Doctoral Thesis for the degree of Doctor of Medicine

Artificial blood vessels

Studies on endothelial cell and blood interactions with bacterial cellulose

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A Doctoral Thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted, or in manuscript).

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EXPERTISSUES

To my mother and brother

Artificial blood vessels

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Abstract

Cardiovascular disease is still the number one cause of death or invalidity in the Western world today. Atherosclerotic plaques and restenosis can result in severe occlusions of peripheral and coronary arteries. Treatment depends on the severity of the disease and includes drug therapy and bypass surgery. Generally, autologous vessels are used as replacement grafts and are the first choices as vascular graft materials. However, if the patient does not have vessels with sufficient quality as a result of previous operations or other diseases, artificial grafts may be used to replace vessels. Available materials are limited to substitution of large vessels (>5 mm) because of frequent thrombosis and occlusion of small diameter grafts. About 10% of patients with coronary artery disease are therefore left untreated. Considering the large number of patients in the need of replacement grafts, the demand for an alternative small-caliber graft is enormous and has driven scientists to search for new materials. Bacterial cellulose (BC) has unique qualities and is an interesting material for vascular grafts.

In this Thesis, bacterial cellulose has been investigated as a potential new vascular graft material by evaluating cell and blood interactions with BC. The specific aims were to evaluate whether surface modifications could promote adhesion of human endothelial cells and to investigate the thrombogenic properties of BC as compared with conventional graft materials.

Modification of BC with a novel technique, using xyloglucan as a carrier molecule for the adhesion-promoting peptide RGD resulted in increased cell adhesion, metabolism and cell spreading. Luminal coating of BC tubes with fibrin glue resulted in increased cell adhesion in static experiments and good cell retention under physiological shear stress.

The evaluation of thrimbogenicity in human blood plasma revealed that BC induces slower coagulation than clinically available materials such as Gore-Tex® and Dacron®. In addition, BC induced the least contact activation evaluated by XIIa generation.
A Chandler loop system with freshly drawn blood showed that BC consumed low quantities of platelets and generated low thrombin values as compared with Dacron® and Gore-Tex®.

This Thesis shows that BC is a promising, novel vascular graft material with low thrombogenicity and promising endothelial cell adhesion.

Keywords: bacterial cellulose, endothelial cells, vascular grafts, cell adhesion, thrombogenicity, RGD, xyloglucan, bioreactor, imaging of coagulation, contact activation

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Populärvetenskaplig sammanfattning

Hjärtsjukdomar är idag en av de vanligaste dödsorsakerna i den vuxna befolkningen. Åderförkalkning är den mest frekventa orsaken till hjärt-kärlskjukdom och kan leda till igentäppning, s.k. förträngningar, av blodkärl i både armar och ben, men även i de små kärlen som försörjer hjärtat med blod. Den ledande behandlingsmetoden vid svåra förträngningar är idag by-pass-kirurgi. I ett sådant ingrepp tas en bit ven från exempelvis patientens ben som sedan används för att leda blodet förbi förträngningen. Problem uppstår då patienten inte har några lämpliga kärl. Då kan man använda sig av konstgjorda ”blodkärl” som är tillverkade av syntetiska material som Gore-Tex® eller Dacron®. De fungerar bra som reservdelar för större kärl, men inte för mindre, exempelvis kranskärlen, som försörjer hjärtat med blod. I dessa kärl bildas nämligen proppar och nya förträngningar. Med tanke på det stora antalet hjärtsjuka patienter som finns idag är behovet av konstgjorda blodkärl stort.

Syftet med min forskning är att undersöka om bakteriell cellulosa (BC) kan användas för att göra konstgjorda blodkärl som kan användas i bypass-operationer för att ersätta små blodkärl. Cellulosa som produceras av bakterien *Acetobacter Xylinum* har många speciella egenskaper vilket gör det till ett lämpligt material för ersättning av kärl. Det är starkt nog att klara blodtrycket och växer in bra i kroppens egen vävnad.

Det är viktigt att material inte ger upphov till blodproppar när det kommer i kontakt med blod. I avhandlingen undersöktes hur blod fungerar i kontakt med BC. Då BC jämfördes med två andra syntetiska material, som idag främst används för bypass-operationer, visade det sig att BC knappt ger någon som helst bildning av blodproppar. Koaglen bildas också mycket långsammare än på de andra materialen. Det kan vara mycket fördelaktigt, då kroppen ges tid att bryta ner små koagel.

Blodkärlen täcks av celler som ser till att blodet normalt inte bildar proppar. Därför är det viktigt att dessa celler växer på materialet. En del av avhandlingen utgörs av utvärdering av hur dessa celler växer på BC. För att utvärdera om ett material är lämpligt som konstgjort blodkärl måste man undersöka hur cellerna, som normalt finns i ett kärl, fungerar på det konstgjorda materialet. Endotelceller är i detta sammanhang mycket viktiga. De täcker blodkärlens insida och utgör gränsskiktet mellan blodet och resten av blodkärlsväggen och ser till att blodet normalt inte bildar proppar.

I den här avhandlingen har BC modifierats för att dessa celler bättre ska fästa till materialet. Det är en helt ny metod som visat öka mängden celler som fäster och som växer på BC utan att förändra strukturen på cellulosan.

Sammanfattningsvis visar avhandlingen att BC är ett intressant material för användning som konstgjorda blodkärl.

List of Papers

This Thesis is based upon the following papers, referred to in the text by their roman numerals:

I. Modification of Nanocellulose with a Xyloglucan-RGD Conjugate Enhances Adhesion and Proliferation of Endothelial Cells: Implications for Tissue Engineering.

Bodin A., Bäckdahl H., Fink H., Brumer H., Risberg B., Gatenholm P.
Biomacromolecules. 2007 Dec;8(12):3697-704.

II. Bacterial cellulose modified with xyloglucan bearing the adhesion peptide RGD promotes endothelial cell adhesion and metabolism - a promising modification for vascular grafts

Fink H., Ahrenstedt L., Bodin A., Brumer H., Gatenholm P., Krettek A., Risberg B.
Submitted to Journal of Tissue Engineering and Regenerative Medicine

III. Real time measurements of coagulation on bacterial cellulose and conventional vascular graft materials

Fink H., Faxälv L., Drotz K., Risberg B., Lindahl T., Sellborn A.
Acta Biomaterialia. Sept 2009 [Epub ahead of print]

IV. An in vitro study of blood compatibility of bacterial cellulose

Fink H., Hong J., Drotz K., Risberg B., Sanchez J., Sellborn A.
In manuscript

V. In vitro evaluation of endothelial cells on fibrin-coated Bacterial cellulose exposed to shear stress

Fink H., Skog A., Drotz K., Redl H., Risberg B., Gatenholm P.
In manuscript

Contribution to papers not included in the Thesis:

Influence of cultivation conditions on mechanical and morphological properties of bacterial cellulose tubes
Bodin A., Bäckdahl H., Fink H., Gustavsson L., Gatenholm P.
Biotechnol Bioeng. 2007 Jun 1;97(2):425-34

Effect of shear stress on the expression of coagulation and fibrinolytic factors in both smooth muscle and endothelial cells in a co-culture model
Helenius G., Hagvall S.H., Esguerra M., Fink H., Soderberg R., Risberg B.
Eur Surg Res. 2008;40(4):325-32.

Intravital fluorescent microscopical evaluation of bacterial cellulose as scaffold for vascular grafts.
Esguerra M., Fink H., Laschke M., Delbro D., Jeppsson A, Menger M, Gatenholm P and Risberg B.
J Biomed Mater. Res. 2009 Jun 17. [Epub ahead of print]

Implantable Materials comprising cellulose and the glycopeptides xyloglucan-GRGDS
Fink H., Bodin A., Ahrenstedt L., Risberg B., Gatenholm P., Brumer H.
Appl No WO/2008/104528

Abbreviations

AT	Antithrombin
BC	Bacterial cellulose
CS	Complemen system
BrdU	5-bromo-2'-deoxyuridine
DAPI	4´,6-diamidino-2-phenylindole
ECs	Endothelial cells
ECM	Extra cellular matrix
ELISA	Enzyme-Linked Immuno Sorbent Assay
ePTFE	extended Poly(tetrafluoroethylene)
FITC	Flourescein isothiocyanate
Hep	Heparin
HSVEC	Human saphenous vein endothelial cells
NO	Nitric oxide
PAI-1	Plasmingoen activator inhibitor 1
PECAM-1	Platelet endothelial cell adhesion molecule 1
PET	Poly(ethylene terephtalate)
PFP	Platelet free plasma
QCM-D	Quarts Crystal Microbalance with Dissipation monitoring
RGD	(Arg–Gly–Asp) peptide
SEM	Scanning electron microscopy
TAT	Thrombin-antithrombin complex
TEBV	Tissue engineered blood vessel
TCC	Terminal complement complex (sC5b-9)
TCP	Tissue culture plastic
TF	Tissue factor
TM	Thrombomodulin
t-PA	tissue-type plasminogen activator
vWF	von Willebrand Factor
XG	Xyloglucan
XG-RGD	xyloglucan-GRGDS-conjugate

Table of contents

1. Introduction

Cardiovascular disease is a major health problem resulting in suffering for the individual and a high economic burden for society. The need for artificial vessels has motivated scientists to develop new materials and, in later years, even completely biological vessels which however, have to be grown for many months and are not a solution to acute situations such as heart infarction, where a substitute is needed immediately.

The development of biomaterials is not a new area of science, having existed for half a century, and has already resulted in successful replacement of heart valves, hip and knee joints, intraocular lenses and development of dental implants.

In recent decades, the field of biomaterials has grown, and together with advances in cell and molecular biology, tissue engineering (TE) has evolved as its own scientific discipline. The challenge is to design scaffolds that interact with biological systems and promote cell attachment and function, e.g. cellular differentiation.

Bacterial cellulose (BC) is an interesting material for biomaterial applications. It has unique properties that make it an exciting candidate as a vascular graft material: strength, good integration into host tissue, and flexibility of production in various shapes and sizes.

Materials intended as vascular grafts must satisfy many important features such as blood compatibility, cell interactions and mechanical properties.

The present Thesis comprises two different approaches to enhance endothelial cell adhesion to BC and the evaluation of the thrombogenic properties of BC as compared with conventional graft materials.

1.1. Why are artificial blood vessels needed?

Cardiovascular diseases (CVDs) are still the number one cause of death or invalidity in the western world today. Cardiovascular diseases alone account for approximately 30% of all global deaths, and in 2005 an estimated 17.5 million people died from CVDs. The World Health Organisation (WHO) estimates, that if current trends are allowed to continue, by 2015 20 million people will die [1, 2]. The CVDs are a group of disorders that affect the heart and

blood vessels including coronary heart disease, cerebrovascular disease and peripherial arterial disease, deep vein thrombosis and pulmonary embolism. The main cause of these acute life-threatening conditions is atherosclerosis. Atherosclerotic plaques and restenosis can result in severe occlusions of peripheral and coronary arteries [3]. Treatment depends on the severity of the disease and includes drug therapy, coronary artery angioplasty and bypass surgery. Generally, autologous saphenouse veins or mammary arteries are used as replacement grafts and are the first choices as vascular graft materials. However, if the patient does not have vessels with sufficient quality, as a result of previous operations or other diseases, artificial grafts may be used.

Today, biomaterials such as polytetrafluorethylene (ePTFE) and polyethylene terephtalate fibre (Dacron®) are in use in the clinic as prosthetic grafts for reconstructive vascular surgery. In small diameter vessels (>6mm) like coronary arteries their performance is dismal, resulting in early thrombosis and intimal hyperplasia. They only function satisfactorily in large-diameter, high flow vessels. Approximately 10% of patients with coronary artery disease are therefore left untreated [4]. Tissue engineered blood vessels could be a solution to this problem.

1.2. The native blood vessel

Human blood vessels consist of three different layers from the luminal side outwards: *tunica intima, tunica media and tunica adventitia* (Figure 1).

The composition and structure of these layers depend greatly on the position of the vessel in the vascular tree, e.g. size and type of vessels. Since arteries transport blood under high pressure in contrast to veins, the walls of arteries are thicker and more elastic and muscular than those of veins. The capillaries, the smallest vessels, are an exception from the general structure. Their function is to supply the surrounding tissue with oxygen. To permit oxygen diffusion, the capillary walls are only one cell thick.

The *intima* is the innermost layer of arteries and veins and consists of a monolayer of endothelial cells, called the endothelium, with a thin underlying basal lamina of connective tissue, the *lamina propria intimae*. In large elastic arteries such as the aorta, the intima has a third component called the *sub-endothelial* layer, which contains smooth muscle cells,

collagen, elastic fibrils and a few fibroblasts. Endothelial cells in the intima are inter-connected with tight occluding junctions (*zonulae occludentes*) that regulate the transport of molecules across the endothelial monolayer as well as with in-plane communication junctions (*gap junctions; maculae communicantes*), which allow cell-to-cell-communication via the transport of ions and metabolites. Other structures involved in transendothelial transport are the pinocytic vesicles, or *Weibel-Palade bodies,* that are mainly connected to the luminal and the basal cell membranes. In arteries, the endothelial cells are flat, elongated and oriented in the direction of the blood flow. Endothelial cells communicate with underlying smooth muscle cells directly through processes that extend through the basal lamina and into the media. The main function of the basal lamina is to provide an adherent network, which consists of an extracellular matrix (ECM) of type IV collagen, laminin, fibronectin and proteoglycans on which endothelial cells can grow. It also provides structural support to the arterial wall.

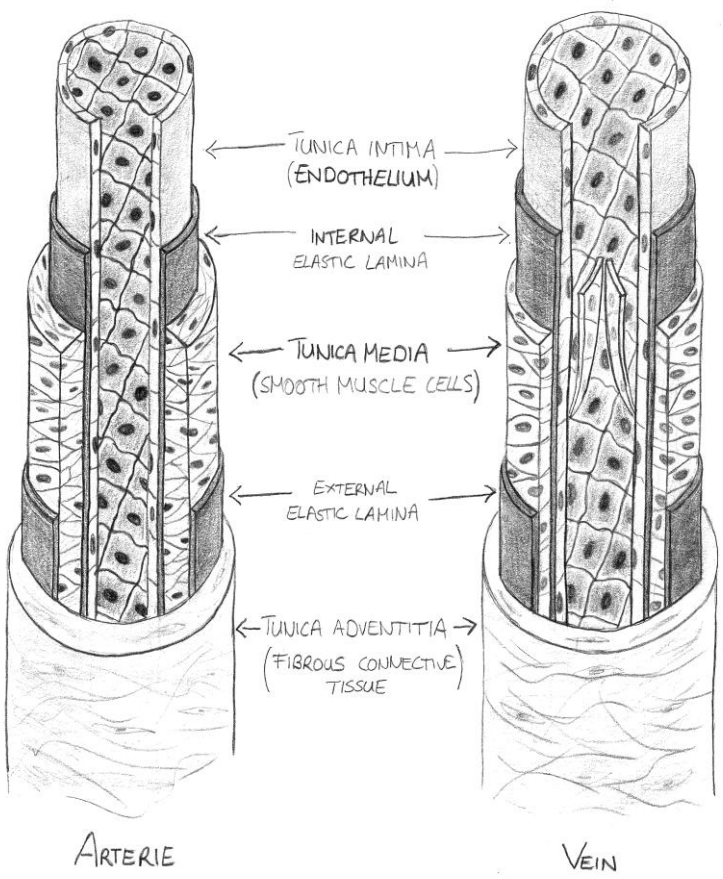

Figure 1. Schematic illustration of blood vessels.

