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**BLOOD BIOCOMPATIBILITY OF POLYVINYL CHLORIDE
SURFACE-MODIFIED WITH MULTI-WALLED CARBON
NANOTUBES**

by

Alan Michael Gaffney

being a thesis submitted for the degree of
Doctor of Philosophy (Pharmacology)

at

**UNIVERSITY OF DUBLIN
TRINITY COLLEGE**



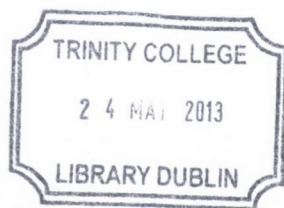
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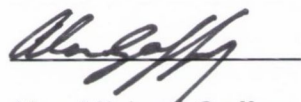
2012



Thesis 10015

DECLARATION

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Alan Michael Gaffney

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1 SUMMARY

Blood-surface interaction in extracorporeal circuits (for example, cardiopulmonary bypass) requires the infusion of a systemic anticoagulant to prevent devastating thrombus formation. Platelet activation upon contact with foreign circuit surfaces is the primary mechanism underlying thrombus formation. Research into nano-enabled approaches to blood-surface biocompatibility is at a very preliminary stage, however, carbon nanotubes are being developed as potential modulators of blood-surface biocompatibility. The **general objective** driving this research was to study the effects of nanomodification of medically relevant surfaces on blood biocompatibility. For the purpose of my PhD research, I **hypothesised** that the modification of polyvinyl chloride surfaces with carbon nanotubes affects blood biocompatibility. The **specific aims** explored in this investigation focused on studying the effects of polyvinyl chloride surface-bound carbon nanotubes on platelet function in *in vitro* (using the quartz crystal microbalance with dissipation flow model, platelet aggregometry, scanning electron microscopy, and phase contrast microscopy) and *in vivo* (rabbit) models of extracorporeal circulation. Furthermore, the platelet biocompatibility of polyvinyl chloride-carbon nanotube surfaces functionalised with a potent antiplatelet drug, iloprost, was investigated using the above *in vitro* techniques as well as mass spectrometry. Finally, proteomics and surface protein adsorption experiments (using the quartz crystal microbalance with dissipation flow model, platelet aggregometry and ELISA) were used to study the mechanisms of nanomodified surface-platelet interactions. Surface bound multi-walled carbon

nanotubes cause platelet activation *in vitro* and devastating thrombosis *in vivo* in an animal model of extracorporeal circulation. The mechanism of the pro-thrombotic effect is uncertain, but likely involves direct nanomaterial-platelet interactions and the interactions mediated by plasma proteins. Anti-platelet drug functionalisation of MWCNTs with the platelet-inhibitory iloprost attenuates MWCNT-induced platelet activation. My experiments provide, for the first time, proof-of-concept evidence that modification of medically relevant surfaces with nanomaterials modulates blood-surface biocompatibility.

2 LIST OF ABBREVIATIONS

$\alpha 2\beta 1$	Integrin $\alpha 2\beta 1$
ACD	Acid citrate dextrose
ACT	Activated clotting time
ADP	Adenosine diphosphate
aPTT	Activated partial thromboplastin time
ABG	Arterial blood gas
AT III	Antithrombin III
cAMP	Cyclic adenosine monophosphate
CCVD	Catalytic chemical vapour deposition
CNT	Carbon nanotube
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
CPB	Cardiopulmonary bypass
CD61	IIIa subunit of GP IIb/IIIa
CD62P	P-selectin
<i>D</i>	Energy dissipation
ddH ₂ O	Double-distilled water
DMAP	4-(N,N- dimethylamino)pyridine
dPBS	Dulbecco's phosphate-buffered saline
DTI	Direct thrombin inhibitor
DTT	Dithiothreitol
ECC	Extracorporeal circulation
ECLS	Extracorporeal life support

ECMO	Extracorporeal membrane oxygenation
EDC	Ethyl-(N',N'-dimethylamino)propylcarbodiimide hydrochloride
ELISA	enzyme linked immunosorbent assay
<i>f</i>	Frequency
FACS	Fluorescent-activated cell sorting
FDA	Food & Drug Administration
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GP Ib	Glycoprotein Ib
GP IIb/IIIa	Glycoprotein IIb/IIIa
GP VI	Glycoprotein VI
HBSS	Hank's balanced salt solution
Hb	Haemoglobin
HIT	Heparin-induced thrombocytopenia
HIT I	Heparin-induced thrombocytopenia type 1
HIT II	Heparin-induced thrombocytopenia type 2
HRTEM	High-resolution transmission electron microscopy
LC-MS/MS	The liquid chromatography tandem mass spectrometry
LMWH	Low molecular weight heparin
MFI	Mean fluorescent intensity
MMP	Matrix metalloproteinase
MMP-2	Matrix metalloproteinase-2
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MWCNT	Multi-walled carbon nanotube

NO	Nitric oxide
PaCO ₂	Arterial carbon dioxide partial pressure
PaO ₂	Arterial oxygen partial pressure
PAR1	Protease activated receptor 1
PAR4	Protease activated receptor 4
PBS	Phosphate buffered saline
PCI	Percutaneous coronary intervention
PE	R-phycoerythrin
PGI ₂	Prostacyclin
PLGA	Poly(lactic-co-glycolic-acid)
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PVC	Polyvinyl chloride
QCM-D	Quartz crystal microbalance with dissipation
QSoft ₄₀₁	Q-Sense software 401
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SIRS	Systemic inflammatory response syndrome
SOCl ₂	Thionyl chloride
SSC	Side scatter
SWCNT	Single-walled carbon nanotube
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis
THF	Tetrahydrofuran

TIMP	Tissue inhibitor of matrix metalloproteinases
TXA ₂	Thromboxane A ₂
TXA ₂ R	Thromboxane A ₂ receptor
UFH	Unfractionated heparin
VAD	Ventricular assist device
vWF	von Willebrand factor
WBC	White blood cell
WCC	White blood cell count
WP	Washed Platelets
XPS	X-ray photoelectron spectroscopy

3 INTRODUCTION

3.1 BIOMATERIALS, BIOCOMPATIBILITY & BLOOD COMPATIBILITY

3.1.1 *What is a biomaterial and what do the words biocompatible and blood compatible mean?*

A *biomaterial* is defined as “a non viable material used in a medical device, intended to interact with biological systems” and *biocompatibility* is “the ability of a material to perform with an appropriate host response in a specific application”⁽¹⁾. Because of the unique interaction between blood and exogenous materials and devices, *blood compatibility* is the term more commonly used in lieu of *biocompatibility* in this particular area of biomaterials science.

3.1.2 *What examples are there of biomaterials in current use and do they all elicit an “appropriate host response”?*

Examples of such materials and their applications include ceramic hip joint prostheses, titanium dental implants and poly(methyl methacrylate) implantable lenses used in cataract surgery. The host response is such that these materials are “accepted” and integrated into the body’s own tissues.

On the other hand, there are many examples of materials in use today that do not elicit an “appropriate host response” but are tolerated by the body only

when the host response is manipulated or suppressed, usually by drugs. This is the case for most materials that come into contact with blood such as those materials at the blood-surface interface in coronary artery stents, mechanical heart valves, vascular grafts, intravascular catheters and the tubing and membranes of extracorporeal circulation (ECC) technologies.

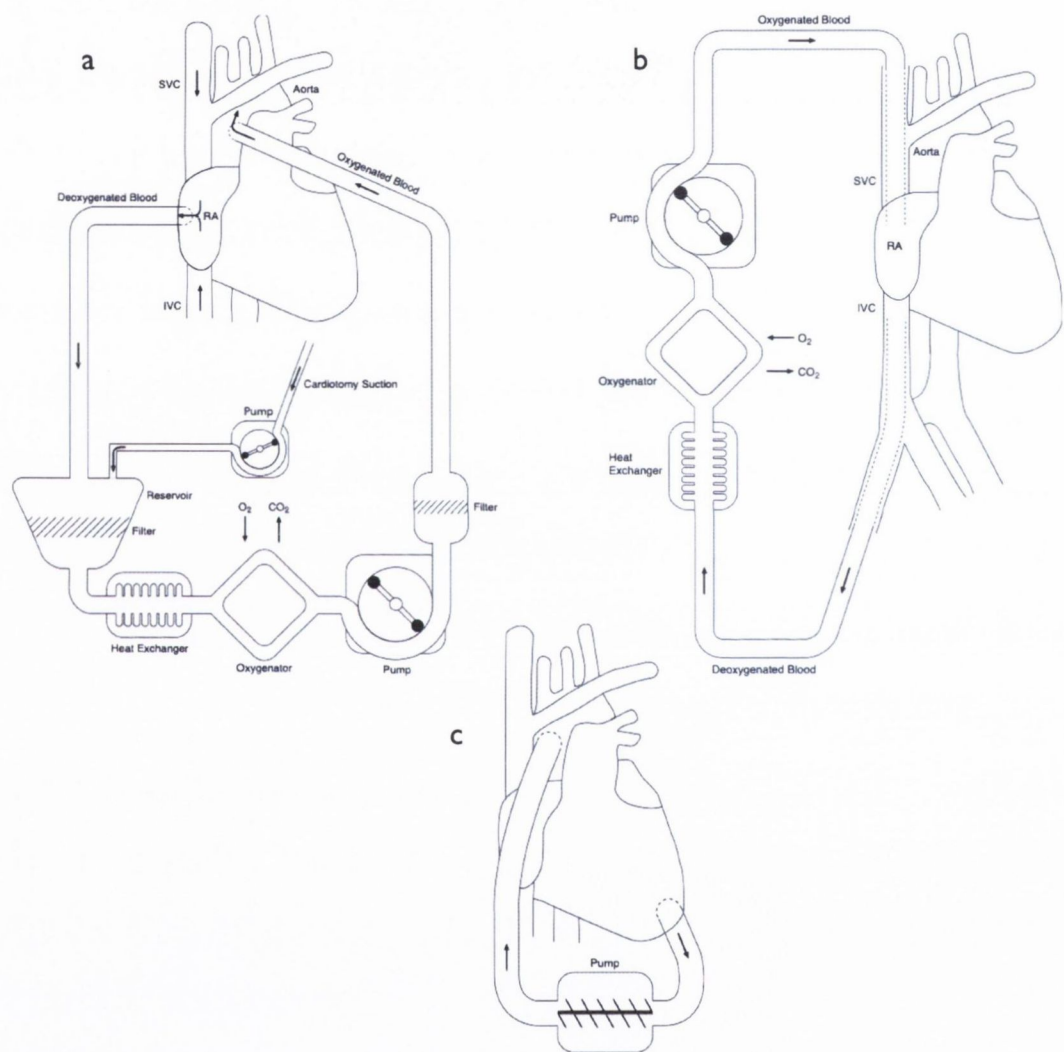


Figure 1
Examples of extracorporeal circulation technologies.
(a) Cardiopulmonary bypass (b) Extracorporeal life support (c) Left ventricular assist device. [SVC = superior vena cava; IVC = inferior vena cava; RA = right atrium; O_2 = Oxygen; CO_2 = carbon dioxide]

Extracorporeal circulation technologies include haemodialysis machines, cardiopulmonary bypass (CPB) machines (used during open heart surgery), extracorporeal life support (ECLS) machines (used as long-term cardiopulmonary bypass in the intensive care unit, for example, to support the functions of the lungs in respiratory failure due to H1N1 influenza)⁽²⁾ and ventricular assist devices (VADs) (figure 1). **It is the interaction of blood with the modified and non-modified surfaces of CPB and ECLS polyvinyl chloride (PVC) tubing that will be the focus of this thesis.**

3.2 BLOOD-SURFACE INTERACTION: THE PROBLEM

3.2.1 *Why is blood-surface interaction important in CPB and ECLS?*

Each year, thousands of cardiovascular surgeries are performed throughout the world using CPB. In addition, increasing numbers of patients with severe heart and lung disease are being supported with ECLS.

Because of the pro-thrombotic interactions between blood and artificial surfaces, and our attempts to counteract thrombosis with anticoagulants, thrombotic and haemorrhagic complications are frequently seen.

The risk of significant bleeding after cardiac surgery is high with transfusion of blood and blood products necessary in 9-100% of patients (depending on the centre and country surveyed) and with 2-6% of patients requiring re-

exploration for post-operative bleeding⁽³⁾⁽⁴⁾. Blood transfusion and re-exploration are both associated with increased morbidity and mortality. Bleeding (7-34%) and thrombosis (8-17%) (despite anticoagulation) account for the majority of serious extracorporeal life support-related complications⁽⁵⁾.

In order to discuss what happens when blood comes into contact with the foreign surface of extracorporeal circulation tubing, it is necessary to appreciate the unique physiology and pharmacology of the platelet.

The multiple roles of platelets in the pathogenesis of atherosclerotic and thrombo-occlusive cardiovascular diseases are well recognized. Antiplatelet therapy forms the cornerstone of their acute management and secondary prevention. Therefore, many patients presenting for cardiac surgery have received some form of anti-platelet therapy in the pre-operative period.

With their wide-ranging physiological and pathological interactions (figure 2), it is unsurprising that platelets are a major component of the body's complex response to ECC. Bleeding, thrombotic and inflammatory complications can be better understood, managed and potentially avoided by studying the effects of ECC on platelets.

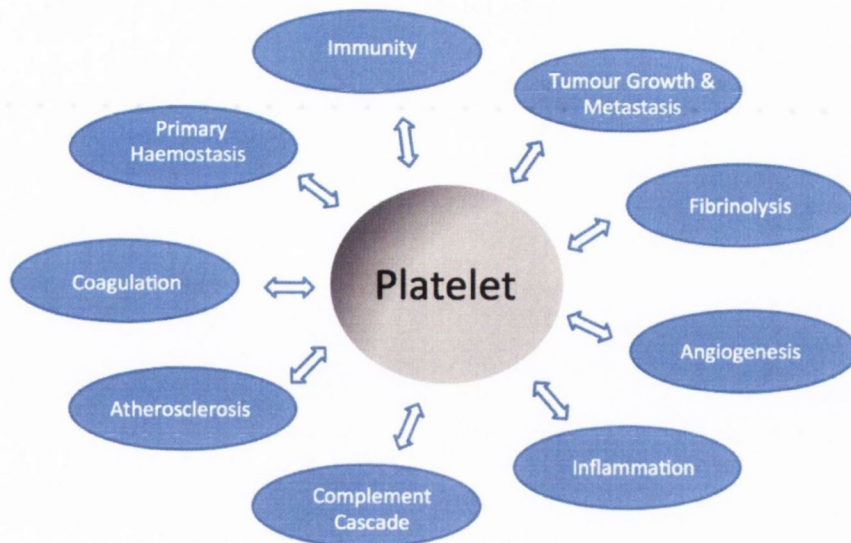


Figure 2
The multiple physiological and pathological interactions of platelets

3.3 PLATELETS

3.3.1 *What are the unique features of platelet physiology?*

Platelets are non-nucleated cell fragments. Their main physiological role is in primary haemostasis where they act to preserve the integrity of the vascular wall through the formation of platelet plugs. They interact intimately with the coagulation cascade, the complement system, the fibrinolytic system and the inflammatory process⁽⁶⁾. Their significant role in many other biological processes including atherosclerosis⁽⁷⁾, tumour growth and metastasis, antimicrobial immunity⁽⁸⁾ and angiogenesis⁽⁹⁾, is being increasingly recognised.

Each litre of adult whole blood contains $150 - 400 \times 10^9$ platelets that measure $2-4 \mu\text{m}$ in diameter and $0.5 \mu\text{m}$ in thickness. They are released from the bone marrow into the circulation as fragments of megakaryocyte precursor cell cytoplasm where they have a lifespan of 7 – 10 days⁽¹⁰⁾.

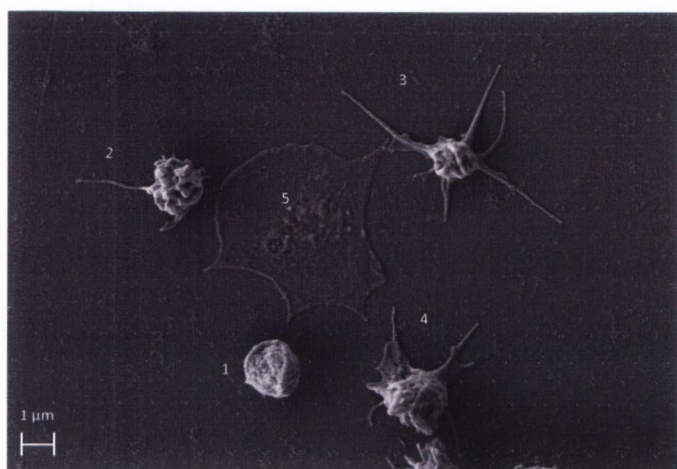


Figure 3
Scanning Electron Microscopy image of platelet-surface interaction on a fibrinogen-coated surface. Various stages of platelet activation can be seen from resting (1) through to the 'fried-egg' appearance of a fully activated platelet (5)

Resting and activated platelets are very different (figure 3). The plasma membrane surface of the discoid-shaped resting platelet is relatively smooth apart from small openings leading to the canalicular system. The cytoplasm contains the cytoskeleton, cellular organelles and storage granules (dense granules and α granules containing Ca^{2+} , adhesion molecules, chemokines and chemical mediators of the inflammatory response) but no nucleus. Because they are anucleate and contain only a tiny amount of mRNA, platelets are effectively unable to produce proteins *de novo*. Platelets must, therefore, perform their various physiological adaptive roles by utilizing their pre-existing complement of biomolecular resources.

Many receptors are expressed on the surface of resting platelets⁽¹¹⁾. Initiation of platelet activation follows binding of agonist molecules to these surface

The diagram illustrates the complex signaling pathways of platelet activation and aggregation. It shows a 'Resting Platelet' on the left and an 'Activated Platelet' on the right, with a 'Damaged Vessel Wall' at the bottom. Key components include:

- Resting Platelet:** Contains ADP receptors (P2Y₁ and P2Y₁₂) and TXA₂ receptors (TXA₂R). It also shows the 'Coagulation Cascade' leading to 'Thrombin' (PAR 1/PAR 4).
- Activation Pathways:**
 - ADP:** Binds to P2Y₁ and P2Y₁₂ receptors, leading to activation.
 - Thrombin:** Binds to PAR 1/PAR 4 receptors, leading to activation.
 - TXA₂:** Binds to TXA₂R receptors, leading to activation.
 - Proinflammatory Factors, Prothrombotic Factors, Adhesion Proteins:** These factors also contribute to platelet activation.
- Activated Platelet:** Upon activation, the platelet releases contents from its α -Granules (containing ADP and TXA₂) and Dense Granules (containing ADP and ATP). It also expresses surface receptors for aggregation and adhesion.
 - Aggregation:** Mediated by GP IIb/IIIa (fibrinogen receptor) and GP VI/ α 2 β I (collagen receptor). NO inhibits this process.
 - Adhesion:** Mediated by GP Ib (vWF receptor) and GP IIb/IIIa (fibrinogen receptor). NO inhibits this process.
- Drugs and Antagonists:**
 - Thromboxane Formation Inhibitor:** Aspirin.
 - ADP-Receptor Antagonists:** Clopidogrel, Prasugrel, etc.
 - GP IIb/IIIa Antagonists:** Abciximab, Eptifibatide, etc.
- Regulatory Factors:** NO (Nitric Oxide) and Prostacyclin are shown as inhibitors of both aggregation and adhesion. MMP-2 (Matrix Metalloproteinase-2) is shown as an activator of the α -Granule.

Platelet adhesion, activation & aggregation at the site of vascular injury. Sites of action of the main classes of anti-platelet drugs are also shown.

The ensuing G protein-coupled transmembrane and intracellular signalling leads to a host of rapid conformational changes and biochemical responses. The discoid shape is lost and the platelet becomes spheroid. Granules protrude out of cytoplasm then finger-like projections grow outwards and the

cell flattens and spreads (figure 3). The final common pathway for all agonists is the Ca^{2+} -dependent activation of the platelet glycoprotein IIb/IIIa (GP IIb/IIIa) receptor (integrin $\alpha\text{IIb}\beta 3$) by conformational alteration⁽¹²⁾. Activation of this receptor allows the binding of adhesive proteins such as fibrinogen and vWF to form cross-links between adjacent platelets leading to the process of platelet aggregation (figure 5).

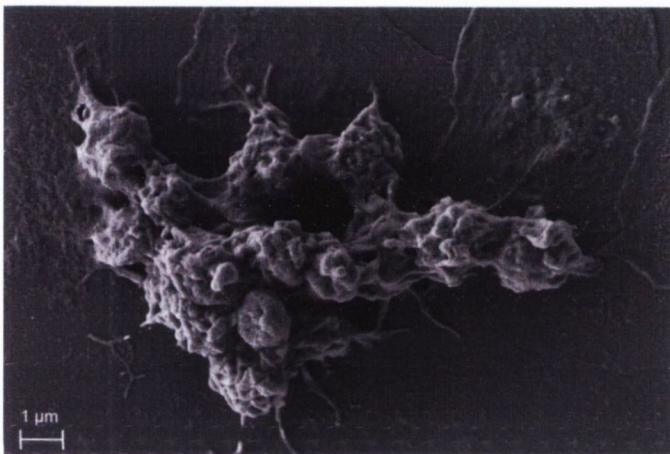


Figure 5
Scanning electron
micrograph of
aggregated platelets on
a fibrinogen-coated
polystyrene surface.

Thromboxane A_2 (TXA_2) is released from the activated platelet cytosol and adenosine diphosphate (ADP) is released from dense granules. These bind to nearby resting platelet TXA_2 and P2Y_{12} surface receptors, respectively. Thrombin is formed by exposure of tissue factor to coagulation factors. This is further promoted by the exposure of acid phospholipids on the surface of activated platelets. Thrombin then binds to the Protease Activated Receptor 1 and 4 (PAR1 and PAR4) of resting platelets⁽¹³⁾. Binding to any of these groups of receptors leads to an increase in intracellular Ca^{2+} , activation of the GP IIb/IIIa receptors and further platelet recruitment to the site of aggregation.

This platelet plug now forms the basis for fibrin deposition, the final product of the coagulation cascade.

Matrix metalloproteinases (MMP) belong to a family of zinc-dependant proteinases. They are involved in normal physiological as well as pathological degradation and remodelling of the extracellular matrix. Activated platelets release both MMPs and their inhibitors, tissue inhibitors of MMPs (TIMPs)⁽¹⁴⁾ regulating aggregation (figure 4).

Platelets interact with the coagulation system (e.g. localization of its response to the site of injury, provision of a process of stimulus amplification, provision of expressed surface receptors and protection for coagulation enzymes and co-factors, release of intracellular fibrinogen and coagulation factors V, XI and XIII); the fibrinolytic system (e.g. provision of a surface for the assembly of proteins of the plasminogen activating system⁽¹⁵⁾); and the inflammatory system (e.g. P-selectin-induced initiation of adhesion to leukocytes causing them to roll and arrest on activated platelets).

Initial arterial haemostasis is achieved when platelets aggregate to form a plug at the site of bleeding. In the low shear stress venous system, platelets release coagulation factors and provide the necessary surfaces for coagulation system reactions. Fibrin-rich clots are formed in response to vascular damage in the venous system.

Normal, intact vascular endothelium prevents inappropriate platelet activation. Even at the site of vascular injury endothelium limits the size and growth of the haemostatic plug a process known as endothelial thromboregulation⁽¹⁶⁾. There are at least three endothelial thromboregulatory pathways: nitric oxide (NO), prostacyclin and CD39 ecto-nucleodase⁽¹⁷⁾. All are potent inhibitors of platelet activation.

Nitric oxide is, itself, produced by platelets where it regulates granule release, promotes disaggregation and inhibits recruitment and adhesion ⁽¹⁸⁾⁽¹⁹⁾.

3.3.2 *What are the unique features of platelet pharmacology?*

Antiplatelet agents are used in the prevention and treatment of arterial thrombotic occlusive disease including acute coronary syndromes, coronary artery stent thrombosis, stroke and peripheral vascular disease. Many patients presenting for cardiac surgery are taking some form of antiplatelet therapy. However, as the main side effect of antiplatelet drugs is bleeding, the benefits of perioperative antiplatelet therapy need to be weighed against this risk. Factors to be considered in this assessment include the choice of drug, its dose and timing of administration, patient specific risk factors and procedure specific risk factors.

From the preceding description of platelet physiology, the sites of action of the most commonly used antiplatelet drugs can be appreciated (figure 4).

Although endogenous regulators of platelet activation such as NO and prostacyclin can prevent the initial activation of platelets by the interaction of cell surface receptors with endothelial collagen or vWF, these drugs are not currently used primarily for this purpose. Drugs which act on adhesion receptors are in development⁽²⁰⁾. There are, however, drugs in clinical use that target molecules and receptors involved in other activation pathways and in platelet aggregation. Some of the most commonly used drugs are discussed.

Aspirin

Aspirin inhibits the generation of TXA₂. It does this by irreversibly inhibiting the activity of cyclooxygenase (COX), predominantly COX-1, the constitutive isoform of the enzyme. COX catalyses the conversion of arachidonic acid to prostaglandin H₂, the immediate precursor to TXA₂⁽²¹⁾. Less TXA₂ produced by activated platelets results in less ligand for the TXA₂ receptors on nearby resting platelets. The GP IIb/IIIa receptor is not, therefore, converted from the inactivated to the activated form by the TXA₂ receptor-signalling pathway. Without GP IIb/IIIa activation there is reduced binding of fibrinogen and decreased platelet aggregation. As platelets are anucleate, COX cannot be synthesized *de novo*, and so, the inhibitory effect lasts for the whole of their 7 – 9 day lifespan.

The benefits of aspirin in acute therapy and in the secondary prevention of cardiovascular disease are well established⁽²²⁾. On the other hand, there is no

proven overall benefit in primary prevention where the risk of serious bleeding appears to offset any modest reduction in cardiovascular events⁽²³⁾⁽²⁴⁾. Although recommendations vary, aspirin is generally continued preoperatively and reinstituted again as soon as possible postoperatively unless contraindicated⁽²⁵⁾⁽²⁶⁾⁽²⁷⁾.

Thienopyridines (ADP Receptor Antagonists)

Clopidogrel, a thienopyridine, is an ADP receptor antagonist. Ticlopidine is an older thienopyridine with haematological side effects. Prasugrel is a newer, more potent drug but carries an increased risk of bleeding⁽²⁸⁾.

Clopidogrel is a prodrug which requires hepatic metabolism to convert it to its active metabolite that irreversibly binds to the P2Y₁₂ ADP-receptor on the platelet surface. This pharmacologically antagonises platelet activation by ADP and prevents amplification of the platelet response to TXA₂ and thrombin⁽²⁹⁾. Again, the effect lasts for the lifetime of the platelet.

Clopidogrel is used in the treatment of acute coronary syndromes⁽³⁰⁾⁽³¹⁾, in the secondary prevention of cardiovascular disease⁽³²⁾ and in the prevention of coronary stent thrombosis in combination with aspirin⁽³³⁾⁽²⁷⁾. Because clopidogrel acts on a different activating pathway to aspirin, the effect of using both in combination is thought to be greater than the individual effects resulting in amplification of the antithrombotic effects of single drugs. It may also be used in patients who do not tolerate aspirin⁽²²⁾. Clopidogrel is usually

discontinued 5 – 7 days before surgery due to the increased risk of bleeding although it should not be withheld in the case of acute coronary syndrome requiring emergency coronary artery bypass grafting⁽²⁵⁾⁽²⁶⁾⁽³⁴⁾⁽³⁵⁾.

GP IIb/IIIa Receptor Antagonists

The conformational change of the GP IIb/IIIa receptor from the inactivated to the activated form is achieved through many different signalling pathways. It is the final common step in platelet activation which allows the binding of fibrinogen and vWF. The GP IIb/IIIa antagonists are highly specific for the receptor. They are very potent antiplatelet agents that are only used intravenously (the oral form may have unwanted partial agonist action at the GP IIb/IIIa receptor and is associated with increased mortality)⁽³⁶⁾⁽³⁷⁾.

Abciximab is a monoclonal antibody that binds to the GP IIb/IIIa receptor. Tirofiban and eptifibatide are highly specific non-antibody receptor antagonists with shorter half-lives and weaker affinity than abciximab. GP IIb/IIIa antagonists are used in the medical treatment of acute coronary syndromes and during percutaneous coronary intervention (PCI)⁽³⁸⁾⁽³⁹⁾. Patients may present for urgent cardiac surgery after recent treatment with these drugs. There is an increased risk of bleeding especially following abciximab administration in this situation⁽⁴⁰⁾.

Dipyridamole

Dipyridamole is mainly used, in combination with aspirin, for the secondary prevention of cerebrovascular disease⁽⁴¹⁾⁽⁴²⁾⁽⁴³⁾. It exerts antiplatelet effects by multiple biochemical mechanisms, not just inhibition of cAMP phosphodiesterase as previously thought⁽⁴⁴⁾.

3.4 MECHANISMS OF BLOOD-SURFACE INTERACTION

3.4.1 What happens when blood comes into contact with the foreign surface of extracorporeal circulation tubing?

Apart from endothelium, every other surface that comes in contact with blood promotes thrombosis and inflammation. There are, as yet, no non-thrombogenic biomaterials available for clinical use.

During ECC, the total body blood volume flows time and again over vast areas of reactive surfaces. When blood comes in contact with the ECC circuit, plasma proteins are immediately adsorbed onto its surfaces⁽⁴⁵⁾. Protein adsorption occurs prior to any blood cell interaction with the artificial surfaces⁽⁴⁶⁾. Platelets are activated to varying degrees by interaction between these adsorbed proteins and platelet surface receptors. Further platelets are recruited to the site by granule-releasing activated platelets where they adhere and aggregate. This platelet aggregate then forms the basis for fibrin deposition and thrombus formation⁽⁴⁷⁾(figure 6).

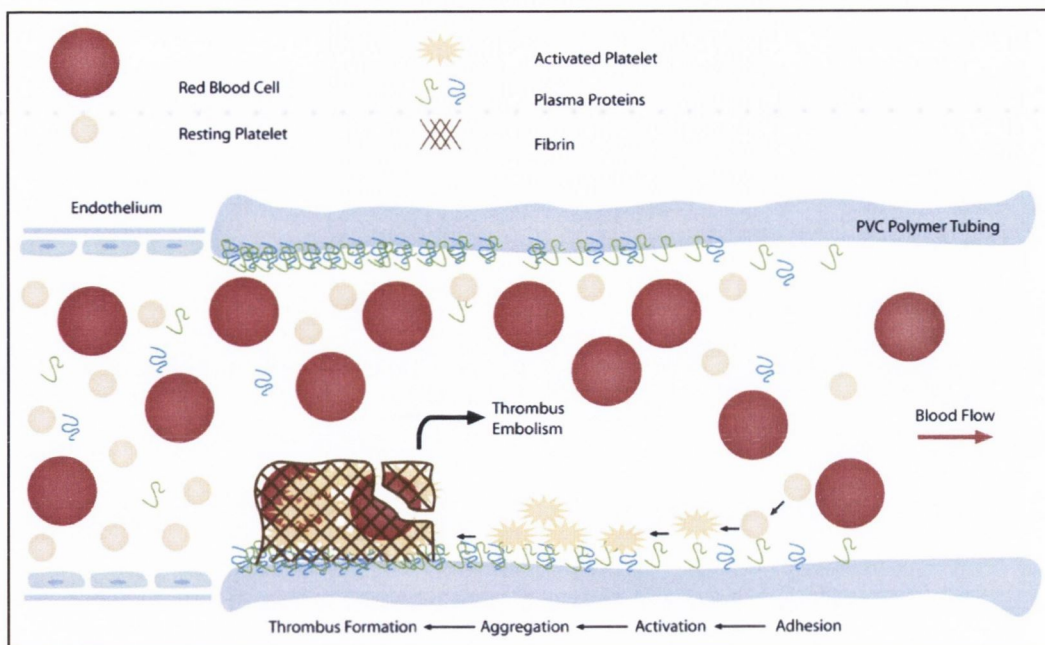


Figure 6

Plasma protein deposition, platelet activation and thrombus formation on an artificial PVC surface.

Protein film constituents include fibrinogen, albumin, globulins, high molecular weight kininogen (HMWK), clusterin, fibronectin, vitronectin, hemoglobin, factor XII and vWF among many others⁽⁴⁸⁾. The composition of the surface protein film is not constant but changes over time. For example, some fibrinogen that is initially adsorbed may be later replaced by HMWK. This dynamic process is known as the Vroman effect⁽⁴⁹⁾⁽⁵⁰⁾. Which proteins are adsorbed and at what concentrations is a function of the physical and chemical properties of the surface layer of the biomaterial as well as the plasma protein composition of the particular patient⁽⁵¹⁾.

Fibrinogen is thought to play a critical role in the initial adherence of platelets to the surface protein film⁽⁵²⁾. Under normal circumstances fibrinogen does not cause activation of platelets. It usually interacts, instead, with the already activated GP IIb/IIIa receptor forming cross bridges between adjacent

activated platelets. It is postulated that the fibrinogen molecule attached to the biomaterial surface undergoes a conformational change allowing it to interact with the inactivated GP IIb/IIIa receptor and, in this way, leads to platelet activation⁽⁵²⁾⁽⁵³⁾. Platelets may be activated by adsorbed vWF in the usual GP Ib-mediated fashion⁽⁵⁴⁾.

Complement is activated initially by the classical pathway and then predominantly by the alternate pathway through contact with the artificial and protein-covered surfaces of the ECC circuit. Complement components are also known to cause platelet activation⁽⁵⁵⁾⁽⁵⁶⁾.

Activated platelets at the biomaterial surface, as well as facilitating clot formation through the coagulation cascade, promote the recruitment and binding of inflammatory cells⁽⁵⁷⁾⁽⁵⁸⁾. Inflammatory mediators are released from platelet granules and lysosomes⁽⁵⁹⁾. Thus, platelet activation contributes to the “whole-body inflammatory response” or “systemic inflammatory response syndrome” (SIRS) induced by cardiopulmonary bypass⁽⁶⁰⁾.

3.5 CONDUCT OF CARDIOPULMONARY BYPASS AND EXTRACORPOREAL LIFE SUPPORT

3.5.1 *What is the state of the art in the conduct of Cardio Pulmonary Bypass?*

The basic cardiopulmonary bypass circuit consists of a pump, an oxygenator, a heat exchanger, a reservoir, a filter and cannulae with associated tubing (figure 1(a), figure 7). Added to this basic circuit are suctioning systems and a cardioplegia delivery system as well as monitoring and safety devices. Adjuncts such as cell salvage and washing systems or ultrafiltration devices are sometimes employed⁽⁵¹⁾. The circuit is assembled and primed, usually with a crystalloid solution. This predictably leads to haemodilution, which, if calculated to be excessive, requires addition of packed red cells. Various other priming solution additives commonly used include heparin, calcium and mannitol. All air is expelled from the circuit.

When the heart and great vessels have been exposed and ancillary surgery such as vein graft preparation is complete, unfractionated heparin (UFH) is administered.

Systemic anticoagulation with UFH is necessary to prevent devastating thrombosis within the CPB circuit once blood comes in contact with the foreign, non-endothelial surfaces. Venous and arterial cannulae are placed

and only when the blood is adequately anticoagulated as measured by a prolonged activated clotting time (ACT), is CPB commenced.

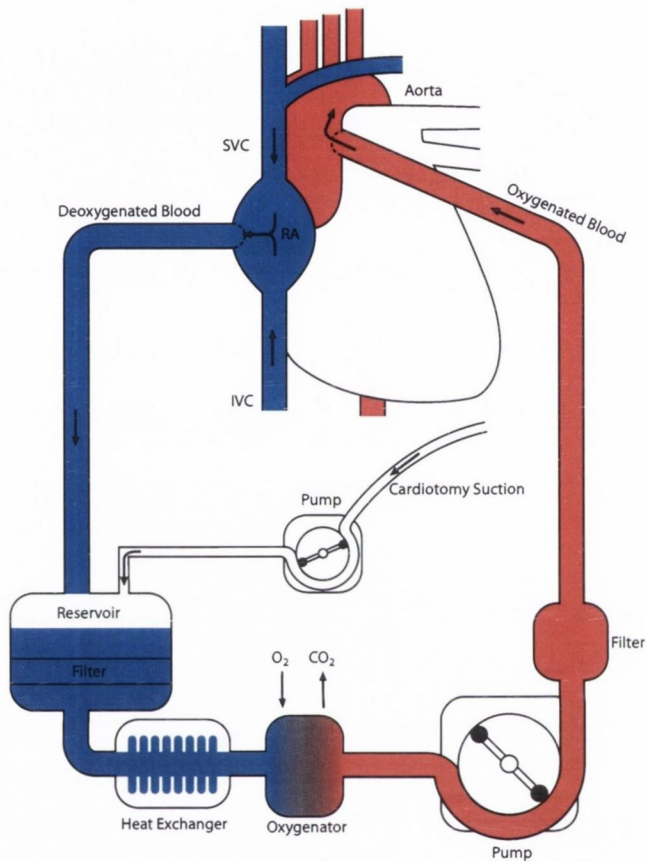


Figure 7
Cardiopulmonary
Bypass Circuit
[Blue deoxygenated
blood; red - oxygenated
blood; IVC - Inferior
Vena Cava; SVC -
Superior Vena Cava]

During CPB, any blood which collects in the operating field is filtered and returned to the venous reservoir of the CPB circuit (cardiotomy suction). Where aortic cross clamping is necessary (thereby preventing coronary artery perfusion), cardioplegia solution is infused into the myocardium through the coronary arteries and/or in a retrograde fashion through the coronary sinus. This prevents myocardial contraction and confers protection from ischemic

injury. Local and systemic hypothermia may also be employed to protect against organ ischemia.

Prior to discontinuation of CPB, the patient is re-warmed, metabolic derangements are corrected, ventilation is recommenced and heart rate, rhythm and function are normalized and supported as necessary.

When CPB has been successfully withdrawn, the heparin antagonist protamine is administered. ACT is checked to confirm that it has returned to its pre-heparin level. There is a shift in focus towards haemostasis at this stage. Large surgical sites and deranged haemostatic responses to CPB combine to increase the risk of bleeding and its complications from this point onwards.

Antifibrinolytic drugs are frequently administered, especially in patients deemed to have a higher risk of post-operative bleeding⁽⁶¹⁾. Transfusion of red blood cells, plasma and platelets in patients requiring cardiopulmonary bypass is common but variable⁽³⁾.

During the process of CPB, coagulation must be suppressed to prevent circuit thrombosis and embolization to the patient. Stroke, delirium and neurocognitive dysfunction are, in part, clinical manifestations of the intraoperative embolization of cellular aggregates, gas and particulate material to the patient's arterial circulation (however, given the failure of off-pump coronary artery bypass grafting in clinical trials to reduce these complications,

it has been suggested that patient factors associated with the need for cardiac surgery may contribute to a larger degree than previously thought⁽⁶²⁾). There is a systemic inflammatory response syndrome (SIRS) reaction to surgery and CPB affecting all organ systems. Once CPB is discontinued haemostasis is prioritised. Efforts to maintain wound haemostasis must be balanced against the risk of potential thrombosis in the area of new graft placement. Post-CPB SIRS continues into the postoperative period.

3.5.2 What is the state of the art in the conduct of Extracorporeal Life Support?

ECLS is a form of cardiopulmonary bypass used in acute severe respiratory or cardiac failure where short-term recovery is expected⁽⁶³⁾. It can also be used as a bridge to VAD placement or to cardiac transplantation. ECLS has mostly been used in the treatment of neonates and children, however its use in adult patients is increasing⁽⁶⁴⁾⁽⁶⁵⁾.

The ECLS circuit is similar to that of the CPB machine, however there is no venous reservoir so the system is described as 'closed'. Cannulae are usually placed in more peripheral vessels using bedside percutaneous or surgical techniques although central cannulation may be used after cardiac surgery. Veno-venous cannulation is used for isolated respiratory support whereas veno-arterial cannulation is necessary where circulatory support is required.

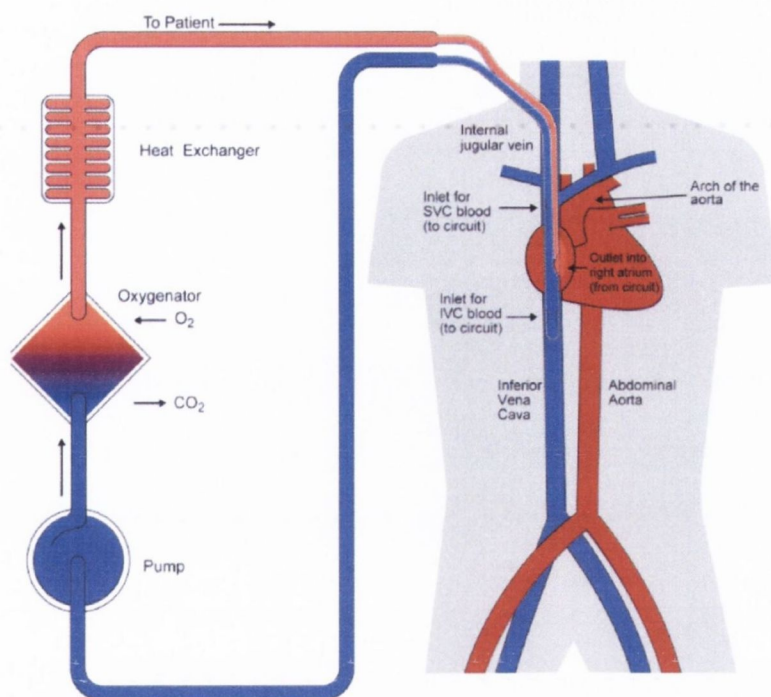


Figure 8

Double lumen cannula version of venous-venous ECLS for respiratory failure.
 [Blue - intravascular deoxygenated blood; red - intravascular oxygenated blood;
 dark red - intravascular and intracardiac mixed oxygenated and deoxygenated
 blood; IVC - Inferior Vena Cava; SVC - Superior Vena Cava]

The basic ECLS circuit consists of tubing taking deoxygenated blood from the patient, a pump, an oxygenator, a heat exchanger and tubing returning oxygenated blood back to the patient (figure 1(b), figure 8).

