanoparticles as controlled release drug delivery and gene transfection carriers, 1278–1288, Copyright (2008), with permission from Elsevier.(115)

Other strategies have sought to use porous silica to deliver key procoagulant proteins such as thrombin. Mesoporous SNPs are easily loaded with procoagulant materials such as thrombin to selectively promote coagulation.(14) Mesocellular foam silica (MCF) was synthesized with pore sizes varying from 6-33 nm in size (Figure 22). TEG studies showed that MCF with pore sizes at 20 nm (MCF-20), 26 nm (MCF-26), and 33 nm (MCF-33) greatly reduced clotting time, while MCF-26 and MCF-33 also rapidly accelerated α or the rate of clot formation. 2 mg unadulterated MCF reduced clotting time nearly as effectively as packaged QuikClot. However, when bovine thrombin was immobilized in MCF-33, the clot time was reduced nearly in half when compared to commercial QuikClot, using smaller amounts of clotting agent. While exciting for topical wound dressings, the large MCF-33 micron particle size and the strong clotting initiator nature of thrombin as a deliverable therapeutic prevents its use as an intravenous agent.(14)

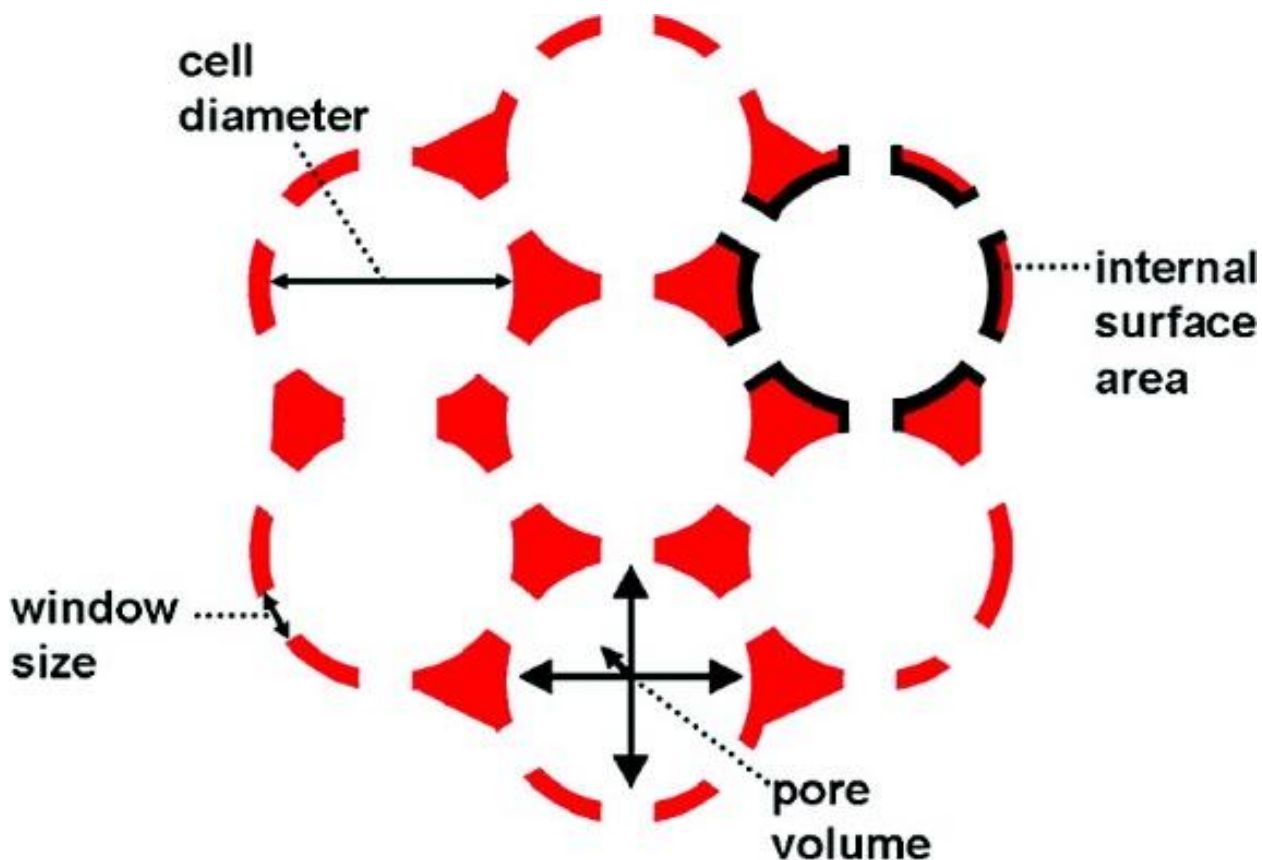


Figure 22. Diagram of porous silica mesocellular foam (MCF). MCF-26 corresponds to a 26 nm window (pore) size. Reprinted with permission from Langmuir, Vol. 24, Issue 24, SE Baker, AM Sawvel, J Fan, Q Shi, N Strandwitz, GD Stucky, Blood Clot Initiation by Mesocellular Foams: Dependence on Nanopore Size and Enzyme Immobilization, 14254–14260. Copyright 2008 American Chemical Society.(14)

In cytotoxicity studies, MCF-26 far outperformed clays such as kaolin, bentonite, and montmorillonite. MCF-26's inability to adhere strongly to cell surfaces played an integral role in improved biocompatibility. The biocompatibility variability of a series of aluminosilicate and silica particles was greatest when tested on human umbilical vein endothelial cells (HUVEC). Kaolin, bentonite, and montmorillonite all had IC₅₀ values well below 250 µg/ml on all three HUVEC cell lines. In comparison, the pure silica MCF-26 had an IC₅₀ value above 1 mg/ml for all cell lines. Pure silica MCF-26 did damage HUVEC cells at a concentration of 7 mg/ml, nearly 30 times more concentrated than kaolin to produce the same effect. Even at 7 mg/ml, MCF-26's did not kill 50 % of human epidermal keratinocytes (HEK) cells (Table 6).(104) In all samples tested, MCF-26 remained 10-30 times less cytotoxic than its clay counterparts.(104) The extremely low IC₅₀ for kaolin and other clays suggests that their use should be avoided in brain injuries.

Table 6. IC50 values and cell viability for kaolin, montmorillonite, and MCF-26.
Reprinted from Toxicology Research, Vol. 2, Y Li, AM Sawvel, Y-S Jun, S Nownes, M Ni, D Kudela, GD Stucky, D Zink, Cytotoxicity and potency of mesocellular foam-26 in comparison to layered clays used as hemostatic agents, 136-144, Copyright (2013), with permission from the Royal Society of Chemistry.(104)

	Kaolin		Montmorillonite		MCF-26	
Cell type	IC50 (µg/ml)	Cell viability 250 µg/ml (%)	IC50 (µg/ml)	Cell viability 1000 µg/ml (%)	IC50 (µg/ml)	Cell viability 1000 µg/ml (%)
HUVEC 1	125 ± 47	39 ± 5	65 ± 3	18 ± 0	>1000	75 ± 2
HUVEC 2	48 ± 4	35 ± 2	33 ± 2	25 ± 3	>1000	73 ± 12
HUVEC 3	51 ± 2	21 ± 2	95 ± 13	33 ± 4	>1000	65 ± 3
HDF 1	>250	138 ± 14	272 ± 99	29 ± 1	>1000	66 ± 13
HDF 2	>250	119 ± 8	335 ± 32	38 ± 5	>1000	130 ± 4
HDF 3	>250	107 ± 3	454 ± 120	57 ± 1	>1000	122 ± 3
HEK 1	>250	58 ± 2	>1000	78 ± 6	>1000	100 ± 3
HEK 2	>250	51 ± 12	>1000	62 ± 2	>1000	66 ± 8
HEK 3	181 ± 44	46 ± 2	>1000	69 ± 2	>1000	102 ± 12
NIH/3T3	>250	96 ± 5	461 ± 22	38 ± 2	>1000	93 ± 9
HK-2	>250	62 ± 6	>1000	66 ± 2	>1000	71 ± 13
HPTC	>250	77 ± 9	280 ± 27	34 ± 2	>1000	89 ± 11

In combining silicate nanoplatelets with gelatin, Gaharwar et al. recently have sought to develop a biocompatible(123) laponite-based sealant that can be injected directly into non-compressible injuries to halt bleeding (Figure 23). Addition of the gelatin-silicate gel to human whole blood reduced clotting time by 77 % compared to untreated blood (Figure 24). In a lethal liver injury in rats, both the laponite-gelatin gel and kaolin-based Combat Gauze led to 100 % survival rates (Figure 25). As an added benefit, 200 μ l gel was injected to better simulate treatment of a non-compressible injury in which topical agents like Combat Gauze are not applicable. Long-term exposure of kaolin and other aluminosilicate topical agents can result in serious complications that threaten the patient's life. In terms of biocompatibility, the injected gel degraded within 28 days, caused less inflammation at the injury site than Combat Gauze, and did not lead to any systemic issues or mortality in rats.(124) While the gel successfully decomposed without side effects, the gel matrix needs to be injected intravenously with an unknown injury in order to determine the laponite-gelatin gel's true potential as an injectable, battlefield agent.

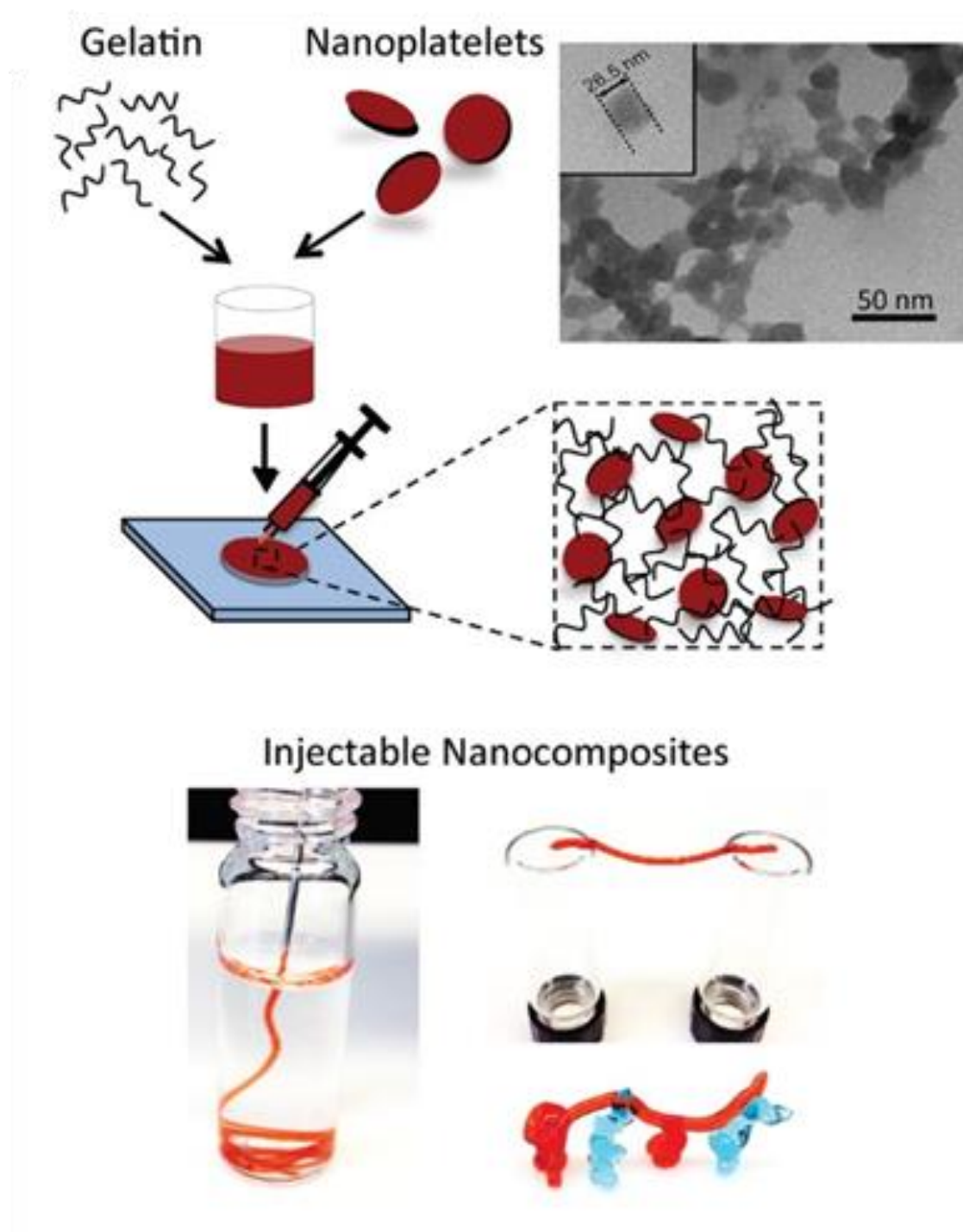


Figure 23. Gel prepared by combining silicate nanoplatelets and gelatin. Reprinted with permission from ACS Nano, Vol. 8, Issue 10, AK Gaharwar, RK Avery, A Assmann, A Paul, GH McKinley, A Khademhosseini, BD Olsen, Shear-Thinning Nanocomposite Hydrogels for the Treatment of Hemorrhage., 9833–9842. Copyright 2014 American Chemical Society.(124)

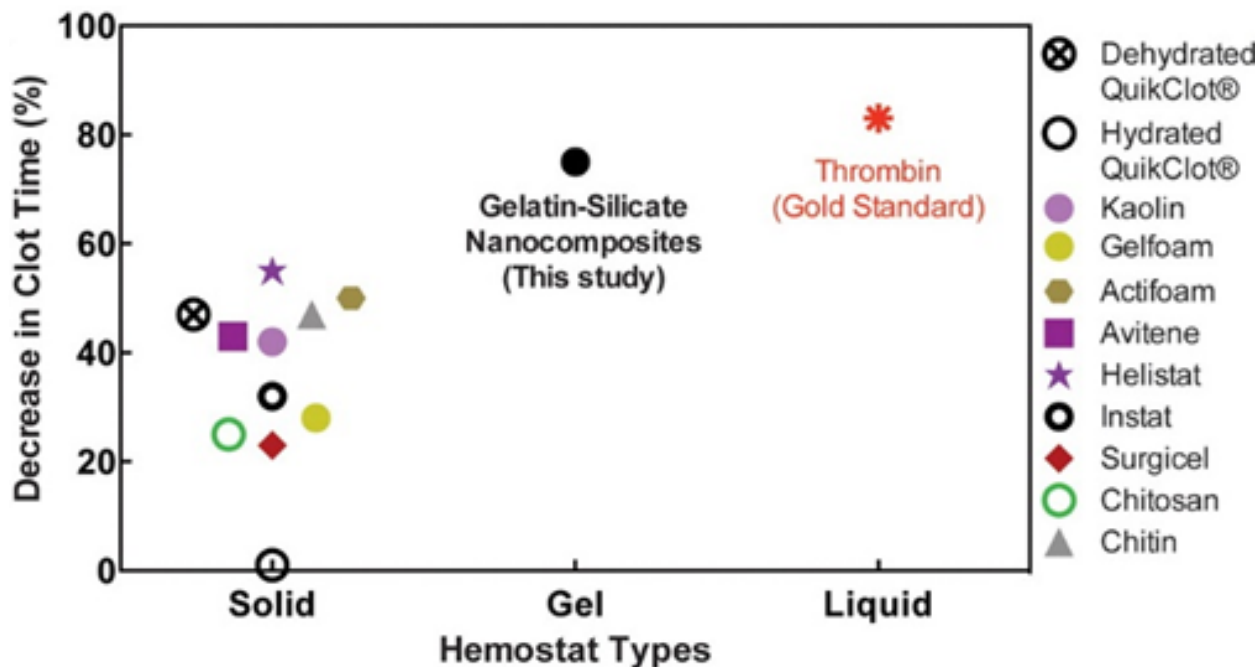


Figure 24. Gelatin-silicate gel was nearly as effective as thrombin and more effective than current commercial topical hemostatic agents in in vitro studies. Reprinted with permission from ACS Nano, Vol. 8, Issue 10, AK Gaharwar, RK Avery, A Assmann, A Paul, GH McKinley, A Khademhosseini, BD Olsen, Shear-Thinning Nanocomposite Hydrogels for the Treatment of Hemorrhage., 9833–9842. Copyright 2014 American Chemical Society.(124)

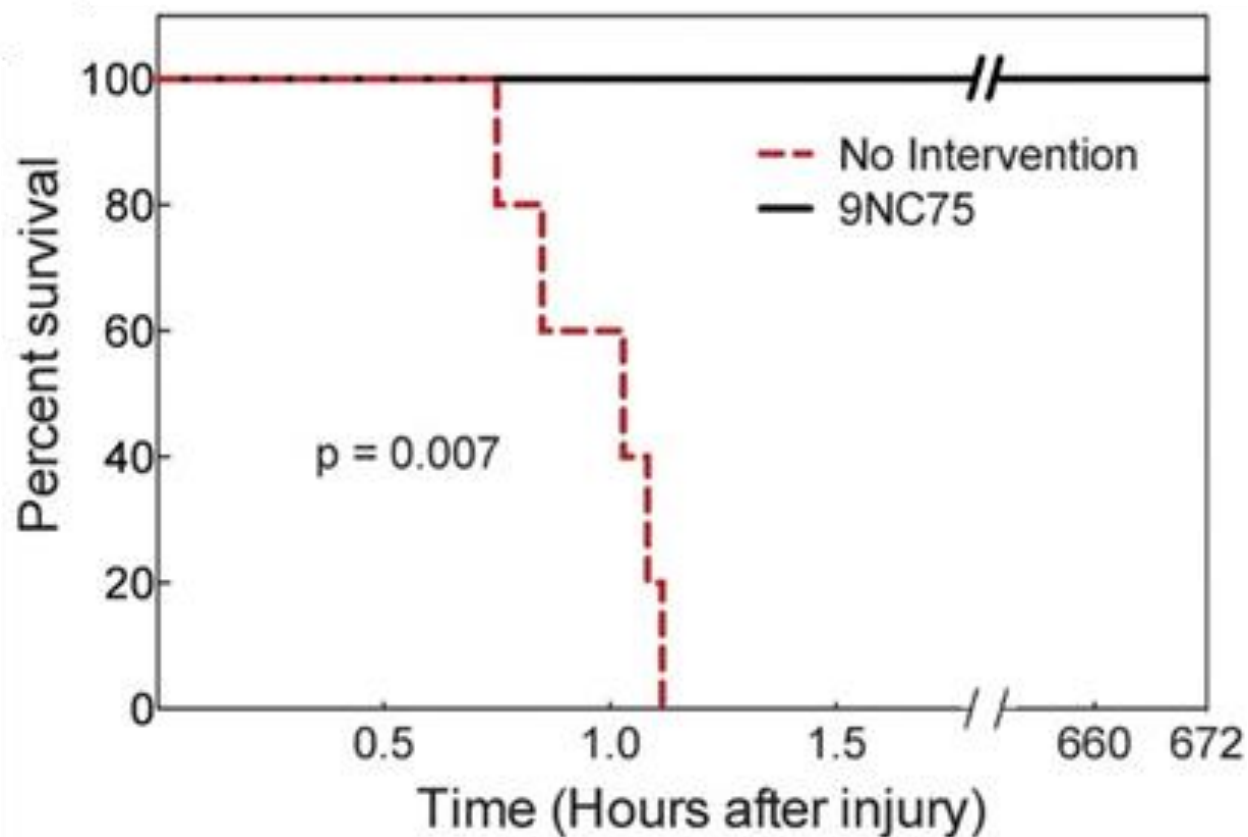


Figure 25. Gelatin-silicate gel (9NC75) injected into rats increased survival rates in a lethal liver injury model. Reprinted with permission from ACS Nano, Vol. 8, Issue 10, AK Gaharwar, RK Avery, A Assmann, A Paul, GH McKinley, A Khademhosseini, BD Olsen, Shear-Thinning Nanocomposite Hydrogels for the Treatment of Hemorrhage., 9833–9842. Copyright 2014 American Chemical Society.(124)

E. Conclusion

Though knowledge of silica's effect on coagulation through FXII and the intrinsic pathway dates back to the 1950s, the use of silica to stop bleeding was only adopted early in the 21st century. The increase of preventable deaths due to compressible, extremity bleeding in Iraq and Afghanistan led to the development of impregnated gauzes designed to stop bleeding within minutes, even when standard issue gauzes proved ineffective. Researchers at UCSB in collaboration with emergency medical personnel from the Office of Naval Research (ONR) experimented with zeolites, smectites, and other silicates until kaolin emerged as the best topical treatment to prevent patients from bleeding to death prior to hospital arrival. Today, US military personnel still carry QuikClot Combat Gauze, a kaolin-impregnated gauze, as a first-response treatment; kaolin-based gauzes are also used in the civilian sector. Though concerns about side effects, particularly in application to head wounds, remain, kaolin remains the battlefield standard to stop major extremity bleeding.

However, silica's procoagulant effects are not only tied to its activation of FXII. Silica is a common drug delivery agent. Though much of the focus on silica has been with an eye toward cancer therapeutics, in the research described here we have explored how SNPs might be used as a synergistic agent to deliver procoagulant payloads to injury sites while at the same time inducing clotting through FXII activation. Silica also improves emergency treatment due to its long-term shelf-life stability at ambient conditions. Preventing the spread of thrombi to healthy vessels and the quick removal of SNPs from the body remain the two most difficult challenges in blood clotting drug delivery. Though there are currently no FDA approved silica-based treatments for non-compressible hemorrhage, recent publications on cancer therapeutics illustrate silica's ability to combine with other agents to form a

synergistic therapeutic. The studies described in this dissertation show promise towards the development of an intravenous agent that will revolutionize treatment of life-threatening bleeding.

III. Accelerating coagulation through polyphosphate-laden silica

nanoparticles¹

Hey man of science with your perfect rules of measure,
Can you improve this place with the data that you gather?
- Bad Religion, *I Want To Conquer The World*

A. Introduction

Previously, I have covered the issues medical personnel face in managing hemorrhage both on the battlefield and in civilian trauma centers. The current treatments available fall into three broad categories: mechanical devices that compress the wound,(125) topical hemostatic agents,(64, 126) and intravenous hemostatic agents.(8, 79, 127) Each treatment has its benefits and limitations(100, 104, 128, 129) depending on the locus and scope of the injury. Researchers and clinicians are still developing and refining treatments that fit the guidelines for the ideal hemostatic agent (Chapter II). In this chapter, I propose that targeted short-chain polyphosphate-laden silica nanoparticles (polyP-SNPs) have the potential to fulfill these requirements.

Because it is a strong clotting accelerator (Scheme 2), intrinsically biocompatible,(18) and a poor initiator of endogenous coagulation enzyme production, short-chain polyP is a reasonable candidate for the management of hemorrhage.(18, 130) Delivery of a polyP payload on a nanoparticle carrier could potentially be optimized to target the site of injury while minimizing the impact on the systemic circulation. Effective targeting and control

¹ Adapted, with permission from John Wiley and Sons, from *Angewandte Chemie International Edition*, Vol. 54, Issue 16, D Kudela, SA Smith, A May-Masnou, GB Braun, A Pallaoro, CK Nguyen, TT Chuong, S Nownes, R Allen, NR Parker, HH Rashidi, JH Morrissey, GD Stucky, *Clotting Activity of Polyphosphate-Functionalized Silica Nanoparticles*, 4018-22, Copyright (2015).

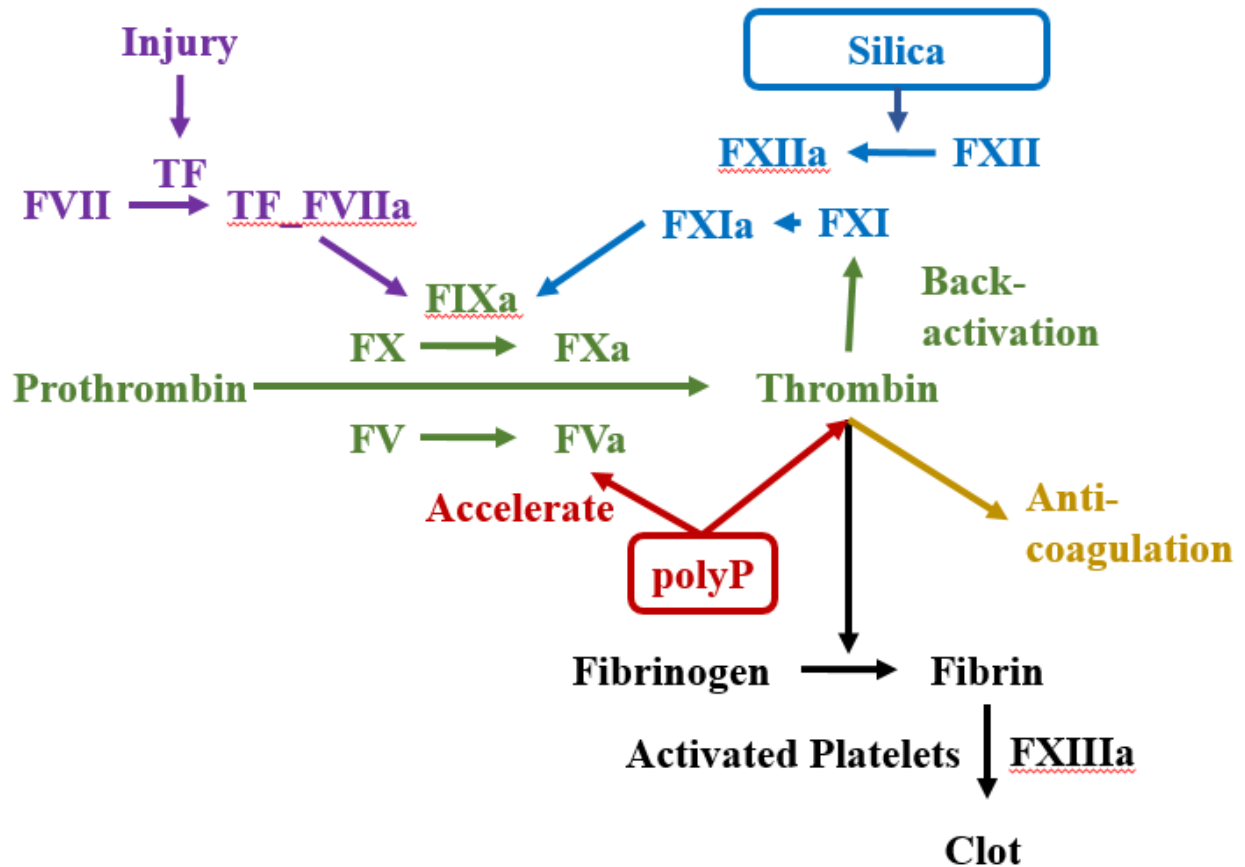
would in theory minimize the risk of thrombotic complications that limit procoagulant therapy. In addition to the potential for better biosystem safety, the production cost for polyP is low compared to that of recombinant proteins and other intravenous clotting agents. Upon attachment to inorganic oxides, polyP also has the potential for long-term stability under a variety of storage conditions.

In response to an injury, human platelets secrete short-chain polyPs of approximately 60-100 monomers. Platelet-secreted polyP has a variety of wound-healing therapeutic effects, including enhanced activation of factors XI and V, which ultimately leads to enhanced factor X activity, and limitation of the activity of tissue factor pathway inhibitor, which results in accelerated thrombin generation.(131) PolyP also creates a stronger clot structure and resistance to fibrinolysis.(20, 132) PolyPs with a chain length of 50-100 mer have been shown to not be strong clot initiating agents, although longer chain polyP (e.g. 500 mer) is adept at initiating clotting by activating FXII.(18) In addition, because of a ~90 minute half-life in plasma due to the normal presence of phosphatase enzymes in the blood,(133, 134) the procoagulant effects are further limited.(20, 131, 134) These properties and the endogenous metabolism of polyP offers excellent biocompatibility that is much better than currently available topical agents such as kaolin, which are not metabolized.

