

Covalent coupling of growth factors to collagen matrices: a novel
development towards a tissue substitute
with enhanced angiogenesis

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1. Introduction

1.1 Plastic surgery

The art of plastic surgery is the restoration of form and function with the resultant improvement in patient's quality of life and aesthetic outcome. It often involves the formation of flaps (a section of living tissue that carries its own blood supply and is moved from one area of the body to another to repair a deficit of tissues) to reshape or mold the defect and thus to approximate the original shape.

Plastic surgery has always been a technology-driven surgical discipline, and there has been a remarkable evolution over the last 25 years with an increased understanding of anatomy leading to a whole host of new and more reliable flaps. Meanwhile, a better understanding of the blood supply of the tissues and the development and maturation of microsurgery have led to the full fruition of anatomic principles (Mustoe and Han, 1999).

However, restoration without destruction still challenges plastic surgeons. Current methods for the repair and replacement of diseased tissues meet formidable barriers in concept, donor scarcity, impairment to the donor site, morbidity and economics. Projection indicates that the gap between tissue supply and demand will even continue to widen in the future (Peters et al., 1998).

1.2 Tissue engineering

Major advances in surgery often come from a marriage of basic and clinical sciences. Tissue engineering may provide an alternative to existing therapies, which would allow the restoration of function by creating a completely natural tissue substitute, with the necessary mechanical and metabolic features, *in vivo*.

Tissue engineering is a relatively new and emerging interdisciplinary field that applies the knowledge of bioengineering, life sciences, and the clinical sciences towards solving critical medical problems of tissue loss and organ failure, thus to create functional substitutes for damaged tissues by combining engineering principles with life sciences (Patrik et al., 1999) (Nerem, 1992). It involves applying engineering principles of transport and reaction phenomena as well as methods of analysis

towards understanding the complex biological processes that occur in tissue development and repair. Frequently, knowledge of molecular phenomena and cellular interactions with surface, biochemical, and mechanical environments are employed. Though still in its experimental stages, the use of tissue engineering to regenerate organs and tissues has the potential to affect the length and quality of life for millions.

The essence of tissue engineering is the use of living cells together with either natural or synthetic extracellular components in the development of implantable parts or devices to replace the diseased tissue function, result in the growth of functioning tissue and the eventual integration of such a device into a host.

Investigators have attempted to engineer almost every human tissue by using various kinds of biomaterials (Mooney and Mikos, 1999). Among them, Integra (Integra LifeScience, Plainsboro, NJ) is a notable example. Integra ® Dermal Regeneration Template is a bilayer membrane system for skin replacement. Upon adequate vascularization of the dermal layer, a thin, meshed layer of epidermal autograft is placed over the “neodermis”, thereby closing the wound.

However, formation of the neodermis typically takes about 4-6 weeks by clinical observation (Integra Users Symposium, Dublin, 2000), which seriously limits the number of patients who can be treated successfully.

1.3 Angiogenesis and growth factors

The kinetics and extent of the neovascularization process, are critical for the survival and subsequent function of autologous grafts. The same is no less true for any tissue-engineered substitutes. Engineered tissues more than a few millimeters thick require blood vessels to grow into them and supply nutrients. A vascular network must be rapidly formed throughout the new tissue to ensure an adequate supply of essential elements (e.g., oxygen). This process of generating new microvasculature, termed angiogenesis, is a process observed physiologically in development and wound healing.

Angiogenesis is mediated by growth factors, polypeptides that transmit signals to control cell growth and activity. Growth factors are capable of exerting their influence at concentrations within the picomole range. The use of growth factors has become

an important aspect of tissue engineering because it allows modulation of cellular function and tissue formation at the afflicted site (Lu et al., 2000). With the increased availability of growth factors derived from cultured human cells and expanded through recombinant technologies, coupled with an increasing understanding of their functions and clinical applications, the need for useful pharmaceutical forms is becoming more and more apparent. Although the applications of growth factors have the potential to enhance the tissue substitute survival, the delivery of growth factors poses a major challenge. Controlled studies of their action in research and clinical work have been hampered, because it is difficult to administer the growth factors over long periods of time in bioactive form at a physiological dose. Previous efforts at incorporating growth factors (e.g. basic fibroblast growth factor, bFGF) within a sustained release vehicle have only been partially successful (Edelman et al., 1991).

1.4 Aim of this study

In this study, we focused on taking collagen sponge as the substance of tissue substitute. It has been widely reported that biodegradable collagen sponges with a porous and fibrillar structure can be used as scaffold for wound tissue (Doillon et al., 1994) (Boyce et al., 1988), because collagen-based materials in the design and preparation of tissue substitutes offer a high degree of plasticity that is needed to meet application criteria.

However, as mentioned before, a primary factor which limits engineering tissues of substantial size is the lack of nutrients of blood supply (Peters et al., 1998). We suppose that one solution to this limitation is to stimulate the rapid development of a vascular network within three-dimensional tissue engineering matrices. A variety of angiogenesis-inducing growth factors have been identified. Among them, vascular endothelial growth factor (VEGF) is an endothelial cell specific mitogen and a potent stimulator of angiogenesis *in vivo* (Peters et al., 1998) (Plate and Warnke, 1997). We suppose that it might be possible to promote rapid angiogenesis in the implanted tissue substitute by modulating the local concentration of VEGF. Thus VEGF can stimulate the endothelial cells, those are already present in the patient's body, to migrate from the surrounding tissue, to proliferate, and finally differentiate into blood vessels.

A major challenge is to achieve a high VEGF loading efficiency sufficient for prolonged therapeutic effect. To overcome rapid diffusion and clearance from the implant site and also to increase stability, VEGF is covalently bound to collagen sponges by means of the homobifunctional cross-linking agent bis-(succinimidylsuccinate)-polyethyleneglycol (SS-PEG₃₄₀₀-SS; Shearwater Polymers). Collagen sponges for controlled release are furthermore cross-linked with SS-PEG₃₄₀₀-SS for slowing down rapid degradation. The biological behavior of collagen sponges, as well as tissue and cell reactions after the treatment with SS-PEG₃₄₀₀-SS will be discussed.

In conclusion, the long term aim of the present study is to develop a tissue substitute that can be used by the plastic surgeons and other specialist. This tissue substitute should be able to selectively induce a growth factor mediated angiogenic response. It should help to determine the three-dimensional morphology, to increase cell survival, to provide initial mechanical stability, to support tissue ingrowth, and to aid in the formation of tissue structure, and ultimately it should be replaced by a regenerated extracellular matrix (ECM) to form normal, completely natural tissue.

2. Research background

2.1 Collagen as a tissue substitute

The success of the tissue substitute is highly dependent on the properties of the implant material, requiring minimally that it should be biocompatible, easily sterilizable, and degradable into harmless products that can be metabolized or excreted. In the search for biomaterials that are versatile and compatible with human tissues, considerable interest has been maintained in collagen based materials for repair and replacement of soft body tissues (Babensee et al., 1998).

Collagen represents the chief structural protein accounting for approximately 30% of all vertebrate body protein. More than 90% of the extracellular protein in the tendon and bone and more than 50% of the skin consists of collagen. The main function of collagen is mechanical reinforcement of the connective tissues of vertebrates.

2.1.1 Medical applications of collagen

Collagen is of great interest as a biomaterial because of the following features (Anselme et al., 1990) (Matton et al., 1985) (Chvapil, 1977):

1. It represents one of the major components of natural extracellular matrices with low toxicity, low immunogenicity and high biocompatibility which is attributed to the similarity in the amino acid sequence between species and the low content of aromatic amino acids
2. It has abundant sources
3. It is a suitable substrate for cell attachment through integrin clusters
4. Its chemotactic effect accelerates infiltration of surrounding cells
5. It affects homeostasis through inducing platelet aggregation
6. It can be biodegraded without toxic byproducts

With the development of processing technology collagen can now be made into different forms like gels, films, tubes, particles or sponges (Friess, 1998), all of which

have found use in medical practice, for example as a scaffold for skin, connective tissue and peripheral nerve tissue engineering. Black *et al.* (Black *et al.*, 1999) (Black *et al.*, 1998) seeded fibroblasts with or without endothelial cells onto collagen/chitosan sponges to construct dermal equivalent. Nehrer *et al.* (Nehrer *et al.*, 1997) used porous collagen matrices as a scaffold for cultured chondrocytes to engineer cartilage and found a marked influence of collagen type and pore characteristics on the phenotypic expression of seeded chondrocytes. Furthermore, attempts have been made to apply these forms of collagen as a drug delivery system in a variety of applications such as in ophthalmology (Rubin *et al.*, 1973), wound and burn dressing. Friess recently reviewed the use of collagen for delivery of antibiotics, local anesthetics, steroids, chemotherapeutics, growth factors as well as large proteins (Friess, 1998). Controlled release of bioactive molecules such as cytokines and growth factors has become an important aspect of tissue engineering because it allows modulation of cellular function and tissue formation at the afflicted site (Friess *et al.*, 1999).

2.1.2 Structure of collagen and natural cross-linking

The individual polypeptide chains of collagen contain all the 20 different amino acids and the precise composition varies between different tissues. The variation in specific amino acid sequences which leads to the variation of the length of the helix and the nature and size of the non-helical portions giving rise to different types of collagen labeled as Type I, Type II up to Type XIX. Among them, collagen type I is the most abundant and the majority of collagen materials for biomedical applications are based on this type (Friess, 1998). Due to these reasons, the paragraph below is limited to collagen type I.

The name collagen is used as a generic term to cover a wide range of protein molecules that form supramolecular matrix structures. They share the basic texture of three individual alpha-helices that are cross-linked biosynthetically and fold to form a triple helix (tertiary structure) with a molecular weight of approximately 300 kD, a length of approximately 300 nm and a diameter of 1.5 nm.

This triple-helix generates a symmetrical pattern of three left-handed helical alpha-chains, which consist of about 1000 amino acid residues. These chains are twisted

with a distance of 2.91 Å, with a relative angle of -110° , making the number of residues per turn 3.27 and the distance between each third glycine 8.7 Å. Collagens are generally characterized by a relatively high content of hydroxylysine and hydroxyproline. These amino acids are formed post-translationally. The presence of the cyclic amino acids, Proline (Pro) and Hydroxyproline (Hyp) imparts rigidity and stability of the coil. Glycine (Gly), the smallest amino acid, must be in every third position in order to create the right-handed triple helix (Figure 1).

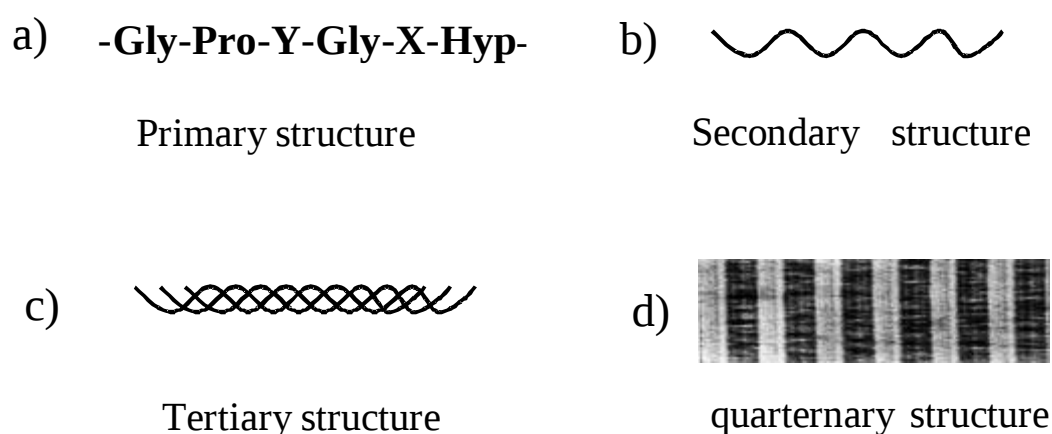


Figure 1 The molecular architecture of the fiber forming collagen.

Furthermore, the hydroxyl groups of Hyp residues are involved in hydrogen bonding and are important for stabilizing the triple-helix structure and two hydrogen bonds per triplet are found. The two hydrogen bonds formed are: one between the NH-group of a glycy residue and the CO-group of the residue in the second position of the triplet in the adjacent chain, and one via the water molecule participating in the formation of additional hydrogen bonds with the help of the hydroxyl group of Hyp in the third position. Such a 'water-bridged' model of the triple helix has been confirmed by physicochemical studies of the collagen molecule in solution and is supported by the observation that the thermal stability of the helix is dependent on the content of Hyp and not of Pro. In addition, model studies showed that the Gly, Hyp and Pro are the triple-helix forming amino acids and that only molecules which contain the triplets Gly-Pro-Hyp are able to form this special helical structure (Zeeman, 1998a).

The mechanical and chemical stability of collagen derives from intra- and intermolecular cross-links. Intramolecular cross-links form between two α -chains of the non-helical section of the same molecule by aldol condensation of two aldehydes, which are formed by oxidation of lysyl amino groups through the action of lysyl oxidase. Intermolecular cross-links occur between the telopeptide region of one collagen molecule and the helical region of a quarterly staggered, adjacent molecule. The bridges between two different tropocollagen molecules result from aldimine formation between aldehyde residues and ϵ -amino groups presented by lysine and hydroxylysine. Thus, through specific self-aggregation and cross-linking, collagen can form fibers with unusual strength and stability (Friess, 1998).

2.1.3 Collagen degradation and exogenous cross-linking of collagen

Collagen is particularly resistant to attacks by neutral proteases, probably due to its function as the primary structural protein in the body. At neutral pH only specific collagenases cleave the native helix. Fibrils as aggregates of collagen are degraded starting from the exterior. Collagenase binds tightly to triple-helices at or near the surface, whereas molecules in the interior become accessible to enzymes in the course of the progressive degradation from the outside. After the triple-helix is cracked, further degradation of the collagen molecules is facilitated by enzymes such as gelatinases and non-specific proteinases which cleave the primary fragments into small peptides and amino acids. Elastases and cathepsins assist in degradation based on their ability to digest non-helical telopeptide regions. Absorption of exogenous collagen *in vivo* is therefore a complex multi-enzyme process and it is not always clear which is the predominant route. Usually the *in vivo* degradation is simulated *in vitro* by incubation with bacterial collagenase, cathepsin, pepsin or trypsin.

Collagen which did not receive a special treatment degrades immediately. The biodegradation rate of collagen has to be customized based on the specific application, in order to maintain the mechanical strength of the transplant and to avoid collapse or stress shielding (van Wachem et al., 1999) (Zeeman et al., 1999a) (Zeeman et al., 1999b). For tissue augmentation and drug delivery system an implant has to maintain its scaffolding properties while it is gradually replaced by host

collagen, and the degradation rate should be optimized to allow transplanted or ingrown cells to proliferate and secrete their own extracellular matrix, while the polymer scaffold can vanish after a desired time period. Thus it is necessary to introduce exogenous cross-links into the collagen molecular structure.

The homobifunctional agent glutardialdehyde (GDA) has been widely used to cross-link biomaterials, but it may release toxic components during *in vivo* biodegradation, and it may modify the biological properties of collagen (Zeeman et al., 1999) (Doillon et al., 1994).

2.2 Angiogenesis and Vascular Endothelial Growth Factor (VEGF)

Angiogenesis is controlled through the interaction between soluble growth factors and insoluble extracellular matrix (ECM) molecules. Soluble mitogens act over long distances to trigger capillary endothelial cell growth whereas ECM acts locally to modulate cell sensitivity to these growth stimuli in the local tissue microenvironment (Ingber et al., 1995) (Ingber and Folkman, 1989).

Investigations by Judah Folkman (Folkman et al., 1979) have shown that cells already in the body could be coaxed into producing new blood vessels. His work aimed at the prevention of cellular growth in the form of cancerous tumors, but today this concept is exploited by tissue engineers: since the engineered tissues as vascular grafts do not have angiogenic capability by themselves. The introduction of biological factors could enhance angiogenesis in the prosthesis tissues (Fournier and Doillon, 1996). For example, exogenous growth factors may be applied to polymers specifically designed to ultimately yield the most efficacious microenvironment to optimize the histologic tissue response.

Growth factors can generally be defined as proteins that promote proliferation and migration of cells, by interaction with specific cell-membrane receptors (Wissink et al., 2000a). They can either stimulate or inhibit cell division, differentiation, migration or gene expression, depending on the cells involved (Nimni, 1997). Among them, Vascular Endothelial Growth Factor (VEGF) plays a central role in the regulation of vasculogenesis.

VEGF is a potent and specific mitogen for endothelial cells derived from small or large vessels but is apparently devoid of appreciable mitogenic activity for other cell

types (Ferrara and Alitalo, 1999) (Neufeld et al., 1999) (Vernon and Sage, 1999) (Senger et al., 1996) (Keyt et al., 1996). The reported activities of VEGF include stimulation of endothelial cell growth, angiogenesis, and capillary permeability. VEGF is synthesized and secreted by many differentiated cells in response to a variety of stimuli including hypoxia. VEGF is expressed in a variety of tissues as multiple homodimeric forms (121, 165, 189, and 206 amino acids/monomer) resulting from alternative RNA splicing (Keyt et al., 1996). Little is known about the regulation of VEGF isoform expression but these isoforms are not necessarily produced coordinately. VEGF₁₆₅ and VEGF₁₂₁ appear to be the most abundant forms of VEGF (Klagsbrun and D'Amore, 1996). In our study we focus on the application of VEGF₁₆₅ since, this variant is up to 100-fold more potent than VEGF₁₂₁ (Keyt et al., 1996). Human VEGF₁₆₅ secreted by a variety of vascularized tissues is a 38.2 KDa homodimeric protein consisting of two polypeptide chains with 165 amino acids each. The sequence of VEGF₁₆₅ is shown in Figure 2.

MNLLSWVHW	SLALLYLHH	AKWSQA ¹ APMA	EGGGQNHHEV ¹¹
1	11	21	31
VKFMDEVYQRS	YCHPIETLVD	IFQEYPDEIE	YIFKPSCVPL
41	51	61	71
MRCGGCCNDE	GLECVPTES	NITMQIMRIK	PHQGQHIGEM
81	91	101	111
SFLQHNKCEC	RPKKDRARQE	KKSVRGKGKG	QKRKRKKSRY
121	131	141	151
KSWSVPCGPC	SERRKHLFVQ	DPQTCCKCCK	NTDSRCKARQ
161	171	181	191
LELNERTCRC	DKPRR		
201	211		

Figure 2 Translated sequence of VEGF-gene, amino acids 1-26 represent the signal peptide, whereas the amino acids 142-165 are removed by alternative splicing from the primary transcript. The amino acids in the shaded sequence parts form the VEGF₁₆₅ polypeptide.

VEGF binds to high affinity receptors on endothelial cell surfaces. Two so-called tyrosine kinase receptors have been shown to be high affinity receptors for VEGF. They are the 180 kDa fas-like tyrosine kinase (Flt-1) and the 200 kDa kinase insert

domain-containing receptor (KDR). Of the two tyrosine kinase VEGF receptors, KDR appears to be the major transducer of the VEGF signal in endothelial cells (Shen et al., 1998) (Klagsbrun and D'Amore, 1996).

We speculate that it may be possible to promote angiogenesis at the site where the collagen sponge is implanted by enhancing the local concentration of VEGF. Since VEGF is the only growth factor known to act solely on endothelial cells (Neufeld et al., 1999) (Dvorak et al., 1995), it will not stimulate non-endothelial cells, such as the proliferation of fibroblast, which are suspected to cause scar formation.

A high VEGF loading capacity that is sufficient for attaining a sustained and controlled VEGF release is desired for attaining an enhanced angiogenesis.

2.3 Controlled release of proteins from various carrier materials

Engineering new tissues utilizing biodegradable polymer matrices as the carrier of angiogenic growth factors is an attractive approach. And the localized, controlled delivery of these growth factors from a matrix may allow an enhanced vascularization of engineered tissues (Gaertner and Offord, 1996) (Cote and Doillon, 1992).

To prolong the effect of protein drugs, many studies are being conducted to control the release of proteins from various carrier materials:

King and Patrick (King and Patrick, 2000) took Poly(DL-Lactide-co-glycolide) (PLGA)/polyethylene glycol (PEG) microspheres as the carrier to control delivery of VEGF or transforming growth factor beta1 (TGF-beta1) (Lu et al., 2000). They coencapsulated VEGF or TGF-beta1 and bovine serum albumin (BSA) into microspheres fabricated from PEG and PLGA using a solid-encapsulation/solvent extraction technique. The microspheres released biologically active VEGF or TGF-beta1 (King and Patrick, 2000) (Lu et al., 2000) (Peters et al., 1998).

Sano et al. (Sano et al., 1998) took collagen as the carrier of the growth factors and they used minipellet to retain growth factors in the collagen.

It is known that some growth factors are heparin-binding proteins. Wissink, et al. (Wissink et al., 2000a) (Wissink et al., 2000b) used N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC)/N-Hydroxysuccinimide (NHS) cross-linked and heparin immobilized collagen as the carrier of basic fibroblast growth factor (bFGF), and endothelial cells were seeded onto the collagen.

Sheridan et al. (Sheridan et al., 2000) (Sheridan and Tompkins, 1999) reported that they developed a gas foaming polymer processing approach that allows the fabrication of three-dimensional porous matrices from bioabsorbable materials. Angiogenic factors (e.g., vascular endothelial growth factor) were subsequently incorporated into matrices during the fabrication process. It has been claimed that the growth factor is released in a controlled manner and retains over 90% of its bioactivity.

Peters et al. (Peters et al., 1998) took spherical alginate beads as a means of delivering biologically functional VEGF at a controlled rate over an extended period of time.

Kawai et al. (Kawai et al., 2000) incorporated bFGF-impregnated gelatin microspheres into an artificial dermis for the regeneration of dermis-like tissues. The radioisotope study revealed that incorporation of bFGF-impregnated gelatin microspheres prolonged *in vivo* retention of bFGF in the artificial dermis.

In our study, we aim to develop a drug delivery system that will result in a controlled VEGF release as a function of time. This VEGF delivery system will improve angiogenesis, while require fewer and smaller dosages, and cause fewer side effects.

In general, a controlled release system utilizes a polymer matrix as a rate-controlling device to deliver the drug in a fixed, predetermined pattern for a desired time period. Controlled-release polymers offer a number of potential advantages when compared to present methods of drug administration (i.e. injection, oral ingestion, eye drops, ointments). These potential advantages include the following; (1) the drug concentration may be continuously maintained in a therapeutically desirable range; (2) harmful side effects from systemic administration can be reduced or eliminated by local administration from a controlled release system; (3) drug administration may be improved and facilitated in underprivileged areas where good medical supervision is not available; (4) administration of drugs that have short *in vivo* half lives may be greatly facilitated; (5) drug delivery by this method is potentially less expensive and less wasteful of the drug; (6) the polymer used to deliver the drug can function as the fill-in material or tissue scaffold at the same time.

In order for the drug to be taken up by the desired part of the body, the drug must firstly be released from the device. The most common mechanism is diffusion,

whereby the drug migrates from its initial position in the polymer to the outer surface and then to the body. It must then diffuse from the surface of the device to the surrounding environment, and finally it must reach its target. Ideally, the first of these steps should be rate-limiting so that release is mainly dependent on the device itself. To be more desirable in most situations, a controlled release in a relative long period might be achieved by tethering the proteins to the collagen matrices via a specific linkage strategy: covalent coupling of drugs to collagen sponges.

