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The Development of a Novel Native Prothrombin Assay for the

More Efficient Management of Oral Anticoagulation Therapy

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the degree of Doctor of Philosophy

Abstract

Disturbances of the natural balance between pro-coagulant and anticoagulant systems, due to hereditary or acquired factors may result in haemorrhagic or thrombotic diseases.

Currently the INR/ISI coagulation monitoring system, introduced in 1983 by the World Health Organisation (WHO), is that of choice for most anticoagulation management clinics. Patients undergoing anticoagulation therapy must regularly attend specialised outpatient clinics for the close monitoring and maintenance of their INR. The automated laboratory caters for rapid online simultaneous analysis of multiple blood samples, resulting in the calculation of a patient's INR from the recorded prothrombin time. The insensitivity of the prothrombin time test has been well documented, requiring a reduction in the prothrombin concentration of 45 % prior to the materialisation of clinically significant prothrombin times.

The project aims to employ three avenues of biotechnology to aid in the development of an immuno or molecular imprinted polymer (MIP) based anticoagulation assay. The project will utilise computational molecular modelling in an attempt to visualise the tertiary structure of human prothrombin, which will allow the rational selection of antigenic sites for molecular imprinting or antibody production.

An aqueous, prothrombin-imprinted homo-polymer was grafted to the gold surface of a surface plasmon resonance biosensor (Biacore). The Biacore allowed the real time monitoring of imprinted polymer binding characteristics to

homogeneous protein solutions. As a direct comparison of two technologies, molecular imprinting and immuno technology, polyclonal antibodies showing specificity towards the same prothrombin antigen were immobilised onto Biacore chips. The imprinted polymer graft and polyclonal antibody based assays recognised homogeneous solutions of prothrombin at a concentration range of 0.01 nM to 14.2 nM and 0.01 nM to 0.5 nM respectively. A randomised preliminary clinical trial was initiated to compare the two assays' ability to differentiate plasma samples with a variety of INR values.

The results thus far show promise for the development of a new anticoagulation assay using molecularly imprinted polymer technology. The ultimate aim for this project is to develop a consistently more accurate point-of-care anticoagulation therapy monitoring kit, incorporating this new technology, which can replace or be used concomitantly with the INR/ISI system currently in use. This thesis raises more questions regarding the efficacy of oral anticoagulation therapy (OACT) and argues for and against the necessity of a novel OACT management assay.

Acknowledgements

I would firstly like to thank my PhD supervisor Professor Anthony Woodman for giving me the opportunity in the first place and for his support throughout. I would like to thank Professor Sergey Piletsky for his expert 'to-the-point' advice regarding MIP production (and life). I would like to thank Dr Kal Karim for his expert opinions and advice throughout the project. I would like to thank Dr Judith Taylor for her expert training on the Biacore and for reading some of my thesis Chapters even if they were out of context. Thanks go to numerous other Cranfield colleagues, too many to name.

I would like to acknowledge that this thesis is in part, dedicated to the memory of the late Dr Bob Dalton; this was his brainchild! Thanks go to Dr Gill Rouse, the staff who took part in plasma sample collection and preparation and to all the anonymous patients whose sample donation became integral to the project.

Thanks go to my parents who have always been there to say, "if you aim for number 1, you might not get there, but you'll get higher than you would if you aimed for number 2 or 3". Your support is always appreciated, and I know what you mean now!

Finally and most importantly to my fiancé Jo Simcox to whom this thesis is dedicated, you have always been there to provide your love, support and understanding, for which I am eternally grateful. I would not have achieved so much so quickly without your brilliant organisational skills. It will be my turn to provide the financial support soon!

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Coagulation Factors

Serine Proteases

Factor II	- Prothrombin
Factor VII	- Proconvertin
Factor IX	- Christmas Factor
Factor X	- Stuart-Prower Factor
Factor XI	- Plasma Thromboplastin Antecedent
Factor XII	- Hageman or Contact Factor
Prekallikrein	- Fletcher Factor

Cofactors

Factor III	- Tissue Factor or TF
Factor V	- Labile Factor
Factor VIII	- Antihaemophilic Factor
HMWK	- High Molecular Weight Kininogen or Fitzgerald factor
Factor XIII	- Transglutaminase

Amino Acids

Aliphatic and Hydroxyl Aliphatic

Ala (A)	- Alanine
Gly (G)	- Glycine
Val (V)	- Valine
Leu (L)	- Leucine
Ile (I)	- Isoleucine
Ser (S)	- Serine
Thr (T)	- Threonine

Acidic and Amino Derivatives

Asp (D)	- Aspartic Acid
Glu (E)	- Glutamic Acid
Asn (N)	- Asparagine
Gln (Q)	- Glutamine

Sulphur Containing

Cys (C)	- Cyteine
Met (M)	- Methionine

Aromatic

Phe (F)	- Phenylalanine
Tyr (T)	- Tyrosine
Trp (W)	- Tryptophan

Secondary Amine Group

Pro (P)	- Proline

Basic

Lys (K)	- Lysine
Arg (R)	- Arginine
His (H)	- Histidine

Other Abbreviations

AA	- Acrylamide
ACT	- Activated clotting time
AIDS	- Acquired immune deficiency syndrome
AMS/C	- Anticoagulation management services/clinics
APA	- Anti phospholipid antibody
APBA	- 3'-aminophenylboronic acid
APC	- Activated protein C
aPTT	- Activated partial thromboplastin time
Ba_2CO_3	- Barium carbonate
Ba_2SO_4	- Barium sulphate
BHT	- Butylated hydroxytoluene
BSA	- Bovine serum albumin
cAMP	- Cyclic adenosine monophosphate
Ca^{2+}	- Calcium II Ion

cDNA	-	Complementary DNA
DCM	-	Dichloromethane
DEAEMA	-	Diethyl aminoethyl methacrylate
DIC	-	Disseminated intravascular coagulation
DMEM	-	Dulbeccos modified eagles medium
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribo nucleic acid
DVB	-	Divinylbenzene
Ecar	-	Echis carinatus (Snake)
EDTA	-	Ethylenediaminetetraacetic acid
EGDMA	-	Ethylene glycol dimethacrylate
EGF	-	Epidermal growth factor
EIA	-	Enzyme immuno assay
ELISA	-	Enzyme linked immuno sorbent assay
FCS	-	Foetal calf serum
F1	-	Prothrombin fragment 1
F2	-	Prothrombin fragment 2
GCA	-	Giant cell arteritis
Gla	-	Gamma-carboxy glutamic acid
GLP	-	Good laboratory practice
G6PD	-	Glucose-6-phosphate dehyrogenase
HEMA	-	Hydroxyethyl methacrylate
HIV	-	Human immuno-deficiency virus
HPLC	-	High performance liquid chromatography
IA	-	Itaconic acid
IgG	-	Immunoglobulin G
INR	-	International normalised ratio
ISI	-	International standard index
ITP	-	Immune thrombocytopenic purpura
I.U.	-	Intravenous units
^{125}I	-	Iodine 125
LA	-	Lupus anticoagulant
LDL	-	Low-density lipoprotein
MAA	-	Methacrylic acid
MBAA	-	N,N′-methylenebisacrylamide
MCTD	-	Mixed connective tissue disease
MK	-	Menaquinone (Vitamin K)
MIA	-	Molecular imprinted assay
MIP	-	Molecular imprinted polymer
NAD(H)	-	Nicotinamide adenine dinucleotide (Hydrogen)
NADP(H)	-	Nicotinamide adenine dinucleotide phosphate (Hydrogen)
7'-OHC	-	7'-hydroxycoumarin
PBS	-	Phosphate buffered saline
PDB	-	Protein databank
PIVKA	-	Proteins induced by vitamin K absence
PMR	-	Polymyalgia rheumatica
PT	-	Prothrombin time
RA	-	Rheumatoid arthritis
RDA	-	Recommended daily allowance
RMC	-	Routine medical care
RO	-	Reverse Osmosis
SLE	-	Systemic lupus erythematosus

SOP	- Standard operating procedure
SPE	- Solid phase extraction
TAFI	- Thrombin activated fibrinolysis inhibitor
TFA	- Trifluoroacetic acid
TFPI	- Tissue factor pathway inhibitor
TGT	- Thromboplastin generation test
TIS	- Triisopropylsiilane
TRIM	- 1-(2-Trifluoromethyl phenyl) imidazole
TT	- Thrombin time
vWF/D	- von Willebrand factor/disease
VLDL	- Very low-density lipoproteins
WHO	- World health organisation
XaPC	- Factor Xa, phospholipid and calcium (prothrombin to thrombin)

Chapter 1

Review of the Literature

1.1 Haemostasis

The circulatory system is an independent organ within the body; it has to be self-sealing to prevent life threatening injury. Haemostasis comprises three independent processes and culminates in the prevention of substantial blood loss from the enclosed coagulation system.

1. Initially, platelets adhere to damaged blood vessels and to themselves to form a platelet plug, which can stem the flow of blood from the damaged area. This process is mediated by von Willebrand factor (vWF), a 10^4 kDa multimeric plasma protein comprising 225 kDa subunits, which binds to collagen, a specific platelet membrane receptor and to other components of the subendothelial membrane.

2. The platelet aggregation activates the release of physiologically active substances, such as serotonin (5-hydroxytryptamine) and thromboxane A_2, which stimulate vasoconstriction thereby reducing the flow of blood to damaged areas.

3. The aggregation of platelets and damaged endothelium initiate the major defence against bleeding, blood coagulation.

1.1.1 Von Willebrand factor

Von Willebrand factor is a 300-kDa polypeptide that is processed in the Golgi apparatus to approximately 250-kDa. These 250-kDa monomers can join together to form polymers of between 1000-kDa and 20000-kDa. After synthesis in the endothelial cells and the megakaryocytes, vWF is stored in the Weibel-Palade bodies and the α-secretory granules of platelets

respectively.   The smaller molecular weight vWF polymers are secreted constitutively into the circulation where they act as carrier proteins for factor VIII, protecting it from premature degradation ($^1/_2$-life with vWF=8-12 hrs, without vWF=2-3 hrs) and delivering it to the site of vascular injury. Thrombin activates breaks down the non-covalent interaction between factor VIII and vWF thereby allowing factor VIII to participate in the coagulation cascade on the platelet surfaces.

Apart from the carrier protein role it plays in haemostasis, vWF has several other important primary haemostatic roles:

- Platelet adhesion. VWF acts as a bridge or glue between the platelet receptor GPIb-IX and subendothelial collagen or elastin microfibrils to mediate the binding of platelets to the subendothelium in areas of high shear rate, i.e. microvasluature.

- Platelet spreading. VWF attaches to matrix proteins and the platelet integrin receptor GPIIb/IIIa on platelet surfaces, which promotes platelet spreading on the subendothelial matrix.

- Platelet aggregation. Essentially a fibrinogen role, however vWF binds GPIIb/IIIa on adjacent platelets, essentially acting as a bridge between platelets, important in afibrinogenemic patients.

- Platelet plug stabilization. This is done by vWF by binding to GPIb-IX on platelets and onto fibrin, therefore incorporating fibrin into the platelet plug.

Because of the close interaction of vWF and factor VIII, von Willebrand disease (vWD) shows similar bleeding characteristics to the haemophilia's.

However, vWD can be determined by measurement of the vWF activity (Ristocetin or Botrocetin), vWF antigen (vWF:Ag ELISA), factor VIII activity (one stage coagulation assay using FVIII deficient plasma) and bleeding time. Classification of the severity of the disease is achieved by determination of the multimeric composition of plasma vWF.

1.1.2 Platelets and the platelet plug

Circulating platelets are maintained in a non-reactive state by three methods.

- Aggregation inhibition compounds, such as nitric oxide and prostacyclin are released from endothelial cells.

- The negatively charged surface of platelets prevents their interaction with erythrocytes, leucocytes and the vascular endothelium.

- The intact endothelium provides a barrier to the platelets, preventing their interaction with the adhesive substrates in the subendothelial connective tissue matrix, i.e. collagen.

Endothelial disruption, by trauma or disease allows platelets to come into contact with and adhere to the subendothelial matrix. Platelets tend to adhere to any rough surface, such as glass, the exposed ends of injured blood vessels or particularly to the collagen in the subendothelial layer surrounding the blood vessel. Platelets are an integral part of the haemostatic process as they initiate via adhesion to the vessel wall, they form the platelet aggregate and they provide the activated surface for the acceleration of coagulation, culminating in the stabilisation of the fibrin/platelet aggregate. These processes are regulated by the platelet surface located

glycoprotein receptors, namely GP-1b-IX-V, which through its interaction with vWF mediates the initial deposition of platelets on the subendothelium (Andrews *et. al., 1997*).

1.1.3 Integrins

Integrins are extracellular, transmembrane bound, heterodimeric glycoprotein receptors that are of crucial importance in the correct functioning of inter and extra-cellular communication and interaction. They are comprised of one alpha and one beta subunit out of 24 and 9 subunits respectively. Integrin ligand specificity varies partly due to the large number of different alpha and beta subunits, which creates a large amount of functional redundancy, and also due to alternative splicing of integrin messenger RNA. Diffuse distribution of integrins over the cell surface prevents ligand binding; adhesion occurs due to a stimulus (thrombin or adrenalin) that promotes the migration of integrins to a hemidesmosome or focal contact point on the cell surface (*Luscher and Weber, 1993*). The low binding affinity (10^6 – 10^9 litres mol^{-1}) and the migration of integrins to a focal contact promotes multiple weak interactions, which allow strong binding for the cell to remain in contact with the extracellular matrix; but also weak enough to allow appropriate cellular flexibility. Common ligands for integrins include fibronectin and laminin, which are both part of the extracellular matrix, they are both recognised by multiple integrins. In the platelets resting state the integrin GP-IIb-IIIa binds fibrinogen only to a limited extent (Kieffer *et. al., 1992*), however when agonists (such as thrombin) activate the platelet, the integrin receptor changes conformation and binds fibrinogen, vWF and fibronectin (Sims *et. al., 1991*). There is a

clustering effect of the GP IIb/IIIa fibrinogen receptor, which inevitably causes an aggregation of fibrinogen on the platelet surface; this has been seen using immunocytochemical (Hourdille *et. al.*, 1985) and electron microscopy (Suzuki *et. al.*, 1991; Painter *et. al.*, 1985) methods.

1.1.4 Biochemical coagulation cascade

Blood coagulation involves the proliferation of a biochemical cascade of circulating proteolytic enzymes and cofactors. This process culminates in the cleavage of thrombin from prothrombin by a complex comprising of activated factor X and V with calcium on phospholipid surfaces. The formation and stabilisation of the platelet aggregates at the site of injury with fibrin monomers occurs due to the action of the activated Factor Xa/Va complex. Thrombin is a serine protease that converts soluble plasma fibrinogen into fibrin, which in turn converts the unstable primary platelet occlusions into firm, stable haemostatic plugs at the sites of cellular and/or vascular damage. Disturbances of the natural balance between the procoagulant and anticoagulant systems due to genetic or an acquired factor usually results in varying severity of bleeding or thrombotic diseases (Dahlback, 2000).

Circulating proteolytic enzymes involved in the coagulation cascade include serine proteases such as factor II (Prothrombin), factor VII (proconvertin), factor IX (Christmas factor), factor X (Stuart-Prower factor), factor XI (plasma thromboplastin antecedent), factor XII (Hageman or contact factor) and Prekallikrein (Fletcher factor). All have serine at their active centre, which confers the ability to hydrolyse peptide bonds. Cofactors include tissue factor (TF or factor III), factor V (labile factor), factor VIII (antihaemophilic factor) and

high molecular weight kininogen (HMWK or Fitzgerald factor). Factors VIII and V are involved in the amplification of the cascade, once activated by thrombin the cofactors amplify the cleavage of factor X and prothrombin respectively. Factor XIII is a transglutaminase, which when activated (XIIIa) by thrombin acts to stabilise fibrin polymers by forming covalent cross-links between glutamine residues.

Initiation of the cascade *in-vivo* occurs through the exposure of TF to blood due to cellular and/or vascular damage, the extrinsic pathway. The serine protease factor VIIa (a small fraction circulates as the active form in the blood) when incorporated into a complex with TF and Ca^{2+} on a phospholipid surface activates factor X. Factor Xa alone triggers the small-scale cleavage of thrombin as well as the promotion of factor IX activation in the intrinsic pathway. Thrombin now has several major roles in the completion and regulation of the cascade:

1. Thrombin cleaves fibrin subunits A and B from fibrinogen.

2. Thrombin activates factor XIII, the transglutaminase that converts the immature platelet aggregate into a stable haemostatic plug.

3. Thrombin also has a positive feedback effect on the cascade by activating co-factors V and VIII. The prothrombinase and the tenase complexes, factors Va/Xa and factors VIIIa/IXa respectively, together with Ca^{2+} on the phosphatidylserine-phospholipid surface, amplify the cascade by promoting further cleavage of prothrombin and the activation of factor X.

4. Thrombin also has a stimulatory role in the promotion of factor XI activation in the intrinsic or contact pathway. This pathway assumes the dominant role in the development of the stable fibrin clot.

5. Thrombin also has an anticoagulant role in an intact vascular system, as it binds to thrombomodulin, which activates protein C (Dahlback, 2000).

Contact reactions between HMWK and kallikrein promote the activation of factor XII; factors XI and IX are subsequently activated. Factor IXa promotes the activation efficiency of factor VIIIa, which further activates factor X. The coagulation process is further regulated using certain physiological protective mechanisms.

Circulating plasma inhibitors bind to active serine proteases, such as thrombin, to localise their effect to the site of injury. Antithrombin (or antithrombin III), the most potent plasma inhibitor binds to free serine proteases i.e. those that have strayed from the site of thrombus formation, forming a stable high molecular weight complex, which eliminates their proteolytic effect (Rosenberg and Damus, 1973). Antithrombin is in itself a relatively inefficient protease inhibitor therefore the heparin-like proteins on the endothelial cell surface potentiate antithrombin's effect markedly as does heparin itself (Triplett, 2000; Dahlback, 2000; Hoffbrand *et al.*, 1993). Other inhibitors include heparin cofactor II (accentuated by dermatan sulphate), α_2-macroglobulins, α_2-antiplasmin and α_2-antitrypsin (Triplett, 2000; Hoffbrand *et al*, 1993), which create inactive, stable, high molecular weight complexes with the active serine protease using peptide bonding.

Circulating plasma inhibitors of factors V and VIII also work to control blood coagulation (Hoffbrand *et al.*, 1993). Thrombomodulin is an endothelial cell surface receptor, which when complexed with thrombin activates the vitamin K dependent serine protease protein C. Activated protein C (APC) has the ability to modulate the activity of factors Va and VIIIa when associated with another vitamin K dependent protein; protein S attached to the platelet cell surface. APC and protein S form the membrane bound complex, which cleaves factors Va and VIIIa, even when they are elements of the prothrombinase and tenase active complexes. Only 30% of plasma protein S is free, the rest is associated with the complement regulatory protein C4b-binding protein.

Factor	Half Life (Hours)
II (Prothrombin)	60
VII	4-6
IX	24
X (Stuart Prower Factor)	48-72
Protein C	~8
Protein S	~30

Table 1.1.1 represents the plasma half lives in hours of some of the major coagulation factors; prothrombin has the longest plasma half-life (adapted from Erban, 1999).

The APC pathway is not so straightforward. Factor VIII and VIIIa are complexed with Von Willebrand factor or in the tenase complex with factor IXa. Whereas factor Va as well as factor V, binds to phospholipids allowing APC to cleave the intact form of factor V. Factor V is cleaved by APC and functions in synergy with protein S as an anticoagulant cofactor to APC in the cleavage of factor VIIIa from its tenase complex. Therefore factor V has both procoagulant and anticoagulant properties, and is thought to be an important factor in the regulation of the tenase complex. Homozygous deficiency in the APC pathway leads to severe even lethal thrombotic disease shortly after birth in both mice and humans. Mice lacking normal thrombomodulin gene functionality have a more severe disease and die during embryogenesis before the functional cardiovascular system has fully developed (Dahlback, 2000).

Figure 1.1.1 the human blood coagulation cascade (Adapted from Hoffbrand *et al.*, 1993)
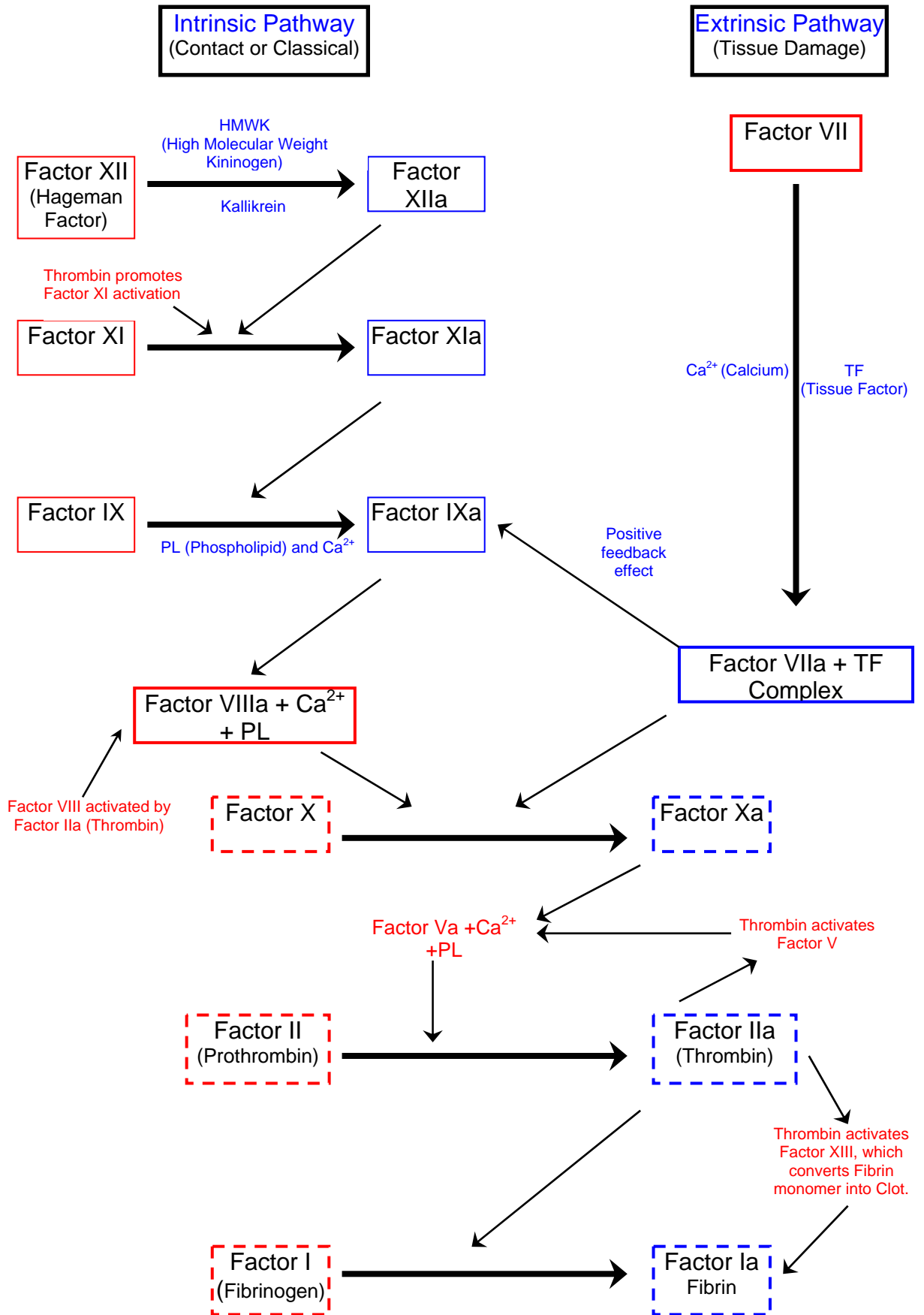
Tissue factor pathway inhibitor (TFPI) can also regulate the *in-vivo* pathway by binding to factors Xa and VIIa (Hoffbrand *et al.*, 1993; Triplett, 2000; Dahlback, 2000). TFPI is mostly bound to LDL in plasma or to heparan sulphate when interacting with the endothelial cell surfaces. There is no evidence available, which suggests that lack of TFPI activity is compatible with human life, this is supported by the fact that TFPI-gene knockout mice have a lethal phenotype (Dahlback, 2000). Therefore, TFPI is an important factor in the regulation of the intrinsic pathway and coagulation.
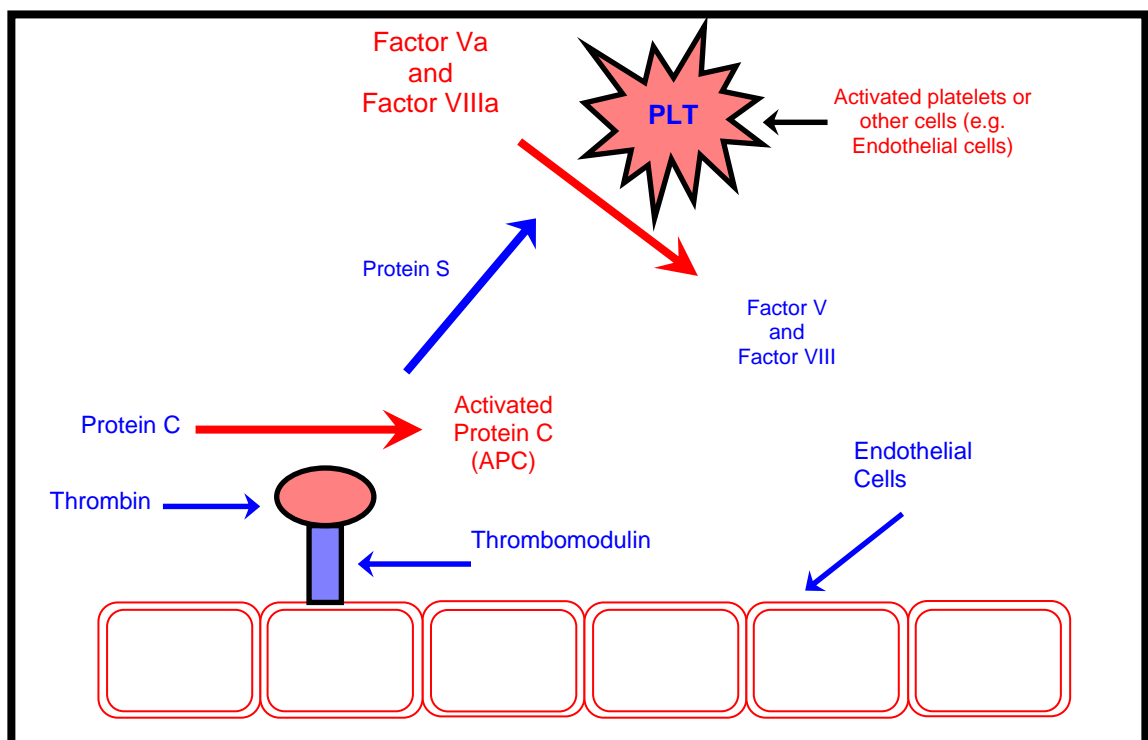
Figure 1.1.2 natural down regulation of activated factors V and VIII by the protein C and S system (Adapted from Hoffbrand *et al.*, 1993)

Blood flow is another physiological mechanism that regulates coagulation; the rapid dilution of activated factors is achieved before fibrin production away from the site of thrombus formation. Liver parenchymal cells, kupffer cells and other reticuloendothelial cells degrade activated factors and remove circulating particulate matter, such as platelet aggregate (Hoffbrand *et al.*, 1993). Many of the factors and cofactors in the coagulation cascade have similar structural and biological properties. Some of the serine proteases e.g.: factors II, VII, IX, X, protein C and protein S are dependent on vitamin K for their activity. Vitamin K is responsible for the post-translational carboxylation of amino-terminal glutamate residues, which allows the vitamin K dependent proteins to bind Ca^{2+}. This is important for the correct folding of the 'Gla' domain (Dahlback, 2000) and the localisation and complexation on platelet surface phospholipid. Termed the Gla region, the terminal carboxyglutamyl residues are essential to the coagulation factors' orientation and concentration within the cascade. A major biochemical abnormality with patients deficient in vitamin K metabolism is the minimal rate of prothrombin to thrombin conversion.

Other important inter-factor structural homologies within the cascade, especially the serine proteases, include epidermal growth factor (EGF) like kringle domains and aromatic amino acid stack domains, which function to localise and orientate the proenzyme prior to activation. Structural and functional regions of prothrombin and similar coagulation factors will be discussed later in the report.

Disturbances of the natural balance between pro-coagulant and anticoagulant systems due to hereditary or acquired factors may result in haemorrhagic or thrombotic diseases.
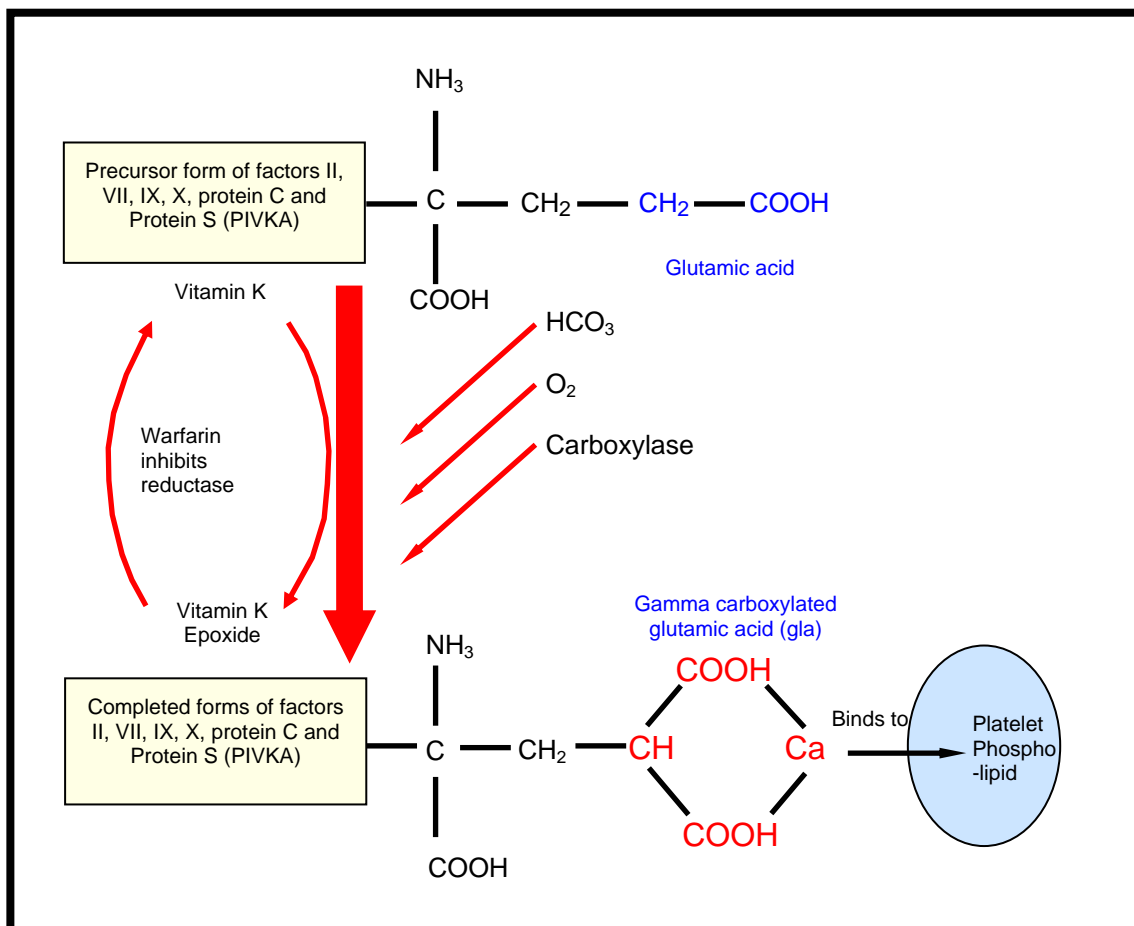
Figure 1.1.3 shows the action of Vitamin K in the γ-carboxylation of glutamic acids in coagulation factors, which are then able to bind calcium and attach to the platelet phospholipid.

1.2 Prothrombin and thrombin

Prothrombin (P00734) is a single chain glycoprotein, which plays a central role in the mammalian blood coagulation cascade, both stimulative and suppressive. Having a molecular weight of 72,000 Da, prothrombin is synthesised in the liver and has a normal plasma concentration in the range 0.11-0.23 mg ml^{-1}. Thrombin (EC 3.4.21.5) has a molecular weight of 39,000 Da is composed of two polypeptide chains A and B, 49 and 259 residues in length respectively, which are held together by a single disulphide bond. Prothrombin and the enzymatically active thrombin have diverse roles, which include the stimulation of platelets to change shape and the regulation of endothelial and other cell proliferation. Thrombin is involved in the haemostatic inflammatory and proliferative responses to wounding, indicating a prominent role in the mediation of wound healing. Prothrombin is also a chemotaxic agent and has been shown to be mitogenic towards macrophages and monocytes, also able to increase DNA synthesis of such cells. The addition of thrombin to monocytes has been shown to increase the cytosolic Ca^{2+} and promote the rapid cytoskeletal association of cytosolic actin; both are initial signs characteristic in chemotaxic stimulation of inflammatory cells (Degen *et al.*, 1983). Thrombin's procoagulant roles include proteolytic cleavage of fibrino-peptides A and B from fibrinogen, the stimulation of factors V and VIII and the activation of factor XIII, which initiates cross-linkage of fibrin clot and platelet aggregation (chemotaxis) (Degen and Sun, 1998).

The proteolytic conversion of prothrombin to thrombin is catalysed by the prothrombinase enzyme complex, which comprises the serine protease activity of factor Xa, its cofactor Va, phospholipids and Ca^{2+}. The reaction splits prothrombin into three fragments: fragment 1 (F1) (consisting of the Gla domain and kringle 1), fragment 2 (F2) (consisting of kringle 2) and the catalytic domain (thrombin). Intermediate fragments such as prethrombin 1 (also termed meizothrombin) and prethrombin 2 (single chain thrombin) are produced during the proteolytic cleavage of prothrombin. Prethrombin 1 remains after the cleavage of fragment 1 from prothrombin, further proteolytic cleavage produces prethrombin 2 and fragment 2. The cleavage of prethrombin 2 between residues 320 and 321 (prothrombin numbering) liberates disulphide bonded, double chain thrombin (Butowski *et al*., 1977). Studies have suggested that the interaction of F2 with factor Va is necessary for the enhancement of its catalytic efficiency within the prothrombinase complex (Kotkow *et al*., 1994), however it has recently been shown that this is not the case (Liaw *et al*., 1998). The proposed function of F2 in prothrombin is the alteration of proenzyme conformation to allow factor Xa easier access to prothrombins scissile bonds (Liaw *et al*., 1998). Thrombin interacts with and binds to several macromolecules already present in plasma including fibrinogen, the PAR-1 receptor, thrombomodulin, thrombin activated fibrinolysis inhibitor (TAFI), heparin cofactor II and anion binding exosites I and II. PAR-1 is a seven-transmembrane spanning G-coupled protein receptor to which thrombin binds and is located in the cellular membranes of platelets, endothelial cells and nervous system cells. The activation of PAR-1 through the specific proteolytic cleavage of an extracellular (basic) peptide bond allows circulating thrombin to mediate its procoagulant haemostatic

responses. Thrombomodulin is an endothelial membrane receptor that mediates thrombin's anticoagulant response, through the activation of protein C in the presence of protein S (Degen and Sun, 1998).

Vitamin K is required for the synthesis of the active protein; its oxidation and reduction pathway provides the microsomal carboxylase activity (***Figure 1.4.3, Page 36)*** that enables the correct synthesis of prothrombins Gla (γ-carboxyglutamic acid) domain (***Figure 1.4.2, Page 35***). Mature human prothrombin is 8 % carbohydrate and contains 579 amino acids, 10 of which are γ-carboxylated glutamyl (gla) residues. Protein sequence analysis and the analysis of cDNA clones enabled groups such as Magnusson *et al.*, Butowski *et al.*, Degen and Sun, and MacGillivray and Davie to deduce the entire primary sequence of human prothrombin (Degen and Sun, 1998; MacGillivray and Davie, 1984; Degen *et al.*, 1983; Butowski *et al.*, 1977; Magnusson *et al.*, 1975). Mature prothrombin contains several tertiary structure functional domains, which include the Gla domain, two kringle structures and a serine protease domain. The gla domain in prothrombin is required for its correct binding to Ca^{2+} ions and hence its localisation to phospholipid surfaces (Li *et al.*, 1995), therefore in the absence of vitamin K prothrombin circulates in an under/non-carboxylated form with reduced, often minimal functionality. There are regions of homology in other vitamin K dependent proteins containing between 8 and 13 gla residues (see ***Figure 1.4.2***), the Gla domains of such haemostatic proteins have been shown to possess phospholipid binding properties (Kotkow *et al.*, 1995).

The kringle domains, of which there are two in prothrombin, consist of approximately 80 amino acids with internal homology around three disulphide bonds. Kringle domains, first discovered in prothrombin, are also present in other proteins and exhibit homologies of 50 % and above. The primary role of the kringle domain appears to be that of interaction with proteins (Kotkow *et al.*, 1995), cofactors, substrates or receptors, the second kringle in prothrombin binds to factor Va (Degen and Sun, 1998). The catalytic serine protease domain, which shows homology to trypsin is located in the carboxy-terminal of prothrombin and is activated by factor Xa. Thrombin is liberated from the Gla and kringle-containing amino-terminal of prothrombin, which stays attached to the phospholipid membrane. The active site of thrombin is composed of the amino acid triad His-363 (H), Asp-419 (D) and Ser-525 (S) as illustrated in ***Figure 1.2.1***; the serine protease activity allows cleavage at basic amino acids (Degen and Sun, 1998).

The anion binding exosites are areas of positively charged amino acids located on the protein surface some distance away from the active site. Anion binding exosite I is believed to be responsible for the substrate specificity of thrombin. Anion binding exosite II is responsible for heparin binding and for thrombin's localisation to specific areas on cell surfaces, whilst keeping the active site available to substrates, cofactors and inhibitors (Degen and Sun, 1998). Conformational changes, which alter the catalytic environment at thrombins active site and anion binding exosite 1, develop through an interaction with prothrombin fragment 2. F2 binding also inhibits heparin catalysed thrombin inactivation by antithrombin four-fold; it enhances the esterolytic activity but also inhibits the clotting activity of thrombin (Liaw *et*

al., 1998). The amino acid sequence DGDEE, residues 68-72 of the inner loop of the kringle domain of prothrombin fragment 2 forms non-covalent salt bridges with Arg residues in thrombins heparin binding region. Co-crystallisation studies have shown that the complete kringle structure is not necessary for the above interaction, the binding domain has been localised to the inner loop (residues 64-93) and a terminal carboxy connecting peptide (residues 94-116) (Liaw *et al.*, 1998).
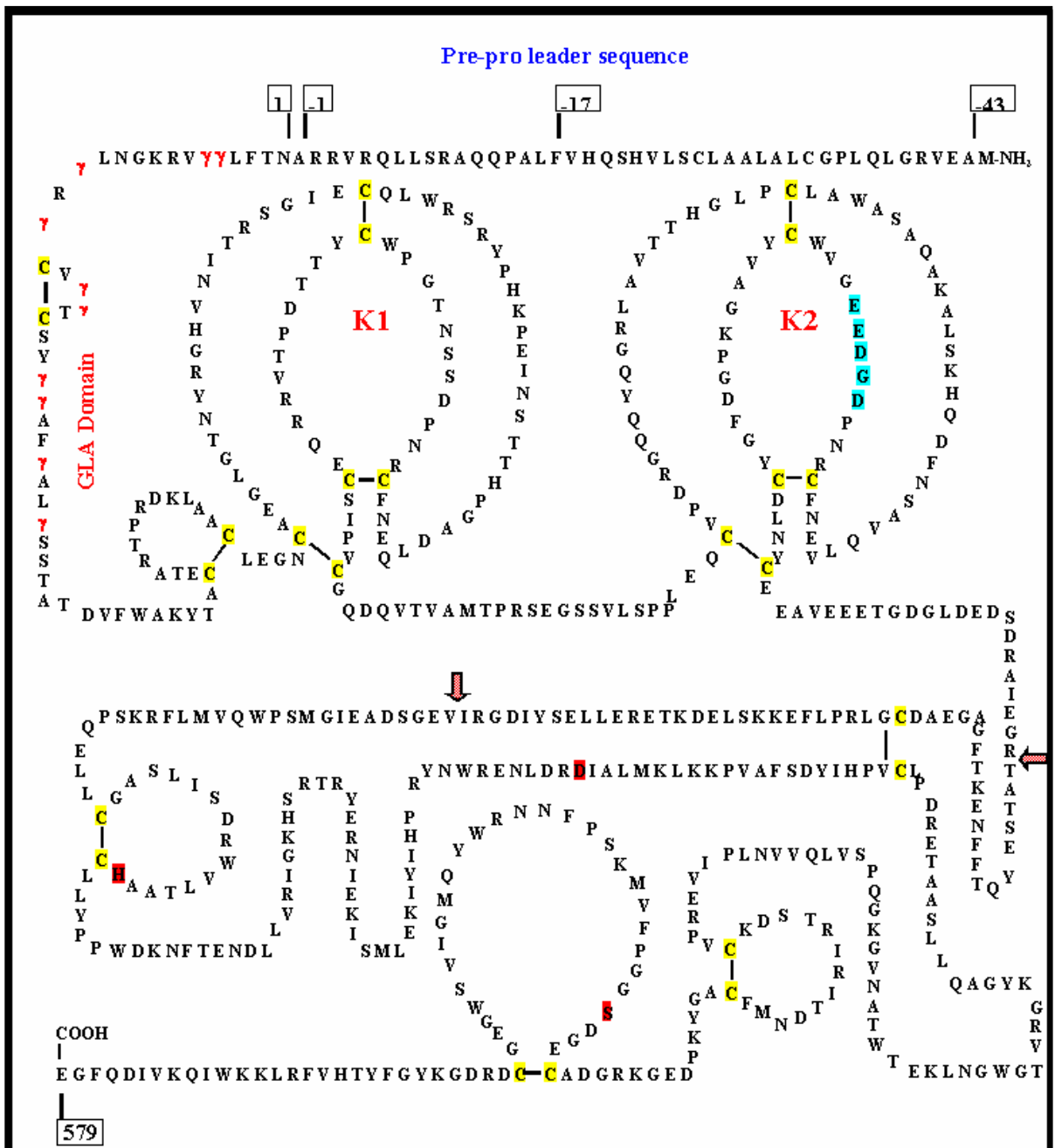
Figure 1.2.1 is a schematic diagram showing the primary structure of human prothrombin. The Gla domain contains 10 γ-carboxyglutamate residues. The kringle structures are labelled K1 and K2, the sequence DGDEE is highlighted in kringle 2 to show the region that forms salt bridges with thrombins heparin binding region (not shown). Cysteine residues are highlighted to show 12 disulphide bonds, 3 in each kringle, 2 in fragment 1, 3 in thrombin chain B and 1 holding thrombin chain A to B. The active site amino acid triad H-363, D-419 and S-525 is labelled as shown. Factor Xa cleavage sites are marked with ⬅. (Data adapted from Degen and Sun, 1998).