The *media* contains only smooth muscle cells embedded in an ECM consisting of elastin, collagen, fibronectin and proteoglycans. The smooth muscle cells are spindle-shaped, with their myofibrils orientated along the long axis of the cell and covered by a thin basement membrane. This layer gives the vessels their contracting and relaxing properties and is thicker in arteries than in veins. In the aorta, the media may reach a thickness of 500 μm in contrast to 20-50 μm thickness in medium-sized veins.

The *tunica adventitia* is the outermost layer and consists of soft connective tissue, mainly containing type I collagen mixed with elastin, nerves, fibroblasts and *vasa vasorum*. The vasa vasorum is a network of arterioles, capillaries and venules that supply the outer vessel walls of thick arteries with oxygen. The adventitia may serve as a protective sheet and allows for innervation of smooth muscle cells from the outer media [5-7].

1.3. Tissue engineering of blood vessels (TEBV)

1.3.1. Tissue engineering

Tissue engineering is a relatively new scientific discipline that combines cells, engineering and materials to improve or replace biological functions. Tissue engineering is described by Langer and Vacanti, two pioneers in the field, as an interdisciplinary area which applies the principles of engineering and life sciences to the development of biological substitutes that can restore, maintain or improve tissue formation [8].

The basic concept of tissue engineering includes a physical support (3D-scaffold) composed of synthetic polymers or natural materials (collagen, elastin or fibrin) that initially serves as a scaffold or template mimicking the ECM on which cells can organize and mature *in vitro* prior to implantation at the appropriate location.

Initial research in the mid twentieth century focused on developing bioinert materials, eliciting a minimal host response, characterized by passive blood transport and minimal interactions with blood and tissues. These were widely available industrial materials such as Teflon and silicone which were not specifically developed for medical applications. Later, it became unrealistic to produce completely non-reactive substances.

Today, other biomaterials are being developed to stimulate reactions between proteins and cells at the molecular level in a manner that is highly precise and controllable. The key concept underpinning development of these biomaterials is that the scaffold should contain chemical or structural information that mimics cell-cell-communication and controls tissue formation, such as growth factors, the adhesion peptide RGD (Arg–Gly–Asp) and other molecules mimicking the ECM components. This peptide is the minimal sequence in basement membrane proteins such as fibronectin, fibrinogen and von Willebrand Factor, which is required for cell adhesion [9].

1.3.2 Biomaterials/Biomaterial scaffolds

Williams defines a biomaterial as any material, natural or man-made, that comprises the whole or part of a living structure or biomedical device which performs, augments, or replaces a natural function [10].

Many different materials have been investigated for biomaterial applications. They can be divided into natural materials and synthetic polymers (Table 1). The required properties for a biomaterial vary with cell type, implantation site and strategy for tissue formation. Common demands for all biomaterials are biocompatibility, e.g., avoiding foreign body reactions, capsule formation and chronic inflammatory reactions. Additionally, for materials intended to be in contact with blood, thrombogenicity has to be evaluated. Mechanical properties are important and depend on the target tissue. For vascular grafts, the material has to withstand the blood pressure and hence burst pressure, compliance, suture strength and fatigue have to be investigated before they can be used as implants.

Electrospinning of different materials to create nano fibre constructs has over the last years grown to become a popular approach. Both electrospun synthetic polymers and native ECM proteins have been used for cell seeding to construct vascular grafts [11-14].

1.3.3 Materials for vascular grafts

Jaboulay and Briau made the first arterial transplantation in 1896, but the anastomoses were imperfect, resulting in thrombosis [15]. Since then, more sophisticated techniques have been developed and today arterial, and and even more so venous autografts, are routinely used in surgery to create bypasses for patients with peripheral or coronary occlusive vascular diseases. However, the availability of autografts is limited, for arteries in particular. The

search for arterial vascular grafts began in 1952, when Voorhees discovered the first fabric graft, Vinyon N (nylon) [16]. A few years later, DeBakey discovered Dacron® in 1958 [17].

Today, Dacron® and ePTFE are still widely used as arterial replacements. Despite their successful replacement of large diameter (>6mm) high-flow vessels, they show thrombogenicity and compliance mismatch in low-flow or small diameter vessels. Sophisticated techniques have been evaluated to enhance patency, including chemical modifications as well as coatings and seeding of the surface with different cells. Synthetic materials in contrast to natural materials often lack adhesion sites. Although sufficient physiological mechanical strength can be reproduced with passive materials, proper metabolic function and cellular signalling requires intact cellular machinery.

Table 1. Examples of biomaterials investigated for TEBV applications.

	Biomaterials	Reference
Natural materials	collagen	[18, 19]
	fibrin	[20, 21]
	Hyaluronic acid (Hyaff)	[22, 23]
	Small intestine submucosa (SIS)	[24]
	Silk fibroin (SF)	[25]
	Bacterial cellulose (BC)	[26, 27] Papers I-V
Synthetic polymers	Poly gycolic acid (PGA)	[28, 29]
	PGD-carpolatone-lactic acid (PGA-CL/LA)	[30]
	PGA-poly-4-hydroxybutyrate (PGA-P4HB)	[31]
	Polyhydroxyalkanoate-PGA (PGA-PHA)	[32]
	extended Poly(tetrafluoroethylene) (ePTFE)	[33, 34]
	Poly(ethylene terephtalate) (PET)	[35, 36]
	polyhedral oligomeric silsesquioxane poly(carbonate urea)urethane (POSS-PCU)	[37]

1.3.4. Different approaches to engineered blood vessels

Weinberg and Bell developed the first completely tissue engineered blood vessel as a multilayered vascular construct by culturing bovine aortic endothelial cells, smooth muscle cells and fibroblasts on an animal collagen gel matrix, but they noted that the construct was weak [18].

L´Heureuz developed a tissue engineered blood vessel based on a "cell self-assembly model" of cocultured human endothelial cells, smooth muscle cells and fibroblasts. The different cells were cultured as sheets and wrapped around a tubular support resulting in a construct with a similar structure and matrix as native vessels and with good strength properties [19].

Many different groups have successfully continued work on tissue engineered blood vessels. In the 1980s, the development of bioresorbable materials such as polyglycolic acid (PGA) began. When the host degrades the foreign material it is replaced with new host-derived tissue. Niklason and colleagues produced small-calibre vessels using a biomimetic system with pulsatile flow. A suspension of smooth muscle cells isolated from the media from bovine aorta was seeded onto a tubular biodegradable PGA scaffold and cultured for 8 weeks. Thereafter, bovine aortic endothelial cells were seeded onto the luminal surface. The resulting blood vessels had a rupture strength greater than that of native veins and showed contractile response to pharmacological agents [28].

Campbell implanted silicon tubing into the peritoneal cavity of rats and rabbits. After two weeks the tubing was harvested and a vessel like tissue of myofibroblasts (smooth muscle like cells - "media"), collagen matrix ("adventitia") and mesothelium had formed around the silicone tube. The tubing was removed and the tissue was everted such that it resembled a blood vessel with the single layer of mesothelial cells on the inside [38].

1.3.5. Cell source

The goal and major challenge is to find an optimal cell source. Large quantaties of cells have to be produced in a short period of time. Autologous cells are preferred since problems with immunological rejection can be avoided, but from a biopsy only a limited quantaty of cells can be obtained in considerable time. The prolonged culture of cells *in vitro* can affect their phenotype. Endothelial cells isolated from blood vessels, for example, can change spontaneously into smooth muscle cells [39]. Thanks to high plasticity of cells, the source may not be the most important factor. The culture time to obtain satisfactory cell quantities and providing the right environment (e.g. cell stimuli) seem to be more important [40].

Endothelial cells and stem cells from human and other species have been investigated as potential sources of seeding biomaterials including ECs from human umbilical vein [19, 31], human saphenous vein (Papers I, II and V), human aorta [18, 41], bovine aorta [28], bovine pulmonary arteries [42], dog saphenous vein [43], human microvascular ECs from medintestinal fat [44], adult mesenchymal stem cells (MSCs) [45] and endothelial progenitor cells (EPCs) [46].

Circulating progenitor cells can be isolated from human peripheral blood [47]. Although research shows promising results for EPCs, it is not clear whether sufficient cell quantaties can be obtained without substantial cultivation time. Adult MSCs can be isolated from bone marrow as well as from adipose and other tissues and are another interesting source of cells. They have a high proliferative potential and can be differentiated into multiple mesenchymal lineages [48]. Adipose tissue is an abundant and easily obtainable source of cells, but laborious procedures are still required for *in vitro* differentiation and expansion of ECs before graft seeding.

In an effort to circumvent the limitation of cell culturing before graft seeding, anti-CD34 antibody-coated prosthetic grafts have been evaluated. These grafts are thought to bind bone marrow derived CD34 positive EPCs *in vivo*. By promoting adherence of these endogenous circulating EPCs, the need for *in vitro* expansion and seeding procedures can be avoided. Such grafts show almost complete coverage with endothelial-like cells after implantation, but in spite of cellular coverage of the luminal surface, intimal hyperplasia is dramatically increased [49, 50].

On the other hand, adipose and amniotic derived stem cells can suppress allogeneic immunological responses and could therefore potentially be extensively expanded *in vitro* and used as a universal source of cells [51-53].

1.3.6. The ideal vascular graft?

Numerous qualities must be combined to construct the ideal small-calibre graft for replacement surgery. Some criteria are entirely essential, others are desirable [54].
The replacement graft must:

- be biocompatible (elicit no foreign body reaction, non-toxic),
- have appropriate mechanical properties (strength, burst pressure, compliance, good suturability),
- be non-thrombogenic and resistant to infection.

Furthermore the construct should:

- be an “off the shelf product” or be readily available,
- have low manufacturing costs and be simple to use,
- be available in different specifications (diameter, length, etc.).

1.4. Blood compatibility

What is blood compatibility? Thrombogenicity is defined by Williams as the ability of a material to induce or promote the formation of thromboemboli [10]. Non-thrombogenic materials should have a low thrombin production rate constant, low platelet consumption and low degree of platelet activation, perhaps some platelet spreading and low complement and leukocyte activation [55].

Since the blood-biomaterial interactions are complex and not yet fully understood, it is not surprising that many studies are contradictory concerning what non-thrombogenic material really is and why no non-thrombogenic material has yet been found.

1.4.1. Hemostasis

The maintenance of a normal and healthy circulatory system requires several mechanisms that can uphold normal functions and respond to a wide range of physiological conditions such as tissue damage, healing of wounds, alteration of blood composition, and inflammatory responses.

The coagulation cascade of hemostasis is often divided into two phases; primary hemostasis where platelets form an initial clot at the site of injury and secondary hemostasis where fibrin is generated through a complex pathway of plasma proteins, the coagulation factors, that strengthen the initial clot. These coagulation factors interact with each other in a Y-shaped pathway that join into a common pathway that ultimately leads to the formation of thrombin, which plays a central role in the coagulation cascade. Thrombin facilitates the cleavage of fibrinogen to fibrin, which can then polymerize and form a fibrin network, a vital part of the haemostatic clot that restricts bleeding after vessel injury.

The enzymes in the coagulation cascade are termed coagulation factors, usually abbreviated with an “F” and assigned specific roman numerals. To distinguish between the activated factor from the zymogen, the activated factor is suffixed with an “a”. Five of the zymogens involved in the coagulation cascade are vitamin K-dependent serine proteases, FVII, FIX, FX, prothrombin and protein C [56]. For coagulation reactions to occur in both pathways, several cofactors are needed including Tissue factor (TF), calcium ions, platelets and membrane surfaces.

1.4.2. Primary hemostasis – platelet adhesion, aggregation, activation and clot formation

Damage to the endothelium exposes the subendothelial matrix, e.g. collagen, which induces rapid platelet adhesion. This initiates the first step in the hemostatic response that leads to platelet plug formation. The initial "rolling" adhesion of platelets to collagen is mainly mediated by glycoprotein (GP), GPIb-XI-V. The binding of platelets to GP is promoted by von Willebrand Factor (vWF), a plasma protein that binds rapidly to exposed collagen and contains several binding sites for the GPIb-XI-V adhesion receptor. Binding to this receptor leads to rapid signal transduction and platelet activation.

Following initial platelet adhesion to exposed subendothelial matrix, platelets that have slowed down can bind directly to collagen via the GPVI receptor. This procedure plays an important role in platelet activation [57]. The $\alpha_2\beta_1$ integrin adhesion receptor (also known as GPIa/IIa), which is unique to platelets, can also bind to collagen. Although this receptor may not be involved in the initial adhesion under high shear rates, it is still considered important for firm adhesion and securing platelets. Following initial platelet adhesion to exposed subendothelial matrix, subsequent adhesion continues as platelet aggregation where platelets from the blood adhere to already adherent platelets. The aggregation of platelets is mainly attributed to the $\alpha_{IIb}\beta_3$ integrin (also known as GPIIb/IIIa). Its main ligand is fibrinogen, but it also binds to other proteins such as vWF, vitronectin, fibronectin and thrombospodin [58].

In order to maintain the recruitment of circulating platelets to the forming clot, it is important to pass on the activated state from already adherent cells to newly arrived platelets. This is achieved by potent autocrine and paracrine signalling pathways.

Platelet activation induces a shape change through cytoskeletal changes, and secretion of granules. The granules contain platelet agonists such as ADP and ATP, thromboxane A_2 (TxA_2), FVa and serotonin, all of which further stimulate coagulation and vasoconstriction. The TxA_2 molecule is synthesised by arachidonic acid metabolism. This synthesis can be inhibited effectively with aspirin treatment [59].

