HEMOSTATIC EFFICIENCY OF AMPHIPHILIC PEPTIDE SOLUTION IN WISTAR RAT MODEL

by

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Abstract

One of the leading causes of death following traumatic injury is exsanguination. The body addresses bleeding through the process of hemostasis which includes the formation of a fibrin mesh structure that holds a blood clot together. During traumatic injury, hemostasis may be unable to stop excess bleeding. Fibrin based hemostatic agents have been developed, however, these studies often use fibrin obtained from biological sources, which poses risk of infection. A novel amphiphilic peptide (h9e) has been studied to form three dimensional nanofibers networks. In this research, we studied the ability to form a synthetically produced, fibrin-mimic, hemostatic material from the h9e peptide sequence. The objective of this study was to determine the blood gelation strength of the h9e peptide necessary to arrest bleeding in the Wistar Rat model.

Commercial mouse blood was used for blood gelation in vitro studies. Dynamic rheometer was used to determine the gelation kinetics at varied h9e peptide concentrations ranging from 1-5% wt. By directly mixing the h9e peptide with blood, we observed that the blood gelation strength right after mixing increased as the h9e peptide weight % concentration increased, from 67 to 1086 Pascals in the peptide concentration from 1 to 5%, respectively. After 24 hours, final gelation strength of all concentrations with commercial mouse blood was lower than the instantaneous strength but consistent throughout testing. Similar testing was conducted using commercial Wistar Rat blood with weight % concentrations of 1, 3, and 5% of h9e peptide. The gelation strength was 500, 1665, and 1914 Pascals, respectively. We also determined the gelation strength of Wistar Rat blood components, such as red blood cells, serum, and plasma

with 1% h9e peptide. We observed the gelation response induced with individual blood components; however, the strength is weaker than whole blood.

In vivo, we applied the cut-tail method by dipping the cut-tail of Wistar Rats into the h9e peptide solutions for 10 seconds and then took it out for blood lost collection. We observed that h9e peptide solution at 1, 3, and 5% weight concentrations can all generate hemostatic function. The h9e peptide solution at 5% weight concentration (1914 Pa) was able to outperform a commercial hemostatic material (Moore Medical CELOX* Hemostatic Granules), significantly reducing both bleeding time and blood lost: h9e peptide at 5% had a bleeding time of 94 sec and 0.75 mL blood lost, while the Celox hemostatic granules had a bleeding time of 225 sec and 1.5 mL blood lost.

Transmission Electron Microscopy and Spinning Disk Confocal Microscope imaging indicated a blood component reinforced, web-like, h9e nanofiber structure similar to the structure formed by fibrin in a blood clot. This study showed that h9e peptide has the potential to be used to induce hemostasis.

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Dedication

This thesis is dedicated to my beautiful daughter, Kaylee. Know that the world is yours for the taking and you can achieve anything in life that you want to. With God on your side NOTHING is impossible.

Chapter 1 - Introduction and Literature Review

1.1 Overview

One of the leading causes of death following a traumatic injury is exsanguination [1-3]. Among U.S. military personnel, hemorrhage is one of the greatest threats to survival as most deaths related to combat injuries occur before military personnel have the opportunity to reach a medical facility [4-5]. Among American civilians, bleeding is the leading cause of death in operating rooms [6]. Extensive blood loss due to hemorrhage is also a worldwide concern. Because exsanguination is responsible for a large percentage of deaths, over the past 40 years, there has been a significant amount of research conducted. To address this issue, several hemostatic materials have been developed.

1.2 Hemostasis

The body naturally addresses the issue of bleeding by the process of hemostasis. Hemostasis is the formation of a blood clot at the wound site [7]. Hemostasis occurs in two phases: Primary and Secondary. During the primary phase of hemostasis, platelets adhere to the wound site. In damaged vascular tissue, platelets release chemical signals that initiate the second phase of hemostasis: The coagulation cascade.

The coagulation cascade is a sequential activation of enzymes that proceeds through two enzymatic pathways: Intrinsic (contact activation) and extrinsic (tissue factor) [7-8]. The intrinsic pathway begins upon damage to endothelial tissue. It activates factor XII which activates a sequence of additional factors. This leads to the activation of factor X [8]. The extrinsic pathway is initiated at the site of the wound as a response to the exposure of tissue factor to blood [8]. This activates factor VII, leading to the step-wise activation of factor X. Both the intrinsic and

extrinsic pathways join at the activation of factor X, which is referred to as the common pathway (Figure 1.1). Factor X is a prothrombinase that cleaves prothrombin into thrombin. Thrombin then activates fibrinogen into fibrin. Fibrin then cross-links at the site of the wound, forming a web, which maintains the structure of the platelet plug, forming a blood clot [8]. Figure 1.2 provides a visual schematic of the formation of a blood clot.

1.3 Hemostatic Agents

Though the body has a process by which to address the issue of bleeding, during a traumatic injury, the body may be unable to stop or slow the amount of bleeding caused by the injury. When the body is unable to control the amount of blood lost, survival is threatened due to hemorrhaging and exsanguination may occur. To address these issues, several hemostatic agents, employing a variety of mechanisms, have been developed. Hemostatic agents stop bleeding by either their own mechanical methods or by reinforcing the coagulation cascade [7].

Hemostatic materials can be classified a number of different ways. Generally they are classified as either biologically based or synthetic. Biologically based materials are those derived directly from living organisms. Biologically based materials are effective in controlling hemorrhage but may pose a risk of infection if derived from a contaminated source [7,9]. Synthetic materials are those chemically derived from sources other than living organisms. Synthetic materials are also effective in controlling hemorrhage and do not pose a risk of infection. However, synthetic materials may not be designed to control hemorrhage in severe wounds [8]. Table 1.1 provides an overview of selected hemostatic materials and evaluates some of their strengths and weaknesses.

1.4 Biologically Based Hemostatic Agents

1.4.1 Fibrin Based Materials

Toward the end of coagulation cascade, fibrinogen is converted to fibrin in the presence of thrombin. Fibrin is an insoluble, fibrillar protein, formed at the site of an injury in response to the induction of the coagulation cascade [10]. Fibrin is arranged in long chains that cross-link to form a mesh network. The fibrin mesh entraps platelets, forming a clot that prevents further blood loss [7, 10].

Fibrin based products are usually derived from either human or bovine thrombin found in blood plasma. Fibrin based products include two main components generally packaged in syringes: One containing a fibrinogen suspension and one containing an activation solution (thrombin or calcium). Fibrin based products are applied by releasing the contents of both syringes simultaneously, combining to form a fibrin matrix used to arrest bleeding when applied to the wound site [7, 11]. Fibrin adhesives can be applied as an aerosol spray or as a liquid fibrin glue. Some examples of fibrin sealants include *Tisseel* (Baxter International Incorporated, Deerfield, IL, USA), *Beriplast P* (CSL Behring, King of Prussia, PA, USA), and *Evicel* (Ethicon US, LLC, Somerville, NJ, USA).

Fibrin based products have some advantages over other products. Mainly, fibrin based products are fast acting and very effective due to the natural mechanical role of fibrin in the coagulation cascade. Fibrin based products are also biodegradable [7, 11]. There are also risks involved with Fibrin based products. They can be very expensive. Fibrin based products may also pose the risk of infection. Though plasma used for the formation of fibrin based products is screened for viruses, the risk of infection cannot be fully eliminated [7, 11].

1.4.2 Thrombin based products

At the start of the common pathway, activated factor X cleaves prothrombin into thrombin (Figure 1.1). Thrombin (activated factor II) is the pivotal enzyme within the coagulation cascade and is responsible for several functions. Thrombin activates several clotting factors and encourages platelet adhesion [12]. However, the main function of thrombin is to convert fibrinogen to fibrin.

Thrombin based products are derived from one of three main sources: Bovine thrombin, human thrombin, or human recombinant thrombin. Bovine thrombin is obtained by extracting prothrombin from bovine plasma and activating it into thrombin [12]. Human thrombin is obtained by extraction from human plasma [12]. Human recombinant thrombin is obtained from recombinant DNA procedures performed on a genetically modified Chinese hamster ovary cell line [12-13]. Thrombin based products should be applied directly to the wound. Some examples of Thrombin based products include *Thrombin-JMI* (Pfizer Incorporated, Peapack, NJ, USA), *Recothrom* (ZymoGenetics Incorporated, Seattle, WA, USA), and *Evithrom* (Ethicon US, LLC, Somerville, NJ, USA).

There are also a few combination products including *Evicel* (mentioned in the fibrin based materials section) and *FloSeal* (Baxter Healthcare Corporation, Deerfield, IL, USA). *Evicel* uses thrombin to activate fibrinogen into fibrin for hemostatic use. *FloSeal* is a gelatin matrix and thrombin combination product. FloSeal is composed of a thrombin component and a cross-linked gelatin [7, 14, 17]. The thrombin component activates the coagulation cascade while the matrix simultaneously serves as a sealant to stop bleeding [15].

Thrombin based products have some advantages over other products. Mainly, thrombin based products are fast-acting, can be applied directly to the wound site, and are effective due to

the natural role of thrombin in the coagulation cascade [12,15,17]. Though thrombin based products have some advantages over other products, there are also risks involved. Thrombin based products may also pose the risk of immunological response, particularly those derived from bovine thrombin [7, 16]. Though all types of plasma used for the formation of thrombin based products are either screened for viruses, purified, or both, the risk of infection cannot be fully eliminated [7, 17].

1.4.3 Other Noteworthy Biologically Based Hemostatic Agents

While a majority of biologically based hemostatic materials include fibrin or thrombin components, there are a few additional types of biologically based hemostatic materials available, particularly *BioGlue* (CryoLife Incorporated, Kennesaw, GA, USA) and *Vitagel*, previously known as *CoStasis* (Stryker, Kalamazoo, MI, USA).

BioGlue is a bovine serum albumin and gluteraldehyde cross-linked adhesive. Glutaraldehyde molecules cross-link with bovine serum albumin molecules within the tip of the *BioGlue* applicator. *BioGlue* is then applied to the tissue surface and bind to surface proteins of the target tissue [7, 18]. *BioGlue* is an ideal material to seal the area around staples and sutures, requiring only a thin layer to reinforce the closure, to prevent excess bleeding [11, 19]. *BioGlue*'s main advantage over other biologically based hemostatic materials is its ability to function independently of the coagulation cascade [7, 18]. There are however a few drawbacks to using *BioGlue*. *BioGlue* must be applied to a dry surface [7, 18]. This may be very inconvenient as it may be difficult to keep a bleeding surface dry. It is also important to apply only a small amount of *BioGlue* as larger quantities of product may pool and lead to infection [19].

Vitagel is a combination of thrombin, fibrinogen, platelets, and microfibrillar collagen. *Vitagel* is supplied in a dual syringe with a mixing tip. One side of the syringe contains the microfibrillar collagen and thrombin component. The other side contains fibrinogen and platelets obtained from blood plasma extracted from the patient [20-21]. The components combine as they are released from the syringe and applied topically. The thrombin, fibrinogen, and platelet components promote hemostasis and initiate the coagulation cascade.

Vitagel has few advantages over other hemostatic materials. *Vitagel* is derived from the patient's own blood plasma. This eliminates the risk of infection from donor plasma sources [21]. The use of *Vitagel* has also been reported to reduce the need for blood transfusions after total knee arthroplasty [22]. While there are some advantages to the use of *Vitagel*, there are also several disadvantages. The use of the patient's blood plasma requires the use of a centrifuge and thus requires some additional preparation prior to use. The thrombin in *Vitagel* is obtained from bovine sources. There is a risk of infection and immunological response with the use of bovine obtained products [20, 22]. *Vitagel* can also be expensive and the success of its use is dependent upon the experience of the individual applying it [23].

1.5 Synthetic Hemostatic Agents

1.5.1 Chitosan based hemostatic products

Chitin is a naturally occurring poly-N-acyl glucosamine found in arthropod shells [11, 24]. When chitin reaches a certain percent of deacetylation, it becomes a chitosan polymer. Chitosan based products interact directly with red blood cells. Through electrostatic interactions, positively charged chitosan attracts negatively charged red blood cells and platelets, forming a cross-linked seal that is independent of natural blood clotting factors and the coagulation cascade

[2, 11, 24]. The most common chitosan based hemostatic materials are *Celox* (Medtrade Products LTD, Crewe, UK) and *HemCon* (HemCon Medical Technologies incorporated, Portland, OR, USA).

Chitosan based products have several advantages over other hemostatic materials products. Chitosan based materials operate independently of the coagulation cascade. Because the body's natural clotting factors are not involved, chitosan based products can clot heparinized blood [25]. Chitosan based products are biocompatible, as they are composed of glucosamine [25]. At a low pH, chitosan based products also have a polycationic nature and natural antibacterial properties [11, 23]. Though there are several advantages to using chitosan based products, chitosan based bandages (*HemCon*) may not fit properly on atypically shaped wounds and may need to be cut in order to fit [25]. This wastes valuable time and may not be possible during a traumatic situation.

1.5.2 Mineral Zeolite based products

Zeolite is a natural mineral that is composed of a porous, crystalline structure [5, 11]. Mineral zeolite products induce hemostasis by promptly absorbing water when the zeolite material comes in contact with blood [2,5,11]. Water molecules are held within the material by hydrogen bonds. The absorption of water leads to an increased concentration of natural blood coagulation elements, which assist in the rapid formation of a clot [2,5,11]. Currently *QuikClot* (Z-Medica, LLC, Wallingford, CT, USA) is the primary mineral zeolite hemostatic material available.

There are a few benefits to the use of *QuikClot*. *QuikClot* can be highly effective in low-pressure bleeding wounds [23]. *Quikclot* is also easy to use and fairly inexpensive [11]. Though

there are several benefits to the use of *QuikClot*, there are also some drawbacks. QuikClot is not very effective in high-pressure bleeding situations [23]. In some instances, *QuikClot* induces an exothermic reaction that produces high temperatures [2,5,11]. Though the manufacturer has reformulated the product to address exothermic reaction concerns, the high temperatures produced by *QuikClot* may pose a significant burn risk and may require additional training in order to properly handle the material [2,5,11].

1.5.3 Polyethylene Glycol Hydrogel Products

Polyethylene glycol hydrogel products are composed of two synthetic polyethylene glycol polymers, a hydrogen chloride solution, and a sodium phosphate/sodium carbonate solution [23, 27]. The primary Polyethylene glycol Hydorgel material is *CoSeal* (Baxter Healthcare Corporation, Deerfield, IL, USA). *CoSeal* crosslinks with proteins on the surface of the wounded tissue and forms a synthetic hydrogel seal [23, 26]. *CoSeal* was developed as a hemostatic material for use in cardiac and other thoracic procedures [26].

