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# Introduction

## ***Tissue engineering***

Tissue engineering (TE) is the application of engineering sciences in medicine. Research fields associated with TE are bioengineering and regenerative medicine. They are all an approach to replace, repair and regenerate diseased, injured or missing tissues and organs. The term *tissue engineering* originates from 1987 at a meeting at the National Science foundation and is a relatively new research field. Engineering tissues is however not a new concept. The earliest mention of a biological TE approach dates back to 1938 [1].

Williams defines TE as “*the creation (or formation) of new tissue for therapeutic reconstruction of the human body, by the deliberate and controlled stimulation of selected target cells through a systemic combination of molecular and mechanical signals*” [2].

Tissue engineering and regenerative medicine fields require knowledge from several disciplines such as biology, biotechnology, medicine and engineering. They necessitate biomaterial design, living cells, as well as cell–cell and cell/tissue–biomaterial interactions. Compatibility and remodelling of the transplanted engineered graft *in vivo* and non-immunoreactivity to the transplant are further prerequisites.

Engineered tissues and organs could reduce the scarcity of transplant material. Advances in the medical engineering science have improved existing artificial organs; however these need further development regarding biocompatibility and functionality.

Over the past 5 decades, several studies have aimed at creating vascular conduits to replace diseased or damaged blood vessels through bypass surgery. Now, scientists aspire to design tissue-based and preferably completely biological vascular constructs.

### ***The need for engineered blood vessels***

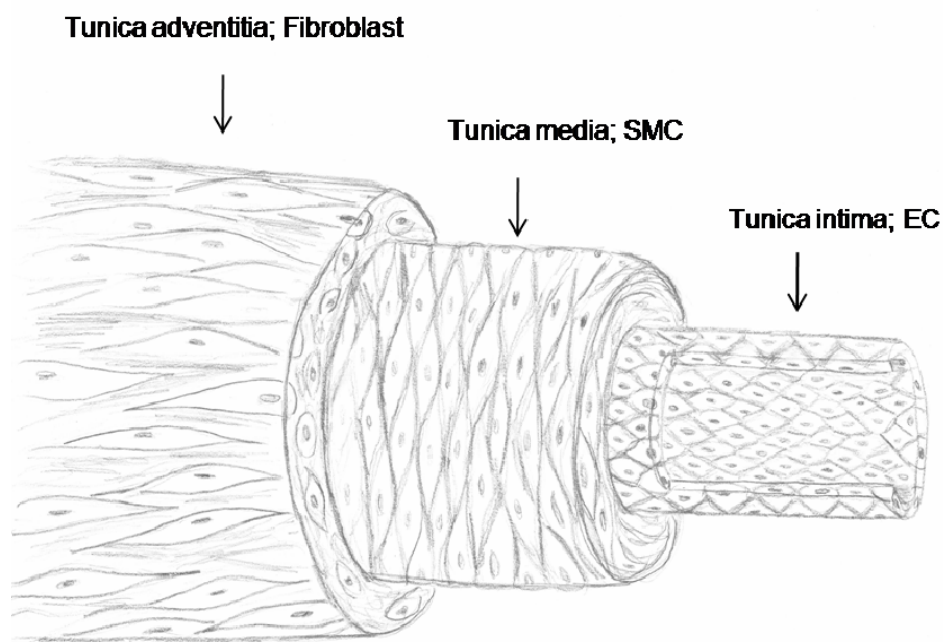
Cardiovascular diseases are a major cause of morbidity, representing 30% of all deaths in the world [3]. Two common diseases are atherosclerosis and hypertension [4]. Atherosclerosis is an inflammatory disease in the arterial wall. It leads to plaque formation and narrowing of the blood vessel lumen [5-6], which results in impaired blood flow and possible tissue damage due to restrained oxygen and nutrition supply [6]. Furthermore, a severe consequence is that atherosclerotic plaques can rupture and clot. Pieces of the clot can detach and occlude smaller vessels and cause myocardial infarction (heart attack) and stroke (cerebrovascular insult) [4].

Blood flow in occluded blood vessels is restored by percutaneous techniques (angioplasty, thrombectomy and stents) or bypass surgery. Severely occluded coronary arteries are routinely replaced through bypass surgery. Autologous, i.e., the patient's own vessels e.g., internal mammary arteries or saphenous veins are usually used as replacement grafts. Internal mammary arteries have excellent long-term performance. Vein grafts, in contrast, undergo structural changes such as wall thickening and neointimal hyperplasia, which result in graft failure. Furthermore, veins are subjected to long-term increasing atherosclerotic degeneration [7-10].

Due to previous vascular grafting and/or non-adequate vessels owing to other diseases, many patients lack autologous replacement vessels. For these patients synthetic grafts are used [7]. Currently used synthetic grafts such as expanded polytetrafluoroethylene (ePTFE) and poly (ethylene terephthalate) are not suitable for replacing small (<6 mm) blood vessels due to thrombogenicity [11]. Tissue engineered small calibre blood vessels may therefore provide an important source for vascular replacement material.

## ***The vasculature***

Veins and arteries are the largest blood vessels in the body with walls consisting of connective tissue and layers of cells. The vascular wall is organized in three layers; the innermost layer, tunica intima, the middle, tunica media and the outermost layer, tunica adventitia (Fig. 1). These layers have different components and properties; interconnection, distribution and orientation of these give rise to the mechanical properties of the vessel wall [4].



**Figure 1.** A schematic drawing of the three layers of a blood vessel wall and the cells comprised in each layer. SMC = Smooth muscle cell, EC = Endothelial cell.

The tunica intima controls the passage of cells, nutrients and waste products through the vascular wall. It includes a monolayer of endothelial cells (ECs), that line the lumen of the entire vascular tree, and an underlying basal lamina [12]. Smooth muscle cells (SMCs) are concentrically arranged and embedded in elastin, collagen and proteoglycans in the tunica media. The primary role of vascular SMCs is to maintain adequate blood flow through regulation of vascular tone [4]. The main role of the tunica adventitia is to act as a protective

sheath against acute overpressure. It consists of fibroblasts surrounded by a dense network of type I collagen fibres, nerves and vasa vasorum [4].

### ***Smooth muscle cell biology and physiology***

The current Thesis focuses on the vascular SMCs since these are often used as one component in engineered blood vessels. The role of SMCs in the vessel is discussed below, their role in TE is discussed in section “Cells employed for vascular engineering”.

#### **Smooth muscle cell plasticity**

Smooth muscle cells are spindle shaped and typically 100  $\mu\text{m}$  long and approximately 5  $\mu\text{m}$  in diameter [4]. Vascular SMCs are divided into two phenotypes; the synthetic and the contractile. The synthetic phenotype is highly proliferative, while the contractile phenotype controls vascular diameter. Smooth muscle cells can switch between these two phenotypes and transient phenotypes of these extremes, a process called phenotypic modulation [4, 13-16].

#### **Regulation of vascular tone**

In the vasculature, SMCs communicate with each other via gap junctions to spread hyperpolarisation and synchronise vasoactivity [17-18] in response to microenvironmental signals. As previously mentioned, there is a difference in the long-term outcome of bypass surgery, depending on which autologous vessel is used as graft. This discrepancy may be explained by the difference in regulation of vascular tone accomplished by neurotransmitters, hormones and a variety of local factors [19-25].

Vascular tone is regulated through contraction and relaxation of SMCs. Contraction is carried out by innervations of the sympathetic nervous system through the neurotransmitter norepinephrine and  $\alpha_1$  and  $\alpha_2$  adrenoceptors. Nitric oxide produced by the ECs in response to luminal stimuli, like e.g., acetylcholine (ACh) and mechanical effects relax SMCs and lead to

vasodilation [4]. Conversely, in the absence of the endothelium, ACh causes contraction of SMCs [26].

Acetylcholine is synthesized in cholinergic nerve terminals and released by exocytosis. Cholinergic innervations, however, is very limited in the vasculature and is not present in the saphenous vein [27]. It is, nevertheless, becoming increasingly evident that non-neuronal cells and organs devoid of cholinergic innervation, express components needed for the synthesis and degradation of ACh; this is called the non-neuronal cholinergic system.

### The non-neuronal cholinergic system

Acetylcholine is mainly known as the major neurotransmitter in both the central and peripheral nervous systems. The biology of the non-neuronal cholinergic system has recently been reviewed [28-30]. Acetylcholine participates in biological processes such as gene expression, proliferation, differentiation, intercellular communication, cell motility and immune functions. It acts in both an auto- and paracrine manner.

Recent studies indicate that the non-neuronal cholinergic system may be present even in human and rat vascular ECs [30] and arterial SMCs [31-32]. Its function in the vasculature is not yet determined. Acetylcholine is thought to be involved in a local regulatory loop in vascular tissue by affecting nitric oxide release from ECs [33].

### Components of the cholinergic system

The components of the cholinergic system include factors for synthesizing and degrading ACh, uptake of choline into cells and the receptors for ACh.

Acetylcholine is derived from choline and acetylcoenzyme A (acetyl CoA). Choline is transported into the neurons by the high affinity choline transporter (CHT1), which was previously thought to be unique for cholinergic neurons. It has however been demonstrated in human and rat keratinocytes [34] and arterial SMCs and ECs [32]. The ACh synthesis occurs in the cytoplasm of the axon terminal in cholinergic nerves through the action of choline acetyltransferase (ChAT). In neural tissue, this enzyme is produced only in cholinergic

neurons and is therefore a good marker for cells using ACh as a neurotransmitter. After synthesis, ACh is transported by the vesicular acetylcholine transporter (VACHT) into synaptic vesicles where it is stored. It is not yet determined how ACh is stored in non-neuronal cells. Thus expression of VACHT, previously reported in human melanocytes and keratinocytes [35], may indicate vesicular storage. Acetylcholinesterase (AChE) is an ACh degrading enzyme. It is located on the effector cells where its role is to terminate the ACh stimuli [36]. There are two receptor families for ACh located in the cell membrane, the nicotinic (ion channels, nAChRs) and the muscarinic (G-protein coupled, mAChRs) ACh receptors [37]. The subtype  $\alpha 7$ nAChR is expressed in e.g., rat vascular SMCs and ECs [31].