1.4.3. Secondary hemostasis – Coagulation

1.4.3.1. Two pathways of the coagulation cascade

The two pathways, named after the respective type of activation, are called the contact activation pathway (extrinsic pathway) and the tissue factor pathway (intrinsic pathway). These pathways join into a common pathway that leads to the generation of a stable blood clot [60].

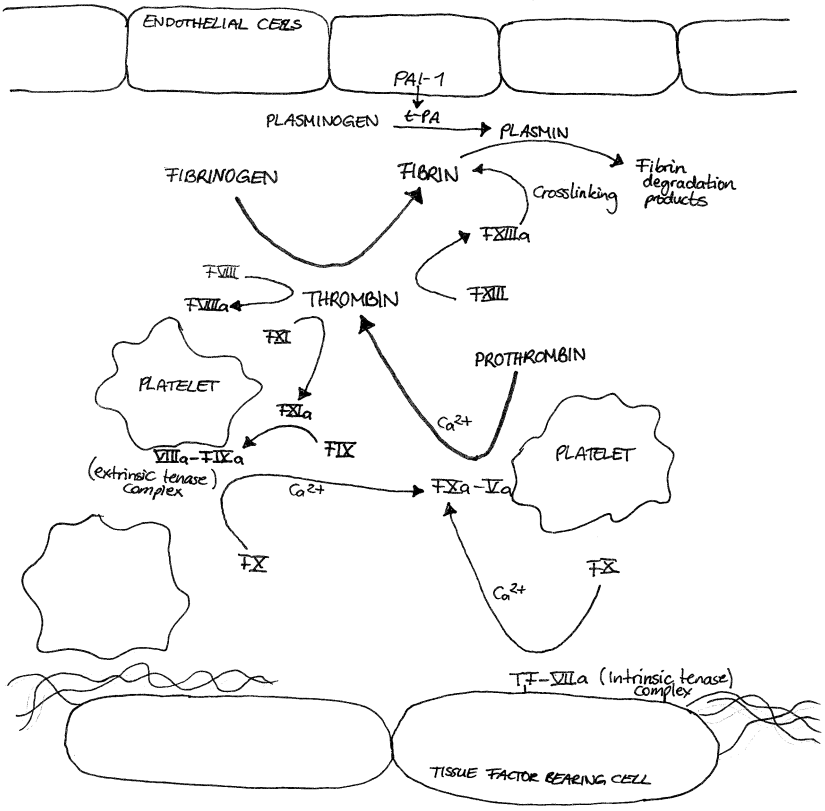

Figure 2. Schematic illustration of the coagulation and fibrinolytic system.

1.4.3.2. Tissue factor pathway (extrinsic pathway)

The first protection from thrombosis is the endothelial cell layer that lines the inner lumen of the vessel and hides the underlying subendothelium and TF. The coagulation process begins almost immediately after cell damage and exposure of TF, which is regarded as the main initiator of coagulation under normal physiological conditions. Tissue factor is expressed by platelets and leukocytes, and in the subendothelial tissue but not on healthy EC. The extrinsic pathway is initiated when activated factor VII comes into contact with TF. Under non-pathological conditions, picomolar concentrations of FVIIa circulate in the blood and act as primer in the initiation of coagulation in the presence of exposed TF. The tenase complex TF-FVIIa cleaves Factor X (FX), the factor that links the intrinsic and extrinsic pathways, into FXa in the presence of calcium.

The common pathway sbegins when Factor X is activated either by FIXa-VIIIa or by FVIIa-TF to Xa in the presence of calcium (Figure 2). The cofactor FV, like FVIII, is activated by thrombin to FVa, which together with FXa, forms the prothrombin complex (FXa-Va) that cleaves prothrombin (FII) to thrombin (FIIa) in the presence of calcium and phospholipids. Thrombin then cleaves soluble fibrinogen into fibrin monomers which polymerise and form long fibrin strands. In addition, thrombin activates Factor XIII into FXIIIa, which stabilizes the fibrin polymer network by cross-linkage that strengthens the initial unstable clot and produces an insoluble fibrin gel [61].

When the first trace amounts of thrombin are formed it activates FV, FVIII and FXI which leads to a positive feedback loop and rapid amplification of coagulation factors at the site of the injury.

1.4.3.3. Contact activation (intrinsic pathway)

The contact activation, also known as the intrinsic pathway, is initiated when factor FXII undergoes autoactivation triggered by negatively charged surfaces (Figure 3). The contact activation system is regulated by the three proteins prekallekrin (PK), high-molecular weight kininogen (HMWK) and FXII (Hageman factor). This pathway is initiated when FXII binds to surfaces such as artificial materials, leading to spontaneous cleavage of factor FXII into its active form. Activated factor XII converts both prekallikrein and FXI into their active forms, kallikrein and FXIa respectively, with HMWK as a cofactor. Factor XII, in turn, is a substrate for kallekrein, creating a short reciprocal activation loop, which leads to rapid contact activation. Factor XI is also activated by thrombin and FXIa. The activation by thrombin is thought to be more physiologically important since a deficiency of FXI but not FXII leads to an increased tendency toward bleeding [62]. The dual action of HMWK is important for its procoagulant role in coagulation. It forms a complex with both FXI and prekallikrein and binds to surfaces, thereby anchoring the coagulation factors to the surface.

Kallekrein also cleaves HMWK, liberating bradykinin, a potent endothelium-dependent vasodilatating agent. The activated factor XI converts factor IX to FIXa and forms the intrinsic tenase complex with factor VIIIa, which is an essential cofactor in the intrinsic activation and requires modification by thrombin. The intrinsic tenase complex (FIXa-FVIIA) on platelet surfaces activates factor X and forms the prothrombinase complex (FXa-FVa) in

the presence of calcium and phospholipids. This process is slow in the absence of appropriate phospholipid surfaces and serves to localise the coagulation to the cell surface.

Small amounts of thrombin can be formed from prothrombin by Xa alone, but this process is slow without the cofactors; factor VIIIa and factor Va. However, even minute amounts of thrombin can activate these cofactors, facilitating the formation of the FIXa-FVIIIa (intrinsic Xase) and FXa-FVa complexes, leading to massive thrombin production. The next step is the common pathway, where prothrombin is converted into thrombin as described in relation to the extrinsic pathway [63].

1.4.3.4. Inhibition of the coagulation system

The coagulation cascade possesses extensive amplification mechanisms to ensure fast arresting of bleeding and to prevent extensive blood loss. Such powerful mechanisms require control systems to avoid massive thrombus formation throughout the circulatory system once coagulation is initiated. One important mechanism is the dilution of coagulation factors by the bloodstream and their subsequent removal by the liver. The rates of several clotting reactions are surface dependent, and these reactions do not occur in the bulk. Another control system is direct inhibition of coagulation enzymes.

Antithrombin III (AT) can bind thrombin, thereby inhibiting its enzymatic activity. The formed thromin-antithrombin-complexes can be measured to assess thrombin production. Heparin binding enhances the activity of AT-mediated thrombin inhibition by colocalisation of both proteins. Thrombin is also removed by binding to thrombomodulin, a protein present on the surface of endothelial cells. The thrombin-thrombomodulin-complex can convert Protein C into active Protein C (APC), which can degrade FVa and FVIIIa together with its cofactor Protein S [64]. Plasmin degrades fibrin to fibrin monomers but can also inactivate FV and FVIII. Another inhibitor of the coagulation system is the tissue factor pathway inhibitor (TFPI), a protein which, together with FXa, inhibits the TF-FVIIa complex [65].

1.4.4. Fibrinolysis

During vessel repair, the blood clot is removed by enzymatic degradation, termed fibrinolysis. The fibrinolytic system is a short enzymatic cascade that leads to the degradation of fibrin to soluble products. The key enzyme of the fibrinolytic pathway is plasmin, which circulates in

an inactive form as plasminogen. Initiation can be achieved by either urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA). tPA is secreated by endothelial cells whereas uPA is mostly found in urine. The fibrinolytic system is regulated in numerous ways to avoid interference with the coagulation process. The most important inhibitors are plasminogen activator inhibitor (PAI-1), α2-macroglobulin and thrombin-activated fibrinolysis inhibitor (TAFI) that promotes stabilisation of fibrin clots [66].

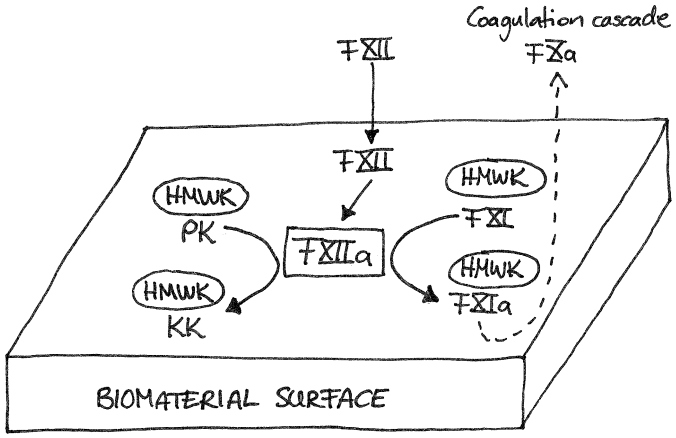

Figure 3. Schematic illustration of material induced coagulation.

1.4.5. Material interactions with blood

1.4.5.1. Blood biocompatibility

When a material comes into contact with blood, its ability to resist the initiation of thrombus formation is of substantial importance. This is particularly relevant for long-term implantable cardiovascular devices including vascular grafts, venous catheters, stents and artificial heart valves. Blood-material contact is also constantly present in treatments where blood is handled in extracorporal devices, e.g. during dialysis, cardiopulmary bypass, blood transfusion or when blood is drawn for analysis or *in vitro* experiments. The artificial surface may activate coagulation, leading to unwanted thrombus formation. This is a serious adverse effect that is usually prevented by aggressive antiplatelet and/or anticoagulation therapy. Such therapies include high dose intravenous heparin administration during cardiac surgery for cardiopulmary bypass, antiplatelet therapy after implantation of vascular stents and warfarin treatment after prosthetic heart valve surgery [67, 68]. These treatments have serious adverse effects that are potentially life-threatening as they confer an increased tendency toward bleeding.

The extent of coagulation induction by a biomaterial is highly dependent on the biomaterial's properties and design. Surface modifications can lower the coagulation activation induced by biomaterials [69, 70].

1.4.5.2. Surface modifications to increase blood compatibility and decrease thrombogenicity

Many strategies have been deployed to decrease material thrombogenicity, such as coating with albumin [71, 72], phosholipid mimicking molecules [73], hydrogels, PEG [74, 75], immobilisation of anti-platelet agents [76] and endothelialisation (Figure 4). Heparinisation by covalently binding heparin to the material has been the most successful surface modification in introducing surface molecules, which has made its way to the clinic to date. The most effective approaches are the Corline [77] and Carmeda techniques [78].

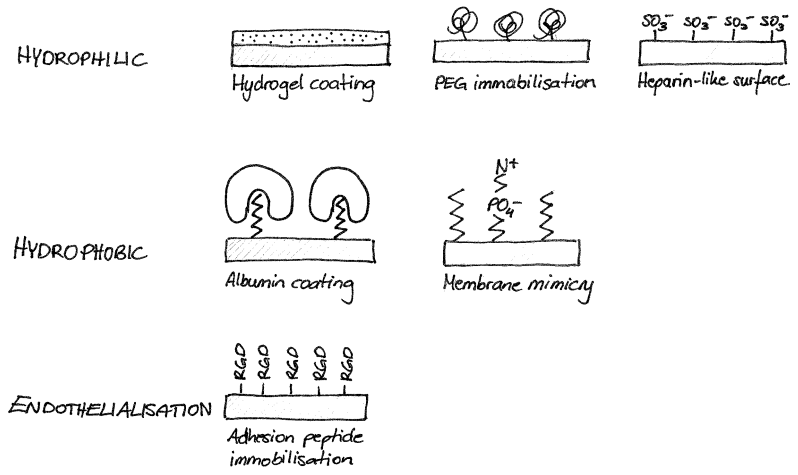

Figure 4. Schematic illustration surface modifications.

Herring and colleagues were the first researchers to isolate endothelial cells and transplant them onto synthetic vascular grafts [79]. Canine vascular endothelial cells were isolated from the saphenous vein and used to pre-coat 6mm weft-knitted Dacron® grafts before implantation into a canine model. The patency rate (percentage of clot-free surface) for seeded grafts was 76%, as compared with 22% in unseeded grafts [79]. Endothelialisation of biomaterials has long been a challenge, since many materials do not support cell adhesion and therefore have to be surface modified. Many different materials have been used for endothelial cell seeding, and studies have employed graft materials seeded with ECs to lower thrombogenicity [33, 36, 79-81]. The most elegant strategy was the adhesion peptide RGD incorporation method

developed by Massia and Hubble [82]. Many other strategies such as protein coatings, introducing electrical charge and chemical modifications have been investigated [83-86].

Despite promising results, EC seeding on synthetic grafts has not yet had a clinical breakthrough. Although this procedure can make the lumen less thrombogenic, the compliance mismatch between the stiff graft material and the native tissue remains a problem [87].

1.4.5.3. Plasma protein adsorption

The initial interaction between blood and an artificial material consists mainly of a rapid plasma protein adsorption phase that occurs within seconds. The amount and composition of adsorbed plasma proteins are both highly dependent on the surface properties of the material and the protein composition and are subject to change over time. Proteins in high concentrations, for example fibrinogen, initially adsorb on the surface, but can subsequently be displaced by secondary proteins in low concentrations with higher affinities for the surface, i.e., the Vroman effect [88-90]. These secondary proteins may include coagulation factor XII and HMWK [91], both involved in the surface induced contact activation of coagulation (intrinsic pathway). Adsorbed proteins can be recognised by platelet receptors, subsequently leading to platelet adhesion and activation.

Although the physiological role of factor XII in coagulation is debatable, as factor deficiency does not result in increased bleeding, it is clear that factor XII has a major impact on coagulation during the contact between blood and artificial surfaces. The mechanism of autoactivation of factor XII is still not very well understood, but autoactivation is facilitated by conformational changes upon surface adsorption [92, 93]. The C1-inhibitor (C1inh) has previously been reported to be the most important inhibitor of the contact activation enzymes FXIIa and FXIa [94, 95]. In a new study, however, AT inhibited these enzymes when activation was induced by activated platelets. This suggests that contact activation triggered by activated platelets is regulated by AT, whereas activation triggered by material surfaces is regulated by C1inh. The FXIIa-AT complex formed upon platelet-mediated contact activation *in vivo* could therefore be a potential marker for distinguishing contact activation on platelet surfaces from biomaterial-induced activation [96].

