ENDOTHELIALIZATION OF COLLAGEN MATRICES

EFFECTS OF CROSSLINKING, HEPARIN IMMOBILIZATION AND bFGF LOADING

PROEFSCHRIFT

Ter verkrijging van
de graad van doctor aan de Universiteit Twente,
op gezag van de rector magnificus,
prof. dr. F.A. van Vught,
volgens het besluit van het College voor Promoties
in het openbaar te verdedigen
op vrijdag 18 juni 1999 te 15.00 uur

door

Marcel Johan Bernhard Wissink

geboren op 19 juli 1968
te Lichtenvoorde
Dit proefschrift is goedgekeurd door:

Promotor: Prof. Dr. J. Feijen
Promotor: Prof. Dr. W.G. van Aken
Assistent promotor: Dr. A.A. Poot
Referent: Dr. Ir. G.H.M. Engbers
That is not dead
which can eternal lie,
and with strange eons
even death may die.

The call of Cthulhu
H.P. Lovecraft, 1890-1937.
Endothelialization of collagen matrices. Effects of crosslinking, heparin immobilization and bFGF loading / M.J.B. Wissink
Thesis University of Twente, Enschede, The Netherlands
With references- With summary in English, met samenvatting in het Nederlands
ISBN: 90-36513154

The research described in this thesis was financed by the Dutch Technology Foundation (STW)

Financial support by the Netherlands Heart Foundation and the Dutch Society for Biomaterials (NVB) for the publication of this thesis is gratefully acknowledged

© M.J.B. Wissink, 1999

Press: FEBODRUK BV, Enschede, The Netherlands
# CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 1</td>
<td>General introduction</td>
<td>1</td>
</tr>
<tr>
<td>Chapter 2</td>
<td>Endothelial cell seeding of collagen-coated vascular grafts</td>
<td>7</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>An EDC/NHS-crosslinked collagen substrate for endothelial cell seeding: Preparation, characterization and the effect of γ-sterilization</td>
<td>39</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>Immobilization of heparin to EDC/NHS-crosslinked collagen. Characterization and in vitro evaluation</td>
<td>61</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>Binding and release of basic fibroblast growth factor from heparinized collagen matrices</td>
<td>87</td>
</tr>
<tr>
<td>Chapter 6</td>
<td>Endothelial cell seeding on crosslinked collagen: effects of crosslinking on endothelial cell proliferation and functional parameters</td>
<td>105</td>
</tr>
<tr>
<td>Appendix to chapter 6</td>
<td>Relation between cell density and the secretion of von Willebrand factor and prostacyclin by human umbilical vein endothelial cells</td>
<td>125</td>
</tr>
<tr>
<td>Chapter 7</td>
<td>Endothelial cell seeding of (heparinized) collagen matrices: Effects of bFGF pre-loading on proliferation (after low density seeding) and pro-coagulant factors</td>
<td>137</td>
</tr>
<tr>
<td>Chapter 8</td>
<td>In vivo biocompatibility of EDC/NHS-crosslinked collagen matrices: Effects of crosslink density, heparin immobilization and bFGF loading</td>
<td>159</td>
</tr>
<tr>
<td>Summary</td>
<td></td>
<td>181</td>
</tr>
<tr>
<td>Samenvatting</td>
<td></td>
<td>187</td>
</tr>
<tr>
<td>Curriculum vitae</td>
<td></td>
<td>193</td>
</tr>
</tbody>
</table>
CHAPTER 1

GENERAL INTRODUCTION

In the western world, diseases of the cardiovascular system are the main cause of death. Prominent degenerative arterial disorders include atherosclerosis and aneurysm formation. Atherosclerosis results in progressive accumulation of smooth muscle cells and lipids within the intima of arteries. This may cause occlusion of arteries, resulting in gangrene, stroke, or myocardial infarction. An aneurysm is a permanent defect or swelling in the wall of the e.g. the aorta and arteries in the brain. Aneurysms are mostly associated with atherosclerosis, and may result in life threatening rupture of the vessel wall. Recognized risk factors for atherosclerosis include increased cholesterol levels, lack of exercise, hypertension and smoking [1].

Treatment of atherosclerotic or aneurysmal disease may involve bypass or replacement of one or more affected arteries, using e.g. autologous veins or synthetic vascular grafts. Although for aortic replacement satisfactory vascular prostheses have been available for more than 30 years [2], no satisfactory small vessel substitute has yet been developed. Patency rates of small-diameter vascular grafts (diameter less than 5 mm) are unacceptably low when compared to autologous saphenous vein grafts. Therefore, autologous saphenous veins are preferentially used to replace small-diameter vessels [3-6]. However, suitable autologous veins are often absent due to disease or previous utilization [7]. Therefore, there is a need for alternative small-diameter vascular grafts, with improved patency rates.

Endothelial cell seeding is a recognized strategy to improve the blood compatibility of synthetic vascular grafts [8, 9]. The process of autologous endothelial cell seeding involves isolation of endothelial cells from one of the veins of a patient undergoing vascular surgery. Subsequently, harvested cells are transferred to the lumen of a vascular graft, and after a limited period of cell attachment the seeded graft is utilized. However, to facilitate successful endothelialization of vascular grafts in humans, two major problems have to be dealt with. A suitable substrate for endothelial cell seeding has to be developed, and problems due to limited availability of autologous endothelial cells for cell seeding have to be resolved, as discussed below.

Currently available synthetic vascular grafts, made of Dacron or expanded Teflon, are unsuitable as substrate for endothelial cell seeding [10]. Pre-clotting, or coating with fibrin glue or fibronectin results in improved endothelialization of graft materials, both in vitro and
in animal models [11-13]. Pre-clotting of vascular grafts, however, is elaborate, and problematic in patients on anticoagulant drugs. Furthermore, the use of fibrin glue or fibronectin in humans carries a risk of transmission of viral diseases. Since non-crosslinked collagen is a suitable substrate for endothelial cells in vitro, application of a collagen coating on synthetic vascular grafts may result in a matrix suitable for endothelial cell seeding. In commercially available collagen-coated vascular grafts, however, the collagen matrix is crosslinked with glutaraldehyde or formaldehyde. These crosslink agents induce cytotoxic reactions, thus hampering endothelialization of the graft [14, 15]. As a consequence, there is a need for a non-cytotoxic collagen substrate, which supports adherence and growth of seeded endothelial cells.

Animal studies have shown significantly improved patency rates after endothelial cell seeding of small-diameter vascular grafts [13, 16]. In clinical trails, however, the results of endothelial cell seeding have been variable and disappointing, most likely due to low cell seeding densities [17, 18]. Using currently available techniques for the isolation and seeding of cells, it is expected that a donor vessel of least 50% to 75% of the length of the prosthetic graft is needed to obtain a sufficient number of endothelial cells for direct seeding techniques. In general, the use of autologous donor vein is this length is unacceptable [19]. To cope with the limited availability of autologous endothelium, alternative cell seeding techniques have been developed [20]. After autologous vein harvest, in vitro cell culture can be applied to expand the number of endothelial cells after isolation, allowing high-density cell seeding prior to graft implantation during a second operation. Alternatively, endothelial cells can be grown to confluence in vitro after seeding in vascular prosthesis, allowing implantation of grafts pre-lined with endothelium. Major disadvantages of these techniques are the need of two surgical procedures, the long interval between the need of an endothelialized graft and its availability, and the increased risk of bacterial infection [21].

AIM OF THIS STUDY

The aim of the studies presented in this thesis was to develop a collagen coating for small-diameter synthetic vascular grafts, able to support adhesion and proliferation of endothelial cells after cell seeding at low densities. To achieve this goal, three individual strategies were combined:

1. A non-cytotoxic crosslinked collagen substrate, suitable for endothelial cell seeding was developed. For this purpose, insoluble type I collagen from bovine achilles tendon was
crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) in combination with N-hydroxysuccinimide (NHS).

2. Immobilization of heparin to this substrate, since collagen is a thrombogenic material. Immobilization of the potent anticoagulant heparin to EDC/NHS-crosslinked collagen may prevent thrombus formation on the collagen surface in the period after cell seeding when the collagen substrate is not yet completely covered by endothelial cells, thus preventing graft failure due to early occlusion. Heparin was immobilized also using EDC and NHS.

3. Local, sustained release of basic fibroblast growth factor (bFGF, a heparin binding endothelial cell growth factor) from the heparinized, EDC/NHS-crosslinked collagen matrix. Local sustained release of bFGF is expected to improve the proliferation of seeded endothelial cells, and possibly might reduce the cell seeding density necessary to induce proliferation of endothelial cells.

![Diagram of a heparinized, collagen-coated synthetic vascular graft with basic fibroblast growth factor (bFGF) as a substrate for endothelial cell seeding.]

Figure 1: Schematic representation of a heparinized, collagen-coated synthetic vascular graft containing basic fibroblast growth factor (bFGF), as substrate for endothelial cell seeding.

**OUTLINE OF THIS THESIS**

In chapter 2 a literature survey is given regarding endothelial cell seeding of vascular grafts. The suitability of collagen-coated vascular grafts as a substrate for endothelial cell seeding is discussed. Furthermore, various aspects concerning immobilization of heparin onto collagen are addressed, and an overview of the use of bFGF in biomedical applications is given.

In chapter 3, crosslinking of collagen using EDC and NHS is described. The crosslink reaction itself, the influence of crosslinking on mechanical properties, the resistance to *in vitro* enzymatic degradation of collagen and the effect of gamma-sterilization were studied.
The immobilization of heparin to EDC/NHS-crosslinked collagen, also using EDC and NHS, is described in chapter 4. To determine whether heparin immobilization improved the blood compatibility of the collagen matrix, contact activation, thrombin inhibition and deposition of blood platelets was studied in vitro.

In chapter 5, the binding of bFGF to, and the in vitro release of bFGF from (heparinized) EDC/NHS-crosslinked collagen is described.

In chapter 6, the effect of EDC/NHS-crosslinking of collagen on the in vitro adhesion and growth of seeded human umbilical vein endothelial cells (HUVECs) is discussed. Furthermore, the effects of EDC/NHS-crosslinking of collagen on various endothelial cell functions were determined. To resolve some questions raised in chapter 6, the relation between endothelial cell density and the secretion of von Willebrand factor and prostacyclin by endothelial cells was determined and described in an appendix to chapter 6.

In the in vitro study presented in chapter 7, the effect of heparin immobilization onto EDC/NHS-crosslinked collagen on the proliferation of seeded human umbilical vein endothelial cells is described. Furthermore, the effect of pre-loading of (heparinized) EDC/NHS-crosslinked collagen with bFGF on the proliferation of endothelial cells and their pro-coagulant properties were studied. In addition, the ability of matrices pre-loaded with bFGF to induce proliferation of HUVECs after seeding at very low cell densities was determined.

Possible cytotoxicity and (longer term) tissue reactions of (heparinized) EDC/NHS crosslinked collagen was determined following subcutaneous implantation in rats, as described in chapter 8. The effects of the crosslink density, the amount of heparin immobilized to an EDC/NHS-crosslinked matrix with a selected crosslink-density, and the effect of pre-loading of (heparinized) EDC/NHS crosslinked collagen with bFGF were studied.

REFERENCES


CHAPTER 2

Endothelial cell seeding of collagen-coated vascular grafts

M.J.B. Wissink, A.A. Poot, G.H.M. Engbers, W.G. van Aken, J. Feijen
Institute for Biomedical Technology, Polymer Chemistry and Biomaterials Group, Department of Chemical Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

1. ENDOTHELIAL CELL SEEDING

1.1. Introduction

Diseases of the circulatory system are the main cause of mortality in the western world, in the Netherlands attributing to approximately 44% of the deaths over the last years [1]. Degenerative arterial diseases like atherosclerosis and aneurysm formation are prominent circulatory disorders. Atherosclerosis is a slowly progressive process starting at relatively young age, which leads to gradual narrowing of arteries due to deposition of atherosclerotic plaque. Eventually, arterial occlusion may result in complications such as myocardial infarction, stroke, and gangrene of the extremities [2]. Aneurysm formation results in a local increase in arterial diameter, accompanied by a decreased thickness of the arterial wall (e.g. the aorta) and thrombosis. When an aneurysm ruptures, acute and severe blood loss occurs which may be life threatening.

Treatment of atherosclerotic or aneurysmal disease may involve bypassing or replacement of affected arteries, using autologous veins, synthetic vascular grafts or crosslinked umbilical veins or xenografts [3]. Synthetic vascular grafts, made of Dacron (polyethylene terephthalate) or expanded Teflon (polytetrafluoroethylene, ePTFE) (fig. 1) have been used successfully for many years in large-diameter applications like the aorta or the aortoiliac region. The long-term patency rates of small-diameter synthetic vascular grafts (diameter 5 mm or less), however, are disappointing, primarily due to (re-) stenosis and thrombus formation [4-7]. Autologous saphenous veins and iliac arteries have been used to repair small- and medium-diameter arteries [8]. In the lower extremities, long-term results of autologous saphenous vein grafts, used either as in situ or as reversed grafts, have been very good [9]. However, suitable autologous veins are often absent,
Figure 1: Scanning electron micrographs of (A) Dacron (Cooley knitted Dacron, Meadox Medicals, Oakland, NJ, 100 ×) and (B) expanded Teflon (Gore-Tex, Gore & Associates, Flagstaff, AZ, 500 ×)

for example due to previous utilization or disease [10]. Furthermore, there are arguments for the preferential use of synthetic graft materials in the upper legs, to preserve autologous veins for later date [11]. Therefore, there is a need for alternative small-diameter vascular prostheses with improved patency rates.

Endothelial cell seeding is a recognized method to improve blood compatibility of small-diameter vascular grafts [12-14]. Endothelium is often described as the perfect natural blood-compatible surface [15, 16] which secretes various substances affecting platelet adhesion and aggregation, blood coagulation and fibrinolysis. Furthermore, expression of cell surface components like heparan sulfate or tissue thromboplastin (tissue factor) regulate interactions with blood components. Endothelial cell function thus directly affects the balance of hemostasis and thrombosis in the cardiovascular system [16, 17]. Upon creating a functional inner lining of endothelial cells in (small diameter) vascular grafts, implants with similar non-thrombogenic surface characteristics as normal blood vessels might be obtained.

In most animal models, endothelialization of vascular grafts will occur spontaneously [18, 19]. In contrast, reports of spontaneous endothelialization of vascular grafts in humans are anecdotal [20]. In humans endothelialization is in general confined to a few millimeters pannus-ingrowth from both anastomoses [21]. To obtain continuously endothelium-lined vascular grafts in humans, endothelial cell seeding has to be applied. The potential of this strategy was demonstrated in a number of animal studies. Significantly increased patency rates
[22-26] and a strong reduction in platelet adhesion and thrombus formation [27-29] was observed after endothelial cell seeding of vascular grafts.

1.2. Endothelial cell seeding and substrate compatibility

Commercially available synthetic vascular graft materials are generally poor substrates for endothelial cell seeding [30-32]. Modification of existing graft materials, by chemical surface treatment [33], glow discharge modification [30, 34-36] or application of a suitable protein coating may improve the potential of currently available graft materials for endothelial cell adhesion and proliferation. Regarding protein coating, substances like fibrin glue [37, 38], non-crosslinked collagen [28, 39] and fibronectin [40, 41] have been shown to result in improved in vitro endothelialization of vascular graft materials. Furthermore, immobilization of RGD-peptides [42] or antibodies against endothelial cell membrane antigens [43] may improve adherence and growth of seeded endothelial cells. Pre-clotting [23, 25, 44, 45], fibrin glue [27] or coating with fibronectin are often used before endothelial cell seeding in animal models [26, 28, 46]. In patients, the use of either one of the above techniques may face certain disadvantages. Pre-clotting of vascular grafts (prior to cell seeding) is elaborate, and problematic notably in patients using anticoagulant treatment. The use of human fibrin glue or human fibronectin introduces a risk of transmitting viral diseases. Therefore, the use of human fibronectin as substrate for endothelial cell seeding is no longer allowed in most countries [12]. In addition, both fibrin glue and fibronectin coatings may cause increased thrombogenicity of vascular graft surfaces. Since non-crosslinked collagen is a suitable matrix for the growth of endothelial cells in vitro [47-49], application of a collagen coating on synthetic vascular graft materials may result in a matrix suitable for endothelial cell seeding. In commercially available collagen-coated vascular grafts, glutaraldehyde or formaldehyde are commonly used to crosslink the matrix [8]. These agents are incorporated in the collagen matrix during crosslinking. During in vitro and in vivo degradation, notably glutaraldehyde-crosslinked collagen evokes cytotoxic reactions due to release of (unreacted) glutaraldehyde and glutaraldehyde derivatives [50-52], which hamper endothelialization of the luminal graft surface [52, 53], as is discussed below. Development of a non-cytotoxic collagen substrate, therefore, is of great interest.

1.3. Approaches to cell seeding

To prevent graft rejection due to differences in histocompatibility and blood group antigens, and to prevent transmission of (viral) diseases between donor (of endothelial cells) and recipient, there is a strong preference for the use of autologous cells for seeding of vascular
grafts. As a consequence, the supply of cells directly after isolation is limited because cells have to be harvested from pieces of vascular tissue from the patient undergoing vascular surgery. The number of cells harvested is usually not sufficient to obtain a confluent covering at the inside of the graft immediately after seeding.

Three basic methods for endothelialization of vascular grafts can be recognized: immediate seeding, delayed seeding or implantation of pre-lined grafts [14]. Delayed seeding is a two stage procedure. After source tissue removal and endothelial cell harvesting during a first operation, standard cell culture techniques are applied to expand cell numbers. Subsequently, the vascular graft is seeded and implanted during a second operation. The second approach, i.e. implantation of pre-lined grafts, also involves a two-staged operation. Successful implantation of grafts pre-lined with endothelial cells has been reported by Zilla et al. [54, 55]. After endothelial cell isolation, cells were seeded directly into fibrin-glue coated ePTFE grafts. Thereafter, the cells were cultured in situ for an average time of 25 days, to obtain vascular grafts lined with a monolayer of endothelial cells. This approach has been successfully used clinically for eight years [12]. An excellent primary patency rate of 74% after 7 years has been reported for pre-lined grafts used for femoropopliteal reconstruction, which is comparable to the patency rate of autologous vein grafts [7, 56].

Drawbacks of delayed seeding or the use of pre-lined grafts are the long interval between the need of an endothelialized vascular graft and its availability, limiting its use to non-emergency situations, and the increased risk of bacterial infection due to prolonged in vitro endothelial cell culture [38]. For those reasons, immediate seeding is the preferred method for endothelialization of synthetic vascular grafts, although low seeding densities have to be accepted and dealt with. Studies with immediate cell seeding in humans, however, have mostly been disappointing. Most likely, seeding densities were insufficient to lead to the formation of a confluent endothelial lining in situ [24, 54, 57, 58].

1.4. Strategy for successful immediate endothelialization

To facilitate successful low density cell seeding of synthetic vascular grafts, vascular grafts with optimal cell seeding characteristics have to be developed. In the research described in this thesis, three strategies have been combined to achieve this objective:

1. An optimal matrix for endothelial cell attachment and proliferation has to be provided. A synthetic vascular graft with a non-cytotoxic crosslinked collagen matrix is a possible candidate. As discussed hereafter in section 2, this can be accomplished by using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxy-succinimide (NHS) for collagen crosslinking.
2. Early graft occlusion due to thrombus formation, in the critical post-operative period when a large part of the graft surface is not endothelialized, has to be prevented [46]. This may be achieved by heparin immobilization onto collagen coated grafts, as discussed in section 3.

3. The outgrowth of low density seeded endothelial cells to form a monolayer should be enhanced by e.g. local, sustained release of basic fibroblast growth factor (bFGF) from the cell seeding matrix, as discussed in section 4.

2. COLLAGEN

2.1. Introduction

Collagen is a structural protein which properties make it suitable for various biomedical applications. Collagen has good mechanical properties, a good biocompatibility (depending on e.g. the type of crosslinking, as discussed below), and as a matrix protein it can be used as a scaffold for cell seeding or for regeneration of host tissue [59, 60]. When purified, collagen is only weakly antigenic [61]. Antigenicity of collagen can be further reduced by (enzymatic) removal of the non-helical telopeptide regions of the molecule [62] or by crosslinking [63, 64].

Preparation of collagen, of human or animal origin, can be classified in two major approaches [62]. First, purified collagen can be solubilized or dispersed and subsequently reconstituted in a pre-designed form or shape, with or without further crosslinking. Examples are injectable collagen preparations for dermal augmentation [65] or collagen-coated synthetic vascular grafts [66]. Secondly, biological structures like umbilical veins, tendons or heart valves are treated intact to remove non-collagenous components and the remaining collagen material can be crosslinked before implantation [67, 68].

2.2. Collagen structure

Collagen is the most abundant protein in mammals. It constitutes about 30% of the total body protein, providing mechanical and structural stability to tissues and organs. Skin, bones, tendon and cartilage all consist largely of collagen [69]. Furthermore, collagen is an important component of extracellular matrix and basement membranes [70]. Collagen is synthesized by a variety of cells, like fibroblasts, osteoblasts, chondrocytes and endothelial cells [59], and exists in a variety of forms like filaments, sheets and fibers. Until now, over 16 different types of collagen have been described [71]. All collagen molecules contain a triple helix structure.
However, the amino acid composition, the length of the helix and the nature and size of the non-helical parts of the collagen molecule differ from type to type [71]. Collagens of type I, II and III are fiber-forming collagens, while collagen type IV is membrane-forming (basement membranes). Collagen type I comprises 80-90% of the total body collagen, and is mainly found in skin, tendons and bones [72]. Type III collagen is found in smaller quantities in the same tissues as type I collagen, except for tendons and bones. In the extracellular matrix of blood vessels type III collagen is estimated to be 30% to 45% of the total collagen content, depending on the localization of the vessel [70].

Figure 2: Schematic representation of the collagen structure.

Fiber-forming collagens are structurally very similar. The collagen molecule (tropo-collagen), the fundamental unit of collagen fibers, has a molecular weight of approximately 300 kD, a length of 300 nm, and a diameter of 1.5 nm. It consists of three peptide chains (α-chains) of approximately 1,000 amino acid residues each. The amino-acid composition of α-chains differs between different types of collagen, and a specific type of collagen may consist of different α-chains. For example, type I collagen is made up of two α1(I) chains and one α2(I) chain, type III collagen consists of three α1(III) chains.

The primary structure of α-chains is a repeating tri-peptide of Gly-X-Y, where proline is mainly found in the X-position and hydroxyproline in the Y-position. Hydroxyproline is essentially unique to collagen [72, 73]. α-Chains form a left handed helix with 3.3 amino-acid residues per turn. Three α-chains combine to a right handed super-helix, forming a rod-like collagen molecule with two short non-helical (telopeptide-) regions at both ends of the molecule. The formation of a triple helix is possible because of the positioning of glycine as every third amino acid residue, and stabilization by steric repulsions caused by proline- and hydroxyproline residues, hydrogen bonds and intra-molecular crosslinks [74, 75].

Electrostatic and hydrophobic interactions between tropo-collagen molecules result in the formation of microfibrils, a parallel array of 5 collagen molecules with the same N → C orientation. Collagen fibrils, with a diameter of 10 to 100 nm are formed by spontaneous lateral and end-to-end aggregation of collagen microfibrils. In a fibril, collagen molecules in one row are separated by 40 nm gaps, and adjacent rows are displaced by 67 nm. The staggered conformation of the tropo-collagen molecules in fibrils, which results in overlap- and gap regions, leads to the characteristic periodic pattern observed by electron microscopy. Further aggregation of collagen fibrils results in formation of fibers and fiber bundles [69, 76]. Collagen fibrils and fibers are stabilized by formation of intermolecular crosslinks, between...
lysine residues in the telopeptide region of the molecules. The number of crosslinks is dependent of the source of the collagen, and increases with age [72].

2.3. Crosslinking of collagen biomaterials

Crosslinking of collagen biomaterials is often applied to control or reduce the \textit{in vivo} resorption rate or to improve mechanical properties of materials [77-80]. In general, there are two methods for chemical crosslinking of collagen [81-83]. Reagents like glutaraldehyde, formaldehyde and diisocyanates introduce crosslinks between two $\varepsilon$-amino groups of lysine and/or hydroxylysine residues. Secondly, crosslinking can be carried out by introducing amide bonds between carboxylic acid groups from aspartic or glutamic acid residues and $\varepsilon$-amino groups. Examples are cyanamide crosslinking, acyl azide crosslinking and the use of carbodiimides.

During the last years, several studies have been directed towards the use of novel epoxy compounds for crosslinking of collagen [84-86]. It has been recently demonstrated that, depending on pH, bis-epoxy compounds introduce crosslinks between either carboxylic acid groups, or primary amino groups of collagen [87].

Figure 3: Schematic representation of collagen crosslinking using glutaraldehyde (upper part) or formaldehyde (bottom part).
Crosslinking of collagen in commercial collagen-coated synthetic vascular grafts is commonly carried out using glutaraldehyde or formaldehyde [8, 66]. The mechanism of crosslinking by these reagents is complex and poorly understood. Using glutaraldehyde (II) it is assumed that Schiff base intermediates (III) are formed by reaction of aldehyde groups of glutaraldehyde with amino groups of collagen (I). Subsequent reactions of these intermediates results in the formation of a large variety of possible crosslink-moieties (IV, simplified), as reviewed by Olde Damink et al. [88].

During crosslinking glutaraldehyde uptake is up to 3 molecules per reacted amino group [88]. A proposed crosslink-mechanism for formaldehyde (V), which is a mono-functional reagent, involves a collagen-imine intermediate (VI), formed by the reaction of formaldehyde with ε-amino groups of collagen. The subsequent reaction with for example a glutamine residue (VII) results in a reversible crosslink (VIII) [81]. Durability of formaldehyde crosslinked materials was found to be inferior to glutaraldehyde crosslinked materials [89].

The use of both glutaraldehyde and formaldehyde for collagen crosslinking has an important drawback: both crosslink-agents are incorporated in the collagen material. Formaldehyde, and notably glutaraldehyde are known to induce local cytotoxicity by the release of (unreacted) crosslink-agents or derivatives thereof, during both in vitro and in vivo application of collagen biomaterials [51, 52, 90]. It has been shown that residual glutaraldehyde completely inhibits in vitro fibroblast proliferation at concentrations as low as 3 ppm [91]. Cytotoxicity was also observed for formaldehyde-crosslinked pericardium [52, 90] and formaldehyde-crosslinked tissue heart valves [89]. It can be concluded that upon implantation, cytotoxic reactions hamper endothelialization of currently available collagen-coated synthetic vascular graft materials [52, 53].

In addition, glutaraldehyde crosslinking of collagen-based biomaterials is associated with enhanced calcification of the implant, which has an adverse effect on the mechanical properties of the material [92, 93].

Crosslinking of collagen using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) is based on a well known method in peptide synthesis [94, 95]. The reaction of EDC (X) with carboxylic acid groups of collagen (IX) results in the formation of O-acylisourea (XI). When O-acylisourea reacts with NHS (XII) reactive NHS-esters (XIII) are formed [96], and release of water-soluble 1-ethyl-3(3-aminopropyl)urea (EDU, XIV) occurs. Subsequently, reaction of NHS-esters with free ε-amino groups of (hydroxy) lysine residues results in the formation of peptide crosslinks (XV) and liberated NHS. Direct reaction of O-acylisourea with free ε-amino groups also results in formation of peptide crosslinks. NHS, however, prevents side reactions of O-acylisourea groups like hydrolysis and N-acyl shift to the stable N-acylisourea.
EDC/NHS-crosslinked collagen is reported to be non-cytotoxic in vitro [50], and biocompatibility was demonstrated in animal models [97, 98]. When subcutaneously implanted in rats, calcification of EDC/NHS-crosslinked collagen proved to be very low compared to glutaraldehyde crosslinked collagen [97, 99].

2.4. Collagen thrombogenicity

Collagen is highly thrombogenic, as is demonstrated from its use as a hemostatic powder or sponge [59]. Collagen induces both platelet adhesion and aggregation as well as activation of the intrinsic coagulation cascade. Fibrillar collagen preparations are used as aggregation agent in diagnostic platelet function tests [100]. Whereas contact of blood with an injured vessel wall will result in extrinsic blood coagulation, contact of blood with purified collagen induces activation of the intrinsic pathway, initiated by adsorption of (activated) factor XII [101, 102]. Furthermore, platelet adhesion and aggregation onto collagen provides a source of phospholipids, thus further stimulating coagulation.

Direct platelet adhesion onto collagen is mediated by platelet membrane glycoproteins (i.e. GP Ia/IIa), and requires collagen triple helix structures [103]. Direct platelet-collagen adhesion can be divided in a divalent cation dependent and an independent mechanism. Although not fully understood, the divalent cation dependent mechanism supports direct platelet adhesion to collagen to a rate and extent far greater than that supported by the divalent-cation independent mechanism [104, 105]. Studies employing chemical modification of collagen demonstrate that for direct adhesion of platelets to fibrillar collagen, carboxylic acid groups of aspartic and glutamic acid are essential. During subsequent platelet activation and secretion, ε-amino groups of (hydroxy) lysine residues have an important role [106].

Indirect binding of platelets to collagen is mediated by von Willebrand factor, fibronectin and thrombospondin [105]. Especially von Willebrand factor plays an important role in platelet
interactions with collagen. vWF is a (multimeric) protein with binding sites for factor VIII, heparin, collagen and glycoproteins Ib and IIb/IIIa (GP Ib, GP IIb/IIIa) on platelet membranes [107]. vWF mediates platelet adhesion to collagen or subendothelial structures under conditions of high shear rates (e.g. 600-3000 s\(^{-1}\)) [108-110]. Binding to collagen induces a conformational change in vWF molecules, facilitating binding of GP Ib to collagen-bound vWF [59].

Fibrillar collagen is a strong inducer of platelet aggregation and release [103]. In vitro platelet aggregation by collagen can be reduced by chemical modification of collagen [111, 112], by glutaraldehyde crosslinking [113], by distortion of the quaternary fibril structure of collagen [114, 115], or by immobilization of heparin [116]. In vitro platelet deposition on fibrillar collagen was also decreased after endothelial cells were seeded on the collagen matrix. It was shown that confluent as well as pre-confluent covering of collagen by endothelial cells resulted in a non-adhesive surface for blood platelets. Endothelial cells produce prostacyclin (PG\(I_2\)), which inhibits the formation of thromboxane A\(2\) (TxA\(2\)), and prevents platelet aggregation and platelet secretion, even when the coverage of collagen by endothelial cells is not confluent [117, 118].

2.5. Collagen-coated vascular grafts

Commercially available collagen-coated vascular grafts have been developed to eliminate the time-consuming procedure of clotting of the vascular graft before implantation [8]. Concerns regarding the use of (animal) collagen as coating of vascular grafts include reduced patency rates due to thrombus formation (see above), the possibility of complications due to immune-reactions and complement activation and more recently the possible risk of transmission of Creutzfeld-Jakob disease.

Comparison of the five year patency rates of uncoated and collagen-coated Dacron prostheses used for aortic (bifurcation) replacement in humans showed that patency was not reduced by pre-sealing the graft with collagen [119, 120]. However, patency rates of collagen-coated grafts, when inserted at small-diameter positions, are reported to be lower when compared to autologous saphenous venous tissue [121]. Despite collagen thrombogenicity, however, patency rates of small-diameter collagen-coated grafts were comparable to or somewhat higher than patency rates of non-coated synthetic grafts [4, 121]. Platelet activation and fibrin formation in patients who either received a collagen-impregnated or a non-impregnated Dacron aortoiliac graft was not significantly different. This indicates that collagen-coated Dacron does not stimulate the coagulation cascade any more than conventional Dacron prostheses [122]. It was therefore suggested, that crosslinking of collagen results in decreased thrombogenicity [123, 124].
Complement activation after exposure of various biomaterials to blood has been associated with platelet activation and cell and organ damage [125]. In a randomized trial, significant activation of the complement system was observed after implantation of both collagen-coated vascular grafts and non-impregnated Dacron grafts. However, no differences in complement activation between the coated and the non-coated prostheses were observed. Complement activation was initiated via both the classical and the alternative pathway [126]. When studying the immune-response to collagen-coated Dacron grafts implanted in humans, no cell-mediated immune response was observed, and none of the recipients developed a reactivity to human collagen [127].

3. HEPARIN IMMOBILIZATION ONTO COLLAGEN COATED GRAFTS

3.1 Introduction

Blood coagulation can be initiated by two distinct mechanisms: the intrinsic pathway and the extrinsic pathway (fig. 5). The intrinsic pathway (or contact activation) is activated whenever blood comes into contact with a foreign substance, like purified collagen. Initially, coagulation factor XII is converted to activated factor XIIa. Together with kallikrein and high molecular weight kininogen, XIIa triggers a cascade of enzymatic reactions, resulting in the formation of thrombin and conversion of soluble fibrinogen into fibrin. Damage to the vascular wall exposes tissue factor (tissue thromboplastin) located on cells in the media and the adventitia, and initiates coagulation through the extrinsic pathway [128]. Tissue factor is a transmembrane glycoprotein which functions as a membrane cofactor in the activation of factor VII, and the activation of factor X and factor IX by factor VIIa [129, 130]. Subsequent thrombin generation results in the formation of fibrin.

As discussed above, the patency rates of small-diameter vascular grafts, either collagen coated or not, need to be improved. When a collagen coating is used as a substrate for low density endothelial cell seeding of small diameter vascular grafts, thrombus formation may result in graft failure in the post-operative period when large parts of the collagen-coated graft surface are not endothelialized. Improvement of the blood compatibility of collagen-coated vascular grafts, therefore, may result in improved graft performance.

A frequently applied approach to improve the blood compatibility of biomaterials is covalent immobilization of heparin [15, 131]. Heparin is extremely effective in the prevention of coagulation. However, a major side effect of heparin, when administered systemically, is bleeding [132]. Covalent attachment of heparin to (collagen) surfaces may prevent such side effects to occur.
Heparin is a negatively charged, single chain mucopolysaccharide, with a strong anticoagulant activity. It consists of alternating (1→4) linked uronic acid residues, and glucosamine residues which are either N-sulfated or N-acetylated [133]. Heparin preparations, e.g. from porcine mucosa, are polydisperse, with approximately 15 to 100 monosaccharide units per heparin-chain and a molecular weight ranging from 3,000 to 30,000 gram per mol [134, 135].

**Figure 5:** Simplified representation of the coagulation cascade. TF: tissue factor, PI: phospholipids, HMWK: high molecular weight kininogen.

Heparin increases the activity of the major inhibitor of the coagulation cascade, antithrombin III (AT III), by a factor 2000, resulting in a fast inactivation of thrombin and an effective inhibition of blood coagulation. Besides thrombin and factor Xa, other serine proteases like VIIa, IXa, XIa and XIIa are inhibited by AT III to a lesser extent [136, 137]. AT III binds with high affinity to a specific pentasaccharide unit of heparin (fig 6). Upon binding of AT III to heparin, a conformational change in AT III is induced rendering the active site of AT III more accessible to thrombin [138, 139]. Following non-specific binding of thrombin [140] adjacent to AT III on the heparin molecule, an inactive, irreversible thrombin-AT III (TAT) complex is formed. Subsequently, the TAT-complex can be displaced from the heparin chain by AT III, which has a higher affinity for heparin than the inactive TAT-complex [141]. Binding of both AT III and thrombin to heparin requires at least 18 heparin-saccharide units.

**Figure 6:** Antithrombin binding site of heparin[133].

Covalent immobilization of heparin to various polymeric surfaces has been reported to result in immobilized anticoagulant activity and increased thromboresistance, both *in vitro* and *in vivo* [142-147]. The anticoagulant activity of immobilized heparin is believed to result from similar mechanisms as observed for heparin in solution: immobilized heparin was demonstrated to inactivate thrombin in the presence of AT III [148]. Inactivation of F XIIa by AT III, however, may contribute significantly to the observed improved blood compatibility of heparinized surfaces [149, 150].

Besides inhibition of blood coagulation, heparin also affects platelet function. Heparin in solution is reported to inhibit [151, 152] as well as to induce [153, 154] platelet activation and
aggregation. Pro-aggregating effects of heparin seem to be dependent on molecular weight, low molecular weight heparin fractions being less active in induction of platelet aggregation [155]. Heparin did not influence in vitro platelet aggregation stimulated by a fibrillar collagen suspension [156], and heparin did not significantly inhibit adhesion of washed human platelets to collagen-coated glass at a concentration of 20 U/ml [154]. In pigs, however, a large reduction in platelet deposition onto denuded vessel walls was observed after systemic heparinization [110].

The mechanisms involved in compatibility of surfaces with immobilized heparin towards platelets are not fully understood. Platelets contain heparin binding sites [157-159], which may mediate binding of platelets to immobilized heparin. In addition, heparin binding proteins like fibronectin or thrombospondin can mediate indirect platelet-binding to heparinized surfaces [160-163]. However, in general heparin immobilization of blood contacting surfaces is reported to reduce in vitro and in vivo platelet adhesion and aggregation [116, 142, 146, 164, 165], although increased platelet adhesion after heparin immobilization has been reported as well [166].
3.2 Heparin immobilization to collagen

Immobilization of heparin to collagen based vascular graft materials can be accomplished by ionogenic binding and by covalent binding. Heparin and fibrillar collagens show a strong ionogenic interaction [167]. Binding experiments indicate a Kd varying from $4 \times 10^{-7}$ to $6 \times 10^{-8}$ M [168, 169]. High affinity binding sites for heparin in both the C-terminal [170] and the N-terminal region of collagen molecules [169] have been identified. Dacron vascular grafts coated with a non-crosslinked collagen-heparin dispersion have been described by Weadock et al. [171]. Protamine sulfate, covalently attached to collagen, is often used for ionogenic binding of heparin to collagen [172-175]. Furthermore, cationic surfactants (e.g. benzylalkoniumchloride) adsorbed to glutaraldehyde-crosslinked collagen were described to improve ionogenic binding of heparin [176]. A major drawback of ionic binding of heparin to collagen-coated vascular graft materials is the gradual elution of heparin, which reduces anti-thrombogenicity in time and might possibly result in systemic heparinization (depending on the amount of heparin eluted and the release kinetics).

