POROUS TUBULAR POLY(TRIMETHYLENE CARBONATE) SCAFFOLDS FOR VASCULAR TISSUE ENGINEERING

Yan Song

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by Yan Song

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Dissertation

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the doctor's degree at the University of Twente,
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by

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1| General Introduction

INTRODUCTION

Atherosclerosis is a main cause of death and morbidity in the Western society [1]. There have been some successful synthetic constructs that were commercial available for large-diameter vascular reconstruction. However, until now, no functional small-diameter (< 6 mm) artificial blood vessels are available. Main issues relate to thrombus formation or internal hyperplasia which occur soon after implantation [2, 3]. Although endothelial cell seeding in small-diameter vascular prosthesis improves the patency to some extent, it does not guarantee implant survival [4]. Therefore, tissue engineering of small-diameter vascular constructs is a very relevant research topic [5, 6]. Construct should be prepared from a biocompatible material that degrades and resorbs at a rate that matches the cell and tissue in-growth *in vitro* and/or *in vivo* [7]. The material should be processable into porous tubular scaffolds The pore network should be interconnected to allow cell ingrowth, adhesion and proliferation, and transfer of nutrients, gases and metabolic end products [8]. The mechanical properties of the cell-containing constructs (especially their compliance) should match those of a natural artery to avoid the development of intimal hyperplasia and subsequent graft failure upon implantation [9].

In the past, many attempts to produce a successful tissue-engineered small-diameter vascular construct have been made using natural or synthetic materials [10]. Natural polymers, especially collagen and elastin which are components of natural arteries, have frequently been used [11, 12]. However, most scaffolds based on natural materials have insufficient mechanical strength, and can burst in experiments when perfusion is conducted at physiological conditions. On the other hand, facile processing of synthetic polymers allows the fabrication of structures with high burst pressures [13]. However, achieving efficient cell seeding, good adhesion and proliferation in synthetic matrices remains a challenge. Elastic PTMC materials have been used to create porous scaffolds with good cell adhesion and proliferation characteristics [14]. Additionally, the mechanical properties of these flexible materials should allow their use in dynamic cell culturing bioreactors [15].

AIM AND STRUCTURE OF THIS THESIS

This thesis aims at evaluating the potential of tubular PTMC scaffolds in the tissue engineering of small-diameter blood vessels. Because of its biocompatibility, biodegradability and flexibility, PTMC has been employed before [16, 17]. PTMC is an amorphous polymer with a relatively low T_g of approximately -15 to -20 °C, therefore preparing (form) stable porous structures is not trivial. Gamma irradiation of high molecular weight PTMC polymers results in crosslinked structures [18, 19]. Porous crosslinked PTMC structures can also be prepared, and creep resistant networks with mechanical properties similar to those of soft tissues can be prepared. Such flexible and elastic scaffolds are resistant to repeated dilation, and should be most suited for use in long term pulsatile flow cell culturing experiments.

In this research, versatile porous tubular scaffolds were prepared from high molecular weight PTMC, and their three dimensional structure was investigated. Their performance in a pulsatile flow system set up to mimic physiological conditions was evaluated. Smooth muscle cells (SMCs) were perfusion seeded and cultured in PTMC scaffolds with suited mechanical properties and pore structures. A biological blood vessel construct was obtained after incubation of the cell-seeded structure in a pulsatile flow bioreactor for 7 to 14 days.

This thesis is divided into three parts. In the introductory part, **Chapter 2** provides an overview of the literature addressing the different tissue engineering approaches to address the problem of atherosclerosis in small-diameter blood vessels. The requirements of tissue-engineered vascular constructs and the motivation for using crosslinked PTMC-based scaffolds are presented. The working mechanisms of bioreactor cell culturing systems and clinical application of tissue engineered grafts are also addressed.

The second part of this thesis deals with the preparation and characterization of flexible and elastic, porous tubular PTMC scaffolds that were crosslinked by gamma irradiation. In **Chapter 3**, the fabrication of porous PTMC scaffolds prepared by dip-coating, irradiation and particulate leaching is described. The morphological and physical properties of the scaffolds were evaluated and compared with natural arteries.

In **Chapter 4**, the crosslinked porous PTMC scaffolds were evaluated in a pulsatile flow system (PFS) operating under conditions that mimic physiological conditions. Their distention behavior with increasing intraluminal pressures, their compliance and stiffness values and their long term form stability and mechanical behavior were assessed.

Chapter 5 deals with the seeding and culturing of SMCs in the porous PTMC tubular scaffolds under static conditions. First, two dimensional cell culturing studies were performed using smooth muscle cells, endothelial cells and mesenchymal stem cells (SMCs, ECs and MSCs. Confluent cell layers were obtained in 3 days, which indicated that the different cells adhere and proliferate well on crosslinked PTMC surfaces. To efficiently seed cells into porous matrices, a thin porous outer layer of PTMC was applied to the tubular PTMC scaffolds. This did not significantly affect the pore structure and the compliance of the scaffolds. In the experiments, the cells were then cultured for 7 to 14 days under static conditions.

A pulsatile flow bioreactor was used for dynamic cell culturing, as described in **Chapter 6**. The SMCs were seeded and incubated for 24 hours to ensure good adherence of the cells to the matrix within the porous PTMC scaffolds. After this time period, the cell-containing scaffolds were connected to the circulating pulsatile medium flow in the bioreactor. In this dynamic environment, the cells were cultured for time periods of 7 and 14 days. The generation of extra-cellular matrix and the development of the mechanical properties of the cell constructs were evaluated.

The behavior after implantation of porous tubular PTMC scaffolds either unseeded or seeded with human mesenchymal stem cells is described in **Chapter 7.** In this preliminary study, the short-term patency of these partial replacements of the rat abdominal aorta was evaluated. Unseeded scaffolds showed extensive leakage of blood, while MSC-seeded scaffolds showed only minor leakage which stopped a few minutes after restoration of blood flow.

In the **Appendix**, the fabrication of porous PTMC structures by electro-spinning solutions of PTMC in $CHCl_3$ is described. Highly porous oriented scaffolds with high excellent mechanical properties were obtained.

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2| Tissue engineering of small diameter vascular grafts: a literature review

BLOOD VESSELS: STRUCTURE AND FUNCTION

The main function of a blood vessel is to carry blood from the heart and to supply tissues and organs with nutrients. In order to serve every part of the body, blood vessels form a branched system of arteries and veins with a complex structure that varies from site to site within the circulatory system.

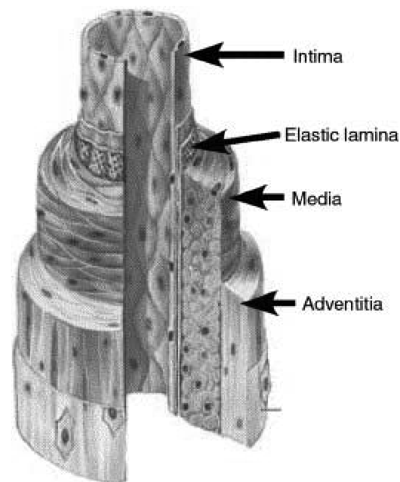

Figure 2-1. Schematic diagram of an arterial wall, showing the intimal, medial, and adventitial layers.

Arteries consist of three layers: these are (from the luminal side outwards) the tunica intima, the tunica media and the tunica adventitia. Depending upon the size and type of vessel the thickness of each layer can vary significantly [1].

The tunica intima forms the layer that contacts the blood, and consists of a lining of endothelial cells (ECs) attached to a connective tissue bed of basement membrane and matrix molecules. ECs prevent the activation of coagulation and complement factors, and inhibit the adherence of leukocytes and platelets [2, 3]. Moreover, it acts as a mechanical barrier to solutes and solvents in plasma and takes part in the regulation of vasomotor tone (dilation and constriction of the blood vessel), growth and vascular remodeling [1,4].

The tunica media is the middle layer in the blood vessel wall, and is predominantly composed of smooth muscle cells (SMCs) reinforced by organized layers of elastic tissue and a small amount of collagen. This layer contributes to the ability of the blood vessel to resist repetitive dilation and constriction resulting from physiological pulsations of the blood flow and intraluminal pressures. The cells are arranged in sheets or bundles and connected by gap junctions. In order to contract and to be able to regulate blood pressure and flow, these cells contain actin and myosin filaments.[1].

The tunica adventia consists of collagenous extracellular matrix (ECM) that contains fibroblasts, capillary blood vessels and nerves. Its main function is to give rigidity and integrity to the blood vessel. In these three layers, it is especially the SMCs, the collagen and the elastin fibers that contribute to the mechanical strength and elasticity of the blood vessels[1,5].

ARTERIAL DISEASE

Atherosclerosis is a disease that affects large, medium and small sized arteries. It is the main cause of coronary occlusion, stroke, aortic aneurysms and gangrene. Atherosclerotic lesions in the arterial wall are characterized by excessive deposition of lipids that are surrounded by extracellular matrix (ECM), smooth muscle cells (SMC) and covered with a fibrous cap[6, 7]. The sizes of these deposits can become large enough to inhibit the flow of blood. Atherosclerosis is one of the leading causes of death in western countries[3].

Autologous arteries or veins are the most commonly used blood vessel substitutes in coronary and peripheral bypass procedures. However, in more than 10% of the patients suitable autologous vessels are not available due to trauma, vessel disease or previous surgery [8,9]. Early attempts to develop blood vessel substitutes have focused on the use of grafts prepared from synthetic materials like Dacron® and Teflon®. Although large and medium sized grafts remain patent for more than 10 years after implantation[10,11], small-diameter synthetic grafts with inner diameter smaller than 6 mm fail rapidly due to thrombotic occlusion and intimal hyperplasia [12]. Currently, many researchers are investigating this field, but until now no ideal solution has been found yet. One of the most promising approaches is the preparation of vascular grafts by tissue engineering.

TISSUE ENGINEERING

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences towards the reconstruction or development of biological substitutes that restore, maintain or improve tissue functions[13]. In the generation of new tissue, three different approaches are generally chosen: (I) guided tissue

regeneration using engineered matrices, (II) injection of cells or (III) implantation of cells seeded within matrices[14]. In the most frequently used approach, cells are seeded within a degradable scaffold that provides the three-dimensional space needed for the development of new structured tissue, and subsequently cultured *in vitro* [15]. The resulting tissue engineered construct is then implanted in the appropriate anatomic location.

A schematic diagram of vascular tissue engineering is shown in Figure 2-2.

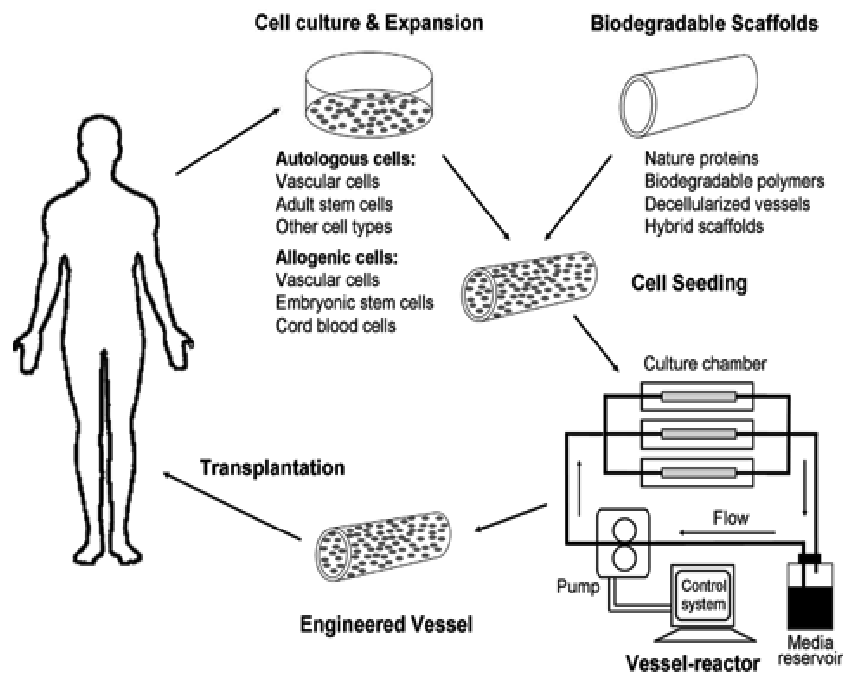

Figure 2-2. Scheme illustrating the tissue engineering approach to prepare vascular grafts for the replacement of diseased blood vessels.

SCAFFOLDS

Scaffolds are very important in tissue engineering. The three dimensional pore structure of a scaffold allows the cells to migrate into, to adhere, proliferate and differentiate and to secrete ECM. Ideally, the scaffold is slowly degrading into degradation products that are non-toxic and can be excreted by the kidneys. In the end then, only functional tissue remains.

As the scaffold needs to replace the artery in the first weeks, its properties should match those of natural arteries as much as possible. According to Baguneid *et al.*[16], the ideal arterial substitute material should be elastic, mechanically durable, degradable and biocompatible. To date, no tubular scaffold for the preparation of small diameter blood vessel grafts possesses all these qualities. Over the past 50 years, many studies investigating the preparation of tubular structures and scaffolds that under physiological conditions behave similarly to natural blood vessels have been carried out. The (polymeric) materials used for this, can be divided into three classes: natural polymers, synthetic polymers and decellularised natural tissues and blood vessels (see Table 2-1).

Table 2-1. Overview of different materials used in the preparation of scaffolding structures for blood vessel tissue engineering.

Material	Examples	Advantages	Disadvantages
Natural polymers	Collagen Elastin Hylaronic acid Chitosan	Good cell attachment Good cell signaling Components of blood vessels	Mechanically weak Expensive
Decellularised blood vessels		Good biocompatibility Mechanical properties of native vessels	Difficult cell seeding Poor cell migration due to ECM structure Laborious cleaning procedures
Synthetic polymers	PGA PLA PCL PHA PTMC	Cheap and readily available Tunable physical and chemical properties	Toxicity of degradation products Sub-optimal cell attachment and proliferation Mechanical properties for vascular tissue engineering not yet optimized

Scaffolds based on natural polymers

Collagen

Because of the excellent biological properties of collagen and its biocompatibility and biodegradability this protein has frequently been used in biomedical applications [17-21]. Weinberg and Bell were the first to report on the preparation of functional biological substitutes as vascular grafts[22]. Their model demonstrated the possibility to create tubular structures with layers that match the intima, media and adventia in natural blood vessels. However, to be able to withstand physiological pressures, these constructs needed to be supported with non-degradable sleeves made from Dacron™. A main limitation in the use of collagen fibers and gels in some clinical applications is their limited strength and rigidity. To obtain tissue engineered blood vessels with adequate mechanical properties, Girton *et al.* used glycation[24] to increase the strength and stiffness of the collagen scaffolds. Nevertheless, their burst pressures were still limited to approximately 225 mmHg.

Buttafoco *et al.* prepared hybrid scaffolds of a P(DLLA-co-TMC) polymer and collagen[25]. This hybridization with collagen conferred structural stability to the fiber-spun scaffolds at 37°C in culture medium, and permitted SMC seeding and culturing under dynamic conditions. Despite the numerous efforts made to improve the mechanical properties of collagen scaffolds for tissue engineering of small diameter blood vessels, the majority of these scaffolds are still too weak to be applied successfully.

Interestingly, L'Heureux *et al.* were successful in creating the first biologically vascular graft without using a scaffolding structure. They prepared layers of cells that were able to fuse together to form tubular structures with burst strengths comparable to those of human blood vessels[23]. These grafts had burst strengths of approximately 2000 mmHg, but cell culturing times were extremely long.

Elastin

Elastin is a protein in the ECM that can be associated with the resilience of tissues. Elastin fibers maintain their elastic properties up to extensions of approximately 140%. In the large arteries that are subjected to high pulsatile pressures generated by cardiac contraction, it is the most abundant protein [1, 26]. The mechanical properties of elastin contribute to the compliance of blood vessels, and allow the vessels to return to their original dimensions after each pulsation of the blood flow. Nevertheless, in the preparation of tissue engineering scaffolds, elastin has been used much less often than collagen due to the laborious purification procedures required for this protein [27]. Scaffolds were successfully prepared by Kurane *et al*. and Leach *et al*. [28, 29].

Elastic fibers were only present in a small number of engineered vascular constructs, and it is assumed that the absence of elastin, and therefore the lack of (visco)elasticity in the grafts, could be one of the major reasons for failure.

Scaffolds based on synthetic polymers

To obtain scaffolds with appropriate mechanical properties that maintain their strength for relatively long times and allow the regeneration of new tissue, scaffolds based on synthetic materials have frequently been used. Compared to natural materials, the mechanical properties of synthetic polymers can be controlled better, thereby allowing the creation of tissue engineered constructs of greater mechanical strength [30]. Synthetic polymers that have often been used in research include poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(ε-caprolactone) (PCL) and poly(hydroxylalkanoate)s (PHA) amongst others [31, 32, 33]. (It should be noted that in some cases the long term effects of implanting synthetic polymers is unknown. Furthermore, some biodegradable synthetic polymers release acidic degradation products that can accumulate at the implantation site and hamper natural tissue growth [34].) Synthetic scaffolding materials that have frequently been used for vascular tissue engineering are listed in Table 2-2. It can be seen from the table that vascular grafts that remain patent for longer than 1 year have not yet been tissue engineered, probably due to their limited physical properties. The development of a fully functional implantable tissue engineered blood vessel graft still remains a most relevant research aim.

Table 2-2. Different synthetic polymers used in the preparation of scaffolds for the tissue engineering of blood vessels

Authors	**Polymer**	**Scaffold fabrication method**	**Patency**
Mooney *et al*.[35]	PGA	Non-woven	4 weeks
Niklason *et al* l.[36]	PGA	Sewn into tubular scaffold	8 weeks
Langer *et al*.[37]	PGA-PHA	Sewn into tubular scaffold	5 months
Hoerstrup *et al.* [38]	PGA-P4HB	Non-woven	32 days
Matsuda *et al*.[39]	PLGA	Frozen and lyophilized	6 months
Jeong *et al*.[40]	PCLLA	Extrusion and particulate leaching	Not reported
He *et al*. [40]	PCLLA	Electro spinning	Not reported
Sarasam *et al*.[41]	Chitosan-PCL	Frozen and lyophilization	2 days
Stitzel *et al*.[42]	PLGA-elastin	Electrospinning	Not reported
Ramakrishna *et al*.[43]	PCL collagen	Electrospinning	3 days
Shin'oka *et al*.[44]	PCLLA	Not reported	1 year
Feijen *et al*.[25]	P(TMC-co-LA)	Melt spinning	Not reported

Poly(glycolic acid) (PGA)

PGA is polyester obtained by the melt ring opening polymerization of glycolide. This gives a biodegradable polymer that degrades through hydrolysis of the ester bonds in the main chain. PGA degrades *in vivo* to glycolic acid in around four weeks and can be metabolized in the human body within six months [45]. Fibers of PGA are quite stiff, with high tensile strength and modulus and are particularly stiff. Mooney *et al*. first investigated PGA structures for blood vessel engineering [46]. Later, Niklason *et al*. described the use of PGA scaffolds reinforced with Dacron® sleeves[47]. After eight weeks of cell culture, the blood vessel constructs had burst pressure strengths of 2150 mmHg. Although these results were very promising, some problems remained:

The ECs were not confluently seeded and their morphology was much rounder than in the natural situation. Moreover, SMCs in the proximity of residual PGA fragments displayed an undifferentiated phenotype. Finally, elastin could not be found in the tissue engineered blood vessels.

Poly(lactic acid) (PLA)

PLA is a biodegradable thermoplastic polyester that is also be produced by ring opening polymerization of lactide in the melt. Lactic acid is a chiral molecule, therefore and thus two crystallizable forms of PLA can be obtained poly(L-lactic acid) (PLLA) and poly(D-lactic acid) (PDLA). Polymerization of a racemic mixture of L- and D-lactide leads to the formation of an amorphous poly(D,L-lactic acid) (PDLLA). PLLA and PDLA are highly crystalline and melt at approximately 180 $^{\circ}$C, while PDLLA is amorphous with a glass transition temperature of approximately 55 $^{\circ}$C. PDLLA also has a somewhat lower tensile strength than PLLA and PDLA.

PLA is hydrophobic and degrades slowly into the naturally occurring lactic acids, making it an interesting material for tissue engineering. PLA itself has not been used in blood vessel engineering, most often copolymers with glycolide (PLGA) or other compounds that render the polymer more flexible or hydrophilic have been prepared [48,49]. Here, the PLA component contributes most to the strength and stiffness of the material [25, 46].

Poly(hydroxylalkanoate)s (PHA)

PHA are polyesters produced *in vitro* by PHA polymerase-catalyzed polymerization. Of the different PHAs, poly(3-hydroxybutyrate) (PHB), copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), poly(4-hydroxybutyrate) (P4HB), copolymers of 3-hydroxybuturate and 3-hydroxyhexanoate (PHBHHx), and poly(3-

hydroxyoctanoate) (PHO) are the polymers that have been produced in sufficiently large quantities for research purposes. PHAs can be modified to yield a wide range of mechanical properties and degradation rates[50].

Poly(ε-caprolactone) (PCL)

PCL is a biodegradable, semi-crystalline polyester prepared by the ring opening polymerization of ε-caprolactone. PCL degrades slowly *in vivo* by hydrolysis of main chain ester bonds, followed by fragmentation and release of oligomeric species of ε-hydroxycaproic acids. These components are eliminated by macrophages and giant cells. Due to the flexible nature of PCL, materials that closely match the physical properties of blood vessels can be prepared. When block copolymers consisting of PCL and PLA blocks are prepared, flexible and elastic materials can be obtained. Scaffolds prepared from these PCLLA block copolymers show significantly less plastic deformation than PLA/PGA scaffolds, while the PCLLA scaffolds are much more flexible [51]. Recently, PCLLA scaffolds have been employed in vascular tissue engineering research due to their flexibility and elasticity [44, 52, 53]. The matching of the mechanical properties of the scaffolding materials and the vascular tissues still needs to be optimized.

Poly(trimethylene carbonate) (PTMC)

Compared to the relatively rigid lactide and glycolide polyesters, use of elastic materials like PTMC might be advantageous in the tissue engineering of blood vessels. PTMC is a flexible, biodegradable and biocompatible polymer and its use in soft tissue engineering applications has been proposed [54]. At body temperature, PTMC is a soft polymer with a low E-modulus and thus can be used in soft tissue

engineering [55, 56]. Buttafoco *et al*. first used a TMC and lactide copolymer to obtain tubular scaffolds [25]. These scaffolds showed good cell adhesion, by culturing relevant cells within these structures and constructs with properties that resembled natural blood vessels were obtained.

Due to its low Tg of approximately -15 to -20 ^{o}C, however, PTMC homopolymers will show creep and need to be crosslinked to obtain form stable materials. Interestingly, Pêgo *et al.* showed that PTMC can readily be crosslinked during sterilization by gamma irradiation [57]. These characteristics allow the polymer to be applied in the preparation of blood vessel scaffolds [59]used in a long term pulsatile cell culturing systems, as they will be able to resist the repeated dilations and contractions of the constructs. In addition, Zhang *et al*. demonstrated that PTMC degrades enzymatically *in vivo* by a surface erosion process [58]. The polymer showed excellent biocompatibility and no toxic side effects were observed upon implantation. Currently, other investigations with PTMC-based materials are also ongoing.

Other scaffolding structures

Decellularized natural blood vessels are entirely composed of ECM. They have good biocompatibility and have mechanical properties which are similar to those of natural blood vessels[60-62]. The process of decellularization is usually done by treating the tissues with a combination of detergents, enzyme inhibitors and buffers. Although several research groups are seeding ECs into the lumen of decellularized arteries, it is found that cell migration into these scaffolds is inadequate. This is likely due to the very tight structure of the matrix [63], although knowledge on the cell migration

process itself is limited. It can be expected that it will take several years before vascular constructs prepared with these materials will be used in the clinic.

Fabrication of tissue engineering scaffolds

It is known that biological and chemical compounds can guide cell differentiation and tissue growth [64], and many research groups have focused on these parameters in preparing functional biomaterials. In recent years it has also been suggested that the structural parameters of a scaffold are also important factors, as this network of pores defines the three-dimensional shape of the tissue and its function [65]. Furthermore, although cells are able to influence and modify their local micro-environment, they are rarely capable of organizing at the size scales of tissues. Achieving this level of organization requires a template with appropriate spatial (and biological) building blocks that enable cells to organize throughout the scaffold as a whole. An overview of often-used scaffold fabrication techniques is given in Table 2-3.

Table 2-3. Overview of often-used scaffold fabrication techniques, and the advantages and disadvantages of their specific characteristics and architectures.

Method	Scaffold characteristics	Advantages	Disadvantages
Solvent casting, dip coating and particulate leaching [66]	Pore sizes of 50 to 1000 µm Porosities of 30 to 95%	Controlled porosities up to 93% Independent control of porosity and pore size	Limit in thickness is 3mm Potentially harmful solvent residues
Phase separation methods [67, 68]	Pore sizes <200 µm Porosities of 70 to 95%	Simple, versatile and cost effective process	Limited control of scaffold morphology Potentially harmful solvent residues
Fiber self-assembly [67]	Structure depends on macromolecules used	Mimics the biological process	Complex process, that is limited to few polymers Impossible to produce continuous fibers in a controlled manner
Electro- spinning [67]	Fiber diameters of 200 nm to 5 µm	Simple, versatile and cost effective process	Use of high voltages
Melt molding and particulate leaching[68]	Pore sizes of 50 to 1000 µm Porosities of 30 to 90%	Independent control of porosity and pore size Mold determines specimen shape	High temperatures required for semi-crystalline polymer
Membrane lamination [69]	Pore sizes <200 µm Porosity of 70 to 95%	Three dimensional matrix with adjustable pore characteristics	Complex process Potentially harmful solvent residues
Decellularised vessels [70]	Natural extra-cellular matrix	Correct architecture of the pore network	Immune-rejection Difficulties in cell seeding
Laser sintering [71]	Pore sizes 200 to 1000 µm Porosities above 50%	Three dimensional matrix with adjustable pore characteristics	Process is limited to few polymers as particles need to be fused by heating
Gas foaming [72]	Pore sizes of 50 to 1000 µm Porosities of 30 to 95%	Simple, versatile and cost effective process	Poor control of pore network characteristics, especially regarding pore connectivity
Fiber knitting [73]	Interconnected channels of 20 to 100 µm in diameter	Good control of fiber orientation	Limited rigidity

Solvent casting and particulate leaching: This method consists of dispersing mineral or organic particles in a polymer solution. This dispersion can then be cast or freeze-dried to remove the solvent. The porosifying particles are then leached with a suitable solvent that does not dissolve the polymer, resulting in a porous polymer matrix. In this manner, highly porous scaffolds with porosities up to approximately 95% and median pore diameters up to 1000 µm can be prepared. The characteristics of the pore

network can be independently varied by adjusting the size, size distribution and amount of porogen particles.