1.4.6. The Complement system

The immune complement system (CS) is part of the innate immune system and its main task is to protect the body from pathogenic agents like bacteria, viruses and fungi. On contact with a foreign surface, e.g. a bacterial surface, the complement system is activated in a cascade that results in either destruction of the bacterial surface or release of bioactive degradation products or both. This causes inflammatory reactions in the surrounding tissue.

The immune complement system is present in blood and serum and consists of more than 30 different cell bound and soluble proteins that circulate as inactive zymogens under non-pathological conditions. The most important factor is complement factor 3 (C3). The cleavage of C3 by C3 convertase creates C3a and C3b and causes a cascade of further cleavage and activation events. Three different pathways lead to the creation of C3 convertase: the classical pathway, the alternative pathway and the mannose-binding lectin pathway. The classical complement pathway typically requires antibodies for activation, whereas the alternative and lectin pathways can be activated by C3 hydrolysis or antigens without the presence of antibody (Figure 5).

1.4.6.1 Classical pathway

Classical convertase is initiated by binding of antibodies to a surface such as a bacterium. Factor C1 binds to an antibody, C1 is then cleaved into C1r and C1s. Together they form the C1 complex, which binds to factor C4 and C2 and splits them into C4a,C4b and C2a,C2b, respectively, forming the classical convertase (C4b2a) [97].

1.4.6.2. Alternative pathway

The alternative pathway is triggered either by spontaneous C3 hydrolysis to form C3a and C3b or by covalent binding of C3b from the classical- and lectin pathways to a surface.

The C3b molecule is capable of covalently binding to a pathogenic membrane surface in its vicinity. If there is no pathogen in the blood, the C3a and C3b protein fragments will be deactivated by rejoining with each other again. Upon binding with a cellular membrane, C3b is bound by factor B to form a C3bB complex. Factor D cleaves this complex into Ba and Bb. The Bb molecule remains covalently bound to C3b to form the alternative pathway C3-convertase (C3bBbP). The binding of factor P to C3bBb stabilises the enzyme complex. A

characteristic feature of the alternative pathway is a feedback mechanism that leads to accelerated C3 activation. Such mechanisms are not present in the classical pathway [98, 99].

1.4.6.3 Mannose-binding lectin pathway

The Mannose-binding lectin pathway is a variant of the classical pathway, but does not require antibodies. This pathway is activated when mannose-binding lectin (MBL) binds to mannose residues on the pathogen surface, which then activates the MBL-associated serine proteases 1 (MASP-1) and 2 (MASP-2). The MBL complex can subsequently split C4 and C2, which generates C3-convertase, as in the classical pathway [100].

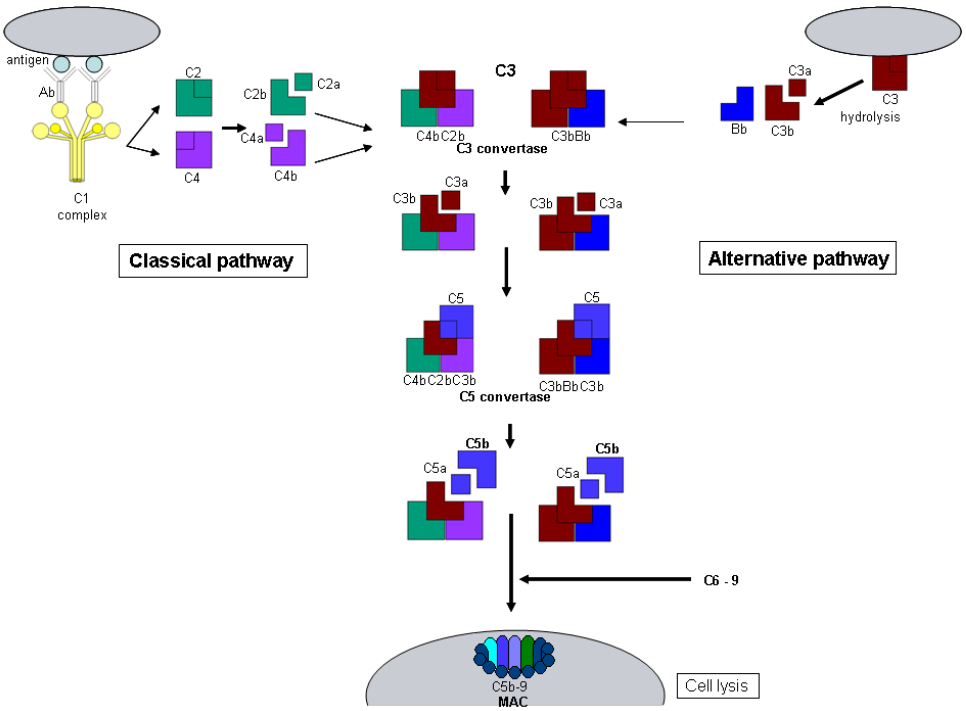

Figure 5. Schematic illustration of complement activation.

1.4.6.4. Terminal Complement Complex

The convertases from both the classical and alternative pathways cleaves C5 into C5a and C5b. The C5b molecule associates with C6, C7, C8, and C9 to form the C5b-9 membrane attack complex (MAC), which is inserted into the cell membrane and initiates cell lysis. The C5b-9, also called Terminal Complement Complex (TCC), may exist as a soluble active form denoted sC5b-9. This soluble form can be measured to assess complement activation (Paper IV). The C5a and C3a fragments are anaphylatoxins that are involved in the recruitment of inflammatory cells and trigger mast cell degranulation. These anaphylatoxins are therefore involved in many forms of acute and chronic inflammation including sepsis [101, 102].

1.4.6.5. Complement activation by biomaterials

Extracorporal treatments such as haemodialysis and cardiopulmonary bypass activate the CS. When blood comes in contact with biomaterials, degradation fragments of complement C3a and C5a and soluble C5b-9 may be generated. These fragments result in chemotaxis of leukocytes, cytokine release and generation of prostaglandins. The result is a life-threatening condition termed "whole body" inflammation. Biomaterial induced CS is activated by both the classical and alternative pathways [103].

1.5. Endothelial cells

The endothelium is composed of a monolayer of squamous epithelial cells that line the inside of blood vessels in a confluent layer with a total area of 350-1000 m^2 and a weight of 0.5-1.5 kg [104, 105]. These cells have a flat morphology that resembles a cobblestone pattern. This morphology is essential to maintaining good blood flow without turbulence. Endothelial cells not only function as a physiological barrier separating the blood from surrounding tissues, as previously believed. In fact, the endothelium is a dynamic layer of cells which, in its resting state, display antithrombotic properties. This is achieved by physically preventing elements in the blood to come into contact with prothrombotic elements in the subendothelium and by active synthesis of various mediators. These endothelial functions help maintain blood vessel function.

The endothelium upholds delicate balances in the vasculature; i.e., vasoconstriction/ vasodilatation, anticoagulant/procoagulant properties (Table 2), blood cell adherence/ nonadherence and growth promotion/inhibition. This regulates vascular tone, maintains hemostasis, controls vascular structure and mediates inflammatory and immunological responses (Figure 6).

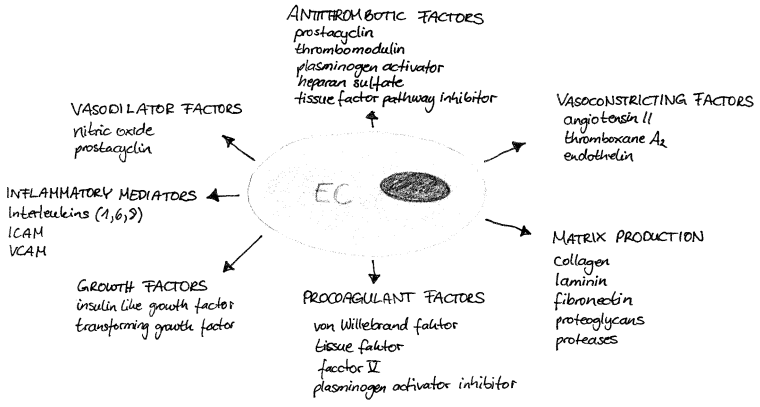

Figure 6. Overview of endothelial cell functions.

1.5.1 Hemostatic control

Hemostatic control is the most important function of the endothelium in relation to biomaterials. Under normal physiological conditions, the endothelial cells express thromboresistant molecules but must be able to switch to a procoagulant state upon injury to initiate coagulation and clot formation. Since the blood is transported under high pressure, this response must be rapid in order to minimise blood loss. Some molecules are continuously secreted by endothelial cells while others are only produced upon stimulation. Molecules can either be expressed on the surface or secreted into the blood stream.

Table 2. Overview of pro- and anticoagulant factors produced by endothelial cells.

Anticoagulant	Procoagulant
Thrombomodulin	Tissue Factor
Heparan sulfate proteoglycans	vWF
PGI_2	Collagen
t-PA/u-PA	factor V
TFPI	PAI-1
NO	HMWK
Antithrombin III	Factor VII
Protein S	

1.5.2 Anticoagulation

1.5.2.1. Inhibition of platelet adhesion, activation and aggregation

Platelet activation, aggregation and platelet-wall-interaction are suppressed by prostacylin I_2 (PGI_2), nitric oxide (NO) and adenosine diphosphatase (ADPase). Both NO and PGI_2 are secreted and act in a paracrine manner, whereas ADPase is expressed on the endothelial cell surface. Platelet inhibition by PGI_2 is mediated through a guanosine nucleotide binding receptor. This receptor-mediated signal transduction leads to increased cyclic adenosine monophosphate (cAMP) levels and inhibition of platelet activation and release of proaggregatory compounds such as thromboxane A_2 [106]. The production of PGI_2 is stimulated by diverse agonists such as thrombin, histamine and bradykinin, and are synthesised via arachidonic acid (AA) and prostaglandin (PGG_2) [107].

Endothelial cells produce endothelial-dependent relaxing factor (EDRF), which is responsible for acetylcholine-induced vasorelaxation. The most important EDRF is NO, which is synthesised by nitric oxide synthase (NOS) through conversion of L-arginine. Since NO is a small molecule it diffuses easily. When NO enters platelets, it inhibits their adhesion and

activation via guanylyl cyclise [108]. Prostacyclin and NO have synergistic effects on inhibition of platelet adhesion, activation and aggregation as well as in reversing platelet aggregation [109, 110]

1.5.2.2. Inhibition of coagulation

The endothelium physically separates coagulation factor VIIa from TF and prevents platelet exposure to collagen and vWF.

Thrombomodulin (TM) is expressed on the surface of ECs. Thrombin binds to TM and thereby undergoes conformational change which results in enhanced affinity for protein C. Thrombin is the only enzyme capable of activating protein C. Activated protein C cleaves and inactivates clotting factor Va and VIIIa [111]. Through the thrombin-TM complex, thrombin is effectively removed from the blood and internalised, which leads to its degradation (Figure 7). The TM molecule can also bind FXa and thereby inhibit the activation of prothrombin [112]. Protein S, also synthesised by ECs, binds to the endothelial surface and protein Ca to form a complex, enhancing FVa and FVIIIa inhibition.

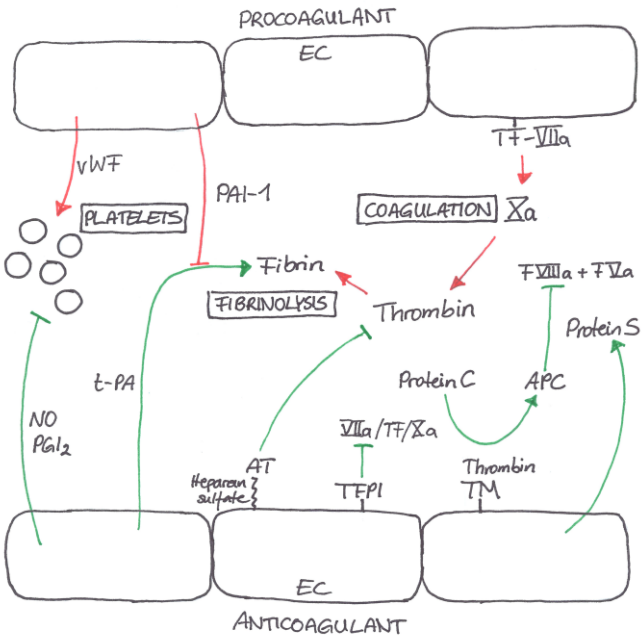

Figure 7. Schematic illustration of the regulation of coagulation by ECs.

The endothelium expresses heparin sulphate proteoglycans with anticoagulant activity on its surface. Heparin is a cofactor for antithrombin III, a protein present in plasma capable of inhibiting thrombin, IXa, FXa and XIIa. The complex binding of thrombin to antithrombin III

occurs at a slow rate. This process is accelerated by the interaction with heparin, which has many binding sites for antithrombin, and serves to localise and increase its activity more than a thousandfold. The β-isoform of antithrombin demonstrates the highest effect compared to the α-isoform. This form is also an effective inhibitor of thrombin-induced proliferation of smooth muscle cells [113].

Tissue factor pathway inhibitor (TFPI), synthesised in the liver and by ECs, forms a complex with Xa and inactivates the VIIa-tissue factor complex by binding to it.

1.5.3. Procoagulation factor

The endothelium is also important in initiating coagulation to arrest bleeding. It therefore expresses a variety of procoagulant factors, including vWF, coagulation factors V and VII, tissue factor (TF) and high molecular weight kininogen (HMWK) (Figure 7).

Von Willebrand Factor, which is an adhesion molecule for platelets, is synthesised by EC and stored in vesicles (Weibel-Palade bodies) and secreted upon stimulation by thrombin. It possesses binding sites for coagulation factor VIII, collagen (exposed after injury) and platelets (GPIb- XI-V) and acts as a bridging molecule in platelet aggregation and activation [114]. The important role of vWF is evident since its absence leads to severe bleeding disorders.

Tissue factor is synthesised by endothelial cells and is mainly found in the subendothelium, sites that are not normally exposed to the bloodstream. The basal production of TF is low in comparison with that of the underlying smooth muscle cells and fibroblasts but can be 10- to 40-fold increased upon stimulation. In addition, ECs have binding sites for factor VII, IX, IXa, X and Xa. By binding factor IXa, its decay is inhibited in the presence of factor VIII and X, which provides an additional feedback mechanism for cell bound procoagulant activity [105, 115].

