Investigating Potential Interactions Between Neurohypophyseal Peptides, their Drug Analogs and Coagulation Factor VIII: A Novel Approach to Achieving Hemostasis

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complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Investigating Neurohypophyseal Modulation of the Coagulation Cascade

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The neurohypophysis is known to secrete vasopressin during times of severe haemorrhage, and oxytocin during labour and delivery, events in which blood loss also occurs. Evidence suggests that there may be an interaction between these peptides and the coagulation cascade, and currently, desmopressin, a drug analog of vasopressin, is a first line therapy for mild Hemophilia A, a common coagulopathy. In this work, interactions between vasopressin and oxytocin with a crucial member of the coagulation cascade, the cofactor Factor VIII, were investigated using fluorescence spectroscopy, interference immunoassays, commercially produced Factor VIII activity kits, and equilibrium dialysis. The results suggest not only that the peptides interact with the coagulation factor, but do so in a way that enhances its activity and prolongs its active form half-life, as seen through the enhancement of the activity of the FXase complex by assay. This theory was then expanded to study the analogs of vasopressin and oxytocin, desmopressin and carbetocin, drugs currently used clinically in the treatment of hemorrhagic conditions. Similar results were obtained. On the whole, these results indicate a potential newly discovered role for the neurohypophyseal hormones, as well as their drug counterparts being used in clinical practice today and may suggest an expanded clinical use in the control of severe haemorrhage.

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"It always seems impossible until it is done."

- **Nelson Mandela**

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List of Abbreviations

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide	EDC
Activated Coagulation Factor IX	FIXa
Activated Coagulation Factor VII	FVIIa
Activated Coagulation Factor VIII	FVIIIa
Activated Coagulation Factor X	FXa
Arginine Vasopressin	AVP
Bovine Serum Albumin	BSA
Capillary Electrophoresis	CE
Carbetocin	Car
Coagulation Factor VII	FVII
Coagulation Factor VIII	FVIII
Coagulation Factor VIII Activity	FVIIIC
Coagulation Factor VIII Concentration	FVIIIAg
Coagulation Factor IX	FIX
Coagulation Factor X	FX
Desmopressin	dDAVP
Fibrinogen Binding Inhibitor Peptide	FBIP
Formic Acid	FA
Heavy Chain of Factor VIII	HC
Human Serum Albumin	HAS
Immune Tolerance Induction	ITI
Immunoglobulin G	IgG
Light Chain of Factor VIII	LC
Lipoprotein Receptor like Protein II	LRPII
Liquid Chromatography Mass Spectrometry	LC-MS
Oxytocin	Oxy
Polyethylene oxide	PEO
Sulfo-*N*-Hydroxysuccinimide	Sulfo-NHS
Tenase Complex	Xase
Von Willebrand Factor	vWF

Chapter 1 Introduction

1.1 The Impact of Hemorrhage

The human body's survival depends on the maintenance of a specific blood volume within the vasculature, as it enables adequate perfusion to the vital organs, delivering nutrients and removing waste products. The importance of the maintenance of this blood volume is evident in the field of trauma medicine. In this field, studies have shown that approximately 30 to 40% of preventable deaths occur because of hemorrhagic shock and exsanguination, with the loss of sufficient circulating volume leading to multi-organ dysfunction, tissue hypoxia and death [1-4]. Trauma, however, is not the only pathology associated with significant risks of bleeding. Liver disease, chronic kidney disease, hematologic malignancies, severe infections, the peripartum state, and vasculopathies, for instance, are all pathologies associated with significant bleeding risks.

The clinical consequences of hemorrhage are dependent on the degree of blood volume lost, and range from asymptomatic to multi-organ dysfunction. As shown in Table 1, there are a variety of physical signs and symptoms that develop as the blood losses progress. The human body, which stores an average of blood volume of 7% of the body weight, can lose up to 15% of this volume before physiological adaptations such as increased heart rate and respiratory rate begin in order to maintain perfusion to the vital organs [5]. Ultimately, unopposed hemorrhage will result in severe shock and eventual death as the vital organs are no longer adequately perfused to receive sufficient oxygen to maintain cellular respiration and life.

Table 1. Classification of Hemorrhage and Clinical Consequences. Adapted from [5].

Parameter	Class of Hemorrhage			
	I	II	III	IV
Blood Loss (mL)	<750	750-1500	1500-2000	>2000
Blood loss (%)	<15	15-30	30-40	>40
Heat Rate (bpm)	<100	>100	>120	>140
Blood Pressure	Normal	Decreased	Decreased	Decreased
Respiratory Rate	8-15	15-20	20-30	>30
Signs & Symptoms	None	Anxious	Confused, pale, clammy, decreased urine output	Lethargic, pale, little to no urine output

Mammalian evolution has, however, led to the development of a variety of mechanisms designed to limit blood loss after injury. Among these is the hemostatic system, a coordinated response of the vasculature, circulating platelets and specific plasma proteins designed to limit hemorrhage by ultimately forming seals at breaks in blood vessels, as depicted in Figure 1.1

The process of hemostasis is composed of two components, the primary hemostatic system and the secondary hemostatic system, also known as the coagulation cascade. The primary system, the initial defense mechanism to be activated, involves activation, aggregation and adhesion of platelets to the endothelial surface at the site of tissue damage, resulting in the formation of the platelet plug [6]. On its own, however, the platelet plug that forms at the site of

vascular injury is weak and cannot withstand the shear stress of blood flow for a prolonged period of time prior to breaking apart. Primary hemostasis thus relies on the coagulation cascade to further strengthen the thrombus [7].

The actions of the coagulation cascade result in the conversion of fibrinogen to fibrin. Fibrin is a protein that crosslinks the platelets together and solidifies the blood clot, or thrombus, ensuring that it can remain in place and resist the shear forces of blood flow. After spontaneous lysis of the platelets in the plug within several hours, the remaining cross-linked fibrin mesh acts as the definitive clot until it is degraded through plasminogen once the seal in the blood vessel is closed. Thus, through the coordination of both hemostatic systems, blood loss can be stemmed within minutes except in the most severe lesions.

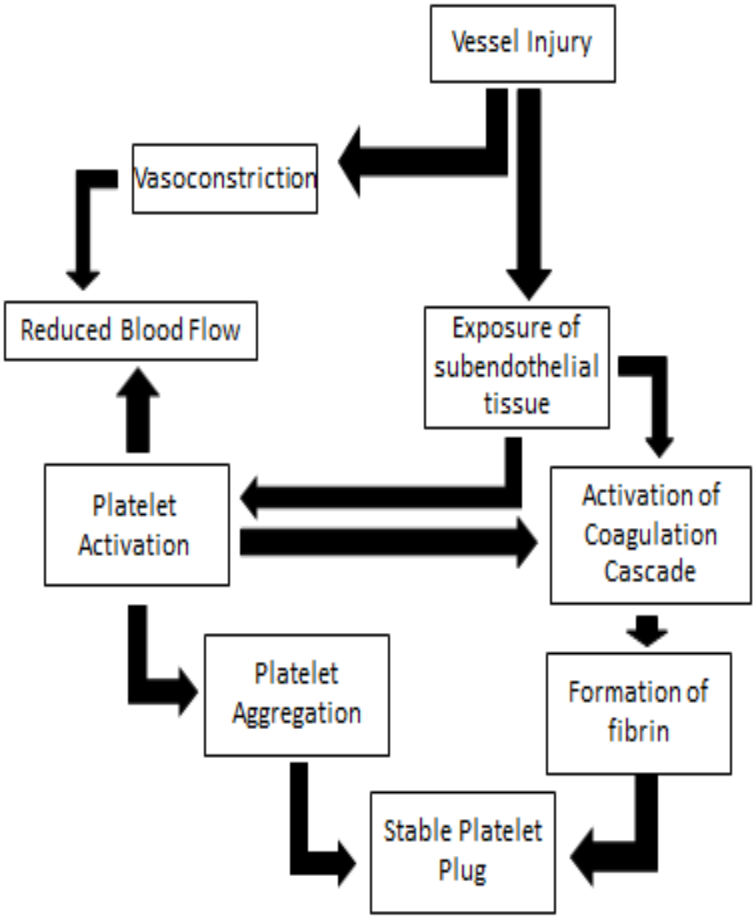

Figure 1.1 Simplified Overview of Hemostasis. The coordinated events of hemostasis begin with injury to blood vessels, which exposes the subendothelial matrix to circulating blood. Platelets are activated in response, and release vasoconstricting products to slow blood flow to the region. They are also activated and begin aggregating. Simultaneously, part of the exposed matrix activates the coagulation cascade, which ultimately produces fibrin to crosslink platelets and solidify the platelet plug [8].

1.2 The Coagulation Cascade

The coagulation cascade is a complex series of proteolytic reactions mediated by the action of a group of serine proteases, which ultimately result in the conversion of the zymogen fibrinogen into fibrin. Initially conceptualized as a "waterfall" model of proteases with two

distinct branches, the “intrinsic” and “extrinsic” pathway, that converged at a “common” pathway to generate thrombin [9], coagulation is now considered as a unified system composed of three phases, initiation, propagation and maintenance, as shown in Figure 1.2 [10].

While relatively complex, the coagulation cascade can be described in a simplified manner. Under normal circumstances, the vascular endothelium has anti-thrombotic properties, ensuring that blood clots to not spontaneously form within undamaged blood vessels and ensuring fluidity to allow adequate flow. However, upon damage to blood vessels, a protein known as Tissue Factor (TF) that is normally sequestered from circulating blood, as well as subendothelial connective tissue matrix, become exposed to circulating platelets and inactive coagulation factors. Tissue Factor then reacts with a small amount of circulating coagulation Factor VIIa (FVIIa), formed through auto-activation from FVII, to form a TF-FVIIa complex. This complex can only activate the Coagulation Factor X (FX) to FXa inefficiently, but enough FXa is generated to cleave proThrombin (FII) into thrombin. This small amount of thrombin can then cleave and activate FVIII and FV, which serve to accelerate the Coagulation Factor IXa’s activation of FX, and FXa’s cleavage of thrombin, respectively. Ultimately, the thrombin that is generated catalyzes the conversion of fibrinogen into fibrin, which can then crosslink the platelets to solidify the thrombus and stem blood loss [11].

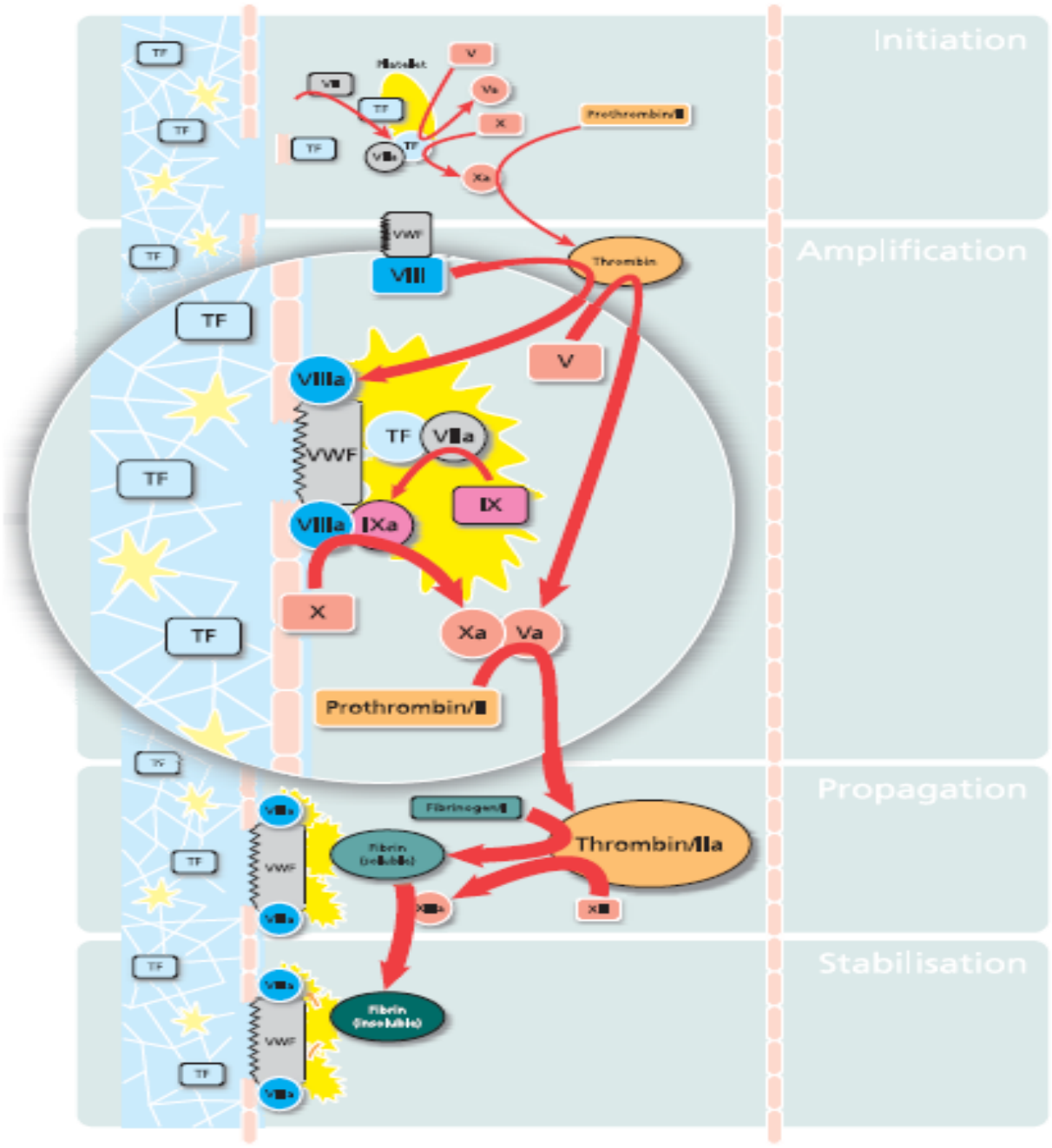

Figure 1.2 The Coagulation Cascade. Composed of separate steps, initation, amplification, propagation and stabilization, the coordinated effects of the coagulation factors, bound to platelet phospholipid membrane and vascular endothelium, mediate a series of catalytic reactions that ultimately result in the formation of fibrin monomers, that can then become crosslinked, as well as serve to crosslink the platelets within the platelet plug, thereby solidifying it. [12]

1.3 Hemophilia A

Though the human body has numerous defense mechanisms to prevent blood loss, many of these are subject to pathologies, including the coagulation cascade. Often times, these are genetic disorders resulting in the decreased production of one or more components of the coagulation cascade. As the cascade proceeds in steps, the absence of one of the coagulation factors can have dire consequences. An important example of this is Hemophilia A, the most common of the severe inherited bleeding disorders [13]. It can be found in approximately 1 per 5000 males [14], and has an estimated frequency of 1 in 10000 births in the general population [15]. The disease is characterized by prolonged bleeding times to both major and minor injuries, as well as by spontaneous hemorrhages, such as in joint spaces (hemarthroses) [16, 17] or, in severe cases, spontaneous intracranial hemorrhages. It also adds risks to patients undergoing even minor procedures such as dental extractions, in which they may bleed profusely. Hemophilia A results from the reduced activity of Factor VIII in circulation, through genetic mutations within the FVIII gene found within the X chromosome, explaining its increased phenotypic expression in males. The percentage of FVIII activity remaining relative to that of normal plasma determines the severity of the disease. If the activity of factor VIII in blood is greater than 25% of the average, then the disease is clinically silent and will not result in pathologies. Mild disease is seen with FVIII levels of 5-25% of the normal range, and moderate disease with 1-5%. Severe hemophilia on the other hand is characterized by an FVIII activity level of less than 1% of the average, and commonly results in problems with spontaneous bleeding [18].

The treatment of Hemophilia A is dependent on the degree of FVIII activity present within circulation and whether or not there is active bleeding. In the setting of active

hemorrhage, FVIII concentrates are given immediately if available. If they are not, fresh frozen plasma, which contains all of the coagulation factors, can be used to restore coagulation function and reduce bleeding events. In the setting of prophylaxis, FVIII concentrates are infused several times per week, with levels post infusion verified to ensure normal hemostatic ability has been restored [15].

While Hemophilia A is relatively rare, it nevertheless represents an important burden on the healthcare system, as "hemophiliacs" tend to require closer medical follow up and can put a strain on blood banks with the recurrent need for transfusions. One important consideration in the treatment of hemophilia A is the cost. In Germany for example, the annual cost for healthcare for hemophiliacs ranges from €40000 to 120000 [19], compared with the cost for the remainder of the population, which averages around €2500 [20]. A large part of this cost relates to the short half-life of FVIII, that of 12 hours. As a result of this, infusion of the missing coagulation factor, the standard treatment for hemophiliacs, only provides therapeutic benefit for a limited amount of time. Consequently, people with Hemophilia A require numerous infusions, often 2-4 times per week [14]. Novel methods of prolonging FVIII half-life have been studied. Presently, PEGylated versions of the recombinant protein have demonstrated significant promise in prolonging FVIII half-life in serum, and therefore reducing the frequency of infusions required [14].

Until the advent of recombinant FVIII, FVIII concentrate infusions tended to be derived from human serum, and consequently, represented important vectors for infection [17]. In fact, many hemophiliacs became infected with the hepatitis viruses B and C, as well as by HIV [21]. The importance of this infection cannot be understated, as 38% of patients with hemophilia A in the United Kingdom from 1985-1996 died as a result of AIDS-related illness, presumably due to

contaminated blood products [22]. Fortunately, the recombinant versions of the protein, though still hampered by the short half-life of the product, do not carry the same risk of infection.

Another important limitation in the treatment of severe Hemophilia A is that repeated infusions of FVIII can engender an immune response to the protein. As patients with severe Hemophilia A have minimal amounts of active, or mutated, FVIII, normal FVIII can be recognized as a foreign protein by their immune system. Thus, these patients can develop the so-called "inhibitors" to FVIII, which are polyclonal antibodies against the FVIII antigen, that bind and remove the infused FVIII from circulation [17, 23]. This leads to significant morbidity and a decrease in overall quality of life. Overcoming these inhibitors is an important area in hemophilia research [24]. Currently, the most widely studied method is the eradication of the inhibitor through inducing immune tolerance (ITI) [25], though other methods of mutating FVIII to reduce its antigenicity are also underway.

1.4 Coagulation Factor VIII

The clinical consequences of Hemophilia A make structural defects in FVIII resulting in a loss of activity an important pathology, and a target of significant research interest. Coagulation Factor VIII (FVIII) is a key component of the coagulation cascade, specifically the intrinsic arm of it, and an important cofactor in the formation of FXa, as mentioned above. It is a 300 kDa glycoprotein heterodimer, produced within the liver sinusoidal endothelial cells, as well as by the pulmonary endothelium [12]. It is released into circulation in its inactive state, with a circulating concentration of 0.5-1 nM [26]. Within the circulatory system, it binds to a large multimeric carrier protein, von Willebrand Factor (vWF), and forms a tight, non-covalent complex, which greatly increases its half-life [27].

FVIII is initially synthesized as a polypeptide composed of 6 distinct domains, termed A1, A2, A3, B, C1 and C2, with conserved acidic amino acid bridges, termed a1, a2, a3, connecting the domains. Thus, the sequence of the initial polypeptide domain structure can be described as A1-a1-A2-a2-B-a3-A3-C1-C2 [28]. After synthesis, this polypeptide undergoes specific cleavages within the B-domain prior to release into circulation, as well as within the segment connecting the B and C1 domains [29]. Together, the A1, A2 and B domains remain complexed, forming the so-called Heavy Chain (HC) of Factor VIII, while the A3, C1 and C2 domains form the Light Chain (LC). The B domain however is noted to be unnecessary for FVIIIa functionality, as B-domain deleted recombinant FVIII maintains its cofactor activity [30], and the cleavage of the B-domain during the protein synthesis can occur at numerous regions within the domain [31, 32]. The ribbon structure of FVIII is depicted in Figure 1.3, while the life cycle of FVIII is shown in Figure 1.4 in order to better illustrate its synthesis and its function.

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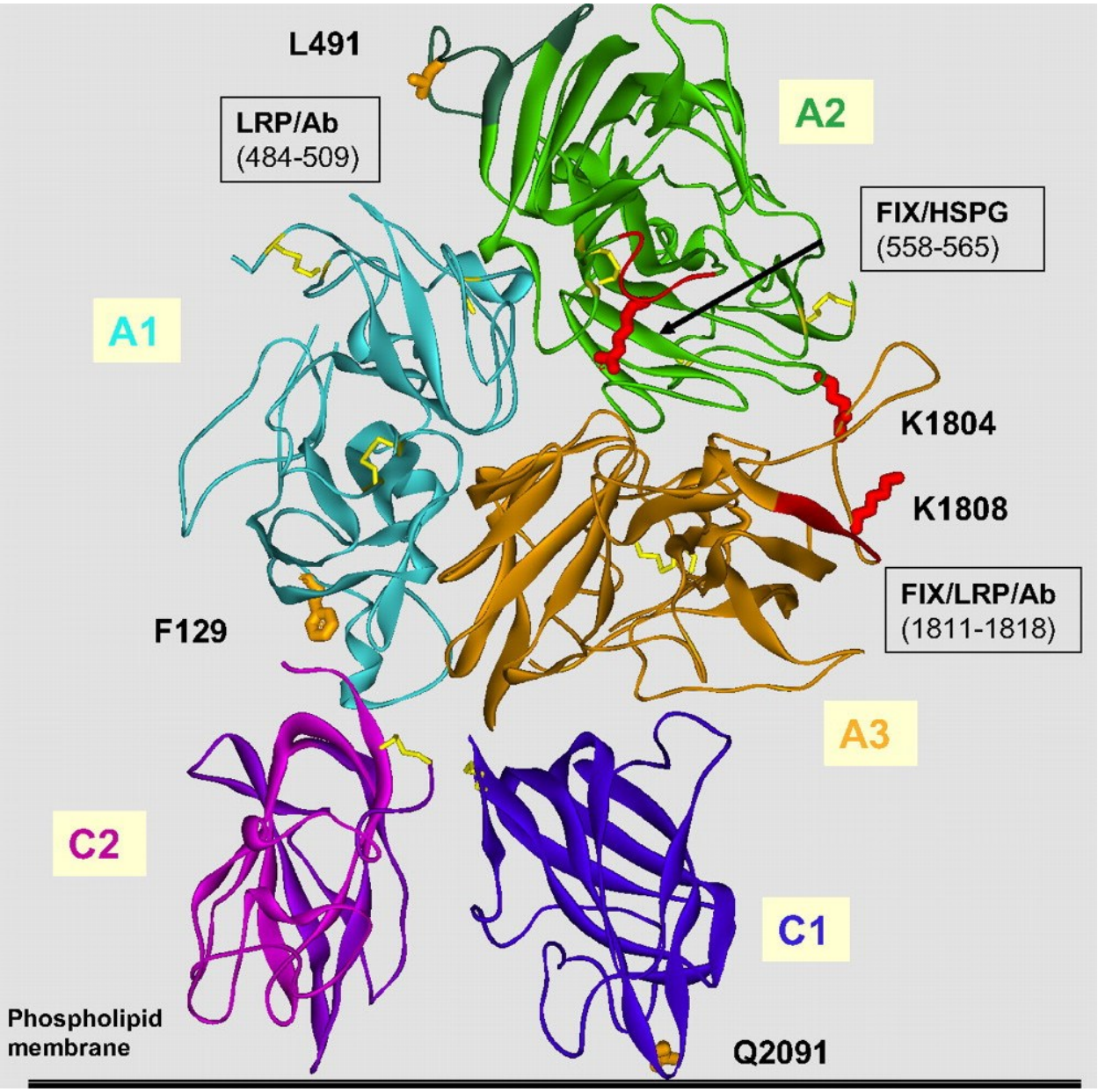

Figure 1.3 The Ribbon Structure of FVIII. This figure shows the theorized structure of coagulation FVIII as well as various important sites of interaction [27]

The HC and LC are non-covalently bound together via a divalent metal ion dependent mechanism, with copper being the most common metal involved [33]. Studies have shown that chelation of this metal ion results in dissociation of Factor VIII and resulting loss of

procoagulant activity. The heavy chain of FVIII is the portion of the protein which binds to FIXa, and its A2 domain is the primary site of this interaction [33]. The light chain of FVIII is the site of binding with the lipoprotein like receptor protein II (LRPII), which is the primary receptor implicated in endocytosis and removal of FVIII from circulation [34-37]. It is also the primary site of binding of FVIIIa to the phospholipid membrane upon which the Xase complex assembles and where the coagulation factors concentrate after vessel injury [38].

Unbound, the half-life of the FVIII protein in circulation is short, on the order of 2.5 hours. Bound to vWF, its half-life increases to 12 hours through inhibition of early proteolysis and by inhibiting cellular uptake of the protein [39, 40]. Factor VIII's clearance from blood is mediated by several systems. Firstly, it is cleared from circulation via endocytosis by Lipoprotein Receptor like Protein II (LRPII), a multiligand endocytic receptor, with a K_d of 116 nM [41]. These proteins are found throughout the human body in various tissues, but are importantly concentrated within the liver cells [41]. As mentioned, the LC of FVIII is the primary site of interaction between LRPII and FVIII. This site of interaction is within the acidic region of the LC [42].

The second important mediator of FVIII clearance from circulation is the spontaneous dissociation of subunit A2 from the remainder of the HC, which results in the loss of procoagulant activity [43, 44]. While the half-life of the FVIII zymogen is approximately 15 to 19 hours [29, 45], that of the activated form, FVIIIa, is approximately 2 minutes [46]. The short half-life of the activated form is a result of the spontaneous dissociation of the A2 domain previously discussed. The interaction between the A2 unit and the remainder of the FVIIIa is considered relatively weak, with a K_d of approximately 260 nM at physiologic pH [47]. Once FVIII has been converted to FVIIIa, the vWF multimer loses its affinity and releases FVIIIa. One

of the effects of the association of vWF on FVIII is also to provide stability to the association of A2 with the remainder of the heavy chain, and once vWF has dissociated, the A2 subunit will dissociate more readily.

The role of FVIII in coagulation is that of a cofactor for FIXa to catalyze the conversion of FX to FXa. In order to function as a cofactor, FVIII must be converted to FVIIIa through cleavage by thrombin. Thrombin cleaves at very specific residues within the HC to release the A2 subunit from the A1 subunit, as well as within the B domain and the light chain. The net result is that FVIIIa forms as a heterotrimer, composed of the heavy chain of A1 and A2 non-covalently bound, interacting with the light chain. The FVIIIa is then free to bind to the phospholipid membrane of platelets and vascular endothelium, as well as interact with FIXa to form the Xase complex [48]. Within the Xase complex, the mechanism through which FVIIIa affects the catalytic activity is complex and remains unclear [13].