Venous-venous ECLS

A double lumen cannula is commonly placed into the right internal jugular vein. Deoxygenated blood flows from the venae cavae into the ECLS circuit and oxygenated blood returns to the right atrium (figure 8). Alternatively, two or more peripheral cannulation sites may be used (figure 1, figure 9(a)).

Venous-arterial ECLS

Cannulae are placed in an artery and in one or more veins. Femoral arterial cannulation is common in adults (figure 9(b)), while the carotid artery is often cannulated in infants (figure 9(c)). The circuit may be attached to existing trans-thoracic cardiopulmonary bypass cannulae where ECLS is required immediately after cardiac surgery (Figure 9(d)). Oxygenated blood is pumped back into the systemic circulation, bypassing both the heart and lungs.

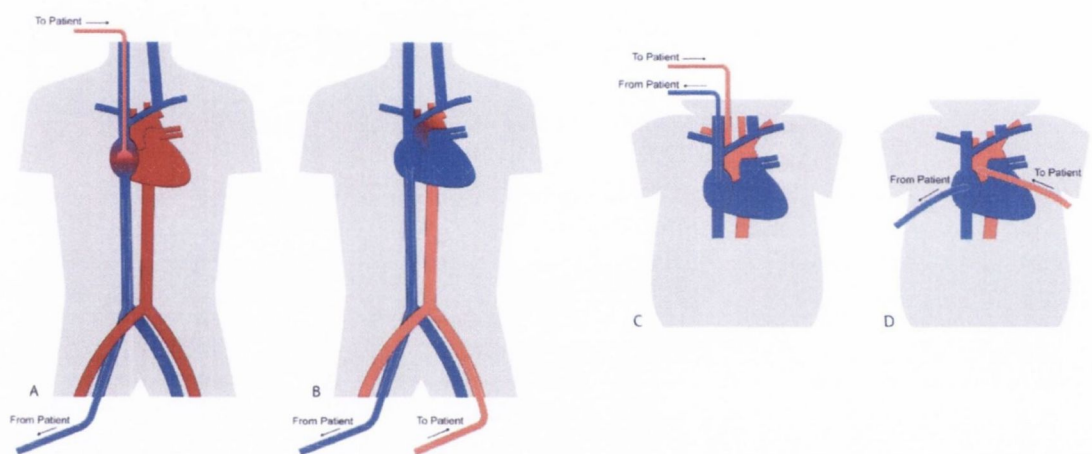


Figure 9
(a) alternate venous-venous ECLS cannulation. (b), (c), (d) various venous-arterial configurations. (a), (b) adults; (c), (d) children.
[Blue - intravascular and intracardiac deoxygenated blood; red - intravascular oxygenated blood; dark red - intravascular and intracardiac mixed oxygenated and deoxygenated blood]

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Anticoagulation

Because of blood-surface interaction, an infusion of unfractionated heparin is necessary to prevent thrombosis within the circuit and embolism to the patient. An activated clotting time of about 1.5 times normal is usually sufficient when using heparin-bonded circuits although this may need to be adjusted depending on clinical signs of bleeding or thrombosis⁽⁶⁶⁾.

Management of the haemostasis/thrombosis balance is particularly difficult during ECLS. Circuit thrombosis and embolization must be prevented concomitantly with the avoidance of haemorrhage from surgical and cannulae sites as well as spontaneous intracranial haemorrhage. This is further complicated by SIRS and the higher potential for disseminated intravascular coagulation and heparin-induced thrombocytopenia (HIT) Type II in this patient population.

3.6 EFFECTS OF ECC ON PLATELETS

3.6.1 *What are the quantitative platelet abnormalities seen in ECC?*

Thrombocytopenia is a quantitative platelet defect and is caused, for the main part, by a combination of haemodilution and platelet adhesion to artificial surfaces⁽⁶⁷⁾⁽⁴⁸⁾. Platelets are also fragmented into microparticles during CPB⁽⁶⁸⁾. Some other causes of thrombocytopenia following CPB and during ECLS include heparin induced thrombocytopenia (HIT) (discussed below), disseminated intravascular coagulation, drug-induced thrombocytopenia and pre-existing thrombocytopenia from other underlying causes.

3.6.2 *What are the qualitative platelet abnormalities seen in ECC?*

Although platelet numbers are mostly preserved above levels that are normally associated with bleeding, it is the functional defects of these platelets that contribute to postoperative bleeding⁽⁶⁹⁾⁽⁷⁰⁾⁽⁷¹⁾. It is postulated that not only is a significant proportion of previously activated platelets now hypofunctional but also that the remaining unactivated platelets are less responsive to activating agonists, express fewer adhesive receptors and produce less clot retraction⁽⁷²⁾⁽⁷³⁾⁽⁷⁴⁾. In addition, effects of pre-operative antiplatelet therapy may continue into the post CPB period.

3.7 COMPLICATIONS OF MANIPULATING THE HOST RESPONSE TO ECC

3.7.1 *What are the complications of modifying the host response to ECC with anticoagulants?*

Complications of unfractionated heparin use

Thrombin cleaves fibrinogen to fibrin. This is the final common step in the coagulation cascade. Fibrin deposition on activated platelet aggregates leads to thrombus formation. Unfractionated heparin inhibits fibrin formation by indirectly inhibiting thrombin. It achieves this by potentiating the activity of antithrombin III (AT III), an endogenous thrombin inhibitor⁽⁵¹⁾. Unfractionated heparin acts, for the most part, as an anticoagulant to prevent fibrin deposition on platelet aggregates and, therefore, thrombus formation in the circuit. It does, however, have additional significant effects on platelets and also on the fibrinolytic system.

Despite high doses of UFH administered during cardiopulmonary bypass some thrombin continues to be produced⁽⁷⁵⁾⁽⁷⁶⁾. Thrombin causes platelet activation. Unfractionated heparin is also known to bind to platelets enhancing activation and aggregation in some studies⁽⁷⁷⁾⁽⁷⁸⁾⁽⁷⁹⁾ but causing the opposite effects in others⁽⁸⁰⁾⁽⁸¹⁾⁽⁸²⁾. The clinical significance of such UFH-platelet binding remains uncertain. As the heparin fragments decrease in size, such as with low molecular weight heparin (LMWH), there is less platelet binding.

High dose UFH induces platelet dysfunction and fibrinolysis⁽⁸³⁾. This may be especially seen following the administration of doses of heparin used to overcome “heparin resistance” (defined as the need for higher-than-normal doses of UFH to induce sufficient anticoagulation for the safe conduct of CPB⁽⁵¹⁾). The cause is usually multifactorial but may include AT III deficiency, thrombocytosis, pre-operative heparin therapy or sepsis to name a few.

Heparin-induced thrombocytopenia (HIT) is a complication of heparin administration and is divided into HIT I and HIT II. Despite their similar names, these represent two very different patho-clinical entities.

Mild thrombocytopenia occurs in 5% to 28% of patients who receive UFH⁽⁵¹⁾. The mechanism is thought to relate to the pro-aggregatory effect of heparin in some patients⁽⁸⁴⁾⁽⁸⁵⁾. Thrombocytopenia usually resolves over time, even if heparin administration is continued. This is a non-immune phenomenon and is termed HIT I.

HIT II, on the other hand, is a much more serious disorder. Antibodies directed against the heparin-platelet factor 4 complex leads to a clinical syndrome characterized by one or more of the following: (1) an otherwise unexplained platelet count fall of at least 30 – 50%, (2) venous or arterial thrombosis, (3) anaphylactoid systemic reactions to heparin infusion and (4) skin lesions at heparin injection sites⁽⁸⁶⁾. Although the overall platelet count is low, there are high numbers of procoagulant platelet microparticles which are associated with the thrombotic complications⁽⁸⁷⁾. The overall risk of antibodies

forming after 4 days of continuous UFH administration is about 2.6%⁽⁸⁸⁾. Patients requiring ECC may often need UFH infusions prior to, during and after ECC thereby increasing the risk of HIT II. In those with measurable antibody levels requiring urgent CPB it may be necessary to use non-heparin anticoagulants. This brings its own sizable risks.

Complications of protamine use

Protamine sulphate is used to neutralize heparin at the end of cardiopulmonary bypass after all cannulae have been removed and it is almost certain that a return to bypass will not be required. Although heparin has many disadvantages as outlined previously, one of its main advantages is the ability of protamine to quickly reverse its anticoagulant effects when they are no longer needed.

Protamine has two active sites. Heparin binds ionically to one of these sites producing a stable precipitate. The other active site exerts a mild anticoagulant effect independently of heparin⁽⁵¹⁾.

Protamine is not without its own adverse effects. Histamine release following rapid administration may lead to transient systemic hypotension. Anaphylactic reactions have been described as have anaphylactoid reactions including severe pulmonary vasoconstriction⁽⁸⁹⁾.

Protamine is an inhibitor of platelet activation and aggregation which may, in part, explain its anticoagulant effects. It was previously thought that platelets were not inhibited so much by protamine alone (unless given in very large doses) than by protamine-heparin complexes⁽⁹⁰⁾⁽⁹¹⁾⁽⁹²⁾. A more recent study suggests, however, that protamine itself may cause platelet inhibition and at much lower doses than previously documented⁽⁸²⁾. Protamine may inhibit platelets by blocking the fibrinogen binding site of the GP IIb/IIIa receptor⁽⁹¹⁾, by inhibiting the release of platelet factor IV, by inhibiting platelet GPIb/vWF interaction⁽⁹³⁾, by attenuating the platelet response to thrombin or by directly inhibiting thrombin⁽⁹⁴⁾⁽⁹⁵⁾.

Protamine is cleared more rapidly than heparin and so may result in unneutralised heparin reappearing in the circulation after surgery, a phenomenon known as "heparin rebound"⁽⁹⁶⁾.

Complications of antifibrinolytics use

Antifibrinolytics are given as prophylaxis during cardiac surgery in an attempt to prevent post CPB bleeding. They are mostly effective at reducing total blood loss and the need for packed red blood cells transfusion.

Tissue plasminogen activator (TPA) is the main activator of fibrinolysis during cardiac surgery⁽⁹⁷⁾. Its production is driven by the presence of thrombin, which continues to be produced during CPB despite the use of high dose UFH. TPA

cleaves plasminogen to plasmin. Plasmin then attaches to fibrinogen and fibrin at its lysine binding sites causing proteolysis and clot breakdown.

The three main antifibrinolytic agents used are the lysine analogues tranexamic acid and aminocaproic acid, and until recently, aprotinin, a serine protease inhibitor. Since the publication of the BART trial (Blood conservation using Antifibrinolytics: a Randomized Trial in high-risk cardiac surgery patients), marketing of aprotinin has been discontinued although this is the subject of current FDA and MRHA review⁽⁶¹⁾⁽⁹⁸⁾. This trial showed a relative increase in all-cause 30-day mortality in high risk cardiac surgical patients receiving aprotinin compared to tranexamic acid or aminocaproic acid with only the possibility of a modest reduction in the risk of massive bleeding. Aprotinin has been more extensively studied than the lysine analogues and so attention is now turning to their safety profiles.

The exact mechanism of action of aprotinin is poorly understood. It is a non-specific serine protease inhibitor with a lysine molecule as part of its structure which has a high affinity for plasmin. Aprotinin has an indirect effect on platelet function by inhibiting plasmin (a serine protease). Plasmin causes platelet activation through its interaction with the PAR4 receptor on the platelet surface. It also has antithrombin activity which leads to less PAR1 platelet activation⁽⁹⁹⁾⁽¹⁰⁰⁾⁽¹⁰¹⁾. Its anti-inflammatory effects achieved by decreasing the production of kallikrein (a serine protease) may also prevent platelet activation⁽¹⁰²⁾. Despite these platelet-preserving effects of aprotinin within the circulation platelets may still be activated in the wound by other mediators through non-PAR pathways⁽¹⁰³⁾. There are conflicting opinions as to

whether aprotinin has any direct effects on platelets⁽¹⁰⁴⁾⁽¹⁰⁵⁾⁽¹⁰⁶⁾. Aprotinin may also block the adverse effects of heparin-platelet binding⁽¹⁰⁷⁾.

Tranexamic acid and aminocaproic acid are lysine analogues that competitively bind to the lysine-binding sites of plasmin preventing the proteolysis of fibrinogen and fibrin. They are often administered before, during and after CPB without any, as yet, known clinical increase in thrombotic complications⁽¹⁰⁸⁾. Tranexamic acid and aminocaproic acid, like aprotinin, block plasmin-induced platelet activation⁽¹⁰⁹⁾⁽¹¹⁰⁾⁽¹¹¹⁾⁽¹¹²⁾. Platelet function does not appear to be preserved as well by tranexamic acid and aminocaproic acid as it is by aprotinin⁽¹⁰⁵⁾. Clinically, there was only a trend towards a greater reduction in postoperative bleeding in the aprotinin group compared to the tranexamic acid and aminocaproic acid groups in the BART trial⁽⁶¹⁾. In the absence of aprotinin, tranexamic acid and aminocaproic acid continue to be used as adjuncts in the prevention of postoperative bleeding.

3.8 REDUCING THE EFFECTS OF THE HOST RESPONSE TO ECC

3.8.1 *If so many complications are attributed to heparin and protamine, can their use be avoided?*

Despite the problems associated with its use, UFH it has many properties that allow the safe conduct of ECC. UFH produces adequate anticoagulation to prevent gross thrombosis during ECC; it is an intravenous preparation with a rapid onset of action; its anticoagulant effects can be monitored intraoperative;

an antidote exists to rapidly and completely reverse its anticoagulant effects and it has a known safety profile. These are the properties that a potentially superior alternative anticoagulant should possess above any other putative benefits⁽¹¹³⁾.

There are, however, clinical scenarios where it may be necessary to avoid the use of heparin/protamine. The most common condition requiring the administration of an alternative anticoagulant is HIT Type II. Because non-heparin anticoagulants are used infrequently in ECC, few randomized, controlled trials have been conducted to evaluate their efficacy and safety.

Low Molecular Weight Heparin

LMWH causes less platelet activation and less platelet retraction than UFH⁽⁷⁹⁾⁽¹¹⁴⁾⁽¹¹⁵⁾. As the heparin fragments decrease in size there is less platelet binding. LMWH is about 8 to 10 times less likely to cause HIT Type II than UFH. Given that LMWH may still lead to HIT Type II, has a long half-life and is only up to 60% neutralized by protamine, it is rarely used as an anticoagulant in CPB.

Heparinises

These are heparin analogues with mainly anti-factor Xa activity. They are even less likely than LMWHs to cause HIT Type II and may cause even less platelet activation⁽¹¹⁶⁾. However, heparinoids have no reversing antidote,

plasma levels poorly correlate with ACT and activated partial thromboplastin time (aPTT) and they have a very long half-life. Again, these are rarely used in ECC.

Direct thrombin inhibitors

Direct thrombin inhibitors (DTIs), unlike UFH, do not require antithrombin III as a co-factor. They not only inhibit circulating thrombin, but also inhibit fibrin-bound thrombin. DTIs reduce thrombin-mediated activation of platelets⁽¹¹⁷⁾. Although their half-lives are short, lepirudin and bilvalirudin may accumulate in renal failure but are 70% cleared by hemofiltration. Argatroban, on the other hand, may accumulate in liver failure. There is no antidote for the DTIs. Experience of using these agents in patients with and without HIT Type II is steadily increasing⁽¹¹⁸⁾⁽¹¹⁹⁾⁽¹²⁰⁾.

Antiplatelet agents

Antiplatelet drugs have been used in combination with lower doses of UFH⁽¹²¹⁾. Prostacyclin and its analogues are administered as an infusion and so may lead to systemic hypotension secondary to vasodilatation. Nitric oxide has been delivered directly into the oxygenator of the CPB circuit with encouraging results in initial studies⁽¹²²⁾. GP IIb/IIIa antagonists can block HIT antibody-induced platelet aggregation. Tirofiban, however, may accumulate in renal failure and, if not removed by ultrafiltration, can lead to platelet transfusion-resistant post-operative bleeding⁽¹²³⁾. GP IIb/IIIa inhibitors may

also paradoxically induce platelet aggregation under certain conditions such as deep hypothermia⁽¹²⁴⁾.

“Platelet anaesthesia” where platelet activation is temporarily blocked during ECC is an experimental strategy to protect platelets during ECC⁽¹²⁵⁾. Prostacyclin, nitric oxide and short-acting GP IIb/IIIa inhibitors are all potentially useful agents to achieve this.

Nafamostat mesilate

This is a synthetic protease inhibitor that inhibits coagulation and fibrinolysis by inhibiting thrombin, plasmin, trypsin, kallikrein, factors XIIa and Xa⁽¹²⁶⁾. It has been used as an alternative to heparin in only a few patients requiring urgent cardiac surgery with recent intracranial haemorrhage secondary to infective endocarditis⁽¹²⁷⁾.

3.8.2 *If thrombocytopenia is attributed, in part, to haemodilution during CPB, can haemodilution be avoided?*

Miniaturized ECC consists of a closed system with no cardiectomy suction or venous reservoir. The result is the avoidance of haemodilution, air-blood contact and the direct return of unwashed cardiectomy suction blood to the circuit. A recent meta-analysis showed a reduction in postoperative red blood cell transfusion in patients undergoing coronary artery bypass grafting using this technology⁽¹²⁸⁾. Randomized studies have shown that the use of these

systems has comparable outcomes to off-pump procedures for coronary artery bypass surgery⁽¹²⁹⁾. Others have found a reduction in platelet consumption and myocardial cell damage⁽¹³⁰⁾.

3.8.3 If there are quantitative and qualitative platelet defects, *why not transfuse platelets?*

Platelet structure and function is altered during collection, processing and storage⁽¹³¹⁾⁽¹³²⁾. Special materials and methods are used to limit the degree of unwanted platelet activation and the accompanying "storage lesion". Alteration in structure and function leads to decreased platelet survival after transfusion although there is some recovery of platelet function *in vivo* compared with the *ex vivo* stored state⁽¹³³⁾.

Platelets are transfused during cardiac surgery somewhere between 0% and 40% of the time⁽¹³⁴⁾⁽¹³⁵⁾⁽¹³⁶⁾. Intraoperative platelet transfusion practices vary considerably between institutions and are infrequently based on laboratory indices. Pre-operative antiplatelet therapy, CPB effects on platelets, the clinical condition of the patient and institutional and physician-specific preferences are all taken into account when deciding to empirically transfuse platelets.

There is no evidence to support the prophylactic transfusion of platelets in cardiac surgery patients⁽¹³⁷⁾. Where microvascular bleeding continues despite adequate surgical haemostasis platelets are often transfused. Even if platelet

numbers are within normal limits platelet dysfunction is expected. There are currently no laboratory tests which can be reliably used in this clinical scenario to evaluate qualitative platelet defects.

Risks of platelet transfusion include viral transmission, allergic reactions, all immunisation, bacterial sepsis, graft versus host disease (GVHD), transfusion-related acute lung injury (TRALI), renal failure, volume overload, and immunosuppression. Platelet transfusions during coronary artery bypass graft surgery may be associated with serious adverse outcomes however studies are sparse and conflicting ⁽¹³⁸⁾⁽¹³⁶⁾⁽¹³⁹⁾. It is unknown whether platelet transfusion may contribute towards these adverse outcomes or if platelet transfusion may be a surrogate marker for sicker patients.

Platelet transfusion is essentially universal in patients on ECLS⁽¹⁴⁰⁾.

3.8.4 What about plateletpheresis?

This intraoperative technique involves the removal of platelets from the circulation prior to cardiopulmonary bypass and their subsequent re-infusion after surgery has been completed. Although the principle would appear to be sound, meta-analysis and more recent studies have not yet provided evidence of sufficient benefit to support its widespread use⁽¹⁴¹⁾⁽¹⁴²⁾⁽¹⁴³⁾⁽¹⁴⁴⁾. Its main potential use is most likely in selected high-risk patient groups in combination with other blood conservation techniques.

3.8.5 *Can cardiopulmonary bypass be avoided in any situations?*

Off-pump coronary revascularization removes the need for CPB, high dose heparinisation, haemodilution, hypothermia, myocardial ischemia and in a proportion of cases, manipulation of the ascending aorta. On the other hand, the technique still requires varying degrees of cardiac manipulation with hemodynamic consequences and, at times, the use of intraluminal shunts, passive distal perfusion or distal perfusion using a conduit and pump. Although small randomized, controlled clinical trials have thus far failed to show an overall decrease in mortality using this technique the rate of postoperative bleeding requiring transfusion appears to be lower⁽¹⁴⁵⁾. Better preservation of platelet function is likely to be associated with decreased release of inflammatory mediators such as MMP-9⁽¹⁴⁶⁾⁽¹⁴⁷⁾⁽¹⁴⁸⁾.

3.8.6 *Can the surfaces that come into contact with blood be modified to attenuate the adverse effects of blood-surface interaction?*

The ideal extracorporeal surface would be completely covered by physiologically functioning endothelium. As no such surface exists as of yet, the intermediate goal is to develop surfaces with as many of the non-thrombogenic and non-inflammatory properties of endothelium as possible. Very few clinically applicable advances have been made in this area in many years.

Heparin can be bonded to circuit surfaces and released locally. Hypothetically, less systemic heparin should be necessary which, in turn, should result in fewer bleeding complications. The use of heparin-bonded surfaces is not, however, routine⁽¹⁴⁹⁾. While some studies have shown lower systemic heparin requirements and lower postoperative bleeding, others have shown little additional clinical benefit when compared to non-coated surfaces⁽¹⁵⁰⁾⁽¹⁵¹⁾⁽¹⁵²⁾.

Phosphorylcholine, which resembles the phospholipid envelope of cells, has been used to coat surfaces. Promising results of *in vitro* studies have not, however, been reproduced in a consistent or significant way in clinical studies⁽¹⁵³⁾⁽¹⁵⁴⁾⁽¹⁵⁵⁾.

Various surface-modifying agents may be added to bulk biomaterials during production. These agents then migrate to the surface where they reduce surface energy in an attempt to alter the properties of the surface and the adsorbed protein layer. Again studies showing appreciable clinical benefit are lacking⁽¹⁵⁶⁾.

Platelets in close proximity to the endothelial surface are temporarily inactivated by physiologic amounts of prostacyclin and nitric oxide (NO) produced by endothelial cells. The incorporation of NO into the surface of extracorporeal circuits reduces platelet consumption and eliminates the need for systemic heparinisation in a rabbit model of extracorporeal circulation⁽¹⁵⁷⁾⁽¹⁵⁸⁾.

Although the expense and modest positive clinical outcomes associated with the use of surface-coated and surface-modified circuits has discouraged widespread use, most studies have been performed in isolated coronary revascularization patients. It may be that higher risk patients and patients undergoing complex cardiac surgery may benefit most from the use of these circuits. Studies in such cardiac surgery sub-groups are lacking at present.

Some circulatory assist devices have micro-textured surfaces which lead to the formation of well-anchored clot and, thus, a biological pseudo intima. It has been suggested that this layer may be nonthrombogenic. On the other hand it has also been suggested that this layer is more likely to be prothrombotic (based on its usually prothrombotic protein composition) and that the body's fibrinolytic response causes sufficient autoanticoagulation to prevent larger thrombus formation with embolic potential⁽¹⁵⁹⁾⁽¹⁶⁰⁾. These VADs do not require chronic anticoagulation.

A question that remains to be answered is whether a **nanotechnological approach** can be used to modify blood-surface interaction (figure 10).

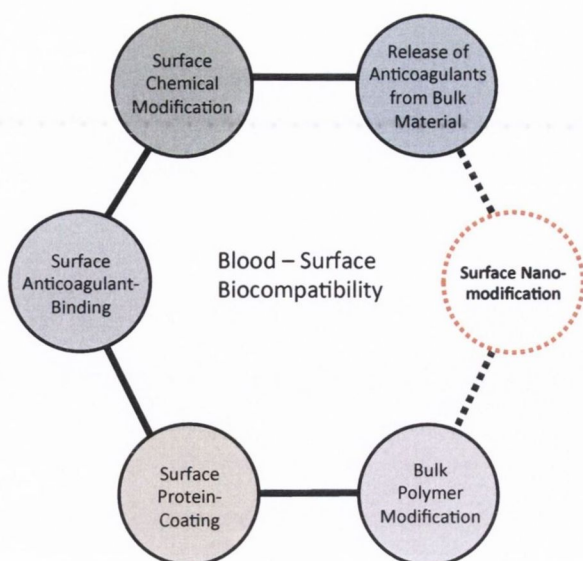


Figure 10
Technologies that attempt to increase blood-surface biocompatibility.

3.9 NANOTECHNOLOGY & NANOMEDICINE

3.9.1 *What is Nanotechnology?*

In 1959 the physicist and Nobel Laureate, Dr Richard Feynman, delivered a lecture entitled “There is Plenty of Room at the Bottom” at the annual meeting of the American Physical Society at the California Institute of Technology⁽¹⁶¹⁾. In the course of his lecture he asked, “What would happen if we could arrange the atoms one by one the way we want them?” This is probably the beginning of what has subsequently become known as “nanotechnology”. Although the original term referred to building nanoscale objects from the “bottom-up”, nanotechnology now refers to both “bottom up” and miniaturization (“top-down”) approaches.

There is a lack of consensus surrounding the definition of nanotechnology. Bawa *et al.* in an article in *Nanomedicine* in 2005, suggested the following definition “The design, characterization, production, and application of structures, devices, and systems by controlled manipulation of size and shape at the nanometre scale (atomic, molecular, and macromolecular scale) that produces structures, devices, and systems with at least one novel/superior characteristic or property⁽¹⁶²⁾.” The International Organization for Standardisation is in the development stage of defining nanotechnology. Although not published yet, they suggest the following definition: “ [the] application of scientific knowledge to manipulate and control matter in the nanoscale in order to make use of size- and structure-dependent properties and phenomena, as distinct from those associated with individual atoms or molecules or with bulk materials”⁽¹⁶³⁾.

Essentially, a material may have different properties and/or structures at nanoscale compared to the same material at a larger scale. For example, materials at nanoscale have larger surface areas that may result in an alteration in electrical, chemical and/or physical properties when compared to the same material in bulk. In addition, quantum effects of nanomaterials may become more pronounced thus affecting electrical, magnetic and/or optical properties. It is these changes in properties that nanotechnology aims to exploit.

A nanomaterial is generally considered to be such if it is composed of structures that measure less than 100nm in at least one dimension. Again

there is no consensus on a precise definition and many structures developed mainly for pharmaceutical applications with dimensions larger than 100nm (but less than 1000nm) have been, and continue to be, referred to as nanostructures⁽¹⁶⁴⁾.

Nanotechnology is an exploding worldwide industry. It affects and influences practically all scientific disciplines in one way or another. In a report by Roco in 2011 looking at the National Nanotechnology Initiative (launched at the Californian Institute of Technology in 2000) at ten years from its inception, the number of researchers and workers employed in some domain or other of nanotechnology had increased from 60,000 to 400,000 worldwide between 2000 and 2008; the numbers of science citation index papers had increased from 18,000 to 70,000 in the same period; the market for products made in some part using nanotechnology increased from \$30 billion worldwide to \$200 billion during that period; and the amount spent on research and development worldwide during that same period increased from \$1.2 billion to \$15 billion⁽¹⁶⁵⁾.

3.9.2 *What is Nanomedicine?*

In a review article on nanomedicine in the New England Journal of Medicine in 2010, Kim et al. describe how “The field of nanomedicine aims to use the properties and physical characteristics of nanomaterials for the diagnosis and treatment of diseases at the molecular level”⁽¹⁶⁶⁾. Indeed, iron oxide nanoparticles have been Food & Drug Administration (FDA) approved for use

as contrast agents in MRI scanning while various liposome, protein and polymer nanomaterials have been FDA approved for use in cancer chemotherapy. In addition to these, many other nanoparticles and materials are being evaluated for potential clinical applications including gold nanorods, quantum dots, fullerenes and **carbon nanotubes (CNTs)**.

3.9.3 Has nanotechnology been used in an attempt to modify blood-surface interaction?

Research in this area is at a very rudimentary stage.

On a micro, rather than a nanoscale, geometric micropatterning of culture surfaces has been shown to alter cell behavior such as migration, apoptosis and differentiation⁽¹⁶⁷⁾⁽¹⁶⁸⁾⁽¹⁶⁹⁾⁽¹⁷⁰⁾. Fan et al. recently described reduced platelet activation on a micro-patterned, multi-scale surface compared to a smooth control surface in an *in vitro* flow model. The experimental surface was structured to mimic the multi-scale inner surface of blood vessels as seen on scanning electron microscopy⁽¹⁷¹⁾.

Moving to the nanoscale, Chen et al. from the same group, in an attempt to reduce the contact area for platelets (and thus lead to reduced platelet activation), coated carbon nanotube arrays with fluorinated poly(carbonate urethane) to form a surface of nanoscale pillars⁽¹⁷²⁾. Again, platelet activation was reduced when compared to smooth control surfaces.

Milner et al. described the patterning of polyether(urethane urea) with order arrays of surface pillars with sub- micron geometries. The adhesion of platelets was reduced compared to smooth controls. The authors concluded: "Localization of platelets in interpillar spaces suggests that optimization of the pillar geometries may lead to further reductions in platelet adhesion"⁽¹⁷³⁾.

Koh et al. have done initial work on composite films of poly(lactic-co-glycolic-acid) with multi-walled carbon nanotubes (PLGA–MWCNT) in both random and vertical alignments. The vertically aligned MWCNTs showed reduced platelet activation when compared to the randomly aligned or smooth control surfaces ⁽¹⁷⁴⁾⁽¹⁷⁵⁾⁽¹⁷⁶⁾. In addition, the authors investigated the influence of high aspect ratio surface features on PLGA films in the submicron to nanometer range. "The lowest ... platelet activity was observed on surfaces with specific topographical features in the submicron range [rather than the micron range] with a significant reduction in adhesion when compared to the pristine PLGA films...The results signify that topographical manipulation of thrombogenic surfaces of biodegradable polymers is a feasible approach for reducing their thrombogenicity".

3.10 CARBON NANOTUBES

3.10.1 What are the various allotropes of carbon?

Diamond, graphite and amorphous carbon (e.g. coal, soot) are probably the best-known allotropes of carbon. Diamond and graphite have crystalline structures whereas amorphous carbon does not. Carbon, however, has multiple additional allotropes to these. In fact, of all atoms, carbon has the greatest number of allotropes. Examples include pyrolytic carbon, graphene (a single layer of graphite), single-walled carbon nanotubes (a layer of graphene formed into a tube), multi-walled carbon nanotubes (layers of graphene tubes inside each other), fullerenes, lonsdaleite, aggregated diamond nanorods and T-carbon, among many others.

3.10.2 *Are any of these carbon allotropes in clinical use as biomaterials?*

Of the above allotropes, pyrolytic carbon and carbon fibre graphite are already in use as biomaterials.

The majority of prosthetic mechanical heart valves are coated with pyrolytic carbon⁽¹⁷⁷⁾. This form of carbon, produced by the thermal decomposition of hydrocarbons in the absence of oxygen, has excellent blood biocompatibility (although systemic anticoagulation is still required to prevent thrombus formation). It has a structure similar to graphite but covalent bonds between layers prevent them from slipping over one another as they do in graphite.

In addition, pyrolytic carbon has been used on the surfaces of some interphalangeal joint and hip joint prostheses⁽¹⁷⁸⁾⁽¹⁷⁹⁾.

Carbon fibres can be added to other materials to form composites of greater strength. This was done in the case of orthopaedic joint prostheses where polymer-carbon fibre composites were implanted into patients. Unfortunately, poor results were obtained due to an inflammatory reaction to the carbon fibres⁽¹⁸⁰⁾. Despite these discouraging results, carbon fibre composites continue to be investigated as biomaterials.

Diamond-like carbon is being evaluated *in vitro* as a coating for orthopaedic implants and coronary vascular stents. Gutensohn et al. have shown that coronary stents coated with diamond-like carbon lead to reduced platelet activation and thrombogenicity when compared to control stents *in vitro*⁽¹⁸¹⁾.

Graphene, fullerenes and carbon nanotubes are not yet in clinical use but are also in the initial stages of biomedical development.

3.10.3 What are the unique features and properties of carbon nanotubes?

Carbon nanotubes are man-made allotropes of carbon that can be produced by a variety of techniques including arch vapourisation (where a current is passed between two carbon electrodes surrounded by an inert gas), laser

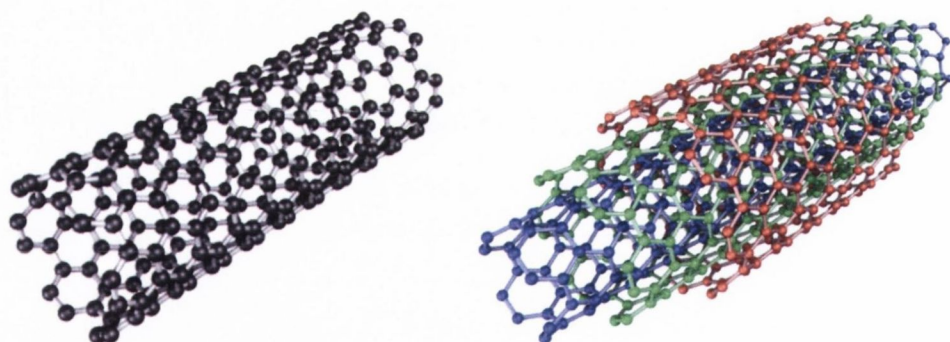


Figure 11 Graphical representation of a single-walled carbon nanotube (left) and a multi-walled carbon nanotube (right) (By Arnero [CC-BY-SA-3.0 (www.creativecommons.org/licenses/by-sa/3.0/)], via Wikimedia Commons)

vapourisation of graphite, chemical vapour deposition or ball milling among others. They have a high aspect ratio, often with diameters less than a few nanometers but with lengths up to as much as 1mm. Carbon nanotubes are very light but extremely strong and stiff. They possess high electrical as well as thermal conductivity.

It is due to the above characteristics and the fact that they can be functionalized along their length that carbon nanotubes are being used as optically transparent electrodes, organic light-emitting diodes, electrodes for lithium ion batteries, supercapacitors, nanowires, contacts in organic transistors, field effect transistors, catalysts in fuel cells, catalysts of organic reactions, filters, membranes, sensors, high strength ropes, electromechanical actuators and structural composites⁽¹⁸²⁾.

The worldwide market for carbon nanotubes is expected to reach more than \$7 billion by 2015⁽¹⁸³⁾.

3.10.4 What are the potential clinical applications of carbon nanotubes in medicine?

Carbon nanotubes are in the early stages of evaluation as biomedical materials. They can be easily functionalized (meaning that chemical groups can be attached to their side-walls) and, because carbon is such an ubiquitous atom in the human body, there was, at least initially, a relatively low expectation of toxicity⁽¹⁸⁴⁾⁽¹⁸⁵⁾. Studies have shown the potential for functionalised carbon nanotubes to be used as drug and gene delivery systems⁽¹⁸⁶⁾⁽¹⁸⁷⁾. Carbon nanotubes have very strong optical absorbance in near infrared (NIR) light range that may enable the use of NIR light to trigger drug or gene release from nanotubes⁽¹⁸⁸⁾⁽¹⁸⁹⁾. They may be used as components of extremely sensitive biosensors and are being investigated for use as antimicrobial surfaces⁽¹⁹⁰⁾⁽¹⁹¹⁾. Like carbon fibre, they are being incorporated into composites⁽¹⁹²⁾. In the fields of tissue engineering and regenerative medicine carbon nanotubes are showing promise as scaffold materials and surfaces for osteocyte bone growth and neuronal proliferation and regeneration⁽¹⁹³⁾⁽¹⁹⁴⁾. Some initial work has been done on their usefulness in blood-surface interaction (Section 1.9.3).

3.10.5 Are carbon nanotubes toxic?

The initial expectation of carbon nanotube biocompatibility without toxicity has been challenged in recent times. This is a subject of some debate with studies showing both toxic and non-toxic effects of carbon nanotubes.

Beginning with Lam et al. in 2004, a number of investigators have demonstrated the development of inflammatory granulomas in the lungs of animals exposed to CNTs (either by instillation or by inhalation)⁽¹⁹⁵⁾. On the other hand, other research has failed to confirm these pathological findings⁽¹⁹⁶⁾. The reasons for this are probably due to a number of factors including differences in the doses, exposure times, purities and structures of the nanoparticles investigated. It is not possible, therefore to generalize about pulmonary toxicology of CNTs and so it is likely that each new form of CNT should be tested independently for toxic effects⁽¹⁹⁷⁾.

Poland et al. injected carbon nanotubes into the abdominal cavity of mice and found resultant pathological similar to those caused by asbestos in the lungs of humans⁽¹⁹⁸⁾. On the other hand, Muller et al., using shorter CNTs in rats, found no evidence of a mesothelioma-like response⁽¹⁹⁹⁾. Again, the structure of the particular type of CNT tested seems to be important.

Work by Singh et al. demonstrated that, where carbon nanotubes functionalized with radio tracers were injected intravenously and then imaged over time, the blood half-life of these carbon nanotubes was less than 3 hours,

there was no retention in the reticuloendothelial system and they were excreted by the kidneys⁽²⁰⁰⁾. The functionalization, however, may have conferred alterations in CNT properties that could have lead to increased clearance because in other studies biopersistence was demonstrated, especially in the liver, lung and spleen⁽²⁰¹⁾.

Radomski et al. demonstrated increased thrombogenicity and platelet activation of CNTs injected intravenously⁽²⁰²⁾.

Neuronal cells grown on CNTs have shown improved signaling and growth⁽²⁰³⁾.

These examples serve to illustrate the unpredictable nature of CNT – host interaction and the necessity to thoroughly test each new CNT considered for biomedical use as generalisations cannot be made with regard to likely toxicological effects.

Where a particular CNT has been shown to be toxic, work has also been done to show how functionalisation may render them more biocompatible and more biodegradable⁽²⁰⁴⁾. The ideal method of functionalisation that allows enhanced biocompatibility and biodegradability without attenuating the desired therapeutic effect remains elusive. It would appear, however, that carboxylated CNTs may be more biocompatible than pristine CNTs⁽²⁰⁵⁾.

3.10.6 Have carbon nanotubes ever been functionalized with anticoagulant or antiplatelet drugs?

Murugesan et al., in 2006, functionalized MWCNTs with heparin as a proof of concept and showed a prolonged APTT *in vitro*⁽²⁰⁶⁾. Apart from an abstract describing aspirin functionalization of carbon nanotubes by Yosuf et al. in 2009, there are no other reports of carbon nanotubes as anti-platelet drug delivery systems.⁽²⁰⁷⁾

3.11 BLOOD BIOCOMPATIBILITY OF POLYVINYL CHLORIDE SURFACE-MODIFIED WITH CARBON NANOTUBES IN EXTRACORPOREAL CIRCULATION

The focus of my PhD research was to investigate the interactions of carbon nanotube-modified polyvinyl chloride surfaces with human blood with particular emphasis on platelet effects.

3.11.1 What is the rationale for choosing to investigate blood biocompatibility in extracorporeal circulation?

Extracorporeal circulation of high volumes of blood provides the greatest test of a surface's biocompatibility. Whether an experimental surface is found to be more or less blood compatible, the result is likely to represent the true biocompatibility of that surface when tested approaching the conditions seen in extracorporeal circulation.

Despite more than 50 years of research in this area the state of the art remains as it was when extracorporeal circulation first began – PVC tubing with systemic anticoagulation of blood. As described above, the morbidity and mortality associated with this approach is significant. Any research that adds to the body of knowledge and understanding of blood-surface interaction is to be welcomed, especially if this involves a novel approach. A nanotechnology approach is both timely and novel.

3.11.2 What is the rationale for choosing PVC as the surface to modify?

PVC is the most common biomaterial used in extracorporeal tubing and is licenced for biomedical use. Its physiochemical properties are favourable to surface modification. In addition, there are published animal models of extracorporeal circulation that use PVC tubing as the default conduit⁽¹⁵⁷⁾.

3.11.3 What is the rationale for choosing CNTs as the surface-modifying nanoparticles to investigate?

Although Radomski et al. demonstrated thrombogenicity and platelet activation following intravenous administration of soluble carbon nanotubes, the mode of exposure has been shown to impact the variability of the host response to these particles (section 1.10.5)⁽²⁰²⁾. In fact, Radomski et al. postulated that CNTs may act as intercellular bridges thus enhancing platelet-platelet communication and activation. It is unlikely that surface-bound CNTs would be able to form such bridges. In addition to this there is some evidence

that CNT-polymer composites may attenuate platelet activation⁽¹⁷⁶⁾. The question remains as to whether surface-bound carbon nanotubes have increased, decreased or no affect on blood compatibility. Furthermore, as carbon nanotubes can be functionalised with inhibitors of platelet function, this process can modulate the blood biocompatibility of nanomaterials.

Because of the large volume of research and development into carbon nanotubes as potentially useful biomedical materials on one hand, but the relative paucity of toxicology data on the other hand, exposure of carbon nanotubes to blood and blood components (especially platelets) while anchored to a surface will allow important toxicological measurements of carbon nanotube-blood interaction to be made where this interaction is, indeed, toxic.

The biocompatibility of surface-anchored CNTs has not been investigated in both *in vitro* flow, and *in vivo* animal models of extracorporeal circulation. The results of such a comprehensive investigation are likely to have far-reaching ramifications for carbon nanotube biomedical development into the future.

4 HYPOTHESIS & AIMS OF PROJECT

4.1 General Objective

The general objective driving this research is to study the effects of nanomodification of medically relevant surfaces on blood biocompatibility.

4.2 Hypothesis

For the purpose of my PhD research, I hypothesised that the modification of polyvinyl chloride surface with carbon nanotubes affects blood biocompatibility.