1.5.4. Fibrinolysis

The endothelium also participates in the regulation of fibrinolysis (Figure 7). Plasmin is needed for the degradation of fibrin. Plasminogen binds to the cell surface and facilitates conversion to plasmin by the two plasminogen activators, tissue type plasminogen activator (tPA) and urokinase (uPA)[116]. The physiologically most important plasminogen activator

in vascular fibrinolysis is tPA. The conversion of plasminogen to plasmin is enhanced 100-fold when tPA is bound to fibrin. The release of tPA is either constitutively or pathway-mediated. Thrombin, FVa, bradykinin and platelet-activating factor as well as shear stress all induce synthesis and release of tPA from ECs [117-119]. When tPA is bound to the EC surface it is protected from degradation by the two plasminogen activator inhibitors (PAI), PAI-1 and PAI-2, also released by ECs. The PAI-1 requires vitronectin, present in the extracellular matrix, to maintain its activity and is the main inhibitor to tPA. Recombinant t-PA (rt-PA) is the most frequently used substance for inducing thrombolysis by pharmacological means [120, 121].

1.5.5. Angiogenesis and vessel remodeling

Endothelial cells regulate vessel structure by producing both growth promoting and inhibiting factors. Growth of smooth muscle cells is stimulated by platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor alpha (TGF-α), endothelin and angiotensin II. Growth is inhibited by NO, PGI_2, some FGFs, insulin-like growth factor 1 (IGF-1) and thrombospondin.

Angiogenesis is regulated by a variety of growth factors. Hypoxia and inflammatory cytokines such as FGF induce upregulation of vascular endothelial growth factor-A (VEGF-A) through autocrine and paracrine mechanisms. VEGF-A is an endothelial specific growth factor that consists of a heparin-binding homodimer, and is a major regulator of endothelial cell function and angiogenesis. A variety of endothelial cell functions such as proliferation, migration and NO-release are activated by VEGF-A. These processes are important in the formation of new blood vessels. It also increases the permeability of the vessel wall. Both VEGF and FGF induce endothelial cells to produce proteases such as metalloproteinases (MMPs) and plasminogen activator (PA). At least twenty metalloproteinases are involved in angiogenesis [122, 123].

Proteases digest the basement membrane, which allows endothelial cells to invade surrounding tissue where they proliferate and migrate to form a sprout. The sprout elongates and the endothelial cells differentiate to form a lumen. Endothelial cells in the newly formed vessel produce PDGF-BB that attracts mural cells (pericytes to capillaries/smooth muscle cells to larger arteries and veins) to stabilise the newly formed vessel. Heparin sulphate

proteoglycans and their glycosaminoglycans (GAG) side-chains, expressed on ECs, play an important role in angiogenesis since they bind circulating growth factors like VEGF [122].

1.5.6. Regulation of vascular tone

Endothelial cells regulate vessel tone, and consequently local blood flow, by managing the communication between the blood and the underlying smooth muscle cells, and by releasing substances that influence smooth muscle cells to relax or contract. Endothelial cells synthesise both vasodilating and vasoconstricting agents.

Vasodilatation is mediated through PGI_2 and NO/EDRF and endothelium-derived hyperpolarizing factor (EDHF), where NO plays a central role. Vasoconstricting agents released by ECs are endothelin, angiotensin II and TXA_2.

Shear stress, bradykinin, thrombin, serotonin and various drugs stimulate endothelial cells to release prostacyklin. Prostacyklin stimulates adenylate cyclase, which increases cAMP in smooth muscle cells. Nitric oxide is synthesized from L-arginine by NO synthase and diffuses to smooth muscle cells, where it activates guanylate cyclase to produce cGMP. This leads to a decrease of intracellular calcium and muscle relaxation. Mechanical induction of NO is mediated by f-actin, sensing mechanical changes in the environment leading to signal transduction into the cell.

The eNOS gene contains a shear stress regulatory element (SSRE) that allows up or downregulation of eNOS activity [124]. Endothelium-derived hyperpolarizing factor (EDHF) is released upon stimulation of M1 muscarinic receptors by acetylcholine and causes changes in membrane potential [125]. Endothelin consists of three isoforms, ET-1, ET-2 and ET-3. Endothelial cells produce endothelin-1, which is the most potent for vasoconstriction. Two receptors for endothelins are found in the vasculature; ET_A on smooth muscle cells and ET_B on endothelial cells. Binding of endothelin-1 to the ET_A-receptor leads to signal transduction and smooth muscle relaxation. Activation of ET_B on ECs on the other hand leads to stimulation of NO and PGI_2 production. Angotensin II in contrast is a much weaker vasoconstrictor. Renin cleaves angiotensinogen to angiotensin I, which is then converted to angiotensin by endothelial angiotensin-converting enzyme (ACE) [126].

1.5.7. Inflammation

Under inflammatory conditions, the endothelium responds by regulating its own permeability and by releasing a variety of substances. Inflammation is mediated by pro-inflammatory mediators such as cytokines like the interleukins (e.g., IL-1β, IL-6, IL-8), platelet activating factor (PAF) as well as expression of endothelial cell leukocyte adhesion molecules 1 (ELAM-1) and inter-cellular adhesion molecule 1 (ICAM-1). These inflammatory mediators control the interaction between EC and circulating blood cells, leukocytes, leading to extravasation of leukocytes.

During an inflammatory response, adhesion molecule P-selectin is expressed on ECs after exposure to leukotrine B4 or histamine, which is produced by mast cells. Tumor necrosis factor alpha (TNF-α) and lipopolysaccharides (LPS) induce P-selectin expression as well as the synthesis of E-selectin, another selectin that appears a few hours after the inflammatory process is initiated. The interactions between these selectins and their corresponding glycoprotein ligands (sialyl-Lewis^x moiety) on leukocytes are relatively weak and reversible, thus the leukocytes are unable to attach firmly to the endothelium. Instead, the leukocytes "roll" along the surface of the vessel wall. The interactions are enhanced as other integrins are induced on the endothelium.

The leukocyte integrins LFA-1 and Mac-1 normally only adhere weakly to the leukocytes. On the other hand, IL-8 and other chemokines bound to the endothelial surface trigger a conformational change in LAF-1 and Mac-1 on the rolling leukocytes, which increases the adhesiveness and consequently firmly anchors the leukocytes to the endothelium. Rolling is arrested and the leukocytes squeeze between the endothelial cells into the subendothelial tissue, a process known as diapedesis.

1.6. Bacterial synthesized cellulose

Cellulose is the most abundant biopolymer on earth; is insoluble in water and degradable by microbial enzymes. It can be produced by several organisms such as plants, algae and bacteria. Some members of the bacterial genus *Acetobacter*, especially *Acetobacter Xylinum*, synthesize and secret cellulose extracellularly [127]. Bacterial cellulose is composed of linear nanosized fibrils of D-glucose molecules [128]. The network structure of cellulose fibrils is very similar to that of collagen in the ECM of native connective tissue (Figure 8) [26].

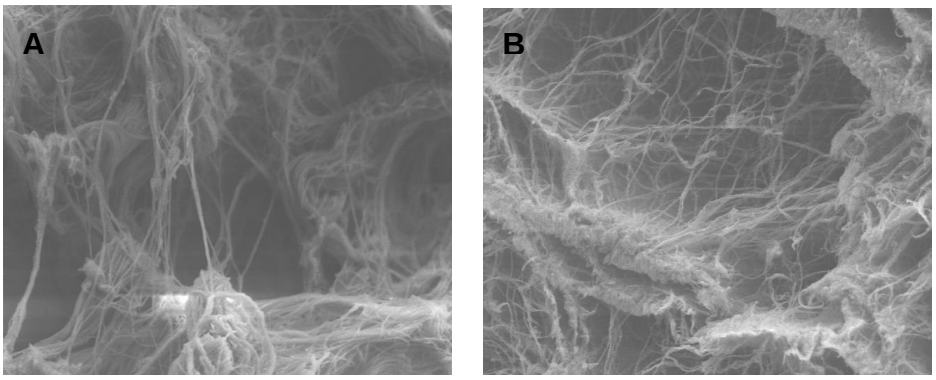

Figure 8. SEM images of (A) collagen and (B) BC.

Bacterial cellulose is not a hydrogel in the true sense of the meaning; it is often referred to as such because of the high water content, 99%, its insolubility in water and highly hydrophilic nature. Since BC consists of a highly entangled network of fibrils, it also provides the material with strong mechanical properties which are essential for tissue engineered blood vessels to withstand mechanical forces and to prevent rupture. The BC can be designed and shaped into three dimensional structures such as tubes or sheets [26]. A major advantage of using BC instead of cellulose produced by any other organism is that BC is completely free from biogenic compounds such as lignin, pectin and arabinan found in e.g. plant cellulose. During the production process, it is also possible to modify several other properties including pore size, surface properties and layering of the material [129].

1.6.1. Structure and morphology

Cellulose synthesis begins with the water-soluble monosaccharide D-glucose and is produced extracellularly as pellicles at the air/liquid interface. Glucan chains of BC are extruded from several enzyme complexes and aggregated by van der Waals forces to form sub-fibrils, approximately 1.5 nm wide. The BC sub-fibrils are crystallized into microfibrils, then into bundles. The bundles form a dense reticulated structure stabilized by hydrogen bonding (Figure 9). In culture medium, the bundles assemble into ribbons to form a network of cellulose. The network of cellulose nano-fibrils provide BC with high mechanical strength and a water retention capacity of about 99% [130].

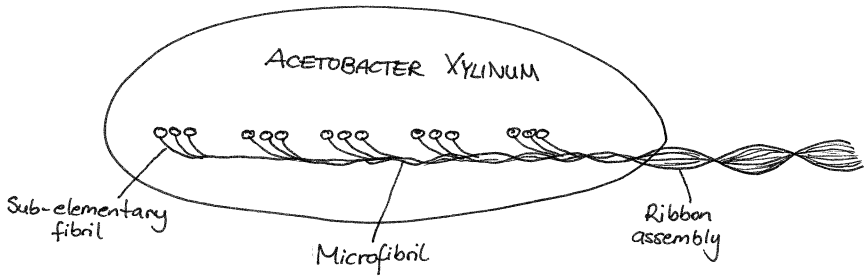

Figure 9. Fermentation of BC by *Acetobacter xylinum*.

The macroscopic morphology of BC varies with different culture conditions. In static conditions, BC accumulates on the surface of nutrient-rich culture medium, at the oxygen-rich air-liquid interface. Statically cultured BC has lower crystallinity than BC fermented during agitation.

Due to the production process, BC consists of two distinctly different layers, which can be seen in detail in SEM pictures (Figure 10): one side has a compact network of fibrils with few if any pores. The other side has a porous network structure. Between these sides a density gradient arises.

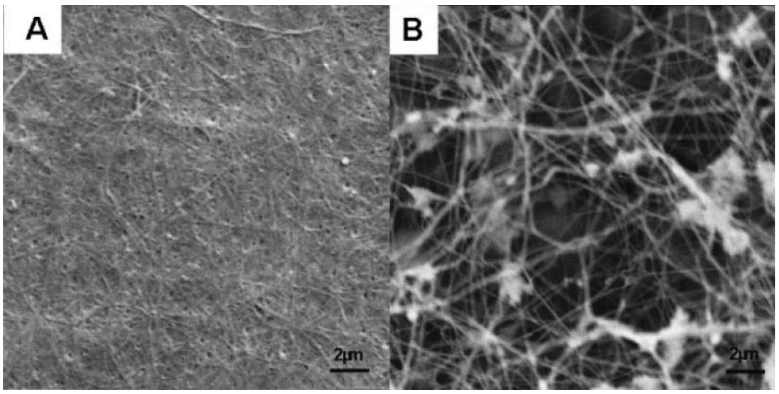

Figure 10. SEM images of the inner side (A) and outer side (B) of BC tubes.

1.6.2. Mechanical properties

The optimal scaffold is a biocompatible biomaterial that can provide proper mechanical and physical properties to promote cell adhesion and tissue formation. Bacterial cellulose tubes have been extensively mechanically tested (burst strength, compliance and tensile strength) prior to implantation into animals. Films or sheets of bacterial cellulose show remarkable mechanical strength, which is explained by the high crystallinity, the high planar orientation

of the ribbons, the ultrafine structure and the complex network [130]. The mechanical properties of BC tubes are similar to those of pig carotid arteries [26]. The BC has a compliance curve resembling that of a native artery more than any other synthetic material on the market, which is advantageous (Figure 11). Material density can be altered by variation of culture conditions or by post-culture modifications.

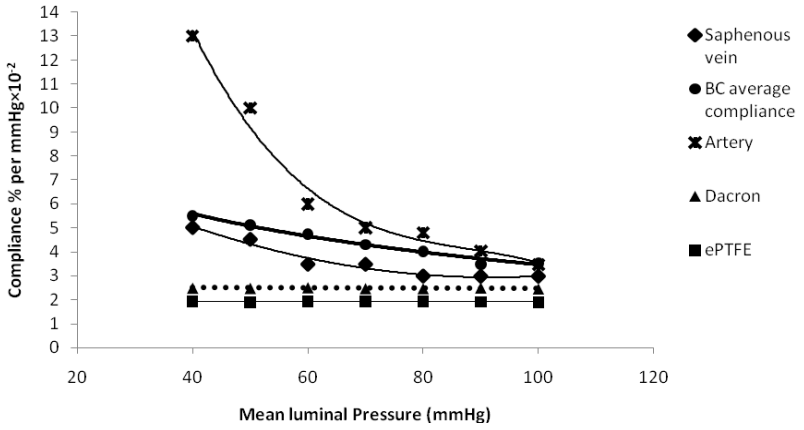

Figure 11. Compliance of BC, native artery and vein and conventional vascular graft materials.

1.6.3. Biocompatibility

Integration of a material with the host tissue is essential for the success of tissue engineered blood vessels. According to Williams, the biocompatibility of a material can be defined as the ability of a material to perform with an appropriate host response in a specific application" [10]. An appropriate host response would then be a biomaterial that induces a very low inflammatory and foreign body response in the host tissue.

A study performed by Helenius et al. showed that BC is well integrated into the host tissue and does not induce any inflammatory or foreign body response [131]. In that study, BC pieces were implanted subcutaneously in rat and explanted after 1, 4 and 12 weeks. The incorporation of the implant in the host tissue made it difficult to distinguish a clear interface between the implant and the host tissue [131]. These results are supported by another *in vivo* study, where BC tubes have been implanted into the carotid arteries in pigs [132]. Therefore, it is clear that BC has good biocompatibility and promising potential as scaffold material.

1.6.4. Bacterial cellulose as biomaterial

Bacterial cellulose has found many applications in a variety of different areas: food matrix (nata de coco), dietary fibres, acoustic or filter membranes and ultra-strength paper. As for biomaterials, BC has been suggested as potential material for tissue engineering in several areas, e.g. as scaffold for tissue engineering of cartilage, blood vessels (BASYC®) and successful treatment of second and third degree burns, stomach ulcers and other situations where a temporary skin substitute is needed (Biofill®, Gengiflex®, XCell®) or to recover periodontal tissue (Gengiflex®) [133-135].