Dip coating and particulate leaching: This method is very similar to solvent casting and particulate leaching. In this case a substrate, for example a glass mandrel, is (repeatedly) dipped into a dispersion of leachable particles in a polymer solution. The method allows the facile preparation of porous tubular scaffolds.

Compression molding and porogen leaching: This method resembles the solvent casting and particulate leaching method, except that in this case the mixtures of polymer and porogen particles are heated to temperatures above the glass transition or melting temperature of the polymer. After molding and cooling, the particles are leached out with a suitable solvent, and a porous polymeric structure is obtained. Here too, the pore size is directly controlled by shape and size of the particles and the porosity can be varied by adjusting the polymer to particles ratio. Polymer scaffolds with different sizes and geometries can be prepared by changing the geometry of the used mold.

Membrane lamination: Membrane lamination is a method in which porous membranes, created for example by particulate leaching methods, are bonded together to create a larger three dimensional structure. The bonding can take place with use of small amounts of a suitable solvent. The contacting surfaces are coated with solvent, and stacked. The fused stacked layers form a three dimensional tissue engineering scaffold that can be used for cell seeding.

Micro patterning: Engineering tissues with structural control at the nano- or microscale requires biomaterial surfaces that have chemical or topological features at these length scales. An effective approach is soft lithography [74]. This technology makes use of a stamp or a mold, which is prepared by casting an elastomer on a

silicon template with a patterned relief structure. This template is prepared by conventional lithography techniques. An advantage of this technique is the rapid generation of substrates with features ranging from 2-500 μm in size. These precisely defined patterns can be transferred to a substrate via micro-contact printing, and used to control *e.g.* cell growth [18]. The pattern can be designed to form micro-channels. By stacking such micro-printed layers, three-dimensional scaffolds can be created that allow liquid flow throughout the structure. This makes transports of nutrients and exchange of metabolic products possible, but simultaneously allows patterning and preferential alignment of cells according to a specific topography [75].

Electro spinning: Electro spinning is a relatively inexpensive technique that allows the manufacturing of sub-micron and micron sized diameter fibers from polymer solutions or melts [67]. With this technique both synthetic and natural polymers have been processed. A liquid polymer solution or melt is pumped through a spinneret that faces a collector, a high voltage is applied to the spinneret and collector. Upon reaching a critical voltage, the surface tension of the liquid polymer at spinneret tip is counterbalanced by localized charges generated by the electrostatic force, and the droplet elongates and stretches into a Taylor cone, from which a continuous jet is rapidly ejected [76]. The forces and the path of the jet are extremely dynamic. Such high speeds, and the long, spiraled traveling distances, make accurately controlled deposition of the electrospun fiber difficult.

When compared to flat surfaces, cells may adopt significantly different morphologies on electrospun fabrics: SMCs orient along the length of multiple fibers and deform them to create their own micro-environment [77]. Thin-layered tissue engineered constructs can be prepared, and the potential of electrospinning in vascular tissue engineering applications has been demonstrated [20, 26, 78].

CELLS USED IN VASCULAR TISSUE ENGINEERING

The ideal cells used in the engineering of vascular tissue are non-immunogenic and functional. They are easy to obtain, and they can readily be expanded in culture as well. Until now researchers have used different types of cells in vascular tissue engineering: mature vascular cells, embryonic stem cells and adult stem cells. ECs and SMCs can be obtained by differentiation of stem cells.

Autologous ECs and SMCs

Non-immunogenic, autologous ECs and SMCs that have been isolated from the patients themselves are the first choice for engineering blood vessel grafts. These cells have been isolated from autologous vessels by several groups [22-23, 36, 79]. Although functional blood vessels have been constructed with these cells, there are several drawbacks: The majority of cells in adult blood are terminally differentiated, and the limited proliferation potential of harvested cells makes it impossible to obtain large amounts of cells from a small biopsy [80].

Many attempts have been made to improve the proliferation capacity of ECs and SMCs. Mckee *et al.* for example, tried genetic manipulation and introduced human telomerase reverse transcriptase subunit into human SMCs [81]. Their work showed that the cells could proliferate far beyond their normal life span and retained the characteristics of normal SMCs. Shao *et al.* used the same technique to increase the life span of ECs [82]. Although the results are promising, it should be mentioned that long term effects of these genetic manipulations remain unknown.

Another approach is to make use of allogeneic ECs and SMCs. Sufficient cells can then be obtained, however, the main drawback is immune rejection. Immune rejection of ECs is especially problematic, as these cells are in direct contact with blood. A

solution to these problems has not yet been found, and research now focuses on dedifferentiation of cells and on controlling cell phenotype with growth factors [83-85].

Stem cells

In the last years stem cells have become a major cell source in tissue engineering. Based on their origin, stem cells can be classified as embryonic stem cells or adult stem cells. Embryonic stem cells are totipotent and thus in principle suitable to produce any tissue, while adult stem cells are pluripotent and differentiation is limited to cells of certain lineages. The utilization of stem cells is attractive; when cultured under the appropriate conditions, these cells can yield the necessary large numbers of the cells required for regeneration of the specific tissue.

Murine embryonic stem cells have been investigated thoroughly with regard to their differentiation into ECs and SMCs [86]. Others have also shown that embryonic stem cells could be differentiated into SMC and EC and formed tube like structures [87, 88]. This shows that embryonic stem cells could indeed be a suitable source of cells for the engineering of blood vessels. However, major obstacles to application in the clinic are the ethical issues involved and difficulties regarding immunogenicity and tumourogenicity that need to be overcome first.

Adult stem cells are a good alternative, as these cells can be obtained from the patients themselves. The problems regarding immune-rejection and ethical issues are absent, but a major drawback is their lineage specificity. Mesenchymal stem cells (MSCs) are needed for differentiation into SMCs. Differentiation of MSCs into SMCs can be done using growth factors and applying mechanical stresses [89, 90].

Endothelial progenitor cells

Endothelial progenitor cells (EPC) are adult stem cells that have the ability to proliferate, migrate and differentiate into ECs [91]. EPCs have been utilized in the endothelization of synthetic vessels [92]. Kaushal *et al.* isolated EPCs from the peripheral blood of sheep and expanded and seeded them in decellularised porcine iliac vessels [93]. After 130 days *in vivo*, the EPC grafts exhibited contractile activity and nitric oxide-mediated vascular relaxations that were similar to those of natural carotid arteries. This indicates that EPCs can function similarly to arterial ECs, and are therefore a suitable source of cells in the engineering of blood vessel grafts. The differentiation of EPCs into SMCs is not yet well-established.

Co-cultures of ECs, SMCs and MSCs

ECs line the lumen of blood vessel, and play very important roles in vasodilatation, preventing platelet coagulation, the immune response and in determining the impermeability of the vessels. For this, ECs can influence the phenotype SMCs present in the second layer of the blood vessel. Despite the abundant knowledge on this bidirectional phenotypic modulation of SMCs, which can be either proliferative or contractile, cell culture systems that model the interactions of ECs with SMCs resulting in this behavior are limited. A number of groups have researched the interactions of ECs with SMCs by culturing the cells on opposite sides of a membrane [94], by culturing ECs on a gel that contains SMCs [95] or by culturing ECs directly on SMCs [96]. It is likely that co-culturing ECs directly on SMCs is the model that is most representative in the tissue engineering of blood vessels, as this mimics the spatial arrangement of the different cell types in the artery.

Lavender *et al.* developed a technique to form a stable direct contact co-culture of confluent porcine arterial endothelium on top of a single layer of porcine arterial SMCs [96]. It was found that ECs attached and spread better on quiescent SMCs than on proliferating SMCs. In addition, ECs were able to form a confluent monolayer on quiescent SMCs.

Although there are quite some papers on co-culturing ECs with SMCs, only few publications address co-culturing with stem cells. Lee *et al*. have done co-culturing experiments with MSCs and anterior cruciate ligament (ACL) cells. They suggest that ACL cells release specific regulatory signals that support selective differentiation of MSCs into ACL cells [97].

The cells most often used for seeding and *in vitro* culture of tubular scaffolds for generating blood vessel grafts are still SMCs and ECs. However, the time required to culture these cells is too long. Therefore, despite the difficulties in differentiating stem cells into the required cells, it remains a major research goal to generate sufficiently large amounts of SMCs and ECs that are needed for vascular tissue engineering purposes [98].

BIOREACTORS FOR VASCULAR TISSUE ENGINEERING

A bioreactor is an apparatus that attempts to mimic and reproduce physiological conditions in order to maintain or encourage cell culture for tissue regeneration [99]. In the human body, cells are constantly subjected to and stimulated by mechanical, chemical and electrical signals that influence the response of cells regarding phenotype, proliferation rate, shape and other properties. Cell culturing parameters such as temperature, pH, biochemical gradients and mechanical stresses should be continuously controlled during the maturation period. It is also necessary to be able to

modify each parameter to study its influence on the growth of different tissues, and on the final properties of a regenerated construct [100]. If signals are inappropriate or absent, certain cells will not proliferate and form organized tissues, they can then dedifferentiate and become disorganized, this then can lead to cell death [64]. Therefore, it is essential that bioreactors are designed and fabricated following the specifications required for each different tissue.

Pulsatile flow bioreactors

When Weinberg and Bell obtained a well-differentiated artery structure, its burst strength was only approximately 90 mmHg [22]. As this is less than normal systolic pressures, the implant would immediately fail upon implantation. After 1 month of culturing, the burst strength further decreased significantly due to dedifferentiation of the cells. The main difference between a natural artery and Weinberg and Bell's arterial graft was the orientation of the SMCs: in natural vessels SMCs are located perpendicular to the flow of blood making the artery relatively rigid in the circumferential direction while this was not the case in the engineered vessels. This led to the hypothesis that mechanical stresses during culture are essential in the formation of vascular tissue. This was eventually proven by Niklason *et al.* [47], who seeded a tubular biodegradable scaffold with SMCs and cultured the construct in a bioreactor under pulsatile flows of 165 pulsations per minute. After 8 weeks, the arteries had a thickness that was twice that of arteries cultured under constant medium flow in control experiments. The burst strength of grafts cultured under pulsatile conditions was also much higher: 2000 mmHg instead of 300 mmHg in the control experiments.

Later, Hoerstrup *et al.* designed a pulsed perfusion bioreactor specifically suited for small vessel culture [38]. After a 1 month culturing period they obtained 5 mm diameter arteries with burst strengths of 326 mmHg, this compared favorably to burst pressures of only 50 mmHg for the constant flow controls. In the first week of culture, arteries cultured under pulsatile flow conditions and constant flow conditions had comparable burst strengths of 180 mmHg. After the second week, the strength of grafts cultured under pulsatile flow conditions increased, while the strength of grafts cultured under constant flow had decreased.

Following these observations, Mironov *et al.* applied an additional longitudinal strain to the grafts, and a two-media perfusion flow system that perfused the inside and the outside of the tubular constructs [101]. In this manner, they imitated the biomechanics of the embryonic vascular environment and accelerated vascular wall maturation. In their publications, the behavior of the blood vessels under these stresses was described, but no burst pressures were reported.

IN VIVO EXPERIMENTS AND CLINICAL APPLICATIONS

As described earlier, mechanical stimuli regulate the phenotype of SMCs in a three-dimensional culture system. During *in vivo* experiments, the scaffolds must maintain their mechanical integrity and deliver mechanical signals to the SMCs. To achieve this, the scaffolds must be elastic and capable of withstanding long term cyclic mechanical strains without failure or severe permanent deformation. In Table 2-4 an overview of recent *in vivo* experiments in the engineering of vascular tissues is presented, indicating scaffold type, scaffold production method, used cells and duration of the *in vivo* experiment.

Table 2-4. Overview of recent scientific publications describing in vivo vascular tissue engineering experiments using tubular scaffolds.

Author	Material	Production method	Cell type	Implantation time	Implantation site
Jeong *et al.* [40, 102]	P(LA-co-CL)	Particulate leaching	SMC	15 weeks	Nude mice
Shin'oka *et al.* [103]	P(LA-co-CL)	-	BMC	17 months	Humans
Martin *et al*. [104]	Decellularized canine carotid	decellularization using SDS	-	2 months	Mongrels
Keun Han *et al.* [105]	PU-PEO-SO_3	Dip-coating	-	39 days	Mongrels
Chaouat *et al.* [106]	Polysaccharide hydrogel and decellularised blood vessel	-	-	8 weeks	Adult Wistar rats
Matsumura *et al.* [107]	PLCL and PGA	Sponge	BMC	6 months	Beagles
Kurane *et al.* [28]	Elastin scaffold	Porcine carotid	-	28 days	Sprague-Dawley rats
Zhang *et al.* [70]	PLGA and PU	PU coated woven PLGA	BMC	24 weeks	Beagles
Hashi *et al*. [108]	PLLA	Electro-spinning	MSC	60 days	Athymic rats

The results of the research presented in Table 2-4 were all very favorable. But especially the work of Shin'oka *et al.* is of great interest. They were the first to describe the clinical application of an engineered blood vessel graft, and successfully reconstructed part of the peripheral pulmonary artery of a 4 year old girl. For this, they seeded autologous cells on reinforced biodegradable P(LA-co-CL) scaffolds[44, 103]. After this successful surgery, they treated another 23 people with the same approach and reported a 95% patency after 1 year of implantation and without any sign of aneurysms, thrombosis, stenosis or calcification.

Another clinical experiment was done by L'Heureux *et al*. [109]. They used cell sheet vascular constructs in haemodialysis patients, where the vascular construct served as an arterial shunt for dialysis access. During 5 months of implantation in three patients,

they observed no failure; the grafts functioned well and allowed adequate access for haemodialysis.

The results obtained until now are very encouraging, although the culturing is still very much time consuming. Advanced bioreactors will be developed to be able to optimize culturing conditions that allow efficient cell differentiation and expansion. To enhance the properties of formed tissue engineered vascular grafts, a better understanding of cell migration and proliferation within scaffolds will prove to be important. The design of novel biomaterials and structures that can be used as tubular scaffolds will be essential.

CONCLUSIONS

Despite years of research in the tissue engineering of vascular grafts, no suitable graft is available yet. The scaffolding material plays an important role, as it determines biocompatibility and biodegradability. Furthermore, its mechanical properties and durability during culturing will determine the applicability of the tissue engineered vascular constructs both *in vitro* and *in vivo*. We believe poly(trimethylene carbonate) (PTMC) is a promising material for application in vascular tissue engineering, as it is a very flexible polymer that is biocompatible and biodegradable. Furthermore, it can be crosslinked by gamma irradiation to yield an elastic, creep resistant structure ideally suited for application under long term pulsatile deformations. Such properties are essential to be able to culture vascular cells under relevant physiological conditions in a bioreactor.

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3| Flexible and elastic porous poly(trimethylene carbonate) structures for use in vascular tissue engineering

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ABSTRACT

Biocompatible and elastic porous tubular structures based on poly(1,3-trimethylene carbonate) (PTMC) were developed as scaffolds for tissue engineering of small-diameter blood vessels. High molecular weight PTMC (M_n = 4.37x10^5) was crosslinked by gamma irradiation in an inert nitrogen atmosphere. The resulting networks (50-70% gel content) were elastic and creep-resistant. The PTMC materials were highly biocompatible as determined by cell adhesion and proliferation studies using various relevant cells types (human umbilical vein endothelial cells (HUVECs), smooth muscle cells (SMCs) and mesenchymal stem cells (MSCs)).

Dimensionally stable, tubular scaffolds with an interconnected pore network were prepared by particulate leaching. Different crosslinked porous PTMC specimens with average pore sizes between 55 μm and 116 μm, and porosities ranging from 59 to 83%. were prepared. These scaffolds were highly compliant and flexible, with high elongations at break. Furthermore, their resistance to creep was excellent and under cyclic loading conditions (20 deformation cycles to 30% elongation) no permanent deformation occurred.

Seeding of SMCs into the wall of the tubular structures was done by carefully perfusing cell suspensions with syringes from the lumen through the wall. The cells were then cultured for 7 days. Upon proliferation of the SMCs, the formed blood vessel constructs had excellent mechanical properties. Their radial tensile strengths had increased from 0.23 to 0.78 MPa, which approaches that of natural blood vessels.

INTRODUCTION

Diseased coronary arteries and peripheral blood vessels often require replacement with small-diameter grafts. Autologous arteries or veins are the best substitutes, but approximately one third of the patients does not have suitable veins for grafting [1, 2]. Due to the occurrence of thrombogenesis and intimal hyperplasia [3], it is not yet possible to use synthetic vascular grafts with inner diameters smaller than 6 mm [4, 5]. In tissue engineering of small-diameter blood vessels, grafts can be constructed in a bioreactor using tubular scaffolds, cells and growth factors [6]. The scaffolding material should allow cell adhesion and proliferation and have adequate mechanical properties.

To allow mechanical stimulation of the cells during culturing *in vitro*, the scaffold should be sufficiently strong, flexible and elastic. The cell-scaffold constructs should resist physiological blood pressures, and recover from repeated dilations during the relatively long culturing times [7, 8]. Preferably the scaffold should degrade, but the mechanical properties of the construct should remain at an acceptable level, implying that the newly formed tissue will compensate for the degradation of the scaffold.

Natural polymeric materials have been used in preparing such scaffolding structures. Tubular constructs that mimic the native structure of an artery have been prepared by incorporating smooth muscle cells (SMCs) in collagen and elastin matrices [6]. Swartz *et al.* have engineered blood vessels with an inner diameter of 4 mm entirely from fibrin as a scaffolding material. *In vivo*, these grafts remained patent for approximately 15 weeks [9]. Unfortunately, as a result of their limited mechanical strength, these scaffolds needed to be supported initially, and could not be used in the culturing of cells under dynamic flow conditions. Decellularized (human) blood arteries, which are

only composed of extracellular matrix, have suitable mechanical properties [10, 11], but the necessary decellularization procedures are very involved and complicated.

Synthetic polymers have also often been used in tissue engineering. Much research has been conducted using well-known rather rigid polymers like poly(glycolic acid) (PGA) [2, 3, 12, 13] and poly(lactic acid) (PLA) [14]. Shin'oka *et al.* prepared pulmonary arteries using tubular scaffolds based on woven biodegradable poly(lactic-co-glycolic acid) meshes sealed with a non-woven PGA mesh [15], which remained patent in the ovine body for 11 to 24 months. In their research, large arteries with a diameter of 15 mm were prepared, which function at relatively low pressures. Later, these researchers used flexible copolymers of L-lactide and ε-caprolactone (at a ratio of 50:50) in preparing their scaffolds [16]. These porous structures were implanted into 23 patients after seeding with bone marrow cells, and remained patent for an average time period of 16.7 months. Still, these structures needed to be reinforced with woven PGA fabrics to allow implantation *in vivo*.

Poly(1,3-trimethylene carbonate), PTMC, is an amorphous material with a low glass transition temperature (T_g) between –14 to –20 °C. High molecular weight PTMC is a flexible material with a Young's modulus of 5 to 6 MPa [17], that crosslinks during sterilization by gamma irradiation [18]. This biocompatible polymer degrades *in vivo* by a surface erosion process [19] at a rate of approximately 10 to 20 μm per day, its erosion rate is mainly determined by the initial polymer molecular weight.

Upon crosslinking, an elastic network is obtained that can effectively resist creep during long term cyclic deformations, like cell culturing under dynamic flow condtions. This would make PTMC an interesting material for the preparation of scaffolds for vascular tissue engineering.

To engineer small-diameter blood vessels, smooth muscle cells (SMCs) and endothelial cells (ECs) have to be cultured to create a blood vessel construct. Collagen and elastin produced by SMCs will provide the mechanical strength of the vascular graft, while an endothelialized lumen will reduce the risk of thrombus formation. Developments in stem cell technology will allow a second approach: the seeding and culturing of mesenchymal stem cells that can differentiate into SMCs and ECs. It has been shown that PTMC is an excellent substrate for culturing of several different cell types [18].

The aim of this study is to prepare porous tubular PTMC structures and to evaluate their applicability for the tissue engineering of small diameter blood vessels. The morphologies and the physical and mechanical properties of porous and non-porous networks were assessed. Also the seeding and the adhesion and growth of the relevant cells (SMCs, ECs and MSCs) on irradiated PTMC structures were investigated.

MATERIALS AND METHODS

Materials

Trimethylene carbonate (1,3-dioxane-2-one, TMC) was obtained from Boehringer Ingelheim, Germany. Stannous octoate (stannous 2-ethylhexanoate, $SnOct_2$) was used as received from Sigma, USA. Powdered sugar (Van Gilse, the Netherlands) and NaCl salt particles (Acros Organics, Belgium) were sieved using stainless steel sieves to particle sizes of 106 to 250 μm. All solvents used (Biosolve, The Netherlands) were of analytical grade.

Natural ovine and porcine carotid arteries were obtained from a local slaughterhouse, and kept in an ice-cooled phosphate buffered solution (pH 7.4). Tensile tests were performed at room temperature within 12 hrs of harvesting. Human arteria mesenterica

inferior were obtained after approval from the medical ethical committee of the Medical Spectrum Twente Hospital, and stored and tested as described before.

Synthesis and characterization of PTMC

In an argon atmosphere, amounts of 50 to 100 g of TMC monomer were charged into freshly silanized (Serva Solution, Boehringer Ingelheim, Germany) and dried glass ampoules, after which 2×10^{-4} mol $SnOct_2$ per mol monomer was added. The ampoules were vacuum sealed and polymerizations were carried out at 130 ± 2 °C for 3 days.

The monomer conversion was determined by ^{1}H-NMR spectroscopy using a 300 MHz Varian Inova (USA) apparatus. Spectra were recorded using solutions of polymers in $CDCl_3$ (Sigma, USA).

The obtained polymers were purified by dissolution in chloroform (2-3% wt/vol), filtration and precipitation into a ten-fold volume of methanol. The precipitated polymers were washed with fresh methanol and dried under vacuum at room temperature until constant weight [2].

Number average and weight average molecular weights ($\overline{M}_n$ and $\overline{M}_w$, respectively), molecular weight distributions and intrinsic viscosities of purified PTMC were determined by gel permeation chromatography (GPC, Viscotek, USA) equipped with TDA302 triple detection columns. This triple detection comprises a refractometer, a viscometer and a light scattering detector, and allows the determination of absolute molecular weights. Chloroform was used as an eluent at a flow rate of 1 ml/min at 30°C. Narrow polystyrene standards were used for calibration, sample concentrations ranged from 0.8 to 1.0 mg/ml.

PTMC network formation

PTMC films (500 μm thick) were prepared by compression molding at 140 °C using a laboratory press (Fontijne THB008, The Netherlands). PTMC networks were formed [18] by vacuum sealing these films in polyethylene pouches and exposing them to ^{60}Co gamma-irradiation (Isotron, Netherlands). The specimens were irradiated at doses of 25, 50 or 100 kGy.

Disks (ϕ = 10 mm) were punched out from irradiated and non-irradiated polymer films. The gel content and degree of swelling (DS) of the irradiated films were determined using chloroform as a solvent and swelling agent.

The gel contents and the volume degrees of swelling (q) of the networks were determined in duplicate at room temperature using chloroform. A single disk (weight w_0) was swollen in 100 ml chloroform for a week, and then the gel fraction was collected and weighed (w_{wet}). Subsequently, the specimen was dried under vacuum at room temperature until constant weight (w_{dry}). The gel contents and the degrees of swelling were calculated using:

$$\text{Gel content} = w_{dry}/w_0 \times 100\% \qquad \text{Equation 1}$$

$$q = 1 + \rho_p \times \left(\frac{w_{wet}}{w_{dry} \times \rho_s} - \frac{1}{\rho_s} \right) \qquad \text{Equation 2}$$

In Equation 2, ρ_s is the density of chloroform (1.48 g/cm^3) and ρ_p is the density of PTMC (1.31 g/cm^3).

Mechanical evaluation of PTMC networks

From the 500 μm thick compression-molded (and crosslinked) PTMC films, specimens measuring 100 mm × 5 mm (according to ASTM D882-91) were punched out for mechanical testing at room temperature [20].

Tensile tests were carried out in triplicate using a Zwick Z020 universal tensile tester equipped with a 500 N load cell. The specimen deformation was derived from the grip-to-grip separation. The initial grip-to-grip separation was 50 mm and the crosshead speed was 50 mm/min. The Young's modulus was determined from the initial slope of the stress-strain curves and is an indication of the stiffness of the materials. The yield stress, maximum tensile strength and elongation at break were determined as well. In cases where a true yield point could not be observed, a value for the yield stress and elongation at yield was estimated from the intersection of tangents to the curves.

The permanent deformation in dynamic creep experiments was determined by carrying out cyclic tensile tests to 50% elongation (20 cycles). After a recovery period of 2 hours, the permanent deformation was determined from the stress-strain diagram of the 21st cycle.

Static creep experiments were carried out according to ASTM D2990-95. A load corresponding to 50% of the yield stress of the specimens was applied, and the elongation in time was measured using a traveling microscope. The creep rate was determined from the linear part of the strain versus time plot. After approximately two days the load was removed and after 24 hrs the recovery of the specimens was evaluated.

Porous tubular PTMC structures

Porous tubular structures were prepared by dip coating glass mandrels (ϕ = 3.0 mm) into solutions of PTMC in chloroform (2.5-3.0% wt/vol) that contained dispersed sugar or salt (NaCl) particles. The process was repeated several times to reach the

desired outer diameter of 7 to 8 mm. The salt- or sugar-containing tubular structures were dried at room temperature for 2 to 3 days.

To obtain a crosslinked (creep-resistant) structure, the coated mandrels were subjected to gamma irradiation as described above. A porous structure was then obtained by leaching out the salt or sugar particles with demineralized water. Before use, the specimens were stored in water at 4 °C.

Crosslinked porous structures were prepared using leachable particles sieved to a size range of 106-250 µm and a polymer to porogen weight ratio of 10:90. This corresponds theoretically to volume fractions of 85.6 vol% in the case of sugar particles and 84.2 vol% in the case of salt particles.

The porosity of the tubular structures (in the hydrated state) was determined gravimetrically according to equation 3:

$$\text{Porosity} = 1 - \left(\frac{W_{dry}}{\rho_{PTMC}}\right) / V_{wet} \qquad \text{Equation 3}$$

Here W_{dry} = dry weight of the tubular structure and V_{wet} = volume of the tubular structure in wet condition.

Micro-computed tomography (micro-CT) was used to obtain three-dimensional visualizations of the pore structure of the prepared tubular scaffolds. A General Electric Explore Locus SP apparatus was used at a resolution of 8 µm. Pore size distributions and porosities were determined from the data as well [21, 22, 23]. Measurements were performed with hydrated scaffolds, adhering water was removed and the surface was blotted with blotting paper.

Scanning electron microscopy (SEM) of the porous tubular PTMC structures was performed using a Hitachi S800 field emission scanning electron microscope operating at 6 kV. The specimens were dehydrated with methanol, fractured in liquid nitrogen and sputter coated with a gold-platinum layer (Polaron E5600 sputter-coater). SEM

was also employed to determine the size distribution and the average size of the leachable sugar and salt particles used in preparing the porous structures.