4.3 Specific Aims

The specific aims explored in this investigation focused on studying the effects of polyvinyl chloride surface-bound carbon nanotubes on platelet function in *in vitro* and *in vivo* models of extracorporeal circulation. Furthermore, the platelet biocompatibility of polyvinyl chloride-carbon nanotube surfaces functionalised with a potent antiplatelet drug, iloprost, was investigated. Finally, proteomics and surface protein adsorption experiments were used to study the mechanisms of nanomodified surface-platelet interactions.

5 MATERIALS AND METHODS

5.1 REAGENTS

All reagents were purchased from Sigma-Aldrich (Dublin, Ireland) unless otherwise indicated.

5.2 BLOOD COLLECTION AND PLATELET ISOLATION

Blood was donated by healthy volunteers. Volunteers who had taken any agents known to affect platelet function within the previous two weeks were excluded from donating blood. Voluntary, informed, written consent was obtained. Platelet rich plasma (PRP), platelet poor plasma (PPP), and washed platelets (WP) were prepared using prostacyclin as described by Radomski & Moncada⁽²⁰⁸⁾. Briefly, 9 parts whole blood was withdrawn and added to 1 part 3.15% sodium citrate (anticoagulant). This was centrifuged at 250g for 20 minutes at room temperature. PRP was removed, platelets were counted in a Coulter Counter ® and phosphate buffered saline (PBS) was added to dilute PRP to a platelet concentration of 210,000 platelets/ μ l. PPP was prepared by PRP centrifugation at 900g for 10 minutes at room temperature. PBS was added to PPP to dilute plasma proteins to the same concentration as that of donor-matched diluted PRP. Washed platelets were prepared by differential centrifugation and washing with Tyrode's solution and platelet concentrations were adjusted using the same solution to 250,000 platelets/ μ l. Re-suspended platelets were allowed to 'rest' for a period of 1 hour and were not used in experiments until collagen (Lab Medics, UK) stimulation resulted in at least

70% light transmission by platelet aggregometry (see section 5.7 - platelet aggregometry).

5.3 Q-SENSE™ E₄ SENSOR PVC COATING

Gold-coated quartz crystals (QSX 301, Gold 100nm, Q-Sense, Sweden) were spin-coated (Laurell WS-400BX-6NPP/Lite) at 6000 revolutions per minute for 15 seconds with 4 x 25µl layers of a 0.5% w/v solution of PVC (Tygon R3603, Fischer, Dublin) in tetrahydrofuran (THF) with or without dispersed carbon nanotubes (see section 5.4.1). See figure 12.

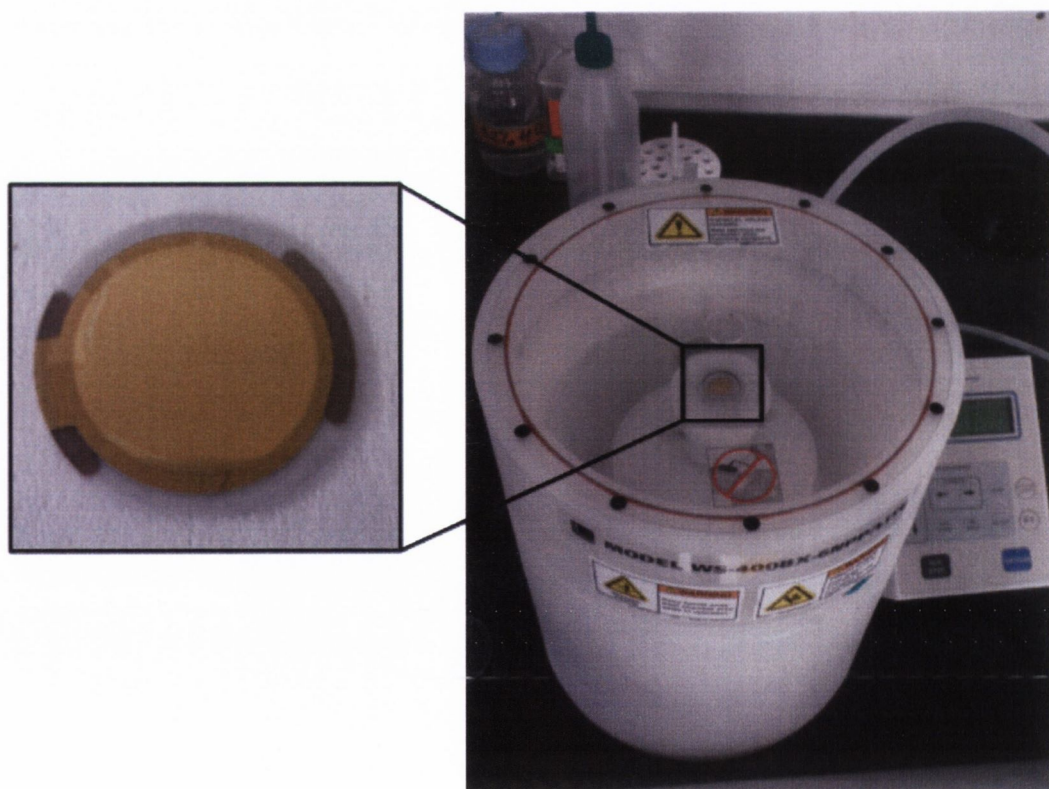


Figure 12 Quartz crystal sensor (left) *in situ* in spin-coater (right).

5.4 CARBON NANOTUBE PREPARATION

5.4.1 Non drug-functionalised carbon nanotubes

Curly thin multi-walled carbon nanotubes (MWCNT, Nanocyl™ 3100 and Nanocyl™ 3101 functionalised with -COOH) were purchased from Nanocyl, Belgium. They were produced via catalytic chemical vapour deposition (CCVD) and purified to > 95% carbon (table 1).

Straight MWCNTs were purchased from MER Corporation, Arizona, USA (calalogue no. MRGC). These are arc-produced MWCNTs (table 2).

Carbon nanotubes at concentrations 0.5mg/ml were added to a solution of PVC 0.5% in THF and tip-sonicated for 5 minutes. This solution was then used to directly coat Q-sense sensors by spin-coating as per section 5.3.

Carbon nanotubes at a concentration of 0.5% w/v in toluene were sonicated using a sonic tip with a 1 second pulse and power regulated at 20% of 750W for 5 minutes to ensure homogenous dispersal in the solvent. This solution was used for PVC swelling as described below.

Property	Unit	Value	Method of Measurement
Average Diameter	nm	9.5	TEM
Average Length	µm	1.5	TEM
Carbon Purity	%	>95	TGA
Metal Oxide	%	<5	TGA
Amorphous Carbon	-	*	HRTEM
-COOH Functionalisation (3101)	%	<4	XPS

Table 1 Characteristics of Nanocyl 3100 & 3101. [* = Pyrolytically deposited carbon on the surface of the CNT; TEM = Transmission Electron Microscopy; TGA = Thermogravimetric analysis; HRTEM = High-resolution transmission electron microscopy; XPS = X-ray Photoelectron Spectroscopy] (Adapted from Nanocyl™ 3100 series datasheet)

Property	Unit	Value
Diameter	nm	6-20
Length	µm	1-5
Carbon Purity	%	>99

Table 2 Characteristics of MER MRGC

5.4.2 Drug-functionalised carbon nanotubes

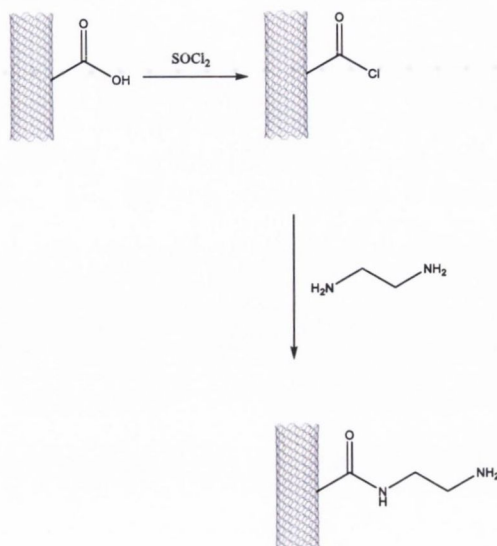
MWCNT functionalisation with iloprost (a stable analogue of prostacyclin) was carried out by Dr Amro Satti. Iloprost (Ilomedin®) was purchased from HE Clissman, Ireland. Briefly, for step 1, Nanocyl™ 3101 thin MWCNTs (MWCNT-COOH) were suspended in thionyl chloride (SOCl_2) and refluxed for 24 hours at 70°C (*Figure 13*). SOCl_2 was evaporated and the nanotubes were dried under vacuum. MWCNTs were then suspended in ethylene diamine and left to stir overnight. The MWCNTs were then filtered and washed with ethanol.

For step 2, Iloprost was dissolved in water. Ethyl-(N',N'-dimethylamino)propylcarbodiimide hydrochloride (EDC) and 4-(N,N-dimethylamino)pyridine (DMAP) were added to the solution and left to stir for 10 min. MWCNTs from step 1 were suspended in water and added to the Iloprost solution. The suspension was left to stir overnight. Following this, the MWCNTs functionalised with iloprost were filtered and washed with water. MWCNTs were then resuspended for experimentation.

For platelet aggregometry experiments, MWCNTs functionalised with iloprost were resuspended in PBS.

For attachment to PVC by ultrasound swelling (described below), swelling was performed after completion of all steps for one set of experiments and between steps 1 and 2 above, with the second step taking place on the

Step 1



Step 2

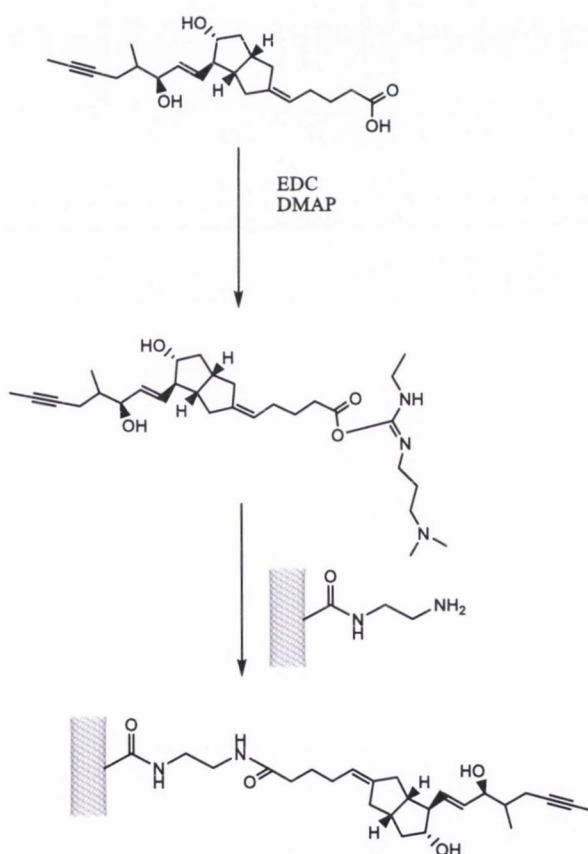


Figure 13 Schematic of the covalent binding of iloprost to COOH -functionalised MWCNTs. [SOCl_2 = thionyl chloride; EDC = Ethyl-(N',N'-dimethylamino)propylcarbodiimide hydrochloride; DMAP = 4-(N,N-dimethylamino)pyridine]

surface of the swelled MWPVC-CNT (from step 1) sensor coating for a second set of experiments. This was to avoid ultrasonification with potential detachment of iloprost from MWCNTs.

5.5 PVC SWELLING & CARBON NANOTUBE ATTACHMENT

This was performed as described by O'Connor et al⁽²⁰⁹⁾.

PVC-coated Q-sense sensors were sonicated for 30 seconds (Cole Parmer 8890) in a solution of MWCNTs in toluene (see section 5.4.1). They were then washed immediately with double-distilled water (ddH₂O), ethanol and ddH₂O again before being allowed to dry at room temperature.

PVC tubing (Tygon R3603, Fischer, Dublin), for use in the rabbit model of extracorporeal circulation, was sonicated for 20 minutes in a solution of MWCNTs in toluene (see section 5.4.1). Again, upon completion of the swelling process, the tubing was washed immediately with double-distilled water (ddH₂O), ethanol and further ddH₂O before being allowed to dry at room temperature.

PVC coated onto individual microtiter wells (96-well microtiter plates (Nalge Nunc #437111, black) (see section 5.16.1) was swelled by immersing in a solution of MWCNTs in toluene (see section 5.4.1) and ultrasonicing (Branson 3510, Fischer, US ultrasonic bath) for 1 minute.

5.6 PROTEIN SURFACE-ATTACHMENT

Fibrinogen, vitronectin, and albumin were purchased from Millipore Ireland. Protein solutions were prepared using PBS as diluent. 200µl of protein solution was placed on the surface of each sensor (that had previously been coated with PVC +/- surface swelling with MWCNTs) for 20 minutes and then removed. Sensors were immediately transferred to the Q-SENSE™ system for experimentation.

5.7 PLATELET AGGREGOMETRY

A Chrono-log platelet light aggregometer was used to test platelet function in PRP or WP. The principle is based on Born's turbidimetric method as previously described⁽²¹⁰⁾. Prior to QCM-D experimentation, the platelet agonist collagen 4µg/ml was added to PRP or WP and aggregation was recorded using aggregometry to determine whether the platelets were, indeed, functional (see section 5.2). In addition, platelet aggregometry was used to test for platelet inhibition or activation in PRP and/or WP in the presence of carbon nanotubes (either functionalised or non-functionalised). Chronolog Aggrolink software was used to display and analyse the results.

5.8 Q-SENSE™ E₄ QUARTZ CRYSTAL MICROBALANCE WITH DISSIPATION

5.8.1 Overview

The Q-Sense™ E₄ system (Q-Sense AB, Sweden) quartz crystal microbalance with dissipation (QCM-D) was used to assess platelet-surface interaction under flow conditions and protein-surface interaction under static conditions. A sensor is placed in a module that allows solutions (in this case PRP, PPP, WP or proteins) to be pumped through the module and to flow over the sensor surface. Any attachment of solution components to the sensor surface (or detachment of previously attached solution components) results in a change in the mass on the surface of the sensor. In addition, the deposited surface layer will have certain viscoelastic properties. Module temperature can be controlled.

An alternating current is passed across the sensor causing it to resonate at its natural frequency (about 5MHz). As mass is deposited onto the surface of the sensor there is a reduction in oscillation frequency (Δf). Material deposited on the sensor surface also causes changes in energy dissipation (ΔD) or dampening of the oscillations depending on the viscoelasticity or stiffness of the deposited surface layer.

Measurements of f and D are recorded and displayed in real time either under flow or static conditions.

The Sauerbrey equation can be used to calculate mass on the surface if that mass is deposited as a thin, rigid, uniformly distributed layer and is small compared to the mass of the crystal: $\Delta m = \frac{-C \times \Delta f}{n}$ (where Δm is the change in mass; Δf is the change in frequency; $C = 17.7 \text{ ng Hz}^{-1} \text{ cm}^{-2}$ for a 5 MHz quartz crystal and n is the overtone). In this case, frequency is proportional to mass⁽²¹¹⁾⁽²¹²⁾.

The Sauerbrey equation cannot, however, be used for platelet deposition mass calculations because platelets are thick and non-rigid and are not uniformly distributed across the sensor surface. Furthermore, because of high energy dissipation, D , changes in f are likely to underestimate the mass of platelets deposited on the surface of the crystal⁽²¹³⁾. Frequency is, therefore, not proportional to mass.

Although f cannot be used to directly quantify the mass of platelets deposited, additional information can be gained from the measurement of D . D increases as more platelets attach to the sensor surface. In cases where f is the same for two surfaces to which platelets have attached in different quantities, D will be greater for the surface to which more platelets have attached (due to an increase in viscoelasticity of the layer). Therefore, a combination of changes in both f and D is used to assess platelet aggregation on the sensor surfaces and results are presented in terms of both f and D . A number of overtones are recorded. Results for the 3rd overtone are presented as this overtone represents the most appropriate balance between over-sensitivity (as can be the case where the 1st overtone is used) and under-sensitivity (Δf and ΔD are

both lower with higher overtones). Data are acquired and analysed using QSoft₄₀₁TM.

5.8.2 Platelet-surface interaction under flow conditions

The QCM-D was used to measure platelet-surface interaction (platelet aggregation) in real time under flow conditions of low shear stress (<4 dyne/cm²) *in vitro* as described by Santos-Martinez et al⁽²¹⁴⁾⁽²¹²⁾. Gold quartz crystal sensors coated with study surface materials as described in section 5.3 were placed into individual flow modules (up to 4 modules were used in parallel with measurements recorded and displayed in real time) (figure 14). The system was primed with either PBS or Tyrode's salt solution depending on whether PRP, PPP and/or WPs were to be perfused (see section 5.2). Baseline frequencies and dissipation values were analysed to confirm that they lay within the manufacturer's recommended parameters. PRP, PPP and/or WPs were perfused at a rate of 100µl/min using a peristaltic microflow perfusion system (Ismatec, IMS 935). All experiments were carried out at 37°C. A block heating system (Grant QBD1, Grant instruments, UK) was used to heat samples prior to perfusion. Between experiments, the system was cleaned by perfusing with 2% sodium dodecyl sulphate (SDS) for 20 minutes and then with ddH₂O for 20 minutes.

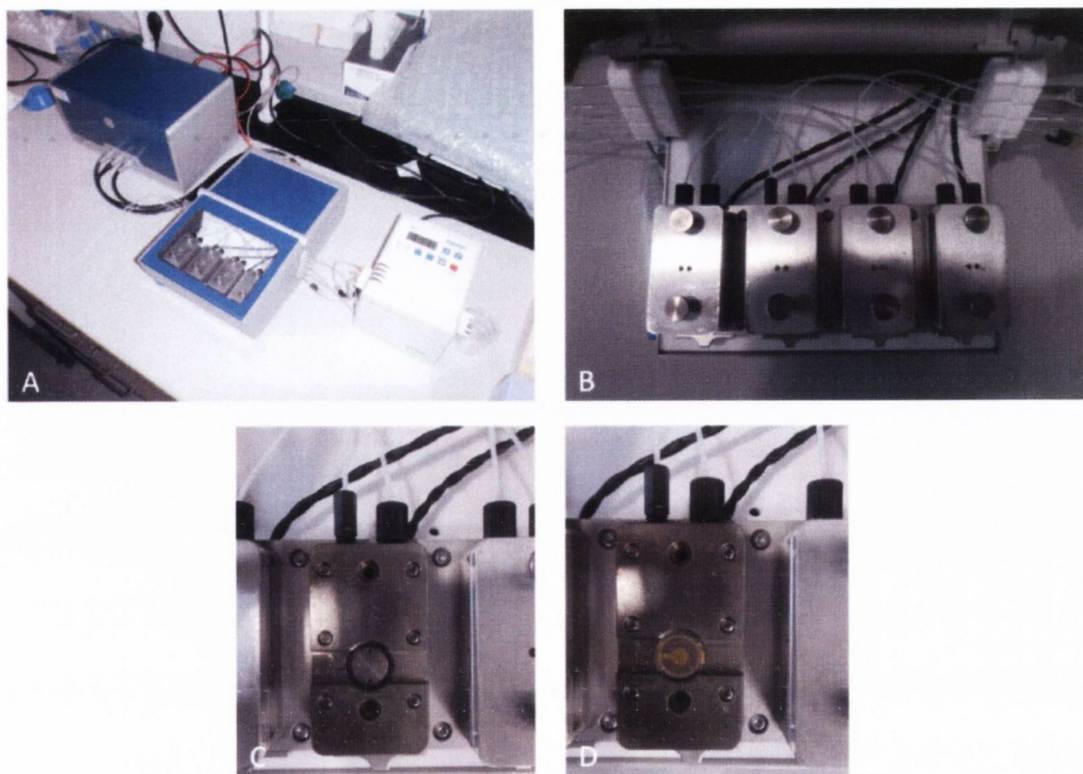


Figure 14 (A) Q-Sense QCM-D system showing control module on (left), flow modules (centre) and peristaltic pump (right); (B) Flow modules (4) *in situ*. (C) Open flow module showing o-ring onto which sensor is seated and inflow and outflow apertures; (D) Sensor seated on o-ring.

5.8.3 Protein-surface interaction under static conditions

Sensors coated with PVC +/- surface swelling with CNTs were perfused with PBS at $\times 30 \mu\text{l}/\text{min}$. Protein solutions were injected into a $100\mu\text{l}$ loop of a manual selector (Cheminert C1, VICI Valco Instruments). The loop valve was opened and QCM-D measurements began. Once the protein solution arrived at the sensor the flow was stopped 1 minute later and measurements were of changes in f and D made under static conditions for a period of 20 minutes. Only one flow module was used at any time. Experiments were carried out at

room temperature. Between experiments the system was cleaned by perfusing with PBS, ethanol and water.

5.9 PHASE CONTRAST MICROSCOPY

Once measurements of f and D were completed following platelet perfusion over sensor surfaces, the flow was stopped. Sensors were removed and placed on slides for examination by phase contrast microscopy using a Zeiss microscope (Axiovert 200M, UK) and 20x magnification objective. Photomicrographs were captured using a digital camera and Axiovision 4.7 Zeiss software.

5.10 SCANNING ELECTRON MICROSCOPY

5.10.1 Non-Platelet Perfused QCM-D Sample Preparation

Surface-modified quartz crystal sensors were mounted directly onto microscopy stubs. MWCNT-swelled PVC tubing was immersed in liquid nitrogen, physically shattered and then mounted directly onto microscopy stubs (to provide a clean break in the polymer tubing for side-on section examination). To investigate whether MWCNTs were present in water circulated in MWCNT-swelled PVC tubing (see section 5.17) 250 μ l of water was deposited on the surface of a SEM stub, allowed to evaporate and repeated 4 times (1ml water evaporated in total per stub). All stub-attached

samples were sputter-coated (Polaron E5100, UK) with gold/palladium prior to scanning electron microscopy (SEM) examination.

5.10.2 Platelet Perfused QCM-D Sample Preparation

Once measurements of f and D were completed following platelet perfusion over sensor surfaces, the flow was stopped. Sensors were removed and placed in wells of a 24-well plate. Samples were fixed using 2.5% gluteraldehyde for 30 minutes at 37°C and then dehydrated with ethanol 60% for 20 minutes, 80% for 20 minutes, 90% for 20 minutes, 100% for 30 minutes and 100% for a further 30 minutes.

Sensors were then prepared for SEM as per section 5.10.1.

After *in vivo* blood perfusion, PVC tubing was stored in 2.5% gluteraldehyde to fix cells. The tubing was cut into small sections, attached directly to microscopy stubs and sputter coated with gold/palladium.

5.10.3 Microscopy

Either a Zeiss Supra (Zeiss, Germany), Tescan Mira XMU (Tescan, Czech Republic) or a Philips XL30ESEM (Philips, Netherlands) scanning electron microscope was used to examine samples. The Philips XL30ESEM was used in environmental mode to examine fixed (but not dehydrated) biological

material and cells on ECC tubing after *in vitro* perfusion. Images were obtained using the ET (Everhart-Thornley) detector.

SEM was performed with the assistance of Dr Heath Bagshaw (Trinity College Dublin) and Dr Kagya Amoako (University of Michigan).

5.11 PROTEOMICS BY MASS SPECTROMETRY

5.11.1 Protein-coating of sensor surfaces

PPP was perfused at a rate of 100µl/min through the QCM-D module(s) as described in section 5.8.2. Experiments were carried out at 37°C. Following perfusion with PPP, PBS was perfused for 5 minutes to remove any unbound proteins from the surface of the sensor. The sensor was then removed and placed in a well containing PBS prior to proceeding to the next step of protein stripping.

5.11.2 Protein stripping of sensor surfaces

6M urea/50mM ammonia bicarbonate, 200mM dithiothreitol (DTT)/50 mM ammonia bicarbonate, at pH 8.0, was added to each well containing a single sensor. This was incubated for 1 hour at room temperature. 200mM Iodoacetamide/50 mM ammonia bicarbonate, pH 8.0, was added and incubated in the well for 1 hour at room temperature in the dark. 200mM DTT/50 mM ammonia bicarbonate, pH 8.0, was added to consume any

unreacted iodoacetamide. The mixture was incubated for 1 hour at room temperature in the dark. 50 nM ammonia bicarbonate, 1 mM CaCl_2 (pH 7.6) was added to reduce the urea concentration to ~0.6M. Trypsin solution was added to a final ratio of 1:50 (w/w, trypsin: protein). The mixture was gently vortexed and incubated at 37°C for 16-20 h. After incubation, the solution was removed and buffer (97% water, 3% acetonitrile, 0.1% formic acid) was added to the well containing the disc. This was shaken gently for 10 minutes to remove the remaining peptides. These solutions were combined and dried down using a vacuum centrifuge before being re-suspended in 1% formic acid. Where more than one surface had been perfused in order to increase the yield of stripped proteins, the re-suspended peptides from each of these crystals were pooled at this stage. Peptides were concentrated using a Zip Tip® (Millipore, Ireland) pipette tip.

5.11.3 Mass Spectrometry

The samples were run on a Thermo Scientific LTQ ORBITRAP XL mass spectrometer connected to an Dionex Ultimate 3000 (RSLCnano) chromatography system. Tryptic peptides were resuspended in 0.1% formic acid. Each sample was loaded onto a Dionex C18 Pepmap (0.075mm internal diameter, 150mm length)/ Biobasic C18 Picofrit™ column (100 mm length, 75 mm internal diameter) and was separated by an increasing acetonitrile gradient, using a 30 min reverse phase gradient at a flow rate of 30 nL min⁻¹. The mass spectrometer was operated in positive ion mode with a capillary temperature of 200°C, a capillary voltage of 9V, a tube lens voltage of 100V

and with a potential of 1800V applied to the frit. All data was acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution mass spectrometry (MS) scan (300-2000 Dalton) was performed using the Orbitrap to select the 7 most intense ions prior to tandem mass spectrometry (MS/MS) analysis using the ion trap.

5.11.4 Analysis

TurboSEQUENT (Bioworks Browser 3.3.1 SP1; Thermo Finnigan, UK) searching the human subset of the Uniprot Swissprot database for peptides cleaved with trypsin was used. Each peptide used for protein identification met specific SEQUEST parameters, i.e. a cross-correlation values of ≥ 1.9 , ≥ 2.5 , ≥ 3.2 and ≥ 3.2 for single-, double-, triple- and quadruple-charged peptides, respectively, and a peptide probability of < 0.001 .

Proteomics work was performed with the assistance of Dr Kieran Wynn and Professor Giuliano Elia (University College Dublin).

5.12 MASS SPECTROMETRY OF ILOPROST

5.12.1 Sample Preparation

Iloprost was covalently bound to COOH-functionalised CNTs (MWCNT-Iloprost) as per figure 13, section 5.4.2. MWCNT-Iloprost was swelled into the PVC surface of PVC spin-coated QCM-D sensors as described in section 5.5.

Prior to perfusion with PRP or WP, sensors were perfused with PBS. The first 200 µl of PBS to pass over the surface was collected and analysed for the presence of free iloprost. Samples for analysis were diluted to 50% v/v with methanol.

5.12.2 Mass Spectrometry

An Accela ultra performance liquid chromatography system coupled to a LTQ Orbitrap Discovery mass spectrometer (Thermo Scientific) was used. Chromatographic separation was accomplished on a Waters Acquity HSS T3 column (150 x 2.1mm, 1.8µm) maintained at 30°C. Separation was achieved by an increasing acetonitrile gradient. The capillary temperature was maintained at 320 °C. An ion spray voltage of 3.0 arbitrary units (AU), a sheath gas of 60 AU, an auxiliary gas of 5 AU, a sweep Gas of 0 AU, a capillary voltage of -5 AU and a tube lens of -73 AU were set.

5.12.3 Analysis

Thermo Scientific Xcalibur software (version 2.1) was used to control the liquid chromatography system and mass spectrometer. Data analysis and assay performance was evaluated using the same software. A calibration curve was constructed by plotting the analyte-to-internal standard ratio versus a known concentration of Iloprost using linear regression analysis (least squares method).

Mass spectroscopy work was performed with the assistance of Mr Brian Talbot.

5.13 WESTERN IMMUNOBLOTTING

5.13.1 Sample Preparation

Western immunoblotting was performed to detect the presence of clusterin, fibronectin and vitronectin in PPP from healthy volunteer donors. Platelet poor plasma was prepared as per section 5.2. Total protein content was measured using the Bradford assay⁽²¹⁵⁾. PPP was diluted with ddH₂O to ensure that an equal quantity of protein was placed into each electrophoresis well. Antibodies were purchased from Abcam, UK: Anti-Clusterin (Anti-Apolipoprotein J) mouse monoclonal antibody (abcam ab16077); Anti-Fibronectin Mouse Monoclonal Antibody (Abcam ab6328); Anti-Vitronectin Mouse Monoclonal Antibody (Abcam ab11591); Rabbit Polyclonal Antibody to Mouse IgG (Abcam ab6728-1).

5.13.2 Process

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using gel acrylamide concentrations of 12% (clusterin, vitronectin) and 8% (fibronectin) at 150V for 90 minutes in a Bio-Rad (Bio-Rad, US) electrophoresis system. After SDS-PAGE, proteins were transferred to membranes using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, US) at 25V for 30 minutes. Membranes were gently rocked in

blocking buffer (10mM TrisHCl, 100 mM NaCl, 0.1% Tween at pH 7.4 + 5% w/v non-fat dry milk) for 1 hour. The membranes were then incubated with primary antibodies (anti-clusterin 1:2000; anti-fibronectin 1:2000; anti-vitronectin 1:1500) and gently rocked for 16 hours at 4 °C. After washing 4 times for 5 minutes, secondary antibodies (rabbit polyclonal antibody to mouse IgG, 1:5000) were added and the membranes were incubated for 1 hour at room temperature while gently rocking. The membranes were washed a further 4 times for 5 minutes. Immobilon™ chemiluminescent western horseradish peroxidase substrate (Millipore, Ireland) was added to each blot and then imaged in a chemiluminescence imager (Bio-Rad Gel Doc, USA).

5.14 RABBIT MODEL OF EXTRACORPOREAL CIRCULATION

5.14.1 Preparation of ECC Circuits

ECC circuits were prepared as described by Skrzypchak *et al*⁽¹⁵⁷⁾. Two 15cm lengths of PVC tubing (Tygon R3603, Fischer, Dublin), with an internal diameter of 6.35mm and one 7cm length of tubing with an internal diameter of 0.53mm were prepared as per figure 15. These pieces were assembled as per figure 16 using THF to weld the tubing together. The central, larger internal diameter piece of tubing serves as a “thrombogenicity chamber” where laminar flow is disrupted (to mimic the non-laminar flow conditions in and in-line oxygenator or filter). PVC male luer-lock to barb connectors were attached to both ends (Qosina, US). A 16 gauge angiocath (Jelco®, Johnson & Johnson, US) was connected to one end and a 14 gauge angiocath to the

other (figure 16). The same procedure was followed for CNT-swelled PVC tubing.



Figure 15 Cut lengths of PVC tubing

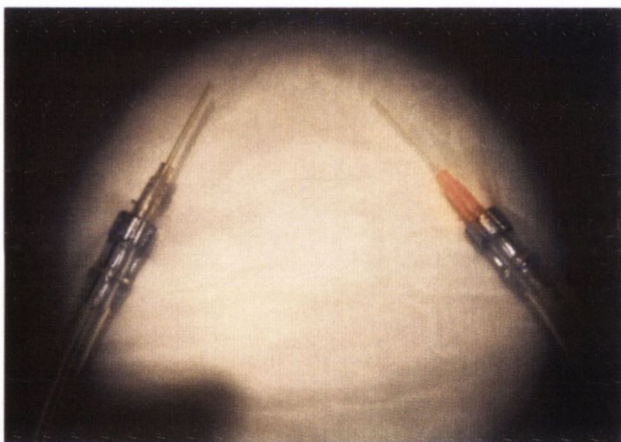
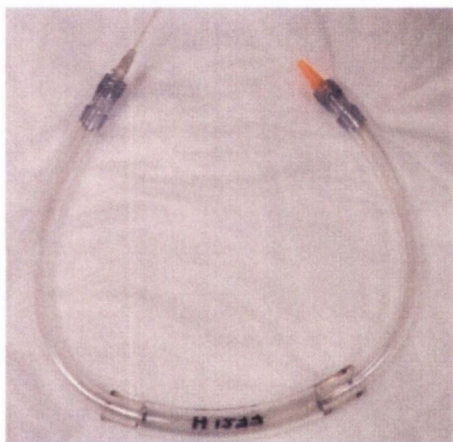


Figure 16 Assembled ECC circuit (left) with close-up of attached angiocaths (right)

5.14.2 Process of ECC

New Zealand white rabbits between 2.5kg and 3.5kg were purchased from Myrtle's Rabbitry, Thompson's Station, Tennessee, US and housed at the University of Michigan animal facility. Ethical approval was granted by the University Committee on the Use and Care of Animals in accordance with

university and federal regulations; all animal handling and surgical procedures were approved and all personnel involved in animal experimentation underwent appropriate training in animal welfare prior to the conduct of any experiments. The minimum number of animals required was used based on the principles of replace, reduce and refine. 14 rabbits in total were used for this study. All procedures were conducted under general anaesthesia and the experiments were non-recovery. One animal was used per circuit tested.

Intramuscular anaesthesia using 5 mg/kg xylazine (AnaSed®, Lloyd Laboratories, Iowa, US) and 30 mg/kg ketamine hydrochloride (Hospira Inc. Illinois, US) was given to allow hair from the neck area to be clipped, positioning of the rabbit in the supine position and intravenous access to be gained in the right ear vein. Ketamine infusion of 2.6 mg/kg/hour was commenced once venous cannulation was successful. A second intravenous cannula was placed in the left ear to allow the delivery of fluids (Lactated Ringer's Solution) at a rate of 10mls/kg/hour.

2% lidocaine was injected subcutaneously in the anterior neck. A midline incision of 6cm was made and the trachea was exposed using blunt dissection. A tracheotomy was formed through which an endotracheal tube was placed and secured (figure 17). Mechanical ventilation was instituted using a Sechrist Infant Ventilator Model IV-100 (Sechrist, California, US) set to deliver 14 breaths per minutes with a peak inspiratory pressure of 18 mmHg, positive end-expiratory pressure of 5 mmHg and an inspiratory time of 0.7 seconds. Once mechanical ventilation had commenced, the muscle relaxant

pancuronium 0.33 mg/kg was administered and repeated every 30 minutes thereafter. Anaesthesia was maintained by continuous ketamine infusion throughout the procedure. A rectal temperature probe was placed and body temperature was maintained at 37 °C using a water-jacketed heating blanket.

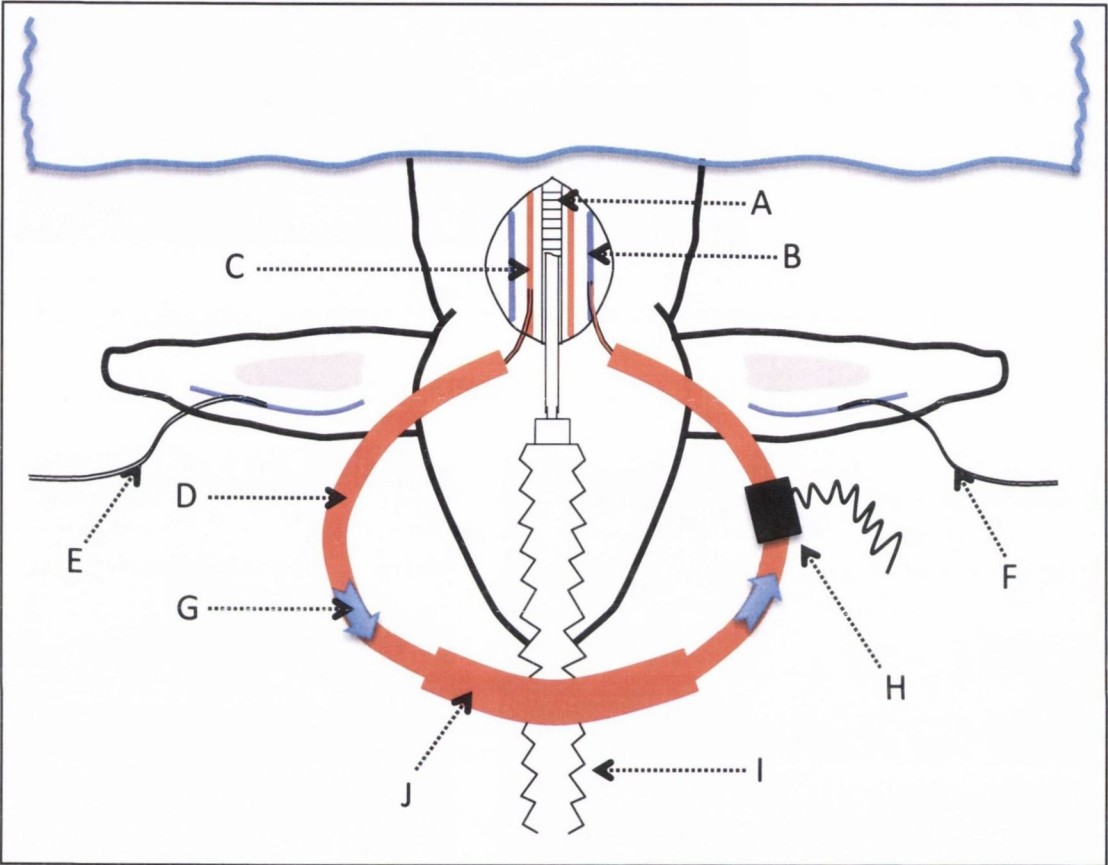


Figure 17 Graphical representation of ECC set-up. (A) Trachea; (B) Right internal jugular vein; (C) Left internal carotid artery; (D) Inflow limb of ECC circuit; (E) intravenous fluid administration; (F) Intravenous anaesthesia administration; (G) Direction of blood flow in ECC circuit; (H) Blood flow monitor; (I) Ventilator tubing; (J) Thrombogenicity chamber.

Blunt dissection was used to reveal the right and left internal carotid arteries and the right internal jugular vein. A 16 gauge angiocath (Jelco®, Johnson & Johnson, US) was placed in the right carotid artery and was connected to an arterial pressure transducing set for continuous blood pressure and heart rate

monitoring (Series 7000 Monitor, Marquette Electronics Wisconsin, US) and intermittent blood sampling. Samples of blood for baseline arterial blood gas (ABG) analysis (pH, PaO₂, PaCO₂, total haemoglobin (Hb) concentration), platelet count, white blood cell count (WCC), platelet aggregometry, fibrinogen concentration, activated clotting time (ACT) and flow cytometry analysis were withdrawn. Blood sampling was repeated hourly except for flow cytometry where only baseline and 4 hour samples were withdrawn. Ventilation was adjusted to maintain a PaCO₂ of 30-35 mmHg.

The ECC circuit was filled with normal saline 0.9%, de-aired and clamped. The 16G cannula was inserted into the left internal carotid artery and secured. The 14 cannula was placed in the right internal jugular vein and secured. A Doppler flow meter (Transonic HT207, US) was attached to the circuit tubing and calibrated to read 0 mls/min. The clamps from the ECC circuit were then removed and ECC began.

For heparin experiments, animals were anticoagulated with an initial bolus of 400 units/kg heparin sodium (APP Pharmaceuticals, US) to a target ACT of >400 seconds. Heparin 100 units units per hour was continuously infused for the duration of the experiment. Where the ACT fell to <400 seconds at any stage, a further 200 units/kg was administered as a bolus and the ACT re-checked.

After 4 hours of ECC (or less in the case where flow in the ECC circuit reduced to 0 indicating complete circuit thrombosis) the circuit was clamped.

For non-heparinised animals, a dose of 400 units/kg heparin sodium was administered to prevent necrotic thrombosis and misinterpretation of necroscopy findings. Euthanasia was performed by administering a bolus of sodium pentobarbital 130mg/kg (Vortech Pharmaceuticals, US). The ECC circuit was removed, unclamped and flushed gently with 60 mls of normal saline 0.9%. The circuit thrombogenicity chamber was photographed and the area of thrombus remaining was determined using Image J imaging software from the National Institutes of Health (Maryland, US). Gross necroscopy was performed after euthanasia specifically to examine the lungs, heart, liver and spleen for evidence of thrombosis (thromboembolic events from the ECC).

5.14.3 Blood Sampling & Analysis

Whole blood was withdrawn into 1 ml syringes containing 40 units/ml heparin sodium for ABG analysis. Arterial blood gas analysis was performed using an ABL 725 blood gas analyser (Radiometer, Denmark). Whole blood was withdrawn into 3 ml syringes containing the anticoagulant acid citrate dextrose (ACD) (9 parts blood : 1 part ACD) for platelet count, WCC, platelet aggregometry, fibrinogen concentration and fluorescent-activated cell sorting (FACS) analysis. Platelet and WCC were measured on a Coulter Counter Z1 (Coulter Electronics, US). Platelet function was assessed using a Chrono-Log optical aggregometer model 490 (Chrono-log, US). PRP was incubated for 10 min at 37 °C after which 40µg/ml collagen (Chrono-PAR #385, US) was added. The percentage of aggregation was determined 3 min after the addition of collagen using Chrono-Log Aggrolink software. Fibrinogen

concentration was measured using a Dade Behring BCS Coagulation Analyser (Siemens, US). Platelet P-selectin and monocyte CD11b expression were determined using a (FACS) with a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, US). Whole blood was withdrawn into non-anticoagulated 1 ml syringes for ACT measurement using a Hemochron Blood Coagulation System Model 801 (International Technidyne Corp., US).

5.15 FLOW CYTOMETRY

5.15.1 Platelet Preparation

Flow cytometry was used to determine the expression of platelet P-selectin (CD62P) and CD61 (IIIa subunit of GPIIb/IIIa). Aliquots of 100µl whole blood diluted 1:100 with Hank's Balanced Salt Solution (HBSS) (without CaCl₂ and MgCl₂) were prepared. 40 µg/ml collagen (Chrono-Par 385) was added to half of the samples and 4µl saline was added to the other half (table 3). Saturating concentrations (10 µl) of monoclonal anti-human IIIa fluorescein isothiocyanate (FITC) (Serotec #MCA728F), monoclonal anti-human CD62P R-phycoerythrin (PE) antibodies (Serotec #MCA2420PE), were added as per table 3 as were anti-mouse IgG1 FITC (Serotec #MCA928F) and PE-mouse IgG1 negative control (Serotec #MCA928PE) nonbinding isotype controls and allowed to incubate for 15 minutes at room temperature in the dark. 10µl PRP was added to 100µl HBSS as a control for the detection of platelets. 700 µl of freshly prepared 1% formaldehyde buffer in Dulbecco's Phosphate-Buffered Saline (dPBS) was added and samples were stored at 4 °C.