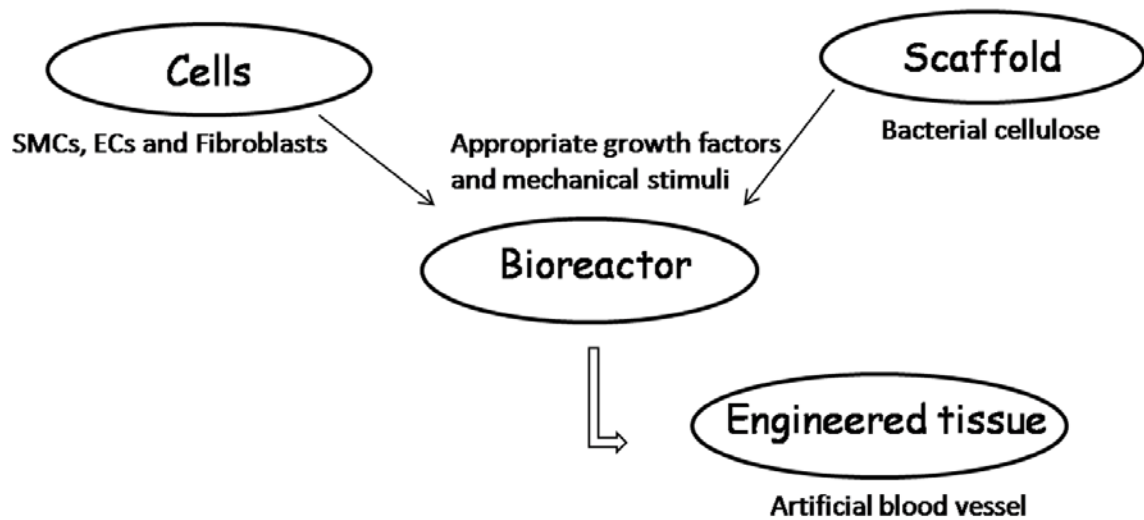
### ***Elements of tissue engineering***

An engineered tissue should be similar to the native tissue and mimic its functions. It should also be non-immunogenic i.e., not elicit an immune response in the host and as well be non-toxic. Furthermore, the construct should also integrate well into the host tissue through cellular infiltration and neovascularisation, and possess appropriate mechanical properties. Additional to these main properties, an engineered tissue should also be readily obtainable and easy to handle for the surgeons [7].

The basic elements in TE *in vitro* are (Fig. 2): cells, biomedical scaffold materials and bioreactors that provide appropriate environments in which the tissues are grown [38]. Each tissue possesses its own challenges regarding cell source, scaffold design and environmental requirements for growth. It is not necessary to use scaffolds in TE. However, most approaches for TE employ biomedical materials to guide tissue growth into the right shape and to provide mechanical stimulation to the growing tissue. Engineering tissue *in vitro* often takes several weeks, due to prolonged cell culturing to obtain a sufficient amount of cells, and for the tissue to form and mature. The problem is that acute transplantations require tissue immediately. Accessibility can be solved by constructing off-the-shelf products.

An approach to satisfy the acute need of a graft may be to construct an acellular device or tissue that can be easily stored and that recruits cells *in vivo* after grafting [1].





**Figure 2.** A schematic figure of the elements in vascular tissue engineering *in vitro*. Cells, scaffold and bioreactor are the fundamentals. The specific elements that are used depend on which tissue that is created.

### ***Engineering blood vessels***

To meet all the requirements above, the tissue engineering researcher faces challenges, i.e., what cell source to use, appropriate biomaterial design, biocompatibility, as well as production time and storage conditions of the product.

It is a common opinion in the vascular engineering field that an optimal vascular graft, in addition to the properties mentioned, should be:

- Sufficient in its mechanical integrity and withstand *in vivo* hemodynamic forces exerted by the cardiovascular system, i.e., resist dilations and rupture.
- Matched in compliance, i.e., resist recoil/collapsing when distending and compressing forces are withdrawn, with native blood vessels.
- Completely incorporated in the tissue to resemble a native blood vessel in structure and function.
- Resistant to both thrombosis and infections.
- Patent (stay open) for a long period of time.
- Easy to suture and sterilize.

## ***Cell source***

The cell source and the phenotype of the cells (differentiated/undifferentiated) for engineered tissues are of utmost importance. Cells for TE should be easily procured and expanded, survive initial expansion, be non-immunogenic, function normally and not become malignant [39]. Much research is undertaken to finding the optimal cell source. To date, cells are derived from autologous differentiated cells; i.e., the patient's own cells, or allogenic differentiated cells; cells from a human donor, adult stem cells or progenitor cells, or even embryonic stem cells [1].

Host rejection to a cellularised engineered tissue may be prevented by using autologous differentiated or progenitor cells. Employing allogenic cells (differentiated or progenitor) or embryonic stem cells (when differentiated), however, requires induced immune acceptance in the host. Applying xenogenic cells in tissue reconstruction is controversial due to immunogenicity and the risk of transmitting pathogens to humans from the donor species [40].

Autologous cells are the most appropriate choice for TE. However, issues arise regarding isolating and culturing these cells. The methods for obtaining cells may be invasive, painful to the patient and costly. Furthermore, it may provide only a limited amount of cells and thus requires cell isolation months in advance and a long culturing period to obtain sufficient quantities. This affects both cell phenotype and age. Within the tissue, cells are subjected to unique mechanical and environmental stimuli through interaction with the extracellular matrix (ECM) and other cells. Taking cells out of their original milieu and culturing them in an artificial environment inflicts trauma and may lead to changes in cell phenotype.

## **Cells employed for vascular engineering**

To create a vascular conduit that mimics the native vessels, researchers often use the cells which constitute the vascular wall. Thus, the cells used for vascular engineering are usually ECs, SMCs and fibroblasts from the vasculature of humans or various animals. Cell sources from animals include bovine aorta [41-42] and pulmonary arteries [43], canine [44] and ovine

[45] carotid arteries, and mongrel dog femoral veins [46]. Human cells are from the umbilical vein [47-48], saphenous vein [49], coronary artery [50] and aorta [51]. These cells are nevertheless acquired through invasive methods, and are thus not optimal. Both ECs and SMCs may however be obtained from other tissue sources. Endothelial progenitor cells (EPCs) and smooth muscle progenitor cells can be found in the blood [52-53]. Progenitor cells differentiate into ECs in culture when stimulated with vascular endothelial growth factor (VEGF) [52]. These cells, however, are closely associated to age and the presence of cardiovascular risk factors such as hypercholesterolemia and diabetes mellitus. Occurrence of these risk factors and aging reduces the level of EPCs in the blood and also impairs the migratory capacity of the cells [53].

Circulating smooth muscle progenitor cells differentiate into SMC *in vitro* when stimulated with platelet-derived growth factor BB (PDGF-BB) [52]. Whether these cells can be used in regenerative medicine is uncertain. They are immature, highly proliferative and exhibit a pro-inflammatory phenotype, resembling dedifferentiated SMCs in atherosclerotic plaques. Mesenchymal stem cells (MSCs) are found in the bone marrow and adipose tissue. These cells are multipotent and differentiate into SMCs *in vitro* in the presence of appropriate growth factors [54]. Adipose tissue may present the optimal autologous cell source. It is easy to obtain with minimal discomfort to the patient and yields a high amount of cells. In addition to MSCs, adipose tissue also provides endothelial progenitor cells [55-56].

Independently of the cell source, it is essential that the cell culture provides suitable conditions for the cells to grow and differentiate/stay differentiated. This includes proper culture containers, medium and growth factors.

### ***Biomedical scaffolds guide tissue formation***

Scaffolds are biomaterials that provide a three-dimensional structure which cells can be seeded onto/into and cultured. Appropriate scaffold design is important for the material interaction with cells and tissues. A biomaterial is defined by Williams as “*a substance that has been engineered to take a form which, alone or as a part of a complex system, is used to*

*direct, by control of interactions with components of living system, the course of any therapeutic or diagnostic procedure, in human and veterinary medicine” [2].*

Materials used in TE are categorized into synthetic polymers or naturally derived materials. These can be either biodegradable or non-degradable [57]. The TE and regenerative medicine fields move towards creating completely biological artificial tissues and organs. Although biological alternatives are favourable, synthetic degradable materials may be used for this purpose by allowing the scaffold to degrade during tissue formation *in vitro*. This approach, however, demands that the degradation rate is synchronized to the tissue growth to ensure adequate mechanical properties.

Whether the biomaterial is degradable or not, for a successful outcome of a graft, it is required to be biocompatible, i.e., “perform with an appropriate host response in a specific application“ [57]. The biocompatibility of materials is enhanced by altering porosity [58], shape and mechanical properties such as strength and elasticity. By binding growth factors to the structure, or designing a structure that slowly emits growth factors, cells may be promoted to adhere to, proliferate on and migrate into the scaffold [59].

Another issue for a successful graft is the transportation of oxygen and nutrients to the engineered construct, and transportation of waste products from it. This is done by perfusion of the tissue or organ. Oxygen diffusion is limited to ~150-200  $\mu\text{m}$  from the vessel wall [11]. Larger organs would therefore need to be vascularised. Angiogenesis may be induced by the release of growth factors (VEGF, PDGF and basic fibroblast growth factor) from the engineered organ [60-61]. Accelerated angiogenesis occurs in materials with pore size >250  $\mu\text{m}$  compared to those with pore size <250  $\mu\text{m}$  [62]. Consequently, porosity and pore size may be critical for the survival of an engineered graft.

### Biomaterials applied for engineered blood vessels

The materials used for constructing artificial blood vessels are mainly collagen matrices and biodegradable polymers. Approaches for engineering vascular grafts will be discussed later in the text. Table 1 presents an overview of some materials applied in vascular engineering.