In our study, we have chosen collagen sponges as the carrier of VEGF, since collagen is highly biocompatible. Collagen formed into sponges may be used as a tissue substitute and as a 3-D matrix for tissue regeneration. We coupled VEGF to collagen sponges by covalent cross-linking. The non-cross-linked VEGF will be released by diffusion only, while the covalently bound VEGF will be released simultaneously with the degradation of collagen. We hope that we can control the degradation process of the collagen sponge by controlling the cross-linking degree of the collagen, thereby controlling the rate of VEGF release.

2.4 The chemistry of protein coupling by Disuccinimidyl-Disuccinate Polyethylene Glycol (SS-PEG₃₄₀₀-SS)

Protein conjugation techniques are dependent on two interrelated chemically reactive groups: the functional groups present on cross-linking reagents (e.g. SS-PEG₃₄₀₀-SS, SS = Succinimidyl Succinate) and the functional groups present on the target molecules (e.g. HRP, VEGF, and collagen). Choosing the correct reagent system forms the basis for successful chemical modification. A careful understanding of the structure of the target molecules and a smart choice of functional groups provide the foundation for the successful use of all modification and conjugation techniques.

2.4.1 The chemistry of functional groups

Every chemical modification or conjugation process involves the reaction of one functional group with another, resulting in the formation of a covalent bond. In our study, the reactive groups involved are the amino group of proteins (HRP, VEGF and collagen), and the SS group of SS-PEG₃₄₀₀-SS.

2.4.1.1 Amino acids and amino groups

Peptides and proteins are composed of amino acids polymerized together through the formation of peptide (amide) bonds. The peptide-bonded polymer that forms the backbone of polypeptide structure is called the alpha-chain. The peptide bonds of the alpha-chain are rigid planar units formed by the reaction of the alpha-amino group of one amino acid with the alpha-carboxyl group of another.

The sequence and properties of the amino acid constituents determine protein structure, reactivity, and function. There are 20 common amino acids found throughout the nature, each containing an specific side chain of particular chemical structure, charge, hydrogen bonding capability, hydrophilicity (or hydrophobicity), and reactivity. The side chains do not participate in polypeptide formation and are thus free to interact and react with their environment.

Amino acids may be grouped depending on the characteristics of their side chains. There are seven amino acids that contain aliphatic side chains that are relatively nonpolar and hydrophobic: glycine, alanine, valine, leucine, isoleucine, methionine, and proline. There is another group of amino acids which contain relatively polar constituents and are thus hydrophilic in character.

The most significant amino acids for modification and conjugation purposes are the ones containing ionizable side chains (Hoffman et al., 2000): e.g. aspartic acid (-COOH group), glutamic acid (-COOH group), lysine (-NH₂ group) (Figure 3).

Reactive groups able to couple with amino group-containing molecules are by far the most common functional groups present on cross-linking or modification reagents. An amine-coupling process can be used to conjugate with nearly all protein or peptide molecules as well as a host of other macromolecules. The primary coupling chemical reactions for modification of amines proceed by one of two routes: acylation or alkylation. Most of these reactions are rapid, mild and occur in high yield to give stable amide or secondary amine bonds.

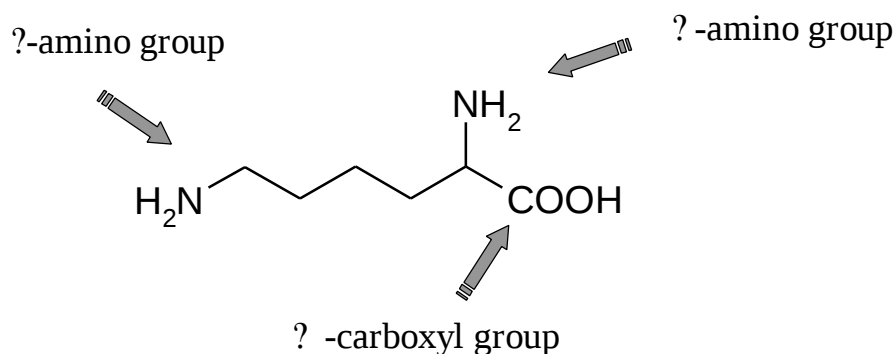


Figure 3 Molecular structure of lysine.

2.4.1.2 N-Hydroxysuccinimide (NHS) ester

N-Hydroxysuccinimide (NHS) reacts with carboxyl groups to form NHS ester derivatives which may be considered as the most common functional groups for creating reactive acylating agents. NHS esters were first introduced as reactive ends of homobifunctional cross-linkers (Lomant and Fairbanks, 1976) (Bragg and Hou, 1975). Today, the majority of commercially available amine-reactive cross-linking or modification reagents utilize NHS esters.

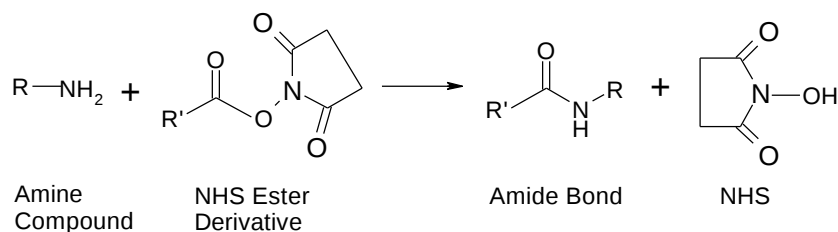


Figure 4 Reaction of amine compound with NHS ester derivative.

NHS ester containing reagents react with nucleophiles thereby releasing N-Hydroxysuccinimide to form an acylated product. The reaction of such esters with sulfhydryl or hydroxyl groups, forming thioesters or ester linkages, respectively, does not yield stable conjugates. These bonds rapidly hydrolyze in aqueous environments. Histidine side-chain nitrogens of the imidazolyl ring also may be acylated with a NHS ester reagent, but they also hydrolyze rapidly. The presence of imidazole in reaction buffers only serves to increase the hydrolysis rate of the active ester. Reaction with

primary and secondary amines, however, creates stable amide and imide linkages, respectively, that do not break down so readily (Figure 4). Thus, in protein molecules, NHS ester couples principally with the N-terminal α -amino and the γ -amino groups of lysine side chains (Hermanson, 1996a).

2.4.2 Homobifunctional cross-linker SS-PEG₃₄₀₀-SS

In this work, the covalent immobilization is achieved by using the homobifunctional cross-linking agent SS-PEG₃₄₀₀-SS (Disuccinimidyl-disuccinate-polyethyleneglycol), which is one of the derivatives of PEG (Polyethyleneglycol) (Figure 5).

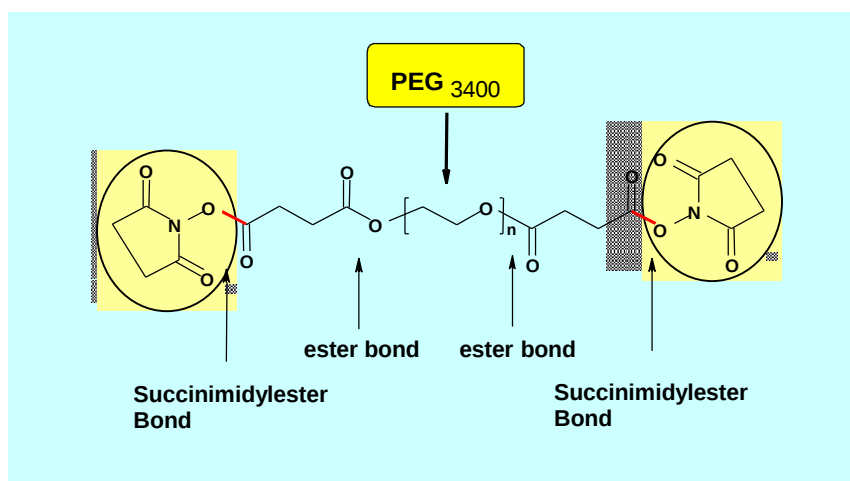


Figure 5 Chemical structure of the homobifunctional cross-linking reagent Disuccinimidyl-disuccinatepolyethyleneglycol (SS-PEG₃₄₀₀-SS).

2.4.2.1 Properties of the Poly Ethylene Glycol (PEG): spacer of the homobifunctional cross-linker

PEG is a polyether-diol with the general structure $OH-(CH_2CH_2O)_n-H$. PEG has a wide range of solubility. It is soluble in most organic solvents as well as in aqueous solutions.

It has been widely reported that PEG is quite safe to be used *in vivo*. The biological activities of PEG-conjugates are typically dominated by the non-PEG part of the molecule (Dittmann, 1973). For the purpose of modification of peptides and proteins

the useful molecular weight range is 2,000- 20,000 daltons. The toxicity of PEGs with molecular weights above 1000 daltons is very low. Extensive toxicity studies on PEG-4000 showed that this polymer even can be safely administered intravenously in 10% solution to rats, guinea pigs, rabbits, and monkeys at a dose level of 16 g per kg body weight (Johnson et al., 1971). It is also reported that when administered intravenously to humans, PEGs with a molecular weight between 1000 and 6000 are readily excreted mainly via the kidney.

Since the first reports by Abuchowski and co-workers in 1977 concerning the alteration of immunological properties toward BSA that had been modified with PEG (Abuchowski et al., 1977), the interest in polymer modification of biological molecules by PEG has grown almost exponentially.

The polymer itself, even with a molecular weight of 5.9×10^6 , is a very poor immunogen (Johnson et al., 1971). Because the polymer backbone of PEG is not of biological origin, its polyether backbone is not degradable by mammalian enzymes. This property results in a slow degradation of the polymer when used *in vivo*, thus extending the half-life of modified substances. PEG modification serves to mask any molecule to which it is coupled- the “pegylated” molecule being protected from immediate breakdown or from being complexed and inactivated by immunoglobulins in the bloodstream.

PEG in solution is a highly mobile molecule that creates a large exclusion volume for its molecular weight, much larger in fact than proteins of comparable size. Whether in solution or attached to other insoluble supports or surfaces, PEG has a tendency to exclude other polymers. This property forms a protein-rejecting region that is effective in preventing nonspecific protein binding. Thus, PEG-modified molecules display low immunogenicity, have good resistance to proteolytic digestion, and survive in the bloodstream for extended periods (Dittmann, 1973) (Abuchowski et al., 1977). The properties of PEG in solution are especially unusual, frequently displaying amphiphilic tendencies, having the ability to solubilize in both aqueous and hydrophobic or organic phases.

2.4.2.2 Applications of PEG derivatives

PEG coupled to other molecules can be used for altering solubility characteristics in aqueous or organic solvents to increase the stability of proteins in solution, to enhance the half-life of substances *in vivo*, to aid in penetrating cell membranes, to alter pharmacological properties, and to increase biocompatibility, especially toward implanted foreign substances (Catalog Shearwater Polymers, 1997). The hydrophilic, biocompatible nature of PEGs and their mild, well-defined chemistry make them ideal for coupling or tethering protein molecules to protein molecules or protein molecules to surface of biomaterial (Abuchowski et al., 1977). Research has shown that use of PEGs as a cross-linker to bind molecules to other molecules and surfaces provides highly active materials:

In 1977, Davids, Abuchowski, and co-workers demonstrated that covalent attachment of PEG to a protein gave minimal loss of activity and rendered the protein non-immunogenic and non-antigenic, thus imparting greatly increased serum lifetime.

Doillon *et al.* demonstrated that PEGylation of collagen films and sponges resulted in improved resistance against enzyme degradation *in vitro* and in a marked delay of disappearance *in vivo* (Doillon et al., 1994a).

Bentz *et al.* used difunctional PEG derivatives for the immobilization of the growth factor TGF-beta2 (Transforming Growth Factor-beta2) to injectable collagen and could demonstrate that covalent binding via PEG potentiated and prolonged *in vivo* TGF-beta 2 response and stabilized the TGF *in vitro* (Bentz et al., 1998).

Covalent attachment of PEG derivatives in the vast majority of cases has been achieved utilizing amino groups of polypeptide molecules as sites of modification. The first step in this process is substitution of hydroxyl end-groups of the polymer by electrophile-containing functional groups. This process is often referred to as 'activation' of PEG molecule. Some of the methods suffer from various disadvantages such as toxicity. Those shortcomings were overcome by using - difunctional N-hydroxysuccinimidyl (or NHS) active ester of PEG succinate. This form of activated PEG was first used for cross-linking of proteins. Due to the reagent's ease of preparation and excellent reactivity under mild aqueous conditions it was later adapted for protein modification and still remains one of the most popular reagents of this class.

In our study, we choose SS-PEG₃₄₀₀-SS as the cross-linker of VEGF and collagen. As a NHS active ester of PEG succinate, it has two identical functional succinimidyl succinate groups (SS) at both ends and a polyethyleneglycol spacer with a molecular mass of about 3400 in-between. As we have introduced before, the succinimidyl esters are characterized by a high reactivity towards NH₂ groups which are present on the surface of most hydrophilic proteins and which usually are provided by lysine side-chains. Moreover the cross-linker has two ester bonds that tend to hydrolyze slowly under physiological conditions thereby releasing the cross-linked protein (Figure 5).

2.4.3 Model protein: Horseradish Peroxidase (HRP)

In order to achieve a high VEGF delivery efficiency, many reaction parameters need to be investigated. Since VEGF is still too expensive to be used in experiments which have to be repeated several times, we decided to perform pilot experiments with horseradish peroxidase (HRP), which is characterized by the advantageous features outlined below:

1. Low cost.
2. Activity can be easily monitored with high sensitivity (≈ 10 pg/ml) by 3,3',5,5'-tetramethylbenzidine (TMB) (Figure 6).
3. HRP has about the same molecular size (44 KDa) as the dimer of VEGF₁₆₅.
4. HRP is a bioactive enzyme, and can thus serve as a good model for studying the effect of SS-PEG₃₄₀₀-SS on the bioactivity of VEGF.

The function of HRP includes removal of H₂O₂, oxidation of toxic reductants, biosynthesis and degradation of lignin, defense response toward wounding and metabolism of auxin. These functions might be dependent on each other. HRP catalytic activity: donor + H₂O₂ = oxidized donor + 2 H₂O. TMB is oxidized during the enzymatic degradation of H₂O₂ by horseradish peroxidase.

The oxidized product of TMB has a deep blue color at pH>7. A clear yellow color is formed after addition of the acidic 2 N H₂SO₄ which stops the reaction. For detection

of oxidized TMB, the OD of the yellow color is determined with a spectrometer at 450 nm.

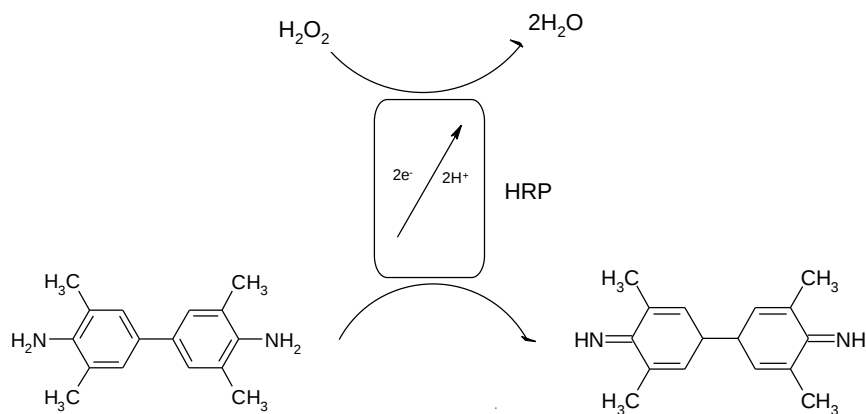


Figure 6 TMB is oxidized during the enzymatic degradation of H_2O_2 by horseradish peroxidase (HRP). The oxidized product of TMB has a deep blue color. A clear yellow color is formed after addition of the acidic stop solution.

3. Materials and Methods

3.1 Collagen sponge

Collagen sponges were prepared according to a directional freeze-drying procedure to achieve sponges with a uniform porous microstructure (Helmholtz Institute for Biomedical Engineering, Aachen, Germany). The basic material is a collagen suspension containing 1.8 wt.-% of type I collagen isolated from bovine skin (Dr. Suwelack Skin & Health Care AG, Billerbeck, Germany). To adjust the pore size of the collagen sponge, 3.8 wt.-% acetic acid was added to the basic material resulting in pH = 2.8. The suspension was then directionally solidified under thermally constant freezing conditions succeeded by a freeze-drying (Heschel et al., 1996) (Heschel and Rau, 1997) (Schoof et al., 1998). Thus, collagen sponges with a uniform microstructure and a defined pore size of 40 μm were prepared for this study (Figure 7). The dimension of the sponges used throughout this study in biochemical experiments is: 0.5 cm $\times$ 0.5 cm $\times$ 0.25 cm, ca. 2 mg in weight. Collagen sponges were sterilized with EO (ethylene oxide) for 8 h (Griffith Microscience, Zoetermeer, The Netherlands). EO sterilized sponges used in this study were subjected to subsequent aeration for at least 2 months.

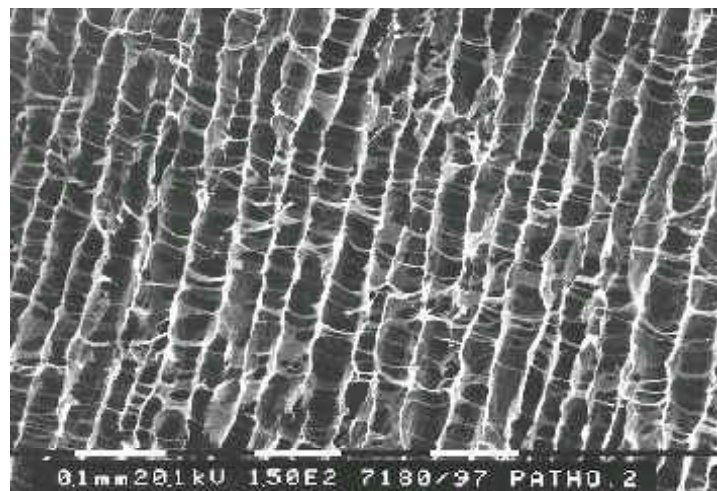


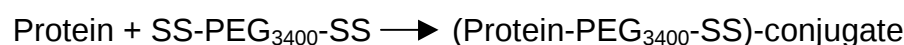
Figure 7 Electron-microscopic picture of collagen sponge with defined pore structure. (white & black bars represent 0,1 mm)

3.2 Protein coupling to collagen

3.2.1 Step 1: conjugation of protein with SS-PEG₃₄₀₀-SS

In the first-step reaction, or conjugation reaction, HRP (Sigma, St. Louis, USA) or VEGF (Chemicon International, Temecula, USA) is allowed to react with SS-PEG₃₄₀₀-SS (Shearwater Polymers, Huntsville, USA) in PBS. One of the two SS groups of the homobifunctional cross-linker will react with the amino groups of protein and PEG-modified protein conjugates, (Protein-PEG₃₄₀₀-SS)-conjugates form, while the second SS group will be still available for the next step reaction with collagen (Figure 8).

The reaction can be described as:



The solution is then added to the sample reservoir of the centricon centrifugal filter device (Millipore, Bedford, USA, YM 10,000 MW), centrifugated at 5000 * g for 0.5 hour. The non-reacted SS-PEG₃₄₀₀ -SS (about 3500 Da) will pass through the membrane and the reaction stops (Figure 9). The retentate, which contains the (protein-PEG₃₄₀₀-SS)-conjugate and the protein which has not reacted with SS-PEG₃₄₀₀-SS is recovered by a 2-minute centrifugation at 5000 * g.

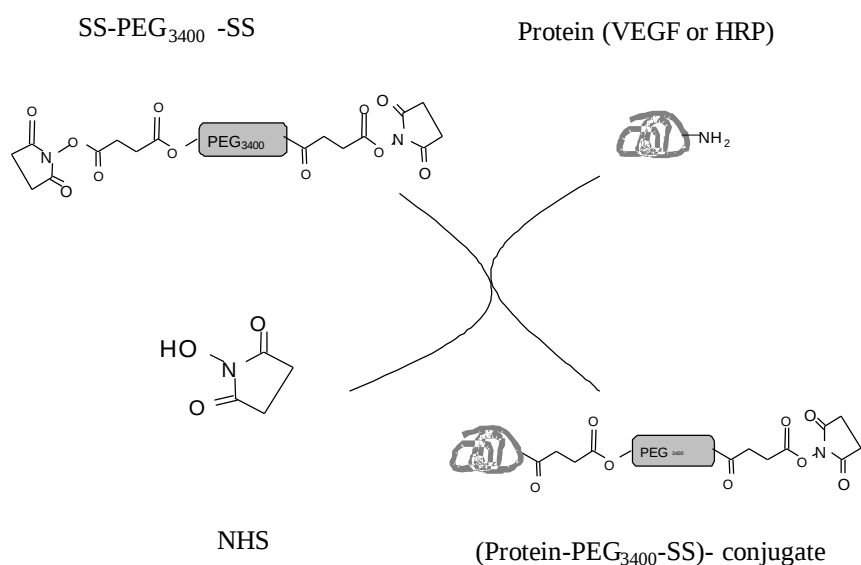


Figure 8 In the first reaction, SS-PEG₃₄₀₀ -SS reacts with protein, (Protein-PEG₃₄₀₀-SS)-conjugate forms.

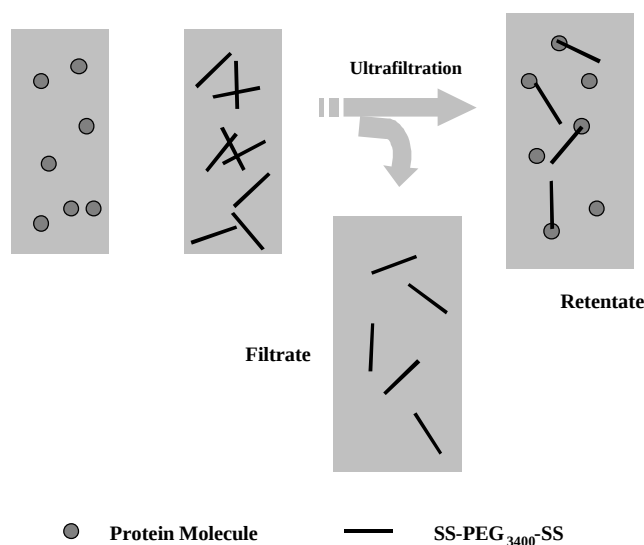


Figure 9 After the conjugation reaction, ultrafiltration is performed to remove of the excess SS-PEG₃₄₀₀-SS .

3.2.2 Step 2: cross-linking of the (protein-PEG₃₄₀₀-SS)-conjugate to collagen

In the second reaction step, or cross-linking reaction, (protein-PEG₃₄₀₀-SS)-conjugate reacts with collagen for overnight. By this step the second SS group on the conjugate may react with the amino group on collagen thus to cross-link the (protein-PEG₃₄₀₀ – SS)-conjugate to collagen (Figure 10).

