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MODIFICATION OF POLYMERIC BIOMATERIALS FOR IMPROVED BLOOD COMPATIBILITY: INVESTIGATION OF PROTEIN ADSORPTION AND IN VITRO BLOOD RESPONSE

by

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ABSTRACT

Polymeric biomaterials are widely used in blood contacting devices. The inevitability of thrombus formation on biomaterial surfaces in contact with blood requires the presence of an antithrombotic agent to enable clinical use, with possible complications. Therefore, the development of biomaterials with improved blood compatibility is an important objective. For polymeric biomaterials, options for improving blood compatibility are polymer synthesis, formulation and modification, with modification the focus of this project. Modification approaches studied in this project were the increase in polymer hydrophilicity by utilisation of poly(ethylene oxide), alterations to cellulose by chemical modification and the attachment of the anticoagulant heparin and the preparation of biomembrane-mimetic surfaces.

Material assessment covered protein adsorption and an in vitro blood compatibility evaluation. The protein adsorption study investigated the influence of materials on fibrinogen and albumin, as determined by radioactivity measurement of ^{125}I-labelled single protein. The emphasis in the blood compatibility evaluation was on complement activation, as represented by measurement of C3a generation, and the contact phase activation of blood coagulation, as represented by measurement of factor XII-like activity (FXIIA). The blood compatibility evaluation required the establishment of a modified incubation test cell capable of providing a consistent area of material in contact with blood and a response appropriate to the selected parameter measurement. The protein adsorption studies demonstrated that both the type of polymer surface and the modification procedure influence the adsorption. A pronounced reduction in protein adsorption was obtained with a biomembrane-mimetic surface. Increase in hydrophilicity by the utilisation of poly(ethylene oxide) can increase albumin adsorption and markedly reduce fibrinogen adsorption. The blood compatibility assessment indicates that alterations to polymer surfaces can strongly influence complement activation but have little influence on contact activation. The evidence confirms the potential of polymer modification as a means of improving blood compatibility and supports the further investigation of the relationship between modification and different features of the blood response.

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DEDICATED TO MY HUSBAND

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CHAPTER 1

INTRODUCTION

1.1 BIOMATERIALS

A biomaterial has been defined as a material of synthetic or natural origin, used in contact with tissue, blood or biological fluid and intended for use in prosthetic, diagnostic, therapeutic or storage applications (Courtney et al 1993a). Biomaterials can be grouped into different types. Table 1.1 lists types of biomaterial and their major applications.

Polymeric biomaterials have a widespread application and are particularly important for artificial organs. The advantages of polymeric biomaterials include lightness in weight, ease of processing, and versatility, in that polymerisation and modification enable wide possibilities for obtaining desired structures. However, polymers are not ideal, as they have low thermal conductivity and heat sensitivity. Additionally, it is difficult to achieve a high level of product consistency, since polymers may have to undergo ingredient incorporation, purification, fabrication and sterilisation.

The utilisation of polymers as biomaterials is a subject of continued clinical, scientific and industrial interest (Szycher 1983; Sharma and Szycher 1991), and polymeric biomaterials formed the focus of this project.

Table 1.1 Typical Classes of biomaterial *

Type of Biomaterial	Application
Polymer	Widespread, e.g. artificial organs
Metal	Implants
Ceramic	Implants, special membranes
Carbon (inert pyrolytic carbon)	Mechanical type heart valve
Composite	Fibre-reinforced polymers

* From Courtney et al 1993b

1.2 BLOOD COMPATIBILITY

When biomaterials are brought into contact with biological fluids of the human body, such as blood or tissues, the key issue is generally to minimise thrombogenic, toxic or allergic reactions. The ability of a biomaterial to perform without a clinically significant host response has been considered under biocompatibility. In the case of a biomaterial contacting blood, the focus is on the blood response or "so-called" blood compatibility. Blood compatibility has been regarded as an aspect of the overall biological response and relevant information can be derived from the knowledge of blood-biomaterial interactions, the influence of biomaterials on blood constituents and the events which induce thrombus formation on biomaterial surfaces (Andrade et al 1981; Szycher 1983; Murabayashi and Nosé 1986; Ringoir and Vanholder 1986; Forbes and Courtney 1987; Forbes et al 1989; Vanholder and Ringoir 1989).

Although the application of biomaterials extends over a period of about 100 years (Williams 1987), the biocompatibility of biomaterials is still not well resolved. Unlike human endothelium, all biomaterials initiate blood coagulation when in contact with blood. Consequently, the application of biomaterials in clinical situations generally requires the administration of an antithrombotic agent, which could result in the risk of bleeding. Therefore, improvement in the compatibility of biomaterials to reduce and perhaps to eliminate the use of these antithrombotic agents is highly desirable for the development of blood-contacting devices, such as the artificial kidney (Nosé 1988; Ringoir and Vanholder 1990) and the artificial heart (Didisheim et al 1989; Kambic and Nosé 1991).

1.3 OPTIONS FOR BLOOD COMPATIBILITY IMPROVEMENT

Biomaterials for blood-contacting devices are dominated by the use of polymeric biomaterials. Factors influencing the properties of polymeric biomaterials are polymerisation methods, polymer ingredients, purification techniques, polymer fabrication and sterilisation procedures (Courtney et al

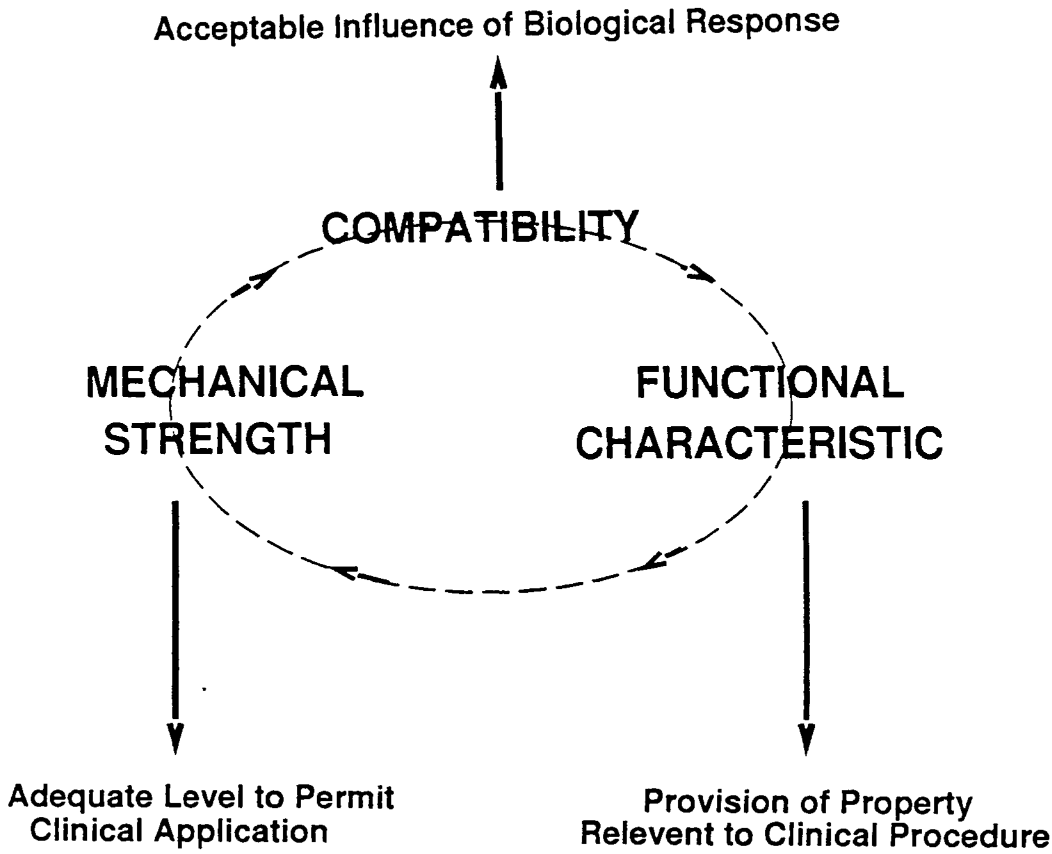


Figure 1.1 Fundamental Properties of a Biomaterial
(From Courtney et al 1993a)

1993b).

The options for improving the blood compatibility of polymeric biomaterials are polymer synthesis, polymer formulation and polymer surface modification. An example of polymer synthesis in order to improve blood compatibility is the preparation of polyurethanes (Grasel and Cooper 1986; Liu et al 1989). An example of utilising polymer formulation in order to improve the blood compatibility is the selection of a plasticiser for poly(vinyl chloride) (PVC) blood tubing (Blass et al 1991; Blass 1992).

The development of biomaterials with improved blood compatibility based on novel polymer synthesis is hindered by establishment of a manufacturing process capable of fulfilling the production level necessary for clinical utilisation. Improving biomaterials with better blood compatibility based on different polymer formulations is generally restricted to the polymers where a polymer ingredient is a major constituent. Therefore, polymer surface modification offers a more widespread option.

It is important that the consideration of blood compatibility should not be isolated from the fact that the biomaterial must possess a functional characteristic in order to fulfil its intended clinical purpose. This functional characteristic may be flexibility for blood tubing or catheters, solute permeability for a membrane to be used in an artificial kidney, or gas permeability for a membrane to be used in an artificial lung. In addition, the biomaterial must also possess an acceptable degree of mechanical strength for practical application, which includes the stability of a biomaterial in biological environment. Together with the biocompatibility, the functional characteristic and the mechanical strength can be regarded as the fundamental properties of a biomaterial (Figure 1.1). In general, it is very difficult for polymer synthesis and polymer formulation to obtain the desired blood compatibility and the required mechanical and functional characteristics at same time, but this can be achieved by a more advantageous option, polymer surface modification. Polymer surface modification concentrates on alteration of the surface properties of an existing biomaterial, which possesses the satisfactory functional characteristic and mechanical strength for clinical application. The potential advantage of polymeric biomaterial modification is that of enhancing compatibility with minimal alteration to material production and its other properties. Polymer modification was the main research interest of this project.

1.4 MODIFICATION TECHNIQUES

The surface properties of biomaterials can be altered by various methods, many of which have proved capable of improving blood compatibility to some extent. The effective techniques for polymer modification can be grouped into the following categories:

1. Alteration to surface physical properties
2. Treatment of surfaces with protein
3. Increase of surface hydrophilicity
4. Surface chemical modification
5. Attachment of antithrombotic agents onto surface
6. Preparation of biomembrane-mimetic surface

These techniques have been developed on the basis of knowledge derived from the investigation of the blood response to the biomaterials. The techniques are reviewed in detail in Chapter 3.

1.5 SELECTED BIOMATERIALS

The biomaterials selected in this research programme were used to investigate the influence on blood compatibility of polymer modification techniques. These techniques covered increasing hydrophilicity, chemical modification, the attachment of antithrombotic agents and the preparation of biomembrane-mimetic surfaces.

To study the influence of increased hydrophilicity, the biomaterial selected was a novel polyurethaneurea hydrogel based on poly(ethylene oxide). An advantage of this hydrogel is solubility in common solvents, thereby promoting its application as a modification material (Chapter 5).

Regenerated cellulose was selected as the basis for investigating chemical modification and the attachment of antithrombotic agents. The standard cellulose membrane Cuprophan was compared with (a) a chemically modified cellulose membrane, Hemophan, and (b) Cuprophan on to which the anticoagulant heparin was covalently attached (Chapter 6).

Biomembrane-mimetic surfaces were studied by selecting the copolymer, poly(2-methacryloyloxyethyl phosphorylcholine (MPC) --co--n-butyl methacrylate (BMA)) (poly(BMA-co-MPC)) and cellulose membrane onto which methacryloyloxyethyl phosphorylcholine (MPC) was grafted (Chapter 7).

1.6 SELECTED PARAMETERS

To monitor the blood response to biomaterials, many parameters have been proposed as indicators of blood interactions with biomaterials (Chapter 2). In general, the advantages of multiparameter assessment have to be offset against the need to acquire proficiency with respect to measurement of any particular parameter. In this project, the parameters selected were based on three relevant features. These were protein adsorption, complement activation and the contact phase activation of blood coagulation.

Protein adsorption plays a key role in the interactions of blood with biomaterials (Chapter 2). The adsorption of fibrinogen and albumin was selected as the basis of this study (Chapter 4). Both proteins are considered important for blood-biomaterial interactions, particularly with respect to platelet reactions.

The relationship between biomaterials and complement activation has become a subject of great interest. This interest has grown because of the following:

1. Clinical data relating to the immune system (Chapter 2)
2. Materials believed to be blood compatible on the basis of reactions of platelets and the coagulation system induce complement activation (Payne and Horbett 1987)

3. Complement activation has been shown to be influenced by the chemical modification of polymers (Chapter 3)

Complement activation was determined by the measurement of the component C3a generation using a radioimmunoassay (Chapter 4).

Contact phase activation caused by blood-biomaterial interactions can be considered as the initial step for the intrinsic pathway of coagulation in which Hageman factor (FXII) plays a central role (Chapter 2). In this project, contact activation was determined by the measurement of FXII-like activity using a modified chromogenic substrate assay (Chapter 4).

1.7 TEST PROCEDURE

The protein adsorption studies carried out in this project were regarded as an initial stage in the study of the relationship between modification and the protein adsorption. Therefore, the decision taken was to perform measurements of the adsorption of single proteins, with concentrations of ^{125}I-fibrinogen or albumin on biomaterial surfaces determined by ^{125}I-radio-activity. Since a range of materials was examined, a test procedure suitable for polymers varying in hydrophilicity was established (Chapter 4).

The blood compatibility assessment was also envisaged as a primary evaluation. Test procedures based on incubation test cells were considered appropriate and selected as the basis for optimisation of blood response for a given biomaterial area. Since the utilisation of an anticoagulant can alter blood response to a biomaterial (Robertson et al 1990), human whole blood containing no anticoagulant was used to study blood compatibility in this project (Chapter 4).

1.8 THESIS OBJECTIVES

The overall objective was to establish suitable procedures for monitoring protein adsorption and the in vitro blood response and to utilize these procedures in an investigation of the influence of the following approaches to modification

1. Preparation of polyurethaneurea hydrogels based on poly(ethylene oxide)
2. Chemical modification of cellulose by partial replacement of hydroxyl groups with diethylaminoethyl (DEAE) groups
3. Covalent attachment of heparin onto cellulose membrane
4. Biomembrane-mimetic surfaces applied to n-butyl methacrylate and cellulose

CHAPTER 2

INTERACTIONS OF BLOOD WITH BIOMATERIALS

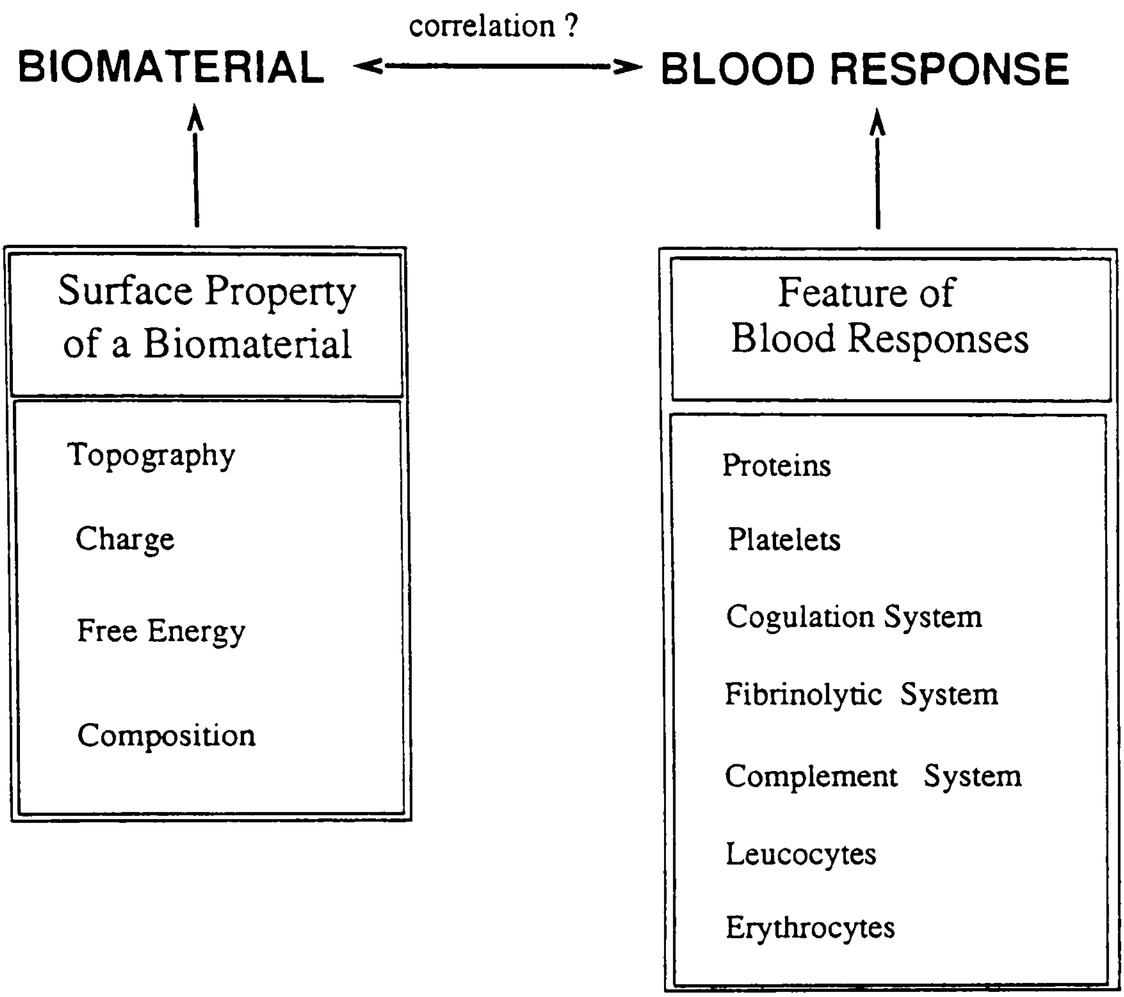


Figure 2.1 Possible correlation between a biomaterial and a blood response

2.1 INTRODUCTION

The investigation of blood interactions with biomaterials has a long history (Brücke 1857; Lister 1863) and has been promoted by the increasing clinical applications of blood-contacting devices (Klinkmann 1989).

The influence of biomaterial surface properties, such as surface charge, surface free energy, chemical composition and surface topography, on blood constituents is one of the aspects of investigating blood interactions with biomaterials. Knowledge obtained from such investigations has been used in attempts to correlate blood response with the properties of the biomaterial surface (Figure 2.1), which is an useful guide for producing surfaces with enhanced blood compatibility (Matsuda 1989; Courtney et al 1993a).

However, the interactions of blood with biomaterials are complex and this complexity may be increased in the clinical situation by device components, the nature of the application and patient status (Klinkmann et al 1987; Courtney et al 1993b). While acknowledging the difficulties, it is believed that a better understanding of blood-biomaterial interactions is important and necessary for optimum utilisation of existing materials and the development of improved blood-contacting materials (Klinkmann 1984; Forbes and Courtney 1987).

2.2 HAEMOSTASIS AND THROMBOSIS

The consideration of blood-biomaterial interactions can be assisted by first examining blood coagulation in relation to haemostasis and thrombosis.

The processes of haemostasis and thrombosis include the complex reactions between the endothelium, blood platelets, the coagulation system and the fibrinolytic system (Ogston 1983) and can be considered in terms of following aspects:

Platelets

Blood coagulation system

Fibrinolytic system

Complement system

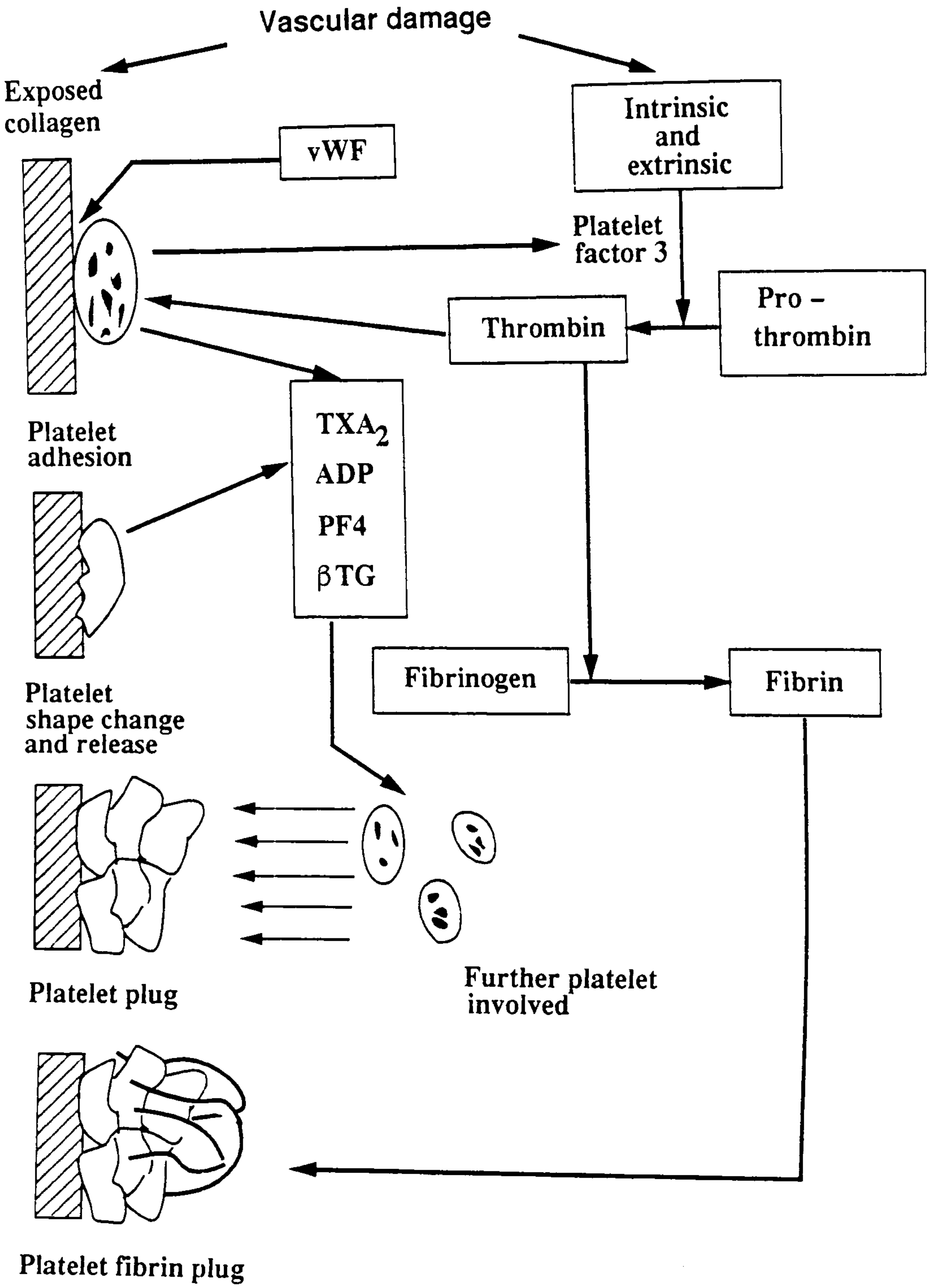


Figure 2.2 Interaction between platelets, plasma factors and vessel wall in haemostasis

2.2.1 Platelets

The action of platelets in the process of haemostasis can be considered in terms of platelet adhesion, platelet release and platelet aggregation.

Normally, platelets do not adhere onto the luminal surface of endothelial cells but only following vascular injury (Stemerman et al 1971; Mason 1972; Mason et al 1976). When platelets come into contact with parts of a damaged blood vessel, such as collagen or endothelium, they change from their normal disc shape to a more rounded form, extend pseudopods and become adherent. The factors important for the adherence of platelets to the site of injury include subendothelial collagen, a platelet membrane glycoprotein and plasma von Willebrand factor (vWF). Collagen is required to achieve full release and aggregation following adhesion (Baumgartner 1977). The glycoprotein of the platelet membrane is the specific receptor on platelets for adhesion of platelets onto collagen (Nurden and Caen 1976, 1977). The vWF helps to induce platelet adhesion after a conformational change by the interactions between vWF and subendothelium (Kao et al 1979).

The adhesion of platelets initiates a further sequence of reactions, leading to platelet release and aggregation. In the release reaction, the contents of platelet granules are released into the circulating blood, including platelet factor 4, β-thromboglobulin and adenosine diphosphate (ADP), which stimulates new platelets to aggregation. Stimulated platelets also produce thromboxane A_2 (TXA_2), a very potent aggregating agent. The mechanism of platelet aggregation is complex and requires extracellular fibrinogen, calcium (Ca^{2+}) and ADP (Bennett and Vilaire 1979; Bennett et al 1981; Marguerie and Plow 1981).

Platelet adhesion, release and aggregation act to prevent blood loss by forming a platelet plug which is initially loose. To stop bleeding, a mechanically stable thrombus has to be formed, which requires the participation of blood coagulation factors. Interaction between platelets, coagulation factors and the vessel wall is illustrated in Figure 2.2. Platelets

Table 2.1 Plasma coagulation factors and their synonyms*

Factor	Synonym
I	Fibrinogen
II	Prothrombin
III	Tissue thromboplastin
IV	Calcium ions
V	Proaccelerin, labile factor, or accelerator globulin
VII	Serum prothrombin conversion accelerator (SPCA), stable factor, or proconvertin
VIII	Antihaemophilic factor (AHF), antihaemophilic factor A, or antihaemophilic globulin (AHG)
IX	Christmas factor, plasma thromboplastin component(PTC), or antihaemophilic factor B
X	Stuart factor or Stuart-Prower factor
XI	Plasma thromboplastin antecedent(PTA) or antihaemophilic factor C
XII	Hageman factor or glass factor
XIII	Fibrin stabilizing factor (FSF)
3	Platelet phospholipid clotting activity
4	Platelet antiheparin activity

* Modified from Szycher (1983)

contribute significantly to the reactions of the coagulation factors by the provision of membrane phospholipids (platelet factor 3). Additional examples of interactions between platelets and coagulation factors include the fact that fibrinogen is an essential cofactor for platelet aggregation and platelet stimulation can result in a rapid reversible binding of fibrinogen to receptors on the platelet surface.

2.2.2 Blood Coagulation System

The processes of blood coagulation involve various proteins known as coagulation factors, which are listed in Table 2.1. The reactions of those factors are complex and can be summarized in terms of the intrinsic, extrinsic and common pathways. The cascade of blood coagulation is shown in Figure 2.3.

2.2.2.1 Intrinsic Pathway

The intrinsic pathway is so named because the formation of prothrombin activation is initiated by the substances contained within the blood itself (Tortora and Anagnostakos 1987), which can be described as follows:

The intrinsic pathway is initiated when blood comes into contact with a foreign surface, which will cause the activation of factor XII. The activation of factor XII in the presence of prekallikrein and high molecular kininogen (HMWK) transforms factor XII to the activated form XIIa . This transformation leads to the conversion of factor XI into its activated form, XIa, which in turn activates factor IX. Subsequently, factor X is converted to Xa in the presence of a complex consisting of factor IXa, factor VIII, calcium ions and platelet factor 3 (PF3). Once factor X is activated, it reacts with platelet phospholipids, factor V, and Ca^{2+} ions to form prothrombin activator. This completes the intrinsic pathway.

2.2.2.2 Extrinsic Pathway

The extrinsic pathway is so named because the formation of prothrombin

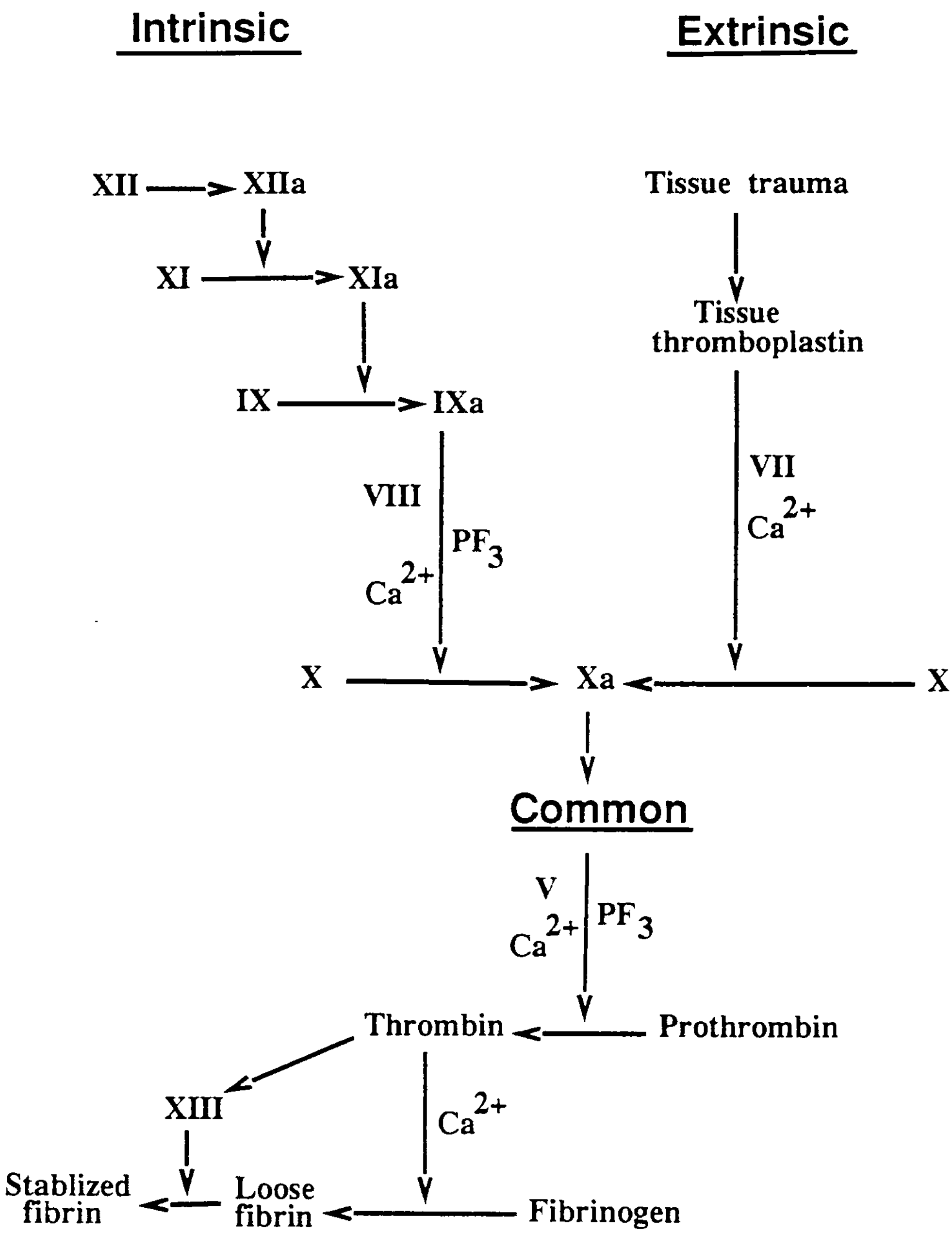


Figure 2.3 Cascade of blood coagulation

activator is initiated by substances released by the damaged blood vessels, or surrounding tissues, instead of those in blood itself (Tortora and Anagnostakos 1987).

The extrinsic pathway is initiated when blood contacts an injured vessel. Damaged blood vessels or tissues release a complex of substances called tissue thromboplastin. Among the components of tissue thromboplastin are an enzyme and membrane phospholipids. Tissue thromboplastin, together with coagulation factor VII and Ca^{2+} ions activate factor X. Once factor X is activated, it reacts with the membrane phospholipids, factor V, and Ca^{2+} ions to form prothrombin activator. This completes the extrinsic pathway.

2.2.2.3 Common Pathway

In the common pathway, the prothrombin activator, together with Ca^{2+} ions, catalyses the conversion of prothrombin into thrombin. In the next stage, thrombin, in the presence of Ca^{2+} ions, converts soluble fibrinogen to insoluble fibrin. Thrombin also activates coagulation factor XIII, which strengthens and stabilizes the fibrin clot.

The process of blood coagulation is autocatalylic and thrombin plays a central role in a positive feedback manner. Once thrombin is formed, it causes platelets to aggregate, resulting in the release of more platelet phosphlipids necessary for the coagulation process. Another positive feedback effect of thrombin is the acceleration of the formation of prothrombin activator through factor V, which in turn accelerates the production of more thrombin.

2.2.3 Fibrinolysis

The process of fibrinolysis involves dissolution of insoluble fibrin. The key reaction of fibrinolysis (Figure 2.4) is the conversion of plasminogen by plasminogen activators into the protease plasmin, which acts within the thrombus, digesting fibrin to induce thrombus dissolution.

Plasminogen can be activated through two different routes. Firstly, both body tissues and blood contain substances that can activate plasminogen to

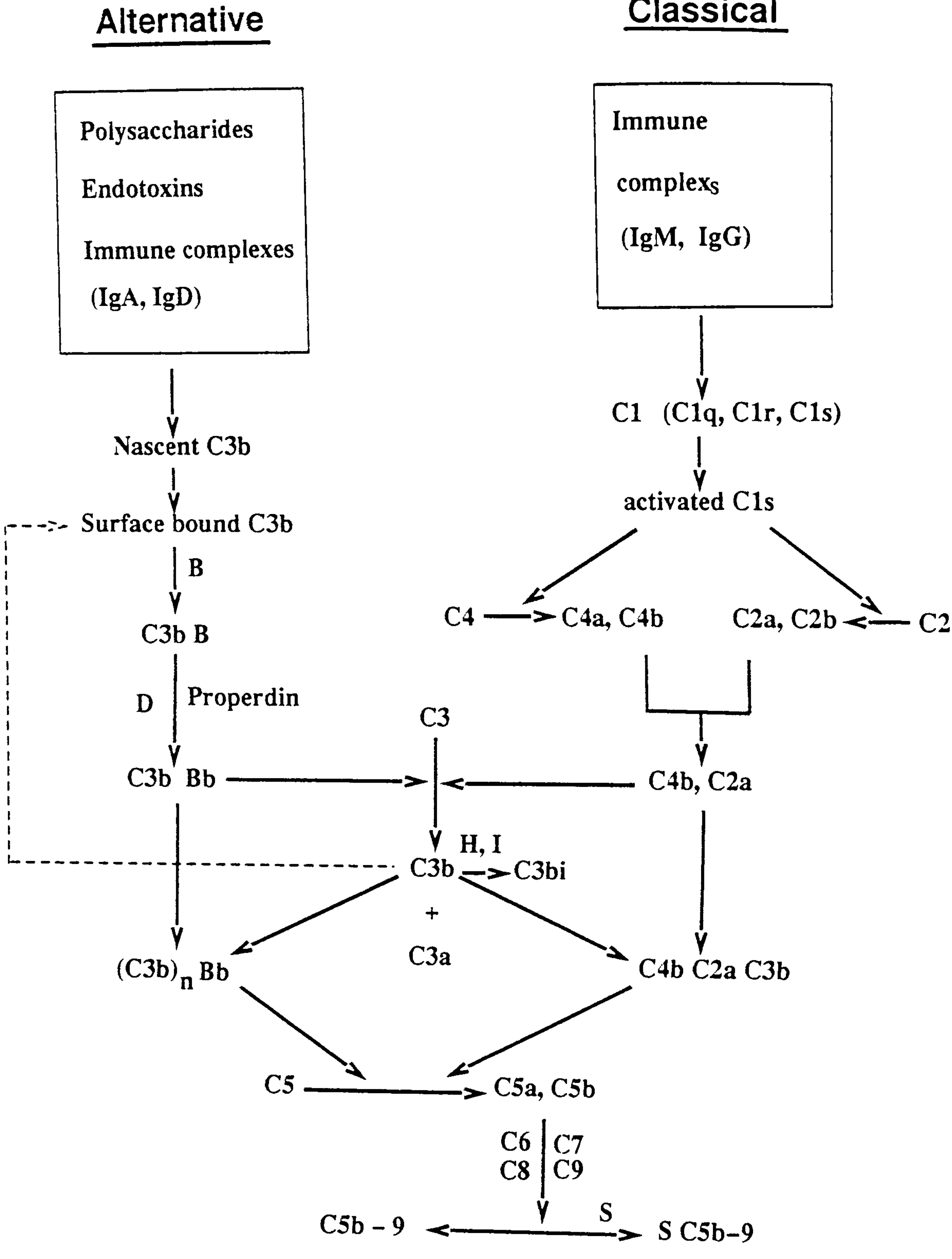


Figure 2.5 The Complement pathway

plasmin. Among these substances are thrombin, activated factor XII, lysosomal enzymes from damaged tissues, and substances from the endothelial lining of blood vessels. In another route, foreign proteins, e.g. urokinase and streptokinase, which have the ability to activate plasminogen, are added.

2.2.4 Complement System

The human complement system comprises nine major plasma proteins, numbered C1 to C9. The activation of complement involves a series of enzymatic reactions in a cascading manner, similar to the coagulation system. Complement activation may occur via two pathways, the classical and the alternative pathways, shown in Figure 2.5. In both pathways, the component C3 is pivotal.