5.15.2 White Blood Cell Preparation

To determine the expression of monocyte CD11b and CD14, whole blood was divided into 100µl aliquots. Saturating concentrations (10 µl) of rat anti-mouse CD11b Alexa Fluor 488 (Serotec #MCA74A488) and monoclonal anti-human CD14 PE antibodies (Serotec #MCA1568PE) were added as per table 4 as were anti-rat IgG2b Alexa Fluor 488 (Serotec #MCA1125A488) and anti-mouse IgG2a PE (Serotec #MCA929PE) nonbinding isotype controls. These were incubated for 30 minutes at 4 °C in the dark. After incubation, red blood cells were lysed by adding 2 ml of FACS Lysing Buffer (Becton Dickinson, US), gently vortexing and then incubating for 10 minutes at room temperature in the dark. The samples were centrifuged at 250g for 5 min at 4 °C to form stained leukocyte pellets. The supernatant was aspirated, the sample was washed with wash buffer containing dPBS, 0.1% sodium azide and 0.5% bovine serum albumin, the sample was centrifuged again and the supernatant was aspirated. The cells were then re-suspended in 250 ml of freshly prepared 1% formaldehyde buffer and stored at 4°C.

5.15.3 Flow Cytometry Process

A Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, US) was used for the acquisition of data and CellQuest software (Becton Dickinson, US) was used for data analysis. Cell populations were identified by their forward scatter (FSC) and side scatter (SSC) light profiles. 30,000 events were analysed per sample. The Fluorescence intensity was quantitated by

histogram log plot analysis. Mean fluorescent intensity (MFI) was expressed as the geometric mean channel fluorescence minus the isotype control.

	Baseline				Post 4 hours on ECC				Baseline
	4µl Saline	4µl Collagen (40µg/ml)	4µl Saline	4µl Collagen (40µg/ml)	4µl Saline	4µl Collagen (40µg/ml)	4µl Saline	4µl Collagen (40ug/ml)	10µl PRP Plus 100µl HBSS
Sample No.	1	2	3	4	5	6	7	8	9
FITC-mouse IgG1 negative control	+	+	-	-	+	+	-	-	-
PE-mouse IgG1 negative control	+	+	-	-	+	+	-	-	-
FITC-mouse antihuman CD61	-	-	+	+	-	-	+	+	-
PE- mouse antihuman CD62P	-	-	+	+	-	-	+	+	-

Table 3 Sample preparation for flow cytometry (Platelet CD61 and CD62P expression) (from T Major’s protocol, University of Michigan)

	Baseline		Post 4 hours on ECC	
Sample No.	10	11	12	13
A488-mouse IgG2b negative control	+	-	+	-
PE-mouse IgG2a negative control	+	-	+	-
A488-rat antimouse CD11b	-	+	-	+
PE-mouse antihuman CD14	-	+	-	+

Table 4 Sample preparation for flow cytometry (White Blood Cell CD11b and CD14 expression) (from T Major’s protocol, University of Michigan)

5.16 FIBRINOGEN ADSORPTION ELISA

5.16.1 Preparation of PVC-coated Microwells

PVC was dissolved in THF (5% w/v) and added to individual polypropylene microtiter wells (96-well microtiter plates (Nalge Nunc #437111, black). The THF was allowed to evaporate overnight with resultant thin, smooth PVC films adherent to the well walls. Half of the plates were placed in a solution of Toluene with 0.5% w/v CNTs where the PVC was swelled as previously described in section 5.5.

5.16.2 Protein adsorption

Serotec BLOCK ACE (#BUF029) was reconstituted by adding 100ml distilled water to each 4g vial. This was diluted a further 10-fold and 0.05% Tween 20 was added to prepare wash buffer. The reconstituted solution was diluted 4-fold to prepare blocking buffer and 10-fold to prepare antibody diluent.

Human fibrinogen (Calbiochem #341576) was diluted in dPBS to a final concentration of 3 mg/ml. 100 μ L of 3 mg/ml fibrinogen solution was added to each polymer-containing microtiter well and incubated for 1.5 hours at 37°C. Each plate was washed 8 times using wash buffer. Wells were incubated with 100 μ L Blocking buffer for 30 minutes at 37°C to block nonspecific antibody binding. Each plate was washed a further 3 times with wash buffer.

A background measurement of the plates was performed at 485/20 nm (excitation) and 528/20 nm (emission).

100 μ L goat anti human fibrinogen FITC (MP Biochem #55169 CAPPEL, US) was diluted 10-fold in diluent buffer, added to each well and allowed to bind to surface-adsorbed fibrinogen for 1.5 hours at 37°C. Each plate was then washed 3 times using wash buffer.

5.16.3 Immunofluorescence

A Biotek Synergy 2 fluorescence reader was used. As mentioned above, a background measurement of the plates was performed at 485/20 nm (excitation) and 528/20 nm (emission). Human fibrinogen adsorption to non-coated polypropylene was used as an internal control to normalise the fluorescence signals within different plates.

5.17 *In vitro* ECC PERFUSION

In order to test for the presence of dislodged carbon nanotubes from PVC-CNT swelled ECC circuits, circuits were perfused with 1000 ml of sterile water at a flow rate of 200 ml/minute for 4 hours. This was accomplished by connecting the ECC circuit to PVC tubing attached to a Stockert Shiley Roller Pump and using a 1L bottle of sterile water as a reservoir (figure 18). Samples of water were withdrawn from the circuit at 4 hours and examined by SEM for the presence of CNTs as per section 5.10.1.

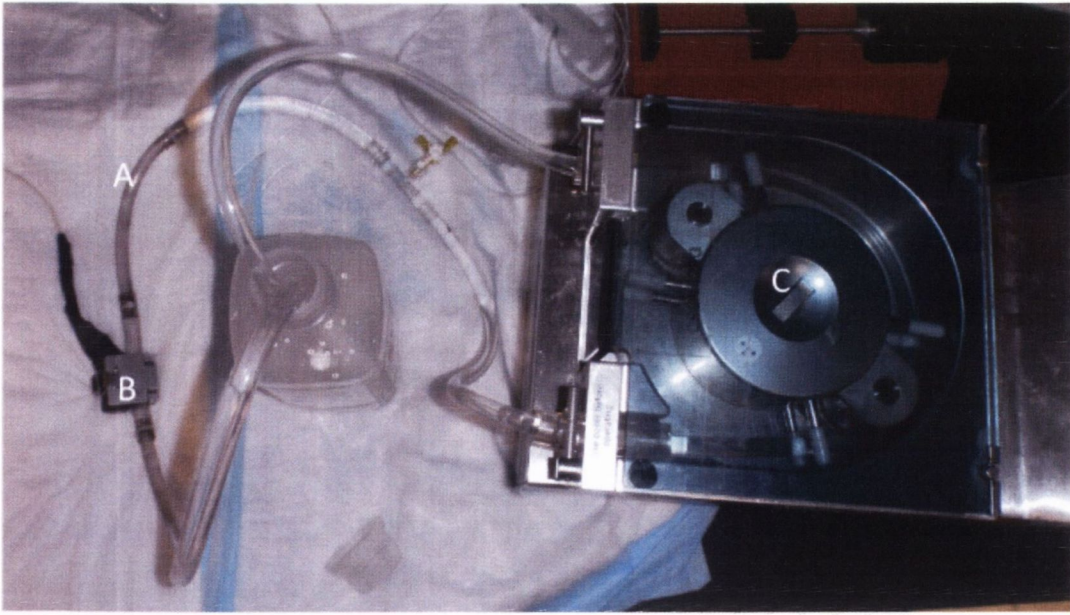


Figure 18 *In vitro* ECC perfusion. A PVC-CNT swelled ECC circuit; B Flow meter; C Roller pump.

6 STATISTICS

QCM-D results are expressed as frequency shift from baseline f (Hz) and dissipation shift from baseline D (1E6) (from the 3rd overtone) or relative shifts from baseline from at least three independent experiments (except for protein adsorption QCM-D measurement where 2 independent experiments were performed for each protein and concentration). Platelet aggregometry experiments are expressed as percentage aggregation or relative aggregation rather than percentage light transmission or relative light transmission. All means are reported with standard deviations or errors. The data were analysed using GraphPad Prism 5 software. Paired and un-paired Student's *t*-tests, one-way analyses of variance, repeated measures analyses of variance and Tukey–Kramer's multiple comparisons post tests were performed as appropriate. Statistical significance was considered where $P < 0.05$.

7 RESULTS - PART I

7.1 Spin-coating of PVC/MWCNT solutions results in sparse surface coverage of attached MWCNTs as demonstrated by scanning electron microscopy.

Initially, sensors were spin-coated with a PVC solution (control), PVC/Straight MWCNT and PVC/Curly MWCNT solutions. Scanning electron microscopy was performed to assess the surface coverage of MWCNTs. As can be seen in figure 19, surface coverage of both straight and curly MWCNTs was sparse.

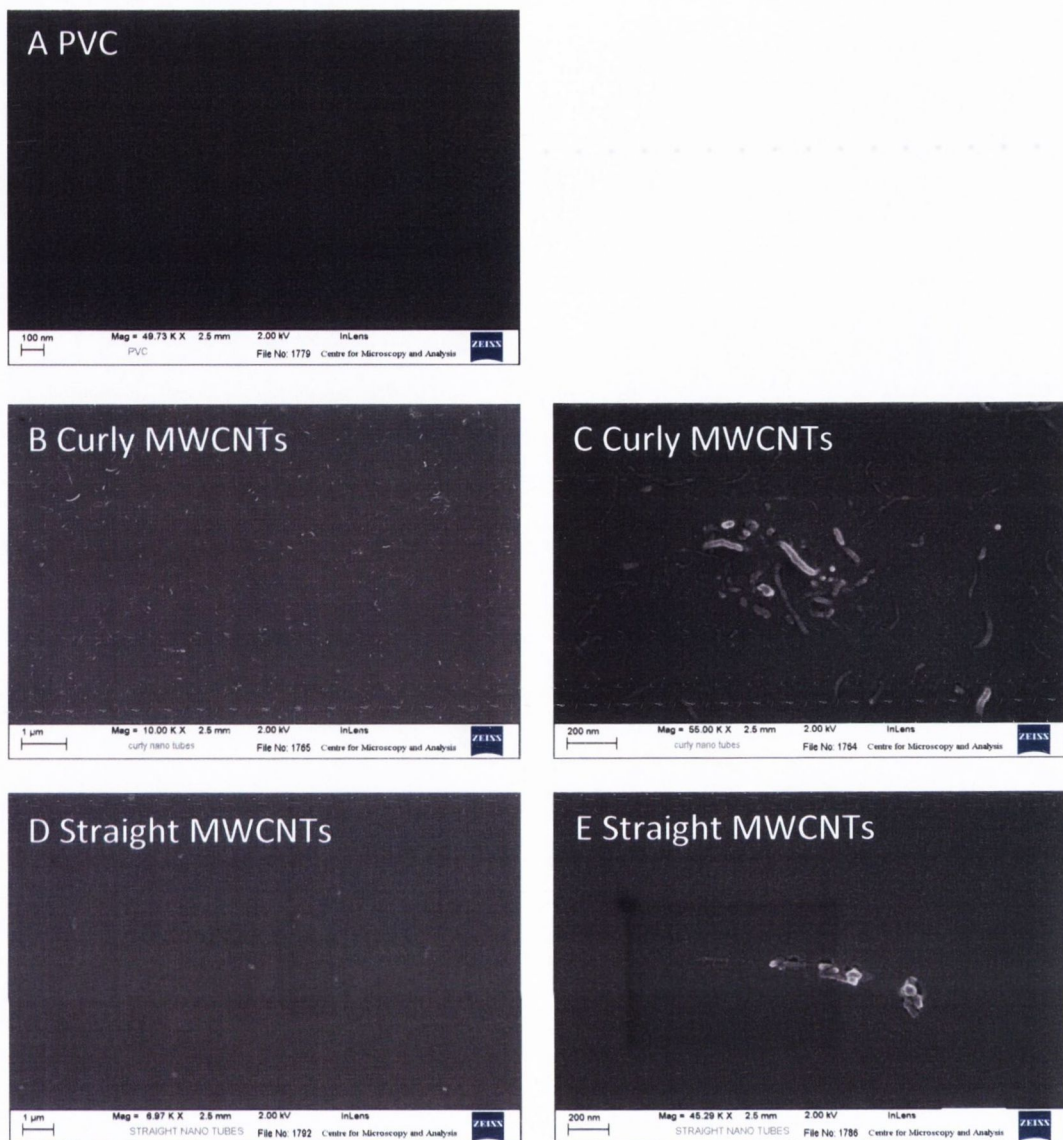


Figure 19 Scanning electron micrographs of spin-coated crystal sensor surfaces. **A** PVC; **B** Curly MWCNTs low magnification; **C** Curly MWCNTs high magnification; **D** Straight MWCNTs low magnification; **E** Straight MWCNTs high magnification. Although there appears to be a higher surface coverage of attached curly MWCNTs compared to straight MWCNTs, overall there is low surface coverage of both.

7.2 There is no difference in surface platelet aggregation between PVC spin-coated surfaces, PVC/Straight MWCNT spin-coated surfaces and PVC/Curly MWCNT spin-coated surfaces as demonstrated by QCM-D.

Although surface coverage of spin-coated CNTs was sparse, PRP at a concentration of 210,000 platelets/ μ l was perfused across PVC spin-coated sensor surfaces, PVC/Straight MWCNT spin-coated surfaces and PVC/Curly MWCNT spin-coated surfaces to assess for differences in platelet aggregation on these surfaces. There were no differences found in terms of f or D between any of the surfaces, either control or experimental. The following is a representative Q-Sense experimental output (figure 20) and statistical analysis of the results (figure 21):

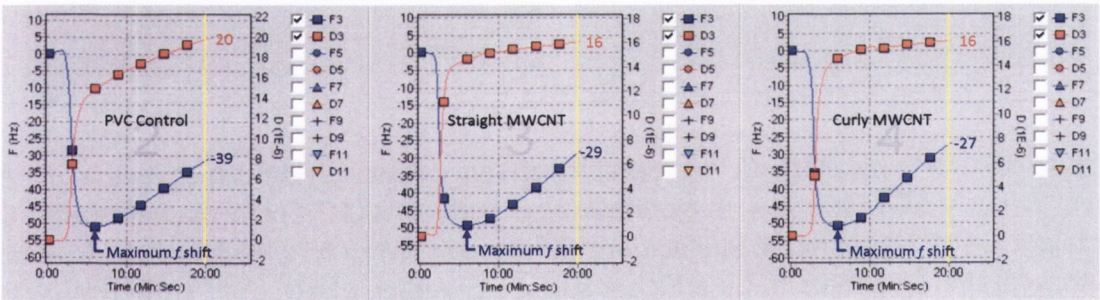


Figure 20 Representative example of a Q-sense experimental output comparing PVC spin-coated control surfaces, PVC/Straight MWCNT spin-coated surfaces and PVC/Curly spin-coated surfaces. Frequency is in blue, dissipation in red. Two frequency shifts are used in data analysis – the shift from baseline at 20 minutes and the maximum shift from baseline between 0 and 20 minutes.

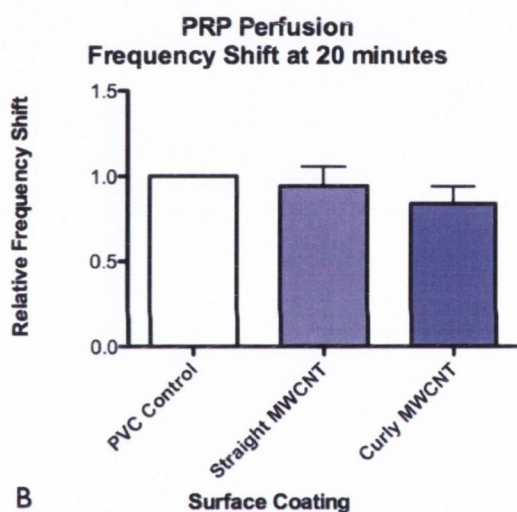
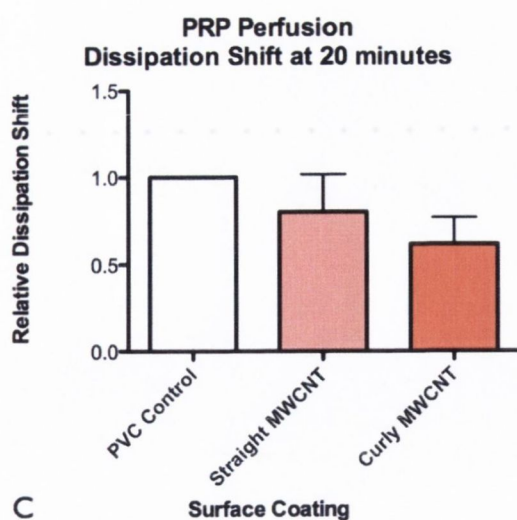
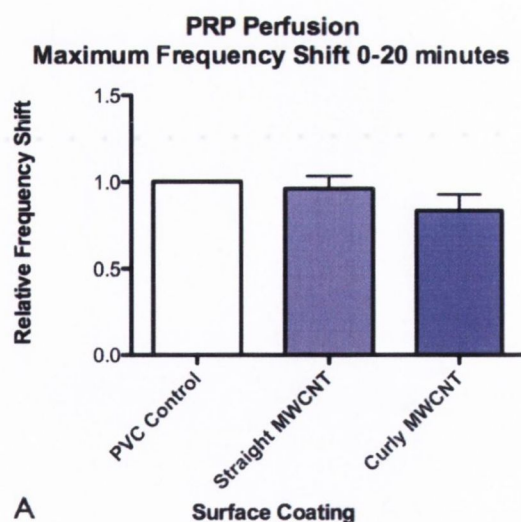


Figure 21 Matched frequency and dissipation shifts from baseline of PRP-perfused PVC/Straight MWCNT spin-coated surfaces and PVC/Curly MWCNT spin-coated surfaces relative to control PVC spin-coated surfaces. **A** shows no significant difference between the maximum frequency shifts across surfaces. **B** shows no significant difference between the frequency shifts at 20 minutes across surfaces. **C** shows no significant difference between the dissipation shifts at 20 minutes across surfaces. Data are mean $\pm$ SE, $n=3$. One-way ANOVA: $P>0.05$.

7.3 Ultrasonification and swelling of PVC with MWCNTs results in high density, confluent surface attachment of Curly MWCNTs but not Straight MWCNTs

Because of poor surface coating of MWCNTs using the spin-coating technique, a swelling technique was used in an attempt to increase the density of MWCNTs attached to PVC-coated surfaces. SEM micrographs below demonstrate that it was possible to coat surfaces with a confluent, dense matrix of curly MWCNTs. Swelling of straight MWCNTs was not successful with only occasional upright MWCNTs seen on SEM-examined surfaces. There also appeared to be a large proportion of amorphous carbon on straight MWCNT-swelled surfaces.

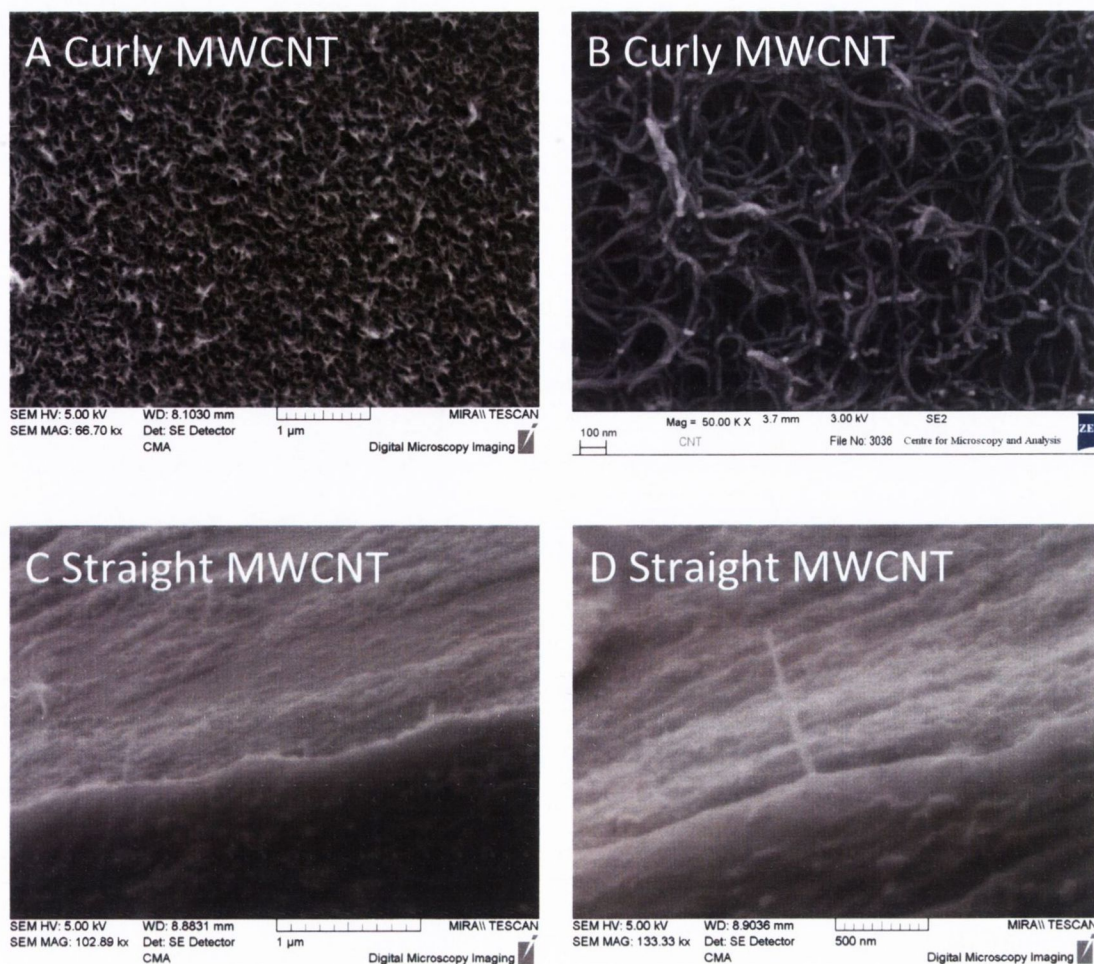


Figure 22 Scanning electron micrographs of MWCNT ultrasound-swelled PVC. **A** Curly MWCNTs low magnification; **B** Curly MWCNTs high magnification; **C** Straight MWCNTs low magnification; **D** Straight MWCNTs high magnification. Only Curly MWCNTs show high density, confluent surface attachment of MWCNTs. Straight MWCNTs are only sparsely attached to the PVC surface. Micrographs of PVC swelled with Straight MWCNTs show large quantities of amorphous carbon deposition whereas none is seen with Curly MWCNT swelling.

7.4 There is an increase in surface platelet aggregation and protein deposition on PVC swelled with curly MWCNTs compared to PVC control as demonstrated by QCM-D.

Straight MWCNT coated surfaces were not evaluated further. From this point onwards, the term MWCNT will refer to curly MWCNTs only. PRP and PPP were perfused across PVC spin-coated sensor surfaces, and MWCNT PVC-swelled sensor surfaces. There were significantly higher frequency shifts and dissipation shifts (indicating increased platelet aggregation - increased frequency shift corresponding to an increased mass on the surface of the sensor and increased dissipation shift indicating a more viscoelastic surface layer) for the MWCNT surfaces compared to the control PVC surfaces perfused with PRP. For PPP perfusion, there was a significantly higher frequency shift for the MWCNT surfaces compared to the control PVC surfaces but there was no difference in dissipation shifts between control and experimental surfaces (indicating an increase in the mass of proteins on the sensor surfaces but no change in the viscoelasticity of this layer even in the face of increased mass deposition). The following is a representative Q-Sense experimental output (figure 23) and statistical analysis of the results (figures 24 & 25):

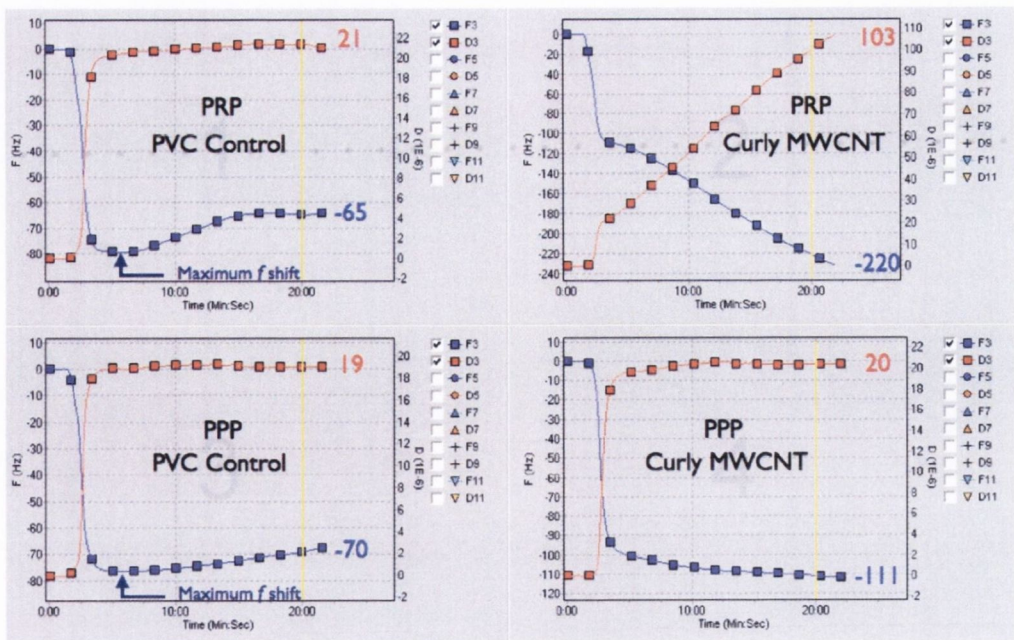


Figure 23 Representative example of a Q-sense experimental output comparing spin-coated PVC control surfaces v MWCNT PVC-swelled surfaces perfused with PRP and PPP. Frequency is in blue, dissipation in red. Two frequency shifts are used in data analysis where the maximum f shift is greater than the shift at 20 minutes – the shift from baseline at 20 minutes and the maximum shift from baseline between 0 and 20 minutes.

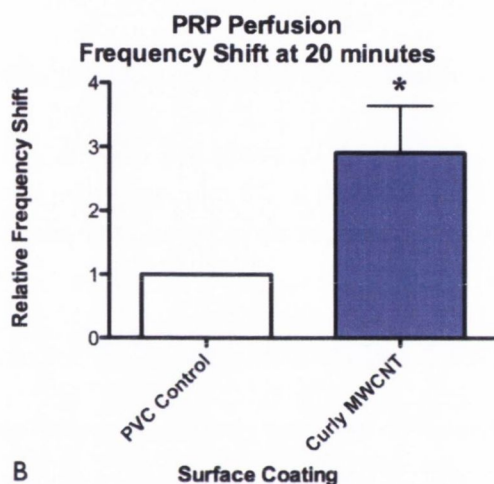
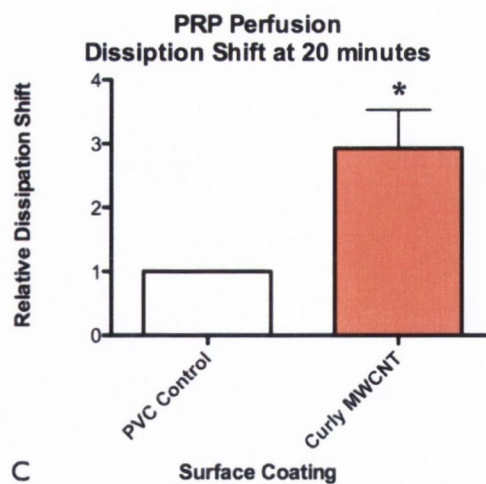
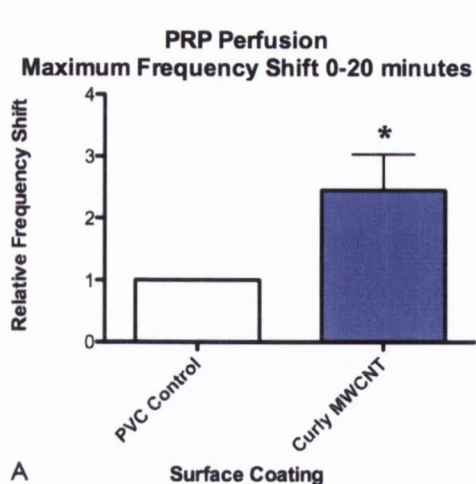


Figure 24 Matched frequency and dissipation shifts from baseline of PRP-perfused MWCNT PVC-swelled surfaces relative to PVC control surfaces. There are statistically significant differences between MWCNT surfaces and PVC control surfaces in terms of maximum frequency shift (A), frequency shift at 20 minutes (B) and dissipation shift at 20 minutes (C). Data are mean $\pm$ SE, n=8. Paired t-test: *P<0.05 compared to PVC Control.

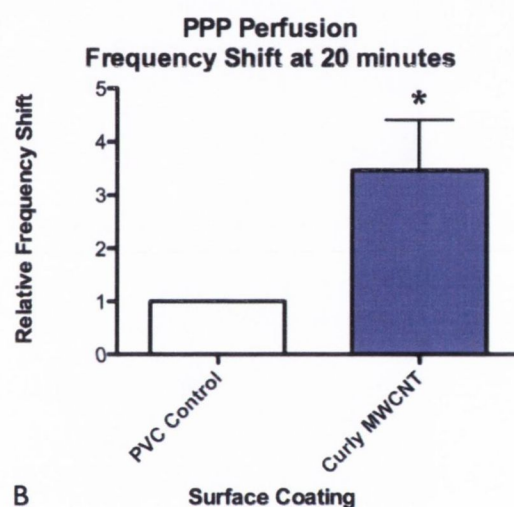
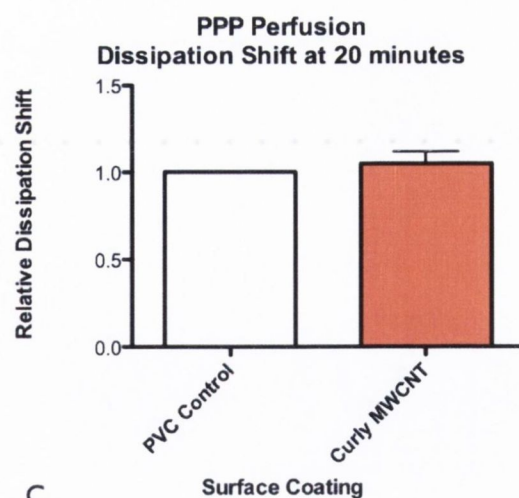
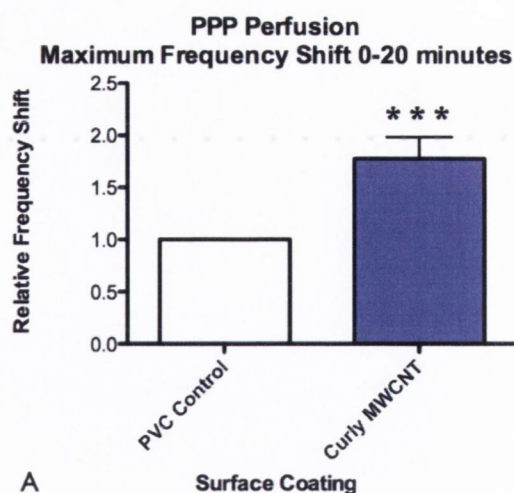


Figure 25 Matched frequency and dissipation shifts from baseline of PPP-perfused Curly MWCNT PVC-swelled surfaces relative to PVC control surfaces. There are statistically significant differences between MWCNT surfaces and PVC control surfaces in terms of maximum frequency shift (A) and frequency shift at 20 minutes (B). There is no difference between surfaces of dissipation shift at 20 minutes (C). Data are mean $\pm$ SE, n=8. Paired t-test: *P<0.05 compared to PVC Control, ***P<0.001 compared to PVC Control.

In addition to sensor perfusion with PRP and PPP, sensors were perfused with washed platelets (WP) at a concentration of 2.5×10^8 platelets/ml suspended in Tyrode's salt solution. There was a significant increase in frequency shift for the MWCNT-swelled PVC surface compared to the PVC control surface. There was no difference in dissipation shift. This is likely explained by an increase in mass of platelets on the MWCNT-swelled PVC surfaces (leading to an increase in frequency shift) but less of a change in the viscoelastic properties of the surface layer of platelets presumably due to the lack of large platelet aggregates. The following is a representative Q-Sense experimental output (figure 26) and statistical analysis of the results (figure 27):

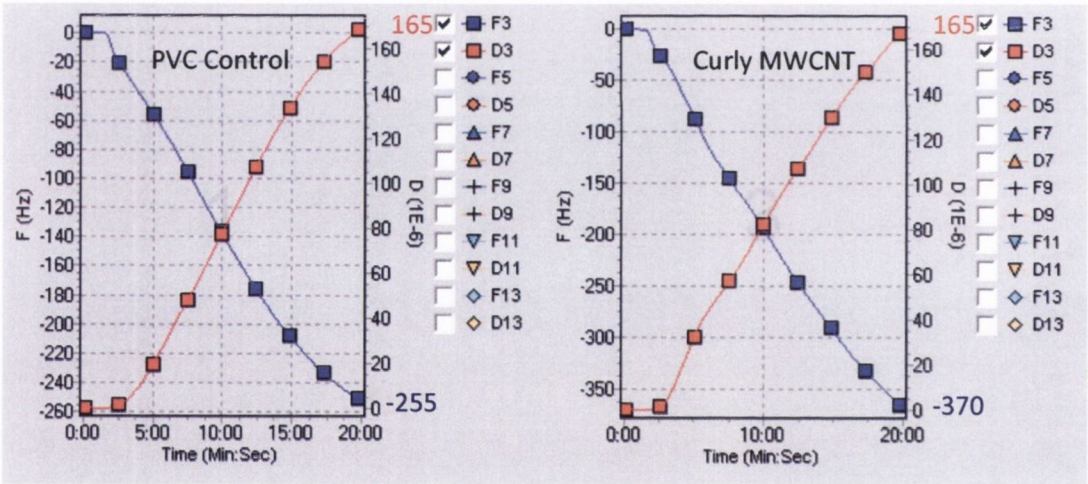


Figure 26 Representative example of a Q-sense experimental output comparing spin-coated PVC control surfaces v MWCNT PVC-swelled surfaces perfused with washed platelets (WP) at 20 minutes. Frequency is in blue, dissipation in red.

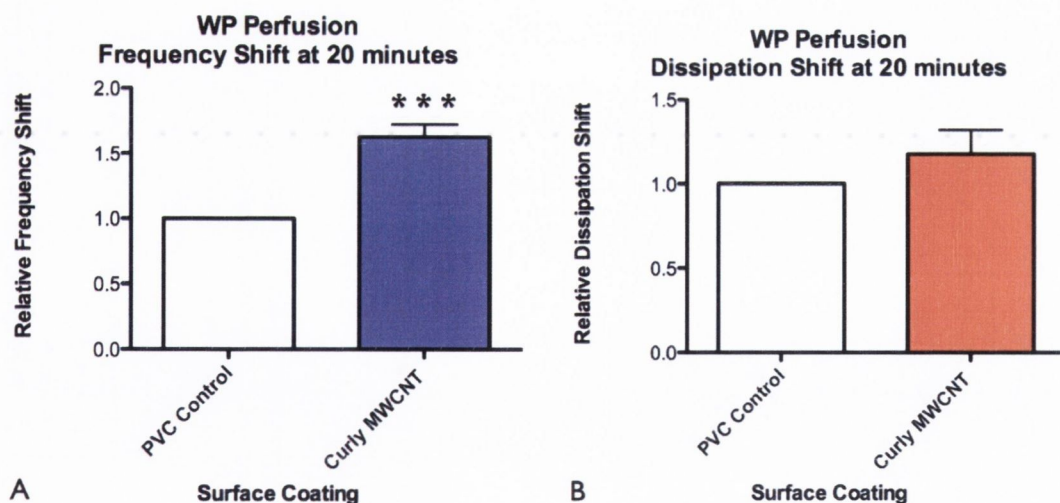


Figure 27 Matched frequency and dissipation shifts from baseline of WP-perfused Curly MWCNT PVC-swelled surfaces relative to PVC control surfaces. There is a statistically significant difference between MWCNT surfaces and PVC control surfaces for frequency shift at 20 minutes (**A**). There is no difference between surfaces for dissipation shift at 20 minutes (**B**). Data are mean $\pm$ SE, n=11. Paired t-test: ***P<0.001 compared to PVC Control.

7.5 There is qualitative confirmation of an increase in surface platelet aggregation on PVC swelled with MWCNTs compared to PVC control as demonstrated by phase contrast microscopy of PRP-perfused sensors as described in section 7.4.

After PRP perfusion of PVC control and MWCNT-coated surfaces, sensors were removed and examined by phase contrast microscopy. Where increased frequency and dissipation was noted following perfusion with PRP, phase contrast microscopy confirmed visually that there was increased platelet aggregation as expected. Phase contrast microscopy examination corresponded in all cases with the results obtained by QCM-D measurement of surface platelet aggregation (*Figure 28*).

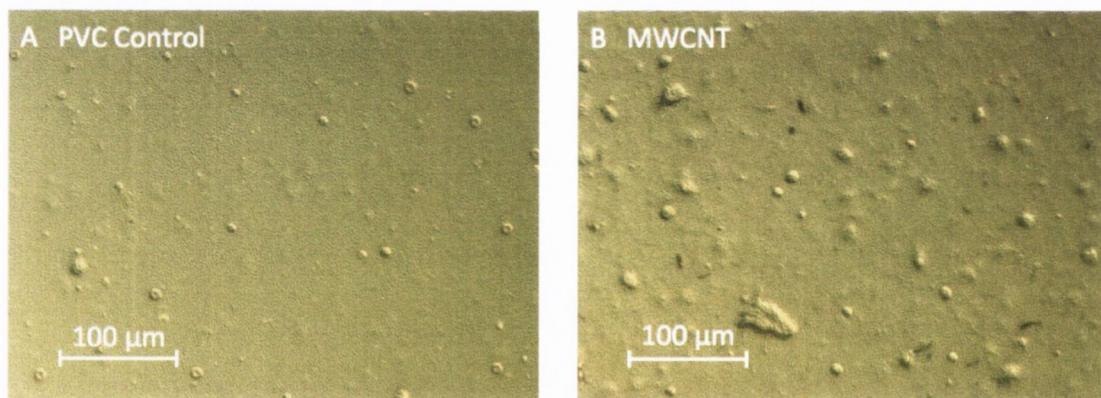


Figure 28 Confocal micrographs of sensor surfaces following perfusion with PRP. Smaller aggregates covering smaller areas are associated with smaller shifts in frequency and dissipation **A**, whereas larger aggregates covering larger areas are associated with larger shifts in frequency and dissipation **B**. This is a representative example of surfaces examined in one of eight experiments from section 7.4.

7.6 MWCNTs cause increased platelet aggregation in washed platelets as demonstrated by light aggregometry.

Light aggregometry was used to confirm the platelet aggregatory effects of MWCNTs. As expected collagen significantly increased platelet aggregation in PRP and WP (figure 29 & 30). In contrast, the aggregating effects of MWCNTs were significant only in WP (figure 29B & 30).

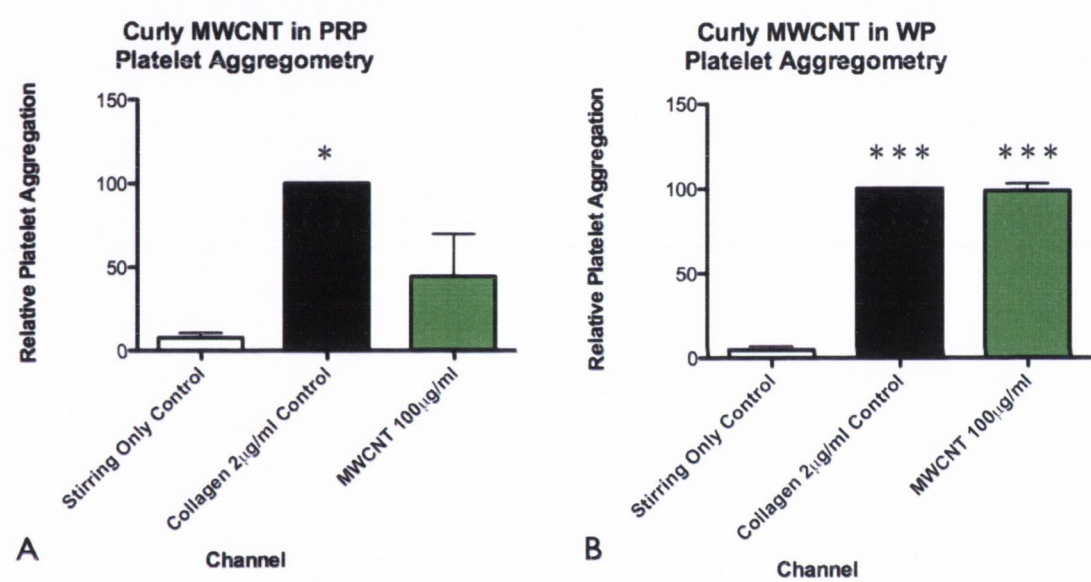


Figure 29 Light aggregometry studies of curly MWCNT 100µg added to PRP **A** and WP **B** and compared to maximum collagen-stimulated aggregation (normalised to 100%) and stirring only control. Collagen significantly increased platelet aggregation in PRP and WP. In contrast, the effects of MWCNT were significant only in WP. Data are mean $\pm$ SE, n=3. One-way ANOVA, Tukey's Multiple Comparison Test: *P<0.05 compared to stirring only control, ***P<0.001 compared to stirring only control.