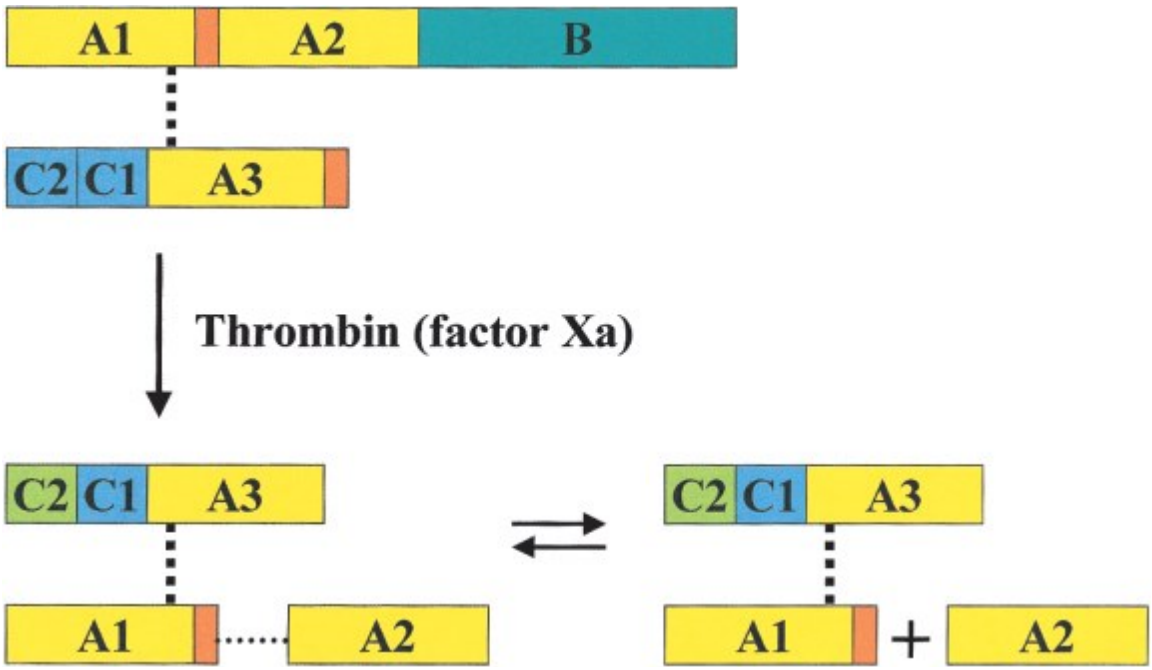

Figure 1.4 Activation of FVIII to FVIIIa by Thrombin. The Heavy Chain is cleaved in the B domain and between the A1 and A2 regions, causing the A2 to dissociate. Meanwhile, cleavage also occurs within the C2 domain, yielding a conformation change. The FVIIIa molecule then is composed of A1 and A2 non-covalently bonded, interacting with a modified light chain through a metal ion bridge. [49]

1.5 Vasopressin and Desmopressin

One of the treatments for mild hemophilia A is with a peptide analog of the hormone Vasopressin (AVP), known as Desmopressin (dDAVP), as administration of it has been noted to increase FVIII concentration in blood [50]. AVP is a nonapeptide produced by the posterior hypothalamus and released via the neurohypophysis (i.e. the posterior pituitary). It has a distinct ring like structure, common to all the neurohypophyseal peptides and their synthetic drug analogs as a result of an intramolecular disulfide bridge (See Appendix A). Its release is governed by several physiological stimuli, notably serum hyperosmolality, severe hypovolemia with hypotension, nausea, and pain among others [51]. It is also found to increase significantly during hemorrhagic states, and is being carefully studied for potential hemostatic benefits [52].

AVP circulates at a basal concentration of 1.6 ng/L, or approximately 1.5 pM , and can increase by at least one order of magnitude in concentration during states of shock, if not more [53].

Vasopressin has multiple physiological roles, related to its activation of different receptors located in different tissues throughout the body. It is currently thought to have three main receptor types, V1, V2 and V3, noting two distinct subtypes of V1 receptors, V1a and V1b [40, 54]. The V1 receptors are found in vascular smooth muscle and the platelet phospholipid membrane, and their activation leads to vasoconstriction and platelet aggregation respectively. The V2 receptors are primarily located within the kidneys and the activation of these receptors causes an increase in renal collecting duct water permeability through the insertion of aquaporins into the duct, allowing retention of more water in the circulatory system and thus increasing blood pressure and decreasing serum osmolality. This receptor is, however, also found in the vascular endothelium. Once these endothelial V2 receptors are activated through binding with AVP, it triggers the release of vWF and FVIII from stores within the endothelium [55, 56]. This is primarily thought to be the method of action for the transient increase in FVIII concentration seen with the infusion of AVP noted since the 1970s [55, 57].

As a therapeutic agent, AVP is primarily used in critical care medicine [58-60]. The activation of the V1 receptors leading to vasoconstriction allows vasopressin to be used in states of shock, whether septic, hemorrhagic or hypovolemic [58]. However, the ability of vasopressin to mitigate hemorrhaging has been demonstrated in multiple studies and is now being considered as a hemostatic agent, though the mechanism through which this occurs is unclear. Stadlbauer *et al.* [61] demonstrated the survival of pigs with liver lacerations receiving vasopressin infusion compared to placebo which died within 20 minutes. Similarly, there have been case reports of trauma victims whom have been successfully resuscitated using arginine vasopressin to improve

blood pressure and control hemorrhage. Similarly, many reviews have established a trend toward significantly less blood transfusion requirements in human patients with hemorrhagic shock when treated with vasopressin [62]. However, to date, there has been a paucity of studies investigating the importance of the effects of AVP on FVIII concentrations within blood and the outcomes of these patient groups, with many studies focusing solely on mortality and hemodynamic parameters, as opposed to net blood loss and need for transfusions [63]. Given the utility of vasopressin in hemorrhagic shock, the implication that the peptide is impacting the hemorrhage itself and not simply the hypoperfusion element is raised. The question of whether or not the peptide is interacting with the coagulation cascade in some way was further supported through the recognition that desmopressin (dDAVP), its synthetic analog, has been utilized in the treatment of Hemophilia A. Desmopressin is also a nonapeptide, with a very similar structure to vasopressin, including the intramolecular disulfide bridge, except that it has been deaminated at the N-terminus, and the Arg^8 residue is a D-isomer. It is now commonly used in the treatment of many bleeding disorders, such as in the treatment of bleeding secondary to platelet dysfunction brought on by uremia and renal failure [64]. While this mechanism is not fully understood, its activity on promoting platelet aggregation and the increase in FVIII levels without inducing significant hemodynamic changes have led to its use in this patient population [65]. Similarly, desmopressin is now used commonly to reduce bleeding in patients without innate coagulation disorders, such as in post cardiac surgery patients who often require medical treatment to reduce bleeding [66], and the theorized mechanism remains that of the release of the stores of pro-coagulants from the vascular endothelium discussed earlier. Desmopressin is a first line treatment for mild Hemophilia A [15]. It had long been noted that the administration of desmopressin resulted in a measurable increase in FVIII activity within the blood of patients with

Hemophilia A, first shown to enable them to undergo minor procedures [27]. It was subsequently shown that the stores of FVIII and vWF within the vascular endothelium which are released through activation by vasopressin, are also released through activation with dDAVP. One critical question raised by the observation of the effects of AVP and dDAVP on FVIII levels is do these peptides directly increase the activity of FVIII. Despite numerous publications demonstrating the *in vivo* response of FVIII to AVP and dDAVP infusion, a study investigating the *in vitro* effects of the peptides on FVIII activity could not be found. However, of note, there has been at least one study investigating the response of FVIII to the peptides in patients with diabetes insipidus, a condition characterized by an absence of the vasopressin receptors [67]. This study demonstrated no FVIII response to dDAVP if the receptor was absent, suggesting that the mechanism of FVIII change with dDAVP was dependent on one of the V receptor subtypes. With this publication, attention shifted away from any potential interaction between the peptides and the protein. However, recently, there has been increasing literature questioning the reliability of FVIII activity kits, with many noticing increased interassay variability and a disconnect between FVIII measured activity and actual concentration [26], suggesting that previous reports on FVIII activity that utilized some of these earlier activity kits may have been subject to imprecise measurements of activity. As a result, one must wonder whether, or not, there could be an interaction between AVP, dDAVP and FVIII that was missed. The possibility that these peptides have an effect on coagulation outside of that noted through activation of the V2 receptor is further supported by the finding of other neurohypophyseal peptides, oxytocin and carbetocin, which may also have hemostatic effects and yet do not interact with the V2 receptor.

1.6 Oxytocin and Carbetocin

The posterior pituitary also releases another hormone, known as Oxytocin (Oxy). Like AVP and dDAVP, oxytocin is a nonapeptide with an internal ring structure resulting from the presence of an intramolecular disulfide bridge. Oxytocin is implicated in many roles. It is crucial to parturition, and helps to stimulate uterine contractions. It is also implicated in the limitation of maternal blood loss post-partum. This property is thought to occur as a result of the tonic contractions of the uterus after placental loss that its administration causes [68]. Post-partum hemorrhage is the leading cause of maternal mortality worldwide, with 67-80% of cases being accounted for by uterine atony [69, 70]. This function of oxytocin has led to the synthesis of a drug analog, known as carbetocin. This drug is now commonly used in obstetrical medicine to both stimulate contractions and aid in fetal deliveries, while also limiting blood loss post-partum [69]. Importantly, carbetocin is often given in the setting of post-partum hemorrhage, as a method of achieving hemostasis quickly though this is believed to be through the treatment of uterine atony as opposed to any direct effect on the hemostatic system [71].

A previous study has demonstrated that oxytocin does in fact have some anti-hemophilic properties that are not well explained through the release of FVIII from the endothelium. Oxytocin does not typically interact with the V2 receptor on the vascular endothelium but is rather more targeted to receptors within the uterine muscle stimulating contraction. [72]. Similarly, carbetocin does not have the V2 receptor activity either, and its therapeutic properties are also thought to be a result of its activation of the oxytocin receptors [73].

Recognizing the utility of vasopressin in shock brought on through hemorrhage, the effect of dDAVP in the treatment of hemophilia and uremic bleeding disorders, and the therapeutic value of oxytocin and carbetocin on the post-partum hemorrhage, one must wonder

whether or not these peptides are interacting with the coagulation cascade in some manner. This possibility is further supported by recognizing that oxytocin and carbetocin impact the outcomes of hemorrhagic states similarly to vasopressin but without acting on the V2 receptors to release FVIII stores. This leads to the question, are the neurohypophyseal peptides and their drug analogs interacting with FVIII, and if so, could this interaction explain the benefits seen in the bleeding patient?

1.7 Thesis Objectives

The goals of this thesis were to investigate if there is an interaction between the neurohypophyseal peptides, their drug analogs, and coagulation Factor VIII, and to qualify this interaction by determining if it is specific, to identify the probable chain of FVIII that the peptides bind, as well as to show if the interaction enhances FVIII's half-life. Ultimately, this thesis' main objective was to elucidate the possibility that the increase in FVIII activity, seen through the administration of desmopressin and vasopressin to patients, not only results from the release of endothelial stores of the protein but also that they interact with FVIII and that this interaction may also contribute to the finding of increased FVIII activity in serum. Furthermore, this thesis will suggest that the interaction between desmopressin and FVIII is not limited to dDAVP and AVP, but due to their similar structures, also includes oxytocin and carbetocin, and may represent alternative explanations for the ability of these peptides to reduce rates of post-partum hemorrhage.

In order to probe if there are protein-peptide interactions between FVIII and the neurohypophyseal peptides, fluorescence spectroscopy binding experiments were performed to assess changes in tryptophan fluorescence from the FVIII with the addition of peptide. The

interactions were further characterized by filtration experiments demonstrating retention of peptide on one side of the filter when FVIII was present. Furthermore, equilibrium dialysis experiments were performed demonstrating a shift in the equilibrium concentration of vasopressin across a semi-permeable dialysis membrane when FVIII was present on one side of the membrane.

In order to assess the location of the peptide binding site on the protein, an attempt was made to develop a microsphere based immunoassay in the hopes of showing that the presence of the peptides within the assay would lead to failure of binding of the antibodies required to detect the FVIII in solution.

Finally, a commercially available FVIII activity kit was used to confirm that the peptides do not enhance FVIII activity in plasma, and assess if they increase the half-life of the activated FVIII. Using these activity kits, it will also be shown that the peptides can rescue FVIII inhibition by heavy chain antibodies.

Chapter 2 Experimental

2.1 Materials and reagents

Human purified FVIII was obtained from ProSpec Bio (Rehovot, Israel). Vasopressin, Desmopressin, Oxytocin, Polyethylene Oxide and Bovine Serum Albumin were all obtained from Sigma Aldrich (Oakville, ON, Canada). Carbetocin was obtained from Creative Peptides (Shirley, NY, USA). Fibrinogen Binding Inhibitor Peptide (FBIP) was obtained from Anaspec Inc (Fremont, CA, USA). The Technochrom factor VIII activity kits and Chromogenix FVIII Coamatic Test Kit were obtained from Diapharma (Ohio, OK, USA). Tosylated paramagnetic microspheres were obtained from Invitrogen Life Sciences (Ottawa, ON, Canada). Fluorescent microspheres were obtained from Bangs Laboratories (Fishers, IN, USA). The anti-FVIII heavy chain (HC) and light chain (LC) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Water was obtained from a Barnstead EASYpure® II UV Ultrapure water system (Dubuque, IA, USA) at 18.2 MΩ-cm.

2.2 Measuring FVIII-peptide interactions

2.2.1 Fluorescence Spectroscopy

The fluorescence spectrum of FVIII and FVIII with the peptides was measured using a Varian fluorimeter with an excitation wavelength of 295 nm, and emission was monitored from 305 to 450 nm. The voltage for the photomultiplier tube was set at 700 V. For this, and all other fluorescence experiments, an excitation bandwidth of 5 nm and an emission bandwidth of 10 nm were utilized. The experiments were performed at 37 °C. A solution of 22 nM FVIII in 10 mM

phosphate buffered saline (PBS), pH 7.4, was brought to temperature over 3 minutes and had its initial fluorescence spectra determined. Then single microliter additions of 22 μM peptides (AVP, DDAVP, Oxytocin, Carbetocin, FBIP) were added and the mixture was incubated for 2 minutes before a new emission spectrum was measured. After 15 μL had been added, further additions of 5 μL were made until no further changes in the spectra were recorded. The results were then analyzed with Origin Pro, in which the EC50 was determined.

2.2.2 Thermal denaturation of FVIII in the presence of peptides

In order to assess possible stabilizing effects of the peptides on FVIII, the thermal denaturation of FVIII with/without peptides was assessed using fluoroscopy. Briefly, the experiments were conducted in the Varian fluorimeter using a Pelletier device to control the temperature. Refrigerated solutions (1 mL) of 22 nM of FVIII with/without 1.32 μM of peptides were heated to 20°C and then heated gradually to 85°C over a period of 2 hours, with recordings performed at increments of 5°C. The fluorescence spectra of the tryptophans in FVIII were monitored through excitation at 295 nm and emission at 334 nm.

2.2.3 Determination of photodegradation of FVIII

Solutions (1 mL) of 22 nM FVIII in 10 mM PBS pH 7.4 were heated in the fluorimeter to 37 °C and then continuously irradiated for 30 minutes at 295 nm with the emission at 334 nm continuously recorded.

2.3 Factor VIII Activity

2.3.1 Determination of FVIII activity after prolonged freezing time

The activity of Factor VIII stored at -80 °C for a period of 3 years was determined using a Chromogenix COATEST SP4 FVIII Activity Kit (Diapharma, US). Briefly, solutions of FVIII initially at a concentration of 10 IU/mL in 10 mM PBS at pH 7.4, were thawed on ice and then diluted down to concentrations ranging from 1 mIU/mL to 10 mIU/mL in 50 mM Tris buffer, pH 7.3, containing 1% (m/v) BSA and 10 mg/L ciprofloxacin. 100 µL of these samples were added to 200 µL of a solution containing a proprietary concentration of phospholipids, FIXa and 2.7 IU of FX with BSA. These solutions were incubated for 5 minutes at 37 °C. 100 µL of $CaCl_2$ at 37 °C was then added and incubated again for 5 minutes. Afterwards, 200 µL of a proprietary chromogenic substrate was added and the reaction was allowed to proceed for 5 minutes before being terminated through the addition of 100 µL of 20% (v/v) acetic acid. The samples were then measured using a UV spectrophotometer plate reader at 405 nm and 490 nm.

2.3.2 FVIII activity in the presence of peptides

Factor VIII activity, in the presence and absence of the peptides, was monitored using the Technochrom FVIII Activity Kit (Technoclone, Austria). The kit was performed as per the provided protocol except for the addition of 25 µL of either dilution buffer, or of a 22 µM solution of the peptides dissolved in the kit's standard dilution buffer, composed of 50 mM imidazole with 100 mM NaCl and 0.2% (m/v) BSA at pH 7.4. Briefly, samples of human plasma containing 75% of the normal FVIII activity (750 mIU/mL) were diluted in the aforementioned dilution buffer. Then, 25 µL of these solutions were incubated with either 25 µL of AVP/dDAVP/Oxytocin/Carbetocin/FBIP or of buffer for 5 minutes at 37 °C. Afterwards, 25 µL

of phospholipids and albumin (proprietary concentration unknown) and 25 µL of FIX, FX, calcium, albumin and thrombin were added and the solutions were incubated at 37 °C for 5 minutes. Then, 125 µL of a proprietary chromogenic substrate dissolved in 50 mM Tris buffer pH 8.3 with 10 mM Disodium ethylenediamine tetraacetate (Na_2EDTA) and 427 mM NaCl was added and the solution was incubated for 5 minutes. The reaction was then quenched with 50 µL of 20% (v/v) acetic acid. Absorbance of 405 nm and 490 nm was then measured.

2.3.3 Determination of the effect of phospholipids on the half-life of FVIIIa in the presence of peptides

The half-life of activated FVIII was compared in the presence and absence of peptides using a modified protocol of the Technochrom FVIII activity kit. Briefly, the solutions of phospholipids and albumin, FIX and thrombin, and plasma with known activities of FVIII were mixed and allowed to incubate for periods of 7, 9 and 11 minutes and 13 minutes at 37 °C. Afterwards, the chromogenic substrate was added to the reactions and incubated for 5 additional minutes. Finally, the reaction was terminated through the addition of acetic acid. Similar experiments were performed in which the FIX, thrombin and FVIII were first mixed and allowed to incubate for periods of 2, 4 and 8 minutes, prior to the addition of the phospholipid solution. Afterwards, these were allowed to incubate at 37°C for 5 minutes prior to the addition of the chromogenic substrate. This solution was then allowed to incubate for 5 additional minutes, followed by termination of the reaction through the addition of 20% acetic acid. The absorbance of each solution was then measured at 405 nm.

2.3.4 Determination of the effect of peptides and anti-heavy chain and anti-light chain antibodies on FVIII activity

The activity of FVIII in the presence of antibodies was measured using the COAMATIC SP4 Factor VIII Activity Kit produced by Chromogenix, but with a modified procedure. Briefly, 50 µL of 100 mIU/mL of FVIII was pre-incubated for 15 min with 50 µL of either 22 nM of the individual peptides or 25 mM Tris Buffer, pH 7.9, with 1% (m/v) BSA at 37°C. Then, 22 nM of polyclonal antibodies directed against either the light chain or the heavy chain of FVIII was added and the solution was incubated for 5 min at 37°C. The remainder of the kit was performed as per the manufacturer's instructions. Technochrom kit was then performed as per the instructions provided by the manufacturer, as described previously. The results were measured at 405 nm with the baseline absorbance at 490 nm subtracted.

2.4 Microsphere Immunoassay

2.4.1 Preparing capture spheres

In order to prepare the capture spheres, paramagnetic polystyrene microspheres with tosyl-activated surfaces (Dynabeads, Invitrogen, USA) were used to react with the amine groups of the antibodies as shown in Figure 2.1. An initial concentration of 4 x 10^{12} beads/mL was washed twice with 0.1 M Na-Phosphate buffer, pH 7.4. They were then resuspended in the same buffer. Then, 37.5 µg of antibodies were added to this, and the total volume was increased to 150 µL with Na-Phosphate buffer. The solution was then vortexed for 10 seconds. Then, 100 µL of 3 M ammonium sulfate in 0.1 M Na-Phosphate buffer was added to the solution and allowed to react at 37°C for a period of 16 hours while being shaken on a slow rotating vortex. The

spheres were then pulled down to the bottom of the tube using a rare-earth magnet and the supernatant was removed and replaced with 10 mM PBS pH 7.4, with 0.5% mono-amine terminated PEO. The solution was incubated at 37 °C on a vortex for one hour prior to the spheres being pulled down again. The supernatant was removed and the spheres were washed twice with the PBS with 0.5% (m/v) PEO and then resuspended to a final concentration of 10^{10} beads/mL.

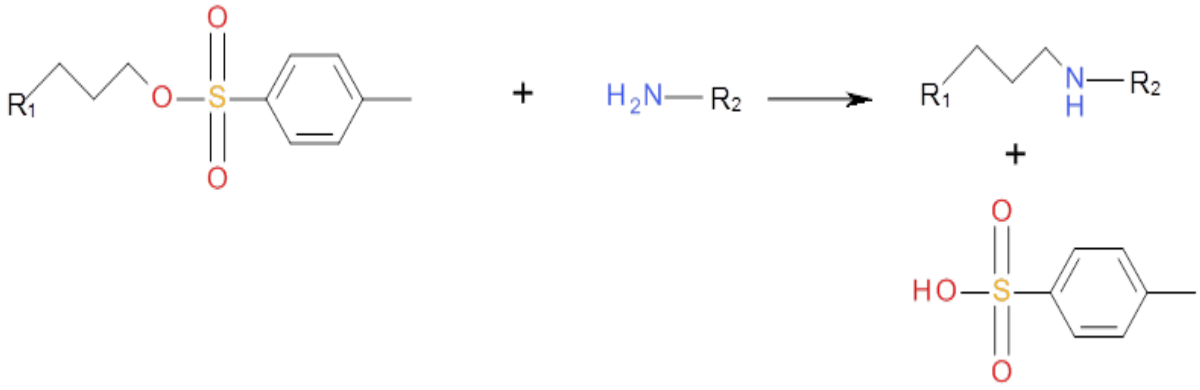

Figure 2.1 Reaction of the microsphere surface tosyl group with the free amine of the immunoglobulin to form the covalent bond between the immunoglobulin and the microsphere surface.

2.4.2 Preparing detection spheres

Carboxyl modified polystyrene microspheres containing Dragon Green fluorescent dye were reacted with a carbodiimide and covalently bound with immunoglobulins (see Figure 2.2) to create the detection spheres [74, 75]. Solutions (100 µL) of the microspheres were first washed twice in activation buffer (0.1 M MES and 0.5 M NaCl, pH 7.4) and then resuspended in 1 mL of the same. 1 mg of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 2.8 mg of *N*-hydroxysulfosuccinnimide (Sulfo-NHS) were added to reach final concentrations of

5.2 mM and 13 mM respectively. This mixture was allowed to react at room temperature with constant shaking for 15 minutes on a vortex at low speed. The microspheres were then divided into two separate aliquots, washed twice with PBS, pH 7.4, and then resuspended in 500 µL of the same. Antibodies (35.7 µg) directed against either the Light chain or the Heavy chain of FVIII were purified from a 200 µg/mL stock solution containing 1% gelatin using a Centricon Ultracentrifugation 100 kDa MW cutoff membrane as per the manufacturer instructions. These purified antibodies were added to the activated microsphere aliquots along with 150 µg of BSA, Fraction V (Sigma Aldrich, Canada). These mixtures were allowed to react with constant mixing for 4 hours at room temperature. Afterwards, these were washed once in PBS and then resuspended in 500 µL of 4 mM amino-terminated Polyethylene oxide (Min MW 5000, Max MW 5400 g/mol) and allowed to react for 30 minutes at room temperature.

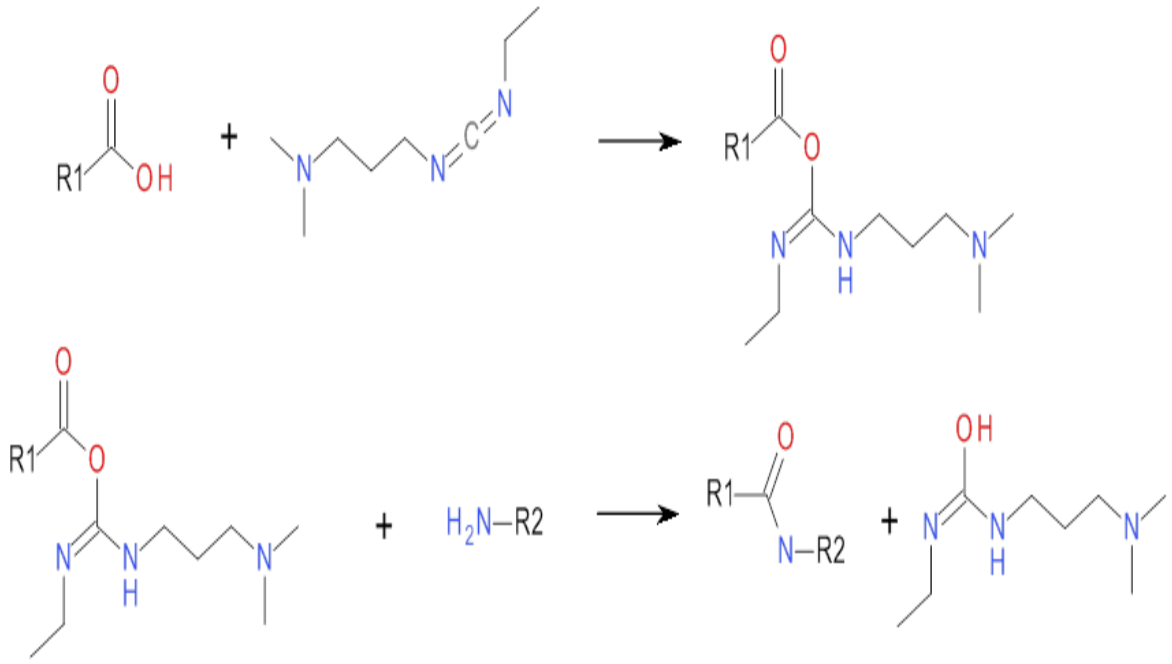

Figure 2.2 Covalent coupling of the immunoglobulins to the detection sphere surface. The reaction begins with the conversion of thecarboxyl group on the sphere surface to a carbodiimide via EDC. This amine-reactive group is then replaced with the free amine of the immunoglobulins.

2.4.3 Conducting the microsphere-based immunoassay to quantify FVIII

The microsphere based immunoassay was performed to quantify FVIII concentrations as shown in Figure 2.3 and establish a calibration curve to be utilized in subsequent interference immunoassays. The assays were conducted on 96-well clear bottom microplates. Initially, 50 µL of capture spheres coated with either anti-heavy chain or anti-light chain antibodies and blocked with polyethylene oxide were pipetted into the wells. Then, 50 µL of FVIII diluted in PBS, with concentrations ranging from 0 to 2.2 nM, were added to the wells. These were allowed to react for 15 minutes, with continuous shaking on a Vortex run at minimal speeds. Then, the plates were placed over a plate containing 0.5 Tesla rare earth magnets arranged in a such a way that

each magnet was centred in between 4 wells. The magnets pulled down the paramagnetic capture spheres and any antigen bound to them. The supernatant was then removed. The plate was taken off the magnetic surface and the spheres-antigen complexes were resuspended in PBS with 4% polyethylene oxide. The plate was then replaced on the magnetic plate and the spheres were pulled down again. This washing procedure was repeated. The spheres were then resuspended in PBS with 4% polyethylene oxide. Detection spheres (50 µL) were then added to the wells. The plate was shaken on a Vortex at minimal speed for 15 minutes. The above mentioned washing procedure was then repeated to a total of six washes in order to minimize non-specific interactions. Finally, the microspheres were resuspended in 100 µL of PBS. The plates' fluorescence was then read using a Victor microplate reader at an excitation wavelength of 480 nm and an emission wavelength of 520 nm, with bandwidths of 15 nm.

2.4.4 Microsphere Interference immunoassay

The effect of the presence of the neurohypophyseal peptides and their drug analogs in solution with FVIII on the microsphere immunoassay's ability to quantify the FVIII accurately was determined, as diagramed in Figure 2.4. Briefly, 50 µL aliquots of 0 to 0.375 nM FVIII in 10 mM PBS buffer, pH 7.4, were incubated with 50 µL of 2.2 nM peptides in 10 mM PBS at pH 7.4 at 37°C for 5 minutes. Following this, the remainder of the microsphere assay was performed as described above. Figure 2.4 depicts the principle behind the microsphere immunoassay interference.