2.2.4.1 Classical Pathway

The main proteins involved in the classical pathway are C1, C2 and C4. The activation of the classical pathway is initiated by antigen-antibody complexes, including IgG and IgM, which provide a binding site for component C1, a macromolecular complex of the three proteins C1q, C1r and C1s. Activated component C1s cleaves C4 to C4a, C4b and C2 to C2a, C2b. C4b and C2a subsequently form the C3 convertase. This C3 convertase changes the specificity of the C3 cleaving enzymes into C5 convertases, resulting in the cleavage of C5, and the generation of C5a and C5b. Subsequently, C5b interacts with C6, C7, C8 and C9 to form either C5b-9 complex or with protein S to form an inactive circulating SC5b-9 complex (Bhakdi and Tranum 1983).

2.2.4.2 Alternative Pathway

The activation of the alternative pathway is initiated by polysaccharides, immune complexes of IgA and IgD, as well as endotoxins. In this route, the main components are C3, factor B, factor D and properdin. The process of activation is initiated by the binding of few C3b molecules, which are derived from the low grade cleavage of C3 that continuously occurs in normal plasma (Haeffner-Cavaillon et al 1988). Onto surface-bound C3b, factor B binds to form a C3bB complex. The cleavage of B within the C3bB complex by factor D results in the formation of C3 convertase, C3bBb. This complex cleaves additional C3 molecules into C3a and C3b, followed by the binding of more C3b molecules and the formation of new convertase complexes on the surface. This convertase triggers the activation of the terminal complement complex C5b-9 in the same manner, as described in section 2.2.4.1.

The function of the alternative pathway C3 convertase is regulated by properdin, factor I and factor H. The C3 convertase, C3bBb, is unstable and spontaneously dissociates. Properdin has the effect of stabilizing the dissociation, while factor H activates the dissociation of the C3bBb complex. In addition, factor H is the cofactor for irreversible inactivation of C3b into C3bi by factor I (Kazatchkine and Carreno 1987; Haeffner-Cavaillon et al 1988).

2.3 BLOOD RESPONSE TO BIOMATERIALS

Consideration of the blood response to biomaterials can be assisted by adopting a similar approach to that used for examining blood coagulation in relation to haemostasis and thrombosis (Mustard and Packham 1977; Courtney et al 1992). As described in section 2.2, the processes of haemostasis and thrombosis are complicated but can be examined in terms of particular aspects such as platelet activation, intrinsic and extrinsic coagulation, fibrinolysis and complement activation. The events following the contact of blood with the biomaterial can be considered in a similar manner (Forbes and Countney 1987). However, distinctions in behaviour towards blood between natural endothelium and artificial surfaces must be accepted.

2.3.1 Natural Endothelium and Artificial Surfaces

The vascular endothelium is nature's blood-compatible container (Tortora and Anagnostakos 1987). The blood compatible properties of endothelium are

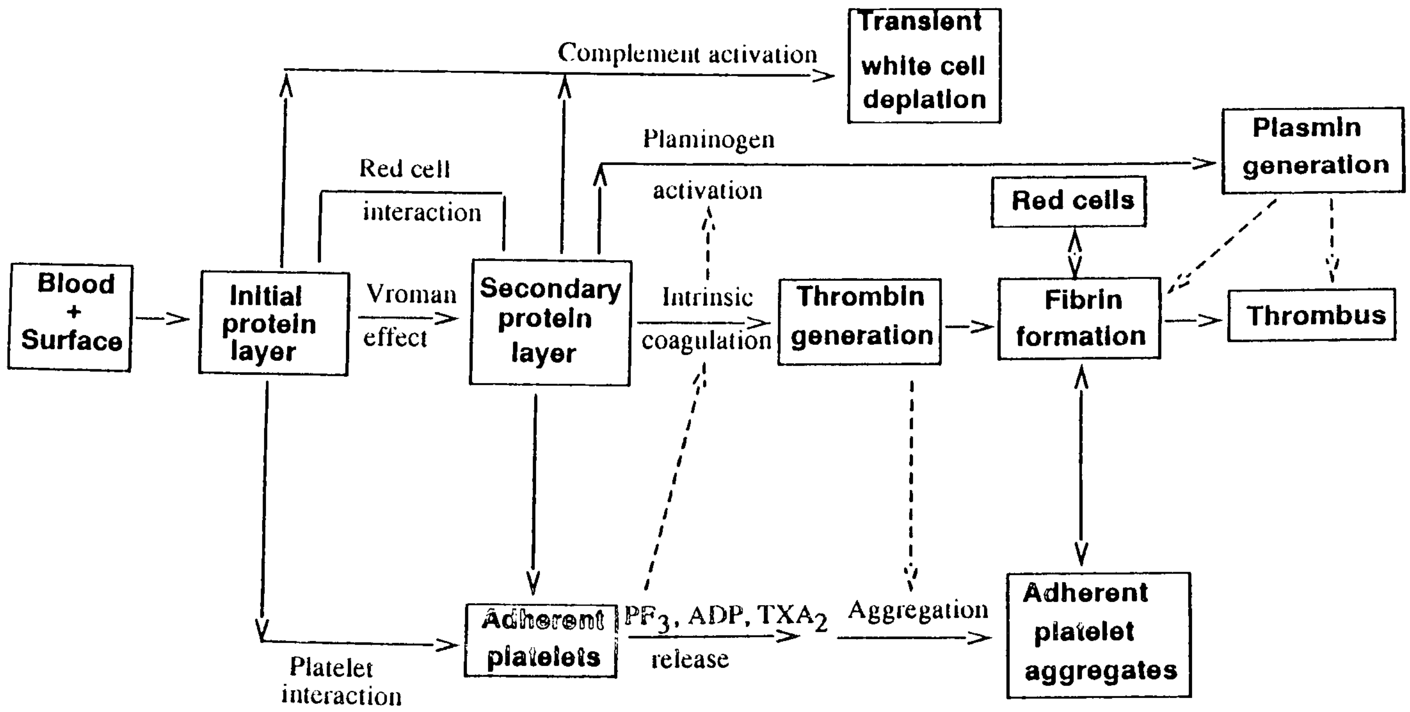


Figure 2.6 Sequence of events during blood – biomaterial interaction (modified from Brash 1987)

based on a so-called "natural anticoagulant mechanism" (Rosenberg and Rosenberg 1984). First of all, endothelium can synthesis prostacyclin, which is an inhibitor of platelet aggregation (Moncada et al 1977). It has been shown that endothelium contains heparin-like proteoglycans, which can take up thrombin to accelerate antithrombin-mediated inhibition of the enzyme (Busch and Owen 1982; Dryjski et al 1983). Also, endothelium contains thrombomodulin, which can activate protein C to mediate the inhibition of coagulation factor V and VIII (Owen and Esmon 1981). Furthermore, it has been reported that endothelium contains a plasminogen activator, which can result in the dissolution of the precipitated fibrin (Todd 1964).

In comparison with natural endothelium, artificial surfaces cannot perform the active role achieved by the endothelium. In addition, the endothelium does not appear to adsorb protein under physiological conditions (Bruck 1980), while protein adsorption is a very important feature of the interaction of blood with artificial surfaces (Szycher 1983).

Figure 2.6 shows a sequence of events during blood-biomaterial interactions. Thrombus formation as a result of blood-biomaterial interactions is generally inevitable, and the clinical use of artificial surfaces requires the utilization of anticoagulants, platelet aggregation inhibitors or plasminogen activators.

2.3.2 Protein Adsorption

Plasma protein adsorption is generally regarded as the first event to occur following the contact of blood with artificial surfaces and the composition of the adsorbed protein layer strongly influences subsequent interactions (Brash 1991). The adsorbed protein has been termed a "conditioning layer". However, this adsorbed protein layer should not consider as "passive" (Bruck 1980), since possibilities exist for transient adsorption, denaturation or changes in conformation (Lee and Hairston 1971; Brash 1983; Klinkmann 1984).

The rapid adsorption of protein onto the biomaterial surface is promoted by the characteristics of proteins, which are the limited solubility of proteins in plasma due to the large molecular weight and chemical heterogeneity (polar/nonpolar and negative/positive). Since the surfaces of the biomaterial are also heterogeneous, binding of proteins onto surfaces is possible (Brash et al 1983, 1991).

The manner and extent of protein adsorption are influenced by the surface properties. It is generally concluded that hydrophobic surfaces induce greater protein adsorption than hydrophilic surfaces, e.g. a hydrogel has minimal protein interaction (Gregonis et al 1984; Ratner and Horbett 1984) and this has been utilised to enhance blood compatibility by increasing the hydrophilicity of biomaterial surfaces (Chapter 3).

Many different plasma proteins have been found to be adsorbed onto artificial surfaces, with albumin, gamma globulin and fibrinogen the most widely studied. The general conclusion is that fibrinogen is more surface active than albumin and gamma globulin and can be considered as a major component of the adsorbed protein layer (Lee et al 1974; Horbett 1981; Uniyal and Brash 1982).

The investigation of protein adsorption has focused on the relationship with platelet reactivity. It can be summarized in that platelet adhesion onto a biomaterial surface is promoted by the prior adsorption of fibrinogen or gamma globulin and inhibited by the prior adsorption of albumin (Evans and Mustard 1968; Packham et al 1969; Whicher and Brash 1978; Young et al 1983). The ability of albumin to inhibit platelet adhesion has been utilized in the preparation of artificial surfaces with improved blood compatibility (Chapter 3).

A close relationship between fibrinogen and platelets has been reported in that the deposition of fibrinogen on artificial surfaces is associated with platelet receptors on adherent platelets and may not be independent of platelet deposition (Young et al 1983).

A further important aspect of fibrinogen adsorption is the replacement of adsorbed fibrinogen by other proteins, notably high molecular weight kininogen (HMWK) (Vroman et al 1980), a protein involved in the contact phase of blood coagulation. This phenomenon of transient fibrinogen adsorption has

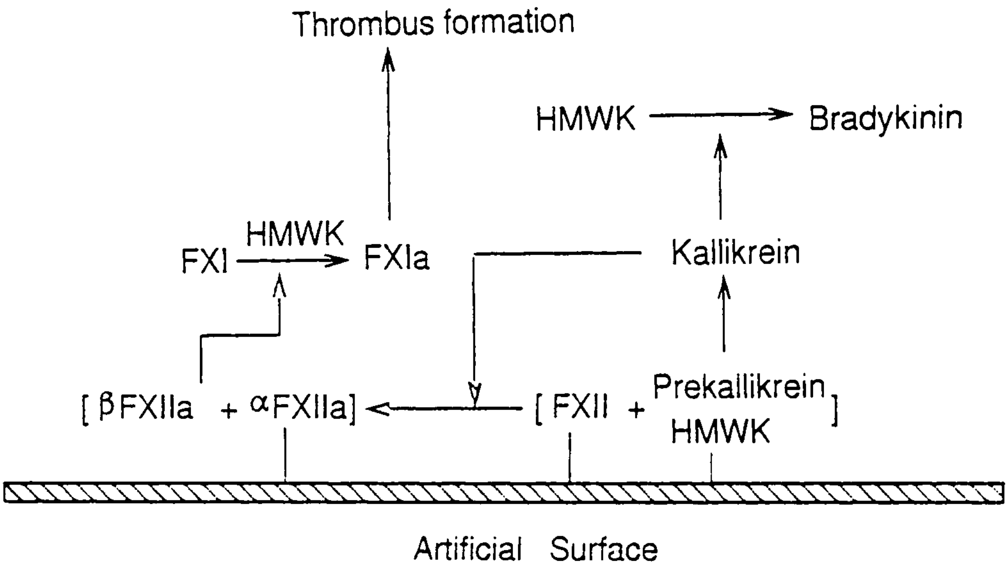


Figure 2.7 Contact phase activation trigged by blood–biomaterial contact

been named "Vroman Effect" (Brash et al 1984; Horbett 1984). The significance of the Vroman Effect in relation to surface thrombogenesis is not clear. A high Vroman Effect with fibrinogen displacement by HMWK might induce less platelet reactivity and more activation of the intrinsic coagulation, while a low Vroman Effect with fibrinogen retention might induce less activation of the intrinsic coagulation and more platelet reactivity (Brash 1991).

Protein adsorption is also relevant to the contact phase activation of the intrinsic coagulation pathway. The contact phase activation, which is shown in Figure 2.7, involves the interaction of contact proteins factor XII, HMWK and prekallikrein (PKK). A sequence of enzymatic reactions is initiated by the adsorption of factor XII and HMWK. Surface-bound HMWK forms a complex with prekallikrein. This complex activates FXII to form FXIIa, which is the activator of conversion of prekallikrein into kallikrein. Since kallikrein activates factor XII, an autoactivation process leads to the rapid production of factor XII on or near the artificial surface. Surface-bound HMWK also forms a complex with factor XI, making factor XI available to FXIIa and ensuring the progression of the coagulation cascade.

It is generally believed that contact phase activation is increased by negatively charged surfaces. However, some negatively charged surfaces have been reported to have good blood compatibility (Fougnot et al 1979a, 1979b; Boffa et al 1984). Therefore, it has been suggested that even if the contact phase is strongly activated, the later phases of coagulation can be inhibited to give an overall anticoagulant effect (Brash 1991). However, it should not be assured that contact phase activation by negatively charged surface is unimportant.

In summary, the adsorption of protein plays a key role in the interactions of blood with foreign surfaces. In order to obtain improved information on the composition of the adsorbed protein layer, studies on protein adsorption require to extend beyond albumin, fibrinogen and gamma globulin (Brash et al 1986) and consideration of the possible influence of trace proteins (Brash et al 1983). It is believed that better understanding of protein adsorption is important for guiding the development of blood-compatible materials.

2.3.3 Platelet Reactions

Platelet adhesion and aggregation are inevitable consequences of blood interactions with an artificial surface (Forbes and Courtney 1987). As observed in section 2.3.2, the adhesion of platelets to artificial surfaces is influenced by protein adsorption, in that platelet adhesion is promoted by prior adsorption of fibrinogen or gamma globin and decreased by prior adsorption of albumin.

The adhesion of platelets onto an artificial surface changes its shape from discoid to spiny spheres. These coalesce to form an irregular monolayer and, as more platelets adhere, they form mounds with erythrocytes and leucocytes trapped in fibrin (Salzman et al 1977).

Following platelet adhesion onto an artificial surface, platelets undergo a release reaction. These released substances relevant to blood-biomaterial interactions include ADP, serotonin (Brash and Whicher 1977), platelet factor 4 (Dudczak et al 1979), ß-thromboglobulin (BTG) (Adler and Berlyne 1981; Needleman and Hook 1982; Bowry et al 1984) and thromboxane (Davies et al 1979; 1980).

Considering the relationship between platelets and intrinsic pathway, an interaction exists (Feijen 1977). Initiation of the intrinsic pathway can be resulted from the activation of factor XII due to platelets stimulated by released ADP. Platelets have receptors for FXI and FXIa (Lipscomb and Walsh 1979). Also, platelet membranes present a surface for FXII activation in the presence of PKK and HMWK (Walsh and Griffin 1981). The formation of thrombin during intrinsic pathway enhances platelet adhesion, aggregation and the platelet release reaction (Shuman and Levine 1980; Patrono et al 1980; Phillips et al 1980).

Platelet activation is also produced by turbulence of the blood flow and is a function of the flow rate (Forbes and Countney 1987).

2.3.4 Erythrocytes

The interactions of blood with artificial surfaces can result in erythrocyte adhesion onto the adsorbed protein layer (Feijen 1977) and under certain conditions, haemolysis occurs, with the platelet release reaction induced by liberated ADP and erythrocyte ghosts (Stormorken 1971). The nature of the adsorbed protein layer and erythrocytes may influence each other due to the erythrocyte adhesion or the competitive adsorption of released haemoglobin with plasma proteins (Uniyal et al 1982). The addition of erythrocytes to protein solutions leads to a reduction in the amount of protein adsorbed (Brash and Uniyal 1979). The adsorption of haemoglobin on biomaterial surfaces has been detected (Owen et al 1985; Coleman et al 1986).

Blood-surface contact leads to significant changes in the metabolism of erythrocyte membranes. There is a possibility of shear-induced haemolysis and in coagulation under low shear forces, entrapped erythrocytes and fibrin form the red thrombus (Bruck 1980).

2.3.5 Leucocytes

Human leucocytes can be grouped into two types: granular leucocytes and agranular leucocytes. Granular leucocytes include neutrophils or polymorphs, eosinophilis and basophils. Agranular leucocytes include lymphocytes and monocytes (Tortora and Anagnostakos 1987).

The interaction of leucocytes with artificial surfaces has been long recognised (Kaplow and Goffinet 1968). Investigation has shown that leucocytes adhere onto biomaterial surfaces, with preferential adsorption of granulocytes in comparison to lymphocytes (Wright et al 1978; Lederman et al 1978; Absolom et al 1979). Leucocyte adhesion is influenced by the adsorbed protein layer, with enhanced adhesion reported for surfaces coated with IgG, thrombin and prothrombin (Altieri and Edgington 1989). The possession of endogenous procoagulant activity and proaggregatory activity by granulocytes (Harrison et al 1966; Niemetz 1972; Saba et al 1973) can promote thrombus formation on artificial surfaces by granulocyte adhesion and by its effect on platelet aggregation (Cumming 1980).

The contact of blood with an artificial surface can change the function of leucocytes. It has been reported that leucocyte damage caused by blood-biomaterial contact (Kusserow et al 1971) produces an impairment in phagocytic activity and a reduced ability to combat infection (Bruck 1980). Furthermore, the activation of leucocytes results in monocytes producing interleukin 1 (Betz et al 1988; Shaldon et al 1985; Lonnemann et al 1988), neutrophils changing in their oxidative metabolism (Lewis and Van Epps 1987) and phagocytes not only decreasing in numbers, but also increasing in metabolic activity (Nguyen et al 1985; Vanholder et al 1989).

Recently, the investigation of the response of leucocytes to artificial surfaces has been linked with the activation of complement system. It is believed that complement activation mediates leucocytes adhesion to artificial surfaces (Herzlinger and Cumming 1980).

With respect to sensitivity to mechanical trauma, similar to platelets, leucocyte damage and aggregation are influenced by shear stress (Dewitz et al 1977).

2.3.6 Complement Activation

Complement activation induced by blood contact with artificial surface was reported by Craddock et al (1977a), who demonstrated that complement activation can be detected during haemodialysis performed with cellulosic membranes. This initial observation has encouraged the study of complement activation induced by different types of biomaterials. It is generally accepted that complement activation initiated by biomaterials occurs via the alternative pathway mechanism and depends highly on the chemical composition of artificial surfaces. Recently, it has been reported that some biomaterials can activate complement system via the classical pathway mechanism (Matsuda 1989).

Clinical investigation has demonstrated that cellulose membranes significantly activate the complement system in comparison with synthetic biomaterials, since complement activation is reduced when cellulose membranes are replaced by the membranes derived from synthetic biomaterials (Ivanovich et al 1983; Hakim et al 1984; Wegmuller et al 1986; Amoto et al 1988; Moll et al 1990). Complement activation by cellulose membranes has been attributed to the presence of hydroxyl groups, which can activate complement system through a transesterification mechanism (Kazatchkine and Carreno 1987). Investigations have demonstrated that modification of cellulose by the partial substitution of hydroxyl groups reduces complement activation (Akizawa et al 1986; Bosch et al 1987; Lucchi et al 1989).

Interest in investigating complement activation has focused on the relationship between complement activation and the leucopenia occurring in haemodialysis. It is believed leucopenia occurring in haemodialysis with cellulose membranes relates to complement activation (Chenoweth 1984).

The processes of complement activation generate the anaphylatoxins C3a and C5a, which lead to granulocyte alteration in chemotaxis, adherence, aggregation, degranulation and production of toxic radical oxygen (Chenoweth 1986). In addition, anaphylatoxins C3a and C5a stimulate the histamine release from mast cells, induce smooth muscle contraction and increase vascular permeability (Haeffner-Cavaillon et al 1988). In plasma, C3a and C5a are rapidly transformed to stable desArg derivatives by removing the carboxyl-terminal arginine. All of them can induce interleukin 1 release from monocytes (Craddock et al 1977b; Haeffner-Cavaillon et al 1987, 1988). Therefore, the activation of the complement system plays an important role in the blood response.

Considering the relationship between complement activation and blood coagulation, it has been reported that complement mediates the activation of blood platelets, since decomplemented blood significantly reduced platelet aggregation on nylon surfaces (Herzlinger and Cumming 1980) and also greatly reduces thromboxane generation and platelet loss during extracorporeal membrane oxygenation (Wonders et al 1983).

Table 2.2 Parameters used to monitor the blood responses

Parameter	**Example**
Protein adsorption	Albumin, fibrinogen
Platelet response	Adhesion, aggregation, release, morphology
Intrinsic coagulation	FXII activation
Fibrinolysis	Fibrinogen degradation products (FDP)
Complement activation	C3a, C5a
Leucocytes	Leucocyte count

Modified from Irvine (1989)

2.4 BLOOD COMPATIBILITY ASSESSMENT

Blood compatibility assessment is the investigation of the interactions of blood with biomaterials, the features of which include:

Selection of the parameters to be studied

Method for achieving blood - material contact

Nature of the blood used

2.4.1 Selection of Parameters

Parameters used to monitor the blood response are based on relevant features, such as protein adsorption, platelet reactions, intrinsic coagulation, fibrinolysis, complement activation and leucocyte alterations. Table 2.2 lists typical parameters that have been used.

Multiparameter assessment facilitates the achievement of a broad perspective and reduces potential errors in biomaterial development, e.g. detection of materials exerting little influence on platelets but inducing complement activation (Payne and Horbett 1987). However, the benefits of multiparameter assessment have to be offset by practical difficulties.

It is generally considered that plasma protein adsorption is the first event to occur following blood-surface contact and that the subsequent phenomena are to a large extent determined by interactions of blood with the adsorbed protein layer. Therefore, as a first stage in vitro, a protein adsorption test is important and useful. It is believed that the investigation of protein adsorption is fundamental to an improved understanding the blood-biomaterials interaction (Brash 1991).

The first parameter selected in this project was protein adsorption, as represented by fibrinogen and albumin single protein adsorption determined by the measurement of radioactivity of radio-labelled protein. The method is presented in Chapter 4.

Following the initial protein deposition, events occurring include platelet adhesion and activation, coagulation and complement system activation. To study complement system activation, the well established parameter C3a generation was selected and determined using a radioimmunoassay (Chapter 4).

To investigate contact phase activation, a novel parameter FXII-like activity was selected. FXII-like activity was determined using a modified chromogenic substrate assay (Chapter 4).

The parameters chosen for this study provide information on a range of considerable importance in the blood response to a biomaterial.

2.4.2 Achievement of Blood-Material Contact

The options of blood-biomaterial contact can be classified as in vitro, ex vivo, in vivo (Courtney et al 1993b).

In vitro evaluation generally involves the donation of blood from healthy individuals with blood-material contact made separate from the donor. The advantages of in vitro test procedures are that they are versatile, rapid and relatively low in cost. Normally, in vitro tests are the first stage in the monitoring of the blood response to a biomaterial. However, in vitro test procedures cannot take into account the interrelationships between the patient and the clinical use of the material (Lindsay et al 1980; Klinkmann et al 1987; Courtney et al 1993b) and do not normally consider the influence of the disease state (Andrade et al 1981).

In ex vivo evaluation, blood (human or animal) passes to the test material extracorporeally and may or may not be returned. If these tests are carried out in animals, the differences in blood components between human and animal need to be taken into account (Grabowski et al 1977).

In vivo evaluation involves implantation of the test material or the evaluation during clinical procedures. Such evaluation approximates more closely to the clinical situation but can be expensive.

Although the final evaluation of artificial surface compatibility with blood has to be performed in vivo, a rapid selection of potentially suitable materials with the aid of a less expensive, simpler and faster combination of in vitro tests still remains necessary.

In this study, the evaluation of blood compatibility was carried out in vitro using an improved incubation test cell. The methodology is detailed in chapter 4.

2.4.3 Nature of Blood Used

The nature of blood used for the compatibility study has an effect on the results. Generally, the blood used for the compatibility assessment can be characterised by three features:

Human or animal blood

'Normal' blood or 'disease state' blood

Absence or presence of antithrombotic agents

It has been shown that the presence of antithrombotic agents can alter the behaviour of blood-biomaterial interactions (Robertson et al 1990). In order to avoid the impact of the coagulation process by the utilisation of antithrombotic agents, whole fresh healthy human blood was used in this study.

CHAPTER 3

POLYMER MODIFICATION TO IMPROVE BLOOD COMPATIBILITY

3.1 INTRODUCTION

As indicated in Chapter 1, the principal advantage of polymer surface modification is the ability to improve blood compatibility with minimal alteration to the other fundamental properties of a polymeric biomaterial. Approaches to polymer modification (Gilchrist and Courtney 1980; Fougnot et al 1984; Forbes and Courtney 1987) can be divided into the following categories:

1. Alteration to surface physical properties
2. Treatment of surfaces with protein
3. Increase of surface hydrophilicity
4. Surface chemical modification
5. Attachment of antithrombotic agents onto surfaces
6. Preparation of biomembrane-mimetic surfaces

It should be noted that the properties of a biomaterial surface are not independent. An approach altering one surface property can also change other properties at same time.

The physical nature of a surface may strongly influence on blood compatibility. An adverse blood response can result from excessive surface roughness which causes mechanical damages, or produces deleterious effects on blood flow due to material shape and presentation. Therefore, an initial simple step in enhancing blood compatibility may be to ensure a smooth surface. However, blood compatibility may be improved by the formation of a neointima induced by a porous surface (Szycher et al 1980; Snyder and Botzko 1982; Hood et al 1984).

Treating polymer surfaces with protein to improve blood compatibility has been achieved by utilising the fact that the adsorption of albumin leads to reduce platelet adhesion. It has been reported that precoating albumin onto polymer surfaces leads to substantial reduction of platelet deposition (Packham et al 1969; Andrade and Hlady 1986). The ability of some polymers to adsorb albumin (Lyman et al 1975) has been enhanced by the preparation of alkyl derivatised polyurethane (Munro et al 1981; 1983) and cellulose acetate

(Frautschi et al 1983). In the processes of treating polymer surfaces with protein, the term "biolisation" has been introduced (Nosé et al 1971), which applies to the chemical and thermal treatment of tissue components, such as proteins, either coated onto a polymer or blend with a polymer (Kambic et al 1983). Therefore, biolised materials include polymers coated with protein, polymers blended with protein, and polymer-protein blends laminated to a base polymer (Courtney et al 1993a).

The approaches of increasing in the hydrophilicity of polymer surfaces to enhance blood compatibility are based on the fact of that protein adsorption and cellular adhesion decreases with increasing hydrophilicity of surfaces.

Surface chemical modification involves the substitution or replacement of one type of functional group by another. The influence on the blood response of the modified surface can be expected to be determined by the nature of the substitution group and the degree of substitution.

Polymeric biomaterials can be modified by the attachment of anticoagulants, such as heparin, platelet aggregation inhibitors, such as dipyridamole (Marconi et al 1979) and prostaglandin (Grode et al 1974; Ebert et al 1982), or plasminogen activators, such as urokinase (Kusserow et al 1973, Sugitachi et al 1980; Ohshiro and Kosaki 1980; Ohshiro 1983).

The concept of biomembrane-mimetic polymers is the preparation of a polymer surface designed to mimic biologically inert interfaces of blood cells and thereby avoid the recognition by the blood as "foreign".

In this thesis, four approaches to polymer modification were studied. These approaches were:

Increase of surface hydrophilicity

Surface chemical modification

Attachment of heparin

Preparation of biomembrane-mimetic surface

The background to each of these approaches is now considered in more detail.

3.2 INCREASE OF SURFACE HYDROPHILICITY

Increasing the hydrophilicity of polymer surfaces is generally achieved by coating with hydrophilic polymers, such as hydrogels which have low interfacial tension in biological environments, onto the substrate biomaterials. Various hydrogels have been investigated and shown to diminish the amount of blood cell adhesion (Ratner et al 1975a, 1975b; Donetski et al 1975; Jansen and Ellighorst 1979; Jansen et al 1985). In this respect, the coating of poly(ethylene oxide) (PEO) on a biomaterial surface is a widely used method (Whicher and Brash 1978; Brash and Uniyal 1979; Golander and Kiss 1988; Lee et al 1989; Desai and Hubbell 1990, 1991; Sa da Costa et al 1980, 1981; Nagaoka 1984; Brinkman et al 1989, 1990) and will be reviewed in this thesis.

Investigation has demonstrated that the application of PEO significantly reduces protein adsorption and platelet adhesion. The achievement of improved blood compatibility with PEO has been explained by its special properties (Nagaoka et al 1984; Lee et al 1989). Firstly, PEO has very low interfacial free energy with water. Therefore, PEO has low driving forces for the deposition of blood components. Also, PEO has unique solution properties (Bailey and Koleske 1976; Merrill and Salzman 1983) and hydrophilicity, which combined with the polymer chain exhibiting considerable flexibility, are believed to prevent protein adsorption or cell adhesion (Nagaoka et al 1984). Additionally, PEO's passivity can be explained by the steric stabilization effect (Lee et al 1989), which includes volume restriction and osmotic repulsion effects. The volume restriction effect is based on the repulsive force resulting from a loss in configurational entropy of long chain PEO when blood elements approach the surface. The osmotic repulsion effect is based on the repulsive force resulting from the rise in the local osmotic pressure occurring when PEO chains interpenetrate each other when blood elements approach the surface.

The techniques utilising PEO generally can be divided as follows:

(a) Direct adsorption of PEO-containing surfactants

(b) Covalent grafting of PEO onto the substrate polymer

(c) Copolymerization of PEO

3.2.1 Direct Adsorption of PEO-containing Surfactants

The technique of direct adsorption of PEO-containing surfactants involves the immersion of hydrophobic polymer in the PEO-containing surfactant solution (Lee et al 1989, 1990). The achievement of a hydrophilic surface is via hydrophobic interactions between the polymer surface and the hydrophobic segments of surfactants. It is reported that protein adsorption is significantly decreased on the surfactant-treated surface, as compared with the untreated surface.

3.2.2 Covalent Grafting of PEO

Techniques of covalent grafting of PEO onto a substrate polymer cover different approaches, which include photo-induced grafting procedures (Mori et al 1982; Nagaoka et al 1984), crosslinking agent-induced grafting procedures (Brinkman et al 1989, 1990) and grafting procedures induced by direct reaction between PEO and the substrate polymer after pretreatment (Golander and Kiss 1988; Nojiri 1990; Desai and Hubbell 1991).

In the photo-induced grafting procedure, PEO has been coated onto a poly(vinyl chloride) (PVC) surface (Mori et al 1982). The procedure involves the introduction of photo active dithiocarbamate groups on the PVC surface, then photo-reaction with monomer containing PEO. Investigation of blood compatibility in vitro, in vivo and ex vivo demonstrates that a PEO-treated PVC surface can reduce both platelet adhesion and protein adsorption (Nagaoka et al 1984).

In crosslinking agent-induced grafting procedures, PEO has been coated onto a polyurethane (PU) surface using dicumyl peroxide crosslinking agent (Brinkman et al 1989, 1990). The increased hydrophilicity of the modified surface has been demonstrated by contact angle measurements. Recalcification times of plasma incubated in PEO-treated PU-coated glass tubes are longer than those obtained with untreated PU-coated tubes. Also, platelet adhesion onto the modified surfaces is lower than onto the unmodified PU surfaces.

A PVC surface has been modified by grafting PEO after pretreatment

(Golander and Kiss 1988). During the pretreatment, PVC is carboxylated and exposed to an aqueous solution of poly(ethylene imine) (PEI). In the presence of a reducing agent, PEO-derivative with one terminal aldehyde group is covalently coupled to the amine groups of PEI-pretreated PVC surface. ESCA demonstrated that the treated surface is completely covered with PEO. All protein adsorption tests have shown exceptionally low results.

With a similar procedure, PEO has been grafted onto poly(ethylene terephthlate) (PET) (Desai and Hubbell 1990, 1991; Gombotz et al 1989) and Biomer (Nojiri 1990) surfaces. As an example of PET surface modification, PEO-modified PET surface is achieved by the reaction between PEO activated with cyanuric chloride and the amine group on PET film after pretreatment with ethylene diamine. The investigation shows that the improvement of blood compatibility using PEO depends on the molecular weight of PEO. The PEO of molecular weight about 18,500 and higher is reported more effective in reducing protein adsorption, attachment and growth of fibroblasts (Desai and Hubbell 1991).

3.2.3 Copolymerization of PEO

Diisocyanate, methylmethacrylate (MMA) (Nagaoka 1984), polyethylene terephthalate (PET) (Lyman 1964), and polystyrene (Furasawa et al 1977; Grainger et al 1989) have been utilised to copolymerize with PEO. It has been shown that copolymers achieve improved blood compatibility with increasing PEO content. Among these copolymers, segmented polyurethane (SPU) and polyurethane urea (SPUU) have attracted the most attention of the numerous approaches based on copolymerization between diisocyanate and PEO with diol or diamine as a chain extender (Lyman and Loo 1967; Whicher and Brash 1978; Brash and Uniyal 1979; Sa da Costa et al 1980, 1981; Merrill et al 1982; Takahara et al 1985, 1991; Goodman et al 1989; Yu et al 1991). Protein adsorption studies demonstrate that SPU based on PEO is significantly less adsorptive (Whicher and Brash 1978; Brash and Uniyal 1979). The investigation of platelet retention has been correlated with the surface

Table 3.1 Approaches of chemical modification cellulose membrane

Approach	Reference
Cellulose acetate	Ivanovich et al 1983; Chenoweth 1984; Lucchi et al 1989; Johnson 1989; Moll et al 1990
Hemophan	Cheung et al 1986; Falkenhagen et al 1987; Lucchi et al 1989
Cellulose anhydride	Johnson 1989; Johnson et al 1990
Cellulose PEO	Akizawa et al 1989; Kishida et al 1992
Cellulose sulphate	Brown 1990

composition of SPU, which are determined by x-ray photoelectron spectroscopy (Sa da Costa et al 1980, 1981). It is found that SPU containing the highest fraction of ethereal carbon in the surface has the lowest platelet retention.

All previous investigations have shown that PEO has little reaction with biological macromolecules and cells, especially with blood proteins and platelet. Therefore, it is believed that the application of PEO will remain significantly interesting to the biomaterial scientists.

In this project, a novel polyurethaneurea hydrogel based on PEO was selected to study the influence on protein adsorption and blood response by hydrophilic polymers.

3.3 SURFACE CHEMICAL MODIFICATION

The possibility of altering the blood response to a polymeric biomaterial by chemical modification has been well established (Courtney et al 1976). The recent emphasis in biomaterials research has been on the chemical modification of regenerated cellulose membranes.

Regenerated cellulose membranes, such as Cuprophan, are the most widely used in haemodialysis for treating renal failure patients. The preparation of these membranes involves the dissolution of purified cellulose (cotton fibres) in an ammonia solution of cupric oxide and regeneration with an alkaline solution (Hakim et al 1984; Courtney et al 1984).

In clinical application, it is found that dialyzers containing Cuprophan induce complement activation in a greater degree than these with other membranes (Hakim et al 1984; Chenoweth 1986) and this has become a central event in dialyser-related blood incompatibility. It is generally considered that the presence of hydroxyl groups on cellulose membranes initiates complement activation (Chenoweth 1984, 1986). To achieve better blood compatibility, chemical modification of cellulose membrane by substitution of hydroxyl groups with other chemical groups has been investigated. Approaches to chemical modification of cellulose membranes are listed in Table 3.1.

In cellulose acetate membranes, hydroxyl groups are partially replaced by

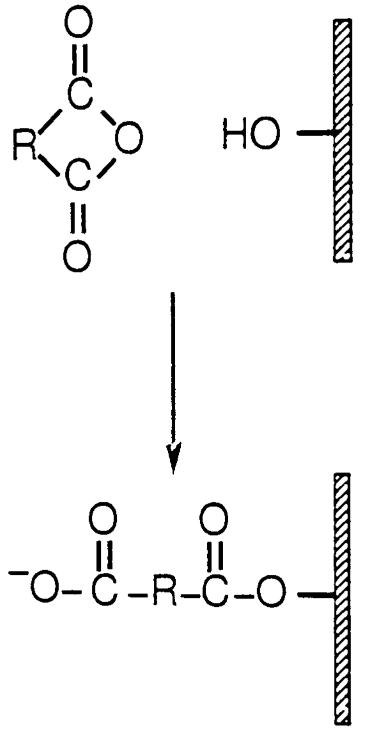


Figure 3.1 Cellulose anhydride modification

acetate groups. Clinical investigation has indicated that cellulose acetate hollow fibre dialyzers possess only about half the complement activating potential of Cuprophan dialyzers (Chenoweth 1984). This observation was interpreted by the fact that cellulose acetate surfaces present fewer sites for attachment of C3b than Cuprophan. Since complement activation is initiated by the covalent attachment of C3b to the surface containing hydroxyl groups, reduced production of C3a and C5a should be achieved by cellulose acetate dialyzers.

Hemophan membranes are cellulose membranes with 5-10% of hydroxyl groups substituted by diethylaminoethyl groups. Investigation of complement activation induced by Hemophan has been compared with Cuprophan and cellulose acetate (Cheung et al 1986; Falkenhagen et al 1987; Lucchi et al 1989). It was found that both Hemophan and cellulose acetate reduced complement activation, with a much greater reduction achieved by Hemophan. These observations suggest that the effectiveness of chemical modification of cellulose membranes depends on both the degree of substitution and the nature of the modifying group. Further investigation of the mechanism of complement activation induced by Hemophan indicated that Hemophan bound both factor B and factor H to a greater degree than Cuprophan, with bound factor H to significantly greater extent than factor B. Since factor B activates the generation of C3a and factor H inhibit this generation, as indicated in Chapter 2, it is suggested that Hemophan displays improved biocompatibility by augmenting factor H mediated inhibition of C3 convertase activity (Johnson 1989).