Covalent immobilization of heparin to collagen results in a more stable heparinized material. Glutaraldehyde is often used for covalent binding of heparin (or glycosaminoglycans in general) to collagen, or collagen-coated vascular graft materials [177-179]. Alternatively, epoxy ether [180] or dialdehyde starch [181] have been used for covalent immobilization of heparin to collagen-based vascular graft materials.

With respect to endothelial cell seeding, cytotoxicity of heparinized materials should be avoided. Analogous to collagen crosslinking, this can be accomplished using EDC and NHS for immobilization of heparin to collagen, as has been described by various authors. When heparin was immobilized onto collagen, by incubation of equal amounts of heparin, collagen powder and EDC, the immobilized heparin partly retained its anticoagulant activity [182]. Senatore immobilized heparin to collagen powder or collagenous tubes (i.e. pretreated animal carotid arteries or human umbilical vein grafts). Heparin was activated with EDC, and activated heparin was subsequently immobilized to collagen. After pretreatment with NH$_4$OH, to increase the number of free amino groups of collagen, heparinization of lamb carotid arteries resulted in a matrix containing 30 mg of immobilized heparin per cm$^2$ of graft material [183, 184]. Immobilization of heparin onto collagen was also carried out using a cyclohexyl-carbodiimide [185, 186], resulting in 15.2 mg immobilized heparin per gram of collagen. After chemical modification of collagen, by introducing additional amino groups, 47.1 mg heparin was immobilized per gram of collagen using the same immobilization procedure.

Several studies have shown that immobilization of heparin on collagen-coated vascular grafts reduces in vivo thrombogenicity. In a rabbit model, epoxy ether crosslinked heterografts
Endothelial cell seeding of collagen-coated vascular grafts showed an improved patency rate after heparin immobilization (96%, versus 0% patency for a non heparinized graft, after one week) [180]. In dogs, comparable results were found for gelatin-coated Corethane (100% vs. 60% patency after 6 months) [187], and collagen or gelatin-coated ePTFE (92% vs. 77% patency after 3 months) [180, 181, 187]. After implantation in the carotid arteries of dogs, the heparinized grafts remained patent for a period of 15 ± 5 days, while control grafts occluded in 4 ± 3 days [116, 183, 184, 188].

3.3. Influence of heparin immobilization on cell proliferation

Heparin immobilization to collagen coated vascular grafts may affect cell or tissue interactions. When added to in vitro endothelial cell cultures, heparin is reported both to enhance and inhibit endothelial cell proliferation [189-191]. Endothelial cells in culture preferentially bind higher molecular weight heparin fractions [192, 193]. Such binding is saturable and reversible [194]. Bound heparin is partially internalized and degraded [195, 196].

The effect of heparin immobilization on the proliferation of seeded endothelial cells is uncertain. Heparin immobilization is both reported to inhibit [197] as well as promote [198] proliferation of endothelial cells when compared to corresponding matrices without immobilized heparin. Heparin immobilization is reported to inhibit attachment and growth of fibroblasts in vitro [199]. In addition, immobilized heparin inhibits proliferation of cultured smooth muscle cells [200], thereby preventing the formation of a thickened tissue layer (intimal hyperplasia) near the anastomoses of vascular grafts, which often leads to graft failure.

4. BASIC FIBROBLAST GROWTH FACTOR

4.1. Introduction

Peptide growth factors can generally be defined as proteins which promote proliferation and migration of cells, after interaction with specific cell-membrane receptors [201]. Several peptide growth factors, or growth factor families can be identified, including epidermal growth factors, platelet-derived growth factor, insulin-like growth factors and fibroblast growth factors [202]. There are at least 9 different, structurally related, human fibroblast growth factors. In terms of interactions with endothelial cells, acidic FGF (aFGF) and basic FGF (bFGF) are the best characterized [203]. aFGF and bFGF have a structural homology of 55%. However, aFGF is far less potent in stimulating proliferation of endothelial cells [204].
bFGF is a 18 kD protein which induces, *inter alia*, the proliferation of endothelial cells, fibroblasts, smooth muscle cells and chondrocytes [205, 206]. bFGF is also known as heparin-binding growth factor 2 (HBGF-2), because of its high affinity for heparin and heparan sulfate. At physiological pH and temperature, the *in vitro* half-life time of bFGF activity is approximately 12 hours [207]. Binding of bFGF to heparin induces a conformational change in the bFGF molecule [208], resulting in an increased resistance against thermal denaturation and enzymatic degradation [207-210]. Binding of bFGF and heparin is mediated by ionic interaction between both 2-0-sulfate groups and N-sulfate groups of heparin molecules [211-213] and specific lysine and arginine residues on bFGF [214, 215]. Depending on its molecular weight, heparin can bind up to 13 molecules of bFGF per molecule of heparin in solution [210, 216].

bFGF is localized in almost any tissue examined [217]. Despite the overall presence of bFGF, suggesting that it is needed continuously, the turnover rate of target cells like for example endothelium is measured in years. Heparan sulfate, a heparin-like glycosaminoglycan, is abundantly present in the extracellular matrix of endothelial cells. The high affinity of bFGF for glycosaminoglycans suggests that the ECM may function as a storage pool for bFGF [218, 219]. When the vascular wall is damaged, bFGF can be released through several mechanisms [220] and proliferation of endothelial cells will be induced. Endothelial cells in culture need a minimal (“critical”) seeding density for induction of proliferation and subsequent formation of a confluent endothelial monolayer. Using bFGF, this minimal seeding density can be significantly lowered [14, 221, 222].

4.2. Mitogenic activity of bFGF

The mechanism of action of bFGF is not completely elucidated. Induction of mitogenesis by bFGF requires interaction of bFGF with both cell surface heparan sulfate (HS) and FGF-receptors (FGFR). HS, a heparin-like glycosaminoglycan, is on cell surfaces covalently linked to cell surface glycoproteins like perlecan or syndecan [223, 224]. Four fibroblast growth factor receptors (FGFR-1 to FGFR-4, Kd 2-20*10⁻¹¹ M) have been identified [203, 225, 226]. Binding of bFGF to HS (Kd 2*10⁻⁹ M) induces a conformational change in the bFGF molecule, facilitating the binding of bFGF to high affinity FGFR [208, 226]. The stoichiometry of this receptor system is still a point of debate, and may involve either a 1: 1: 1 complex of HS, bFGF and FGFR (HS:bFGF:FGFR) [227], or may contain dimers of bFGF and/or FGFR (HS:bFGF:2FGFR, HS:2bFGF:2FGFR [228-230]). Binding of bFGF to FGFR induces tyrosine-kinase activity on the cytosolic part of the receptor. The phosphorylation cascade from the Ras-protein to a variety of substrates leads to rapid activation of the *fos* and *myc*-genes, which is an early key event in the cellular response to a growth factor signal. The
activation of these "early genes" is considered to be essential for later events in cell division and proliferation [225, 231-233].

After receptor binding and internalization, bFGF or bFGF fragments are partially translocated to the cell nucleus [234-236], and following uptake in the nucleus, DNA synthesis and cell proliferation is stimulated [232, 237, 238]. Fibroblasts lacking cell surface heparan sulfate demonstrated substantially reduced binding of bFGF to cell surface receptors, thus inhibiting bFGF-induced fibroblast growth. Binding of bFGF to these cells was restored by low concentrations of exogenous heparin (1 ng/ml) [239, 240].

bFGF internalized via FGFR-independent pathways is directed to lysozomal vesicles and is subsequently degraded [235, 241, 242].

4.3. bFGF release

When injected intravenously, bFGF is rapidly cleared. In rats, half-life times of 1.5 to 3 minutes are reported [243, 244]. Subcutaneous injection of bFGF-containing gelatin hydrogels strongly induced local neo-vascularization in mice, whereas subcutaneous injection of either bFGF or gelatin hydrogel had no effect [245]. This suggests that local sustained release from a suitable matrix is the most effective method of in vivo bFGF-administration.

Controlled release of peptide growth factors, which is widely explored in tissue engineering, is used to improve angiogenesis of implanted matrices [246, 247] or proliferation of cells seeded on certain matrices [248-251]. Tefft et al. described a wound dressing prepared by reaction of EDC-activated heparin with a mixture of collagen and starch, displaying bFGF binding with an efficiency of 85% [252]. Silver made a wound dressing of a cyanamide-crosslinked collagen sponge containing bFGF. When applied on full thickness dermal wounds, an increased degree of re-epithelialization was observed when compared to collagen sponges without bFGF [248, 253]. Thompson et al. seeded hepatocytes in a matrix of gelatin or collagen pre-loaded with aFGF. After implantation in rats, the matrix was rapidly vascularized, thus providing a source of nutrients and oxygen for the seeded cells. It was suggested that this technique might be used for the development of new organoid structures in vivo [247, 254, 255].

A connective tissue substitute was described by Doillon et al. Fibrin-coated collagen sponges, containing heparin and bFGF, were used in an in vivo model. Subcutaneous implantation in mice resulted in a fast infiltration of fibroblasts [219, 256]. Edelman et al. developed a system, consisting of bFGF-loaded heparin-sepharose embedded in alginate microspheres. When used in combination with heparinase, bFGF-release is controlled by the amount of added heparinase. Heparinase cleaves heparin, resulting in a release of a complex of bFGF with a heparin fragment [257, 258].
Local sustained bFGF release has been shown to improve spontaneous endothelialization of vascular grafts in vivo. A fibrin glue sealant containing heparin and aFGF was developed to induce spontaneous endothelialization when applied as coating of synthetic vascular graft materials [259, 260]. By adjusting the ratio of heparin and growth factor in the fibrin glue, proliferation of seeded endothelial cells was accelerated while proliferation of smooth muscle cells could be inhibited in vitro [261]. Spontaneous endothelialization of these vascular grafts was found to be increased when implanted in rats, dogs and rabbits [259, 262, 263]. Polyurethane grafts coated with a mixture of photoreactive gelatin, heparin and bFGF, demonstrated spontaneous endothelialization as a result of transmural in-growth of endothelial cells, when implanted in rat aortas [264]. Until now, local sustained release of bFGF from vascular grafts in combination with endothelial cell seeding has not been described.

5. CONCLUSIONS

Patency rates of small-diameter synthetic vascular grafts are disappointing. Endothelial cell seeding is an appreciated strategy to improve the performance of vascular grafts. Immediate endothelial cell seeding of vascular grafts is preferred above delayed seeding techniques, to minimize the risk of bacterial infection and to provide a graft that is directly available for implantation. The consequence of immediate seeding is incomplete coverage of the graft surface due to a limited supply of autologous endothelial cells. Most likely because of low seeding densities, immediate seeding caused disappointing results in clinical studies. The work described in this thesis is directed to the development of a small diameter vascular graft substrate with characteristics optimal for low density cell seeding. A literature survey demonstrated that a non-cytotoxic, biocompatible collagen matrix, containing immobilized heparin and basic fibroblast growth factor, might provide such properties. Commercially available collagen-coated grafts are crosslinked using glutaraldehyde or formaldehyde, rendering the matrix unsuitable for cell seeding. Various studies demonstrate that EDC/NHS-crosslinked collagen matrices are non-cytotoxic and biocompatible. An EDC/NHS-crosslinked collagen therefore is expected to be a suitable matrix for endothelial cell seeding. Collagen, however, is a thrombogenic material. Early graft occlusion in the period after cell seeding, when a large part of the graft surface is not yet endothelialized, leads to low patency rates. Immobilization of heparin to the crosslinked collagen coating might prevent thrombus formation and improve graft performance. Although the effect of heparin immobilization on endothelial cell proliferation remains unclear, heparin immobilization may prevent graft failure due to intimal hyperplasia, by inhibition of smooth muscle cell proliferation.
Local sustained release of bFGF from heparinized collagen is expected to improve the proliferation of endothelial cells. Furthermore, bFGF is likely to allow lower seeding densities than grafts without bFGF pre-loading, thus increasing the chance of successful endothelialization of vascular grafts after immediate, low density endothelial cell seeding.

REFERENCES


Endothelial cell seeding of collagen-coated vascular grafts


Endothelial cell seeding of collagen-coated vascular grafts


Endothelial cell seeding of collagen-coated vascular grafts


Endothelial cell seeding of collagen-coated vascular grafts


CHAPTER 3

An EDC/NHS-crosslinked collagen substrate for endothelial cell seeding: Preparation, characterization and the effect of γ-sterilization

Institute for Biomedical Technology, Polymer Chemistry and Biomaterials Group, Department of Chemical Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

ABSTRACT

As part of the development of a suitable, non-cytotoxic substrate for endothelial cell seeding, type I collagen matrices were crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS). The objectives of this study were to optimize the crosslink reaction and to study the relation between crosslink density and material properties. Furthermore, the effect of γ-sterilization on crosslinked collagen was studied.

Crosslinking of collagen was demonstrated by a decrease in the number of free primary amino groups, and an increase in the shrinkage temperature of the material. Compared to non-crosslinked collagen (N-Coll, 27 free primary amino groups per 1,000 amino acid residues), EDC/NHS-crosslinked collagen showed an improved tensile strength, E-modulus and resistance to in vitro collagenase degradation, which increased with increasing crosslink densities (e.g. 23, 16 and 13 free primary amino groups per 1,000 amino acid residues after crosslinking, E/N23C; E/N16C and E/N13C). After 24 hours incubation in collagenase solution, the weight loss of E/N13C was only 4 % whereas 80 % of N-Coll was solubilized within 5 hours.

Gamma-irradiation of E/N23C, E/N16C and E/N13C at a dose of 25 kGy did not affect the number of free primary amino groups, while the shrinkage temperature was decreased by approximately 15°C. Both E-modulus and tensile strength of crosslinked collagen were not affected by γ-irradiation, except for the tensile strength of E/N13C which was decreased by 20%. Whereas before sterilization no relation between crosslink density and elongation at
break was observed, the elongation at break of EDC/NHS-crosslinked collagen decreased with increasing crosslink-density after γ-irradiation. After γ-irradiation of (crosslinked) collagen matrices, the susceptibility to in vitro collagenase degradation was increased especially for N-Coll and E/N23C, while the degradation rate of E/N13C was not affected.

It is concluded that EDC/NHS-crosslinked collagen with relatively high crosslink densities, like E/N13C, demonstrates improved material properties, which are largely retained after γ-sterilization. Therefore, these matrices will be further evaluated as substrates for endothelial cell seeding of vascular grafts.

1. INTRODUCTION

Synthetic vascular grafts are porous structures, generally made of knitted or woven Dacron or expanded Teflon [1]. For many years, vascular grafts have been successfully used for the replacement of large-diameter vessels. In contrast, small-diameter vascular grafts (diameter 5 mm or less) have disappointing patency rates, primarily due to thrombus formation [2-4]. Endothelial cell seeding is a recognized strategy to improve the performance of small-diameter vascular grafts [5, 6]. Dacron or Teflon substrates, however, are not suitable for endothelial cell seeding [7, 8]. Since non-crosslinked collagen is a suitable matrix for the growth of endothelial cells in vitro [9, 10], application of collagen coating on synthetic vascular graft materials may result in a matrix suitable for in vivo endothelial cell seeding.

Enzymatic degradation of non-crosslinked collagen after implantation is usually very fast, but can be delayed and controlled by chemical crosslinking [11], thus maintaining structural stability [12] and providing a scaffold for endothelial cells over a prolonged period of time. A crosslinked-collagen coating for synthetic vascular grafts, however, has to meet several requirements. Crosslinking should not affect the suitability of collagen as a substrate for endothelial cell seeding. Secondly, application of a crosslinked-collagen coating should not or only slightly modify the mechanical properties (i.e. stiffness and compliance) of a synthetic vascular graft. Gamma-sterilization of (crosslinked) collagen, necessary for in vivo applications, should not affect the degradation rate, the mechanical properties and the suitability of the material as a matrix for endothelial cell seeding.

Collagen, gelatin and albumin impregnated synthetic vascular grafts originally have been developed to eliminate the procedure of preclotting of the porous Dacron prior to implantation [13]. Glutaraldehyde or formaldehyde, which are commonly used in commercially available collagen-coated vascular grafts [13], are incorporated in the collagen matrix during crosslinking. During in vitro and in vivo degradation, especially glutaraldehyde-crosslinked collagen evokes cytotoxic reactions due to release of (unreacted) glutaraldehyde and
glutaraldehyde derivatives [14-16], thus hampering endothelialization of the luminal graft surface [16, 17]. Crosslinking of collagen using the water soluble N-(3-dimethyl-aminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) results in a material that has shown to be non-cytotoxic in vitro and biocompatible in vivo [14, 18]. The reaction of EDC with carboxylic acid groups results in the formation of O-acylisourea. Subsequently, the reaction of O-acylisourea with NHS will give rise to NHS-activated esters, which in turn react with primary amino groups to form amide bonds and releasing NHS [19, 20]. When used to crosslink collagen, peptide bonds are formed between glutamic- or aspartic acid residues, and lysine- or hydroxylysine residues. The use of NHS reduces side reactions of the EDC-activated groups such as hydrolysis or N-acyl shift to form stable N-acylisoureas [19].

In this study, EDC/NHS-crosslinking of a collagen matrix for endothelial cell seeding was investigated. The crosslink reaction itself, the influence of crosslinking on mechanical properties, the resistance to in vitro enzymatic degradation of collagen and the effect of gamma-sterilization was studied. It was shown that the proliferative characteristics of endothelial cells seeded on collagen may be influenced by physical and chemical alterations introduced by crosslinking and γ-sterilization.

2. MATERIALS AND METHODS

2.1. Materials

Unless otherwise stated, chemicals were obtained from Merck (Darmstadt, Germany), and were of the highest purity available.

2.2. Collagen films

Type I insoluble collagen (1 g) derived from Bovine Achilles Tendon (Sigma, St.Louis, MO, C 99879, lot 23H7065) was swollen overnight in 0.52 M acetic acid solution (50 ml) at 4°C. The mixture was dispersed with 50 g of crushed ice for 4 minutes in a Philips Blender and thereafter homogenized for 30 minutes at 4°C using an Ultra-Turrax T25 (IKA labortechnik, Staufen, BRD). The resulting slurry was filtered through a series of filters (Cellctor screen, Bellco, Feltham, UK), with a pore size decreasing from 140 µm to 10 µm, mounted in 47 millimeter diameter Swinnex disc filter holders (Millipore, Etten-Leur, The Netherlands). After de-aeration at a pressure of 0.06 mbar, the resulting suspension was casted as a film with a thickness of 3 millimeters on a flat poly(ethylene terephthalate) (PET) surface, using a
casting knife. After drying at room temperature, a collagen film with a thickness of approximately 50 μm was obtained.

2.3. Collagen crosslinking

Collagen films were crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). In order to minimize hydrolysis of EDC, crosslinking was carried out in a buffer of 2-morpholinoethane sulfonic acid (MES buffer, 0.05 M, pH adjusted using 10 M NaOH) [21]. Dried collagen films were incubated with MES buffer for 30 minutes. Subsequently, the films were immersed in a solution of EDC and NHS in MES buffer, and crosslinking was carried out under gentle shaking. Typically, crosslinking was carried out at pH 5.40, using 0.731 g EDC and 0.415 g NHS in 215 ml MES-buffer per gram of collagen (1.29 mmol carboxylic acid groups, Coll-COOH, per gram of collagen [22], resulting in a molar ratio of EDC : NHS : Coll-COOH of 7.0 : 2.8 : 1). At selected times (15 minutes to 4 hours), crosslinking was stopped by washing the collagen film with 0.1 M Na₂HPO₄ solution for 2 hours. This treatment results in hydrolysis of both activated carboxylic acid groups and remaining EDC [21, 23]. After repeated washing with demineralized water (four times for 30 minutes) collagen was stored over KOH in a desiccator. Crosslinking of collagen was investigated as a function of reaction time (up to 4 hours), reaction volume (100 to 500 ml), pH (4.5 to 6.5), and amounts of EDC (molar ratio of EDC to Coll-COOH up to 12.5) and NHS (molar ratio of NHS to EDC of 0.2 to 1.0) used.

2.4. Assay for primary amino groups

The residual number of free primary amino groups in collagen after crosslinking was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS, Fluka, Buchs, Switzerland), according to a procedure described by Wang et al. [24, 25]. Samples of approximately 4 mg of (crosslinked) collagen were incubated with NaHCO₃ solution (4 wt.%, 1 ml) for 1 hour at room temperature. Thereafter, TNBS solution in water (0.5 wt.%, 1 ml) was added and the mixture was incubated at 40°C for 2 hour. Collagen samples were hydrolyzed at 60°C for 90 minutes after addition of 3.0 ml 6 M HCl. The absorbance was measured at 345 nm, after dilution with water (5 ml) and cooling to room temperature, using an Uvikon 930 spectrophotometer (Kontron Instruments, Switzerland). A reference sample for UV measurements was prepared using the same procedure, except that HCl was added prior to the TNBS solution. For calculation of the number of unreacted amino groups a molar absorption coefficient of 14,600 l mol⁻¹ cm⁻¹ for trinitrophenyl lysine was used.
Results were expressed as numbers of free primary amino groups per 1,000 amino-acid residues.

2.5. Shrinkage temperature

The shrinkage temperature of (crosslinked) collagen was determined using Differential Scanning Calorimetry (DSC) [26]. A sample of approximately 2.5 mg collagen was incubated with 50 µl phosphate buffered saline (PBS) (NPBI, Emmer Compascuum, The Netherlands) for 1 hour at room temperature in a sealed volatile sample pan (Perkin-Elmer, Norwalk, CT). Thereafter, the DSC thermogram was recorded on a Perkin Elmer DSC 7 DSC-apparatus. Samples were heated from 20°C to 95°C at a heating rate of 5°C per minute. A sample containing 50 µl PBS was used as a reference. The onset of the endothermic peak, which indicated denaturation of collagen, was recorded as the shrinkage temperature.

2.6. Swelling

Dried collagen samples were weighed and incubated with PBS for 24 hours at room temperature. Swollen samples were gently blotted on filter paper, and weighed again. The swelling of the samples was expressed as (wet weight/dry weight)×100%. Since γ-irradiation of collagen (see below) may induce denaturation, the swelling of denaturated collagen was determined as well. Dried collagen samples were weighed and incubated with demineralized water for 24 hours. Subsequently, the samples were denaturated by immersion for 60 seconds in boiling demineralized water. Following incubation with PBS (2 times for 24 hours) at room temperature collagen swelling was determined as described above.

2.7. Scanning electron microscopy

The surface morphology of (crosslinked) collagen films was examined using scanning electron microscopy. Collagen films were washed in distilled water, and dehydrated using a graded series of ethanol. After drying in vacuo at room temperature the specimens were sputter coated with gold and examined using a Hitachi S-800 field emission scanning electron microscope (Hitachi, Tokyo, Japan) at an acceleration voltage of 7 kV.
2.8. Mechanical properties

Dried collagen films were incubated with PBS for 24 hours at room temperature. Test samples (4 cm length, 4.0 mm width) were prepared with the intended direction of elongation during tensile testing parallel to the casting direction of the collagen films. The thickness of the wet samples in the test area was measured at three points, using an electronic micrometer (Mitutoyo, Tokyo, Japan). Stress-strain curves were obtained using a Zwick Z020 tensile tester (Zwick, Ulm, Germany), with a load cell of 500 N, a sample length of 10.0 mm and an elongation speed of 2 mm per minute.

2.9. In vitro collagen degradation

Collagen samples (10.0 ± 0.2 mg) were incubated at 37°C with 0.5 ml of Tris-HCl buffer (0.1 M, pH 7.40, containing 0.005 M CaCl₂ and 0.05 mg/ml NaN₃). After 1 hour, collagenase solution (0.5 ml, in the same Tris-buffer) was added, giving a final collagenase concentration of 100 U/ml. The collagenase used (Sigma, EC 3.4.24.3 from Clostridium Hystolyticum) had an activity of 289 U/mg solid. Degradation was stopped at selected times (up to 24 hours) by adding EDTA solution (Titriplex III, 0.1 ml, 0.25 M in Tris-buffer without CaCl₂) and cooling on ice. After centrifugation of the supernatant (600 g, 10 minutes), the amount of solubilized collagen was determined using a spectrophotometric assay for hydroxyproline. Degradation of the collagen samples was expressed as the percentage of weight remaining after different times of degradation.

2.10. Hydroxyproline-assay

From the filtered supernatants of in vitro collagen degradation experiments, 0.1 ml was added to 0.9 ml of 6 M HCl in a test-tube. After evacuation to 0.06 mbar, the test-tubes were sealed, and the soluble collagen fragments were hydrolyzed for 20 hours at 110°C. Hydrolysates were dried in vacuo at room temperature, and were redissolved in citrate/phosphate buffer (0.005 M citric acid, 0.01 M Na₂HPO₄, pH 6.0), giving estimated hydroxyproline concentrations between 2 and 10 mg/ml. Of these solutions, 1.00 ml was reacted with Chloramine-T solution (0.5 wt.%, 1.0 ml) in citrate/phosphate buffer containing 2-propanol (5%, v/v), at room temperature. After 18 minutes, Ehrlich’s reagens (2.0 ml) was added, and the temperature was raised to 60°C. Ehrlich’s reagens consisted of a solution of 7 g dimethylaminobenzaldehyde and 15 ml 60% perchloric acid in 2-propanol, with a total volume of 100 ml. The reaction was stopped after 12.5 minutes by cooling the solution to room temperature, and the absorbance at 555 nm was read using an Uvikon 930 spectrophotometer. A blank was prepared from 1.00 ml
citrate/phosphate buffer without hydrolyzed collagen fragments. The collagen content of the samples was calculated from a calibration curve obtained from hydrolyzed collagen samples with predetermined weight [27].

2.11. Gamma sterilization

For γ-sterilization, dried collagen films were placed in 50 ml centrifugation tubes (Costar, Cambridge, MA) which were exposed to a 60Co-source of 900,000 Ci (Gammaster, Ede, The Netherlands), until a total dose of 25 kGy was reached [28].

2.12. Statistical analysis

Results were expressed as mean ± standard deviation. For statistical analysis, a Student t-test (using GraphPad InStat, GraphPad Software, San Diego, CA) was used. Results were considered significantly different with p < 0.05.

3. RESULTS

Crosslinking of collagen using EDC and NHS results in the formation of peptide-like crosslinks between ε-amino groups, from lysine or hydroxyllysine residues, and carboxylic acid groups from aspartic or glutamic acid residues. For non-crosslinked collagen (N-Coll), the number of free primary amino groups per 1,000 amino acid residues (n/1,000) amounts to 27 [29]. Maximal crosslinking, defined as a maximal decrease in primary amino groups was observed at pH 5.40 (as determined in a pH range from 4.5 to 6.5, data not shown). At pH 5.40, crosslinking using increasing ratios of EDC to collagen carboxylic acid groups (Coll-COOH) resulted in decreasing numbers of primary amino groups (fig 1A). Maximal crosslinking was observed using a molar ratio of EDC : Coll-COOH of more than 5, resulting in crosslinked collagen containing approximately 15 primary amino groups per 1,000 amino acid residues. Crosslinking of collagen at pH 5.40 at a molar ratio of EDC : Coll-COOH of 7.0 was maximal when using a molar ratio of NHS to EDC of 0.4 (EDC : NHS : Coll-COOH = 7.0 : 2.8 : 1.0); higher molar ratios of NHS to EDC resulted in slightly higher numbers of free amino groups after crosslinking (fig. 1B). At these conditions, crosslinking was complete within 4 hours (fig. 1C). When crosslinking was carried out in 100 ml buffer per gram of collagen, the number of free primary amino groups after crosslinking was 9.6 ± 0.4 per 1,000 amino acid residues (fig. 1D). With increasing reaction volume, crosslinking decreased. The largest increase in free primary amino groups after crosslinking was observed upon the initial
Figure 1: Crosslinking of collagen as function of the ratio of EDC to collagen carboxylic acid groups (A), the ratio of EDC to NHS (B), the reaction time (C) and the reaction volume (D), expressed as the number of free primary amino groups per 1,000 amino acid residues after crosslinking (n = 4, mean ± SD).

Crosslink-conditions: (fig. 1A) pH = 5.40. EDC : NHS = 1.0 : 1.0 (mol : mol), 4 hours reaction in 215 ml buffer per gram collagen; (fig. 1B) EDC : Coll-COOH = 7.0 : 1.0 (mol : mol), 4 hours reaction in 215 ml buffer per gram collagen; (fig. 1C) EDC : NHS : Coll-COOH = 7.0 : 2.8 : 1 (mol : mol : mol), in 215 ml buffer per gram collagen; and (fig. 1D) EDC : NHS : Coll-COOH = 7.0 : 2.8 : 1 (mol : mol : mol), 4 hours reaction.
An EDC/NHS-crosslinked collagen substrate for endothelial cell seeding

Figure 2: Shrinkage temperature versus the number of free primary amino groups of EDC/NHS-crosslinked collagen (n = 2, mean ± SD).

![Shrinkage temperature versus amino groups](image)

Figure 3: SEM image of non-crosslinked collagen (N-Coll, fig. 3A) and E/N14-collagen (E/N14C, fig 3B), original magnification 500 x.

volume increase from 100 to 200 ml per gram of collagen. When crosslinking was performed in 215 ml MES-buffer per gram of collagen, a crosslinked material containing 14 free primary amino groups per 1,000 amino acid residues (E/N14C) was obtained. The shrinkage temperature showed an inverse correlation with the number of free primary amino groups of EDC/NHS-crosslinked collagen (fig. 2).
Using scanning electron microscopy, no morphological differences were observed between N-Coll (fig. 3A) and EDC/NHS-crosslinked collagen (fig. 3B, representative image, E/N14C). Both materials showed a relatively flat surface without defects.

For further experiments, collagen was crosslinked using a reaction volume of 215 ml MES buffer (pH 5.40) per gram of collagen and a molar ratio of EDC : NHS : Coll-COOH of 7.0 : 2.8 : 1. To obtain EDC/NHS-crosslinked collagen matrices with different degrees of crosslinking, the crosslinking-time was used as a variable. When crosslink-times between 15 minutes and 4 hours were applied, crosslinked collagen containing 22 to 14 free primary amino groups per 1,000 amino acid residues was obtained. Alternatively, to obtain EDC/NHS-crosslinked collagen matrices with higher crosslink-densities a reaction volume of 100 ml per gram of collagen and a crosslink time of 4 hours was applied.

Materials containing 23, 16 and 13 free primary amino groups per 1,000 amino acid residues after crosslinking (Table I, E/N23C, E/N16C and E/N13C, respectively) were used for γ-sterilization. Sterilization did not significantly affect the number of free primary amino groups. The shrinkage temperature, however, significantly decreased (13 to 15°C) as a result of sterilization.

**Table I**: Shrinkage temperature and number of free primary amino groups per 1,000 amino acid residues (n = 3, mean ± SD) of non-sterilized collagen and gamma sterilized collagen.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Free amino groups (n/1,000)</th>
<th>Shrinkage temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not sterilized</td>
<td>γ-sterilized</td>
</tr>
<tr>
<td>N-Coll</td>
<td>27.3 ± 0.3</td>
<td>28.2 ± 0.6</td>
</tr>
<tr>
<td>E/N23C</td>
<td>22.8 ± 0.1</td>
<td>23.3 ± 1.0</td>
</tr>
<tr>
<td>E/N16C</td>
<td>16.4 ± 0.5</td>
<td>14.7 ± 1.2</td>
</tr>
<tr>
<td>E/N13C</td>
<td>12.8 ± 0.5</td>
<td>13.7 ± 0.7</td>
</tr>
</tbody>
</table>

n.d.: not detectable.

**Table II**: Mechanical properties of non-sterilized and gamma sterilized collagen (n = 6, mean ± SD).

<table>
<thead>
<tr>
<th>Sample</th>
<th>E-Modulus (MPa)</th>
<th>Tensile strength (MPa)</th>
<th>Elongation at break (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not sterilized</td>
<td>γ-sterilized</td>
<td>Not sterilized</td>
</tr>
<tr>
<td>E/N23C</td>
<td>12.1 ± 1.8</td>
<td>11.0 ± 1.8</td>
<td>10.4 ± 0.5</td>
</tr>
<tr>
<td>E/N16C</td>
<td>16.2 ± 0.8</td>
<td>16.9 ± 2.2</td>
<td>13.4 ± 2.1</td>
</tr>
<tr>
<td>E/N13C</td>
<td>27.9 ± 4.1</td>
<td>28.5 ± 1.1</td>
<td>16.3 ± 1.9</td>
</tr>
</tbody>
</table>

†: Significantly different from non-sterilized sample (p < 0.05).
In table II the mechanical properties of crosslinked collagen before and after γ-sterilization, are given. Non-crosslinked collagen was not included because of poor mechanical properties. The low strain E-modulus and the tensile strength significantly increased with increasing crosslink densities. For non-sterilized E/N13C a modulus of 27.9 MPa and a tensile strength of 16.3 MPa were found. In contrast, the elongation at break of crosslinked collagen was not dependent on the crosslink density of the material. Gamma-sterilization had no significant effect on the low strain E-modulus and the tensile strength, except for the tensile strength of E/N13C which was decreased after γ-irradiation. The influence of γ-irradiation on the elongation at break was not consistent. After γ-irradiation the elongation at break of E/N23C and E/N16C was increased, whereas E/N13C demonstrated a decrease.

**Figure 4:** In vitro collagenase degradation of non-sterilized (EDC/NHS-crosslinked) collagen (A) and γ-sterilized (EDC/NHS-crosslinked) collagen (B) as a function of degradation time (n = 3, mean ± SD).
With increasing crosslink densities, the resistance to \textit{in vitro} collagenase degradation of non-sterilized collagen was increased (fig. 4A). After exposure to a solution of collagenase, N-Coll was degraded within hours, whereas for E/N16C and E/N13C the weight loss after 24 hours degradation was only 8% and 4%, respectively. Gamma irradiation of all collagen samples, but especially samples with a low crosslink density (E/N16C, E/N23C), resulted in an increased weight loss after 24 hours of incubation in the collagenase solution (figure 5). Gamma irradiation hardly influenced the rate of collagenase degradation of materials with
higher crosslink densities (E/N13C, 4% after 24 hours). During the first 6 hours of degradation, the rate of weight loss of γ-irradiated N-Coll and EN23C was remarkably higher as compared to non-sterilized samples (fig. 4A, B). Crosslinking of collagen resulted in a large reduction of swelling in PBS (324% versus 230%, for N-Coll versus E/N13C, respectively) (fig. 6). Upon sterilization of collagen, a small but significant (p < 0.005) decrease in swelling was observed for all samples. Denaturation of sterilized and non-sterilized samples resulted in increased swelling, this effect was most pronounced for N-Coll.

4. DISCUSSION

Commercially available protein-coated synthetic vascular grafts are crosslinked using formaldehyde or glutaraldehyde [13]. Due to cytotoxicity [14], these grafts do not support proliferation of seeded endothelial cells [16, 30]. To provide a suitable substrate for in vivo endothelial cell seeding, collagen was crosslinked using EDC and NHS which leads to the formation of peptide crosslinks between carboxylic acid groups and free primary amino groups of collagen. EDC/NHS-crosslinked collagen is reported to be non-cytotoxic, both in vitro [14] and upon implantation in experimental animals [18]. Crosslinking of collagen using EDC and NHS was rather inefficient with regard to the amount of EDC needed to obtain a maximal decrease in free amino groups (fig. 1A), as also observed by others [20]. Hydrolysis of EDC may in part be responsible for this observation: in 0.05 M MES buffer, pH 5, the half life time of EDC is reported to be 3.9 hours [21]. Crosslinking was maximal using a molar ratio of NHS to EDC of 0.4 (fig. 1B). Increased crosslinking using increasing ratios of NHS : EDC resulted from conversion of EDC-activated carboxylic acid groups to reactive NHS-esters, thus preventing side reactions like hydrolysis of EDC activated groups and O-N-acyl shift. However, a possible side reaction between NHS and EDC has been reported, especially at high molar ratios (i.e. above 0.4) [20, 31]. The resulting depletion of EDC and NHS from the crosslink solution adversely affects crosslinking of collagen, which might explain the slight increase in the number of free amino groups observed when collagen was crosslinked at a higher NHS to EDC ratio (i.e. above 0.4). Crosslinking was completed within 4 hours (fig. 1C). Formation of NHS-activated esters in collagen, however, is reported to be much faster [20]. Therefore, the time necessary to obtain maximal crosslinking is determined by subsequent formation of crosslinks by the reaction of NHS-esters with primary amino groups. Furthermore, depletion of EDC due to hydrolysis may account in part for the observation that no further crosslinking occurred after longer reaction times.
For experimental reasons crosslinking of collagen was first carried out using a buffer volume of 215 ml per gram of collagen. A maximal decrease in free primary amino groups from 27 per 1,000 amino acid residues for non-crosslinked collagen (N-Coll) to 14 for EDC/NHS-crosslinked collagen (E/N14C) was observed. Concomitantly the shrinkage temperature, a parameter for the resistance of (crosslinked) collagen against thermal denaturation, increased from 55.4 \(\pm\) 0.6 °C for N-Coll to 79.3 \(\pm\) 0.7 °C for E/N14C. When reducing the buffer volume in which crosslinking was carried out (e.g. below 200 ml per gram of collagen), materials with higher crosslink densities were obtained, probably due to a higher concentration of the crosslink agents. When using 100 ml buffer per gram of collagen, the number of free primary amino groups after crosslinking was 9.6 \(\pm\) 0.4 per 1,000 amino acid residues.