There are some benefits associated with the use of *CoSeal*. The effectiveness of *CoSeal* is not dependent upon the participation of the coagulation cascade [26]. Without reliance on the coagulation cascade, *CoSeal* may be useful in patients with blood coagulation deficiencies [26]. *CoSeal* works rapidly and is not known to instigate an inflammation response [11, 23]. There are however, a few drawbacks to the use of *CoSeal*. *CoSeal* can swell up to four times its original volume within 24 hours [23, 27]. This may pose a problem if areas that may be negatively affected by excessive material swelling. *CoSeal* is not a versatile hemostatic material, as it was designed to specifically assist with cardiac and thoracic procedures.

1.6 Nanotechnology in Medicine

The use of nanotechnology in the field of medicine has been rapidly increasing over the past few years. The main goal of research in nanotechnology is to construct novel materials on a nanoscale. In order to meet the changing demand for materials needed within the medical field, several research groups have begun working with nanomaterials.

Protein nanotechnology has been used in the field of medicine for drug delivery, tissue engineering, 3-D cell culture, and scaffolds [28-30]. Proteins can be used as the structural framework for these nanomaterials [28]. Protein polymers are composed of chains of amino acids. Chains of amino acids, shorter then proteins, are called peptides. Peptides can be rationally designed and synthesized to serve a particular function, or to form desirable shapes, by several processes including, but not limited to, self-assembly.

Self-assembly is a process by which weak interactions form reversible associations to achieve a structure that is energetically optimized [28]. Several molecules including DNA, proteins, and peptides, are known to self-assemble [28]. The process of self-assembly requires favorable thermodynamic conditions. These conditions can be directed by hydrogen bonding, dipole-dipole interactions, other electrostatic interactions and hydrophobic interactions [28]. The aforementioned interactions can result in several types of nanostructures, including a hydrogel.

1.6.1 Peptide Hydrogels

Peptide hydrogels are attractive for use in biomaterials. Because of their high water content and structural polymer framework, peptide hydrogels can be used for a variety of materials, including 3-D cell culture matrixes and injectable materials, without the loss of the molecule's original biological function [29-32]. To use a peptide hydrogel as an injectable

material, it is beneficial if the peptide exhibits shear-thinning and rapid recovery characteristics [29]. Shear thinning hydrogels are a special type of material. The storage modulus of this type of hydrogel decreases rapidly under shear force but also rapidly recovers when left undisturbed [29]. Generally the self-assembly of an injectable hydrogel occurs under specific conditions. Sometimes these conditions involve a certain temperature or pH requirement [29]. A hydrogel forming peptide may be increasingly useful if, in addition to being injectable and shear thinning, the peptide can be injected into the body as a liquid solution and form a hydrogel triggered by a condition within the body such as pH, temperature, or concentration of a particular trigger substance [29].

1.6.2 Peptide based hemostatic research

There are several types of biological and synthetic hemostatic materials available to meet a variety of hemorrhage control needs. However, as previously mentioned, there are several drawbacks and unresolved issues. The use of self-assembling peptides has been investigated to serve as an alternative to current hemostatic materials and to address the shortcomings of previous hemostatic materials. Self-assembling peptides that possess the ability to form nanofibers and/or hydrogels have shown promise as use in wound healing and hemostasis [33-34]. Peptide hydrogels offer several benefits over the preceding hemostatic agents, specifically, reduced risk of negative immune response, reduced risk of infection, and stable nanoweb structures that serve as a hemostatic seal. To date, the ability of several peptide sequences to induce hemostasis has been studied. Though these studies appear to address the shortcomings of previous hemostatic materials, few, if any, have been approved for use by the US Food and Drug Administration. Table 1.2 provides an overview of peptide based hemostatic materials.

1.6.3 N-Pro-Ser-Phe-Cys-Phe-Lys-Phe-Glu-Pro-C

The N-Pro-Ser-Phe-Cys-Phe-Lys-Phe-Glu-Pro-C peptide is a sequence that forms a fishnet-like nanoweb at certain concentrations [33]. The sequence is amphiphilic because it contains the ionic residues Lys and Glu toward the C terminus and the non-ionic residues Phe, Cys,and Phe, toward the N terminus. Proline residues at both terminals to cause turns in the sequence that can be used for tethering peptide components together [33]. The N-Pro-Ser-Phe-Cys-Phe-Lys-Phe-Glu-Pro-C peptide contains both β-sheet and β-turn structures [33]. The sequence also has self-healing abilities and it behaves as an elastic gel [33]. The exact mechanism used by the peptide sequence to stop bleeding appears to need additional research.

1.6.4 KOD

Collagen is a structural protein found in the connective tissue of mammals. In addition to providing structural support in the bodies of mammals, collagen also has the ability to activate the coagulation cascade. The KOD sequence was designed to mimic collagen. It is composed of 36 amino acids, including a proline-hydroxyproline-glycine repeating sequence, similar to that of collagen [35]. The KOD sequence forms triple helix nanofibers. The triple helix nanofibers add end-to-end and can interweave into a hydrogel [35]. The KOD peptide is able to arrest bleeding by adhering to and activating platlets [35]. This induces the coagulation cascade in a similar manner as collagen.

1.6.5 TDM-621

TDM-621 is a peptide that is composed of alternating hydrophilic aspartic acid, hydrophilic arginine, and hydrophobic alanine amino acid residues, chemically named acetyl-

(arginyl-alanyl-aspartyl-alaninyl)$_4$-amide [36]. The TDM-621 peptide forms a hydrogel with a collagen-like fibrous network at a pH of 7 in the presence of sodium and potassium ions [36]. When the acidic TDM-621 peptide solution comes in contact with blood, the solution is neutralized and the β-structure of the peptide forms fibers that assemble into a hydrogel [36]. The peptide hydrogel serves as a hemostatic plug, allowing for coagulation to occur [36].

1.6.6 SPG-178

Self-assembling peptide gel, amino sequence #178 (SPG-178) is a chemically synthesized peptide sequence composed of arginine (R), leucine (L), alanine (A), and aspartic acid (D) residues [37]. The SPG-178 peptide was designed to have an isoelectric point of 11.5 so that stability in a neutral pH environment can be maintained [37]. In aqueous solution, the SPG-178 peptide has an antiparallel β-sheet structure. The SPG-178 self-assembles to form nanofibers that are less than 10 nm in diameter [37]. The nanofibers possess the ability to further form a stable net structure that covers the surface of a wound, thus controlling hemorrhage [37].

1.6.7 IAP

The IAP sequence is an amphipathic peptide composed of a series of repeating leucine (L) and lysine (K) residues (KLLKLL) [34]. The IAP peptide forms a helical structure with hydrophilic lysine residues on one side and hydrophobic leucine residues on the other [34]. It is believed that IAP facilitates hemostasis by accelerating several enzymatic functions within the coagulation cascade [34]. Though the exact mechanism used by IAP is still unknown, its hemostatic properties are likely due to its amphipathic nature and helical structure [34]. The IAP peptide sequence can be combined with certain biocompatible hydrogels. Because hydrogels

absorb large amounts of water and possess the ability to conform to atypically shaped wounds, the hemostatic functions of the IAP peptide can be increased [34].

1.6.8 EAK-16

EAK-16 is a peptide sequence that is derived from a segment of the Z-DNA binding yeast protein, Zuotin [38]. There are several derivatives of EAK-16 peptides being studied for the hemostatic properties. In general the types of EAK-16 peptides being studies have the same amino acid composition but are arranged into different amino acid sequences [39]. EAK-16 peptides are generally self-assembling and possess the ability to form nanofibers. EAK-16 nanofibers form net-like scaffolds. It is suspected that ions and coagulation factors absorbed by EAK-16 peptides contribute to their ability to rapidly control hemorrhage [38]. It is important to note however, that different types of EAK-16 peptides may possess different properties [39].

1.6.9 $RADA_{16}$

One of the most widely researched peptide sequences is $RADA_{16}$. The $RADA_{16}$ peptide consists of alternating hydrophobic alanine (A), and hydrophilic arginine (R) or aspartic acid (D) residues (AcN-RADARADARADARADA-$CONH_2$) [38, 40-41]. In an aqueous environment, the $RADA_{16}$ peptide forms a stable β-sheet structure and nanofibers [40]. At neutral pH, the $RADA_{16}$ peptide self-assembles into a hydrogel scaffold. It is believed that the $RADA_{16}$ peptide induces hemostasis when it reacts with ions present in the blood [41]. However, the exact mechanism for hemostasis is not fully understood [42]. The $RADA_{16}$ peptide has been found to be biodegradable and can be absorbed by the body [40]. $RADA_{16}$ hydrogel is also able to control hemorrhage in a-typically shaped wounds [40]. Several modified versions of the original

$RADA_{16}$ peptide sequence have been studied. Some of them appear to be just as effective as the original $RADA_{16}$ sequence at controlling hemorrhage [40].

1.7 Peptide Sequence Development within the Bio-Materials and Technology Lab in the Department of Grain Science at Kansas State University

The Biomaterials and Technology lab in the department of Grain Science and Industry at Kansas State University has identified two unique peptide sequences, h9 and eD_2, that possess similar qualities as the previously mentioned peptide sequences.

The h9 peptide sequence (FLIVIGSII), was derived from a subunit of human muscle L-type calcium channel [28]. The h9 peptide sequence exhibits a high hydrophobicity and can form a β-like structure, by self-assembly [28]. The h9 peptide also forms a viscous gel-like substance when mixed at certain concentrations [28].

The eD_2 peptide sequence was designed from a combination of spider flagelliform silk protein and the Ca^+ binding portion of Lipase Lip A from *Serratia marcescens*, a species of gram positive, rod-shaped bacteria [31].

The spider flagelliform silk sequence (GPGGX; where X represents any amino acid) was selected because it forms a β-spiral motif that provides the elasticity in the high tensile strength in spider silk [31]. Each amino acid provides specific properties of the sequence. The Glycine (G) provides flexibility. The Proline (P) influences mechanical properties and structure by affecting cross-linking and hydrogen bonding [31].

The Ca^+ binding portion of Lipase Lip A from *Serratia marcescens* sequence (GXGXDXUX; where X represents any amino acid and U represents any hydrophobic amino acid) was selected for its calcium binding ability. As in the spider silk sequence, individual

amino acids provide specific properties to the sequence. Namely the Aspartate (D) is necessary to facilitate ion bonding [31].

The eD_2 sequence (GPGGDGPGGD) was formed by rationally combining the two aforementioned sequences through a process of "hiding" the sequences in each other [31]. The sequences were "hidden" by substituting unnecessary amino acids such that the new sequence retained vital properties of both of the parent peptide sequences [31].

The eD_2 peptide sequence is self-assembling, forms a β-spiral structure in an aqueous environment, and forms nanofibers triggered by the addition of Ca^+ [31].

1.7.1 h9e peptide sequence development

The h9e peptide sequence was designed by rationally combining the hydrophobic h9 sequence and the hydrophilic eD_2 sequence [29]. The novel h9e peptide sequence contains several physical properties that are considered ideal for peptides developed for use in nanotechnology. The h9e peptide sequence is amphiphilic due to its hydrophobic and hydrophilic segments [29]. The h9e peptide has a helical structure with the C-terminal amino acids bent into the structure [43]. The nanofibers formed by h9e are about 5nm in diameter without a specific orderly shape [43]. When Ca^+ is added to h9e peptide solution, nanofibers rapidly assemble into a peptide hydrogel matrix [43]. The hydrogel formed by h9e is both shear-thinning and recoverable [29, 43].

To date, the h9e peptide has been successfully used for a variety of applications including as an influenza H1N1 vaccine adjuvant, a 3-D cell culture material for drug testing, and as an adjuvant for modified live virus vaccines [29-30, 32]. The success of the h9e peptide sequence in

these applications, along with favorable physiological properties, indicates the h9e peptide sequence may be a useful component in other biomaterials within the field of medicine.

1.8 Objectives

The main objective of this study was to determine the blood gelation strength of the h9e peptide necessary to arrest bleeding in the Wistar Rat model. Specific objectives were to: (1) study the gelation kinetics of h9e peptide solution with commercial mouse blood, commercial rat blood, and commercial rat blood components using the Dynamic Rheometer; (2) conduct hemostatic efficiency study with Wistar rat model and compare to a commercial hemostatic agent.