The principal route of cellulose anhydride modification is shown in Figure 3.1. Investigation has demonstrated that cellulose anhydride significantly reduced complement activation (Johnson 1989; Johnson et al 1990) and platelet activation (lower levels of thromboxane A_2). The ability of diminishing the complement-activating potential by cellulose anhydride was explained as follows: Firstly, the modification limits the binding sites of C3b, thereby reducing C3 and C5 convertase activity. Secondly, the modified surface

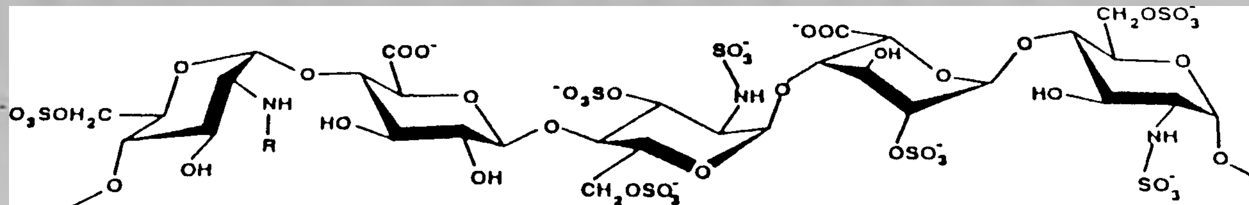


Figure 3.2 Structure of Heparin

facilitates factor H control of C3 and C5 convertase activity. Finally, since the modification results in a negatively charged surface, the modified surface could adsorb the cationic C5a molecule to inhibit its interaction with the cellular elements of the blood.

Cellulose PEO denotes the cellulose membranes coated with poly(ethylene oxide). Cellulose sulphate denotes cellulose membranes with hydroxyl groups replaced by sulphate groups. All these approaches demonstrated that modified cellulose membranes can achieve lower complement activation than Cuprophan.

In summary, it has been demonstrated that chemical modification of cellulose membranes by displacing hydroxyl groups by other chemical groups can reduce complement activation. However, since the complement system reaction is only one aspect of blood-biomaterial interactions, to make modified cellulose membranes clinically applicable, further investigations covering blood coagulation pathway, blood cell activation and permeability are required.

In this project, Hemophan was selected to study the chemical modification of cellulose membrane. The influence on protein adsorption, contact phase activation and complement activation was investigated (chapter 6).

3.4 ATTACHMENT OF HEPARIN

Heparin (Figure 3.2) is a negatively charged polysaccharide, mainly composed of alternating residues of sulphated glucoronic, iduronic acid and glucosamine derivatives linked in the 1-4 position.

Heparin is an anticoagulant widely used in clinical practice. The main anticoagulant effect of heparin is that of acting as a catalyst of the inactivation of thrombin, or some other coagulation proteases, by the major inhibitor antithrombin III (AT-III), which can bind onto the specific sites on the pentasaccharide sequence of heparin (Lindahl et al 1979; Thunberg et al 1982; van Boeckel et al 1985). In the case of thrombin inhibition, thrombin also binds onto heparin, reacts with the bound AT-III, and the inactive complex is then released (Migonney et al 1988). The biological activity of heparin depends on the binding of both thrombin and AT-III and the dominant role of

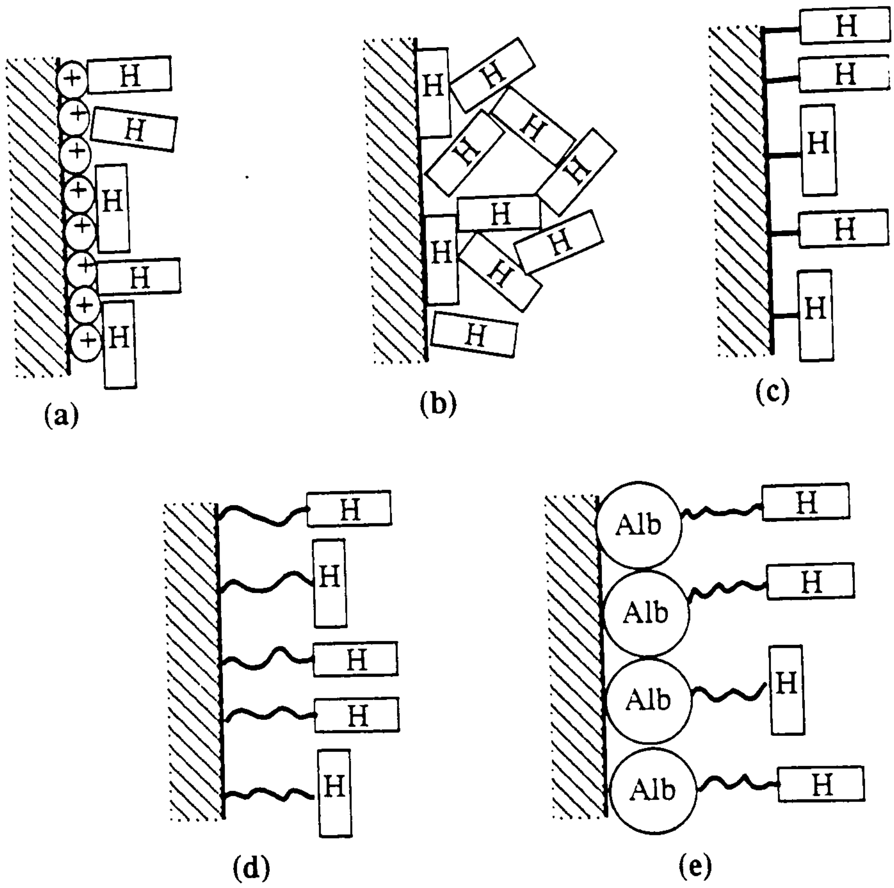


Figure 3.3 Methods of Attachment Heparin

(a) heparin bound ionically on positively charged surface

(b) heparin cross–linked surface

(c) heparin–attached surface

(d) heparin attachment via pspacer arms

(e) heparin–albumin conjugate–coated surface

(modified from Hoffman 1987)

AT-III-binding sites on heparin (Larm et al 1986).

To improve the blood compatibility of biomaterials, different methods for the attachment of heparin onto a biomaterial surface have been developed. These methods for heparinization of sufaces are illustrated in Figure 3.3. Generally, the approaches for heparinization of a biomaterial surface can be divided into ionic and covalent binding (Gilchrist and Courtney 1980; Kim et al 1983; Fougnot et al 1984; Larm et al 1989; Engbers and Feijen 1991).

3.4.1 Ionic Binding of Heparin

Ionic binding of heparin is based on the strong anionic character of heparin and can be achieved on cationic surfaces or polymer surfaces rendered cationic by special treatment or copolymerization. The first preparation of heparinized surface by ionic attachment (Gott et al 1963) comprised the sequential treatment of a polymer with colloidal graphite, the cationic surfactant benzalkonium chloride and heparin. This procedure is generally unsuitable for flexible materials, since the graphite layer is brittle. Therefore, to eliminate the use of graphite, an alternative method of providing groups for heparin attachment is required.

It has been found that the quaternary ammomonium salt, tridodecyl-methylammonium chloride (TDMAC) is an effective substance for heparin attachment and has been widely used in the preparation of heparinised surface (Grode et al 1969). The procedure involves contacting the polymer with TDMAC solution, followed by contacting with heparin. With this method, silicone rubber, polyurethane, polyethylene, polypropylene and poly(vinyl chloride) have been treated. TDMAC, with three long alkyl chains, is water insoluble and believed to be more stable on the surface (Falb et al 1977).

Preparation of heparinized surfaces by ionic attachment has also been achieved by incorporation of a polymer-heparin complex. Several complexes have been made, including these of heparin with benzalkonium chloride (Fourt et al 1966; Usdin and Fourt 1969), hexadecylpyridinium bromide (Hersh et al 1971), TDMAC (Leininger et al 1972; Grode et al 1972), cetylpyridinium chloride (Schmer et al 1976). PVC bypass tubes were coated with TDMAC/heparin complex and showed better thromboresistance than the uncoated surface (Brenner et al 1974).

The ionic attachment of heparin can be accomplished with polymers containing tertiary amine or quaternary ammonium groups. This approach has been utilised for elastomers (Falb et al 1966; Yen and Rembaum 1971), cellulose acetate (Martin et al 1970), methyl methacrylate (Courtney et al 1978) and cellulose (Holland et al 1978; Schmitt E et al 1983).

In the preparation of a more stable ionic binding of heparin, a glutaradehyde crosslinking agent has been used (Lagergren and Eriksson 1971; Barbucci et al 1985, 1987). The investigation of such a surface using S-labelled heparin demonstrated a greater stability of the heparin layer and good antithrombogenicity (Larsson et al 1977; Olsson et al 1977).

A common characteristic of heparinized surfaces by ionic attachment is the removal of the heparin when in contact with blood or plasma, and this leaching effect has been utilised in the preparation of polymers designed to provide a controlled release of heparin (Tanzawa et al 1973; Mijama et al 1977). However, the heparinized biomaterials prepared by ionic binding are unsuitable for long-term application.

3.4.2 Covalent Binding of Heparin

To achieve more stable heparinized surfaces, covalent attachment of heparin onto polymer surfaces has been developed. Normally, the heparinisation of polymer surfaces by covalent binding involves the functional activation of treated surfaces to produce groups such as OH or NH_2, which are capable of reacting with COOH groups in the heparin molecules.

With classical activation techniques, heparin has been covalently immobilised onto poly (vinyl alcohol) through an acetal bridge (Merrill et al 1970), onto agarose through cyanogen bromide and carbodiimide activation (Schmer et al 1972; Danishefsy and Tzeng 1974), and onto cellulose through radiochemical activation (Hasenfratz and Knaup 1981). The preparation of amine or hydroxy derivative surfaces followed by heparin attachment has been applied to silicone rubber through cyanuric chloride activation (Grode et al 1972; Falb et al 1977), to poly(etherurethane) catheters through hydrolysis in NaOH solution (Heyman et al 1985), to styrene-butadiene-styene elastomers through peracetic acid treatment (Goosen and Sefton 1989; Sefton and Merrill 1976).

Considering the biological activity, covalently heparinized surfaces do not always show an improved blood response (Merrill et al 1970; Hoffman et al 1972). The effectiveness of the activity of heparin bound on surfaces is considered dependent on the possible utilisation of the functional groups in the active sites of heparin molecule during the attachment procedure, on the inhibition of blood-heparin contact by protein adsorption (Larm et al 1989) and on the mobility of the heparin chain on surfaces.

It is reported that heparin contains specific sites on the pentasaccharide chain, which could form a complex with AT-III and be able to catalyse the thrombin inhibiting reaction (Lindahl et al 1979; van Boeckel 1985). On the basis that a satisfactory surface must maintain the ability of heparin to bind and activate AT-III (Larsson et al 1980), procedures for covalent attachment of heparin have taken into account the effect of the attachment process on AT-III binding sites. Protection of the AT-III binding sites during heparin immobilisation has been achieved by a technique producing end-point attachment of heparin (Larm et al 1989). In this technique, heparin is partially degraded with nitrous acid to produce at the reducing terminal residues, fragments with reactive aldehyde groups, which are coupled to an aminated surface by reductive amination. Other functional groups in heparin are not involved in the immobilisation reactions and the AT-III binding sequence is not influenced.

Another approach to achieving better anticoagulant activity of heparinized surfaces has employed a spacer through which heparin is immobilised onto a polymer matrix (Ebert and Kim 1982). With this approach, heparin was covalently immobilized onto agarose with an alkyl spacer and the anticoagulant activity of heparinized agarose increased with increasing the spacer length (Ebert et al 1982). Based on this investigation, the achievement of better anticoagulant activity may result from the increasing mobility of the heparin chain on surfaces by utilizing the spacer arm. Since the hydrophobic alkyl spacer may activate platelets, the hydrophilic poly (ethylene oxide) chains have been preferred as spacers for the covalent attachment of heparin to polyurethane (Park et al 1988), polysiloxane (Grainger and Kim 1988) and polystyrene (Vulic et al 1988) in triblock copolymers.

A variation to heparin immobilisation involves the covalent binding of an albumin-heparin conjugate (Hennink et al 1983). This approach utilises the benefits of the albumin adsorption reducing the platelet activation and heparin activating the AT-III to prevent thrombus formation.

The interactions of heparinized surfaces with blood have been widely investigated (Larsson et al 1980; Goosen et al 1980; Hennink et al 1984; Pasche et al 1986). Generally, the investigation of the interactions of heparinized surfaces with blood are carried out with covalently heparinized surfaces, since the ionically heparinized surfaces release heparin in contact with blood or plasma, making it difficult to decide if the achievement of anticoagulant effects result from the heparin binding or the released heparin. With covalently heparinized surfaces, it has been shown that both thrombin (Goosen et al 1980; Hatton et al 1983) and AT-III (Hennink et al 1984; Pasche et al 1986) are adsorbed onto heparinized surfaces. The thrombin inhibition reaction occurs between adsorbed proteins to form the inactive thrombin-AT-III complex. This complex is released and the binding sites on heparin are available for binding thrombin and AT-III again (Migonney et al 1988). This mechanism emphasises the importance of protecting the binding sites in heparin moleculares during the preparation of heparinized surfaces.

The successful usage of heparinized materials is dependent on the nature of the application. The focus has been towards catheters (Heyman et al 1985; Eloy et al 1987) and extracorporeal blood purification procedures. In clinical application, the process of extracorporeal blood purification requires the systemic use of heparin, which has the potential disadvantages of a risk of haemorrhage (Leonard et al 1969) and an adverse effect on platelets (Lindsay et al 1977; Kelton 1986). To eliminate these disadvantages of the systemic use of heparin, the immobilization of heparin onto the components of extracorporeal blood purification has been evaluated. In haemodialysis, systems have been evaluated based on ionic binding (Schmer et al 1976, 1977) and covalent binding (Lins et al 1984), but regular application has not yet been achieved. In cardiopulmonary bypass, membrane oxygenators have been heparinized by ionic binding through TDMAC-heparin complex stabilized by glutaraldehyde (Rea et al 1972; Hagler et al 1975), but covalent binding (Mottaghy et al 1989; Nilsson et al 1990; Palatianos et al 1990; Tong et al 1990; Videm et al 1991) appears to be the approach most likely to gain clinical utilisation (Courtney et al 1993a). It has been reported that heparinized membrane surfaces not only achieve better antithromgenicity but also reduce granulocyte activation (Videm et al 1991) and complement activation (Mottaghy et al 1991).

3.5 PREPARATION OF BIOMEMBRANE-MIMETIC SURFACE

The approach of biomembrane-mimetic surface is the preparation of polymer surfaces designed to mimic biologically inert interfaces of blood cells and thereby avoid the recognition by the blood as "foreign".

The development of such an approach has arisen from the studies of the cell membrane structure and function. The investigation has established that erythrocytes and platelets are built upon an asymmetric fluid bilayer of phospholipid (Zwaal et al 1977) and choline-containing phospholipids make up the major fraction of the lipid exposed at the cell surface (Zwaal and Beavers 1983). It is reported that the phosphorylcholine (PC) head group forms 88% of erythrocyte and 78% of platelet outer membrane surfaces (Durrani and Chapman 1987). The importance of the PC head group in preventing the activation of blood coagulation is supported by the fact of that the PC-containing lipid induces minimal protein adsorption and platelet activation

(Ishihara et al 1990, 1991).

To develop the biomembrane-mimetic polymers, diacetylenic phospholipids polymers containing PC groups have been synthesised (Durrani and Chapman 1987). The coating of this polymer onto hydrophobic surfaces, such as PVC, polyethylene, polypropylene polystyrene, has been shown to reduce the adsorption of fibrinogen and platelets (Chapman and Charles 1992).

In an alternative approach, functionally active PC-containing compounds have been synthesised. It is believed that these compounds could covalently attach onto polymer surfaces containing hydroxyl, carboxylic acid or acid chloride groups (Durrani et al 1986; Hayward et al 1986a, 1986b; Hall et al 1989) and similar improvement proposed could be achieved.

In another technique for the preparation of biomembrane-mimetic surfaces, the monomer containing PC, 2-methacryloyloxyethyl phosphorylcholine (MPC), has been synthesed and successfully copolymerized with hydrophobic monomers, such as butyl methacrylate (BMA) (Ishihara et al 1990) and styrene (Kojima et al 1991), and coated onto cellulose membrane (Ishihara et al 1992). Investigations of blood compatibility indicated that poly(MPC-co-BMA) reduces protein adsorption (Ishihara et al 1991) and platelet adhesion (Ishihara et al 1990), with the effect dependent on MPC composition. Investigation of MPC-coated cellulose membrane. indicated suppressed platelet adhesion (Ishihara et al 1992).

In this project, the copolymer of poly(BMA-co-MPC) and MPC grafted cellulose membrane were selected to study the influence on protein adsorption and blood response of biomembrane-mimetic surfaces (Chapter 7).

3.6 CONCLUSION

The development of blood-compatible biomaterials is important for successful clinical application. Although biomaterials with improved blood compatibility have been achieved by surface modification, an ideal blood compatible biomaterial resembling the endotheluim is far from sight. It is believed that improved knowledge of blood-biomaterial interactions can promote the development of blood-compatible biomaterials. For this purpose, the influence on protein adsorption and blood responses by modified polymers has been studied in this thesis.

CHAPTER 4

METHODOLOGY

4.1 INTRODUCTION

To investigate the blood compatibility improvement induced by polymer modification, in vitro test procedures were selected in this project. The advantages of in vitro tests are versatility, rapidity, simplicity of performance and relative inexpense. In the process of biomaterial assessment, in vitro tests can play an important role in the initial stage of evaluation.

In this project, blood (protein) - biomaterial contact was achieved by an incubation test procedure, which is suitable for evaluating materials in flat sheet form. To achieve a consistent area of a biomaterial in contact with blood (protein), a modified incubation test was established and developed to provide a response appropriate to the selected parameter measurement.

A multiparameter assessment programme is desirable in the process of biomaterial evaluation but this has to be offset by the practical limitations. The parameters selected in this study focused on three relevant features, which are important aspects in the blood responses to a biomaterial. These were protein adsorption, complement activation and the contact phase activation of the intrinsic coagulation. Protein adsorption was determined by the measurement of radioiodine-labelled fibrinogen and albumin single protein adsorption. Complement activation was monitored by measurement of C3a generation using a radioimmunoassay. Finally, contact phase activation of the intrinsic coagulation was measured by determining a parameter of FXII-like activity (FXIIA) with a chromogenic substrate assay.

4.2 REFERENCE MATERIALS SELECTED FOR INVESTIGATION

The reference materials selected in this project for blood compatibility evaluation were the regenerated cellulose membrane Cuprophan 150 PM, the synthetic membrane AN69S (acrylonitrile-sodium methallyl sulphonate copolymer) and a negatively charged polyamide membrane. All these materials have well-defined polymer composition, surface characteristics and high quality of manufacture.

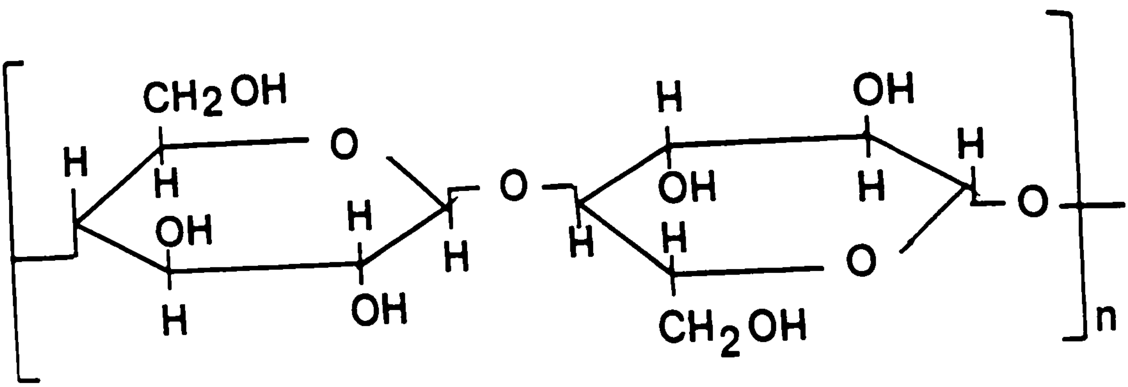


Figure 4.1 Chemical structure of Cuprophan (Akzo)

4.2.1 Cuprophan

Cuprophan, the most widely used regenerated cellulose membrane for haemodialysis, is manufactured by Akzo (Wuppertal, Germany). The structure of Cuprophan is shown in Figure 4.1.

The manufacture of Cuprophan is based on the properties of cellulose. Cellulose consists of a large number of anhydroglucose units joined together by 1-4 and 1-6 beta-glucosidic linkages (Brydson 1975). A strong intermolecular attraction, resulting from hydrogen bonding, is a feature of cellulose. This characteristic is of great importance to membrane formation and utilisation. The hydrogen bonding is promoted by the regular arrangement of hydroxyl groups. It ensures that cellulose is insoluble in solvents and cannot be melted since thermal degradation occurs before flow. Therefore, the manufacture of cellulose membranes requires the production of a soluble or thermoplastic derivative as an intermediate (Briston and Katan 1974). The manufacture of cellulose membranes involves three steps:

1. Chemical modification to produce a soluble or thermoplastic cellulose derivative.
2. Membrane formation by solvent casting or melting.
3. Treatment of the cellulose derivative to achieve regeneration into cellulose.

Production of the cellulose derivative is a key factor in the process. Most of the cellulose membranes used for haemodialysis are produced by the cuprammonium process. A soluble complex is formed by dissolution of cellulose in cuprammonium solution and regeneration is achieved by reaction with alkaline solution. Membranes can also be produced by the viscose process. This process involves the formation of a soluble xanthate by reaction of cellulose with alkali and carbon disulphide, followed by acidic regeneration. Another technique used in membrane production is the production of thermoplastic cellulose acetate, which is hydrolysed to cellulose by reaction with alkali (Courtney et al 1984).

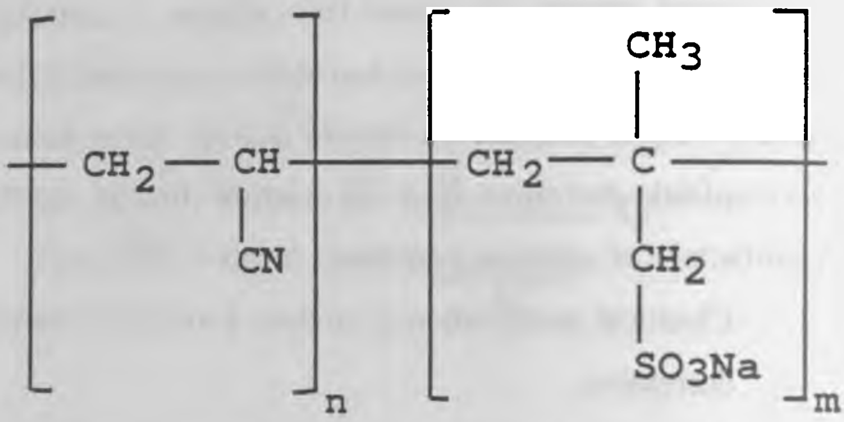


Figure 4.2 Chemical structure of AN69S

Cellulose films are generally identified by a system of figures and letters derived from a code used in the manufacture of regenerated cellulose films as wrapping materials (Briston and Katan 1974). The figures refer to the nominal weight in grams of 10 m^2 of film and therefore are indicative of thickness. The letters denote the condition of the film by indicating whether it is coated or uncoated, since cellulose films may be coated with cellulose nitrate in order to reduce moisture sensitivity.

Regenerated cellulose films used as membranes for extracorporeal blood purification use the letters PM, denoting that the membrane is uncoated and suitable for medical application. The most important regenerated cellulose membranes for haemodialysis is Cuprophan, which is prepared by the cuprammonium process. The availability of Cuprophan membranes and their clinical application have led to these membranes serving as reference membranes. Cuprophan 150 PM is the reference membrane selected in this project.

4.2.2 Polyacrylonitrile - based membrane AN69S

Polyacrylonitrile - based AN69S membranes are produced from a copolymer of acrylonitrile and sodium methallyl sulphonate, under the tradename AN69S. Figure 4.2 shows the chemical structure of this polymer. AN69S membranes are clinically used in extracorporeal blood purification and have a significantly higher permeability to solutes in the 1000 to 2000 molecular weight range as compared with Cuprophan 150 PM.

In general, AN69S membranes have been considered to provide improved blood compatibility in that their influence on leucocytes and the complement system is significantly less than that of regenerated cellulose. However, these materials require specialised monitoring equipment, because of high ultrafiltration rates and excessive fluid removal from patients, and hence their clinical application has been hindered.

4.2.3 Polyamide

There has been a long interest in the use of polyamides in medical applications. Polyamide or nylon has been used as fabrics and sutures, but is normally too reactive to be suitable for long-term implantation (Courtney and Gilchrist 1980).

Polyamide - based membranes were originally developed for use in extracorporeal blood purification. In one attempt to improve on the Cuprophan 150 PM haemodialysis membrane, a polyamide - epoxide resin copolymer was studied by Luttinger and Cooper (1970). However, no significant improvement was achieved. In 1977, Streicher and Schneider produced an asymmetric hollow fibre membrane from an aromatic polyamide. The application of these membranes was in haemofiltration.

Polyamide membranes (NRZ and NR) supplied by Pall Process Filtration Ltd (England, UK) were selected for evaluation in this project. These materials had the same porosity (0.2 μm) and zeta potentials are -17.0 mv for NRZ and -28.6 mv for NR at physiological pH value. They were chosen principally for their well characterised electronegativity, and secondly because they have been previously utilised as control materials in blood compatibility assessment (Travers 1987; Irvine 1989).

4.3 BLOOD RESPONSE MEASUREMENT

4.3.1 Introduction

The basic features of blood compatibility assessment, which were considered in Chapter 2, are nature of the blood, parameters selected and the method of achieving blood-material contact. Since the utilisation of anticoagulant can modify the response of blood to biomaterials (Robertson et al 1990), healthy human whole blood was used in this study. The parameters selected to monitor blood response were C3a generation and FXII-like activity (FXIIA). To achieve blood-material contact, an incubation test cell was utilised and modified.

4.3.2 Blood Collection

Blood was obtained from the median cubital vein of healthy volunteers who had taken no medicine likely to effect platelets for 14 days. Good venepuncture technique ensured minimal trauma to blood. Collected blood was transferred into a polyproprene vial and then aliquoted into test cell using a pipette. Since the blood would contact other surfaces, viz the syringe, needles, vial and pipette, prior to contact with the test material, it was essential to transfer the blood to the test cell as quickly as possible.

4.3.3 C3a Measurement

4.3.3.1 Introduction

The activation of the complement system resulting from blood contact with biomaterials is known to play a key role in the production of inflammatory mediators (Chapter 2). To monitor blood complement activation induced by contact with artificial surface, C3a generation, a major component of the complement cascade, is measured. In plasma, C3a is unstable and can be rapidly converted to C3a des Arg by a serum carboxypeptidase removing arginine. Therefore, the assay is a measure of the concentration of C3a des Arg by a radioimmunoassay.

The standard haemodialysis membrane Cuprophan was selected as the reference material throughout C3a measurement. This was chosen principally for the fact that such membranes are the most widely used haemodialysis materials and have been widely studied. The control material selected was the polystyrene (PS) incubation plate.

4.3.3.2 Principle of Assay

The measurement of C3a was achieved by a radioimmunoassay (Amersham International plc) kit. The assay was based on the competition between unlabelled C3a des Arg and a fixed quantity of [^{125}I]-labelled C3a des Arg for a limited number of binding sites on a specific antibody. With fixed amounts of antibody and [^{125}I]-labelled C3a des Arg, the amount of labelled C3a des

Arg bound was inversely proportional to the concentration of sample non-radioactive C3a des Arg. The antibody bound C3a fraction was then reacted with a second antibody reagent. The second antibody bound fraction was removed from the solution by centrifugation, followed by decantation of the supernatant and counted in a gamma counter. Each assay included a standard curve from which unknown C3a concentrations were calculated. The standard curve and samples were prepared simultaneously.

The standards supplied with the kit can measure samples with concentration in the range of 40-1000 ng/ml. Samples which were expected to exceed the limit of accuracy were diluted 50% by mixing the supernatant with isotonic saline containing sodium EDTA.

4.3.3.3 Procedure of Assay

Plasma sample was assayed in duplicate with following procedure:

1) The frozen plasma samples and assay kit were thawed at room temperature
2) Adding 200 µl of precipitating reagent into 200 µl of plasma sample tube to removal of all C3a in sample
3) Mixing the tube well, incubating at room temperature for at least 5 min and then centrifuging at 2500g for 15 min at 4^0C
4) 25 µl of supernatant or standard were transferred to another assay tube which contained 25 µl assay buffer
5) Adding 25 µl of assay tracer and 25 µl of antiserum into the tube
6) Mixing the tube well, incubating at room temperature for 30 min
7) Adding 25 µl of second antibody into the tube
8) Mixing the tube well, incubating at room temperature for 30 min
9) Adding 1 ml of isotonic saline into the tube and then centrifuging at 2000g for 10 min at 4^0C
10) Decanting off the supernatant and inverting the tube to drain for 5-10 min
11) Determining the radioactivity of the sample by counting in a gamma

counter

4.3.3.4 Calculation

For each standard and unknown sample, the percentage of antibody bound labelled C3a des Arg is calculated using the following equation:

$\% \ B/B_0 = 100 * (\text{Standard/Sample cpm} - \text{NSB cpm})/(B_0 \text{ cpm} - \text{NSB cpm})$

NSB: no specific binding

B_0: zero standard

The standard curve was generated by plotting the percent B/B_0 against the log C3a des Arg concentration. The concentration of C3a des Arg of the samples was obtained from the standard curve after account for dilution.

4.3.3.5 Statistics

Statistical analysis was performed using the minitab package (version 7.1, VMA/VAX Version). The numbers of measurements for each type of polymers, at certain time, were 2*n (n is number of donors, duplicate C3a assays on each donor's plasma). In order to represent the patterns of a polymer influence on C3a generation, the actual mean values were used.

Comparisons of the difference between two means were carried out by the twosample student's t-test and these are reported at 95% confidence intervals ($P<0.05$).

4.3.4 FXII-like Activity Measurement

4.3.4.1 Introduction

Factor XII, also known as the Hageman factor (FXII), is present as an inactive precursor in normal human plasma at a concentration of between 23-40 μg/ml (Saito et al 1976). When blood is in contact with an artificial surface, FXII is activated into an active serine protease FXIIa by the interaction between artificial surface, high molecular weight kininogen and kallikrein. The production of FXIIa is an autoactivation process and enables the progression of the coagulation cascade.

In addition to the initiation of intrinsic coagulation pathway, which has been described in Chapter 2, FXII and its activated form can also initiate the kinin system, fibrinolysis and complement activation.

The activation of kinin system, induced by the action of kallikrein (Colman et al 1975), results in the production of bradykinin, which can induce pulmonary damage, increase vascular permeability and result in interstitial oedema (Gustafson and Colman 1987).

The influence of FXII on fibrinolysis is due to the activation of plasminogen to plasmin. The production of plasminogen activator induced by kallikrein and bradykinin was reported (Egberg et al 1988). Therefore, the effect of FXII on fibrinolysis can be direct and indirect.

The relationship between FXII and complement system is also direct and indirect. β-FXIIa has been shown to activate the first component of complement (Ghebrehiwet et al 1983). As the activation of FXII leads to both plasmin and thrombin generation and both of these enzymes can activate C1 and plasmin can convert C3 to C3a, FXII activation can lead indirectly to activation of the complement system (Fuhrer et al 1990).

FXIIa exists in two forms, which are α-FXIIa and β-FXIIa. α-FXIIa has a molecular weight of 80,000 and is mainly bound onto the biomaterial surface. Specific cleavage of α-FXIIa results in the formation of β-FXIIa. This enzyme has a molecular weight of 28,000 and is free to diffuse away from the surface (Silverberg et al 1980). The measurement of FXII-like activity in this project included both forms of FXIIa.

The reference materials selected for FXIIa measurement were polyamides (NRZ and NR). It has been indicated that a polyamide with negative charge induced higher FXII activation (Irvine 1989). The control polymer was a polystyrene incubation plate.

4.3.4.2 Principle of Assay

Measurement of FXII-like activity was achieved with the chromogenic substrate (2-AcOH-H-D-CHT-Gly-Arg-pNA) (Channel Diagnostics, Walmer, Kent) assay (Sundaram 1992). The assay was based on that FXIIa attacked the substrate to release the substance of p-nitroaniline (pNA) with yellow colour, which gave the absorbance at 405 nm in a spectrophotometer. The amount of FXIIa was proportional to the absorbance. To specify the assay, inhibitors were utilised to protect the other enzymes in plasma from attacking the substrate. Kallikrein inhibitor was utilised to inhibit kallikrein, plasmin, trypsin and FXa. Hirudin was utilised to inhibit thrombin.

4.3.4.3 Procedure of Assay

Prior to assay, the substrate was reconstituted in 10 ml of sterile water to obtain a final concentration of 1 µmol/ml. Kallikrein inhibitor (KI) (Channel Diagnostics, Walmer, Kent) was reconstituted in 10 ml of distilled water and diluted 1/50 in buffer. The stock solution of hirudin (CGP-39393) (Ciba Geigy) was prepared by dissolving hirudin in saline to obtain a concentration of 10 AT (antithrombin) units/ml. The buffer (Tris-HCl 0.025 M, NaCl 0.025 M, pH=8) was used throughout the experiment.

Plasma sample was assayed in duplicate on a microtitre (96 well polystyrene microplate, Limbra Ltd) with following procedure:

1. Adding 25 µl of plasma sample into each well
2. Adding 75 µl of buffer containing KI and 25 µl of hirudin stock solution, then incubating for 2 min at 37°C
3. Adding 50 µl substrate, then incubating for 10 min at 37°C
4. Reaction stopped by adding 50 µl of 50% acetic acid
5. Measuring the absorbance of the mixture at 405nm in a spectrophotometer against a blank, which was the plasma sample at t=0 without the addition of substrate

4.3.4.5 Statistics

Statistical analysis was performed using the minitab package (version 7.1, VMA/VAX Version). The numbers of measurements for each type of polymers, at certain time, were 2*n (n is number of donors, duplicate FXIIA assays on each donor's plasma). In order to represent the patterns of a polymer influence on FXII activation, the actual mean values were used. Comparisons of the difference between two means was carried out by two sample student's t-test and these are reported at 95% confidence intervals ($P<0.05$).

4.3.5 Test Cell and Procedure

4.3.5.1 Introduction

An important feature of blood compatibility assessment is the method used to achieve blood-material contact. Various procedures have been employed in the evaluation of flat sheet materials. These include the rocking test cell (Lindsay et al 1973), the oscillating test cell (Bowry 1981; Travers 1987; Robertson 1988) and a test cell permitting control of blood flow and wall shear (Weng et al 1991).

An alternative approach to the evaluation of flat sheet materials is the use of incubation test cells. The original method utilised a Petri dish (Lemke 1985) but the use of multiwell plates has become more common (Payne and Horbett 1987; Allen 1988; Irvine 1989). Therefore, a 24 well polystyrene microplate (diameter 1.6 cm, shown in Figure 4.3) was selected as the basis for the first phase of the blood compatibility assessment.

