DEVELOPMENT AND VALIDATION OF ~~ANTICOAGULANT PEPTIDE FOR BONDING~~ ONTO ~~PROSTHETIC GRAFT~~

PEPTIDES FOR SURFACE MODIFICATION APPLICATION OF VASCULAR POLYMER

A thesis submitted for the degree of Doctor of Philosophy of the University of London

By

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Abstract

Polymeric biomaterials used for applications such as coronary and vascular bypass grafting have demonstrated poor patency due to their surface thrombogenicity, initiation of chronic inflammation and unfavourable host tissue responses.

The aim of this thesis has been to develop a peptide which would demonstrate an inhibitory effect on blood coagulation and/or improved endothelial cell adhesion. Employing the RGD (Arginine-Glycine-Aspartate) peptide as a base, GRGD, GRGDS and $GRGD(AhxGRGD)_3$ were produced. In order to allow incorporation of the peptide into the polymer matrix the corresponding lauric acid (LA) conjugated peptides were synthesised. *In vitro* determination of blood clotting time and tissue factor activity was utilised to determine the optimum peptide concentration for an anti-thrombogenic effect. Cytotoxicity and cell adhesion were assessed on endothelial cells. The results obtained suggest that LA-GRGD offered the best anti-thrombogenic effect whilst LA-GRGDS had the most improved cell adhesive effect. These two peptides were then used to investigate the surface modification of poly(carbonate-urea)urethane (PCU).

The PCU surface was modified by passive peptide coating or peptide incorporation into the polymer matrix. Cell adhesion and activity studies showed that the incorporated LA-GRGDS peptide produced a significant ($P<0.05$) improvement. Biocompatibility studies demonstrated no adverse effects with respect to either platelet adhesion or haemolysis. The inhibition of platelet factor 4 obtained with coated GRGD, GRGDS and incorporated LA-GRGD was comparable to that obtained with heparin coating. An *in vitro* flow study showed that significantly ($P<0.005$) more incorporated peptide (42.6%) was retained on the surface of the polymer after 8 hours flow compared to coated (20%).

In conclusion the direct incorporation of an LA conjugated peptide into the matrix of the polymer was successful with the peptide retaining its activity. This process of incorporation by solvent casting is attractive from a commercial viewpoint and shows the potential for future development and use in a clinical situation to produce a surface modified PCU polymer.

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ABBREVIATIONS USED

AB	Alamar Blue™
ADP	Adenosine diphosphate
Ahx	Aminohexanoic acid
cAMP	Adenosine monophosphate
ATIII	Antithrombin III
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
BTG	β-thromboglobulin
CPB	Cardiopulmonary bypass
CVD	Cardiovascular disease
DAB	3, 3' Diaminobenzidine
Dacron	Polyethylene-terephthalate
DCM	Dichloromethane
DMAC	Dimethylacetamide
DMF	Dimethylformamide
ECC	Extracorporial circuits
ECM	Extracellular matrix
ECs	Endothelial cells
EDT	1,2-ethanedithiol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ePTFE	Expanded Polytetraflouroethylene
FAB-MS	Fast-atom-bombardment mass analysis
FEPP	Fibronectin Engineered Protein Polymer
FGF-1	Fibroblast growth factor type 1
FITC	Fluorescein isothiocyanate
FTIR	Fourier transform infrared
GPIIb/IIIa	Glycoprotein IIb/IIIa

GRGD	Gly-Arg-Gly-Asp
GRGDS	Gly-Arg-Gly-Asp-Ser
GRGDSY	Gly-Arg-Gly-Asp-Ser-Tyr
GRGDVY	Gly-Arg-Gly-Asp-Val-Tyr
HBTU	2-(1H-Benzotriazole-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate
HIT	Heparin-induced thrombocytopenia
HOBt	1-hydroxybenzotriazole
HRP	Horseradish peroxidase
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
IH	Intimal hyperplasia
IR	Infrared
IV	Intravenous
KGD	Lys-Gly-Asp
LA	Lauric acid
LMWH	Low Molecular Weight Heparin
ma	Maximum amplitude
MC	Mesothelial cells
MD	Magnetic device
MI	Myocardial Infraction
MW	Molecular weight
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
OD	Optical density
PBS	Phosphate buffered saline
PCU	Poly(carbonate-urea)urethane
PECAM	Platelet endothelial cell adhesion molecule
PEG	Polyethylene glycol
PEO	Polyethylene oxide

PF4	Platelet factor 4
PRP	Platelet-rich plasma
PT	Prothrombin time
PVD	Peripheral vascular disease
RGD	Arg-Gly-Asp
RGDS	Arg-Gly-Asp-Ser
RGDV	Arg-Gly-Asp-Val
SEM	Scanning electron microscopy
TEG	Thrombelastography
TEG-r	Thrombelastograph reaction time
TF	Tissue factor
TFA	Trifluoroacetic acid
TIA's	Transient ischemic attacks
TPA	Tissue plasminogen activator
TXA2	Thromboxane A_2
UFH	Unfractionated heparin
vWF	von Willebrand factor

CHAPTER ONE:

INTRODUCTION

1.1 Introduction

Cardiovascular disease (CVD) is the number one killer in Europe, accounting for nearly half of all deaths. In the majority of European countries, approximately 40 percent of people who die before the age of 74 are killed by cardiovascular disease [1]. Based on current disease trends and the growing number of elderly people in the European population, CVD is expected to continue to be the major killer disease in Europe in the foreseeable future.

Atherosclerosis, commonly referred to as "hardening of the arteries", is responsible for most cardiovascular disease [2]. Atherosclerosis is the build up of fatty materials (plaque) on the inside of the arteries. The inner surface of arteries can be made rough by fatty deposits or plaques and blood flow through them can become reduced. As blood passes more slowly through these rough, narrowed arteries, blood clots are more likely to form. In some cases, blood clots or thrombi can become so large that they block an artery completely. Narrowing, loss of elasticity or blockage of an artery can have serious effects on the part of the body that depends on that artery for a steady supply of blood. Such effects include claudication (due to poor circulation to the leg muscles), angina (due to poor circulation to the heart muscle or coronary arteries), and narrowing or blockage of the arteries that supply the brain with blood which can result in a stroke or transient ischemic attacks (TIAs).

The common procedures used to treat a narrowing or blocked artery are angioplasty and bypass surgery. Angioplasty uses a catheter with a small inflatable balloon to open a narrowed or partially blocked artery. The balloon is pushed into the area of the blockage, and then inflated to squeeze the plaque back against the artery's wall. After repeated inflations have completely cleared the artery, a stent is pushed to the site of blockage and left there to keep the artery from closing up again. Bypass surgery is performed to treat a potentially occluded artery by creating a new route along which the blood can flow. Since its introduction in the early 1950s, bypass surgery has become one of the most

common surgeries performed in the western world. The procedure can improve the quality of life and even add years to it, especially when combined with a healthy lifestyle.

The ideal source of material for bypass surgery is autologous veins or arteries which have been shown to have a high success rate [3, 4]. However in many patients adequate autologous vein is lacking leading to the necessity of using synthetic or prosthetic materials instead [5, 6]. Although metal, glass and rubber have been tried as blood conduits, the greatest attention has been devoted to various polymers [7]. Many polymers have the advantage of being essentially inert within the human body and they have the additional advantages of durability, flexibility and moldability. Although plastics are ideal from many standpoints for the construction of prosthetic devices, their thrombogenic characteristics significantly limit their use as long-term implants. The first polymer utilised as a synthetic graft to bridge arterial defects in humans was made of polyethylene terephthalate (Dacron™) in 1954. Expanded polytetrafluoroethylene (ePTFE) is the other material commonly used for synthetic grafts currently in clinical practice. Both Dacron™ and ePTFE are rigid which can result in a compliance mismatch at the anastomosis. Polyurethane polymers have been popularly used in biomedical applications due to their mechanical properties such as compliance, strength, durability and tolerance within the body [8, 9]. The clinical use of polyurethanes has been limited currently due to their tendency to suffer from biodegradation [10]. The recent development of a novel stress-free compliant poly(carbonate-urea)urethane (PCU), with similar compliance to lower limb arteries, has demonstrated an improved resistance to chemical and environmental degradation that may overcome these limitations [11, 12].

At the present time thrombosis on prosthetic surfaces and also compliance mismatch between polymeric graft and native vessel is considered to be a major hindrance to the continued progress and ultimate solution of the use of prosthetic replacements for vascular vessels. Vascular prostheses have

been used with high success rates in large artery substitutions. However grafts in small calibre vessels (<6mm diameter) for replacement of artery or vein segments suffer from high failure rates and lack long-term functionality [13, 14].

Rudolf Virchow (1845) first suggested the important factors leading to thrombosis on prosthetic surfaces are: 1) hypercoagulable blood factors 2) stasis of flow and 3) loss of the normal vascular lining. Any one of these factors alone usually will not produce thrombosis; however, any two of the factors together will ordinarily lead to thrombus formation (Figure 1.1). Prosthetic vascular graft, in small calibre vessels will definitely provide the latter two factors and the surgical trauma required to replace or bypass a vessel may provide the first factor.

The search for more effective small diameter vascular grafts has increased greatly in recent years. It is assumed that the low procoagulant activity and significant antithrombotic activity of endothelial cells lining the lumen of a normal vascular vessel contribute to the maintenance of blood fluidity [15]. It may be that no prosthetic material devoid of endothelial cells will ever be satisfactory for small-diameter vascular prostheses since it cannot provide a hypothetical critical minimal level of antithrombotic activity in a low flow situation. However, since large-diameter grafts do successfully replace large segments, research continues on the design of prosthetic materials that will have reduced thrombogenecity relative to current material or an anti-thrombogenic (blood compatible) surface.

A variety of devices can be successfully accepted by most patients through the administration of systemic anticoagulants such as heparin and warfarin. However, the risk of complications or drug intolerance related to these anticoagulants is ever present, and only adds to the inherent risk of device-associated complications. Some patients cannot tolerate any pharmaceutical regimen used to counteract the clotting that can be caused by synthetic materials, and are therefore ineligible to receive certain blood-contacting

devices. For these reasons, research into blood-compatible materials has been increasingly pursued

Another process to improve blood compatibility is to covalently bind agents known to inhibit blood coagulation and/or platelet adhesion to the surface of the polymer. The first successful step taken towards this study was by Gott in 1963 [16]. His surface modification of a polymer by chemically binding a drug (heparin) known to inhibit blood coagulation has received much attention. Since then many discoveries have been made demonstrating that surface modification can improve the blood compatibility of prosthetic materials in vitro experiments.

Tissue engineering is another approach which has extensively been studied by several research groups to improve blood compatibility of synthetic materials. An initial attempt at tissue engineering a blood vessel substitute involved seeding the lumen of a synthetic graft with endothelial cells (ECs). Seeding involves extracting autologous ECs and then lining these cells onto the graft lumen. Herring together with Mansfield[12] and co-workers, suggested that this would provide a more biocompatible surface and thereby decrease thrombosis and intimal hyperplasia (IH) [17, 18]. Herring and co-workers then showed clinical evidence in humans that when a graft was seeded, an extensive lining of endothelial cells was possible in addition endothelial cell seeding that also results in fewer graft-based infections [19, 20].

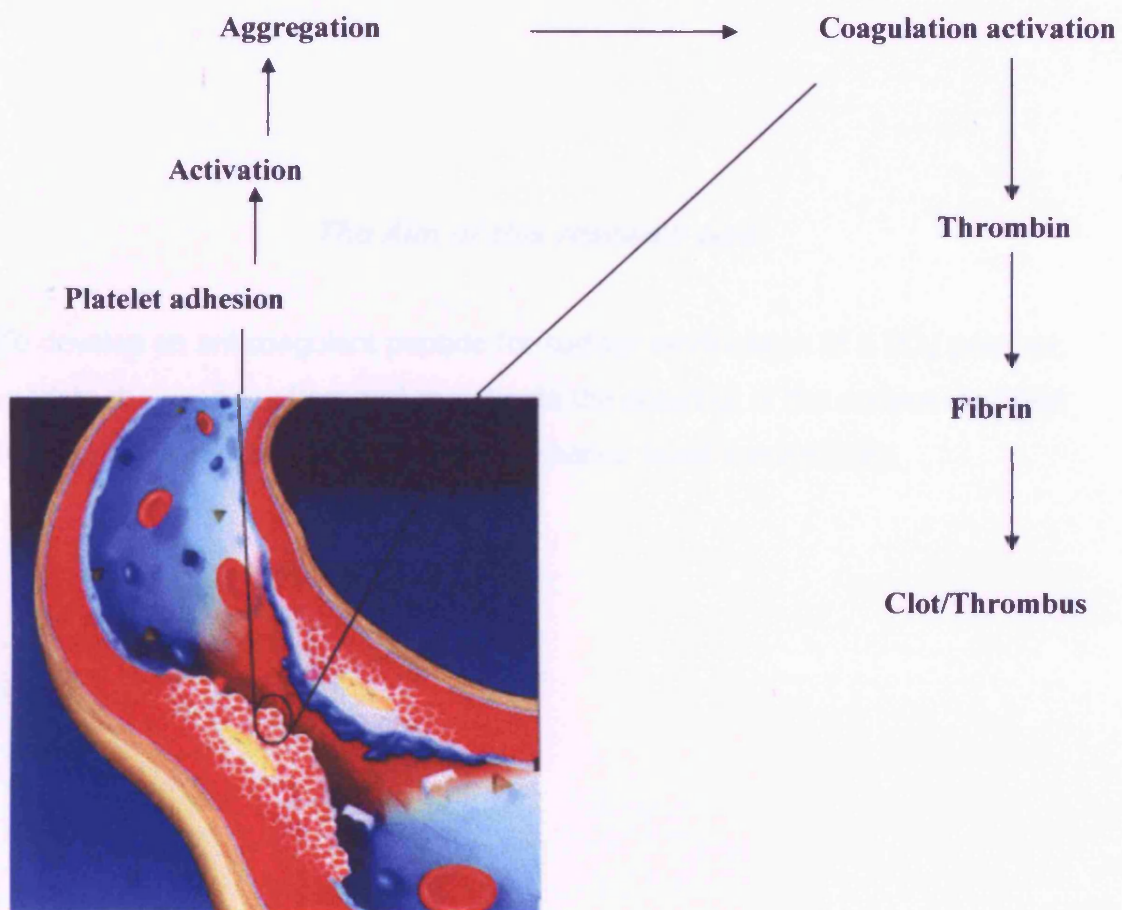


Figure 1.1 Schematic diagram of thrombus formation

The Aim of this research was:

To develop an anticoagulant peptide for surface modification of a PCU polymer, validate the peptide effect and investigate the potential of the surface modified polymer combined with the peptide to enhance blood compatibility.

CHAPTER TWO:

ANTICOAGULANT AND ANTIPLATELET AGENTS: USAGE AND CURRENT APPLICATIONS IN SURFACE MODIFICATION OF BIOMATERIALS USED IN CARDIOVASCULAR TISSUE ENGINEERING: A REVIEW

2.1 Introduction

This chapter is a background study and literature review on usage and current application of antiplatelet and anticoagulant agents in the surface modification of biomaterials [21]. Primarily the current status of antiplatelet and anticoagulant agents is highlighted from a clinical perspective, their current and potential clinical usages and their modes of respective action during the coagulation cascade process. It is then focused on these agents' roles in improving the blood-compatibility of biomaterials as used in cardiovascular applications.

Diseases of the cardiovascular system remain the leading cause of morbidity and mortality worldwide. Cardiovascular diseases include myocardial infarction, stroke, and peripheral vascular diseases. The traditional strategies of prevention and treatment of these cardiovascular diseases are classified as medical or surgical. The commonly used and routinely available agents for antiplatelet and anticoagulation therapy are aspirin and heparin.

Over the past several years, controlled trials totalling more than 100,000 subjects have shown that antiplatelet therapy mainly aspirin, reduces the risk of vascular death by about one sixth and the risk of non-fatal myocardial infarction and stroke by about one third in 'high risk' subjects with clinical vascular disease [22]. Unfractionated heparin (UFH) has also been one of the established anticoagulant therapy of choice for prevention and treatment of thrombotic disorders for many years. However, in the past several years, newer anticoagulants and antiplatelets such as the direct thrombin inhibitors, thienopyridines and intravenous platelet glycoprotein IIb/IIIa (GPIIb/IIIa) inhibitors have been found to be important adjunctive therapy for reduction of vascular death and non-fatal myocardial infraction (MI) [3, 23-25].

A significant proportion of high-risk patients with arterial occlusive diseases undergo open surgery with coronary and peripheral occlusions being bypass grafted. Vascular grafts are used to bypass or replace occluded (narrowed)

arteries, both coronary and peripheral [26-28]. The ideal biological replacement for blood vessel should be able to properly function, repair, remodel and grow.

Venous autographs, which are usually taken from patient's saphenous vein, are used to replace small to medium sized arteries [3, 4, 29]. These vein grafts have a high success rate, however about one third of the patients in need of a vascular graft have poor saphenous veins, usually because of peripheral vascular disease (PVD), too small, or non-existent because they have already been removed for another bypass or vessel replacement [5, 30]. For these patients artificial or prosthetic grafts are used [6, 31]. Currently artificial grafts include treated natural tissue, laboratory-engineered tissue, synthetic polymer fabrics and synthetic grafts such as polyethylene-terephthalate (Dacron) and expanded polytetraflouroethylene (ePTFE).

Dacron and ePTFE are the two commonly used vascular prosthetic graft materials. These prostheses have been used with high success rates in large artery substitutions, but grafts in small calibre vessels (<6mm diameter) suffer from high failure rates, lacking long-term functionality. The inherent thrombogenicity of the graft material and the development of stenotic lesions or intimal hyperplasia around the anastomosis mainly due to a compliance mismatch between the graft and the native blood vessel [32-34] are the main reasons behind the graft failure.

In order to reduce surface thrombogenicity of such prosthetic materials numerous research groups including ours have been investigating a wide variety of approaches. These include surface modification and tissue engineering or cell seeding of the prosthetic material [35-41]. Cell seeding is a process of lining ECs to the lumen of any cardiovascular device. ECs lining the lumen of a normal vascular vessel prevent platelet adhesion and blood coagulation or formation of thrombosis [42]. Hence to encourage the growth of a layer of endothelial cells over the device surface has been an alternative method to prevent thrombogenicity and improve blood compatibility.

Different surface modification agents such as the anticoagulant agent heparin or the anti-adherent agent polyethylene oxide (PEO) have been used. In a number of laboratory and clinical studies heparin-coated biomaterial devices have been shown to enhance various aspects of blood compatibility. Heparin-coated cardiopulmonary bypass circuit reduces platelet adhesion, platelet and complement activation [43-47]. However, heparin coating can degrade (or leech) [48] over time and is therefore not suitable for use on long-term blood-contacting devices such as pacemaker leads or heart valves. PEO prevents platelet attachment also repels other cells [49], making these coatings inappropriate for devices such as vascular grafts or coronary stents, onto which cell overgrowth is desired.

The anticoagulant agent dipyridamole and the direct thrombin inhibitor recombinant hirudin (r-hirudin) have been successfully coupled to synthetic graft surfaces. Dipyridamole treated polyurethane grafting has reduced thrombogenicity and platelet adhesion in vitro [50] but provided no beneficial evidence in vivo. Covalently bound r-hirudin to Dacron or polyurethane has also inhibited thrombin and reduced thrombogenicity in vitro [51-53].

2.2 The clinical uses of antiplatelet and anticoagulant agents

2.2.1 Thrombogenesis

In normal healthy non-disrupted vascular endothelium platelets and blood coagulation factors are not activated. Endothelial cells synthesize several inhibitors of thrombosis; plasminogen activators, thrombomodulin and heparan [54]. These molecules modulate coagulation and promote fibrinolysis. The matrix of the vessel wall contains thrombogenic elements including adhesive proteins, such as collagen and von Willebrand factor (vWF) (both of which promote platelet adhesion), and tissue factor (TF) that triggers blood coagulation cascade (Figure 2.1) [55].

Thrombosis however, may occur if the haemostatic stimulus becomes unregulated for example if the capacity of the inhibitory pathway is impaired, or more commonly, the capacity of the natural anticoagulant mechanism is overwhelmed by the intensity of the stimulus [56]. One example being acute stroke [57]. Important predisposing conditions to thrombosis are low flow state, disturbed flow [58]and altered endothelial coverage (ulceration or endarterectomy). Injury of the vessel wall plays a major role in vascular thrombosis [59]. However, it is more important in the pathogenesis of arterial thrombosis than its venous counterpart.

Arterial thrombi are predominantly composed of platelets, a scanty amount of fibrin, and a few red blood cells, hence the term "white thrombi." Because of the high platelet composition of these thrombi, antiplatelet agents, rather than anticoagulants, have been used in the treatment and prevention of arterial thrombosis [60]. However, venous thrombi are mainly composed of red blood cells in a fibrin mesh, hence the term "red thrombi" [58] and anticoagulant agents are used in the treatment of venous thrombosis.

When a blood vessel injury occurs (Figure 2.2), a critical event in platelet aggregation is the expression of surface membrane receptor GPIIb/IIIa that has the capacity to bind fibrinogen as well as vWF, fibronectin, and vitronectin [61]. Fibrinogen appears to be the most important in aggregation by virtue of its divalent structure that allows it to form a bridge from platelet to platelet, thereby mediating aggregation [62]. While vWF and collagen can interact with resting platelets, fibrinogen forms a high-affinity bond only with the integrin GPIIb/IIIa on activated platelets.

Many agonists, such as thrombin, adenosine diphosphate (ADP) [63], collagen, and arachidonic acid, have the ability to induce platelet aggregation and secretion [64]. Specific receptors exist on the platelet surface for these agonists.

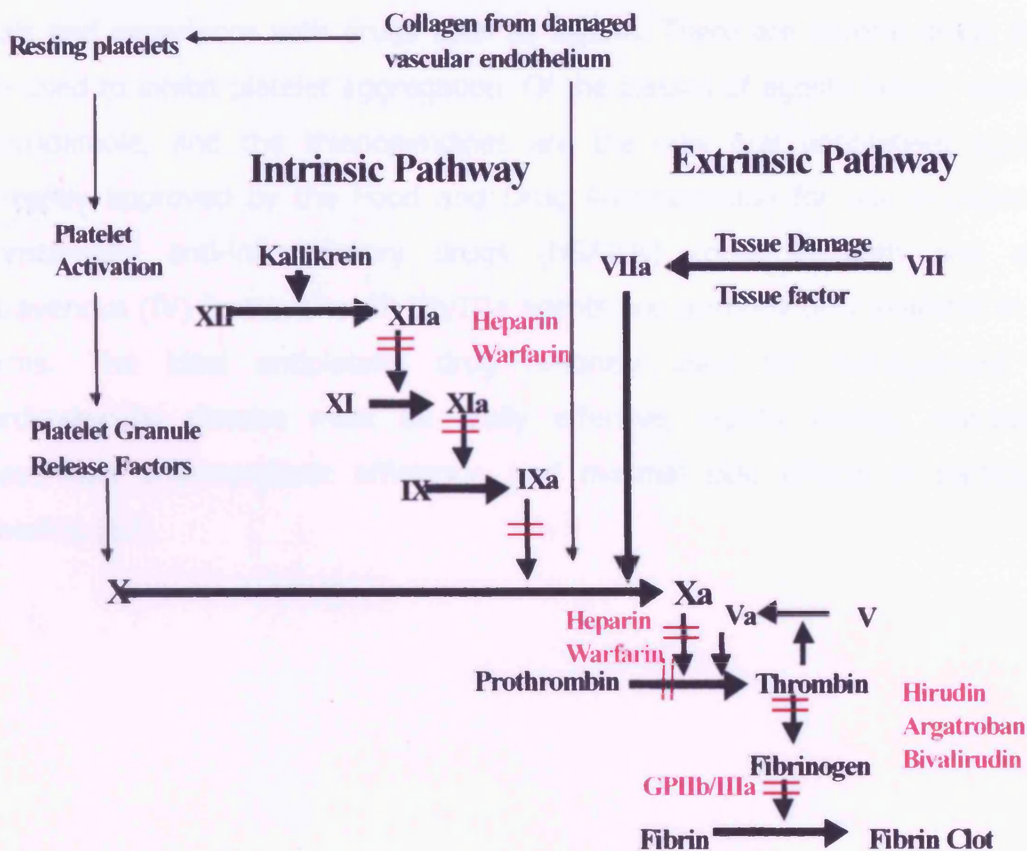


Figure 2.1 The blood coagulation cascade with sites of action of the anticoagulant drugs. The classic coagulation system is divided into extrinsic and intrinsic pathways. An intrinsic system that is activated by coagulation factors that is already present in the blood and an extrinsic system that is initiated outside of blood vessels in the presence of injury to a vessel. In the extrinsic system, factor VII, which is present in whole blood, is converted into its activated form factor VIIa, by binding to (TF). The TF/VIIa complex formed then converts factor X into its activated form Xa. In turn this forms a complex with factor Va and so brings about cleavage of prothrombin in order to form thrombin. Thrombin can then cleave fibrinogen to form fibrin, which polymerises to form fibrin sheets.

2.2.2. Platelet inhibiting agents

The relevance of antiplatelet drugs has been firmly established by clinical trials and experience with drugs such as aspirin. There are several drugs that are used to inhibit platelet aggregation. Of the classes of agents below, aspirin, dipyridamole, and the thienopyridines are the only oral antiplatelet agents currently approved by the Food and Drug Administration for use in-patients. Nonsteroidal anti-inflammatory drugs (NSAIDs) come in both oral and intravenous (IV) forms. The GP IIb/IIIa agents are currently only available in IV forms. The ideal antiplatelet drug routinely used for management of cardiovascular disease must be orally effective, rapidly acting, non-toxic, reasonable antithrombotic efficiency, and minimal side effects in particular bleeding [65].

Figure 2.2 The mechanism of platelets in response to injury of blood vessel with sites of action of antiplatelet drugs. When a blood vessel injury occurs, platelets exhibit a sequence of events. These events include 1) adhesion of platelets to the injury site, 2) spreading of adherent platelets over the exposed subendothelial surface, 3) secretion of platelet granule constituents, 4) platelet aggregation, and 5) thrombus formation.

2.2.2.1 Cyclooxygenose inhibitors: Aspirin and aspirin-like drug

As a general class nonsteroidal anti-inflammatory drugs (NSAIDs), typically acetyl salicyclic acid (Aspirin™), indomethacin, and ibuprofen, interferes with the binding of arachidonic acid in the cyclooxygenase active site of the enzyme. Aspirin (Figure 2.3) is the most widely used inhibitor of platelet function. It interferes with platelet aggregation by *inhibiting* the synthesis of thromboxane A_2 (TXA2) through the irreversible acetylation of cyclooxygenase [66]. Other NSAIDs compete reversibly with arachidonic acid for binding to the cyclooxygenase site.

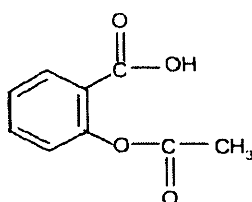


Figure 2.3 Chemical structure of Aspirin

2.2.2.2 ADP receptor blockers: thienopyridine derivatives

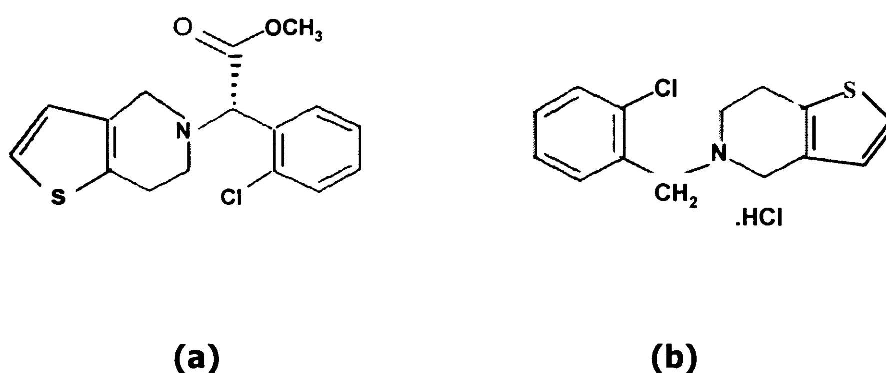
Ticlopidine (Ticlid™) and its more recently developed analog clopidogrel (Plavix™), are thienopyridine derivatives. They inhibit the binding of ADP to its platelet receptor (Figure 2.2); this ADP receptor blockade leads to direct inhibition of the binding of fibrinogen to the glycoprotein IIb/IIIa complex [67, 68]. Ticlopidine may also interfere with vWF, resulting in less binding of vWF factor to platelet receptors.

Ticlopidine and clopidogrel (Figure 2.4) can both be administered orally. Both agents are inactive in vitro, requiring breakdown to an unidentified active

metabolite or metabolites to achieve in vivo activity [69]. Activation seems to occur in the liver, and the active metabolites are primarily excreted renally.

Ticlopidine has been reported to improve the long- term patency of saphenous vein bypass graft in patients with PVD [70]. It is used often concurrently with aspirin to prevent thrombosis in patients who have had coronary artery stents implanted [71]. Ticlopidine has a number of potentially serious side effects; it has been associated with a low rate of severe neutropenia, which requires the monitoring of white cell counts during the first few weeks of treatment [72].

Treatment with clopidogrol has resulted in a slightly greater reduction in endpoint of patients with PVD. Because it apparently has fewer side effects than ticlopidine, clopidegrol has been substituted increasingly for ticlopidine to prevent subacute thrombosis in intracoronary stents [73-75]. Combined clopidogrel and aspirin are frequently used in the prevention of subacute thrombosis following coronary stent implantation, and appear to be a safe and effective therapy [76-78]. Further, clopidogrel is associated with a reduction in gastrointestinal haemorrhage, making it a valuable therapeutic alternative to aspirin in oral, long-term prevention of atherothrombotic vascular occlusion [69].



(a) **(b)**

Figure 2.4 Chemical structure of thienopyridine derivatives a) Clopidegrol and b) Ticlopidine

2.2.2.3 Adenosine uptake inhibitor: dipyridamole

The pyrimidopyrimidine derivative dipyridamole (Persantine™) is a phosphdiesterase inhibitor that has been used as an antiplatelet agent, almost always concurrently with either aspirin or warfarin.

Elevation of intracellular cyclic adenosine monophosphate (cAMP) levels by agents that activate adenylate cyclase or that inhibit the cyclic phosphodiesterases results in inhibition of platelet responses [64]. Dipyridamole (Figure 2.5), a weak phosphodiesterase inhibitor, appears not to inhibit aggregation responses to collagen, epinephrine, and ADP at usual doses but has a synergistic effect with aspirin in preventing platelet aggregation in thromboembolic disorders (Table 2.1). Its phosphodiesterase inhibitory activity potentiates the effect of adenosine on platelets. As a result, dipyridamole may have an effect on the initial phase of platelet adhesion as well as platelet aggregation [59].

Dipyridamole used concurrently with aspirin has increased coronary blood flow and graft patency following coronary bypass surgery [79-81]. It was as effective in reducing smooth muscle cell proliferation as the combination of aspirin and dipyridamole [82].

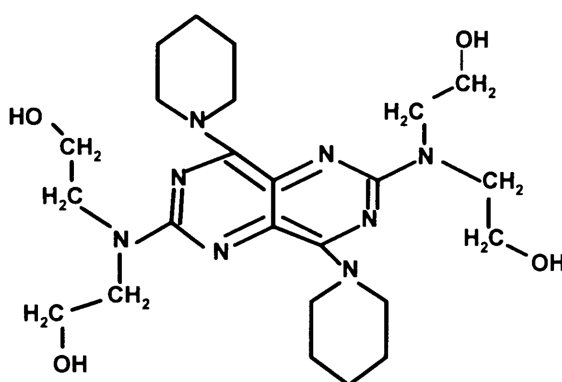


Figure 2.5 Chemical structure of Dipyridamole

2.2.2.4 Platelet glycoprotein IIb/IIIa – inhibitors

Ligand binding to GP IIb/IIIa receptor on activated platelets is a pre-request for platelet aggregation and formation of a platelet thrombosis [83, 84]. Thus the GP IIb/IIIa receptor has been a target for the development of drugs to inhibit platelet-mediated thrombus formation (Figure 2.2). Several intravenous medications directed specifically at this receptor (called platelet GP IIb/IIIa receptor antagonists) have emerged. These include the human-murine chimeric monoclonal antibody Fab fragment abciximab, the peptide antagonist eptifibatide and the peptidomimetics tirofiban (Table 2.1).

2.2.2.4.1 Abciximab - Abciximab (c7E3 Fab, Reopro™) is the Fab fragment of a human-murine chimeric monoclonal antibody that inhibits agonist-stimulated fibrinogen binding of GP IIb/IIIa receptor and in vitro platelet aggregation [85]. It was the first agent of this class to demonstrate clinical effectiveness (Table 2.1) [86]. Several of the specific properties of abciximab, such as its long half-life, lack of receptor-blocking specificity, and some tendency for antigenicity, have prompted the development of alternative GP IIb/IIIa inhibitors with distinct pharmacological profiles [87].

Primarily, the drug inhibits platelet aggregation, but it may also have anticoagulant activity and other beneficial effects, such as inhibiting migration and promoting apoptosis of smooth muscle cells [88]. The drug is used in conjunction with heparin and aspirin to prevent ischaemic complications associated with percutaneous coronary revascularisation in-patients with coronary heart disease [89]. Large and well designed clinical studies have shown abciximab, as an adjunct to aspirin and heparin, to reduce by around one-third to one-half, the incidence of ischaemic complications within 30 days of percutaneous coronary revascularisation [90].

2.2.2.4.2 Eptifiban - Eptifiban (Integrelin™) is a synthetic cyclic heptapeptide based on the Lys-Gly-Asp (KGD) motif of the snake venom disintegrin barbourin. It has shown high specificity and high affinity for GPIIb/IIIa and a short half-life [91]. Eptifiban (Figure 2.6) affords rapid, competitive and reversible platelet inhibition when administered with concomitant aspirin and heparin in-patients undergoing elective percutaneous coronary intervention [88]. A large, multicentre study designed to assess the use of eptifiban on patients undergoing coronary intervention supported the notion that the drug does not increase the risk of bleeding [92]. During coronary bypass surgery, eptifibatide did significantly decrease the incidence of perioperative MI.

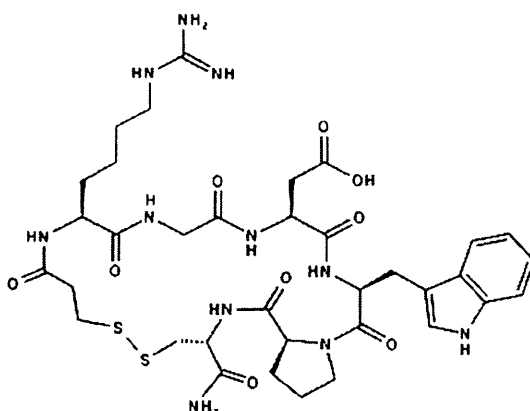


Figure 2.6 Chemical structure of Eptifiban
(Image adapted from www.pharmazeutische-zeitung.de)

2.2.2.4.3 Tirofiban - Tirofiban (Aggrastat™) is a tyrosine derivative that inhibits fibrinogen binding to GP IIa/IIIb. This particular drug is an Arg-Gly-Asp (RGD)-based peptidomimetic that effectively blocks the surface glycoprotein

GPIIb/IIIa receptor reducing thrombin generation and subsequently platelet aggregation and secretion [93, 94]. The drug is used intravenously administered, together with heparin for coronary applications (unstable angina, non-Q-wave MI, and angioplasty) but not in-patients who have hypertension or have had haemorrhagic stroke or suffered trauma. The use of tirofiban (Figure 2.7) with heparin has resulted in a significant decrease in the composite endpoints of death, MI and refractory ischemia [44, 95, 96]. However, the benefit has been short term for patients with acute coronary syndromes. The most common complication with this drug is excessive bleeding and in ~5% of such patients pelvic pain and slowing of the heart rate together with dizziness.

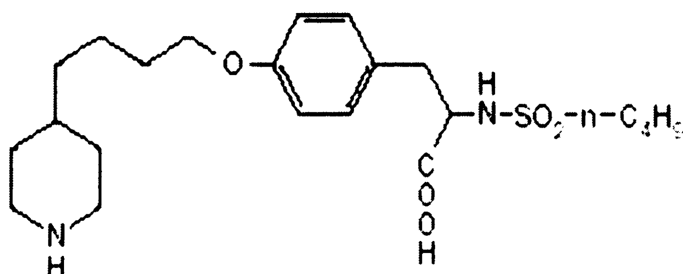


Figure 2.7 Chemical structure of Tirofiban
(Image adapted from www.pharmazeutische-zeitung.de)

2.2.3 Anticoagulant agents

Anticoagulant drugs represent a wide group of natural agents, recombinant agents' equivalent to some of the naturally occurring proteins and synthetic agents. This group of drugs is characterized by marked structural and functional heterogeneity. The coagulation cascade reaction result in the formation of thrombin and subsequently fibrin. The thrombogenic effects of thrombin can be

inhibited by inactivation of the enzyme or by preventing thrombin generation from precursor coagulation proteins (Figure 2.1). Agents can inactivate thrombin indirectly, by activating naturally occurring thrombin inhibitors or directly by binding to thrombin and preventing it from interacting with its substrates.

2.2.3.1 Coumarin derivatives

The coumarin compounds in common clinical uses are warfarin (Coumadin), acenocoumarol and phenprocoumon. These oral anticoagulants induce their anticoagulant effect by inhibiting the hepatic synthesis of 4 vitamin K–dependent coagulation proteins: factors II (prothrombin), VII, IX and X that act sequentially to produce thrombin (Figure 2.1) [97, 98].

Warfarin (Figure 2.8) is used to prevent and treat patients with venous thrombosis and pulmonary embolism (Table 2). It is also used to treat and prevent dangerous blood clotting in-patients with atrial fibrillation and in some cases, to prevent stroke. Since warfarin is an oral anticoagulant, it is used for long term anticoagulation therapy. However it has several disadvantages in use. It interacts with a wide range of drugs, which inhibit or induce liver metabolism, reduce binding to serum proteins, affect vitamin K dependent factors or absorption of warfarin. Furthermore, risk of haemorrhage is significantly increased in elderly patients and with concurrent administration of aspirin [99].

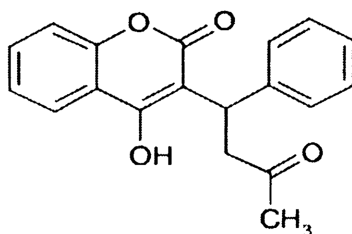


Figure 2.8 Chemical structure of Warfarin

2.2.3.2 Heparin

Heparin (Figure 2.9) is a family of glycosaminoglycans of various molecular weights. A specific pentasaccharide in the heparin molecule is the crucial structural element for the high-affinity binding of heparin to ATIII, and thus for heparin anticoagulant activity. Heparin is an indirect thrombin inhibitor. ATIII is the molecular target of heparin and when it is activated it binds either thrombin (activated factor II) or activated factor X (Figure 2.1).