Table 1.1 Selected Hemostatic Agents

Hemostatic Material	**Material Type**	**Mechanism**	**Advantages**	**Disadvantages**	**Examples**
Fibrin Based Materials	Biological	Cross-links to form a matrix that supports clotting factors [7, 10].	Effective, Fast acting, takes advantage of natural coagulation cascade and Biodegradable [7, 11].	Can be expensive and Risk of infection if derived from contaminated sources [7,11].	*Tisseel*, *Beriplast P* and *Evicel*.
Thrombin Based Materials	Biological	Activates the coagulation cascade, forming a hemostatic plug	Effective, fast acting, can be applied directly to the wound, and takes advantage of natural coagulation cascade process [12-14, 16].	Can be expensive, Risk of immunological response, and Risk of infection if derived from contaminated sources [12-13, 16].	*Thrombin-JMI, Recothrom, Evithrom,* and *Evicel.*
Bovine Serum Albumin and Gluteraldehyde	Biological	Glutaraldehyde cross-links with bovine serum albumin and creates an adhesive that and binds to surface proteins of target tissue [7,18].	Ideal for sealing area around sutures and staples, only requires thin layer, and functions independently of coagulation cascade [7,11,18-19].	Requires dry surface, large amounts of product may pool and lead to infection [7, 18-19].	*BioGlue*
Platelet based Sealant	Biological	Thrombin, fibrinogen, platelets, and microfibrillar collagen combine and initiate the	Derived from patient's own plasma (no donor source) providing reduced risk of infection and	Requires centrifuge, Risk of infection and immunological response from bovine obtained	*Vitagel*

		coagulation cascade	may reduce need for blood transfusions in certain cases [21-22].	components, can be expensive, and may require experience to handle [20, 22-23]	
Chitosan Based Hemostatic Products	Synthetic	Attracts red blood cells and platelets to form a cross-linked seal [2, 11, 24].	Functions independently of Coagulation cascade, can clot heparinized blood, biocompatible, and some antibacterial properties [11, 23, 25].	Bandages may not fit atypically shaped wounds [25].	*Celox* and *HemCon*
Mineral Zeolite Based Products	Synthetic	Absorbs water concentrating blood coagulation elements [2, 5, 11].	Highly effective in low-pressure bleeding wounds, easy to use, and inexpensive [11, 23].	Not very effective in High pressure bleeding wounds, exothermic reaction that leads to burn risk, may require special training to handle [2, 5, 11].	*QuikClot*
Polyethylene Glycol Hydrogel Products	Synthetic	Crosslinks with proteins on surface of target tissue and forms a synthetic hydrogel seal [23, 26].	Functions independently of Coagulation cascade, may work in patients with blood coagulation deficiencies, and is not known to instigate inflammation response [11, 23, 26].	Can swell up to 4 times its orginial volume within 24 hours and designed specifically for cardiac and thoracic procedures [23, 27].	*CoSeal*

Table 1.2 Peptide based hemostatic materials

Peptide	Sequence	Mechanism	Notes
N-Pro-Ser-Phe-Cys-Phe-Lys-Phe-Glu-Pro-C	N-Pro-Ser-Phe-Cys-Phe-Lys-Phe-Glu-Pro-C	Unknown	Amphiphilic; May work faster than chitosan on liver cut rat model; mechanism unknown
KOD	36 residues; Proline-hydroxyproline-glycine (repeating)	Adheres to and activates platelets	Collagen mimic; Induces coagulation cascade
TDM-621	Aspartic acid, arginine, alanine (repeating)	Forms hydrogel and serves as hemostatic plug	Alternating hydrophobic and hydrophilic residues; Forms hemostatic plug
SPG-178	Arginine, leucine, alanine, aspartic acid residues	Forms nanofibers web to cover wound surface	------
IAP	Repeating Leucine and Lysine residues (KLLKLL)	Unknown; Possibly accelerates enzymatic functions of coagulation cascade	Helical structure with hydrophilic lysine on one side and hydrophobic leucine on the other; can conform to atypically shaped wounds
EAK-16	Derived from Z-DNA binding yeast protein, Zuotin	Ions and coagulation factors absorbed by EAK-16 peptides contribute to rapid hemorrhage control	Multiple types of EAK-16 peptides; Self-assembling; form nanofibers
$RADA_{16}$	(AcN-RADARADARADARADA-$CONH_2$)	Unknown; Reacts with ions in blood	Biodegradable; conforms to atypically shaped wounds; Several Variations of $RADA_{16}$ peptide

Figure 1.1 Schematic of the Coagulation Cascade (adapted from Behrens et al.) [8].

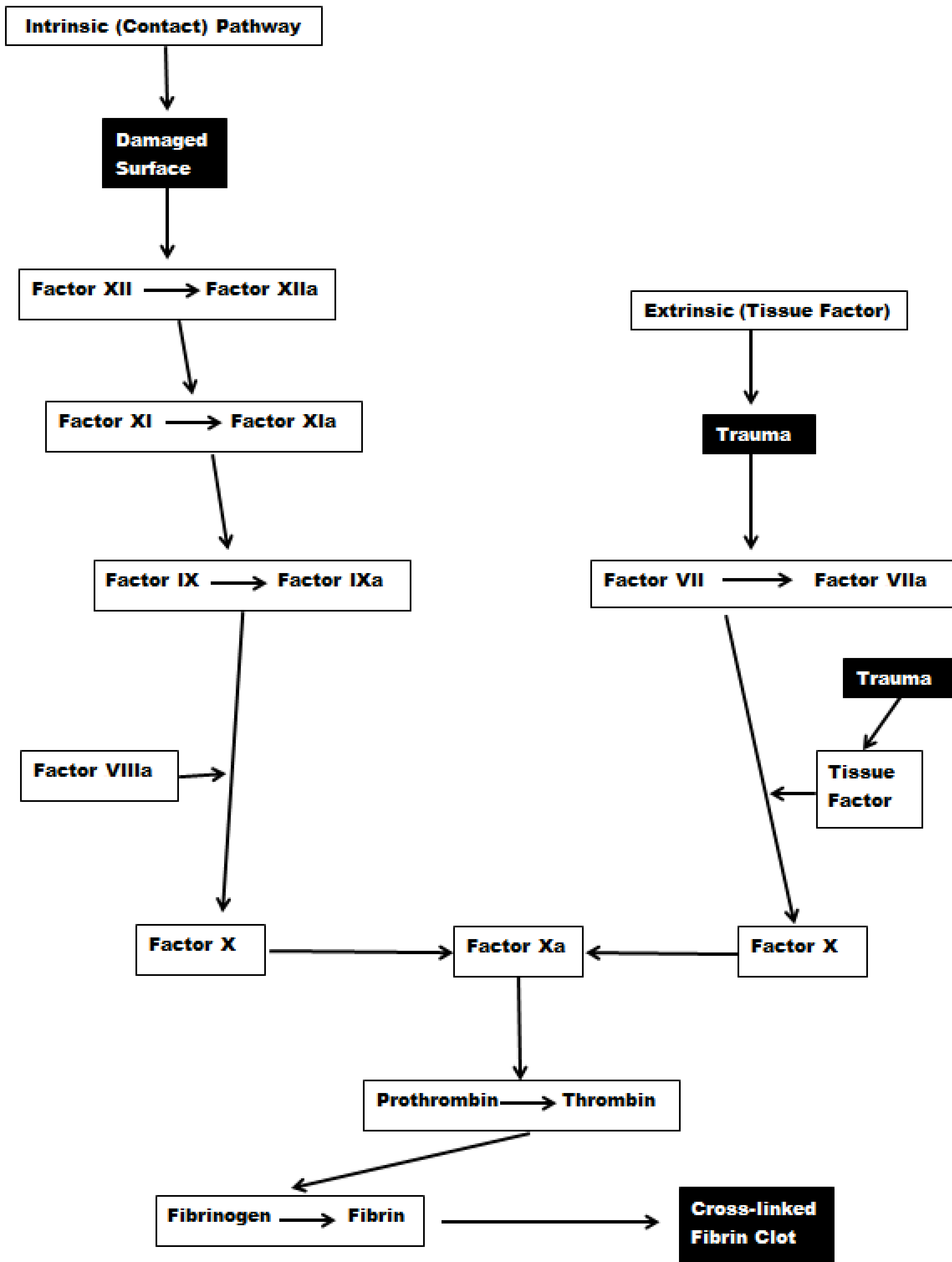
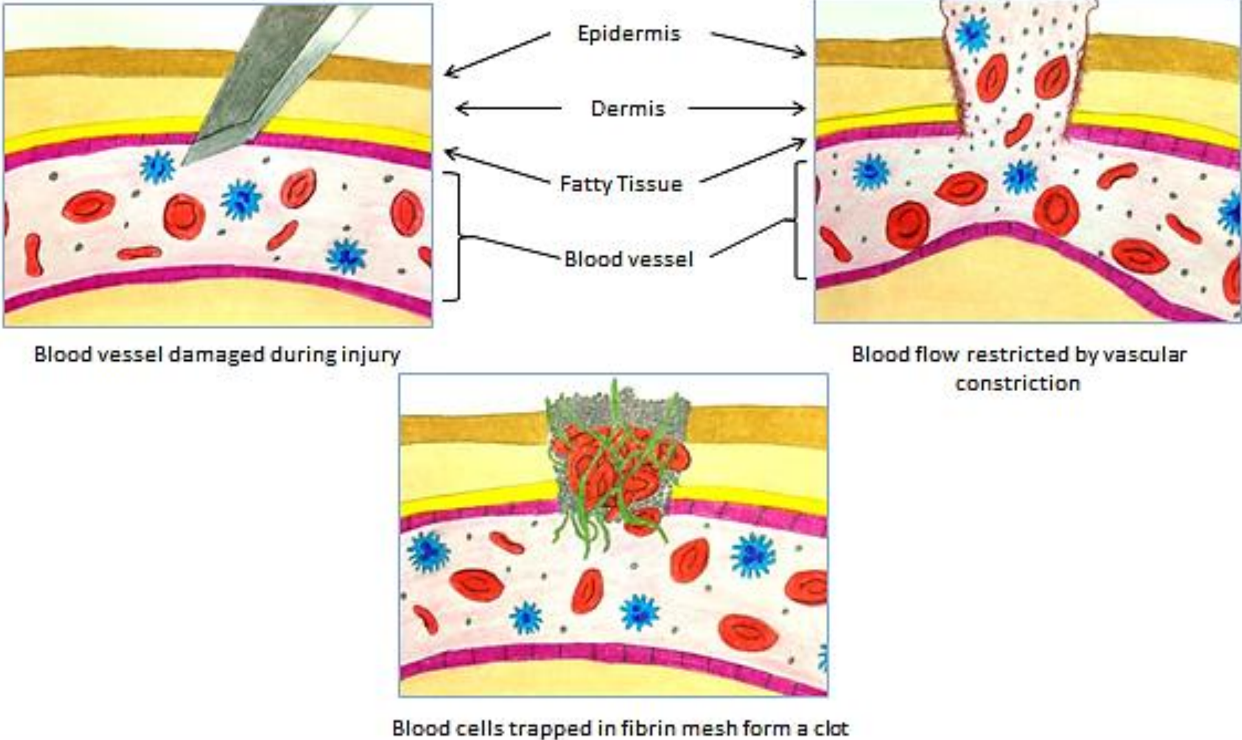


Figure 1.2 Schematic of blood clot formation



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Chapter 2 - Hemostatic Efficiency of the h9e Peptide in Wistar Rats

2.1 Introduction

One of the leading causes of death following a traumatic injury is exsanguination [1-3]. Among U.S. military personnel, hemorrhage is one of the greatest threats to survival [4-5]. Most deaths related to combat injuries occur before military personnel have the opportunity to reach a medical facility [4]. A vast majority of exsanguination deaths are due to injuries to the torso area, where it may be difficult to control bleeding in a timely fashion [4,6]. Among American civilians, bleeding is the leading cause of death in operating rooms [7]. Extensive blood loss due to hemorrhage is a worldwide concern.

In order to prevent or significantly reduce the number of deaths associated with exsanguination, research has been conducted over the past 40 years. The body naturally addresses the issue of bleeding by the process of hemostasis (coagulation cascade) [8]. However, during a traumatic injury, the body may be unable to stop or slow the amount of bleeding caused by the injury. To address these issues, several hemostatic agents have been developed.

Hemostatic agents stop bleeding by either their own mechanical methods or by reinforcing the coagulation cascade [8]. Traditionally, medical personnel have used gauze and pressure to assist with the body's natural process of hemostasis. Aside from the traditional gauze and pressure method, there are several types of hemostatic agents that are currently used to address medical needs. All of the hemostatic agents currently on the market offer their own set of benefits over other types. Therefore, it is usually necessary to select a hemostatic agent for use on a case by case basis. Though all hemostatic agents offer benefits, there are several unaddressed risks involved with hemostatic agent use.

Currently a majority of the hemostatic agents available on the market are derived from biological sources including purified bovine serum albumin, bovine thrombin, and crustacean chitin [8]. Because the components of these hemostatic agents are obtained from biological sources, the risk of allergic reaction or infection from contaminated biological systems is a concern [8,9].

Recently self-assembling peptide sequences have been suggested for use as alternative biomedical materials [10]. Self-Assembling peptides that possess the ability to form nanofibers and hydrogels have shown promise for use in wound healing and hemostasis [11-12]. Peptide hydrogels offer several benefits over the preceding hemostatic agents, specifically, reduced risk of negative immune response and infection.

Within the past few years, the Bio-Materials and Technology Lab, in the Department of Grain Science and Industry, at Kansas State University has developed a new amphiphilic peptide sequence, the h9e peptide. The sequence was produced by rationally combining two native peptide sequences, h9 and eD_2, after comparing their primary functions. The h9 peptide sequence (FLIVIGSII), was derived from a subunit of human muscle L-type calcium channel [13]. The h9 peptide sequence exhibits a high hydrophobicity and can form a β-like structure, by self-assembly [13]. The h9 peptide also forms a viscous gel-like substance when mixed at certain concentrations [13].

The eD_2 sequence (GPGGDGPGGD) was formed by rationally combining a sequence of spider flagelliform silk protein and the Ca^+ binding portion of Lipase Lip A from *Serratia marcescens*. The sequences were combined through a process of "hiding" the sequences in each other [14]. The sequences were "hidden" by substituting unnecessary amino acids such that the new sequence retained vital properties of both of the parent peptide sequences [14]. The eD_2

peptide sequence is self-assembling, forms a β-spiral structure in an aqueous environment, and forms nanofibers triggered by the addition of Ca^+ [14].

The conformation, morphology, and mechanical properties of the h9e peptide have been studied extensively [14-15]. The h9e peptide has proven to be a useful as an adjuvant for both the influenza H1N1 vaccine and the porcine reproductive and respiratory syndrome virus vaccine [16-17]. The h9e peptide hydrogel proved to be biologically safe and performed 70% better than a commercial adjuvant [16]. It has also been shown that the h9e peptide has the potential to be used as a 3D cell culture matrix for drug testing [18].

While exploring the many possible applications of this innovative peptide, it was determined that if mixed with serum albumin, a hydrogel would form. Hydrogels have the potential to be used for several applications including, but not limited to, the formation of a hemostatic agent. Because of its elasticity and high tensile strength properties, the h9e peptide hydrogel has the potential for use in biomaterials for medical uses and possibly the formation of a hemostatic agent.

The main objective of this study was to determine the blood gelation strength of the h9e peptide necessary to arrest bleeding in the Wistar Rat model.

2.2 Materials and Methods

Preparation of h9e peptide solution

H9e peptide was synthesized at the Bio-Materials and Technology Lab, in the Department of Grain Science and Industry at Kansas State University. After synthesis the peptide was cleaved, washed with anhydrous ether, dissolved in acetonitrile and distilled water (1:1 ratio), frozen at -80°C, and freeze dried. Stock solutions of peptide were made by dissolving

freeze-dried peptide in 100mM $NaHCO_3$ to the appropriate weight percentage. All solutions were autoclaved before use. The purity of the h9e peptide used was ≥ 85% confirmed by high performance liquid chromatography (HPLC).

Rheological analysis of commercial mouse blood gelation

A time sweep was conducted on h9e peptide solution and commercial mouse blood at the following wt % concentrations of h9e peptide dissolved in 100mM sodium bicarbonate: 1%, 2%, 3%, 4%, and 5%. Equal volumes of the appropriate wt% of h9e peptide solution and commercial mouse blood (Innovative research, Novi, Michigan, USA) were combined in a microcentrifuge tube. The mixture was homogenized and either immediately added to the rheometer for evaluation or placed in a carbon dioxide incubator at 37°C for 24 hours (Nuaire, Plymouth, MN, United States). Samples placed in the incubator were removed after 24 hours and were added to the rheometer for evaluation. The elastic modulus, G', of the h9e/blood hydrogel was determined using a C-VOR 150 rheometer (Malvern Instruments, Malvern, Worcestershire, United Kingdom). A plate 20mm in diameter was used with 1 % strain and frequency of 1 Hz at a 37°C temperature for 30 minutes.