The tensile properties of the hydrated porous tubular scaffolds (0.8 to 1 cm in length) were determined in the radial direction according to standards of the American National Standards Institute and the Association for the Advancement of Medical Instrumentation (ANSI/AAMI VP20: 1994) at a rate of 1 mm/min. The initial stiffness was determined from the slope of the tensile curve from 2.5% to 5% of strain. The permanent deformation in cyclic tensile tests was evaluated by first repeatedly (20 times) stretching the specimens in the radial direction to 3 mm (depending on the wall thickness, this corresponds to 30 ± 3% elongation) and then determining the permanent deformation after 2 hours relaxation in these experiments The absolute error in the elongation is approximately 0.1 %.

The suture retention strength (SRS) of hydrated specimens was also determined according to (ANSI/AAMI VP20: 1994) at a rate of 50 mm/min. It is defined as the maximum force needed to tear the wall of the tubular specimen. Values are normalized by dividing the force by the wall thicknesses.

The properties of non-porous PTMC tubes that were prepared analogously without leachable particles, and of natural blood vessels, were determined for comparison. All experiments were conducted in triplicate.

Cell culturing

Mesenchymal stem cells (MSCs) were obtained from human bone marrow. The isolation was performed following a procedure developed by Both *et al*. [24]. The MSCs were cultured in αMEM (Invitrogen) medium containing 10% FBS (South American Origin from Cambrex), confluent cultures were detached and subcultured up

to passage 15. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical veins by the method of Jaffe *et al*.[25], and cultured on fibronectin (2mg/ml) coated tissue culture polystyrene (TCPS) flasks using EGM 2 medium (Cambrex). Confluent cultures were sub-cultured for up to passage 8 after detachment. Smooth muscle cells (SMCs) were isolated from human umbilical veins too, and cultured on gelatin-coated (0.5% w/v) TCPS flasks using Dulbecco's modified Eagle medium (DMEM) containing 20% (v/v) heat-inactivated (30 min, 56 $^{\circ}$C) fetal bovine serum. Confluent cells were detached, and sub-cultured up to passage 10. All media were refreshed every 2-3 days and the cell cultures were maintained in a fully humidified atmosphere at 37 $^{\circ}$C and 5% CO_2.

The different cells were cultured on flat PTMC surfaces, which were prepared by spin-coating solutions of PTMC in chloroform (3% wt/vol) onto glass disks with a diameter of 10 mm. The PTMC coated disks were dried, vacuum-sealed and gamma irradiated (and crosslinked) as described above. The behavior of the different cells on non-irradiated specimens was evaluated as well. These samples were disinfected with 70% EtOH, rinsed with phosphate buffered saline (PBS) and placed in medium overnight.

MSCs and SMCs were seeded at cell densities of 10.000/cm^2, HUVECs were seeded at a density of 20.000/cm^2. The adhesion and proliferation of the cells on the polymer surfaces were studied by light microscopy.

The adhesion and proliferation of SMCs in porous tubular PTMC structures was investigated after disinfecting with 70% EtOH, rinsing with PBS and placing the scaffolds overnight in DMEM containing 20% v/v FBS. The seeding of the scaffolds with SMCs was done in triplicate by perfusing SMC suspensions (2.5 million cells per cm of scaffold length) through the walls of the scaffolds with two syringes at both ends. During the first 2 hours, the seeded scaffolds were rotated 90 degrees every 10

min to ensure a homogeneous distribution of the cells. The SMCs were cultured for a period of 7 days.

For histological analyses after culturing for 1 day and 7 days, the scaffolds were rinsed with PBS, fixed using a 4% paraformaldehyde (PFA) solution and embedded in glycol methacrylate (GMA). Transverse sections with a thickness of 5 μm were cut, stained with hematoxylin and eosin (HE) and observed by light microscopy.

RESULTS AND DISCUSSIONS

High molecular weight PTMC ($M_n = 4.37\times10^5$, MWD = 1.32) was prepared by ring opening polymerization of TMC. The monomer conversion was higher than 99%. The glass transition temperature (T_g) determined by DSC was approximately -17°C.

Formation of PTMC networks

PTMC networks with different crosslinking densities were prepared by gamma irradiation of high molecular weight PTMC in vacuum. To evaluate the effect of irradiation dosage on the physical properties of the obtained networks, compression molded PTMC films were irradiated with 25, 50, or 100 kGy. Figure 3-1 presents the gel fractions and the degrees of swelling (q) of the irradiated specimens using chloroform as a swelling agent. It can be seen that the gel content of the PTMC samples increased with increasing irradiation dose, whereas the degree of swelling of the networks decreased with increasing irradiation dose. This indicates that the crosslinking density increased with increasing irradiation dose.

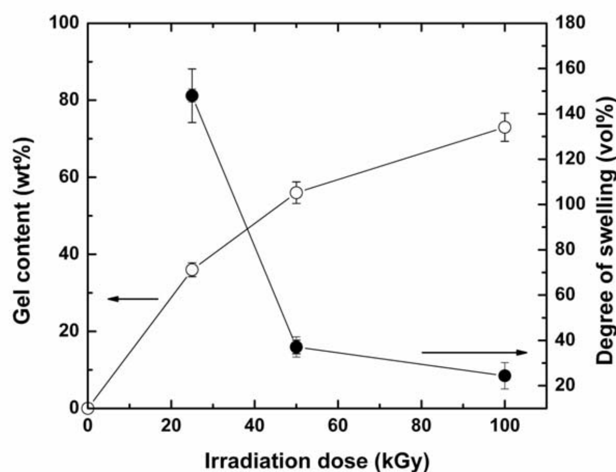

Figure 3-1. Gel content (○) and degree of swelling in chloroform (●) of PTMC networks prepared by gamma irradiation at 25, 50 and 100 kGy. (n=2)

Mechanical properties of PTMC networks

The different irradiation doses applied result in different crosslinking densities of the PTMC networks, which will affect the mechanical properties of the materials. With the final aim of using these networks as scaffolding materials for cell culturing under dynamic pulsatile conditions, the materials must be flexible and elastic (creep-resistant). Tensile tests and creep tests under static and dynamic conditions were performed to evaluate the mechanical behavior of PTMC networks. The results are given in Table 3-1.

After gamma irradiation, flexible PTMC networks with a modulus of 4.6 to 5.8 MPa, a yield stress of 1.1 to 1.6 MPa, a maximum tensile stress of 1.8 to 2.0 MPa and high elongations at break of approximately 600%-800% were obtained. The values of the Young's modulus, the yield stress and the maximum tensile stress of the networks

were lower than those of non-crosslinked PTMC. With increasing radiation dose, gel content increases and the modulus and the yield stress values decrease, while maximum tensile stress and elongation at break do not change very much.

To evaluate the elasticity (resistance to creep) of (crosslinked) PTMC films, their performance under static and dynamic loading conditions was determined. The table shows that under static loading conditions, the rate of creep of crosslinked PTMC networks is much lower than that of non-crosslinked PTMC. With an increase of the irradiation dose from 0 to 100 kGy, the plateau creep rate decreased from 1.09×10^{-4} to 1.16×10^{-7} s^{-1}. The permanent deformation of the networks (4.6 to 5.1%) was much lower than that of linear PTMC which failed after two days of loading.

In cyclic tensile tests, the permanent deformation after 20 cycles to 50% elongation decreased significantly from 4.1% for non-crosslinked PTMC to less than 1.0% for the crosslinked samples. Evidently, the crosslinked PTMC specimens are highly elastic and resistant to dynamic loading.

Table 3-1. The mechanical properties of PTMC (non-irradiated) and PTMC networks obtained by gamma irradiation at different doses (average values ± standard deviations, n=3).

Irradiation dose (kGy)	Gel content (%)	Tensile properties				Static creep [a]		Dynamic creep [b]
		Young's modulus (MPa)	Yield stress (MPa)	Maximum stress (MPa)	Strain at break (%)	Constant creep rate (mm/s)	Permanent deformation (%)	Permanent deformation (%)
0	0	6.8 ± 0.2	2.1 ± 0.3	3.3 ± 0.6	640 ± 30	$1.09 \pm 0.12 \times 10^{-4}$	failed in 2 d	4.1 ± 0.5
25	33 ± 4	5.8 ± 0.1	1.6 ± 0.3	1.8 ± 0.1	780 ± 35	$5.64 \pm 0.27 \times 10^{-7}$	5.1 ± 0.4	0.2 ± 0.1
50	56 ± 6	5.7 ± 0.1	1.5 ± 0.2	1.8 ± 0.1	580 ± 35	$3.35 \pm 0.24 \times 10^{-7}$	4.8 ± 0.4	0.4 ± 0.1
100	74 ± 8	4.6 ± 0.1	1.1 ± 0.2	2.0 ± 0.1	820 ± 45	$1.16 \pm 0.11 \times 10^{-7}$	4.6 ± 0.4	1.0 ± 0.3

[a] Specimens were loaded to 50 % of the yield stress for 3 days, the permanent deformation was determined after a 1 day recovery period.

[b] Specimens were cyclically deformed for 20 times to 50% elongation, after a relaxation period of 2 hours the permanent deformation was determined from the 21st cycle.

Porous tubular PTMC scaffolds

To be used as a substrate for the culturing of cells in blood vessel tissue engineering, porous tubular structures are required. Creep resistant, porous PTMC scaffolds can be prepared by crosslinking porogen-containing tubular PTMC specimens, followed by leaching of the porogen. First, glass mandrels were dip coated with PTMC solutions containing dispersed salt or sugar particles. After evaporation of the solvent and crosslinking by gamma irradiation under vacuum, the particles were leached out with water and the tubular scaffolds were removed from the mandrel.

As the polymer network has a low glass transition temperature (T_g is approximately -17 ^oC), shrinkage and undesired collapse of the pore structure can occur. However, by maintaining the specimens in the wet state, this could be prevented to a great extent.

An overview of different PTMC scaffolds prepared in this manner is presented in Table 3-2. These structures were prepared using glass mandrels with a diameter of 3 mm. After dip coating, crosslinking and leaching with water, the porous tubular structures have inner diameters ranging from 2.8 to 3.2 mm and wall thicknesses of 0.8 to 1.0 mm. To reach these latter values, the number of times the mandrels were dipped in the polymer solutions was varied from 8 to 10. To assess the porosities of the scaffolds, both gravimetric and micro-CT determinations were performed. It can be seen from Table 3-2, that porosities obtained by gravimetry were close to the theoretical porosities expected from the leachable particle to PTMC ratios. Porosities determined from micro-CT were somewhat lower than those determined from gravimetry, especially in the case of porous structures prepared using sugar particles. Although the resolution of the micro-CT apparatus was 8 μm, only pores larger than 25 μm were considered in our determinations as these sizes are most relevant for cell

seeding. Pores with smaller diameters were disregarded, which results in the determination of too low porosity values for specimens A-0 and A-50.

Table 3-2: Characteristics of porous tubular PTMC scaffolds prepared by dip-coating, crosslinking by gamma irradiation and porogen leaching. The specimens were kept in the wet state at all times. The values are presented as average values of at least 5 specimens ± the standard deviation.

Sample code		**Inner diameter [c] (mm)**	**Wall thickness [c] (mm)**	**Porosity (vol%) [d]**	**Porosity (vol%) [e]**	**Average pore size (µm) [e]**	**Range of pore sizes (µm) [e]**
Sugar	A-0 [a]	2.87 ± 0.27	0.78 ± 0.30	82.6 ± 1.9	58.8 ± 2.9	60	0-150
	A-50 [a]	2.75 ± 0.25	0.76 ± 0.31	84.9 ± 1.3	59.1 ± 1.5	55	0-150
NaCl	B-0 [b]	3.02 ± 0.28	0.90 ± 0.16	81.1 ± 2.8	82.6 ± 0.6	116	0-308
	B-25 [b]	3.05 ± 0.21	0.92 ± 0.19	81.3 ± 2.3	78.0 ± 0.8	110	0-308
	B-50 [b]	3.15 ± 0.28	0.83 ± 0.25	82.2 ± 2.0	77.8 ± 1.7	108	0-308

[a] Sugar particles (sieved to 106-250 µm) were used in preparing the porous structures. The porogen content was 90 wt%. The number indicates the irradiation dose (kGy) employed.
[b] Salt particles (sieved to 106-250 µm) were used in preparing the porous structures. The porogen content was 90 wt%. The number indicates the irradiation dose employed.
[c] Inner diameter and wall thickness of hydrated porous tubular structures. Measurements were performed using calipers.
[d] Porosity was determined gravimetrically
[e] Porosities, average pore sizes and approximate pore size ranges were determined by micro-CT.

Evaluation of the pore structure by micro-CT, showed that the average pore size (approximately 110 µm) and the largest pore size (approximately 308 µm) of tubular PTMC structures prepared using NaCl particles were larger than those of tubular structures prepared using sugar particles. These had an average pore size of approximately 60 µm and a largest pore size of approximately 150 µm. The difference in pore structure is evident from the pore size distribution curves presented in Figure 3-2A.

The larger average pore sizes of scaffolds prepared with salt particles compared to those prepared with sugar particles is due to the different sizes and size distributions of the porogen particles. In Figure 3-2B, it can be seen that after sieving the particles to sizes between 106 and 250 μm, the average size of the sugar particles was 142 μm and the average size of NaCl particles was approximately 207 μm. After leaching of the porogen, porous PTMC tubes prepared with sugar particles will have smaller average pore sizes than porous tubes prepared using salt particles. Furthermore, for both porogens the average sizes of the resulting pores is smaller than the average sizes of the porogen particles due to shrinkage of the polymer matrix. As the glass transition temperature of PTMC is below room temperature, this flexible polymer network is able to deform significantly to its relaxed equilibrium state. In the case of NaCl porogen particles, small numbers of pores with sizes larger than the salt particles were determined (Table 3-2, Figure 3-2A). This can be the result of aggregation of the salt particles.

The fabrication method use to prepare the porous PTMC structures was very reproducible: In a comparative experiment, where PTMC composites containing 90 wt% salt particles ranging from 106-250 mm was irradiated at 25 kGy and leached out with water, micro-CT analysis showed that the porosity and average pore size of a single specimen evaluated in triplicate respectively were 82.8± 0.8% and 114.7±0.5 μm, while analysis of three different specimens gave respective values of 82.3±1.3% and 114.3±0.3 μm.

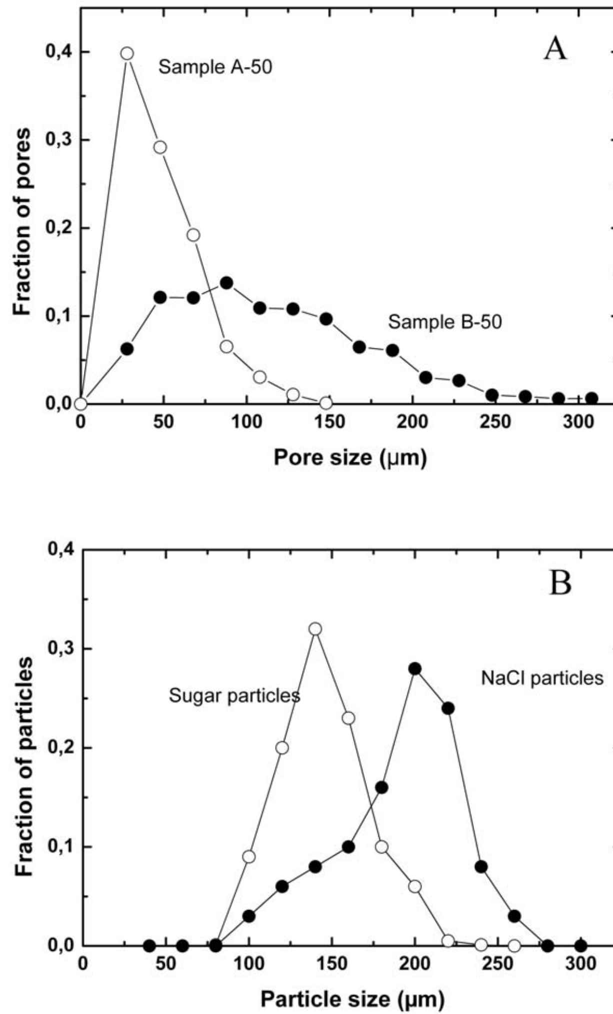

Figure 3-2. The pore size distributions of hydrated porous tubular PTMC scaffolds (samples A-50 and B-50 in Table 2) as determined by micro-CT (A) and the distribution of particle sizes of the used leachable sugar and NaCl particles (B). The porogen particles were sieved to sizes between 106 μm and 250 μm, the particle size distribution was determined from SEM.

Cross-sections of the porous tubular PTMC scaffolds were also visualized by SEM. Figure 3-3 shows cross-sections of the samples. It can be seen that in both cases an open porous structure was obtained: the pores in the walls of the tubular scaffolds are connected and the pores in the lumen of the tubes are open without being covered by a skin. From SEM images, porous PTMC structures prepared with sugar particles had an average pore size of 48 μm, and structures prepared with NaCl particles had an average pore size of 101 μm. These values are slightly lower than those obtained from micro-CT analysis, likely due to shrinkage occurring during the drying step in the sample preparation process and the vacuum applied during SEM analysis. Nevertheless, SEM confirms that the pore sizes of the porous PTMC structures prepared using NaCl as leachable particles were larger than those prepared using sugar particles.

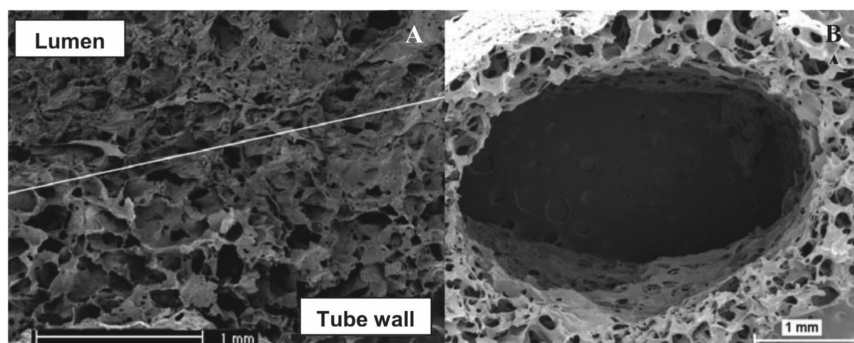

Figure 3-3: SEM images of porous tubular PTMC scaffolds crosslinked at 50 kGy. In (A) a porous PTMC specimen prepared with sugar as the porogen (sample A-50) was cut in the longitudinal direction. In (B) a porous PTMC specimen prepared with NaCl as porogen (sample B-50) was cut in the transverse direction. In both cases, the

images show the open pore surface of the lumen and the cross-section of the porous tube wall with connected pores.

Mechanical properties of porous tubular PTMC scaffolds

The dimensions of the porous tubular PTMC structures we prepared (inner diameter of 3.0 mm and wall thickness of 0.7 to 0.8 mm) are quite similar to those of ovine-, porcine carotid arteries and the human arteria mesenterica inferior (inner diameters ranged from 2.4-3.1 mm, wall thicknesses ranged from 0.4-0.6 mm). Figure 3-4 shows a photograph of a porous PTMC scaffold and a porcine carotid artery.

Figure 3-4: Photograph of a porous tubular PTMC structure (left, sample A-50) and a porcine carotid artery (right).

In Table 3-3, the results of tensile measurements in the radial direction of different natural arteries and of PTMC scaffolds are presented.

The radial tensile strength and elongation at break of the natural tissues are approximately 1.6 to 1.9 MPa and 200 to 350 %, respectively. The properties of non-porous PTMC tubes compare favorably with those of the natural blood vessels with a maximum tensile strength of 1.8 MPa and an elongation at break of 480 %.

Table 3-3. Tensile properties and permanent deformation upon dynamic cyclic loading of porous PTMC tubular scaffolds and natural arteries. Experiments were done in triplicate the radial direction of the tubular structures unless mentioned otherwise. Data is presented as average values ± standard deviations.

Sample	Initial stiffness (MPa)	Maximum radial tensile strength (MPa)	Elongation at break (%)	Permanent deformation [c] (%)
ovine carotid artery	1.21± 0.14	1.65 ± 0.08	255 ± 21	1.3
porcine carotid artery	3.89 ± 0.33	1.55 ± 0.10	207 ± 16	0.6
human arteria mesenterica inferior [a]	5.7	1.89	345	not determined
Non-porous PTMC [a, b]	4.1	1.78	479	0 [c]
A-0	1.84 ± 0.25	0.28 ± 0.05	721 ± 85	0 [c]
A-50	1.61± 0.32	0.23 ± 0.04	1156 ± 140	0 [c]
B-0	1.11± 0.04	0.19± 0.04	843 ± 95	0 [c]
B-25	1.30 ± 0.36	0.18± 0.05	1549 ± 105	0 [c]
B-50	1.10 ± 0.08	0.17 ± 0.04	1214 ± 120	0 [c]

[a] Single measurement

[b] Non-porous PTMC tubes had an inner diameter of 2.6 mm and a wall thickness of 0.5 mm.

[c] The error in these measurements is approximately 0.1%.

Evidently, the porous PTMC tubular scaffolds have lower maximal strengths, but significantly higher elongations at break (800-1200%) than the natural blood vessels. Depending on the porosity of the porous structures, the maximum tensile strength in the radial direction varied between 0.17 and 0.30 MPa. It is to be expected, that the maximum tensile strength of the porous structures will significantly increase upon seeding, culturing and expansion of cells in the scaffolds. As seeded cells proliferate and produce extra-cellular matrix, the load-bearing capacities of the constructs will increase and approach those of the natural arteries.

The unseeded porous PTMC tubes with inner diameters of 3.0 mm and wall thicknesses of 0.7 to 0.8 mm could readily be anastomosed to natural blood vessels (typical inner diameters of 2.4-3.1 mm and wall thicknesses of 0.4-0.6 mm). Their suture retention strengths were 1.94 N/mm and 1.64 N/mm for samples B-25 and B-50, respectively. These values are lower than those of non-porous PTMC tubes with an inner diameter of 2.6 mm and a wall thickness of 0.5 mm, which have suture retention strengths of 4.09 N/mm. The suture retention strengths of native ovine- and porcine arteries were higher, with values of 8.9 N/mm and 10.8 N/mm, respectively. It is to be expected, that upon cell culturing under appropriate dynamic conditions that allow for the formation of an oriented extra-cellular matrix, suture retention strengths of the cell-polymer constructs will increase significantly as well.

In long-term cell culturing where the cells are mechanically stimulated for time periods of days to weeks, *e.g.* in a bioreactor with a pulsatile flow of the cell culture medium, resistance to creep of the supporting scaffold structures is essential. In the tissue engineering of blood vessels, the typical mechanical strains that are applied vary between 5% and 10% elongation [26]. We evaluated the resistance to creep of PTMC scaffolds under dynamic loading conditions and compared their performance with that of natural arteries. The scaffolds were cyclically deformed (20 times) to approximately 30% elongation, then after a recovery period of 2 h, the permanent deformation due to creep was determined in the next cycle. While the natural tissues showed permanent deformations of 0.6 to approximately 1.3 % under these conditions, all tubular PTMC scaffolds were completely elastic with 0% permanent deformation. Therefore, these crosslinked porous structures should be well-suited for the dynamic culturing of relevant cells under physiological conditions.

Based on the results of the polymer network formation experiments and the mechanical evaluations of the different PTMC materials, the network films and porous tubes prepared by irradiation at 50 kGy were chosen for further cell culturing experiments.

Cell culturing on PTMC films irradiated at 50 kGy

In the different approaches to the tissue engineering of blood vessels, the relevant cells are either mesenchymal stem cells (MSCs) or smooth muscle cells (SMCs) and endothelial cells (ECs). To evaluate the compatibility of PTMC films crosslinked at 50 kGy with these cells, cell adhesion and proliferation experiments were performed.

As shown in Figure 3-5A human umbilical chord endothelial cells (HUVECs) were cultured on spin coated and gamma-irradiated PTMC films. After 1 day a confluent layer of cells could be observed. A confluent layer of SMCs was obtained after 3 days, as presented in Figure 3-5B. Both cell types show their characteristic morphology: the HUVECs showed a typical cobble-stone morphology, while the SMCs displayed a two-polar spindle shape and a “hill and valley” appearance. The morphology and the rates of proliferation were similar to those of cells seeded and cultured on tissue culture polystyrene (TCPS) (data not shown).

MSCs could also be cultured on these PTMC networks. In Figure 3-5D it can be seen that MSCs adhere and proliferate well and confluency is reached after 3 days. For comparison, on the non-irradiated PTMC films presented in Figure 3-5C, confluency was not yet reached in the same time period.

From these data it can be concluded that PTMC irradiated at 50 kGy is a suitable substrate for culturing of ECs, SMCs and MSCs.

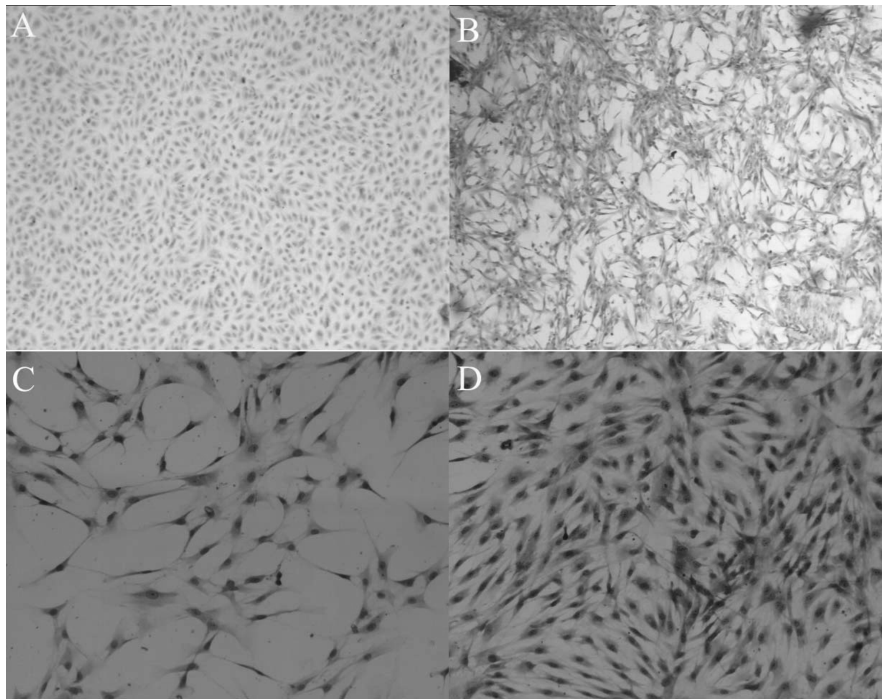

Figure 3-5. Characteristic morphologies of HUVECs (A) and of SMCs (B) cultured on the surface of PTMC films irradiated at 50 kGy (100×). Confluency was reached at 1 day and 3 days respectively. The morphology of MSCs cultured for 3 days on non-irradiated PTMC films (C), is similar to that of cells cultured on irradiated (50kGy) PTMC films (D), and characteristic for MSCs. MSCs and SMCs were seeded at cell densities of 10.000/cm^2, HUVECs were seeded at a density of 20.000/cm^2. Magnification is 100×.