The reaction can be described as:



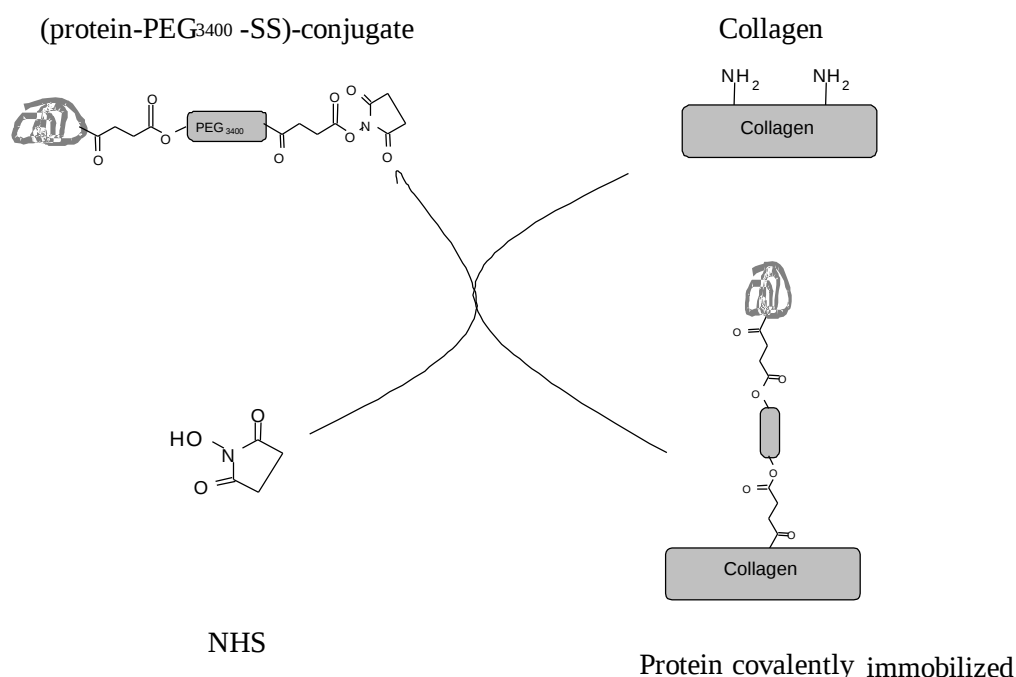


Figure 10 In the second reaction, the protein-PEG conjugate reacts with collagen which leads to the covalent immobilization onto collagen.

3.2.3 General experimental procedures

3.2.3.1 Effect of SS-PEG₃₄₀₀ -SS on protein (HRP or VEGF) coupling to collagen sponge

In order to study the effect of the homobifunctional cross-linker SS-PEG₃₄₀₀ -SS on coupling proteins to collagen sponge, experiments were performed as outlined in Table 1.

The experiment group 1 was a control group in which no cross-linker was present. In experiment group 2 the protein is allowed to react with the cross-linker. After a certain reaction period the first-step reaction is stopped by ultrafiltration and the excess cross-linker is removed. The ultrafiltration step is followed by a procedure to recover the Protein-PEG₃₄₀₀ -SS from the retentate, then the conjugate is added to collagen sponge.

To correctly quantify the amount of protein immobilized to collagen sponge, an extensive washing procedure is necessary, after which the non-cross-linked protein

will be washed out, whereas the cross-linked, and the physically adsorbed protein will stay in the sponge.

For quantification of HRP or VEGF, collagen sponges are degraded with 0.5 ml collagenase PBS solution (10 units collagenase, Worthington Biochemical Corporation, Lakewood, USA) from *Clostridium histolyticum* at pH 7.2, 37 °C. The released protein in the solution is then determined with a specific and sensitive spectrophotometrical procedure (HRP) or with an ELISA assay (VEGF).

In order to achieve optimal protein coupling efficiency, several parameters such as the effect of reaction pH, reaction temperature, the molar ratio of the cross-linker to the protein, and the necessity of ultrafiltration were studied by taking HRP as a model protein (Table 1).

Table 1 General experimental procedure for investigating the coupling of HRP to collagen sponges with SS-PEG₃₄₀₀-SS.

		Experiment group 1	Experiment group 2	
HRP	(5µg)	+	+	First step reaction
SS-PEG-SS	(20µg)	-	+	
PBS	(0,5 ml)	+	+	
Ultrafiltration	(10,000MW)	+	+	Second step reaction
Collagen sponge	(2mg)	+	+	
Washing procedure		+	+	
Collagenase	(10U, 0.25ml)	+	+	Quantification
HRP activity test		+	+	

3.2.3.2 Effect of SS-PEG₃₄₀₀-SS on HRP coupling to soluble collagen

The first-step reaction or conjugation reaction was the same as in the experiments with the collagen sponge. In the second-step reaction, (HRP-PEG₃₄₀₀-SS)-conjugate and non-cross-linked HRP (if there is any) were added to soluble collagen (Fluka, Buchs, Switzerland) instead of collagen sponges. To quantify the amount of HRP immobilized, the second-step reaction was followed by another ultrafiltration step with a membrane with a different cut off (100,000 MW), the HRP (44 kDa) which did not couple to collagen will pass the membrane, but the collagen molecule (300 kDa) and the HRP coupled collagen (>300 kDa) will stay in the retentate. The HRP coupling efficiency can be quantified by testing the HRP activity in the retentate and the filtrate (Table 2).

Table 2 General experimental procedure for investigating the coupling of HRP to soluble collagen with SS-PEG₃₄₀₀-SS.

		Experiment group 1	Experiment group 2	
HRP	(5µg)	+	+	First step reaction
SS-PEG-SS	(20µg)	-	+	
Ultrafiltration	(10,000MW)	+	+	
Soluble Collagen	(0.2mg)	+	+	Second step reaction
Ultrafiltration	(100,000MW)	+	+	Quantification
HRP activity test		+	+	

3.2.4 Test of protein cross-linking

3.2.4.1 Determination of HRP by spectrophotometric method

To determine the HRP amount, 10 µl TMB (Merck, Darmstadt, Germany) solution (10 mg TMB in 1 ml Dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany)) and 10 µl 0.3% H₂O₂ was added into each 1ml sample. The reaction was then stopped with 1 ml 10% H₂SO₄ 15 minutes later. The HRP activity of samples can be quantified by measuring the absorbance at 450 nm wavelength then comparing the data with the OD (Optical Density) value of a standard concentration of HRP.

3.2.4.2 Determination of VEGF by Enzyme-linked Immunosorbent Essay (ELISA)

ChemiKine Human VEGF kit (Chemicon International, Temecula, USA) is a sandwich enzyme immunoassay (ELISA), which determines the amount of free VEGF. With the ChemiKine assay system, pre-coated mouse monoclonal antibodies generated against human VEGF are used to capture human VEGF in a sample. Simultaneously, biotinylated VEGF specific rabbit polyclonal antibodies detect VEGF in the sample. With the addition of streptavidin-alkaline phosphatase (which binds to the biotinylated rabbit anti-human polyclonal cytokine antibody), followed by the addition of the color generating solution the amount of cytokine is detected. The standard curve demonstrates a direct relationship between OD and VEGF concentration. By comparing the OD data of the samples with the standard curve, the VEGF concentration of the samples can be quantified.

3.2.4.3 Test of the conjugation reaction: HRP binding to SS-PEG₃₄₀₀-SS

3.2.4.3.1 Reversed Phase High Performance Liquid Chromatography (RP-HPLC)

Coupling of SS-PEG₃₄₀₀-SS to HRP was monitored by RP-HPLC (Hewlett-Packard System 1050, Waldbronn, Germany) experiments performed. To prepare the samples, HRP 50 µg was allowed to react with SS-PEG₃₄₀₀-SS 200 µg in 500 µl PBS solution (pH 7.2) for 10 min, then stop the reaction by ultrafiltration. HRP (50 µg) dissolved in 500 µl PBS solution was taken as the control. Samples were analyzed on a water/millipore HPLC system equipped with a 600s controller, 626 quaternary pump system. After reaction of the proteins with the cross-linkers, samples were immediately acidified to pH 3.0, diluted with 1 vol of 0.1% trifluoroacetic acid (TFA) containing an excess of glycine, and fractionated on a Vydac 218TP54, 0.46 * 25 cm, C18 reverse phase column. Solution A was 0.1% TFA in water, and solvent B was 0.1% TFA in acetonitrile. The column was run at room temperature. At a flow rate of 1ml/min, a two-step gradient with a plateau at 38% and 52% solvent B was applied.

3.2.4.3.2 MALDI-TOF (Matrix Assisted Laser Desorption/Ionization Time-Of-Flight) spectrometry

Molecular mass determination was performed on a Bruker BiflexTM III Maldi time-of-flight mass spectrometer (Bruker-Franzen Analytik GmbH, Bremen, Germany) equipped with a multiprobe gridless delayed extraction ion source and an ion-mirror. The MALDI-TOF technique operates on the principle that when a temporally and spacially well defined group of ions of differing mass/charge ratios are subjected to the applied electric field and allowed to drift in a region of constant electric field, they will traverse this region in a time which depends upon their mass/charge ratios (Lennon and Glish, 1997).

For MALDI analysis, HRP (50 µg) was allowed to react with SS-PEG₃₄₀₀-SS (200 µg) for 10 minutes in 500 µl PBS, pH 7.2. Then the sample was mixed with matrix DHB (2,5-Dihydroxybenzoic acid) and applied to the sample support for measurement. A HRP solution (50 µg in 500 µl PBS) was taken as the control.

3.2.4.3.3 SDS-PAGE (Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis)

Almost all analytical electrophoresis procedures of proteins are carried out in polyacrylamide gels under conditions that ensure dissociation of proteins into their individual polypeptide subunits and minimize aggregation. Sodium dodecyl sulphate (SDS, Boehringer Ingelheim, Germany) is a strongly anionic detergent. The denatured polypeptides bind SDS and become negatively charged. SDS-polypeptide complexes migration through polyacrylamide go entirely in accordance with the size of the polypeptides. SDS-PAGE is carried with a discontinuous buffer system in which the buffer in the reservoirs is of a different pH and ionic strength from the buffer used to cast the gels. The sample and the stacking gel contains Tris. HCL (pH 6.8), the resolving gel contains Tris. HCL (pH 8.8), and the upper and lower buffer reservoirs contain Tris-glycine (pH 8.3). All components of the system contain 0.1% SDS. The ability of discontinuous buffer system to concentrate all of the complexes in the sample into a very small volume greatly increases the resolution of SDS-PAGE.

To prepare the samples, HRP (500 µg) was allowed to react with SS-PEG₃₄₀₀-SS (2000 µg) in PBS solution (500 µl, pH 7.2) for 10 minutes, 1 hour or 24 hours, then the reaction was stopped by ultrafiltration, retentates were recovered and adjusted to HRP concentration 1mg/ml. Samples were further diluted 1:1 with 2x sample buffer and incubated in a 50 °C water bath for 20 minutes. Standard HRP sample was treated in the same way. Samples were run on a 12% SDS-PAGE gel overnight (150V).

The gels were stained with Coomassie blue to visualize binding of the HRP to SS-PEG₃₄₀₀-SS .

Discontinuous buffer system:

Sample buffer

125 mM Tris-HCL pH 6.8
4% SDS
10% 2-mercaptoethanol
20% glycerol
0.04% bromphenol blue

Running buffer

0.25 m Tris pH 8.3
1% SDS
1.92 M glycine

5% Stacking gel

Solution component	Volume (ml)
H ₂ O	2.7
29.2% acrylamide and 0.8%	
N',N'-Methylenbisacrylamide	0.67
1.5 M Tris-HCL pH 8.8	0.5
10% SDS	0.04
10% APS	0.04
TEMED	0.004

12% Separating gel

Solution component	Volume (ml)
H ₂ O	3.3
29.2% acrylamide and 0.8%	
N',N'-Methylenbisacrylamide	4.0
1.5 M Tris-HCL pH 8.8	2.5
10% SDS	0.1
10% APS	0.1
TEMED	0.008

3.2.4.4 Test of the cross-linking reaction: (HRP-PEG₃₄₀₀-SS) or (VEGF-PEG₃₄₀₀-SS)- conjugate binding to collagen sponges

After the second-step reaction, as shown in the schematic illustration below (Figure 11), there should be free protein (loosely absorbed in the collagen sponge), cross-linked protein (part of which has been covalently immobilized onto collagen) in the collagen sponge. To test the covalent coupling efficiency of the protein with collagen sponge, a washing procedure was used. After reacted with HRP or cross-linked HRP overnight, the collagen sponge samples were extensively washed with PBS 5-7 times, then the sponges are degraded by collagenase. HRP or VEGF left is thought to be covalently immobilized.

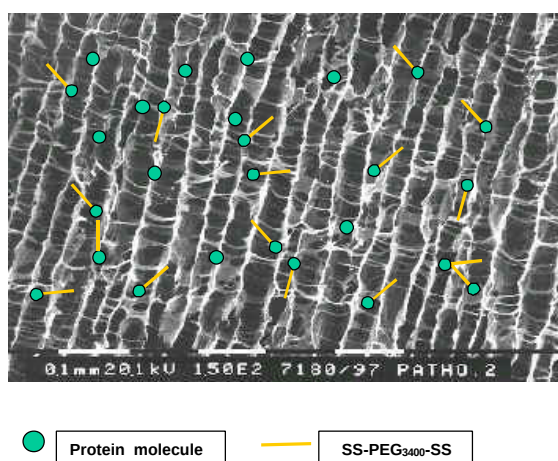


Figure 11 After the second-step reaction, there is free protein, (Protein-PEG₃₄₀₀-SS)-conjugate (part of which has been covalently immobilized onto collagen) in the collagen sponge.

3.2.4.5 Test of (HRP-PEG₃₄₀₀-SS)-conjugate binding with soluble collagen

To correctly quantify the amount of HRP coupled to soluble collagen, an ultrafiltration procedure was performed, which will cut off the molecules smaller than 100 kD. The non-cross-linked HRP and (HRP-PEG₃₄₀₀-SS)-conjugate molecules which did not couple to the collagen molecule will pass through the membrane into the filtrate, while the cross-linked or non-cross-linked collagen molecules will stay in the retentate. The HRP coupling efficiency can thus be quantified by measuring the HRP activity in the retentate.

3.3 Exogenous cross-linking of collagen

Collagen sponges (0.5 cm × 0.5 cm × 0.25 cm, 2 mg) were soaked in SS-PEG₃₄₀₀-SS PBS solution with a concentration of 4 mg/ml or 40 mg/ml. The reaction was carried out at pH 7.2, room temperature for 1 hour. After cross-linking, the sponges were immersed in PBS and extensively rinsed with deionized water. Collagen sponges of the control group were immersed in PBS solution for the same time period and then washed with deionized water.

3.3.1 Resistance against enzyme degradation

2 mg collagen sponges (0.5 cm × 0.5 cm × 0.25 cm) were treated with 0.25 ml collagenase (40 U/ml) solution. Specimen were incubated at 37 °C and observed at 15-min intervals during incubation. The incubation time for complete disappearance of collagen sponges was considered to correlate to the resistance of the modified collagen against enzymatic degradation.

3.3.2 Amino group assay

The primary amino group content of the collagen sponge samples was determined spectrophotometrically (492 nm wavelength) after reaction of primary amine groups with STDB (Sulfosuccinimidyl-4-O-(4,4'-dimethoxytrityl) butyrate (Sigma, St. Louis, USA), and subsequent hydrolysis of the reaction products by HClO₄ (Figure 12). The amount of amino groups and the degree of cross-linking, can be calculated by using

the equations:

$$A \text{ (mol/l)} = (\text{OD}-0.01)/0.007;$$

(A : number of amine groups)

$$\text{Cross-linking degree (\%)} = (A \text{ of control group} - A \text{ of sample}) * 100 / (A \text{ of control group}).$$

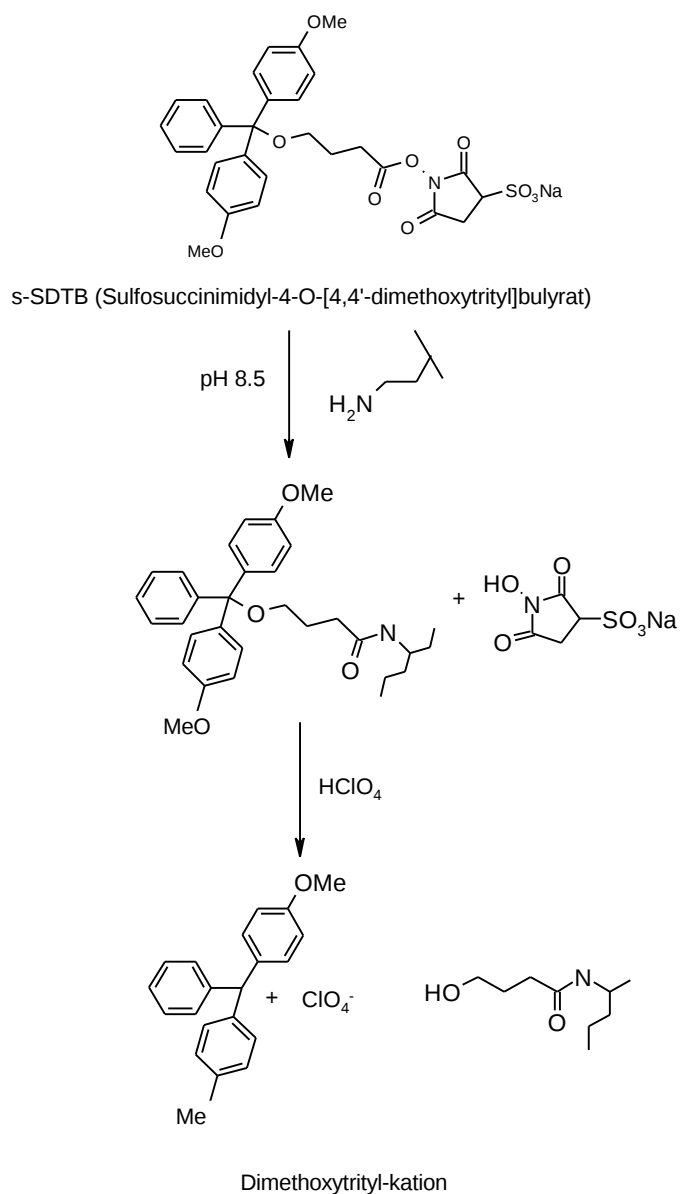


Figure 12 The principle of the amino group assay of collagen sponge cross-linking.

3.4 Determination of HRP or VEGF releases from collagen sponges

Proteins (HRP or VEGF) were coupled to collagen sponges according to the protocols in 2.2.2, then the collagen sponges were immersed in 0.5 ml diluted collagenase solution (0.04 U/ml). All samples were maintained at 37 °C with shaking in a thermoshaker.

At 1-5 days in HRP experiments and at 1-10 days in VEGF experiments, the collagenase solutions were changed and collected daily, then the samples were stored at –20 °C for future testing. After all the samples were collected, the HRP or VEGF amounts were determined.

3.5 Test of cytotoxicity of SS-PEG₃₄₀₀-SS

3.5.1 Cell proliferation assay

Human dermal fibroblasts obtained from skin were cultured in DMEM (Dulbecco's Modified Eagle Medium, Life Technologies GmbH, Karlsruhe, Germany) supplemented with 10% FBS (fetal bovine serum, Life Technologies GmbH, Karlsruhe, Germany), 0.02 mol/L of L-glutamine, 100 U/ml of penicillin, and 100µg/ml of streptomycin in a moist atmosphere of 5% CO₂ at 37 °C. The culture medium was changed every 3 days. At confluence, the cells were removed from the flasks by trypsinization (0.05% trypsin, 0.02% EDTA salt solution). Cultures at passages 3 to 4 were used for the experiments.

To test the cytotoxic effect of SS-PEG₃₄₀₀-SS, fibroblasts proliferation in SS-PEG₃₄₀₀-SS solution was tested with a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) cell proliferation assay kit (Boehringer, Mannheim, Germany).

SS-PEG₃₄₀₀-SS PBS solution was sterilized by injecting the solution through sterilization filter (Millipore, Molsheim, France) with a pore size of 0,2 µm.

Fibroblasts were seeded at a concentration of 20000 cells/well in 100 µl DMEM culture medium (with 10% FBS and antibiotics) containing various amounts of SS-PEG₃₄₀₀-SS with the final concentration of 0.1 µg/ml-10000 µg/ml into microtiter plates (tissue culture grade, 96 wells, flat bottom, Becton Dickinson European HQ, Belgium). Incubate cell cultures for 24 h at 37 °C and 7.5% CO₂. Then 10 µl MTT (tetrazolium salts) solution (5 mg/ml) was added to each well and incubated for 4

hours. The yellow MTT can be cleaved into a purple formazan dye only by metabolically active cells. After incubation the converted formazan crystals were solubilized in 100 μ l 100% isopropanol in 0.1N HCl per well for 12 h. Solubilized formazan dye was spectrophotometrically quantified using an ELISA reader at the measurement wavelength of 570 nm and the reference wavelength of 690 nm. The absorbance directly correlates to the metabolic activity and thus correlates to the number of living cells.

3.5.2 Fibroblast seeding onto collagen sponges

In order to study the effects of collagen cross-linking on the cell-ingrowth behavior, fibroblasts are seeded onto cross-linked or non-cross-linked collagen sponges.

To prepare the cross-linked collagen sponges, EO sterilized sponges were immersed in SS-PEG₃₄₀₀-SS solution (400 μ g/ml) at pH 7.2, room temperature for 1 hour, then the collagen sponges were washed extensively with PBS. The sponges just immersed in PBS were taken as the control.

Collagen sponges pieces (1 cm $\times$ 1 cm $\times$ 0.25 cm) were put into in 6-well plates (Becton Dickinson European HQ, Erembodegem-Aalst, Belgium) for cell seeding. Primary fibroblasts isolated from human dermis were cultured in a medium supplemented with 10% (FBS) and antibiotics (100 IU/ml penicillin and 100 μ g/ml streptomycin). Passaged fibroblasts were seeded onto collagen sponges at a cell density of 2×10^5 cells/sponge/well in 150 μ l medium. 4 hours after seeding 1.5 ml medium was added to each well. Small volume seeding medium and delayed addition of culture media are to ensure fibroblasts complete seeding onto sponges instead of the well-plate. Cells were cultured in an incubator at 37°C under a humid atmosphere in 5% CO₂. Culture medium was changed every 3 days.

The specimens were prepared and fixed in 4% phosphate-buffered paraformaldehyde for 48 hours, then washed, dehydrated in graded alcohol, embedded in paraffin. Serial 6 μ m sections were stained with H&E Method.

3.5.3 Subcutaneous implantation and histological examination

To show the animal tissue reaction towards the cross-linked collagen sponges, cross-linked and the non-cross-linked collagen sponges were implanted

subcutaneously in Lewis rats. The cross-linked collagen sponges were prepared following the protocol mentioned in previous paragraph.