Low molecular weight heparins (LMWH) are produced by enzymatic or chemical degradation of unfractionated heparin (UFH), and consist of smaller polysaccharide chains with higher ratios of anti-Xa: anti-IIa activity than UFH (Table 2.2) [91].

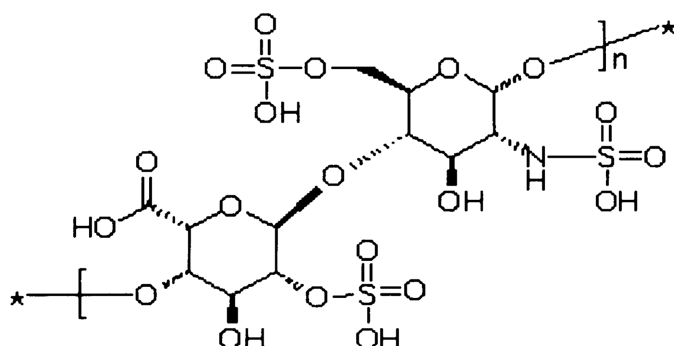


Figure 2.9 Chemical structure of Heparin (n = number of polysaccharide chains)

UFH has been the established therapy for prevention and treatment of thrombotic disorders for many years. Although safe and effective, there are substantial problems, including the need for regular laboratory monitoring, wide differences in responses between patients and the small risk of a potentially life-threatening, heparin-induced thrombocytopenia (HIT). LMWH differ from those

of UFH, because they have greater bio-availability after subcutaneous administration (about 100%), longer half-life, dose-independent clearance and more predictable anticoagulant response due to these factors as well as less binding to plasma proteins and vascular cells. At a higher dose these drugs are used to treat active thrombotic disease and at lower dose to prevent thrombosis. The LMWHs, dalteparin, enoxaparin, and tinzaparin were evaluated in unstable angina and have been found to be safe and effective alternative to heparin therapy or aspirin alone for patients with unstable coronary artery disease [70, 88, 100]. Recent trials have shown, however, that differences exist in safety and efficacy between different LMWHs [101, 102].

Although heparin (UFH and LMWH) can inhibit thrombin function, it has a number of drawbacks as a therapeutic agent. Heparin requires ATIII as a cofactor for anticoagulation. In addition, heparin has no affinity for clot-bound thrombin and thus is ineffective in dissolving pre-existing clots [103]. A small but important percentage of patients (1-10%) develop HIT, a potentially severe complication that can lead to limb amputation or death.

2.2.3.3 Hirudin and its derivatives

Natural hirudin is not a single entity but rather a generic name for a group of structurally similar single-chain polypeptides ('hirudins') of a length of 65 or 66 amino acids. Initially it is isolated from the salivary glands of the medicinal leech, *Hirudo medicinalis* [104, 105], and it is now available through recombinant DNA technology (r-hirudins, including lepirudin and desirudin) (Table 2.2)

Anticoagulant action of hirudin is the result of potent, direct and specific inhibition of the enzymatically active site of thrombin. Hirudin inhibits free and fibrin (clot)-bound thrombin (Figure 2.1). Several recent trials have evaluated the safety and efficacy of hirudin when given in the setting of acute MI, unstable angina, or percutaneous transluminal coronary angioplasty.

The data from organisation to assess strategies for ischemic syndromes (OASIS-2) suggest that r-hirudin is superior to heparin in preventing cardiovascular death, myocardial infarction, and refractory angina with an acceptable safety profile in patients with unstable angina or acute MI without ST elevation [56].

Hirudin unlike heparin, the anticoagulant action is not dependent on circulating ATIII or heparin cofactor II. Thus, r-hirudin can be used effectively in ATIII- and heparin cofactor-deficient patients. In addition, anti-heparin proteins do not inactivate hirudin, e.g. platelet factor 4, which neutralize the anti-coagulant activity of heparin. Other benefits of hirudin include a more uniform anticoagulant effect, much weaker allergenicity and a relative lack of effect on the endothelium.

2.2.3.4 Bivalirudin

Bivalirudin (Hirulog™) is a 20 amino-acid peptide containing the two active sites of hirudin separated by a (Gly) 4 bridge. It is a direct-acting irreversible thrombin inhibitor (Figure 2.1), which can inactivate both soluble and clot-bound thrombin. When bound to thrombin, all effects of thrombin are inhibited, including activation of platelets, cleavage of fibrinogen, and activation of the positive amplification reactions of thrombin XI. Advantages over heparin include activity against clot-bound thrombin, more predictable anticoagulation, and no inhibition by components of the platelet release reaction.

Drug-drug interaction studies have found no clinically relevant interactions between bivalirudin and ticlopidine, abciximab, tirofiban or eptifiban. Bivalirudin is well tolerated by patients previously receiving LMWH and switching from heparin to bivalirudin reduces ischemic and bleeding event.

In patients undergoing percutaneous coronary interventions, bivalirudin has been associated with equivalent efficacy but lower bleeding rates than UFH [106, 107]. During coronary angioplasty for unstable angina, bivalirudin has reduced ischemic complications and bleeding after angioplasty [108].

2.2.3.5 Argatroban

Argatroban is a direct thrombin inhibitor that reversibly binds to the thrombin active site. It is derived from L-arginine and is capable of inhibiting the action of both free and clot-associated thrombin and it does not interact with heparin-induced antibodies.

Argatroban is the first synthetic direct thrombin inhibitor approved for the prevention and treatment of thrombosis in patients with HIT (Table 2.2). Argatroban anticoagulation, compared with historical control subjects, improved clinical outcomes in patients who had HIT, without increasing bleeding risk [109].

The safety and efficacy of the drug as an adjunctive treatment is tolerated well in patients with acute MI compared to heparin [110]. It is indicated that argatroban is an effective and safe drug for the treatment of acute cerebral thrombosis [111] and easy to monitor and control with little potential for underdosing or overdosing, regardless of age, gender, or renal function [112]. Argatroban, as compared with heparin, appeared to enhance reperfusion with tissue plasminogen activator (TPA) in patients with acute MI, particularly in those patients with delayed presentation and the incidences of major bleeding and adverse clinical outcome were reduced[113].

Antiplatelet agents	Date approved	Dose	Ligand	Mode of action	Indication
Salicyclic acid (Aspirin™)	1988	75-300mg daily	COX	Irreversibly inhibit enzyme COX to block the formation of thromboxane A2	Primary & secondary prevention of thrombosis in patients with established vascular disease
Dipyridamole (Persantine™)	1997	300-600mg daily in 3-4 divided doses	Phosphodiest erase	Inhibit phosphodiesterase enzyme result in increase intracellular cAMP	Secondary prevention of stroke, for maintenance of patency of coronary bypass grafts
Abciximab (Reopro™)	1994	0.25mg/Kg IV bolus 0.125μg/Kg/min IV	GPIIb/IIIa	Blocks receptor GPIIb/IIIa from binding to fibrinogen & inhibit platelet recruitment	Prevention of cardiac ischemic complications in patients undergoing PCI and with unstable angina
Tirofiban (Aggrastat™)	1998	0.4μg/kg/min IV for 30min, then 0.1μg/kg/min	GPIIb/IIIa	Blocks receptor GPIIb/IIIa from binding to fibrinogen & inhibit platelet recruitment	Treatment of acute coronary syndrome
Eptifibatide (Integrilin™)	1998	0.18mg/Kg IV bolus 2μg/Kg/min IV	GPIIb/IIIa	Blocks receptor GPIIb/IIIa from binding to fibrinogen & inhibit platelet recruitment	Treatment of acute coronary syndrome (unstable angina and non-Q-wave MI)
Ticlopidine (Ticlid™)	1991	250mg twice daily	ADP-induced GPIIb/IIIa complex	Inhibits platelet aggregation induced by thrombin, collagen, arachidonic acid & platelet activating factor	Secondary prevention of thrombosis (MI, stroke & vascular death) in patients with established vascular disease
Clopidogrel (Plavix™)	1997	75mg once daily	ADP-receptor GPIIb/IIIa	Inhibit ADP & thrombin induced platelet aggregation.	Prevention of atherosclerotic events in patients with history of ischemic stroke, MI

Table 2.1 Antiplatelet agents clinically used their mode of action and indication.

Dates approved FDA and drugs doses from BNF

Anticoagulant Agents	MW (Da)	Half-life (hour)	Mode of action	Dose	Indication
Warfarin (Coumadin™)	308	6	Inhibit synthesis of vitamin-K dependent coagulation factors, factor II, VII, IX and X	2.5-10 mg daily	Treatment of venous thrombosis and prevention of venous thromboembolism in patients with MI
Heparin (UFH)	15,000*	1-2	Binds to & activates AT-III to inhibit factor IIa and Xa	5000-10000U IV bolus, followed 15-25U/Kg/h	Prevention & treatment of arterial & venous thrombosis, prophylaxis in general surgery
LMWHs	5,000*	4-6	Activates AT-III to inhibit factor IIa & Xa		
Enoxaparin (Lovenox™)				2000-4000U daily	Treatment of DVT, Unstable angina & MI
Dalteparin (Fragmin™)				2500-5000U daily	Prophylaxis of postoperative DVT & pulmonary embolism.
Certoparin (Alphaparin™)				3000U daily	Prophylaxis of postoperative DVT
Hirudin and its derivatives	7000*	1-2	Direct thrombin inhibitor that reversibly binds to thrombin active site	0.4mg/kg bolus	For the prophylaxis of postoperative DVT in patients undergoing hip replacement
	6980	1.3		0.15mg/kg/hr IV	
Lepirudin (Refludan™)					
Bivalirudin (Hirulog™)	2180	0.5	Direct thrombin inhibitor that irreversibly binds to thrombin active site	1.0mg/kg bolus followed 2.5mg/kg/h daily	Treatment of thromboembolic diseases in patients with HIT
Argatroban	527	0.5-1	Direct thrombin inhibitor that reversibly binds to thrombin active site	2μg/kg/min	Prevention of unstable angina, MI, and PCI

Table 2.2 Anticoagulant agents clinically used, their mode action and indication.

Drug doses from BNF

2.3 Current applications of Antiplatelet and Anticoagulant Agents in surface modification of biomaterials

2.3.1 Biologically active coatings

The heparinisation of biomaterials was first reported in 1963[16]. As heparin has a strongly anionic, ionic bonding is readily achieved on surfaces pre-treated with a cationic substance such as colloidal graphite. A general disadvantage of this method has been the rapid release of heparin upon exposure to blood or plasma[114]. Despite this a number of studies on heparin-coated biomaterial devices have been shown to enhance various aspects of blood compatibility. Several coating techniques including covalent immobilisation (Table 3) have been investigated and commercialized by various companies.

The two most commonly used heparin-coated systems are the Carmeda Bioactive Surface® and Duraflo II®. Heparin coating using the Carmeda Bioactive Surface uses the so-called "End-point immobilization". This involves covalent binding of heparin to the substrate resulting in a chemical modification[115]. The reaction in heparin occurs only at one end so that the overall structure particularly on the antithrombogenic site is not changed. This enables the heparin molecule to be tied to the surface only at one end and the remainder is free and still relatively bioactive. Duraflo II heparin coating is an ionically bound benzalkonium-chloride complex which enables relatively firm connections with the surface compared to Carmeda. Other techniques for heparin coating include the Bioline® (Jostra), AOThel®, Corline® and 3M methods. The Bioline coating method employs natural surface substances such as polypeptides to bind the heparin to the polymer. As a result stable bonding of the heparin molecule is achieved by formation of covalent bonds and ionic interactions between the heparin molecule and the immobilized polypeptide[116]. The recent and newly developed heparin-coated technique from the 3M company is processed in a similar way to the Carmeda Bioactive coatings. It involves a covalent binding of an oxidized

heparin to a layer of coated biomaterial. Currently this procedure is still under preclinical evaluation and awaiting clinical introduction in the near future.

Name and Approved Date	Coating techniques	Clinical Uses
Carmeda Bioactive Surface® (Carmeda), 1983	Covalent binding of heparin "End-point immobilization"	Vascular graft, coronary stent, oxygenation systems and extracorporeal device.
Duraflo II® (Baxter), 1988	Ionic binding of heparin-benzalkonium- chloride complex	Aortocoronary bypass operations
BioLine coating® (Jostra), 1992	Covalent and ionic binding of heparin to an immobilized polypeptide	All components of extracorporeal device including silicon
AOThel® (Artificial Organ Technology), 1997	LMWH	All components of extracorporeal device and oxygenator,
Corline system AB® (corline), 1997	Covalent binding of macromolecular heparin conjugate to an inert polyamine chain	Glass, metal and synthetic polymers,
3M, under pre-clinical evaluation	Covalent binding of oxidized heparin by the addition of cyanoborohydride, to a layers of coated surface	Metal surfaces and polymers

Table 2.3 Heparin coating techniques approved for clinical purpose and their uses.

Keys: LMWH, Coating with certified low molecular weight heparin.

2.3.1.1 Extracorporeal circuits

In vitro, heparin-coated extracorporeal circuits (ECC) reduce formation of C3 complement activation products and soluble C5b-9 complexes[117, 118], granulocyte activation[119] and reduce leucocyte activation and adhesion[120]. The Carmeda Bioactive heparin coated ECC has significantly reduced platelet adhesion[121], platelet, granulocyte and complement activation. In addition post operative blood loss during cardiopulmonary bypass has been reduced significantly[122-124]. The heparin-coated (Duraflo II) cardiopulmonary bypass circuit combined with full systemic heparinisation was found to limit both pro-inflammatory responses and anti-inflammatory responses to cardiopulmonary bypass (CPB) and ischaemia[45, 125-127]. This may significantly contribute to a reduction in myocardial ischaemia-reperfusion damage that has been subsequently observed. It also showed significant reduction of C3 and C4 complement activation[45-47] with reduced systemic heparin[128] and the formation of kallikrein-C1-inhibitor complexes (contact system activation) during cardiac operations[129] compared with the control group.

An *in vitro* study of heparin coated Duraflo II circuit showed higher plasma heparin concentration than the Carmeda Bioactive Surface indicating unstable heparin bonding this resulting in leaching of the coating[48]. However a comparison of the Carmeda Bioactive heparin-coated system and the Duraflo II heparin-coated system showed no clinical differences after coronary artery bypass operations in combination with reduced systemic anticoagulation[44].

In low-risk coronary artery bypass surgery the heparin-coated circuit, Bioline has significantly reduced inflammatory responses such as neutrophil and complement activation and pro-inflammatory cytokine production. However it did not affect platelet activation, coagulation or the fibrinolyis cascade under full systemic heparinization. As a result, no improved clinical outcome was observed[116]. The AOThel coating method uses LMWH instead of UFH. The clinical and coagulatory effects of AOThel coated ECC have been studied randomly in patients undergoing cardiopulmonary bypass

graft. Thrombin generation was elevated significantly and platelet activation decreased. Significantly less post-operative bleeding and a correspondingly lesser need for blood replacement occured only if AOThel coated ECC use was combined with low doses of systemic heparin[130] being administered.

The Corline heparin surface is produced by means of a uniform macromolecular heparin conjugate. This conjugate consists of multiple heparin molecules, which are covalently bound by specific linkers to an inert polyamine chain. The conjugate binds to those surfaces of the medical device that come into contact with blood[131]. Corline-coated ECC systems have been in clinical use, however it is still to early to comment on their clinical outcomes.

2.3.1.2 Stents

For the past several years intravascular stents have been in use to manage acute occlusion and restenosis after coronary angioplasty[132]. A restenosis rate of 30-50% after balloon angioplasty has been reduced to a current rate of 10-30% after stenting. One limitation of stent implantation, however, is acute or subacute thrombotic occlusion. Research has shown that successful therapy with antiplatelet drugs reduced stent thrombosis or occlusion rates to 0.8-1.9%[133, 134]. In addition the application of bio-compatible coating of stents was introduced to prevent thrombosis.

It has been hypothesised that heparin coating of stents lowers the stent thrombosis rate, minimizes the adhesion and activation of platelets and granulocytes, and decreases the activation of coagulation and complement. Experimental studies have demonstated the ability of heparin coating stents (such as Palmaz-Schatz, Duraflo II) to reduce platelet adhesion[135-137]. Correspondingly, clinical trials with heparin-coated stents showed a lower rate of subacute thrombosis[138, 139]. However, comparison of the Corline heparin coated stent versus uncoated stent, showed no influence on clinical outcome and stent thrombosis or restonosis to the uncoated version of the stent[48].

The same techniques that have been applied to improve the blood compatibility of vascular grafts have also been shown to enhance the quality of stents. In addition to heparin coating, Phosphorylcholine-based polymer (Biodiv Ysio™) and carbofilm (Sorin Carbostent™) coated stents provided high biocompatibility and were developed to further reduce the risk of stent thrombosis[140-143].

2.3.1.3 Bypass grafts

Synthetic conduits (especially those of <5mm diameter) used for blood vessel replacement have certain disadvantages which greatly limit their application for long-term usage as discussed earlier. Extensive research has been carried out to overcome these problems. Covalent bonding or immobilisation of heparin improved thromboresistance of polyethyleneimine *in vitro*[115]. When heparin-bonded Dacron was used for a femoral-popliteal bypass graft in human, improved patency was achieved compared to uncoated ePTFE[144].

It has been shown in a number of *in vitro* studies that a heparinised matrix loaded with basic fibroblast growth factor (bFGF) improves proliferation of human umbilical cord vein endothelial cell (HUVEC). This proliferation will probably lead to a more rapid formation of a confluent monolayer of ECs on a bypass graft surface *in vivo*[145-147]. In a canine model ePTFE coated with fibrin glue containing fibroblast growth factor type 1 (FGF-1) and heparin improved retention of seeded ECs[148] and showed significantly less platelet deposition than uncoated ePTFE[149, 150]. However, this decrease in platelet deposition was suggested to be due to the fibrin glue rather than the heparin[148] itself.

Biochemical experiments *in vitro* have indicated reduced thrombogenicity and lowered adhesion of blood platelets after the dipyridamole treatment of a polyurethane vascular graft (Chronoflex)[50, 70]. *In vivo* experiments, in goat and sheep models have not provide evidence for a beneficial effect of the dipyridamole coating. Moreover covalent immobilisation of dipyridamole to polyurethane graft via photo-modification showed a virtually undisturbed

lumen, on which a confluent layer of endothelial-like cells was observed[151] further studies also demonstrated improved thromboresistance [152].

The direct thrombin inhibitor, r-hirudin has also been studied *in vitro* for surface modification of biomaterials. Whole blood sample containing hirudin showed about 50% reduction in platelet deposition to tetrafluoroethylene-propylene co-polymer during low-stress shear flow[153]. It has been found that increasing Angstrom distance between a compound and the biomaterial surface increased biological activities[154]. Based on this foundation, one such approach has been the covalent binding of r-hirudin to an intermediate `basecoat` compound, bovine serum albumin (BSA)[52, 53]. Binding sites for r-hirudin were generated on BSA via the cross-linker sulphosucinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate [sulpho-SMCC] that was reacted in various molar ratios (1:50) with BSA. These complexes, BSA-SMCC were then covalently linked to a sodium hydroxide-hydrolysed Dacron surface[53] or polyurethane surface containing surface bound carboxylic acid groups[52]. Both studies demonstrated that *in vitro*, r-hirudin could be covalently bound to a biomaterial surface while maintaining its ability to bind and inhibit thrombin. *In vivo* assessment of the Dacron surfaces with covalently bound r-hirudin, showed no gross thrombus and a thin pseudointima of platelets and plasma proteins. In contrast, the control patches without r-hirudin had a thick pseudointima composed of fibrin-rich thrombus. It was found that covalently bound r-hirudin to Dacron patches, helped its biological activity as well as preventing thrombus formation on the graft surface[51].

Extensive research over the last decade has been performed on the incorporation of adhesion promoting peptides onto biomaterial surfaces. Since the identification of the RGD peptide sequence as mediating the attachment of cells to several plasma and ECM proteins, including fibronectin and vitronectin, researchers have been incorporating RGD-containing peptides onto biomaterials to promote cell attachment.

An RGD-containing peptide, GRGD, in solution inhibited fibrinogen binding to ECs and fibrinogen-induced ECs migration[155]. When this peptide was

photochemically grafted onto the surface of polyethylene glycol modified polyurethane (PU-PEG) to form PU-PEG-GRGD, it improved ECs adhesion and growth on the surface. The enhancement efficiency was well correlated with GRGD content[156]. It has also been shown that coating an ePTFE graft surface with RGD-containing synthetic peptides significantly improved ECs seeding of ePTFE grafts[157]. Other studies have shown that covalent bonding of RGD-containing peptides based on cell-adhesive regions of fibronectin, Arg-Gly-Asp-Ser (RGDS) and vitronectin, Arg-Gly-Asp-Val (RGDV) to a polyurethane graft backbone via amide bonds enhanced cell adhesion and spreading[158]. Another study showed that a Gly-Arg-Gly-Asp-Val-Tyr (GRGDVY) grafted substrate supported a larger number of adherent cells and a higher extent of cell spreading than a Gly-Arg-Gly-Asp-Ser-Tyr (GRGDSY) - grafted substrate[159]. Recently covalent immobilization of RGD and heparin onto the surface of a poly(carbonate-urea)urethane (MyoLink™) graft have been shown to result in a significant improvement of cell retention of ECs after seeding[37, 160, 161].

Percutaneous trans-luminal angioplasty (PTCA) has become a very commonly practiced clinical procedure to treat diseased vessels. During PTCA guide wires and catheters are required in order to deliver the required stent. However, a high restenosis rate (30-50%)[162] post 6 months remains a serious problem despite a variety of therapeutic schemes being implemented[163]. The chronic complications include IH and mural thrombosis[164]. The systemic administration of anti-thrombotic drugs post PTCA has been widely advocated clinically and has reduced the restenosis rates, but it is associated with systemic toxic effects and haemorrhage[165]. Local delivery of anti-platelet and anti-thrombogenic has become a means of therapeutic manipulation of the angioplastic site[166].

Richey and co-workers[167] attached argatroban to two anionic monomers to high-density polyethylene balloon catheters, acrylic acid at 70μg/cm^2 and 2(dimethylamino)ethyl methacrylate at 48μg/cm^2 by UV polymerisation. Surface grafting was verified by contact angle, X-ray photoelectron spectroscopy and zeta potential measurements. In the rabbit common carotid artery animal model 280nmol/g tissue argatroban were

found within the ballooned arterial segment immediately after angioplasty, followed by a decrease after blood flow was restored[167]. Hydrogels have been used to attach nadroparin, a low molecular weight heparin to balloon catheters, and in a pig iliac artery model it was found that 98IU were delivered to the angioplasty site and distal vessel[168]. A trend towards decreased platelet deposition was observed but statistical significance was not achieved (p=0.1563), coupled with medial SMC proliferation in nine of ten pigs (p=0.0137). In addition heparin (40000U/ml) was attached in a hydrogel to balloon catheters and it was found in a small clinical trial (n=33) that a mean primary patency of (p=0.0174) was obtained at 143 days. However, it was concluded that local delivery of heparin did not lead to a significant reduction in restenosis[169].

The Carmeda process has been used to covalently attach heparin to the surface of balloon catheters and in a rat jugular vein animal model it was found that patency lasted 30 days, with fewer lesions at the site of angioplasty being observed and lower levels of bacteremia. It was concluded that covalent coating of heparin significantly prolonged patency compared to either ionic or hydrogel type coatings[170]. In the HEPACOAT (Hepacoat and an antithrombotic regimen of aspirin alone) study of two hundred patients were the Carmeda covalently coated balloon catheter was used the primary end point of stent thrombosis at 30 days occurred in 2 of 200 patients (1%) and it was concluded that the procedure was safe in patients with de novo or restenotic lesions in native coronary arteries and that the heparin coating provided additional protection against stent thrombosis[171].

The guide-wires used in PTCA have also been coated a recent study by Hanssen and colleagues showed that copolymers of N-vinyl-2-pyrrollidinone and n-butylmethacrylate a type of hydrogel when incorporated with heparin the release obeyed first-order kinetics and lasted >50m in a goat animal model and left them virtually clear of thrombus[172].

2.4 Usage in development of antithrombogenic elastomers for novel scaffolds in tissue engineering

Scaffolds play a prominent role in tissue engineered cardiovascular devices since they provide a three dimensional framework for cells to attach, proliferate and lay down ECM. They also provide initial mechanical stability, support tissue and may serve as carriers for cells, growth factors and other biomolecular signals[173]. Biodegradable materials used as scaffolding for tissue engineering fall into two main categories. The first category includes natural ECM molecules such as collagen[174], chitosan[175], gelatine[176] and alginate[177]. Secondly there are the synthetic materials, examples of which include poly(ethylene glycol), dextran, poly(vinyl alcohol), polylactide, polyester and polyacrylamide. Natural ECM molecules have the potential advantage over the synthetic materials of inducing specific cell interactions. However, they are not easily available in large amounts and are mechanically weak which limits their biomedical applications[178]. On the other hand synthetic materials are more reproducible in their manufacture and microstructure. In addition their degradation rate can be manipulated and controlled.

An ideal scaffold should bio-absorb in vivo at a pre-defined rate so that the three-dimensional space occupied by the initial scaffold is replaced by generated host. Thus the materials used in their fabrication serve as temporal conduits whilst simultaneously allowing the complex interactions between the arterial wall, host macrophges and biomaterial to occur. A variety of natural and synthetic derived hydrogels have shown great promise as scaffold for tissue engineering (providing growth factor and drug incorporation and entrapment of viable cells). Hydrogels are formed by modifying the mechanical and physical properties of the polymers. By introducing various chemical crosslinkers[179, 180] (i.e. glutaraldehyde, formaldehyde, carbo-diimide) by crosslinking with physical treatments[181] (i.e. UV irradiation, freeze-drying, heating), and by blending it with other polymers[182-185] (i.e. hyaluronic acid (HA), poly(lactic acid) (PLA),

poly(glycolic acid), (PGA), poly(lactic-co-glycolic acid) PLGA), chitosan and PEO).

Adhesion proteins (fibronectin, collagen, albumin and fibrin), their peptide motifs (RGD peptides), growth factors and polysaccharides and other closely related anticoagulant-platelet agents (heparin, hirudin and salicyclic acid) have been covalently bound to biomaterial surfaces. Matsuda and Magoshi[186] derivatised heparin with a styryl or methacryloyl group by condensation with either 4-vinylaniline or –benzoic acid. The vinylated heparin was then copolymerised with styrenated gelatin and diacrylated poly(ethylene glycol) (PEG) and photo-cured this resulting in a tubular scaffold. Mizutani[187] used coumarin to end-cap tetra-branched copolymers of ε-caprolactone and trimethylene carbonate with pentaerythritol or four-branched PEG as the initiator after, which UV irradiation resulted in photo-cured solid biodegradable polymers. The group of Erdmann[188] synthesised a degradable poly(anhydride-ester) by melt condensation polymerisation of an acetylated monomer to, which was attached salicyclic acid. It was found that at pH7 the salicyclic acid was released with 50% content by day 20 and polymer degradation being complete by 90 days. It was found that these polymeric materials resulted in reduced inflammatory reaction and so had possibilities as scaffolds for engineering purposes.

The coating of adsorbed silyl-heparin, benzyl-bis(dimethylsilylmethyl) oxycarbamoyl-heparin[189]has been shown to be a good system to attach to polymer surfaces and allow local delivery of growth factors such as basic fibroblast growth factor (bFGF). After only 4 days capillary tube formation was observed in human ECs. In a similar study bFGF was immobilised and shown to increase the proliferative potential of ECs when immobilised onto albumin-heparin-poly(acrylic acid) constructs. Using UV irradiation this was attached to a polyurethane surface[190]. This growth factor has also been immobilised in heparin, poly(lactic-co-glycolic acid), alginate scaffolds[191, 192]and when implanted in a rat animal model shown to increase matrix revascularisation, whereby at day 10 capillary density was 45+/-3/mm^2 and it increased to 7045+/-3/mm^2 by day 21. It was concluded these scaffolds showed promise as scaffolds for tissue engineering.

It has been shown that the immobilization of r-hirudin with glutaraldehde as a coupling agent improves the blood contacting properties of the biodegradable polymer poly (D-L –lactide-co-glycolide) RG756[193]. The polymer surface was first activated by incubation with glutarldehyde in distilled water, followed by incubation with r-hirudin in phosphate buffered saline. The results of this *in vitro* study indicated that the effect of hirudin was limited by the fact that the amount of hirudin directly bound to thrombin reached saturation and therefore was unable to bind more in contrast to heparin which acted as an indirect thrombin inhibitor. On the contrary, hirudin immobilization may lead to a passivation of the surface, which is restricted by thrombin generation on the artificial surface.

NSAIDS have also been used as starting materials for the development of three-dimensional engineered scaffolds[192]. Liquid acrylate-endcapped poly(ε-caprolactone-co-trimethylene carbonate) was prepared the acrylate group allowing the subsequent terminal capping attachment of the NSAID indomethacin. This polymer was then converted into 3D constructs using stereo-lithography and UV irradiation together with a computer aided design programme. On implantation in a rat animal model histological haematoxylin and eosin (H&E) and periodic acid-schiff (PAS) staining showed that the slow diffusion of the NSAID into the surrounding tissue significantly reduced the foreign body inflammatory reaction. It was concluded this approach showed great promise as a scaffold for biomaterial and engineering applications[192].

A summary of investigations into the surface modification of cardiovascular polymers is included in Table 2.4 showing the type of modification carried out, the polymer type, if the polymer was seeded with cells or not and providing references to the appropriate publications. The data showed that the surface modification will enhance cell adhesion and product compatibility.

Table 2.4 Overview of employed surface modification techniques for cardiovascular polymers application.

Keys: Argatroban (ARG), Carotid artery (CA), Fibrin glue (FG), Hirudin (rHir), Monomethoxy poly(ethylene glycol) (MPEG), Plasminogen Activator Inhibitor-1 (PAI-1), poly(dimethyl siloxane) (PDMS), Polyethylene (PE), Poly (ethylene oxide) (PEO), Poly(lactic acid) (PLA), poly(L-lysine) (PLL), Prostaglandin E-1(PGE1), Prostoglandin Inhibitor 2 (PGI2), tissue-Plasminogen Activator (t-PA), Decrease/inhibited (↓), Increase/enhanced (↑).

Year Pub.	Moeities	Polymer	SM technique	Test/cells	Outcome
1983[115]	Heparin	Polyethyleneimine	Covalent immobilisation.	In vitro	Improved thromboresistance compared to control.
1988[194]	Fibronectin	PTFE	Coating	Canine Jugular vein ECs	↑ ECs coverage & platelet reactivity of SM group at 2 weeks patency not adversely affected
1990[195]		PTFE	Ammonia plasma modified surface	In vitro Bovine ECs	ECs attachment to after 24 hrs on control and SM surfaces were 36% and 92%
1991[196]		ePTFE	Surface hydroxylation using aluminum deposition and removal with sodium hydroxide	In vitro (14 days) Rat ECs	↓ Hydrophobicity of ePTFE & ↑ cell adhesion with SM
1992[159]	GRGDSY & GRGDVY	PU	Covalent immobilisation by surface carboxylation or via amide bond	In vitro HUVECs	Both supported cell adhesion and spreading
1992[197]	RGD & GRGDSY	Poly(tetramethylene oxide)-based PU	Covalent immobilisation by surface carboxylation or via amide bond	In vitro	RGD-containing was successfully grafted onto the polymer & confirmed by Sakaguchi assay and amino acid analysis.
1995[149]	Heparin with FG & FGF-1	ePTFE	Surface coating	Canine (Bilateral aortoiliac)	Platelet deposition ↓ on coated group ($p<0.05$) after 120 min. of flow circulation
1996[157]	Fibronectin & RGD	ePTFE	Surface coating	In vitro human vein ECs	↑ ECs attachment & retention with SM after shear stress
1997[50]	Dipyridamole	PU	Covalent immobilisation via photo-modification (UV)	In vitro	Dipyridamole retains its inhibitory activity with respect to activation and aggregation of blood platelets with SM

1997[53]	rHir	Dacron	Covalent immobilisation via carboxylic acid groups	In vitro	Improved thrombin inhibition in SM
1997[193]	rHir	Poly(D-L-lactide-co-glycolide)	Covalent immobilisation via glutaraldehyde	In vitro (30 min with blood)	SM ↓ clotting time, platelet adhesion and activation
1997[150]	Heparin, with FG & FGF-1		Surface coating	Canine (Carotid balloon injury)	SM with FG/FGF-1/heparin ↓ by 45% platelet deposition on balloon injured canine carotid arteries after 2 hours. No difference at 30 days patency
1998[52]	rHir	PCU	Covalent immobilisation via carboxylic acid groups	In vitro	Improved thrombin inhibition in SM
1998[198]		ePTFE	Ammonia plasma modified surface	In vitro Bovine ECs	SM enhanced the ECs lining under both constant and pulsatile flow conditions and ECs monolayer on SM graft surface was observed
1998[199]	Heparin	Dacron & PTFE	Surface-binding	In vitro	Fibrinogen levels in SM group ↑ compared with control >30min human blood. No difference between SM PTFE & Dacron
1999[51]	rHir	Dacron	Covalent immobilisation via carboxylic acid groups	Canine (thoracic aorta)	rHir maintained its biologic activity as well as preventing thrombus formation on the graft surface post 2 hrs implantation
1999[200]	MPEG	PU based on PDMS	Covalent immobilisation by allophanate and esterification reactions	In vitro	↓ Platelet adhesions with PDMS-based PUs incorporation of MPEG ↓ platelet adhesion
1999[201]	PEO	Silastic, PE , glass & ePTFE	Covalent immobilisation by gamma-irradiation	In vitro/Ex-vivo (Canine)	↓ Platelet deposition by 35% & ↓ fibrinogen adsorption by 70-95% in Silastic, polyethylene and glass but only 30% in ePTFE
2000[167]	ARG	PE balloon catheter	Ionic binding to UV modified surface	Rabbit (Carotid artery)	Local delivery of ARG (280 nmol/g tissue) was achieved immediately after angioplasty.

2000[202]	L-lactide	PU	Plasma glow modification & immobilisation of bio-active molecule	In vitro HUVECs	↑ Surface hydrophilicity and cell attachment with SM ↓ Platelet adhesion with SM
2000[203]	PEO	Nitinol stent / Dacron / ePTFE	Covalent immobilisation by gamma-irradiation	In vitro/In vivo (Canine)	↓ Fibrinogen adsorption by 70-95% with SM in vitro ↓ Thrombus formation by 85% on metallic stent in vivo
2000[204]	PEG	PTFE	Covalent immobilisation by photo-modification (UV) or coupling of hydroxyl groups	In vitro	↑ Surface hydrophilicity with SM
2000[205]	PGE1, heparin or phosphatidyl choline	PTFE/Teflon / Dacron	Plasma glow modification & immobilisation of bio-active molecule	In vitro	Fibrinogen adsorption and platelet adhesion on modified grafts were significantly ↓ in both static and pulsatile flow.
2001[151]	Dipyridamole	PU (5mm diameter)	Covalent immobilisation via photo-modification (UV)	Goat (CA) & sheep (CA)	Graft patency improved in goat (3/8) but not sheep in which deterioration of the polyurethane material was observed
2001[79]	Heparin	Dacron & ePTFE	Bonding	Human (Femoropopliteal bypass)	Improve patency over 50% with SM after a mean follow-up of 42 months
2001[156]	GRGD	PU	Covalent immobilisation via photochemical immobilisation of PEG	In vitro HUVECs	ECs were well adhered & growing on the SM Cells showed ↑ viability with increasing GRGD
2001[206]	Fibronectin , Collagen & Gelatin	PTFE	Ammonia plasma modified surface	In vitro HUVECs	All SM showed similar secretions of PGI2 and ↓ levels of PAI-1 secretion. Secreted t-PA activity ↑ with SM
2001[207]	GRGDS	PLA	Covalent immobilisation via PLL	In vitro Bovine ECs	↑ ECs spreading with SM
2001[143]	Phosphorylcholine	Stainless steel	Coating	Porcine (Coronary artery)	No significant difference in patency at 28 days

2002[208]	Gelatin, Albumin & RGD	PU	Covalent Immobilisation by cleavage of the mesyl end groups via PEO coupling	In vitro HUVECs	↑ Cell adhesion & proliferation with Gelatin & RGD
2002[209]		PTFE / Dacron	Ammonia plasma modified surface	In vitro HUVECs	↑ Adhesion and growth of ECs. SM did not exhibit a direct inflammatory effect in terms of monocyte adhesion
2002[210]		PTFE / Dacron	Ammonia plasma modified surface	In vitro HUVECs	↑ Cell adhesion in SM after 1 day with PTFE but not Dacron
2002[211]	Protein conjugated to PEG	Hydrogel	Covalent immobilisation by photo polymerization	In vitro Fibroblasts	Protein demonstrated integrin-binding capability based on the RGD. In addition heparin bound strongly to the protein's anti-thrombin III-based region and supported 3D outgrowth of human fibroblasts.
2002[160]	Heparin, RGD	PCU	Chemically bonded onto polymer at graft manufacture & extrusion using a modified ceric ion technique with two spacer arms	In vitro HUVECs	SM groups significantly improved ECs retention to flow shear stress ($P<0.01$)
2003[212]	Sulfobetaine	PU	Immobilisation by treatment with hexamethylene diisocyanate in toluene at 50° C in the presence of di-n-butyl tin dilaurate as a catalyst	In vitro	↑ Surface hydrophilicity & ↓ platelet adhesion.
2003[213]	Sulfonated polyrotaxane	PU	Blending followed by solution casting	In vitro	↑ Surface hydrophilicity, ↓ platelet activation, proteins & bacteria with SM
2003[214]	PEO conjugated with amino acids & RGD	PU	Non-covalent introduction by physical blending	In vitro HUVECs	Lysine & arginine demonstrated similar performance in cell adhesion and proliferation (at 96hrs) to that of RGD
2003[215]	Silyl-heparin	ePTFE	Adsorption onto a carbon coated surface	Canine (Bilateral aortoiliac)	Short lived (2hrs) improvement in thromboresistance. Patency in 7-days was 87.5% for heparin coated & 50% for control grafts

2003[216]		PTFE	Photochemical (UV) modification by UV at wavelength of 172 nm	In vitro (3-8 days) ECs	↑ Cell densities with SM & ↑ cell adhesion & proliferation with irradiation time
2003[152]	Dipyridamole with hydrophilic spacer chain	PU	Covalent immobilisation via photo-modification (UV)	In vitro	Improved thromboresistance in SM group

2.5 Conclusions

Interest in surface modification is growing and the techniques involved have applications in a wide variety of areas. The enhancement of surface properties has rapidly become an essential element in the developmental process for biomedical devices and scaffolds for tissue engineering. This is especially true of devices used for cardiovascular applications where the blood response to these artificial materials, including thrombosis and platelet deposition, continues to limit the long-term efficacy of these implants by causing vessel restenosis or wall reclosure. The surface modification of these materials is an attempt to increase the working life span of these implants by increasing the biocompatibility of the materials.

