Development and Optimization of Hemostatic Peptides for Hemorrhage Control on Battlefields

Hemostatic Peptides

Sophie Charbonneau, Mark D. Blostein
Lady Davis Institute of Medical Research
Jewish General Hospital, McGill University

Prepared By:
Dr. Mark D. Blostein
3999 Chemin de la Cote-St-Catherine
Montreal, Quebec
Lady Davis Institute of Medical Research
W7711-057957/001/TOR
Contract Project Manager: Dr. Mark D. Blostein, 514-340-8214
2009-014
CSA: Henry Peng, Defence Scientist, 416-635-2129

Defence R&D Canada – Toronto

Contract Report
DRDC Toronto CR 2009-014
February 2009

Principal Author

Original signed by Dr. Mark D. Blostein

Dr. Mark D. Blostein

Associate Professor of Medicine

Approved by

Original signed by Dr. Bob Cheung

Dr. Bob Cheung

Acting Head, Individual Readiness Section

Approved for release by

Original signed by K. C. Wulterkens

K. C. Wulterkens

for Chair, Document Review and Library Committee

In conducting the research described in this report, the investigators adhered to the 'Guide to the Care and Use of Experimental Animals, Vol. I, 2nd Ed.' published by the Canadian Council on Animal Care.

Abstract

The goal of the research contract conducted for the Defence Research and Development Canada – Toronto, was to develop a hemostatic biocompatible surface to treat hemorrhagic injuries sustained on the battlefield. During the first year of the contract, a candidate hemostatic amphipathic helical peptide (an ideal amphipathic helical peptide or IAP) was developed (published in Biochemical Journal 412: 545-51 2008) and different biocompatible surfaces were tested (published in Journal of Biomaterials Research, Part B: Applied Biomaterials, 2008 Sep 5). Once the peptide and biomaterial were identified, the second year was dedicated to testing IAP coupled to different surfaces and we finally settled on IAP coupled to polyethylene glycol and poly(allylamine) (PEG-PAA) for further testing. *In vivo* work testing this material was carried out on small animals (rabbits) and subsequently on large animals (pigs) during the second and third year. We have data that demonstrates the feasibility of such material to arrest bleeding *in vivo* by reducing hemorrhage by 40-50%.

Résumé

Ce contrat de recherche fait pour le Département de Recherche Nationale avait pour but de développer une surface hémostatique biocompatible pour traiter les blessures externes ayant lieu sur des champs de bataille. Durant la première année du projet, un peptide amphipatique et hélitique (IAP : ideal amphipathic peptide, ou peptide amphipatique idéal) a été développé (publication : Biochemical Journal 412: 545-51 2008) et différentes surfaces biocompatibles ont été testées (publication : Journal of Biomaterials Research, Part B: Applied Biomaterials, 2008 Sep 5). Une fois le peptide et le biomatériau identifiés, la deuxième année du projet a servi à tester le couplage du peptide (IAP) à différentes surfaces. Nous avons finalement conclu que le peptide couplé au polyéthylène glycol- poly(allylamine) (PEG-PAA) offrait les meilleurs résultats. La troisième année du contrat a été dédiée aux tests *in vivo*. Des modèles hémorragiques ont été développés chez des lapins et des cochons afin de tester le

peptide (IAP) couplé à la surface biocompatible (PEG-PAA). Les résultats ont démontrés que le peptide réduisait le temps de saignement de 40 à 50% chez les petits et gros animaux.

Executive summary

Development and Optimization of Hemostatic Peptides for Hemorrhage Control on Battlefields: Hemostatic Peptides

Sophie Charbonneau; Mark D. Blostein; DRDC Toronto CR 2009-014; Defence R&D Canada – Toronto; February 2009.

Introduction or background: Hemorrhage is the leading cause of death on the battlefield. In modern conflicts, over 90% of combat deaths occur before evacuation of the casualties and a little more than half of those are caused by uncontrolled hemorrhage. A variety of products have been designed to control hemorrhagic events caused by trauma, surgery, or in battlefields, and include injectable compounds as well as biocompatible hemostatic materials that could be applied at the site of injury [1]. Recombinant factor VIIa is a successful injectable hemostatic agent in patients with severe bleeding [2]. However, in trauma, randomized clinical trials have shown efficacy in primarily blunt, not penetrating injuries. Therefore, the need for local measures for penetrating injuries continues to be a major focus of investigation [3]. The use of Zeolites (QuikClot®) is a local measure that is used by the United States military. It is an inert material that absorbs water and creates an exothermal process that can physically concentrate the components of hemostasis. It has proven to be effective in massive bleeding in *in vivo* models of hemorrhage but causes thermal damage to tissues [1]. Another compound, poly-N-acetyl glucosamine (HemCon®) also referred to as chitin or chitosan, has been shown to be effective in ameliorating external hemorrhage although its mechanism of action is not entirely clear but hypothesized to be due to its ability to concentrate red blood cells resulting in a 'sticky' adhesive [4]. It would thus be interesting to test the hemostatic properties of a procoagulant peptide that is attached to a biomaterial that is easy to use, effective and accessible. The aim of the current three-year project is to develop and optimize such a hemostatic agent.

Resources: The work was conducted in the laboratory of Dr. Mark Blostein at the Lady Davis Institute for Medical Research at McGill University in Montreal, Quebec, Canada. The resources utilized were standard *in vitro* biochemical techniques using state of the

art technologies as well as *in vivo* animal models, approved by the institution's animal care committee.

Results: During the first year of the contract, a candidate procoagulant peptide (called an ideal amphipathic helical peptide or IAP) was developed and different biocompatible surfaces were tested for attachment. Once the peptide and biomaterial were identified, the second year was dedicated to testing IAP coupled to different surfaces and we finally settled on IAP coupled to polyethylene glycol and poly(allylamine) (PEG-PAA). *In vivo* work testing of this material was carried out on small animals (rabbits) and subsequently on large animals (pigs) during the third year. We have data that demonstrates the feasibility of such material to arrest bleeding *in vivo*, fulfilling the requirements outlined at the start of the three year project.

Significance: At the completion of our three-year project for Defence Research and Development Canada - Toronto, we have identified a biocompatible matrix that has *in vivo* activity to arrest bleeding in animal models of hemorrhage. This matrix is composed of PEG-PAA coupled to an amphipathic helix, IAP. This material is readily produced and has been shown *in vivo* to reduce bleeding times by 40-50%.

Future plans: The goal of future experimentation would be to further characterize this material in animal models of trauma with the penultimate goal of testing this material in humans.

Sommaire

Development and Optimization of Hemostatic Peptides for Hemorrhage Control on Battlefields: Hemostatic Peptides

Sophie Charbonneau; Mark D. Blostein; DRDC Toronto CR 2009-014; R & D pour la défense Canada – Toronto; Février 2009.