Seeding and culturing of SMCs in tubular PTMC scaffolds

To tissue engineer an artery, the relevant cells should be seeded and cultured in the porous tubular scaffolds. The culturing of SMCs and ECs can lead to the formation of cell constructs that mimic a natural blood vessel. First SMCs are seeded and cultured

in the wall of the porous tube, this is followed by endothelialisation of the lumen with ECs in a next step. In our experiments, we first seeded and cultured SMCs in tubular PTMC scaffolds which were irradiated at 50 kGy.

Seeding was done by perfusing SMCs from the lumen through the wall of the tubular scaffolds. Then the seeded cells were cultured under static conditions for different periods of time.

It has been reported that in suspension at 37 ºC aortic SMCs have a length of 54.5 ± 1.5 µm and a diameter of 7.5 ± 0.3 µm [27, 28]. Although an optimal pore size for the seeding of SMCs was determined to be approximately 100 µm, we found that perfusion seeding of SMCs in the salt leached (sample B-50) PTMC scaffolds with an average pore size of 108 µm (Table 3-2) was not efficient (in this case the seeding efficiency was only 10-20%). Much better cell seeding results were obtained using the tubular scaffolds with an average pore size of 55 µm which were prepared with sugar particles (sample A-50); cell seeding efficiency was approximately 45%.

Figure 3-6 illustrates the adhesion (after 1 day) and the growth (after 7 days) of SMCs in the seeded porous PTMC scaffolds (sample A-50). It can be seen that SMCs have penetrated the wall of the porous PTMC tube through the interconnected pore network and are well attached to the pore surfaces. In 7 days they have proliferated significantly, forming connected layers of cells.

The proliferation of SMCs and the generation of extra-cellular matrix (ECM) components will further increase the mechanical integrity of these cell-containing scaffold constructs [29]. Indeed, already under the static culturing conditions we employed, we found that after 7 days of culturing of seeded SMCs, the maximum tensile strength in the radial direction had increased from 0.23 MPa for the initial

scaffold to 0.78 MPa for the cell-containing construct. Also the elongation at break had a very high value of 1360 %.

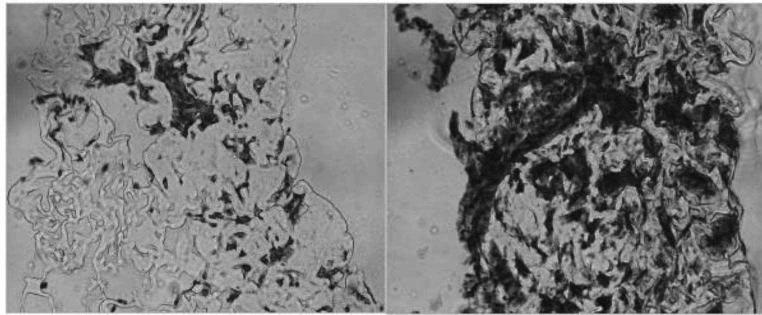

Figure 3-6. SMCs (stained with hematoxylin and eosin) cultured in porous tubular PTMC scaffolds (sample A-50) for 1 day (left) and 7 days (right). Images were obtained by phase-contrast microscopy (at a magnification of 10x).

To obtain cell-polymer constructs with mechanical properties that resemble natural vessels, it will be necessary to perform the cell culturing under dynamic physiological conditions [30]. Although the ultimate tensile strengths of the tubular PTMC scaffolds are lower than those of natural blood vessels, the scaffolds will not be subjected to such extreme forces during cell culturing conditions that mimic the natural environment. Under physiological conditions, the distention of blood vessels is less than 10% (26), the high extensibility of these porous PTMC structures (elongations at break of 600-800%) should make it therefore possible to use these scaffolds in bioreactors operating under the appropriate culturing conditions.

As we did not observe degradation of the PTMC scaffolds during the static culturing experiments, it will be possible to have vascular cells proliferate and produce extra-cellular matrix within the scaffolds before implantation of the constructs in the host.

Currently we are seeding SMCs in porous tubular PTMC scaffolds and culturing the cell-scaffold constructs in a pulsatile flow bioreactor. The results will be published in a forthcoming paper.

CONCLUSIONS

Upon gamma irradiation of high molecular weight PTMC, crosslinked networks are formed. These networks are flexible, elastic and resistant to creep when subjected to static and dynamic loading. Smooth muscle cells, endothelial cells, and mesenchymal stem cells adhered and proliferated very well on these PTMC networks.

Porous tubular PTMC structures can be fabricated by dip-coating using dispersions of leachable particles in polymer solutions. After evaporation of the solvent and crosslinking by gamma irradiation, the porogen particles are leached out with water yielding form-stable porous structures. Due to the low glass transition of the PTMC network, some shrinkage of the pore structure occurs.

Tubular PTMC scaffolds with suitable porosities, pore sizes and pore interconnectivity for the seeding and culturing of smooth muscle cells were prepared. These porous tubular scaffolds are highly flexible and elastic as well. This allows their use in pulsatile flow bioreactors where tissue engineered vascular substitutes are made by culturing relevant cells under dynamic physiological conditions.

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4| Evaluation of PTMC tubular scaffolds in a pulsatile flow system (PFS)

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ABSTRACT

Tubular scaffolds (internal diameter approximately 3 mm and wall thickness approximately 0.8 mm) with a porosity of approximately 83 % and an average pore size of 116 μm were prepared from flexible poly(trimethylene carbonate) (PTMC) polymer by dip-coating and particulate leaching methods. PTMC is a flexible and biocompatible polymer that crosslinks upon irradiation; porous network structures were obtained by irradiating the specimens in vacuum at 25 kGy before leaching soluble salt particles. To assess the suitability of these scaffolds in dynamic cell culturing for cardiovascular tissue engineering, the scaffolds were coated with a thin (0.1 to 0.2 mm) non-porous PTMC layer and its performance was evaluated in a closed pulsatile flow system (PFS). For this, the PFS was operated at physiological conditions at liquid flows of 1.56 ml/s with pressures varying from 80-120 mmHg at a frequency of 70 pulsations per minute.

The mechanical properties of these coated porous PTMC scaffolds were not significantly different than non-coated scaffolds. Typical tensile strengths in the radial direction were 0.15 MPa, initial stiffness values were close to 1.4 MPa. Their creep resistance in cyclic deformation experiments was excellent. In the pulsatile flow setup, the distention rates of these flexible and elastic scaffolds were approximately 0.10 % per mmHg, which is comparable to that of a porcine carotid artery (0.11 % per mmHg). The compliance and stiffness index values were close to those of natural arteries.

In long-term deformation studies, where the scaffolds were subjected to physiological pulsatile pressures for one week, the morphology and mechanical properties of the PTMC scaffolds did not change. This suggests their suitability for application in a dynamic cell culturing bioreactor.

INTRODUCTION

Obtaining functional small-diameter synthetic vascular grafts for clinical application has been an important goal [1-3]. In small-diameter blood vessel reconstructions, in which the diameters of the blood vessels are smaller than 6 mm, thrombosis is still the main problem encountered. In coronary bypass operations, grafting is carried out with autologous internal mammary arteries or saphenous veins. This surgical approach, however, has significant drawbacks such as a limited supply of grafts and mismatch in dimensions.

Tissue engineering is considered to be a promising approach to prepare blood vessel substitutes *in vitro*. To successfully engineer a functional blood vessel, biocompatible and biodegradable tubular scaffolds are required. Ideally these scaffolds are compliant and elastic, and mechanically durable to allow the dynamic culturing of cells under relevant physiological culturing conditions, for example in a cell culturing bioreactor operating under pulsatile flow conditions.

As scaffolding materials, both natural and synthetic polymers have been used. Natural polymers like collagen and elastin are present in blood vessel walls. As a rule, porous scaffolds made from natural materials show good cell adhesion and proliferation. However, they often lack sufficient mechanical strength, especially in terms of compliance and burst strength, which significantly limits their use[4-6].

The use of synthetic polymers may obviate these problems. These polymers are relatively easy to process, and can be tailored to mimic the mechanical properties of natural blood vessels. For this, lactide and glycolide based polymers have been used most often for the preparation of blood vessel tissue engineering scaffolds. These polymers are quite rigid, and achieving the necessary flexibility and compliance remains a challenge[7-11].

In vivo, the blood vessel wall is continuously exposed to hemodynamic forces[12]. As a result of these forces smooth muscle cells (SMC) align perpendicular to the direction of blood flow, and the formation of extracellular matrix is specifically directed to allow the vessel to withstand the pulsating pressure generated by the heartbeat. Mechanical stimuli contribute to the orientation of the blood vessel wall tissue and enhance the production of extracellular matrix to yield a functional construct[13,14]. Therefore, culturing vascular grafts in a bioreactor operating under dynamic pulsatile flow conditions is considered to be effective in preparing functional tissue engineered blood vessels. Compliant scaffolds are essential to be able to culture cells in an environment that mimics the physiological conditions. For this, a cell culturing system operating under physiological pulsatile flow conditions system is needed. A detailed analysis of such a PFS (PFS) was given by Conklin *et al*.[15]. In their system, physiological heamodynamics were simulated and the effect of pressure and shear stress on intact vascular tissues was investigated.

We have recently reported on the development of crosslinked porous tubular scaffolds based on rubber-like PTMC networks, which showed excellent mechanical properties. Adhesion and proliferation of endothelial cells, smooth muscle cells and mesenchymal cells (cells relevant in blood vessel tissue engineering) was excellent and the structure of the pore network was very well suited to allow efficient cell seeding [16].

The aim of this research was to evaluate the mechanical performance of crosslinked tubular PTMC scaffolds in a flow system that mimics the pulsatile flow of blood in vessels, thereby assessing their suitability in the dynamic culturing of cells in small-diameter blood vessel tissue engineering.

MATERIALS AND METHODS

Pulsatile flow system

A closed pulsatile flow system (PFS) for the dynamic testing of small-diameter blood vessel tissue engineering scaffolds was constructed. The setup was based on the design of Buttafoco *et al*.[17] and Webb *et al*.[18]. A peristaltic roller-pump (Sci-Q-323, Watson and Marlow, Belgium) was used to circulate phosphate buffered saline (PBS) from a three-port glass reservoir via PVC tubing (with 8mm inner diameter and 11 mm outer diameter) to a custom-made glass chamber. The peristaltic roller-pump generates a pulsatile flow with pressure pulses of 40 mmHg. In the glass chamber, see also Figure 3C, tubular scaffolds with lengths up to approximately 10 cm can be mounted and with use of an LED optical micrometer (Keyence LS 7600, Germany) their distention behavior as a function of pressure could be evaluated with an error smaller than 2 μm. The pressure was monitored distal to the tubular scaffolds using pressure sensors (Edwards Lifesciences, USA) and a scope meter oscilloscope (Fluke 199BM, The Netherlands). The data was recorded using LabView 8.5.1 (National Instruments, USA).

The glass reservoir contained 60 ml PBS, pressurization of the closed circulating flow system with air or nitrogen gas was done using an electronically controlled Venturi valve (Fairchild T5200-50, USA).

The characteristics of the PFS closed circulating flow system flow were assessed using a 4 cm length of silicone tubing with an inner diameter of 3.0 mm and wall thickness of 1.5 mm (Watson and Marlow) as scaffold and PBS as the circulating medium. It was assumed in the calculations that the diameter of the tubular structures did not vary, that entrance effects could be neglected, and that the liquid flow within the tubular structures had equilibrated.

The PBS flow rates (φ in ml/s) were determined at different settings of the peristaltic roller pump resulting in flow pulsation frequencies between 30 and 130 pulsations per minute. This data was used in estimating to a first extent the nature of the flow, the shear rate. The laminar or turbulent nature of the liquid flow in the tube is characterized by its Reynolds number (Re):

$$Re = \frac{d * \overline{V} * \rho}{\mu} \qquad \text{Equation 1}$$

Where d is the inner diameter of the scaffold (0.3 cm), ρ is the density of the circulating liquid (1 g/cm^3) and μ is its viscosity ($8.9x10^{-3}$ (dynes x s)/cm^2 at 25 ^oC). The mean velocity of the liquid ($\overline{V}$) was calculated from the determined flow rates using: $\varphi = 1/4(\overline{V} * \pi * d^2)$.

The shear rate ($\dot{\gamma}$ in s^{-1}) was calculated using:

$$\dot{\gamma} = \frac{\varphi}{2\pi * d^3} \qquad \text{Equation 2}$$

It should be noted that although the average flow rate could be varied in our experimental setup, the pressure difference between the systolic and diastolic intraluminal fluid pressures was a constant 40 mmHg.

Preparation of tubular PTMC scaffolds

High molecular weight PTMC (M_n= $636x10^3$ g/mol, PDI=1.38) was synthesized according to previously described methods[16]. Porous tubular PTMC structures were prepared by dip-coating and salt-leaching. Glass mandrels (diameter = 3.0 mm) were dipped into solutions of the PTMC in chloroform (2.5% wt/vol) containing homogeneously dispersed NaCl salt particles (Acros Organics, Belgium), after which the solvent was evaporated. The water-leachable salt particles were sieved to a size range of 106-250 μm, the PTMC to salt particle weight ratio in the suspension was

10:90. The dip-coating process was repeated several times to reach a desired outer diameter of approximately 8 mm. The coated mandrels were then dried at room temperature for 2 to 3 days, packaged in vacuum and subjected to a 25 kGy ^{60}Co gamma irradiation (Isotron, Netherlands) to obtain crosslinked structures[16].

The salt particles were leached with demineralized water and the scaffold was removed from the mandrel, in this manner a porous creep-resistant tubular scaffold based on a PTMC network was obtained. To prevent excessive shrinkage and collapse of the pores, the scaffold was kept in the hydrated state unless mentioned otherwise.

To allow their evaluation under pulsatile flow conditions, tubular PTMC scaffolds with an additional non-porous outer layer were prepared. This was done by dipping the dried and irradiated mandrels coated with the PTMC polymer and salt composite into a PTMC solution in chloroform (2.5 % wt/vol) for 4 times. After evaporation of the solvent, the salt particles were leached with demineralized water and a porous PTMC scaffold with a closed outer layer was obtained.

The morphology of the porous tubular scaffolds was observed by scanning electron microscopy (SEM). The samples were cut in liquid nitrogen, and dried after rinsing with methanol. Cross sections, inner and outer surfaces of the samples were gold-sputtered using a Polaron E5600, the thickness of the gold layer was approximately 120 nm. The experiments were performed using a Hitachi S800 field emission scanning electron microscope operating at 2.5 or 5 kV.

Micro-computed tomography (micro-CT) was also employed to investigate the three-dimensional pore morphology of the tubular PTMC scaffolds. The scaffolds were kept in a moisturized state, and scanned using a General Electric eXplore micro-CT operating at an X-ray voltage of 60kV. The resolution was 8 μm.

In the analysis of the pore network, pore voxels are defined as voxels corresponding to void space and polymer voxels as voxels corresponding to the polymer phase. To evaluate the characteristics of the pore network, void voxels were filled with modeled spheres of different diameters. The pore diameter assigned to a pore voxel is the diameter of the largest sphere that fits inside the pore[19]. The average pore size was then calculated by averaging the product of the pore voxels with their assigned pore diameter over the total number of pore voxels, according to:

$$average\ pore\ size = \frac{\sum_i (pore\ voxel_i * pore\ size_i)}{\sum_i pore\ voxel_i} \qquad \text{Equation 3}$$

The accessible pore volume was calculated using an algorithm that mimics mercury porosimetry[20]. With a thresholding operation, all pores not accessible for a simulated sphere of a certain diameter or not (inter)connected to the outsides of the scaffold are discarded. The volume of the remaining pore network is calculated and plotted versus the diameter of the simulated sphere; this results in a graph of the accessible pore volume (as a fraction of total volume) versus sphere diameter.

The accessible pore surface area was calculated using a triangularization algorithm[21]. The calculated surface consists of triangular surfaces that contact the scaffold and triangular surfaces that do not contact the scaffold. Triangular surfaces not contacting the scaffold are suppressed, resulting in a surface area of the pore volume of the scaffold that is accessible for simulated spheres of a certain diameter.

Mechanical properties of PTMC scaffolds

The tensile properties of porous tubular PTMC scaffolds were measured in the radial direction according to the American National Standards Institute Inc., and the Association for the Advancement of Medical Instrumentation (ANSI/AAMI) standards

VP20: 1994 (paragraph 8.3). Tensile experiments were carried out using a Zwick Z020 universal tensile testing machine (Germany) at room temperature. The tensile testing machine was equipped with a 500 N load cell, and was operated at a crosshead speed of 1 mm/min. The specimen deformation was derived from the grip to grip separation.
Repeated cyclic loading experiments were performed at a crosshead speed of 50 mm/min. The tubular structures with a length of 5 mm were stretched circumferentially 3 mm for 20 times, after a 2 hr relaxation period the permanent deformation was determined from the 21st testing cycle.
The mechanical properties of porcine carotid arteries were evaluated as well. These arteries were obtained from a local slaughterhouse and had inner diameters ranging from 2.4 to 3.1 mm and wall thicknesses of 0.4 to 0.6 mm. They were kept in an ice-cooled phosphate buffered solution (pH 7.4), and mechanical tests were performed at room temperature within 12 hrs of harvesting.

Performance of PTMC scaffolds in a PFS

To assess the possibility for the coated tubular PTMC scaffolds to resist different pressures *in vivo*, changes in diameter of the scaffolds at increasing pressures were recorded optically. In static experiments, the pressure of the liquid was increased from 20 to 140 mmHg in steps of 10 mmHg. When equilibrium was reached, the distension of the scaffolds was recorded using LabVIEW software. In total, the scaffolds were subjected to increasing pressures for approximately 5 hrs. The distension of the scaffolds was normalized with respect to their initial (external) diameters.
The dimensional recovery of the scaffolds after removing the pressure was also determined after 30 minutes and after 24 hrs relaxation.

To evaluate the response of the scaffolds to dynamic pulsatile flows (70 pulsations per minute), compliance and wall stiffness indices of the scaffolds were determined [18, 22]. The changes in scaffold diameter resulting from the difference in systolic and diastolic pressures (40 mmHg) were determined using the optical micrometer setup. The measurements were carried out under pulsatile flows with average pressures of 50, 80, 100, 120 or 150 mmHg.

The compliance is expressed as the percent change in tubular scaffold diameter per 100 mmHg:

$$C = \frac{10^4 (d_{sys} - d_{dia})}{d_{dia}(p_{sys} - p_{dia})} \qquad \text{Equation 4}$$

The stiffness index, β, can be calculated using equation 5:

$$\beta = \frac{\log_e \left(\frac{p_{sys}}{p_{dia}} \right) d_{dia}}{d_{sys} - d_{dia}} \qquad \text{Equation 5}$$

In these equations, d_{sys} and d_{dia} are systolic and diastolic scaffold diameters (in mm), respectively, and p_{sys} and p_{dia} are the systolic and diastolic intraluminal fluid pressures (in mmHg), respectively. Please note that in this study the difference between the systolic and diastolic intraluminal fluid pressures was 40 mmHg.

The long term mechanical integrity and dimensional stability of the porous coated PTMC scaffolds was investigated by subjecting the scaffolds to this pulsatile flow for a period of 7 d. After this time, the tensile properties and scaffold morphology were evaluated as described earlier.

RESULTS AND DISCUSSIONS

Pulsatile flow system (PFS)

A pulsatile flow system (PFS) that mimics physiological conditions was built to evaluate the mechanical performance of tubular PTMC scaffolds, which are to be used in the tissue engineering of small diameter (approximately 3 mm) blood vessels under dynamic cell culturing conditions. In this setup a peristaltic roller pump generates a continuous pulsatile flow of medium, while the resulting changes in diameter of the tubular scaffold are recorded with an optical micrometer. To establish adequate and physiologically relevant operating conditions of the PFS, flow dynamics of the system were first evaluated using a silicone rubber tube with an inner diameter of 3.0 mm as the tubular scaffold.

When the roller pump is operated using flexible silicone tubing of inner diameter 8.0 mm and outer diameter of 11.0 mm a pressure pulse of 40 mmHg is generated. By applying an additional static pressure of 80 mmHg to the closed circulating flow, a pulsatile flow of 80 to 120 mmHg is created. These values are close to physiological pressures which range from 70 to 130 mmHg [23].

Increasing the rotational speed of the pump from 30 to 130 pulsations per minute led to a linear increase of the average flow rate from 0.63 to 2.94 ml/sec in our PFS. At 70 pulsations per minute, the average flow rate was 1.56 ml/s and the average Reynolds number was 91, indicating laminar flow in the tubular scaffold which allows efficient transfer of nutrients and waste products. In human carotid arteries of varying diameters, flow rates between 1.59 ml/s and 6.16 ml/s have been determined, and Reynolds numbers varied from 2 to 560 [24].

In addition, when the PFS was operated under the given conditions, the average shear rate[25] in the silicone scaffold was determined to be 590 s^{-1} dyne $\times$ s /cm^2. This

value also fits within the range found in human carotid arteries, where the shear rate is 60-775 s^{-1}. This parameter is important, as it determines the biochemistry of endothelial cells and the permeability of the arterial wall to macromolecules [27,28].

Table 4-1. Flow dynamics in human carotid arteries determined by ultrasound imaging and pressure measurements in volunteers[23], and in a silicone scaffold in a PFS operating at 80 to 120 mmHg and 70 pulsations per minute.

	Human carotid artery[a]	**Silicone scaffold in PFS**[b,c]
Flow rate (ml/s)	1.59- 6.16 [24]	1.56
Reynolds number	2-560 [26] [d]	91
Shear rate (s^{-1})	60-775 [25] [d]	590

[a] The inner diameter of human carotid arteries varied between 4.05 and 6.77 mm
[b] As a tubular scaffold, silicone tubing with inner diameter 3.0 mm and length 4 cm was used
[c] Values for Reynolds number and shear rate are estimated using equations 1 and 2.
[d] Values are for diastole and systole, respectively

In Table 4-1, an overview is given in which the fluid dynamics in human carotid arteries are compared with those in the silicone tubing in our PFS operating at pulsatile pressures of 80 to 120 mmHg and 70 pulsations per minute. It follows from the table, that these flow conditions are relevant to evaluate the performance of our porous PTMC scaffolds.

Tubular PTMC scaffolds

Porous tubular PTMC scaffolds were prepared as described in the experimental part. The gamma irradiation process at 25 kGy leads to the formation of a crosslinked structure. The scaffolds had lengths of 90 ± 5 mm, inner diameters of 3.0 ± 0.3 mm and wall thicknesses of approximately 0.8 mm. In the tissue engineering of blood

vessels, endothelial cells and smooth muscle cells will be seeded and cultured in the scaffolds. However, before seeding and production of extracellular matrix by the cells, the porous scaffolds will be permeable to fluids. Therefore, in evaluating the mechanical performance of porous PTMC scaffolds in a PFS in the absence of cells, the porous scaffolds were coated with a thin closed PTMC layer with a thickness of 25 to 40 μm.

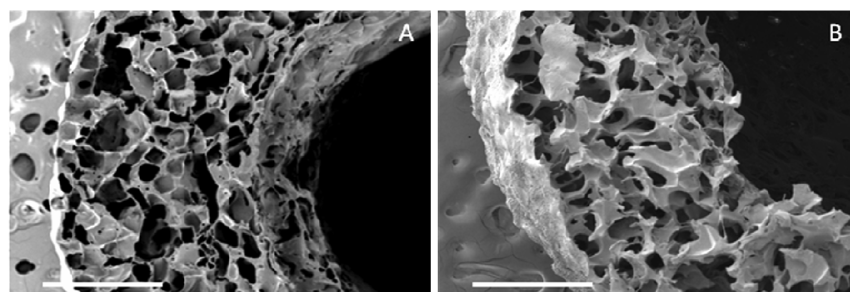

Figure 4-1. SEM images of cross-sections of crosslinked tubular PTMC scaffolds before (A) and after coating with a non-porous outer layer (B). The scale bar is 1 mm.

Figure 4-1 illustrates the formation of a closed, non-permeable outer PTMC layer on porous PTMC scaffolds. It can be seen in the SEM images, that the creation of the closed outer layer has not affected the pore structure within the scaffold. All scaffolds were quite stable, and could be handled with ease in the dry and hydrated state.

Analysis of the pore structure of the scaffolds in the hydrated state was done by micro-CT. A three-dimensional images of tubular PTMC scaffolds non-coated and coated with a non-porous outer layer is presented in Figures 4-2 A and B. The visualizations indicate excellent adherence of the coating to the porous PTMC structure. The porosity of the non-coated PTMC scaffold was 83.0 % ±1.3 %, the overall porosity of the coated scaffolds was 82.3 % ±1.3 %. In Figures 4-2 C and D quantitative data

regarding the structure of the pore network of the coated and non-coated PTMC scaffolds is given. The characteristics of the pore structure are quite comparable for the different specimens. Pore sizes range from 0 to 250 μm, with very similar pore size distributions. The average pore sizes determined for the non-coated and the coated PTMC scaffolds were respectively 114 μm and 121 μm. Most importantly, the interconnectivity of the pores was not much influenced by the coating process.

Smooth muscle cells from the aortic wall have a length of 54.5±1.5μm and a diameter of 7.5±0.3μm that in suspensions at 37 ºC, and an optimal pore size for smooth muscle cell migration was determined to be 100 μm[28]. Figure 4-2D shows that for spherical particles with a diameter of 50 μm, approximately 70 % of the pore volume of the coated and the non-coated PTMC scaffolds are accessible. These high values would allow excellent migration and seeding of smooth muscle cells into the structures. Furthermore, analysis of the micro-CT data (not presented) indicates that the surface area to which the cells can attach (and on which they can proliferate) would be higher than approximately 17.5 mm^2 per mm^3 of scaffold. Coating the porous PTMC structures with a thin closed PTMC layer does not influence the inner pore structure of the scaffold to a significant extent.

