IMPROVING THE EFFICACY OF HEMOSTATIC AGENTS

BY INCREASING THEIR TRANSPORT INTO WOUNDS

by

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Abstract

Uncontrollable hemorrhage remains a leading cause of mortality in many situations, including during surgery and following trauma. Many hemostatic interventions have been developed, but mortality and morbidity remain high because they are ineffective or difficult to use in cases of severe bleeding. Blood flow can rapidly wash away topically applied hemostatic agents and prevents them from reaching leaking vessels and forming robust clots. We hypothesized that increasing the transport of hemostatic agents into wounds could improve their ability to manage bleeding. To test this hypothesis, I developed self-propelling particles that can transport through flowing blood and into wounds. These particles, consisting of calcium carbonate and an organic acid, delivered two hemostatic agents, thrombin and tranexamic acid (TXA), and improved their ability to stop bleeding in mice (Chapter 3). Next, I showed that self-propelling particles loaded with thrombin applied with gauze (PTG) significantly improved survival in a swine model of massive traumatic bleeding without compression, compared to those agents on gauze without propulsion (Chapter 4). This demonstrates that increasing transport of hemostatic agents could increase their utility for managing clinically relevant hemorrhage, such as from battlefield trauma. In two sheep models of surgical hemorrhage, PTG’s ability to stop bleeding was compared to two clinical standard interventions (Chapter 5). In a model of endoscopic surgical bleeding, PTG significantly reduced bleeding time compared to control gauze. In a model of massive open surgical bleeding, PTG achieved hemostasis in more cases than a standard thrombin-containing hemostatic agent. These demonstrate that increased transport of hemostatic agents could enable improved management of surgical bleeding. Finally, formulating TXA with self-propelling
particles increased its ability to inhibit fibrinolysis in vitro and reduce bleeding in mice in vivo, demonstrating that these particles could transport a variety of hemostatic agents and increase their efficacy too (Chapter 6). The findings of this thesis suggest that improving the transport of hemostatic agents into wounds, such as by using self-propelling particles, is a promising approach for increasing the efficacy of those agents, and may present an opportunity to reduce mortality, morbidity, and the clinical burden associated with bleeding.
Lay Summary

Bleeding kills many people, in hospitals and on battlefields. Many agents that treat bleeding don’t work because blood flow washes them out of the wound. Stopping these agents from being washed away could increase their ability to bleeding. Here, we show that micro-rockets made of calcium carbonate and an organic acid can propel through flowing blood and deliver drug cargoes into wounds. When loaded with an enzyme that clots blood (thrombin), these rockets could stop bleeding in multiple animal experiments better than thrombin alone. Gauze containing thrombin-loaded micro-rockets stopped deadly bleeding in two sheep models of surgical bleeding, and in a pig model of battlefield bleeding. These micro-rockets were also able to deliver another drug that treats bleeding, TXA, and improved its effectiveness. These results show that moving these drugs deeper into wounds, such as by using micro-rockets, could make these drugs more effective at treating bleeding.
Preface

All projects and associated methods were approved by the University of British Columbia Animal Care Committee [certificates #A12-0130, #A14-0172, #A16-0176] and performed in accordance with the guidelines established by the Canadian Council on Animal Care.


A version of Chapter 3 has been published. Baylis, J. R. *et al.* Self-propelled particles that transport cargo through flowing blood and halt hemorrhage. *Sci. Adv.* **1**, e1500379–e1500379 (2015). I conceived of the hypotheses, methods, and applications, conducted experiments, collected and analyzed the data, and the manuscript. Dr. Christian Kastrup was the corresponding author on this work and conceived of the hypotheses methods, and applications, contributed to writing the manuscript and oversaw the research.

A version of Chapter 4 has been published. Baylis, J. R. *et al.* Self-Propelled Dressings Containing Thrombin and Tranexamic Acid Improve Short-Term Survival in a Swine Model of Lethal Junctional Hemorrhage. *Shock* **46**, 123–128 (2016). I conceived of the hypothesis, prepared samples, analyzed data, and the manuscript. Surgeries and data collection were performed at University of Washington under supervision and clinical input of Dr. Nathan White and Dr. Alex St. John, who was co-first author and who led the surgical team and animal experiments.
A version of Chapter 5 has been published. Baylis, J. R. et al. Rapid hemostasis in a sheep model using particles that propel thrombin and tranexamic acid. *Laryngoscope* **127**, 787–793 (2017). I conceived of the hypotheses, methods, and applications. I collected and analyzed data, and wrote the manuscript. Surgeries were performed at UBC under supervision and clinical input of Dr. Amin Javer and his team from St. Paul’s Hospital, including Dr. Andres Finkelstein-Kulka who was co-first author and who led the surgical team and animal experiments. Surgeries were assisted by UBC Animal Care Services, who also performed the histopathological analyses.

A version of Chapter 6 is in preparation for submission, tentatively titled “Topical tranexamic acid is more effective when formulated with self-propelling particles.” Baylis, J.R., Lee, M.M., St. John, A.E., Wang, X., Simonson, E., Cau, M., Kazerooni, A., Gusti, V., Yoon, J.S.J., Liggins, R.T., White, N.J., Kastrup, C.J. (2018). I conceived of the hypotheses, methods, and applications, analyzed data, and I wrote the manuscript. I am co-first author with Michael Lee, who performed many of the experiments and conceived of their design.
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List of Symbols

\[ [\text{TXA}]_{\text{plasma}} \] Concentration of TXA in plasma

\( A \) Frontal area of a bubble

\( a^\text{gauze}_{\text{thrombin}} \) Activity of thrombin on gauze

\( C_D \) Coefficient of drag

\( D^\text{systemic}_{\text{thrombin}} \) Systemic dose of thrombin

\( F_{\text{buoy}} \) Buoyant force

\( F_{\text{drag}} \) Drag force

\( g \) Acceleration due to gravity (9.8 m/s\(^2\))

\( m^\text{gauze}_{\text{TXA}} \) Mass of TXA on gauze

\( m_{\text{pig}} \) Bodyweight of pig

\( \nu \) Kinematic viscosity

\( r \) Radius of a bubble

\( \text{Re} \) Reynolds number

\( \rho_{\text{water}} \) Density of water (1000 kg/m\(^3\))
$V_{\text{blood}}$  Total blood volume

$V_{\text{bubble}}$  Bubble volume
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>α2-AP</td>
<td>α2-antiplasmin</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>APTT</td>
<td>Activated partial thromboplastin time</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>KG</td>
<td>Kaolin-based gauze, a.k.a. QuikClot Combat Gauze&lt;sup&gt;R&lt;/sup&gt; (Z-Medica)</td>
</tr>
<tr>
<td>FESS</td>
<td>Functional endoscopic sinus surgery</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluoroscein isothiocyanate</td>
</tr>
<tr>
<td>GBS</td>
<td>Glycine-buffered saline</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HBS</td>
<td>HEPES-buffered saline</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>ivTXA&lt;sub&gt;11&lt;/sub&gt;</td>
<td>Intravenous TXA (11 mg/kg)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ivTXA$_{33}$</td>
<td>Intravenous TXA (33 mg/kg)</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>nonpropelled thrombin</td>
<td>A powder mixture of CaCO$_3$, TXA, and thrombin</td>
</tr>
<tr>
<td>NPTG</td>
<td>Non-propelled thrombin and TXA gauze</td>
</tr>
<tr>
<td>npTXA</td>
<td>Non-propelled TXA: a powder mixture of CaCO$_3$ and TXA-HCl</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>pdTXA</td>
<td>Pre-dispersed TXA: TXA in solution dispersed throughout the system</td>
</tr>
<tr>
<td>PFO</td>
<td>1$H_1$,1$H_2$,2$H_2$-Perfluoro-1-octanol</td>
</tr>
<tr>
<td>PG</td>
<td>Plain gauze</td>
</tr>
<tr>
<td>$P_{\text{Interaction}}$</td>
<td>p-value of interaction between time and treatment</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-lysine</td>
</tr>
<tr>
<td>propelled thrombin</td>
<td>A powder mixture of CaCO$_3$, TXA-HCl, and thrombin</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin time</td>
</tr>
<tr>
<td>PTG</td>
<td>Propelled thrombin and TXA gauze</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PTime</td>
<td>p-value comparing changes over time</td>
</tr>
<tr>
<td>PTreatment</td>
<td>p-value comparing changes between treatments</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>RM-ANOVA</td>
<td>Repeated measures ANOVA</td>
</tr>
<tr>
<td>spTXA</td>
<td>Self-propelling TXA: a powder mixture of CaCO3 and TXA-HCl</td>
</tr>
<tr>
<td>TEG MA</td>
<td>Thromboelastogram maximum amplitude</td>
</tr>
<tr>
<td>TIC</td>
<td>Trauma-induced coagulopathy</td>
</tr>
<tr>
<td>toTXA</td>
<td>Topical TXA, administered in an aqueous solution</td>
</tr>
<tr>
<td>TXA</td>
<td>Tranexamic acid</td>
</tr>
<tr>
<td>TXA-HCl</td>
<td>Tranexamic acid hydrochloride</td>
</tr>
</tbody>
</table>
Background

1.1 Bleeding is a substantial cause of mortality and morbidity

Bleeding kills over 2 million people each year.\textsuperscript{1–3} Uncontrollable hemorrhage remains a leading cause of mortality in many situations, including in combat, emergency, and hospital settings. Bleeding is identified as the leading cause of preventable death in trauma.\textsuperscript{4–6} Hemorrhage is responsible for 50\% of deaths in the military, and noncompressible hemorrhage is the single leading cause of mortality, often deemed potentially survivable.\textsuperscript{7–9} Reports estimate that effective control of junctional hemorrhage could have prevented 5\% of combat fatalities in Afghanistan.\textsuperscript{7,10–12} In emergency, hemorrhage causes 33\% of prehospital mortality, and this number has remained constant since 1977.\textsuperscript{13}

Bleeding following surgery is responsible for 47\% of surgical deaths.\textsuperscript{14,15} Poorly-controlled intraoperative bleeding can lead to complications such as infection, procedural error, increased operating time, and transfusion of blood products.\textsuperscript{16,17} Excessive blood loss in surgery is associated with increased mortality, morbidity, and intensive care stay.\textsuperscript{18–20} Bleeding also contributes

significantly to mortality in other scenarios; postpartum hemorrhage (PPH) causes maternal death in 1–2% of all births in low-resource settings.\(^\text{21}\)

Current conventional treatments include fluid resuscitation, blood/platelet transfusion, intravenous recombinant proteins, or compression of wounds combined with external delivery of hemostatic agents. Though mortality rates due to bleeding remain high, there have been few major clinical developments of new hemostatic interventions.\(^\text{22}\) Multiple reviews of modern hemostatic strategies identify a need for new hemostatic interventions.\(^\text{23–26}\)

1.2 Hemostatic molecules must reach sites of damaged vasculature

Convective and diffusive transport play important roles in regulating blood coagulation through delivery of coagulation factors to growing hemostatic plugs and through removal of activated factors to prevent thrombosis.\(^\text{27}\) To form a stable clot that can stop bleeding, coagulation factors must be delivered to vascular damage and must be maintained at high enough concentrations to trigger coagulation. These concentration thresholds that must be reached are increased when blood flow rates are high, such as during severe bleeding.\(^\text{28,29}\) Improving the ability of hemostatic agents to transport and localize to sites of damaged vasculature could increase their ability to manage bleeding. Conversely, when hemostatic agents cannot reach their sites of action, threshold concentrations of coagulation factors are not produced, a robust clot does not form, and death can result. While many agents exist which can initiate and stabilize clot growth during bleeding, such as endogenous clotting factors (e.g., thrombin), small molecule therapeutics (e.g. tranexamic acid (TXA)), and inorganic materials (e.g., silica, kaolin), methods of increasing their transport to sites
of vascular damage are still under development.\textsuperscript{30–32} The roles of two particular hemostatic agents, thrombin and tranexamic acid, in mediating coagulation are described in Figure 1.1.

\textbf{Figure 1.1 Enzymatic reactions of blood coagulation and fibrinolysis.}

\textit{Thrombin and thrombin-mediated cleavages are in red. TXA and TXA-mediated inhibitions are in blue.}
1.3 Targeted delivery of systemically administered hemostatic agents

Many hemostatic agents are administered systemically—orally, intravenously, intramuscularly, or intraosseously. In hospitals, bleeding is often managed by intravenously administered agents, such as TXA or recombinant factor VIIa.\textsuperscript{16,17,33} However, systemic administration results in uniform plasma concentrations of hemostatic agent to be uniform throughout circulation, thereby increasing potential risks of thrombosis and immunologic responses when their action cannot be adequately localized after injection.\textsuperscript{22,34,35} For example, large multi-centre clinical trials have shown that intravenous TXA, for managing hemorrhage following trauma, increased risks of thromboses.\textsuperscript{36} Enhancing the targeted delivery of hemostatic agents specifically to sites of bleeding could greatly increase both their safety and efficacy.

Many technologies are being developed that can increase the localization and transport of systemically administered hemostatic agents to sites of bleeding.\textsuperscript{37,38} Among these are agents that mimic endogenous components of coagulation by responding to biochemical signals and localizing at sites of bleeding. Some of these agents, which can be soluble or particles, bind extracellular matrix components such as collagen, and to plasma components such as von Willebrand factor and fibrin, to mediate platelet clustering and clot initiation and adhesion.\textsuperscript{35,39–41} Synthetic polymers have also been described that are activated specifically by coagulation enzymes and mediate coagulation at sites of bleeding and thrombosis.\textsuperscript{42} Similarly, particle-based agents have been designed which respond to mechanical stimuli, such as changes in shear rate, to release bioactive molecules which mediate coagulation.\textsuperscript{40,43} For example, particles have been developed that release fibrinolytic enzymes at sites of thrombosis in response to high shear; this approach may be useful
for targeted release of coagulants at sites of hemorrhage. Agents that respond to regions of low shear, such as within pooling blood, have not been reported to our knowledge, but could also be useful.\textsuperscript{44} A wide array of creative and sometimes exotic drug delivery technologies have been produced for non-hemostatic indications and these may be useful for treating hemorrhage in the future, such as technologies that can be systemically administered and then controllably triggered to release therapeutic cargos.\textsuperscript{45–48}

1.4 Topical hemostatic agents could benefit from increased transport into wounds

One method of localizing hemostatic agents to the site of bleeding, while reducing risks of immunologic responses or thromboses, is to apply them topically. Compared to intravenous, intramuscular, or intraosseous administration, topical application of such hemostatic agents can also reduce the delay between injury and treatment and can require less sophisticated medical infrastructure and fewer trained personnel to administer.\textsuperscript{49} For example, many reports have investigated administering tranexamic acid topically for managing surgical and traumatic bleeding, and have found it can potentially reduce blood loss or reduce the number of blood products required.\textsuperscript{50–52} Topical administration could also be used in in low resource settings, such as low- and middle- income countries, in austere environments, or in settings where evacuation of the casualty is paramount, such as during the Care Under Fire phase of Tactical Combat Casualty Care.

Topical hemostatic agents are often applied following trauma, where the need to control bleeding and stabilize a patient for transport is paramount. Some of these agents are applied as powders,
such as chitosan or kaolin (e.g. Celox™ (Celox Medical), QuikClot™ (Z-Medica)). These powders can also be loaded upon absorbable or nonabsorbable matrices and applied as hemostatic dressings. However, these dressings are typically indicated for use with application of external compression for three minutes, which is infeasible in many trauma scenarios such as during combat or during transport, and the efficacy of these dressings is highly dependent on the training of the responder administering them. Additionally, hemostatic dressings are difficult to apply when the bleeding tissue is not immediately accessible; for example, managing abdominal hemorrhage using hemostatic sponges requires damage-control laparotomy, which is highly invasive and carries risks of infection. Recent preclinical studies have shown that these hemostatic dressings may not be more effective than plain gauze at stopping bleeding. The limitations of these dressings is discussed further in Chapter 4.

Topical hemostatic agents are also used during surgery. Thrombin, a potent coagulation enzyme, can be administered in aqueous solutions or within flowable matrices, such as gelatin. Other surgical hemostatic dressings include foams and sponges made from gelatin, collagen, or oxidized cellulose. Synthetic and fibrin-based sealants are also used but are limited in their ability to control bleeding because they can be difficult to apply to wounds of irregular shapes, and can also be flushed from the wound site due to high flow rates. Many applicator devices, such as endoscope- and catheter-based devices, are being developed to enhance delivery of hemostatic agents, such as embolic agents and hemostatic sprays. The risks and limitations of interventions for perioperative hemorrhage are discussed further in Chapter 5.
In both trauma and surgery, while many topical hemostatic agents exist they have limited efficacy in many clinical scenarios, such as when bleeding originates deep within a wound, when damaged vessels cannot be located, or when wounds cannot be compressed.\textsuperscript{22,60} In these situations, blood flow rapidly transports external agents away, preventing their delivery and delaying initiation of clotting at compromised vessels.\textsuperscript{22} Puncture or severance of arteries can create dramatic pressure gradients – hundreds of mmHg per cm – which drive flow away from damaged vasculature.\textsuperscript{27} Preclinical experiments which use Doppler sonography to measure flow velocities from damaged arteries show bleeding velocities of \(\sim 150\ \text{cm/s} \) at peak systole.\textsuperscript{61} Additionally, following injury and subsequent vasospasm, blood flow velocities from wounds can increase three- to ten-fold.\textsuperscript{62} Since topically agents cannot reach damaged vessels, they instead form superficial clots form at wound surfaces, and these clots are susceptible to rupture during patient transport and resuscitation, causing rebleeding,\textsuperscript{63–65} which is correlated with poor clinical outcomes.\textsuperscript{66,67} While much progress has been made towards increasing the transport of systemically administered hemostatic agents to sites of bleeding, reports are lacking which describing efforts to increase the transport of locally applied external hemostatic agents into bleeding wounds (Figure 1.2).
These concerns could be addressed by quickly delivering therapeutics such as coagulants, anti-fibrinolytics, antimicrobials, or growth factors to the damaged vasculature. However, systemic delivery of these therapeutics via injections or local delivery via intravascular catheters is often impractical. Delivering agents topically is also limited by biophysical barriers; it is difficult to transport agents against blood flow, especially far enough upstream to reach the leaking vessels. A fast-acting topical agent that actively delivers therapeutics deep within difficult-to-reach, incompressible or massively bleeding wounds has the potential to reduce fatal hemorrhage.
Chapter 2  Hypothesis and rationale\textsuperscript{2}

2.1  Specific hypothesis

The specific hypothesis tested by this thesis is that increasing the transport of hemostatic agents into wounds, so that greater mass of the agent is deposited proximal to the injured vessel, will improve their ability to stop bleeding.

2.2  Self-propelling particles are known to transport cargo through aqueous fluids

To test this hypothesis, self-propelling particles were used as a tool for improving the transport of agents into wounds. Self-propelling particles are a promising candidate for improving transport of agents, for managing bleeding, and for drug delivery in general. Many self-propelling particle systems have been developed, with proposed applications in targeted drug delivery.\textsuperscript{69–72} Though few of these systems have yet advanced to \textit{in vivo} testing (delivering biologically active cargo in live animals to achieve a therapeutic effect), their diversity and ingenuity make self-propelling particles promising candidates for biomedical applications.\textsuperscript{70,71,73} The work described here includes represents the first reports of self-propelling particles delivering therapeutic cargos \textit{in}

vivo, the first self-propelling particles that function in blood, and the first that function in vivo using an exogenous fuel.