A wide variety of agents which prevent thrombosis or improve biocompatibility have been investigated. Several research groups have attempted to use antiplatelet and anticoagulant agents for surface modification purposes. However, the chemical and pharmacokinetic properties of these agents limit their uses for surface modification. For example, antiplatelet agents such as the thienipyridine derivatives require breakdown in the liver to become an active metabolite and induce antiplatelet action. This can also be the case for anticoagulant agents like the coumarin derivatives, which also induce their anticoagulation effects by inhibiting the hepatic synthesis of coagulation factors in the liver. To date heparin-coated devices are the only ones used in surgical practice. Although heparin can inhibit thrombin function, it has a number of drawbacks. Heparin requires antithrombin III as a cofactor for anticoagulation. In addition, heparin has no affinity for clot-bound thrombin and thus is ineffective in dissolving pre-existing clots. Finally heparin can degrade and be washed off over time when immobilized on material surfaces and therefore is not suitable for use on long-term blood-contacting devices. There is still scope for much further investigation in this area.

Endothelial cell seeding or tissue engineering is attracting growing interest and will have uses across a wide range of applications. In the case of

blood-compatible bio-mimetic coatings, a key objective is to encourage the growth of a layer of ECs over the device surface and prevent platelet attachment and blood coagulation. Comprehensive research has been performed on the incorporation of ECs adhesion promoting peptides into biomaterial surfaces; such as including RGD-containing peptides and ECM proteins including fibronectin and vitronectin.

In conclusion it can be said that no single approach, either through the use of antiplatelet or anticoagulant therapies or for surface modification or through cell seeding is likely to satisfactorily solve the problem of the lack of long term patency of blood-contacting biomaterials. However a combination of the various approaches may prove more successful in the future.

CHAPTER THREE:

MATERIALS AND METHODS

3.1. Cell Culture

3.1.1 Endothelial cell extraction from human umbilical cord

Human umbilical vein endothelial cells (HUVECs) were harvested from human umbilical cord vein [217]. All procedures were carried out using aseptic techniques in a flow hood. Human umbilical cords were obtained from the labour ward of the Royal Free Hospital, Hampstead and stored in 40ml of collecting medium consisting of 29.8ml basic medium, 10ml foetal bovine serum (Invitrogen, Paisley, U.K.) and 0.2ml gentamycin (Sigma Chemical Company, Dorset, U.K.). The basic medium was obtained from a stock solution made up from 500ml M199 medium (Invitrogen, Paisley, U.K.), 15ml of 7.5% sodium bicarbonate solution and 5ml penicillin/streptomycin solution consisting of penicillin 10,000U/ml and streptomycin 10mg/ml (Invitrogen, Paisley, U.K.). Cords were collected within 24 hours of delivery and used if free of clamp marks or needle holes. Each end of the umbilical vein was cannulated with 4cm lengths of nasogastric tubing and then secured with sterile silk ties. The cord was flushed several times with warm PBS to remove all clotted blood prior to instillation of 25ml of warm, filtered collagenase solution (consisting of 12.5mg collagenase A) suspended in 25ml of basic medium). Both ends of the vein were clamped and the cord incubated at 37°C for 10 minutes. The cord was massaged gently prior to collecting the collagenase/cell suspension into a 50ml centrifuge tube. The collagenase/cell suspension was then neutralised by the addition of an equal volume of complete medium obtained from stock made up of 157ml basic medium (40ml Foetal bovine serum and 3.6ml of 200mM L-glutamine solution (Invitrogen, Paisley, U.K.). The cell suspension was centrifuged at 300g for 7 minutes after which the supernatant medium was removed and the cell pellet resuspended in 6ml of warm complete medium. The cell suspension was then transferred to a 25cm^2 tissue culture flask and incubated at 37°C/5%CO_2. 24-hours later, the flasks were gently washed with 8ml PBS to remove red blood cells and fed with 6ml of complete medium. The flask was viewed daily under high power transilluminated

microscopy and the presence of EC's verified by confirmation of their characteristic cobblestone morphology. Once a confluent monolayer was achieved cultures were passaged by removing the cell culture medium, washing with 8ml PBS and then adding 3ml of 10% trypsin solution (Invitrogen, Paisley, U.K.). The flask was then incubated for 3 minutes prior to gentle tapping in order to loosen all the cells. The trypsin was then neutralised by the addition of 10ml complete medium. The cell suspension was spun at 300g for 7 minutes before discarding the supernatant, resuspending the cell pellet in 10ml complete medium, and placing in a gelatine coated 75cm^2 flask. Cultures were passaged every 2-3 days at a ratio of 1:2 and fed every other day.

3.2 Assessment of cell metabolism and survival

3.2.1 Alamar Blue™ assay

Alamar Blue™ (AB; Serotec Ltd., Kidlington, U.K.) is an assay designed to measure quantitatively cell metabolism and viability by incorporating resazurin and resarufin as colorimetric oxidation reduction indicators that change in colour in response to chemical reduction resulting from cell metabolism. The data may be collected with either fluorescence based or absorbance-based instruments. In this study absorbance was monitored at 570nm and 630nm. Resazurin has a much higher electrochemical potential than the carriers on the cell membrane, and on contact with the membrane, it is reduced to resarufin. Resazurin acts as an intermediate electron acceptor in the electron-transport chain between the final reduction of O_2 and cytochrome oxidase by substituting for molecular oxygen as an electron acceptor.

The rate of bio-reduction is related to the level of redox potential on the cell membrane, which in turn characterises the constitutive part of the metabolic activity of a given cell type. AB has certain properties that make this assay attractive. It is soluble in culture media, stable in solution, and

minimally toxic to cells and produces changes that are easy to measure. AB has been used as a measure of cell viability in tumor neurosis factor hyper-sensitive cell lines [218], studies of apoptotic neuronal death [219], and studies of lymphocyte proliferation [220].

Various approaches to the assessment of EC viability have been undertaken. Foremost are methodologies looking for known morphological factors, including ultrastructural studies that require substantial effort, skilled personnel, and often expensive equipment but during the extensive processing required do not yield quantifiable results and destroy the sample [221]. The assessments of EC membrane integrity with dye uptake and vital stains has been used as an indirect measure of viability, but they are terminal assays that destroy the cell or interfere with its function [222-224]. Continuous monitoring of EC viability is achievable by the measurement of glucose uptake and lactic acid release into an incubation medium, but such methodologies are labour intensive and relatively insensitive [225]. The reduction of colourless tetrazolium salts by mitochondrial succinate deyhdrogenase activity into an intensively colored formazan product uses hazardous reagents and requires washing, fixing, and extraction steps that destroy the cell [226-228]. Furthermore, the insoluble, intracellular crystals that disrupt the cellular membrane result in an extracellular precipitate attached to the polymeric substrate that on polyurethanes remains bound to the substrate even after extraction by detergent, thus affecting the outcome of this particular assay [229]. [3H]-thymidine incorporation has been used as a measure of viability and cell metabolism in EC [230] but radioisotopes have many disadvantages, including the terminal nature of the measurement, labour-intensive handling and disposal (along with expense), and excessive processing time with 3H-thymidine incorporation.

AB is not a new assay because it was first developed to determine how susceptible microorganisms are to various growth-inhibition products and has been used to examine bacterial antibiotic susceptibility [231] and yeast antifungal receptivity[232] and to analyze the cytotoxicity of drugs and chemotherapeutic agents in mouse fibroblasts, macrophages, and human tumour cells [233]. AB allows a continuous assessment of the metabolism

and viability of seeded cells, is simple to perform, and does not destroy the cells [217]. Limitations of AB are few. If prolonged incubation times are used (>24 h), reversal of the reduction process occurs via a secondary redox step, resulting in a colourless solution, particularly when very high cell concentrations are used. Microbial contamination would also reduce AB, thus yielding erroneous results, but this would affect any other assay of ECs as well.

AB was added to cell culture medium at a concentration of 10%. At each AB assay time point wells were washed with 1 ml phosphate buffered saline (PBS) and 1 ml of the AB/medium mixture added to each well. After 4 hours a 100µl sample of the AB/medium mixture was removed and the absorbance at 570 nm and 630 nm measured in a 96-well plate (Helena Biosciences, Sunderland, U.K.) using a Multiscan MS UV visible spectrophotometer (Labsystems, Somewhere, U.K.). The absorbance at 630 nm (background) was subtracted from that at 570 nm and results expressed as a percentage of the control (untreated) value. Four wells per treatment at each time point were measured and each experiment repeated four times.

3.3 Assessment of peptide purity and characterisation

3.3.1 High performance liquid chromatography (HPLC)

RGD-containing peptides were prepared manually using a solid-phase peptide synthesis and were assessed with reverse-phase HPLC for their purity and fourier transform infrared spectroscopy for characterisation. HPLC is a versatile form of chromatography used in a wide variety of applications to separate individual compounds of a particular class of molecule on the basis of size, polarity, solubility or adsorption characteristics. It is a very sensitive technique which can detect small differences in either absorbance or fluorescence as the elutate from the HPLC column passes through an appropriate detector.

The purity of each peptide was assessed by reverse-phase HPLC. The peptide sample (1mg/ml) was re-suspended in 0.1% TFA in water and

injected into a C_{18} (semi-preparative) TSK ODS 120T column (Pharmacia, Uppsala, Sweden) equilibrated in 100% buffer A (HPLC grade water containing 0.1% TFA) and 0% buffer B [90% (v/v) HPLC grade acetonitrile (Rathburn Scotland, UK) in water containing 0.1% TFA]. The peptide was purified on a linear gradient of buffer B (from 0% to 70% in 30 minutes) at a flow rate of 2.5 ml/minute. Sample peaks were detected spectrophotometrically by monitoring the absorbance of the eluent at 220nm using a Varian 5000 Liquid Chromatograph (Waters Limited, Hertfordshire, U.K.) equipped with a variable wavelength UV detector.

3.3.2 Fourier transform infrared (FTIR) spectroscopy

Infrared spectroscopy is a well-established method for investigating the secondary structure of proteins. The energy vibrations between chemical bonds (for example stretching, rotating and twisting) can be linked to the corresponding infrared (IR) regions of electromagnetic spectra. Vibrations may be localised to particular groups or bonds, examples of which include the O-H group or the C=O bond. In most cases vibrational modes are not caused by a single bond but are coupled to neighbouring bonds as well. In the case of proteins IR spectra are characterised by amide modes (bands) which represent set absorption regions. In the infra red region nine absorption bands, known as A, B and I to VII are produced by proteins. The most useful band for studying proteins is the amide I (~1630-1690 cm^{-1}) as it is closely correlated with protein secondary structure. This band is seen around 1650-1660 cm^{-1} for an α-helical conformation and at 1630-1640 cm^{-1} in the case of a β-sheet confirmation. Due to the fact that water absorbs strongly in the 1640 cm^{-1} region it is necessary to utilise a ^2H_2O solution, which does not cause such interference, when carrying out conformational studies.

FTIR spectra were obtained using a Perkin-Elmer 1750 FTIR spectrometer equipped with a fast recovery TGS detector and Perkin-Elmer 7300 computer for data acquisition and analysis. Samples were placed in a Beckman FH-01 CFT micro-cell fitted with CaF_2 windows and a 50μm Teflon

spacer for measurements in ^2H_2O. Temperature control of 30°C was achieved by means of a cell jacket of circulating water. The spectrometer was continuously purged with dry air to eliminate water vapour absorptions from the spectral region of interest. A sample shuttle was employed to permit the sample to be signal- averaged with the background. Measurements in ^2H_2O at 30°C were recorded at a peptide concentration of 10mg/ml and 200 scans were signal averaged.

All spectra were recorded at a resolution of 4 cm^{-1}. Aqueous buffer spectra were recorded under identical conditions as the sample spectra. Absorption spectra were obtained by digitally subtracting the solvent spectrum from the corresponding sample spectrum. Where appropriate, water vapour contributions were subtracted from the absorption spectrum using a previously recorded water vapour spectrum. Second derivatives were calculated over a 13 data-point range (13 cm^{-1}) using the Perkin-Elmer DERIV function to assign features of the composite amide I band to structural features present in the polypeptides.

3.3.3 Mass Spectrometry

Mass spectroscopy by Fast-atom-bombardment mass analysis (FAB-MS) (VG-70SE positive ion) was carried out by the UCL Mass Spectrometry Service (Department of Chemistry, University College, London U.K.) on the peptides synthesised.

3.4. Assessment of whole blood coagulation

3.4.1 Thrombelastography (TEG)

Thrombelastography (TEG) has been used in many clinical settings since its introduction in 1948 and has been shown to be a reliable technique for diagnosis and monitoring treatment of various blood disorders. The TEG measurements correlate well with the coagulation profile (Zuckerman et al 1981)[234] and have the advantage of being a rapid technique which

requires small blood volumes. Most blood clotting tests do not provide sufficient information regarding the quality of the clot formed or the speed and intensity of fibrin formation. A number of thrombelastographic patterns have been correlated with abnormal blood clotting states such as clinical thrombotic and haemorrhagic conditions.

TEG is carried out using venous blood rather than capillary blood with the first few millilitres being discarded to reduce any effect due to tissue thromboplastin which may be released on venepuncture. The blood or plasma sample is placed in a pre-warmed cuvette fitted into a moving device which is oscillated over an angle of 4 degrees 45 minutes (1/12 radian) around a vertical axis in a 10 second cycle. A piston which is freely suspended by a fine torsion wire and connected to a pen recorder chart, is lowered into the blood, resulting in a uniform clearance of 1 mm between the piston and cuvette. The blood is then covered with a thin layer of liquid paraffin to prevent drying and a resulting pH change due to atmospheric oxygen.

Initially the blood in the cuvette is in a fluid state and therefore the piston remains stationary, producing a straight line on the thrombelastogram (Figure 3.1). As the blood clots fibrin strands are formed between the surface of the piston and the cuvette. The motion of the cuvette is coupled to the piston causing it to oscillate along with the cuvette. The motion of the piston is transmitted to the torsion wire which results in a deflection of the pen recorder. During the course of coagulation the shear elasticity of the fibre strands increases and the rotation of the cuvette enables the elasticity of the clot to be measured without detaching the fibrin strand. As fibrin formation and the elasticity of the coagulum progress the deflection of the pen recorder increases progressively, providing a graphic representation of the fibrin build up and subsequent fibrinolysis. This technique can therefore be used to follow the entire process of blood coagulation from the initial fluid state, through the gradual increase in clot strength and polymerisation of the fibrin strands to the dissolution of the clot following fibrinolysis.

CTEG Model # 3000

Figure 3.1 Thrombelastograph coagulation analyser

3.4.2 Interpretation of the thrombelastogram

The output from the TEG records the elasticity of the blood clot (Figure 3.2). A number of important parameters can be measured and quantified which reflect the nature of the coagulum produced. In the TEG the reaction time denoted `r` (TEG-r) is measured from the start mark (recalcification point for a citrated sample) until an amplitude of 2mm is obtained. During this time there is no resistance in the blood and so the piston remains motionless. The r-time corresponds to the time taken for the formation of the first fibrin strands, and is therefore indicative of the enzymatic sequence of events which occur in the blood coagulation cascade prior to fibrinogen conversion to fibrin. The time from the measurement of r (the beginning of the clot formation) until a fixed level of clot firmness is reached (amplitude 20mm) is the k-time. It is a measure of the speed or clot kinetics to reach a certain level of clot strength. Angle (α) is closely related to k-time. The angle is more comprehensive than the k-time, since there are hypocoagulable conditions in which the final level of clot firmness does not reach an amplitude of 20mm. The maximum strength or stiffness of the developed clot is described as `ma` (Maximum Amplitude).

In general, the shape of the curve (k, α and ma) is determined by fibrinogen and platelet activity whereas the r-time is highly dependent on the functional aspects of the clotting factors.

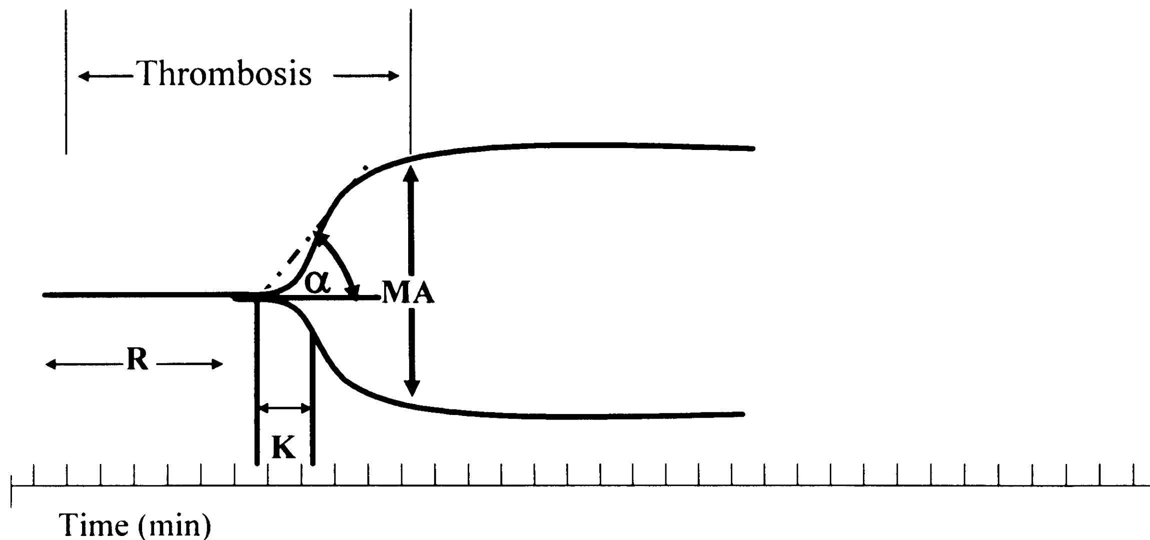


Figure 3.2 A typical TEG analysis tracing;

Where

R = Initial fibrin formation (a period of time from initiation of the test to the initial fibrin formation

K = Dynamic clot formation (a measure of time from beginning of clot formation until the amplitude of TEG reaches 20mm)

α = The acceleration of fibrin build up & cross linking (an angle between the line in the middle of the TEG(r) tracing and the line tangential to the "body" of the TEG® tracing)

MA = Maximum amplitude (reflect strength of a clot which is dependent on number and function of platelets and its interaction with fibrin)

3.4 3 Measurement of procoagulant activity

The procoagulant activity of blood or plasma can be determined by the one-stage prothrombin time assay (Figure 3.3). The one-stage prothrombin time assay relies upon the initiation of the extrinsic pathway of coagulation. Tissue factor in the presence of calcium and phospholipids, acts as a cofactor for factor VII to facilitate the activation of factor X and subsequent coagulation factors. This culminates in the formation of a clot. An unknown quantity of tissue factor activity in a sample can be determined through examination of the time taken to produce a clot. The higher the concentration of tissue factor present within a sample the less time is taken for clot formation.

Figure 3.3 Coagulation analyser

3.4.3 One stage prothrombin time assay

The one-stage prothrombin time assay was performed on a Cascade - M-coagulometer (Helena Laboratories). The assay was carried out at 37°C using 50 nM recombinant tissue factor (TF) in 12.5 mM $CaCl_2$ to which standard plasma was added (2:1 ratio), and the clotting time was measured using a

coagulometer (Figure 3.4). Clotting was initiated by the addition of 100µl reconstituted plasma, containing clotting factors in their inactive form, thus initiating the extrinsic pathway of coagulation through the formation of the tissue factor VIIa complex. Clot formation was detected via a change in optical density of the sample, and a reading of clotting time supplied.

From the time taken for clot formation to occur, tissue factor activity was quantified by reference to recombinant tissue factor standard, which at a concentration of 1000 units/ml clotted plasma in 14 sec. A standard curve was constructed using recombinant tissue factor diluted with distilled water, and expressed log tissue factor concentration (units/ml) versus log clotting time (seconds) (Figure 3.4). The concentration of tissue factor was calculated using straight line regression constants. The tissue factor activity was then calculated from a standard curve prepared previously, and the percentage of inhibition due to the moiety being tested was calculated as:

$$Inhibition(\%) = 100x\frac{A_i - A_r}{A_i}$$

Where *A* is activity and subscript *i* and *r* are initial and residual respectively

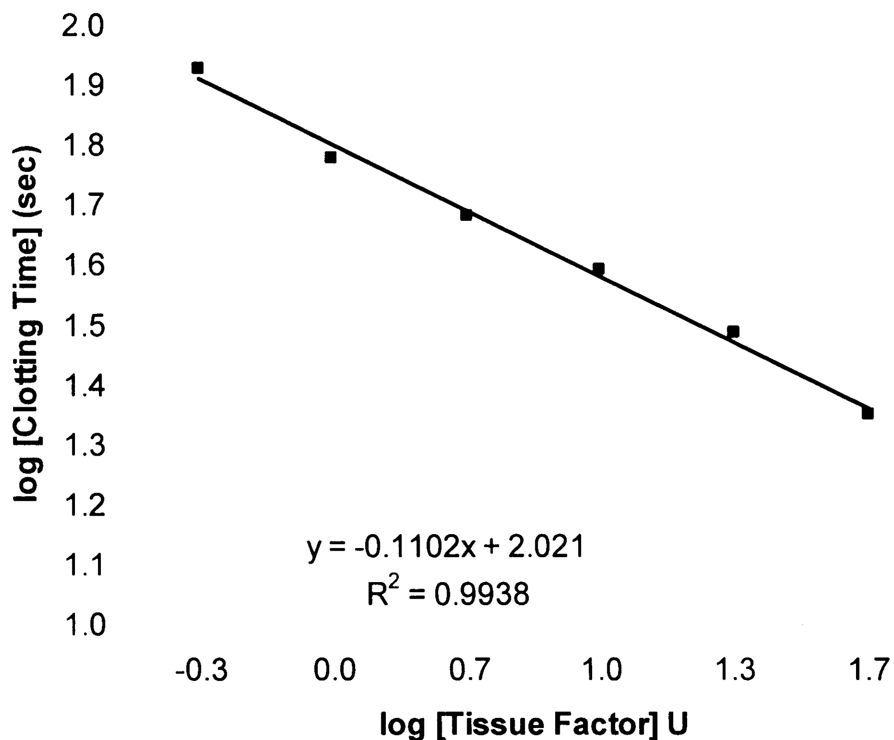


Figure 3.4 A representative tissue factor activity standard curve.
The standard curve was constructed using (0.5 – 50 units/ml) recombinant tissue factor, and expressed as log tissue factor concentration (units/ml) versus log clotting time (seconds).

3.5 Data analysis and statistical methods

Results were analysed using the statistical software package GraphPad Prism. Students t-test or one way Anova and pair-wise multiple comparison procedures (student Newman Keuls method) were used during the data analysis. Data are presented as mean ± SD or mean ± SEM. $p < 0.05$ was considered as statistically significant.

CHAPTER FOUR:

EXTRACTION OF CELLS FOR SEEDING VASCULAR BYPASS GRAFTS

4.1 Introduction

The vascular endothelium is versatile and multifunctional with many synthetic and metabolic properties. These include the regulation of thrombus and platelet adhesion as well as modulation of vascular tone and blood flow. EC secrete and express numerous growth factors, extracellular matrix products, anti-thrombotic and pro-coagulant factors. ECs are intimately involved in maintaining a non-thrombogenic blood-tissue interface[235, 236]. The absence of an intact EC lining therefore predisposes prosthetic grafts to platelet deposition, thrombosis and graft failure. As a result, investigators have developed methods to promote the endothelialisation of vascular grafts prior to implantation by transplantation of ECs *in vitro*, a process called EC seeding.

Seeding of EC onto the lumen of prosthetic bypass grafts has been attempted with the aim of improving their biocompatibility and patency [237]. Seeding of grafts has followed two strategies, single and two-stage. Single-stage seeding refers to EC extraction and seeding all within the timeframe of a typical bypass operation [36]. This avoids the need for cell culture and reduces operative time. Two-stage seeding involves cell extraction typically from an autologous vein, culture until adequate cell numbers are available, and seeding in a rotating device until confluence is reached on the lumenal surface at which time the graft is implanted. To date only two-stage seeding has been carried out successfully in large clinical trials. There are however difficulties with taking a two-stage seeding approach. Firstly the patient is required to undergo two surgical procedures with a relatively long wait between them to obtain sufficient cells to seed the graft. This makes the procedure unsuitable for patients with critical ischaemia. Secondly in around 30% of patients the cells extracted in the first stage of the procedure are not suitable for use due to either poor cellular proliferation resulting in an inadequate number to seed the graft or the development of infection in the cell cultures [238]. For these reasons single-stage seeding of prosthetic vascular grafts remains the ultimate aim for

cardiac and vascular surgeons and further investigation to achieve this goal continues.

4.1.1 Sources of endothelium for seeding

Early investigators used animal tissue such as bovine, porcine or canine endothelium as their source of ECs [239]. In order to make the transition from laboratory research to clinical practice it is necessary to obtain a source of cells from the patients themselves. In humans there is increasing evidence to show that cells of the same type from different tissues are morphologically, biochemically and functionally diverse. These findings of endothelial heterogenecity have resulted in debate regarding which type and source of EC are suitable for *in vitro* and *in vivo* studies and it has become accepted that this is a major consideration in tissue engineering. As a result of this numerous sources of cells have been investigated, including non-essential vessels such as the saphenous vein or umbilical vein and omentum or subcutaneous adipose tissue [81, 240, 241].

Herring and co-workers first reported the use of venous-derived endothelium for subsequent transplantation. The yield of cells from a suitable piece of saphenous vein segment was approximately 1×10^4 cells [17, 242]. This amount of cells would only provide a sparse coating of cells in a single-stage seeding procedure, requiring an extensive cell proliferation post-seeding to achieve a monolayer. In addition blood flow-induced shear stress could also result in the loss of a large number of seeded cells [243]. Thus whilst venous-derived endothelium may be suitable for a two-stage seeding process the need to explore different EC sources with higher cell yields remains to achieve successful single-stage seeding.

In 1986 Jarrell et al reported methods for the isolation of autologous microvessel EC from adipose tissue using enzymatic digestion similar to the methods for autologous vein [241]. The ECs could then be separated from the adipocytes by density gradient centrifugation.

Another potential source of EC investigated for use in single-stage seeding is subcutaneous fat. Published results in this area have been varied

as some groups have claimed that no EC could be extracted from subcutaneous fat using a conventional collagenase digestion process whilst others have claimed that up to 80% of the cells extracted are EC using the same technique [244-246]

Omentum has also been utilised as a cell source. The cells extracted have been characterised as mesothelial cells (MC). Both EC and MC have a similar function producing substances such as tissue-type plasminogen activator (tPA) and urokinase plasminogen activator [247, 248]. In animal experiments most trials involving the seeding of grafts using cells derived from omentum have shown good results[249-253], except those reported by Verhagen and co-workers[254]. Based on this work the possible use of MC isolated from peritoneal lavage has been considered. Peritoneal lavage is a simple technique with a minimal morbidity for the patient. Ivarsson and co-workers reported that it was possible to extract between 3×10^6 and 8×10^6 cells from around 500ml of lavage fluid [255].

4.1.2 Isolation of autologous human endothelial cells.

Two techniques have been developed in order to harvest ECs from autologous veins such as the long saphenous vein. Firstly, mechanical scraping [256] and secondly enzymatic digestion using collagenase or trypsin [250]. Mechanical scraping uses an abrasive action to remove EC from the vascular wall which leads to significant EC damage, the possibility of contamination with smooth muscle cells[250] and provides a poor harvest of EC[250]. Enzymatic digestion using collagenase or trypsin to remove the endothelium avoids the problem of mechanical damage to the cells and provides much improved EC recovery. Digestion is also suitable for use with tissue samples such as omentum and subcutaneous fat and has been applied to them. However in the case of subcutaneous fat and omentum, contamination with other cell types has been a significant problem [238].

Many groups have suggested that the only way to obtain a pure culture of EC from subcutaneous fat or omentum would be to use purification techniques such as Percoll gradient centrifugation, filtration or magnetic

beads [36]. This is because it is believed that collagenase digestion of fat leads to the extraction of a mixed population of EC, MC, fibroblasts and smooth muscle cells. Percoll gradient centrifugation and filtration techniques have the disadvantage that they reduce the number of EC extracted. In addition Percoll is also thought to be detrimental to the subsequent proliferation of the extracted EC[36].

The use of magnetic beads to isolate ECs from contaminants has been suggested as a more suitable method of purification, particularly where large numbers of cells are involved. In this method the magnetic beads are coated with an antibody specific for the cells required. These beads are then mixed with the cell suspension resulting in the beads binding to all the cells expressing the correct antigen. The cell suspension is then passed through a magnetic field, which traps the magnetic beads and therefore the positive cells allowing the negative cell fraction to pass through. By removing the magnetic field the positively selected cells can then be collected.

Magnetic beads coated with platelet endothelial cell-adhesion molecules (PECAM; CD31) and Dynabeads have been used for extraction of EC for subsequent culture and cell characterisation but have never been used for seeding in a clinical situation. The effect of these microbeads on EC behaviour is however poorly understood.

4.1.3 Characterisation of endothelial cells

Mature ECs in culture have a very distinctive and well described cobblestone appearance upon confluence. Typically, they appear as flat, 1-2μm thick, cells about 10-20μm in diameter with a central nucleus. ECs also exhibit contact inhibition and can be seen growing in patches or clusters in sparsely seeded cultures.

Immunohistochemistry is one of the most widely used tools for characterising cell types by using antibodies specific to the cell of interest. However, one significant difficulty of this technique has been the relative lack of a dependable marker for endothelial cells. Two of the most commonly used antibodies are vWF and CD31. vWF has been routinely used for

endothelial identification in adult cells. It is particularly useful for venous-derived endothelial cells possessing large numbers of Weibel-Palade bodies, cytoplasmic inclusions containing high concentrations of vWF which results in a characteristic punctate cytoplasmic staining. However not all ECs possess Weibel-Palade bodies or express vWF[257]. Microvascular endothelium for example contains relatively few Weibel-Palade bodies and can therefore produce a negative reaction when stained for vWF. CD31 is expressed at the cell/cell junction of confluent endothelium resulting in positive staining around the periphery of the cell. Again although characteristic of ECs positive staining is not guaranteed in all cases[257].

The aims of this section of the study were:

I) To extract EC from subcutaneous fat using three different techniques: firstly conventional collagenase digestion and culture, secondly removal of fibroblasts using CDw90 beads followed by culture and finally the use of CD31 beads to positively extract EC.

II) To assess the impact of the source of MC and to consider the feasibility of extracting cells from peritoneal lavage for use in tissue engineering.

III) To investigate the effect of Dynabeads™ on cell population and metabolism.

IV) To study the process of seeding the extracted cells on to a cardiovascular bypass graft, compliant poly(carbonate-urea)urethane and assess cell metabolism and proliferation.

4.2 Materials and Methods

4.2.1 Endothelial cell extraction from subcutaneous fat

Subcutaneous fat was removed from patients undergoing abdominal aortic surgery after informed consent. Fat removed from the patient was weighed, washed with phosphate buffered saline and finely minced in a petri dish with a scalpel until a fine paste was achieved. This was incubated with collagenase A (Roche Diagnostics, Lewes, U.K.) at 2mg/ml at 37°C for 25 minutes. At the end of the incubation period, the enzyme was neutralised with complete medium: 157 ml M199 medium, 40 ml fetal calf serum, 4.8 ml sodium bicarbonate 7.5% solution (Invitrogen, Paisley, U.K.), 5 ml L-glutamine (200mM) and 1.57 ml penicillin/streptomycin (5,000 units penicillin and 5 mg streptomycin/ml) (Sigma Chemical Company, Dorset, U.K.) and centrifuged at 300g for 7 minutes. The upper layer of fat was discarded and the mixture centrifuged at 650g for a further 7 minutes. The supernatant was removed and the cell pellet resuspended in 10 ml complete medium. A cell count was performed and the cell suspension divided into two equal portions. One portion was plated in a 25cm^2 flask and incubated at 37°C/ 5% CO_2 in an air humidified chamber.

The other portion was subjected to either cell purification by a negative isolation technique with dynabeads using a modification of a method used previously for endometrial tissue [258] or a positive isolation technique using CD31 beads to positively remove endothelial cells (EC) using a modification of the method employed previously by Hewett and co-workers [244] as described below:

4.2.1.1 Removal of fibroblasts (negative cell isolation)

Dynabeads Pan Mouse IgG (Dynal, Wirral, U.K.) were resuspended thoroughly and the required number of beads pipetted into a 50 ml centrifuge tube. The centrifuge tube was placed in a magnetic device (MD)

(Dynal, Wirral, U.K.) and the fluid removed from the tube. The tube was then removed from the MD and the Dynabeads resuspended in 1 to 2 ml PBS.

The DM Pan Mouse IgG beads were then coupled to Thy-1 (CDw90) mouse anti-human monoclonal antibody (Serotec, Kidlington, U.K.) at a concentration of 0.1-1μg antibody/10^7 beads. The antibody/bead mixture was incubated on a roller for 30 minutes at 2-8°C. The excess antibody was then washed off and the coated beads resuspended in PBS.

The coated dynabeads were added to the cell suspension at a concentration of 4 beads per target cell. The cell/bead mixture was incubated for 10-30 minutes at 2-8°C with tilting and rotation. The MD was then used to remove dynabead positive cells which were then cultured as were the dynabead negative cells remaining.

4.2.1.2 Removal of endothelial cells (positive cell isolation)

CD31 coated dynabeads (Dynal Ltd, Wirral, U.K.) were washed and mixed with the cell suspension at a concentration of 4 beads/cell. This mixture was incubated at 2-8°C for 30 minutes. The CD31 positive cells were removed using the MD. The cells were cultured at 37°C in complete medium which was changed every 48-72 hours.

4.2.2 Mesothelial cell extraction from human lavage

Patients undergoing elective open abdominal aortic surgery were suitable for the study. Ethical approval was obtained from the local ethical committee and informed consent was taken of all patients taking part.

In all patients, the abdomen was opened using a standard midline laparotomy incision. Once the peritoneum was opened, 1 litre of warm saline was used for the lavage. The abdomen was gently shaken and the lavage fluid was collected after 2 minutes with a 50 ml syringe until no further fluid extraction was possible. The isolation of cells was similar to the method employed by Ivarsson and co-workers [255]. Briefly, the collected fluid was transferred into 50ml centrifuge tubes and centrifuged at 650g at 20°C for 10

minutes. The supernatant was discarded and the cell pellet counted. As a control, in three of the patients a small amount of subcutaneous fat was also removed. Cells were extracted after mincing and digestion with collagenase A at 4mg/ml for 30 minutes at 37°C [259]. The cells were extracted by centrifugation and cultured as per our standard protocol.

4.2.3 Effect of magnetic beads on endothelial cell metabolism

HUVECs were cultured as described above and used at 3rd passage. On the day of experiment, HUVECs were trypsinised and a portion of the cells was incubated with CD31 coated beads at 2-8°C on a roller for 30 minutes. The HUVECs with attached dynabeads (D+ve EC) were removed using a magnetic particle counter and counted at 48, 72, 120 and 144 hours to assess cell metabolism whilst cells not attached to the beads were discarded. Control was an equal number of HUVECs. Experiments were repeated four times.

4.2.4 Assessment of cells extracted from subcutaneous fat and peritoneal lavage

Equal numbers of D+ve EC and HUVECs were cultured overnight in gelatin coated 24 well plates as described in chapter 3 (Section 3.1.1). After 24 hours of incubation, AB was added and this was further changed every 48 hours. Cells were trypsinised after 48, 72, 120 and 144 hours from initial plating and a cell count performed. Cells were counted using standard techniques with a haemocytometer. To assess the cell metabolism and viability the AB assay was applied as described above. As a control AB/medium added to empty wells was measured.

4.2.5 Cell seeding of compliant poly(carbonate-urea)urethane bypass graft

The compliant poly(carbonate-urea)urethane (PCU) graft was cut flat and segments mounted in cryovials. The graft was coated with fibronectin engineered protein polymer (FEPP) (Sigma, Dorset, U.K.) to improve cell adherence. To seed cells extracted from subcutaneous fat onto vascular graft firstly the graft was coated with the FEPP solution at 37°C. After 24 hours the excess solution was washed off with PBS and the graft was ready for use. The cells still coated with the beads were seeded onto the graft for 1 hour and then washed with PBS three times to remove any non-adherent cells. The seeded graft was then cultured in complete medium with AB (10%) (Serotec, Kidlington, U.K.) for 72 hours. Every 24 hours a 50µl sample of the AB/medium mixture from the graft was removed and measured as above.

To assess the effect of dynabeads on EC metabolism, experiments were also performed after one hour seeding of the cells on to a prosthetic graft made of PCU. Cryogenic vials (2ml; Nalgene, New York, U.S.A) were used in which the ends were removed to obtain a long graduated cylinder. A standardized (1cm x 1cm) sample of PCU graft was then stretched over the other end of the cylinder and screwed down firmly using the screw cap fixing the graft firmly in place and not allowing any cell suspension to leak out. The graft material therefore formed a flat floor on which EC could be seeded. To enable use of the system in a CO_2 incubator for a period of days seeding chambers were placed in a 24 well tissue culture plate (Marathon, London, U.K.) with another placed on top to allow gases to perfuse readily and prevent contamination. Graft segments were then seeded with dynabead coated EC (at a bead: cell ratio of 50: 1) and normal HUVEC at a cell density of $2x10^5$ cells/cm^2. The cryovials were then washed three times with PBS, the washings collected and cell retention calculated from the number of cells lost in the washings. AB/medium was then added into the cryovials and readings were taken at 4, 24, 48 and 72 hours as previously.

4.3 Results

4.3.1 Endothelial cell extraction from subcutaneous fat

Subcutaneous fat was removed from patients undergoing abdominal aortic surgery. 7 patients took part in this study. The average age of the patients was 68.6±3.8 years (63-71). The number of cells extracted per gram of fat was $0.75 \times 10^5 \pm 0.49 \times 10^5$ cells (0.26×10^5-1.08×10^5 cells/gram).

The percentage of cells which were removed using the CDw90 beads (negative cell isolation by removal of fibroblasts) ranged from 9-75% (Table 4.1). On culture the cells extracted using the conventional technique, the cells positive for CDw90 and the cells negative for CDw90 all grew to confluence and resembled either smooth muscle cells or fibroblasts in morphology (Figure 4.1). On staining, the cells stained positively for fibroblast antibody whilst there was negative staining for CD31, actin and vWF (Figure 4.1).

The cells extracted using CD31 (positive cell isolation of EC) were confluent and resembled the typically cobblestone appearance of EC (Figure 4.1). These cells stained positively for vWF.

4.3.2 Single stage seeding of extracted cells onto PCU graft

SEM confirmed cells still adhered to the graft 72 hours after seeding (Figure 4.1). The seeded cells were viable as confirmed by the Alamar blue™ viability assay with cell metabolism of 214.6% ± 6.6% compared to unseeded graft segments. The results of the Alamar blue™ assay are presented as a percentage as the assay is measured in arbitrary units.

4.3.3 Mesothelial cell extraction from lavage

10 patients (9 males) took part. The average age of the patients was 71.7±1.4 years. All the patients at the time of surgery were either non-

smokers or ex-smokers. 5 patients had renal impairment defined as a blood creatinine level higher than the normal values taken at a pre-operative visit. Peripheral vascular disease was defined as either symptomatic disease or ankle brachial pressure less than 0.9 similar to previously published papers.

The results of the cell yields are summarised in Table 4.2. The average volume of fluid collected was 622±40 ml (400-750 ml). The average cell count for all the patients was 5898 ± 1384 cells/100 ml of peritoneal fluid collected.