In vivo Hemostatic Efficiency in Rat Model

Similar to the method used by Mortazavi et al., in this study 30 rats were divided randomly into 5 groups of 6 animals each [5]. Figure 2.1 shows the schematic for the in vivo experiment. Male Wistar rats were maintained on an AIN-93 pellet diet with free access to food and water, under 12-h light-dark cycles, for one week. Before excising the tail, rats were first anesthetized and maintained with 3% isoflurane (Figure 2.1 A) until the eyelid closure reflex was

lost and there was no reactive reflex in response to a toe pinch. In order to maintain an appropriate body temperature during anesthesia, a circulating water blanket was used at a temperature of 41-43°C (Figure 2.1. B). Following anesthesia, animals' tails were cut off at a thickness of 5 mm (Figure 2.1. C) using a pair of mortuary scissors. The cut tail was immediately inserted into a small vial containing the appropriate treatment (Figure 2.1. D). The 1st group received topical saline solution (negative control) and the 2nd to 4th groups received topical h9e peptide solution at 1, 3, or 5 wt%, respectively. The 5th group received an application of a commercially available hemostatic agent as a positive control (Moore Medical CELOX* Hemostatic Granules). After treatment with the above mentioned agents, the volume of blood lost was measured using a scaled test-tube. The bleeding time was measured using a stop watch. Before the procedure was performed, the weight of each animal was recorded. The mean weight of animals used in each testing group was also recorded before the start of the procedure. In an attempt to be as accurate as possible, animals used were chosen at random. At the conclusion of the study, the rats were sacrificed using a carbon dioxide chamber. All animal testing was done in accordance to procedures approved by the Institutional Animal Care and Use Committee (IACUC protocol #3276). Statistical analysis of rat clinical trial data was performed using one-way ANOVA. P-values of less than 0.05 were considered statistically significant. There was no significant difference in body weight variance between the treatment groups.

Rheological analysis of instantaneous gelation of whole rat blood and blood components

A time sweep was conducted on h9e peptide solution and Wistar rat blood components (red blood cells, blood plasma, or blood serum). H9e peptide was dissolved in 100mM sodium bicarbonate to yield desired wt% concentration according the aforementioned methods. Equal

volumes of the appropriate wt% of h9e peptide solution and whole wistar rat blood or the appropriate wistar rat blood component (Innovative research, Novi, Michigan, USA) were combined in a microcentrifuge tube. The mixture was homogenized and immediately added to the rheometer for evaluation. The elastic modulus, G', of the h9e/blood hydrogel was determined using a C-VOR 150 rheometer (Malvern Instruments, Malvern, Worcestershire, United Kingdom). A plate 20mm in diameter was used with 1 % strain and frequency of 1 Hz at a 37°C temperature for 30 minutes.

Transmission electron microscopy (TEM)

The morphology of Rat blood and h9e solution was observed using transmission electron microscopy (TEM). Exactly six mL of the appropriate sample was placed onto a negative glow discharged carbon-coated copper grid for two minutes. The remaining sample was removed by blotting with a kimwipe (Kimberly Clark Professional, Roswell, Georgia, USA). The grid was then placed into 2% uranyl acetate for two minutes. Extra stain was removed by blotting with tissue paper. The wet grids were allowed to air-dry for several minutes prior to being examined under TEM.

Samples were imaged using a FEI Technai G2transmission electron microscope at an electron acceleration voltage of 200 KeV. Images were captured using a standardized, normative electron dose and a constant defocus value from the carbon-coated surfaces. The images presented are presented in the absence of any further processing or image filtering.

Spinning Disk Confocal Microscope

Ten µl of the appropriate sample was placed onto a glass slide and covered with a cover slip. The sample was observed under Olympus spinning disk confocal inverted microscope and images were captured by using slide book version 5.5 (3i-Intelligent Imaging Innovations, Inc.).

2.3 Results

Rheological analysis of commercial mouse blood gelation

All weight % concentrations of h9e peptide tested induced a gelation response in commercial mouse blood. The gelation strength of the instantaneous samples increased as both time and weight % concentration of the h9e peptide increased (Fig. 2.2). With the exception of the 1% wt concentration, all other % wt concentrations were above 100 Pa within 1-3 seconds. The 1% wt concentration reached the 100 Pa level after about 10 minutes.

Compared to the instantaneous samples, the gelation strength of all concentrations decreased when allowed to sit 24 hours post homogenization (Fig. 2.3). Similar to the instantaneous samples, as the % concentration of h9e solution increased, the gelation strength increased.

In vivo Hemostatic Efficiency of h9e Peptide Solution in Rat Model

In order to observe the ability of h9e solution to control hemorrhage in a live, bleeding model, in vivo testing was conducted. The hemostatic efficiency test showed that all percent concentrations of h9e peptide solution were effective in controlling hemorrhage in comparison to the sterile saline negative control (Table 2.1). Though all concentrations of h9e solution were effective in reducing blood lost, the 5% h9e solution was able to control bleeding most effectively. The 5% h9e peptide solution outperformed the commercial positive control Celox™

granules, reducing both bleeding time and blood lost. Similar to the results obtained by the rheology studies, as the concentration of h9e solution increases, the hemostatic efficiency also increases.

Rheological analysis of instantaneous gelation of whole rat blood and blood components

The rheological analysis of commercial rat blood was used to compare gelation strength to that of the commercial mouse blood. Similar to the commercial mouse blood, the gelation strength increased as both time and concentration of the h9e peptide increased (Fig. 2.4).

To further investigate the ability of 1% h9e to induce a gelation response when mixed with rat blood, rheological analysis of individual blood components (red blood cells, serum and plasma) was conducted. Figure 2.5 shows that a gelation response is induced with all three components tested, though the gelation strength of the blood serum with 1% h9e solution is much lower than the gelation strength of the red blood cells and blood plasma with 1% h9e solution.

Transmission electron microscopy (TEM)

TEM imaging indicates that observed h9e nanofibers have a width of about 10 nm. TEM evaluation of both blood plasma and blood serum showed few significant differences (Figures 2.7 and 2.8). Both the blood plasma and blood serum show aggregation of blood proteins and other components into agglomerate clusters. TEM evaluation of 0.5% h9e with blood serum clearly illustrates the binding of blood serum components to the h9e fibers within the samples (Figure 2.9). TEM evaluation of 0.5% h9e with blood plasma indicates a similar binding of plasma components to the h9e fibers (Figure 2.10).

Spinning Disk Confocal Microscope

Confocal imaging of red blood cells only, in Figure 2.11, illustrates the red blood cells suspended freely in saline. When red blood cells are added to 0.5% h9e peptide solution, as in Figures 2.12 and 2.13, the cells become trapped within the h9e fiber nanoweb. The arrows within the images indicate the immobilized cells within the nanoweb compared to several cells floating freely in the background.

2.4 Discussion

This study is the first report of the h9e peptide's ability to induce hemostasis. Similar to other peptide sequences used for hemostasis, the h9e peptide possesses characteristics that offer several advantages over biologically derived hemostatic agents. The h9e peptide sequence is amphiphilic. Amphipilicity as a physiochemical property has been known to induce hemostatic response by activating some of blood's natural clotting factors [12]. The h9e peptide self-assembles into nanofibers that have the ability to form a nanoweb structure. Nano web-like structures have been known to seal bleeding surfaces preventing hemorrhage and rapidly inducing hemostasis [19-20]. Nano web-like structures have also been known to be efficient in a typically shaped wounds [12]. The h9e peptide sequence contains no biological elements. The lack of biological elements eliminates the risk of infection from contaminated biological sources [19, 21].

The h9e peptide induces hemostasis by forming a nano-web structure in the presence of blood. The h9e fibers are reinforced by components within the blood plasma and blood serum, as shown in TEM images. Red blood cells are entangled in h9e fibers, as shown in confocal images. Collectively, this causes a strong gelation response and the formation of a structure similar to

that of a blood clot. The aforementioned structure is then acts as both a preliminary thrombus and a surface upon which a thrombus can continue to develop until bleeding is arrested. The nano-web structure formed by the h9e peptide is similar to that of fibrin within the coagulation cascade. The h9e peptide sequence seems to mimic fibrin in its ability to maintain a blood clot structure.

The success of the h9e peptide was confirmed in vivo. We used the tail-clip method in a Wistar Rat because it is easy to obtain reproducible results. In general, as the weight % concentration of h9e peptide solution increased, the hemostatic efficiency increased. Other peptide studies have been determined that higher peptide concentration may be beneficial to nanoweb formation [20].

Despite the decreasing amount of blood lost, from low concentration to high concentration of h9e solution used, the 3% h9e solution had a slightly increased time of hemostasis induction compared to the 1% h9e solution (Table 2.1). There are two factors that may have contributed to this observation. The first is that the time recorded for all treatment groups was based on the visual judgment that bleeding in the wound had stopped. Though we were consistent with each animal tested, visual judgment is never flawlessly accurate. The second is that the animals used in the 1% h9e solution testing group weighed less than the animals used in the 3% h9e solution testing group (Table 2.1). To be as accurate as possible, animals were randomized and statistical analysis showed there was no significant difference in weight between animal treatment groups.

The 5% h9e solution induces the process of hemostasis much faster than the commercial Celox™ granules. On advertised packaging, Celox granules boast the ability to induce hemostasis in the presence of an anticoagulant. This claim has been confirmed by an

experimental study performed by Koksal et al [22]. In their study, they found that Celox induces hemostasis in the presence of a warfarin anticoagulant. Warfarin is an orally administered anticoagulant used to prevent thrombosis. Their study also indicates that Celox has the ability to clot blood under hypothermic conditions [22].

The blood samples used in this study were drawn using Alsevers, a balanced isotonic salt solution, as an anticoagulant. Though the rats used in our study were not being treated with an oral anticoagulant, because the rheology data demonstrated that h9e peptide solution forms a stable hydrogel with the commercial blood, we speculate that h9e may also be able to induce hemostasis in the presence of an anticoagulant. Further clinical trials would be needed to confirm this theory. If our speculation is correct, this may prove significant in treating patients on blood thinners or possibly treating patients with blood clotting disorders.

As mentioned before, high % concentrations of h9e peptide solution induced a faster hemostatic response. With the commercial mouse blood testing, it is important to note that the points plotted on the instantaneous gelation graph are not parallel with the X-axis. This indicates that if the assay were allowed to continue to run after the allotted 30 minutes, the Pa strength of the sample may still increase. The samples allowed to sit for 24 hours indicated decreased gelation strength in comparison to the instantaneous gelatinization results. The points plotted on the 24 hour gelation study graph are more parallel with the X axis. This indicates that within 24 hours the samples may have reached a stable and homogeneous Pascal strength by sitting undisturbed. This particular aspect of the study is vital to future uses of the h9e peptide as it would be undesirable for the hydrogel to weaken or become unstable within a short period of time.

Consistent with the commercial whole mouse blood rheological examination, the points plotted on the whole rat blood graph are not parallel with the X axis. This indicates that if the assay were allowed to continue, the gelation strength of those samples would also increase. Overall, the gelation strength of the rat blood samples was significantly higher than that of the mouse blood samples. This is probably due to a difference in blood protein and clotting factor between the two species.

Previous research indicated that h9e forms a hydrogel when triggered by bovine serum albumin. It is well knows that albumin is abundant in whole blood and it is, therefore rational that blood components would contain some level of albumin and trigger a similar gelation response.

The gelation strength of the red blood cell and blood plasma samples was significantly higher than that of the blood serum sample. It is well known that blood serum is obtained by intentionally removing the body's natural clotting factors from blood plasma. The absence of clotting factors may be the cause of the reduced the gelation strength of the blood serum sample. Though all blood components tested triggered a gelation response, the reduced gelation strength of the blood serum sample may indicate that clotting factors play a role in h9e's ability to induce hemostasis. The gelation response of all blood components was also lower than that of the 1% whole rat blood observed in Figure 2.4. This may indicate that each blood component plays a role in the gelation response and their interactions are interdependent.

2.5 Conclusions

We have demonstrated the h9e peptide's ability to induce hemostasis in the Wistar Rat model by mimicking fibrin's ability to provide the framework for a blood clot. Both the bleeding

time and amount of blood lost were lower than that of commercial Celox™ granules with the 5% h9e peptide solution. The h9e peptide is amphiphilic, nano-web forming, and contains no biological elements that may induce an immunological response or cause infection. Based on these findings, we consider the h9e peptide a useful component of a hemostatic material.