Introduction ou contexte: L'hémorragie est la première cause de décès sur les champs de bataille. Au cours des conflits modernes, plus de 90% des morts au combat se produisent avant l'évacuation des blessés, et un peu plus de la moitié de ces morts sont dues à une hémorragie incontrôlée. Divers produits ont été fabriqués pour contrôler les hémorragies dues aux traumatismes, aux chirurgies ou aux blessures sur les champs de bataille. On retrouve sur le marché des produits injectables et des matériaux hémostatiques biocompatibles pouvant être appliqués directement sur la blessure [1]. Le facteur VIIa recombinant est utilisé comme agent hémostatique injectable et offre de bons résultats chez les sujets ayant une hémorragie importante [2]. Par contre, lors de traumatismes, des études cliniques ont démontrées que ce produit n'est pas efficace sur des blessures profondes. Il est donc primordial de trouver un moyen efficace de soigner les blessures profondes [3]. L'utilisation des zéolites (QuickClot®) est répandue dans l'armée Américaine. Ce matériau est inerte et absorbe l'eau en créant une réaction exothermique qui concentre les composés nécessaires à la coagulation. Les zéolites ont prouvé être efficaces *in vivo* dans des modèles d'hémorragie extrême. Par contre, ce matériau cause des dommages thermiques aux tissus [1]. Un autre produit composé de poly-N-acétyle-glucosamine (HemCon®) est disponible. Ce matériau, aussi appelé chitine, a prouvé être efficace pour contrôler les hémorragies externes. Son mécanisme d'action n'est pas clair, mais il est supposé que la chitine exerce ses actions en concentrant les globules rouges et en agissant comme une sorte de colle au site de la blessure [4]. Il serait intéressant de tester les propriétés hémostatiques d'un peptide pro coagulant qui serait attaché à un biomatériau. Ce nouveau produit devrait être facile à utiliser, efficace et accessible. Le but de ce contrat de trois ans est de développer et d'optimiser un tel peptide hémostatique.

Ressources : Cette recherche a été effectuée dans le laboratoire du Dr. Mark Blostein, à l'institut de recherches Lady Davis de l'université McGill, à Montréal, Québec, Canada. Les expériences conduites sont toutes des techniques de biochimie *in vitro* standards. Les tests *in vivo* conduits ont été approuvés par le comité étique de l'institut.

Résultats: Durant la première année de ce contrat, a peptide pro coagulant potentiel (appelé IAP : ideal amphipathic peptide, ou peptide amphipatique idéal) a été développé. Différentes surfaces biocompatibles ont aussi été testées pour y attacher le peptide. Une fois le peptide et le matériau identifiés, la deuxième année de recherche a permis de tester le peptide couplé à différentes surfaces. Nous avons finalement décidé d'utiliser le peptide couplé au polyéthylène glycol-poly(allylamine) (PEG-PAA). Des tests *in vivo* ont ensuite été fais sur des petits animaux (lapins) et ensuite sur des plus gros animaux (cochons). Nos résultats démontrent que le peptide couplé au PEG-PAA est un produit hémostatique prometteur.

Importance: À la fin de ce contrat de trois ans avec le Département de Défense du Canada, nous avons identifié une matrice biocompatible qui peut arrêter l'hémorragie *in vivo* sur des modèles animaux. Cette matrice est composée de PEG-PAA couplé à un peptide hélitique et amphipatique, l'IAP. Ce matériau est facilement produit et a démontré des réductions du temps de saignement de 40 à 50% *in vivo*.

Perspectives: Dans le futur, il serait important de tester ce matériau chez l'humain.

Table of contents

List of figures

.

1 Introduction

The leading cause of death for trauma patients in both civilian and combat settings is uncontrolled hemorrhage [1]. The methods used to stop hemorrhage are not always appropriate and effective. The need for a hemostatic agent easy to use, effective and accessible is important. The aim of this three-year project was to develop and optimize a hemostatic agent.

In the recent years, different products have been designed to control hemorrhagic events [2]. These different hemostatic agents include injectable compounds as well as biocompatible materials that can be applied directly at the site of injury. QuikClot®, composed of zeolites, is a local hemostatic agent used by the U.S military. This inert material acts by absorbing water and thus creates an exothermal reaction that can physically concentrate the components of hemostasis. This material has proven to be effective in *in vivo* model of hemorrhage. However, QuikClot® causes thermal damage to tissue due to the exothermal reaction [5]. Another compound, poly-N-deacetylglucosamine (HemCon®), also known as chitosan, has been shown to be effective in ameliorating external hemorrhagic wounds. This compound works mainly by concentrating red blood cells, resulting in a sticky adhesive [3]. Factor VIIa seems to be an effective hemostatic agent for controlling internal bleeding; however, its relative high cost and instability at ambient temperature are main drawbacks for optimal use on the battlefield. Therefore, there is a general consensus that further research and development is required to develop new hemostatic materials for the treatment of both external and internal hemorrhage on the battlefield.

Blood coagulation is the result of a cascade of enzymatic reactions that lead to the generation of a fibrin clot. Most of the enzymes participating in blood coagulation, namely, coagulation factors VIIa (FVIIa), IXa (FIXa), Xa (FXa), and thrombin (FIIa) belong to a family of vitamin-K dependent serine proteases. Importantly, the tenase complex, composed of the enzyme, factor IXa, and its cofactor, factor VIIIa, assembled on a phospholipid membrane in the presence of calcium, catalyzes factor Xa generation from its zymogen factor X (FX). In addition, the prothrombinase complex, composed of

factor Xa with its cofactor factor Va, catalyses thrombin generation from prothrombin (FII). The proper functioning of the tenase and prothrombinase complexes is necessary for proper hemostasis. The penultimate goal of these reactions is the generation of fibrin and subsequent blood clot.

The use of an amphipathic helical peptide as a cofactor of coagulation factor IXa (FIXa) in the generation of activated factor X (FXa), was first described by Blostein *et al*, (2000) [4]. These authors reported that a membrane binding peptide from the C2 domain of coagulation factor VIII, $FVIII_{2303\text{-}23}$, enhances proteolytic efficiency of factor IXa in the absence of phospholipid membranes. This improvement results from a reduction in the K_M for the substrate, factor X, and a modest increase in k_{cat}. The same work showed that the enhancement of factor IXa cleavage of factor X is independent of the peptide sequence, but is tightly related to its helicity and its amphipathic properties, since similar results were obtained with Melittin, an amphipathic helical peptide derived from bee venom. The data from these experiments also prove that peptide binding to the gamma-carboxyglutamic containing (Gla) domains of factor X and factor IX is fundamental in factor X activation. These results support the possibility of a practical application of amphipathic helical peptides as coagulation enhancers for hemorrhage control.

DRDC Toronto has initiated a project to address the issues on hemostatic biomaterial development and internal bleeding mitigation, for the treatment of non-compressible hemorrhage sustained by front-line casualties. Our laboratory has been awarded a contract entitled 'Development and optimization of hemostatic peptides for hemorrhage control on battlefield'. The contract includes 3 phases. The objective of the first phase was to identify, prepare and evaluate peptides with hemostatic properties using *in vitro* assays, with the intent of further developing the peptides into a product to be evaluated *in vivo* to stop external and internal bleeding. The objectives of second and third phases were to test hemostatic properties of peptide-based biomaterials in small and large animal bleeding models relevant to hemorrhagic penetrating wounds on the battlefield. This purpose also includes the advancement of our understanding of *in vivo* issues for topical application of the biomaterials. The outcome of this contract is expected to generate effective hemostatic peptides and to establish *in vivo* bleeding

models, allowing the further development of hemostatic products to save the lives of soldiers from uncontrolled severe bleeding.

This report summarizes our work on development and optimization of new hemostatic peptides that exploit the procoagulant properties of amphipathic helices by attaching them to a biocompatible matrix for use to treat non-compressible hemorrhagic injuries.