The decrease in free amino groups after EDC/NHS-crosslinking of collagen was inversely related to the shrinkage temperature (Ts) (fig. 2). This indicates, that the shrinkage temperature of EDC/NHS-crosslinked collagen is an independent measure for the degree of crosslinking. When hydrated collagen is heated, the material will denature at a specific temperature. Denaturation is characterized by non-reversible endothermic transition of the triple helix of the collagen molecule to a random coil, a process which is accompanied by a macroscopic shrinkage of the material. Crosslinking results in stabilization of the triple helix structure, thus increasing the shrinkage temperature [32].

Depending on the form of collagen (purified collagenous tissues, reconstituted collagen fibers or films, pepsin solubilized collagen), and the type of crosslinking used, materials with a wide range of mechanical properties are obtained. In the present study, EDC/NHS-crosslinked collagen films demonstrated increasing low strain E-moduli with increasing crosslink-densities (table II). This is in agreement with results obtained by others, for EDC/NHS-crosslinked collagen [20] as well as collagen biomaterials crosslinked using other techniques [12, 33]. EDC/NHS-crosslinked collagen films demonstrated increasing tensile strength with increasing crosslink densities. The tensile strength of E/N13C, which was lower than reported for glutaraldehyde crosslinked collagen biomaterials, was comparable to or higher than that of carbodiimide crosslinked collagens [12, 34, 35]. There was no significant influence of EDC/NHS-crosslinking on the elongation at break. In general, the relation between crosslink density and elongation at break is not well-defined. Decreased as well as an increased elongation at break with increasing crosslink densities have been reported [20, 25, 36].

In fibrillar collagens, collagen molecules are assembled into microfibrils, which in turn are organized into fibrils and fibers [37]. During stress-strain measurements (randomly) oriented collagen fibers are realigned in the direction of strain. The low strain E-modulus is determined by resistance of collagen fibers against this alignment, and maximal reorientation of fibers in the direction of strain will largely determine the elongation at break. At high strain, slippage and break of fibers will result in failure of the material [25]. Crosslinking of collagen using
EDC and NHS results in intramolecular and intramicrofibrillar crosslinks [38], therefore not contributing to increased E-modulus, elongation at break and tensile strength.

The reconstituted collagen used in the present study has very poor mechanical properties, as was also observed by others [39, 40]. The observed increased E-modulus and tensile strength after EDC/NHS-crosslinking during this study, therefore, is due to other mechanisms than described above. Mechanical dispersion of collagen can introduce a large fraction of fiber- and fibril fragments with split or fractured ends. Furthermore, a small fraction of bovine achilles tendon collagen molecules is solubilized during swelling in diluted acetic acid [22]. In theory, acidic dispersion therefore may result in a swollen mixture of collagen fiber fragments interconnected or penetrated by split fiber/fibril-ends and single collagen molecules (length approximately 300 nm). Upon drying during film formation, aggregation of this mixture results in a matrix of fiber-fragments with tight interfibrillar or inter-fiber connections or entanglements. When crosslinked, such a matrix would demonstrate increased resistance to fiber realignment and decreased slipping of fibers because of covalent interfibrillar connections.

Regarding crosslinked collagen biomaterials, the type of crosslink, the crosslink density, and site of implantation of the biomaterial may influence the resorption rate of the collagen implant [41]. Native collagen is degraded to smaller fragments by specific, extracellular tissue collagenases [42], which cause solubilization of collagen molecules at the outside of collagen fibrils and fibers by cleavage at specific sites in the triple-helix region. In vitro incubation with bacterial collagenase is a well established method to determine the degradation behavior of collagen biomaterials.

N-Coll was degraded rapidly, but upon EDC/NHS-crosslinking the degradation rates decreased with increasing crosslink densities. Yannas et al. reported a correlation between the rate of collagenase degradation in vitro and the extent of in vivo resorption [43]. This indicates that EDC/NHS crosslinking of collagen results in a matrix which is resistant to resorption under in vivo conditions for prolonged periods of time.

Denaturation of (crosslinked) collagen led to increased swelling by conversion of the collagen triple helix structure to a random coil conformation, because of the increased accessibility of collagen peptide chains to hydration [32]. The effect of denaturation of collagen was most pronounced for non-crosslinked collagen. Increased crosslinking of collagen causes a decrease of the peptide chain length between crosslinks, resulting in decreased swelling of (denaturated) samples when crosslink density increases.

Gamma irradiation or ethylene oxide (EO) sterilization is widely used to sterilize biomaterials before implantation. However, EO may cause extensive chemical modification of functional groups in collagen [44]. It has been reported that EO is antigenic [45], and EO-sterilized materials can induce (severe) toxic effects by the release of residual EO [46]. Therefore, EO-
sterilization of EDC/NHS-crosslinked collagen is likely to affect the proliferation of cells seeded on these matrices. Consequently, γ-irradiation is the preferred method of sterilization. Endothelial cell proliferation on EDC/NHS-crosslinked collagen was not influenced by γ-sterilization of the collagen substrate (data not shown). However, sterilization of collagen-based biomaterials using γ-irradiation may affect the physical and mechanical properties of the material, as well as the in vitro and in vivo degradation rate [47]. The collagen source [48], the presence and type of crosslinks [49], the radiation dose [50] and the water content of the collagen sample [51] have been shown to play a role in the extent of the effects of ionizing radiation on collagen. In general, γ-irradiation of structural proteins may lead to three types of reactions: scission of the peptide backbone, disruption of secondary hydrogen bonding and the formation of inter- and intramolecular crosslinks. Irradiation of collagen samples results predominantly in chain scission, leading to poly-peptide chains with amide- and ketone-end groups (in the presence of traces of oxygen and/or water), and in the formation of radicals. Recombination of radicals may result in the formation of inter- or intramolecular crosslinks [51]. Formation of new crosslinks by recombination prevails over chain scission in wet collagen samples. In dry samples, formation of new crosslinks by recombination is hampered by the limited chain mobility [52].

In the present study, γ-irradiation of (crosslinked) collagen at a dose of 25 kGy did not significantly affect the number of free primary amino groups. Formation (or absence) of amide groups as result of chain scission (see above) is, however, not detected using TNBS. Gamma irradiation of crosslinked collagen resulted in a decrease of the shrinkage temperature varying from 13°C to 15°C (table I), which is consistent with observations by others [49, 53]. A decreased shrinkage temperature indicates that disruption or denaturation of the collagen triple helix structure has occurred, most likely by chain scission of the peptide chains. Denaturation of (crosslinked) collagen in boiling water resulted in increased swelling, both for γ-irradiated and non-sterilized samples. In contrast, γ-irradiation of (crosslinked) collagen resulted in decreased swelling, indicating that material modifications additional to chain scission alone had occurred. This suggests the formation of new crosslinks by recombination of cleaved peptide chains, resulting in a material with a somewhat higher crosslink density than the non-irradiated sample.

The E-modulus and the tensile strength of EDC/NHS-crosslinked collagen were hardly affected by gamma sterilization (table II). In contrast, irradiation of for example glutaraldehyde-crosslinked dermal sheep collagen at a dose of 25 kGy resulted in a reduction of tensile strength and low strain E-modulus [49, 53]. This underlines once more the significance of the type of collagen and the type of crosslink for the effect of γ-sterilization on the crosslinked collagen, making comparisons between different materials very difficult. At a
higher radiation dose (e.g. 100 kGy), more severe effects on mechanical properties of crosslinked collagen are to be expected [51, 53]. For γ-irradiated E/N23C, the elongation at break was increased compared to non-irradiated E/N23C. Increasing crosslink densities resulted in a lower elongation at break (table II) after γ-irradiation. Crimp of collagen materials as a result of glutaraldehyde crosslinking is associated with increased elongation at break during tensile testing of non-irradiated materials [54]. Possibly, irradiation of EDC/NHS-crosslinked collagen samples induced a similar crimp. Reduced crimp with increasing crosslink densities would account for a reduced elongation at break. During the present study crimp of EDC/NHS-crosslinked collagen samples was not determined.

Irradiation of N-Coll and E/N23C resulted in an increased rate of in vitro degradation when exposed to collagenase, whereas the degradation rate of E/N16C and E/N13C was hardly influenced (fig. 4B). The higher rate of weight loss during the first 6 hours of degradation, especially of gamma-irradiated N-Coll and N23C, was not due to release of soluble peptide fragments. Only negligible amounts of hydroxyproline containing peptide fragments were detected in degradation medium without collagenase after 24 hours of incubation with irradiated samples (control experiment, results not shown). In vitro collagenase degradation measurements were carried out using bacterial collagenase from Clostridium Histolyticum, a non-specific collagenase which cleaves collagen-molecules at more than 200 sites, before every Gly-Pro sequence. γ-Sterilization of collagen may also result in chain scission, which would result in initially fast degradation when subsequently exposing sterilized collagen samples to a collagenase solution. This agrees with observations made for non-crosslinked collagen and EN23C. At higher crosslink densities gamma irradiation hardly influences the rate of collagenase degradation, possibly due to the stabilizing effect of the increased number of crosslinks.

5. CONCLUSIONS

Using EDC in combination with NHS for crosslinking of collagen, a maximal reduction in free primary amino groups from 27 per 1,000 amino acid residues for non-crosslinked collagen to approximately 10 for EDC/NHS-crosslinked collagen was observed. The decrease in primary amino groups was inversely related to the shrinkage temperature. Low strain E-modulus and tensile strength of crosslinked collagen increased with increasing crosslink densities; the elongation at break was not influenced by the crosslink density. The resistance to in vitro collagenase degradation also increased as a result of crosslinking, the weight loss of
E/N13C was only 4% after 24 hours of degradation whereas N-Coll was almost completely degraded (≈ 80%) within 5 hours.

Gamma sterilization decreased the shrinkage temperature of crosslinked collagen by 13 to 15°C, whereas the number of free primary amino groups was not affected. Results suggest the formation of a material with a somewhat higher crosslink density than the non-irradiated sample. Gamma-sterilization hardly affected the E-modulus and the tensile strength of EDC/NHS-crosslinked collagen, except for the tensile strength of E/N13C which was decreased by 20%. The elongation at break after γ-irradiation showed an inverse correlation with crosslink density. In vitro collagenase digestion of E/N13C was not affected by γ-irradiation. However, with decreasing crosslink densities a progressively increased susceptibility for collagenase degradation was observed.

EDC/NHS-crosslinked collagen with relatively high crosslink densities, like E/N13C, demonstrates both improved material properties and preservation of notably resistance to degradation after γ-sterilization. These properties favor collagen matrices like E/N13C as a substrate for endothelial cell seeding of synthetic vascular grafts. The crosslink density which is optimal for endothelial cell seeding, as well as the mechanical properties of EDC/NHS-crosslinked collagen required for application as a coating of synthetic vascular grafts, however, remain subject of further research.

REFERENCES

An EDC/NHS-crosslinked collagen substrate for endothelial cell seeding


CHAPTER 4

Immobilization of heparin to EDC/NHS-crosslinked collagen. Characterization and in vitro evaluation

M.J.B. Wissink¹, R. Beernink¹, J.S. Pieper², A.A. Poot¹, G.H.M. Engbers¹, T. Beugeling¹, W.G. van Aken¹, J. Feijen¹

¹Institute for Biomedical Technology, Polymer Chemistry and Biomaterials Group, Department of Chemical Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands.
²Department of Biochemistry, Faculty of Medical Sciences, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

ABSTRACT

In the present study, heparin immobilization to a non-cytotoxic crosslinked collagen substrate for endothelial cell seeding was investigated. Crosslinking of collagen using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) resulted in a material containing 14 free primary amino groups per 1,000 amino acid residues (E/N14C). At a fixed molar ratio NHS : EDC of 0.6, the amount of heparin covalently immobilized to E/N14C increased with increasing molar ratios of EDC to heparin carboxylic acid groups (Hep-COOH), to a maximum of approximately 5-5.5 wt. % at a ratio of 2. Upon incubation in cell culture medium of endothelial cells, 4 to 7% of the immobilized heparin was released during 11 days.

Immobilization of increasing amounts of heparin to E/N14C progressively reduced activation of contact activation proteases. Optimal anticoagulant activity, as measured by thrombin inhibition, was obtained after heparin immobilization using a ratio of EDC to Hep-COOH of 0.2 to 0.4 (14 to 20 mg heparin immobilized per gram of collagen). Platelets deposited to (heparinized) E/N14C showed only minor spreading and aggregation, although heparin immobilization slightly increased the number of adherent platelets. The results of this study suggest that heparin immobilization to EDC/NHS-crosslinked collagen may improve the \textit{in vivo} blood compatibility of this material.
1. INTRODUCTION

Synthetic vascular grafts, made of Dacron or expanded Teflon, are widely used to replace occluded or diseased arteries in man. When used in large diameter applications, synthetic vascular grafts show satisfactory patency rates. In small diameter applications (inner diameter less than 5 mm), however, graft performance is disappointing due to stenosis and thrombus formation [1-4].

Endothelial cell seeding is an accepted approach to improve (small-diameter) graft performance. For successful endothelial cell seeding, a suitable substrate is required, which is not provided by Dacron or expanded Teflon grafts [5-7]. Since non-crosslinked collagen is a suitable matrix for the growth of endothelial cells in vitro [8-10], application of a collagen coating on synthetic vascular graft materials may result in a matrix suitable for in vivo endothelial cell seeding.

Collagen-coated vascular grafts have been developed to eliminate the procedure of pre-clotting of porous Dacron prior to implantation. In commercially available collagen-coated vascular grafts, collagen is crosslinked using glutaraldehyde or formaldehyde to reduce the in vivo resorption rate [11]. Both crosslink agents are incorporated in the collagen coating during crosslinking. Especially glutaraldehyde is known to induce cytotoxic reactions by release of (unreacted) glutaraldehyde or glutaraldehyde derivatives during in vitro or in vivo degradation [12-15], thus hampering endothelialization of the graft [15, 16].

We have previously developed an alternative collagen coating for synthetic vascular grafts, crosslinked by N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) [17]. These crosslink-agents introduce “zero length” amide-crosslinks between carboxylic acid groups from aspartic and glutamic acid residues, and ε-amino groups from (hydroxy-) lysine residues [18]. EDC/NHS-crosslinked collagen is reported to be non-cytotoxic in vitro [12], and biocompatibility was observed in animal studies [19, 20].

The supply of autologous endothelial cells for seeding is limited. Although vascular grafts with a confluent lining of endothelial cells can be obtained after expansion of cell numbers by in vitro culture, this approach introduces a long interval between the need of an endothelialized graft and its availability, and increases the risk of bacterial infection due to prolonged culture [21]. Therefore, per-operative cell seeding is the preferred method for endothelialization. This implies application of low cell seeding densities, leaving (large) parts of the collagen-coated graft surface exposed to blood in the period after cell seeding.

Collagen is a highly thrombogenic material, as is demonstrated from its use as a hemostatic powder or sponge [22]. Collagen induces platelet adhesion and aggregation as well as activation of intrinsic blood coagulation. To prevent graft failure resulting from thrombus...
formation when collagen is not yet completely covered by (proliferating) endothelial cells, development of a collagen matrix for endothelial cell seeding with improved blood compatibility is required.

A generally applied approach to improve the blood compatibility of biomaterials is covalent immobilization of heparin [23, 24]. Heparin is an effective inhibitor of blood coagulation. Furthermore, immobilization of heparin is reported to reduce *in vitro* and *in vivo* platelet adhesion and aggregation [25-29], although increased platelet adhesion after heparin immobilization has been reported as well [30]. The effect of heparin immobilization on endothelial cell proliferation is not consistent: heparin immobilization is both reported to inhibit [31] as well as promote [32] proliferation of endothelial cells.

In the present study, heparin was immobilized to EDC/NHS-crosslinked collagen, also using EDC and NHS. Carboxylic acid groups of heparin were converted to reactive NHS-esters using EDC and NHS, and thereafter heparin was immobilized by reaction of NHS-activated carboxylic acid groups of heparin with residual primary amino groups in the EDC/NHS-crosslinked collagen matrix. The immobilization of heparin to EDC/NHS-crosslinked collagen was investigated, as well as the stability of the resulting heparinized matrices. To determine whether heparin immobilization improved the blood compatibility of the collagen matrix, contact activation, thrombin inhibition and deposition of blood platelets was studied *in vitro*.

2. MATERIALS AND METHODS

2.1. Materials

Unless otherwise stated, chemicals were obtained from Merck (Darmstadt, Germany), and were of the highest purity available.

2.2. Collagen crosslinking

All experiments were carried out using flat collagen films as model substrates. Collagen films were prepared from Type I insoluble collagen derived from Bovine Achilles Tendon (Sigma, St.Louis, MO, C 99879, lot 23H7065), as described in chapter 3 of this thesis. These matrices, with a thickness of approximately 50 μm, were crosslinked using N-(3-dimethylaminopropyl)-N'-ethyldiaminecarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). In order to minimize hydrolysis of EDC, crosslinking was carried out in 0.05 M buffer of 2-morpholinoethane sulfonic acid (MES buffer, pH 5.40) [33]. Before crosslinking, dried
collagen films were washed with MES buffer. Subsequently, the films were immersed in a solution of EDC and NHS in MES buffer, and gently shaken. For the crosslinking reaction, 1.731 g EDC and 0.415 g NHS in 215 ml MES buffer were used per gram of collagen (molar ratio EDC : NHS : collagen-carboxylic acid groups = 7.0 : 2.8 : 1.0). After 4 hours, when the reaction was completed, the collagen was washed with 0.1 M Na2HPO4 solution (2 hours) and demineralized water (four times for 30 minutes) [33, 34]. The residual number of free primary amino groups in collagen after crosslinking was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS, from Fluka, Buchs, Switzerland), according to a slightly modified procedure described by Wang et al. [35-37]. The shrinkage temperature of (crosslinked) collagen, indicating the resistance against thermal denaturation, was determined using Differential Scanning Calorimetry (DSC) [38].

2.3. 3H-Labeling of heparin

Heparin sodium salt (Bufa Chemie, Castricum, the Netherlands) was used. This heparin preparation from porcine mucosa has the following characteristics [39]: Mn = 12,500 g/mol (molecular weight distribution 3,000-30,000 g/mol), activity = 195 IU/mg, 18.75 mol of carboxylic acid groups (Hep-COOH) per mol of heparin.

Heparin was tritiated using the method described by Hatton et al. [40], with slight modifications. Briefly, heparin (3.00 g) was dissolved in distilled water (400 ml), and the pH was adjusted to 8.0 with NaOH solution (4 M). NaB3H4 (100 mCi, 7.5 Ci/mmol, Amersham, Amersham, UK) was added, and the reaction was allowed to proceed for 3 hours at room temperature. The resulting 3H-labeled heparin was purified by dialysis at 4°C against subsequently 2M NaCl in phosphate buffered saline (PBS, from NPBI, Emmer Compascuum, the Netherlands, pH 7.40), PBS (2 times) and distilled water (3 times). 3H-heparin was isolated by lyophilization and stored in a desiccator at room temperature. The yield was 2.80 g, with a specific activity of 76.9 kBq/mg. Free label content was less than 0.5%, as determined by gel filtration using a PD10-Sepharose column (Pharmacia, Upsala, Sweden).

2.4. Heparin immobilization

Typically, heparin immobilization onto crosslinked collagen was performed as described below. Crosslinked collagen films were equilibrated with MES-buffer (0.05M, pH 5.60) for at least 30 minutes. Carboxylic acid groups of heparin (Hep-COOH) were activated by adding EDC and NHS to a 2% (w/v) solution of (3H-labeled) heparin in 0.05 M MES-buffer (pH 5.60), at a molar ratio of EDC : NHS : Hep-COOH of 0.4 : 0.24 : 1.0. After pre-activation for 10 minutes, 1 g of crosslinked collagen (containing 14 free primary amino groups per 1,000
Immobilization of heparin to EDC/NHS-crosslinked collagen

Amino acid residues, E/N14C) was added to 188.3 ml of EDC/NHS-activated heparin solution, giving a molar excess of heparin to free collagen primary amino groups (Coll-NH$_2$, 151 μmol/gram collagen, calculated from 10.76 mol amino acids per gram of collagen [41]) of 2.0. After 2 hours of reaction, the heparinized E/N14C, designated as E/N14C-H, was washed with 0.1 M Na$_2$HPO$_4$ (2 hours), 4 M NaCl (4 times for 24 hours) and distilled water (3 times for 24 hours).

Immobilization of heparin was investigated as a function of heparin pre-activation time (up to 60 minutes), immobilization time (up to 4 hours), pH (4.5 to 6.5), the amount of heparin (concentrations up to 4% (w/v), molar ratio of heparin to Coll-NH$_2$ up to 10), and amounts of EDC (molar ratio of EDC to Hep-COOH up to 5) and NHS (molar ratio of NHS to EDC of 0.2 to 1.0) used.

2.5. Determination of immobilized heparin

$^3$H-Heparinized collagen films were dissolved in Luma Solve (2 ml, Lumac, Olen, Belgium) for 24 hours at 60°C. After addition of scintillation cocktail (20 ml, Optiphase HiSafe 3, Wallac, Milton Keynes, UK) the radioactivity of the samples was measured using a 1414 Winspectral liquid scintillation counter (Wallac, Türku, Finland). The amount of immobilized heparin was calculated from the specific activity of $^3$H-heparin.

The amount of immobilized non-radiolabeled heparin was determined using toluidine blue [42]. Circular samples with a diameter of 8 mm were incubated with 5 ml aqueous solution of toluidine blue (0.1 M HCl, 2 mg/ml NaCl, 0.4 mg/ml toluidine blue O zinc chloride double salt) for 4 hours at room temperature, resulting in complexation of toluidine blue with heparin. Thereafter, samples were washed with distilled water (twice for 5 minutes). Subsequently, toluidine blue complexed to heparin was solubilized in 5 ml of a 1:4 (v/v) mixture of 0.1 M NaOH and ethanol. The extinction of the resulting solution was determined at 530 nm using an Uvikon 930 spectrophotometer (Kontron Instruments, Switzerland). The amount of immobilized heparin was calculated from a calibration curve obtained from EDC/NHS-crosslinked collagen containing various amounts of immobilized $^3$H-labeled heparin.

2.6. Localization of immobilized heparin

Alcian Blue staining was used for localization of immobilized heparin in crosslinked collagen. Samples were processed for paraffin sectioning using standard histological techniques. Briefly, after dehydration in a graded series of ethanol (50%, 70%, 96% and 100%) and xylene, the specimen were embedded in paraffin. The paraffin embedded samples were sectioned at 6 μm, and mounted on gelatin coated glass slides. Sections were deparaffinized in
xylol (three times for 5 minutes), and rehydrated in a graded series of ethanol and demineralized water. After incubation in acetic acid solution (3% v/v, pH 2.5) for 3 minutes, sections were stained during 30 minutes using a solution of Alcian Blue 8GX (2% w/v) in 3% acetic acid solution [43]. After washing with demineralized water (15 minutes), the specimen were examined using a Zeiss Axioscop (Zeiss, Jena, Germany).

2.7. Stability of heparinized collagen

$^3$H-heparinized E/N14C discs with a diameter of 10 mm (thickness $54 \pm 6$ μm, weight $2.5 \pm 0.2$ mg) were washed with PBS (twice for 10 minutes). Thereafter, the samples were transferred to endothelial cell culture medium (CM) containing 5% human serum, as used for endothelial cell culture, which for this experiment was supplemented with NaN$_3$ (50 μg/ml). CM consisted of a mixture of equal volumes of RPMI 1640 and M199 (both standard synthetic cell culture media), containing penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml) and glutamax (2 mM) (all from Gibco, Paisley, UK). Release of $^3$H-heparin was measured upon incubation in CM/5% serum (5 ml) at 37°C for 11 days on an orbital shaker. Supernatant medium was replaced every 24 hours by 5 ml of fresh medium, after which $^3$H-heparin in the culture medium was determined. Scintillation cocktail (9 ml, Optiphase HiSafe 3) was added to culture medium (1 ml), and subsequently the radioactivity of the sample was measured using liquid scintillation counting.

2.8. Contact activation assay

Activation of intrinsic coagulation by (heparinized) E/N14C was measured using a chromogenic substrate for activated factor XII and plasma kallikrein (S2302, Chromogenix, Mölndal, Sweden) [44, 45]. (Heparinized) E/N14C discs with a diameter of 15 mm (weight 5.6 ± 0.5 mg) were fixed in a 24 wells microtiter plate (Costar, Cambridge, MA) using silicon-rubber rings (Eriks, Alkmaar, the Netherlands). Glass was used as a reference substrate. After incubation with PBS for 24 hours at 37°C, samples were washed with PBS (37°C) twice for 1 minute. Fresh frozen, pooled citrated plasma from 12 healthy donors (Bloedbank Twente en Achterhoek, Enschede, the Netherlands) was thawed at 37°C, and diluted with PBS (37°C) to 25% (v/v). Test samples were incubated with 200 μl of diluted plasma at 37°C on an orbital shaker. After 15 minutes, contact activation proteases in the diluted plasma were assayed. A sample of 50 μl was incubated with 50 μl Tris-buffer (50 mM trishydroxy-methylaminoethane, 0.15 M NaCl, pH 7.40) and 50 μl substrate solution (2.5 mg/ml S2302 in distilled water). The optical density of the solution at 405 nm was recorded as a function of time for 20 minutes using an ELISA reader (340 ATTC, SLT, Austria). The
enzymatic activity of the contact-activation proteases in the sample was expressed as the slope of the resulting straight line. Plasma diluted with PBS, which had not been in contact with an activating surface, was used as control.

After removal of the diluted plasma, surfaces were washed three times with Tris-buffer. Thereafter, the films were incubated at 37°C with 50 μl Tris buffer and 50 μl substrate solution. After 45 minutes, 50 μl samples were transferred to wells of a 96 wells microtiter plate (Costar) containing 50 μl stop solution (acetic acid in water, 50% (v/v)). The optical density at 405 nm was taken as a measure for contact activation proteases present on the sample surface. Wells containing 50 μl Tris-buffer and 50 μl substrate solution but no sample were used as a control.

2.9. Thrombin inactivation assay

The anticoagulant activity of immobilized heparin was determined using a thrombin inhibition assay, based on a method described by Chandler [46]. Briefly, the ability of immobilized heparin to mediate the inactivation of thrombin by ATIII was measured using a chromogenic substrate for thrombin (S2238). Heparinized collagen disks with a diameter of 10 mm (weight 2.5 ± 0.2 mg), were incubated with PBS for 24 hours. After blotting on filter paper, the films were transferred to wells of a 48 well tissue culture cluster (Costar, Cambridge, MA) containing 50 μl PBS and 250 μl Tris-buffer. The Tris buffer used for this assay consisted of 50 mM Tris (pH 8.40) containing 1 g/l PEO 6,000, 1 g/l BSA (Sigma) and 150 mM NaCl. The substrate solution consisted of 0.2 mg/ml S2238 (Chromogenix, Mölndahl, Sweden) and 70 mU/ml purified human AT III (gift from CLB, Amsterdam, the Netherlands) in Tris-buffer. After addition of 150 μl substrate solution, the assay was started by adding 50 μl of a 1.2 U/ml solution of bovine thrombin (Sigma, T 4265) in Tris-buffer. After incubation for 10 minutes at 37°C on an orbital shaker, the reaction was stopped by adding acetic acid solution (50 μl, 40% v/v). Subsequently, 250 μl of the supernatant was transferred to a 96 well tissue culture cluster (Costar), and the absorbance at 405 nm was measured using an ELISA reader (340 ATTC). The activity of the heparinized collagen films was calculated using a calibration curve obtained from heparin solutions in PBS with concentrations ranging from 0 to 250 mU/ml.

2.10. Preparation of platelet suspensions

Fresh “buffy coats” derived from whole blood units collected from healthy volunteers (Bloodbank Twente en Achterhoek) were diluted 2.5 times with Krebs-Ringer buffer (107 mM NaCl, 20 mM NaHCO3, 2 mM Na2SO4, pH 7.3) containing 19 mM trisodiumcitrate and
27 mM α-D-glucose, after which platelet-rich plasma (PRP) was obtained by centrifugation at 1700 g during 4 minutes. Subsequently, PRP was diluted with an equal volume of Krebs-Ringer buffer (pH 5.0) containing trisodiumcitrate (19 mM) and α-D-glucose (27 mM), giving a final pH of 6.1. Thereafter, platelets were washed according to the method described by Cazenave and labeled with \(^{111}\)Indium (Amersham, Amersham, UK) as previously described [47]. Platelets were finally suspended in ABO-compatible platelet poor plasma (Bloodbank Twente en Achterhoek) at a concentration of 100,000/\(\mu\)l. The radioactivity of the platelets was 457 ± 15 cpm per million platelets.

2.11. Deposition of \(^{111}\)In-labeled platelets

Samples were fixed with a silicon rubber ring (Eriks) in custom-made Teflon sample holders, giving an exposed (flat and circular) surface area of 4.10 cm\(^2\) (calculated weight 13 ± 1 mg). Surfaces were equilibrated overnight at 37°C with PBS. After two additional washes with PBS (37°C), the surfaces were incubated with 485 \(\mu\)l \(^{111}\)Indium-labeled platelets suspended in plasma, under static conditions at 37°C. After 1 hour the platelet suspensions were removed, and the substrates were washed carefully with PBS (three times, 37°C). The number of deposited platelets was determined by measuring the radioactivity on the washed substrates, using a Compugamma 1282 \(\gamma\)-counter (LKB, Stockholm, Sweden).

An EDC/NHS-crosslinked albumin gel (bovine serum albumin, Sigma), prepared as described by Bos et al. [48], was used as a control surface. Briefly, to a solution of albumin in MES-buffer (200 mg/900 \(\mu\)l, pH 5.3) a solution of EDC (16.6 mg) and NHS (2.0 mg) in MES-buffer (100 \(\mu\)l, pH 5.3) was added. The mixture was pipetted in a petridish. After 4 hours of crosslinking, the resulting albumin-gel (E/N-albumin) was extensively washed with PBS.

2.12. Scanning electron microscopy

Samples for scanning electron microscopy (SEM) analysis were fixed in a glutaraldehyde solution in PBS (2% w/v, 4°C) for at least 24 hours. Thereafter, samples were dehydrated in a graded series of ethanol and air-dried. After sputter-coating with gold/palladium (10 nm), the samples were examined using an S-800 field emission SEM (Hitachi, Tokyo, Japan) at an acceleration voltage of 7 kV.
2.13. Statistical analysis

Results were expressed as mean ± standard deviation. For statistical analysis, a Student-T test (using GraphPad InStat, GraphPad Software, San Diego, CA) was used. Results were considered statistically different with p < 0.05.

3. RESULTS

3.1. Collagen crosslinking

Upon crosslinking with EDC and NHS, the number of free primary amino groups per 1,000 amino acid residues, which in native collagen amounts to 27 [49], decreased while the shrinkage temperature increased. At the conditions used, collagen crosslinking resulted in a material containing 14 free primary amino groups per 1,000 amino acid residues (E/N14C). The shrinkage temperature increased from 55.4°C for non-crosslinked collagen (N-Coll) to 76.1°C for E/N14C.

3.2. Heparin immobilization

Immobilization of \(^3\)H-heparin to E/N14C was maximal at pH 5.60 (as determined using a pH range from 4.5 to 6.5) in the presence of a heparin concentration of 2% (w/v) or higher (determined for heparin concentrations up to 4%). The immobilization reaction was complete within 2 hours (data not shown).

Using these conditions, pre-activation of heparin with EDC and NHS for 5 to 30 minutes resulted in maximal heparin immobilization (fig. 1A). Using a pre-activation time of 10 minutes and a molar ratio EDC : NHS : Hep-COOH of 0.4 : 0.24 : 1.0, the amount of immobilized heparin increased when the molar ratio of heparin to free primary amino groups of collagen (Coll-NH₂) was increased, leveling off above a ratio of 2 (fig. 1B). Heparin immobilization using a molar ratio of heparin : Coll-NH₂ of 2.0 (10 minutes pre-activation, EDC : Hep-COOH = 0.4) was maximal at a molar ratio of NHS to EDC in between 0.4 to 0.6 (fig. 1C). At a fixed molar ratio of NHS : EDC of 0.6 (10 minutes pre-activation, EDC : Hep-COOH = 0.4), the amount of immobilized heparin increased with increasing molar ratio of EDC to heparin-carboxylic acid groups (Hep-COOH), to a maximum of approximately 5.5% heparin (w/w) at a ratio of 2 (molar ratio EDC : NHS : Hep-COOH = 2.0 : 1.2 : 1.0) (fig. 1D). Based on these results a standard procedure for heparin immobilization to E/N14C was adopted, using 2% (w/v) heparin solution (pH 5.60), a fixed molar ratio of NHS to EDC of...
Figure 1: Immobilization of $^3$H-heparin to E/N14 collagen as a function of pre-activation time of heparin (A), molar ratio of heparin to free primary amino groups in collagen (B), molar ratio of NHS to EDC (C), and molar ratio of EDC to heparin carboxylic acid groups (D) (n = 4, mean ± SD).

Heparin concentration 2% (w/v), pH = 5.60, immobilization time 2 hours. Heparin : Coll-NH$_2$ = 2, EDC : NHS : Hep-COOH = 0.4 : 0.24 : 1.0 (fig. 1A); 10 minutes pre-activation, EDC : NHS : Hep-COOH = 0.4 : 0.24 : 1.0 (fig. 1B); heparin : Coll-NH$_2$ = 2; 10 minutes pre-activation, EDC : Hep-COOH = 0.4, (fig. 1C); Heparin : Coll-NH$_2$ = 2, 10 minutes pre-activation, NHS : EDC = 0.6 (fig. 1D).

0.6, a variable molar ratio of EDC : Hep-COOH of 0 to 2.0, heparin activation for 10 minutes, a molar ratio of heparin to free collagen primary amino groups of 2.0, and 2 hours of immobilization reaction. In further experiments, the amount of heparin immobilized to E/N14C was predetermined by the molar ratio of EDC to Hep-COOH used for immobilization.
3.3. Localization of immobilized heparin

Alcian Blue is a cationic dye which can be used for quantitative determination of glycosaminoglycans in solution [43] and for selective staining of glycosaminoglycans in tissue sections [50]. Light-microscopic images of Alcian Blue stained sections of collagen-heparin films demonstrated homogeneous staining through the entire thickness of the specimen, in contrast to the non-heparinized crosslinked collagen which showed no staining using the same procedure (fig. 2). This indicates that heparin is immobilized homogeneously throughout the entire thickness of the film.

![Figure 2](image_url)

**Figure 2**: Light microscopic images of Alcian Blue stained sections of crosslinked collagen (E/N14C, fig. 2A) and heparinized crosslinked collagen (E/N14C-H(0.4), fig. 2B) (original magnification 10 ×).

3.4. Stability of heparinized collagen

Upon incubation of heparinized E/N14C matrices (E/N14C-H) with endothelial cell culture medium supplemented with 5% human serum partial release of immobilized heparin occurred (fig. 3). When the molar ratio of EDC to Hep-COOH for heparin immobilization was increased, the release of $^3$H-heparin from E/N14C-H also increased. E/N14C heparinized using a molar ratio of EDC : Hep-COOH of 0.1 (E/N14C-H(0.1)) showed 7% heparin release after 11 days (0.54 ± 0.02 mg heparin per gram of E/N14C-H(0.1)), whereas E/N14C-H(0.4) and E/N14-H(1.0) showed a release of approximately 5% of the immobilized heparin (1.01 ± 0.03 and 2.1 ± 0.1 mg of heparin released per gram of heparinized E/N14C, respectively). Heparin release leveled off after longer incubation times, but a plateau value was not observed during 11 days. Release of free $^3$H label, as determined using a PD10-column, was negligible.
Contact activation generated when diluted plasma was incubated with E/N14C was significantly lower compared to glass (fig. 4A). Immobilization of heparin to E/N14C using molar ratios of EDC : Hep-COOH increasing from 0 (control, without EDC and NHS being used during the immobilization reaction) to 1.0 reduced contact activation. Significantly less contact activation was observed after immobilization of heparin at a ratio of EDC : Hep-COOH of 0.4 or higher.

The enzymatic activity of contact activation proteases adsorbed to glass after incubation with diluted plasma was lower compared to E/N14C or E/N14C-H (p < 0.05) (fig. 4B). Collagen onto which heparin was immobilized did not contain significantly more protease activity than when no heparin was immobilized, except for E/N14C-H(0.6) and E/N14C-H(1.0). Results are expressed as mOD, measured after 45 minutes of reaction of surface adsorbed contact activation proteases and chromogenic substrate solution.

3.6. Thrombin inhibition

Inactivation of thrombin by heparinized E/N14C was determined as function of the molar ratio of EDC : Hep-COOH used for heparin immobilization (fig. 5). Maximal thrombin
Heparin was immobilized onto E/N14C using molar ratios of EDC : Hep-COOH of 0, 0.2, 0.4, 0.6 and 1.0 (E/N14C-H(n), n represents the molar ratio of EDC : Hep-COOH), resulting in 2.8 ± 0.4; 13.8 ± 2.6; 23.3 ± 2.1; 28.9 ± 2.5 and 41.9 ± 3.1 mg immobilized heparin per gram of E/N14C, respectively. Fig. 4A: contact activation in solutions of plasma in PBS (25%) after contact for 15 minutes at 37° with various substrates. Background activity in a plasma solution in PBS (25%) was 2.2 ± 0.6 mOD/min. Fig. 4B: contact activation proteases adsorbed to various surfaces after incubation with solutions of plasma in PBS (25%) for 15 minutes at 37°. Background activity measured for the chromogenic substrate solution was 73 ± 4 mOD.