Table 2.1 Measured values of average animal body weight, blood lost, and total bleeding time

Test Group	Animal Body weight ±SD (grams)	Blood Lost ± SD (mL)	Bleeding Time ± SD (seconds)
Saline (Negative Control)	245.85 ± 10.36	2.76 ± 0.66	521.33 ± 54.67
1% h9e solution	239.33 ± 12.22	2.20 ± 0.81	203.33 ± 57.39
3% h9e solution	252.05 ± 9.42	1.87 ± 0.90	226.00 ± 58.79
5% h9e solution	253.45 ± 17.39	0.75 ± 0.29	94.00 ± 37.05
Celox™ (Positive Control)	254.91 ± 14.31	1.53 ± 0.87	225.20 ± 56.21
	P= 0.24	P= 0.001	P= 7.18 x 10^-12

Figure 2.1 Scheme procedure of the in vivo experiment

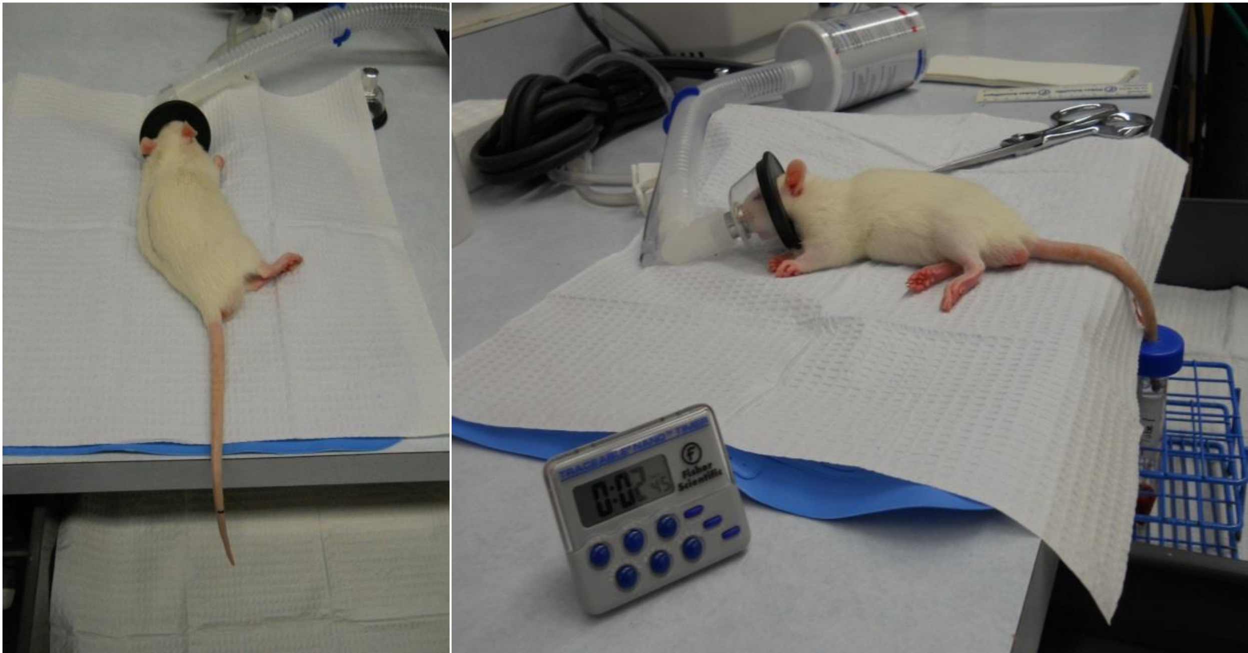


Figure 2.2 Storage modulus vs time of mouse whole blood samples with various h9e peptide concentrations mixed and measured instantaneously

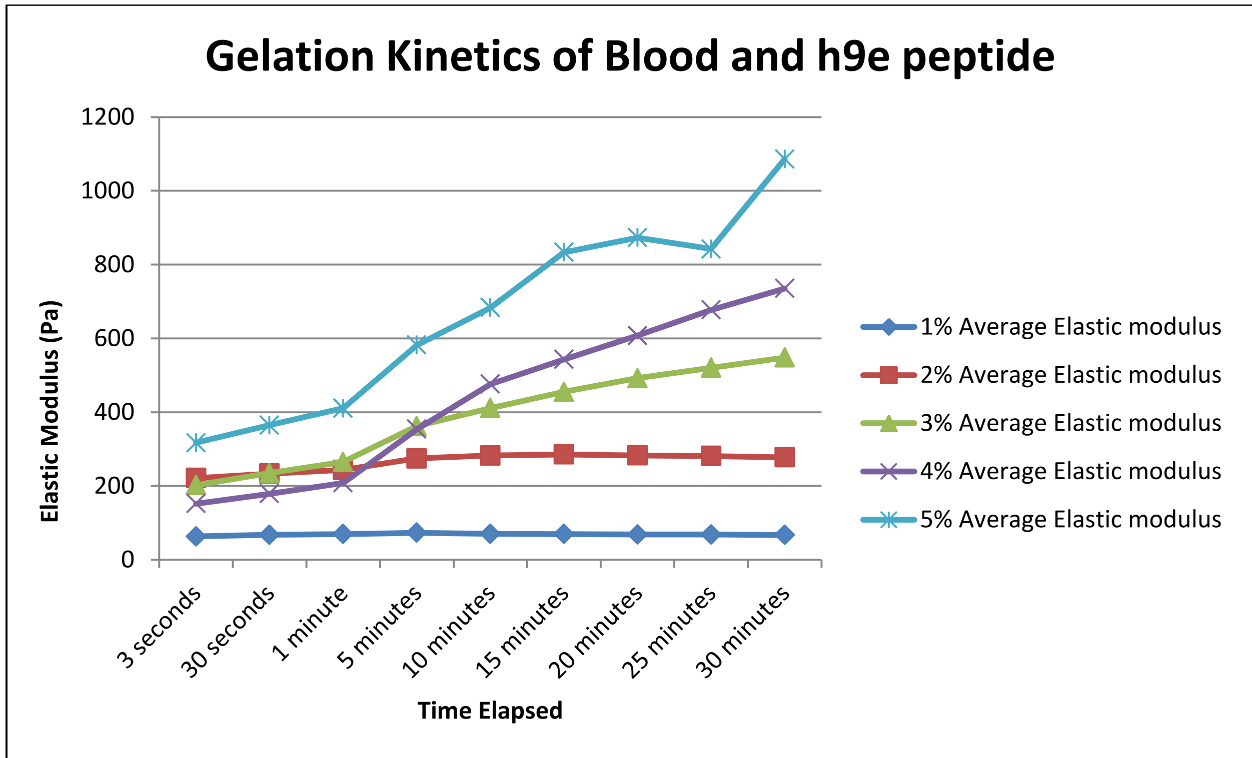


Figure 2.3 Storage modulus vs time of mouse whole blood samples with various h9e peptide concentrations mixed and measured 24 hours post gelation

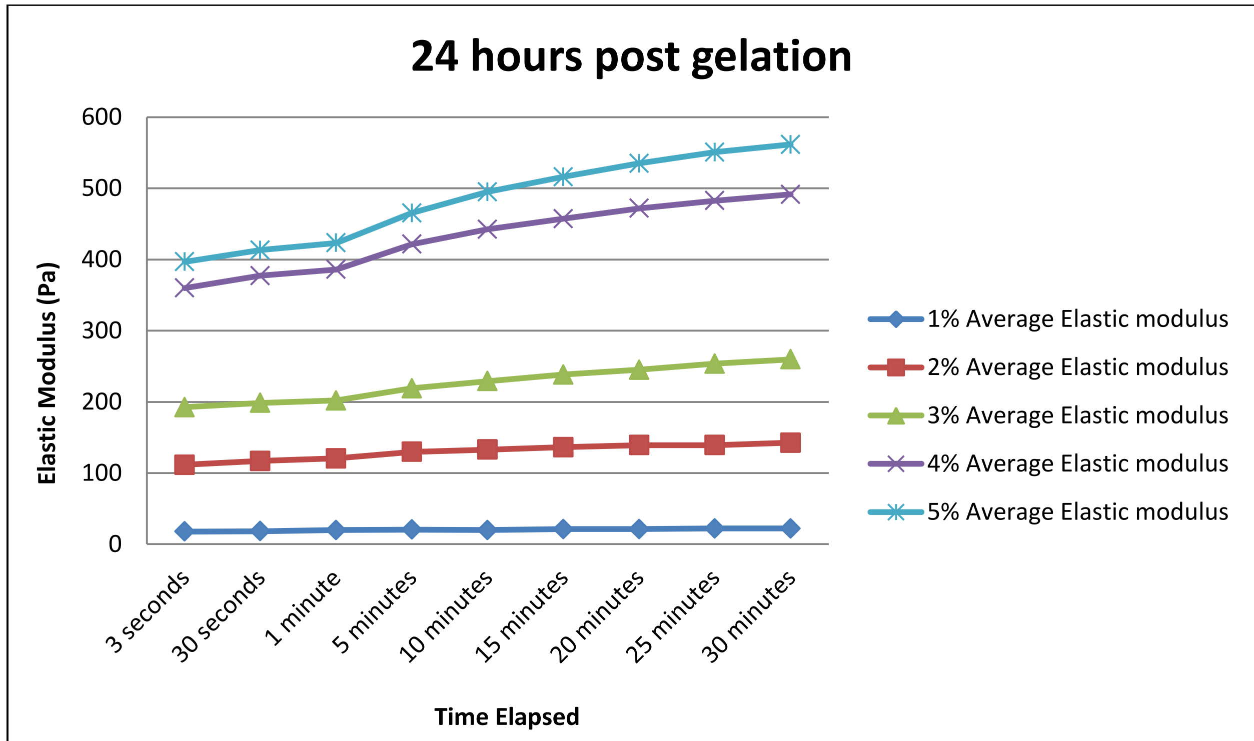


Figure 2.4 Storage modulus vs time of rat whole blood samples with various h9e peptide concentrations mixed and measured instantaneously

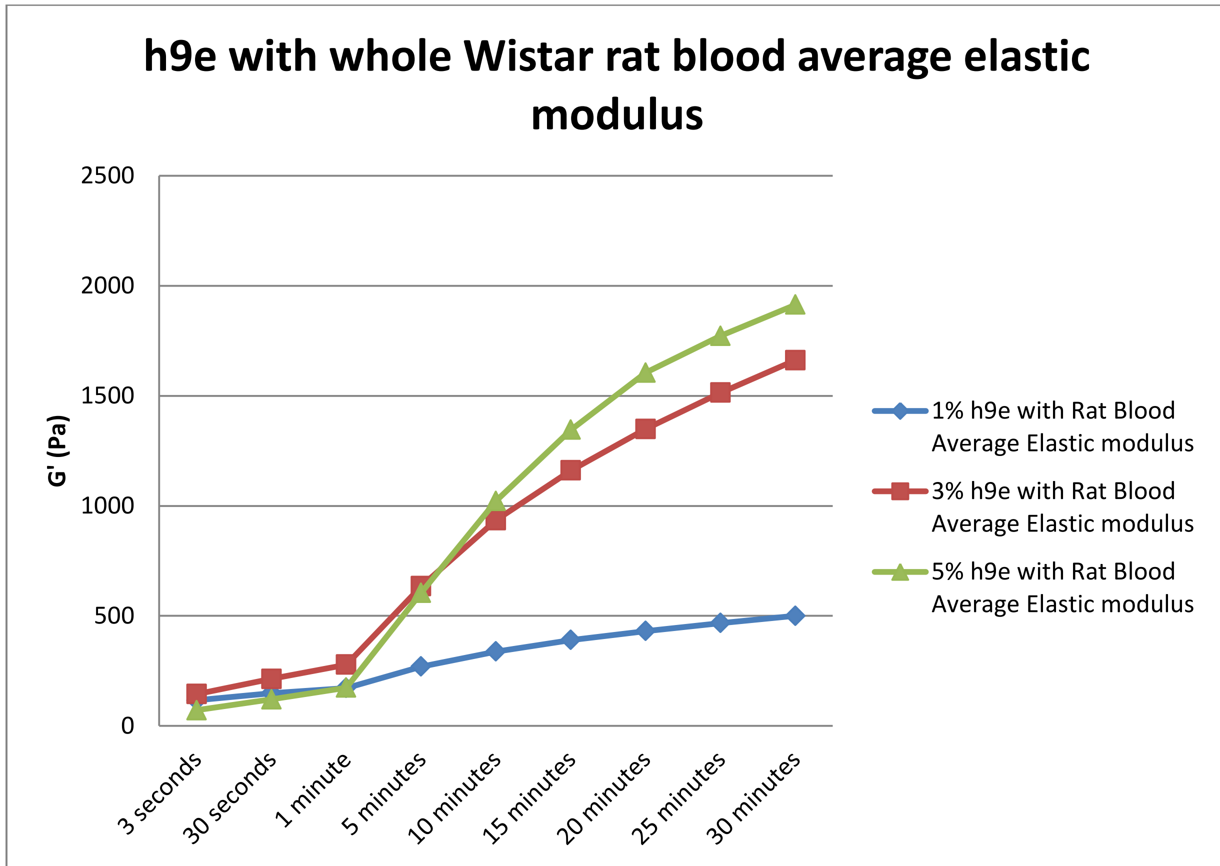


Figure 2.5 Storage modulus vs time of rat blood components with 1% h9e peptide solution mixed and measured instantaneously