2 Materials and Methods

2.1 Materials

IAP, and the negative control (ConGly) (LKRTIRTRLDDREGGEDGWGGTAA), and scrambled-IAP (LKKLKKLLLLKLLLLKLLLLKL), (HPLC purified, >90% pure) were synthesized by the Alberta Peptide Institute (API) (Edmonton, AB, Canada). Prothrombin, thrombin, factor IXa, factor Xa, factor X, Gla-domainless factor X, factor Va, and anti-factor X were purchased from Haematologic Technologies Inc. (Essex Junction, VT, USA). Factor VIII was from Bayer. Chromogenic Substrate for factor Xa, Chromogenix S2238, S2765 Chromogenic plasmin substrate, and Chromogenix S-2251 were purchased from Diapharma, (Columbus, OH, USA). Bovine Serum Albumin (BSA), and inorganic salts were purchased from Sigma Biochemicals (St. Louis, MO, USA). PBS was purchased from Wisent Inc. (St-Bruno, QC, Canada). The Polysorp plates were purchased from Nunc (Rochester, NY). The Surgicutt templates were purchased from VWR. Four-arm poly(ethylene glycol) succinimidyl glutarate (PEG), with a number-averaged molecular weight of 10,000 was obtained from Polymer Source (Dorval, QC, Canada). Poly(allylamine hydrochloride) (PAA), with a weight-averaged molecular weight of approximately 15,000 and 70,000, was purchased from Sigma-Aldrich (Mississauga, ON, Canada). DuraSeal® sealant system was obtained from Confluent Surgical, Inc. (Waltham, MA, USA).

2.2 Activation of factor VIII

A dialysis of FVIII into 0.15 M NaCl/20 mM HEPES/5 mM $CaCl_2$ pH 7.4 was conducted using centricon 100. FVIII was then activated using 3.6 μM of human thrombin. FVIII and thrombin reacted for 10 minutes at 37°C. Thrombin was then inhibited using 0.3 μM PPACK (D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone). Thrombin and PPACK were finally removed from the FVIIIa by centrifugation using 0.1 M sodium acetate/0.5 M NaCl pH 5.5 and 0.025 M sodium acetate/ 0.5 M NaCl/ 5 mM $CaCl_2$ pH 5.5 and centricon 100.

To verify that FVIII was activated to FVIIIa, a SDS-PAGE was run. The concentration of FVIIIa obtained was calculated using absorbance at 280 nm and an extinction coefficient of 1.6.

2.3 Factor Xa generation assay

The generation of factor Xa by the serine protease factor IXa and different peptides, as cofactors, was followed by a two-step amidolytic assay. Factor IXa (20 nM, final concentration) was incubated with an optimal concentration of peptide in assay buffer (150 mM NaCl, 20 mM Hepes, pH 7.4, 1.5 mM $CaCl_2$, and 0.1% BSA). The reactions were started with the addition of factor X, or Gla-domainless factor X, at varying concentrations ranging from 15 nM to 1000 nM (final concentrations). The reactions were stopped 30 minutes after the addition of factor X, or Gla-domainless factor X, with an EDTA solution (5 mM final concentration) containing a factor Xa-specific chromogenic substrate S2765 (0.2 mg/ml final concentration). The amount of generated factor Xa was determined immediately after the addition of the stopping solution with S2765 by continuous OD readings at 405 nm in a Molecular Devices ELISA plate reader, sampling every 10 seconds for a total period of 15 minutes. Concentration of generated factor Xa was calculated based on a calibration curve run on the same assay plate, simultaneously with the assay. Factor Xa or Gla-domainless factor Xa concentrations in the calibration curve varied from 0.39 nM to 25 nM. Alternatively, the assays were performed on plates coated with different peptides at varying concentrations. The experimental procedure remained the same, with the exception that the peptide was not added to the reaction solution because it was previously attached to the microtest plate surface. For experiments using FVIIIa as a cofactor, 12.5 nM of FVIIIa were added to each well.

2.4 Thrombin generation assay

Thrombin generation from Prothrombin by factor Xa in the presence of IAP was followed by a two-step amidolytic assay, in a similar fashion to the factor Xa assay. Briefly, 3 nM factor Xa was incubated with 5 μM IAP, or no peptide, and prothrombin varied from 0.08 μM to 5 μM in assay buffer. Reactions were stopped 30 minutes after the addition of Prothrombin with 5 mM EDTA and a Thrombin-specific chromogenic substrate S2238

(0.2 mg/ml final concentration). The amount of generated Thrombin was determined immediately after the addition of the stopping solution with S2238 by continuous OD readings at 405 nm in a Molecular Devices ELISA plate reader, sampling every 10 seconds for a total period of 5 minutes. Concentration of generated Thrombin was calculated based on a calibration curve run on the same assay plate, simultaneously with the assay. Thrombin concentrations in the calibration curve varied from 0.94 nM to 60 nM. For experiments using FVa as a cofactor, 1.25 nM of FVa were added to each well.

2.5 Coating of plates

Peptides were diluted to the desired concentration in PBS (10 mM phosphate, pH 7.4, 150 mM NaCl, 3 mM KCl) and 100 µl of the resulting solution were added to each well of a 96-well polystyrene microtest plate (BD Falcon 353279, BD Falcon 353910, Sarstedt 82.1582, Nunc Apogent Polysorp or Nunc Apogent Maxisorp) and incubated overnight at 4°C. The following morning, the plates were washed 5 times with PBS (500 µl per well). The plates were blocked with 1% BSA in PBS or 5% dry milk in PBS for 2 hours at room temperature and washed 5 times with PBS.

2.6 Clot lysis and Plasminogen activation

5 µl of thrombin (2.42 µM final concentration) and 5 µl t-pA (tissue-type plasminogen activator) (147 nM final concentration) were placed on opposite sides of wells in a flat-bottom 96-well plate. 90 µl of a solution containing fibrinogen (2.35 µM final concentration), lysine-plasminogen (0.55 µM final concentration), S-2251 (0.44 mM final concentration), $CaCl_2$ (2.22 mM final concentration) and different concentrations of IAP were then added to each well. Absorbance values at 405 and 490 nm were continuously read in a Molecular Devices ELISA plate reader, sampling every 10 seconds for a total period of two hours. Absorbance values at 490 nm were subtracted from those at 405 nm to measure S-2251 hydrolysis corrected for turbidity.

2.7 *In vivo* experiments on rabbits models of hemorrhage, ear bleeding model

New Zealand white rabbits weighing 2.5-3 kg were used to establish a bleeding model. No anesthesia is required for these experiments. Incisions were made in the central ear artery, at 3.5 cm from the tip of the ear, using Surgicutt templates. DuraSeal®, pure or containing IAP is then applied directly on the wound, and the time for bleeding to stop is recorded.

2.8 *In vivo* experiments on rabbits models of hemorrhage, liver laceration model

New Zealand white rabbits weighing 2.5-3 kg were used. The animals were anesthetised with ketamine and xylazine. An incision of 2 cm in length and 0.5 cm in depth was made using a scalpel blade #11 on the liver median lobe. This wound produces an adequate amount of blood suitable for reproducible measurements. PEG-PAA, pure or with IAP, is then applied directly on the wound, and the time for bleeding to stop is recorded.

2.9 *In vivo* swine liver bleeding model

Male domestic swine weighing between 35 and 40 kg were used. All pigs were anesthetised with a Pre-mix cocktail and anesthesia was maintained with isoflurane in oxygen. The liver was exposed, and a scalpel blade #11 was used to create a wound 0.5 cm deep and 2 cm long on the liver median lobe. PEG-PAA (alone or with 30 μM IAP in the PAA solution) was then applied directly on the wound. The time needed for the bleeding to stop was recorded. On every swine, two different wounds were done on the median lobe, one to test the PEG-PAA alone and the other to test PEG-PAA with IAP.