The mesothelial cells were only viable for up to 6 days after extraction whilst the subcutaneous fat extracted cells were easily grown to confluence.

Age (years)	Cells (no/gm)	CDw90+ve cells (%)
71	0.46×10^5	13.1
63	1.08×10^5	17.0
68	1.42×10^5	19.2
73	0.50×10^5	20.0
68	0.26×10^5	8.9
71	0.16×10^5	75.1
72	0.80×10^5	58.4

Table 4.1 CDw90 positive cell extraction from subcutaneous fat

Age (years)	Sex	PMH	TLV (ml)	TCC (x10^5)	Cells (no/100ml)
63	M	IHD, COAD	730	0.61	8470
68	M	PVD, IHD, RI	450	0.50	1120
69	M	IHD, CVA, RI	600	0.08	1333
71	M	IHD	700	0.76	10900
71	M	IHD, RI	530	0.24	4528
72	F	IHD, CVA	730	0.65	9000
74	M	IHD	750	0.22	2933
75	M	IHD, PC, RI	400	0.80	2000
77	M	IHD, RI	600	0.30	5000
77	M	IHD	730	1.00	13700

Table 4.2 Profile and cell extraction in patients undergoing abdominal aortic surgery.

Keys: CVA, cerebrovascular accident; COAD, chronic obstructive airway disease; IHD, ischaemic heart disease; PC, prostate cancer; PVD, peripheral vascular disease, RI; renal impairment, PMH: past medical history, TCC, total cell count; TLV, total lavage volume.

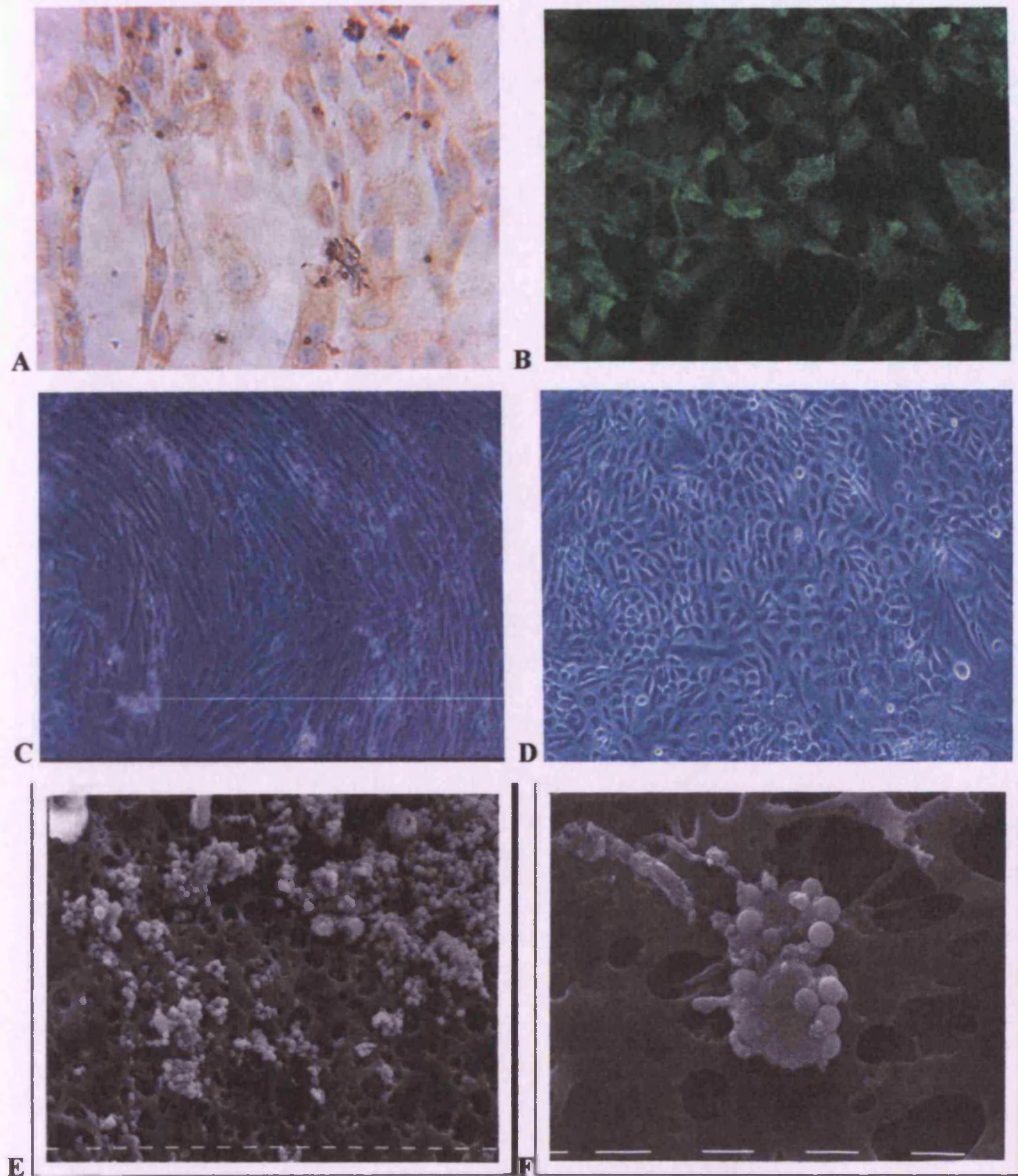


Figure 4.1 CDw90 cells stained with mouse anti-fibroblast antibody (A) and mouse anti-vWF antibody (B) (Both magnification x40). Cells extracted from subcutaneous fat showing long spindle shaped cells (C) and cells extracted using CD31 beads showing typical cobblestone appearance (D) (Both magnification x20). Scanning electron microscopy of single stage seeding of CD31 coated cell extracted from subcutaneous fat at on PCU graft material (E) and (F) at magnification x220 (E) and x820 (F). Scale bar = 10 μm.

4.3.4 Assessment of Dynabead cytotoxicity

The total number of EC attached to Dynabeads™ is summarised in Figure 4.2. It is shown that as the number of magnetic beads increases so does the proportion of EC attached to the beads with 4 beads having cell attachment of 64%± 3.83 whilst the corresponding result for 50 beads was 83%±5.3. There was no significant difference in attachment of EC between the 4 beads per cell and 10 beads per cell, between 10 beads per cell and 50 beads per cell but there was a statistically significant difference between the 4 beads per cell and 50 beads per cell ($p<0.05$).

The % cell population from the original plating for the D+ve EC for each time period post culture is summarised in Table 4.3. The results are given as a percentage by comparing D+ve EC to the normal EC. The results show that by the end of the 6th day the % of D+ve EC in culture compared to controls was 30.7 when 50 beads per cell was used whilst this figure was 41.3 and 59.2 for 10 and 4 beads respectively. This shows that using 4 beads per cell leads to nearly twice as many available cells compared to 50 beads per cell. The cell viability assay using Alamar blue™ results are summarised in Table 4.4. The corresponding Alamar Blue results for 50 beads per cell at 6 days was 43.7% whilst for the normal cells it was 72.1%. Figure 4.3 shows D+veEC cells at (a) 24 and (b) 96 hours post culture showing fewer cells in culture with prolonged culture time.

4.3.5 Assessment of D+ve EC seeded on PCU graft

The cell retention for (50beads/cell) D+ve EC and normal EC was not statistically significant (unpaired t-test, $p=0.3087$). The Alamar blue viability assay results are summarised in Table 4.5 showing that there was significantly less cellular metabolism in D+ve cells. Figure 4.1 shows a scanning electron microscopy picture of D+ve EC coated onto PCU graft showing cells still attached to the graft 72 hours after seeding.

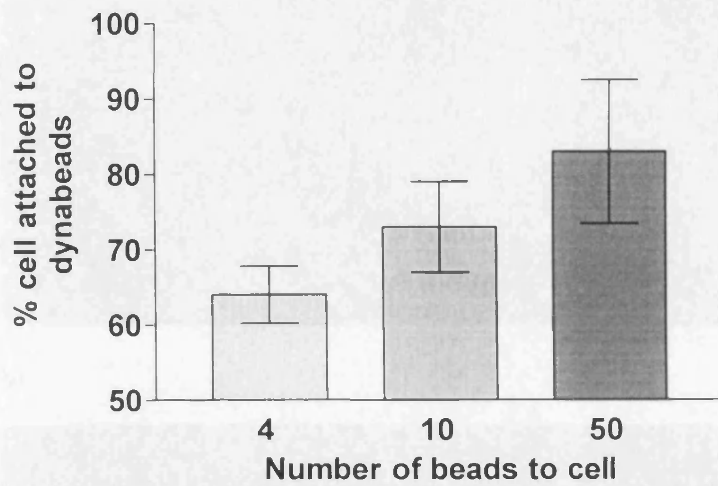
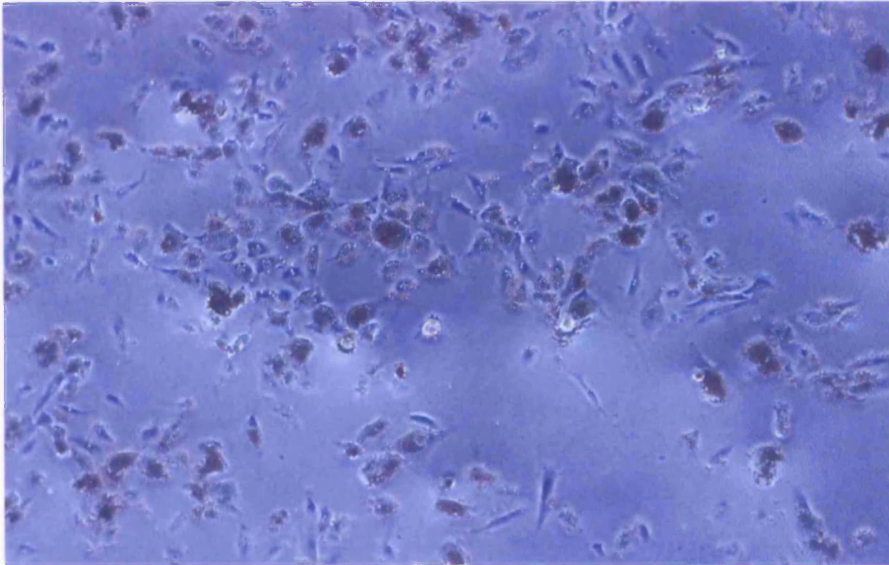


Figure 4.2 % attachment of cell to Dynabeads™ with different bead to cell ratio

a)



b)

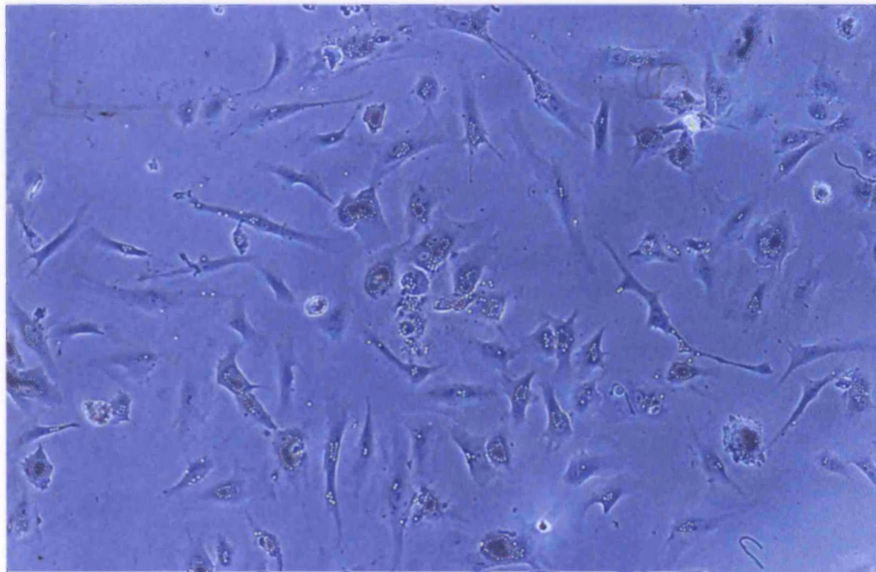


Figure 4.3 A typical image of cell with Dynabeads™ (50 beads/cell) at (a) 24hours and (b) 96hours post culture as culture time increases.

CDC	TPC (hours)	% of D+ve EC compared to normal EC	p value
50	48	82.0 ± 11.4	0.224
	72	66.3 ± 11.0	0.005
	120	61.0 ± 6.5	0.001
	144	30.7 ± 2.6	<0.001
10	48	54.4 ± 5.9	0.001
	72	70.4 ± 15.2	0.097
	120	54.5 ± 7.4	0.007
	144	41.3 ± 9.8	0.005
4	48	76.2 ± 12.8	0.064
	72	72.5 ± 9.5	0.037
	120	78.1 ± 20.7	0.082
	144	59.2 ± 7.3	0.026

Table 4.3 Cell population with Dynabeads. The results are given as a percentage (%) of control cells. Data are Mean ± SEM. **Keys:** D+ve EC (cell coated with Dynabeads™); normal EC (cells without Dynabeads™); TPC (time post culture); CDC (concentration of Dynabeads™ per endothelial cell).
$p < 0.05$ was considered statistically significant compared to normal EC.

CDC	TPC (hours)	D+ve EC	p value
50	48	77.8±3.7	0.004
	72	87.3±1.4	0.002
	120	50.9±2.5	<0.001
	144	43.7±1.2	<0.001
10	48	61.7±3.8	<0.001
	72	60.9±1.8	<0.001
	120	58.2±1.0	<0.001
	144	61.8±1.4	<0.001
4	48	84.1±6.7	0.169
	72	76.6±4.7	0.0001
	120	77.3±3.2	<0.001
	144	72.1.±4.3	<0.001

Table 4.4 Alamar blue viability assay results. Results are in percentage cell metabolism compared to the control. Data are Mean ± SEM. **Keys:** D+ve EC (cell coated with Dynabeads™); TPC (time post culture); CDC (concentration of Dynabeads™ per endothelial cell). $p < 0.05$ was considered statistically significant compared to normal ECs without Dynabeads™.

Time(hours)	%D+ve EC metabolism compared to control	p value
4	82.3 ± 3.4	0.0001
24	62.5 ± 3.9	<0.0001
48	70.6 ± 2.8	<0.0001
72	71.1 ± 3.6	<0.0001

Table 4.5 Alamar blue cell viability results after seeding on grafts.
Results are given in % compared to controls and as Mean ± SEM.
Keys: D+ve EC; Dynabeads™ coated endothelial cells

4.4 Discussion

Finding a suitable source of EC and the purification of EC once extracted is still a vitally important challenge for research scientists. In this study, peritoneal lavage and subcutaneous fat were investigated as a potential source of cells. Since MC from peritoneal lavage have been shown to release similar antithrombogenic substances to EC, the suggestion has been made that they could provide an ideal source for tissue engineering[255]. The major advantages of employing peritoneal lavage as a cell source are that it requires only minimally invasive surgery and no enzymatic digestion is needed to extract the cells.

In our study, the number of MC extracted from peritoneal lavage was much lower compared to Ivarrson and co-workers. There are a number of factors that may explain this. In the Ivarrson study the age or the past medical history of the patients who underwent the lavage was not specified. This has a major influence on the extraction of the cells and their subsequent proliferation[260]. In order to ascertain if this was a potential reason for the lower number of cells extracted the authors of the study were contacted and confirmed that they undertook their work on patients undergoing colorectal surgery and as such were not typical vascular patients (personal communication). We used patients undergoing abdominal aortic surgery with evidence of atherosclerotic disease including ischemic heart disease, peripheral vascular disease, carotid artery disease and renal impairment. These patients fit the typical profile of patients who require peripheral vascular bypass. The effect of using older patients with other co-morbidities may well have affected the ability to extract cells and account for the lower number of cells extracted. This was particularly evident in patients with abnormal serum creatinine levels where fewer cells were extracted compared to patients with normal creatinine; due to the small numbers of patients further data and investigation is required to confirm our observations.

A report by van Westreenen and co-workers showed that different lavage fluids could have a detrimental effect on MC survival. This study suggested

that normal saline would kill 20% of MC though this was the safest of all the lavage fluids tested[261].

The second potential source of EC investigated in this study was subcutaneous fat. Many authors have reported large harvests of pure EC from fat using conventional enzymatic techniques whilst others have only had success using cell purification techniques similar to those used in this study. EC were extracted from subcutaneous fat using three different techniques: firstly conventional collagenase digestion and culture, secondly removal of fibroblasts using CDw90 beads followed by culture (negative cell extraction) and finally the use of CD31 beads to positively extract EC (Figure 4.1). One factor which can affect the success of EC extraction is the source of the enzyme used, enzyme obtained from different manufacturers may not all behave in the same manner and there may be further differences in activity between batches of enzyme from the same manufacturer. To investigate this two different collagenases (Sigma Chemical Co. Ltd, Poole, U.K. and Roche Diagnostics, Lewes, U.K.) at concentrations of 2 and 4 mg/ml were utilised to extract EC in a preliminary experiment and showed no obvious differences between the EC extracted. This preliminary experiment was only done in two patients because of the difficulty in obtaining limited human samples and it was then decided to use one brand of collagenase (Roche Diagnostics, Lewes, U.K.) in the rest of the study. However the numbers of cells extracted were similar to previously published reports so it is unlikely that the variety of collagenase employed was responsible for the low number of EC and high numbers of fibroblasts obtained.

In previous work, Arts and co-workers have used magnetic beads to remove both fibroblasts and smooth muscle cells for cell purification[262]. Our experience with CDw90 beads has shown that fibroblasts can be removed but the results are inconsistent and currently this is not a reliable technique for clinical use. EC extracted with CD31 beads produced cells which had the typical cobblestone appearance of EC and stained positively for vWF. Therefore for use in a clinical situation we would recommend the use of the more reliable CD31 magnetic beads for positive EC extraction.

When using magnetic beads it has been suggested that large numbers of beads are required to ensure that as many as possible of the available EC have been extracted. However it has been previously suggested that utilising a large number of magnetic beads may cause toxic effects to EC[263]. In order to investigate if this was the case the effect of Dynabeads™ at varying concentrations on cell population and metabolism was investigated using HUVECs. The recommended number of beads to extract cells is 4 beads /cell but this results in only 64% of all possible cells being attached to the beads. When this is increased to 10 beads per cell only 73% of the cells were attached whilst by greatly increasing the bead to cell ratio to 50, 83% of the cells were attached to the beads (Table 4.2).

The results on table 4.3 and table 4.4 have also demonstrated that by using higher numbers of beads more cells can be extracted. However there is a decrease in cellular proliferation especially associated with the use of higher numbers of beads with increased culture time. This is particularly evident by day 6 showing that the toxic effect of Dynabeads™ increases with increasing time. The reasons for this are unclear and further investigation is needed to determine if this is just a physical effect of the presence of the beads or a functional effect which can be looked at by measurement of parameters of cell function such as secretion of vWF.

These results have shown that using a large number of beads to improve cell extraction, whilst producing a larger pool of cells initially for use, results in reduced cell population and metabolism. Thus it is necessary to strike a balance between maximising cell extraction and ensuring that cell survival and proliferation occurs. In the future it may be worthwhile investigating the use of either detachable beads or smaller beads (such as Miltinyi) to see if they have a similar significant detrimental effect to the results obtained in this study.

This study highlights the problems faced by researchers in attempting to extract cells for tissue engineering for typical vascular patients. MC extracted from peritoneal lavage remain a potential alternative source for cells for tissue engineering but due to the limited number of cells extracted from patients with vascular problems probably only in non-vascular patients. With

regard to extracting cells from subcutaneous fat it can be concluded that for single-stage seeding successful harvesting will require the use of magnetic beads with the drawback that using such beads in high concentrations is detrimental to EC proliferation and metabolism whilst using lower number of magnetic beads will reduce the number of EC extracted.

CHAPTER FIVE

DEVELOPMENT AND CHARACTERISATION OF NEW PEPTIDES

5.1 Introduction

Among the numerous biological molecules in the living tissues, extracellular matrix (ECM) proteins that bind to integrin receptors have attracted tremendous attention and resources in the research environment. ECM proteins are macromolecules; large molecules that are assembled from simple subunits or monomers. Each protein has a unique structure, and as a consequence of that structure, it has a unique shape and function in any given set of environmental conditions. The physical and chemical properties are determined by the nature of the constituent amino acid side chains and by the polyamide peptide backbone itself. In order to comprehend protein structure and function in detail, the composition and properties of individual amino acids must be examined.

5.1.1 Amino acids

Amino acids Arginine (R), Glycine(G) and Aspartic acid (D) as a tripeptide sequence RGD is known to be the active site of adhesive proteins of the ECM. As their name suggests, amino acids are organic acids, which also contain an amino group. These biologically important amino acids belong to the group called α-amino acids. As indicated by the general formula of amino acid (Figure 5.1), any distinctive properties of a particular amino acid must be dependent on the nature of the R-group attached to its α-carbon. Since there is considerable diversity among the naturally occurring amino acids it is convenient to classify them according to the characteristics of their R groups or side chain [264].

Amino acids vary in their structure, size and electric charge and these also influence their solubility in water. Amino acids with polar R groups are generally water soluble, and their hydroxyl (-OH) or sulfhydryl (-SH) groups often play a key functional role in the proteins of which they form a part. The polar R groups of these amino acids do not ionize in the physiological pH range.

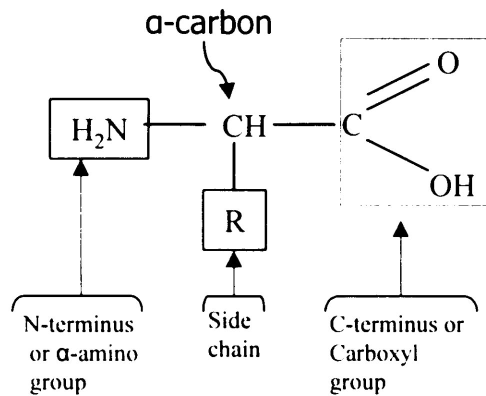


Figure 5.1 General chemical formula of amino acids

The amino acids can generally be grouped into hydrophobic and hydrophilic residues (Table 5.1). The hydrophilic residues can be categorised conveniently into three groups: 1) amino acids with neutral or polar side chains such as serine, 2) acidic side chains, such as aspartic acid and glutamic acid, and 3) basic side chains such as arginine.

Glycine is the simplest amino acid, which has just a hydrogen atom at its side chain. Due to the small size of its side chain, glycine permits the peptide backbone a great deal of conformational flexibility and doesn't fit into any of the above categories. When present as a residue in the chain it is neutral and can be accommodated into polar or non-polar environments.

Amino acid	R-group	Property	MW
NH_2, NH (=) $C - N(H) - CH_2 - CH_2 - CH_2 - C(H)(NH_2) - COOH$ Arginine (Arg) **R**	Basic side chain	Hydrophilic	174
$H - CH(H)(NH_2) - C - COOH$ Glycine (Gly) **G**	Non-polar	Hydrophobic	75
O (=), OH $C - CH_2 - C(H)(NH_2) - COOH$ Aspartic acid (Asp) **D**	Acidic side chain	Hydrophilic	133
$HO - CH_2 - C(H)(NH_2) - COOH$ Serin (Ser) S	Neutral side chain	Hydrophilic	105

Table 5.1 Structure, functional group (R-group), property and molecular weight (MW) of amino acids Arginine, Glycine, Aspartic acid and Serine.

5.1.2 6-Aminohexanoic acid

Aminohexanoic acid (MW=131.2) is an amino acid derivative with an amino (NH_2) group at one end and a carboxyl (-COOH) group at the other end. It acts as a fibrinolysis inhibitor with a mechanism of action that is thought to be inhibition of plasminogen activators. Aminohexanoic acid (Figure 5.2) is found to have penetrated through an extra vascular and intravascular body compartments and into red blood cells and other tissue cells, when introduced intravenously. It is used to help maintain homeostasis when fibrinolysis leads to bleeding. It is used medically to reduce or prevent bleeding [265, 266].

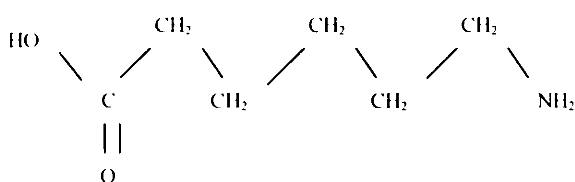


Figure 5.2 Chemical structure of 6-Aminohexanoic acid

5.1.3 Lauric acid

Lauric acid (also called Dodecanoic acid) is aliphatic carboxylic acid with 11 hydrocarbon lengths at one end of the chain joined to a terminal carboxyl (-COOH) group at the other end. The general formula of lauric acid is CH_3-$(CH_2)_{10}$-COOH (Figure 5.3). As one of the medium-length long-chain fatty acids, lauric acid is part of the class of organic compounds known as lipids, which are vital in the construction of cellular membranes and act as a source of food under starvation conditions. It is insoluble in water and has a melting point of 40-44^{0}C. Lauric acid is also believed to possess antimicrobial properties and is frequently exploited by pharmaceutical companies.

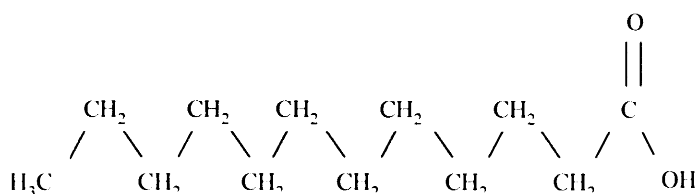


Figure 5.3 Chemical structure of Lauric acid

5.2 Peptide synthesis: The solid phase

Merrifield in1963 first described solid-phase peptide synthesis. The essential feature, which distinguishes solid-phase synthesis from the solution-phase technique, is the use of a solid resin support [267]. The resin support is often a polystyrene suspension polymer cross-linked with 1% of divinylbenzene as a cross-linking agent. Solid-phase peptide synthesis strategies retain the proven solution-phase chemistry while adding a covalent attachment step that links the peptide chain to an insoluble polymeric support (Merrifield in 1963). The chain elongation is initiated in the carboxyl → amino direction where the carboxyl residue of the selected sequence is attached to the support either directly or by means of an appropriate "handle". For peptide synthesis to proceed successfully, without unwanted side reactions, the supports and amino acid derivatives must be protected at the reactive N^{α}-amino groups and side chain functionalities. The carboxyl group of each amino acid is unprotected and must be activated prior to coupling.

The carboxyl group of each incoming amino acid is activated by one of several strategies and couples with the N^{α}-amino group of the proceeding amino acid. The N^{α}-amino group of the incoming amino acid is temporarily protected to prohibit peptide bond formation at that site. The "temporarily" protecting group of the N^{α}-amino group is removed (deprotected) at the beginning of the next synthesis cycle.

In addition, reactive side-chains on the amino acids are protected with "permanent" protecting groups. Repeating the synthesis cycle extends the polypeptide chain. Excess reagents at high concentrations are used to drive the reaction as close to completion as possible. This generates the maximum possible yield with high quality final products.

When the peptide has fully assembled, the "permanent" side chain protecting groups are removed and the peptide is cleaved from the solid support, using conditions compatible with the labile residues.

5.2.1 Amino-protecting groups

In the early stages of peptide synthesis, it was realised that urethane derivatives were particularly suitable for the protection of amino groups. These derivatives were easily prepared and chemically stable. The urethane nitrogen atom is usually inert to the subsequent peptide synthesis reaction conditions. The Fmoc group (ring-subtituted benzyl urethane) is labile to bases and thus makes the Fmoc group ideal for temporary protection.

5.2.2 Carboxy-protecting groups

Amino acid benzyl-$CO_2CH_2C_6H_5$ and t-butyl-CO_2CMe_3 ester are more popular in peptide synthesis since their cleavage conditions were analogous to the corresponding urethanes used for amino protection.

5.2.3 Side chain protecting groups

Most of the amino acids commonly encountered in proteins contain functional side chains. The need for protecting these side chains depends upon the severity of the reaction conditions. Since in solid phase synthesis the reaction conditions tend to be rather severe, side chain protection is almost always required. It is also convenient in solid phase synthesis if the majority or all of the permanent protecting groups are cleaved simultaneously in a single step at the end of the synthesis.

5.2.4 Peptide-resin bond

The most important step in solid phase synthesis is the chemical linkage of the growing peptide to the resin support. It has to be easily formed, stable to repeat cycles of acrylation and deprotection reactions and yet easily cleaved at the end of the synthesis without damage to newly formed peptide bonds.

5.2.5 Formation of the peptide bond

A peptide bond is formed when the amino group of one amino acid reacts with the carboxyl group of a second amino acid, eliminating a water molecule and resulting in a peptide (amide) bond. In order to form a peptide bond between two amino acids it is necessary to activate the carboxyl group of one of the amino acids. Simply alkyl esters of protected amino acids undergo aminolysis at too slow a rate to be generally useful for peptide bond synthesis. Phenyl esters are more reactive, and when electronegative substituents are present in the aromatic ring it makes them even more reactive.

1-Hydroxybenzotriazole (acylation catalyst) is commonly used as a catalyst in these reactions. In this study pentafluorophenyl ester of Fmoc amino acids were used. Pentafluorophenyl esters are efficient acylating agents and their chemical structures provide little opportunity for side reactions. They are cleaved cleanly and rapidly by solutions of secondary

bases in DMF, conditions that leave even particularly sensitive *t*-butyl derivatives entirely unaffected. Hence selectivity between amino acid side chain deprotection reactions is obtained.

The aims of this study were:

I) To develop an RGD-containing peptide with an anti-thrombotic effect and endothelial cell adhesion property using solid phase peptide synthesis.

II) To modify the peptide developed to enhance its activity and suitability for surface modification application of poly(carbonate urea)urethane polymer.

III) To characterise the peptides developed.

5.3 METHODS AND MATERIALS

5.3.1 Solid phase peptide synthesis

The synthesis of GRGD, GRGDS, LA-GRGD, LA-GRGDS, repeat GRGD(AhxGRGD)$_3$ and repeat LA-GRGD(AhxGRGD)$_3$ was carried out manually by a stepwise solid phase method on a Rink-amide resin. Briefly to synthesise a peptide the first amino acid was coupled to the resin followed by the sequential coupling of the remaining F-moc amino acids with side chain protected groups as appropriate. On completion the peptide was then detached from the resin. In the case of LA-conjugated peptides the same process was followed by conjugation of lauric acid prior to detachment of the peptide. Rink-amide resin and F-moc amino acids were supplied by Novabiochem, Beeston, U.K. and all other chemicals by Rathburn, Walkerburn, Scotland.

***Procedure* I:** *Fmoc deprotection.*

0.5g Fmoc-protected resin was deprotected by suspending in 10ml of 20% piperidine in DMF and agitating with N_2 for 5 minutes twice at room temperature. The resin was then washed five times with dimethylformamide (DMF; 10ml).

Procedure II: *Coupling with HBTU/HOBt.*

A reaction solution was prepared consisting of 2.5 equivalents of the initial Fmoc-amino acid, an equivalent amount of 2-(1H-Benzotriazole-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate(HBTU) and 1-hydroxybenzotriazole (HOBt), and 1M N,N-diisopropylethylamine (1.6ml in 10mls DMF). This mixture was then added to the deprotected resin, allowed to couple for 30 to 40 minutes at room temperature, then washed with DMF under vacuum.

Procedure III: *Deprotection of Fmoc-amino acid coupled to resin*

The N-terminal of the Fmoc-amino acid coupled to the resin was subjected to two deprotection cycles with 10ml of 20% piperidine in DMF and agitated for 5 minutes. It was then thoroughly washed five times with 10ml DMF for 1minute each.

This process of deprotection and coupling was then repeated to add the remaining Fmoc-amino acids resulting in a peptide-resin complex.

Procedure IV: *coupling of aminohexanoic acid to GRGD-resin complex*

Aminohexanoic acid (Ahx) was also deprotected and coupled to the GRGD-resin complex as above. A further GRGD sequence was then added using the process above and this procedure continued until four repeat GRGD sequences had been synthesised.

Procedure V: *Conjugation of Lauric acid to the peptide-resin complex.*

In the case of the preparation of LA-conjugated peptides, LA (153mg) was coupled to the peptide-resin complex using the same deprotection and coupling process above.

Procedure VI: *Detachment and deprotection of peptide and LA-conjugated peptide from the solid phase.*

The resin was washed with dichloromethane (DCM) and vacuum dried. 10% trifluoroacetic acid (TFA) in DCM was added and agitated for 60 minutes. Every 20 minutes the filtrate was collected. The TFA and DCM were removed from the combined filtrates by rotary evaporation until an oily mixture remained. To this oily mixture 50ml 95% TFA in H_2O (containing 5% scavengers (1,2 ethanedithiol (EDT), phenol and thioanisole)) was added followed by incubation at room temperature for 5 hours for complete deprotection. The TFA and scavengers were removed by rotary evaporation

and cold diethyl ether was added to precipitate the peptide and remove any residual scavengers. The precipitated peptide was further rinsed with diethyl ether and centrifuged to obtain a pellet which was dried under vacuum. An example of the solid phase peptide synthesis scheme is shown in Figure 5.4 for LA-GRGDS with the other peptides synthesised in a similar manner.

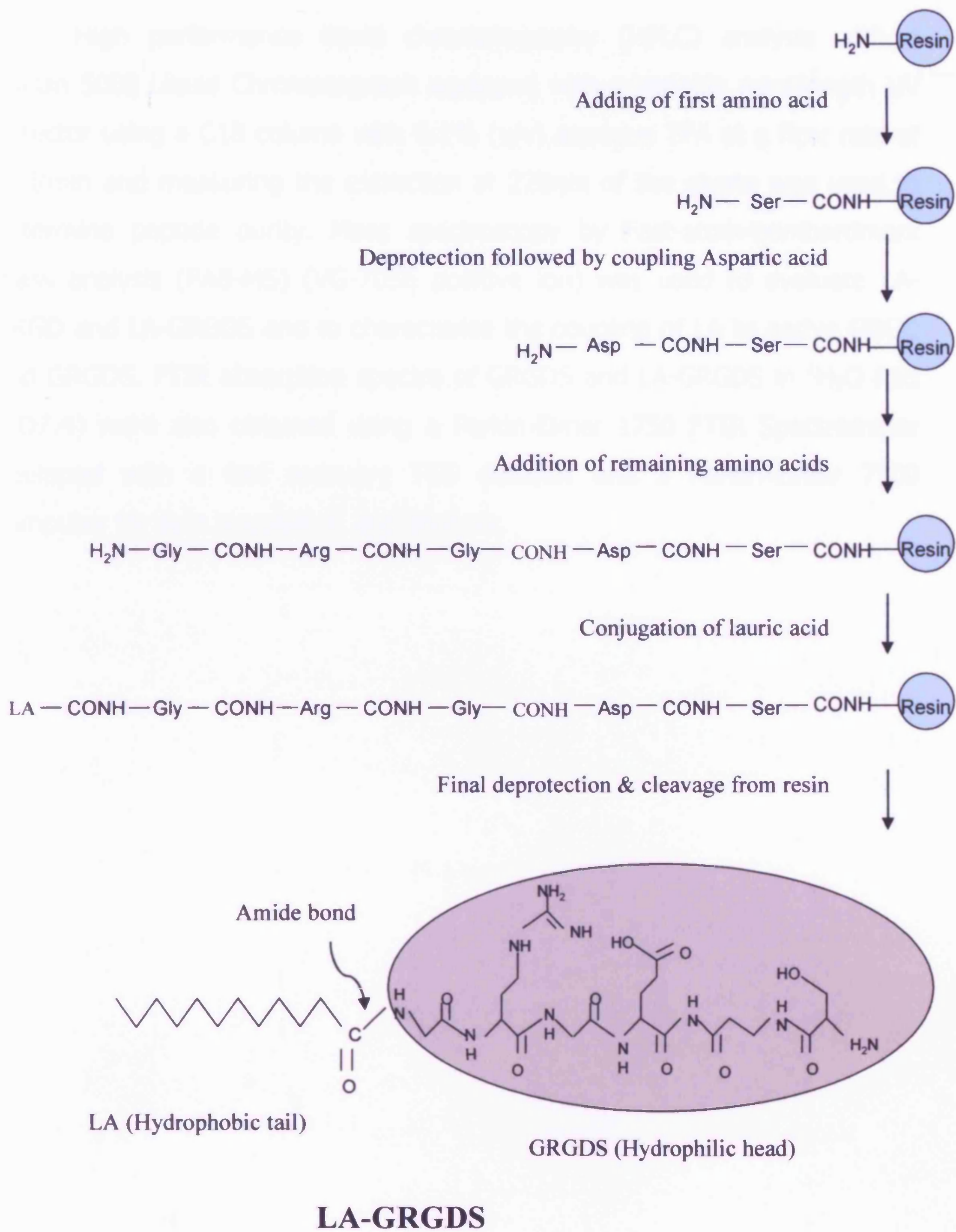


Figure 5.4 Typical solid phase peptide synthesis scheme: example shown for LA-GRGDS.

5.3.2 Characterisation and analysis of peptides

High performance liquid chromatography (HPLC) analysis with a Varian 5000 Liquid Chromatograph equipped with a variable wavelength UV detector using a C18 column with 0.1% (v/v) aqueous TFA at a flow rate of 1ml/min and measuring the extinction at 220nm of the eluate was used to determine peptide purity. Mass spectroscopy by Fast-atom-bombardment mass analysis (FAB-MS) (VG-70SE positive ion) was used to evaluate LA-GRGD and LA-GRGDS and to characterise the coupling of LA to native GRGD and GRGDS. FTIR absorption spectra of GRGDS and LA-GRGDS in ^2H_2O PBS (pD7.4) were also obtained using a Perkin-Elmer 1750 FTIR Spectrometer equipped with a fast recovery TGS detector and a Perkin-Elmer 7300 computer for data acquisition and analysis.

5.4 Results

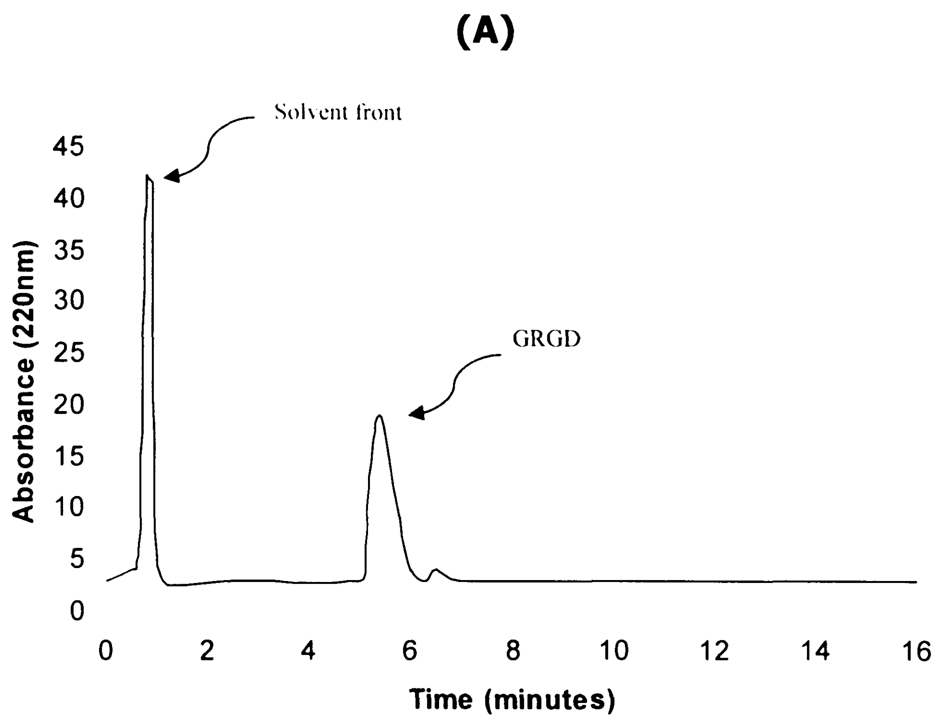
Hydrophilic GRGD, GRGDS and repeat GRGDAhx and their corresponding lauric acid (LA) conjugated peptides listed on Table 5.2 were produced via a solid phase peptide synthesis. The yield of peptide obtained from a typical synthesis was approximately 150mg.

