Modulation of the angiogenic potential in collagen matrices by immobilisation of heparin and loading with vascular endothelial growth factor

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1 Introduction

1.1 General Introduction

1.1.1 Plastic surgery

The aim of plastic surgery is the restoration of form and function with resultant improvement in patient's quality of life and esthetic outcome. Accordingly, two main tasks of plastic surgery are: 1. to improve the quality of wound healing in the repairing area of a tissue deficit. 2. to reduce as much as possible the destruction in the donor area.

Wound healing is fundamental to surgery. As to plastic surgery, one of the main objectives is to reduce the scar formation of healing which will influence bothfunctional restoration and the esthetic outcome. At the same time, restoration with less or without destruction still challenges plastic surgery. Projection indicates that the gap between tissue supply and demand will even continue to widen in the future.

Naturally, the question arises whether a tissue substitute may be developed which can not only replace the deficit tissues, but can also improve the wound healing simultaneously was proposed to meet the above demands. Fortunately, the rapid development of modern science and the new findings, in biological materials and factors involved with cell development gave birth to the interdisciplinary field of research termed "Tissue Engineering".

1.1.2 Tissue engineering

Major advances in surgery often come from a cooperation of fundamental and clinical research. To solve the problem of a significant shortage of tissues and organs suitable for clinical transplantation (Koshland, 1990), tissue engineering was developed for providing alternative therapies which can maintain, restore or improve lost tissue functions by developing biological artificial tissue substitutes (Langer and Vacanti, 1993).

Tissue engineering is a relatively new and emerging interdisciplinary field that applies the knowledge of bioengineering, life science and the clinical sciences for creating new functional substitutes for damaged host tissues (Nerem, 1992; Patrik *et al*., 1999). Thus, it has been proposed as a therapeutic approach to treat patients suffering from the loss or failure of organs and tissues.

With the approach of tissue engineering, many human tissues were developed by using various kinds of biomaterials (Mooney and Mikos, 1999), which includes the replacement of skin, soft tissue (Hollander *et al*., 2001), hard tissues (Hutmacher *et al*., 2001), blood vessel and heart valve (Sodian *et al*., 2000; Watanabe *et al*., 2001). As for the ideal biomaterials which serve as a temporal scaffold in the body, they should be biocompatible, biodegradable, highly porous with a large surface to volume ratio, mechanically strong, and capable of being formed into desired shapes, which play a positive role in manipulating host cell functions by secreting their own extracellular matrix proteins to form gradually normal, completely natural tissue. Collagen as a natural extracellular matrix component, meets all the demands above. Therefore, it is not surprising that collagen is the most commonly used biomaterial in skin, connective tissue, peripheral nerve, blood vessel tissue engineering (Doillon *et al*., 1994b; Friess, 1998; Sheridan and Tompkins, 1999).

After synthetic biomaterials being implanted, a critical question is how to supply the cells which grow into the biomaterials with sufficient oxygen and nutrients to sustain their survival, proliferation and allow for the integration of the developing tissue with the surrounding tissue. A rapid and high level of vascularization in transplanted matrices is essential in tissue engineering approaches to meet this challenge.

1.1.3 Tissue engineering and Angiogenesis

Tissues with a high cell density require a vascular network of arteries, veins and capillaries for the delivery of nutrients to each cell. Thus, the development of efficient methods for enhancing angiogenic effects of biomaterials is critical for a successful outcome. Generally, three approaches may lead to this goal: 1. Incorporation of angiogenic factors in biomaterials. 2. Seeding of endothelial cells (ECs) along with other cell types in biomaterials (Park *et al*., 2002). 3. Prevascularization of matrices prior to cell seeding.

It should be kept in mind that some fundamental guidelines must be followed (Soker *et al*., 2000): 1. The support matrix for biomaterials must be compatible with EC growth and capillary formation. It should have a high degree of porosity to allow the penetration of blood vessel into the implant. 2. Angiogenic growth factors should be applied for enhancing positive effects. 3. The combination of EC seeding with insertion of a constitutive source for angiogenic factor secretion could be advantageous. 4. The angiogenic process in biomaterials should be controlled before a functional vascular network can be obtained, which is important since it was reported that sustained overproduction of VEGF may result in deformed, non-functional blood vessels (Springer *et al*., 1998).

1.2 Collagen as a biomaterial

1.2.1 Collagen matrices as tissue substitutes

Among biological materials, collagen, particularly type 1 collagen, is a major component of various connective tissues, such as duramater, fascia, intestine, tail tendons, etc. The replacement of human tissues with biomaterials, results in the improvement of the wound-healing process because of the presence of collagen. Therefore, the application of collagen-derived products as biomaterials has tremendous advantages in biomedicine because of these products' natural structure as a biological support for cells and scaffold for tissue repair or regeneration, their biodegradability that obviates removal of implants, and their biocompatibility. It is not surprising that collagen has been used to design numerous biomaterials in plastic surgery, such as wound dressings, artificial dermis, tissue engineered devices, and injectable materials (Hutmacher *et al*., 2001; Sodian *et al*., 2000; Watanabe *et al*., 2001; Yannas *et al*., 1982).

In order to understand the good safety profile of collagen as a biomaterial and its role in wound healing, its structure should be discussed first. The name collagen is used as a generic term to cover a wide range of protein molecules, which form supra-molecular structures. 14 types of collagen have been identified to date (van der Rest *et al*., 1990), the most abundant being type 1 collagen (van der Rest *et al*., 1990). Because

of its abundance in nature (more than 90% of all fibrous protein is Type 1 collagen) and its unique physical and biological properties, Type 1 collagen has been used extensively to formulate medical devices. Therefore I concentrate my depiction to Type 1 collagen.

The primary structure of collagen is unique, showing a strong internal sequence homology, which places glycine at every third amino acid portion, and has a high content of proline and hydroproline which contributes to forcing each collagen subunit into helical structure. The three subunits are arranged in the form of a triple helical procollagen, similar to a triple stranded rope (tertiary structure) (Zeeman, 1998a). The procollagen is then exported into the extracellular space to form the collagen monomer, with short non-helical ends of 15 to 25 amino acid residues (N-and C-terminal telopeptides). Cross-linking renders these fibers stable and provide them with an adequate degree of tensile strength and visco-elasticity to perform their structural role. Two types of natural cross-links in collagen can be distinguished and are based on the aldehyde groups formed from (hydroxy)lysine residues in the telopeptides, by enzymatic oxidation by lysy/oxidase, yielding allysin. The collagen may be further modified to form intra- and intermolecular cross-links which are formed by an aldol condensation reaction of two aldehyde groups, or by the reaction of the aldehyde group with the ε-amino group of a (hydroxy)lysine residue of an adjacent helix respectively (Nimni, 1988). With the aid of cross-linking, collagen can form fibers, and then macroscopic bundles used to form tissue.

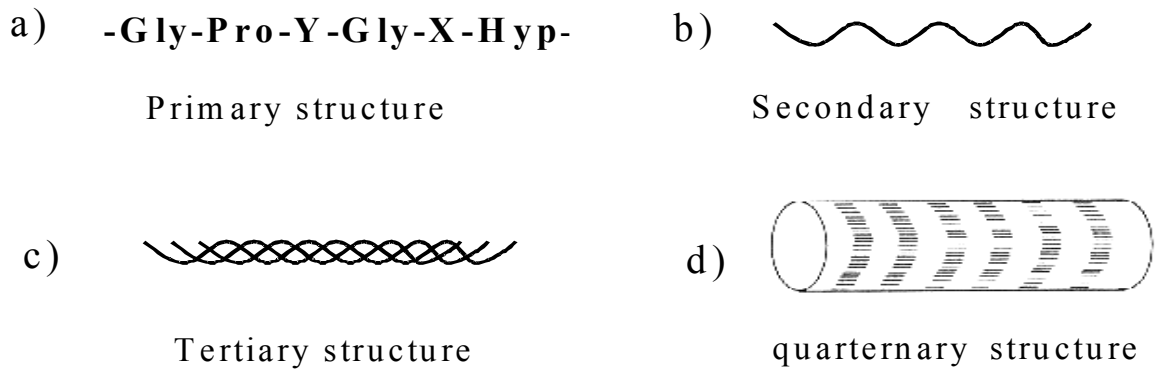


Figure 1 The molecular architecture of the fiber forming collagen.

From the structure of collagen, we know that collagen, the primary constituent of the extracellular matrix, essentially consists of amino acids. The amino acids in collagen contain side-chain groups such as amines (NH_2), carboxylic acids (COOH) and hydroxyls (OH), which together with the amide bond of the protein polymer, are sites for possible chemical reaction on collagen. At the most basic level, a collagen molecule consists of three chains of polypeptides arranged in a trihelical configuration ending in non-helical carboxyl and amino terminals, one at each end.

These non-helical ends, which can be enzymatically or chemically removed and can also be masked by cross-linking which minimizes non-helical ends (Furthmayr and Timpl, 1976), are believed to contribute most to its antigenicity. In addition, when cleaving the peptides bonds of collagen with the enzymes secreted from cells, collagen can be biodegraded. Therefore, biomaterials based on collagen may be modified to have properties as: high tensile strength, low extensibility, fiber orientation. Their porous structure further allows for cell migration and proliferation, inhibition of the wound contraction and acceleration of wound repair (Chvapil, 1982; Doillon *et al*., 1984; Furthmayr and Timpl, 1976; Yannas and Burke, 1980). It is therefore not surprising that collagen based biomaterials have been widely applied for medical use and have been produced in a variety of forms such as film, gel, powder and sponge (Pachence, 1996).

Collagen sponges or matrices are reconstituted from pure collagen. After forming a 0.5-2% slurry and frozen, collagen is dehydrated either by lyophilization or by extraction with organic solvents to form sponges. The formed sponge can then be cross-linked to stabilize the three-dimensional meshwork. In scanning electron microscopy, the pore size within the matrices surface of the sponge is uneven and irregular, showing the continuity of the pores ranging in size from 10 to 40 µm (Lau & Richter, unpublished results). The pore size is crucial for cell permeation (Gey *et al*., 1974), promotion of wound healing (Nathan *et al*., 1974) and regeneration of tissues (Holmes *et al*., 1975; Hutmacher *et al*., 2001). In contact with aqueous liquid, collagen sponges bind large volumes of liquid, which has a beneficial effect on cell adhesion and mobility (Salzman, 1971).

The research on collagen matrices as a scaffold for tissue engineering may be classified into 4 aspects:

1 Cell seeding experiments: various types of cells, such as HUVEC cells (Wissink *et al*., 2000c), human menses cells (Nakatake *et al*., 2001), hepatocytes (Glicklis *et al*., 2000), chondrocytes (Glicklis *et al*., 2000), fibroblasts (Doillon *et al*., 1994a) have been seeded into collagen matrices for testing the ability of functioning as a scaffold. Generally these cells grow and proliferate successfully within collagen scaffolds. Furthermore, stem cells, such as mesenchymal stem cells (Hori *et al*., 2002), have also been applied in order to differentiate into the desired mature cells. Some gene transfected cells, such as bone morphogenetic protein-2-expressing muscle-derived cells (Lee *et al*., 2001) have been used in order to express desired proteins or growth factors favorable to tissue regeneration.

2 Incorporating growth factor experiments: The application of growth factors to biomaterials has become an important aspect of tissue engineering because it allows modulation of cellular function and tissue formation at the affiliated site (Lu *et al*., 2000). As for the biomaterials based on collagen sponge, they have been applied as a sustained released vehicle for various kinds of growth factors, including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF)(Fujisato *et al*., 1996), VEGF (Dellian *et al*., 1996).

3 Experiments focussing on incorporating bioactive molecules. Some bioactive molecules (e.g. heparin, hyaluronic acids, glycosaminoglycans) may have substantial effects on the cell growth and may also stabilise the activity of growth factors. Therefore, they were also incorporated into the collagen sponges and their effects were evaluated *in vitro* and *in vivo* (Park *et al*., 2002; Pieper *et al*., 2000a; Wissink *et al*., 2001a; Wissink *et al*., 2000b).

4 Experiments directed towards the modification of structural characteristics of the collagen matrices. The internal structure of the matrices may have effects on tissue ingrowth and response. Some investigations are focussing on modifying the porous structure, for instance by treating them with alkaline (Rocha *et al*., 2002).

As a result of the above mentioned investigations, a number of devices have been developed to commercial products for wound healing or as an artificial skin (Gao *et al.*, 2002).

1.2.2 Collagen matrices as biomaterial with enhanced angiogenic potential

Angiogenesis is a multi-step process in which new blood vessels grow from existing vessels. The sequential steps required for angiogenesis are extracellular matrix remodeling, endothelial cell migration and proliferation, and capillary differentiation and anastomosis (Ribatti *et al*., 1997).

A large number of angiogenic factors have been identified to be involved in the development of new vessels in a variety of assays. Angiogenesis plays a pivotal role in the survival of implant biomaterials *in vivo*, therefore, many investigations focus on developing appropriate angiogenic scaffolds by implementing angiogenic factors.

One approach is to modify the collagen matrices. Collier et al. (Collier *et al*., 2000) synthesised a biomaterial with polypyrrole-hyaluronic acid, which improves both the cell compatibility and the vascularisation. Pieper et al. cross-linked glycosaminoglycans to collagenous materials to preserve porous integrity, promote angiogenesis and reduce foreign body reaction (FBR) (Pieper *et al*., 2000b). Another approach is to deliver growth factors such as FGF, VEGF and ECGF for increasing angiogenesis, enhancing epithelialization and reducing contraction of wound healing (Andrade *et al*., 1987). The third approach is to administrate the various peptides to the implant biomaterials to study their angiogenic activity in the biomaterials. Both vasoactive intestinal peptide (VIP) and angiotensin II show angiogenic activity *in vivo* and the effect is activated according to the level of their receptors' level (Hu *et al*., 1996). Heparin affinity regulatory peptides were found to have properties that can stimulate endothelial cell tube formation, migration, and induction of angiogenesis (Papadimitriou *et al*., 2000). Substance P, a member of the neurokinin family of regulatory peptides and acts through cell surface receptors, is able to enhance angiogenesis through its action on microvascular NK receptors (Walsh *et al*., 1996). The last kind of assay is to utilise the ligand of chemokine receptors to bind the

objective cells to activate its angiogenic effects. The CC chemokine I-309 was found to bind to endothelial cells, thereby stimulating chemotaxis and invasion of these cells, as well as enhancing their differentiation into capillary-like structures and inducing angiogenesis *in vivo* (Bernardini *et al*., 2000).

With the change of the structure of the biomaterials, administration of a variety of growth factors, vasoactive peptides, and certain kind of receptor ligands of the receptors, angiogenic effects were acquired in the biomaterials various aspects of angiogenesis were addressed. It may be speculated that by immobilization of heparin, - several angiogenic growth factors, such as bFGF, VEGF have a high affinity towards heparin -within the collagen matrices, should be a suitable approach for developing angiogenic biomaterials for tissue engineering. In addition, heparinized collagen matrices can enhance their angiogenic effects *in vivo* and *in vitro* (van Wachem *et al*., 2001; Wissink *et al*., 2000a). Therefore, investigations focussing on binding growth factors to heparinized biomaterials could be a way to search for angiogenic biomaterials. In order to immobilize heparin to collagen matrices, cross-linking agents have to be added first to activate carboxylic acid of heparin (Hep-COOH) with highly active N-hydroxysuccinimidyl esters which subsequently react with NH_2-groups of the collagen.

1.3 Cross-linking of collagen matrices

1.3.1 Introduction

Untreated collagen matrices are biodegradable and can be degraded by enzymes (e.g. collagenase) quickly after implantation. In order to control its degradation rate to fulfill the various demands needed in tissue engineering, many approaches have been developed to cross-link collagen matrices. In general, these approaches can be classified into two kinds of cross-linking methods, namely physical and chemical method. As to the physical treatment of cross-linking, dehydrothermal treatment (DHT) and ultraviolet (UV) irradiation were developed to increase the cross-linking rate of collagen matrices. The primary advantage of such treatments is that they do not introduce chemicals that cause potential harm. The shortcoming is that the collagen matrices modified by physical treatment becomes rigid and shrink in form. Chemical treatment concentrates on creating new additional chemical bonds between the

collagen molecules by reaction with chemical agents. Most of collagen matrices modified with chemical cross-linking treatment can maintain much of the original character of the tissue, such as flexibility and mechanical properties (Khor, 1997). Among the different cross-linking agents, glutaraldehyde has been investigated thoroughly. The application of glutaraldehyde leads to high degrees of cross-linking when compared with other known agents such as formaldehyde, epoxy compounds, cyanamide and acyl-azides (Jayakrishnan and Jameela, 1996; Olde Damink *et al*., 1996). However, cytotoxicity and calcification of glutaraldehyde modified collagen matrices were observed upon *in vivo* implantation (van Luyn *et al*., 1992). Therefore, researchers embarked on attempts to replace glutaraldehyde as a cross-linking agent. Alternatively the water soluble carbodiimide is known to activate the carboxylic acid groups of collagen, followed by a reaction with an adjacent amino group. The reaction products of the cross-linking agent may be washed out and will no longer remain inside the collagen matrices. For these reasons I prefer to apply carbodiimide as the cross-linking agent in our investigation.

1.3.2 EDC/NHS as a cross-linking agent

The water soluble agent N-(3-Dimethylaminopropyl)-N'-Ethylcarbodiimide (EDC) offers a method based on the concept of cross-linking by activation of carboxylic acid groups to generate cross-links between carboxyl and amine groups, without being incorporated (Nimni *et al*., 1987). Addition of the nucleophile N-hydroxysuccini-mide (NHS) to the EDC solution during cross-linking is based on the suppression of two side reactions which produce hydrolysis and N-acyl shift (Olde Damink *et al*., 1996). The general reaction scheme of EDC cross-linking collagen is as follows (see Figure 2): 1. activation of the carboxylic acid groups of Asp or Glu residues by EDC to give O-acylisourea groups. 2. conversion of the O- acylisourea groups into a NHS activated carboxylic acid group. 3. yielding a so-called zero length cross-link between activated carboxylic acid groups and amine groups of (hydroxy)lysine (Grabarek and Gergely, 1990). Therefore, EDC/NHS does not remain as part of the cross-link in the matrix which otherwise can possible release of potential cytotoxic agent, such as glutaraldehyde (GA). The by-products during cross linking of EDC/NHS (EDC-urea) are water soluble and can be easily removed by rinsing (Gratzer and Lee, 2001) (Figure 2).

After cross-linking with EDC/NHS, collagen matrices in general have a higher shrinkage temperature as well as an increased resistance towards proteolytic enzymes as compared to GA. Furthermore, cross-linked collagen shows a lower tendency to calcify and a better biocompatibility (van Wachem *et al*., 1994a; van Wachem *et al*., 1994b). In addition, it was also shown that this modification promotes the proliferation of HUVECs *in vitro* (Wissink *et al*., 2000c). Finally, EDC/NHS cross-linked collagen matrices absorb more water than non-cross-linked matrices, which is beneficial to cell migration (Park *et al*., 2002).

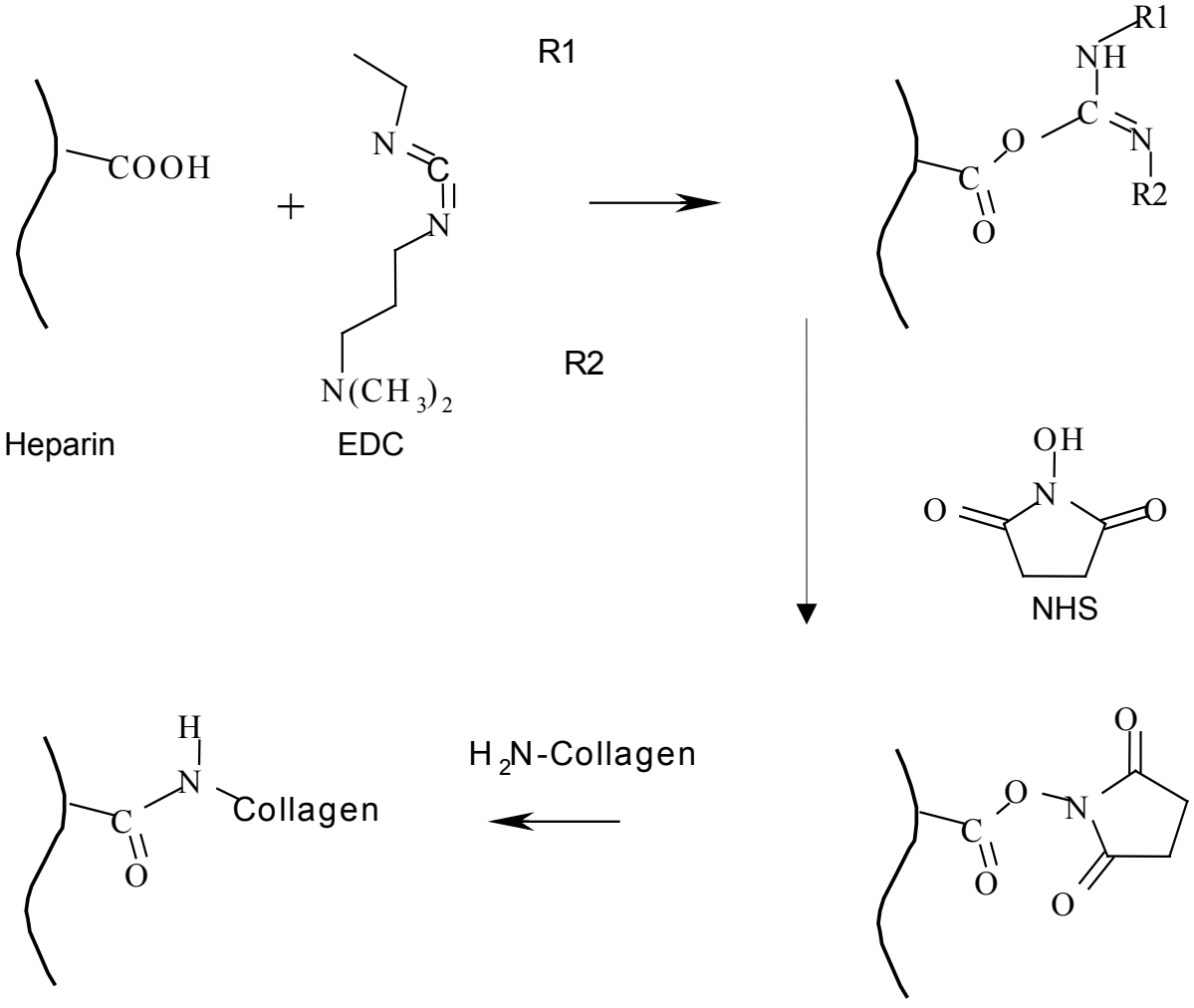


Figure 2 Sequence of reactions in the cross-linking of heparin to collagen

It was reported that EDC/NHS is stable at pH 7.0, while it is considerably more reactive at pH 5.0 (Gilles *et al*., 1990). When used for cross-linking of collagen, it was observed that the optimal pH of the reaction solution is 5.5 (Olde Damink *et al*., 1996). Also heparin has carboxylic groups (Hep-COOH), which enables EDC/NHS to cross-link heparin to the amine functions of collagen.