2.10 *In vivo* swine femoral artery bleeding model

Following the liver experiment and with the animal still under anesthesia, femoral artery bleeding experiments were performed on the same animals. The femoral artery was exposed for approximately 4 cm. A stab wound was made with a needle (23G). PEG-PAA (alone or with 30 μM IAP in the PAA solution) was then applied directly on the wound. The time needed for the bleeding to stop was recorded. On every animal, one

femoral artery was used to test the PEG-PAA alone and the other was used to test PEG-PAA with IAP.

3 Results

During the first year of the project, the *in vitro* biochemical development of a hemostatic peptide that can be attached to a biocompatible surface for future *in vivo* applications was optimized. The peptide that was developed was based on an ideal amphipathic peptide structure (named IAP, ideal amphipathic peptide) capitalizing on the biophysical properties of the $fVIII_{2303-23}$ that was initially described by Blostein et al. (2000) [4]. We focused on analyzing IAP as a potential hemostatic agent by performing different *in vitro* assays. Factor X activation assays were conducted and gave promising results by improving the catalytic efficiency of factor IXa. We also tested the IAP attached to a surface and showed that the surface-bound peptide maintained its catalytic enhancing properties for factor IXa.

CD (circular dichroism) spectroscopy experiments were performed to confirm the helical structure of IAP and the variant peptides described below. We showed that the secondary structure of IAP was preserved, consistent with the preservation of its activities in *in vitro* activity assays (see figure 1 below).

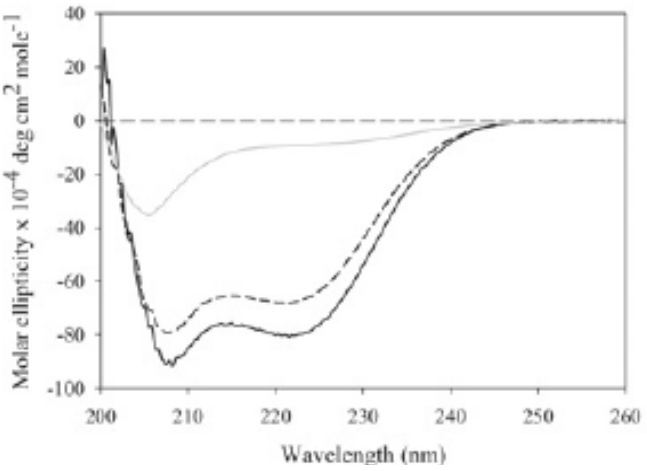
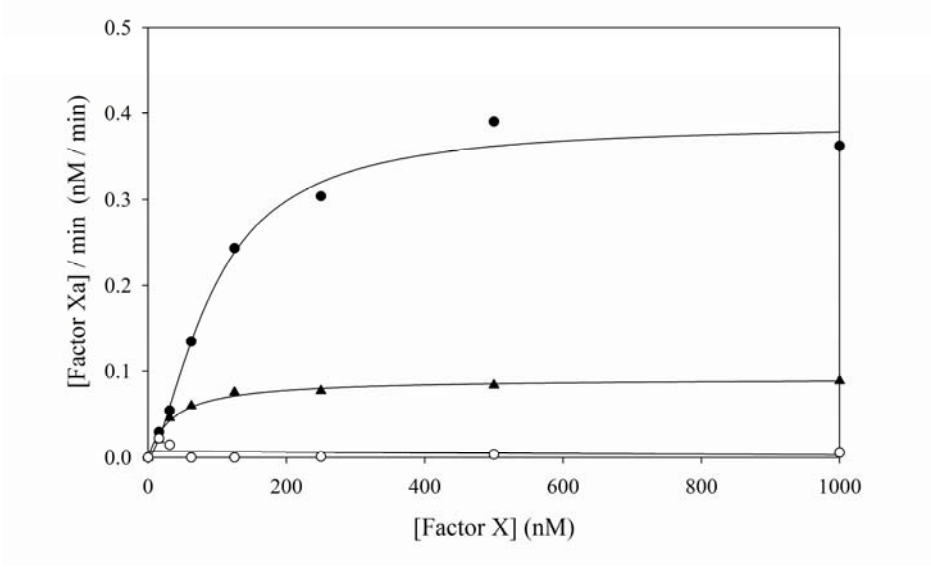


Figure 1: CD spectra of 200 µM of the three different peptides in aqueous buffer. IAP (black continuous line), scrambled-IAP (broken line), and ConGly (grey continuous line).

We also analyzed IAP activity in enzymatic reactions of the blood coagulation cascade by performing factor Xa and thrombin generation assays. We first conducted these experiments with the peptide in solution, see figure 2 below.

A



B

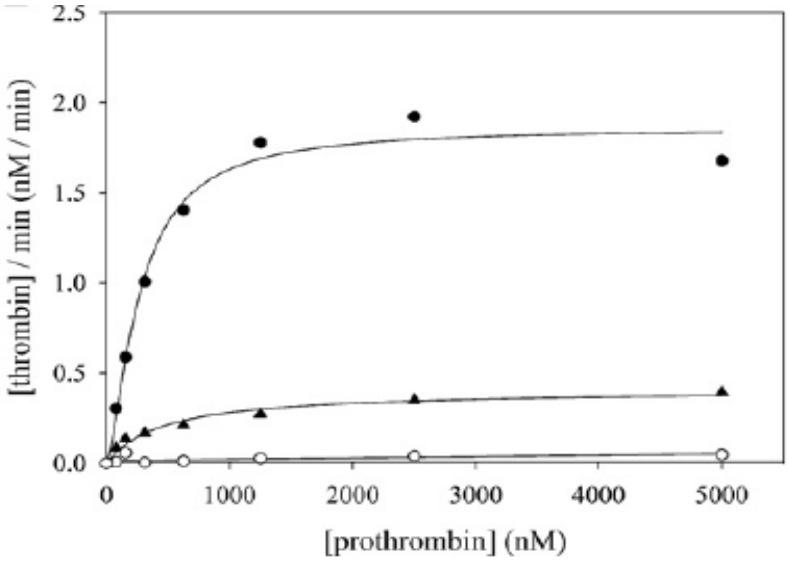


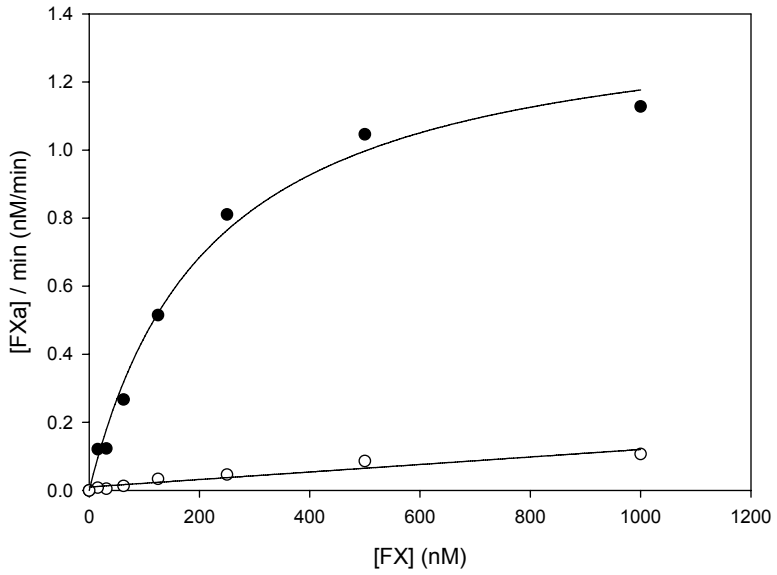
Figure 2: The activity of the tenase and the prothrombinase complexes is enhanced in the presence of IAP. A) Factor Xa generation was measured with various concentrations of factor X in the presence of 20 nM factor IXa and 2.5 μM IAP (black dots), 2.5 μM scrambled-IAP (black triangles), or 2.5 μM ConGly (white dots). B) Thrombin generation was measured with various concentrations of prothrombin in the presence of 3 nM factor Xa and 2.5 μM IAP(black dots), 2.5 μM scrambled-IAP (black triangles), or 2.5 μM ConGly (white dots).