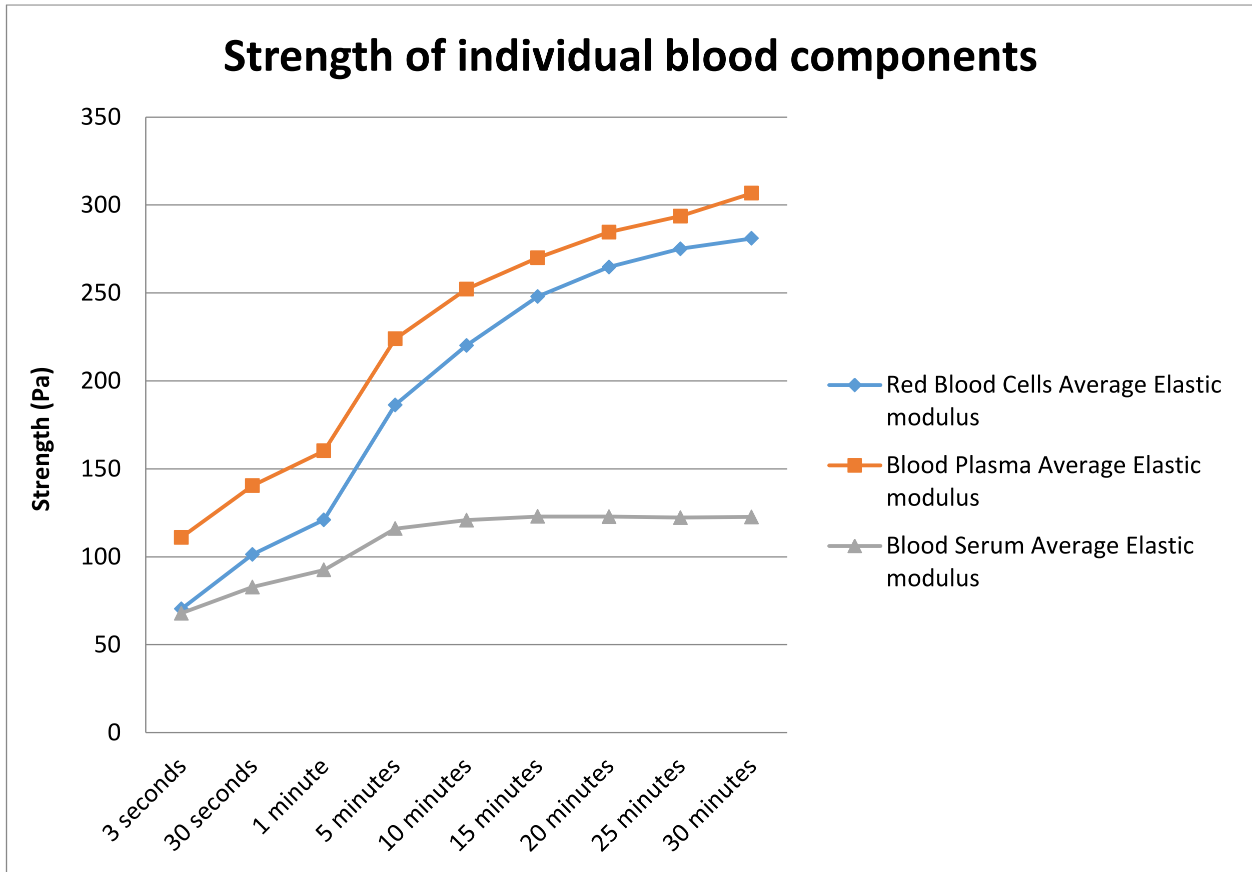


Figure 2.6 TEM imaging of 0.5% h9e peptide solution

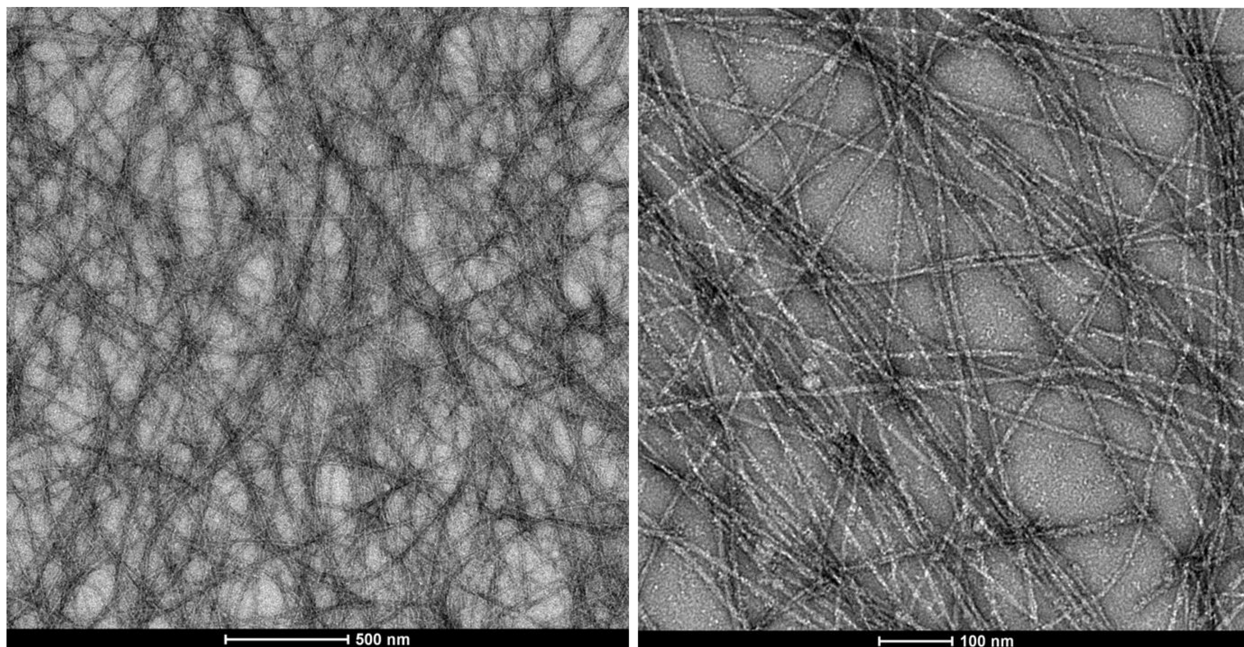


Figure 2.7 TEM imaging of rat blood serum

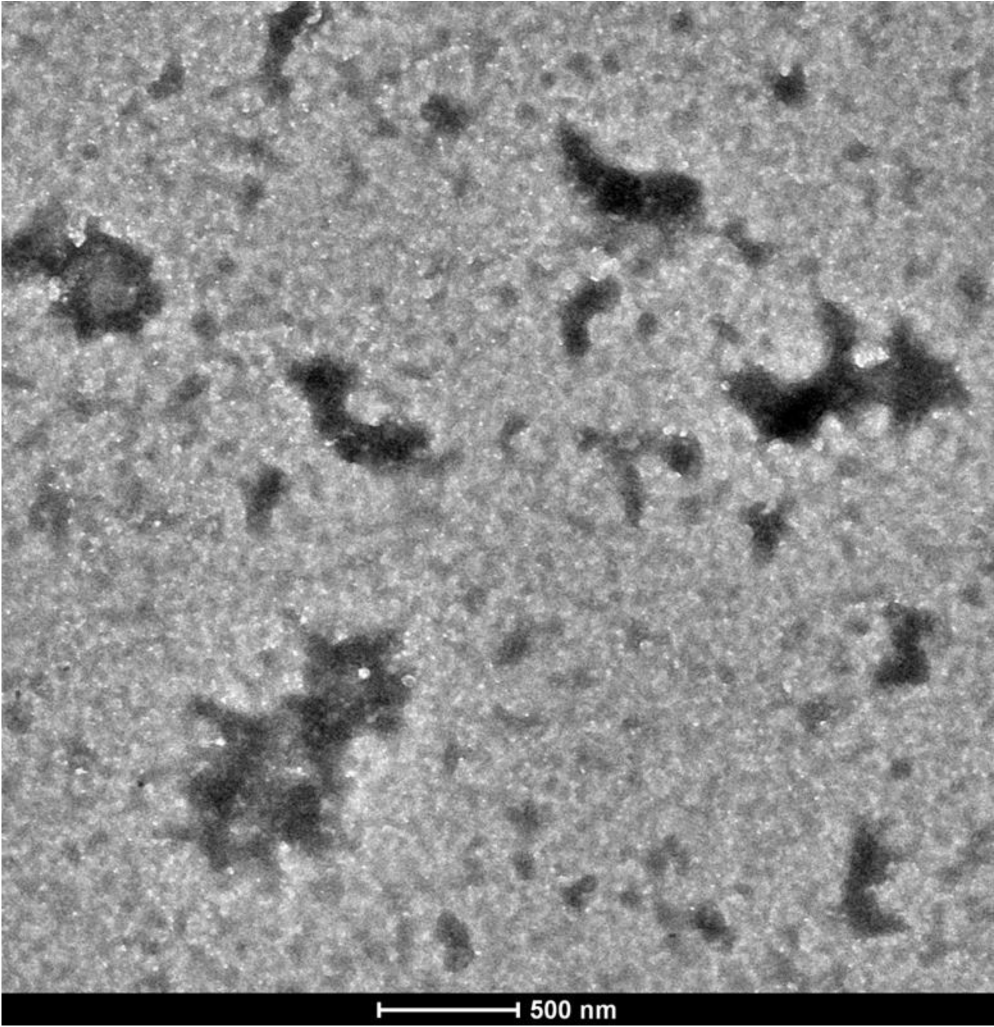


Figure 2.8 TEM imaging of rat blood plasma

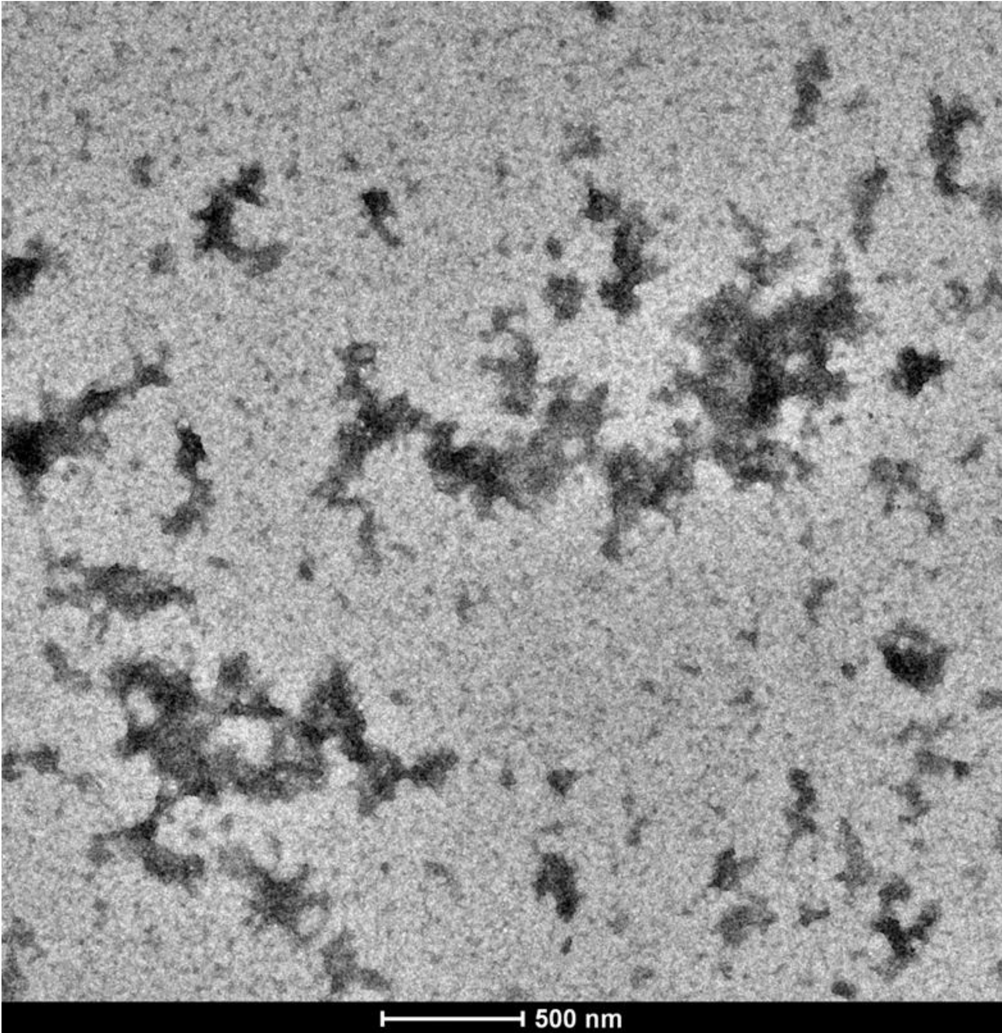


Figure 2.9 TEM imaging of 0.5% h9e with blood serum

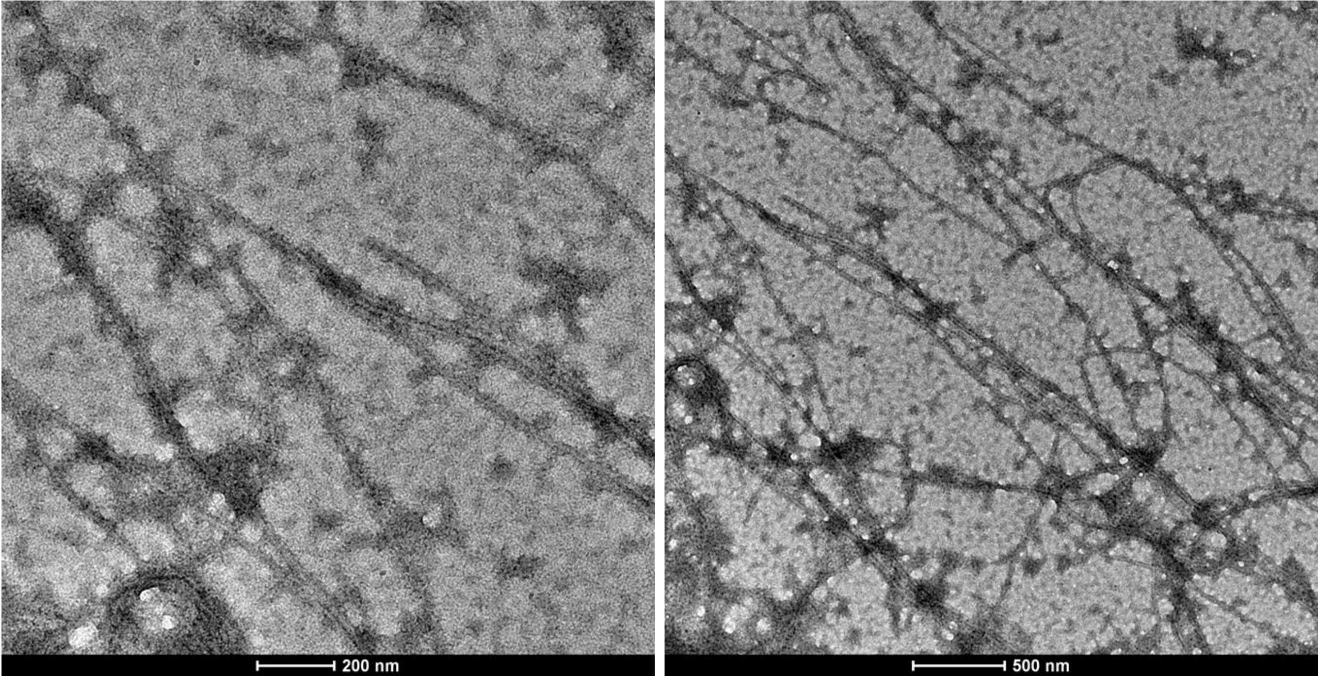
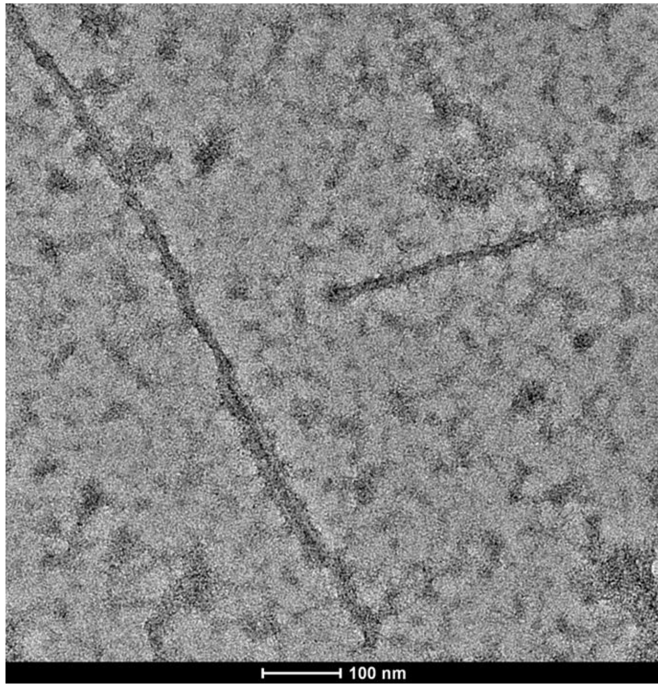