**Table 1.** Biomaterials employed for engineering vascular grafts

	<b>Biomaterial scaffolds</b>	<b>References</b>
<b>Natural</b>	Collagen Collagen-Elastin Decellularized tissue Hyaluronic acid Bacterial cellulose	[42, 63] [48] [64-68] [69-70] [71-73]
<b>Synthetic</b>	Poly (ethylene terephthalate) Poly (glycolic acid) Polyurethane Expanded polytetrafluoroethylene	[42] [41, 44, 49, 74-76] [50, 77] [78]
<b>Without any scaffold</b> Sheet-based-tissue engineering Bioprinting The body as bioreactor		[47, 79] [80] [81-82]

The current Thesis investigates poly (glycolic acid) (PGA), ePTFE and bacterial cellulose (BC) as biomaterials.

### Poly (glycolic acid)

Poly (glycolic acid) is the most investigated biodegradable material. It was used in the first absorbable synthetic suture and has, since 1970, been available commercially. Other products include membranes for dentistry, bone fixation devices and implantable drug delivery systems [57]. Poly (glycolic acid) is currently examined as scaffold for engineering blood vessels by several research groups and is promising for this application [41, 45-46, 49, 83]. Due to hydrolytic degradation, the mechanical strength of PGA is lost over a period of 2-4

weeks after implantation. To prolong the degradation period, PGA is often used in copolymers with poly (lactic acid), polyethylene glycol or polyhydroxyalkanoate [38].

### Expanded polytetrafluoroethylene

Polytetrafluoroethylene (PTFE) is a synthetic biologically inert and non-biodegradable polymer. It is flexible, resilient and durable. Due to these properties it is used as vascular grafts [57].

Expanded polytetrafluoroethylene is woven PTFE. It is a strong, although soft and porous material. The pores allow cell and tissue ingrowth into the structure. Though biocompatible, it is prone to thrombosis due to lack of ECs in the lumen. Ongoing efforts are investigating the possibility of *in vitro* endothelialisation by coating ePTFE grafts with extracellular matrix components and seeding ECs onto them [78]. A study by Tai *et al.* showed that ePTFE is less compliant compared to the human artery [84]. Compliance mismatch of a prosthetic graft and native artery cause distal anastomotic myointimal hyperplasia. Myointimal hyperplasia is the abnormal proliferation of SMCs in the vascular wall. The cells migrate into the subendothelial area and form a hyperplastic lesion, which causes stenosis and obstruction of the lumen [85].

### Bacterial cellulose

Cellulose is a natural polysaccharide produced by plants, algae and bacteria. It consists of a linear chain of D-glucose units [86]. Cellulose has been used in biomedical applications. By altering the physical and chemical properties of cellulose, it can become biocompatible and even absorbed *in vivo* [87]. In contrast to plant cellulose, BC, also known as microbial cellulose, is pure and does not contain compounds such as lignin, pectin or arabinan that are found in plant cellulose [88]. Furthermore, the BC is highly crystalline, has fine and highly pure nano-fibril network, high mechanical strength and water content (~99%).

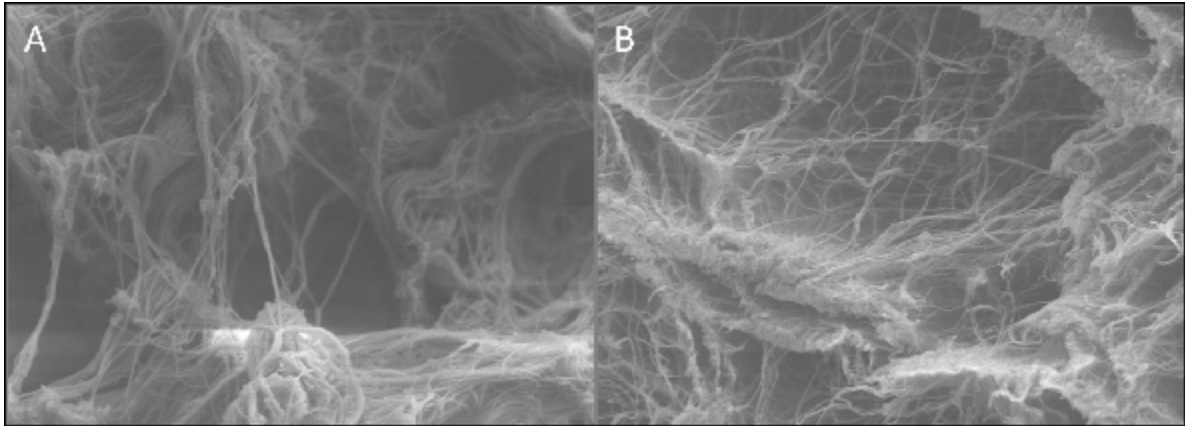
*Acetobacter xylinum* is a rod-shaped, gram-negative aerobic bacterium that produces and excretes BC extracellularly [89]. The bacterium is found on fruits and flowers. Why *Acetobacter xylinum* produces BC is not yet known, however, it is hypothesised that it is a

means of protection against UV-light and competitors that use the same substrate as a source of nutrients [90]. The cellulose also provides protection against natural enemies [91]. It may also be used to retain moist or as a floating device that allow contact with the atmosphere [92].

Bacterial cellulose can be shaped into 3D-structures *in vitro* during culture. Structural variations such as thickness and network density can be controlled by changes in culture conditions. The BC is produced in a layered and asymmetric structure in the interface of air and liquid, resulting in a denser surface facing the air and a more gelatinous network formation in the liquid. The BC network structure is highly porous (94.1%) with microchannels in the network of varying size [93].

Bacterial cellulose is used in the medical sector for wound care treatment. It is degraded in nature by microbial and fungal enzymes. These enzymes are not present in the human body; thus BC could be used as a non-biodegradable biomaterial for engineered grafts. Although BC cannot be degraded enzymatically in the human body, it may be degraded by the inflammatory process. Moreira et al. studied whether BC nanofibres are toxic to cells i.e., mouse fibroblasts, and if the fibres affect the cell proliferation. The study showed that cellulose is not toxic to cells. Although the proliferation rate is reduced in the presence of BC fibres the cell morphology was not altered [94]. Several research groups are investigating applications for BC as biomaterial for engineering replacement of both hard and soft tissues such as blood vessels [71-73, 95], bone [96-97], cartilage [98-100] and dura mater [101].

Similarly to blood vessels, BC has high water content and has cellulose fibres resembling collagen in size (Fig. 3). Previous studies from our laboratory indicate that BC is biocompatible [102] and that tubular BC can be produced with sufficient mechanical properties for application as vascular grafts [103]. Additionally, ECs and SMCs adhere to and proliferate on the BC [72, 103]. Interestingly, coagulation studies show that BC does not activate the coagulation cascade to the same extent and speed as currently used graft materials [73].



**Figure 3.** Electron microscopic images of A) collagen from umbilical cord and B) bacterial cellulose produced by *Acetobacter xylinum*. Magnification x 10,000. Collagen and BC have similar fibre structure [72].

### ***Bioreactors control the environment and provide physical signalling***

The long-term success of an engineered tissue depends on its ability to respond to biological and biomechanical signals in the body. To meet the demands of correct tissue architecture and proper functionality, tissues are usually grown in bioreactors that mimic the environment of native blood vessels. Reactors provide the appropriate dynamic culture conditions and supply nutrients, growth factors and signalling molecules necessary for the cells to generate a tissue [64, 104-105].

Bioreactors may also provide monitoring of tissue formation and allow observation of the reaction of the tissue to physical stimuli [106]. Thus, the bioreactors do not only provide a milieu for tissue growth, but also a means to test the outcome of the tissue construct.

A common bioreactor setup for engineering blood vessels is a container in which tubular structures with seeded cells can be mounted. The reactor is connected to a loop system, including a medium reservoir and a peristaltic pump that allows perfusion of the tissue construct and provides mechanical stimulation [44, 49, 64, 106-107].



### ***Inflammation, foreign body response and healing***

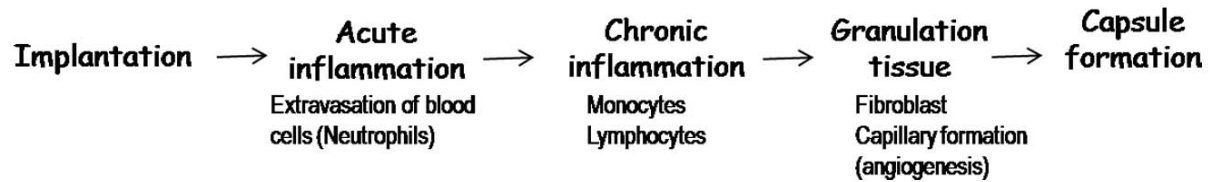
Implantation procedures of a biomaterial or tissue cause injury to the involved tissues or organs, which elicits inflammatory reactions. The reaction in the tissue is similar to the healing response, the difference being the presence of an implant (Fig. 4). Healing mechanisms are initiated by an acute inflammation as a response to injury, by the activation of inflammatory cells such as neutrophils, monocytes and mast cells. Inflammation is characterized by changes in vascular permeability, exudation of plasma protein and extravasation of blood cells (mainly neutrophils) [57]. Macrophages together with mast cells produce inflammatory mediators that cause vasodilation and increased vascular permeability. This leads to exudation of blood plasma and allows leukocytes to migrate to the site of injury. Inflammatory signals induce the expression of cellular adhesion molecules in the endothelium including selectins. These tether circulating leukocytes that are attracted to the site of injury by chemokines. Binding to the endothelium slows the leukocytes and causes rolling. Simultaneously, chemokines activate the leukocytes which results in tighter adhesion to the endothelium and subsequent migration through the vascular wall [108].