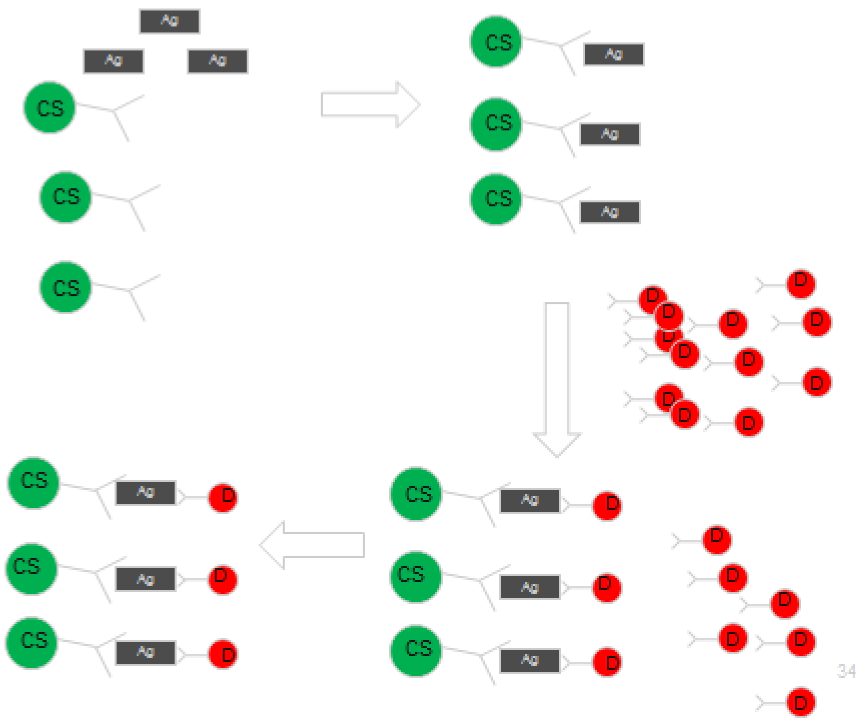

Figure 2.3 The Microsphere Immunoassay. An excess of capture microspheres (CS) are incubated with the antigen (Ag), FVIII in this case, allowed to bind and pulled to the bottom of the titre well. The supernatant is then washed away and detection spheres (D) are added to the sample. These are allowed to bind to the FVIII. The excess detection spheres are then washed away and the sample is measured using a plate reader equipped with a fluorescence detector.

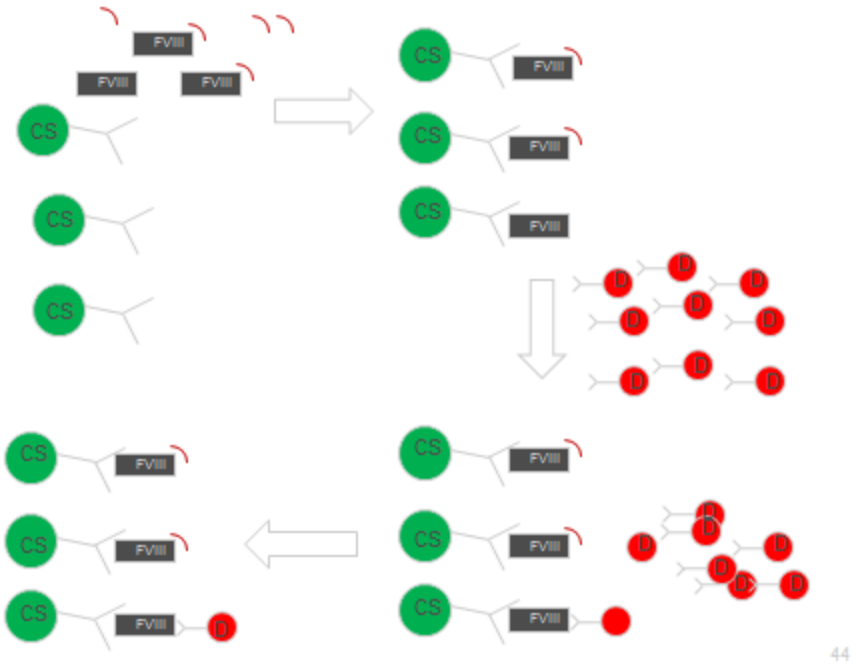

Figure 2.4 The Microsphere Interference Immunoassay. The FVIII is preincubated with the neurohypophyseal antibodies and allowed to interact. This sample is then added to the capture spheres and the remainder of the assay procedure is followed. Here it is shown that the presence of the peptides block an epitope for the immunoglobulins bound to the detection spheres and therefore reduce detection sphere retention and will lead to a reduced fluorescent signal.

2.5 Equilibrium dialysis

The effect of FVIII on the equilibrium concentration of the peptides across a dialysis membrane was assessed. Briefly, 500 µL of 21.7 nM FVIII, diluted in PBS buffer, pH 7.4, was placed in a microfuge tube that had been cut in half. Into this solution was placed a Slide-a-Lyzer MINI dialysis unit (Thermo Fisher, Illinois, USA) with a size cutoff of 2 kDa. The dialysis chamber contained 500 µL of 221 nM peptides also in PBS buffer. These solutions were allowed to equilibrate across the dialysis membrane for a period of 8 hours at 37°C. The solution on top of the membrane was then removed. To these solutions, Glu-fibrinopeptide was added to a

concentration of 50 nM to act as an internal standard. The sample was then analyzed by Liquid Chromatography-Mass Spectrometry as follows. 10 μL of the sample was applied to a C4 column (Grace, USA), and eluted with a gradient of water with 0.1% formic acid (FA) to acetonitrile (ACN), starting with 100% H_2O + FA, 0% ACN, and gradually converting to 5% H_2O + FA and 95% ACN over 10 minutes, with a flow rate of 0.2 mL/min and a pressure limit of 400 bar. The separated solution then underwent analysis in a Waters Micromass Q-Tof Ultima API Mass Spectrometer (Mississauga, ON), in MS Scan mode with a mass/charge (m/z) range of 100-1999 Da, using positive ionization mode, and a cone voltage of 80V, with a scan duration of 0.9 seconds.

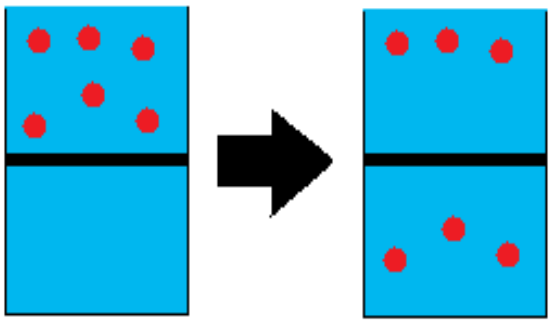

Figure 2.5 Equilibrum dialysis calibration curve design. The equilibrium concentration of the neurohypophyseal peptides was first determined by incubating them on one side of the dialysis membrane, with only PBS buffer on the other side. The peptides were allowed to equilibrate over several hours, and then a sample was collected and measured using mass spectrometry.

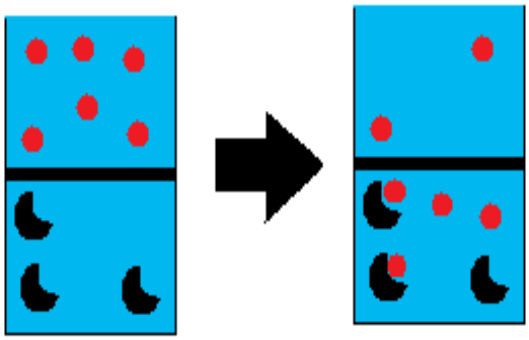

Figure 2.6 Equilibrium dialysis experimental setup. The neurohypophyseal peptides were dialyzed against FVIII for several hours and allowed to equilibrate across the membrane as well as with the protein for 8 hours. The peptide fraction was then probed using mass spectrometry.

Chapter 3 Results and Discussion

3.1 Demonstrating the peptide-protein interaction

Demonstration of peptide-protein interactions can be performed in numerous ways [76, 77]. The key feature is that the interaction between the two species can result in conformational changes in one or the other, or both. These conformational changes can be probed via techniques such as circular dichroism [78, 79], or fluorescence spectroscopy [80, 81]. Alternatively, the heats of interaction between the two species can be measured through isothermal titration calorimetry [82]. Still other techniques can analyze the stoichiometry of the interacting species, such as with analytical ultracentrifugation [83]. Fluorescence spectroscopy is one of the simpler methods. Several of the amino acids, tryptophan, phenylalanine, tyrosine, are fluorescent, absorbing light in the 280-295 nm range and emitting in the 320-380 nm range. The emission profile and intensity is highly dependent on the relative environment of these amino acids and therefore can be used as a reflection of the overall conformation of a protein, should there be sufficient residues present. As a result, one can detect alterations in a protein's higher order structure by monitoring the fluorescent emission of these residues. Therefore, if the neurohypophyseal peptides and their drug analogs interact with FVIII in a manner that alters the environment of the aforementioned residues in the protein, it should be detectable by fluorescence spectroscopy. The primary structures of the neurohypophyseal peptides (See Appendix A) lack tryptophan residues, thus allowing for it to be an ideal target to assess the tryptophan residues within the FVIII.

3.1.1 Determination of FVIII-peptide interactions via fluorescence spectroscopy.

Solutions of FVIII were incubated at 37 °C, and then had increasing amounts of the peptides added to them, all the while monitoring the fluorescence spectrum of the local tryptophan residues with an excitation wavelength of 295 nm and monitoring the spectrum from 305 nm to 450 nm. The following results were obtained, as shown in Figures 3.1 to 3.4, for the addition of vasopressin, desmopressin, carbetocin and oxytocin, respectively.

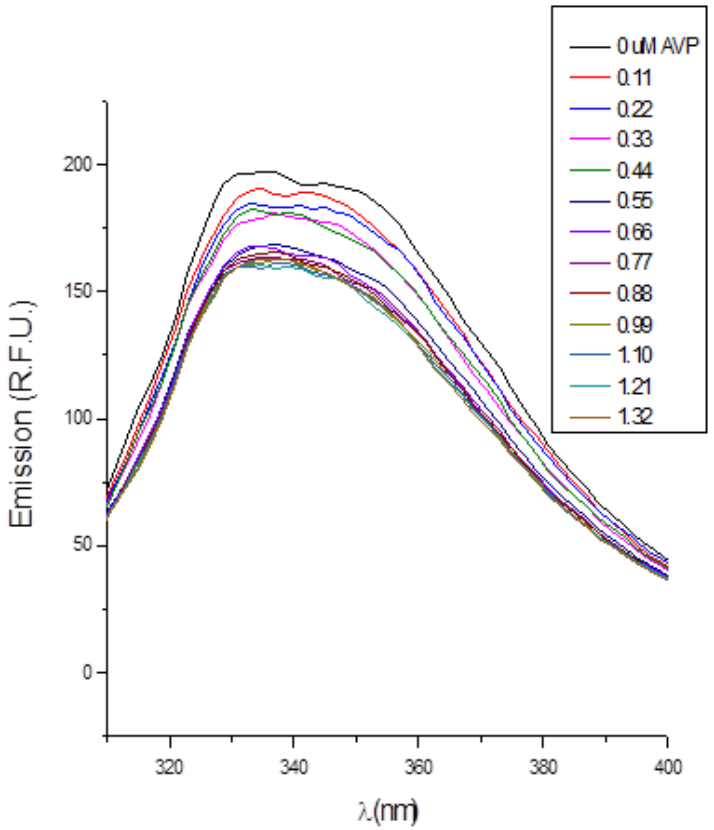

Figure 3.1 Fluorescence spectrum of 21. 7 nM FVIII titrated with 1.32 µM AVP in PBS.

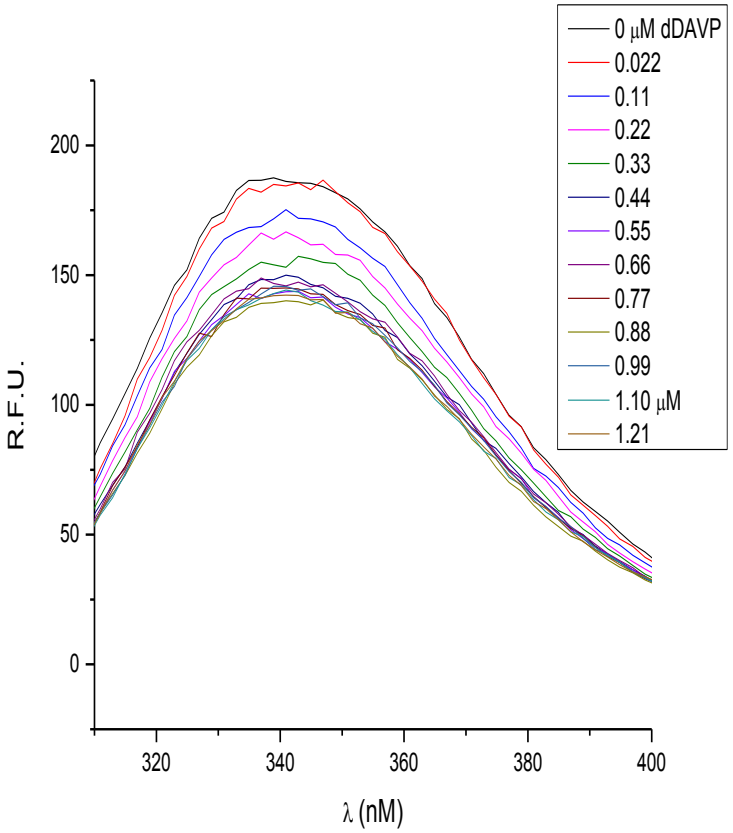

Figure 3.2 The fluorescence spectrum of 21.7 nM FVIII in PBS, pH 7.4, with the addition of dDAVP.

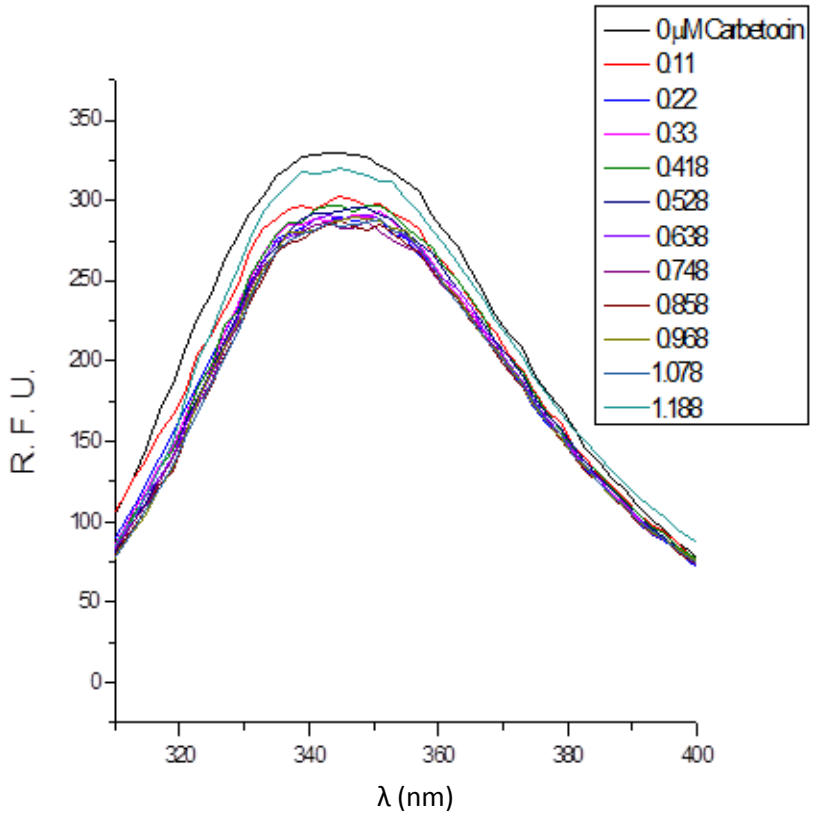

Figure 3.3 Fluorescence spectrum of 21. 7 nM FVIII titrated with 1.32 µM Carbetocin in PBS.

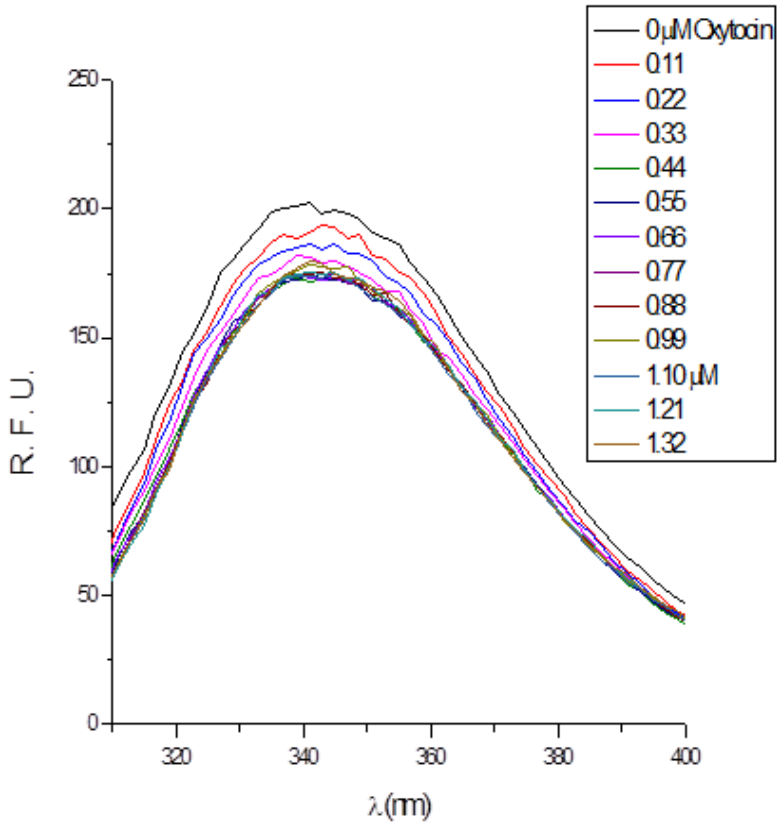

Figure 3.4 Fluorescence spectrum of 21.7 nM FVIII titrated with 1.32 µM Oxytocin in PBS.

Upon examination of the results, there were several important points to note. Firstly, the fluorescence spectra obtained for the FVIII alone, prior to the addition of the peptides, were similar to those previously published, suggesting that the FVIII was in its native conformation [84]. Secondly, it appeared that increasing concentrations of the peptides resulted in quenching of the FVIII amino acid residues' fluorescence. It also appeared that the extent of the quenching differed amongst the various peptides, with AVP producing the greatest quenching. Furthermore, there was no demonstrable quenching observed with the addition of the control peptide, Fibrinogen Binding Inhibitor Peptide (data not shown). This latter molecule is structurally different from the other peptides in both primary sequence and in that it does not contain the conserved disulfide bridge that the neurohypophyseal peptides and their drug analogs contain which provide them a ring like structure. These results were suggestive of a selective, possibly specific, interaction between the peptides and the FVIII.

It was interesting to note that the degree of fluorescence emission from each of the native FVIII samples varied. This was thought to be due the fact that the FVIII utilized within these experiments was isolated from human blood and aliquoted by the manufacturer based upon co-enzymatic activity. This led to batch-to-batch variation in the correlation between enzymatic activity and protein concentration, meaning that some batches may have had more FVIII activity in them but had more total protein because of a smaller activity to mass ratio. While these experiments utilized concentration of protein as opposed to activity to control for this variation, there nevertheless was a clear variation in the fluorescence signal from the FVIII samples. This could account for some variation in the amount of peptide needing to be added to the FVIII solution to obtain a complete quenching effect and may represent a potential confounder in this experiment. The fact that different concentrations of the protein in the different samples had

different activities suggested the possibility that the concentration of FVIII in its native conformation was variable. Thus, it could not be said for certain that the quenching occurring was strictly from the FVIII in its native conformation as opposed to that in a different state.

One important consideration in these experiments was that they were performed at 37 °C in order to mimic the conditions in the human body. It is known that the fluorescence of proteins is temperature dependent, with higher temperatures causing a decrease in the quantum yield from the aromatic residues [85]. This raised concern that the results obtained may have been secondary to the heating of the FVIII solution, and not simply a result of the addition of the peptides. However, this was not thought to be the case for several reasons. Firstly, the control experiments performed with FBIP showed no significant quenching in the fluorescence signal, despite being conducted under identical conditions to the other peptide experiments. Had the quenching seen been a thermal effect, it would have been seen in the FBIP experiments as well. Secondly, the FVIII solution was allowed to thermally equilibrate for a period of 3 minutes, which should have been sufficient for the solution to reach a steady state in terms of temperature. Thirdly, a set of control experiments were performed in which the fluorescence of FVIII was monitored for a period of 30 minutes at 37 °C, These experiments failed to show any loss in fluorescence signal, suggesting that the temperature was not likely contributing to the quenching seen in the peptide experiments.

The quenching effect at 334 nm, the emission maxima, was analyzed using Origin in order to plot dose-response curves and obtain the EC50 of the interaction. The following results were obtained and are displayed in the following Figures 3.5-3.8.

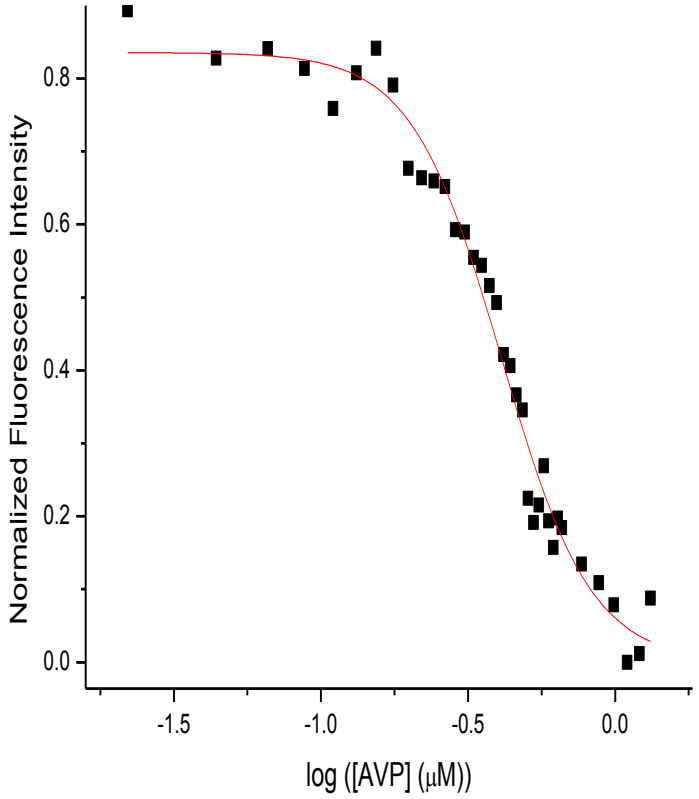

Figure 3.5 Dose response curve of AVP with FVIII fluorescence quenching.
The point at which 50% of the quenching is achieved is at 0.375 μM

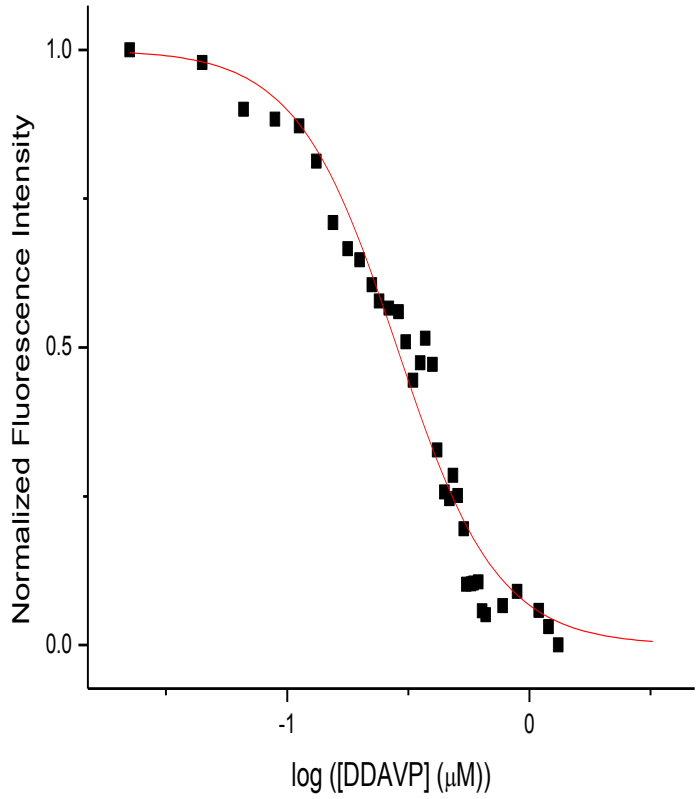

Figure 3.6 Dose response curve of dDAVP with FVIII fluorescence quenching. The point at which 50% of the quenching is achieved is 0.308 μM.

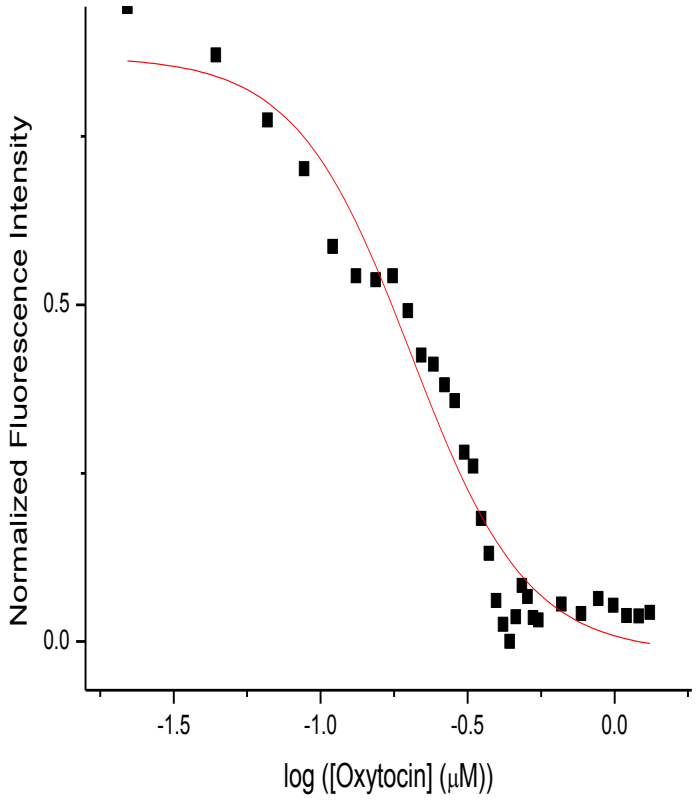

Figure 3.7 Dose response curve of Oxytocin with fluorescence quenching of FVIII.
The point at which 50% of the quenching is achieved is at 0.155 μM.

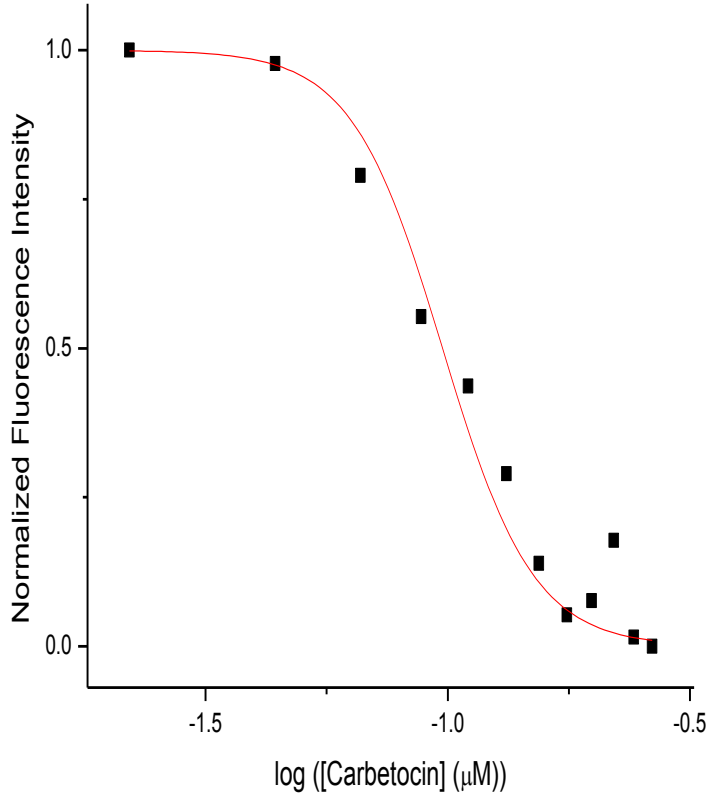

Figure 3.8 Dose response curve of carbetocin with FVIII fluorescence quenching.
The point at which 50% of the quenching is achieved is 0.098 μM.

Table 2. EC50 values for peptide quenching of FVIII as determined by fluorescence spectroscopy. The concentration of AVP needed to achieve 50% quenching was higher than the other peptides. The drug analogs for AVP and oxytocin showed similar ranges of EC50 to their endogenous counterparts.