1.4 Immobilization of heparin to collagen matrices

1.4.1 Structure and general functions of heparin

Heparin is a biopolymer belonging to the class of mucopolysaccharides (glycosaminoglycans, GAGs). It is widely distributed in animal tissues and well known for its anticoagulant effect. More than 70% of the structure of “conventional” heparin can be accounted for by repeating disaccharide units consisting of 1.4-linked L-iduronic acid and D-glucosamine. The iduronic acid residues are O-sulfated at position 2, and glucosamine residues are N-sulfated at position 6, which was unequivocally confirmed by nuclear magnetic resonance (NMR) spectroscopy (Perlin, 1977). At physiological pH, the carboxyl and sulfate groups of heparin are ionized. Therefore, heparin is a strong polyelectrolyte and can easily interact with basic sites of proteins. Some plasma proteins which normally occur in low concentration were shown to be retained on heparin affinity columns with high specificity. The well-known time-honoured antithrombotic effect of heparin may be explained by binding to antithrombin via a specific high affinity pentasaccharide sequence that is only present in a minor portion of heparin chains. Binding of the pentasaccharide to antithrombin causes a conformational change of antithrombin that accelerates by a factor of approximately one thousand its intensity with thrombin and factor Xa (factor X) (Hirsh *et al*., 1998).

Additional to these functions, recently an independent function has been recognized, which is the involvement of heparin in the regulation of angiogenesis (see Table 1) (Bombardini and Picano, 1997). Since then, much evidence has been accumulated pointing to an important role of heparin in the control of angiogenesis.

Table 1

Classical antithrombotic *versus* novel angiogenic effects of heparin

Therapeutic Role	Antithrombotic (Established)	Angiogenic (Hypothetical)
Molecular target	Factor Xa, Factor IIa	Heparin-binding growth factor
Cellular target	None	Endothelial and smooth muscle cell
Therapeutic effect	Antithrombotic	Angiogenic
Molecular cofactor	Antihrombin III	Adenosine
Timing of effect	Minutes to hours	Weeks to months
First demonstration	1916 Mclean	1981 Folkman

1.4.2 Immobilization of heparin to collagen matrices by using the cross-linking agent EDC/NHS

Heparin can be covalently immobilized to collagen matrices by activating the carboxylic acid groups by using water-soluble carbodiimide EDC and NHS. After the activation of carboxyl groups, heparin immobilizes to collagen matrices by a covalent linkage between the carboxyl groups of heparin and the free amino groups of collagen (Nimni, 1983). Since there are many carboxyl groups in heparin, the immobilisation of heparin on collagen matrices most likely occurs through a multi-point attachment. Therefore, after immobilization, heparin will be released upon degradation of the collagen matrices. It was reported that the optimal reaction buffer for activating the heparin carboxyl groups is 2-morpholinoethane sulfonic acid (MES), at a pH of 5.6. The reaction time is found for about 5 to 30 minutes. The longer time will possibly lead to hydrolysis of EDC/NHS activated carboxyl groups, which results in decreased amounts of immobilized heparin (Wissink *et al*., 2001b). After the activation of the heparin carboxyl groups, collagen biomaterials should be added for immobilizing the heparin to the collagen. Afterwards, the modified collagen must be extensively washed with sodium chloride and water to remove the reaction products of EDC/NHS and non -immobilized heparin (Wissink *et al*., 2001b).

Immobilization of heparin in biomaterials has been applied to enhance the thromboresistance of artificial surface exposed to blood or plasma for many years. Examples of heparin immobilized biomaterials are artificial vessels, heart valves and catheters. These devices are not biodegradable in the body (Kang *et al*., 1996). The applications of immobilization of heparin to biodegradable biomaterials were most on drug delivery systems (Ahola *et al*., 2001; Yang *et al*., 1999), which can be coated on vascular prostheses to promote the blood compatibility (Bos *et al*., 1999a).

Collagen is one of the most widely used biomaterials applied for tissue engineering. There are a number of investigations focussing on the immobilization of heparin into collagen matrices, because of the latter being a highly thrombogenic material which introduces platelet adhesion and aggregation as well as activation of intrinsic blood coagulation. It was found that immobilization of heparin can reduce platelet adhesion and aggregation as well as inhibit contact activation (Senatore *et al*., 1990). Since endothelial cells perform a key regulatory role in hemostasis, some studies have been focused on the effects of heparinized materials on endothelial cell proliferation. The results were not always consistent, some report on an inhibitory effect (Nojiri *et al*., 1987) whereas other reports favour the (Bos *et al*., 1998) proliferative effect on endothelial cells. The immobilization of heparin into collagen films which were previously cross-linked lead to a slight inhibition of the endothelial cell proliferation as compared to collagen matrices which were cross-linked only (Wissink *et al*., 2001b). The above observations can be ascribed to the proliferative effects of immobilized heparin *in vitro*. It is of interest that when heparinized collagen matrices are applied *in vivo*, most reports conclude that they slightly promote vascularization around the implant sites (van Wachem *et al*., 2001) and that they improve wound healing (Kratz *et al*., 1998)

Recently a number of angiogenic growth factors, such as aFGF, bFGF, VEGF have been found to have a high affinity towards heparin. Therefore the loading of these growth factors to heparinized matrices which can protect them against denaturation and allow for a sustained release *in vitro* and *in vivo*, has received much attention (Bos *et al*., 1999b; Doi and Matsuda, 1997; van Wachem *et al*., 2001). Results so far show that heparinized matrices loaded with bFGF promote the proliferation of endothelial cells *in vitro* and increase vascularization *in vivo* (Wissink *et al*., 2001b).

In summary, when heparin is immobilized with biomaterials, it still has the effect of an anticoagulant (Senatore *et al*., 1990). Although the results about its proliferative effects on endothelial cells *in vitro* are not consistent, it seems that the heparinization may enhance vascularization *in vivo* (van Wachem *et al*., 2001). Further, when loaded with angiogenic growth factors, results show that this loading promotes angiogenesis substantially both *in vitro* and *in vivo*. Therefore, cross-linking agents such as EDC/NHS may be applied for covalent binding of heparin into matrices in order to realize a sustained release, which is governed by the degradation of such modified matrices.

1.5 Angiogenic Growth Factors

1.5.1 Introduction

Growth factors are a family of proteins that act on cells in a hormone-like fashion to regulate cell activity. They attach to specific cell membrane receptors and direct the cells intercellular activity. They regulate cell migration, proliferation, matrix synthesis, and remolding. Among these growth factors, there is a family of growth factors which are angiogenic they enhance vascularization and angiogenesis *in vitro* and *in vivo*. Though there are quite some growth factors found to have angiogenic effects, only the basic Fibroblast Growth Factor (bFGF), the Vascular Endothelial Growth Factor (VEGF), the Platelet-Derived Growth Factor (PDGF), and the Transforming Growth Factor ß (TGF-ß) are being investigated intensively.

bFGF belongs to the family of heparin-binding growth factors that has been widely researched to have the ability of vascular tube formation (Abraham *et al*., 1986), enhancing vascular endothelial cells proliferation *in vitro* and increasing the number of blood vessels *in vivo* (Pieper *et al*., 2000b). VEGF, a mitogen specific to endothelial cells *in vitro* and a potent stimulator of angiogenesis *in vivo* (Peters *et al*., 1998), also has a high affinity towards heparin and will be applied in this study. Therefore I will introduce its properties in the next paragraph.

1.5.2 Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is a 34- to 42- kD homodimeric glycoprotein (Senger *et al*., 1993). It has at least three naturally occurring splice variants: $VEGF_{189}$, $VEGF_{165}$, $VEGF_{121}$. The three vary in their affinity for heparin, with $VEGF_{121}$ displaying a minimal, $VEGF_{165}$ an intermediate and $VEGF_{189}$ a strong heparin affinity. Two so-called tyrosine kinase receptors have shown to function as high affinity receptors for VEGF, they are the 180 kd fas-like tyrosine kinase (flt-1 or VEGFR-3) and the 200 kd kinase inset domain-containing receptor (KDR or VEGFR-2). Of the two tyrosine kinase VEGF receptors, KDR appears to be the major transducer of the VEGF signal in the endothelial cells (Klagsbrun and D'Amore, 1996; Shen *et al*., 1998). Upon binding to these receptors, VEGF can play a central role in the regulation of vasculogenesis and angiogenesis.

Studies show that VEGF is a specific mitogen for endothelial cells derived from small or large vessels (Ferrara and Alitalo, 1999) and is the only growth factor known to act solely on endothelial cells (Neufeld *et al*., 1999). VEGF has been shown to be able to promote the vascular proliferation, migration, and tube formation *in vitro* and not only increase the number of blood vessels, but also increase their permeability *in vivo* (Clauss *et al*., 1990).

We speculate that by using VEGF's high affinity to heparin, it may be possible to bind the VEGF to heparin immobilised within the collagen matrix. After physical binding of VEGF to heparin, VEGF may be protected in a thermo-stable and protease-resistant form and a sustained release of VEGF could go along with the degradation of the collagen matrix. Therefore, the angiogenic potential of modified collagen matrix may be enhanced by incorporation of VEGF for obtaining a sustained release *in vitro* and *in vivo*.

1.6 Incorporation of VEGF into collagen matrices

1.6.1 Introduction

To overcome the problem of rapid diffusion and clearance from the implant site and also to increase stability after implantation, VEGF may be bound to collagen matrices

by means of several methods. To our knowledge, at least three approaches of immobilizing VEGF to collagen matrices have been investigated to date. One approach is to covalently bind VEGF to collagen matrices with a homobifunctional cross-linking agent e.g. bis-(succinimidylsuccinate)-polyethyleneglycol (SS-PEG-SS). The method has been applied on binding VEGF to collagen matrices in our laboratory before and it can successfully covalently bind VEGF to collagen with sustained release *in vitro* (Chen, 2001; Chen *et al*., 2002). A second approach is to incorporate VEGF into biodegradable microspheres obtained with the emulsion/solvent extraction technique, subsequently these spheres are homogeneously mixed in the collagen suspension and lyophilized (Peters *et al*., 1998). These two approaches for incorporation of VEGF could be defined as chemical method because of the cross-linking and the encapsulate agents are inside the matrices which may have some influence after implantation. A third approach is to incorporate VEGF into collagen matrices by physical binding to heparin which was immobilized inside before. In this approach the growth factors will not be immobilized covalently. I will apply this approach in our study.

1.6.2 Physical binding of VEGF to heparinized collagen matrices

As mentioned before, VEGF is a angiogenic growth factor with a high affinity towards heparins (Shing *et al*., 1984). Therefore, it might be possible to preserve VEGF inside collagen matrices as a reservoir immobilized to heparin covalently linked to the collagen. Another advantage of this approach is that after binding to heparin, the binding of VEGF to receptors on vascular endothelial cells may be potentiated by prevention of the inactivation of VEGF by α-2 macroglobin (Soker *et al*., 1993).

There are reports demonstrating that bFGF binds to heparinized collagen films (Wissink *et al*., 2001b; Wissink *et al*., 2000a). In this study the collagen film was first cross-linked with EDC/NHS. The heparin was subsequently immobilised in the cross-linked collagen films. Thereafter, the heparinized collagen films were immersed in different concentrations of bFGF solutions and incubated for 90 min at room temperature. The results show that as compared with non-heparinized cross-linked collagen films, the amount of bFGF binding in heparinized collagen film is much higher and the release is sustained at a relative low rate. In addition, the bioacitivity of bFGF

in the heparinized collagen film was - as evaluated by the vascular endothelial cell proliferation assay - better than that of bFGF simply admixed to non-heparinized collagen. Their experiment results encouraged us that it may be possible to another growth factor with a high affinity towards heparin i.e. VEGF, the action of which is restricted to vascular endothelial cells (Ferrara *et al*., 1992). To our knowledge, no such investigations have been reported to date.

1.7 Methods to evaluate the characteristics of modified collagen matrices

1.7.1 Quantification of immobilized heparin

For the determination of the amounts of immobilized heparin I made use of the procedures as published in 1996 (Walsh *et al*., 1996). The assay is based on the capacity of heparin to complex with toluidine blue and can be solubilized in a mixture solution of NaOH and ethanol. By measurement of the absorbance of the solution at 530 nm using a spectrophotometer, the amount of immobilized heparin can be calculated from a calibration curve obtained with the toluidine blue solution assay (van Wachem *et al*., 2001). Indeed, an alternative method to evaluate the amount of immobilized radio active labeled heparin (^{3}H-Heparin) has been applied (Walsh *et al*., 1996). This method can determine very low amounts of heparin in solution and is, however, usually applied for the determination of heparin release from heparinized biomaterials.

1.7.2 Moisture uptake

Collagen molecules are known to bind a large amount of water. Cell adhesion and blood coagulation are two fundamental events that can occur on the surface of implant materials (Jansen *et al*., 1989). Cell adhesion is required for incorporation of implants into tissues, while thrombogenicity is less desirable. Unfortunately, collagen is a thrombogenic substrate (Silverstein and Chvapil, 1981). The adhesion of anchorage-dependent cells and the induction of a thrombosis depend on numerous factors, among which, the equilibrium aqueous content seems to be the most important (Lydon *et al*., 1985). Different cross-linking treatments can modify the surface of collagenous

materials and thus change their thrombogenicity (Cote and Doillon, 1992). It has been reported that heparinization and cross-linking of collagenous materials promote the moisture uptake, reduce the adhesion of platelets and increase albumin absorbance (Chuang *et al*., 2001).

There are two methods to test the moisture uptake of modified materials. One is aqueous contact which was measured by using a goniometer and surface tension which was developed by Kaelble and Moacanin (Yang *et al*., 1997). The other is by measurement of the different weights before and after the materials being immersed into an aqueous phase and after having reached equilibrium (Gao *et al*., 2002). Generally, the results are in agreement with each other.

1.7.3 Determination of the *in vitro* degradation

Collagen based materials is biodegradable into body because of varies enzymes will cleave the peptide bond within the triple helical of collagen. The stability of collagen materials towards enzymatic degradation can be improved by cross-linking (Lee *et al*., 1996). Through evaluation of their degradation degree by enzymes, the cross-linking degree of collagen materials can be detected. Several enzymes may be used for evaluating the degradation behaviour of modified collagen matrices. They are the bacterial collagenase, cathopsin and trypsin (Zeeman, 1998b).

The bacterial collagenase isolated from *Clostridium histolyticum* is capable of cleaving peptide bonds within the triple helical structure of collagen and has a specificity for Pro-X-Gly-Y sequences, cleaving between X and Gly. X and Y are predominantly apolar amino acid residues (Harper *et al*., 1972). The molecular weight of collagenase is between 68 and 125KD. In a first approximation of a spherical shape, the radius of gyration will be between 2.8 and 3.9 nm, which implies that collagenase probably will start at surface of fibrils (Olde Damink *et al*., 1996). Investigations so far have shown that collagenase initially absorbs onto fibers of collagen and during degradation, collagenase can penetrate into the fiber. After being treated by EDC/NHS, cross-links are formed among polypeptide chains of the collagen. They hinder penetration of collagenase into fiber, which results in decreasing the degradation by collagenase.

From the above, it may be concluded that the evaluation of the degradation behaviour of modified collagen matrices by collagenase will allow for a prognosis for the resistance against the enzyme attack after implantation in vivo. This prognosis may be meaningful for controlling the degree of biodegradation of engineered tissues.

1.7.4 Determination of free amino groups

Collagenous materials are proteinaceous materials whose lysine is one of the essential amino acids. Several chemical cross-linking agents, such as GA, EDC/NHS , modify the structure of collagen by the reaction between carboxylic acid groups and ε-amino groups. Heparin immobilized in the collagenous materials by EDC/NHS is through activation of carboxylic acid groups of heparin and then react with ε-amino groups of collagen. Therefore, by determination of the number of ε-amino groups in the modified collagenous materials, the cross-linking degree can be evaluated.

A method which is often applied is the quantification of the ε-amino groups in the collagenous materials is by using trinitrobenzenesulfonic acid (TNBS) (Cayot and Tainturier, 1997). TNBS, as a UV-chromophore, reacts with primary amino groups of proteins at an alkaline pH to form a trinitrophenyl derivative and a sulfate ion. By measurement of the yellow absorbance of the trinitrophenyl derivation, the number of ε-amino groups in protein can be calculated. It needs to be pointed out that an extraction step should be introduced to remove both the excess unreacted TNBS and TNP-α-amino derivatives (Kakade and Liener, 1969).

1.8 Evaluation of the angiogenic potential of modified matrices

1.8.1 *Chorioallantoic* Membrane (CAM) Assay

The *chorioallantoic* membrane (CAM) of the chicken embryo, which serves as the embryonic respiratory organ (Billett *et al*., 1965), is a highly vascularized membrane which lines inside the surface of egg shell and is relatively thin and transparent. Its two dimensional vascular structure can be seen entirely with minimal preparation. This is one of the major reasons it has become a popular assay tissue for putative angiogenic and antiangiogenic substances (Weis *et al*., 1991). In addition, the CAM

assay is relatively cheap, easy to operate and has less ethical concerns as compared to other *in vivo* angiogenesis assays .

However, the methods to quantify the extent of the angiogenesis or vascularization in the *chorioallantoic* membrane are somewhat troublesome. They have been many efforts developed at quantifying the number. For example, one method is to estimate the DNA and protein synthesis in the *chorioallantoic* membrane for the reason of the more neovascularization, the more DNA and protein synthesis in the *chorioallantoic* membrane (Thompson *et al*., 1985). The other method is morphometric measurement of microvessels of the *chorioallantoic* membrane by counting the number of "vessel endpoints " with or without the assistance of a computerized image analysis system (Neufeld *et al*., 1999). Later, a fractal analysis was applied on the method of morphometric measurement which was reported to be more accurate, reproducible and objective since the morphometric form of the vessels in the *chorioallantoic* membrane poses hierarchical branching patterns (Kirchner *et al*., 1996).

Many angiogenic factors have been investigated by using the CAM assay, they include bFGF and VEGF. The results show that bFGF stimulates angiogenesis associated with hyperplasia of chorion and fibroblast cell proliferation and its antibodies inhibit such effects (Ribatti *et al*., 1997). In general, VEGF has the same effects as that of bFGF. In addition, VEGF transfectants were found to be able to induce neovasculature with open junctions and a fenestrated endothelium (Ribatti *et al*., 2001). This means that VEGF may have a strong angiogenic potential *in vivo* in this model.

However, to my knowledge, up to date, there is no report investigating the angiogenic potential of modified collagen matrices with a sustained release of VEGF by applying the CAM assay. I think it should be of prime interest to evaluate the angiogenic potential by this method.

1.8.2 Animal experiments

Although the CAM assay has some advantages in the evaluation of the angiogenic potential of biomaterials *in vivo*, there are still several limitations by using the assay. For example, the time of this assay is limited to 21 days because of the development

of the embryo. The total volume of blood inside the *chorioallantoic* membrane of embryonated eggs is relatively small as compared to that of animals because of the limited weight of the embryos. Moreover it is rather difficult to quantify the hemoglobin content and blood flow inside implants. Therefore, the CAM assay cannot completely replace the animal experiments to date.

Several animal model experiments have been developed to evaluate the angiogenic potential of biomaterials *in vivo*. They include the rabbit corneal model (Gimbrone *et al*., 1974), the hamster cheek pouch model and the rodent subcutaneous model (Andrade *et al*., 1987). Among them, the rodent subcutaneous model is the most widely applied. With the help of recent subcutaneous model experiments, the morphometric neovasculature analysis (Mayhew and Sharma, 1984), the quantification of hemoglobin content of angiogenesis (Teixeira and Andrade, 1999) and the measurements of the pharmacological reactivity of the neovasculature in the vascularized implants (Andrade *et al*., 1997) have been successfully applied. Among these, I think that the hemoglobin content inside implants may represent the amount of blood flow inside such implants to supply the nutrients for cell survival. Therefore, the hemoglobin content of the explants may be an important parameter for evaluating the angiogenic potential of implants *in vivo*.

1.9 Aim of the thesis

In the present thesis, I embark on taking collagen as the starting material for developing a tissue substitute. The biodegradable collagen matrix, which is characterised by a porous and fibrillar structure, is - since its properties meet many of the required criteria - one of the most widely used materials for tissue engineering (Boyce *et al*., 1988; Doillon *et al*., 1994a).