Upon embarkment of the work described in this thesis, there had been no reports of use of self-propelling particles in vivo (experiments using live animals), and such reports remain rare. The first reports of self-propelling microparticles used catalytic degradation of aqueous hydrogen peroxide to generate gas and thrust.\textsuperscript{74–76} Similar systems were described wherein bimetal rods consisting of gold and platinum can reduce and oxidize hydrogen peroxide, respectively, generating an anisotropic ion distribution and an electrical field-generated thrust.\textsuperscript{77} While these systems provided an important proof-of-principle for self-propelling particles, they have limited utility in biological environments, where hydrogen peroxide may be present only at sparing concentrations and where the high ionic strength and conductivity of the medium decrease the effective strength of the generated electric field. Recent reports have built upon these catalytic micromotors to enable external manipulation of their trajectories, such as through magnetic steering or through thermally responsive speed control.\textsuperscript{78,79}

Since then, particles have been developed which utilize multiple forms of propulsion, such as magnetic swimming, ultrasound-driver motion, and bioelectrochemical reactions.\textsuperscript{70,80–82} Self-propelling particles have been loaded with a range of cargoes, including sugars, drugs, such as doxorubicin, and whole cells.\textsuperscript{71,83–86} Self-propelled nanomotors have been developed which can localize at sites of damage in electrical circuits, and this yields interesting future prospects for particles which can sense sites of tissue damage to specifically deliver therapeutics to wound sites.\textsuperscript{87} Some reports previously suggested that propulsion of micromotors through biological
fluids, such as undiluted blood, may be very difficult or impossible to achieve due to the presence of cells, such as red blood cells, and high viscosity caused by high protein concentrations, but this was recently accomplished with simple self-propelling particles, including those developed in this thesis. These factors which prevent propulsion through blood are particularly limiting when using catalytic micromotors, which generate small thrust and propel at only microns per second. Additionally, there are no reports to our knowledge of micromotors propelling against pressure gradients, such as those which drive blood flow.

2.3 Self-propelling particles can deliver drugs in vitro

Though self-propelling particles have been built and studied for over eighteen years, their uses in vivo and for drug delivery have only been reported since 2015. Self-propelling particles are proposed to be well-suited to increasing of drug delivery; for example, they are proposed to enable drugs which are encapsulated in in liposomes, polymersome, dendrimers, and micelles, to cross hydrophobic cell membranes and access diseased tissues. Because of this, much attention has been directed towards developing self-propelling particles which rely on fuels which are nontoxic and/or available in physiological environments, such as lactic acid. Developing self-propelling particles driven by biocompatible fuel sources is an ongoing challenge. Many reports have tested the ability of different self-propelling particles to deliver therapeutic payloads to mammalian cells in vitro as preliminary steps towards functional in vivo drug delivery systems.

In 2015, Gao et al. described zinc-based micromotors which can react in gastric acid to produce hydrogen gas and enhance transport and penetration of the particles into the stomach linings of
mice. More recently, de Avila et al. described magnesium-based micromotors loaded with clarithromycin that were as effective as clarithromycin co-administered with proton pump inhibitors at reducing gastric infection in mice infected with *Heliobacter pylori*. These results, combined with the findings presented in this thesis, indicate that self-propelling particles can be used to improve transport of therapeutics *in vivo*. Those two reports and the four reports contained in this thesis are, to our knowledge, the only reports to date showing successful use of self-propelling particles for to deliver drugs in live animals and achieve therapeutic effects. These three self-propelling particle systems are similar in that they are each relatively simple, and generate propulsion using only non-catalytic, gas-generating reactions; these particles do not require external stimuli, such as ultrasound, or exogenous fuel sources, such as hydrogen peroxide.

### 2.4 Self-propelling particles could increase transport into bleeding wounds

In this thesis, I developed self-propelling particles that can deliver therapeutic cargo *in vivo*, and the first report that they can propel through whole blood. The formulation utilizes carbonate salts mixed with a solid organic acid to release CO$_2$ upon contact with aqueous solutions, such as blood. For the organic acid, we used TXA, because it is used clinically to stabilize clots during trauma by inhibiting tissue-type plasminogen activator and plasmin, but almost any organic acid can be used to propel these particles. During the reaction, the particles dissolve, the organic acid is buffered and CO$_2$ is produced, which is highly soluble in blood. The rapid production of gas bubbles made particles transport through blood in all directions at velocities up to centimetres per second. Propulsion occurred from a combination of particles rising buoyantly, propelling laterally, and the
large convection generated by the release of gas. Because of their propulsion velocities, which are comparable to those found in bleeding scenarios, propulsion of particles greatly increased their delivery and accumulation in wounds and local microvasculature in mice with either transected tails or lacerated livers. The ability of these particles to propel against flowing blood, and to transport cargo into bleeding wounds, enabled this thesis to test the hypothesis that increasing transport of hemostatic agents into bleeding wounds will improve their ability to stop bleeding.

2.5 Purpose and overview for this thesis

To demonstrate that the efficacy of hemostatic agents can be improved by increasing their transport into wounds, using self-propelling particles, four different studies (presented in Chapters 3-6) were designed and conducted:

- Can self-propelling particles transport cargo upstream through flowing blood and increase their transport into bleeding wounds? (Chapter 3)

In this study, we developed the carbonate-based self-propelling particle formulation for delivering cargo through flowing aqueous solutions. These particles can increase the transport of cargoes into wounds. Using these particles, we delivered two clinically used hemostatic agents, thrombin and TXA, and increased their ability to stop bleeding in multiple animal models compared to thrombin administered without propulsion.
Can increasing the transport of thrombin and tranexamic acid into wounds, increase their ability to prevent death in a swine model of non-compressible battlefield junctional hemorrhage? (Chapter 4)

In this study, we used a swine femoral artery model of junctional hemorrhage which is used by the United States Army to mimic bleeding scenarios encountered in combat. In this model, propelled particles loaded with thrombin and tranexamic acid on gauze (PTG) significantly improved survival compared to the same agents without propelled particles and compared to clinical standards for treating combat trauma-associated hemorrhage.

Can increasing the transport of thrombin into wounds increase its ability to bleeding in a sheep model of massive surgical hemorrhage? And is PTG potentially safe and effective at stopping bleeding, compared to clinical interventions, in two sheep models of endoscopic and open surgical bleeding? (Chapter 5)

In this study, PTG was superior to clinical standard (control) gauze at reducing bleeding in a model of endoscopic nasal surgery. PTG also achieved hemostasis in more cases than a gold-standard thrombin-containing surgical hemostatic agent in a carotid artery puncture model of catastrophic surgical hemorrhage. PTG did not cause any adverse events, as measured by histopathological examination of exposed tissues and biomarkers of thrombosis.

Can increasing the transport of TXA increase its ability to inhibit fibrinolysis in vitro and reduce bleeding in vivo? (Chapter 6)
In this study, we demonstrated that increasing hemostatic efficacy by increasing transport into wounds is not specific to thrombin and is applicable to other hemostatic agents, including TXA alone. Here, we showed that delivering TXA with self-propelling particles increased its ability to inhibit fibrinolysis \textit{ex vivo}, and to reduce bleeding in two animal models of hemorrhage compared to non-propelling TXA administered via other routes.
Chapter 3  Self-propelled particles transport through flowing blood into wounds, and deliver hemostatic cargo³

3.1 Introduction

Uncontrolled bleeding occurs in many clinical conditions, including surgical and dental procedures, severe nosebleeds, post-partum hemorrhage (PPH), trauma, and in patients with hemophilia.⁹⁶–⁹⁸ The primary concern during severe bleeding is controlling blood loss, although controlling secondary bleeding, infection, and tissue repair are also important. These concerns could be addressed by quickly delivering therapeutics such as coagulants, anti-fibrinolytics, antimicrobials, or growth factors to the damaged vasculature.⁷,⁶⁸ However, as discussed in Chapter 1, systemic delivery of these therapeutics via injections or local delivery via intravascular catheters is often impractical. Delivering agents topically is also limited by biophysical barriers; it is difficult to transport agents against blood flow, especially far enough upstream to reach the leaking vessels. This chapter tests the hypothesis that by formulating therapeutics into water-reactive particles that release gas to transport themselves, therapeutics can be delivered upstream through blood flow and directly address hemorrhaging.

As discussed in Chapter 2, many particle systems that exhibit active autonomous and collective movement have been developed for broad potential uses.\textsuperscript{70,71,74,75,88,90} Chemically-driven self-propelling particles, sometimes termed catalytic microengines, often rely on gas generation for propulsion, although other mechanisms, such as ultrasound or magnetically-driven swimmers, have been used.\textsuperscript{80,81} Several limitations have prevented these self-propelling particles from being utilized \textit{in vivo} to deliver therapeutics. These particles typically propel at velocities many orders of magnitude slower than blood flow, or rely on having fuels, often hydrogen peroxide, dispersed in the solution.\textsuperscript{70,71} The transport of particles through whole blood by catalytic microengines was recently reported to be unachievable, although non-catalytic, self-fueled particles have been developed that can transport through gastric fluids.\textsuperscript{88,89,91} To develop self-fueled particles suitable for transport through blood, we chose materials that react vigorously to produce gas in any aqueous solution. Calcium carbonate (CaCO\textsubscript{3}) is commonly used in antacid tablets and drug formulations, rapidly produces carbon dioxide (CO\textsubscript{2}) gas in acidic solutions, and forms porous microparticles that adsorb protein, making this substance ideal for self-fueled particles.\textsuperscript{99} In this chapter, we demonstrate that CaCO\textsubscript{3}–based microparticles actively transport through blood and can carry a functional protein cargo through wounds and into the vasculature. This is the first report of self-fueled particles being utilized \textit{in vivo} to transport through blood, and the findings demonstrate that propulsion is a viable mechanism for targeting the delivery and increasing the transport of therapeutics into wounds.
3.2 Methods

3.2.1 Sources of materials

Inorganic salts and buffers, including CaCO$_3$ particulates, citric acid and sodium citrate were purchased from Thermo Fisher Scientific (Waltham, MA). Sodium heparin, bovine thrombin, malic acid, tranexamic acid (TXA) poly-L-lysine (PLL), antifoam 289, $1H,1H,2H,2H$-Perfluoro-1-octanol (PFO) and silica gel were purchased from Sigma Aldrich (St. Louis, MO). FITC-dextran (4 kDa) and green-fluorescent polystyrene microspheres (1 $\mu$m diameter) were purchased from Polysciences, Inc. (Warrington, PA). Dark red-fluorescent, carboxylate-modified polystyrene nanoparticles (660/680, 0.2 $\mu$m diameter) were purchased from Life Technologies (Burlington, ON, Canada). Tween-20 was purchased from Amersco LLC (Solon, OH). Fluorogenic thrombin substrate (Boc-Val-Pro-Arg-MCA) was purchased from Peptide Institute, Inc. (Minoh-shi, Osaka, Japan). Human blood plasma was purchased from Affinity Biologicals, Inc. (Ancaster, ON, Canada). Corn trypsin inhibitor and human alpha-thrombin were purchased from Haematologic Technologies, Inc. (Essex Junction, VT). Fluorocarbon oil (Fluorinert FC-770) was purchased from 3M (London, ON, Canada). Poly(dimethylsiloxane) (PDMS) was purchased from Dow Corning (Midland, MI). BALB/c mice (18–25 g) were purchased from Charles River Laboratories (Wilmington, MA). C57Bl/6 mice (~30 g) were purchased from Jackson Laboratories (Bar Harbor, ME). CaCO$_3$ microparticles used in porcine experiments were purchased from American Elements (Los Angeles, CA). Kerlix™ (Covidien) gauzes was purchased from Stevens (Delta, BC, Canada).
3.2.2 Preparing propulsion reagents

To generate porous CaCO$_3$ microparticles, a solution of Na$_2$CO$_3$ (0.33 M in 50 mL of water) was quickly added to a rapidly stirring solution of CaCl$_2$ (0.33 M in 50 mL of water), based on methods in previous reports.$^{99,100}$ Those reports also describe alterations of the method for producing microparticles of different sizes, and with different encapsulation efficiencies when coprecipitating compounds. The precipitated CaCO$_3$ microparticles were collected by centrifugation at 3,000 g for 10 min and washed with de-ionized water. To make fluorescent microparticles loaded with FITC-dextran or dark red-fluorescent (660/680) polystyrene nanoparticles (200 nm), the fluorescent agent was added to the CaCl$_2$ solution prior to precipitation at 50 µM for FITC-dextran (4 kDa) or 0.2 mg/ml for nanoparticles. Particle size and morphology were measured using microscopy. To generate TXA-HCl, concentrated HCl was added to 0.5 M tranexamic acid (TXA) until the pH reached 4.3, and then the solid TXA-HCl was collected following lyophilization. To adsorb thrombin onto the particles, particles were suspended at 10% w/v in a solution of bovine thrombin (447 µM) buffered with 10 mM HEPES. The suspension was incubated at 4°C for 1 hr. Particles were purified by centrifugation at 10,000 g for 5 min and then lyophilized. To obtain mixtures capable of propelling, the dry solid CaCO$_3$ particles were then added to an equal molar ratio dry solid protonated tranexamic acid (TXA-HCl).

3.2.3 Measuring velocities of CaCO$_3$ particulates and porous CaCO$_3$ microparticles

We tested both the velocities of porous CaCO$_3$ microparticles and of CaCO$_3$ particulates as obtained from the manufacturer. Particle velocities were measured following injection into 1 M
citric acid, 16 mm below the surface. Particles were imaged at 35 ms time intervals. Particulates and porous microparticles were separately mixed with TXA-HCl at a 1:1 molar ratio, yielding mixtures that propelled when injected into solutions with neutral pH. To measure the velocities of the particles without hindrances of cells or proteins, propelling mixture was injected similarly into phosphate-buffered saline containing 50 mM sodium citrate (PBS-citrate). To measure the velocity of particles in the presence of red blood cells and blood proteins, propelling particle mixture was injected into heparinized and citrated whole blood, or a solution of washed and concentrated red blood cells (RBCs) in PBS-citrate. Citrate was used to prevent clotting of blood when Ca\(^{2+}\) was added, and it also prevented precipitation of calcium phosphate. The surface of the blood or buffer was imaged, and the time at which particles were observed at the surface was recorded to calculate the average upward velocity of the mixture. To measure if CaCO\(_3\) and TXA-HCl mixture could propel a larger hull, a steel hull was made from a 2 mm piece of an 18-gauge steel needle that was closed on one end. Approximately 1 mg of the CaCO\(_3\)+TXA-HCl mixture was loaded into the hull and it was submerged into an aqueous solution containing 1.5 M citric acid, 33% glycerol (v/v) and bovine serum albumin (16.4 mM BSA).

### 3.2.4 Measuring propulsion of CaCO\(_3\) microparticles against flow using microfluidics

An *in vitro* microfluidic system was employed to assess the particles’ ability to propel through flowing water. Water containing 0.1% Tween-20 was flowed at velocities between 0.06 and 5.9 mm/s using a syringe pump. The solution was fed through a microfluidic device made of PDMS, which contained a “Y-shaped” junction. At the ends of the junction, polytetrafluoroethylene
(PTFE) tubing connected the device to glass capillaries (10 cm long, I.D. of 0.8 mm). The propelled particle mixture was applied at the end of one of the capillaries and the transit and accumulation of particles and bubbles was imaged over the length of the capillary. For the data shown in Figure 3.5E, the cross-sectional area of particles and bubbles was recorded over 30 sec in the region 4 cm to 7 cm upstream of the site of particle application.

To determine the ability of particles to propel against flowing whole blood, citrated whole blood was flowed at velocities between 0 and 1.8 mm/s through a PDMS microfluidic channel positioned at 0°, 40°, and 90°. The channel was parallel to the direction of gravity when at 90°. Blood was flowed downward while the movement of particles upstream was measured. Particles were injected into the middle of the channel via a catheter, and the movement of particles and bubbles was imaged by optical microscopy. In experiments testing propulsion in horizontal devices, steps were taken to minimize any error in leveling. The microscope stage was leveled using a spirit level. The microfluidic devices were flat, as the silicon wafer templates were flat. Glass capillaries, used in the clotting experiments, were flat. To further minimize systematic errors in levelling and unintended irregularities in device construction, devices were rotated 180 degrees in a plane normal to gravity between measurements. Velocities and displacements of particles were quantified in both directions and these measurements were averaged. The maximum distance particles travelled against flow and the fraction of particles that travelled against flow were measured. The microfluidic channel extended 6 mm past the injection site, so 6 mm was the maximum distance that particles were monitored in this experiment.
3.2.5 Quantifying the amount of thrombin adsorbed to particles

To determine the total thrombin content adsorbed to porous CaCO₃ microparticles, dried CaCO₃-thrombin particles were first resuspended at 10% (w/v) in 10 mM HEPES. Both the total amount of thrombin in the mixture and the amount of thrombin strongly adsorbed to the particles were determined. To determine the amount of thrombin adsorbed to the interior of the porous microparticles, particles were washed with HBS at 10% CaCO₃ (w/v) and isolated by centrifugation (5 min, 10,000 g). Both washed and unwashed particles were solubilized in 100 mM HCl (0.5% CaCO₃ w/v). The HCl-solution was then diluted to 0.025% CaCO₃ in HBS containing 25 µM of a fluorogenic substrate for thrombin (Boc-Val-Pro-Arg-MCA). Standards with known thrombin concentrations were prepared in an identical fashion to the unknown samples and contained the equivalent buffer and quantities of dissolved CaCO₃ and HCl. Immediately after the fluorogenic substrate was added to the thrombin solution the fluorescence intensity at 460 nm was measured for 30 min using a Tecan M200 Infinite plate reader (Tecan Group Ltd., Maennedorf, Switzerland) and compared to a standard curve generated from known concentrations of thrombin.

3.2.6 Measuring flow and occlusion times in a microfluidic model of bleeding

To test if the propelled thrombin particles could clot flowing plasma, particles were applied to the bottom of a glass capillary tube with plasma flowing between 0.006 and 3.4 mm/s to mimic blood pooling in wounds or flowing through capillaries and low-pressure vessels (Figure 3.7A). A microfluidic system was used to control the flow of plasma and detect occlusion of flow through the capillary tube (Figure 3.8). Citrated human blood plasma was re-calcified by adding calcium-
rich saline (40 mM CaCl₂, 90 mM NaCl) to plasma in a volumetric ratio of 1:3. Re-calcified blood plasma was flowed through glass capillaries (10 cm long, I.D. of 0.8 mm) coated with poly-L-lysine (PLL) and the propelling particles (20 mg) were applied to the end of one of the capillaries. The capillaries were coated with 0.01% PLL for 1 hr and dried for 1 hr at 60°C, as PLL enhanced adhesion of the clot to the capillary and increase background occlusion times. Plasma was held in plastic syringe reservoirs and flowed into the capillaries via polyethylene tubing. Particles (20 mg) containing combinations of CaCO₃, thrombin, and TXA-HCl were applied while plasma was flowing through. In all cases, “propelled thrombin” and “nonpropelled thrombin” treatments contained equal doses of thrombin and particles. A microfluidic system loaded with an oil was used upstream of the syringes of plasma to control the flow rates though the capillaries and detect when the capillaries became occluded with clotted blood plasma. The oil contained 10% PFO as a surfactant. The movement of tracer particles was monitored with optical microscopy to determine when occlusion and clotting occurred. Times of clot initiation between groups were compared using a Mann-Whitney U test.

3.2.7 Analyzing bleeding times in a mouse model of amputation and hemorrhage

All procedures involving animals were approved by the University of British Columbia Animal Care Committee and performed in accordance with the guidelines established by the Canadian Council on Animal Care. BALB/c mice weighing 20-25 g were anaesthetized via isoflurane inhalation. Tails were transected 8 mm from the tip followed by no treatment or application of either non-propelling thrombin or propelled thrombin particles for 30 sec. Doses were
approximately $1.8 \times 10^{-5}$ mg/g bodyweight of thrombin and 0.5 mg/g each of CaCO$_3$ and TXA, which equates to ~1,000,000 particles. Bleeding was recorded over a 10 min observation period while the tails were immersed in a citrated saline. Since some mice did not stop bleeding during the 10 min observation, total time clotted was plotted for each treatment group. For example, each mouse that did not stop bleeding had a total time clotted of zero. To determine statistical significance of bleeding time, total times clotted were compared by Mann-Whitney U test.