While long-chain polyP is a potent activator of the contact pathway,(18) short-chain polyP released from platelets has relatively poor capacity to activate factor XII.(18, 130) I employed relatively short-chain polyP for these studies in order to maximize the enhancement of downstream coagulation enzymatic steps (common pathway), while minimizing contact pathway activation.

As noted above, platelets serve as a polyP delivery agent, secrete procoagulants and clotting factors that promote blood coagulation, and initiate the formation of a clot-dissolving enzyme that degrades blood clots during the healing process. In my studies following up on the extensive research originally carried out by the Morrissey group,(18, 20, 130, 131, 134) I use free polyP as a benchmark. In the research reported here, delivery agent bifunctionality is introduced by using SNPs as a procoagulant carrier for the polyP.

Scheme 2. Coagulation cascade. Silica initiates clotting by activating FXII of the intrinsic pathway (blue). PolyP (red) accelerates clotting by increasing FVa and thrombin, the key factor in the coagulation cascade, production. Short-chain polyP (red) binds to thrombin and enhances the rates of activation of FV and FXI leading to an earlier thrombin burst. Rapid thrombin generation leads to increased conversion of fibrinogen to fibrin, which combines with activated platelets to form the physical clot. Finally, thrombin production also facilitates increased activity in the anticoagulation pathway, which limits the spread of coagulation to uninjured vessels.(135)



B. Materials

Ethanol (200 proof), tetraethyl orthosilicate (TEOS), ammonia NH₄OH (28 wt%), (3-aminopropyl)triethoxysilane (APTES) were supplied by Sigma Aldrich. Polyphosphate was purified from P70 (BK Giulini GmbH, Germany). Deionized water was obtained using a Milli-Q water purification system (Millipore). Frozen citrated pooled normal plasma (PNP) and Factor XII-deficient plasma were purchased from George King Biomedical (Overland Park, KS) and handled according to package instructions. Phospholipid solutions in chloroform were purchased in Avanti Polar Lipids: L- α -phosphatidylcholine (PC) and L- α -phosphatidylserine (PS). Sodium chloride, potassium chloride, sodium phosphate, dibasic and potassium phosphate were also purchased from BK Guilini GmbH (Germany).

Polyphosphate was obtained from the Morrissey lab by solubilizing the P70 in 250 mM LiCl/50 mM LiBO₃, pH 10.5 at 100 °C for 10 min. The resulting crude material was then purified by isopropanol precipitation, with fractions characterized by Western blotting.

C. Synthesis of silica nanoparticles and polyphosphate coated silica nanoparticles

Silica is generally considered to be a non-toxic material, and it is often used in drug delivery studies.(111, 112) However, due to its negative surface charge, silica is also a contact activator.(64, 95) Consequently, the ideal construction of a polyP-bearing silica nanoparticle would shield the silica from exposure to the systemic circulation, with targeting and exposure of the silica carrier surface and polyP at the site of internal hemorrhage. Current materials used for treating external hemorrhage generally contain particles in the micrometer range, which are too large to easily traverse capillaries and unsuitable for use as intravenous therapeutics.(118) I consequently developed an approach for the synthesis of 50 - 100 nm diameter particles (Figures 29 and 30, Table 7).(64, 118)

SNPs were synthesized following a modified Stöber method.(136) In a typical synthesis, TEOS and ammonia were added consecutively, dropwise into 57 mL of ethanol (EtOH) while stirring at 300 rpm at room temperature. Stirring was continued for 24 h. Differing amounts of TEOS (0.5 – 5 mL) and ammonia (0.5 – 4 mL) were used to produce a library of selectively sized nanoparticles with different diameters. To produce the desired 55 nm SNP, 5 mL TEOS were added dropwise to 57 mL EtOH followed by 3 mL NH₄OH (28 wt%). pH and particle size were measured directly after synthesis, in ethanol. The materials were recovered by centrifugation (14 k, 30 min) and washed three times with ethanol to remove impurities. After redispersing in ethanol by sonication (bath sonicator, Model FS20 Fisher Scientific), the products were dried overnight at 60 °C. The powder was homogenized then calcined at 550 °C for 4 h.

In order to attach the highly anionic polyP to an oxide, I followed the model of Lorenz et al. who used zirconia, which like silica has a negative surface potential, as the scaffold for applications in protein separation and purification.(137) This attachment strategy exploits the Lewis acid properties of an oxide surface to bind polyP, overcoming the electrostatic repulsion.(138)

To prepare the polyP coated SNP (polyP-SNP) the synthesized silica nanoparticles were first dispersed by sonication in Milli-Q water and placed at 30 °C. Polyphosphate was dissolved in a separate Milli-Q water solution and was added dropwise under vigorous stirring. The solution was stirred overnight. The functionalized materials were recovered by two rounds of centrifugation (14 k, 30 min), each time washed with water and redispersed by sonication. PolyP-SNP products were dried overnight at 60 °C. Successful modification was determined using dynamic light scattering (DLS) and zeta potential. Using phosphate

buffered saline solution (PBS) as the solvent at pH 7.4, functionalizing the SNP with P70 increased particle size roughly 15 nm.

D. Characterization of the particles

1. Zeta potential and particle size determination

Particle sizes and zeta potentials were measured by laser diffractometry using a Zetasizer Nano ZS instrument (ZEN 3600, Malvern Instruments) at 20 °C with an incident wavelength of 633 nm and 173 ° backscattering angle. Zeta potentials were measured in water at different pH values and in PBS buffer (approximately 137 mM NaCl, 2.7 mM KCl, and 12 mM phosphate, pH 7.4). Particle size was measured just after synthesis (with ethanol as a solvent) and again after the calcination step, in ethanol at 1 mg/ml. Disposable cuvettes were cleaned with ethanol and water prior to sample loading.

The change in surface charge suggested the presence of polyP in undigested polyP-SNP (Table 1, SI Figure. 2). At physiological pH in PBS, both SNPs and polyP display a negative surface charge. In deionized water, the SNP surface charge ranged from -15 to -25 mV (Figures 27 and 28, Table 7).⁽¹³⁵⁾ In simulated body fluid (SBF) at physiological pH, SNPs had a surface charge of -50 to -60 mV. PolyP is negatively charged at physiologic pH due to a pKa1 between pH 1-2 (for all internal phosphates) and pKa2 between pH 7.2 and 8.2 (for the two terminal phosphates).⁽¹³⁹⁾ Upon functionalization of polyP to the SNP surface, the zeta potential of the polyP-SNP decreased from -20 to -30 mV to roughly -40 to -50 mV in water, confirming the attachment of the polyP. In SBF, both particles exhibited a strongly negative charge below -45 mV.

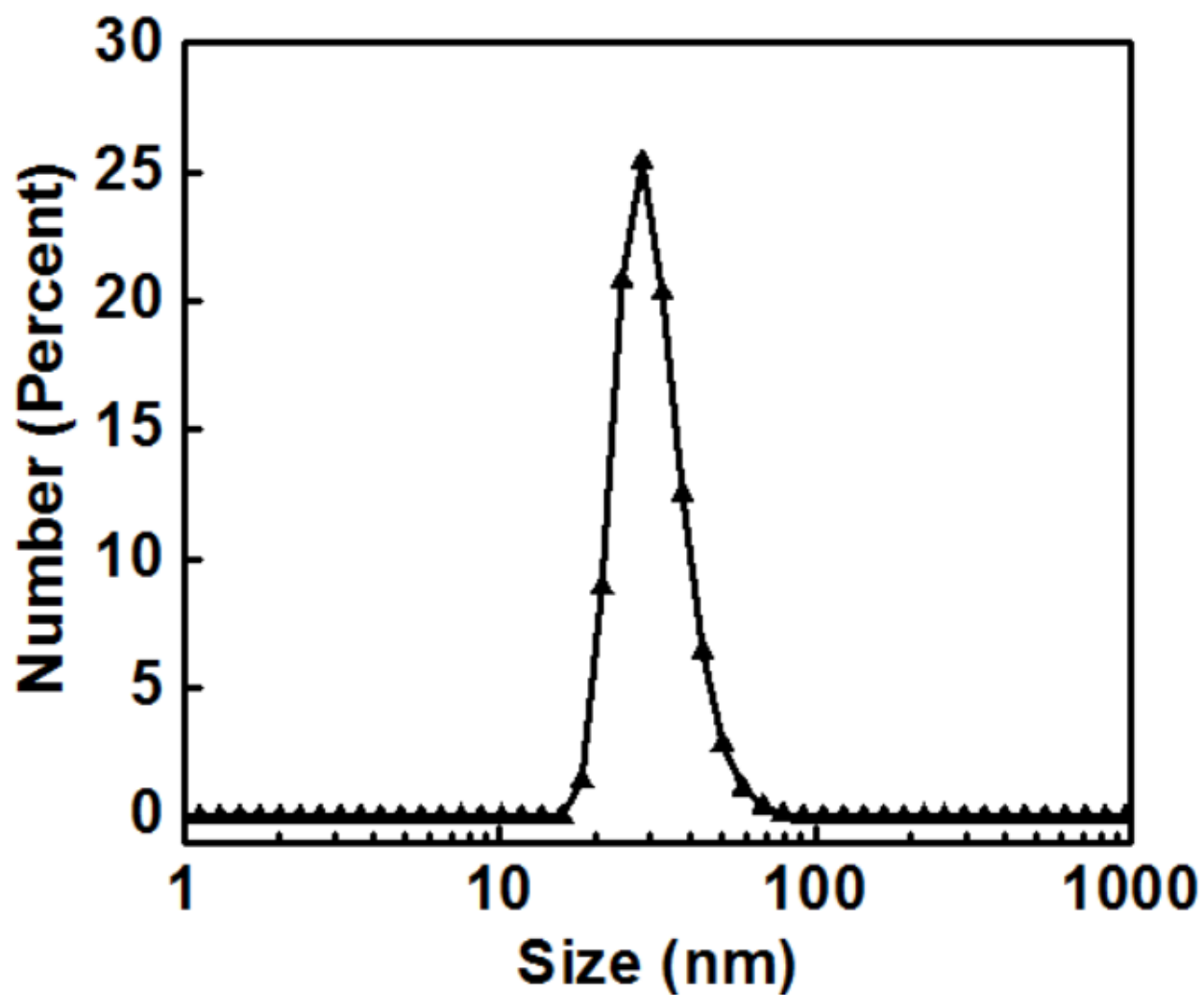


Figure 26. Size of SNPs after synthesis in ethanol measured using DLS.(135)

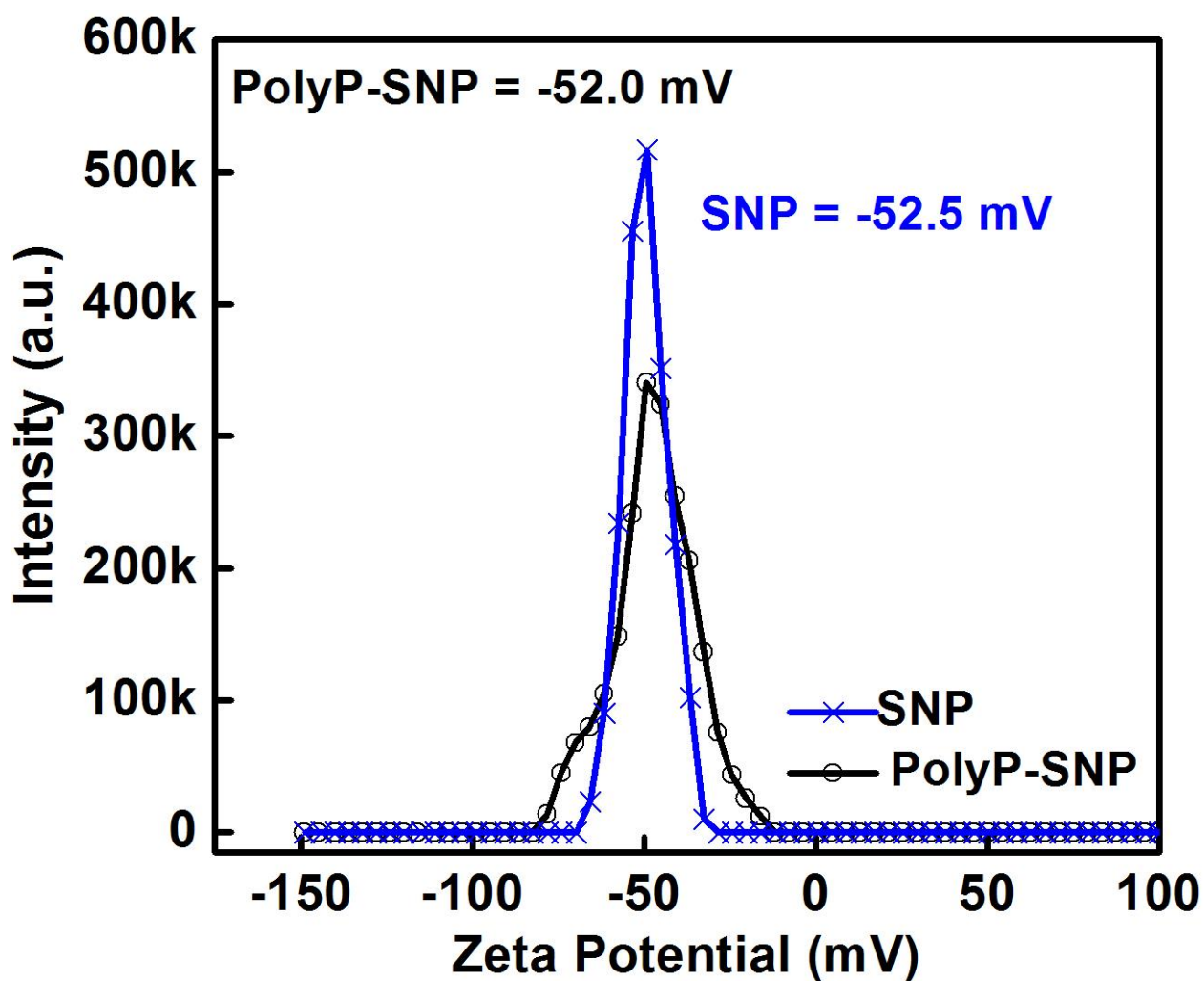


Figure 27. Both SNPs and polyP-SNP have a Zeta potential of roughly -50 mV in PBS, pH 7.4.(135)

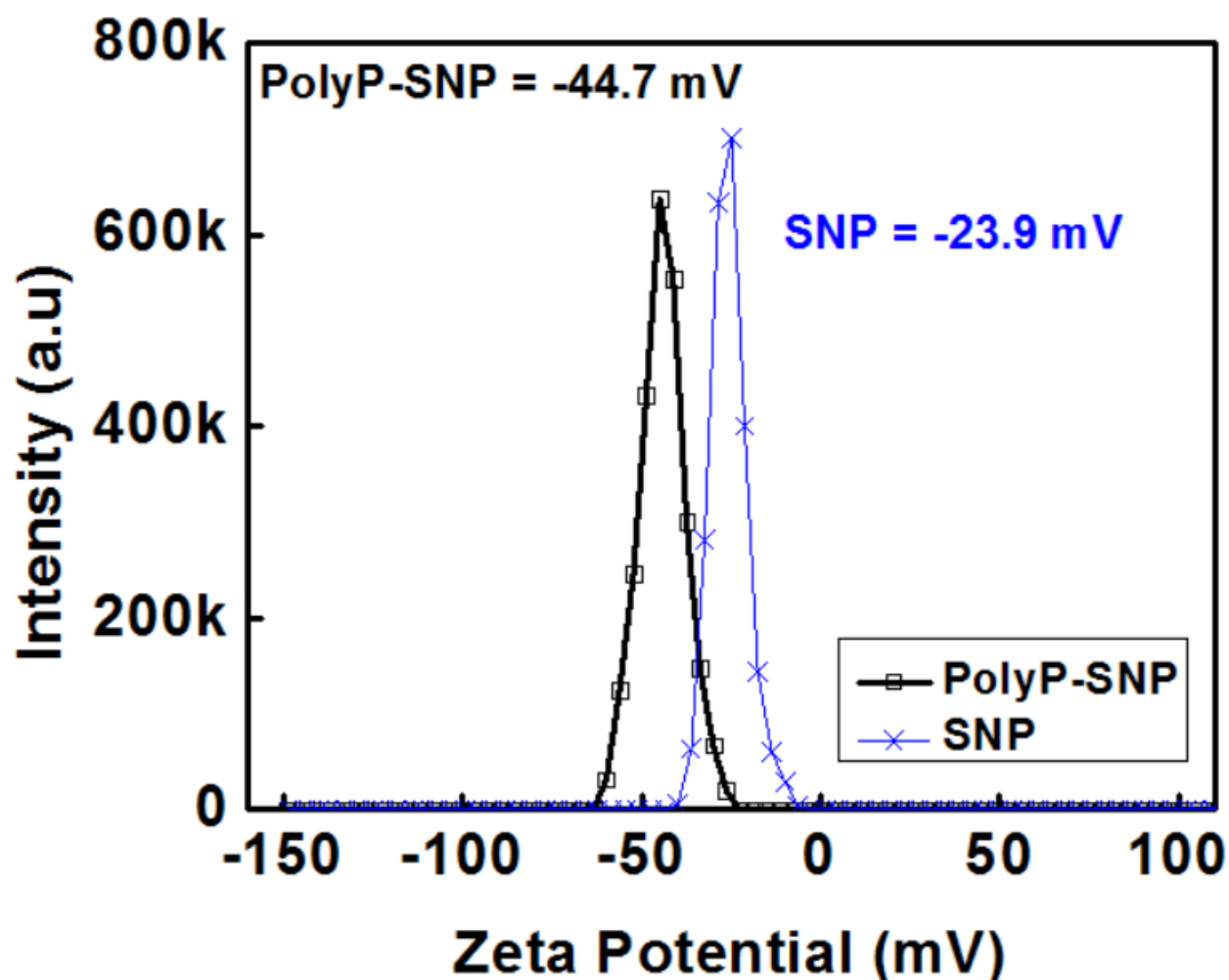


Figure 28. Zeta potential of polyP-SNP and SNP in deionized water.(135)

Table 7. The zeta potential and surface charge of the particles change when dispersed in water or phosphate buffered saline solution, pH 7.4.(135)

Compound, Medium	Zeta Potential (μV)	Size (nm)
SNP, H ₂ O	-24.4 ± 0.3	55.97 ± 2.19
SNP, PBS	-52.5 ± 2.1	74.00 ± 6.24
PolyP-SNP, H ₂ O	-44.3 ± 0.3	53.79 ± 1.75
PolyP-SNP, PBS	-52.0 ± 3.2	78.18 ± 2.86

2. Morphology and structure of the particles

The size, morphology, and structure of representative samples were observed via transmission electron microscopy (TEM). TEM micrographs (Figures 29, 30) were obtained on a FEI Tecnai G2 Sphera electron microscope with an accelerating voltage of 200 kV.

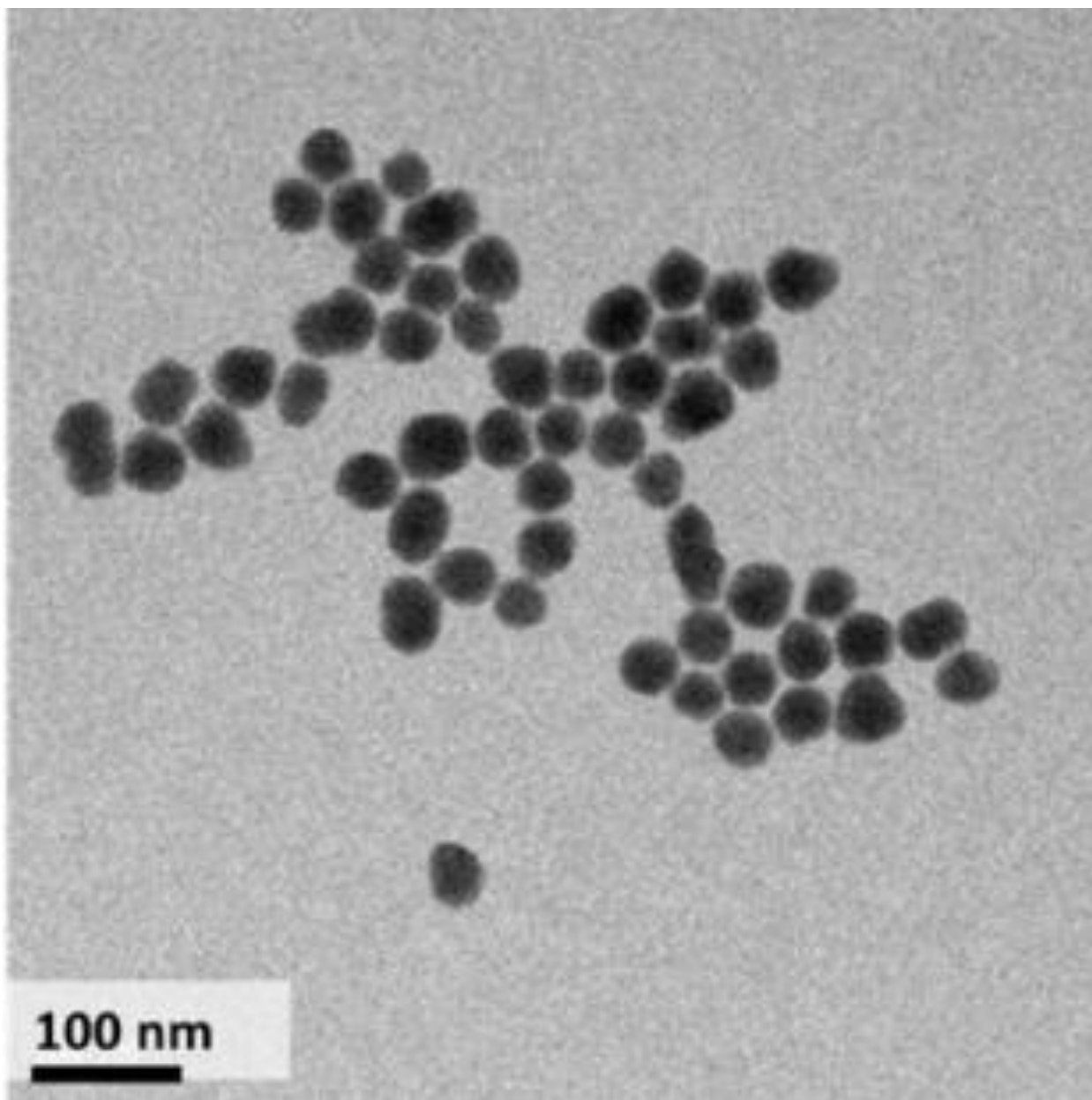


Figure 29. TEM images of polyP-SNPs.(135)

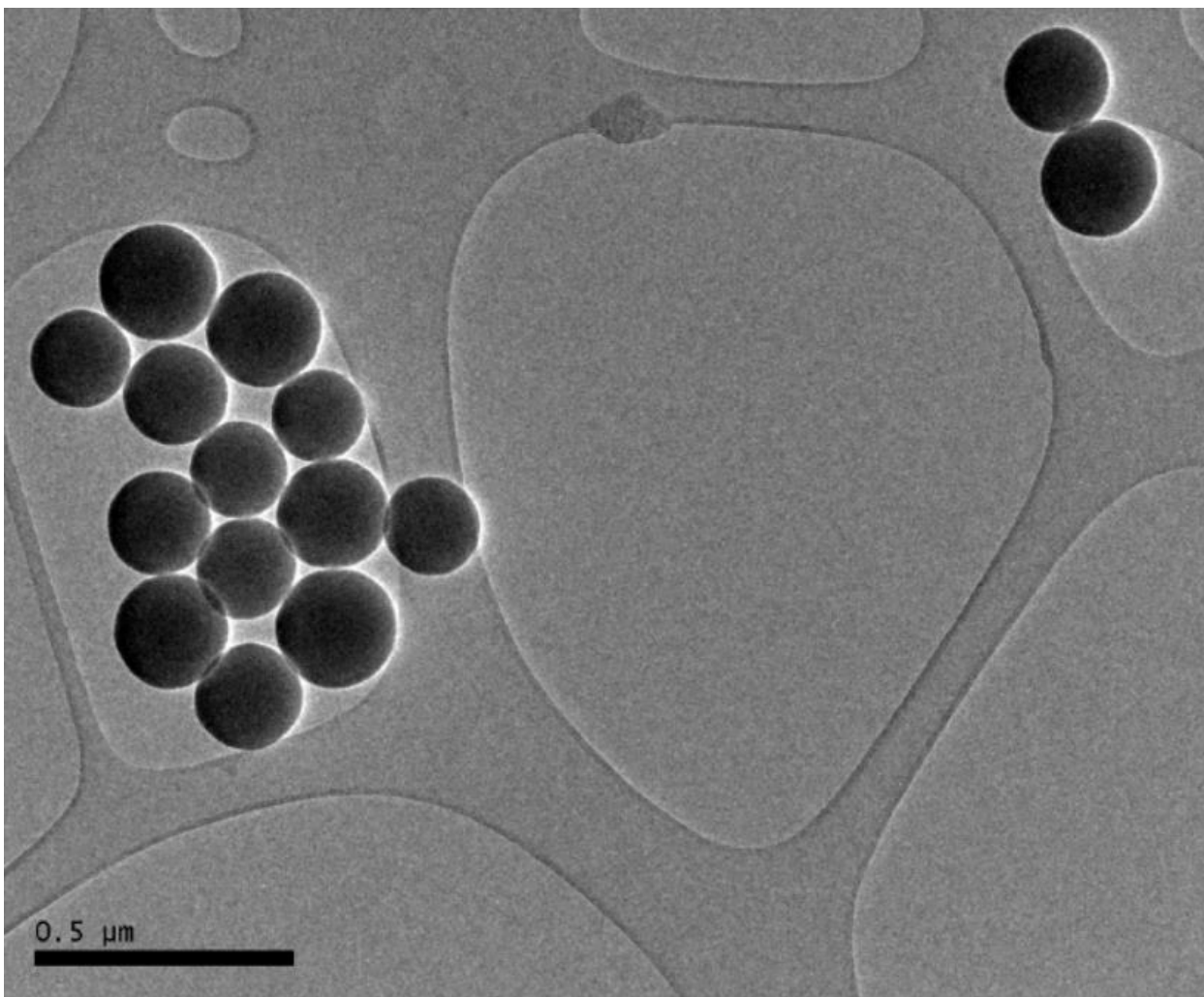


Figure 30. TEM images of SNPs.(135)

3. Digestion of polyP-SNP particles into phosphate monomers for malachite green assay

PolyP content on the particles was quantified by the Morrissey lab by hydrolyzing polyP to monophosphate. Calf intestinal alkaline phosphatase (CIAP, a potent exopolyphosphatase) was added to PolyP at 37 °C, followed by phosphate analysis using malachite green microassay. The polyphosphate is quantified in units of phosphate monomer (MW 102). Malachite green identified concentrations of 56, 26, and 23 nmol PO₃/mg SNP.

4. Quantification of the polyphosphate on the nanoparticles

Further quantification used inductively coupled plasma atomic emission spectroscopy (ICP-AES). 170.35 mg polyP-SNP particles were digested in 1M hydrochloric acid at 100 °C for several hours to break polyP into phosphate ions. The resulting solution was decanted to remove the phosphorous-rich supernatant from solid SNPs. ICP-AES tests on the supernatant concentration revealed 1.5624 ppm P. The ppm ratios were converted to nmol PO₃/mg SNP and the resulting ratio was found to be 29.6 nmol PO₃/mg SNP, which correlates to the 26 nmol PO₃/mg SNP determined through malachite green assay. Digestion of other polyP-SNP samples in hydrofluoric acid also identified the presence of phosphorous in the silica nanoparticles (Table 2, 3). However, the samples digested in HF were too dilute to reliably report the phosphorous concentration quantitatively.

To quantify the polyP loaded on the SNP surface, the digested phosphate solutions were measured using both a malachite green assay and inductively coupled plasma atomic emission spectroscopy (ICP-AES). ICP-AES determined the 26 nmol PO₃ sample (malachite green) had 29.6 nmol PO₃/mg SNP. Assuming each polyP chain has 70 PO₃ monomers, these data suggest 100 – 200 polyP molecules are attached to each SNP.