1.3 Diseases of haemostasis

1.3.1 Hereditary haemorrhagic diseases

Haemophilia A is one of the most common hereditary haemostatic disorders, it is a bleeding disorder which has a UK population incidence of 1 in 10, 000 (Peyvandi *et. al.*, 2002). Although showing sex-linked inheritance, 33% of patients do not show any family history. The defect manifests itself through the absence or reduced levels of plasma factor VIII, which normally combines with factor IXa on the phospholipid surface together with Ca^{2+} to propagate the activation of factor X and the common coagulation pathway (See *Figure 1.1.1*).

Clinical features of the disease include recurrent painful haemarthroses and muscle haematomas in severely affected individuals becoming painful and destructive if not treated (Bolton-Maggs and Pasi, 2003). Haematuria is more common than gastrointestinal bleeding; dental extraction and post-operative trauma can often cause prolonged bleeding occurrences in mildly and severely affected patients (Hoffbrand *et al*, 1993), consequently intra-cerebral and intra-cranial haemorrhage can account for 25% of haemophiliac morbidity. The disease correlates well with the extent of coagulation protein deficiency where factor VIIIa activities vary from <1% to 20% of the normal activity. The severity of the disease is maximal when the activity of factor VIIIa is <1%, large gene deletions or other gene inactivation mutations must predominate. Milder forms of the disease are characterised by factor VIIIa activities between 1%-5% and 5%-20% (Hoffbrand *et al.*, 1993).

Coagulation products are used to control haemorrhages in haemophiliacs, concentrates of factors VIIIa and IX are produced from donated blood for transfusion. However many haemophiliacs show clinical features of hepatitis C and some infected adults show slow progression to cirrhosis of the liver (Makris *et. al.* 1996; Lee and Dusheiko 2002; Goedert *et. al.* 2002). Due to infected blood and coagulation products from the late 1970s early 1980s, it is estimated that up to 50% of haemophiliacs treated in the USA and Europe have HIV and hepatitis antibodies in their blood. Therefore, AIDS is now thought to be responsible for a large proportion of haemophiliac morbidity, in the UK between 1970 and 1985 more than 1200 individuals were infected with HIV through blood transfusions. More than half of the patients infected between '79-'85 have now died but no new transfusion borne HIV infections have been detected since 1986 (Bolton-Maggs and Pasi, 2003). Donor testing and two viral inactivation steps during concentrate preparation and the use of genetic engineering to produce inherently infection-free recombinant coagulation concentrates now prevent HIV and hepatitis infection during therapy (Hoffbrand *et al.*, 1993). There is suspicion that the variant Creutzfeldt-Jakob disease could be transmitted through infusion of plasma products, however there has not been any evidence of such transmission to date.

Haemophilia B is a similar disease of coagulation affecting factor IX, it is also known as Christmas disease or just factor IX deficiency. The clinical features of the disease are almost identical to haemophilia A; however, the incidence of affected people is only 1 in 50, 000 (Peyvandi *et. al.*, 2002).

Von Willebrand disease (vWD) is a very common hereditary haemostatic disorder showing autosomal dominant inheritance; its prevalence in the general world population is estimated to be 1% (Rodeghiero, 2002). Pre-pubertal clinical presentations include bruising and epistaxis (nosebleeds). Epistaxis decreases during and after puberty however females can become menorrhagic, which can often be incorrectly diagnosed as uterine cancer. Better patient management and earlier diagnoses will prevent the need for unnecessary hysterectomies.

vWD affects the multifunctional multimeric protein von Willebrand factor (vWF), which promotes platelet-to-platelet cohesion under high shear stress and the adhesion of platelets to exposed endothelial surfaces. The A1 domain of vWF contains binding sites for the Platelet Glycoprotein 1b along with binding sites for the snake venom protein botrocetin, heparin and minor binding sites for collagen (Sadler *et. al.* 2000). Platelet Glycoprotein IIb-IIIa on activated platelets interacts with an RGDS sequence at the carboxyl-terminus of the C2 domain of vWF. The 2050 amino acid mature vWF also prevents the premature degradation of factor VIII prior to its activation and complexation with factor IXa; therefore the activity of factor VIIIa is also compromised in vWD patients (Rodeghiero, 2002). The low factor VIIIa and vWF activities combined with a lack of platelet adhesion causes varying severity of bleeding, mainly due to post-traumatic and operative haemorrhages.

As with haemophilia A, there are three distinguishable states of disease severity, termed types I, II and III; type III presenting as the most severe form

of the disease. Type I vWD is the most predominant form of the disease, generally comprising between 60-80% of all vWD cases (Sadler *et. al.*, 2000). Type I vWD is characterised by a partial, quantitative deficiency of vWF, nevertheless the distribution of vWF multimers appears normal or nearly normal and the residual plasma vWF appears to have normal activity.   A number of different subgroups of type I have been identified; these are based on the different levels of plasma and platelet vWF (Hoyer *et. al.*, 1983).

Type II vWD is concerned with qualitative defects of vWF and is divided in to four variants IIA, IIB, IIM and IIN. Type IIA vWD provides patients with an increased number of smaller vWF subunits with compromised functionality. There are two mechanisms that produce type IIA characteristic vWF multimers.   The mechanism of group 1 causes defective intracellular transportation together with impairment of assembly and storage of vWF multimers in the plasma and platelet compartments. Group 2 renders the vWF multimers ultra-sensitive to proteolysis in plasma, therefore transforming vWF into small non-functional vWF multimers (Reviewed in Sadler, 1994 and Sadler *et. al.* 2000).   The classification of group II vWD phenotypes is summarised in ***Table 1.3.1*** below.

Type 2A		Qualitative defects causing decreased platelet dependent function due to absence of HMW vWF multimers caused by their impaired synthesis or increased degradation.
Type 2B		Qualitative defects due to multimers possessing increased affinity for platelet glycoprotein Ib. VWF antigen usually higher than in IIA.
Type 2M		Multimer: Qualitative defects causing decreased platelet dependent function **not** due to lack of HMW vWF multimers. Possible mutations in vWF domain A1.
Type 2N		Normandy: Corresponds to all qualitative variants with markedly decreased affinity for factor VIII. Factor VIII binding site mutants!

Table 1.3.1 summarises the group II vWD subtypes and their corresponding defects. Type IIA vWD includes the previous type IIA category as well as types IB, IIC, IID, IIE, IIF, IIG, IIH, II-I, IIA-1, IIA-2 and IIA-3 (Reviewed in Sadler, 1994 and Sadler *et. al.* 2000).

Type 3 vWD is pathophysiolgically similar to vWD type 1 in that it is a quantitative defect. However, due to its symptoms being so much more severe and having much more distinct therapy requirements it was assigned to a completely different category. A total deficiency of vWF is the determining characteristic of type 3 vWD, whereby the vWF activity is reduced to less than 10%, which leads to spontaneous bleeding into joints and soft tissues. As in type 2N the absence of vWF-FVIII binding may introduce blood clotting as a secondary haemostatic defect in more severe cases. Its inheritance is usually autosomal recessive; hence symptoms are normally only present in homozygous or compound heterozygous patients, heterozygous relatives may present with normal or mildly reduced levels of vWF and thus asymptomatic or occasionally present with mild bleeding symptoms. In many cases of type 3 vWD patients develop alloantibodies to

vWF due to the vWF-containing plasma they are given; these antibodies render this therapy ineffective and can increase the risk of anaphylactic shock on introduction of therapy.

1.3.2 Acquired haemorrhagic diseases

Vitamin K deficiency may be caused by inadequate diet, malabsorption of Vitamin K in the gut or the use of coumarins and inanediones, which act as vitamin K antagonists during anticoagulation therapy. The disease is associated with the decrease in the functional activity of factors II, VII, IX, X and proteins C and S, however immunological methods show normal levels of these proteins in haemorrhagic patients. The non-functional proteins, termed PIVKA – Proteins Induced by Vitamin K Absence, are present due to the fact that absence of vitamin K inhibits the post-translational carboxylation of specific glutamate residues in the amino terminal domain of each protein. The carboxylation process involves the recycling of Vitamin K to vitamin K epoxide and back, using a carboxylase and reductases respectively. Warfarin is thought to interfere with the reduction of vitamin K epoxide, which produces a functional vitamin K deficiency, however this concept may need some revision (Hoffbrand *et al.*, 1993).

Haemorrhagic disease of the newborn is an acquired vitamin K haemorrhagic disease, which may manifest itself in the first 4 days of life. Contributing factors include poor placental transfer of vitamin K, immature liver cells, and a sterile gut, which precludes the utilisation of bacterial

sources of the vitamin and breast milk is also relatively deficient in vitamin K (Suttie, 1992).

Prophylaxis of 1 mg vitamin K (phytomenadione) is given intramuscularly to all newly born babies immediately after birth, except those likely to be glucose-6-phosphate dehydrogenase (G6PD) deficient. Bleeding infants are given 1 mg intramuscular vitamin K every 6 hours and initially fresh frozen plasma if bleeding is severe. Children and adults with obstructive jaundice (impaired absorption of vitamin K), pancreatic or small bowel disease may have resulting vitamin K deficiency, which may also increase the risk of haemorrhagic episodes (Hoffbrand *et al.*, 1993). Treatment is again with daily oral prophylaxis of vitamin K. The prothrombin time (PT) is prolonged in newly born and in adult cases, low plasma levels of the active vitamin K dependent factors would also be expected.

Liver disease is the broad term used to describe symptoms manifested due to disfunctionality of the liver. The liver is the largest gland in the body weighing in at approximately 1.2-1.6 kg. Being very complex it has many varied functions including the production and secretion of bile into the gall bladder and duodenum, the liver is also an important site for metabolism of carbohydrates, proteins and fats. The liver regulates the blood glucose levels converting excess glucose to glycogen, and removes excess amino acids by breaking them down into ammonia and finally urea. It is also the site of synthesis of fibrinogen and vitamin K dependent coagulation factors and heparins, as well as a site for metabolism and storage of vitamin K itself. Therefore, due to the liver's essential role in control of normal blood

coagulation, deficiency in all or part of its function leads to among others, coagulation disorders. Decreased synthesis of vitamin K dependent proteins may be brought about by biliary obstruction. In severe hepatocellular disease factor V and fibrinogen levels are depleted and there are increased levels of tissue plasminogen activator, leading to the suppression of normal coagulation processes.

Haemorrhagic complications may occur due to **overdose with oral anticoagulants**. These can be vitamin K antagonists and may result in severe deficiency of coagulation factors II, VII, IX and X. Poorly controlled heparin or systemic thrombolytic agent therapies may result in coagulation difficulties and subsequent bleeding episodes. Due to its poor preservation properties, massive transfusion of stored blood may serve to dilute the already depleted levels of platelets, coagulation factors and inhibitors. Platelets aggregate and function poorly after 24 hours at 4 ^{o}C, the labile coagulation factors V and VIII function poorly after 3-4 days storage. Minor activation of coagulation factors, microaggregates and degenerate cells all contribute to **massive transfusion syndrome**.

	Platelet Count	Prothrombin Time (PT)	Activated Partial Thromboplastin Time (aPTT)	Thrombin Time (TT)
Liver Disease	Low	Prolonged	Prolonged	Normal (rarely prolonged)
DIC	Low	Prolonged	Prolonged	Grossly Prolonged
Massive Transfusion	Low	Prolonged	Prolonged	Normal
Oral Anticoagulants	Normal	Grossly Prolonged	Prolonged	Normal
Heparin	Normal (rarely low)	Mildly Prolonged	Prolonged	Prolonged
Circulating Anticoagulant	Normal	Normal or Prolonged	Prolonged	Normal

Table 1.3.2 a summary of the typical haemostatic test results obtained in screening for acquired bleeding disorders (Taken from Hoffbrand *et al.*, 1993).

Thrombosis is the condition in which the blood changes from a liquid to a solid state producing a clot or thrombus (*pl.* thrombi). Pathogenic implications of abnormal thrombus formation arise due to narrowing (ischaemia) of blood vessels through local vascular obstruction or embolization and obstruction of a distal part of the circulation. Assuming that thrombosis forms a dominant role in the pathogenesis of conditions such as myocardial infarction, cerebrovascular disease and deep vein occlusion, thrombosis becomes one of the most important medical problems facing the western world today (Hoffbrand *et al.*, 1993). The incidence of thrombosis increases with age and is usually associated with certain risk factors such as, operations and pregnancy, although some thrombophilic individuals may develop thrombi at a younger age through acquired or hereditary predispositions. Multiple thrombi in the microcirculation result in the disorder known as disseminated intravascular coagulation (DIC) as discussed below.

The pathogenesis of thrombi in different parts of the blood system is similar, although formation of thrombi is different. In **arterial thrombosis** (atherosclerosis), thrombi develop from platelet reaction and aggregation due to vessel wall damage, atherosclerosis and plaque rupture. The major predictive risk factors related to the development of atherosclerosis are high plasma factor VII and fibrinogen levels, a positive family history and being male. **Venous thrombosis** is brought about due to the accumulation of thrombin in areas of lethargic blood flow, leading to coagulation of blood in these areas.

1.3.3 Acquired haemostatic/thrombotic diseases

Disseminated intravascular coagulation (DIC) (or intravascular coagulation and fibrinolysis (ICF) (Bowie *et al.*, 1983) is a condition resulting from over stimulation of the blood clotting mechanisms in response to disease or injury. Examples include severe infection, liver disease, promyelocytic leukaemia, asphyxia, hypothermia, *abrupto placentae* (premature separation of the placenta) and intra-uterine foetal death. Gram-negative and meningococcal septicaemia, certain virus infections (purpura fulminans) and severe burns cause widespread endothelial damage and platelet aggregation. Patients present with generalised coagulation throughout the blood system, especially in the small vessels, and excessive consumption of coagulation factors due to the release of procoagulant material into the blood stream, such as amniotic fluid, some snake venoms and mucins from adenocarcinomas. Inevitably, the consumption of platelets due to bleeding episodes associated with DIC leads to a reduction in blood platelet count or thrombocytopenia. Fibrinogen levels

in DIC associated patients are generally low, circulating fibrin monomers are produced by intravascular thrombin that form fibrinogen-fibrin complexes, which eliminates the fibrin monomers and fibrinogen from normal coagulation. Intravascular thrombin also causes intense fibrinolysis with the release of split products that interfere with fibrin polymerisation; these products may be detected in the blood serum and urine. The prothrombin time and aPTT are prolonged and the activities of factors V and VIII are reduced (Fruchtman *et al.*, 1986).

Coagulation deficiency caused by circulating antibodies is occasionally seen; IgG antibodies to factor VIII, which may produce a bleeding syndrome, are seen in 5-10 % of haemophiliacs. These antibodies are associated with certain immunological disorders such as rheumatoid arthritis and old age, but are seen rarely in newly born infants. The most common acquired inhibitor of coagulation is the *lupus anticoagulant* (LA), which is a member of the anti-phospholipid antibody (APA) family (Triplett, 2000). LA is detected in 10 % of patients with systemic erythematosus (SLE) and in patients with other autoimmune diseases frequently presenting with antibodies to other lipid containing antigens, e.g. cardiolipin (Hoffbrand *et al.*, 1993). Most APAs seen in the setting of infections have no clinical complications (Triplett, 2000), but a large percentage of patients with underlying autoimmune disease present with arterial and venous thrombotic complications, and recurrent spontaneous abortion in women (Triplett, 2000; Hoffbrand *et al.*, 1993). The laboratory diagnosis of LA is based on the presentation of the clinical complications mentioned above and three laboratory screening procedures to test for LA and/or anti-cardiolipin antibodies (Triplett, 2000).

Normal healthy people		1% - 14% (age related)
Infection		Syphilis, AIDS (93 %), Lyme disease (39 %), infectious mononucleosis (20 %), tuberculosis (20 %), Measles, chickenpox, mumps, hepatitis A
Drug induced		Phenothiazines, procainamide, chlorpromazine, oral contraceptives etc
Malignancy		Melanoma, renal cell carcinoma, lung carcinoma, lymphoma, and leukaemia
Autoimmune diseases		SLE (15-50 %), Sj★gren's syndrome (42 %), RA ($\leq$ 33 %), psoriatic arthritis (28 %), scleroderma (25 %), MCTD (22 %), GCA/PMR (20 %), Beh◇ets disease (0-50 %), ITP (30 %), juvenile chronic arthritis (55 %), haemolytic anaemia, idiopathic thrombocytopenic purpura, Crohn's disease
Neurologic diseases		Myasthenia gravis (68 %), multiple sclerosis (29 %), Guillain-Barr■ syndrome, transverse myelitis

Table 1.3.3 prevalence of antiphospholipid antibodies (Taken from Fessler, 1997): SLE = systemic lupus erythematosus, RA = rheumatoid arthritis, MCTD = mixed connective tissue disease, GCA = giant cell arteritis, PMR = polymyalgia rheumatica, ITP = immune thrombocytopenic purpura.

1.3.4 Hereditary haemostatic diseases

The prevalence of hereditary haemostatic disorders is at least as high as that of hereditary bleeding disorders. Young patients suffering from spontaneous thrombosis or recurrent deep vein thromboses may be diagnosed with hereditary or familial thrombophilia, usually without an easily identifiable underlying cause.

Antithrombin III deficiency was first recognised in 1965 as an autosomal dominant inherited disorder, further research has lead to the discovery of many different variants of the antithrombin III mutation. At a young age patients present with recurrent venous thromboses with arterial thrombosis

only occasionally.   Antithrombin III concentrates' are available for use to prevent thrombosis during childbirth or general surgery.

Protein C deficiency is arguably the most common form of hereditary thrombophilia, having autosomal dominant inheritance and variable penetrance, occasional homozygous individuals present with severe DIC or purpura fulminans in early infancy. Skin necrosis may develop in the first two days of warfarin therapy in some patients. This is believed to be due to further reduction in plasma protein C concentration, before the levels of vitamin K dependent coagulation proteins are reduced under the influence of warfarin.

Factor V deficiency, Leiden gene mutation is a very common cause of increased risk of thrombosis responsible for approximately 20 % of all cases. The factor V gene mutation confers a resistance to protein C inactivation upon factor V (Hoffbrand *et al*., 1993), which leads to the uncontrolled propagation of the coagulation cascade.

Protein S deficiency also autosomal dominant in its inheritance has been recognised in some families with thrombophilia. Abnormal fibrinogens and plasminogens have also been recognised as being causative of rare hereditary or acquired thrombophilia, as well as elevated levels of fibrinogen and factor VII.

1.4 Vitamin K

1.4.1 The Quinones

Henrik Dam; a Danish nutritional biochemist, discovered vitamin K in the 1930s as an anti-haemorrhagic agent capable of curing a certain bleeding disorder in chicks fed on a lipid free diet (Suttie, 1992). The majority of vitamin K in the human diet is contained in green plants and is in the phylloquinone (*Figure 1.4.1*) or vitamin K_1 form, vitamin K_2 or menaquinone (*Figure 1.4.1*) is bacterially synthesised and comprises an unknown (but small) portion of the human diet. In 1970, Rietz and associates demonstrated the presence, in human livers, of menaqinone-7, 8, 9(2H), 9(4H), 10 and 11 in addition to phylloquinone; suggesting that the presence of vitamin K homologs within the mammalian liver reflects the dietary and the bacterial sources of the vitamin in the gut (Olson, 1984).

A: Phylloquinone - vitamin K_1
2-methyl-3-phytyl-1, 4-napthoquinone

B: Menadione - vitamin K_2
2-methyl-1, 4-napthoquinone
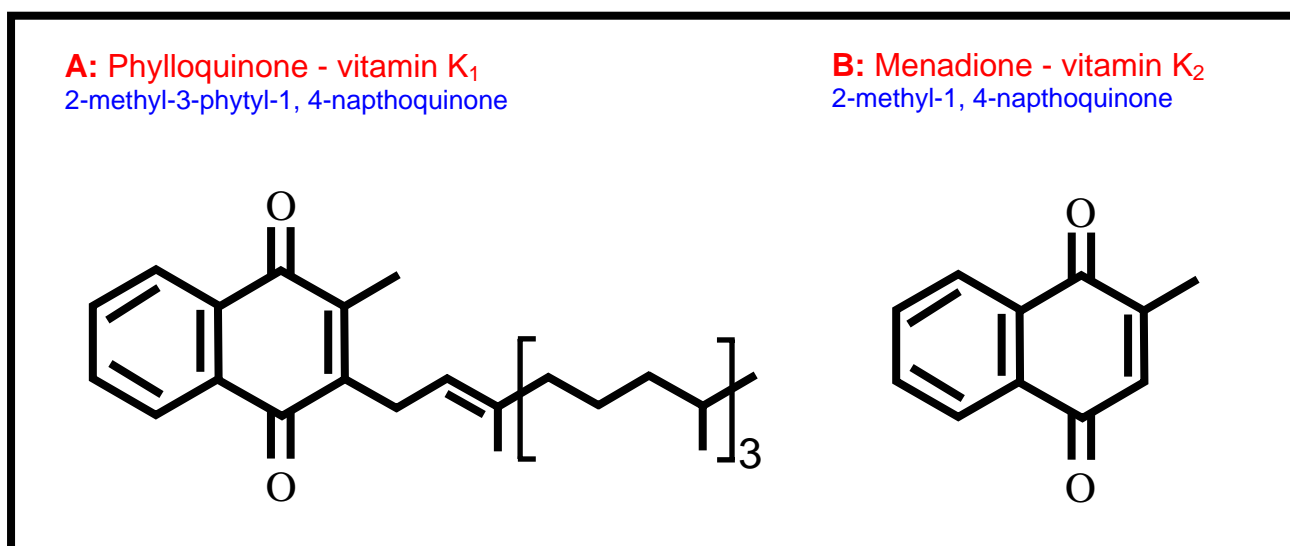
Figure 1.4.1 shows the structures of the biologically active forms of vitamin K, phylloquinone from green plants and menadione from bacteria (now used only in animal feed). Vitamin K_2 was originally characterised as a series of multiprenyl menaquinones with unsaturated side chains (structures taken from Suttie, 1992).

The biochemical function of vitamin K was not revealed until the 1970s when it was discovered that a dietary lack of vitamin K not only led to the increased dysfunction of prothrombin but of clotting factors VII, IX and X. Investigations also led to the discovery of the previously uncharacterised amino acid γ-carboxyglutamic acid or gla, which arises through a vitamin K dependent post-translational carboxylation of glutamic acid residues through the action of a liver microsomal carboxylase enzyme. There are 10 gla residues present in the amino terminal of factor II and VII, 11 gla residues in the homologous region of factor X and there are 12 gla residues present in the N terminal region of factor IX, these regions are illustrated in *Figure 1.4.2*.

	1				5					10					15					
Prothrombin	Ala	Asn	Lys	Thr	Phe	Leu	Gla	Gla	-	Val	Arg	Lys	Gly	Asn	Leu	Gla	Arg	Gla	Cys	Val
Factor X	Ala	Asn	Ser	Phe	Phe	Leu	Gla	Gla	-	Lys	Lys	-	Gly	His	Leu	Gla	Arg	Gla	Cys	Met
Protein C	Ala	Asn	Ser	-	Phe	Leu	Gla	Gla	-	Leu	Arg	Pro	Gly	Asn	Val	Gla	Arg	Gla	Cys	Ser
Factor IX	Tyr	Asn	Ser	Gly	Lys	Leu	Gla	Gla	Phe	Val	Gln	-	Gly	Asn	Leu	Gla	Arg	Gla	Cys	Met
Factor VII	Ala	Asn	-	Ala	Phe	Leu	Gla	Gla	Leu	Arg	-	Pro	Gly	Ser	Leu	Gla	Arg	Gla	Cys	Lys

	20				25					30					35					
Prothrombin	Gla	Gla	Thr	Cys	Ser	Tyr	Gla	Gla	Ala	Phe	Gla	Ala	Leu	Gla	Ser	Ser	Thr	Ala	Thr	Asp
Factor X	Gla	Gla	Thr	Cys	Ser	Tyr	Gla	Gla	Ala	Arg	Gla	Val	Phe	Gla	Asp	Ser	Asp	Lys	Thr	Asn
Protein C	Gla	Gla	Val	Cys	?	Phe	Gla	Gla	Ala	Arg	Gla	Ile	Phe	?	Asn	Thr	?	?	Thr	
Factor IX	Gla	Gla	Lys	Cys	Ser	Phe	Gla	Gla	Ala	Arg	Gla	Val	Phe	Gla	Asn	Thr	Gla	Arg	Thr	Thr
Factor VII	Gla	Gla	Gln	Cys	Ser	Phe	Gla	Gla	Ala	Arg	Gla	Ile	Phe	Lys	Asp	Ala	Gla	Arg	Thr	Lys

	40				45	
Prothrombin	Val	Phe	Trp	Ala	Lys	Tyr
Factor X	Gla	Phe	Trp	Asn	Lys	Tyr
Protein C						
Factor IX	Gla	Phe	Trp	Lys	Gln	Tyr
Factor VII	Leu	Phe	Trp	Ile	Ser	Tyr

Key:

Gla	Gamma – carboxyglutamic acid
Amino Acids	Regions of Homology
Amino Acids	Non Homologous regions
?	Amino Acid not known
-	No Amino Acid

Figure 1.4.2 shows the amino acid homology in the NH_2-terminal portions of human prothrombin, factor VII, IX and X and protein C. The numbering is that of prothrombin and the other proteins have been aligned to show the homology. The sequences of prothrombin, factor VII, X and IX were taken from the "ExPaSY SWISS-PROT" web site. Accession numbers for the proteins are prothrombin - P00734, factor VII – P08709, factor IX – P00740 and factor X – P00742. The sequence for protein C was taken from Stenflo *et al.*, 1977.
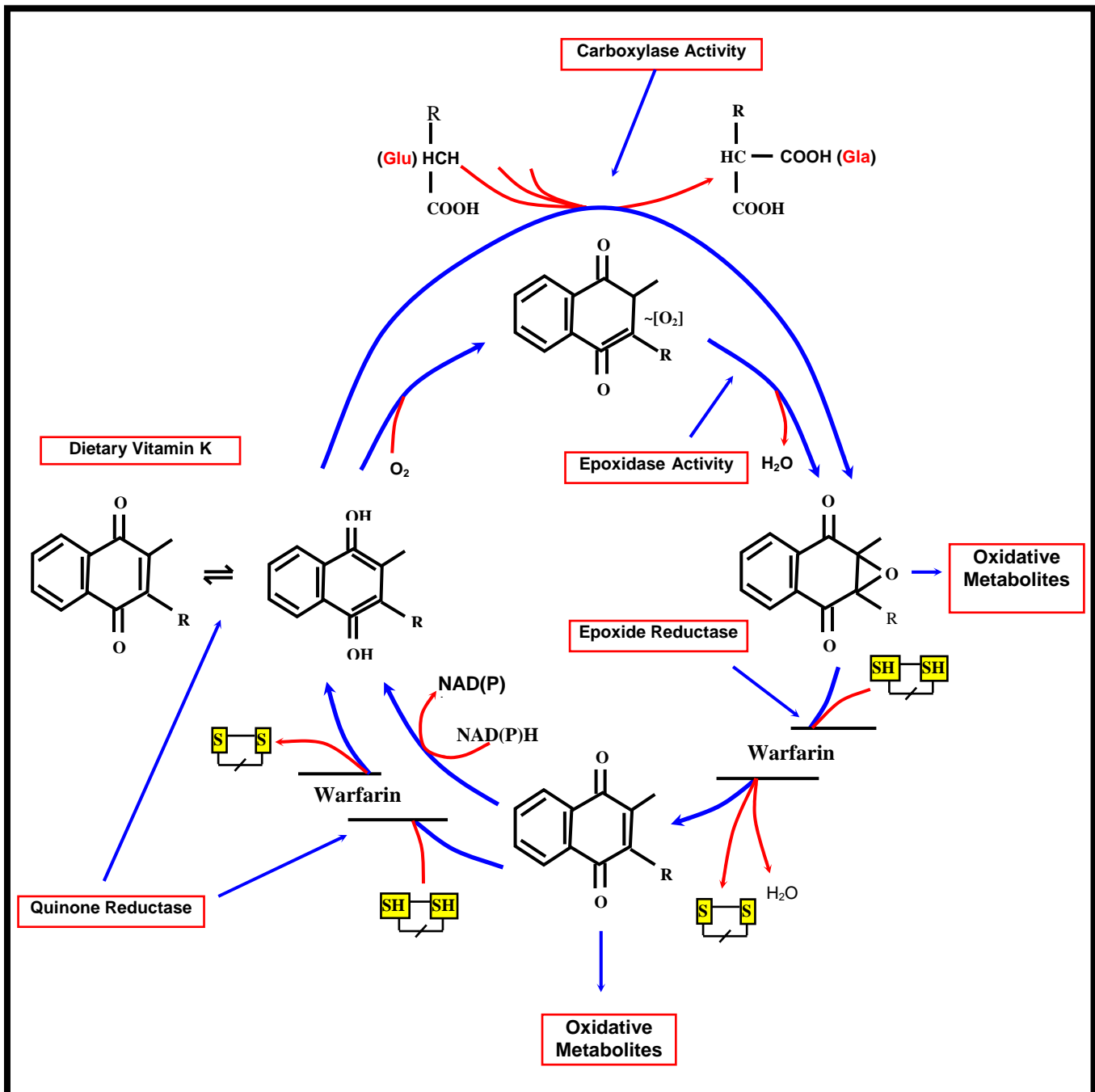
Figure 1.4.3 shown below, **the vitamin K epoxide cycle** is a salvage pathway for vitamin K, a vitamin present in nanomolar concentrations in the liver, skin and muscles. It suggests that vitamin K is converted to vitamin K-epoxide by an epoxidase activity that may be identical or linked to the glutamate carboxylase activity. The epoxide is converted back to the quinone (vitamin K) using an epoxide reductase activity, which is a site of inhibition by warfarin. The cycle continues with the reduction of the quinone to the hydroquinone, which in turn reacts with oxygen forming the epoxide; this step brings about the carboxylation of glutamic acid (adapted from Olson, 1984).

Absorption of vitamin K requires bile and pancreatic juices for maximum effectiveness, dietary sources are absorbed in the small bowel and they are incorporated into chylomicrons that enter the lymphatic system. Absorption efficiency has been measured at between 40-80 % depending on the absorption vehicle. Orally administered vitamin K may appear in the plasma within 20 minutes and as much as 20 % may appear in the liver within 2 hours, whereas 50 % of parenterally administered vitamin K may appear in the liver within 1 hour (Olson, 1984). During the period 48-72 hours post absorption the plasma concentration of vitamin K declines exponentially, this is due to its transfer from chylomicrons to the liver, where it is incorporated into very low-density lipoproteins (VLDL) and distributed to tissues via low density lipoproteins (LDL). The turnover of the human vitamin K body-pool was estimated to be 2 ½ hours (Bjornssen *et al.*, 1979), body-pool sizes were estimated from approximate daily intake to be 50-100μg, which is exceptionally low for a fat-soluble vitamin.

Vitamin K 2,3-epoxide was reported and characterised as a metabolite of vitamin K in 1970. In 1941, it was shown that the epoxide rapidly converts to vitamin K in normal animals and in 1970 Matschiner demonstrated the accumulation of warfarin in treated rats. Matschiner also demonstrated that the biological activity of vitamin K was equal to that of its epoxide, except in warfarin treated animals (Matschiner *et al.*, 1970). The suggestion was that warfarin inhibited the reductase, which converted vitamin K-epoxide back to its quinone form; this hypothesis was established in the early 1970s through the work of Bell, Sadowski, Matschiner and Caldwell (Bell *et al.*, 1972; Bell *et al.*, 1973).

Vitamin K-2,3-epoxide reductase activity converts vitamin K-2,3-epoxide back to vitamin K, this activity is repressed by the action of warfarin. DT diaphorase was purified from liver microsomes by affinity chromatography and was shown to be the physiologically relevant vitamin K (quinone) reductase in the epoxide cycle, later shown to be more sensitive to warfarin (Wallin *et al.*, 1978). The cyclic interconversion of the vitamin to its epoxide and back is required for its glutamate carboxylation activity; warfarin exerts its inhibitory effect on prothrombin synthesis through the inhibition of epoxide and quinone reductases within the cycle. Warfarin resistance has been demonstrated in rats in northern Europe and the activity of vitamin K epoxide in these rats was shown to be less sensitive to warfarin, however more hydrophobic 4-hydroxy-coumarin drugs (e.g. difenacoum) inhibit epoxide reduction equally well in resistant rats (Olson, 1984).

1.4.2 Nutritional vitamin K requirements

Human requirement for vitamin K, up to 50 % of which may derive from gut microflora, has been calculated to be approximately 2 μg kg^{-1} (body weight) day^{-1}, therefore the dietary requirement is 1 μg kg^{-1} day^{-1} (Olson, 1984; Suttie, 1992; Booth *et al.*, 1998). This was calculated using the assumptions; an intravenous dose of 1 μg kg^{-1} is needed to raise depressed vitamin K levels to normal for 1 day, and the gut absorbs approximately 50 % of dietary vitamin K. In the US a normal mixed diet has been calculated to contain approximately 300-500 μg (of vitamin K) day^{-1} (Olson, 1984), which far exceeds the recommended daily allowance for the vitamin. However, more recent studies have suggested that the mean intake of vitamin K in young

(<45 yrs) and old (>55 yrs) adults range from 60-110 μg and 80-210 μg respectively per day in the US (Booth *et al.*, 1998). The increase in vitamin K content in the diet of older adults may be attributed to the increased vegetable content of their diet. These estimates are closer to the RDA; however some individuals fail to meet even this lower level on a daily basis, which may lead to vitamin K deficiencies and possible haemorrhagic complications.

The two sources of vitamin K, as mentioned previously, are the diet and intestinal microflora. Menaquinones have been reported to comprise up to 50 % of the daily supply of vitamin K (Olson, 1984), however a recent publication states that the microflora of the gut provide only a minor portion of the vitamin K RDA (Booth *et al.*, 1998). Another publication states that the menaquinones, originally thought to provide the complete vitamin K RDA, are now thought to be of rather less nutritional significance (Suttie, 1990). Numerous case reports of antibiotic induced vitamin-K responsive hypoprothrombinaemias have argued for the importance of menaquinones in the human diet and others argue against their nutritional significance. However, more recent evidence suggests that menaquinones only partially satisfy the human nutritional requirement for vitamin-K (Suttie, 1995). Because of the interest in haemorrhagic disease of the newborn, the phylloquinone content of human breast milk has been studied. The estimated requirement of 1 μg kg^{-1} cannot be met with a diet of breast milk alone, taking into consideration the variability between vitamin K content of the mother, ranges from 1-3 μg L^{-1}. Milk-formula-fed infants show a lower incidence of haemorrhagic complications due to the higher phylloquinone content of cows-

milk; the vitamin is concentrated within the lipid portion of the milk, making high-fat dairy products also high in vitamin K (Suttie, 1992).

Green leafy vegetables such as kale and parsley contain the highest vitamin K concentrations, reported to be in excess of 500 μg 100 g^{-1}, which may comprise 40-50 % of daily intake. Oils such as soybean, cottonseed, canola and olive contain between 200 and 400 μg 100 g^{-1}, whereas fruit and cereals have low concentrations and dairy products contribute intermediate amounts of vitamin K. Skeletal meat may contribute less than 5 μg 100 g^{-1} and in some cases less than 1 μg 100 g^{-1}, however the liver being the major vitamin K storage organ may contribute between 20 and 100 μg 100 g^{-1}, depending on the vitamin K intake of the animal prior to slaughter. Mixed dishes and certain meals, due to the high phylloquinone content of the oils used in their preparation, comprise 15 % of the total dietary phylloquinone intake (Booth *et al.*, 1998). Some foods such as tomatoes and carrots do not contain large amounts of phylloquinone, but they are ranked as good providers due to the large amounts consumed. Due to its low dietary requirement it is difficult to compile a daily diet adequate in other nutrients that contributes less than 80 μg of vitamin K, therefore satisfying the daily requirement (Suttie, 1992). Tobacco is one of the richest sources of vitamin K known, containing up to 5 mg in 100 g; a small percentage of this vitamin K is volatised when smoking and absorbed through the mucous membrane in the pharynx and bronchi.

Menaquinones, supplied by the microflora of the gut, are also reported to be present in certain animal products including chicken egg yolk and butter; various cheeses contain MK-8 and MK-9 at concentrations of 5-20 μg 100 g^{-1}.

Fermented soybean products, such as Natto, contain large amounts of MK-6, MK-7 and MK-8, which may be of high nutritional value to populations consuming this class of food e.g. the Japanese (Suttie, 1995). Menaquinone absorption in the lower bowel however is rather limited; therefore, the contribution of dietary menaquinone to total vitamin K intake is less well understood (Booth *et al.*, 1998). MK-4 has been indicated to possess a unique role in vitamin K metabolism; this short chain menaquinone is not produced in significant amounts by the intestinal microflora, it has been reported however to be present in significant amounts in extra-hepatic tissues such as brain, pancreas, salivary gland and sternum. It was concluded in the 1960s that the bacteria in the gut remove the phytyl chain of phylloquinone releasing menadione, which was absorbed and converted to MK-4. This tissue specific localisation of MK-4 and pathway for its metabolism from phylloquinone suggest it has a unique and yet unknown function that is detached from that currently recognised for vitamin K (Booth *et al.*, 1998).

1.4.3 Oral anticoagulants and drug therapy

Warfarin is a soluble vitamin K antagonist that was developed through the discovery of dicoumarol (bis-hydroxy-coumarin) (Campbell *et* al., 1941) as the active agent in spoilt sweet clover, which caused a haemorrhagic disease in cattle in 1922 (Olson, 1984; Suttie, 1990). Derivatives of 4-hydroxy-coumarin or phenindandione have been synthesised and tested on humans and animals as vitamin K antagonists, many more hydrophobic hydroxy-coumarins have been used as rodenticides, such as difenacoum and brodifacoum. Oral anticoagulants, which regulate the synthesis of prothrombin and other vitamin K dependent proteins in the liver, also induce hypoprothrombinaemia and other factor deficiencies at specific rates when given in saturating doses. The effective concentration of the antagonist causes the synthesis of vitamin K dependent proteins to cease. Hydroxylated inactive products are created through the degrading action of liver microsomal enzymes, a process that may take hours or days depending on the half-life of the hydroxy-coumarin. Hydroxy-coumarins exert their indirect inhibitory effect on prothrombin synthesis by blocking the recycling of vitamin K epoxide back to the quinone form of the vitamin; both reductase enzymes are inhibited by warfarin (Olson, 1984).

Other commonly used anticoagulants include unfractionated heparin (UH) (a mixture of anionic glycosaminoglycans with molecular weights in the range 3000-30,000 kDa) and low molecular weight heparin (LMWH) (molecular weights in the range 4500-6000 kDa). The heparins exert their anticoagulant effect by catalysing the inactivation of factor Xa (UH and LMWH) and

thrombin (LMWH) by antithrombin III. The catalysis of antithrombin III inactivation of factor Xa occurs due to the conformational change induced upon its binding to UH or LMWH, which causes an increase in the rate of reaction with factor Xa. The catalysis of the antithrombin III/thrombin inactivation occurs due to the stabilisation of a ternary inhibitory complex, in which thrombin is tethered to antithrombin III. LMWH is usually administered subcutaneously in an unmonitored fixed-dose fashion, whereas due to its lower subcutaneous bioavailability, UH is administered intravenously and requires frequent monitoring and dose adjustment (Shafer, 1998).

Salicylate is another drug that may be used to control the coagulative state of a patient; hypoprothrombinaemia is induced in the use of doses greater than 6 g per day. The mechanism of action is different to that of the hydroxy-coumarins in that DT-diaphorase is inhibited, although there is indirect elevation of epoxide concentration. *In-vitro* however there is no effect of salicylate on vitamin K epoxide reductase. Ticrynafen is a nonsulfonamide diuretic, uricosuric and antihypertensive agent, which causes hypoprothrombinaemia in humans; the mechanism of action is similar to that of salicylate in that it markedly inhibits DT-diaphorase and tends to increase hepatic epoxide: quinone ratios. Toxic doses of butylated hydroxytoluene (BHT) have been reported to cause haemorrhagic death in rats; BHT is thought to prevent the uptake of vitamin K by the liver and intestinal cells (Olson, 1984).

Vitamins A and E are known vitamin K antagonists, hypervitaminosis A in the rat lead to haemorrhages and hypoprothrombinaemia, which can be

prevented by administration of vitamin K. Similarly a patient undergoing warfarin (5 mg per day) and megavitamin E therapy (1200 I.U. per day) showed bleeding tendencies and prolonged prothrombin times, which were alleviated upon discontinuation of megavitamin E therapy. Smaller doses of vitamin E (42 I.U per day for 30 days) do not potentiate the effects of warfarin (Olson, 1984). Sterilisation of the bowel with sulpha drugs, neomycin and other broad-spectrum antibiotics may induce a haemorrhagic syndrome due to the reduction in vitamin K supply from gastrointestinal flora (Olson, 1984). In addition, cephalosporin antibiotics inhibit DT-diaphorase, which reduces the amount of quinone to hydroquinone conversion in the vitamin K recycling/salvage pathway, thus further reducing the supply of active vitamin K.