### 3.2.8 Quantifying blood loss in a mouse model of liver puncture and hemorrhage

Livers were accessed via a transverse incision approximately 3 cm in length. Two lacerations, each 2 mm-long and 2 mm-deep, were made to each liver using a 2 mm ophthalmic knife. Blood loss was quantified from each laceration independently. Blood was collected on pre-weighed filter papers immediately following injury until bleeding stopped. Filter papers were approximately 2 cm x 2 cm and were arranged to line the site of puncture prior to incision. Each laceration bled for 30 sec before either 2-3 mg of the CaCO$_3$ mixture or 20 µl of freshly reconstituted recombinant thrombin solution were applied. Doses were approximately $1.8 \times 10^{-6}$ mg/g bodyweight of thrombin and 0.05 mg/g each of CaCO$_3$ and TXA, which equates to ~100,000 particles. The particles were applied as a powder in this liver model of bleeding and no pressure was applied. Particles moved freely in any direction through blood. Blood losses were compared by the Mann-Whitney U test. To confirm that changes in filter paper mass correlated with the volumes of blood soaked, known volumes of fresh blood were soaked onto pre-weighed filter papers.
To measure the delivery of propelled CaCO$_3$ into the sites of liver injury, the livers of a separate cohort of mice were accessed and injured similarly. Immediately following injury, 2-3 mg of propelled or non-propelled CaCO$_3$ microparticles (~100,000 particles) containing the dark red-fluorescent nanoparticles and thrombin were applied. Once bleeding stopped, livers were collected. Histological sections were viewed with an epi-fluorescence microscope, and the amount of fluorescent nanoparticles was quantified using ImageJ software. Fluorescent intensities were compared to a standard containing a known mass of the particles to calculate the mass of CaCO$_3$ delivered to each wound.

3.2.9 Measuring local toxicity of propelled thrombin in a mouse model of hemorrhage

To test single-dose toxicity of propelled thrombin, mice received tail clips followed by administration of propelling thrombin or no treatment as described above. This model was chosen to reflect toxicity following intralesional administration, which would likely be propelled thrombin’s route of administration in a clinical scenario. Mice were monitored for 3 days for any signs of pain, thrombosis or tail necrosis. Tail tissues were collected, sectioned and stained using hematoxylin and eosin (H&E) and Masson’s trichrome. The degree of infiltration of inflammatory cells was qualitatively assessed to measure inflammation caused by propelled thrombin compared to control.
3.2.10 Assessing toxicity of IV-injected CaCO$_3$ microparticles

A group of mice were given intravenous injections of either suspensions of CaCO$_3$ microparticles, microparticles with TXA-HCl, or saline (control). Animals were monitored for twenty-four hours for signs of pain or distress, then blood was collected and concentrations of alanine aminotransferase (ALT) or aspartate aminotransferase (AST) were measured. ALT and AST are markers of liver toxicity. ALT and AST concentrations were compared by Mann-Whitney U test and showed no statistical significance between groups. A separate cohort of mice receiving comparable, IV doses of microparticles and TXA-HCl (25 mg/kg of each agent, ~50,000 particles) were observed for 7 d post-injection observed for signs of pain or distress.

3.2.11 A pilot study in a porcine model of lethal, traumatic hemorrhage

To prepare gauze loaded with CaCO$_3$ and adsorbed thrombin, 8 g of CaCO$_3$ microparticles were suspended in 8 mL glycine-buffered saline (GBS) (40 mM glycine, 171 mM NaCl, pH 7.2) containing 0.06 mg/ml human thrombin (specific activity ~4000 U/mg) and incubated on ice for 20 minutes. Suspensions were diluted with an additional 8 mL GBS and poured onto 3 g strips of Kerlix™ gauze and lyophilized. To prepare propelled thrombin gauze (PTG) or non-propelled thrombin gauze (NPTG), these dressings were combined with 3 g Kerlix™ gauze containing 4.7 g of either TXA-HCl or TXA, respectively. Doses were approximately 267 mg/kg CaCO$_3$, 157 mg/kg TXA and $1.67 \times 10^{-2}$ mg/kg (67 IU/kg) human thrombin. The PTG and NPTG were identical formulations, except that PTG contained TXA-HCl and produced CO$_2$, while NPTG contained TXA and did not produce CO$_2$. Doses of CaCO$_3$ and TXA reflected the maximum
amount of material that could be loaded on the gauze. The dose of thrombin was similar to the therapeutic doses of commercially-available thrombin products. Gauzes were trimmed to a standard length and width using a gauze template, which was previously found to tightly pack the bleeding wound cavity.

A modified porcine bleeding model developed by the United States Military for testing of topical hemostatic agents was used for these experiments. After induction of anesthesia with inhaled isoflurane, the left femoral artery and vein were cannulated for arterial blood pressure monitoring, and administration of resuscitation fluids. The injury site was prepped in the right hind limb by a 4 cm skin incision to access the femoral artery, which was exposed while keeping adductor muscles intact. The exposed artery was bathed in 2% lidocaine to dilate and prevent vasospasm after injury. The artery was then clamped proximally and distally and a 5 mm biopsy punch was used to create an arteriotomy in the anterior wall of the artery. The clamps were removed to allow for 30 sec of free bleeding. Bleeding wounds were then packed with the selected gauze to tightly fill the wound cavity. There was no compression of the wound, no repacking was performed, and CO₂ freely escaped the wounds. At 3.5 min after onset of hemorrhage, all animals received one bolus of hydroxyethylstarch solution (15 ml/kg) (Hextend™, Hospira Inc. Lake Forest, IL) infused over 15 min. Lactated Ringers electrolyte solution (Hospira Inc.) was then infused as needed at 3 ml/kg/min to achieve or maintain MAP ≥60 mmHg to a maximum of 100 ml/kg. Animals were observed for 3 hr and blood was collected using pre-weighed gauze sponges. Blood loss and mean arterial pressures (MAP) were measured serially. Animals were euthanized under anesthesia if
they survived to 3 hr, or if the arterial pressure waveform was lost, indicating loss of cardiac activity.

3.3 Results and Discussion

3.3.1 CaCO$_3$ particles propel in water

When CaCO$_3$ microparticles were mixed with a solid organic acid, they rapidly traveled through aqueous solutions (Table 3.1). CaCO$_3$ particles were mixed in a 1:1 molar ratio with an organic acid, protonated tranexamic acid (TXA-HCl), and injected into a buffered saline solution or whole blood (Figure 3.1A,B). The particles reacted vigorously, rising buoyantly and spreading laterally both below and at the surface within seconds (Figure 3.1C,D). The transport of particles, referred to here as “propulsion”, occurred from a combination of particles rising buoyantly, propelling laterally, and the large convection generated by the rapid production of gas bubbles.$^{103}$

Propulsion also occurred when other solid organic acids, such as citric acid and malic acid, were substituted for TXA-HCl (Figure 3.2, Figure 3.3, Table 3.2). When CaCO$_3$ particles were mixed with neutral tranexamic acid (TXA), which was not able to protonate carbonate, they did not propel. Similar to other types of gas-releasing fuels used in propelled particles, this CO$_2$–releasing mixture could be incorporated into a larger object and propel it.$^{70,71,104}$ A 10 mg steel hull containing the fuel propelled at 0.2 mm/s despite having 10-times greater mass than the fuel (Figure 3.1E). When larger particles were formed, such as calcium carbonate aggregates or when using sodium carbonate, these particles were capable of persistent movement with velocities of 3
mm/s, potentially due to their increased inertia and increased potential for anisotropy of the gas generation reaction across their surfaces.
Table 3.1 Velocities of CaCO$_3$ particulates and porous microparticles through blood.

Velocities of CaCO$_3$ particulates and porous microparticles mixed with tranexamic acid propelling in PBS-citrate, citrated whole blood or washed RBCs, due to combined contributions of buoyancy and convection caused by CO$_2$ generation. $n = 6-20$. Error bars indicate standard deviation.

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<th>CaCO$_3$ particulates</th>
<th>Porous CaCO$_3$ microparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBC concentrate, PBS-citrate</td>
<td>PBS-citrate</td>
</tr>
<tr>
<td>Velocity (mm/s)</td>
<td>3.84 ± 0.46</td>
<td>6.32 ± 0.86</td>
</tr>
</tbody>
</table>
Figure 3.1 CaCO₃ particles combined with an organic acid travel through aqueous solutions.

(A) Schematic showing CaCO₃ particles releasing CO₂ and propelling themselves and their cargo when placed in water. (B) Schematic showing how particle movement was measured in buffer and whole blood. (C and D) Images of particles appearing at the surface of a buffered solution (C) and whole blood (D). Scale bars, 2 mm. (E) Schematic and images of a steel hull propelled by CaCO₃ and TXA-HCl. Scale bar, 2 mm. (F) Images of immobilized CaCO₃ particles containing a fluorescently tagged cargo, FITC-dextran. Scale bars, 30 μm (green fluorescent particles) and 0.5 μm (scanning electron micrographs). (G) Schematic showing a mouse tail being amputated and treated with propelling CaCO₃ particles. Red rectangle denotes the field of view in (H). (H) Histological section of a treated tail showing particles located 6 mm inside the tail, blood vessels,
and caudal vertebrae (CV). Fluorescence staining shows actin (red), nuclei (blue), and CaCO3 particles (green). Scale bar, 200 μm.

**Figure 3.2 Imaging CaCO₃ particulates in acidic solution.**

CaCO₃ particulates transporting in all directions in 1 M citric acid solution. Scale bar is 1 mm.
Table 3.2 Upward and downward velocities of CaCO$_3$ particulates and porous microparticles.

Average particle size, bubble volume, and velocities of particles propelling in 1 M citric acid.

<table>
<thead>
<tr>
<th></th>
<th>Bubble volume (mm$^3$)</th>
<th>Particle diam. (mm)</th>
<th>$v_y$ (mm/s)</th>
<th>$v_x$ (mm/s)</th>
<th>$v_T$ (mm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upward</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCO$_3$ particulates</td>
<td>0.21 ± 0.18</td>
<td>0.18 ± 0.10</td>
<td>71.6 ± 22.1</td>
<td>3.6 ± 3.5</td>
<td>71.8 ± 22.1</td>
</tr>
<tr>
<td>Porous CaCO$_3$ microparticles</td>
<td>0.26 ± 0.26</td>
<td>0.45 ± 0.17</td>
<td>77.6 ± 21.5</td>
<td>3.4 ± 3.3</td>
<td>77.7 ± 21.5</td>
</tr>
<tr>
<td><strong>Downward</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCO$_3$ particulates</td>
<td>0.03 ± 0.02</td>
<td>0.30 ± 0.07</td>
<td>4.1 ± 1.1</td>
<td>0.8 ± 0.4</td>
<td>4.2 ± 1.1</td>
</tr>
<tr>
<td>Porous CaCO$_3$ microparticles</td>
<td>0.04 ± 0.07</td>
<td>0.42 ± 0.09</td>
<td>10.3 ± 4.3</td>
<td>0.7 ± 0.5</td>
<td>10.3 ± 4.3</td>
</tr>
</tbody>
</table>
Figure 3.3 Dense and aggregated carbonate particles propel horizontally but not upward.

In this image a large $\text{Na}_2\text{CO}_3$ particulate is propelling at 3 mm/s along the bottom of a Petri dish filled with 0.4 M malic acid in water.
3.3.2 CaCO$_3$ particles propel through blood and into wounds

To initially test if porous microparticles could propel and carry fluorescent cargos through blood in vivo, they were applied to a site of bleeding in a mouse model of hemorrhage. A portion of the tail of a mouse was amputated and propelling microparticles containing fluorescent nanoparticles or FITC-dextran were applied. Histological sections of the intact tail showed fluorescent particles up to 1 cm deep in the vasculature, confirming transport and delivery through blood in vivo (Figure 3.1G,H). The location of particles inside blood vessels, and their co-localization with red blood cells demonstrates that propelled particles transported through blood vessels and past the surface of the tail wound (Figure 3.4). When non-propelling CaCO$_3$ microparticles were tested, particles were not found past the surface of the tail. Due to the multiple mechanisms of propulsion and the complex nature of blood flow in wounds it is not expected that particles maintained their velocity in a single direction. Although the propulsion of catalytic microengines in blood was previously considered unachievable,$^{90,105}$ our results show that a simple method of generating gas from particles can achieve this.
Figure 3.4 Histological analysis of mouse tails treated with propelling CaCO₃ microparticles.

(A-C) Additional histological sections of three tails treated with self-propelling particles. Images were taken 6 mm (panel A), 4 mm (panel B) and 10 mm (panel C) from the site of injury. Fluorescence staining indicates smooth muscle actin (red), nuclei (blue), and fluorescent polystyrene nanoparticles (panels A and B) or FITC-dextran (panel C) in CaCO₃ (green). (D,E) Histological sections of tails treated with propelled (panel D) or non-propelled (panel E), thrombin-loaded, fluorescently-labeled particles. Corresponding sections are unstained (left of each pair) or stained with H&E (right of each pair). Black or white arrowheads denote particles
localized within blood vessels. Scale bars are 50 µm in (A), 5 µm in (B) and (C), and 200 µm in (D) and (E).

3.3.3 Velocities of CaCO$_3$ particles propelling through still water

To understand the range of potential applications for propelling CaCO$_3$ particles, the velocities of particles in stagnant and flowing solutions were measured (Fig. 2). The CaCO$_3$ particles were injected at the bottom of a container containing aqueous organic acid and particle trajectories were imaged (Fig. 2A). Particles usually reacted as aggregates with a diameter of 0.4 ± 0.2 mm (mean ± standard deviation), breaking apart into individual particles as they traveled (Figure 3.2, Table 3.1, Table 3.2). Particles had upward velocities of 71 ± 23 mm/s and lateral velocities of 3.6 ± 3.5 mm/s, among the fastest reported for self-propelling particles.$^{70,71}$ This upward velocity of particles mimics the gravitactic movement that some microorganisms use to propel upwards against gravity.$^{104}$ The particle velocity increased with bubble volume (Figure 3.5B). Images showed that CaCO$_3$ particles were attached to the bottom of CO$_2$ bubbles and were buoyantly carried upward as they reacted (Figure 3.5C). When particles were intentionally clumped together into larger, heavier aggregates, they sank and propelled laterally with similar horizontal velocities of ~3 mm/s (Figure 3.3). These results may imply that other gas-releasing self-propelled particles of low-density may rise buoyantly if the reaction rate is high and large bubbles are produced.
Figure 3.5 CaCO$_3$ particles travel upstream and at high velocities through stagnant and flowing solutions.

(A) Images of CaCO$_3$ particles transporting upward through a stagnant acidic solution. (B) Particle velocity increased as a function of the volume of attached bubbles. The red line denotes the velocities of bubbles predicted by a model equating buoyant and drag forces. The black solid line denotes a one-half power regression of the data. Dashed lines denote the 99% confidence band (black) and 90% prediction band (gray) of the regression. (C) Images of particles carried upward by CO$_2$ bubbles. (D) Schematic showing how propulsion of particles with TXA-HCl through flowing water was measured. (E) The particles traveled against flow velocities of up to 3 mm/s. (Inset) Fraction of particles that accumulated for each flow velocity at 20 s. $n = 1$. (F) Schematic showing how propulsion of particles through a channel of flowing whole blood was measured at three angles. (G) Maximum distances that particles traveled upstream through flowing and stagnant blood at various angles. $n = 2$. Scale bars, 2 mm.
3.3.4 A mathematical approximation of the forces responsible for propulsion

Particles appeared to become attached to CO$_2$ bubbles, which may cause their large upwards velocities. To understand the dynamics of particle propulsion and their attached bubbles, the forces that are expected to cause the upward transport were considered, i.e. the buoyant force of CO$_2$ bubbles and the drag force opposing motion. Bubbles were modeled as rigid oblate spheroids and were assumed to be at terminal velocity at the time that bubble radius and velocity were measured. In this model, we hypothesized that the predominant driving force to be the buoyant force acting upon the attached bubbles. The equation describing the buoyant force is given in Eq. 3.1, where $\rho$ is the density of the aqueous solution (considering the density of CO$_2$ to be zero), $g$ is the acceleration due to gravity, and $V_{\text{bubble}}$ is the volume of the CO$_2$ bubbles.

\[
E_{q. 3.1} \quad F_{\text{buoy}} = \rho g V_{\text{bubble}}
\]

As bubbles reached terminal velocity, this force was balanced by an opposing drag force. The drag force ($F_{\text{drag}}$) is given in Eq. 3.2, where $A$ is the frontal area of the bubble, $v$ is the upward velocity, and $C_D$ is the coefficient of drag which for an oblate spheroid at high Reynolds numbers is considered a constant value of 0.6.

\[
E_{q. 3.2} \quad F_{\text{drag}} = \frac{1}{2} \rho A C_D v^2
\]

Assuming the bubbles reach terminal velocity, the forces become balanced. Equating the two forces gives the relationship in Eq. 3.3 relating the velocity of a particle to the radius of its attached bubble.


\[ \text{Eq. 3.3} \quad v = \sqrt{\frac{4gr}{3C_D}} \]

Despite simplifying assumptions and deriving them from first principles, this relationship predicts the particle velocities of bubbles of measured radii within a factor of two (Figure 3.5B). Here, we examine these assumptions and qualitatively estimate their contribution to discrepancies between predicted and measured bubble velocities.

First, this model assumes that bubbles are rigid nondeformable spheroids, which is likely untrue at these velocities. Air bubbles are known to behave as spheres only up to a critical Reynolds number, which for solutions no containing surfactants is approximately 40.\textsuperscript{106} The bubbles observed in this experiment had radii ranging from 0.1 mm to 0.6 mm, and had measured velocities of 30 mm/s to 135 mm/s. Reynolds number for a sphere moving relative to a fluid is defined by Eq. 3.4, where \( \nu \) is the kinematic viscosity of the medium (for water \( \nu \approx 10^{-6} \text{ m}^2/\text{s} \)).

\[ \text{Eq. 3.4} \quad Re = \frac{2\nu r}{\nu} \]

Using this equation, the Reynolds numbers for these bubbles range from 10 to 150. At the high velocities measured, bubbles are likely deforming which could vary drag coefficients by a factor of two (0.4 to 1.1). Bubbles also likely coalesced to minimize their surface energies, which would increase variability in the measured velocities since it is not known whether velocities were quantified soon relative to a coalescence event. This increase in variability of bubble velocities for larger, potentially coalesced bubbles, is seen in the measured data.
Additionally, while coefficients of drag likely vary due to bubble deformation, it is also known that coefficients of drag for rigid spheres vary with Reynolds numbers within the ranges measured. For example, it has been numerically modeled that for spheres in flow with Reynolds numbers between 20 and 260 are given by Eq. 3.5 where Re is the Reynolds number for a particular bubble and its attached particles.

\[ C_D = \frac{24}{Re} (1 + 0.1935 Re^{0.6305}) \]

Using Eq. 3.4 to calculate Reynolds numbers for each bubble and inputting those values into Eq. 3.5 gives drag coefficients ranging from 1, for the largest bubbles, to 4, for the smallest bubbles. Because our model assumes \( C_D \) for each bubble is 0.6, this model probably overestimates velocities for the smallest bubbles observed, and this difference is reflected in the data. Similarly, this model would still overestimate velocities of bigger faster bubbles but, as discussed above, larger variation is seen in velocities in this range, potentially due to bubble coalescence. To account for the variability of Reynolds numbers in this range, Eq. 3.5 could be substituted into Eq. 3.3 giving the following implicit relation:

\[ v = \sqrt{\frac{4gr}{3} \left( \frac{12v}{vr} + 3.595 \left( \frac{v}{vr} \right)^{0.3695} \right)} \]

A more thorough analysis of the system could utilize solutions of Eq. 3.6.

Another potential refinement of this model would be to include surfactants in the aqueous media. Surfactants are present in many biological media, including blood, so their inclusion would increase the biological relevance of this model. Surfactants decrease surface tension, which stabilizes small bubbles and prevents their coalescence. Surfactants can also increase the critical
Reynolds number under which bubbles behave as spheres, mentioned above, to approximately 130.\textsuperscript{108} For both these reasons, including surfactants when experimentally measuring and when mathematically predicting bubble velocities could decrease variability due to bubble coalescence, and could ensure small bubble velocities and Reynolds numbers to increase the probability that bubbles behave as spheres in this system.