However, as mentioned before, one of the primary factors that challenge the prospects of tissue engineering is the insufficient supply of blood and nutrients in the engineered tissue after implantation into the body. This supply is necessary for the living cells to grow and survive. A variety of angiogenic growth factors have been discovered recently, which induce vascular endothelial cell proliferation and migration, as well as vascularization. Among them, vascular endothelial growth factor (VEGF) is specific for

endothelial cells and a potent stimulator of angiogenesis *in vivo* (Peters *et al*., 1998; Plate and Warnke, 1997). Therefore, the incorporation of angiogenic growth of VEGF represents a promising approach to enhance the angiogenic potential of engineered tissue substitutes. Thus, VEGF may stimulate endogenous vascular endothelial cells to proliferate and migrate into the tissue substitute, and finally form blood vessels.

It is known that the *in vivo* half life of VEGF is very short (Veikkola *et al*., 2001) since it can be denatured easily. Therefore, the realization of collagen matrices which bind, protect and release angiogenic growth factors, such as VEGF, in a therapeutic manner should be one of the major goals on our way to develop a tissue substitute with enhanced angiogenic capabilities.

It has been reported, that the angiogenic growth factors bFGF and VEGF have high affinities for heparin and that they become more stable and more active after binding to the heparin (Gospodarowicz and Cheng, 1986; Sommer and Rifkin, 1989).

It seemed therefore desirable to develop collagen matrices with different amounts of immobilised heparin using the cross-linking agent EDC/NHS. By applying different EDC/NHS to heparin ratios one expects to obtain collagen matrices with different amounts of immobilized heparin and different resistances towards degradation. I speculated that the release of VEGF from these differently modified collagen matrices depends on their amounts of immobilized heparin and their degradation behavior.

Thus, the aim of the presented work was to develop collagen matrices which differ in the amounts of immobilized heparin and in the *in vitro* degradation behaviors. In order to evaluate the biochemical properties, the number of primary free amino groups and the moisture uptake was investigated. Finally, the angiogenic potential of these modified matrices was investigated by exposure to the *chorioallantoic* membrane of the chicken embryo (CAM assay) and to subcutaneous tissue in animal model experiments.

In overview, the main objectives of this study are:

1 Modification of collagen matrices by cross-linking with appropiate cross-linking agents.

2 Incorporation of heparin into collagen matrices for providing binding sites for immobilizing the angiogenic factor VEGF.

3 Chemical and physical characterization of the modified collagen matrices.

4 Evaluation of the angiogenic potential of the modified collagen matrices with the chorioallantoic membrane of the chicken embryo.

5 Evaluation of the angiogenic potential of the modified collagen matrices by subcutaneous implantation in rats.

2 Materials and Methods

2.1 Collagen Matrices

Collagen matrices were prepared according to a freeze-drying procedure to achieve matrices with a non-directed porous microstructure. The basic material is a collagen suspension containing 1.8 wt.-% of type 1 collagen isolated from bovine skin (Dr. Suwelack Skin & Health Care AG, Billerbeck, Germany) in acetic acid (pH 2,8) (Matristypt: Kollagen-Matrix, Ch: 226911). For the cell culture, CAM assay and animal model experiments, the matrices were sterilized with 70% ethanol. Briefly, the (non-) modified matrices were immersed into 70% ethanol for 24 h. Then under sterilized condition, the samples were transferred into 0.9% NaCl (24 h) and cell culture medium (24 h).

2.2 Modification Procedures

The collagen matrices were cut into cubes of either 5*5*5 mm (3.8–4.2 mg) for determination of the modification parameters, or into dics of 10 mm of diameter, 2 mm and 5 mm thick (3.8– 4.2 mg and 11.5-12.5 mg rep.) for determination of their angiogenic potential with CAM-assays and animal model experiments respectively.

The modification procedure was in principle performed as described by Wissink et al (Wissink *et al*., 2001b). The main difference is that the procedure of Wissink et al. is described for collagen films and that in their procedure the collagen matrices were in all cases cross-linked with EDC/NHS prior to the cross-linking of the heparin.

Then carboxylic acid groups of heparin (Hep-COOH) were activated with EDC/NHS, whose weight ratio was fixed at 1 : 0.6. Defined amounts of EDC/NHS were added to a solution of heparin at various weight ratios. The EDC to heparin ratio was varied form 0.2 to 4.

The modification parameter H1E1 refers to 1 mg heparin and 1 mg EDC/0.6 mg NHS per 500 µl reaction mixture, H4E3 refers to 4 mg heparin and 3 mg EDC/1.8 mg NHS

and H0E0 refers to non-modified collagen. Heparin (sodium salt, 170 USP units/mg), EDC and NHS were purchased from Sigma-Aldrich.

Heparin is activated with EDC/NHS for 10 minutes at 37°C in 0,05 M buffer of 2 – morpholinoethane sulfonic acid (MES buffer, pH 5.6) (K 28866628 131, Merck KgaA, 64271 Germany). After activation, collagen matrices are - under gentle shaking - immersed into the reaction mixture and incubated for 4 h at 37°C. After this reaction, collagen matrices are intensively washed with 0.1 M $Na_2H_2PO_4$ (2 h), 4 M NaCl (4 times in 24 h) and distilled water (5 times in 24 h). Thereafter, the modified collagen matrices are frozen at –80°C overnight and then lyophilized. After lyophilization, the collagen matrices are stored at room temperature until use.

2.3 Determination of immobilized heparin in collagen matrices

The amount the heparin immobilised in the modified collagen matrices is determined by using the toluidine blue assay (Hinrichs *et al*., 1997). Cubic collagen matrices are incubated with 5 ml aqueous solution of toluidine blue (0.1 M HCl, 2 mg/ml NaCl, 0.4 mg/ml toluidine blue zinc chloride double salt (Sigma)) for 4 h at room temperature, resulting in complexation of toluidine blue with heparin. Afterwards, the collagen matrices are washed with distilled water (10 ml per sample) 5 times overnight. Subsequently, toluidine blue complexed to heparin of one sample is solubilized with 5 ml of a 1:4 (v/v) mixture of 0.1 M NaOH and ethanol. The absorbance of the resulting solution is determined at 530 nm after 1:5 dilution with the sodiumhydroxide/ethanol solution. Standard curves were obtained with the heparin solution assay (Hinrichs *et al*., 1997) which is described briefly as follows.

0, 0.1, 0.2, 0.4, 0.8, 1.0, 1.2 mg of heparin are dissolved in 2 ml aqueous of 0.1N HCl/0.2% NaCl respectively. Then add 2 ml of 0,04 wt% toluidine blue to each of the samples and allow to react for 4h at room temperature under gentle shaking, this reaction results in complexation of toluidine blue with heparin leading to a precipitating, non-soluble complex. Afterwards, the samples are centrifuged at 3000 g for 10 minutes and then the supernatant is decanted. The precipitate is rinsed with aqueous 0.1N HCl/0.2% NaCl for three times and centrifuged after each time of rinse.

Thereafter, the precipitate is dissolved with 5 ml of 4:1 (v/v) solution of ethanol to 0.1M NaOH. The absorbance of the resulting solution is read at 530 nm after 1:10 dilution with the sodiumhyroxide/ethanol solution.

2.4 *In vitro* Degradation

The degradation of collagen matrices is evaluated through the difference of their weights before and after the *in vitro* degradation by the bacterial collagenase (*Clostridium histolyticum* type 1, 232 u/mg, CLS 1, Worthington Biochemical Corporation) at different concentrations for a defined period of time. First, the weight of the modified collagen matrices (size: 5*5*5 mm) are determined immediately after lyophilization. Then each of such samples are immersed into 500 µl of the concentrations of either 40 units or 200 units collagenase /ml and incubated at 37°C under gentle shaking for variable periods of time. The degradation is terminated by adding 20 µl of 0.25 M ethylenediamine-tetraacetate (EDTA) (pH 6.7) (Sigma) and cooling on ice. After 10 minutes, the solution of EDTA is decanted and washed with 10 ml of phosphate buffer solution (PBS) (pH 7.2) per sample 3 times for 30 minutes, followed with 10 ml of distilled water 3 times for 30 minutes. After the washing procedure, the samples are frozen and lyophilized again. After lyophilization, the remaining weight of each sample is determined by the same electronic balance. The weight loss of collagen matrices, expressed as the percentage of initial weight remaining which represent the degradation percentage, calculated as the formula as below:

$$\text{Degradation (\%)} = (W_i - W_d) / W_i * 100$$

W_i represents the original weight of each sample, W_d represents the weight of the corresponding sample after the degradation in the presence of collagenase.

2.5 Determination of free amino groups

The primary amino group content (lysine and hydroxylysine residues) of modified collagen matrices is determined using 2,4,6–trinitrobenzenesulfonic acid (TNBS). To a modified collagen sample, 1.0 ml of 4 wt% $NaHCO_3$ solution (pH 9.0) and 1.0 ml of a freshly prepared 0.5 wt% TNBS (P-2297, Sigma-Aldrich) solution in distilled water is added. After reaction overnight at 40°C, 3.0 ml of 6 M HCl is added and the temperature is raised to 60°C for 90 minutes to solubilize the collagen matrices. The blank is prepared by the procedure except for the HCl which is added before the addition of TNBS to prohibit any reaction of TNBS with the protein. The resulting solution is extracted with 20 ml portions of diethylether (Merck Schuchardt, 85662 Hohenbrunn, Germany) for five times to remove excess untreated TNBS and TNP-α-amino groups, followed by evaporating the residual ether in a hot water bath for 15 minutes. Thereafter, the 1 ml of the aliquots is diluted with 9 ml of distilled water and the absorbance is measured at 345 nm with a spectrophotometer. The number of free amino groups per 1000 amino acids in collagen matrices is calculated by the formula below:

$$\text{Free amino groups} = (\text{Absorbance at 345 nm}) * (0.05 \text{ liters}) (MW) / (1.46 * 10000 \text{ liters/mole .cm}) * (b) * (x)$$

Where MW is the molecular mass of the collagen in g/mol, 1.46 ∗ 10000 liter/mol.cm is the molar absorptivity of TNP-lysine, b is the cell path length in cm, and x is the sample weight in gram.

2.6 Determination of the moisture uptake

The moisture uptake of the modified collagen matrices are determined by the measurement of their ability to keep the amount of liquid. First, the initial weight each sample using for moisture determination is determined with a electronic balance immediately after lyophilization, followed by immersion in 1 ml of PBS buffer (pH 7.2) at 37°C and incubation for 2 h. Subsequently the samples are taken out and exposed to a piece of filter paper until no water was soaked out of the matrix any more. Then the weight of each sample is determined. The moisture uptake of each sample is calculated with the formula:

$$\text{Moisture uptake} = (W_{wet} - W_{dry}) / W_{dry}$$

W_{wet} represents the sample weight after moisture uptake and exposure to the tissue paper. W_{dry} denotes the weight of the lyophilized specimen before uptake of the moisture.

2.7 Evaluation of the angiogenic potential of the modified collagen matrices with the *chorioallantoic* membrane assay (CAM)

The angiogenic potential of the modified collagen matrices is determined with *chorioallantoic* membrane assay (CAM). The eggs for the experiment are cleaned and disinfected with 70% ethanol, followed by incubation at 37°C for 7 days. Afterwards, the fertilized eggs are selected for further experiments. First, one hole is drilled on the larger end of egg with the membrane beneath shell being thrusted broken, followed by drilling another hole on top of horizontally placed egg in opposite to the embryo with the membrane intact. Subsequently, a small amount of ice is put on the top hole for 5 min, followed by ethanol disinfecting and thrusting the membrane beneath the top hole. Thereafter, a triangular window is opened by using an electronic drill knife without damaging the *chorioallantoic* membrane. The window should be immediately covered with a piece of parafilm to prevent dehydration and contamination. Next, the sterilized modified collagen matrices were placed on the CAM in laminar flow. Aqueous (100 µl) solutions containing 30 ng or 100 ng of rhVEGF is pipetted inside the sample if necessary, followed by covering the window and taking the eggs out of the laminar flow. Thereafter, sealing tape and another hole of the shell on the larger end of the egg are sealed by using liquid wax. At last, all the eggs are put into an incubation container for another 7 days at 37°C for the angiogenic potential evaluator. Below is the scheme of the CAM experiment:

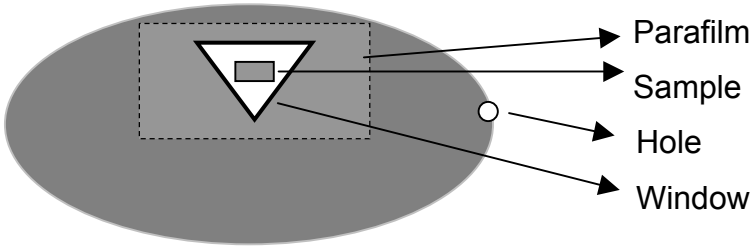


Figure 3 Scheme of the CAM experiment before incubation

After incubation for 7 days, the following steps ensued :

1. The parafilm was remove from the window of each egg and the window was enlarged up to the border of *chorioallantoic* membrane without damaging the membrane. Below is the scheme of this step:

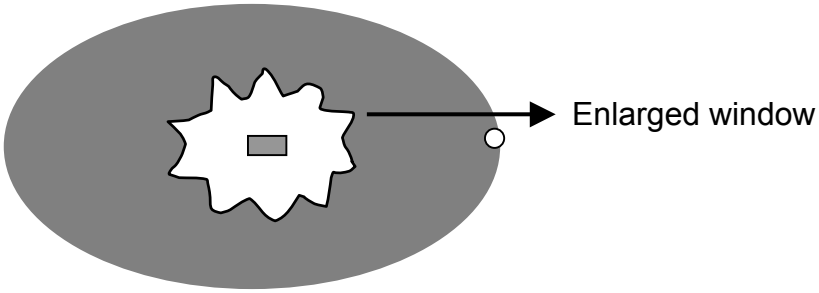


Figure 4 Situation of the *chorioallantoic* membrane experiment with enlarged window after the incubation period

2. Photos were taken of on each enlarged window. The macroscopic changes of each sample in the CAM were recorded, including the death rate of eggs, area reduction of the (non-) modified samples, vessels form beside the samples, the overgrowth granumatous tissue engulfed by the CAM.

3. 3.5% formaldehyde was dropped on the top of CAM under the window for fixing of CAM in situ. After 3 minutes, 3.5% formaldehyde was sucked with a paper and the CAM was dissected near the implant (collagen matrices). The area is about 1.5 cm * 1.5 cm. Then the implant was excised carefully and put into a tube with 3 ml of 3.5% formaldehyde for histological examination.

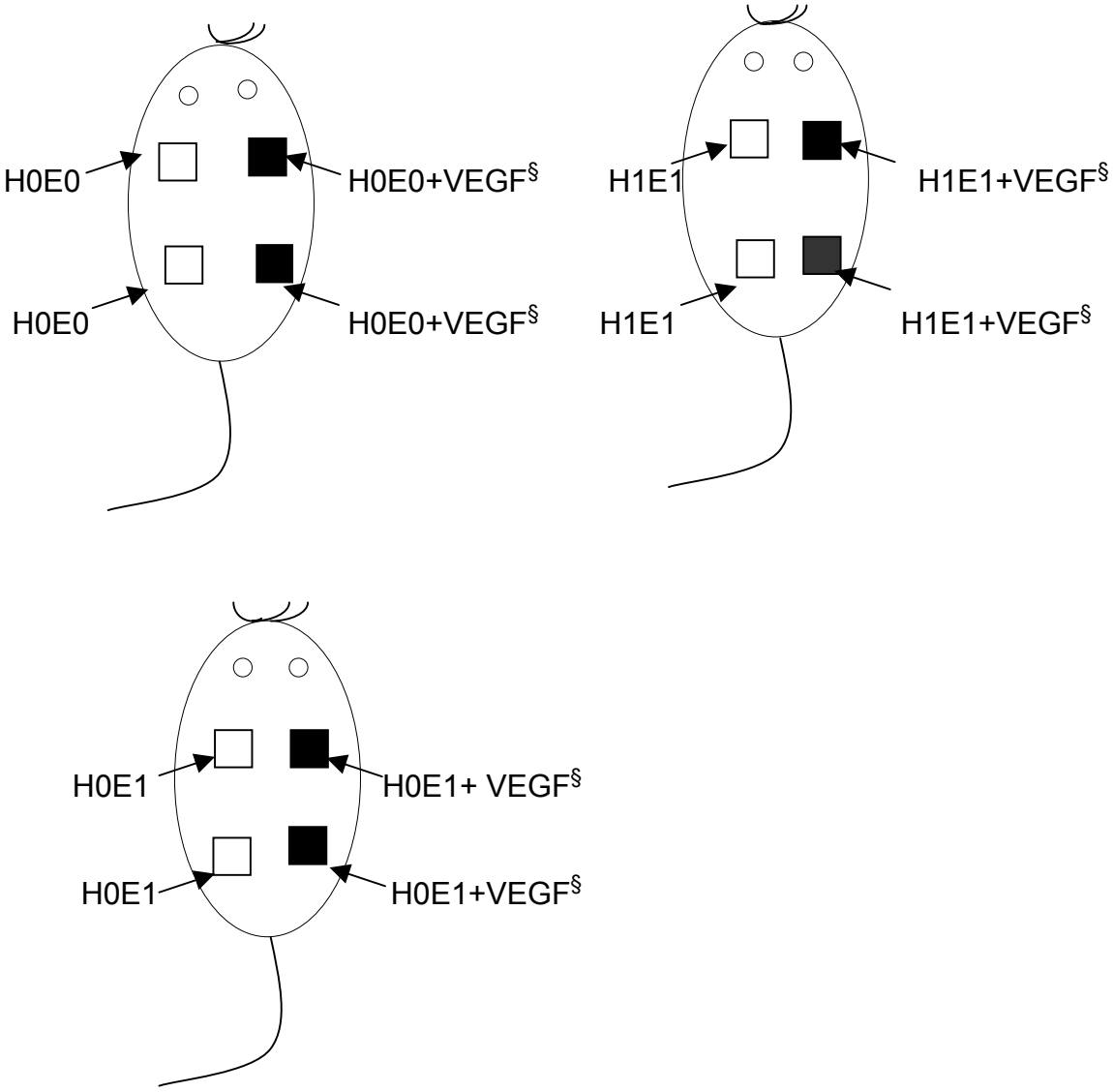
4. The excised CAM was immersed into water in order to obtain a flat excision, then the excised membrane was mounted on a microscope glass slide and fixed with a fixative solution (Hydromount).

5. The number of capillaries was counted with a light microscope equipped with a 121 points grids under 50 times magnification.

2.8 Evaluation of the angiogenic potential by determination of the hemoglobin content of explanted matrices after implantation into rats for 15 days

The collagen matrices of 10 mm in diameter, 5 mm in thickness was made for animal experiments. The samples were first modified with heparin immobilisation, EDC/NHS cross-linking, whose procedure was the same as that of H1E1 and H0E1. Afterwards, the weight of each sample was evaluated immediately after lyophilization. Thereafter, the samples were sterilized with 70% of ethanol (see the procedure of sterilization) before implantation in the Lewis rats. Rats of approximately 300 mg weight were ether anaesthetized. Then the rat's back was shaven and disinfected, four beside midline incisions of 1 cm were created. A total of four pockets were created laterally to the incisions. One sample was placed in each pocket, care was taken to implant the materials completely flat. Before explantation, rats were ether anaesthetized to be sacrificed. Implants were taken out after 15 days implantation. For the determination of hemoglobin, the fibrotic capsules adhering to the samples were excised carefully. Figure 5 shows a schematic representation of the animal experiment.

Hemoglobin determinations were done by spectrophotometer. First the standard curve was made by correlation of different amounts of standard myoglobin (29895, SERVA; Feinbiochemica, Heideberg) which replaces standard myoglobin in our experiment and their maximal OD at around 410 nm. Then the implants were taken out, they were first immersed into 1 ml of distilled water for 5 minutes to wash out the hemoglobin attached to the surface, then the samples were



§: each of modified collagen matrices were loaded with 300 ng of rr-$VEGF_{165}$

Figure 5 Schematic representation of the animal experiments. Implantation site on dorsum of rat.

put into another 1 ml of distilled water for 24 h under gentle shaking to wash out the hemoglobin contained in the samples. Afterwards, these samples were digested completely with 1 ml of 1000 units of collagenase. The absorbances of water and

collagenase solutions of the hemoglobin were determined at 410 nm (Soret band). The absorbances were used for calculation of the hemoglobin contents in microgram per ml with the help of a standard curve obtained with sperm whale myoglobin (29895,SERVA, Feinbiochemica, Heidelberg, Deutschland). For this purpose 1 mg sperm whale myoglobin was dissolved in 1 ml of water, aliquots with 0.05 – 0.3 mg myoglobin per ml were used for obtaining the standard curve.

3 Results

3.1 Immobilisation of heparin

3.1.1 Search for optimal immobilization conditions

The main aim of this thesis was to develop a collagen matrix with enhanced angiogenic capabilities. This goal may be achieved by incorporating angiogenic factors into these matrices. Since the most potent angiogenic growth factor $VEGF_{165}$ disposes of a domain which binds with high affinity to heparin (Tessler *et al*., 1994), Idecided to covalently link heparin to collagen by means of the cross-linking agent EDC/NHS. For this purpose Iadopted the procedure of Wissink et al. (Wissink *et al*., 2001b). In contrast to their procedure I performed the modification in one single step.