†: p < 0.05 compared with glass; ‡: p < 0.05 compared with E/N14C.
Figure 5: Thrombin inactivation by (heparinized) E/N14-collagen (n = 4, mean ± SD).
Heparin was immobilized onto E/N14C using molar ratios of EDC : Hep-COOH of 0, 0.05, 0.1, 0.2, 0.4, 0.6 and 1.0 (E/N14C-H(n), n represents the molar ratio of EDC : Hep-COOH), resulting in 2.6 ± 0.5; 4.4 ± 1.3; 7.6 ± 1.8; 14.2 ± 2.3; 20.3 ± 3.7; 30.6 ± 3.3 and 38.6 ± 2.2 mg immobilized heparin per gram of E/N14C, respectively. Thrombin inactivation by various surfaces during incubation with a solution of purified human ATIII and bovine thrombin for 10 minutes at 37°C.

Figure 6: Deposition of 111Indium labeled platelets onto various substrates (n= 3, mean ± SD).
Heparin was immobilized onto E/N14C using molar ratios of EDC : Hep-COOH of 0.2, 0.4 and 1.0 (E/N14C-H(n), n represents the molar ratio of EDC : Hep-COOH), resulting in 13.8 ± 2.6; 23.3 ± 2.1 and 41.9 ± 3.1 mg immobilized heparin per gram E/N14C, respectively. As reference, EDC/NHS-crosslinked albumin was used. Substrates were incubated with platelets (100,000/ml) resuspended in human ABO-compatible platelet-poor plasma, for 1 hour at 37°C.
†: p < 0.05 compared with N-Coll; ‡: p < 0.05 compared with E/N14C.
Immobilization of heparin to EDC/NHS-crosslinked collagen

inhibition was observed when heparin was immobilized using a molar ratio of EDC : Hep-COOH of 0.2 to 0.4 (14 - 20 mg heparin/gram collagen), which resulted in an anticoagulant activity equivalent to 3.1 to 3.5 mU heparin/cm$^2$. For E/N14C without immobilized heparin (E/N14C as well as E/N14C-H(0)) low anticoagulant activity of 0.29-0.34 mU/cm$^2$ was observed.

3.7. Platelet deposition

Deposition of $^{111}$In-labeled platelets was measured under static conditions, using platelets resuspended in human plasma. When compared to N-Coll, platelet deposition to E/N14C was significantly lower (fig. 6). Platelet adhesion to E/N14C without immobilized heparinized was comparable to platelet deposition onto heparinized E/N14C, except for E/N14C-H(0.4), onto which platelet deposition was significantly higher. The number of platelets deposited from plasma to E/N14C-H(0.4) and N-Coll were comparable. Platelet deposition onto E/N-Alb, which was used as a reference matrix, was very low compared to these substrates.

The morphology of platelets deposited onto (heparinized) E/N14C was studied using scanning electron microscopy. Platelets deposited on E/N14C and heparinized E/N14C demonstrated similar morphology (fig. 7A-D). Most adherent platelets had developed pseudopodia. Only few completely spread platelets were seen, on both E/N14C and E/N14C-H. Platelet activation (pseudopod formation) had occurred on all surfaces and deposited platelets were mainly observed in clusters of activated platelets. On E/N-Alb, adherent platelets demonstrated limited pseudopodia formation. No spreading or platelet aggregates were observed (not shown).

4. DISCUSSION

To obtain a non-cytotoxic collagen substrate suitable for endothelial cell seeding, collagen was crosslinked using EDC and NHS, which results in formation of amide crosslinks between carboxylic acid groups and free primary amino groups from amino acid residues in collagen [18]. Crosslinking of collagen was demonstrated by a decrease in free amino groups after crosslinking, and a corresponding increase in the shrinkage temperature. Denaturation of collagen is characterized by transition of the triple helix of the collagen molecule to a random coil, accompanied by a macroscopic shrinkage. Crosslinking results in stabilization of the triple helix structure, thus increasing the shrinkage temperature [51].

In the present study, heparin immobilization to EDC/NHS-crosslinked collagen was performed using EDC and NHS as well, in order to prevent cytotoxicity due to heparin immobilization reagents. Collagen crosslinking and heparin immobilization were carried out
Figure 7: Representative SEM images of platelets deposited from human plasma onto various substrates during 1 hour incubation at 37°C.

Substrates: E/N14C (fig. 7A), E/N14C-H(0.2) (fig. 7B), E/N14C-H(0.4) (fig. 7C) and E/N14C-H(1.0) (fig. 7D). Original magnification 3,000 ×.
Immobilization of heparin to EDC/NHS-crosslinked collagen

in two successive procedures, to allow monitoring of both the crosslink density of the EDC/NHS-crosslinked collagen matrix as well as the heparin immobilization reaction. This control is needed, because we observed in earlier studies that the crosslink-density of the collagen matrix influences the proliferation of endothelial cells when seeded on EDC/NHS-crosslinked collagen [52].

After carboxylic acid groups of heparin (Hep-COOH) were activated using EDC and NHS, heparin was immobilized to E/N14C by formation of peptide bonds between activated carboxylic acid groups of heparin and free primary amino groups of crosslinked collagen. The heparin pre-activation time was not very critical with respect to the amount of heparin immobilized (fig. 1A). Pre-activation times between 5 and 30 minutes resulted in comparable heparin immobilization. Longer pre-activation times resulted in decreased amounts of immobilized heparin, possibly due to hydrolysis of EDC- or NHS activated carboxylic acid groups [33]. The increase in immobilized heparin, observed when the ratio of heparin to Coll-NH_2 was increased (fig. 1B), can be explained by simple reaction kinetics. The depletion of heparin from the pre-activated heparin solution was very low (< 0.1%), at all ratios of heparin to Coll-NH_2 studied. The heterogeneous system, immobilization of heparin in solution onto a solid surface, may account for inefficiency of the immobilization reaction. Electrostatic repulsion between (negatively charged) immobilized heparin and heparin in solution may determine maximal heparin immobilization at given reaction conditions, resulting in a plateau value of heparin immobilization at ratios of heparin to Coll-NH_2 above 2. Increased heparin immobilization using increasing ratios of NHS : EDC (fig. 1C) probably resulted from conversion of EDC-activated carboxylic acid groups to reactive NHS-esters, thus preventing side reactions like hydrolysis of EDC activated groups or an O-N-acyl shift. When increasing the ratio of EDC to Hep-COOH (fig. 1D), more reactive NHS-esters or EDC-activated carboxylic acid groups are introduced per molecule of heparin, resulting in increased heparin immobilization. This is probably accompanied by increased numbers of covalent bonds introduced per molecule of immobilized heparin. Because of the gradual increase in the amount of immobilized heparin with increasing ratios of EDC to Hep-COOH, this ratio was used to control the amount of immobilized heparin. Materials with different amounts of immobilized heparin were used in blood compatibility studies described below.

Maximal heparin immobilization to E/N14C was approximately 5-5.5 wt. %, which is higher than reported when using EDC [53] or 1-cyclohexyl-3-(2-(N-methylmorpholino)ethyl)-carbodiimide, an alternative water soluble carbodiimide [54, 55], for immobilization of heparin to non-crosslinked collagen (0.4 - 1.5 wt %). This indicates that the use of EDC in combination with NHS is a very efficient method for immobilization of heparin to collagen.

Alcian blue staining (fig. 2) demonstrated heparin immobilization through the entire thickness of E/N14C-H(0.4). The heparin content of E/N14C-H(0.4) was approximately 2.2 wt % (fig.
1C-D), which corresponds to 70 μg of heparin immobilized per cm² of crosslinked collagen (calculated from a weight of 2.5 mg/10 mm disc, see materials and methods section). Bokros et al. have determined that for a densely packed monolayer of immobilized heparin orientated “side on” on a solid surface, a surface concentration of 0.1 to 0.2 μg heparin/cm² is needed [56]. For a solid surface with a monolayer of “end on” orientated heparin, a heparin surface concentration of 2 μg heparin/cm² was calculated. Consequently, immobilization of 70 μg of heparin per cm² of E/N14C can not be only limited to the outer surface of the material, but has to occur throughout the bulk of the crosslinked collagen matrix, which confirms the results of the alcian blue staining assay.

After heparinization, the films were washed with 4 M NaCl solution until elution of heparin from the matrices was negligible (i.e. after 3-4 days, determined using ³H-heparin). During subsequent incubation of E/N14C-H for 11 days in endothelial cell culture medium (CM), however, 5 to 7% of the heparin was released (fig. 3). This is most likely due to release of a residual fraction of non-covalently bound heparin not removed by washing with 4 M NaCl. Possibly, the high salt concentration used decreased the swelling of the heparinized collagen to such an extent, that part of the unbound heparin was entrapped within the collagen matrix during the washing procedure.

Increased heparin immobilization resulted in significantly increased swelling of heparinized E/N14C in PBS (data not shown). Increased swelling may facilitate a faster diffusion of non-covalently bound heparin out of the heparinized E/N14C matrix, explaining the faster (absolute) heparin release with increasing amounts of heparin immobilized.

The relatively slow and decreasing release rates indicate that a substantial amount of immobilized heparin will be present on or in the E/N14C-H matrix for prolonged periods of time. A continuous release of heparin from a matrix results in a micro-environment of heparinized blood near the blood-biomaterial interface, thus inhibiting thrombus formation. A minimal heparin release of 40 ng cm⁻² min⁻¹ is reported to create thromboresistant catheter surfaces or thromboresistant tubes, even at relatively high flow rates [57-59]. It is proposed that actual release can be much lower, because the remaining immobilized heparin also contributes to the thromboresistance of the surface [59, 60]. The average rate of heparin release from E/N14C-H during the first hours of incubation with CM was calculated to be in the range of 0.1-0.4 ng/cm²/min, and release decreased with time. It is not likely that such low heparin release contributes to thromboresistance.

The effect of heparin immobilization to E/N14C on blood coagulation depends on both the extent of contact activation induced by heparinized EDC/NHS-crosslinked collagen and the anticoagulant properties of the heparinized material. Glass is a strong activator of intrinsic coagulation [61]. Compared to glass surfaces, contact activation by E/N14C was considerable (fig 4). Immobilization of heparin decreased the activation of the contact system significantly,
although contact activation by E/N14C-H was still high when compared for example to heparinized polystyrene surfaces [62] or surface-modified polyethylene [63]. The exact mechanism underlying reduced contact activation after heparin immobilization is not clear. Immobilized heparin is reported to inactivate factor XIIa in the presence of ATIII [64, 65]. Alternatively, decreased non-specific adsorption of coagulation factors (FXII, HMWK) due to a modified surface chemistry after immobilization of heparin may result in reduced factor XII activation [66].

The enzymatic activity of surface adsorbed contact activation proteases did not differ much between E/N14C and the heparinized E/N14C matrices (fig. 4B), although a slight but significant increase was observed for E/N14C-H(0.6) and E/N14C-H(1.0). Results were comparable to the enzymatic activity of surface adsorbed contact activation proteases observed in other studies [48, 62, 63].

Immobilization of heparin to E/N14C resulted in matrices with improved anticoagulant activity (fig. 5). However, thrombin inactivation by heparinized E/N14C did not progressively increase with increasing amounts of immobilized heparin. E/N14C with 10.3 - 23.2 mg heparin immobilized per gram of collagen (obtained using a molar ratio EDC : Hep-COOH of 0.2 - 0.4) demonstrated maximal thrombin inhibitory activity. When using higher ratios of EDC : Hep-COOH, the anticoagulant activity of the obtained matrix decreased despite more immobilized heparin. Decreased thrombin inhibition using ratios of EDC : Hep-COOH > 0.4 may be due decreased accessibility of the ATIII binding site and/or decreased thrombin binding to the immobilized heparin, caused by an increased number of covalent bonds introduced between heparin and collagen.

The anti-thrombin activity of E/N14C-H(0.2) and E/N14C-H(0.4) was higher than the anti-thrombin activity of surfaces with an immobilized albumin-heparin conjugate [48, 62] or solid catheter surfaces to which heparin was covalently immobilized [46]. On the other hand, the thrombin inhibitory activity of E/N14C-H(0.2) and E/N14C-H(0.4), was lower than observed for surfaces that were heparinized via flexible spacers and surfaces containing “end point” immobilized heparin (10-30 mU/cm²) [46, 62]. This indicates, in the present study direct immobilization of heparin onto collagen rendered a surface with relatively high anti-thrombin activity, although heparinized collagen with a higher anti-thrombin activity may be obtained using alternative immobilization techniques.

In vitro platelet aggregation by collagen can be reduced by chemical modification of collagen [67, 68], by distortion of the quaternary fibril structure of collagen [69], or by immobilization of heparin [25]. Compared to N-Coll, platelet deposition was decreased for EDC/NHS-crosslinked collagen (fig. 6). Crosslinking of collagen decreased platelet deposition probably due to a decrease in the number of free carboxylic acid groups of aspartic and glutamic acid residues during crosslinking [70]. Heparin immobilization to various surfaces was
demonstrated to result in both increased [30] as well as decreased in vitro platelet deposition [25, 26]. In the present study, heparin immobilization to E/N14C did not influence the morphology of adherent platelets (fig. 7). However, significantly increased numbers of deposited platelets were observed on E/N14C-H(0.4). Striking is the positive correlation between platelet deposition and thrombin inhibitory activity of heparinized E/N14C (fig. 5 versus fig. 6), which is in good agreement with results of Bos et al. [48]. In addition, Lindon et al. reported increased platelet deposition onto heparinized polyethylene surfaces with higher ATIII-binding capacity [30]. Platelets contain heparin binding sites [71], which may mediate binding of platelets to immobilized heparin. Therefore, maximal platelet deposition onto E/N14C-H(0.4) might result from the same steric considerations as discussed above for the observed antithrombin activity. Alternatively, steric considerations may determine the ability of plasma proteins to bind to immobilized heparin. Maximal binding of plasma proteins like von Willebrand factor, fibronectin and thrombospondin [72], therefore may be responsible for the observed maximal platelet deposition onto E/N14C-H(0.4).

The effect of heparin release from E/N14C-H (fig. 3) on the in vitro studies described above was not determined experimentally. Heparin release is, however, not likely to interfere with contact activation measurements (fig. 4). It is reported that high heparin concentrations in plasma exposed to a heparinized surface did not influence activation of FXII by heparinized polyethylene [64]. Heparin release, however, may in part contribute to the observed in vitro thrombin inactivation (fig. 5). The activity of heparin released from E/N14C-H(0.1), E/N14C-H(0.4) and E/N14C-H(1.0) during the assay was calculated to be approximately 0.1, 0.3 and 0.5 mU/cm², respectively. Although the actual activity of the immobilized heparin may be somewhat lower than depicted in fig. 5 because of heparin release, the position of the observed maximum is not changed. Heparin in solution is reported to show variable results with regard to in vitro platelet function tests [73]. Heparin may induce platelet aggregation and release. However, heparin did not influence platelet aggregation stimulated by a fibrillar collagen suspension [74]. Furthermore, heparin did not significantly inhibit adhesion of washed human platelets to collagen-coated glass at a concentration of 20 U/ml [75]. Therefore, heparin release from E/N14C-H is not expected to influence platelet deposition (fig. 6) during the present study.

Several studies have shown that immobilization of heparin on collagen-coated vascular grafts reduces thrombogenicity in vivo. In a rabbit model, epoxy ether crosslinked heterografts show improved patency after heparin immobilization [76]. In dogs, comparable results have been found for gelatin-coated Corethane [77], and collagen or gelatin-coated ePTFE [76-78]. Although one has to be careful in extrapolating results of in vitro blood compatibility studies to actual in vivo thromboresistance, the results from the in vitro study presented here suggest that, compared to E/N14C, especially for E/N14C-H(0.4) improved in vivo blood
compatibility might be expected. However, the results of the present study are not conclusive. Resuming, E/N14C-H(0.4) combined highest thrombin inhibition with lowest contact activation for all heparinized E/N14C surfaces. Although on E/N14C as well as E/N14C-H only limited platelet spreading or aggregation was observed, platelet deposition on E/N14C-H(0.4) was somewhat higher than observed for E/N14C, E/N14C-H(0.2) and E/N14C-H(1.0).

5. CONCLUSIONS

Heparin was immobilized to E/N14C using EDC and NHS. Using increasing molar ratios of EDC to heparin-carboxylic acid groups, increasing amounts of heparin were immobilized, up to a maximum of 5-5.5 wt. %. Increased heparin immobilization onto E/N14C resulted in a progressive decrease in contact activation. Thrombin inhibition was maximal after heparin immobilization using a molar ratio of EDC to heparin-carboxylic acid groups of 0.2 to 0.4. E/N14C-H(0.4) demonstrated lowest contact activation in combination with the highest thrombin inhibition. Heparin immobilization resulted in a slightly increased platelet adhesion, platelet deposition was maximal on E/N14C-H(0.4). Immobilization of heparin did not affect the morphology of platelets deposited, and platelets adherent onto (heparinized) E/N14C demonstrated only limited spreading or aggregation. It is concluded that compared to E/N14C, especially for E/N14C-H(0.4) improved in vivo blood compatibility may be expected.

REFERENCES


Immobilization of heparin to EDC/NHS-crosslinked collagen


Immobilization of heparin to EDC/NHS-crosslinked collagen


CHAPTER 5

Binding and release of basic fibroblast growth factor from heparinized collagen matrices

Institute for Biomedical Technology, Polymer Chemistry and Biomaterials Group, Department of Chemical Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

ABSTRACT

Endothelial cell seeding is a promising method to improve the performance of small-diameter vascular grafts. Growth of endothelial cells seeded on the luminal surface of synthetic vascular grafts, coated with a matrix suitable for cell seeding (e.g. collagen), can be accelerated by local, sustained release of basic fibroblast growth factor (bFGF).

In this study two potential matrices for in vivo endothelial cell seeding were studied with respect to bFGF binding and release: collagen crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS), as well as heparinized EDC/NHS-crosslinked collagen. bFGF binding was determined after incubation of circular samples (10 mm diameter) with 0.25 ml bFGF solution for 90 minutes. Immobilization of increasing amounts of heparin, also using EDC and NHS, to crosslinked collagen containing 14 free primary amino groups per 1,000 amino acid residues (E/N14C) resulted in binding of increasing amounts of bFGF. A plateau in bFGF binding was observed for heparinized E/N14C containing approximately 2.0 to 3.0 wt. % of immobilized heparin which was obtained using a molar ratio of EDC to heparin-carboxylic acid groups of 0.4 during heparin immobilization (E/N14C-H(0.4)). At concentrations up to 840 ng bFGF/ml, 10% of the added bFGF bound to E/N14C, while binding of bFGF to E/N14C-H(0.4) amounted to 22%. Both E/N14C and E/N14C-H(0.4) pre-loaded with bFGF showed sustained bFGF release. A burst release of 30% in endothelial cell culture medium (CM) was observed for E/N14C during the first 6 hours, compared to 2% release from E/N14C-H(0.4). After 28 days, the bFGF release from E/N14C and E/N14C-H(0.4) in CM amounted to 100% and 65%, respectively.
Combined results of binding and release of bFGF indicate that compared to E/N14C, E/N14C-H(0.4) is the substrate of choice for bFGF pre-loading and subsequent endothelial cell seeding.

1. INTRODUCTION

Vascular grafts made of Dacron or expanded Teflon are successful in large-diameter applications [1]. In small-diameter positions (inner diameter less than 5 mm), however, graft performance is disappointing. Due to thrombus formation and stenosis especially long term patency rates are low [2-5].

Endothelial cell seeding is a recognized strategy to improve the performance of small-diameter vascular grafts [6, 7]. However, for successful endothelialization of synthetic vascular grafts a number of problems have to be dealt with. Dacron and expanded Teflon are poor substrates for endothelial cell seeding [8-10]. Regarding commercially available albumin-, gelatin- and collagen coated synthetic vascular graft materials, glutaraldehyde or formaldehyde used for crosslinking of the protein coating evokes cytotoxic reactions, thus hampering endothelialization of the luminal graft surface [11, 12]. Secondly, the supply of autologous endothelial cells is limited, leaving (large) parts of the thrombogenic graft surface exposed to blood directly after cell-seeding. Vascular grafts with a confluent lining of endothelial cells can be obtained after expansion of cell numbers by cell culture in vitro. Drawbacks of this approach are the time interval between the need of an endothelialized vascular graft and its availability, as well as the increased risk of bacterial infection, limiting its use to non-emergency situations [13]. Per-operative seeding therefore is the preferred method for endothelialization of vascular grafts. As a result, low seeding densities have to be coped with.

We have previously developed a new collagen coating for existing vascular graft materials, crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS). EDC/NHS-crosslinked collagen can be heparinized using EDC and NHS as well. During EDC/NHS-crosslinking of collagen, carboxylic acid groups of aspartic and glutamic acid residues in collagen react with EDC and NHS. This results in formation of NHS-activated carboxylic acid groups, which upon reaction with ε-amino groups from lysine and hydroxy lysine residues form peptide-like crosslinks and release NHS. Although EDC/NHS-crosslinked collagen is reported to be non-cytotoxic, both in vitro [14] and in vivo [15, 16], collagen is a thrombogenic material. Immobilization of the anticoagulant heparin is a widely applied approach to reduce thrombus formation at blood-biomaterial interfaces [17, 18]. Immobilization of heparin to collagen, therefore, may prevent platelet
adhesion and blood coagulation [19, 20], notably when the collagen coating is not yet completely covered by seeded endothelial cells. We have previously shown that both EDC/NHS-crosslinked collagen and heparinized EDC/NHS-crosslinked collagen are suitable substrates for endothelial cells in vitro [21, 22].

In addition to providing a suitable substrate for endothelial cell seeding, proliferation of seeded endothelial cells might be further improved by local, sustained release of basic fibroblast growth factor (bFGF, a heparin binding protein). Controlled release of growth factors like bFGF is widely explored in tissue engineering. Vascularization of implanted matrices [23, 24] or proliferation of cells (seeded) on various matrices [25-28] could be improved by local release of bFGF.

Local, sustained bFGF release has been shown to improve the proliferation of (seeded) endothelial cells in synthetic vascular grafts implanted in experimental animals. A fibrin glue sealant containing bFGF was developed to induce spontaneous endothelialization when applied as coating on synthetic vascular graft materials [29]. Greisler et al. studied endothelialization of ePTFE grafts coated with a mixture of fibrin glue, heparin and either aFGF or bFGF. By adjusting the ratio of heparin and growth factor in the fibrin glue, proliferation of endothelial cells was accelerated while proliferation of smooth muscle cells could be inhibited in vitro [30]. Spontaneous endothelialization of these vascular grafts was observed when implanted in rats, dogs and rabbits [31-33]. Polyurethane grafts coated with a mixture of photoreactive gelatin, heparin and bFGF, also demonstrated spontaneous endothelialization as a result of transmural in-growth of endothelial cells, when implanted in rat aortas [34].

In the present study, the binding of bFGF to and the release of bFGF from (heparinized) EDC/NHS-crosslinked collagen was determined.

2. MATERIALS AND METHODS

2.1. Materials

Unless otherwise stated, chemicals were obtained from Merck (Darmstadt, Germany), and were of the highest purity available.

2.2. Collagen crosslinking

All experiments were carried out using flat collagen films as model substrates. Collagen films were prepared from Type I insoluble collagen derived from Bovine Achilles Tendon (Sigma,
St. Louis, MO, C 99879, lot 23H7065), as previously described [35]. These collagen films, with a thickness of approximately 50 μm, were crosslinked using N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). In order to minimize hydrolysis of EDC, crosslinking was carried out in a 0.05 M buffer of 2-morpholinoethane sulfonic acid (MES buffer, pH 5.40) [36]. Before crosslinking, dried collagen films were incubated with MES buffer for at least 30 minutes. Subsequently, the films were immersed in a solution of EDC and NHS in MES buffer under gentle shaking. For the crosslinking reaction, 1.731 g EDC and 0.415 g NHS in 215 ml MES buffer was used per gram of collagen, corresponding to a molar ratio of EDC : NHS : collagen-carboxylic acid groups of 7.0 : 2.9 : 1.0. Using these conditions, crosslinking was completed in 4 hours. Thereafter, the collagen was washed with 0.1 M Na₂HPO₄ solution (2 hours) and demineralized water (four times for 30 minutes) [36, 37]. The residual number of free primary amino groups in collagen after crosslinking was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS, from Fluka, Buchs, Switzerland), according to a slightly modified procedure as described by Wang et al. [38-40]. The shrinkage temperature of (crosslinked) collagen, indicating the resistance against thermal denaturation, was determined using Differential Scanning Calorimetry (DSC) [41].

2.3. Heparin immobilization

Heparin sodium salt (Bufa Chemie, Castricum, the Netherlands) was used. This heparin preparation from porcine mucosa has the following characteristics: Mn = 12,500 g/mol (molecular weight distribution 3,000-30,000 g/mol), activity = 195 IU/mg, 18.75 mol of carboxylic acid groups (Hep-COOH) per mol of heparin [42]. Crosslinked collagen films were incubated with 0.05 M MES-buffer (pH 5.60) for at least 30 minutes. Carboxylic acid groups of heparin (Hep-COOH) were activated using EDC and NHS at a fixed molar ratio of 0.6, and a molar ratio of EDC : Hep-COOH of 0 to 8.0. To a 2% (w/v) solution of (³H-labeled) heparin in 0.05 M MES-buffer (pH 5.60), EDC and NHS were added. After 10 minutes, 1 g of crosslinked collagen (containing 14 free primary amino groups per 1,000 amino acid residues, E/N14C) was reacted with 188.3 ml of EDC/NHS-activated heparin solution, giving a molar ratio of heparin to free primary amino groups of 2. After 2 hours of incubation, the heparinized E/N14C (E/N14C-H) was washed with 0.1 M Na₂HPO₄ (2 hours), 4 M NaCl (4 times for 24 hours) and distilled water (3 times for 24 hours). The amount of immobilized heparin was determined either by measurement of the radioactivity of the collagen samples after immobilization of ³H-labeled heparin, or using toluidine blue as described elsewhere [43, 44].
2.4. $^{125}$I-labeling of bFGF

Human recombinant basic fibroblast growth factor (bFGF, Gibco, Paisley, UK) was labeled with $^{125}$I using Iodobeads [45, 46]. Briefly, to 3 Iodobeads (Iodobeads iodination reagens, Pierce, Rockford, IL) 500 $\mu$Ci $^{125}$I-Na (Amersham) in 100 $\mu$l phosphate buffer (100 mM, pH 7.0) was added. After 5 minutes, bFGF solution in phosphate buffer (1 ml, 100 $\mu$g bFGF/ml) was added and iodination was carried out under gentle shaking at room temperature. After 15 minutes, the beads were removed and rinsed in 150 $\mu$l phosphate buffer. To the total aliquot of phosphate buffer, 600 $\mu$l bovine serum albumin (BSA, Sigma A7030) solution in phosphate buffer was added, giving a final albumin concentration of 1 mg/ml. Residual $^{125}$I was removed from the $^{125}$I-labeled bFGF solution by purification over a series of 3 PD10-columns. Thereafter, the bFGF solution (with a specific activity of $36.4\pm1.7$ kBq/$\mu$g bFGF) was aliquotted and stored at -20°C until use.

2.5. $^{125}$I-bFGF binding studies

Circular films of E/N14C and heparinized E/N14C with a diameter of 10 mm were incubated overnight in 5 ml PBS. After blotting dry, the films were incubated with 0.25 ml bFGF solution (0-320 ng bFGF/ml) in phosphate buffered saline (PBS) (NPBI, Emmer Compascuum, The Netherlands) containing 1 mg/ml BSA, for 90 minutes at room temperature. Thereafter, the samples were washed in 5 ml PBS (2 times for 5 minutes, removing all non-bound bFGF as determined previously), and the radioactivity of the samples was measured using a Compugamma 1282 $\gamma$-counter (LKB, Stockholm, Sweden).

Alternatively, bFGF binding was investigated using bFGF solutions in PBS (1 mg/ml BSA) containing 0-2.5 M NaCl. In order to mimic endothelial cell culture conditions, (heparinized) E/N14C samples were incubated overnight in 5 ml PBS containing penicillin (100 U/ml) and streptomycin (100 $\mu$g/ml) (both from Gibco, Paisley, UK) before bFGF binding.

2.6. bFGF release experiments

Circular collagen films with a diameter of 10 mm were loaded with bFGF as described above, either using unlabeled bFGF (840 ng/ml) or $^{125}$I-labeled bFGF (280 ng/ml). After washing with PBS, the samples were transferred to release medium (5 ml), which consisted of endothelial cell culture medium (CM) containing 5% human serum as used for endothelial cell culture. Endothelial cell culture medium (CM) consisted of a mixture of equal volumes of RPMI 1640 and M199, containing 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 2.5 $\mu$g/ml fungizone, 2 mM L-glutamax (all from Gibco). Release of bFGF was measured at 37°C.
Medium was replaced every 24 hours, and stored at -20°C until assayed. The release of unlabeled bFGF was studied for 10 days. The bFGF concentration in the supernatant was determined using a sandwich ELISA (Quantikine bFGF ELISA, R&D Systems, Abingdon, UK). The release of $^{125}$I labeled bFGF was studied for 28 days.

3. RESULTS

3.1. Collagen crosslinking

Upon crosslinking with EDC and NHS, the number of free primary amino groups per 1,000 amino acid residues decreased while the shrinkage temperature increased. At the conditions used, collagen crosslinking resulted in a material containing approximately 14 free primary amino groups per 1,000 amino acid residues (E/N14C), compared to 27 in native collagen [47]. The shrinkage temperature increased from 55.4°C for native collagen to 75.9°C for E/N14C.

3.2. Heparin immobilization

Heparin was immobilized to E/N14C, using reaction conditions optimized in a previous study [44]. In figure 1, the amount of immobilized heparin is given as a function of the molar ratio of EDC to carboxylic acid groups of heparin (Hep-COOH) used for immobilization. The amount of immobilized heparin increased with increasing ratios of EDC : Hep-COOH to a maximum of approximately 5 to 5.5% heparin (w/w) per gram of collagen at a molar ratio of EDC : Hep-COOH of 2. Heparin was immobilized homogeneously through the entire thickness of the crosslinked collagen film, as shown using Alcian Blue staining of paraffin coupes of the material [44].

3.3. bFGF binding

Binding of $^{125}$I-labeled bFGF from PBS (1 mg/ml BSA) to E/N14C and heparinized E/N14C reached a plateau value within 90 minutes of incubation (data not shown). Immobilization of increasing amounts of heparin to E/N14C led to increased binding of bFGF (figure 2). A plateau value in bFGF binding was observed for materials obtained after heparin immobilization using a molar ratio EDC : Hep-COOH of 0.4 to 0.6. This corresponds to approximately 20-30 mg heparin immobilized per gram of collagen, which is lower than the maximally achievable amount of immobilized heparin (55 mg heparin/g collagen).
Figure 1: Immobilization of $^3$H-heparin to E/N14C, as a function of the molar ratio of EDC to HepCOOH used for immobilization (n = 4, mean ± SD).

Figure 2: Binding of $^{125}$I-labeled bFGF to heparinized E/N14 collagen, as a function of the amount of heparin immobilized (n = 4, mean ± SD).

Circular films with a diameter of 10 mm were incubated for 90 minutes with 0.25 ml $^{125}$I-bFGF solution (360 ng/ml).
For further experiments, E/N14C and E/N14C heparinized using a molar ratio EDC : Hep-COOH of 0.4 (E/N14C-H(0.4)) were used. Binding of bFGF to E/N14C and E/N14C-H(0.4) showed a linear correlation with the concentration of bFGF used for incubation (figure 3). Up to concentrations of 320 ng/ml bFGF, bFGF binding to E/N14C and E/N14C-H(0.4) amounted to 10% and 22% respectively. In separate experiments, similar observations were made for bFGF concentrations up to 840 ng/ml (data not shown).

![Figure 3: Binding of $^{125}$I-labeled bFGF to E/N14 collagen and E/N14 collagen-heparin(0.4), as a function of the bFGF concentration before incubation (n = 3, mean ± SD).](image)

Circular films with a diameter of 10 mm were incubated for 90 minutes with 0.25 ml $^{125}$I-bFGF solution (0-320 ng/ml).

In a control experiment, bFGF binding from PBS containing 20 mg/ml BSA was studied. Higher albumin concentrations are usually applied to prevent loss of bFGF e.g. due to adsorption to the walls of test-tubes during experiments. When using a solution of bFGF in PBS containing 20 mg/ml BSA, bFGF binding to both E/N14C and E/N14C-H(0.4) showed a linear correlation with bFGF concentration. Binding of bFGF to both matrices, however, was approximately 25% lower when compared to bFGF binding from PBS containing 1 mg/ml BSA (data not shown).

In a second control experiment, bFGF solutions (100 ng bFGF/ml, 1 mg BSA/ml in PBS) used for incubation with E/N14C or E/N14C-H(0.4) were used for a second incubation with fresh substrates. In this experiment bFGF-binding to E/N14C as first substrate was $11.1 \pm 0.7 \%$. Subsequent incubation of the partially depleted bFGF solution with E/N14C or E/N14C-H(0.4) resulted in additional bFGF binding of 3.6 % and 9.2 % respectively, when bFGF-binding was expressed as percentage of the bFGF present in the original 100 ng/ml bFGF
solution (table I). When using E/N14C-H(0.4) as first substrate, bFGF binding amounted to 22.6 ± 0.5 %. The second incubation of this bFGF solution with E/N14C and E/N14C-H(0.4) resulted in bFGF binding of 1.0 % and 2.8 % respectively. Substantial bFGF binding to a second substrate was only observed after subsequent incubation of a bFGF solution with E/N14C and E/N14C-H(0.4) (9.2%). Combined bFGF binding to both substrates was 20.3 ± 2.6%, which was not significantly different from bFGF binding to E/N14C-H(0.4) alone (22.6±0.5 % in this experiment). These results indicate that only a fraction of the bFGF was capable of binding to (heparinized-) EDC/NHS-crosslinked collagen.

**Table I:** Binding of $^{125}$I-labeled bFGF to E/N14 collagen and E/N14 collagen-heparin (0.4) during second incubation.

<table>
<thead>
<tr>
<th>First substrate</th>
<th>Binding to second substrate</th>
<th>E/N14C</th>
<th>E/N14C-H(0.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E/N14C</td>
<td>3.6 ± 0.4 %</td>
<td>9.2 ± 0.6 %</td>
<td></td>
</tr>
<tr>
<td>E/N14C-H(0.4)</td>
<td>1.0 ± 0.1 %</td>
<td>2.8 ± 0.9 %</td>
<td></td>
</tr>
</tbody>
</table>

Overnight incubation of E/N14C and E/N14C-H(0.4) with a solution of penicillin and streptomycin in PBS, a procedure used prior to seeding of HUVECs on these substrates, did not influence binding of bFGF from PBS (1 mg/ml BSA) (data not shown).

**Figure 4:** Binding of $^{125}$I-labeled bFGF to E/N14 collagen and heparinized E/N14 collagen as function of the NaCl concentration (n = 3, mean ± SD).

Circular films with a diameter of 10 mm were incubated for 90 minutes with 0.25 ml $^{125}$I-bFGF solution (100 ng/ml).
Binding of bFGF to E/N14C and E/N14C-H(0.4) was dependent on the NaCl concentration (figure 4). Solutions of bFGF dissolved in phosphate buffer (1 mg/ml BSA) containing increasing NaCl concentrations were used for loading of E/N14C and E/N14C-H(0.4). For E/N14C, maximal bFGF binding was observed at a physiological NaCl concentration. bFGF binding from PBS (0.14 M NaCl) to E/N14C was approximately 2-fold higher when compared to solutions without NaCl. Compared to bFGF binding from PBS, bFGF binding gradually decreased with increasing NaCl concentration. For E/N14C-H(0.4) maximal bFGF binding was observed at 0 to 0.6 M NaCl; at higher NaCl concentrations bFGF binding also gradually decreased.

3.4. Release of bFGF

In figure 5, the release of bFGF from E/N14C and E/N14C-H(0.4) at 37°C in endothelial cell culture medium (CM) supplemented with 5% human serum is plotted. E/N14C and E/N14C-H(0.4) films were incubated with bFGF solutions, resulting in $27.2 \pm 1.3$ ng and $49.0 \pm 1.9$ ng bFGF bound per film, respectively. The initial release of bFGF from E/N14C-H(0.4) was slower, compared to the release of bFGF from E/N14C. During the first 6 hours, $9.3 \pm 0.3$ ng bFGF (34%) was released from E/N14C, while in the same period only $1.0 \pm 0.1$ ng bFGF (2%) was released from E/N14C-H(0.4). After 10 days, however, the cumulative absolute bFGF release from E/N14C equaled $22.4 \pm 4.0$ ng, $(83 \pm 14\%)$ which was comparable to the absolute bFGF release from E/N14C-H(0.4) $(20.5 \pm 2.6$ ng, $42 \pm 5\%)$.

In a separate experiment the release of $^{125}$I-labeled bFGF from E/N14C and E/N14C-H(0.4) at 37°C in CM supplemented with 5% human serum was measured for 28 days (figure 6). E/N14C and E/N14C-H(0.4) films were pre-loaded with $8.1 \pm 1.2$ and $19.4 \pm 1.6$ ng bFGF respectively. Regarding $^{125}$I-labeled bFGF release, results were similar to results obtained using unlabeled bFGF in combination with a bFGF ELISA. Initial release of $^{125}$I-labeled bFGF from E/N14C was faster compared to the bFGF release from E/N14C-H(0.4). During the first 4 hours $3.6 \pm 0.2$ ng bFGF was released from E/N14C, while $1.6 \pm 1.1$ ng bFGF was released from E/N14C-H(0.4). After 168 hours, cumulative absolute bFGF release from E/N14C $(6.2 \pm 1.1$ ng, $77 \pm 22\%)$ was comparable to absolute bFGF release from E/N14C-H(0.4) $(7.3 \pm 0.7$ ng, $38 \pm 5\%)$.