Table 8. Concentration of P (20x dilution) in a 170.35 mg polyP-SNP sample digested in 1 M HCl as measured by ICP-AES.(135)

Element	Wavelength (nm)	Units	Avg	Std Dev	RSD
Phosphorous	177.495	ppm	1.574	0.0105	0.6675
Phosphorous	178.284	ppm	1.563	0.0038	0.2463
Phosphorous	178.766	ppm	1.56	0.0054	0.344
Phosphorous	213.618	ppm	1.565	0.0123	0.7855
Phosphorous	214.914	ppm	1.55	0.0066	0.4243
Average		ppm	1.562		

Table 9. Concentration of P (9x dilution) in a 79.10 mg polyP-SNP sample digested in 1 M HCl as measured by ICP-AES.(135)

Element	Wavelength (nm)	Units	Avg	Std Dev	RSD
Phosphorous	177.495	ppm	11.06	0.0105	0.6675
Phosphorous	178.284	ppm	11.08	0.0038	0.2463
Phosphorous	178.766	ppm	11.08	0.0054	0.344
Phosphorous	213.618	ppm	11.06	0.0123	0.7855
Phosphorous	214.914	ppm	11.04	0.0066	0.4243
Average		ppm	11.06		

5. ^{31}P NMR (Nuclear Magnetic Resonance)

The decanted phosphate sample was dried and resuspended in deuterium oxide. The resulting suspension was measured using an Agilent Technologies 400 MHz, 400-MR DD2 Spectrometer. A ^{31}P NMR spectra showed evidence of phosphate from the digested polyP-SNP sample.

^{31}P NMR and surface charge change qualitatively demonstrated polyP attachment. PolyP-SNPs were digested in acid to break down polyP into phosphate monomers. ^{31}P NMR tests on the digested sample detected the presence of phosphorous.

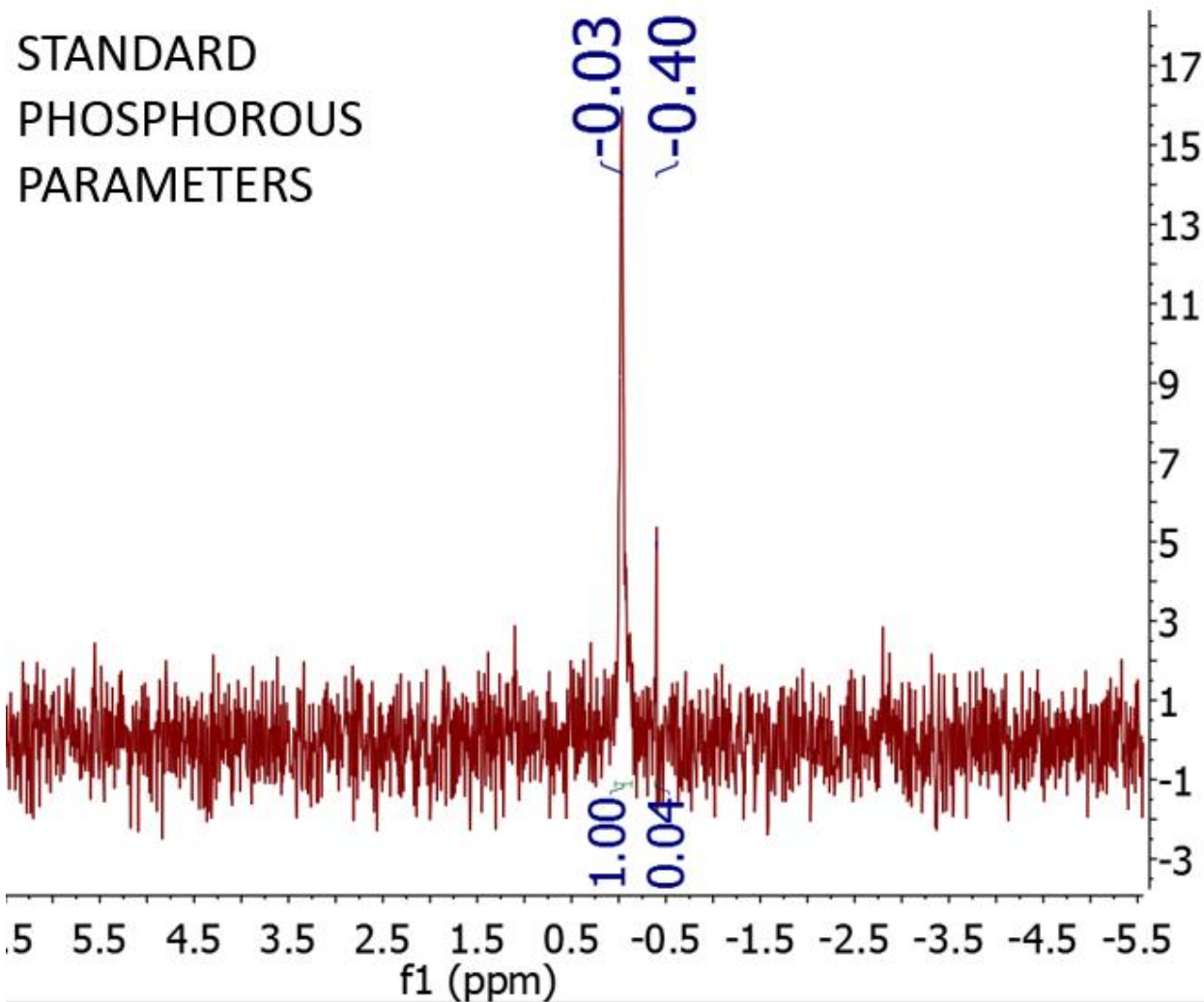


Figure 31. ^{31}P NMR spectrum of digested polyP-SNPs shows evidence of phosphorous.(135)

6. Determination of the clotting activity

The clotting activity was determined by two methods: standard coagulometry and thromboelastography (TEG) (Thromboelastograph model TEG® 5000, Haemonetics). TEG is an accepted point of care test used by clinicians.⁽¹⁴⁰⁾ These tests measure several parameters that are relevant to coagulation, including initial time for clot formation (R, min), rate of clot formation (α , deg), time until clot reaches 20 mm (K, min) and clot strength (maximum amplitude (MA), mm). For these tests, the particles were dispersed in HEPES buffered saline (HBS) containing phospholipid (PL) vesicles and sonicated. The phospholipids were a mixture of 80 % phosphatidylcholine (PC) and 20 % phosphatidylserine (PS) prepared by sonication of chloroform stocks in HBS, following the protocol by Morrissey et al. All subsequent dilutions were made by diluting the stock dispersion in this same solvent. HBS is 100 mM HEPES (Sigma-Aldrich), 20 mM HEPES/NaOH buffer (pH 7.5), and 0.02 % (w/v) sodium azide (Sigma-Aldrich).⁽¹⁴¹⁾

For the coagulometry tests, 50 μ L of the particles were placed into a pre-warmed coagulometer cuvette followed by 50 μ L of pooled normal plasma. After incubating for 33 minutes at 37 °C, the contact pathway was activated and the mixture equilibrated to the chosen temperature, then 50 μ L of pre-warmed 25 mM CaCl_2 was added into the cuvette. The results are the average values from duplicate runs.

In the TEG experiments, first 340 μ L of plasma and 10 μ L of the clotting agent were added in the TEG cup and incubated at 37 °C. After 3 minutes, 20 μ L of 0.2 M CaCl_2 were added to the cup and the test was started immediately. Concentration- and size-dependent analyses were performed. The results shown are the average value of, typically, 4 to 6 replicates.

I also monitored the formation of thrombin by fluorescence plate assay, using a thrombin-sensitive dye T-butyloxycarbonyl-b-benzyl-L-aspartyl-L-prolyl-L-arginine-4-methylcoumaryl-7-amide (Boc-Asp(OBzl)-Pro-Arg-MCA)Boc-Asp(OBzl)-Pro-Arg-MCA (Peptides International, Louisville, KY). This assay is used to calculate the thrombin burst time, which is related to clot formation time.

E. Discussion

By not using tissue factor to initiate clotting, I focused my clotting assays on the intrinsic and contact activation pathways. Negatively charged particles, such as the aluminosilicate kaolin (QuikClot® Combat Gauze™) used for external injuries, activate the contact pathway.(64, 125) Because it is a poor clot initiator, polyP attachment shields SNPs in the systemic circulation, which is beneficial for use to control hemorrhage intravenously. I measured the impact of both bare SNPs and polyP-SNPs on clot time using thromboelastography (TEG, Figures 7 - 10) on pooled normal plasma (PNP). Both particles decreased the time to initial clot formation (R) in a concentration-dependent manner, with polyP-SNP being more potent at concentrations below 0.5 mg/ml. The clot time reported in figures 2 and 4 refers to the time to initial clot formation, which is key in illustrating how polyP-SNP successfully accelerates clotting. The agent used did not affect the overall clot size formed, only the time required to reach peak clot size. However, polyP has previously been shown to improve overall clot formation, in part by limiting the effect of fibrinolysis in plasma containing tissue plasminogen activator.(20, 134)

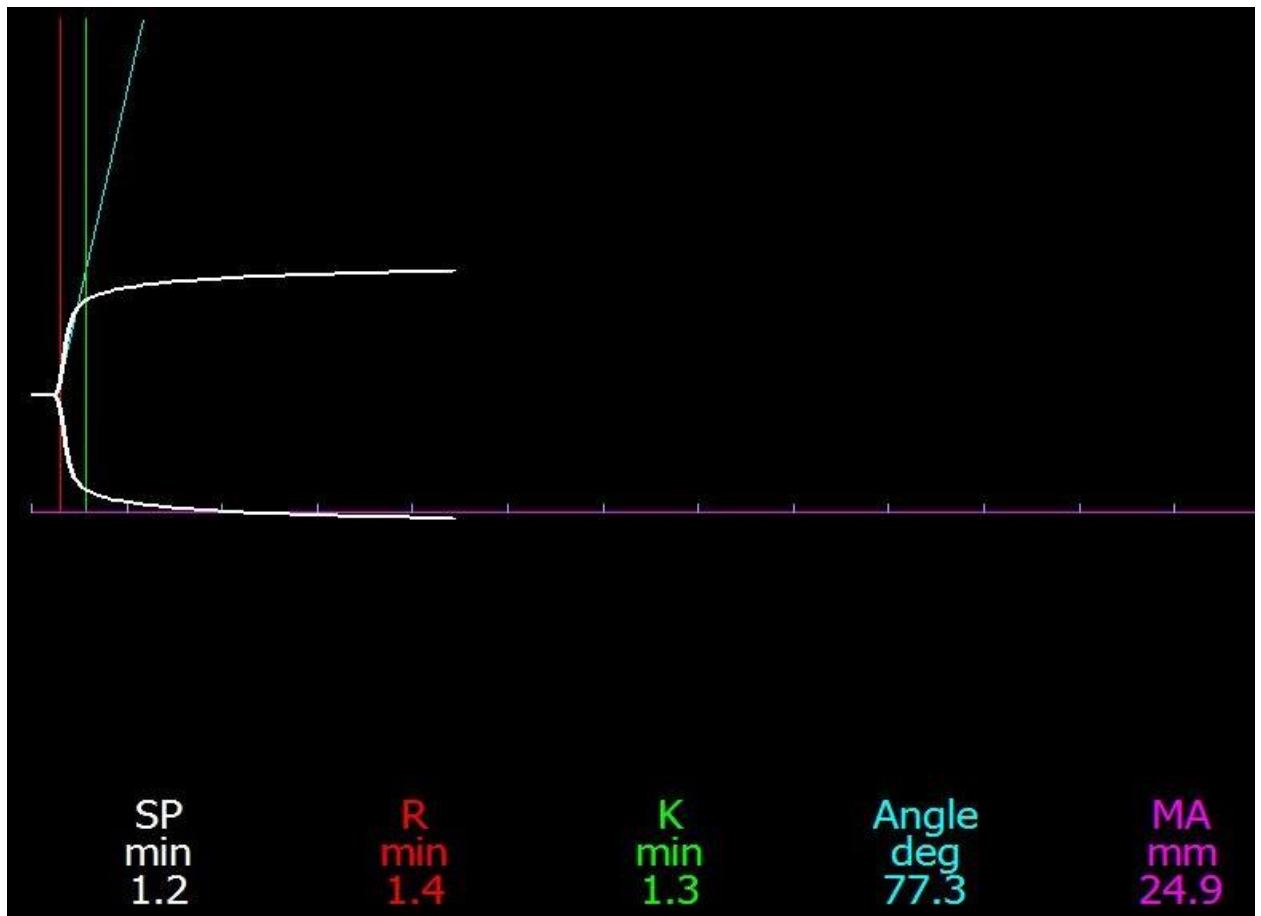


Figure 32. Sample thromboelastography curve.(135)

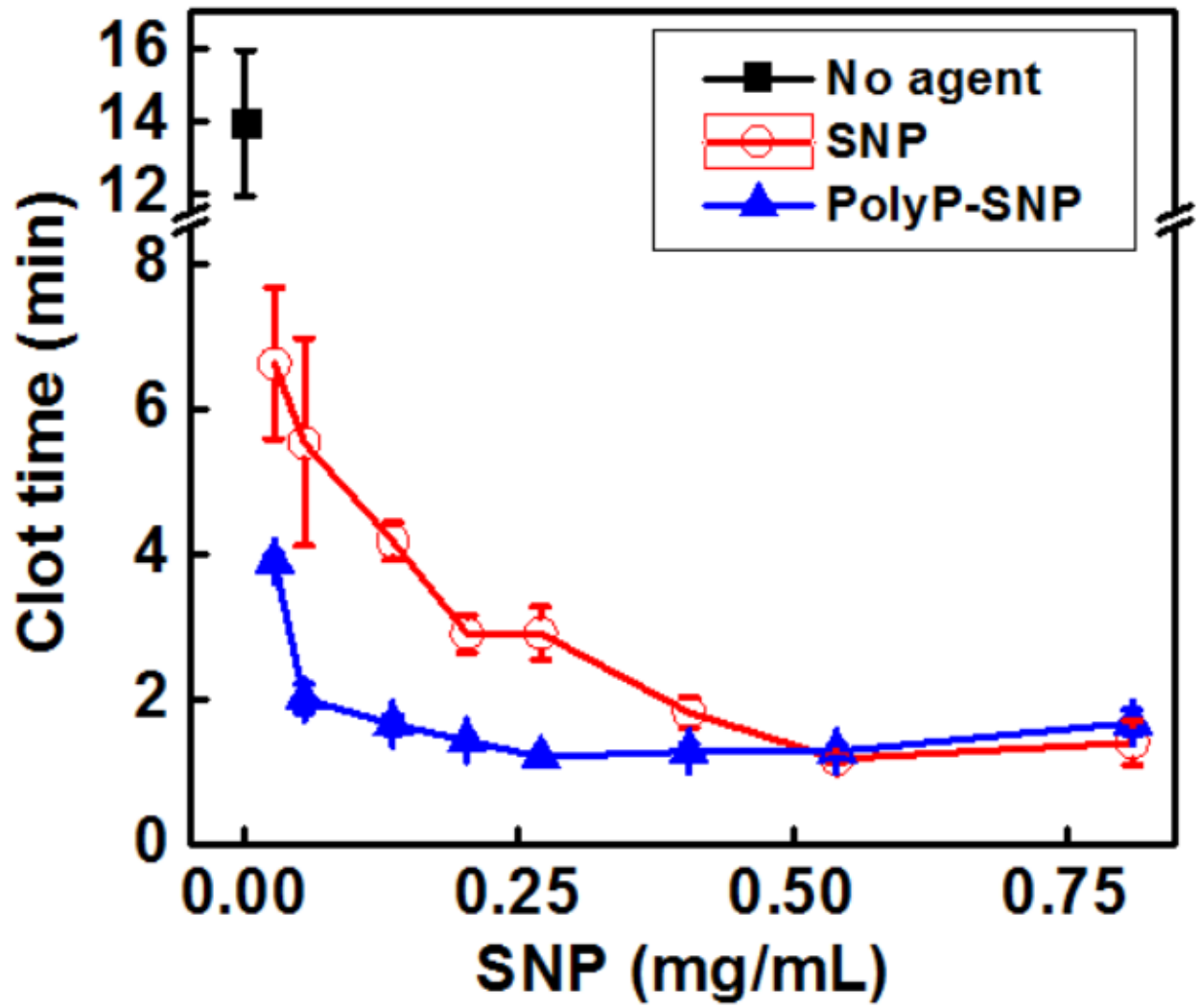


Figure 33. PolyP-SNP cuts clot time (R value using TEG) roughly in half versus SNP below 0.3 mg/ml.(135)

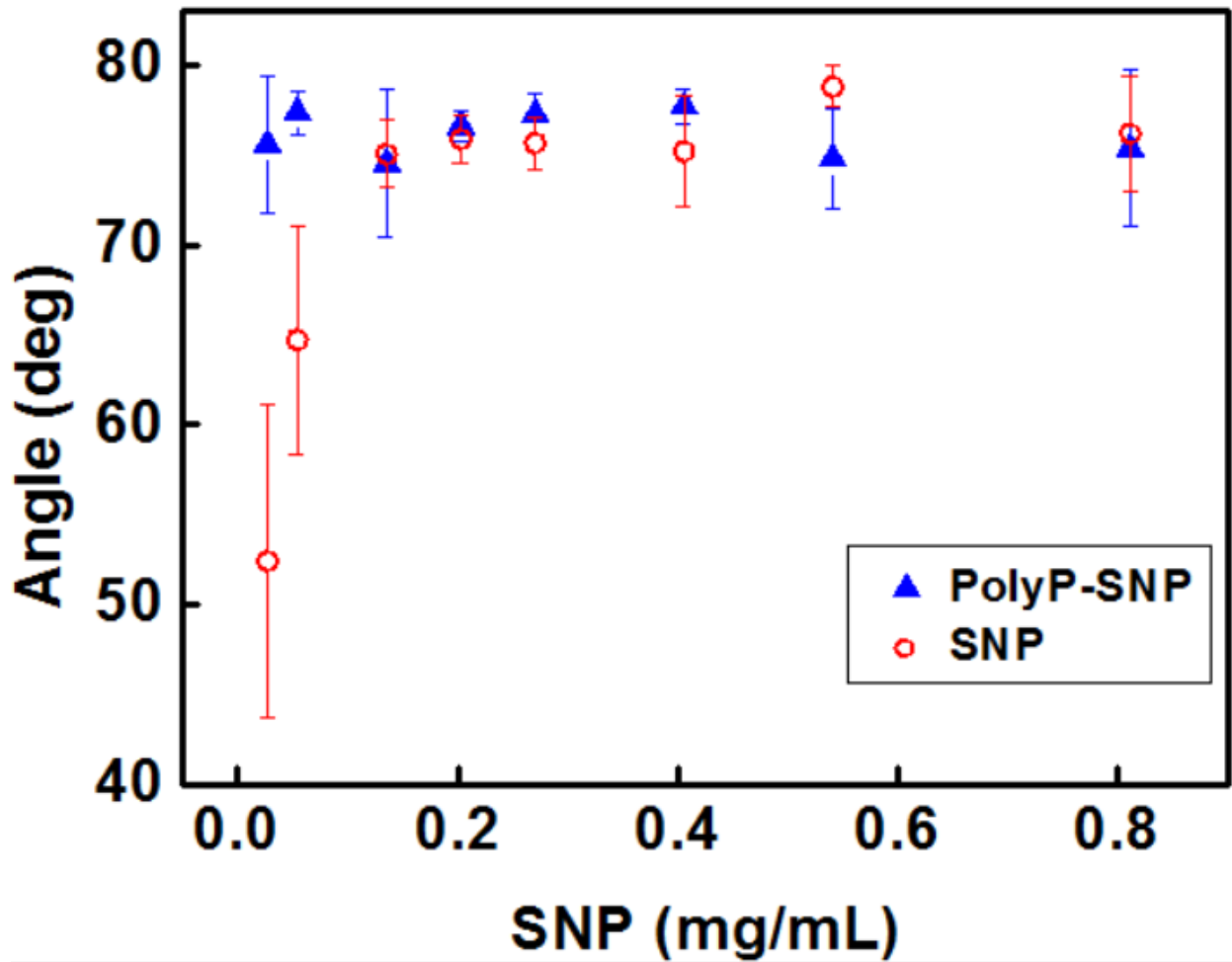


Figure 34. The Angle value for thromboelastography measurements correlates to the rate of clot growth. At low concentrations, polyP-SNP induced clots grow faster than SNP induced clots.(135)

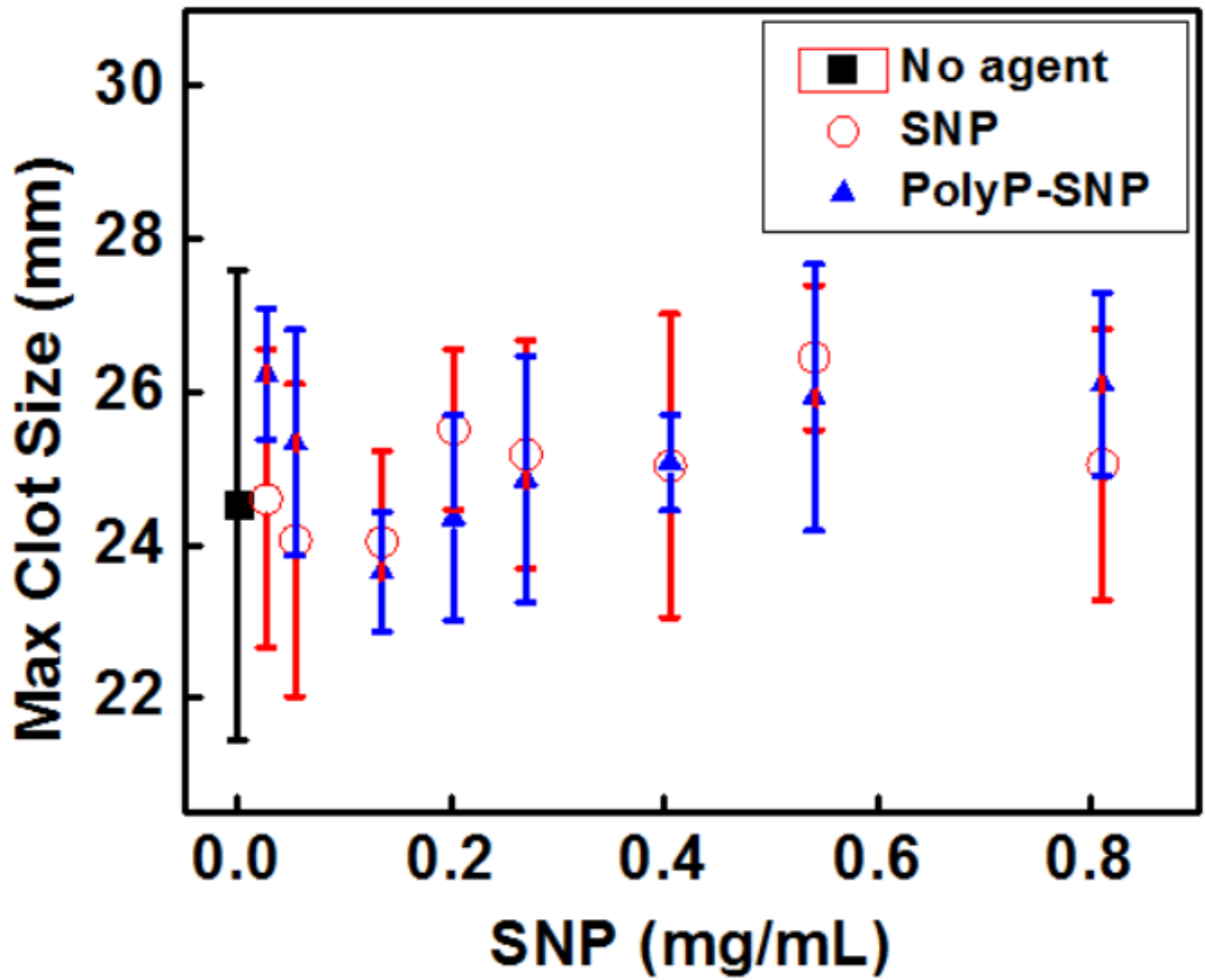


Figure 35. The agent used had minimal impact on the maximum size of the clot formed in plasma.(135)

Next, the Morrissey lab evaluated the procoagulant activity of polyP-SNPs formed with differing loads of polyP (Figure 36). PolyP-SNPs of varying polyP loading were synthesized and shipped for analysis using the Morrissey lab's coagulometer. The ability to promote coagulation was measured by adding polyP-SNP or polyP in solution to PNP. Coagulation was evaluated by measuring time to clot formation on a fibrometer (Figure 29). In comparing polyP payload, polyP-SNPs were more potent at activating the contact pathway than polyP in solution.