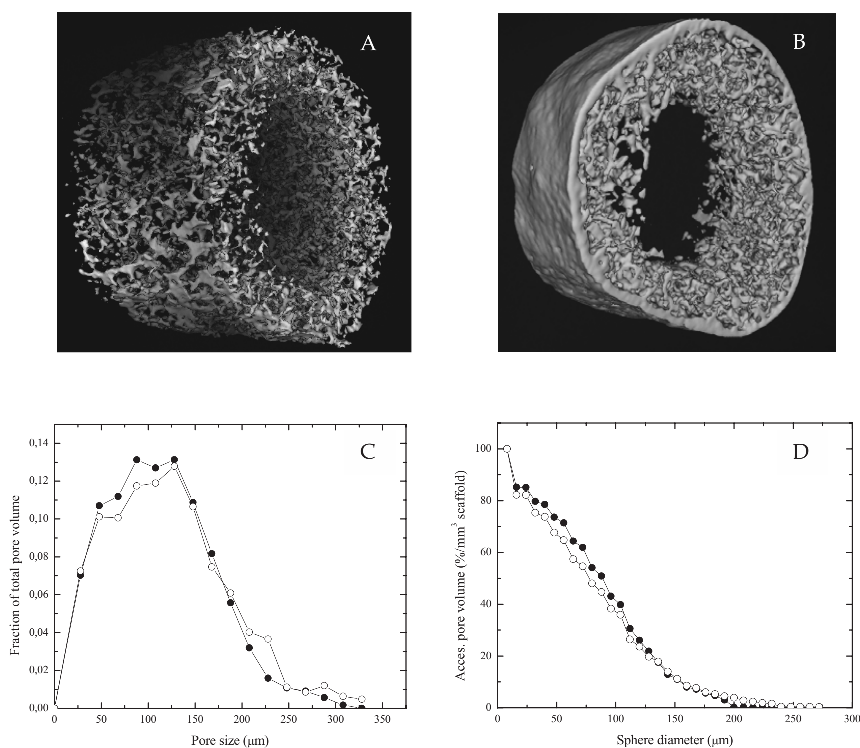

Figure 4-2. Three dimensional visualizations of a porous tubular PTMC scaffold (A), and of a porous tubular PTMC scaffold coated with a non-porous PTMC layer (B). In (C) pore size distributions are shown, while (D) indicates the accessible pore volume of porous tubular PTMC structures for spheres of different diameters. The properties of non-coated PTMC scaffolds (●) and coated PTMC scaffolds (○) are presented.

Comparisons were also made with regard to the mechanical properties of the PTMC scaffolds. Tensile testing experiments and repetitive cyclic tensile tests were performed on coated and non-coated porous PTMC scaffolds. In Table 4-2 an overview is given of the mechanical properties determined in the radial direction. It is seen that once again the differences between the coated and the non-coated porous PTMC structures is minimal.

Table 4-2. Tensile properties and resistance to cyclic deformation of tubular PTMC scaffolds determined in the radial direction. For comparison, the properties of porcine carotid arteries are given as well.

	Tensile testing			**Cyclic tensile testing**
	Initial stiffness (MPa)[a]	**Maximum strength (MPa)**	**Strain at break (%)**	**Permanent deformation (%)[b]**
Tubular PTMC scaffold	1.32 ± 0.28	0.15 ± 0.02	1644 ± 198	0
Coated tubular PTMC scaffold	1.42 ± 0.31	0.14 ± 0,03	1428 ± 254	0
Porcine carotid artery[c]	3.89 ± 0.33	1.55 ± 0.21	207 ± 16	0.6

[a] The initial stiffness was determined at strains between 0.05% and 0.5%.
[b] The permanent deformation was determined after 20 cycles to an elongation of 3 mm and 2 hrs relaxation
[c] The porcine carotid arteries had inner diameters ranging from 2.4 to 3.1 mm and wall thicknesses of 0.4 to 0.6 mm

In both cases, highly flexible scaffolds (initial stiffness of 1.3 to 1.4 MPa) are obtained with maximum radial tensile strengths of 0.14 to 0.15 MPa) and very high values of the elongation at break of approximately 1500%. In addition, both crosslinked scaffolds were extremely resistant to creep in cyclic deformation tests. In comparison, freshly harvested non-porous porcine arteries which contain cells are more rigid and stronger, but are less extensible and show some permanent deformation upon repeated cyclic testing.

Based on these results, we conclude that it will be possible to use the coated porous PTMC structures in a PFS to assess the mechanical behavior of PTMC scaffolds under (long term) dynamic flow conditions which mimic physiological conditions.

Mechanical evaluation of coated PTMC scaffolds in a PFS

Adequate compliances and values of stiffness of grafts are key issues in determining the viability of cells in the tissue engineering of vascular grafts. If mechanical cell signaling is improper, extracellular matrix production will be limited resulting in atherosclerotic behavior of the vascular construct *in vivo*. Poor compliance and a mismatch in stiffness could lead to rupture of the graft during culturing in pulsatile bioreactors or in the failure of the implant after application [4,29].

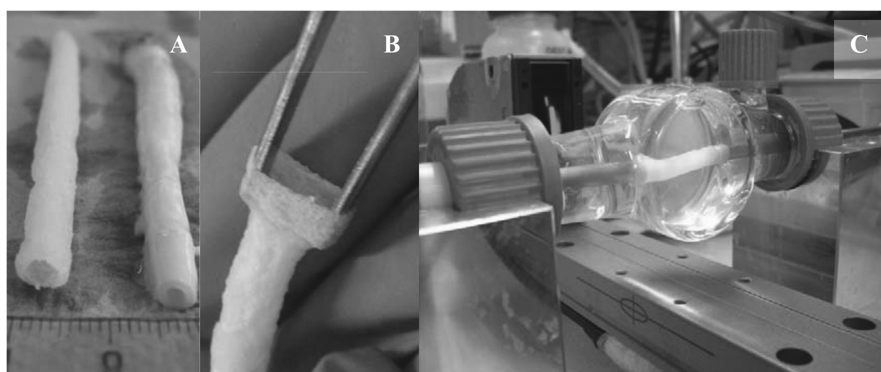

Figure 4-3. In (A) a non-coated crosslinked porous tubular PTMC scaffold (left) is compared with a porcine carotid artery (right). It is shown in (B) that these resilient tubular scaffolds are highly flexible and elastic. The photograph in (C) shows a coated scaffold mounted in the perfusion flow system. With an optical micrometer, the distension of the scaffold as a function of the applied pulsatile pressure can be measured.

In our experiments, highly flexible and elastic porous PTMC scaffolds were prepared to match the dimensions of small diameter blood vessels. Photographs of these tubular grafts are presented in Figures 4-3A and 4-3B. The dimensions were very similar to those of porcine carotid arteries with inner diameters ranging from 2.4 to 3.1 mm and

wall thicknesses of 0.4 to 0.6 mm were measured in the same condition. These highly flexible and resilient porous PTMC structures were coated with a thin PTMC layer. The inner diameter of the coated scaffolds was 3.0 mm, and wall thicknesses ranged from 0.7 to 0.8 mm. Figure 4-3C shows the scaffold mounted in the PFS.

It was confirmed that when the PFS was operating under physiologically relevant conditions (70 pulsations per minute, pressures varying between 80 and 120 mmHg), the fluid flow characteristics through porous PTMC scaffolds coated with a non-porous outer layer were essentially the same as through silicone scaffolds. Furthermore, their distention behavior was compared with that of porcine carotid arteries. As indicated in Figure 4-4, the diameters of the coated tubular PTMC structures and the porcine carotid arteries increased with increasing pressure. The average distention rate of the scaffolds (0.1% per mmHg) was comparable to that of the native carotid arteries (0.11% per mmHg). After removal of the applied pressure, the diameters of both the coated crosslinked tubular PTMC scaffold and the porcine carotid artery returned to their original values. Within 30 minutes, the remaining deformation was less than 1%.

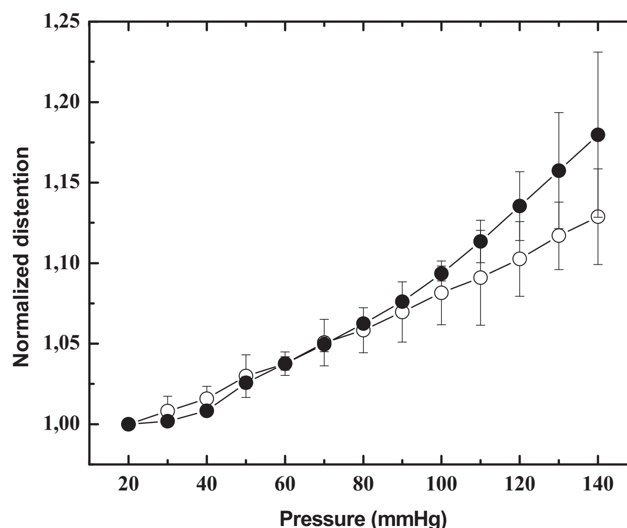

Figure 4-4. Distension behavior (expressed in relation to the initial diameter) of porcine carotid arteries (●) and coated crosslinked PTMC tubular scaffolds (○) as a function of the mean intraluminal fluid pressure.

The compliance and stiffness indices of coated porous tubular PTMC scaffolds and porcine carotid arteries were determined at different mean pressures at different pressures (Figure 4-5). Arterial tissue is an anisotropic material, and the relationship between radial distension and intra-luminal pressure is non-linear[30]. We also found that the compliance of the arteries decreased when the mean pressure increased. Although the PTMC scaffolds were somewhat less compliant than the porcine arteries at low pressures, their compliance was comparable at higher pressures. Analogously, the stiffness indices of the PTMC scaffolds were somewhat higher than that of the arteries at low pressures and lower at higher pressures.

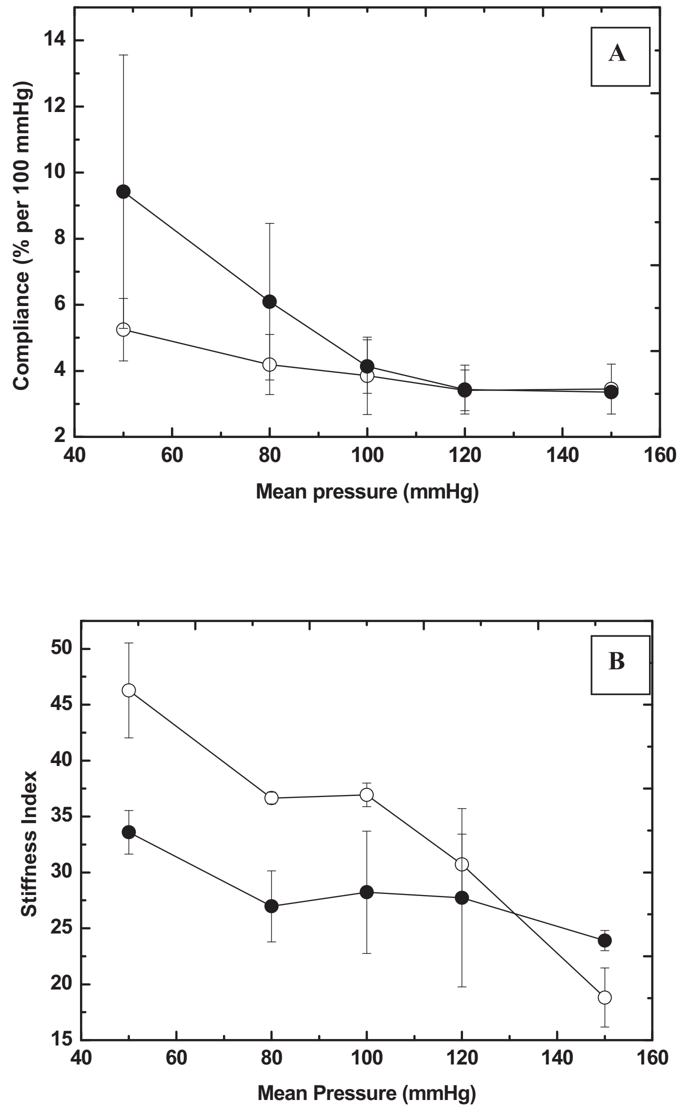

Figure 4-5. Compliance (A) and stiffness index (B) as a function of mean pulsatile intraluminal fluid pressure for porcine carotid arteries (○) and coated tubular PTMC scaffolds (●). The porcine carotid arteries had inner diameters ranging from 2.4 to 3.1 mm and wall thicknesses of 0.4 to 0.6 mm, the tubular PTMC scaffolds had inner diameters of 3.0 mm and wall thicknesses of 0.7 to 0.8 mm.

Table 4-3. Compliance values and stiffness indices of human arteries and veins commonly used in coronary bypass surgery. The corresponding values of commercially available e-PTFE arterial grafts, porcine carotid arteries and coated crosslinked PTMC scaffolds are presented as well.

	Compliance[a] (% change/ 100 mmHg)	**Stiffness[a] (ß stiffness index)**
Human iliac artery[32-35] [b]	2.6±0.8	41.8±20.0
Human internal mammary artery[36] [b]	5.3±0.9	-
Human saphenous vein[31] [b]	1.5±0.4	67.9±16.7
e-PTFE graft [31,37] [d]	0.5±1.2	156.0±6.0
Porcine carotid artery [c]	4.1±0.8	28.1±5.5
Coated tubular PTMC scaffolds [c]	3.9±1.2	37.1±1.1

[a] The compliance and stiffness indices were determined at pressures ranging from 80 to 120 mmHg, which were created by superimposing a pulsatile pressure of 0 to 40 mmHg (70 pulsations per min) onto a static pressure of 80 mm.
[b] The dimensions of human arteries depend on age, weight and gender: Human iliac artery diameters varied between 1.98 and 9.26 mm, human internal mammary artery diameters varied between 0.99 and 2.55 mm, human saphenous vein diameters varied between 1.60 and 5.70 mm.
[c] The porcine carotid arteries had inner diameters ranging from 2.4 to 3.1 mm, and wall thicknesses of 0.4 to 0.6 mm. The coated tubular PTMC scaffolds had inner diameters of 3.0 mm and wall thicknesses of 0.7 to 0.8 mm.
[d] The internal diameters of the ePTFE grafts varied and were larger than 1.04 mm, their wall thicknesses varied from 0.02 to 0.25mm

Table 4-3 gives an overview of data on the compliance and stiffness of small-diameter (autologous) arteries and veins that are commonly used in coronary bypass surgery. It shows that for the scaffolds, the values of compliance and stiffness lie between those of the natural arteries and the human saphenous vein. It is likely that upon culturing cells in the scaffolds and the creation of an extracellular matrix by the cells, the properties of the constructs will further improve and optimal grafts will be obtained. Most importantly the scaffolds were much more compliant and significantly less stiff

than the e-PTFE currently which is used in large diameter vascular grafts, thereby reducing the risk of graft failure and atherosclerosis.

The long-term dimensional stability of the coated tubular PTMC scaffolds was investigated by subjecting the scaffolds to a pulsatile flow of 70 pulsations per minute, with diastolic and systolic pressures of 80 and 120 mmHg for a continuous time period of 7 days. The specimens were then analyzed for changes in dimensions, pore structure, and mechanical properties. The change of the external diameters was less than 0.50%, this corresponds to a very low distention rate of approximately 4.28×10^{-3} mm/day.

Figure 4-6 shows SEM micrograph images of cross-sections of the scaffolds before and after being subjected to the pulsatile flow. It is clear from the figure that changes in scaffold wall thickness, porosity, pore size and pore size distribution are minimal after long-term pulsatile deformation. It was also found that the mechanical properties remained unchanged after this 7 day period. For the coated scaffolds, a maximum tensile strength of 0.16 MPa and en elongation at break of 1650% was determined. These values are similar to the values before the pulsatile conditioning (see Table 4-2).

The excellent form stability of these highly compliant scaffolds upon pulsatile deformation for long times should allow their use in dynamic cell culturing bioreactors. As their mechanical properties are similar to those of natural blood vessels and are maintained as well, it would be especially attractive to apply them in the tissue engineering of small diameter vascular grafts.

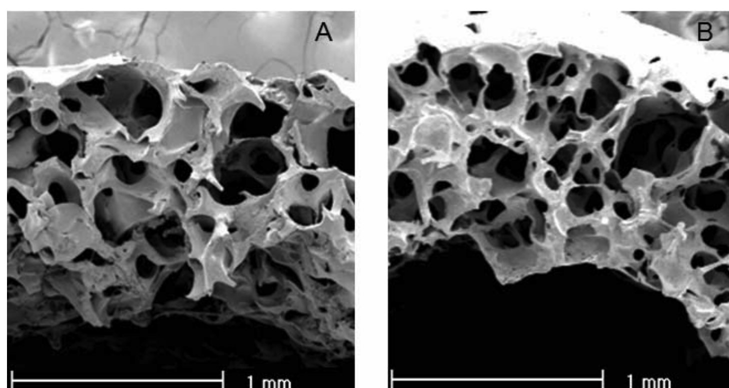

Figure 4-6. SEM images of coated tubular PTMC scaffolds before (A) and after (B) being subjected to pulsatile flow conditions (70 pulsations per minute, diastolic and systolic pressures of 80 and 120 mm Hg) for 7 days.

CONCLUSIONS

A closed flow system operating under pulsatile conditions was used to evaluate the mechanical performance of tubular porous PTMC structures. At 70 pulses per minute, 80 to 120 mmHg pressure pulses of phosphate buffered saline flowing through tubular scaffolds with inner diameters of approximately 3 mm resulted in dynamic flow conditions similar to those in human carotid arteries.

Crosslinked porous PTMC scaffolds with porosities of 83% and average pore sizes of 114 μm were prepared by dip coating and salt leaching methods. These porous PTMC scaffolds were highly compliant and flexible. To allow their use in the PFS, the porous scaffolds were coated with a non-porous outer PTMC layer. It was shown that this outer layer did not significantly affect the pore network or the mechanical properties of the scaffolds, and therefore these scaffolds could be used to investigate the long-term distention behavior of porous PTMC scaffolds.

The results indicate that the scaffolds were highly resistant to creep under physiological flow conditions. This allows their use in the tissue engineering of small-diameter blood vessels, where cells are cultured under dynamic pulsatile conditions in a bioreactor.

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5| Effective Seeding of Smooth Muscle Cells into Tubular Poly(Trimethylene Carbonate) Scaffolds for Vascular Tissue Engineering.

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ABSTRACT

Porous tubular poly(trimethylene carbonate) (PTMC) scaffolds for vascular tissue engineering, with an inner diameter of 3 mm and a wall thickness of 1 mm, were prepared by means of dip-coating and subsequent leaching of NaCl particles. The scaffolds, with an average pore size of 110 μm and a porosity of 85%, showed a smooth muscle cell (SMC) seeding efficiency of only 10%. To increase the efficiency of cell seeding, these scaffolds were coated with a micro-porous PTMC outer layer with a thickness of 0.1-0.4 mm, an average pore size of 28 μm and a porosity of 65%. Coating of the scaffolds with the micro-porous outer layer did not influence the inner pore structure or the mechanical properties of the scaffolds to a significant extent. The intrinsic permeability of the scaffolds decreased from 60×10^{-10} m^2 to approximately 5×10^{-10} m^2 after coating with the micro-porous outer layer. The latter value is still relatively high, indicating that these scaffolds may facilitate sufficient diffusion of nutrients and waste products during cell culturing. The efficiency of SMC seeding determined after 24 h cell adhesion in the scaffolds increased from less than 10% to 43% after coating with the micro-porous outer layer. The cells were homogeneously distributed in the scaffolds and cell numbers increased 60% during culturing for 7 days under stationary conditions. It is concluded that coating of porous tubular PTMC scaffolds with a micro-porous PTMC outer layer facilitates effective cell seeding in these scaffolds.

INRODUCTION

For the treatment of vascular diseases, functional small-diameter blood vessel prostheses are not available. Although synthetic vascular grafts made from Dacron or Teflon perform reasonably well in large-diameter applications, these grafts fail in

small-diameter reconstructions (inner diameter less than 5 mm) due to thrombus formation and intima hyperplasia [1,2].

Tissue engineering is a promising technique to fabricate functional small-diameter arterial replacements. To this end, autologous vascular cells are seeded in biodegradable (tubular) scaffolds which are subsequently cultured in a bioreactor or immediately implanted [3]. The ideal scaffold should be biocompatible, flexible, elastic and biodegradable [4]. To facilitate formation of vascular tissue, the porous scaffolds should provide a three-dimensional space for adhesion and proliferation of cells, allow diffusion of nutrients and metabolic waste products and maintain suitable mechanical properties until maturation of the newly formed tissue [5,6].

In general, porous scaffolds prepared from natural polymers such as collagen and elastin facilitate cell adhesion and proliferation but show deficiencies in terms of mechanical properties [7]. Therefore, synthetic polymers or hybrid structures of natural and synthetic polymers have been applied in vascular tissue engineering [4,8-10]. With regard to synthetic polymers, mainly lactide- and glycolide-based polymers have been used. However, scaffolds prepared from these polymers generally do not meet the required compliance and elasticity for vascular tissue engineering applications [11].

Our approach to engineer small-diameter arteries is based on cell seeding in flexible and elastic tubular poly(trimethylene carbonate) (PTMC) structures. This material shows excellent biocompatibility and enzymatic degradation by surface erosion in vivo [12-14]. Compliant and creep-resistant PTMC networks are obtained by means of gamma irradiation [15] and interconnected pores are formed by particulate leaching [12]. PTMC supports the culturing of human smooth muscle cells (SMCs), endothelial cells (ECs) and mesenchymal stem cells (MSCs). Cell seeding by means of perfusion

of a cell suspension from the lumen through the wall of a tubular scaffold, however, resulted in a low seeding efficiency due to the relatively large pore sizes of around 110 μm [16].

The aim of the present study was to increase the cell seeding efficiency by providing these porous tubular PTMC structures with a micro-porous outer layer. The thickness of this outer layer was optimized in terms of fluid permeability and seeding efficiency. Human smooth muscle cells were cultured in these scaffolds for 7 days under stationary conditions after which histology, cell proliferation and mechanical properties were evaluated.

MATERIALS AND METHODS

Preparation of porous tubular poly(trimethylene carbonate) scaffolds

High molecular weight PTMC (M_w = 8.78 × 10^5 g/mol) was synthesized from trimethylene carbonate (1,3-dioxane-2-one, Boehringer Ingelheim, Germany) as previously described [16]. Glass mandrels (ϕ = 3.0 mm) were dipped in a PTMC solution in chloroform (2.5% w/v) containing homogeneously dispersed NaCl particles (Acros Organics, Belgium) sieved to a size range of 106-250 μm. The polymer to porogen weight ratio was 10:90. The dip-coating process was repeated several times until an outer diameter of 7-8 mm was reached. Subsequently, the coated mandrels were dried at room temperature for 2 to 3 days, vacuum sealed and subjected to ^{60}Co gamma irradiation (25 kGy, Isotron, The Netherlands) to obtain crosslinked structures. Finally, the salt particles were leached out with demineralized water and the scaffolds were removed from the mandrels. To prevent shrinkage and collapse of the pores, the scaffolds were stored in water at 4 ^{o}C.

In order to increase the cell seeding efficiency, the gamma-irradiated salt-containing PTMC-coated mandrels were dipped 1-4 times in a PTMC solution in chloroform (1.5% w/v) containing homogeneously dispersed NaCl particles sieved to a size range of 0-60 μm. The polymer to porogen weight ratio was 30:70. Subsequently, the coated mandrels were dried at room temperature for 1 day, the salt particles were leached out after which the porous tubular PTMC scaffolds with a micro-porous outer layer were stored in water as described above.

Analysis of porous tubular poly(trimethylene carbonate) scaffolds

Cross-sections of the porous tubular PTMC scaffolds were evaluated by means of scanning electron microscopy (SEM). The samples were cut in liquid nitrogen, rinsed with methanol and dried. After coating of the specimens with a gold-platinum layer using a Polaron E5600 sputter-coater, images were taken with a Hitachi S800 field emission scanning electron microscope operating at 6 kV.

The three-dimensional pore structure of the tubular PTMC scaffolds was also visualized by means of micro-computed tomography (micro-CT). Hydrated specimens were scanned at a resolution of 8 μm with a General Electric Explore Locus SP apparatus operating at an X-ray voltage of 80 kV. The pore size distribution and porosity of the scaffolds were determined from the data as well [17-18].

Because of the limited resolution of the micro-CT measurements, the pore size distribution and porosity of the micro-porous outer layer were determined by SEM and gravimetry, respectively. With the NaCl-containing PTMC solution that was used to prepare the micro-porous outer layer as described above, separate tubular micro-porous layers with a thickness of 1 mm were prepared by means of dip-coating and salt leaching. Samples were processed for SEM as described above, after which the

dimensions of 50 pores were measured. The porosity in the hydrated state was determined gravimetrically according to equation 1 using samples of 5x5x1 mm:

$$\text{Porosity} = 1 - \left(\frac{W_{dry}}{\rho_{PTMC}}\right) / V_{wet} \qquad \text{Equation 1}$$

Here W_{dry} = weight of the structure in dry condition, V_{wet} = volume of the structure in wet condition and ρ_{PTMC} = density of PTMC (1.31 g/cm^3).

The permeabilities of the tubular PTMC scaffolds with and without a micro-porous outer layer were determined by measuring the flow rate of phosphate-buffered saline (PBS) during perfusion through the wall of a scaffold at known pressures [19]. One end of a tubular PTMC scaffold was connected to a reservoir by means of a silicone tube, the other end of the scaffold was closed. Subsequently, the reservoir was filled with PBS, pressurized at 80, 100 or 120 mmHg and the flow rate of PBS through the scaffold wall was measured. The length of the perfused segments and the inner diameter of the PTMC scaffolds were 10 mm and 3 mm, respectively.

The intrinsic permeability k of the porous tubular PTMC scaffolds was determined according to Darcy's law [20,21]:

$$k = Q.l.\mu/\Delta P.A \quad (m^2) \qquad \text{Equation 2}$$

Here Q = volumetric flow rate (m^3/s), l = wall thickness (m), μ = fluid viscosity (0.001 Pa.s for PBS), ΔP = pressure drop across the specimen (Pa) and A = luminal surface area (m^2).

The tensile properties of hydrated porous tubular scaffolds with a length of 5 mm were measured in the radial direction according to standards of the American National Standards Institute and the Association for the Advancement of Medical Instrumentation (ANSI/AAMI, VP20: 1994, paragraph 8.3). Tensile tests were carried

out using a Zwick Z020 universal tensile testing machine (Ulm, Germany) at room temperature. The tensile testing machine was equipped with a 500 N load cell and was operated at a crosshead speed of 1 mm/min. The specimen deformation was derived from the grip to grip separation. The initial stiffness was determined from the slope of the tensile curve from 2.5% to 5% of strain. Tensile properties of freshly harvested porcine and ovine carotid arteries and cell-seeded scaffolds after 7 days of culturing were determined in the same way.

Seeding and culturing of SMCs in porous tubular PTMC scaffolds

SMCs were isolated from human umbilical veins as previously described [22]. The cells were cultured in gelatin-coated (0.5% w/v) tissue culture polystyrene flasks using Dulbecco's modified Eagle medium (Invitrogen, The Netherlands) containing 20% (v/v) heat-inactivated (30 min, 56 ^{o}C) fetal bovine serum (Lonza, The Netherlands), 50 units/ml penicillin and 50 μg/ml streptomycin. Cell culturing was carried out in a humidified atmosphere at 37 ^{o}C and 5% CO_2 in an incubator. Culture medium was refreshed every 2 or 3 days. When cultures were almost confluent, SMCs were detached with 0.125% (w/v) trypsin/0.05% (w/v) EDTA and subcultured with a split ratio of 1:3 up to 10 passages.