During experiments with the 24 well incubation test cell, the material to be tested was cut as a disc (diameter 1.5 cm) and placed into the well. Then 1 ml blood was added into each well in which the contact of blood with material was achieved. To achieve adequate mixing, the incubation plate was placed on an orbital shaker. After the selected incubation time, blood sample was collected. The disadvantage of this incubation test cell is that the contact area of material with blood is not constant, since it was found the disc of material

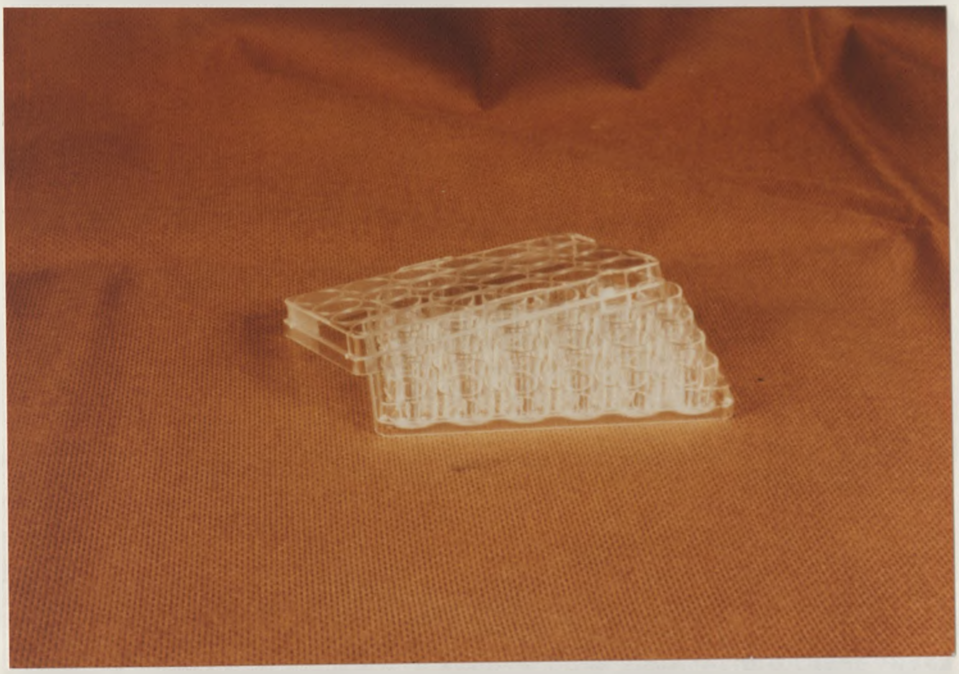
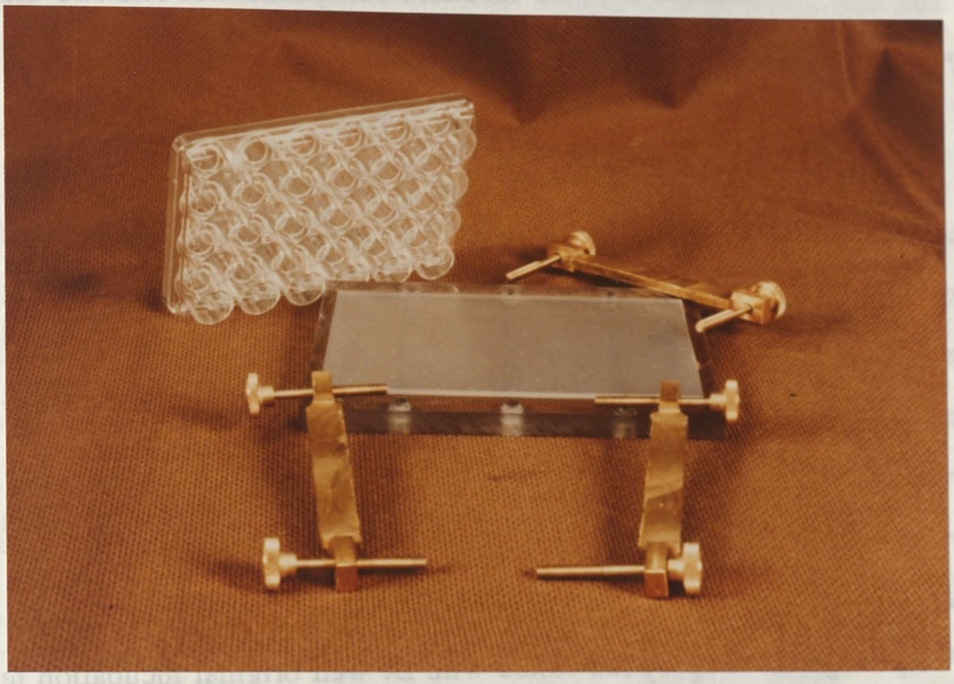


Figure 4.3 Original 24 well incubation test cell



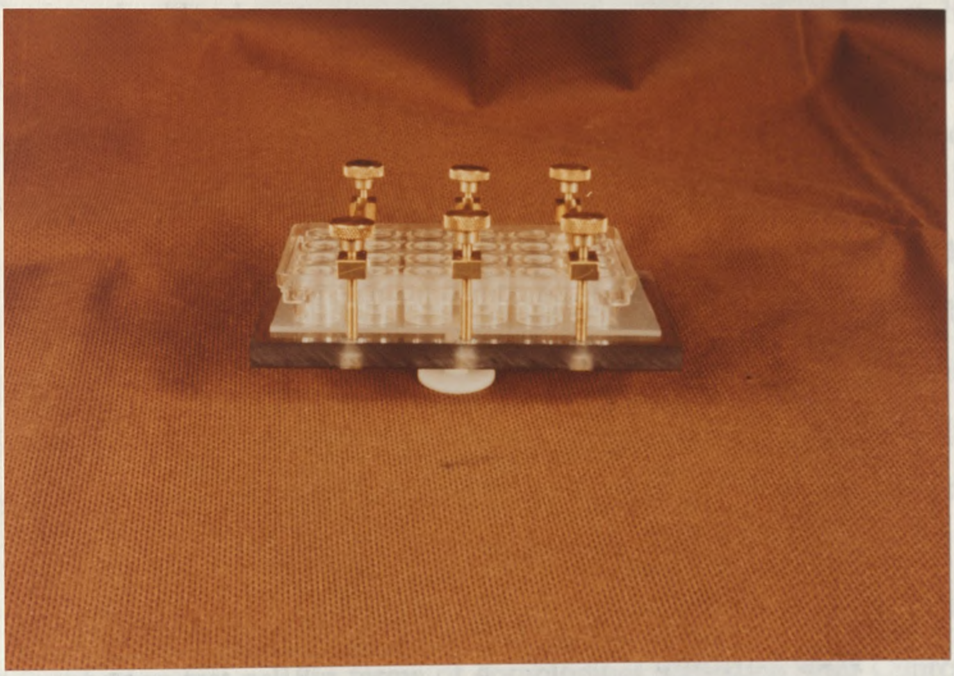


Figure 4.4 Modified 24 well incubation test cell

Table 4.1 Comparison of C3a concentration levels of Cuprophan obtained with the 24 well modified incubation test cell and the 24 well original incubation test cell

Time (min)	C3a desArg Concentration Mean ± S.D.(ng/ml)		
	Material		
	Control n=3	Cup-original * n=3	Cup-modified * n=5
Pre	195 ± 32	192 ± 32	201 ± 24
3	216 ± 35	425 ± 158	305 ± 44
6	266 ± 72	512 ± 174	371 ± 76
9	311 ± 28	645 ± 166	473 ± 50
12	368 ± 83	851 ± 308	568 ± 92

* Cup-original: Cuprophan tested with 24 well original incubation test cell
Cup-modified: Cuprophan tested with 24 well modified incubation test cell

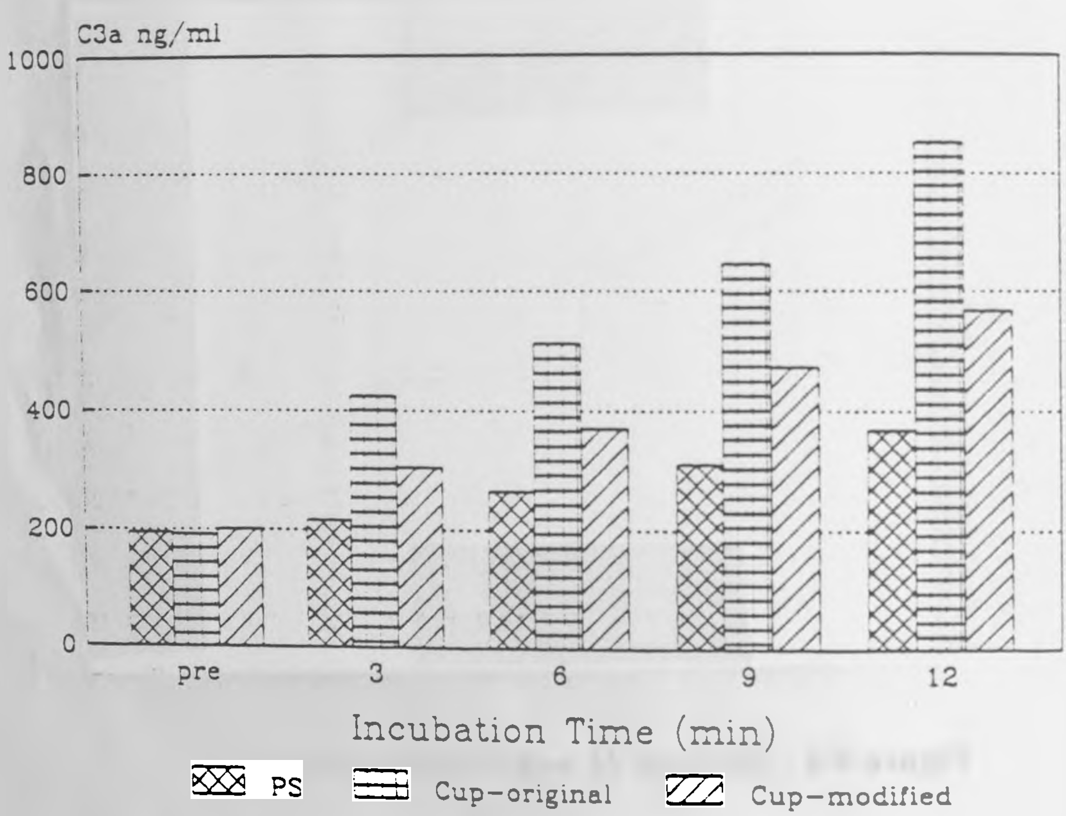


Figure 4.5 Comparison of C3a concentration levels of Cuprophan obtained with the 24 well modified incubation test cell and the 24 well original incubation test cell

was not at the bottom of the well and could float up during the incubation. To overcome this disadvantage, a 24 well modified incubation test cell was established.

4.3.5.2 Modified 24 Well Incubation Test Cell and Procedure

The 24 well modified incubation test cell is shown in Figure 4.4. It contains a base plate made of polymethylmethacrylate (Perspex), a piece of soft silicone rubber, a 24 well (diameter 1.6 cm) polystyrene microplate opened at the top and bottom, three bars for fixing and covers (Figure 4.4.a). To set up the new modified incubation test cell, the material to be tested (which has been soaked in saline overnight) was placed in between the 24 well polystyrene plate and the silicone rubber sheet fixed on the Perspex base plate. The test cell was clamped together with the three bars (Figure 4.4.b). Since the silicone rubber is soft, blood does not leak from the bottom after clamping.

During the blood test experiment, the test cell was set up before blood collection. After collection, 1 ml whole blood was immediately added into each well and incubated with the material at 37°C. To achieve adequate mixing, the incubation plate was placed on an orbital shaker. Samples were collected from the test cell at 3, 6, 9 and 12 min. Blood (500µl) was added to the C3a sample tubes containing 50µl 1.5% disodium EDTA (450 µl blood were added to the FXIIa sample tubes containing 50 µl 3.2% sodium citrate) as anticoagulant. Samples were centrifuged at 2000g at 4 °C (room temperature for FXIIa samples) for 15 min and plasma was separated and stored at -70 °C until assayed.

The comparison of blood test results between the modified and original 24 well incubation test cell, in terms of complement activation with Cuprophan, is shown in Table 4.1 and Figure 4.5.

The results in Table 4.1 and Figure 4.5 show that the standard deviation of the C3a levels of Cuprophan tested with the modified incubation test cell are much less than those with the original incubation test cell. This can be interpreted on the basis that a consistent area of biomaterial contact with blood

Table 4.2 Comparison of C3a concentration levels obtained with the 6 well and the 24 well modified incubation test cells

Time (min)	C3a DesArg Concentration Mean ± S.D.(ng/ml) (n=5)				
	Modified Incubation Test Cell				
	24 Well ($2cm^2$)		6 Well ($9.5cm^2$)		
	PS*(n=3)	Cup *	PS	Cup	AN69S
Pre	195 ± 32	201 ± 24	98 ± 35	98 ± 35	98 ± 35
3	216 ± 35	305 ± 44	115 ± 42	526 ±201	243 ±117
6	266 ± 28	371 ± 76	156 ± 64	1015 ±303	427 ±165
9	311 ± 28	473 ± 50	312 ± 189	1383 ±309	731 ±248
12	368 ± 83	568 ± 92	409 ± 211	2506 ±522	1076 ±140

* PS : Polystyrene; Cup: Cuprophan

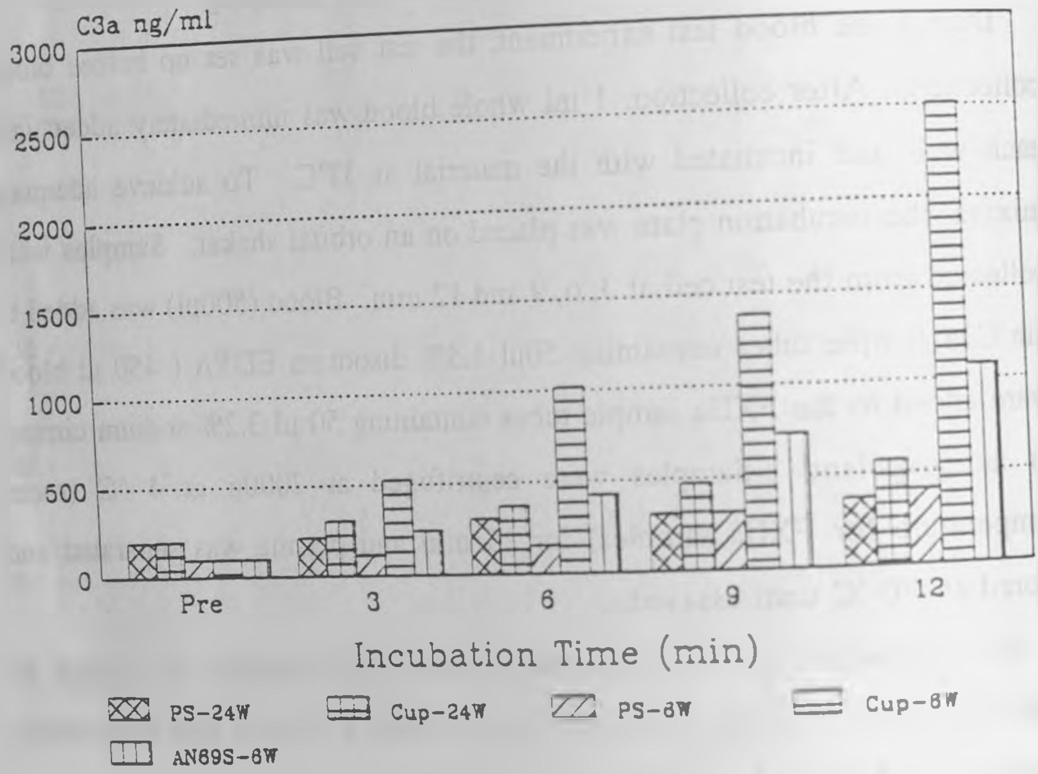


Figure 4.7 Comparison of C3a concentration levels obtained with the 6 well and the 24 well modified incubation test cells

is achieved by the modified incubation test cell. The results in Table 4.1 also show the C3a concentration levels of Cuprophan tested with the modified incubation test cell are much lower than those with the original incubation test cell. This is because the C3a generation is dependent on the surface area of biomaterial in contact with blood (Mahiout et al 1987), and with the original incubation test cell, blood is likely to contact both sides of the biomaterial.

However, statistical analysis with the paired t-test reveals that the C3a concentration levels of Cuprophan obtained with the modified 24 well incubation test cell are not significantly ($P>0.05$) different from those of control material polystyrene (PS). This can be explained on the basis that the area of material in contact with blood is insufficient to provide an appropriate response of C3a. In order to overcome this problem, the test cell was developed.

4.3.5.3 Modified 6 Well Incubation Test Cell and Procedure

The purpose of replacing the 24 well modified incubation test cell was to increase the area of material contact with blood. This was achieved with a 6 well polystyrene plate instead of the 24 well polystyrene microplate. The area of material contact with blood is 9.5 cm^2 for the 6 well test cell and 2 cm^2 for the 24 well test cell.

Figure 4.6 shows the 6 well modified incubation test cell system. It is similar to the system of 24 well modified incubation test cell. The blood test procedure using this 6 well modified incubation test cell was the same as that for the 24 well modified incubation test cell, except 1.5 ml whole blood were added into each well. The ratio of the area of material in contact with blood to the volume of blood added is 6.3 for the 6 well test cell and 2.0 for the 24 well test cell.

The comparison of blood test results between these two modified incubation test cells, in terms of complement activation with Cuprophan and AN69S, is shown in Table 4.2 and Figure 4.7.

Statistical analysis of the results with the paired t-test reveals that the C3a

Figure 4.6 Modified 6 well incubation test cell

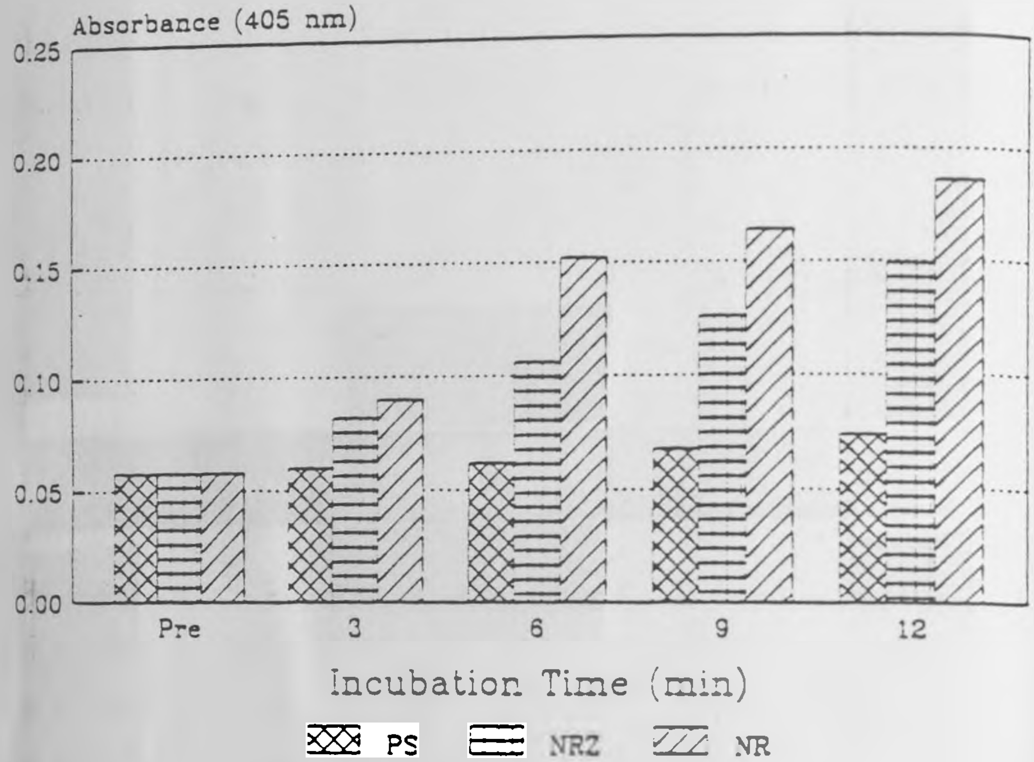


Figure 4.8 FXII-like activity measured with the modified 24 well incubation test cell

concentration levels of Cuprophan are significantly higher (P<0.05) than those of the control material polystyrene (PS) at any time interval. C3a concentration levels of AN-69S are significantly lower (P<0.05) than those of Cuprophan after 6 min incubation, which is in agreement with the previous indication of AN69S being more compatible than Cuprophan in the respect of complement activation.

Advantages of the modified 6 well incubation test cell are both the achievement of a consistent area of material contact with blood and the provision of an appropriate response to the parameter selected.

4.3.5.4 Influence of The Modified Incubation Test Cell on FXII-like Activity (FXIIA) Measurement

The FXIIA results with the polyamides (NRZ and NR) and polystyrene (PS), measured with the 24 well modified incubation test cell, are shown in Table 4.3 and Figure 4.8. Statistical analysis reveals that both polyamides induces significantly greater (P<0.05) FXIIA throughout the incubation period when compared with the control polymer polystyrene (PS). Comparing the two polyamides, the more electronegative polymer NR induces significantly higher (P<0.05) FXIIA than the less electronegative polymer NRZ after 6 min incubation. These results demonstrate that FXIIA is dependent on the electronegativity of the biomaterial surface, with the more negative NR having a greater (P<0.05) effect than less negative NRZ. This observation is consistent with the accepted view (Colman et al 1987) that negatively charged surface induces higher contact phase activation. These results also show that the assay of FXII-like activity measurement is material-dependent.

Table 4.4 and Figure 4.9 show FXIIA results with polyamide (NR) and control polymer polystyrene (PS) measured with the 6 well modified incubation test cell, which are comprised with the results obtained with the 24 well modified incubation test cell. Statistical analysis with the paired t-test reveals that negatively charged NR induces significantly greater FXIIA than polystyrene after immediately incubation. Comparing FXIIA between the two

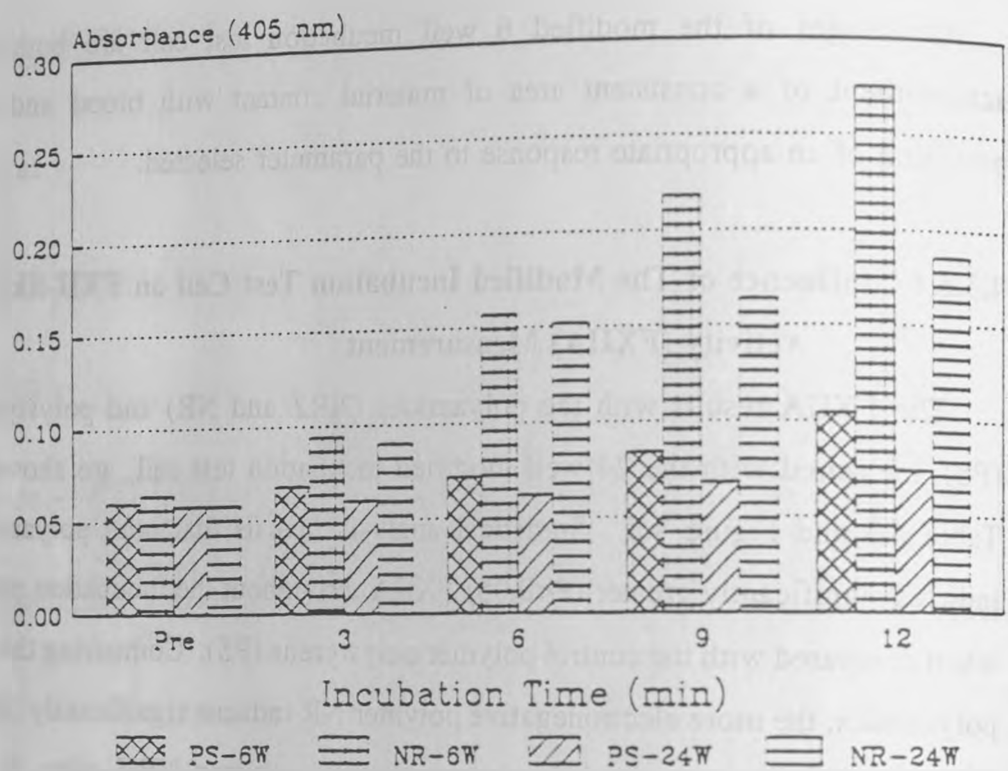


Figure 4.9 FXII-like activity measured with the 6 well and the 24 well modified incubation test cells

modified incubation test cells, analysis reveals that FXIIA is dependent on the surface area of material in contact with blood.

For the consistency of blood testing, the 6 well modified incubation test cell was utilised throughout the blood test experiment.

Table 4.3 FXII-like activity measured with the 24 well modified incubation test cell

Material	Absorbance (405 nm) ± S.D. (n=3)				
	Incubation Time (min)				
	Pre	3	6	9	12
Control PS	0.058 ± 0.012	0.060 ± 0.009	0.062 ± 0.014	0.068 ± 0.008	0.074 ± 0.013
Polyamide NRZ	0.058 ± 0.012	0.082 ± 0.006	0.106 ± 0.005	0.126 ± 0.009	0.149 ± 0.012
Polyamide NR	0.058 ± 0.012	0.090 ± 0.008	0.152 ± 0.009	0.164 ± 0.013	0.186 ± 0.016

Table 4.4 FXII-like activity measured with the modified incubation test cell

Time (min)	Absorbance (405 nm) ± S.D. (n=3)			
	Modified Incubation Test Cell			
	6 Well (9.5cm^2)		24 well (2cm^2)	
	PS	NR	PS	NR
Pre	0.060 ± 0.010	0.064 ± 0.011	0.058 ± 0.012	0.058 ± 0.012
3	0.068 ± 0.013	0.094 ± 0.007	0.060 ± 0.009	0.090 ± 0.008
6	0.072 ± 0.008	0.157 ± 0.010	0.062 ± 0.014	0.152 ± 0.009
9	0.084 ± 0.011	0.218 ± 0.017	0.068 ± 0.008	0.164 ± 0.013
12	0.104 ± 0.010	0.275 ± 0.011	0.074 ± 0.013	0.186 ± 0.016

4.4 PROTEIN ADSORPTION MEASUREMENT

4.4.1 Introduction

Protein adsorption is the initial event when blood contacts an artificial surface and subsequent reactions of blood with a biomaterial are strongly influenced by the nature of adsorbed protein layers (Chapter 2). Therefore, the investigation of protein adsorption is important in understanding the blood response to biomaterials and for developing blood compatible biomaterials.

Protein adsorption has been studied from single protein (Brash and Davidson 1976; Weathersby et al 1977; Schmitt et al 1983; Brash et al 1983; Cheng et al 1987), protein mixture (Lee et al 1974; Brash and Uniyal 1979), plasma (Vroman et al 1980; Horbett 1981; Brash and Hove 1984) and whole blood (Ihlenfeld and Cooper 1979; Horbett 1986; Lelah et al 1986). The fundamental knowledge of protein-biomaterial interaction was derived from the studies of single protein adsorption. Furthermore, single protein adsorption study is versatility and easy to control. Therefore, for the basis of protein adsorption study, single protein adsorption of radioiodine-labelled fibrinogen and albumin was investigated in this project. These proteins are generally considered important for platelet reactions.

The materials involved in this project cover both hydrophilic and hydrophobic polymers, which have different flexibility. Therefore, the measuring protein adsorption procedure was established to be suitable for the hydrophilicity of material . The more hydrophilic cellulose membrane and PUU hydrogels were tested using the 24 well modified incubation test cell and the standard haemodialysis membrane Cuprophan was used as reference material. The brittle PBMA materials (at 25^0C) were tested by a stirring system.

4.4.2 Procedure for Hydrophilic Materials

Direct single fibrinogen or albumin adsorption measurements of hydrophilic materials (cellulose membrane and PUU hydrogel) were carried out with the 24 well modified incubation test cell. A square piece (2cm x 2cm) of material

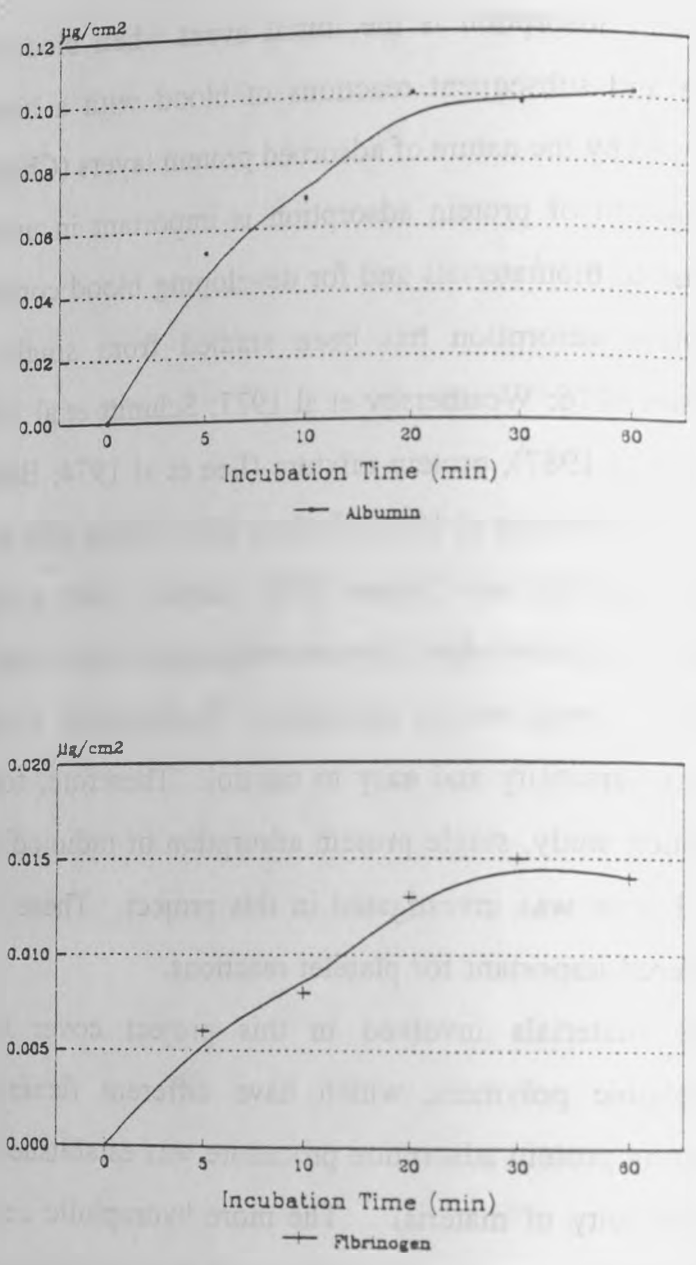


Figure 4.11 Protein adsorption on Cuprophan against time

to be tested was fixed in the test cell after being soaked overnight in buffer (5mM tris, 0.9% NaCl, PH=7.4), which was utilised throughout the protein adsorption experiment.

Iodinated (^{125}I) human fibrinogen (Amersham Int Plc) was reconstituted with 1 ml distilled water as a stock solution containing 0.8 mg/ml iodinated fibrinogen in an isotonic solution containing 0.75% sodium citrate and 0.65% sodium chloride.

Iodinated (^{125}I) human albumin (Amersham Int Plc) was used as received as a stock solution containing 20mg/ml of iodinated albumin in an isotonic solution containing 0.75% sodium chloride and 0.9% benzyl chloride.

For the adsorption experiments, 40 µl of the fibrinogen stock solution (200µl of albumin) were added into the vial containing 20ml of buffer and stirred at 25 °C (see Figure 4.10a). The concentration of fibrinogen in this solution was 1.6 µg/ml (200 µg/ml for albumin). When the temperature reached to 25 °C, 1ml of this solution was added into the well, fixed with the material to be tested and incubated at 25 °C. The area of material in contact with protein solution was 2 cm^2. The incubation was achieved by placing the test cell on an orbital water bath shaker, which was set at 25 ^{0}C. After the selected incubation time, protein solution was removed out and 1 ml of buffer added for rinsing. The material was then taken out and dried with absorbent tissue. For counting the amount of radioactive fibrinogen (albumin) adsorbed on the material, the material was placed in a vial and counted three times in a standard gamma well counter.

The levels of protein adsorption on Cuprophan against time are shown in Table 4.5 and Figure 4.11. Results are represented by the actual mean of nine values (three occasional measurements and three time count readings), in order to represent the pattern of protein adsorption against time. From Figure 4.11, it can be seen that the levels of protein adsorption reach a steady level after 20 min incubation. On this basis, the standard incubation time as 20 min for the protein adsorption experiments was selected.

Figure 4.10 The system for protein adsorption measurement

Table 4.5 Protein adsorption on Cuprophan against time

Protein	Protein Adsorption on Cuprophan (μg/cm²)				
	Incubation Time (min)				
	5	10	20	30	60
Albumin	0.053	0.069	0.101	0.098	0.102
Fibrinogen	0.006	0.008	0.013	0.015	0.014

4.4.3 Procedure for Poly(BMA-co-MPC)

Since poly(BMA-co-MPC) material is too brittle to clamp into the modified incubation test cell at the 25°C, an alternative procedure was established to determine protein adsorption on poly(BMA-co-MPC) materials (Figure 4.10b).

The proteins used were again fibrinogen and albumin.

For the adsorption experiments, 40 μl of the fibrinogen stock solution (200μl of albumin) were added into the vial containing 20ml of buffer and stirred at 25 °C (Figure 4.10a). The concentration of fibrinogen in this solution was 1.6 μg/ml (200 μg/ml for albumin). The sample, which had been pre-soaked in the same buffer overnight, was placed vertically in this stirred solution when the temperature reached 25 °C. To achieve a constant area of material in contact with the protein solution, a paper clip was used to hang the sample in the solution (Figure 4.10b). The sample, 1.5 cm in width, was immersed to a depth of 2 cm, giving an area of 6 cm^2 in contact with the solution. After 20 min adsorption at 25 °C, the sample was taken out and rinsed by immersion in buffer. Excess buffer was removed by gently contacting with adsorbent tissue. The radioactive protein adsorbed on the sample was counted three times in the gamma counter.

4.4.4 Calculation

Protein adsorption was calculated by dividing the retained radioactivity on the sample, corrected for background, by the specific activity of the original protein stock solution and the known surface area of the sample.

Amount of protein adsorption = (Radioactivity on sample cps)/ (Radioactivity of original stock solution cps/μg)/(Area of sample cm^2)

4.4.5 Statistics

Statistical analysis was performed using the minitab package (version 7.1, VMA/VAX Version). The number of values for each type of polymer was nine (three time measurements, three time count reading for each measurement). In order to represent the patterns of protein adsorption to a polymer, the actual mean values were used. Comparisons of the difference between two means was carried out by one-way analysis of variance and these are reported at 95% confidence intervals ($P<0.05$).

Where materials are compared the following procedure can be adopted:

1. The values of fibrinogen and albumin adsorption, obtained as single protein determinations, are normalised for a selected or reference material to 100%.
2. The term "ratio" is used to express the normalised value of albumin in comparison to that of fibrinogen, with the reference material having a value of 1.

CHAPTER 5

HYDROPHILIC POLYMER POLYURETHANEUREA BASED ON PEO

5.1 INTRODUCTION

Recently, block copolymer polyurethanes have been used as biomaterials in several blood contacting devices such as catheters, vascular grafts and heart valves (Ihlenfeld et al 1979; Graham and Hercules 1981) due to their suitable thromboresistant and mechanical properties (Lelah and Cooper 1986). However, problems still remain in achieving ideal blood compatibility, especially in developing long term patency of small-calibre vascular grafts. To improve the blood compatibility of polyurethane block copolymers, increasing polymer hydrophilicity by the incorporation of poly(ethylene oxide) chains as part of the polymer matrix has been utilized, which are achieved either through direct grafting to the main chain (Park et al 1988; Nojiri et al 1990; Barbucci et al 1991; Bamford et al 1992), or synthesized as a segment of a block copolymer (Bots et al 1986; Grasel and Cooper 1986).

To investigate the influence of the increase in polymer hydrophilicity achieved by the utilization of PEO on the blood response, novel polyurethaneurea (PUU) block copolymers were selected in this project. These polyurethaneurea block copolymers are hydrogels and were supplied by Professor N.B.Graham, Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow, in a collaborative programme.

The influence of those polyurethaneurea hydrogels on protein adsorption (albumin/fibrinogen), complement activation as represented by the measurement of C3a concentration levels and contact phase activation as represented by the measurement of FXII-like activity (FXIIA) levels were studied, with the methods previously described (Chapter 4).

The results from these studies are presented in this chapter and have been divided into following sections:

(a) Section 5.2, Materials

(b) Section 5.3, Results and discussion

- 5.3.1 Protein adsorption
- 5.3.2 Complement activation
- 5.3.3 Contact phase activation

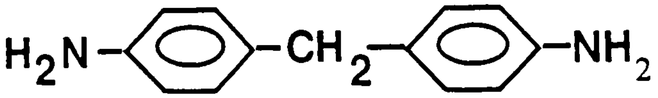
(c) Section 5.4, Summary

$$HO-(CH_2CH_2O)_n-H$$

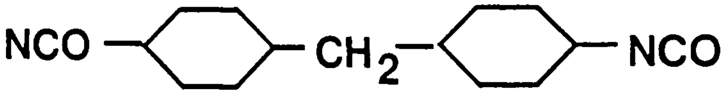
Poly(ethylene oxide) (PEO), 5830 MW

$$HO-(CH_2CH_2CH_2O)_n-H$$

Poly(propylene glycol) (PPG), 425 MW



4,4' –diphenylmethanediamine



4,4–dicyclohexylmethanediisocyanate

Figure 5.1 Chemical structure of chemicals used for the preparation of PUU hydrogels

5.2 MATERIALS

5.2.1 Polyurethaneurea hydrogels

The polyurethaneurea (PUU) hydrogels selected in this project were prepared from a high molecular weight poly(ethylene oxide) (PEO) of MW 5830 and a low molecular weight poly(propylene oxide) (PPO) of MW 425, with the polyurethaneurea chain extension carried out with 4,4'-dicyclohexylmethanediisocyanate and 4,4'-diphenylmethanediamine. Figure 5.1 shows the chemical structure of these chemicals. The PUU hydrogels containing no PEO and containing 20% and 40% PEO were selected in this project.

The PUU hydrogel films were prepared by a solvent evaporation method from a methanol solution of PUU on a polyethylene plate. The hydrophilicity of these hydrogel films was determined by the equilibrium water content after 24 h of swelling in saline at 37°C. Table 5.1 lists the water content of PUU hydrogels selected in this project. The water content (% H_2O) was calculated using the following equation:

$$\% \ H_2O = 100 * (\text{wt.swollen polymer-wt.dry polymer})/(\text{wt.swollen polymer})$$

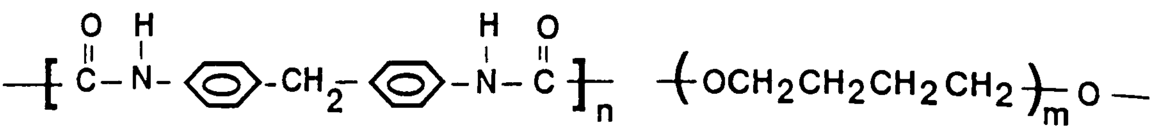
Table 5.1 Water content of PUU hydrogels selected

PUU hydrogels	PUU-PEO 0%	PUU-PEO 20%	PUU-PEO 40%
% H_2O	≈ 10%	≈ 40%	≈ 60%

5.2.2 Biomer

Biomer has been used in a number of blood-contacting devices such as catheters (Gilding and Taylor 1977) and artificial heart (Kolff et al 1974; Boretos et al 1975), due to its better blood compatibility (Hanson et al 1980; Lelah et al 1983) and excellent physical and mechanical properties (Boretos 1980; Lelah et al 1983).