1.5 Oral anticoagulation and its monitoring

1.5.1 Warfarin

Warfarin sodium anticoagulation induces an anticoagulative state by causing a reduction in the vitamin K dependent modification of certain coagulation factors, such as prothrombin, factor X and factor VII, thereby lowering the potential for blood coagulation. The precise monitoring of patients on oral anticoagulation therapy (OACT) is of critical importance in maintaining an effective course of treatment, predominantly due to the narrow therapeutic range of the drugs used. Current monitoring techniques employ the functional, prothrombin time (PT) and the activated partial thromboplastin time (aPTT) tests, which measure the clotting time of blood/plasma on addition of thromboplastin, phospholipid and calcium. The warfarin dose can be titrated by maintaining the patients INR between 2 and 3 with a target of 2.5, or between 2 and 3.5 with a target of 2.7 or above (Pengo *et al.*, 2001).

Developments of the clotting time monitoring system and coagulation management since the 1980s, such as the introduction of the international normalised ratio (INR) and the international standard index (ISI), and the implementation of dedicated coagulation monitoring clinics have reduced some of the problems associated with early coagulation monitoring. Due to the multi-conglomerate manufacture of thromboplastins and reagents for the functional tests, the World Health Organisation introduced the INR/ISI system in 1983, with the aim to standardise and improve the oral anticoagulation therapy (OACT) risk/benefit profile. Each thromboplastin produced was given an ISI relative to the reference thromboplastin rTF/95; the prothrombin time

index (PTI) could then be converted to the INR (Kahn *et al.*, 1998). The INR is the ratio between the patients PT and the mean laboratory normal PT, raised to the power of the international standard index (ISI) of the thromboplastin used. This standardisation of PT thromboplastins relative to the reference material attempted to limit the effects of their varying sensitivities, especially apparent in orally anticoagulated patients. Even with the strictest monitoring regimen there is still a 20 % incidence of thrombotic or haemorrhagic complications with patients on long-term OACT. Low dose anticoagulation treatments are still not controlled well using the INR system suggesting that the INR/ISI system is far from perfect.

1.5.2 Thromboplastins

Thromboplastins used in clotting time tests are produced from the brain tissue of humans and animals, and in the 1980s the use of human brain and plasma derived anticoagulation reagents declined. This was due to elevated fears over the increase in the incidence of Creutzfeld Jacob Disease (CJD) and the fact that the HIV antibody was regularly found in some of the plasma derived reagents (O'Donnell *et al.*, 1987). Efforts were made to produce reagents without viral contaminants and to explore new methods of anticoagulation monitoring. Prothrombin is the most important factor in the coagulation pathway, it is the largest protein in the cascade at 70 KDa and it has the longest blood/plasma half-life. Thrombin generation is determined by the concentration of prothrombin in the plasma, therefore addition of factors VII, IX or X to factor deficient plasma has no influence on thrombin generation (Xi *et al.,* 1989). With these facts in mind it is not unreasonable to consider that prothrombin concentration or activity may be a good indicator of coagulative

state. Therefore, in the search for new approaches to anticoagulation control, it might be wise to develop a method whereby the concentration/activity of prothrombin in the blood/plasma could be quantified.

1.5.3 Alternative functional tests

A functional test that uses the native biochemical transformation of normal prothrombin to thrombin, the XaPC method, may be used to monitor the coagulative state of blood/plasma samples. This method uses already present factors Xa and V together with phospholipid and calcium to convert normal prothrombin into thrombin, the active serine protease. Thrombin can subsequently be quantified by adding a chromogenic substrate such as S2238, which is catalytically degraded by thrombin (Widdershoven *et al.*, 1987). A reduction in the concentration of native, fully carboxylated and calcium bound prothrombin, caused by warfarin therapy could be detected using the XaPC method.

A similar test based on the principal of native prothrombin to thrombin conversion, is the Carcinactivase-1 (CA-1) method. Carcinactivase was isolated from the snake venom of *Echis carinatus leucogaster* because of its ability to specifically cleave the native calcium bound conformer of prothrombin to thrombin. The amount of p-nitroaniline liberated by the cleavage of the amino acid and nitroaniline-derived substrate Boc-Val-Pro-Arg-pNA by Carcinactivase, is directly proportional to the concentration of thrombin, and thus native prothrombin present in the sample (Iwahashi *et al.*, 2001).

The functional tests, i.e. those that do not measure the factor in question (prothrombin), but an endpoint of a biochemical pathway, such as the clot or thrombin itself, are not ideal for anticoagulation control. This is due to a loss in accuracy due to the relatively high number of variables in each test; therefore there are more points at which errors may occur than in a single step/factor direct assay. The ideal situation would present a specific and accurate test for the direct quantification of the factor in question i.e. prothrombin and also the detection of those patients with the hypercoagulable state.

1.5.4 The native prothrombin antigen

The use of the native prothrombin antigen in coagulation monitoring has achieved much more efficient control and prediction of the hypercoagulable state, with up to an 85 % reduction in complications compared to prothrombin time (Furie *et al.*, 1990). It had already been demonstrated that the prothrombin antigen correlated closely with prothrombin coagulant activity both with normal and warfarin anticoagulated plasma (Blanchard *et al.*, 1983), therefore potentially useful in the control of OACT. Furie and colleagues in 1984 developed a radioimmunoassay specific for the native prothrombin antigen, using monospecific polyclonal antibodies (Furie *et al.*, 1984). The assay performed much better than the established PT in randomised clinical trials, with greater accuracy in the prediction of bleeding and thrombotic complications. This study also demonstrated that abnormal prothrombin concentration is less closely correlated to complications arising from OACT than native prothrombin (Furie *et al.*, 1984), possibly due to the generation of heterogeneous abnormal prothrombin species.

The radioimmunoassay detected native prothrombin levels in non-anticoagulated patients of 108 $\pm$19 μgml^{-1}, compared to the therapeutic range of 12-24 μgml^{-1} in anticoagulated patients (Furie *et al.*, 1990). Patients monitored with the NPA assay developed complications with a rate of 1.5 % per patient year, which contrasted greatly with the complication rate of 9.5 % per patient year, using the PT assay (Furie *et al.*, 1990). The NPA assay monitored patients on OACT with 85 % less complications than the established PT assay (Furie *et al.*, 1990). Analysis of complications associated with INR monitored OACT showed that 30 % occurred in patients with INRs in the therapeutic range of 1.5-2.0, 25 % of the complications associated with low dose OACT were also within the therapeutic range of 1.5-1.8 (Furie *et al.*, 1990, Kornberg *et al.*, 1993).

Fatal	Major	Major/Minor	Reference
1.6 (Vascular death + Fatal bleed)	1.8 (Major bleed + Non fatal Ischeamic stroke)	4.4 (Minor bleed)	Pengo *et al.*, 2001
0.8	4.9	15	Landefeld *et al.*,1993
0-4.8	2.4-8.1	-	Levine *et al.*, 1992

Table 1.5.1 shows some haemorrhagic complication rates associated with oral anticoagulation therapy as monitored using standard prothrombin time and international normalised ratios.

Due to methodological limitations, accurate data on true OACT complication rates are lacking. Studies have been undertaken however, and figures for OACT complication rates have been calculated, some are shown in **Table 1.5.1**.

In 1993, Abraham Kornberg and his group compared the NPA with the PT for OACT monitoring, using a similar assay to that developed by Furie and colleagues in 1984 (Kornberg *et al.*, 1993). The radioimmunoassay (Furie *et al.*, 1990) was replaced with an enzyme-linked immunoassay (ELISA) specific for native Ca^{2+} prothrombin (Kornberg *et al.*, 1993) and used in the comparison of the NPA with PT for OACT monitoring. The mean normal prothrombin concentration was estimated using the ELISA to be 164 $\pm$32 μgml^{-1} (Kornberg *et al.*, 1993), which was in contrast to the much lower concentration of 108 $\pm$19 μgml^{-1}, measured by radio-immunoassay (Furie *et al.*, 1990). The therapeutic range (TR) of the NPA concentration during OACT measured by ELISA (50-80 μgml^{-1}) was much higher than that measured by radioimmunoassay (Kornberg *et al.*, 1993). During surgery however, it was recommended that the NPA concentration should be raised above the TR, due to 3 patients in the study suffering post surgical haemorrhages, with mean NPA concentrations of 66 μgml^{-1} (Kornberg *et al.*, 1993).

As the antibodies are similar in both assays, the higher therapeutic range for NPA measured by ELISA compared to RIA may be explained by the detection method. The signal intensity in the RIA is time dependent (i.e. exposure of photographic film to radioactivity), therefore an increase in the exposure time may potentially lead to an increase in the TR. The NPA/ELISA measures a parameter more closely related to thrombotic predisposition than the PT (Kornberg *et al.*, 1993). Prevention of thrombosis requires a reduction in the concentrations of Factor X and II, which both have relatively long half-lives. A prolonged PT however, may be caused by the acute reduction of Factor VII

(short half-life), rather than the more effective, chronic reduction of factor II or X (Kornberg *et al.*, 1993). Low dose (LD) OACT is monitored relatively inefficiently with the PT, this is due to the relatively small TR of 1 to 1.25 that has to be maintained during this course of therapy. As the INR increases from 0.8-1.5 the NPA concentration decreases fourfold from 200-50 μgml^{-1} (Kornberg *et al.*, 1993), therefore increasing the potential sensitivity of the NPA assay for LD-OACT. Variations in the NPA concentration over the whole TR and beyond for OACT are detected extremely well by the NPA/ELISA, therefore making it a very sensitive alternative to the more established PT. All of the above-mentioned benefits suggest that the NPA assay would confer much safer monitoring and control over OACT than current PT/INR methods, including LD-OACT.

1.5.5 Laboratory techniques for coagulation monitoring

Morawitz in 1905 postulated that fibrinogen, prothrombin and calcium ions are required to form a blood clot; this theory was later found incomplete as more coagulation factors were discovered (Morawitz, 1905). In 1913 the whole blood clotting time test was developed by Lee and White, this was to form the original backbone of the clotting investigation, however it is only used nowadays as a modified activated clotting time test (ACT) (Lee and White, 1913). Based on Morawitz's theory, Armand Quick in 1935 developed the Quick's time assay, now called the prothrombin time (PT), as prothrombin was the only known plasmatic factor to act on fibrinogen at the time (Quick *et al.*, 1935). Quick also noticed that the prolonged clotting times of aged plasma reverted back to normal when fresh plasma was added, this lead to the discovery of the labile factor, later named, using standard roman numeral

nomenclature, factor V. Factor VII was discovered by Alexander *et al.* soon after, which was then followed by the discovery of factor X (Alexander *et al.*, 1951). In 1953 Biggs and Douglas developed the thromboplastin generation test (TGT), which allowed the diagnosis of haemophilia types A, B and eventually C. The TGT detected deficiencies in factors VIII, IX and XI respectively; TGT is not now used, as it is too technically complex to perform (Biggs and Douglas, 1953). The Brinkhous group in 1953 developed the activated partial thromboplastin test (aPTT), which is one of the tests used to date (Langdell *et al.*, 1953). The use of plasma from patients known to have single coagulation factor defects in the aPTT allowed the simple diagnosis of the major haemophilic diseases and other major coagulation deficiencies. The discovery of the aPTT lead to the identification of other clotting factors in the contact phase or intrinsic coagulation pathway, such as prekallikrein and high molecular weight kininogen (HMWK) (Khan *et al.*, 1998).

1	1905	Morawitz's theory: fibrinogen, prothrombin and calcium in blood lead to clot formation
2	1935	Quick *et al* introduced Prothrombin Time
3	1962	International committee in haemostasis and thrombosis revises clotting factor nomenclature
4	1953	Biggs *et al* develop TGT illustrating the intrinsic system
5	1953	Brinkhaus's group describes aPTT, which at the time was not as popular as TGT
6	1964	Macfarlane et al and Ratnoff and Davie suggest enzyme cascade hypothesis
7	1995	Rappaport discovers that tissue factor can directly activate factor VII

Table 1.5.2 represents a summary of the development of oral anticoagulation therapy and it's monitoring (Khan *et. al.*, 1998).

1.5.6 The activated clotting time (ACT)

The ACT is a test that measures the clotting time of non-anticoagulated whole blood; it is very useful in situations requiring a rapid turn around time for the result, situations where continuous monitoring and adjustments of heparin dosage are necessary. ACT is used in cardiac catheterisation laboratories where the clotting time needs to be kept between 300-500 seconds and in monitoring heparin efficacy during open-heart surgery where the clotting time is kept within 400-800 seconds also in haemodialysis.

Prothrombin time (PT) and aPTT are the most commonly used tests in coagulation therapy monitoring and for the detection of coagulation defects today. They are both considered as being functional tests as they measure enzymatic activities that lead to clot formation. The modern PT and aPTT tests are processed using automated technology in the laboratory, allowing approximately 10-15 minutes per sample (automated technology allows multiple and simultaneous sample processing).

1.5.7 The activated partial thromboplastin time (aPTT)

The aPTT is a test that determines the clotting time of citrated plasma (plasma with trisodium citrate), when activated by adding a partial thromboplastin or phospholipid reagent and calcium chloride. The test is insensitive to factor VII due to the lack of tissue factor in the reagents. This makes the test sensitive therefore to the factors in the intrinsic and common coagulation pathways and useful in the detection of deficiencies in factors VIII, IX, XI and XII and severe deficiencies in factor V, X, II or fibrinogen.

Modern determination of the aPTT involves the use of automated clot detection methods such as a fibrometer or photo-optical equipment, which measures the decrease in light transmission through a sample as the clot forms. Many different reagent types can vary in the type of activator, the platelet/phospholipid mixture, the calcium chloride concentration used and the times of incubation of plasma with activator phospholipid mixture. The variety of reagents available on the market may lead to inter-laboratory discrepancies in clotting time results, due to varying sensitivities of the reagents to heparin and detection of coagulation factor deficiencies. This problem may be overcome by the implementation of a standardisation of thromboplastin reagents against an international reference material, currently however such a standard is not available for use with the aPTT.

The aPTT is used widely to monitor the effects of heparin, certain considerations need to be taken into account however when processing such test results. The time of sample collection in relation to heparin administration is critical, as unfractionated heparin has a relatively short half-life of $1\frac{1}{2}$ hrs. Using aPTT therefore, to monitor heparin therapy requires swift determination due to the rapid diminishing of heparins effect with time. Other factors that may give erroneous results include the variability of the aPTT reagents with the type of heparin (unfractionated vs. low molecular weight heparins), the mode of administration of heparin (intravenous vs. subcutaneous) and the clot detection methodology used. The aPTT may however be prolonged with out any clinical defect, coagulation inhibitor or heparin. In a prospective study of 100 prolonged aPTT cases referred for consultation, only 50 % showed any coagulation factor deficiency. Of the remaining 50 % the degree of abnormality did not correlate sufficiently with the haemostatic defect; 36 %

were of no clinical significance and 14 % were artefactual (Khan *et al.*, 1998).

The clinical significance of a shorter aPTT or PT is however currently not fully

understood.

	aPTT test
1	Heparin Monitoring
2	Work-up of patients with a history suggestive of hereditary or acquired coagulation defects
3	Pre-operative testing with a positive bleeding history
4	Haemorrhage after surgery or invasive procedure with no clear aetiology
5	Suspicion of coagulation inhibitors especially lupus inhibitor and factor VIII inhibitor
	PT test
1	Warfarin monitoring
2	Pre-operative testing with a positive bleeding history
3	Haemorrhage after surgery or invasive procedure with no clear aetiology
4	Patients with liver disease

Figure 1.5.3 summarises the clinical implications for using the prothrombin time (PT) and the activated partial thromboplastin time (aPTT) (Khan *et. al.*, 1998)

1.5.8 The prothrombin time (PT)

The PT is another coagulation time determining test that is sensitive to the

extrinsic and common coagulation pathways, i.e. to factor VII and factor II, V,

X and abnormally low fibrinogen. As indicated in *Table 1.5.3* the PT is used

for monitoring patients on warfarin therapy or long-term coagulation inhibition,

it is not used for the monitoring of heparin therapy due to a negative

correlation between the results and the degree of heparinisation. As with

aPTT, there are many thromboplastic reagents on the market specific for use

with the PT.   In contrast with the aPTT, PT reagents have all been

standardised against an international reference thromboplastin (rTF/95),

which has an international standard index (ISI).   Due to the multi-

conglomerate manufacture of widely varying sensitivity thromboplastins, the

INR/ISI system, introduced by the World Health Organisation (WHO) in 1983, allowed the standardisation and improvement of the oral anticoagulation therapy risk/benefit profile (WHO, 1983). Therefore, all PT results are expressed as an international normalised ratio (INR), which is the PT ratio that would be observed if the thromboplastin used was the international reference material. This standardisation of thromboplastins relative to a reference material was necessary due to the varying sensitivities of reagents, especially in orally anticoagulated patients. In the laboratory situation computerised automated equipment allows the rapid online calculation of the INR from the recorded PT (Khan *et al.*, 1998).

Indications		Prophylaxis and/or treatment of venous thrombosis and its extension, and pulmonary embolism
		Prophylaxis and/or treatment of thromboembolic complications associated with atrial fibrillation and/or cardiac valve replacement
		Reducing the risk of death, recurrent myocardial infarction, and thromboembolic events such as stroke or systemic embolization after myocardial infarction

Table 1.5.4 illustrates the indications for the initiation of warfarin therapy (Information taken from Erban, 1999).

Contra-indications		Pregnancy	Warfarin crosses the placenta and can cause fatal haemorrhage or embryopathy
		Active bleeding or haemorrhagic tendencies	Especially GI or GU tracts, CNS haemorrhage, aortic or cerebral aneurysms, pericarditis or pericardial effusion and bacterial endocarditis
		Recent or contemplated surgery	Especially surgery of CNS, eye, or major trauma
		Other invasive procedures	Especially lumbar puncture or other procedures with potential for uncontrollable bleeding
		Prior sensitivity to warfarin	E.g. warfarin skin necrosis (Homozygous)

Table 1.5.5 illustrates the contraindications for the initiation of warfarin therapy; warfarin therapy is contraindicated whenever the potential risks outweigh the potential benefits (Information taken from Erban, 1999).

Prior to the initiation of warfarin therapy, a risk-benefit assessment profile must be performed. The indications and the existing or potential contraindications should all be taken into consideration, together with the patient's concerns and understanding of the treatment and its potential adverse reactions. Regardless of the final decision, the rationale should be clearly communicated to the patient and documented in the medical record (Erban, 1999). The measurement of a patient's PT is achieved by adding a specific amount of thromboplastin (to initiate clot formation) to a sample of a patient's plasma, which was obtained from the centrifugation of a sample of whole blood. The time taken for the thromboplastin to form the clot, usually 11-15 seconds in normally coagulated individuals, is the PT.

1.5.9 The international normalised ratio (INR)

This value is then used in the INR equation as follows:

$$INR = (\text{Patients PT (sec)} \div \text{laboratory mean normal PT (sec)})^{ISI}$$

An INR value of 1.0, assuming the ISI is also 1.0, indicates that the individual is normally coagulated; INRs of 2 and 3 indicate clotting times twice and three times that of the normal. For patients on anticoagulation therapy the INR is maintained within the range 2-3, any lower and the effects of the warfarin are impaired, any higher and bleeding complications can occur. Using the PT system without the ISI exponential can lead to very varied results.
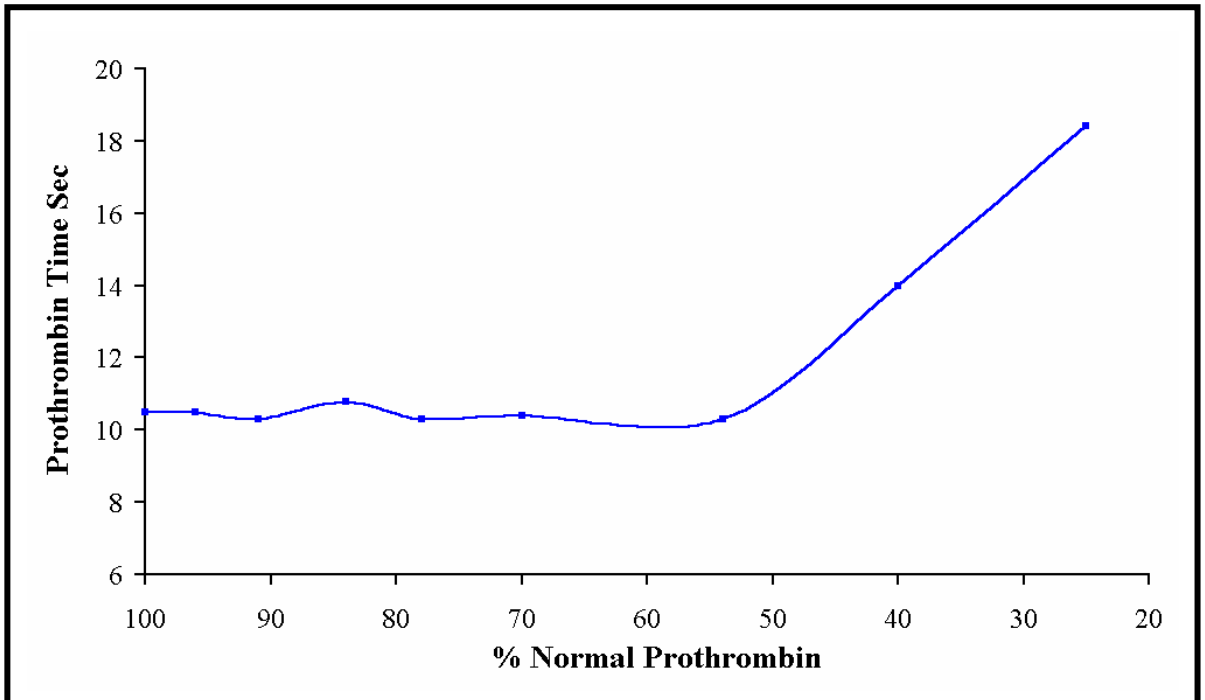
Figure 1.5.1 shows the dependence of prothrombin time on prothrombin concentration, with the threshold concentration illustrated at 50 % of the normal. Normal human plasma was mixed with plasma from a warfarin treated patient (25 % of normal prothrombin) in varying amounts to produce a series of plasma samples containing 25-100 % of the normal concentration of prothrombin. Standard clinical prothrombin times were then determined (adapted from Suttie, 1992).

The insensitivity of the prothrombin time test has been well documented and is illustrated in ***Figure 1.5.1***. There is a threshold prothrombin concentration, above which the prothrombin time stays constant; therefore, the prothrombin concentration has to be reduced to 55 % before the prothrombin time starts to be clinically significant. As can be seen from ***Figure 1.5.1*** a plasma sample with only 50 % prothrombin (compared to the human normal plasma concentration of 1.1-2.3 mg ml^{-1}) still has a normal prothrombin time.

Much more sensitive chromogenic and immunologic vitamin K dependent protein assays have been developed for the quantification of certain individual factors. Clot based assays are functional modifications of the aPTT and the PT therefore they can be used to detect both quantitative and qualitative coagulation factor defects. To qualitatively detect a factor defect, patients plasma is mixed with commercially available plasma deficient in a single factor; if the defects do not match then there will be a reciprocal correction of the prolonged clotting time. In the quantitative test, the plasmas are mixed together in equal known volumes and the clotting time is compared to that of a serially diluted normal control, the percentage factor defect may be estimated by comparing the clotting times. Tests used to detect factors VII, II, X and V are PT based assays and aPTT based assays are used to detect factors VIII, IX, XI and XII.

1.5.10 Developments in oral anticoagulation therapy

Three major developments have decreased the risks of oral anticoagulation therapy over the last few years. These include the introduction of the ISI/INR standard, the definition of the indications of anticoagulation and improvement of its management by the development of anticoagulation management services. However it still has a high risk/benefit profile due to three further factors: warfarin has a narrow therapeutic index, clinical outcomes are influenced by patient-specific characteristics, and dose adjustment and patient communication are often mismanaged. Excessive or inadequate anticoagulation is proven to lead to serious consequences in the warfarinised patient. Fluctuations in the systemic warfarin concentration or changes (due to other factors) in the pharmacodynamic response to warfarin, in spite of the

stable warfarin concentration can lead to haemorrhagic or thrombotic complications.

Hylek *et al* in 1994 demonstrated a 5-fold increase in intracranial haemorrhage as the INR exceeded 4.5, compared to a 17-fold increase in stroke as the INR falls below 2.0 and approaches 1.0 (Hylek *et al*., 1994). Cannegieter's group in 1995 discovered a broader INR therapeutic range of 2.5-4.9 in over 1608 patients with mechanical heart valve prostheses; increasing incidences of haemorrhage or thrombosis were observed above and below the range respectively (Cannegieter *et al*., 1995). Patient specific factors may also be responsible for thrombotic and haemorrhagic events during anticoagulation. Positive correlations exist between risk of major haemorrhage and morbidities such as heart, liver or kidney dysfunction, cancer or severe anaemia, stroke and gastrointestinal bleed history, atrial fibrillation or combinations of three or more.

It has been observed that anticoagulation patients cared for in specialised *anticoagulation management services* (AMS/C) or *anticoagulation clinics* fare between 50-75 % better than patients under routine medical care (RMC). The AMS/C model of care provides efficient dosing management and determination of the therapies appropriateness; it also serves to communicate with other providers of a patients health care whilst providing ongoing patient evaluation and education. Savings of $800-1000 per patient, per year of therapy have been made in the USA, due to a decrease in the incidence of anticoagulation related complications by the implementation of AMS health care models (Ansell, 1999). The ongoing development of AMS care models

will lead to the reduction of adverse coagulation events, a reduction in the use of hospital resources and a saving of vital health care funds.

Many reliable and accurate yet considerably more expensive point-of-care devices have been developed for the measurement of aPTT and PT. Whole blood samples may be taken up into the device by capillarity, as anticoagulation of samples in heparinised tubes is unnecessary prior to testing. This allows the use of 'fingerstick' sampling of blood (pinprick sampling), which is much more comfortable for the patient and allows for less complicated processing. Described below are some of the major point-of-care devices available on the market. The Ciba Corning 512 system (Ciba Corning Diagnostics Corp., Medfield, MA) uses a drop of whole blood that is placed on a disposable plastic cartridge. Capillary action draws the blood into a reaction chamber and clot detection is by a laser optical system. The Hemochron 801 system (International Technidyne Corp., Edison, NJ) uses a small magnet containing glass tube system in which the whole blood sample is mixed with the reagents. The tubes are rotated and heated until a firm clot is formed, displacing the magnet within the tube and triggering a proximity switch, which stops the time measurement. The bedside coagulation monitors listed below are similar in theory to the standard laboratory tests, however they use different detection methodology, reagents, activators and sample volumes (Reich *et al.*, 1993). The bedside monitors were calibrated through their clinical comparison with standard laboratory tests before they were more widely used preoperatively, where efficient and accurate coagulation monitoring is necessary.

Four alternative tests that may be utilised in the monitoring of a patients warfarin therapeutic range are described in further detail below. The tests described below provide quantitative and qualitative data on the amount of abnormal prothrombin (also termed PIVKA II (protein induced by vitamin K absence II) des-γ-carboxyprothrombin, isoprothrombin, paraprothrombin, dicoumarol-induced prothrombin, atypical-prothrombin and acarboxyprothrombin (Olson, 1984) in a blood sample. Measurements of abnormal prothrombin may involve calculation of the total prothrombin combined with subtraction of the functional prothrombin; this will give an indirect measurement of the abnormal prothrombin. To measure total prothrombin the snake venom from *Echis carinatus* is used to convert prothrombin, including inactive prothrombin, to thrombin. The resulting thrombin proteolytically cleaves the p-nitroaniline derived chromogenic substrate S2238, the amount of p-nitroaniline liberated correlates to the amount of thrombin present. Functional prothrombin is measured using the XaPC method, where factor V, already present in the plasma sample, converts functional prothrombin into thrombin using already present factor Xa, phospholipid and calcium. The name of the test derives from the latter three substrates mentioned. The active thrombin is then chromogenically measured again using Kabi Vitrum or substrate S2238. The abnormal prothrombin measurement is then calculated as the ratio XaPC/ECar; a ratio of ≤ 0.86 conclusively establishes the presence of abnormal prothrombin, whether it is due to vitamin K deficiency or problems with the liver (Widdershoven *et al*., 1987).

The XaPC method may also be used to calculate total prothrombin. Normal plasma has been proven to be abnormal prothrombin free, therefore pooled normal plasma maybe used in place of the total prothrombin measurement, as all the prothrombin present will theoretically be active. So if the prothrombin activity in a patients' plasma is ≤ 86 % of that of the pooled normal plasma or that calculated using the ECar method then further tests should be implemented to determine the cause of the low prothrombin activity (Widdershoven *et al.*, 1987). The relative accuracy of the XaPC ratio or Xa/ECar ratio methods of abnormal prothrombin detection may be calculated as 0.95 x 0.95 = 0.90 = 90 %. This implies that if the true ratio of Xa/ECar is 1, then due to the precision of the method alone a variability of 0.90 – 1.10 would be produced. Therefore, abnormal prothrombin concentrations of less than 10% of the total prothrombin may escape detection using this method (Widdershoven *et al.*, 1987; Von Kries *et al.*, 1992). The question now arises; are such abnormal prothrombin concentrations cause for concern and indicative of future bleeding complications during anticoagulative therapy?

The second method for assaying des-Υ-carboxyprothrombin is by removing active carboxylated prothrombin from the plasma sample and measuring the remaining prothrombin. Carboxylated prothrombin is adsorbed onto barium carbonate (Ba_2CO_3), sulphate ($BaSO_4$) or aluminium hydroxide gel columns. The remaining under carboxylated prothrombin, which is not always completely de-carboxylated (Olson 1984), may be assayed using the ECar or XaPC methods as described above, quantitative/qualitative immunochemical methods may also be implemented (Widdershoven *et al.*, 1987).

A method using electrophoresis and subsequent immunofixation may be used to provide semi-quantitative measurements of abnormal prothrombin in serum. After electrophoresis of plasma on a Cellogel® membrane, a cellulose strip coated with rabbit antiserum to human prothrombin may be placed over the gel and left overnight. Immunofixative strips can then be placed on the gel with goat antiserum to rabbit immunoglobulin, which intensifies the primary immunofixative reaction. After thorough washing in saline solutions for two days and a staining/destaining procedure of Amido Black B10/methanol: acetic acid: water (5:1:4 by vol.) together with clearing and drying procedure, presence of abnormal prothrombin is indicated by two bands on the gel. This procedure was described above as being only semi-quantitative; the amount of immunoprecipitate is not only sensitive to the amounts of abnormal prothrombin and prothrombin but also to the quality of the antisera used. The procedure as described by Widdershoven *et al.*, 1987 also had a three and a half day time scale, which is unsuitable for use as a quantitative/qualitative diagnostic test for abnormal prothrombin.

1.5.11 Snake venom proteins

Snake venom activator proteins are classified into four different groups according to their structures and functions. Group A consists of the metalloprotease-type activators, whose action is not reliant on any other coagulation factors, they are also widely distributed in many genera of viper e.g. *Echis* and *Bothrops* species. Group 1 enzymes are also considered to be the most toxic, due to their resistance to serpins (natural coagulation inhibitors) such as Antithrombin-III. Group B enzymes resemble those of group A in that they are also metalloproteases, however they do require

calcium ions for their activity. Group C enzymes physiologically resemble factor Xa, in that they contain Gla residues and require factor Va, phospholipids and calcium for their activation. Group D enzymes are hybrids, in that they contain a catalytic subunit similar to that in factor Xa, and a regulatory subunit resembling that from factor Va, they also require phospholipids and calcium for their activation.

Group B enzymes possess a metalloprotease catalytic subunit (similar to that of Ecarin A) and a regulatory subunit (similar to that of the Habu snake venom protein IX/X-bp), which confers the Ca^{2+} and Gla-domain binding specificity of these proteins. Snake venom proteins such as the disintegrins and C-type lectins do not have any enzymatic activity, however they still have haemostatic properties.   The disintegrins are low molecular weight polypeptides present within snake venom, they posess RGD sequence motifs that allow them to specifically bind and interact with endogenous integrins (i.e. GPIIb-IIIa), therefore occupying platelets and preventing aggregation (Kamiguti *et. al.*, 1996). C-type lectins are approximately 30 kDa in size and comprise of a pair of homologous polypeptides also containing a characteristic carbohydrate recognition domain. The lectins are known to interact with proteases; therefore it is likely that they play a major role in the targetting of the protease to key proteins with critical haemostatic function (Braud *et. al.*, 2000).

Studies of the metalloprotease group of venom proteins have shown that they play an important part in the degradation of all the major protein components of the extracellular matrix (ECM) (Baramova *et. al.*, 1989; Baramova *et. al.*, 1990). Haemorrhage caused by metalloprotease venom proteins is effective

within minutes, however the degradation of the ECM proteins takes much

longer, suggesting that the mechanism of haemorrhagic action is quite

complex. Many snake venom proteins have haemostatic related properties,

which make them interesting for use in studies of haemostasis and blood

coagulation control.

Class	Cofactor Requirement	Examples	Former Class
Group A	None	Ecarin	Group 1A
Group B	Ca^{2+}	Carcinactivase and Multactivase	Group 1B
Group C	Ca^{2+} + phospholipids	Oscutarin, Pseutarin	Group II
Group D	Ca^{2+}, phospholipids + factor Va	Trocarin, Notecarin, Hopsarin.	Group III

Table 1.5.6 Shows the classification of the snake venom prothrombin activator proteins (Kini

et. al., 2001).

Various venom proteins have been utilised in the development of diagnostic

tools for diseases and problems of haemostasis as described in **Table 1.5.7**.

Diagnostic Test		Biological function	
Test	Diagnostic Test	Protein	Function
Reptilase™	Titration of fibrinogen-fibrin reaction	Batroxobin	Release of fibrinopeptide A from fibrinogen
Ecarin	Detection of abnormal prothrombin species	Ecarin	Activation of Prothrombin
Stypven®	Measurement of FX level	RVV-X	Activation of Factor X
Protac®	Titration of protein C and S	ACC-C	Activation of protein S
Botrocetin	Diagnostic of vWD and Bernard-Soulier syndrome	Botrocetin	Agglutination of platelets in the presence of vWF

Table 1.5.7 lists some of the diagnostic tests based on the incorporation of snake venom

proteins that influence haemostasis (RVV, Russell's Viper Venom; ACC, *A.c.Contortrix*)

(adapted from Braud *et. al.*, 2000).

Snake venom proteins have various diagnostic uses, due to the direct influence they have on haemostasis, this has been reviewed in Marsh, 2001. Prothrombin is an interesting target for characterisation and diagnostic determination with regard to snake venoms and the fact prothrombin is one of the main controlling factors of the human coagulation cascade. To develop an assay for prothrombin, that could distinguish between normal and native prothrombin species, would assist in the diagnostic evaluation of abnormal prothrombin species, and potentially more important allow the rapid monitoring of oral anticoagulation therapy. Commercially available snake venom prothrombin activator proteins exist such as Ecarin from the saw scaled viper (*Echis carinatus*), textarin from the Australian brown snake (*Pseudonaja textilis*) and an enzyme from the taipan (*Oxyuranus s. scutellatus*) (Marsh, 2001). These venom proteins however are not so useful for the monitoring of wafarin anticoagulation, because they activate all species of prothrombin including the decarboxylated forms present in warfarin treated patients.

The group B snake venom prothrombin activators carinactivase-1 from *Eachis carinatus leucogaster* (Yamada *et. al.*, 1996) and multiactivase from *Echis multisquamatus* (Yamada and Morita, 1997) are potentially very useful in the monitoring of warfarin-oral anticoagulation therapy. Their calcium dependent prothrombin activation properties allow the activation of fully carboxylated native prothrombin, even in a sample also containing partially or non-carboxylated abnormal prothrombin species, i.e. warfarin treated blood samples (Yamada and Morita, 1999). The gla-domain binding region of carinactivase-1 displays calcium dependent properties, which also shows substantial homogeneity to the snake venom protein IX/X-bp (Yamada *et. al.*,

1996). The IX/X-bp protein from the habu snake *Trimeresurus flavoviridis* has strong anticoagulant activity that effects the neutralisation of factors IX and X, by binding to their calcium complexed gla domains (Sekiya *et. al.*, 1995).

The saw scaled viper protein carinactivase-1 (CA-1) was incorporated into a native prothrombin assay designated the CA-1 method, for the fast and specific monitoring of native prothrombin levels in blood samples of warfarin treated patients. Thrombin produced by the calcium dependent activation of native prothrombin by carinactivase-1, cleaved the thrombin specific chromogenic substrate *t*-butoxy-Val-Pro-Arg-*p*-nitroanilide. The velocity of *p*-nitroaniline production determined the level of native prothrombin in the sample. Prothrombin levels determined by the CA-1 method correlated strongly with levels determined by the standard thrombotest and prothrombin time (Yamada and Morita, 1999; Iwahashi *et. al.*, 2001). The CA-1 therefore provides a novel method in which native prothrombin levels can be monitored in warfarin treated patients.

1.6 Immunoassay methods

Immunoassay methods may be considered as an interesting approach to the evaluation of the functional properties of specific vitamin K dependent blood coagulation proteins in complex biological fluids such as blood, plasma and serum (Blanchard *et al.*, 1983). Ganrot and Nilehn demonstrated in 1968 the presence of a protein fraction in the plasma of warfarin treated human patients that reacted to antibodies against human prothrombin, but showed little or no biological activity in the standard assay using factor X and thromboplastin (Olson, 1984). Antibodies towards abnormal prothrombin species have been created (Blanchard *et. al.*, 1979; Owens *et. al.*, 1984; Motohara *et. al.*, 1985; Furie *et. al.*, 1993; Belle *et. al.*, 1995) for the development of ELISA's and potential diagnosis of vitamin K deficiency/antagonism.

Reduction of plasma vitamin K by dietary or chemical antagonism brings about the impairment of N-terminal glutamic acid post-translational modification of prothrombin and other vitamin K dependent proteins. The decarboxylation of prothrombin by vitamin K antagonism often introduces partially decarboxylated 'abnormal prothrombin' species into the circulation (Esnouf and Prowse, 1977; Friedman *et. al.*, 1977), each of which possesses heterogeneous antigenic properties. In 1979 Blanchard and colleagues produced antibodies specific for abnormal prothrombin (Blanchard *et. al.*, 1979), similar antibodies produced by Muntean had specific emphasis for the diagnosis of vitamin K deficiency in newborns (Muntean *et. al.*, 1979). The proof that abnormal prothrombin was not stabilised by calcium ions as with

native prothrombin came about when Owens and colleagues developed monoclonal antibodies specific for the calcium free conformer of prothrombin (Owens *et. al.*, 1984). This work indicated that the only antigenic difference between native and abnormal prothrombin was the calcium stabilisation and subsequent conformational change in the gla domain.

1.6.1 Abnormal prothrombin

Abnormal prothrombin is that which is produced when hepatic vitamin K levels are depleted and the gamma carboxylation of the N-terminal region of prothrombin is repressed. In the plasma of patients on long-term warfarin therapy, abnormal prothrombin species are present that do not bind, or bind poorly to anti-abnormal prothrombin antibodies. These forms of abnormal prothrombin are thought to be partially decarboxylated as opposed to the abnormal prothrombin completely devoid of all its 10-gla residues. Whether the fluctuation of these abnormal prothrombin species occurs at initiation or sometime during warfarin therapy is unclear, it is also unclear as to whether there is any marked individual variation between the concentration of the abnormal prothrombin species and a patients PT.

Monoclonal antibodies specific for abnormal prothrombin have been isolated, also showing minimal cross reactivity with native prothrombin and other coagulation proteins. Using these antibodies together with anti-prethrombin-1 monoclonal antibodies, which bound abnormal prothrombin and normal prothrombin equivalently, assays were developed that may be used in qualitative/quantitative diagnostic tests for abnormal prothrombin in plasma

and serum. Prothrombin and abnormal prothrombin were shown to bind equivalently to the prethrombin-1 antibodies; they also produced identical displacement curves in a competition assay against ^{125}I-labelled abnormal prothrombin bound to anti-prethrombin-1 antibodies (Blanchard *et al.*, 1983). Therefore, the anti-prethrombin-1 antibodies could be used in an EIA to quantify total prothrombin levels in plasma or serum samples. The antibodies that were specific for native/functional prothrombin with minimal cross reactivity may be used in a separate assay to quantify normal prothrombin levels. Theoretically, total prothrombin minus the normal prothrombin leaves the abnormal prothrombin. The development of an EIA, using antibodies such as those mentioned above, allowing the direct quantification of abnormal prothrombin may be considered as a new approach to monitoring oral anticoagulant therapy alongside the coagulation time tests aPTT and PT.

A diagnostic test for the detection of severe vitamin K deficiency was produced using a single antibody showing specificity for the most decarboxylated forms of prothrombin (Motohara *et. al.* 1985). This abnormal prothrombin test, although beneficial for the diagnosis of severe vitamin K deficiency in newborns where the levels of carboxyglutamic acid (gla) have decreased below 5 gla mol^{-1}, becomes less useful in the case of warfarin treated adult patients due to the increased plasma gla content of approximately 7.7 gla mol^{-1} (Motohara *et. al.* 1985). A peroxidase linked EIA, similar to that developed by Motohara *et al.* in 1985, is the most sensitive abnormal prothrombin determination assay, requiring only 100 μl of plasma per test and providing a detection limit of 0.13 mg of prothrombin per litre of plasma (Widdershoven *et al.*, 1987). A monoclonal antibody (C4B6), again

specific for abnormal prothrombin, was developed by Belle and colleagues, and showed greater specificity and enhanced ELISA results compared to two other commercial antibodies from Stago and Esai Co. The application here was also aimed at the diagnosis of newborn vitamin K deficiency (Belle *et. al.*, 1995).

1.6.2 Native prothrombin

Antibodies have been produced that show specificity towards the calcium stabilised native prothrombin antigen and show minimal cross reactivity with abnormal prothrombin (Tai *et. al.*, 1980; Lewis *et. al.*, 1983; Malhotra and Sudilovsky, 1987; Owens *et al.*, 1984) for the specific application of native prothrombin assay.

This observation may hinder the development of direct abnormal coagulation factor immuno-assays, due specific monoclonal antibodies being sensitive to the lack of structural consistency with abnormal prothrombin species'. For this reason, an indirect calculation of the abnormal prothrombin within a sample, through subtraction of normal prothrombin from total prothrombin, may be a primary development route for this new type of assay. In a preliminary report 89 % of "at risk" patients were identifiable using an immuno-assay method compared to 33 % using the standard PT ("at risk": increased potential for bleeding or thrombotic complications during anticoagulative therapy) (Blanchard *et al.*, 1983).