Using E/N14C matrices with varying amounts of heparin immobilized (obtained using a ratio of EDC : Hep-COOH of 0.2 to 2.0, figure 1), release profiles similar to the release profile of bFGF from E/N14C-H(0.4) were obtained (data not shown).
Binding and release of basic fibroblast growth factor from heparinized collagen matrices

Figure 5: Release of bFGF from E/N14 collagen and E/N14 collagen-heparin(0.4) in culture medium supplemented with 5% serum (n = 3, mean ± SD).
Circular films with a diameter of 10 mm were loaded for 90 minutes with 0.25 ml unlabeled bFGF solution (840 ng/ml). bFGF concentrations in supernatant were determined using a bFGF-ELISA.

Figure 6: Release of $^{125}$I-labeled bFGF from E/N14 collagen and heparinized E/N14 collagen (n = 3, mean ± SD).
Circular films with a diameter of 10 mm were loaded for 90 minutes with 0.25 ml $^{125}$I-labeled bFGF solution (280 ng/ml).
4. DISCUSSION

Growth factors can generally be defined as proteins which promote proliferation and migration of cells, by interaction with specific cell membrane receptors. Basic fibroblast growth factor (bFGF) is an 18 kDa protein, which induces, *inter alia*, the proliferation of endothelial cells, fibroblasts, smooth muscle cells and chondrocytes [48, 49]. Basic FGF is also known as heparin-binding growth factor, because of its high affinity for heparin and heparan sulfate. At physiological pH and temperature, the *in vitro* half-life time of bFGF activity is approximately 12 hours [50]. Binding of bFGF to heparin induces a conformational change in the bFGF molecule [51], resulting in an increased resistance against thermal denaturation and enzymatic degradation, and a reduced inactivation at acidic pH [50-53]. Furthermore, binding of bFGF to heparin or heparan sulfate facilitates the binding of bFGF to high affinity cell membrane receptors [51, 54]. Although bFGF is localized in almost any tissue examined [55], suggesting that it is needed continuously, the turnover time of target cells like for example endothelium is measured in years. Heparan sulfate is abundantly present in the extracellular matrix (ECM) of endothelial cells. The high affinity of bFGF for glycosaminoglycans suggests that the ECM may function as a storage pool for bFGF [56, 57]. When the vascular wall is damaged, bFGF can be released through several mechanisms [58] and proliferation of endothelial cells will be induced. By binding of bFGF to a heparinized collagen-coated vascular graft, this process of induction of cell growth can be mimicked.

Increased binding of radio-labeled bFGF was observed with increasing amounts of immobilized heparin, giving maximal bFGF binding for materials obtained after heparin immobilization using a molar ration of EDC : Hep-COOH of 0.4 to 0.6. In a previous study the anticoagulant activity of heparin immobilized to E/N14C was determined [44]. E/N14C matrices heparinized using a molar ratio of EDC : Hep-COOH of 0.4 showed maximal thrombin inhibitory activity and a maximal reduction in contact activation. Combining these results, for further experiments E/N14C matrices heparinized using a molar ratio of EDC to Hep-COOH of 0.4 (E/N14C-H(0.4)) were used.

Binding of bFGF to E/N14C and E/N14C-H(0.4) showed a linear correlation with bFGF concentrations up to 840 ng/ml. Depending on molecular weight, heparin can bind up to 13 molecules of bFGF per molecule of heparin in solution [53, 59]. In case of E/N14C-H(0.4), maximal one molecule of bFGF was bound per 1,000 molecules of immobilized heparin, indicating that saturation of binding will occur only at much higher bFGF concentrations. The low Kd reported for binding of bFGF to cell surface heparan sulfate or heparin in solution (10⁻⁸ to 10⁻⁹ M) [54] led us to expect high efficiency binding of bFGF from solution to heparinized crosslinked collagen matrices. Low binding of bFGF was observed, however, which was most likely not caused by material characteristics, but probably due to depletion of
the fraction of bFGF capable of binding to E/N14C and E/N14C-H(0.4) (table I). When both substrates were incubated with fresh bFGF solution after a first incubation with bFGF, during both first and second incubation an illustrative bFGF binding of 10% and 22% was observed for E/N14C and E/N14C-H(0.4), respectively (data not shown). One might speculate that low binding is caused by e.g. deficient folding of the recombinant protein during manufacturing, resulting in a fraction of bFGF not capable of binding [60].

Binding of bFGF and heparin is mediated by ionic interaction between both 2-0-sulfate groups and N-sulfate groups of heparin molecules [61, 62] and certain lysine and arginine residues in bFGF [63, 64]. Studies concerning binding of bFGF to collagen are not reported in literature, but binding of bFGF to collagen is likely to involve non-specific ionic interactions. Additionally, hydrophobic interactions might account for the increased bFGF binding from PBS to E/N14C observed when increasing the NaCl concentration from 0 to 0.15 M. This, however, is speculative, and a conclusive explanation can not be given. In this study, binding of bFGF to E/N14C and E/N14C-H(0.4) decreased with increasing NaCl concentrations, indicating the presence of ionic interactions between bFGF and (heparinized) E/N14C. The gradual decrease in bFGF binding with increasing NaCl concentrations suggests the involvement of multiple binding sites with a range of binding strengths located on both E/N14C and E/N14C-H(0.4).

When used for cell culture in our laboratory, (heparinized) crosslinked collagen is incubated overnight in a solution containing penicillin and streptomycin, a procedure used to decrease the risk of bacterial infection during cell culture. Following incubation with penicillin and streptomycin, matrices are pre-loaded with bFGF and subsequently endothelial cells are seeded. Incubation of these matrices with antibiotics did not influence bFGF binding despite the fact that these antibiotics are positively charged and capable of binding to heparin.

Release of bFGF was measured in CM supplemented with 5% serum, resembling the medium used for culture of HUVECs seeded on bFGF-loaded (heparinized) collagen matrices [22]. Release of bFGF from E/N14C was initially faster than bFGF release from E/N14C-H(0.4), probably due to the higher affinity of bFGF for heparin. However, because loading of E/N14C-H(0.4) with bFGF was approximately two-fold higher compared to E/N14C, after 10 days in CM 5% comparable amounts of bFGF were released from both materials.

As there was some doubt regarding the stability of radio-labeled bFGF, its release was also measured using unlabeled bFGF in combination with an ELISA. Release profiles of unlabeled bFGF and 125I-labeled bFGF were in good agreement. Although initial release from E/N14C-H(0.4) as measured using 125I-bFGF was somewhat higher compared to release measured using unlabeled bFGF (deviation maximal 10%) results expressed as percentage release show no difference after longer periods of time (e.g. 4 days). These observations are in agreement
with results obtained by others when measuring bFGF release using $^{125}$I-bFGF, in combination with additional techniques [65, 66].

When injected intravenously, bFGF is cleared rapidly from blood. In rats, half-life times of 1.5 to 3 minutes are reported [67, 68]. When E/N14C or E/N14C-H(0.4) are used in vivo as matrix for endothelial cell seeding of vascular grafts, a rapid clearance of bFGF released from these matrices is to be expected too. For bFGF bound to E/N14C, a burst-release of 30-35% was observed during the first 6 to 20 hours, in contrast to E/N14C-H(0.4) from which bFGF release was more gradual, resulting in a substantial loss of bFGF pre-loaded to E/N14C. In addition to a slower release rate of bound bFGF, heparin immobilization to E/N14C resulted in approximately two-fold higher bFGF loading. Therefore, compared to E/N14C, improved proliferation of seeded HUVECs on E/N14C-H(0.4) over a prolonged period of time is to be expected. Our results indicate that E/N14C-H(0.4) is a candidate matrix for bFGF pre-loading and subsequent endothelial cell seeding.

5. CONCLUSIONS

Immobilization of 2 to 3 wt % heparin to E/N14C results in approximately two-fold increase of bFGF binding. Both E/N14C and E/N14C-H(0.4) pre-loaded with bFGF show sustained bFGF release in endothelial cell culture medium over a prolonged period of time. Because of the increased bFGF binding and the slower, more gradual release of bFGF from E/N14C-H(0.4), E/N14C-H(0.4) pre-loaded with bFGF is expected to be a better substrate for endothelial cell seeding than E/N14C without immobilized heparin.

REFERENCES


This thesis, Chapter 6.

This thesis, Chapter 7.


Chapter 5


CHAPTER 6

Endothelial cell seeding on crosslinked collagen: Effects of crosslinking on endothelial cell proliferation and functional parameters

M.J.B. Wissink¹, M.J.A. van Luyn², R. Beernink¹, F. Dijk³, A.A. Poot¹, G.H.M. Engbers¹, T. Beugeling¹, W.G. van Aken¹, J. Feijen¹

¹Institute for Biomedical Technology, Polymer Chemistry and Biomaterials Group, Department of Chemical Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands.
²Faculty for Medical Sciences, Cell Biology and Biomaterials, Groningen University, Bloemsingel 10, 9712 KZ, Groningen, The Netherlands.
³Cell Biology and Electron Microscopy, Groningen University, Oostersingel 69-2, 9713 EZ, Groningen, The Netherlands.

ABSTRACT

Endothelial cell seeding, a promising method to improve the performance of small-diameter vascular grafts, requires a suitable substrate, such as crosslinked collagen. Commonly used crosslinking agents such as glutaraldehyde and formaldehyde cause, however, cytotoxic reactions and thereby hamper endothelialization of currently available collagen-coated vascular graft materials.

The aim of this study was to investigate the effects of an alternative method for crosslinking of collagen, using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) in combination with N-hydroxysuccinimide (NHS), on various cellular functions of human umbilical vein endothelial cells (HUVECs) in vitro. Compared to non-crosslinked type I collagen, proliferation of seeded endothelial cells was significantly increased on EDC/NHS-crosslinked collagen. Furthermore, higher cell-numbers were found with increasing crosslink-densities. Neither the morphology of the cells nor the secretion of prostacyclin (PGI₂), von Willebrand factor (vWF), tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1) was affected by the crosslink-density of the collagen substrate. Therefore, EDC/NHS-crosslinked collagen is candidate substrate for in vivo application such as endothelial cell seeding of collagen-coated vascular grafts.
1. INTRODUCTION

Whereas vascular grafts have been successfully used to replace large-diameter blood vessels, the long-term patency of small diameter vascular grafts is disappointing, primarily due to thrombus formation [1-4]. The bio-compatibility of small-diameter vascular grafts can be improved by endothelial cell seeding, which provides optimal non-thrombogenic surface characteristics [5]. Synthetic vascular grafts, which are made of expanded Teflon or Dacron, are generally poor substrates for cell-seeding [6]. Chemical surface modification [7], gas plasma treatment [6, 8-10], or application of a suitable protein coating may improve the potential of graft materials for cell adhesion and proliferation. Since non-crosslinked collagen appears to be a suitable substrate for the growth of vascular endothelial cells in vitro [11-13], application of a collagen coating on synthetic vascular graft materials may result in a matrix suitable for endothelial cell seeding. Commercially available collagen-coated vascular grafts have been developed to eliminate the time-consuming procedure of clotting of the graft before implantation. Crosslinking of this matrix by agents such as glutaraldehyde and formaldehyde, to prevent rapid in vivo resorption, is commonly used [14, 15]. Of these crosslinking agents, which are incorporated in the protein coating during crosslinking [16], notably glutaraldehyde is known to evoke cytotoxic reactions by release of unreacted glutaraldehyde or glutaraldehyde residues, during in vitro as well as in vivo degradation [17-20]. Upon implantation, cytotoxic reactions hamper endothelialization of currently available collagen-coated graft materials [20, 21]. As a consequence there is a need to develop a non-cytotoxic crosslinked coating of collagen, which supports adherence and proliferation of seeded endothelial cells.

The use of N-(3-dimethylaminopropyl)-N’-ethyldenediisocarbodiimide (EDC) in combination with N-hydroxysuccinimide (NHS) as crosslink agents is well known from literature concerning peptide synthesis [22, 23]. The reaction of EDC with carboxylic acid groups from proteins results in the formation of O-acylisourea. Subsequently, reaction of O-acylisourea with NHS gives rise to NHS-activated carboxylic acid groups, which react with amino groups to form peptide bonds together with liberated NHS. When used to crosslink collagen, activated glutamic- and aspartic acid residues react with lysine- or hydroxylysine residues. The use of NHS prevents side reactions of the EDC-activated groups such as hydrolysis, or N-acyl shift to form stable N-acylisourea [23].

The aim of the present study was to investigate the effect of crosslinking of collagen, using EDC and NHS, on the adhesion and growth of seeded human umbilical vein endothelial cells in vitro. Furthermore, the effects of EDC/NHS-crosslinking of collagen on various endothelial cell functions including $^3$H-thymidine incorporation and the secretion of tissue plasminogen
activator (t-PA), plasminogen activator inhibitor (PAI-1), von Willebrand factor (vWF) and prostacyclin (PGI₂) were determined.

2. MATERIALS AND METHODS

2.1. Materials

Unless otherwise stated, chemicals were obtained from Merck (Darmstadt, Germany), and were of highest purity available.

2.2. Collagen films

All experiments were carried out using flat collagen films as model substrates. Type I insoluble collagen derived from Bovine Achilles Tendon (Sigma, St.Louis, Missouri, USA, C99879, lot 23H7065) was swollen overnight in 0.52 M acetic acid solution (1 g collagen/50 ml) at 4°C. The mixture was dispersed with 50 g of crushed ice for 4 minutes in a Philips Blender and thereafter further homogenized for 30 minutes at 4°C using an Ultra-Turrax T25 (IKA labortechnik, Staufen, BRD). The resulting slurry was filtered through a series of filters (Cellctor screen, Bellco, Feltham, UK), with pore sizes decreasing from 140 μm to 10 μm, mounted in 47 millimeter diameter Swinnex disc filter holders (Millipore, Etten-Leur, The Netherlands). After de-aeration at a pressure of 0.06 mbar, the resulting suspension was casted as a film with a thickness of 3 millimeters on a flat poly(ethylene terephthalate) (PET) surface, using a casting knife. After drying at room temperature, a collagen film with a thickness of approximately 50 μm was obtained.

2.3. Collagen crosslinking

Collagen films were crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS), in 0.05 M buffer of 2-morpholinoethane sulfonic acid (MES buffer, pH 5.40) [24]. Before crosslinking, dried collagen films were equilibrated with MES buffer for 30 minutes. Subsequently, the films were immersed in a solution of EDC and NHS in MES buffer, under gentle shaking. For the crosslinking reaction, 1.731 g EDC and 0.415 g NHS per gram of collagen were used. At selected times, crosslinking was stopped by washing the collagen film with 0.1 M Na₂HPO₄ solution for 2 hours. This treatment resulted in hydrolysis of both activated carboxylic acid groups and residual EDC [24, 25]. After repeated washing (four times for 30 minutes) with demineralized water collagen was
either lyophilized and dried over KOH in a desiccator for further analysis, or used for endothelial cell seeding.

2.4. TNBS assay

The residual number of free primary amino groups in collagen after crosslinking was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS, Fluka, Buchs, Switzerland), according to a procedure described by Wang et al. [16, 26, 27]. The reaction of TNBS with primary amino groups leads to the formation of trinitrophenyl lysine residues. Samples of approximately 4 mg of (crosslinked) collagen were incubated with 1.0 ml of a 4 % (w/v) NaHCO$_3$ solution for 1 hour at room temperature. Thereafter, 1.0 ml of a 0.5 % (w/v) TNBS solution in water was added and the mixture was heated at 40°C for 2 hours. Collagen samples were hydrolyzed at 60°C for 90 minutes upon incubation in 3.0 ml of 6 M HCl. The absorbance was measured at 345 nm, after addition of 5 ml water and cooling to room temperature. A control sample was prepared using the same procedure, except that HCl was added prior to the TNBS solution. For calculation of the number of unreacted amino groups a molar absorption coefficient of 14,600 l mol$^{-1}$ cm$^{-1}$ for trinitrophenyl lysine was used [27]. Results were expressed as numbers of free primary amino groups per 1,000 amino-acid residues.

2.5. Shrinkage temperature

The shrinkage temperature of (crosslinked) collagen was determined using Differential Scanning Calorimetry (DSC) [28]. A sample of approximately 2.5 mg collagen was incubated with 50 µl phosphate buffered saline (PBS) (NPBI, Emmer Compascuum, The Netherlands) for 1 hour at room temperature, in a sealed volatile sample pan (Perkin-Elmer, Norwalk, CT). Thereafter, the DSC thermogram was recorded on a Perkin Elmer DSC 7 DSC-apparatus. Samples were heated from 20°C to 95°C, at a heating rate of 5°C/minute. A sample only containing 50 µl PBS was used as a reference. The onset of the endothermic peak, which indicated denaturation of collagen, was recorded as the shrinkage temperature.

2.6. Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured according to van Wachem et al. [29]. After harvesting, cells were cultured to the third passage in tissue culture polystyrene (TCPS) flasks (Costar, Cambridge, MA), precoated with a solution of partially purified human fibronectin (Fn$^c$, 2 mg/ml, in serum-free culture medium). Fn$^c$ was a
gift of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam, the Netherlands). Endothelial cell culture medium (CM) consisted of a mixture of equal volumes of RPMI 1640 and M199 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 2 mM Glutamax (all from Gibco, Paisley, UK), supplemented with 20 % human serum derived from a pool of 12 healthy donors, who had not taken aspirin during 3 days before blood donation (CM 20%). Cell culturing was carried out at 37°C, in a humidified atmosphere of 5% CO2 and 95% air. HUVECs, used for subcultures and for experiments were detached from TCPS by incubation with trypsin (Gibco, 0.05 % trypsin with 0.02% EDTA in PBS). Residual trypsin was inactivated by addition of culture medium.

2.7. HUVEC proliferation experiments

For proliferation experiments, endothelial cells were seeded on collagen with varying crosslink densities, as well as on a non-crosslinked collagen matrix and fibronectin-coated TCPS. Before use, collagen films were incubated overnight at 37°C in a solution of penicillin (200 U/ml) and streptomycin (200 μg/ml) in PBS. The films were placed in wells of tissue culture dishes (Costar, Cambridge, Massachusetts), and fixed with steam sterilized Viton O-rings (Eriks, Alkmaar, the Netherlands). HUVECs were seeded at a density of 10,000 cells/cm². Culture medium consisted of CM supplemented with 5% human serum and bFGF (human recombinant basic fibroblast growth factor, Gibco, 0.3 ng/ml) (CM 5%/H/F). Culture medium was replaced three times per week. At selected times, the number of HUVECs was determined using a Bürker chamber. After removal of supernatant culture medium, HUVECs were detached from collagen films by incubation with 400 μl of a solution containing collagenase (Sigma C 6885, type 2, 1 mg/ml) and bovine serum albumin (BSA, Sigma A6003, 5 mg/ml) in PBS.

2.8. 3H-Thymidine incorporation

Supernatant culture medium of the HUVEC cultures was replaced by 0.5 ml culture medium containing 3H-thymidine (Amersham, Amersham, UK, 1 mM, 1.75*10⁶ dpm/ml). After 6 hours incubation, medium was removed and cells were washed with warm PBS. Subsequently, the cells were incubated for 10 minutes using cold 10% trichloroacetic acid solution (TCA), followed by two washes with cold 10% TCA. The precipitate was solubilized overnight in NaOH (1 ml, 1 M). After washing of the surface with 1 M NaOH (0.5 ml), radioactivity in the total volume of 1.5 ml NaOH was measured upon addition of 18.5 ml scintillation cocktail (Optiphase HiSafe, Wallac, Milton Keynes, UK), using a 1414 Winspectral liquid scintillation counter (Wallac, Turku, Finland).
2.9. Electron microscopy

Cell samples were washed for 1 minute using a 6.8% (w/v) of sucrose solution in cacodylate buffer (0.1 M, pH 7.4). Pre-fixation was carried out in situ, i.e. in tissue culture dishes, using 2% glutaraldehyde solution in cacodylate buffer, at 4°C. After post-fixation with OsO₄, the samples were dried using a graded series of ethanol, followed by critical point drying from CO₂. Following sputter-coating with gold/palladium (10 nm), SEM images were taken using a Jeol 6301F (Jeol, Tokyo, Japan) scanning electron microscope.

For transmission electron microscopy, after pre-fixation with glutaraldehyde, cell samples were rinsed with cacodylate buffer and postfixed in OsO₄ with 1.5% K₂Fe(CN)₆ for 2 hours at room temperature. Samples were dehydrated by graded series of ethanol and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips EM201 transmission electron microscope (Philips, Eindhoven, The Netherlands), operated at 60 kV.

2.10. t-PA and PAI-1 secretion

Culture medium of HUVECs was replaced by 1 ml of freshly prepared culture medium. After culturing for 24 hours the supernatant medium was collected, centrifuged (10 min, 400g, 4°C) and stored at -20°C. Concentrations of t-PA and PAI-1 in the samples were determined using ELISAs for each of these proteins [30] (Innogenetics, Zwijnaarde, Belgium).

2.11. vWF and PGI₂ secretion

Cell cultures were washed with a solution of BSA in culture medium without serum (CM/1% BSA, 37°C), followed by 30 minutes incubation in respectively CM/1% BSA (“non-stimulated”) or CM/1% BSA with 10 µM calcium ionophore (A23187, “stimulated”). Subsequently, supernatant medium was collected, centrifuged (10 min, 400g, 4°C) and stored at -20°C until assayed. vWF was determined using an ELISA [31] (Gradiopore, North Ryde, Australia). PGI₂ concentrations were determined using a competitive EIA for the stable hydrolysis product of PGI₂, 6-keto-prostaglandin F₁α (Amersham, Amersham, England) [32].
3. RESULTS

3.1. Collagen crosslinking

Upon crosslinking with EDC and NHS, activated carboxylic acid groups of collagen react with free ε-amino groups to form peptide-like crosslinks. As a result, the number of free primary amino groups per 1,000 amino acid residues, which in native collagen amounts to 27 [33], decreases while the shrinkage temperature increases.

In a previous study, the reaction time and the reaction volume proved to be useful parameters in controlling the final crosslink-density of collagen [34]. At the conditions used in the present study, crosslinking was completed within 4 hours. Crosslinking in 215 ml MES buffer per gram of collagen resulted in materials with 22, 18 and 14 free primary amino groups per 1,000 amino acid residues (E/N22C, E/N18C and E/N14C) after respectively 15 minutes, 30 minutes and 4 hours of reaction. The shrinkage temperature (Ts) increased correspondingly from 55.4°C for native collagen to 78.2°C for E/N14C. Using a buffer volume of 100 ml per gram of collagen, crosslinked collagen containing 10 free primary amino groups (E/N10C, Ts = 82.0°C) was obtained after 4 hours of reaction. A linear correlation between the increase in shrinkage temperature and the decrease in the number of free primary amino groups was observed (data not shown), which is in agreement with data from the literature [16].

3.2. Proliferation of HUVECs

At three days after seeding, the number of HUVECs on TCPS and N-Coll was higher than on EDC/NHS-crosslinked collagen (fig. 1). Whereas HUVECs plated on TCPS continued to proliferate for at least 10 days, on native collagen cell growth stopped after approximately 7 days. On crosslinked collagen, the number of cells at three days was about half of the number present on TCPS. Thereafter, cell proliferation on crosslinked collagen continued for at least 10 days. The number of HUVECs on the EDC/NHS-crosslinked collagen matrices at day 7 were comparable to, or somewhat higher than cell numbers on TCPS and native collagen. At 10 days after seeding the number of HUVECs on crosslinked collagen was higher than on TCPS and native collagen, except for E/N22C.

In figure 2, SEM images are shown of HUVECs on E/N14C and E/N10C after 3, 7 and 10 days proliferation (fig. 2a-f) and on TCPS and N-Coll after 10 days of proliferation (fig. 2g-h). SEM showed a (nearly) confluent layer of HUVECs on all substrates after 7 days of proliferation (E/N22C and E/N18C not shown). Confluent layers of HUVECs were found on all substrates 10 days after cell seeding. No morphological differences were observed when comparing HUVECs on crosslinked collagen substrates with different crosslink-densities and...
HUVECs on TCPS and N-Coll. Transmission electron microscopy (TEM) revealed a monolayer of endothelial cells with comparable morphology on all substrates after 10 days of proliferation. Cell edges occasionally showed some overlap. Cross-sections of HUVECs on both (crosslinked-) collagen (fig. 3a, representative image, E/N14C) and TCPS (fig. 3b) showed a close, continuous contact with the underlying matrix. At day 10, cells on both TCPS and crosslinked collagen were still active. Chromatin in the cell nuclei mainly consists of euchromatin (i.e. non-condensed, fig. 3), considered transcriptionally active in providing RNA molecules. In the collagen matrix (fig. 3a) the distinct periodicity of longitudinally cut, intact collagen fibers could clearly be observed.

![Figure 1](image-url)  
**Figure 1:** Proliferation of HUVECs on fibronectin-coated TCPS, non-crosslinked collagen (N-Coll) and EDC/NHS-crosslinked collagen (E/N22C to E/N10C) during 10 days after seeding (n = 4, mean ± SD).

### 3.3. $^3$H-thymidine incorporation

Thymidine incorporation was investigated after cells were cultured for respectively 3, 7 and 10 days. Except for E/N10C, $^3$H-thymidine incorporation by endothelial cells behaved similarly, and decreased as a function of time during which cells had been in culture. Cells cultured on EDC/NHS-crosslinked collagen (especially on E/N18C and E/N10C) showed a higher $^3$H-thymidine incorporation 3 days after seeding when compared to cells cultured on TCPS and N-Coll.
Figure 2: Continued on next page.
Figure 2: Representative SEM-images of HUVECs on EDC/NHS-crosslinked collagens E/N14C and E/N10C, cultured for 3 days (2a and 2b), 7 days (2c and 2d) and 10 days (2e and 2f), and HUVECs cultured for 10 days on fibronectin-coated TCPS and N-Coll (2g and 2h, overview) (original magnification 400× (a-f) or 100× (g, h)).

Figure 3: Representative TEM-images of HUVECs on EDC/NHS crosslinked collagen E/N14C (3A) and fibronectin-coated TCPS (3B) after 10 days of proliferation (original magnification 10,750 ×). N: nucleus, *: euchromatin, arrow: collagen periodicity.
Figure 4: \(^3\)H-Thymidine incorporation by HUVECs cultured on fibronectin-coated TCPS, non-crosslinked collagen (N-Coll) and various EDC/NHS-crosslinked collagens (E/N22C to E/N10C) (n = 3, mean ± SD).

\(^3\)H-Thymidine incorporation after 6 hours of incubation with 0.5 ml culture medium containing 1 \(\mu\)M tritiated thymidine (1.75\(\times\)10\(^6\) dpm/ml).

3.4. t-PA secretion

Secretion of tPA by HUVECs on TCPS and N-Coll increased upon culturing for 10 days (fig. 5). tPA secretion by HUVECs on crosslinked collagen was initially (day 3) up to 10 fold higher compared to cells cultured on TCPS and N-Coll. However, during the period of 3 to 10 days of culturing the initially high tPA secretion by HUVECs plated on crosslinked collagen decreased to values comparable to secretion of cells cultured on TCPS and N-Col.

3.5. PAI-1 secretion

Secretion of PAI-1 by HUVECs decreased as a function of culture time on all substrates except N-Coll (fig. 6), which remained stable. PAI-1 secretion by HUVECs cultured for 3 days on EDC/NHS-crosslinked collagen was substantially higher compared to cells cultured on TCPS and N-Coll. In contrast, secretion of PAI-1 by HUVECs cultured for 10 days on EDC/NHS-crosslinked collagen (except E/N22C) was significantly lower compared to cells cultured on N-Coll and TCPS.
Figure 5: t-PA secretion by HUVECs cultured on fibronectin-coated TCPS, non-crosslinked collagen (N-Coll) and various EDC/NHS-crosslinked collagens (E/N22C to E/N10C) (n = 4, mean ± SD).
Culture medium was replaced by 1 ml of freshly made culture medium. After 24 hours of incubation (after 3, 7 and 10 days of culture, respectively) the concentration t-PA was determined in cell supernatants using an ELISA.

Figure 6: PAI-1 secretion by HUVECs cultured on fibronectin-coated TCPS, non-crosslinked collagen (N-Coll) and various EDC/NHS-crosslinked collagens (E/N22C to E/N10C) (n = 4, mean ± SD).
Culture medium was replaced by 1 ml of freshly made culture medium. After 24 hours of incubation (after 3, 7 and 10 days of culture, respectively) the concentration PAI-1 antigen was determined in cell supernatants using an ELISA.
Figure 7: vWF secretion by HUVECs cultured on fibronectin-coated TCPS, non-crosslinked collagen (N-Coll) and various EDC/NHS-crosslinked collagens (E/N22C to E/N10C) (n = 4, mean ± SD).

Cell cultures were washed with a solution of BSA in culture medium (CM/1% BSA, 37°C), followed by 30 minutes incubation at 37°C in CM/1% BSA (fig. 7A, “non-stimulated”) or CM/1% BSA with 10 μM calcium ionophore (A23187, fig. 7B, “stimulated”). vWF concentrations in cell supernatants were determined using an ELISA.
3.6. vWF secretion

Non-stimulated vWF secretion by HUVECs cultured for 3 days on EDC/NHS-crosslinked collagen was 4 to 6 fold higher when compared to HUVECs cultured on TCPS and N-Coll (fig. 7). After 10 days of proliferation, non-stimulated vWF secretion of HUVECs cultured on crosslinked collagen decreased to values comparable to TCPS and N-Coll. A23187-stimulated vWF secretion by HUVECs cultured on all substrates was significantly higher compared to basal secretion of vWF, except for 3 days culture time at which stimulated and non-stimulated vWF-secretion was comparable. Although A23187-stimulated secretion of vWF by HUVECs cultured for 3 days on EDC/NHS-crosslinked collagen was significantly higher compared to HUVECs cultured on TCPS and N-Coll, this difference disappeared after 10 days of culture, as was observed for non-stimulated vWF secretion. A23187-stimulated vWF secretion by HUVECs cultured for 3 days on all substrates was not significantly different compared to basal secretion of vWF

3.7. PGI₂ secretion

Irrespective of the culture substrate and the culture time, secretion of PGI₂ by A23187-stimulated HUVECs was significantly higher compared to non-stimulated PGI₂ secretion. Both non-stimulated and A23187-stimulated PGI₂ secretion by HUVECs cultured for 3 days on EDC/NHS-crosslinked collagen were higher compared to cells cultured on TCPS and N-Coll. In contrast, secretion of PGI₂ by HUVECs cultured for 10 days on EDC/NHS-crosslinked collagen was substantially lower compared to cells cultured on TCPS and N-Coll, especially for A23187-stimulated secretion. This difference, however, was not observed in additional experiments (see discussion).

4. DISCUSSION

From commercially available collagen- or gelatin-coated vascular grafts, formaldehyde or glutaraldehyde residues are released. Therefore, these commercially available grafts will not support the proliferation of seeded endothelial cells [17, 20, 35]. EDC and NHS are well known from peptide synthesis [22, 23]. By means of EDC and NHS, peptide bond-like crosslinks are formed in collagen. These crosslink agents in theory are not incorporated in the matrix. After crosslinking, (non)reacted reagents can be readily removed by washing. EDC/NHS-crosslinked collagen has been reported to be non-cytotoxic in vitro [17], and biocompatibility was observed in animal models [36-38]. In the present study,
Figure 8: Prostacyclin secretion by HUVECs cultured on fibronectin-coated TCPS, non-crosslinked collagen (N-Coll) and various EDC/NHS-crosslinked collagens (E/N22C to E/N10C) (n = 4, mean ± SD).

Cell cultures were washed with a solution of BSA in culture medium (CM/1% BSA, 37°C), followed by 30 minutes incubation at 37°C in CM/1% BSA (fig. 8A “non-stimulated”) or CM/1% BSA with 10 μM calcium ionophore (A23187, fig. 8B, “stimulated”). PGI₂ concentrations in cell supernatants were determined using an assay for the stable hydrolysis product of PGI₂, 6-keto-prostaglandin F₁α.

Materials with 22, 18, 14 and 10 free amino groups per 1,000 amino acid residues were prepared (E/N22C, E/N18C, E/N14C and E/N10C). The functional characteristics of endothelial cells seeded on these substrates as well as non-crosslinked collagen (N-Coll) and fibronectin-coated TCPS were compared.
Chapter 6

At 3 days after seeding, the number of endothelial cells on crosslinked collagen was about half the number present on TCPS and N-Coll. This can be due to the lower initial adherence of seeded HUVECs on the crosslinked collagen substrates. $^3$H-thymidine incorporation by endothelial cells plated on crosslinked collagen was higher than found for N-Coll and TCPS, due to the higher proliferation rate of cells on EDC/NHS-crosslinked collagen. At confluency, the number of rapidly dividing HUVECs decreased and as a result $^3$H-thymidine incorporation declined irrespective of the seeding surface. It is not clear why crosslinking of collagen causes improved proliferation of seeded endothelial cells. Possibly, the improved proliferation of HUVECs is a result of physical and chemical alterations introduced by EDC/NHS-crosslinking of collagen, like a decreased number of free amino- and carboxylic acid groups, or a decreased swelling of the collagen matrix.

The secretion of various factors affecting platelet adhesion and aggregation (PGI$_2$), blood coagulation (vWF) and fibrinolysis (t-PA, PAI-1) was measured to compare the function of endothelial cells on various substrates. Compared to N-Coll and TCPS, secretion of these factors was increased after 3 days of culture due to activation of HUVECs on EDC/NHS-crosslinked collagen shortly after seeding. Upon prolonged culturing the secretion of these substances gradually decreased.

Dekker et al., who studied the effect of cell density on the release of tPA, PAI-1, PGI$_2$ and vWF by endothelial cells seeded on fibronectin-coated TCPS observed that at low density endothelial cells secreted more of the various factors than at high density [39]. This effect was most pronounced for tPA and vWF, the release of tPA and vWF decreased sharply when the number cells increased. At cell numbers higher than 10,000 - 20,000 cells/cm$^2$ the release was almost independent of cell density, which confirms observations by others [40, 41].

To verify if culturing of HUVECs on EDC/NHS-crosslinked collagen at low density also resulted in increased vWF and prostacyclin secretion, HUVECs were seeded at densities ranging from 2,500 to 50,000 cells/cm$^2$ on fibronectin-coated TCPS, N-Coll and on E/N14C. Basal- and stimulated secretion of prostacyclin and vWF showed a marked increase after one day of proliferation for the lowest cell densities on EDC/NHS-crosslinked collagen (data not shown) [42]. The same observation was made for fibronectin-coated TCPS and non-crosslinked collagen. After longer times of culture, the initially higher secretion of PGI$_2$ and vWF at the lowest seeding densities decreased, to values comparable to secretion found after seeding at higher densities. The observed low PGI$_2$ secretion by HUVECs after 10 days of culture on EDC/NHS-crosslinked collagen (fig. 8) was not reproduced in these additional experiments, which is probably due to biological variations between different isolations of HUVECs.

These results clearly indicate that the initially high secretion of the endothelial cell factors tested in the present study is due to low cell densities, instead of activation of HUVECs by the
EDC/NHS-crosslinked collagen substrate. This implies that seeding density is critical, when endothelial cell seeding is applied *in vivo*. The overall effect of (a low) cell density on the balance of hemostasis in endothelial cell seeded vascular grafts remains to be investigated.

5. CONCLUSIONS

In this study, we used EDC/NHS-crosslinked collagen as an *in vitro* model for collagen-coated vascular graft materials. EDC/NHS-crosslinking of collagen resulted in a substrate which supported proliferation of seeded HUVECs *in vitro*, indicating non-cytotoxicity. HUVECs cultured on crosslinked substrates showed increased cell proliferation with increasing crosslink-densities. Cell structure was not altered compared to HUVECs cultured on non-crosslinked collagen and TCPS. After 10 days of proliferation, cell numbers on crosslinked collagen matrices with higher crosslink densities (i.e. E/N18C, E/N14C and E/N10C) were higher than on TCPS and N-Coll. The results of this study indicate that the initially high secretion of tPA, PAI-1, vWF and PGI2 by HUVECs cultured on EDC/NHS-crosslinked collagen is due to low cell densities shortly after seeding rather than material characteristics.

Concluding, the results obtained with EDC/NHS-crosslinked collagen suggest it is suitable as a matrix for endothelial cell seeding of synthetic vascular grafts.

REFERENCES


[34] This thesis, Chapter 3.
APPENDIX TO CHAPTER 6

Relation between cell density and the secretion of von Willebrand factor and prostacyclin by human umbilical vein endothelial cells

Institute for Biomedical Technology, Polymer Chemistry and Biomaterials Group, Department of Chemical Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

ABSTRACT

In this study, the relation between the density of human umbilical vein endothelial cells (HUVECs) cultured on TCPS and (crosslinked) collagen, and the secretion of von Willebrand factor (vWF) and prostacyclin (PGI\textsubscript{2}) was determined. Collagen was crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) in combination with N-hydroxysuccinimide (NHS), resulting in a matrix containing 14 free primary amino groups per 1,000 amino acid residues after crosslinking (E/N14C). HUVECs were seeded on E/N14C, non-crosslinked collagen (N-Coll) and fibronectin coated TCPS at densities ranging from 2,500 to 50,000 cells/cm\textsuperscript{2}. After one day of culture, both basal and A23187-stimulated secretion vWF (expressed per 1,000,000 cells) was considerably increased at low cell densities (i.e. below 5,000 cells/cm\textsuperscript{2}) on all substrates. Secretion of PGI\textsubscript{2} gradually increased with decreasing cell densities below 10,000 cells/cm\textsuperscript{2}. After 10 days of proliferation, cell numbers on all substrates exceeded 50,000 cells/cm\textsuperscript{2}, irrespective of the seeding density. Concomitantly, the initially higher secretion of PGI\textsubscript{2} and vWF at the lowest seeding densities was decreased after longer times of culture, to values comparable to those obtained for higher seeding densities.