Within the conditions tested, no correlation was found between particle size and the velocity of buoyant rise, although it is expected that the mass of the particle may influence transport of particles under other conditions. This simplified model only accounts for the buoyant rise we observed in a large volume of stagnant, aqueous solution; it considers bubbles as isolated objects and does not account of bubble interactions or entrainment. It also does not account for the transport of particles due to local convection or lateral propulsion from bubble generation. The model does not describe many important aspects of the behavior of particles in flowing solutions or wounds, such as turbulent or pulsating flow, or in heterogeneous solutions such as blood.\textsuperscript{109} This model also does not consider how the formation of bubbles can generate surface flows or affect the progression of blood coagulation, such as how the blood-gas interfaces can contribute to the formation of thrombin films which can enhance clot stability.\textsuperscript{110}

\subsection*{3.3.5 Propulsion of particles through microfluidic flow systems}

To determine if the particles were capable of transport upstream against flow, CaCO\textsubscript{3} and TXA-HCl were mixed together and applied to an aqueous solution flowing between 0.06 and 5.9 mm/s through a glass capillary (Figure 3.5D). When particles were applied at the bottom of the tube
they travelled upward, and the fraction of the tube occupied by particles and accompanying bubbles was measured (Figure 3.5E). The propelling particles successfully opposed flow velocities up to 3.0 mm/s. To determine the flow velocity of blood through which particles could travel, they were injected into a stream of whole blood in a microfluidic channel (Figure 3.5F,G). The maximum distance that particles traveled through flowing and stagnant blood was measured. In stagnant blood oriented perpendicular to gravity, particles travelled at 0.54 mm/s to a maximum distance of 3.9 mm. Through blood flowing at 0.4 mm/s, a large portion of particles consistently traveled upstream at various angles relative to gravity. When flow was 1.8 mm/s and parallel to the direction of gravity, 13% of particles propelled upstream to maximum distances over 6 mm. Physiological flow velocities are approximately 1 mm/s in capillaries, and up to 10-100 mm/s in larger arteries, but are variable and can be much lower in wounds when bleeding originates from these vessels. Based on these values, CaCO₃ particles appear capable of propelling through flow velocities present in wounds and small vessels, but likely not against blood flowing through large arteries. This may help contain the particles near wound sites and prevent them from entering systemic circulation.

### 3.3.6 Thrombin can be loaded onto particles

The ability to transport molecular cargos through blood could address an unmet need for materials that halt severe bleeding effectively. Minimizing bleeding can sometimes be achieved with anti-fibrinolytics, such as tranexamic acid, or with intravascular catheters that embolize vasculature feeding damaged vessels. However, when vessels cannot be targeted with catheters, or when
bleeding must be stopped immediately, more traditional approaches are attempted. The wound can be packed with dressings or powders containing gelatin, modified cellulose, or zeolites. Intraoperatively, thrombin can be applied topically, in solution or with gelatin. However, these methods are not highly effective during severe bleeding or when external blood loss originates inside a cavity.

To create propelling particles that clot blood, thrombin (a serine protease that activates the coagulation system and directly cleaves fibrinogen), was adsorbed onto the porous particles. Particles were loaded with 0.9 μmol of active thrombin per gram of CaCO₃, of which approximately 0.6 μmol was loosely bound and could be removed upon washing with low stringency buffer (Figure 3.6A). These thrombin-loaded particles rapidly clotted blood, when added alone or combined with either TXA-HCl ("propelled thrombin") or TXA ("non-propelled thrombin"), and the activity of thrombin in propelled and non-propelled formulations was similar (Figure 3.6B,C,D).
Figure 3.6 Thrombin can be immobilized on CaCO₃ particles and clot stagnant plasma.

(A) Quantifying the amount of thrombin adsorbed inside and outside of particles. (B) Quantifying clot initiation times in stagnant blood plasma. Either ~2.5 mg of particles and tranexamic acid were applied or 2.5 μl of thrombin solution (447 μM in 10 mM HEPES buffer, equal in thrombin activity to other groups) to 200 μL of recalcified plasma. The plasma contained 5 μg/ml of corn trypsin inhibitor (CTI), to decrease nonspecific activation of clotting by contact activation of factor XIIa, and antifoam 289, to reduce foaming which sometimes formed blockages in the vessel. n = 3-6. (C) Quantifying clot initiation times in stagnant blood plasma containing various concentrations of propelled or nonpropelled thrombin. n=1. (D) Quantifying clot initiation times in stagnant blood plasma containing 0.1 μg/ml of particles. n=3. (E) Clotting of flowing human plasma ex vivo by particles combined with thrombin. Plasma was flowed at 0.25 mm/s in a vertical orientation. n=3.*P<0.05 Error bars indicate S.E.M.
3.3.7 Thrombin-loaded particles can clot flowing plasma in vitro

To test if the propelled thrombin particles could clot flowing plasma, particles were applied to the bottom of a glass capillary tube with plasma flowing between 0.006 and 3.4 mm/s (Fig. 3A). A microfluidic system was used to control the flow of plasma and detect occlusion of flow through the capillary tube (Figure 3.8). The propelled thrombin occluded flow at velocities approximately 10 times higher than non-propelled thrombin. At a physiologically-relevant flow velocity of 3 mm/s, the particles propelled upwards against flow, accumulated high within the tubing, and initiated clotting (Figure 3.7C). Non-propelled thrombin only occluded at very slow velocities, up to 0.06 mm/s, and showed no difference from controls at 0.6 mm/s, where the background occlusion times were 30 min. Without propulsion, thrombin was able to form a clot at the exit of the tubing, but it was easily pushed out and unable to permanently occlude flow. Thrombin clotted flowing plasma four times faster when it was adsorbed onto propelled particles compared to when it was non-absorbed and mechanically mixed with the particles, which when wetted yields soluble thrombin was applied alongside particles without any adsorbed thrombin, however both gas-generating formulations clotted faster than thrombin adsorbed on non-propelled particles (Figure 3.6E). In a separate experiment, when plasma was flowed perpendicular to gravity at 0.125 mm/s, propulsion still significantly decreased time to occlusion, which occurred within 4 min with propelled thrombin, but took 14 min with non-propelled thrombin (Figure 3.7B,D). Together, these results show that thrombin initiates clotting faster when propelled because it is delivered upstream both by direct transport (as cargo loaded onto the propelled particles) and likely by the convective effects of gas generation.
Figure 3.7 Propelled thrombin clots flowing blood plasma and halts severe hemorrhage.

(A and B) Schematic of clotting and occlusion of flowing blood plasma ex vivo in vertical (A) and horizontal (B) orientations. (C and D) Clotting of flowing human plasma ex vivo by thrombin-loaded particles at various flow rates in a vertical orientation (C) and at 0.13 mm/s in a horizontal orientation (D). n = 3. *P < 0.05. Error bars indicate SEM.
Figure 3.8 Full apparatus used to detect occlusion of flowing blood plasma.

Images show tracer particles flowing through the junction of microfluidic channels before and after the capillary was occluded with clotted blood plasma. At 200 sec, flow is occluded in the clotted channel and beads are diverted to the top (control) channel. Scale bar is 350 μm.

3.3.8 Thrombin-loaded particles stop bleeding in mouse tail clips

The self-propelling particles were then tested for the ability to halt hemorrhaging in two murine models. First, in a model of amputation, mouse tails were transected 8 mm from the tip, causing severe hemorrhage. The propelling or non-propelling thrombin-loaded particles were applied for 30 sec at the site of amputation and bleeding was monitored for 10 min in warm saline. Control mice were not treated after the amputation. The propelled thrombin significantly decreased the bleeding time compared to both other groups (Figure 3.9A). In mice that received the propelled thrombin, 7 out of 9 (78%) stopped bleeding during observation. In contrast, only 3 mice out of 9
(33%) and 2 out of 8 (25%) stopped bleeding in the groups receiving non-propelled thrombin and no treatment, respectively.

### 3.3.9 Thrombin-loaded particles stop bleeding in mouse liver punctures

In spatially unconfined systems, the data presented shows that these particles typically transport upward due to buoyant rise. If transport of particles in wounds only occurred upward, this could potentially limit the use of particles to very specific clinical scenarios where bleeding is in the direction of gravity. To test if propelled thrombin could be efficacious in a wide variety of wound geometries, including wounds with unfavorable orientation relative to gravity where transport by convection and lateral propulsion would play a role, a second murine model and a porcine model were used. In the second murine model of hemorrhage, particles were applied to punctured livers. Mice were laid supine, incisions were made to the median lobe of each liver and bleeding occurred opposite the direction of gravity (Figure 3.9B). Wounds received propelled thrombin, a clinically used solution of recombinant thrombin, propelled particles without thrombin, or no treatment. Propelled thrombin significantly decreased blood loss compared to all other groups, reducing mean blood loss by approximately four-fold (Figure 3.9C).
Figure 3.9 Propelled thrombin is delivered deep into wounds and halts hemorrhage in vivo.

(A) Bleeding times in vivo after the tails of mice were amputated. (B) Schematic showing a mouse liver punctured and treated with propelled thrombin. (C) Volume of blood loss in a separate cohort of mice after their livers were punctured and treated. (D and E) Histological sections of livers treated with propelled thrombin (D) or nonpropelled thrombin (E). Fluorescence staining shows actin (red), nuclei (blue), and CaCO₃ particles (green). Scale bar is 200 μm. (F) Mass of CaCO₃ delivered to sites of liver puncture. (G) Schematic showing a pig’s punctured femoral artery being
treated with gauze impregnated with propelled thrombin. (H) Survival of pigs after treatment. \( n = 5 \). *\( P < 0.05 \), **\( P < 0.01 \). Error bars indicate SEM.

### 3.3.10 Propulsion increases penetration into mouse livers

To verify that propulsion increased the transport of thrombin into the wound sites, self-propelling CaCO\(_3\) microparticles were loaded with fluorescent nanoparticles, and administered in the same liver puncture model of bleeding but using a separate cohort of mice. Due to substantial sample variability in blood loss and tissue damage in tail wounds, the accumulation of particles was rigorously quantified, by histopathology, using this liver puncture model of bleeding.\(^{117}\) Ten times more particles were localized in the liver wound with propelled thrombin than with non-propelled thrombin (Figure 3.9D-F). Enhanced transport and delivery downward into the wounds occurred because the reaction of ~100,000 particles, and the resulting local convection, rapidly distributed them throughout the blood and wound. These results demonstrate that self-propelling particles can increase transport of cargo, including thrombin, into bleeding wounds, and this likely contributed to its increased ability to stop bleeding.

### 3.3.11 Propelled thrombin and CaCO\(_3\) microparticles were well-tolerated in mice

For three days following tail clips and application of propelled thrombin, mice showed no signs of pain, thrombosis or tail necrosis. Histopathological inspection of tail tissues showed no increase in infiltration of inflammatory cells between groups, which demonstrates that propelled thrombin
did not cause substantial increase in inflammation following tissue injury compared to no treatment (Figure 3.10). Further testing will be required to fully determine safety and toxicity.
Figure 3.10 Histological sections of two treated tails at different distances from the site of amputation.

Tails were treated with propelling thrombin or no treatment following injury. Sections were stained with hematoxylin and eosin (H&E, left of each pair) or Masson’s Trichrome (right of each pair). Sections are oriented similarly with arrows denoting dorsal veins (DV), lateral veins (LV) and ventral arteries (VA). Scale bar is 500 μm.

Twenty-four hours following IV injections of either suspensions of CaCO₃ microparticles, microparticles with TXA-HCl, or saline (control), mice showed no signs of pain or distress, and no differences in serum levels of alanine aminotransferase (ALT) or aspartate aminotransferase (AST) (Figure 3.11A,B). Seven days following IV doses of microparticles and TXA-HCl (25 mg/kg of each agent, ~50,000 particles), mice showed no signs of pain or distress. These results suggest the embolic risk caused by topical administration of the propelling microparticles is low. If signs of embolism or toxicity are observed at higher doses or with other formulations in the
future, reported methods of fabricating nano-scale, amorphous CaCO₃ particles may be used.¹¹⁺

No signs of pain or distress were observed, even when calcium carbonate particles and TXA-HCl were injected intravenously, and histology of lung tissues showed no difference from controls, suggesting that major embolism or severe toxicity did not occur (Figure 3.11C). However, additional work is required to rigorously evaluate safety and toxicity for specific applications.
Figure 3.11 Mice remain healthy after intravenous injection of particles.

(A-B) Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in mice 3 days after IV-injections of either saline, CaCO3 microparticles or a reacted mixture of CaCO3 microparticles with TXA-HCl. Differences between groups were not significant (p>0.20). Error bars indicate S.E.M. (C) Histological sections of lungs, stained with H&E (top) or Masson’s Trichrome (bottom) do not show evidence of emboli. Scale bar is 500 \(\mu m\).
3.3.12 Propelled thrombin increases survival in pigs, a pilot study

To test if self-propelling particles could prevent death from catastrophic bleeding, a porcine model of traumatic femoral artery hemorrhage was used.\textsuperscript{102} The femoral arteries of pigs were surgically exposed and 5 mm diameter punch-holes were inflicted to induce rapid high-pressure arterial bleeding. In previous studies, without hemostatic intervention at the bleeding site, pigs quickly died from hemorrhagic shock (n=0/5, 0% survival at 3 hours).\textsuperscript{119} Alternatively, when PTG was packed above the bleeding artery, there was 100% (5/5) survival, whereas only 40% (2/5) survived to 3 hours when treated with NPTG (\textbf{Figure 3.9H}). Blood loss volumes and MAP of surviving animals are given in Figure 3.12. These results further support that increasing the transport of thrombin and tranexamic acid into a wound, using self-propelling particles, could increase their ability to stop bleeding – even when challenged by massive traumatic bleeding where outward flow rates are high.
Figure 3.12. Blood loss and MAP in a porcine model of lethal femoral artery hemorrhage.

(A-B) Blood loss volumes and MAP of pigs receiving NPTG. Ref fields represent changes in the number of animals surviving over time. (C-D) Blood loss volumes and MAP of pigs receiving PTG. Error bars indicate standard deviation. Points without error bars represent measurements which include two or fewer animals, for example at a time interval where only one animal lost blood. Total hemorrhage volumes are discussed further in Chapter 4.

3.4 Conclusion

Here we have developed a simple, self-fueled particle system for delivering cargo through flowing aqueous solutions, including for increasing transport of cargo into bleeding wounds. This system is functional, and in three animal models of severe hemorrhage the propelled particles were able to deliver thrombin and tranexamic acid and increase their ability to halt bleeding, compared to non-propelling controls. The efficacy seen in these models demonstrates that gas-generation can effectively transport particles throughout blood in diverse scenarios. In Chapter 4 and Chapter 5, the utility of this material is tested for increasing the ability of hemostatic agents to manage traumatic and surgical bleeding. Additionally, this material may be particularly useful in treating external or intraoperative bleeding that originates internally, such as in the uterus, gastrointestinal tract, or abdomen, where traditional topical agents are less effective; these future directions are explored further in Chapter 7. In addition to coagulants, this system may have far reaching applications for increasing the delivery of other classes of therapeutics into sites of injury and
hemorrhage. CaCO₃ adsorbs biomolecules non-specifically, and therefore a wide-range of therapeutics can be loaded and propelled. CaCO₃ particles are also scalable to sub-micron diameters and larger quantities. The by-products of the reaction, including CO₂, are inert and soluble in blood. Initial applications in drug delivery may include locally distributing therapeutics within complex vascular geometries when injected via drug-eluting intravascular catheters, or transporting therapeutics through blood and into exposed tissue during surgery, such as tumor resection. Numerous medical applications have been suggested for systems of self-propelling particles, and the findings here provide evidence that these applications may be achieved.
Chapter 4    Increasing transport of thrombin and tranexamic acid increases their ability to prevent death in a swine model of massive traumatic hemorrhage

4.1 Introduction

Trauma remains a major source of morbidity and mortality in the United States and worldwide.\textsuperscript{120,121} Hemorrhage is the leading cause of trauma-related death in the military and the second-leading cause in the civilian sector, and it is the leading cause of preventable trauma death.\textsuperscript{4,7,60,122} Early control of hemorrhage is necessary to improve outcomes for trauma patients.\textsuperscript{4,60,123}

In combat settings, the second most common sites of lethal hemorrhage are junctional anatomical locations, such as the groin, neck, and axilla.\textsuperscript{4} Junctional hemorrhage is particularly difficult to control in the prehospital setting because limb tourniquets cannot be applied proximal to these sites.\textsuperscript{124} Reports estimate that almost 5\% of combat casualties among Canadian forces in Afghanistan could have been saved by effective control of junctional hemorrhage.\textsuperscript{125}

Field management for junctional wounds includes wound compression, gauze packing, and/or indirect vascular compression using external compressive devices applied proximal to the site of bleeding (including several types of junctional tourniquets). Manufacturers of hemostatic gauzes recommend at least 3 min of direct wound compression after packing, but the use of external compressive devices is questionable in prehospital and combat settings, because they are often cumbersome, require extensive training, and may completely occlude distal blood flow. These limitations also exclude their use during care-under-fire scenarios, leaving few viable options for rapid hemorrhage control during this urgent and immediate phase of care. Thus, there is a need for an appropriate gauze dressing that is effective without requiring compression. A gauze dressing that remained effective despite the outward flow of blood would be ideal in time-sensitive scenarios, such as during care-under-fire, as well as in non-compressible anatomic locations.

In Chapter 3, we developed self-propelling particles for drug delivery and which could transport hemostatic agents into wounds to stop bleeding. This approach could be particularly useful for managing traumatic hemorrhage, because particles loaded with procoagulant agents can be applied to a site of bleeding to deliver their cargo upstream against the flow of blood and self-disperse within wounds to promote hemostasis at the site of injury even in actively hemorrhaging arterial wounds (Figure 4.1A). The particles are composed of calcium carbonate (CaCO₃) with adsorbed thrombin and solid TXA-HCl, which causes the particles to release carbon dioxide gas and transport thrombin and TXA into the wound (Figure 4.1B). Transporting thrombin and TXA against flow deep into sites of bleeding could induce clot formation even during massive junctional
bleeding which is not amenable to compression. In our Chapter 3 these particles increased the
efficacy of these hemostatic agents as demonstrated in mouse models of tail amputation and liver
injury. Also in that chapter, pilot data was presented testing the ability of propelled thrombin gauze
(PTG) to stop bleeding in a combat-relevant large animal model is required to mimic the
hemorrhage volumes and pressures seen in humans after combat injury. In that pilot study, PTG
increased survival following femoral artery injury and hemorrhage in a small cohort of swine. In
this chapter, we extended this study to a larger cohort, measured additional clinical parameters,
and tested against the kaolin-based QuikClot Combat Gauze® (KG) bandage (Z-Medica,
Wallingford, CT) currently approved by the Committee on Tactical Combat Casualty Care for
battlefield use. We hypothesized that the 3-hr survival would be improved compared to KG
through the propelling action of the carbonate particles in this swine model of otherwise lethal
junctional hemorrhage.
Figure 4.1 Self-propelling CaCO$_3$ particles loaded with thrombin rapidly propel throughout wound sites, deposit their procoagulant cargos, and stop bleeding.

(A) Schematic showing application of propelled particle gauze to a wound pocket. Particles react with water to propel and deliver thrombin throughout the bleeding area. (B) Photograph of propelled thrombin gauze in a wound pocket. Scale bar is 1 cm.