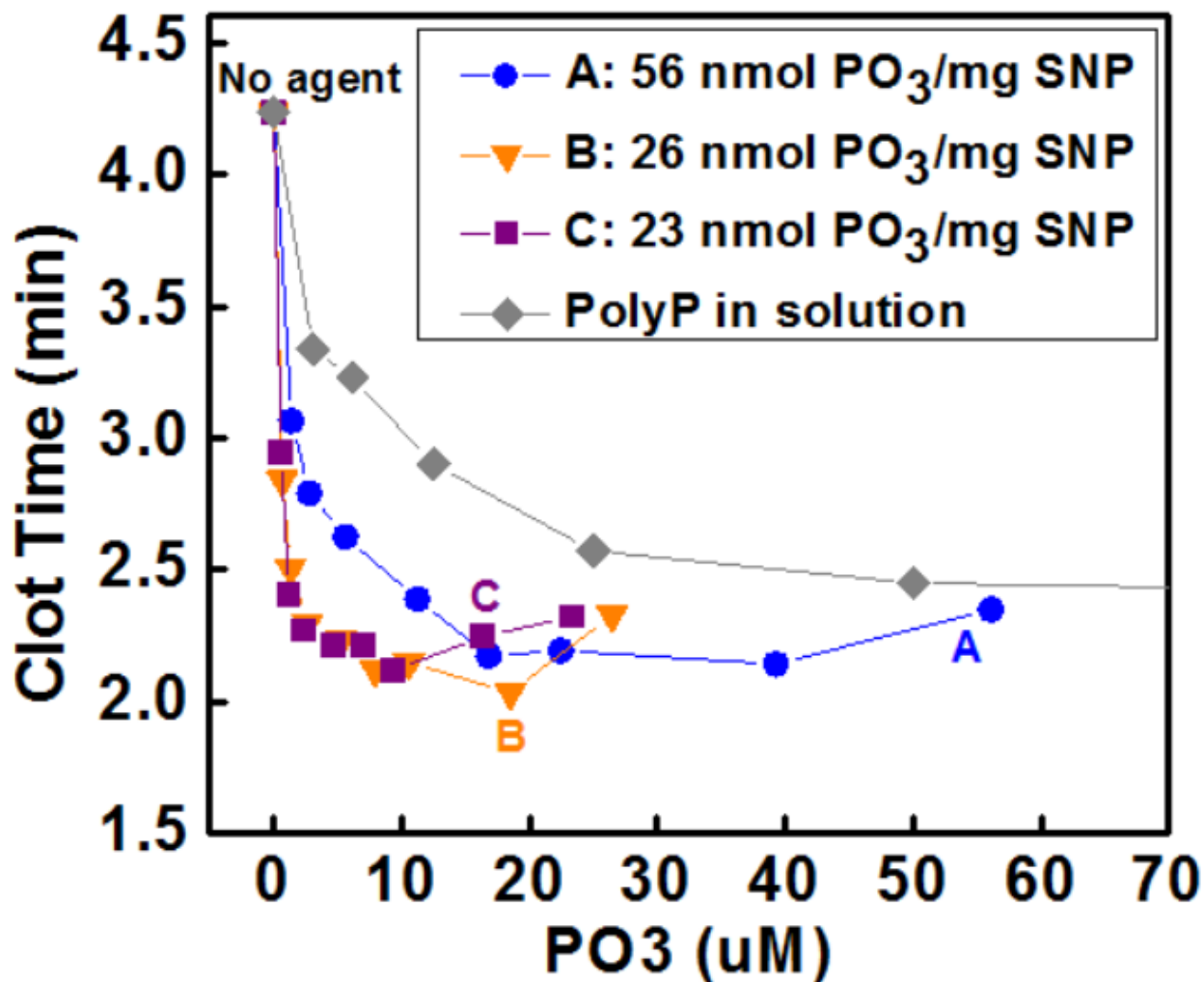


Figure 36. PolyP loaded onto silica lowers clot time compared to bare polyP when measured by fibrometry.(135)



Figure 37. Upon addition of polyP-SNP to recalcified plasma, rapid thrombin generation is seen. The blue color indicates cleavage of the fluorescent dye by thrombin, signifying active coagulation.(135)

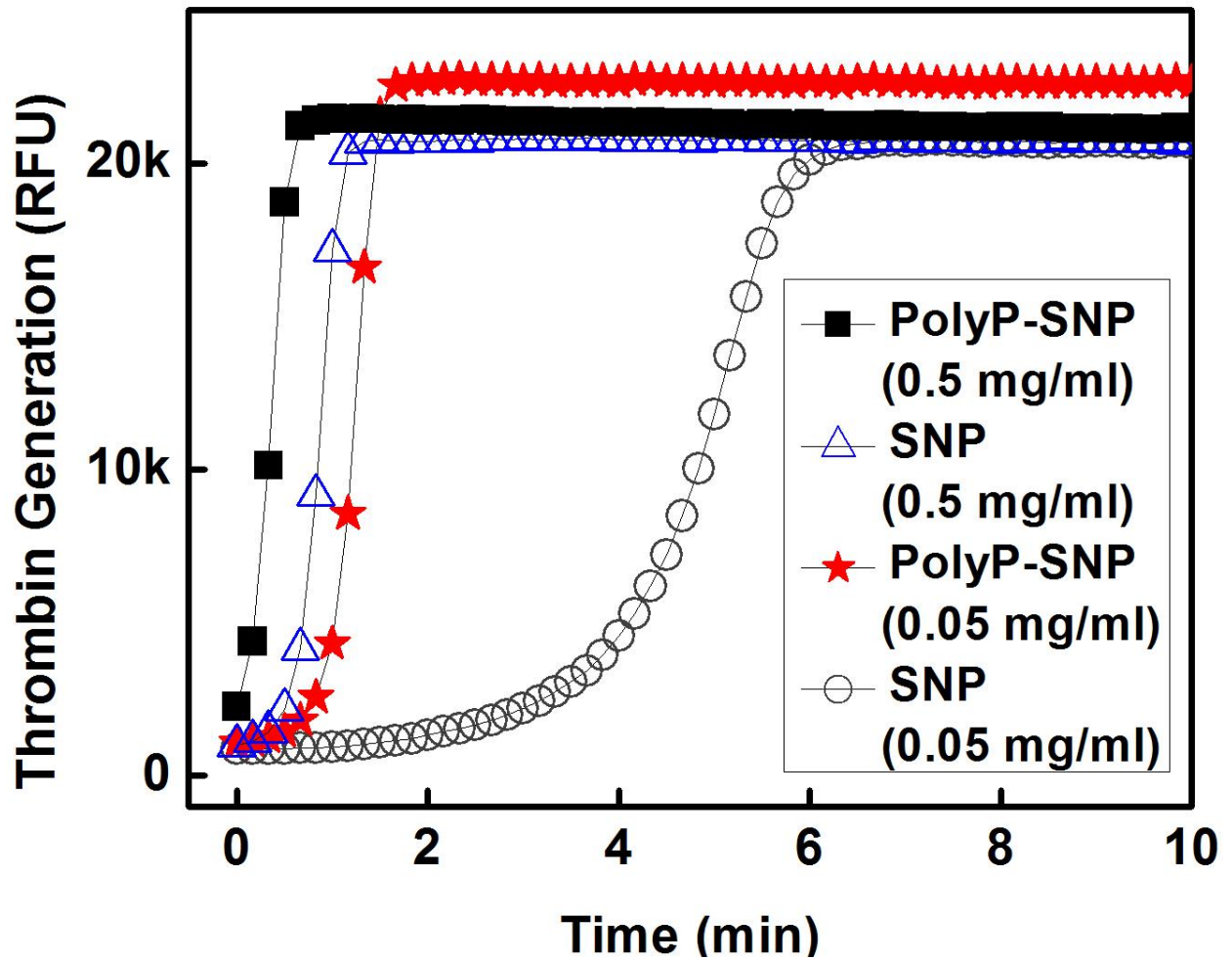


Figure 38. PolyP-SNPs are superior to bare SNPs in their ability to generate a rapid thrombin burst. This difference becomes even more pronounced at lower concentrations (0.05 mg/ml). Thrombin generation is measured using a thrombin-sensitive fluorescent dye.(135)

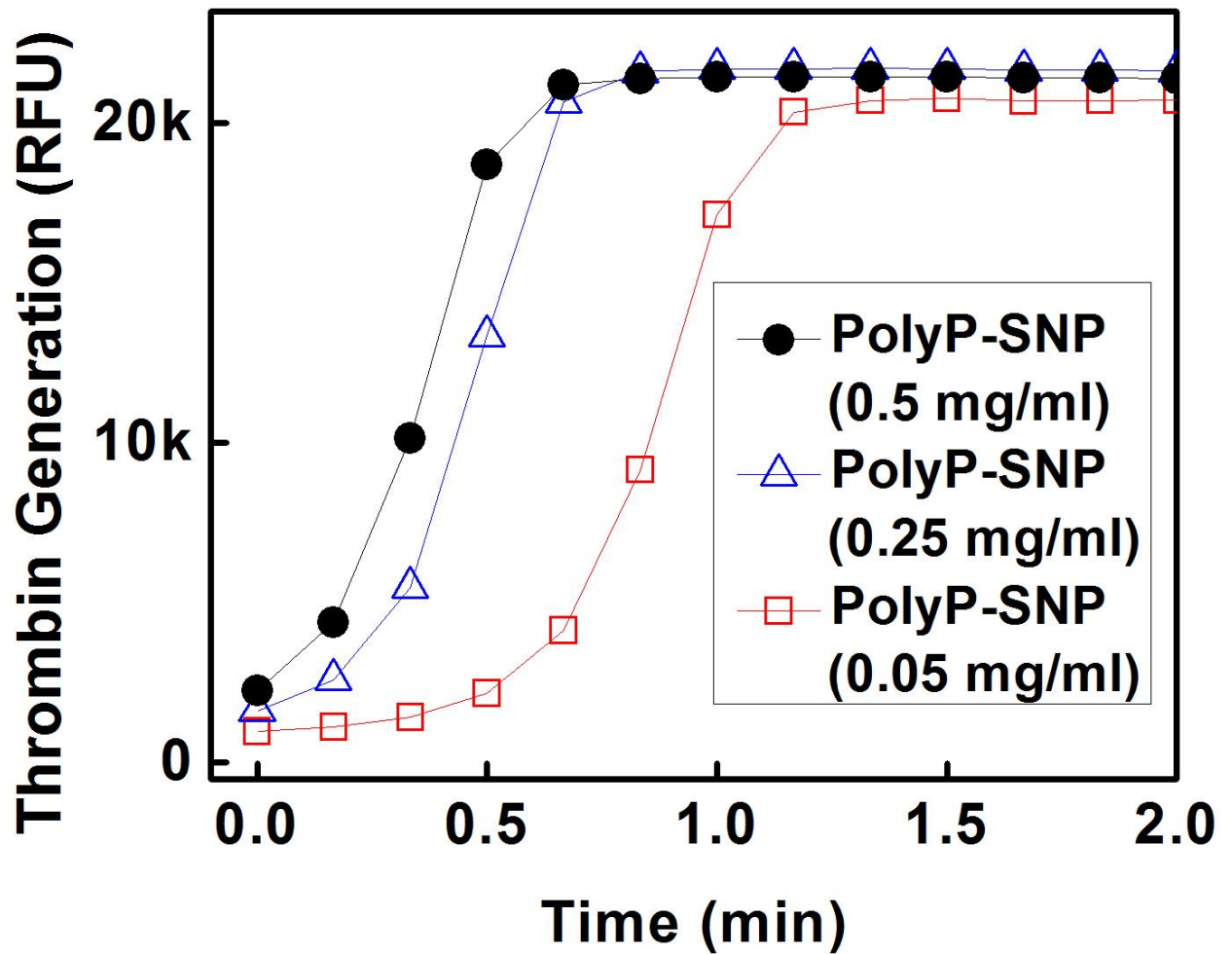


Figure 39. PolyP-SNPs are able to generate a rapid thrombin burst even at low concentrations. Thrombin generation is measured using a thrombin-sensitive fluorescent dye.(135)

In order to further explore the relative activities of the new materials, I evaluated the ability of the materials to generate thrombin, the terminal enzyme of the coagulation cascade and the primary determinant of the rate of fibrin formation.(18) PolyP-SNP again substantially outperformed its bare counterpart (Figures 37-39).

I next evaluated whether polyP-SNPs were able to enhance the generation of downstream coagulation enzymes (common pathway). I eliminated any potential impact on contact activation by utilizing factor XII-deficient plasma and initiating coagulation using a small amount of lipidated tissue factor (LTF, 63 pM). As expected, bare SNPs did not affect TEG clot time in this system (Figure 40). In contrast, polyP-SNP did shorten the time to physical clot formation, though it did not change the overall clot growth (Figures 40, 41). This indicates that the polyP is accessible for binding to the relevant downstream coagulation proteins. Additionally, this response could also be evaluated in the clinical setting by comparing tests such as prothrombin time (PT) and partial thromboplastin time (PTT).

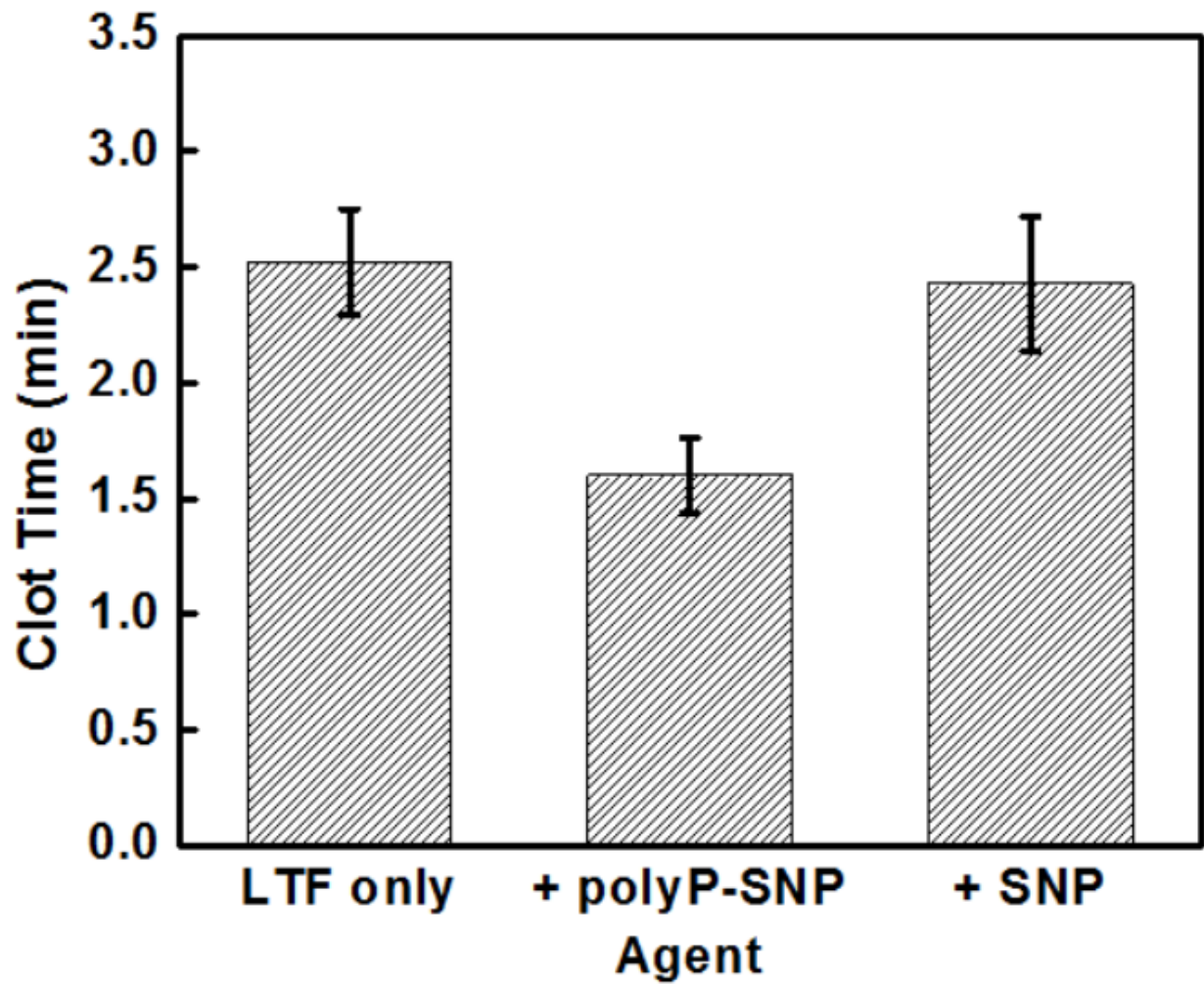


Figure 40. Adding PolyP-SNP to LTF shortens clot time in FXII-deficient plasma compared to LTF only or LTF + SNP.(135)

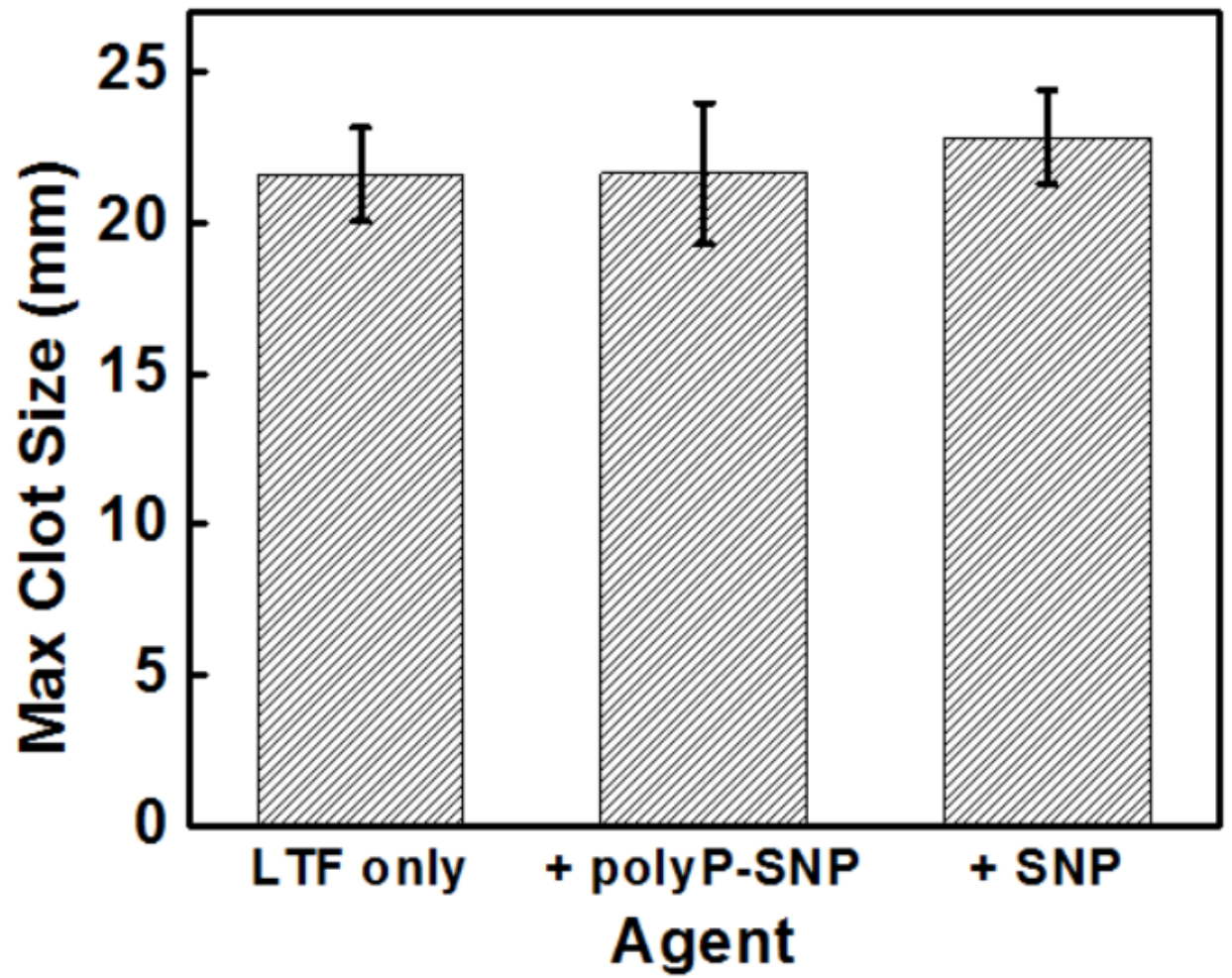


Figure 41. The maximum clot size formed in FXII deficient plasma was nearly identical regardless of the agent used.(135)

One of the problems facing emergency medical personnel is that current intravenous treatments have a significantly short half-life at ambient temperature. Even pure polyP nanoparticles only remain stable for hours.⁽¹³⁰⁾ In comparison, attaching polyP to silica greatly enhanced the stability and procoagulant function of polyP from hours to weeks. After bench-top storage at room temperature in both powder and aqueous suspensions, polyP-SNP clotting times remained constant for at least 24 weeks (Figure 42). The strong negative surface charge of polyP-SNPs also minimized aggregation in aqueous suspensions over the same time period. Injectable drugs with long-term shelf-life can be used by emergency medical personnel prior to hospital arrival without concern that the particles will degrade without refrigeration. This suggests that the polyP-SNP system is a candidate to be the first prehospital intravenous injection designed to treat internal injuries by accelerating the clotting system at bleeding sites.

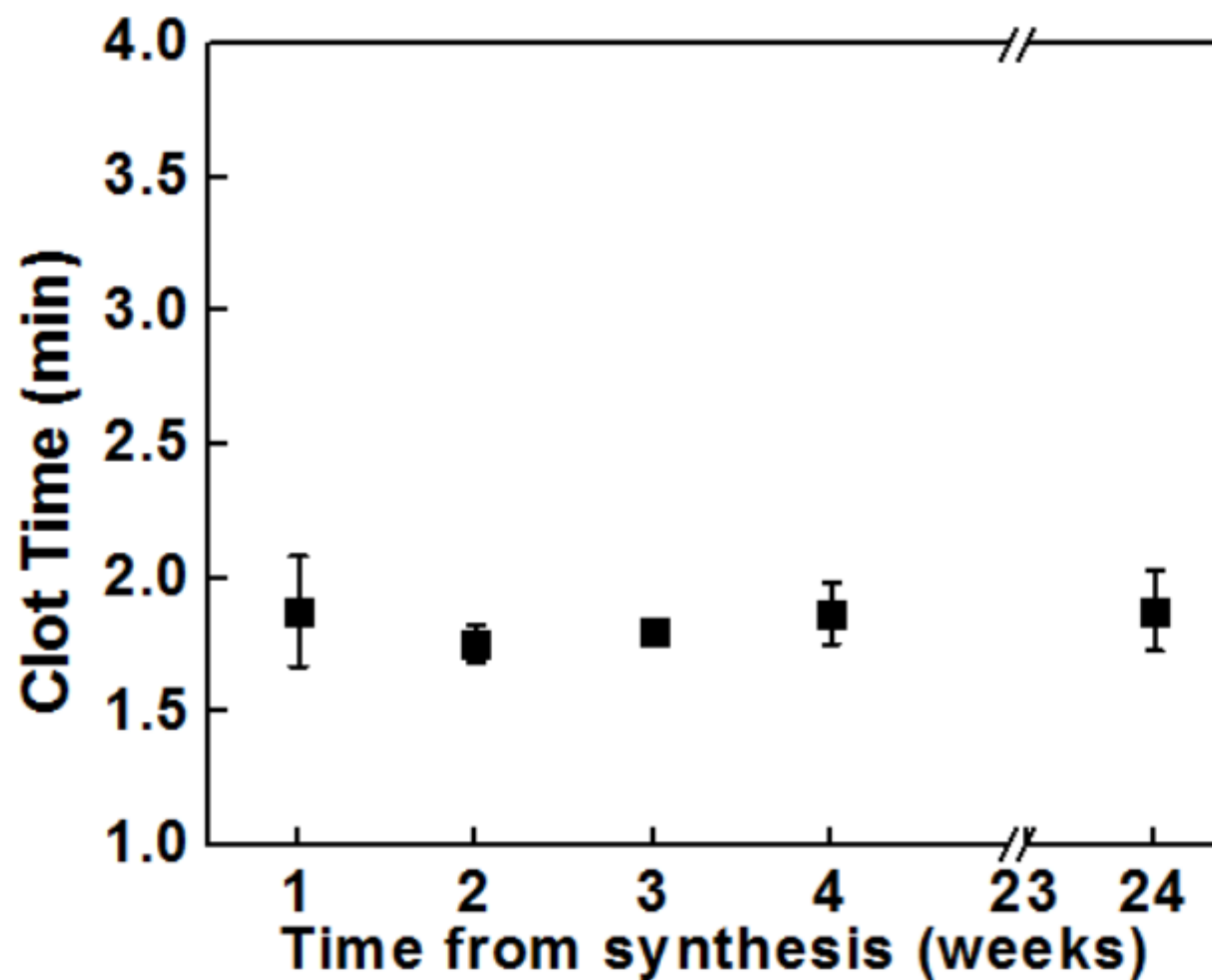


Figure 42. PolyP-SNP suspended in aqueous solution retains its procoagulant function after weeks of storage at ambient conditions.(135)

F. Conclusion

In this study, I successfully attached polyP to the surface of small-diameter SNPs and demonstrated that these polyP-SNPs are more potent than bare SNPs at promoting coagulation, likely due to polyP's ability to accelerate the common pathway for active clotting processes. PolyP-SNPs, like polyP in solution, are able to enhance downstream coagulation reactions resulting in a shorter time to clot formation. Even after long-term storage at regular room temperature, the polyP-SNP system retained its procoagulant ability. The polyP-SNP construct is consequently promising as a prohemostatic agent. Further *in vitro* exploration of polyP-SNP's ability to induce clotting in simulated trauma conditions are required prior to translation to *in vivo* preclinical trials. (142)

IV. Accelerating Clotting in the Presence of the Lethal Triad with Polyphosphate-Laden Silica Nanoparticles

It's all right, man
I'm only bleeding, man
Stay hungry, stay free
And do the best you can

- The Gaslight Anthem, *We're Getting A Divorce, You Keep The Diner*

A. Introduction

Clinicians have revised their definition of coagulopathy. Once thought to be one part of the lethal triad (factor-depleted coagulopathy, hypothermia, and acidosis),(143) acute traumatic coagulopathy (ATC) alone is now accepted as the integral factor for severe clotting impairment (Chapter 1, Figure 5).(33) As such, the lethal triad is now de-emphasized as a group of symptoms that develop in the critically injured patient after, but are not directly tied, to the onset of ATC.(33) However, the lethal triad is still a strong predictor that the patient is suffering from ATC (Tables 1, 2).(143) Furthermore, the lethal triad still impacts the coagulation cascade negatively and must be attenuated if the patient is to return to homeostasis.

Table 10. Risk factors for developing coagulopathy. Reprinted from The Journal of Trauma and Acute Care Surgery, Vol. 42, Issue 5, N Cosgriff, EE Moore, A Sauaia, M Kenny-Moynihan, JM Burch, B Galloway, Predicting Life-threatening Coagulopathy in the Massively Transfused Trauma Patient: Hypothermia and Acidoses Revisited, 857-862, Copyright (1997), with permission from Wolters Kluwer Health, Inc.(143)

Factor	Coagulopathy	No Coagulopathy	<i>p</i> Value
Age (years)	34.5 ± 2.2	36.2 ± 3.3	0.67
Blunt	48%	45%	0.62
ISS	35.8 ± 3.4	26.1 ± 2.7	0.03
ISS > 25	70%	35%	0.01
SBP mm Hg	71.3 ± 3.6	81.5 ± 3.2	0.04
SBP < 70 mm Hg	52%	19%	0.02
PRBC units/24 h	26.2 ± 2.7	22.4 ± 1.9	0.24
PRBC units/6 h	16.6 ± 2.6	14.8 ± 1.4	0.55
>15 U PRBC/6 h	44%	55%	0.60
Lactate (mmol/L)	6.31 ± 0.65	5.04 ± 0.37	0.10
pH	7.02 ± 0.03	7.15 ± 0.02	0.0004
pH < 7.10	78%	32%	0.0014
Temperature (°C)	33.8 ± 0.3	34.6 ± 0.2	0.05
Temperature < 34°C	59%	23%	0.01

ISS, Injury Severity Score; SBP, systolic blood pressure; PRBC, packed red blood cells.