20 male Lewis rats, 200-220 g, received implants of collagen sponge. We used ether inhalation for induction of anesthesia and pentothal intraperitoneally as the main anaesthetic. The back of the rats were closely shaved and swabbed with alcohol, then covered with sterile towels. A midline incision 1 cm long was made in the skin in the dorsal zone, an extending pocket was dissected bluntly beneath the *panniculus carnosus* at the right side (Figure 13), and a square of collagen sponge (1 * 1 * 0,4 cm) was inserted into the pocket without fixation. The skin was closed with interrupted absorbable sutures (Deknalon, Lübeck, Germany). Rats were sacrificed at 3,7,14,28,42 days after implantation. The dorsal skin with the attached sponges and muscles beneath was excised. The specimens were prepared and fixed in 4% phosphate-buffered paraformaldehyde for 48 hours, then washed, dehydrated in graded alcohol, embedded in paraffin. Serial 6 µm sections were stained with H&E Method.

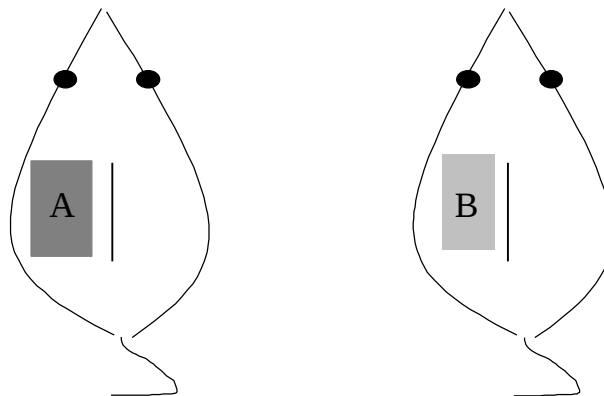


Figure 13 A square of collagen sponge (1 * 1 * 0,4 cm) was inserted into the pocket on the backs of the rats. (A: SS-PEG₃₄₀₀-SS cross-linked collagen sponge, B: non-cross-linked collagen sponge.

Collagen sponges were fixed in 70% ethanol and prepared further for paraffin section. 8 µm thick slides from every specimen were stained with hematoxylin and eosin. The haematoxylin component stain the cell nuclei blue-black, with good intra-nuclear detail, while the eosin stains cell cytoplasm of most connective tissue fiber in varying shades and intensities of pink and red. The samples were examined under a light microscope.

4. Results

4.1 Cross-linking of proteins to collagen

4.1.1 Cross-linking of HRP

The major aim of this thesis is the development of a collagen sponge with immobilized angiogenic factors for enhancing angio- and vasculogenesis. Thus the immobilization of proteins within collagen sponges represents an important issue.

One of the possibilities to immobilize proteins is the covalent cross-linking with bifunctional agents.

In this study we investigated the cross-linking of the VEGF to collagen by using the bifunctional reagent Disuccinimidylsuccinatepolyethyleneglycol (SS-PEG₃₄₀₀-SS). Since VEGF is still very expensive, we took horseradish peroxidase (HRP) as a model protein. As already mentioned in the introduction (1.5.2) this agent is characterized by the presence of two succinimidylester groups at both ends. These groups are responsible for the formation of covalent bonds with available amino functions in the side-chains of lysines. Furthermore the agent comprises two ester bonds which will hydrolyze slowly and are therefore – in concert with the degradation of the collagen - expected to play an important role in the long-term release of the proteins from the collagen.

4.1.1.1 Coupling of HRP to SS-PEG₃₄₀₀-SS and formation of (HRP-PEG₃₄₀₀-SS)-conjugate

The efficiency of the covalent coupling of HRP to collagen was investigated by three different methods: Reversed Phase-HPLC, MALDI-TOF Mass Spectrometry and SDS-Polyacrylamidegel electrophoresis (PAGE).

Figure 14 shows the RP-HPLC chromatograms of a series of experiments in which the coupling of the cross-linking agent to HRP was investigated as a function of time. In the experiment corresponding to figure 14 A non-modified HRP was injected, the peak appearing at a retention time of 23 minutes thus represents native HRP. The

chromatogram of Figure 14 B corresponds to an experiment in which HRP and the bifunctional agent were allowed to react for 10 minutes. The result of this experiment strongly suggests that after 10 minutes almost all native HRP-molecules have disappeared and that instead two new peaks have emerged at 26 and 28 minutes. These new peaks most likely represent newly formed conjugates of HRP and the bifunctional agent. One could argue that the peak eluting at 26 minutes represents the HRP-conjugate with one cross-linking molecule and that the peak eluting at 28 minutes corresponds to a conjugate in which two cross-linking molecules are bound. This experiment strongly suggests that already after a reaction period of only 10 minutes all HRP-molecules have been conjugated to one or two molecules of the cross-linker.

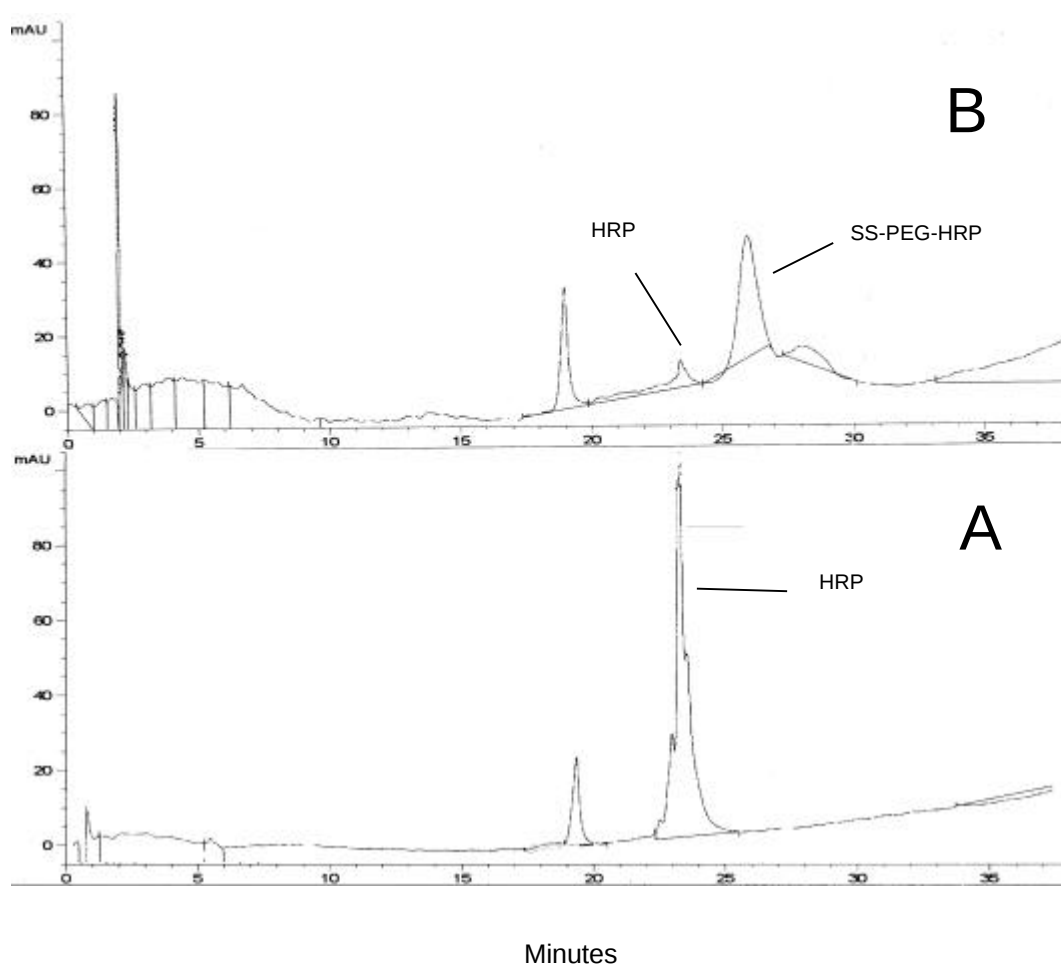


Figure 14 Reversed Phase-HPLC chromatograms of HRP before (A) and after a 10 minute reaction of HRP with the bifunctional cross-linking agent SS-PEG₃₄₀₀-SS (B). See Materials and Methods for details of the reaction and chromatographic procedure.

The cross-linking was further investigated by the mass-spectrophotometric method MALDI-TOF. This technique allows the determination of mass of the conjugates before and after reaction. MALDI-TOF experiments were performed in order to determine the molecular mass of HRP and of the (HRP-PEG₃₄₀₀-SS)-conjugate. Figure 15 (A) and (B) show the molecular mass of HRP (43.4 kD) and of the product of the reaction between HRP and cross-linker (47.1 kD). Deduced from the latter molecular mass, we can demonstrate the formation of (HRP-PEG₃₄₀₀-SS)-conjugate. The reaction parameters are the same as described by the RP-HPLC experiments. The calculated molecular mass of (HRP-PEG₃₄₀₀-SS)-conjugate (47 kD) is in good agreement with the mass experimentally determined.

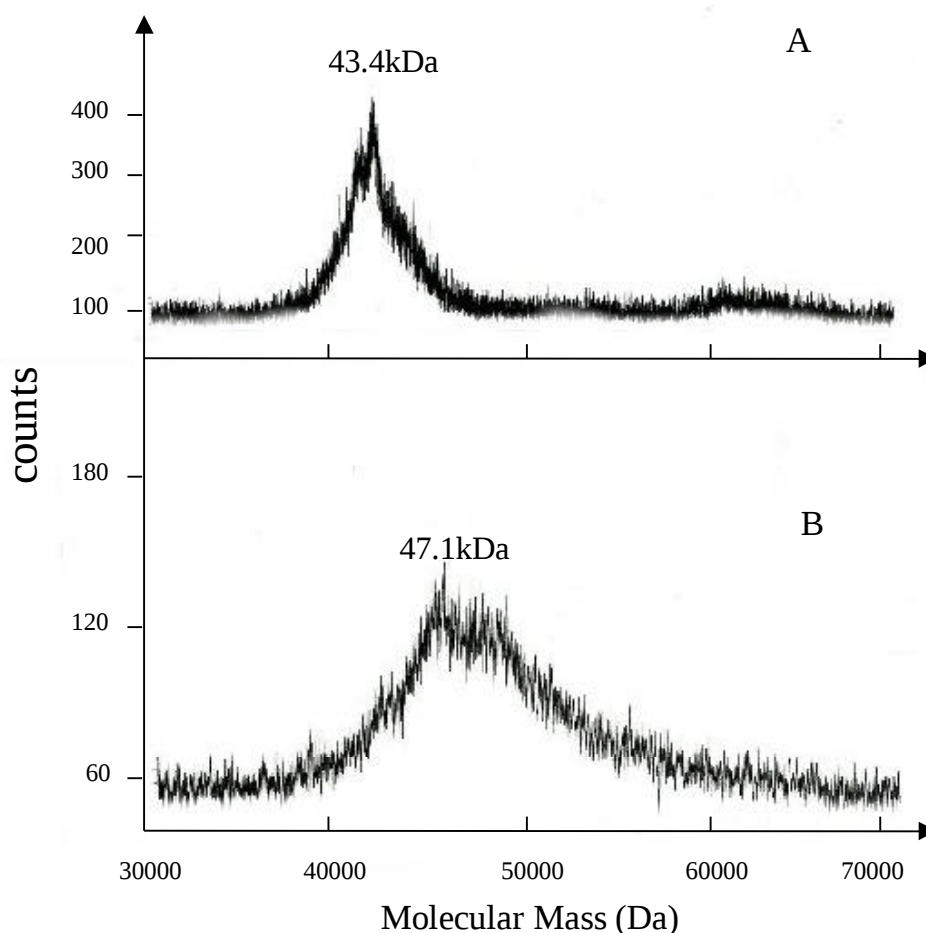


Figure 15 MALDI-TOF mass spectra of native (non-modified) HRP (A) and of the HRP-PEG₃₄₀₀-SS-conjugate obtained after a reaction time of 10 minutes. See Materials and Methods for details of the reaction and mass spectroscopic procedure.

The efficiency of the coupling between the protein and the coupling agent was further investigated by SDS-PAGE. The apparent molecular mass of the non-modified HRP (lane 4) corresponds to the theoretical mass deduced from the biochemical characteristics of this glycoprotein (mass protein entity: 34 kD; mass carbohydrate entity: 12 kD). Lanes 1-3 correspond to reaction times from 24 hours to 10 minutes. The result of lane 3 again confirms that after 10 minutes all HRP-molecules are already conjugated. The apparent molecular mass of the conjugate is estimated to 48 kD, this mass corresponds to the sum of the masses of the glycoprotein and the cross-linking molecule. The experiment furthermore shows that longer reaction times do not lead to conjugates in which more than two cross-linking molecules are bound.

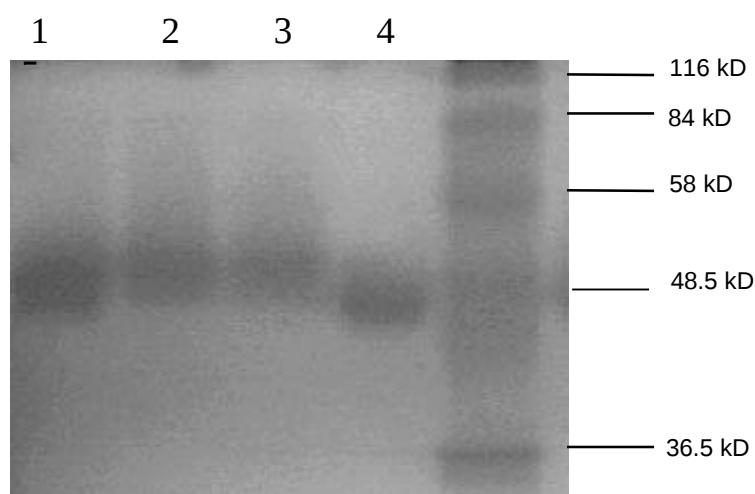


Figure 16 SDS-Polyacrylamidegel electrophoresis of native HRP and of reaction mixtures of HRP and SS-PEG₃₄₀₀-SS taken after various reaction times. The following proteins and protein mixtures were applied: lane 1: HRP-conjugate after a reaction time of 24 h; lane 2: HRP-conjugate after 1 h; lane 3: HRP-conjugate after 10 minutes; lane 4: native (non-modified) HRP and lane 5: Mixture of marker proteins. See Materials & Methods for experimental details.

4.1.1.2 Coupling of the (HRP-PEG₃₄₀₀-SS)-conjugate to collagen and the reaction parameters influencing it

In the previous paragraphs we have demonstrated the formation of (HRP-PEG₃₄₀₀-SS)-conjugate after the reaction between HRP and SS-PEG₃₄₀₀-SS. In this

paragraph, we report on the coupling of (HRP-PEG₃₄₀₀-SS)-conjugate to collagen sponge.

In order to achieve a high efficiency and to study the influence of several parameters such as temperature, pH, and reaction time, on the coupling reaction between the (protein-PEG₃₄₀₀-SS)-conjugate and collagen, experiments were performed under varying conditions.

4.1.1.2.1 Effect of exposure to collagenase, SS-PEG₃₄₀₀-SS and PBS on activity of HRP

As mentioned in materials and methods, collagenase solution is used in every experiment to degrade the collagen matrix leading to the release of the immobilized HRP-PEG₃₄₀₀-conjugate. It is therefore necessary to investigate the effect of collagenase on HRP activity. At the same time, it is important to know the effect of the homobifunctional cross-linker on HRP activity.

Before we investigate the effect of the different parameters on (Protein-PEG₃₄₀₀-SS)-conjugate coupling efficiency, experiments have been performed to test the influence of collagenase, SS-PEG₃₄₀₀-SS and PBS on the activity of HRP as a function of time. The activity of HRP in PBS was taken as a control.

Figure 17 demonstrates that an exposure of 1 hour to a collagenase containing solution (40 U/ml) does not have a big impact on the activity of HRP.

Exposure to a SS-PEG₃₄₀₀-SS solution (80 µg/ml), however, leads to a substantial reduction of the HRP activity. Similar results are obtained when HRP is exposed for 1 hour to a PBS solution (buffer).

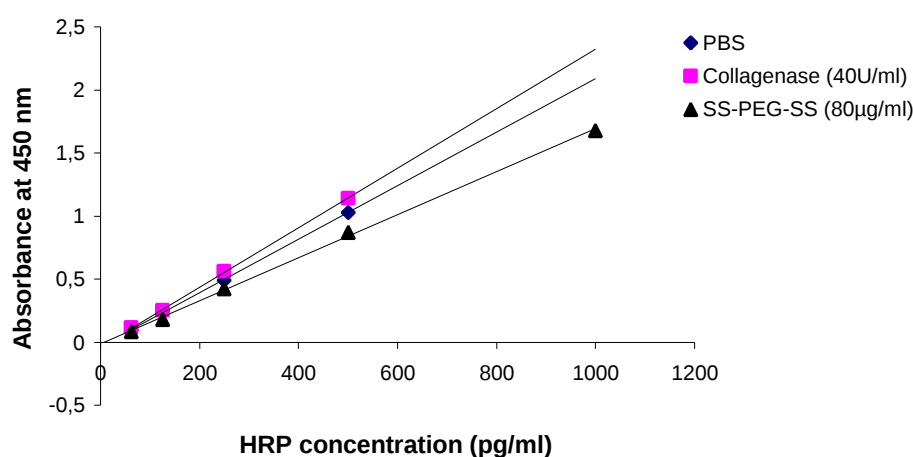


Figure 17 The activity of HRP after treatment with PBS, SS-PEG₃₄₀₀-SS (80 µg/ml) or collagenase solution (40 U/ml) for 1 hour. Results shown represent average values. The error bars are too low to be seen in this experiment (n=4).

The results of Figure 18 show that the activity of HRP (500 pg/ml) decreases as a function of time after exposure in PBS or SS-PEG₃₄₀₀-SS solution at pH 7.2, room temperature. After 24 hours, HRP lost most of its activity in PBS, while its activity is maintained in SS-PEG₃₄₀₀-SS solution (Table 3).

Table 3 Effect of PBS and SS-PEG₃₄₀₀-SS on HRP activity

	10 min	1 hour	24 hours
with PBS	1.06±0.007	1.03±0.015	0.325±0
with SS-PEG ₃₄₀₀ -SS	0.811±0.001	0,87±0.011	0.735±0,001

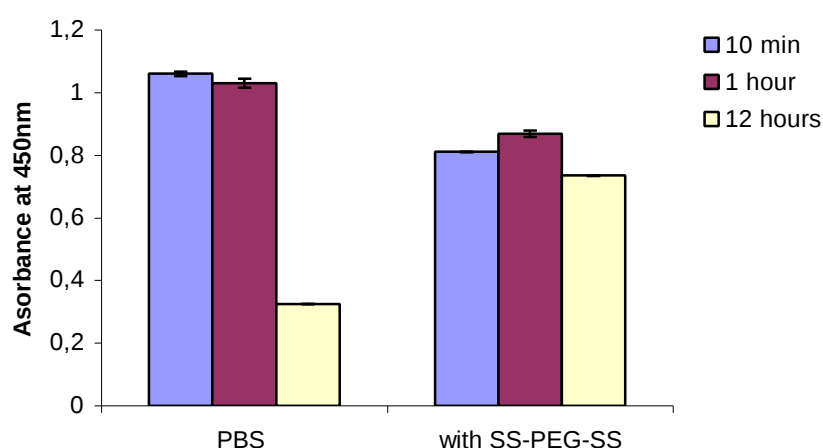


Figure 18 The dependency of the HRP activity on exposure to PBS and SS-PEG₃₄₀₀-SS solution (80 µg/ml) for exposure times varying from 10 min to 24 hours. The concentration of HRP is 500 pg/ml. Results shown represent average values and error bars represent the standard deviations (n=4).

4.1.1.2.2 Effect of the conjugation time on the coupling efficiency

In this paragraph the influence of the reaction time of the conjugation of HRP to SS-PEG₃₄₀₀-SS on the overall efficiency of the coupling of HRP to the collagen was reported.

Table 4 Effect of the reaction time of the conjugation step (first reaction step) on the overall coupling efficiency

HRP	(5µg)	+	+	+	+
SS-PEG-SS	(20µg)	-	+	+	+
PBS	(0.5ml)				
pH		7,2	7,2	7,2	7,2
Reaction time		-	10 min	1h	24h
Ultrafiltration		-	+	+	+
CS	(2mg)	+	+	+	+
HRP immobilized	(ng)	1,023±0,22	14,533±0,4	3,037±0,46	0,623±0,1

Table 4 summarizes a series of experiments in which HRP was allowed to react for various times (10 min, 1 hour or 24 hours) with the cross-linking agent. The conjugation reaction was stopped after the given reaction period by ultrafiltration. The retentates, which contain the HRP-PEG₃₄₀₀-conjugates, were added to the collagen. The conjugates were allowed to react with the collagen for a fixed period of time (15

h). The results of HRP coupling (HRP immobilized [ng]) are shown graphically in a column diagram (Figure19).

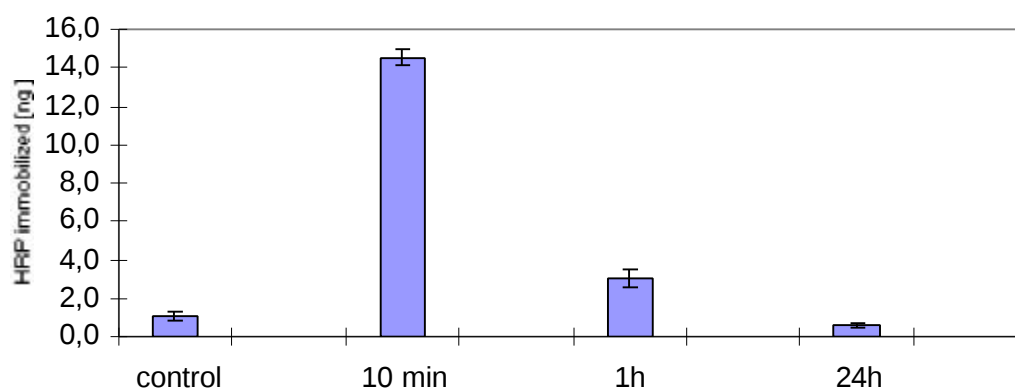


Figure 19 Effect of the reaction time of HRP and SS-PEG₃₄₀₀-SS on HRP coupling. The reaction conditions are listed in Table 4. Column heights correspond to the mean values and the error bars to the standard deviations (n=4).

The results of Figure 19 clearly demonstrate that a reaction time of 10 minutes leads to the highest amounts of immobilized HRP. Longer reaction times result in lower coupling efficiencies.

4.1.1.2.3 Effect of conjugation pH on the coupling efficiency

The effect of the pH in the conjugation reaction on the coupling efficiency was investigated in a series of experiments as explained (shown) in Table 5. The results at the various pH-values are compared with the results of control experiments which were carried out in the absence of the cross-linker. The results of HRP coupling efficiency (HRP immobilized [ng]) are shown graphically in the column diagram of Figure 20. Coupling efficiencies are highest at pH 7.2 and 8.5, lower and higher pH-values lead to lower binding efficiency.