Persistent inflammatory reactions lead to chronic inflammation, distinguished by infiltration of macrophages and lymphocytes simultaneously as tissue is destructed. Tissue repair involves formation of granulation tissue, fibrosis and neovascularisation. Formation of granulation tissue is considered a normal wound healing response to implanted biomaterials; it is also a normal foreign body reaction [57].

Fibroblasts and myofibroblasts are critical participants in the wound healing process. These cells produce extracellular matrix (ECM) components, and contract the tissue which results in wound closure. Fibroblasts exhibit both contractile and non-contractile phenotypes, and regulate the turnover of the ECM during normal tissue conditions. However, in injured tissues, fibroblasts become activated and differentiate into myofibroblasts.

Myofibroblasts are an intermediate between SMCs and fibroblasts. Similarly to SMCs, these cells express the early SMC differentiation marker smooth muscle  $\alpha$ -actin. The myofibroblasts are also contractile. Although important in the wound healing process, these

cells may cause pain, tissue immobilization and loss of function, attributable to excessive actions that results in tissue fibrosis, i.e., scar tissue and contraction of the tissue [109].



**Figure 4.** Common events in both the healing process and the host reaction to implants.

### ***Engineered blood vessels***

Wineberg and Bell were the first to construct a multilayered vascular structure based on collagen, SMCs, fibroblasts, ECs and Dacron mesh. The model demonstrated differentiated SMCs and functional ECs. The mechanical properties however were poor and not adequate for a vascular graft [42]. Others have used similar techniques both with and without synthetic mesh [47, 110-112]. Improvement of the mechanical properties, of constructs without synthetic mesh support, was undertaken by applying pulsatile flow during tissue culturing. The pulsatile flow results in circumferential alignment of SMC and EC parallel to the flow direction [110, 113].

Several researchers have created completely biological vessels based on the degradable, synthetic polymer PGA [41, 45-46, 49, 83]. Niklason *et al.* seeded bovine aortic SMCs and ECs on a tubular PGA scaffold and cultured the tissue in a bioreactor with a pulsatile flow for 8 weeks. The rupture strength ( $2150 \pm 709$  mm Hg) had the same magnitude as native human saphenous veins ( $1680 \pm 307$  mm Hg). Implanted in swine, these engineered vessels were patent for four weeks [41]. Hoerstrup *et al.* produced a vascular graft based on a PGA scaffold and ovine myofibroblasts and ECs. Grafted in the main pulmonary artery of lambs, the artificial vessels showed good performance for up to two years [83].

Decellularised tissues are also investigated for application in vascular engineering. One such is the small intestine submucosa (SIS) which can withstand a pressure up to 3517 mm Hg which is satisfactory for a vascular graft [65].

Completely biological artificial blood vessels were constructed without the use of scaffolds by L'Heureux *et al.* [47, 79]. Cultured sheets of human umbilical SMCs and fibroblasts were wrapped around a tubular mandrel, thus creating a medial and an adventitial layer. After maturation of about 7 weeks, the mandrel was removed and the lumen was seeded with human umbilical ECs. The vessel resembled a human artery and had a burst pressure of  $2598 \pm 5012$  mmHg. Grafted in dogs, the vessels displayed a patency rate of 50% 7 days after implantation [47]. Similar vascular grafts were made with fibroblasts and ECs isolated from patients who had undergone bypass surgery. Grafted in rats as abdominal interpositional grafts for up to 225 days, the grafts showed 86% patency. Furthermore, the grafts had integrated into the surrounding tissue, and displayed smooth lumen and intact anastomoses. There were no signs of aneurysm formation or narrowing of the lumen. Similar features were displayed of these engineered vascular conduits after arterial grafting in macaques, where these vessels were patent up to 8 weeks [79].

Another approach to create autologous vascular grafts has been presented by Campbell *et al.* [82] and Chue *et al.* [81]. In these studies, tubings were inserted in the peritoneal or the pleural cavities of rats, rabbits and dogs, to act as irritants for the induction of granulation tissue and capsule formation. Harvested tubes displayed capsules that resembled native blood vessels with an endothelium-like layer of mesothelial cells that stained positive for the von Willebrand Factor specific for ECs, a layer of spindle shaped myofibroblasts that expressed the smooth muscle marker SM  $\alpha$ -actin and an adventitia of connective tissue. In vivo performance showed 72% patency 6.5 months after grafting in dog [81] and 67% patency up to four months in rat [82]. Furthermore, six weeks after tube implantation, the grafts demonstrated response to contractile and relaxing agents [82].

It is well known that long-term patency of a vascular conduit is enhanced by an endothelial lining. However, the entire lumen of grafts does not endothelialise *in vivo*. The

transanastomotic endothelialisation is limited to 1-2 cm, which is not sufficient for grafts that are usually 40-60 cm long [114]. Interestingly, transmural ingrowth of microvessels may provide ECs to the lumen. Therefore, porous vascular grafts can enhance patency [115].

## Aims

The principal aim of this Thesis was to investigate the BC-tissue and BC-cell interaction to evaluate whether BC can be used as a biomaterial, primarily for the production of vascular conduits. Since the SMCs are fundamental for several vascular functions, some focus was also directed at control mechanisms of these cells.

The specific aims of this Thesis were to test the hypotheses whether:

- Bacterial cellulose is biocompatible (*Paper I*).
- It is possible to produce BC with defined pores, and if these pores will enhance SMC migration into the cellulose (*Paper II*).
- Bacterial cellulose with engineered micropores is more biocompatible than control (i.e., conventional) BC (*Paper III*).
- Vascular SMCs display the non-neuronal cholinergic system (*Paper IV*).

## Methodological Considerations

This section provides an overview of the methods applied in the current Thesis. Detailed information is given in the individual *Papers I-IV*.

### ***Production of bacterial cellulose – Papers I-III***

Bacterial cellulose can be fermented in different shapes. In the current Thesis, two forms of BC have been evaluated; as sheet (*Paper I*) and tube (*Papers II-III*). Tubular BC was fermented with a novel technique [116]. By adding porogens we were able to produce BC with defined pores, denoted porous BC (*Paper II*).

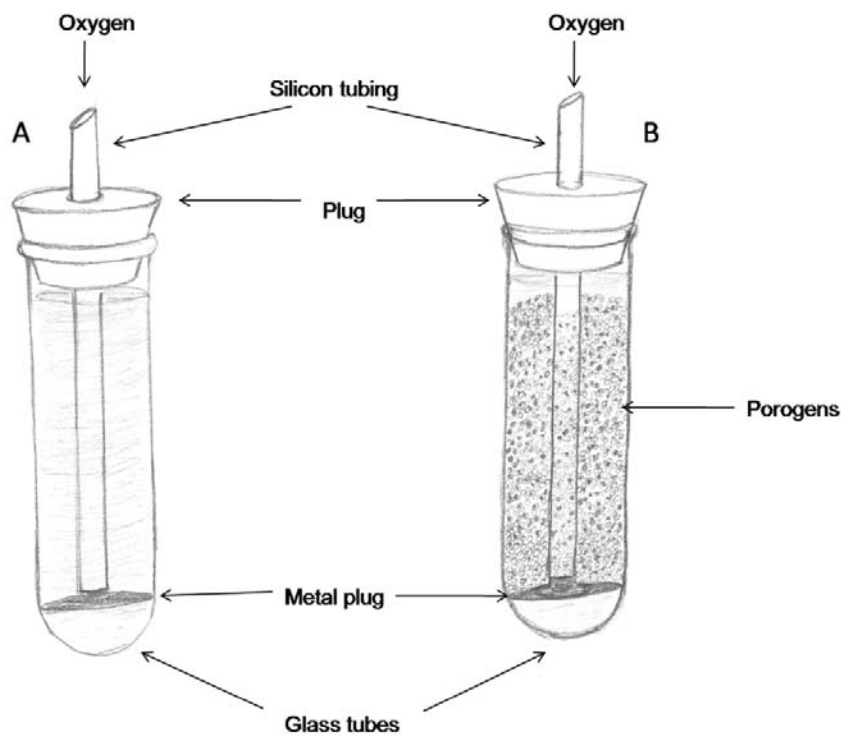
#### Bacterial cellulose – Papers I-III

The BC used in *Paper I* was fermented in a culture flask for 2 days to produce a 0.5-1 mm thick sheet. Tubular BC was produced in annular glass bioreactors with oxygen permeable silicon tubing (Fig. 5A). The silicon tube was mounted in the centre of the bioreactor which was filled with medium containing *Acetobacter xylinum*. Oxygen was blown through the silicon tube. Due to *Acetobacter xylinum* being aerobic, they accumulate near the silicon tube and produce the cellulose around it, which results in a tubular BC. The BC tubes were fermented for 7 days before harvest and subsequent purification by washing and boiling in 1M NaOH and water. Before analysis, the tubes were autoclaved (120°C, 1bar).

#### Porous BC – Papers II and III

Porous BC was produced by the same set up as tubular BC (Fig. 5B) with the addition of porogens in the medium. In *Paper II* we sought to create BC with defined pores by applying either potato starch (5-100 µm in diameter) or paraffin (90-500 µm in diameter) particles. A third porous BC structure was made with fused paraffin particles. Paraffin particles were made by melting paraffin wax and pouring into a PVA solution during rotational stirring. The solution was then cooled and the particles collected in sieves. In addition to the purification

procedure, paraffin particles were removed by cyclic washing in a surfactant solution and ethanol. Starch particles were removed by enzymatic actions and then also by surfactant and ethanol. Scaffolds were autoclaved prior to subsequent analysis and SMC culture on the biomaterials. In *Paper III*, we employed fused paraffin particles that were 150-300  $\mu\text{m}$  in diameter to produce porous BC scaffolds.



**Figure 5.** Schematic figure of the bioreactor setup for A) ordinary BC and B) porous BC.

Depending on the preference, paraffin porogens can be made in different sizes by controlling the stirring speed of the wax and the PVA solution. Additionally, more optimal and narrowed pore range may be achieved by optimizing the sieves. However, separation of the paraffin particles is difficult since the wax is trapped in the holes of the sieves. This may lead to production of porous BC with a wider span of pore sizes than the preferred range. Non-optimal pore size could result in, for example, impaired cell migration into the BC.