Peptide Synthesised	**Peptide Sequence**
GRGD	Gly-Arg-Gly-Asp
LA-GRGD	LA- Gly-Arg-Gly-Asp
GRGDS	Gly-Arg-Gly-Asp-Ser
LA-GRGDS	LA- Gly-Arg-Gly-Asp-Ser
Repeat GRGDAhx	Gly-Arg-Gly-Asp-(Ahx-Gly-Arg-Gly-Asp)$_3$
Repeat LA-GRGDAhx	LA-Gly-Arg-Gly-Asp- (Ahx-Gly-Arg-Gly-Asp)$_3$

Table 5. 2 Amino acid sequence for peptide preparation by solid phase synthesis.

5.4.1 HPLC analysis of peptides

The purity of the peptides synthesised was analysed by using reverse-phase HPLC which revealed the presence of a well-resolved single major peak as shown in Figures 5.5, 5.6 and 5.7.



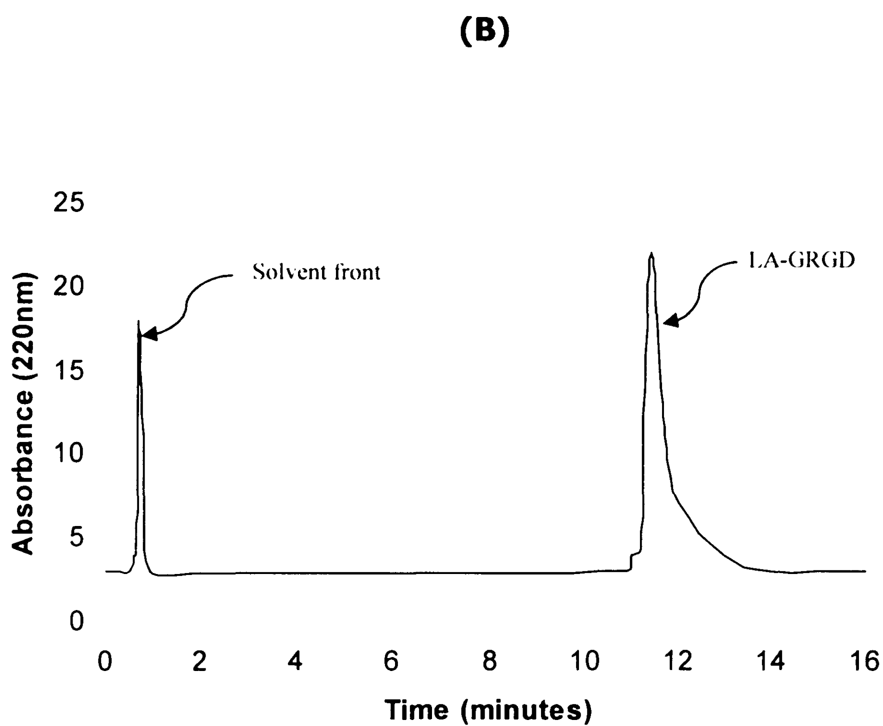
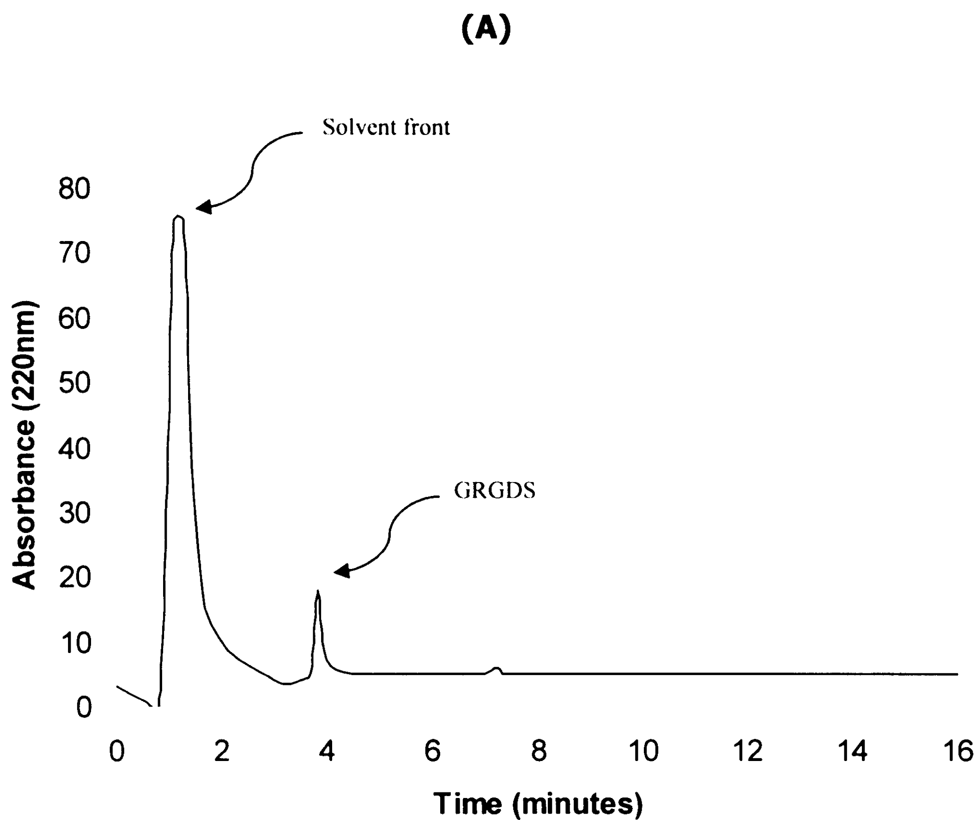


Figure 5.5 HPLC profile of (A) GRGD and (B) LA-GRGD.

HPLC chromatography obtained from the extinction at 220nm of the eluate in 0.1% (v/v) aqueous TFA at a flow rate of 1ml/min from 300A pore-size, C18 column.



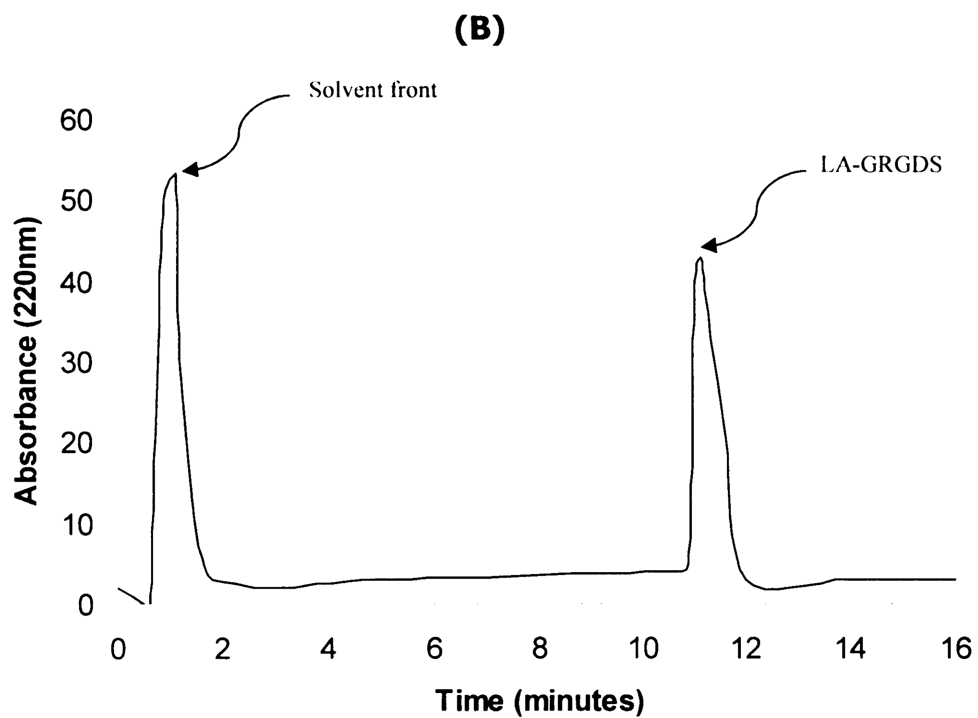
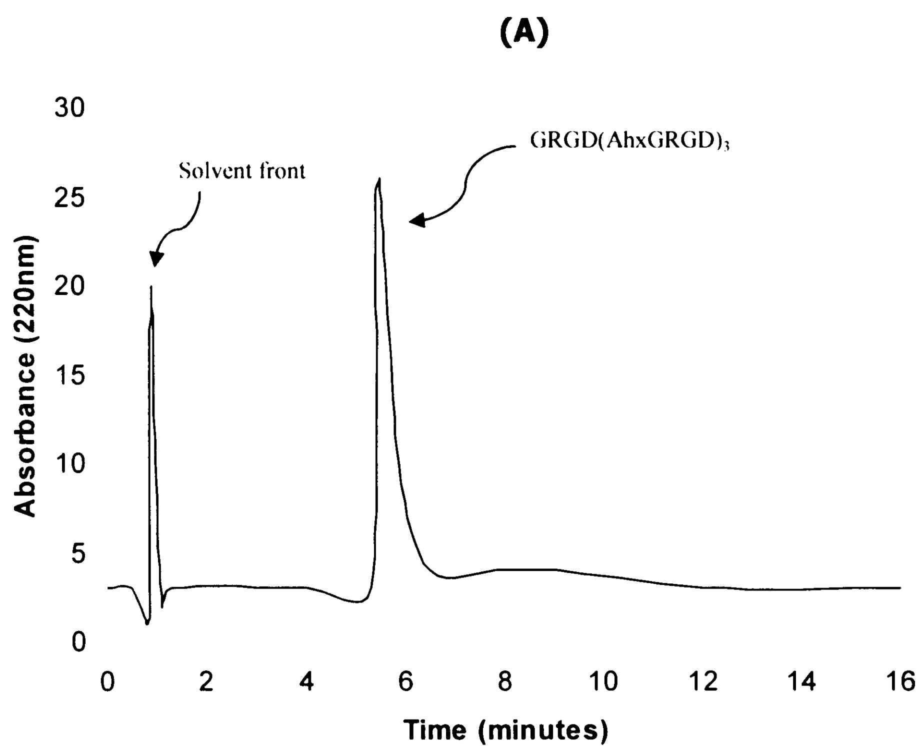


Figure 5.6 HPLC profile of peptides (A) GRGDS and (B) LA-GRGDS.
HPLC chromatography obtained from the extinction at 220nm of the eluate in 0.1% (v/v) aqueous TFA at a flow rate of 1ml/min from 300A pore-size, C18 column.

(A)



(B)

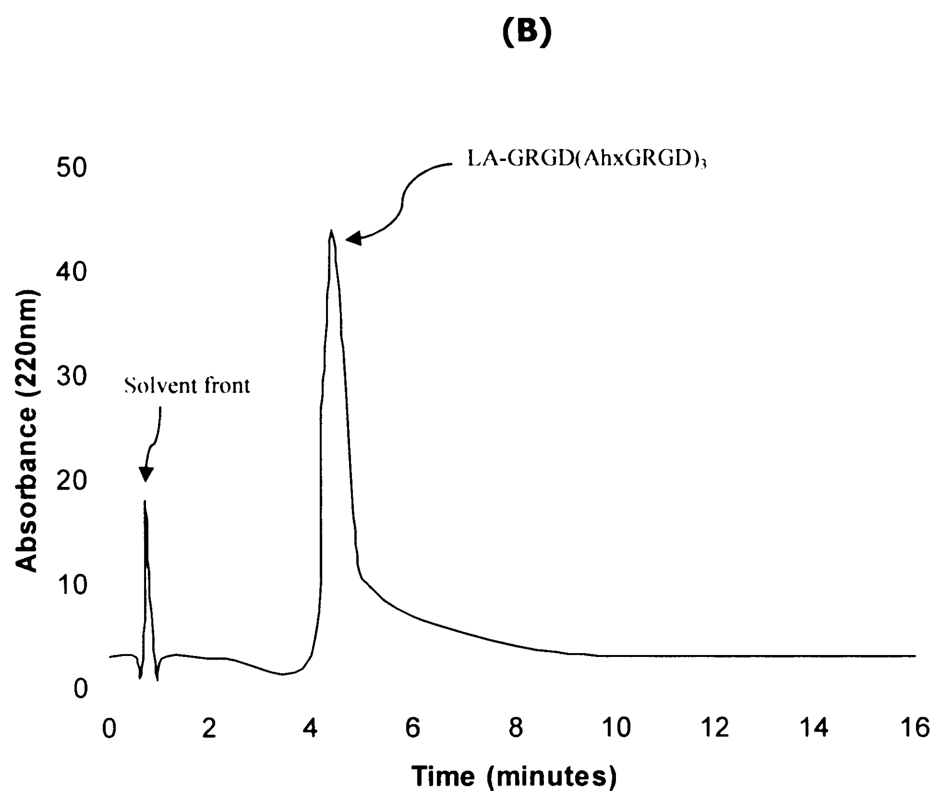


Figure 5.7 HPLC profile of peptides (A) $GRGD(AhxGRGD)_3$ and (B) LA-$GRGD(AhxGRGD)_3$.

HPLC chromatography obtained from the extinction at 220nm of the eluate in 0.1% (v/v) aqueous TFA at a flow rate of 1ml/min from 300A pore-size, C18 column.

5.4.2 Mass Spectrometry

The effectiveness of the conjugation of LA to GRGD and GRGDS was confirmed by Fast-atom-bombardment mass analysis (FAB-MS) (VG-70SE positive ion). The value determined by FAB-MS for LA-GRGD was 585 which was in agreement with the predicted molecular weight of 585 (Figure 5.8). In the case of LA-GRGDS the determined value was 672 which was again in agreement with the predicted molecular weight of 672 (Figure 5.9).

Although FAB-MS produces very accurate analysis of a wide range of synthetic peptides, a major limitation was its upper mass limit. Hence due to their high molecular weigh, it was impossible to evaluate the LA-GRGD(AhxGRGD)$_3$ peptide.

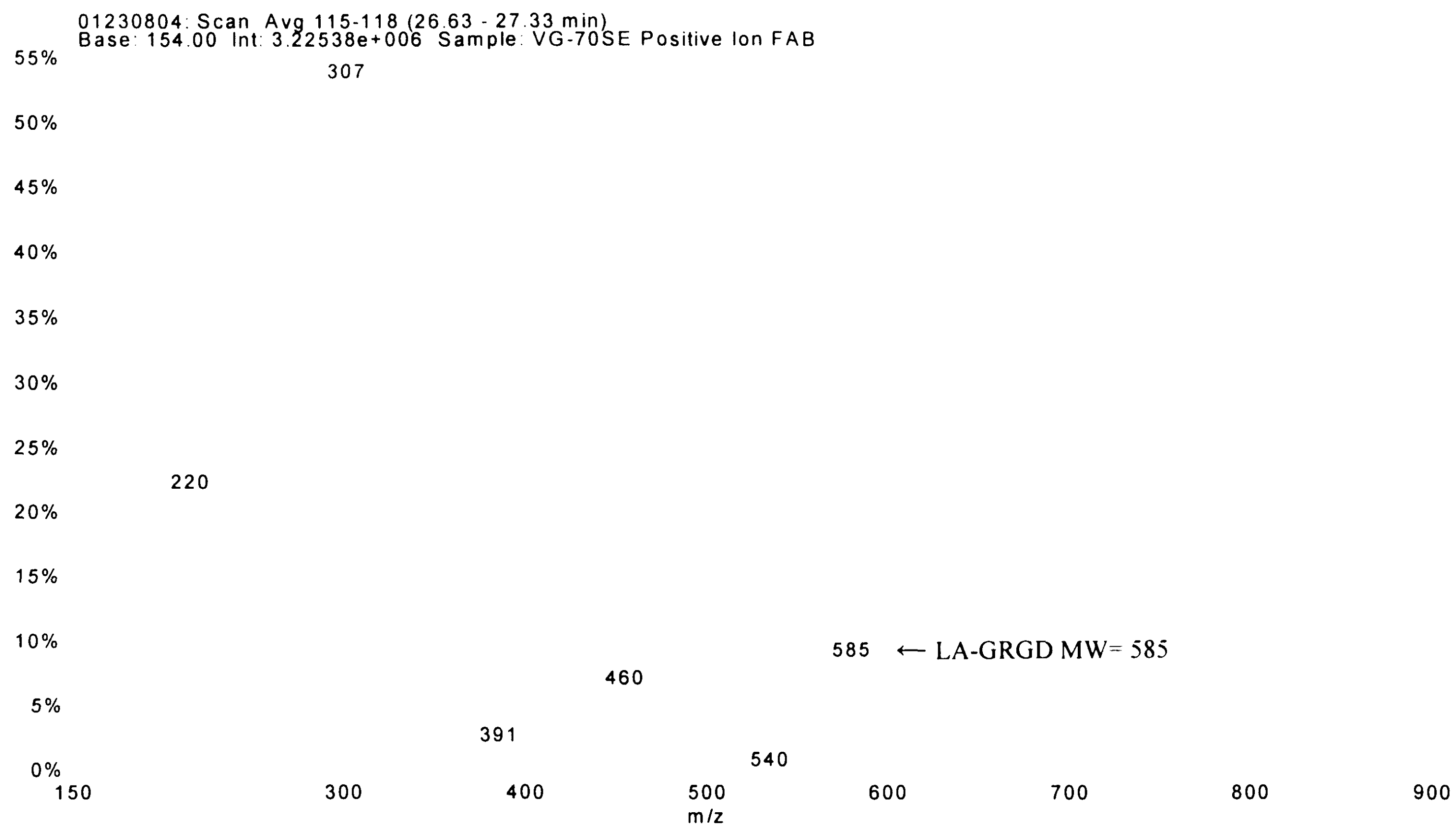


Figure 5.8 Mass Spectra trace of LA-GRGD

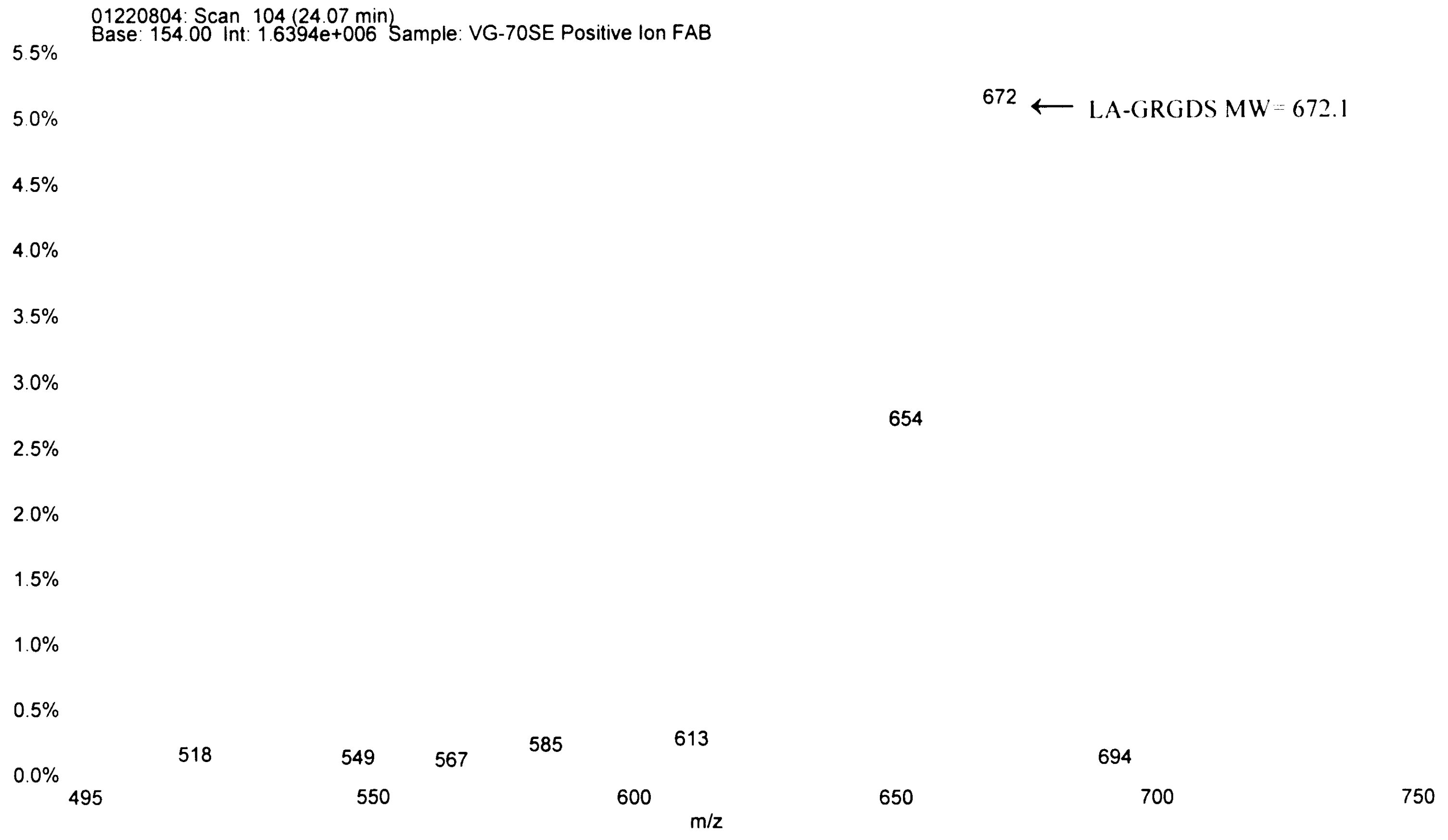


Figure 5.9 Mass Spectra trace of LA-GRGDS

5.4.3 FTIR analysis

FTIR absorption spectrum of GRGD, LA-GRGD, GRGDS, LA-GRGDS, $GRGD(AhxGRGD)_3$ and $LA\text{-}GRGD(AhxGRGD)_3$ in ^2H_2O PBS (pD 7.4) are shown in Figure 5.10, 5.11, 5.12, 5.13 5.14 and 5.15 respectively. The amide I band maximum for GRGD, LA-GRGD, GRGDS and LA-GRGDS was observed at 1650 cm^{-1}. The 1650cm^{-1} band is assigned to α-helical structure and demonstrates the formation of a protein-like secondary structure. Despite the conjugation of LA to the GRGD and GRGDS, the peptides remain stable and maintain their structure. The amide I band maximum for $GRGD(AhxGRGD)_3$ and $LA\text{-}GRGD(AhxGRGD)_3$ was found to be 1643.

The amide I vibration, which is principally C=O stretching, can vary according to the nature of the hydrogen bonding found in α-helical and β-sheet structures. As a result, the frequency of the amide I band can be applied to distinguish between the secondary structures, which occur in proteins and peptides. Bands in the spectral range 1620-1640 cm^{-1} are attributed to the β-sheet accompanied by minor component bands at 1680-1690 cm^{-1}. This vibration in absorption may be attributed to the protein water interactions which is minimal in hydrophobic membrane environment and hence in increase in the amide I maximum.

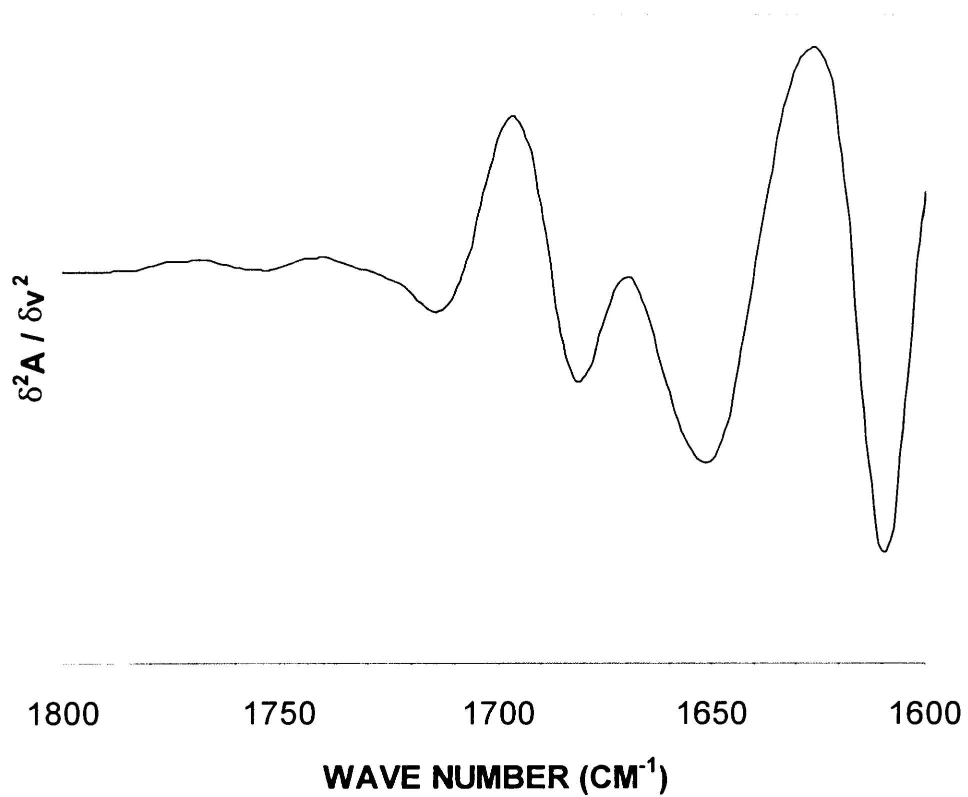


Figure 5.10 FTIR spectrum of GRGD in ^2H_2O PBS (pD 7.4) at 30°C

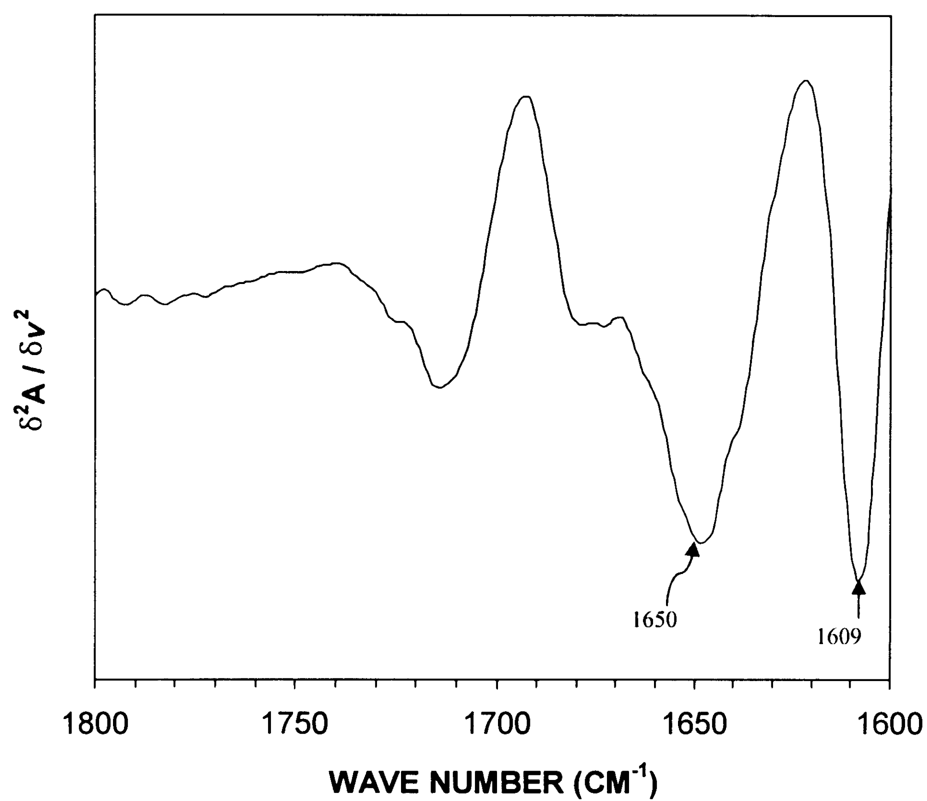


Figure 5.11 FTIR spectrum of LA-GRGD in ^2H_2O PBS (pD 7.4) at 30°C

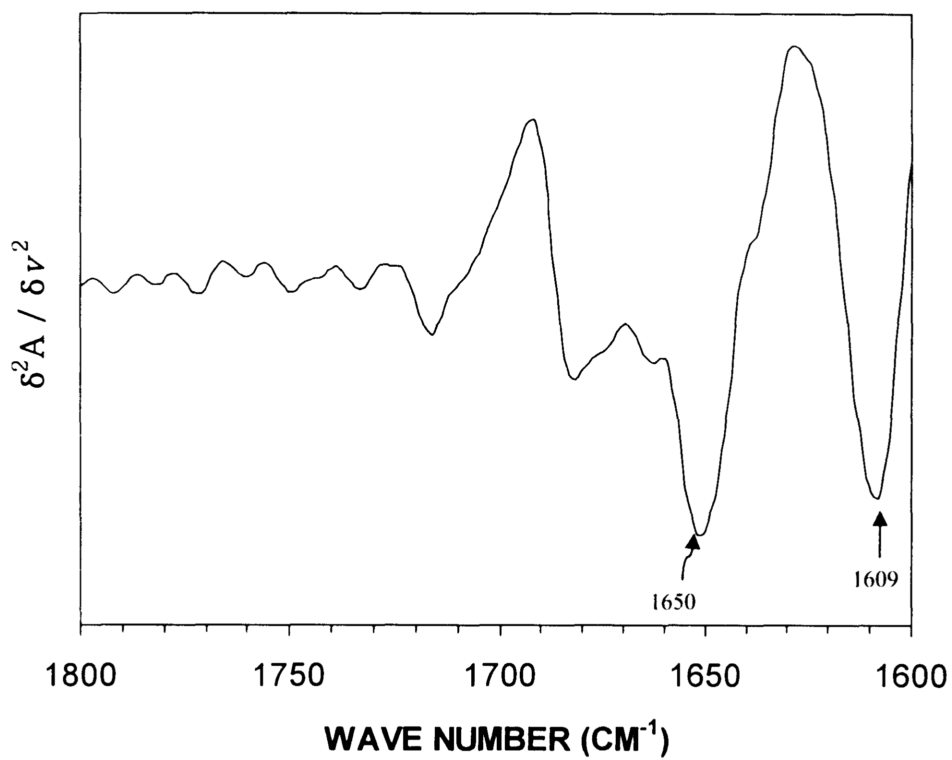


Figure 5.12 FTIR spectrum of GRGDS in $^{2}H_2O$ PBS (pD 7.4) at 30°C.

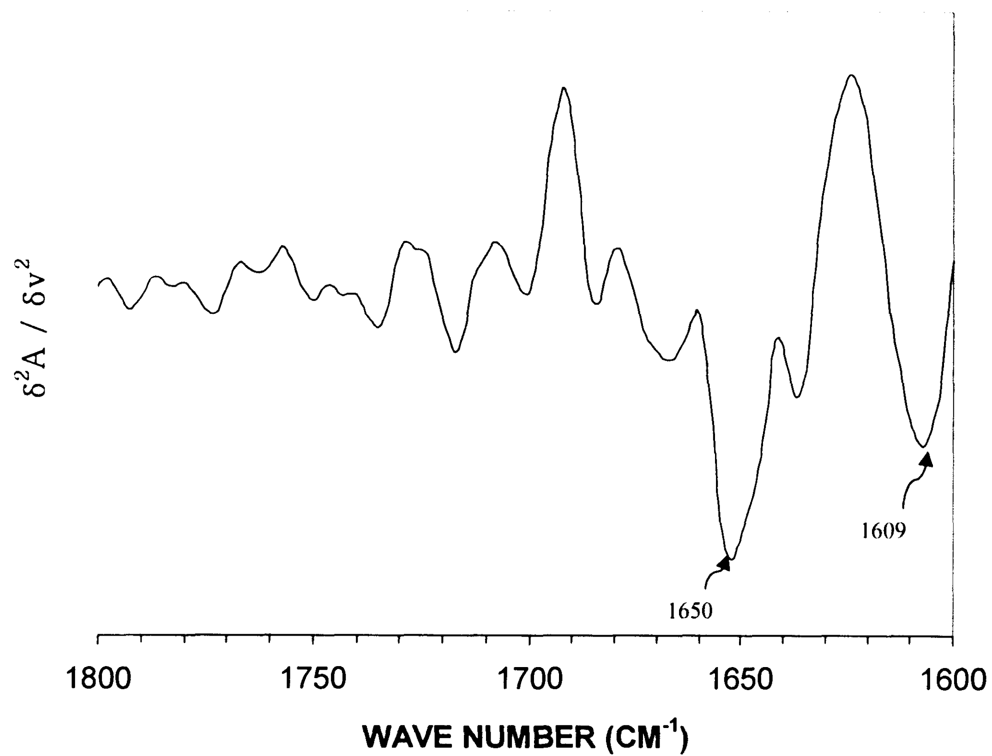


Figure 5.13 FTIR spectrum of LA-GRGDS in ^2H_2O PBS (pD 7.4) at 30°C

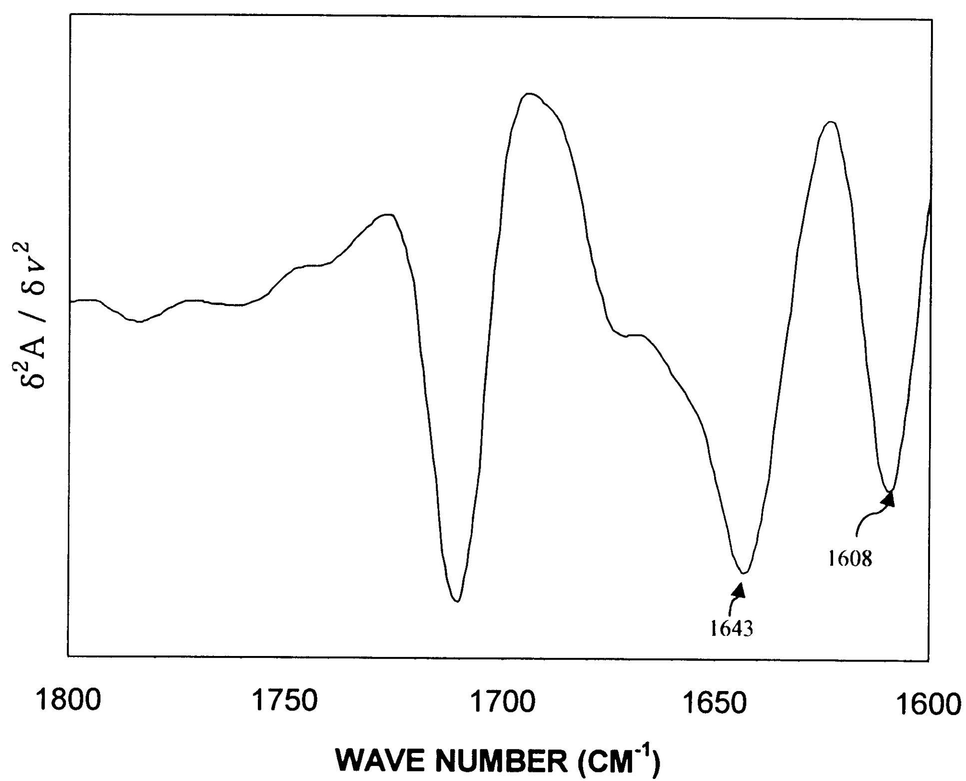


Figure 5.14 FTIR spectrum of GRGD(AhxGRGD)$_3$ in ^2H_2O PBS (pD 7.4) at 30°C

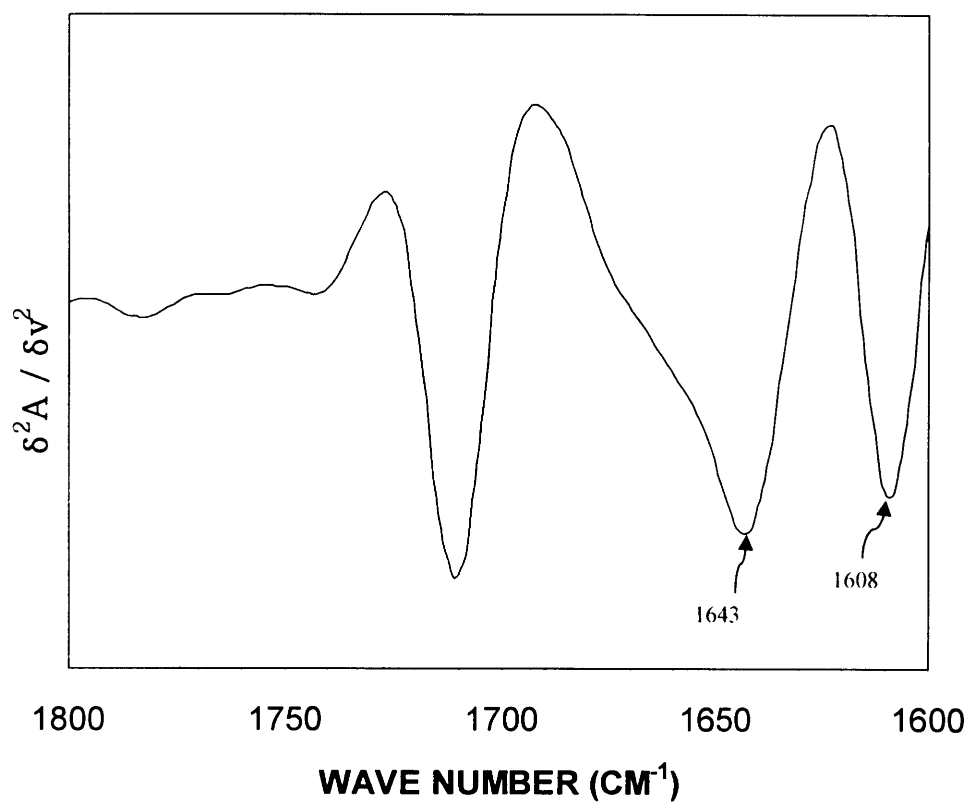


Figure 5.15 FTIR spectrum of LA-GRGD(AhxGRGD)$_3$ in ^2H_2O PBS (pD 7.4) at 30°C.