An enzyme linked sandwich immuno-assay was developed by Motohara *et al.* in 1985 that detected abnormal prothrombin in 50 μl of plasma from vitamin K deficient subjects, in the range 0.5-0.5 $x10^{-2}$ μg of protein. The immuno-assay also detected pure decarboxylated prothrombin in the range 0.5 $x10^{-1}$ - 0.5 $x10^{-3}$ μg of protein, this discrepancy was attributed to the heterogeneity of abnormal prothrombin de-carboxylation in vitamin K deficient and warfarin treated individuals. The monoclonal antibody, specific for des-carboxy prothrombin, used in the assay was isolated and characterised from a hybridoma cell line. The cell line was created through the fusion of spleen cells, from an antigenated female BALB/C mouse, and P3U-1 myeloma cells with polyethylene glycol (Motohara *et al.*, 1985). The favoured monoclonal antibody for abnormal prothrombin detection was that which recognised prothrombin containing the least Gla residues or the antibody showing the greatest specificity.

1.7 Artificial Antibodies Prepared by Molecular Imprinting

1.7.1 Definition of molecular imprinting

"*A template induces the self assembly of molecular components to allow reaction or transformation to take place in forming a novel molecular structure; thereafter the template is separated from the novel structure*" (Steinke *et. al*., 1995).

1.7.2 General introduction

Molecular imprinting can be regarded as a templating approach, it is the process of template-induced formation of specific recognition sites (binding or catalytic) in a material where the template directs the positioning and orientation of the material's structural components by a self-assembly mechanism (Piletsky *et. al*., 2001b). These sites are created when functional monomers, bound to certain binding/recognition sites on a template molecule polymerise in the presence of a cross-linker. The polymerisation can be initiated by the addition of a free radical forming initiator, for example 1,1'-azobis (cyclohexanecarbonitrile), heating, or exposing the monomer mixture to UV irradiation. Polymers, containing specific well-defined recognition cavities, predetermined by the chemical nature of the template, are produced on template removal as illustrated in *Figure 1.7.1*. After polymerisation the reaction vessel is smashed and the polymer removed for grinding, typically 40-60 % of the polymer particles are recovered as usable particles, 60-40 % is lost as particle fines due to sedimentation. Attempts to improve particle

shape have been made by using the pores of pre-formed beaded silica and TRIM for imprinting, or by dispersion-polymerisation-techniques, using a solvent based continuous phase (Andersson, 2000).

The choice of monomers contributes significantly to the success of imprinting polymerisation. Functional monomer screening procedures including thermodynamic calculations and combinatorial screening approaches have produced polymers with good affinity and selectivity properties (Chianella *et. al.*, 2002). However, due to the increasing sizes of functional monomer databases current manual screening procedures have become uneconomical. Computational molecular modelling, which has been extensively utilised in rational drug discovery and design, has now been adapted for use in functional monomer screening (Piletsky *et. al.*, 2001a). Virtual functional monomer databases can be rapidly screened on all possible interactions of the molecular model of the template using molecular modelling software. This screening process calculates a binding score for each monomer in the database, and those with the highest scores are considered for use in the molecular imprinting of the target molecule. This screening method has yielded polymers with the affinity and specificity similar or even superior to those of monoclonal and polyclonal antibodies (Chianella *et. al.*, 2002). A computationally designed MIP has separated ephedrine from its enantiomers with a separation factor higher than that obtained from commercially available chiral phases (Piletsky *et. al.*, 2001a).
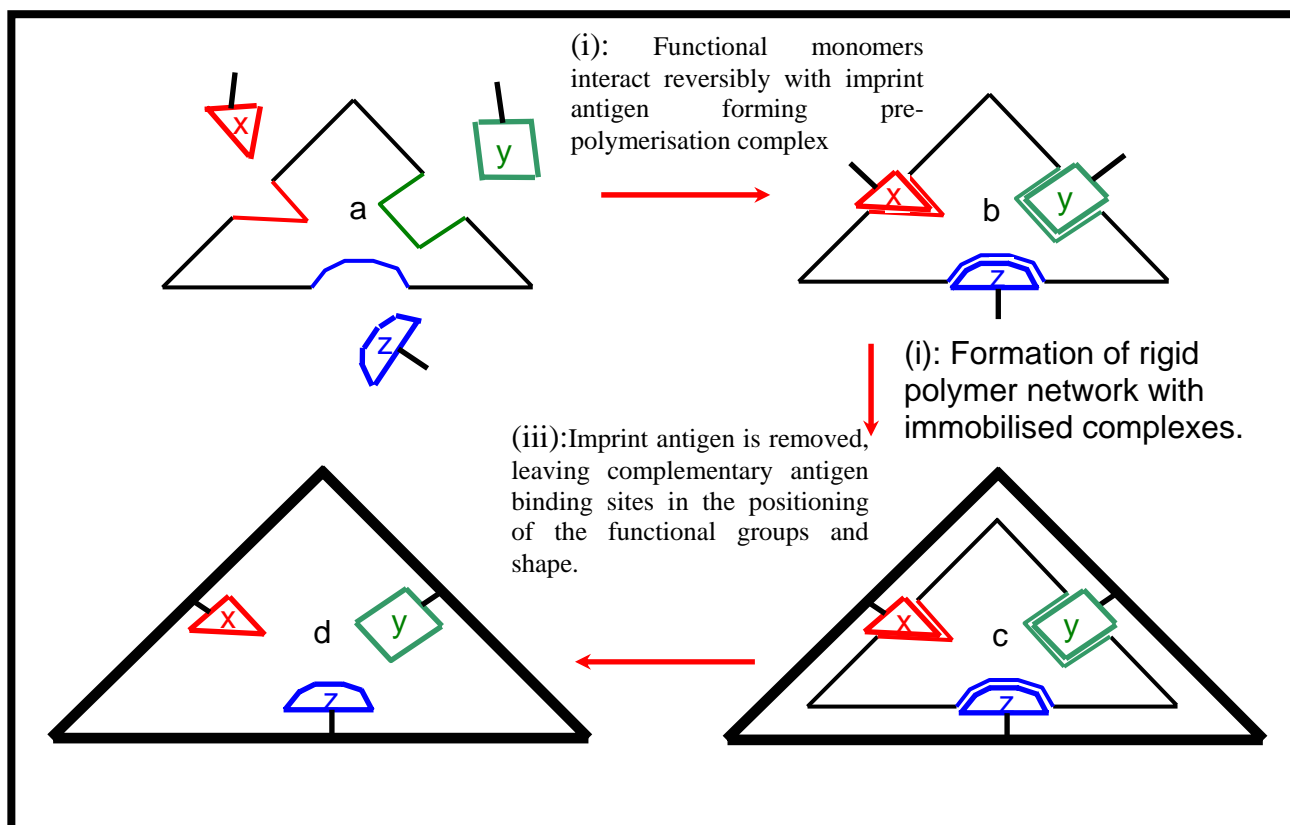
Figure 1.7.1 represents schematically the molecular imprinting process: **i**, **ii** and **iii** depict respectively complexation, polymerisation and template removal. The functional monomers are represented by x, y and z. (Diagram adapted from Allender *et. al.*, 1999; Ansell *et. al.*, 1996).

Among the monomers identified as best suited for the preparation of imprinted polymers are: methacrylic acid (MAA) and itaconic acid (IA), acrylamide (AA), Diethyl aminoethyl methacrylate (DEAEMA), and hydroxyethyl methacrylate (HEMA).   The typical cross-linkers used in molecular imprinting are ethylene glycol dimethacrylate (EGDMA), N,N'-methylenebisacrylamide (MBAA) and divinylbenzene (DVB).

Natural bio-molecules	MIPs
Low stability	MIPs are stable at low/high pHs, pressure and temperature
High cost of enzymes and receptors	Inexpensive and easy to prepare
Poor performance in organic solvents	Good performance in organic solvents
Good performance in aqueous media	Poor performance in aqueous media as yet
Can be used in the aqueous and solid phases	For use in the solid phase only, as yet
Preparation protocols have been optimised due to extensive usage	There is currently an absence of a general MIP preparation protocol
Natural molecules can emit signals on binding which can be easily translated and measured	Difficulties have arisen in the translation of the MIP binding event into a readable signal and integration of the MIP binding signal with the transducer
Integration of natural bio-molecules in multi sensor unit is difficult due to different operational requirements of each molecule (pH, ionic strength, temperature and substrate)	Due to minimal operational requirements of MIPs, the design of a MIP multi-sensor is very easy
Natural receptors and enzymes exist for a limited number of practically important analytes	MIPs could be prepared for practically any compound
Poor compatibility with micro-machine technology and miniaturisation	The polymers are fully compatible with almost any compound

Table 1.7.1 represents a comparison of the benefits of MIPs compared with natural molecules as they are applied for use in clinical, environmental and industrial sensor technology (Anonymous 2001).

A comparison of the benefits of MIPs and natural molecules is depicted in **Table 1.7.1.** Advantages of MIPs include their impressive long-term stability at low and high temperatures; they can also be treated with extreme acid or alkaline solutions without any degradation of their recognition properties (Piletsky *et. al.*, 2001b). The imprinting procedure negates the use of animals, and is very time, energy and cost efficient in comparison to the generation of natural antibodies. Excellent thermal, chemical and mechanical stability; their adaptability makes MIPs potential candidates for use as artificial

antibodies in affinity chromatography, solid phase extraction, solid phase binding assays, and sensors. MIPs are also capable of recognising small variations in template structure, therefore enabling them to distinguish between different enantiomers or analogues of the same template (Kempe *et. al.*, 1995).

Currently, MIPs cannot be used in immuno-assays in which the antibodies are in their soluble form, such as immuno-diffusion, immuno-electrophoresis, immuno-blotting and tissue immuno-fluorescence. Problems arose due to the polymeric nature of these materials, which make them insoluble in both organic and aqueous solvents. Nevertheless polymeric suspension can be used successfully in solid phase based assays. Radioligand assays were developed in aqueous media using morphine and [Leu^5] enkephalin as the imprint molecule (Andersson *et. al.*, 1995). This study addressed two problems associated with adaptation of the relatively immature MIA technology to clinical analyses. Among these problems is the need for additional extraction steps for transferring analyte from aqueous to organic phase. As it was shown in this work, binding cross-reactivity of [Leu^5] enkephalin-imprinted polymer in water was significantly higher than in organic solvents. A second problem was a large amount of print molecule necessary for assay development, e.g. up to 12.5 mg of theophylline is needed to make an assay for this template (Mullett *et. al.*, 1998).

Some antibody immunoassay techniques currently require a clean-up step; therefore, the first criticism with the [Leu^5] enkephalin-imprinted polymer was not a great problem. MIPs possess certain inherent problems such as, the

low compatibility of the procedure with the aqueous environment, and the necessary use of large amounts of template in the imprinting procedure. To this list, we may add the absence of a general preparation procedure for rational MIP design and the difficulty of macromolecular and protein imprinting. All of these problems necessitate resolution before MIPs may replace natural antibodies in certain assays.

1.7.3 Protein and macromolecular imprinting

Macromolecular recognition, using MIPs has been achieved by the development of two different approaches. The first, more common, utilises metal chelating agents, such as bis-imidazoles or functional monomers that form a small number of strong electrostatic interactions with a few specific binding sites on the template, usually with an organic or mixed solvent as the porogen. The second approach to macromolecular imprinting utilises the ability of MIPs to recognise molecular shape. The monomers used for development of shape-recognisable polymers are capable of forming multi point weak interactions such as hydrophobic or hydrogen bonds. Bio-analytical applications of MIPs usually necessitate aqueous conditions, however due to the strong hydrophobic interactions present in aqueous polymer systems, hydrophobic adsorption to the polymer surface can lead to strong non-specific binding. Although it is less convenient, it is possible to prepare imprinted polymers in organic solvents, which promote strong non-covalent/polar interactions such as hydrogen bonds (Andersson, 2000). Bovine serum albumin (BSA) and the enzyme urease have been imprinted using mixtures of 3-aminopropyltriethoxy-silane and tetraorthosilicate. The

polymers showed some specificity towards their target, however they lacked the stability of traditional cross-linked polymers, and the method itself was non-reproducible (Burow and Minoura, 1996; Rachkov and Minoura, 2001). In another approach, acrylamide was used for imprinting glucose oxidase in water (Burow and Minoura, 1996). The experiments showed that although the polymer had specificity initially, this was lost during drying and re-swelling; this was attributed to the lack of sufficient cross-linking in the polymer. On the other hand, higher degrees of cross-linking might increase the level of protein denaturation.

Critical issues in the imprinting procedure suited for protein imprinting include the stabilisation of the monomer-template complex in the pre-polymerisation mixture, removal of the template molecules and the choice of an appropriate format of the polymer. Steric and thermodynamic effects hinder the synthesis of MIPs selective for macromolecules such as proteins. The steric effect is because bulky proteins cannot slip in and out of the polymer network easily. The thermodynamic consideration indicates that the use of non-rigid protein templates yields less well-defined recognition sites (Rachkov and Minoura, 2001). One method of overcoming such hindrances, as well as the loss of yield and the destruction of affinity sites by grinding is to use a surface grafting technique. Copper II chelating imidazole polymers have been grafted onto activated silica beads suitable for HPLC (Plunkett and Arnold, 1995). Polystyrene microtiter plates were grafted with a polymer prepared in aqueous solution in the presence of various protein templates (Bossi *et. al.*, 2001). Such a polymer was prepared by the chemical oxidation of 3'-aminophenylboronic acid (APBA). The polymerisation product was grafted

tightly to the surface of the plates by aromatic ring electron pairing interactions.   Proteins such as horseradish peroxidase, lactoperoxidase, haemoglobin and microperoxidase have been imprinted by this method, and the resulting MIPs showed good specificity and affinity towards their templates.   The *in-situ* molecular imprinting of the triazine herbicide, desmetryn, in an aqueous system, onto commercial porous polypropylene membranes was achieved.   These membranes showed affinity towards the template and to other triazine herbicides, therefore presenting a quick and easy solid-phase extraction of triazine herbicides in water (Piletsky *et. al.*, 2000).

The epitope approach is another method in which many of the hindrances of macromolecular imprinting are avoided. As in nature, an antibody recognises and interacts with only the epitopes of an antigen; similarly, the imprinting of a small exposed fragment of a protein allows the recognition of not only the template, but also larger fragments containing the imprint fragment.   The recognition of the neurophyseal nona-peptide hormone, oxytocin was achieved through the imprinting of the tetra-peptide (YPLG), which was homologous to the hormones C-terminal region. Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) were copolymerised in acetonitrile. Acetonitrile was used to promote the formation of intramolecular hydrogen bond interactions, which are known to stabilise the conformational folding of oxytocin. In aqueous solutions, oxytocin is extremely flexible and it does not appear that intramolecular hydrogen bond plays a significant role in determining a molecule structure (Rachkov and Minoura, 2001).

1.7.4 Clinical applications of MIPs

MIPs have been prepared against the steroids, cortisol and corticosterone and used in the analysis of the functional status of the adrenal cortex (Ramstrom *et. al.*, 1996). Due to the rigid fused ring system of the corticosteroids, the number of conformations produced by molecules interacting with recognition matrices is small, which is good for narrowing binding site diversity. The few polar interaction points on steroid molecule contributed to the complex formation making non-covalent molecular imprinting specific, and increasing the binding strength of such polymers. In aqueous buffer systems, the polymers showed severely diminished specificities towards their targets, and the affinity of the reference polymers to the imprint molecules was similar to that of the imprinted polymers. In the solvent-based assays, the cortisol and corticosterone imprinted polymers gave detection limits in the range 10^{-7} and 10^{-8} M respectively, which may be improved by the development of a more sensitive assay (Ramstrom *et. al.*, 1996).

The imprints of drugs such as the anticoagulative metabolite 7'-hydroxycoumarin (7'-OHC) (Walshe *et. al.*, 1997) and bronchodilator theophylline (Mullett and Lai, 1998) have been used in solid-phase extraction and competition radioligand assay, respectively, to measure analytes in urine and blood samples. A MIP specific for 7'-OHC was capable of extraction of template present in 10-50 μl ml^{-1} concentrations (Walshe *et. al.*, 1997). Detection limits for the polymers used in the radioligand assays were defined as the concentration of ligand required for the displacement of 10 % bound

radio labelled compound. The calculated detection limit for theophylline was 3.5 μmol L^{-1}. Cross reactivities of the MIPs were highly comparable with those of their natural antibody counterparts (Ansell *et. al.*, 1996; Wulff, 1995). Molecular imprints of sugar derivatives have shown good enantiomeric selectivity and minimal cross-reactivity with similar compounds. The imprint polymer of the β-adrenergic blocker, (S)-(-)-timolol was also used in chromatographic separation of the print molecule from similar structures (Ansell *et al.*, 1996).

The polymer produced from the imprint of octyl-α-D-glucoside preferentially bound methyl-α-D-glucoside with binding energy two orders of magnitude greater than that for methyl-α-D-galactoside, which differs only by the orientation of one hydroxyl group (Ansell *et al.*, 1996). Berglund and colleagues in 1996 (unpublished results) imprinted the α_2-adrenoceptors yohimbine and coryanthine and the resulting polymers acted as receptor mimics that were both used in MIA analysis (reported in Ansell *et al.*, 1996). The two structures differ only by the orientation at the carboxyl-bearing stereogenic centre, yet the mimics showed good selectivity towards the template molecule in solvent and aqueous solutions.

MIP hydro-gels that displayed isomerically resolved glucose binding have been produced. Binding capacities of 0.5 g of glucose per gram of dried gel were observed, such polymers worked well in aqueous media (Wizeman and Kofinas, 2001). In 1997, Arnold's group (Chen *et.* al., 1997) prepared a glucose imprinted metal complexing polymer, which preferentially bound glucose in porcine plasma. The polymer functioned at alkaline pH and

released protons in proportion to the concentration of glucose bound, therefore had the potential to be adapted for use in a non-expensive, simple glucose assay. The incorporation of the signal recognition element into the bulk of the composition matrix is an exciting area of MIP sensor development. As with the glucose imprinted polymer and some optical sensing systems, binding of the target analyte produces a change in the properties of a reporter monomer, such as proton release in the glucose polymer and fluorescence quenching in a cAMP imprinted polymer (Haupt and Mosbach, 2000).

1.8 Biosensors

1.8.1 Introduction

A typical biosensor assay comprises of a surface immobilised ligand to which a ligand in solution is bound. A detector converts a signal from this binding event into readable electrical information that can be represented using computer software. In terms of biosensors, a recognition element is usually one of a pair of biospecific interaction molecules, such as antibodies and antigen, hormones and receptors or drugs and proteins (Lundstrom, 1994). Molecular recognition elements can be divided into two different categories

1. Catalytic, which include enzymes, microbes, cells and tissues

2. Affinity, which includes antibodies, MIPs, nucleic acids and receptors.

A surface-bound bioorganic derived or analogous recognition element coupled with a physico-chemical transducer is what constitutes a biosensor. They work by translating a recognised biochemical signal into an electronic output that can be read by simple computational methods. The surface, to which the template specific material is bound, should be able to produce a signal in proportion to the amount of recognised template. Five basic classes of transducer exist; optical (e.g. SPR based Biacore chips, (Biacore AB, Uppsala, Sweden)), electrochemical (e.g. glucose sensors (Chen *et. al.*, 1997)); thermometric, piezoelectric (quartz crystal microbalance) and magnetic transducers, which have not been researched as much as the latter two types.

1.8.2 Molecular recognition elements (MRE's)

Catalytic recognition elements form a smaller group of MRE compared to affinity based recognition group, but expansion in this part of the industry has become a scientifically exciting as the affinity group. Enzymes and their substrates have been used as catalytic recognition elements in biosensors to the point that their engineering and purification have become paramount to the current stability and sensitivity of the biosensors they are incorporated into. Examples include enhancement or introduction of thermostability into specific dehydrogenase enzymes in an attempt to lengthen enzyme biosensor shelf life, which may allow the more cost effective production of stable biosensors e.g. glucose biosensor (Kojima *et. al.*, 2000; Sode *et. al.,* 2000; Helianti *et. al.,* 2001). The isolation of proteases from various bacteria located in soil at the top of mountains (Morita *et. al.,* 1997) or in salmon intestines (Morita *et. al.,* 1998; Yamaura *et. al.,* 1999) may aid biosensing in cold environments, such as the food or agricultural industry. Modified β-cyclodextrins (oligosaccharides) have been incorporated into biomimetic biosensors for use as synthetic dehydrogenase enzyme mimics (Kataki and Morgan, 2003), a nicotinamide group allowed the electron transfer to occur while the cyclodextrin allowed the hydrophobic reaction to take place. DNA has been used as a catalytic tool for the real time specific biosensing of metal ions in solution; such a biosensor could be useful for environmental, clinical toxicology and industrial process monitoring (Lu *et. al.*, 2003). Cell based biosensors can incorporate the catalytic recognition elements of a cell, in an in-vivo array type system (reviewed in Durick and Negulescu, 2001).

Affinity recognition elements form a larger group of MRE and have been extensively used in biosensing applications. Antibodies and their antigens have been used expansively as immunosensing tools (covered in chapter 1.7), and molecularly imprinted polymers have also become an important type of synthetic catalytic recognition element in the past few decades (covered in chapter 1.8). The four categories of natural receptors, summarised in *Table 1.8.1*, have been integrated into biosensors for a variety of applications (reviewed in Subrahmanyam *et. al.*, 2002). Natural recognition elements such as enzymes, or those receptors that are enzyme linked are favoured for biosensor integration due to the number of measurable parameters that can be generated including protons, ions, electrons, heat, light and mass (Lowe, 1989).

Characteristics	Ion Channel Receptors	G-protein linked receptors	Receptors with a single transmembrane domain	Enzyme linked receptors
Endogenous Ligands	Neurotransmitters	Neurotransmitters, hormones, autocoids, chemotactic factors	Receptors with a single transmembrane domain	Atrial natriuritic peptide ligands, growth factors
Structure	Several proteins with a pore	1-2 proteins	1-2 proteins with catalytic domain	Individual protein linked with enzyme
Transmembrane Segments	Four	Seven	One	Single-pass transmembrane proteins
Function	Regulation of ion transport	Activation of G-proteins, regulation of cellular functions and protein expression	Catalytic	Suppress proliferation, stimulate synthesis of extracellular matrix, stimulate bone formation, attract cells by chemotaxis
Cellular Response	Depolarisation/ hyperpolarisation	Depolarisation/ hyperpolarisation	Regulation of cellular functions, proliferation and differentiation	Regulation of cyclase, production of cyclic GMP, cell signalling and regulation of cell cycle

Table 1.8.1 represents the classification of natural receptors (adapted from Subrahmanyam *et. al.*, 2002).

The enzyme-based biosensor generally produces an amplified measurable signal, whereas the non-enzyme linked receptor may produce a transducer-detectable conformational change. Generally this type of conformational change is difficult to measure without the specific attachment of fluorescent labels (Kostrzynska *et. al.*, 2002), the linkage to another amplification system (Baeumner *et. al.*, 2002; Pei *et. al.*, 2001; Lisdat *et. al.*, 1997) or very sensitive detection devices such as the piezoelectric crystals or quartz-crystal microbalance (Lin and Tsai, 2003; Wu, 1999; Wong *et. al.*, 2002; Steegborn and Skladal, 1997; Halamek *et. al.*, 2002). One optical transducer technique termed surface plasmon resonance (SPR) allows the real-time analysis of biospecific interaction or BIA (see chapter **4.1**), i.e. without labels or additional amplification system.

1.8.3 Transducers

The optical transducer category, which includes surface plasmon resonance (Biacore [www.biacore.com], IBIS [www.ibis-spr.nl], Nippon Laser Electronics [www.nle-lab.co.jp/English/ZO-HOME.htm], Texas Instruments [www.ti.com/sc/docs/products/msp/control/spreeta]) and evanescent wave biosensors (Affinity Sensors [www.affinity-sensors.com], Farfield Sensors [www.farfield-sensors.co.uk]) monitors the change in refractive index of the running buffer near the sensor surface as complexes form and break down in real time (Squillante, 1998; Huber et al., 1999). Optical transducer systems allow for labelless real-time biomolecular interaction analysis (BIA), even in crude preparations (Baird and Myszka, 2001).

Electrochemical transducer systems are those that produce an electrical signal due to a direct interaction with the recognition element and generally comprise of a biorecognition element incorporated into the electrode material. This enables for fast responses to the involved substrates because of the lack of recognition element-bound supporting membrane and the close proximity of the substrate to the electrode material. Interesting reviews detailing the materials used for and types of biochemical sensors have been produced in recent years (Zhang *et. al.*, 2000; Serra *et. al.*, 2002). The monitoring of glucose in diabetic patients has received much assistance with the development of the glucose oxidase (GOD) based glucose biosensor. As the nature of diabetes is such that continuous monitoring on blood glucose is essential, patients have been able to monitor their own glucose levels using a simple "pin prick" method. However, the 'idea' that a GOD based glucose sensor could be implanted into the patient to provide continuous online blood glucose monitoring has not yet been fully realised, due to biocompatibility problems causing inflammation and immune responses (Abel and Woedtke, 2002). A non-invasive glucose biosensor based on impedance spectroscopy has recently been through primary stage human clinical trials (Caduff *et. al.*, 2003), it is the size of a wristwatch and may prove constructive in the realisation of the continuous blood glucose monitoring 'idea'.

Thermometric biosensors measure the total heat energy absorbed or evolved during a biochemical reaction. This absorbed or evolved heat energy is proportional to the molar enthalpy and to the product stoichiometry of the biochemical reaction (Reviewed in Ramanathan and Danielsson, 2001). Piezoelectric transducers (or quartz crystal microbalance -QCM) comprise of

a specifically cut thin disc of quartz crystal with electrodes plated onto either one or both of its surfaces. If an oscillating electrical potential is passed through these electrodes then an acoustic wave propagates perpendicular to the surface of the crystal. If this crystal is placed into an electrical circuit and the current is oscillated at a similar frequency to the fundamental mechanical oscillation frequency of the crystal then a resonant oscillation may be achieved. In simple terms, the resonant oscillatory frequency is dependent on several factors including chemical structure, density and thickness of the crystal and similar physical properties of the surrounding medium. Therefore this frequency is also directly related to the amount or mass of material immobilised onto the surface of the crystal (Principles and applications reviewed in O'Sullivan and Guilbault, 1999).

Magnetic and pressure transducers are both small categories of transducers used in biosensing and have not been researched extensively. Magnetic bacterial particles have been incorporated into an automated ELISA type format for the detection and discrimination of single nucleotide polymorphisms (Tanaka *et. al.*, 2003). Another example of the magnetic technology was the integration of magnetic micro-beads with DNA hybridisation to detect pathogenic bioanalytes (Edelstein *et. al.*, 2000). Theodore Sand and colleagues have developed an immuno-sensor coupled to a high sensitivity pressure transducer, which measures a small pressure change due to the production of CO_2 in an airtight chamber. The use of a thin membrane, into which a sandwich immunoassay comprising of haptens and antibodies bound with catalase is incorporated, creates a surface for the progression of the assay and detection of the gas produced (Sand *et. al.*, 2003).

Chapter 2

Computational Ligand Design, Peptide Synthesis and Production of MIP for Solid Phase Extraction

This preliminary work came about as a result of the prothrombin molecular modelling. The aim was to identify a region of the computational prothrombin model that would act as an epitope for a synthetic adsorbent, based on an imprinted peptide. This region would be required to be specific for the native form of prothrombin and ideally locate to an accessible area of the protein, i.e. in a binding pocket or on the surface. An 8 amino acid region located in the thrombin fragment 1 region of the surface of prothrombin presented some interest, and was synthesised as an 8-mer peptide. The imprinted bulk polymer synthesised using the peptide as a template, showed a small amount of specific binding towards prothrombin at concentrations below 0.05 mgml^{-1}. It is known that prothrombin undergoes a conformational transition when complexed with calcium ions (Bloom and Mann, 1978); this calcium-binding gla domain was also of particular interest.

2.1 Introduction

2.1.1 Thermodynamics

The basis for the memory of molecular imprinted polymers lies in the formation of template-monomer associations in the pre-polymerisation mixture. These critical interactions can range from weak van der Waals forces to reversible covalent bonds (Nicholls et. al. 2002). The relative strengths of the monomer-monomer, template-template and monomer-template interactions govern the affinity of the subsequent polymer to its template. The affinity of the polymer to its template during the rebinding step is an area undergoing much development. It is known that high monomer

concentrations, lower polymerisation temperatures and the use of non-competitive solvents promoted increased affinity and selectivity towards the template. Increased pressure also has a beneficial effect on the stability and affinity of a polymer with its template (Sellergren et. al. 1996).

A thermodynamic treatment (Equation 1) can be applied as a basis for the better understanding of molecular recognition as harnessed by molecular imprinting.

Equation 1

$$\Delta G_{bind} = \Delta G_{t+r} + \Delta G_r + \Delta G_h + \Delta G_{vib} + \Sigma \Delta G_p + \Delta G_{conf} + \Delta G_{vdW}$$

The Gibbs free energy parameters in equation 1 above describe the following terms. Complex formation, ΔG_{bind}; translational and rotational forces, ΔG_{t+r}; restriction of rotors upon complexation, ΔG_r; hydrophobic interactions, ΔG_h; residual soft vibrational modes, ΔG_{vib}; the sum of the interacting polar group contributions, $\Sigma \Delta G_p$; adverse conformational changes, ΔG_{conf}; unfavourable van der Waals interactions, ΔG_{vdW}.

Translational and rotational energy ΔG_{t+r} is a term that describes the change in energy due to the rotation and translation (change of coordinates) of components within a molecular complex. The restriction of rotors ΔG_r, e.g. by the rigid alkaloid templates morphine (Fischer et. al. 1991) and yohimbine (Berglund et. al. 1996), during complexation promotes the formation of stable polymers. This is because the restriction of rotors also restricts the number of possible conformations the polymer can form around the template during complexation. The sum of all the polar group interactions $\Sigma \Delta G_p$, determines

the selectivity of the resulting polymer. As with the relatively strong binding of covalent imprinting methods (Khasawneh et. al., 2001), more electrostatic interactions between the monomers and template enables stronger, more stable complex formation and increased selectivity of the resultant polymer. In the work by Matsui and Takeuchi, the more acidic functional monomer TFMAA produced a nicotine-imprinted polymer that showed much more selectivity than a similar polymer created using MAA (Matsui and Takeuchi, 1997).

Hydrophobic and strong electrostatic interactions (such as those elicited by metal chelation monomers) are important in aqueous imprinting procedures. These interactions denoted by the term DGh, contribute to the thermodynamics of aqueous or polar pre-polymerisation complex formation. Aqueous imprinting procedures are necessary when biomedical or environmental templates are used (Kempe and Mosbach, 1995; Kempe et. al. 1995), where non-polar (organic) solvents cannot be used to dissolve the template. As aqueous imprinting requires hydrophobic interaction or electrostatic interaction in a polar environment, monomers (such as cyclodextrin) have been specifically designed for the job and have been utilised effectively in the recognition of steroids (Asanuma et. al. 1997) and amino acids (Piletsky et. al. 1999).

The soft vibrational modes term, DGvib is directly related to the temperature of polymerisation, a higher temperature provides the polymerisation complex with more energy and therefore more vibration. In order for solution adducts to form effectively, a compromise of template conformation and effective

solvation is required in the polymerisation complex, this is reflected in the terms ΔG_{conf} and ΔG_{vdW}. As we can assume that the template-polymer pre-arrangement and rebinding processes are under thermodynamic control and that rebinding take place in the polymerisation solvent, the unfavourable van der Waals forces and adverse conformational terms are dismissed. Therefore equation 1 can be simplified to equation 2, which relates to the "central dogma of molecular imprinting"; i.e. the recognition sites formed during pre-polymerisation are preserved in the final polymer.

Equation 2

$$\Delta G_{bind} = \Delta G_{t+r} + \Delta G_r + \Delta G_h + \Delta G_{vib} + \Sigma \Delta G_p$$

The thermodynamic parameters described above are essential considerations for the rational design of MIPs. The general principles illustrated above provide the general basis for the more empirical approaches applied to MIP development, despite the fact that complex polymer systems do not perform well on the application of complex thermodynamic principles. Molecular modelling, which performs very well, provides the computational theoretical platform to which the physical parameters of thermodynamics can be applied to complex model systems, such as molecularly imprinted polymers.

2.1.2 Modelling

The definition of a 'model' in the Oxford English dictionary is 'a simplified or idealised description of a system or a process, often in mathematical terms, devised to facilitate calculations and predictions'. 'Molecular modelling'

therefore would be concerned with ways in which molecular systems can be simplified and predicted.

For a computational program to be able to modify a molecule, it is obviously important to determine the atom positions within molecules. Cartesian coordinates could be used, however internal coordinates, usually written as a Z-matrix are an alternative. Internal coordinates are useful in describing the spatial relationship corresponding to atoms within a single molecule, however Cartesian coordinates may be more useful for a collection of discrete molecules (Leach, 2001). The information presented in a Z-matrix (see *Figure 2.2*) of internal coordinates describes the atom number, the atom symbol, the bond length (measured in angstroms, $1\text{Å} \equiv 10^{-10}\text{m} \equiv 100$ pm) to the next atom and the atom number of that atom. The angle formed by the atom and two other attached atoms, and the torsion angle produced by the atom and three other attached atoms.
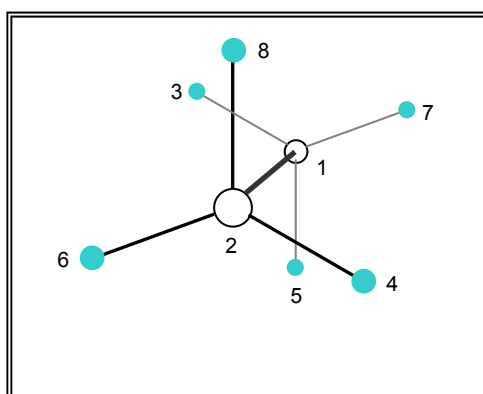
Figure 2.1.1 represents the staggered conformation of ethane.

The internal coordinates of each atom in the ethane molecule as in ***Figure 2.1.1*** can be represented in the following Z-matrix.

```
1   C
2   C       1.54    1
3   H       1.0     1       109.5   2
4   H       1.0     2       109.5   1       180.0   3
5   H       1.0     1       109.5   2        60.0   4
6   H       1.0     2       109.5   1       -60.0   5
7   H       1.0     1       109.5   2       180.0   6
8   H       1.0     2       109.5   1        60.0   7
```

Figure 2.1.2 represents the Z matrix configuration of the internal coordinates of ethane as depicted in *Figure 2.1.1*.

The overall aim of molecular modelling methods is to try to relate biological activity to the structure of the molecule. The computation of the potential energy of a molecule as a function of the positions of the atoms is essential (Forster, 2002). The approximation that nuclear and electronic motions of molecules can be separated is applied to standard molecular modelling. Therefore the energy of a molecule in its ground electronic state can be considered as a function of its nuclear coordinates. So if a nucleus within a molecule is moved e.g. by a reaction or simple bond rotation process, then the energy of the system will change. The 'potential energy surface' is a term used to describe the changes in energy of a molecular system (Leach, 2001).

Many of the problems considered in molecular modelling are too large to be tackled by quantum mechanics. In quantum mechanics, the electrons within a system are considered. As the calculations are so time consuming, such a large amount of particles cannot be considered using quantum mechanics. For large molecules such as proteins, the approximation known as 'molecular mechanics' or 'force fields' can be used (Burkert and Allinger, 1982). Molecular mechanics cannot therefore provide information on the electronic distribution of a molecule, but can be as accurate as quantum mechanics with

problem evaluation in a fraction of the time. In this approach there are energy terms that describe the deformation of bond lengths, bond angles and torsion angles away from their equilibrium values. Even when simple functions such as F_s= -kx (Hookes law) are applied to the system, molecular mechanics force fields perform adequately well.

Molecular mechanics, essentially serves to predict the energy of a molecule or complex of interacting molecules in a particular conformation. These energies can then be considered as relative values to compare and contrast between different molecules or complexes. The total energy of a particular conformation of a complex can be calculated by comparison to the accepted bond parameters stored in the molecular mechanics 'force field' database. The equations that describe the energetic parameters of a complex (Equation 1) are also stored in the molecular mechanics force field; some force field examples include TRIPOS (Clark *et. al*. 1989), MM2 (Allinger, 1977), and AMBER (Weiner *et. al.* 1984).

Equation 3

$$E_{\text{Potential Energy}} = E_{\text{stretching}} + E_{\text{bending}} + E_{\text{torsional}} + E_{\text{electrostatic}} + E_{\text{vdW}}$$

Equation 3 describes the energetic parameters used to represent molecular structure in molecular mechanics calculations.   The potential energy parameter is comprised of the five different force parameters that describe the movements of all the bonds in a molecular complex.

2.1.3 Ligand design

Dramatic increases in computational power, improvements of methodology and algorithms for the calculation of empirical binding energies, has increased the rate and quality of biomolecular systems analysis and their atomic interactions, beyond that which can ever be achieved manually (Joseph-McCarthy, 1999). The application of molecular modelling software such as Sybyl 6.9 (Tripos Inc., St. Louis Missouri, USA) or Quanta (Accelrys Ltd. Cambridge, UK), can introduce an altogether new 'rational' methodology, which can greatly enhance ligand design and subsequent production of synthetic biochemical adsorbents.

Computational modelling and ligand design involves the simulated fitting of functional monomers from a virtual library (Piletsky *et. al.*, 2001a), into specific areas of the target biomolecular computational model with computer algorithms such as LEAPFROG (Ash *et. al.*, 1996). Prior to the monomer probing, the target molecular model is refined by charging the atoms (e.g. Gasteiger-Huckel) and application of molecular mechanics algorithms to 'minimise' the 'strain' energy imposed on the atoms in the molecule (e.g. MAXIMIN2) (Labanowski *et. al.*, 1986). Inhibitors, specific for coagulation factor Xa have recently been identified and analysed relative to their quantitative structure-activity relationships (QSAR's) using SYBYL® molecular modelling software. Literature values of known interactions between factor Xa and established inhibitors provided the basis for the docking of novel potential inhibitor molecules from a virtual library (Matter *et. al.*, 2002).

Molecular modelling techniques have been used to simulate human protein molecular dynamics, with limited use of NMR data and atomic coordinates specific for the protein in question.   Currently, NMR crystal structure data regarding human prothrombin is limited and centres around predictive molecular modelling of human prothrombin fragment-1 based on its significant homology (82 %) with bovine prothrombin.   This model was created using literature data from the NMR crystal structure of bovine prothrombin fragment-1 (Soriano-Garcia *et. al.*, 1992) and Sybyl 6.0 (Tripos Inc., St. Louis Missouri USA) (Li *et. al.*, 1995).

The rational design of ligands using a computational approach can be applied to the design of molecularly imprinted polymers, for use in biomolecular assays in various environments. The computational approach has been used to design selective MIPs for ephedrine enantiomers (Piletsky *et. al.*, 2001a), creatinine (Subrahmanyam *et. al.*, 2001), microcystin-LR (Chianella *et. al.*, 2002) and synthetic adsorbents for coagulation factor VII (Morrill *et. al.*, 2002).   The computational approach can be applied to the generation of synthetic adsorbents, based on interactions of ligands with specific regions of biomolecules.   A region, analogous to an antibodies epitope, can provide a 'handle' for the synthetic recognition and capture of a complete biomolecule. This approach can be termed "the epitope approach", and is based on the imprinting of a short peptide template, which resembles a region (epitope) of the protein under investigation (Rachkov and Minoura, 2001).

The peptide FNEKTFGA was selected from initial computational studies of a human prothrombin model, as a template for the synthesis of MIPs for the

potential recognition of human prothrombin. This peptide was screened against a 'virtual' functional monomer database (*Figure 2.2.1*) using the Leapfrog algorithm within Sybyl 6.7. The two best monomers were selected for the production of a MIP that showed selectivity for the synthesised peptide template and potentially human prothrombin. Investigations into the synthesis of a second, potentially more interesting region of the human prothrombin 'gla' domain, proved fruitless due to financial and logistical barriers. However evidence exists that makes this one of the most interesting avenues for future studies in the development of a novel native prothrombin assay, as will be discussed later.

2.2 Materials and methods

2.2.1 Molecular modelling of human prothrombin

A Silicon Graphics Octane workstation running IRIX 6.5 was used together with Sybyl 6.9 molecular graphics software from Tripos Inc., USA (http://www.tripos.co.uk).   The   protein   data   bank   (PDB) (http://www.rcsb.org/pdb/) was used to acquire the tertiary structure of bovine prothrombin   and   the   expert   protein   analysis   server   (ExPASy) (http://ca.expasy.org/sprot/) was used to search the Swiss-Prot/TrEMBL database for the primary (amino acid) sequence of human prothrombin. The primary and tertiary structures of human and bovine thrombin were also sourced using the same search engines.

The protein models were charged using Gasteiger-Huckel charges throughout and the MAXIMIN2 command was used to refine the molecular mechanics of the molecules. A target minimum energy of 0.05 Kcal mol^{-1} was used for all minimisations.   The Amber95 force field, incorporated into the Sybyl 6.9 Biopolymer package and corresponding metal parameters (contained within a Sybyl programming language script, Amber_metals.spl) were employed in an attempt to minimise the calcium complexed gla domain of bovine and human prothrombin.

2.2.2 Synthesis of rationally designed peptides: *FNEKTFGA*

Fmoc amino acids utilised for the synthesis of FNEKTFGA included alanine (A), 2x phenylalanine (F) and glycine (G) and those with protected side chains included O-tertiary-Butyl asparagine (N), glutamic acid (E) and threonine (T) and Boc lysine (K).

Alanine preloaded (0.367 g or 0.7 mmol g^{-1}) 2-chlorotrityl-chloride resin was used for the attachment of the C-terminal portion of the peptide. Synthesis was carried out using 0.25 mmol Fmoc chemistry on a Perkin Elmer (Applied Biosystems-433A) solid phase peptide synthesiser; the nature of the chemistry and synthesiser control software consumed four equivalents of each amino acid.