Table 11. Probability a patient will develop coagulopathy based on injury profile, systolic blood pressure, blood pH, and body temperature. Reprinted from The Journal of Trauma and Acute Care Surgery, Vol. 42, Issue 5, N Cosgriff, EE Moore, A Sauaia, M Kenny-Moynihan, JM Burch, B Galloway, Predicting Life-threatening Coagulopathy in the Massively Transfused Trauma Patient: Hypothermia and Acidoses Revisited, 857-862, Copyright (1997), with permission from Wolters Kluwer Health, Inc.(143)

No risk factor			=	1%
ISS > 25			=	10%
ISS > 25	+	SBP < 70 mm Hg	=	39%
ISS > 25	+	pH < 7.10	=	58%
ISS > 25	+	Temp < 34°C	=	49%
ISS > 25	+	SBP < 70 mm Hg + Temp < 34°C	=	85%
ISS > 25	+	SBP < 70 mm Hg + pH < 7.10 + Temp < 34°C	=	98%

Because ATC is a systematic condition, it can only be studied *in vitro* using shed blood from trauma patients. In the absence of a supply of shed blood, the best method for simulating trauma *in vitro* is through the lethal triad. In this vein, pooled normal plasma (PNP) was diluted with isotonic saline to deplete factor levels, chilled below normal body temperature (37° C) during assays to produce hypothermia, and acidified with lactic acid to achieve metabolic acidosis.(33)

While treatment is always designed to improve a patient's outcome, the use of resuscitative fluids can actually worsen coagulopathy.(9) A study on bypass patients who suffered major bleeding either during or after surgery identified a risk factor when the amount of crystalloids exceeded that of whole blood in blood vessels, a 50 % diluted whole blood marker (Figure 43).(144) In patients who have developed ATC, this critical dilution by crystalloids and colloids of factors can be fatal.(9)

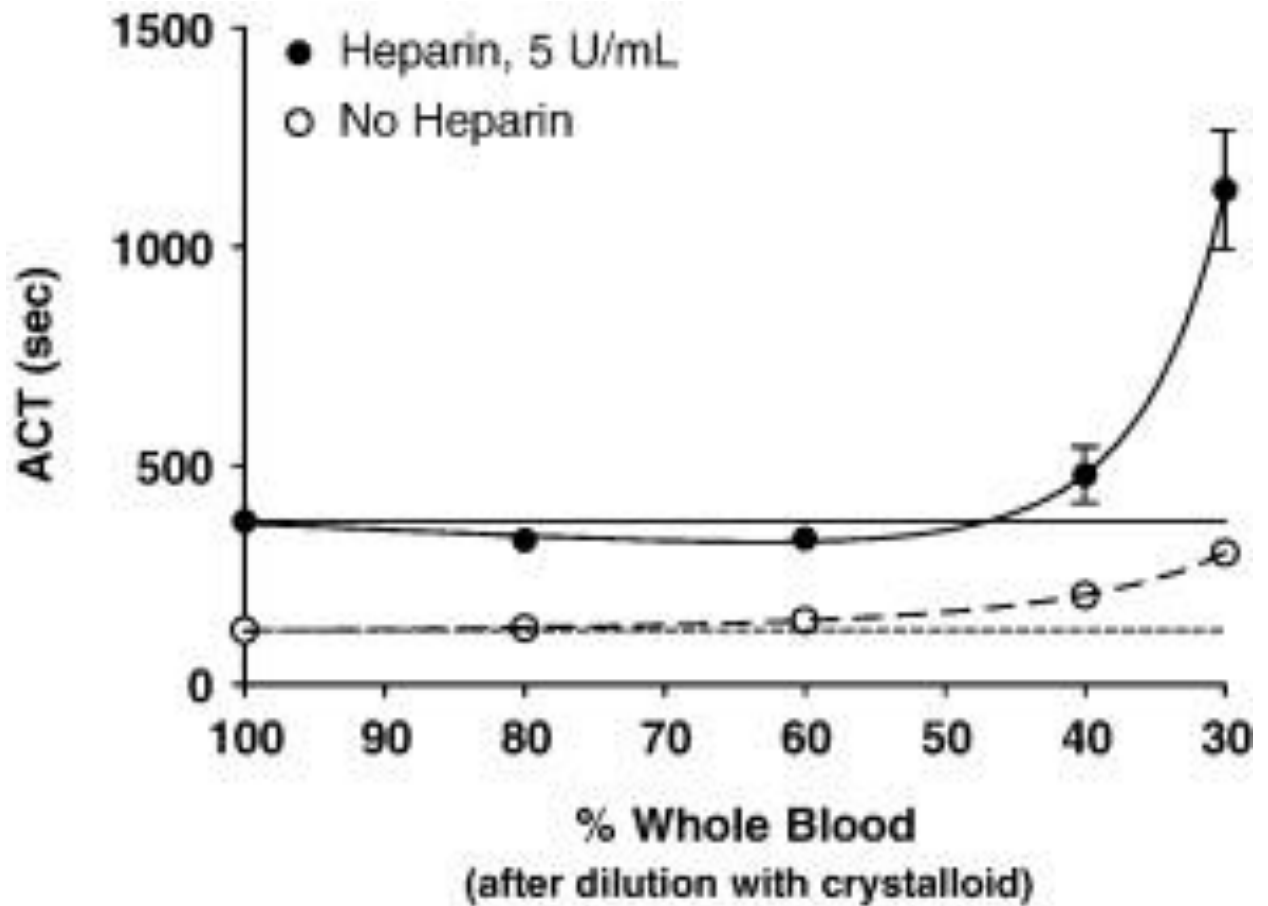


Figure 43. Study of activated clotting time (ACT) in patients showed that the use of crystalloids to maintain blood vessel integrity began to inhibit the patient's ability to clot once half the whole blood volume was replaced with crystalloids. Reprinted from *Blood Cells, Molecules, and Diseases*, Vol. 43, Issue 3, BS Bull, KL Hay, PC Herrmann, Postoperative bypass bleeding: A bypass-associated dilutional (BAD) coagulopathy?, 256-259, Copyright (2009), with permission from Elsevier.(144)

Of the triad, hypothermia has the smallest effect on clot impairment.(145) The decrease in temperature appears to inhibit the initiation of coagulation the most, delaying factor activation but not affecting overall factor levels (Figure 44).(146) This is also shown on the thromboelastograph, where clot initiation and rate of clot formation are delayed, but overall clot strength remains unperturbed.(145)

Unlike hypothermia, acidosis' effects are more severe and widespread.(145) Normal blood has a pH of 7.4, which drops as a result of trauma. As the pH drops, both the rate of thrombin production and the overall level of thrombin decreases as well (Figure 44).(147) While sodium bicarbonate can return the blood's pH to a normal pH level, effects from acidosis continue to inhibit coagulation for roughly 24 hours (Figure 45).(148, 149) Acidosis' greatest effect is on thrombin production,(146, 147, 150) which then significantly slows both the rates of clot formation and clot growth.(145)

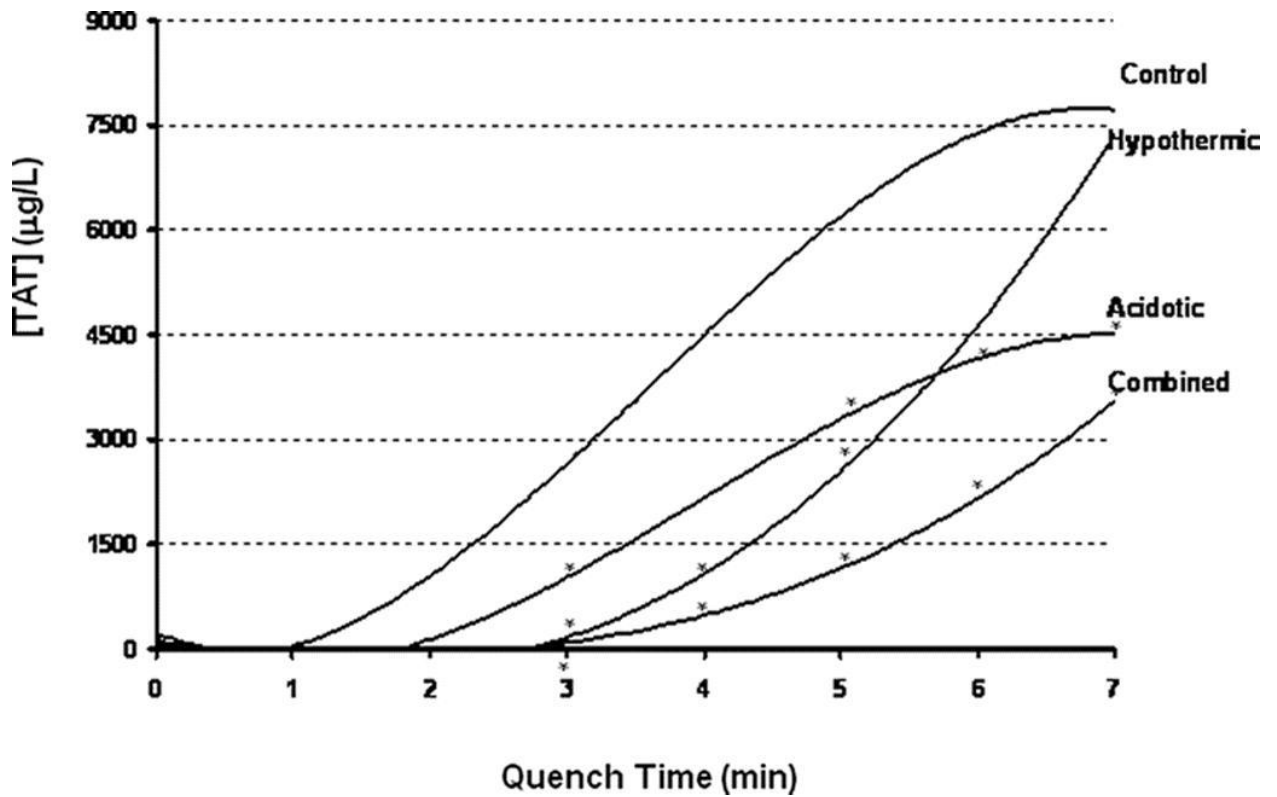


Figure 44. Thrombin production in a splenic injury model under normal (control), hypothermic, acidotic, and combined hypothermic and acidotic conditions. Thrombin-Antithrombin III (TAT) complex is a marker for production of thrombin. Reprinted from The Journal of Trauma and Acute Care Surgery, Vol. 58, Issue 5, W Martini, A Pusateri, J Uscilowicz, A Delgado, J Holcomb, Independent Contributions of Hypothermia and Acidosis to Coagulopathy in Swine., 1002-1010, Copyright (2005), with permission from Wolters Kluwer Health, Inc.(146)

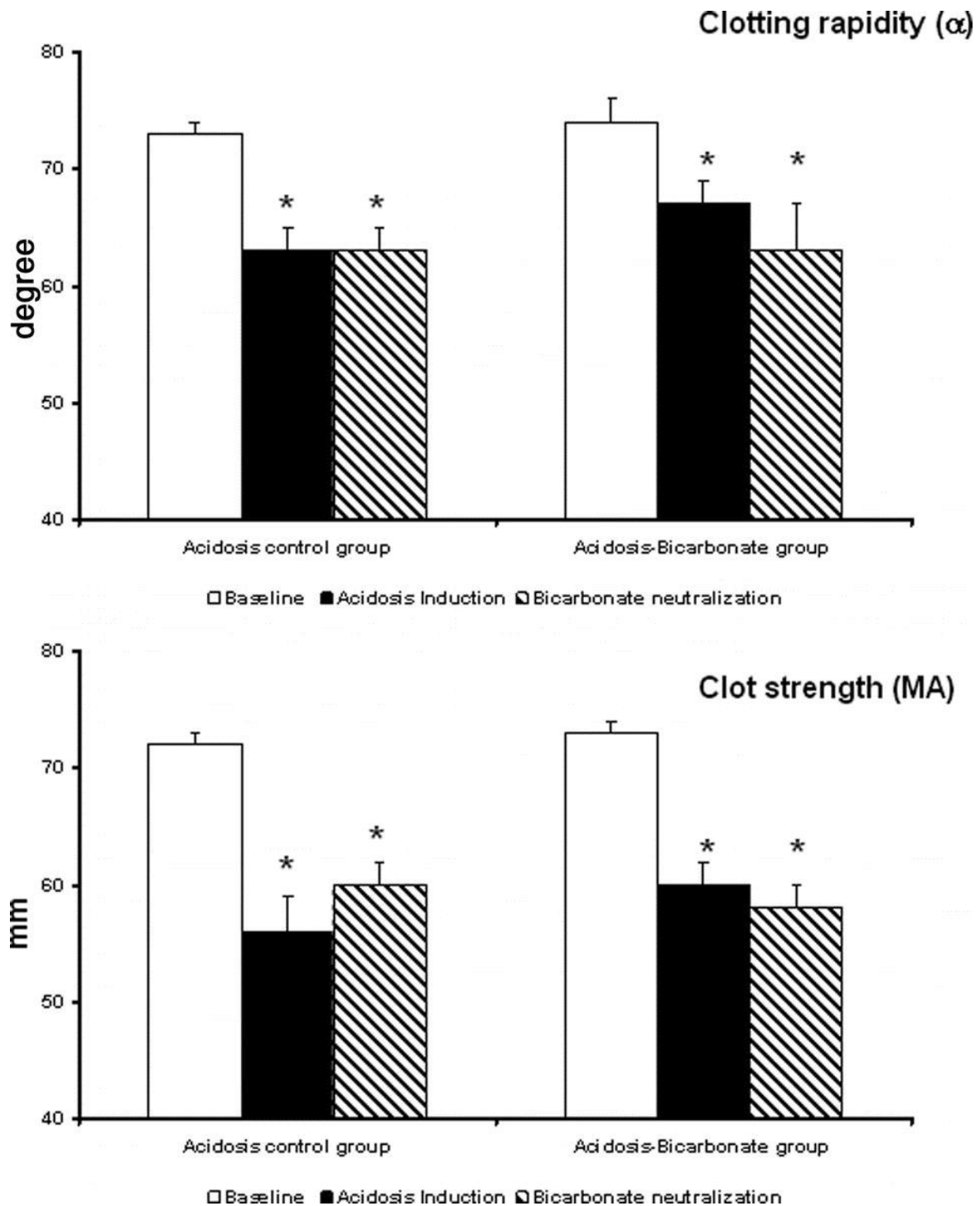


Figure 45. Even after neutralization with sodium bicarbonate, acidosis still inhibits the rate of clot formation (acceleration) and the overall clot size. Reprinted from *The Journal of Trauma and Acute Care Surgery*, Vol. 67, Issue 1, W Martini, Coagulopathy by Hypothermia and Acidosis: Mechanisms of Thrombin Generation and Fibrinogen Availability., 202-208, Copyright (2009), with permission from Wolters Kluwer Health, Inc.(148)

While great advances have been made in the resuscitation of the injured patient, attenuating bleeding and correction of coagulopathy remain vexing clinical problems. Mortality remains high in ATC patients; the best available resuscitation and surgical practices often are unsuccessful in adequately controlling bleeding. ATC and the lethal triad are currently countered by hemorrhage resuscitative methods, including transfusion of blood and blood products and the use of hemostatic agents. Indeed, there remains a need for adjunctive agents aimed at stopping bleeding in many patients. To date there has been some success in this area and a wide array of hemostatic dressing agents have been successfully deployed. For noncompressible hemorrhage however there is a dearth of available agents.

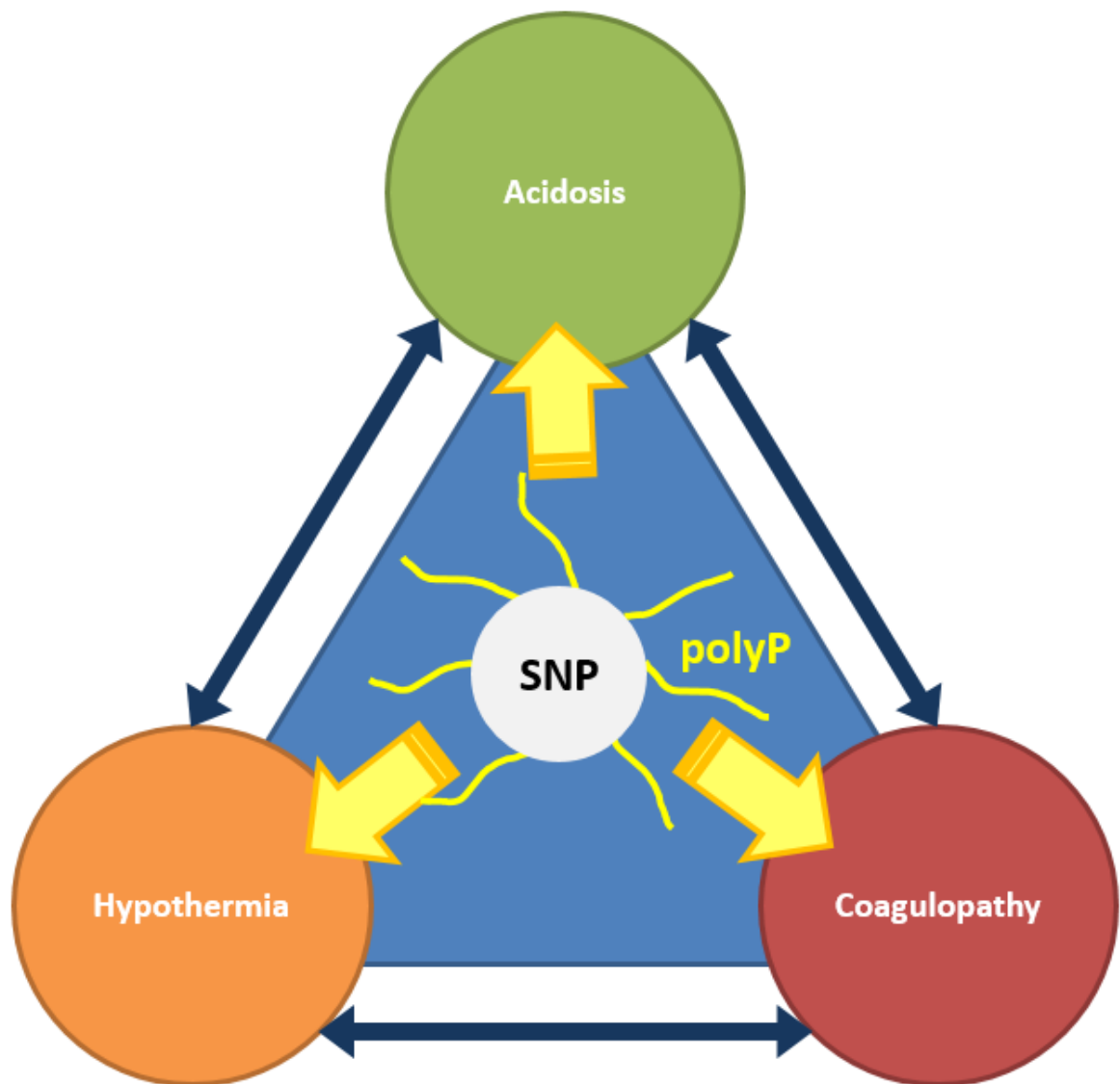
In 2010, Kheirabadi et al. called for the need for “a new class of hemostatic agent that can function independently of host coagulation activity”.⁽⁶⁷⁾ Such an agent would be particularly beneficial to trauma patients who become coagulopathic at the point of injury, or who acquire coagulopathy after clinical treatment as a result of resuscitation therapy and excessive surgical bleeding. The use of an endogenous hemostat that induces clotting without occluding the injured vessel would alleviate safety issues. If successful, such a hemostat could help treat internal bleeding. Even better, replacing recombinant human proteins with inorganic agents that have longer shelf lifetimes, cost significantly less to produce, and therefore have lower treatment costs, is highly desirable.

Human recombinant factor proteins suffer from ineffectiveness, side effects, and steep cost per dose.⁽⁷⁹⁾ Recombinant factors also require refrigeration, which preclude their use in certain prehospital and austere far forward military environments delaying essential lifesaving care. The delay from time of injury to administration allows the patient to develop

coagulopathy as a result of significant blood loss. Tranexamic acid has a strict window of viability after injury and a disputed mechanism.(92)

Among its many benefits, the mechanism of thrombin generation and long term stability of polyP-SNP at ambient conditions has the potential to ameliorate treatment at any point where the patient is at risk for exsanguination. I have previously shown that attaching polyP to SNPs to create polyP-SNP forms a synergistic effect (Chapter III Scheme 1) that improves the coagulant ability significantly over bare SNPs.(135) The polyP-SNP's ability to stimulate the intrinsic and common pathways in patients with life-threatening bleeding might be a viable alternative to what is currently available in management of these patients. The long-term stability of the polyP-SNP system at ambient conditions(135) could allow for prehospital use. Minimizing the time between injury and treatment may enable the body to successfully generate and maintain healthy clots prior to or upon initial onset of ATC.(151, 152)

Scheme 3. To be an ideal clotting agent, polyP-SNP must enable clotting even in the face of the lethal triad - coagulopathy, hypothermia, and acidosis. Diagram courtesy of Dr. Anna May-Masnou.



B. Materials, Synthesis of silica nanoparticles and polyphosphate coated silica nanoparticles, Clotting assays

Both the Materials, Synthesis, and Clotting sections of this chapter are identical to the sections published in the previous chapter (Chapter III).

C. Modification of plasma

1. Hemodilution

Pooled normal plasma (PNP) was stored at -80°C and thawed less than 1 h before conducting the assay. Iatrogenic coagulopathy was reproduced *in vitro* through hemodilution with isotonic saline or phosphate buffered saline solution to reach the desired dilution level – i.e., 100 µl saline added to 300 µl PNP produced 400 µl 75 % plasma. 100 %, 75 %, 50 %, 40 %, 33 %, and 25 % PNP levels were tested.

2. Hypothermia

Hypothermia was induced through temperature variation: 37 °C, 35 °C, 32 °C, and 30 °C. Plasma was incubated at the desired temperature for 20 min prior to conducting the assay. Both instruments (thromboelastograph and plate reader) used had temperature settings. Prior to the assay, temperature on the instrument was set at the desired temperature (e.g., 32 °C for moderate hypothermia) prior to the experiment. This ensured that the reaction took place at the desired temperature and that the plasma did not warm up to 37 °C during the course of the assay.

3. Acidosis

PNP was acidified using lactic acid. Because PNP was purchased from George King Biomedical instead of procured fresh, I determined to use base deficit or acid excess as the marker for acidosis instead of pH for my studies. Base deficit – the amount of base required to counteract the acid in blood - is an excepted clinical marker for acidosis.(32) 2 mmol lactic acid/L, 5 mmol lactic acid/L, and 10 mmol lactic acid/L blood plasma were tested.

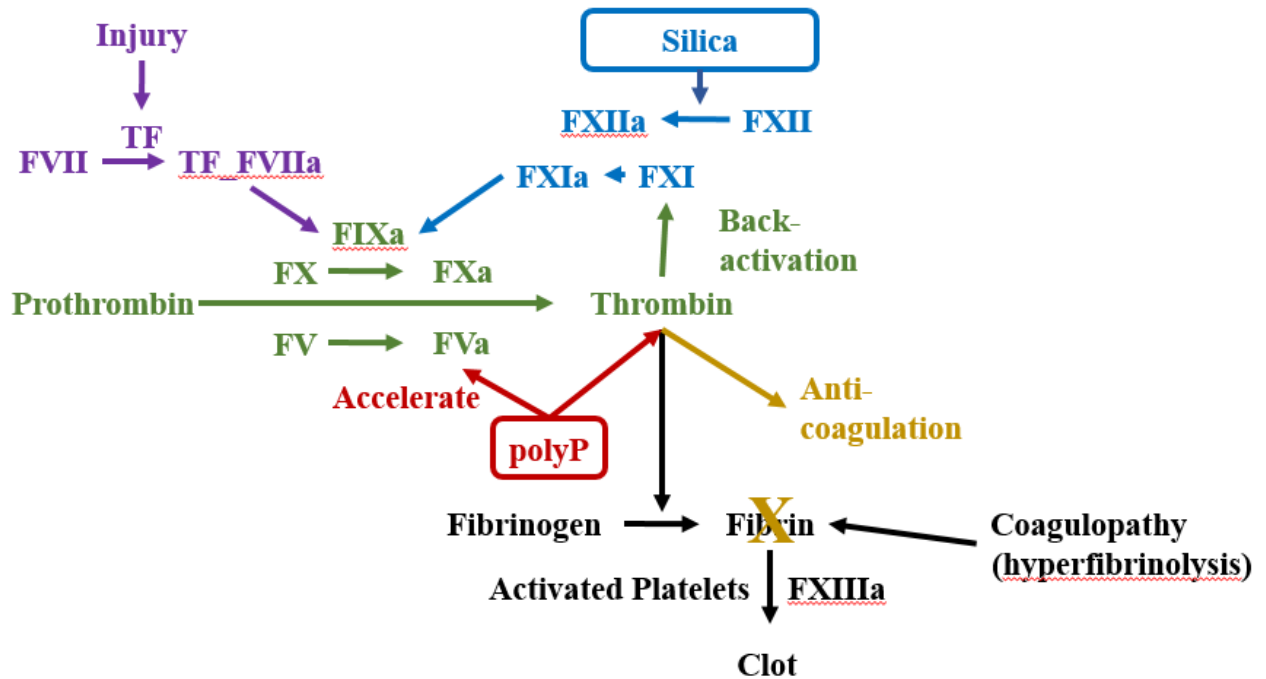
D. Discussion

In previous studies, I have illustrated the efficacy of polyP-SNP under non-traumatic conditions.(135) Using ~70 nm SNPs as the core for polyP functionalization, I compare the effectiveness of the polyP-SNP system to that of known initiators of blood clotting: bare SNP and lipidated tissue factor (LTF). Plasma was modified to simulate the lethal triad. Clotting activity was determined by two different methods: thrombin activity using a thrombin-specific fluorescent dye and thromboelastography (TEG). Coagulation was assessed both by the production of the key protein (thrombin) as well as development of the clot from its inception through full clot size (TEG). The data given below show that polyP-SNP retains its clotting function even under critical conditions, where other pieces of the clotting cascade cease to function.

My focus on coagulopathy was due to its presence in the severely injured trauma population as well as its ties to worsening outcomes. Having previously identified 0.25 mg/ml as the ideal polyP-SNP concentration,(135) I tested this concentration of polyP-SNP against the LTF and SNP baselines. Due to the loss of both procoagulant and anticoagulant factors, prior research suggested hemodilution only begins to inhibit clotting locally at the 50 % level.(144) Using TEG (Figure 46) and thrombin generation tests (Figure 56), I established

a dilution baseline in line with the findings of Herrmann et al. using the body's main defense against vessel injury, lipidated tissue factor (LTF) as well as bare SNPs. The baseline confirmed that clot time and thrombin formation decreased below the 50 % threshold. As the plasma become more dilute, all factor levels, especially fibrinogen, dropped. As a result, using the same clotting agent (polyP-SNP, Figure 6), clot time decreased slowly while both clot acceleration and clot size decreased significantly.

Scheme 4. ATC mainly inhibits coagulation through hyperfibrinolysis, preventing the formation of stable and healthy clots to stop bleeding as well as lowering the concentration of fibrinogen, the protein that supplies fibrin for the clot.



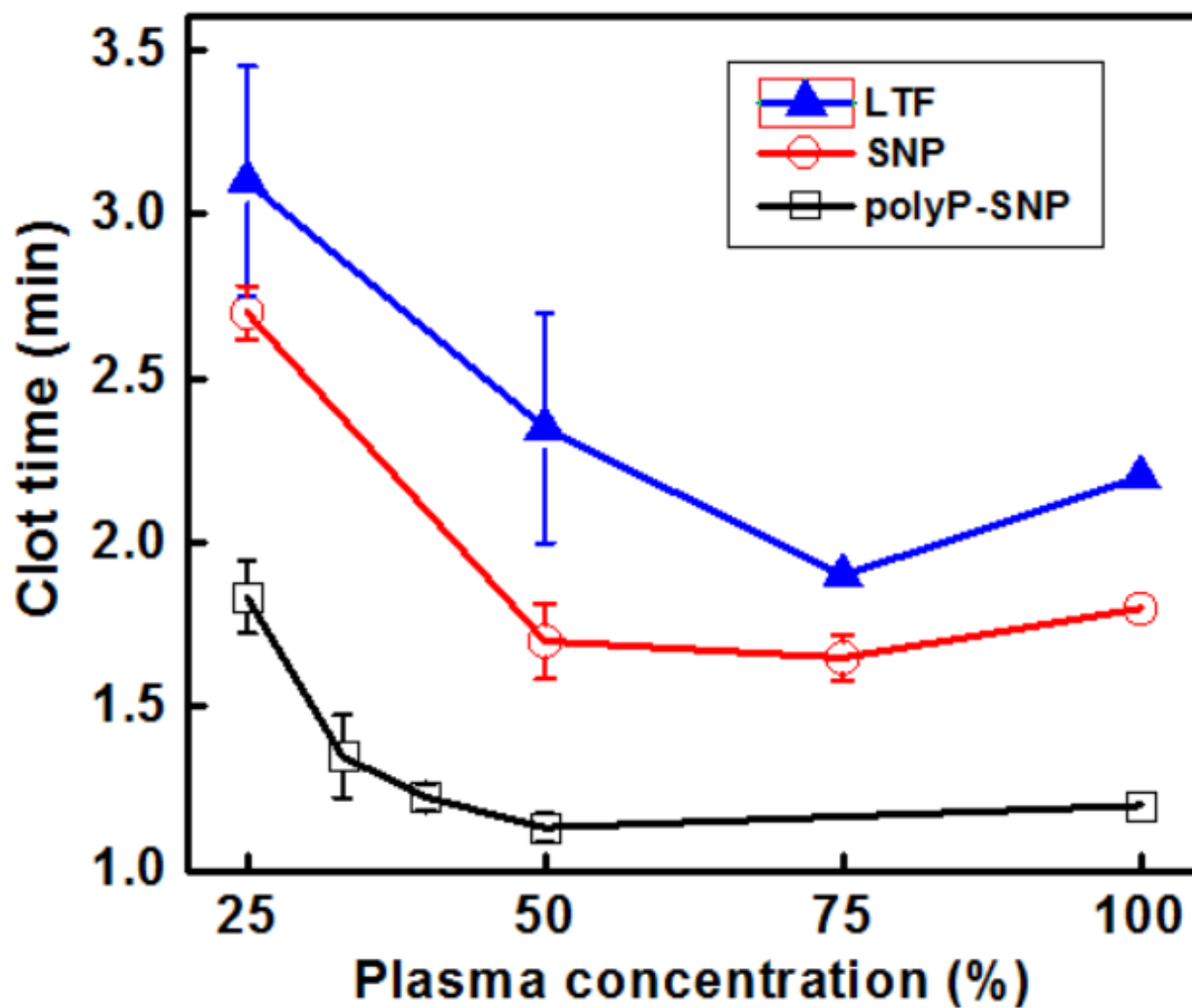


Figure 46. TEG clot time for several agents based on PNP hemodilution. PolyP-SNP outperforms all agents regardless of plasma hemodilution.