Biomer is a block copolymer of a segmented polyurethane. The soft segments consist of polytetramethyleneoxide (PTMO). The hard segments



HARD SEGMENT SOFT SEGMENT

Figure 5.2 Chemical structure of Biomer

consist of 4,4'-diphenylmethanediisocyanate (MDI), chain-extended with a mixture of diamines, primarily ethylene diamine (Lelah and Cooper 1986). Figure 5.2 shows the repeat unit chemical structure of Biomer.

Biomer (Ethicon Inc.) was selected in this chapter to serve as a reference material for the protein adsorption study.

5.3 RESULTS AND DISCUSSION

5.3.1 Protein Adsorption

The influence of PUU hydrogels on albumin and fibrinogen adsorption is shown in Table 5.2 and Figure 5.3, in comparison with Biomer and Cuprophan.

Statistical analysis of variance reveals that PUU hydrogels containing PEO induce significantly lower fibrinogen adsorption than Biomer and Cuprophan. Compared with the PUU hydrogel containing no PEO, fibrinogen adsorption is markedly and significantly reduced on PUU hydrogels containing PEO. Between PUU hydrogels containing 20% PEO and 40% PEO, fibrinogen adsorption is not significantly different.

Table 5.2 Protein adsorption on PUU hydrogels compared with Biomer and Cuprophan

Polymer	Protein adsorption on polymer surface (μg/cm^2) Mean ± S.D. (N=9)*	
	Albumin	Fibrinogen
Biomer	0.192 ± 0.015	0.043 ± 0.006
Cuprophan	0.101 ± 0.005	0.013 ± 0.002
PUU-PEO 0%	0.176 ± 0.010	0.018 ± 0.005
PUU-PEO 20%	0.190 ± 0.005	0.003 ± 0.001
PUU-PEO 40%	0.203 ± 0.012	0.003 ± 0.001

* N: Number of values

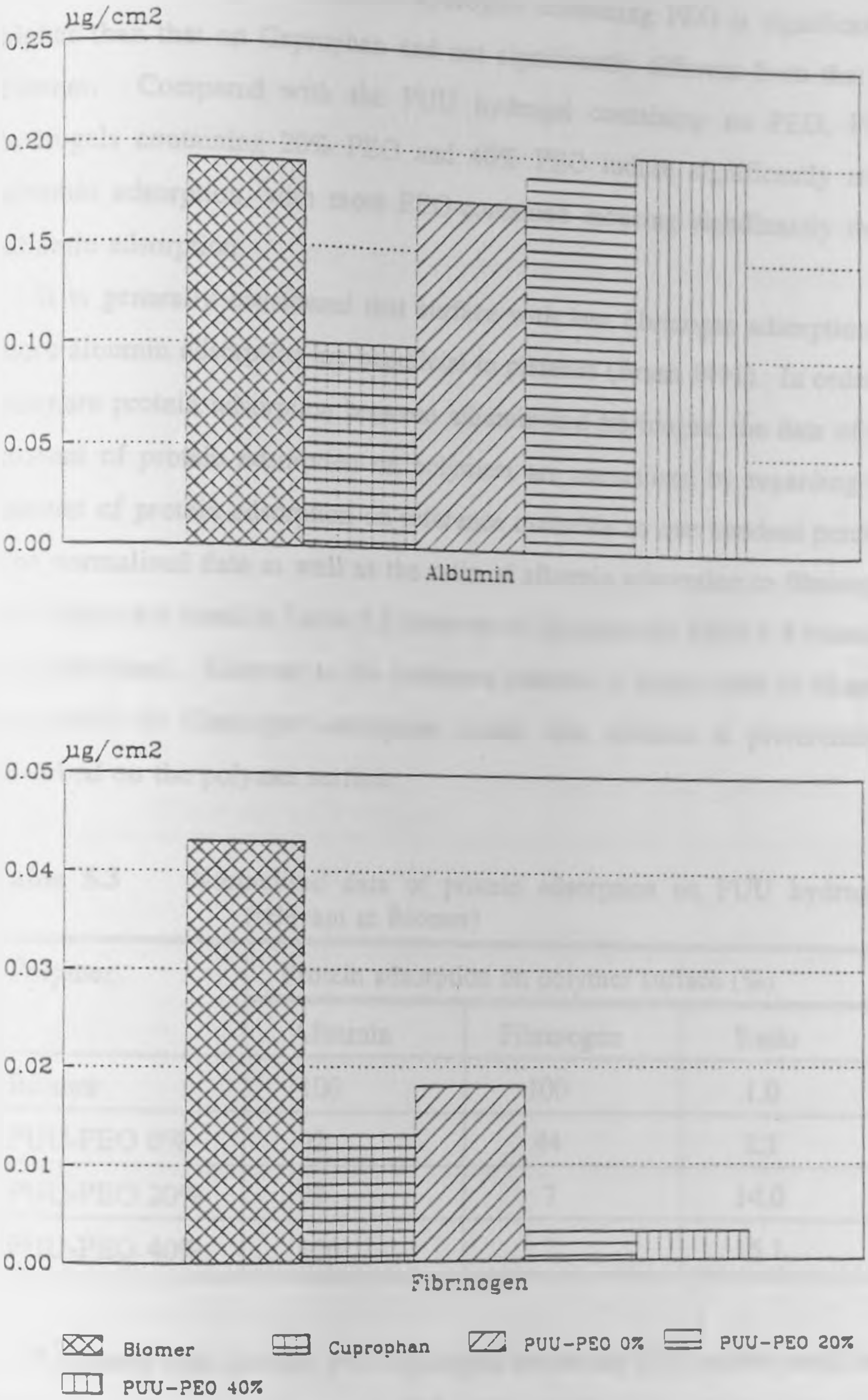


Figure 5.3 Protein adsorption on PUU hydrogels compared with Biomer and Cuprophan

Albumin adsorption on PUU hydrogels containing PEO is significantly higher than that on Cuprophan and not significantly different from that on Biomer. Compared with the PUU hydrogel containing no PEO, PUU hydrogels containing 20% PEO and 40% PEO induce significantly more albumin adsorption, with more PEO-contained inducing significantly more albumin adsorption.

It is generally considered that surface with less fibrinogen adsorption or more albumin adsorption are beneficial to platelets (Brash 1991). In order to compare protein adsorption between albumin and fibrinogen, the data of the amount of protein adsorption on polymers are normalised by regarding the amount of protein adsorption on reference materials as one hundred percent. The normalised data as well as the ratio of albumin adsorption to fibrinogen adsorption are listed in Table 5.3 (relevant to Biomer) and Table 5.4 (relevant to Cuprophan). Relevant to the reference material, a higher ratio of albumin adsorption to fibrinogen adsorption means that albumin is preferentially adsorbed on the polymer surface.

Table 5.3 Normalised data of protein adsorption on PUU hydrogels (relevant to Biomer)

Polymer	Protein adsorption on polymer surface (%)		
	Albumin	Fibrinogen	Ratio
Biomer	100	100	1.0
PUU-PEO 0%	92	44	2.1
PUU-PEO 20%	99	7	14.0
PUU-PEO 40%	106	7	15.1

Compared with Biomer, PUU hydrogels containing PEO induce much less fibrinogen adsorption (about only 10% of that on Biomer) and about the same amount of albumin adsorption. PUU hydrogel containing no PEO also induce less fibrinogen adsorption (about 55% of that on Biomer) and the same amount of albumin adsorption than Biomer. The ratio of albumin adsorption to fibrinogen adsorption is in the order: PUU hydrogels containing PEO > PUU hydrogel containing no PEO > Biomer. This could mean that albumin in comparison to fibrinogen is preferentially adsorbed on polyurethane polymers in following order: PUU containing PEO > PUU containing no PEO > Biomer. The soft segment phases of these polyurethane polymers are polyethylene oxide, polypropylene oxide and polytetramethylene oxide, respectively. Therefore, these results indicate that polyurethane polymers preferentially adsorbing albumin in comparison to fibrinogen, in which polyethers are incorporated as the soft segment phase, should be in the following order: polyethylene oxide > polypropylene oxide > polytetramethylene oxide. This observation is in agreement with the evidence of platelet reaction increasing in the opposite order when polyethers are incorporated as the soft segment phase : polyethylene oxide < polypropylene oxide < polytetramethylene oxide (Pekala et al 1986).

Table 5.4 Normalised data of protein adsorption on PUU hydrogels (Relevant to Cuprophan)

Polymer	Protein adsorption on polymer surface (%)		
	Albumin	Fibrinogen	Ratio
Cuprophan	100	100	1.0
PUU-PEO 0%	174	138	1.3
PUU-PEO 20%	188	23	8.2
PUU-PEO 40%	201	23	8.7

Compared with Cuprophan, PUU hydrogels containing PEO induce much less fibrinogen adsorption (about 30% of that on Cuprophan) and about 90% more albumin adsorption. The ratio of albumin adsorption to fibrinogen is much higher for PUU hydrogels containing PEO than Cuprophan. These results indicate that PUU hydrogels based on PEO preferentially adsorb albumin in comparison to fibrinogen when compared with Cuprophan. This observation of albumin preferentially adsorbing on PUU hydrogels containing

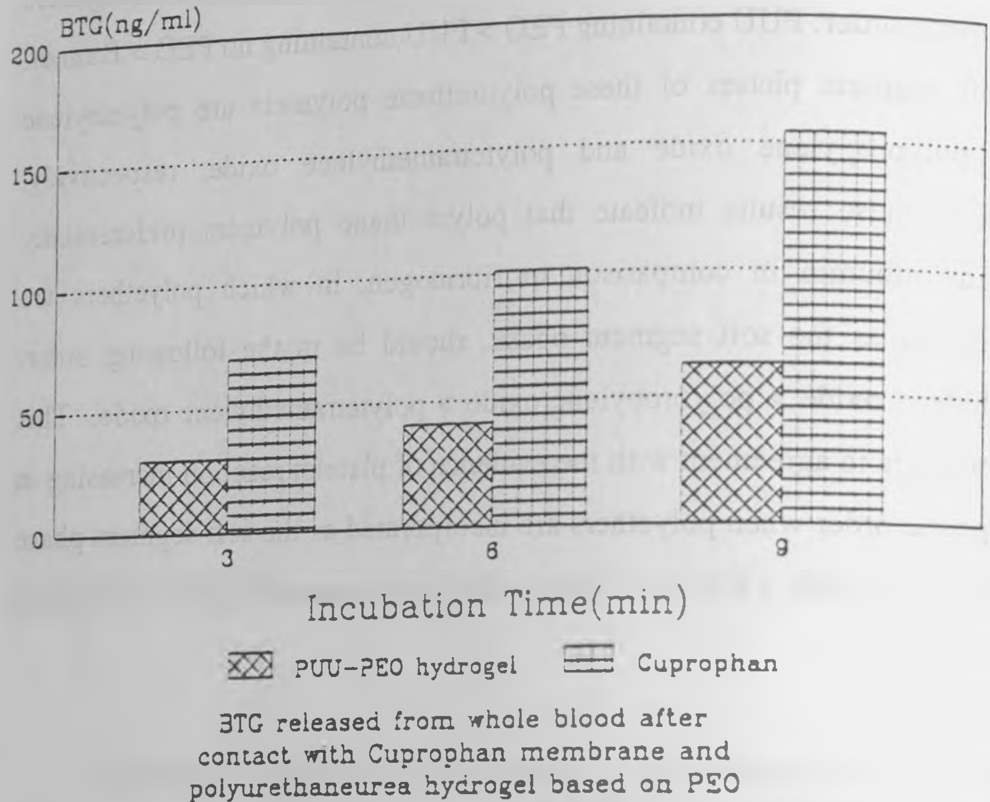


Figure 5.4 Alteration in beta thromboglobulin concentration with time following blood contact with PEO-contained PUU hydrogel and Cuprophan

PEO is in agreement with the evidence that a lower platelet response can be obtained with the PUU hydrogel containing PEO than Cuprophan (Travers et al 1986). Figure 5.4 illustrates the influence of PEO-containing PUU hydrogel, compared with Cuprophan, on the platelet release reaction, determined by measurement of the platelet-specific protein beta thromboglobulin (BTG) (Travers et al 1986).

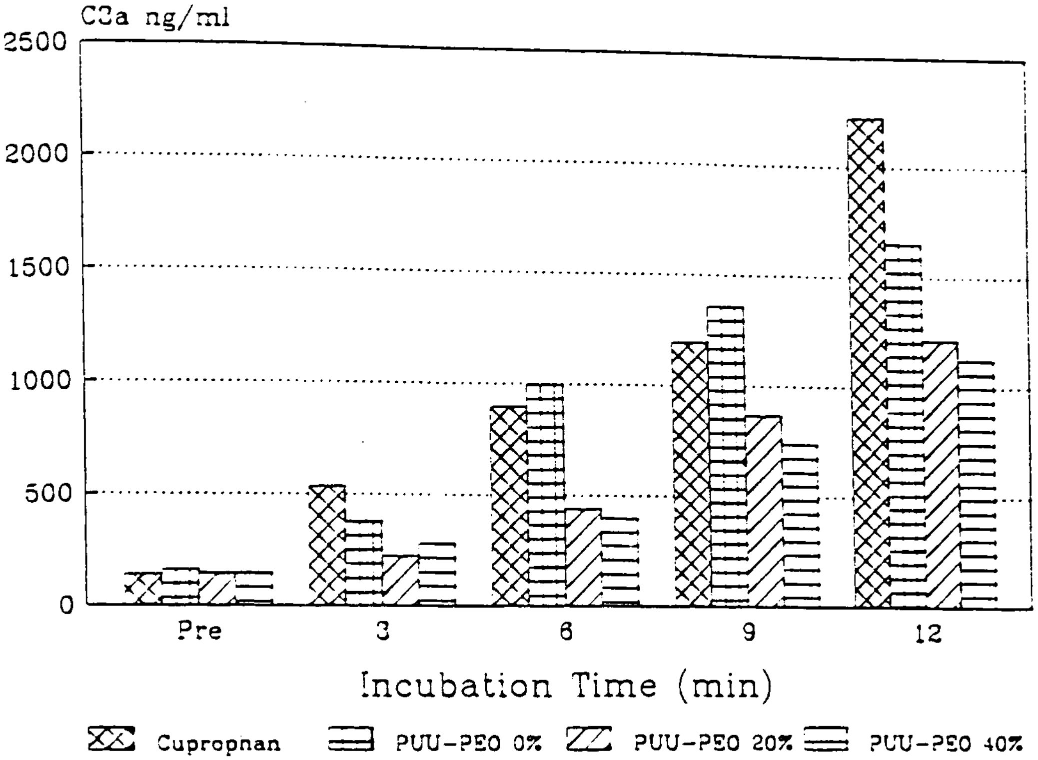
5.3.2 Complement Activation

The influence of PUU hydrogels on complement activation, as represented by the measurement of C3a concentration levels at different time following blood-polymer contact, is shown in Table 5.5 and Figure 5.5, compared with Cuprophan and polystyrene (PS).

Statistical analysis reveals that PUU hydrogels significantly increase C3a concentration levels after 6 min incubation, compared with C3a concentration levels prior to blood-polymer contact.

Table 5.5 C3a concentration levels obtained with PUU hydrogels in comparison to Cuprophan and polystyrene (PS)

Polymer	C3a des Arg concentration levels (ng/ml) Mean ± S.D.				
	Incubation Time (min)				
	Pre	3	6	9	12
PS (n=5)	98 ± 35	115 ± 42	156 ± 64	312 ± 189	409 ± 211
Cuprophan (n=10)	139 ± 38	534 ± 155	896 ± 126	1196 ± 162	2225 ± 315
PUU-PEO 0% (n=4)	160 ± 26	375 ± 126	997 ± 322	1359 ± 320	1652 ± 208
PUU-PEO 20% (n=5)	144 ± 23	221 ± 28	437 ± 144	868 ± 139	1216 ± 78
PUU-PEO 40% (n=5)	144 ± 23	276 ± 141	397 ± 190	739 ± 131	1126 ± 309



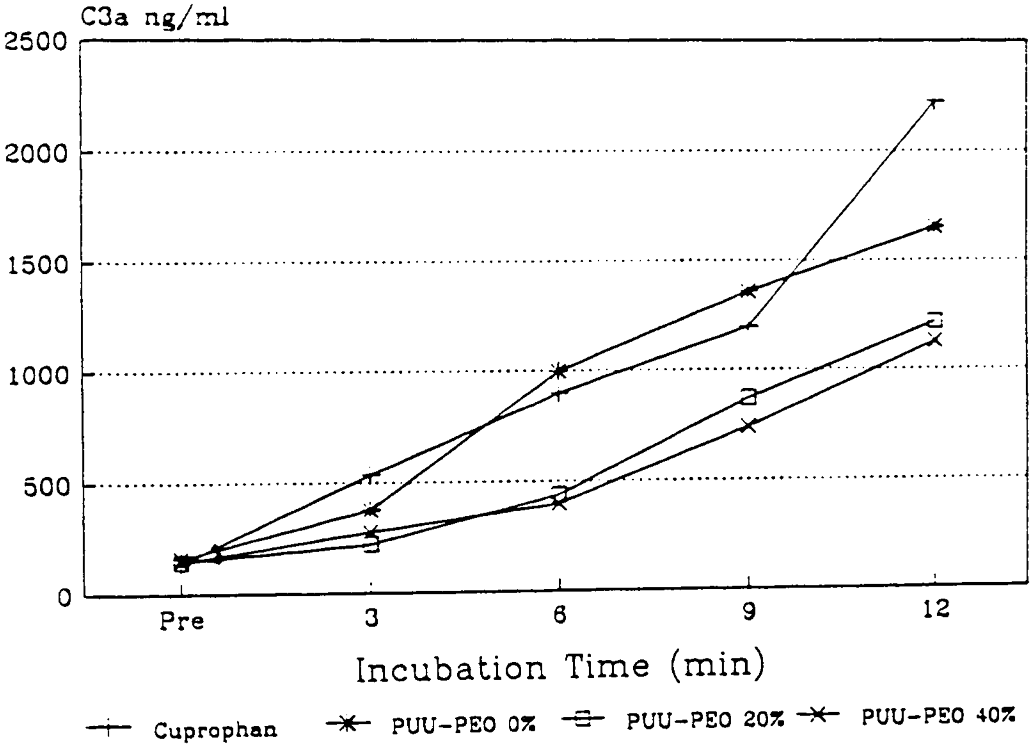
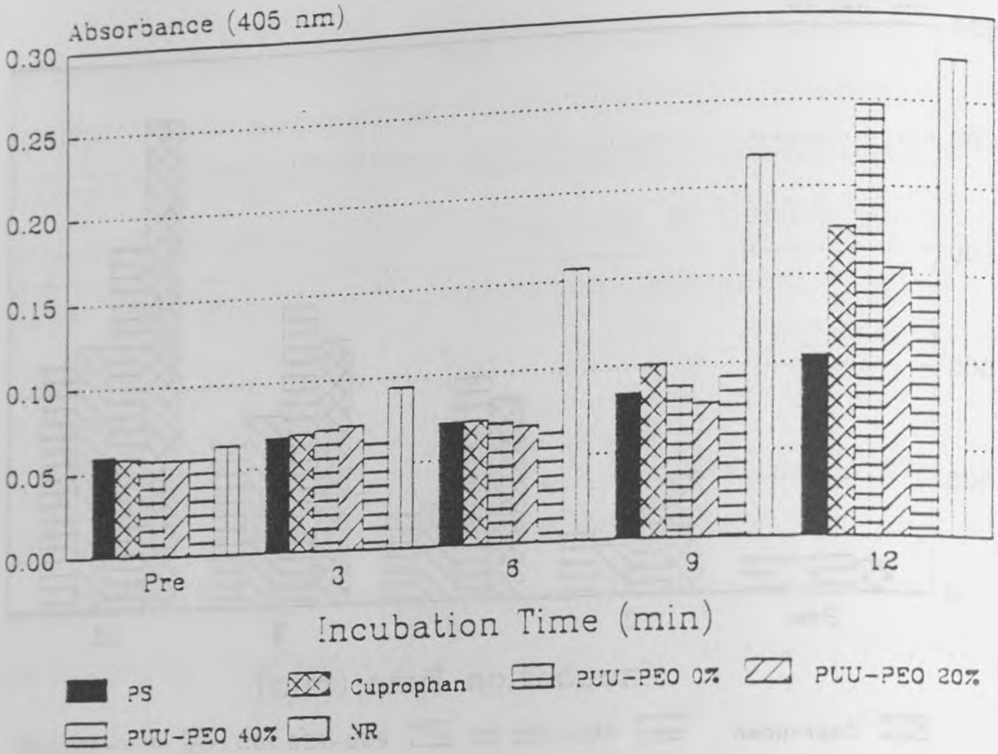


Figure 5.5 Alteration in C3a concentration levels with time following blood contact with PUU hydrogels and Cuprophan



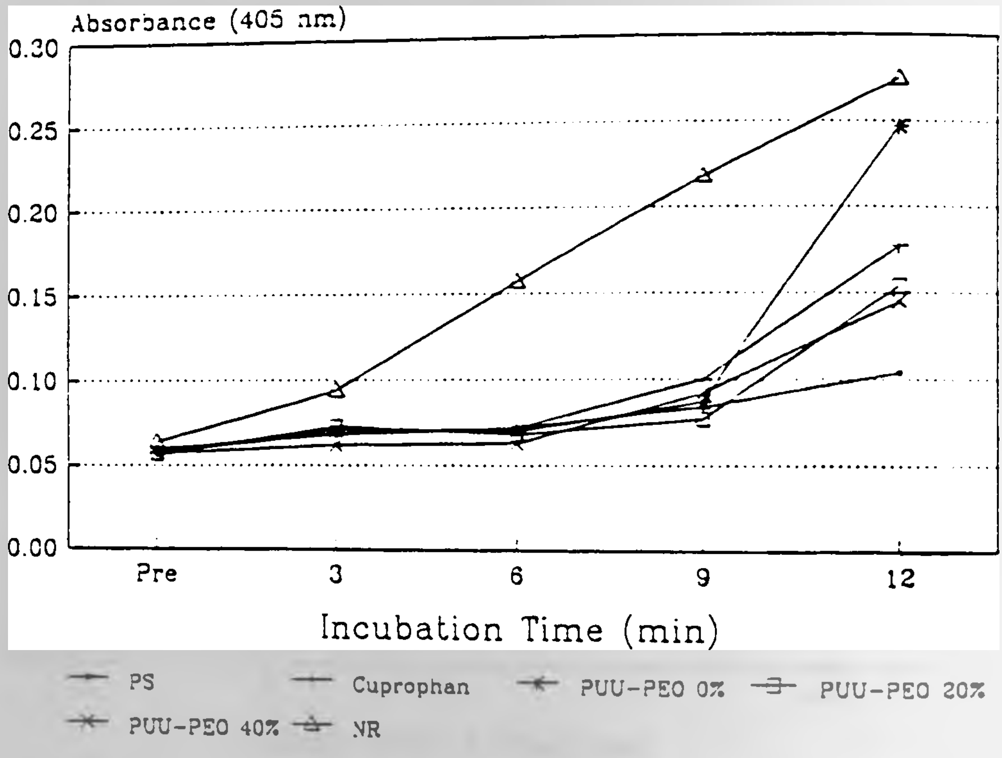


Figure 5.6 Alteration in FXIIA Levels with time following blood contact with PUU hydrogels, Cuprophan, NR and PS

Compared with polystyrene, PUU hydrogels containing PEO induce significantly more C3a concentration levels after 6 min incubation. Compared with Cuprophan, PUU hydrogels containing PEO induce significantly less C3a concentration levels after 6 min incubation, and the PUU hydrogel containing no PEO induces significantly less C3a concentration levels at t=12 min. Compared with the PUU hydrogel containing no PEO, PUU hydrogels containing PEO induce significantly less C3a concentration levels after 6 min incubation. Between PUU hydrogels containing 20% and 40% PEO, C3a concentration levels are not significantly different at any incubation time.

These results indicate that increasing the hydrophilicity of polymer by the utilization of PEO can reduce complement activation. Although the mechanism for this achievement is uncertain, the following postulation can be made:

Protein adsorption behaviour can be altered by the incorporation of PEO. As it has been reported that PEO has very low interfacial free energy with water, flexible polymer chain and the steric stabilization effect (Chapter 3), these special properties of PEO may prevent complement protein adsorption and adhesion, therefore inducing less complement activation.

5.3.3 Contact Phase Activation

The influence of the PUU hydrogels on the contact phase activation, as represented by the measurement of FXII-like activity (FXIIA) levels at different time following blood contact, is shown in Table 5.6 and Figure 5.6, compared with polystyrene (PS), Cuprophan and polyamide (NR).

Statistical analysis of variance reveals that FXIIA levels obtained with PUU hydrogels before 9 min incubation show no significant difference, when compared with FXIIA levels prior to blood-polymer contact. At t=12 min, all polymers activate FXII to a significant degree when compared with FXIIA levels prior to blood-polymer contact. As no anticoagulant was used, this rise may be partially associated with thrombin formation. Evidence of thrombin formation at about 9 min was observed (Sundaram 1992b). Also, reducing contact phase activation by adding the anticoagulant heparin into the blood has

Table 5.6 FXII-like activity (FXIIA) levels obtained with PUU hydrogels in comparison to Cuprophan, polystyrene (PS) and polyamide (NR)

Material	Absorbance (405 nm) Mean ± S.D.				
	Incubation Time (min)				
	Pre	3	6	9	12
PS (n=3)	0.060 ± 0.010	0.068 ± 0.013	0.072 ± 0.008	0.084 ± 0.011	0.104 ± 0.010
Cuprophan (n=9)	0.058 ± 0.015	0.069 ± 0.021	0.072 ± 0.022	0.099 ± 0.040	0.177 ± 0.061
PUU-PEO 0% (n=3)	0.057 ± 0.013	0.071 ± 0.033	0.070 ± 0.023	0.087 ± 0.038	0.247 ± 0.012
PUU-PEO 20% (n=3)	0.057 ± 0.013	0.073 ± 0.039	0.068 ± 0.019	0.076 ± 0.048	0.154 ± 0.021
PUU-PEO 40% (n=3)	0.057 ± 0.013	0.062 ± 0.016	0.063 ± 0.024	0.092 ± 0.046	0.145 ± 0.019
NR (n=3)	0.064 ± 0.011	0.094 ± 0.007	0.157 ± 0.010	0.218 ± 0.017	0.275 ± 0.011

been reported (Irvine 1989).

FXIIA levels obtained with NR polyamide are significantly greater than all other polymers after 6 min incubation. Compared with polystyrene (PS), PUU hydrogels do not induce significantly more FXIIA levels until t=12 min incubation. Compared with Cuprophan, PUU hydrogels containing PEO induce no significant difference in FXIIA levels at any time incubation. Compared with the PUU hydrogel containing no PEO, PUU hydrogels containing PEO induce significantly less FXIIA levels at t=12 min. Between PUU hydrogels containing 20% and 40% PEO, FXIIA levels are not significantly different at any incubation time.

These results reveal that contact phase activation is immediately activated after blood contact with negatively charged surface. For PUU hydrogels, the contact phase system is not activated to a significant degree until thrombin formation.

5.4 Summary

The investigation of the influence of PUU hydrogels based on PEO on protein adsorption, complement activation and contact activation reveals that PEO-containing PUU hydrogels can increase albumin adsorption and markedly reduce fibrinogen adsorption, reduce complement activation and little influence on contact phase activation. The results of protein adsorption are in agreement with the evidence of platelet reactivity reported previously. These observations indicate that the incorporation of PEO into a polymer can produce biomaterials with reduced platelet reactivity and a reduced immune response, with little influence on the contact phase activation.

An important feature of biomaterials research is the synthesis of polymers which either form the basis of biomaterials with improved blood compatibility or have the ability to modify the blood response to other polymers and thereby promote the development of improved composite biomaterials. The polyurethaneurea hydrogels studied, whilst insoluble in water, are soluble in a number of organic solvents, including methanol, ethanol, chloroform and dichloromethane. This solubility is an important advantage for these hydrogels in manufacturing composite biomaterials.

CHAPTER 6

CELLULOSE MODIFICATION

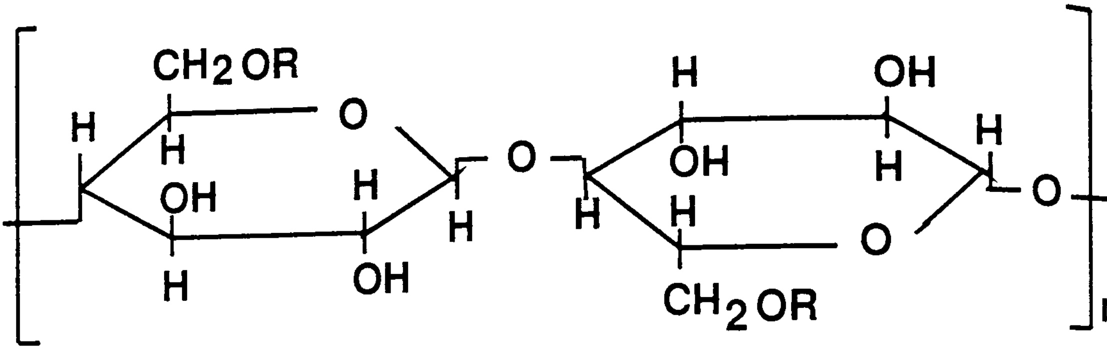
6.1 INTRODUCTION

Membranes used in haemodialysis can be divided into two types. These are cellulose-based membranes, such as Cuprophan, cellulose acetate and Hemophan, and synthetic membranes, such as polyacrylonitrile-based polymer, poly(methyl methacrylate), polysulphone, polyamide and polycarbonate. The most widely used membrane in haemodialysis is regenerated cellulose Cuprophan, due to its reasonable blood compatibility, high dialysis efficiency for small molecules, tolerance to steam autoclave sterilization, low blood leakage and low product cost (Akizawa et al 1986).

The preparation and structure of Cuprophan have been fully described in Chapter 4.

Clinical utilization of Cuprophan still requires the systemic application an anticoagulant agent such as heparin to prevent thrombus formation. This may result in the risk of bleeding. Also, as indicated in Chapter 2 and Chapter 3, these membranes induce complement activation and result in the production of the anaphylatoxins C3a and C5a, which function as inflammatory mediators. These inflammatory mediators may be responsible for a number of manifestations noted clinically, such as neutropenia, hypoxia and leucocyte activation (Craddock et al 1977a, 1977b; Chenoweth et al 1983; Ivanovich et al 1983; Cheung et al 1987; Haeffner-Cavaillon et al 1989). Therefore, modification of Cuprophan to improve blood compatibility could be beneficial for the clinical utilization. The considerable advantage of modified cellulose, over synthetic materials, is the use of production conditions similar to those for well-established regenerated cellulose membranes.

The modification of cellulose membranes has been investigated for a long time as a possible means of obtaining membranes with improved blood compatibility in the respect of complement activation (Chapter 3). However, cellulose modification may alter other characteristics of membranes, such as permeability, response of platelets and contact phase system.



R : $-CH_2CH_2N(C_2H_5)_2$

Figure 6.1 Chemical structure of Hemophan

In this project, selected cellulose modifications for investigation were:

(a) Chemical modification of Cuprophan with partial replacement of hydroxyl groups with diethylaminoethyl (DEAE) groups (Hemophan)

(b) Cuprophan on which heparin is immobilized

The influence of these cellulosic membranes on protein adsorption (albumin or fibrinogen), complement activation, as represented by the measurement of C3a concentration levels, and contact phase activation, as represented by the measurement of FXII-like activity (FXIIA) levels were studied, using the methods previously described (Chapter 4).

The results from these studies are presented in this chapter, which has been divided into following sections:

(a) Section 6.2, Materials

(b) Section 6.3, Results and discussion

- 6.3.1 DEAE-cellulose (Hemophan)
  - 6.3.1.1 protein adsorption
  - 6.3.1.2 complement activation
  - 6.3.1.3 contact phase activation
- 6.3.2 Heparinized-Cuprophan
  - 6.3.2.1 protein adsorption
  - 6.3.2.2 complement activation
  - 6.3.2.3 contact phase activation

(c) Section 6.4, Summary

6.2 MATERIALS

6.2.1 DEAE-cellulose (Hemophan)

Hemophan, manufactured by Akzo, is a cellulosic membrane with hydroxyl groups partially displaced by diethylaminoethyl groups, with the degree of hydroxyl substitution is 0.01. Figure 6.1 shows the chemical structure of Hemophan.

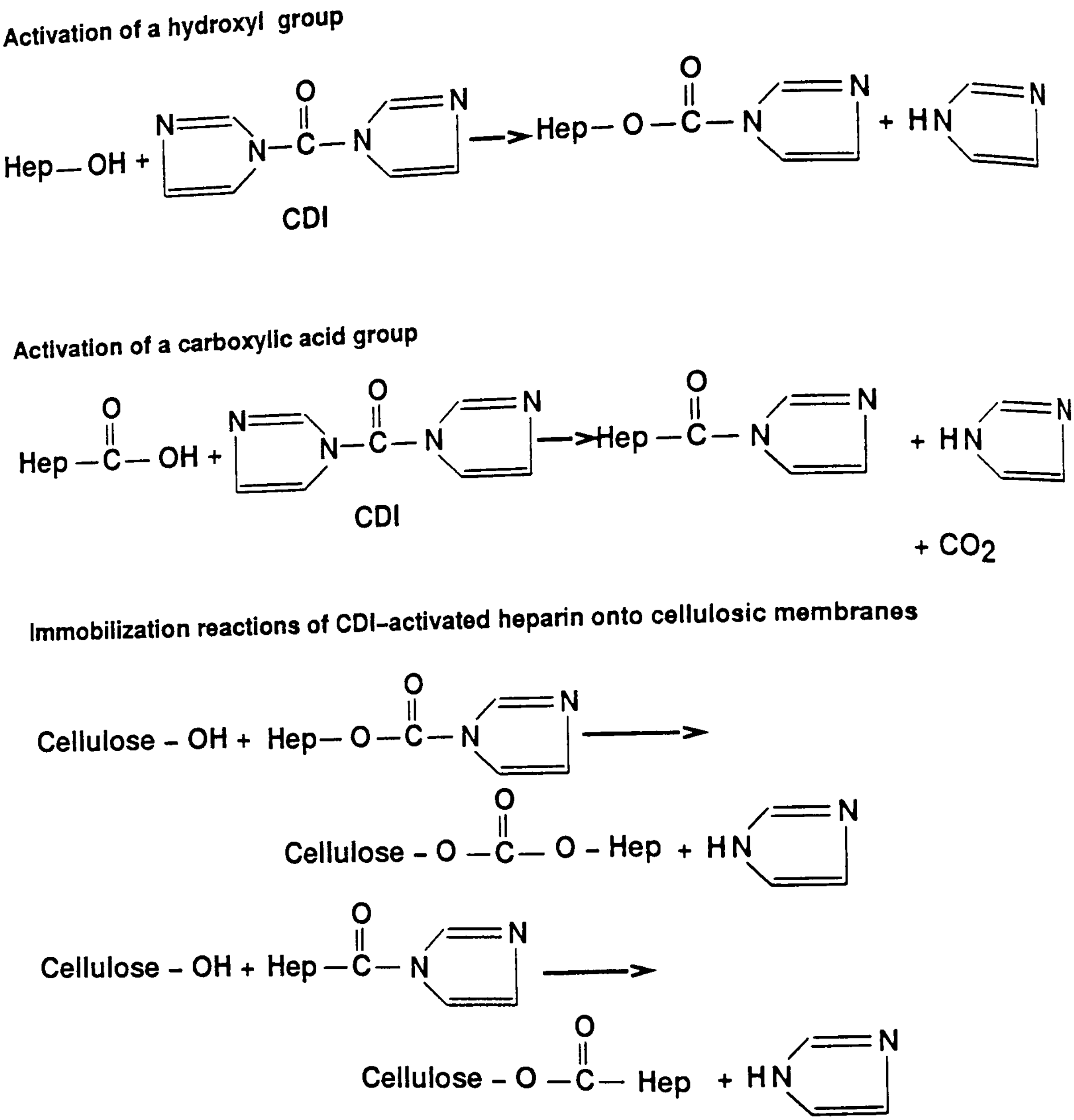


Figure 6.2 Reaction routes of Cuprophan heparinization

6.2.2 Heparinized-Cuprophan

Cuprophan heparinization was carried out in Twente University, Enschede, The Netherlands, by Dr. Gerard Engbers, in a collaborative programme. The principal modification reactions of Cuprophan heparinization processes are illustrated in Figure 6.2.

Before heparinization, Cuprophan membranes were rinsed with purified formamide to remove glycerol and water from membranes, since they attack the diimidazole used in the process. During the process of heparinization, dried heparin (Triton-B) dissolved in purified formamide was activated by the addition of 1,1'-carbonyl diimidazole (CDI). After 30 min activation, Cuprophan membrane, pre-rinsed with formamide, was added into the activated heparin solution. The heparinization reaction was continued for 48 h.