2.2.3 Cleavage of resin

The resin (0.62 g) was treated with 5 ml 95 % TFA in 2.5 % TIS and 2.5 % water for 2 hours at room temperature. After this time the resin was filtered and washed with 5 ml 95 % TFA in DCM. Approximately 100 ml of ether was added to the resulting filtrate and significant amounts of precipitate were obtained. This mixture was allowed to stand at 4 ^{o}C for 1 hour, which afforded a white residue. The ether solution was decanted and the solid residue was repeatedly washed with ether until all the TFA had been removed resulting in a white solid.

The product was obtained and a small sample was sent for mass spectrum analysis. The expected mass was 913 .44185 and the measured mass was 913.62134, see appendix for mass spectrograph output.

2.2.4 Molecular imprinting

Gasteiger Huckel charges were again used to charge the molecule and the bond energies were then minimised to approximately 0.01 kcal mol^{-1}. The peptide (*FNEKTFGA*) was computationally screened using the LEAPFROG algorithm within SYBYL 6.9, against a database containing 20 common functional monomers; the database is illustrated in *Figure 2.2.1*. This was carried out to determine the strongest possible binding interactions with the monomers, monomers that interacted strongly with the template were chosen for the imprinting process.

Acrylamide was predicted to have a high binding efficiency and therefore considered to be the best monomer for imprinting; ethylene glycol dimethacrylate (EGDMA) was used as the cross-linking monomer. A 5 % template solution was made by dissolving 50 mg of the peptide (template), 50 mg of acrylamide and 900 mg of cross-linker in 1057 μl (1 g) of DMF. Sonication produced a near clear solution to which 9 mg of initiator (1,1,azobis (cyclohexanecarbonitrile)) was added. Continuous rotation of the reaction vessel under a 300 W Cermax, UV-Xenon fibre-optic light source (ILC Technology, CA94089, USA) for approximately two hours allowed the polymerisation to occur.

F1 1-VINYLIMIDAZOLE

F2 2-VINYLPYRIDINE

F3 ACRYLAMIDO-2-METHYL-1-PROPANESULFONIC ACID (AMPSA)

F4 2-HYDROXYETHYL METHACRYLATE

F5 4-VINYLPYRIDINE

F6 ACROLEIN

F7 ACRYLAMIDE

F8 ACRYLIC ACID

F9 ACRYLONITRILE

F10 ALLYAMINE

F11 p-DIVINYLBENZENE

F12 ETHYLENE GLYCOL DIMETHACRYLATE (EGDMA)

F13 UROCANIC ACID ETHYL ESTER

F14 ITACONIC ACID

F15 m-DIVINYLBENZENE

F16 N,N-METHYLENE BIS ACRYLAMIDE

F17 METHACRYLIC ACID

F18 STYRENE

F19 UROCANIC ACID

F20 N,N-DIETHYLAMINO ETHYL METHACRYLAT (DEAEM)

Figure 2.2.1 is an illustration of the structures of the functional monomers within the database. The LEAPFROG algorithm within SYBYL 6.9 was used for the screening process, between the functional library and the peptide template (*FNEKTFGA*). The binding energy of each monomer with the template was calculated, and the top four are represented in *Table 2.3.1*. The monomers with the most non-covalent interactions also possessed the highest binding energies (Piletsky *et al.*, 2001a).

2.2.5 Solid phase extraction

The polymer was carefully ground, after separation from the broken reaction vessel, using a pestle and mortar. The polymer was wet sieved through a 106 μm and a 38 μm filter using methanol; the 38 - 106 μm and the <38 μm (fines) fractions were both collected and a yield of 270 mg of 38-106 μm particles was obtained. An SPE cartridge with 50 mg of polymer was prepared with 38 - 106 μm particles. An SPE cartridge was packed with 50 mg of the 38 - 106 μm fraction of the MIP. The template was eluted from the SPE cartridge using alternate washes of varying concentrations of acid (10 mM, 75 mM, 100 mM and 500 mM HCl) and base (10 mM, 75 mM, 100 mM and 500 mM NaOH) and RO (Reverse Osmosis) water.

Solutions containing 0.005 mgml^{-1} to 0.4 mgml^{-1} of prothrombin, thrombin and albumin made using 100 mM phosphate buffer solution (comprising 3.5 g Na$_2$HPO$_4$ and 3 g of NaH$_2$PO$_4$ in 500 ml of RO H$_2$O at pH 7.4) were used to measure the affinity and specificity of the MIP. Prior to SPE, calibration curves of each protein's spectral absorbance at 280 nm over the concentration range 0.005 mgml^{-1} to 0.4 mgml^{-1} were constructed. Subsequently 2 ml of each protein solution was filtered through the SPE cartridge and the spectral absorbance of the eluant was measured at 280 nm. The percentage absorbance of the protein at each concentration was calculated using the calibration and the eluant absorbencies. After each filtration the cartridge was washed, to remove any bound protein, it was found that water removed the bound protein.

2.3 Results

2.3.1 Modelling

The tertiary structure of human prothrombin as determined by NMR or X-ray crystallography has not, as yet been submitted to the protein database. Therefore in order to construct a molecular model of human prothrombin, the homologies between human and bovine prothrombin species had to be taken into consideration. Bovine prothrombin shows approximately 80% homology with human prothrombin, determined by comparison between the two native forms as deposited in the Swiss-Prot/TrEMBL databases (Figure 2.3.1). Similarly the complete X-ray crystal structure of bovine prothrombin has yet to be recorded in the PDB. Therefore the crystal structure of bovine meizothrombin (1A0H) as determined by X-ray diffraction (Martin et. al., 1997), and the Ca2+-complexed structure of bovine prothrombin fragment 1, also determined by X-ray diffraction (Soriano-Garcia et. al., 1992) were both used to create a bovine prothrombin molecular model using SYBYL 6.9.

Using the human prothrombin primary sequence (ExPASy accession number: P00734), extracted from the Swiss-Prot/TrEMBL databases, the primary sequence of bovine prothrombin (ExPASy accession number: P00735) was mutated (Figure 2.3.2). The resulting sequence was minimised until the bond energies approximated to 0.05 kcal mol-1; this produced a structure that was considered to be a molecular model of human prothrombin based on its homology relationship with bovine prothrombin. After minimisation the structure of the newly formed prothrombin model was checked against data

from the Swiss-Prot view of human prothrombin (P00734) on the ExPASy website. The human prothrombin structure was then altered to represent the established data as necessary and minimised again.

Figure 2.3.1 illustrates the homology between the amino acid sequences of human (P00734) and bovine (P00735) prothrombin. The red boxes represent areas of dissimilarity between the two sequences, which equates to approximately 81.1% homology between human and bovine prothrombin. This homology data was acquired from a BLAST search comparison on the SIB BLAST network service using the Uniprot database, where the two sequences were aligned using the CLUSTAL W sequence alignment algorithm (http://ca.expasy.org/sprot/). The European Bioinformatics Institute (EBI) produced a similar output when the two sequences were aligned (http://www.ebi.ac.uk/).

Prothrombin fragment 1 mutations: Mutations 1-25

Mutation No:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Residue No:	3	19	22	23	35	36	40	53	56	59	60	70	72	77	79
Change From	K	L	P	R	L	S	A	N	E	N	E	V	M	N	S
Change To:	#	V	T	Y	S	T	V	T	D	A	A	L	LGT	H	N

Mutation No:	16	17	18	19	20	21	22	23	24	25	# = null mutation				
Residue No:	80	111	120	122	131	137	141	162	163	164	LGT = multiple mutation				
Change From	V	R	G	I	S	E	V	E	V	I					
Change To:	I	Q	S	T	D	Q	I	A	M	T					

Prothrombin fragment II mutations: Mutation numbers 1-39. Thrombin chains A+B: Mutation numbers 40-71

Mutation No:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Residue No:	3	6	13	14	16	26	27	32	34	42	52	66	72	73	78
Change From	L	T	R	E	R	S	R	S	E	D	A	A	D	Q	E
Change To:	P	Q	Q	Q	Q	L	P	A	A	H	V	V	G	K	G

Mutation No:	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Residue No:	88	90	91	92	93	96	101	103	105	112	113	114	115	116	118
Change From	P	D	G	D	L	R	P	P	A	S	E	D	H	F	P
Change To:	A	E	E	E	T	G	E	S	R	A	T	S	E	Y	T

Mutation No:	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Residue No:	122	123	127	142	143	144	146	152	157	5	9	11	56	57	74
Change From	E	K	A	Q	V	Q	Q	F	E	Q	V	L	V	D	K
Change To:	P	R	G	S	L	E	K	L	D	S	I	M	E	N	N

Mutation No:	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Residue No:	75	82	93	103	107	109	110	111	123	124	128	131	134	142	143
Change From	V	D	K	L	R	I	E	L	K	Q	K	H	F	G	N
Change To:	I	E	R	M	K	V	A	F	R	E	S	Q	Y	N	L

Mutation No:	61	62	63	64	65	66	67	68	69	70	71				
Residue No:	144	145	151	153	154	175	193	215	256	257	259				
Change From	R	R	S	A	E	A	G	Y	R	L	S				
Change To:	K	#	N	G	K	D	D	F	Q	F	E				

Figure 2.3.2 represents the mutations imposed on bovine fragment 1 and meizothrombin sequences in order to produce a humanised prothrombin molecular model.

Glutamic acid was modified to produce g-carboxyglutamic acid and was assigned specific Amber95 parameters and atom types, represented in Figure 2.3.3 below.

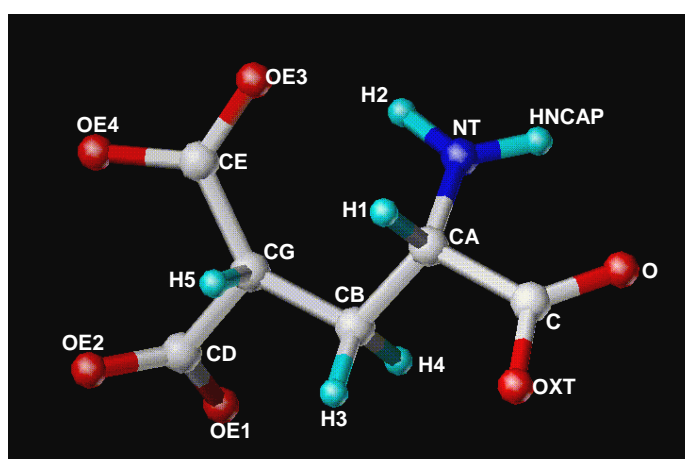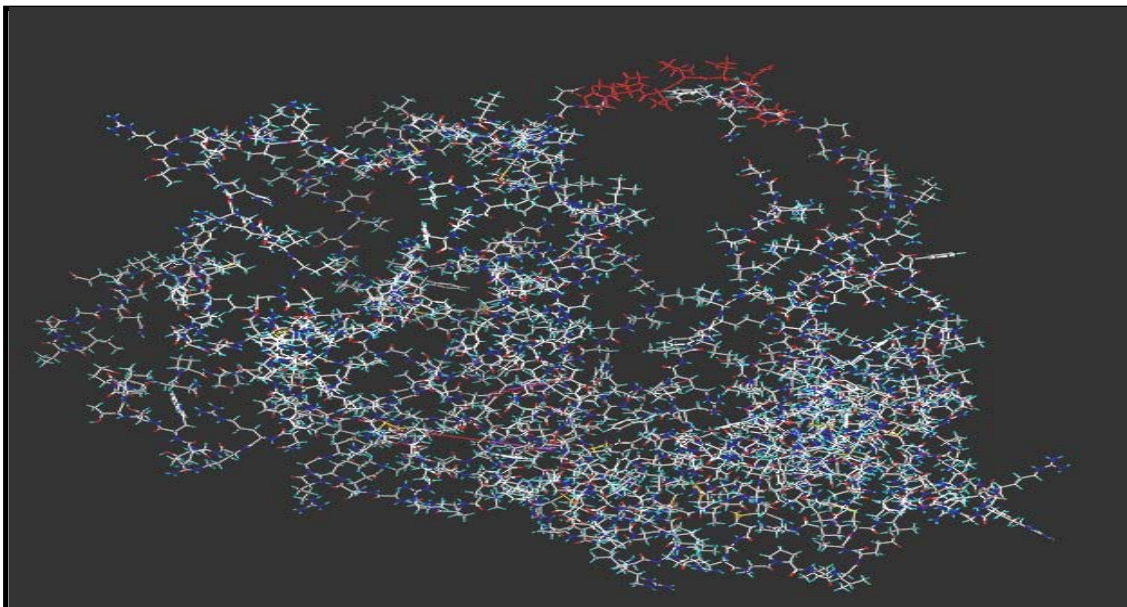**Figure 2.3.3**. γ- **carboxyglutamic acid.**   The extra carboxyl group attached to the Gamma carbon CG comprising of CE, OE3 and OE4 was charged with the following respective point charges 0.8054, -0.8188 and –0.8188.

Figure 2.3.4 represents a graphical output of our computational model of humanised bovine prothrombin. The amino acid sequence of bovine prothrombin was extracted from the PDB (http://www.rscb.org/pdb/). It was mutated manually to the human prothrombin sequence, which was retrieved from the ExPaSY molecular biology server (http://www.expasy.ch/). The bovine and human prothrombin primary sequences show 80% homology with each other, therefore increasing the potential of producing a valid protein model after minimisation. Minimisation was undertaken using Gasteiger Huckel charges and the Tripos general-purpose force field. The exposed area in red represents the 8-mer peptide chosen for molecular imprinting (right to left ***FNEKTFGA***).

As the 3D-structure of human thrombin and prothrombin have not yet been elucidated, the bovine structures, which show 80 % homology to the human structures, were used as templates for the modelling work. The primary structure of prothrombin fragment 1 was mutated 27 times, which included 1 deletion, 3 insertions and 23 replacement mutations; 40 amino acids were also replaced in prothrombin fragment 2. Thrombin underwent 31 mutations, which included 30 single amino acid replacements and 1 deletion. The bond energies in each structure were minimised over 100 thousand iterations, with the minimum target set at 0.05 kcal mol-1. As the structures were to be used

as guides to the identification of potential imprinting sites, 100 thousand iterations was considered sufficient. The bond energies in the last 1000 iterations of the prothrombin minimisation were in the range 0.4-0.15 kcal mol^{-1}.

2.3.2 Molecular Imprinting of Peptide, FNEKTFGA

The imprinted peptide (**FNEKTFGA**), representing 8 residues close to the exposed factor Xa cleavage site on the surface of prothrombin (**Figure 1.2.1**), was considered to be an interesting preliminary imprinting target. It was also considered that the surface location of the site would allow the 8-mer imprinted polymer to bind prothrombin relatively easily from solution. The screening of the peptide template (**FNEKTFGA**) with the 20 common imprinting monomers using the LEAPFROG algorithm within the SYBYL 6.7 package, allowed us to visualise the strongest monomer-template interactions, and therefore select the best monomers for imprinting. The binding energies for the strongest interacting four monomers with the template are represented in **Table 2.3.1**. Acrylamide was chosen as the best monomer for imprinting the peptide template, due to the strong binding energy of 44.31 kcal mol^{-1}, and therefore predicted strong interaction with the template. A graphical representation of the interaction between the template and acrylamide is depicted in **Figure 2.3.4.**

Structure				
	LPF_3	LPF_17	LPF_24	LPF_1
Monomer Name	Acrylamide	Itaconic Acid	2-(Trifluoro) Propenoic Acid	Bisacrylamide
Binding Energy (Kcal/mol)	44.31	41.16	36.54	36.06

Table 2.3.1 represents part of the table produced by the LEAPFROG algorithm, and shows the monomer name, structure and binding energies of the four strongest interacting monomers with the template (**FNEKTFGA**).
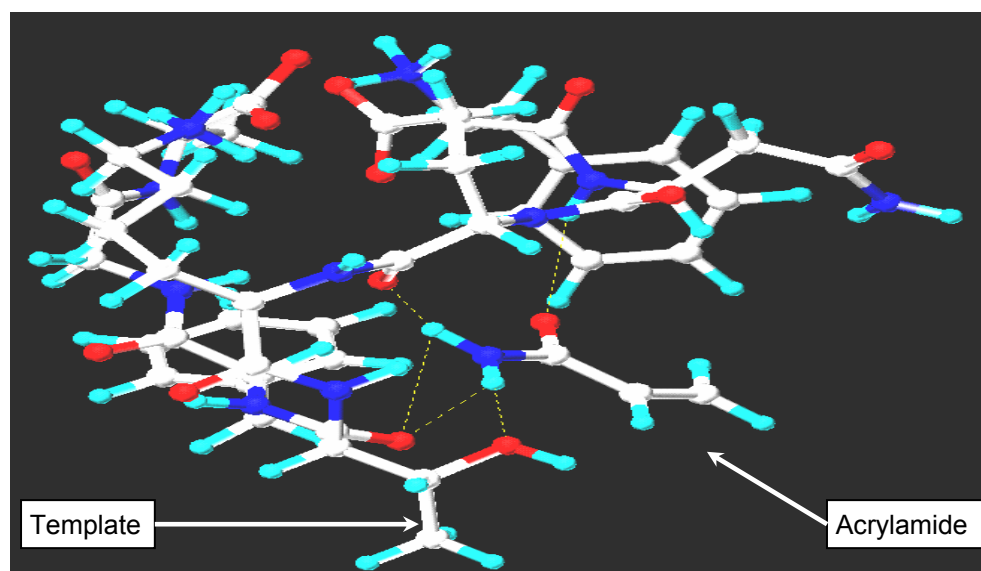
Figure 2.3.5 is a computational representation of the interactions between acrylamide and the peptide template (**FNEKTFGA**), as predicted by the LEAPFROG algorithm within SYBYL 6.7. The nitrile group (NH_2) and the carboxy group (CO) of acrylamide interact non-covalently with various residues of the peptide template.

The strongest binding energies are possessed by monomers that produce multiple non-covalent interactions with the template; the multiple hydrophobic interactions between acrylamide and the peptide are represented above in **Figure 2.3.5**.

There were initial concerns over the specificity of the MIP; therefore its affinity was assessed by assaying the SPE cartridge packed MIP's affinity with varying concentrations of prothrombin, thrombin and albumin in solution. The results of initial tests with prothrombin and thrombin using the concentration range of 0.02 - 0.4 mg ml^{-1} (or 2.78 x10^{-7} - 5.56 x10^{-6} M) are represented in *Figure 2.3.6*. The experiments were repeated using the protein concentration range 0.1 - 0.005 mg ml^{-1} (or 1.39 x10^{-6} - 6.94 x10^{-8} M) and including albumin as a control protein to which minimal cross reactivity was expected.
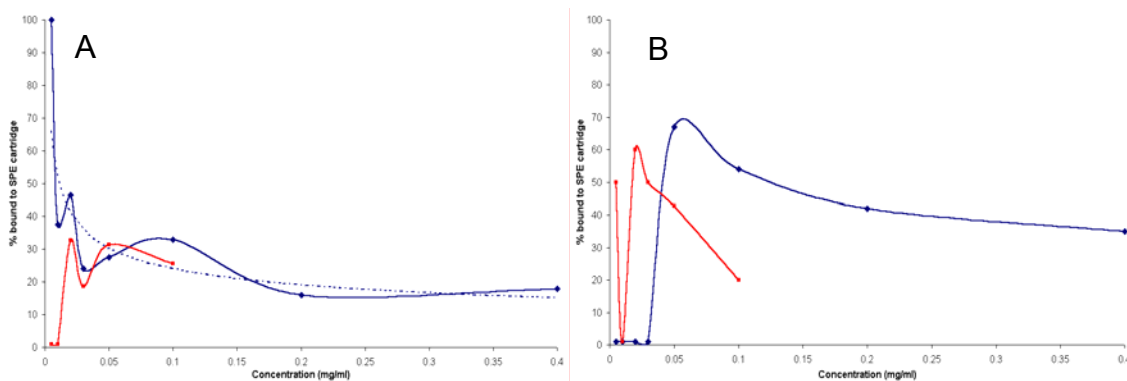
Figure 2.3.6 represents initial data describing the solid phase extraction of prothrombin (A) and thrombin (B) using the peptide (FNEKTFGA) imprinted MIP, which was ground and packed into an SPE cartridge. Red represents the data corresponding to protein injections through the cartridge packed with the blank polymer and blue represents the peptide imprinted MIP. Concentrations of prothrombin below 0.05 mg ml^{-1} seem to bind more strongly than the higher concentrations. The blank polymer however seems not to show any trend in binding, and all concentrations of prothrombin and thrombin show trend-less binding to the blank polymer.

As shown in *Figure 2.3.6* the peptide imprinted polymer showed a small amount of selectivity towards prothrombin, however only at very low concentrations. The lower concentration that would be expected in plasma

samples is approximately 0.1 mg ml^{-1}, and this concentration of thrombin and prothrombin binds equally well in to the blank polymer.   However, the percentage of thrombin, compared to the percentage of prothrombin bound to the peptide imprinted MIP is higher at all concentrations above 0.05 mg ml^{-1}.

2.4 Discussion

The molecular modelling of a reliable human prothrombin structure was attempted, with the aim of providing theoretical information on potentially interesting structural characteristics of human prothrombin. This information would have been useful for the identification of structural regions of the prothrombin molecule, to provide potential templates for molecular imprinting experiments. The application of molecular imprinting for the recognition of specific epitopes has been documented previously (Rachkov and Minoura, 2001), and shares an analogous relationship with natural biomolecular recognition by monoclonal antibodies. Native human prothrombin shares over 90% homology with inactive prothrombin and contains normal glutamic acid residues in place of the g-carboxyglutamic acid residues in the gla domain. The modelling of the native human prothrombin gla domain therefore could have provided us with a graphical representation of a specific epitope. Which may have provided the necessary target for the synthesis of a synthetic recognition element and its integration into an assay for native human prothrombin.

Our computational experiments using a humanised form of bovine prothrombin produced a structure that possessed limited reliability as a

human prothrombin model. However, useful information was obtained that lead to the determination of several interesting sites, which may have been useful epitopes for molecular imprinting experiments. The prothrombin fragment 2/thrombin cleavage site was synthesised, and the peptide template was used to produce a bulk-imprinted polymer. This site was considered useful because it possessed high prothrombin specific homology and a lack of homology with other blood borne proteins, as deduced by a homology BLAST-P search (ExPaSY: http://ca.expasy.org/sprot/). The minimisation of our human prothrombin computational model, using the Gastieger-Huckel force field together with Tripos atom types and charges, did not accommodate long-range ionic forces and protein-metal interactions; therefore the gla domain was inaccurately modelled. The gla domain of native human prothrombin contains 10 g-carboxyglutamic acid residues that complex with 8 calcium ions in a specific structural conformation. Long-range ionic forces restrain the calcium ions with the carboxyl-oxygen atoms of carboxyglutamic acids in the gla domain (Li et. al., 1995), which promotes binding of the gla domain to phospholipid membranes and the activation of prothrombin to thrombin.

The modelling of bovine (Hamaguchi et. al., 1992) and human prothrombin fragment 1 (Li et. al., 1995), using the coordinates of corresponding residues in the structure of bovine prothrombin has been attempted previously. In 1995, Leping Li and colleagues modelled the calcium coordinated 'gla' and fragment 1 domain of human prothrombin, using the previously elucidated crystal structure of bovine prothrombin fragment 1 (Soranio-Garcia et. al., 1992) as a template. Careful simulation protocol, which accommodated long-

range ionic forces between calcium and carboxyl-oxygen atoms, was applied to solve the highly ionic gla domain of human prothrombin. Li et. al., used a modified version of the Amber 3 force field for the energy minimisation of the gla domain of human prothrombin, however when we attempted to apply the Amber 4.1 force field, (Amber95) (Weiner et. al., 1984), modified to include parameters for calcium ions, minimisation did not proceed. The more general MMFF94 and Gastieger-Huckel force fields included within the Sybyl 6.9 package again did not minimise the prothrombin molecule correctly. The parameterisation of calcium ions was a major problem associated with the unmodified force fields, as they were only recognised as dummy atoms without the correct charges and electrostatic parameters. Therefore minimisation of the metal complexed prothrombin molecule produced an unfolded gla domain much like that of abnormal prothrombin, which contains glutamic acid residues in place of the g-carboxyglutamic acid residues. It was also found that the Amber95 force field experienced difficulties in assigning atomic parameters, such as point charges and atom types to the extra carboxyl group of the g-carboxyglutamic acid residues. Parameters were assigned to g-carboxyglutamic acid using glutamic acid as a template; therefore both carboxyl groups received identical parameters and sequential atom types, which may not have been correct.

The imprinting of protein templates has been achieved many times by methods such as surface grafting (Kempe *et. al.*, 1995; Piletsky *et. al.*, 2000a and 2000b; Bossi *et. al.*, 2001) and the use of hydrogels (Burow and Minoura, 1996; Liao *et. al.*, 1996; Hjerten *et. al.*, 1997). These two methods allowed the use of hydrophilic macromolecular templates such as peroxidase

enzymes (Bossi *et. al.*, 2001), Ribonuclease A, Myoglobin and Growth Hormone (Kempe *et. al.*, 1995; Hjerten *et. al.*, 1997) to generate template specific synthetic adsorbents, which could be used in solid phase separation. The use of a peptide that represents only part (or an epitope) of a protein in molecular imprinting is an attractive idea, due to the removal of the need for stringent protein treatment and aqueous imprinting conditions. The epitope approach to MIP production (Rachkov and Minoura, 2001) shares an analogous relationship with antibody recognition of protein epitopes, and combined with computational methods can enable the synthesis of synthetic adsorbents without the use of expensive and chemically cumbersome protein templates.

The design of the peptide (FNEKTFGA) imprinted MIP was considered rational, due to the completion of initial molecular modelling of human prothrombin that allowed the selection of the peptide through the visualisation of surface located regions of the protein. A BLAST search for homology relationships with other proteins using the Expert Protein Analysis Server website (ExPaSY: http://ca.expasy.org/sprot/), showed that the peptide was unique to prothrombin. The MIP did not yield good separation results however, which may have been due to the relatively small size of the representative peptide compared with the macromolecular size of prothrombin. For this reason it is now considered that the peptide would not have allowed the production of sufficient specific covalent binding sites in the MIP. To attempt the capture of such a large protein, within a complex medium such as plasma, using such a small specific recognition area would be difficult. Previously the epitope approach has yielded good protein and

template binding, however a relatively large proportion (4 amino acids out of 9) of a small target molecule was used (Rachkov and Minoura, 2001). But the epitope approach to protein imprinting has not been applied to many other areas of protein recognition.

It is known that the gla domain of native prothrombin is complexed with calcium ions that impose a conformational structure on the N-terminal region of prothrombin, which is absent in abnormal prothrombin (Bloom and Mann, 1978). Studies have emphasised the fact that the gla domain possesses much of the antigenic and structural variation between native and abnormal prothrombin (Blanchard *et. al.*, 1983). Therefore the proposal that the gla domain, or a peptide representing a specific region of the gla domain could act as a native prothrombin specific antigen is an interesting one. This idea was pursued to the point of contracting out the synthesis of a peptide region of the gla domain, incorporating four γ-carboxyglutamic acid residues. However this contract could not be fulfilled due to logistical and financial complications associated with obtaining modified glutamic acid residues. The target, representing the gla domain, was still considered to be interesting with respect to potential prothrombin recognition of the 'gla'-peptide molecular imprinted polymer. However, research has now shown that the gla domain may not be the ideal antigenic target for the development of a specific native prothrombin assay, due to the significant homology it shares with other gla containing proteins in the blood. This research indicated that antibodies directed to the prothrombin gla domain, also recognised proteins such as protein C, factor VIII and calcitonin (Brown *et. al.*, 2000), which would potentially give false positive results in any assay. One alternative could be

to reliably model the human gla domain and computationally identify synthetic ligands, which show strong electrostatic binding to the calcium dependent prothrombin conformation. This has been achieved previously with human Factor VII, and a reliable assay was developed from such research (Morril *et. al.*, 2002).

A narrow toxic-therapeutic ratio associated with warfarin oral anticoagulation therapy (OACT), may induce thrombotic and haemorrhagic complications, therefore strict monitoring of such therapy is essential. Currently the monitoring of OACT is achieved through the regular sampling of blood and subsequent measurement of the INR (***Chapter 1.5***). The current monitoring methods however, do not predict all of the complications associated with OACT (Palarati *et. al.*, 1996; Palarati *et. al.*, 1997; Pengo *et. al.*, 2001), therefore more accurate OACT monitoring methods must be investigated. The monitoring of the native prothrombin antigen has been shown to predict OACT associated complications with increased accuracy, compared with the INR (Furie *et. al.*, 1984; Furie *et. al.*, 1990; Kornberg *et. al.*, 1993). The production of prothrombin specific antibodies, involves the immunisation of a laboratory animal with prothrombin. Therefore the production of a synthetic prothrombin antibody, which involves the imprinting or templating effect of the complete protein, is also a valuable proposal. However, molecular imprinting is a technology that is not so broadly applicable to aqueous imprinting methods or protein recognition.

Molecular modelling of human prothrombin, using bovine prothrombin as an initial protein structure, did not produce a reliable model of the calcium-

complexed human prothrombin conformer.   We have produced a novel synthetic polymer, using a computationally identified prothrombin specific peptide sequence, located at the thrombin cleavage site.   However, as described above, the polymer did not recognise prothrombin as we had first anticipated, therefore we will move on to the imprinting of the complete prothrombin molecule.

Chapter 3

Poly-aminophenylboronic acid (poly-APBA) and aqueous imprinting of prothrombin

Here, the aims include the production of polymeric adsorbents in aqueous environments that have specificity for prothrombin in homologous solutions. The selectivity of these adsorbents should be such, that they should selectively bind prothrombin in heterogeneous solutions such as plasma. The prothrombin selective adsorbents should be incorporated into a sensor system whose interface would provide comprehensive real time data on the binding of prothrombin from various solutions. The same sensor system and interface should be used to incorporate an antibody recognition element directed towards the same target protein as with the polymeric absorbents. This would allow the direct comparison of biological and synthetic adsorbents in the aqueous/biological environment. The majority of the work will involve the use of the monohydrate boronic acid derivative, 3-aminophenylboronic acid (APBA), but acrylamide gel will also be used in an aqueous prothrombin imprinting study.
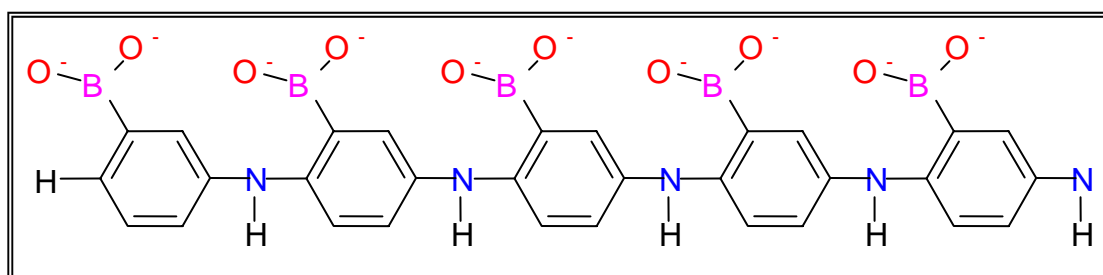
Figure 3.1 is an illustration of the two dimensional structure of poly-aminophenylboronic acid. The boronic acid residues are negatively charged and interact with any positively charged regions of proteins or target molecule.

3.1. Introduction

3.1.1 Surface plasmon resonance

Surface plasmon resonance (SPR) is an optical transducer based technique that relies on the total internal reflection of light within a prism. Light energy passing beyond the optical interface forms an evanescent field, which can be absorbed by free electrons in a thin metal coating on the surface of the optical interface. The angle of incidence at which SPR occurs can be used to monitor the rate and extent of biological interactions with surface bound materials in real-time. For example, it is possible to directly visualise the binding of injected template to transducer-surface immobilised antibodies; the change in the angle of incidence is directly related to the binding of additional material to the transducer surface and the medium passing over the surface.

Therefore SPR can be characterised as a label-less real time biospecific interaction analysis system, which thus derives the term Biacore. A surface plasmon is a longitudinal or transverse charge density wave propagating along the interface between a metal surface and a dielectric (good insulator). The electrons in the metal must exhibit free-electron (Boardman, 1982) (or gas) like behaviour for optical excitation of the surface plasmon to occur. The electrostatic potential of the metal ion cores must have negligible effect on the movement of the electrons in the metal lattice (Liedberg *et. al.,* 1993), therefore only metals such as gold, silver, copper and aluminium are useful for SPR.

$$k_{sp} = \frac{\omega}{c} \sqrt{\frac{\varepsilon(\omega)\varepsilon_a}{\varepsilon_a + \varepsilon(\omega)}} \qquad \textbf{\textit{Equation 1}}$$

$$\varepsilon(\omega) \approx 1 - \frac{\omega_p^2}{\omega^2} \qquad \textbf{\textit{Equation 2}}$$

$$\sqrt{\varepsilon_g}\, sin\theta_{sp} \approx \sqrt{\frac{\varepsilon'(\omega)\varepsilon_a}{\varepsilon'(\omega) + \varepsilon_a}} \qquad \textbf{\textit{Equation 3}}$$

Figure 3.1.1 illustrates the equations relating to surface plasmon propagation and excitation.

Equation 1 describes the dispersion relationship for a plasmon propagating along the interface between a metal with a complex dielectric function $\varepsilon(\omega)$ and a dielectric with dielectric constant ε_a, where c is the speed of light and k_{sp} is the wave vector for the propagating plasmon. ***Equation 2*** describes the complex dielectric function of the metal film. As the propagating wave vector is always larger than the ambient wave vector ($k_a = (\omega/c)\sqrt{\varepsilon_a}$), it is not possible to excite a surface plasmon through direct reflection of light on the metal film. The use of an attenuated total reflection (or Kretschmann) configuration solves this problem, which is illustrated in ***Figure 3.1.2***. The evanescent wave present outside the prism after total reflection sets up a surface plasmon at a given angle θ_{sp} (Boardman, 1982; Kretschmann, 1971).
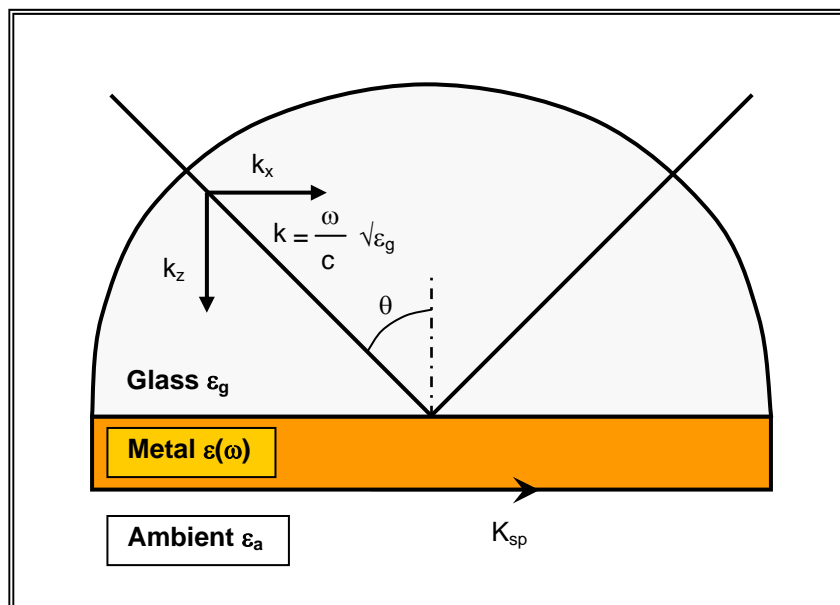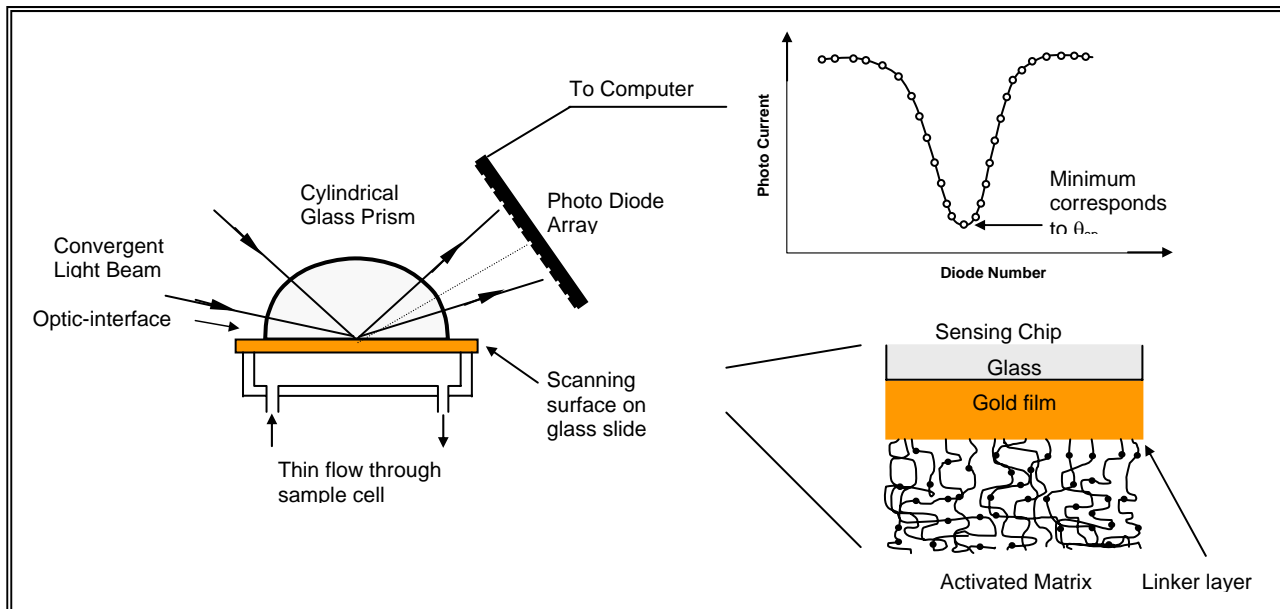
Figure 3.1.2 is a schematic representation of the Kretschmann (or attenuated total reflection) configuration used for optical excitation of a surface plasmon (k_{sp}), which is set up at the interface between the metal and the ambient (less dense medium), when k_x is equal to k_{sp} (adapted from Lundstrom, 1994).

The thin metal layer is coupled to the high refractive index prism either by direct deposition, or by deposition onto a glass slide that is optically coupled to the prism by an index matching polymer or liquid. The part of the system that interacts with the biological molecules and allows the biomolecular interaction analysis to occur is the ambient side of the metal layer. As the surface plasmon propagates along the metal/ambient interface an evanescent wave extends into the ambient. This wave, which is influenced by changes in the refractive properties of the ambient in turn, influences the surface plasmon resonance angle and thus creates a measurable response. It has also been found that changes in the optical properties closer to the metal/ambient interface influence the evanescent wave and produce larger effects than changes further away. The mathematical explanation of the evanescent wave is described in Boardman, 1982.

As specific binding of biomolecules is important in biomolecular interaction analysis, it becomes problematic when the bare metal surface does not promote specific adhesion of biomolecules. One method to solve this problem is to use a coupling matrix, which may consist of a hydrogel such as carboxymethylated dextran. This hydrogel (illustrated in *Figure 3.1.3*) allows the covalent attachment of specific biomolecules to the metal surface using standardised coupling chemistries (see materials and methods) (Liedberg *et. al.,* 1993 and Lundstrom, 1994). The use of a coupling matrix also allows for a greater number of interaction sites compared to a monolayer and their increased accessibility, all due to the increase in available surface area. The hydrogel matrix and coupling chemistries have been described in detail in the following references Lofas and Johnsson, 1990 and Johnsson *et. al.*, 1991.

The SPR based laboratory biosensor system developed by Biacore AB (Uppsala, Sweden) is one such system that can accurately monitor the binding of template to recognition element in real time. Molecular recognition elements such as antibodies can be amine-coupled to the surface of a gold chip coated with carboxymethyl-dextran hydrogel, whereas a molecularly imprinted polymer such as poly-APBA can be grafted directly to an unmodified gold surface. A simple schematic of the Biacore SPR system is illustrated in *Figure 3.1.3* below.

Figure 3.1.3 represents a schematic of the Biacore system from Pharmacia Biotech (Uppsala, Sweden). The sensing chip is also represented in an exploded view, which details the activated hydrogel matrix allowing specific covalent attachment of ligands. The graph depicted in the figure represents the information produced by the diode array; the surface plasmon resonance angle corresponds to the minimum in the graph. This information is then translated into response units by the system software.

A major benefit that the Biacore system possesses is its ability to allow the regeneration of ligands covalently attached to the chip surface, which can generally be achieved through the changing of conditions such as pH, Ionic strength and the presence of surfactants in a wash buffer (Dillon *et. al.*, 2003; Quinn *et. al.*, 1997). This regeneration allows for prolonged use of the same immobilised ligands and therefore study of kinetics and affinity analysis through subsequent injections of varied concentrations of analyte. A change in the resonance signal of 1000 RU corresponds to an increase in the surface plasmon resonance angle (1000 RU $=0.1^{\circ}$).

3.1.2 Aqueous protein imprinting

Molecular imprinting, as a technology has grown rapidly over the last 20 years, the production of synthetic adsorbents that recognise targets ranging from pharmaceuticals (ampicillin, erythromycin A, tylosin) to food components, additives and contaminants (amino acids, carbohydrates, menthol, caffeine, listeria and staphylococcus sp.) to herbicides, pesticides and trace metals (atrazine, triazine and Co^{2+}) to name but a few (Reviewed in Ramstrom *et. al.*, 2001). Originally imprinting was achieved through the production of bulk polymers, which were ground into smaller particles and packed into columns for solid phase extraction purposes. This strategy however is relatively limited with regard to the incorporation of MIPs into biosensor-based formats, which can be utilised without the need for complex laboratory analysis and methodology. The incorporation of an aqueous imprinted hydrogel comprising of acrylamide and its derivatives, into a solid phase extraction cartridge yielded good separation of template molecules from a heterogeneous mixture (Hjerten *et. al.*, 1997). In this study horse myoglobin was selectively removed from a mixture also containing whale myoglobin by adsorption to the column of horse-myoglobin imprinted polyacrylamide gel beads.