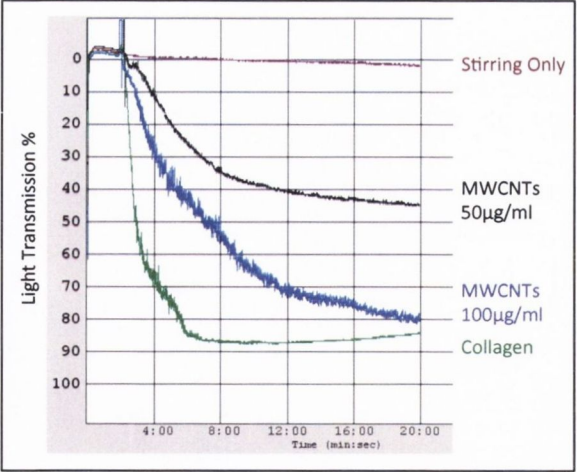


Figure 30 Light aggregometry of platelet aggregation in WP showing the effects of stirring alone (control to confirm that platelets are not aggregating spontaneously), MWCNTs 50 µg/ml, MWCNTs 100µg/ml and Collagen (control agonist to confirm maximum platelet aggregation) on washed platelets.

7.7 *In vivo* ECC with MWCNT-swelled PVC tubing results in increased circuit thrombosis and decreased flow rates compared to control PVC tubing.

All control 4 PVC ECC circuits remained patent throughout the 4-hour duration of each experiment (figure 31). Because experiments in the MWCNT group did not progress beyond 1 hour in 3 out of four animals, in-depth statistical analysis of differences in vital signs, platelet number and function, white cell count, fibrinogen concentration and ACT was not possible. Examination of the data as represented below does not suggest, however, that there were significant differences in these parameters (figures 32, 33 & 34). On the other hand, there was a significant difference in the volume of clot formed in the thrombogenicity chamber. As it was not possible to accurately determine thrombus volume, the area of thrombus in two-dimensional images of the thrombogenicity chambers of perfused ECC circuits was used as an index to compare control and experimental circuits. The area of thrombus in the MWCNT-swelled circuits was twice that of the control circuits. Importantly, the clot formed in 3 of the 4 experimental circuits was so great that circuit flow fell to zero within one hour of extracorporeal circulation (figure 31).

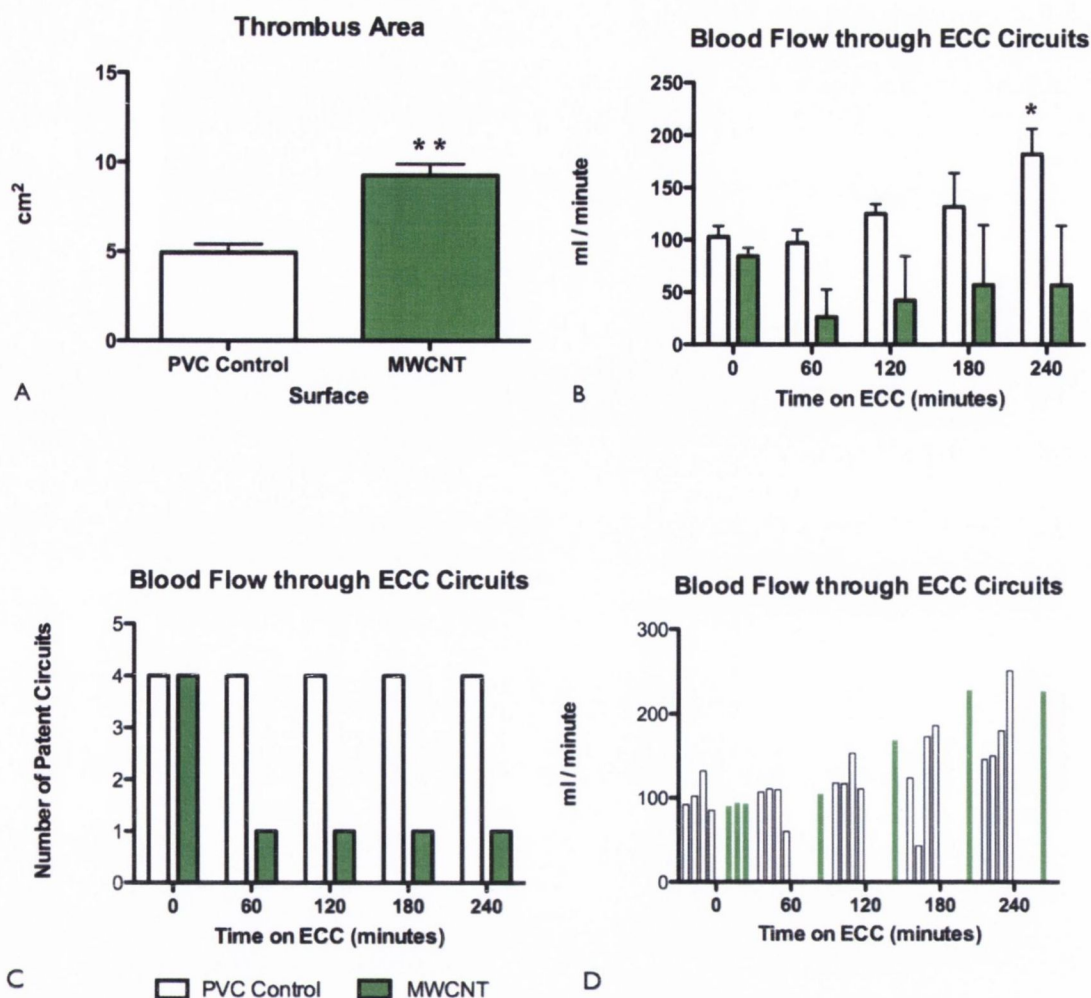


Figure 31 Thrombus formation in the thrombogenicity chamber of the ECC circuit was significantly increased in MWCNT-swelled circuits compared to PVC controls (A). Thrombus formation in MWCNT-swelled circuits led to circuit occlusion in three out of 4 circuits within 60 minutes of ECC compared to no occlusion for the 4-hour duration of the experiment in any of the 4 PVC control circuits (B), (C) and (D). In (A) data are mean $\pm$ SE, $n=4$. Unpaired t-test: ** $P < 0.01$ compared to PVC control. In (B) data are mean $\pm$ SE, $n=4$. One-way ANOVA: * $P < 0.05$. Raw data are presented in (C) and (D).

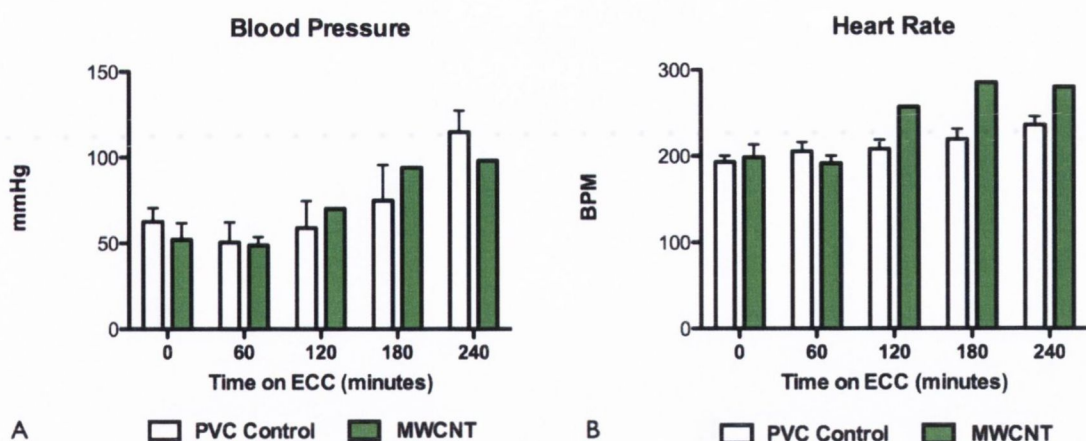


Figure 32 Vital signs were recorded continuously for the duration of each experiment. A and B show blood pressure and heart rate measurements, respectively, for each group. Data are mean $\pm$ SE, $n=4$. Statistical comparison analysis was not possible due to occlusion of 3 out of 4 MWCNT-swelled circuits.

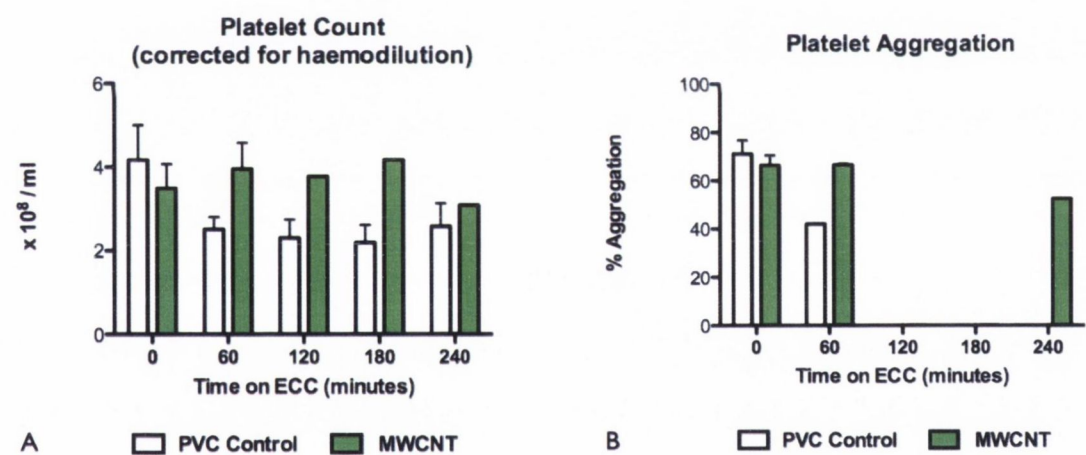


Figure 33 Blood was withdrawn for platelet count at baseline (time 0) and every hour thereafter where the ECC remained patent. Where the platelet concentration in PRP was at least 2×10^8 platelets/ml, platelet aggregometry was performed. A shows platelet counts in whole blood for control and experimental circuits over time. B shows the results of platelet aggregometry where enough sufficient numbers of platelets were present to allow aggregometry to be performed. Data are mean $\pm$ SE, $n=4$. Statistical comparison analysis was not possible due to occlusion of 3 out of 4 MWCNT-swelled circuits.

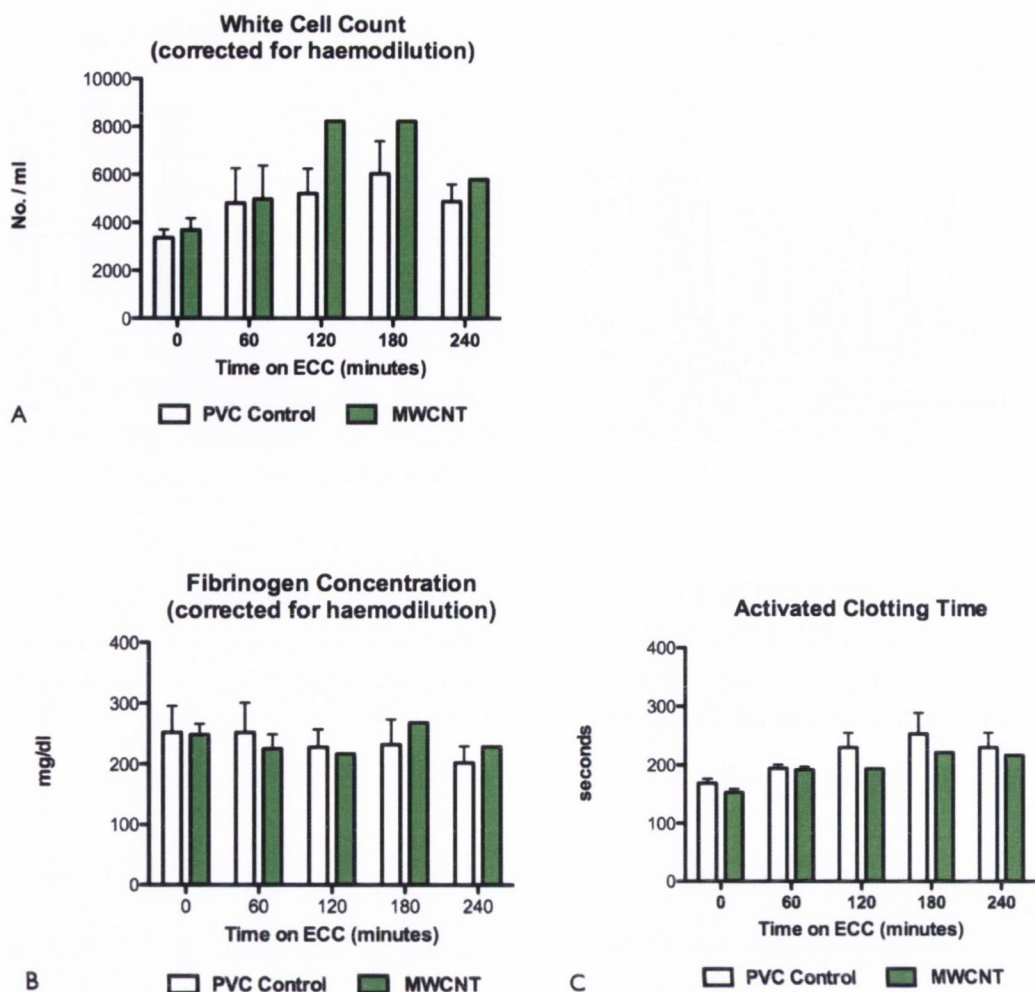


Figure 34 Blood was withdrawn for white cell count, fibrinogen concentration and activated clotting time at baseline (time 0) and every hour thereafter where the ECC remained patent. **A** shows white cell counts in whole blood for control and experimental circuits over time. **B** shows fibrinogen concentration in whole blood for control and experimental circuits over time. **C** shows the activated clotting time of whole blood for control and experimental circuits over time. Data are mean $\pm$ SE, n=4. Statistical comparison analysis was not possible due to occlusion of 3 out of 4 MWCNT-swelled circuits.

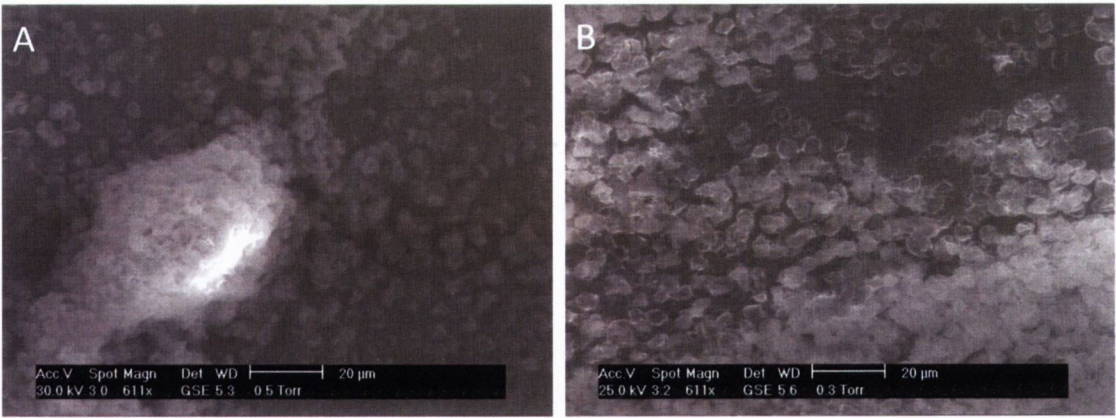


Figure 35 Representative example of SEM images of thrombus formation on a PVC ECC circuit (A) and a MWCNT-swelled circuit (B) after *in vivo* experimentation.

SEM imaging of ECC circuits after perfusion failed to show differences between blood-surface interaction on control and experimental surfaces. An example of images obtained can be seen in figure 35.

7.8 When systemic heparin is administered to both groups of animals, there are no differences between PCV control and MWCNT-swelled surface ECC circuits in any measured parameters.

In order to assess the potential effects of MWCNT-swelled PVC surfaces on platelet count, platelet function, white cell count, fibrinogen levels, platelet p-selectin and CD 61 expression and monocyte CD 11b and CD 14 expression separate to local thrombotic effects, and in order to allow all experiments to extend to 4 hours in duration, systemic heparin was administered to all animals to maintain an ACT of >400 seconds. All 3 control PVC and 3 experimental MWCNT ECC circuits remained patent throughout the 4-hour duration of each experiment and no thrombus formed in the thrombogenicity chambers of either the control or experimental surfaces (figure 36). There was no statistical difference between groups in vital signs, platelet number and function, white cell count, fibrinogen concentration platelet p-selectin expression, platelet CD 62P expression, monocyte CD 11b expression and monocyte CD 14 expression (figures 37 - 41). There was no statistical difference in the ACT achieved (on heparin) between groups (figure 39). Two baseline blood samples were taken for all experiments except flow cytometry. The first was taken just prior to heparin administration (Time 0 Pre-Heparin) and the second 3 minutes after heparin administration (Time 0 Post-Heparin).

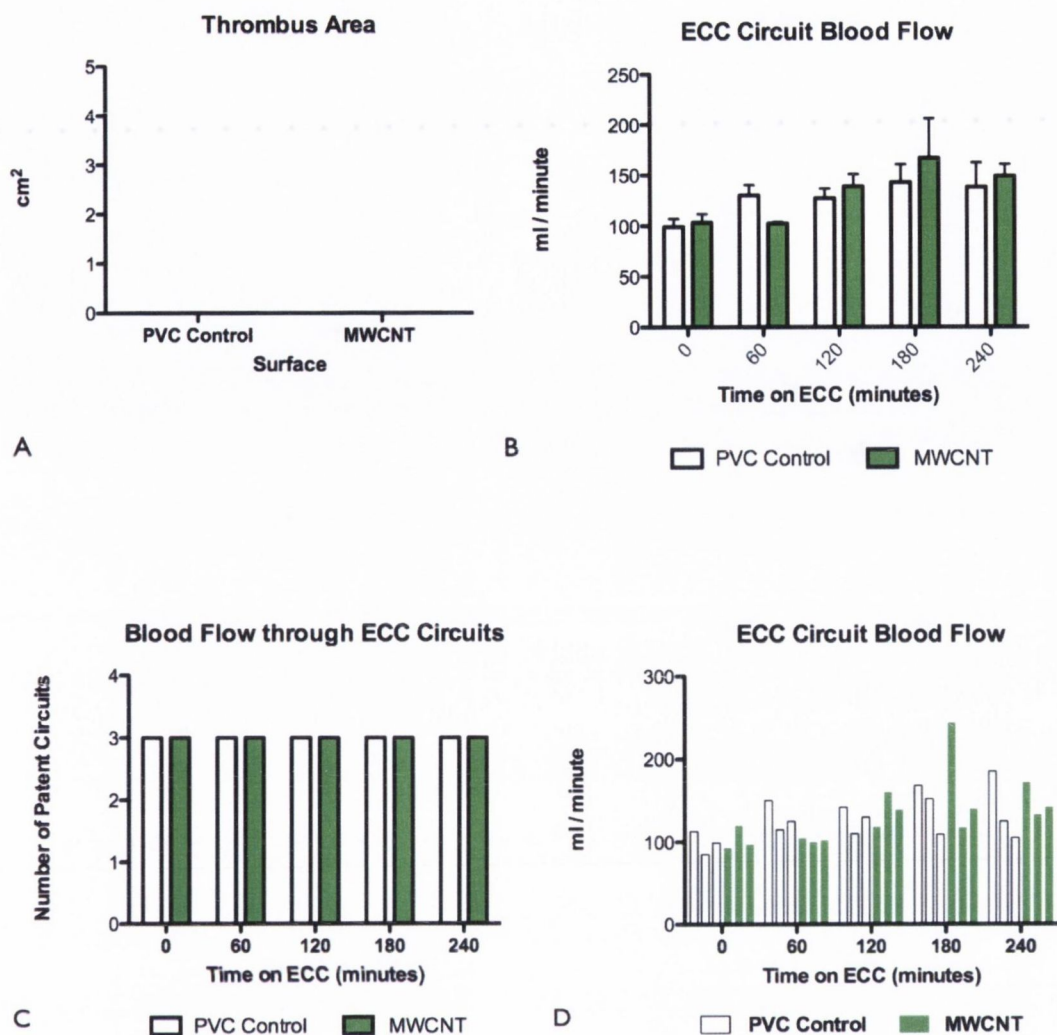


Figure 36 There was no thrombus formation in the thrombogenicity chambers of either the MWCNT-swelled circuits or the PVC control circuits (A). All circuits remained widely patent for the duration of the experiments (B), (C) and (D). In (B) data are mean $\pm$ SE, $n=3$. One-way ANOVA: $P > 0.05$. Unanalysed raw data are presented in (C) and (D).

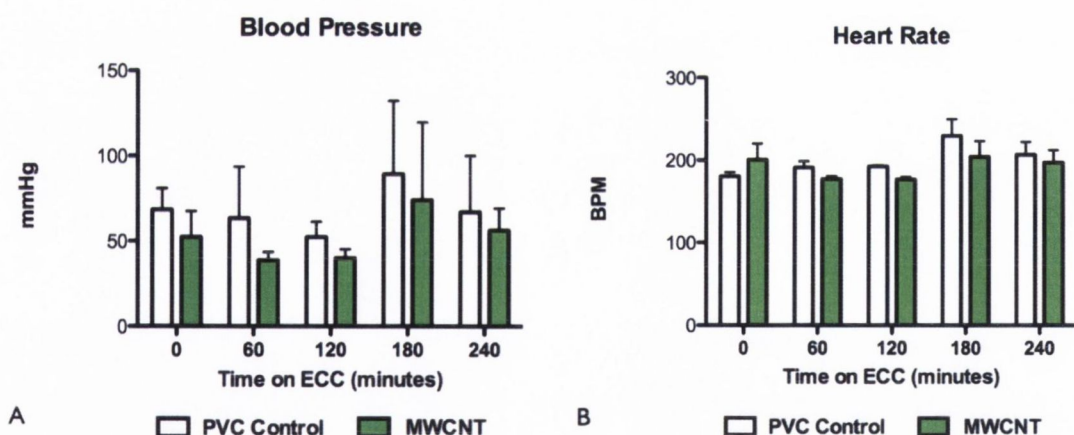


Figure 37 Vital signs were recorded continuously for the duration of each experiment. A and B show blood pressure and heart rate measurements, respectively, for each group. Data are mean $\pm$ SE, $n=3$. One-way ANOVA: $P > 0.05$.

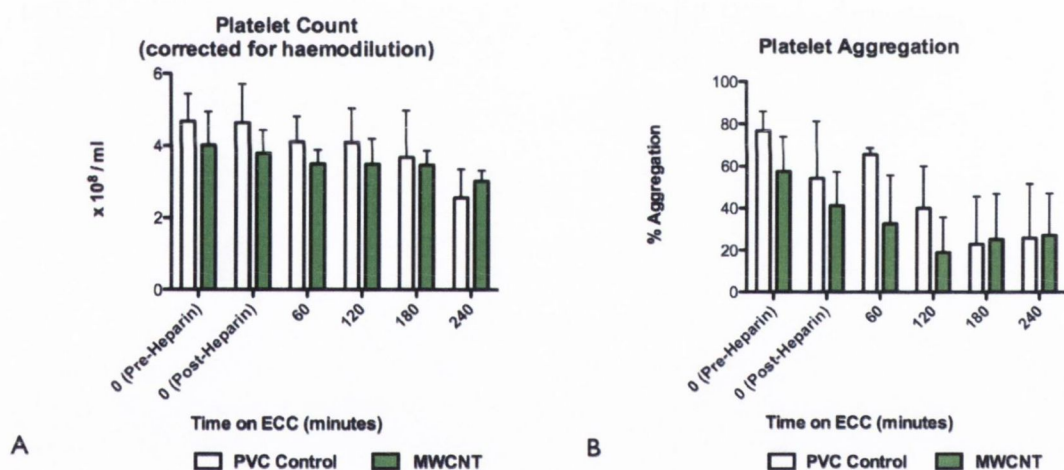
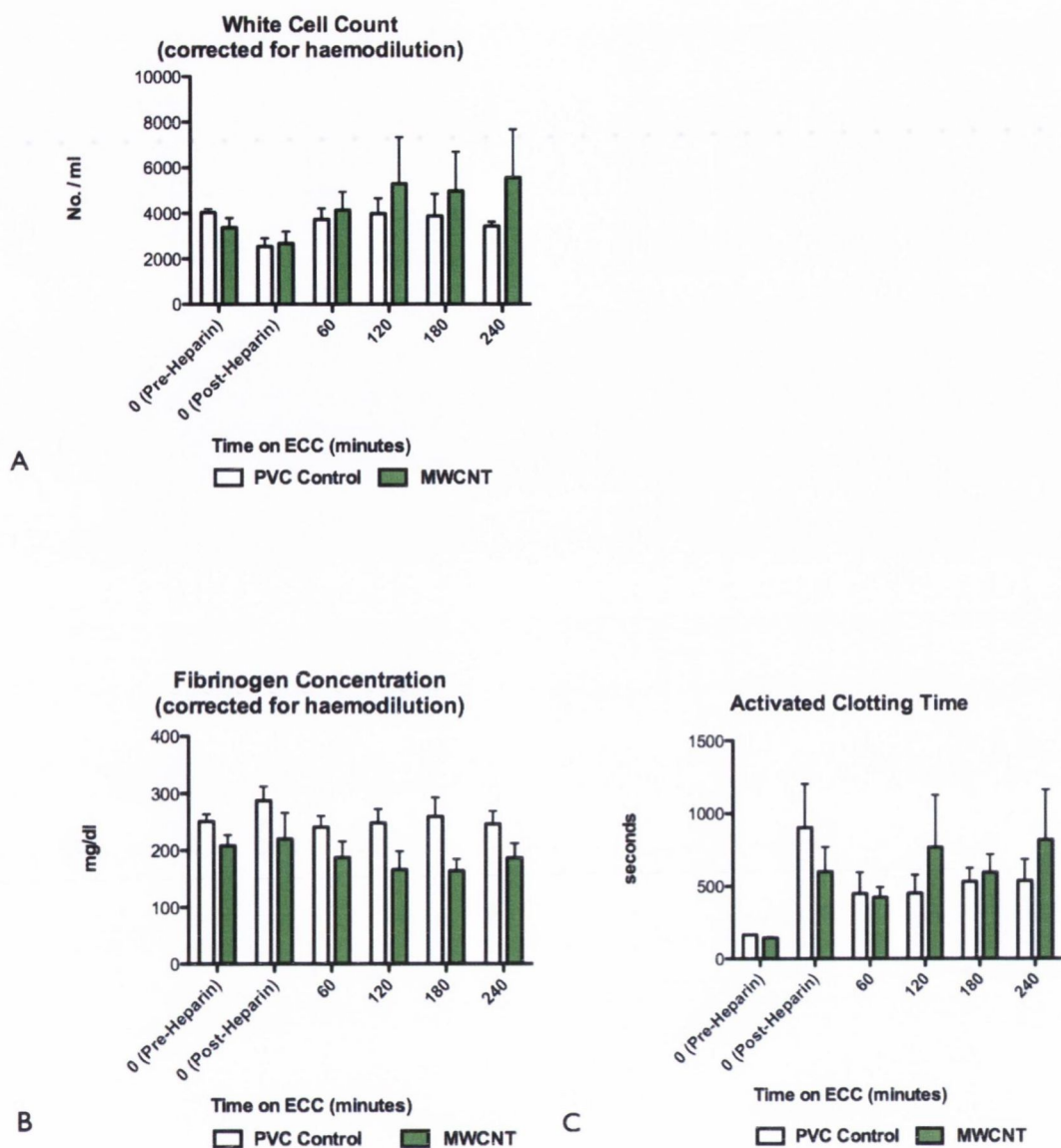


Figure 38 Blood was withdrawn for platelet count at baseline (time 0, immediately prior to and 3 minutes after heparin administration) and every hour thereafter. Where the platelet concentration in PRP was at least 2×10^8 platelets/ml, platelet aggregometry was performed. A shows platelet counts in whole blood for control and experimental circuits over time. B shows the results of platelet aggregometry where enough sufficient numbers of platelets were present to allow aggregometry to be performed. Data are mean $\pm$ SE, $n=4$. One-way ANOVA: $P > 0.05$.



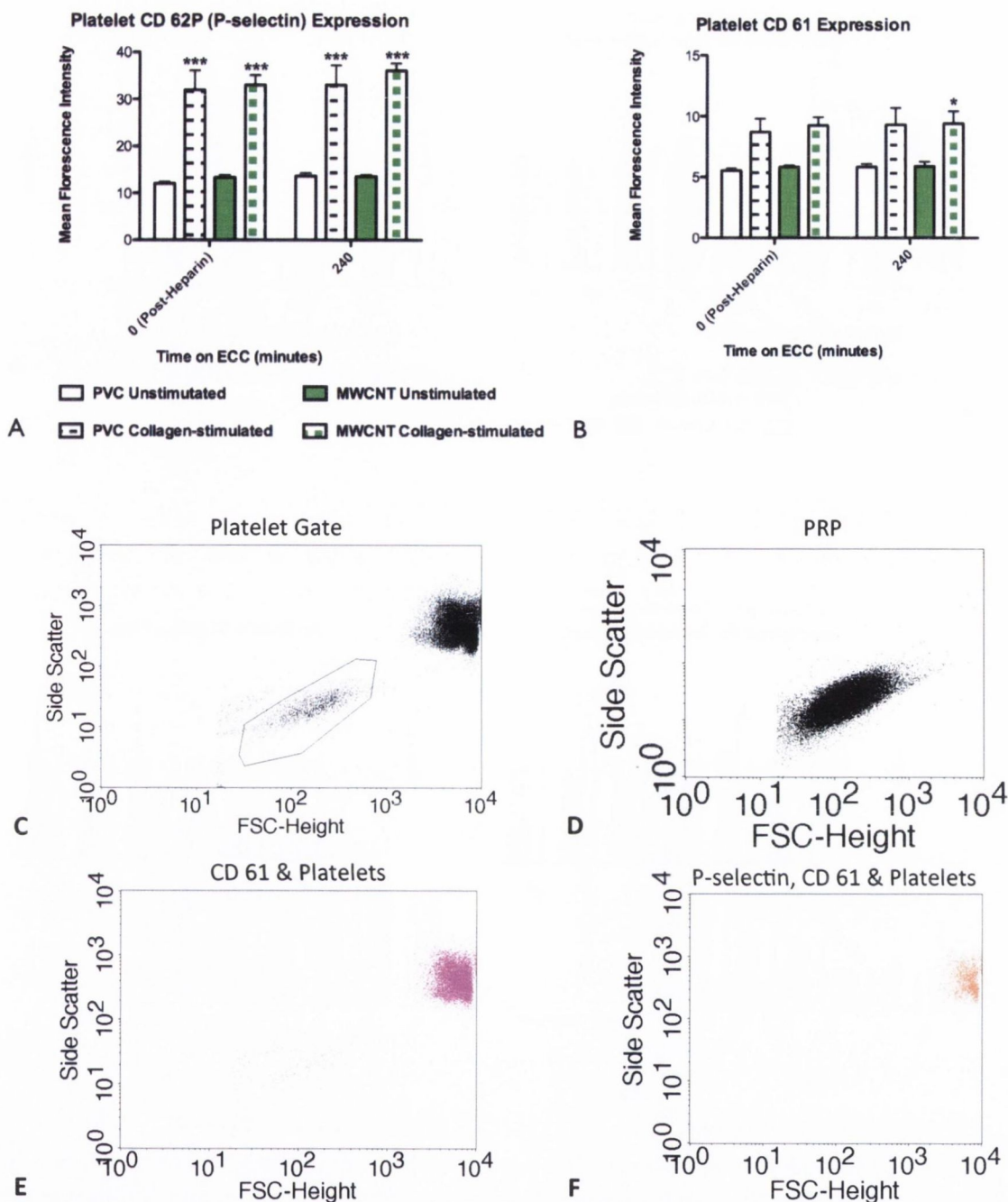


Figure 40 Blood was withdrawn for measurement of platelet CD 62P (p-selectin) and CD 61 expression by FACS analysis at baseline (3 minutes post heparin administration) and at 240 minutes post initiating ECC. Unstimulated and collagen stimulated (40µg/ml) expression was measured. There were no differences between control and experimental surfaces for platelet p-selectin or CD 61 expression at baseline or at 240 minutes, either unstimulated or collagen-stimulated (A) & (B). Data are mean $\pm$ SE, n=3. One-way ANOVA: *P < 0.05 compared to PVC Unstimulated at time 0, ***P < 0.001 compared to all unstimulated. Example of flow cytometry gate setting and output (C)-(F).

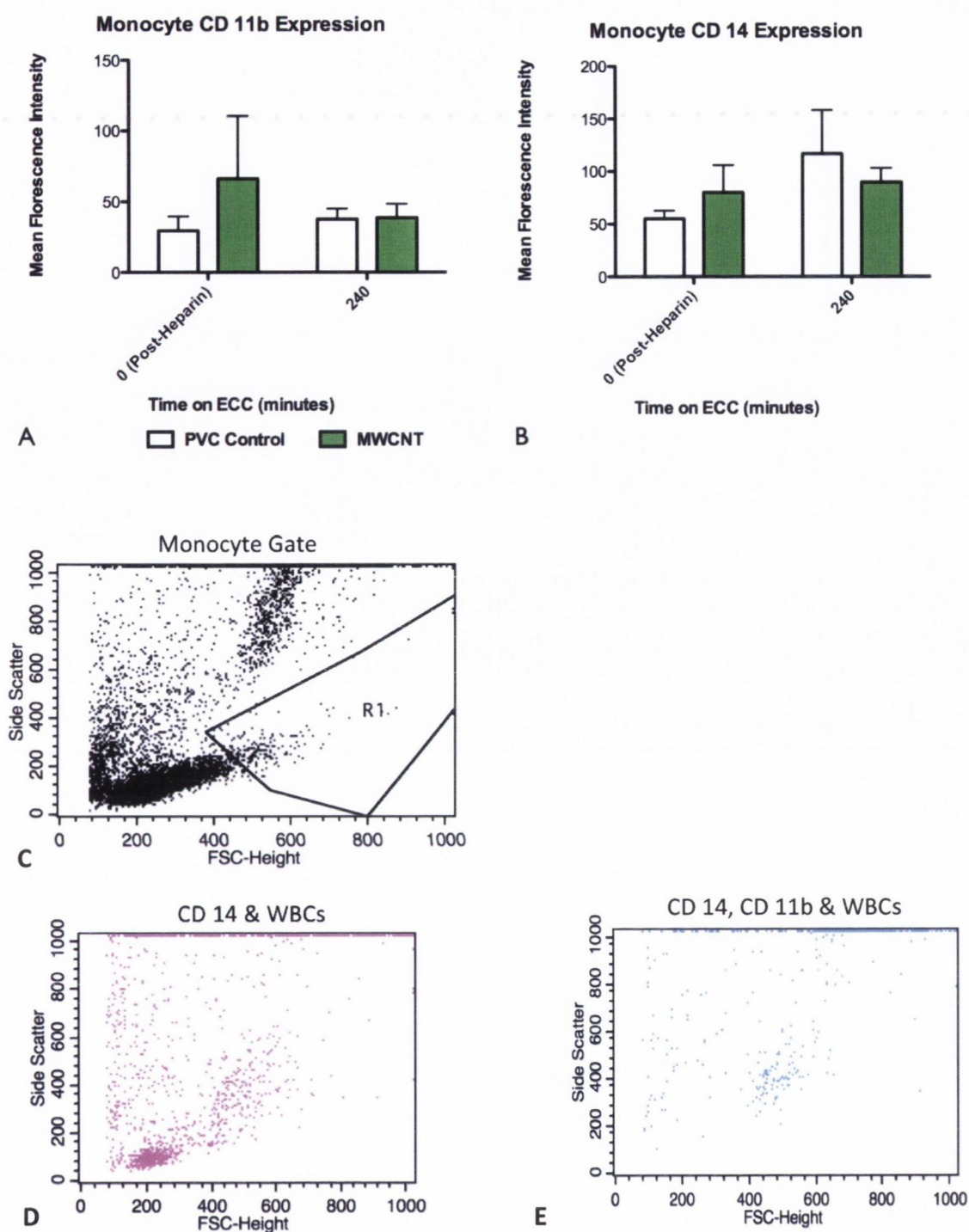


Figure 41 Blood was withdrawn for measurement of monocyte CD 11b and CD 14 expression by FACS analysis at baseline (3 minutes post heparin administration) and at 240 minutes post initiating ECC. There were no differences between control and experimental surfaces CD 11b or CD 14 expression at baseline or at 240 minutes. (A) & (B). Data are mean $\pm$ SE, n=3. One-way ANOVA: $P > 0.05$. Example of flow cytometry gate setting and output (C)-(E).

7.9 These was no scanning electron microscopy evidence of carbon nanotubes liberated into water perfused through MWCNT-swelled ECC circuits for 4 hours.

Because MWCNT concentration in solution cannot be measured by light, x-ray or any other spectroscopy technology, scanning electron microscopy was used to search for MWCNTs that may have become liberated from the ECC surface during perfusion. One L water perfusate was used in a closed circle system to continuously perfuse MWCNT-swelled ECC circuits at a flow rate of 200ml/minute. Three circuits were evaluated. No evidence of liberated MWCNTs in solution could be found.

7.10 There was no difference in the quantity of fibrinogen adsorbed to PVC control surfaces and MWCNT-swelled surfaces as measured by fibrinogen adsorption ELISA.

Figure 42 shows two scanning electron micrographs of MWCNT-swelled PVC QCM-D crystals prior to and after PPP perfusion. The MWCNTs appear to be coated in plasma proteins following PPP perfusion. In order to test the hypothesis that plasma protein – MWCNT interaction may be a component of the underlying mechanism for MWCNT-induced platelet activation and thrombosis, a number of experiments were performed to investigate the make-up and quantity of proteins adsorbed to control and experimental surfaces. The first of these was a fibrinogen adsorption ELISA.

Because fibrinogen is known to adsorb to foreign surfaces, change its conformation and cause platelet adhesion, activation and aggregation, a fibrinogen adsorption assay was carried out to examine for differences in the quantity of fibrinogen adsorbed to control and experimental surfaces in an attempt to ascertain the reasons for excess thrombosis on experimental surfaces (figure 43). No differences were found.

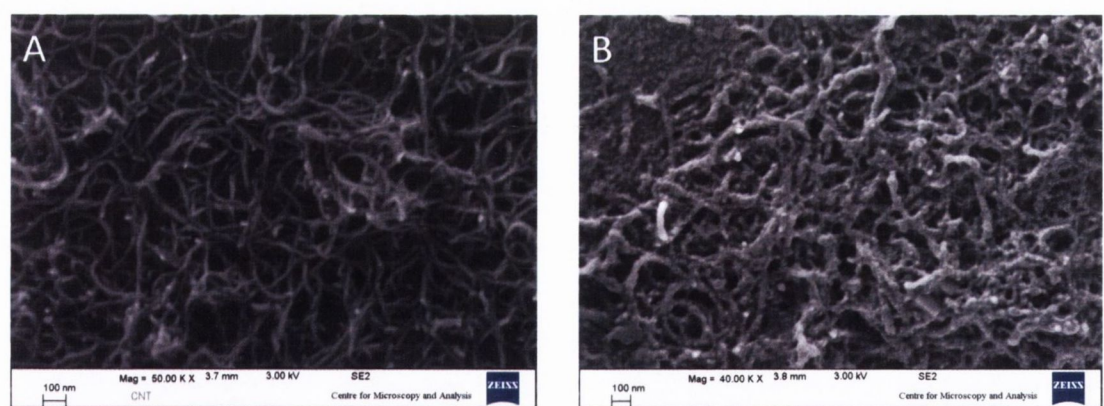


Figure 42 Scanning electron micrographs of MWCNT-swelled PVC QCM-D crystals prior to (A) and after (B) PPP perfusion. The MWCNTs appear to be coated in plasma proteins following PPP perfusion.

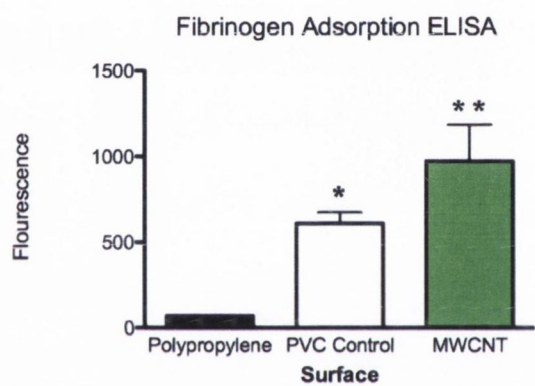


Figure 43 Although there was a statistical difference in fibrinogen adsorption to both PVC control and MWCNT-swelled surfaces compared to uncoated polypropylene (the material from which the 96 well plates are fabricated), there was no difference in fibrinogen adsorption to PVC control compared to MWCNT-swelled surfaces. Data are mean $\pm$ SE, n=3. Repeated measures ANOVA: Tukey's Multiple Comparison Test: *P<0.05 compared to polypropylene, **P<0.01 compared to polypropylene.

7.11 Proteomic examination of proteins stripped from control and experimental surfaces reveals deposition of fibrinogen, clusterin, fibronectin and vitronectin on both PVC control surfaces and MWCNT-swelled surfaces.

PVC control and MWCNT-swelled surfaces were perfused with PPP from three different donors. In order to ensure a sufficient quantity of protein to analyse, PPP was perfused over 1-4 crystals and stripped proteins were pooled by donor prior to analysis. Of those proteins identified, only those known to cause cell or platelet adhesion or platelet activation were considered as potential targets for further study. Table 5 lists those proteins identified and considered to be potentially important in understanding the mechanism of MWCNT platelet activation and thrombosis. The percentage area of the count peak area was used as a semi-quantitative measure of relative protein abundance. Although the 4 proteins listed below were present on surfaces, there was no obvious pattern showing a relative abundance of any particular protein on one surface compared to the other.