After heparinization, the membrane was rinsed with demineralized water to remove formamide and non-reacted species and to hydrolyse non-reacted activated hydroxyl and carboxylic acid groups of heparin. Non-covalently bound heparin was removed by washing with sodium chloride solution, which was then rinsed away with demineralized water.

The control membrane (Cup-treated) was the Cuprophan treated with a heparin solution in formamide without the addition of CDI.

The amount of heparin immobilized onto the membrane was determined using the toluidine blue assay, which was principally based on the fact that heparin can form a complex with toluidine blue. After toluidine blue solution was contacted with heparinized Cuprophan, the toluidine blue depletion of this solution was determined by measuring the extinction at 634 nm in a spectrophotometer. The amount of heparin immobilized onto the membrane was calculated from the calibration line of extinction at 634 nm against the concentration of heparin (Engbers 1990). The heparinized Cuprophan selected in this project contained 20 $\mu g/cm^2$ heparin on the surface. Measurement of the control membrane (Cup-treated) indicated that no heparin was present.

The advantage of this Cuprophan heparinization is that it avoids crosslinking of the membrane. Most of the other cellulose heparinization

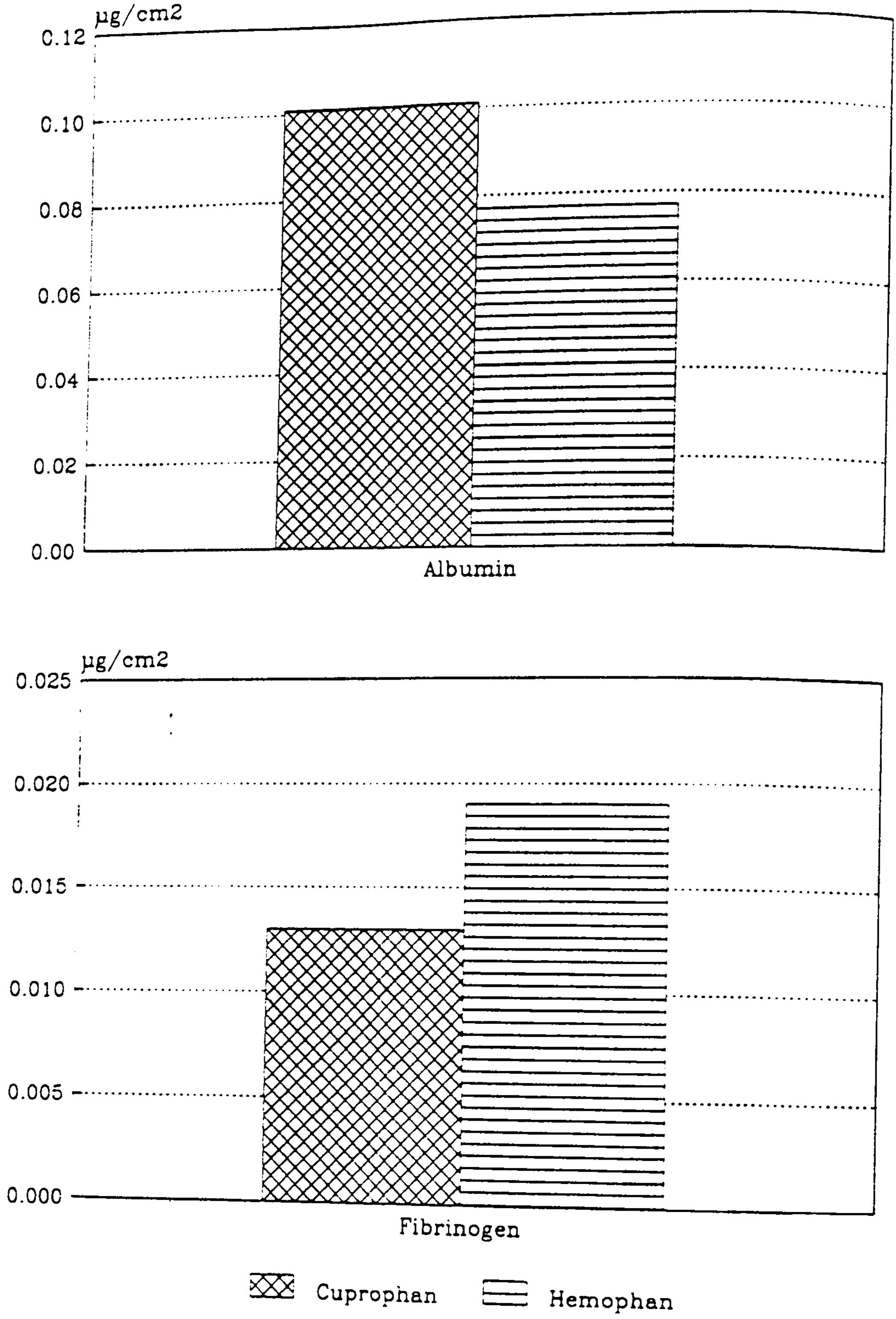


Figure 6.3 Protein adsorption on Hemophan compared with Cuprophan

procedures involve the utilisation of bi- or trifunctional reagents. These reagents are able to react with two or more hydroxyl groups on the membrane, which may induce membrane crosslinking. Therefore, the permeability of membrane may be affected. The heparinized Cuprophan studied in this project has been shown to have a similar permeability to that of untreated Cuprophan (Engbers 1990).

6.3 RESULTS AND DISCUSSION

6.3.1 DEAE-cellulose (Hemophan)

6.3.1.1 Protein Adsorption

The influence of Hemophan on albumin and fibrinogen single protein adsorption is shown in Table 6.1 and Figure 6.3, in comparison to that obtained with Cuprophan.

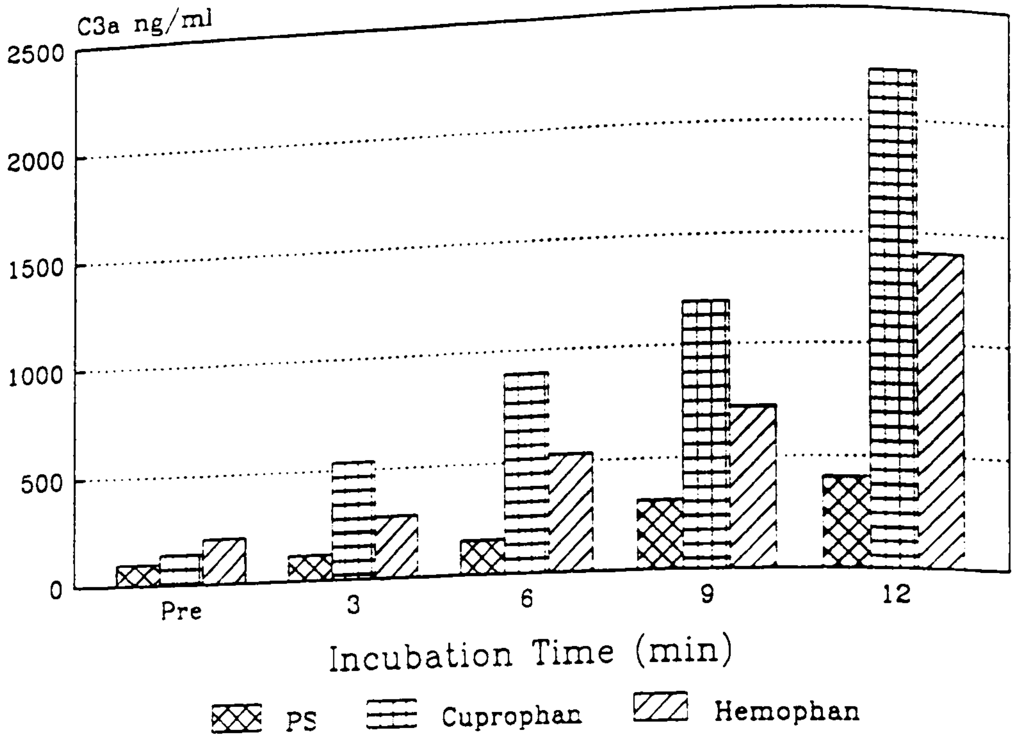
Statistical analysis of variance reveals that Hemophan induces significantly less albumin adsorption and more fibrinogen adsorption when compared with Cuprophan.

Table 6.1 Influence of Hemophan on protein adsorption compared with Cuprophan

Material	Protein Adsorption (μg/cm²) Mean ± S.D. (N=9)*	
	Albumin	Fibrinogen
Cuprophan	0.101 ± 0.004	0.013 ± 0.002
Hemophan	0.077 ± 0.005	0.019 ± 0.004

* N: Number of values

It is generally considered that surfaces with less fibrinogen adsorption or more albumin adsorption are beneficial to platelets (Brash 1991). In order to compare protein adsorption between albumin and fibrinogen, with the same method used in Chapter 5, the data of the amount of protein adsorption on



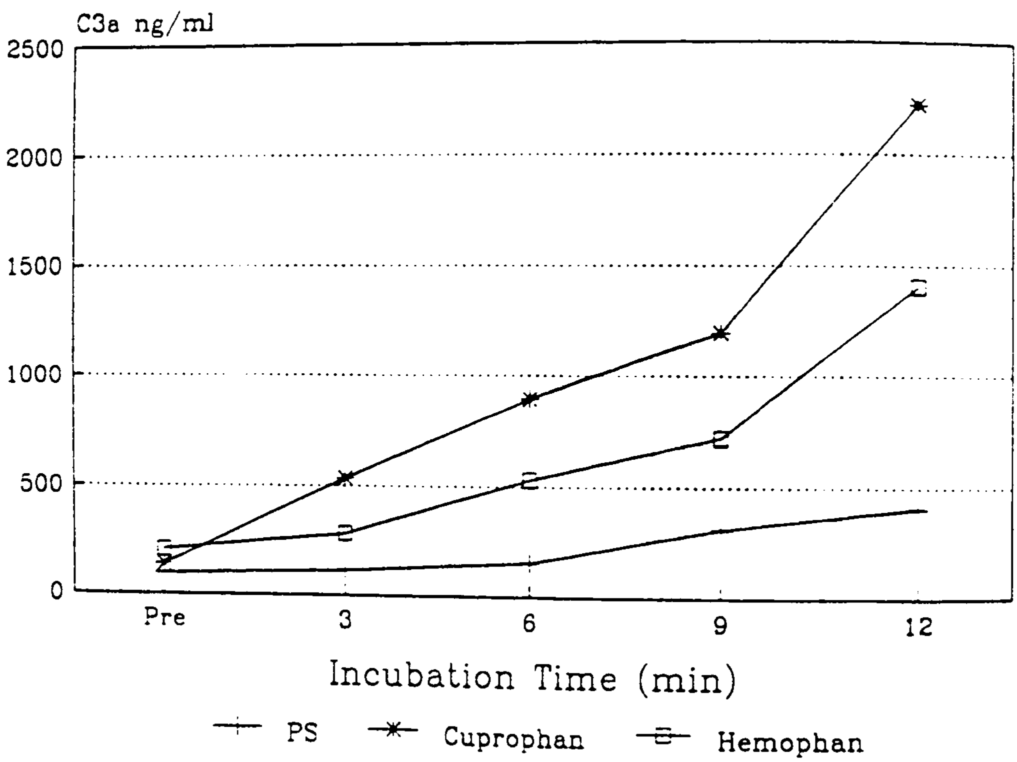


Figure 6.4 Alteration of C3a concentration levels at different time following blood contact with Hemophan compared with Cuprophan and polystyrene (PS)

Hemophan are normalised by regarding the amount of protein adsorption on Cuprophan as one hundred percent. The normalised data as well as the ratio of albumin adsorption to fibrinogen adsorption are listed in Table 6.2.

Table 6.2 Normalised data of protein adsorption on Hemophan (relevant to Cuprophan)

Material	Protein Adsorption (%)		
	Albumin	Fibrinogen	Ratio
Cuprophan	100	100	1.0
Hemophan	76	146	0.5

From Table 6.2, it can be seen that Hemophan reduces albumin adsorption by about 25% and increases fibrinogen adsorption by about 45% when compared with Cuprophan. The ratio of albumin adsorption to fibrinogen adsorption is less for Hemophan in comparison to Cuprophan. This result could mean that fibrinogen is preferentially adsorbed by Hemophan, relevant to Cuprophan. This observation is in agreement with the fact that some types of Hemophan have been reported to induce higher platelet activity than Cuprophan (Robertson 1988).

6.3.1.2 Complement Activation

The influence of Hemophan on complement activation, as represented by C3a concentration levels at different time following blood-membrane contact, are shown in Table 6.3 and Figure 6.4, compared with Cuprophan and polystyrene (PS).

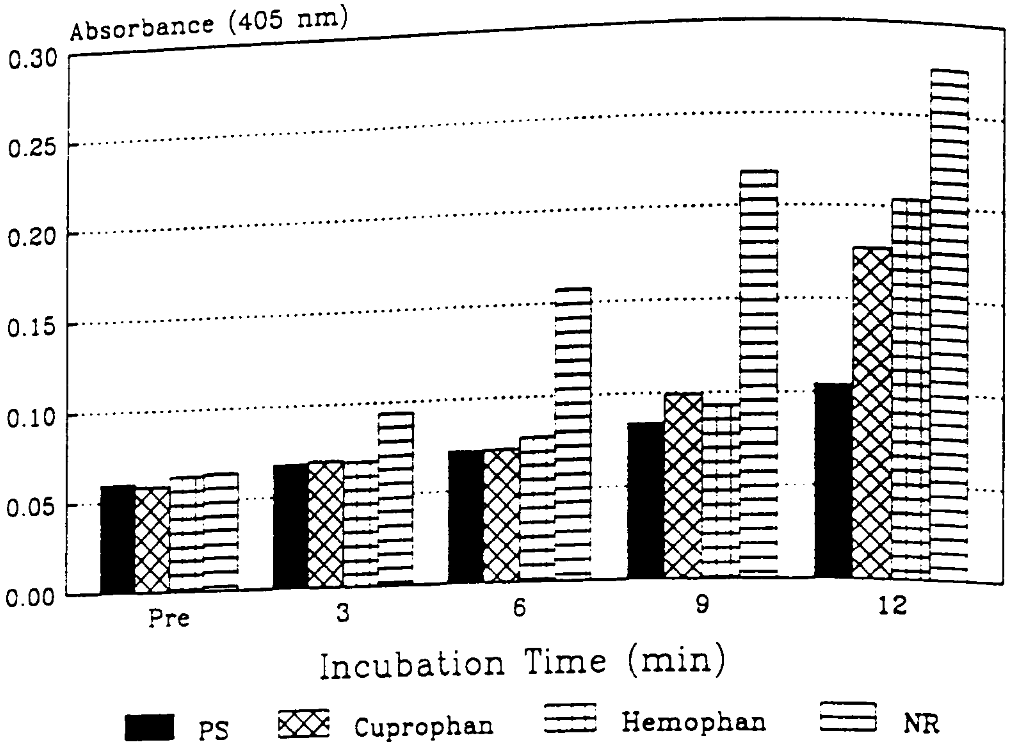
Statistical analysis reveals that C3a concentration levels are significantly increased by Hemophan after 6 min incubation, when compared with the C3a concentration level prior to blood-membrane contact. Compared with the control polymer polystyrene, Hemophan induces significantly more C3a concentration levels at any time incubation. Compared with Cuprophan, Hemophan induces significantly lower C3a concentration levels after 6 min

Table 6.3 C3a concentration levels obtained with Hemophan compared with Cuprophan and polystyrene (PS)

Material	C3a desArg Concentration Mean ± SD (ng/ml)				
	Incubation Time (min)				
	Pre	3 min	6 min	9 min	12 min
PS (n=5)	98 ± 35	115 ± 42	156 ± 64	312 ± 189	409 ± 211
Cuprophan (n=10)	139 ± 38	534 ± 155	896 ± 126	1196 ± 162	2225 ± 315
Hemophan (n=4)	208 ± 63	284 ± 61	530 ± 148	722 ± 239	1406 ± 297

incubation. These results indicate that Hemophan can induce less complement activation in comparison to Cuprophan. This observation is in agreement with the previous reports that cellulose chemical modification by partial replacement of hydroxyl groups reduces complement activation (Chapter 3).

As indicated in Chapter 3, Cuprophan inducing complement activation is initiated by covalent attachment of C3b to hydroxyl groups on the surface. Hemophan has less hydroxyl groups than Cuprophan, since hydroxyl groups are partially replaced by DEAE groups. Therefore, Hemophan induces lower complement activation than Cuprophan. The fact of Hemophan inhibiting complement activation can be alternatively explained by that of Hemophan augmenting factor H interaction. In the alternative pathway of complement, factor H is an inhibitor of C3 activation, while factor B is an activator of C3 activation, which has been fully described in Chapter 2. The surface preferentially binding factor H than factor B induces lower C3a generation. Evidence of Hemophan augmenting factor H binding has been observed (Johnson 1989).



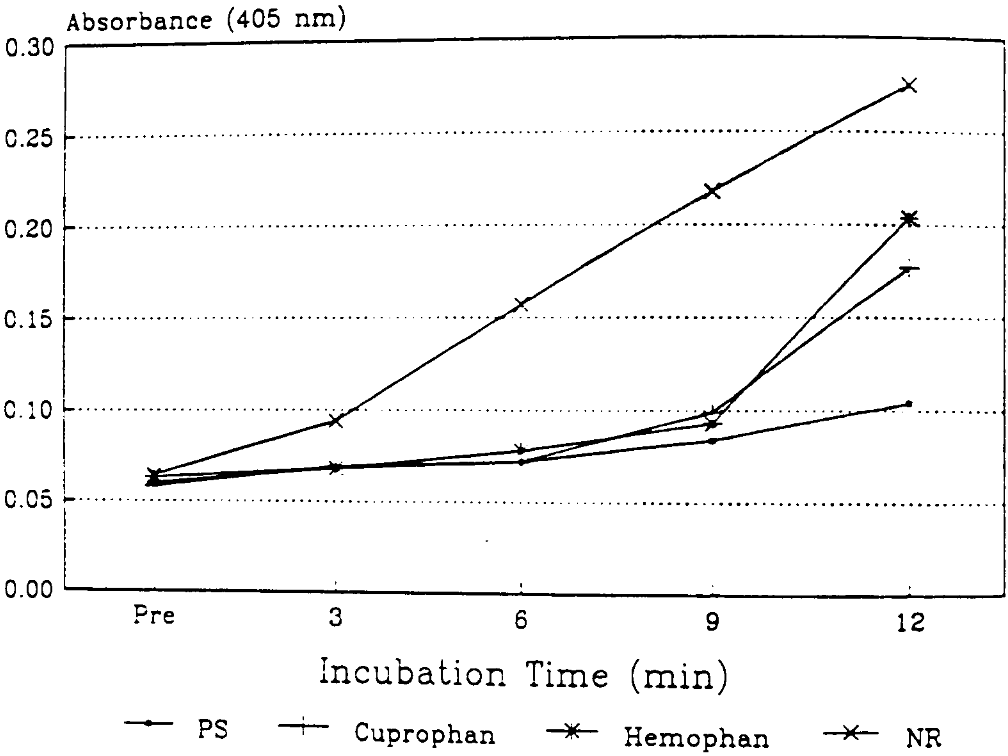


Figure 6.5 Alteration of FXII-like activity (FXIIA) levels at different time following blood contact with Hemophan compared with Cuprophan, polystyrene (PS) and polyamide (NR)

6.3.1.3 Contact Phase Activation

The influence of Hemophan on the contact phase activation, represented by the FXII-like activity (FXIIA) levels at different time following blood contact, is shown in Table 6.4 and Figure 6.5, compared with Cuprophan, polystyrene (PS) and polyamide (NR).

Statistical analysis reveals that FXIIA levels obtained with Hemophan, Cuprophan and polystyrene before 9 min incubation show no significant difference, when compared with FXIIA levels prior to blood-membrane contact. At t=12 min, all polymers induce significantly more FXIIA levels when compared with FXIIA levels prior to contact with blood. These results have a similar pattern to those obtained with PUU hydrogels. As no anticoagulant was used, this rise must be partially associated with thrombin formation (Chapter 5).

Table 6.4 FXII-like Activity (FXIIA) levels obtained with Hemophan compared with Cuprophan, polystyrene (PS) and polyamide (NR)

Material	Absorbance (405 nm) Mean ± S.D.				
	Incubation Time (min)				
	Pre	3	6	9	12
PS (n=3)	0.060 ± 0.010	0.068 ± 0.013	0.072 ± 0.008	0.084 ± 0.011	0.104 ± 0.010
Cuprophan (n=9)	0.058 ± 0.015	0.069 ± 0.021	0.072 ± 0.022	0.099 ± 0.040	0.177 ± 0.061
Hemophan (n=4)	0.063 ± 0.020	0.068 ± 0.014	0.078 ± 0.014	0.093 ± 0.011	0.204 ± 0.078
NR (n=3)	0.064 ± 0.011	0.094 ± 0.007	0.157 ± 0.010	0.218 ± 0.017	0.275 ± 0.011

FXIIA levels obtained with NR polyamide are significantly greater than all other polymers after 6 min incubation. Compared with polystyrene (PS) and

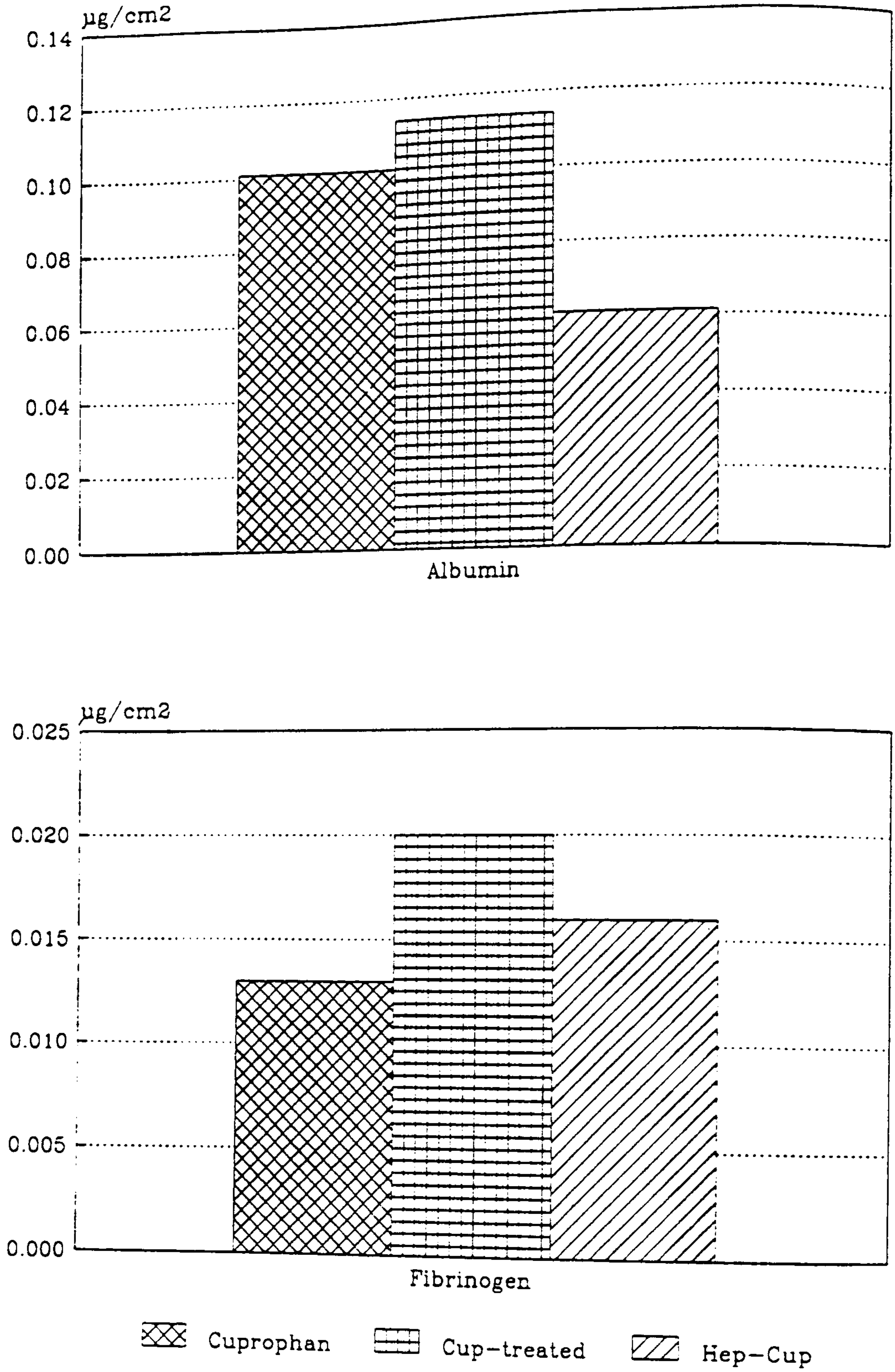


Figure 6.6 Protein adsorption influenced by Cuprophan heparinization compared with Cuprophan

Cuprophan, Hemophan induces no significant difference in FXIIA levels before 9 min incubation. At t=12 min, Hemophan induces significantly more FXIIA level than polystyrene and no significantly different than Cuprophan.

These results revealed that contact phase system is immediately activated after blood contact with a negatively charged surface. For Cuprophan and Hemophan, contact phase system is not activated to a significant degree until thrombin formation. Hemophan, while reducing complement activation, has little influence on contact phase system in comparison to Cuprophan.

6.3.2 Heparinized-Cuprophan

6.3.2.1 Protein Adsorption

The influence of Cuprophan heparinization on albumin and fibrinogen single protein adsorption is shown in Table 6.5 and Figure 6.6, in comparison to that obtained with Cuprophan.

Statistical analysis of variance reveals that the heparinized-Cuprophan (Hep-Cup) induces significantly less albumin adsorption and more fibrinogen adsorption, when compared with Cuprophan. Treated-Cuprophan significantly increases both albumin and fibrinogen adsorption, when compared with Cuprophan.

Since Cuprophan was pre-treated before the heparinization, the study of the protein adsorption influenced by heparinization should compare the heparinized surfaces with the treated-Cuprophan. Compared with treated-Cuprophan, heparinized-Cuprophan significantly reduces both albumin and fibrinogen adsorption. These results indicate that protein adsorption is influenced not only by the heparin immobilised on membrane but also by the modification procedure. This observation is agreement with a previous observation (Sanada et al 1986), in which protein adsorption (albumin, fibrinogen and gamma-globulin) was found to be less on heparinized polymer (polyaminoetherurethane) than the pre-treated-polymer, and more on the pre-treated-polymer than the untreated polymer.

Table 6.5 Influence of Cuprophan heparinization on protein adsorption compared with Cuprophan

Material	Protein Adsorption ($\mu g/cm^2$) Mean ± S.D. (N=9)*	
	Albumin	Fibrinogen
Cuprophan	0.101 ± 0.004	0.013 ± 0.002
Cup-treated	0.114 ± 0.003	0.020 ± 0.003
Hep-Cup	0.061 ± 0.010	0.016 ± 0.001

* N: Number of values

In order to compare protein adsorption between albumin and fibrinogen, with the same method previously used, the data of the amount of protein adsorption on heparinized-Cuprophan and treated-Cuprophan are normalised by regarding the amount of protein adsorption on Cuprophan as one hundred percent. The normalised data as well as the ratio of albumin adsorption to fibrinogen adsorption are listed in Table 6.6.

Table 6.6 Normalised data of protein adsorption on heparinized-Cuprophan and treated-Cuprophan (relevant to Cuprophan)

Material	Protein Adsorption (%)		
	Albumin	Fibrinogen	Ratio
Cuprophan	100	100	1.0
Cup-treated	113	154	0.7
Hep-Cup	60	131	0.5

From Table 6.6, it can be seen that heparinized-Cuprophan reduces albumin adsorption by about 40% and increases fibrinogen adsorption by about 30% when compared with untreated-Cuprophan. Treated-Cuprophan increases albumin adsorption by about 13% and fibrinogen adsorption by about 55%

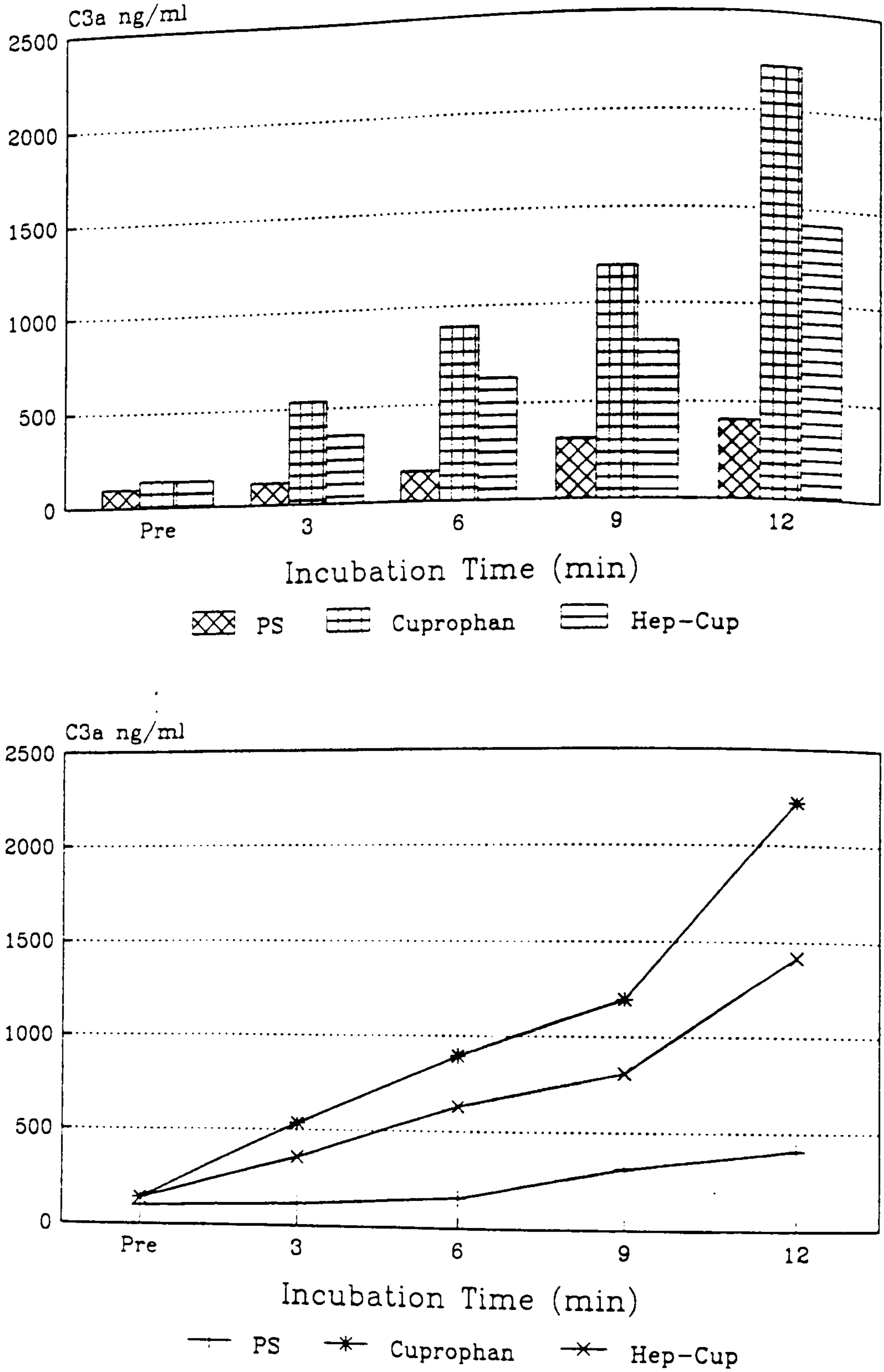


Figure 6.7 Alteration of C3a concentration levels at different time following blood contact with heparinized-Cuprophan compared with Cuprophan and polystyrene (PS)

when compared with Cuprophan. The ratio of albumin adsorption to fibrinogen adsorption is less for heparinized-Cuprophan in comparison to Cuprophan. This result could mean that fibrinogen is preferentially adsorbed by heparinized Cuprophan, relevant to Cuprophan. Since platelet reactivity influenced by the heparinized-Cuprophan has not been reported, the significance of the protein adsorption on the heparinized- Cuprophan studied is uncertain. It has been known that heparin in blood can induce platelet release and aggregation, since thrombocytopenia complications may occur in patients receiving heparin therapy (Blockmans et al 1986; Godal 1989). However, investigations on the effect of immobilized heparin on platelets are controversial. Suppressed (Lagergren et al 1974; Larsson et al 1979; Larm et al 1983; Han et al 1989) as well as enhanced (Lindon et al 1978; Plate and Valuev 1983; Llanos and Sefton 1992) platelet deposition on heparinized surfaces has been measured. It should be indicated that differential protein adsorption of albumin and fibrinogen adsorption is only one of factors influencing platelet reaction. Another important factor influencing platelet reaction by heparinized material to be considered is thrombin, since thrombin is a strong activator of platelet reaction. Thrombin formation inhibited by polymer heparinization has been observed (Goosen and Sefton 1982; Hennink et al 1984; Pasche et al 1986; Arnander et al 1986). The heparinized-Cuprophan, studied in this project, has shown to increase blood recalcification times when compared with Cuprophan (Engbers 1990).

6.3.1.2 Complement Activation

The influence of heparinized-Cuprophan on complement activation, as represented by C3a concentration levels at different time intervals following blood-membrane contact, is shown in Table 6.7 and Figure 6.7, compared with Cuprophan and polystyrene (PS).

Statistical analysis reveals that C3a concentration levels are significantly increased by heparinized-Cuprophan after 6 min incubation, when compared with the C3a concentration level prior to blood-membrane contact. Compared

Table 6.7 C3a concentration levels obtained with heparinized-Cuprophan compared with Cuprophan and polystyrene (PS)

Material	C3a desArg Concentration Mean ± SD (ng/ml)				
	Incubation Time (min)				
	Pre	3 min	6 min	9 min	12 min
PS (n=5)	98 ± 35	115 ± 42	156 ± 64	312 ± 189	409 ± 211
Cuprophan (n=10)	139 ± 38	534 ± 155	896 ± 126	1196 ± 162	2225 ± 315
Hep-Cup * (n=3)	137 ± 12	356 ± 99	630 ± 162	810 ± 95	1417 ± 80

* Hep-Cup: Heparinized-Cuprophan

with control polymer polystyrene, heparinized-Cuprophan induces significantly more C3a concentration levels at any time incubation. Compared with Cuprophan, heparinized-Cuprophan induces significantly lower C3a concentration levels after 6 min incubation. These results indicate that heparinized-Cuprophan can induce less complement activation in comparison to Cuprophan. This observation is in agreement with the previous report that the heparin-coated surface for extracorporeal circulation can induce less complement action than non-coated surfaces (Mottaghy et al 1991).

The reduction of complement activation by Heparinized-Cuprophan can not be explained by less activation sites of hydroxyl groups due to heparin contains hydroxyl groups. Since heparin is a negatively charged polysaccharide, the heparinization of Cuprophan produces a membrane with negative charge. Reduction of complement activation by the heparinized-Cuprophan may be explained by the adsorption of cationic anaphylatoxin C3a through ionic interaction (Johnson et al 1990). An alternative explanation for the reduction of complement activation by the heparinized-Cuprophan may arise from an increase in factor H binding on the heparinized-Cuprophan. The evidence of

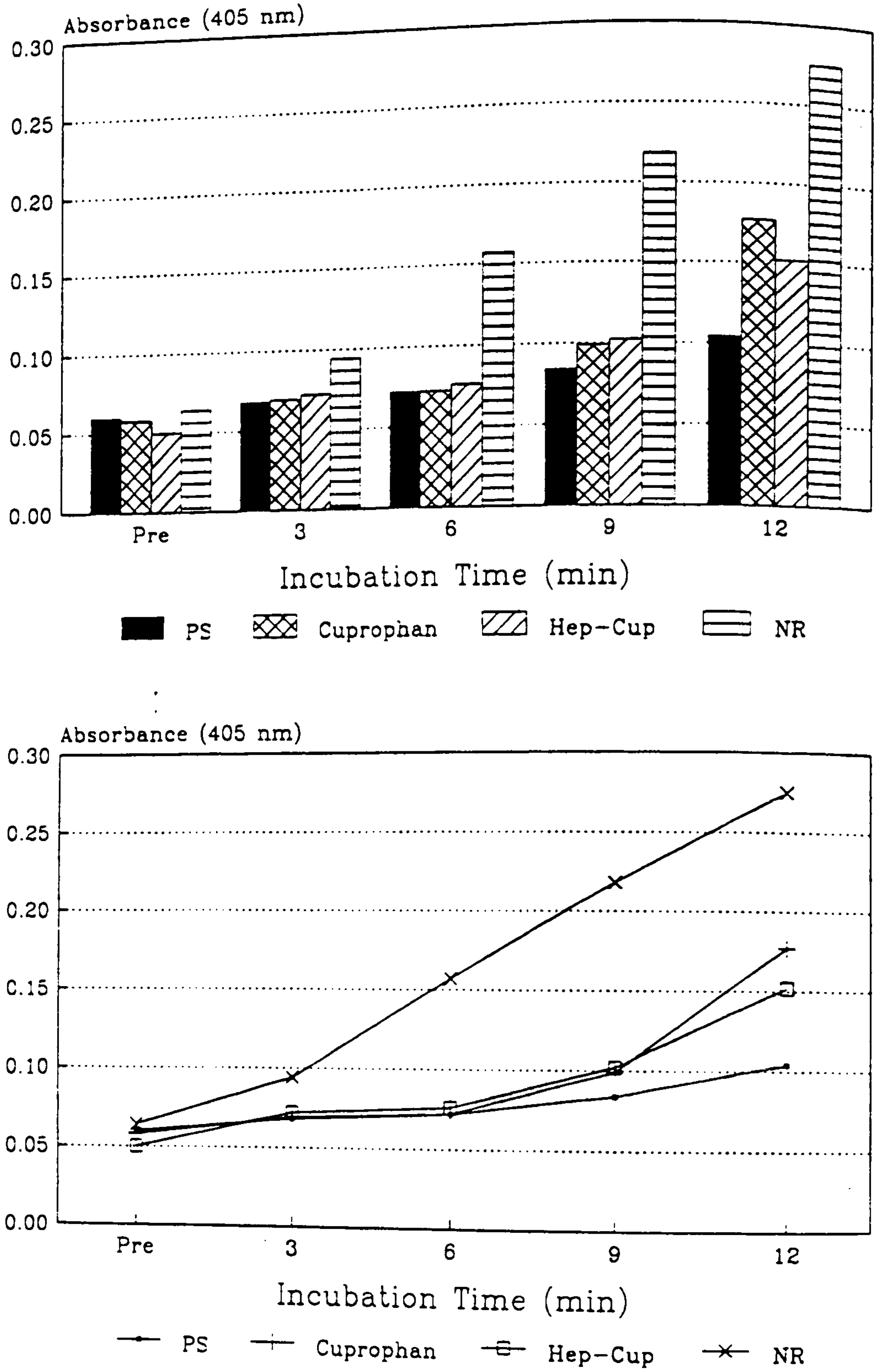


Figure 6.8 Alteration of FXII-like activity (FXIIA) levels at different time following blood contact with heparinized-Cuprophan compared with Cuprophan, polystyrene (PS) and polyamide (NR)

negatively charged surfaces, such as heparinized-zymosan and heparinized-Sepharose (Kazatchkine et al 1979), carboxymethyl-Sephadex (Carreno et al 1989), Cuprophan modified with maleic anhydride (Johnson et al 1990), adsorbing cationic C3a anaphylatoxin and augmenting factor H binding has been reported.