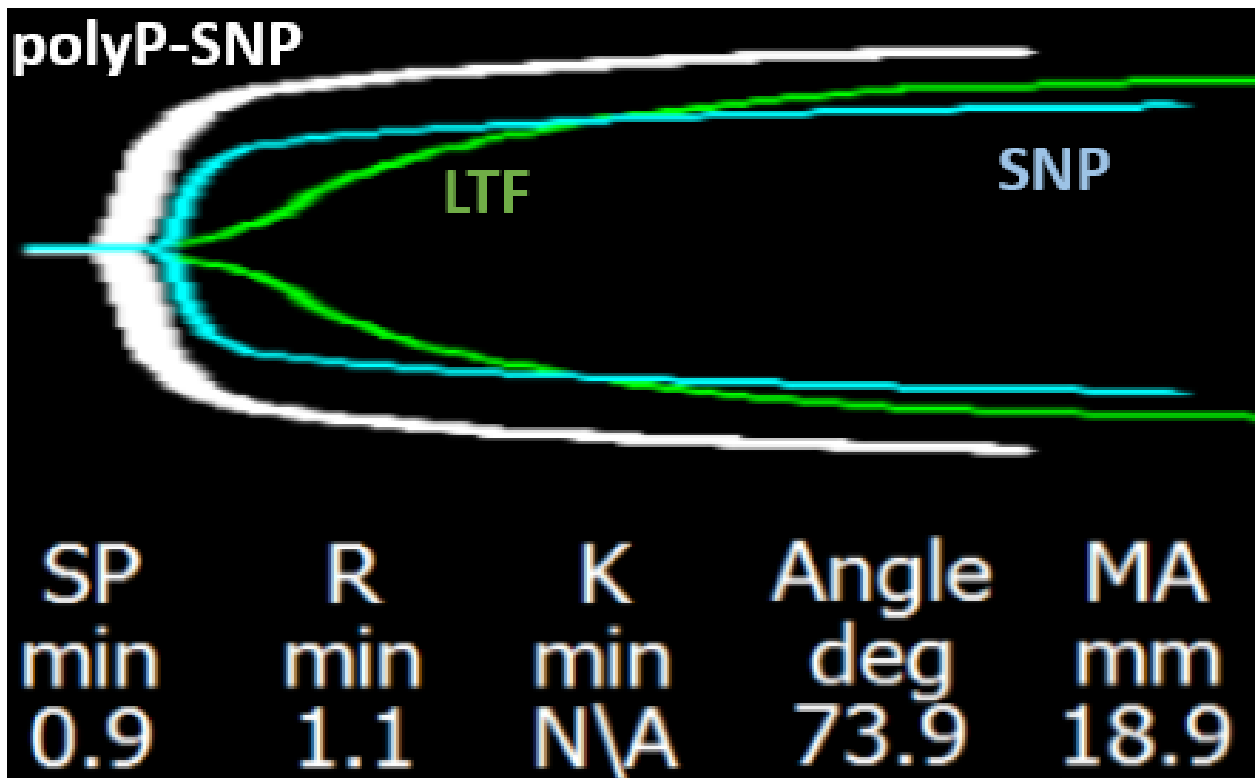


Figure 47. Actual TEG curve showing all three agents at 50 % hemodilution. PolyP-SNP has quickest clot time, largest rate of clot growth, and peak clot size.

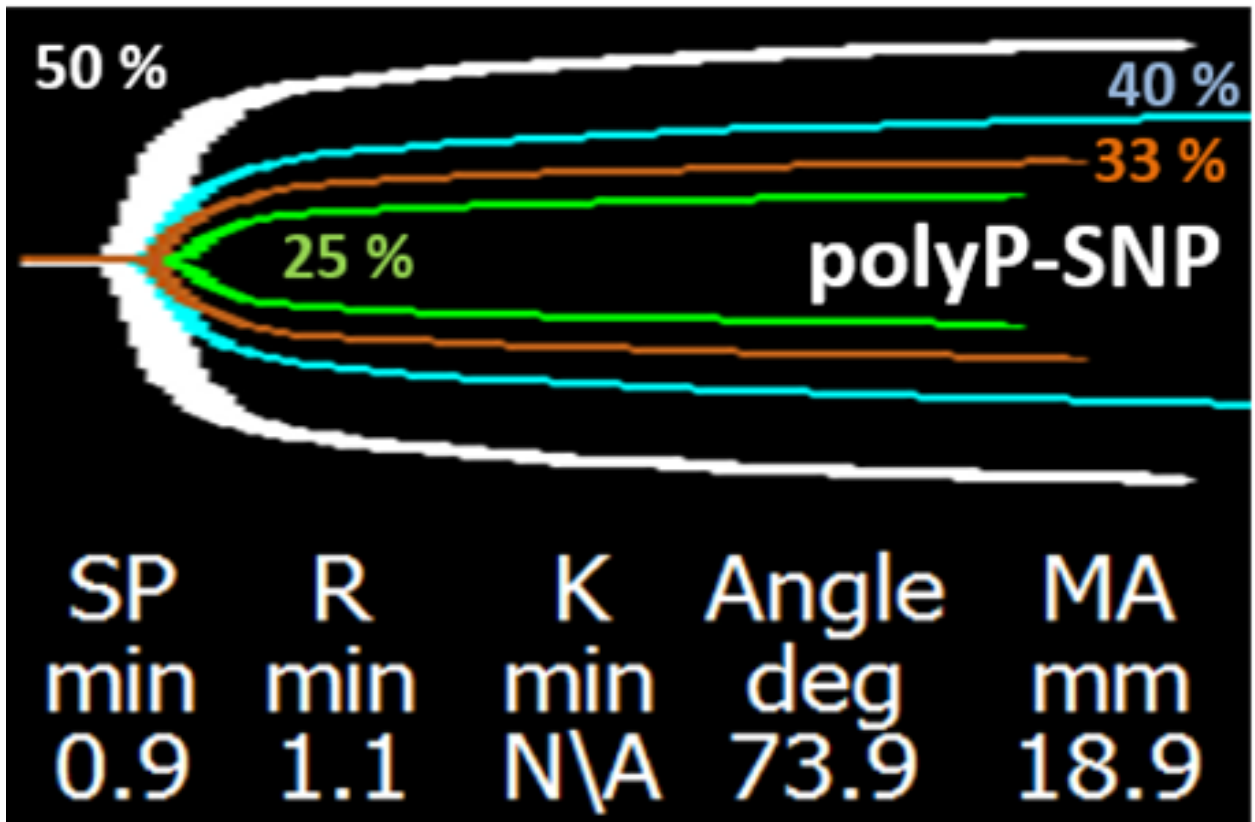
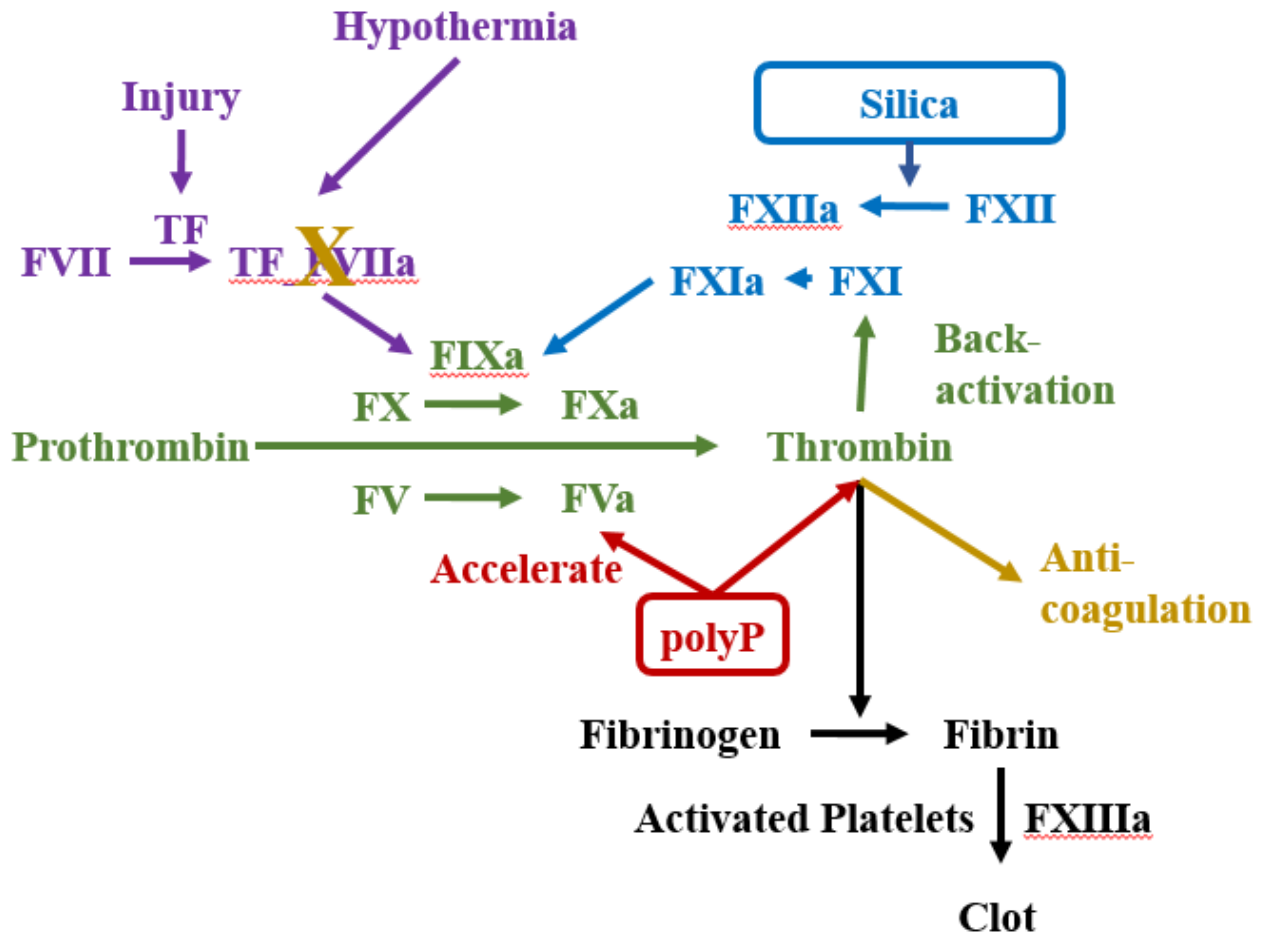


Figure 48. As the factor concentrations decrease in hemodilute plasma (e.g., 40 % is 40 % PNP and 60 % dilutant), clot time decreases slightly and the clot growth rate and overall clot size both decrease significantly even when polyP-SNP is used.

Furthermore, the TEG curve (Figure 47) shows that polyP-SNP also accelerated clot growth to reach a maximum clot size faster than the other agents. PolyP-SNP's ability to quickly grow clots is extremely valuable given Hagemo et al.'s findings that fibrinogen levels and fibrin clot size after 5 minutes were the best predictors of coagulopathy and need for massive transfusion in trauma patients.(153) While polyP-SNP cannot counteract decreases in fibrinogen levels, the accelerated clot growth suggests that polyP-SNP can help convert more fibrinogen into the fibrin needed for the physical clot. Overall, these data suggest that quicker intervention during blood loss should improve clotting function, which could prevent or help mitigate the effects of coagulopathy.

After testing polyP-SNP on hemodilution, my next tests focused on hypothermic and acidotic plasma. The TEG was set to hypothermic conditions to ensure that the plasma did not reach normal body temperature during the assay. At moderate to severe hypothermia, 32 °C and 30 °C, both SNPs, and LTF showed significant prolongation in TEG clot time, with LTF clot times rising above 4 min (Figures 50, 51). The loss of clotting ability by LTF may be explained by Ramaker et al.'s theory, which states that reduced body temperature results in decreased rate kinetics of many of the coagulation factors, especially formation of the tissue factor-FVIIa complex.(145) Decreasing temperature did not significantly alter clot time or overall clot size, but it did slow the rate of clot formation (Figures 50, 51).

Scheme 5. Hypothermia primarily impairs coagulation by slowing activation of the TF_FVIIa complex, the mechanism by which the extrinsic pathway initiates clotting.



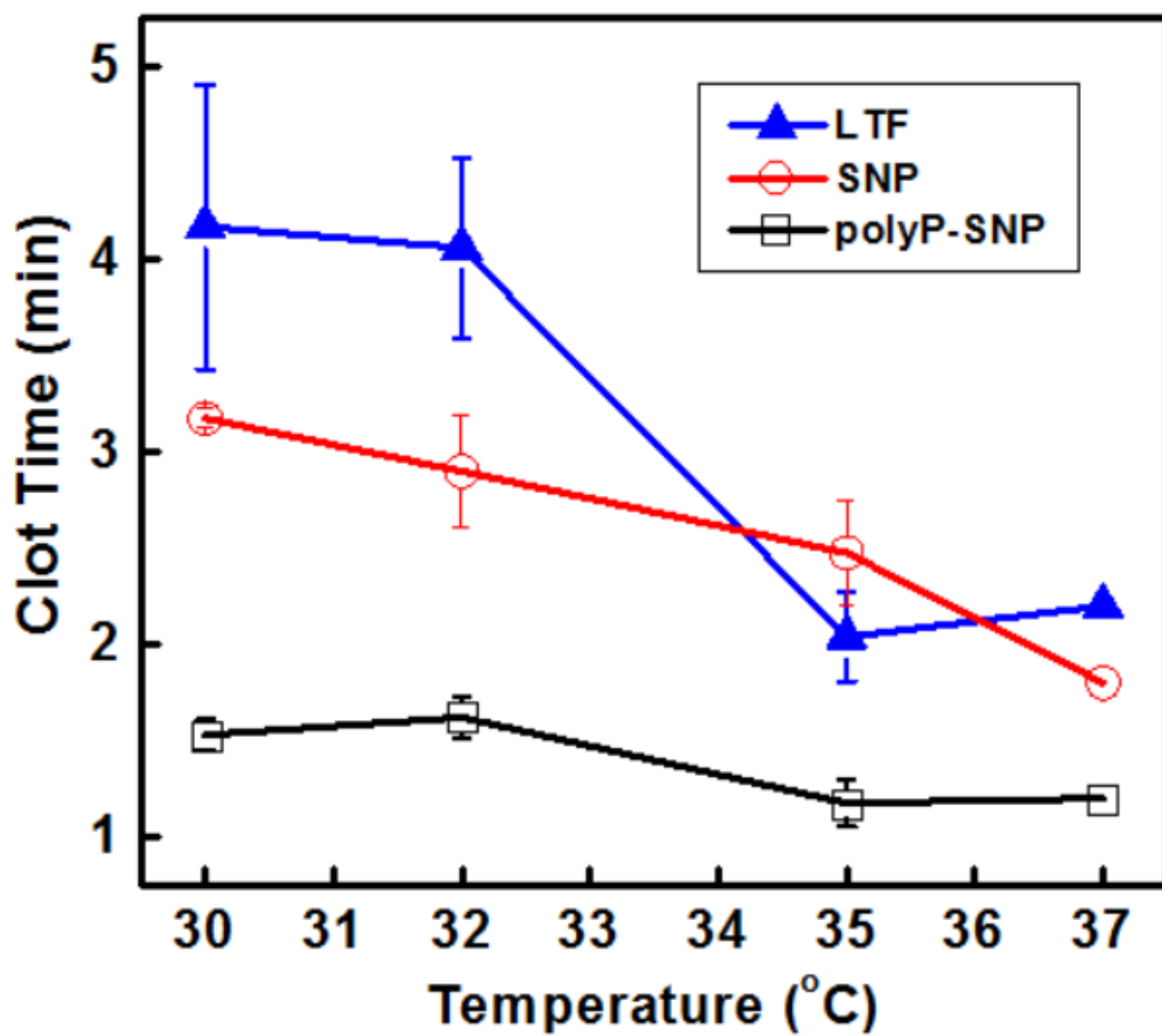


Figure 49. TEG clot time for hypothermic plasma. PolyP-SNP is superior to both LTF and bare SNP.

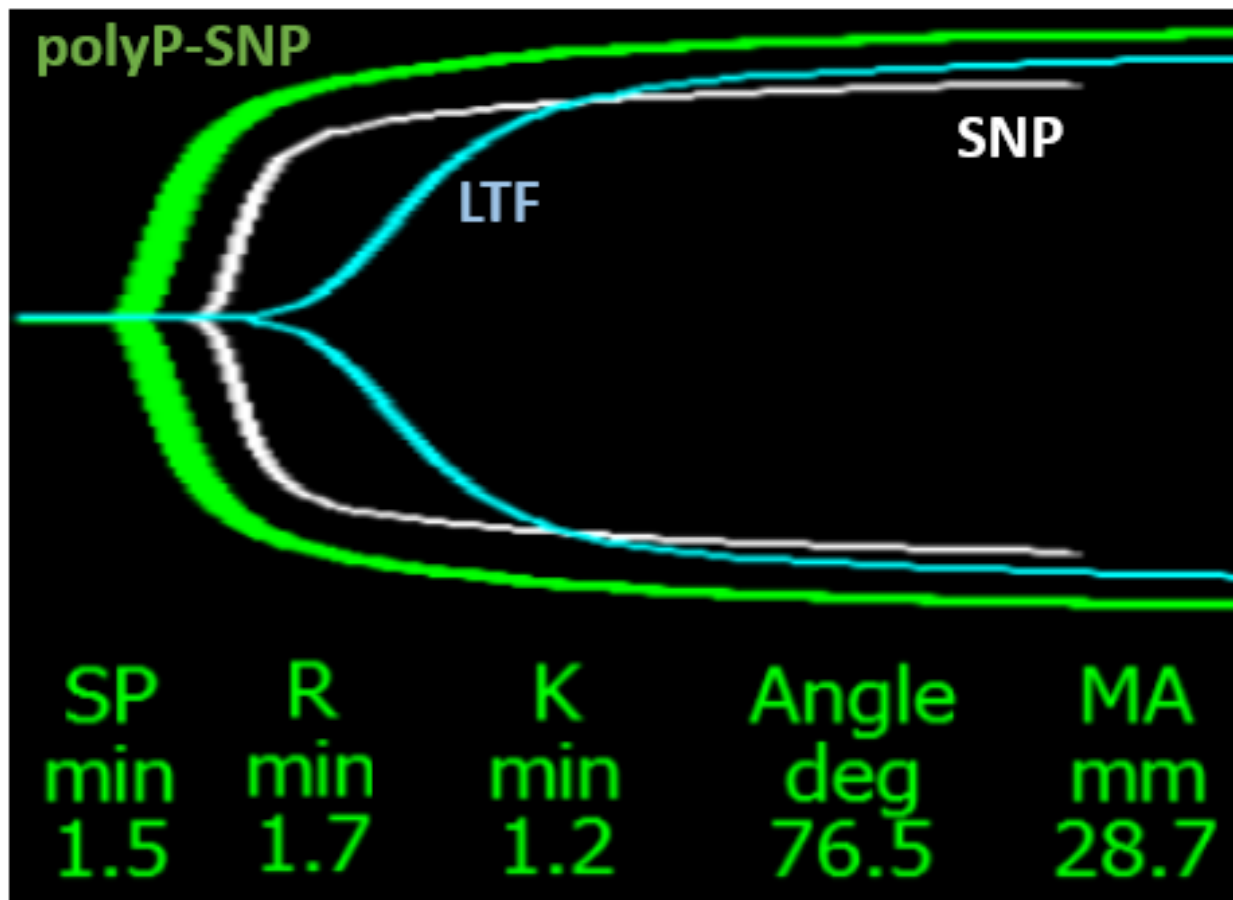


Figure 50. Actual TEG curve comparing all three agents at 32 °C or moderate hypothermia and 10 mmol/L lactic acid.

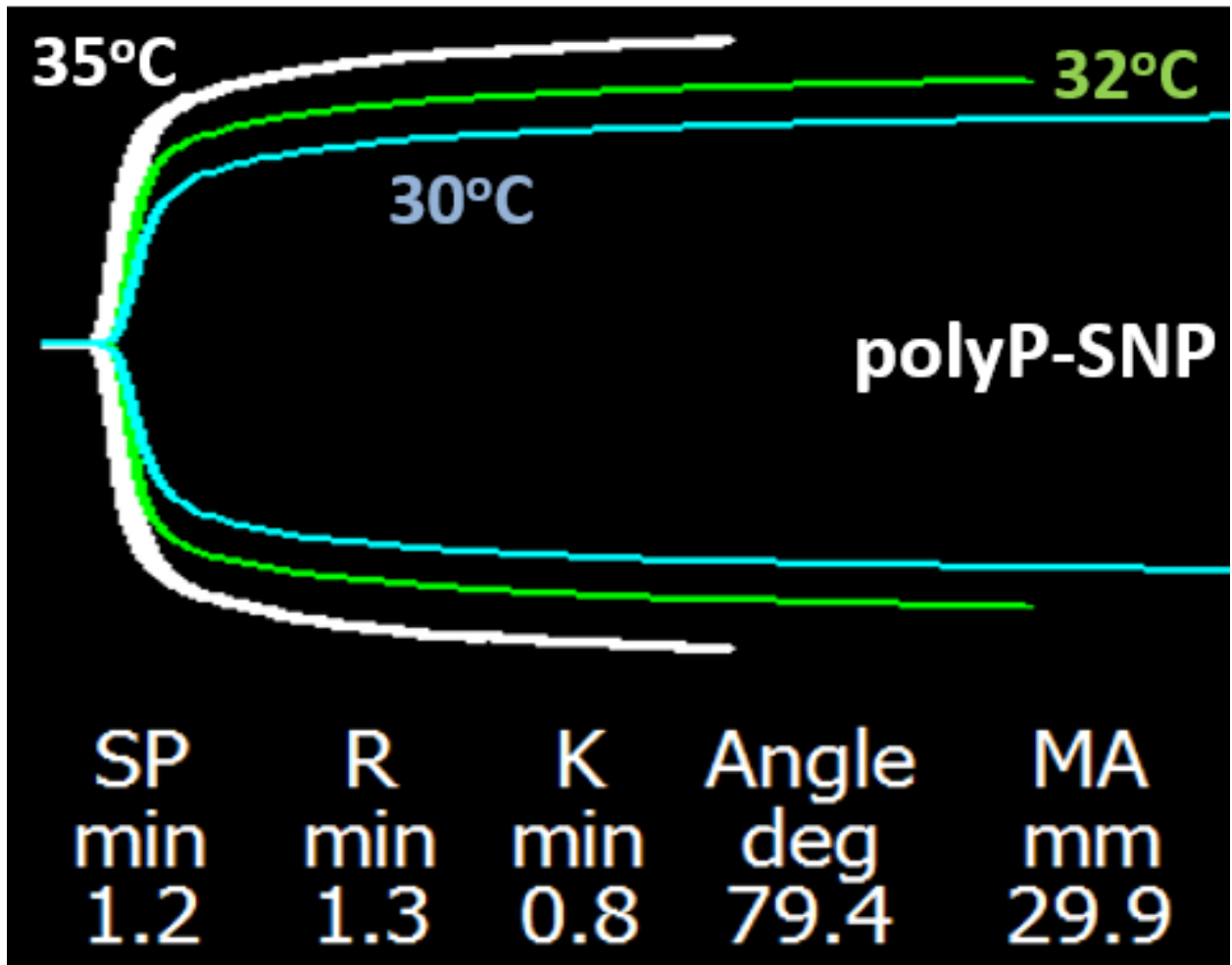
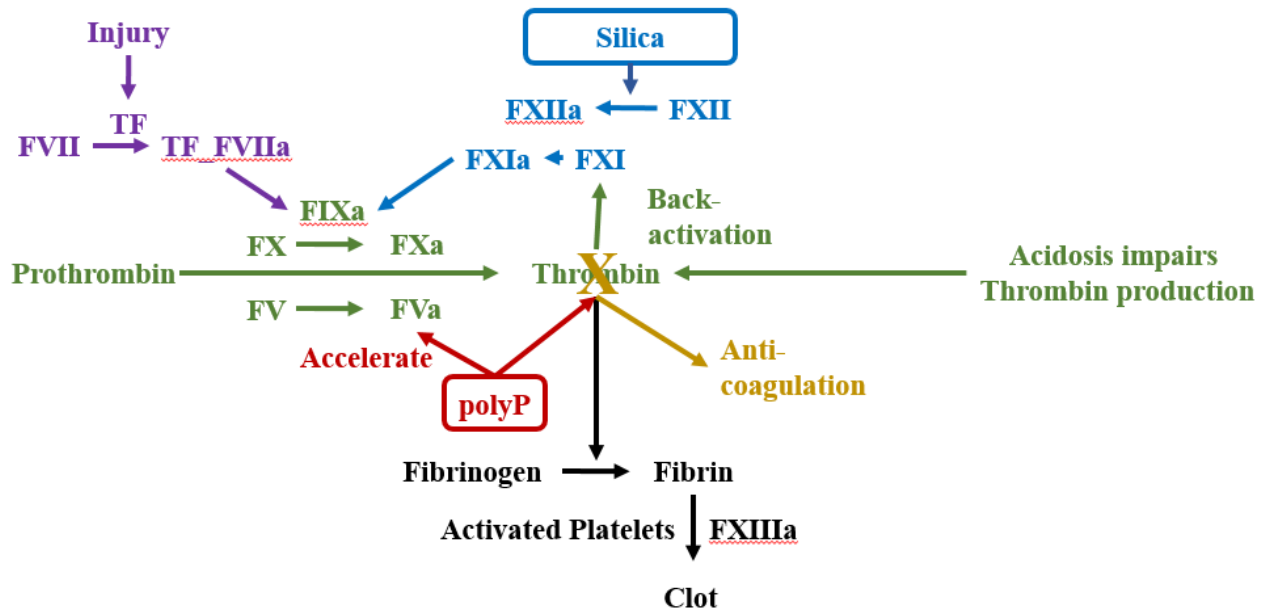


Figure 51. As temperature decreases, clot time remains stable, but rate of clot growth and final clot size decrease even when polyP-SNP is used.

Scheme 6. Acidosis primarily impairs thrombin generation, the main pathway by which polyP-SNP drives coagulation.