		PVC Control		MWCNT	
Sample	Protein	Peptide Hits	% Area Count Peak Area	Peptide Hits	% Area Count Peak Area
1	Fibrinogen	4	2.9	8	8.4
	Clusterin			6	6.98
	Fibronectin				
	Vitronectin			3	6.59
2	Fibrinogen	7	4.91	19	7.4
	Clusterin	1	1.36	6	4.21
	Fibronectin	1	0.05	4	0.54
	Vitronectin			4	2.48
3	Fibrinogen	28	11.69	3	1.49
	Clusterin	5	2.65		
	Fibronectin	4	0.48	1	0.31
	Vitronectin	3	0.56	1	4.16

Table 5 Proteins associated with cell adhesion and/or platelet activation on PVC control and MWCNT-swelled surfaces following perfusion with platelet poor plasma. The percentage area of the count peak area for vitronectin is highlighted as this is the only protein that may be semiquantitatively more abundant on MWCNT-swelled PVC surfaces compared to PVC control surfaces. Each sample is from a different donor. Raw data.

7.12 Western immunoblotting of donor plasma confirms that clusterin, fibronectin and vitronectin are present prior to perfusion across control and experimental surfaces.

To confirm by an additional method that the cell adhesion and platelet activation proteins identified by proteomic analysis are, indeed, present in donor plasma, western immunoblots were prepared using monoclonal antibodies to clusterin, fibronectin and vitronectin. It was possible to identify these three proteins as seen in figure 44.

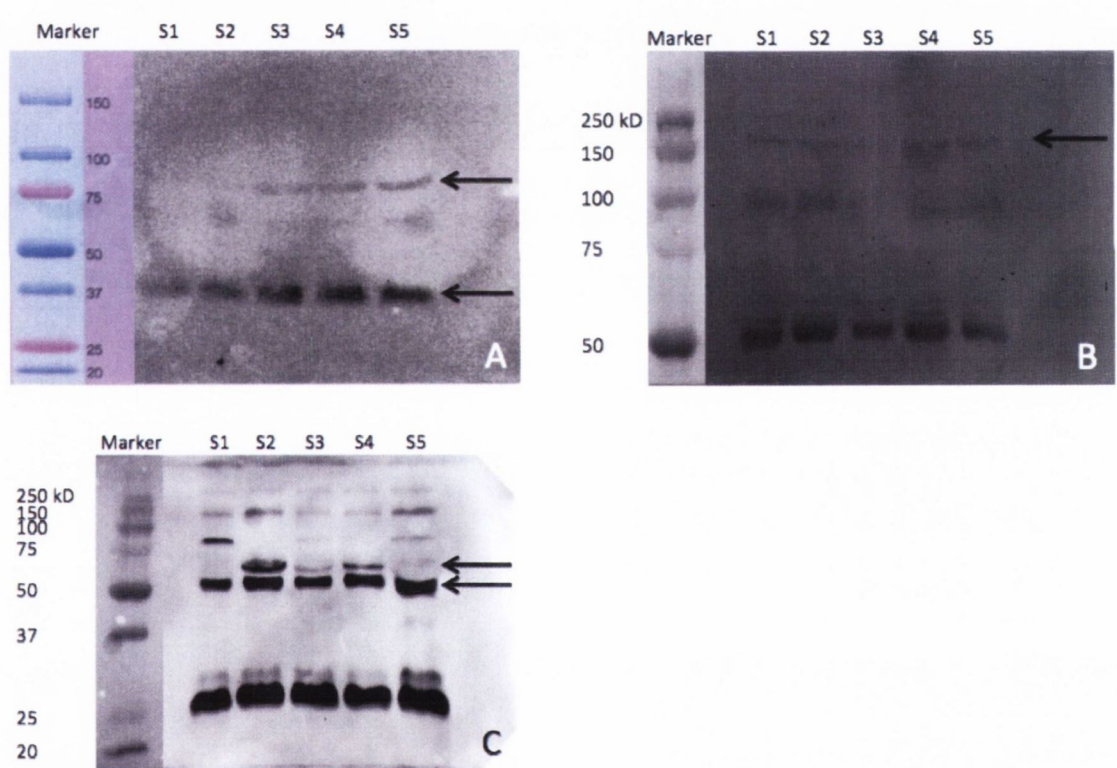


Figure 44 Western immunoblots. PPP from 5 individual donors (S1 – S5). **(A)** Clusterin is a heterodimeric protein of about 80 kDa. Here bands are seen at about 38 and 78 kDa (arrows). **(B)** Fibronectin bands seen at 200-250 kDa (arrow). **(C)** Vitronectin bands seen between 65-75 kDa (arrows).

7.13 Fibrinogen, albumin and vitronectin are shown to adsorb to PVC and MWCNT-swelled PVC sensor surfaces as measured by QCM-D.

To test for interaction between washed platelets and surfaces pre-adsorbed with individual proteins, it was first necessary to show that proteins are, in fact, adsorbed to PVC and MWCNT-swelled PVC surfaces on QCM-D sensors. Fibrinogen is known to cause platelet activation after a change in conformation when adsorbed to a foreign surface. Albumin adsorption protects against platelet-surface interaction. Vitronectin adsorption was studied because it was the only cell adhesion/platelet activating protein to be identified as being potentially semiquantitatively more abundant on MWCNT surfaces compared to PVC as seen in section 7.11 above (proteomics).

All three proteins were found to bind to PVC and MWCNT-swelled PVC surfaces (figures 45 – 48) with each one reaching maximum adsorption (as demonstrated by no further shifts from baseline of f and D over time) within 20 minutes.

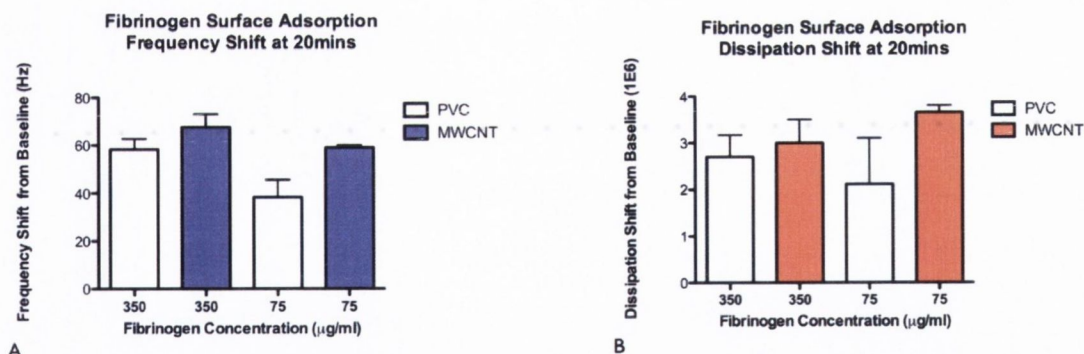


Figure 45 QCM-D measurement of fibrinogen adsorption to PVC and MWCNT-swelled PVC sensor surfaces. Data are mean $\pm$ SE, n=2.

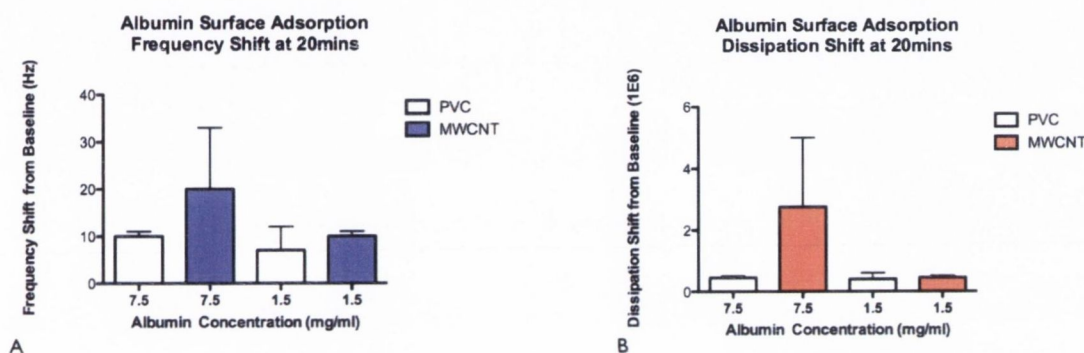


Figure 46 QCM-D measurement of Albumin adsorption to PVC and MWCNT-swelled PVC sensor surfaces. Data are mean $\pm$ SE, n=2.

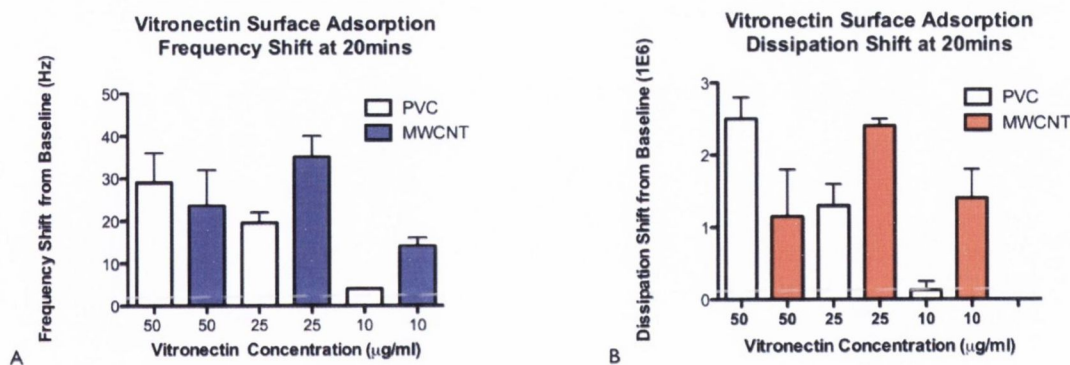


Figure 47 QCM-D measurement of vitronectin adsorption to PVC and MWCNT-swelled PVC sensor surfaces. Data are mean $\pm$ SE, n=2.

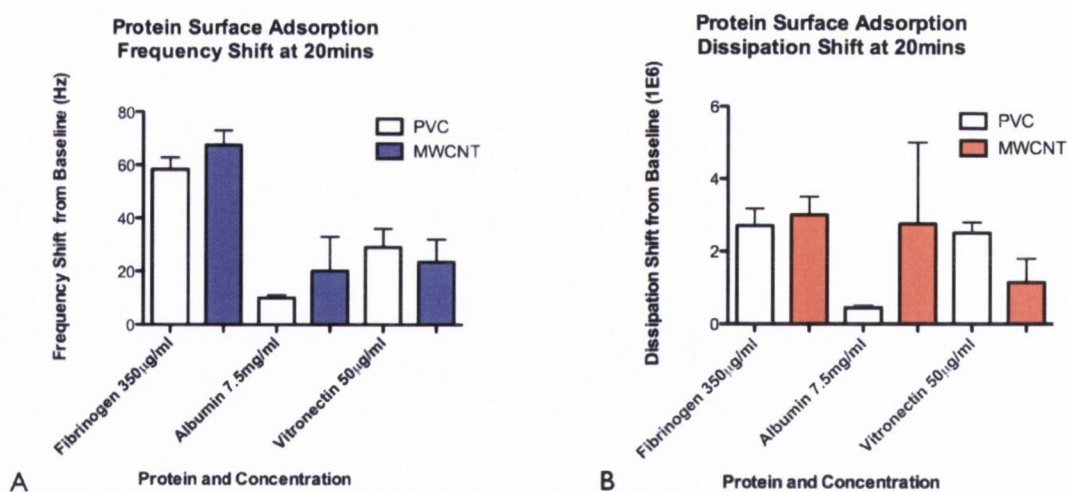


Figure 48 Comparison of frequency and dissipation shifts for the highest concentration of each protein adsorbed to PVC control and MWCNT-swelled PVC sensor surfaces. Data are mean $\pm$ SE, n=2.

7.14 There is less platelet activation when WPs are perfused across PVC and MWCNT-swelled PVC surfaces to which either fibrinogen, albumin or vitronectin has been adsorbed compared to the same surfaces without protein adsorption as demonstrated by QCM-D.

Figures 49 to 52 show the statistically significant reduction in platelet activation (as evidenced by reductions in baseline shifts of both frequency and dissipation) that occurs when fibrinogen, albumin and vitronectin are adsorbed to control and experimental surfaces. This can be seen to be concentration-dependent where the effects of concentration gradients were examined in the case of vitronectin (figure 51).

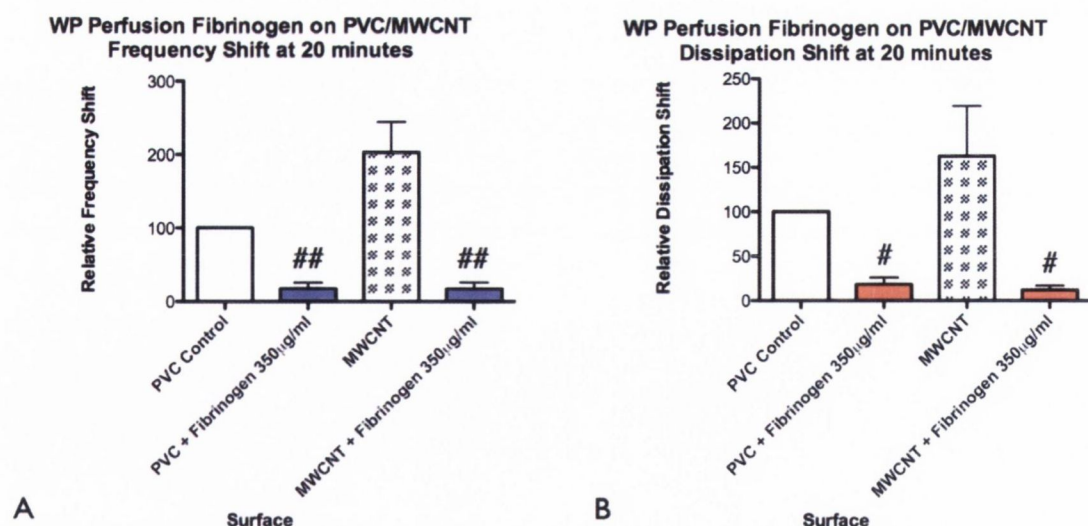


Figure 49 Perfusion of washed platelets over PVC and MWCNT-swelled PVC surfaces coated and uncoated with fibrinogen 350 µg/ml. Data are mean ± SE, n=3. Repeated measures ANOVA: Tukey's Multiple Comparison Test: #P <0.05 compared to MWCNT, ##P <0.01 compared to MWCNT.

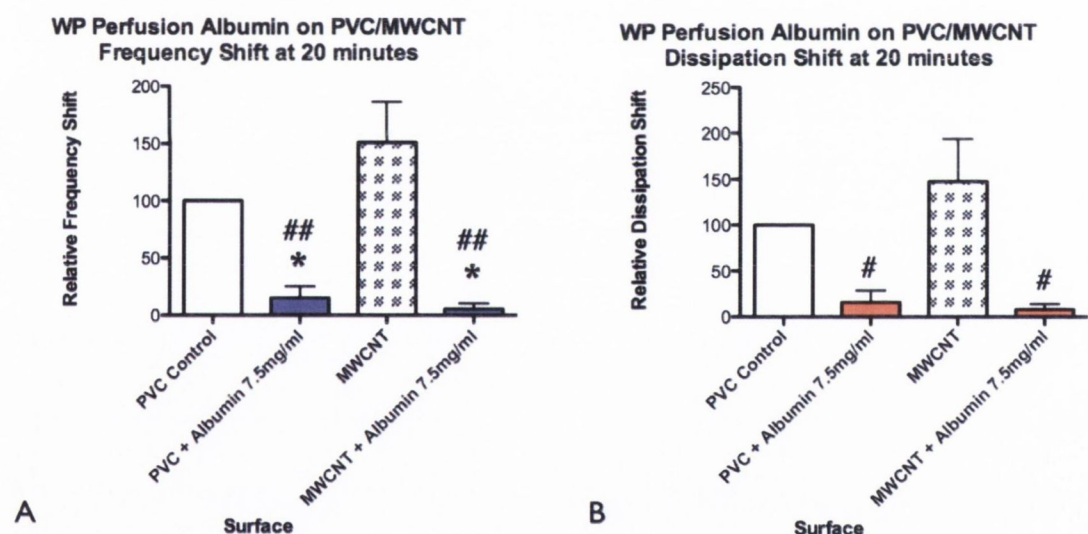


Figure 50 Perfusion of washed platelets over PVC and MWCNT-swelled PVC surfaces coated and uncoated with albumin 7.5mg/ml. Data are mean ± SE, n=3. Repeated measures ANOVA: Tukey's Multiple Comparison Test: *P <0.05 compared to PVC Control, #P <0.05 compared to MWCNT, ##P <0.01 compared to MWCNT.

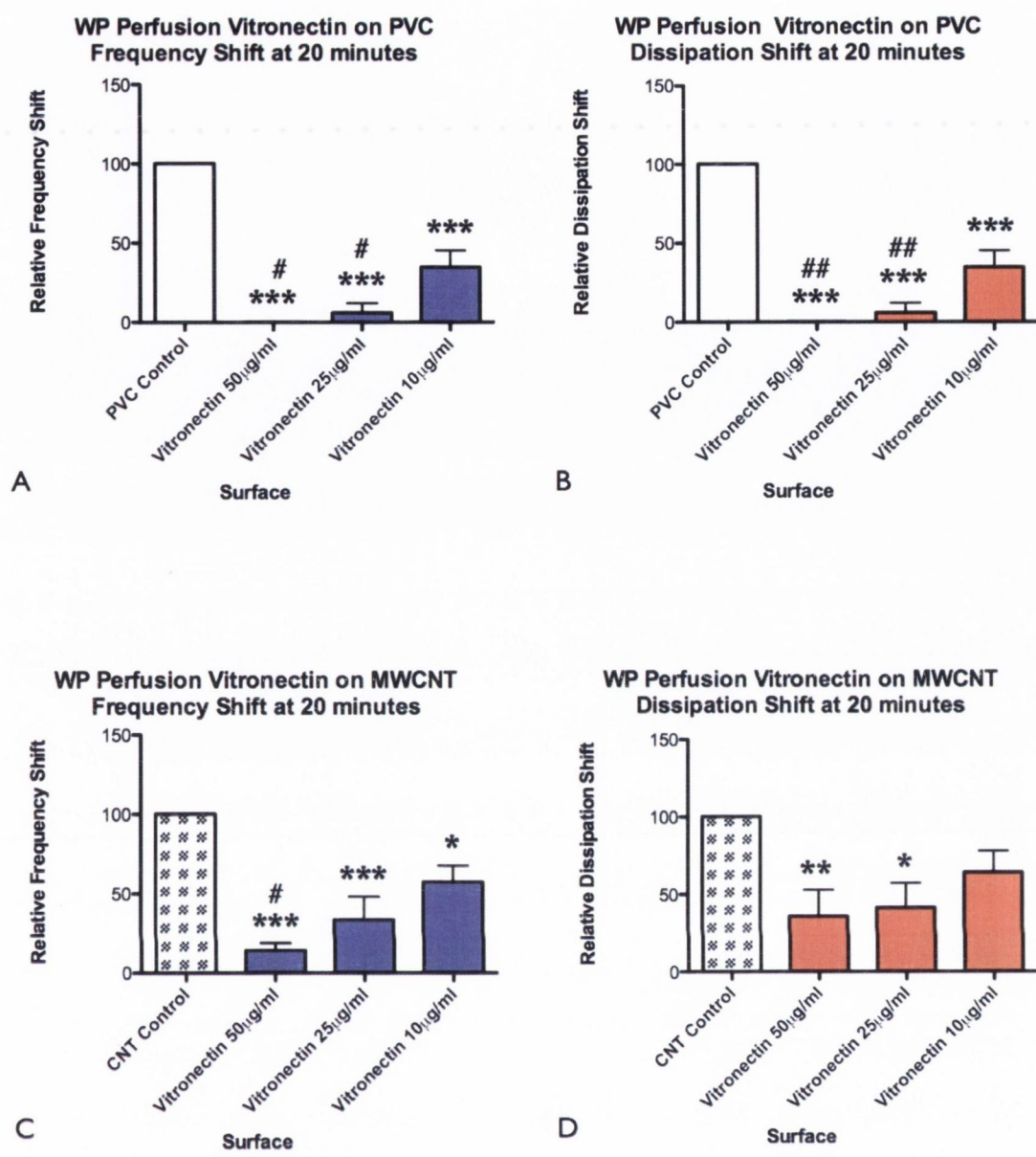


Figure 51 Perfusion of washed platelets over PVC (A) & (B) and MWCNT-swelled PVC (C) & (D) surfaces coated with increasing concentrations of vitronectin. Data are mean $\pm$ SE, n=3. Repeated measures ANOVA: Tukey's Multiple Comparison Test: *P <0.05 compared to CNT Control, **P <0.01 compared to CNT Control, ***P <0.001 compared to CNT Control, #P <0.05 compared to vitronectin 10µg/ml, ##P <0.01 compared to vitronectin 10µg/ml.

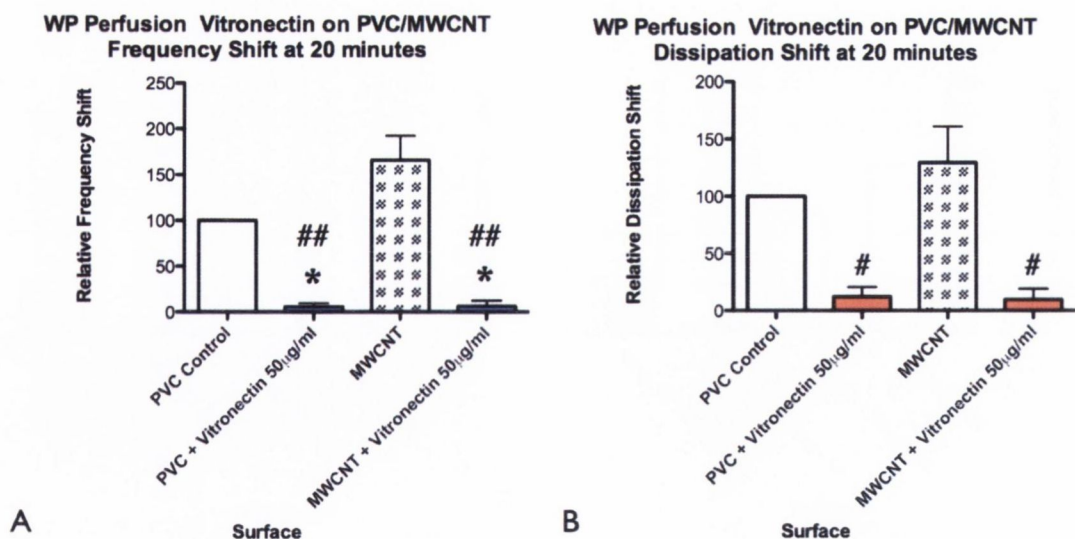


Figure 52 Perfusion of washed platelets over PVC and MWCNT-swelled PVC surfaces coated and uncoated with vitronectin 50µg/ml. Data are mean $\pm$ SE, n=3. Repeated measures ANOVA: Tukey's Multiple Comparison Test: *P <0.05 compared to PVC Control, #P <0.05 compared to MWCNT, ##P <0.01 compared to MWCNT.

8 RESULTS - PART II

8.1 Soluble MWCNTs functionalised with iloprost (MWCNT-Iloprost) cause significantly less platelet aggregation than MWCNTs functionalised with -COOH (MWCNT-COOH) as demonstrated by light aggregometry.

MWCNTs functionalised with -COOH (MWCNT-COOH) is the raw material used to functionalise MWCNTs with iloprost (MWCNT-Iloprost) (see section 5.4.1). For this reason, MWCNT-COOH is used as control for platelet aggregometry studies (and QCM-D experiments in sections 8.2-8.4) to compare drug with non-drug functionalised CNTs.

Light aggregometry studies comparing the effects of MWCNT-COOH and MWCNT-Iloprost on platelet aggregation in WPs showed significantly less platelet aggregation for MWCNT-Iloprost functionalised CNTs (figure 53).

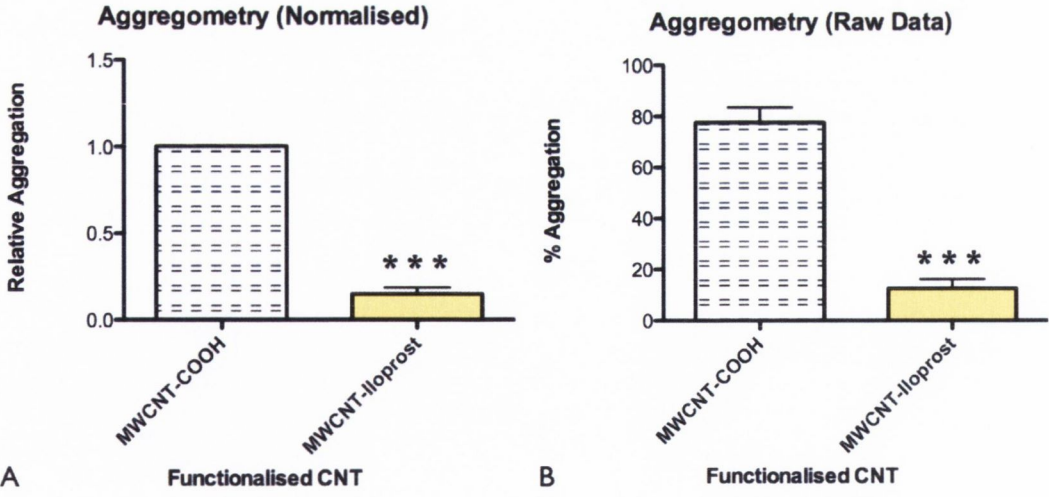


Figure 53 Comparison of platelet aggregatory effects of MWCNT-COOH and MWCNT-Iloprost both at 100µg/ml. Data are mean ± SE, n=6. Paired t-test: ***p < 0.001.

8.2 Mass Spectroscopy of MWCNT-Iloprost demonstrates the presence of iloprost associated with the preparation.

Mass spectroscopy was performed on samples of MWCNT-Iloprost (100µg/ml) and MWCNT-COOH (100µg/ml) used for platelet aggregometry studies in section 8.1. No iloprost was found in samples of MWCNT-COOH whereas iloprost was detected in samples of MWCNT-Iloprost (*Figure 54*).

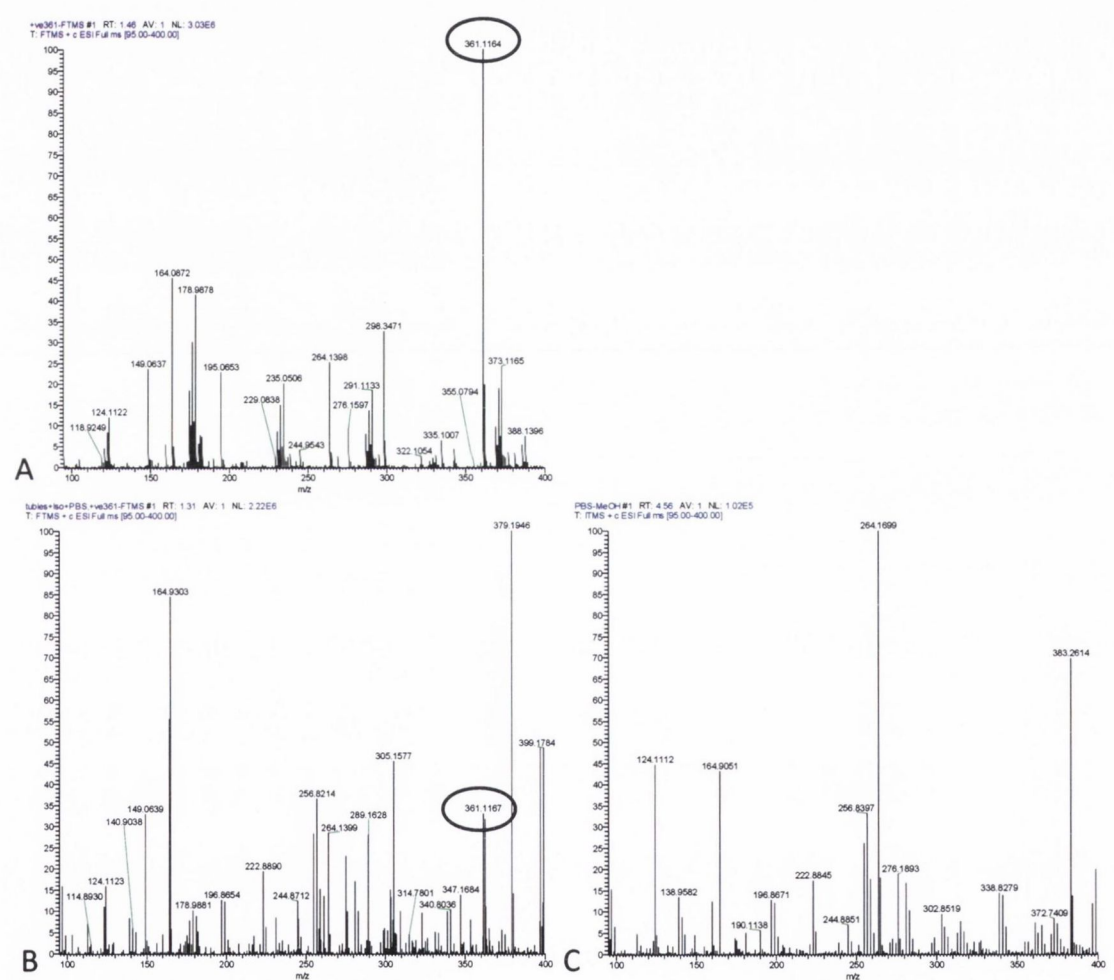


Figure 54 Mass spectroscopy. (A) Full scan mass spectra of 50ng/ml Iloprost in 50:50 MeOH:PBS. Detection of sample ion at m/z 361.1164 (B) Full scan mass spectra of MWCNT-Iloprost in 50:50 PBS:MeOH. Detection of sample ion at m/z 361.1164. (C) Full scan mass spectra of MWCNT-COOH 50:50 MeOH:PBS. Iloprost ion at m/z 361 is not detected.

8.3 Incubation of PRP and WP with Iloprost prior to perfusion across MWCNT-swelled PVC and PVC control surfaces leads to decreased platelet aggregation as demonstrated by QCM-D.

To demonstrate the platelet inhibitory effects of soluble iloprost on MWCNT-swelled PVC and PVC surfaces, iloprost at 200nM was added to PRP and WP immediately prior to perfusion across PVC control surfaces and MWCNT-swelled PVC surfaces. Figure 55 shows that platelet aggregation for WP is significantly decreased in the presence of iloprost compared to iloprost-free controls (significant differences in baseline shifts for both frequency and dissipation). Platelet aggregation for PRP incubated with iloprost is also significantly decreased when frequency shift, but not dissipation shift, is analysed (figure 55).

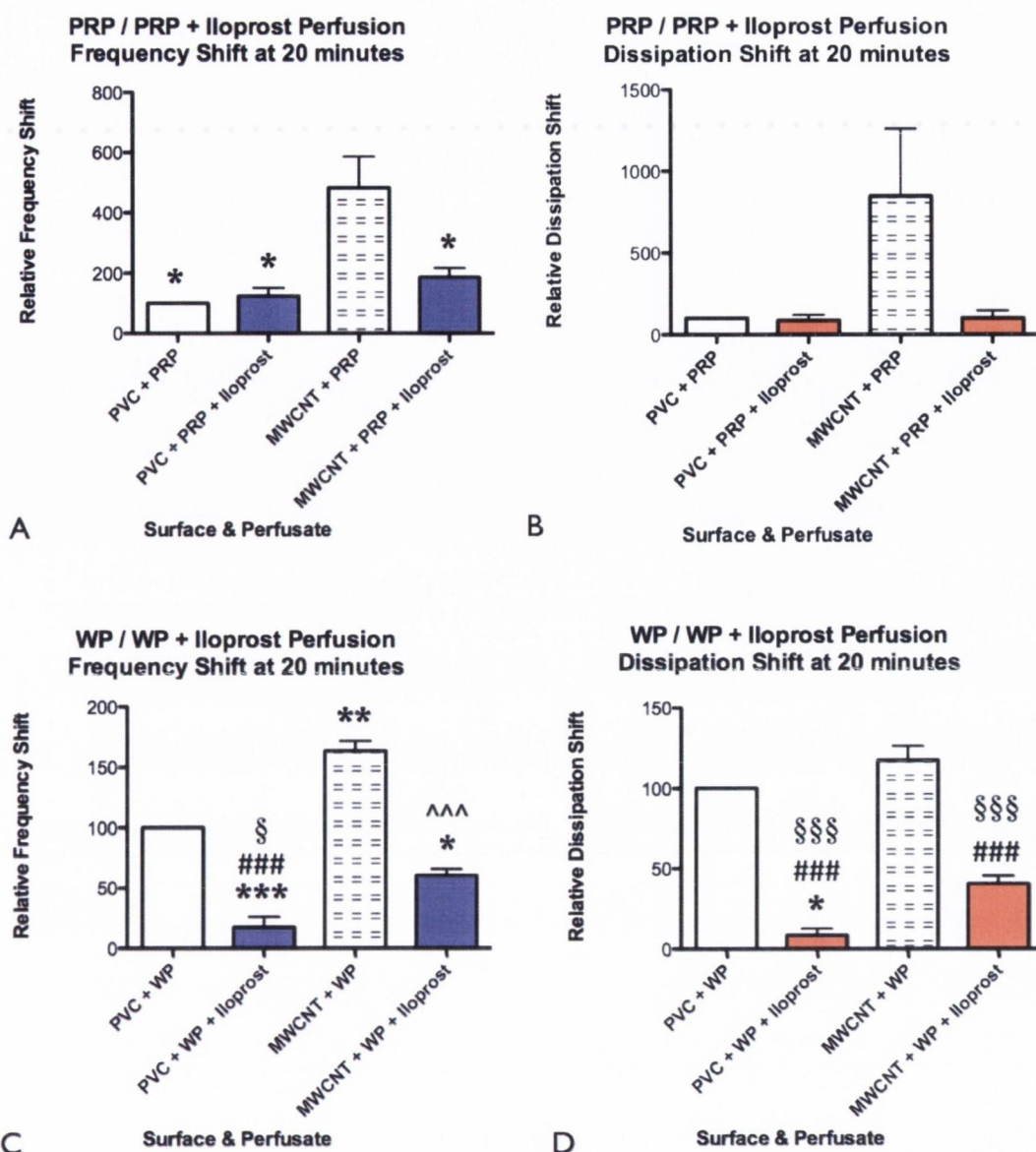


Figure 55 Platelet aggregation in PRP and WP in the presence or absence of iloprost (200nM) perfused across PVC and MWCNT-swelled PVC QCM-D sensor surfaces for 20 minutes. Data are mean $\pm$ SE, n=3. (A) Repeated measures ANOVA. Tukey's Multiple Comparison Test: *P < 0.05 compared to MWCNT and non-incubated PRP. (B) Repeated measures ANOVA: P > 0.05. (C) Repeated measures ANOVA. Tukey's Multiple Comparison Test: *P < 0.05 compared to PVC and non-incubated WP, **P < 0.01 compared to PVC and non-incubated WP, §P < 0.05 compared to MWCNT and iloprost-incubated WP, ***P < 0.001 compared to PVC and non-incubated WP, ###P < 0.001 compared to MWCNTs and non-incubated WP, ^^P < 0.001 compared to MWCNTs and non-incubated WP (D) *P < 0.05 compared to MWCNT and iloprost-incubated WP, ###P < 0.001 compared to PVC and non-incubated WP, §§§P < 0.001 compared to MWCNT and non-incubated WP.

8.4 There is no difference in platelet aggregation between surface-bound MWCNTs functionalised with iloprost (MWCNT-Iloprost-swelled PVC) and surface-bound MWCNTs functionalised with -COOH (MWCNT-COOH-swelled PVC) perfused with PRP or WP as demonstrated by QCM-D.

In these experiments, MWCNT-Iloprost was swelled onto PVC spin-coated sensors in the usual way. No differences were found in platelet aggregation between control and MWCNT-Iloprost surfaces (figure 56).

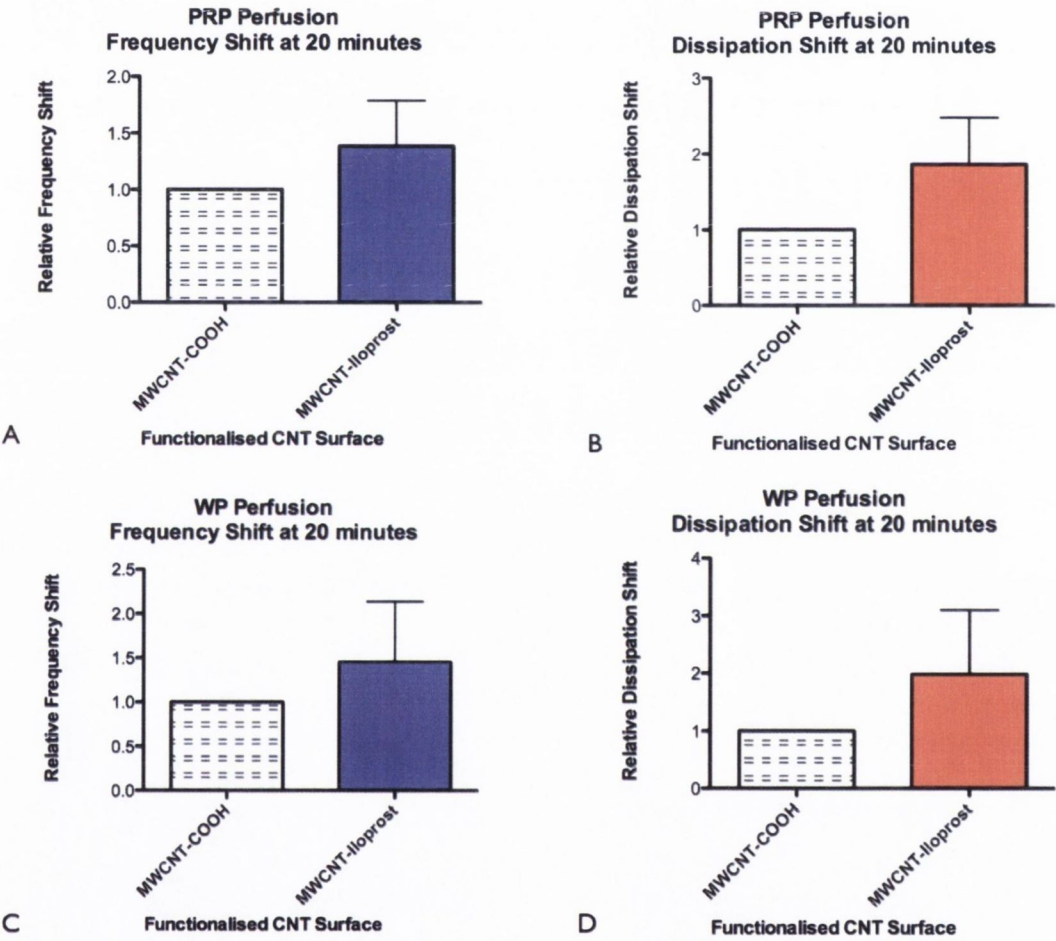


Figure 56 Matched frequency and dissipation shifts from baseline of PRP (A) & (B) and WP-perfused (C) & (D) MWCNT-COOH-swelled PVC surfaces compared to MWCNT-Iloprost PVC-swelled surfaces. Data are mean $\pm$ SE, n=7 (A) & (B), n=4 (C) & (D). Paired t-test: P >0.05.

8.5 No free iloprost is detected in initial PBS perfusate across MWCNT-Iloprost-swelled PVC sensor surfaces.

Prior to perfusing MWCNT-Iloprost-swelled PVC sensor surfaces with PRP and WP (section 8.4) the first 200-250µl of PBS perfused across the surfaces while calibrating the QCM-D to baseline was collected and analysed by mass spectrometry for the presence of iloprost that may have become detached from the sensor surfaces during initial PBS perfusion.

No iloprost was found in any samples.

8.6 There is no difference in platelet aggregation between surface-bound MWCNTs functionalised with iloprost (MWCNT-NH₂-swelled PVC with iloprost binding *in situ* thereafter) and surface-bound MWCNTs functionalised with -NH₂ (MWCNT-NH₂-swelled PVC) perfused with PRP as demonstrated by QCM-D.

Because of the failure of MWCNT-Iloprost-swelled PVC to attenuate platelet aggregation compared to MWCNT-COOH-swelled PVC (section 8.4), MWCNT-NH₂ was first swelled onto PVC and then iloprost was covalently bonded to form MWCNT-Iloprost. This was done to avoid potential disruption of the MWCNT-Iloprost bond by ultrasonification (which may account for the failure to attenuate platelet aggregation). MWCNT-NH₂ was the appropriate control surface for these experiments.

Again, there was no difference in platelet aggregation between control and MWCNT-Iloprost surfaces (figure 57).