	AVP	**DDAVP**	**Oxytocin**	**Carbetocin**
Approximate EC50 (μM)	0.375 (-/+ 0.009)	0.308 (-/+0.007)	0.155 (-/+ 0.005)	0.098 (-/+ 0.008)

These results demonstrated that AVP has the highest EC50 value, meaning that a higher concentration of the peptide was required to achieve 50% of the maximal quenching effect. The fact that the other peptides required less should not be mistaken as being suggestive that the other peptides were more potent in their interaction with FVIII however, as the EC50 values do not characterize the magnitude of the effect achieved, only the concentration required to achieve 50% of the total effect. As shown in Table 2, the EC50s of AVP and dDAVP were similar to each other, as were those of oxytocin and carbetocin. As these are the endogenous hormones and their synthetic counterparts, their structures are more similar to each other than to the other pair of peptides, which may have explained the correlation. Though they are all nonapeptides and may modulate coagulation in some manner, possibly through interaction with FVIII, the peptides have different affinity profiles for the vasopressin and oxytocin receptors, and therefore, could be expected to interact with FVIII with variable degrees of affinity and thereby producing differences in the fluorescence quenching profile.

Interpreting these results in the context of pharmaceutical application was difficult as the therapeutic dosing of vasopressin, oxytocin and carbetocin were all based on the activity of the peptides, not in terms of mass. As a result, one cannot compare the EC50s obtained to the therapeutic dosing of the peptides. In the case of desmopressin however, typical doses used are 0.3 μg/kg intravenously, which would represent a dose of approximately 20 nmoles, divided into the plasma volume, which would lead to results of approximately 4 nM for a 70 kg person with a blood volume of 5 L. The EC50 derived was for a solution of 22 nM FVIII, approximately 20 to 40 fold higher than the standard concentration of FVIII in serum [39]. As a result, for a physiological concentration of FVIII, one would expect the EC50 to range roughly between 7.7 to 14 nM, which is within range of the values used therapeutically. However, one must be very

cautious when making assertions regarding the transferability of results from a simple *in vitro* setting to a complex *in vivo* one. These experiments were conducted in PBS buffer with a pH of 7.4 to mimic the normal pH of human blood. Blood however, is a complex solution consisting of numerous proteins, salts, carrier-bound fats, and a variety of cells. Thus, the interactions within circulation could easily be affected by the numerous other constituents present, and thus the *in vitro* results may not represent what is occurring *in vivo*.

3.2 Investigating photodegradation of FVIII

Photodegradation is the process through which protein, irradiated with a specific wavelength of light, may undergo photolytic cleavage of various covalent bonds within its structure, or through which oxidation occurs and results in the formation of aggregates of the proteins through free radical mediated mechanisms [86]. This process can lead to substantial structural changes within the protein, resulting in measurable changes within the protein's fluorescence spectrum. As a result, fluorescence experiments can be strongly influenced by this process and this possible confounder should be assessed in positive fluorescence quenching experiments. This was particularly relevant in the case of FVIII, in which a previous study has shown FVIII to be susceptible to photodegradative processes, resulting in measurable losses of co-enzymatic activity [87]. In order to investigate whether or not this photodegradative process could be occurring in the fluorescence experiments with FVIII and the peptides, a solution of FVIII in PBS buffer, the same used in the protein-peptide interactions experiments, was continuously irradiated with 295 nm light for a period of 30 minutes and its emission at 334 nm continuously measured. The results below, displayed in Figure 3.9 were obtained.

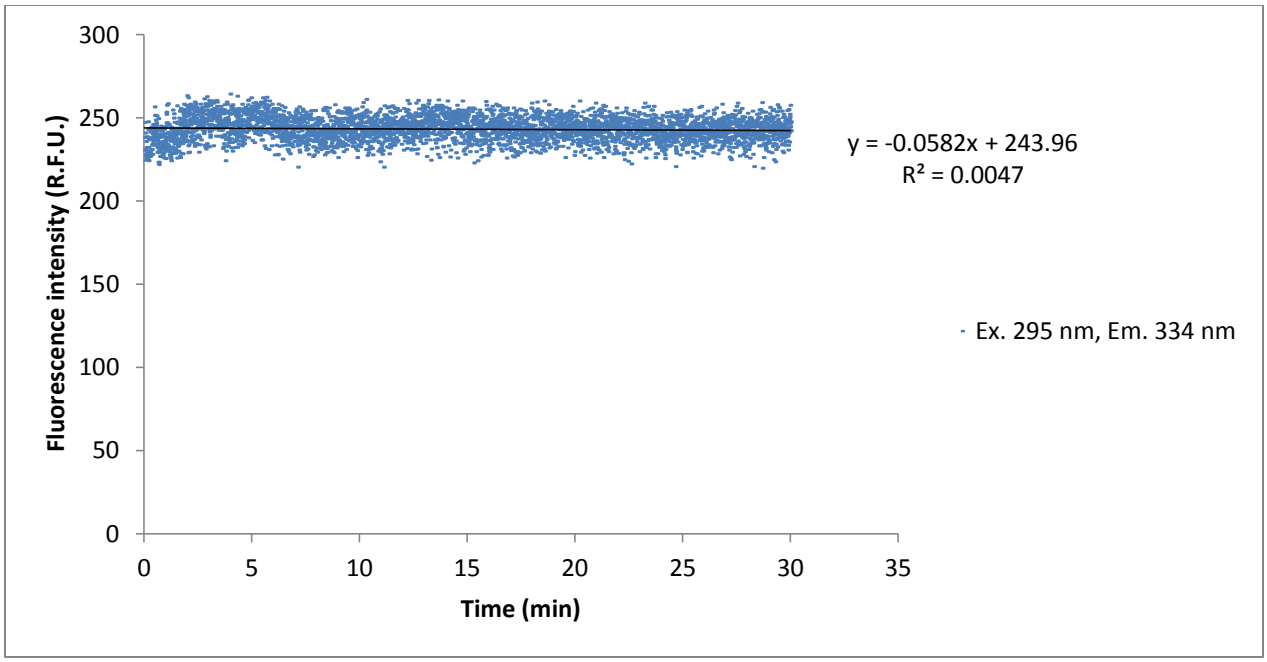

Figure 3.9 Effect of continuous irradiation of FVIII at 295 nm on fluorescence emission of tryptophan residues. There is no loss of fluorescence intensity after 30 minutes of continuous irradation to support a photoegradative process is occurring.

These results demonstrated that there was no significant change in the fluorescence emission of the native FVIII resulting from continuous irradiation with 295 nm light. There was no evidence to suggest any form of photodegradation of FVIII occurred within the time frames of the other fluorescence experiments performed. This supported that the results obtained from the protein-peptide interaction experiments using fluorescence spectroscopy were the result of the interactions and not a photodegradation process.

3.3 Thermal denaturation of FVIII in the presence of the peptides

Factor VIII is known to have specific transition points in its thermal denaturation [88], and is used as a method of characterizing FVIII, notably with circular dichroism spectroscopy. Should the neurohypophyseal peptides be interacting with FVIII, then they may cause a change

in the thermal stability of the FVIII molecule at higher temperatures that may be detectable by monitoring the fluorescence emission of the tryptophan residues while increasing the temperature. Thus, the temperature stability of FVIII was ascertained using fluorescence spectroscopy. Essentially, solutions of FVIII were heated from 20 °C to 85 °C with their fluorescence spectrum measured at 5 °C intervals, focusing on emission at 334 nm, the maxima found during the titration experiments previously described. Similarly, the FVIII solutions pre-incubated with the neurohypophyseal peptides were also heated to 85 °C and had their fluorescence measured. Given the difference per sample in terms of fluorescence intensity discussed previously, the data was normalized to the average of the intensity at 70-85 °C, at which point the vast majority if not all of the proteins would be in the same conformational state and aggregated. The following results were obtained.

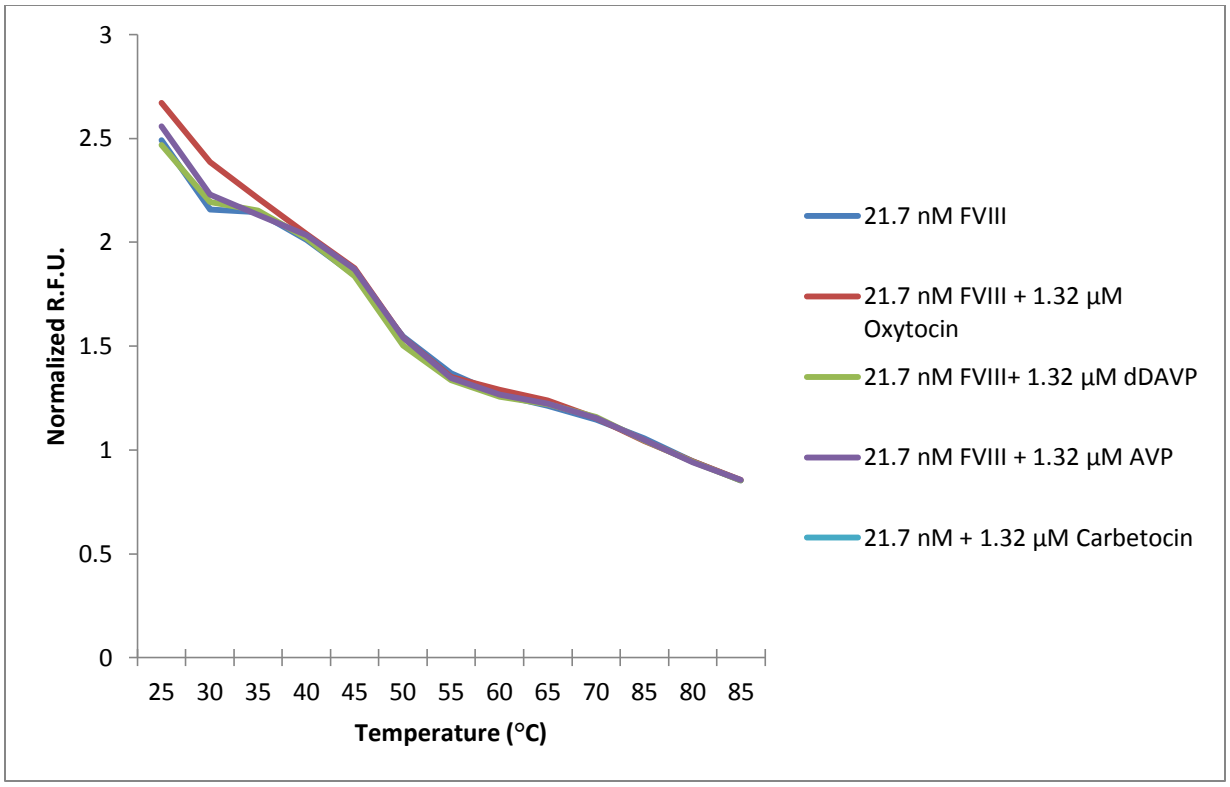

Figure 3.10 Thermal denaturation curve of FVIII with/without peptides. The fluorescence spectrum of FVIII from 25-85 °C shows no difference in the presence or absence of the peptides.

These results indicated several important points. Firstly, the FVIII showed similar transition points to what has already been reported in the literature [84]. This supported that it was in its native state. Secondly, the presence of the neurohypophyseal peptides pre-incubated with the FVIII resulted in no specific change in the shape or the transition points of the thermal denaturation of FVIII.

The implications of this data are ambiguous as not all protein-peptide interactions will affect thermal stability of the protein. As a result, the absence of a measurable effect in these experiments did not exclude the possibility of an interaction. It merely did not support it. Had a clear change in the thermal denaturation of FVIII in the presence of the peptides been found, it

would have been further supportive of an interaction. While the temperature of the human body is tightly regulated and will not significantly vary by more than a few degrees, the proteins in circulation can still be studied at various temperatures in order to assess interactions. While an effect on transition points at the higher temperatures are of interest, they would have no physiological relevance. Nevertheless, had the peptides altered the transitions of the FVIII at higher temperature, it would have supported a strong interaction between the two species, as otherwise, one would have expected the species to dissociate at lower temperatures should their interactions be weaker and the protein return to its native thermal denaturation pattern.

3.4 Factor VIII activity

3.4.1 Evaluating activity of FVIII after prolonged storage at -80 °C

To draw conclusions regarding the interactions of the neurohypophyseal peptides and FVIII, it must be demonstrated that the FVIII samples retain native conformation and activity after prolonged storage, as the proteins utilized in this thesis were subject to prolonged storage times at -80 °C. To this end, FVIII samples in PBS buffer, pH 7.4, were routinely tested for activity using a commercially available FVIII kit. The following calibration curve shown in Figure 3.11 was obtained using a sample frozen for 3 years at -80° C. Note that the results are presented as percentages of FVIII activity as is the standard for the activity kits, where 100% FVIII activity refers to 1 IU/mL activity.

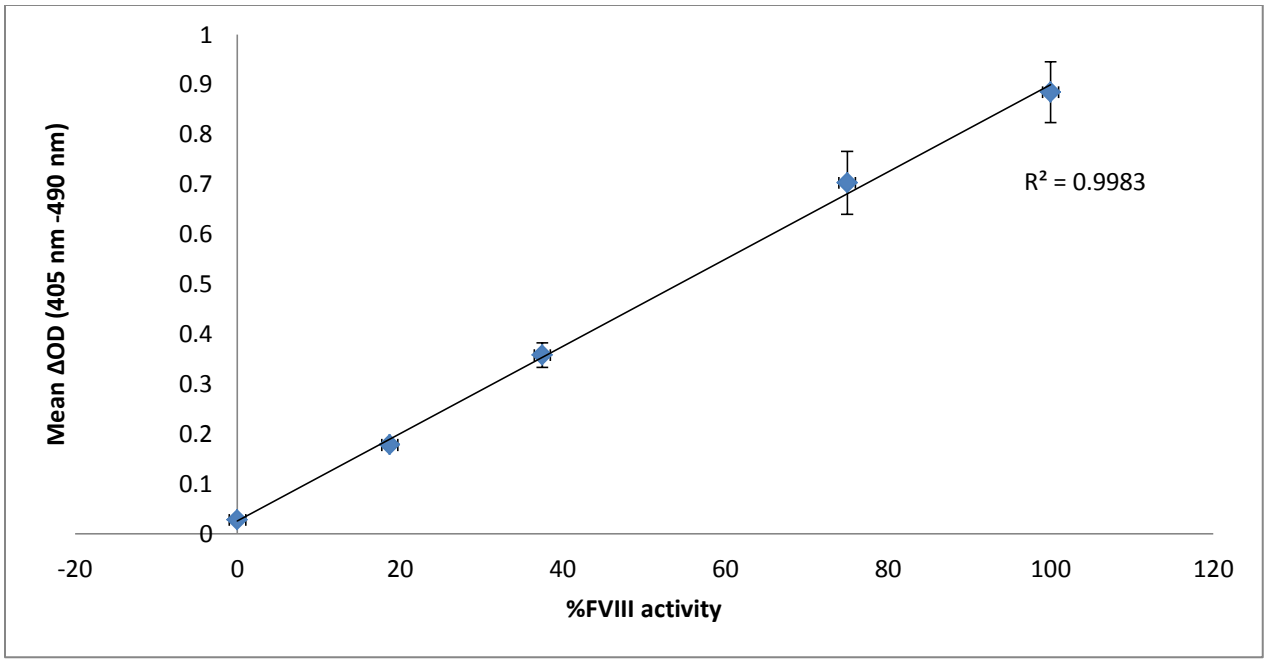

Figure 3.11 Activity of FVIII stored at -80 °C for 3 years (one freeze thaw).

The FVIII sample demonstrated the expected activity as per data supplied by the manufacturer of the kit. There was no evidence of loss of activity of the FVIII samples after prolonged storage. It also further supported that the FVIII utilized throughout the experiments maintained its activity. However, it should be noted that comparison of activity of the particular samples were not performed as no activity was measured on arrival. As a result, though the samples retained a correlation between concentration and measured activity, conclusions cannot be clearly drawn as to whether or not there could have been a loss of activity over time suggestive of an alteration in protein structure.

3.4.2 Evaluating the effect of the presence of neurohypophyseal peptides on FVIII activity

Current theory regarding any potential interaction between FVIII and vasopressin and desmopressin holds that the increase in FVIII activity noted post infusion of the peptides results from the release of stores of FVIII and vWF from the endothelium in response to peptide binding to surface receptors [50]. There appeared to have been no definitive study isolating the FVIII protein and measuring any direct effect of the peptide on the isolated FVIII's activity. As most studies correlated increases in FVIII activity (FVIIIC) with increases in FVIII antigen (FVIIIAg), the line of thinking regarding the effects of the peptides focused upon the increase in the measured antigen and assumed that the change in activity was purely related to that, as opposed to a change in the FVIIIC/FVIIIAg ratio [27, 89, 90]. Others recognized that as the concentration of all proteins in circulation is the result of the balance between production and removal, the increase in FVIIIAg may be the result of protection of the FVIII from proteolytic degradation [91]. However, most attribute this possibility to the resulting increase in circulating vWF after dDAVP and AVP administration [92]. Consequently, the possibility of a protein-peptide interaction leading to the increase in the FVIIIC appeared to have been overlooked. Thus, in order to assess this possibility, the activity of isolated FVIII in the presence or absence of the neurohypophyseal peptides and their drug analogs was assessed. Solutions of varying concentrations of FVIII were pre-incubated for 2 minutes at 37 °C with 100-fold excess peptide and then subjected to the commercial activity kit determination. Figure 3.12 demonstrates the results, which are also summarized in Table 3.

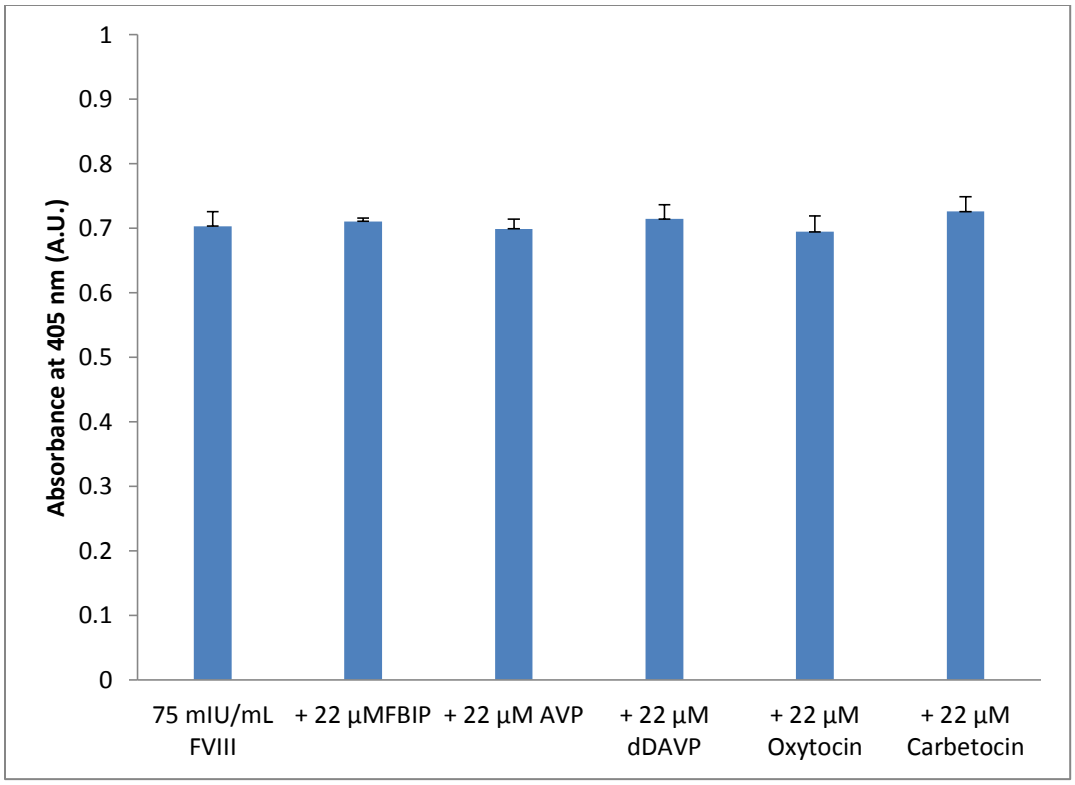

Figure 3.12 FVIII Activity with/without AVP/dDAVP/Oxytocin/carbetocin/FBIP. These results show no significant difference in the activity of FVIII with the addition of the peptides as compared to its baseline activity without them.

Table 3. Measured activity of FVIII in the presence of the neurohypophyseal peptides. There was no significant change in the measured activity of FVIII in the presence of the peptides

	1.667 nM FVIII	1.667 nM FVIII + 167 nM peptide	p value
Mean ΔOD (405 nm– 490 nm) (A.U.)	0.7032 (+/- 2.234×10^{-2})	AVP: 0.6989 (+/-1.513×10^{-2})	0.6571
		DDAVP: 0.714 (+/- 2.242×10^{-2})	0.8044
		Oxytocin: 0.6944 (+/- 2.452×10^{-2})	0.8533
		Carbetocin: 0.7259 (+/- 2.246×10^{-2})	0.524
		FBIP: 0.7104 (+/- 5.419×10^{-3})	0.7889

These results indicated no significant change in the activity of FVIII in the presence of the neurohypophyseal peptides. While these results were consistent with previously published data, they did not substantiate, or refute, the theorized interaction between FVIII and the peptides. These results suggested that, should there be an interaction between the FVIII and the peptides that explains the therapeutic utility of dDAVP in the treatment of Hemophilia A and of the peptides in the treatment of various causes of hemorrhage, it is not likely through a direct effect on FVIII activity.

One caveat to the use of commercially available kits is that their ability to approximate in vivo settings is questionable. This assay utilizes FVIIIa's coenzymatic properties to catalyze the cleavage of a chromogenic reactant, yielding a measurable product, and does so in a linear fashion correlating to the FVIII activity present. Whether or not this accurately reflects the activity of FVIII in serum in catalyzing the conversion of FX to FXa, and then the action of Xa in the cleavage of the chromogenic substrate can be subject to debate [93]. The manufacturer could not provide any further data on the substrate or its interaction with the Xa complex. As discussed earlier, blood is a complex system, with many circulating species. The ability of *in vitro* experiments to capture *in vivo* findings, such as the noted increase in FVIIIC post infusion of the neurohypophyseal peptides into test subjects, is doubtful and as a result, the absence of a response could not rule out an important response seen with the complexities of blood. Future experimentation could attempt to assess the potential increase in FVIII activity by performing the assay in blood that has been depleted of platelets, which are a source vWF [94]. This would allow for the further investigation of the potential interaction in the complex system but also removing the possible confounder of the release of VWF from platelets.

3.4.3 Measuring the effect of the peptides on the $t_{1/2}$ of FVIIIa

The half-life of FVIIIa is known to be short, on the order of 2 minutes, and primarily dictated by the spontaneous dissociation of the A2 subunit from the remainder of the FVIIIa molecule [95]. One can imagine then the possibility that the neurohypophyseal peptides may be affecting the half-life of FVIIIa, and by prolonging the half-life of the protein, lead to an increased measurement of protein activity in serum after the peptide administration. In order to assess this theory, a commercially available FVIII activity kit was purchased, which allowed for the activation of FVIII independent of the remainder of the assay. Utilizing this fact, the FVIII was pre-incubated with the various peptides and then activated via the addition of thrombin to the FVIII-peptide mixture. This activated sample was then allowed to incubate at 37 °C for specific durations of time, followed by completing the assay and measuring the FVIII activity remaining after the prolonged activation period. The following results were obtained.

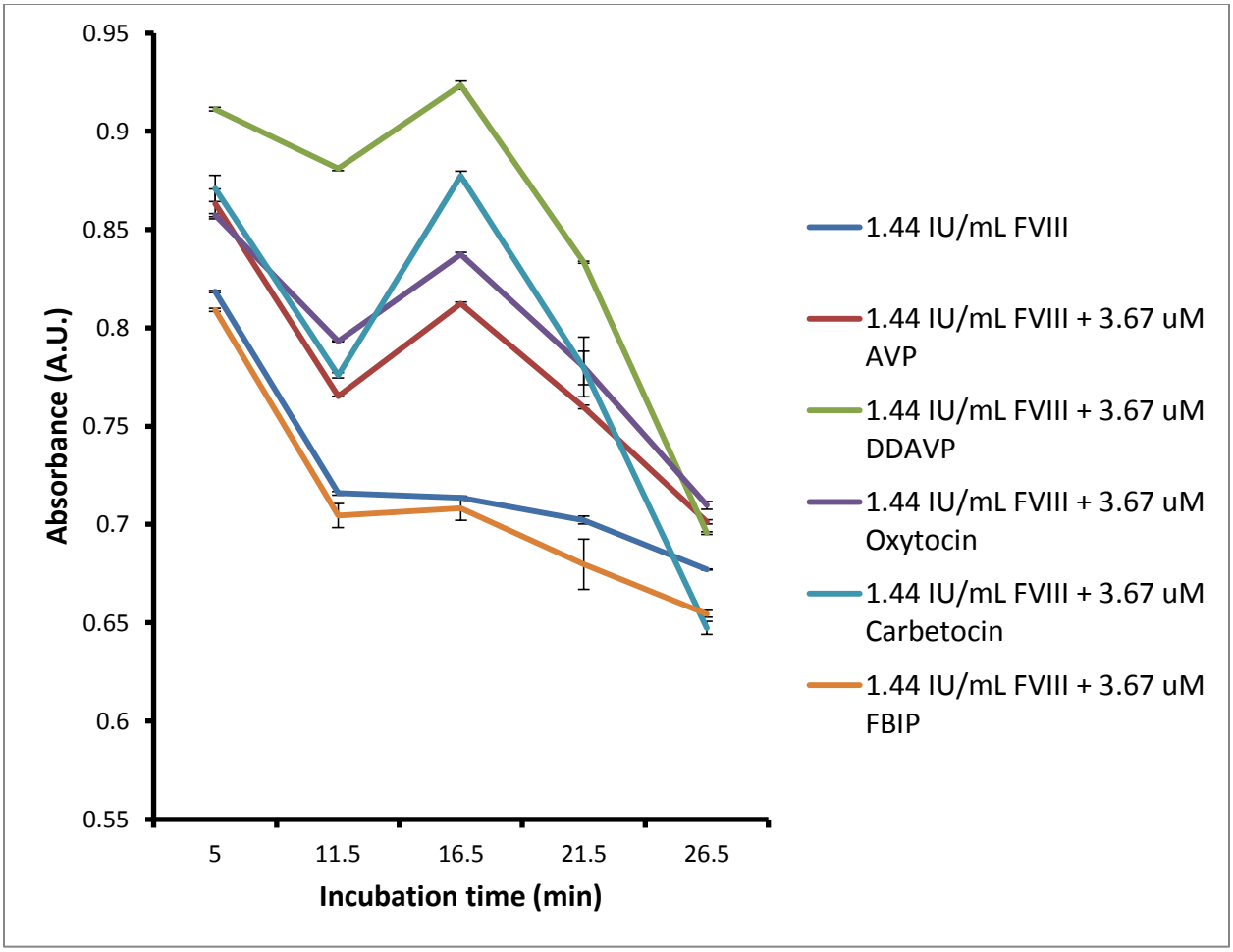

Figure 3.13 The activity of FVIIIa over time with/without peptides. The FVIII and the FVIII + FBIP solutions demonstrated less baseline activity than the FVIII + AVP/ dDAVP/ Oxytocin/ Carbetocin, which persisted over time post activation of FVIII. Note the error bars resulted from the measurement of the same solutions 3 times.

There are a few important features to the data presented in Figure 3.13. Firstly, it was noted that the baseline activity of FVIII was significantly higher in the peptide groups, with dDAVP providing the highest amount of measured activity. This phenomenon was not duplicated utilizing the Chromogenix COAMATIC SP4 activity kit however, as shown in Figure 3.12, in which there was no significant difference in the FVIII activity in the presence or absence of the peptides. This difference in activity from assay to assay could be explained by the difference in

assay method, though this seemed unlikely as the two assays are essentially identical except in the order in which the reactants are mixed in the solution. These assays all function upon the same principle that at specific concentrations of FIXa and FX, in the presence of adequate Ca^{2+} and phospholipids, the generation of the FXa enzyme and its activity is strictly dependent on the FVIII activity itself. Thus, they should all demonstrate the same activity profiles. There has been published literature noting discrepancies in the measured activity of FVIII samples across assay types, such as the clotting assays versus the chromogenic assays [26], but not between the different chromogenic assays themselves. These findings suggested that there may be an interassay variability. As a result, one could not determine which assay results were reflective of the actual FVIII activity. If the results presented in Figure 3.13 were in fact reflective of the real FVIII activity, then it would suggest that the peptides *in vitro* increased the FVIII activity and further support that there is an interaction between the peptides and the protein.