Porous tubular PTMC scaffolds with a length of 2.5 cm were disinfected with 70% (v/v) ethanol, rinsed with PBS and placed overnight in culture medium. SMCs were seeded in a scaffold by perfusing 20 ml SMC suspension in culture medium ($0.3x10^6$ cells/ml) from the lumen through the wall with two syringes connected to both ends of the scaffold. Cell retentions were determined by counting cell numbers in the suspensions after passage through the scaffolds. During the first 2 hrs, the seeded scaffolds were rotated 90 degrees every 10 min to ensure a homogeneous distribution

of cells. Cell culturing was carried out under stationary conditions in the incubator for 1 to 7 days. Culture medium was refreshed every 3 days.

Analysis of cell-seeded scaffolds

PTMC scaffolds seeded with SMCs and cultured up to 7 days were rinsed with PBS, fixed overnight in a 4% (w/v) paraformaldehyde solution and embedded in glycol methacrylate. Subsequently, transverse sections with a thickness of 5 μm were cut, stained with hematoxylin and eosin and observed by light microscopy.

Numbers of SMCs present in the constructs were quantified by means of the CyQuant cell proliferation assay (Molecular Probes, The Netherlands) [23]. After culturing up to 7 days, constructs were rinsed with PBS, tubular samples with a length of 7 mm were cut and digested with 200 μl proteinase K solution (1 mg/ml) for 16 h at 56 ^{o}C. Subsequently, the solutions were diluted various times with cell-lysis buffer (Molecular Probes) containing 1.35 Kunitz units/ml RNAse and incubated for 1 h at room temperature to remove RNA and single-stranded DNA. Finally, the samples were mixed with CyQuant® dye and after 2 min fluorescence was measured with a Victor fluorescence analyzer (Perkin-Elmer, Finland). Excitation and emission wavelengths were 480 and 520 nm, respectively. The measured fluorescence intensities were correlated to cell numbers by means of a calibration curve made from suspensions with known concentrations of SMCs.

RESULTS AND DISCUSSIONS

Recently, we have reported on the fabrication of porous, tubular, flexible and elastic PTMC scaffolds for vascular tissue engineering [16]. NaCl or sugar particles were used as porogen. Because of different size distributions of the NaCl and sugar

particles, scaffolds with average pore sizes of 110 μm and 55 μm were obtained, respectively. Highly flexible structures with porosities of approximately 85% were obtained which could be made creep-resistant by means of crosslinking at 25 kGy gamma irradiation. Initial cell seeding experiments using human SMCs showed cell retentions of approximately 10% for the scaffolds prepared with NaCl particles with much better results for the scaffolds prepared with sugar particles. Cells were seeded by perfusion of a cell suspension from the lumen through the wall of a tubular scaffold which led to higher cell retentions for the sugar-leached scaffolds with smaller pores and lower porosity. In view of the diffusion of nutrients and waste products and space available for cell proliferation, however, the more open structure of the NaCl-leached scaffolds is to be preferred. Therefore, we provided these tubular structures with a micro-porous outer layer.

Glass mandrels were coated with a PTMC layer containing NaCl particles (90 wt%) sieved to a size range of 106-250 μm. After gamma irradiation at 25 kGy, the PTMC-coated mandrels were dipped 1-4 times in a PTMC solution containing NaCl particles (70 wt%) sieved to a size range of 0-60 μm in order to create the micro-porous outer layer. After drying and salt leaching, porous tubular PTMC scaffolds were obtained with a length of 8 cm, an inner diameter of 3 mm and a total wall thickness of 0.9-1.2 mm. The thickness of the micro-porous outer layer varied from 0.1-0.4 mm.

The pore structures of the scaffolds in the hydrated state were analyzed by means of micro-CT. Three-dimensional images of tubular PTMC scaffolds without and with a micro-porous outer layer are shown in Figures 5-1A and B, respectively. Because of the 8 μm resolution of the measurements, the micro-porous outer layer appears almost

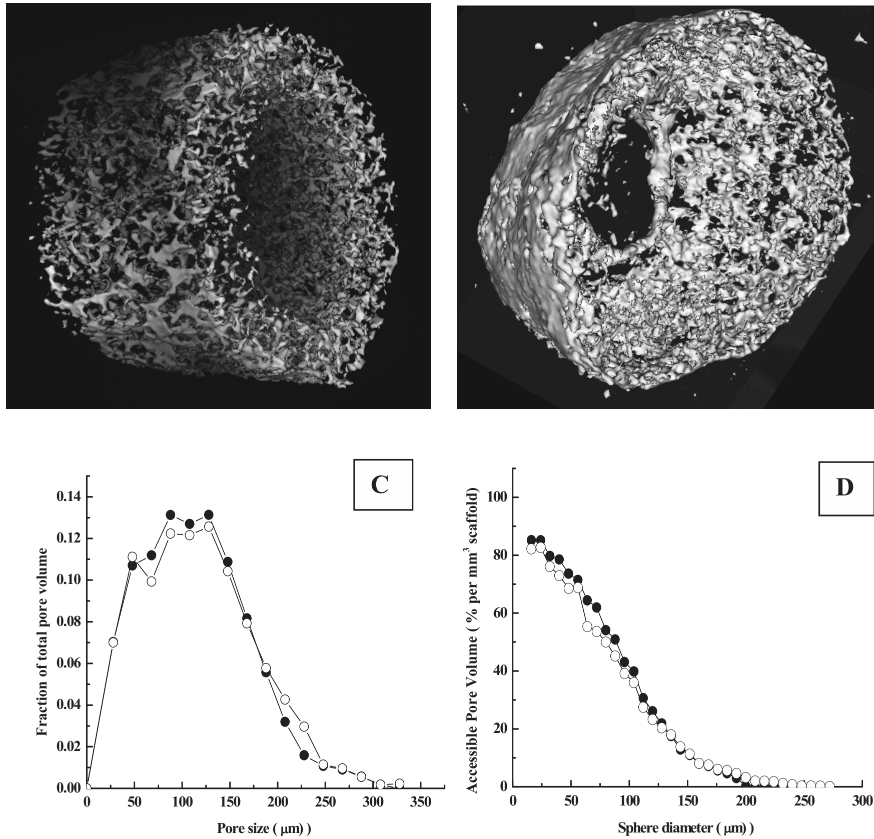

Figure 5-1. Three dimensional images of crosslinked tubular PTMC scaffolds without (A) and with (B) a micro-porous outer layer, their pore size distributions (C) and accessible pore volumes (D) as determined by micro-CT. ●: scaffolds without and ○: with a micro-porous outer layer which was prepared by dipping twice as described in the experimental part.

closed. Pore sizes of both types of scaffold ranged from 0-250 μm with an average of 110 μm (Figure 5-1C) and porosities were approximately 85%. Given the dimensions of human smooth muscle cells in suspension (length 55 μm and diameter 8 μm [24]), Figure 5-1D indicates that approximately 70% of the pore volumes of both types of

scaffold would be available for seeding of smooth muscle cells into the structures. In both cases the surface areas available for adhesion and proliferation of the cells would be greater than approximately 17.5 mm^2 per mm^3 of scaffold (data not shown). It can be concluded that coating of the porous tubular PTMC scaffolds with a micro-porous outer layer does not influence the inner pore structure of the scaffolds to a significant extent.

As shown in Figure 5-2, the micro-porous outer layer was firmly attached to the rest of the porous PTMC structure. By means of SEM, the average pore size of the micro-porous outer layer was determined to be 28±5 μm. Because of shrinkage of the specimens during drying and the vacuum applied during SEM analysis, this value is probably slightly higher in the wet state. Nevertheless, given the dimensions of SMCs in suspension (55 by 8 μm), these sizes indicate the potential of the micro-porous outer layer to increase the cell seeding efficiency. By means of gravimetry, the porosity of the micro-porous outer layer was determined to be 65%.

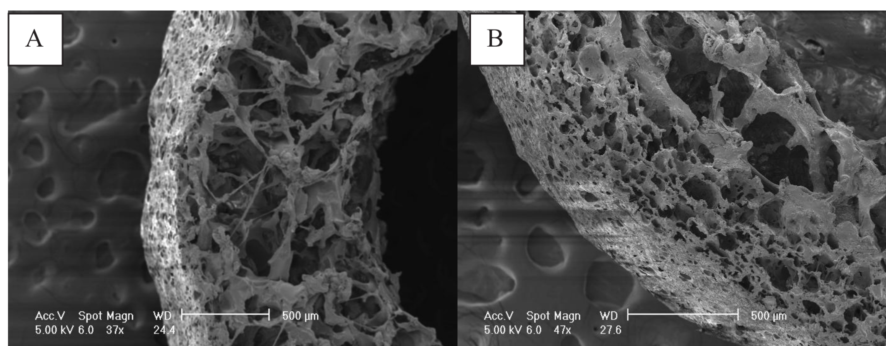

Figure 5-2. SEM images of cross-sections of crosslinked tubular PTMC scaffolds with microporous outer layers prepared by coating 2 times (A) and 4 times (B). Scale bars are 500 μm.

The mechanical properties of tubular PTMC scaffolds without and with micro-porous outer layers varying in thickness were investigated by means of tensile testing of hydrated samples in the radial direction. As shown in Table 5-1, the initial stiffness and maximum tensile strength of the scaffolds in the radial direction tended to increase with increasing thickness of the micro-porous outer layer. The differences, however, were not significant. In all cases, highly flexible scaffolds were obtained with initial stiffnesses of 1.3-1.4 MPa, maximum tensile strengths of 0.15-0.19 MPa and very high elongations at break of approximately 1500%. The cell and extracellular matrix-containing arteries were stronger but less extensible than the porous tubular PTMC scaffolds. Coating of the porous tubular PTMC scaffolds with a micro-porous outer layer does not influence the mechanical properties of the scaffolds

Table 5-1. Tensile properties, determined in the radial direction, of porous tubular PTMC scaffolds and porcine and ovine carotid arteries (n = 3, ± s.d.).

Sample code	Wall thickness (mm)	Initial stiffness (MPa)	Maximal tensile strength (MPa)	Elongation at break
Porcine carotid artery[a]	0.76 ± 0.06	3.89 ± 0.33	1.55 ± 0.10	207 ± 16
Ovine carotid artery[b]	0.45 ± 0.05	1.21± 0.14	1.65 ± 0.08	255 ± 21
Scaffold without outer layer[c]	0.92 ± 0.19	1.32 ± 0.28	0.15 ± 0.02	1644 ± 198
Scaffold with outer layer (dipped 1 time)	0.98 ± 0.12	1.32 ± 0.30	0.15 ± 0.03	1376 ± 181
Scaffold with outer layer (dipped 2 times)	1.02 ± 0.10	1.37 ± 0.39	0.16 ± 0.03	1628 ± 129
Scaffold with outer layer (dipped 4 times)	1.12 ± 0.12	1.42 ± 0.38	0.19 ± 0.03	1527 ± 133

[a]inner diameter 3.1 mm, [b]inner diameter 2.4 mm, [c]all scaffolds inner diameter 3.0 mm.

to a significant extent. The permeability of a fluid through a porous structure is dependent on structural parameters such as porosity, pore size, interconnectivity and pore orientation. A high permeability may result in a good diffusion of nutrients and metabolic waste products through the structure, which are prerequisites for successful tissue engineering. The intrinsic permeability describes the porous structure independent of the sample size and the fluid used [25].

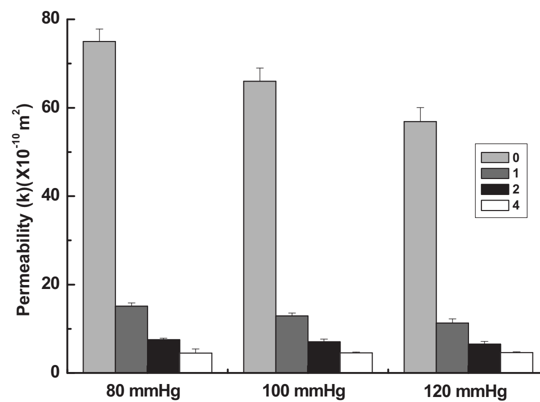

Figure 5-3. Intrinsic permeability (k) of porous tubular PTMC scaffolds with or without micro-porous outer layer, determined at 80, 100 and 120 mmHg (n = 3, ± s.d.). 0, 1, 2, 4 refer to PTMC scaffolds without outer layer and with micro-porous outer layers prepared by dipping 1, 2 and 4 times, respectively.

As shown in Figure 5-3, the intrinsic permeability of the porous tubular PTMC scaffolds substantially decreased after coating of the scaffolds with a micro-porous outer layer and further decreased with increasing thickness of this layer. At pressures of 80-120 mmHg, the intrinsic permeabilities of all tested scaffolds were higher than 4.5×10^{-10} m^2. In other tissue engineering applications, intrinsic scaffold permeabilities in the order of 10^{-17} m^2 (decellularized small-intestinal mucosa for

cartilage regeneration [26]), 10^{-13} m^2 (collagen-based scaffolds for bone regeneration [25]) and 10^{-11} m^2 (lactide-based scaffolds for vascular tissue engineering [27]) have been used. Thus, compared to other tissue engineering scaffolds, our porous tubular PTMC scaffolds show high intrinsic permeability values even after coating with a micro-porous outer layer.

SMCs were seeded in porous tubular PTMC scaffolds with or without a micro-porous outer layer by perfusion of a cell suspension from the lumen through the wall with two syringes connected to both ends of the scaffold. As shown in Table 5-2, the cell retention in the scaffold as well as the time needed for seeding increased with increasing thickness of the micro-porous outer layer. Based on these data it was decided to carry out further experiments with scaffolds provided with a micro-porous outer layer prepared by dipping two times.

Table 5-2. Seeding times and cell retentions in porous tubular PTMC scaffolds with or without a micro-porous outer layer (n = 3, ± s.d.).

Sample code	Seeding time (min)[a]	Cell retention directly after seeding (%)
Scaffold without outer layer	10 ± 2	<10
Scaffold with outer layer (dipped 1 time)	12 ± 3	25 ± 5
Scaffold with outer layer (dipped 2 times)	15 ± 3	63 ± 12
Scaffold with outer layer (dipped 4 times)	32 ± 4	78 ± 8

[a]Time required to perfuse 20 ml SMC suspension through the wall of a porous tubular PTMC scaffold by applying gentle manual pressure.

To investigate the distribution of seeded SMCs in the scaffolds, transverse sections were made of constructs cultured for 1 day under stationary conditions and

subsequently stained with hematoxylin and eosin. As shown in Figure 5-4A, the cells were homogeneously distributed throughout the wall of the tubular PTMC scaffolds. After culturing for 7 days, substantially more cellular material could be observed (Figure 5-4B).

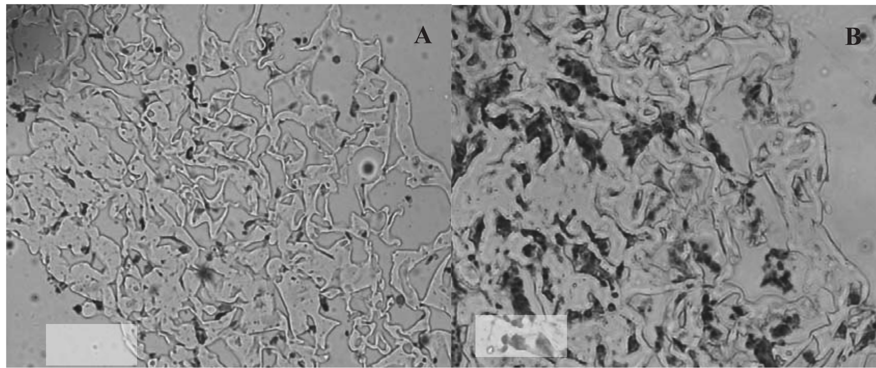

Figure 5-4. Histological analysis of SMC-seeded porous tubular PTMC scaffolds, provided with a micro-porous outer layer prepared by dipping two times, cultured under stationary conditions for 1 day (A) and 7 days (B). Magnification 100x.

The seeding efficiency determined 24 h after cell adhesion as well as cell proliferation were quantified by means of the CyQuant assay. In tubular PTMC scaffolds with a length of 2.5 cm, 6.3×10^6 SMCs were seeded. After 1 day, 2.7×10^6 cells/scaffold were determined indicating a seeding efficiency of 43%. Cell retention directly after seeding for this type of scaffold was 63% (see Table 5-2). Apparently, not all SMCs which were retained inside the porous structure adhered properly. After culturing for 7 days, cell numbers had increased to 4.3×10^6 cells/scaffold indicating moderate proliferation of the seeded SMCs. Culturing the constructs under dynamic conditions in a bioreactor will further increase cell proliferation rates [23].

Tensile testing of the SMC-seeded scaffolds cultured for 7 days under stationary conditions showed that the maximum tensile strength had increased from 0.16 MPa (unseeded scaffold) to 0.38 MPa, which was accompanied by a decrease of the elongation at break from 1628% to 1008%. The improved tensile properties of the constructs were likely due to the presence of SMCs within the porous structure and deposition of extracellular matrix proteins.

CONCLUSIONS

To increase the efficiency of cell seeding in highly porous tubular PTMC scaffolds for vascular tissue engineering, the structures were coated with a micro-porous PTMC outer layer. Coating of the scaffolds with the micro-porous outer layer did not influence the inner pore structure as well as the mechanical properties of the scaffolds to a significant extent. Although the intrinsic permeability of the scaffolds decreased after coating with the micro-porous outer layer, permeability values remained relatively high. Coating with the micro-porous outer layer significantly increased the efficiency of SMC seeding in the scaffolds. The cells were homogeneously distributed and proliferated during culturing for 7 days under stationary conditions. It is concluded that coating of porous tubular PTMC scaffolds with a micro-porous PTMC outer layer facilitates effective cell seeding in these scaffolds.

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6 | Dynamic Culturing of Smooth Muscle Cells in Tubular Poly(trimethylene carbonate) Scaffolds for Vascular Tissue Engineering

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ABSTRACT

Porous, tubular, flexible and elastic poly(trimethylene carbonate) (PTMC) scaffolds (length 8 cm, inner diameter 3 mm) for vascular tissue engineering were prepared by means of a dip-coating and particulate leaching procedure. Using NaCl as porogen, scaffolds with an average pore size of 110 μm and a porosity of approximately 85% were obtained. Before leaching the salt, the structures were made creep-resistant by means of crosslinking at 25 kGy gamma irradiation. To increase the efficiency of cell seeding, the scaffolds were provided with a micro-porous outer layer of 0.2 mm with an average pore size of 28 μm and a porosity of around 65% (total wall thickness 1 mm). Human smooth muscle cells (SMCs) were seeded in these scaffolds with an efficiency of approximately 45%, as determined after 24 h cell adhesion.

One day after cell seeding, culturing of the constructs was continued up to 14 days under stationary conditions or under pulsatile flow conditions in a bioreactor (pressure 70-130 mmHg, 69 pulsations/min, average wall shear rate 320 s^{-1}). Although SMCs proliferated under both conditions, cell numbers were 3-5 times higher in case of dynamic culturing. This was qualitatively confirmed by means of histology.

Also in terms of mechanical properties, the dynamically cultured constructs performed better than the statically cultured constructs. After culturing for 14 days, the maximum tensile strengths of the constructs, determined in the radial direction, had increased from 0.16 MPa (unseeded scaffold) to 0.48 MPa (dynamic culturing) and 0.38 MPa (static culturing). The results indicate that suitable constructs for vascular tissue engineering can be prepared by dynamic culturing of human SMCs seeded in porous tubular PTMC scaffolds.

INTRODUCTION

(Cardio) vascular disease is the leading cause of death in the Western society, claiming half a million lives every year in the United States alone [1]. For small-diameter arterial reconstructions (inner diameter less than 6 mm), functional blood vessel prostheses are still not available to date.

Tissue engineering is a promising technique to prepare functional small-diameter arterial replacements. To this end, autologous vascular cells are seeded in biodegradable (tubular) scaffolds and subsequently cultured in a bioreactor or immediately implanted [2]. Amongst others, Niklason et al. [3] implanted bioreactor-cultured blood vessel equivalents based on poly(glycolic acid), smooth muscle cells and endothelial cells in experimental animals. Although the constructs remained patent for several weeks, the mechanical properties of these grafts are considered to be insufficient for implantations in humans [4]. Shin'oka et al. [5] implanted porous scaffolds (patches or tubes), prepared from a copolymer of L-lactide and ε-caprolactone, directly after seeding with autologous mononuclear bone marrow cells in the pulmonary artery of 1-24 year old patients with a congenital defect. Follow-up during 30 months showed no complications and all arteries remained patent. However, the pulmonary artery is a relatively large-diameter low-pressure artery, indicating that additional research is necessary for replacement of arteries in the systemic circulation. L'Heureux et al. introduced the concept of cell sheet-based tissue engineering without the use of a polymer scaffold [6]. Several layers of fibroblast sheets are wound around a mandrel and subsequently matured. After removal of the mandrel, the luminal surface is seeded with endothelial cells. Although these grafts show good mechanical properties and perform well as arterio-venous fistulas in humans, the main draw-back of this approach is the long preparation time of 6-9 months [4].

Our vascular tissue engineering approach is based on cell seeding in flexible and elastic tubular poly(trimethylene carbonate) (PTMC) structures [7]. This material shows excellent biocompatibility and enzymatic degradation by surface erosion in vivo [8-10]. Compliant and creep-resistant PTMC networks are obtained by means of gamma irradiation [11] and interconnected pores are formed by particulate leaching [12]. To prepare the medial layer of the vascular graft, human smooth muscle cells (SMCs) are seeded by perfusion of a cell suspension from the lumen (inner diameter 3 mm) through the wall of a tubular scaffold [7]. To increase the cell seeding efficiency, the scaffolds are provided with a thin micro-porous outer layer (0.2 mm, total wall thickness 1.0 mm) [13].

Mechanical stimulation of SMCs seeded in vascular tissue engineering scaffolds, promotes the circumferential orientation of the cells as well as the deposition of extracellular matrix [14,15]. SMCs can be subjected to cyclic mechanical strain by pulsatile perfusion of culture medium through the scaffold. Long-term perfusion of our tubular PTMC scaffolds in a pulsatile flow system mimicking physiological conditions (average wall shear rate 590 s^{-1}, pressure 80-120 mmHg, 70 pulsations/min), showed that the scaffolds are able to withstand the pressure pulses and are completely creep-resistant. Moreover, the compliance of the scaffolds is comparable to that of native carotid arteries [16]. The aim of the present study was to subject SMCs seeded in the tubular PTMC scaffolds to cyclic mechanical strain in a pulsatile flow bioreactor in order to evaluate the histology, cell proliferation and mechanical properties of the constructs up to14 days of culturing.

MATERIALS AND METHODS

Preparation of porous tubular poly(trimethylene carbonate) scaffolds

High molecular weight PTMC (M_w = 8.78 × 10^5 g/mol) was synthesized from trimethylene carbonate (1,3-dioxane-2-one, Boehringer Ingelheim, Germany) as previously described [7]. Glass mandrels (ϕ = 3.0 mm) were dipped in a PTMC solution in chloroform (2.5% w/v) containing homogeneously dispersed NaCl particles (Acros Organics, Belgium) sieved to a size range of 106-250 μm. The polymer to porogen weight ratio was 10:90. The dip-coating process was repeated several times until an outer diameter of 7-8 mm was reached. Subsequently, the coated mandrels were dried at room temperature for 2 to 3 days, vacuum sealed and subjected to ^{60}Co gamma irradiation (25 kGy, Isotron, The Netherlands) to obtain crosslinked structures. Finally, the salt particles were leached out with demineralized water and the scaffolds were removed from the mandrels. To prevent shrinkage and collapse of the pores, the scaffolds were stored in water at 4 °C.

In order to increase the cell seeding efficiency, the gamma-irradiated salt-containing PTMC-coated mandrels were dipped 2 times in a PTMC solution in chloroform (1.5% w/v) containing homogeneously dispersed NaCl particles sieved to a size range of 0-60 μm. The polymer to porogen weight ratio was 30:70. Subsequently, the coated mandrels were dried at room temperature for 1 day, the salt particles were leached out after which the porous tubular PTMC scaffolds with a micro-porous outer layer were stored in water as described above.

Analysis of porous tubular poly(trimethylene carbonate) scaffolds

Cross-sections of the porous tubular PTMC scaffolds were evaluated by means of scanning electron microscopy (SEM). The samples were cut in liquid nitrogen, rinsed

with methanol and dried. After coating of the specimens with a gold-platinum layer using a Polaron E5600 sputter-coater, images were taken with a Hitachi S800 field emission scanning electron microscope operating at 6 kV. Specimens cut from cell-seeded scaffolds cultured up to 14 days were dried using a series of incremental ethanol solutions (60-100%), sputter-coated and evaluated in the same way.

The tensile properties of hydrated porous tubular scaffolds with a length of 5 mm were measured in the radial direction according to standards of the American National Standards Institute and the Association for the Advancement of Medical Instrumentation (ANSI/AAMI, VP20: 1994, paragraph 8.3). Tensile tests were carried out using a Zwick Z020 universal tensile testing machine (Ulm, Germany) at room temperature. The tensile testing machine was equipped with a 500 N load cell and was operated at a crosshead speed of 1 mm/min. The specimen deformation was derived from the grip to grip separation. The initial stiffness was determined from the slope of the tensile curve from 2.5% to 5% of strain. Tensile properties of porcine carotid artery, human arteria mesenterica inferior and cell-seeded scaffolds cultured up to 14 days were determined in the same way.

Seeding and culturing of smooth muscle cells in porous tubular PTMC scaffolds

Smooth muscle cells (SMCs) were isolated from human umbilical veins as previously described [17]. The cells were cultured in gelatin-coated (0.5% w/v) tissue culture polystyrene flasks using Dulbecco's modified Eagle medium (Invitrogen, The Netherlands) containing 20% (v/v) heat-inactivated (30 min, 56 °C) fetal bovine serum (Lonza, The Netherlands), 50 units/ml penicillin and 50 μg/ml streptomycin. Cell culturing was carried out in a humidified atmosphere at 37 °C and 5% CO_2 in an incubator. Culture medium was refreshed every 2 or 3 days. When cultures were

almost confluent, SMCs were detached with 0.125% (w/v) trypsin/0.05% (w/v) EDTA and subcultured with a split ratio of 1:3 up to 10 passages.

Porous tubular PTMC scaffolds with a length of 4 cm were disinfected with 70% (v/v) ethanol, rinsed with PBS and placed overnight in culture medium. SMCs were seeded in a scaffold by perfusing 20 ml SMC suspension in culture medium ($0.5x10^6$ cells/ml) from the lumen through the wall with two syringes connected to both ends of the scaffold. During the first 2 hrs, the seeded scaffolds were rotated 90 degrees every 10 min to ensure a homogeneous cell distribution. The cells were allowed to adhere to the scaffolds for 1 day. Subsequently, cell culturing was continued under stationary conditions in the incubator or under dynamic conditions in a bioreactor (see below) for 7 or 14 days. Culture medium was refreshed every 3 days.