5.5 Discussion

The theory behind designing peptidomimetic agents is based on a knowledge of the amino acids as the basic building blocks that constitute the protein. These provide the crucial functional groups to establish interaction with the active site of the receptor and result in a functioning protein. Therefore in this study, the amino acids arginine, glycine and aspartic acid are used as building blocks for the synthesis of different RGD-containing peptides.

As described previously (CHAPTER 2) the tri-peptide Arg-Gly-Asp (RGD) sequence is known to be the active sequence of adhesive proteins of the extracellular matrix (ECM) [268-270] that binds to integrin receptors. However RGD tri-peptides are small peptides that lack a defined structure, which is necessary for their recognition by receptors. Hence to ensure adequate binding of the peptide segment to the cell surface receptors and to prolong the degradation time, the RGD tri-peptide was lengthened to include two additional amino acids (Glycine and Serine) from the fibronectin sequence.

A variety of RGD-containing peptides have been developed previously to improve the bio-compatibility properties of cardiovascular devices. In this context, the aim of this work is to develop RGD-derivative peptides which demonstrate an anti-thrombotic and EC adhesion effect (targeting the $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ integrins) when immobilised onto the surface of a PCU polymer. The RGD-containing peptides are functionalised in order to provide stable linking to the polymer via a functional (amino) group.

One method of achieving this aim is to conjugate the RGD-containing peptide to a fatty acid such as LA. This conjugation of the peptide to LA is crucial to allow its solubility in organic solvents. Ordinarily RGD peptides do not readily dissolve in the organic solvents used to dissolve the polymer itself during the commercial synthesis process due to their hydrophilic nature and thus are unsuitable for incorporation into the polymer during production. The difficulty

can be potentially overcome by employing the conjugation of LA to the RGD peptide to provide an amphiphilic property to the hydrophilic RGD peptide.

It has been suggested that larger molecules are more likely to interact with surfaces as they are able to contact the surface at more sites. Conjugating many copies of a ligand to a single-polymer backbone has also been proven to be a successful method for increasing ligand affinity and specificity through multivalent interactions. Previously several researchers have developed multimeric compounds in which the RGD sequence is locally enriched and can bind polyvalently to the cells to enhance the affinity of the receptor-ligand interactions [271-274]. Maynard et al synthesised a polynorbones polymer with multidentate ligands by the conjugation of a synergistic peptide Pro-His-Ser-Arg-Asn (PHSRN) in addition to GRGDS to the polymer backbone. Since PHSRN enhances cell binding to the RGD domain in fibronectin, a copolymer substituted with both GRGDS and PHSRN should exhibit a higher competitive inhibitory activity than a polymer containing only GRGDS. The results presented in the study showed that the copolymer substituted with GRGDS and PHSRN ligands was the most potent inhibitor of cell adhesion when compared to the homopolymer containing only the GRGDS ligand [272].

The increased affinity of RGD ligands due to multivalent interactions has also been demonstrated by Kok et al [275]. Multivalent derivatives of a cyclic RGD-peptide were prepared by covalent attachment of the peptide to the side chain amino groups of a HuMab protein. EC adhesion assays and radiobinding studies into the affinity of these derivatives to the $\alpha_v\beta_3/\alpha_v\beta_5$ integrins demonstrated that the RGD-peptide-protein conjugates inhibited $\alpha_v\beta_3$-mediated EC adhesion and that the amount of RGD-peptide coupled per protein affected the affinity of the RGD-peptide-protein conjugates to EC compared to the free peptide. Based on this work in this study repeat GRGD peptide sequences have been synthesised to enhance and prolong the peptide activity by incorporating an amino acid derivative, aminohexanoic acid (Ahx) into the peptide structure.

The Ahx acts as a spacer between the GRGD sequences and may also provide an additional function as an anti-fibrinolytic agent [265, 266].

Solid phase peptide synthesis was used to produce all the RGD-containing peptides and their corresponding LA conjugated peptides by attaching a LA to an RGD-containing peptide via an amide bond (Table 5.2). Following synthesis the peptides produced were characterised using HPLC, FTIR and mass spectrometery. The HPLC analysis demonstrated the peptides homogenecity revealing the presence of a well resolved single major peak in each chromatogram. The FTIR absorbance results confirmed the formation of a protein-like secondary structure with all the peptides showing an amide I band maximum in the range of 1615-1695 cm^{-1}. GRGD, LA-GRGD, GRGDS and LA-GRGDS showed an amide I band maximum at 1650 cm^{-1} band which is assigned to α-helical structure. Both repeat GRGD peptides and LA-GRGD demonstrated an amide I band maximum at 1643 cm^{-1}. Mass spectrometry analysis confirms that the solid phase peptide synthesis was successful and that the successful conjugation of LA to the peptides GRGD and GRGDS had been achieved.

In conclusion the results presented in this study show that the synthesis of the RGD-containing peptides GRGD, GRGDS and repeat GRGD peptides was achieved by utilising a solid phase peptide synthesis process. The synthesis of the corresponding LA conjugation peptides has also been demonstrated to be successful. Finally the results of this study also show that the conjugation of LA to the various peptides produced no effect on the structure or stability of the original RGD-containing peptide.

CHAPTER SIX

EVALUATION OF NEW PEPTIDES

6.1 Introduction

This study was designed to evaluate the anticoagulation effect of RGD-derivative peptides prepared using solid phase peptide synthesis (Chapter 5) upon tissue factor activity (in control plasma and fresh whole blood), to define the likely cytotoxic concentrations on EC metabolism and also assess their EC binding effect. As previously discussed, these peptides were developed and synthesised for surface modification application of a poly(carbonate-urea)urethane (PCU) polymer. In this department, a variety of research including this study, has been performed to improve the surface anti-thrombotic property of PCU to be used for small diameter vascular grafts [35, 38, 39, 161, 276]. The surface modification using RGD-derivative peptides is aimed to reduce surface thrombosis by inhibiting blood coagulation and enhance EC adhesion to provide an endothelial layer. Therefore it is important to investigate peptide anticoagulant activity using plasma and whole blood. It is also necessary to evaluate the optimal concentration and cytotoxic concentration of these peptides. To assess the peptides effect on EC metabolism and EC adhesion, EC extracted from human umbilical vein (HUVEC) were used.

6.1.1 Measurement of procoagulant activity

The one-stage prothrombin time assay is a simple and quick technique which can be used to evaluate the effect of different agents and varying concentrations on inhibition of plasma coagulation. Prothrombin time analyses prolonged clotting time of plasma by an agent which possesses an inhibitory effect on the coagulation cascade. A prolonged clotting time may result from the inhibition of one or more of the following, proaccelerin (V), proconvertin (VII), Stuart factor (X), prothrombin (II) or fibrinogen (I). The one-stage prothrombin time assay relies upon the initiation of the extrinsic pathway of coagulation. Tissue factor in the presence of calcium and phospholipids, acts as a cofactor

for factor VII to facilitate the activation of factor X and subsequent coagulation factors. This culminates in the formation of a clot. An unknown quantity of tissue factor activity in a sample can be determined through examination of the time taken to produce a clot. The higher the concentration of tissue factor present within a sample the less time is taken for clot formation.

6.1.2 Assessment of whole blood coagulation (thrombelastograph analysis)

Thrombelastography (TEG) is used to measure the clotting time of whole blood. TEG has been used in many clinical settings since its introduction in 1948 and has been shown to be a reliable technique for diagnosis and monitoring treatment of various blood disorders. The TEG measurements correlate well with the coagulation profile[234]and have the advantage of being a rapid technique which requires small blood volumes. The time it takes for whole blood to clot is a measure of the efficiency of all stages of the intrinsic clotting pathway. A prolonged clotting time of whole blood may result from the inhibition of platelet activation and inhibition of one or more of the following clotting factors XII, XI, IX, X, II or I.

The output from the TEG records the elasticity of the blood clot (Figure 3.3). A number of important parameters can be measured and quantified which reflect the nature of the coagulum produced. In the TEG the reaction time denoted `r` (TEG-r) is measured from the start mark (re-calcification point for a citrated sample) until an amplitude of 2mm is obtained. During this time there is no resistance in the blood and so the piston remains motionless. The r-time corresponds to the time taken for the formation of the first fibrin strands, and is therefore indicative of the enzymatic sequence of events which occur in the blood coagulation cascade prior to fibrinogen conversion to fibrin. The time from the measurement of r (the beginning of the clot formation) until a fixed level of clot firmness is reached (amplitude 20mm) is the k-time. It is a measure of the

speed or clot kinetics to reach a certain level of clot strength. Angle (α) is closely related to k-time. The angle is more comprehensive than the k-time, since there are hypocoagulable conditions in which the final level of clot firmness does not reach an amplitude of 20mm. The maximum strength or stiffness of the developed clot is described as `ma` (Maximum Amplitude).

In general, the shape of the curve (k,α and ma) is determined by fibrinogen and platelet activity whereas the r-time is highly dependent on the functional aspects of the clotting factors.

6.1.3 Assessment of cell metabolism and cytotoxicity

Alamar blue™ (AB) is an assay designed to measure quantitatively cell metabolism, cytotoxicity, and viability by incorporating resazurin and resarufin as colorimetric oxidation reduction indicators that change in colour in response to chemical reduction resulting from cell metabolism. The data may be collected with either fluorescence based or absorbance-based instruments. AB has particular properties that make this assay attractive to be used in this study. It is soluble in culture media, stable in solution, minimally toxic to cells and produces changes that are easy to measure. AB has the advantage of allowing a continuous assessment of metabolism and viability of seeded cells. It is simple to perform, and does not destroy the cells [217]. Limitations of AB are few. If prolonged incubation times are used (>24 h), reversal of the reduction process occurs via a secondary redox step, resulting in a colourless solution, particularly when very high cell concentrations are used.

6.2 Method and Materials

6.2.1 Moieties

RGD-containing peptides prepared by solid phase peptide synthesis (CHAPTER 5), GRGD, LA-GRGD, GRGDS, LA-GRGDS, $GRGD(AhxGRGD)_3$, LA-$GRGD(AhxGRGD)_3$ were to give 1000µg/ml concentration and diluted to 1:10, 1:20, 1:100, 1:200, 1:1000.

6.2.1.1 Fibronectin engineered protein polymer

Fibronectin engineered protein polymer (FEPP) contains a multiple repeated sequence of VTGRGDSPAS incorporating the GRGD sequence (Sigma Chemical Company, Poole, UK). FEPP (MW= approximately 72,000) was prepared to give 1000µg/ml and further diluted to 1: 10, 1: 20, 1: 100, 1: 200 1: 1000, and 1:10,000.

6.2.1.2 Heparin

Heparin (MW= approximately 15,000, Monoparin®, 1000 units/ml) from CP Pharaceuticals Ltd (Wrexham, UK) was used. The stock concentration was diluted with phosphate buffer saline (PBS) (from Sigma Poole, Dorset, UK) to1: 1000, 1:2000, 1:10,000, and 1:100,000.

6.2.1.3 Lauric acid

A stock solution of Lauric acid (LA) (Sigma Chemical Company, Poole, U.K. MW= 200) at 1000µg/ml was prepared and further diluted to 1: 10, 1: 20, 1: 100, 1: 200 1: 1000, and 1:10,000.

6.2.2 Thromboelastograph analysis

The action of peptides was assessed in fresh whole blood using a computerised thromboelastograph (TEG) coagulation analyser (Launch diagnostics, UK). TEG was carried out using venous blood with the first few millilitres discarded to reduce any effect due to tissue thromboplastin which may be released on venepuncture. The blood sample was placed in a pre-warmed cuvette. A piston which is freely suspended by a fine torsion wire and connected to a pen recorder chart, is lowered into the blood resulting in a uniform clearance of 1 mm between the piston and cuvette. The blood is then covered with a thin layer of liquid paraffin to prevent drying and a resulting pH change due to atmospheric oxygen.

Selected blood donors were normal healthy volunteers, who had taken no medication known to influence platelet function or blood coagulation for 14 days prior to blood donation. Their TEG parameter values were within the normal range.

Fresh venous whole blood was taken from 10 volunteers by venepuncture with a 21 gauge butterfly needle (M Abbott, Medical system) from an uncuffed arm vein using the syringe technique. The first 2ml were discarded to eliminate the effects of tissue thromboplastine released on venepuncture. 18ml of blood was collected into a second syringe or blood tube with 3.8% sodium citrate (1:10, v/v, at pH 7.4) (Sarstedt, Leicester, UK). The citrated blood was incubated at room temperature for 30 min. 20µl 0.2 M of calcium chloride (Medicell, London, UK) was used to initiate the coagulation and 50 nM recombinant tissue factor (Gamidore, Abingdon, Oxfordshire, UK) was added to activate the clot. Experiments were repeated four times.

6.2.3 Prothrombin time assay

The one-stage prothrombin time (PT) assay was performed on a Cascade - M- coagulometer (Helena Laboratories). The assay was carried out at 37°C using

50 nM recombinant tissue factor (TF) in 12.5 mM $CaCl_2$ to which standard plasma was added (2:1 ratio), and the clotting time was measured using a coagulometer. Clotting was initiated by the addition of 100µl reconstituted plasma (Sigma Chemical Company, Dorset, U.K.) containing clotting factors in their inactive form, thus initiating the extrinsic pathway of coagulation through the formation of the tissue factor VIIa complex. Clot formation was detected via a change in optical density of the sample, and a reading of clotting time supplied.

From the time taken for clot formation to occur, tissue factor activity was quantified by reference to recombinant tissue factor standard, which at a concentration of 1000 units/ml clotted plasma in 14 sec. A standard curve was constructed using recombinant tissue factor diluted with distilled water, and expressed log tissue factor concentration (units/ml) versus log clotting time (seconds) (Figure 3.5). The concentration of tissue factor was calculated using straight line regression constants. The tissue factor activity was then calculated from a standard curve prepared. Experiments were repeated four times.
The percentage of inhibition due to the moiety was calculated as:

$$Inhibition(\%) = 100x\frac{A_i - A_r}{A_i}$$

Where *A* is activity and subscript *i* and *r* are initial and residual respectively

6.2.4 Assessment of cell metabolism and cytotoxicity

Cytotoxicity of all the RGD-containing peptides, LA and heparin to EC metabolism and survival was assessed using AB assay (Serotec Ltd., Kidlington,

Oxford). AB is a reagent which is incorporated and metabolised by EC [217]. AB was added to cells grown in 24-well plates at a final concentration of 10% for 4 hours. 50-100 µl of the AB/medium mixture was then added to 96-well plate and measured using a Multiscan MS spectrophotometer (Labsystems, Finland) at wavelengths of 530 nm and 630 nm. EC metabolism and cytotoxicity was defined in terms of optical density units reflecting the absorbance values. Five wells per treatment at each time point were measured. Experiments were repeated four times.

6.2.5 Assessment of cell binding

The binding effect of RGD-containing peptides, LA and heparin to ECs was assessed in solution. All peptide including heparin were individually added at a concentration of 10µg/ml or 10U/ml to 2 x 10^5 trypsinised ECs and mixed gently. As a control 2 x 10^5 untreated EC were used. The cells were then plated in 24-well plates and incubated overnight. Following this the EC were washed with PBS to remove cells bound to moeties. 10% AB was added to the cells remaining in 24-well plate and absorbance was measured after 4 hours as above. Experiments were repeated four times.

EC binding was calculated as:

$$Binding(\%) = 100 \times \frac{C' - T}{C}$$

Where *C* and *T* are the absorbance value of the control and peptide respectively.

6.3 Result

6.3.1 Pro-coagulant activity and inhibition of tissue factor

The comparative effect of all synthesised RGD peptides, fibronectin engineered protein polymer (FEPP), the standard anticoagulant drug heparin and the thrombin inhibitor hirudin on the pro-coagulant activity is shown in Table 6.1. All the moieties inhibited the pro-coagulant activity of TF to different degrees at a different concentration following 2 minute incubation at 37°C. The standard anticoagulant heparin inhibited tissue factor activity by >90% at 1 U/ml concentration. Similar effects were noted with FEPP and the lauric acid conjugated GRGD (LA-GRGD) at 100μg/ml and 200μg/ml respectively. GRGD, GRGDS and the repeated sequence of GRGD, GRGD(AhxGRGD)$_3$ showed <50% inhibition of tissue factor at 1000μg/ml concentration. Like LA-GRGD, the lauric acid conjugated GRGDS also demonstrated better (57.4%) inhibition of tissue factor than the corresponding non-conjugated GRGDS (37.3%). The inhibitory effect of the agents was found to be concentration dependent. LA alone had no effect on pro-coagulant activity and TF (results not shown).

6.3.2 Initial fibrin formation

The effect of all RGD peptides, FEPP and heparin on the initial fibrin formation (TEG-r time) is shown in Table 6.1. At 1 U/ml, heparin exhibited a relatively stronger inhibitory effect compared to hirudin, FEPP and all the RGD-derivative peptides developed. Heparin increased the TEG-r time by 87.5 ± 7.8 min. At 10U/ml hirudin increased the TEG-r time by only 40.6 ± 7.7min. At 10μg/ml, FEPP showed a comparable increase to heparin in TEG-r time (73.7 ± 8.4 min). LA-GRGD increased the TEG-r time by only 8.2 ± 3.3 min. All the rest of RGD-peptides, GRGD, GRGDS, LA-GRGDS and the repeated sequence of GRGD demonstrated no effect on the initial fibrin formation of whole blood. LA alone had no effect on the TEG-r time (results not shown).

Moieties	Concentration	% Inhibition	(TEG-r)(min)
Heparin	0.01 U/ml	-11.8 ± 8.2	-0.6 ± 1.2
	0.1 U/ml	17.5 ± 18.0	4.3 ± 1.6
	0.5 U/ml	88.1 ± 8.3	73.5 ± 16.1
	1 U/ml	97.4 ± 1.6	87.5 ± 7.8
FEPP	0.1 μg/ml	-24.8 ± 23.1	-0.6 ± 0.9
	10 μg/ml	-3.0 ± 17.9	1.3 ± 1.1
	50 μg/ml	55.1 ± 15.7	6.2 ± 3.7
	100 μg/ml	91.5 ± 5.3	73.7 ± 8.4
Hirudin	0.1 U/ml	-24.8 ± 23.1	-0.2 ± 1.0
	1 U/ml	18.8 ± 11.3	4.7 ± 1.3
	5 U/ml	74.5 ± 6.9	18.9 ± 4.0
	10 U/ml	90.3 ± 3.9	40.6 ± 7.7
LA-GRGD	10 μg/ml	18.7 ± 12.6	1.4 ± 1.3
	50 μg/ml	31.2 ± 2.7	4.4 ± 1.4
	100 μg/ml	39.6 ± 8.9	6.2 ± 1.1
	200 μg/ml	90.5 ± 11.3	8.2 ± 3.3
GRGD	50 μg/ml	33.46 ± 3.0	0.23 ± 2.0
	100 μg/ml	31.54 ± 4.2	0.1 ± 1.8
	500 μg/ml	31.01 ± 5.9	-0.53 ± 0.4
	1000 μg/ml	37.32 ± 4.9	-0.7 ± 0.2
GRGDS	50 μg/ml	-0.2 ± 1.0	-13.0 ± 8.3
	100 μg/ml	4.7 ± 1.3	-16.0 ± 10.0
	500 μg/ml	18.9 ± 4.0	-21.0 ± 3.3
	1000 μg/ml	40.6 ± 7.7	-5.9 ± 7.05
LA-GRGDS	50 μg/ml	2.8 ± 4.5	-0.3 ± 0.8
	100 μg/ml	1.31 ± 6.6	-0.3 ± 1.0
	500 μg/ml	26.3 ± 2.8	-0.95 ± 0.3
	1000 μg/ml	57.4 ± 7.1	-0.43 ± 0.6
$GRGD(AhxGRGD)_3$	50 μg/ml	6.95 ± 6.8	0.33 ± 0.3
	100 μg/ml	6.17 ± 3.5	0.75 ± 0.4
	500 μg/ml	44.1 ± 23.3	-0.18 ± 0.4
	1000 μg/ml	44.1 ± 21.3	-0.45 ± 0.8
$LA\text{-}GRGD(AhxGRGD)_3$	50 μg/ml	7.66 ± 3.2	-1.5 ± 1.7
	100 μg/ml	7.35 ± 8.5	-1.78 ± 0.6
	500 μg/ml	12.1 ± 11.4	-1.0 ± 0.3
	1000 μg/ml	16.1 ± 9.8	-1.15 ± 0.4

Table 6.1 The effect of Heparin, Hirudin, FEPP, GRGD, GRGDS, LA-GRGD, LA-GRGDS, $GRGD(AhxGRGD)_3$ and $LA\text{-}GRGD(AhxGRGD)_3$ on tissue factor activity and on the initial fibrin formation (TEG-r). The data are presented as the Mean ± SD.

6.3.3 Endothelial cell metabolism and cytotoxicity

The standard anticoagulant drug heparin and hirudin showed no cytotoxic effect on EC metabolism and viability at all concentrations tested (Figure 6.1 and Figure 6.2). There was steady progress in cell metabolism up to day 3 with concentration ranging from 0.1 to 100 U/ml ($P<0.005$) compared to control (Figure 2). At day three the highest metabolic activity was observed at a concentration of 10 U/ml. All the synthetic RGD-derivative peptides including FEPP had no significant cytotoxic effect on cell metabolism and viability at concentrations ranging from 0.1µg/ml to 50µg/ml, but at higher concentrations (100µg/ml and 1000µg/ml) had a detrimental effect (Figures 6.2, 6.4, 6.5, 6.6, 6.7, 6.8 and 6.9). LA showed on cytotoxic effect (results not shown).

6.3.4 Endothelial cell binding

The binding effect of RGD-derivative peptides and heparin to EC is shown in Figure 6.10. A concentration of 10µg/ml FEPP showed a highly significant binding effect ($P<0.001$) to EC (57.8%) compared to heparin (23.8%), LA-GRGD (25.8%), GRGD (23.5%), LA-GRGDS (27.3%), GRGDS (28.3%), LA-GRGD(AhxGRGD)$_3$ (13.0%), and GRGD(AhxGRGD)$_3$ (16.0%). LA-GRGD (25.8%), LA-GRGDS (27.3%) and GRGDS (28.3%) showed an increase in % of EC binding, but not significantly different compared to heparin (23.8%). Both the repeat sequence of GRGD showed significantly less EC binding ($P<0.05$) than all the RGD-derivatives. LA had no effect on cell binding (results not shown).

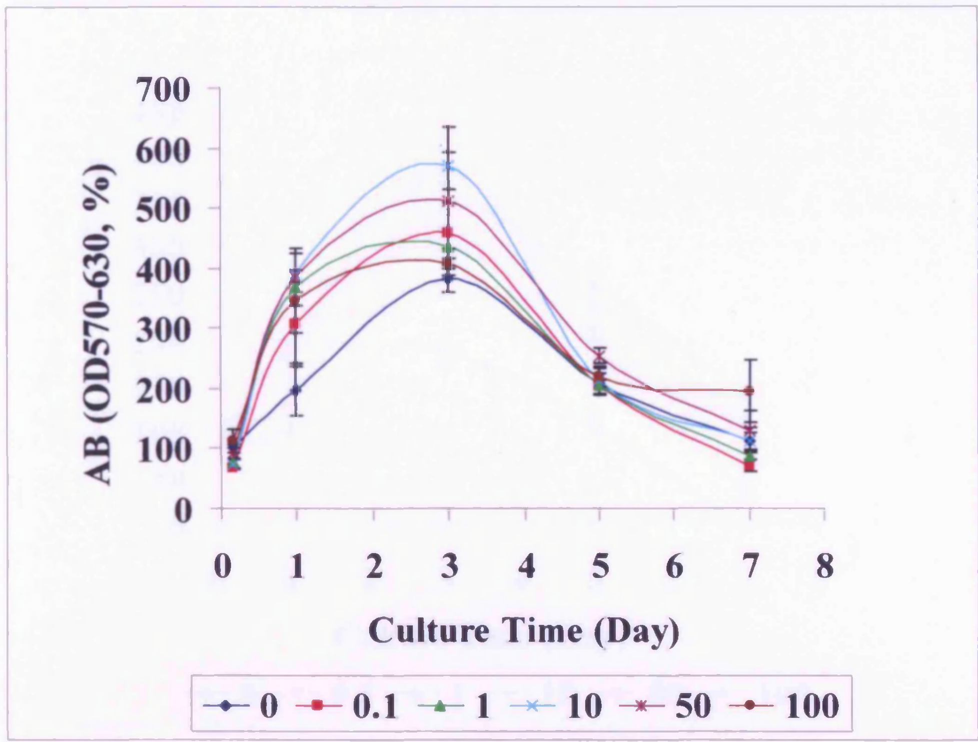


Figure 6.1 Effect of Heparin on survival and metabolism of endothelial cells. Data are presented as mean ± SEM. AB = Alamar Blue absorbance and is % of control.

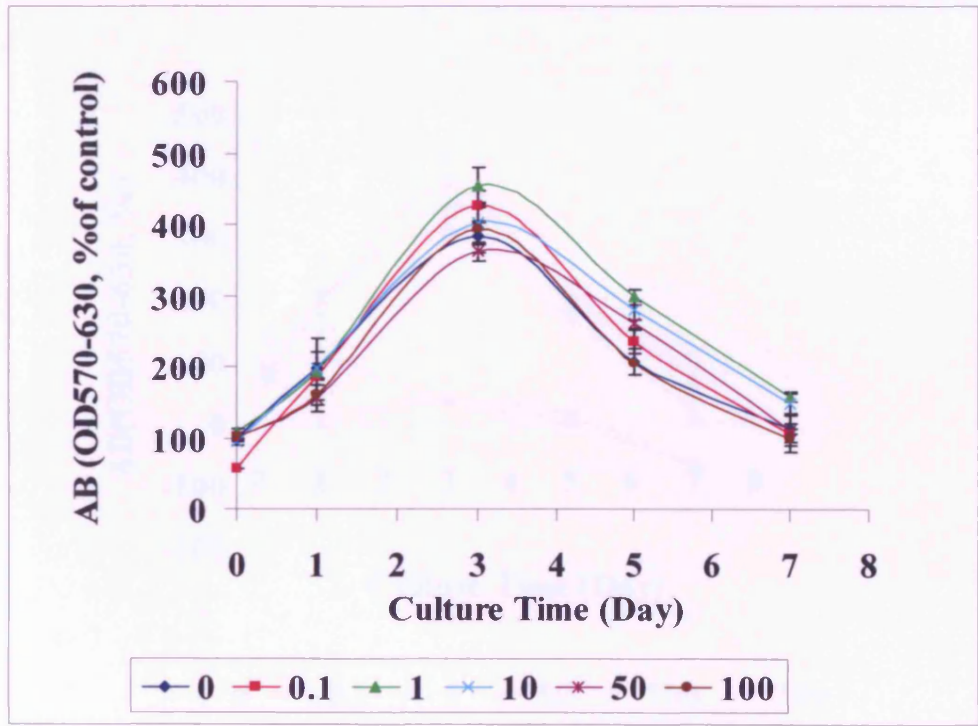


Figure 6.2 Effect of Hirudin on survival and metabolism of endothelial cells. Data are presented as mean ± SEM. AB = Alamar Blue absorbance and is % of control.

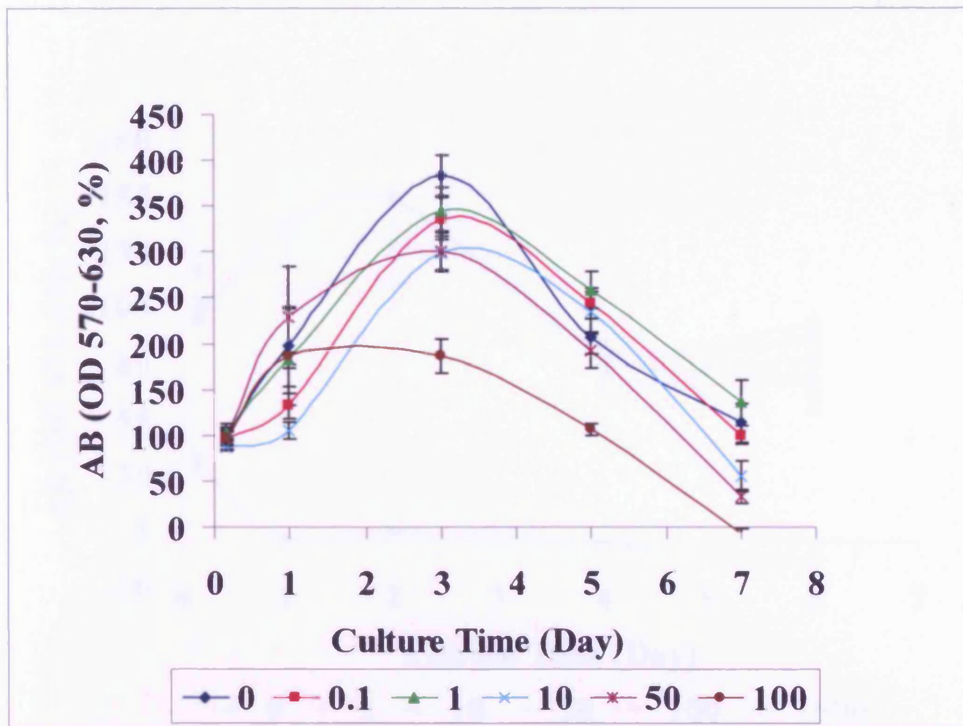


Figure 6.3 Effect of FEPP on survival and metabolism of endothelial cells. Data are presented as mean ± SEM. AB = Alamar Blue absorbance and is % of control.

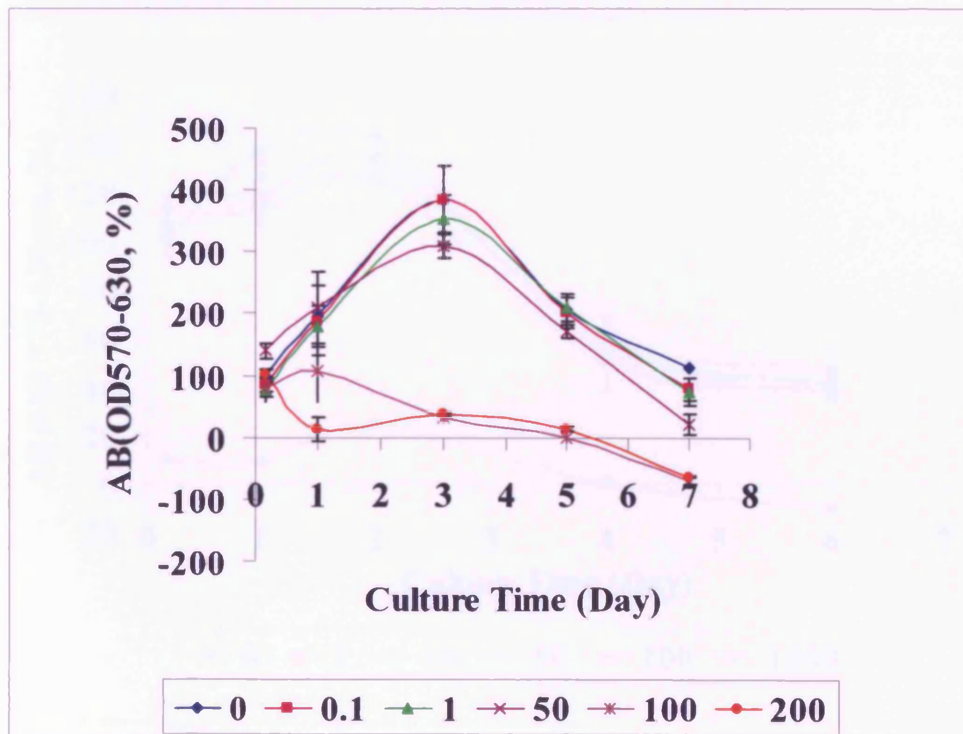


Figure 6.4 Effect of LA-GRGD on survival and metabolism of endothelial cells. Data are presented as mean ± SEM. AB = Alamar Blue absorbance and is % of control.

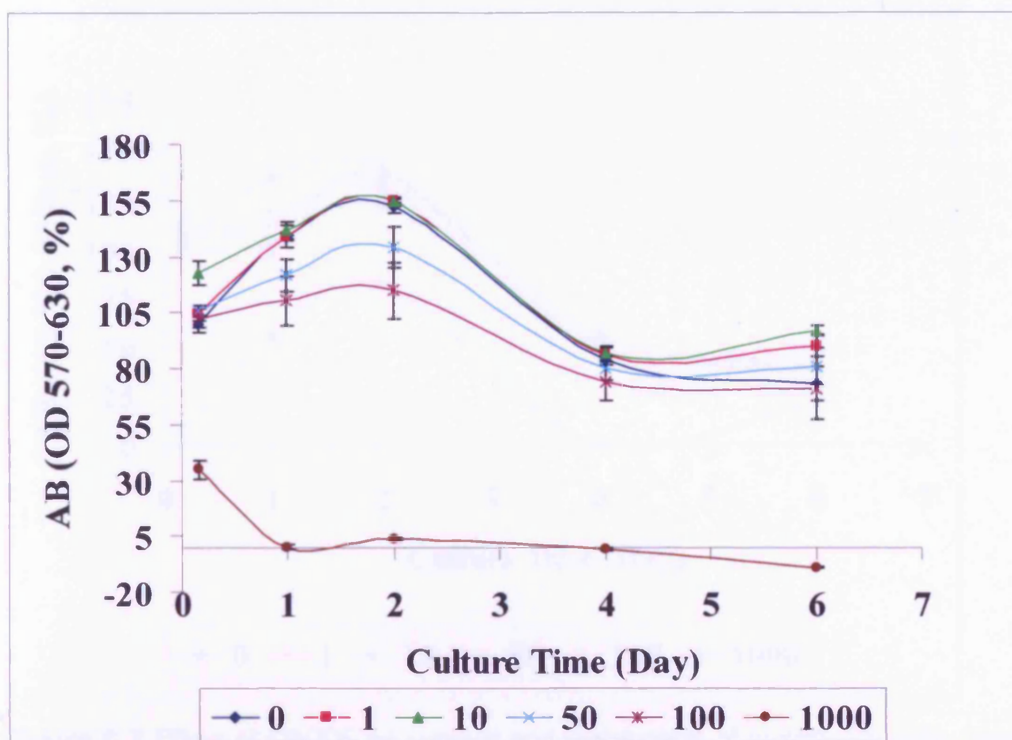


Figure 6.5 Effect of GRGD on survival and metabolism of endothelial cells. Data are presented as mean ± SEM. AB = Alamar Blue absorbance and is % of control.

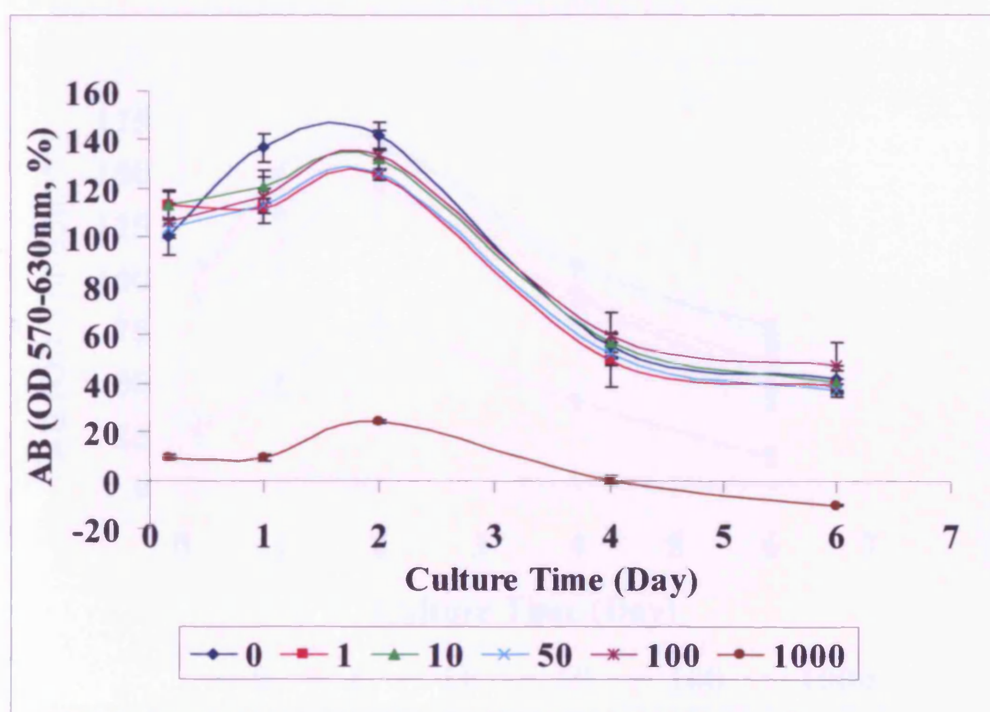


Figure 6.6 Effect of LA-GRGDS on survival and metabolism of endothelial cells. Data are presented as mean ± SEM. AB = Alamar Blue absorbance and is % of control.

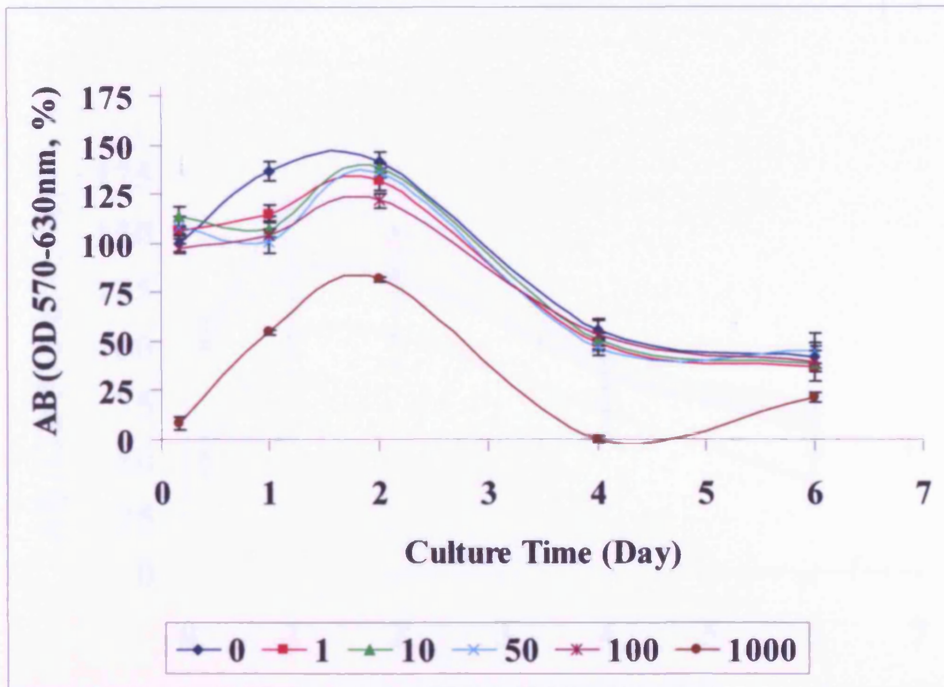


Figure 6.7 Effect of GRGDS on survival and metabolism of endothelial cells. Data are presented as mean ± SEM. AB = Alamar Blue absorbance and is % of control.