Figure 20 shows that the pH of buffer used in reactions plays an important role in (HRP-PEG₃₄₀₀-SS)-conjugate coupling process. Figure 20 shows that the coupling efficiency is optimal at the pH-values 7.2 and 8.5. At more acidic (pH 5.5) or more

alkaline pH-values (pH 10) the amount of covalently cross-linked HRP is substantially reduced.

Table 5 Effect of pH on HRP coupling efficiency.

HRP (5µg)	+	+	+	+	+	+	+	+
SS-PEG-SS (20µg)	-	-	-	-	+	+	+	+
Reaction time	-	-	-	-	10 min	10 min	10 min	10 min
pH	5,5	7,2	8,5	10	5,5	7,2	8,5	10
Ultrafiltration	-	-	-	-	+	+	+	+
CS (2mg)	+	+	+	+	+	+	+	+
HRP immobilized (ng)	1,42±0,15	1,583±0,62	0,667±0,1	0,90±0,03	1,2±0,13	6,767±0,13	6,667±0,007	3,22±0,18

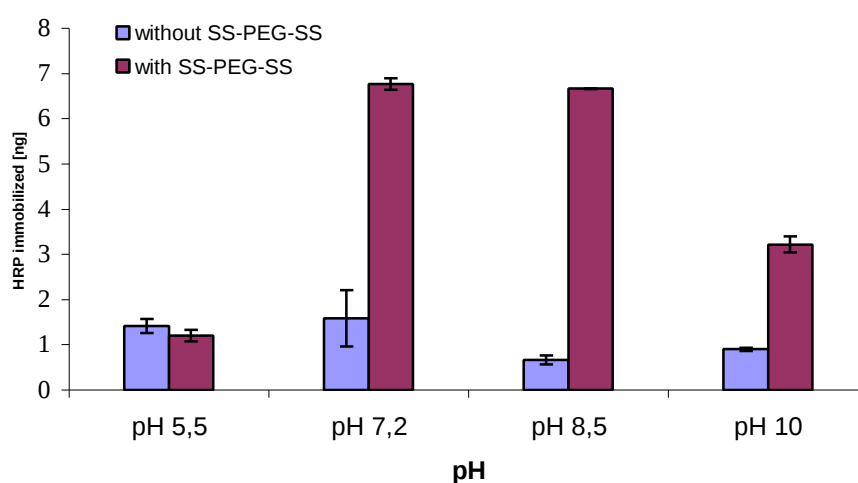


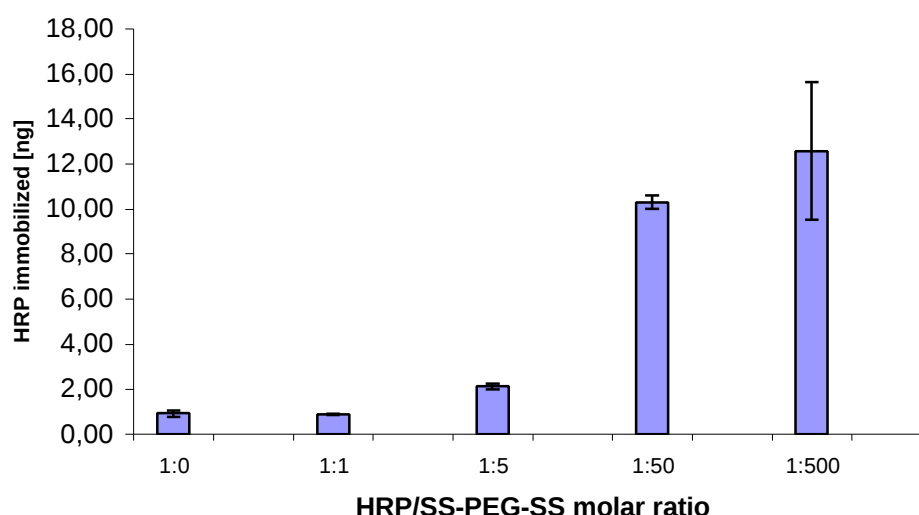
Figure 20 Effect of the reaction pH of HRP and SS-PEG₃₄₀₀-SS on HRP coupling efficiency. Column heights correspond to the mean values and the error bars to the standard deviations (n=4).

4.1.1.2.4 Effect of conjugation HRP/ SS-PEG₃₄₀₀-SS molar ratio on the coupling efficiency

Table 6 summarizes the experimental procedures and the results of a series of experiments in which the influence of HRP/cross-linker molar ratios on the coupling efficiency has been investigated. Again the essential results are shown graphically in the column diagram of Figure 21. Although higher HRP/SS-PEG₃₄₀₀-SS ratios give rise to higher coupling efficiencies, a molar ratio of 1:50 appears to be optimal.

Table 6 Effect of HRP/SS-(PEG₃₄₀₀)-SS molar ratio on HRP coupling efficiency.

Molar ratio	1:0	1:1	1:5	1:50	1:500
HRP (250µl)	5µg	5µg	5µg	5µg	5µg
SS-PEG-SS (250µl)	-	0,4µg	2µg	20µg	200µg
HRP immobilized	0,92±0,14	0,87±0,0	2,12±0,11	10,32±0,29	12,58±3,05

**Figure 21** Effect of various HRP/SS-PEG₃₄₀₀-SS molar ratios on HRP coupling efficiency. Column heights correspond to mean values, error bars to standard deviation (n=4).

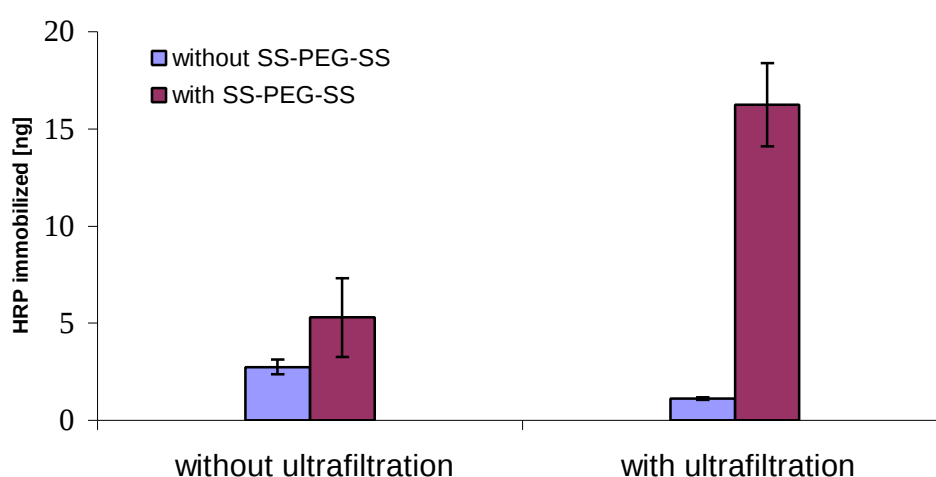
4.1.1.2.5 Effect of the ultrafiltration step on the coupling efficiency

In principal the coupling of HRP via conjugation with the bifunctional cross-linking agent SS-PEG₃₄₀₀-SS can be performed in two ways. In the first procedure the conjugation reaction is stopped by a ultrafiltration step. In this step the cross-linker molecules which have not reacted with the protein within the given reaction time are removed by ultrafiltration through a filter membrane with a cut-off of 10 kDa. In the second procedure the ultrafiltration step is omitted, this means that in this case in addition to the conjugate, also the cross-linker molecules, which have not reacted with the protein, are given to the collagen sponge. Experimental parameters and results are summarized in Table 7.

Table 7 Effect of ultrafiltration on HRP coupling efficiency.

HRP (5µg)	+	+	+	+
SS-PEG-SS (20µg)	-	-	+	+
pH	7,2	7,2	7,2	7,2
Reaction time	-	-	10 min	10 min
Ultrafiltration	-	+	-	+
CS (2mg)	+	+	+	+
HRP immobilized (ng)	2,75±0,382	1,12±0,08	5,29±2,013	16,25±2,145

The essential results are also shown in the column diagram of Figure 22. The results of Figure 22 clearly demonstrate that the ultrafiltration step strongly improves the coupling efficiency. Apparently the removal of cross-linker molecules, which have not reacted, has a positive effect on the coupling efficiency. A possible explanation for this phenomenon will be given in the Discussion.

**Figure 22** Effect of ultrafiltration on HRP coupling efficiency. Column heights correspond to the mean values and the error bars to the standard deviations (n=4).

4.1.1.2.6 Effect of conjugation temperature on the coupling efficiency

In this study, the influence of the reaction temperature of the conjugation step on the coupling efficiency has also been studied. Experimental parameters and results are shown in Table 8.

Table 8 Effect of reaction temperature on HRP coupling efficiency.

CS	(2mg)	control, 4°C	control, 22°C	control, 37°C	NHS-PEG-NHS, 4°C	NHS-PEG-NHS, 22°C	NHS-PEG-NHS, 37°C
HRP	(250µl)	+	+	+	+	+	+
SS-PEG-SS	(250µl)	5µg	5µg	5µg	5µg	5µg	5µg
Reaction temperature (°C)		-	-	-	20µg	20µg	20µg
HRP immobilized [ng]		4	22	37	4	22	37
		2,32±0,32	2,52±1,45	2,49±0,7	16,62±4,03	16,52±1,67	10,4±1,41

The final results are shown graphically in the column diagram of Figure 23. The conjugation step was performed at three different temperatures: 4 °C , 22 °C or 37 °C. The coupling efficiencies at 4°C and 22 °C are similar, the efficiency at 37°C, however, is substantially reduced.

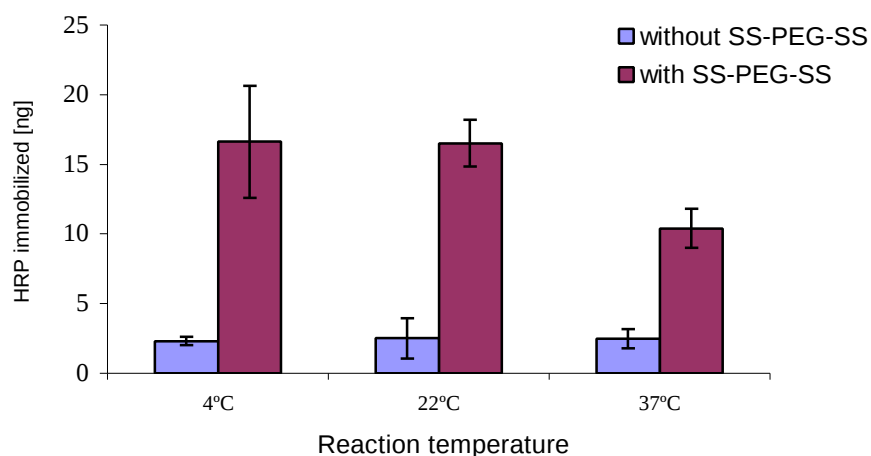


Figure 23 Effect of the reaction temperature on HRP coupling. Column heights correspond to the mean values and the error bars to the standard deviations (n=4).

4.1.1.3 HRP coupling efficiency under optimized conditions

Table 9 reports on a set of coupling experiments which were performed under optimized conditions. These optimized conditions were deduced from the results of experiments described in previous paragraphs. These optimized conditions are: pH of the conjugation reaction 7.2, protein and cross-linker were allowed to react for only 10 minutes, the reaction was then stopped by ultrafiltration and the reaction was performed at room temperature. Under these conditions an 8 times higher amount of HRP was immobilized in collagen sponges as compared with the group of control

experiments, which were performed in the absence of the cross-linking agent (Figure 24).

Table 9 General experimental protocol with HRP.

HRP	(5 μ g)	+	+
SS-PEG-SS	(20 μ g)	-	+
PBS	(0.5ml)	+	+
pH		7,2	7,2
Reaction time		-	10'
Ultrafiltration		-	+
CS	(2mg)	+	+
HRP immobilized (ng)		2,2 $\pm$ 0,16	16,8 $\pm$ 0,83
HRP in the sixth washout (ng)		0,038 $\pm$ 0,009	0,099 $\pm$ 0,0005

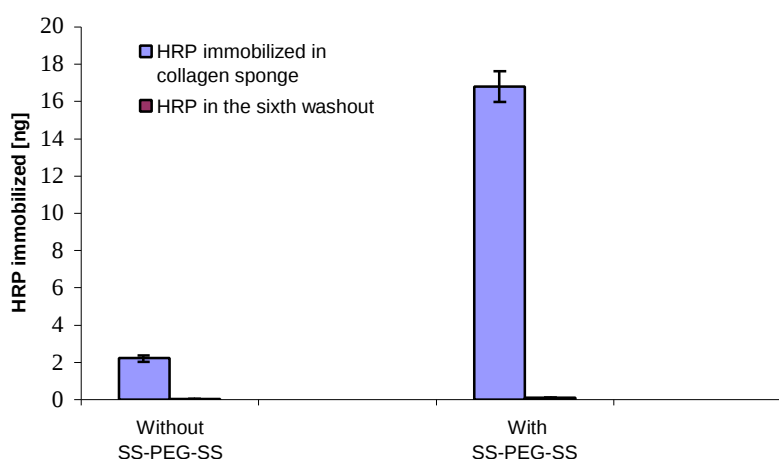


Figure 24 Binding of HRP to collagen sponges in the presence and absence of the SS-PEG₃₄₀₀-SS. Column heights correspond to the mean values and the error bars to the standard deviations (n=4).

4.1.1.4 Coupling of HRP to soluble collagen (with SS-PEG₃₄₀₀-SS)

Figure 25 shows that by using optimized reaction conditions, pH 7.2, 10' reaction of HRP with SS-PEG₃₄₀₀-SS, then followed by ultrafiltration, a 3 times higher HRP amount was bound to soluble collagen than to the control group.

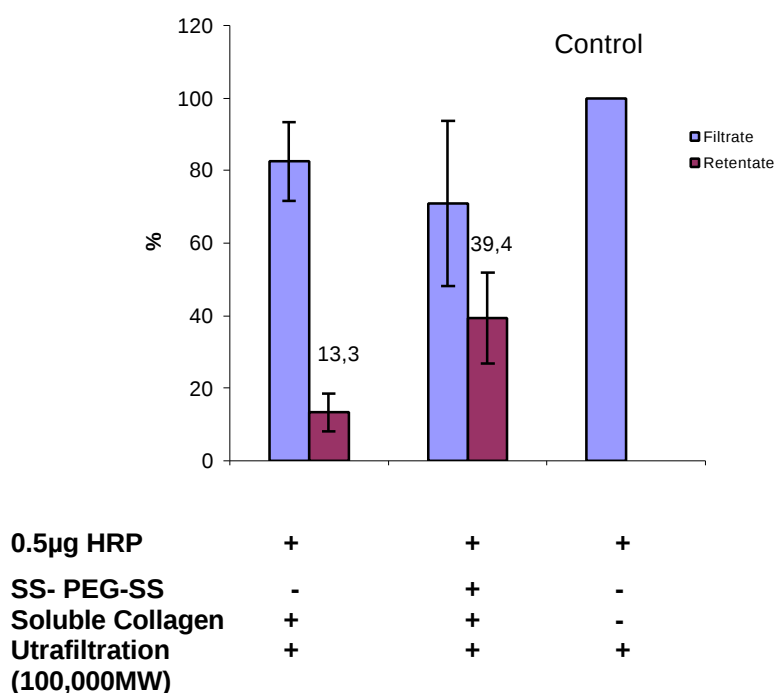


Figure 25 Effect of SS-PEG₃₄₀₀-SS on HRP coupling to soluble collagen. Column heights correspond to the mean values and the error bars to the standard deviations (n=4).

4.1.2 Cross-linking of VEGF to collagen

In the previous paragraphs, HRP was taken as a model protein, and the effect of SS-PEG₃₄₀₀-SS on HRP coupling to collagen has been demonstrated. In this paragraph, the effect of SS-PEG₃₄₀₀-SS on VEGF coupling to collagen will be investigated.

Before we study the effect of SS-PEG₃₄₀₀-SS on VEGF coupling, experiments have been performed to show the influence of SS-PEG₃₄₀₀-SS and collagenase on the antigenicity of VEGF. Figure 26 shows that the antigenicity of VEGF does not change obviously in the collagenase solution but was decreased in the SS-PEG₃₄₀₀-SS solution.

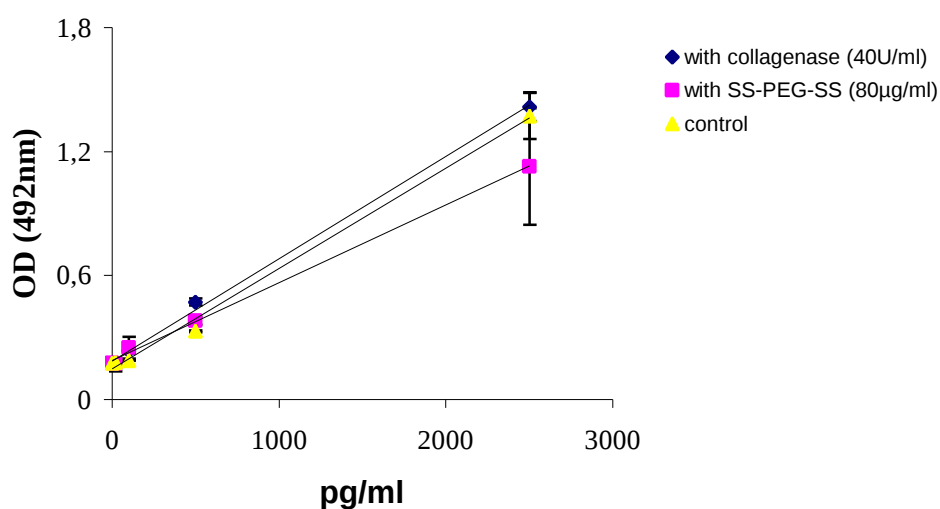


Figure 26 VEGF standard curves after treatment of VEGF with SS-PEG₃₄₀₀-SS or collagenase for 1 hour. Results shown represent average values and error bars represent the standard deviations (n=4).

Table 10 summarizes the experimental parameters and results of a series of experiments in which we investigated the coupling of VEGF to collagen with SS-PEG₃₄₀₀-SS. The optimal conditions are deduced from the coupling experiments with HRP (Paragraph 3.1.1.3). The results are shown as column diagram in Figure 27: a six time higher VEGF coupling efficiency was achieved when compared with the control group in which the cross-linker was omitted.

Tabel 10 General experimental protocol of VEGF coupling to collagen sponges.

VEGF	(0.5µg)	+	+
SS-PEG-SS	(2µg)	-	+
PBS	(0.5ml)	+	+
pH		7,2	7,2
Reaction time		-	10'
Ultrafiltration		-	+
CS	(2mg)	+	+
VEGF immobilized	(ng)	1,19±0,09	6,66±0,08

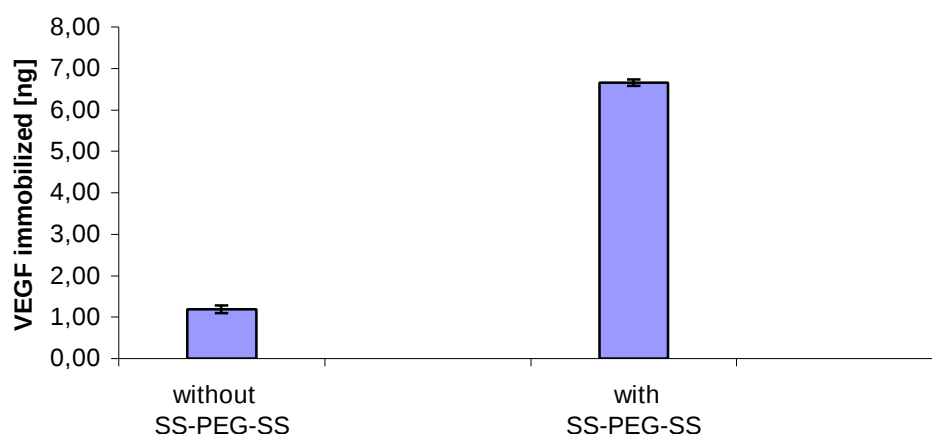


Figure 27 VEGF left after the washing procedure, the VEGF concentration was determined by ELISA. Column heights correspond to the mean values and the error bars to the standard deviations (n=4).

4.2 Exogenous cross-linking of collagen

As we have mentioned in the introduction, SS-PEG₃₄₀₀-SS may also be used to cross-link the collagen molecules within the collagen sponge. By this procedure collagen sponges may become more resistant against enzyme attack, leading to longer degradation time which may be necessary for a controlled release system. In this part, we investigate the cross-linking of collagen sponges by SS-PEG₃₄₀₀-SS.

4.2.1 *In vitro* degradation of collagen sponge with collagenase

The *in vitro* degradation of collagen sponges was studied by exposing the materials to collagenase from *Clostridium histolyticum*. As shown in Figure 28, the results show that when the concentration of the cross-linker was higher than 4mg/ml, the degradation of SS-PEG₃₄₀₀-SS treated collagen sponges was delayed in comparison to non-treated collagen sponges. While strikingly, longer cross-linking times did not lead to a longer degradation time.

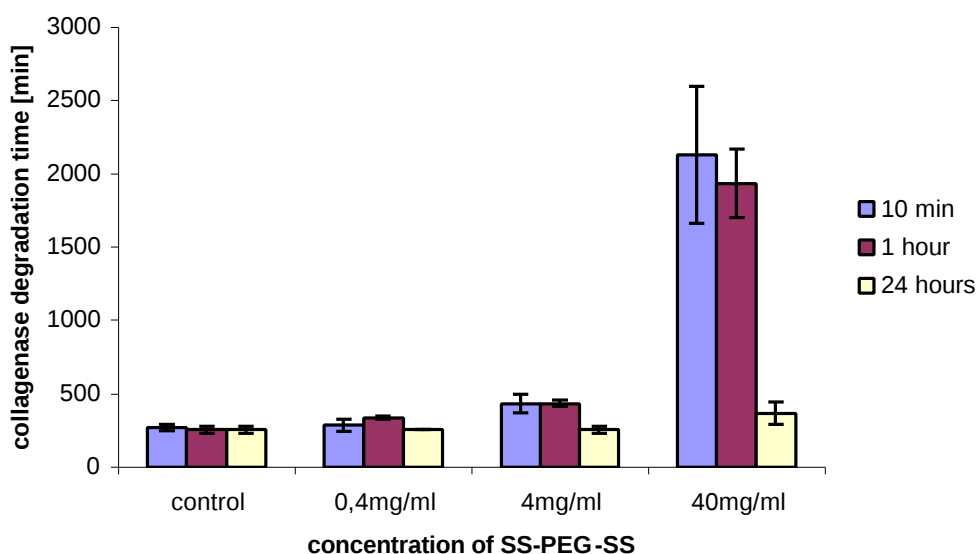


Figure 28 Dependence of the collagenase degradation time from the SS-PEG₃₄₀₀-SS-concentration and the cross-linking reaction time. Column heights correspond to the mean values and the error bars to the standard deviations (n=4).

4.2.2 Quantitative determination of collagen cross-linking

Cross-linking of the collagen using SS-PEG₃₄₀₀-SS results in a decrease of the amino groups. As shown in Figure 29, collagen sponge cross-linking degree rises as the increase of SS-PEG₃₄₀₀-SS concentration, but it decreases when reaction lasts longer which is in good agreement with the results of *in vitro* degradation experiments.