4.2 Methods

4.2.1 Preparation of Gauze

PTG and NPTG were prepared for this study as described in 3.2.11. Kerlix was chosen because it is the standard of care for non-hemostatic gauzes for wound treatment. Combat Gauze is the current standard of care hemostatic gauze used by the U.S. Military and is impregnated with kaolin by the manufacturer. Gauze loaded with CaCO$_3$ and thrombin was layered with TXA-HCl gauze, yielding propelled thrombin&TXA gauze (PTG), or with neutral TXA gauze, yielding non-propelled thrombin&TXA gauze (NPTG). Gauzes were stored individually in sealed containers until, just prior to application, gauzes were layered and trimmed to a standard length and width using a gauze template, which was previously found to tightly pack the bleeding wound cavity.
4.2.2 Animal Preparation and Instrumentation

This protocol (Figure 4.2) was adapted from one previously published and widely used by the U.S. Army to evaluate topical hemostatic agents.\textsuperscript{102,132,133} This protocol is also described in 3.2.11 but is repeated here with greater procedural detail and description of additional outcomes which were measured. Previously, studies performed by these collaborators have shown that this model is 100% lethal without treatment, with median survival time of 36 min.\textsuperscript{119} All animal handling and procedures were approved by the University of Washington Office of Animal Welfare or the University of British Columbia Animal Care Committee and performed in accordance with the guidelines established by the Canadian Council on Animal Care and the National Institutes of Health guidelines for the use of laboratory animals. Immature female Yorkshire mix swine (25-30 kg) were fasted overnight with water \textit{ad libitum} prior to the study. In the morning, animals were sedated with ketamine (30 mg/kg IM) and anesthetized with a mixture of isoflurane (2-3\%) and oxygen (33\%) via nose cone. They were orotracheally intubated and given a single dose of buprenorphine (0.01 mg/kg IM) for analgesia. The isoflurane concentration was then maintained at 1-1.5\%.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{timeline.png}
\caption{Timeline of experimental protocol. MAP indicates mean arterial pressure.}
\end{figure}
Animals were mechanically ventilated (Anesco Anesthesia Ventilator) to achieve normal pH, pCO₂, and hemoglobin oxygen saturation. End-tidal CO₂ was monitored continuously (Datex Capnomac Ultima, Datex Instrumentarium Corp). The bilateral femoral sites were shaved and prepared with povidone-iodine solution. The left femoral artery and vein were isolated and cannulated for central blood pressure monitoring, blood sampling, and fluid and drug administration. The left femoral artery catheter was advanced to the distal aorta. The Biopac MP150 monitoring and data acquisition system (Biopac System, Inc., Santa Barbara, CA) was used to continuously record vital signs and hemodynamics.

A 4 cm longitudinal incision extending distally from the inguinal crease was made in the right femoral region, and the femoral artery was exposed. The femoral artery was bluntly dissected from the surrounding tissue, and all small arterial branches were ligated. The exposed artery was then bathed in 5 mL of 2% lidocaine solution to dilate and paralyze the artery to prevent vasospasm after injury.

4.2.3 Injury Protocol

Following 30 min equilibration, baseline measurements were obtained. Proximal and distal artery clips were placed on the isolated right femoral artery, and a 5 mm diameter arteriotomy was created using a circular punch biopsy tool in the anterior wall of the vessel, with care taken not to transect the artery. The wound cavity was kept free of any pooling blood to prevent spontaneous clot formation prior to removal of the vascular clips. The vascular clips were removed to initiate hemorrhage, and the artery was allowed to bleed freely for 30 seconds. After free bleeding, the
wound was finger-packed by a single operator with a standardized amount of either PTG, NPTG or KG (in rolled form) to completely fill the wound cavity. PTG and NPTGs were slightly stiffer than KG, due to the lyophilized products, but there were no differences in their ability to fully insert into the wound cavity. The skin was left open with packing in place. Manual compression was not applied.

4.2.4 Fluid Resuscitation

Three and a half minutes after onset of bleeding, all animals received one dose of Hextend™ (6% Hetastarch in a balanced salt solution, Hospira, Lake Forest Park, IL) 15 mL/kg IV over 15 min, followed by infusion of 3 mL/kg/min Ringer’s Lactate solution to a maximum of 100 mL/kg as needed to maintain a goal mean arterial pressure (MAP) of 60 mmHg. Animals were observed for 3 hr following initiation of hemorrhage or to the time of death. Death was defined as loss of pulse pressure on arterial waveform. At time of death or at 3 hr for survivors, animals were euthanized under anesthesia using a pentobarbital overdose (100 mg/kg).

4.2.5 Outcome Measurements

Survival time was measured up to 3 hr. Blood loss was measured by collecting all hemorrhaged blood using pre-weighed sponges. Given that the protocol allowed only one gauze application, the time to hemostasis was not recorded because, unlike other published protocols, no interventions were allowed in the event of rebleeding. MAP was recorded from the arterial catheter in the distal aorta and arterial blood lactate concentration and coagulation profiles were also measured serially.
and compared between groups. Blood samples were taken at baseline and at 3, 15, 30, 60, 90, 120, 150, and 180 minutes after injury, or at time of death. The START-4 coagulation analyzer (Diagnostica Stago, Asnières, France) was used to measure fibrinogen concentration by the modified method of Clauss in platelet-poor plasma after centrifugation. Thrombelastography by simple recalcification of citrated blood was used to measure clot formation parameters (TEG, Haemonetics, Braintree, MA). Fibrinogen concentrations were measured and thromboelastography was performed to assess hemodilution and potential fibrinolysis caused by fluid resuscitation and hypotension. Plasma TXA concentrations were measured in a subset of PTG pigs throughout the observation period and in a subset of NPTG pigs at 0 min and 120 min. Plasma TXA concentrations were measured following sample cleanup by solid phase extraction then analysis by ultra performance liquid chromatography – tandem mass spectrometry employing hydrophilic interaction liquid chromatography and multiple reaction monitoring detection. The concentrations of TXA measured in the plasma of PTG and NPTG animals ([TXA]_{plasma}) were used to estimate systemic thrombin doses $D_{thrombin}^{systemic}$ using the following equation:

$$D_{thrombin}^{systemic} = \frac{[TXA]_{plasma} V_{blood}}{m_{TXA}^{gauze}} \times \frac{a_{thrombin}^{gauze}}{m_{pig}}$$

where $V_{blood}$ is the animal’s total blood volume (assumed to be 65 ml/kg bodyweight), $m_{TXA}^{gauze}$ is the mass of TXA in the gauze, $a_{thrombin}^{gauze}$ is the activity of thrombin in the gauze and $m_{pig}$ is the animal’s bodyweight. This equation assumes that the fraction of total thrombin that was absorbed is equal to the fraction of TXA absorbed, and thus likely overestimates the systemic thrombin dose.
4.2.6 Statistical Analysis

Based upon previous reports, it was calculated that a sample size of 8 animals in each group was required to detect a difference of 50% in 3-hr survival between groups. MAP, volumes and average rates of hemorrhage, volumes and average rates of Lactated Ringer’s infusion, serum lactate concentration, hemoglobin concentration, platelet count, fibrinogen concentration and thromboelastogram maximum amplitude (TEG MA) were measured to explain any differences in survival and to direct future studies involving larger numbers of animals. However, this study was not designed to detect significant differences in these parameters.

All statistical analyses were performed with JMP (SAS Software, Cary, NC). Survival time was compared using Kaplan-Meier survival analysis and log-rank test. Cumulative hemorrhage volumes per minute of survival and fluid requirements per minute of survival were compared by one-way ANOVA. MAP and laboratory values were compared using repeated measures ANOVA (RM-ANOVA) to test for changes over time ($p_{Protocol Time}$), differences between treatment groups ($p_{Treatment}$) and if gauze treatment affected how these parameters varied with time ($p_{Interaction}$). One fibrinogen concentration (NPTG at baseline) could not be measured because the blood sample clotted and could not be analyzed, so the value was interpolated as the average fibrinogen concentration of all other animals at baseline. *Post hoc* pairwise comparisons were performed for variables with $p_{Treatment}<0.05$ and at each timepoint for variables where $p_{Interaction}<0.05$. Tukey adjustment was made for multiple comparisons. An overall $p$ value of less than 0.05 was considered statistically significant for all analyses.
4.3 Results

4.3.1 PTG significantly increased survival

PTG increased survival compared to NPTG and KG groups (Figure 4.3A, p<0.01). At 3 hr, 100% (8/8) of pigs receiving PTG survived, while only 25% (2/8) and 37.5% (3/8) of pigs receiving NPTG or KG survived, respectively. Compared to the NPTG and KG animals, PTG animals tended towards lower mean hemorrhage volumes, lower mean rates of hemorrhage, and lower mean rates of infusion of Lactated Ringer’s required to maintain MAP of 60 mmHg, but these differences were not significant (Figure 4.3B-C, Table 4.1). The large number of deaths in NPTG and KG groups did not allow sufficient statistical power to detect differences in MAPs of surviving animals ($p_{\text{Treatment}} = 0.145$ and $p_{\text{Interaction}} = 0.967$) or volume of Lactated Ringer’s infused.

4.3.2 PTG significantly decreased serum lactate compared to NPTG

There was a significant group effect of gauze treatment on serum lactate concentration (Figure 4.3D, $p_{\text{Treatment}} < 0.001$); animals receiving PTG had lactates that were lower than those receiving NPTG ($p < 0.001$) but were not significantly different from those receiving KG ($p = 0.938$). There was also a significant group effect of gauze treatment on hemoglobin concentration ($p_{\text{Treatment}} = 0.005$), with PTG animals having hemoglobin concentrations that were higher than NPTG ($p = 0.012$) but were not significantly different from KG ($p = 0.921$). There was no significant interaction between time and treatment in hemoglobin concentrations ($p_{\text{Interaction}} = 0.724$). Platelet
counts, fibrinogen concentrations and TEG MA at time of death or sacrifice are given in Table 4.1 and for all time points in Figure 4.4.
Figure 4.3 Propelled thrombin gauze increased survival in pigs that received lethal injuries to the femoral artery.

(A) Kaplan–Meier survival plot of pigs treated with three different hemostatic agents. *P < 0.01. (B) Mean arterial pressures of surviving animals. (C) Mean arterial pressures of all animals, where the MAP of deceased animals was included as 0 mmHg. Lactated Ringer was infused to maintain target MAP of 60 mmHg. (D) Blood lactate of pigs receiving three different hemostatic agents. **P < 0.001 comparing PTG and NPTG. n = 8 at time = 0. Error bars represent SEM. KG indicates QuikClot Combat Gauze; NPTG, non-propelled thrombin+TXA gauze; PTG, propelled thrombin+TXA gauze.
Table 4.1 Fluid balance and hematological parameters which were measured but not statistically powered to detect differences between groups and did not account for dead animals.

Values are mean ± SEM. n=8. *Last measured value for each animal. p-values were calculated by one-way (†) or repeated-measures (‡) ANOVA.

<table>
<thead>
<tr>
<th></th>
<th>PTG</th>
<th>NPTG</th>
<th>KG</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total hemorrhage volume (ml/kg)</td>
<td>29 ± 6</td>
<td>36 ± 10</td>
<td>45 ± 11</td>
<td>0.475†</td>
</tr>
<tr>
<td>Post-treatment hemorrhage volume (ml/kg)</td>
<td>12.1 ± 6.0</td>
<td>17.2 ± 8.6</td>
<td>23.0 ± 10.6</td>
<td>0.407†</td>
</tr>
<tr>
<td>Average rate of hemorrhage (ml/kg/min)</td>
<td>0.16 ± 0.03</td>
<td>0.59 ± 0.28</td>
<td>0.82 ± 0.29</td>
<td>0.163†</td>
</tr>
<tr>
<td>Lactated Ringer's infused (ml/kg)</td>
<td>57 ± 16.1</td>
<td>58 ± 16.9</td>
<td>46 ± 19.3</td>
<td>0.864†</td>
</tr>
<tr>
<td>Average rate of infusion (ml/kg/min)</td>
<td>0.53 ± 0.21</td>
<td>0.72 ± 0.2</td>
<td>0.72 ± 0.31</td>
<td>0.817†</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)*</td>
<td>6.8 ± 0.5</td>
<td>4.4 ± 0.8</td>
<td>6.7 ± 0.8</td>
<td>0.005‡</td>
</tr>
<tr>
<td>Platelet count (10⁹/L)*</td>
<td>261 ± 25</td>
<td>218 ± 31</td>
<td>250 ± 25</td>
<td>0.388‡</td>
</tr>
<tr>
<td>Fibrinogen (mg/dL)*</td>
<td>103 ± 14</td>
<td>77 ± 14</td>
<td>125 ± 31</td>
<td>0.747‡</td>
</tr>
<tr>
<td>TEG MA (mm)*</td>
<td>67.1 ± 1.4</td>
<td>56.2 ± 4.2</td>
<td>62.9 ± 2.7</td>
<td>0.259‡</td>
</tr>
</tbody>
</table>
Figure 4.4 Hematological parameters of animals following wound packing.

Hemoglobin concentrations (A), fibrinogen concentrations (B), thromboelastography maximum amplitudes (C), and platelet counts (D). n=8 at time = 0. *p<0.05 at specific timepoints comparing PTG and NPTG. Error bars represent SEM. KG indicates QuikClot Combat Gauze; NPTG, non-propelled thrombin+TXA gauze; PTG, propelled thrombin+TXA gauze.

4.3.3 Plasma concentrations of pigs receiving NPTG and PTG

Plasma concentrations of TXA in PTG animals increased to approximately 6 µg/mL (38 µM) at 2 hr and 8 µg/mL (51 µM) at 3 hr post-injury (Figure 4.5). NPTG animals had mean TXA concentrations of 3 µg/mL (19 µM) and 9 µg/mL (54 µM) at 2 hr and 3 hr, respectively. There was no significant difference in the plasma concentrations of TXA between PTG and NPTG. Femoral arteries for all groups were excised and examined following sacrifice. They were all patent and were neither occluded nor thrombosed.
Figure 4.5 Plasma TXA concentrations and estimated systemic thrombin doses for pigs receiving PTG or NPTG.

Plasma concentrations of TXA (left y-axis) in pigs receiving PTG or NPTG and estimated, corresponding systemic doses of absorbed thrombin (right y-axis). n = 2 to 8. Error bars represent SEM for timepoints and treatment groups where n ≥ 3. NPTG indicates non-propelled thrombin+TXA gauze; PTG, propelled thrombin+TXA gauze.

4.4 Discussion

Animals treated with gauze loaded with self-propelling, thrombin-loaded particles and TXA had significantly higher survival compared to those treated with QuikClot Combat Gauze or a non-propelling control in a model of junctional hemorrhage without compression. Therefore, these results expand upon those presented in Chapter 3 and further support that the increased transport of thrombin and tranexamic acid into wounds enabled them to significantly improve survival in this swine model of incompressible junctional hemorrhage. Additionally, self-propelling gauze may be useful in combat-relevant wounds that are not amenable to direct compression due to anatomical location or time.
Animals treated with PTG had the lowest mean hemorrhage volumes, though the differences were not significant, and significantly better lactate and hemoglobin concentrations compared to NPTG. These are consistent with the most likely mechanism by which PTG increases survival, hemorrhage control. The difference in hemoglobin concentration also suggests that hemodilution caused by hemorrhage and crystalloid fluid resuscitation may have contributed to the mortality seen in control groups. A similar trend was seen when comparing lactates and hemorrhage volumes of PTG and KG animals, but the difference was not significant. Since volumes of Ringer’s infused were similar between groups, the increased lactate in the NPTG group is likely due to worsened shock and not due to exogenously infused lactate. Decreased clearance of lactate is a predictor of mortality in trauma patients, and patients with initial lactate concentrations ≥ 4.0 mg/dL have an approximately 3.8 times increased risk of death. These results suggest that animals that received NPTG and survived to 3 hr would be more likely to die if the observation time were increased. Therefore, we suspect that the PTG’s main contribution to survival in this study was decreasing hemorrhage. However, studies involving larger numbers of animals would be required to conclusively verify this.

Since this study was powered to detect differences in survival, it was limited in its ability to detect differences in secondary outcomes, given in Table 4.1, many of which had very high variability even within treatment groups largely due the large number of deaths in NPTG and KG groups. Additionally, our statistical models do not account for censoring the animals that died before 3 hr and only considered surviving animals at each timepoint. Therefore, these comparisons likely overestimate the health of NPTG and KG animals at later timepoints. For example, animals who
died early in the protocol did not continue hemorrhaging or receiving Ringer’s infusion. Ultimately, the rapid death of animals in the KG and NPTG groups limited the ability to discriminate potential differences in blood loss and metabolic measurements over time. As stated above, we expect that with a greater sample size or a non-lethal model of hemorrhage, differences in other secondary outcomes, such as MAP, hemorrhage volume, and Lactated Ringer’s requirement, would reach significance. With the observed means and variance in hemorrhage volume per minute of survival time, 63 animals (21 per intervention group) would be required to detect heterogeneity across groups with 80% power and α=0.05, and this is beyond the scope of the study presented in this chapter.

Absorption of TXA and thrombin were low in both PTG and NPTG groups. In both groups, plasma concentrations of TXA were much lower than clinical targets for oral or intravenous TXA (1 mM). Similar concentrations of TXA in both groups suggests generalized absorption into the wound tissues that was independent of gas-generation and propulsion.\(^{21}\) Micromolar concentrations of TXA inhibit fibrinolysis in vitro and the local concentration of TXA in the wound may be considerably higher, therefore it is possible that PTG and NPTG could inhibit fibrinolysis both locally and systemically.\(^{138}\) The efficacy of PTG or NPTG formulations containing thrombin or TXA alone have not been tested; therefore, the relative hemostatic contributions of thrombin and TXA have not been isolated. Although thrombin is expected to have a much greater immediate contribution, TXA could be effective in situations where fibrinolysis is expected to have greater impact, such as during prolonged care. Assuming TXA and thrombin are absorbed at an equal stoichiometric ratio, we estimate that a maximum of 0.3 IU/kg thrombin enters systemic circulation
following packing with PTG and NPTG. However, equal absorption is unlikely because thrombin's high molecular weight (36,000 Da compared to 157 Da for TXA) would diminish its absorption intramuscularly. This dose of thrombin is 0.1% the maximum tolerable dose of intravenous thrombin (400 IU/kg), which is known not to cause thrombosis because it is rapidly inhibited by antithrombin within minutes of administration. These data suggest that risks of thrombosis associated with PTG or NPTG’s thrombin content are low. However, further work is required to rigorously test toxicity and pharmacology of the individual components of the PTG formulation.

Overall, there are multiple advantages to using this novel dressing. First, these results suggest this dressing could reduce blood loss from non-compressible wounds and in time-sensitive scenarios, such as care-under-fire, where the only currently recommended hemostatic intervention is tourniquet application. This could be critical in preventing major blood loss in the first few minutes after injury. Second, decreasing the degree of lactic acidosis is an indicator of improved tissue perfusion which may minimize later morbidity and mortality. Finally, this dressing shows the feasibility of creating a therapy with self-propelled microparticles that could potentially be used to deliver a variety of prothrombotics or other wound-healing cargoes throughout a wound cavity.

### 4.5 Conclusion

In conclusion, we have shown that PTG improves early survival in a swine model of lethal, junctional hemorrhage compared to gauze containing the same active ingredients but without propulsion, and compared to a clinical standard – QuikClot Combat Gauze. Coagulation parameters showed no obvious signs of thrombosis or toxicity, though further safety testing is
needed to translate the technology from its current experimental formulation to a product that is suitable for humans. If proven safe, self-propelling dressings may be a promising alternative to current dressings for managing bleeding and reducing mortality in combat and in prehospital, emergency, and clinical settings, and present an opportunity to increase the efficacy of hemostatic agents already used in trauma or for adapting other hemostatic agents for new utilities.
Chapter 5  Propelled thrombin gauze safely stops bleeding in a sheep model of endoscopic surgical and open surgical bleeding

5.1 Introduction

Poorly controlled intraoperative bleeding can lead to complications such as infection, procedural error, increased operating time, blood product transfusion, and death. In endoscopic surgery, intraoperative bleeding is especially problematic, given the narrow field of view; even moderate bleeding can sufficiently obstruct the surgical field. Intraoperative bleeding during functional endoscopic sinus surgery (FESS) can be reduced by perioperative measures, including preoperative corticosteroids, controlled hypotension, the reverse Trendelenburg position, vasoconstrictors, and warm saline irrigation. However, these techniques are limited and not suited for managing acute severe bleeding.

When inadvertent injury to the internal carotid artery, anterior or posterior ethmoid, or sphenopalatine artery occurs during FESS, management must be swift as the patient can decompensate within minutes. Immediate tamponade with nasal packing can be applied in these scenarios but this is associated with high morbidity and mortality if the ruptured vessel becomes stenosed or occluded. Electrocauterization can also be effective, but is associated with

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severe, unfavourable tissue reactions. Recently, more powerful topical hemostatic agents such as FLOSEAL matrix containing thrombin (Baxter International Inc), Surgicel (Ethicon, Johnson & Johnson), chitosan gel, fibrin-based products and muscle patches have been proposed as effective interventions with fewer adverse events. Fortunately, such bleeds are rare, occurring in approximately 1% of patients undergoing pituitary surgery, and more frequently in complex skull base procedures.