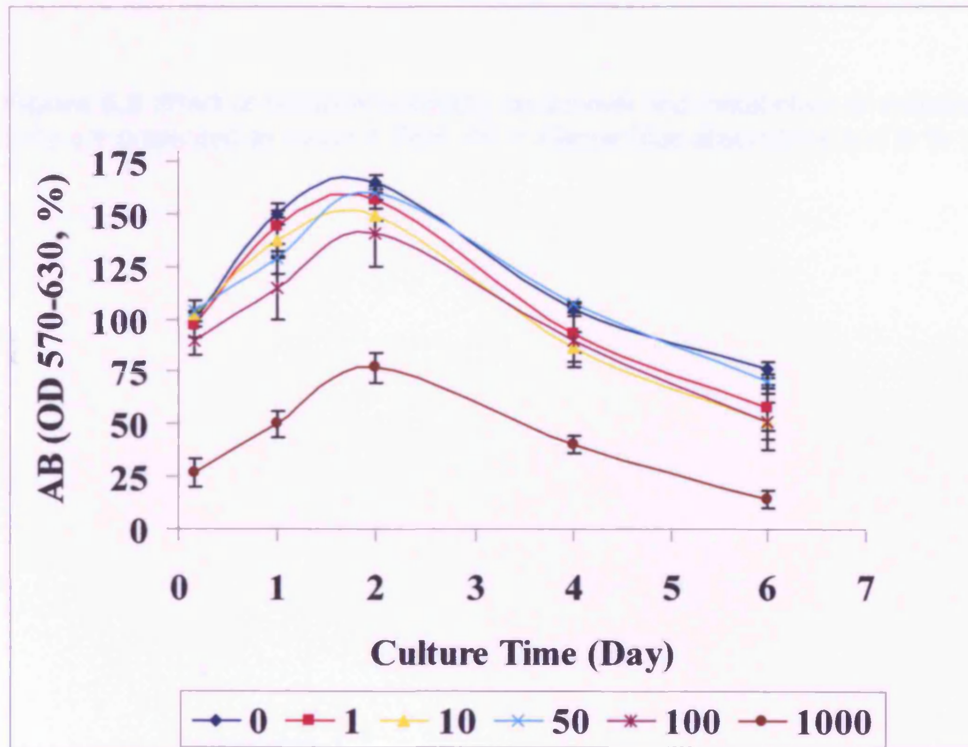


Figure 6.8 Effect of repeat LA-GRGD(AhxGRGD)$_3$ on survival and metabolism of endothelial cells. Data are presented as mean ± SEM. AB = Alamar Blue absorbance and is % of control.

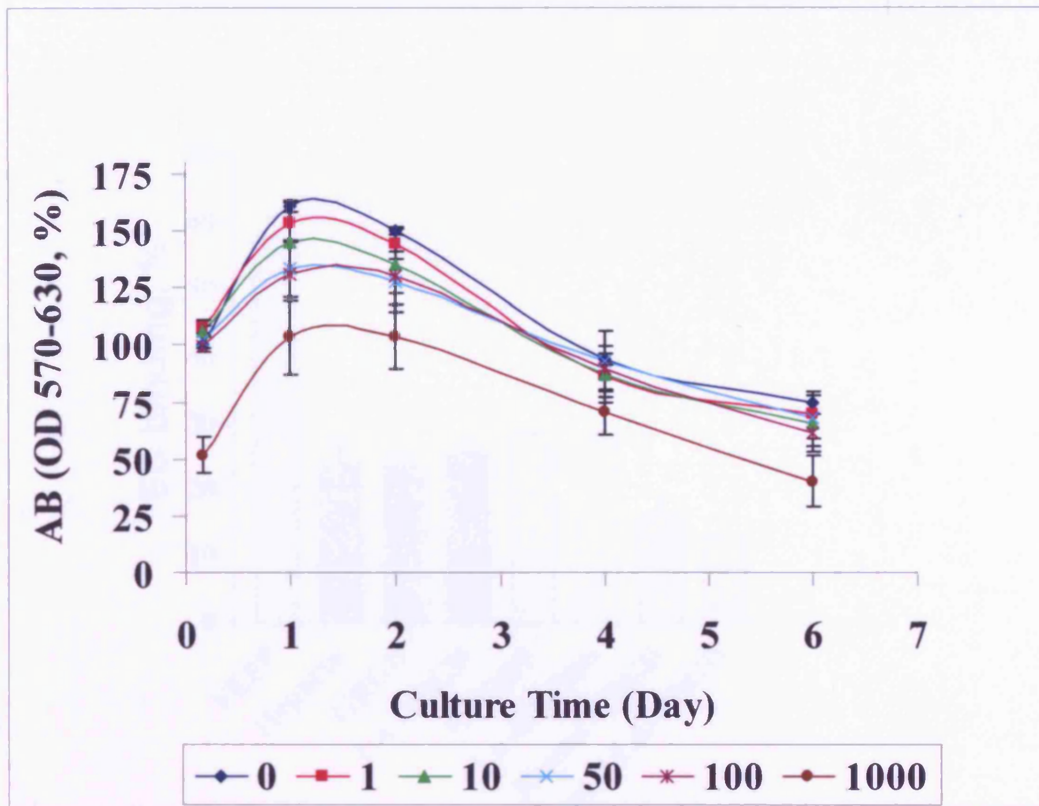


Figure 6.9 Effect of $GRGD(AhxGRGD)_3$ on survival and metabolism of endothelial cells. Data are presented as mean ± SEM. AB = Alamar Blue absorbance and is % of control

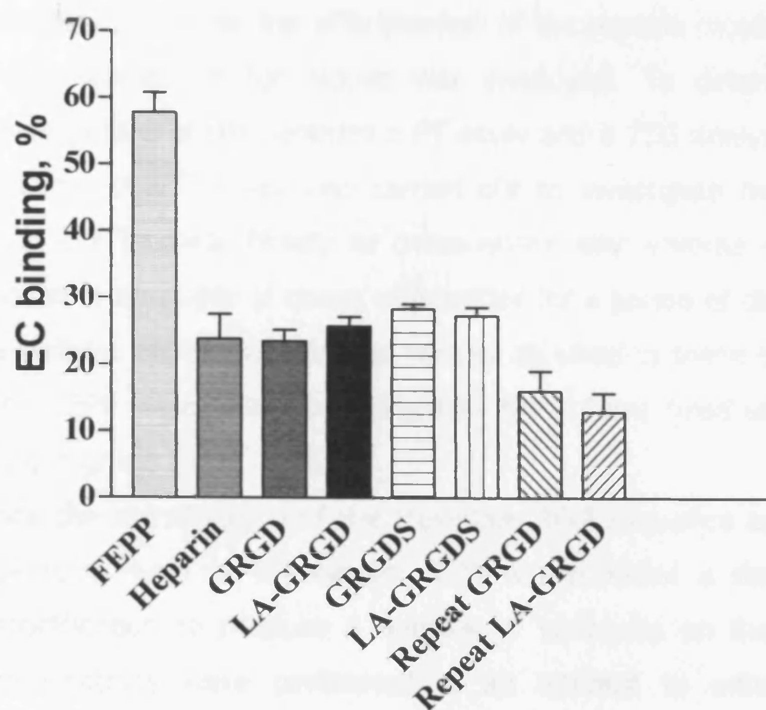


Figure 6.10 Endothelial cell binding effect of FEPP, Heparin, GRGD, LA-GRGD, GRGDS, LA-GRGDS, GRGD(AhxGRGD)$_3$ and LA-GRGD(AhxGRGD)$_3$.

6.4 Discussion

In order to evaluate the effectiveness of the peptide moeties described in Chapter 5, a variety of techniques was employed. To determine the anti-coagulant properties of the peptides a PT assay and a TEG analysis were carried out. An Alamar blue™ assay was carried out to investigate the effect of the peptides on cell binding. Finally to demonstrate any adverse cytotoxicity, EC were exposed to a variety of doses of peptides for a period of days. In order to provide a suitable comparison for the results obtained in these studies heparin, hirudin and FEPP were also investigated as these have been used in previous studies by our group [161, 277].

Since the identification of the tripeptide RGD sequence as an active site of ECM proteins such as fibronectin, RGD has provided a starting point for rational modification to produce a number of variations on the RGD peptide. These modifications were performed in an attempt to enhance the anti-thrombotic and cell adhesive properties of the peptide and also to lengthen the degradation time *in vivo*[278-280].One approach to modifying the RGD peptide is to add extra amino acids to the peptide sequence as it has been demonstrated that this can have an important effect on the peptide activity, in particular at the C-terminus[280]. It has been found that the tetra-peptide GRGD (the RGD peptide with an extra glycine amino acid added) inhibited fibrinogen binding to EC and fibrinogen-induced EC migration [155]. It has also been reported that the introduction of the amino acid serine (S) to the RGD moiety (RGDS) enhanced the inhibition of fibrinogen to platelets and platelet aggregation[279, 281-283]. It has also been suggested that significant GPIIb-IIIa specificity can be achieved either by substitution of Arg by Lys or by substituted Lys side chains into the RGD recognition sequence [284].

In this study the standard anti-coagulant drug heparin, which is commonly used both in research and therapeutically, was used as a comparison for the peptides synthesised. At 1U/ml TF activity was inhibited by >90% and

the TEG-r time by 87.5±7.8 minutes. Heparin also produced a 23.8% cell binding effect. At 1U/ml no cytotoxic effect was observed on EC, and indeed this dose produced a significant increase in cell metabolism and proliferation at day-2 and day-3. Hirudin also produced a >90% inhibition of TF activity at 10U/ml but only increased TEG-r time by 40.6±7.7 minutes. Again no cytotoxic effects were observed at this dose.

Of the peptides synthesised in this study GRGD produced a 33% inhibition of TF activity at 50µg/ml but had no effect on TEG-r time. The cell binding effect of GRGD was 28.3% which is comparable to heparin. LA-GRGD had a 31.2±2.7% inhibition of TF activity and again had no effect on TEG-r time. LA-GRGD produced a 25.8% cell binding effect, again comparable to heparin. Thus it can be seen that the conjugation of LA to GRGD had no significant effect when compared to GRGD at 50µg/ml. At this dose GRGD or LA-GRGD both showed no cytotoxic effect to EC. GRGDS and LA-GRGDS did not exihibit any effect on TF activity or TEG-r time at 50µg/ml but did retain an effect on cell binding at 28.3% and 27.3% respectively.

A further potential development of RGD-containing peptides is to produce a peptide in which the RGD sequence is repeated a number of times. In theory this should provide a method for increasing the ligand binding efficiency via multivalent interactions. Several researchers have developed RGD-containing peptides in which the RGD sequence is locally enriched and can bind polyvalently to cells resulting in an improved effectiveness [271-274]. $GRGD(AhxGRGD)_3$ and $LA\text{-}GRGD(AhxGRGD)_3$ peptides were developed in an effort to enhance the affinity of the receptor ligand interactions due to multivalent interaction. When compared to GRGD there was a 4-fold loss of TF activity and 2-fold loss of cell binding effect in the case of both $GRGD(AhxGRGD)_3$ and $LA\text{-}GRGD(AhxGRGD)_3$. In contrast fibronection engineered protein polymer (FEPP) with multi copies of the RGD attachment ligand of human fibronectin interspaced between repeated structural peptide

units which is prepared by polymerisation, has been shown to improve cell adhesion in previous studies [161, 285].

In this study FEPP demonstrated an anti-coagulant effect comparable with that of heparin at high concentrations. At 100μg/ml, FEPP inhibited >90% of the TF activity and prolonged the TEG-r time by 73.7 ± 8.4 minutes. However at this concentration a cytotoxic effect was seen on EC. A non-cytotoxic dose of 50μg/ml reduced TF inhibition to 55.1% and had no effect on TEG-r time. The cell binding result demonstrated that FEPP showed a significant increase ($P<0.001$) in binding effect to EC (60%) compared to heparin (22%) and all the RGD peptides synthesised. Such an improvement in cell binding was not observed in the two repeat-RGD peptides synthesised in this study. $GRGD(AhxGRGD)_3$ and $LA\text{-}GRGD(AhxGRGD)_3$ both showed no significant effect on TF activity or TEG-r time at 50μg/ml. Unlike FEPP there was no improvement in cell binding compared to either GRGD or GRGDS. One potential reason for this is that the linear structure of the repeated RGD peptides as a result of their synthesis by solid phase peptide synthesis may have reduced their effectiveness. This effect has been investigated by the use of disintegrins, a group of polypeptides from snake venom containing the RGD sequence due to their ability to bind to integrin receptors and inhibit integrin-ligand interactions in a competitive manner. It has been postulated that the conformation of the RGD amino acid sequence within the disintegrin structure accounts for the fact that they are up to 1000 times more potent than linear RGD-containing peptides. Consistent with this hypothesis are the observations that snake venom-derived peptides are rich in disulfide bridges and that their inhibitory activity is greatly diminished upon disulfide reduction [286, 287].

In an attempt to make use of this discovery the incorporation of the RGD recognition sequence into a disulfide-containing, cyclic octapeptide ring system demonstrated that this could increase the affinity of these modified analogue compared to the corresponding unmodified RGD-containing peptide as measured by the inhibition of attachment of normal rat kidney cells to

vitronectin[288]. In another study snake venom-derived peptides were compared with the corresponding linear or cyclic RGD-containing peptides. Both linear or cyclic RGD-containing peptides lacked significant specificity for GPIIb-IIIa[278] when compared to the snake venom-derived peptide.

In many cases an improvement in the inhibition of TF activity was achieved at high peptide concentrations, but these high concentrations proved to have detrimental cytotoxic effects. This may be due to a mechanism suggested by Buckley et al. who discovered that short hexapeptides containing an L-argininylglycyl-L-aspartic acid motif were found to induce apoptosis in an analysed cell line. According to the mechanism proposed by the authors, RGD peptides enter the cell and convert procaspase 3 into the reactive form of the enzyme by intramolecular interaction. Since caspase 3 is an effector caspase that mediates the proteolysis of proteins essential for cell survival, the RGD peptides may directly initiate apoptosis [289].

In summary it can be shown that from the results obtained GRGD and LA-GRGD produced the most favourable overall effect of all the peptides synthesised inhibiting TF activity and affecting cell binding. GRGDS and LA-GRGDS enhanced cell binding alone but had no inhibitory effect on TF activity. The repeat-RGD peptides GRGD(AhxGRGD)$_3$ and LA-GRGD(AhxGRGD)$_3$ had only a limited effect on cell binding and no inhibitory effect on TF activity. In conclusion it was decided to employ GRGD, LA-GRGD, GRGDS and LA-GRGDS for surface modification studies and to proceed no further with the repeat-RGD peptides due to their relatively poor performance with regard to inhibiting TF activity and enhancing cell binding.

CHAPTER SEVEN

SURFACE MODIFICATION OF POLY(CARBONATE-UREA)URETHANE POLYMER USING RGD-CONTAINING PEPTIDE

7.1 Introduction

Polyurethanes comprise a large family of polymers. The presence of a repeating urethane [-NH(CO)O-] group in the polymer chain is the characteristic feature of this family of polymers, which are highly divergent in terms of their physical properties. PUs have been popularly used in cardiovascular and other biomedical applications firstly due to the broad variety of compositions which can be synthesised and secondly their different mechanical properties such as elasticity, strength, durability, compliance and acceptance or tolerance in the body during the healing process[8, 290, 291]. Medical polyurethanes generally consist of two linked polymeric components: a "hard" segment consisting of the aromatic or aliphatic urethane and a "soft" segment of poly(ester), poly(ether) or poly(carbonate). The polyurethane is generated by the reaction of the soft segment, or macroglycol, with an isocyanate prior to extension of the polymeric chain with a chain extender. The ratio of macroglycol to chain extender/isocyanate determines the relative hardness and elasticity of the urethane. The material itself can be formed into shape by solution or melt processes and can be spun, cast into porous or solid structures and extruded; as such a broad variety of polyurethane compositions can be synthesised and this has lead to their application as conduits for bypass grafts and as replacement valves, pacemaker connectors and scaffolds for tissue engineering[9, 292-295].

The clinical use of polyurethanes has been limited to date due to the tendency of the soft segment to suffer from biodegradation by hydrolytic or oxidative mechanisms [10]. Grafts using a poly(ester) soft segment polyurethane have tended to degrade rapidly by hydrolysis [296]. Poly(ether) soft segment grafts have been shown to be susceptible to oxidative degradation [297-299]. The development of a graft based on a poly(carbonate) soft segment which does not suffer from such problems has been carried out by our group in collaboration with an industrial concern. This PCU graft has been subjected to *in vitro* degradation studies using glass wool, hydrogen peroxide and cobalt

chloride [12] and by hydrolytic, peroxidative and blood-based methods [11, 300] which resulted in only minor degradation of the material. These *in vitro* findings were confirmed by an *in vivo* study involving implantation of graft into the aorta-iliac position of dogs and demonstrated the retention of compliance and patency for up to 3 years with only minimal hydrolysis [301]. The PCU graft is produced by coagulation at low temperature resulting in a honeycomb structure and displays similar compliance to lower limb arteries [302] and has been shown to be suitable for EC seeding [303].

The surface composition and structure of the PU selected plays an important role in determining the biocompatibility of the polymer. One persistent problem which limits the usage of PU is their generally poor compatibility with blood. Another difficulty which has limited use is the generally unsatisfactory cell adherence and proliferation when seeded on PU[161, 304]. In order to overcome these problems surface modification of the PU has been attempted to overcome the thrombogenic nature of the PU surface and improve its suitability for cell seeding.

A number of methods have been developed to promote endothelialisation and tissue repair on PU materials. These include modification of the chemical structure, surface modification by UV irradiation or plasma treatment [202, 305-307] and coating or grafting adhesive proteins that mediate cellular attachment [166, 208, 308, 309].

To enhance EC adhesion, survival, and proliferation on the polymeric matrices attempts have been made to optimise the cell-polymer interaction. The main focus of such modifications has been on RGD and its derivatives. RGD is the minimal sequence in fibrinogen which leads to recognition and binding to the glycoprotein IIb/IIIa (GPIIa/IIIb) platelet receptor during aggregation. This sequence has also been shown to promote endothelial cell (EC) and smooth muscle cell adhesion[160, 161, 214]. There are two broad methods for utilising such derivatives, firstly to immobilise the derivative on the surface of the PU using methods such as chemical bonding[159, 160, 208]or photochemical

immobilisation[156] and secondly to incorporate them within the PU in order to permit gradual release out of the polymeric matrix[214].

Examples of the first approach include studies such as photochemically grafting the RGD-containing peptide GRGD onto the surface of a polyethylene glycol modified polyurethane (PU-PEG) to form PU-PEG-GRGD. This improved EC adhesion and growth on the surface and the enhancement efficiency was well correlated with GRGD content[156]. Other studies showed that covalent bonding of RGD-containing peptides based on the cell-adhesive regions of fibronectin (Arg-Gly-Asp-Ser (RGDS) and vitronectin (Arg-Gly-Asp-Val (RGDV)) to a PU graft backbone via amide bonds enhanced cell adhesion and spreading[197]. Another study showed that a Gly-Arg-Gly-Asp-Val-Tyr (GRGDVY) grafted substrate supported a larger number of adherent cells and a higher extent of cell spreading than a Gly-Arg-Gly-Asp-Ser-Tyr (GRGDSY) - grafted substrate[159]. Recently covalent immobilization of RGD and heparin onto the surface of a poly(carbonate-urea)urethane graft has been shown to result in a significant improvement of cell retention in EC seeding[37, 160, 161].

However this general methodology has several drawbacks from a commercial viewpoint. These include the multi-step nature of the process and the expensive synthetic routes required. An example of these difficulties was shown by the study of Massia and colleagues[310] who grafted RGD with dextran which resulted in high levels of bioactivity but affected long-term polymer stability due to the harsh oxidation procedures required. Furthermore in some cases the covalently coated polymer surfaces may exhibit low levels of RGD bioactivity.

In order to produce an incorporated surface-modified polymer which contains RGD derivatives the most commercially viable procedure is to employ the solvent casting method[311]. Previously the solvent casting method could not be used for preparing polymers enclosing RGD derivatives, since RGD derivatives do not readily dissolve in the organic solvents used to dissolve the polymer itself during the synthesis. The recent development of amphiphilic RGD

derivatives which allow dissolution in both water and organic solvents and facilitate use of a solvent casting process should allow such derivatives to be readily loaded into the matrix of the polymer.

In Chapter-5 and Chapter-6 the synthesis and evaluation of amphiphilic RGD derivatives which demonstrated potential for use as a controlled release system and retained the anti-thrombogenic and cell binding qualities required were described [312]. The aim of this study was to investigate if these amphiphilic RGD derivative peptides retain their activity when immobilised onto a poly(carbonate-urea)urethane (PCU) surface. LA-GRGD, LA-GRGDS and the corresponding non-conjugated GRGD and GRGDS were used for this study. Peptides were immobilised onto surface by incorporation into polymer using a solvent that could dissolve both the polymer and the peptide with the solvent leaving by evaporation. This method was used on the basis that the addition of amphiphilic peptides to the polymer may result in the more hydrophilic portion being expressed on the polymer surface, anchored by the more hydrophobic end entangled in the polymer surface. This would be simple to produce with no major chemical changes to the polymer production process and result in peptide immobilised on the polymer surface which should increase the stability of the peptide.

7.2 Materials and Methods

7.2.1 Preparation of modified poly(carbonate-urea)urethane films

PCU polymer was synthesised from methylene diisocyanate, polycarbonate diol, a pre-polymer and chain extended into polymer by ethylene diamine in dimethylacetamide (DMAC) in molar ratios of 2:1:0.97.

The PCU polymer was modified by two different methods. Firstly to produce coated films 3ml of polymer solution was poured into a glass dish (10cm diameter). A film was obtained upon casting the solution at 55-65°C for 18 hrs in a circulating air oven. 5ml of 100µg/ml solution of peptide (GRGD or GRGDS) in PBS was added to each dish and left for 6 hours to coat. The solution was then removed and the coated polymer rinsed with three times with 5ml PBS.

Secondly incorporation of LA-GRGD and LA-GRGDS was achieved by adding 2mg of the peptide dissolved in 1ml DMAC to 19ml of polymer solution. The solution was mixed well and left to stand at room temperature for 1 hour and then 3ml poured to a glass dish and cast as above. Control films were produced as above by using polymer solution alone. By using the casting method to produce films a clear film was produced unlike with the extrusion method of production which results in an opaque film. This allows the seeded cast films to be examined by phase contrast microscopy in addition to the use of techniques such as scanning electron microscopy (SEM) and fluorescent confocal microscopy unlike extruded films which can only be examined by SEM.

7.2.2 Assessment of peptide stability after exposure to flow

Surface modified PCU films ($5cm^2$) with LA-GRGD were prepared as above. A flow system was used to assess the stability of peptide coated and peptide incorporated polymer. Briefly, the model consisted of a pump, flow

waveform conditioner, hollow-fiber oxygenator, outflow resistance, transonic medical flowmeter system and a Millar Mikro-tip catheter transducer. The films were subjected to flow of phosphate buffered saline at 33ml/minute for 1hr, 2hrs and 8hrs. A mean shear stress of 7.51 ± 0.3 dyne per cm^2 was applied. PCU films were then prepared into $1cm^2$ segments and placed into a 24 well tissue culture plate for protein analysis using a BSA protein assay (Sigma Chemical Company, Dorset, U.K.). 400µl of dye was added to the wells/films and incubated at 37^0C for 15 minutes. Following incubation films were removed from the wells and the absorbance of the remaining solution measured at 550nm. Percentage concentration remaining after exposure to flow was then calculated from a standard curve (0-800µg/ml, data not shown).

$$Concentration(\%) = 100 - [(\frac{C_i - C_r}{C_i})x100]$$

Where C is concentration and subscript i and r are initial and residual concentrations respectively.

7.2.3 Polymer seeding and assessment of cell metabolism

PCU modified films were sterilised by incubation in 70% ethanol solution for 1min, followed by two rinsing steps of sterile water and PBS. HUVEC at passage three were then seeded at a density of 2 x 10^5 cells in 5ml of culture medium per dish and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. EC metabolism was assessed using an AB assay as described in Chapter 3.2. 10% AB was added to each dish and absorbance was measured at 24, 48 and 72 hours. Experiments were repeated four times.

7.2.4 Assessment of cell morphology

7.2.4.1 Toludine Blue

HUVECs at P_3 stage were seeded onto polymer films as above and incubated for 24 hours. Briefly, after fixation with 10% buffered formaline and 4% formaldehyde for 10 minutes at room temperature, seeded polymer were rinsed with distilled water once. Cells were then incubated with the 200µl Toludine Blue (Sigma; 0.1% in PBS) for 5minutes and rinsed with distilled H_2O three times. Seeded polymers were examined using a phase contrast microscope.

7.2.4.2 Electron microscopy

HUVECs at P_3 stage were seeded onto polymer films as above and incubated for 24 hours. Polymer films were removed and SEM performed. Films were fixed in 1.5% glutaraldehde (VWR International, Lutterworth, U.K.) for a minimum of 2 hours, washed with PBS and post-fixed using 1% osmium tetraoxide /1.5% potassium ferricyanide (VWR International, Lutterworth, U.K.) for 1½ hours. The specimens were then washed with distilled water and dehydrated through high performance liquid chromatography (HPLC) graded acetone (VWR International, Lutterworth, U.K.) ranging from 30 to 100% for a period of 30 minutes. Then they were transferred to tetramethylsilane (VWR International, Lutterworth, U.K.) for 10 minutes and then allowed to air dry. The grafts were attached to aluminium stubs with double-sided sticky tabs (TAAB Laboratories, Reading, U.K.) and coated with gold using an SC500 (EMScope) sputter coater. The stubs were examined and photographed using a Philips 501 scanning electron microscope.

7.2.4.3 Immunohistochemistry

One of the most widely used endothelial cell marker for studying angiogenesis and neovascularization is von Willebrand Factor (Factor VIII related antigen). von Willebrand Factor synthesised by endothelial cells, causes adhesion of platelets to injured vessel walls and functions as a carrier and stabilizer for coagulation of Factor VIII. Anti-von Willebrand Factor antibody reacts specifically with the endothelial cells of blood vessels and is a useful marker for the identification of endothelial lineage of tumours.

Immunohistochemistry was performed to assess the EC seeded onto PCU polymer films using standard procedures. HUVECs (2×10^5 cell per well) at passage 3 were cultured onto coverslips in 24 well tissue culture plate at for 24 hours. Complete medium was removed and wells were washed twice with PBS. After fixation in ice-cold acetone for 15 minutes, nonspecific binding was blocked with 5% normal goat serum (in PBS for 20 minutes). The cells were then incubated with the primary rabbit polyclonal antibody anti-von Willebrand Factor (DAKO; 1:50 in PBS containing 0.1% Triton X-100) overnight at 4°C. After washing in PBS, cells were then incubated with the biotinylated secondary goat anti-mouse antibody IgG (Sigma; 1:200) followed by the avidin-streptavidin FITC complex (Sigma; 1:75), each for 90 min at room temperature. Films were mounted with Vectashield (Vector Laboratories Ltd, Peterborough, U.K.) and examined using a confocal microscope equipped with appropriate filters for fluorescein.

7.3 Results

7.3.1 Polymer seeding and cell metabolism

EC metabolism on peptide coated and peptide incorporated PCU after 24hrs, 48hrs and 72hrs is shown in Figure 7.1. At 24 hrs post-seeding the GRGDS coated surface, EC had a significantly higher activity ($p<0.05$) compared to control, incorporated LA-GRGD and incorporated LA-GRGDS values. All peptide coated and incorporated treatments increased EC activity compared to control after 48 hrs and 72 hrs. In the case of the incorporated LA-GRGDS peptide this increase was significant ($p<0.05$) at both time points. In general, the incorporation of LA-GRGDS showed a higher cell activity compared to all LA-GRGD incorporated, GRGD and GRGDS coated polymers.

7.3.2 Peptide stability after exposure to flow

The percentage of LA-GRGD peptide concentration remaining after exposure to flow for 1hr, 2hrs and 8hrs is shown in Figure 7.2. Over the time course of the experiment the amount of peptide remaining on the coated films fell significantly at each time point. In the case of the incorporated peptide films the amount of peptide remaining fell significantly between 1 and 4 hours post-flow ($p<0.05$) following which there was no significant change between 4 hours and 8 hours. By 8 hours post-flow there was significantly ($p<0.005$) more peptide remaining on the incorporated films (42.6%) compared to the coated films (20%).

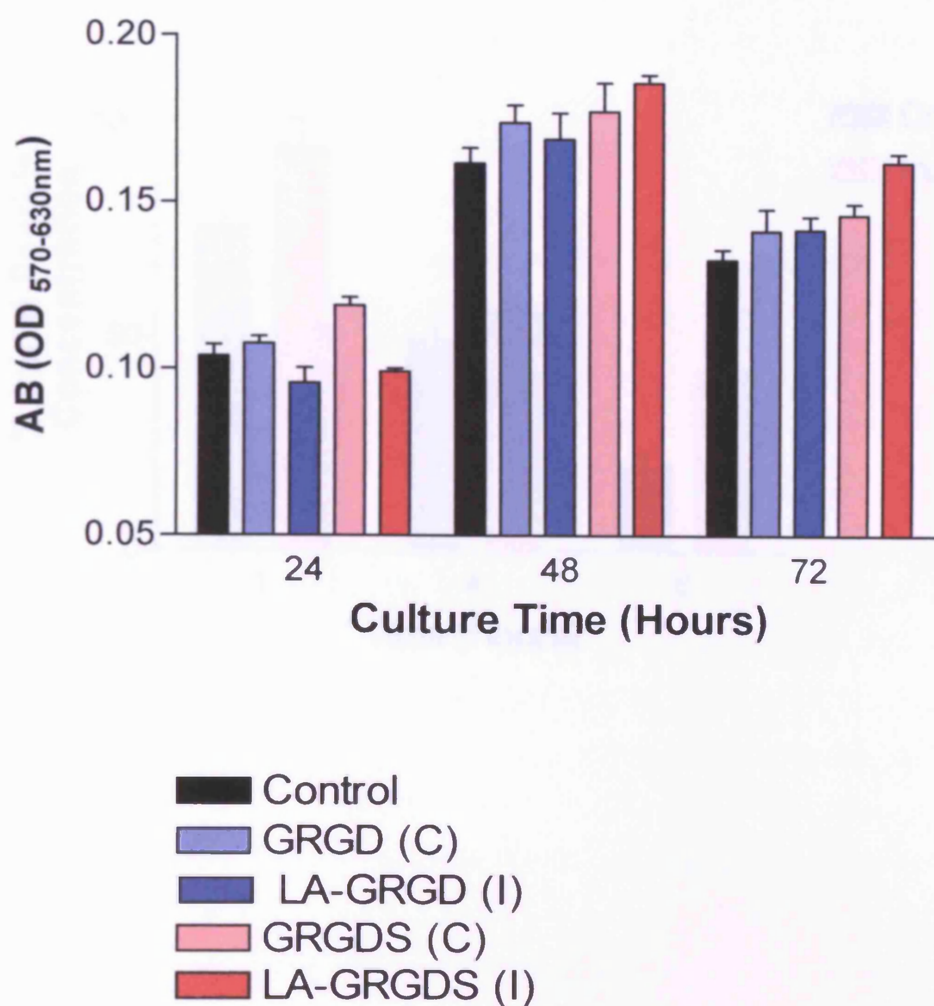


Figure 7.1 Cell metabolism of EC on poly(carbonate-urea) urethane films: unmodified PCU (control); GRGD, GRGDS coated and LA-GRGD and LA-GRGDS incorporated. Data are presented as mean ± SEM.

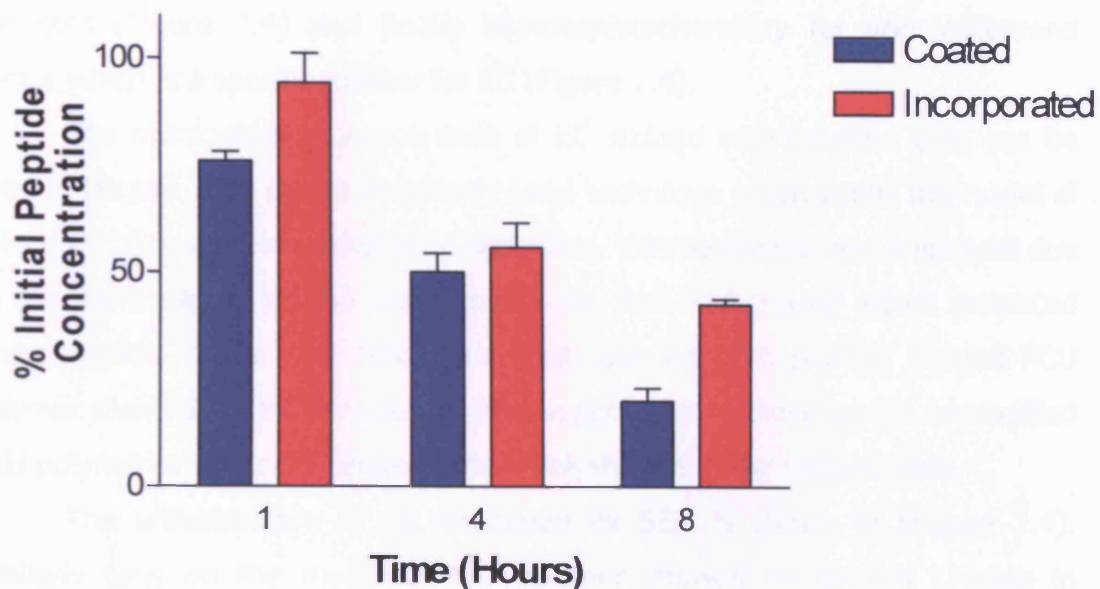


Figure 7.2 Comparison of percentage of initial peptide concentration for coated LA-GRGD and incorporated LA-GRGD after 1, 4 and 8 hours exposure to flow. Data are presented as mean ± SEM.

7.3.3 Cell morphology

Morphology of ECs seeded onto the surface of modified and unmodified PCU polymer was investigated using three different techniques: firstly toludine blue staining (Figure 7.3), a rapidly obtained general view of the cell population, secondly scanning electron microscopy (SEM) which shows the ultrastructure of the cells (Figure 7.4) and finally immunohistochemistry for von Willebrand Factor which is a specific marker for EC (Figure 7.4).

The morphological appearance of EC stained with toludine blue can be seen in (Figure 7.3). It is a commonly used technique which stains the nuclei of cells dark blue and the background light blue. This technique was employed due to the rapid nature of the staining and the low background signal produced when applied to the cast PCU films. Cells seeded onto peptide treated PCU polymer showed a similar morphological appearance to those on the unmodified PCU polymer and to cells seeded onto a polystyrene tissue culture plate.

The ultrastructure of EC examined by SEM is shown in (Figure 7.4). Similarly cells on the modified PCU polymer showed no evident change in morphological appearance compared to those on the unmodified PCU polymer and showed the typical appearance of cobblestone morphology and rounded morphology in all PCU surfaces. The morphology of EC seeded to RGD-containing peptides appear the same as EC seeded to fibronectin coated glass slides and vascular graft demonstrated by Curti et al [246].

Confocal microscopy view of ECs seeded on polymer is shown in Figure 7.5. Immunocytochemical analysis of human endothelial cell adhesion on surface modified PCU polymer demonstrated that the cells were expressing the specific EC marker vWF.

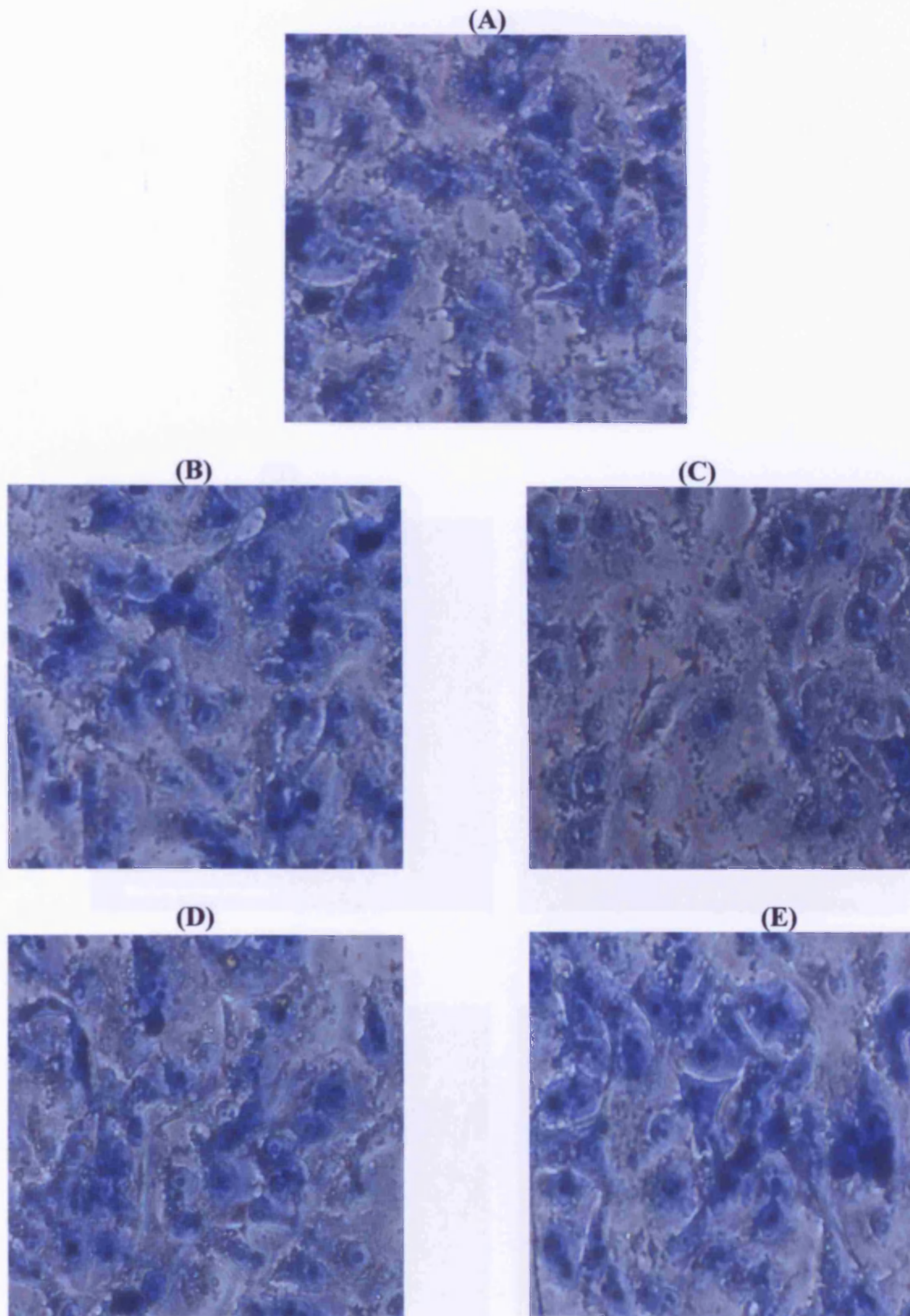


Figure 7.3 Phase-contrast microscopy showing toludine blue stained ECs seeded on PCU polymer surface at day 1 (a) Unmodified or native (b) GRGD coated (c) LA-GRGD incorporated (d) GRGDS coated (e) LA-GRGDS incorporated polymers (Magnification x 40).