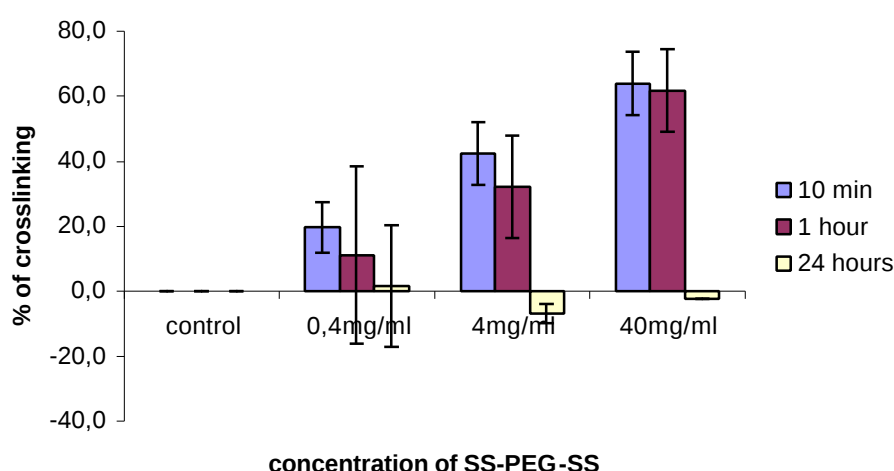


Figure 29 Dependence of the cross-linking degree from the SS-PEG₄₀₀-SS concentration and the cross-linking reaction time. Column heights correspond to the mean values and the error bars to the standard deviations (n=4).

4.3 Release of proteins from collagen sponges

4.3.1 Release of HRP

Realizing a controlled release of proteins from collagen sponges represents a major challenge within the field of tissue engineering and is also one of the main goals of this investigation.

Figure 30 compares the release of covalently coupled HRP from non-cross-linked collagen sponges and collagen sponges cross-linked with SS-PEG₄₀₀-SS. The release is initiated by immersing the sponges into a collagenase containing solution (0.1U/ml). Both release profiles are characterized by two phases: (a) An initial burst release occurring during the first 24 hours. The burst observed from the non-cross-linked collagen sponge was greater (about 95% in 24 hours) than that from the cross-linked collagen sponge (about 82% in 24 hours). (b) A longer linear steady release phase that lasts for 5 days. As expected the release of HRP from the cross-linked collagen sponge was slower.

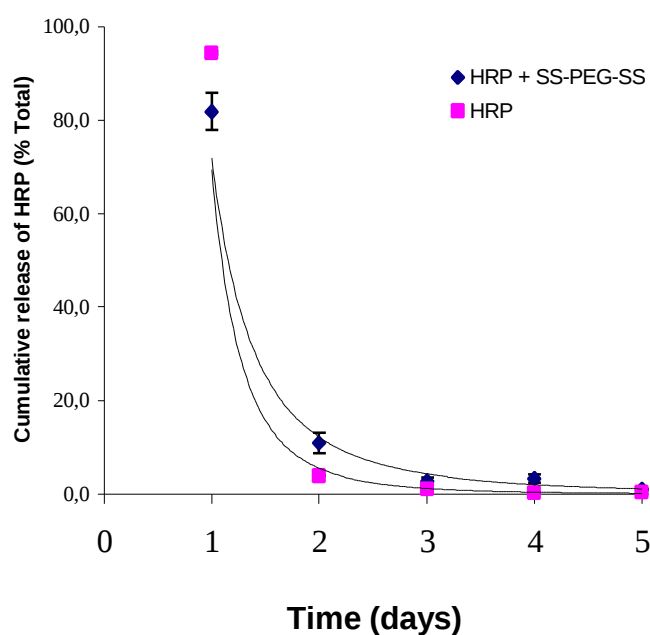


Figure 30 Release profiles of cross-linked or non-cross-linked HRP from collagen sponges degraded with collagenase (0.1 U/ml) at 24-hour intervals. Results shown represent average values and error bars represent the standard deviations (n=4).

4.3.2 Release of VEGF

The release of VEGF from cross-linked and non-cross-linked collagen sponges is shown in figure 32. In the control group which was non-cross-linked with SS-PEG₄₀₀-SS, almost all the VEGF released on the first day, and from the second day, VEGF became undetectable. In the case of the cross-linked sponges VEGF was released slower, even after 9 days VEGF could still be detected (Figure 31 shows the standard curve of VEGF).

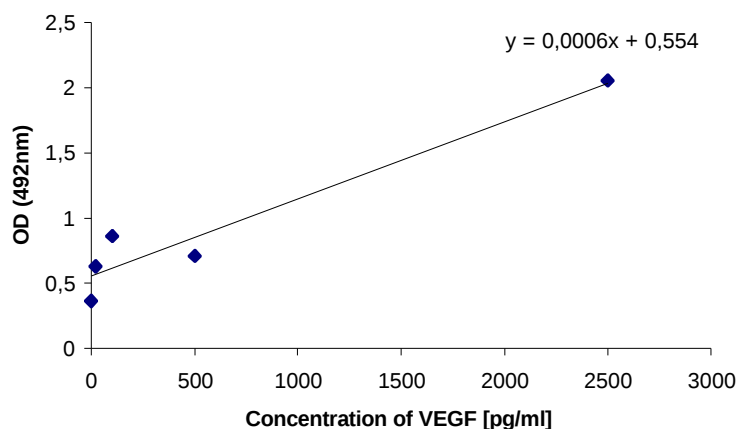


Figure 31 Standard curve of VEGF

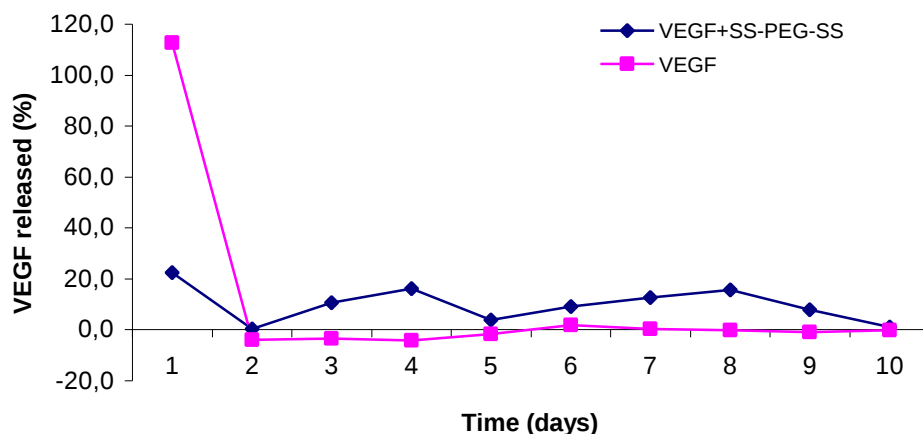


Figure 32 Cumulative release of VEGF from collagen sponges with or without treatment of SS-PEG₃₄₀₀-SS at 24-hour intervals.

4.4 Cytotoxicity tests

Although the cytotoxicity of polyethyleneglycol and a variety of derivatives has already been investigated, the cytotoxicity of the specific derivative SS-PEG₃₄₀₀-SS has so far not been tested. It remains therefore important to demonstrate that also SS-PEG₃₄₀₀-SS does not induce cytotoxic reactions. Fibroblast cell culture experiments were performed to study the effect of SS-PEG₃₄₀₀-SS on cells.

4.4.1 Effect of SS-PEG₃₄₀₀-SS on the proliferation of fibroblasts (MTT- test)

Fibroblasts were cultured in media containing varying concentrations of the cross-linker (concentration from 0,1 µg/ml to 10 mg/ml) The results as shown in figure 33 clearly demonstrate that according to the MTT-test the cross-linker has no effect on proliferation of fibroblasts. Only at very high concentrations a minor effect is observed.

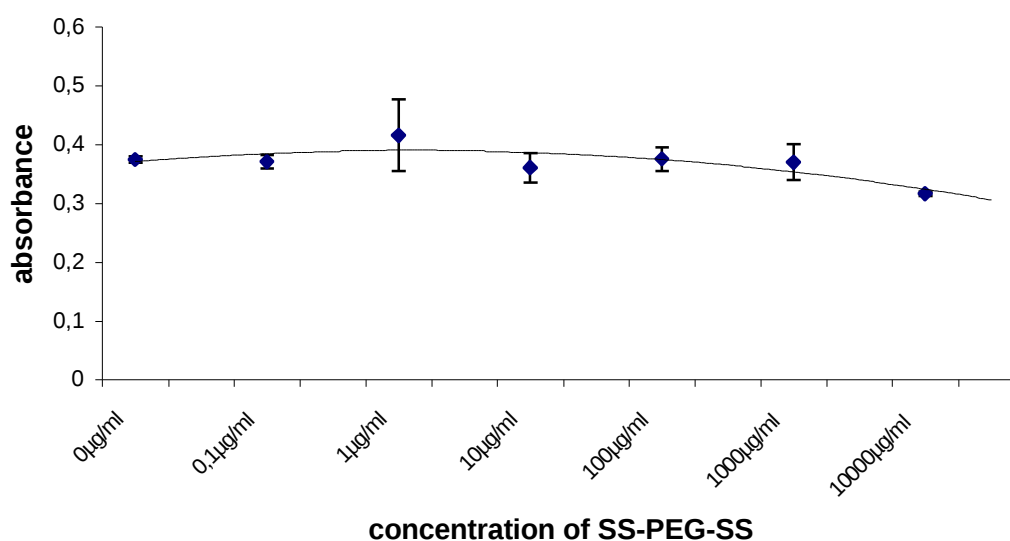


Figure 33 Proliferation of fibroblast in response to different SS-PEG₃₄₀₀-SS concentration. Results shown represent average values and error bars represent the standard deviations (n=4).

4.4.2 Effect of SS-PEG₃₄₀₀-SS cross-linking on fibroblast ingrowth into collagen sponges

Fibroblasts were seeded onto non-cross-linked and cross-linked collagen sponges to study the response of cells to the cross-linked materials. Results show that fibroblasts ingrowth was not significantly changed by the cross-linking procedure as compared to cells ingrown in non-cross-linked collagen sponges (Figure 34, 35).

Figure 34 show that one week after seeding, fibroblasts attached on the surface of the both groups of collagen sponges with formation of a monolayer of cells covering the entire sponges surface and the cells began to grow inward. Two weeks after seeding, histological sections showed that the cells had deeply penetrated into the

sponges and were spread over the collagen network (Figure 35). Ingrown fibroblasts were seen in the center of the sponges, and the cell density or cell morphology showed no obvious difference between the two groups, suggesting that SS-PEG₄₀₀-SS does not suppress the ingrowth of fibroblasts into collagen sponges.

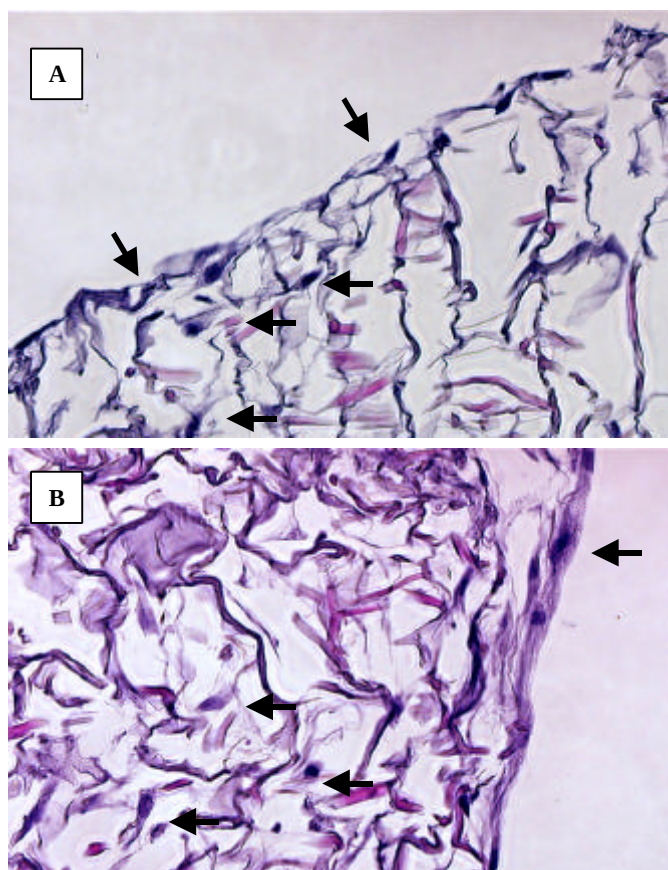


Figure 34 Light micrographs showing histological section of collagen sponges seeded with 2×10^5 fibroblasts/cm², 1 week after seeding: (A) non-treated collagen sponge; (B) collagen sponge treated with SS-PEG₄₀₀-SS (400 µg/ml). Arrows identify fibroblasts. Magnification: 200x.

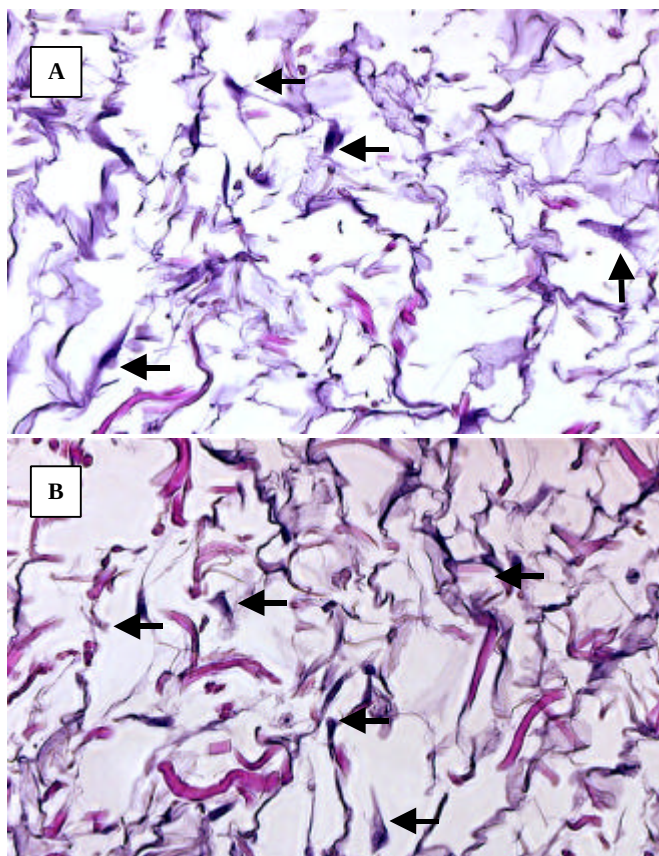


Figure 35 Light micrographs showing histological section of collagen sponges seeded with 2×10^5 fibroblasts/cm², 2 weeks after seeding: (A) non-treated collagen sponge; (B) collagen sponge treated with SS-PEG₄₀₀-SS (400 µg/ml). Arrows identify fibroblasts. Magnification: 200x.

4.4.3 Tissue responses to collagen sponge implantation

In order to evaluate the various tissue responses to non-cross-linked or cross-linked collagen sponges, samples of both types were implanted subcutaneously in rats for up to 6 weeks. The implanted collagen sponges produced a bulge under the skin, which disappeared progressively in two weeks, leaving the surface of the skin absolutely normal. At day 7, all implants were encapsulated by fibrous capsules, in which capillaries, fibroblasts and inflammation cells were observed. It was also noted that the capsules of the non-cross-linked collagen sponges were thicker than those of the cross-linked collagen sponges. Few polymorphonuclear cells (PMN) infiltrated the implants. One week after implantation, mononuclear cells had replaced the PMN within the implanted collagen sponges. Newly formed capillaries were observed 2

weeks after implantation in the center part of both non-cross-linked (Figure 36A) and cross-linked collagen sponges (Figure 36B). The non-cross-linked sponges began to collapse 2 weeks after implantation (Figure 37A). The pore structure of the cross-linked sponges, however, remained intact for at least 4 weeks (Figure 37B).

Accumulation of giant cells was observed close to the collagen/tissue interface, their number was, however, smaller in the case of the cross-linked collagen sponges (Figure 38 AB). A larger fibroblast ingrowth and an increase of the deposition of the extracellular matrix material was observed in the non-cross-linked collagen sponges (Figure 38 A).

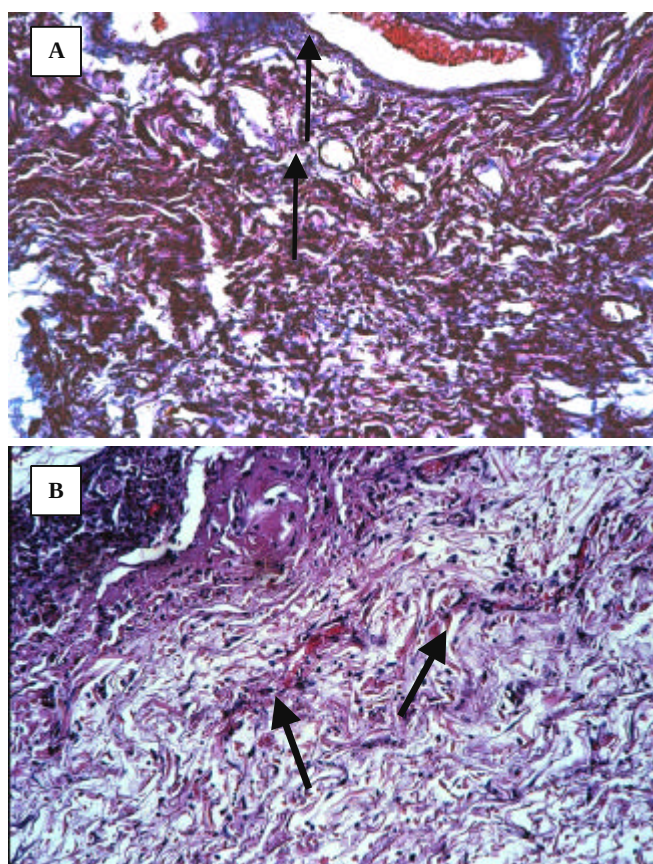


Figure 36 Collagen sponges after 2 weeks of subcutaneous implantation. (A) non-treated collagen sponge; (B) collagen sponge treated with SS-PEG-SS (400 µg/ml). Arrows identify ingrown vessels. Magnification: 50x.

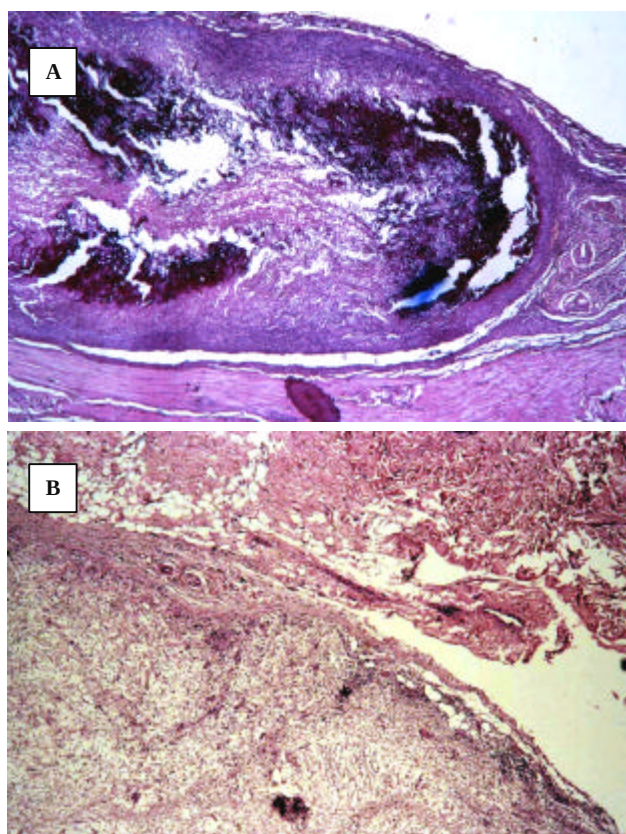


Figure 37 Collagen sponges: (A) 2 weeks after subcutaneous implantation, non-treated collagen sponge; (B) 4 weeks after subcutaneous implantation, collagen sponge treated with SS-PEG₃₄₀₀-SS (400 µg/ml). Magnification: 50x.

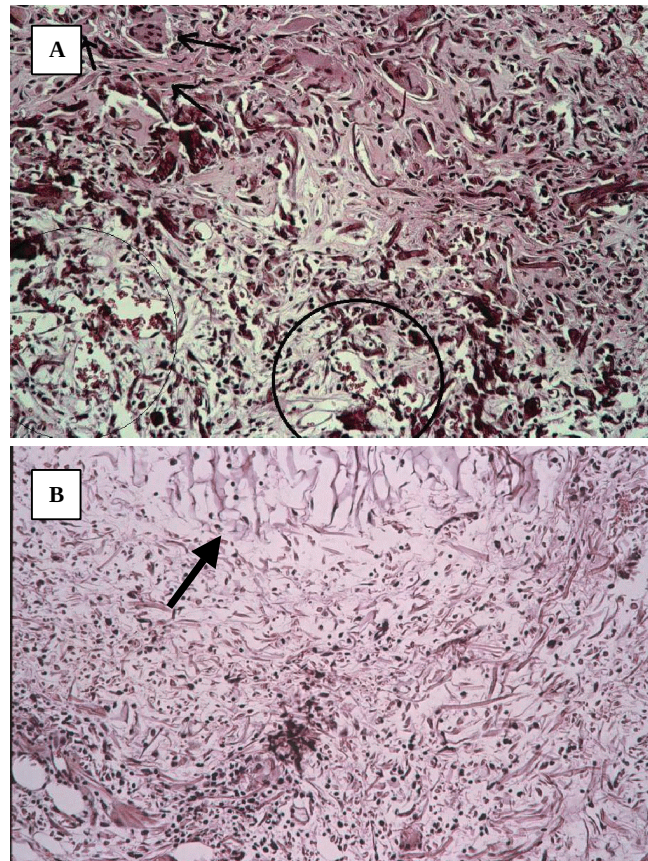


Figure 38 Collagen sponges at week 4 after implantation.
(A) non-treated collagen sponge. Circles identify angiogenesis, arrows identify foreign body giant cells; (B) collagen sponge treated with SS-PEG₄₀₀-SS (400 µg/ml). Arrows identify original collagen sponge structure. Magnification: 50x.

5. Discussion:

Traumatic injuries are often associated with tissue loss. Restoration of normal structure and function in these cases requires replacement of missing tissue which may be accomplished by surgical transfer of healthy and natural tissue from an uninjured location elsewhere in the body (Kaihara and Vacanti, 1999) (Peter et al., 1998). However, this method is seriously limited by tissue availability and secondary deformities at the donor site.

To overcome the limitations of the conventional reconstructive approaches, our objective was to develop a tissue substitute based on biodegradable collagen matrices. This study aims at an increased ingrowth of cells (e.g., endothelial cells, fibroblasts) from the host bed into this optimized tissue substitute. These cells should attach to the seeded scaffold, replicate, differentiate, and organize into normal healthy tissue as the scaffold degrades.