1.6.5. Surface modification of bacterial cellulose

The challenge in the field of vascular grafts is to promote endothelial cell attachment, since many biomaterials similar to BC exhibit limited support for cellular adhesion [136]. Over the years, many strategies have therefore been developed to modify material surfaces. To optimize cell-biomaterial interactions, synthetic scaffolds have been coated with cell adhesive proteins such as collagen, fibronectin or laminin. The difficulty with such passive protein adsorption is that biofilms with varying composition are formed, which is also subject to modification by exchange with other proteins (Vroman effect). The extent of cell adhesion is difficult to predict in such a system. Cell adhesion peptide RGD (Arg–Gly–Asp) and its derivatives have attracted much attention as an alternative for stimulating reproducible and predictable cell adhesion [137-140].

Known modifications of cellulose involve chemical modifications where surface hydroxyl groups are used as reactive sites. These are the very same hydroxyl groups that are involved in the hydrogen bonding that holds the cellulose fibre network together. Disruption of these bonds is associated with loss of fibre ultrastructure [141, 142]. Dry films of BC have been modified with carboxymethyl and acetyl groups [143]. However, surface modifications to BC in a wet state have not been studied extensively. Thus, modification of a BC hydrogel is especially challenging, since solvent exchange and cellulose modification typically destroy the hydrogel morphology.

2. Objectives

The major aim of this Thesis was to explore EC and blood interactions with BC to evaluate whether this material could be a potential new vascular graft.

The specific aims of the Thesis were to test the following hypotheses:

- Surface modification of bacterial cellulose can promote cell adhesion and proliferation. (Papers I, II and V)
- Bacterial cellulose is less thrombogenic than conventional vascular graft materials. (Papers III and IV)

3. Materials and Methods

For a detailed description of Materials and Methods used in this Thesis, please refer to Papers I-V. This section gives an overview of the different methods used.

3.1. Bacterial cellulose production

Acetobacter xylinum was used for cellulose synthesis (ATCC 1700178, American Type Culture Collection). Cellulose tubes were grown in corn steep liquid media at 30°C for 7 days. The cellulose was then purified by boiling first in 0.1 M NaOH at 60°C for 4h and then in Millipore™ water. Finally, the cellulose was sterilised by autoclaving for 20 minutes.

3.2. Cells and cell culture (Papers I, II and V)

Endothelial cells were isolated from non-diseased human saphenous veins, by-products of coronary bypass surgery. Cells were isolated using an enzymatic technique and cultured at 37°C in a humidified incubator with 5% CO_2. The ECs had the characteristic morphological cobblestone pattern when grown as a confluent monolayer, were positive for PECAM-1 (Abcam, Cambridge, MA) and von Willebrand factor (Dako, Glostrup, Denmark) and negative for α-actin (Dako). All cells were used in passage 4.

3.3. Cell adhesion (Papers II and V)

The amount of cells attached to the BC was determined by quantifying DNA content with a standard CyQuant-kit (Molecular Probes, Eugene, OR). This assay is a convenient, rapid and sensitive procedure for determining cell density in cell culture. It is based on the fluorescent dye CyQuant GR, which exhibits fluorescence enhancement when bound to nucleic acids and measured at an excitation wavelength of 480 nm and mission wavelength of 520 nm. The advantage with this method is that cell numbers can be quantified without depending on cell metabolism. A disadvantage is that only endpoint measurements are possible.

3.4. Cell proliferation (Papers II and V)

Cell proliferation was determined both as DNA content and using a 5-bromo-2'-deoxyuridine (BrdU) assay (Roche Diagnostics, Mannheim, Germany). For DNA-content, the same protocol was applied as for the adhesion study described above. The BrdU-assay is a

nonradioactive alternative to [^{3}H]thymidine incorporation and takes advantage of the incorporation of the pyrimidine analog BrdU instead of thymidine into the newly synthesized DNA of proliferating cells. The incorporated DNA is detected with an ELISA against BrdU.

3.5. Cell metabolism (Paper II)

Cell metabolism was determined using an Alamar Blue™ assay (Serotec Ltd Scandinavia, Hamar, Norway). AlamarBlue is a dye that uses the natural reducing power of living cells to convert resazurin to the fluorescent molecule, resorufin. The active ingredient of AlamarBlue® (resazurin) is a nontoxic, cell permeable compound, dark blue in color and with virtually little intrinsic fluorescence. Upon entering cells, resazurin is chemically reduced to resorufin, which produces red fluorescence. Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of metabolism.

The reduction can be measured either by fluorescence at excitation wavelength 544 nm and emission wavelength 590 nm or by absorbance at 570 nm. For this study, dye reduction was measured by fluorescence. It is possible to perform measurements on the same cells over a period of several days in culture.

3.6. Morphology (Papers I, II and V)

For analysis of cell morphology, living ECs were either imaged with light microscopy (Zeiss Axiovert 20) digitally captured with a Canon Powershot G5 (Canon Inc., Japan) or fixed for further analysis of cell morphology by scanning laser confocal microscopy or scanning laser microscopy (SEM).

3.7. Blood compatibility (Papers III and IV)

For coagulation experiments (Papers III and IV), PVC tubing, metal connectors and O-rings were heparin-coated using the Corline method according to the manufacturer's recommendations (Corline Systems AB, Uppsala, Sweden). The surface concentration of heparin was 0.9 μg/cm^2, with an antithrombin (AT) binding capacity of 10–12 pmol/cm^2 [77].

Fresh blood was drawn from healthy volunteers and collected either in 7.5 mL Monovette® sampling tubes with 0.13 M citrate (Paper III) or in a heparinised 50 mL Falcon® tube

containing soluble heparin with a final concentration of 0.5 IU heparin/mL (Paper IV). Although a citrate concentration of 130 mM is reported to induce higher variations in thrombin generation, subsequent control experiments showed no difference in variation between the 130 mM and 105 mM citrate concentrations when thrombin generation was triggered by a contact activating agent.

3.7.1. Chandler-loop model (Paper IV)

A modified Chandler Loop model was used to investigate the response of freshly drawn blood to different biomaterial surfaces [144]. Fifty mm long tubes of BC, PET and ePTFE with internal diameters of 4 and 6 mm and an average blood exposed surface of 5.7 cm^2 and 8.5 cm^2 respectively, were fitted on metal connectors and assembled to loops with PVC tubing coated with immobilized heparin (Figure 12). The loops had a total length of 450 mm and a total volume of 7 and 16 mL, respectively. After assembly, the loops were rinsed with saline and immediately filled with 4 and 4.5 mL blood, respectively, leaving airspace. Heparinised PVC tubes (hep. PVC) were used as negative control. The blood-filled loops were secured on a platter and rotated at 14 rpm for 60 min vertically at 37°C. At the end of each experiment, the blood was carefully removed from the tubing for further analysis.

Figure 12. Chandler Loop system of 4mm tubes filled with whole blood rotating in 37°C.

3.7.2. Enzyme-linked immunoassay (ELISA) (Paper IV)

Detection of coagulation markers was performed using enzyme-linked immunoassays (ELISAs). Plasma levels of thrombin-antithrombin (TAT), C3a, and sC5b-9 were determined using a sandwich ELISA technique where a capture antibody against the protein of interest is pre-coated on a microtiter plate. The plates are washed between each step. Plasma and standards of known concentrations are added, the plates incubated with biotinylated detection antibody for C3a, followed by HRP-conjugated streptavidin. Directly conjugated antibodies were used for detection of TAT and sC5b-9. The addition of a substrate and its subsequent reaction with the HRP creates a blue coloured solution. A stop solution of sulphuric acid is added, yielding a yellow colour. The absorbance can be measured at 450nm. The intensity of the colour is proportional to the concentration of the molecule of interest. Comparing the values with those of a standard curve gives the amounts of TAT, C3a and sC5b-9.

3.7.3. Calibrated automated thrombography (Paper III)

The levels of thrombin and factor XIIa were measured using calibrated automated thrombography, which displays the concentration of thrombin and factor XIIa respectively in clotting plasma with or without platelets (platelet-rich plasma/platelet-free plasma, PRP/PFP). The splitting of a fluorogenic substrate is monitored for either thrombin or factor XIIa and compared with a constant known thrombin or factor XIIa activity, respectively, in a parallel non-clotting sample.

The fluorescence (excitation: 390 nm, emission: 460 nm) was measured with a Fluoroscan Ascent plate reader. This thrombin generation results in a thrombogram (Figure 13) with the parameters lagtime, endogenous thrombin potential (ETP), peak and time-to-peak (ttPeak) by analysis with the Thrombinoscope software (Thrombinoscope BV)[145]. The “lagtime” is the time it takes for thrombin generation to start, “ttPeak” is the time to where the maximum thrombin generation occurs, “peak” is the maximum amount of thrombin generated and “ETP” is the total amount of thrombin generated.

To evaluate thrombin and factor XIIa generation exclusively induced by the biomaterial surfaces, the material samples were fixed with heparinised O-rings and analysed by calculating the average rate of fluorescence increase over a period of 60 min.

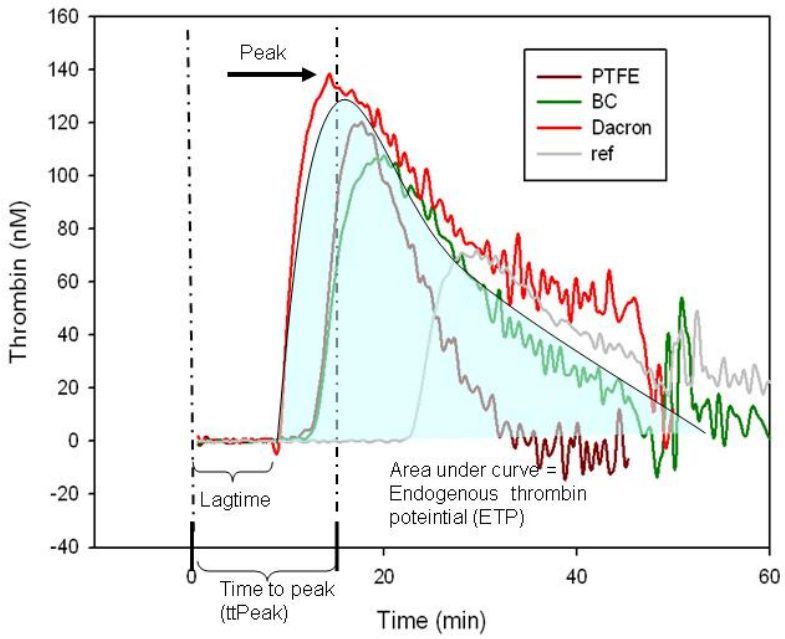

Figure 13. Thrombogram generated from thrombin generation assay displaying lagtime, time to peak (ttPeak), peak and endogenous thrombin potential (ETP).

3.7.4. Imaging of coagulation (Paper III)

In order to measure biomaterial-induced coagulation exclusively at the inner surface of the materials, custom made stainless steel clips were manufactured by Riboverkstan (Gothenburg, Sweden) according to our specification to fit 4.5 mL spectrophotometry cuvettes (Figure 14). To avoid activation of coagulation by the clips themselves, they were treated with 0.1% (w/v) poly-L-lysine water solution (Sigma-Aldrich).

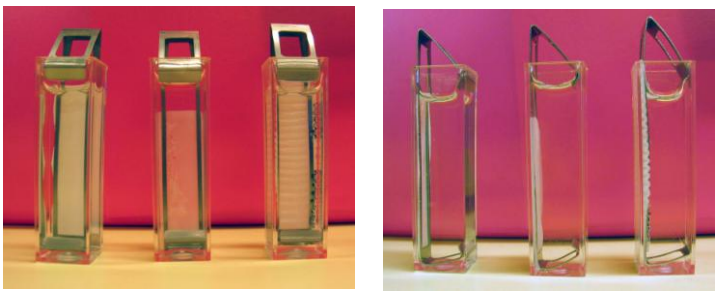

Figure 14. Custom made metal clips for video imaging of coagulation.

For imaging the coagulation process, test materials were placed along one side of the cuvette wall and secured with clips. The plasma filled cuvettes were then placed in a custom made holder and illuminated from beneath with white light emitting LEDs. Time-lapse images of all materials were simultaneously captured with a digital camera. The captured images were

then transferred to a computer and converted into video sequences using our own software. Coagulation was detected in the video as an increase in light intensity attributed to light scattering from the insoluble fibrin network.

The captured images were analyzed using custom made software that calculates the coagulation time of the plasma at the surface (surface coagulation time) and into the bulk (propagation).

Most methods, available to study the coagulation process, measure the coagulation in the bulk without regards to where coagulation was initiated or the kinetics describing the propagation from the initiation point. With this method, it is possible to visualize and determine the exact point where the coagulation is initiated and how coagulation propagates [146], highly relevant questions in the study of material interactions with blood.

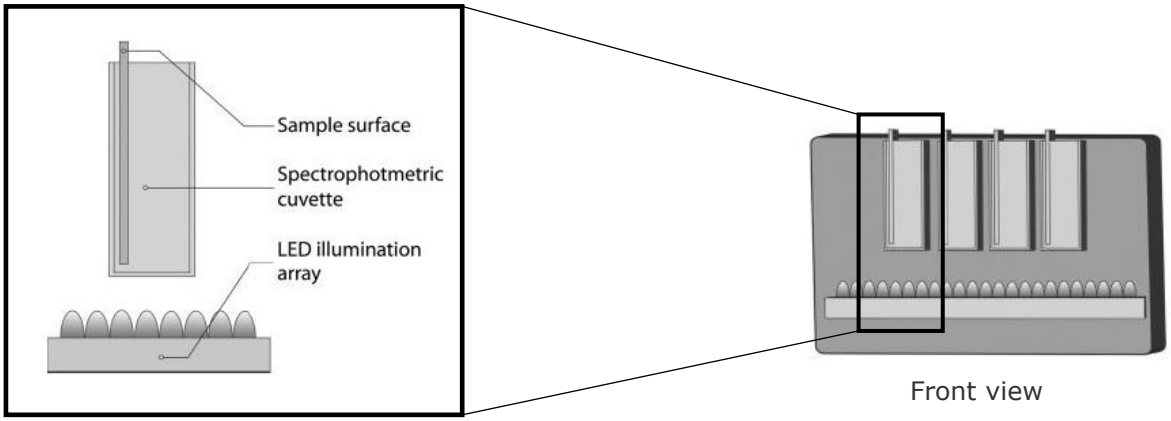

Figure 15. Schematics of imaging of coagulation

3.8. Bioreactor set-up (Paper V)

A bioreactor (Figure 16) and bioreactor system was designed and set up in our laboratory. The system was equipped with a dynamic pump (Cole-Parmer EW-75211-55) and a flow meter (Transonic T106), which delivered pulsatile (60 bpm) laminar shear stress (12 dyn/cm2). In order to reach appropriate shear rates and ensure laminar flow, the viscosity of the medium was adjusted by addition of dextran (Pharmacia, 70 kD). A bioreactor without flow was used as a static control.