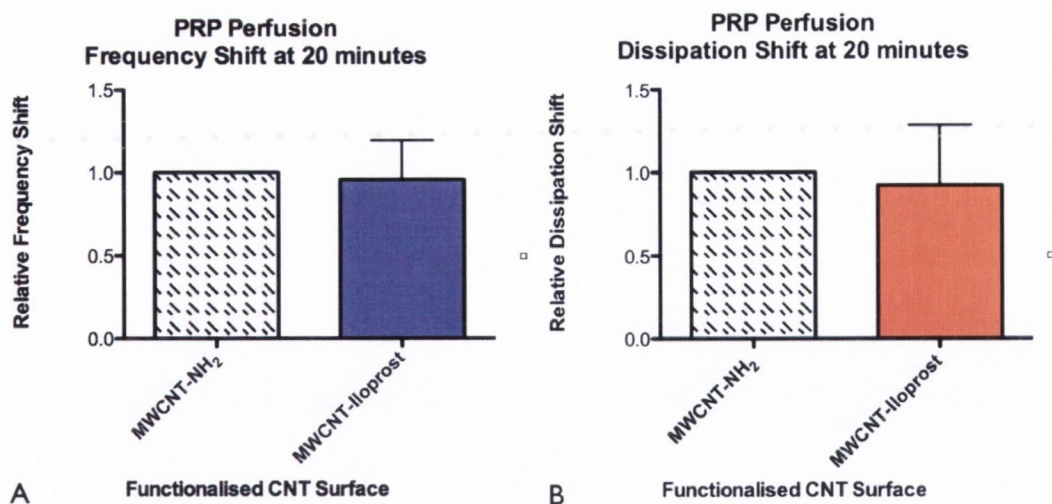


Figure 57 Matched frequency and dissipation shifts from baseline of PRP-perfused MWCNT-NH₂-swelled PVC surfaces compared to MWCNT-Iloprost-swelled PVC surfaces. Data are mean $\pm$ SE, n=3. Paired t-test: P >0.05.

9 DISCUSSION

9.1 Choice of control surface.

In order to test the blood biocompatibility of surface-bound carbon nanotubes it was necessary to choose a control surface material to which all results would be compared. The following criteria guided the selection of control surface: (1) the tested material had to be approved for clinical applications and currently in use in medical devices; (2) it would form a sufficiently large proportion of the blood-contacting component of the device; (3) it would represent a “gold standard” biomaterial, comparisons to which would be clinically meaningful and, finally; (4) it was important that blood-surface interaction was the main biological interaction of the device in which the material was used and that the clinical consequences of this interaction was such that blood biocompatibility of any experimental material could be judged to be greater, less or the same when compared to it.

The material chosen was polyvinyl chloride (PVC) used in extracorporeal circulation technologies. This material fulfils all of the above criteria for the appropriate control biomaterial. It is an approved, licensed biomaterial from which the tubing of ECC devices is manufactured. It is the most commonly used ECC tubing material, has been used for this purpose for over 50 years and is, as such, the “gold standard” ECC tubing biomaterial. PVC is in direct contact with the equivalent of the whole cardiac output blood volume every minute. The adverse results of blood-surface interaction can be devastating

as described in sections 3.2 and 3.4. Without systemic heparin administration, blood-PVC interaction causes thrombus formation. This material, however, causes the least amount of thrombus formation compared to any other material capable of being formed into tubing and in current clinical use. It is possible, therefore, that the blood biocompatibility of an experimental surface material could be less, more or equally biocompatible as PVC.

9.2 Choice of carbon nanotube.

Carbon nanotubes have been grown as “forests”, with tightly packed carbon nanotubes arranged side-by-side in vertical alignment⁽²¹⁶⁾. Although such a surface would be very interesting to study, and indeed has been studied to some degree previously, it would not be practical to grow such forests on the inside of PVC tubes or on the surfaces of sensor crystals as the total surface area required would be excessive using current nanoforest-growing technologies⁽¹⁷⁵⁾. It was decided, instead, to use MWCNTs in powdered form and to attach these to the inside of PVC tubing and to the surfaces of PVC-coated QCM-D sensors. MWCNTs are the most commonly produced, commercially available carbon nanotubes and are significantly cheaper than single-walled carbon nanotubes. Although either MWCNTs or SWCNTs could have been studied it was decided to begin, initially by examining the potential use of MWCNTs. MWCNTs can generally be subdivided into straight and curly forms. Both of these forms were tested initially.

9.3 Methods of carbon nanotube surface attachment.

Two methods of MWCNT surface attachment to PVC were tested. In the first, MWCNTs (straight and curly) were suspended in a 0.5% w/v solution of PVC in THF and spin-coated onto sensor surfaces. Results of SEM analysis clearly show such a technique to result in surfaces sparsely covered with MWCNTs. This finding is unsurprising for a number of reasons. Firstly, because of surface tension, carbon nanotubes tend to clump together. Although ultrasonification is used to help disperse MWCNTs, it is unlikely that ultrasonification in a solution of PVC would result in adequate dispersal compared to a MWCNTs suspended in a solvent. Clumps of MWCNTs in PVC solution are likely to be displaced from the surfaces of sensors to the edges and beyond by the high centrifugal forces generated by the spin-coating process. Secondly, any MWCNTs present in the PVC film are unlikely to protrude far beyond this layer. This can be seen in figure 19 where the majority of any MWCNTs seen appear to lie below the surface.

Despite the lack of MWCNT surface coverage, it was important to test for differences in platelet aggregation on PVC/MWCNT (both straight and curly) spin-coated QCM-D sensors. There were no significant differences in the changes from baseline of either frequency or dissipation between these surfaces as measured by QCM-D. The QCM-D Q-Sense instrument is an exquisitely sensitive instrument for detecting platelet activation at the nanoscale and allows for careful quantification of experimental results ⁽²¹²⁾. Therefore, I concluded that sparsely coated MWCNTs do not elicit a

measurably different platelet aggregatory response compared to a PVC control surface.

In the second method of MWCNT surface attachment to PVC, a patented technique of “swelling” was used. Here, PVC is “swollen” with a solvent while ultrasound is used to attach MWCNTs to the swelled PVC surface. The solvent then evaporates leaving the MWCNTs firmly fixed into the topmost layer of polymer. When the spin-coated layer of PVC attached to the surface of QCM-D sensors was swelled with a solution of MWCNTs 0.5% w/v in toluene and ultrasound applied, a much more confluent, densely coated layer of curly MWCNTs was produced on the sensor surfaces as evidenced by scanning electron microscopy. Importantly, the process was reproducible. In contrast to PVC-swelled curly MWCNTs, straight MWCNTs did not produce an acceptable coating. It is likely that most attached straight MWCNTs were lying horizontally due to their shape and high aspect ratio. Even where curly MWCNTs were also more likely to lie horizontally, their shape ensured that at least part of the MWCNT protruded above the uppermost layer of the PVC to the surface.

Because of the success of manufacturing nanomaterial-modified surfaces using the curly MWCNT PVC-swelling method and the relative failure of the straight MWCNT PVC-swelling approach, it was decided to continue further experimentation with curly MWCNTs only. **From now on, the term MWCNT will refer to curly MWCNTs only.**

9.4 QCM-D *in vitro* flow model to measure platelet aggregation in real-time.

Platelet light aggregometry is the “gold standard” for testing platelet activation and aggregation. This method can only be used, however, under essentially static conditions (although a stirring bar used to keep platelets in suspension causes some fluid mechanical shear stress) where platelet agonists and antagonists are added to a suspension of PRP or WPs. To assess platelet-surface interaction most methods have previously involved only post-perfusion microscopic examination of the surface. Using the QCM-D method, however, surface platelet aggregation was measured, for the first time, in the nanoscale range, in a flow model, in real-time.

9.5 MWCNTs cause an increase in platelet aggregation *in vitro*.

The most important observation made during the course of these experiments was that platelets (as PRP or WPs) perfused across MWCNT-swelled PVC QCM-D sensor surfaces aggregated to a significantly greater degree than the same suspensions perfused across PVC control surfaces. Indeed, significant differences were seen in both frequency and dissipation shifts from baseline. In addition, phase contrast microscopy performed on all perfused crystals confirmed this increase in platelet aggregation on MWCNT-swelled PVC surfaces compared to PVC control surfaces.

I also used light aggregometry to study the interactions between soluble (surface-unbound) MWCNTs and platelets. Previous experiments from our group have shown that soluble MWCNT have the ability to aggregate WPs⁽²⁰²⁾. Therefore, I studied the effects of MWCNT used for PVC surface nanomodification. As expected, I found that MWCNT, when added to PRP and WP, significantly increased aggregation of WPs at concentration of 100 µg/ml, an effect similar to the maximally effective concentrations of collagen.

In contrast to WPs, the addition of MWCNT (100 µg/ml) to PRP did not significantly increase platelet aggregation. Why, then, is there a difference in the results obtained between light aggregometry and QMC-D measurement of platelet aggregation for PRP? A number of possibilities can be considered. It has previously been shown that PRP diluted with PPP leads to less platelet aggregation following the addition of agonist compared to the same agonist added to PRP diluted with buffer⁽²¹⁷⁾. It was suggested by the authors that substances (such as ADP) released into PPP by the centrifugation process could desensitise platelets to the subsequent aggregatory challenge. However, in my experiments, collagen stimulation of platelet aggregation was unaffected and PRP used in QCM-D experiments was diluted with buffer prior to perfusion. Another explanation could be that plasma proteins attached to MWCNTs in PRP could prevent direct interaction between MWCNTs and platelets, thus attenuating the aggregatory response of platelets. This is again unlikely as PRP used for perfusion over MWCNT-swelled PVC surfaces also contained plasma proteins capable of binding to MWCNTs (see next section, 9.6). It may be that MWCNTs are more likely to clump together when in

suspension and less likely when surface-bound. Clumping would lead to an overall reduction in surface area for MWCNT-platelet interaction to take place and thus result in decreased platelet aggregation. Clumping is prevented by swelling MWCNTs to the surface of PVC. The final, and possibly most likely, explanation for this observed difference in aggregation seen in QCM-D experiments compared to aggregometry could be the increased sensitivity of QCM-D to detect platelet activation compared to light aggregometry. Indeed, Santos-Martinez et al. found QCM-D measurement of silica, polystyrene, and gold nanoparticle-induced platelet aggregation to be more sensitive than light transmission aggregometry⁽²¹²⁾.

9.6 Protein deposition on PVC and MWCNTs

Perfusion of PPP across PVC control and MWCNT-swelled PVC surfaces resulted in significantly higher frequency shifts for the MWCNT experimental surfaces compared to control PVC surfaces. However, there was no difference in dissipation shifts between control and experimental surfaces. Differences in frequency shifts between surfaces can be explained by a difference in the mass of proteins deposited on those surfaces. Indeed, MWCNT-swelled PVC surfaces appear to attract more plasma protein deposition compared to PVC controls. This evidences only that a greater mass of proteins is deposited on MWCNT-swelled PVC, but not the composition or thickness of proteins deposited on the surface. For example, due to the likely increased surface area of each sensor crystal because of the high surface area to mass ratio of attached MWCNTs, the surface layer of

proteins deposited may be equally thick on each surface but deposited over a larger total area compared to PVC controls.

Viscoelasticity of the attached surface layer is measured by changes in dissipation from baseline. Protein attachment appears to result in a maximum dissipation shift that is not altered by changes in the mass of proteins attached (within the ranges of protein deposition seen in these experiments). An interesting phenomenon is noted when graphical representations of frequency and dissipation shifts for PVC control-perfused surfaces are examined. It can be seen that frequency shifts from baseline over time decrease in PVC control sensors perfused with either PRP or PPP. This phenomenon is reproduced in all QCM-D experiments using PVC control surfaces. It can also be seen that dissipation values do not change over the same time periods. The most likely explanation for this phenomenon is the Vroman effect previously discussed⁽⁴⁹⁾. Surface protein adsorption is a dynamic process whereby initially adsorbed proteins are replaced by other proteins over time. A decrease in frequency shift, in this case, may be explained by the replacement of proteins initially adsorbed by proteins of lesser mass. The adsorbed protein layer is maintained and so there is no change in dissipation shift. The same phenomenon does not occur for PVC control sensors perfused with WPs as no significant amount of plasma proteins is present in platelets suspended in Tyrode's salt solution.

Because the frequency shift at 20 minutes for PVC-control surfaces perfused with PRP or PPP is less than the maximum frequency shift attained, it may be

considered reasonable to use the value of the maximum frequency shift in comparisons between PVC control and MWCNT-swelled PVC surfaces where the frequency shift of the MWCNT-swelled PVC surfaces is greater (using the 20 minutes frequency shift could overestimate differences in frequency shifts between PVC control and MWCNT-swelled PVC surfaces). Both of values of frequency shift are presented.

Protein coating of nanoparticles, i.e. the so called “protein corona”, will be discussed in section 9.8.

9.7 MWCNT-swelled PVC ECC circuit thrombosis *in vivo*.

Although convincing *in vitro* evidence for surface-attached MWCNT-induced platelet activation and aggregation was obtained, it remained to be seen whether this would be mirrored by systemic, cellular, biochemical or biomolecular differences in an animal model of ECC comparing MWCNT-swelled PVC circuits to PVC control circuits. Both rabbit and pig models of extracorporeal circulation have been used previously⁽¹⁵⁷⁾⁽²¹⁸⁾. Rabbit models of ECC permit extracorporeal circuit perfusion for up to 4 hours whereas porcine models have been used successfully for up to 48 hours of ECC. The rabbit model was considered most appropriate for initial experimentation.

The results from the rabbit model of ECC were clear-cut. Within 60 minutes of commencing ECC, 3 out of 4 MWCNT-swelled PVC circuits had become completely thrombosed and occluded. Flow in all four PVC control circuits, on

the other hand, increased over time. Two-dimensional measurement of thrombus area in the thrombogenicity chamber was used to compare the amount of thrombus formed in control and experimental surfaces. Overall, the area of clot in the MWCNT-swelled PVC thrombogenicity chamber was almost twice that of the PVC control chamber.

There were no significant differences in vital signs recorded between animal groups up to one hour. Thereafter, only one circuit survived, precluding statistical analysis. It was similarly impossible to assess for difference in platelet count, platelet aggregation, white cell count, fibrinogen concentration or ACT.

Although it was quite apparent that MWCNT-swelled PVC caused thrombosis within the circuit, effects on the animal as a whole beyond the circuit could not be assessed because of the overwhelming local thrombotic response. An attempt was made, therefore to separate the two processes by infusing systemic heparin for the duration of the experiment in a further two groups of animals. The rationale for this was as follows:

1. Heparin administration would prevent thrombus formation by potentiating the effect of antithrombin III⁽²¹⁹⁾.

Indeed, when heparin was used all circuits survived with no reduction in blood flow over time and there was no thrombus seen in any of the thrombogenicity chambers of circuits from either group. Because of the infusion of heparin to a

target ACT, there was no difference in ACT between groups, as expected. Furthermore, since the major component of thrombus is fibrin, only when thrombus is formed is fibrinogen polymerised to fibrin and plasma levels are thus depleted. It was not expected, therefore, that there would be differences in blood fibrinogen levels between groups. This was, indeed, confirmed confirmed by the appropriate analysis.

2. Heparin administration would not prevent the interaction of platelets with the ECC circuit surface and, therefore, differences in platelet count, aggregometry and p-selectin and CD 61 expression could potentially occur between groups where platelets were affected (either in number or in function) by platelet-surface interaction.

There were, however, no differences between groups in the above parameters. Platelet count fell over time for MWCNT-swelled PVC circuits, but not significantly more so than for PVC control circuits. This was also the case for platelet aggregometry.

P-selectin (CD 62P) is a cellular adhesion molecule, the expression of which is increased by activated platelets when it is translocated to the platelet membrane from cytoplasmic storage granules. CD 61 is the IIIa subunit of the platelet GP IIb/IIIa fibrinogen receptor, the expression of which is also increased by the activation of platelets. The expression of this adhesion molecule and receptor subunit was measured by flow cytometry at baseline (3 minutes after heparin administration) and at 4 hours after commencing ECC.

There was no difference in the magnitude of expression of either of these two proteins between groups at baseline or at 4 hours. In order to show that an increase in expression could be induced *ex vivo*, collagen was added to matched samples from both groups at baseline and at 4 hours. Platelet activation was, indeed, seen in all samples to which collagen was added. There were, however no significant differences in expression between groups. This raises some interesting points. Firstly, the results of flow cytometry do not concur with the results of platelet aggregometry. In aggregometry studies, platelets aggregated less to collagen stimulation as ECC progressed. In flow cytometry, however, collagen stimulation was just as effective in activating platelets at 4 hours as it was at baseline. Secondly, if the results of flow cytometry are considered to be more reliable, then circulating platelets appear to function just as well at the end of ECC as they do prior to ECC. This would be contrary to findings from previous studies that platelets tend to be hypofunctional after ECC^(72–74). Notwithstanding this dilemma, there were no differences between groups in any of these platelet-related parameters.

3. Heparin administration would not prevent the systemic inflammatory response to ECC and, therefore, differences in vital signs, white cell count and monocyte CD 11B and CD 14 expression could potentially occur between groups.

There were, however, no differences between groups in the above parameters. The systemic inflammatory response syndrome (SIRS) could be expected to cause a raise in white cell count over time; however this was not

the case in my experiments. CD 14 is a constitutive monocyte surface receptor and has also been shown to increase in SIRS⁽²²⁰⁾. CD 11B is expressed when monocytes are activated. Monocyte activation as part of the SIRS response to ECC may result in the detection of an increase in CD 11B and CD 14 expression detected by flow cytometry⁽²²¹⁾. Again, there were no differences between baseline and 4 hours on ECC within groups nor were there differences between groups (figure 41). SIRS would also be expected to result in an increase in vasodilation causing hypotension and tachycardia (both intrinsic and as a response to vasodilation). There were no differences between groups in heart rate or blood pressure measurements throughout the duration of the experiments.

In non-heparinised animals, the complex interaction between surface-induced platelet activation and the consequent further interaction between activated platelets and the coagulation cascade led to a massive thrombotic response, so severe as to cause total ECC circuit occlusion in 3 out of 4 animals. Circuit thrombosis and occlusion are the most important and clinically relevant endpoints for the comparison of MWCNT-swelled PVC ECC circuits to PVC control circuits. This is the culmination of the interaction between many individual but interdependent processes. In an attempt to break down this overall process into its component parts, further studies were carried out. In this case, heparin was infused to prevent a thrombotic response and to allow the assessment of the potential systemic effects of blood-surface interaction. No differences between groups in these effects were found. There are a number of possible reasons for this. Firstly, in relation to SIRS, four hours of

ECC might not be long enough for the SIRS response to peak to such a degree that differences between groups become more apparent or more significant. Furthermore, fluid bolus injections were administered to counteract hypotension during ECC thus preventing the non-treated SIRS response to be compared between groups i.e. the lack of differences between groups in heart rate and hypotension more likely reflect the adequate resuscitation of both groups throughout the experiments than the lack of a SIRS response. Unfortunately, an accurate record of total fluid volume administered to both groups was not kept to analyse. Secondly, changes in platelet number and function of those platelets collected systemically may not accurately represent what is actually occurring at the blood-surface interface. If all interaction occurs locally and few activated platelets are released into the circulation, then examination of circulating platelets may not reflect the true nature of platelet-surface interaction. Thirdly, the removal of one component of the thrombotic process (namely, the production of thrombin and its catalysis of the conversion of fibrinogen to fibrin) affects the interaction of many systems as a whole. It is more likely that this 'whole' will result in systemic effects than the component parts acting individually. **Although experiments using systemic heparinisation were not enlightening from a mechanistic standpoint, the overriding message is that MWCNT-swelled PVC causes significantly more thrombosis compared to PVC controls.**

9.8 Evaluation of the role of adsorbed proteins in surface-induced platelet activation.

In order to further explore potential underlying mechanisms for the increase in platelet activation caused by MWCNT-swelled PVC compared to PVC control, proteomics was used for comprehensive analysis of protein content associated with these surfaces.

It has been well documented that nanoparticles in serum attract a protein 'corona' i.e. proteins coating the surface of various nanomaterials, a concept first advanced by Dawson's group⁽²¹⁸⁾. Like the Vroman effect, protein constituents of this corona change over time in a dynamic process. It is suggested that the proteins attached to nanoparticles have a significant impact on the interaction between nanoparticles and cells⁽⁽²²²⁾⁾.

In order to test the hypothesis that the particular protein coating of surface-attached MWCNTs could be a potentially important component of the underlying mechanism of MWCNT-induced platelet activation, proteomic analysis of surface proteins attached to both MWCNT-swelled PVC and PVC control surfaces was performed. The results of this analysis (as it is the case with all proteomics approaches) were semi-quantitative only, as protein retrieval from individual samples was variable and donor plasma from different individuals was used in the preparation of each set of samples. As proteomics generated massive amounts of data, only those proteins that were known to be involved in cell adhesion or platelet activation were identified as potential

targets. There were no obvious differences in the protein constituents between control and experimental surfaces except for vitronectin which had a greater percentage area of the count peak area in all 3 MWCNT-swelled PVC samples. All identified proteins were confirmed by western immunoblotting to be present in donor plasma in order to increase the observation power of my experiments.

In addition to vitronectin, I focused also on fibrinogen and albumin. Fibrinogen was selected because it is known to cause platelet activation after a change in conformation when adsorbed to a foreign surface⁽⁵³⁾. Albumin was selected because it appears to protect against platelet-surface interaction when adsorbed to a foreign surface⁽²²³⁾. Although only two replicates were performed for each experiment, the aim was only to confirm that proteins were actually being absorbed using the chosen technique. Even in the case where multiple replicates allowed statistical analysis of differences in adsorbed masses, interpretation of these data would be difficult as the surface area of the MWCNT-swelled PVC surface is unknown. A fibrinogen binding ELISA was, however, performed in triplicate and showed no difference between MWCNT-swelled PVC surface fibrinogen adsorption and PVC control surface fibrinogen adsorption.

Next, a concentration of each protein corresponding to 1/6 of the concentration of that protein in human plasma was used for testing PRP and WP perfusion under flow conditions.

There were no differences in the effect of proteins on platelet aggregation between experimental and control surfaces. Equally as interesting, however, is the fact that the adsorption of all three proteins had the same effect – attenuation of platelet aggregation. Whereas this was an expected finding for albumin, this was an unexpected finding for fibrinogen. Perhaps, for some reason, fibrinogen did not change conformation when adsorbed and, therefore, acted like soluble free fibrinogen which does not cause platelet activation in plasma when circulating with resting platelets ⁽²²⁴⁾. There have been conflicting results published on the effects of vitronectin on platelets with some studies suggesting that it prevents platelet activation and others suggesting that it promotes platelet activation⁽²²⁵⁾⁽²²⁶⁾. The results of PRP and WP perfusion across vitronectin-adsorbed surfaces clearly demonstrate its attenuating effects on platelet activation in a concentration-dependent manner.

Although the proteomics analysis did not result in a clear-cut mechanistic explanation for MWCNT-swelled PVC surface-induced platelet activation, the effects of plasma proteins cannot be ruled out. Where I have shown that the three selected proteins above can attenuate the surface activation of platelets *in vitro*, it is quite possible that either (1), platelet-activating proteins or combinations of proteins attach to MWCNTs *in vivo* and promote platelet activation or (2), the platelet-attenuating effects of the protein corona *in vivo* is too weak to prevent **direct MWCNT-induced platelet activation**. Either way, plasma proteins appear to be important modulators in MWCNT-platelet interaction.

9.9 Drug-functionalised MWCNTs

It is shown here that surface-immobilised non-functionalised MWCNTs clearly cause platelet activation *in vitro* and thrombosis *in vivo*. The underlying mechanism for the above effects is unclear but may involve direct MWCNT-platelet interaction, platelet activation by proteins attached to MWCNTs or a combination of both. Either way, it would appear that the use of non-functionalised MWCNTs in the body should be avoided.

It has been suggested, however, that chemical functionalisation of MWCNTs could improve their biocompatibility. For example, some studies have shown that carboxylated MWCNTs are more biocompatible than pristine MWCNTs⁽²²⁷⁾⁽²²⁸⁾. Also, functionalised MWCNTs as drug delivery systems are undergoing investigation⁽²²⁹⁾.

Iloprost is a stable synthetic analogue of prostacyclin and is a potent inhibitor of platelet activation⁽²³⁰⁾. Here, MWCNTs were functionalised with iloprost to study the effect that this would have on platelet activation *in vitro*, both in aggregometry and QCM-D studies. Iloprost was chosen because of its potency, stability and chemical structure (to allow relatively straightforward functionalization to MWCNTs)⁽²³¹⁾⁽²³²⁾⁽²²⁹⁾.

Because the first step in iloprost functionalisation is carboxylation, ready-carboxylated MWCNTs were purchased and the drug was attached to these nanomaterials. Indeed, mass spectrometry confirmed the presence of iloprost

in functionalised MWCNTs. As hypothesised, compared to carboxylated MWCNTs, iloprost-functionalised MWCNTs caused significantly less platelet aggregation as demonstrated by platelet aggregometry studies of washed platelets. These experiments show that direct platelet-MWCNT interactions are of great importance in the mechanism of pro-thrombotic effect of these nanomaterials.

Unfortunately, these results could not be replicated when iloprost-functionalised MWCNTs were either swelled onto PVC sensor surfaces after functionalisation or whether functionalisation with iloprost was completed after MWCNT swelling.

There are a number of possible reasons for these results:

1. Although iloprost functionalisation of MWCNTs was successful as evidenced by light aggregometry studies, the ultrasonification necessary for the swelling process may have disrupted the iloprost-MWCNT covalent bond resulting in the absence of iloprost on the sensor surfaces.
2. Iloprost may have attached non-covalently to the MWCNTs as a salt rather than covalently as planned. This bond would almost certainly not survive the ultrasonification process. Indeed, it might not even survive perfusion with PBS (however, should iloprost have been present in the salt form on crystal surfaces, any dislodged iloprost would likely have

been detected by mass spectrometry interrogation of initial PBS perfusate (section 8.5))

3. It is more likely that the tips of the MWCNTs are easier to functionalise than the sidewalls. That may lead to the scenario where adequate covalently bound iloprost that is available to interact with platelets in platelet aggregometry studies becomes 'buried' along with the MWCNT tips in the swelled PVC and is therefore not available to platelets in the QCM-D model. Buried COOH-functionalised tips would also not be available for *in situ* binding of iloprost after ultrasonification.

Despite the lack of technological success with the functionalisation of surface-bound MWCNTs, the proof of principle that MWCNTs can be functionalised with an antiplatelet agent and that this functionalisation attenuates platelet is clearly demonstrated.

10 CONCLUSIONS

Surface bound multi-walled carbon nanotubes cause platelet activation *in vitro* and devastating thrombosis *in vivo* in an animal model of extracorporeal circulation.

The mechanism of the pro-thrombotic effect is uncertain, but likely involves direct nanomaterial-platelet interactions and the interactions mediated by plasma proteins.

Anti-platelet drug functionalisation of MWCNTs with the platelet-inhibitory iloprost attenuates MWCNT-induced platelet activation.

My experiments provide, for the first time, proof-of-concept evidence that modification of medically-relevant surfaces with nanomaterials modulates blood-surface biocompatibility.

11 FUTURE DIRECTIONS

In section 3.9.3 the very preliminary research carried out to date in the area of nanotechnology and blood-surface interaction was outlined. In particular, nanopatterning of surfaces was discussed. Recently, an application was submitted by our group, in conjunction with nanotechnology experts in cell biology, physics and chemistry disciplines, for a European Research Council Synergy Grant. I have largely contributed to the concept and development of this proposal and I would like to be involved in this research, if funded. The main aim of this project is to develop novel nano-enabled approaches for surface modification and to design new nanostructured materials with improved blood compatibility. This will include the imprinting of PVC with nanopatterned dyes. The project design ensures a multidisciplinary approach to this interdisciplinary problem of blood-surface interaction. The experience gained from my work in terms of nanoparticle coating technique, QCM-D experimentation, animal experimentation, proteomics etc. will form the central *in vitro*, *ex vivo* and *in vivo* evaluation components of the project (figure 58).

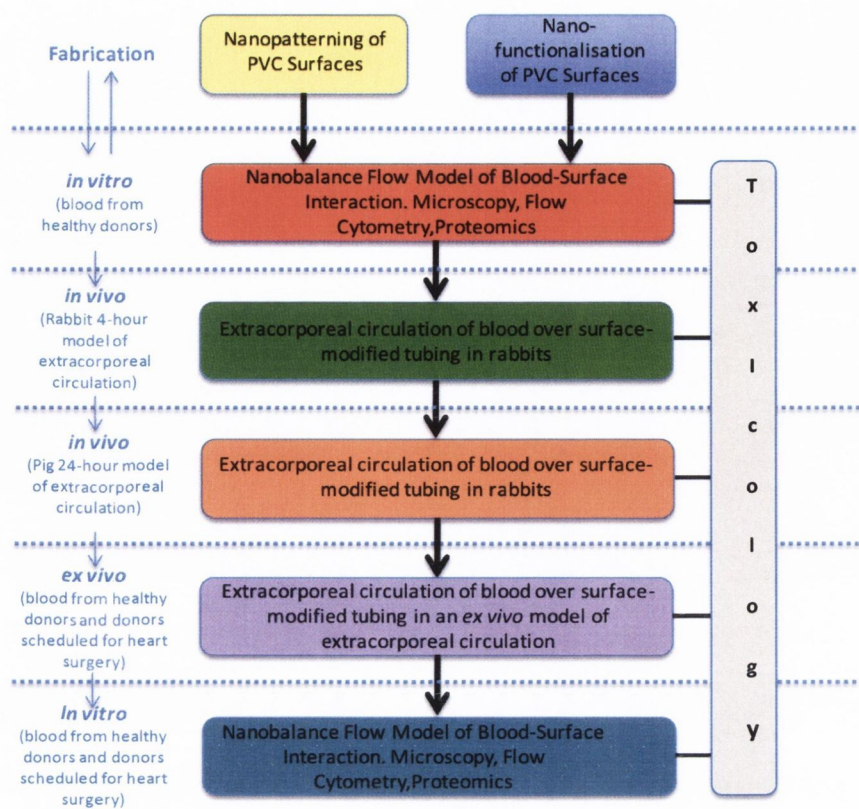


Figure 58 ERC Synergy Project Flow.

12 REFERENCES

1. Williams DF (David F, European Society for Biomaterials. Definitions in biomaterials : proceedings of a consensus conference of the European Society for Biomaterials, Chester, England, March 3-5, 1986 / edited by D.F. Williams. Amsterdam ; New York : Elsevier; 1987.
2. Gaffney AM, Wildhirt SM, Griffin MJ, Annich GM, Radomski MW. Extracorporeal life support. *BMJ*. 2010;341:c5317.
3. Snyder-Ramos SA, Möhnle P, Weng Y-S, Böttiger BW, Kulier A, Levin J, et al. The ongoing variability in blood transfusion practices in cardiac surgery. *Transfusion*. 2008 Jul;48(7):1284–99.
4. Ranucci M, Bozzetti G, Ditta A, Cotza M, Carboni G, Ballotta A. Surgical Reexploration After Cardiac Operations: Why a Worse Outcome? *The Annals of Thoracic Surgery*. 2008 Nov;86(5):1557–62.
5. Extracorporeal Life Support Organization (ELSO) Extracorporeal Life Support (ECLS) Registry Report International Summary. July 2010 Ann Arbor, MI,.
6. Müller F, Mutch NJ, Schenk WA, Smith SA, Esterl L, Spronk HM, et al. Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. *Cell*. 2009 Dec 11;139(6):1143–56.

7. Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC, et al. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. *J. Am. Coll. Cardiol.* 1998 Feb;31(2):352–8.
8. McNicol A, Israels SJ. Beyond hemostasis: the role of platelets in inflammation, malignancy and infection. *Cardiovasc Hematol Disord Drug Targets.* 2008 Jun;8(2):99–117.
9. Kisucka J, Butterfield CE, Duda DG, Eichenberger SC, Saffaripour S, Ware J, et al. Platelets and platelet adhesion support angiogenesis while preventing excessive hemorrhage. *Proc. Natl. Acad. Sci. U.S.A.* 2006 Jan 24;103(4):855–60.
10. Michelson AD. *Platelets.* Second. Elsevier; 2007.
11. Rivera J, Lozano ML, Navarro-Nunez L, Vicente V. Platelet receptors and signaling in the dynamics of thrombus formation. *Haematologica.* 2009 May 1;94(5):700–11.
12. Davi G, Patrono C. Platelet Activation and Atherothrombosis. *N Engl J Med.* 2007 Dec 13;357(24):2482–94.
13. Chung AWY, Jurasz P, Hollenberg MD, Radomski MW. Mechanisms of action of proteinase-activated receptor agonists on human platelets. *Br. J. Pharmacol.* 2002 Mar;135(5):1123–32.

14. Santos-Martínez MJ, Medina C, Jurasz P, Radomski MW. Role of metalloproteinases in platelet function. *Thromb. Res.* 2008;121(4):535–42.
15. Loscalzo J, Pasche B, Ouimet H, Freedman JE. Platelets and plasminogen activation. *Thromb. Haemost.* 1995 Jul;74(1):291–3.
16. Marcus AJ, Safier LB, Kaminski WE, Islam N, Hajjar KA, Jendraschak E, et al. Principles of thromboregulation: control of platelet reactivity in vascular disease. *Adv. Prostaglandin Thromboxane Leukot. Res.* 1995;23:413–8.
17. Marcus AJ, Broekman MJ, Pinsky DJ. COX inhibitors and thromboregulation. *N. Engl. J. Med.* 2002 Sep 26;347(13):1025–6.
18. Radomski MW, Palmer RM, Moncada S. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc. Natl. Acad. Sci. U.S.A.* 1990 Jul;87(13):5193–7.
19. Gkaliagkousi E, Ritter J, Ferro A. Platelet-derived nitric oxide signaling and regulation. *Circ. Res.* 2007 Sep 28;101(7):654–62.
20. Denis CV, Wagner DD. Platelet adhesion receptors and their ligands in mouse models of thrombosis. *Arterioscler. Thromb. Vasc. Biol.* 2007 Apr;27(4):728–39.
21. Musumba CO, Walley T. Aspirin: old and new. *J R Coll Physicians Edinb.* 2006;36:308–11.

22. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *BMJ*. 2002 Jan 12;324(7329):71–86.
23. Baigent C, Blackwell L, Collins R, Emberson J, Godwin J, Peto R, et al. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. *Lancet*. 2009 May 30;373(9678):1849–60.
24. Fowkes FGR, Price JF, Stewart MCW, Butcher I, Leng GC, Pell ACH, et al. Aspirin for Prevention of Cardiovascular Events in a General Population Screened for a Low Ankle Brachial Index. *JAMA: The Journal of the American Medical Association*. 2010 Mar 3;303(9):841 – 848.
25. Ferraris VA, Ferraris SP, Moliterno DJ, Camp P, Walenga JM, Messmore HL, et al. The Society of Thoracic Surgeons practice guideline series: aspirin and other antiplatelet agents during operative coronary revascularization (executive summary). *Ann. Thorac. Surg*. 2005 Apr;79(4):1454–61.
26. Antman EM, Hand M, Armstrong PW, Bates ER, Green LA, Halasyamani LK, et al. 2007 focused update of the ACC/AHA 2004 guidelines for the management of patients with ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *J. Am. Coll. Cardiol*. 2008 Jan 15;51(2):210–47.

27. Becker RC, Meade TW, Berger PB, Ezekowitz M, O'Connor CM, Vorchheimer DA, et al. The primary and secondary prevention of coronary artery disease: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest*. 2008 Jun;133(6 Suppl):776S–814S.
28. Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, et al. Prasugrel versus Clopidogrel in Patients with Acute Coronary Syndromes. *N Engl J Med*. 2007 Nov 15;357(20):2001–15.
29. Meadows TA, Bhatt DL. Clinical aspects of platelet inhibitors and thrombus formation. *Circ. Res*. 2007 May 11;100(9):1261–75.
30. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *N. Engl. J. Med*. 2001 Aug 16;345(7):494–502.
31. Sabatine MS, Cannon CP, Gibson CM, López-Sendón JL, Montalescot G, Theroux P, et al. Addition of clopidogrel to aspirin and fibrinolytic therapy for myocardial infarction with ST-segment elevation. *N. Engl. J. Med*. 2005 Mar 24;352(12):1179–89.
32. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. *Lancet*. 1996 Nov 16;348(9038):1329–39.

33. King SB, Smith SC, Hirshfeld JW, Jacobs AK, Morrison DA, Williams DO, et al. 2007 focused update of the ACC/AHA/SCAI 2005 guideline update for percutaneous coronary intervention: a report of the American College of Cardiology/American Heart Association Task Force on Practice guidelines. *J. Am. Coll. Cardiol.* 2008 Jan 15;51(2):172–209.
34. Mehta RH, Roe MT, Mulgund J, Ohman EM, Cannon CP, Gibler WB, et al. Acute clopidogrel use and outcomes in patients with non-ST-segment elevation acute coronary syndromes undergoing coronary artery bypass surgery. *J. Am. Coll. Cardiol.* 2006 Jul 18;48(2):281–6.
35. Ebrahimi R, Dyke C, Mehran R, Manoukian SV, Feit F, Cox DA, et al. Outcomes following pre-operative clopidogrel administration in patients with acute coronary syndromes undergoing coronary artery bypass surgery: the ACUITY (Acute Catheterization and Urgent Intervention Triage strategY) trial. *J. Am. Coll. Cardiol.* 2009 May 26;53(21):1965–72.
36. Chew DP, Bhatt DL, Sapp S, Topol EJ. Increased mortality with oral platelet glycoprotein IIb/IIIa antagonists: a meta-analysis of phase III multicenter randomized trials. *Circulation.* 2001 Jan 16;103(2):201–6.
37. Mackman N. Triggers, targets and treatments for thrombosis. *Nature.* 2008 Feb 21;451(7181):914–8.

38. Kong DF, Califf RM, Miller DP, Moliterno DJ, White HD, Harrington RA, et al. Clinical outcomes of therapeutic agents that block the platelet glycoprotein IIb/IIIa integrin in ischemic heart disease. *Circulation*. 1998 Dec 22;98(25):2829–35.
39. Stone GW, Grines CL, Cox DA, Garcia E, Tcheng JE, Griffin JJ, et al. Comparison of angioplasty with stenting, with or without abciximab, in acute myocardial infarction. *N. Engl. J. Med*. 2002 Mar 28;346(13):957–66.
40. Cheng DKF, Jackevicius CA, Seidelin P, Feindel C, Rouleau JL. Safety of glycoprotein IIb/IIIa inhibitors in urgent or emergency coronary artery bypass graft surgery. *Can J Cardiol*. 2004 Feb;20(2):223–8.
41. Albers GW, Amarenco P, Easton JD, Sacco RL, Teal P. Antithrombotic and thrombolytic therapy for ischemic stroke: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. *Chest*. 2004 Sep;126(3 Suppl):483S–512S.
42. Halkes PHA, van Gijn J, Kappelle LJ, Koudstaal PJ, Algra A. Aspirin plus dipyridamole versus aspirin alone after cerebral ischaemia of arterial origin (ESPRIT): randomised controlled trial. *Lancet*. 2006 May 20;367(9523):1665–73.

43. Halkes PHA, Gray LJ, Bath PMW, Diener H-C, Guiraud-Chaumeil B, Yatsu FM, et al. Dipyridamole plus aspirin versus aspirin alone in secondary prevention after TIA or stroke: a meta-analysis by risk. *Journal of Neurology, Neurosurgery & Psychiatry*. 2008 Nov 1;79(11):1218 –1223.
44. Aktas B, Utz A, Hoenig-Liedl P, Walter U, Geiger J. Dipyridamole enhances NO/cGMP-mediated vasodilator-stimulated phosphoprotein phosphorylation and signaling in human platelets: in vitro and in vivo/ex vivo studies. *Stroke*. 2003 Mar;34(3):764–9.
45. Uniyal S, Brash JL. Patterns of adsorption of proteins from human plasma onto foreign surfaces. *Thromb. Haemost.* 1982 Jun 28;47(3):285–90.
46. Feuerstein IA, Sheppard JI. States in adherent platelet morphology and the processing of adsorbed protein on biomaterials. *Biomaterials*. 1993;14(2):137–47.
47. DiFazio LT, Stratoulis C, Greco RS, Haimovich B. Multiple platelet surface receptors mediate platelet adhesion to surfaces coated with plasma proteins. *J. Surg. Res.* 1994 Jul;57(1):133–7.
48. Rao G, Chandy T. Role of platelets in blood-biomaterial interactions. *Bulletin of Materials Science*. 1999 May 1;22(3):633–9.
49. Vroman L, Adams AL, Fischer GC, Munoz PC. Interaction of high molecular weight kininogen, factor XII, and fibrinogen in plasma at interfaces. *Blood*. 1980 Jan;55(1):156–9.

50. Andrade JD, Hlady V. Vroman effects, techniques, and philosophies. *J Biomater Sci Polym Ed.* 1991;2(3):161–72.
51. Gravlee GP, Davis RE, Davis RF, Stammers AH, Ungerleider RM. *Cardiopulmonary Bypass.* 2007.
52. Ratner BD. *Biomaterials science.* Academic Press; 2004.
53. Lindon JN, McManama G, Kushner L, Merrill EW, Salzman EW. Does the conformation of adsorbed fibrinogen dictate platelet interactions with artificial surfaces? *Blood.* 1986 Aug;68(2):355–62.
54. Wu Y, Zhang M, Hauch KD, Horbett TA. Effect of adsorbed von Willebrand factor and fibrinogen on platelet interactions with synthetic materials under flow conditions. *J Biomed Mater Res A.* 2008 Jun 1;85(3):829–39.
55. Rinder CS, Rinder HM, Smith BR, Fitch JC, Smith MJ, Tracey JB, et al. Blockade of C5a and C5b-9 generation inhibits leukocyte and platelet activation during extracorporeal circulation. *J. Clin. Invest.* 1995 Sep;96(3):1564–72.
56. Chen JC, Rollins SA, Shernan SK, Boyce S, Allen K, Wallace A, et al. Pharmacologic C5-complement suppression reduces blood loss during on-pump cardiac surgery. *J Card Surg.* 2005 Feb;20(1):35–41.
57. Rinder CS, Bonan JL, Rinder HM, Mathew J, Hines R, Smith BR. Cardiopulmonary bypass induces leukocyte-platelet adhesion. *Blood.* 1992 Mar 1;79(5):1201–5.