In order to be able to evaluate and determine the extent of incorporated heparin Idetermined a calibration curve (Figure 6) by means of the method of Hinrichs et al. (Hinrichs *et al*., 1997)

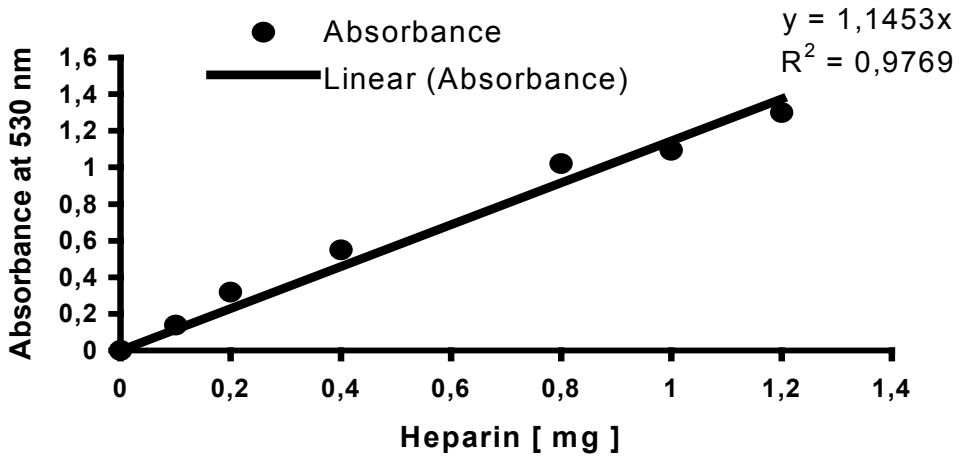


Figure 6 Calibration curve used for the determination of immobilized heparin.

My primary goal was to look after optimal reaction conditions. Optimized reaction conditions should lead to maximal heparin immobilisation in combination with an optimized degradation behaviour. Initially, 4 different reaction procedures were investigated for evaluation of their heparin immobilisation and their resistance *versus* degradation. These procedures are 1. (H+E)+C: 1 mg heparin/500 µl were allowed to react for 10 min with 1 mg EDC/NHS /500 µl , the collagen specimens wereimmersed into this solution and incubated for 4 h at 37°C under gentle shaking, 2. (H+E+C): specimen were immersed into the solution of 1mg heparin/500 µl and 1 mg EDC/NHS/500 µl and incubated for 4h and 10 minutes. 3. (H+C)+E: specimens were immersed into mg heparin/500 µl for 10 minutes, subsequently 1 mg EDC/NHS/500 µl was added and incubated for 4 h. 4. (H+C+L)+E: collagen specimen were immersed into a solution of 1 mg heparin/500 µl and lyophilized,

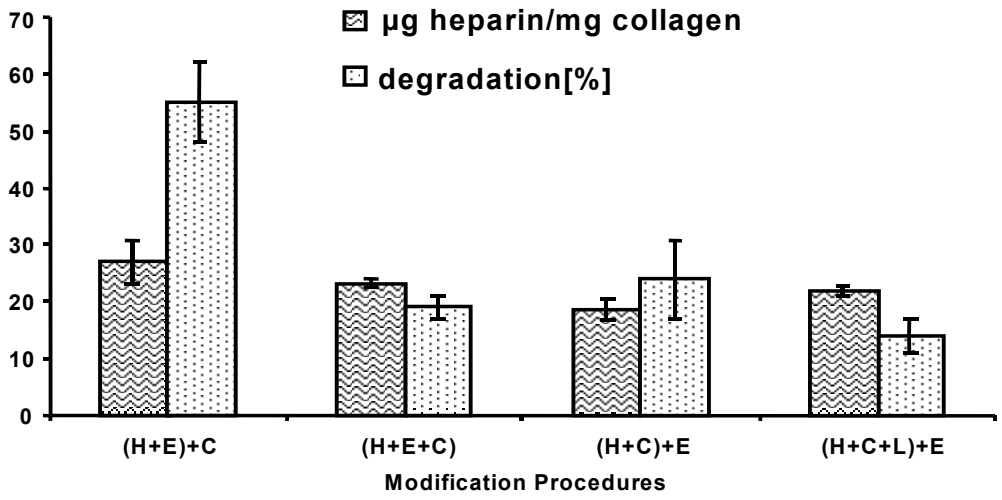


Figure 7 Heparin immobilisation and *in vitro* degradation obtained with the different reaction procedures. Reaction procedures were performed as described in Materials and Methods and explained in the text. Degradation was carried out with 40 units collagenase in 1 ml of PBS buffer for 2 h at 37°C. Columns show mean values, error bars represent the corresponding standard deviations (n = 5).

After lyophilization the dry specimens were allowed to react in a solution of 1 mg EDC/0,6 mg NHS per 500 µl. The results of Figure 7 show that the amount of heparin immobilisation is maximal with procedure 1. The different procedures do not lead to large differences in the *in vitro* degradation behaviour among the procedure 2, 3, 4, I therefore selectedprocedure 1 (H+E)+C for further experiments. I then optimized the reaction time, the results of Figure 8 demonstrate that an incubation time of 4 h led to maximal heparin immobilisation. Based on these results, I selected the procedure (H+E)+C and reaction time for 4 h as the optimized reaction condition for further experiments.

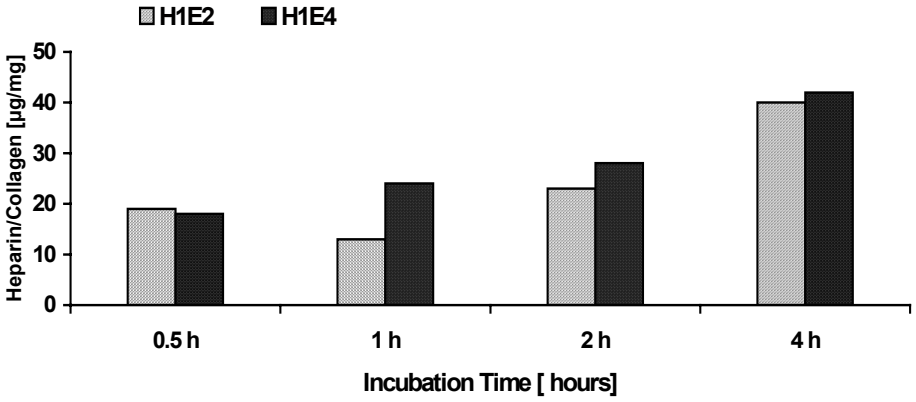


Figure 8 Heparin immobilisation in collagen matrices H1E2 and H1E4 as a function of reaction times varying from of 0.5 to 4 h (n=1).

3.1.2 Heparin immobilisation as a function of varying EDC/NHS to heparin ratios.

As shown in Figure 9, heparin immobilisation increases with increasing weight ratios of EDC/NHS to heparin, it reaches a plateau at a ratio of 2:1. Further increase of the ratio to 4:1, does not lead to higher immobilisation. Maximal heparin immobilisation amounts to 40 µg heparin immobilised per 1 mg collagen. Figure 10 furthermore shows that virtually no heparin was bound in the absence of the cross-linking agent (H1E0). This demonstrates that virtually no heparin is adsorptively bound and the

extensive washing procedure has apparently removed all non-covalently bound heparin.

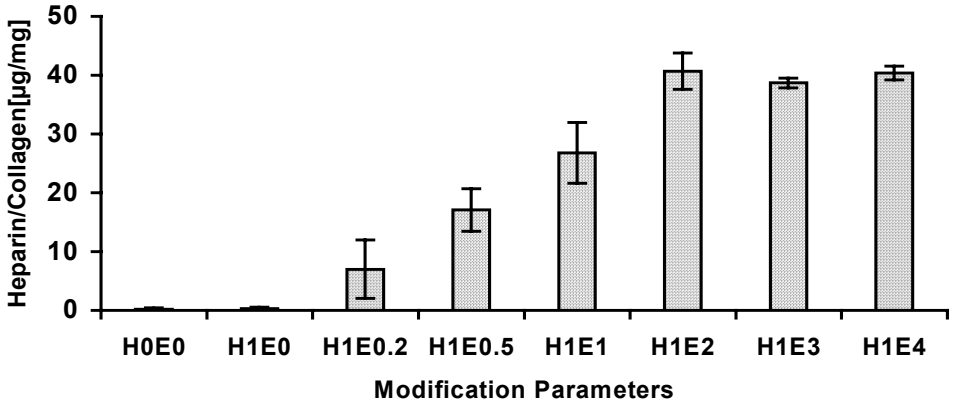


Figure 9 Heparin immobilisation assay as function of increasing weight ratio of EDC/NHS to heparin. The columns show the mean values, the error bars represent the corresponding standard deviations (n=5).

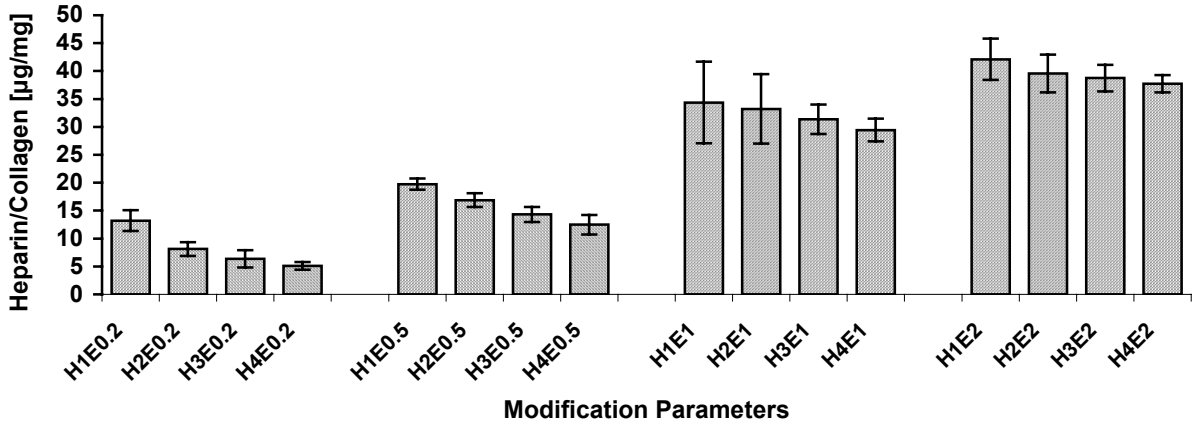


Figure 10 Heparin immobilisation as a function of increasing weight ratios of heparin to EDC/NHS. The columns show the mean values, the error bars represent the corresponding standard deviations (n=5).

3.1.3 Heparin immobilisation as a function of different ratios of heparin to EDC/NHS

What Figure 10 shows are in contrast with our expectations. By increasing the ratio of heparin to EDC/NHS, no additional heparin immobilisation was achieved. The higher ratios (w/w) of heparin to EDC/NHS led to lower extents of heparin immobilisation. The trend of this reduction slowed down with increasing amount of EDC/NHS involved in reaction. From H1E0.2 to H4E0.2, the amount of immobilized heparin was reduced by 50 %, from H1E0.5 to H4E0.5, the heparin immobilisation was reduced by about 30 %. This trend became less prominent by comparing the heparin immobilisation of H1E1 and H4E1, as well as H1E2 and H4E2.

3.2 *In vitro* degradation of modified collagen matrices

Figure 11 shows the results of a series of *in vitro* degradation experiments. Modified collagen matrices were immersed into a solution of 40 units collagenase/1ml and incubated at 37°C for 2 h and the weights before and after the degradation were determined. From these weights, the degradation percentages were calculated as described in Materials & Methods. The results show that with increasing EDC/NHS to heparin ratios, the degradation percentages decrease gradually. Whereas the matrices H0E0 and H1E0.2 were almost 70 % degraded, the matrices H1E0.75 to H1E4 were degraded to percentages < 10 . The relatively sharp dro between H1E0.2 and H1E0.75 will be discussed in chapter 5.

Under the conditions used, only small differences in degradation behaviour were observed for the modification parameters H1E1, H1E2, H1E3, H1E4 (all less than 10% degraded). In the next set of experiments, I changed the collagenase concentration to 200 units/ml and the incubation times to 2, 6, 24 and 48 h. The results are shown in Figure 12. Collagen matrices H1E1 are under these conditions degraded to

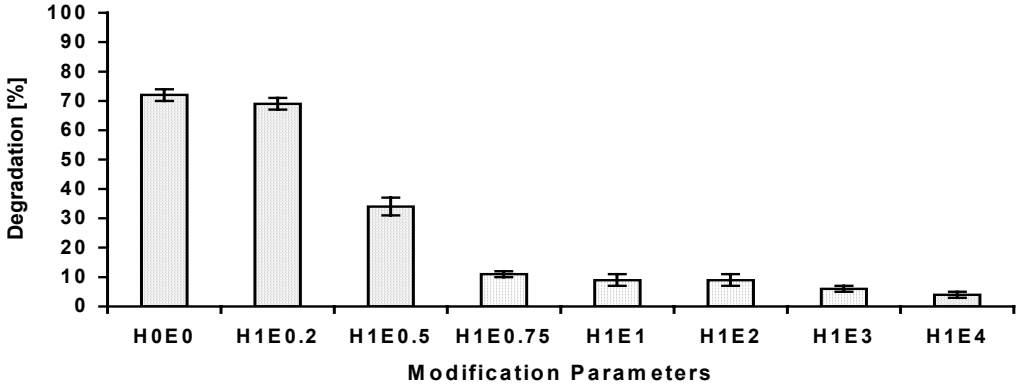


Figure 11 *In vitro* degradation of collagen matrices modified according to parameters specified in Materials & Methods. Degradation was carried out with 40 units collagenase in 1 ml of PBS buffer for 2 h at 37°C.

substantially larger extents: after 24 h these matrices were almost completely degraded. H1E2 matrices were degraded to about 54.5% ± SD 4.3 % after 48 h. The matrices H1E3 and H1E4 were very resistant to the action of collagenase, even after 48 h the matrices H1E4 were only degraded to about 16.5% ± SD 2.4 % .

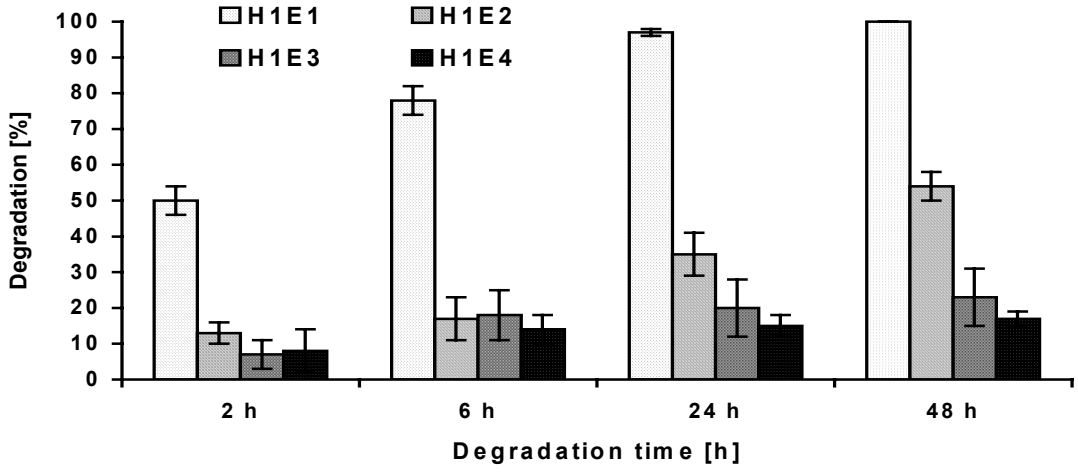


Figure 12 *In vitro* degradation of heparinized and cross-linked collagen matrices as a function of time. Degradation was carried out with 200 units collagenase per 1 ml of PBS buffer for different periods of time at 37°C. Columns show mean values, error bars represent the corresponding standard deviations (n=5).

In order to detect if there is any influence of the heparin immobilisation on the cross-linking of the collagen matrices, I further evaluated the degradation of collagen matrices which were modified by EDC/NHS cross-linking only. Primarily, I determined

the degradation of H0E0, H0E0.2, H0E0.5, H0E1 with 40 units collagenase/ml and an incubation period of 2 h at 37°C. The results are shown in Figure 13. The degradation of H0E0.2 dramatically decreased as compared to H0E0. When, however, the cross-linking was performed in the presence of heparin the degradation percentage of H1E0.2 did not not differ very much from H0E0 (see Figure 12). This observation will be dealt with in the Discussion (chapter 4).

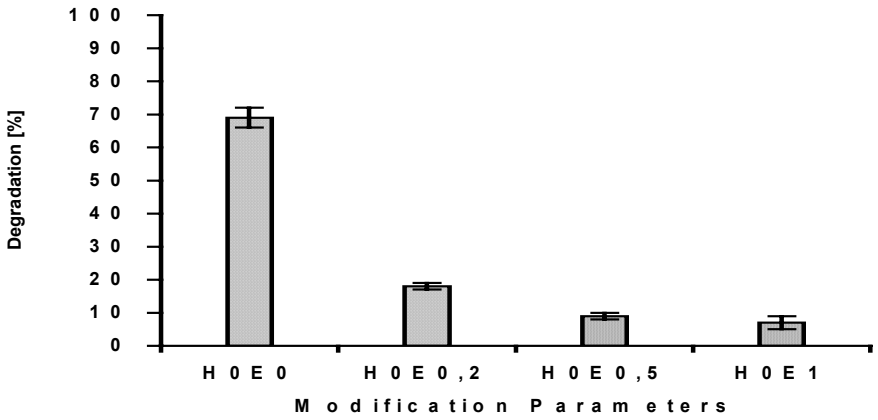


Figure 13 *In vitro* degradation of non-heparinized, cross-linked collagen matrices. Degradation was carried out with 40 units collagenase in 1 ml of PBS buffer for 2 h at 37°C. Columns show mean values, error bars represent the corresponding standard deviations (n=5).

For the same reason as discussed earlier, I applied higher collagenase concentrations (200 units/1 ml) and longer periods of incubation (24 h) for better discrimination between the various degradation behaviours. Figure 15 demonstrates that the resistance versus degradation increases with increasing EDC/NHS to heparin ratios (w/w).

Based on the results of Figure 14, I may conclude that the presence of heparin in the reaction solution has important consequences for the cross-linking by EDC/NHS. Therefore, I thought that it was of interest to investigate the influence of different heparin to EDC/NHS ratios (w/w) on the cross-linking of modified collagen matrices.

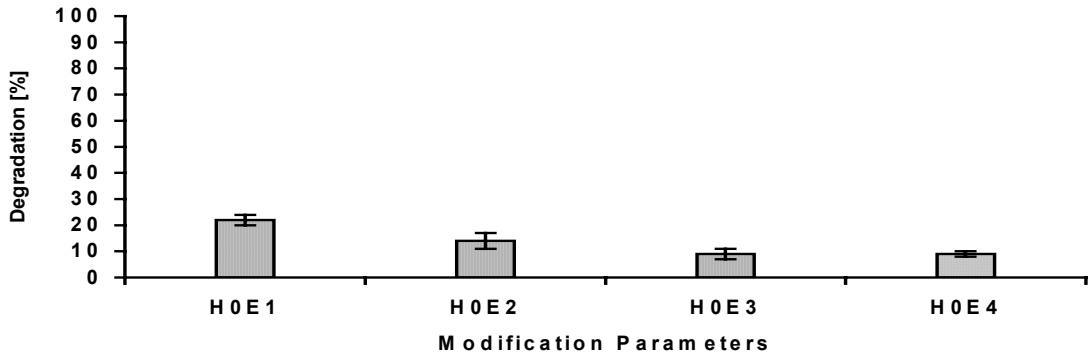


Figure 14 *In vitro* degradation of collagen matrices which were cross-linked only. Degradation was carried out with 200 units collagenase in 1 ml of PBS buffer for 24 h at 37°C (n=5)

I selected the following modification parameters: H1E0.2 H1E0.5, H1E1, H1E2 to H4E0.2 H4E0.5, H4E1, H4E2. Within the series H1E0.5 to H4E0.5, the degradation increased from about 60 % to 75 %, within the series H1E2 to H4E2, the degradation increased from about 10 % to 65 % (see Figure 15). Here again, the heparin to EDC/NHS ratios (w/w) during the activation played a major role in the degree of cross-linking.