Topical hemostatic agents that contain thrombin, which is an endogenous coagulation enzyme, have emerged at the forefront for management of catastrophic surgical bleeding as well as intractable epistaxis. One of these agents, FLOSEAL, is known to worsen long-term surgical outcomes, such as by increasing granulation tissue and adhesions, but these are often considered acceptable losses in the context of severe bleeding scenarios. However, the applicability of these agents are inherently limited during FESS or during severe epistaxis. These topical agents are indicated only when bleeding sites can be visualized, which is often difficult. When blood loss is severe, conventional topical agents cannot reach the damaged vasculature due to the rapid outflow of blood. We hypothesize that a self-propelling hemostatic agent, which can move through flowing blood to increase the transport of its active hemostatic into the bleeding wounds, would be more effective than current static hemostatic strategies used in epistaxis, FESS, and in cases of catastrophic bleeding.

In Chapter 3, we described a self-propelling hemostatic agent that vigorously effervesces upon contact with blood. This agent consists of calcium carbonate (CaCO₃) microparticles, thrombin, and tranexamic acid (TXA). When applied to a wound, CaCO₃ propels and disperses thrombin and
TXA against flow, thereby improving contact with the bleeding vasculature. In Chapter 4, these particles and agents were formulated upon gauze, and significantly increased survival in a swine femoral artery bleeding model of fatal junctional hemorrhage in combat settings.\textsuperscript{131,157} Here, we tested to see if this agent, a self-propelling formulation of thrombin and tranexamic acid on gauze (PTG), was safe and effective in producing hemostasis in a sheep model of minor and major surgical bleeding.

5.2 Methods

5.2.1 Preparation of PTG

PTG for application to turbinate injuries was prepared based on methods described in Chapter 3 and Chapter 4.\textsuperscript{131,157} To adsorb CaCO\textsubscript{3} and thrombin onto gauze, 188 mg of CaCO\textsubscript{3} microparticles (American Elements, Los Angeles, CA), 188 µL glycine-buffered saline (GBS) (40 mM glycine, 171 mM NaCl, pH 7.2), and 0.34 mg human thrombin (specific activity \~3650 IU/mg, Haematologic Technologies, Essex Junction, VT) were co-incubated on ice for 20 min. Suspensions were diluted by addition of 188 µl GBS, and pipetted onto 2.5 x 2.5 cm\textsuperscript{2} pieces of Kerlix\textsuperscript{TM} gauze (Covidien, Dublin, Ireland), which were then lyophilized. To load gauze with TXA-HCl, 375 µL TXA solution (2 M, pH 4.3, Chem-Impex International, Wood Dale, IL) was applied to an equal amount of Kerlix\textsuperscript{TM} gauze, which was then baked for 30 min at 120\textdegree C. CaCO\textsubscript{3}-thrombin gauze and TXA gauze were layered to form PTG immediately prior to application to the turbinate.
PTG for application to carotid injuries were prepared similarly; Kerlix™ gauze measuring 10 x 10 cm² was loaded with 6.5 g CaCO₃, and 0.34 mg (1250 IU) human thrombin, and a separate gauze of equal dimensions was loaded with 3.9 g of TXA-HCl.

5.2.2 Animal preparation and instrumentation

All procedures involving animals were approved by the University of British Columbia Animal Care Committee (protocol #A14-0172) and performed according to Canadian Council on Animal Care guidelines. Dorset cross sheep (51 kg average bodyweight) were induced using 4-6 mg/kg of intravenous propofol. Animals were then intubated and maintained on isoflurane anesthesia (2-5%). Ketoprofen (3 mg/kg) was given subcutaneously to manage post-operative pain and an esophageal tube was placed to prevent bloat. Heart rate, EKG, non-invasive blood pressure, expired carbon dioxide, inspired and expired isoflurane, oxygen saturation and temperature were monitored throughout all procedures.

5.2.3 Initial biopsy and initiation of hemorrhage

Six sheep were included in this part of the study. Nasal cavities were explored with a 0-degree rigid endoscope, and inferior turbinates were identified. Using right and left angled 3 mm through-cutting Toffel punches (Access 11-371, 11-372), a turbinate biopsy/injury was performed in the medial aspect of the middle third (posterior injury). PTG or plain gauze, which was de-identified to the operator, was applied to the site of the injury for 60 seconds using Blakesley forceps. After gauze removal, bleeding was monitored endoscopically for 10 min. Neither gauze contained any
other agents to reduce nasal trauma during gauze removal. Attempts to quantify volumetric blood loss were unsuccessful, as suction could not be applied concomitantly with compression and endoscopic visualization of the injury site. Additionally, it was not possible to quantify any blood that may have flowed posteriorly into the nasopharynx. This procedure was replicated to produce a posterior injury on the contralateral side. After bleeding from each posterior injury had abated, the posterior injury sites were covered with gauze to prevent anterograde flow of any PTG-residue. Following this, similar injuries were repeated anteriorly and placed 1 cm posterior to the head of the inferior turbinate (anterior injury). Four injuries were completed per animal, for a total of 24 injuries over six sheep. Following randomization of the first injury, the remaining three injuries were assigned such that each turbinate received both treatments, and treatment assignments on the contralateral turbinate were reversed anterior-posterior. Animals were recovered and monitored for two days.

To test if PTG was associated with any medium-term adverse events, four separate sheep were used. Bleeding was initiated and treated as described above except that each turbinate received only one biopsy/injury (two total per sheep), and each sheep was treated with either PTG containing 156 IU of thrombin or FLOSEAL containing an equal dose of thrombin. Animals were recovered and monitored for 15 or 17 days for any changes to behavior or vitals.
5.2.4 Carotid artery injury

Eight sheep were included in this part of the study. While anaesthetized, a midline vertical neck incision was performed, and dissection was completed until identification of the STRAP muscles. The deep neck facial layers were followed until the right carotid sheath was identified. The common carotid artery was dissected away from surrounding tissues to obtain a minimum of 5 cm of artery exposure. The artery was clamped proximally and punctured using an 11-blade scalpel, to enable a standard arteriotomy using a 4 mm endoscopic mushroom punch. Punch injuries are regularly used for testing the efficacy of hemostatic agents in large animal models of hemorrhage, although a linear vascular injury could have been used to cause even greater bleeding.\textsuperscript{21,22} Clamps were released to initiate bleeding. After 3 seconds of free bleeding and with the operator blinded, PTG or gauze containing FLOSEAL with an equal amount thrombin (1250 IU) was directly applied to the puncture site. Plain gauze pack was mounted above the PTG or FLOSEAL gauze and 36 N (8 lb) of force was applied for 10 min in a direction parallel to gravity using a spring-loaded compression device with diameter \textasciitilde{}3 cm. Local pressure to the internal carotid is a maneuver clinically accepted in neck surgery and endoscopic procedures; however, the magnitude and direction of pressure may differ between clinical scenarios. Bleeding was assessed after 10 min by releasing pressure and visualizing the injury without removal of the PTG or FLOSEAL gauze. If hemostasis was achieved, the final gauze was removed and recurrence of hemorrhage was recorded. Carotid arteries were then ligated and animals were euthanized by sodium pentobarbital overdose (120 mg/kg IV). Once again, bleeding volumes could not be accurately
measured in this model due to the high pressure and projectile nature of the bleed, which flooded the surgical field rapidly after injury.

5.2.5 **Histology, coagulation analysis and necropsy**

Biopsies of turbinate injury sites at baseline and at 48 hours post-treatment were examined histologically. These images were scored by a blinded, certified pathologist for inflammation, ulceration, infiltrate and inflammatory debris using the following scale: 0=normal microscopic findings, 1=minimal, 2=mild, 3=moderate, 4=severe. Blood was collected at baseline, 48 hours post-turbinate injury and approximately 20 min post-carotid injury to measure semi-quantitative fibrinogen concentration, D-dimer levels, hemoglobin, hematocrit, platelet count, prothrombin time (PT) and activated partial thromboplastin times (APTT). Following sacrifice, carotid arteries were excised and grossly examined for any vessel occlusion. Cadavers were necropsied and brains, lungs and hearts were grossly examined for ischemic or infarctive lesions.

5.2.6 **Statistical analysis**

All statistical analyses were performed with GraphPad Prism 5 (GraphPad Software, CA). Bleeding times, pathologist scores and coagulation parameters were compared by Mann-Whitney U test. When a group of baseline pathologist scores or semi-quantitative fibrinogen concentrations were each the same value, comparisons were made with a Wilcoxon signed-rank test.
5.3 Results

After turbinate injuries, sites receiving PTG bled for 5.09 ± 2.25 min (mean ± standard error) compared to 8.21 ± 2.16 min for plain gauze sites (p=0.002, Figure 5.1B). Four out of the twelve control sites did not achieve hemostasis within 10 min, whereas all PTG sites did. To confirm that the location of turbinate injury did not significantly affect bleed times, anterior and posterior sites that had received the same treatment were compared. Anterior and posterior PTG sites were not significantly different, bleeding for 5.32 ± 2.85 min and 4.85 ± 1.70 min respectively (p=0.76). Similarly, anterior and posterior control sites were not significantly different, bleeding for 7.94 ± 2.26 min and 8.47 ± 2.22 min respectively (p=0.43).
Figure 5.1 Propelled thrombin gauze (PTG) decreases bleed times following turbinate injury.

(A) Schematic showing PTG being applied paranasally and propelled thrombin particles rapidly delivering cargo through blood flow. (B) Bleed times following turbinate injury and application of hemostatic agent. n = 12. *P < .01. Error bars represent standard error of the mean.

At baseline, all pathologist scores for ulceration, infiltration, inflammation and inflammatory debris scores were 0 except for one inflammation score of 1 (Figure 5.2). Two days post-turbinate injury, mean scores for each parameter increased for PTG and control sites, however significant differences were only seen when comparing baseline inflammation with PTG (p=0.01) and baseline ulceration with control (p=0.05, Table 5.1). There were no significant differences in pathologist scores between PTG sites and control sites.
Figure 5.2 Assessing propelled thrombin gauze (PTG)’s local tissue damage in the inferior turbinate following injury and treatment.

(A) Representative histopathological images of turbinate epithelium at baseline and 2 days postinjury. Scale bar is 150 μm. (B–E) Pathologist scores for inflammation, ulceration, infiltrate, and inflammatory debris of turbinate biopsies 2 days postinjury. Error bars represent standard error of the mean. n = 7–23. *P < 0.05.
Table 5.1 Probability values comparing pathologist scores of turbinate biopsies at baseline and 2 days after injury was treated.

PTG = propelled thrombin gauze.

<table>
<thead>
<tr>
<th></th>
<th>Baseline v. control</th>
<th>Baseline v. PTG</th>
<th>PTG v. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>0.060</td>
<td>0.012</td>
<td>0.744</td>
</tr>
<tr>
<td>Ulceration</td>
<td>0.048</td>
<td>0.057</td>
<td>0.146</td>
</tr>
<tr>
<td>Infiltrate</td>
<td>0.500</td>
<td>0.346</td>
<td>0.574</td>
</tr>
<tr>
<td>Inflammatory debris</td>
<td>0.174</td>
<td>0.149</td>
<td>0.661</td>
</tr>
</tbody>
</table>

Following carotid artery injury and compression, PTG stopped bleeding in 100% (4 out of 4) cases when dressings remained in place (Table 5.2). When the dressings were fully removed, re-bleeding occurred in 75% (3/4) of these injuries. FLOSEAL stopped bleeding in 25% (1/4) injuries, and this injury did not re-bleed when the dressing was removed. In animals that did not achieve hemostasis following removal of dressings, bleeding was severe and not expected to halt without further intervention.
Table 5.2 The proportion of carotid artery injuries that achieved hemostasis within 10 minutes of treatment.

Each agent was loaded onto gauze and compressed in the wound with 36 N of force. PTG = propelled thrombin gauze.

<table>
<thead>
<tr>
<th></th>
<th>Dressing on wound</th>
<th>Dressing removed</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PTG</td>
<td>FLOSEAL</td>
<td></td>
</tr>
<tr>
<td>Achieved hemostasis</td>
<td>100% (4/4)</td>
<td>25% (1/4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PTG</td>
<td>FLOSEAL</td>
<td></td>
</tr>
<tr>
<td>Achieved hemostasis</td>
<td>25% (1/4)</td>
<td>25% (1/4)</td>
<td>25% (1/4)</td>
</tr>
</tbody>
</table>

Baseline hemoglobin concentrations were 93 ± 6 mg/ml, hematocrits were 0.28 ± 0.02, and platelet counts were 266 ± 96 10⁹/L (Figure 5.3). PT and APTT were 13.7 ± 0.9 sec and 35.5 ± 6.5 sec, respectively. Semi-quantitative fibrinogen concentration was 1.0 mg/ml for each sample. D-dimer concentrations were 176 ± 78.7 ng/ml (Figure 5.4). Two days following turbinate injury, no parameter changed significantly except hemoglobin. Additionally, D-dimer levels did not differ significantly between any time points or between sheep who received PTG or FLOSEAL to their carotid injuries (p>0.05).
Figure 5.3 Hematological analysis of venous blood samples at presurgical baseline and 48 hours after turbinate injury and hemostatic treatment.

Parameters measured include hemoglobin, hematocrit, platelet count, prothrombin time, activated partial thromboplastin time, and semiquantitative fibrinogen. Error bars represent standard error of the mean. n = 5–6.
Figure 5.4 D-dimer concentrations in venous blood samples at baseline, 2 days following turbinate injury, and 20 to 30 minutes following carotid injury and hemostatic treatment.

Error bars represent standard error of the mean. n = 3–6. PTG = propelled thrombin gauze.

No occluded vessels were found in any animal postmortem. No signs of thromboembolism or ischemia were found during gross inspection and histopathology of heart, brain and lungs. No animal showed any major changes in behavior or vitals within 15 or 17 days following turbinate injury.

5.4 Discussion

During FESS and cases of epistaxis, mild to moderate bleeding is common and can be managed easily without long-term sequelae; however, profuse blood loss can be very difficult to manage and could increase morbidity and mortality. Various hemostatic agents have been developed for sinus surgery and primary epistaxis, but blood flow pushes these agents away from the bleeding site, decreasing their contact times and their abilities to form stable clots. The sheep model used here was a remarkable replication of the most relevant clinical bleeding events faced by
rhinologists. The turbinate injury model accurately represents common FESS bleeding and primary epistaxis events, while the carotid bleed model reflects the most catastrophic scenarios. Both injuries were easily induced and highly reproducible and thus work is ongoing to validate this sheep model for research in FESS.\textsuperscript{158,159}

In this study, we show that gauze containing self-propelling thrombin and tranexamic acid stopped both protracted surgical epistaxis and massive carotid bleeding, and we detected no adverse events. Therefore, because of its ability to transport hemostatic agents to damaged vasculature, PTG may become an invaluable agent to reduce bleeding and improve patient outcomes in a variety of FESS and difficult epistaxis scenarios where current treatments are slow or fail to achieve hemostasis.

In the turbinate injury model, PTG significantly decreased and nearly halved bleeding times compared to controls that received only local pressure with plain gauze. In sinus surgery, PTG could improve field of view to allow for more complete surgery while reducing operating time and incidence of complications associated with profuse and continued bleeding.

Treatment of turbinate injuries with PTG did not cause any more local tissue damage than plain gauze alone, even without irrigation or use of corticosteroids.\textsuperscript{160} Some packing agents have been shown to increase granulation tissue and adhesions in patients, which require subsequent lysis.\textsuperscript{155,156} Specifically, gelatin-containing resorbable hemostatic agents, such as FLOSEAL and Gelfoam (Pfizer), have been shown to worsen inflammation, elicit foreign body reactions, and cause fibrosis potentially leading to adhesions.\textsuperscript{161–163} While some of these agents also contain thrombin, thrombin is suggested to be uninvolved in adhesion formation.\textsuperscript{156} Therefore, a thrombin-
containing gelatin-free hemostatic agent, such as PTG, which can achieve rapid hemostasis without causing increased inflammation, would be very desirable. However, further studies are required to determine how PTG compares to other hemostatic agents regarding long-term sequelae relating to inflammation and healing, such as fibrosis or adhesions over months following application.

Powerful hemostatic agents, such as thrombin, carry risks of thrombosis, especially following application to major vessels such as carotid arteries.\textsuperscript{164} To determine if PTG caused thrombosis, systemic D-dimer concentrations were measured. D-dimers concentrations are measured to clinically rule out thrombosis. D-dimer levels that can rule out thrombosis have not been established for sheep; however, all concentrations measured here are within normal ranges for sheep, humans and other laboratory animals, such as dogs.\textsuperscript{165,166} Furthermore, D-dimer concentrations at 48 hours post-turbinate injury did not differ significantly from baseline, suggesting no incidence of thromboembolism.\textsuperscript{167}

Similarly, of the other blood count parameters measured, only hemoglobin significantly differed between baseline and 48 hours post-application, and all are similar to reference values.\textsuperscript{168} No signs of pain or adverse events were observed up to 17 days after treatment. Together, these results suggest that no significant thrombotic events occurred and that the risk of thrombosis associated with paranasal application of PTG is low. However, further preclinical studies involving larger numbers of animals are required to demonstrate this rigorously, and to translate this technology for use in humans.
In this model, PTG also effectively stopped bleeding from the common carotid artery, which is a high-flow, high-pressure bleed that is a catastrophic complication of surgery. There have been conflicting reports regarding which hemostatic agent should be used in the event of an endoscopic carotid artery bleed. One study, which used a live sheep endoscopic carotid artery injury model, concluded that the U-clip and the muscle patch are superior to FLOSEAL, oxidized regenerated cellulose, and chitosan gel.\textsuperscript{169} However, a systematic review that included different endoscopic and open surgical approaches showed that FLOSEAL was superior in reducing blood loss.\textsuperscript{55} Given that our carotid injury model was an open approach, we chose to compare PTG to FLOSEAL. When clot disruption was avoided, by allowing the gauze to remain adhered to the bleeding site, PTG achieved hemostasis in more sheep than FLOSEAL. Both interventions contained thrombin, but PTG can transport that thrombin into the wound and make it more effective. PTG’s ability to transport thrombin into the wound, and also outwards into the gauze dressing, may create stable clotting throughout the wound and dressing materials, which could explain why these clots were dislodged upon removal of the dressing. This strongly suggests that PTG may be an effective clinical treatment of catastrophic intraoperative bleeding. However, further studies are required to demonstrate PTG’s efficacy and superiority to clinical standards, such as the muscle patch, and to optimize PTG’s formulation and method of application.