Pulsatile flow bioreactor

The SMC-seeded tubular PTMC scaffolds were cultured under dynamic conditions in a Bose-Electroforce pulsatile flow bioreactor (Figure 6-1). Three culture chambers, each containing one construct, were operated in parallel. Culture media were independently perfused through the chambers using three gear pumps (G1 in Figure 6-1). Pulses were generated by a linear displacement pump (Dynamic pump in Figure 6-1), which was shared between the culture chambers. The shape of the intraluminal pressure profile and other flow conditions were programmed with WinTest software. The bioreactor was placed in a 5% CO_2 incubator operating at 37 $^{\circ}$C.

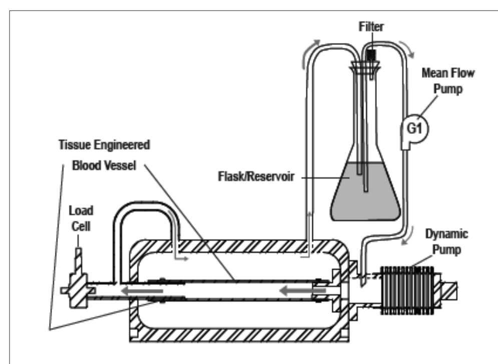

Figure 6-1. Schematic representation of the Bose-Electroforce pulsatile flow bioreactor. The perfusion of culture medium is shown through one culture chamber containing a cell-seeded tubular scaffold.

Analysis of cell-seeded scaffolds

PTMC scaffolds seeded with SMCs and cultured up to 14 days were rinsed with PBS. Transverse sections of 2 mm were cut, fixed overnight in a 4% (w/v) paraformaldehyde solution, rinsed with demi-water and incubated for 1 h in a 1% methylene blue solution. Subsequently, the samples were rinsed with demi-water until the water was clear and evaluated using a stereomicroscope. Alternatively, samples were fixed as described above and embedded in glycol methacrylate. Transverse sections with a thickness of 5 μm were cut, stained with hematoxylin and eosin and observed by light microscopy.

Numbers of SMCs present in the constructs were quantified by means of the CyQuant cell proliferation assay (Molecular Probes, The Netherlands) [18]. After culturing up to 14 days, constructs were rinsed with PBS, tubular samples were cut with a length of 7 mm and digested with 200 μl proteinase K solution (1 mg/ml) for 16 h at 56 °C.

Subsequently, the solutions were diluted various times with cell-lysis buffer (Molecular Probes) containing 1.35 Kunitz units/ml RNAse and incubated for 1 h at room temperature to remove RNA and single-stranded DNA. Finally, the samples were mixed with CyQuant® dye and after 2 min fluorescence was measured with a Victor fluorescence analyzer (Perkin-Elmer, Finland). Excitation and emission wavelengths were 480 and 520 nm, respectively. The measured fluorescence intensities were correlated to cell numbers by means of a calibration curve made from suspensions with known concentrations of SMCs.

RESULTS AND DISCUSSIONS

As recently reported, porous, tubular, flexible and elastic PTMC scaffolds for vascular tissue engineering were prepared by means of a dip-coating and particulate leaching procedure [7]. Using NaCl as porogen, scaffolds with an average pore size of 110 µm and a porosity of approximately 85% were obtained, which were made creep-resistant by means of crosslinking at 25 kGy gamma irradiation. The length of the tubular scaffolds was 8 cm, the inner diameter 3 mm and the wall thickness 0.8 mm. To increase the efficiency of cell seeding, the scaffolds were provided with a micro-porous outer layer of 0.2 mm with an average pore size of 28 µm and a porosity of around 65%. As shown in Figure 6-2, the porous inner and outer layers are firmly attached to each other. The efficiency of seeding human SMCs in these scaffolds is approximately 45%, as determined after 24 h cell adhesion [13].

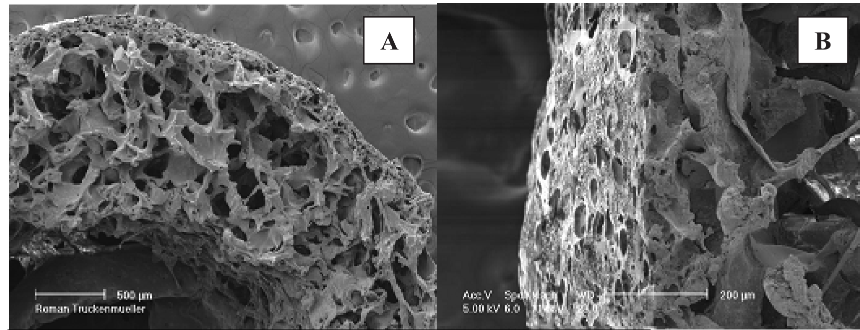

Figure 6-2. SEM images of cross-sections of tubular PTMC scaffolds provided with a micro-porous outer layer.

In this study, we used a Bose-Electroforce pulsatile flow bioreactor to culture the SMC-seeded tubular PTMC scaffolds under dynamic conditions (Figure 6-3A). The intraluminal pressure waveform mimicked that of the human carotid artery (Figure 6-3B). In the SMC-seeded tubular PTMC scaffolds, the intraluminal pressure ranged from 70-130 mmHg, the number of pulsations was 69/min and the flow rate was 50 ml/min, corresponding to an average wall shear rate of 320 s^{-1}. These values fit within the range found in human carotid arteries [19, 20].

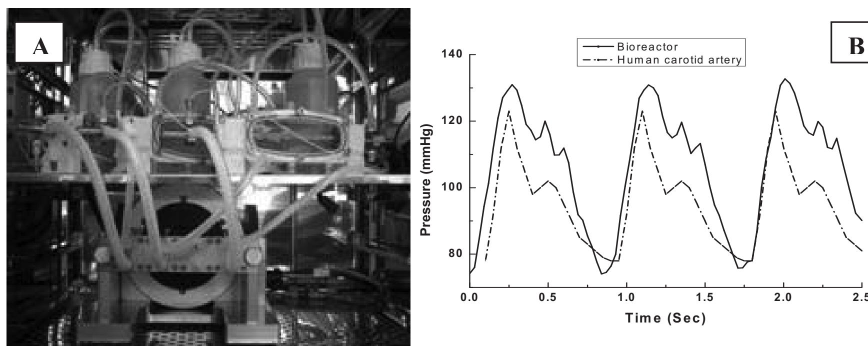

Figure 6-3. A: The Bose-Electroforce pulsatile flow bioreactor placed in an incubator. B: Intraluminal pressures in the SMC-seeded tubular PTMC scaffolds (—) and the human carotid artery (- -) [21].

After seeding of human SMCs by perfusion of a cell suspension from the lumen through the wall of a tubular PTMC scaffold, the constructs were cultured up to 14 days under stationary or dynamic conditions. In contrast to the former conditions, dynamic culture conditions resulted in a "tissue-like" appearance of the constructs after 14 days (Figure 6-4).

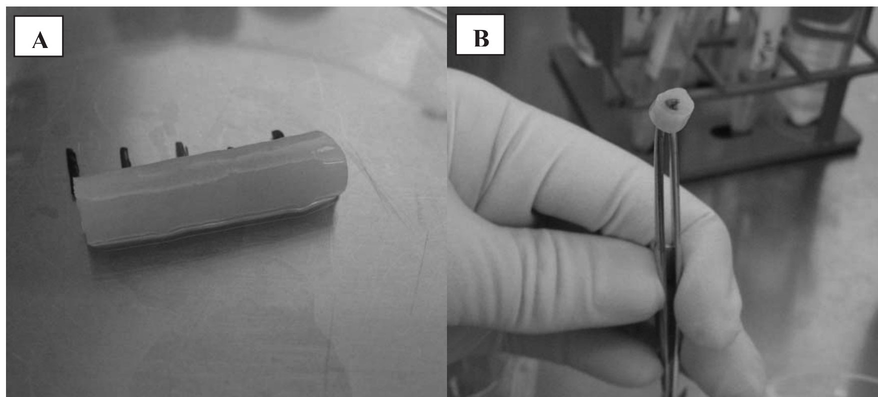

Figure 6-4. A: Overview of a 4 cm long SMC-seeded tubular PTMC scaffold cultured under dynamic conditions for 14 d. B: Sample cut from this construct for further analysis.

During the first week of culturing, the seeded SMCs proliferated both under stationary and dynamic conditions (Figure 6-5). Cell numbers were significantly higher in case of dynamic culturing, indicating that improved transport of nutrients and waste products and/or cyclic mechanical strain stimulates SMC proliferation. Cell numbers after 1 day cell adhesion corresponded to a seeding efficiency of 43%.

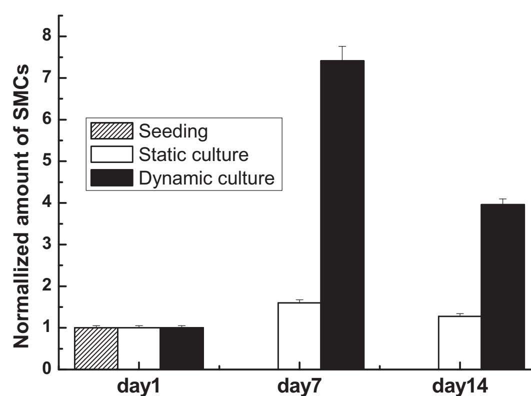

Figure 6-5. Numbers of SMCs present in the tubular PTMC scaffolds during culturing, normalized to the amount of cells present on day 1. Cell numbers were determined with the CyQuant cell proliferation assay (n = 3, ± s.d.).

During the second week of culturing, SMC numbers decreased both under stationary and dynamic conditions (Figure 6-5). Cell numbers were still significantly higher in case of dynamic culturing. Possibly, increased cell mass and/or pore occlusion resulting from SMC proliferation adversely affected the diffusion of nutrients and waste products [22, 23]. These data indicate that this approach may benefit from an adventitial-like layer containing small channels or capillaries protruding into the medial layer, facilitating sufficient transport of nutrients and waste products [24].

Methylene blue staining of cross-sections of SMC-seeded tubular PTMC scaffolds showed homogeneous cell adhesion in the scaffolds after 1 day of culturing under stationary conditions (Figure 6-6B). Moreover, methylene blue staining confirmed significant SMC proliferation after 7 and 14 days of culturing under pulsatile flow conditions (Figure 6-6C, D).

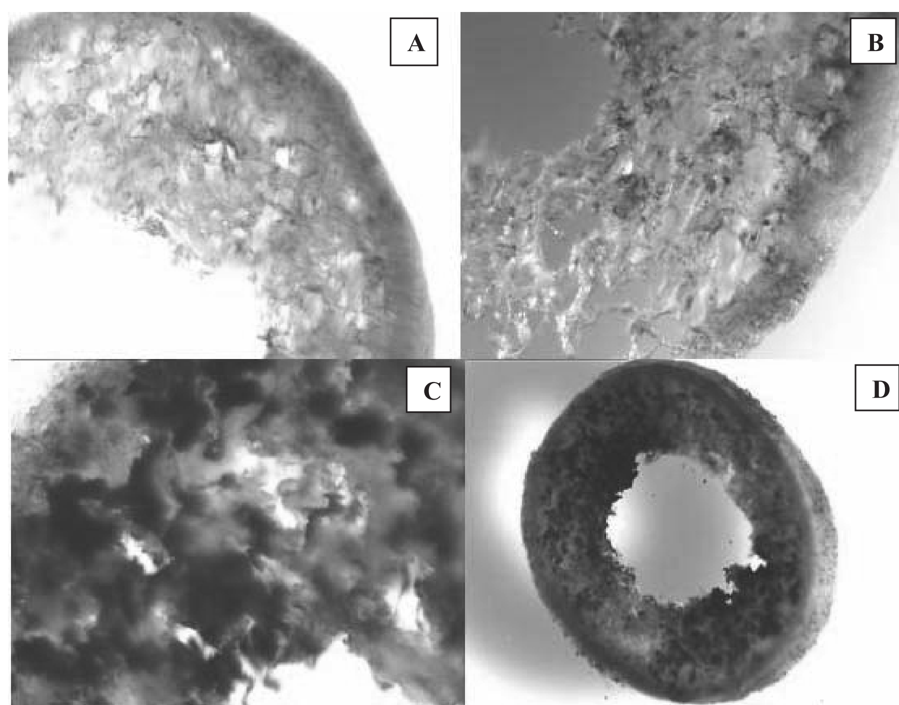

Figure 6-6. Stereomicroscopic images of cross-sections of tubular PTMC scaffolds stained with methylene blue. A: Control without cells, B-D: SMC-seeded and cultured for 1 day under stationary conditions (B), 7 days under dynamic conditions (C) and 14 days under dynamic conditions (D).

Homogeneous adhesion of SMCs in the tubular PTMC scaffolds one day after seeding was confirmed by means of histological staining with hematoxylin and eosin (Figure 6-7A). Subsequent culturing under stationary conditions until day 7 showed SMC proliferation predominantly on the adventitial side (outside) of the scaffolds (Figure 6-7B). In contrast, culturing under pulsatile flow conditions resulted in a more homogeneous cell distribution, although some parts of the scaffolds contained more cells than other parts (Figure 6-7C, D). After 7 days of culturing under dynamic

conditions, SMCs could be observed on the luminal side of the constructs by means of SEM (Figure 6-7E,F).

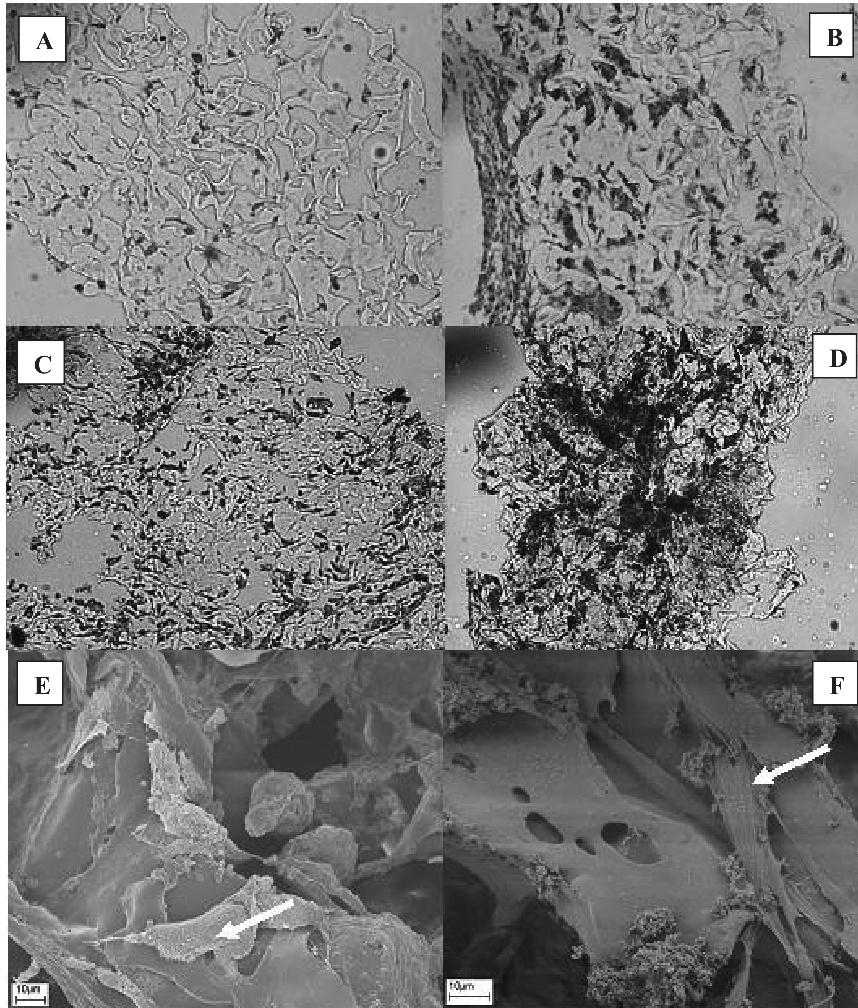

Figure 6-7. A-D: Hematoxylin and eosin staining of cross-sections of SMC-seeded tubular PTMC scaffolds cultured under stationary conditions for 1 day (A) and 7 days (B) and under dynamic conditions for 7 days (C, D). Magnification 100x. E,F: SEM images of the luminal sides of constructs cultured for 7 days under dynamic conditions. Arrows indicate SMCs, scale bars 10 µm.

The mechanical properties of SMC-seeded tubular PTMC scaffolds cultured up to 14 days were investigated by means of tensile testing of freshly recovered samples in the radial direction. The maximum tensile strengths of constructs cultured for 7-14 days under stationary conditions were higher than that of hydrated scaffolds without cells (Figure 6-8). The highest tensile strengths were measured with constructs cultured for 7-14 days under dynamic conditions. With increasing tensile strength, the elongation at break decreased.

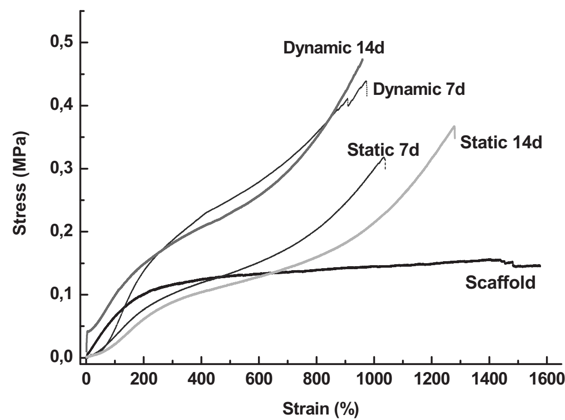

Figure 6-8. Tensile properties (determined in the radial direction) of SMC-seeded tubular PTMC scaffolds cultured up to 14 days under static or dynamic conditions. Scaffolds without cells were used as reference.

Combining this data with the histological data, it can be concluded that during the first week of culturing the tensile properties of the constructs increased with increasing numbers of SMCs present in the constructs. The decrease in cell numbers from day 7 to 14, as determined with the CyQuant assay, did not adversely affect the tensile properties of the constructs, indicating that the cells had deposited extracellular matrix proteins, which mainly determine the mechanical properties of the constructs.

In Table 6-1, the mechanical properties of the constructs cultured under pulsatile flow conditions are summarized and compared to those of porcine carotid artery and human arteria mesenterica enferior. In addition to the data discussed above, Table 6-1 shows that the initial stiffness of the SMC-seeded tubular PTMC scaffolds tended to decrease somewhat upon dynamic culturing. Probably, the PTMC was degraded to some extent. As shown in Figure 6-8, the stress-strain curves of the constructs display a toe-region at low strain values, which may also be an indication for the presence of extracellular matrix. The toe-region, which is also present in stress-strain curves of native blood vessels and tendons, is characteristic for the removal of macroscopic crimp in collagen fibres [25, 26].

Table 6-1. Mechanical properties of porcine carotid artery, human arteria mesenterica inferior and SMC-seeded tubular PTMC scaffolds cultured under dynamic conditions (n = 3, ± s.d., except human arteria mesenterica inferior n = 1).

	Culture time (d)	**Thickness (mm)**	**Inner diameter (mm)**	**Initial stiffness (MPa)**	**Maximal strength (MPa)**	**Elongation at rupture (%)**
Porcine carotid artery	----	0.76 ± 0.06	3.03 ± 0.57	3.89 ± 0.33	1.55 ± 0.21	207 ± 16
Human arteria mesenterica inferior	----	0.44	2.65	5.7	1.89	345
SMC-seeded PTMC scaffolds	0	1.02± 0.10	3.23 ± 0.03	1.37 ± 0.39	0.16 ± 0.03	1628 ± 129
	1	1.08 ± 0.06	3.24 ± 0.05	1.36 ± 0.35	0.16 ± 0.03	1588 ± 143
	7	1.10 ± 0.06	3.32 ± 0.09	1.02 ± 0.32	0.44 ± 0.09	1130 ± 143
	14	1.11± 0.05	3.39 ± 0.08	1.06 ± 0.29	0.47 ± 0.06	928 ± 211

Table 6-1 also shows that the values of the maximum tensile strength and initial stiffness of the constructs cultured for two weeks under dynamic conditions were

lower than those of native arteries. After seeding with cells, in vivo studies will be performed to investigate if the mechanical properties of the constructs are sufficient for implantation and if maturation of the vascular grafts will subsequently take place.

CONCLUSIONS

Porous tubular PTMC scaffolds were seeded with human SMCs and subsequently cultured up to 14 days under stationary conditions or pulsatile flow conditions in a bioreactor. Both determination of cell numbers and histology showed that dynamic culture conditions significantly improved SMC proliferation, as compared to stationary culture conditions. Also in terms of mechanical properties, the dynamically cultured constructs performed better. The results indicate that suitable constructs for vascular tissue engineering can be prepared by dynamic culturing of porous tubular PTMC scaffolds seeded with human SMCs.

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7| A preliminary study on the in vivo performance of cell-seeded tubular poly(trimethylene carbonate) scaffolds for vascular tissue engineering

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ABSTRACT

A preliminary implantation study was carried out using porous tubular PTMC scaffolds, either unseeded or seeded with human mesenchymal stem cells (MSCs), as partial replacement of the rat abdominal aorta. The study showed that due to a mismatch in wall thickness of the tubular PTMC scaffolds and the rat abdominal aorta, end-to-end suturing was problematic. Therefore, two poly(propylene) connectors were used to connect a scaffold to the aorta. Unseeded scaffolds showed extensive leakage of blood upon implantation. However, MSC-seeded scaffolds showed minor leakage which stopped a few minutes after restoration of blood flow. The grafts remained patent until the end of the experiment, 4 h after implantation. It is concluded that for long-term experiments, another suturing technique has to be used. To prevent blood loss, the PTMC scaffolds have to be seeded with cells.

INTRODUCTION

In the previous chapters we have reported on the preparation of porous tubular poly(trimethylene carbonate) (PTMC) scaffolds for vascular tissue engineering [1-4]. The scaffolds are compliant and creep-resistant and show good performance during long-term perfusion in a pulsatile flow system mimicking physiological conditions. The efficiency of cell seeding can significantly be improved by providing the scaffolds with a micro-porous PTMC outer layer. Human smooth muscle cells (SMCs) are able to adhere and proliferate on the PTMC material. SMCs seeded in the tubular PTMC scaffolds and subsequently cultured in a pulsatile flow bioreactor, show improved proliferation as compared to cells cultured under stationary conditions. Moreover, the tensile properties of constructs cultured under dynamic

conditions are better than those of cell-seeded scaffolds cultured under stationary conditions.

In view of these promising results, it was decided to initiate in vivo experiments. As animal model, partial replacement of the abdominal aorta of athymic rats was used. Athymic rats are immuno-deficient, which allows implantation of scaffolds seeded with human cells. Given the dimensions of the rat abdominal aorta, porous tubular PTMC scaffolds with an inner diameter of 1.5 mm were prepared by means of dip-coating and NaCl salt leaching. The architecture and thickness of the scaffold wall were the same as described in previous chapters [3, 4]. The aim of the present study was to investigate the handling, suturability and possible leakage of the porous tubular PTMC scaffolds upon implantation. For this initial study, MSCs were chosen as cell type to seed the scaffolds. Although the approach of first seeding SMCs and after some time endothelial cells (ECs) is still valid, the procedure could possibly be shortened because MSCs are able to differentiate into smooth muscle cells as well as endothelial cells [5-8]. Moreover, MSCs have antithrombogenic properties [9]. In the present study, MSC-seeded tubular PTMC scaffolds were implanted 24 h after cell seeding as partial replacement of the rat abdominal aorta. Unseeded scaffolds were implanted as control. The performance of the grafts in terms of patency and leakage was monitored up to 4 hours.

MATERIALS AND METHODS

Using glass mandrels with a diameter of 1.5 mm, porous tubular PTMC scaffolds with a length of 8 cm were prepared as described [3]. The scaffolds were provided with a micro-porous outer layer of 0.2 mm, total wall thickness was 1.0 mm.

MSCs were isolated from human bone marrow provided by Medical Spectrum Twente Hospital Group (Dept. of Orthopaedic Surgery), as described by Both et al. [10]. The cells were cultured in αMEM medium (Invitrogen) containing 10% (v/v) fetal bovine serum (Cambrex) in an incubator at 37 ^{o}C/5% CO_2. Confluent cultures were detached with trypsin/EDTA and sub-cultured with a split ratio of 1:3 up to 15 passages.

Porous tubular PTMC scaffolds with a length of 4 cm were disinfected with 70% (v/v) ethanol, rinsed with PBS and placed overnight in culture medium. $10x10^6$ MSCs were seeded in a scaffold by a perfusion technique as described [3]. The cells were allowed to adhere for 24 h in the incubator. The seeding efficiency after 24 h cell adhesion is approximately 45% [3].

The implantations were carried out in the central animal facility at the University of Groningen and were approved by the Institutional Animal Care and Use Committee. Athymic rats (WAG-nude, Harlan, The Netherlands) were anaesthetized with isoflurane/O2 after which the abdominal cavity was opened. The aorta was clamped below the renal arteries and above the bifurcation to the legs, after which a 1 cm segment of the aorta was removed and replaced with a tubular PTMC scaffold, either unseeded or seeded with human MSCs. The animals were not treated with anticoagulant drugs. An end-to-end anastomosis technique was intended to be used. However, due to a mismatch in wall thickness of the scaffold and the artery (see below) they were connected with two polypropylene connectors with an inner diameter of 1.5 mm, outer diameter of 1.9 mm and a length of 5 mm. The two ends of a connector were placed in the lumen of the scaffold and the aorta, respectively, after which two sutures around the scaffold and aorta were tightened. After restoration of

circulation, the performance of the grafts in terms of patency and leakage was monitored up to 4 hours after which the animals were euthanized.

RESULTS AND DISCUSSIONS

Porous tubular PTMC scaffolds with an inner diameter of 1.5 mm were successfully prepared. In order to allow adequate handling of the scaffolds and to prevent collapse of the lumen, the scaffolds were kept in the wet state. A rat abdominal aorta is shown in Figure 7-1. Compared to this artery, the tubular PTMC scaffold wall was substantially thicker. For the short-term experiments described in this study, the mismatch in wall thickness could be solved by using two poly(propylene) connectors. However, for long-term experiments another approach should be used such as the parachute inlay technique, which is also used to suture Dacron and Teflon prostheses to blood vessels in case of a mismatch in wall thickness [11,12].

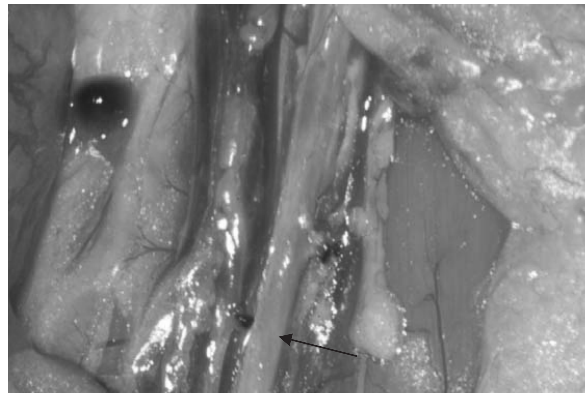

Figure 7-1. The rat abdominal aorta (arrow).