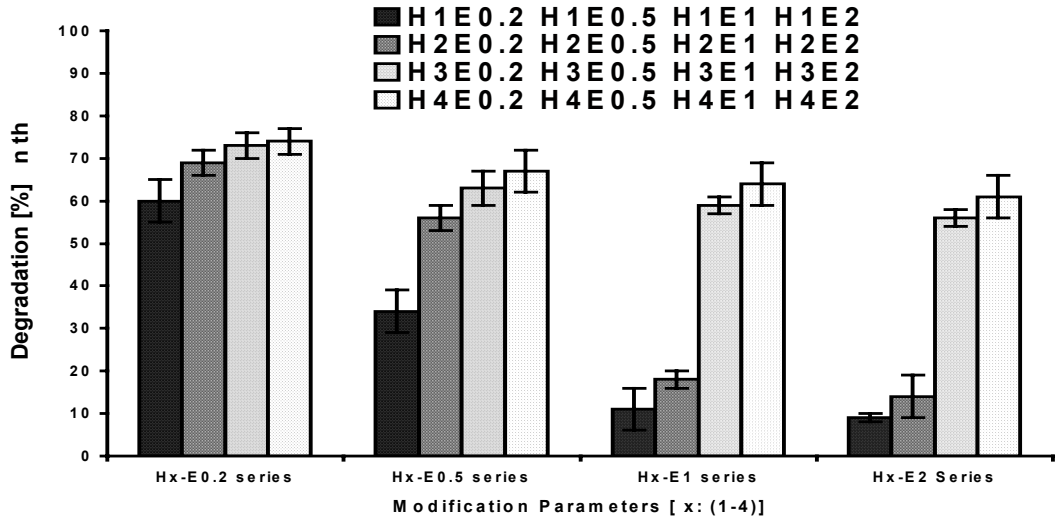


Figure 15 *In vitro* degradation of collagen matrices modified according to parameters specified in Materials & Methods. Degradation was carried out with 40 units collagenase per ml PBS buffer for 2 h at 37°C. Columns show the mean values, error bars represent the corresponding standard deviations (n=5)

The next two figures inform about the comparison of the cross-linking effect of EDC/NHS in the presence and absence of heparin in the reaction solution. Figure 16 shows the degradation under the following conditions: 40 units collagenase/ml and

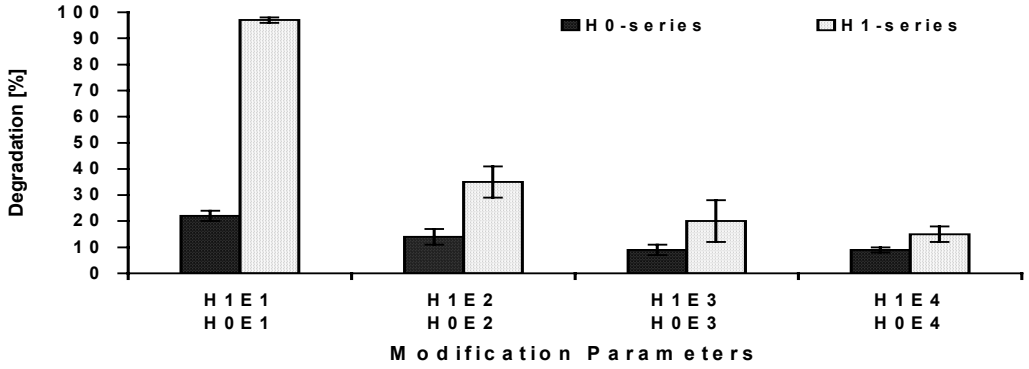


Figure 16 Comparison of the *in vitro* degradation of matrices, which were both heparinized and cross-linked and matrices, which were cross-linked only. Degradation was carried out with 40 units collagenase per ml PBS for 2 h at 37°C. Columns show the mean values, error bars represent the corresponding standard deviations (n=5).

incubation at 37°C for 2 h. The presence of heparin in the reaction solution appears to have is a dramatic effect on the degradation behaviour of the modified matrices H1E0.2 and H0E0.2 as well as H1E0.5 and H0E0.5. Figure 17 shows the degradation under the condition of 200 units collagenase/ml and incubation at 37°C for 24 h. More pronounced differences of the degradation are observed between H0E1 and H1E1 as well as between H0E2 and H1E2. The degradation percentages range from 10 to 95 and 15 to 35, respectively

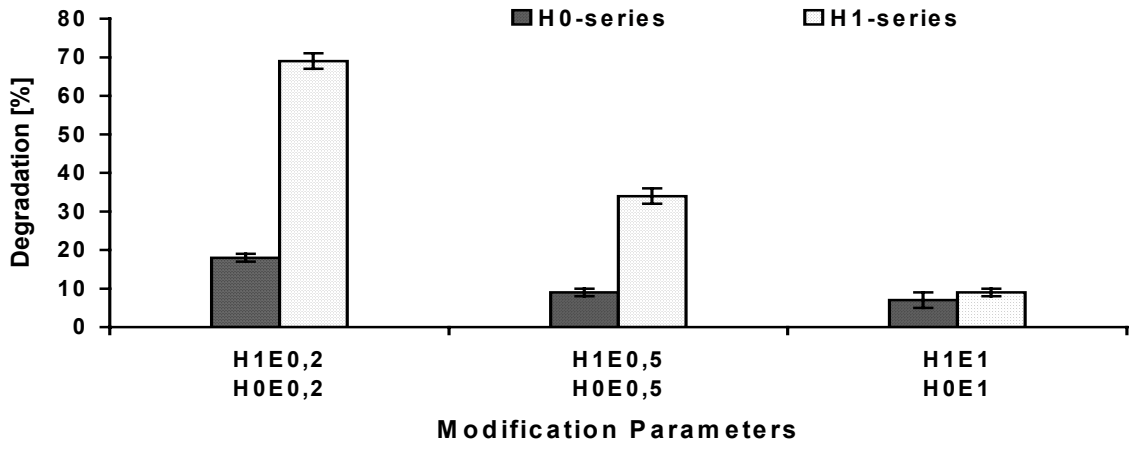


Figure 17 Comparison of *in vitro* degradation of heparinized and cross-linked matrices and matrices which were cross-linked only. Degradation was carried out with 200 units collagenase/ml for 24 h at 37°C. Columns show the mean values, error bars represent the corresponding standard deviations (n=5).

3.3 Determination of free amino groups in the modified collagen matrices

As discussed before, the modified collagen matrices are cross-linked between Hep-COOH and NH_2-collagen due to reactions induced by EDC/NHS. Due to the cross-linking process, the number of free primary amino groups of collagen matrices will be reduced whereas the reaction products of EDC/NHS will be washed out. Therefore the number of free amino groups directly correlates with the degree of cross-linking. Figure 18 shows the number of free amino groups per 1000 amino acids as a function of the modification parameters. For the non-modified collagen matrix (H0E0) I determined 36 free amino groups per 1000 amino acids, upon modification the number gradually decreased to 34 in H1E0.5, 32 in H1E1, 28 in H1E2 and 21 in H1E4. The results are in fairly good agreement with the results of the *in vitro* degradation experiments. The number of 36 free amino groups in non-modified collagen matrices nicely fits to the number of lysines and hydroxylysines present in collagen Type 1 from bovine sources (Bubnis and Ofner, 1992). This means that only a small number of lysines and hydroxylysines may be involved in the dehydrothermal treatment.

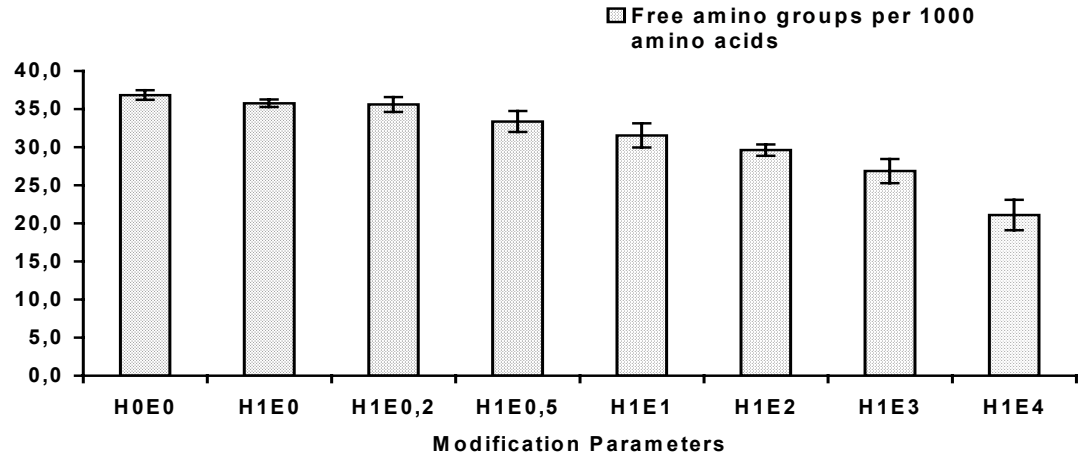


Figure 18 Free amino groups of collagen matrices modified according to parameters specified in Materials and Methods. Free amino groups were determined with trinitrobenzenesulfonic acid (TNBS). Columns show the mean values, error bars represent the corresponding standard deviations (n=5).

3.4 Determination of moisture uptake

Collagen is a porous hydrophilic material, which can bind large amounts of liquid. Heparin is also hydrophilic and it was reported that after heparin immobilisation in the collagen matrices, its water binding capacity will increase (Tsai *et al.*, 2001). I therefore investigated the moisture uptake of the modified collagen matrices. As expected, the moisture uptake (Figure 19) increased with increasing EDC/NHS to heparin ratios, thus with increasing additional cross-linking and increasing extents of immobilized heparin. Hence the results correlate with the degree of cross-linking and the extent of immobilized heparin which both reach a plateau with H1E2.

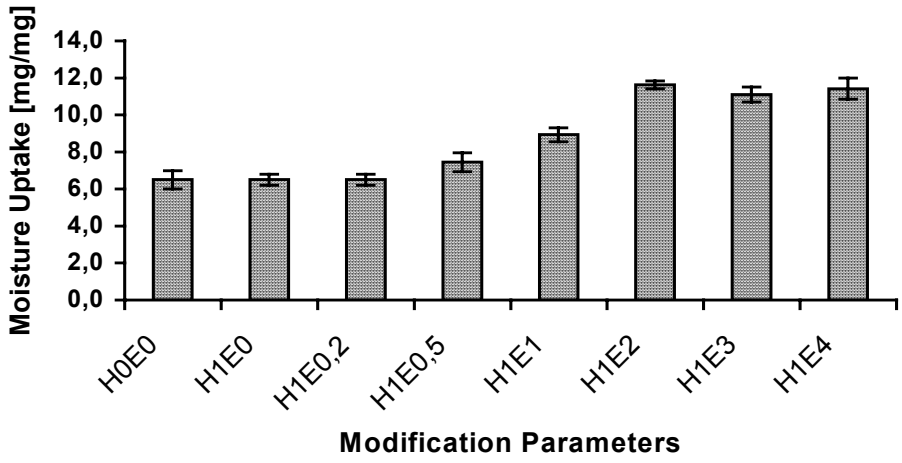


Figure 19 Moisture uptake of heparinized and cross-linked collagen matrices. Columns show the mean values, error bars represent the corresponding standard deviations (n=5).

In order to investigate whether different degrees of cross-linking lead to a change in moisture uptake, I investigated the effect the EDC/NHS cross-linking in the absence of heparin in the reaction solution. The results show that the moisture uptake increased with increasing cross-linking degrees, leading to moisture uptake of 6.8 and 9.5 mg/mg for H0E0.2 and H0E4 resp. (see Figure 20).

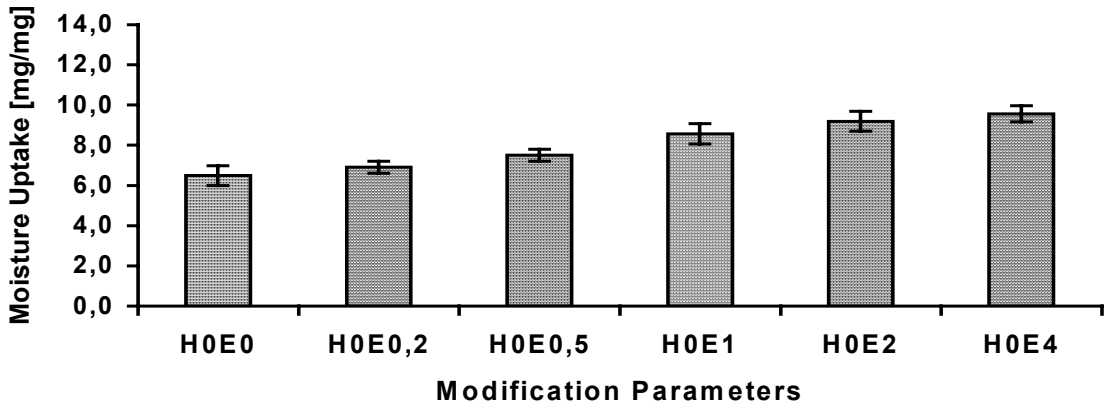


Figure 20 Moisture uptake of collagen matrices which were cross-linked only. Columns show the mean values, error bars represent the corresponding standard deviations (n=5).

3.5 Correlation of heparin immobilisation and *in vitro* degradation percentage among the modified collagen matrices

The extent of heparin immobilisation increases with the increased of the ratio of EDC/NHS to heparin, the *in vitro* degradation of the modified matrices, however, decreased (Figure 21). The amount of immobilized heparin in H1E0.2 was less than 10 µg/mg collagen and its degradation percentage reached 69% which was nearly the same as that of H0E0, whereas, the amount of heparin in H1E2, H1E4 was more than 40 µg/mg collagen, but their degradation percentages were less than 10 % for H1E2 and less than 5% for H1E4, which means that with the high amount of heparin immobilisation accompanied with lower degradation degree *in vitro*, the immobilised heparin release willkeep a long time after implanting into the body.

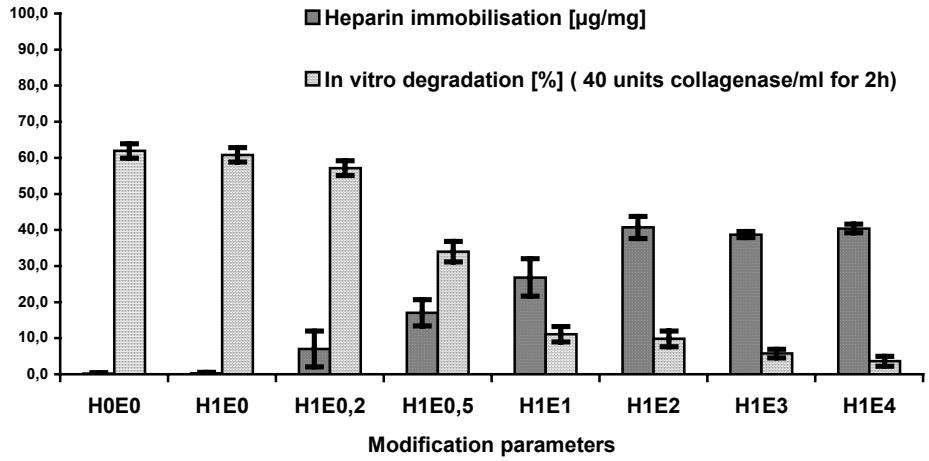


Figure 21 Relationship between the immobilized heparin and the *in vitro* degradation of collagen matrices modified at varying heparin to EDC/NHS ratios (40 units collagenase/ml of PBS at 37°C for 2 h) (n=5).

Oberviously, I showed that higher heparin to EDC/NHS ratios result in lower resistance to *in vitro* degradation, whereas, the heparin immobilisation of such modification only resulted in a bit less (Figure 9, 10). Furthermore, I analysed the amount of heparin immobilisation 33 µg/mg of H2E1, 40 µg/mg of H2E2, 38 µg/mg of H3E2, whose *in vitro* degradation percentages are 20 %, 30 %, and 55 % respectively. As we know, after modified collagen matrices degraded, the immobilised heparin and their binding growth factors will release into the surrounding tissues. This results shows that a large range of heparin and their binding growth factors release could be chosen with the different ratio of heparin to EDC/NHS and EDC/NHS to heparin.

Furthermore, these analyses show that it might be possible to produce matrices which bind the same amount of heparin, but are characterized by different degradation behaviours (Figure 22).

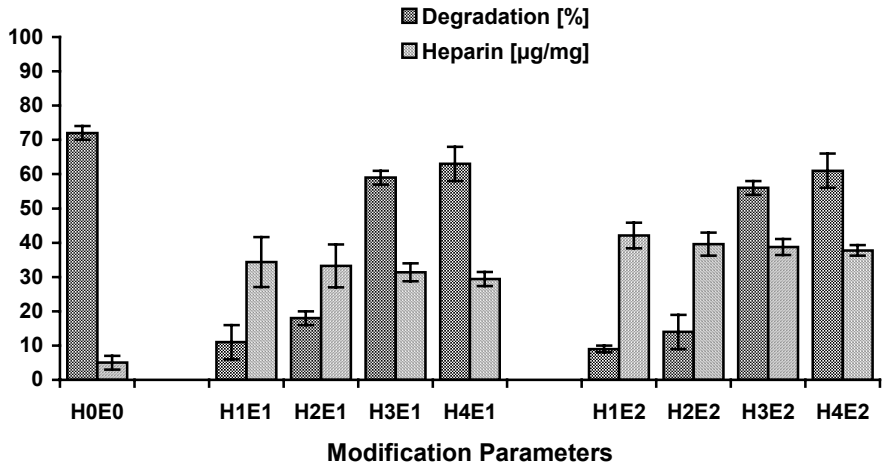


Figure 22 Comparison of the extent of the heparin immobilisation and the *in vitro* degradation with of 40 units collagenase /1ml of PBS buffer, under 37°C for 2 h (n=5).´

3.6 Angiogenic potential

3.6.1 Angiogenic potential determined by the *chorioallantois* membrane assay

3.6.1.1 The macroscopic evaluation with CAM assay

After inculation of the specimen onto the *chorioallantoic* membrane for 7 days, the macroscopic changes were observed for each collagen sample and the surrounding

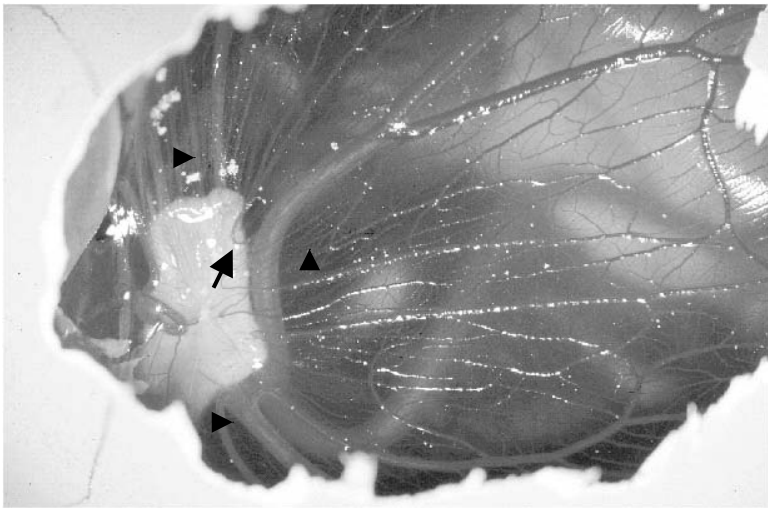


Figure 23 Macroscopic evaluation of specimen implanted on chorioallantoic menbrane of chicken embryo for 7 days. Vessels grow towards the implantant in a spoke-like manner (arrow head). About 80% of implantant are overgrown by granulomatous tissue of membrane (arrow).

chorioallantoic membrane are excised and mounted on a slide. Photographs of the excisions are taken. Figure 23 shows a picture of an excised part of the *chorioallantoic* membrane, the collagen implant (H0E0) is almost engulfed from the *chorioallantoic* membrane and the vessels are clearly oriented in a spoke-like manner.

Table 2 compiles the characteristics investigated. The data of 10 eggs of each experimental group were used for calculatiion of the percentages. Death rates represent of percentage of dead embryos during the whole incubation period. Area reduction means the reduction of surface area of implants after contact. As for the vessel form, spoke-like means that the vessels grow towards the implantant (see Figure 23, arrow heads) and undirected means that the vessels grow without a such direction. Finally, overgrowth by granulomatous tissue of the specimen represents the part of the specimen engulfed by the surrounding *chorioallantoic* menbrane (Figure 23, arrow) .

Table 2 Macroscopic evaluation of CAM assay experiments

Modification parameters	Death incidence [%]	Area reduction [%]	Vessel structure [%]		Overgrowth by granulomatious tissue [%]
Control	20	-	undirected (100)	-	-
H0E0	20	50	undirected (33,3)	spoke-like (66,7)	70
H0E0.2	20	30	undirected (33,3)	spoke-like (66,7)	80
H0E0.5	10	20	undirected (25)	spoke-like (75)	70
H0E1	10	10	undirected (0)	spoke-like (100)	60
H0E2	25	5	undirected (0)	spoke-like (100)	60
H1E0.2	20	40	undirected (50)	spoke-like (50)	75
H1E0.5	15-20	30	undirected (25)	spoke-like (75)	80
H1E1	20	15	undirected (0)	spoke-like (100)	75
H1E2	20	10	undirected (25)	spoke-like (75)	75
Control+VEGF	20	-	undirected (100)	spoke-like (0)	-
H0E0+VEGF	20	50	undirected (33)	spoke-like (67)	60
H1E0.2+VEGF	20	35	undirected (25)	spoke-like (75)	75
H1E1+VEGF	15	20	undirected (25)	spoke-like (75)	75
H1E2+VEGF	20	10	undirected (0)	spoke-like (100)	75

Regarding the death rates of the various modification parameters, we did not find the obviously different from the death rates of the control group. The percentage of spoke-like vessel organisation in the surrounding tissue increases with increasing degrees of cross-linking, heparin immobilisation and VEGF loading. The percentages of the overgrowth by granulomatous tissue - by the *chorioallantoic* membrane - of modified samples are not significantly different from the control group.

3.6.1.2 The microscopic evaluation with CAM assay

The angiogenic potential of an implant is now derived from the number of microvessels in three defined areas in the vicinity of the implant. Vessels were counted as described by Zwadlo-Klarwasser (Zwadlo-Klarwasser *et al*., 2001) and in Materials and Methods. Figure 24 shows a microscopic photograph of the *chorioallantoic* membrane at a site in close proximity of an implant, the rectangle restricts the area in which the microvessels were counted.

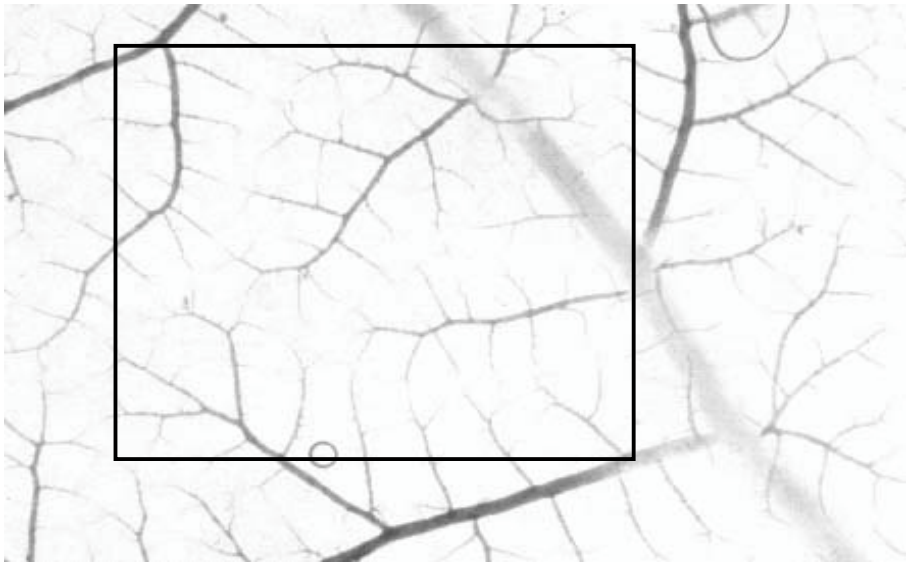


Figure 24 Microscopic evaluation of angiogenic potential of collagen matrices by counting mcirovessels in the chorioallantoic membrane of the chicken embryo (Magnification 22,5).