Factor Xa generation assays were also conducted with the cofactor, factor VIIIa, as well as thrombin generation assays with the cofactor, factor Va [2]. The similar results obtained again show that IAP is a potential hemostatic agent.

We also attached the peptide IAP or ConGly, our negative control, to the test plates before performing factor Xa and thrombin generation assays in order to test the peptide's hemostatic properties when it is attached to a surface as opposed to being in solution (see figure 3 below).

Again, we performed the same experiment adding the cofactors, factor VIIIa or factor Va, and obtained similar results, again confirming the hemostatic potential of IAP (see figure 4 below).

A



B

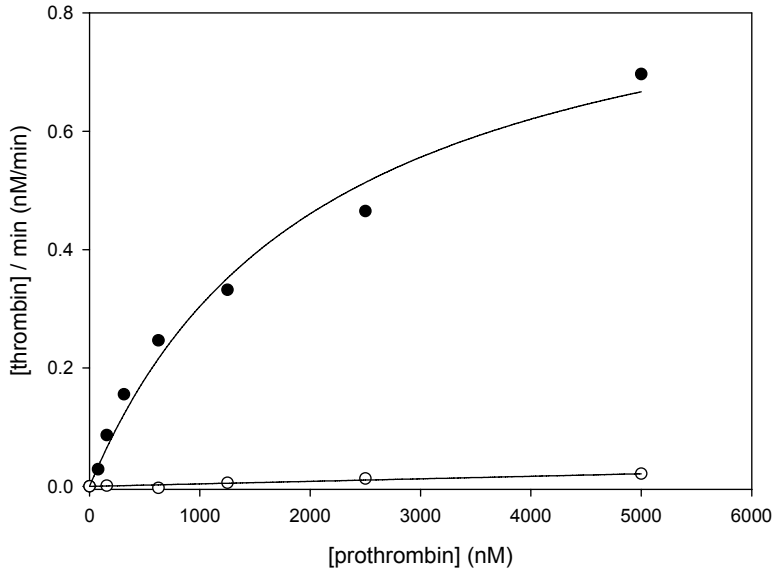
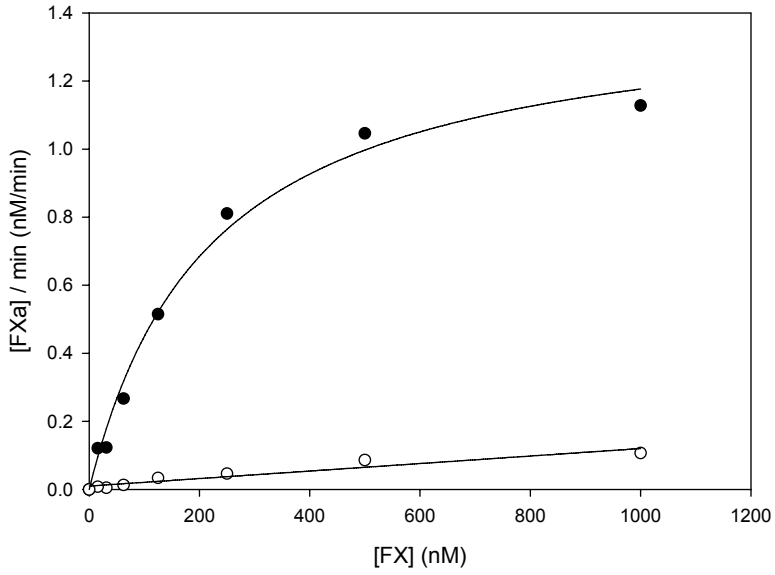


Figure 3: The activity of the tenase and the prothrombinase complexes is enhanced in the presence of surface-bound IAP. A) Factor Xa generation was measured with various concentrations of factor X in the presence of 20 nM factor IXa and 30 μM IAP (black dots), or 30 μM ConGly (white dots). B) Thrombin generation was measured with various concentrations of prothrombin in the presence of 3 nM factor Xa and 30 μM IAP (black dots), or 30 μM ConGly (white dots).

A



B

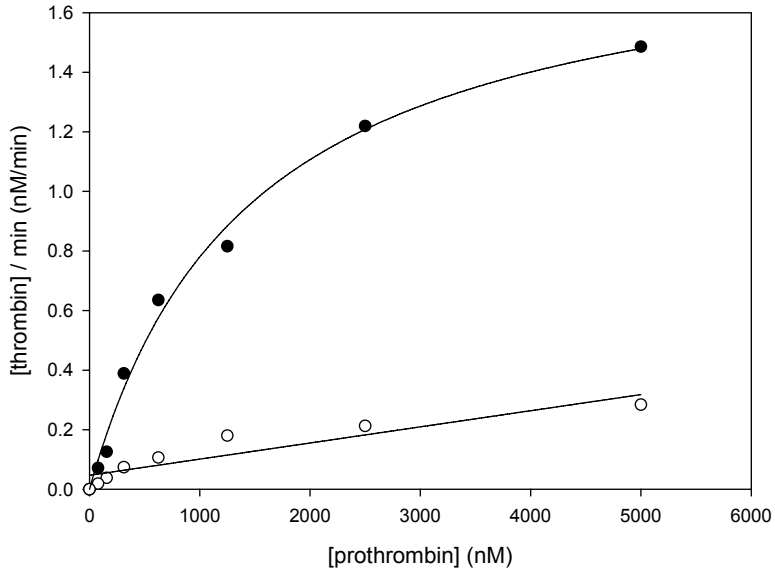


Figure 4: The activity of the tenase and the prothrombinase complexes is enhanced in the presence of surface-bound IAP and in the presence of the cofactors. A) Factor Xa generation was measured with various concentrations of factor X in the presence of 20 nM factor IXa and its cofactor factor VIIIa, and 30 μM IAP (black dots), or 30 μM ConGly (white dots). B) Thrombin generation was measured with various concentrations of prothrombin in the presence of 3 nM factor Xa and its cofactor factor Va, and 30 μM IAP(black dots), or 30 μM ConGly (white dots).

Given the known anti-fibrinolytic (and hence prothrombotic) nature of lysines and given the presence of several lysine residues in IAP, we conducted an assay for clot lysis in a purified system in order to test the affect of IAP on fibrinolysis. The results show that IAP retards clot lysis as shown in figure 5 below.