6.3.2.3 Contact Phase Activation

The influence of heparinized-Cuprophan on the contact phase activation, as represented by the FXII-like activity (FXIIA) levels at different time following blood-membrane contact, is shown in Table 6.8 and Figure 6.8, compared with Cuprophan, polystyrene (PS) and polyamide (NR).

Statistical analysis reveals that FXIIA levels obtained with heparinized-Cuprophan before 9 min incubation show no significant difference, when compared with FXIIA level prior to blood-membrane contact. At t=12 min, heparinized-Cuprophan induces significantly higher FXIIA level when compared with FXIIA level prior to contact with blood. These results have similar pattern to these obtained with PUU hydrogels, Cuprophan, Hemophan.

FXIIA levels obtained with NR polyamide are significantly greater ($P<0.05$) than all other polymers after 6 min incubation. Compared with polystyrene (PS) and Cuprophan, heparinized-Cuprophan induces no significant ($P>0.05$) difference in FXIIA levels at any incubation time. These results revealed that heparinized-Cuprophan, while reducing complement activation, has little influence on contact phase system in comparison to Cuprophan.

Table 6.4 FXII-like Activity (FXIIA) obtained with heparinized-Cuprophan compared with Cuprophan, polystyrene (PS) and polyamide (NR)

Material	Absorbance (405 nm) Mean ± S.D.				
	Incubation Time (min)				
	Pre	3	6	9	12
PS (n=3)	0.060 ± 0.010	0.068 ± 0.013	0.072 ± 0.008	0.084 ± 0.011	0.104 ± 0.010
Cuprophan (n=9)	0.058 ± 0.015	0.069 ± 0.021	0.072 ± 0.022	0.099 ± 0.040	0.177 ± 0.061
Hep-Cup * (n=3)	0.050 ± 0.011	0.072 ± 0.020	0.076 ± 0.022	0.102 ± 0.060	0.152 ± 0.079
NR (n=3)	0.064 ± 0.011	0.094 ± 0.007	0.157 ± 0.010	0.218 ± 0.017	0.275 ± 0.011

* Hep-Cup: heparinized-Cuprophan

6.4 SUMMARY

The investigation of the influence of cellulose chemical modification (Hemophan) and Cuprophan heparinization on protein adsorption reveals that Hemophan and heparinized-Cuprophan increase fibrinogen adsorption and reduce albumin adsorption, when compared with Cuprophan. And protein adsorption on the heparinized-Cuprophan can be influenced by theheparinization procedure. The investigation of the influence on complement activation and contact phase activation reveals that Cuprophan heparinization and chemical modification can greatly reduce complement activation but has little influence on contact phase activation.

It is generally considered that the increase in fibrinogen adsorption and the decrease in albumin adsorption can increase platelet reactivity (Brash 1991). Therefore, the blood compatibility improvement of Cuprophan with respect to complement activation, achieved by hydroxyl substitution, may be offset by the increase of platelet reactivity. As the previous indication (Courtney et al 1993a), polymer surface modification involves a balance between achieving better performance in one parameter and altering the performance of other parameters.

CHAPTER 7

BIOMEMBRANE-MIMETIC POLYMERS

7.1 INTRODUCTION

The introduction of phosphorylcholine to a polymer represents a novel approach towards the development of biomaterials with improved blood compatibility (Chapter 3). To examine the influence of phosphorylcholine groups on the blood compatibility improvement, two approaches were studied:

(a) copolymer of butyl methacrylate and 2-methacryloyloxyethyl phosphorylcholine (MPC), poly(BMA-co-MPC), was compared with poly(butyl methacrylate) (PBMA)

(b) MPC grafted Cuprophan was compared with Cuprophan

The influence of phosphorylcholine groups on protein adsorption (albumin or fibrinogen), complement activation as represented by the measurement of C3a concentration levels and contact phase activation as represented by the measurement of FXII-like activity (FXIIA) levels were studied, using the methods previously described (Chapter 4).

The results from these studies are presented in this chapter, which has been divided into following sections:

(a) Section 7.2, Materials

(b) Section 7.3, Results and discussion

- 7.3.1 Copolymer of poly(BMA-co-MPC)
  - 7.3.1.1 protein adsorption
  - 7.3.1.2 complement activation
  - 7.3.1.3 contact phase activation
- 7.3.2 MPC grafted Cuprophan
  - 7.3.2.1 protein adsorption
  - 7.3.2.2 complement activation
  - 7.3.2.3 contact phase activation

(c) Section 7.4, Summary

7.2 MATERIALS

The polymers containing phosphorylcholine groups were supplied by Professor N. Nakabayashi and Dr. K. Ishihara, Tokyo Medical and Dental University, Japan, under a collaborative programme.

7.2.1 Preparation of MPC Compound

The synthetic route and structure of MPC (compound 4) are shown in Figure 7.1. 2-chloro-2-oxo-1,3,2-dioxaphospholane (COP) (compound 1) was synthesized by the condensation reaction between glycol and PCl_3 and then oxidation. COP was condensed with hydroxyethyl methacrylate (HEMA) (compound 2) in tetrahydrofuran (THF) solvent to obtain 2-(2-oxo-1,3,2-dioxaphospholoyloxy)ethyl methacrylate (OPEMA) (compound 3). OPEMA was converted to MPC by opening the ester moiety with trimethylamine in acetonitrile solvent. ^{1}H NMR and ^{13}C NMR spectra confirmed the structure of MPC (Ishihara et al 1990).

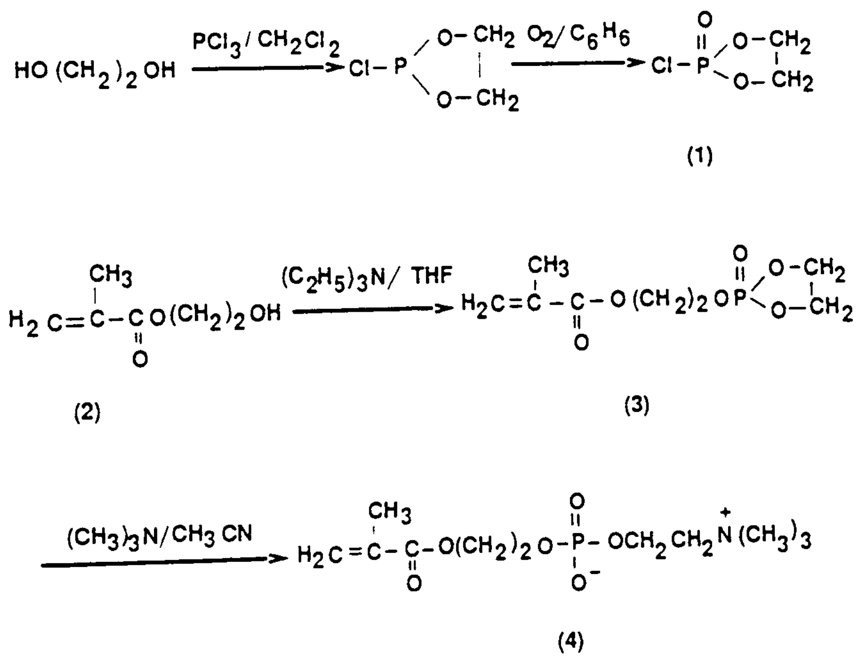


Figure 7.1 Synthetic route and structure of MPC (compound 4)

7.2.2 Preparation of Poly(BMA-co-MPC)

Poly(BMA-co-MPC) was copolymerized from the monomers BMA and MPC in ethanol at 60^{0}C using 2,2'-azoisobutyronitrile as the initiator. The structure of the poly(BMA-co-MPC) is shown in Figure 7.2, which was confirmed by IR and ^{1}H NMR spectra (Ishihara et al 1990).

Poly(BMA-co-MPC) membrane was prepared by a solvent evaporation method from a tetrahydrofuran solution on a polyethylene plate. The MPC composition on the surface was determined with an X-ray photoelectron spectroscope (XPS) and was calculated from the average ratio of phosphorus atoms against carbon atoms (P/C) (Ishihara 1990). The poly(BMA) and poly(BMA-co-MPC) membranes with 0.20 mole fraction and 0.30 mole fraction of MPC were studied in this project.

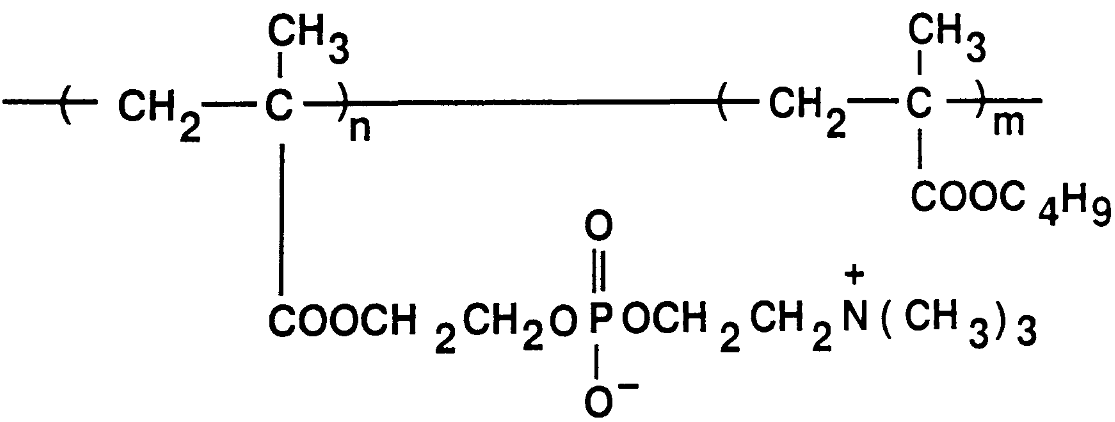


Figure 7.2 Chemical structure of copolymer of butyl methacrylate with MPC [poly (BMA–co–MPC)]

7.2.3 Preparation of MPC grafted Cuprophan

The standard haemodialysis membrane, Cuprophan 150 PM (Akzo Germany), was used to graft MPC. MPC grafting on Cuprophan was initiated by cerium ammonium nitrate (Ce^{4+}). Ce^{4+} ions attacked the oxygen atoms to form radicals on them, then grafting reaction was initiated by the radicals, since MPC is vinyl monomer. Figure 7.3 illustrates the mechanism for the formation of radicals which can initiate the graft polymerization of the vinyl monomer on to Cuprophan by cerium ions.

The MPC grafted on Cuprophan membrane was confirmed by XPS and (FT-IR) (Fourier transform infrared) spectroscopy (Ishihara and Nakabayashi 1992). The MPC composition on the Cuprophan surface was calculated from the average ratio of phosphorus atoms against carbon atoms (P/C). Cuprophan grafted MPC with 0.20 mole fraction and 0.30 mole fraction were studied in this project.

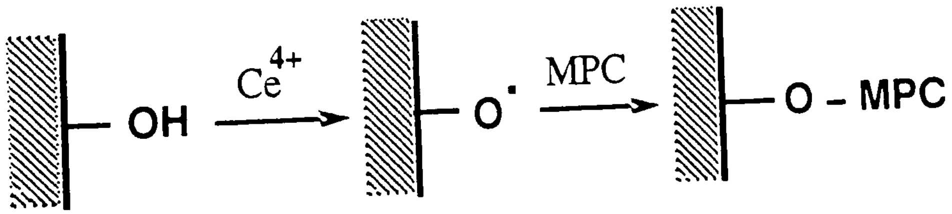


Figure 7.3 Cuprophan grafted with MPC (Cuprophan–MPC)

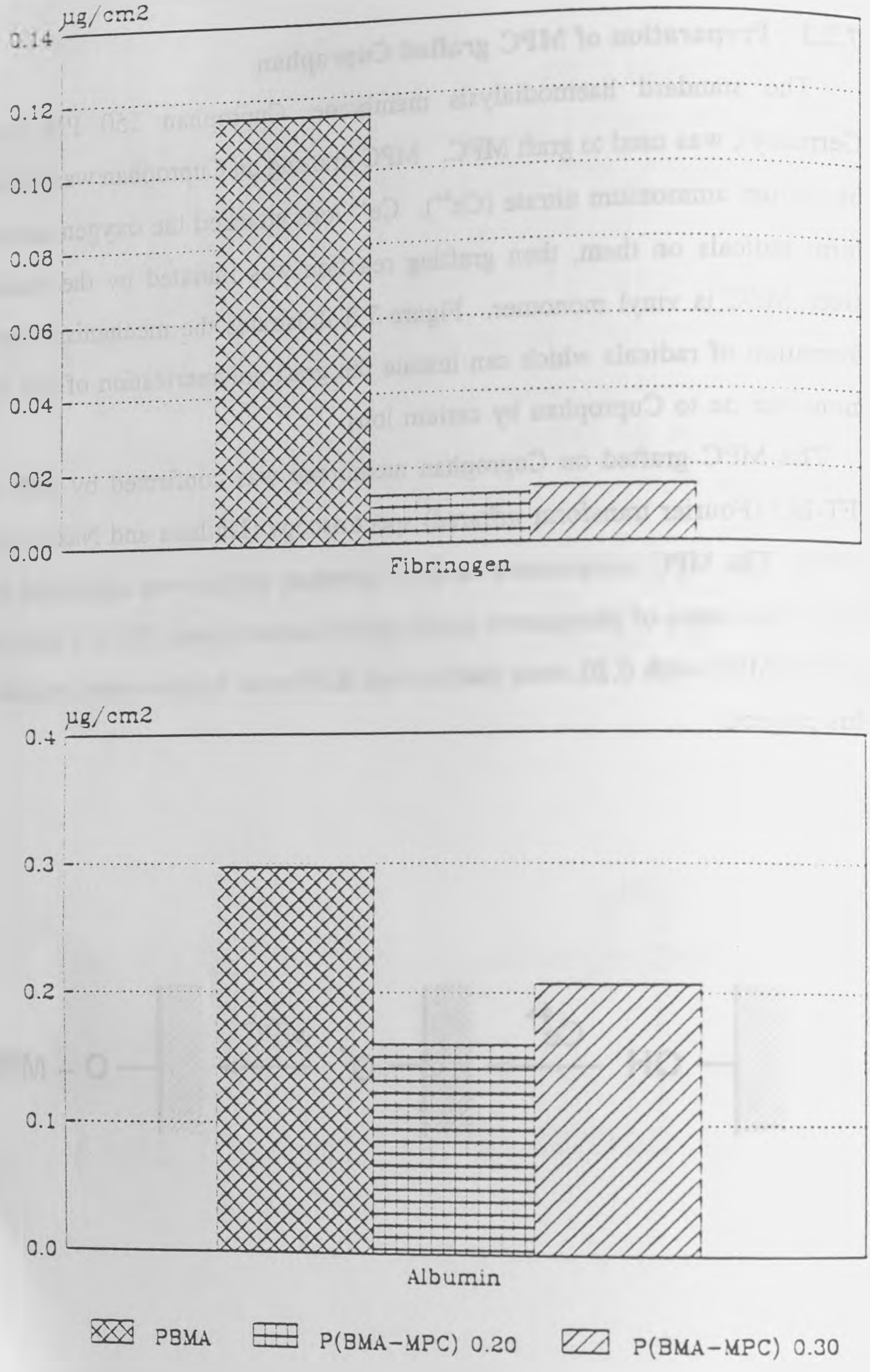


Figure 7.4 Protein adsorption on PBMA and poly(BMA-co-MPC) polymers

7.4 RESULTS AND DISCUSSION

7.4.1 Copolymer poly(BMA-co-MPC)

7.4.1.1 Protein Adsorption

The influence of copolymer poly(BMA-co-MPC) on fibrinogen and albumin single protein adsorption is shown in Table 7.1 and Figure 7.4, compared with PBMA.

Statistical analysis reveals that poly(BMA-co-MPC) copolymers significantly reduce both albumin and fibrinogen adsorption when compared with PBMA. Compared with poly(BMA-co-MPC) with 0.30 mole fraction of MPC, poly(BMA-co-MPC) with 0.20 mole fraction of MPC induces significantly lower albumin and fibrinogen adsorption. Increasing the concentration of fibrinogen solution, the difference in fibrinogen adsorption between poly(BMA-co-MPC) copolymers is clearly seen (Table 7.2 and Figure 7.5).

Table 7.1 Protein adsorption on poly(BMA-co-MPC) copolymers compared with PBMA

Polymer	Protein Adsorption (μg/cm^2) Mean ± S.D. (N=6) *	
	Albumin	Fibrinogen
PBMA	0.297 ± 0.001	0.115 ± 0.011
P(BMA-MPC)* 0.20	0.160 ± 0.002	0.013 ± 0.001
P(BMA-MPC) 0.30	0.207 ± 0.004	0.015 ± 0.001

N: Number of values
P(BMA-MPC): poly(BMA-co-MPC)

The observation of poly(BMA-co-MPC) copolymers markedly reducing protein adsorption is in agreement with the previous indication that phosphorylcholine-contained polymer can reduce protein adsorption (Ishihara et al 1991; 1992; Chapman and Charles 1992). The reduction of protein adsorption by phosphorylcholine-contained polymer may be associated to the

Table 7.2 Fibrinogen adsorption on PBMA and poly(BMA-co-MPC) copolymers with increasing fibrinogen solution concentration (µg/cm^2) Mean ± S.D. (N=6) *

Polymer	Fibrinogen Solution Concentration (µg/ml)	
	1.6	3.2
PBMA	0.115 ± 0.011	0.146 ± 0.004
P(BMA-MPC)* 0.20	0.013 ± 0.001	0.027 ± 0.003
P(BMA-MPC) 0.30	0.015 ± 0.001	0.041 ± 0.006

* N: Number of values
P(BMA-MPC): poly(BMA-co-MPC)

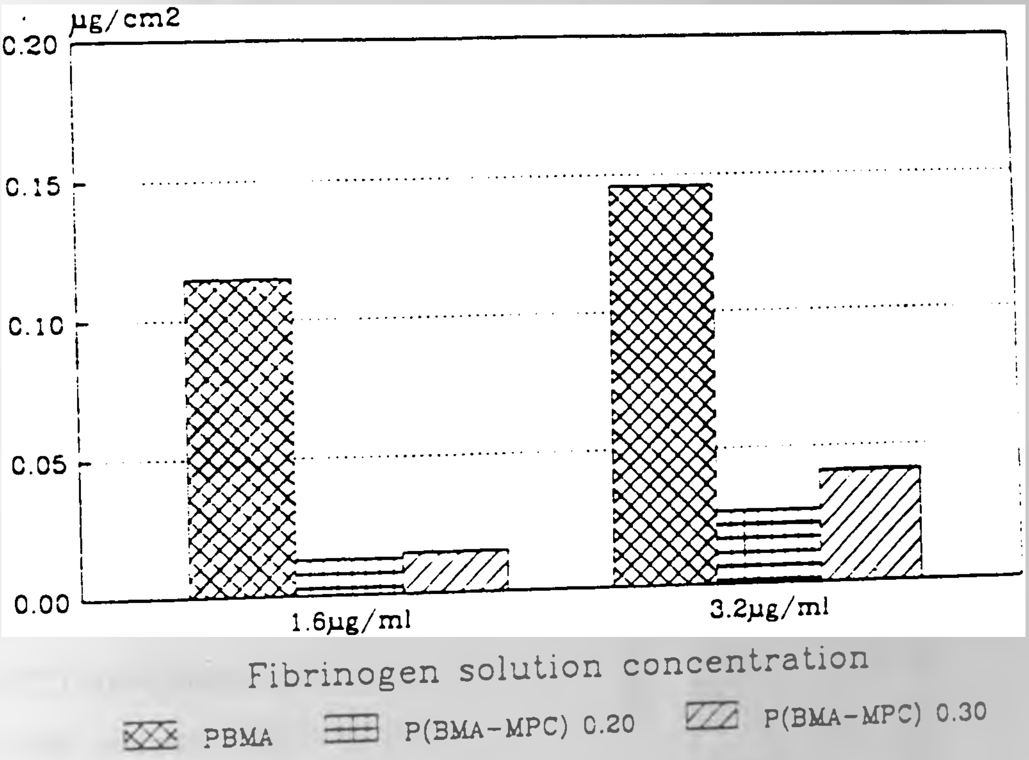


Figure 7.5 Fibrinogen adsorption on PBMA and poly(BMA-co-MPC) polymers with increasing fibrinogen solution concentration

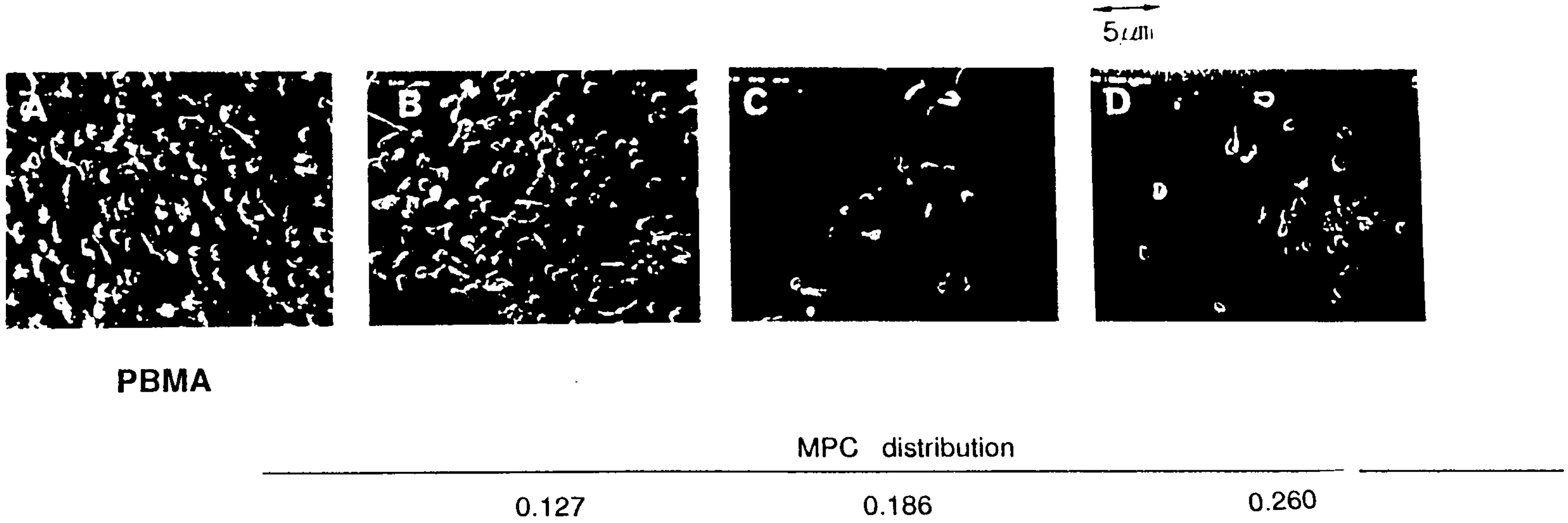


Figure 7.6 SEM picture of poly(BMA-co-MPC) coated beads after contacting citrated platelet-rich plasma for 20 min.

zwitterionic (positive/negative) and hydrophilic properties of phosphorylcholine. The observation of the poly(BMA-co-MPC) with more MPC content inducing more protein adsorption is contrary to the previous indication (Ishihara et al 1991).

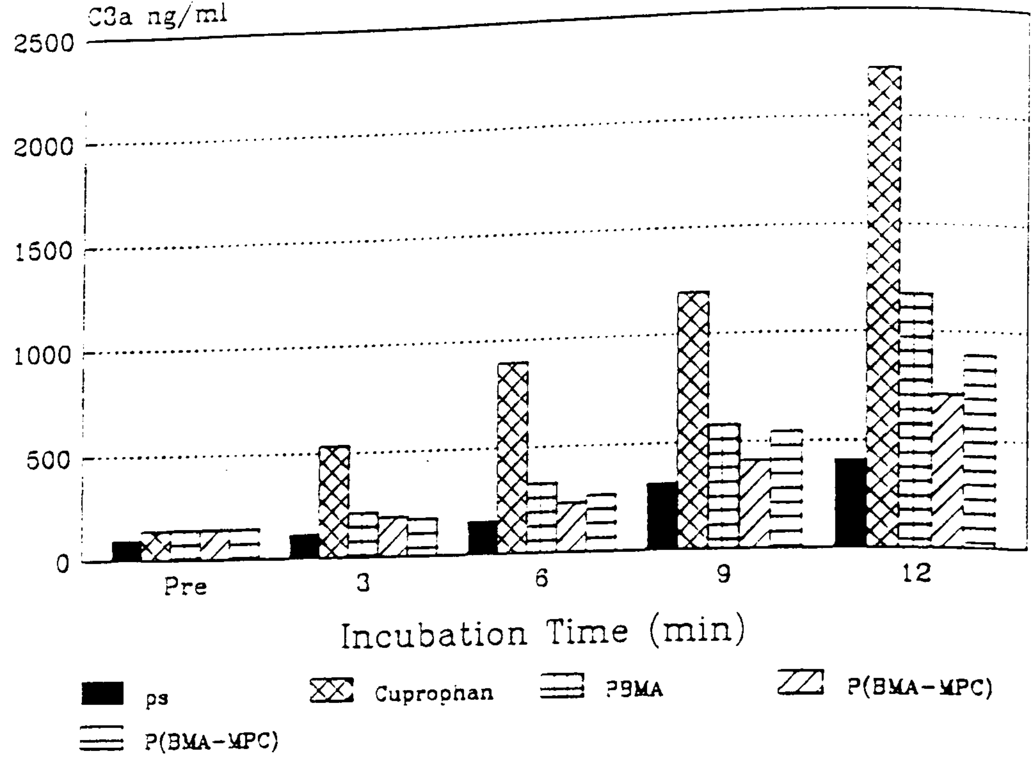
In order to compare protein adsorption between fibrinogen and albumin, the data of the amount of protein adsorption on polymers are normalised by regarding the amount of protein adsorption on PBMA as one hundred percent. The normalised data as well as the ratio of albumin adsorption to fibrinogen adsorption are listed in Table 7.3. Relevant to the PBMA, a higher ratio of albumin adsorption to fibrinogen adsorption means that albumin is preferentially adsorbed on the polymer surface.

Table 7.3 Normalised data of protein adsorption on poly(BMA-co-MPC) copolymers (relevant to PBMA)

Polymer	Protein adsorption on polymer surface (%)		
	Albumin	Fibrinogen	Ratio
PBMA	100	100	1.0
P(BMA-MPC)* 0.20	54	11	4.9
P(BMA-MPC) 0.30	70	13	5.4

*: P(BMA-MPC): poly(BMA-co-MPC)

From Table 7.3, the marked reduction in protein adsorption by poly(BMA-co-MPC) copolymers can be clearly seen. Compared with PBMA, higher ratio of albumin adsorption to fibrinogen adsorption is achieved with poly(BMA-co-MPC) copolymers and the ratio increases with increasing phosphorylcholine moiety content. This could mean albumin in comparison to fibrinogen is preferentially adsorbed on poly(BMA-co-MPC) copolymers even the adsorption of both proteins is reduced. This observation of the poly(BMA-co-MPC) copolymers preferentially adsorbing albumin than fibrinogen is in agreement with the evidence on the suppression of platelet adhesion with increasing MPC composition (Ishihara et al 1990; Xi et al 1992). Figure 7.6 shows SEM



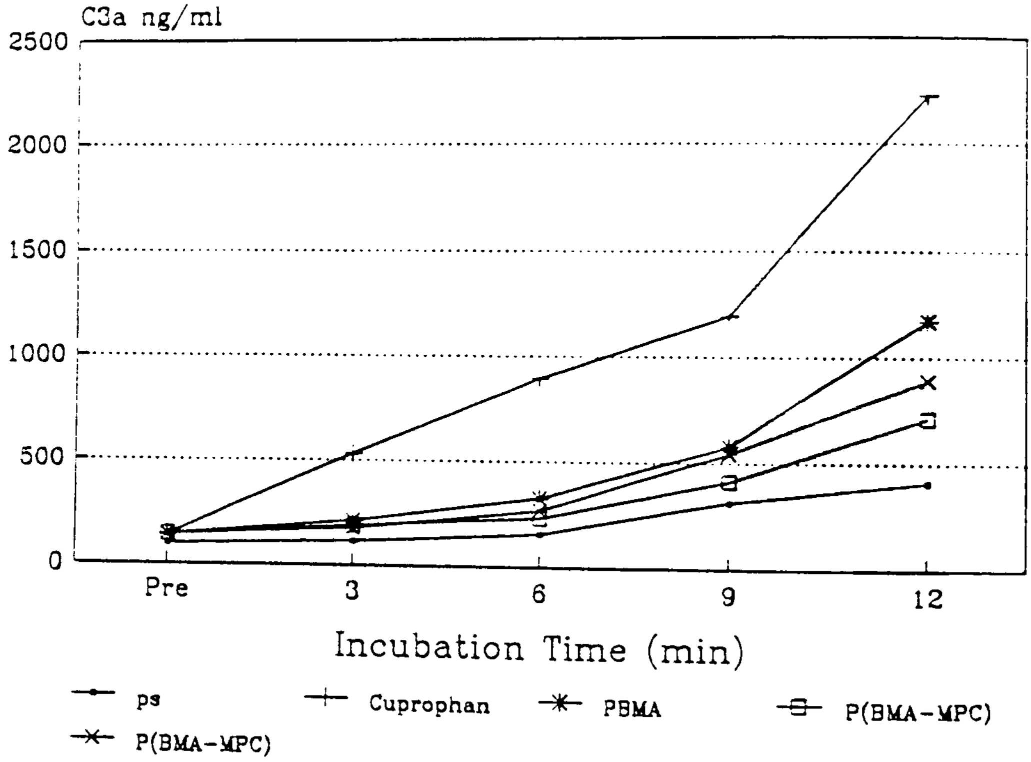


Figure 7.7 C3a concentration levels obtained with poly(BMA-co-MPC) copolymers compared with polystyrene (PS), PBMA and Cuprophan

pictures of platelets adhering to beads coated with poly(BMA-co-MPC) after 20 min of contact with citrated PRP (Ishihara et al 1990).

7.4.1.2 Complement Activation

The influence of poly(BMA-co-MPC) copolymers on complement activation, represented by C3a concentration levels at different time following blood contact, is shown in Table 7.4 and Figure 7.7, compared with PBMA , polystyrene (PS) and Cuprophan.

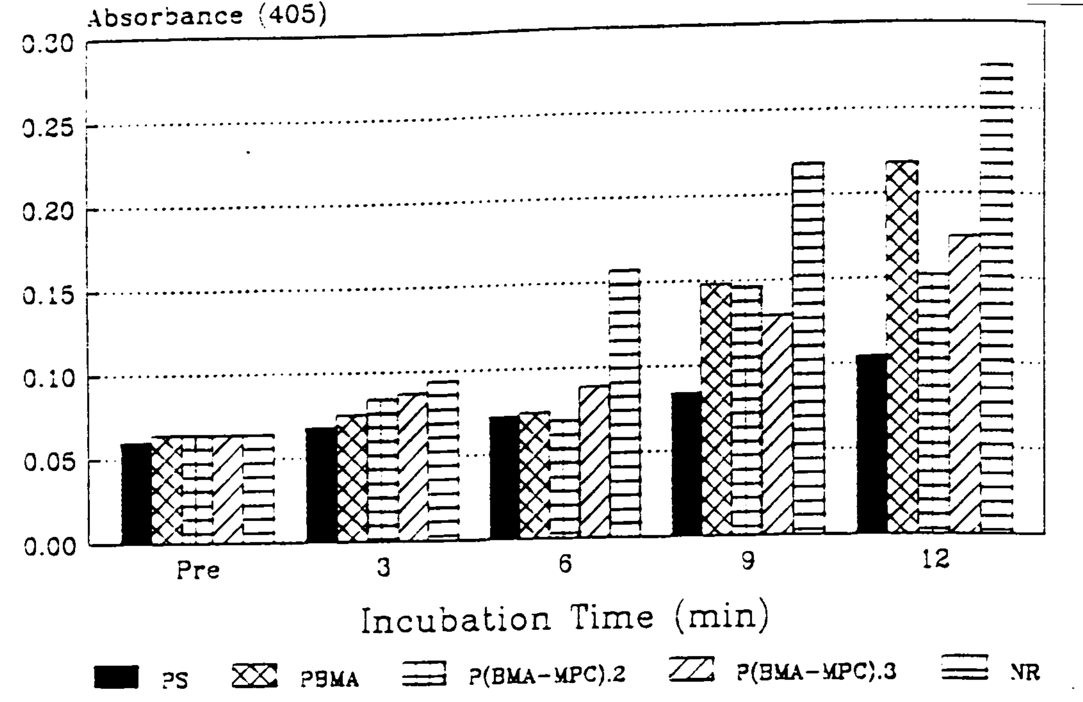
Statistical analysis reveals that poly(BMA-co-MPC) copolymers induce significantly more C3a concentration levels at the end of incubation time when compared with the C3a concentration levels prior to blood-polymer contact, and PBMA induces significantly more C3a concentration levels after 6 min incubation time when compared with the C3a concentration level prior to blood-polymer contact. Compared with control polymer polystyrene, poly(BMA-co-MPC) copolymers do not induce significantly ($P<0.05$) more C3a concentration levels until at t=12 min. Compared with Cuprophan, poly(BMA-co-MPC) copolymers induce significantly less C3a concentration levels at any time incubation. Less complement activation achieved by poly(BMA-co-MPC) copolymers in comparison to Cuprophan is associated with the fact that no hydroxyl groups exist on poly(BMA-co-MPC) copolymers.

Compared with PBMA, poly(BMA-co-MPC) copolymers induce less C3a concentration levels but not significantly different before 9 min incubation. At t=12 min, poly(BMA-co-MPC) with 0.20 mole fraction induces significantly less C3a concentration levels when compared with PBMA, and poly(BMA-co-MPC) with 0.30 mole fraction does not induce significantly less C3a concentration levels when compared with PBMA. These results indicate that poly(BMA-co-MPC) with less MPC content induces less C3a concentration levels, even the C3a concentration levels are not significantly different between the two poly(BMA-co-MPC) copolymers, which may result from insufficient donors. The observation of poly(BMA-co-MPC) copolymers inducing lower

Table 7.4 C3a Concentration levels obtained with poly(BMA-co-MPC) compared with PBMA, polystyrene (PS) and Cuprophan

Material	C3a DesArg Concentration (ng/ml) Mean ± S.D.				
	Incubation Time (min)				
	Pre	3	6	9	12
PS (n=5)	98 ±35	115 ± 42	156 ± 64	312 ±189	409 ±211
Cuprophan (n=10)	139 ±38	534 ±155	896 ±126	1196 ±162	2225 ±315
PBMA (n=3)	142 ±58	211 ±38	325 ±112	578 ±96	1174 ±129
Poly(BMA-co-MPC) 0.20 (n=3)	142 ±58	188 ±39	230 ±75	411 ±199	713 ±183
Poly(BMA-co-MPC) 0.30 (n=3)	142 ±58	175 ±28	266 ±66	544 ±170	892 ±175

complement activation than PBMA may be associated with less protein adsorption achieved with the poly(BMA-co-MPC) copolymers. The evidence of poly(BMA-co-MPC) copolymers adsorbing less complement protein (C5) than that of PBMA has been reported (Ishihara et al 1991). The observation of poly(BMA-co-MPC) with less MPC content inducing less complement activation corresponds the results of protein adsoption (Section 7.4.1.1). The reason for this observation is uncertain.