After restoration of circulation, the unseeded PTMC scaffolds showed extensive leakage of blood (Figure 7-2). This could not be stopped by re-clamping the artery to allow the blood to clot.

Figure 7-2. Unseeded PTMC scaffold after implantation. On both sides of the scaffold a tampon is placed to absorb the blood.

The MSC-seeded scaffolds, however, showed minor leakage which stopped after a few minutes (Figure 7-3). Apparently, the pores of the scaffolds were sufficiently filled with cells making the constructs almost impervious to blood. As judged by the presence of blood pulse in the limbs, the grafts remained patent until the end of the experiment, 4 h after implantation.

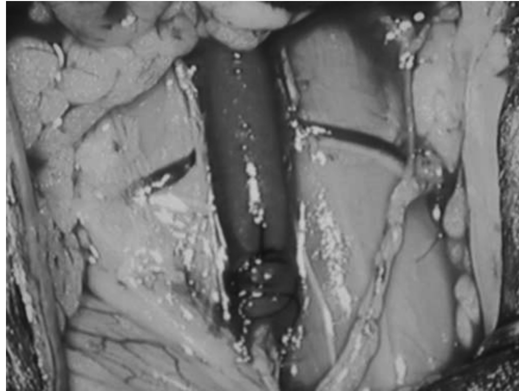

Figure 7-3. MSC-seeded scaffold after implantation.

CONCLUSIONS

This preliminary implantation study showed that due to a mismatch in wall thickness of the tubular PTMC scaffolds and the rat abdominal aorta, end-to-end suturing was problematic. Other techniques, such as the parachute inlay technique, will be evaluated in subsequent experiments. To prevent leakage upon implantation, the porous tubular PTMC scaffolds have to be seeded with cells.

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Poly(trimethylene carbonate) Porous Tubular Structures Made by Electrospinning

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ABSTRACT

Using electro-spinning technique or dip-coating method, porous PTMC tubular structures with different morphological and mechanical properties were prepared. The mechanical strength and elasticity of the porous PTMC structures are superior to those of scaffolds fabricated by dip-coating. The results allow us to construct functional scaffolds for tissue engineered small diameter blood vessels.

INTRODUCTION

Poly(trimethylene carbonate) (PTMC) degrades rapidly in vivo by surface erosion [1]. By gamma irradiation of high molecular weight PTMC, networks with excellent elasticity and creep-resistance can be formed [2]. Smooth muscle cells (SMCs), human umbilical vein endothelial cells (HUVECs), and mesenchymal stem cells (MSCs) attach well on (irradiated) PTMC surfaces. Previously, the applicability of electro-spun poly(TMC-DLLA) was evaluated as blood vessel scaffolds [3]. Based on that, PTMC are interesting scaffolding materials for tissue engineering of vascular grafts.

The aim of this study was to prepare PTMC porous tubular structures by respectively electro-spinning technique or dip-coating method, and to compare the morphological (pore size and porosity) and mechanical properties of these scaffolds. The mechanical properties of the tubular structures were evaluated in both static and pulsatile conditions, and compared with those of sheep and human blood vessels of similar dimensions.

MATERIALS AND METHODS

High molecular weight PTMC (M_n= 6.36 × 10^5, DPI= 1.38) was synthesized by ring-opening polymerization. Porous scaffolds were obtained by electro-spinning of fibers from polymer solutions in a mixture of dichloromethane and trifluoethanol. Alternatively, crosslinked porous tubular scaffolds were prepared by repeatedly dipping glass mandrels (diameter = 3 mm) in polymer solutions (2.5-3.0 wt/vol% in CH_3Cl) containing dispersed, sieved NaCl particles (size range 106-250 µm, sugar to polymer ratio was 90:10) until reaching the required outer diameter (7-8 mm), followed by gamma irradiation (25 kGy) under vacuum, and leaching in demineralized water.

Porosities of the electro-spun and the dip-coated tubular structures were measured by gravimetry. Morphology of the scaffolds was observed by SEM. Tensile properties of the porous tubular scaffolds in the radial direction were determined by stretching the structures with homemade clamps via a Zwick Z020 tensile tester (500N load cell, 1 mm/min) according to standards of the American National Standards Institute and the Association for the Advancement of Medical Instrumentation (ANSI/AAMI VP20: 1994). Native arteries from ovine carotid tissue and from the mesenterica inferior of a patient with similar dimensions were tested as references.

RESULTS AND DISCUSSION

Flexible tubular structures were obtained by both electro-spinning technique and dip-coating method. By electro-spinning technique, thin tubular structures (c.a. 0.3 mm wall thickness) were formed with porosities between 60 and 65 vol%. The morphology of the tubular structures was investigated by SEM (Fig. A-1 A and B):

the surface is smooth; the tubes were composed of fiber-like structures with diameter 1-5 μm, and the pore size ranged from 8 to 10 μm.

When dip-coating at appropriate dipping and rotation speeds, homogeneous coatings of the glass mandrels were obtained. Gamma irradiation followed by leaching of the particles resulted in the formation of crosslinked porous structures. The porosity of the dip-coated porous structures in the wet state was 81.3 ± 2.3 vol%. The porous structures were up to 90 mm in length and had wall thicknesses of 0.8-1.0 mm thick.

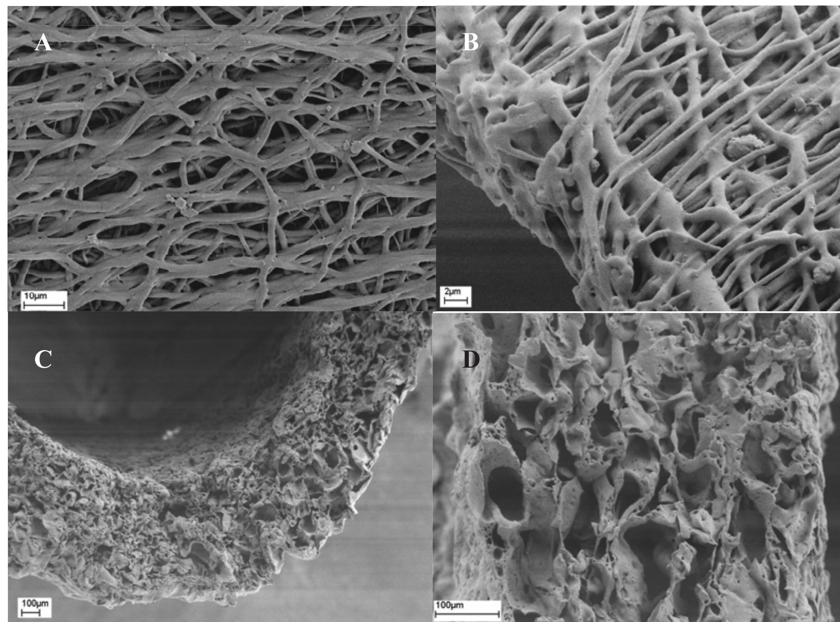

Figure A-1. SEM images of PTMC porous tubular scaffolds prepared by electro-spinning (A and B) and dip-coating (C and D): A and B were from the out-surface and cross section of an electro-spun sample. C and D were from cross section of samples made with 90 wt% sugar and crosslinked at 50 kGy.

Both the outer and the inner surfaces were porous with open connected pores. The average pore sizes ranged from 100-120 μm depending on different irradiation dosage (Fig. A-1 C and D). Clearly, the morphologies of the electro-spun and the dip-coated porous structures are different.

Although the pore size of the electro-spun tubular scaffold is much smaller than that of the dip-coated ones, it was reported that MSCs could readily be seeded in electro-spun PCL porous tubes with similar morphology [4, 5]. Furthermore, the elastic nature of PTMC can make dynamic seeding of cells possible through enlarged pores.

The tensile properties of these porous structures in radial direction were compared with those of ovine- and human arteries. The results are listed in Table A-1. Electro-spun tubes have the highest maximum strength among all specimens tested, while the dip-coated PTMC tubular structures show lowest maximum strength. On the other hand, the elongation at rupture of dip-coated tubes was the highest, followed by that of the electro-spun tubes. In both parameters, the porous electro-spun tubes were better than the native blood vessels. It should be noted that the mechanical strength of the porous tubes can further be enhanced after culturing of cells.

Table A-1. Tensile properties of tubular scaffolds and native ovine- and human arteries in radial direction.

	Initial stiffness (MPa)	**Maximum strength (MPa)**	**Elongation at break (%)**
Ovine carotid arteries	2.12± 0.14	1.65 ± 0.08	255 ± 21
Human arteria mesenterica inferior [a]	5.7	1.89	345
Electro-spun porous structure	4.1	0.86	480
Dip-coated porous structure [b]	1.10 ± 0.08	0.33 ± 0.04	1214 ± 120

[a] Single measurement

[b] The sample was prepared by dip-coating a suspension with 90wt% of sugar particles (106-250μm) and irradiated at 50 kGy afterwards. The sample code corresponding to the formal chapters was B-50.

Even without crosslinking, electro-spun porous structures were much stronger than dip-coated ones. This may be related to their different morphology: the electro-spun tubes possess smaller pore size and more regular structure as compared to the dip-coated ones.

As the morphological and mechanical properties of the dip-coated and the electro-spun porous structures are very different, it is possible to investigate the effects of these factors on the seeding and culturing of different cells (e.g. SMCs and MSCs) that are relevant for tissue engineering of small diameter blood vessels.

CONCLUSIONS

Porous tubular structures based on PTMC were prepared by electro-spinning technique and dip-coating method. Morphological and mechanical properties of these porous structures are quite different. Electro-spun porous structures showed higher mechanical strength due to orientation of fiber structures which can be advantageous in application of vascular scaffolds.

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Summary

Atherosclerosis is a vascular disease that affects medium and small diameter blood vessels. It is the main cause of coronary occlusions, aortic aneurysms and gangrene. Although synthetic grafts like Dacron and Teflon grafts are effective in large diameter vascular reconstructions, these materials can not be used as small diameter blood vessels substitutes. Autologous veins and arteries are currently used as main substitutes for these replacements, but often the lack of grafts of suitable size and their limited supply are a major problem. In recent years, more and more research is focused on tissue engineering to construct functional small diameter vascular grafts. Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences towards the reconstruction or development of biological substitutes that restore, maintain or improve tissue function. The aim of the research presented in this thesis is to develop tissue engineered small diameter blood vessels using poly(trimethylene carbonate) scaffolds. These materials can withstand pulsating physiological pressures and allow the culturing of smooth muscle cells under dynamic conditions. This should allow the constructs to be successfully implanted.

Chapter 2 gives an overview of grafting approaches in the replacement of atherosclerotic blood vessels. To realize this by tissue engineering, tubular scaffolds with suitable micro structures that allow adhesion and growth of relevant cells are to be prepared. Many polymers have been used to prepare these scaffolding materials, and include both natural and synthetic polymers. However, several challenges remain to be solved: the mismatch in compliance, occurrence of thrombosis, and the long culture times in vitro limit the success of tissue engineered small-diameter blood vessels. To address these issues, in **Chapter 3**, we developed biodegradable, biocompatible and elastic porous tubular scaffolds based on poly(trimethylene carbonate) (poly(TMC)). High molecular weight poly(TMC) could be crosslinked by gamma irradiation, and the resulting networks were elastic and creep-resistant. Poly(TMC) materials proved to be biocompatible as determined by cell adhesion

and proliferation studies. Stable, tubular scaffolds with interconnected pores were fabricated and their micro structures were characterized. Compared with natural blood vessels, the porous scaffolds had good compliance and high flexibility. Smooth muscle cells (SMCs) were seeded by perfusion and subsequently cultured for 7 d. The formed tissue engineered blood vessel constructs had high mechanical strength due to the proliferation of SMCs.

Using electro-spinning or dip-coating methods, porous PTMC tubular structures with different morphological and mechanical properties were prepared (**Appendix**). The mechanical strength and elasticity of the electro-spun porous PTMC structures were superior to those of scaffolds fabricated by dip-coating and could be compared with native arteries. The applicability of electro-spun poly(TMC) tubular scaffolds in tissue engineering vascular grafts needs to be explored.

In order to use the scaffolds in in vitro culturing under pulsatile conditions and to allow long-term implantation studies, their form stability and durability needs to be evaluated. In **Chapter 4**, a pulsatile flow system (PFS) was constructed to provide liquid flows, pressures and pulsations that mimic the physiological conditions. Thin non-porous outer layers were used to coat the scaffolds and allow the porous structures to be evaluated in the pressurized liquid flow system. The distention behavior of the scaffolds was similar to that of natural porcine carotid arteries. The compliance and stiffness values of the scaffolds was similar to that of native arteries, this could provide a well-suited mechanical environment for cell growth. The morphology and integrity of the scaffolds remained unchanged after dynamic perfusion for 7 d in the PFS.

Although porous tubular poly(TMC) scaffolds were produced with high porosities and large pore sizes, the cell seeding efficiencies were not very satisfactory. To keep the cells within the structure, the porous tubular scaffolds were coated with a fine porous outer layer as described in **Chapter 5.** In these double layered structures, the inner porous layer had a porosity of 86% and average pore size of 116 μm that allowed cell adhesion and proliferation. The porous outer layer had a lower porosity of 65% and a smaller average pore size of 28 μm that held the cells during perfusion seeding while

still allowing sufficient permeation of medium. The thickness of the porous outer layer could easily be regulated to tune the permeability and strength of the scaffolds. When SMCs were seeded in the scaffolds, seeding efficiencies significantly increased as could be observed by methylene blue staining after 1 day.

Poly(TMC) tubular scaffolds with a porous outer layer were used to seed and culture the cells to form tissue engineered vascular substitutes (**Chapter 6**). After seeding and culturing human umbilical smooth muscle cells for 1, 7 or 14 d in a pulsatile flow bioreactor, significant proliferation of the cells and formation of tissue was observed. This resulted in an engineered vascular construct with good mechanical properties. The success of these dynamic in vitro culturing experiments, holds great promise for application of these constructs in vivo.

Chapter 7 presents the results of the first implantation studies where porous poly(TMC) tubular scaffolds were applied. For the in vivo studies in rats, tubular poly(TMC) scaffolds with inner diameters of 1.5 mm and wall thicknesses of 0.6 mm were prepared as described in **Chapters 5** and **6**.

The research described in this PhD thesis covers many of the aspects that need to be considered when constructing functional tissue engineered small-diameter blood vessels. However, many other investigations, including endothelialization of the lumen with endothelial cells and long-term in vivo studies, still need to be conducted.

Samenvatting

Atherosclerose is een vasculaire ziekte die bloedvaten met een kleine en middelkleine diameter aantast. Het is de hoofdoorzaak van coronaire occlusies, aorta aneurysma's en gangreen. Hoewel synthetische bloedvatprothesen van Dacron en Teflon met een grote diameter goed functioneren, kunnen ze niet gebruikt worden voor kleine-diameter vasculaire reconstructies. Hiervoor worden momenteel autologe venen en arteriën gebruikt, die echter niet altijd de juiste afmetingen hebben of niet beschikbaar zijn. Daarom wordt er de laatste jaren veel onderzoek gedaan naar de weefselengineering van bloedvaten met een kleine diameter. Weefselengineering is een interdisciplinair onderzoeksgebied waarin de principes van engineering en levenswetenschappen worden gecombineerd om constructen te ontwikkelen die de normale weefselfunctie herstellen, handhaven of verbeteren. Het doel van het onderzoek beschreven in dit proefschrift is het ontwikkelen van kleine-diameter bloedvatprothesen door middel van weefselengineering gebruikmakend van poly(trimethyleencarbonaat) scaffolds. Deze scaffolds kunnen pulserende fysiologische drukken weerstaan en gladde spiercellen kunnen er onder dynamische condities in worden gekweekt. Dit zou het mogelijk moeten maken deze constructen succesvol te implanteren.

In **Hoofdstuk 2** wordt een overzicht gegeven van verschillende strategieën voor het vervangen van atherosclerotische bloedvaten. Om dit te realiseren door middel van weefselengineering, moeten buisvormige scaffolds met geschikte microstructuren worden vervaardigd waarin relevante cellen kunnen hechten en groeien. Hiervoor zijn vele polymeren gebruikt, zowel natuurlijke als synthetische. Verscheidene problemen die het succes van deze kleine-diameter vaatprothesen belemmeren moeten echter nog worden opgelost, zoals de ongelijke compliantie, het optreden van trombose en de lange kweektijden in vitro.

Om een bijdrage te leveren aan de oplossing hiervan, hebben we zoals beschreven in **Hoofdstuk 3** biodegradeerbare, biocompatibele en elastische poreuze buisvormige scaffolds ontwikkeld op basis van poly(trimethyleencarbonaat) (poly(TMC)). Hoog moleculair gewicht poly(TMC) werd vernet door middel van gamma bestraling, de gevormde netwerken waren elastisch en kruipvrij. Het poly(TMC) materiaal was biocompatibel, zoals bleek uit celadhesie en -proliferatie studies. Vervolgens werden

stabiele buisvormige scaffolds met doorverbonden poriën vervaardigd waarvan de microstructuur werd gekarakteriseerd. De poreuze scaffolds hadden vergeleken met natuurlijke bloedvaten een goede compliantie en hoge flexibiliteit. Gladde spiercellen werden in de scaffolds gezaaid door middel van een perfusie-techniek en vervolgens gedurende 7 dagen gekweekt. De mechanische sterkte van de gevormde constructen bleek toegenomen te zijn ten gevolge van de proliferatie van de spiercellen.

Poreuze buisvormige poly(TMC) structuren met verschillende morfologische en mechanische eigenschappen werden vervaardigd door middel van electro-spinning of dip-coating methoden (**Appendix**). De mechanische sterkte en elasticiteit van de scaffolds gemaakt door middel van electro-spinning bleek beter te zijn dan die van de andere scaffolds en waren vergelijkbaar met die van natieve arteriën. De toepasbaarheid voor de weefselengineering van bloedvaten van buisvormige poly(TMC) structuren vervaardigd door middel van electro-spinning dient nader onderzocht te worden.

Om de scaffolds gemaakt door middel van dip-coating te kunnen gebruiken voor in vitro weefselkweek onder dynamische perfusie condities alsmede voor implantaties in vivo, diende de vorm-stabiliteit en duurzaamheid van de scaffolds onderzocht te worden. Zoals beschreven in **Hoofdstuk 4**, werd er een pulsatiel flowsysteem (PFS) geconstrueerd voor het nabootsen van fysiologische vloeistofstromingen, drukken en pulsaties. De scaffolds werden voorzien van dunne niet-poreuze buitenlaagjes om evaluatie in het PFS mogelijk te maken. Het verwijden van de scaffolds met toenemende druk bleek vergelijkbaar te zijn met dat van varkens carotis arteriën. De compliantie en stijfheid van de scaffolds waren vergelijkbaar met die van natieve arteriën, hetgeen zou kunnen zorgen voor een goede mechanische omgeving voor celgroei. De morfologie en integriteit van de scaffolds veranderden niet gedurende 7 dagen dynamische perfusie in het PFS.

De efficiëntie van het zaaien van gladde spiercellen in de poreuze poly(TMC) scaffolds bleek niet erg hoog te zijn. Om de cellen tijdens het zaaien binnen de poreuze structuur te houden, werden de scaffolds voorzien van een dun microporeus buitenlaagje zoals beschreven in **Hoofdstuk 5**. In deze dubbellaag structuur had de binnenste laag een porositeit van 86% en een gemiddelde porie-grootte van 116 μm, hetgeen celadhesie en -proliferatie mogelijk maakten. De microporeuze buitenlaag had een lagere porositeit van 65% en een kleinere gemiddelde porie-grootte van 28 μm, waardoor de cellen tegengehouden werden tijdens de zaaiprocedure maar er toch nog voldoende permeabiliteit van kweekmedium was. Door de dikte van de microporeuze buitenlaag te

variëren konden de permeabiliteit en sterkte van de scaffolds worden gereguleerd. Ten gevolge van het aanbrengen van de microporeuze buitenlaag, nam de efficiëntie van het zaaien van gladde spiercellen significant toe, hetgeen bleek uit methyleenblauw kleuring na 1 dag.

Door het zaaien en kweken van humane gladde spiercellen in buisvormige poly(TMC) scaffolds voorzien van een microporeuze buitenlaag werden vasculaire constructen gevormd (**Hoofdstuk 6**). Na het zaaien en vervolgens kweken van de cellen gedurende 1, 7 of 14 dagen in een bioreactor met pulserende perfusie van kweekmedium, werd een significante celproliferatie alsmede weefselvorming waargenomen. Dit resulteerde in vasculaire constructen met goede mechanische eigenschappen. Het succes van deze kweekexperimenten onder dynamische condities is veelbelovend voor de toepassing van deze constructen in vivo.

De eerste implantatiestudies met deze poreuze buisvormige poly(TMC) scaffolds worden beschreven in **Hoofdstuk 7**. Voor deze in vivo studies in de rat werden scaffolds met een binnendiameter van 1.5 mm en een wanddikte van 0.6 mm gemaakt, zoals beschreven in **Hoofdstukken 5** en **6**.

Het onderzoek beschreven in dit proefschrift omvat vele aspecten die in overweging moeten worden genomen voor het construeren van functionele kleine-diameter bloedvaten door middel van weefselengineering. Een aantal aspecten, zoals het bekleden van de binnenwand met endotheelcellen en langere in vivo studies, moeten echter nog worden uitgevoerd.

论文摘要

在现代社会中，由动脉硬化而产生的血管疾病，是一些较典型的损伤如动脉硬化性闭塞症、多发性大动脉炎、动脉栓塞、动脉瘤等疾病的主要原因。尽管一些合成材料如膨体聚四氟乙烯（e-PTFE）和达克龙（Dacron）能有效用于大尺径血管再生，这些材料显然不能适用于需要更高血液动力学要求的小尺径血管移植再生。

目前，治疗血管疾病的主要方法为静脉移植和动脉分流法。目前小管径血管(< 6mm)替代物主要是应用自体的动、静脉移植物，但可用于替代的自体动静脉却由于尺寸的差异和数量的匮乏而无法移植。自体动静脉替代的中短期效果尚可，但不能满足永久替代物的要求，往往需要再次手术，给病人带来额外的痛苦。临床上的这种需求也使得越来越多的科研工作集中到了通过组织工程制造小尺径血管替代物。

组织工程学是应用生命科学和工程学的原理和方法，以分子生物学、细胞生物学、生物工程学和临床医学为基础，设计、构造、改良、培育和保养活组织，用以修复或重建组织器官的结构，维持或改善组织器官的功能的一门新兴的边缘科学。本课题旨在使用聚三甲基碳酸酯（PTMC）均聚物作为支架材料，运用组织工程开发新型小血管移植材料。

第二章全面综述了主要在研究的各种可用于替代病变血管的移植材料。合适的血管组织工程的支架需要有合适的微观构造和尺寸，从而有效促进细胞粘着和生长。尽管天然材料和合成材料广泛地应用于各种血管组织工程支架的研究中， 多数研究的难点仍然集中在材料柔韧性的缺乏，血栓的形成和长期体外细胞培养。在**第三章**中，我们以聚三甲基碳酸酯（PTMC）均聚物为原料制造可降解高弹性的管状支架。通过gamma射线实现交联后的PTMC可以明显的提高弹性和抵抗形变的能力。PTMC具有足够生物相容性，细胞能在材料表面顺利附着并繁殖。稳定的多空隙管状支架可以通过多次浸镀技术获得，其支架微观结构也得到进一步表征。在研究中我们发现，和天然血管相比，这类多孔隙管状支架具有较好的柔韧性和弹性，平滑肌细胞（SMC）能通过内壁压力灌注的方式进入支架并且附着。通过 7 天的体外静态培养，细胞的生长使得支架的机械强度得到进一步的提高。

PTMC多孔隙管状支架不仅可以通过多次浸镀技术得到，也能运用电纺丝技术制造。不同技术可以获得不同尺寸，厚度和不同微孔密度的多孔管状支架，可适用于不同部位血管的移植需要，也可适用于植入不同细胞用于其它开发研究（**附录**）。通过电纺丝技术得到的支架具有更高的弹性和机械强度，和人体血管相似，这类支架的应用将得更广阔的发展。

人体血管具有高度强度和柔韧性，为了测试我们制备的交联 PTMC 多孔支架的能否适应长期脉冲压力的环境，在研究中，我们建立了一个生物反应器 （PFS）（**第四章**）。该生物反应器能够模拟人体内环境的流体力学，管壁的剪切压强，脉冲信号等等。通过在多孔隙支架外镀上一层薄膜，使得支架的力学表现可以通过生物反应器得到模拟。通过测试，我们发现，这种 PTMC 管状支架具有高强弹性，勘比人体自身血管的脉动表现。在长时间测试中，我们制备的这种 PTMC 管状支架可以承受上万次脉冲力而不发生变形。

研究认为管状支架具有较高孔隙率和孔径能为细胞的附着和生长提供空间，但是也容易导致细胞在内壁压力灌注的植入同时通过孔隙流出支架。在**第五章**中，我们通过在原有多孔隙管状支架外表面覆盖上一层细密孔隙的 PTMC 层 （POL），成功地解决了这一问题，提高了整体细胞植入率。在这种双层多孔隙管状支架中，内层具备了较高孔隙率（平均 86%）和较大孔径（平均 116 μm），适于细胞生长；外层（POL）具有相对较低的孔隙率（平均 65%）和小孔径（平均 28 μm），能有效阻止细胞流失并且维持支架内的养分循环。实验证明 POL 确实有效提高了细胞的植入率，在培养一天后，显微镜观察支架的染色切片可以清晰地观察到细胞的附着。

尽管在血管组织工程方面，研究人员尝试过很多支架材料，但其中很多都存在力学性能上的问题，无法耐用于长时间血液流动，脉冲压力的环境。而这一点，在通过体外细胞培养实现血管可移植物的过程中，是成功的关键之一。通过前几章的研究工作，我们发现交联的PTMC为材料的管状支架具有匹配的力学性能，在接下来的**第六章**，我们通过细胞的植入和培养来构建组织工程血管移植替代物。在人体平滑肌细胞（SMCs）植入并在生物反应器中培养 7 和 14 天后，我们观察到细胞的显著增长和组织层的行成，从而更提高了整个血管替代物的强度。在体外细胞培养的成功也为支架的进一步体内移植实验奠定了基础。

第七章主要介绍了PTMC多孔隙管状支架在体内移植的情况。为适用于替换小鼠腹部大动脉，

我们按照同等工艺制造了内径仅 1.5mm，厚度仅 0.6mm的较小管状支架。

在整个 PhD 的研究工作中，我们参考和覆盖了小尺径血管组织工程中的很多关键要点，使研究工作体现出了高度的真实性和可参考度。然而，更广泛的有关于内皮细胞生长，生长因子作用和长期体内实验表现等等仍然有待更多努力。

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