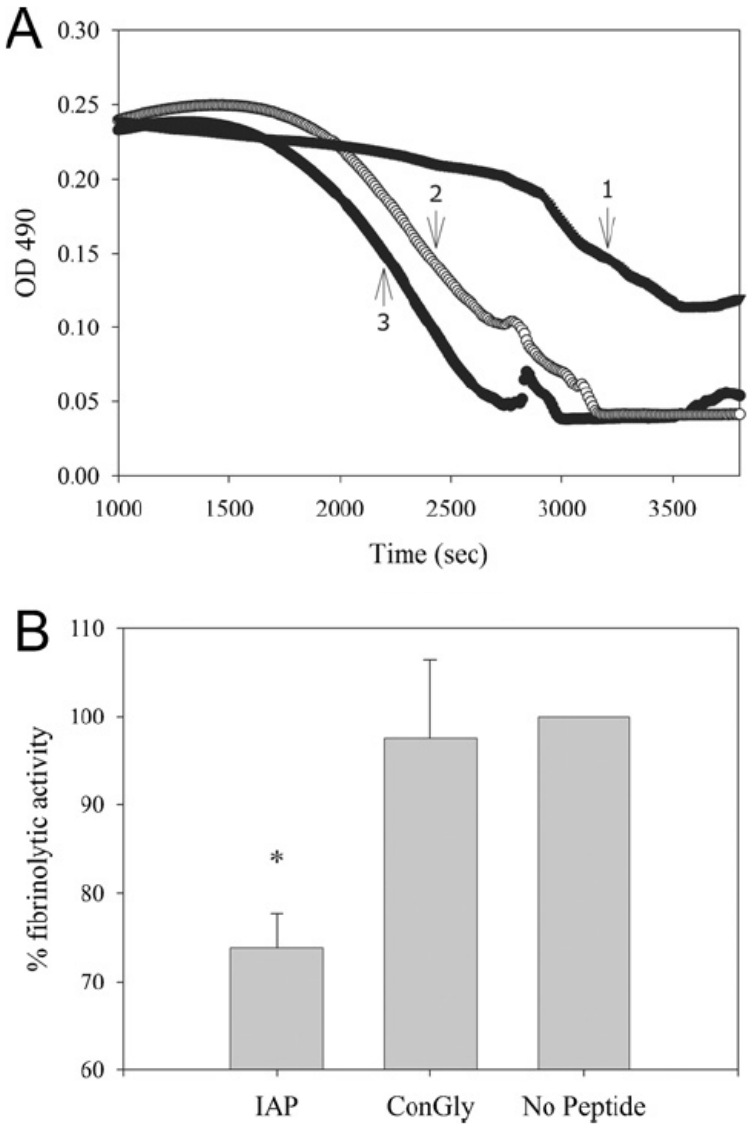


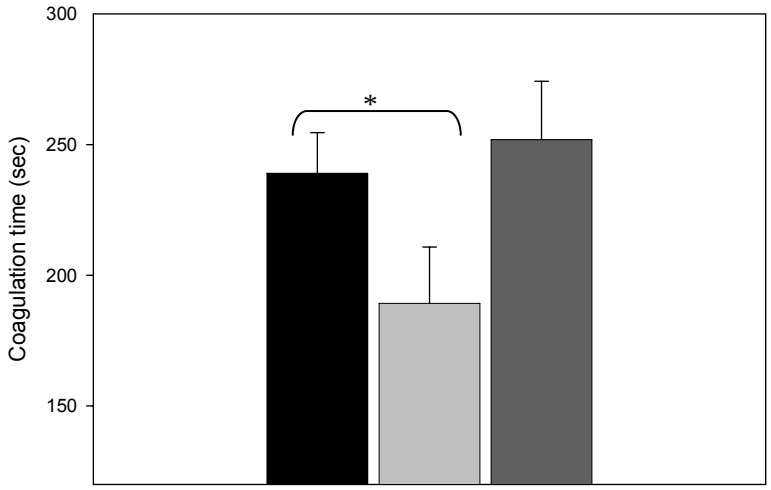
Figure 5: IAP inhibits fibrinolysis by delaying clot lysis. A) Representative plot of the effect of IAP (1), ConGly (2) or no peptide (3), in tPA-dependent clot lysis as determined using a purified system. Time at half lysis is indicated by the arrows. OD 490, absorbance at 490 nm. B) Reduction of fibrinolysis as related to no peptide was calculated from the half-lysis times (n=5; P=0.02).

During this first phase of the project, other *in vitro assays* were conducted and are presented and are published [6] or outlined in the phase 1 interim reports and the first annual report.

The second year of the project involved the *in vivo* testing of our peptide IAP on small animal models, rabbits. We developed two distinct bleeding models using these animals, an ear bleeding model and a liver laceration model. IAP was attached to a commercially available sealing agent (DuraSeal®), containing polyethylene glycol-trilysine amine, for the ear bleeding model. For the liver laceration model, polyethylene glycol-poly(allylamine) (PEG-PAA) in-situ forming hydrogels were developed to combine with IAP. The obtained results are presented below.

These results in Figure 6 show that IAP, when incorporated to DuraSeal, decreases bleeding time by 25% in ear bleeding models in rabbits. We also showed that when incorporated to PEG-PAA, IAP decreases bleeding time by 40% in liver laceration models in rabbits.

A



B

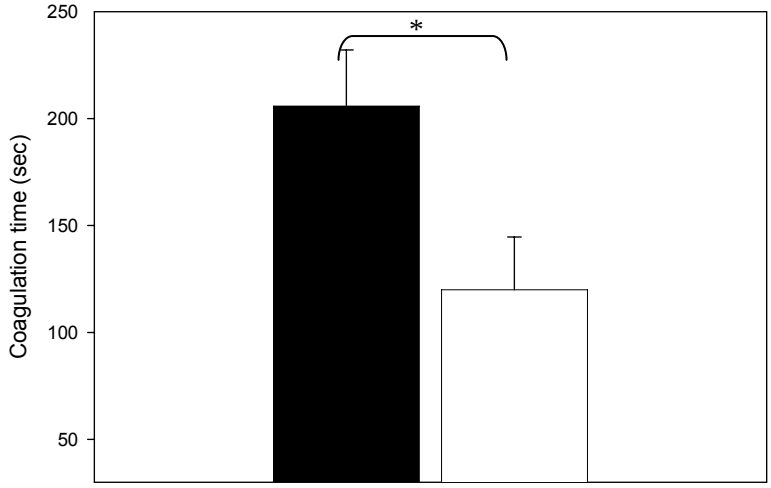


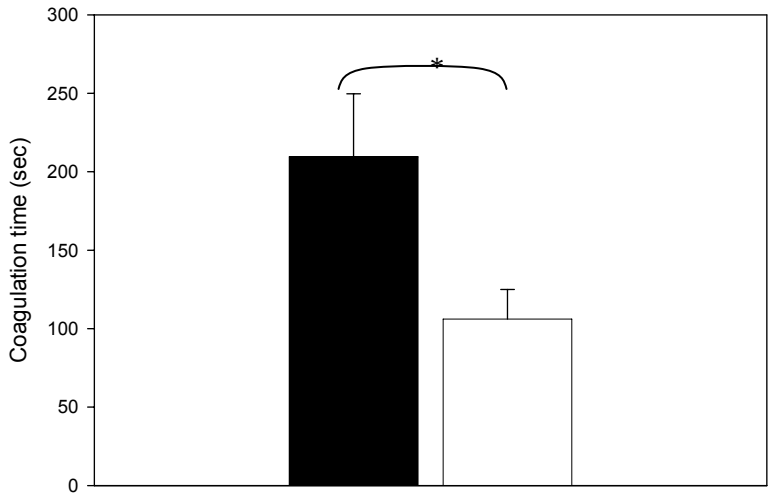
*Figure 6: IAP decreases bleeding time in rabbit hemorrhage models. A) DuraSeal® was used either alone (black bar), or with IAP (light gray bar), or with ConGly (dark gray bar) on ear bleeding model in rabbits. (*P < 0.03, n=20). B) PEG-PAA was used either alone (black bar), or with IAP (white bar) on liver laceration model in rabbits. (*P < 0.02, n=12).*

The third and last year of this research project involved the development and optimization of *in vivo* hemorrhage models in large animals, namely swine. Again, we

developed two distinct models in those animals: a liver laceration model, similar to the one conducted in the rabbits, and a femoral artery injury model. In both of these models, we compared pure PEG-PAA to PEG-PAA containing IAP. The results obtained are presented below.