3.6.1.3 Angiogenic effect of immobilized heparin as determined by the CAM assay

We first tested the angiogenic potential of collagen matrices modified with different ratios of EDC/NHS and heparin. The modified specimen matrices were mounted on the *chorioallantoic* membrane of the embryonated eggs under sterile conditions and incubated for 7 days at 37°C. The results show that a higher ratio of EDC/NHS to heparin increase the angiogenic potential. The number of the capillaries counted in the CAM increased gradually from 58 (control) to 110 (H1E2) (Figure

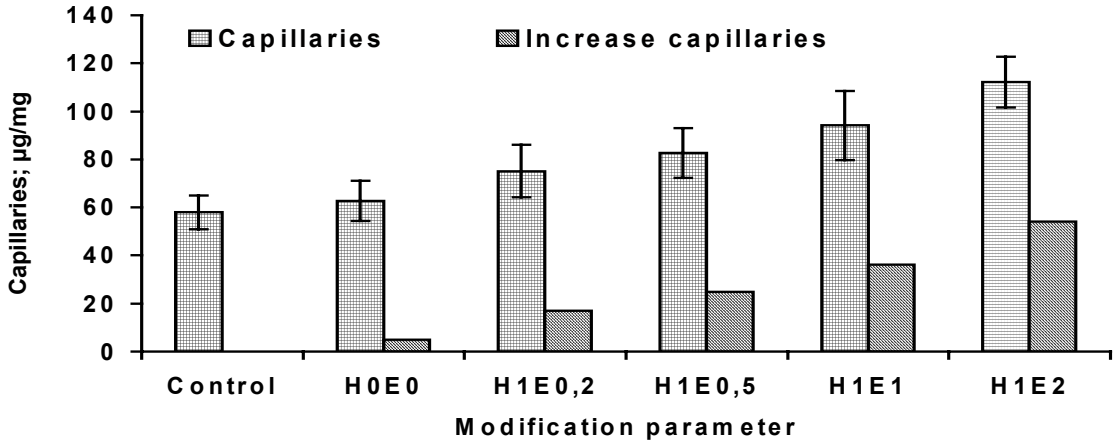


Figure 25 Number of capillaries counted in the vicinity of the collagen matrices. The increase of capillaries was obtained by subtracting the number of capillaries of the control. Columns show the mean values, error bars represent the corresponding standard deviations (n = 5).

As shown above, the amount of immobilized heparin increases with higher EDC/NHS to heparin ratios (see Figure 9). Comparing the correlation of the amounts of heparin immobilisation and the angiogenic potential of the same modification, it is obvious that there is a trend of the effect of angiogenic potent and the amount of heparin immobilisation as well. This suggests that the angiogenic potential of modified matrices is caused by immobilised heparin (Figure 26).

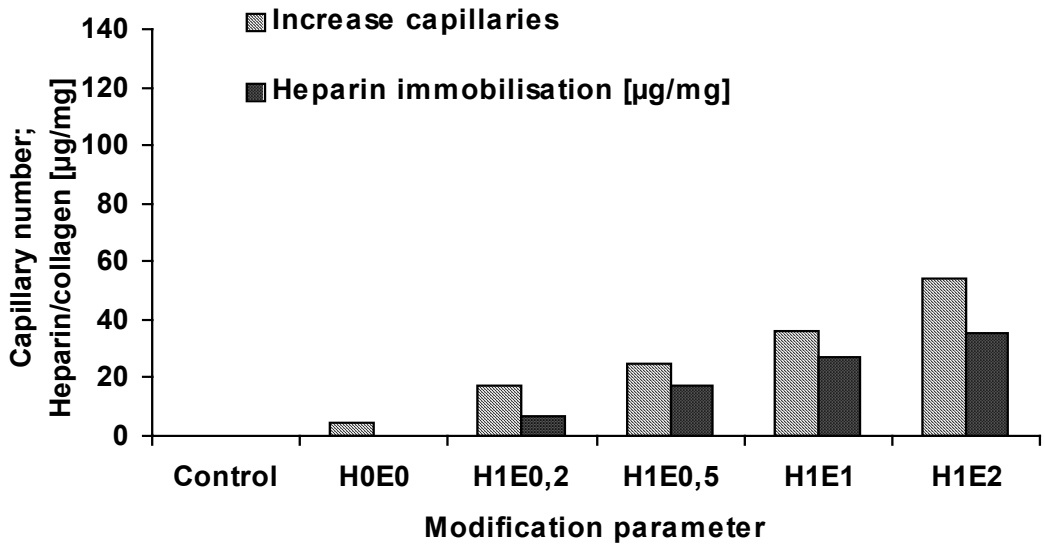


Figure 26 Correlation of the increase of capillaries of heparinized, cross-linked collagen matrices with the amounts of immobilized heparin in these matrices. Results are taken from Figures 10 and 24 respectively.

3.6.1.4 Angiogenic effect of EDC/NHS cross-linking as determined by CAM assay

To further evaluate the heparin effect on angiogenesis observed in CAM assay, I evaluate the possible angiogenic potential of the collagen matrices modified with EDC/NHS alone in the samples the modification procedures were the same as those of heparin immobilisation, except heparin application.

In the CAM assays, I showed that cross-linking of collagen matrices leads to a substantially improved angiogenic potential and this effect becomes more evident with increasing degrees of cross-linking. The number of the capillaries counted gradually increases from 48 for the control group to 64 for H0E0 and 100 for H0E2 (Figure 27).

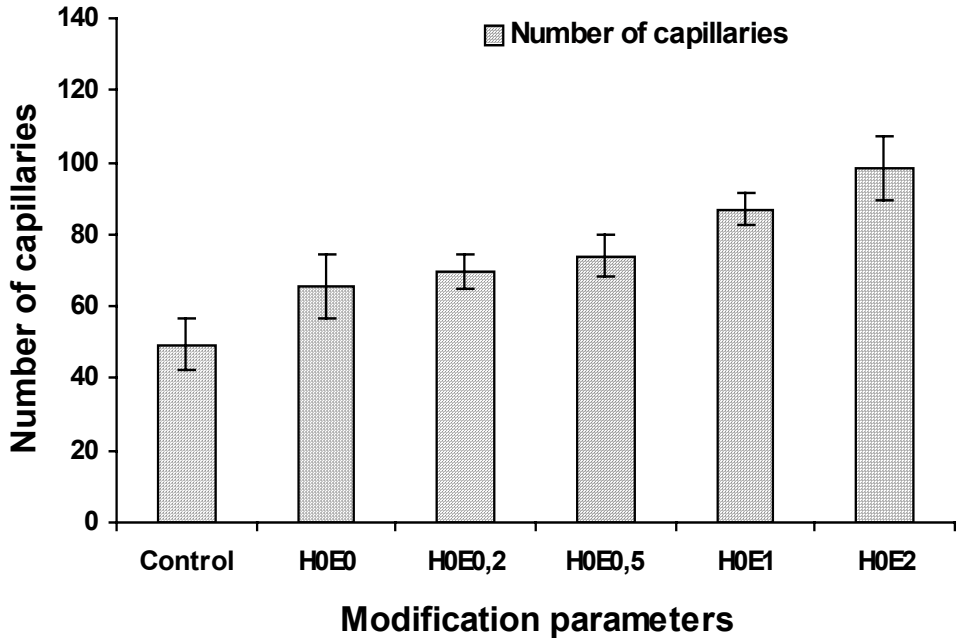


Figure 27 Angiogenic effect of collagen matrices which were cross-linked only. Columns show the mean values, the error bars represent the corresponding standard deviations (n = 5).

Considering that the increase of the angiogenic potential might be due solely to the cross-linking effect, I compared the angiogenic potential of collagen matrices which were cross-linked in the absence of heparin (H0-series) with collagen matrices which were both heparinized and cross-linked (H1-series). I observe that upon comparison of H1E0.2 to H0E0.2 and of H1E1 to H0E1, the differences are relatively small, demonstrating that the effect on the angiogenic potential is mainly due to the cross-linking effect and that the immobilized heparin only has a small additional influence (Figure 28).

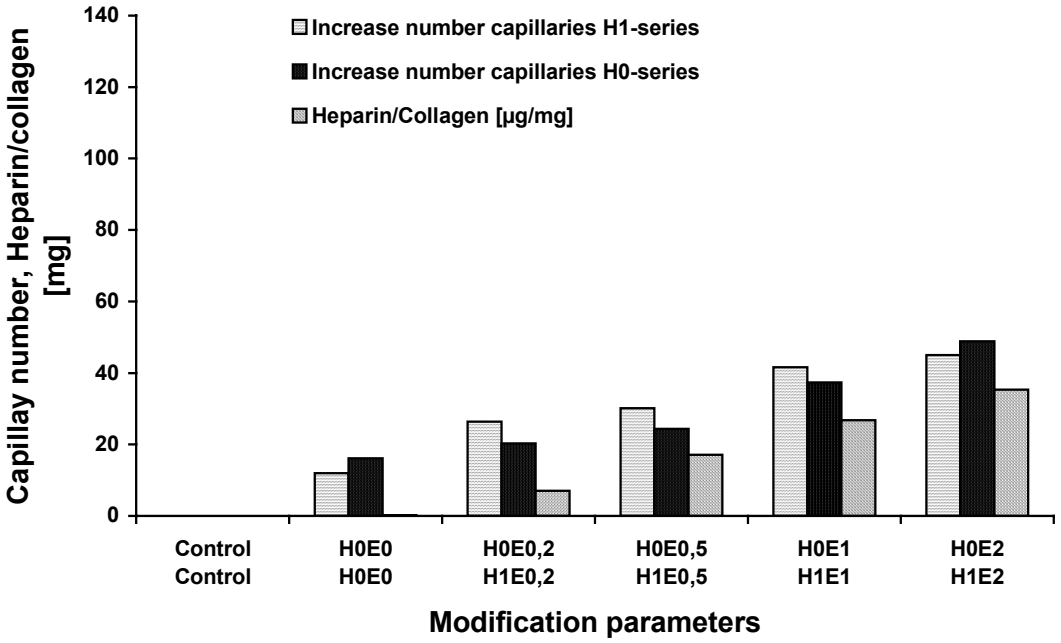


Figure 28 Correlation of the angiogenic effects of modified collagen matrices: H1Ex-matrices were both heparinized and cross-linked, H0Ex-matrices were cross-linked only. For comparison the corresponding amounts of immobilized heparin are also shown. Columns show the mean values of the increases, the data on the heparin immobilization were taken from Figure 24, 26 and 10 resp. .

3.6.1.5 The angiogenic effect of loading VEGF to heparinized and non-heparinized collagen matrices as evaluated by the CAM assay

As mentioned in the introduction, VEGF is a potent angiogenic growth factor specifically acting on vascular endothelial cells and which is characterized by a high affinity towards heparin. I therefore modified collagen matrices by incorporation of heparin within the matrices by covalent immobilisation. In a series of experiments I evaluated the angiogenic effect of loading VEGF to heparinized and non-heparinized collagen matrices by the CAM assay.

The results show that the number of capillaries increases as a result of the loading of rhVEGF165 to both heparinized and non-heparinized collagen matrices. As compared

to the increase of the number of capillaries of the control group loaded with 30 ng VEGF (13,5), that of H1E0.2 with 30 ng VEGF reaches 36,8, that of H1E1 and H1E2 with VEGF reaches 67,6 and 63,6 respectively (Figure 29).

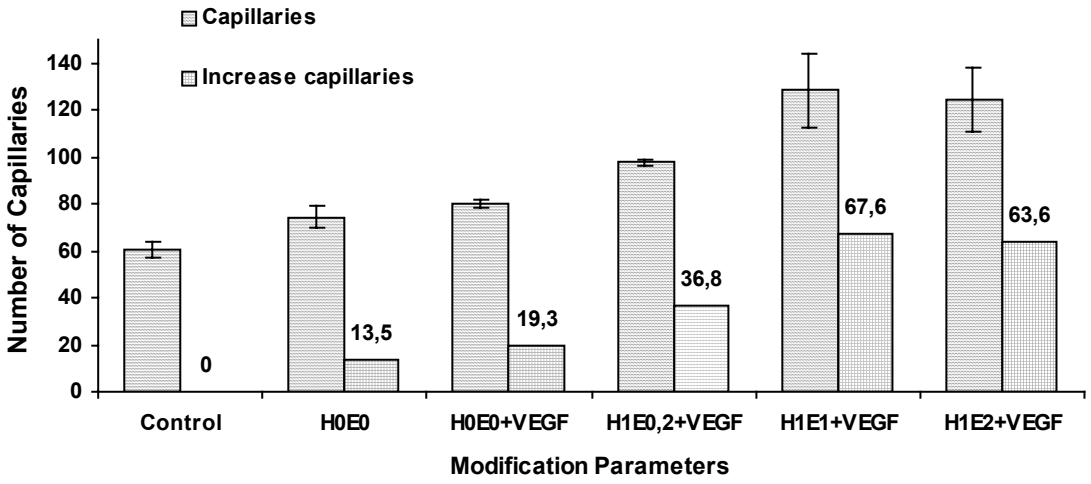


Figure 29 Angiogenic effects of loading VEGF to heparinized collagen matrices (30 ng VEGF/collagen matrix). Columns show the mean values, error bars represent the corresponding standard deviations (n=5). Numbers indicating the corresponding increases were obtained by subtracting the number of capillaries of the control from each value.

To evaluate the angiogenic potential of all modification parameters, I subsequently investigated the following set of modified collagen matrices in a single CAM experiment: 1. matrices modified by EDC/NHS cross-linking only, 2. matrices with EDC/NHS cross-linking and heparin immobilisation, both in the presence and absence of VEGF.

The results of Figure 30 confirm that the number of increase of capillaries of H1E1 (70/40) in the presence and absence of VEGF preloading is more than those of H0E0 (15/5) and H0E1(55/35). It could be clearly found from Figure 31 that the strongest angiogenic potential was acquired in the modification of H1E1 with VEGF preloading. Furthermore, the additional effect exerted by VEGF loaded in H1E1 was about 1,5 to2 times stronger compared to that with H0E1 and H0E0.

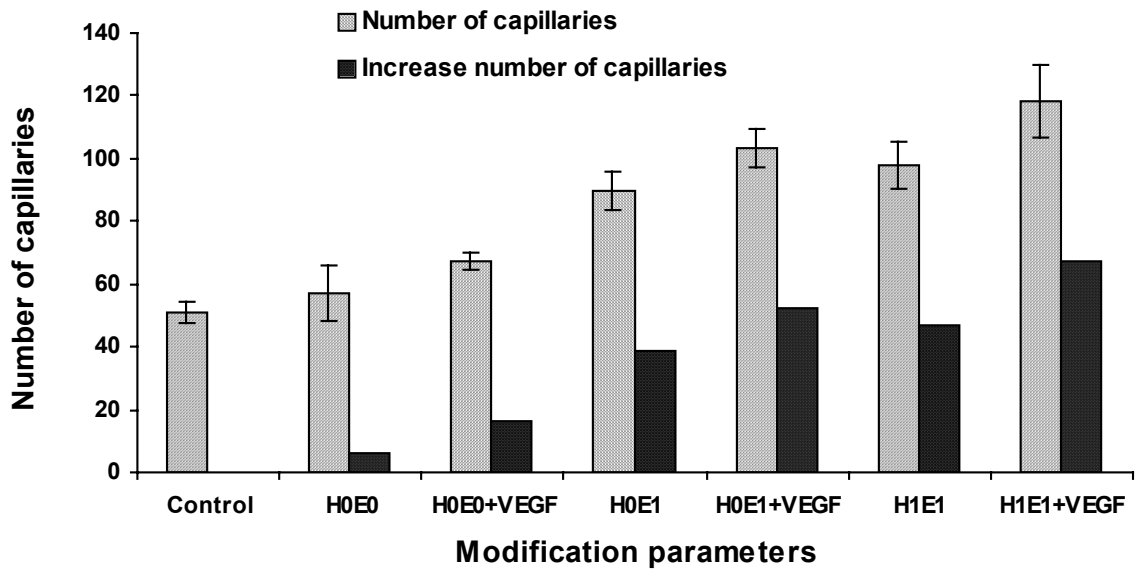


Figure 30 Evaluation of the angiogenic effect of loading of 100 ng rhVEGF165 to non-modified (H0E0), heparinized and cross-linked matrices (H1E1), as well as matrices which were cross-linked only (H0E1). Columns show the mean values, error bars represent the corresponding standard deviations (n=5).

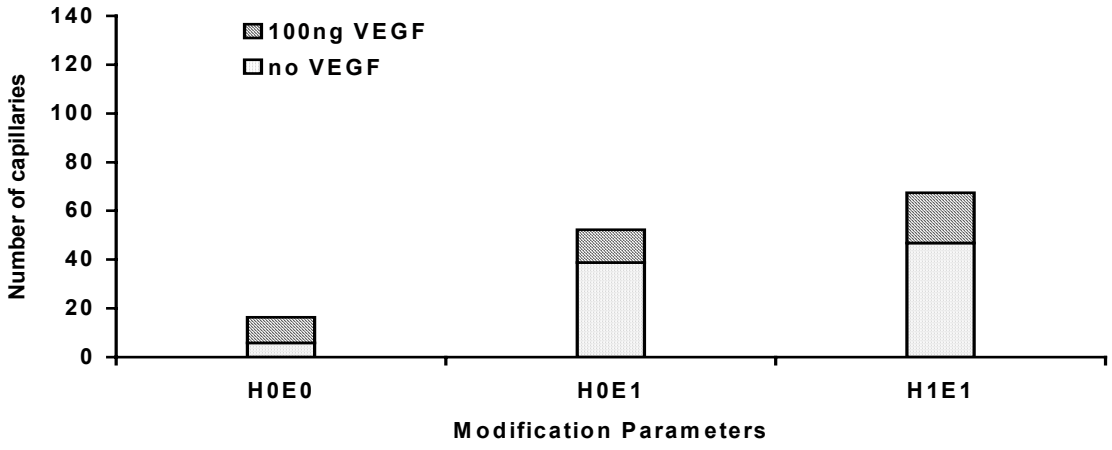


Figure 31 Additional angiogenic effects of loading of VEGF to heparinized and non-heparinized collagen matrices. The upper parts of the columns represent the additional effect exerted by VEGF loading. Data are taken from Figure 30.

3.6.2 Angiogenic effect of VEGF loading as determined by the hemoglobin content of explanted collagen matrices

For the evaluation of the angiogenic effect of loading of rrVEGF$_{165}$ to heparinized and non-heparinized collagen matrices I implanted these matrices subcutaneously on the back of Lewis rats. Details of the procedures applied in these animal model experiments are given in chapter 2 (Materials & Methods).

The macroscopic observations of the collagen specimen explanted after 15 days are shown in Figure 31. The colour intensity of the explanted specimen increases with increasing degrees of cross-linking. The loading with VEGF has in all cases an additional angiogenic effect. The colour intensity of the collagen matrix H0E0 is low, whereas the colour of H1E1 loaded with VEGF is dark red. The colour intensities of the other modifications are intermediate.

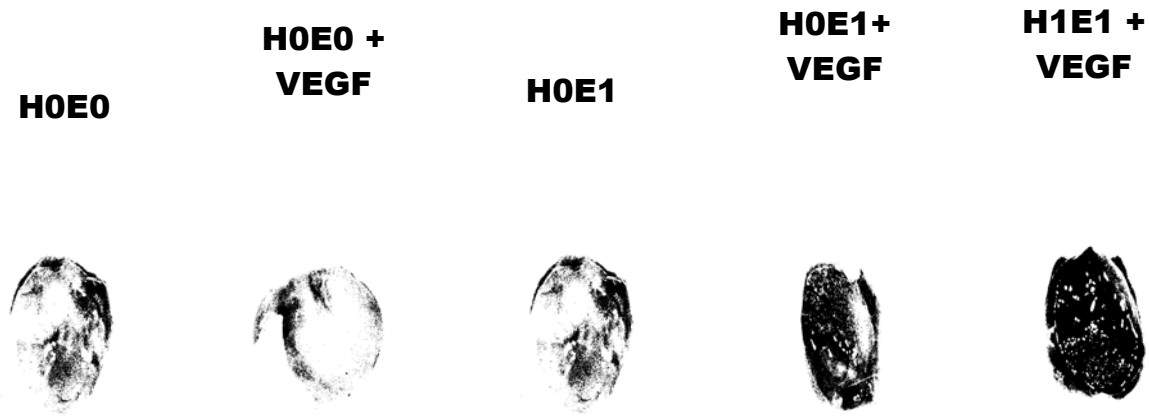


Figure 31 Photographs of explanted collagen matrices after 15 days implantation. Indicated specimen were loaded with 300 ng rrVEGF$_{165}$

The angiogenic effect was investigated also by determining the hemoglobin content in the explanted matrices. The hemoglobin content was determined after washing and degrading the matrices with collagenase by difference VIS-spectroscopy at wavelength around 410 nm (Soret band) using a calibration curve obtained from sperm whale myoglobin. The calibration curve used is shown in Figure 32.