The shape of the curves in Figure 3.13 demonstrated a difference in the activity of the FVIIIa over time. Within the first five minutes of activation, there is a clear decline in FVIII activity regardless of the presence or absence of the peptides. However, at 11.5 minutes, there is a higher amount of FVIII activity seen in the peptide groups, though not in the FVIII alone or the FVIII incubated with FBIP. One explanation for this possibility would be that the continued activity of thrombin in solution allowed for the generation of more FVIIIa over time. In the presence of the peptides, this generation of new FVIIIa exceeded that of the spontaneous dissociation of the FVIIIa seen in the FVIII only samples and the FVIII and FBIP samples. This suggested that the peptides may be prolonging the half-life of FVIII. As mentioned, the half-life of FVIIIa is thought to be dependent on the spontaneous dissociation of the A2 subunit from the remainder of the protein complex. The prolongation of the FVIIIa half-life in the presence of the

neurohypophyseal peptides was suggestive of the possibility that the interaction between the peptides and the proteins may act in some way to stabilize the multimer. However, no conclusions could be drawn regarding the specific subunits within FVIIIa or the mechanism through which the activity is prolonged. It was noted that the FVIII activity at 16.5 minutes was much higher than that at 11.5 minutes and then subsequently lower at 21.5 minutes. This was suggestive of the possibility of an experimental error at this time point. This assay was conducted by incubating different solutions for different period of times, and measuring the absorbance of single samples in triplicate, which could lead to experimental error if one solution had slight more FVIII aliquoted into it. Future work should utilize one large solution for each condition that would then have aliquots removed from it at the various time points. This would control for any sample to sample variation.

The results here were limited in several ways. Firstly, the sample used in these experiments was human serum with 144% of the average FVIII activity (1.44 IU/mL) found in the population and is provided by the manufacturers of the activity kit. This activity was chosen in order for a sufficient activity to remain after several half-lives to be quantifiable. It is noted then that after 5 minutes, or two half-lives, there still remains a substantial amount of activity detected in all samples. This may indicate that this range of activity is on the flat portion of the %FVIII activity *vs.* absorbance curve for this particular assay. As a result, the difference in activity of the sample over time was difficult to assess as changes in FVIII activity may not be readily detectable. However, this seemed unlikely as the manufacturer has suggested that the assay's linear range extends to 144%.

Overall, while these findings supported an interaction between FVIII and the peptides, the interassay variability noted, and the potential for experimental error, rendered the results questionable in validity.

3.4.4 Evaluating effect of FVIII-peptide interactions on FVIII-antibody binding

One potential way to evaluate for potential protein-peptide interactions is to assess the effect of the peptide on the formation of an antibody-antigen complex. This often requires specific knowledge of where the peptide is likely to bind on the protein in order to choose the appropriate antibody whose epitope is found in the same region of the protein. Otherwise, a lack of rescue of the protein from antibody binding would not rule out an interaction between the peptide and the protein as there would be no way of knowing if the antibody was not binding in a different location of the protein. Using polyclonal antibodies allows for the probing of the protein-peptide interaction in the case where the binding site is unknown. As the polyclonal antibodies will have epitopes that span the protein, it is likely that the binding of at least one clone will be blocked by the peptide binding in that location. Thus, solutions of FVIII, pre-incubated with the peptides were then mixed with polyclonal antibodies directed against either the light chain or the heavy chain of FVIII. The FVIII activity within those solutions was then assessed. The results are presented in Figure 3.14.

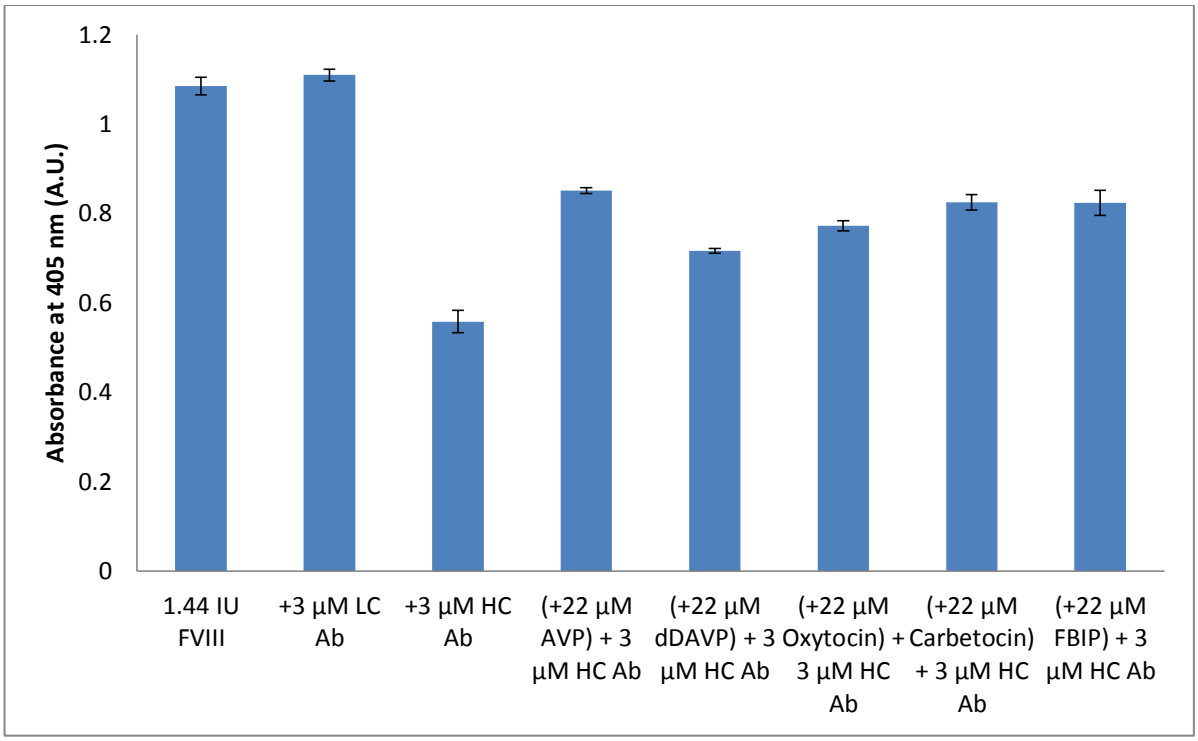

Figure 3.14 The effect of pre-incubation of the peptides with FVIII on the antibody-mediated FVIII inactivation. The LC antibody did not produce any clear loss of measurable FVIII activity in contrast to that of the HC antibody, which reduced it significantly. The pre-incubation of the peptides with FVIII resulted in the FVIII activity being retained in the presence of then HC antibody.

The results demonstrated that the peptides could "rescue" FVIII activity from HC antibody-mediated inhibition. However, the control peptide FBIP also caused a retention of FVIII activity in the solution as well, suggesting that this effect was not selective. The HC antibodies likely prevented the formation of the Xase complex by inhibiting the binding of IXa to the HC of FVIII. The K_d of the interaction between FIXa and FVIIIa has been reported to be approximately 0.8 nM [96]. While this is a strong interaction, it was likely overcome by the binding of the antibodies to the FVIII. The fact that incomplete recovery of activity was observed despite an excess of peptides compared to antibodies was likely a result of the polyclonal nature of the antibodies. As the peptide likely only bound a small region of the protein, they would have

only been able to block the epitopes within that region. The antibodies however, likely had Fc regions whose epitopes fell outside of that portion of the protein and as a result, were able to interact and bind to the HC, and as a result, prevent assembly of the Xase complex.

In contrast, the LC antibody did not produce any measurable inhibition of FVIII activity. The FVIII LC is the surface upon which the protein binds to the phospholipid membrane, in contrast to the HC upon which FIXa binds. It was expected that both antibodies would neutralize FVIII activity in solution as the Xase complex is typically assembled on the phospholipid membrane, which is why all activity kits contain phospholipids. Previous studies have also demonstrated that antibody binding to the LC would result in a significant loss of FVIII function [38]. In order to assess the importance of the phospholipids on measurable FVIII activity, experiments were done in which the FVIII activity was measured in the absence of phospholipids. These showed a significant decrease in detectable FVIII activity (data not shown). This further supported that the LC Ab could not have been preventing the phospholipid binding to the FVIII as there were normal levels of measurable activity detected when the LC antibodies were present.

The absence of effect of the LC antibodies on FVIII activity was suggestive of a few possibilities. Firstly, it could be that the antibodies were binding and simply not inhibiting the assembly of the Xase complex and as a result, not affecting cofactor activity of FVIII. If this were the case, there would be no way to determine if the peptides were inhibiting antibody binding via this particular assay. A second possibility is that the phospholipid-LC interaction was stronger than that of the antibody-LC interaction. The K_d for the interaction between the phospholipids and the LC of FVIII is 0.24 nM, indicating a very high affinity interaction [97]. While the antibody-antigen interaction is also usually a high affinity interaction [98], it may have

been outcompeted by that of the phospholipids-LC binding, especially as the phospholipids were abundant in the reaction. This possibility, however, conflicts with previous data, which established that phospholipid binding to the LC could be inhibited by anti-LC antibodies [99]. However, the aforementioned study focused on antibodies directed against the C2 domain of the LC, as opposed to the polyclonal ones here. It is possible that despite their polyclonal nature, there were few antibodies within the solution whose epitope fell within the C2 domain, where the phospholipids bind specifically. As a result, it was possible that the phospholipid outcompeted the antibodies and as such, any potential inhibition of antibody binding by the peptides would not have yielded any measurable results. A third possibility would be that the antibodies were inactive, and no longer able to bind the FVIII. However, given that these antibodies were successfully used in the synthesis of the microsphere immunoassay, this is unlikely. On the whole, the results with the LC antibody do not substantiate nor refute the research thesis.

While the results with the HC antibody supported that the peptides were likely to be binding on the HC of FVIII, the complex nature of the activity kit, with multiple protein species present, casted some doubt in the interpretation of the results. While the kit was designed to measure FVIII activity, it nevertheless relied on the functionality of numerous different proteins, such as FIX, FX, thrombin, as well as the chromogenic substrate, as shown in the following chemical reactions.

$$\text{FVIII} + (\text{FIX-Phospholipid-Ca}^{2+}) \xrightarrow{\text{FII}_a} (\text{FVIII}_a\text{-FIX}_a\text{-Phospholipid-Ca}^{2+})_{complex}$$

$$\text{FX} \xrightarrow{(\text{FVIII}_a\text{-FIX}_a\text{-Phospholipid-Ca}^{2+})_{complex}} \text{FX}_a$$

$$\text{Substrate} \xrightarrow{\text{FX}_a} \text{pNa} + \text{peptide}$$

As shown, if the HC antibody were to interfere with any portion of this assay, it would cause a loss of measured cleavage of chromogenic substrate. While antibodies are typically considered as having a high specificity for particular antigens, patients with Hemophilia A have demonstrated that this is not always the case. There have been reports of inhibitors which cross-react with FIX and FX [74]. Though the antibodies used in this experiment were not derived from hemophiliacs, they are developed through a similar process to the generation of inhibitor antibodies, as they are created through the infusion of human FVIII into non-human species whose immune system recognizes the protein as foreign. Thus, though the anti-HC antibodies used here were directed against the HC, it was conceivable that they were reacting against the other components of within the system of the activity kit. As a result, in order to further assess the possibility that the peptides could interfere with antibody binding, the microsphere immunoassay, with its mono-antigen solution, was designed.

3.5 Microsphere Immunoassay

The microsphere-based immunoassays were originally designed with multiplexing capabilities in mind [100]. These polystyrene beads are small in size, ranging from <0.1 μm to ≈100 μm. They can contain paramagnetic iron oxide to allow magnetic retention and concentration of the spheres for use as a pull-down assay [101]. They can also be loaded with fluorescent dyes, allowing for multiplexing capabilities as well as a high signal/sphere ratio, allowing for small concentration analyte detection. Given these properties, the microspheres were utilized to detect the low concentration FVIII as well as investigate for further evidence of peptide-mediated antibody-FVIII binding inhibition.

3.5.1 Evaluating coating methods for minimization of microsphere agglomeration

Microspheres, through their hydrophobic polystyrene surfaces, are known to agglomerate non-specifically [102]. This is problematic for many uses of microspheres, which frequently require that beads be distinct unless an antigen is present to hold them together in close proximity. As a result, many methods have been investigated to reduce non-specific adsorption of the microspheres. These include the use of dissolved blocking agents such as BSA within the solution, or with the covalent bonding of blocking agents to the bead surface [103]. In the current work, different combinations of dissolved blockers as well as covalently linked blocking agents were investigated. The level of non-specific adsorption was then determined by direct visualization via microscopy, counting the number of single microspheres in a field of vision, relative to the number of dimers and higher order agglomerates. Selected results are presented in Figure 3.15 through Figure 3.18, in which prepared solutions of microspheres either uncoated, or coated with BSA, or in solution with PEO, were aliquoted onto a glass slide and photographed under a microscope at 50X magnification.

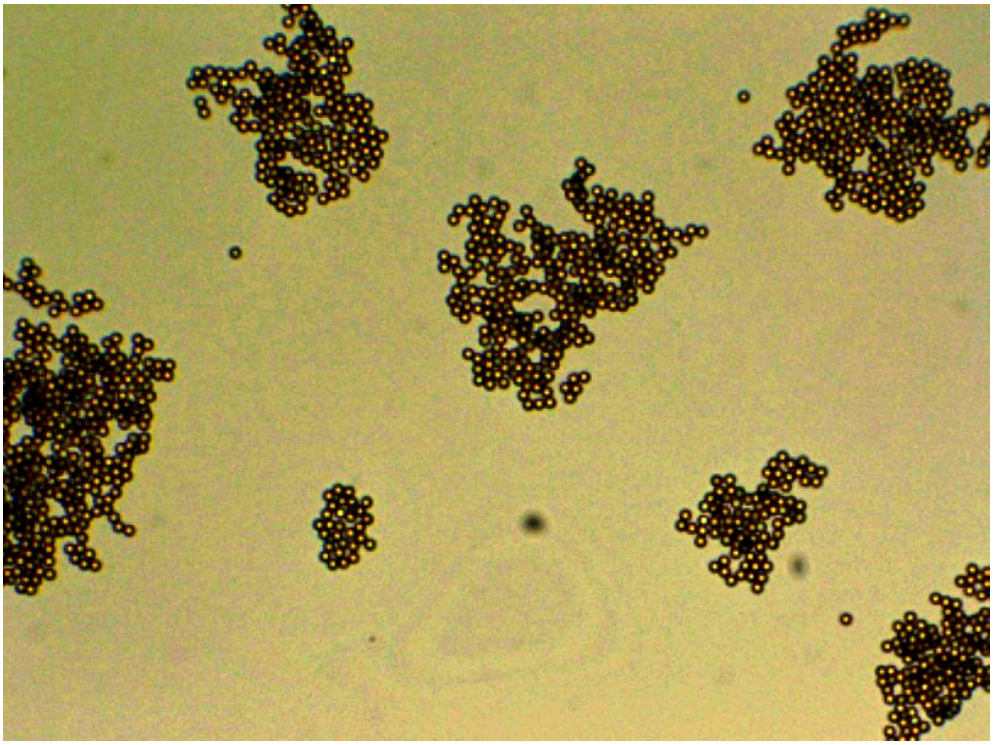

Figure 3.15 Polystyrene microspheres suspended in PBS, pH 7.4

The agglomeration of non-blocked microspheres was well illustrated under microscopy, as shown in Figure 3.15, where large multimers were clearly visible. There were few single microspheres. In Figure 3.16, in which the spheres had been suspended in a solution containing 4% PEO, it was clear that the proportion of single microspheres had increased. Furthermore, there were fewer appearing in the microscope field of view, indicating that they were on the whole more dispersed. There still remained a number of larger agglomerates however.

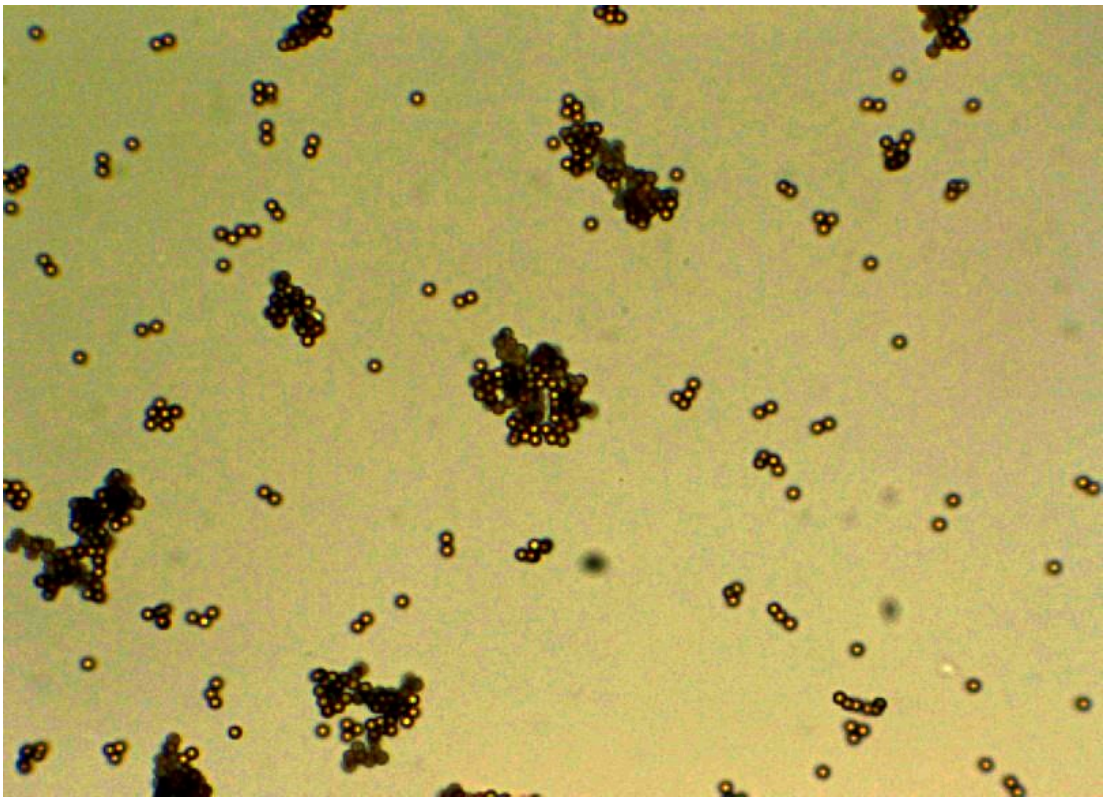

Figure 3.16 Uncoated Polystyrene microspheres suspended in 4% PEO (m/v) in PBS, pH 7.4, seen at 50X magnification

As mentioned, an alternative strategy to reducing the non-specific adsorption is to coat the spheres with a blocking agent, such as BSA. Thus, another sample of microspheres was chemically modified to be coated with BSA. As shown in Figure 3.17, the microspheres formed far fewer agglomerates compared to the uncoated spheres, and the agglomerates that did form tended to be smaller in nature, consisting of only a few microspheres interacting.

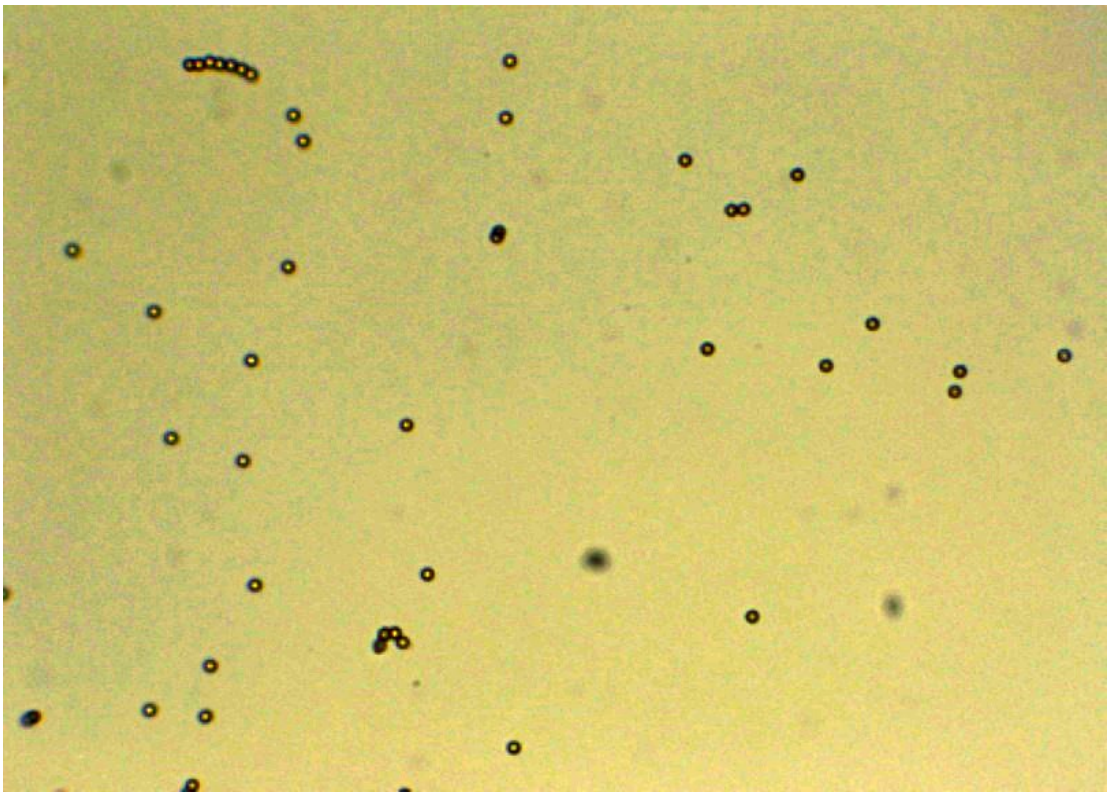

Figure 3.17 Polystyrene microspheres blocked with BSA in PBS, pH 7.4, at 50X magnification

The BSA blocked microspheres were then suspended in PBS, pH 7.4, containing 4% PEO, demonstrating improved dispersal, with few doublets noted in the microscope's field of view (Figure 3.18). The concentration of microspheres used was the same as in the other experiments; they were simply more dispersed on the slide as a result of the decrease in non-specific interactions. The lack of large agglomerates and the paucity of even doublets was indicative of little non-specific adsorption of the microspheres. These conditions were well-suited for use in the immunoassay.

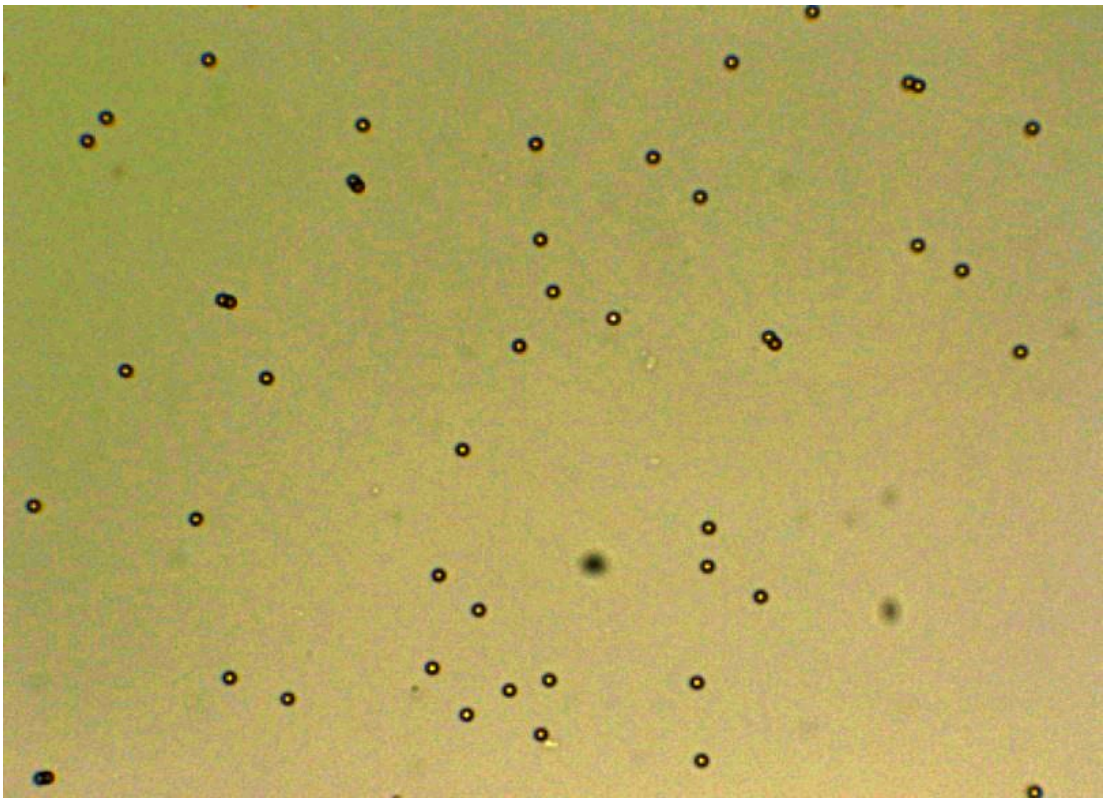

Figure 3.18 BSA blocked polystyrene microspheres suspended in 4% PEO in PBS, pH 7.4, at 50X magnification

3.5.2Demonstrating successful formation of a microsphere sandwich assay

One of the difficult characteristics in the development of a microsphere sandwich immunoassay is that the only method to monitor the successful coupling of antibodies to the surfaces of the microspheres in appropriate quantities is by successfully performing an immunoassay. To do so, one needs to establish a calibration curve which demonstrates the assay's capability of correlating concentration of antigen with retained detection spheres as seen by fluorescent signal. Below in Figure 3.19, we demonstrated that a calibration curve was obtained showing linear correlation between increasing concentration of FVIII (0.2-2 nM) with increasing relative fluorescence units.

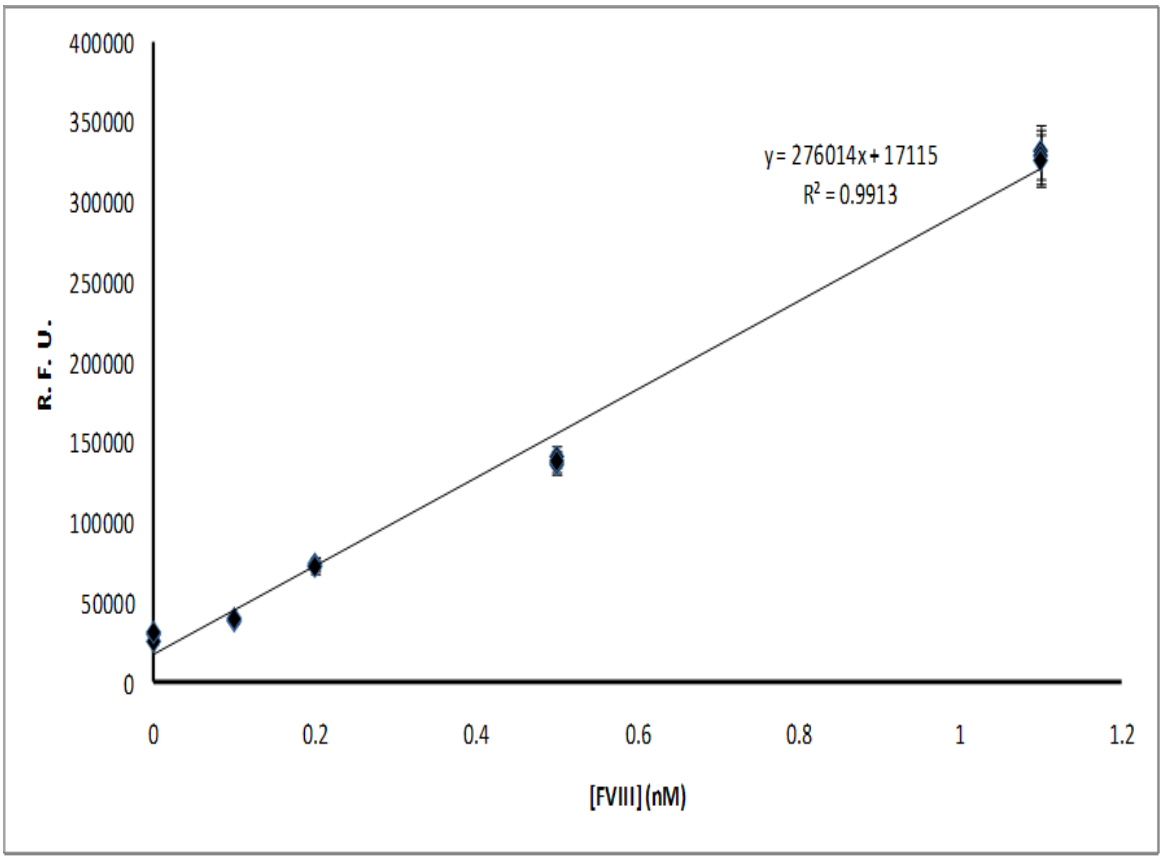

Figure 3.19 Calibration curve of the microsphere sandwich immunoassay with the FVIII analyte. The assay showed a linear correlation at low concentrations of FVIII.