No signs of thromboembolism were seen by gross inspection or histopathology of heart, brain and lungs in any animals treated with PTG or FLOSEAL. However, additional studies that investigate long-term embolic, thrombotic or ischemic risks associated with using PTG for major arterial bleeds are required. These studies are especially pertinent to test PTG’s safety in carotid injuries,
as the model presented here did not allow recovery of the animals or time for neurological deficits and secondary ischemic strokes to manifest.\textsuperscript{170}

5.5 Conclusion

This study has demonstrated that self-propelling, thrombin and tranexamic acid loaded particles can effectively treat bleeding in an endoscopic sinus surgery and open carotid injury model. Following turbinate injury, PTG achieved hemostasis significantly faster than standard gauze packing. There was no indication that PTG caused any local tissue damage or systemic thrombosis. Moreover, PTG remarkably stopped catastrophic bleeding following carotid artery injury and preliminary results suggest its efficacy is comparable or superior to clinical standards for managing severe bleeding. Although this self-propelled particle-based hemostatic agent was efficacious and did not have adverse effects in this model, further preclinical studies are required to fully understand its longer term safety risks prior to clinical testing.
Chapter 6  Increasing transport of TXA increases its ability to inhibit fibrinolysis and reduce bleeding

6.1  Introduction

Bleeding is the leading cause of preventable death following trauma, and the second-leading cause of death worldwide.4-6 Rapid and effective management of hemorrhage is critical to improving patient outcomes, and prevents development of secondary coagulopathies such as fibrinolytic shutdown and hyperfibrinolysis, which occur in many cases of polytrauma and Prolonged Field Care.6,7,35,171-173 This trauma-induced coagulopathy (TIC) is both common, occurring in 25-35% of trauma patients, and lethal, accounting for a four-fold higher mortality than in patients without TIC.174

In combat settings, junctional hemorrhage, particularly from groins, necks and axillae, is the second most common type of lethal bleeding175, and as many as 5% of combat fatalities in Afghanistan could have been prevented with effective control of this type of injury.7,10-12 This high mortality is due to the inability to apply a tourniquet proximal to the site and logistical difficulty compressing the wound. Truncal hemorrhage is the main cause of potentially survivable fatalities,


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and is even more challenging to treat because the injury cannot often be fully accessed and compressed.\textsuperscript{7,176–178} Existing strategies for managing bleeding following trauma are often ineffective in cases of TIC, and infeasible in remote locations or austere environments, which can delay access to medical facilities and administration of blood products.\textsuperscript{179} Strategies that require complex or time-consuming interventions are logistically impracticable and unavailable during field care situations.\textsuperscript{24,180,181}

Endogenous fibrinolytic activation contributes to coagulopathy after injury and its inhibition by antifibrinolytic agents is one strategy to improve hemostasis.\textsuperscript{182} Tranexamic acid (TXA) is one such agent that is safe and effective at improving survival after trauma when given intravenously.\textsuperscript{36,183} TXA is more efficacious when given quickly after injury. It loses its effectiveness entirely and becomes harmful if given after 3 hours from the time of injury.\textsuperscript{183,184} It is estimated that over 120,000 lives could be saved annually if TXA could be given more quickly.\textsuperscript{185}

Delivering TXA directly to the wound would decrease the time to treatment and would enable delivery of TXA both locally and systemically. This approach would directly deliver high concentrations to the wound surface and provide antifibrinolytic therapy to prevent systemic hyperfibrinolysis without the need for intravenous delivery. Therefore, delivering a systemic dose of TXA immediately via the wound could potentially reduce trauma mortality by reducing the time to TXA therapy. However, topical delivery of hemostatic agents, such as TXA, is complicated in severe bleeding by the brisk outward flow of blood, which prevents therapeutics from reaching the damaged vasculature and systemic circulation.\textsuperscript{22,131} A mechanism that transports topical TXA
against blood flow deep into wounds and into the systemic circulation could improve its ability to inhibit fibrinolysis and reliably stop bleeding.

Throughout this thesis, we have described the development of self-propelling particles that transport against flow to deliver therapeutic cargos. Formulations of self-propelling particles that included a combination of thrombin and TXA increased the efficacy of thrombin and were effective in reducing bleeding times and improving survival in several animal models, including endoscopic sinus surgery in sheep and junctional hemorrhage without compression in swine. These particles have successfully propelled cargo deep into bleeding wounds. While transporting thrombin into wounds is highly effective, thrombin is not always a desired hemostatic in point of care applications. Thrombin is a natural biologic and powerful coagulant, which is expensive and has a perceived risk of thromboses. Here, we tested the efficacy of self-propelling TXA (spTXA) alone (without thrombin) in in vitro models of fibrinolysis and in murine and swine models of hemorrhage without compression. We hypothesized that a topically applied propelled TXA could improve clot durability under flow, decrease blood loss in preclinical bleeding models and achieve therapeutic blood concentrations (Figure 6.1).
Figure 6.1 Schematic of self-propelling TXA transporting against the flow of blood, dispersing through a wound, and inhibiting fibrinolysis.

Following injury, blood flows outward from the ruptured vessels. When no intervention is applied (top), the clot is susceptible to lysis and rebleeding, particularly during TIC. When agents are applied that cannot disperse against blood flow (middle), they are pushed outward, and cannot penetrate deep into the wound to stabilize the entire clot from rupture. Self-propelling TXA (bottom) effectively disperses TXA into the wound against flow, thus forming a robust fibrin clot that resists fibrinolysis and prevents rebleeding.
6.2 Methods

6.2.1 Sample preparation

TXA-HCl was prepared as previously described, with modifications;\textsuperscript{131} concentrated HCl was added to 0.5 M TXA (Chem-Impex, Wood Dale, IL) and the solution was lyophilized to yield solid TXA-HCl. To make spTXA, CaCO\textsubscript{3} microparticles (3 µm diameter, American Elements, Los Angeles, CA), TXA-HCl and Na\textsubscript{2}CO\textsubscript{3} (Thermo Fisher Scientific, Waltham, MA) were mechanically mixed together in a 6:3:3:1 mass ratio. Non-propelling TXA (npTXA) was prepared by mechanically mixing TXA (not TXA-HCl), as obtained from the manufacturer, with CaCO\textsubscript{3} and Na\textsubscript{2}CO\textsubscript{3} in the same mass ratio. Because of the neutral pH of npTXA, these particles do not convert carbonate to CO\textsubscript{2}, and thus do not self-propel. To prepare topical TXA solution (toTXA) for \textit{in vitro} and \textit{in vivo} use, TXA was dissolved to 1 M in pH 7.4 phosphate-buffered saline (PBS) (Life Technologies, Burlington, ON, Canada); for \textit{in vivo} use, TXA was dissolved in water. For swine experiments, spTXA was used on gauze (spTXA gauze), and was prepared as previously described, with modifications;\textsuperscript{157,186} 4.8 g of TXA-HCl was dissolved at 300 mg/ml in water and poured onto Kerlix cotton gauze (Covidien, Dublin, Ireland). Separately, 4.0 g of CaCO\textsubscript{3} was suspended at 250 mg/ml in water and poured onto an equivalent amount of Kerlix gauze. To make the final gauze preparation, gauzes were lyophilized separately and layered upon one another immediately prior to application.
6.2.2 Measuring clot lysis in vitro

To test if spTXA can stabilize clots in vitro under stagnant conditions, plasma clots were prepared in glass capillary tubes (1.5 mm inner diameter, Kimble Chase, Vineland, NJ) by mixing normal plasma or plasma deficient in α2-antiplasmin (α2-AP) (Affinity Biologicals, Ancaster, ON, Canada) with calcium-rich saline (40 mM CaCl₂, 90 mM NaCl) in a 3:1 volumetric ratio, and suspending 10 μL recalcified plasma inside the capillary, 2 cm from the outlet. Plasma deficient in α2-AP was used to mimic hyperfibrinolysis. Hyperfibrinolysis is triggered clinically by substantial tissue damage, hypotension or by consumptive coagulopathy. Approximately one hour after recalcification, 50 μL PBS containing 0.4 μM (3125 U/mL) human urokinase-type plasminogen activator (uPA, Sigma-Aldrich, St. Louis, MO, USA) (for normal plasma) or 0.2 μM (1563 U/mL) uPA (for α2-AP-deficient plasma) was added on both sides of the clot, which was found to lyse clots reliably within two hours. The top end of the capillary was then sealed, and to the bottom end, 0.5 mg of spTXA, 0.5 mg of npTXA, 0.81 μL of toTXA, or nothing was applied. To simulate intravenously administered, pre-dispersed TXA (pdTXA), TXA was pre-mixed with the uPA solution at a final concentration of 1 mM and added to the bottom end of the capillary and nothing else was applied. As a positive control for total inhibition of fibrinolysis, the 1 mM TXA-uPA mixture was added throughout the system on both ends of the clot and nothing else was applied. Clot lysis was monitored at regular time intervals using digital photography and quantified using ImageJ software (NIH, Bethesda, MD). To test if spTXA could inhibit clot lysis when applied to opposing flow, such as in where bleeding is oozing or pooling or originates from capillaries, the in vitro system was nested within a secondary tube of 2 mm diameter through which
PBS flowed at 0.4 mm/s opposite the direction of TXA application. In this flow system, the clot was formed using 5 μL α2-AP-deficient plasma, and then 2 mg of spTXA, 2 mg of npTXA, 3.23 μL of toTXA, or nothing was applied to the outlet of the external tube (3 cm from the clot) for 10 sec.

### 6.2.3 Mouse tail transection assay

All experimental procedures involving animals were approved by the University of British Columbia Animal Care Committee and performed in accordance with the guidelines established by the Canadian Council on Animal Care. To compare the efficacy of spTXA, npTXA, toTXA and two doses of intravenously administered TXA in mice, female WT C57BL/6 mice aged 6-16 weeks weighing 18-26 g were used (Jackson Laboratory, Bar Harbor, ME, and Charles River Laboratories, Wilmington, MA). Mice were anesthetized with isoflurane, placed in prone position, and the distal 3 mm tip of their tails was transected using a fresh scalpel blade. The bleeding tails were immediately placed over 31 mg of spTXA, 31 mg of npTXA, 50 μL of toTXA, or nothing, and 50 μL PBS was added to solubilize the spTXA and npTXA particles. After 30 sec of applying the agent, tails were bled into 10 mL PBS containing 5 mM sodium citrate (pre-warmed to 36 °C) for 30 min. Mice treated with intravenous TXA received a single bolus of either 14 mM TXA (11 mg/kg, “ivTXA11”) or 42 mM TXA solution (33 mg/kg, “ivTXA33”) in 0.9% NaCl via tail vein injection 15 min prior to the tail amputation. The ivTXA11 dose was chosen since this is a typical dose of intravenous TXA clinically. The ivTXA33 dose was estimated to be the upper limit for systemic absorption of TXA following topical application of 31 mg of spTXA, and is also a dose
that is used clinically. Blood collected in citrated PBS (10 mL) was centrifuged at 250 x g for 15 min at 22 °C. Then 8.2 mL of the supernatant was removed. The pellet was resuspended with 200 μL erythrocyte lysis buffer (8.3 g/L NH₄Cl, 1.0 g/L KHCO₃, and 0.037 g/L EDTA in water) and incubated on ice for 5 min. Hemoglobin concentrations were measured spectrophotometrically at 590 nm using a GENios microplate reader (Tecan, Männedorf, Switzerland). Blood loss was calculated based on a standard curve with known amounts of blood and normalized by body weight.

6.2.4 Measuring acute toxicity in mice

To evaluate the safety and tolerability of spTXA in mice, CD-1 mice (Charles River Laboratories, Wilmington, MA) weighing 23.5 – 26.9 g were injected subcutaneously with spTXA (0, 30, 100, 300, or 1000 mg/kg TXA) combined with saline (10 mL/kg). Body weight for each mouse was monitored for 14-days. Plasma concentrations of TXA and calcium were measured at 1 hr, 4 days, and 14 days post-administration in mice receiving 300 mg/kg TXA.

6.2.5 Swine model of junctional hemorrhage without compression

Swine experiments, including femoral artery injury, resuscitation, and measurement of hemorrhage volume, plasma concentration of TXA, and survival via mean arterial pressure, were conducted exactly as previously described in Chapter 4, and is described in brief here. This protocol was adapted from one used by the US Army to evaluate topical hemostatic agents and has
reached widespread use. Approval for all pig handling and procedures was obtained from the University of Washington office of animal welfare.

Animals were sedated, anesthetized, and mechanically ventilated via orotracheal intubation. The right femoral artery was exposed via a 4-cm longitudinal incision made just distal to the inguinal crease, and the artery was bluntly-dissected from the surrounding tissue. The artery was bathed in 2% lidocaine to dilate the artery and to prevent vasospasm following injury. Proximal and distal artery clips were placed on the isolated right femoral artery and a 5-mm diameter arteriotomy was created in the anterior wall using a circular punch biopsy tool, and the vascular clips were removed to initiate hemorrhage. The artery bled freely for 30 sec, after which packing was initiated. In this study, all six animals were packed with spTXA gauze, with the amount standardized to completely fill the wound cavity. The skin was left open with packing in place and manual compression was not applied, to simulate a non-compressible wound.

Three and a half minutes after onset of bleeding, animals received one dose of Hextend (6% Hetastarch in a balanced salt solution, Hospira, Lake Forest Park, IL) 15 ml/kg IV over 15 minutes, followed by infusion of 3 ml/kg/min Ringer’s Lactate solution to a maximum of 100 ml/kg as needed to maintain a MAP of 60 mmHg. Animals were observed for 3 h following initiation of hemorrhage or to the time of death. Death was defined as loss of pulse pressure on arterial waveform. At the time of death or at 3 h for survivors, animals were euthanized using a pentobarbital overdose. Hemorrhage volume was measured throughout by collecting and weighing all hemorrhaged blood using pre-weighed sponges. To measure plasma concentrations of TXA, blood was collected from the systemic circulation at 30-min intervals throughout the observation
period, plasma was isolated, and TXA concentration was measured as previously described, using liquid chromatography-tandem mass spectrometry.\textsuperscript{157}

6.2.6 Statistical analysis

All statistical analyses were performed using Prism 5 (GraphPad Software, La Jolla, CA). For all experiments, $P$-values of $<0.05$ were considered statistically significant. Error bars indicate SEM in all figures. Individual clot size measurements in the \textit{in vitro} clot lysis experiments were rounded down to every 10 min for normal human plasma and every 5 min for $\alpha2$-AP-deficient plasma. Percent lysis measurements were compared by two-way ANOVA comparing each treatment group to spTXA at every timepoint. Bonferroni adjustments were made for multiple comparisons. Mean values for each condition in the tail transection experiments were compared to spTXA using a two-tailed unpaired $t$-test with Welch’s correction to account for unequal variances between groups.

6.3 Results

6.3.1 Self-propelling TXA increases its ability to inhibit fibrinolysis \textit{in vitro}

We first evaluated the ability of spTXA to inhibit clot lysis \textit{in vitro} under stagnant conditions. Clot sizes decreased over time in all conditions, except in the “no lysis control” group, in which both sides of the clots were saturated with TXA before the start of the experiment (\textbf{Figure 6.2B}). npTXA, toTXA and no treatment groups lysed throughout the period, and had $52 \pm 3\%$, $52 \pm 3\%$, $45 \pm 4\%$ (mean $\pm$ SEM) clot remaining at 110 min, respectively. In contrast, spTXA and pdTXA
had pronounced effects on preventing clot lysis by maintaining the clots at 77 ± 5%, 84 ± 2% at 110 min, respectively. spTXA and pdTXA were each significantly higher than all of npTXA, toTXA, and no treatment samples but were not significantly different from one another. The no lysis control group was significantly higher than all other groups. These same statistical trends were seen in both experiments below, using α2-AP-deficient plasma and under flow.

Figure 6.2 Propulsion increases TXA-mediated inhibition of fibrinolysis in vitro.

(A) Schematic showing how clot lysis was measured under stagnant conditions. (B-C) The proportion of clot remaining over time for clots formed from normal human plasma (B) or α2-antiplasmin deficient plasma (C) under stagnant conditions. (D) Schematic showing how clot lysis was measured under flowing conditions. (E) The proportion of clot remaining over time using α2-antiplasmin deficient plasma under flow. *P < 0.05 compared with spTXA at the last timepoint. Data indicate mean ± SEM, n = 3-5.
Next, we repeated the experiments using plasma deficient in α2-AP, an endogenous inhibitor of fibrinolysis. α2-AP-deficient clots lysed more quickly than normal clots (Figure 6.2C), though the trends were consistent with those obtained using normal human plasma. toTXA, npTXA, and no-treatment groups lysed continuously throughout observation, and had 41 ± 1%, 34 ± 1%, 18 ± 5% clot remaining at 110 min, respectively. spTXA and pdTXA both enhanced clot retention, maintaining clots at 76 ± 2%, 77 ± 1%, respectively.

To determine if spTXA could prevent clot lysis in vitro when applied against flow, saline was flowed around the clot and treatments were applied downstream. Similar to stagnant conditions, clots decreased over time in all groups, except for the no lysis control (Figure 6.2E). npTXA, toTXA and no treatment groups lysed continuously throughout observation, and lysed completely by 60 min. When clots were treated with spTXA and 1 mM uniform TXA, the amounts retained were 62 ± 1% and 71 ± 5%, respectively.

6.3.2 spTXA reduces bleeding in mice

To test the efficacy of spTXA in vivo, we examined its ability to reduce bleeding in mice following tail transection. Mice were treated with saline, spTXA, npTXA, toTXA, ivTXA11, or ivTXA33, and their total bleeding volumes measured via hemoglobin assay. We observed marked reductions in blood loss with spTXA compared to other treatment groups (Figure 6.3A): the mean blood loss, normalized to body weight were for saline (7.4 ± 2.9 μL/g), spTXA (1.4 ± 0.4 μL/g), npTXA (5.7 ± 2.2 μL/g), toTXA (6.1 ± 2.9 μL/g), ivTXA11 (8.0 ± 2.8 μL/g), and ivTXA33 (8.43 ± 2.43 μL/g). Blood loss following spTXA treatment was significantly lower than saline, npTXA, ivTXA11, or
ivTXA_{33} (P<0.05). Although the mean blood loss of the toTXA group was higher than that of the spTXA group, this difference was not significant (P = 0.15).

Figure 6.3 Self-propelled TXA stops bleeding and is well tolerated in mice.

(A) Blood losses following tail transection and treatment with various formulations of TXA. n = 6-8. *P <0.05. (B) Bodyweights of mice after subcutaneous administration of spTXA at various doses. Mice with 1000 mg/kg were sacrificed prior to the first timepoint. n = 3. (C) Plasma TXA concentrations in mice that received 300 mg/kg spTXA subcutaneously. n = 3. b.d. indicates below detection. Data indicate mean ± SEM.

6.3.3 spTXA has low acute toxicity when subcutaneously administered in mice

To evaluate the tolerability of spTXA in vivo, healthy euvolemic mice were injected with spTXA suspended in saline (30 – 1000 mg/kg TXA) or saline vehicle alone, and body weight was monitored. Animals in all groups, except the 1000 mg/kg group, gained ~8% weight over the 14-day observation period, which is typical for this strain and age of mice (Figure 6.3B). Mice
receiving spTXA at doses of up to 300 mg/kg showed no signs of pain or distress. Mice that received 1000 mg/kg showed signs of local pain and irritation within hours of administration and were sacrificed in accordance with ethics protocols. Mice that received 300 mg/kg spTXA had plasma TXA concentrations of 1366 ± 18 µg/ml one-hour post-administration, which decreased to 0.036 ± 0.024 µg/ml at 96 hr, which equates to half-life of approximately 8 hr (Figure 6.3C). Plasma calcium concentrations were elevated to approximately 1.6-fold over baseline one-hour post-administration, and returned to baseline by 14 days, and no adverse events or calcium-associated toxicity were observed.

6.3.4 spTXA gauze stops bleeding in swine without compression

The effects of spTXA powder gauze on blood loss, survival time, and systemic TXA levels were characterized in a simulated non-compressible junctional bleeding swine model with femoral arteriotomy. Sixty-seven percent (4 out of 6) of pigs receiving spTXA gauze survived to 3 hr with a mean survival time of 161 min (Figure 6.4A). The proportion of animals receiving spTXA that survived was less than animals receiving PTG, which contained a similar formulation except with thrombin and a different preparation of TXA-HCl (Figure 6.5). Total hemorrhage volumes were 33.3 ± 13.3 ml/kg (Figure 6.4B). Plasma TXA concentrations were approximately 6 mg/l within the first hour and rose to 8-12 mg/l from 60 min to 180 min (Figure 6.4C).
**Figure 6.4** Gauze loaded with spTXA can stop bleeding and can deliver therapeutic doses of TXA in a swine model of junctional hemorrhage without compression.

(A) Kaplan-Meier survival plot of pigs treated with spTXA, compared to historical data from hemostatic agents that were previously reported.\textsuperscript{119,157} (B) Total hemorrhage volumes of animals receiving spTXA, compared with historical data. (C) Plasma concentrations of TXA in pigs receiving propelled TXA. Dotted lines represent literature values of plasma concentrations which are seen during clinical IV TXA regimens, and which are known to inhibit lysis in vitro.\textsuperscript{138} Data indicate mean ± SEM, n = 5-8. † data from reference \textsuperscript{119}. ‡ data from reference \textsuperscript{157}. # clinical values from reference \textsuperscript{138}. 
Figure 6.5 Survival of all animals receiving PTG, NPTG, spTXA gauze, Combat Gauze, and plain gauze and control (no treatment).