The incorporation of enzymes into electrochemical biosensor electrodes first demonstrated in 1962 (Clark and Lyons, 1962) has generated new ideas whereby MIP technology could in principle, be applied to the development of simple biosensors. Currently however, the immobilisation of macromolecular biomolecules onto transducer microsurfaces using conventional methods

such as cross-linking, covalent attachment and hydrogel or membrane entrapment suffers low reproducibility and poor spatially controlled deposition (Cosnier, 1999). This remains to be a major problem for the production of synthetic biosensors. Electropolymerisation of acrylamide and phenylboronic acid based monomers onto SPR, QCM and standard gold electrochemical electrode surfaces has been achieved, in an attempt to develop a glucose sensor based on the swelling properties of such immobilised hydrogels (Gabai *et. al.*, 2001). Boronic acid monomers and the swelling properties of hydrogels were used together with a QCM resonator to detect nucleotide binding (Kanekiyo *et. al.*, 2000).

Aminophenylboronic acid (APBA) was coated on the inner surface of microplate wells in an attempt to produce an affinity matrix, which showed specificity for the protein templates, microperoxidase, horseradish-peroxidase, lactoperoxidase and haemoglobin. The polymerisation process used aqueous ammonium persulfate (APS $_{(aq)}$) to coat the microplate wells with a thin graft of the protein-specific homopolymer. The stabilising function of the polystyrene wells, to which the polymer was grafted, and the spatial orientation of template functional groups were critical factors affecting polymer formation and recognition of the template (Bossi *et. al.*, 2001). Boronic acid functionalised monomers have been used in aqueous molecular imprinting, with application to nucleotide detection (Ozdemir and Tuncel, 2000; Tuncel and Ozdemir, 2000; Kanekiyo *et. al.*, 2000), glycated-protein affinity sorbents for HPLC (Koyama and Terauchi, 1996), extraction of B-blocking drugs from plasma (Martin *et. al.*, 1993) and glucose detection (Gabai *et. al.*, 2001). The boronic acid monomer has desirable functionality

with regard to non-covalent template recognition. Non-covalent imprinting is specifically useful when dealing with proteins, as covalent attachment to proteins may involve degradation or modification of the target; hence a large number of electrostatic and hydrophobic interactions are desirable in large molecule or protein imprinting. Advantages of the APBA approach to protein imprinting include its simple integration with the SPR transducer system, as polymerisation readily occurs on the gold chip surface. Due to the real-time monitoring capabilities of the SPR transducer system, polymer thickness can easily be regulated, which aids in the monitoring of polymer homogeneity and template affinity.

3.2. Materials and methods

The 96 well Maxisorp® plates were supplied by Fisher UK (Loughborough, UK). Prothrombin, thrombin, albumin, acrylamide, N, N-methylene bisacrylamide, ammonium persulphate, Ecarin (from Echis carinatus) and p-nitroanilide were all supplied by Sigma (Dorset, UK). Acros organics (Loughborough, UK) supplied the 3-Aminophenylboronic acid monohydrate and the UV initiator, 2, 2'-azobis(2-methylpropionamide) dihydrochloride. Biacore International SA UK (Stevenage, Hertfordshire, UK) supplied Gold J1 Biacore chips in a self-assembly kit (BR-1004-05).

3.2.1 Acrylamide gel protein imprinting

A solution comprising of N N-methylene bisacrylamide (16 mM), acrylamide (810 mM), 2, 2'-azobis(2-methylpropionamide) dihydrochloride (15 mM) and 1

ml of $NaHCO_3/Na_2CO_3$ buffer pH 6.0 was produced for the blank polymer gel. For the prothrombin imprinted polymer gel, the carbonate buffer was replaced with 1ml of 2.0 mgml^{-1} prothrombin solution in pH6.0 $NaHCO_3/Na_2CO_3$ buffer. The mixtures were sonicated under vacuum to remove the majority of dissolved gas. A high intensity UV light source (UV Aprint 100 CVI, *Dr. Hölne AG*) was used to polymerise 40 µl of the acrylamide solution placed into the wells of a 96-well microtiter plate; polymerisation took between 5-10 minutes. The template was washed from the gel disks overnight with NaH_2PO_4/Na_2HPO_4 buffer + 1% Tween 20 pH 6.0. The incubation of serial dilutions of prothrombin and thrombin for 90 minutes in the wells containing gel disks, was followed by 3 1 hr washes of NaH_2PO_4/Na_2HPO_4 buffer to remove excess.

Prothrombin activation was achieved using 100 µl of 5.4 Uml^{-1} of Ecarin solution dissolved in pH 8.0 NaH_2PO_4/Na_2HPO_4 buffer, subsequently 100 µl of 0.25 mgml^{-1} chromogenic substrate T1637 was added to each gel disk. Activated prothrombin and thrombin quantification was then achieved by the kinetic monitoring of the optical absorbance at 405 nm of the solution in the wells containing the gel disks.

3.2.2 Poly-aminophenylboronic acid imprinting of prothrombin

A Technobiochip Libra mod α (10 MHz) piezoelectric crystal reader using the Libra 3.1 control software was used in conjunction with 14 mm piezoelectric crystals from Technobiochip for the preliminary stage of this study.   The second stage involved the use of a Biacore 3000 (Biacore AB, Uppsala.

Sweden) SPR platform with standard gold surface J1 Biacore chips (Biacore UK). The grafting of prothrombin imprinted poly-APBA onto the surface of a J1 Gold unmodified chip surface was achieved as follows. Prothrombin was diluted in pH 6.0 phosphate buffer (NaH_2PO_4/Na_2HPO_4) and 10 μl was added to 10 μl of 100 mM aminophenylboronic acid $_{(aq)}$ (15.5 mg ml^{-1}), 10 μl of this mixture was added to 10 μl of 100 mM ammonium persulphate solution (22.8 mg ml^{-1}). The mixture was placed on the gold surface of the Biacore chip, making sure the four channels were covered, and allowed to polymerise for 25 - 30 minutes. This procedure was first implemented to the piezoelectric crystal grafting. After polymerisation the chip/crystal was washed excessively with deionised H_2O and 10 mM HCl and conditioned *in-situ* until a stable baseline was achieved. Calibrations using increasing concentrations of prothrombin, thrombin and albumin were subsequently achieved.

3.3. Results

The grafting of the prothrombin imprinted poly-aminophenylboronic acid (APBA) onto the non-modified gold surface of a J1 Biacore chip yielded interesting results with homogeneous solutions of prothrombin, thrombin and albumin, with a concentration range of 0.01 – 15 nM. Polymerisation of imprinted ABPA for 20 minutes produces an increase in the relative SPR response of between 20000-40000 RU. However the material deposited on the surface, quickly washes off to a level that is approximately 10000 RU higher than a blank chip. The resulting monolayer surface displayed quantitative recognition of homogeneous solutions of prothrombin at a concentration range of 0.01 nM to 14.2 nM as shown in *Figure 3.3.1A*. The

same prothrombin imprinted polymer monolayer displayed a significantly

reduced recognition of homogeneous solutions of thrombin at a similar

concentration range of between 0.01nM to 10nM as shown in *Figure 3.3.1A*.
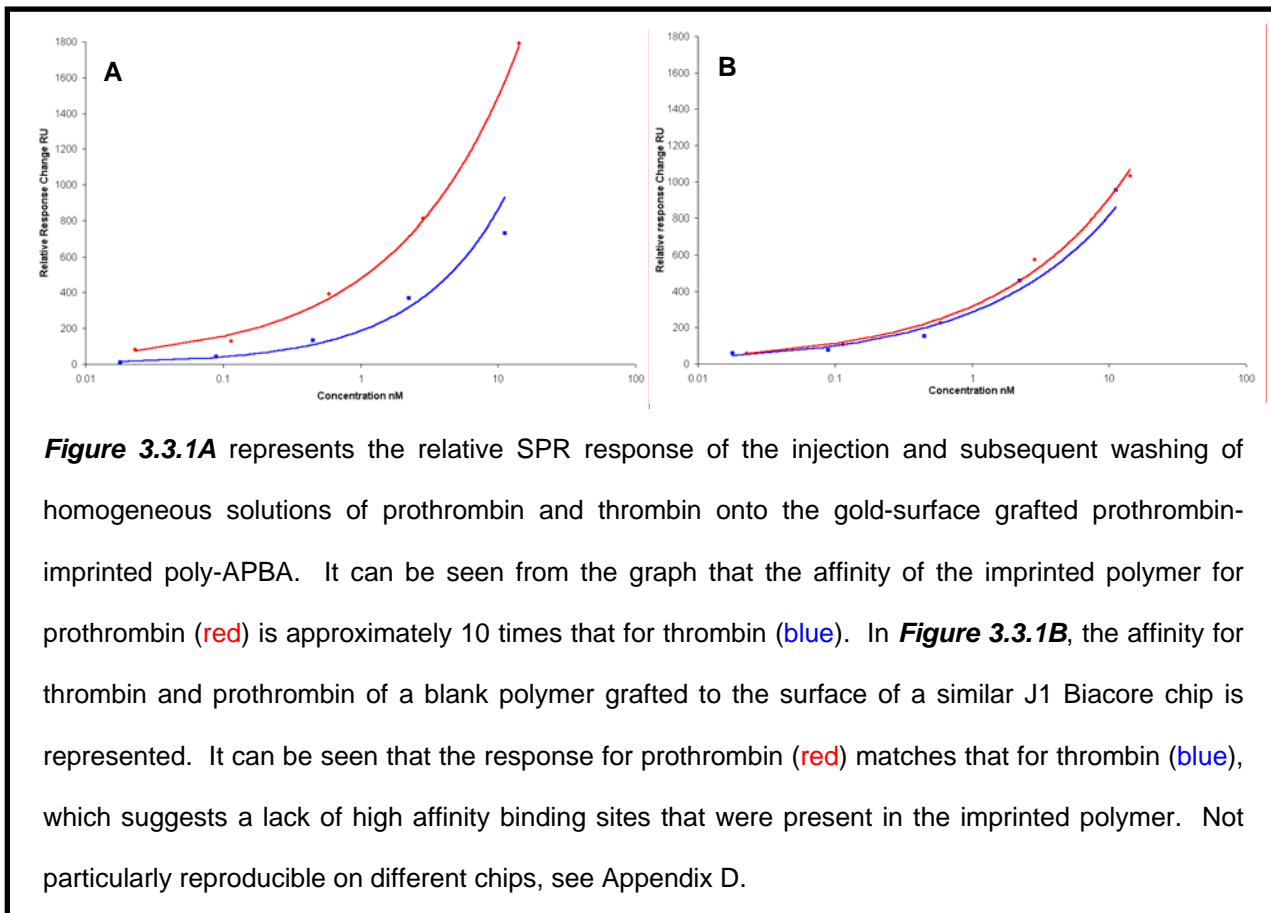
Figure 3.3.1A represents the relative SPR response of the injection and subsequent washing of homogeneous solutions of prothrombin and thrombin onto the gold-surface grafted prothrombin-imprinted poly-APBA. It can be seen from the graph that the affinity of the imprinted polymer for prothrombin (red) is approximately 10 times that for thrombin (blue). In *Figure 3.3.1B*, the affinity for thrombin and prothrombin of a blank polymer grafted to the surface of a similar J1 Biacore chip is represented. It can be seen that the response for prothrombin (red) matches that for thrombin (blue), which suggests a lack of high affinity binding sites that were present in the imprinted polymer. Not particularly reproducible on different chips, see Appendix D.

The wash procedure for the removal of homogeneous protein samples from

the imprinted polymer surface utilised 10mM and 100mM HCl. The majority

of the homogeneous sample was removed by a single 10 μl injection of 10mM

HCl, and further injections return the response to the preinjection level. More

stubbornly bound protein could be removed with 10 μl injections of 100mM

HCl.

Injections of homogeneous concentrations of albumin at concentrations similar to the concentration range used for prothrombin and thrombin, suggested minimal non-specific recognition of albumin by the prothrombin-imprinted polymer.   The non-specific recognition of albumin at plasma concentrations i.e. 150 - 750 μM, was as high as that for prothrombin concentrations of 10 nM (i.e. 1400 RU), as represented in *Figure 3.3.2B*.
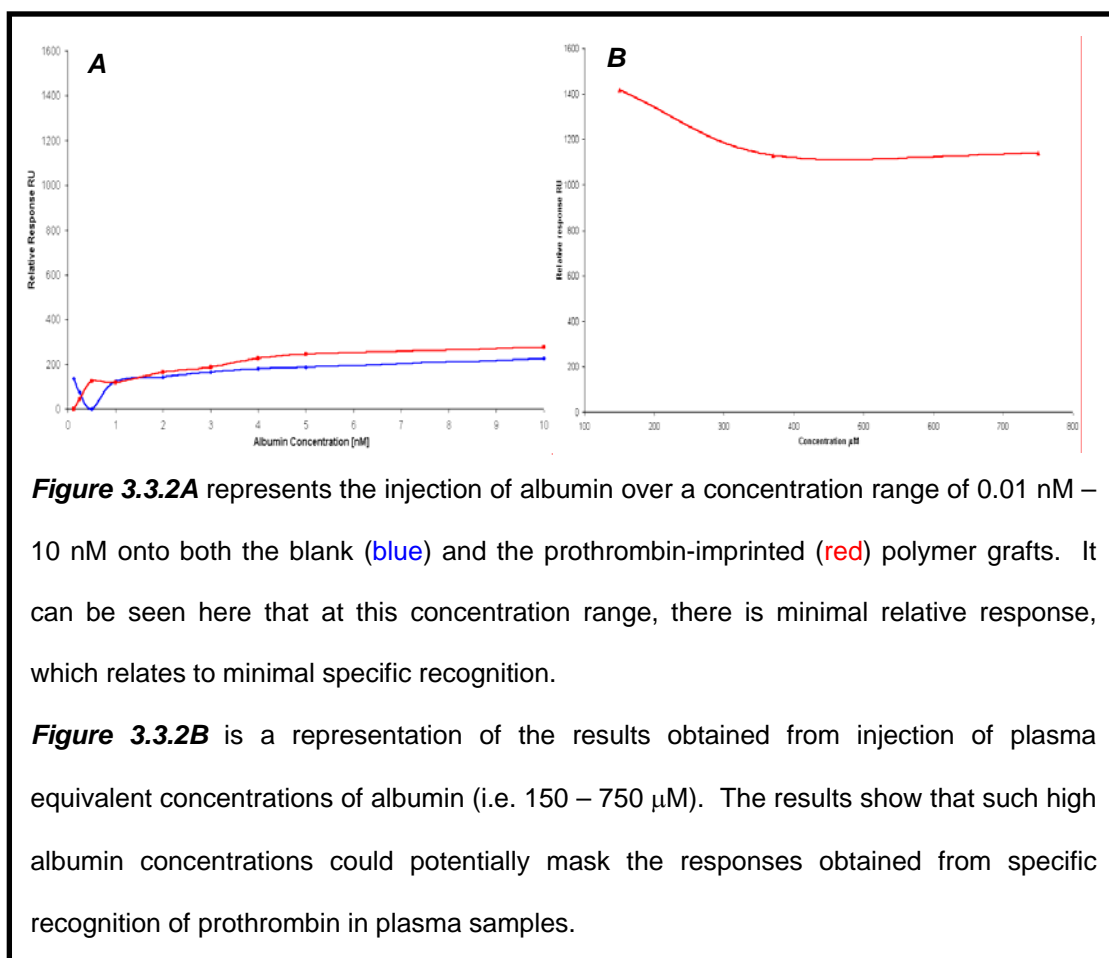
Figure 3.3.2A represents the injection of albumin over a concentration range of 0.01 nM – 10 nM onto both the blank (blue) and the prothrombin-imprinted (red) polymer grafts. It can be seen here that at this concentration range, there is minimal relative response, which relates to minimal specific recognition.

Figure 3.3.2B is a representation of the results obtained from injection of plasma equivalent concentrations of albumin (i.e. 150 – 750 μM). The results show that such high albumin concentrations could potentially mask the responses obtained from specific recognition of prothrombin in plasma samples.

The polymers were also tested for their affinity for prothrombin in more complex solutions such as plasma and 100 kDa protein depleted plasma. The results of these experiments were somewhat disappointing with respect to the response elicited from the injection of prothrombin-spiked plasma onto the prothrombin-imprinted polymer. A bulk increase in the SPR response was

created on injection of spiked 1:10 diluted whole plasma, indicating that there

was a deposition of material on the polymer or on the chip surface itself.

Calibrations using spiked 1:10 diluted whole plasma therefore produced

insignificant results, due to the bulk deposition effect. The wash procedure in

this case did not remove all of the injected plasma sample, and the addition of

detergents such as 0.1% SDS and 0.1% Tween20 to 10 mM HCl did not

alleviate the situation with regard to removal of the surface bound material.

Figure 3.3.3 represents the poor quality of the results obtained when spiked filtered

plasma is injected onto the imprinted polymer graft (red). The concentration range with

which the polymer shows any recognition for prothrombin seems to have reduced from

0.01 nM – 10 nM with homogeneous solutions to 1 nM – 10 nM with spiked filtered

plasma samples. The relative response elicited by the polymer due to a prothrombin

spike of 6 nM is approximately four times smaller than that elicited from an injection of

a 6 nM prothrombin homogeneous solution. The blank polymer (blue) gave reduced

responses to equivalent prothrombin spike concentrations.

A filtration method was employed to remove the fraction of plasma greater than 100 kDa; plasma samples were deposited into a centrifugal cartridge, which was spun at 9000 xg for 1 hour and 20 minutes. Dilutions (1:10) of the <100 kDa fraction were spiked with prothrombin and injected onto the prothrombin-imprinted polymer graft on the Gold J1 Biacore chip. The quality of the response elicited by spiked filtered plasma injections, does not reflect the response elicited due to the injection of homogeneous prothrombin solutions.

3.4. Discussion

The use of the piezoelectric crystal platform for the aqueous imprinting of prothrombin with poly APBA monohydrate, yielded results that suggested affinity of prothrombin imprinted pAPBA for its target. However the instability of this detection platform caused difficulties in obtaining and interpreting these results. Therefore, it was decided to utilise the distinctly more stable and time efficient Biacore 3000 platform for the pAPBA imprinting studies.

Gaining useable results from acrylamide imprinting of prothrombin in the 96 well micro titre plates also proved to be difficult. Gel discs were produced in the wells of the micro titre plates during the imprinting/polymerisation process, however gas bubbles were created inside the gel disks, which caused the plate reader to give ambiguous results. When the assay reagents (Ecarin and chromogenic substrate T1937) were added, the disks lifted from the base of the wells, which amplified the ambiguity of the result from the plate reader. It may be suggested that the affinity of the prothrombin imprinted-acrylamide

disk surface was not sufficient enough for the capture of prothrombin, and its subsequent quantification.

Hjerten and colleagues however showed that acrylamide gel could show selective recognition for proteins. In contrast to our work however, the acrylamide gel used here was granulated and then packed into SPE beds (Liao *et. al.*, 1996; Hjerten *et. al.*, 1996 and 1997). This granulation allowed for an increase of surface area and hence of the higher affinity selective protein recognition sites, created during the imprinting process. Packing of the prothrombin-imprinted polymeric gel granules into SPE columns increased the efficiency of specific protein recognition, by essentially forcing the proteins of interest closer to the high affinity sites contained within the gel.

Molecularly imprinted acrylamide has been used to create hydrogels capable of distinguishing between isomerically distinct forms of glucose (Wizeman and Kofinas, 2001). These hydrogels were dried (at 50°C) and washed extensively (up to 5 days), the addition of a measured amount of dried hydrogel to a solution containing glucose allowed investigators to measure the amount of glucose bound to the hydrogel. This method however would not lend itself to simple quantification of prothrombin concentration in complex aqueous solutions such as plasma or blood. The dried hydrogel however would promote the efficient uptake of solution, and promote the efficient binding of target proteins.

The adaptation of the Biacore platform to acrylamide imprinting of prothrombin was unsuccessful. The gold surface of the Biacore chip allowed

for attachment of a monolayer of the imprinted material, which as discussed previously did not provide enough specific high affinity sites for prothrombin binding. We then directed our attention to the aminophenylboronic acid (APBA) imprinting of prothrombin, which showed some encouraging results with the piezoelectric crystal detection system. The grafting of a thin film of imprinted poly-APBA onto an undocked Biacore chip was simple to achieve, the mixture of APBA, ammonium persulphate and prothrombin was simply placed onto the gold surface and allowed to polymerise. A rapid polymerisation time associated with poly-APBA would cause major fluidic damage to the Biacore system. Therefore, in contrast to the immobilisation of antibodies, this procedure was not controlled using the surface immobilisation wizard available within the Biacore control software.

Aminophenylboronate has been used previously to distinguish between glycosylated and non-glycosylated proteins such as haemoglobin (Middle *et. al.*, 1983), albumin, macroglobulin and transferrin (Gould and Hall, 1987). APBA has also been use in copolymer studies with acrylamide; the recognition of nucleotides and monosaccharides via imprinting was achieved here (Sallacan *et. al.*, 2002). Therefore the use of the phenylboronate ligand in affinity recognition/separation studies has produced interesting results in the past.

The work here was inspired by the work of Bossi and colleagues, where the molecular imprinting of various peroxidase enzymes using APBA, produced efficient selective recognition of each target on its reintroduction (Bossi *et. al.*, 2001). They showed selective recognition of target proteins by thin layer

imprinted APBA grafts on the inner surface of micro plate wells, showing that imprinted cavities need not be created to provide selective recognition of protein targets. They also demonstrated the benefits of the imprinting effect, when they confirmed that the blank polymer only showed non-specific binding of the protein targets. Similar work using chemical grafting of molecularly imprinted poly-APBA has achieved good selective recognition of epinephrine and horseradish peroxidase (Piletsky *et. al.*, 2000a).

As a direct comparison to the previous poly-APBA imprinting work (Piletsky *et. al.*, 2000; Bossi *et. al.*, 2001), our prothrombin imprinting has produced similar results with regard to the rebinding of homogeneous template in aqueous solution. As can be seen from the results in *Figures 3.3.1A* and *B* the injection of prothrombin onto the imprinted graft generates a much larger response than thrombin or prothrombin onto the blank graft, which demonstrates the imprinting effect. The preparation of homologous prothrombin solutions in 10 mM sodium acetate buffer pH 4.0 conferred the largest responses on reapplication to the immobilised prothrombin imprinted polymer. However the fact that homogeneous solutions of prothrombin could be recognised by a prothrombin imprinted MIP is only a starting block for the development of a prothrombin diagnostic test. Such a diagnostic test should be able to quantify prothrombin from complex solutions such as plasma or blood.

The mechanism of recognition of the prothrombin-imprinted polymer is most likely to be via interaction of many weak ionic interactions with the target protein. This explains why the pH of both the polymerisation mixture (pH 6.0)

and of the rebinding buffer (pH 4.0), were both critical for selective recognition of prothrombin. At pH 4.0 there is an overall positive charge on prothrombin, therefore exposed boronic acid ligands are able to bind more easily to prothrombin. The imprinting effect in this situation is of obvious importance for prothrombin selectivity, as the boronic acid ligands would be arranged in such a manner to facilitate prothrombin rebinding. This mechanism however does not lend itself to the specific recognition of large proteins in complex media, where the potential for non-specificity is high due to the presence of various proteins that posses many similar structural and ionic/electrostatic characteristics. This reason would therefore explain why the injection of prothrombin spiked plasma samples did not produce such encouraging results with respect to the specific recognition of prothrombin. A major source of non-specific binding could have been due to a potentially high concentration of fibrinogen, which is known to efficiently bind to gold surfaces through strong electrostatic and/or chemical binding upon incubation with plasma (Kanagaraja *et. al.*, 1995; Eriksson and Nygren, 1997).

Another reason for the poor response changes on injection of plasma and spiked plasma samples maybe due to a buffering effect gained from the plasma proteins, which may have created a non favourable pH for prothrombin binding, even though the plasma was diluted in buffer. A buffer composed of 10 mM sodium acetate at pH 4.0, would allow prothrombin and other plasma proteins of comparable pI (approximately 4.6) to become charged in the same way, and therefore possess similar affinities towards the imprinted polymer.

Chapter 4

Polyclonal Antibodies Towards Native Prothrombin and their Incorporation into a Real-Time Assay Format

This chapter describes the production of polyclonal antibodies with specificity and affinity for the native prothrombin antigen (NPA), and how they could aid in the development of an assay for the monitoring of oral anticoagulation therapy using vitamin K antagonists such as warfarin. The immobilisation of these polyclonal antibodies onto an SPR based gold chip surface would allow a direct comparison of their template recognition properties with the prothrombin imprinted aminophenylboronic acid polymer, as described in chapter 3. Importantly, the antibody should possess the specificity and sufficient affinity to recognise and bind to the native form of prothrombin in a complex mixture also containing various heterogeneous species of abnormal prothrombin.

4.1 Introduction

The routine monitoring of oral anticoagulation therapy in UK hospital laboratories is achieved through automated application of the prothrombin time (PT). However, despite current automation technology, point of care devices, more stringent regulation of thromboplastin manufacture and efforts to improve monitoring standardisation (Reviewed in Poller, 1987; Talstad, 2000), haemorrhagic and thrombotic complications associated with prothrombin time (PT) monitored oral anticoagulation therapy (OACT) patients are unacceptably high. Antibodies possess specificity and affinity qualities that make them desirable capture phases for their incorporation into highly sensitive assays. However, the complexity of blood provides a stumbling block to most blood-protein assays. The application of native-prothrombin specific monoclonal antibodies in the development of a

monitoring assay, could potentially reduce the complication rate associated with OACT and its' monitoring. Antibodies for use with such an assay should be specific enough to bind native prothrombin in a complex solution such as blood or plasma, as well as in homogeneous solutions.

Studies have shown that the Native Prothrombin Antigen (NPA) could provide a more efficient method in the monitoring of OACT using vitamin K antagonists. Antibodies directed towards the NPA were incorporated into a radioimmunoassay RIA to measure the native prothrombin antigen in warfarin treated patients (Blanchard *et. al.*, 1983). A study using these antibodies showed that the native prothrombin concentration correlated well with the occurrence of haemorrhagic or thrombotic complications associated with OACT. The results also indicated that the state of anticoagulation of a patient could be assessed with greater accuracy using the NPA-RIA, rather than the standard INR methodology (Furie *et. al.*, 1984). Following the preliminary work undertaken in the early 80s, Furie *et. al.* demonstrated, in a randomised controlled clinical trial, that the use of their NPA-RIA for the monitoring of OACT could reduce the associated complications from 8.8% by 85% to 1.3% (Furie *et. al.*, 1990). These findings were reiterated in 1993 when an ELISA, using a similar mono-specific polyclonal antibody for the calcium stabilised conformer of native prothrombin, was used to demonstrate decreased OACT-associated complications compared with the INR (Kornberg *et. al.*, 1993). Native prothrombin concentrations in non-anticoagulated individuals as determined by the ELISA were 164 ±32 mgml^{-1} as compared with 108 ±19 mgml^{-1}, determined by RIA (Kornberg *et. al.*, 1993).

The INR has contributed significantly to OACT monitoring and the methodology and accuracy has been developed over the years, however the associated complication rate is still unacceptably high. This makes the detection of haemorrhagic and thrombotic complications during INR-OACT monitoring, difficult for the clinician and for the patient in terms of needless stress and worry. Evidence from the clinical trial carried out by Kornberg and his group actually suggested that the probability of the prediction of a thrombotic episode by the INR was statistically insignificant, whereas the NPA showed statistical significance, where $P<0.001$ on three out of five days during the trial (Kornberg *et. al.*, 1993). An Italian multicentre inception cohort study to assess the risk of bleeding associated with OACT, showed that there was a risk of bleeding associated with OACT in elderly patients and those with complications, such as diabetes and previous thromboembolism. However, the major bleeding risk to benefit ratio of OACT, with regard to the treatment of Non-rheumatic Atrial Fibrillation (NRAF), is still very low and the treatment efficacy was confirmed (Pengo *et. al.*, 2001).

The adaptability of the Biacore sensor chip surface has been demonstrated and can be useful for the immobilisation of many types of ligand. Antibodies may account for a large proportion of the immobilisations, but ligands such as recombinant proteins (Yamaguchi *et. al.*, 2003), synthetic receptor mimics (Gomes *et. al.*, 2000; Gomara *et. al.*, 2000; Bracci *et. al.*, 2001), synthetic enzyme mimics (Kataki and Morgan, 2003), saccharides (Kuziemko *et. al.*, 1996) and carbohydrates (Maegawa *et. al.*, 2002) have also been used as recognition elements on Biacore chip surfaces.
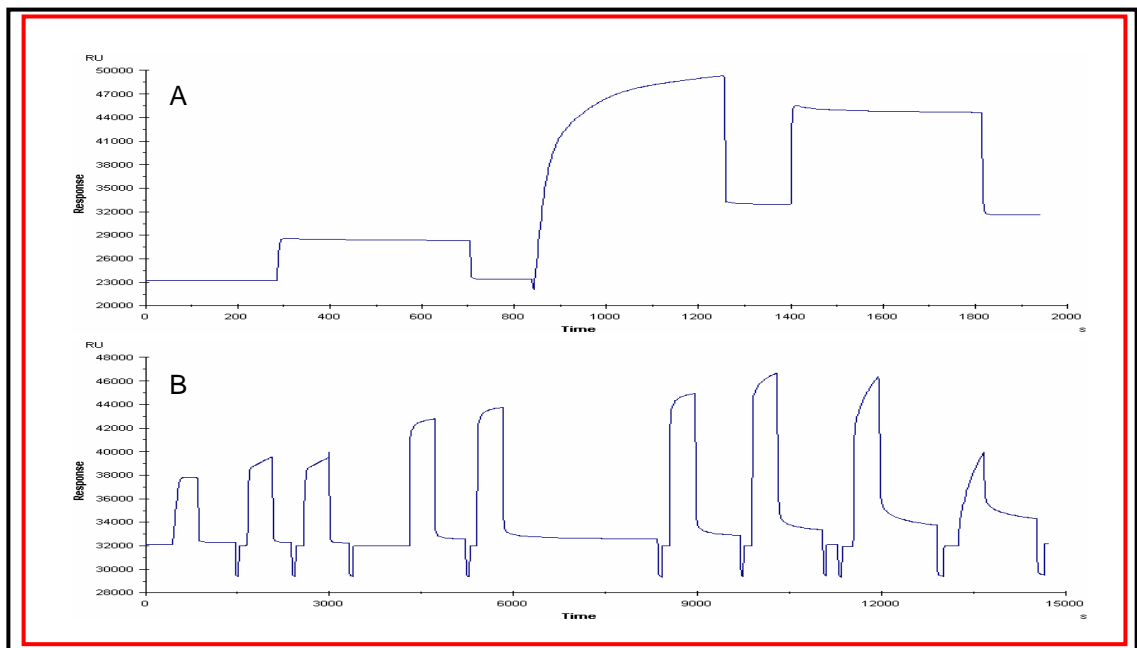
Figure 4.1.1A is a graphical output representing the specific chemical (amine) coupling of prothrombin polyclonal antibodies one of four channels on the surface of a Biacore chip. The Chip (CM5) is coated with a carboxymethyl dextran hydrogel to allow the covalent attachment of the antibodies to the chip surface, using a standard EDC/NHS coupling method (refer to ***3.3*** for details). ***Figure 4.1.1B*** is the graphical output produced by the Biacore 3000 software when increasing concentrations of thrombin are injected through the channel containing immobilised prothrombin antibodies. This figure demonstrates the regenerability of the antibodies and the hydrogel dextran matrix to which the antibodies are covalently bound, due to the washing of the antibodies with 10mM HCl, allowing the response curve to continually return to the value prior to the injection of protein sample. This can be achieved generally between 50-100 times (Jönsson and Malmqvist, 1992).

Antibody immobilisation onto the carboxymethyl-dextran coated gold surface of a CM5 Biacore chip was by the Biacore standard EDC/NHS amine coupling method (Johnsson *et. al.*, 1991). A summary of the methods available for ligand immobilisation using the Biacore platform is represented in ***Figure 4.1.2***.

Covalent Surface Derivatisation for Ligand Attachment
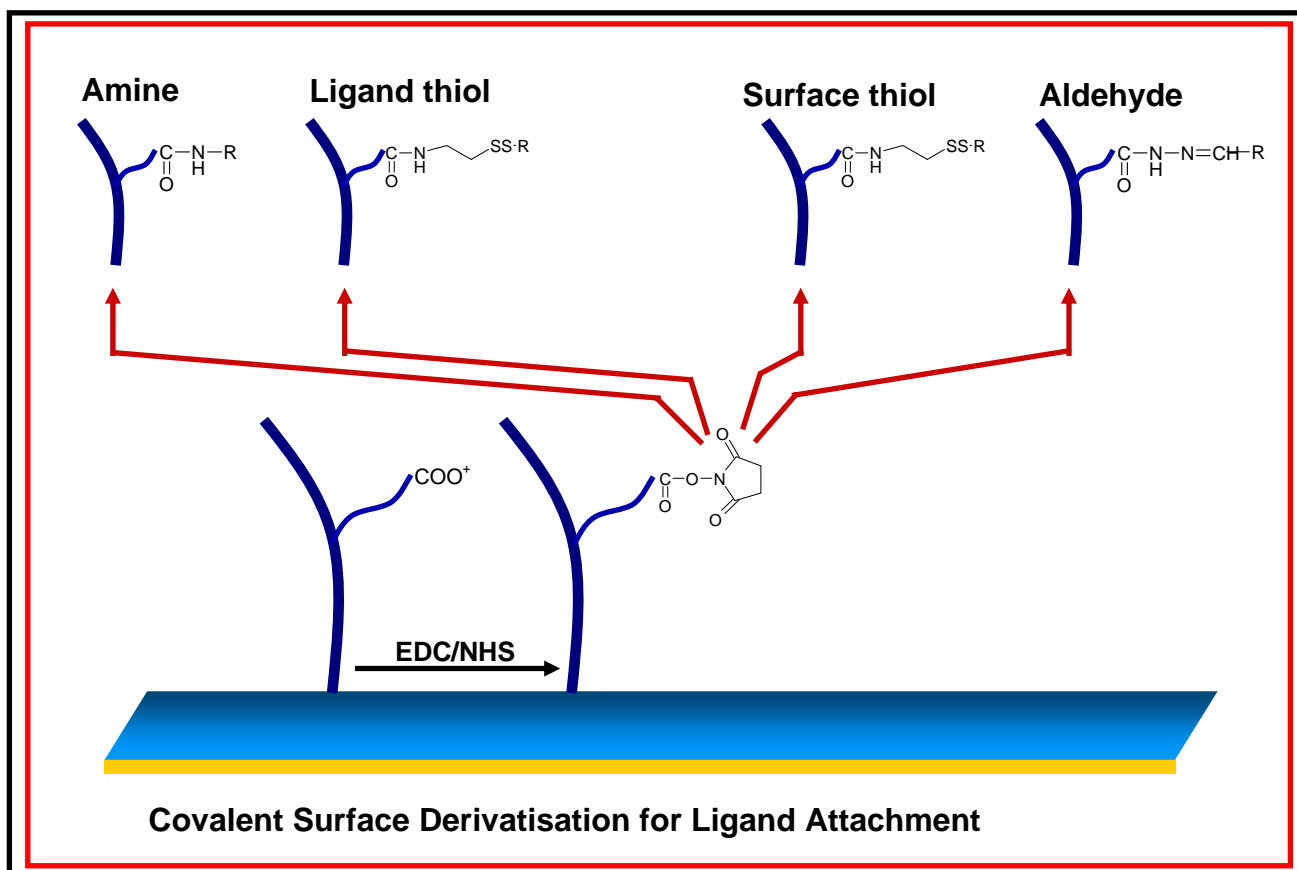
Figure 4.1.2 represents the coupling chemistry available for application with ligand immobilisation onto the modified gold surface of the Biacore chips. The Amine coupling chemistry utilises free carboxymethyl groups on the CM5 chip surface, by activating them with a mixture of EDC/NHS and allowing the attachment of antibody ligands to the new free amine (Johnsson *et. al.*, 1991).

4.2 Materials and methods

CM5 research grade Biacore chips (BR-1000-72), HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20) (BR-1001-88), 7 mm diameter plastic vials (0.8 ml) (BR-1002-12) and the Amine coupling kit (BR-1000-50) were obtained through Biacore UK (Stevenage, Hertfordshire, SG1 2EF). Vivaspin 2 cartridges were purchased from Fisher Scientific UK (product number VS0242).

4.2.1 Antibody production

Antibody production was achieved through the standard methodology used by Immune Systems Limited (IS-L, Paignton UK.   http://www.is-l.com).   A preparation of native prothrombin antigen [2 mgml^{-1}] was prepared using the Newfa adjuvant with phosphate buffered saline at physiological pH. A rabbit (IS-L, Paignton UK) was immunised with approximately 0.25 ml of the antigen/adjuvant emulsion on days 0, 14, 28 and 42 of the antibody production. (A small sample (1.5 ml) of the serum was taken at day 49 to assess the titre of the antibody. A larger sample of serum (60-80 ml) was taken subsequent to the test sample possessing a high antibody titre and prior to a terminal bleed of approximately 60-80 ml after 8 weeks of maintenance. Pre and post immune serum samples were screened against the target immunogen and a negative control using an ELISA, which allowed the cross reactivity of the antibodies to be assessed.

4.2.2 Affinity purification

Approximately 70 ml of serum collected from the rabbit was loaded onto a PBS pH 7.4 equilibrated, 3ml agarose linked native prothrombin affinity column, at a rate of approximately 8 mlh^{-1}. The column was then washed with 50ml 2M NaCl, pH 7.5, 50ml 0.1M Borate, pH 9.1 and 50ml 0.1M PBS, pH 4.5. Serum containing native prothrombin specific polyclonal antibodies was collected by elution using 20mM glycine-HCl/200mM NaCl. Fractions of 0.8 ml were collected into tube containing 0.4 ml, 0.1M Tris-HCl, pH 8.5, after which the absorbance of each fraction was determined at 280 nm.   The

fractions containing peak antibody concentrations were pooled and stored at 4°C in 0.05% sodium azide (NaN$_3$) solution.

4.2.3 Antibody immobilisation

All Biacore work was undertaken on a Biacore 3000 automated surface plasmon resonance (SPR) platform (Biacore AB, Uppsala, Sweden). Prothrombin specific polyclonal antibodies were immobilised onto the surface of carboxymethyl-dextran coated (CM5) Biacore chips, using Biacore standard amine coupling chemistry. The flow rate was set at a constant 5 μlmin^{-1} throughout the antibody coupling procedure, using HBS-EP buffer. The CM5 chip was activated using a 7-minute pulse of 200 mM EDC/50 mM NHS mix, which allowed the activation of approximately 40% of the carboxymethyl groups on the chip surface. Following activation of the CM5 chip a 7-minute injection pulse of prothrombin specific antibodies at a concentration of approximately 25 μgml^{-1} in 10 mM sodium acetate buffer pH 5.0, were injected. The target immobilisation range of the antibodies was approximately 10,000 RU. A 7-minute injection pulse of 1 M ethanolamine hydrochloride pH 8.5 deactivated the chip surface after antibody immobilisation. Similarly, non-specific antibodies were immobilised on a separate channel on the same chip, for negative control purposes.

4.2.4 Protein injections

The flow rate varied between 5-10 μlmin^{-1} during the protein injections. Running buffer in this instance was standard Biacore prepared HBS-EP (10

mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20).

Prothrombin (0.01-14.7 μM), thrombin (0.01-10 μM) and albumin (75-757 μM)

were dissolved in 10 mM sodium acetate pH 4.0 and injected through the

antibody-immobilised channels.   The concentration ranges reflected those

present in human plasma.

4.2.5 Plasma injections

Informed consent for the collection of plasma was gained from patients

undergoing routine INR determination for the strict monitoring of oral

anticoagulation therapy at Cheltenham general hospital INR clinic.   Plasma

were frozen on collection and stored at -20°C until required. The response of

the immobilised antibodies to serial dilutions of the plasma samples was

assessed, by the injection of 35 μl of undiluted, 1:10, 1:100 and 1:1000

plasma concentrations, diluted in pH 5.0 acetate buffer.

4.3 Results

The final yield of native prothrombin specific antibodies was approximately 18.9 mg, which was presented as a 702 μgml^{-1} solution of approximately 13 ml. This solution was separated into 1 ml aliquots and stored at –20 ^{o}C until required.
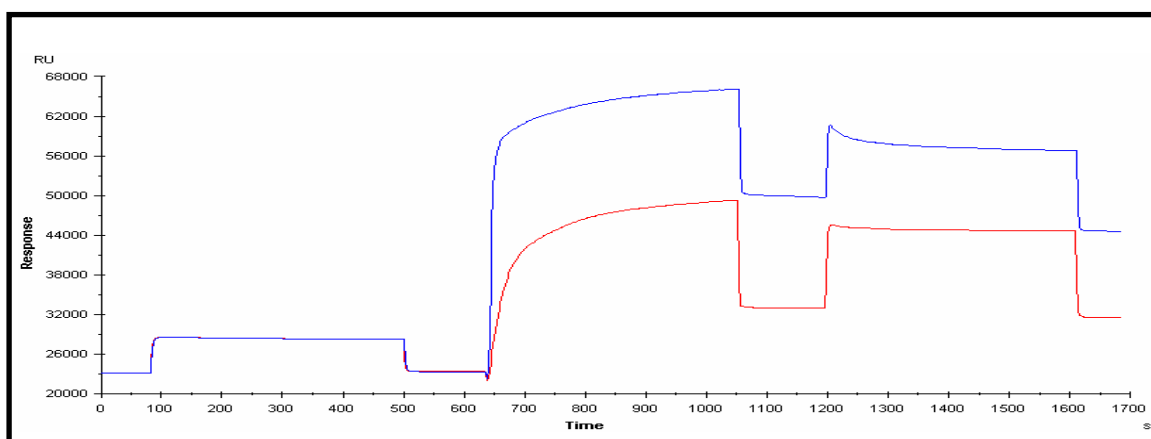
Figure 4.3.1 represents the graphical output of the Biacore 3000 during immobilisation of prothrombin (PT) (red) and microcystin-LR (MC-LR) (blue) antibodies onto the dextran modified surface of a CM5 chip. The level of surface immobilisation depended on the concentration and quality of the antibody, but for the prothrombin antibodies varied between 8,000 and 10,000 RU and between 18,000 and 25,000 RU for the microcystin-LR antibodies. The microcystin antibodies were immobilised for the control, or non-specific binding element of the rebinding experiments.