1. INTRODUCTION

In chapter 6, the functional characteristics of endothelial cells seeded on fibronectin-coated TCPS, non-crosslinked collagen (N-Coll) and collagen matrices crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) were determined. Human umbilical vein endothelial cells (HUVECs) were seeded at 10,000
cells/cm² on TCPS, N-Coll and EDC/NHS-crosslinked collagen matrices with increasing crosslink densities. After 3 days of culture, secretion of tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1), von Willebrand factor (vWF) and prostacyclin (PGI₂) by HUVECs cultured on EDC/NHS-crosslinked collagen was much higher than secretion of these substances by HUVECs on N-Coll and TCPS. Cell numbers found on EDC/NHS-crosslinked collagen after 3 days of culture were lower than cell numbers found on N-Coll and TCPS.

Dekker et al. reported that both non-stimulated and thrombin-stimulated human saphenous vein endothelial cells at low cell densities secreted more t-PA, PAI-1, vWF and PGI₂ than cells at higher cell densities [1]. At cell numbers higher than about 10,000 - 20,000 cells/cm² secretion was almost independent of cell density. Lower cell densities, however, resulted in a significantly increased secretion of especially t-PA and vWF. Basal and thrombin-stimulated PAI-1 and PGI₂ secretion increased more gradual with reducing cell densities. Boutherin-Falson et al. also reported gradually decreasing (thrombin-stimulated) PGI₂ secretion by HUVECs at increasing cell densities in the presence of culture medium either supplemented with endothelial growth factor or not [2].

Comparison of published data concerning secretion of substances synthesized by endothelial cells is hampered by the fact that there is no consensus regarding the experimental setup. The source of endothelial cells [3, 4], the substrate on which endothelial cells are cultured [5-7], and the composition of the endothelial cell culture medium used [8, 9] may influence the secretion of various substances by endothelial cells.

In the present study, the relation between cell density of HUVECs cultured on EDC/NHS-crosslinked collagen as well as N-Coll and TCPS, and the secretion of vWF and PGI₂ by HUVECs on these substrates was determined.

2. MATERIALS AND METHODS

2.1. Materials

Unless stated otherwise, materials were obtained from Merck (Darmstadt, Germany), and were of the highest purity available.

2.2. Collagen crosslinking

Experiments were carried out using flat collagen films as model substrates. Preparation of collagen films, crosslinking of these films using N-(3-dimethylaminopropyl)-N'-ethyl-
carbodiimide (EDC) and N-hydroxysuccinimide (NHS) and subsequent characterization was carried out as described in the materials and methods section of chapter 6. EDC/NHS-crosslinked matrices containing 14 free primary amino groups after crosslinking (E/N14C) were used for the experiments described in this appendix.

2.3. Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured according to van Wachem et al. [10], as described in chapter 6. After harvesting, cells were cultured to the third passage before being used for experiments.

2.4. HUVEC proliferation experiments

For proliferation experiments, endothelial cells were seeded on E/N14C, as well as on non-crosslinked collagen (N-Coll) and fibronectin-coated TCPS. Before use, collagen films were incubated overnight at 37°C in a solution of penicillin (200 U/ml) and streptomycin (200 μg/ml) (both from Gibco, Paisley, UK) in PBS (NPBI, Emmer Compascuum, The Netherlands). The films were placed in wells of tissue culture dishes (Costar, Cambridge, MA), and fixed with steam sterilized Viton O-rings (Eriks, Alkmaar, the Netherlands). HUVECs were seeded at a density of 2,500 to 50,000 cells/cm².

Endothelial cell culture medium (CM) consisted of a mixture of equal volumes of RPMI 1640 and M199 containing penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml) and Glutamax (2 mM) (all from Gibco, Paisley, UK). CM was supplemented with 5% human serum, derived from a pool of 12 healthy donors who had not taken aspirin during 3 days before blood donation, and bFGF (human recombinant basic fibroblast growth factor, Gibco, 0.3 ng/ml). Culture medium was replaced three times a week. After 1, 2, 3, 7 and 10 days the number of HUVECs was determined using a Bürker chamber. After removal of supernatant culture medium, HUVECs were detached from collagen films by incubation with 400 μl of a solution containing collagenase (Sigma C 6885, type 2, 1 mg/ml) and bovine serum albumin (BSA, Sigma A6003, 5 mg/ml) in PBS.

2.5. vWF and PGI₂ secretion

Cell cultures were washed with a solution of BSA in culture medium without serum (CM/1% BSA, 37°C), followed by 30 minutes incubation in respectively CM/1% BSA ("non-stimulated") or CM/1% BSA with 10 μM calcium ionophore (A23187, Boehringer, Mannheim, Germany, "stimulated"). Subsequently, supernatant medium was collected,
centrifuged (10 min, 400g, 4°C) and stored at -20°C until assayed. vWF was determined using an ELISA [11] (Gradiopore, North Ryde, Australia). PGI₂ concentrations were determined using a competitive EIA for the stable hydrolysis product of PGI₂, 6-keto-prostaglandin F₁α (Amersham, Amersham, England), a generally accepted measure for quantification of PGI₂ [12].

3. RESULTS

3.1 HUVEC proliferation

When plated at a density of 50,000 cells/cm², the number of cells on TCPS and N-Coll were found to be comparable (approximately 53,000 cells/cm² after 2 days of proliferation), which were lower than the cell number found on E/N14C (69,000 cells/cm²) (fig. 1A-C). After cell seeding at a density of 10,000 cells/cm, proliferation of HUVECs on TCPS continued for 7 days, resulting in cell numbers comparable to cell numbers found on TCPS 2 days after seeding 50,000 cells/cm². On N-Coll and E/N14C, proliferation continued for at least 10 days, resulting in 70,000 and 94,700 cells/cm², respectively. After plating at 5,000 or 2,500 cells/cm², an initial lag in proliferation was observed on all substrates during the first 3 days after seeding. At 7 days after seeding, cell numbers were considerably increased compared to 3 days after seeding. When seeded at a density of 5,000 cells/cm², proliferation was observed for at least 7 days (cell numbers on N-Coll at 7 and 10 days after seeding at a density of 5,000 cells/cm² were not determined), giving cell numbers comparable to those found after seeding at 10,000 cells/cm². When seeded at a density of 2,500 cells/cm², proliferation continued for at least 10 days on all substrates, giving cell numbers lower than found after plating at 5,000 or 10,000 cells. Irrespective of the seeding density, the number of cells on E/N14C after 10 days of proliferation were higher than on TCPS and N-Coll.

3.2. vWF secretion

HUVECs were seeded at a density of 2,500 to 50,000 cells/cm² on TCPS, N-Coll and E/N14C, as described above. After one day of proliferation, on all substrates basal and A23187-stimulated vWF secretion increased with decreasing cell numbers (fig. 2A-B). Considerably elevated vWF secretion was observed, notably when the number of cells was lower than approximately 5,000/cm². Non stimulated vWF secretion by HUVECs seeded at 2,500 cells/cm² on E/N14C, N-Coll and TCPS was respectively 5-fold, 4-fold and 8-fold higher when compared to HUVECs seeded at a density of 5,000 cells/cm². Depending on
Figure 1: Proliferation of HUVECs on fibronectin-coated TCPS, non-crosslinked collagen and E/N14-collagen as function of cell seeding density during 10 days of proliferation. (n = 3, mean ± SD).

*: not determined.
Figure 2: vWF secretion by HUVECs on TCPS, non-crosslinked collagen and E/N14-collagen as a function of cell density, after 1 day of culture. (n = 3, mean ± SD).

HUVECs were seeded at a density of 2,500 to 50,000 cells/cm². After 1 day of culture, cells were washed with a solution of BSA in culture medium (CM/1% BSA, 37°C), followed by 30 minutes incubation at 37°C in CM/1% BSA (fig. 2A “non-stimulated”) or CM/1% BSA with 10 μM calcium ionophore (A23187, fig. 2B, “stimulated”). vWF concentrations in cell supernatants were determined using an ELISA.

Substrate and cell density, stimulation of HUVECs with Ca²⁺-ionophore resulted in a 1.5 to 2.1-fold increased vWF secretion. One day after seeding, stimulated vWF release by HUVECs plated at 2,500 cells/cm² on TCPS or N-Coll was increased 2-fold, when compared to stimulated vWF secretion after seeding at a density of 5,000 cells/cm². Similarly, a 6 fold increased vWF secretion was observed for HUVECs on E/N14C. Both non-stimulated and A23187-stimulated vWF secretion by HUVECs on TCPS was lower than vWF secretion by HUVECs on N-Coll at comparable cell densities. vWF secretion was highest on E/N14C. Basal and stimulated vWF secretion by HUVECs cultured on TCPS, N-Coll and E/N14C decreased with increasing proliferation times (fig. 3A-F). After 10 days of proliferation on a specific substrate, basal vWF secretion was comparable for all seeding densities (10,000,
Figure 3: vWF secretion by HUVECs on TCPS, non-crosslinked collagen and E/N14-collagen as a function of cell seeding density and proliferation time (n = 3, mean ± SD, note the different y-axis scales).

HUVECs were seeded at a density of 2,500 to 50,000 cells/cm² on TCPS (fig. 3A, B), N-Coll (fig. 3C, D) and E/N14C (fig. 3E, F). After different times of proliferation, cell cultures were washed with a solution of BSA in culture medium (CM/1% BSA, 37°C), followed by 30 minutes incubation at 37°C in CM/1% BSA (fig. 3A, C, E, “non-stimulated”) or CM/1% BSA with 10 μM calcium ionophore (A23187, fig. 3B, D, F, “stimulated”). vWF in cell supernatants were determined using an ELISA. *: not determined.
5,000 or 2,500 cells/cm$^2$), which was also observed for stimulated vWF secretion. Compared to HUVECs on TCPS, vWF secretion of HUVECs on N-Coll was 1.5-fold higher, whereas vWF secretion by HUVECs on E/N14C was 2-fold higher. After 10 days of culture on a specific substrate, stimulated vWF secretion was approximately 2 fold higher than basal vWF secretion.

![Graph of PGI$_2$ secretion by HUVECs on TCPS, non-crosslinked collagen and E/N14-collagen as a function of cell density, after 1 day of culture. (n = 3, mean ± SD, note the different y-axis scales).](image)

**Figure 4:** PGI$_2$ secretion by HUVECs on TCPS, non-crosslinked collagen and E/N14-collagen as a function of cell density, after 1 day of culture. (n = 3, mean ± SD, note the different y-axis scales).

HUVECs were seeded at a density of 2,500 to 50,000 cells/cm$^2$. After 1 day of culture, cells were washed with a solution of BSA in culture medium (CM/1% BSA, 37°C), followed by 30 minutes incubation at 37°C in CM/1% BSA (fig. 4A “non-stimulated”) or CM/1% BSA with 10 μM calcium ionophore (A23187, fig. 4B, “stimulated”). PGI$_2$ concentrations in cell supernatants were determined using an assay for the stable hydrolysis product of PGI$_2$, 6-keto-prostaglandin F$_{1α}$.
3.3. PGI\textsubscript{2} secretion

In fig. 4, basal and A23187-stimulated PGI\textsubscript{2} secretion are plotted as a function of the HUVEC density after one day of culturing. Non-stimulated PGI\textsubscript{2} secretion increased with decreasing cell numbers on TCPS and E/N14C (i.e. below 10,000 cells/cm\textsuperscript{2}). Compared to PGI\textsubscript{2} secretion at higher cell densities, a 2-2.5 fold increased PGI\textsubscript{2} secretion was observed, except for HUVECs cultured on N-Coll which hardly showed an influence of cell density on non-stimulated PGI\textsubscript{2} secretion.

Stimulated PGI\textsubscript{2} secretion demonstrated a more distinct inverse correlation with cell density than basal PGI\textsubscript{2} secretion. Below a cell density of approximately 10,000/cm\textsuperscript{2}, a gradual increase in PGI\textsubscript{2} secretion was observed with decreasing cell numbers, for HUVECs on TCPS, E/N14C as well as N-Coll. When compared to higher cell densities, a 2-2.5 fold increase in PGI\textsubscript{2} secretion was observed at the lowest cell densities. In general, stimulated PGI\textsubscript{2} secretion was 10 to 50-fold higher than basal PGI\textsubscript{2} secretion.

PGI\textsubscript{2} secretion of HUVECs on TCPS, N-Coll and E/N14C was determined for a seeding density of 10,000 cells/cm\textsuperscript{2} as a function of proliferation time (fig. 5A-B). Non-stimulated PGI\textsubscript{2} secretion of HUVECs, which did not change during 10 days of culture on TCPS,
decreased on N-Coll and E/N14C. After 10 days of culture, non-stimulated PGI₂ secretion of HUVECs cultured on E/N14C and TCPS was comparable, while PGI₂ secretion by HUVECs cultured on N-Coll for the same period of time was still 4-fold higher. Stimulated PGI₂ secretion did not change significantly during 10 days of culture, and comparable PGI₂ secretion was observed for all substrates. For HUVECs cultured on E/N14C and TCPS, stimulated PGI₂ secretion after 10 days of culture was 35 respectively 45-fold higher than basal PGI₂ secretion. Stimulated PGI₂ secretion of HUVECs cultured for 10 days on N-Coll was approximately 9 fold higher when compared to basal PGI₂ secretion.

4. DISCUSSION AND CONCLUSIONS

Endothelial cells in vivo secrete various substances affecting platelet adhesion and aggregation, blood coagulation and fibrinolysis. Endothelial cell function thus directly affects the balance of hemostasis and thrombosis in the cardiovascular system [13, 14]. Endothelial cell seeding of vascular grafts is a recognized strategy to improve the patency of small-diameter synthetic vascular grafts [15, 16]. Seeding of endothelial cells on artificial substrates, however, may affect endothelial cell metabolism. Reduced PGI₂ secretion, increased vWF secretion, and, for example increased tissue factor expression, can result in enhanced pro-coagulant properties of seeded endothelial cells.

In chapter 6 of this thesis, the effect of EDC/NHS-crosslinking of collagen on the secretion of t-PA, PAI-1, vWF and PGI₂ by HUVECs cultured on these matrices after seeding at 10,000 cells/cm² is described. The number of cells found on fibronectin-coated TCPS and N-Coll 3 days after seeding was 20,000 and 15,000 cells/cm², respectively. After 3 days of culture, cell densities on EDC/NHS-crosslinked collagens with varying crosslink densities, however, were below 10,000 cells/cm². Compared to HUVECs seeded on TCPS and N-Coll, secretion of endothelial cell products was increased after 3 days of culture on EDC/NHS-crosslinked collagen. Secretion of t-PA, PAI-1 and vWF by HUVECs on EDC/NHS-crosslinked collagen decreased upon prolonged culturing (e.g. 10 days) to levels comparable to that of HUVECs on TCPS and N-Coll. In contrast to the present study, secretion of PGI₂ by HUVECs cultured for 10 days on EDC/NHS-crosslinked collagen was lower compared to cells cultured on TCPS and N-Coll.

In the present study, an inverse relation between cell density and basal as well as stimulated secretion of vWF and PGI₂, for HUVECs cultured on E/N14C as well as TCPS and N-Coll, was shown. This observation offers a very likely phenomenological explanation for the initially high secretion of vWF and PGI₂ by HUVECs cultured on EDC/NHS-crosslinked collagens, as reported in chapter 6.
When crosslinking agents were added exogenously to endothelial cell culture medium, notably NHS and MES caused a significantly increased secretion of vWF by HUVECs seeded at 10,000 cells/cm² on TCPS, N-Coll and E/N14C (data not shown). Despite these findings, the initially high secretion of vWF and PGI₂ by HUVECs on EDC/NHS-crosslinked collagen observed in the present study is not likely to be caused by release of traces of these substances from the crosslinked matrix. HUVECs on TCPS and N-Coll also showed increased secretion of vWF and PGI₂ at low cell densities, although crosslinking reagents were not present in these cultures.

After seeding at low densities, proliferation of HUVECs resulted in cell numbers exceeding 50,000 cells/cm² on all substrates after 10 days of proliferation. Concomitantly, the initially higher secretion of PGI₂ and vWF at the lowest seeding densities was decreased when cell numbers increased after longer times of culture, to values more comparable to those obtained for higher seeding densities. However, after 10 days of culture basal PGI₂ secretion and both basal and stimulated vWF secretion of HUVECs cultured on (crosslinked) collagen was still somewhat higher than observed for HUVECs cultured on fibronectin-coated TCPS. As reported by others, this might indicate an influence of the substrate on the secretion of substances by adherent HUVECs. Compared to fibronectin-coated ePTFE, culture of human saphenous vein endothelial cells on ePTFE coated with non-crosslinked bovine collagen resulted in reduced t-PA secretion (both basal and stimulated), but did not significantly affect PGI₂ secretion [6]. In another study, comparing fibronectin-coated ePTFE and ePTFE coated with non-crosslinked rat tail tendon collagen, human saphenous vein endothelial cells demonstrated increased stimulated PGI₂ secretion on the latter substrates as well as increased basal t-PA and PAI-1 secretion [7].

Although not determined, it is to be expected that the initially high secretion of t-PA and PAI-1 by HUVECs cultured on EDC/NHS-crosslinked collagens, as described in chapter 6, was also caused by the initially low cell densities on these substrates, an assumption validated by the results of Dekker et al. [1].

REFERENCES


Appendix to chapter 6


CHAPTER 7

Endothelial cell seeding of (heparinized) collagen matrices: Effects of bFGF pre-loading on proliferation (after low density seeding) and pro-coagulant factors

Institute for Biomedical Technology, Polymer Chemistry and Biomaterials Group, Department of Chemical Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

ABSTRACT

Endothelial cell seeding to improve the performance of small-diameter vascular grafts requires a suitable substrate, such as crosslinked collagen. In addition to providing a suitable substrate for adhesion and growth of endothelial cells, proliferation of seeded endothelial cells can be enhanced by local, sustained release of basic fibroblast growth factor (bFGF, a heparin-binding growth factor for endothelial cells).

We have previously shown that collagen crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) supports adhesion and proliferation of human umbilical vein endothelial cells (HUVECs). In the present study, HUVECs were seeded on (heparinized) EDC/NHS-crosslinked collagen, pre-loaded with bFGF. Proliferation of HUVECs on (heparinized) crosslinked collagen increased with increasing amounts of pre-loaded bFGF. The minimal cell-seeding density required for proliferation proved to be very low after pre-loading the substrates with bFGF, and was 4-fold lower for heparinized crosslinked collagen compared to crosslinked collagen (250 versus 1,000 cells/cm²). Pro-coagulant properties (von Willebrand factor secretion and tissue factor expression) of HUVECs seeded on (heparinized) crosslinked collagen, with or without pre-loading of bFGF, were comparable to those of HUVECs on TCPS.

It is concluded that heparinized, EDC/NHS-crosslinked collagen pre-loaded with bFGF is a candidate matrix for in vivo endothelial cell seeding of synthetic vascular grafts materials.
1. INTRODUCTION

Treatment of obstructive atherosclerotic disease, which is a major cause of mortality in the western world, may involve replacement or bypassing of affected arteries using synthetic vascular grafts. Whereas vascular grafts have been used successfully to replace large-diameter blood vessels, the long term patency of small-diameter vascular grafts is still disappointing, primarily due to stenosis and thrombus formation [1-4]. Endothelial cell seeding has been proposed to improve the blood compatibility of small-diameter vascular grafts by creating an inner lining with similar non-thrombogenic surface characteristics as native blood vessels [5]. Autologous endothelial cells are required for endothelial cell seeding, which means that the supply of endothelial cells is limited. As a consequence, cells are seeded either at (very) low seeding densities, or alternatively, after the number of cells has been increased by cell culture in vitro. Important drawbacks of expanding cell numbers in vitro are the time interval between the need of an endothelialized vascular graft and its availability, which limits its use to non-emergency situations, and the increased risk of bacterial infection [6]. Peroperative seeding is the preferred method for endothelialization of synthetic vascular grafts, notably when the surface characteristics allow adhesion of seeded cells without activation processes like platelet adhesion and thrombin formation.

Synthetic vascular graft materials such as Dacron and expanded Teflon are poor substrates for cell seeding [7-9]. To eliminate the time-consuming procedure of clotting of vascular grafts before implantation, collagen-, gelatin- and albumin-coated vascular grafts have been developed. Non-crosslinked collagen appears to be a suitable substrate for the growth of endothelial cells in vitro [10-12]. In order to prevent rapid in vivo resorption of the protein coating, collagen-coated vascular grafts are commonly treated with crosslinking agents like glutaraldehyde and formaldehyde [13, 14] These crosslinkers, however, are incorporated in the crosslinked matrix. Upon implantation of currently available collagen-coated graft materials, cytotoxic reactions evoked by release of non-reacted glutaraldehyde or glutaraldehyde residues [15-17], hamper endothelialization of the luminal graft surface [17, 18].

Crosslinking of collagen using N-(3-dimethylaminopropyl)-N’-ethylenecarboxydiimide (EDC) and N-hydroxysuccinimide (NHS), results in the formation of peptide-bond like crosslinks [19]. Crosslinking of collagen using EDC and NHS renders a matrix which is non-cytotoxic, in vitro as well as in experimental animals [20, 21]. We have previously shown that, compared to non-crosslinked collagen, EDC/NHS-crosslinked collagen matrices show enhanced in vitro proliferation with increasing crosslink densities [22]. As collagen is a thrombogenic material, immobilization of the anticoagulant heparin may prevent platelet adhesion and blood coagulation, notably when the collagen coating is not yet completely covered by seeded
endothelial cells. In addition for providing a good substrate for cell seeding, proliferation of seeded cells might be improved by local release of basic fibroblast growth factor (bFGF) bound to the heparinized collagen matrix.

In the present in vitro study, the effect of heparin immobilization to EDC/NHS-crosslinked collagen on the proliferation of seeded human umbilical vein endothelial cells was determined. Furthermore, the effect of pre-loading of (heparinized) EDC/NHS-crosslinked collagen with bFGF on the proliferation of endothelial cells and their pro-coagulant properties were studied. In addition, the ability of matrices pre-loaded with bFGF to induce proliferation of HUVECs after seeding at very low cell densities was determined.

2. MATERIALS AND METHODS

2.1 Materials

Unless otherwise stated, chemicals were obtained from Merck (Darmstadt, Germany), and were of the highest purity available.

2.2. Collagen crosslinking

All experiments were carried out using model substrates composed of flat collagen films prepared from Type I insoluble collagen derived from Bovine Achilles Tendon (Sigma, St.Louis, MO, C 99879, lot 23H7065), as previously described [23]. Collagen films, with a thickness of approximately 50 μm, were crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxy-succinimide (NHS). In order to minimize hydrolysis of EDC, crosslinking was carried out in a 0.05 M buffer of 2-morpholinoethane sulfonic acid (MES buffer, pH 5.40) [24]. After incubation of dried collagen films in MES buffer for at least 30 minutes, films were immersed in a solution of EDC and NHS in MES buffer under gentle shaking. For the crosslinking reaction, 1.731 g EDC and 0.415 g NHS in 215 ml MES buffer was used per gram of collagen. At the conditions used, the reaction was completed within 4 hours. Thereafter, the collagen was washed with 0.1 M Na₂HPO₄ solution (2 hours) and demineralized water (four times for 30 minutes) [24, 25]. The residual number of free primary amino groups in collagen after crosslinking was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS, Fluka, Buchs, Switzerland), according to a procedure described by Wang et al., with slightly modifications [26-28]. The shrinkage temperature of (crosslinked) collagen, indicating the
resistance against thermal denaturation, was determined by Differential Scanning Calorimetry (DSC) [29].

2.3. Heparin immobilization

Heparin sodium salt (Bufa Chemie, Castricum, the Netherlands) was used. This heparin preparation from porcine mucosa has the following characteristics [30]: Mn = 12.500 g/mol (molecular weight distribution 3,000-30,000 g/mol), activity = 195 IU/mg, 18.75 mol of carboxylic acid groups (Hep-COOH) per mol of heparin. Crosslinked collagen films (containing 14 free primary amino groups per 1000 amino acid residues after crosslinking (E/N14C) were incubated with 0.05 M MES-buffer (pH 5.60) for at least 30 minutes. Carboxylic acid groups of heparin were activated using a molar ratio of EDC : NHS: HepCOOH of 0.4 : 0.24 : 1. Heparin activation was started by adding EDC and NHS to a solution of heparin in 0.05 M MES-buffer (2% w/v, pH 5.60). After 10 minutes, 1 g of crosslinked collagen (E/N14C) was added to 188.3 ml of EDC/NHS-activated heparin solution, giving a molar ratio of heparin to free primary amino groups of 2. After reaction for 2 hours the heparinized E/N14C (E/N14C-H(0.4), 0.4 indicates the molar ratio of EDC : HepCOOH used for heparin immobilization) was washed with 0.1 M Na2HPO4 (2 hours), 4 M NaCl (4 times for 24 hours) and distilled water (3 times for 24 hours).

2.4. bFGF labeling and binding studies

Human recombinant basic fibroblast growth factor (bFGF, Gibco, Paisley, UK) was labeled with 125I using Iodobeads [31, 32]. Briefly, to 3 Iodobeads (Iodobeads iodination reagens, Pierce, Rockford, IL) 500 μCi 125I-Na (Amersham) in 100 μl phosphate buffer (100 mM, pH 7.0) was added. After 5 minutes, 1 ml 100 μg/ml bFGF solution in phosphate buffer was added, and iodination was carried out under gentle shaking at room temperature. After 15 minutes, the beads were removed and rinsed in 150 μl phosphate buffer. To the total aliquot of phosphate buffer, 600 μl bovine serum albumin (BSA, Sigma A7030) solution in phosphate buffer was added, giving a final albumin concentration of 1 mg/ml. Residual 125I was removed from the 125I-labeled bFGF solution by purification over a series of 3 PD10-columns. Thereafter, the bFGF solution (with a specific activity of 36.4 ± 1.7 kBq/μg bFGF) was aliquotted and stored at -20°C until used. E/N14C and heparinized E/N14C samples were incubated overnight in 5 ml PBS. After blotting dry, the samples were incubated with bFGF solution (0-840 ng/ml) diluted in phosphate buffered saline (PBS, NPBI, Emmer Compascuum, the Netherlands) containing 1 mg/ml BSA, for 90 minutes at room temperature. Thereafter, the samples were washed in 5
ml PBS (2 times 5 minutes, removing all non-bound bFGF as determined previously), and the radioactivity of the samples was measured using a Compugamma 1282 γ-counter (Wallac, Stockholm, Sweden).

2.5. Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured according to van Wachem et al. [33, 34]. After harvesting, cells were cultured to passage three in tissue culture polystyrene (TCPS) flasks (Costar, Cambridge, MA), which were precoated with a solution of partially purified human fibronectin (Fn^c, 2 mg/ml, in serum-free culture medium). Fn^c was a gift of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam, the Netherlands). Endothelial cell culture medium (CM) consisted of a mixture of equal volumes of RPMI 1640 and M199, containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 2 mM L-glutamax (all from Gibco, Paisley, UK), which was supplemented with 20% human serum derived from a pool of 12 healthy donors who had not taken aspirin during 3 days before blood donation (CM 20%). Cell culturing was carried out at 37°C, in a humidified atmosphere of 5% CO₂ and 95% air. HUVECs used for subculture and for experiments were detached from TCPS by incubation with trypsin (Gibco, 0.05% trypsin with 0.02% EDTA in PBS). Residual trypsin was inactivated by addition of culture medium.

2.6. HUVEC proliferation experiments

2.6.1. Effect of bFGF release

Before use, E/N14C and E/N14C-H(0.4) films were incubated overnight at 37°C in a solution of penicillin (200 U/ml) and streptomycin (200 μg/ml) in PBS. Thereafter, the films were washed thoroughly in PBS and placed in wells of tissue culture dishes (Costar), and fixed with steam sterilized Viton O-rings (Eriks, Alkmaar, the Netherlands), exposing a surface area of 2.77 cm². Before cell seeding, both E/N14C and E/N14C-H(0.4) films were incubated for 90 minutes at room temperature with 0.44 ml bFGF solution, at concentrations ranging from 0 to 96 ng/ml. Alternatively, HUVECs were seeded directly on E/N14C and E/N14C-H(0.4), without pre-loading with bFGF (control). HUVECs were seeded at a density of 10,000 cells/cm². Culture was carried out using culture medium supplemented with only 5% serum (CM 5%, films pre-loaded with 0-96 ng/ml bFGF), or CM supplemented with 5% serum, 5 U/ml heparin and 0.3 ng/ml bFGF (CM 5%/HF, control). Culture medium was replaced three times a week. At selected times, the number of HUVECs was determined using a Bürker
chamber. After removal of supernatant culture medium, HUVECs were detached from collagen films by incubation in 400 μl of a solution containing collagenase (Sigma, C6885, type 2, 1 mg/ml) and bovine serum albumin (BSA, Sigma A6003, 5 mg/ml) in PBS.

2.6.2. Effect of cell-seeding density

E/N14C and E/N14C-H(0.4) films were incubated for 90 minutes at room temperature with bFGF solution (0.44 ml, 100 ng bFGF/ml). Alternatively, HUVECs were seeded directly on E/N14C and E/N14C-H(0.4) without pre-loading with bFGF. HUVECs were seeded at a density of 100 to 10,000 cells/cm². On E/N14C and E/N14C-H(0.4) films pre-loaded with 100 ng/ml bFGF solution culture was carried out using CM 5%. On E/N14C and E/N14C-H(0.4) films not pre-loaded with bFGF, cell culture was carried out using either CM 5%, or CM supplemented with 5% serum, 5 U/ml heparin and 0.3 ng/ml bFGF (CM 5%/HF, control).

2.7. vWF secretion

Cell cultures were rinsed with a 1% solution of BSA (w/v) in culture medium (CM/1% BSA, 37°C), followed by 30 minutes incubation in respectively CM/1% BSA (“non-stimulated”) or CM/1% BSA with calcium ionophore (A23187, Boehringer, Mannheim, Germany, 10 μM, “stimulated”). Subsequently, supernatant medium was collected, centrifuged (10 min, 400g) and stored at -20°C until assayed. vWF antigen was determined using a sandwich ELISA [35] (Gradipore, North Ryde, Australia).

2.8. Tissue factor expression

2.8.1. Sample preparation

Apical surfaces of HUVECs cultured for 6 or 10 days were incubated for 5 hours with respectively fresh culture medium (“non-stimulated”) or with culture medium containing 10 μg/ml endotoxin (from Escherichia coli, Sigma L8274, “stimulated”). Thereafter, cell surfaces were washed three times for approximately 15 seconds with warm buffer A (10 mM Hepes, 137 mM NaCl, 4 mM KCl, 11 mM α-D-glucose, pH 7.4, 37°C). For the second washing step buffer A was supplemented with EDTA (0.5 mM). Subsequently, substrates with (stimulated) HUVECs were incubated with a serum free medium (a mixture of equal volumes M199 and RPMI 1640, containing 5 mg/ml albumin) for 1 hour, washed once with the same medium, and twice with buffer B (buffer A, containing 5 mg/ml albumin and 25 mM CaCl₂). Cell lysates were prepared after removal of supernatant culture medium and cell detachment by
treatment with 400 μl collagenase solution. 100 μl of the cell suspension was added to 900 μl buffer B, and centrifuged for 10 minutes at 200 g. The resulting cell-pellet was resuspended in buffer B (1 ml), and exposed to three freeze-thaw cycles (-80°C/37°C) [36].

2.8.2. Measurement of tissue factor activity

Apical cell surfaces were incubated for 5 minutes at 37°C with buffer B (320 μl) to which was added 40 μl 10 nM stock solution of activated human factor VII (FVIIa, Kordia, Leiden, the Netherlands) in the same buffer. Alternatively, 320 μl of cell lysate was incubated at 37°C with 40 μl of FVIIa stock solution in 24 wells microtiter plates (Costar). Thereafter, a solution of human factor X (FX, Kordia) in buffer B (40 μl, 494 nM FX) was added. After 10 minutes of incubation, 50 μl samples were transferred to wells of a 96 wells microtiter plate (Costar) containing 100 μl stop solution (buffer A containing 2.5 mM EDTA, 4°C), and assayed for factor Xa [37]. The concentration of FXa was determined spectrophotometrically, as described by Bom et al. [38]. The assay was started by adding 0.1 ml of a 0.2 mg/ml solution of a chromogenic substrate for FXa (S2337, Chromogenix, Mölndal, Sweden) in buffer B. The optical density at 405 nm was measured for 45 minutes at 37°C using an ELISA reader (340 ATTC, STL, Austria). Using a standard curve, employing human FXa (Kordia) solutions (0-55 nM, in buffer B), the concentration of FXa in the samples was calculated.

3. RESULTS

3.1. Matrix preparation

Upon EDC/NHS-crosslinking of collagen the number of free primary amino groups per 1000 amino acid residues decreases while the shrinkage temperature increases. At the conditions used, crosslinked collagen contained approximately 14 free primary amino groups per 1000 amino acid residues (E/N14C), compared to 27 in native collagen [39]. The shrinkage temperature increased from 55.4°C for native collagen to 75.9°C for E/N14C. After immobilization of heparin to E/N14C, the amount of immobilized heparin equaled approximately 1.9% (w/w) as previously determined using tritium-labeled heparin [40]. The heparinized E/N14C demonstrated a thrombin inhibitory activity of 2 IU per gram collagen. Binding of 125I-labeled bFGF to E/N14C and E/N14C-H(0.4) increased linearly with the concentration of bFGF. bFGF binding efficiency to E/N14C was approximately 10% at
After cell seeding at a density of 10,000 cells/cm², culture medium supplemented with 5% serum (CM 5%, films incubated with bFGF), or culture medium supplemented with 5% serum, heparin (5 U/ml) and bFGF (0.3 ng/ml) (CM 5%/HF, films not pre-loaded with bFGF) was added.

concentrations up to 840 ng/ml bFGF, while bFGF binding efficiency to E/N14C-H(0.4) was approximately 22% [41].

Matrices used to determine the effect of bFGF pre-loading on proliferation of HUVECs were incubated with bFGF solutions with a concentration ranging from 0 to 96 ng bFGF/ml.

Figure 1: Proliferation of HUVECs on E/N14 collagen (A) and E/N14 collagen-heparin(0.4) (B) loaded with varying amounts of bFGF (n = 3, mean ± SD).
Assuming bFGF binding of 10% and 22% to E/N14C and E/N14C-H(0.4) respectively, this resulted in E/N14C pre-loaded with 0 to 1.52 ng bFGF/cm², whereas E/N14C-H(0.4) contained 0 to 3.35 ng bFGF/cm². Matrices used to study the effect of cell seeding density and pro-coagulant properties of HUVECs were incubated with either 50 or 100 ng/ml bFGF solution, giving E/N14C with 0.79 or 1.58 ng bFGF/cm², and E/N14C-H(0.4) with 1.74 or 3.49 ng bFGF/cm².

3.2. Proliferation of HUVECs

3.2.1. Effect of bFGF release

Proliferation of HUVECs cultured in CM 5% on E/N14C or E/N14C-H(0.4) pre-loaded with bFGF (fig. 1, 0.10-1.52 ng bFGF/cm² and 0.21-3.35 ng bFGF/cm², respectively), was enhanced compared to HUVECs cultured on the same matrices without bFGF pre-loading (0 ng/cm²). For E/N14C as well as E/N14C-H(0.4), cell numbers, observed 6 and 10 days after seeding, increased with increasing bFGF pre-loading of the films. After 10 days of proliferation, the number of HUVECs found on E/N14C-H(0.4) was comparable to or higher than on E/N14C, for all concentrations of bFGF used for incubation. However, only after pre-loading with bFGF solutions of 24 and 96 ng/ml (E/N14C: 0.38 and 1.52 ng bFGF/cm²; E/N14C-H(0.4): 0.84 and 3.35 ng bFGF/cm²) differences in cell numbers 10 days after seeding were statistically significant (p < 0.05).

Using CM 5%, cell numbers on both E/N14C and E/N14C-H(0.4) not pre-loaded with bFGF increased slightly to approximately 15,000 cells/cm² at 10 days after seeding. Using CM 5% supplemented with 5 U/ml heparin and 0.3 ng/ml bFGF (CM 5%/HF), HUVECs seeded on E/N14C continued to proliferate for at least 10 days, whereas cell growth on E/N14C-H(0.4) using CM 5%/HF stopped after approximately 6 days. As frequently observed, cell growth on E/N14C-H(0.4) tended to be somewhat lower compared to E/N14C. After 10 days, cell numbers amounted to 40,000 and 60,000 cells/cm², respectively.

3.2.2. Effect of seeding density

Irrespective of the cell seeding density, HUVECs seeded on both E/N14C and E/N14C-H(0.4) without pre-loaded bFGF did not proliferate when cultured in CM 5% (figs. 2B and 3B, 0 ng bFGF/cm²). When instead CM supplemented with bFGF (CM 5%/HF) was used, proliferation was observed on both substrates when cells were seeded at a density of at least 1,000/cm² (figs. 2A and 3A). Proliferation continued for at least 9 days, at which time cell numbers on both control-substrates were comparable: i.e. approximately 10,000 and 40,000 cells/cm² after
Figure 2: Proliferation of HUVECs on E/N14 collagen, after seeding at a density of 100, 250, 1,000, 2,500 and 10,000 cells/cm² (n = 3, mean ± SD).