The biological concentration of FVIII, typically said to be between 0.5 and 1.0 nM is found to be at the midpoint of this calibration curve. This assay, which utilized small volumes of sample, 10^{-5} L, allowed for the detection and accurate quantification of FVIII on the pico-gram (10^{-12} g) scale. Though these detection levels are not typically necessary for the assessment of FVIII concentrations *in vivo*, they still represented important goals for any assay designed to measure analytes derived from serum. By reducing the minimal quantifiable mass of the analyte, one can utilize smaller volumes of sample for analysis. In the medical field, where blood sampling can be an important cause of anemia and subsequent morbidity, reducing sample size can be crucial

[104]. The ability of this assay to quantify small concentrations of FVIII was particularly interesting, as some studies have noted difficulty in studying FVIII at low concentrations due to its tendency for non-specific adsorption to surfaces [105]. Achieving low mass detection limits with this assay further supported the efficiency of the microsphere surface blocking with BSA, as well as the inclusion of the polyethylene oxide within the washing methods.

The production of the microsphere assay was challenging for several reasons. Firstly, the covalent coupling of antibodies to the surface of the carboxylated microspheres required a two-step synthesis with an unstable, o-Acylisourea intermediate as shown in Figure 3.20 A) below. This unstable intermediate was readily hydrolyzed, which prevented formation of the desired amide bond linking the antibody to the microsphere functional surface group, shown in Figure 3.20 B). This hydrolysis often limited the production of antibody coated microspheres utilizing carboxyl-activated microspheres and resulted in decreased assay sensitivity. The method used to avoid this was the addition of Sulfo-N-hydroxysuccinimide (Sulfo-NHS) to the initial activation step of the carboxyl group. The Sulfo-NHS reacted with the unstable *o*-Acylsilourea intermediate to form an amine-reactive Sulfo NHS-ester intermediate as shown in Figure 3.20 D). This intermediate was then able to react with the amino groups of the antibodies but was not as readily hydrolyzed, as shown in Figure 3.20 E). Ultimately, this improved the yield of antibody coated microspheres and thus the assay sensitivity.

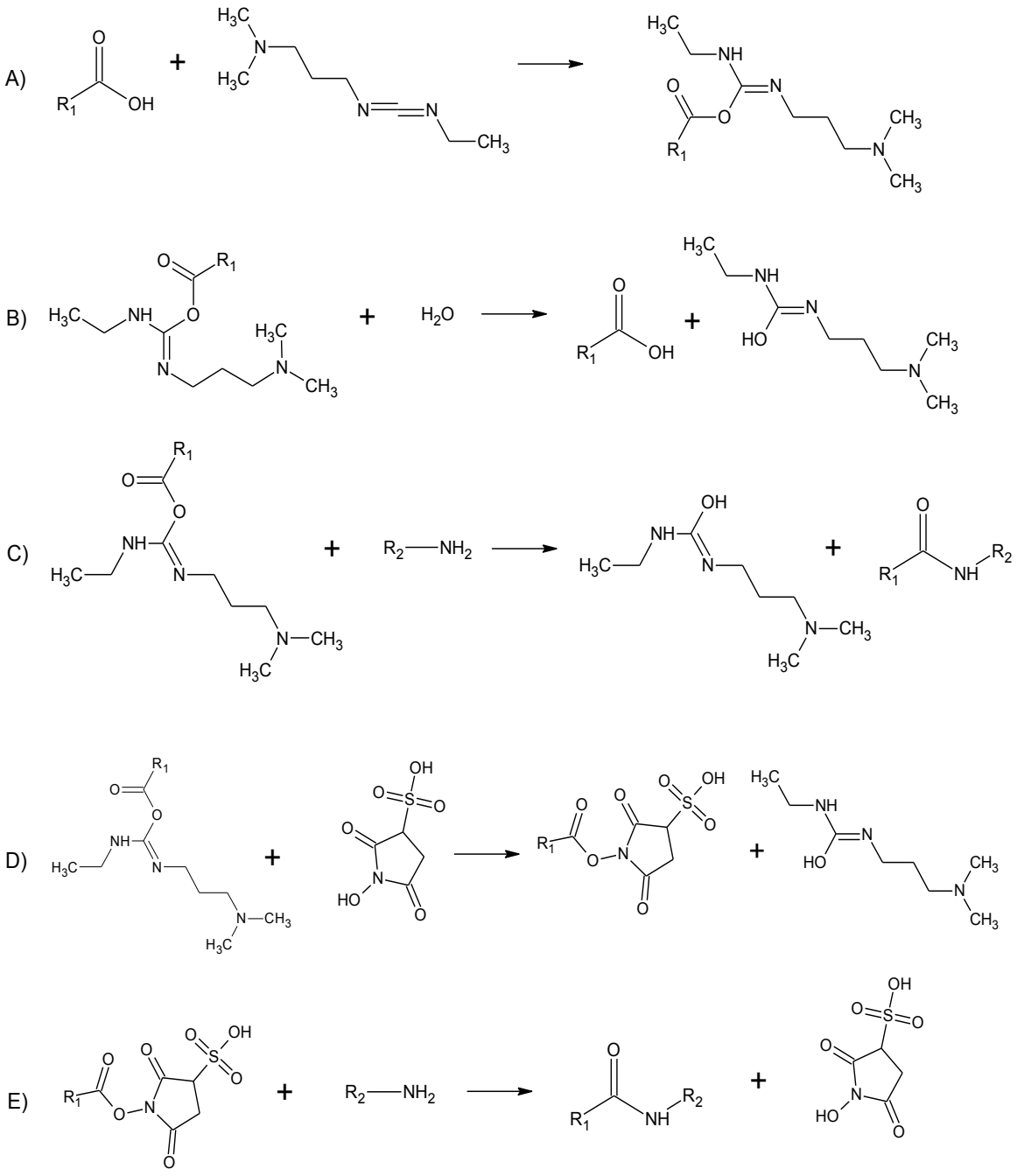

Figure 3.20 Synthesis of antibody coupled microspheres. R1 represents the microsphere surface, and R2 represents the antibody with the free amine residues. The mechanism is dependent on the EDC activation of the carboxyl residues on the microsphere surface, creating the unstable *o*-Acylsilourea (A). This intermediate is prone to hydrolysis (B). In the presence of primary amines, the intermediate can react with the immunoglobulins to covalently link them to the microsphere surface (C). The addition of Sulfo-NHS can allow the formation of a stable, amine reactive, Sulfo-NHS ester (D), that is less likely to be hydrolyzed and more likely to react with the primary amine (E).

Another important limitation in the production of the antibody coated microspheres was the mass of antibody required in the coupling process to saturate the surface of the microspheres, often taken to be approximately ten times the mass required to form a monolayer on the surface [106]. The mass required to form a monolayer is determined by a number of factors, notably the diameter of the microsphere and the capacity of its surface for a particular protein. These factors are represented in the following equation,

$$S = (\frac{6}{pSd})(C)$$

where S is the amount of protein required to saturate the surface of the microsphere, *pS* is the density of the solid sphere, *d* is the diameter of the sphere in μm, and C is the capacity of the sphere surface for the protein in question, IgG in this case, in mg of protein/m^2 of sphere surface [99]. One can see that the smaller spheres, with their greater surface to volume ratio, can require larger amounts of proteins to saturate their surface. As the microsphere assay described herein utilized smaller microspheres, the amount of IgG required to saturate the spheres was higher and as a result, would have been expensive. In these experiments, the amount of IgG utilized was the calculated value of the monolayer, as opposed to the ten-fold excess recommended and based upon previously published data regarding the affinity of protein for polystyrene surfaces [107]. The potential ramification of this was a reduced number of antibodies bound to the surface of the detection spheres, and as a result, a reduced tendency of the spheres to bind to the analyte FVIII. It was thought that this hindrance could be overcome by utilizing a high concentration of detection spheres, such that the reduced ability of a single sphere to capture an antigen would be overcome by the sheer number of spheres within the solution. Another concern regarding the use of a smaller concentration of immunoglobulins to bind was that the amino-reactive surfaces of

the microspheres would then react more often to the less accessible non-terminal amino groups of the antibodies and therefore result in increasing crosslinking of detection microspheres through the antibodies. The use of a blocking agent such as BSA could also contribute to this occurrence, as it would have a variety of amino groups free for crosslinking. This phenomenon was thought to be occurring commonly in the synthesis of the detection spheres as large aggregates of spheres would often form during the coupling steps and could not be separated via thorough washing, sonication or the introduction of a detergent. In order to minimize this, it was important to ensure that at each step of the synthesis process, the microspheres were well suspended and to maximize the amount of antibody used for the synthesis in order to increase the concentration of the amino terminals that are reacting. Another possible solution would be the utilization of a mono-aminated blocking agent such as mono-aminated PEO. This agent only has a single amino terminal and therefore cannot crosslink the detection spheres together. While this agent was utilized in the capture spheres, it was not utilized in the synthesis of the detection spheres mainly as a cost-cutting method as this molecule can be quite costly. Given the benefit however, future work with microspheres would likely benefit from its utilization as a blocking agent.

Further complicating the synthesis of the immunoglobulin functionalized microspheres were the steric requirements for the coupling of the antibodies to the microsphere surface, such that the Fab domains of the antibodies were readily accessible to the FVIII in solution. Figure 3.21 below graphically demonstrates this factor. It was clear that should the covalent coupling of the IgG occur at the Fab domain, then the antibodies would be unable to bind to any of the FVIII antigen present. Furthermore, the microsphere samples were populated by spheres with a variety

of directions of immunoglobulins bound to their surface, potentially causing significant assay variation.

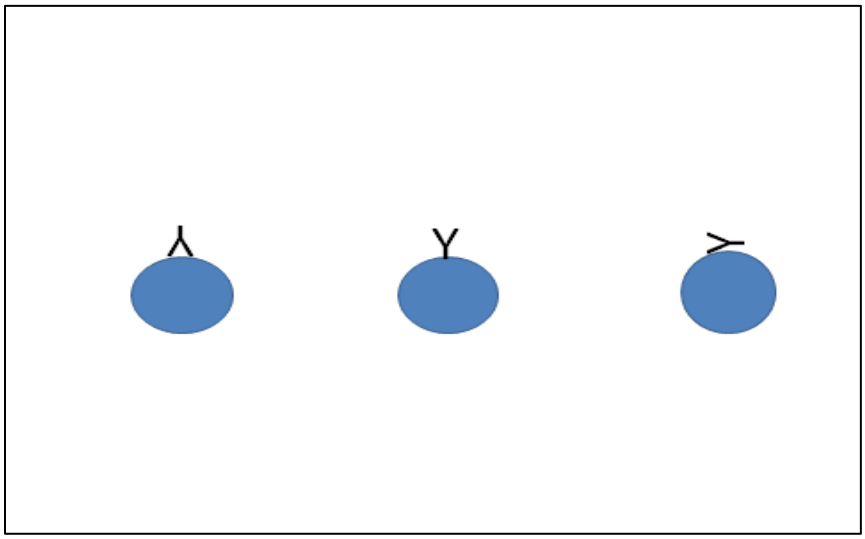

Figure 3.21 The possible orientations of immunoglobulin bound to the microspheres. This diagram illustrates that the binding of the IgG to the microspheres can occur through any free amine, not simply the Fc region terminal amine. As a result, only the centre sphere would be fully active and capable of binding the maximum amount of antigen. Conversely, the far left sphere would be unable to bind any antigen as the Fab region is sterically/chemically blocked off.

While the microsphere assay was successful and did produce low detection limits, the above mentioned issues with the coupling chemistry made the carboxylated spheres unattractive options.

3.5.3 Studying the effect of the peptides on FVIII-antibody interaction

Factor VIII in circulation is essentially composed of two polypeptide chains, the heavy chain and the light chain. Therefore, any peptide binding site must be located on one of these two components. Similarly, the epitopes for any anti-FVIII immunoglobulins must be located on one of the chains. It was therefore assumed that the binding of a peptide to FVIII would likely mask

the epitope for a particular antibody and as a result, prevent its binding to FVIII. The reverse would also be true, that the binding of the antibody to its particular epitope would prevent peptide binding to FVIII if the binding site contained that occupied epitope. These assumptions were thought to allow for the localization of the peptide binding site on FVIII through the utilization of competitive assays between the peptide and the antibodies for FVIII. Thus, to determine the peptide binding site on FVIII, the microsphere immunoassay was used. Essentially, we attempted to show that by incubating the peptides with the FVIII prior to performance of the microsphere assay, the amount of FVIII that could be detected by the assay would be reduced in comparison to the amount detected when no peptides were present. As a proof of concept, we can see this demonstrated in Figure 3.22, which showed the results when FVIII was incubated with arginine vasopressin before quantifying it using the immunoassay in which the capture spheres were coated with anti-HC antibodies and the detection spheres with anti-LC antibodies.

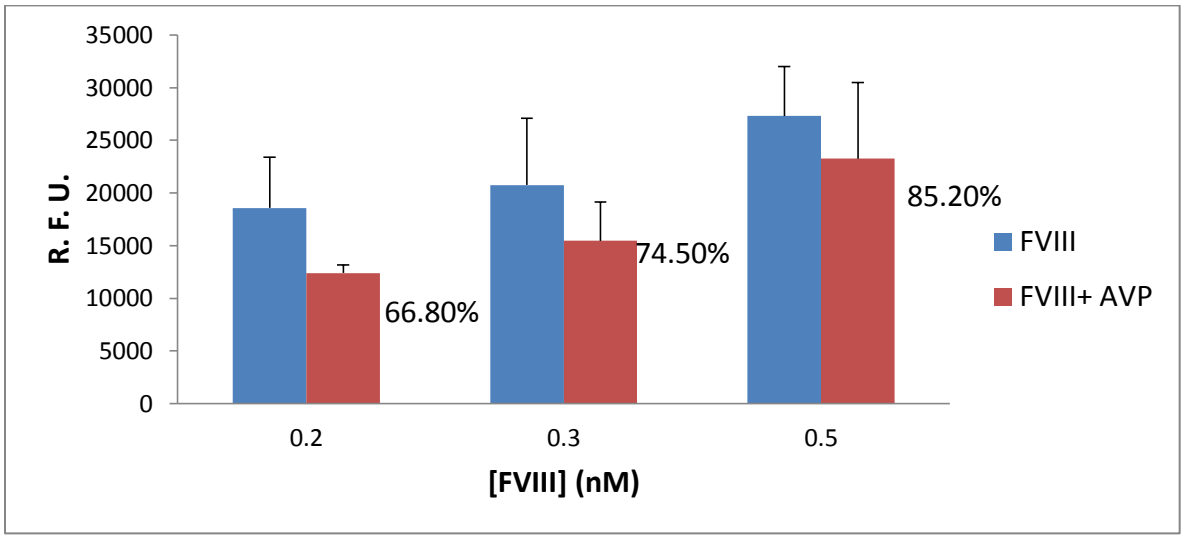

Figure 3.22 Effect of pre-incubation of AVP with FVIII on R.F.U. of the microsphere immunoassay. Pre-incubation of 20 nM of AVP with the FVIII resulted in a reduction in the fluorescence intensity of the assay, though as the concentration of FVIII increased, the R.F.U. increased as well to match that of the FVIII without AVP.

It was evident by these results that the presence of the AVP reduced the detected amount of FVIII, and this inhibition was decreasing with increasing concentrations of FVIII. However, what was not clear was which part of the immunoassay was inhibited. There were two distinct possibilities. It could be that the peptide binding site lies on the heavy chain and, as a result, the blocking of this site by the vasopressin inhibited the FVIII capture by the capture spheres. As a result, a proportion of FVIII would be lost in the washing steps as it was not magnetically retained by the capture spheres. Then, consequently, there would be less FVIII present to retain the detection spheres, reducing the R.F.U. of the assay. The other possibility is that the peptide binding site lies on the light chain. In that case, the FVIII and peptides were captured in the initial steps and retained during the washes. However, during the detection phase, the detection spheres were unable to bind to the FVIII as the light chain binding site was occupied by the peptide. An increased number of detection spheres would be lost in the wash steps, reducing the detected fluorescent signal.

With these distinct possibilities, attempts were made to repeat the microsphere immunoassay and include the remainder of the test peptides. However, the point at which the peptides were added to the FVIII varied. In order to assess the peptides binding site, the assay was performed under 4 different conditions. In the first condition, the FVIII was pre-incubated with the peptide. Then, capture spheres with the anti-HC antibody were added and the remainder of the assay was completed. In the second condition, the peptides were again pre-incubated with the FVIII. However, this time, capture spheres with the anti-LC antibody bound to them were added and the assay was completed. In the third condition, the FVIII was incubated with the capture spheres containing the anti-HC antibody first. This would allow the formation of the capture sphere-FVIII complex. Then, the peptide was added to the reaction and incubated for a

time. This would be followed by the usual remainder of the assay. In the fourth condition, the capture spheres with the anti-LC antibody were pre-incubated with the FVIII, then the peptide added and the assay completed. If the peptide were binding to the HC, then the assay conducted under the first two conditions would fail, as would the assay conducted under the fourth condition (as the detection spheres coupled to anti-HC antibodies would be unable to bind). If the peptides were binding to the LC, the first two conditions would also fail, as would the assay conducted under the third condition. Thus, by seeing the pattern of assay failure, one could ascertain the binding site of the peptide. This concept was predicated on the assumption that the antibody and the peptide binding to FVIII were irreversible. While this may be true to a certain extent for the antibodies given the K_d of the interaction being less than 1 nM, it was unknown if this were the case for the peptide-FVIII interaction. Regardless, in all formats of the assay, no linearity could be achieved between FVIII and fluorescent signal in the presence of the peptides, as demonstrated in Figure 3.23.

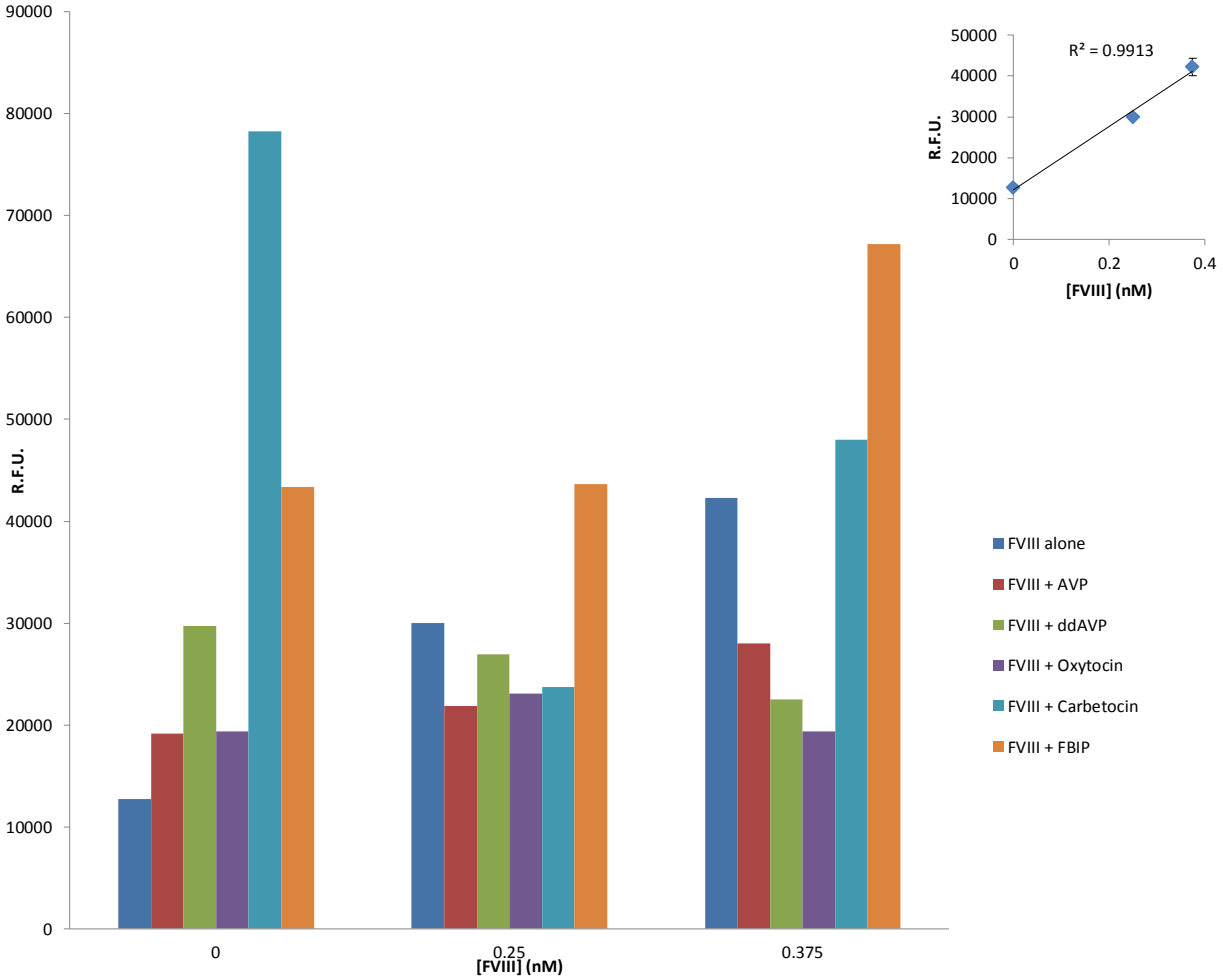

Figure 3.23 Fluorescent signal from microsphere assay in the presence or absence of the peptides at three different FVIII concentrations. Though a linear trend is observed in the performance of this assay with FVIII alone, with an R^2 of 0.9913, addition of the peptides to the assay ultimately resulted in loss of linearity as well as in an important rise in blank signal.

The results presented in Figure 3.23 however demonstrated the important limitation of microsphere based assays, that of non-specific adsorption of the microspheres, as previously mentioned. These results showed an increase in the fluorescence of the blank in the presence of the peptides. This was thought to be related to the peptides mediating the adsorption of the microspheres, despite their surface coating with polyethylene oxide residues. There have been publications supporting the role of proteins in the adsorption of other cells to bio-surfaces [108]

and it was thought possible that a similar phenomenon was occurring with the peptides and the microsphere surface. While the assay produced the expected correlation between FVIII concentration and the fluorescence from retained detection spheres, the addition of the peptides resulted in an increased fluorescence across all concentrations of FVIII and a breakdown in the linearity of the assay. In an attempt to overcome this, an increased number of washes prior to fluorescence reading were performed, with the goal of removing the excess non-specifically adsorbed microspheres. However, the increased number of washes further decreased the correlation coefficient between the FVIII concentration and the number of detection spheres retained. This phenomenon also occurred with the calibration curve in the absence of the peptides, as shown in Figure 3.24, which also noted a loss of correlation between FVIII concentration and detection sphere retention when increasing numbers of washes were performed. This was expected to be the case however. Despite the affinity of antibodies for their antigens, the equilibrium constant between the detection spheres complexed to the capture spheres via the antibodies was not nil. With each successive wash, while more non-specifically bound spheres were removed, there was a re-establishment of equilibrium between spheres bound to FVIII through the antibodies and those free within the sample. With each wash, more detection spheres were lost and the assay lost its correlation. The effect of the washes on the correlation coefficient of the assay is demonstrated in Figure 3.24. Another important point to consider was whether or not the washing methods were optimal. The washing methods were determined based on the suspension of the larger beads within the washing buffer. As seen in Figure 3.18, the spheres blocked with BSA and suspended in 4% PEO had very few higher order aggregates, suggesting that the non-specific interactions between the spheres were minimal. While this may have been the case for the larger capture spheres imaged, these conditions may

not have been optimal for the capture sphere-detection sphere interactions, especially when suspended in a solution containing the analyte as well as the peptides. The development and refinement of the assay method should have included optimizing washing buffers with the actual assay, as opposed to with simple coated capture spheres. In order to accomplish this, one could perform the assay using various washes and determine the effect of the composition of washing buffer with the relationship between the correlation coefficient and the number of washes.

Ultimately, though the concept of the interference assay suggested that it could help determine the location of the peptide binding site, it relied on the assumption of the irreversible binding of the antibodies to the analyte. However, as seen by the loss of linearity of the assay with increasing number of washes, this was not likely to be the case. As a result, the likelihood of the interference assay succeeding seemed low.

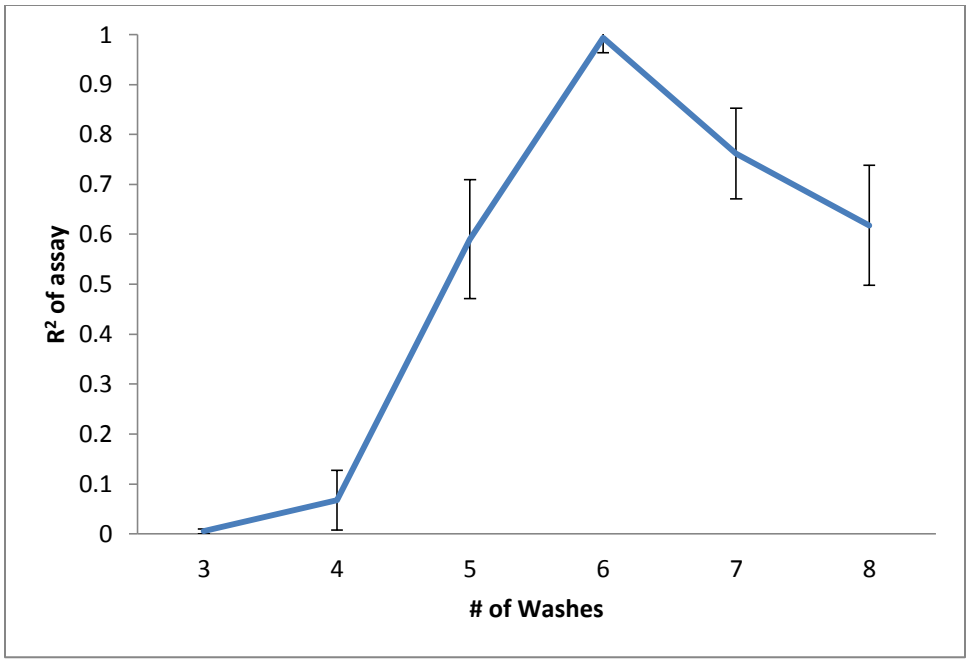

Figure 3.24 Relationship between microsphere assay washing and R^2 of the assay. The figure shows that the washes increase the correlation between concentration and R.F.U., up until the 6th wash. Subsequent washes cause a loss of linearity between the fluorescent signal and the FVIII concentration, suggesting that an increased ratio of specifically bound detection spheres to non-specifically bound ones are being washed away.