A CM5 Biacore chip was prepared by the amine coupling of prothrombin specific antibodies on one channel and microcystin-LR antibodies on a control channel. Microcystin-LR antibodies were used as a control antibody, due to their proven specificity towards microcystin-LR and hence minimal non-specificity for other proteins. The volume and concentration of injected antibody and the velocity of each injection regulated the degree of antibody immobilisation, which varied between 8,000-10,000 RU for prothrombin

antibodies and between 18,000 and 25,000 RU for the microcystin-LR antibodies, as represented in *Figure 4.3.1* above. The antibodies were then subjected to injections of homogeneous protein solutions ranging in concentration from 0.01 – 0.5 nM for prothrombin and 150 – 750 nM for albumin.
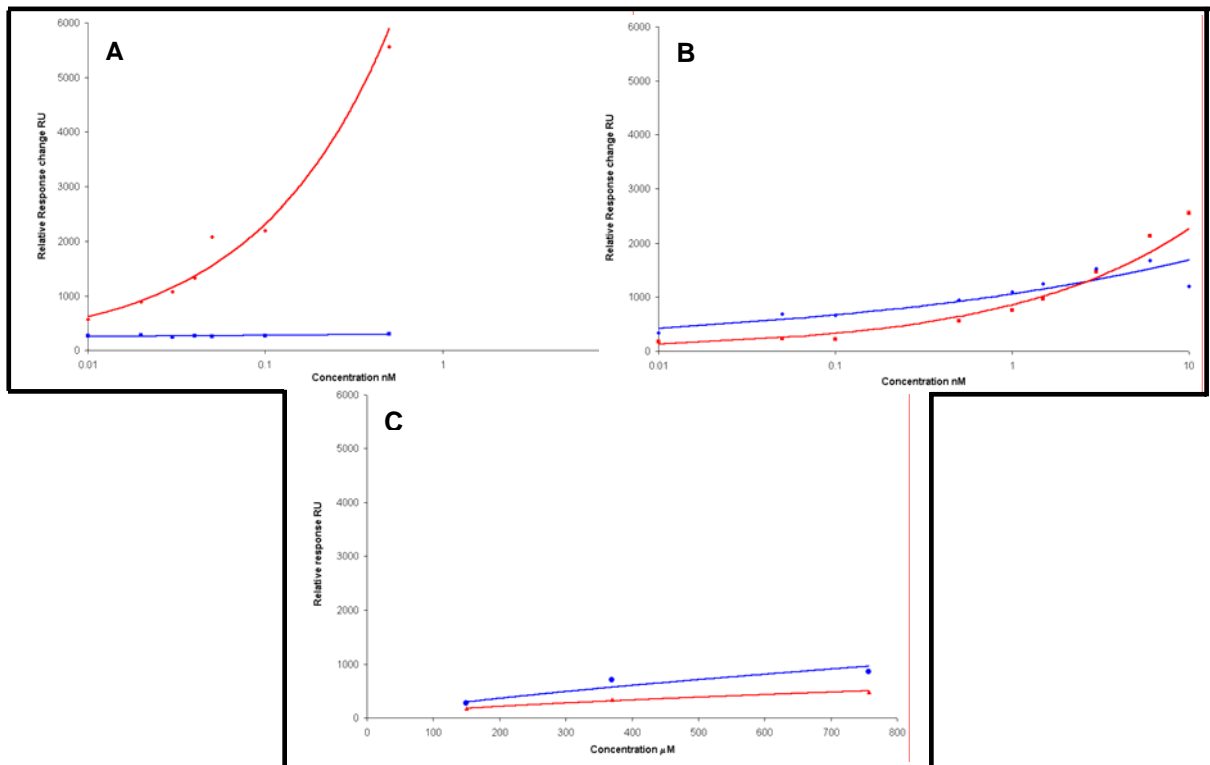
Figure 4.3.2 represents the calibration of the prothrombin (Red) and microcystin-LR (Blue) antibodies with homologous protein solutions ranging in concentration from 0.01–0.5 nM for prothrombin (**A**), 0.01–10 nM for thrombin (**B**) and 150–750 nM for Albumin (**C**). The protein concentrations tested were representative of those found in normal human blood/plasma samples, therefore giving an idea of how the antibodies would react to human samples in a trial situation.

The immobilised prothrombin antibodies bound homologous prothrombin at concentrations of 0.01 nM and seemed to reach a saturation point at approximately 0.5 nM, as represented by the red plot in graph **A** of *Figure 4.3.2*. The response of the prothrombin antibodies to homologous solutions of thrombin, at the concentration range 0.01-0.5 nM, was significantly reduced

compared with that for prothrombin (**Figure 4.3.2 B**). The response obtained

due to the injection of human plasma like (150-750 nM) concentrations of

albumin was also minimal, as represented in **Figure 4.3.2 C**. The response

associated with the reaction of prothrombin with microcystin-LR antibodies is

minimal, and appears to be concentration independent in the concentration

range 0.01-0.5 nM (**Figure 4.3.2 A**).   The microcystin-LR antibodies did

appear to react more strongly to thrombin in the concentration range 0.01-5

nM compared with the prothrombin antibodies, however above 5 nM thrombin

the prothrombin antibodies reacted more strongly, as illustrated in **Figure**

4.3.2 B. This may be explained by the reduction in molecular mass of

thrombin compared with prothrombin, enabling thrombin to bind to more of

the non-specific binding sites. The dissociation constant calculated for the

prothrombin specific antibodies was 1.2 x10^{-10} with a χ^2 value of 3.68 x10^3,

calculated using the BIAevaluation software version 3.2 (Biacore AB).
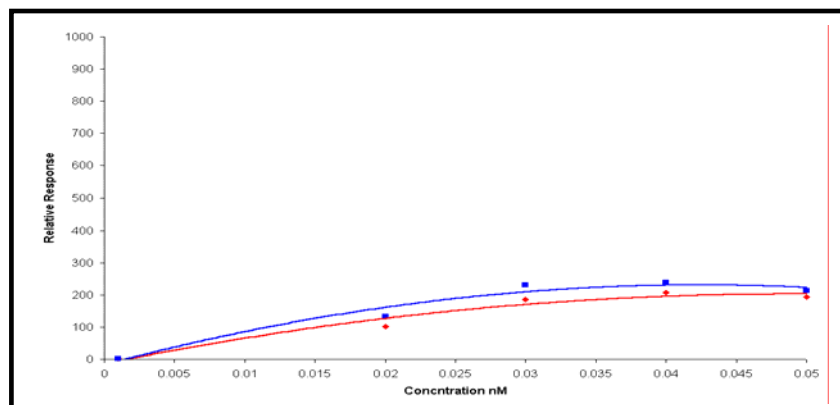
Figure 4.3.3 is a representation of the response obtained due to injection of prothrombin-spiked

plasma, onto the prothrombin and microcystin-LR antibodies. The injection of 1:10 sodium acetate

buffer (pH 4.0) diluted plasma, spiked with a concentration range (0 – 0.05 nM) of prothrombin. It

can be seen from the graph that both of the immobilised antibodies interact similarly to prothrombin

spiked-plasma. The injection of a 0.05 nM concentration of prothrombin produces a response of

only 200 response units, which is 5 times less than the injection of 0.05 nM homologous

prothrombin, as represented in **Figure 4.3.1A**. As with previous figures the blue trace represents

microcystin-LR antibodies and the red trace represents immobilised prothrombin antibodies.

The response elicited from the interaction between immobilised prothrombin antibodies and prothrombin-spiked 1:10 diluted plasma pH 4.0 was much reduced compared with the interaction between prothrombin antibodies and homologous-buffered prothrombin pH 4.0, as seen in *Figure 4.3.3*. The injection of prothrombin-spiked 1:10 diluted plasma over both antibody surfaces seems to elicit a non-specific response, relative to the antibody type, throughout the prothrombin concentration 0-0.05 nM. However the response does increase as the concentration of prothrombin increases (*Figure 4.3.3*).
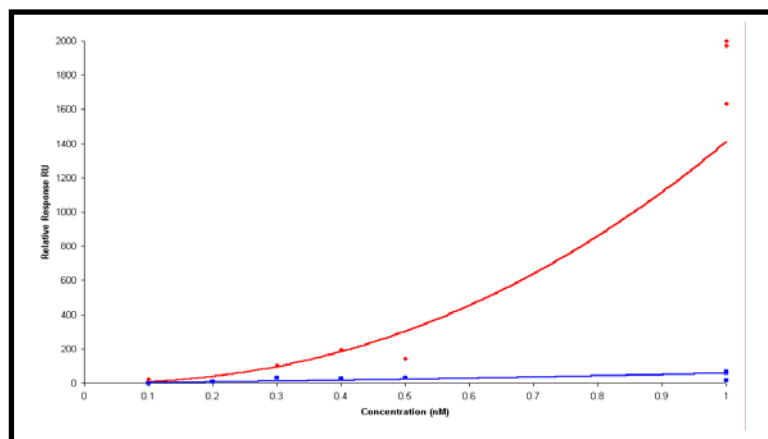
Figure 4.3.4 represents the injection of prothrombin spiked (0.1 – 1 nM) filtered plasma onto the prothrombin specific (red) and the non-specific (blue) antibodies. Centrifugal filtration was employed to remove the >100 kDa fraction from plasma samples, which were then diluted 1:10 with pH 4.0 sodium acetate buffer, and spiked with a (0.1 – 1 nM) range of prothrombin concentrations.

Prothrombin spiked plasma samples and prothrombin spiked filtered plasma samples behaved differently when injected onto the surface of the immobilised prothrombin-specific and non-specific antibodies. When the prothrombin spiked whole plasma was injected there was an apparent deposition of material onto the chip surface. This was characterised by a

general increase in SPR response and a loss of specificity with respect to prothrombin binding.   When spiked filtered plasma was injected, the deposition of material was not visible and the specific binding of prothrombin was more apparent.   The response elicited due to an injection of 0.05 nM prothrombin spiked whole plasma was not produced when spiked filtered plasma was used.

4.4 Discussion

Antibodies towards native human and bovine prothrombin have been synthesised and utilised in various quantitative and qualitative assays (Tai *et. al.*, 1979; Blanchard *et. al.*, 1983; Lewis *et. al.*, 1983; Malhotra and Sudilovsky, 1987).   Antibodies to native human prothrombin have been utilised in assays specifically designed to evaluate the significance of the native prothrombin antigen in monitoring oral anticoagulation therapy (Furie *et. al.*, 1983; Furie *et. al.*, 1990; Kornberg *et. al.*, 1993).   These antibodies were potentially able to monitor OACT much more efficiently than standard clot based assays, however clot based assays such as the prothrombin time and activated partial thromboplastin time are still the method of choice for OACT monitoring.

Initial attempts at producing a synthetic "epitope", using the "gla" domain of native human prothrombin as a template, were unsuccessful due logistical problems associated with the utilization of gamma-carboxyglutamic acid in the synthetic process.   After further research of the literature, this method may not have been ideal for the application in this case. Due to a large number of

various gla-containing proteins in the blood, the use of the prothrombin gla domain could introduce high cross reactivity with respect to the gla domain-specific antibodies (Brown *et. al.,* 2000). A commercial preparation of native human prothrombin was used as the antigen for rabbit polyclonal antibody production; these antibodies were subsequently affinity purified against the same commercial prothrombin antigen preparation. The commercial preparation was used to allow a direct comparison of antibody and molecular imprinting technology (Chapter 3) during the development of a native prothrombin specific assay. The necessary calcium ions required for the correct conformation of native prothrombin would be acquired from the rabbit's blood supply after immunisation, therefore the rabbit would have effectively been immunised with the native form of human prothrombin. However the affinity purification of these antibodies on a column of the commercial prothrombin, may have removed the calcium complexed native prothrombin specific antibodies. Affinity purification of the non-affinity purified antibody sample, against prothrombin complexed with calcium could provide antibodies specific for the calcium dependent conformation of native prothrombin, which could potentially already be present. However this would involve expensive and time-consuming experiments for the development of the affinity column and to acquire the specific antibody fraction, and also to test the antibody fraction against plasma samples.

Antibodies directed towards abnormal prothrombin species have also been produced, and initially it was thought that these could provide the basis of a prothrombin assay for OACT monitoring. The detection of vitamin K deficiency has been achieved through the production of an assay containing

abnormal prothrombin antibodies (Motohara *et. al.*, 1985), and it was for this reason and the fact that vitamin K antagonism is central to the mechanism of OACT, that such an assay was initially considered. Research of the literature subsequently suggested that total abnormal prothrombin would be difficult to detect in the complex plasma medium, due to its heterogeneity in warfarin treated patients (Esnouf and Prowse, 1976; Friedman *et. al.*, 1977). The level of vitamin K dependent protein decarboxylation and the relative proportions of decarboxylated prothrombin species were found to be related to the amount of vitamin K antagonism received by the patient (Tuhy *et. al.*, 1979). This indicated that increased and prolonged warfarin treatment is proportional to the increased levels of completely decarboxylated prothrombin in the blood, therefore adding substance to the argument that completely decarboxylated prothrombin might be a more suitable antigen for our purpose. However, Blanchard and colleagues in 1983 determined that total abnormal prothrombin could not be quantified using a single antibody species, due to the presence of variable concentrations of partially carboxylated prothrombin. They also discovered that the activity of native prothrombin correlated excellently with the native prothrombin antigen, allowing for the development of a more convenient approach to prothrombin quantification (Blanchard *et. al.*, 1983). This discovery more importantly, suggested a novel method of OACT monitoring using immunoassay technology, instead of the relatively inaccurate clot based assays. Evidence from three separate clinical trials, initiated to compare the native prothrombin antigen with prothrombin time and OACT monitoring, demonstrated that native prothrombin was a more accurate predictor of anticoagulative state (Furie *et. al.*, 1984; Furie *et. al.*, 1990; Kornberg *et. al.*, 1993).

The immobilisation of affinity purified prothrombin antibodies, using standard amine-coupling chemistry and Biacore technology, allowed us to produce a real-time immunoassay for human prothrombin. Immobilisation levels of the antibodies, achieved with a standard injection time and flow rate, corresponded with an antibody concentration of 8-10 ng mm^2 for the prothrombin antibodies and 18-25 ng mm^2 for the microcystin-LR antibodies, which correlated with those obtained previously (Vikinge *et. al.*, 1998; Sonezaki *et. al.*, 2000; Dillon *et. al.*, 2003).

Kinetic data corresponding to binding characteristics of the prothrombin antibodies could not be obtained due to badly fitting association and dissociation curves, which may have been due to the polyclonal nature of the prothrombin antibodies. Multiple antigenic sites on the prothrombin molecule would have bound to the CM5 immobilised polyclonal antibodies with variable association and dissociation constants, which may have caused badly fitting curves and therefore inadequate raw data for simple kinetic analysis (Brown *et. al.*, 2000).   The prothrombin antibodies however bound homologous solutions of prothrombin at concentrations (0.01–0.5 nM), much lower than the concentration of prothrombin in the blood or plasma (1.5-3.2 μM).   In keeping with the prothrombin specific properties of the antibodies, 0.1nM homologous solutions of thrombin produced a 10-fold decrease in relative response compared with prothrombin (see *Figures 4.3.2 A* and *B*).   With regard to the potential of albumin interfering with the response from prothrombin in plasma samples, plasma albumin concentrations of 750 μM produced 4-fold decrease in relative response compared to the response from

0.1 nM prothrombin. Thus indicating that there shouldn't be any interfering response from albumin in plasma samples, especially if 1:10 and 1:100 dilutions of plasma are used.

Prothrombin spiked plasma samples however did not produce a specific response, in fact the response of the microcystin-LR antibodies was very similar to that of the prothrombin antibodies during the injection of spiked plasma samples. Indicating that there could have been a non-specific binding mechanism interfering with the specific binding of prothrombin from the samples. An explanation for this non-specific effect maybe down to the high concentrations plasma proteins present in 1:10 diluted plasma, which may be reduced by the use of a higher plasma dilution factor e.g. 1:100. Studies of the initial reactions of blood with gold surfaces have shown that surface bound fibrinogen is predominant after 5 seconds of incubation (Eriksson and Nygren, 1997). In response to this observation, the binding of fibrinogen from plasma samples to the gold CM5 chip surface may block the specificity of the prothrombin antibodies, especially if the antibody is not so specific to start with. Therefore a sample pre-treatment that would remove all proteins above 100 kDa could potentially remove the antibodies' specific inhibitory effect imposed by fibrinogen in the whole plasma samples.

The centrifugal method used for the removal of the >100 kDa fraction from whole plasma did not turn out to be ideal. The instruction sheet provided with the filtration cartridges recommended a 50 kDa difference between the protein of interest (prothrombin at 70 kDa) and the fraction for removal, in this case the fraction above 100 kDa including fibrinogen. However filtration

cartridges with a molecular weight cut off, 50 kDa greater than prothrombin (i.e. 120 kDa) and 50 kDa less than fibrinogen (<200 kDa) were unavailable, which may have impaired the separation of fibrinogen from prothrombin. The sample pre-treatment was employed to remove the non-specific fibrinogen binding from the equation, in an effort to quantify specific prothrombin binding more easily.

As polyclonal serum contains a mixture of antibodies raised against the same antigen, but possess different affinities for a variety of epitopes on the surface of the antigen. If the antigen were a large protein for example, such as prothrombin, there would also be a wide variety of polyclonal antibodies produced with variable specificity towards the antigen. With this in mind, the use of polyclonal antibodies for Biacore affinity studies is not ideal, badly fitting dissociation curves and non-statistically viable dissociation constants would be produced. The dissociation constant of 1.2 $\times 10^{-10}$ indicates that the antibody possesses high affinity for homologous solutions of prothrombin. The statistical significance of the dissociation constant with a χ^2 value of 3.68 $\times 10^3$ is put under question due to heterogeneous binding of the polyclonal antibodies.   Over time, it was apparent that there was considerable degradation of the antibody, which was characterised by a significant loss of affinity to prothrombin, and a reduction in the amount of antibody immobilised onto the surface of a CM5 Biacore chip. As the dissociation constant is a measure of the antibodies affinity, its reduction is therefore characteristic of the antibodies degradation. A dissociation constant of 1.08 $\times 10^{-9}$ with a χ^2 value of 5.52 $\times 10^4$ suggests a reduction of the antibodies affinity. However

due to the association and dissociation curves badly fitting with the Langmuir binding 1:1 model, kinetics calculations were statistically unviable.

What we have presented here is a prothrombin specific polyclonal antibody based assay that displays affinity towards homologous concentrations of prothrombin, and prothrombin spiked processed plasma samples. The objective now is to determine whether this prothrombin assay can detect variable levels of native prothrombin in processed plasma samples, which can be achieved through the injection of clinical processed plasma samples through the assay, as presented in chapter 5.

Chapter 5

Comparison of prothrombin quantification systems using plasma samples and corresponding INR data

5.1 Introduction

Oral anticoagulation therapy monitoring is currently achieved through the implementation of clot-based assays that allow calculation of the international normalised ratio (INR), which is used to determine the dose of oral anticoagulant given to the patient. Despite the development and execution of strict standardisation procedure, patients monitored using the INR method can have an approximate major bleeding risk of between 3 - 8 % (Palareti *et. al.*, 1996; Yuoh *et. al.*, 2001). This is predominantly down to patient individual variation and sensitivity to the reagents of the clot-based assay and the limited success of the general practitioners maintenance of the therapeutic INR (Duxbury and Poller, 2001).

Comparison of oral anticoagulation monitoring methods in the past has provided substance to the argument that quantification of the native prothrombin antigen could provide a more efficient method for oral anticoagulation therapy (OACT) monitoring. The root of this idea originated from prothrombin specific antibody work, pioneered by Furie and colleagues in the early 1980s (Blanchard *et. al.*, 1983; Lewis *et. al.*, 1983). Conformation specific monoclonal antibodies were produced that recognised the calcium dependent conformer of prothrombin, and therefore correlated with functional coagulant activities. Many antibodies directed towards abnormal prothrombin have been developed in the past (Blanchard *et. al.*, 1979; Tai *et. al.*, 1979; Owens *et. al.*, 1984), however it was the native prothrombin antigen that provided increased correlation with a patient's state of anticoagulation (Furie *et. al.*, 1984 and 1990; Kornberg *et. al.*, 1993).

The INR is a valuable and reasonably accurate method for the monitoring of OACT and standard laboratory tests have achieved 97 % accuracy, as reported by a recent clinical trial (Yuoh *et. al.*, 2001). OACT monitoring can be inconvenient for patients due to the recurrent clinical visits required for blood tests and dose adjustment. However the development and introduction of patient self-test devices, has provided relief for the more inconvenienced of patients, by allowing self-OACT management and therefore reducing the number of required clinical visits. The accuracy of the patient self-test devices is not as high as the standard laboratory assays (Yuoh *et. al.*, 2001), however patients monitoring themselves are able to control their own INR more efficiently (Watzke *et. al.*, 2000), and thus able to decrease their general OACT complication rate.

The idea that OACT management can be achieved without complication is a bold and idealistic one. However, all therapies relating to human problems are prone to complication, which is not necessarily the downfall of the therapy itself, but to individual variation associated with the patient. This chapter will describe results obtained from a comparison of two alternative technology prothrombin assays, using randomised plasma samples obtained with consent from orally anticoagulated patients. With the objective of determining whether immuno or molecular imprinting technology could provide a solution to the problems of excessive bleeding or thrombosis, associated with current OACT monitoring using INR.

5.2 Materials and Methods

Vivaspin 2 PES (polyethersulfone) centrifugal filtration cartridges (catalogue number VS0242) with a 100,000 kDa molecular weight cut off were purchased from Vivascience (Hanover, Germany) through Fisher, UK (Loughborough, UK). Biacore UK (Biacore International SA UK, Stevenage, Hertfordshire, SG1 2EF) supplied research grade CM5 (BR-1000-14) and SIA kit Au (BR-1004-05) Biacore chips, together with HBS-EP buffer (BR-1001-88). A Beckman model J2-21 centrifuge was used for the sample preparation. A Biacore 3000 automated SPR instrument was used to carry out the assays for the entire study.

5.2.1 Sample collection

Informed consent (see appendix 5.1) was gained for the collection of plasma samples from patients who routinely attended Cheltenham general hospital's INR clinic for OACT management. The remainder of the plasma samples after the INR had been determined was collected and was stored at -20°C until required; the INR data for each labelled samples was also collected and stored in an MS excel file (see appendix 5.1).

5.2.2 Sample preparation

Samples (7 at a time) were removed from -20°C storage and placed in a 36°C water bath for 2 minutes to thaw. Each sample was aliquoted into the upper compartment of a labelled vivaspin 2 cartridge represented in *Figure*

5.2.1. The cartridges were then centrifuged at 9,000 xg for 75 minutes at room temperature. A 1:10 dilution with 10 mM sodium acetate buffer pH 4.0, was made using the filtrate containing the <100 kDa fraction, which was then aliquoted into vials for use in the Biacore assays. The remainder was stored at −20°C.

Figure 5.2.1. Vivaspin 2 polyethersulfone cartridge schematic

Load sample at the top of the cartridge and centrifuge at 9,000 xg for 75 minutes at 25°C.

Filter with 100,000 kDa Mw Cut off

Filtrate
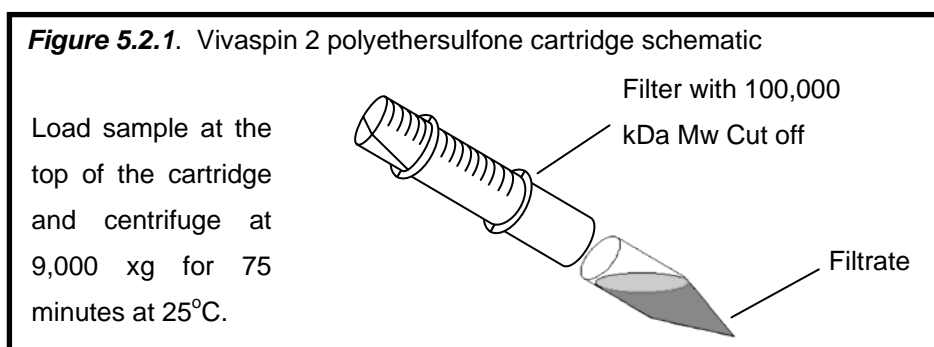
5.2.3 Biacore assays

Unmodified gold Biacore chips were used for the prothrombin imprinted poly-APBA assay and carboxymethyl-dextran modified CM5 chips were used for the antibody assay.   The polymer grafting (*Chapter 3*) and antibody immobilisation (*Chapter 4*) procedures have been described previously. A CM5 Biacore chip with prothrombin specific polyclonal antibodies immobilised on channel 1 and non-specific antibodies (Microcystin-LR specific) immobilised on channel 2 was docked into the Biacore 3000. A sensorgram was started using HBS-EP buffer at a flow rate 5 μlmin^{-1} and temperature 25 ^{o}C. A stable baseline was achieved and 35 μl of 1:10 diluted filtered sample was injected through both channels of the CM5 chip. The change in SPR

response (ΔRI = RI post injection – RI pre injection) for each sample was calculated. After each sample injection, the channel surface was washed with 10 mM HCl to remove any bound material from the immobilised antibodies, in preparation for subsequent sample injections.   The mild regeneration conditions (10 mM HCl) were used in order to preserve the activity of the immobilised antibody and allow the surface to be used repeatedly.

A prothrombin imprinted poly-aminophenylboronic acid (APBA) grafted J1 Biacore chip was docked into the Biacore 3000 and primed with physiological pH phosphate buffered solution, prior to starting a sensorgram on one channel at a buffer flow-rate of 5 μlmin^{-1} and 25 ^{o}C. A stable baseline was obtained through washing with 10 mM HCl, after which 35 μl of 1:10 diluted sample was injected onto a single prothrombin imprinted polymer-immobilised channel of the chip. The SPR response pre-injection was logged so that the change in SPR response 500 seconds post-injection could be calculated. After 500 seconds the channel was washed using a 10 μl injection of 10 mM HCl + 0.1% SDS, followed by a 10 μl injection of 10 mM HCl. The remainder of the samples were processed through the assay in this way, with 1 in 4 samples repeated.

5.2.4 Statistical analysis

The relationship between the INR and ΔRI of each specific sample was plotted on a graph and described using a simple correlation coefficient and linear regression.   The student T-test was used to describe the statistical significance of the correlation.

5.3 Results

Two sets of data were produced from the processing of diluted filtered plasma samples with the antibody and prothrombin imprinted MIP based assays, which are represented in *Figures 5.3.1* and *5.3.2*.

Figure 5.3.1 represents the change in SPR response (**ΔRU**) from injections of plasma 1:10 diluted with 10 mM sodium acetate, onto prothrombin specific antibodies (red) and microcystin-LR specific antibodies (blue), immobilised onto separate channels of a CM5 Biacore chip. The linear trend for the prothrombin specific antibody data can be represented by the equation y= 20.946x + 313.89 and has an r^2 value of 0.0016. The non-specific antibody linear trend line can be represented by the equation y= 10.504x + 85.094 with an r^2 value of 0.005. The correlation coefficients for the two sets of data were calculated as r= 0.0394 and r= 0.0706 for the prothrombin-specific and non-specific antibody data respectively.

Figure 5.3.2 represents **ΔRU** data corresponding to the injection of 39 plasma samples diluted 1:10 with 10 mM sodium acetate, onto the J1 Biacore chip with the prothrombin-imprinted poly-APBA grafted onto its surface. The linear trend line on the graph can be represented by the equation *y= -63.123x + 708.63*, and has an *r²* value of *0.0109*. The *correlation coefficient* relating the INR data with the change in SPR response of the sample injections was calculated as r= *–0.10424*.

The response values obtained for the immobilised prothrombin imprinted polymer and the filtered plasma samples seem to cluster around the INR range 1.5 – 3.5 (***Figure 5.3.2***), whereas the INR range for the antibody based assay varied equally between 1.5 – 4.0 (***Figure 5.3.1***). The high SPR responses during the antibody-based assay do not correspond with either high or low INR values (***Figure 5.3.1***), whereas in the MIP based assay the largest response is obtained from a relatively low INR value of 2.3 (***Figure 5.3.2***). With respect to the INR/prothrombin quantification predictive capabilities of the two assays, according to ***Figure 5.3.2***, the MIP-based assay seems to perform better than the antibody-based assay. The

correlation coefficient relating INR with the relative response obtained from the MIP based assay experiments was calculated as r^2= 0.0109, whereas the antibody-based assay provided an r^2 value of 0.003. The linear trend line drawn on the MIP-based assay filtered plasma results had an r-value of − 0.10424, potentially correlating lower INR plasma samples with higher relative SPR results. The antibody-based assay linear trend possessed a slightly positive r-value of 0.0394 suggesting a potential correlation between high INR and increased SPR response.

5.4 Discussion

The Biacore platform enabled us to follow in real time the performance of our antibody and MIP-based assays, in the analysis of filtered plasma samples for native prothrombin quantification. The fact that the grafted MIP was immobilised on an unmodified Biacore chip surface is in itself novel, however methods need to be developed that allow on-line monitoring of aqueous imprinted polymer surface immobilisation. This would enable the immobilisation of blank and imprinted polymers onto different channels of the same Biacore chip, which would further increase the validity of control polymer experiments. During our experiments blank and imprinted polymers were grafted to the surfaces of different chips, due the unavailability of a suitable off-line channel separation manifold.

The immobilisation of a blank polymer alongside an imprinted polymer (i.e. on the same surface) could provide an internal control channel and hence increase the validation of our results, however this is currently not possible on

our Biacore 3000 system. Biacore have recently developed an attachment for the Biacore 3000 (Biacore 3000 Recovery Upgrade Package, BR-1005-75), which enables the on-line monitoring of experiments that would otherwise cause harm to the internal fluidics of the system. This new attachment allows the separation of a chip surface into four distinct flow channels as with the standard four channels of the normal system, however such an attachment is expensive and beyond budget for this project. The specific order in which the testing of samples was conducted (i.e. prothrombin and non-specific antibodies immobilised on separate channels of a CM5 chip, followed by prothrombin imprinted polymer on a single J1 chip), allowed us to conclude that a control (blank) polymer was not required due to the imprinted polymer already producing non-correlating results. However, the use of an imprinted and control/blank polymer in the preliminary model assay demonstrated increased affinity of homogeneous prothrombin to the imprinted polymer over the blank polymer as represented in *Figure 3.3.1A*.

The question we need to answer now is, from the results of the filtered plasma injections, can we correlate increasing INR with Δ**RU** of the assays, in any way? The statistical information calculated from *Figures 5.3.1* and *5.3.2* suggests that there is no significant correlation between Δ**RU** and the INR value. Our hypothesis proposed an inversely proportional relationship with increasing INR and change in SPR response after injection (Δ**RU**), i.e. as INR increased, Δ**RU** would decrease. This could be explained by the fact that increasing INR corresponds to increased clotting times, which is caused by the removal of native prothrombin from the circulation by the action of warfarin.

Although statistically insignificant (t= 3.94 x10^{-7}), the antibody-based assay in **Figure 5.3.1** potentially shows the opposite of the proposed relationship and suggests instead, a proportional relationship between increased SPR response and INR, which would clearly be illogical. The MIP based assay (**Figure 5.3.2**) shows a closer relationship to the proposed inverse proportion for INR and Δ**RU**, where r= -0.10424.   However there is no statistical significance with this relationship, as the students' t-test gives a value of 1.98 x10^{-8} (insignificant even at the lowest confidence interval p= 0.2). So if the data doesn't correlate with the hypothesis, then it is highly likely that the assays are not measuring the native form of prothrombin.

The antibody-based assay could distinguish between increasing concentrations of homologous prothrombin and increasing concentrations of spiked prothrombin in filtered plasma (**Chapter 4**, **Figures 4.3.2** and **4.3.4** respectively).   We may also conclude that the antibody-based assay was specific enough to detect 0.1 – 1 nM concentrations of prothrombin in filtered plasma samples and 0.01 – 0.5 nM concentrations of homologous prothrombin.   This level of affinity would have been sufficient to detect the levels of prothrombin present in 1:10 diluted plasma (139 – 319 nM). So why did the antibody-based assay function differently to the way we hypothesised it could, i.e. that SPR response would correlate with INR? One option is that the antigen to which the antibody was raised was not specific for the native calcium dependent conformation of prothrombin, which is known to correlate precisely with prothrombin activity (Blanchard *et. al.*, 1983), and therefore anticoagulation status. A second reason why the plasma comparison results did not correlate with INR may have been brought about due to the use of

polyclonal antibodies in the development of the assay. The use of a heterogeneous combination of antibodies all showing specificity for different epitopes of the prothrombin molecule, may not have introduced sufficient specificity for the native conformation of prothrombin into the assay. The control antibody however did confirm that the prothrombin specific antibody was in fact specific for the prothrombin target.

The imprinted polymer graft showed potential for aqueous protein recognition both in this comparison and previously in (**Chapter 3.1.2**). It also demonstrated that the application of synthetic affinity material to the field of biosensors and protein recognition is feasible. Essentially we have created a synthetic affinity ligand that can be easily immobilised to gold surfaces, and shows affinity towards homologous prothrombin at a range 0.1 – 10 nM. However, this was reduced to 1 – 10 nM on application of prothrombin spiked plasma samples to the assay, which was still below the plasma prothrombin concentration range of 139 – 319 nM. The magnitude of the response elicited from such sample injections was also reduced, indicating that there was a loss in affinity, or an increase in non-specificity when plasma was used. This is not surprising, as plasma proteins are designed specifically to participate in acid-base equilibria and counteract electrostatic interferences (Figge *et. al.,* 1991). Non-specific binding of food components and low molecular weight plasma proteins to the surface of Biacore chips has been described previously (Nedelkov *et. al.*, 2000; Nedelkov and Nelson, 2001). The 6.5 kDa plasma proteins were identified as apolipoprotein C-1 and one of its derivatives (Nedelkov and Nelson, 2001), but only after subjecting the SPR-retained material to matrix-assisted laser desorption/ionisation – time-of-flight

mass spectroscopy (MALDI-TOF MS) (Krone *et. al.*, 1997). Such plasma proteins would still be present in the <100 kDa filtered plasma, therefore cause non-specific binding to the chip surface, or unreacted carboxyl groups of the carboxy-dextran matrix. SPR biosensors cannot definitively distinguish between non-specific binding and specific binding to an antibody-immobilised surface, coupled with MALDI-TOF MS however non-specific binding can be detected easily (Nedelkov *et. al.*, 2000). This technology however is beyond the scope of our project, but further direction in the development of a specific native prothrombin assay may incorporate such technology.

It is proposed that a complex mechanism, composed of many small electrostatic interactions together with the imprinting effect may have provided the observed affinity of the MIP towards the target protein. The negatively charged boronic acid groups of APBA would potentially interact with positive regions located on the surface of the protein, and the imprinting effect would specifically arrange these interactions with the target protein. This has been achieved previously using a similar polymer for the specific recognition of a variety of haemoglobin isomers (Middle *et. al.*‚1983; Gould and Hall, 1987), peroxidase enzymes (Piletsky *et. al.*, 2000; Bossi *et. al.*, 2001), and using acrylamide gels for specific recognition of glucose isomers (Liao *et. al.*, 1996; Hjerten *et. al.*, 1997; Wizeman and Kofinas, 2001). From previous studies it does seem that there is the potential for imprinted polymers to provide the necessary affinity for the quantification of prothrombin from plasma, however these results indicate that further studies are required to provide such an assay. Antibody degradation and loss of affinity was apparent when the

antibody-based assay was not stored in 'running' buffer, whereas the imprinted polymer lost no affinity on dry cold (4 ^{o}C) storage. Once stabilised, the MIP-based assay demonstrated increased physical stability over the antibody-based assay, due to its improved ability to maintain affinity for the target throughout the washing procedure.

Initially it was hoped that the plasma samples could be used raw and without any pre-processing, however it became apparent that after each raw plasma injection, a deposition of material onto each chip surface occurred, which subsequently hindered accurate response reading. There were indications that fibrinogen was a predominant causative agent involved in the bulk deposition (Eriksson and Nygren, 1997), therefore a pre-processing step was employed to remove the >100 kDa fraction from each plasma sample. In keeping with the initial design of the study, a rapid and uncomplicated pre-processing step was required, as extensive and complex protein purification techniques such as affinity chromatography and dialysis would utilise large amounts of laboratory time and expense. The Vivaspin-2 cartridges provided a simple solution to the pre-processing problem. The temperature regulation efficiency of the centrifuge used to process the samples was however, insufficient to prevent large fluctuations in temperature during filtration. This problem could not be overcome without significant expense, and it is thought that such temperature fluctuations could potentially have interfered with prothrombin quantification in the plasma samples.

In conclusion therefore, the assays developed here have shown quantifiable affinity for prothrombin (as provided by Calbiochem, UK), in homologous

solution (***Chapters 3 and 4***) and in prothrombin spiked, filtered plasma. The

Δ**RU** from both assays did not correlate significantly with the INR data

obtained for each sample. However, the application of monoclonal antibodies

to the antibody-based assay could potentially remove non-specific plasma

protein interactions and increase specificity and affinity for the native calcium

dependent conformation of prothrombin (Lewis *et. al.*, 1983; Malhotra and

Sudilovsky, 1987).

Chapter 6

Discussion and Final

Conclusions

6.1 General project summary

The global project aim for this project was:

- The development of an assay that shows specificity towards native prothrombin in homologous solutions and in complex matrices such as plasma and blood and the initialisation of a randomised clinical trial using ethically obtained plasma samples from orally anticoagulated patients allowing comparison with patient specific INR data.

This PhD thesis has presented evidence, which directly addresses the project aim and is summarised as:

- A computational model of human prothrombin was constructed using the tertiary bovine prothrombin structure and the primary amino acid sequence of human prothrombin, which was then used to design an epitope specific to prothrombin.

- The surface located peptide epitope was synthesised using standard Fmoc synthetic chemistry and was used as the template for an imprinted bulk polymer, which showed some specificity towards prothrombin, however showed substantial non-specificity at thrombin concentrations above 0.05 mgml^{-1}.

- Subsequently the complete prothrombin molecule was used as the template for polymeric adsorbent synthesis.

- Polymeric adsorbents using acrylamide and its derivatives did not produce the required affinity towards a commercial preparation of prothrombin, despite several previous successful attempts using different protein templates.

- The complete prothrombin molecule was again used as the template for poly-APBA homo-polymer imprint grafting, which allowed simple, but novel integration with the surface plasmon resonance transducer system (Biacore).

- The synthetic poly-APBA adsorbent showed specificity towards homologous prothrombin at concentrations between 0.01 – 14.2 nM, however this affinity concentration range was reduced by a factor of 10 when prothrombin spiked plasma was used.

- The production of polyclonal antiserum showing specificity towards the same commercial preparation of prothrombin was achieved, for the direct comparison of imprinting and immuno technologies with respect to prothrombin specificity and affinity. In comparison with the synthetic MIP adsorbent, the polyclonal antibodies showed affinity towards homologous prothrombin at a concentration range of 0.01 – 0.5 nM, which was again reduced when prothrombin spiked plasma was used.

- Biacore immobilisation technology was used to create synthetic imprinted polymer and immuno based assays for prothrombin.

- A clinical trial was conducted using the new assays, which comprised of 81 plasma samples with corresponding INR data, obtained with informed consent from patients regularly attending the INR clinic at Cheltenham General Hospital.

- The responses obtained from the injection of plasma samples through the prothrombin assays were compared to corresponding INR data. It was found that assay response did not significantly correlate with plasma INR data.

6.2 Global discussion and final conclusions

Why did the two assays not function the way we hypothesised they could?

We developed the MIP based assay with the aim that it would be specific enough to capture or bind prothrombin in plasma. As mentioned previously good recognition of epinephrine and peroxidase enzymes has been achieved using APBA homo-polymeric grafts in a microtiter plate format (Piletsky *et. al.*, 2000; Bossi *et. al.*, 2001). Therefore it was hypothesised that prothrombin could be recognised in complex solutions, such as plasma if the polymeric graft were fused with a highly sensitive transducer such as the Biacore. Recognition of various concentrations of homogeneous prothrombin, resulted in proportional SPR responses relative to the prothrombin concentration, therefore calibration of the assay was possible. The prothrombin imprinted MIP-based assay produced much smaller responses to thrombin than to prothrombin, whereas the blank polymer responded similarly towards both proteins, thus indicating the importance of the imprinting effect. Albumin concentrations of similar magnitude (0.05 – 10 nM) did not produce significant assay response with the prothrombin imprinted MIP, however albumin concentrations similar to those present in plasma produced responses equivalent to 10 nM prothrombin. Hence, the masking effect seen with prothrombin spiked whole plasma samples could potentially be attributed to saturating concentrations of albumin.

The dilution of plasma may have reduced the albumin concentration, however the relative concentration of prothrombin was also reduced, and therefore a dilution alone was insufficient to promote prothrombin specific binding. It was then noted that fibrinogen presented a major non-specific binding problem (Eriksson and Nygren, 1997), due to diluted plasma samples producing a bulk binding effect on the chip surface, which could not be removed by washing. The removal of the >100 kDa fraction of plasma, which contained fibrinogen, was achieved through centrifugal filtration, and the resulting plasma sample filtrate, did not produce the permanent bulk binding effect seen with whole plasma samples. Similar albumin saturation and bulk binding effects were apparent with the antibody-based assay; sample dilution and removal of fibrinogen reduced background non-specificity. The antibody-based assay bound homologous prothrombin solutions with increased affinity compared with the MIP-based assay, which was expected. In contrast to the MIP-based assay, it was apparent that environmental conditions, such as temperature and number of washes had significant effect on the ability of the antibodies to specifically bind prothrombin.

The polyclonal nature of the antibodies may have promoted the binding of abnormal prothrombin species in plasma samples. Due to the relatively large size of the antigen (prothrombin), the antiserum produced by the rabbit would have contained a variety of antibodies that show specificity towards a variety of epitopes located on the prothrombin molecule. And, due to the similar nature of native and abnormal prothrombin, i.e. the calcium dependent conformational change in the N-terminus of the protein, polyclonal antibodies may not have provided adequate specificity. In contrast, the efficiency of the

synthetic adsorbent mechanism providing affinity for prothrombin may not have been adequate in complex solutions. It is proposed that such a mechanism involves many weak electrostatic interactions between the template and the polymer, and that the imprinting process together with the solid gold surface allows their specific arrangement. Higher levels of binding were displayed when the pH of the binding buffer was kept at pH 4.0, indicating that the binding mechanism was strongly influenced by electrostatic interactions and environmental pH. Plasma proteins are involved in pH regulation and are able to control the electrostatic environment in the blood, which could explain the high level of non-specific binding with plasma injections onto the MIP-based assay.