In the following, the non-modified conventional tubular BC is designated control BC.

### ***Bacterial cellulose analysis prior to subsequent experiments***

After synthesis and purification of the BC, it was tested for endotoxin (*Paper III*) as well as porogens and surfactant residues (*Papers II-III*) before application *in vivo* and *in vitro*.

#### **Endotoxin test – Paper III**

*Acetobacter xylinum*, like other gram-negative bacteria, contains lipopolysaccharides (LPS, also known as endotoxin) in the cell wall. Such LPS cause inflammation and activate the immune system. To ensure that bacteria and pathogenic residues were absent, we determined endotoxin levels in BC. The technique used for endotoxin testing is a standardized method. BC was cut into small pieces and incubated in water during agitation. The water was then tested for endotoxin content. This process presumes detachment of any bacterial residues and endotoxin from the cellulose through shaking. Incomplete elimination of endotoxin would result in poor biocompatibility due to immune system activation. The endotoxin level for medical devices should not exceed 0.5 EU/mL. In our studies, endotoxin level in the porous BC was <0.005 EU/mL and thus adequate for *in vivo* studies.

#### **Electron spectroscopy for chemical analysis (ESCA) – Paper II**

Electron spectroscopy for chemical analysis (also known as X-ray photoelectron spectroscopy, XPS) provides information about the chemical composition of a surface. The method is sensitive and can identify almost all elements. It is based on irradiation of surfaces with x-rays and detection of characteristic elemental energies resulting from the emission of photoelectrons. The sampling volume extends from the surface of the sample to a depth of 50-70 Ångström. In *Paper II*, ESCA was employed to detect residues from porogen materials.

Prior to ESCA analysis, the BC was dried into a thin film. Although the sample is thin, the method does not allow detection of chemicals in the entire depth of the material. Furthermore, ESCA only analyses small fractions of the material. Thus, any residues in the rest of the material may be undetectable.



### Fourier transform infrared spectroscopy (FT-IR) – Papers II and III

Fourier transform infra-red spectroscopy may be applied for identification of chemical components in solids, liquids and gas. The technique is based on identification of chemical bonds by irradiation of a sample which results in a characteristic infrared absorption spectrum that is a molecular "fingerprint".

By subjecting the porogen materials, surfactant, porous BC and filter paper to FT-IR analysis, we were able to compare the molecular composition of the purified BC. As previously mentioned, we found no residues of either paraffin or surfactant in the porous BC. For these experiments, small fractions were cut from the BC and were analyzed. Thus, any paraffin or surfactant excess in other parts of the BC tubes were not detectable.

### ***Animal models***

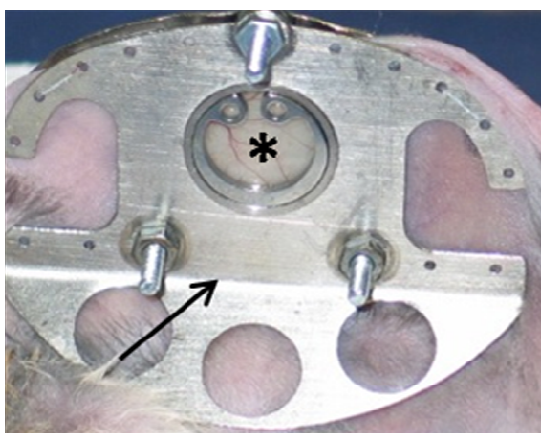
To study *in vivo* biocompatibility of BC we used both the dorsal skinfold chamber in hamster and subcutaneous implants in rat. Both methods are established in the field of TE for the evaluation of biocompatibility and tissue-biomaterial interactions.

### Dorsal skinfold chamber and intravital microscopy – Paper I

The dorsal skinfold chamber is an established method that allows repetitive intravital observation of the reaction of the tissue under investigation to implants. Intravital observations combined with epi-illumination fluorescence microscopy allows for the investigation of angiogenesis. Microvasculature can be analyzed both quantitatively and qualitatively. Repetitive fluorescence microscopic analysis may be carried out over a period of 3-4 weeks. Moreover, cellular responses such as vascular endothelial leakage as well as leukocyte trafficking and adhesion to the endothelium are detectable. Thus, both inflammatory reactions and integration of the material into the tissue may be monitored [117].

In *Paper I* we compared the vascularisation and inflammatory response to BC in comparison with PGA and ePTFE. Syrian golden hamsters, 7 animals per group, were equipped with light titanium frames that sandwiched the extended dorsal skinfold (Fig. 6). Chambers were prepared by removing a skin layer in a circular area on one side of the frame. The chamber was covered by a removable coverslip during the experimental period. Intravital fluorescence microscopic analysis was performed prior to implantation and 30 min as well as 3, 6, 10 and 14 days after implantation. Microhemodynamics such as volumetric blood flow, shear rate, blood velocity and vessel diameter as well as leukocyte-endothelium interaction and macromolecular leakage were analyzed in 1-2 venules at the border zone of the implants. The hemodynamic parameters were stable throughout the entire experimental period, indicating no agitated respiration or hypertensive stress in the animals [118]. Angiogenesis was evaluated in eight regions of interest at the border and in the centre of each implant.

This study was conducted in collaboration with Professor Michael Menger and his group at the University of Saarland, Saarbrücken, Germany. The experiments were performed in Germany in accordance with the German legislation on protection of animals and the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, USA), and were approved by the local governmental animal care committee.



**Figure 6.** An illustration of the titanium frame (arrow) mounted on a Syrian golden hamster and the prepared skinfold chamber (asterisk).

### Subcutaneous implantation – Paper III

Subcutaneous implantation is a conventional method for analyzing the biocompatibility of a biomaterial [99, 119-121]. In this animal model, biomaterials are grafted under the skin. It has previously been used in our laboratory [102]. The study was approved by the Ethics Committee at Gothenburg.

In *Paper III*, we implanted pieces of tubular conventional BC (control) and porous BC in 14 female Wistar rats. A one cm incision was made on the back of the rats and a pocket was prepared on each side. One of each cellulose type was inserted without fixation with sutures into either of the pockets. Grafts were explanted 3 and 7 weeks upon operation for subsequent analyses.

As previously mentioned, the BC becomes asymmetric during culturing. Tubular BC has a dense luminal surface and a loose network structure at the outer surface. In the current Thesis, BC was implanted as tubes to study cellular migration into the outer surface. As a tubular implant, the outer surface will be interacting with the surrounding tissues. Therefore, it is of interest to first evaluate the overall biocompatibility and host reaction to this surface prior to implantation into any circulatory system in the body.

### ***Cell culture – Papers II and IV***

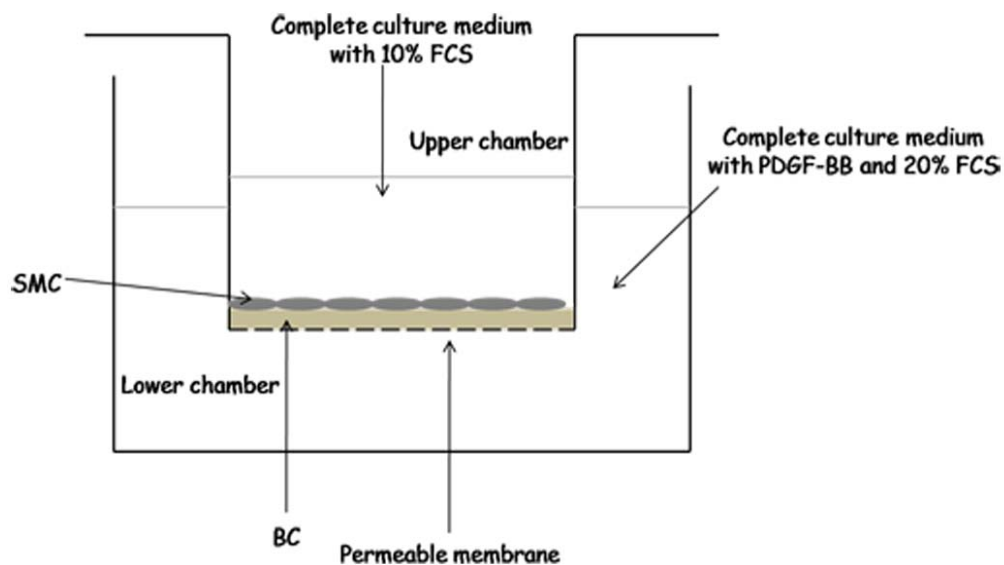
Human venous SMCs (*Papers II and IV*) were isolated from spare parts of saphenous veins from patients undergoing bypass surgery. Human arterial SMCs (*Paper IV*) were either splenic or superior mesenteric from patients undergoing either splenectomy for hypersplenism or resection for colon carcinoma. Donors were anonymous. According to the Swedish law, permission from the ethics committee is not required for research on anonymous surplus specimen

Smooth muscle cells were isolated with the outgrowth-technique. The medial layer of the vessel wall was separated from the intima and the adventitia. Small pieces of the media were cut and placed in cell culture wells. Cells were allowed to migrate out from the biopsies until

confluence. Cultures were tested for bacterial, fungal and mycoplasma contamination. Smooth muscle cells were confirmed through morphology and expression of SM  $\alpha$ -actin detected by immunocytochemical analysis (*Papers II and IV*) and Western blotting (*Paper IV*). The cells were used in passage 5 (*Paper II*) or passage 4-6 (*Paper IV*).

### ***Smooth muscle cell migration – Paper II***

In *Paper II* we studied the migration of SMCs into porous BC using Boyden chambers (Fig. 7). Boyden chambers utilize two chambers that are separated by a permeable membrane. Cells are seeded in the upper chamber (the inserts) and are allowed to migrate through the membrane. The lower chamber (the well) is filled with medium and supplemented with a chemoattractant.