3.6 Studying the protein-peptide interactions via equilibrium dialysis

Equilibrium dialysis is a useful technique in the evaluation of protein-peptide interactions. By utilizing a semi-permeable membrane to isolate the protein, the technique allows for the peptide to equilibrate with a targeted protein while also allowing isolation of the peptide for direct measurement [109]. Thus, the neurohypophyseal peptides were allowed to equilibrate across a 2 kDa Molecular weight (MW) cutoff membrane over 8 hours while 21.7 nM FVIII was retained on the other side. The peptide solution sample was then taken for quantitation via mass spectrometry. As mentioned earlier, one important limiting factor throughout the experiments described here within is the limited concentration of FVIII that could be utilized for this research. As the anticipated stoichiometry of the FVIII-peptide interaction was thought to be small with a

weaker K_d, the anticipated change in peptide concentration was thought to be minimal as well. Thus, in order to successfully conduct these experiments, a higher than physiological concentration of FVIII and peptides were utilized.

Despite the higher concentrations of peptides, the mass spectrometer was unable to detect the oxytocin, FBIP, and dDAVP standards at 221 nM. It was able to detect the AVP and the carbetocin however, as shown in the data in Figure 3.26 and Figure 3.28 respectively. The area under the curve (AUC) for these peaks was however small, representing a low total ion count and suggested that solutions were near the detection limit. Similarly, the mass spectrometer could not detect the presence of the FBIP, oxytocin or dDAVP peptides in the solutions dialyzed against the FVIII. It did detect the AVP and the carbetocin however, just as in the peptide standards, as shown in Figure 3.29 and in Figure 3.30. There was no significant difference between the ion count of the carbetocin solution dialyzed against buffer versus that dialyzed against FVIII. In contrast, there was a significant decrease between the ion counts of the AVP dialyzed against FVIII compared to that dialyzed against PBS. While this could suggest a significant change in the equilibrium concentration of the peptide in the presence of the FVIII, the magnitude of the difference was more suggestive of experimental error, as it was a decrease of approximately 80% of the total ion counts. If that were the truly reflective of a change in the equilibrium concentration, that would indicate a significant number of AVP molecules bound to each FVIII molecule. Given that there was one order of magnitude more peptide than protein, if only 20% of the peptide remained unbound after equilibrium to be detected by the mass spectrometer, then 80% of those molecules must be bound to the factor VIII, or approximately 8 peptides per molecule of FVIII. The FVIII protein is relatively large at 300 kDa and it is conceivable that it could bind that many peptides. This would also be in line with the magnitude

of change in the fluorescence spectrum of FVIII during titration with AVP as shown in Figure 3.1, which suggested that numerous tryptophan residues were being affected by the conformational change being caused by the peptide. However, given that the carbetocin solutions showed no real change in their ion counts, and coupling that with the failure of the mass spectrometer to detect the other peptides at the same concentration, it seemed likely that the changes made were likely a result of experimental error.

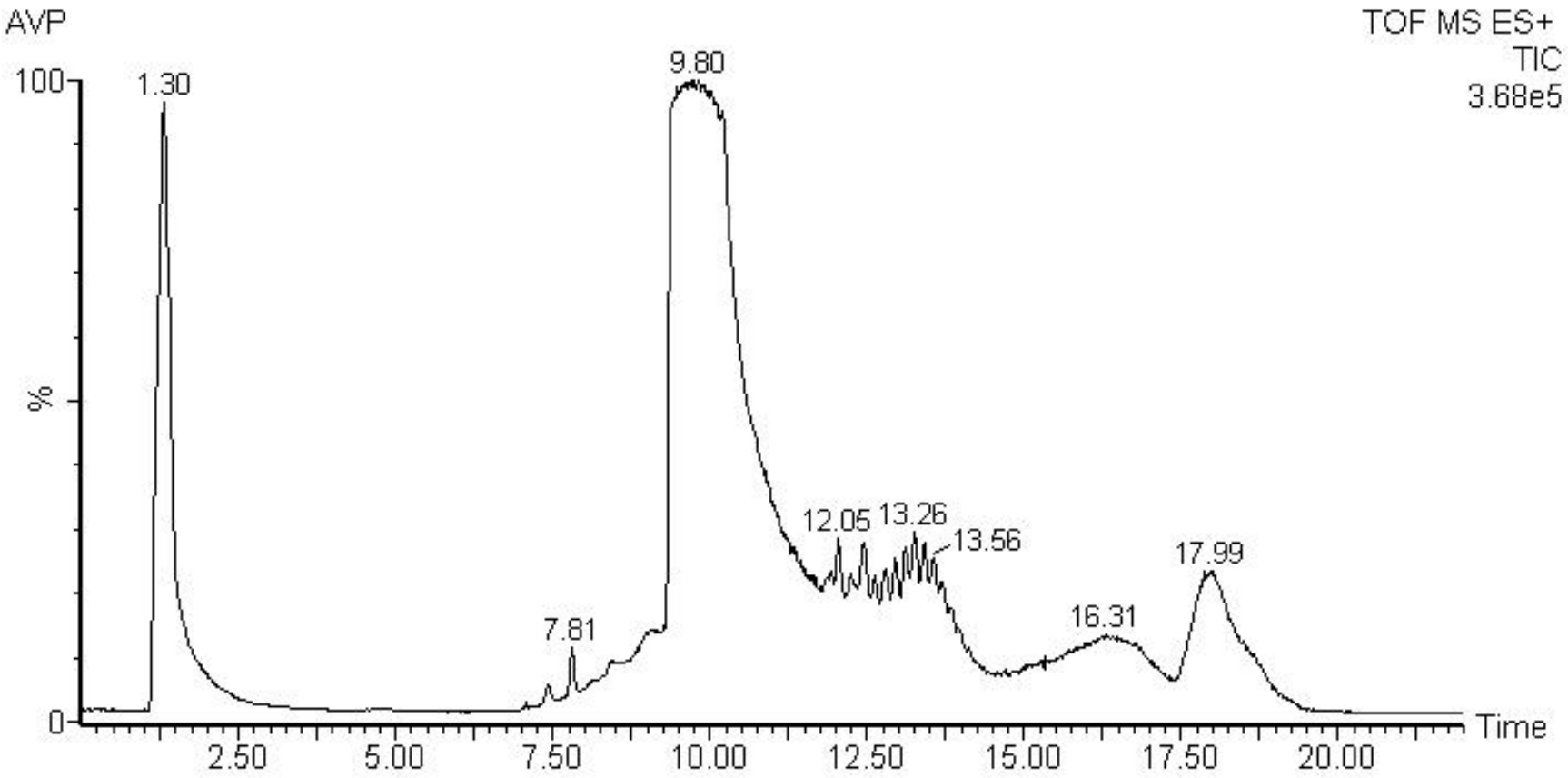

Figure 3.25 Total Ion Chromatogram (TIC) of 221 nM AVP dialyzed against PBS buffer with a 2 kDa restriction membrane. The peptide peak was found to elute between 12.5 and 13.5 minutes. The large peak at 9.8 minutes represents the 66 kDa protein contaminant found within the sample.

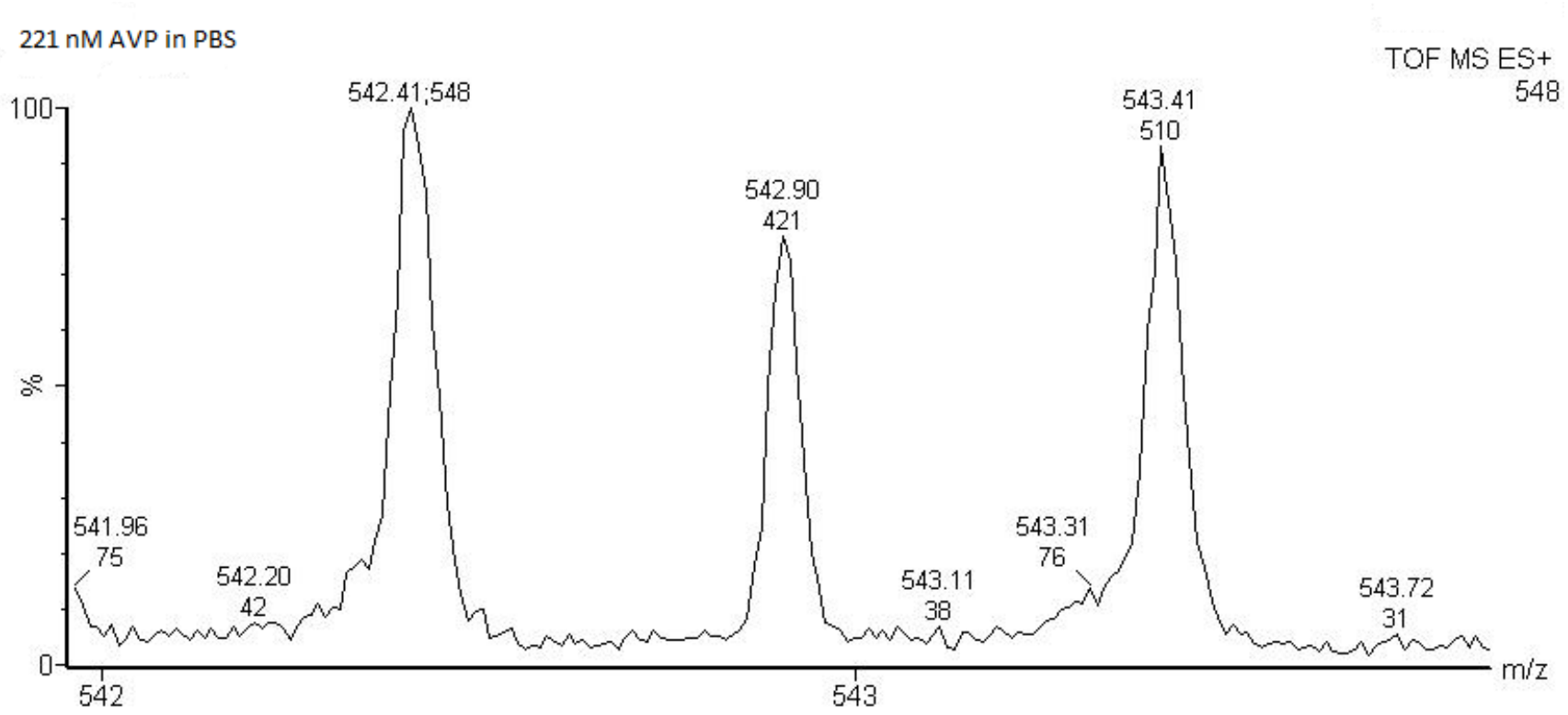

Figure 3.26 Mass spectrum of 221 nM AVP (1084.23 Da) in PBS dialyzed against PBS. The AVP peptide is observed as the doubly charged ion at M/Z = 542.41. The total ion count was 548 for these peaks.

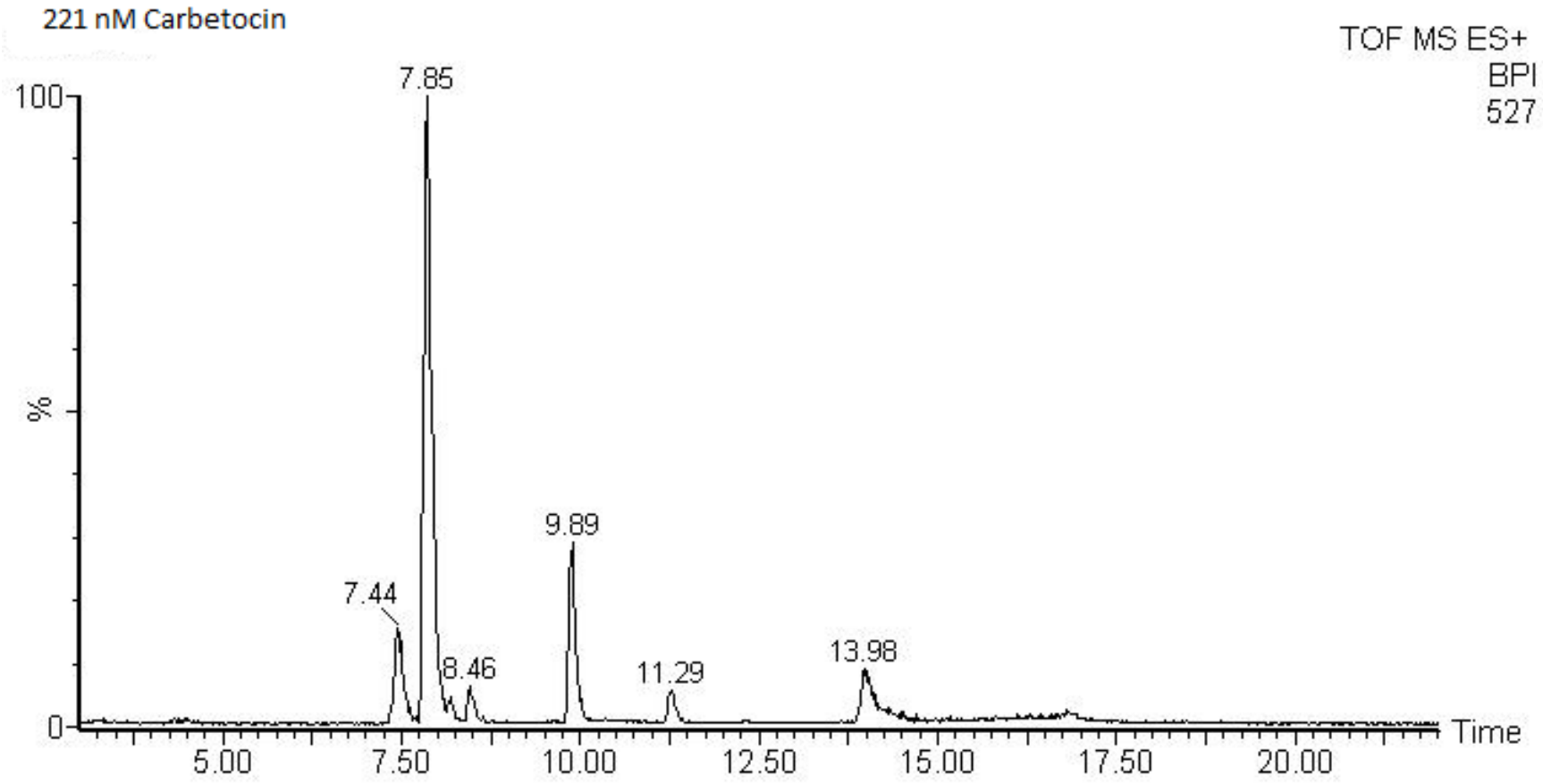

Figure 3.27 Total Ion Chromatogram (TIC) of 221 nM carbetocin in PBS dialyzed against PBS. The peaks at 7.44, 7.85 and 8.46 minutes represent the time of elution of the Glu-fibrinopeptide standard, while the peak at 9.89 minutes represents the elution time of the carbetocin, at a point when the solvent was composed of 60% ACN and 40% of 0.1% FA.

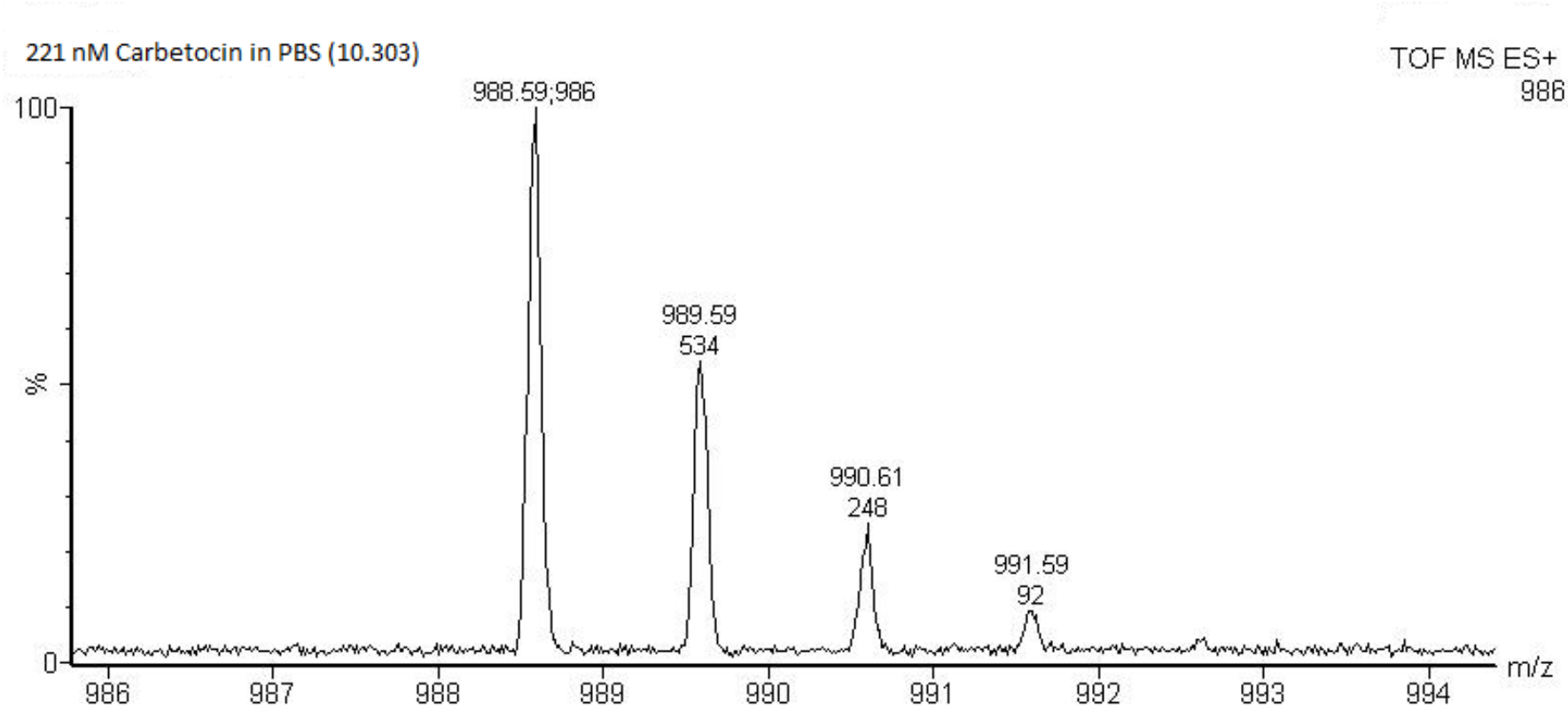

Figure 3.28 Mass spectrum of 221 nM carbetocin (988.20 Da) dialyzed against PBS through a 2kDa MW cutoff membrane. The peaks shown represent the single-charged species of the peptide.

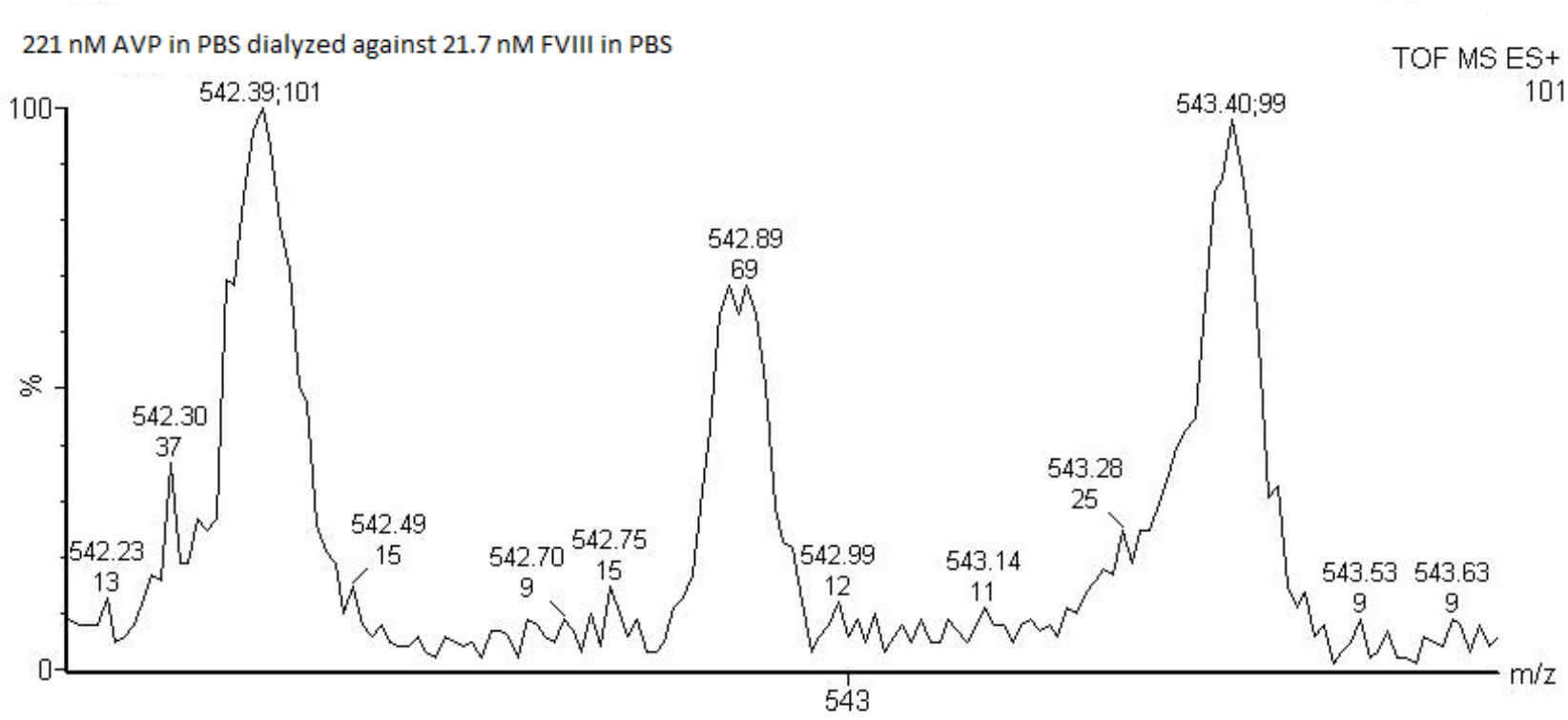

Figure 3.29 Mass spectrum of 221 nM AVP dialyzed against 21.7 nM FVIII. The AUC for the doubly charged species shown here are reduced in comparison to that of the sample of 221 nM AVP in PBS dialyzed against PBS.

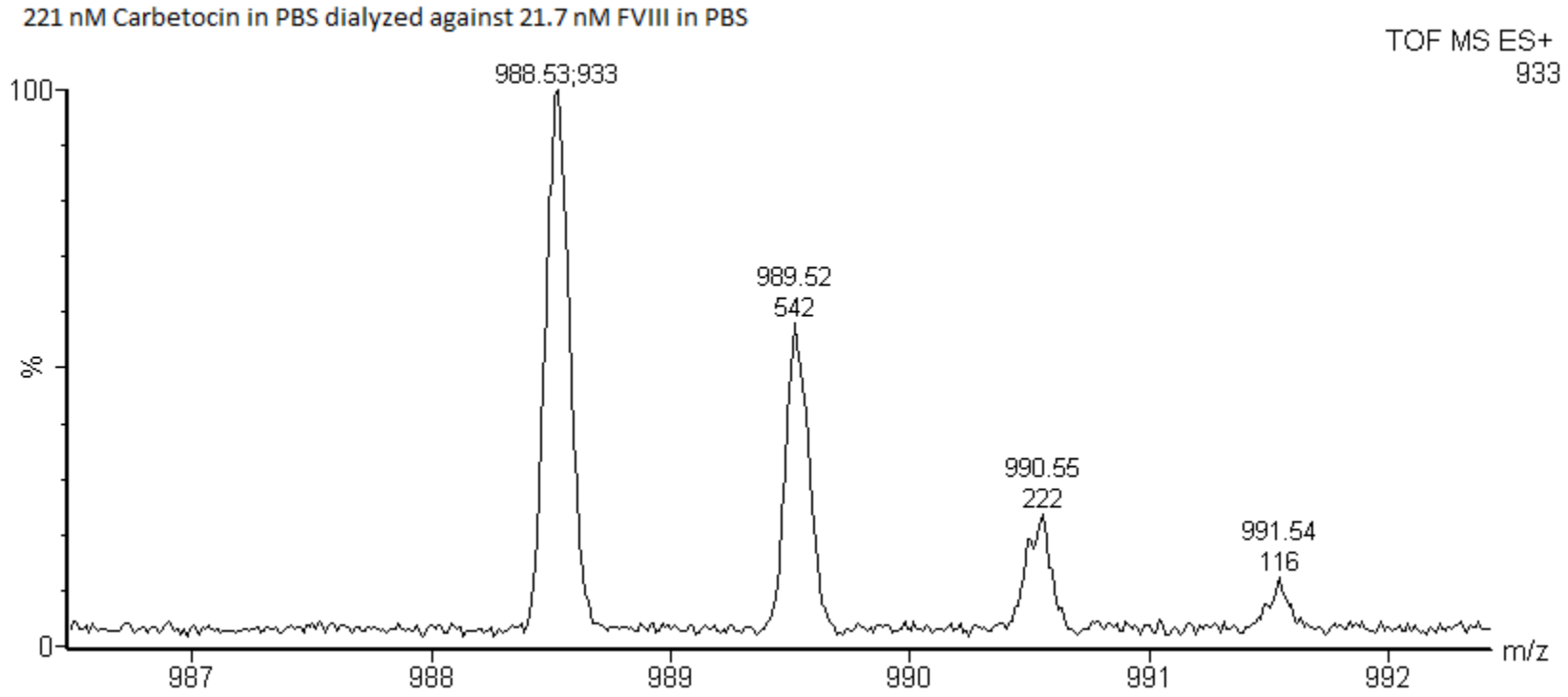

Figure 3.30 Mass spectrum of 221 nM carbetocin dialyzed against 21.7 nM FVIII in PBS. The peaks represent the singly charged species of carbetocin.

The reason behind the inability to detect the remaining peptides at the concentration of 221 nM was unclear. One possibility was that the peptides do not ionize well and therefore mass spectrometry will have a low sensitivity for them. However, studies have been published utilizing mass spectrometry to quantify these peptides and so this possibility seems unlikely, as low concentrations were detected with success [77, 110]. Another possibility was that the use of PBS buffer reduced the ionization of the peptides, and future work should use a more compatible mass spectrometry buffer. Another concern was that, as seen in the chromatograms in Figure 3.25 and Figure 3.27, these peptides had a tendency to bind to hydrophobic compounds, as they required a high concentration of organic solvent to elute. As such, one concern would be that the peptides bound to the dialysis chamber walls and were therefore reduced in the solutions analyzed. This may have explained the significant difference in the detected AVP in the FVIII sample as well, as in that particular sample, a significant amount of the peptide bound to the chamber wall. Future work should utilize organic solvents to wash the dialysis chamber after completion of the equilibrium experiments and then analyzing the wash for peptide. This would enable verification of the possibility that the peptides were being lost to the walls of the device. Should this be the case however, then the utility of the equilibrium dialysis experiments becomes questionable. If the peptides are binding to the wall in a significant proportion, then it would be difficult to determine what concentration is equilibrating across the membrane and with the protein on the other side, and what concentration is binding to the wall, using mass spectrometry. One could theoretically use a control peptide that does not bind to FVIII and assess what proportion of it is lost to the walls and membrane during an equilibrium dialysis experiment. Using this information, one could correct for those losses with the test peptides. However, the question of the differences between the control and test peptides would remain and there would

still be doubt regarding how accurately the losses of the control peptide reflect the losses of the test peptides. Alternatively, one could pre-wash the dialysis containers with a blocking type of peptide that would reduce the unblocked surface area to which the peptides could bind non-specifically. A final alternative would be to perform mass-balance experiments, in which the concentration of peptide on both sides of the dialysis membranes are quantified and compared to the concentration of the original solution. This would provide an indication as to how much peptide is being lost on the walls of the dialysis chamber or to the membrane itself. It is however important to note that this set of experiments would still require a different method of detection.

In order to assess if the lack of signal with the 221 nM peptides was a result of being below the detection limit for the instrument, a sample of 500 nM dDAVP in PBS was probed. The results in Figure 3.32 demonstrated that at this concentration, the peptide could be detected, though again with low ion counts. These results suggested that if future dialysis work were to be done with the intention of determining the K_d, one would need to use significantly higher concentrations of peptides and protein in order to be detected and quantified by the Mass spectrometer. Given the high cost of FVIII, the utilization of mass spectrometry in the analysis of the interactions between the peptides and the protein may not be cost-effective.