58. Kozik DJ, Tweddell JS. Characterizing the Inflammatory Response to Cardiopulmonary Bypass in Children. *Ann Thorac Surg.* 2006 Jun 1;81(6):S2347–2354.
59. Nannizzi-Alaimo L, Rubenstein MH, Alves VL, Leong GY, Phillips DR, Gold HK. Cardiopulmonary bypass induces release of soluble CD40 ligand. *Circulation.* 2002 Jun 18;105(24):2849–54.
60. Ferroni P, Speziale G, Ruvolo G, Giovannelli A, Pulcinelli FM, Lenti L, et al. Platelet activation and cytokine production during hypothermic cardiopulmonary bypass--a possible correlation? *Thromb. Haemost.* 1998 Jul;80(1):58–64.
61. Fergusson DA, Hébert PC, Mazer CD, Fremes S, MacAdams C, Murkin JM, et al. A comparison of aprotinin and lysine analogues in high-risk cardiac surgery. *N. Engl. J. Med.* 2008 May 29;358(22):2319–31.
62. Selnes OA, Gottesman RF, Grega MA, Baumgartner WA, Zeger SL, McKhann GM. Cognitive and neurologic outcomes after coronary-artery bypass surgery. *N. Engl. J. Med.* 2012 Jan 19;366(3):250–7.
63. Gaffney AM, Wildhirt SM, Griffin MJ, Annich GM, Radomski MW. Extracorporeal life support. *BMJ.* 2010;341:c5317.
64. Schuerer DJE, Kolovos NS, Boyd KV, Coopersmith CM. Extracorporeal membrane oxygenation: current clinical practice, coding, and reimbursement. *Chest.* 2008 Jul;134(1):179–84.

65. Peek GJ, Mugford M, Tiruvoipati R, Wilson A, Allen E, Thalanany MM, et al. Efficacy and economic assessment of conventional ventilatory support versus extracorporeal membrane oxygenation for severe adult respiratory failure (CESAR): a multicentre randomised controlled trial. *The Lancet*. 2009 Oct;374(9698):1351–63.
66. ELSO Guidelines for Cardiopulmonary Extracorporeal Life Support. Extracorporeal Life Support Organisation, Version 1:1. Ann Arbor, MI www.else.med.umich.edu. 2009.
67. Holloway DS, Summaria L, Sandesara J, Vagher JP, Alexander JC, Caprini JA. Decreased platelet number and function and increased fibrinolysis contribute to postoperative bleeding in cardiopulmonary bypass patients. *Thromb. Haemost.* 1988 Feb 25;59(1):62–7.
68. Nieuwland R, Berckmans RJ, Rotteveel-Eijkman RC, Maquelin KN, Roozendaal KJ, Jansen PG, et al. Cell-derived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant. *Circulation*. 1997 Nov 18;96(10):3534–41.
69. Beurling-Harbury C, Galvan CA. Acquired decrease in platelet secretory ADP associated with increased postoperative bleeding in post-cardiopulmonary bypass patients and in patients with severe valvular heart disease. *Blood*. 1978 Jul;52(1):13–23.
70. Friedenbergr WR, Myers WO, Plotka ED, Beathard JN, Kummer DJ, Gatlin PF, et al. Platelet dysfunction associated with cardiopulmonary bypass. *Ann. Thorac. Surg.* 1978 Apr;25(4):298–305.

71. McKenna R, Bachmann F, Whittaker B, Gilson JR, Weinberg M. The hemostatic mechanism after open-heart surgery. II. Frequency of abnormal platelet functions during and after extracorporeal circulation. *J. Thorac. Cardiovasc. Surg.* 1975 Aug;70(2):298–308.
72. Mammen EF, Koets MH, Washington BC, Wolk LW, Brown JM, Burdick M, et al. Hemostasis changes during cardiopulmonary bypass surgery. *Semin. Thromb. Hemost.* 1985 Jul;11(3):281–92.
73. Greilich PE, Carr ME, Carr SL, Chang AS. Reductions in platelet force development by cardiopulmonary bypass are associated with hemorrhage. *Anesth. Analg.* 1995 Mar;80(3):459–65.
74. Greilich PE, Brouse CF, Beckham J, Jessen ME, Martin EJ, Carr ME. Reductions in platelet contractile force correlate with duration of cardiopulmonary bypass and blood loss in patients undergoing cardiac surgery. *Thromb. Res.* 2002 Mar 15;105(6):523–9.
75. Brister SJ, Ofosu FA, Buchanan MR. Thrombin generation during cardiac surgery: is heparin the ideal anticoagulant? *Thromb. Haemost.* 1993 Aug 2;70(2):259–62.
76. Hunt BJ, Parratt RN, Segal HC, Sheikh S, Kallis P, Yacoub M. Activation of coagulation and fibrinolysis during cardiothoracic operations. *Ann. Thorac. Surg.* 1998 Mar;65(3):712–8.
77. Horne MK, Chao ES. Heparin binding to resting and activated platelets. *Blood.* 1989 Jul;74(1):238–43.

78. Suda Y, Marques D, Kermode JC, Kusumoto S, Sobel M. Structural characterization of heparin's binding domain for human platelets. *Thromb. Res.* 1993 Mar 15;69(6):501–8.
79. Ajayi AA, Pharmacols FB, Cooper J, Horn EH, Rubin PC. Comparison of the effects of unfractionated heparin and the low-molecular-weight heparins dalteparin and enoxaparin on spontaneous platelet aggregation and adenosine diphosphate activity in platelets during the third trimester of pregnancy. *Methods Find Exp Clin Pharmacol.* 2007 Oct;29(8):539–45.
80. Saba HI, Saba SR, Morelli GA. Effect of heparin on platelet aggregation. *Am. J. Hematol.* 1984 Oct;17(3):295–306.
81. Carr ME, Carr SL, Greilich PE. Heparin ablates force development during platelet mediated clot retraction. *Thromb. Haemost.* 1996 Apr;75(4):674–8.
82. Griffin MJ, Rinder HM, Smith BR, Tracey JB, Kriz NS, Li CK, et al. The effects of heparin, protamine, and heparin/protamine reversal on platelet function under conditions of arterial shear stress. *Anesth. Analg.* 2001 Jul;93(1):20–7.
83. Khuri SF, Valeri CR, Loscalzo J, Weinstein MJ, Birjiniuk V, Healey NA, et al. Heparin causes platelet dysfunction and induces fibrinolysis before cardiopulmonary bypass. *Ann. Thorac. Surg.* 1995 Oct;60(4):1008–14.

84. Salzman EW, Rosenberg RD, Smith MH, Lindon JN, Favreau L. Effect of heparin and heparin fractions on platelet aggregation. *J. Clin. Invest.* 1980 Jan;65(1):64–73.
85. Chong BH, Castaldi PA. Platelet proaggregating effect of heparin: possible mechanism for non-immune heparin-associated thrombocytopenia. *Aust N Z J Med.* 1986 Oct;16(5):715–6.
86. Warkentin TE, Greinacher A, Koster A, Lincoff AM. Treatment and prevention of heparin-induced thrombocytopenia: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest.* 2008 Jun;133(6 Suppl):340S–380S.
87. Warkentin TE, Hayward CP, Boshkov LK, Santos AV, Sheppard JA, Bode AP, et al. Sera from patients with heparin-induced thrombocytopenia generate platelet-derived microparticles with procoagulant activity: an explanation for the thrombotic complications of heparin-induced thrombocytopenia. *Blood.* 1994 Dec 1;84(11):3691–9.
88. Martel N, Lee J, Wells PS. Risk for heparin-induced thrombocytopenia with unfractionated and low-molecular-weight heparin thromboprophylaxis: a meta-analysis. *Blood.* 2005 Oct 15;106(8):2710–5.
89. Horrow JC. Protamine allergy. *J Cardiothorac Anesth.* 1988 Apr;2(2):225–42.

90. Ellison N, Edmunds LH, Colman RW. Platelet aggregation following heparin and protamine administration. *Anesthesiology*. 1978 Jan;48(1):65–8.
91. Carr ME, Carr SL. At high heparin concentrations, protamine concentrations which reverse heparin anticoagulant effects are insufficient to reverse heparin anti-platelet effects. *Thromb. Res.* 1994 Sep 15;75(6):617–30.
92. Mochizuki T, Olson PJ, Szlam F, Ramsay JG, Levy JH. Protamine reversal of heparin affects platelet aggregation and activated clotting time after cardiopulmonary bypass. *Anesth. Analg.* 1998 Oct;87(4):781–5.
93. Barstad RM, Stephens RW, Hamers MJ, Sakariassen KS. Protamine sulphate inhibits platelet membrane glycoprotein Ib-von Willebrand factor activity. *Thromb. Haemost.* 2000 Feb;83(2):334–7.
94. Cobel-Geard RJ, Hassouna HI. Interaction of protamine sulfate with thrombin. *Am. J. Hematol.* 1983 May;14(3):227–33.
95. Okano K, Saito Y, Matsushima A, Inada Y. Protamine interacts with the D-domains of fibrinogen. *Biochim. Biophys. Acta.* 1981 Dec 29;671(2):164–7.
96. Kesteven PJ, Ahmed A, Aps C, Williams BT, Savidge GF. Protamine sulphate and heparin rebound following open-heart surgery. *J Cardiovasc Surg (Torino)*. 1986 Oct;27(5):600–3.

97. Spiess BD, Vocelka C, Cochran RP, Soltow L, Chandler WL. Heparin-coated bypass circuits (Carmeda) suppress the release of tissue plasminogen activator during normothermic coronary artery bypass graft surgery. *J. Cardiothorac. Vasc. Anesth.* 1998 Jun;12(3):299–304.
98. Henry D, Carless P, Fergusson D, Laupacis A. The safety of aprotinin and lysine-derived antifibrinolytic drugs in cardiac surgery: a meta-analysis. *CMAJ.* 2009 Jan 20;180(2):183–93.
99. Poullis M, Manning R, Laffan M, Haskard DO, Taylor KM, Landis RC. The antithrombotic effect of aprotinin: actions mediated via the protease-activated receptor 1. *J Thorac Cardiovasc Surg.* 2000 Aug 1;120(2):370–8.
100. Day JRS, Taylor KM, Lidington EA, Mason JC, Haskard DO, Randi AM, et al. Aprotinin inhibits proinflammatory activation of endothelial cells by thrombin through the protease-activated receptor 1. *J. Thorac. Cardiovasc. Surg.* 2006 Jan;131(1):21–7.
101. Landis C. Why thrombin PAR1 receptors are important to the cardiac surgical patient. *J Extra Corpor Technol.* 2007 Dec;39(4):305–7.
102. Lavee J, Savion N, Smolinsky A, Goor DA, Mohr R. Platelet protection by aprotinin in cardiopulmonary bypass: electron microscopic study. *Ann. Thorac. Surg.* 1992 Mar;53(3):477–81.
103. Landis RC, Asimakopoulos G, Poullis M, Haskard DO, Taylor KM. The antithrombotic and antiinflammatory mechanisms of action of aprotinin. *Ann Thorac Surg.* 2001 Dec 1;72(6):2169–75.

104. Basora M, Gomar C, Escolar G, Pacheco M, Fita G, Rodriguez E, et al. Platelet function during cardiac surgery and cardiopulmonary bypass with low-dose aprotinin. *J. Cardiothorac. Vasc. Anesth.* 1999 Aug;13(4):382–7.
105. Mengistu AM, Röhm KD, Boldt J, Mayer J, Suttner SW, Piper SN. The influence of aprotinin and tranexamic acid on platelet function and postoperative blood loss in cardiac surgery. *Anesth. Analg.* 2008 Aug;107(2):391–7.
106. Shore-Lesserson L. Aprotinin has direct platelet protective properties: fact or fiction? *J. Cardiothorac. Vasc. Anesth.* 1999 Aug;13(4):379–81.
107. Carr ME, Carr SL, Roa V, McCardell KA, Greilich PE. Aprotinin counteracts heparin-induced inhibition of platelet contractile force. *Thrombosis Research.* 2002 Nov 1;108(2-3):161–8.
108. DelRossi AJ, Cernaianu AC, Botros S, Lemole GM, Moore R. Prophylactic treatment of postperfusion bleeding using EACA. *Chest.* 1989 Jul;96(1):27–30.
109. Soslau G, Horrow J, Brodsky I. Effect of tranexamic acid on platelet ADP during extracorporeal circulation. *American Journal of Hematology.* 1991;38(2):113–9.
110. Miyashita T, Kamibayashi T, Ohnishi Y, Kobayashi J, Kuro M. Preservation of collagen-induced whole blood platelet aggregation by tranexamic acid therapy in primary cardiac valve surgery. *Perfusion.* 2000 Nov;15(6):507–13.

111. Greilich PE, Brouse CF, Rinder CS, Smith BR, Sandoval BA, Rinder HM, et al. Effects of epsilon-aminocaproic acid and aprotinin on leukocyte-platelet adhesion in patients undergoing cardiac surgery. *Anesthesiology*. 2004 Feb;100(2):225–33.
112. Watabe A, Ohta M, Matsuyama N, Mizuno K, el Borai N, Tanimoto T, et al. Characterization of plasmin-induced platelet aggregation. *Res. Commun. Mol. Pathol. Pharmacol*. 1997 Jun;96(3):341–52.
113. Riess F-C. Anticoagulation management and cardiac surgery in patients with heparin-induced thrombocytopenia. *Seminars in Thoracic and Cardiovascular Surgery*. 2005 Spring;17(1):85–96.
114. Warkentin TE, Levine MN, Hirsh J, Horsewood P, Roberts RS, Gent M, et al. Heparin-induced thrombocytopenia in patients treated with low-molecular-weight heparin or unfractionated heparin. *N. Engl. J. Med*. 1995 May 18;332(20):1330–5.
115. John LC, Rees GM, Kovacs IB. Different anticoagulants and platelet reactivity in cardiac surgical patients. *Ann. Thorac. Surg*. 1993 Oct;56(4):899–902.
116. Burgess JK, Chong BH. The platelet proaggregating and potentiating effects of unfractionated heparin, low molecular weight heparin and heparinoid in intensive care patients and healthy controls. *Eur. J. Haematol*. 1997 Apr;58(4):279–85.
117. Di Nisio M, Middeldorp S, Buller HR. Direct Thrombin Inhibitors. *N Engl J Med*. 2005 Sep 8;353(10):1028–40.

118. Koster A, Buz S, Krabatsch T, Yeter R, Hetzer R. Bivalirudin anticoagulation during cardiac surgery: a single-center experience in 141 patients. *Perfusion*. 2009 Jan;24(1):7–11.
119. Czosnowski QA, Finks SW, Rogers KC. Bivalirudin for patients with heparin-induced thrombocytopenia undergoing cardiovascular surgery. *Ann Pharmacother*. 2008 Sep;42(9):1304–9.
120. Anand SX, Viles-Gonzalez JF, Mahboobi SK, Heerdt PM. Bivalirudin utilization in cardiac surgery: shifting anticoagulation from indirect to direct thrombin inhibition. *Can J Anaesth* [Internet]. 2010 Dec 7 [cited 2011 Jan 15]; Available from: <http://www.ncbi.nlm.nih.gov.proxy.library.rcsi.ie/pubmed/21136312>
121. Mertzlufft F, Kuppe H, Koster A. Management of urgent high-risk cardiopulmonary bypass in patients with heparin-induced thrombocytopenia type II and coexisting disorders of renal function: use of heparin and epoprostenol combined with on-line monitoring of platelet function. *J. Cardiothorac. Vasc. Anesth*. 2000 Jun;14(3):304–8.
122. Chung A, Wildhirt SM, Wang S, Koshal A, Radomski MW. Combined administration of nitric oxide gas and iloprost during cardiopulmonary bypass reduces platelet dysfunction: a pilot clinical study. *J. Thorac. Cardiovasc. Surg*. 2005 Apr;129(4):782–90.
123. Warkentin TE, Greinacher A. Heparin-induced thrombocytopenia and cardiac surgery. *Ann. Thorac. Surg*. 2003 Dec;76(6):2121–31.

124. Straub A, Schiebold D, Wendel HP, Azevedo R, Dietz K, Ziemer G.
Platelet anaesthesia during extracorporeal circulation: differential effects
of GP IIb/IIIa blockers on platelet activation marker P-selectin
expression at hypothermia. *Thromb Res.* 2008;122(3):383–9.
125. MD YH, BSE NG, MD HLAI, MD JHGI, PhD CM, PhD RJG, et al.
Tirofiban provides “platelet anesthesia” during cardiopulmonary bypass
in baboons, , , ,. *The Journal of Thoracic and Cardiovascular Surgery.*
1997 Jan;113(1):182–93.
126. Hitomi Y, Ikari N, Fujii S. Inhibitory effect of a new synthetic protease
inhibitor (FUT-175) on the coagulation system. *Haemostasis.*
1985;15(3):164–8.
127. Ota T, Okada K, Kano H, Okita Y. Cardiopulmonary bypass using
nafamostat mesilate for patients with infective endocarditis and recent
intracranial hemorrhage. *Interact CardioVasc Thorac Surg.* 2007 Jun
1;6(3):270–3.
128. Benedetto U, Angeloni E, Refice S, Capuano F, Goracci M, Roscitano
A, et al. Is minimized extracorporeal circulation effective to reduce the
need for red blood cell transfusion in coronary artery bypass grafting?
Meta-analysis of randomized controlled trials. *J. Thorac. Cardiovasc.*
Surg [Internet]. 2009 May 22 [cited 2009 Aug 27]; Available from:
<http://www.ncbi.nlm.nih.gov/pubmed/19660383>

129. Mazzei V, Nasso G, Salamone G, Castorino F, Tommasini A, Anselmi A. Prospective randomized comparison of coronary bypass grafting with minimal extracorporeal circulation system (MECC) versus off-pump coronary surgery. *Circulation*. 2007 Oct 16;116(16):1761–7.
130. Castiglioni A, Verzini A, Pappalardo F, Colangelo N, Torracca L, Zangrillo A, et al. Minimally Invasive Closed Circuit Versus Standard Extracorporeal Circulation for Aortic Valve Replacement. *Ann Thorac Surg*. 2007 Feb 1;83(2):586–91.
131. Klinger MH. The storage lesion of platelets: ultrastructural and functional aspects. *Ann. Hematol*. 1996 Sep;73(3):103–12.
132. Cauwenberghs S, van Pampus E, Curvers J, Akkerman J-WN, Heemskerk JWM. Hemostatic and signaling functions of transfused platelets. *Transfus Med Rev*. 2007 Oct;21(4):287–94.
133. Owens M, Holme S, Heaton A, Sawyer S, Cardinali S. Post-transfusion recovery of function of 5-day stored platelet concentrates. *Br. J. Haematol*. 1992 Apr;80(4):539–44.
134. Snyder-Ramos SA, Möhnle P, Weng Y-S, Böttiger BW, Alexander Kulier, Levin J, et al. The ongoing variability in blood transfusion practices in cardiac surgery. *Transfusion*. 2008 Jul;48(7):1284–99.

135. Stover EP, Siegel LC, Parks R, Levin J, Body SC, Maddi R, et al.
Variability in transfusion practice for coronary artery bypass surgery persists despite national consensus guidelines: a 24-institution study. Institutions of the Multicenter Study of Perioperative Ischemia Research Group. *Anesthesiology*. 1998 Feb;88(2):327–33.
136. Spiess BD, Royston D, Levy JH, Fitch J, Dietrich W, Body S, et al.
Platelet transfusions during coronary artery bypass graft surgery are associated with serious adverse outcomes. *Transfusion*. 2004 Aug;44(8):1143–8.
137. Simon TL, Akl BF, Murphy W. Controlled trial of routine administration of platelet concentrates in cardiopulmonary bypass surgery. *Ann. Thorac. Surg.* 1984 May;37(5):359–64.
138. Mangano DT, the Multicenter Study of Perioperative Ischemia Research Group. Aspirin and Mortality from Coronary Bypass Surgery. *N Engl J Med*. 2002 Oct 24;347(17):1309–17.
139. McGrath T, Koch CG, Xu M, Li L, Mihaljevic T, Figueroa P, et al. Platelet Transfusion in Cardiac Surgery Does Not Confer Increased Risk for Adverse Morbid Outcomes. *The Annals of Thoracic Surgery*. 2008 Aug;86(2):543–53.
140. Chevuru SC, Sola MC, Theriaque DW, Hutson AD, Leung WCW, Perez JA, et al. Multicenter analysis of platelet transfusion usage among neonates on extracorporeal membrane oxygenation. *Pediatrics*. 2002 Jun;109(6):e89.

141. Rubens FD, Fergusson D, Wells PS, Huang M, McGowan JL, Laupacis A. Platelet-rich plasmapheresis in cardiac surgery: a meta-analysis of the effect on transfusion requirements. *J. Thorac. Cardiovasc. Surg.* 1998 Oct;116(4):641–7.
142. Wajon P, Gibson J, Calcroft R, Hughes C, Thrift B. Intraoperative plateletpheresis and autologous platelet gel do not reduce chest tube drainage or allogeneic blood transfusion after reoperative coronary artery bypass graft. *Anesth. Analg.* 2001 Sep;93(3):536–42.
143. Christenson JT, Reuse J, Badel P, Simonet F, Schmuziger M. Plateletpheresis before redo CABG diminishes excessive blood transfusion. *Ann. Thorac. Surg.* 1996 Nov;62(5):1373–1378; discussion 1378–1379.
144. Stover EP, Siegel LC, Hood PA, O’Riordan GE, McKenna TR. Platelet-rich plasma sequestration, with therapeutic platelet yields, reduces allogeneic transfusion in complex cardiac surgery. *Anesth. Analg.* 2000 Mar;90(3):509–16.
145. Briffa N. Off pump coronary artery bypass: a passing fad or ready for prime time? *Eur. Heart J.* 2008 Jun;29(11):1346–9.
146. Sokal A, Zembala M, Radomski A, Kocher A, Pacholewicz J, Los J, et al. A differential release of matrix metalloproteinases 9 and 2 during coronary artery bypass grafting and off-pump coronary artery bypass surgery. *J. Thorac. Cardiovasc. Surg.* 2009 May;137(5):1218–24.

147. Møller CH, Steinbrüchel DA. Platelet function after coronary artery bypass grafting: is there a procoagulant activity after off-pump compared with on-pump surgery? *Scand. Cardiovasc. J.* 2003 Jun;37(3):149–53.
148. Valletly MP, Bannon PG, Bayfield MS, Hughes CF, Kritharides L. Quantitative and temporal differences in coagulation, fibrinolysis and platelet activation after on-pump and off-pump coronary artery bypass surgery. *Heart Lung Circ.* 2009 Apr;18(2):123–30.
149. Mejak BL, Stammers A, Rauch E, Vang S, Viessman T. A retrospective study on perfusion incidents and safety devices. *Perfusion.* 2000 Jan 1;15(1):51–61.
150. Gunaydin S, Farsak B, McCusker K, Vijay V, Sari T, Onur MA, et al. Clinical and biomaterial evaluation of hyaluronan-based heparin-bonded extracorporeal circuits with reduced versus full systemic anticoagulation in reoperation for coronary revascularization. *J Cardiovasc Med (Hagerstown).* 2009 Feb;10(2):135–42.
151. Gunaydin S, McCusker K, Vijay V, Isbir S, Sari T, Onur MA, et al. Comparison of polymethoxyethylacrylate-coated circuits with leukocyte filtration and reduced heparinization protocol on heparin-bonded circuits in different risk cohorts. *Perfusion.* 2006 Nov;21(6):329–42.

152. Mirow N, Zittermann A, Koertke H, Maleszka A, Knobl H, Coskun T, et al. Heparin-coated extracorporeal circulation in combination with low dose systemic heparinization reduces early postoperative blood loss in cardiac surgery. *J Cardiovasc Surg (Torino)*. 2008 Apr;49(2):277–84.
153. Agati S, Ciccarello G, Trimarchi ES, Grasso D, Trimarchi G, Di Stefano S, et al. Extracorporeal circulation, optimized: a pilot study. *Artif Organs*. 2007 May;31(5):377–83.
154. Schulze CJ, Han L, Ghorpade N, Etches WS, Stang L, Koshal A, et al. Phosphorylcholine-coated circuits improve preservation of platelet count and reduce expression of proinflammatory cytokines in CABG: a prospective randomized trial. *J Card Surg*. 2009 Aug;24(4):363–8.
155. Lorusso R, De Cicco G, Totaro P, Gelsomino S. Effects of phosphorylcholine coating on extracorporeal circulation management and postoperative outcome: a double-blind randomized study. *Interact Cardiovasc Thorac Surg*. 2009 Jan;8(1):7–11.
156. Gunaydin S. Emerging Technologies in Biocompatible Surface Modifying Additives: Quest for Physiologic Cardiopulmonary Bypass. *Current Medicinal Chemistry - Cardiovascular & Hematological Agents*. 2004 Oct;2(4):295–302.
157. Skrzypchak AM, Lafayette NG, Bartlett RH, Zhengrong Zhou, Frost MC, Meyerhoff ME, et al. Effect of varying nitric oxide release to prevent platelet consumption and preserve platelet function in an in vivo model of extracorporeal circulation. *Perfusion*. 2007 May;22(3):193–200.

158. Annich GM, Meinhardt JP, Mowery KA, Ashton BA, Merz SI, Hirschl RB, et al. Reduced platelet activation and thrombosis in extracorporeal circuits coated with nitric oxide release polymers. *Crit. Care Med.* 2000 Apr;28(4):915–20.
159. Spanier TB, Chen JM, Oz MC, Stern DM, Rose EA, Schmidt AM. Time-dependent cellular population of textured-surface left ventricular assist devices contributes to the development of a biphasic systemic procoagulant response. *J. Thorac. Cardiovasc. Surg.* 1999 Sep;118(3):404–13.
160. Ratner BD, Hoffman AS, Schoen FJ, Lemons JE. *Biomaterials Science: An Introduction to Materials in Medicine.* 2nd ed. Academic Press; 2004.
161. Feynman RP. *Engineering and Science*, Volume 23:5, February 1960. *Engineering and Science.* 1960 Jan 1;23(5):22–36.
162. Bawa R, Bawa SR, Maebius SB, Flynn T, Wei C. Protecting new ideas and inventions in nanomedicine with patents. *Nanomedicine: Nanotechnology, Biology and Medicine.* 2005 Jun;1(2):150–8.
163. ISO Concept Database. Accessed Feb 1 2012 [Internet]. [cited 2012 Feb 1]. Available from:
<https://cdb.iso.org/cdb/termentry!display.action?entry=497541&language=1>
164. Medina C, Santos-Martinez MJ, Radomski A, Corrigan OI, Radomski MW. Nanoparticles: pharmacological and toxicological significance. *Br. J. Pharmacol.* 2007 Mar;150(5):552–8.

165. Roco MC. The long view of nanotechnology development: the National Nanotechnology Initiative at 10 years. *Journal of Nanoparticle Research*. 2011;13(2):427–45.
166. Kim BY, Rutka JT, Chan WC. NanomedicineCurrent Concepts: The New England Journal of Medicine. 2010 Dec 16;363(25):2434–43.
167. Jiang X, Bruzewicz DA, Wong AP, Piel M, Whitesides GM. Directing cell migration with asymmetric micropatterns. *Proc. Natl. Acad. Sci. U.S.A.* 2005 Jan 25;102(4):975–8.
168. Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Geometric control of cell life and death. *Science*. 1997 May 30;276(5317):1425–8.
169. Kilian KA, Bugarija B, Lahn BT, Mrksich M. Geometric cues for directing the differentiation of mesenchymal stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 2010 Mar 16;107(11):4872–7.
170. Yamato M, Kwon OH, Hirose M, Kikuchi A, Okano T. Novel patterned cell coculture utilizing thermally responsive grafted polymer surfaces. *J. Biomed. Mater. Res*. 2001 Apr;55(1):137–40.
171. Fan H, Chen P, Qi R, Zhai J, Wang J, Chen L, et al. Greatly improved blood compatibility by microscopic multiscale design of surface architectures. *Small*. 2009 Oct;5(19):2144–8.

172. Chen L, Han D, Jiang L. On improving blood compatibility: from bioinspired to synthetic design and fabrication of biointerfacial topography at micro/nano scales. *Colloids Surf B Biointerfaces*. 2011 Jun 15;85(1):2–7.
173. Milner KR, Snyder AJ, Siedlecki CA. Sub-micron texturing for reducing platelet adhesion to polyurethane biomaterials. *J Biomed Mater Res A*. 2006 Mar 1;76(3):561–70.
174. Koh LB, Rodriguez I, Venkatraman SS. The effect of topography of polymer surfaces on platelet adhesion. *Biomaterials*. 2010 Mar;31(7):1533–45.
175. Koh LB, Rodriguez I, Venkatraman SS. A novel nanostructured poly(lactic-co-glycolic-acid)–multi-walled carbon nanotube composite for blood-contacting applications: Thrombogenicity studies. *Acta Biomaterialia*. 2009 Nov;5(9):3411–22.
176. Koh LB, Rodriguez I, Zhou J. Platelet adhesion studies on nanostructured poly(lactic-co-glycolic-acid)-carbon nanotube composite. *J Biomed Mater Res A*. 2008 Aug;86(2):394–401.
177. Haubold AD. On the durability of pyrolytic carbon in vivo. *Med Prog Technol*. 1994;20(3-4):201–8.
178. Bernasek TL, Stahl JL, Pupello D. Pyrolytic carbon endoprosthetic replacement for osteonecrosis and femoral fracture of the hip: a pilot study. *Clin. Orthop. Relat. Res*. 2009 Jul;467(7):1826–32.

179. Sweets TM, Stern PJ. Pyrolytic carbon resurfacing arthroplasty for osteoarthritis of the proximal interphalangeal joint of the finger. *J Bone Joint Surg Am*. 2011 Aug 3;93(15):1417–25.
180. Mortier J, Engelhardt M. [Foreign body reaction in carbon fiber prosthesis implantation in the knee joint--case report and review of the literature]. *Z Orthop Ihre Grenzgeb*. 2000 Oct;138(5):390–4.
181. Gutensohn K, Beythien C, Bau J, Fenner T, Grewe P, Koester R, et al. In vitro analyses of diamond-like carbon coated stents. Reduction of metal ion release, platelet activation, and thrombogenicity. *Thromb. Res*. 2000 Sep 15;99(6):577–85.
182. Schnorr JM, Swager TM. Emerging Applications of Carbon Nanotubes†. *Chem. Mater*. 2010;23(3):646–57.
183. Global Industry Analysts, Inc. Carbon Nanotubes - A Global Strategic Business Report. Accessed Feb 3 2012 [Internet]. [cited 2012 Feb 3]. Available from:
http://www.strategyr.com/Carbon_Nanotubes_Market_Report.asp
184. Lacerda L, Bianco A, Prato M, Kostarelos K. Carbon nanotubes as nanomedicines: from toxicology to pharmacology. *Adv. Drug Deliv. Rev*. 2006 Dec 1;58(14):1460–70.
185. Son SJ, Bai X, Lee SB. Inorganic hollow nanoparticles and nanotubes in nanomedicine Part 1. Drug/gene delivery applications. *Drug Discov. Today*. 2007 Aug;12(15-16):650–6.

186. Kostarelos K, Lacerda L, Pastorin G, Wu W, Wieckowski S, Luangsivilay J, et al. Cellular uptake of functionalized carbon nanotubes is independent of functional group and cell type. *Nat Nanotechnol.* 2007 Feb;2(2):108–13.
187. Singh R, Pantarotto D, McCarthy D, Chaloin O, Hoebeke J, Partidos CD, et al. Binding and condensation of plasmid DNA onto functionalized carbon nanotubes: toward the construction of nanotube-based gene delivery vectors. *J. Am. Chem. Soc.* 2005 Mar 30;127(12):4388–96.
188. O'Neal DP, Hirsch LR, Halas NJ, Payne JD, West JL. Photo-thermal tumor ablation in mice using near infrared-absorbing nanoparticles. *Cancer Lett.* 2004 Jun 25;209(2):171–6.
189. Kam NWS, O'Connell M, Wisdom JA, Dai H. Carbon nanotubes as multifunctional biological transporters and near-infrared agents for selective cancer cell destruction. *Proc. Natl. Acad. Sci. U.S.A.* 2005 Aug 16;102(33):11600–5.
190. Wang J. Carbon-Nanotube Based Electrochemical Biosensors: A Review. *Electroanalysis.* 2005 Jan 1;17(1):7–14.
191. Aslan S, Loebick CZ, Kang S, Elimelech M, Pfefferle LD, Tassel PRV. Antimicrobial biomaterials based on carbon nanotubes dispersed in poly(lactic-co-glycolic acid). *Nanoscale.* 2010 Sep 1;2(9):1789–94.
192. White AA, Best SM, Kinloch IA. Hydroxyapatite–Carbon Nanotube Composites for Biomedical Applications: A Review. *International Journal of Applied Ceramic Technology.* 2007 Jan 1;4(1):1–13.

193. Bhattacharya M, Wutticharoenmongkol-Thitiwongsawet P, Hamamoto DT, Lee D, Cui T, Prasad HS, et al. Bone formation on carbon nanotube composite. *J Biomed Mater Res A*. 2011 Jan;96(1):75–82.
194. Fabbro A, Cellot G, Prato M, Ballerini L. Interfacing neurons with carbon nanotubes: (re)engineering neuronal signaling. *Prog. Brain Res*. 2011;194:241–52.
195. Lam C-W, James JT, McCluskey R, Hunter RL. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. *Toxicol. Sci*. 2004 Jan;77(1):126–34.
196. Mitchell LA, Gao J, Wal RV, Gigliotti A, Burchiel SW, McDonald JD. Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes. *Toxicol. Sci*. 2007 Nov;100(1):203–14.
197. Johnston HJ, Hutchison GR, Christensen FM, Peters S, Hankin S, Aschberger K, et al. A critical review of the biological mechanisms underlying the in vivo and in vitro toxicity of carbon nanotubes: The contribution of physico-chemical characteristics. *Nanotoxicology*. 2010 Jun;4(2):207–46.
198. Poland CA, Duffin R, Kinloch I, Maynard A, Wallace WAH, Seaton A, et al. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. *Nat Nanotechnol*. 2008 Jul;3(7):423–8.

199. Muller J, Delos M, Panin N, Rabolli V, Huaux F, Lison D. Absence of carcinogenic response to multiwall carbon nanotubes in a 2-year bioassay in the peritoneal cavity of the rat. *Toxicol. Sci.* 2009 Aug;110(2):442–8.
200. Singh R, Pantarotto D, Lacerda L, Pastorin G, Klumpp C, Prato M, et al. Tissue biodistribution and blood clearance rates of intravenously administered carbon nanotube radiotracers. *Proc. Natl. Acad. Sci. U.S.A.* 2006 Feb 28;103(9):3357–62.
201. Yang S, Guo W, Lin Y, Deng X, Wang H, Sun H, et al. Biodistribution of Pristine Single-Walled Carbon Nanotubes In Vivo†. *J. Phys. Chem. C.* 2007;111(48):17761–4.
202. Radomski A, Jurasz P, Alonso-Escolano D, Drews M, Morandi M, Malinski T, et al. Nanoparticle-induced platelet aggregation and vascular thrombosis. *Br. J. Pharmacol.* 2005 Nov;146(6):882–93.
203. Lovat V, Pantarotto D, Lagostena L, Cacciari B, Grandolfo M, Righi M, et al. Carbon nanotube substrates boost neuronal electrical signaling. *Nano Lett.* 2005 Jun;5(6):1107–10.
204. Bianco A, Kostarelos K, Prato M. Making carbon nanotubes biocompatible and biodegradable. *Chem. Commun. (Camb.).* 2011 Oct 7;47(37):10182–8.

205. Jain S, Thakare VS, Das M, Godugu C, Jain AK, Mathur R, et al. Toxicity of multiwalled carbon nanotubes with end defects critically depends on their functionalization density. *Chem. Res. Toxicol.* 2011 Nov 21;24(11):2028–39.
206. Murugesan S, Park T-J, Yang H, Mousa S, Linhardt RJ. Blood Compatible Carbon Nanotubes – Nano-based Neoproteoglycans. *Langmuir.* 2006;22(8):3461–3.
207. Yusof AM, Buang NA, Yean LS, Ibrahim ML, Rusop M, Soga T. The Use of Multi-Walled Carbon Nanotubes as Possible Carrier in Drug Delivery System for Aspirin. *AIP*; 2009 [cited 2012 Feb 6]. p. 390–4. Available from: <http://eprints.utm.my/13147/>
208. Radomski M, Moncada S. An improved method for washing of human platelets with prostacyclin. *Thromb. Res.* 1983 May 15;30(4):383–9.
209. O'Connor I, De S, Coleman JN, Gun'ko YK. Development of transparent, conducting composites by surface infiltration of nanotubes into commercial polymer films. *Carbon.* 2009 Jul;47(8):1983–8.
210. Born G, Cross M. The aggregation of blood platelets. *The Journal of physiology.* 1963;168(1):178.
211. Sauerbrey G. The use of quartz oscillators for weighing thin layers and for microweighing. *Zeitschrift Für Physik.* 1959;155:206–22.

212. Santos-Martinez MJ, Inkielewicz-Stepniak I, Medina C, Rahme K, D'Arcy DM, Fox D, et al. The use of quartz crystal microbalance with dissipation (QCM-D) for studying nanoparticle-induced platelet aggregation. *Int J Nanomedicine*. 2012;7:243–55.
213. Höök F, Rodahl M, Brzezinski P, Kasemo B. Energy Dissipation Kinetics for Protein and Antibody–Antigen Adsorption under Shear Oscillation on a Quartz Crystal Microbalance. *Langmuir*. 1998;14(4):729–34.
214. Santos-Martinez M, Medina C, Prina-Mello A, Conroy J, Samuels S, Volkov Y, et al. A nanoscale resolution assay of flow-induced platelet microaggregation. *Kardiochirurgia i Torakochirurgia Polska*. 2010;7(4):365–75.
215. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*. 1976;72(1):248–54.
216. Gjerde K, Kjelstrup-Hansen J, Clausen CH, Teo KBK, Milne WI, Rubahn H-G, et al. Carbon nanotube forests: a non-stick workbench for nanomanipulation. *Nanotechnology*. 2006 Oct 14;17(19):4917–22.
217. Cattaneo M, Lecchi A, Zighetti ML, Lussana F. Platelet aggregation studies: autologous platelet-poor plasma inhibits platelet aggregation when added to platelet-rich plasma to normalize platelet count. *Haematologica*. 2007 May 1;92(5):694–7.

218. Tofukuji M, Stahl GL, Metais C, Tomita M, Agah A, Bianchi C, et al. Mesenteric dysfunction after cardiopulmonary bypass: role of complement C5a. *Ann. Thorac. Surg.* 2000 Mar;69(3):799–807.
219. Gravlee GP, Haddon WS, Rothberger HK, Mills SA, Rogers AT, Bean VE, et al. Heparin dosing and monitoring for cardiopulmonary bypass. A comparison of techniques with measurement of subclinical plasma coagulation. *J. Thorac. Cardiovasc. Surg.* 1990 Mar;99(3):518–27.
220. Bouma M, Maessen J, Weerwind P, Dentener M, Fransen E, de Jong D, et al. Release of lipopolysaccharide toxicity-modulating proteins in patients undergoing cardiopulmonary bypass using noncoated and heparin-coated extracorporeal circuits. A clinical pilot study. *Chest.* 1997 Mar;111(3):577–83.
221. Huang W-D, Guo J-T, Liu X, Hong X-O, Xu J-J, Huang S-W, et al. [CD11b expression in neutrophils and lymphocytes of children with systemic inflammatory response syndrome]. *Zhongguo Dang Dai Er Ke Za Zhi.* 2009 Jul;11(7):540–2.
222. Lynch I, Salvati A, Dawson KA. Protein-nanoparticle interactions: What does the cell see? *Nature Nanotechnology.* 2009 Sep 1;4(9):546–7.
223. Amiji, Park H, Park K. Study on the prevention of surface-induced platelet activation by albumin coating. *Journal of biomaterials science Polymer edition.* 1992;3(5):375–88.

224. Endenburg SC, Lindeboom-Blokzijl L, Zwaginga JJ, Sixma JJ, de Groot PG. Plasma Fibrinogen Inhibits Platelet Adhesion in Flowing Blood to Immobilized Fibrinogen. *Arterioscler Thromb Vasc Biol.* 1996 May 1;16(5):633–8.
225. Wu Y-P, Bloemendal HJ, Voest EE, Logtenberg T, de Groot PG, Gebbink MFBG, et al. Fibrin-incorporated vitronectin is involved in platelet adhesion and thrombus formation through homotypic interactions with platelet-associated vitronectin. *Blood.* 2004 Aug 15;104(4):1034–41.
226. Reheman A, Gross P, Yang H, Chen P, Allen D, Leytin V, et al. Vitronectin stabilizes thrombi and vessel occlusion but plays a dual role in platelet aggregation. *J. Thromb. Haemost.* 2005 May;3(5):875–83.
227. Iannazzo D, Piperno A, Ferlazzo A, Pistone A, Milone C, Lanza M, et al. Functionalization of multi-walled carbon nanotubes with coumarin derivatives and their biological evaluation. *Org. Biomol. Chem.* 2012 Jan 12;10(5):1025–31.
228. Burke AR, Singh RN, Carroll DL, Owen JD, Kock ND, D'Agostino R Jr, et al. Determinants of the thrombogenic potential of multiwalled carbon nanotubes. *Biomaterials.* 2011 Sep;32(26):5970–8.
229. Azizian J, Tahermansouri H, Biazar E, Heidari S, Khoei DC. Functionalization of carboxylated multiwall nanotubes with imidazole derivatives and their toxicity investigations. *Int J Nanomedicine.* 2010;5:907–14.

230. Saniabadi AR, Lowe GD, Belch JJ, Forbes CD, Prentice CR, Barbenel JC. The novel effect of a new prostacyclin analogue ZK36374 on the aggregation of human platelets in whole blood. *Thromb. Haemost.* 1983 Oct 31;50(3):718–21.
231. Darius H, Hossmann V, Schrör K. Antiplatelet effects of intravenous iloprost in patients with peripheral arterial obliterative disease. *Klinische Wochenschrift.* 1986 Jun;64(12):545–51.
232. Grant SM, Goa KL. Iloprost. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in peripheral vascular disease, myocardial ischaemia and extracorporeal circulation procedures. *Drugs.* 1992 Jun;43(6):889–924.