Films with an exposed surface area of 2.77 cm² were incubated with 0.44 ml bFGF solution (0 or 100 ng/ml) before cell seeding, resulting in E/N14C loaded with 0 or 1.58 ng bFGF/cm². After cell seeding at densities of 100, 250, 1,000, 2,500 and 10,000 cells/cm² culture medium supplemented with 5% serum (CM 5%, films pre-loaded with 0 or 1.58 ng bFGF/cm²) or culture medium supplemented with 5% serum, heparin (5 U/ml) and bFGF (0.3 ng/ml) (CM 5%/HF, films not pre-loaded with bFGF) was added.
Figure 3: Proliferation of HUVECs on heparinized E/N14 collagen, after seeding at a density of 100, 250, 1,000, 2,500 and 10,000 cells/cm² (n = 3, mean ± SD).
Films with an exposed surface area of 2.77 cm² were incubated with 0.44 ml bFGF solution (0 or 100 ng/ml) before cell seeding, resulting in heparinized E/N14C loaded with 0 or 3.49 ng bFGF/cm². Culture conditions were similar to those described in the legend of fig. 2 (n = 3, mean ± SD).
Figure 4: Von Willebrand factor secretion of HUVECs cultured on fibronectin-coated TCPS, E/N14 collagen and heparinized E/N14 collagen using CM 5%/HF, and on heparinized E/N14 collagen pre-loaded with 1.74 or 3.49 ng bFGF/cm² using CM 5% (n = 3, mean ± SD).

Cell seeding density was 10,000 cells/cm². Cell cultures were washed with a solution of BSA in culture medium (CM/1% BSA, 37°C), followed by 30 minutes incubation at 37°C in CM/1% BSA (4A: “not-stimulated”) or CM/1% BSA with 10 µM calcium ionophore (A23187, 4B: “stimulated”). vWF in the cell supernatant was determined by means of an ELISA.

seeding at a density of 1,000 and 10,000 cells/cm², respectively. When the seeding density was below 1,000 cells/cm², cell proliferation was not observed.

Proliferation of HUVECs on E/N14C pre-loaded with 1.58 ng bFGF/cm², was observed when seeding density was equal to or larger than 1,000 cells/cm² (figure 2C), while on E/N14C-H(0.4) pre-loaded with 3.49 ng bFGF/cm² cell proliferation was observed after seeding at a density of at least 250 cells cm² (figure 3C). After seeding at a density of 250 cells/cm² on E/N14C-H(0.4) pre-loaded with bFGF, 15,000 cells/cm² were found after 9 days of culture. Using a seeding density of 1,000, 2,500 or 10,000 cells/cm², cell numbers after 9 days of culture on E/N14C and E/N14C-H(0.4) pre-loaded with bFGF were comparable (approximately 10,000, 15,000 and 25,000 cells/cm² respectively).
Figure 5: Tissue factor expression of HUVECs cultured on fibronectin-coated TCPS, E/N14 collagen and heparinized E/N14 collagen using CM 5%/HF, and on heparinized E/N14 collagen pre-loaded with 3.49 ng bFGF/cm² using CM 5% (n = 3, mean ± SD). Cell seeding density was 10,000 cells/cm². Cell cultures were incubated for 5 hours with culture medium ("non-stimulated") or culture medium with 10 μg/ml endotoxin ("stimulated"). TF activity was measured as conversion of FX to FXa on apical cell surfaces and by cell lysates.

3.3. vWF secretion

vWF secretion of non-stimulated HUVECs cultured for 3 days on E/N14C and E/N14C-H(0.4) (not pre-loaded, or pre-loaded with 1.74 and 3.49 ng bFGF/cm²) was approximately 5-fold higher when compared to HUVECs on TCPS (fig. 4). vWF secretion of non-stimulated HUVECs cultured on E/N14C and E/N14C-H(0.4) decreased to values comparable to TCPS after 7 and 10 days.

After stimulation of HUVECs with A23187, secretion of vWF by HUVECs cultured for 3 days on E/N14C and E/N14C-H(0.4) was 2 to 3 fold higher compared to HUVECs cultured on
TCPS. After 10 days of culture, stimulated vWF secretion of HUVECs on E/N14C and E/N14C-H(0.4) decreased to values comparable to TCPS.

3.4. Tissue factor expression

After 6 and 10 days of culture, total cell associated TF activity of HUVECs cultured on E/N14C-H(0.4) pre-loaded with 3.49 ng bFGF/cm² (using CM 5%) was lower than TF activity of HUVECs cultured on TCPS, E/N14C and E/N14C-H(0.4) using CM 5%/HF (fig. 5A, B), for both non-stimulated as well as endotoxin stimulated cells. Upon stimulation with endotoxin, total cell-associated TF activity was significantly increased. After 10 days of culture, total cell associated TF activity had decreased for HUVECs cultured on all substrates when compared to 6 days of culture, except for non-stimulated HUVECs on E/N14C-H(0.4) pre-loaded with bFGF (fig. 5B).

Both after 6 and 10 days of culture, apical TF activity of HUVECs cultured on the various test substrates was very low compared to total cell-associated TF activity (fig. 5A and b). There was no significant difference between non-stimulated and stimulated apical TF activity, except for HUVECs cultured on TCPS for 6 days, which did not express any detectable non-stimulated apical TF activity.

4. DISCUSSION

Following implantation of vascular grafts, endothelium from the native artery starts to overgrow the graft surface from both distal and proximal anastomoses. In humans, this process is limited to approximately 1 cm from both anastomoses, whereas in various experimental animals spontaneous endothelialization of the entire graft lumen will occur within a period of weeks to months, depending on animal species and the length of the graft [42, 43]. In humans, cell seeding may support the formation of endothelium-lined vascular grafts. Endothelial cell seeding requires a suitable protein matrix, which is not provided by commercially available vascular grafts due to the presence of cytotoxic crosslinkers. In a previous study we have shown that EDC/NHS-crosslinked collagen substrates provide a suitable matrix for attachment and proliferation of seeded endothelial cells in vitro [22].

Collagen is a thrombogenic material. When used as a substrate for in vivo endothelial cell seeding, parts of the collagen substrate in the lumen of a collagen-coated vascular graft will be exposed to blood until seeded cells reach confluence. Heparin immobilization to collagen may prevent blood coagulation and platelet adhesion in the period after cell seeding when the lumen of the graft is not yet completely covered by endothelial cells. In addition,
immobilization of heparin may prevent intimal hyperplasia [44] near the anastomoses of vascular grafts, which often leads to graft failure.

Heparin immobilization to EDC/NHS-crosslinked collagen (containing 14 free primary amino groups after crosslinking, E/N14C) was carried out using EDC and NHS as well. The immobilized anticoagulant activity obtained was dependent on reaction conditions during heparin immobilization. Using a molar ratio of EDC : heparin-carboxylic acid groups (HepCOOH) of 0.4, a matrix with maximal thrombin inhibitory activity was obtained (E/N14C-H(0.4)) [40].

Controlled release of growth factors such as bFGF, which is widely explored in tissue engineering, is used to improve angiogenesis of implanted matrices [45, 46] or proliferation of cells seeded on certain matrices [47-50]. In addition, local sustained bFGF release has been shown to improve the spontaneous endothelialization of vascular grafts in vivo [51-53].

Culture medium (CM) for culturing HUVECs is commonly supplemented with (human) serum to provide essential nutrients and growth factors. When the serum concentration in CM is diminished (e.g. 5%), proliferation of HUVECs decreases [54, 55]. In contrast, addition of endothelial cell growth factors to HUVEC cultures increases cell proliferation [56] and allows the minimal serum concentration required for cell growth to be lower. In our hands, the proliferation of HUVECs on E/N14C and E/N14C-H(0.4) was dependent on exogenous bFGF, either present in the matrix or in the culture medium, when culture was carried out in CM supplemented with 5% human serum (CM 5%) (fig. 1). Even at the lowest concentration of bFGF used for pre-loading, improved proliferation was observed when compared to proliferation on (heparinized-) collagen matrices not loaded with bFGF. It is concluded that biologically active bFGF is released from the (heparinized) EDC/NHS-crosslinked collagen matrix and subsequently stimulates cell proliferation. This stimulatory effect was dependent on the bFGF concentration used for pre-loading, resulting in maximal proliferation on matrices with the largest amount of bFGF bound.

It has to be noted, that results differed slightly between different isolations of HUVECs. Due to biological variations between different HUVEC isolations, differences between proliferation of HUVECs on E/N14C and E/N14C-H(0.4) in CM 5%/HF, or proliferation on E/N14C and E/N14C-H(0.4) after pre-loading with bFGF, may be smaller than the differences shown in figure 1, in some cases resulting in comparative cell numbers on both substrates. Furthermore, especially on E/N14C-H(0.4) pre-loaded with bFGF, decreased cell numbers were observed after 10 days of proliferation. This may be due to the high cell numbers observed at day 6. Reorganization of such a densely packed, confluent monolayer of endothelial cells can result in larger cells and concomitantly lower cell numbers.

Binding to heparin, or heparin-like molecules, is reported to protect bFGF from inactivation, thus maintaining the biological activity of bFGF [57]. Binding of bFGF to E/N14C without
immobilized heparin, however, also resulted in increased proliferation of HUVECs. This indicates that bFGF is stabilized by binding to (crosslinked) collagen, or alternatively by binding to substances (like heparan sulphate) deposited by endothelial cells in the collagen matrix [58].

Heparin immobilization to E/N14C resulted in approximately two-fold higher bFGF loading, and in a slower release rate of bound bFGF [41]. These combined characteristics result in bFGF release from E/N14C-H(0.4) into culture medium over a longer period of time than from E/N14C. Compared to E/N14C, support of proliferation of HUVECs on E/N14C-H(0.4) over a longer period of time therefore is to be expected.

Endothelial cells in culture need a minimal (“critical”) seeding density for induction of proliferation and subsequent formation of confluent monolayer. Using culture medium supplemented with growth factors, this minimal seeding density can be lowered significantly [5, 54, 56]. After pre-loading with bFGF, the critical seeding density on E/N14C-H(0.4) was 250 cells/cm², which was 4-fold lower than on E/N14C without heparin immobilized (1,000 cells/cm²) (fig. 2, fig. 3). These results create perspectives for low density cell seeding in vivo.

In studies previously performed in our laboratory, HUVECs cultured on E/N14C for 10 days did not show altered secretion of prostacyclin (PGI2), vWF, tissue plasminogen activator (tPA) or plasminogen activator inhibitor (PAI-1) when compared to HUVECs cultured on TCPS. In this study, we focused on the effects of the type of substrate and the release of bFGF from these substrates on two pro-coagulant factors: von Willebrand factor (vWF) and tissue factor (TF). Von Willebrand factor secretion by HUVECs in culture is known to be elevated by agonists such as thrombin, calcium ionophore, phorbol esters, and heparin in combination with bFGF [59]. Irrespective of culture conditions (medium, pre-loading of matrix with bFGF), only shortly after seeding (i.e. 3 days) vWF secretion of HUVECs cultured on E/N14C and E/N14C-H(0.4) was higher than of HUVECs plated on TCPS (fig. 4).

In a previous study, increased secretion of vWF by HUVECs cultured on EDC/NHS-crosslinked collagen was related to low cell densities (i.e. below 10,000 cells/cm²) [60]. In the present study, cell numbers after 3 days of proliferation on (heparinized) collagen, either pre-loaded with bFGF or not, amounted to 8,000 cells/cm², thus explaining the increased secretion of vWF compared to TCPS (17,200 cells/cm²).

Although under normal conditions endothelium in vivo does not show tissue factor expression [61], in culture low basal TF activity can be detected [62]. Furthermore, growth factors and traces of endotoxin [63-65] as well as contamination with smooth muscle cells [66] have shown to result in higher TF expression in tissue culture. In this study, apical TF activity and TF activity in cell lysates was determined. In general, apical TF activity of HUVECs on different substrates was very low compared to TF activity measured in cell lysates (fig. 5). When compared to HUVECs cultured on TCPS, which was used as an arbitrary reference,
apical TF activity did not show elevated levels when cells were cultured on E/N14C and E/N14C-H(0.4) for 10 days. Although the presence of growth factors in culture medium may increase TF expression by endothelial cells in culture, culture of HUVECs on E/N14C-H(0.4) pre-loaded with bFGF resulted in lower TF activity in cell lysates, when compared to culture of HUVECs on E/N14C-H(0.4), using culture medium containing bFGF. Whether this can be ascribed to a different mechanism of action on cells of bFGF released from the substrate over bFGF present in culture medium, or to lower bFGF concentrations in culture medium in case of pre-loading with bFGF was not determined. The observed differences in TF expression can not be explained by differences in cell numbers. After 10 days of culture, cell numbers on all substrates were in the range of 30,000 to 40,000 cells/cm².

5. CONCLUSIONS

E/N14C and E/N14C-H(0.4) substrates pre-loaded with bFGF are able to support the in vitro adhesion and proliferation of HUVECs, by local release of bFGF. After pre-loading with bFGF, E/N14C-H(0.4) showed a 4-fold lower critical seeding density needed to induce cell proliferation, when compared to E/N14C without heparin immobilized. Secretion of von Willebrand factor and apical expression of tissue factor by HUVECs cultured on (heparinized) collagen matrices, with bFGF either present in the culture medium or in the matrix, were not affected compared to HUVECs cultured on TCPS. Pre-loading of E/N14C-H(0.4) with bFGF resulted in a decrease of total cell-associated tissue factor, compared to HUVECs cultured in bFGF-containing medium on (heparinized-) collagen matrices as well as TCPS.

It is concluded that E/N14C-H(0.4) pre-loaded with bFGF is a candidate substrate for in vivo endothelial cell seeding of synthetic vascular grafts materials.

REFERENCES

[23] This thesis, Chapter 3.
[40] This thesis, Chapter 4.
[41] This thesis, Chapter 5.


CHAPTER 8

In vivo biocompatibility of EDC/NHS-crosslinked collagen matrices: Effects of crosslink density, heparin immobilization and bFGF loading

P.B. van Wachem¹, J.A. Plantinga¹, M.J.B. Wissink², R. Beernink², A.A. Poot², G.H.M. Engbers², T. Beugeling², W.G. van Aken², J. Feijen², M.J.A. van Luyn¹

¹University of Groningen, Faculty for Medical Sciences, Cell Biology and Biomaterials, Bloemsingel 10, 9712 KZ Groningen, The Netherlands;
²University of Twente, Institute for Biomedical Technology, Department of Chemical Technology, Polymer Chemistry and Biomaterials Group, P.O. Box 217, 7500 AE Enschede, The Netherlands

ABSTRACT

Collagen matrices, crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS), were previously developed as a substrate for endothelial cell seeding of (small-diameter) vascular grafts. In the present study, the biocompatibility of various EDC/NHS-crosslinked collagen matrices was evaluated following subcutaneous implantation in rats for periods up to 10 weeks. The effects of the crosslink density, the amount of heparin immobilized to an EDC/NHS-crosslinked collagen matrix containing 14 free primary amino groups per 1,000 amino acid residues (EN14) and the effect of pre-loading of (heparinized) EN14 with basic fibroblast growth factor (bFGF) on the induced tissue reactions were studied.

EDC/NHS-crosslinked collagen was biocompatible at both early and late time intervals, and especially matrices with high crosslink densities (i.e. EN14, EN10) demonstrated a significantly decreased antigenic response when compared to non-crosslinked collagen. Furthermore, increased crosslinking resulted in a decreased degradation rate.

Immobilization of heparin onto EN14 resulted in a similar or somewhat reduced tissue reaction, but fibrin formation and vascularization were increased with increasing quantities of immobilized heparin. Matrices pre-loaded with bFGF also demonstrated good biocompatibility, especially in combination with higher amounts of immobilized heparin. After pre-loading with bFGF, the latter matrices demonstrated significantly increased
vascularization for periods up to 3 weeks. Heparin immobilization nor bFGF pre-loading induced an increased antigenic response.

It is concluded that the results of this study justify further evaluation of bFGF pre-loaded, heparin immobilized EN14 collagen, as a matrix for endothelial cell seeding in experimental animals.

1. INTRODUCTION

Endothelial cell seeding is an appreciated method to improve the blood compatibility of (small-diameter) vascular grafts [1, 2]. This strategy is hampered, however, by the poor adhesive properties of synthetic vascular graft materials, e.g. Dacron and expanded Teflon, for endothelial cells [3].

Collagen is a structural protein which is used in a variety of biomedical applications [4, 5]. Since non-crosslinked collagen is a good matrix for endothelial cell seeding in vitro [6], application of a collagen coating onto synthetic vascular grafts is expected to result in a suitable substrate for cell seeding. Commercially available collagen-coated vascular grafts have been developed to eliminate the elaborate procedure of preclotting of the graft before implantation. To prevent rapid in vivo resorption of the protein coating, collagen-coated grafts are commonly crosslinked using formaldehyde or glutaraldehyde [7, 8], which are incorporated in the collagen matrix during crosslinking. Formaldehyde, and especially glutaraldehyde crosslinked collagen are known to induce cytotoxic reactions, by release of (unreacted) crosslink agents or derivatives thereof [9-11], thus hampering endothelialization of currently available collagen-coated vascular grafts [11, 12].

We have developed an alternative collagen coating for synthetic vascular grafts, crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS). Using EDC and NHS to crosslink collagen, amide bonds are introduced between carboxylic acid groups of aspartic or glutamic acid residues, and e-amino groups of (hydroxy-) lysine residues [13, 14]. No reagents are incorporated in the crosslinked collagen matrix. EDC/NHS-crosslinked dermal sheep collagen was shown to be non-cytotoxic in vitro [15] and biocompatibility was demonstrated in animal models [16-18].

As collagen is a thrombogenic material, application as coating of vascular grafts may induce platelet deposition and blood coagulation, which may result in early graft occlusion. Immobilization of heparin is a recognized approach to improve the blood compatibility of biomaterials [19, 20]. We have previously demonstrated, that both EDC/NHS-crosslinked reconstituted collagen of bovine origin and its heparinized counterpart are suitable substrates.
for human endothelial cells *in vitro*. In addition, EDC/NHS-crosslinked collagen matrices exhibited enhanced proliferation of seeded endothelial cells with increasing crosslink-densities [21]. Pre-loading of (heparinized) EDC/NHS-crosslinked collagen with increasing quantities of basic fibroblast growth factor (bFGF, a heparin-binding endothelial cell growth factor) *in vitro* resulted in progressively improved endothelial cell proliferation [22].

To assess suitability for *in vivo* applications, in the present study the biocompatibility of EDC/NHS-crosslinked collagen was determined, following subcutaneous implantation in rats. Variables included the crosslink density, the amount of heparin immobilized to an EDC/NHS-crosslinked collagen matrix with a selected crosslink density, and pre-loading with bFGF.

### 2. MATERIALS AND METHODS

#### 2.1. Materials

Unless otherwise stated, chemicals were obtained from Merck (Darmstadt, Germany), and were of the highest purity available.

#### 2.2. Collagen crosslinking

All experiments were carried out using flat collagen films as model matrices. Collagen films were prepared from reconstituted Type I insoluble collagen derived from Bovine Achilles Tendon (Sigma, St.Louis, MO) [23]. These films, with a thickness of approximately 50 μm, were crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). Dried collagen films were incubated with 0.05 M buffer of 2-morpholinoethane sulfonic acid (MES buffer, pH 5.40) [24] for 30 minutes. Subsequently, the films were immersed in a solution of EDC/NHS in MES buffer, and crosslinking was carried out under gentle shaking. For the crosslinking reaction, 1.731 g EDC and 0.415 g NHS in either 215 or 100 ml MES buffer was used per gram of collagen (1.29 mmol carboxylic acid groups per gram of collagen [25], resulting in a molar ratio of EDC : NHS : Coll-COOH of 7.0 : 2.8 : 1.0). At selected times (15 minutes to 4 hours) crosslinking was stopped by washing the collagen film with 0.1 M Na₂HPO₄ solution for 2 hours. This treatment resulted in hydrolysis of both activated carboxylic acid groups and residual EDC [24, 26]. Thereafter, the films were washed with demineralized water (four times for 30 minutes).

The residual number of free primary amino groups in collagen after crosslinking was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS, from Fluka, Buchs, Switzerland), according to a procedure described by Wang *et al.* [27-29]. The shrinkage temperature of
(crosslinked) collagen (indicating the resistance against thermal denaturation) was determined using Differential Scanning Calorimetry (DSC) [30].

### 2.3. Heparin immobilization

Heparin sodium salt (Bufa Chemie, Castricum, the Netherlands) was used. This heparin preparation from porcine mucosa has the following characteristics [31]: \( \text{Mn} = 12,500 \text{ g/mol} \) (molecular weight distribution 3,000-30,000 g/mol), activity = 195 IU/mg, 18.75 mol of carboxylic acid groups (Hep-COOH) per mol of heparin. Crosslinked collagen films were incubated with 0.05 M MES-buffer (pH 5.6) for 30 minutes. Carboxylic acid groups of heparin were activated using EDC and NHS in a fixed molar ratio of 0.6, and a molar ratio of EDC : Hep-COOH of 0.2, 0.4 or 1.0. To a 2% (w/v) solution of heparin in 0.05 M MES-buffer (pH 5.6) EDC and NHS were added. After 10 minutes, 1 g of crosslinked collagen (containing 14 free primary amino groups per 1,000 amino acid residues, EN14) was added to 188.3 ml of EDC/NHS-activated heparin solution, giving a molar ratio of heparin to free primary collagen amino groups of 2. After 2 hours, the heparin immobilized films were washed with 0.1 M \( \text{Na}_2\text{HPO}_4 \) (2 hours), 4 M NaCl (4 times for 24 hours) and distilled water (3 times for 24 hours).

### 2.4. Assay for immobilized heparin

Circular films with a diameter of 8 mm were incubated with 5 ml aqueous solution of toluidine blue (0.1 M HCl, 2 mg/ml NaCl, 0.4 mg/ml toluidine blue O zinc chloride double salt) for 4 hours at room temperature, resulting in complexation of toluidine blue with heparin [32]. Thereafter, samples were washed with distilled water (twice for 5 minutes). Subsequently, toluidine blue complexed to heparin was solubilized in 5 ml of a 1:4 (v/v) mixture of 0.1 M NaOH and ethanol. The extinction of the resulting solution was determined at 530 nm using an Uvikon 930 spectrophotometer (Kontron Instruments, Switzerland). The amount of immobilized heparin was calculated from a calibration curve obtained using EDC/NHS-crosslinked collagen heparinized with \(^3\)H-labeled heparin.

### 2.5. Sterilization

Dried samples were sterilized using \( \gamma \)-irradiation. Samples were exposed to a \(^{60}\)Co-source of 900,000 Ci (Gammaster, Ede, The Netherlands) until a total dose of 25 kGy was reached [33].
2.6. bFGF pre-loading

A solution of human recombinant basic fibroblast growth factor (bFGF, Gibco, Paisley, UK) in PBS (supplemented with 1 mg/ml albumin) was sterilized using a 0.22 µm filter (Millipore). After determination of the bFGF concentration using a sandwich ELISA (Quantikine bFGF ELISA, R&D Systems, Abingdon, UK), the solution was diluted to 100 ng bFGF/ml with PBS containing 1 mg/ml albumin. Circular EN14 and heparin-immobilized EN14 films with a diameter of 8 mm were incubated with 160 µl bFGF solution for 90 minutes at room temperature. As previously determined, within 90 minutes bFGF binding reaches a plateau value. Subsequently, samples were washed in 5 ml PBS (twice for 5 minutes, removing all unbound bFGF).

2.7. Implantation

Animal experiments were carried out according to NIH guidelines for care and use of laboratory animals (NIH publication # 85-23 rev. 1985). Circular, sterilized films with a diameter of 8 mm were incubated with PBS for 30 minutes at room temperature. After rinsing in PBS (twice for 5 minutes) samples were either implanted directly, or after pre-loading with bFGF, as described above. Male albino Oxford rats of approximately 3 months of age were ether-anaesthetized. After the back of a rat was shaven and disinfected, two mid-line incisions of 1 cm were created. A total of four subcutaneous pockets were created, one to the left and the right of each incision. One sample was placed in each pocket; care was taken to implant the materials completely flat.

Upon explantation, rats were ether-anaesthetized. Implants with surrounding tissue were excised 1, 2, 5, and 10 days, and 3, 6 and 10 weeks after implantation, after which the rats were sacrificed.

2.8. Microscopy

Samples were carefully explanted together with the surrounding capsule, and immediately immersion-fixed in 2% (v/v) glutaraldehyde in PBS (0.1 M, pH 7.4) for at least 24 hours at 4°C. After dehydration in graded alcohols, a mid-part of the fixed samples was embedded in glycolmethacrylate (GMA) for light microscopic (LM) evaluations. GMA-sections (2 µm) were routinely stained with toluidine blue. Sections were evaluated independently by three persons, rating cell numbers (polymorphonuclear cells, macrophages, giant cells and lymphocytes), as well as fibrin formation and vascularization of each sample in arbitrary units, scaled from 0 to a maximum of 3.
3. RESULTS

3.1. Materials

The number of free primary amino groups per 1,000 amino acid residues of non-crosslinked collagen (NX) amounted to 27, which is similar to that of native collagen [34]. Upon EDC/NHS-crosslinking the number of primary amino groups decreased, while the shrinkage temperature increased (Table I). Depending on reaction time and volume, materials with approximately 22, 18, 14 and 10 free primary amino groups per 1,000 amino acid residues (Table I, EN22, EN18, EN14 and EN10) were obtained.

Table I: Number of free primary amino groups per 1,000 amino acid residues (n = 3, mean ± SD) and shrinkage temperature (n = 2, mean ± SD) of EN crosslinked collagen samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Reaction volume (ml/g collagen)</th>
<th>Reaction time</th>
<th>Primary amino groups (/1,000)</th>
<th>Shrinkage temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX</td>
<td>-</td>
<td>-</td>
<td>26.7 ± 0.5</td>
<td>55.4 ± 0.6</td>
</tr>
<tr>
<td>EN22</td>
<td>215</td>
<td>15 minutes</td>
<td>21.5 ± 0.8</td>
<td>64.9 ± 0.6</td>
</tr>
<tr>
<td>EN18</td>
<td>215</td>
<td>30 minutes</td>
<td>18.1 ± 1.2</td>
<td>69.0 ± 0.6</td>
</tr>
<tr>
<td>EN14</td>
<td>215</td>
<td>4 hours</td>
<td>13.6 ± 0.8</td>
<td>79.0 ± 0.5</td>
</tr>
<tr>
<td>EN10</td>
<td>100</td>
<td>4 hours</td>
<td>10.3 ± 0.6</td>
<td>82.4 ± 0.9</td>
</tr>
</tbody>
</table>

When the ratio (r) of EDC : Hep-COOH for covalent immobilization of heparin to EN14 was increased, heparinized collagen matrices (EN14-H(r)) with increasing amounts of immobilized heparin were obtained (Table II). Increased heparin immobilization resulted in more bFGF binding (Table II). A plateau value of bFGF binding was observed for EN14-H(r) with r ≥ 0.4, as previously determined using ¹²⁵I-labeled bFGF [35].

Table II: Heparin immobilization onto EN14 (n = 4, mean ± SD) and bFGF binding (n = 3, mean ± SD).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Immobilized heparin (mg/g collagen)</th>
<th>Sample</th>
<th>Pre-loaded bFGF (ng/8 mm disk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EN14</td>
<td>-</td>
<td>EN14-F</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>EN14-H(0.2)</td>
<td>10.3 ± 0.2</td>
<td>EN14-H(0.2)F</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>EN14-H(0.4)</td>
<td>18.6 ± 2.3</td>
<td>EN14-H(0.4)F</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>EN14-H(1.0)</td>
<td>38.1 ± 2.0</td>
<td>EN14-H(1.0)F</td>
<td>3.6 ± 0.3</td>
</tr>
</tbody>
</table>
In vivo biocompatibility of EDC/NHS-crosslinked collagen matrices

Figure 1: Light-microscopic images of non-crosslinked (NX) collagen.

Fig. a (original magnification 25×): At day 2, the folded film was loosely present in the surrounding capsule (C), with thick layers of adherent PMN and Mϕ. The film did not kink. Framework: Figure 1b.

Fig. b (original magnification 200×): The end point of the film shows first signs of degradation by means of splitting. Adherent are compact layers of primarily Mϕ, while cell accumulations (A) somewhat further away contain more PMN. WF: wound fluid; Fb: fibrin.

Fig. c (original magnification 100×): At day 10, NX was partly degraded. Three parts of the folded film are observed. One part represents the end point of the film with split-wise transition of film collagen into connective tissue collagen. V: blood vessel. Framework: Figure 1d.

Fig. d (original magnification 400×): An accumulation of lymphocytes. Part of the lymphocytes concern the immunoglobulin-producing plasma cells (arrows). NX: remaining film collagen, in between which fibroblasts and Mϕ, but no giant cells are present; C: connective tissue collagen.

3.2. Macroscopic evaluation

Before implantation, all materials had an opaque color, were rigid and strong when pulled at, but easy to tear with e.g. sharp tweezers. Wet samples easily adhered to different surfaces, like glass, polystyrene and connective tissue and had a tendency to flipping double or rolling up. After implantation, materials could always be retrieved easily, with the
Figure 2: Light-microscopic images of EDC/NHS-crosslinked collagen.
Fig. a (original magnification 100 ×): EN22 at day 2, lying flat in between the back muscle (M) and fat (F) tissue, showed a reasonable good adherence of the capsule with low cellular activation.
Fig. b (original magnification 100 ×): EN18 at day 2. At the top, the capsule (C) has detached, and an accumulation (A) of PMN and Mø in fibrin is present underneath the EN18 film. WF: wound fluid.
Fig. c (original magnification 200 ×): EN22 at week 6. Where a part has broken off, the folded film is degraded by active giant cells (G). Blood vessels (V) and a small accumulation (A) of plasma cells were observed.
Fig. d (original magnification 200 ×): EN18 at week 6 showed the highest number of giant cells (G), also alongside straight parts of the EN18 film.

exception of NX from week 3 on. During the first two days after implantation, capsules were still active and fragile. Already at day 5, NX appeared soft, flabby and possibly somewhat decreased in size. EN22 also appeared softer, while EN18, EN14, EN10 and heparin-immobilized and/or bFGF-loaded EN14 remained clearly more rigid. At day 10, NX films had become very small. Some of the crosslinked films clearly were folded. From week 3 on, all crosslinked materials were surrounded by quiet capsules. Flipping double or rolling up of samples was increasingly observed over time. Explants of all samples were (somewhat) decreased in size at week 6 and 10.
In vivo biocompatibility of EDC/NHS-crosslinked collagen matrices

Figure 3: Light-microscopic images of heparinized E/N14 collagen and heparinized E/N14 collagen pre-loaded with bFGF.

Fig. a (original magnification 100 ×): EN14-H(0.2) at day 1, showing hardly any cellular activation at the top side of the film, cellular activation at the bottom side was somewhat higher.

Fig. b (original magnification 100 ×): EN14-H(0.2)F at day 1. Cellular accumulations (A) and concomitant wound fluid (WF) and fibrin (Fb) formation reached levels of NX at day 1.

Fig. c (original magnification 50 ×): EN14-H(1.0)F at week 10. The folded film is quietly encapsulated with some giant cells and primarily connective tissue. Giant cells were most clear at end- or break points. The space inside a fold was often (partly) empty. Framework: Figure 3d.

Fig. d (original magnification 200 ×): The encapsulation at this bottom side appeared to consist of a direct attachment of connective tissue collagen (C) alongside the film. G: giant cells at this top side; V: blood vessel.

3.3. Microscopic evaluation

Microscopic evaluation of materials before implantation confirmed the structural similarity and comparable thickness of different samples. Apart from some dispersed, lengthwise stretched, dark blue particles, homogeneous toluidine blue staining was observed with all films.
3.3.1. Non-crosslinked collagen

Within 24 hours, NX clearly induced the most intense tissue reaction of all materials, with wound fluid, fibrin formation and vascularization, and the largest number of attracted cells (fig. 4A, 5A, 6A and 8A). These concerned predominantly polymorphonuclear cells (PMN) and macrophages (MØ), which both scored as 3. These cells were present in thick layers on the folded film, which itself was not attached to the surrounding tissue. Wound fluid with lightly stained unidentifiable cells left one side of the folded implant. Similar findings were observed at 2 days after implantation (fig. 1A, 1B).

The number of PMN decreased markedly at day 5 and 10, and wound fluid was not present any more. This period is characterized by fibroblast encapsulation which resulted in a strongly adherent capsule. The number of MØ remained relatively high during this period, but giant cells were not observed (fig. 7). Lymphocytes were first seen at day 5. The largest number of lymphocytes within this entire study was observed at day 10 in small accumulations, amongst which plasma cells (i.e. immunoglobulin producing cells) (fig. 1C, 1D). At day 10, fibrin was no longer present. The inserted film was clearly degrading, lying in bends with thinner and thicker sections, broken off fragments and split-wise transitions of film collagen into tissue collagen were observed. From week 3 on, NX was not retrieved.

3.3.2. EDC/NHS-crosslinked collagen

Infiltration of PMN and MØ, fibrin formation and wound fluid presence at day 1 and 2 were less intense for EN22, EN18, EN14 and EN10 than observed for NX (fig. 2A, 2B; fig. 4A, 5A, 6A). PMN numbers, which clearly decreased from day 5 onward, were not anymore observed at week 6 and 10. The number of MØ varied in time. Cell adhesion, as observed with NX, did hardly occur, but occasionally a single row of MØ adhered to the film which itself was often loosely present in the surrounding tissue. Most lymphocytes, amongst which a few plasma cells, were observed at relatively large distance in the surrounding tissue at day 10. Only few lymphocytes were observed at later time intervals. At day 10 a monolayer of giant cells had formed at the interfaces, but invasion of the collagen films was not yet observed. Using EN14 and EN10 giant cell numbers decreased strongly after this time point (fig. 7). At 6 weeks after implantation EN22, i.e. EDC/NHS-crosslinked collagen with the lowest number of crosslinks, had clearly degraded (fig. 2C). This was, in contrast to NX, associated with relatively large numbers of giant cells (fig. 7). The largest number of giant cells was present in case of EN18 at week 6 (fig. 2D). At week 10, EN22 and EN18 had degraded further compared to EN14 and EN10 while the number of giant cells had strongly decreased for all crosslinked collagens. Kinking of the films was hardly observed.
Figure 4: Polymorphonuclear cells (PMN) in the presence of various matrices as a function of implantation time (arbitrary units, ± 0.5).

PMN presence after subcutaneous implantation in rats for periods up to 10 weeks, around (fig. A) non-crosslinked collagen (NX) and EDC/NHS-crosslinked collagens (EN22 to EN10); (fig. B) EN14 collagen and heparinized EN14 collagen (EN14-H(0.2) to EN14-H(1.0)); (fig. C) EN14 collagen, and (heparinized) EN14 collagen pre-loaded with bFGF (EN14-F, EN14-H(0.2)F to EN14-H(1.0)F).
Figure 5: Macrophages (MØ) in the presence of various matrices as a function of implantation time (arbitrary units, ± 0.5).

MØ presence after subcutaneous implantation in rats for periods up to 10 weeks, around (fig. A) non-crosslinked collagen (NX) and EDC/NHC-crosslinked collagens (EN22 to EN10); (fig. B) EN14 collagen and heparinized EN14 collagen (EN14-H(0.2) to EN14-H(1.0)); (fig. C) EN14 collagen, and (heparinized) EN14 collagen pre-loaded with bFGF (EN14-F, EN14-H(0.2)F to EN14-H(1.0)F).
Figure 6: Fibrin formation in the presence of various matrices as a function of implantation time (arbitrary units, ± 0.5).

Fibrin presence after subcutaneous implantation in rats for periods up to 10 weeks, around (fig. A) non-crosslinked collagen (NX) and EDC/NHS-crosslinked collagens (EN22 to EN10); (fig. B) EN14 collagen and heparinized EN14 collagen (EN14-H(0.2) to EN14-H(1.0)); (fig. C) EN14 collagen, and (heparinized) EN14 collagen pre-loaded with bFGF (EN14-F, EN14-H(0.2)F to EN14-H(1.0)F).
Figure 7: Giant cells in the presence of various matrices as a function of implantation time (arbitrary units, ± 0.5).

Giant cell presence around non-crosslinked collagen (NX) and EDC/NHC-crosslinked collagens (EN22 to EN10) after subcutaneous implantation in rats for periods up to 10 weeks.

3.3.3. Heparin immobilized EN14

EN14 was selected to immobilize heparin, resulting in EN14-H(0.2), EN14-H(0.4), and EN14-H(1.0). Compared to EN14, heparin-immobilized films induced similar or somewhat reduced tissue reactions (fig. 3A; fig. 4B, 5B). Less lymphocytes extravasated, the largest number being found with EN14-H(1.0) at day 5 (not shown). At each explantation period, formation of giant cells was similar within this series, and comparable to EN14 (not shown). From week 3 on, heparinized EN14 was always present in bended forms and encapsulated by fibrous capsules with varying thickness, similar to EN14. Within this series, clear differences in degradation were not observed.

Vascularization was judged by the presence of smaller and larger blood vessels. As compared to EN14, vascularization was always slightly increased (fig. 8B), both close to the interface as well as at a distance, especially with EN14-H(0.2) (not shown). The presence of fibrin layers was increased after heparin immobilization (fig. 6B). At day 5 fibrin presence had diminished and was comparable among this series of heparinized EN14, and at day 10 only remnants of fibrin were observed.