Why is INR still the method of choice for anticoagulation clinics?

The INR is still the preferred method for OACT monitoring, due to its level of standardisation and amount of development it has received over the years (Talstad, 2000). Antibody–based assays have been produced that claim to be able to monitor OACT with increased efficiency over INR (Blanchard *et. al.*, 1983; Furie *et. al.*, 1984; Furie *et. al.*, 1990; Kornberg *et. al.*, 1993), however none have been implemented in OACT clinics. The use of bedside INR monitoring devices such as the Ciba Corning 512 system (Ciba Corning Diagnostics Corp., Medfield, MA), Hemochron 801 system (International Technidyne Corp., Edison, NJ) or the Coaguchek plus system (Roche Diagnostics) has enabled the self-monitoring of a patients INR. These devices are in fact less accurate than the laboratory tests (Yuoh *et. al.*, 2001),

however due to the fact that patients self-monitor and are able to dose adjust themselves every week, target INR levels are maintained efficiently (Watzke *et. al.*, 2000).

Complication rates, due to major bleeding episodes associated with OACT monitoring using INR, have recently been calculated at 1.2 – 2.8% in younger patients (<75 years) to 9.6 – 10.8% in patients aged 75 and over (Pengo *et. al.*, 2001). The incidence in younger patients is relatively low and can be attributed to the individual variation in sensitivity to thromboplastins and assay reagents. The higher incidence in older patients can arguably be attributed to a general deterioration of health with age, thinning and stiffening of vasculature and decreased recuperation efficiency with age. However, the complication incidences are still unacceptable and methods should be put in place to reduce them. The INR determining assays are in themselves relatively accurate, and standardisation since 1983 (WHO, 1983) has allowed extensive development of the PT and aPTT assays, but still the complications remain! Dedicated INR clinics have provided an increase in the quality of patient care relating to OACT monitoring in the UK and the Netherlands and some other European countries. Computer assisted dose adjustment and patient home testing have also provided increased efficacy of INR monitoring (Duxbury and Poller, 2001). It may be that the INR monitoring method has become as accurate as it can be, and that further developments of the anticoagulant, or as with the aim of this project further monitoring methods will have to be found.

What have we produced?

We have produced a synthetic adsorbent that shows specificity towards homogeneous prothrombin solutions, at a concentration range of 0.05 – 14.2 nM. The adsorbent also displays specificity towards prothrombin in spiked filtered plasma samples within a range of 1 – 10 nM, however the degree of binding was much reduced. The adsorbent however did not display any significant correlation to corresponding INR data, leading to a conclusion that the adsorbent was not solely specific for native prothrombin, but recognised many prothrombin species. The adsorbent however was considerably more stable than the polyclonal antibodies raised against an identical antigen, and showed comparable specificity towards homogeneous prothrombin and prothrombin spiked plasma samples. The adaptation of MIP technology to a variety of transducer systems, from microtiter plates, to SPR biochips, makes them an interesting and potentially lucrative technology for application in biosensor and diagnostic manufacture.

Further development of MIP technology in synthetic recognition of bio-molecules is required, along with the study of mechanisms that provide specific protein – ligand interactions. Molecular modelling, using NMR and protein crystallography data can provide an important starting block for this development. The molecular modelling of human prothrombin, in the early stages of this project, was initiated to provide structural information relating to potentially antigenic regions of the prothrombin surface. With such computational/structural information, there may have been the potential to model conformational changes of the protein, on calcium binding for instance.

The coupling of such data with a virtual library comprising of functional monomers for use in aqueous molecular imprinting, may have provided a more specific synthetic recognition element for use in OACT monitoring.

Essentially we have raised more questions about the efficacy of OACT monitoring using INR than we have answered in this study, and prior to the production of a synthetic native prothrombin assay that is robust and efficient, we are required to answer at least some of them. INR is not a 'gold standard' of OACT monitoring; it is a simplified ratio relating the coagulation time of a sample with that of a thromboplastin standard. Would the comparison of a native prothrombin assay against an established assay measuring native prothrombin enhance the validity of the study? The antibodies used by Kornberg *et. al.* (1993) were incorporated into an enzyme linked native prothrombin immunoassay by Diagnostica Stago (France), however this ELISA is now not produced and similar assays are rare, or unavailable. However, the work undertaken by Furie *et. al.* (1984 and 1990) and Kornberg *et. al.* (1993) indicated that OACT can be monitored more effectively through quantification of the native prothrombin antigen. So why haven't native prothrombin specific antibodies been incorporated into an assay suitable for the monitoring of OACT? The use and maintenance of animals in the production of antibodies is both costly and time consuming, ranging from £3000 – £10000 (IS-L, Paignton, UK) and a minimum of 12 weeks for the production of antiserum. The relatively large amount of development and standardisation that has been applied to the INR system has also put a restraint on the introduction of new OACT monitoring technology. Such novel assays are required to be cost effective and robust, which generally implies a

synthetic based assay and more importantly, more effective at OACT monitoring than the INR.   Antibodies, generally possess the affinity and necessary specificity for their incorporation into assays, but do not possess the robustness of synthetic adsorbent materials.   So with the rapid development of molecular imprinting technology, the production of a synthetic native prothrombin recognition element, with the specificity and affinity characteristics of antibodies is certainly foreseeable.

Departmental biotechnological tools, such as Sybyl 6.9 computational molecular modelling software, could not successfully model the human prothrombin structure. The necessary software (Sybyl 6.9.2) and force field updates (Amber 7) that accommodate long range ionic forces associated with metal ion-protein complexation are required for successful modelling to be carried out.   Software such as Quanta (Accelrys, Cambridge, UK), may provide an alternative for the computational simulation of prothrombin conformational change and the identification of synthetic binding ligands, for the specific recognition of native prothrombin. Software updates alone may not provide the necessary tools for successful human prothrombin simulation; X-ray crystallographic data of the calcium complexed human prothrombin gla domain is essential for the reliable modelling of such a complex protein structure.   This data would provide the necessary information to allow consistent molecular mechanics simulations of synthetic binding elements with potentially interesting regions of the protein structure, such as the complexation of carboxyglutamate residues within the gla domain.

So currently, INR is still the preferred OACT monitoring method purely due to the high costs incurred from its development and standardisation. One alternative to the development of OACT monitoring could lie with further development of the anticoagulative drugs used in the therapy. The use of computational methods to design a synthetic warfarin analogue, with increased therapeutic benefits such as slow release, may lead to reduced associated complication rates purely due to a potential decrease in the necessity for INR monitoring. The development of oral anticoagulant cocktails with increased anticoagulant potential may lead to a general dose reduction, potentially lowering complication rates. The questions raised by this study lead us to conclude that; the development of a novel native prothrombin assay may provide an answer to the complication rate associated with current anticoagulation therapy. However, it may be that further development to increase the efficacy of anticoagulant therapy could lessen the need for a more accurate monitoring assay; oral anticoagulants have such a narrow therapeutic ratio that any new monitoring method is required to be almost 100% accurate. Such questions require deliberation and research prior to formulating any decision on the future of oral anticoagulation therapy.

Chapter 7

Future Directions

7.1 Future directions

Future directions for the development of a novel native prothrombin assay would initiate with the acquisition of NMR and protein crystallography data of the calcium complexed conformation of prothrombin. I feel that a reliable computational model of the human prothrombin gla domain, based on real data would allow interactions of synthetic ligands with the gla domain to be consistently modelled. This computational data would provide the obvious financial benefits to the research, however will also allow us to visualise the mechanisms of interaction, prior to synthesis in the laboratory. The implementation of upgraded force fields and computational modelling software is an integral part of this recommendation, where the correct parameters for metal ions and modified amino acids are explicitly characterised. After the identification of native prothrombin specific-interaction ligands, rigorous specificity testing using homologous and spiked complex samples must be undertaken. Such investigations would potentially reduce non-specific binding of plasma proteins. With regard to non-specific binding, efforts to utilise more haemo-compatible surfaces in the development of blood-based biosensors, may also alleviate the challenge of non-specific binding from fibrinogen (Grunkemeier and Horbett, 1996, Eriksson and Nygren, 1997).

One major point that was illuminated in the previous chapter was that of the efficacy of oral anticoagulant therapy itself for the majority of patients, i.e. those over the age of 60 years. The therapeutic ratio of warfarin and its

analogues is extremely narrow, which puts increased emphasis on the accuracy of the monitoring methods used for its control. The INR system was developed from Armand Quick's prothrombin time (Quick *et al.*, 1935), which was a crude measurement of the time taken for blood to form a clot. The current INR system developed by the world health organisation (WHO, 1983), has received extensive standardisation and automation since the 1980s, but essentially has not changed since then.   With 20 years worth of biotechnological developments and new scientific research tools, surely the time has come (and gone) to either enhance the INR system, introduce a novel monitoring methodology or introduce a novel anticoagulation drug, or cocktail of drugs.

The brainchild of the late Dr Bob Dalton served initially to fuel this project, but after three years of trying to develop a novel native prothrombin assay to allow the more accurate management of OACT with INR, more questions have been raised regarding the efficacy of such an assay, than have been answered. For example, why hasn't the enzyme-linked assay developed by Kornberg *et. al.* (1993), using the monoclonal antibodies produced by Blanchard *et. al.* (1984) received the standardisation that INR has received, even though it has been suggested as a more efficient OACT management assay? One can only pose the answer that the financial implications of producing such an assay far outweigh the complications associated with the current methods.

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MIP and Antibody Based Prothrombin Assays for Monitoring Oral Anticoagulation Therapy

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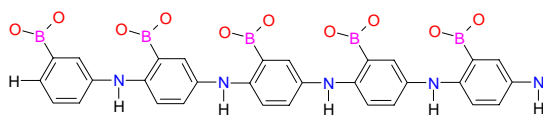
Introduction

The existing online protocol for measurement of the International Normalised Ratio (INR) allows rapid multiple sample analysis, however it suffers from the inaccuracy of the prothrombin time test. It has been proven that native prothrombin concentration is a more accurate predictor of anticoagulation status in warfarin treated patients. The objective of this work is the development of a novel direct native prothrombin assay utilising molecular imprinting technology. However, molecular imprinting of large protein molecules continues to be a challenging task.

Our strategy is based on developing homologous MIPs capable of template recognition through the combination of weak molecular interactions and the geometrical shape of the imprinting cavity, rather than through the strong interactions between the template and a few carefully positioned functional monomers. We used a surface grafting method, which allowed the non-covalent adsorption of prothrombin imprinted poly-aminophenylboronic acid (monohydrate) to the gold surface of a standard J1 Biacore Chip. The selective material described here offers attractive potential for the development of a real time prothrombin biosensor. The MIP performance was also compared with that of monoclonal antibodies.
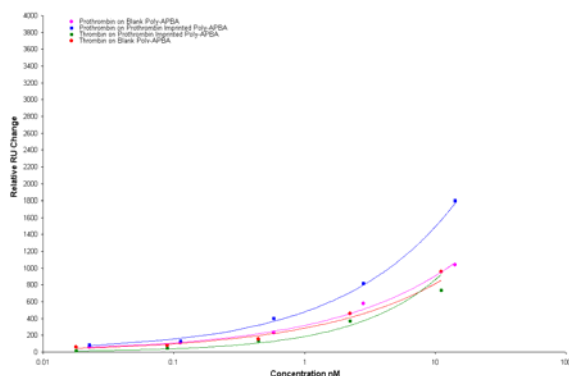
Results

Initial results suggest that prothrombin-imprinted poly-APBA preferentially binds prothrombin over thrombin. The results also show that corresponding blank poly-APBA (prepared in the absence of template) does not bind prothrombin equally well in the same experimental conditions. The cold dry storage capability of the MIP presented an advantage over the antibodies, which required a steady cold flow of running buffer for storage purposes.

**Figure 1:** The 2D molecular structure of poly-aminophenylboronic acid.

Prior to rebinding the removal of excess material from the chip surface was achieved using 10 mM HCl, which was also used to regenerate the polymer after rebinding experiments. The regeneration and washing of immobilised anti human prothrombin polyclonal antibodies was also achieved using 10 mM HCl. Rebinding of the template to the polymer was achieved in 100 mM sodium acetate buffer pH 4.0. The relative SPR response corresponding to the addition of protein samples was monitored using the Biacore® 3000.

Figure 2: Prothrombin imprinted poly-APBA and blank poly-APBA, prothrombin and thrombin rebinding. Prothrombin imprinted polymer can detect prothrombin levels from 0.1 nM.
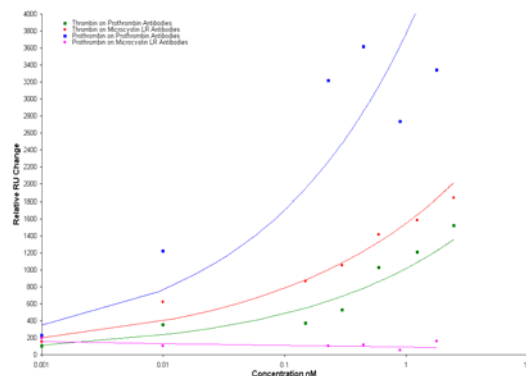
Figure 3: Prothrombin and Microcystin-LR polyclonal antibodies, prothrombin and thrombin rebinding. Prothrombin antibodies can detect prothrombin levels from 0.01 nM.

Conclusions

The rebinding of varying concentrations of homologous prothrombin solution to the surface grafted prothrombin imprinted polymer produced a larger SPR response compared with the blank polymer. The prothrombin polyclonal antibodies showed much higher affinity for prothrombin compared with the prothrombin imprinted MIP. The polymeric material and the polyclonal antibodies also showed (although significantly reduced) non-specific binding to thrombin. The MIP displayed increased stability to 10 mM HCl regeneration compared with the polyclonal antibodies which showed significant denaturation after 30 washes. Preliminary tests show that both the antibodies and prothrombin imprinted polymer can detect prothrombin in prothrombin spiked diluted plasma solutions.

The prothrombin imprinted polymer produced has the ability to detect normal levels of native human prothrombin in plasma samples. A clinical trial is currently underway to assess the potential to detect the levels of native human prothrombin in plasma samples from orally anticoagulated patients.

(*) a.j.sylvester.s00@cranfield.ac.uk

www.cranfield.ac.uk/ibst/cbmc

CRANFIELD BIOMEDICAL CENTRE

<u>Appendices</u>

Appendix A.

Appendix B. IS-L Product information sheets

Appendix C. Evidence for ethical approval

Appendix D. Lack of calibration reproducibility

Appendix E. Poster

Accurate Mass Spectrum of FNEKTFGA (TFA Salt) Synthesised on

Perkin Elmer 433A Peptide Synthesiser
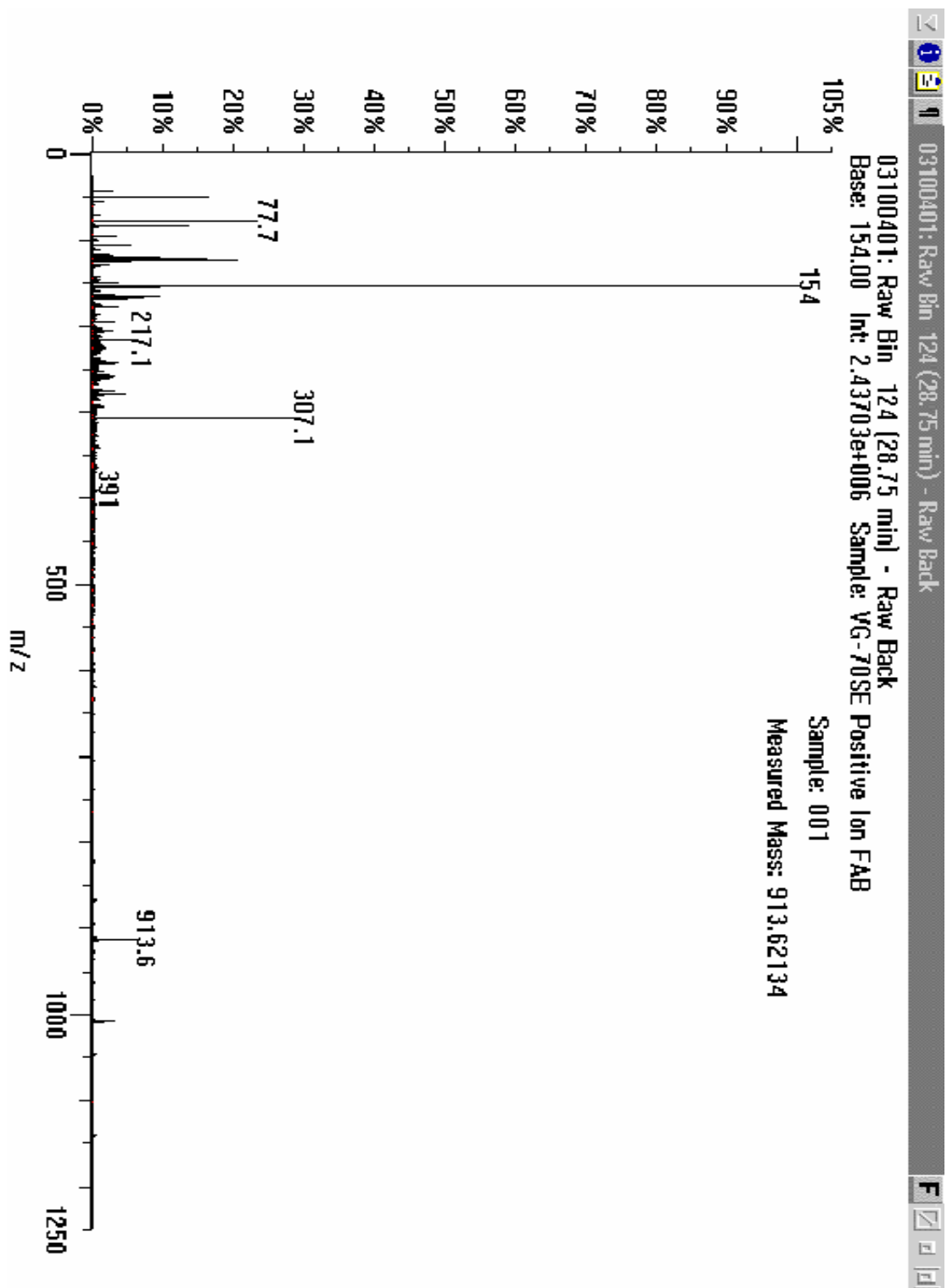
IS-L Product Information Sheets

Product Specification Sheet

Affinity Purified Rabbit anti-Human Prothrombin Antibody.

Antibody Designation:	Human Prothrombin
Immunogen:	Human Prothrombin
Quantity of sera processed:	70ml
Affinity purification, method:	Affinity purification of whole serum on column of immobilised human prothrombin, cross absorption on a human immunoglobulin column, followed by G50 buffer exchange.
Final yield:	Approximately 18.9 mg
Presentation:	702 µg/ml, ml in phosphate buffered saline with 0.05% sodium azide. Recommended for storage at 4°C.
Batch Reference:	36AA03
For ISL	3 June, 2003

Conditions

The products listed herein are for in vitro use only and should not be administered to humans or used for any drug purpose. The information presented is believed to be accurate. The information and products are offered without warranty or guarantee since the ultimate conditions of use and the variability of the materials are beyond our control. We cannot be responsible for patent infringements or other violations that may occur with the use of these products.

03 June 2003

ISL

Product Specification Sheet

Affinity Purified Cross Reactive Human Prothrombin Antibody.

Antibody Designation: Human Prothrombin (cross reactive)

Immunogen: Human Prothrombin

Quantity of sera processed: 70ml

Affinity purification, method: Following affinity purification of whole serum
                                         on a column of human prothrombin, the
                                         purified antibody fraction was run on a human
                                         immunoglobulin column. Cross reactive
                                         antibody was eluted and buffer exchanged on a
                                         G50 column.

Final yield: Approximately 1.16mg

Presentation: 58 μg ml^{-1}, ml in phosphate buffered saline
                                         with 0.05% sodium azide. Recommended for
                                         storage at 4°C.

Batch Reference: 36AB03

For ISL   3 June, 2003

Conditions
The products listed herein are for in vitro use only and should not be administered to humans or used for any drug purpose.
The information presented is believed to be accurate. The information and products are offered without warranty or guarantee
since the ultimate conditions of use and the variability of the materials are beyond our control. We cannot be responsible for
patent infringements or other violations that may occur with the use of these products.

                                                                                         03 June 2003

Material Safety Data Sheet

Name: Purified antibody.

Description: Antibody concentrate purified from guinea pig sera in phosphate buffered saline solution.

Hazards: No hazards are known to be associated with this product.

Material Safety Data Sheet

Name: Sodium Azide.

Chemical Description: $NaN3$, Mw 65.01, + 0.05% solotion.

Hazards: Highly toxic. Fatal if inhaled or swallowed or absorbed through the skin. May cause genetic damage. May cause explosions. May give toxic gases.

Handling protection: Wear protective clothing, gloves, glasses and face mask. Do not swallow. Do not expose to skin and eyes. Avoid prolonged or repeated exposure

First Aid: Flush eyes and skin with large amounts of water. Remove contaminated clothing. If swallowed, rinse mouth and seek medical advice, If inhaled, remove to fresh air.

Handling/storage: Wear protective clothing, gloves, glasses and face mask. Use only in a chemical fume hood. Store in a cool dry place. Keep tightly closed.

Avoid: Sodium azide may react with heavy metals and metal halides to form explosive products. Avoid acids. May explode when heated.

Spills: Clear the area. Clean up wearing suitable clothing, mask, gloves. Place dry contents in bag or bottle for disposal. Wash up any residual.

Fire precautions: Dry powder only. Wear contained breathing equipment. May emit toxic fumes.

Disposal: Approved disposal service.

The above information is for guide-line purposes only and may not be fully comprehensive. All products should only be handled by trained personnel. Immune Systems and affiliated companies are not liable for any damage caused in any way by the above material.

ISL

Product Specification Sheet

Antibody Depleated Rabbit Serum.

Antibody Designation: Human Prothrombin

Immunogen: Human Prothrombin

Original quantity of sera: 71ml

Presentation: Bulk of serum, antibody depleted and diluted
                                                with phosphate buffered saline with 0.05%
                                                sodium azide. Recommended for storage at
                                                4°C.

For ISL   2 June, 2003

ISL

Product Specification Sheet

Rabbit Serum.

Antibody Designation:	Human Prothrombin
Immunogen:	Human Prothrombin
Original quantity of sera:	71ml
Presentation:	Sample of unpurified serum. Bulk of serum, antibody depleted and diluted with phosphate buffered saline with 0.05% sodium azide. Recommended for storage at 4°C.
For ISL	2 June, 2003

Conditions

02 June 2003

Material Safety Data Sheet

Name: Animal Sera.

Description: Animal sera in phosphate buffered saline solution.

Hazards: No hazards are known to be associated with this product.

Material Safety Data Sheet

Name: Sodium Azide.

Chemical Description: NaN3, Mw 65.01, + 0.05% solution.

Hazards: Highly toxic. Fatal if inhaled or swallowed or absorbed through the skin. May cause genetic damage. May cause explosions. May give toxic gases.

Handling protection: Wear protective clothing, gloves, glasses and face mask. Do not swallow. Do not expose to skin and eyes. Avoid prolonged or repeated exposure

First Aid: Flush eyes and skin with large amounts of water. Remove contaminated clothing. If swallowed, rinse mouth and seek medical advice, If inhaled, remove to fresh air.

Handling/storage: Wear protective clothing, gloves, glasses and face mask. Use only in a chemical fume hood. Store in a cool dry place. Keep tightly closed.

Avoid: Sodium azide may react with heavy metals and metal halides to form explosive products. Avoid acids. May explode when heated.

Spills: Clear the area. Clean up wearing suitable clothing, mask, gloves. Place dry contents in bag or bottle for disposal. Wash up any residual.

Fire precautions: Dry powder only. Wear contained breathing equipment. May emit toxic fumes.

Disposal: Approved disposal service.

The above information is for guide-line purposes only and may not be fully comprehensive. All products should only be handled by trained personnel. Immune Systems and affiliated companies are not liable for any damage caused in any way by the above material.

Data protection compliance

Gloucestershire Hospitals **NHS**
NHS Trust

Gloucestershire Royal Hospital
Great Western Road
Gloucester
GL1 3NN
Telephone: 08454 225660
Fax: 08454 224590
Email: SueDennis@gloucr-tr.swest.nhs.uk

5 August 2003

DATA PROTECTION COMPLIANCE

Mr Adrian Sylvester
Cranfield University
Barton Road
Silsoe
Bedfordshire
MK45 4DT

Dear Mr Sylvester

Re: Study - The development of a novel native prothrombin assay for the monitoring of oral and anticoagulation therapy (OACT)

In my capacity as Data Protection Officer for Gloucestershire Hospitals NHS Trust I have received details of the above Study. I do not have any major issues related to Data Protection but would ask you to confirm the details raised in point 3 below and if you could just note and the amend the documentation accordingly as advised in points 1 and 2:

1   Could I suggest that for future reference to the documentation and to assist patients you do not use abbreviations such as on the Protocol, Patient Information Sheet and Consent Form the use of *CGH* or *INR* unless they have previously been written out in full on the documents.

2   **Consent Form**

Please note the above comments and could you record on the bottom of the form where and by whom copies of the Consent Form will be retained.

3   **Security and retention**

You have covered the issues of security and retention related to electronic data but it is not clear as to where the manual cross referencing data will be held, by whom, for how long, who will have access to it and who will take responsibility for its ultimate destruction. Please could you clarify this detail?

I will await your reply but am happy in the meantime for this Study to proceed through for Trust approval and submission to the Ethics Committee.

Yours sincerely

Sue Dennis
Clinical Information and Data Protection Manager

Cc:
Dr Sally Pearson, Director of Clinical Strategy, Gloucestershire Hospitals NHS Trust
Dr Guy Routh, Medical Director, Gloucestershire Hospitals NHS Trust
Dr Colin Roch-Berry, Caldicott Guardian, Gloucestershire Hospitals NHS Trust
Julie Hapeshi, Research & Development Co-ordinator, Gloucestershire Hospitals NHS Trust
Dr Anthony Woodman, Cranfield Biomedical Centre

Ethical approval

NHS

Gloucestershire Local Research Ethics Committee

Gloucestershire Royal Hospital
Great Western Road
Gloucester
GL1 3NN

Tel: 01452 395726
Fax: 01452 395720
Email: sue.starck@glouc-tr.swest.nhs.uk

Our Ref: 03_59G(22_9)

September 22, 2003

Mr Adrian Sylvester
PhD Student
Cranfield BioMedical Centre
Cranfield University at Silsoe
Barton Road, Silsoe
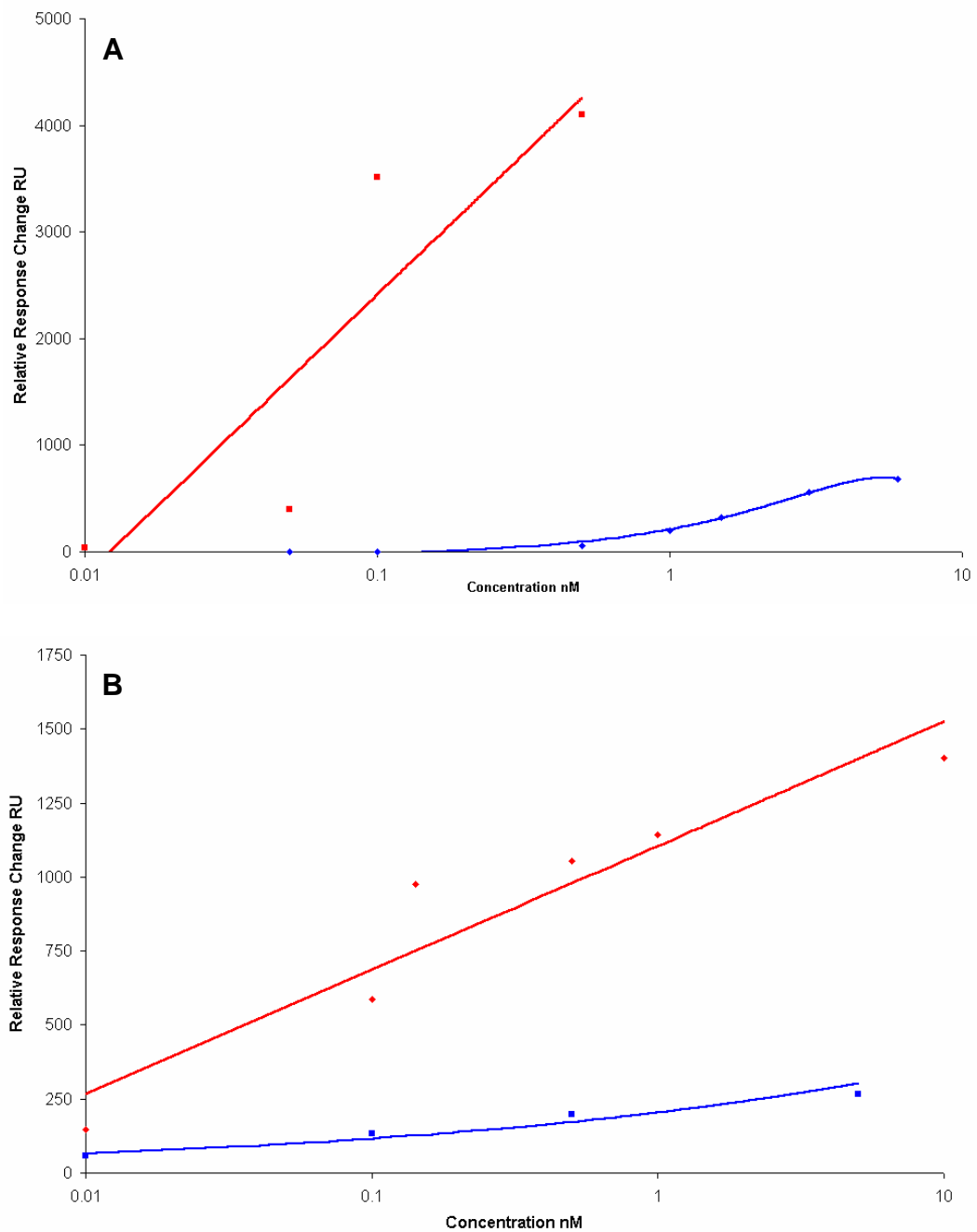Bedfordshire
MK45 4DT

Dear Mr Sylvester

Study No 03/59G : The development of a novel native prothrombin assay for the monitoring of oral anticoagulation therapy (OACT)

I am writing further to recent correspondence received from Dr Anthony Woodman dated September 11, 2003 with regards to the above study. I have now reviewed the amendments and will be advising the committee that in my view there is now no objection on ethical grounds to the proposed study. Therefore, I am happy to give you approval on the understanding that you will follow the conditions of the approval set out below. The following documents were reviewed by the committee: -

- LREC application form
- Protocol (version 1, dated July 21, 2003)
- Email (dated September 4, 2003)
- Email (dated September 3, 2003)
- Consent Form (version 1, dated July 21, 2003)
- Patient Information Sheet (version 1, dated July 21, 2003)
- Emails with Sue Dennis regarding Data Protection Compliance (dated August 7, 2003)
- Data Protection Compliance letter (dated August 5, 2003)
- Curriculum Vitae
- Host Organisation Trust Approval (dated August 11, 2003)

a) It is the responsibility of the investigator to notify the LREC immediately of any information received by him/her, or of which he/she becomes aware which would cast doubt upon, or alter, any information contained in the original application, a later amendment application or verbal resume submitted to the LREC. The committee should be informed immediately if this information would raise questions about the safety and/or continued conduct of the research.

b) The need to comply with the Data Protection Act 1998.

c) You will need to comply with the Research Governance Framework for Health and Social Care (Department of Health 2001). This includes the requirement to make available records for the monitoring of the study by the research sponsor. Further information regarding this document can be obtained from Gloucestershire Research & Development Support Unit on 01452 395726.

d) The need to refer proposed amendments to the protocol to the LREC for further review and to obtain LREC approval thereto prior to implementation (except only in cases of emergency where the welfare of the subject is paramount).

e) The requirement to furnish the LREC with details of the progress of the research project periodically (usually annually) and **failure to do this could result in approval to continue with the study being withdrawn.** Please also inform us of the conclusion and outcome of the research project and inform the LREC should the research be discontinued or any subject withdrawn altogether.

f) It is the responsibility of the person conducting any Trial to ensure that all professional staff and management of NHS Trusts involved are notified that it is taking place.

Gloucestershire LREC is fully compliant with the International Conference on Harmonisation/ Good Clinical Practice (ICH GCP) Guidelines for the Conduct of Trials Involving the Participation of Human Subjects.

Please indicate your agreement to comply with the requirements outlined in this letter by signing both copies of this letter and returning one to Sue Starck. Full approval does not commence until the signed copy is returned.

Yours sincerely

**Mrs Philippa Burgon
Vice Chair, Gloucestershire LREC**

cc   Dr Sally Pearson, Director of Clinical Strategy, Gloucestershire Hospitals NHS Trust
       Sue Dennis, Data Protection Officer, Gloucestershire Hospitals NHS Trust

I agree to comply with the requirements outlined in this letter.

Signed _____   Date 25/9/03

An advisory committee to Avon, Gloucestershire & Wiltshire Strategic Health Authority

Appendix D.

Appendix D represents the results obtained when the prothrombin (red) and thrombin (blue) calibrations are repeated on different chips. Graph **A** represents a calibration on the pAPBA coated J1 chip and graph **B** represents a calibration using immobilised prothrombin antibodies.   The relative response change on injection of 0.5 nM prothrombin on the prothrombin imprinted polymer coated chip is much higher than that in *Figure 3.3.1A*. Similarly there are vast differences with the antibody calibration, thus indicating that the coating of different chips with prothrombin imprinted pAPBA and prothrombin antibodies does not give highly reproducible calibration results.

__Cranfield Biomedical Centre__   Date of sampling:
Institute of Bioscience and Technology   Patient Number:
Cranfield University   Pathology Number:
Silsoe   Study Sample ID Code:
Bedfordshire   INR Data Sent: Yes / No
MK45 4DT   INR Value:

Consent Form

Title of Project: The development of a novel normal prothrombin assay for the more accurate monitoring of oral anticoagulation therapy.

Name of Researchers: Mr Adrian J Sylvester BSc. MSc. and Dr Gill Rouse.

1. I confirm that I have read and understand the information sheet dated 21.07.03 for the above study. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. ☐

3. I am willing to allow access to my medical records by Dr Gill Rouse for the duration of the study but understand that strict confidentiality will be maintained. ☐

4. I agree to take part in the above study ☐

I understand that no information from which I could be recognised will leave Cheltenham General Hospital (CGH).

_____   _____   _____
Name of Patient   Date   Signature

_____   _____   _____
Name of Person Taking Consent   Date   Signature
(If different from researcher)

_____   _____   _____
Researcher   Date   Signature

Copies of the consent form will be held by Dr Gill Rouse (with patient Data), Adrian Sylvester (without patient data) and the patient.

CGH Consent form, Version 1, 21.07.03

Cranfield Biomedical Centre
Institute of Bioscience and Technology
Cranfield University
Silsoe
Bedfordshire
MK45 4DT

Patient Information Sheet

Title:

The development of a novel native prothrombin assay for the more accurate monitoring of oral anticoagulation therapy (OACT)

Invitation to participate:

You are invited to participate in a research project. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information sheet carefully and discuss it with friends and relatives. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

The aim of this study is to use two new methods for the development of a new improved method for monitoring OACT. Current monitoring techniques involve measurement of the clotting time in a patient's blood sample and its comparison to a standard clotting time, which gives the International Normalised Ratio (INR) result. This is then used to calculate the warfarin dose that the patient should receive.

We are looking more closely at prothrombin, which is the major blood clotting protein and is more directly related to the clotting state of the blood. By measuring the levels of prothrombin in a blood sample we believe that we could improve dosing of warfarin.

We are going to use two new methods to measure the levels of prothrombin and then we will compare these results with the INR data from each sample. This study is jointly run between scientists at Cranfield University, Silsoe and staff at Cheltenham General Hospital (CGH).

Why have I been chosen?

You have been chosen because you are undergoing routine anticoagulation monitoring at CGH INR clinic. Blood samples are taken regularly from outpatients to determine the patients INR and subsequent warfarin dose. Once the routine INR data has been obtained the remainder of the sample will analysed at Cranfield Biomedical centre as part of the research.

CGH Information Sheet, Version 1, 21.07.03

Do I have to take part?

It is entirely up to you whether you take part or not. If you do decide to take part you will be asked to sign a consent form, however you are free to withdraw at any time and without reason, and this will not affect the standard of care you receive. If you choose to withdraw, all data collected about you and all your samples will be destroyed.

What will happen to me if I take part?

You will attend the INR clinic consultation as normal and your INR will be calculated as normal from the blood sample you give. However, the remainder of this sample will be analysed in the laboratory in a new novel system that will detect the amount of normal prothrombin in the sample. We will only require part of this one sample from you and this will not be used for any other purpose other than for this research study. **No additional blood samples will need to be taken from you.**

What do I have to do?

We would like you to give your permission to allow some of the routine blood sample to be used for this study. This research will in no way affect the treatment you would normally receive.

What are the side effects of taking part?

There are no side effects of taking part in this study.

What are the possible disadvantages and risks of taking part?

There are no disadvantages or risks associated with agreeing to allow the use of your blood in this study.

What are the possible benefits of taking part in this study?

There is no specific clinical benefit to you from taking part in this study. The results will however, hopefully lead to further research that may better allow the more accurate prescription of oral anticoagulant dose, therefore helping future patients.

What happens when the research study stops?

The information gathered will be analysed and published as a scientific thesis to contribute to further research. Any remaining research material will be destroyed.

What if something goes wrong?

If taking part in this study harms you in any way, there are no special compensation arrangements. Regardless of this, if you wish to complain about any aspect of the way in which you have been approached or treated during the course of the study, the normal National Health Service complaints mechanisms will be available to you.

Will my taking part in this study be kept confidential?

All blood specimens will be kept confidential. If you consent to take part in this study then your medical documents maybe examined by the INR consultant, but of course this information will remain confidential. No personal information about you will leave Cheltenham General Hospital.

What will happen to the results of the research study?

The results will be analysed and written up in a scientific PhD thesis. This will take approximately 1 year to produce and will be available in the libraries of the participating hospital. You will not be identified in any report or publication.

Who is organising the funding?

Cheltenham General Hospital Trust and Cranfield Biomedical Centre, Silsoe have jointly funded the research.

Who has reviewed the study?

The Gloucestershire Research Ethics Committee has reviewed this study.

Contacts for further information:

Mr Adrian Sylvester   Dr Gill Rouse
Cranfield Biomedical Centre   Department of Pathology
Cranfield University   Cheltenham General Hospital
Barton Road   College Road
Silsoe   Cheltenham
Bedfordshire. MK45 4DT   GL55 7AN

Tel: 01525 863002 (Ext: 3776)   Tel: 08454 224057
Email: a.j.Sylvester.s00@cranfield.ac.uk   Email: Gill.Rouse@egnhst.org.uk

Thank you for taking the time to read this information sheet!

Cranfield Biomedical Centre
Institute of Bioscience and Technology
Cranfield University
Silsoe
Bedfordshire
MK45 4DT

Protocol for Sample Analysis

Title: The development of a novel native prothrombin assay for the more accurate monitoring of oral anticoagulation therapy (OACT).

1) Patients will be asked to participate by Dr Gill Rouse or their nurse during a routine International Normalised Ratio (INR) clinic at Cheltenham general Hospital (CGH).

2) The patients will be given an information sheet, which provides relevant information about the study and Dr Rouse or the nurse will explain the study to them. They will then have until their next INR clinic appointment (up to 2 weeks) to decide if they wish to participate.

3) Patients arriving at the next INR clinic will be asked of their decision regarding the study. The study participants will then be given a consent form to sign, and three copies will be made, 1 for the researcher, 1 for the patient and 1 for the health records.

4) The patients' blood sample will be taken for INR determination.

5) The consultant or the nurse will take and label the blood sample tubes using a study ID code sticker.

6) The data obtained from the sample collection at CGH will be stored in an electronic password protected database on Dr Anthony Woodman's computer.

7) The following data will be required: year of birth, gender and the results of the INR determination. Only the following researchers will have access to the data: Mr Adrian Sylvester and Dr Anthony Woodman. All data will be held until the end of the project (PhD) and subsequently destroyed by Dr. Anthony Woodman.

8) A sticker with the following data will be attached to each hospital copy of the consent form for cross-referencing purposes: date of sampling, patient number, pathology number, sample ID code and whether INR data was sent with the sample.

9) The hospital copies of the consent forms will be held by Dr Gill Rouse for a period of 1 year from sampling and will be destroyed after this period. Access will not be provided to anyone else other than Dr Gill Rouse.

10) Samples will be analysed using the newly developed polymer based method and with the antibody based method using a surface plasmon resonance (SPR) based detection device.

11) Statistical analysis and their comparison with the INR data from each sample will enable the calibration of the method with respect to it potential in monitoring oral anticoagulation.

Gloucestershire Hospitals **NHS**

NHS Trust

Gloucestershire Royal Hospital
Great Western Road
Gloucester
GL1 3NN
Telephone: 08454 225660
Fax: 08454 224590
Email: SueDennis@gloucr-tr.swest.nhs.uk

5 August 2003

DATA PROTECTION COMPLIANCE

Mr Adrian Sylvester
Cranfield University
Barton Road
Silsoe
Bedfordshire
MK45 4DT

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Yours sincerely

Sue Dennis
Clinical Information and Data Protection Manager

Cc:
Dr Sally Pearson, Director of Clinical Strategy, Gloucestershire Hospitals NHS Trust
Dr Guy Routh, Medical Director, Gloucestershire Hospitals NHS Trust
Dr Colin Roch-Berry, Caldicott Guardian, Gloucestershire Hospitals NHS Trust
Julie Hapeshi, Research & Development Co-ordinator, Gloucestershire Hospitals NHS Trust
Dr Anthony Woodman, Cranfield Biomedical Centre