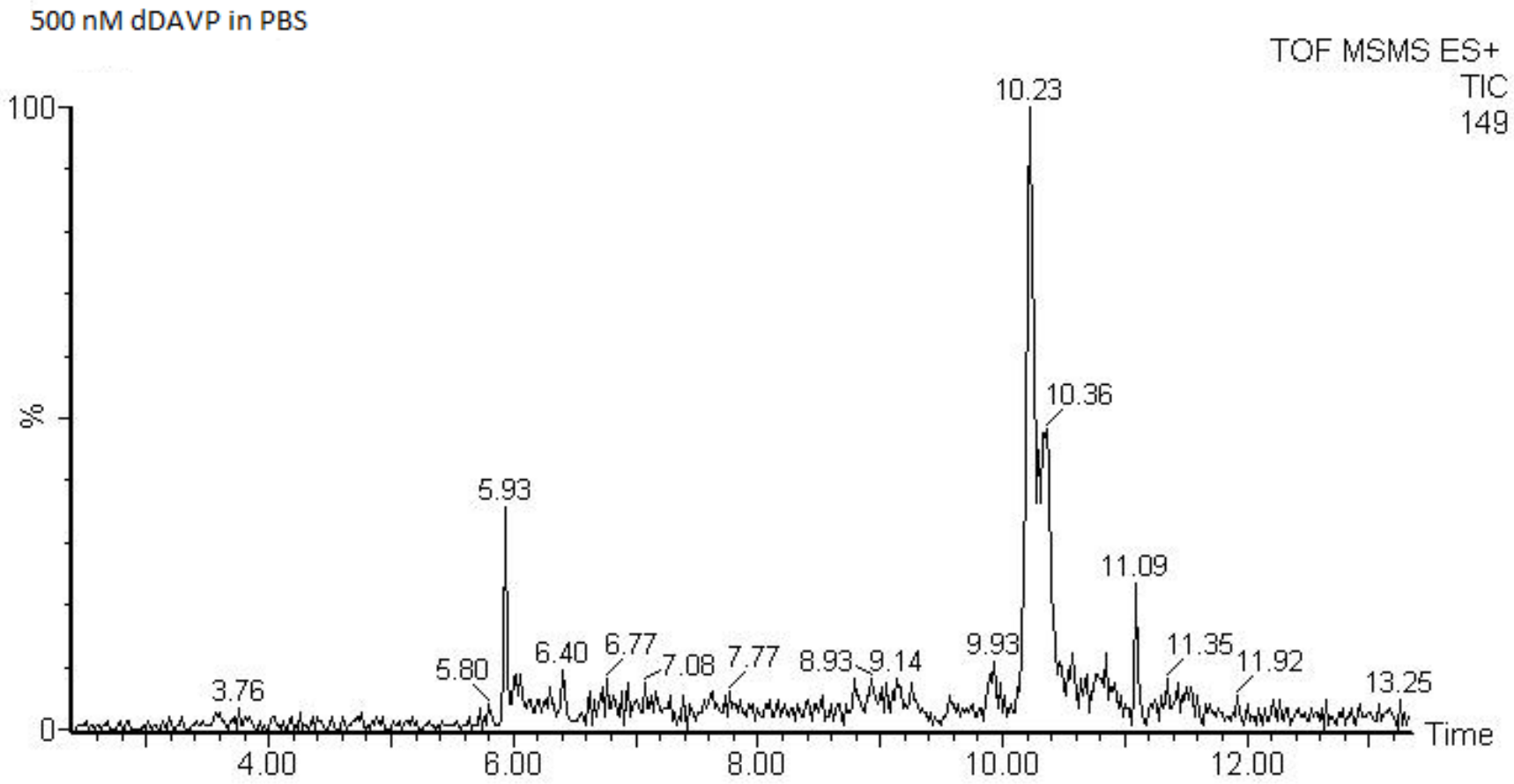

Figure 3.31 Total ion chromatogram of 500 nM dDAVP in PBS separated on a C4 column before injecting into the Q-TOF. The peak at 10.23 minutes is the peak containing the dDAVP ions.

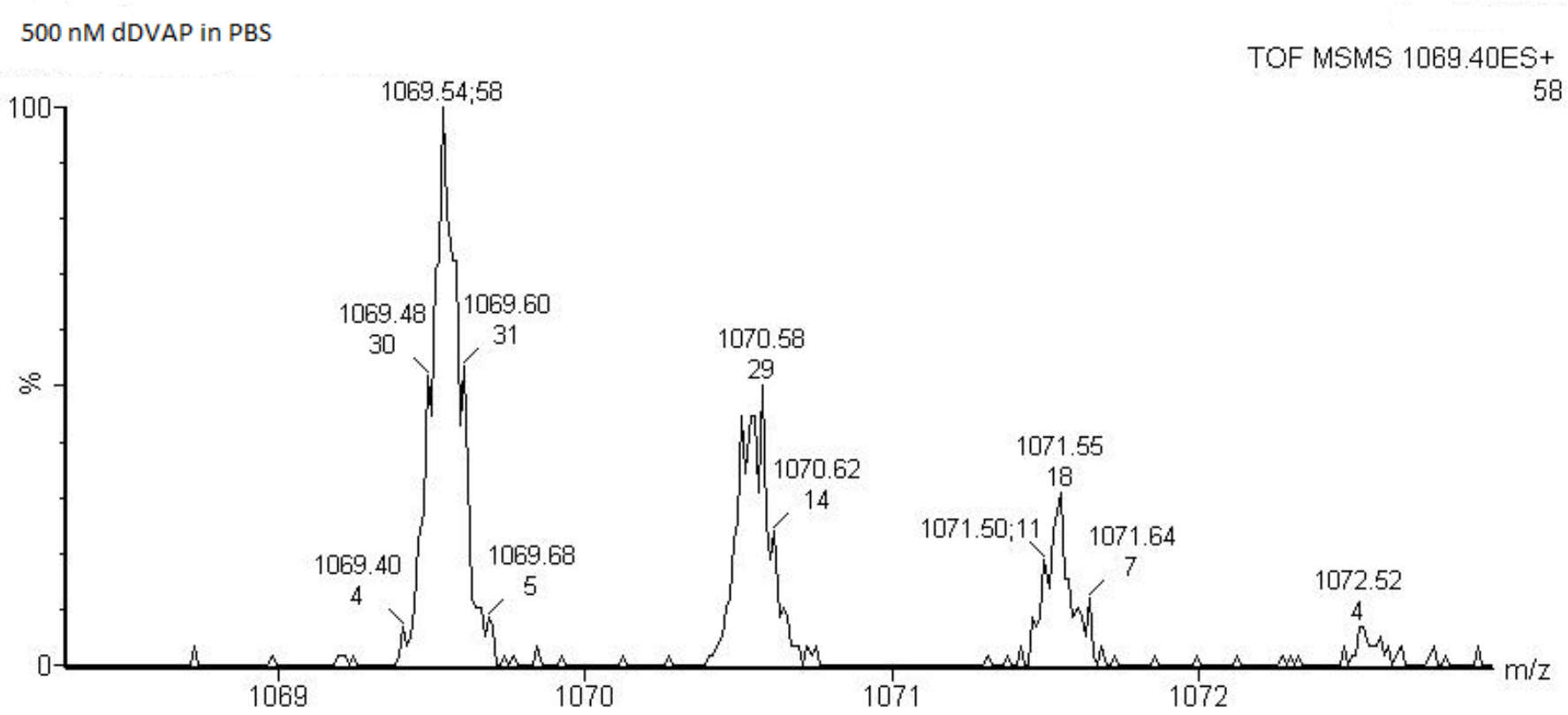

Figure 3.32 The mass spectrum of 500 nM dDAVP (1069.21 Da) in PBS. This spectrum shows the singly charged species of dDAVP.

One important finding from the analysis of the mass spectrum of AVP is that a large protein eluted from the sample at 9.8 minutes, as seen in the chromatogram in Figure 3.25. The mass spectrum for this peak shows a typical charge envelope of a protein, as shown in Figure 3.33. In order to determine the mass of this protein based on the different peaks present, the MaxEnt software of Mass Lynx was utilized, and the mass spectrum of the protein, shown in Figure 3.34 was generated.

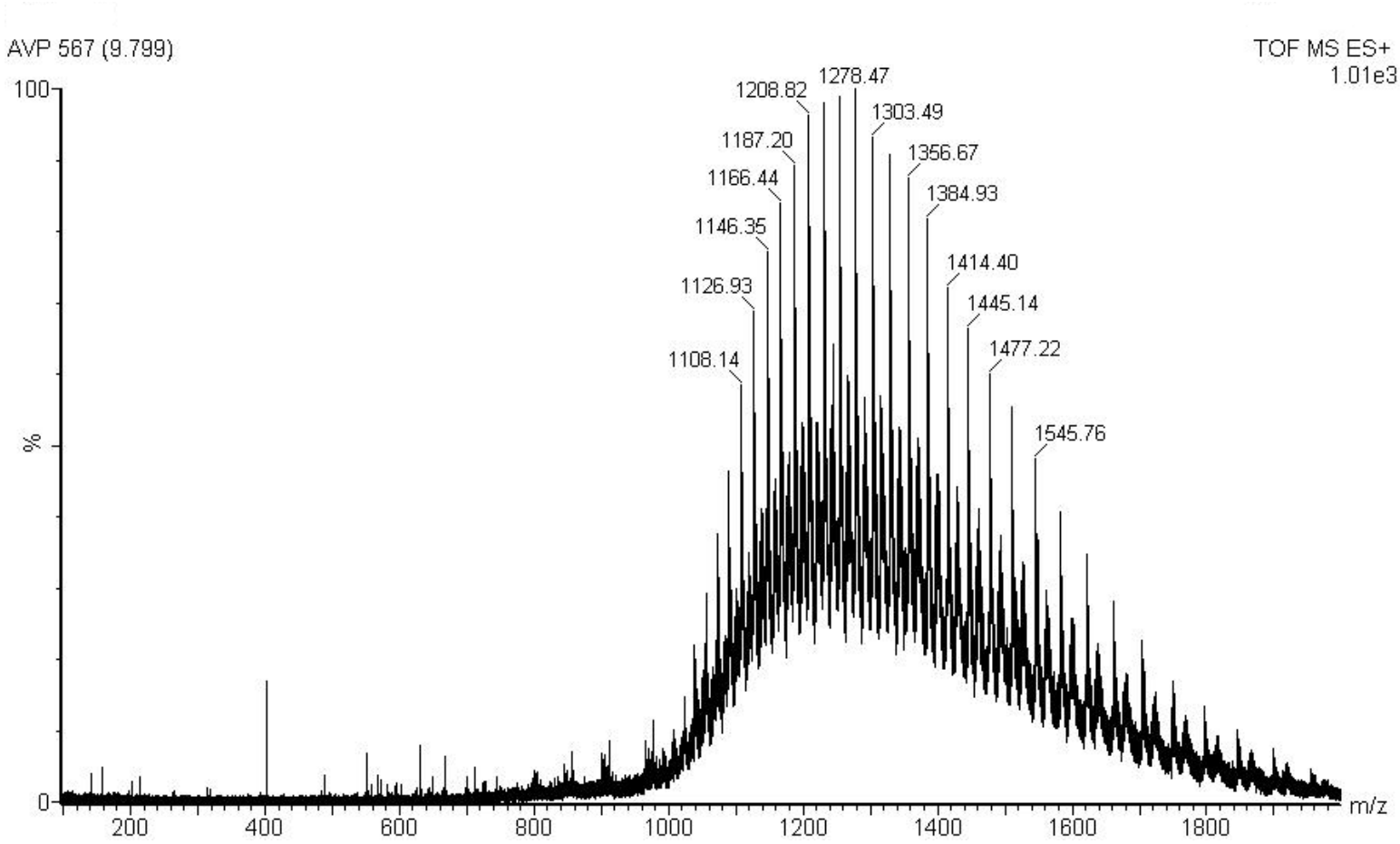

Figure 3.33 The mass spectrum of the protein contaminant of the 221 nM AVP in PBS. The chromatographic separation of the peptide solution demonstrated the elution of a large peak at 9.8 minutes from the C4 column, which contained a variety of m/z peaks in a pattern consistent with the charge envelope of a protein.

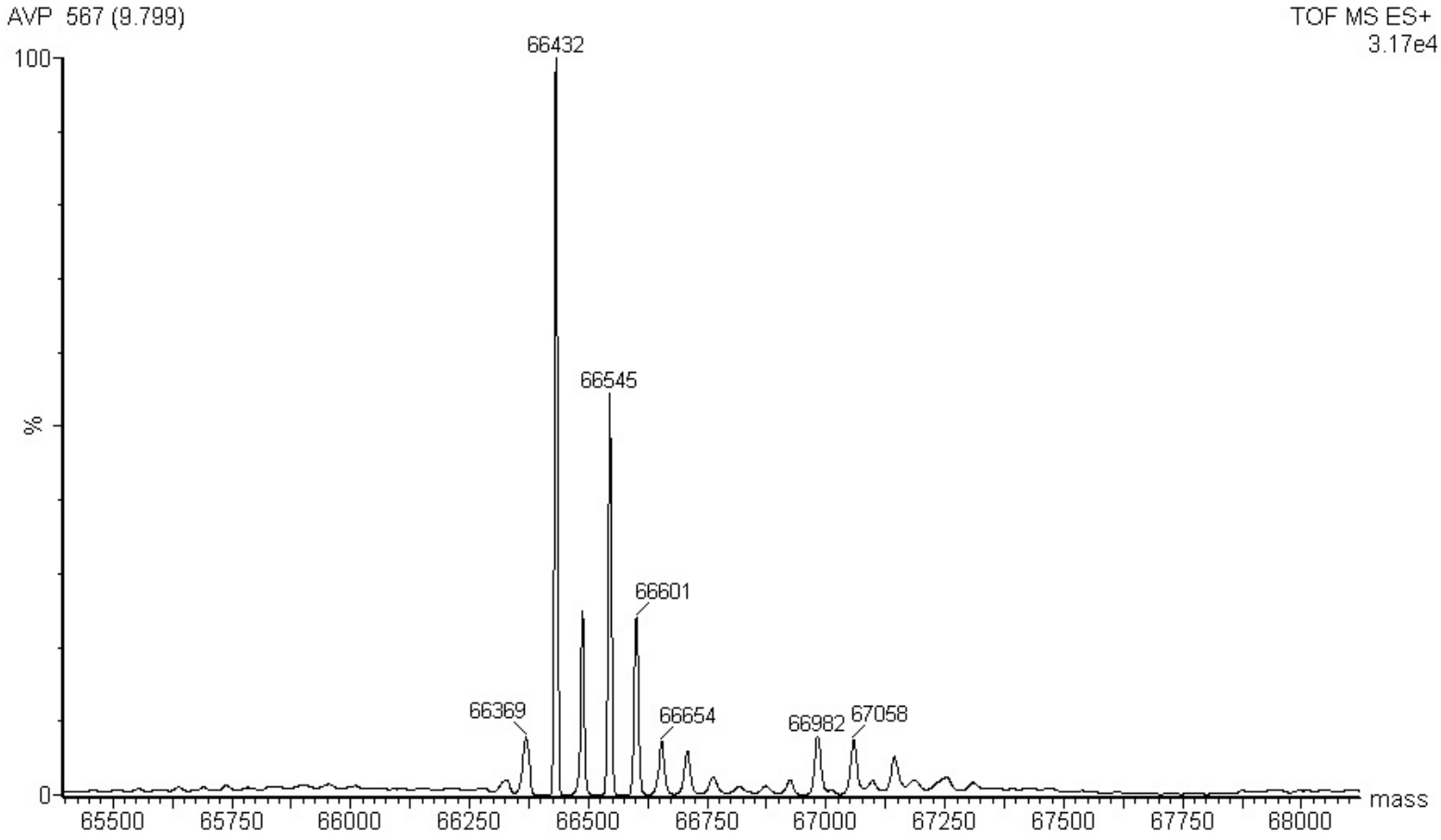

Figure 3.34 Mass of the species eluting at 9.8 minutes from the separation of the 221 nM AVP in PBS. This mass spectrum was determined using the MaxEnt1 function of the Mass Lynx program, using a half peak width of 4 Da, and estimating the mass to be between 50 and 70 kDa.

The predominant peak in the mass spectrum is 66.432 kDa. Given that the AVP was derived from human blood, this could possibly be human serum albumin (HSA), which has an average mass of 66.437 kDa. This protein was not seen in the carbetocin sample, though given this is a synthetic peptide that was not isolated from human blood, this was not surprising. The presence of this protein in the solution was problematic for the interpretation of all of the results that utilized the vasopressin. The presence of a confounder in the samples created uncertainty in the findings, as one could then question whether or not any of the observations were a result of the protein as opposed to the peptide. Furthermore, albumin is known to bind to many different proteins, some selectively and some not. If it were present with the vasopressin, then it would be concerning as the vasopressin may then be in equilibrium between an active unbound state and an inactive bound state. Ultimately, the presence of the albumin may have reduced the overall fraction of vasopressin able to interact with FVIII. There are several counter points to consider however. The fluorescence experiments utilized blank measurements of the PBS buffer with sequential additions of the peptides, and then had these spectrums subtracted from the results of the FVIII titration with the peptides. During this procedure, at no point did the fluorimeter pick up any significant signal from the protein seen during the mass spectrometry experiments. This suggests that the concentration of this protein was minimal. Furthermore, during the FVIII activity kit experiments, both the COAMATIC SP4 kit and the Technochrom kit contained additional albumin to be added during the reaction. Despite this, the experiments nevertheless demonstrated an important ability of the peptides to rescue the FVIII activity in the presence of anti-heavy chain antibodies. Had there been a significant interaction between the peptides and the albumin, one would have expected no rescue to occur, should the albumin have remained

free for peptide binding. Thus, the presence of the albumin, while raising the concern for possible confounding of results, may not have yielded any specific effect on the overall results.

3.7 Summary of results

The sum of the results obtained during investigation of the FVIII-peptide interactions are presented in Table 4 below.

Table 4 Summary of main results of the thesis.

Technique	Main Results
Fluorescence spectroscopy	• Selective quenching of FVIII fluorescence in the presence of the peptides • No evidence of photodegradation • No change to thermal denaturation of FVIII in the presence of the peptides
FVIII activity kits	• Conflicting results between the Technochrom and the Chromogenix kits, with one showing increase in FVIII activity in the presence of the peptides and the other showing no change • Possible prolongation of the $FVIII_a$ half-life • Non-selective peptide mediated blocking of HC antibody inhibition of FVIII activity assay
Microsphere Immunoassay	• Assay can quantify to 100 pM [FVIII] • Failure of microsphere immunoassay to quantify FVIII in the presence of peptides
Equilibrium Dialysis- Mass Spectrometry	• Failure of Mass Spectrometry to detect 3/5 peptides at the concentrations used • Significant shift in AVP equilibrium concentration in the presence of the FVIII

Chapter 4 Conclusions & Future Work

Factor VIII is a critical component of the coagulation cascade, enabling the rapid formation of the Xase complex to cleave prothrombin into thrombin, allowing for the formation of fibrin monomers that can crosslink the platelets of the thrombus and strengthen the seals covering ruptured blood vessels. Factor VIII also represents an important area of human pathology, with Hemophilia A being a common genetically acquired coagulopathy, that if untreated, can have devastating consequences. In the treatment of mild Hemophilia A, desmopressin emerged in the 1970s as a promising method to increase FVIII levels and prevent bleeding complications without the infusion of blood derived products. The role of desmopressin soon expanded into the realm of critical care and control of bleeding resulting from other pathologies. Like desmopressin, vasopressin also began being utilized in critical care, developing an important role in the management of traumatic hemorrhagic shock. In a similar vein, oxytocin and carbetocin were soon being used to control post-partum hemorrhage, an important cause of perinatal mortality of mothers. Given the similarities in structure, the role in controlling hemorrhage and the utility of AVP and dDAVP in the management of Hemophilia A, the possibility that the peptides were interacting with FVIII seemed likely.

The investigations into the possible interactions between FVIII and the neurohypophyseal peptides and their drug analogs presented herein were conflicting. While some of the techniques yielded results supportive of an interaction, others did not. Some of the experiments performed were also discordant, with initial results confirming an interaction while failing to do so upon repetition, and another method, the FVIII activity kits, resulting in conflicting results between different commercially produced kits. Perhaps the most important cause of these discrepancies is the utilization of mainly indirect techniques to assess the presence of the interactions, and doing

so using minimal concentrations of protein and peptide. Obtaining higher concentrations of FVIII was cost prohibitive, and as a result, the more common tools to measure protein-peptide interactions, such as isothermal titration calorimetry and surface plasmon resonance, could not be performed, leading to a reliance on the indirect measurements of protein-peptide interactions used. While the microsphere assay enabled quantitation of physiological and lower concentrations of FVIII, the interference assay was hampered by non-specific adsorption that could not be overcome with washes and therefore was of limited utility in probing the FVIII-peptide interactions. Future work in this area will need to optimize the washing and blocking parameters to cope with solutions containing peptides. It is possible that the use of PEO as the blocking agent may be more successful.

The equilibrium dialysis experiments failed as a result of the peptide concentration being below the mass spectrometry detection limit. An interesting possibility in overcoming the detection limit of the peptides would be to use radiolabeled peptides. These peptides are quantifiable at very low levels and can be utilized in the equilibrium dialysis experiments for a more direct measurement of the protein-peptide interactions [111]. The advantage of the radiolabeled peptide is that it would also allow for detection of the fraction of the peptides retained on the vessel wall and the dialysis membrane. This would allow for a more accurate determination of the K_d and mass balance. These peptides can be prepared commercially as well, rendering the risk of radioactive contamination of the laboratory space minimal. Another important advantage of these peptides would be the ability to use them in *in vivo* conditions, such as in blood. As discussed earlier, one of the limitations of the work presented herein was the poor reflection of *in vivo* conditions by the *in vitro* studies performed. Radiolabeled peptides could be spiked into blood samples and remain easily identifiable and quantifiable. As such, equilibrium

dialysis experiments could be performed with plasma rather than buffer on both sides of the membrane, with radiolabeled peptide added to one side. These would equilibrate with the native FVIII present on both sides of the membrane, yielding a specific equilibrium concentration of the peptide. Afterwards, FVIII would be added to one side of the membrane. If the peptide is interacting with the FVIII, this addition should cause an increased amount of the peptide to shift to the side containing the additional FVIII. This change in the equilibrium concentration as well as the disproportionate amount of peptide on one side of the membrane should be readily detectable. This would support that there is an interaction between the peptides and FVIII.

In order to proceed with further investigation into the potential interactions between the peptides and FVIII, one could utilize higher concentrations of the reagents. The caveat to this however is that one must keep the ratios of the protein to peptides close to physiologic or therapeutic ranges. As the equilibrium constant is a ratio, so long as the concentration of both peptide and protein are increased equally, the ratio will be unchanged. While the purchase of significant quantities of FVIII is costly, this can be bypassed through the cloning of FVIII into a cell lineage that can synthesize the protein. The ability to clone the FVIII gene into Chinese Hamster Ovary cells was an important development in the study of FVIII as it allowed for the production and utilization of higher concentrations of FVIII than what was commercially available from plasma isolate [97]. By synthesizing larger quantities of FVIII, one could then perform the more common methods to measure protein-peptide interactions. Another option would be to use FVIII from another species, such as dogs. This animal model is known to have FVIII responses to dDAVP similar to that of humans and therefore canine FVIII may be a suitable and potentially cheaper option to utilize in future research [112],

An important future goal for this research will be the specific localization of the site of interaction. This could shed light on its therapeutic mechanism. One theory is that it stabilizes the A2 domain's interaction with the remainder of the HC and therefore allows FVIIIa to remain therapeutically active for a longer period of time. Another possibility is that the interacting peptide blocks specific areas on one of the chains necessary for proper interaction with LRPII and therefore delays its endocytosis and removal from circulation. In this thesis, the ability of the peptide to block antibody mediated loss of FVIII activity was used as an indirect method of assessing the binding location. This work utilized polyclonal antibodies however. Future work should utilize monoclonal antibodies in order to narrow down the region of binding. This can be done once it has been determined if the site is on the heavy chain or the light chain. Should there be insufficient monoclonal antibodies available to cover the entirety of the FVIII domains, another possibility could be chemical crosslinking experiments followed by enzymatic digestion and analysis via mass spectrometry. One concern however would be that there may be a certain degree of non-specific adsorption of the peptides to FVIII which would be also become cross-linked to the protein and subsequently interfere with the interpretation of the results. However, if it is assumed that each of the four peptides binds in the same specific location on FVIII, then there should be a pattern to the enzymatically generated protein fragments allowing for the recognition of the specific binding site. X-ray crystallography can also be performed in order to assess the site of binding, though again, this would require high concentrations of the protein and peptides [113].

The theory that the interaction of a small peptide with a small region of FVIII may induce radical changes in its half-life is not novel. A previous study has demonstrated that a synthetic peptide of 8 amino acids found within the acidic region of FVIII's LC, between residues 1670

and 1689, resulted in inhibition of vWF binding to FVIII, despite the large interface between the two proteins [114]. Similar studies have been published [115, 116]. The findings here suggested the possibility that the peptides were increasing the half-life of FVIIIa. However, the results obtained in this thesis suggested that the half-life of FVIII alone was longer than what had previously been published. There was no clear explanation for this discrepancy. An interesting method to study the possible prolongation of the FVIIIa half-life would be through Fluorescence Resonance Energy Transfer experiments utilizing the A1 and A2 domains as the exchanging pair. The native FVIII should show a decrease in the FRET signal of the FVIIIa that approximates the previously published literature as A2 dissociates from the complex, as the half-life of FVIIIa is determined to a certain extent by the spontaneous dissociation of the A2 subunit. Therefore, if the peptides were interfering with the spontaneous dissociation, the FRET signal should be retained for a longer period of time. Another interesting experiment would be to incubate the FVIIIa and peptides on one side of a dialysis membrane and measure the amount of A2 that crosses the membrane over time. If the peptides are increasing the half-life of FVIIIa, there should be a decreased rate of A2 appearance on the other side of the membrane. Finally, the life-span of FVIII is influenced by its endocytosis by the LRPII receptor proteins. For completion, one should address whether or not the peptides are influencing the clearance of FVIII by LRPII. This protein is commercially available and can be utilized in the activity kit experiments described herein, replacing the antibodies. Should the LRPII cause a loss of FVIII activity that can be rescued by the peptides, it would further support that it is through the interaction with FVIII, as opposed to another species within the matrix. Other possible experiments could be the equilibrium dialysis experiments, investigating the effect of the

presence of the peptide on the equilibrium concentration of LRPII across the dialysis membrane in the presence of FVIII alone and in the presence of FVIII pre-incubated with the peptides.

In conclusion, the clinical utility of AVP, dDAVP, oxytocin and carbetocin in the management of hemorrhagic conditions is apparent. The role of AVP and dDAVP in increasing FVIII concentration is also well-established. While the current line of thinking has resulted from the finding that the peptides cause a release of FVIII stores, the possibility that the peptides are also interacting with FVIII appears to have been overlooked. The results herein suggest that not only is there an interaction, but it is one that may directly increase the cofactor activity of FVIII. Given the significant morbidity and mortality associated with hemorrhaging, it is vital that these results be further validated and the interactions studied further as they may lead to important changes in the clinical roles of these drugs and be of benefit to an expanded patient population.

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Appendix A: Peptide Sequences

Arginine Vasopressin

Primary Structure: CYFQNCPRG

Molecular Weight: 1084.2316 g/mol

Desmopressin

Primary Structure: SCH2CH2CO-YFQNCP(d-R)G

Molecular weight: 1069.22 g/mol

Oxytocin

Primary Structure: CYIQNCPLG

Molecular weight 1007.18734 g/mol

Carbetocin

Primary Structure: YIQNCPLG

Molecular Weight: 988.16086 g/mol

Fibrinogen Binding Inhibitor Peptide

Primary Sequence: HHLGGAKQAGDV

Molecular Weight: 1189.28 g/mol