Figure 2.10 TEM imaging of 0.5% h9e with blood plasma



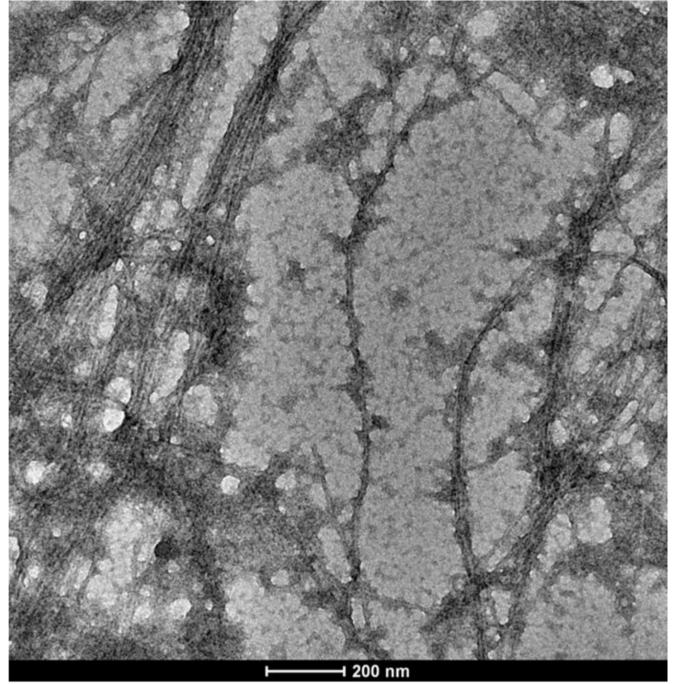


Figure 2.11 Confocal imaging of untreated red blood cells

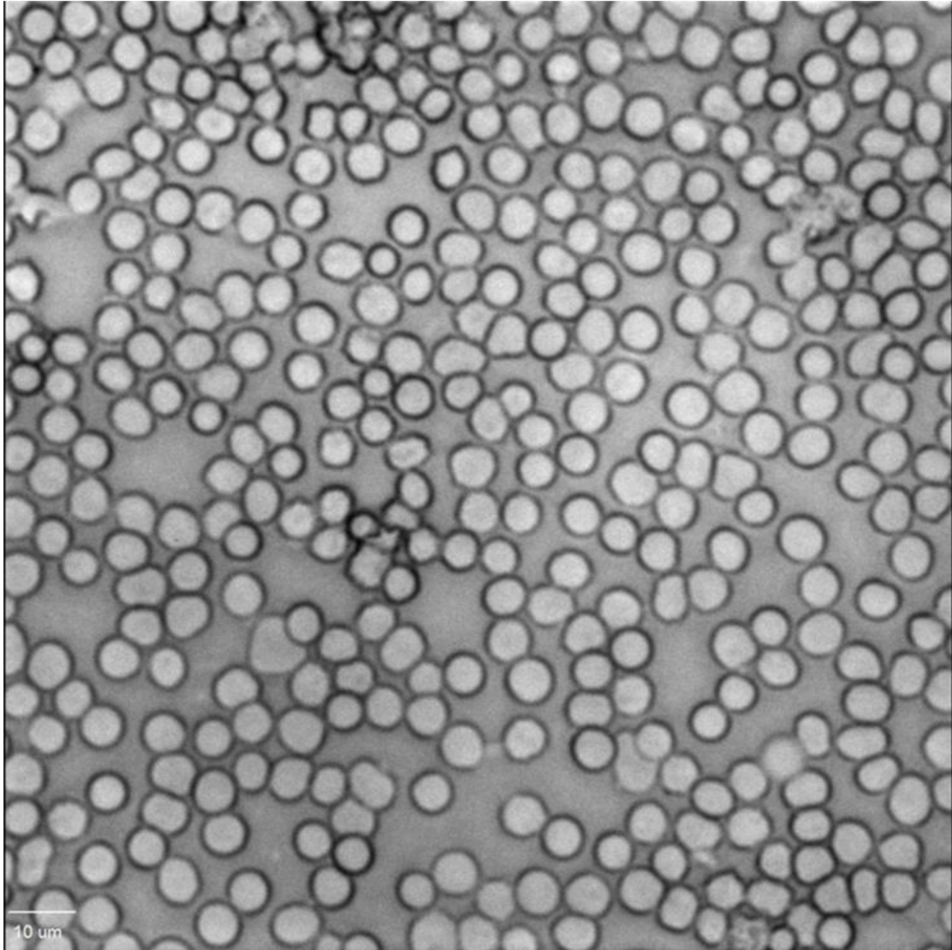


Figure 2.12 Confocal imaging of red blood cells with 0.5% h9e solution (1:2 cells to h9e)

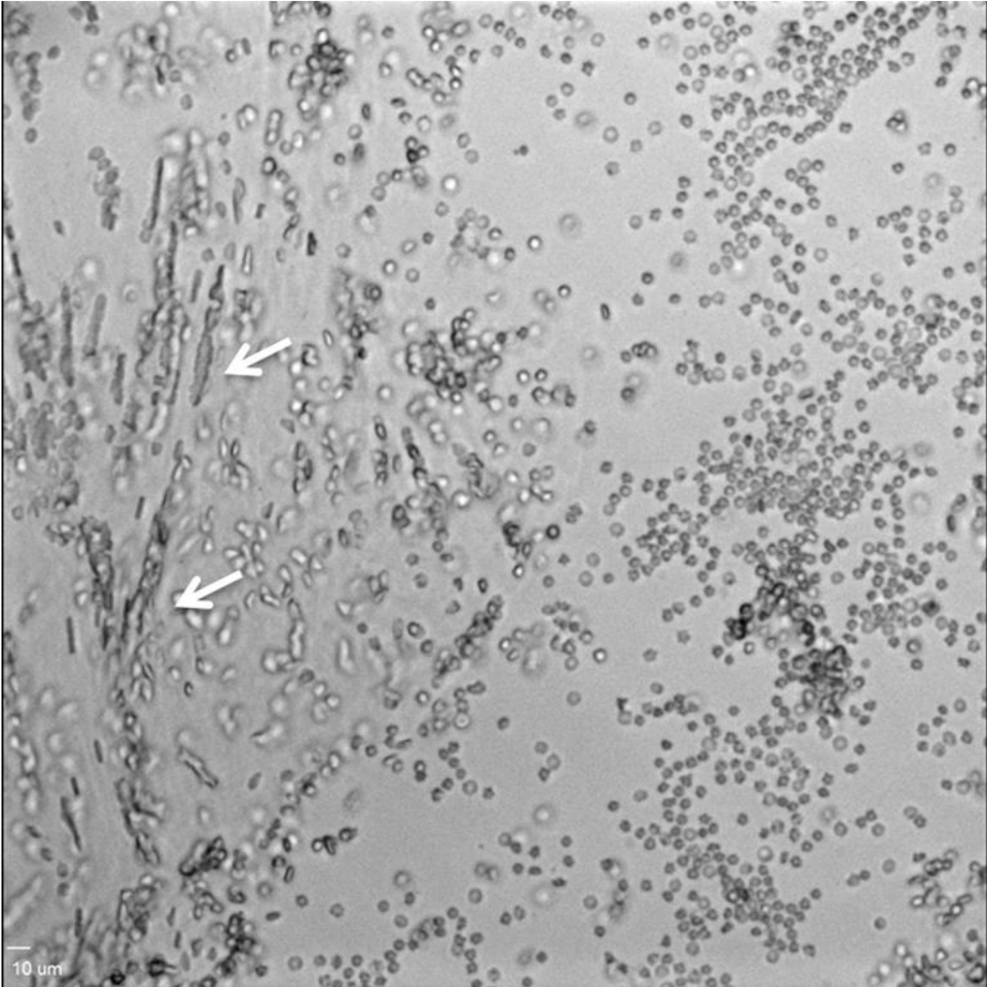
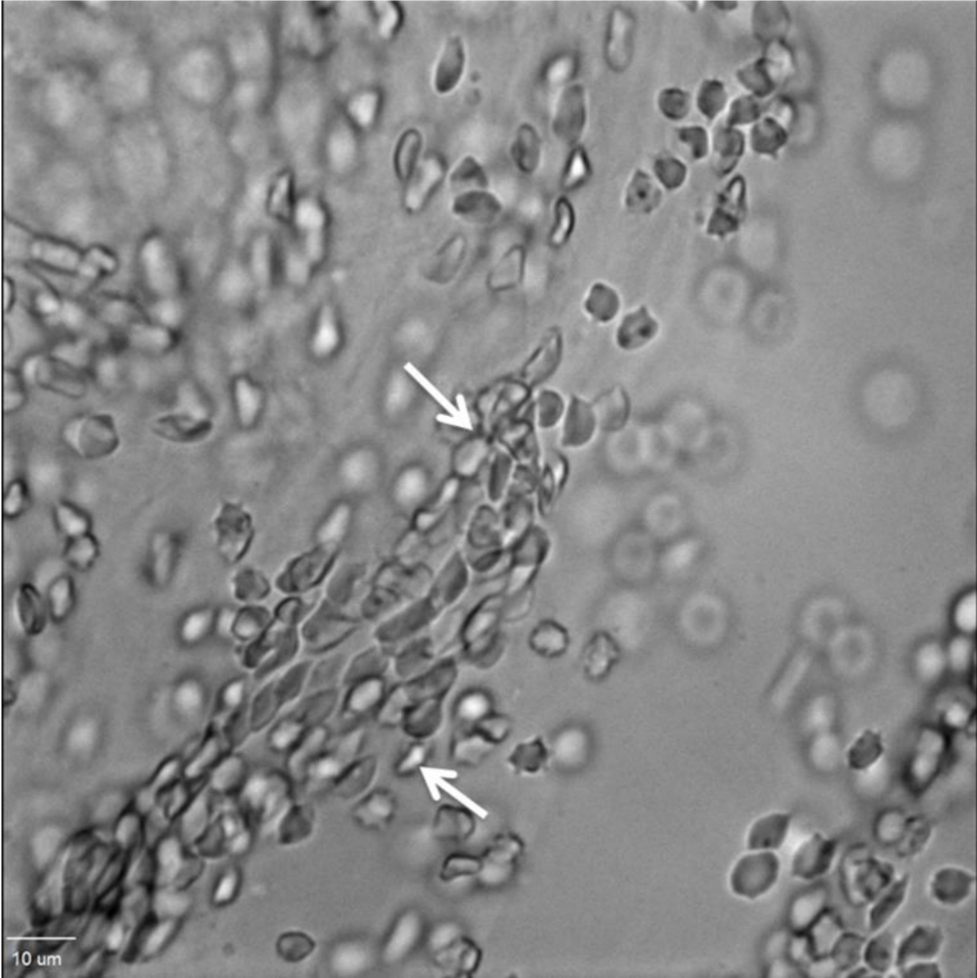


Figure 2.13 Confocal imaging of red blood cells with 0.5% h9e solution (1:2 cells to h9e) higher magnification



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Chapter 3 - Effects of antibiotics on hemostatic efficiency

3.1 Introduction

It is generally accepted that all wounds have some type of bacteria growth but over colonization can prevent wound healing and cause chronic infection [1]. It is well known that in addition to controlling hemorrhage, preventing chronic infection is key to healing [1].
This work is our first report of the h9e peptide's ability to form a stable hydrogel that can be used effectively for hemostasis. We have conducted preliminary research in an attempt to address the issue of chronic infection as well as hemorrhage. We have identified two potential additions to the h9e peptide solution for the purpose of producing an anti-bacterial hemostatic agent: Ampicillin and Neomycin Sulfate.

3.2 Materials and Methods

Preparation of h9e peptide solution

H9e peptide was synthesized at the Bio-Materials and Technology Lab, in the Department of Grain Science and Industry at Kansas State University. After synthesis the peptide was cleaved, washed with anhydrous ether, dissolved in acetonitrile and distilled water (1:1 ratio), frozen at -80°C, and freeze dried. Stock solution of peptide solution was made by dissolving freeze-dried peptide in 100mM $NaHCO_3$ to 5% wt. Solution was autoclaved before use. The purity of the h9e peptide used was ≥ 85% confirmed by high performance liquid chromatography (HPLC).

Rheological analysis of instantaneous gelation performance

A time sweep was conducted on 5% wt. h9e peptide solution, whole rat blood, and antibacterial solution (either 10% ampicillin or 10% neomycin sulfate). Samples were mixed at different ratios to yield a total sample size of approximately 400µl. The mixture was homogenized and immediately added to the rheometer for evaluation. The elastic modulus, G', of the sample was determined using a C-VOR 150 rheometer (Malvern Instruments, Malvern, Worcestershire, United Kingdom). A plate 20mm in diameter was used with 1 % strain and frequency of 1 Hz at a 37°C temperature for 30 minutes.

Transmission electron microscopy (TEM)

The morphology of antibacterial agents and antibacterial agent/h9e mixtures was observed using transmission electron microscopy (TEM). Exactly 6 ml of the appropriate sample was placed onto a negative glow discharged carbon-coated copper grid for 2 minutes. The remaining sample was removed by blotting with kimwipe. The grid was then placed into 2% uranyl acetate for 2 min. Extra stain was removed by blotting with tissue paper. The wet grids were allowed to air-dry for several minutes prior to being examined under TEM.

Samples were imaged using a FEI Technai G2transmission electron microscope at an electron acceleration voltage of 200 KeV. Images were captured using a standardized, normative electron dose and a constant defocus value from the carbon-coated surfaces. The images presented are presented in the absence of any further processing or image filtering.

3.3 Preliminary Research Results and Discussion

3.3.1 Ampicillin

The rheological analysis of 5% wt. h9e peptide solution, 10% ampicillin solution, and whole rat blood was used to compare the gelation strength at different ratios. Figure 3.1 shows that as the amount of ampicillin added to the mixture decreases, the strength of the gelation response slightly increases. Gelation strength is at its highest when ampicillin is not part of the mixture, however, small amounts of ampicillin still induce a gelation response. No gelation response is formed with ampicillin and h9e solution only.

The TEM imaging of ampicillin and 0.5% h9e shows that ampicillin molecules become trapped in the h9e fiber matrix (Figure 3.2). At this time the exact mechanism and how it relates to a decrease in gel strength is unclear. For the purpose of getting clear images, a very low concentration of h9e peptide solution had to be used.