From figure 7, we see that the incorporation of IAP into PEG-PAA reduces by almost 50% the bleeding time in liver laceration models, and by 34% in the femoral artery bleeding model. In Conclusion, we definitely demonstrate that IAP incorporated in PEG-PAA is an effective biocompatible matrix for reducing hemorrhage *in vivo*.

A



B

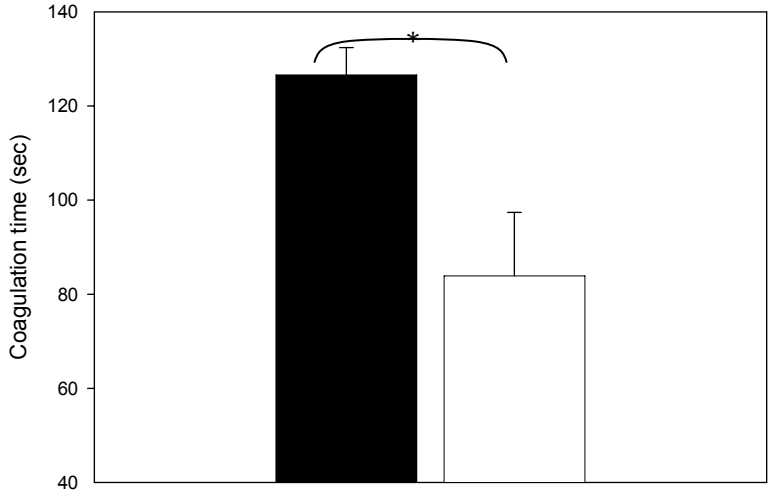


*Figure 7: IAP decreases bleeding time in swine hemorrhage models. A) PEG-PAA was used either alone (black bar), or with IAP (white bar) on the liver laceration model in swines. (*P < 0.02, n=11). B) PEG-PAA was used either alone (black bar), or with IAP (white bar) on the femoral artery injury model in swines. (*P<0.01, n=7).*

4 Discussion

During the current project aimed at developing a hemostatic surface for controlling hemorrhage, we developed and tested a biocompatible matrix containing polyethylene glycol and poly(allylamine) (PEG-PAA) coupled with a hemostatic peptide called IAP. We first conducted *in vitro* experiments assessing the efficacy of IAP on enzyme activity and clotting times. We showed that IAP significantly enhanced procoagulant activities of both factor IXa and Xa. We believe that the efficacy of this peptide is due to its physicochemical properties, namely its amphipathicity and helical structure. It is suggested that IAP exerts its procoagulant function by assisting in the assembly of the coagulation enzymes and substrates through their Gla (γ-carboxyglutamic acid)-rich domains. IAP is also procoagulant by inhibiting fibrinolysis (i.e. clot lysis) (see our first published paper [6]).

With regard to hemostasis, IAP presents several advantages over previously published amphipathic peptides that have been shown to enhance factor IXa activity. First, IAP accelerates both factor IXa and thrombin activity. Secondly, full tenase and prothrombinase activities (in the presence of cofactors) are promoted by IAP. Thirdly, given its simple structure, biophysical properties such as length and charge of the peptide can be easily altered to further refine hemostatic efficacy. Fourthly, IAP contains several positively charged lysine residues that inhibit clot lysis *in vitro*, and therefore can be anti-fibrinolytic. Given the *in vitro* properties of IAP, its effect *in vivo* was then tested in different animal models of hemorrhage.

Animal models in hemorrhage research can be classified into two general categories: small animal models (mice, rats and rabbits) and large animal models (dogs, swine and sheep) [7]. The choice of an animal model depends on the research goal. In our case, we first tested IAP's hemostatic properties in small animal models (rabbits) before moving into larger animals (swine). One of the main advantages of using rabbits instead of other small animals (mice or rats) is the larger blood volume making quantification of blood loss feasible. The rabbit's blood volume has been estimated at 56 ml/kg. Porcine models also provide many advantages. The swine cardiovascular and

hemodynamic responses are closely related to human responses; another important advantage is that wound healing is very similar in pig and human skin.

IAP was coupled to a matrix composed of polyethylene glycol and trilysine amine (DuraSeal®) or PEG-PAA prior to its use to stop bleeding since we demonstrated that IAP attached to a surface is effectively procoagulant *in vitro*. We first used DuraSeal®, a commercially available sealing agent, with IAP. We then applied DuraSeal®-IAP in an ear bleeding model in rabbits to test the hemostatic properties of IAP. The use of Surgicutt templates to create wounds on the ear artery was tested and proved to give reproducible results. To our knowledge, this is the first attempt at using DuraSeal® as a biocompatible matrix to stop hemorrhage *in vivo*. Results shown in figure 6 demonstrate that IAP decreases bleeding time by about 25% when incorporated to DuraSeal®.

A liver laceration model was then developed in order to test IAP on an internal wound which produces significant blood loss. We first attempted to again use DuraSeal® on those wounds. However, because this material alone arrested bleeding quickly, it was impossible to evaluate the hemostatic property of IAP incorporated into this matrix. We hypothesize that the fast action of DuraSeal® on the liver wound as opposed to its relatively slow action on the ear is due to the lower surface temperature on the exposed surface. The liver temperature is around 37°C whereas the temperature of rabbits' ears is relatively lower. DuraSeal® is a sealing agent that is optimized for surgical procedures, and thus it is not surprising that this material works better at 37°C. Because of this, we developed a similar sealing material composed of a solution of polyethylene glycol (PEG) and poly(allylamine) (PAA) (PEG-PAA). When comparing this material alone and with IAP on liver laceration models in rabbits, we found that the incorporation of the peptide reduces the bleeding times by about 40% (see figure 6). Finally, we tested PEG-PAA/IAP on bleeding models in larger animals (pigs). We developed a liver laceration model and a femoral artery bleeding model on those animals. We found that the incorporation of IAP into the PEG-PAA material reduced the bleeding times by almost 50% in the liver bleeding model and by 34% in the femoral artery injury model (figure 7).

In conclusion, at the completion of our three-year project for Defence Research and Development Canada - Toronto, we have identified a biocompatible matrix that has *in vivo* activity to arrest bleeding in animal models of hemorrhage. This matrix is composed of PEG-PAA coupled to a procoagulant peptide, IAP. This material is readily produced and has been shown *in vivo* to reduce bleeding times by 40-50%. The goal of future experimentation would be to further characterize this material in animal models of trauma with the penultimate goal of testing this material in humans.

5 References

[1] Gustafson, S.B., Fulkerson, P., Bildfell, R., Aguilera, L., and Hazzard, T., M. Chitosan dressing provides hemostasis in swine femoral arterial injury model. Prehospital Emergency Care, 2007. 11: p. 172-178.