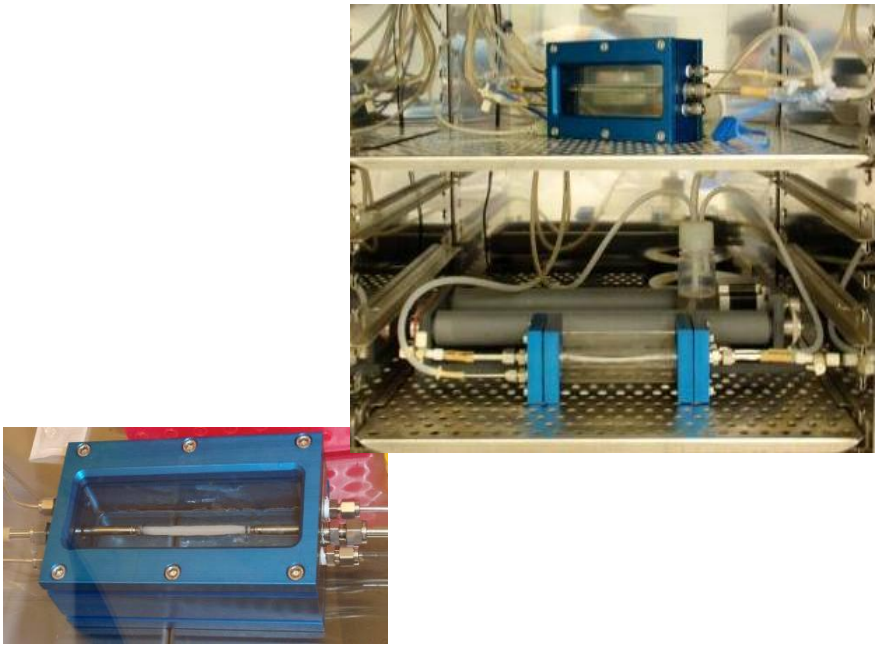

Figure 16. Bioreactor with mounted BC tube.

4. Results and Discussion

4.1. Surface modifications to enhance EC adhesion (Papers I, II and V)

A non-thrombogenic surface is the key to a successful vascular graft. This can be achieved by various surface modifications. Endothelial cells could provide such a non-thrombigenic surface, and endothelialisation has therefore been investigated intensively.

Bacterial cellulose is a very interesting material for biomaterial applications. Its structure resembles that of collagen, the component in arteries and veins that gives the blood vessel its strength. The manufacturing of BC allows a versatility of shapes and sizes including tubes. Studies have shown successful growth of cardiac rat derived myocytes and fibroblasts [147], rat derived hepatocytes [148, 149] and osteoprogenitor cells [150] from mice on cellulose-based materials. However, these matrices are based not on natural cellulose but on derivatives such as cellulose acetate and regenerative cellulose. Bacterial cellulose is biocompatible but does not generally promote cell growth [151]. Thus BC must be modified to support EC adherence. Modification of BC in a wet state is a challenging task, since the fibre structure and thus its strength must be maintained. This is of importance for vascular grafts, as they have to withstand blood pressure.

To optimize cell-biomaterial interactions, synthetic scaffolds have been coated with cell adhesive proteins such as collagen, fibronectin or laminin [83, 152]. The difficulty with such passive protein adsorption is that a biofilm is formed with varying protein composition, and is also subject to modification over time [90]. Additionally, protein adsorption to BC is very low. The cell adhesion peptide RGD and its derivatives have attracted a great deal of attention as an alternative for stimulating reproducible and predictable cell adhesion [137-140]. Most RGD modifications are done by covalent binding to the material [153].

Although cellulose contains reactive hydroxyl groups that can be chemically modified, these very same hydroxyl groups are involved in the hydrogen bonding that holds the cellulose fibre network together. Disruption of these bonds is associated with loss of fibre ultrastructure [141, 142]. Thus, modification of a BC hydrogel is especially challenging, since solvent exchange and cellulose modification typically destroy the hydrogel morphology. Most

modifications to date have been performed on dried BC. Consequently, a new method is needed that would increase cell attachment without altering the structure of the BC network.

Bacterial cellulose was modified with two different methods to enhance EC adhesion: xyloglucan (XG) bearing a GRGDS peptide and fibrin coating.

4.1.1. XG-RGD-modification

Xyloglucan, the most abundant hemicellulose, is present in the primary wall of many plants. In contrast to cellulose it is soluble in water and interacts strongly with cellulose fibres [154]. In this Thesis, I have taken advantage of these properties which provide an elegant means of introducing the cell adhesion peptide RGD with xyloglucan as a carrier molecule to BC (Figure 17). Bacterial Cellulose and cotton linters, as reference material, were modified with XG and XG bearing a GRGDS pentapeptide.

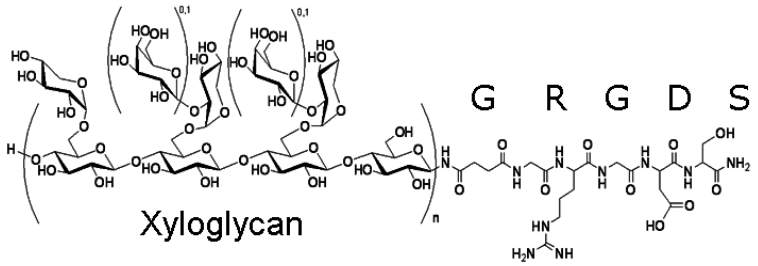

Figure 17. Xyloglucan molecule bearing the adhesionpeptide RGD.

4.1.1.1. Preserved BC morphology

The adsorption maximum of the XG and XG-RGD reached approximately180 mg/g on BC. The adsorption was verified with colorimetric methods. Modification in the water phase, in comparison with organic solvents, was clearly advantageous for preserving the morphology, as observed with SEM (Figure 18).

4.1.1.2. Increased wettability and decreased protein adsorption on modified BC

The adsorption of XG increased the wettability only to a minor extent, which might explain the decreased or undetectable adsorption of adhesive proteins shown by QCM-D. These QCM-D studies further revealed no binding of fibrinogen antibodies to BC, leading to the conclusion that cell enhancement would be a result of the presence of RGD epitopes and not

of unspecific protein adsorption such as fibronectin from the cell culture medium. Xyloglucan has also been used to enhance hepatocyte adhesion [155], and modification of BC with XG itself has shown no adverse effects on ECs.

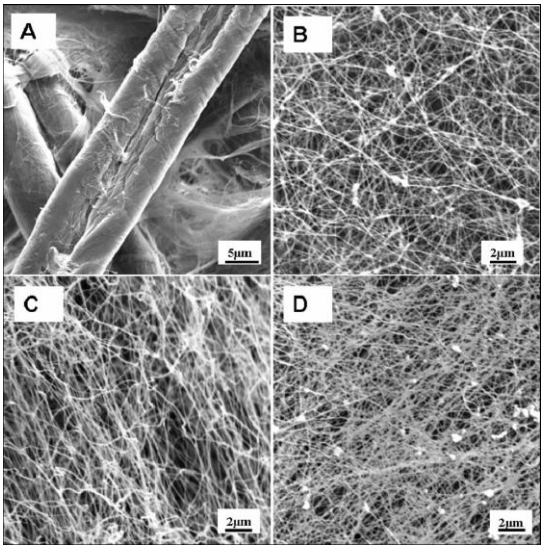

Figure 18. SEM micrograph of BC morphology of (A) cotton linter, (B) unmodified BC, (C) XG-RGD modified BC and (D) acetone treated BC.

4.1.1.3. Increased cell adhesion and metabolism on RGD-modified BC

The XG-RGD-modification increased cell adhesion by 20%, and also increased the metabolism of seeded ECs as compared with unmodified BC (Figure 19). In contrast, the proliferation rate was only affected to a lesser extent, presumably as a result of biological variation between cell donors. Our results are comparable with studies on RGD-grafted regenerated cellulose, where the introduction of an adhesion peptide enhances adhesion by approximately 20% [156].

Absence of serum affected cell adhesion on unmodified BC negatively but it did not have this effect on modified BC, further indicating that the increased adhesion is peptide specific. The introduction of an RGD-peptide to the BC surface also resulted in enhanced cell spreading, with more organised actin fibre formation and a more mature phenotype.

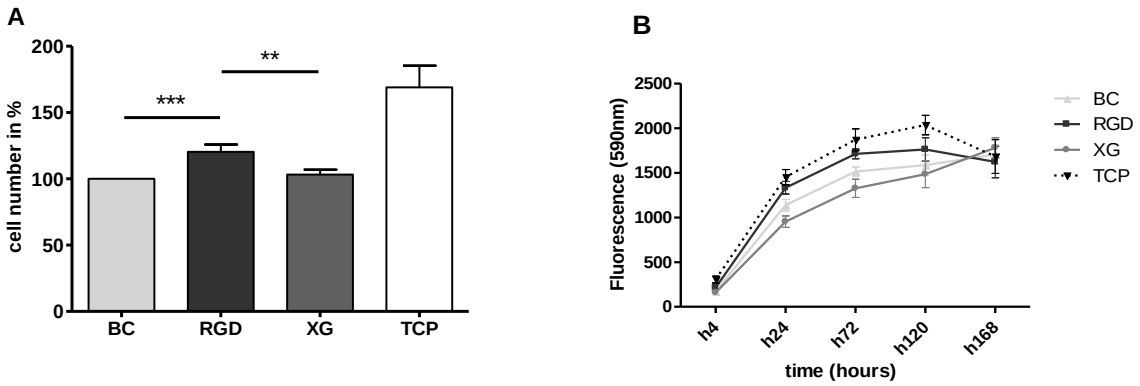

Figure 19. Cell adhesion 4h after seeding the number of cells in % of the control on a) XG-modified (XG), RGD-modified BC (RGD), unmodified BC (BC) and tissue culture plastic (TCP) (A) and cell metabolism determined by fluorescent intensity measured at 4, 24, 72, 120 and 168 h with an Alamar Blue-assay (B).

4.1.1.4. Increased cell spreading on modified BC

The initial cell attachment is crucial to subsequent behaviour such as spreading, proliferation, and differentiation of the cells on substrates. The extent of cell spreading is one of the important parameters of the biocompatibility of materials. Endothelial cell adhesion to the ECM proteins is normally followed by cell spreading, in which the cells reorganize the f-actin cytoskeleton, which results in the flattening and spreading of the cell. These polymerised actin filaments attach the cells to the substrate via focal adhesions. Cells grown on RGD-modified BC spread out and displayed a well-organized actin cytoskeleton with prominent f-actin fibres. They also grew in clusters, which we believe is a step towards achieving a confluent monolayer.

4.1.2. Fibrin coating of BC

Fibrin is produced when thrombin polymerizes fibrinogen monomers. It is the final product of coagulation, and functions not only as a plug to minimize blood loss upon injury but also as a scaffold for tissue repair. Fibrin has previously been used as a scaffold for vascular grafts and as coating for graft materials [157, 158]. Fibrin can be harvested autologously from the patient's blood [159, 160].

The fibrin gel only serves to obtain initial uniform cell distribution and more efficient seeding; the fibrin is allowed to degrade while the BC scaffold provides structural integrity to the developing tissue, which is important during the initial phase of culturing. This gives the cells time to produce their own matrix proteins. The newly formed ECM can then replace the fibrin, over time, when degradation is not inhibited.

This Thesis therefore explored the possibility of coating BC with fibrin in order to enhance endothelial cell (EC) adhesion and retention to shear stress. Tubes were initially luminally coated with different concentrations of fibrinogen to optimise the modification.

4.1.2.1. Fibrinogen concentration important for fibrin modification of BC

The fibrinogen concentration and the modification method were found to be of crucial significance for the properties and integration of the fibrin into the cellulose. The fibrinogen concentration and the modification method are of crucial significance for the properties and integration of the fibrin into the cellulose. With the undiluted fibrinogen, a fibrous surface with poor integration into the cellulose is formed. The fibrin layer detaches easily and folds. If fibrinogen is diluted, the fibrin layer is smooth and well integrated into the cellulose fibre network. The fibrin is denser at the luminal side and gradually becoming thinner in the BC network towards the outer side (Figure 20). At a concentration of 50 mg/ml, the fibrin seals the tubes and hence no fluid leaks out from them. This is not achieved when the fibrinogen is further diluted. The BC tube is modified evenly over the entire tube. The intermediate fibrinogen concentration exhibited best results and was therefore chosen for all experiments.

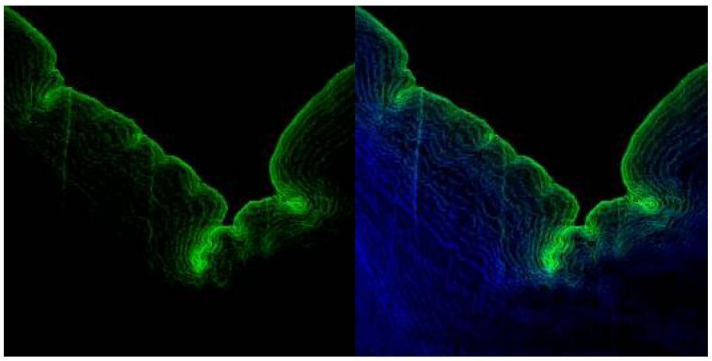

Figure 20. Confocal image of a cross-section of BC (blue) with integrated fibrin (green).

4.1.2.2. Increased cell adhesion and NO production

Fibrin coating of BC increased cell adhesion by 200% as compared with uncoated BC under static conditions. Shear stress experiments revealed an overall cell retention of 60% after 24 h of physiological shear stress. The ECs were elongated and aligned in the direction of the flow. Shear stress, compared with static controls, induced a 3-fold increase in the production of NO, which is a potent anti-coagulation agent. Thus, fibrin coating of BC is a promising modification for promotion of cell adhesion and retention, providing an anti-thrombogenic surface.