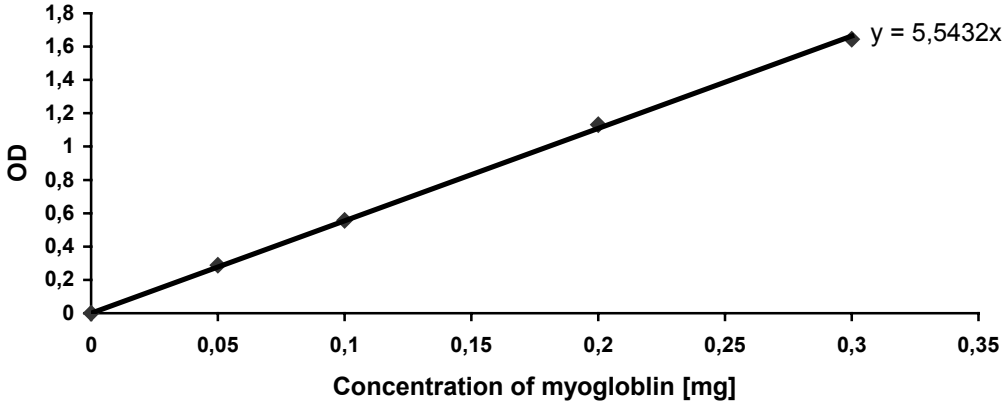


Figure 32 Standard curve for hemoglobin determination

Figure 33 shows that the hemoglobin content of the explanted collagen matrices increases with increased cross-linking and that the loading of rrVEGF$_{165}$ has an additional angiogenic effect. Thus the highest angiogenic effect is obtained with collagen matrix H1E1 loaded with VEGF (Figure 33).

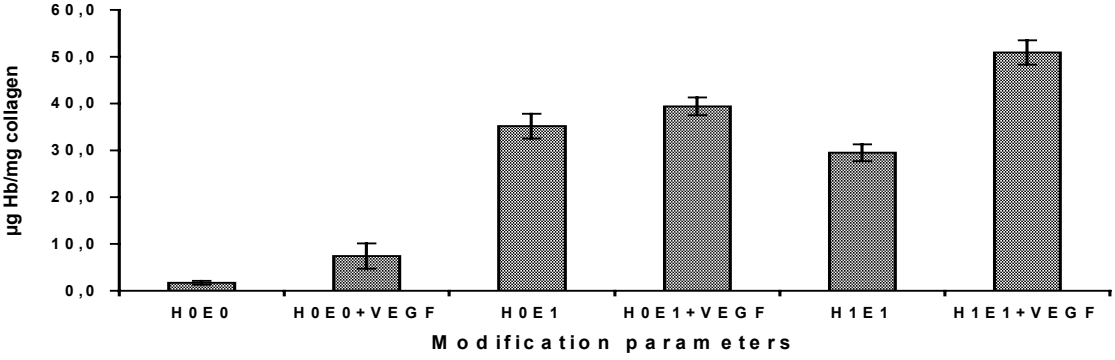


Figure 33 Hemoglobin content of collagen matrices explanted after 15 days implantation. Part of the matrices (as indicated) were loaded with 300 ng rrVEGF$_{165}$ prior to implantation in rats. Columns show mean values, error bars represent the corresponding standard deviations (n=2).

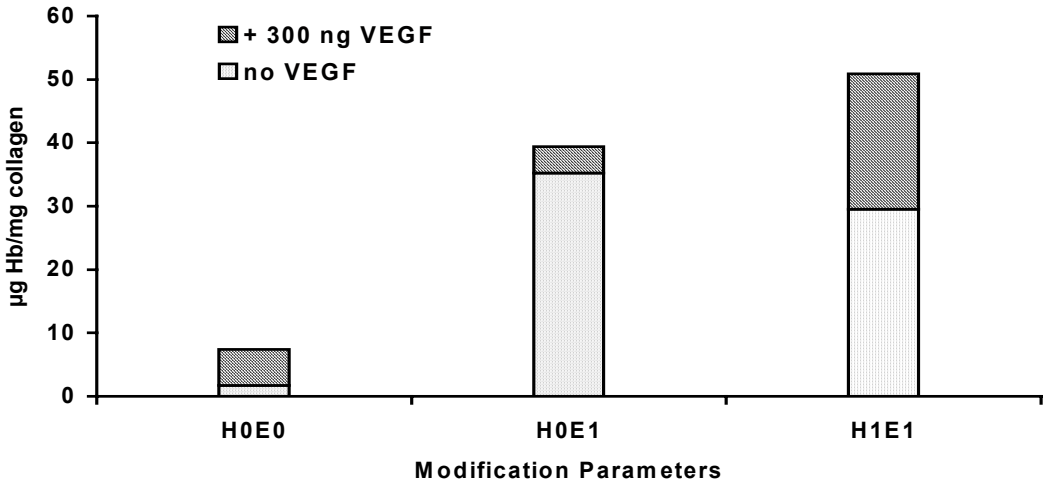


Figure 34 Angiogenic effects of loading 300 ng rrVEGF$_{165}$ to heparinized and non-heparinized collagen matrices. The upper parts of the columns represent the additional effect exerted by VEGF loading. The angiogenic effects were evaluated by determination of the hemoglobin content in the explanted matrices after 15 days implantation (n=2). Data are taken from Figure 33.

4 Discussion

The final goal of tissue engineering is to develop engineered tissues which can replace or regenerate the host tissues completely after implantation. Therefore, it is essential for cells - either seeded or ingrown from the surrounding host tissues – to survive. The formation of a functioning vascular network for the supply of oxygen and nutrients is an important prerequisite. It is not surprising that the development of biomaterials with enhanced angiogenic potential are of primary interest in tissue engineering (Nomi *et al*., 2002).

Many ideas have been put forward to develop biomaterials with improved angiogenic capabilities. One of these methods is to incorporate angiogenic growth factors into biomaterials in order to release these growth factors in a sustained manner. Collagen is of great interest as a biomaterial since it is biodegradable, biocompatible, non-toxic, suitable for cell attachment and ingrowth.

Naturally, much attention has been directed towards the incorporation of angiogenic growth factors into collagen matrices. Several approaches have been applied: in one approach the growth factor TGF-ß was covalently linked to the collagen with a homobifunctional cross-linking agent (Bentz *et al*., 1998), in another approach VEGF was loaded in and released from microspheres (Peters *et al*., 1998). However, these two methods will include the reaction chemicals into the biomaterials which may have some influence on the host after implantation. Therefore, I investigate the physical binding of VEGF to collagen matrices in which heparin molecules were covalently attached. Compared to the two methods mentioned above, at least three advantages of our present approach could be concluded:

1 Heparin is immobilised into collagen matrices with the water soluble cross-linking agent EDC/NHS which leads to a zero length bonding that does not include inside the biomaterials after cross-linking and the supplementary reagents and reactants can be washed out easily after reaction.
2 After binding to heparin, VEGF may be protected from denaturing and its bioactivity may increase (Sommer and Rifkin, 1989).

3 Collagen is a thrombogenic material which induces platelet adhesion and aggregation, as well as activation of intrinsic blood coagulation. Immobilized heparin may decrease the thrombogenic potential of collagen and may be beneficial to vascular endothelial cells of the surrounding tissue and may encourage them to migrate in.

A substantial number of characteristics of modified and non-modified collagen matrices have been subject to our present investigation:

1 the amount of heparin immobilisation
2 *in vitro* degradation rate
3 the number of primary free amino groups
4 moisture uptake
5 the angiogenic potential of (non-)modified samples in the presence and absence of VEGF.

4.1 The mechanism of the modification procedure

Several reports claim that the cross-linking agent EDC/NHS may be used to activate the carboxyl acid groups of collagen to highly active succinimidylesters, which react with free amino groups on neighbouring collagen fibrils, resulting in the cross-linking of collagen. Heparin is an anionic linear polysaccharide consisting of two repeating disaccharide units. It also has carboxyl groups in the two repeating disaccharide units. Thus, EDC/NHS can also be used for activating the carboxyl groups of heparin. Heparin is covalent linked to EDC first, followed by EDC-activated carboxyl groups which react with NHS-esters, which prevent the side reactions like hydrolysis of EDC activated groups or an O-N-acyl shift. After activation of the carboxyl groups on the heparin, dry collagen matrices are immersed into the reaction solution. The activated carboxyl acid groups of heparin now react with free amino groups on the collagen surface leading to „zero length“ covalent bond between the COOH-group on the heparin and the NH2-group on the collagen (see Figure 2). Since having a high solubility in water, the reaction products of EDC/NHS are washed out easily, .

After immobilisation, the modified collagen matrices are washed extensively with sodium chloride and distilled water to remove all the reaction products and non-

immobilized heparin. In the procedure of Wissink et al. (Wissink, *et al*, 2001b), heparin was immobilised to collagen matrices which were previously cross-linked. In our procedure heparin is immobilised to collagen matrices which were not chemically cross-linked. Additionally the cross-linking of the heparin and the additional cross-linking of the collagen were performed in a one step procedure. Since the number of primary free amino groups in non-cross-linked collagen is higher than in cross-linked collagen ones, I think there should be more free amino groups of non-cross-linked collagen available to react with activated carboxyl acid groups of heparin.

The next step will be to physically bind VEGF to heparinized collagen matrices. There are reports that bFGF binds to heparin immobilised in collagen films (Wissink *et al*., 2001a). Therefore I have chosen a reaction time of 90 minutes for binding VEGF to heparin is at room temperature because our collagen matrices is much thicker (5 mm) than that of film (20 µm). Figure 33 shows a schematic illustration of the physical binding of VEGF heparinized collagen matrices and of the additional cross-linking of the collagen fibers.

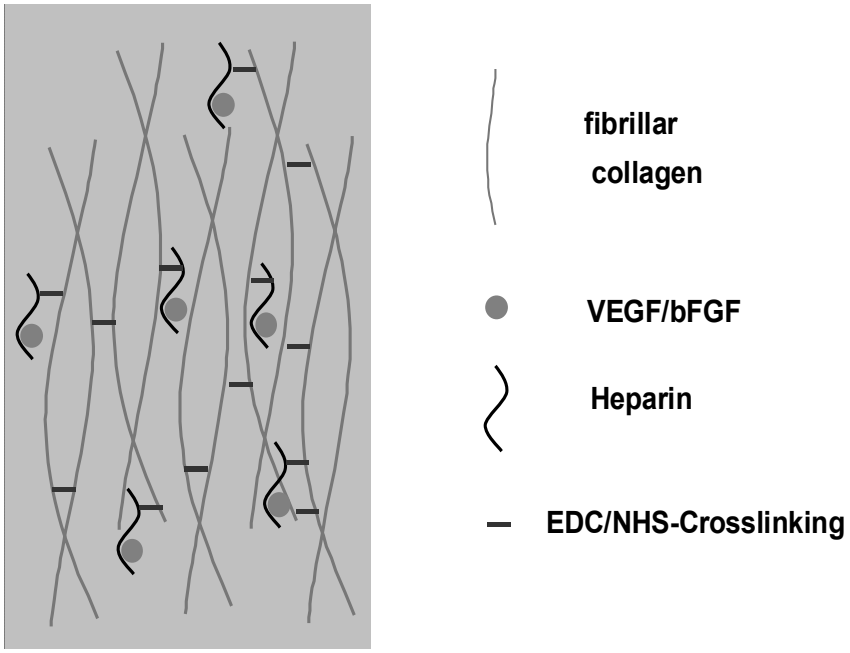


Figure 33 Schematic representation of physical binding of angiogenic growth factors to heparin covalently linked to collagen fibrils by cross-linking with EDC/NHS.

4.2. Immobilisation of heparin

4.2.1. Optimal conditions for immobilisation

One of the objectives of the present investigation is to develop modified collagen matrices which have the ability to physically bind a high amount VEGF and release it in a controlled way. Since VEGF binds with a high affinity to heparin, the binding capacity directly correlates with the extent of heparin immobilisation. Thus our first goal is the development modified the collagen matrices with incorporated high values of heparin.

Heparin was reported to be successfully immobilised into collagen films with the cross-linking agent EDC/NHS (Wissink *et al*., 2001b). This group used MES buffer with pH 5.6, the heparin carboxyl groups were activated from 5 to 30 minutes and the time allocated for heparin immobilisation was 2 h. Taken their results as guide lines for our experiments, I decided to use MES buffer (pH 5.6) as reaction buffer and 10 minutes for the time of heparin pre-activation. In order to achieve an increase of the heparin immobilisation, I first tested whether a reaction time of 2 h is sufficient for obtaining optimal heparin immobilisation. I determined the amount of heparin immobilisation after reaction periods varying from 0.5 to 4 h. Our results show that a reaction time of 2 h is not sufficient for the maximal heparin immobilisation. The amount of immobilized heparin reaches a maximal value at 4 h and does not increase any further (data not shown). This difference between our results and those of Wissink et al. may be explained by different sizes of the collagen matrices. Whereas I use cubic specimen (5*5*5 mm), they use collagen films with a thickness of 50 µlµl. Therefore, the reaction agent might need more time to diffuse into the matrix.

As a next step I investigated four different procedures for the immobilisation of heparin. I obtained the best results with procedure 1 in which is to preactivate carboxyl acid groups of heparin with EDC/NHS for 10 min, followed by reaction with collagen matrix for additional 4 h. (Figure 8). This is not surprising because of the principle of competition. In the case the carboxyl groups of heparin are activated first, they will preferentially react with the free amino groups of collagen. Therefore, procedure 1 results in the highest amount of immobilized heparin and the lowest

resistance to collagenase degradation since less EDC/NHS are left to additionally cross-link the collagen. As compared to procedure 1, procedures 2, 3 and 4 result in lower amounts of immobilized heparin and a slightly higher resistance towards collagenase degradation. The heparin immobilisation in procedure 3 is slightly lower than that of procedure 2, I guess that after the immersion of the specimen into the heparin solution, heparin will be distributed all over the matrix, thus the activation of the carboxyl groups may interfere with the cross-linking of the collagen. Therefore, the resistance to collagenase degradation is slightly lower than that of procedure 2. If the specimens are lyophilised after immersion into the activated heparin solution, the disadvantage caused by the heparin distribution will be compensated for. Therefore, the results of procedure 4 are similar to the results of procedure 2.

The washing procedure, as described by Wissink et al., takes about one week. I tried to reduce the time of the washing procedure. Collagen specimens which were subjected to the washing procedure of Wissink et al. were compared with specimens which were washed within two days. No differences were observed as evaluated from the amount of immobilized heparin and from the death rate of the chicken embryos in the CAM assay (See table 2). I therefore decided to restrict the extensive washing procedure to two days.

In summary, the optimal reaction procedure for cross-linking heparin to collagen is the following: 1. carboxyl groups on the heparin are activated with EDC/NHS for 10 minutes, 2. dry collagen matrices are immersed and evacuated for 5 minutes to be homogeneously equilibrated, 3. reaction period should be 4 h, 4. the heparinized samples are washed extensively over a period of two days.

4.2.2 Methods to determine the amount of immobilized heparin

The toluidine blue assay, applied for our experiment, was reported widely for evaluation of the amount of immobilized heparin. The results are stable and reproducible. In principle I adopted the procedures as published by Hinrichs *et al*. (1997). The washing steps for removing the non-bound toluidine blue are essential for obtaining reliable results. Wissink et al. (Wissink, et al, 2001b) washed their collagen specimen after complexation twice for 5 minutes with water. In our hands this

procedure appeared to be not sufficient. Therefore I washed the specimen with a relatively large volume of distilled water (10 ml per sample) for about 16 h (overnight) and changed the water 5 times. After this extensive washing procedure, virtually no heparin is detected in collagen matrices H1E0. Therefore, I am convinced that all physically absorbed heparin was washed out under the conditions used.

4.2.3 Extent of heparin immobilization as a function of varying EDC/NHS to heparin and heparin to EDC/NHS ratios (w/w)

I first evaluated the heparin immobilisation with gradually increasing weight ratios of EDC/NHS to heparin. Ratios were varied from 0 to 4 with intermediate steps at 0, 0.2, 0.5, 1, 2 and 3. I observed that up to a ratio of EDC/NHS to heparin of 2, higher ratios of EDC/NHS to heparin result in higher the amounts of immobilised heparin. The results are in agreement with the reports of Wissink et al. (Wissink *et al*., 2001b). It seems that by increasing the EDC/NHS-heparin ratio, more reactive N-Hydroxysuccinimidylesters of EDC-activated carboxylic acid groups are introduced per molecule of heparin, which finally results in an increased heparin immobilisation. The maximum level of heparin immobilisation in our experiments reaches 45 µg of heparin per 1 mg of collagen, this was obtained at EDC concentrations of 2 mg per 500 microliter. A further increase of the EDC/NHS to heparin ratio from 2 to 4 does not lead to a further increase of the amount of heparin immobilisation. Apparently the activation of all carboxyl groups of the heparin requires a certain amount of EDC/NHS. This shows that the reaction of EDC activated carboxyl groups of heparin reach a saturation at the ratio of 2:1. Further increases of the ratio do not lead to higher amounts of immobilized heparin. Hence the surplus EDC/NHS will activate carboxyl groups of collagen, leading to an additional cross-linking of collagen instead.

I expected that the increase of heparin concentrations would promote the immobilisation of heparin. The weight ratio of heparin to EDC/NHS was investigated in our experiment. Surprisingly this increase was undeveloped. A possible explanation is that the higher concentrations of heparin may consume more EDC/NHS, therefore, less surplus EDC/NHS for cross-linking. The deduce is confirmed by *in vitro* degradation experiments which demonstrate that with higher heparin to EDC/NHS ratios leads to a smaller resistance to collagenase degradation. In the previous

experiment, the maximal amount of heparin immobilisation is achieved at ratios of EDC/NHS to heparin 2:1.

In conclusion, the extent of the heparin immobilisation largely depends on the increase of EDC/NHS to heparin ratio. Maximal immobilisation is achieved at ratios of 2:1, higher ratios will not promote heparin immobilisation further. Increased heparin to EDC/NHS ratios, however, lead to lower levels of heparin immobilisation and a concomittant decrease of the resistance versus collagenase degradation.

4.3 Evaluation of cross-linking of collagen matrices

Collagen is a degradable material and should stimulate the surrounding tissue to regenerate. It is therefore important to control the degradation of modified collagen matrices. Two methods are applied for evaluation the degradation degree *in vitro*. The first is to determine the resistance of modified samples to bacterial collagenase which is capable of cleaving peptide bonds within the triple helical structure and has a specificity for the Pro-X-Gly-Y sequences, cleaving the peptide bond between X and Gly. In our experiment the in vitro degradation was performed with collagenase from *Clostridium histolyticum*. A gravimetrical method was used to determine the weight-loss of treated collagen matrices. The second method determines the primary free amino acid groups in the (non-)modified collagen matrices by using TNBS. I used the procedure as described by Zeeman et al (Zeeman *et al*., 1999). I introduced a number of modifications due to the different size of our specimens. The reaction period was changed from 2 to 16 h and the introduction of an additional extraction of non-complexed TNBS with ethylether. If the latter extraction step is omitted as reported by Zeeman et al. (Zeeman *et al*., 1999), the observed numbers of free amino groups are far too high.

Our results of the *in vitro* degradation experiments with collagenase show that the resistance towards degradation increases with increasing EDC/NHS to heparin ratios (w/w) (see Figure 11) The relatively sharp drop between H1E0.2 and H1E1 was surprising and might be explained as followed. The degradation percentage of H0E0 and H1E0.2 are almost identical, I conclude that 0.2-0.3 mg of EDC/NHS are consumed for activating all carboxyl groups present in 1 mg heparin. This figure nicely

corresponds to the amount of EDC which I calculated to be required for activating all carboxyl groups. For this calculation I assume that the average molecular mass of heparin is 12500 Da and that the average number of carboxyl groups is 18,75 per molecule. On the basis of these assumptions I calculate that 0,3 mg of EDC are consumed for activating all the carboxyl functions. The presence of 0.5 mg EDC/NHS in the activation mixture of H1E0.5 leaves about 0.2-0,3 mg of EDC for the additional cross-linking of the collagen. This may explain the relatively sharp drop observed between the modification parameters H1E0.2 and H1E1.

The activation of more heparin with the same concentration of EDC, however, leads to less cross-linking since more EDC is consumed for activating the heparin. This implies that heparin to EDC/NHS ratios of 4:1 will result in almost no EDC/NHS left for additional cross-linking of the collagen, although a similar amount of heparin will be immobilised. As the sensitive cross-linking effect is influenced by the ratio of EDC/NHS to heparin, I think it is necessary to compare the net cross-linking effect of EDC/NHS to the results above.

From Figure 14, I conclude that even at the modification parameters H0E0.2 some degree of cross-linking is observed. The degradation percentage of H0E0.2 ranges between H1E0.5 and H1E0.75. This fact may be explained by assuming that about 0.2 mg of EDC/NHS are available for cross-linking with H1E0.5 and H1E0.75. Thus the apparent differences could be produced among the modified sample of H1E0.2, H1E0.5 and H1E1.

The extent of cross-linking can also be evaluated by the method of determination of the number of primary free amino acid groups in the modified collagen matrices (Olde Damink *et al*., 1996). As explained before, the heparin immobilisation and the additional cross-linking occur through the linkage of carboxyl groups of heparin or collagen to adjacent free amino groups in the polypeptide chains of collagen. From Figure19, we can see that there is a gradual decrease of the number of free amino groups. At first sight it may be surprising that the relatively sharp drop in the degradation percentage between the modification parameters is not observed. Since the heparin present in the activation mixture initially will consume about 0.2 mg of EDC/NHS no EDC will be left for additional cross-linking. This explains why there is

almost no change in both the degradation percentage and the number of free amino groups in collagen matrices prepared according to the modification parameters H1E0 (36 per 1000 amino acids) and H1E0.2 (36 per 1000 amino acids). With increasing EDC/NHS concentrations at the constant concentration of 1 mg heparin per 500 microliter, I observe a slow down of the degradation percentage whereas there is a further decrease of the number of free amino groups. Apparently the increase of the EDC/NHS concentration in excess of 1 mg/500 microliter does not lead to a further shrinkage of the collagen fibrils, it does, however, lead to a continuation of formation of intrachenar cross-link bonds. This may explain why the degradation percentage, and the amount of immobilized heparin and the moisture uptake do not change anymore.