The crucial point for any cells to replicate and to proliferate is vascularization. This study demonstrates possible avenues for optimizing matrices via enhancing vascularization by chemical coupling of growth factors into the matrix.

A variety of tissue-inducing factors (e.g., growth factors) that promote cell proliferation have been identified (Nimni, 1997). Among them, growth factors such as TGF- β , EGF (epidermal growth factor) and bone morphogenetic proteins (BMP) have been used with collagen to induce and to promote tissue repair and remodeling (Bentz et al., 1998) (Kuhl and Griffith-Cima, 1996) (Bentz et al., 1991)

The present study focus on the delivery of VEGF, a potent stimulator of angiogenesis *in vivo* which acts specifically on endothelial cells (Neufeld et al., 1999) (Peters et al., 1998) (Plate and Warnke, 1997) (Klagsbrun and D'Amore, 1996) (Senger et al., 1996) (Dvorak et al., 1995)

Cell responses to growth factor do not only depend on the presence of the factor, but on the magnitude and duration of its presence as well (Marshall, 1995). However, if the growth factors are simply mixed to the collagen matrices, they elicit a 'burst release' because of diffusive spread, cell uptake, and degradation (Bentz et al., 1998) (Kuhl and Griffith-Cima, 1996). A burst release of growth factor may increase the local drug concentration to an undesirably high level or at least lead to the loss of

the excess drug. A controlled release of VEGF is desirable in order to maintain the released VEGF at an optimally effective concentration.

These problems might be overcome if VEGF is covalently bound to collagen matrices. In this study, in order to control the release of VEGF and to prolong its bioactivity, VEGF was covalently linked to collagen sponges via the homobifunctional cross-linking agent bis-(succinimidyl succinate) polyethylene glycol (SS-PEG₄₀₀-SS). PEG derivatives have been widely used to modify enzymes and growth factors (Zalipsky et al., 1992)(Katre et al., 1987)(Abuchowski et al., 1977)(Abuchowski et al., 1977), as well as cross-linking collagen(Catalog Shearwater Polymers, 1997) (Doillon et al., 1994) Moreover PEG-attached proteins are typically more stable and usually remain highly active(Katre et al., 1987)

In the present study, the experimental parameters of protein coupling, biological behavior of collagen sponges as well as tissue and cell reactions after the treatment of collagen with SS-PEG₄₀₀-SS have been investigated.

5.1 Cross-linking of proteins to collagen

To achieve the optimal reaction conditions which will lead to a high VEGF coupling efficiency, a large number of experiments had to be performed. However, VEGF is still too expensive to be used for that purpose (500 DM per 10 µg, Chemicon).

HRP has a similar molecular mass as VEGF and its activity can be easily quantified (Internet protein databank SWISS-PROT, <http://www.expasy.ch/cgi-bin/niceprot.pl?P80679>). Due to these reasons, we selected HRP as a model protein.

The coupling or cross-linking procedure was divided into two steps.

In the first step (conjugation reaction), the target protein (VEGF or HRP) was allowed to react with the homobifunctional cross-linker (SS-PEG₄₀₀-SS) and the protein-PEG-SS-conjugate is formed (Figure 8). In the second step, after stopping the conjugation reaction via an ultrafiltration procedure (figure 9), the protein-PEG-SS conjugate is collected and added to collagen sponge in order to realize the covalent coupling (Figure 10).

One-step or two-step reaction:

Even for our purpose it is tempting to just add all reagents (SS-PEG₃₄₀₀-SS, HRP or VEGF and collagen sponge) at the same time. Comparing the easy one-step reaction with the two step procedure in which the first reaction (HRP and PEG) was stopped by ultrafiltration, the one-step procedure shows a 60% less coupling efficiency. (Figure 22).

The necessity of a two step procedure was very well discussed by Hermanson (Hermanson, 1996b) After the conjugation reaction procedure, if the excess cross-linker molecules have not been removed by the ultrafiltration procedure, they might competitively block most of the amino groups in collagen during the cross-linking reaction procedure because of their amount advantage compared to (Protein-PEG₃₄₀₀-SS)-conjugates, while the amino groups are expected to react with HRP or VEGF conjugates.

At the same time, the problems of indeterminate conjugation products are amplified in single-step reaction procedures using homobifunctional reagents (Hermanson, 1996b). Only a small percentage of which represent the desired or optimal conjugate. Excessive conjugation may cause the formation of insoluble complexes that consist of very high molecular weight polymers which have no biological activity (Figure 39). To overcome this shortcoming, a two-step reaction procedure has been used. In the two-step protocols, HRP or VEGF reacts with SS-PEG₃₄₀₀-SS, the excess cross-linking reagent and by-products are removed. In the second stage, the activated protein is mixed with the other protein or molecule to be conjugated, and the final cross-linking process occurs.

The use of homobifunctional reagents in two-step protocols still creates many of the problems associated with single-step procedures (Hermanson, 1996a; Hermanson, 1996b). Homobifunctional reagents by definition have the same reactive group on either end of the cross-linking molecule, the first protein can cross-link and polymerize with itself long before the second protein is added. This inherent potential to polymerize uncontrollably is characteristic of all homobifunctional reagents, even in multi-step protocols.

Conjugation reaction (first step):

In order to standardize our method several basic experiments were performed. Firstly the following steps have to be cleared.

- ? How long will the conjugation reaction last?
- ? How much of the protein will react with SS-PEG₄₀₀-SS?
- ? Which kinds of products will be formed?

In a series of experiments methods as RP-HPLC, MALDI-TOF and SDS-PAGE have been used. The results suggest that already after 10 minutes, all HRP molecules have been reacted with cross-linker molecules.

This was demonstrated by: i) RP-HPLC: after the ten-minute reaction, the peak appearing at 23 minutes which corresponds to original HRP completely disappeared, and there are new peaks appearing at 26 and 28 minutes respectively (Figure 14). ii) MALDI-TOF: after the ten minute reaction, the newly formed product has a molecular mass of 47 kD rather than 44 kD of the HRP molecule (Figure 15). iii) SDS-PAGE: after a reaction lasting longer than 10 minutes, only one band appears at about 48 kD while native HRP migrates corresponding to the expected molecular size of approximately 44 kD. (Figure 16).

We know from the HRP amino acid sequence that there are altogether 9 lysines in 1 HRP molecule (Internet protein databank SWISS-PROT, <http://www.expasy.ch/cgi-bin/niceprot.pl?P80679>). If all the lysines react with SS-PEG₄₀₀-SS, the molecular weight of the newly formed molecule should be around 100 kD. The results of MALDI-TOF and SDS-PAGE suggest that the molecular mass of the (HRP-PEG₄₀₀-SS)-conjugate is only 47 kD to 48 kD, which means that only 1 SS-PEG₄₀₀-SS molecule has reacted with one HRP molecule. A possible explanation for this observation may be that many lysine residues are involved in the dimer formation and not accesible for conjugation.

After the reaction of HRP and SS-PEG₄₀₀-SS, there is no band or peak which corresponds to the original HRP that can be observed in SDS-PAGE or RP-HPLC experiments respectively. This suggests that the efficiency of the first reaction step is high. These data are in good agreement with the results of Bentz et al. which also showed an high combination rate of TGF- β with the bifunctional cross-linkers

(difunctional succinimidyl glutarate (D-SG-PEG) or succinimidyl protionate polyethylene glycol (D-SE-PEG))(Bentz et al., 1998)

Coupling reaction (second step):

Coupling of the (HRP-PEG₃₄₀₀-SS)-conjugate to collagen sponges was demonstrated by testing the HRP activity after extracting the non-coupled HRP from collagen sponges. After the extensive washing procedure, most of the HRP molecules which have not been coupled to the collagen sponges, were washed out. While the HRP molecules covalently coupled to collagen and the HRP molecules, which were physically absorbed, remained in the collagen sponges. The amount of residual HRP activity was quantified after degrading the collagen sponge with collagenase.

As shown in Figure 24, in both control (only HRP) and (HRP-PEG₃₄₀₀-SS)-conjugate treated groups, the HRP activity in the last (sixth) washout liquid was almost zero, demonstrating that most of the physically absorbed HRP was washed out. The residual HRP activity in the (HRP-PEG₃₄₀₀-SS)-conjugate treated groups was about 8 times higher than that in the control group. These results do demonstrate the effect of SS-PEG₃₄₀₀-SS in HRP cross-linking to collagen.

The results of our preliminary experiments showed that different reaction conditions would lead to different final HRP coupling efficiencies. In order to achieve the optimal reaction conditions that can lead to a higher protein coupling efficiency, we performed a series of experiments in which we investigated :

- ? reaction time of conjugation step,
- ? pH,
- ? molar ratio of the protein and the cross-linker,
- ? ultrafiltration,
- ? reaction temperature.

Reaction time:

As the stability of HRP alters with time, the natural deterioration time of HRP and the effect of the homobifunctional cross-linker on it were determined. HRP was allowed to react with SS-PEG₃₄₀₀-SS for 10 minutes, 1 hour or 24 hours, then the activity was

measured and compared with the HRP control groups in which SS-PEG₄₀₀-SS was absent. The results (Figure 18) show that HRP loses its activity as a function of time, but the trend was slower in the group treated with SS-PEG₄₀₀-SS than in the control group. These data are in good agreement with the results of other researchers' which showed that PEG coupled to proteins can increase their stability in solution (Berger and Pizzo, 1988). This may be explained by the fact that PEG is an uncharged hydrophilic polymer and typically increases solubility and stability of molecules to which it is attached (Bentz et al., 1998).

Interestingly the experiments also demonstrate that the HRP coupling efficiency to collagen is obviously higher in the group in which the first step reaction lasted 10 minutes than in the groups where the reaction lasted longer than 1 hour (Figure 19).

There are two possible explanations for this phenomenon.

One possibility was discussed by Avrameas et al. This group postulated that, it was possible that the cross-linking of two proteins with homobifunctional agents results in a system of interconnected conjugates (Avrameas, 1969).

In the first reaction step, the SS-PEG₄₀₀-SS may initially react with one of the proteins, forming an active intermediate. This activated protein may then form cross-links with other proteins. The activated groups may also react intramolecularly with other functional groups on the same polypeptide chain (Hermanson, 1996b). As shown in figure 39, other cross-linker molecules may continue to react with these already conjugated species to form various mixed products. At longer reaction times, the HRP conjugates may even react with each other, leading to high molecular aggregates. Thus even before they have a chance to react with collagen, their activity would already have been lost.

These aggregates then should have a high molecular mass. However, our SDS-PAGE results do not support this explanation. After a reaction time of 24 hours, only one band with an apparent molecular mass of about 48 kDa was observed. This corresponds to the expected mass of a (HRP-PEG₄₀₀-SS)-conjugate. No band corresponding to the HRP-dimer (about 90 kD) or -trimer (about 140 kD) has been observed.

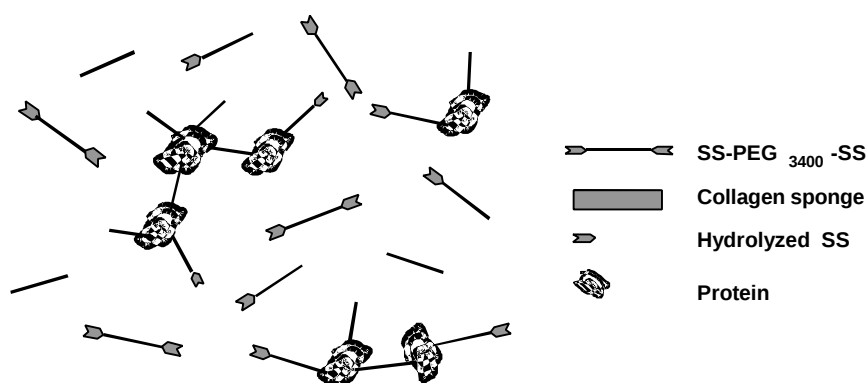


Figure 39 The possible reaction products after the first reaction step.

The second possibility is that after a longer reaction time, most activity of the second functional SS-group on the (HRP-SS₃₄₀₀-SS)-conjugate has been hydrolyzed which is supposed to react with lysines in collagen sponge in the second step reaction. It is known that the hydrolysis half life of SS group at pH 8, 25 °C is 9.8 minute (Catalog Shearwater Polymers, 1997) which means that after the ten-minute conjugation reaction of SS-PEG₃₄₀₀-SS with HRP, half of SS ester groups that are expected to react with amino groups in collagen, have been lost (Figure 40). If the reaction lasts longer, the residual activity of SS groups will be even smaller. This may explain why the coupling efficiency of the HRP-PEG conjugate to the collagen was relatively low.

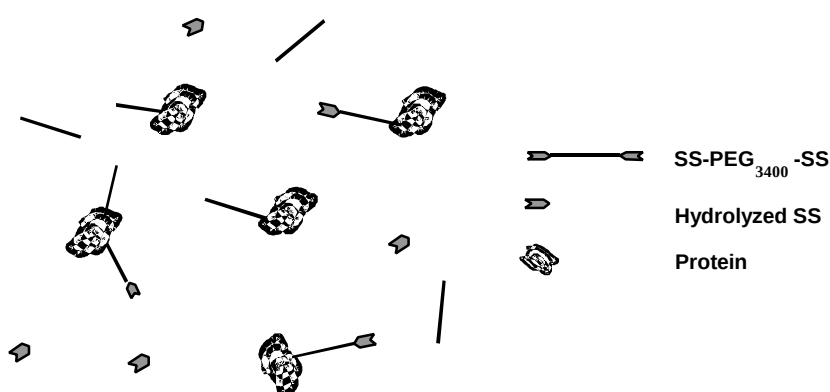


Figure 40 The possible reaction products after the first step reaction.

Effect of pH:

The pH plays an important role in the coupling procedure (Catalog Shearwater Polymers, 1997). HRP coupling efficiency is much higher if the pH range is between 7.2 and 8.5 (Fig. 20).

In order to explain this phenomenon we may consider the fact that ionizable amino groups within proteins can exist in two forms: protonated (NH_3^+) or unprotonated (NH_2). As shown in figure 8, SS groups of the homobifunctional cross-linker react with the nucleophilic nonprotonated free amino groups (NH_2) of lysine in HRP or VEGF (in the conjugation reaction) and collagen (in the cross-linking reaction). To increase the reaction of SS groups with proteins, it is required to convert the free amino groups in proteins into a more strongly nucleophilic agent or to increase the molar proportion of non-protonated to protonated amino groups ($[\text{R}-\text{NH}_2]/[\text{R}-\text{NH}_3^+]$). Increasing the pH of the reaction leads to a higher ratio of $[\text{R}-\text{NH}_2]/[\text{R}-\text{NH}_3^+]$ (Sung et al., 2000), thus the number of non-protonated groups will increase with pH.

Anyhow further increment of the pH results in a higher hydrolysis rate of the succinimidylester bond (Lomant and Fairbanks, 1976) this means that a balance has to be found between the rate of covalent bond formation and hydrolysis.

Temperature:

It is known that the pK values of the ionizable groups in proteins decrease with increasing temperature (White et al., 1973) (McClain and Wiley, 1972). It can therefore be deduced that at the same pH the ratio of $[\text{R}-\text{NH}_2]/[\text{R}-\text{NH}_3^+]$ increases with increasing temperature. Thus at higher temperatures the molecules are more reactive than at lower temperatures, the collision frequency increases, so a higher combination rate may be expected at a higher temperature. But as indicated in Figure 23, the HRP combination rate was lower at 37 °C than at 22 °C or 4 °C. This may be explained by the increased instability of SS-PEG₃₄₀₀-SS at 37 °C than at 4 °C or 22 °C.

Molar ratio:

In the conjugation reaction procedure, excess amount of SS-PEG₃₄₀₀-SS should be used. The half-life of SS-PEG₃₄₀₀-SS is relatively short, and some of the reactive

groups--SS groups will be hydrolyzed even before they can have a chance to react with HRP or VEGF molecules (Figure 40). We know from the amino acid sequence that there are 24 lysines in one VEGF (Internet protein databank SWISS-PROT, <http://www.expasy.ch/cgi-bin/niceprot.pl?P15692>) and 18 lysines in one HRP molecule (Internet protein databank SWISS-PROT, <http://www.expasy.ch/cgi-bin/niceprot.pl?P80679>) which means every molecule of HRP and VEGF has the potential to react with several SS-PEG₃₄₀₀-SS molecules. It might be possible to enhance the protein coupling efficiency by raising the number of SS-PEG₃₄₀₀-SS molecules coupled to the protein. Our results show that within a certain range HRP coupling efficiency increases with the increase of the molar ratio of HRP to SS-PEG₃₄₀₀-SS (Figure 21).

However when too many cross-linker molecules are involved, the active center of the protein may be blocked. We found that HRP activity decreases with increasing the concentration of SS-PEG₃₄₀₀-SS. So it appeared to be necessary to adjust the molar ratio of SS-PEG₃₄₀₀-SS to the target molecule, HRP or VEGF. The level of modification and conjugation may thus be controlled in order to create an optimal product.

Soluble collagen:

In the present study, the protein coupling reaction was also investigated by using soluble collagen. In the experiments that soluble collagen instead of collagen sponges were used for the second reaction, a much higher HRP collagen coupling efficiency has been achieved (Figure. 25).

The collagen sponges we use in the experiments are three-dimensional matrices that have the size of 0,5 * 0,5 * 0,2 cm. The solid three dimensional structure of collagen sponge may be the limitation of the reaction of HRP or VEGF conjugates within the center of the collagen sponge, since it may produce a physical barrier for the HRP- or VEGF-conjugates.

Prolonged penetration time of the HRP- or VEGF-conjugates into the sponge might result in an activity loss of the second SS groups on the protein conjugates. So it is possible that there is a concentration gradient of HRP or VEGF conjugates covalently combined throughout the collagen sponges. And this may be another important reason for the low protein coupling efficiency besides the short half-life of SS ester.

This problem can be partly solved by using soluble instead of fibril collagen (Figure 25). If the (HRP-PEG₃₄₀₀-SS)-conjugate is allowed to react with soluble collagen instead of a collagen sponge the collagen molecules have more chances to collide with the conjugate-molecules than in the solid collagen sponge.

To transform this protein coupled soluble collagen into collagen sponge in order to provide a matrix for tissue engineering is base of a current project.

Bioactivity of proteins:

The goal of most protein modification or conjugation procedures is to create a stable product with good preservation of the native state and activity. Ideally, any derivatization should result in a protein that performs exactly as it would in its unmodified original form.

One of the best ways to ensure preserve of the activity in protein molecules is to avoid chemical reaction at the active center (Hoffman et al., 2000)(Ding et al., 1998)

The active center is the portion of the protein where ligand, antigen, or substrate binding occurs. The interplay of amino acid functional groups and the three-dimensional folding of polypeptide chains is seen as the formation process of the protein activity. It is very important to avoid the active site by selecting functional groups away from that area or by protecting the site through the incorporation of additives.

Our results show that SS-PEG₃₄₀₀-SS does not obviously affect the activity of HRP (Figure 17) or the antigenicity of VEGF (Figure 26) during the cross-linking process.

By checking the amino acid sequence (Internet protein databank: SWISS-PROT, <http://www.expasy.ch/cgi-bin/niceprot.pl?P15692>), we know that there are 24 lysines existing in the dimer molecule of VEGF₁₆₅ (Figure 41). As shown in Figure 42, only lys₈₄ is involved in the binding site of the kinase domain receptor (KDR) (Muller et al., 1997).

We assume that the harmful effect of the bifunctional cross-linker on VEGF bioactivity can be minimized by adjusting the molar ratio of VEGF and SS-PEG₃₄₀₀-SS. Further experiments of our research group will focus on the effects of SS-PEG₃₄₀₀-SS on the bioactivity of VEGF.

1	11	21	31	41
APMAEGGGQ	NHHEVV K FMDV	YQRSYCHPIE	TLVDIFQEYP	DEIEYIF K PS
51	61	71	81	91
<u>CVPLMR</u> CGGC	<u>CNDEGLE</u> CVP	<u>TEESNIT</u> MOI	<u>MRIK</u> PHOGOH	<u>IGEM</u> SFLOHN
101	111	121	131	141
K CECRP KK DR	ARQE K PCGPC	SERR K HLFVQ	DPQTC K CSC K	NTDSRC K ARQ
151	161	165		
LELNERTCRC	D K PRR			

Figure 41 Amino acids sequence shows the position of lysines (red) in a VEGF₁₆₅-monomer. Sequence 8-109 (underlined) was over-expressed to solve the crystal structure shown in Figure 41.

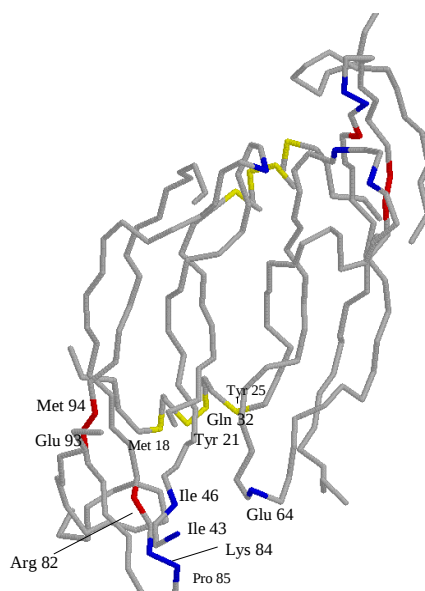


Figure 42 Main-chain representation of the binding determinants on the structure of VEGF 8–109 including the side chains of the binding determinates for KDR (blue) and both antibodies (red for A4.6.1 and yellow for 3.2E3.1.1). This figure was produced using the software program Raswin.

5.2 Exogenous cross-linking of collagen for obtaining controlled degradation

Collagen sponges used as connective tissue scaffold and VEGF carrier must maintain structural integrity and porosity to facilitate tissue ingrowth. Growth factors should be incorporated in a stable manner, and released in a controlled manner with desirable kinetics, thus to promote the vascularization of the developing tissue.

After implantation the collagen sponge is prone to an enzymatic attack which can result in rapid degradation of the material (Doillon et al., 1994a) (Doillon et al., 1994b). Therefore collagen sponges used as VEGF carrier should be stabilized by cross-linking to control the rate of biodegradation.

Our way to couple a growth factor into the sponge has a perfect side effect to this problem. The used linking agent acts as a cross linker on the collagen microstructure as well. In this matter cross-linking means the creation of new additional chemical bonds between the collagen molecules, and thereby reinforce the tissue to give a tough and strong but non-viable material that maintains the original shape of the tissue (Figure 43).

The reaction involved during cross-linking have been extensively studied, but the reaction mechanism is very complex and still not completely understood (Zeeman, 1998b).