[2] Acheson, E.M., et al. Comparison of hemorrhage control agents applied to lethal extremity arterial hemorrhages in swine. The Journal of Trauma, 2005. 59(4): p. 865-875.

[3] Wedmore, I., McManus, J. G., Pusateri, A. E., and Holcomb, J. B. A special report on the chitosan-based hemostatic dressing: experience in current combat operations. The Journal of Trauma, 2006. 60: p. 655-658.

[4] Blostein, M.D., et al. Amphipathic helices support function of blood coagulation factor IXa. Biochemistry, 2000. 39: p. 12000-6.

[5] Alam, H.B., Chen, Z., Jaskille, A., Querol, R.I., Koustova, E., Inocencio, R., Conran, R., Seufert, A., Ariaban, N., Toruno, K., and Rhee, P. Application of a zeolite hemostatic agent achieves 100% survival in a lethal model of complex groin injury in swine. The Journal of Trauma, 2004. 56: p. 974-983.

[6] Ganopolsky, J.G., Charbonneau, S., Peng, H.T., Shek, P.N., Blostein, M.D. Characterization of an Ideal Amphipathic Peptide as a Procoagulant Agent, Biochemical Journal, 2008. 412: p. 545-551.

[7] Majde, J.A. Animal models for hemorrhage and resuscitation research. The Journal of Trauma, 2003. 54: p. S100-S105.

Distribution list

Document No.: DRDC Toronto CR 2009-014

LIST PART 1: Internal Distribution by Centre

0 TOTAL LIST PART 1

LIST PART 2: External Distribution by DRDKIM

1 Library and Archives Canada

1 TOTAL LIST PART 2

1 TOTAL COPIES REQUIRED

DOCUMENT CONTROL DATA
(Security classification of the title, body of abstract and indexing annotation must be entered when the overall document is classified)

1. ORIGINATOR (The name and address of the organization preparing the document, Organizations for whom the document was prepared, e.g. Centre sponsoring a contractor's document, or tasking agency, are entered in section 8.)

Publishing: DRDC Toronto

Performing: Jewish General Hospital, Mc Gill University, 3999 Chemin de la Cote−St−Catherine, Montreal, Quebeci

Monitoring:

Contracting: DRDC Toronto

2. SECURITY CLASSIFICATION (Overall security classification of the document including special warning terms if applicable.)

UNCLASSIFIED

3. TITLE (The complete document title as indicated on the title page. Its classification is indicated by the appropriate abbreviation (S, C, R, or U) in parenthesis at the end of the title)

Development and Optimization of Hemostatic Peptides for Hemorrhage Control on Battlefields (U)
(U)

4. AUTHORS (First name, middle initial and last name. If military, show rank, e.g. Maj. John E. Doe.)

Sophi Charbonneau; Mark D. Blostein

5. DATE OF PUBLICATION (Month and year of publication of document.)

February 2009

6a NO. OF PAGES (Total containing information, including Annexes, Appendices, etc.)

37

6b. NO. OF REFS (Total cited in document.)

7

7. DESCRIPTIVE NOTES (The category of the document, e.g. technical report, technical note or memorandum. If appropriate, enter the type of document, e.g. interim, progress, summary, annual or final. Give the inclusive dates when a specific reporting period is covered.)

Contract Report

8. SPONSORING ACTIVITY (The names of the department project office or laboratory sponsoring the research and development − include address.)

Sponsoring:

Tasking:

9a. PROJECT OR GRANT NO. (If appropriate, the applicable research and development project or grant under which the document was written. Please specify whether project or grant.)

9b. CONTRACT NO. (If appropriate, the applicable number under which the document was written.)

W7711−057957/001/TOR

10a. ORIGINATOR'S DOCUMENT NUMBER (The official document number by which the document is identified by the originating activity. This number must be unique to this document)

DRDC Toronto CR 2009−014

10b. OTHER DOCUMENT NO(s). (Any other numbers under which may be assigned this document either by the originator or by the sponsor.)

11. DOCUMENT AVAILABILITY (Any limitations on the dissemination of the document, other than those imposed by security classification.)

Unlimited distribution

12. DOCUMENT ANNOUNCEMENT (Any limitation to the bibliographic announcement of this document. This will normally correspond to the Document Availability (11), However, when further distribution (beyond the audience specified in (11) is possible, a wider announcement audience may be selected.))

Unlimited announcement

DOCUMENT CONTROL DATA
(Security classification of the title, body of abstract and indexing annotation must be entered when the overall document is classified)

13. ABSTRACT (A brief and factual summary of the document. It may also appear elsewhere in the body of the document itself. It is highly desirable that the abstract of classified documents be unclassified. Each paragraph of the abstract shall begin with an indication of the security classification of the information in the paragraph (unless the document itself is unclassified) represented as (S), (C), (R), or (U). It is not necessary to include here abstracts in both official languages unless the text is bilingual.)

(U) The goal of the research contract conducted for the Defence Research and Development Canada – Toronto, was to develop a hemostatic biocompatible surface to treat hemorrhagic injuries sustained on the battlefield. During the first year of the contract, a candidate hemostatic amphipathic helical peptide (an ideal amphipathic helical peptide or IAP) was developed (published in Biochemical Journal 412: 545−51 2008) and different biocompatible surfaces were tested (published in Journal of Biomaterials Research, Part B: Applied Biomaterials, 2008 Sep 5). Once the peptide and biomaterial were identified, the second year was dedicated to testing IAP coupled to different surfaces and we finally settled on IAP coupled to polyethylene glycol and poly(allylamine) (PEG−PAA) for further testing. In vivo work testing this material was carried out on small animals (rabbits) and subsequently on large animals (pigs) during the second and third year. We have data that demonstrates the feasibility of such material to arrest bleeding in vivo by reducing hemorrhage by 40−50%.

(U) Ce contrat de recherche fait pour le Département de Recherche Nationale avait pour but de développer une surface hémostatique biocompatible pour traiter les blessures externes ayant lieu sur des champs de bataille. Durant la première année du projet, un peptide amphipatique et hélitique (IAP : ideal amphipathic peptide, ou peptide amphipatique idéal) a été développé (publication : Biochemical Journal 412: 545−51 2008) et différentes surfaces biocompatibles ont été testées (publication : Journal of Biomaterials Research, Part B: Applied Biomaterials, 2008 Sep 5). Une fois le peptide et le biomatériau identifiés, la deuxième année du projet a servi à tester le couplage du peptide (IAP) à différentes surfaces. Nous avons finalement conclu que le peptide couplé au polyéthylène glycol−poly(allylamine) (PEG−PAA) offrait les meilleurs résultats. La troisième année du contrat a été dédiée aux tests in vivo. Des modèles hémorragiques ont été développés chez des lapins et des cochons afin de tester le peptide (IAP) couplé à la surface biocompatible (PEG−PAA). Les résultats ont démontrés que le peptide réduisait le temps de saignement de 40 à 50% chez les petits et gros animaux.

14. KEYWORDS, DESCRIPTORS or IDENTIFIERS (Technically meaningful terms or short phrases that characterize a document and could be helpful in cataloguing the document. They should be selected so that no security classification is required. Identifiers, such as equipment model designation, trade name, military project code name, geographic location may also be included. If possible keywords should be selected from a published thesaurus, e.g. Thesaurus of Engineering and Scientific Terms (TEST) and that thesaurus identified. If it is not possible to select indexing terms which are Unclassified, the classification of each should be indicated as with the title.)

(U) Peptides; Hemorrhage control