4.2. Thrombogenic properties of BC (Papers III and IV)

The thombogenicity of a biomaterial is an essential factor for any material intended to be in contact with blood. A non-thrombogenic surface is the key to a successful vascular graft. Although the thrombogenic property of cellulose has been extensively researched as it has been used for haemodialysis membranes [75, 161], the thrombogenicity of BC has not yet been investigated, since it is a relatively new material for vessel grafts.

Evaluation of coagulation induced by biomaterials is mostly studied in terms of platelet adhesion, partial thromboplastin time (PTT), protein adsorption by QCM-D or ellipsometry [161-164]. The QCM-D method is surface sensitive, but the distance from the surface to where measurement is possible is limited. Currently, it is not possible to attach BC to quartz crystals. Therefore, cellulose other than BC must be used. This material could, however, be used for QCM-D measurements as a model surface as a complement to other studies. Since ellipsometry is an optical method it is not possible to use native BC for this assay either. Automated calibrated thrombin generation is very sensitive and has become a widespread method for quantitative analysis of coagulation kinetics in blood plasma [145, 165, 166]. Thrombin generation is also considered the most sensitive method to assay thrombogenicity.

Since BC is used in a wet state it has been challenging to find appropriate analysis techniques. To our knowledge, this is the first time the thrombogenic properties of BC have been investigated and compared with other graft materials. During the course of my work with this Thesis, I have developed a modified automated calibrated thrombin generation assay (Paper III). This makes it is possible to follow thrombin generation, in the presence of a material, in real time instead of using an endpoint assays. This has led to new insights into the kinetics of thrombin generation induced by a material surface, which would otherwise have been missed.

4.2.1. Thrombin generation and propagation of coagulation

4.2.1.1. Slower coagulation on BC

Biomaterial induced coagulation of BC was compared with clinically used graft materials i.e., expanded polytetrafluoroethylene (ePTFE) and polyethyleneterephtalat (PET). In addition, the coagulation propagation at the material surfaces and into the plasma bulk was visualized.

Thrombin generation experiments revealed dramatic differences between the tested materials. Both ePTFE and BC were found to generate longer lagtimes and ttPeak values than PET. Furthermore, BC was found to generate the lowest 'Peak', which indicates a slower coagulation process at the surface (Figure 21). These results are also supported by the measurements of factor XIIa generation and analysis of surface coagulation times, where BC hade the lowest FIIa generation and slowest propagation of coagulation into the bulk (Figure 22 and 23).

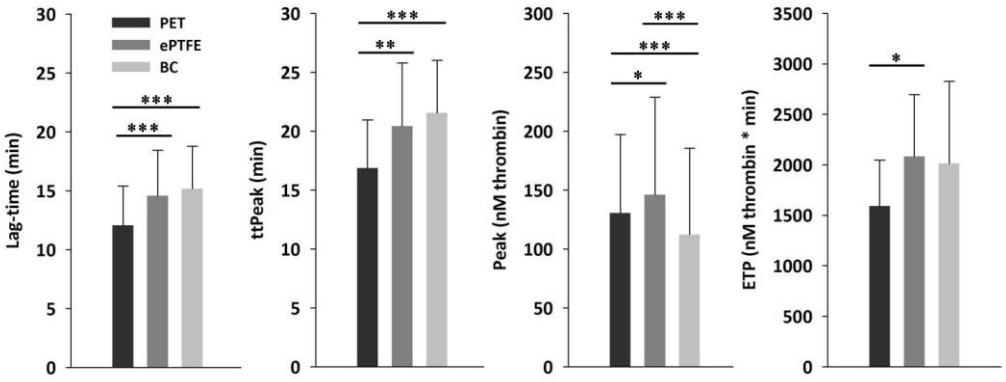

Figure 21. Thrombin generation experiments were performed in PFP on the exposed lumen of PET, ePTFE and BC graft material samples. The thrombin generation parameters lagtime and time-to-peak (ttpeak) both revealed significantly longer times for ePTFE and BC than for PET. The thrombin generation peak was found to be significantly higher for PET and ePTFE than for BC.

Thrombin generation in the whole blood Chandler loop system depicted the same response with lower accumulated TAT levels on both BC and ePTFE as compared with PET. Since the measurements are performed after one hour and not continuously, the difference in coagulation speed cannot be observed. On the other hand, this assay is performed in whole blood during flow conditions closer resembling an *in vivo* situation compared to measurements in platelet-free plasma during static conditions. It is interesting and promising that these two systems show similar results.

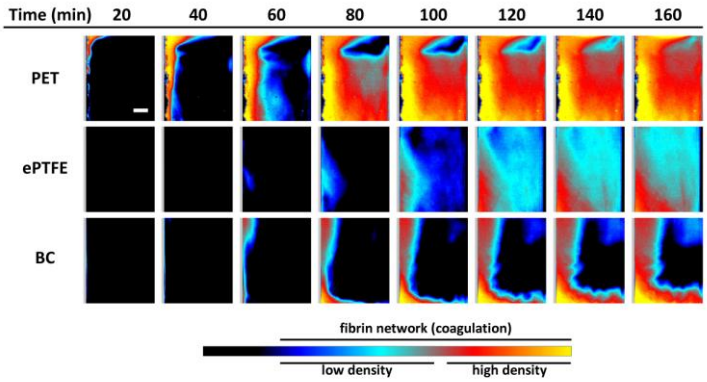

Figure 22. Representative time-lapse images from imaging of coagulation tests in PFP at the exposed lumen of PET, ePTFE and BC graft samples. Graft material samples are attached along the left wall in the images. The colour represents the density of the formed fibrin network.

The slower coagulation process on BC could hypothetically be an advantage when it comes to blood contacting biomaterial applications, as it would provide time for the blood flow to divert and dilute activated coagulation products. Measurements of thrombin generation correlate very well with the XIIa generation assay and visualisation of propagation. Together these methods could be potential fast screening methods for evaluation of the thrombogenicity of biomaterials.

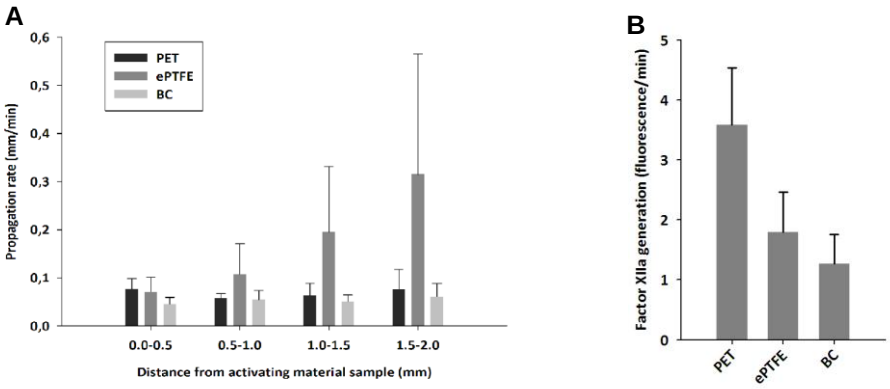

Figure 23. Propagation of coagulation measured in four consecutive 0.5 mm intervals from the PET, ePTFE and BC graft material samples (A) and factor XIIa generation at the material surfaces of PET, ePTFE and BC (B).

4.2.1.2. Good anti-thombogenic properties of 4 mm BC tubes

The whole blood model also shows that 4 mm BC tubes perform very well regarding anti-thrombogenic properties and perform better as compared with ePTFE than 6 mm tubes.

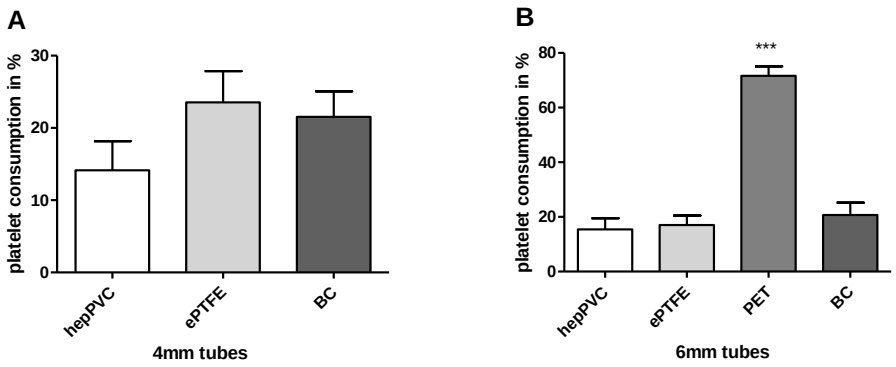

Figure 24. Platelet consumption of (A) 4 mm and (B) 6 mm tubes of control, heparinised PVC (hepPVC), expanded poly(tetrafluorethylene) (ePTFE), Poly(ethyleneterephtalate) fibre (PET) and bacterial cellulose (BC) 1h of exposure to blood in a Chandler Loop system.

The amount of TAT generated depends on the velocity (e.g. shear rate) of the blood in the loop system. Higher velocities are associated with increased TAT generation. In the 4 mm loop system, the shear rates are higher than the 6 mm system and the narrower material also exhibits greater coagulation activation. Interestingly, however, this does not cause increased platelet consumption, which is an indication of platelet activation (Figure 24). The amount of TAT generated on ePTFE increased 18-fold on 4 mm tubes as compared with 6 mm tubes, whereas only a 3-fold increase could be detected for BC tubes (Figure 25). In comparison with the other tested materials, the platelet consumption of BC is remarkably low, especially compared with heparinised PVC, known to have low thrombogenic properties [167]. In addition, there is no visible sign of clotting with the cellulose following one hour of incubation with whole blood containing only small amounts of soluble heparin (Figure 26).

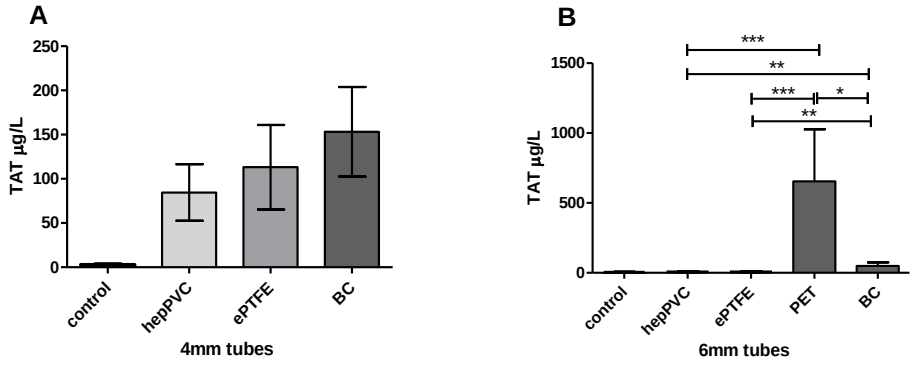

Figure 25. Coagulation measured as TAT generation on on control, heparinised PVC (hepPVC), expanded poly(tetrafluorethylene) (ePTFE), Poly(ethyleneterephtalate) fibre (PET) and bacterial cellulose (BC) 1h of exposure to blood in a Chandler Loop system for (A) 4 mm and (B) 6 mm tubes.

4.2.2. Complement activation by BC

The Chandler Loop system was also used to assess activation of the complement system. The complement activation parameters (C3a and C5b-9) were much higher for BC in comparison with the other materials, for both 4 and 6 mm tubes. Cellulose is known to induce complement activation in hemodialysis membranes [168]. The mechanisms underlying these results for bacterial cellulose are still unclear and need to be further studied. Bacterial fragments could still be present in the material. However the endotoxin values are well within the limit for cardiovascular devices. It is also possible that exposed hydroxyl groups induce complement activation through the alternative pathway [169, 170]. The physiological significance *in vivo* of this complement activation remains to be evaluated. One interesting observation is that platelet activation is low even when the complement activation is high. According to the literature, platelet consumption and complement activation are closely related [75, 171-173].

Figure 26. Representative image of the inner surface of a BC tube after 1h of exposure to blood. No visible signs of clot formation could be detected.

5. Concluding remarks and future perspectives

In this Thesis two possible approaches to modifing BC have been presented. Both enhance EC growth *in vitro*: xyloglucan bearing and RGD-peptide and coating with fibrin.

The XG-method is an elegant technique for modification of BC to promote EC. It is an easy one-step procedure carried out in water. The advantage is the preservation of the fibre structure, thus maintaining its strength.

The modification of BC with the XG-techique is far from limited to the RGD peptide. Different peptide sequences or other active groups and growth factors could be attached to the

XG molecule. Platelets could potentially adhere to exposed RGD peptides. Therefore, other peptides, more specific to ECs, or different combinations of peptides should be explored.

Fibrin coating of BC also show promising results for promotion of cell adhesion and retention, thus providing an antithrombogenic surface. The properties of fibrin can also be changed to optimize fibrin degradation and fibre cross-linking [158, 174]. Since the degradation of fibrin can be changed by incorporation of fibrinolytic inhibitors, different degradation rates can be explored and optimised. For generation of EC-lined grafts for clinical use, a limited number of available autologous cells have to be grown at a faster rate without accenting their normal physiology. Greisler et al. reported a method of immobilizing FGF-1 to the inner and outer surfaces of ePTFE grafts within fibrin glue coats, which resulted in an attachment efficiency of 39% [175]. It would be possible in future experiments to implant such growth factors into BC during the fibrin coating process.

The two methods presented both show good EC growth *in vitro*. However, they have yet to be tested in an *in vivo* situation.

Measurements of thrombin generation correlated very well with the XIIa generation assay and visualisation of the propagation of coagulation. Together, these methods could be potential fast screening methods for evaluation of the thrombogenicity of biomaterials and future surface modifications.

Bacterial cellulose could be used for vascular grafts, with to different approaches. Either BC could be implanted as a tube without cells or seeded prior to implantation. The ideal modification of BC would provide an initial non-thrombogenic surface while promoting endothelialisation in the long run.

Future modifications of BC could include heparinisation or combinations of surface modifications, e.g. different peptides or coatings. Heparin is a potent antithrombotic agent that functions by binding antithrombin. In recent years, heparinized ePTFE grafts (Propaten®) have been developed [176]. Although encouraging outcomes for below-knee bypass are reported, the compliance mismatch of ePTFE grafts still remains. However, this is an exciting modification and preliminary studies on heparinzed BC tubes show considerably lower amounts of thrombin on the hep-BC surface.

6. Conclusions

It is possible to introduce an adhesion peptide to BC that enhances endothelial cell adhesion without altering the fibre network or the mechanical properties. The anti-thrombogenic properties of BC, especially 4 mm tubes, are promising as compared with conventional graft materials. Bacterial cellulose, together with the modification methods presented in this Thesis, has the potential to become a material for artificial blood vessels.

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