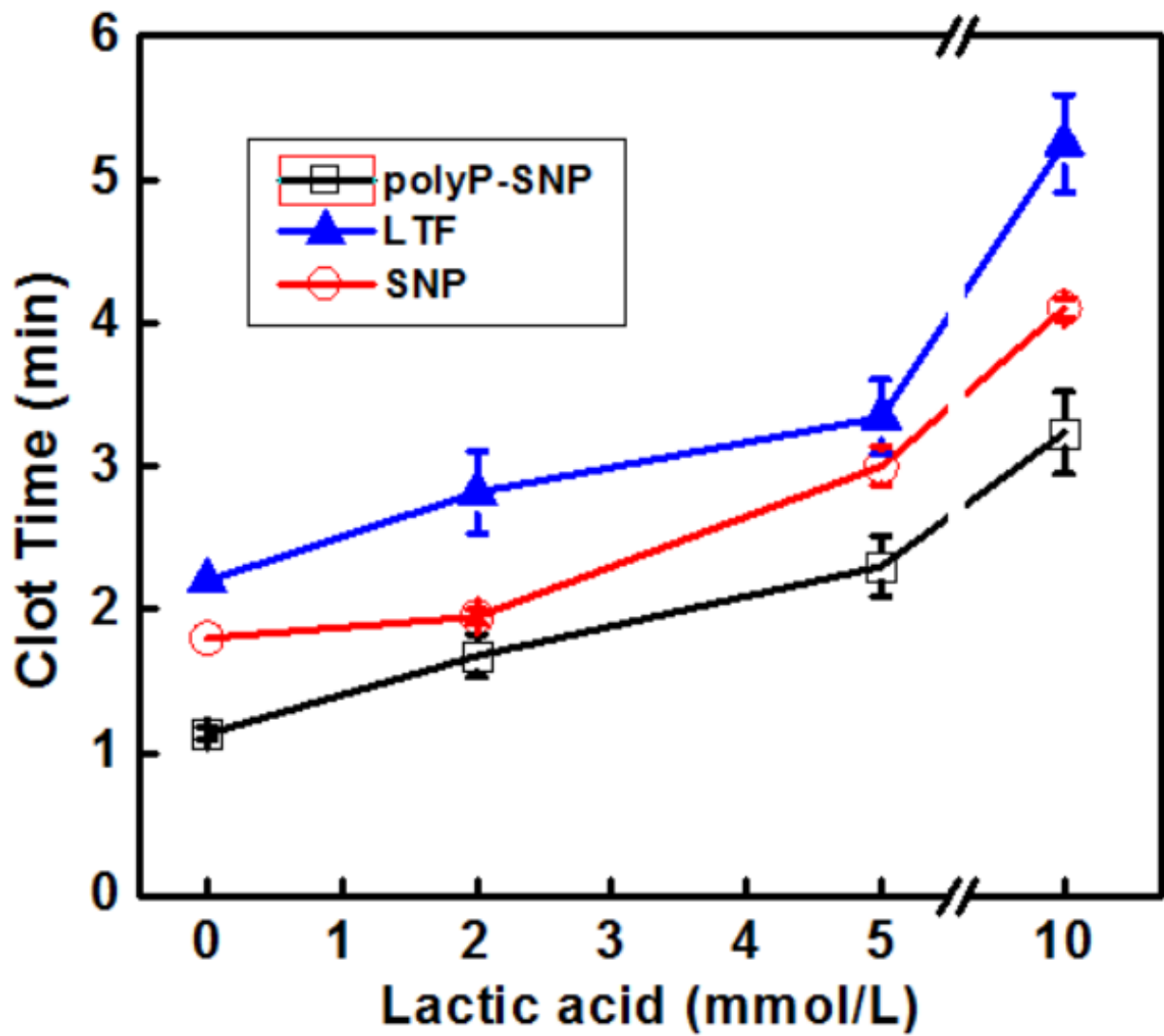


Figure 52. TEG clot time for acidified plasma. PolyP-SNP is superior to both LTF and bare SNP.

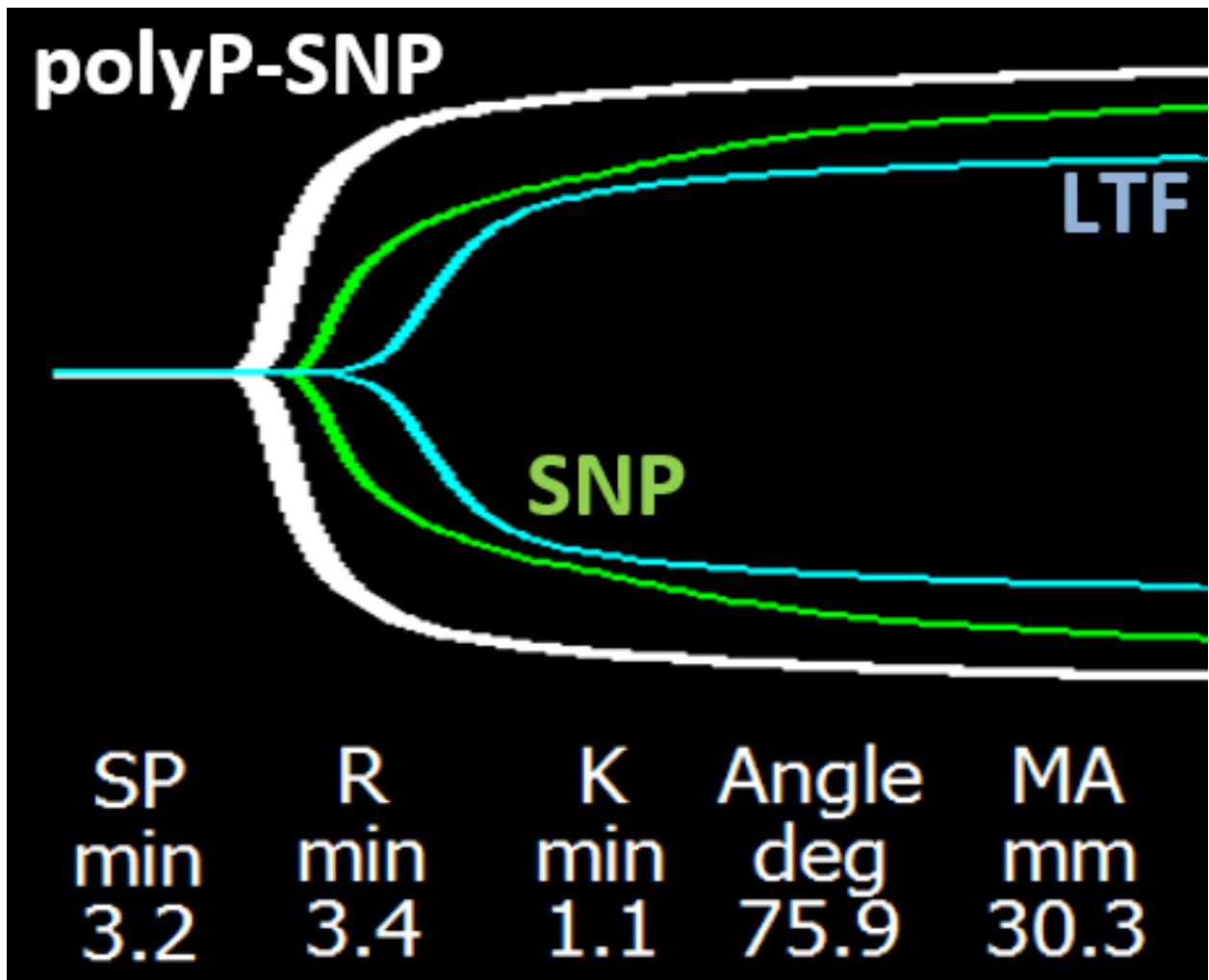


Figure 53. Actual TEG curves comparing all three agents at acidotic (10 mmol lactic acid/L blood plasma) conditions.

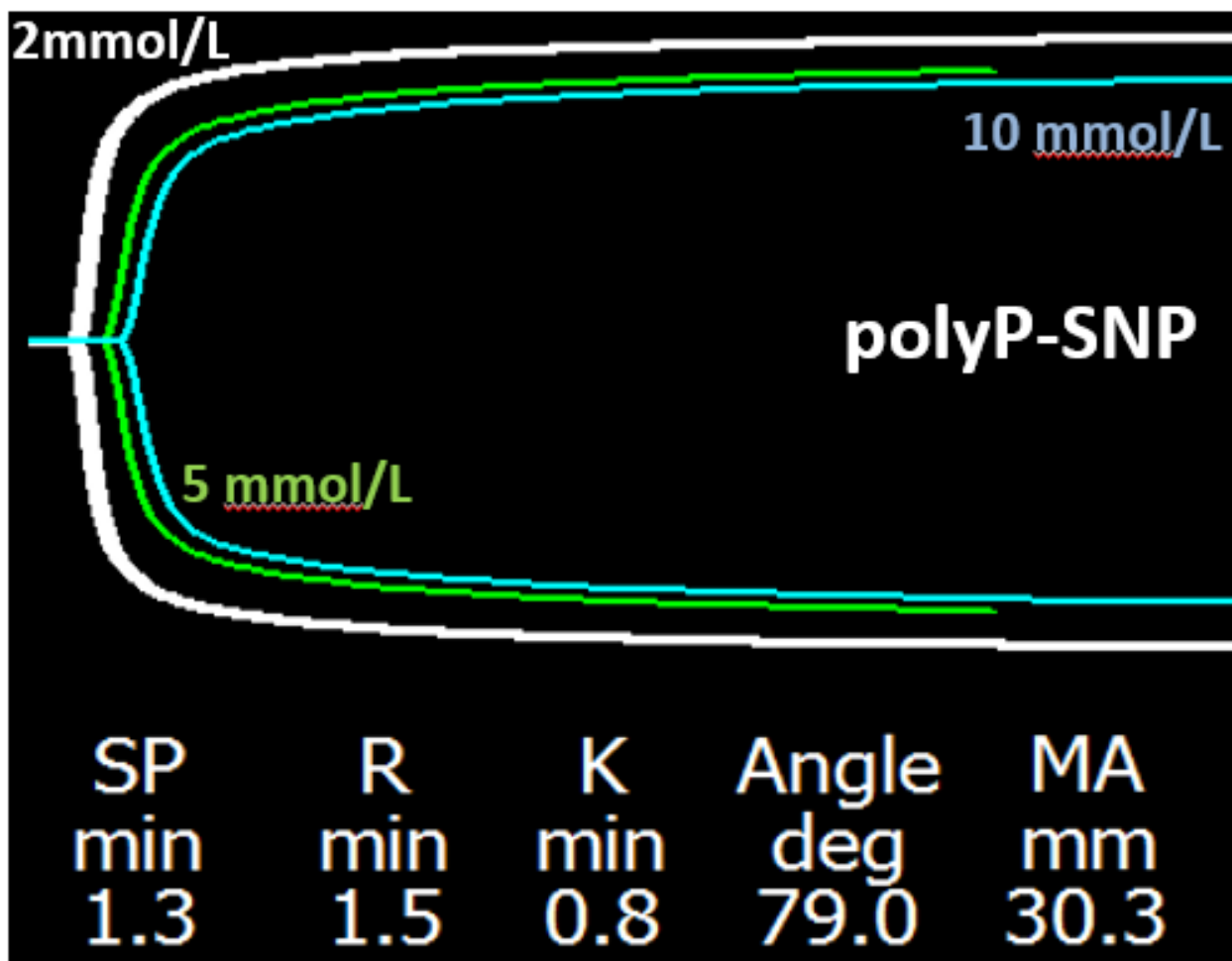


Figure 54. As more lactic acid is added, clot time, rate of growth, and final size all decrease even when polyP-SNP is used.

Acidosis was achieved through the addition of excess lactic acid to simulate metabolic acidosis.(36) Acidosis is treated clinically through the administration of sodium bicarbonate, though it can take 24 h after neutralization for the effects of acidosis to abate.(147) I chose to measure acidosis as the amount of excess lactic acid in the solution because the time between blood draw and assays was too long for an acceptable pH measurement. Of the triad, acidosis has the greatest effect on thrombin generation,(147) which can lead to exsanguination if unchecked. Because it accelerates coagulation through increased thrombin production, acidosis had a greater effect on polyP-SNP (Figure 52 - 54, 58) than the rest of the triad. However, even in the presence of 10 mmol/L excess lactic acid, polyP-SNP still outperformed LTF and SNP in generating thrombin and clots.

Thrombin production is the key role of the common pathway and accelerates growth of the nascent clot. Under all conditions, polyP-SNP outperformed bare SNP and LTF in producing thrombin. At 50 % hemodilution, the thrombin burst occurred roughly 100 seconds quicker for polyP-SNP than bare SNP and even later for that of LTF (Figure 55). More significantly, LTF was unable to generate significant thrombin below 33 % dilution of plasma. This suggests that there is a blood-loss threshold at which the body alone fails to adequately produce the thrombin required for significant, healthy clots. In comparison, polyP-SNP significantly hastens thrombin formation to accelerate clot formation under all hemodilutional conditions.

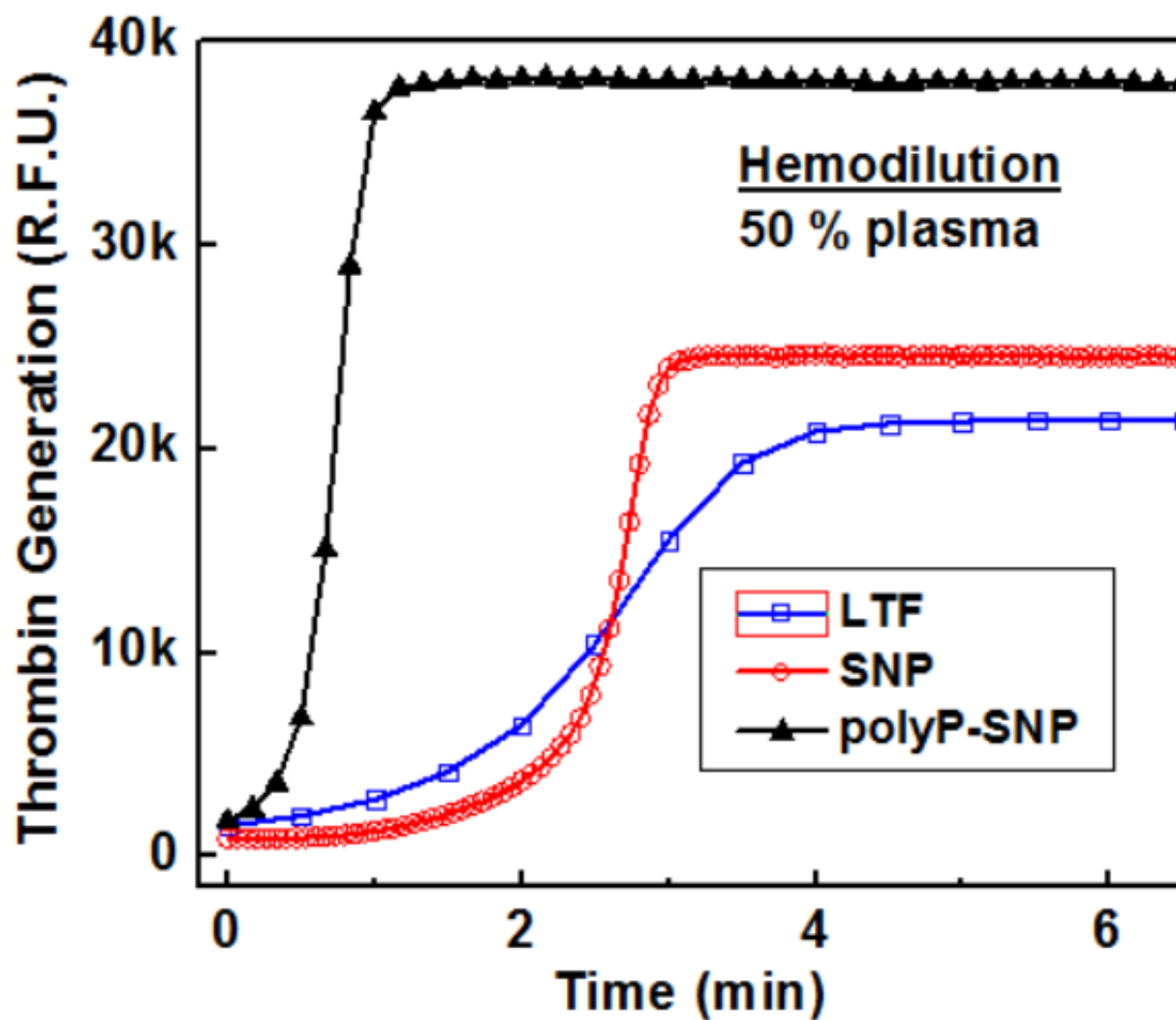


Figure 55. At 50 % PNP hemodilution, polyP-SNP generates thrombin much faster when compared to LTF and bare SNPs.

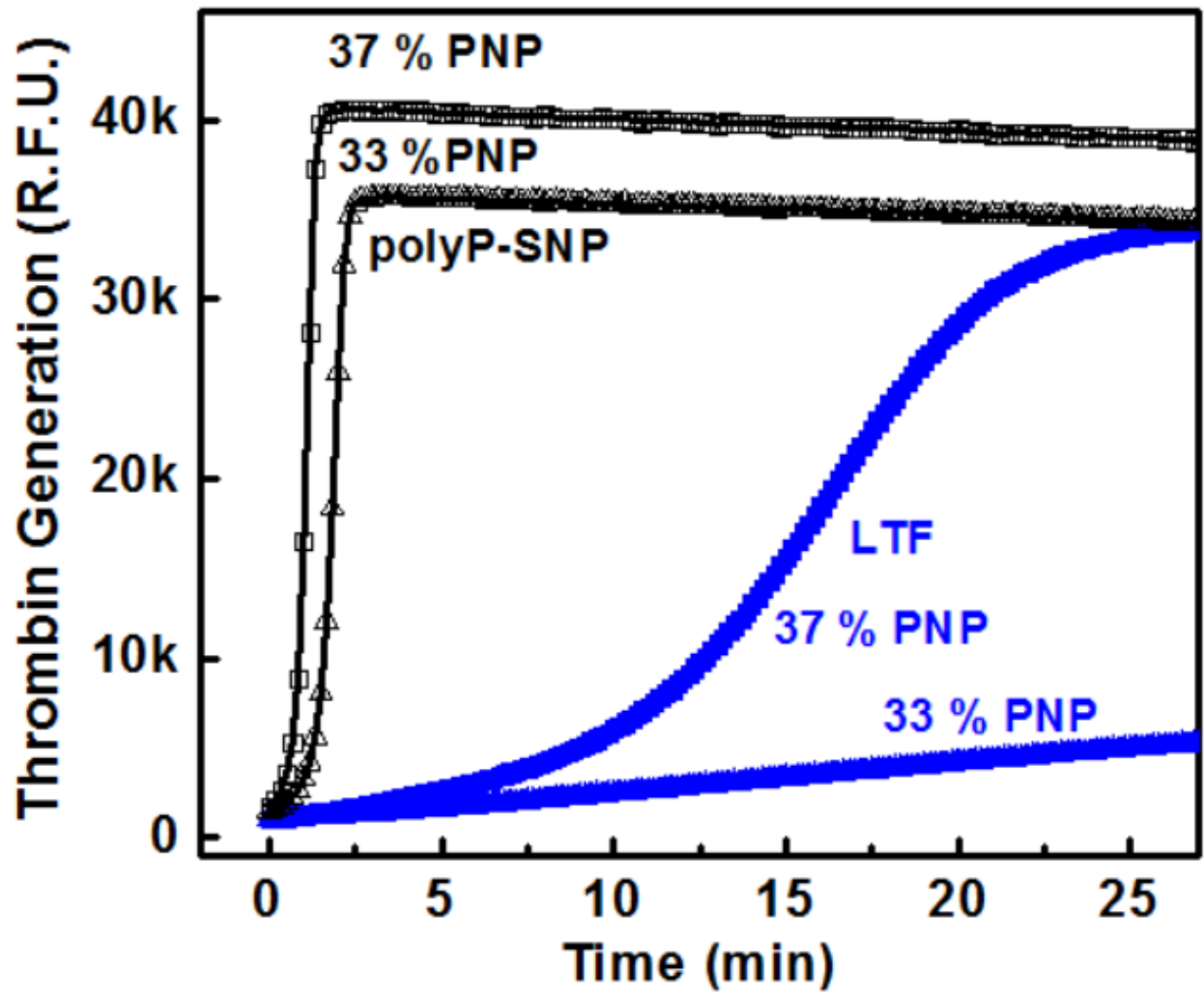


Figure 56. Under increasing hemodilution (33 % PNP), polyP-SNP generates thrombin quickly even when LTF cannot.

As stated by Ramaker, hypothermia leads to a decreased kinetic rate of factor activation.⁽¹⁴⁵⁾ Unlike the coagulopathy simulation, the decrease in activity did not result in complete inhibition of thrombin production. In comparison, polyP-SNP's effect on thrombin production barely decreased, reaching peak thrombin generation at around 2 min under severe hypothermia. When the plasma was chilled to moderate hypothermia, 32 °C, polyP-SNP generated maximum thrombin fluorescence around 1 min, while LTF did not reach maximum thrombin production until 10 min (Figure 58). These data suggest that the polyP-SNP system adjusts to temperature loss better than the clot initiation pathways alone. As described by Martini et al., thrombin production was significantly impaired by excess lactic acid. Yet even at a concentration of 10 mmol/ L excess lactic acid (Figure 58), polyP-SNP's still outperformed LTF and SNP.

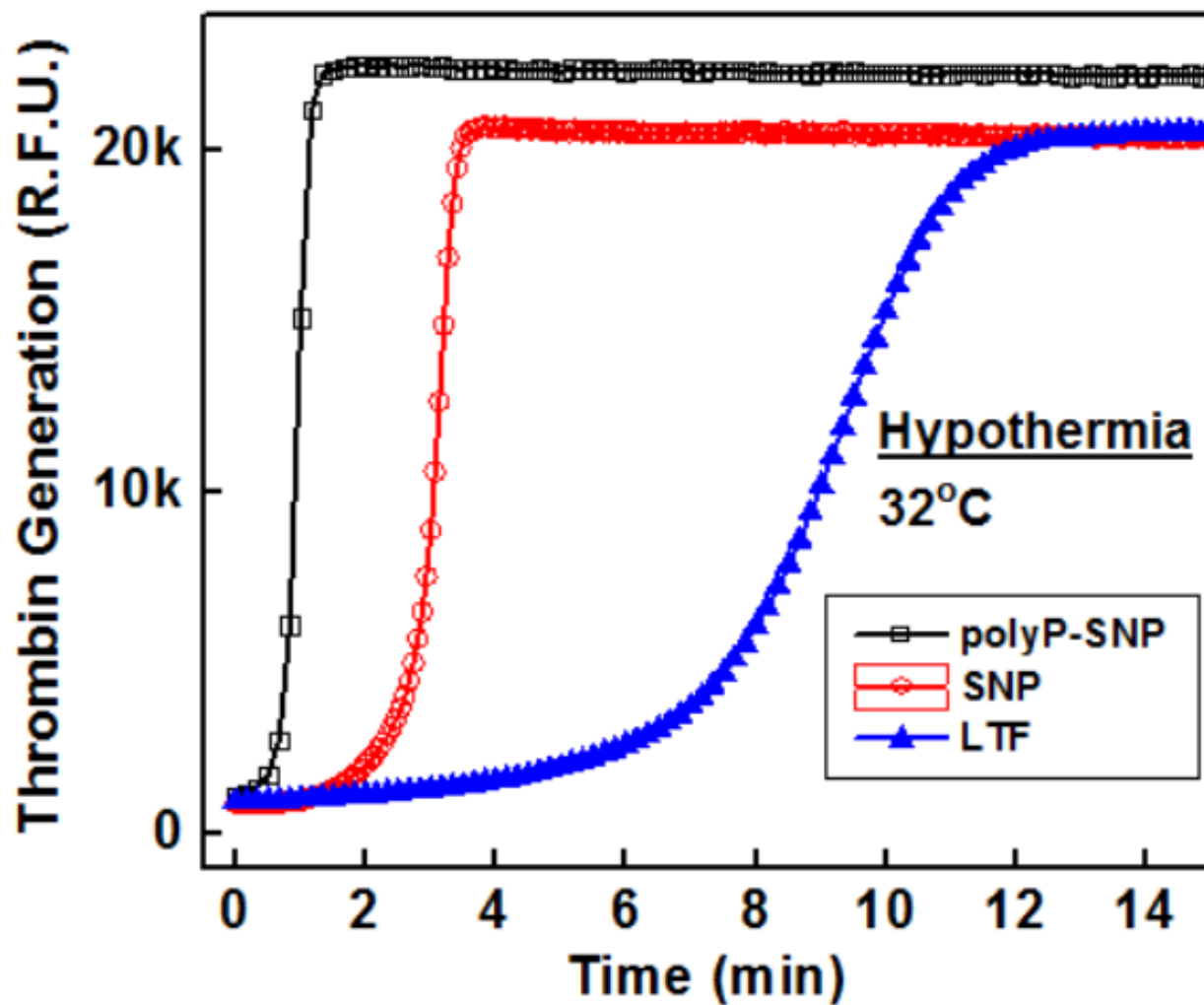


Figure 57. At 32°C, polyP-SNPs generate thrombin at an accelerated rate compared to LTF or bare SNPs.

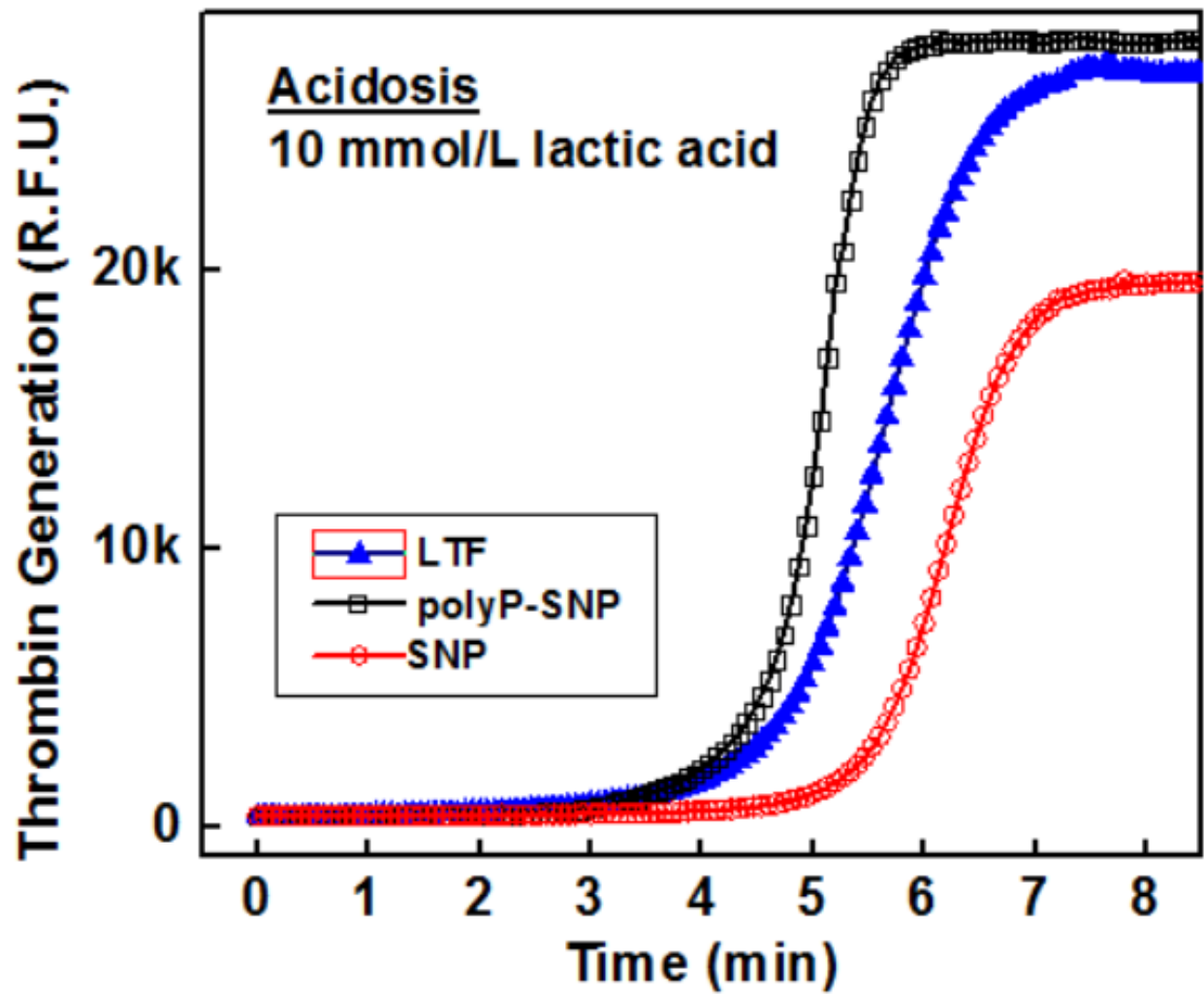


Figure 58. Though excess lactic acid delays thrombin production, polyP-SNP minimizes the time to thrombin burst.