7.4 Discussion

Previous studies have demonstrated that prosthetic graft materials exhibit a uniformly poor surface for the retention of EC which have been seeded onto them by both single- and two-stage seeding processes[36]. It has also been shown that primary human cell cultures show slower cell division and spreading across both graft surfaces and in tissue culture than animal cell lines due to low cell attachment, even under the optimal experimental conditions of tissue culture[313, 314].

These problems are exacerbated by the fact that the availability of autologous venous endothelium for transplantation onto prosthetic vascular implants is limited resulting in the need to explore alternative sources of EC such as omentum[250, 251], subcutaneous fat [262, 285], peritoneal lavage [249, 285] or fat obtained by liposuction.

As a result of the above in order to achieve the maximum EC coverage possible from a limited source of cells for seeding it is vital to optimise the conditions for seeding EC and this has been extensively investigated by many different groups. Over the last two decades it is has been recognised that the extracellular matrix (ECM) plays an integral role in cellular function. Based on this theory one focus of the attempt to optimise EC attachment to vascular prosthesis has been to surface modify the prosthesis to improve cellular attachment and retention as the choice of graft coating material will influence not only cell attachment but also cellular activity.

In an attempt to increase cell attachment to prosthetic vascular graft materials researchers have utilised soluble components of ECM such as fibronectin, collagen and laminin. These all increased cell density and cell attachment rates significantly. Despite the apparent improvement in cell seeding achieved by the use of these proteins there are some drawbacks to their usage. These cell adhesion proteins are complex proteins which are difficult to produce for use in a clinical situation and as a result tend to be expensive to employ.

The use of RGD peptides is attractive commercially, as they are simple to produce due to their shorter amino acid sequence and hence cheaper to employ than more complex protein coatings with considerably longer sequences (such as fibronectin). Researchers have used different techniques to immobilise RGD-peptides onto the surface of vascular graft prosthesis. Here in our department covalent bonding and passive coating have been used previously to immobilise RGD-peptides onto PCU polymers. As mentioned previously there are difficulties inherent in the commercial synthesis method required to produce covalently bonded RGD-peptides on vascular prosthesis. When this is combined with findings which suggest that such coatings may be leached from the graft relatively easily the need to develop an alternative technique in which the RGD-peptides are incorporated is apparent.

In this study the two of the RGD peptides described earlier (LA-GRGD and LA-GRGDS) were used to modify a PCU polymer by direct incorporation (blending) into the polymer solution. This incorporation was made possible by the conjugation of a LA- group to GRGD and GRGDS resulting in amphiphilic peptides suitable for incorporation. As a comparison, the same unconjugated peptides were used for passive coating of the polymer. The effectiveness of the surface modification was investigated by seeding HUVEC onto modified PCU films then monitoring cell metabolism by utilising an Alamar Blue™ assay and examining cell morphology via phase contrast, confocal and scanning electron microscopy.

24 hours after seeding and also coating the graft surface with GRGDS produced a significant increase in EC metabolism compared to the other treatments investigated. At later stages (48 and 72 hours) incorporating LA-GRGDS into the PCU resulted in a significant increase in cell metabolism, with the other treatments showing higher values than the control (unmodified) samples. It has been previously shown that adhesive interactions play a critical role in directing the migration, proliferation and differentiation of cells[315] and that cell migration rates depend in a very sensitive manner on the strength of

cell adhesion [316, 317]. The longer term improved effect (>24hrs) with the LA-GRGDS modified polymer in this study may be due to stronger cell adhesion kinetics and increased cell population in a similar manner. This could also explain the improvement in cell metabolism when LA-GRGDS is incorporated into the matrix of the polymer compared to the coated PCU. A further reason for this difference may be that the coating has been leached from the graft by repeated media changes. This possibility is supported by the data obtained from the peptide stability study which demonstrated a significant loss of peptide from PCU films coated with LA-GRGD compared to those in which the LA-GRGD was incorporated after exposure to flow for 8 hours suggesting that a loss of peptide activity could well result from repeated media changes in the case of coated polymer films.

Previous studies on cell morphology of EC seeded onto vascular grafts have utilized scanning electron microscopy as the major investigative tool. This is an expensive, complex and time consuming technique which may result in significant changes to the cells and/or polymer during the tissue processing necessary to examine a sample under SEM. The reason for this is that the materials and methods used to produce vascular grafts result in a graft which is opaque to light and thus unsuitable for examination by phase contrast microscopy. By producing the PCU discs by casting which results in a clear film this problem is overcome and it is possible to examine EC seeded onto the films directly by phase contrast microscopy and also to stain them with commonly used histological stains. In this study toludine blue, a commonly used technique which stains the nuclei of cells dark blue and the background light blue, was employed due to the rapid nature of the staining and the low background signal produced when applied to the cast PCU films. It has been previously described that following seeding, endothelial cells initially attached to the graft with rounded morphology, later developing a more flattened phenotype responsible for the `cobblestone` appearance of the mature endothelial monolayer[246].

In conclusion it can be seen from the results obtained that incorporating LA-conjugated GRGD and GRGDS into PCU produced a comparable effect to coating PCU with the same peptides, and indeed in the case of LA-GRGDS improved cell metabolism in the longer term (48 and 72 hours). In addition, incorporation of the peptide into the polymer may result in an improvement in the time the peptide remains effective when compared to simply coating onto the surface of the polymer as demonstrated by the significant improvement in peptide retention when exposed to flow. As a result of this the RGD derivatives developed in this study have potential as a surface modification system in which the peptides cell binding activity is retained whilst allowing its solubility into solvents used in the manufacture of polymers for bypass grafts. This study demonstrates that LA conjugated RGD derivatives may have wide applicability for polymer formulation via the commercially used solvent casting methodology for use in coronary, vascular and dialysis bypass grafts, scaffold polymer based tissue regeneration and tissue engineering.

CHAPTER EIGHT

IN-VITRO BLOOD COMPATIBILITY ASSESSMENT OF SURFACE MODIFIED POLYMER

8.1 Introduction

One of the greatest challenges in biomaterial science is the development of improved blood compatibility. Several strategies have been developed in an attempt to overcome this problem. Bio-inert coatings and hydrogels aim at low protein adsorption and cell adhesion to reduce any activation of coagulation or complement cascade reactions in the blood [203]. Biological molecules have been immobilised to trigger anticoagulant, fibrinolytic or anti-inflammatoric reactions[131]. Further, biodegradable scaffolds have been developed to support the regeneration of blood contacting functional tissue [318]. In order to estimate the effectiveness of these varying strategies reliable and standardised methods for blood compatibility testing are required. There is currently no single standard test generally accepted to measure blood compatibility. A variety of techniques have been utilised in an effort to provide a direct correlation of the events occurring during contact of blood with a polymer. These procedures employ selected and relevant indicators of blood-material interactions.

The *in vitro* blood compatibility assessment of polymers involves haematological studies on blood exposed to materials under defined conditions. Although many assays on blood can theoretically be performed, acceptance of test procedures proposed so far has been limited due to several factors in the blood/biomaterial interaction affecting the assays used in haematological studies. This is mainly due to the high degree of complexity of the reaction paths in the blood and the interrelation of different activation cascades [319, 320].

The first *in vitro* parameter investigated for the characterization of the hemocompatibility of materials was investigated by Lee and White in 1913. They looked at the clotting time of whole blood[321]. A test tube was coated with the test material and freshly drawn blood added. The tube was then tilted back and forth until the blood clotted. The varying time taken for this to occur provided a comparison between the different test materials. Many modifications of this

initial test have been made but no test yet developed has entirely overcome problems associated with tissue trauma at the time of venopuncture and the turbulence of blood flow during sample mixing.

An initial simple test which can be carried out to assess blood compatibility is haemolysis testing. This is a widely used primary testing technique for the *in vitro* assessment of blood compatibility of foreign materials. This technique measures the capacity of the polymer surface to cause red blood cells to rupture. Haemolysis testing has been used for the evaluation of blood contacting foreign materials in a variety of areas such as extracorporeal membrane oxygenators[322], biodegradable scaffolds[323] cardiopulmonary bypass circuit[324], vascular grafts[325] and drug delivery systems[326].

One of the most common assessments of the biocompatibility of biomaterials carried out is to measure platelet adhesion, which is often used as an index of blood compatibility. This is a well-established method which has been used to compare the biocompatibility of different blood contacting devices and to assess the effect of surface modification on the biocompatibility of materials. Goodman et al carried out platelet adhesion experiments with surfaces of differing thrombogenicity and showed that the adhesion and morphology or shape change of platelets on a sample surface *in vitro* is strongly linked to the *ex vivo* thrombogenicity of materials [327].

Several researchers have established fundamental aspects of the behaviour of blood or blood components *in vitro*. Vroman, in his pioneering work, emphasised the relevance of protein displacement phenomena at the solid surface for the macroscopically observed effects of blood clotting [328, 329].

When blood comes into contact with a foreign material several proteolytic enzyme systems in the plasma become activated. The most relevant system activated as a consequence of interaction between blood and vascular prosthetic materials in a clinical situation is the coagulation cascade[47, 48, 128, 330].

Activation of the coagulation cascade is dependent upon activation of both platelets and of the plasma coagulation system. The plasma coagulation

system is activated either by the intrinsic or extrinsic pathway as described in Chapter 2. Furthermore there is an interaction at several levels between platelet activation and plasma coagulation and both are necessary for the development of surface thrombosis.

Platelet response to a foreign surface is important in understanding the thrombogenecity of the material (Figure 8.1). The platelet release reaction is an essential part of the process of platelet aggregation during haemostasis and thrombus formation. Platelet Factor 4 (PF4) is believed to be released from a sub-population of α-granules together with β-thromboglobulin (BTG) [331-333]. Platelets respond to various stimuli by becoming activated. Once activated, platelets release α-granules (for example PF4 and BTG) which contain potent coagulation accelerators into the surrounding media. In addition activation exposes coagulation accelerating phospholipids on the platelet surface[334, 335].

The platelet release reaction is directly associated with platelet adhesion and aggregation, which are preliminary events leading to thrombus formation and the primary cause of thrombosis following contact of blood with a foreign material. The release reaction of α-granules from platelets during blood-polymer interactions was examined by Bowry and Courtney in 1984 [336] by measuring BTG levels in order to determine blood compatibility of polypropylene, poly(vinyl chloride), silicone rubber and siliconised glass. This study showed that polypropylene tubes caused a reduced release of BTG compared to those made of silicone rubber. In addition siliconised glass induced less BTG release than poly(vinyl chloride). Another study by Kuragano *et al* [337] looked at BTG and PF4 levels as markers of platelet activation and release to assess and compare the biocompatibility of cellulose triacetate and polysulfone dialysis membranes. This study showed that there was no significant change in platelet counts (measuring platelet adhesion), BTG or PF4 levels between the two membranes investigated.

In this study the effectiveness of both surface modification techniques (passive coating and incorporation) on blood compatibility of the modified poly(carbonate urea)urethane polymer was investigated. Whole blood haemolysis, measurement of adsorbed platelets and the determination of a platelet activation product (PF4) have been selected to investigate the blood compatibility of the surface modified polymer.

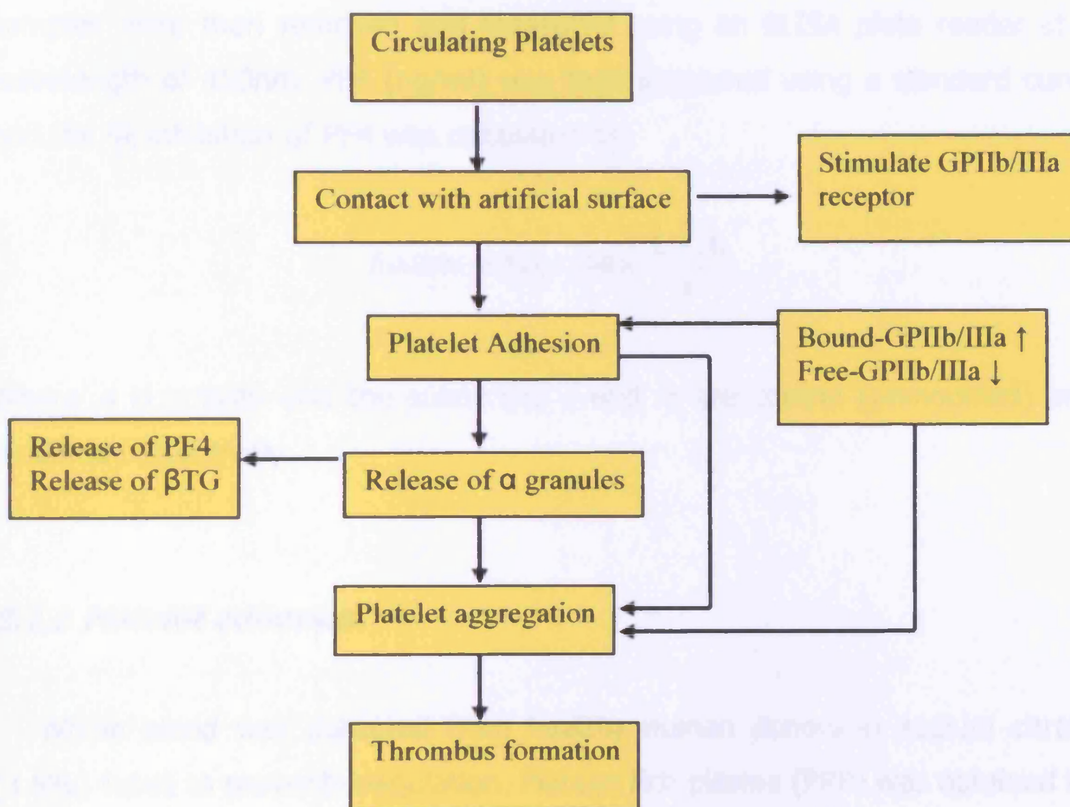


Figure 8.1 Platelet responses associated with blood contact with an artificial surface (from Kuragano *et al* [337]).

8.2 Materials and Methods

8.2.1 Inhibition of Platelet Factor 4 (PF4).

An IMUCLONE® Platelet Factor 4 (PF4) ELISA assay (American Diagnostica Inc., Connecticut, U.S.A.) was used to assess the blood compatibility of modified PCU films. 300μl of platelet poor plasma was added to each polymer film in a tissue culture plate. The plate was then placed on a shaker and incubated at room temperature by gentle mixing. After 30 minutes duplicate 100μl plasma samples were then removed and measured using an ELISA plate reader at a wavelength of 450nm. PF4 (ng/ml) was then measured using a standard curve and the % inhibition of PF4 was calculated as:

$$Inhibition(\%) = 100 \times \frac{A_c - A_m}{A_c}$$

Where A is activity and the subscripts c and m are control (unmodified) and modified respectively.

8.2.2 Platelet adhesion.

Whole blood was collected from healthy human donors in sodium citrate (3.8%) tubes to prevent coagulation. Platelet rich plasma (PRP) was obtained by centrifuging the citrated blood at 1500rpm for 20 minutes. Platelet density was then adjusted to $7x10^3$ platelets/μl. PCU films were cut into $1cm^2$ sections, placed in 1.5ml microcentrifuge tubes, and 1ml of PRP added. PRP incubated without film was used as a reference. Poly-L-lysine-coated (0.1% in pure water, Sigma Chemical Company, Dorset, U.K.) PCU films were used as a positive control. All tubes were then incubated at 37°C for 3 hours with gentle mixing.

After incubation PCU films were removed from the tubes and the platelets remaining counted using a Bayer Advia 120 Haematology System counter. Platelet adhesion was calculated as follows:

$$Platelet\ \ Adhesion(\%) = 100 \times \frac{C - T}{C}$$

Where C is the reference count and T is the test count.

8.2.3 Haemolysis assay.

The haemolytic effect of the material was investigated using an *in vitro* haemolysis assay[338]. A fresh venous blood sample was collected from a human volunteer in an EDTA tube. PCU films were cut into 1cm^2 sections and placed in 1.5ml microcentrifuge tubes. A 1ml aliquot of diluted blood (0.2ml in 10ml 0.9% sodium chloride (Baxter Healthcare Ltd, Norfolk, England)) was then added to each tube. 0.2ml blood in 10ml pure water was used as a positive control. All tubes were then incubated at 37°C for 3 hours with gentle mixing. After incubation PCU films were removed from the tubes and all tubes were centrifuged for 10 minutes at 1000*g*. The absorbance of the resulting supernatant solutions was measured using a spectrophotometer at 550nm. Each experiment was repeated four times.

8.3 Results

8.3.1 Inhibition of Platelet Factor 4

The effect on PF4 inhibition following surface modification of PCU by coating with peptide, incorporating peptide and coating with heparin is shown in Figure 8.2. Polymer coated with GRGD (100μg/ml) produced the highest inhibition in PF4 release (11.3%). Heparin (1U/ml) inhibited PF4 release by 9.5% compared to LA-GRGD (100μg/ml) incorporated polymer and GRGDS (100μg/ml) coated polymer which showed a comparable inhibition in PF4 release (10.0% and 10.3% respectively). LA-GRGDS incorporated polymer inhibited the PF4 by only 4.1% and coated LA-GRGDS showed a significantly lower inhibition of PF4 (0.54%) compared to heparin coated. There was no statistically significant difference in inhibition of PF4 release between heparin and any of the other surface modification techniques.

8.3.2 Platelet adhesion

The percentage of platelets adhering following a three hour incubation with unmodified (native) PCU polymer, poly-L-lysine coated polymer (in order to provide a positive control to which platelets should adhere), GRGD coated polymer; LA-GRGD incorporated polymer, GRGDS coated polymer, LA-GRGDS coated and LA-GRGDS incorporated polymer are shown in Figure 8.3. Polymer coating with poly-L-lysine resulted in a significantly increased number of platelets adhering to the surface (34%) compared to the unmodified polymer. No statistical significance was observed between any of the other modifications and the unmodified polymer.

8.3.3 Haemolysis assay

The results of the haemolysis investigation are shown in Figure 8.4. No statistical difference was observed between the unmodified PCU polymer and any of the surface modified PCU polymers. The finding that the unmodified PCU, peptide coated PCU and peptide incorporated PCU polymers have a haemolysis that is similar to the negative control (saline) and considerably lower than the positive control shows that the native PCU polymer does not cause significant haemolysis and that the different surface modifications carried out to the native polymer had no significant effect on this property.

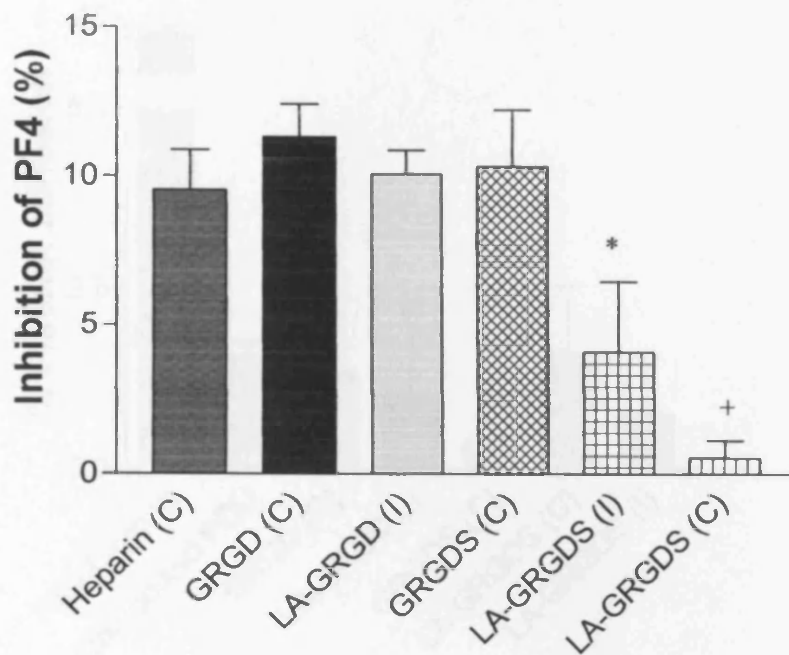


Figure 8.2 Inhibition of Platelet Factor 4 (PF4) on poly (carbonate-urea) urethane films: Heparin coated (C); GRGD coated (C); LA-GRGD incorporated (I); GRGDS coated (C); LA-GRGDS incorporated (I) and LA-GRGDS coated (C).

Data are presented as mean ± SEM. * $p>0.05$ vs. Heparin (not significant). ⁺ $p<0.05$ vs. Heparin (significant).

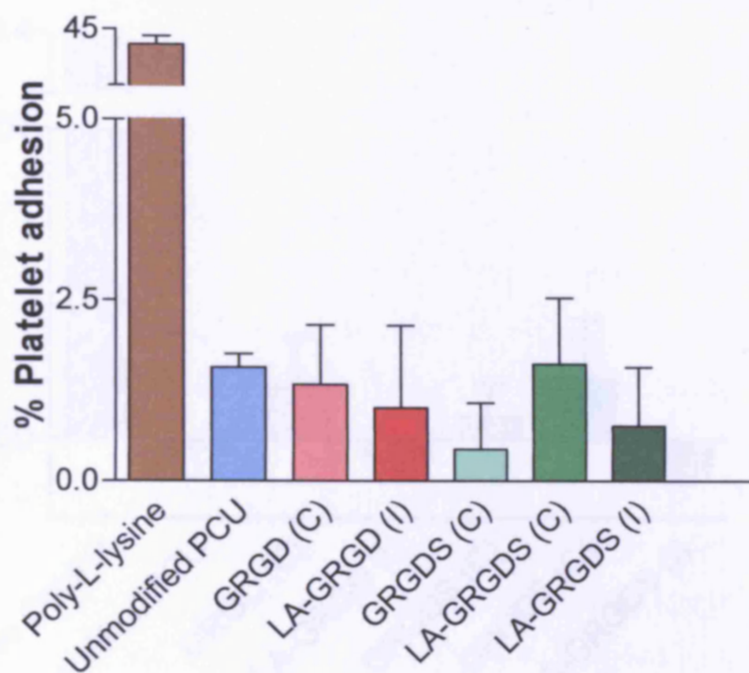


Figure 8.3 Percentage of platelet adhesion on poly (carbonate-urea) urethane films: Poly-L-lysine coated (C); GRGD coated (C); LA-GRGD incorporated (I); GRGDS coated (C); LA-GRGDS coated (C) and LA-GRGDS incorporated (I).

Data are presented as mean ± SEM.

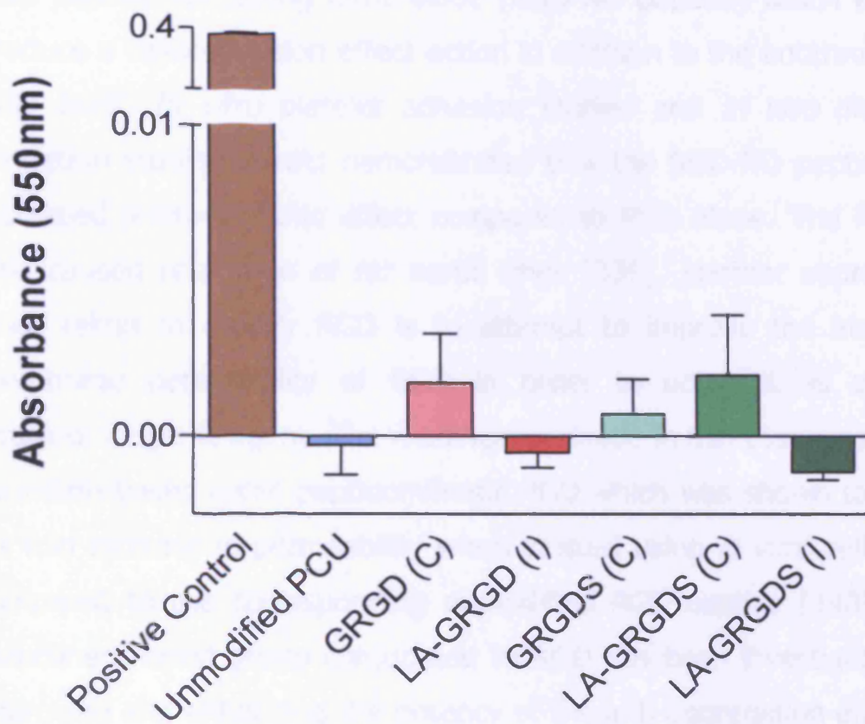


Figure 8.4 Haemolysis assay on poly (carbonate-urea) urethane polymer films: Positive Control, PCU (unmodified); GRGD coated (C); LA-GRGD incorporated (I); GRGDS coated (C); LA-GRGDS coated (C) and LA-GRGDS incorporated (I). Data are presented as mean ± SEM

8.4 Discussion

Several researchers have modified the RGD peptide for a variety of different purposes. One example of such modification was the production of an RGD peptide containing nitric oxide (RGD-NO peptide) which was designed to produce a vasorelaxation effect action in addition to the antithrombotic effect of RGD itself. *In vitro* platelet adhesion studies and *in vivo* platelet thrombus formation studies in rats demonstrated that the RGD-NO peptide produced an increased antithrombotic effect compared to RGD alone. The RGD-NO peptide also caused relaxation of rat aortic rings [339]. Another approach which has been taken to modify RGD is to attempt to improve the intestinal mucosal membrane permeability of RGD in order to utilise it as an orally active antithrombogenic agent. The modification made in this case was to synthesise a coumarin-based cyclic peptidomimetic RGD which was shown to have a five- to six fold increase in permeability when studied using *in vitro* cell culture models compared to the corresponding unmodified RGD peptide [340]. The use of a carbolinecarboxyl group conjugated to RGD has been investigated as a way of improving and enhancing the potency of the anti-aggregation effect of RGD and to prolong the peptide [341].

A study by the group of Moon 2001 [342] synthesised a heparin-DOCA conjugate with an amphiphilic property suitable for incorporation with polyurethane in a co-solvent. This was used to produce a heparin-release system using the solvent casting method. The anti-thrombogenicity of the film was measured and showed that loading heparin-DOCA above 7.5% prevented platelet adhesion and fibrin clot formation. The objective of this study correlates with ours and furthermore indicates that conjugating a hydrophilic agent (heparin) with amphiphilic properties which can be incorporated with polyurethane in an achievable manner and still retain its activity.

We have previously reported that LA conjugated GRGD (LA-GRGD) in solution demonstrated an antithrombotic and cell-binding effect. The

conjugation of LA had no effect to the inhibition of coagulation, cytotoxicity or cell attachment of the GRGD peptide alone [312]. When LA-GRGD, LA-GRGDS and their corresponding non-conjugated GRGD and GRGDS were immobilised onto the PCU surface they retained their cell attachment activity (Chapter-6).

Hence in this study, the effect on blood compatibility of LA-GRGD, LA-GRGDS, GRGD and GRGDS immobilised onto a PCU surface was investigated using measurement of whole blood haemolysis, the assessment of adsorbed platelets and estimation of platelet activation factor (PF4). Two different methodologies were employed to modify the PCU surface: passive coating with peptides onto the PCU surface and incorporation of them by solvent casting. Coating of the peptides was achieved by incubating a 100µg/ml peptide solution with the polymer material for 24 hours. In order to incorporate them the peptides were immobilised onto the surface by incorporation into the polymer material using a solvent that could dissolve both the polymer and the peptide, with the solvent being removed by evaporation. This method was used on the basis that the addition of amphiphilic peptides to polymer may result in the more hydrophilic portion being expressed on the polymer surface, anchored by the more hydrophobic end which is entangled in the polymer surface.

The results obtained in this study (Figure 8.3 and Figure 8.4) demonstrated that peptides immobilised on PCU polymer by incorporation showed an effect comparable to that of the coating technique. The platelet adhesion and haemolysis studies demonstrated that none of the modified PCU films caused adverse effects with respect to either platelet adhesion or haemolysis. The inhibition of PF4 obtained with coated GRGD, GRGDS and incorporated LA-GRGD was comparable to that obtained with heparin coating (1U/ml), while incorporated LA-GRGDS resulted in a lower level of inhibition which was not significantly different. However, coating LA-GRGDS had significantly ($P<0.05$) lower inhibition compared to heparin (Figure 8.2).

From this study it can be concluded that the direct incorporation of LA conjugated peptide into the matrix of the polymer was achieved successfully

with the peptide retaining its activity. Interestingly, incorporated LA-GRGD demonstrated a comparable inhibition of PF4 to heparin which correlated with our previous results[312]. LA-GRGDS incorporation showed the best endothelial cell adhesion whereas LA-GRGD showed the best antiplatelet activation effect.

CHAPTER NINE

SUMMARY

9.1 SUMMARY

At present t thrombosis on prosthetic surfaces and compliance mismatch between polymeric graft and native vessel is considered to be a major hindrance to the continued progress and ultimate solution in the use of prosthetic replacements for blood vessels. In order to reduce the surface thrombogenicity of such prosthetic materials numerous research groups have been investigating a wide variety of approaches. These include surface modification and tissue engineering or cell seeding of the prosthetic material [35-41]. ECs lining the lumen of a normal vessel prevent platelet adhesion and blood coagulation or the formation of thrombosis [42]. Hence to encourage the growth of a layer of endothelial cells over the device surface has been additional approach to prevent thrombogenecity and improve blood compatibility.

Previously in our Department in collaboration with an industrial partner a polyurethane polymer with a poly(carbonate) soft segment has been produced using a low-temperature cast coagulation method in order to prevent stress formation in the material. The rational behind this development was that the polymer produced demonstrated improved visco-elastic properties and resistance to chemical degradation and biodegradation compared to other synthetic materials such as PTFE or Dacron. In addition, it allowed the production of a wide variety of polymer compositions which could therefore be tailored to specific applications [11, 12, 292, 300, 301]. However PCU like PTFE and Dacron suffers from causing surface thrombosis which limits its clinical application [161, 303].

The aims of this thesis were to develop an anticoagulant peptide for surface modification of the PCU polymer, validate the peptide effect and investigate the potential of the surface modified polymer combined with the peptide to enhance blood compatibility. In order to investigate potential sources of EC or MC for utilising in grafts seeding procedures and for testing the efficiency of surface modification, a preliminary study was carried out into the

extraction and isolation of EC from a variety of tissues. Whilst it was possible to obtain MC from peritoneal lavage, a technique which was promising due to the relative ease with which samples could be obtained, the number of cells extracted proved to be insufficient for further studies. When using subcutaneous fat sufficient cells could be obtained via enzymatic digestion of the fat followed by purification using magnetic beads, as has been shown by other groups previously. However further studies carried out into the use of magnetic beads suggest that in order to obtain a high proportion of the potential EC population a high ratio of beads to cells must be employed. The use of such high ratios of beads can cause detrimental effects to cell population and metabolism later on in the culture process [21]. This combined with the relative difficulty involved in obtaining samples of subcutaneous fat resulted in the use of EC isolated from human umbilical cord vein for the studies on the effectiveness of surface modification of PCU.

As a starting point for the development of a suitable peptide for surface modification the tri-peptide RGD was chosen. RGD has been shown to reduce surface thrombosis by inhibiting blood coagulation and enhancing EC adhesion to provide an endothelial layer. RGD has been extensively used in research studies but to date has not been used in a clinical situation. Research has concentrated on either coating or chemical modification to immobilise RGD on the polymer surface. Whilst this is achievable it has the disadvantage that from a manufacturing viewpoint these processes are difficult to scale up. Another potential drawback to such methods, especially coating, is that the peptide may be washed off the graft over time reducing its effectiveness. These problems could potentially be overcome if it was possible to incorporate the RGD peptide in the polymer material. Incorporation of the peptide would require only minor modification to the current manufacturing process and may result in a longer lasting effect. However it is not possible to incorporate simple RGD into a polymer base due to the hydrophilic nature of the peptide.

By developing lauric acid conjugated RGD-containing peptides which provide a hydrophobic tail to the hydrophilic RGD-head (as described in Figure 5.4) and thus show amphiphilic properties the difficulties encountered to date with incorporating RGD-based peptides may be overcome. The LA acts as a spacer and influences the solubility of the LA-peptide conjugate, allowing it to dissolve in both water and an organic solvent (DMAC). Unlike other hydrophilic RGD peptides this amphiphilic nature demonstrated by LA-GRGD and LA-GRGDS makes them highly suitable for incorporation into the matrix of a poly(carbonate-urea)urethane polymer.

Solid phase peptide synthesis was employed to synthesise the RGD-containing peptides GRGD, GRGDS and the multi repeated GRGD ($GRGD(AhxGRGD)_3$) in addition to their corresponding LA-conjugated peptides LA-GRGD, LA-GRGDS and LA-$GRGD(AhxGRGD)_3$ successfully. Further study demonstrated that the conjugation of the LA to the peptides showed no effect on the structure or stability of the original RGD peptides they were based on. Characterisation by HPLC, FTIR and mass spectroscopy was also carried out. HPLC analysis demonstrated that the peptides synthesised were homogenous, revealing the presence of a single well resolved major peak on the chromatogram for each peptide. Investigation by FTIR confirmed the formation of a protein-like secondary structure for each peptide, all of which showed an amide I band maximum in the range of 1615-1695 cm^{-1}. Mass spectrometry analysis confirmed that solid phase peptide synthesis was successful and that the successful conjugation of LA to the peptides GRGD and GRGDS had been achieved.

To evaluate the effectiveness of these peptide moeties, techniques such as the prothrombin time assay and a thrombelastography analysis were carried out to determine the anti-coagulant properties of the peptides. An Alamar Blue™ assay was carried out to indicate their cell binding effect on EC and to investigate any potentially adverse cytotoxicity effect on EC. Both GRGD and LA-conjugated GRGD retained 33% and 31% inhibition of TF respectively at a

concentration of 50μg/ml. At this concentration cell viability was unaffected. Investigation of the cell binding effect of LA-GRGD indicated that LA-GRGD(28.6%) retained its cell binding activity and showed no significant difference to the non-conjugated GRGD (25.8%) peptide and thus the conjugation of LA has not had an influence on the bio-activity of GRGD. LA-GRGDS, like LA-GRGD, also retained its activity and demonstrated no-significant difference in cell binding effect (30%) to the non-conjugated GRGDS (31.1%). At this concentration (50μg/ml) neither peptide had an effect on TF inhibition or blood coagulation. Again the conjugation of LA to the peptide showed no influence on this activity.

Both LA-GRGD and LA-GRGDS were immobilised onto surface by incorporation into polymer using a solvent which was able to dissolve both the polymer and the peptide, with the solvent leaving upon evaporation. This method was used on the basis that the addition of amphiphilic peptides to polymer may result in the more hydrophilic portion being expressed on the polymer surface, anchored by the more hydrophobic end entangled in the polymer surface (figure 9.1). From a commercial viewpoint this would be simple to produce with no major chemical changes to the polymer production process with the additional advantage that to have the peptide immobilised on the polymer surface should increase the stability of the peptide.

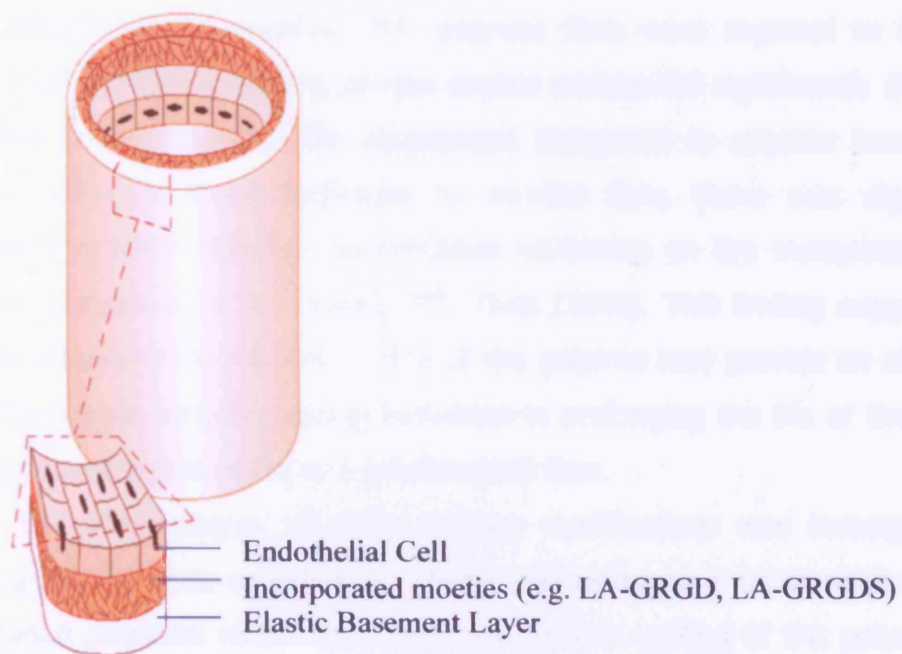


Figure 9.1 Schematic representation of *in vitro* reconstruction of a surface modified vascular wall. The modified graft has a hierarchical arterial structure consisting of a monolayer of endothelial cells, incorporated moieties (e.g. LA-GRGD) and an outer elastic basement layer.

When surface modified PCU polymer films were exposed to flow, the amount of peptide remaining on the coated surface fell significantly (P<0.005) over the time course of the experiment compared to peptide incorporated surface. After 8 hours exposure to *in-vitro* flow, there was significantly (P<0.005) greater peptide concentration remaining on the incorporated films (42.6%) compared to the coated PCU films (20%). This finding suggests that peptide incorporation into the matrix of the polymer may provide an advantage over the simple surface coating technique in prolonging the life of the peptide when polymer is subjected to a physiological flow.

The effectiveness of these surface modifications was investigated by seeding HUVEC onto modified PCU films and using as a comparison the non-conjugated peptides which were used for passive coating of the polymer then monitoring cell metabolism by utilising an Alamar Blue™ assay and examining cell morphology via phase contrast, confocal and scanning electron microscopy. Incorporating LA-conjugated LA-GRGD and LA-GRGDS into PCU polymer produced a comparable effect to coating PCU with the same peptides, and indeed in the case of LA-GRGDS provided improved cell metabolism in the longer term (48 and 72 hours).

In order to further investigate the effects of such surface modifications the blood compatibility of LA-GRGD, LA-GRGDS, GRGD and GRGDS immobilised onto PCU surface was evaluated using measurement of platelet activation factor (PF4) and the assessment of adsorbed platelets and whole blood haemolysis. The inhibition of PF4 obtained with coated GRGD, GRGDS and incorporated LA-GRGD was comparable to that obtained with heparin coating (1U/ml), while incorporated LA-GRGDS resulted in a lower level of inhibition which was not significantly different. However, coating LA-GRGDS had significantly (P<0.05) lower inhibition as compared to heparin. The platelet adhesion and haemolysis studies demonstrated that none of the modified PCU films resulted in any adverse effect with respect to either platelet adhesion or haemolysis.

As a result of this the RGD derivatives developed in this study have potential as a surface modification system in which the peptides cell binding activity is retained whilst allowing its solubility into solvents used in the manufacture of polymers for bypass grafts. This study has demonstrated that LA conjugated to an RGD derivative may have wide applicability for polymer formulation *via* commercially used solvent casting methodology for use in coronary, vascular and dialysis bypass grafts, scaffold polymer based tissue regeneration and tissue engineering.

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