**Figure 7.** Schematic picture of a Boyden chamber. A permeable membrane in the upper chamber supports the bacterial cellulose (BC) with seeded smooth muscle cells (SMCs) and allows diffusion of growth factors between the chambers. FCS = foetal calf serum, PDGF-BB = platelet-derived growth factor BB.

Porous cellulose tubes were cut open and round pieces were punched out. The BC pieces were placed in the inserts with the compact luminal side down towards the permeable membrane as a support. Smooth muscle cells were seeded on the cellulose pieces and were grown for one or two weeks. The inserts were filled with medium supplemented with 10% foetal calf serum (FCS), and the wells with serum supplemented with 20% FCS and PDGF-BB to attract cell migration towards the lower part of the BC.

### ***Paraffin sections – Papers I-IV***

For histological and immunohistochemical analyses, vascular biopsies, implanted BC and BC preparations with cells were fixed in 4% phosphate buffered formaldehyde. The samples were dehydrated, embedded in paraffin and sectioned. Achieving intact 5 µm sections of the BC, especially the porous BC, was challenging (*Papers II and III*). Tissue or cell layers were often detached from the cellulose during sectioning, resulting in sections with fractures and empty spans between the BC and the tissue. To meet these problems and avoid fractured sections, sectioning was performed first through the cell layer and then through the cellulose (*Paper II*). Intact sections were also obtained by thicker sectioning (8 µm) (*Paper III*).

### ***Naphthol AS-D chloroacetate esterase – Paper III***

Naphthol AS-D chloroacetate esterase (CAE) identifies mast cells and granulocytes (eosinophils, basophils and neutrophils) [122]. The lysosomes of these cells contain an esterase that can use the Naphthol AS-D chloroacetate as substrate and release the naphthol. Naphthol reacts with the diazonium salt "Fast Red Violet LB" and is visualized as red depots. We used CAE to investigate the presence of inflammatory cells of the myeloid lineage in the BC and surrounding tissue.

### ***Immunohistochemistry – Papers I, III and IV***

Histology is a well established technique and an essential tool for both diagnostic pathology and research. Both immunohistochemistry (IHC) and immunocytochemistry (ICC) are based on detection of cellular components (antigens) through specific antibody binding to a target of interest, and subsequent labelling of the antibody. Immunohistochemistry is employed for staining tissues, while ICC is used for staining cultured cells. The avidin-biotin-complex method (ABC-method) is widely used. It was applied for IHC analysis in *Paper I* and all ICC analysis in *Paper IV*. The ABC-method was also applied in IHC analysis in *Paper IV* that applied primary antibodies derived from goat. For the IHC analysis when the primary antibody was from mouse or rabbit (*Papers III-IV*), we used the Envision system from Dako Cytomation. It is a more sensitive, easy and less time consuming technique compared to the ABC-method. Unspecific staining due to binding of biotin to endogenous avidin may be a problem with the ABC-method, this is prevented in the Envision-method by the absence of biotin.

We performed IHC on BC implants to evaluate the tissue and cellular reaction to the material (*Papers I and III*). Additionally, sections of veins and cultured vascular SMCs (*Paper IV*) were examined for cholinergic markers.

### ***Western blotting – Paper IV***

Tissue extracts of both venous and arterial SMCs were analysed by Western blotting (WB) for expression of various cholinergic markers. This method involves the detection of proteins through antibodies as in IHC and ICC. For all these methods, antibody specificity is of fundamental importance. In contrast to ICC and IHC, WB utilizes protein extracts that are first separated by size and then the protein of interest is detected with antibodies. Thus, WB has an advantage in not only answering whether or not the antigen is present, but also provides an assurance by presenting protein size. Furthermore, WB also allows a more accurate quantitative evaluation of protein levels by the analysis of band intensity. However this was not performed in this Thesis. Controls were undertaken by excluding the primary antibody and replacing it with serum. For those primary antibodies with commercially

available “blocking peptides” (i.e., the immunogenic antigens), additional controls were performed by absorbing the primary antibody with its antigen. At least ten times more antigen relative to the antibody was used for neutralizing the primary antibodies.

### ***Organ bath technique – Paper II***

The organ bath technique is traditionally applied in pharmacological experiments on muscle preparations *in vitro*, investigating the concentration-response relationship of a certain chemical compound causing contraction or relaxation of the tissue. A strip of tissue containing muscle cells is mounted in a chamber filled with a continuously oxygenated physiological salt solution. The tissue is pre-stretched in order to obtain a preload that ascertains an optimal length-tension relationship. To monitor the mechanical activity of the tissue under investigation, one end of the strip is attached to a force transducer, which, in turn, is connected to a pen recorder or a computerized recording system. In *Paper II* the organ bath was used to analyze the mechanical activity of SMCs grown on BC. Samples were exposed to the muscle relaxatory agents papaverine sulphate and adrenaline, and to the contractile agents KCl, noradrenaline and ACh.

### ***Statistics***

Results were statistically evaluated with GraphPad and SPSS 15.0 for windows. In Paper III, outliers were identified with Grubbs’ test in GraphPad. SPSS was used for analyzing unpaired data with the non-parametric Kruskal-Wallis (*Paper I*) and Mann-Whitney U-test (*Papers I and III*). Paired data on time-dependent effects (*Paper I*) were evaluated with the non-parametric Spearman rank-order correlation coefficient test. A  $p$ -value  $< 0.05$  was considered statistically significant.

## Results and Discussion

### ***In vivo studies: Is bacterial cellulose biocompatible?***

Results from our laboratory have previously demonstrated that BC exerts adequate biocompatibility and integration into rat tissue. Enhanced porosity and pore size may, as previously mentioned, improve the biocompatibility of a graft due to enhanced cellular ingrowth and vascularisation. In *Paper I*, we aimed to further study the biocompatibility of and vascular ingrowth in BC by the skinfold chamber in Syrian golden hamsters. In *Paper III*, we aimed to evaluate the biocompatibility of porous BC in comparison with control BC by subcutaneous implantation in rats.

### **Porous BC elicited an inflammatory reaction and immune activation in contrast to control BC**

Ideally, engineered tissues should not induce inflammation in the host tissue. In *Paper I*, a possible inflammatory response to BC, compared with that to PGA and ePTFE, was evaluated by studying leukocyte rolling and adherence to the endothelium. There was no significant difference in leukocyte trafficking between BC and the control groups. Thus, the BC is equal to PGA and ePTFE regarding this aspect of the inflammatory response. We also observed a significant decrease of adherent leukocyte within the BC and the ePTFE groups with time which suggests a subsiding inflammation during the observation period.

In *Paper III*, there was no indication of an inflammatory reaction to either porous or control BC as no redness, swelling or pus was macroscopically detected during the explantation procedure. Nevertheless, numerous macrophages, lymphocytes and myofibroblasts were identified adjacent to the porous BC which suggests that these implants may provoke chronic inflammation and an immune reaction. This tissue reaction was not observed in the control BC. An initial inflammation is required for the healing mechanism; however, a prolonged inflammation leads to immune activation and a foreign body response [57].



### Why does porous BC invoke inflammation and immune activation?

Inflammation and immune reaction to the porous BC, but not to the control BC may be a result of differences in the structure of the materials. Physical shape and surface properties of the same material may cause differences in experimental studies [123]. Routine histology of the implants demonstrated that the control BC had retained its shape throughout the experimental period, while the porous BC was crumpled. This may depend on porous BC being thinner and more sensitive to breakage at lower stress compared to control BC, as shown in *Paper II*. These features could cause the altered shape as a result of the movements of the rats. Additionally, both inflammation and activation of the immune system may be due to inadequate removal of porogens or surfactant. Prior to subsequent experiments, the porous BC was studied visually by light microscopy and FT-IR. Paraffin residues were not detected with either of the methods. However, light microscopy may not be the optimal tool to detect paraffin droplets inside the BC. If present, such residues should be visible by light microscopy as small black dots in the material; accordingly, very small residues inside the BC may not be detected. Furthermore, FT-IR analysis was performed on small fragments from the BC samples randomly chosen. Therefore, residues in other parts of the BC samples may go undetected.

Although endotoxin tests and FT-IR showed no endotoxin, surfactant or paraffin residues, these tests have their limitations. It is possible that immune activating agents are trapped in the cellulose network and are thus not detected by the tests. Occasionally, entire *Acetobacter xylinum* bacteria and DNA fragments from the bacteria have been detected by scanning electron microscopy and 4',6-diamidino-2-phenylindole fluorescent staining, respectively. This has been noted in both porous and control BC. Why then would the porous constructs elicit inflammation and immune reaction, but not the control ones? The pores may provide a larger surface area which enhances the chance for immunological detection. These observations do not imply that BC would be a poor biomaterial. It indicates however, that the purification process should be further optimized. The tubular shape of the BC might be a challenge, since the water and NaOH used in the purification process may not easily enter the lumen of the tubes when incubated in flasks.

We occasionally observed by light microscopy unidentified structures in the specimens that may be residues (unpublished findings). Evaluations by us (*Paper I and III*) and others [71] have not shown any inflammatory response to conventional BC. Interestingly, Mendes *et al.* noted lymphocyte and granulocyte infiltration into BC up to 30 days after implantation. The BC used was the commercially available Bionext BC membrane that is used in the clinic as wound dressing. After 60 days, these cells were no longer present. This indicates that the reaction was not persisting but resolved over time. Thus, the biocompatibility of porous and control BC should be further examined in longer *in vivo* studies to determine whether the inflammatory and immune response to porous BC declines or if it is persisting. Production and purification procedures of the porous BC must be optimized, and the scaffolds should also be carefully examined for particle and surfactant residues prior to subsequent evaluation.

#### Subcutaneous BC implants were encapsulated

In *Paper III*, we did not distinguish any encapsulation macroscopically during explantation. However, routine histology of the implants demonstrated capsule formation around all implants, as well as a cellular reaction adjacent to the porous BC.