6.4 Discussion

Here we have shown that delivering TXA topically using self-propelling particles is has low toxicity and may have several advantages including supporting local clot formation in bleeding wounds, reduced blood loss from wounds, and providing systemic delivery of TXA.

We found that in vitro clot retention was significantly increased under both static and flow conditions when using spTXA compared to npTXA and toTXA. This suggests that self-propelling particles can increase TXA’s local antifibrinolytic activity at local wound sites. The inability of npTXA and toTXA to inhibit clot lysis is exacerbated under flow due to them being flushed from the application site. The 0.4 mm/s flow rate we used mimics the bulk flow from oozing and slowly bleeding wounds, as well as flow rates at the margins of fast bleeding wounds and in tortuous wound cavities. Potentially, transport of npTXA and toTXA from the application site to the clot in stagnant systems could be accomplished by diffusion if the distance is small, but when flow is introduced, convective transport out of the system dominates. Therefore, our results support that
formulations of TXA which possess no autonomous movement to oppose this flow will not reach the clot to inhibit fibrinolysis. This suggests that even in wounds where bleeding is very minor (<1 mm/s), these flow rates are sufficient to remove externally applied TXA which cannot self-propel. They also imply that propelling TXA using self-propelling particles could increase hemostasis in all types of bleeding wounds. The mouse tail clip bleeding model supports this conclusion by demonstrating that mice receiving spTXA bled significantly less than mice receiving npTXA, ivTXA_{11}, or ivTXA_{33}. Neither ivTXA_{11} nor ivTXA_{33} groups differed significantly from control mice who received no intervention following injury, which suggests that TXA may be required to be concentrated more within the wound to directly reduce blood loss. Recent studies of TXA in cardiac surgery support this notion by demonstrating small, but significantly-decreased post-operative blood loss with systemically-administered TXA^{189}, and further reduction of blood loss when combined with topical TXA^{190}. Mouse models include many physiological aspects relevant to managing hyperfibrinolysis, such as the fibrinolytic system and blood vessels. However, bleeds in mice are not directly translatable to trauma in humans, because mouse vessels, hemorrhage volumes, bleed times, and extent of fibrinolysis are much smaller. To test whether spTXA could stop bleeding that is biophysically similar to clinical scenarios, large experimental animals with coagulopathic conditions would be useful.

To test the tolerability of spTXA in vivo, spTXA was administered subcutaneously in mice. Though TXA is not indicated to be administered subcutaneously, this model was chosen because it emulates acute, prolonged exposure that would be seen using spTXA as a hemostatic agent, and has been used as a route of administration for assessing the toxicity and biocompatibility of
TXA. This could mimic treatment during prolonged care of wounds lasting over several days, such as that required during Prolonged Field Care. The results indicate that the maximum tolerated subcutaneous dose in mice is between 300 and 1000 mg/kg, which equates to approximately 34-112 mg/kg in pigs and to approximately 24-80 mg/kg in humans, when normalizing by body surface area. Since this human equivalent dose is usually well tolerated and similar to clinical doses of intravenous TXA, this acute toxicity of the spTXA formulation may be due to the high osmolarity of the injected solution, since high osmolarity alone is known to cause local toxicity, pain, and tissue damage. spTXA was suspended in saline vehicle at 1000 mg/ml, which yields a solution of approximately 1.3 Osm/L - ~4 times higher than physiological osmolarity (0.3 Osm/L) and higher than recommended maximum tonicities for subcutaneous injectables (1 Osm/L). Because mice cannot be subcutaneously injected with large fluid volumes, this experiment likely overestimates the toxicity of spTXA due to these osmolarity-related effects. Although subcutaneous administration may mimic some aspects of the pharmacokinetics of intralesional spTXA, there will be differences. Both subcutaneous and intralesional administration will have slower absorption compared to intravenous administration. In this study, spTXA had an apparent half-life of 8 hr, while the half-life of intravenously administered TXA in humans is 2-3 hr. Prolonged absorption was also observed in intralesional administration to swine in Chapter 5. To more accurately measure the acute toxicity, pharmacokinetics, and estimate the human equivalent doses of spTXA, future studies involving intralesional administration with extended monitoring periods are required. Together, these data suggest that using spTXA for controlling bleeding, which could involve prolonged acute exposure to spTXA or its reaction products, is
unlikely to cause local or systemic toxicity, but formal preclinical safety studies are required to rigorously test this.

In a swine femoral artery injury model of junctional bleeding without compression, spTXA gauze had comparable efficacy to other previously tested gauzes with the added advantage of providing therapeutic systemic levels of TXA without the need for its intravenous infusion. This provides initial evidence that self-propelling particles loaded with TXA and applied with plain gauze, could address both local mechanical bleeding from junctional wounds while also addressing systemic hyperfibrinolysis induced by shock. We compared the performance of spTXA gauze to the current clinical standard, Combat Gauze (KG), which was tested previously in the same model. Overall, mean blood loss (33.3 ± 13.3 ml/kg with spTXA vs. 45.3 ± 11.2 ml/kg with KG) and the proportion that survived to 3 hours were quite similar (67% with spTXA vs. 38% with KG), although it is difficult to make direct comparisons since the studies were done at different times. This suggests that spTXA gauze may have similar positive effects on local hemostasis compared to other hemostatic gauzes that rely upon activating coagulation for their hemostatic effect. Pigs receiving spTXA gauze also had plasma TXA concentrations approximately equal to minimum concentrations for antifibrinolytic effects; approximately 25% of the plasma concentration typically achieved following conservative intravenous doses of TXA (10 mg/kg). The subsequent effects on clot formation were not evaluated and are not reliable in this model because pigs are hypercoagulable and hyperfibrinolysis is not reliably induced after induction of shock.

There are strong clinical implications for a topical treatment that can reduce blood loss from a high-pressure junctional wound in addition to simultaneously providing systemic antifibrinolytic
therapy. Systemically administering TXA in the field immediately following trauma can be difficult. The current standard for Tactical Combat Casualty Care recommends that bleeding from external wounds be managed by application of a tourniquet or hemostatic dressing, followed by administering TXA intravenously or intraosseously. These multiple steps and the need for vascular access prolongs the time required to give TXA, thus reducing its efficacy. A topical bandage that could achieve both of these aims while reducing the complexity of care would transform prehospital civilian and military trauma care by making TXA therapy faster and applicable more broadly.

There are several limitations that must be considered for this study. One major limitation is that pigs are hypercoagulable compared to humans and are already very resistant to fibrinolysis. For this reason, future studies in pigs with coagulopathy or hyperfibrinolysis may better demonstrate spTXA gauze’s ability to reduce bleeding and improve survival compared to Combat Gauze. A direct concurrent comparison to Combat Gauze is also required to compare outcomes reliably. Additionally, spTXA should be tested in models of prolonged field care, in which animals are monitored for 6-8 hours following injury, to evaluate its effects on longer-term resuscitation and tolerability. In all, more preclinical studies are required to demonstrate the utility and versatility of spTXA for treating both complex mechanical bleeding from junctional wounds in addition to its effect on TIC.
6.5 Conclusion

In conclusion, we have demonstrated that self-propelling TXA microparticles are tolerable and may have several advantages when incorporated into bandages including supporting local clot formation, reducing blood loss from wounds, and providing systemic delivery of TXA. This approach could both improve and greatly simplify prehospital trauma care, and presents an opportunity for field administration of TXA via a new route which enables it to be transported deep into wound sites.
Chapter 7   Conclusions

This thesis has demonstrated that carbonate-based self-propelling particles can increase the transport of hemostatic agents into bleeding wounds, and this in turn improves their ability to stop bleeding. In multiple preclinical studies presented here, hemostatic agents that were loaded onto self-propelling particles were superior at stopping bleeding compared to control formulations which didn’t propel into wounds. Remarkably, self-propelled hemostatic agents outperformed clinical standards at stopping bleedings in both situations of traumatic hemorrhage and of surgical hemorrhage.

7.1   Summary of findings of this thesis

In summary, the efficacy of hemostatic agents can be improved by increasing their transport into wounds. Transporting therapeutics to sites of vascular damage is a major hurdle when managing bleeding. Chapter 1 discussed many diverse technologies that are currently being developed which aim to enhance delivery of hemostatic agents to sites of bleeding and overcome the transport barriers that can render agents with no active delivery or transport mechanisms ineffective. In Chapter 2, rationale was given that supports that self-propelling particles, with their established abilities to deliver drugs in vitro, could be used to test this hypothesis by developing them into a vehicle to deliver hemostatic agents against blood flow into wounds.

In Chapter 3, we developed self-propelling particles that can propel against blood flow to deposit their cargo deeper into wounds. These particles were able to increase the transport of thrombin and
tranexamic acid (“propelled thrombin”) into wounds, which increased the ability of those agents to stop bleeding in multiple mouse models of bleeding.

In Chapter 4, we showed that gauze loaded with propelled thrombin improved survival, compared to gauze loaded with non-propelled thrombin, in a swine model of massive traumatic junctional bleeding. Since PTG and NPTG were identical in all aspects – such as dose of agents, mass, and method of application by the operator – except that PTG could self-propel, this demonstrated that increasing the transport of thrombin and tranexamic acid into wound increased their ability to stop bleeding.

In Chapter 5, we showed that gauze loaded with propelled thrombin reduced bleeding time, compared to plain gauze, in a sheep model of endoscopic surgical bleeding. Additionally, PTG was compared to a clinical hemostatic agent (FLOSEAL™), which contained the same amount of thrombin, in a sheep carotid artery injury model of catastrophic surgical bleeding. PTG achieved hemostasis in more cases than the clinical standard, suggesting that increasing the transport of thrombin may increase its ability to stop surgical bleeding compared to marketed hemostatic devices.

In Chapter 6, increasing the transport of TXA using self-propelling particles increased its ability to inhibit fibrinolysis in vitro, and to reduce bleeding in mice. These results corroborate that increasing the transport of hemostatic agents, other than thrombin, into wounds can increase their efficacy, and that this method could potentially be extended to other hemostatic agents.
7.2 Future development of propelled thrombin gauze

In the studies presented here, PTG was safe and effective at managing traumatic and surgical hemorrhage. Coagulation parameters did not differ significantly from baseline in both sheep and pigs receiving PTG, suggesting no incidence of thrombosis in these studies, and PTG did not cause any excess local damage to nasal tissues compared to plain gauze. These results were corroborated in mice, who showed no excess tissue damage when propelled thrombin was applied to their tails nor when calcium carbonate microparticles were injected IV. In all animals, no adverse events have been detected. However, additional safety studies are required before clinical testing of PTG, including studies preclinical studies to fully understand its longer-term safety risks prior to clinical testing. PTG is similar in its dimensions and physical characteristics to current interventions for managing battlefield hemorrhage, and physicians and medics in Tactical Combat Casualty Care have identified it as a promising candidate for adoption into their treatment protocols. Studies are ongoing for refining the PTG formulation for managing hemorrhage in Tactical Combat Casualty Care. This thesis has also explored two similar formulations where propelled particles enhanced the delivery of hemostatic agents – PTG and spTXA gauze. spTXA gauze may have certain benefits over PTG gauze for managing traumatic hemorrhage; since it doesn’t contain thrombin, spTXA gauze would be less costly and more thermostable than PTG. The swine studies presented here suggest that spTXA gauze may be less effective than PTG gauze at managing severe junctional hemorrhage, so further studies are warranted which could explore the minimum effective dose of thrombin in PTG, and which are powered to detect the differences in survival.
These future studies could direct whether PTG, spTXA, or both should be further developed for Tactical Combat Casualty Care.

7.3 Impact of findings on treating bleeding

The results presented in this thesis demonstrated that the ability to increase the efficacy of hemostatic agents by improving their transport into wounds is not specific to thrombin and may be applied to other hemostatic agents and materials. While thrombin is commonly used as a hemostatic agent during surgery, PTG is quite distinct from typical surgical hemostatic products, which are generally applied using absorbable matrices such as fibrin, gelatin or oxidized regenerated cellulose. Therefore, translating PTG into a useful tool in operating rooms likely requires reformulation using an absorbable matrix instead of its current matrix – nonabsorbable cotton gauze. Since the methods used to produce PTG required only wetting of cotton gauze with aqueous solutions and suspensions of the components of the self-propelling particle system, it is possible that similar methods could be used to load propelled thrombin onto the same absorbable materials used in marketed hemostatic products. These products are also typically applied in parallel with thrombin, so increasing the transport of this thrombin into the site of bleeding may improve the hemostatic efficacy of these products.

The findings of this thesis may impact the management of bleeding by enabling hemostatic agents to addressing bleeding in scenarios where they are not typically used. This possibility is supported by the findings in Chapter 4, where propelling thrombin increased survival, compared to Combat Gauze, at stopping junctional bleeding in a swine model. In this experiment, pigs receiving NPTG
had similar survival compared to Combat Gauze (25% vs. 37.5%) suggesting that dressings which contain thrombin, without the increased transport demonstrated in this thesis, likely would not improve patient outcomes and so the increased cost associated with its use would not be justified. Increasing the transport of thrombin increased survival to 100%, which suggests that thrombin – with propulsion – could be used for treating junctional hemorrhage and may warrant the increased cost per unit of PTG compared to CombatGauze. However, cost analyses are required to support this.

Self-propelling hemostatic agents, including propelled thrombin, may also be developed into interventions for managing incompressible bleeding originating from the abdomen and pelvis, which is responsible for many hemorrhage related deaths. In Afghanistan, 44% of Canadian trauma deaths were attributed to bleeding from these the torso, abdomen, and pelvis. Management of these hemorrhages is majorly limited by the ability to specifically address the site of bleeding, which typically requires damage-control laparotomy or transport to decisive care, such as a Role 2 hospital. Bleeding from these areas makes delivering hemostatic agents to damaged vasculature particularly difficult since these injuries are not amenable to external compression. Some strategies for managing these bleeds, such as aortic compression or resuscitative endovascular balloon occlusion of the aorta (REBOA), focus on stemming flow through major blood vessels, but these strategies are technically challenging, are infeasible during early phases of care, and carry major risks of blocking distal perfusion, potentially necessitating limb amputation. Managing hemorrhage on the frontlines during prolonged field care (6+ hours) will become especially important in future conflicts, where NATO forces are less likely to have
air superiority and medical evacuations will become more challenging. Interventions which can be administered at point of injury and address abdominal hemorrhage could improve survival. Hemostatic agents, such as propelled thrombin as a powder, that could potentially disperse throughout the retroperitoneal space could be administered transperitoneally using minimally invasive techniques, such as via a laparoscopic trocar, and could penetrate through pooling blood to effectively manage torso hemorrhage. Similar methods could potentially be used to deliver similar self-propelling hemostatic agents to manage bleeding within other bodily cavities that are not easily compressed or have complex geometries, such as from the upper gastrointestinal tract or from the uterus during postpartum hemorrhage.\textsuperscript{7,21,204}

New self-propelling hemostatic agents could also use a variety of active ingredients to initiate coagulation or inhibit fibrinolysis. Since the methods described here for loading calcium carbonate microparticles with cargo have been used for a variety of other cargoes, they could potentially be used to combine these particles with hemostatic agents other than thrombin and TXA. These calcium carbonate microparticles could potentially be loaded other enzymes, peptides, or high molecular weight clotting factors,\textsuperscript{99} these could include coagulation factor VIIa (rFVIIa) or factor XIII (rFXIII) or fibrinogen, which are typically administered IV,\textsuperscript{205,206} or other topically applied hemostatic agents, such as kaolin or chitosan.\textsuperscript{113,176}

Future analysis could also be done to use quantitative models, based on solutions of the Navier-Stokes equations, to determine what types of bleeding can be addressed by enhancing transport of hemostatic agents using self-propelling particles. This thesis demonstrated that these calcium carbonate-based self-propelling particles, which propel at millimeters per second against pressure
driven blood flow, can increase transport through slowly flowing blood – *in vitro* and in mouse models of hemorrhage. These particles were also effective at delivering hemostatic agents in situations of brisk arterial bleeding, and the mechanisms by which they achieve this is not quantified in-depth. For directing which bleeding indications could be targeted by self-propelling particle drug platforms, it could be useful to quantify the transport phenomena within bleeding wounds – such as the flux of thrombin into wounds and its removal under shear – to understand the extent that self-propelling particles can increase activation of clotting in different bleeding scenarios.

### 7.4 Impact of findings on non-bleeding applications

Increasing the delivery of therapeutics against flow and into diseased tissue could be useful in many clinical scenarios beyond bleeding. The results presented in this thesis demonstrate that gas generation can effectively transport particles and their cargo throughout blood, and potentially other physiological fluids, to deliver therapeutic cargoes. Since the CaCO₃ microparticles employed in these studies could be loaded with a variety of cargoes, these self-propelling particles could potentially deliver non-hemostatic therapeutics to enhance their delivery to enhance management of other clinical pathologies.

Adding additional therapeutic cargos to the formulations described in this thesis could potentially be useful for addressing secondary pathologies related to bleeding. Therapeutics could be added which address wound healing, such as growth factors, or which prevent infection, such as antibiotics and antimicrobials. Enhanced delivery of antimicrobial agents could enhance their
ability to prevent infections, disrupt biofilms, and combat antibiotic resistance.\textsuperscript{207} Enhancing delivery of topically applied antimicrobials and antibiotics is a promising approach for managing chronic wounds, such as diabetic ulcers,\textsuperscript{208,209} since they are known to be poorly perfused; poor tissue perfusion is associated with greater susceptibility to infection as well as decreased delivery of systemically administered antibiotics.\textsuperscript{210,211}

These particles could also potentially be used upon bloody and exposed tissue during surgery to deliver other therapeutics, such as chemotherapeutics during tumour resection.\textsuperscript{212,213} Recent reports have shown efficient loading of an anti-cancer drug, cisplatin, into calcium carbonate microparticles for delivery to tumour tissues,\textsuperscript{214} which presents an opportunity for them to be incorporated into this formulation for preclinical testing in murine tumour models.

These particles could also potentially deliver thrombolytic agents for managing deep vein thrombosis (DVT), which is highly prevalent and a major cause of morbidity and healthcare burden. DVT has a high case mortality rate (2.6\%) which is generally caused by embolisation of the thrombus to the lung.\textsuperscript{215} In severe, limb-threatening cases, clots can be treated with catheter-directed intrathrombus thrombolysis (CDT) drugs followed by balloon angioplasty, together known as pharmacomechanical thrombectomy. Even with treatment and long-term prophylaxis, recurrence rates of DVT can be as high as 30\% within 10 years.\textsuperscript{215–217} Inadequate clearance of thrombi can also result in post-thrombotic syndrome, which is a substantial cause of morbidity. Computational models have predicted that delivery of thrombolytics to thrombi is diffusion-limited due to stagnating blood, and that the length of the affected vessel plays a central role in the
likelihood of clearance. Therefore, it is highly likely that increasing the spatial distribution of thrombolytic enzymes using self-propelling particles could enhance the effectiveness of CDT.

7.5 Impact on the field of self-propelling particles

Self-propelling particles had long been proposed for applications in drug delivery, and it was during the undertaking of this thesis work that this goal was realized. The reports presented here are among the first to describe self-propelling particles that are effective at transporting therapeutic cargoes in vivo and, to our knowledge, represent four of the six reports to date wherein this is described. This thesis demonstrated the utility of calcium carbonate for developing self-propelling particles for drug delivery. Using calcium carbonate particles for this purpose address major hurdles identified in the literature: that particles and their mechanism of propulsion must be biocompatible and that they must generate thrust and velocities fast enough to overcome the mechanical characteristics of physiological fluids, such as the presence of red blood cells. The results described here will hopefully be the basis for further research in their utility for transporting cargoes with other biomedical applications, such as for generating carbon dioxide bubbles for local controlled release or as an imaging contrast agent.
7.6 Closing

The work presented in this dissertation supports that increasing the delivery of hemostatic agents into sites of bleeding increases their hemostatic efficacy. Improving delivery of hemostatic agents, potentially using the self-propelling particles described here, presents an opportunity to decrease mortality, morbidity, and clinical burden associated with bleeding.
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