One way to investigate the effect is the degradation of the manipulated sponges. The resistance against enzymatic degradation of the cross-linked or non-cross-linked collagen sponge is usually studied by in vitro tests (Zeeman et al., 1999a) (Zeeman et al., 1999b) (Zeeman, 1998a) (Doillon et al., 1994a) using enzymes such as bacterial collagenase from *Clostridium histolyticum*, which is capable of cleaving peptide bonds within the triple helical structure and has a specificity for the Pro-X-Gly-Pro-Y region, splitting between X and Gly (Friess, 1998). The rate of degradation of collagen sponge by collagenase is determined by the cross-link density, the accessibility of the cleavage sites and the extent of denaturation. Cross-links will hinder the collagenase to act at the specific cleavage sites, thus to decrease the degradation rate (Zeeman, 1998a) On the other hand, the stability of the PEG-modified collagen sponges might be linked to the repulsive properties of PEGs after

which their covalent binding to the amino groups of the protein stabilize the tertiary structure of the proteins (Doillon et al., 1994a)

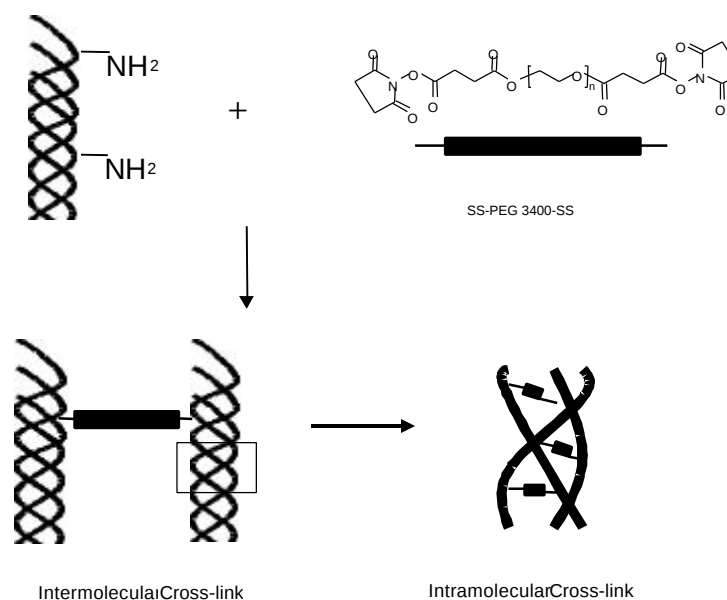


Figure 43 Presumable cross-linking mechanism of collagen with SS-PEG₃₄₀₀-SS.

Our results show that modified collagen sponges have demonstrated a significant increased biostability. The resistance to collagen degradation rises when the concentration of SS-PEG₃₄₀₀-SS is increased higher than 40mg/ml (Figure 28). In the groups where the concentration of SS-PEG₃₄₀₀-SS was lower than 40mg/ml, the collagen degradation time did not show differences when compared with the control group.

The free amino group content decreases as the SS-PEG₃₄₀₀-SS concentration rises (Figure 29). Both the collagen degradation period and degree of cross-linking get lower when the reactions last longer, which indicates that the cross-linking of the collagen sponges by SS-PEG₃₄₀₀-SS is completed after about 10 minutes. This finding is reproducible but the reason for this phenomenon remains unclear. One might speculate that the high activity of SS group leads to a quick reaction with lysine groups, and the SS groups will be hydrolyzed in a short time. At the same time, the cross-linking reaction might be hindered by the 3D structure of the sponges which may be a physical barrier for the reagents to enter the center part of the sponge, as discussed earlier.

5.3 Release of proteins from collagen sponge

Protein drug releasing profiles can be affected by several factors: the hydrophobicity, size and concentration of the drugs, the interaction with collagen, the pore size and degradability of the collagen sponge (Peters et al., 1998) (Friess, 1998) (Bentz et al., 1998). Constant release is desirable in order to maintain the released drug at a constant effective concentration. A large burst release may increase the local drug concentration to an undesirably high level or simply lead to the loss of the excess drug (Peters et al., 1998)

In the present study protein drugs were released in a two-phase fashion (Figure 30 and figure 32). The initial burst release was due to the releasing of proteins absorbed at the collagen sponge surface. The subsequent collagen sponge degradation and protein diffusion lead to a second linear release phase and collagen mass loss.

Proteins exhibited a much more significant initial burst release from the non-cross-linked collagen sponges since all the proteins were just admixed with collagen sponges. While in the cross-linked collagen sponges, part of the proteins were covalently coupled to the collagen, they are released as the collagen sponges degrades. We suppose that it might be feasible to design a system to deliver VEGF over a desired time span via adjusting the degradation process of collagen.

To test the release of the proteins, collagen sponges were immersed in a diluted collagenase solution (0.04 U/ml). The collagenase solution was changed and collected daily. As shown in our results (Figure 32), after one day, VEGF could not be detected in the non-cross-linked collagen sponges, but VEGF could be detected in the cross-linked group, even 10 days later.

Besides controlling the release profiles, another key issue is the preservation of VEGF stability. As a protein, VEGF is especially fragile during formulation and release. It is possible that VEGF may have lost its activity even before it is released from the collagen matrices. However, the half-life of the proteins can be increased by conjugation with inert carrier polymers (Mark Saltzman and Baldwin, 1998). We suppose that besides changing the release kinetics, SS-PEG₄₀₀-SS cross-linking can also prolong the biological half-life of VEGF. Ongoing investigation will focus on the biological activity of the released VEGF.

5.4 Cytotoxicity

The application of cross-linking agents for coupling proteins and for cross-linking collagen implants implies that these agents exert no or little cytotoxicity. Doillon et al. studied the tissue reaction toward activated PEGs conjugated collagen sponges. The results showed that PEGs conjugated onto collagen sponges stabilize the porous structure without deactivating the biological properties of collagen (Doillon et al., 1994a). Although the safety of PEG application has been widely reported (Zalipsky, 1995) (Dittmann, 1973), the cytotoxicity and tissue response to the PEG's derivative used in our investigations needed to be evaluated.

This was the first time SS-PEG₃₄₀₀-SS was used as a coupling agent in collagen sponges. In order to evaluate the cytotoxicity of SS-PEG₃₄₀₀-SS we performed *in vitro* tests with fibroblast cell culture and *in vivo* tests by subcutaneous implantation.

The effect of SS-PEG₃₄₀₀-SS on the proliferation of cells was investigated by fibroblasts cultures in varying concentrations (0-10000 µg/ml) of SS-PEG₃₄₀₀-SS solution. The results of MTT experiments show that SS-PEG₃₄₀₀-SS does not suppress the proliferation of fibroblasts even when the concentration rises to 10000 µg/ml (Figure 33), while the concentration of SS-PEG₃₄₀₀-SS is only 80 µg/ml in the protein cross-linking experiments or 400 µg/ml in *in vivo* exogenous collagen cross-linking experiments.

To know the influence of exogenous collagen cross-linking on cell ingrowth behavior, fibroblasts were seeded onto SS-PEG₃₄₀₀-SS cross-linked or non-cross-linked collagen sponges. Compared with fibroblasts seeded onto non-cross-linked collagen sponges, cells seeded onto cross-linked collagen sponges show a reasonable cell growth and morphology (Figure 34, 35), this suggests that the exogenous cross-linking of collagen sponges with SS-PEG₃₄₀₀-SS has no effect on cell ingrowth behavior.

Before biomaterials can be applied for tissue engineering, the tissue response to these materials has to be evaluated (Pieper et al., 1999). The presence of an implanted biomaterial in the body will generate a series of inter-related reactions, the

nature of which depends greatly on the type of materials and its physical and chemical structures (Boon et al., 1995) In order to investigate the tissue responses against the implanted materials, cross-linked or non-cross-linked collagen sponges were implanted subcutaneously into rats, and explantations were performed 3 days, 1 week, two weeks and four weeks after implantation.

The *in vivo* results of the implants showed that both the SS-PEG₄₀₀-SS cross-linked and uncross-linked collagen sponges were well tolerated by rats after subcutaneous implantation (Figure 36-38). No intense tissue reaction was observed in both groups. The inflammatory reaction was transitory and is clearly different from the reaction to an irritative foreign body (Etherington et al., 1979) Two weeks after implantation, many newly formed blood vessels had appeared in the center part of both groups of collagen sponges with different degree of degradation (Figure 36), These results reinforce the *in vitro* cytotoxicity tests, in which SS-PEG₄₀₀-SS showed no cell growth inhibition and cell ingrowth inhibition (into collagen sponges), but a reasonable cell growth and morphology.

Meanwhile the SS-PEG₄₀₀-SS cross-linked collagen sponges demonstrated a significant increased resistance to *in vivo* degradation, which is in very good agreement with the results of *in vitro* degradation. After treatment with SS-PEG₄₀₀-SS (400 µg/ml), the collagen sponges did not collapse and kept their porous structure for more than four weeks after implantation. In contrast, the non-treated sponges collapsed and were gradually absorbed and replaced by collagenous connective tissues two weeks after implantation (Figure 37,38).

The results of amino group assays and animal experiments show that cross-linking processes already occur when the SS-PEG₄₀₀-SS concentration is lower than 40 mg/ml. This observation is not in agreement with the results of the collagenase assay. This phenomenon may be explained by the extremely low concentration of the proteolytic enzymes *in vivo* as compared with the collagenase concentration applied in the collagenase assay.

5.5 Conclusions

1. The homobifunctional cross-linker SS-PEG₄₀₀-SS is useful to:
 - Covalently immobilize protein to collagen for controlled drug delivery. The optimal reaction conditions for coupling HRP to collagen:
10-minute first step reaction, 1:50 molar ratio of HRP:SS-PEG₃₄₀₀-SS, at pH 7.2, room temperature then stop the first step reaction via ultrafiltration.
 - SS-PEG₃₄₀₀-SS can cross-link collagen within a short period of time, thereby stabilize the structure and slow down its *in vivo* and *in vitro* degradation. Increasing the cross-linker content resulted in a higher cross-linking degree.
2. SS-PEG₃₄₀₀-SS is not cytotoxic and can be safely used *in vivo*.

5.6 Future prospects

The collagen sponge is a solid 3D matrix. It may therefore take some time for the HRP-PEG₃₄₀₀-SS conjugate to be distributed homogeneously into it. And also could there be some residual air in the collagen sponge, which will hinder the contact of HRP-PEG₃₄₀₀-SS conjugate with the amino groups of the collagen sponge. To solve these problems, we have tried to perform a series of experiments in which we used soluble collagen instead of fibril collagen. In preliminary experiment a much higher HRP coupling efficiency (>30%) has been achieved. The possible reason may be that in solution, the collision frequency for the HRP and SS-PEG₃₄₀₀-SS molecules will be much higher than in the solid collagen sponge. Our next step is to figure out a method to incorporate this protein-collagen conjugate into collagen sponges without harming the bioactivity of the proteins.

Our results (RP-HPLC, MALDI-TOF) show that, in the conjugation step, relatively high combination rates of SS-PEG₃₄₀₀-SS and protein can be obtained. The combination rate of (Protein-PEG₃₄₀₀-SS)-conjugate with collagen, however, was still poor. The key point of low coupling efficiency of (Protein-PEG₃₄₀₀-SS)-conjugate with collagen is whether the second SS group is still active at the start of the second reaction period.

Two possibilities may explain the low combination rate:

1. The active –SS groups in the homobifunctional cross-linker we are using—SS-PEG₃₄₀₀-SS have a very short half life of about 10 minutes. Most of them may already have been hydrolyzed during the first reaction.
2. Both the SS groups may react with protein molecules unselectively in the conjugation reaction. Thus it is likely to assume that, in that case no reactive SS groups will be available for the second step reaction with amino groups of collagen.

By using heterobifunctional PEG, this problem might be overcome. SS group still can be used as the first functional group, because it is highly reactive and will usually not harm the bioactivity of protein drug. The second functional group can be maleimide, which will react with sulfhydryl groups of cysteine instead of the amine group of lysine and remain stable in water. But as we know, there is almost no free sulfhydryl group existing in natural collagen sponges. So we need to modify the collagen sponge by using thiolating agents, which modify collagen by introducing free sulfhydryl groups. In preliminary experiments a six time higher HRP coupling efficiency was observed with the hetero-bifunctional cross-linker as compared to experiments with the homobifunctional cross-linker SS-PEG₃₄₀₀-SS.

The extracellular matrix (ECM) is a complex structural entity surrounding and supporting cells and is ubiquitous within mammalian tissues. Besides the soluble molecules like growth factors, the insoluble ECM ingredients (elastin, fibrillin, fibronectin, proteoglycans and laminin etc) also play important roles in angiogenesis (Ingber and Folkman, 1989) ECM may directly alter vascular endothelial cell behavior and it may also function as a local repository for various growth factors (Jerdan et al., 1991) An optimally engineered tissue substitute should consist of collagen sponge plus some of these components. Experiments need to be performed to study the effect of ECM on angiogenesis.

Our results suggest that this method of covalent linkage would also be useful for delivering other kinds of peptide growth factors that are short-living and are important to be kept active over a longer period of time. TGF, FGF (fibro

blast growth factor), BMP (bone morphogenetic proteins) are some of the examples. Since we assume that this technique only involves the functional groups on proteins, it could be transferred from HRP and VEGF to other proteins.

6. Summary

Introduction

Tissue engineering has been recognized in recent years as a viable solution for repair of tissue defects. Since engineered tissues (as vascular graft) do not have angiogenic capability by themselves, the introduction of Vascular Endothelial Growth Factor (VEGF) could be used to enhance angiogenesis into the prosthesis tissues. A major challenge in the development of a sustained-release of protein drugs is to achieve a high drug loading sufficient for a prolonged therapeutic effect. To overcome rapid diffusion and clearance from the implant site and to increase stability, VEGF was covalently bound to collagen sponges by means of the homobifunctional cross-linking agent bis-(succinimidyl succinate) polyethylene glycol (SS-PEG₃₄₀₀-SS), after coupling activity can then be compared with control experiments in which the VEGF was simply admixed. In order to prevent rapid degradation, collagen sponges used as protein drug delivery substrate were cross-linked by SS-PEG₃₄₀₀-SS. The biological behavior of collagen sponges as well as tissue and cell reactions after the treatment of SS-PEG₃₄₀₀-SS are investigated.

Method

Protein coupling: We selected Horseradish Peroxidase (HRP) as a model protein since it has a similar molecular weight as VEGF, and its activity can be easily measured by spectrophotometer with high sensitivity. Covalent binding was achieved in a two-step procedure: 1. proteins were reacted with SS-PEG₃₄₀₀-SS for 10 min, at room temperature, then stop the reaction by ultrafiltration (10,000 MW, 5000 rpm), the non-reacted SS-PEG₃₄₀₀-SS will be cut off; 2. the SS-PEG₃₄₀₀-SS attached protein react with collagen sponge overnight at room temperature. **Collagen sponge cross-linking:** collagen sponges were immersed in SS-PEG₃₄₀₀-SS solution for about 10 minutes, then wash extensively. Collagenase assay, fibroblast cell culture and subcutaneous implantation were performed to evaluate the cross-linking degree and change of biological properties of collagen sponges.

Results

1. The binding of HRP to SS-PEG₃₄₀₀-SS was demonstrated by reversed phase high performance liquid chromatography (RP-HPLC), Matrix-Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry (MALDI-TOF-MS) and Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Significant higher HRP activities and VEGF antigenicity were observed when HRP or VEGF was coupled with SS-PEG₃₄₀₀-SS as a comparison to control groups in which the HRP or VEGF was simply admixed.
2. HRP or VEGF covalently coupled to collagen released in a controlled way when the collagen sponges were degraded by collagenase.
3. The degradation of SS-PEG₃₄₀₀-SS cross-linked collagen sponge with collagenase is delayed in comparison to untreated sponges.
4. In culture, fibroblasts show a normal morphology and interaction in SS-PEG₃₄₀₀-SS treated collagen sponges.
5. *In vivo*, the porous structure of non-cross-linked collagen sponges collapse after two weeks, but the structure of SS-PEG₃₄₀₀-SS cross-linked collagen sponges remain stable and porous for more than 4 weeks.

Conclusion

1. The homobifunctional cross-linker SS-PEG₃₄₀₀-SS is useful to:
 - covalently immobilize protein to collagen for controlled drug delivery. The optimal reaction conditions for coupling HRP to collagen are:
10-minute first step reaction, 1:50 molar ratio of HRP:SS-PEG₃₄₀₀-SS, pH 7.2, room temperature, removal excess cross-linker molecules via ultrafiltration.
 - cross-link collagen within a short period of time, thereby stabilizing the structure, and slow down its *in vivo* and *in vitro* degradation. Increasing the cross-linker concentration resulted in a higher cross-linking degree.
2. SS-PEG₃₄₀₀-SS is not cytotoxic and can be safely used *in vivo*.

7. Zusammenfassung

Einleitung

‘Tissue Engineering’ bietet schon jetzt die Möglichkeit, einige Gewebedefekte reparieren zu können. Da die künstlich gebildeten Gewebe keine angiogenen Fähigkeiten aufweisen, könnte die Verwendung des Wachstumsfaktors VEGF (Vascular Endothelial Growth Factor) die Angiogenese im Ersatzgewebe verbessern. Die größte Herausforderung bei der Entwicklung eines Systems zur nachhaltigen Freisetzung physiologisch aktiver Proteine ist die Bereitstellung ausreichender Mengen bioaktiver Proteine für einen langanhaltenden therapeutischen Effekt. Um eine vorzeitige Freisetzung der Proteine durch Diffusion zu verhindern, wurde VEGF durch das bifunktionelle Reagenz bis-(Succinimidylsuccinat)-Polyethylenglykol (SS-PEG₃₄₀₀ SS) kovalent an Kollagen-Schwämme gebunden, wobei durch das Reagenz gleichzeitig die Stabilität des physiologisch aktiven Proteins erhöht wird. Die Aktivität des kovalent gebundenen Proteins kann dann in Kontrollexperimenten mit der Aktivität von Mischungen aus Kollagen und VEGF verglichen werden. Die für das Protein als ‘drug delivery’ System dienenden Kollagen-Schwämme wurden durch SS-PEG₃₄₀₀-SS quervernetzt, um deren rasche Degradation zu verhindern. Das biologische Verhalten der Schwämme gegenüber Geweben und Zellen wurde ebenfalls diskutiert.

Methoden

Protein-Kopplung: wir haben Meerrettich-Peroxidase (HRP) als Modell-Protein verwendet, da es ein ähnliches Molekulargewicht wie VEGF aufweist und die enzymatische Aktivität bequem photometrisch bestimmt werden kann. Die kovalente Verknüpfung erfolgte in zwei Schritten: Die Proteine wurden mit SS-PEG₃₄₀₀-SS für 10 min bei Raumtemperatur zur Reaktion gebracht. Nach Ultrafiltration (10k MW, 5000 rpm) zum Entfernen des Reagenzes, werden die mit SS-PEG₃₄₀₀-SS umgesetzten Proteine in einem zweiten Schritt über Nacht bei Raumtemperatur mit Kollagen-Schwämmen zur Reaktion gebracht.

Quervernetzung der Kollagen-Schwämme: Die Schwämme wurden für 10 Minuten in

eine SS-PEG₃₄₀₀-SS-Lösung eingebracht und anschließend ausgiebig gewaschen. Um den Grad der Quervernetzung und die Veränderungen bezüglich der physiologischen Eigenschaften zu bestimmen, wurden anschließend Untersuchungen mit Kollagenase, Fibroblasten-Zellkultur und subkutaner Implantation durchgeführt.

Ergebnisse

1. Die kovalente Anbindung von SS-PEG₃₄₀₀-SS an HRP wurde mittels Umkehrphasen Hochleistungs Chromatographie (RP-HPLC), Matrix-Assisted Laser Desorption Time of Flight Mass Spectrometry (MALDI-TOF-MS) und Natriumdodecylsulfat Polyacrylamid Gel-Elektrophorese (SDS-PAGE) nachgewiesen. Die Kollagen-Schwämme mit kovalent gebundenem Protein zeigten für HRP photometrisch eine signifikant höhere Aktivität als die Kontroll-Gruppe mit nicht-kovalent gebundenem Protein. Diese Ergebnisse bestätigten sich auch für VEGF durch den Einsatz eines Enzyme Linked Immuno Sorbent Assays (ELISA).
2. Sowohl kovalent gebundenes HRP als auch kovalent gebundenes VEGF zeigten eine kontrollierte Freisetzung bei Degradation der Schwämme durch Kollagenase.
3. Die mit SS-PEG₃₄₀₀-SS quervernetzten Kollagen-Schwämme wurden durch Kollagenase im Vergleich zu unbehandelten Kollagen-Schwämmen langsamer abgebaut.
4. Kultivierte Fibroblasten zeigten in den quervernetzten Kollagen-Schwämmen sowohl eine normale Morphologie als auch eine normale Interaktion.
5. Die Struktur der nicht quervernetzten Kollagen-Schwämme kollabiert *in vivo* innerhalb von zwei Wochen, die Struktur der quervernetzten Kollagen-Schwämme blieb dagegen auch nach vier Wochen noch erhalten.

Schlußfolgerung

1. Das homobifunktionelle Reagenz SS-PEG₃₄₀₀-SS ist geeignet für:
 - die kovalente Immobilisierung von Proteinen an Kollagen und deren anschließende kontrollierte Freisetzung. Die optimalen Reaktionsbedingungen hierfür sind: Protein : SS-PEG₃₄₀₀-SS im Verhältnis 1:50, pH 7.2, Raumtemperatur. Nach 10

Minuten wird Ultrafiltriert.

- die rasche Quervernetzung des Kollagens und somit die Stabilisierung der Struktur der Kollagen-Schwämme (wobei eine höhere Konzentration des Reagenzes eine höhere Quervernetzung ermöglicht). Daraus resultierend eine verlangsamte Degradation *in vitro* und *in vivo*.

2. SS-PEG₃₄₀₀-SS ist nicht zytotoxisch und kann *in vivo* eingesetzt werden.

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9. Abbreviations

APS	Ammonium Persulfate
BFGF	Basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
CS	Collagen Sponge
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
ECM	Extracellular Matix
EDC	N-(3-Dimethylaminopropyl)-N'-Ethylcarbodiimide
EDTA	Ethylendiamin-Tetraacetate
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked immunosorbent assay
EO	Ethylene Oxide
FBS	Fetal Bovine Serum
Flt-1	Fas-Like Tyrosine kinase-1
GDA	Glutardialdehyde
Gly	Glycine
HE	Haematoxin and Eosin
HRP	Horseradish peroxidase
Hyp	Hydroxyproline
KDR	Kinase insert Domain-containing Receptor
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time-of-Flight
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5diphenyl tetrazolium bromide
NHS	N-hydroxysuccinimide

Abbreviations

OD	Optical Density
PBS	Phosphate Buffer Solution
PEG	Poly Ethylene Glycol
PLGA	Poly-Lactic and Glycolic Acid
Pro	Proline
RP-HPLC	Reversed Phase High Performance Liquid Chromatography
rhVEGF	Recombinant Human Vascular Endothelial Growth Factor
SC	Soluble Collagen
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SS	Succinimidyl Succinate
Sulfo-STDB	Sulfo-Succinimidyl-4-O-(4,4`-Dimethoxytrityl) Butyrat
TEMED	N,N,N',N'-Tetramethylenediamine
TFA	Trifluovoacetic Acid
TGF	Transforming Growth Factor
TMB	Tetramethylbenzidine
Tris	Tris (hydroxyethyl) aminomethane

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