Encapsulation of a biomaterial indicates a foreign-body response, and thus poor biocompatibility. Analysis of capsule thickness in control BC demonstrated no difference between the time points three and seven weeks. Measurements of the capsule surrounding the porous BC could not be performed, due to the crumpled form of the cellulose as length measurements require reference points. For example, we used the skin muscle tissue as reference point for measuring the capsule thickness on the skin side of the control BC, which was parallel to the muscle tissue. The porous BC however, was not parallel to the skin and additionally displayed rifts in the tissue-BC interface. In these samples, it was difficult to perceive the entire width of the capsule.

In our previous study [102], the implants were surrounded by connective tissue. However, this was not recognized as encapsulation. This distinction between the previous and the current study in *Paper III* may result from implantation of BC with different shapes [124]. In the previous study, BC was grown and implanted as a sheet; while in *Paper III* the BC was

fermented and implanted as tubes. Furthermore, the encapsulation may develop due to mechanical irritation as a result from non-fixed implantation rather than to the cellulose itself. To avoid false reactions to the BC implants, due to possible reactions to sutures, the implants were not sutured into the tissue pockets. This resulted however in relocation of some implants and deformation of the porous BC grafts. It may also have contributed to the capsule formation around the implants, owing to constant abrasion against the skin and underlying muscle tissue. Cells in the interface between the graft and the tissue can become activated as a result of mechanical abrasion. This can lead to capsule formation to compensate the mechanical burden which the implant is subjected to [123]. Capsule formation may be avoided by suturing the grafts to the tissue. In the previous study by Helenius *et al.*, the implants were also non-fixed [102]. However, the cellulose was thicker and may therefore not have moved as much as in *Paper III*.

#### New microvessels were formed adjacent to and at the borders of BC implants

Vascularisation of an implant is required for the survival of the graft and the infiltrating cells. Newly formed microvessels were present in the border of and adjacent to the BC in *Paper I* and *III*, respectively. Macromolecular leakage is an indicator of inflammation and angiogenesis. In *Paper I*, increasing leakage was observed in BC, PGA and ePTFE during the entire experimental period. Acute inflammation likely resulted from tissue damage during implantation, and is not an indication to an adverse reaction to the implants. Inflammation is a normal healing process that includes both formation of new tissue and neovascularisation which are necessary for graft survival and integration into the tissue. Additionally, leukocyte activation induces angiogenesis by producing angiogenic factors which lead to increased vascular permeability and leakage [125-127].

In *Paper I*, angiogenesis and microvessel density were analyzed in eight regions of interest in the border and centre zones of the implants. In the border zones of the control groups, these variables were significantly higher compared to BC. Angiogenesis was detected in PGA and in ePTFE 10 and 14 days, respectively, after implantation. Total neovascularisation was not observed in BC. Only PGA displayed angiogenesis in the centre zones. These results indicate that angiogenesis in BC is slower than in PGA and ePTFE. Nevertheless, the angiogenic

response and microvessel density significantly increased in BC over time suggesting that the material may be vascularised during a longer time period.

In *Paper III*, neovascularisation of the capsule was more pronounced in the porous BC. In this aspect, it may be interpreted that porous BC is more biocompatible than control BC. However, the cellular reaction induced by porous BC strongly suggests the opposite.

### Cellular reaction to BC *in vivo*

In *Paper I*, histology showed that PGA was completely populated with granulation tissue. Both BC and ePTFE only displayed granulation tissue at the border of the implant. This can be explained by the fact that PGA is degradable. However, due to this feature, rapid cellular ingrowth, proliferation and tissue formation is necessary. Thus, rapid neovascularization is also required. For example, degradation of a material used for engineering of a vascular conduit must match the period of time it will take for vascular cells i.e., SMCs and fibroblasts to form the tissue. Similar to angiogenesis, a higher degree of cellular ingrowth, of for example SMCs, into BC may need a longer period of time. It would therefore be interesting to perform longer *in vivo* studies to evaluate if BC becomes completely vascularised and infiltrated with cells generating a tissue. Immunohistochemical analysis revealed that all three biomaterials had granulation tissue that stained positive for proliferative cell nuclear antigen, a marker for proliferating cells. Apoptotic cells, staining positive for caspase-3, were not detected. Thus, cells adjacent to the BC and ePTFE and inside PGA were proliferative, indicating that these materials do not induce cell death. However, what kind of cells these were was not evaluated.

In *Paper III*, fibroblasts, identified as elongated spindle shaped cells, were present in the reactive tissue of the porous BC. They were also observed in *Paper I* and in our previous study [102]. Immunohistochemistry demonstrated myofibroblasts at the border of the porous BC implants. Excessive activity of myofibroblasts may contract a tissue to such a degree that the contraction results in tissue hardening and loss of function [109]. Contraction and hardening of the BC would not be optimal due to changes in the mechanical properties. As a vascular replacement, BC implants should be as flexible as native vessels to avoid

compliance mismatch. Hardening would result in a rigid construct that may disturb the blood flow and possibly cause thrombosis [128].

### Limitations with the dorsal skinfold chamber and intravital fluorescence microscopy

A possible drawback with the dorsal skinfold model and intravital microscopy is the impaired contrast in intravital fluorescence microscopic analysis at the later time points. This is the result of non-degraded previously applied fluorescein isothiocyanate (FITC) labelled dextran, which is used to enhance contrast of blood plasma. The problem may be solved by extending the experimental period and performing the analyses less frequently. Also, there is a risk that the chamber is damaged during the surgical procedure. In our study, two animals per group could not be used due to infections or damage of the chamber. These problems were not related to the implants, but rather from the procedure of fitting the chamber.

### Immunohistochemical considerations

A disadvantage with applying IHC, either with the ABC-method or Envision system, is that the BC is also stained. We speculate that unspecific binding or absorbance of the secondary antibodies occurs in our studies. It is however possible to distinguish the cellulose from specific cellular staining due to the structure of the cellulose.

### Summary of the *in vivo* biocompatibility studies

The dorsal skinfold chamber offers the advantage to study implant-tissue response in awake animals; however the experimental period is limited. Subcutaneous implantation provides a model to study long term effects of tissue reactions to a material. Thus these two animal models can be used to complement each other.

The results from *Paper I* indicate that the BC does not integrate into the host tissue as well as PGA or ePTFE due to less angiogenesis. Nevertheless, BC is equal to PGA and ePTFE regarding the inflammatory response. Additionally, cell ingrowth was more pronounced in BC than in ePTFE, however, not as much as in PGA. In *Paper III*, we observed that both

porous and control BC became encapsulated microscopically. In contrast to the control BC, the porous one elicited a marked cellular reaction immediately adjacent to the cellulose, composed of macrophages, lymphocytes and myofibroblasts. This distinction between the BC forms may be the result from differences in shape and structure and/or paraffin and surfactant residues in the porous BC.

Taken together, when investigated as a tube, control BC is biocompatible; however, porous BC is not. Bacterial cellulose is an interesting new biomaterial for tissue engineering applications. Though, to employ BC as a biomedical scaffold for artificial blood vessels requires additional compatibility studies, in which the BC is subjected to the environmental cue that exists in the grafting site in the vasculature. This includes biomechanical testing for hemodynamics and also blood compatibility studies both *in vitro* and *in vivo*.

Ordinary BC tubes can withstand normal blood pressure without the aid of SMCs. However, it has to be determined whether ordinary BC tubes can function as vascular conduits without seeding with cells prior to implantation. Such an approach, i.e., a non-cell seeded graft, would be optimal considering availability, storage and cost. Cellulose tubes are inexpensive to produce and are easily stored in water in the refrigerator.

### ***In vitro studies***

It has previously been shown that a BC substrate improves cell viability compared to cell culture plastics [129]. Additionally, a study in our laboratory demonstrated that SMCs migrate into BC scaffolds [72]. In *Paper II*, our main aim was to produce BC with defined pores, as a means to enhance cellular ingrowth. Secondly, we evaluated SMC ingrowth into the porous scaffolds and the mechanical activity of these in response to chemical stimuli. Additionally, in *Paper IV*, we aimed at determining whether the cholinergic system is expressed in SMCs of intra-abdominal arteries and the saphenous vein.

### Microporous BC with interconnected pores can be made by adding fused paraffin particles while culturing *Acetobacter xylinum*

Paraffin, fused paraffin and starch particles were used as porogen materials in the construction of microporous tubular BC structures in *Paper II*. During the fermentation and subsequent purification procedure, the starch particle swelled and caused damage to the pores, which resulted in a loose network structure in the outermost layer of the BC construct. Paraffin created pores halfway through the wall of the tube and the fused paraffin produced pores throughout the entire wall.

After purification, no porogen residues were detectable in the constructs. Analysis of the fused paraffin pores further revealed that the wall of the pores were more dense compared to the surrounding network, and also that the pores were denser towards the lumen of the BC tube. This could be a result of cellulose synthesis occurring more rapidly when *Acetobacter xylinum* is attached to a surface [89, 130-131]. Additionally, *Acetobacter xylinum* is aerobic and becomes more active when oxygen is provided; consequently the bacteria accumulate near the silicone tubing, thus more bacteria attach to the porogens closer to the silicon which leads to higher cellulose production in that area.

### Smooth muscle cells attach to and migrate into porous BC

A cell layer of cultured SMCs was observed on in all BC samples. The cell layers differed in thickness (one cell layer up to 200  $\mu\text{m}$ ). Only one cell layer grew on scaffolds with pores created with potato starch. Cells grew further into the BC with pores made with fused paraffin compared to those produced with non-fused paraffin. In the latter case, SMCs grew mostly on the surface of the BC and partly into the scaffold. In the former, the cells were unevenly distributed throughout the scaffold. This could be an effect of fused paraffin producing pores that are more interconnective, which allows cells to migrate more freely through the cellulose. Compared to the results from our previous study, SMC migration into porous scaffolds was better than in conventional BC fermented as a sheet [72]. Fluorescence imaging demonstrated collagen production in the cell-BC interface and in the border of the porous BC made with paraffin particles.