In summary, by using the cross-linking agent EDC/NHS, heparin is covalently bound to the polypeptides chains of collagen and the excess reagent additionally cross-links the collagen. The degree of cross-linking of modified collagen matrices increases with increasing EDC/NHS to heparin ratios. The addition of more heparin to the reaction solution leads to less incorporation of heparin as well as less additional cross-linking: whereas the amount of immobilized heparin is reduced to a small extent, the impact on the degradation behaviour is substantial. This means that I am able to produce a wide range of modified collagen matrices incorporating various amounts of heparin and exhibiting different *in vitro* degradation behaviours. For example, if an immobilisation of 35-40 µg heparin/mg collagen is required, I have the option of choosing the following different degradation performances: after a treatment with 40 units collagenase/1ml for 2 h 10% of H1E2, 15% of H2E2, 25% of H2E1, 60% of H3E2 and 65% of H4E2 are degraded (see Figure 23).

Since the extent of physical binding of VEGF may correlate with the amounts of immobilized heparin, the VEGF release may also correlate with the *in vitro* and *in vivo* degradation. I am thus able to control a wide range of VEGF binding and release behaviours which will be of great help for the development of suitably modified collagen matrices with enhanced angiogenic capabilities.

4.4 Moisture uptake

In general, the moisture uptake or the hydrophilicity of collagenous materials decreases with increasing degrees of cross-linking (Weadock *et al*., 1995). In our case, however, the moisture uptake of cross-linked collagen matrices shows increased water binding as cross-linking increased (Figure 20, 21). I postulate that this observation may be due to the modification of the pore structure in the cross-linked collagen matrices. In the absence of additional cross-linking, the degree of cross-links is relatively low and therefore the non-modified collagen matrices have a limited stability. Wet non-modified matrices are easily collapsed after „blotting“ them on a piece of filter paper.

In fact, it was found in cell culture experiments (Marth Markowicz, unpublished) that the non-cross-linked matrices have a higher tendency to collapse. It has been reported that resilience of cross-linked collagen matrices are much better than the non-cross-linked ones (Chvapil, 1977). Comparing the matrices which were cross-linked in the absence of heparin (H0Ex-series) to the matrices which were both heparinized and cross-linked, the latter take up more moisture. Heparin contains a variety of hydrophilic groups such as $R–O^-$, R-OH, $R\text{-}COO^-$, $R\text{-}NH\text{-}SO_3$ and $R\text{-}O\text{-}SO_3$. They may form hydrogen bonds with water molecules, making the heparinized collagen matrices more hydrophilic than their non-heparinized counterparts. These results are in agreement with data reported by Tsai et al., who immobilised heparin to collagen with naturally occurring cross-linking agent genipin (Tsai *et al*., 2001).

It was also reported that increased grafting of hydrophilic groups induces a decrease of fibrinogen adsorption and an increase of albumin adsorption (Papadimitriou *et al*., 2000). Fibrinogen is known to be capable of mediating platelet aggregation and adhesion via interaction with platelet receptors such as GP IIb / IIIa and platelets do not specifically interact with albumin or adhere to albumin-coated surfaces (Amiji and Park, 1993). In addition, it was also reported that the high moisture uptake of sponge-like matrices seems to be dependent on the porosity of the structure (Patel and Amiji, 1996). As far as our experiments are concerned, I find that the cross-linked and heparinized samples are more moisture uptake than the non-modified specimen. Highly hydrophilic matrices, should allow for a more homogeneous seeding of the cells

and a better ingrowth. More hydrophobic matrices will make it more difficult to deliver the cells from the surrounding tissues inside uniformly throughout the matrices (Freed *et al*., 1993). The cross-linked and heparinized samples with high moisture uptake are more stable compared to the untreated ones and therefore may be beneficial for maintaining the desired shape while reserving sufficient free space for formation of new tissue via cell proliferation and extracellular matrix secretion. In addition, it was found that the increased moisture uptake capacity may beneficially influence tissue remodelling by promoting open matrix structures (Cha *et al*., 1991). Therefore, I consider that the moisture uptake in modifying matrices represents an important factor for influencing the angiogenic potential.

4.5 Evaluation of the angiogenic potential of modified collagen matrices

As outlined earlier the main objective of this investigation is to deliver a contribution to the development of collagen matrices with enhanced angiogenesis for a better oxygen and nutrient supply. The angiogenic potential was evaluated with two different in vivo assays: exposure of collagen matrices to the *chorioallantoic* membrane of the chicken embryo (CAM assay) and subcutaneous implantation on the back of rats.

4.5.1 Angiogenic effects determined with the CAM assay

4.5.1.1 Angiogenic effect of cross-linking collagen matrices

I found that cross-linked collagen matrices promote the angiogenic potential *in vivo*. With higher degrees of cross-linking, the stronger angiogenic potential resulted (Figure 26). At the present time it is difficult for us to fully explain this phenomenon. In a collaborative effort, coworkers were able to show that a similar effect is observed when vascular endothelial cells are exposed to the modified collagen matrices *in vitro* (Marth Markowicz, unpublished) . I may, however, postulate that the positive angiogenic effect is due to the lower antigenicity of the cross-linked samples (Furthmayr and Timpl, 1976) and thus will result in less foreign body reaction, which in general creates a layer of non-vascularized structures that encapsulate the implants and hinder the ingrowth of surrounding cells and vessels. In addition, the cross-linked matrices have an improved structural stability, preventing them from collapsing, which may be beneficial for surrounding vascular endothelial cells migrating in. I furthermore

envision that the higher hydrophilicity of the cross-linked samples will allow for a more tissue liquid to flow inside the samples homogeneously and thus should be easier for the surrounding cells migrating inside.

It is true that compared to the control experiments (no matrices being present on the *chorioallantoic* membrane), the non-modified collagen matrices (H0E0) have some angiogenic potential *in vivo*, though the effect is much weaker than the modified ones (Figure 24). I think this is because of the inflammatory response due to the larger antigenicity of non-modified collagen matrices. Upon implantation of the samples, inflammatory cells will gather around and produce angiogenic growth factors such as bFGF, PDGF which in turn stimulate angiogenesis. Therefore, I think that the effect on the angiogenic potential of non-modified samples is different from that of modified ones which decrease the antigenicity and thus inflammatory response *in vivo*. Therefore, it is of interest to investigate the mechanisms which determine the angiogenic potential of cross-linked collagen matrices in the future.

4.5.1.2 Angiogenic effect of immobilizing heparin in collagen matrices

In our experiment, I find that the angiogenic effect of heparinized cross-linked matrices becomes gradually stronger with the increased amount of heparin immobilisation. Our results shown in Figure 25 suggest that the angiogenic potential as expressed as the increase of the number of capillaries correlates with the amount of immobilized heparin in the specimen. In order to test this hypothesis I also investigated the angiogenic effect of cross-linking collagen matrices in the absence of heparin.

Considering that cross-linked collagen matrices can also be angiogenic potential, I compared the results of angiogenic potential by cross-linking alone without heparin immobilization (H0-series), heparin immobilization with cross-linking (H1-series) , and the amount of heparin immobilsation (µg heparin/ mg collagen). I found that the increased number of capillaries correlates more with the amount of cross-linking than the degree of heparin immobilisation. From Figure 27, it seems to be concluded that heparin has almost no angiogenic effect in *chorioallantoic* membrane experiments.

But taking into account the results of the *in vitro* degradation experiments showing that the cross-linking extent of the H0-series is quite higher than that of H1-series (see Figures 15 and 16), I observe that the degradation behaviour of H0E0.2 is similar to that of H1E1. If I compare, the angiogenic potential of matrices H0E0.2 with H1E1, the extent of cross-linking are similar, I conclude that the amount of immobilized heparin may have a small angiogenic effect (see Figure 28). Our results therefore partly support reports of van Wachem et al., who found that the immobilisation of heparin in collagen matrices leads to an increased vascularization when implanted in rats (van Wachem *et al*., 2001). They explained this observation by postulating that the heparinized collagen matrices trap heparin binding growth factors from the surrounding tissue, which results in local induction of capillary vessel formation (Meddahi *et al*., 1994).

In our experiments, the increase in the amounts of immobilized heparin is always accompanied by a concomitant additional cross-linking of the collagen matrices. This complicates the investigation of the angiogenic effect of immobilized heparin. I am currently changing the modification procedure in order to be able to segregate the two effects.

In summary, the modification procedure which incorporates heparin collagen matrices exerts an important angiogenic effect *in vivo*. The effect is most likely due to the additional cross-linking. The immobilised heparin may have a small additional effect. A different modification procedure should make it possible to better discriminate between the two effects.

4.5.1.3 Angiogenic effect of loading VEGF to heparinized collagen matrices

As mentioned in 1.5.2, VEGF is a mitogen specific to vascular endothelial cells, as well as a potent stimulator of angiogenesis *in vivo.* I therefore added VEGF to heparinized and non-heparinized collagen matrices and investigated the angiogenic effect by using the *chorioallantoic* membrane assay. As a first result I observed that the addition of

increasing amounts of VEGF (30 – 300ng) to the collagen matrices prior to the application on the *chorioallantoic* membrane, does not lead to an increase of the number of capillaries. Apparently the addition of 30 ng VEGF is already sufficient for provoking a maximal effect. It was reported that an overdose of VEGF may lead to malformation and nonfunctional capillaries (Springer *et al*., 1998), I , however, did not observe such a development upon addition of 300 ng of VEGF. This may be due to the procedure with which I applied VEGF on the CAM assay.

In our experiments, different amounts of VEGF were loaded to non-modified collagen matrices and then mounted on the *chorioallantoic* membrane for an incubation period of 7 days. I conclude that most of the added VEGF will diffuse out quickly after implantation and may be then denatured rapidly due to the very short *in vivo* half life time. Therefore, the high VEGF loading does not necessarily lead to a corresponding increase of the angiogenic potential and neither results in deformed and nonfunctional capillaries.

In summary, it may be concluded from these experiments that even very small amounts of VEGF (30 ng) apparently produce substantial angiogenic responses *in vivo*. Therefore, it is necessary to preload VEGF on the modified samples to acquire sustained release. I choose 30ng and 100ng of VEGF for further experiments.

Previously, preloading of EDC/NHS cross-linked collagen matrices with and without heparin immobilisation with hVEGF165 was applied to cell culture assay and resulted in the improvement of the proliferation of hUVECs in our laboratory cooperated by Marth Markowicz . In this study, I further evaluated the angiogenic potential of proloading VEGF to (non-)modified samples and compared to the effects of none VEGF preloading.

From the macroscopic observation, I learned that the percentage of spoke-like vessel structure directed towards the implants, increases when collagen matrices H0E1 and H1E1 are loaded with VEGF. I thus conclude, that VEGF induces a directed vessel growth towards the implants .

The loading of VEGF to heparinized matrices results in a substantially angiogenic effect compared to the loading of VEGF to non-heparinized collagen matrices. (see Figure 30). The maximal response was observed when H1E1 matrices were loaded with VEGF. Matrices with higher amounts of immobilized heparin did not lead to stronger angiogenic effects. Apparently the loading of VEGF to heparinized matrices leads – when in contact with the *chorioallantoic* membrane – to a more slowly and sustained release provoking the observed angiogenic effect. Comparing of the results in the H0E1 preloading with VEGF to those of H1E1 preloading with VEGF shows that angiogenic potential is stronger in H1E1 preloading. It was reported that the physical binding of VEGF to the immobilized heparin may promote their bioactivity and protect them from denaturation *in vivo* (Sommer and Rifkin, 1989).

4.5.2 Angiogenic affect as determined by subcutaneous implantation in rats

From the results of the hemoglobin content determination in explanted specimen, I conclude that the H1E1 matrices loaded with 300 ng VEGF showed the largest angiogenic effect. In our view, the hemoglobin content of the explants correlates with the extent of newly formed vessels within the implants. This observation may be more objective as compared to the counting of capillaries in the CAM-assay since it represents the total amount of blood flow within the collagen matrices.

Collagen matrices, which were cross-linked only (H0E1), show - as observed by the increase of the hemoglobin content - a substantially higher angiogenic potential . I deduce that this effect may be due to i) the larger pore size, ii) the more stable and rigid structure and iii) the increase in hydrophilicity. Loading of 300 ng VEGF to these matrices only leads to a relatively small increase of the angiogenic potential. This effect may be explained by the fact that VEGF cannot physically bind to the cross-linked collagen and thus rapidly diffuses into the surrounding tissue.

The collagen matrices which were both heparinized and cross-linked (H1E1) demonstrate the highest angiogenic effects in the *chorioallantoic* assay both loaded or non-loaded with VEGF. Matrices (H1E1) loaded with VEGF show the highest

angiogenic effect. These results were confirmed by the colour intensity of the collagen specimen explanted after 15 days. A clear cut increase in the colour intensity is observed in the following order: H0E0, H0E1 and H1E1. In all cases the intensity of the colour increases when the matrices were loaded with 300 ng VEGF prior to the implantation. (see Figure 31). This means that the vascularization was maximal inside H1E1 loaded with VEGF, the lowest degree of vascularization was observed with the H0E0 specimen in the absence of VEGF.

The general conclusion then is that the loading of VEGF to heparinized collagen matrices results in the highest angiogenic effect, this observation may be due to a slow and sustained release of VEGF and a promotion of the bioactivity *in vivo*.

4.6 Conclusions

4.6.1 Characteristics of modified collagen matrices

(1). Heparin was covalently incorporated into collagen with the cross-linking agents EDC/NHS. The extent of heparin immobilization increases with increasing EDC/NHS concentrations and EDC/NHS to heparin ratios.

(2). The *in vitro* degradation also strongly depends on the EDC/NHS concentrations and EDC/NHS to heparin ratios. Higher EDC/NHS concentrations and EDC/NHS to heparin ratios lead to lower *in vitro* degradation rates. The results are confirmed by determination of the free amino groups in the modified collagen matrices.

(3). The moisture uptake of modified collagen matrices increases with increasing cross-linking of collagen and the extent of heparin immobilisation.

4.6.2 Angiogenic potential

1. The modification of collagen matrices with EDC/NHS generally leads to an increase of the angiogenic potential. The cross-linking apparently has the most important effect, the additional effect of the heparin incorporation is relatively small.

2. The loading of VEGF to the modified matrices results in an additional positive effect on the angiogenesis. The largest additional effect was observed with VEGF loading to heparinized matrices.

3. The loading of increasing amounts of VEGF to non-modified collagen matrices (H0E0) does not lead to an increased number of capillaries.

4. The angiogenic potential as determined by the hemoglobin content of explanted matrices increases with increasing cross-linking. The loading of VEGF has an additional effect on the angiogenesis, again the largest additional effect was observed with the heparinized matrices.

4.7 Future prospects

The development of a tissue substitute that can be used by the plastic surgeons and other specialists is our main goal. Such tissue substitutes should be able to mediate angiogenic responses *in vivo* and thus help to enhance cell survival and tissue ingrowth. Ultimately the tissue substitute should be replaced by regenerated, completely natural tissue.

Based on the results obtained in the present investigation, the following topics further should be investigated in the future.

1. The binding of VEGF to modified collagen matrices as well as its release should be evaluated in more detail by ELISA methods, or if necessary by radioactive labeling of VEGF. It is necessary to better understand the correlation of the release rates and the angiogenic effects *in vitro* and *in vivo*.
2. Since the sterilisation procedures may change the characteristics of modified matrices and as a consequence the angiogenic potential *in vitro* and *in vivo*, it is of interest to investigate the effects of the various sterilisation procedures.
3. I evaluated the angiogenic potential by means of the *chorioallantoic* assay over a period of 7 days. At the end of this period, most of the modified samples are not

degraded, which means that there may still be some VEGF left and their effects are not being evaluated yet. Therefore, longer evaluation periods may be considered in the future.

4. I observed that collagen matrices which were cross-linked only show an increased angiogenic potential *in vitro* and *in vivo*, which means that the change of pore structure may be a factor of importance for modulating angiogenesis. Therefore, I think the porous structure of the various modified collagen matrices should be evaluated by electron microscopy in order to better understand the correlation between the structural changes and their angiogenic effects.
5. Additional animal experiments should be performed in order to quantify the vessel ingrowth into the modified matrices. It may furthermore be interesting to evaluate the degradation behaviour of modified collagen matrices *in vivo* in animal model experiments. If possible, I think the amount of newly formed collagen inside the matrices should be evaluated in the future. The new formed collagen inside matrices means that the ability for such matrices to supply for the survival of tissues ingrowth from surrounding.
6. Finally, the mechanics behind the changes of the angiogenic potential of modified matrices should be investigated. One approach would be to investigate which endothelial cell genes are switched on upon contact of these cells with modified matrices by using RT-PCR.

5 Summary

Introduction

Tissue engineering has been recognized as a viable concept for repair of tissue defects. Ideally engineered tissues should be non-toxic, biocompatible, biodegradable and angiogenic. The enhancement of the angiogenic capabilities is still a challenge to us. Collagen is one of the major components of natural extracellular matrices and is characterized by a high biocompatibility and a biodegradation without toxic byproducts. Heparin, a well-known and time-honored antithrombotic drug, was also found to regulate angiogenesis. Further, it has high affinity to angiogenic growth factors, such as bFGF and VEGF. Based on these knowledge, collagen matrices were modified by incorporation of different amounts of heparin and by cross-linking with EDC/NHS. A variety of collagen matrices were produced according to a restricted number of modification parameters. These matrices were characterized biochemically and physicochemically. Finally their angiogenic potential were evaluated by exposure to the chorioallantoic membrane of the chicken embryo and by subcutaneous implantation in rats.

Methods

Collagen matrices, kindly provided by Dr. Suwelack Skin & Health Care AG, Billerbeck, Germany, were cut into cubes of either 5*5*5 mm (3,8-4,2 mg) or cylinders of 10 mm in diamter and 2 mm in thickness (3,8-4,2 mg). Heparin was covalently incorporated with EDC/NHS. Initially carboxylic acid groups of the heparin are activated with EDC/NHS at fixed EDC to NHS weight ratios of 1 to 0,6. The weight ratios of EDC/NHS to heparin (w/w) varied from 0.2 to 4 and *vice versa*. After activation, collagen specimen were immersed in the activation solution and incubated for 4h to immobilise heparin and washed extensively subsequently. Samples were frozen, lyophilized and stored at room temperature for further characterization: determination of immobilised heparin (toluidine blue assay), free amino groups (TNBS assay), degradation behavior (in vitro degradation with collagenase) and moisture

uptake. VEGF loading was performed under sterile conditions, depending on the application different amounts of VEGF were loaded.

Angiogenic effects were evaluated by exposure of the modified collagen matrices to the chicken embryo *chorioallantoic* membrane (CAM assay) and subcutaneous rat tissue. The number of capillaries in the *chorioallantoic membrane* were counted, the macroscopic responses provoked by the contact with modified matrices were evaluated. In the animal model experiments the angiogenic potential were deduced from the hemoglobin contents in the explants.

Results

1 Characterisation of heparinized matrices

1.1 The extent of immobilized heparin and the moisture uptake increase with increasing EDC/NHS to heparin weight ratios (w/w). The resistance to collagenase degradation and the number free amino groups progressively decrease. Increase of heparin to EDC/NHS ratios did not result in higher extents of immobilized heparin, but in a sharp decrease of the resistance towards degradation.

1.2 The moisture uptake of modified collagen matrices increases both with the extent of immobilized heparin and the degree of cross-linking.

2 Angiogenic potential

2.1 Vessels show a spoke-like structure growing towards the modified matrices. The higher the EDC/NHS to heparin weight ratio, the slower the degradation degree of the matrices, which is in accordance with the *in vitro* collagenase assay.

2.2 The increased number of capillaries in CAM were observed with heparinised collagen matrices and with collagen matrices which were cross-linked only.

2.3 No significant differences of the death rates of embryos were observed between the control group of and the groups of non-modified and modified collagen matrices.

2.4 Loading of VEGF to heparinized matrices results in a strong promotion of angiogenic potential in comparison with loading the same amount of VEGF to non-modified and non-heparinized matrices.

2.5 Heparinized matrices loaded with VEGF had the highest angiogenic potential as deduced from the hemoglobin washed out from these explants.

Conclusions

1

In the present study I developed a procedure by which heparin is covalently incorporated into collagen matrices. Modified collagen matrices are characterized by a controllable biodegradability, heparin immobilisation and an improved moisture uptake. They are furthermore non-toxic, have high affinities towards the angiogenic growth factor VEGF and may represent biomaterials meeting specific requirements of tissue engineering.

2

Modified matrices are characterized by substantially enhanced angiogenic capabilities as evaluated from *in vivo* experiments. This increase apparently results from changes in the microstructure of the collagenous matrix and from a sustained release of VEGF from a VEGF-pool physically immobilized to covalently incorporated heparin.

3

These modifications may be of prime importance for future developments in the field of skin substitutes, in which the rate of vascularization is essential for the survival of seeded autologous or stem cells.

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7 Abbreviations

bFGF	Basic Fibroblast Growth Factor
CAM	*Chorioallantoic* Membrane
DHT	Dehydrothermal Treatment
EC	Endothelial Cell
ECM	Extracellular Matrix
EDC	N-(3-Dimethylaminopropyl)-N'-Ethylcarbodiimide
EDTA	Ethylendiamin-Tetraacetate
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
FBR	Foreign Body Reaction
Flt-1	Fas-Like Tyrosine Kinase-1
GA	Glutaraldehyde
GAGs	Glycosaminoglycans
Hb	Hemoglobin
Hep-COOH	Carboxyl Groups of Heparin
HUVEC	Human Umbilic Vascular Endothelial Cell
KDR	Kinase insert Domain-containing Receptor
MES	2-Morpholinoethane Sulfonic Acid
NHS	N-hydroxysuccinimide
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PBS	Phosphate Buffer Solution
PDGF	Platelet-Derived Growth Factor
PEG	Poly Ethylene Glycol
$rhVEGF_{165}$	Recombinant Human Vascular Endothelial Growth Factor (splice variant with 165 amino acids)
SD	Standard Deviation
SS	Succinimidyl Succinate
TGF	Transforming Growth Factor
TNBS	Trinitrobenzenesulfonic Acid
VEGF	Vascular Endothelial Growth Factor

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7 Modulation of angiogenic potential of collagen matrices by covalent incorporation of heparin and loading with VEGF
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