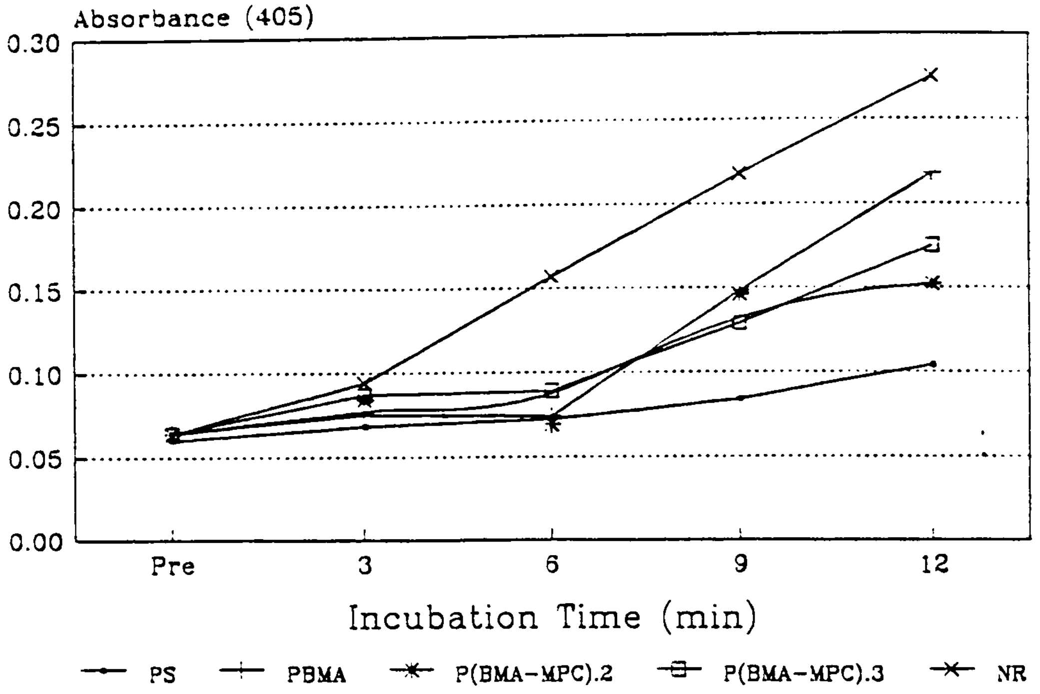


Figure 7.8 FXII-like activity levels obtained with poly(BMA-co-MPC) copolymers compared with polystyrene (PS), PBMA and polyamide (NR)

7.3.1.3 Contact Phase Activation

The influence of poly(BMA-co-MPC) copolymers on contact phase activation, represented by the measurement of FXII-like activity (FXIIA) levels following blood-polymer contact, is shown in Table 7.7 and Figure 7.8, compared with PBMA, polystyrene (PS) and polyamide (NR).

Statistical analysis reveals that poly(BMA-co-MPC) copolymers and PBMA induce significantly more FXIIA levels after 9 min incubation time when compared with FXIIA levels prior to blood-polymer contact. Compared with polystyrene, poly(BMA-co-MPC) copolymers and PBMA induce significantly more FXIIA levels after 9 min incubation time. Compared with polyamide, poly(BMA-co-MPC) copolymers and PBMA induce significantly less FXIIA levels after 6 min incubation time. Between poly(BMA-co-MPC) copolymers and PBMA, FXIIA levels are not significantly different at any time incubation. These results indicate that poly(BMA-co-MPC) copolymers markedly reducing protein adsorption has little influence on contact phase activation.

Table 7.5 FXII-like activity (FXIIA) obtained with poly(BMA-co-MPC) copymers compared with PBMA, polystyrene (PS) and polyamide (NR)

Material	Absorbance (405 nm) Mean ± S.D. (n=3)				
	Incubation Time (min)				
	Pre	3	6	9	12
PS	0.060 ±0.010	0.068 ±0.013	0.072 ±0.008	0.084 ±0.011	0.104 ±0.010
PBMA	0.064 ±0.011	0.075 ±0.025	0.074 ±0.030	0.148 ±0.031	0.209 ±0.049
Poly(BMA-co-MPC) 0.20	0.064 ±0.011	0.084 ±0.030	0.069 ±0.014	0.146 ±0.016	0.152 ±0.035
Poly(BMA-co-MPC) 0.30	0.064 ±0.011	0.087 ±0.019	0.089 ±0.012	0.129 ±0.011	0.175 ±0.053
NR	0.064 ±0.011	0.094 ±0.007	0.157 ±0.010	0.218 ±0.017	0.275 ±0.011

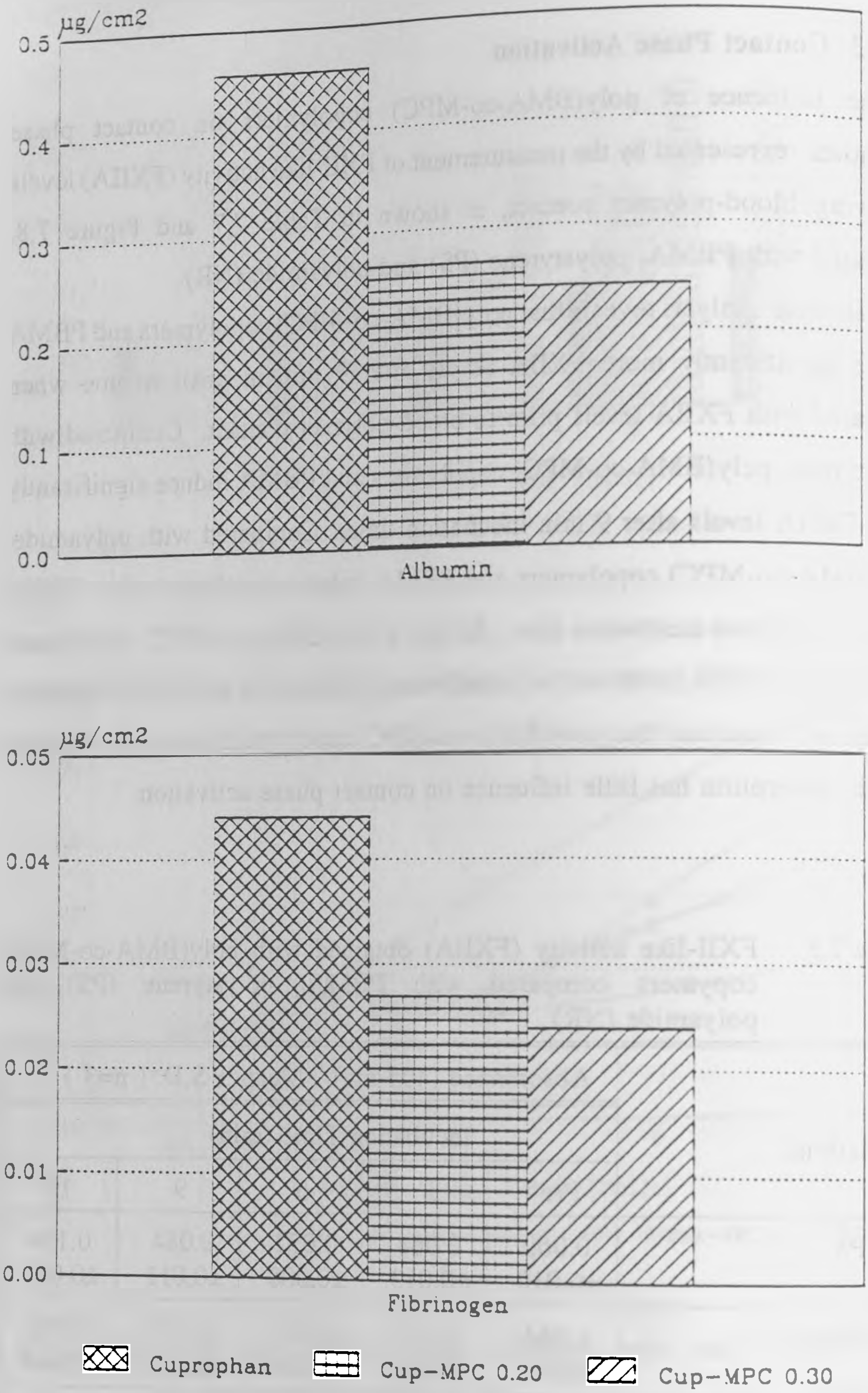


Figure 7.9 Protein adsorption on Cuprophan and MPC-grafted Cuprophan

7.3.2 MPC grafted Cuprophan

7.3.2.1 Protein adsorption

The measurement of protein adsorption on MPC grafted Cuprophan was performed with the modified 6 well incubation test cell in order to provide a appropriate response. The methodology of protein adsorption measurement with the modified 6 well incubation test cell was similar to that with the modified 24 well incubation test cell but 1.5 ml of fibrinogen solution were added into each well.

The influence of MPC grafted Cuprophan on albumin and fibrinogen single protein adsorption, is shown in Table 7.6 and Figure 7.9, compared with Cuprophan.

Table 7.6 Protein Adsorption on MPC grafted Cuprophan compared with Cuprophan

Polymer	Protein adsorption on polymer (μg/cm^2) Mean ± S.D. (N=6) *	
	Albumin	Fibrinogen
Cuprophan	0.458 ± 0.006	0.044 ± 0.001
Cup-MPC 0.20 *	0.267 ± 0.001	0.027 ± 0.002
Cup-MPC 0.30	0.243 ± 0.017	0.024 ± 0.004

* N: Number of values; Cup-MPC: MPC grafted Cuprophan

Statistical analysis reveals that MPC grafted Cuprophan significantly reduce both albumin and fibrinogen adsorption when compared with Cuprophan. These results are in agreement with poly(BMA-co-MPC) copolymers reducing protein adsorption (Section 7.4.1.1). Comparing MPC grafted Cuprophan with different MPC contents, less adsorption of both albumin and fibrinogen is achieved on MPC grafted Cuprophan with more MPC content.

In order to compare protein adsorption between fibrinogen and albumin, the data of the amount of protein adsorption on MPC grafted Cuprophan are

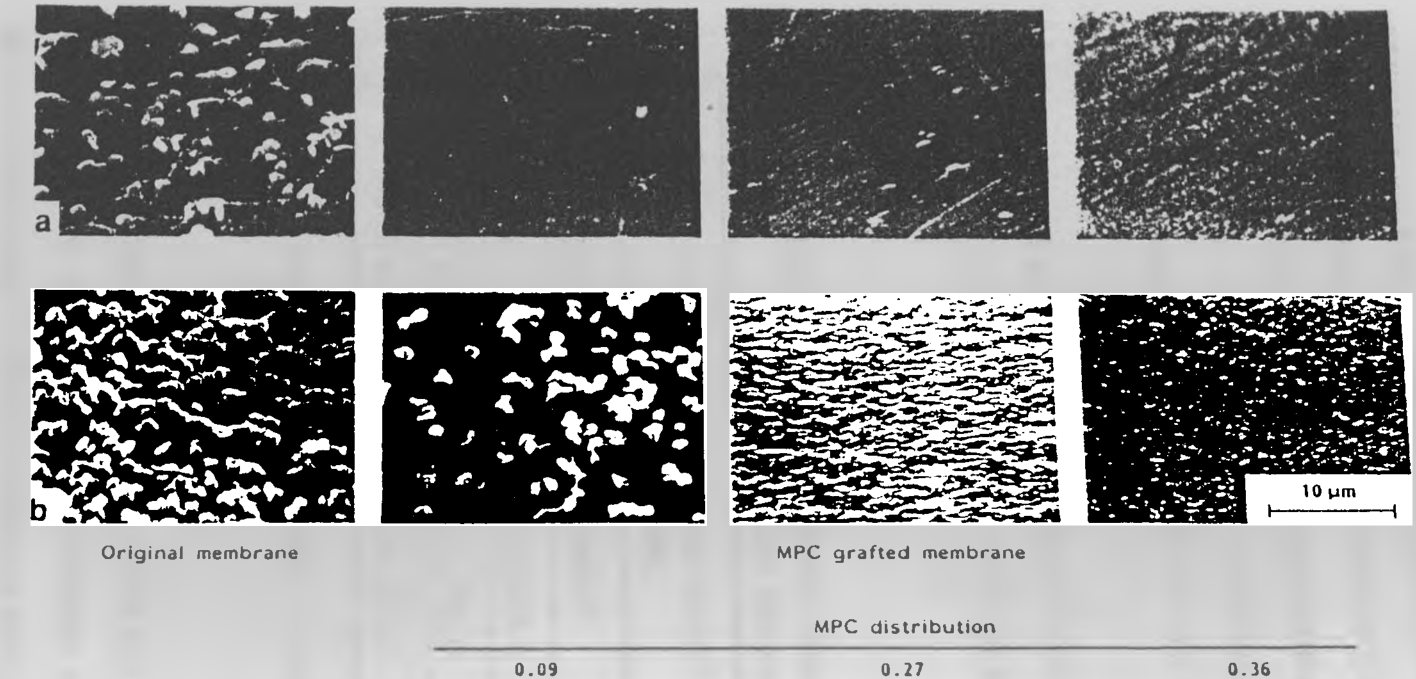


Figure 7.10 SEM pictures of Cuprophan and MPC-grafted Cuprophan after contact with platelet-rich plasma for: a, 30 min; and b, 180 min

normalised by regarding the amount of protein adsorption on Cuprophan as one hundred percent. The normalised data as well as the ratio of albumin adsorption to fibrinogen adsorption are listed in Table 7.7.

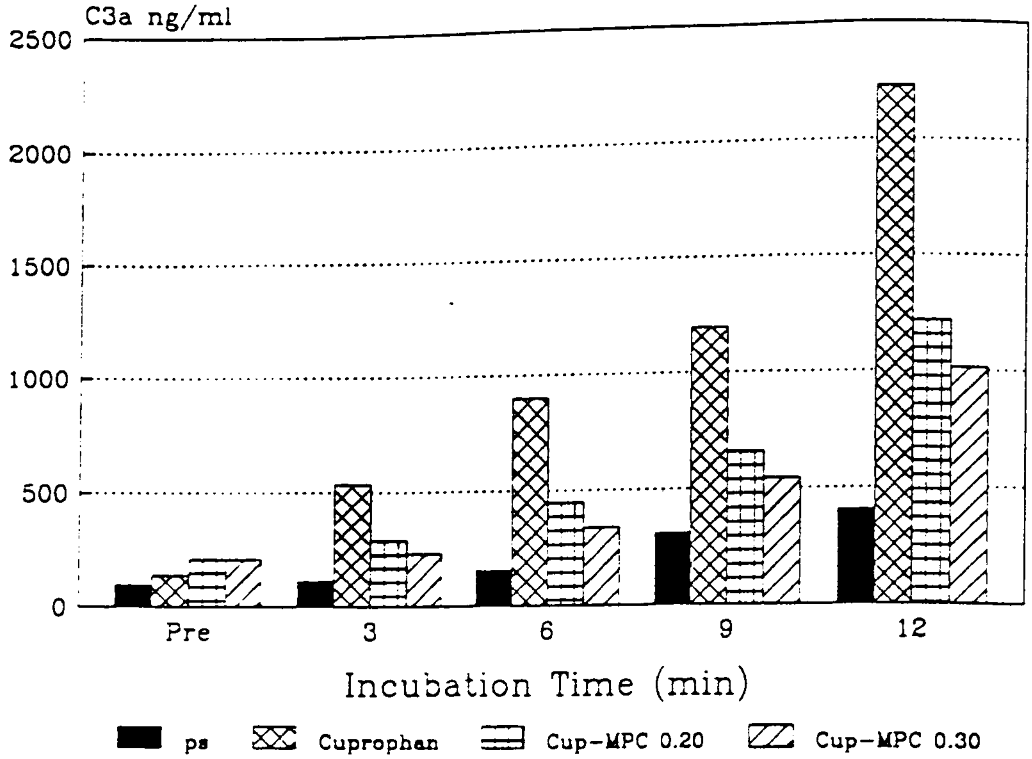
Table 7.7 Normalised data of protein adsorption on MPC grafted Cuprophan (relevant to Cuprophan)

Polymer	Protein adsorption on polymer (%)		
	Albumin	Fibrinogen	Ratio
Cuprophan	100	100	1.0
Cup-MPC 0.20	58	61	≈ 1.0
Cup-MPC 0.30	53	54	≈ 1.0

From Table 7.7, the marked reduction of both albumin and fibrinogen adsorption of MPC grafted Cuprophan can be clearly seen. The ratio of albumin adsorption to fibrinogen adsorption for MPC grafted Cuprophan grafted MPC is the same as that for Cuprophan. On the basis that adsorbed albumin decreases and adsorbed fibrinogen increases platelet adhesion (Brash 1991), this observation could not explain the evidence that MPC grafted Cuprophan suppresses platelet adhesion (Ishihara et al 1992b). Figure 7.10 shows SEM pictures of platelets adhering to MPC grafted Cuprophan after contact with PRP (Ishihara et al 1992b). On the basis that natural endothelium does not adsorb proteins under physiological condition (Bruck 1980) and is compatible with blood, lower protein adsorption induced by MPC grafted Cuprophan can achieve lower platelet adhesion than Cuprophan. The evidence of hydrogels inducing lower protein adsorption and suppressing platelet activity has been reported (Hoffman 1975).

7.3.2.2 Complement Activation

The influence of MPC grafted Cuprophan on complement activation, represented by the C3a concentration levels following blood-membrane contact,



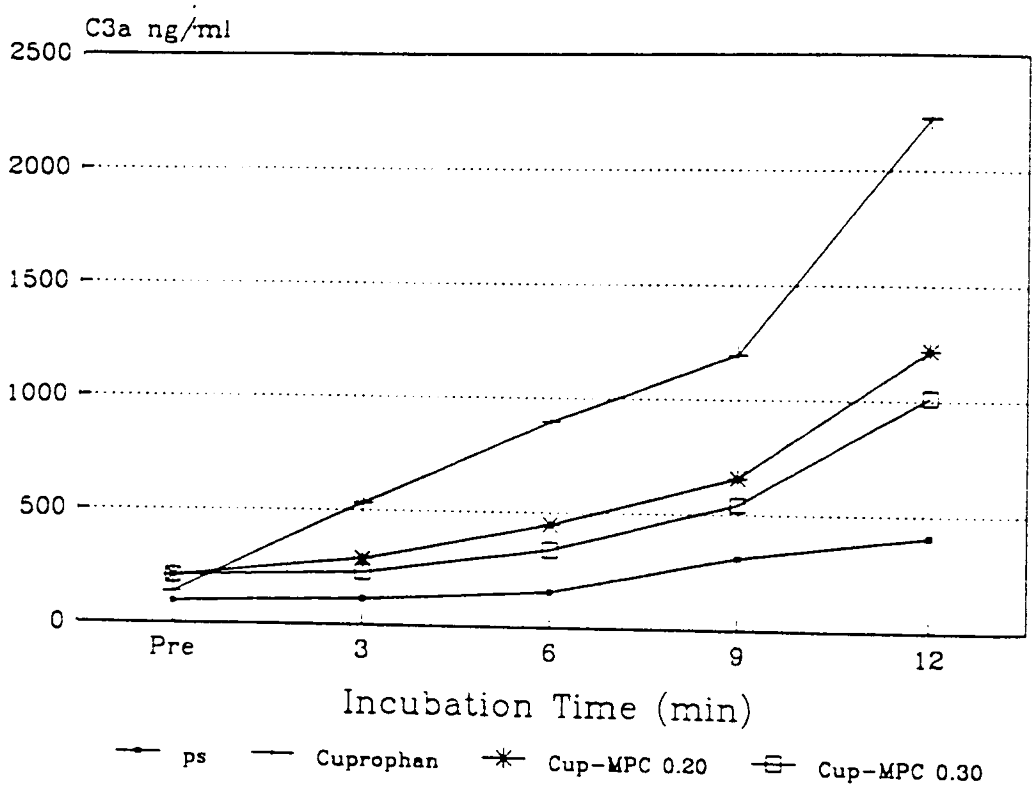


Figure 7.11 C3a concentration levels obtained with MPC-grafted Cuprophan compared with polystyrene (PS) and Cuprophan

is shown in Table 7.8 and Figure 7.11, compared with Cuprophan and polystyrene.

Table 7.8 C3a concentration levels obtained with MPC grafted Cuprophan (Cup-MPC) compared with Cuprophan and polystyrene (PS)

Material	C3a desArg Concentration Mean ± SD (ng/ml)				
	Incubation Time (min)				
	Pre	3	6	9	12
PS n=5	98 ±35	115 ± 42	156 ± 64	312 ± 189	409 ± 211
Cuprophan n=10	139 ± 38	534 ± 155	896 ± 126	1196 ± 162	2225 ± 315
Cup-MPC 0.20 n=4	210 ± 59	290 ± 59	448 ± 146	658 ± 255	1218 ± 341
Cup-MPC 0.30 n=4	210 ± 59	231 ± 25	339 ± 19	543 ± 125	1014 ± 181

Statistical analysis reveals that C3a concentration levels are significantly increased by MPC grafted Cuprophan after 6 min incubation, when compared with the C3a concentration levels prior to blood-membrane contact. Compared with control polymer polystyrene, MPC grafted Cuprophan induce significantly more C3a concentration levels at any time incubation. Compared with Cuprophan, MPC grafted Cuprophan induces significantly ($P<0.05$) lower C3a concentration levels at any incubation time, and C3a concentration levels are not significantly different between MPC grafted Cuprophan with different MPC content. These results indicate that MPC grafted Cuprophan can induce less complement activation in comparison to Cuprophan.

As indicated in Chapter 3, Cuprophan inducing complement activation is initiated by covalent attachment of C3b to hydroxyl groups on the surface. MPC grafted Cuprophan has less hydroxyl groups than Cuprophan, since hydroxyl groups are partially replaced by MPC. Therefore, MPC grafted

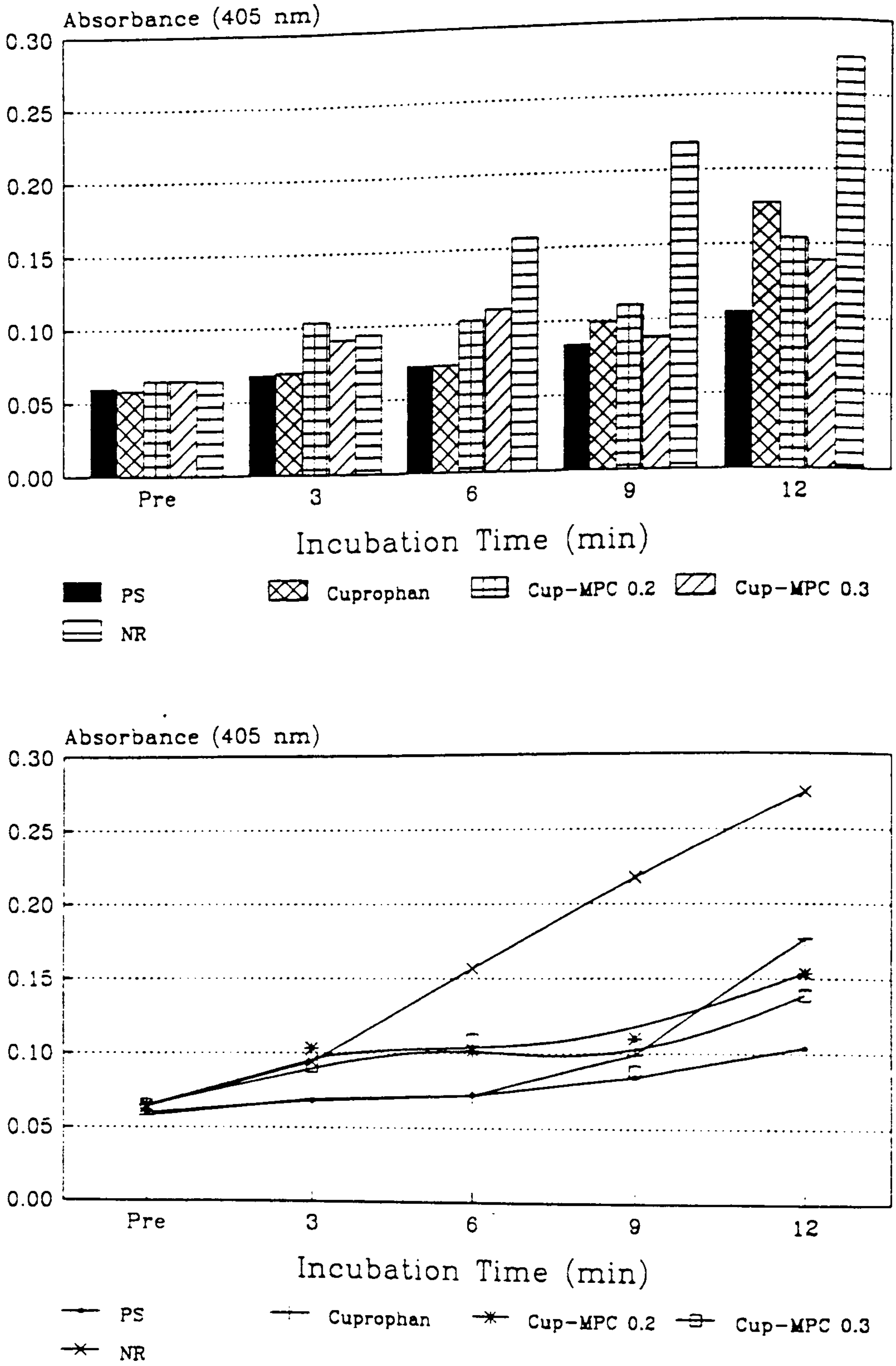


Figure 7.12 FXII-like activity (FXIIA) obtained with MPC-grafted Cuprophan compared with polystyrene (PS), Cuprophan and polyamide (NR)

Cuprophan induces lower complement activation than Cuprophan. An alternative explaination may arise from that MPC-grafted Cuprophan reduce less complement protein adsorption, since the evidence of phosphorylcholine-contained polymers reduce complement protein adsorption has been reported (Ishihara et al 1991).

7.3.2.3 Contact Phase Activation

The influence of MPC-grafted Cuprophan on contact phase activation, represented by the FXII-like activity (FXIIA) following blood-membrane contact, is shown in Table 7.9 and Figure 7.12, compared with Cuprophan, polystyrene (PS) and polyamide (NR).

Table 7.9 FXII-like Activity (FXIIA) obtained with MPC grafted Cuprophan (Cup-MPC) compared with Cuprophan, polystyrene (PS) and polyamide (NR)

Material	Absorbance (405 nm) Mean ± S.D.				
	Incubation Time (min)				
	Pre	3	6	9	12
PS n=3	0.060 ± 0.010	0.068 ± 0.013	0.072 ± 0.008	0.084 ± 0.011	0.104 ± 0.010
Cuprophan n=9	0.058 ± 0.015	0.069 ± 0.021	0.072 ± 0.022	0.099 ± 0.040	0.177 ± 0.061
Cup-MPC 0.20 n=4	0.068 ± 0.017	0.110 ± 0.026	0.100 ± 0.030	0.110 ± 0.025	0.154 ± 0.077
Cup-MPC 0.30 n=4	0.068 ± 0.017	0.081 ± 0.044	0.085 ± 0.027	0.081 ± 0.028	0.130 ± 0.046
NR n=3	0.064 ± 0.011	0.094 ± 0.007	0.157 ± 0.010	0.218 ± 0.017	0.275 ± 0.011

Statistical analysis reveals that MPC grafted Cuprophan induce more FXIIA levels at the end of incubation time but no significant difference when compared with FXIIA levels prior to blood-membrane contact. Compared with polystyrene, MPC grafted Cuprophan do not induce significantly more FXIIA until at t=12 min. Compared with polyamide, MPC grafted Cuprophan induce significantly less FXIIA levels after 6 min incubation time. Compared with Cuprophan, MPC grafted Cuprophan induces no significantly different FXIIA levels at any time incubation, although the mean values of MPC grafted Cuprophan are much lower than that of Cuprophan at t=12 min. These results indicate that the MPC grafted Cuprophan, while reducing protein adsorption and complement activation, has little influence on contact phase activation.

7.4 SUMMARY

The investigation of the influence of phosphorylcholine-contained polymers on protein adsorption has demonstrated that a marked reduction in protein adsorption can be achieved when phospholipid polar groups are introduced into polymers by copolymerization or grafting. However, higher MPC modification inducing higher protein adsorption was observed with Poly(BMA-MPC), but not with MPC grafted Cuprophan. This observation may be explained by the fact that MPC modification not only changed the surface composition, making it biomembrane-mimetic, but also altered to surface hydrophilicity and charge. There may exist a "critical point" for the modification approach, at which different surface properties are balanced to achieve the best blood compatibility. This "critical point" is influenced substrate polymer, parameter selection, parameter measurement technique.

The investigation of the influence of phosphorylcholine-contained polymers on the complement activation and the contact phase activation has demonstrated that phosphorylcholine-contained polymers can reduce the complement activation but have little influence on the contact phase activation.

In summary, the demonstration of protein adsorption and complement activation reduction, as well as little influence on contact phase system activation achieved with phosphorylcholine-contained polymers, supports the utilisation of phospholipid groups as a means of improving blood compatibility of biomaterials.

CHAPTER 8

DISCUSSION AND FUTURE WORK

8.1 INTRODUCTION

The present status of artificial surfaces utilized in blood-contacting applications means that thrombus formation is inevitable and biomaterials require the administration of an antithrombotic agent. Therefore, there is a need for biomaterials with improved blood compatibility. However, it is important that attempts to improve blood compatibility do not have an adverse influence on the other fundamental properties of the biomaterial. In this respect, modification of polymeric biomaterials offers potential advantages.

In this project, different approaches to polymer modification have been studied. These approaches were:

1. An increase in hydrophilicity, as represented by polyurethaneurea hydrogels (PUU) based on poly(ethylene oxide) (PEO)
2. Chemical modification, as represented by the replacement of the hydroxyl groups of regenerated cellulose Cuprophan with diethylaminoethyl (DEAE) groups in the preparation of Hemophan
3. Heparinization, as represented by the covalent attachment of the anticoagulant heparin to Cuprophan
4. Biomembrane-mimetic surfaces, as represented by the copolymer of butyl methacrylate and 2-methacryloyloxyethyl phosphorylcholine (MPC) and MPC grafted Cuprophan

8.2 PARAMETER SELECTION

The investigation of blood-biomaterial interactions necessitates parameter selection, which is limited by the fact that in a given project, it may not be possible to study a wide range of parameters. This project focused on the following :

Protein adsorption

Complement activation

Contact phase activation

8.2.1 Protein Adsorption

In this project, protein adsorption was studied by the measurement of radiolabelled albumin and fibrinogen adsorption from single protein solution. The advantages of this investigation are reliability, rapidity, relative non-expense and simplicity. A major difficulty is to relate such protein adsorption investigations to the clinical situation. The methodology used in this investigation could be augmented by the measurement of different proteins such as coagulation protein, e.g. factor VIII (vWF) (Horbett and Counts 1984), FXII, high molecular weight kininogen (HMWK) (Ziats et al 1990; Ishihara et al 1991), complement proteins, e.g. C3, C5, factor B and factor H (Johnson 1989; Johnson et al 1990; Lin et al 1992), by the measurement of protein adsorption from plasma or blood (Brash 1991). An alternative techniques for the measurement of protein adsorption could involve the utilization of a radioimmunoassay and immunogold labelling with scanning electron microscopy (SEM) (Ziats et al 1990; 1991; Ishihara et al 1991).

8.2.2 Complement Activation

Determination of C3a concentration was used as a measurement of complement activation. The method employed a radioimmunoassay, which has been well established and widely used in vitro and clinical investigation. As an alternative, the determination of C5a (Enia et al 1990; Sundaram 1993) or terminal C5b-9 complexes (Salama et al 1987) could be utilized to measure the complement activation.

8.2.3 Contact Phase Activation

Contact phase activation was determined by a chromogenic substrate assay, which measures a factor XII-like activity (FXIIA) (Sundaram 1993) with the advantage of the enhancement of substrate specificity. Since only enzymes of relevance to the coagulation cascade were inhibited, the disadvantage of this assay is that the proteases splitting the substrate, e.g. protein C and elastase, may influence the substrate specificity. As an alternative, the contact phase activation could be measured with an immunoassay measuring enzyme-inhibitor complex (Kaplan et al 1985) or a radioimmunoassay (Nuijens et al 1987).

The investigation of the contact phase activation influenced by polymer modification, covering increase hydrophilicity, chemical modification of cellulose, heparization and preparation of biomembrane-mimetic surface, indicates that none of modification procedures have a marked influence FXIIA, whereas the PEO-contained PUU hydrogels and phosphorylcholine-contained polymers have been reported to strongly influence platelets and modified cellulose influence complement. If the consequence of modification is an altered influence on platelets or complement, there may be no difference in contact activation. The evidence of the administration of the antiplatelet agent aspirin having little influence of FXIIA levels (Sundaram 1993) may be relevant.

8.3 TEST PROCEDURES

A critical feature in the study of blood-biomaterial interactions is the methods selected for achieving blood-material contact. Such methods range from simple to complex (Forbes and Courtney 1987). In this project, an incubation test cell was utilized. The original design of this test cell proved to be unsatisfactory because the change in shape of the material during blood-material contact, with consequent uncertainty over the ratio of surface area to blood volume. The incubation test cell was modified by splitting the polystyrene plate, introducing a Perspex base plate with silicone rubber and clamping the plates after placing the test material.

In its modified form, the 24 well modified test cell proved satisfactory for the protein adsorption measurement of polyurethaneurea hydrogels, but the 6 well modified test cell was necessary for the blood response measurement. A disadvantage of the modified incubation test cell is the unability to test brittle or porous materials. However, it is suitable for a wide range of materials in flat sheet form, particularly at an early stage of evaluation.

The different blood response can be measured with the 6 well modified incubation test cell between the modified-material and the unmodified-material, but the measurement of the significant difference in blood response, between the modified-materials, may require more donors, which has practical difficulties. More detailed investigation may require a test cell with controlled conditions of blood flow and wall shear rate and with the possibility of increasing the influence of the material on the blood response and ensuring that the response confirms to the accuracy of the measurement methodology.

8.4 INCREASE IN HYDROPHILICITY

The investigation of the influence of increase in polymer hydrophilicity on protein adsorption, the complement activation and contact phase activation has demonstrated that the PUU hydrogels based on PEO can increase albumin adsorption and markedly reduce fibrinogen adsorption, reduce the complement activation, but have little influence on the contact phase activation.

The new biomaterial with a ability to modify the blood response to other polymers has the potential advantage to promote the development of improved composite biomaterials. The PEO-contained PUU hydrogels studied are soluble in a number of organic solvents, such as methanol, ethanol, chloroform and dichloromethane. This solubility is an important advantage for these hydrogels in manufacturing composite biomaterials.

8.5 CHEMICAL MODIFICATION

The investigation of the influence of chemical modification on protein adsorption, the complement activation and the contact phase activation has demonstrated that the chemical modification of regenerated cellulose can reduce the complement activation and has little influence on the contact phase activation. Hemophan induces more fibrinogen adsorption and less albumin adsorption than Cuprophan, which is in agreement with platelet reactivity reported previously for Hemophan (Robertson 1987). A problem in studying Hemophan is that the degree of substitution of hydroxyl groups has not been consistent. It would be advantageous to study a range of DEAE-substituted cellulose, also cellulose acetate or other modified cellulose (Brown 1990).

8.6 HEPARINIZATION

The investigation of the influence of polymer heparinization on protein adsorption, the complement activation and the contact phase activation has demonstrated that protein adsorption can be influenced by both the heparinized-surface and the modification procedure. Heparinized-Cuprophan reduces the complement activation and has little influence on the contact phase activation. An extend studies of the influence of heparinized-Cuprophan on platetlet activity and the inhibition of thrombin formation may be required.

An alternative procedure is the binding of low molecular weight heparin or hirudin on polymer surfaces.

8.7 BIOMEMBRANE-MIMETIC SURFACE

The investigation of the influence of biomembrane-mimetic surface on protein adsorption, the complement activation and contact phase activation has demonstrated that phosphorylcholine-contained polymers can markedly reduce protein adsorption, which is in agreement with the previous indication that phosphorylcholine-contained polymers reducing platelet adhesion (Ishihara et al 1990; 1992b). Phosphorylcholine-contained polymers can also reduce complement activation. The observation supports the utilisation of phosphorylcholine groups as a means of improving blood compatibility of biomaterials. However, the effect of biomembrane-mimetic surface on the blood response may time-dependent. Future study in the influence of biomembrane-mimetic surface on blood response in ex vivo may be necessary.

8.8 SUMMARY

The data generated in this thesis have confirmed the potential of polymer modification as a means of improving blood compatibility. The clinical benefit of modification is uncertain in that in the clinical situation, the biomaterial is only one of the influencing factors on the blood response. Others are the antithrombotic agent, the blood condition and the nature of the application. However, the results obtained support further investigation. Such investigation could focus more closely on the relationship between modification and the different features of the blood response. Polymer modification to improve blood compatibility involves a balance between achieving better performance in one parameter and altering the performance of other parameters.

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