### Smooth muscle cells do not contract porous BC

Analysis of the mechanical activity of the SMC-BC samples with the organ bath technique demonstrated no response to either contractile (KCl, noradrenaline and ACh) or relaxatory (papaverine sulphate and adrenaline) compounds. For a tissue with SMCs to either relax or contract in response to chemical compounds, the cells must be distributed throughout the tissue and also be connected to each other with intercellular junctions. Furthermore, it is essential that the SMCs are of the contractile phenotype. As previously mentioned, SMCs are either synthetic or contractile [13, 132]. When SMCs are isolated from the tissue, they may lose their contractile phenotype. The phenotype of the cells applied in the experiments was not evaluated. Additionally, to achieve a contractile engineered blood vessel the construct with SMCs has to be mechanically stimulated [133]. Furthermore, to enhance the migration of the cells into the cellulose, we used PDGF-BB as chemoattractant. It induces the synthetic and migratory phenotype of SMCs [132, 134]. The application of PDGF-BB to the culture could explain the lack of mechanical activity. Also, since the SMCs were not homogeneously distributed through the scaffolds, they probably had not formed intercellular junctions. Therefore, the cells would not have been able to contract the cellulose. Speculatively, independent cells may be contractile but represent only a limited part of the engineered tissue and thus are unable to contract the entire construct.

### Means to enhance cellular migration into porous BC *in vitro*

Cellular ingrowth into porous BC and the cell performance needs to be further evaluated *in vitro*. The cell migration and proliferation must be enhanced, especially if the porous BC is used as scaffold to create vascular grafts *in vitro*. Cell phenotype and cellular functions should be evaluated, as well as the possible alterations that may appear in the cellulose due to cellular infiltration. Cellular migration into the porous BC and proliferation of the cells may be enhanced by infusing the pores with a biocompatible and degradable gelatinous substrate into which the cells can migrate, such as collagen, hyaluronic acid and fibrin. The pore size that we have used may be too large; instead of migrating into the material, cells may be drawn into the pores by gravitation. However, according to what has previously been reported [62], pore size ranges of 150-300  $\mu\text{m}$  (as used in *Paper III*) should promote angiogenesis. Furthermore, transmural vascularisation is required for endothelialisation of a



vascular graft. Thus, smaller pores would not be optimal. Infusing the pores with a substrate is a better alternative. Additionally, there is a risk of blood leakage through the wall of microporous BC tubes. This also suggests that the pores should be sealed. Possibly, the cells can be mixed with the substrate and be dispersed throughout the tubular BC wall at the same time as the pores are filled. Moreover, growth factors and chemoattractants may also be added into the substrate to facilitate incorporation of the graft into the tissue. A higher degree of ingrowing and proliferating cells could result in a construct with a more homogenously distributed cell population with cell-cell contact and intercellular junctions that may allow vasoactivity.

It must be evaluated whether the porous BC can resist blood pressure. However, this may be difficult due to leakage through the pores. Conventional BC tubes have sufficient mechanical properties to withstand blood pressure [103]. Though, it is a necessity to evaluate in both control and porous BC any changes in the mechanical properties that may arise as a result of *in vivo* remodelling.

*In vitro* studies that demonstrate the properties of a tissue construct may offer an indication of the *in vivo* outcome. However, it is important to consider additional remodelling that takes place in the body. It is therefore necessary to complement such studies with animal models for examining *in vivo* performance of a new graft material. Tissue ingrowth leads to changes in the mechanical properties of a vascular graft [135]. Thus, it is essential to study the graft after implantation. Although the ordinary BC tubes demonstrate adequate mechanical properties, these properties may very well be changed due to cellular infiltration.

### Smooth muscle cells express cholinergic markers

Considering the fact that vascular diseases are still the major cause of morbidity and mortality in the world, the knowledge regarding vascular regulatory systems needs to be improved to fully understand vascular physiology and pathophysiology. Differences in the regulation of vascular tone [19-25] mediated by factors such as ACh may explain why arteries are better vascular grafts than veins [7-9]. The human saphenous vein lacks cholinergic innervation [27]. In *Paper IV*, we examined the non-neuronal cholinergic system

in human saphenous vein and intra-abdominal arteries. We found expression of cholinergic markers in SMCs isolated from these vessels and in biopsies from the saphenous vein. However, the role of the non-neuronal cholinergic system in the vascular wall remains to be determined. It has been suggested to regulate cellular function, such as proliferation, motility and differentiation in a number of tissues, which are also relevant for TE.

### Challenges of SMC isolation and cellular source

In our studies on SMCs, we have used the outgrowth-technique which allows cells to migrate from tissue biopsies. A possible problem with this method is that the culture can become contaminated with ECs and/or fibroblasts. Endothelial cells are removed from the lumen by either scraping or enzymatic detachment. All ECs may not always be removed. The adventitia is removed from the media by hand with forceps and without the use of a microscope. It is therefore possible that some of the adventitial layer is not removed and thus fibroblasts may remain and contaminate the SMC culture. Furthermore, the cells migrating out from the tissue may be dedifferentiated SMC that have lost their contractility. It is important to consider that cells isolated from the body and grown in a 2D-culture *in vitro* may not exhibit the same functions as in the body. Primary SMCs may dedifferentiate *in vitro* which can lead to difficulty in interpreting the results or predicting the outcome of an implant.

Additionally, SMCs have different phenotypes, i.e., contractile and synthetic as mentioned. We have not analysed these cells. These various phenotypes have diverse expression patterns and may differ in expression of the markers that are under investigation. To further complicate this issue, the isolated SMCs from different patients may comprise of unequal amounts of the different phenotypes. However, this matter may not be of importance to predict e.g., SMC infiltration into a biomaterial in the body. An *in vitro* study demonstrates whether the cells attach to, migrate into and proliferate in a biomaterial. It does not predict the performance in the body; for this, *in vivo* studies are needed.

One other possible issue with isolating primary cells is the limited amount that is obtained. To acquire sufficient number of cells, prolonged sub-culturing may be necessary. We have

cultured cells for weeks up to months to acquire enough cells for our studies. During sub-culturing, cells are split into new culture flasks for expansion. This increases the risk of fungal and bacterial contamination and promotes phenotypic alterations.

The quality of the cells is also a critical issue. The patients undergoing bypass surgery are usually elderly and may have underlying diseases. Cells obtained from such patients may not be the optimal cell source for research. On the other hand, by using cells from the patients in need of engineered blood vessels, it is possible to achieve results that are more matched to these patients regarding cellular and tissue function. Cells from younger, healthy individuals that do not need replacement vessels or cell lines that are immortalized may not be representative for elderly patients. However, for TE transplants, the patient's own cells would be the best to employ to avoid graft rejection by the host.

### Summary of the *in vitro* studies

In *Paper II*, we demonstrated that it is possible to produce porous BC tubes with interconnected pores by the use of fused paraffin particles. Smooth muscle cells attach to and grow into these constructs; however, their phenotype remains to be determined. Porous BC is more advantageous to control BC by allowing cells to migrate further into the material. Nevertheless, cells are not evenly distributed which may influence cell function and mechanical properties of the construct. Thus, porous BC needs to be further evaluated, and perhaps modified.

We demonstrated in *Paper IV* that SMCs from both the saphenous vein and intra-abdominal arteries as well as in saphenous vein biopsies express cholinergic markers. When taken together, the findings strongly suggest the existence of the non-neuronal cholinergic system in the vascular SMCs, the biological significance of which remains to be elucidated.

## Conclusions

This Thesis investigated the *in vivo* biocompatibility of BC (*Papers I and III*), the possibility to induce microporosity in the cellulose (*Paper II*) and the non-neuronal cholinergic system in SMCs (*Paper IV*).

Bacterial cellulose presents good biocompatibility through acceptance of and integration into the host tissue. The cellulose elicits no inflammatory reaction, becomes vascularised and cells grow into it. Thus, BC is a promising new biomaterial (*Paper I*). We sought to enhance the biocompatibility of the BC by introducing pores in the biomaterial with paraffin and starch particles (*Paper II*). Cellulose constructs with interconnected pores were successfully achieved with fused paraffin particles. *In vitro* studies indicated improved cellular migration into the porous cellulose compared to the conventional cellulose. Conversely, *in vivo* evaluation demonstrated an adverse tissue reaction to the porous BC compared to the control BC. The porous BC induced a cellular reaction characterized by an assembly of macrophages, lymphocytes and myofibroblasts adjacent to the cellulose (*Paper III*). Therefore the porous BC is less biocompatible than the control BC. For the application of porous BC in tissue engineering it needs to be made more biocompatible. This may be achieved by optimising the cleansing procedure.

Because there is a difference between veins and arteries in the success as vascular replacement, differences in their physiology such as e.g., the regulation of vascular tone, needs to be evaluated. Blood flow is dependent on vascular diameter which is regulated by the contractile state of the SMCs. Therefore, the biology of these cells is of interest for understanding their function in the native vessels and in culture (*Paper IV*). We investigated whether the neurotransmitter ACh also may function as a non-neuronal mediator in the vascular SMCs. Components of the cholinergic system were found in vascular SMCs.

## Future perspectives

The non-neuronal cholinergic system in SMCs may provide means to treat diseases. Speculatively, controlling the cholinergic system in these cells, pathogenic proliferation and migration such as myointimal hyperplasia may be hindered. Because of different important physiological/pathophysiological roles of this system, it is also an interesting topic for TE.

Bacterial cellulose is an attractive biomaterial that is currently investigated by several research groups. However, few studies are focused on the application of BC as a vascular conduit and further studies are required before its clinical application as vascular graft. In view of this, BC may not be introduced into the clinic as a vascular conduit in the near future. Considering that BC is biocompatible when investigated as a subcutaneous implant, its use in plastic surgery as a tissue filler could be a promising *in vivo* application.

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