3.3.4. bFGF pre-loading

EN14 and heparinized EN14 films were also implanted after pre-loading with bFGF. At day 1, EN14-F induced a PMN reaction similar to NX (fig. 3B; fig. 4A, 4C). Compared to EN14, EN14-H(0.4)F and EN14-H(1.0)F demonstrated similar or lower PMN numbers (fig. 4C). At day 5, a small increase of PMN’s was observed with
Figure 8: Vascularization in the presence of various matrices as a function of implantation time (arbitrary units, ± 0.5).

Vascularization after subcutaneous implantation in rats for periods up to 10 weeks, around (fig. A) non-crosslinked collagen (NX) and EDC/NHS-crosslinked collagens (EN22 to EN10); (fig. B) EN14 collagen and heparinized EN14 collagen (EN14-H(0.2) to EN14-H(1.0)); (fig. C) EN14 collagen, and (heparinized) EN14 collagen pre-loaded with bFGF (EN14-F, EN14-H(0.2)F to EN14-H(1.0)F).
EN14-H(1.0)F, which also demonstrated the largest number of \( \text{MØ} \) at this time point (fig. 4C, 5C). bFGF pre-loading did not influence the number of giant cells, lymphocytes or plasma cells (not shown). As observed with heparinized EN14, fibrin formation occurred during the first days after implantation of bFGF pre-loaded films, but only at day 1 fibrin presence was enhanced compared to EN14 (fig. 6C). For a specific heparinized EN14 film, there was no obvious effect of bFGF pre-loading on fibrin formation (fig. 6B versus 6C). At day 1, EN14-H(1.0)F demonstrated less vascularization than EN14, while other bFGF pre-loaded matrices displayed comparable or more vascularization (fig. 8C). EN14-F and EN14-H(0.2)F showed a similar degrees of vascularization when compared to EN14 and EN14-H(0.2). In contrast, EN14-H(0.4) and EN14-H(1.0) demonstrated significantly increased vascularization after pre-loading with bFGF, from 1 or 2 days after implantation persisting until week 3.

Similarly to the crosslinked and heparinized films, in time folding and a quiet encapsulation (fig. 3C), sometimes with direct attachment of connective tissue (fig. 3D), was observed.

4. DISCUSSION

The aim of this study was to evaluate the in vivo biocompatibility of newly developed (heparinized) EDC/NHS-crosslinked collagen matrices, with and without pre-loading of bFGF.

4.1. Non-crosslinked collagen

Non-crosslinked (NX) collagen showed the most intense tissue reaction, both at the level of cellular attraction (PMN’s, MØ, lymphocytes), fibrin and blood vessel formation, and induction of wound fluid. NX demonstrated a high degradation rate which was not due to giant cells. This is not surprising, since our group previously observed absence of giant cell formation after subcutaneous implantation of a non-crosslinked collagen material based on sheep dermis [36]. Accordingly, NX probably degrades by (enzymatic) hydrolysis and pinocytosis by MØ.

4.2. EDC/NHS-crosslinked collagen

Additional crosslinking of collagen was associated with a clearly decreased tissue reaction. Over time films with lower crosslink-densities (EN22, EN18) became surrounded by larger numbers of MØ and giant cells, and appeared to degrade faster than collagen films with higher crosslink-densities (EN14, EN10). All materials could, however, be retrieved after 10 weeks. Although the presence of giant cells was related to the presence of crosslinks, the largest
number of giant cells was found at collagens with the lowest crosslink densities (EN22, EN18). For these materials giant cell numbers, however, were still low when compared to e.g. EDC/NHS-crosslinked sheep dermis [36].

Infiltration of PMN’s and lymphocytes quickly reduced after 5 days. Compared to NX, the presence of plasma cells was strongly reduced after crosslinking, suggesting the involvement of antigenic groups on NX. These findings correlate with the facts that fibrillar collagen is reported to be (weakly) antigenic [37] and that antigenicity can be reduced by crosslinking [38, 39].

4.3. **Heparin immobilization**

Based on previous results [21, 23], EN14 was selected for heparin immobilization to improve blood compatibility. Heparin immobilization may act on fibrin formation and vascularization (as discussed below). Compared to NX, heparin immobilization onto EN14 caused a similar or even further decrease in the tissue reaction than EN14. Compared to the latter, MØ numbers were lower at day 10, but later on there was no clear difference in giant cell formation or degradation. Heparin immobilization resulted in variable fibrin formation at day 1 and 2. EN14-H(1.0), which was expected to release the largest amount of non-covalently bound heparin (chapter 4, fig. 3), showed the largest fibrin formation at day 1. This is likely due to prolonged bleeding (initiated by the implantation procedure) by temporary, local inhibition of coagulation in the presence of released heparin. The enhanced fibrin formation at day 2 by EN14-H(0.2) and EN14-H(0.4) is, however, not readily explained. The increased vascularization observed during the first 3 weeks was expected in view of the reported capacity of collagen-heparin matrices to trap heparin binding growth factors from surrounding tissue, which results in local induction of fibroblast proliferation and capillary blood vessel formation [40, 41].

4.4. **bFGF pre-loading**

Previously, pre-loading of (heparinized) EN-crosslinked collagen films with bFGF was applied to improve the proliferation of seeded endothelial cells. In this study, the effects of bFGF pre-loading remained limited to a short time interval after implantation. bFGF pre-loading of EN14 and EN14-H(0.2) generally resulted in a more intense early tissue reaction than with EN14-H(0.4) and EN14-H(1.0), possibly resulting from a chemotactic effect of released bFGF [42]. The present observations agree with previous *in vitro* studies, which showed that initially bFGF release from EN14 proceeded much faster than bFGF release from EN14-H(0.4) (34% versus 2% release of bound bFGF during the first 6 hours, respectively).
Thus, EN14-F and EN14-H(0.2)F may be considered as less biocompatible shortly after implantation. This is probably due to more extensive release of bFGF and does not concern a cytotoxic effect such as e.g. observed with glutaraldehyde-crosslinked collagen, which gives rise to extensive cell death even at day 10 [9-11, 43]. The largest effect of bFGF pre-loading on vascularization was observed for EN14-H(0.4)F and EN14-H(1.0)F, with a significant increase during the first 3 weeks. This indicates that a slower and more prolonged bFGF release, associated with higher amounts of immobilized heparin, is more efficient in inducing vascularization in the surrounding tissue over prolonged periods of time.

Despite the species differences among the source of heparin and bFGF and the experimental animals, heparin immobilization as well as pre-loading with bFGF did not induce increased lymphocyte attraction, which agrees with other reports [42, 44]. The 97% amino-acid homology [45] between rat bFGF and the recombinant human bFGF used for pre-loading offers an explanation for the absence of lymphocytes.

The immunologic response to the evaluated materials after subcutaneous implantation was slight, and similar as observed for e.g. glutaraldehyde- or EDC/NHS-crosslinked porcine heart valve cusps and walls after subcutaneous implantation [46]. Because the extent of immunologic response to commercial heart valve bioprostheses in an actual situation of blood flow is also quite accepted, problems due to immunologic response of bFGF pre-loaded EN14-H applied as a matrix for endothelial cell seeding in vascular grafts are not to be expected.

The collagen-based materials described in this study were developed as a matrix for endothelial cell seeding of small-diameter vascular grafts. For this purpose, material characteristics such as mechanical and structural stability, flexibility and ease of application to e.g. Dacron are required. In terms of in vivo application, however, biocompatibility, which was demonstrated in the present study, remains the most important aspect.

5. CONCLUSIONS

EDC/NHS-crosslinked collagen matrices demonstrated good biocompatibility after subcutaneous implantation in rats, at both early and late time intervals as demonstrated by their non-cytotoxicity, decreased early tissue reaction compared to NX, and mild foreign body reaction. Especially collagen matrices with high crosslink densities (i.e. EN14 and EN10) demonstrated a significantly decreased antigenic response compared to NX, and a prolonged resistance to in vivo degradation.
In vivo biocompatibility of EDC/NHS-crosslinked collagen matrices

In general heparin immobilization onto EN14 resulted in a slightly reduced early tissue reaction, whereas at later time points reactions were comparably mild as with EN14. bFGF pre-loaded matrices also demonstrated good biocompatibility, especially after immobilization of larger quantities of heparin (i.e. EN14-H(0.4) and EN14-H(1.0)). Pre-loading EN14 and EN14-H(0.2) with bFGF resulted in a relatively intense early reaction, which however decreased rapidly in time. EN14-H(0.4) and EN14-H(1.0) pre-loaded with bFGF demonstrated a mild early reaction, and increased vascularization of the surrounding tissue during the first 3 weeks of implantation. These observations may be explained from a more prolonged release of bFGF from EN14-H(0.4) and EN14-H(1.0).

Together with previous in vitro results described in this thesis, it is concluded that the observed biocompatibility justifies further evaluation of bFGF pre-loaded EN14-H(0.4) and EN14-H(1.0) as a matrix for in vivo endothelial cell seeding in experimental animals.

REFERENCES

[23] This thesis, Chapter 3.
Summary

Diseases of the cardiovascular system are the main cause of death in the western world. Increased cholesterol levels, hypertension, smoking and lack of exercise contribute to the development of degenerative arterial diseases, like atherosclerosis or the formation of aneurysms. Without treatment, these disorders may eventually cause a life threatening situation.

Treatment of obstructive atherosclerotic disease may involve reconstructive vascular surgery. As discussed in chapter 1, affected arteries can be replaced or bypassed using autologous vein grafts (e.g. saphenous vein) or synthetic vascular grafts made of knitted or woven Dacron or expanded Teflon (ePTFE). Synthetic grafts have been successfully used for more than 30 years in large-diameter applications like the aorta or in the aortoiliac region. However, the patency rates of small-diameter vascular grafts (inner diameter less than 5 mm) are unacceptably low due to thrombus formation. Therefore, saphenous veins are preferentially used for replacement of medium- or small-diameter arteries, although suitable autologous veins are often absent for example due to previous utilization or disease.

Endothelial cell seeding is an appreciated method to improve the blood compatibility of synthetic vascular grafts. In animal models, markedly improved patency rates of small-diameter synthetic vascular grafts were observed after endothelial cell seeding. However, until now several problems hamper successful endothelialization of vascular grafts in humans.

The supply of autologous endothelial cells is generally limited, and certainly not sufficient to produce a confluent endothelial lining when cells are seeded in the lumen of the graft directly after cell harvest. In human clinical trials the results of direct endothelial cell seeding have been disappointing, most likely due to low cell seeding densities. Alternatively, after initial endothelial cell harvest large numbers of autologous endothelial cells can be obtained by in vitro cell culture. This technique would allow high density endothelial cell seeding of a vascular graft before implantation in a second operation. Alternatively, endothelial cells can be grown to confluence in vitro after seeding of a graft, allowing implantation of grafts pre-lined with endothelium. An excellent primary patency rate of 74% after 7 years has been reported for fibrin-glue coated ePTFE grafts pre-lined with endothelial cells. Major disadvantages of these techniques are the need of two operations, the time lag between the need of an endothelialized graft and its availability, and the increased risk of bacterial infection. Therefore, direct seeding is preferred for endothelialization of synthetic vascular grafts in humans.

A second problem is that synthetic vascular graft materials are unsuitable as substrates for endothelial cell seeding. Since non-crosslinked collagen is a good substrate for attachment and proliferation of endothelial cells in vitro, application of a collagen matrix in the lumen of
synthetic vascular grafts may result in a matrix suitable for endothelialization. Collagen-coated vascular grafts have been developed to eliminate the elaborate procedure of preclotting of the porous grafts before implantation. Commercially available collagen-coated synthetic vascular grafts are commonly crosslinked using glutaraldehyde or formaldehyde, to reduce the *in vivo* resorption rate of the collagen matrix. These crosslink agents, which are incorporated in the collagen matrix, induce cytotoxic reactions, thus hampering endothelialization of the luminal graft surface. The development of a non-cytotoxic collagen substrate which supports adherence and growth of seeded endothelial cells, therefore is of great interest.

The aim of the study presented in this thesis was to develop a non-cytotoxic collagen-coated, small-diameter synthetic vascular graft which should be able to support adhesion and proliferation of endothelial cells after cell seeding at (very) low densities. These studies were carried out *in vitro*, using flat model substrates prepared from insoluble type I bovine achilles tendon collagen.

In chapter 2, an overview regarding the current status of endothelial cell seeding of vascular grafts is given. Furthermore, based on literature a strategy was formulated to realize the objectives of the study presented in this thesis. To obtain a non-cytotoxic matrix, collagen can be crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) in combination with N-hydroxysuccinimide (NHS). EDC/NHS-crosslinking results in the formation of amide crosslinks between carboxylic acid groups and epsilon-amino groups of amino acid residues in collagen. Since collagen is a thrombogenic material, and after low density cell seeding large parts of the collagen substrate remain exposed to blood, this may result in early graft failure due to thrombus formation. Immobilization of heparin onto collagen is expected to improve the blood compatibility of the matrix. Furthermore, local sustained release of basic fibroblast growth factor (bFGF, a heparin binding endothelial cell growth factor) from heparinized collagen may improve the proliferation of seeded endothelial cells, thereby increasing the potential of low density endothelial cell seeding.

Chapter 3 deals with optimization of the EDC/NHS-crosslink reaction, and the relation between crosslink density and material properties. In addition, the effect of gamma-sterilization on material properties was determined. At the conditions used, crosslinking of collagen resulted in a maximal reduction in free primary amino groups from 27 per 1,000 amino acid residues for non-crosslinked collagen to approximately 10 for EDC/NHS-crosslinked collagen (E/N10C, the number denotes the amount of free primary amino groups per 1,000 amino acid residues). The decrease in primary amino groups was inversely related to the shrinkage temperature. Low strain E-modulus and tensile strength of crosslinked collagen increased with increasing crosslink densities, whereas the elongation at break was not influenced by the crosslink density. The resistance to *in vitro* collagenase degradation also increased as a result.
of crosslinking. Whereas non-crosslinked collagen was almost completely degraded within 5 hours, the weight loss of E/N13C was only 4% after 24 hours of degradation. Gamma sterilization at a dose of 25 kGy decreased the shrinkage temperature of crosslinked collagen by 13 to 15°C, whereas the number of free primary amino groups was not affected. It was shown that EDC/NHS-crosslinked collagen with relatively high crosslink densities maintained their resistance to degradation after γ-sterilization. Sterilization hardly affected the E-modulus and the tensile strength of crosslinked collagen, except for the tensile strength of E/N13C which was decreased by 20%. The elongation at break after γ-irradiation showed an inverse correlation with crosslink density.

The immobilization of heparin onto E/N14C, also using EDC and NHS, is presented in chapter 4. Using increasing ratios of EDC to heparin carboxylic acid groups (Hep-COOH), heparinized E/N14C (EN14C-H(r), r = molar ratio EDC : Hep-COOH) with increasing amounts of immobilized heparin was obtained. Maximal heparin immobilization (5-5.5 wt. %) was obtained using a molar ratio of r = 2.

To determine whether heparin immobilization improved the blood compatibility of the collagen matrix, contact activation, thrombin inhibition and deposition of blood platelets was studied in vitro. Compared to E/N14C, increased immobilization of heparin resulted in a progressive decrease in contact activation. Thrombin inhibition was maximal after heparin immobilization using a molar ratio of EDC to heparin carboxylic acid groups of 0.2 to 0.4. E/N14C-H(0.4) displayed the lowest contact activation in combination with the highest thrombin inhibition. Heparin immobilization resulted in an increased platelet deposition which was maximal on E/N14C-H(0.4). Immobilization of heparin did not affect the morphology of deposited platelets, and platelets adhering onto (heparinized) E/N14C demonstrated only limited spreading or aggregation.

In chapter 5, a plateau in bFGF binding is demonstrated for heparinized E/N14C containing approximately 2.0 to 3.0 wt. % of immobilized heparin, which was obtained using a molar ratio of EDC to heparin carboxylic acid groups of 0.4 (E/N14C-H(0.4)). Therefore, E/N14C and E/N14C-H(0.4) were studied in more detail with respect to bFGF binding and release. Up to concentrations of 840 ng bFGF/ml, 10% of the added bFGF bound to E/N14C, while binding of bFGF to E/N14C-H(0.4) amounted to 22%. Both E/N14C and E/N14C-H(0.4) pre-loaded with bFGF demonstrated sustained release of bFGF. During the first 6 hours, a burst release of 30% in endothelial cell culture medium (CM) was observed for E/N14C, compared to 2% release from E/N14C-H(0.4). After 28 days, the bFGF release from E/N14C and E/N14C-H(0.4) in CM amounted to 100% versus 65%, respectively. Because of the higher bFGF binding and the slower, more gradual release of bFGF from E/N14C-H(0.4), it was concluded that E/N14C-H(0.4) pre-loaded with bFGF is the preferable substrate for endothelial cell seeding.
In chapter 6 it is demonstrated that EDC/NHS-crosslinked collagen is a suitable substrate for human umbilical vein endothelial cells (HUVECs) in vitro. EDC/NHS-crosslinked collagen matrices with 22, 18, 14 and 10 free amino groups per 1,000 amino acid residues were prepared (E/N22C, E/N18C, E/N14C and E/N10C). The functional characteristics of endothelial cells seeded on these substrates as well as non-crosslinked collagen (N-Coll) and fibronectin-coated TCPS were compared. The morphology of HUVECs cultured on EDC/NHS-crosslinked collagen was not altered compared to HUVECs cultured on non-crosslinked collagen and TCPS. After 10 days of proliferation, cell numbers on crosslinked collagen matrices with higher crosslink densities (i.e. E/N18C, E/N14C and E/N10C) were higher than on TCPS and N-Coll. EDC/NHS-crosslinking of collagen did not affect functional properties of endothelial cells like \(^3\)H-thymidine incorporation and the secretion of tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1), von Willebrand factor (vWF) and prostacyclin (PGI\(_2\)). During the first days of culture, a high secretion of tPA, PAI-1, vWF and PGI\(_2\) by HUVECs cultured on EDC/NHS-crosslinked collagen substrates was observed when compared to the secretion of these substances by HUVECs on N-Coll and TCPS. In the appendix to chapter 6, it was demonstrated that this was due to low cell densities on EDC/NHS-crosslinked collagen shortly after seeding rather than material characteristics.

Chapter 7 deals with the seeding of HUVECs on (heparinized) EDC/NHS-crosslinked collagen, pre-loaded with bFGF. Proliferation of HUVECs on both E/N14C and E/N14C-H(0.4) increased with increasing amounts of pre-loaded bFGF. The minimal cell seeding density required for proliferation proved to be very low after pre-loading of the substrates with bFGF, and was 4-fold lower for heparinized crosslinked collagen compared to crosslinked collagen (250 versus 1,000 cells/cm\(^2\)). Pro-coagulant properties (von Willebrand factor secretion and tissue factor expression) of HUVECs seeded on (heparinized) crosslinked collagen, with or without pre-loading of bFGF, were comparable to those of HUVECs on TCPS. It was concluded that heparinized, EDC/NHS-crosslinked collagen pre-loaded with bFGF is a candidate matrix for in vivo endothelial cell seeding of synthetic vascular grafts.

Finally, in chapter 8 possible cytotoxicity and (longer term) tissue reactions of (heparinized) EDC/NHS-crosslinked collagen were determined, following subcutaneous implantation in rats. The effects of the crosslink density, the amount of heparin immobilized to E/N14C and pre-loading of (heparinized) EDC/NHS crosslinked collagen with bFGF were studied. Crosslinked collagen was biocompatible at both early and late time intervals, and especially matrices with high crosslink densities (i.e. E/N14C, E/N10C) demonstrated a significantly decreased antigenic response when compared to non-crosslinked collagen. Immobilization of heparin onto E/N14C resulted in a similar or somewhat reduced tissue reaction, but fibrin formation and vascularization were increased with increasing amounts of immobilized
heparin. bFGF pre-loaded matrices also demonstrated good biocompatibility, especially in combination with larger amounts of immobilized heparin (i.e. E/N14C-H(0.4) and E/N14C-H(1.0)). In addition, these matrices demonstrated significantly increased vascularization in the surrounding tissue for periods up to 3 weeks after pre-loading with bFGF.

In view of the promising results of the studies presented in this thesis, low density endothelial cell seeding of heparinized EDC/NHS-crosslinked collagen matrices was studied in a porcine model. Dacron grafts coated with an E/N14C-H(0.4) matrix, with and without bFGF pre-loading, were directly seeded with autologous endothelial cells (< 8,000 cells/cm²) isolated from the external jugular veins. A bFGF pre-loaded graft and a non-loaded control graft were sutured together at either end to create a common inflow respectively outflow opening. In the infrarenal aorta two aortotomies were made to which the grafts were anastomosed in an end-to-side manner, after which the aorta between the anastomoses was ligated. No complications occurred upon implantation. During implantation for a time period up to 4 weeks, 11 out of 12 seeded grafts remained patent. At one week after implantation, endothelial cells were found on the entire length of the seeded grafts. In non-seeded grafts, presence of endothelial cells at this time point is normally restricted to the anastomoses, indicating that the endothelial cells observed in our study were most likely seeded cells. After 4 weeks, a confluent endothelial lining was observed in all grafts. At the time of printing of this thesis, however, the effect of bFGF pre-loading on the rate of endothelialization was still under evaluation.
Samenvatting

Cardiovasculaire aandoeningen leiden tot een hoog sterftepercentage in de moderne westerse maatschappij. Een verhoogd cholesterol-niveau, te hoge bloeddruk, roken en een gebrek aan lichaamsbeweging dragen bij aan de ontwikkeling van degeneratieve aandoeningen aan bloedvaten, zoals atherosclerose of het ontstaan van aneurysma’s. Zonder behandeling kunnen deze aandoeningen een levensbedreigende situatie veroorzaken.
Obstructieve atherosclerose kan worden behandeld met bijvoorbeeld een reconstructieve operatie. Zoals beschreven in hoofdstuk 1 kunnen aangetaste slagaders worden vervangen of omgeleid, door gebruik te maken van autologe venen (in het algemeen de vena saphena) of kunststof bloedvat-prothesen (gemaakt van gewoven of gebreid Dacron of ge-expandeerd Teflon, ePTFE). Deze bloedvat-prothesen worden al meer dan 30 jaar succesvol toegepast voor de vervanging van bloedvaten met een grote interne diameter, zoals de aorta. Door vorming van bloedstolsels zijn de "patency rates" van prothesen met een kleine diameter (interne diameter kleiner dan 5 mm) echter onaanvaardbaar laag. Daarom wordt de vena saphena preferentieel gebruikt voor vervanging van kleine of middelgrote vaten, alhoewel geschikte autologe venen vaak niet beschikbaar zijn omdat ze bijvoorbeeld al zijn gebruikt in voorafgaande operaties of omdat ze zelf zijn aangetast door atherosclerose.
Het zaaien van endotheelcellen is een erkende methode om de bloedcompatibiliteit van kunststof bloedvat-prothesen te verbeteren. Dierproeven hebben aangetoond dat de “patency rates” van kleine-diameter bloedvat-prothesen opvallend hoger waren na het zaaien van endotheel. Echter, op dit moment staan een aantal problemen succesvolle endothelialisatie van kunststof bloedvat-prothesen in de mens in de weg.
De hoeveelheid autoloog endotheel die kan worden geïsoleerd is beperkt, en zeker niet voldoende om een aaneengesloten, confluente bedekking van de binnenzijde van een implantaat te geven wanneer de cellen direct na isolatie worden gezaaid. Klinische onderzoeken waarin het zogenaamde "direct zaaien" van endotheel werd toegepast, hebben teleurstellende resultaten opgeleverd, waarschijnlijk juist door lage zaaidichtheden.
Als alternatief kunnen na de isolatie van endotheel standaard in vitro weefselkweektechnieken worden toegepast om grote hoeveelheden endotheel te verkrijgen. Op deze manier kan het zaaien van endotheel in hoge dichtheden worden gerealiseerd. Een andere mogelijkheid is het in vitro kweken van prothesen die met een lage dichtheid zijn gezaaid, waardoor prothesen met een confluente laag endotheel kunnen worden geïmplanteerd. Voor ePTFE prothesen, gecoat met een fibrine lijm en op deze manier voorzien van een confluente laag endotheel, is een uitstekende primaire “patency rate” van 74% na 7 jaar gerapporteerd.
Deze alternatieve technieken hebben echter een aantal grote naden: er zijn twee operaties nodig (één voor isolatie en één voor implantatie), het duurt lang voordat een prothese met een
confluente laag endotheel beschikbaar is, en in vitro kweek introduceert een verhoogd risico op bacteriële infectie. Daarom heeft het direct zaaien van endotheel voor endothelialisatie van kunststof bloedvat-prothesen in mensen sterk de voorkeur.

Een tweede probleem is het feit dat kunststof bloedvat-prothesen niet direct geschikt zijn als substraat voor endotheel. Omdat niet-gecrosslinkt collageen een goed substraat is voor hechting en proliferatie van endotheel in vitro, kan worden verwacht dat het aanbrengen van een collageen-coating in het lumen van synthetische bloedvat-implantaten resulteert in een geschikte matrix voor endothelialisatie. Prothesen met een collageen-coating zijn in het verleden al ontwikkeld om de arbeidsintensieve procedure van voorstollen met bloed achterwege te kunnen laten. Commercieel verkrijgbare, collageen-gecoate prothesen worden in het algemeen gecrosslinkt met glutaraaldehydro of formaldehydro, om de in vivo degradatiesnelheid van de collageen-coating te reduceren. Deze reagentia, die in de collageenmatrix worden geïncorporeerd, induceren cytotoxische reacties waardoor endothelialisatie van deze materialen wordt belemmerd. De ontwikkeling van een niet-cytotoxische collageencoating die de hechting en groei van gezaaide endotheelcellen ondersteunt is daarom van groot belang.

Het doel van het onderzoek dat staat beschreven in dit proefschrift was de ontwikkeling van een niet-cytotoxisch, collageen-gecoat kleine-diameter synthetisch bloedvat-implantaat, geschikt voor het zaaien van endotheelcellen in (zeer) lage dichtheden. Dit onderzoek is uitgevoerd in vitro, door gebruik te maken van vlakke collageenfilms (type I collageen, uit achillespezen van runderen) als modelsubstraat.

In hoofdstuk 2 wordt een overzicht gegeven van de huidige stand van zaken met betrekking tot endothelialisatie van bloedvat-prothesen. Op basis van gepubliceerde onderzoeken is een strategie voor realisatie van de doelstellingen van het huidige onderzoek geformuleerd. Een niet-cytotoxische collageenmatrix kan worden verkregen door crosslinking met N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) in combinatie met N-hydroxysuccinimide (NHS). EDC/NHS-crosslinking resulteert in de vorming van amide bindingen tussen carboxylzuurgroepen en ε-aminogroepen van aminozuur residuen in collageen. Collageen is zeer thrombogeen, en na het zaaien van endotheel (in lage dichtheden) blijft er contact tussen grote delen van het collageen en bloed, wat kan leiden tot verstopping van de prothese door thrombusvorming. Een verbeterde bloedcompatibiliteit van de collageenmatrix kan worden gerealiseerd door het immobiliseren van heparine. Daarnaast kan een locale, gereguleerde afgifte van basic fibroblast growth factor (bFGF, een heparine bindende groeifactoor voor endotheel) leiden tot een snellere groei van gezaaide endotheelcellen, waardoor de kans op succesvolle endothelialisatie na zaaien met een lage zaaidichtheid toeneemt.

In hoofdstuk 3 wordt de optimalisatie van de EDC/NHS crosslinking beschreven. Daarnaast is de relatie tussen crosslinkdichtheid en materiaaleigenschappen en het effect van γ-
Samenvatting

sterilisatie op materiaaleigenschappen bepaald. Onder geoptimaliseerde reactiecondities resulteerde crosslinking in een maximale reductie van vrije primaire aminogroepen van 27 per 1.000 aminozuur residuen voor niet-gecrosslinkt collageen naar ongeveer 10 voor EDC/NHS-gecrosslinkt collageen (E/N10C, 10 geeft het aantal vrije aminogroepen per 1.000 aminozuur residuen). De afname in primaire aminogroepen was gerelateerd aan een toename van de krimptemperatuur. De E-modulus bij lage rek en de treksterkte van gecrosslinkt collageen namen toe met toenemende crosslinkdichtheid, de rek bij breuk werd echter niet beïnvloed door crosslinking. De weerstand tegen in vitro collagenase degradatie nam toe door crosslinking: niet-gecrosslinkt collageen werd binnen 5 uur bijna volledig gedegradeerd, terwijl het gewichtsverlies voor E/N13C na 24 uur degradatie slechts 4% bedroeg. Gamma sterilisatie met een dosis van 25 kGy resulteerde in een afname van de krimptemperatuur met 13 tot 15°C, terwijl het aantal vrije aminogroepen ongewijzigd bleef. EDC/NHS-gecrosslinkt collageen met relatief hoge crosslinkdichtheden behield zijn weerstand tegen collagenase. Sterilisatie had nauwelijks invloed op de E-modulus en de treksterkte van gecrosslinkt collageen, met uitzondering van E/N13C dat een 20% reductie in treksterkte te zien gaf. De rek bij breuk na γ-sterilisatie was invers gecorreleerd aan de crosslinkdichtheid.

Immobilisering van heparine aan E/N14C met gebruik van EDC en NHS staat beschreven in hoofdstuk 4. Door gebruik van toenemende ratio’s van EDC ten opzichte van carboxylzuurgroepen van heparine (Hep-COOH) werd gehepariniseerd E/N14C (E/N14C-H(r), r = molaire ratio EDC : Hep-COOH) met toenemende hoeveelheden heparine verkregen. Een maximale hoeveelheid heparine (5-5.5 gewichts %) werd geïmmobiliseerd met een molaire ratio r ≥ 2.

Om te bepalen of immobilisering van heparine daadwerkelijk resulteerde in een verbeterde bloedcompatibiliteit van E/N14C, is de in vitro contact activering, thrombine inhibitie en depositie van bloedplaatjes bepaald. De contact activering van E/N14C-H nam af met toenemende hoeveelheden geïmmobiliseerd heparine. Maximale thrombine inhibitie werd gevonden na heparine immobilisering met een molare ratio EDC : Hep-COOH van 0.2 tot 0.4. Voor E/N14C-H(0.4) werd de laagste contact activering in combinatie met de hoogste thrombine inhibitie gevonden. Immobilisering van heparine resulteerde in een toename van de depositie van bloedplaatjes, die maximaal was op E/N14C-H(0.4). Immobilisering van heparine was echter niet van invloed op de morfologie van de afgezette plaatjes, en plaatjes afgezet op (gehepariniseerd) E/N14C lieten (morfologisch) slechts beperkte activering en spreiding zien.

De binding van bFGF aan gehepariniseerd E/N14C, zoals beschreven in hoofdstuk 5, vertoonde een plateau-waarde voor materialen met (meer dan) 2 tot 3 gewichts % heparine, verkregen door heparine immobilisering met een molaire ratio EDC : Hep-COOH ≥ 0.4.
Daarom zijn E/N14C en E/N14C-H(0.4) geselecteerd voor verdere studies met betrekking tot de binding en release van bFGF. Tot bFGF concentraties van 840 ng/ml bedroeg de binding van bFGF aan E/N14C 10%, terwijl 22% van het bFGF werd gebonden aan E/N14C-H(0.4). Na belading met bFGF lieten zowel E/N14C als E/N14C-H(0.4) een langzame afgifte van bFGF zien. In “endothelial cell culture medium” (CM) werd voor E/N14C gedurende de eerste 6 uur een burst-release van 30% waargenomen, vergeleken met 2% afgifte uit E/N14C-H(0.4). Na 28 dagen was uit E/N14C en E/N14C-H(0.4) respectievelijk 100% en 65% van het oorspronkelijk gebonden bFGF vrijgekomen. Omdat de binding van bFGF aan E/N14C-H(0.4) hoger, en de afgiftesnelheid lager en meer geleidelijk is, is E/N14C-H(0.4) beladen met bFGF een beter substraat voor het zaaien van endotheelcellen dan E/N14C beladen met bFGF.

In hoofdstuk 6 is aangetoond dat EDC/NHS-gecrosslinkt collageen een goed in vitro substraat is voor HUVECs, endotheelcellen geïsoleerd uit humane navelstrengvenen. In deze studie werden EDC/NHS-gecrosslinkt collageen met 22, 18, 14 en 10 vrije primaire aminogroepen per 1.000 aminozuurresiduen (E/N22C, E/N18C, E/N14C en E/N10C) gebruikt. De functionele karakteristieken van HUVECs gezaaid op deze matrices, alsook op niet gecrosslinkt collageen (N-Coll) en “tissue culture polystyreen” gecoat met fibronectine (Fn-TCPS), werden vergeleken. De morfologie van HUVECs gekweekt op EDC/NHS-gecrosslinkt collageen was vergelijkbaar met de morfologie van HUVECs op N-Coll en Fn-TCPS. Na 10 dagen proliferatie was het aantal HUVECs op EDC/NHS-gecrosslinkt collageen met hogere crosslinkdichtheid (met name E/N18C, E/N14C en E/N10C) hoger dan op Fn-TCPS en N-Coll. EDC/NHS-crosslinking van collageen was niet van invloed op functionele eigenschappen van HUVECs, zoals \(^3\)H-thymidine incorporatie en de secretie van tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1), von Willebrand factor (vWF) en prostacycline (PGI\(_2\)). Gedurende de eerste dagen na het zaaien van HUVECs op EDC/NHS-gecrosslinkt collageen was de secretie van tPA, PAI-1, vWF en PGI\(_2\) wel hoog in vergelijking met de secretie van deze stoffen door HUVECs op N-Coll en Fn-TCPS. In de appendix bij hoofdstuk 6 is echter aangetoond dat dit naar alle waarschijnlijkheid werd veroorzaakt door lage cellerdichtheid van HUVECs op EDC/NHS-gecrosslinkt collageen direct na het zaaien, en niet door materiaal karakteristieken van het EDC/NHS-gecrosslinkte collageen.

In hoofdstuk 7 wordt ingegaan op het zaaien van HUVECs op (gehepariniseerd) E/N14C, na beladen van de materialen met bFGF. De proliferatie van HUVECs op zowel E/N14C als E/N14C-H(0.4) nam toe met een toenemende bFGF belading. De minimale zaaidichtheid die nodig is voor inductie van proliferatie was zeer laag na belading van de substraten met bFGF. Vergeleken met E/N14C was de minimale zaaidichtheid voor E/N14C-H(0.4) zelfs 4 maal lager (1.000 versus 250 cellen per cm\(^2\)). Pro-coagulerende eigenschappen (von Willebrand factor secretie en tissue factor expressie) van HUVECs op (gehepariniseerd) E/N14C, met of
Samenvatting

zonder bFGF belading, waren vergelijkbaar met die van HUVECs op TCPS. Er is daarom geconcludeerd dat E/N14C-H(0.4) beladen met bFGF een veelbelovende matrix is voor in vivo endothelialisatie van synthetische bloedvat-implantaten.

Tot slot is de mogelijke cytotoxiciteit en de (langere termijn) weefselreaktie van (gehepariniseerd) EDC/NHS-gecrosslinkt collageen bepaald na subcutane implantatie in ratten, zoals beschreven in hoofdstuk 8. De effecten van de crosslinkdichtheid, de hoeveelheid heparine geïmmobiliseerd aan E/N14C, en belading met bFGF zijn hierbij bestudeerd. Gecrosslinkt collageen was zowel kort na implantatie als op langere termijn biocompatibel, en in het bijzonder matrices met hoge crosslinkdichtheid (E/N14C en E/N10C) lieten een duidelijk verlaagde antigene reactie zien vergeleken met niet-gecrosslinkt collageen. Immobilisering van heparine aan E/N14C resulteerde in een vergelijkbare of iets afgenomen weefselreactie, maar fibrinevorming en vascularisatie namen toe met toenemende hoeveelheden geïmmobiliseerd heparine. Voor matrices beladen met bFGF werd ook een goede biocompatibiliteit aangetoond, in het bijzonder voor matrices met een grotere hoeveelheid geïmmobiliseerd heparine (E/N14C-H(0.4) en E/N14C-H(1.0)). Deze materialen lieten na belading met bFGF ook een significante toename van vascularisatie van het omliggende weefsel zien gedurende de eerste drie weken na implantatie.

Met het oog op de veelbelovende resultaten van het onderzoek dat staat beschreven in dit proefschrift, is het met lage dichtheid zaaien van endotheelcellen op gehepariniseerd EDC/NHS-gecrosslinkt collageen bestudeerd in varkens. Dacron protheses gecoat met een E/N14C-H(0.4) matrix, met en zonder bFGF belading, zijn gezaaid met endotheelcellen (< 8.000 cellen/cm²) direct na isolatie uit de externe vena jugularis. Een prothese beladen met bFGF en een niet-beladen controle-prothese werden aan beide uiteinden aan elkaar gehecht om een gezamelijke instroom- respectievelijk uitstroom opening te creëren. In de infrarenale aorta werden twee openingen gemaakt waarop de grafts end-to-side werden gehecht, waarna de aorta tussen beide anastomosen werd afgebonden. Tijdens de implantaties traden geen complicaties op. Gedurende een periode tot 4 weken na implantatie bleven 11 van de 12 geïmplanteerde protheses doorgankelijk. Eén week na implantatie werden endotheelcellen gevonden over de volledige lengte van de geïmplanteerde prothese. Normaliter is na één week de aanwezigheid van endotheelcellen beperkt tot een gelimiteerde ingroei ter plekke van de anastomosen, wat aangeeft dat de endotheelcellen die in deze studie zijn geobserveerd naar alle waarschijnlijkheid gezaaide cellen waren. Na 4 weken werd in alle grafts een conflente laag endotheel gevonden. Op het moment van verschijnen van dit proefschrift was het effect van belading van de protheses met bFGF echter nog niet geëvalueerd.
Curriculum vitae