3.3.2 Neomycin sulfate

The rheological analysis of 5% wt. h9e peptide solution, 10% neomycin sulfate solution, and whole rat blood was used to compare the gelation strength. Figure 3.3 shows that no gelation response is formed from this mixture. The figure also shows the vast difference between 5% h9e only and whole blood gelation response vs the 5% h9e, 10% neomycin, and whole blood solution.

The TEM imaging of neomycin sulfate and 0.5% h9e shows that neomycin sulfate molecules are breaking down and forming thick fibers in the presence of the h9e peptide solution (Figure 3.4). The fibers observed, are not h9e fibers. When the fibers are broken, a clean break is observed. The fibers formed from the neomycin sulfate and h9e solution are not recovering. The fibers appear to form a new substance. It is important to note that during visual examination, the

combination of h9e solution and neomycin sulfate solution yield a thick, white substance. For the purpose of getting clear images, a very low concentration of h9e peptide solution had to be used.

3.4 Future research suggestions

1. To determine an appropriate amount of ampicillin or neomycin sulfate that will yield a gel forming material that can maintain its antibacterial properties and still induce a hemostasis response.
2. To test the antibacterial ability of the additive with h9e in a manner that will provide quick and accurate results. The Kirby-Bauer disk method used with with a common bacterium found in infected wounds, such as *Staphylococcus aureus* should be sufficient for a conceptual study. The ring of inhibition (zone of inhibition) should be measured and ring size should be used for comparison and formulation of antibacterial solution.
3. To determine if there are additional antibacterial agents that will yield a gel forming material, can maintain its antibacterial properties and still induce a hemostasis response.

Figure 3.1 Storage modulus vs time of 5% wt. h9e peptide solution, 10% ampicillin solution, and whole rat blood at various ratios

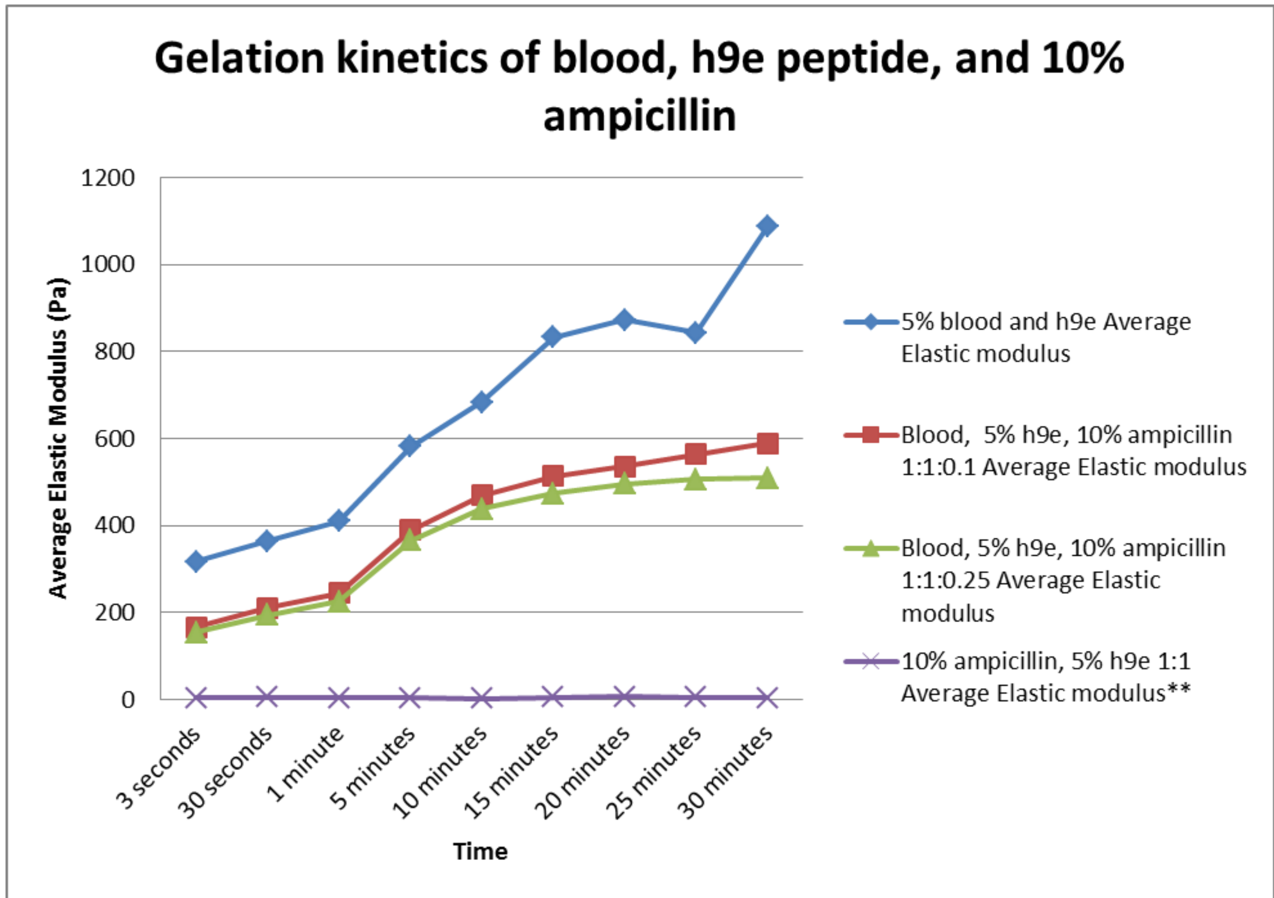
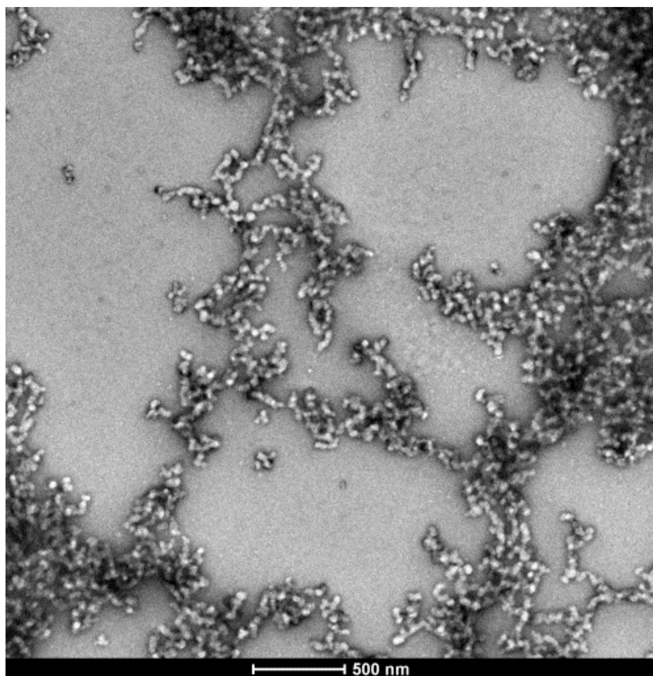


Figure 3.2 TEM imaging of ampicillin only and ampicillin with 0.5% h9e



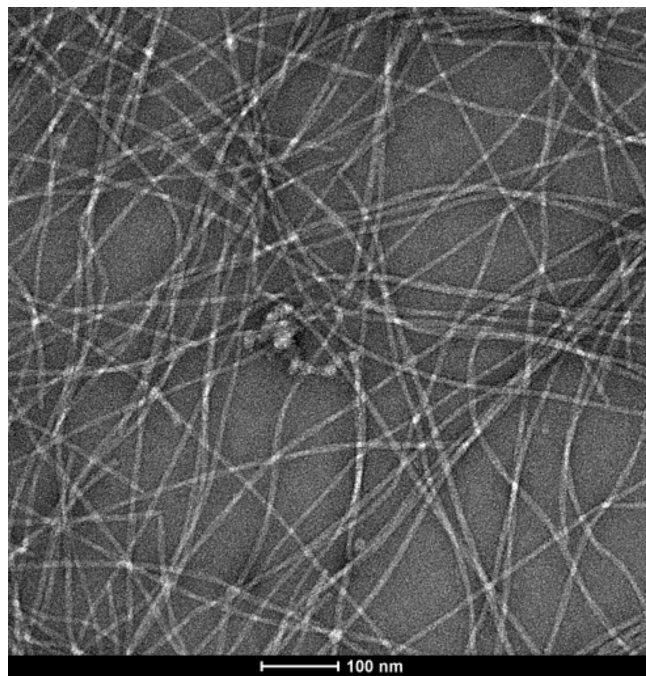


Figure 3.3 Storage modulus vs time of 5% wt. h9e peptide solution, 10% neomycin sulfate solution, and whole rat blood (1:1 ratio).

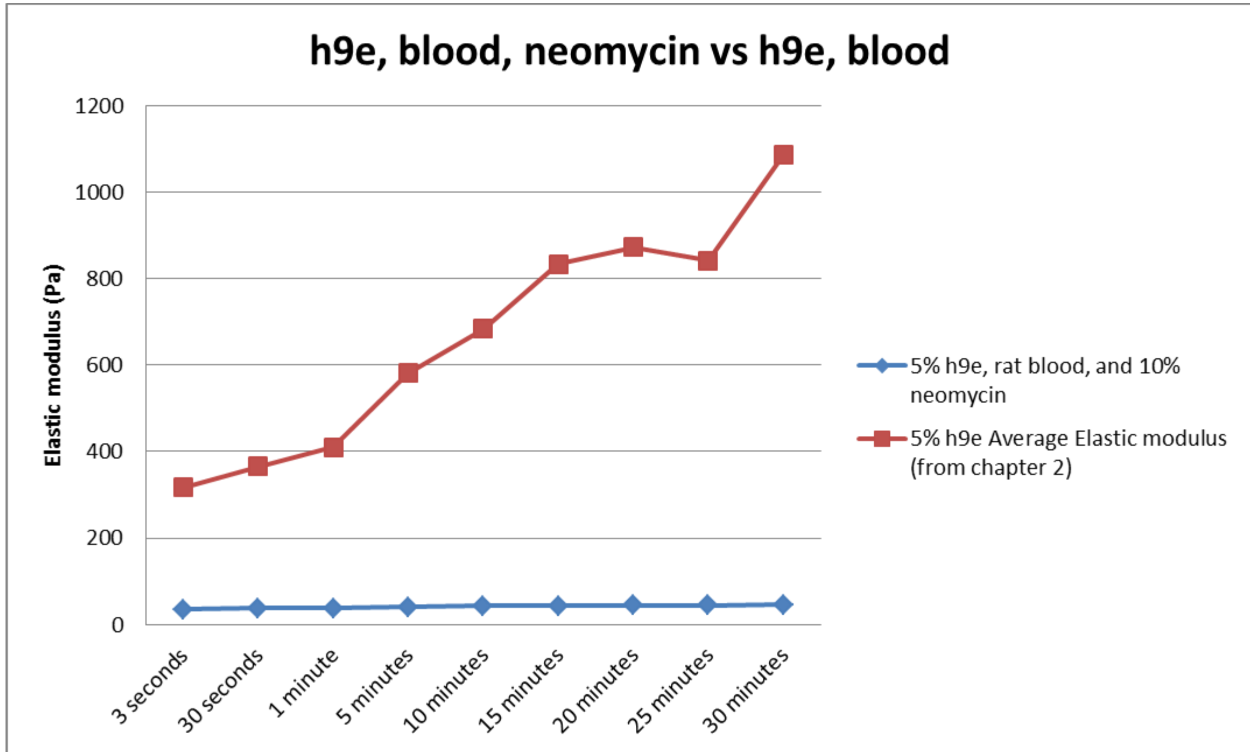
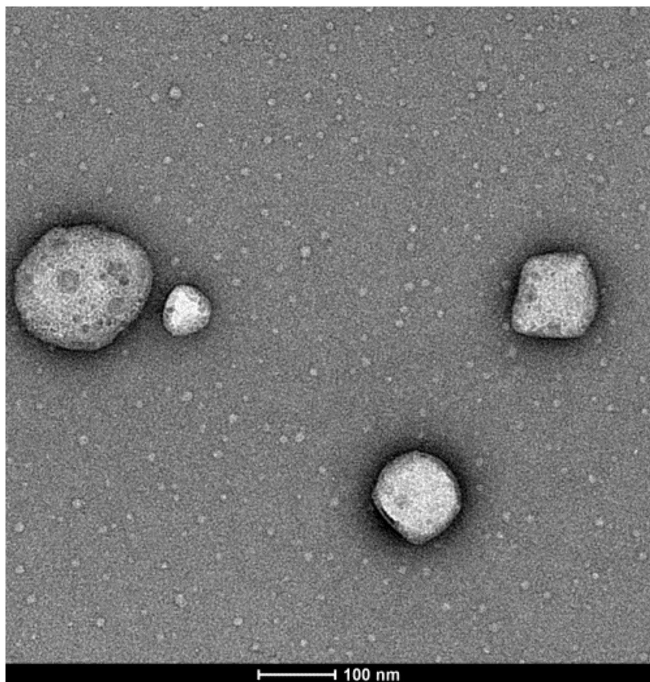
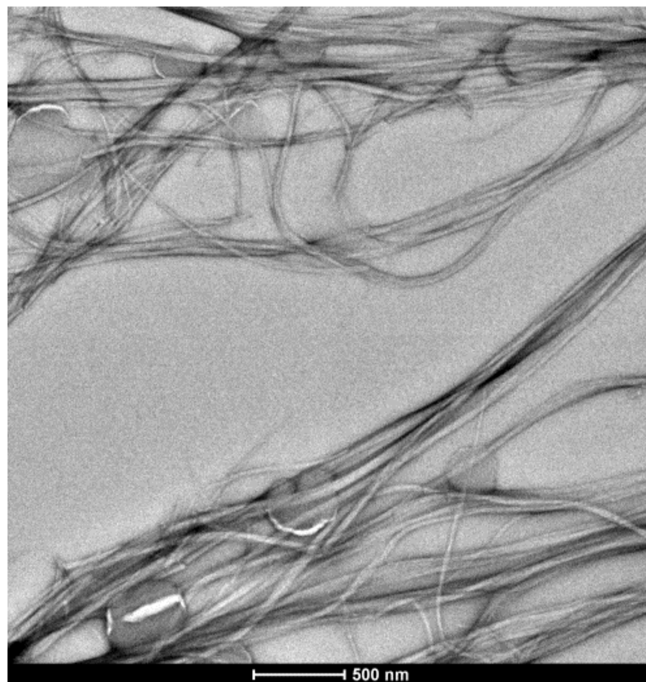


Figure 3.4 TEM imaging of neomycin sulfate only and neomycin sulfate with 0.5% h9e





3.5 References

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