The data produced suggests a first step at developing a novel clotting agent. However, ATC is a multifactorial process involving an endogenous hypocoagulable state occurring nearly immediately after injury complicated by issues such as the injury suffered, clotting abnormalities, cellular interaction, and inflammation.(33) Along with ATC, the onset of an iatrogenic coagulopathy resulting from dilution, hypothermia, and acidosis is a key factor in mortality relating to trauma-induced hemorrhage. The *in vitro* data produced mimics the lethal triad that complicates treatment. However, *in vivo* trials will be required to adequately reproduce ATC conditions.(34) In addition to injury models for effectiveness, *in vivo* trials will also determine if the polyP-SNP system is as safe as theorized through pharmacokinetic and pharmacodynamic studies.

E. Conclusion

Stopping bleeding and correcting coagulopathy remain the key goals in trauma treatment. Rapid effectiveness of both is the key to saving lives and preventing morbidity. PolyP-SNPs are effective, stable at ambient conditions, and far cheaper than recombinant proteins. Attaching polyP to the shell of SNPs significantly reduces clot time and time to thrombin burst despite severe drops in factor levels, temperature, or pH. PolyP-SNP particles may have the ability to serve as a new type of agent for hemorrhage intervention based on its potential to drive coagulation in critical conditions. Further exploration of polyP-SNP's ability to limit contact activation *in vivo* will be necessary for use as a systemic agent. Ultimately, *in vivo* trials(34) will determine whether stable, intravenous polyP-SNP will allow emergency medical personnel to stabilize a patient's clotting function either before or much sooner after the onset of coagulopathy, thereby preventing massive blood loss and lead to greater patient outcomes upon hospital admission.

V. Future work

The Guide and I into that hidden road
Now entered, to return to the bright world;
And without care of having any rest

We mounted up, he first and I the second,
Till I beheld through a round aperture
Some of the beauteous things that Heaven doth
bear;

Thence we came forth to rebehold the stars.

- Dante Alighieri, *Inferno*, Canto XXXIV, 133-139

A. Introduction

There is no ideal hemostatic agent. Even worse there is a lack of intravenous clotting agents to treat the most dangerous wounds. Clinicians have rapidly increased their knowledge of trauma to understand how the body becomes coagulopathic after shock and injury. As modeling becomes more adept, medical personnel will be able to predict which patients will become coagulopathic, leading to improved immediate care for patients and decreasing mortality rates.

A recent trend in coagulopathy has been the development of different treatments for different conditions. External injuries are treated with tourniquets and topical devices to quickly stop the flow of blood. ATC bleeding is now primarily treated with TXA or PCC.(8, 127) Patients with genetically-impaired clotting cascades, such as hemophiliacs, who bleed receive rFVIIa.(154) If the patient is on the vitamin K anticoagulant Coumadin, PCC will be administered.(83) If the patient is on a novel oral anticoagulant (NOAC), there is no currently indicated treatment.(155)

B. Translating research from the benchtop to clinical use

While polyP-SNP's are attractive based for their improved clotting performance *in vitro*, this is only the first step in a long, arduous process of drug development. This process begins with drug discovery in labs, graduates to preclinical trials in simple and complex mammalian systems, and, finally, leads to clinical trials (Figure 59). While preclinical trials are a requirement for clinical application, animal models are not ideal predictors of ATC in humans. In a recent study, 43 animal models (30 swine, 6 rabbit, 5 rat, 1 sheep, 1 mouse, and 0 primate) were compared and none were considered to fully encompass the multiple systems that play a role in ATC.(34) Researchers will likely continue to rely upon swine for injury models because swine have the most similar (pro)coagulation cascade to humans.(156)

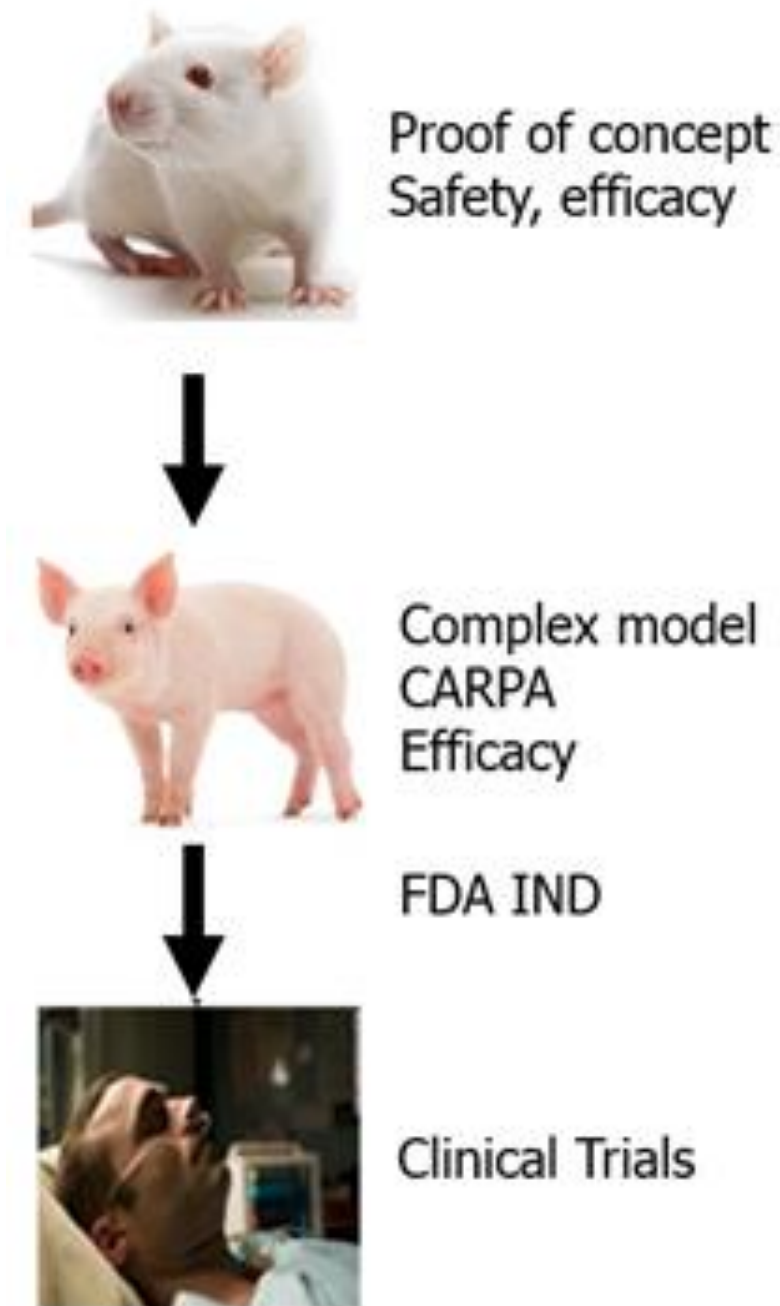


Figure 59. Translation of potential clotting drugs into clinical trials. After drug discovery, potential clotting agents are tested first in rats for proof of concept of safety and efficacy. Swine are used for the complex mammalian model because the circulatory system is similar to that of humans. If successful, the FDA approves the drug as an Investigational New Drug (IND) for use in clinical trials.

A pilot study using Sprague-Dawley rats was initiated in collaboration with Chi Nguyen of Galen Stucky's lab (UCSB Chemistry) and Kyle Ploense of Tod Kippin's lab (UCSB Psychology and Brain Sciences). Particles were suspended in saline and injected into healthy rats at a maximum concentration of 2 mg / kg body weight. Blood was drawn at specific time points and tested using TEG to determine clot time and clot size (Figure 60). The animals were euthanized after 3 hours. Chi Nguyen conducted the post-euthanasia analysis using ICP-MS to track particle accumulation in major organs (Figure 61). In addition, samples were sent to Charles River Laboratories (Wilmington, MA) to test for evidence of thrombosis. Tests results indicated no positive evidence of thrombosis. It is important to stress that these initial results are extremely preliminary and not conclusive. Further trials must be conducted to build a significant population.

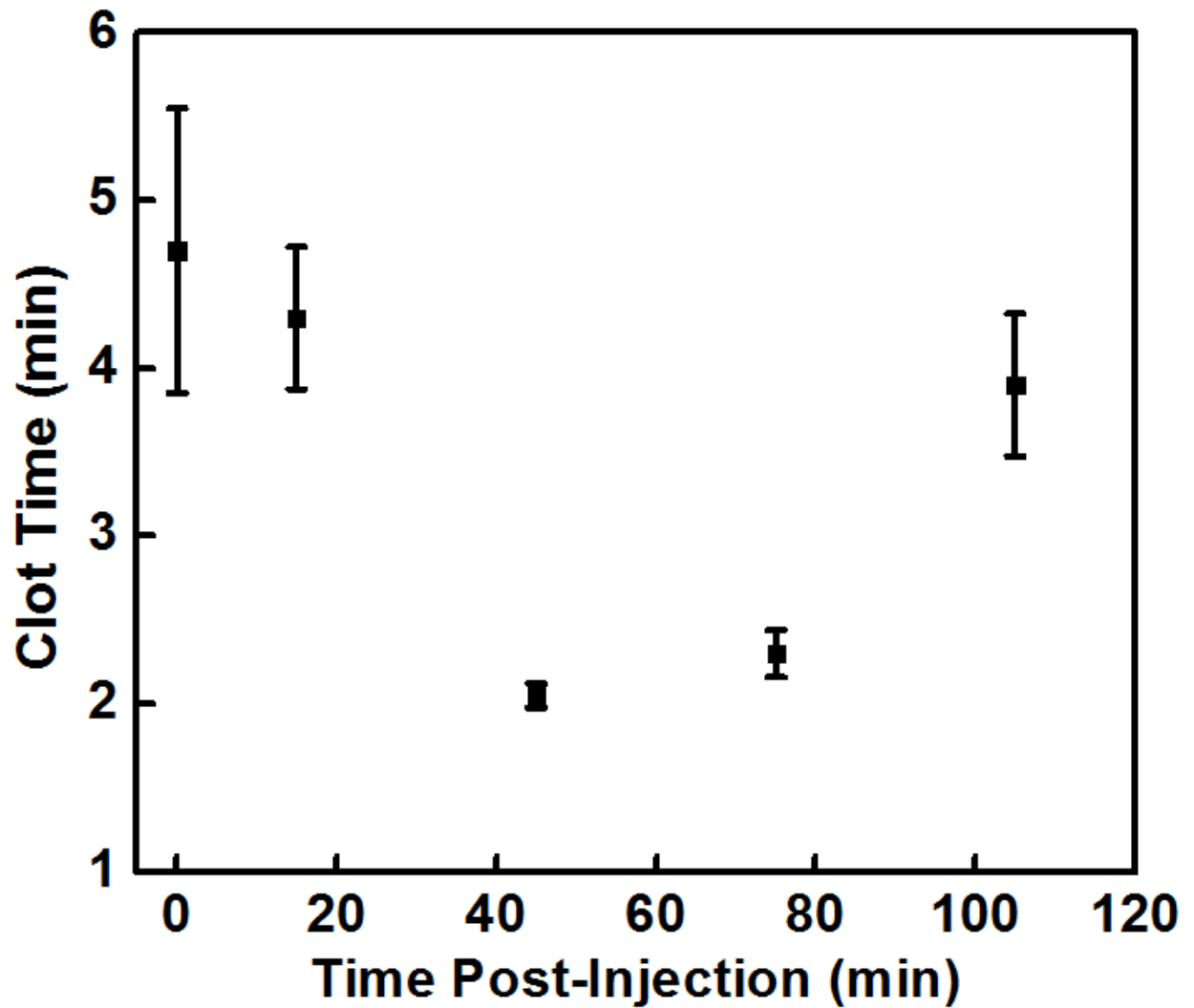


Figure 60. 2 mg / kg polyP-SNP lowers clot time on blood drawn from a Sprague-Dawley rat between roughly 40 -80 minutes after injection. Time 0 corresponds to blood drawn prior to polyP-SNP injection. Trial conducted with Chi Nguyen (Stucky lab) and Kyle Ploense (Kippin lab).

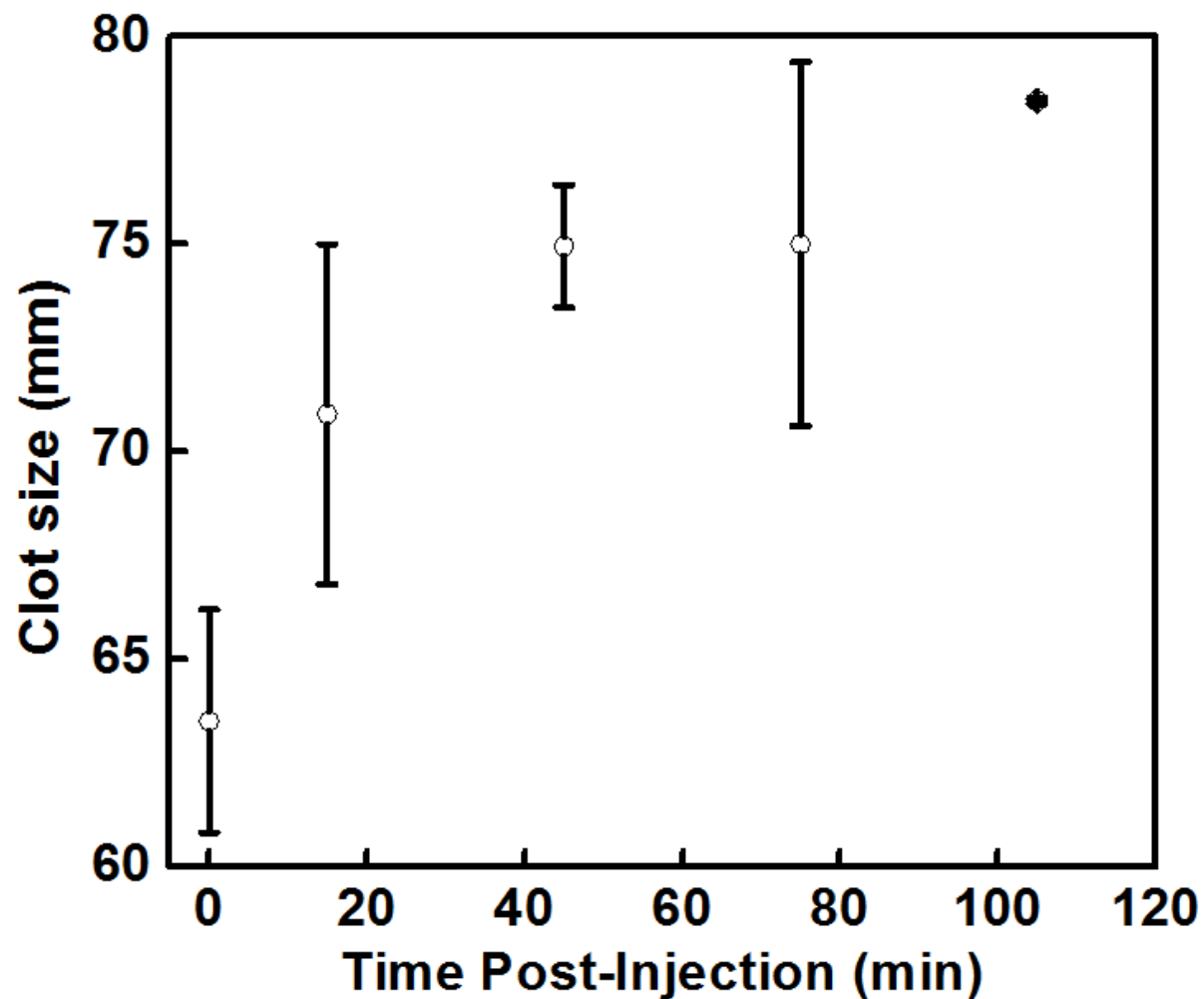


Figure 61. 2 mg / kg polyP-SNP increases maximum clot size increases after injection in Sprague-Dawley rat. Trial conducted with Chi Nguyen (Stucky lab) and Kyle Ploense (Kippin lab).

C. Barriers to translation

1. Complement activation related pseudoallergy (CARPA)

Beginning with cancer treatments,(119) the study of nanoparticles for drug delivery use has increased dramatically in the last decade. Nanomedicine has the potential to provide improved stability,(135) imaging,(157) and targeting(158) to improve treatment for a myriad of conditions. One of the greatest risks with nanomedicine has been the development of a complement activation related pseudoallergy (CARPA). CARPA includes, but is not limited to, cardiopulmonary, hemodynamic, hematological, biochemical and dermatological changes in the patient that present within minutes of injection (Figure 62).(159) While most symptoms dissipate quickly, CARPA can lead to death on its own.(160) In trauma patients, CARPA can accentuate the inflammatory response related to injury, disrupt normal coagulation cascades, and alter hemodynamics.(161) ~10 % of patients have a reaction to doxorubicin and other nanomedicine chemotherapeutics, even when drugs are coated with polyethylene glycol (PEG, PEGylated) and infused slowly.(162) The particle's surface charge, size, and the particle make-up can all increase a patient's chances at hypersensitivity.

CARPA is defined best as a set of symptoms rather than a specific syndrome. CARPA does not affect small mammals such as rodents and mice, the traditional first step in drug discovery. However, Szabeni et al. have identified that swine and canine models are the most effective animal models for predicting CARPA in humans.(159) While primates remain the closest human analogue, non-human primate CARPA models have not been developed. Research is required to determine the pathogenesis of CARPA. This research must begin with more complex mammalian models to study systems that are affected by this condition.

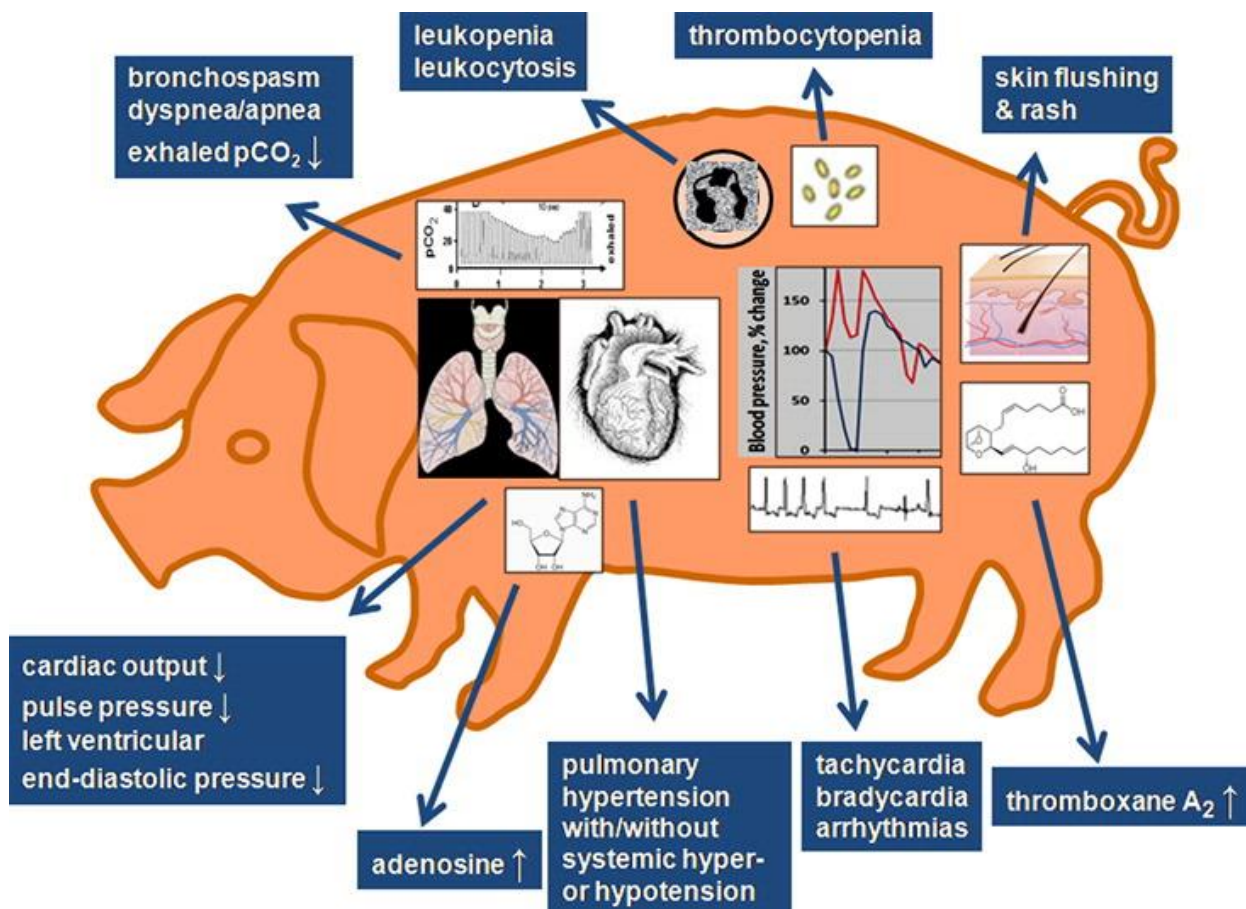


Figure 62. Symptoms seen in a CARPA swine model. Reprinted from Advanced Drug Delivery Reviews, Vol 64, Issue 15, J Szebeni, P Bedocs, D Csukas, L Rosivali, R Bunger, R Urbanics, A porcine model of complement-mediated infusion reactions to drug carrier nanosystems and other medicines, 1706-1716, Copyright (2012), with permission from Elsevier.(159)

D. Anticoagulant reversal

Warfarin, a Vitamin K antagonist, is the most widely prescribed oral anticoagulant drug in America. Warfarin acts to prevent activation of multiple proteins tied to clotting including procoagulant F(actors) X, VII, and II (thrombin) and the anticoagulant protein C. As a result, patients receiving warfarin often suffer from serious side effects, including adverse interactions with a myriad of food and drugs. Patients suffering minor injuries on warfarin can develop uncontrollable hemorrhage. In cases of bleeding, both Vitamin K and prothrombin complex concentrate are FDA-approved antidotes to reverse the effects of warfarin.(163)

Two new classes of anticoagulants have been developed to minimize the side effects seen in warfarin. These single factor inhibitors prevent activation of FXa (rivaroxaban) or thrombin (argatroban). Since FDA approval in 2011, doctors have prescribed rivaroxaban over 6 million times.² In 2012 alone, there were over 30 million chronic prescriptions for warfarin. Hindering further usage, single-factor inhibitors lack an approved antidote.(164) Recently, recombinant FXa has been shown to be a potential antidote for rivaroxaban.(163) Even if it is effective, recombinant FXa will suffer from the same cost and storage issues that all recombinant proteins face.

In addition to its effect on coagulation, short-chain polyP has also been shown to effectively reverse both genetically and medically induced coagulopathy, including the NOACs rivaroxaban and argatroban (Figure 63).(131) While this has not been confirmed in the polyP-SNP system, polyP-SNP increases thrombin production tenfold over bare polyP(165) and, thus, should be effective at overcoming anticoagulation in the event of the

² Data provided by Janssen Pharmaceuticals, Inc.

bleed. PolyP-SNP's easiest path to clinical use may follow the track of PCC: FDA approval as an anticoagulant reversal agent with the potential to also mediate ATC-related hemorrhage. When combined with SNP, polyP has the long-term stability and cost-effectiveness of a topical agent combined with the *in vitro* effectiveness of a recombinant protein. If polyP-SNP can be shown to be safe and effective *in vivo*, this therapeutic could lead to a breakthrough in trauma management by increasing the trauma population that is able to be treated prehospital, significantly decreasing the time between injury and first-treatment that is critical to patient survival.

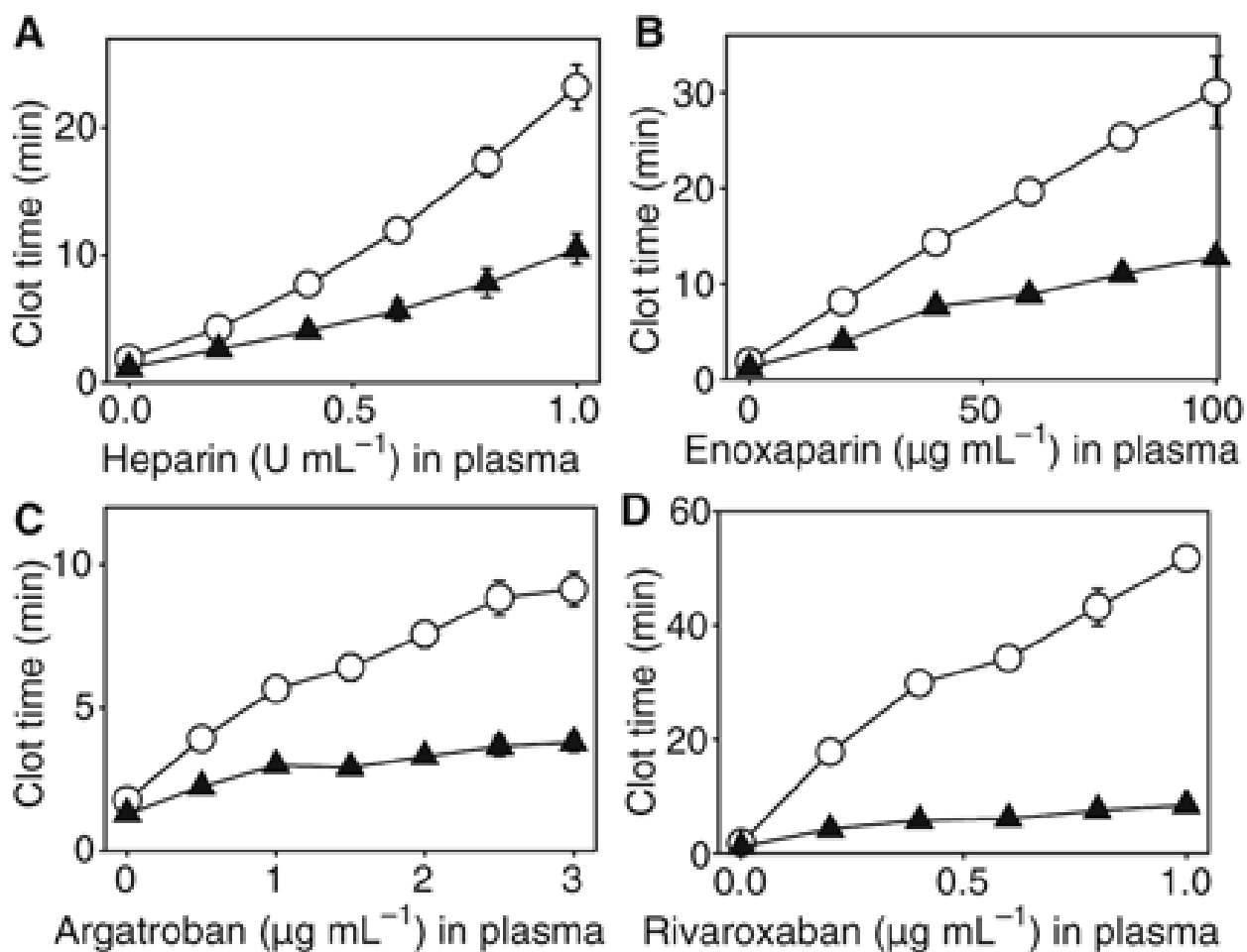


Figure 63. PolyP (black) lowers clot time for the anticoagulants (A) heparin, (B) enoxaparin, (C) NOAC argatroban, and (D) NOAC rivaroxaban. Reprinted from *Journal of Thrombosis and Haemostasis*, Vol. 6, Issue 10, SA Smith, JH Morrissey, Polyphosphate as a general procoagulant agent, 1750-1756, Copyright (2008), with permission from John Wiley and Sons. (131)

While this thesis covers only the drug discovery stage, the results presented within show the exciting potential of the polyP-SNP system. In all *in vitro* assays, polyP-SNP proved an excellent agent at inducing coagulation. The next step is to translate this research into preclinical trials to determine if polyP-SNP is indeed safe and effective in the face of systematic hemostatic damage. If successful, polyP-SNP has the potential to act as an ideal universal procoagulant, able to accelerate clotting in traumatic injuries regardless of current problems such as the patient's condition, injury locus, and inability to rapidly transport the patient to a medical facility. Humans will never eradicate injury, but, with improved knowledge and smarter therapeutics, the damage can be mitigated.

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