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VEGF Carrying Coatings for Enhanced Vascular Graft Endothelialization

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Erklärung

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Ehrenwörtliche Versicherung

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For Susan and Christopher

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Introduction and Objective of the Thesis

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ATR	Attenuated Total Reflectance
bFGF	basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
CLSM	Confocal Laser Scanning Microscopy
DCCI	N,N'-Dicyclohexylcarbodiimide
DMSO	Dimethyl Sulfoxide
DSC	Differential Scanning Calorimetry
EC	Endothelial Cell
EDC	Ethyl(dimethylaminopropyl)-carbodiimide
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EPC	Endothelial Progenitor Stem Cell
ePTFE	expanded PTFE
FACS	Fluorescence Activated Cell Sorting
FITC	Fluorescein Isothyocyanat
FT-IR	Fourier Transform Infrared Spectroscopy
HTS	High Throughput Screening
HUVEC	Human Umbilical Vein Endothelial Cell
IND	Internodual Distance
NHS	N-Hydroxysuccinimide
PEEK	Polyetheretherketone
PEG	Polyethylene Glycol
PET	Polyethylene Terephthalate
PGA	Polyglycolic Acid
PLA	Polylactic Acid
PLGA	Poly(lactic-*co*-glycolic acid)
PMMA	Poly(methyl methacrylate)
PTFE	Polytetrafluoroethylene
rpm	rounds per minute
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy

SMC	Smooth Muscle Cell
T_g	Glass Transition Temperature
T_m	Melting temperature
TNBS	2,4,6-Trinitrobenzenesulfonic Acid Solution
VEGF	Vascular Endothelial Growth Factor
VEGFR-1	Vascular Endothelial Growth Factor Receptor 1
XPS	X-ray Photoelectron Spectroscopy

Chapter 1

Introduction and Objective of the Thesis

Abstract

In the general introduction, vascular grafts for the replacement of blood vessels and approaches for their improvement concerning the main problems graft infection, thrombogenicity, and intimal hyperplasia were discussed. A focus was on the special role of endothelial cells in vessel replacement and graft healing after implantation. The approaches for performance improvement of these prostheses were highlighted concentrating on the problems surrounding the endothelialization of the inner vascular graft surface, due to its importance for the long term patency of prostheses. Furthermore, polymers for the potential modification of vascular grafts were introduced describing their relevance in the field of controlled release applications.

Keywords: vascular graft, Dacron®, ePTFE, collagen, PLGA, endothelial cell

1 Introduction

The use of vascular grafts in vessel replacement is not a modern age occurrence. Vascular surgery, a necessary prerequisite for vessel replacement, dates as far back as 130 AD when first surgeons used vascular ligature to stop and control bleedings from vessels. Early prostheses were made of metal, glass or ivory, which rapidly blocked due to blood clotting [1]. It was not until Carrel reported a reliable method for suturing cut ends of blood vessels together with appropriate patency rates in 1912 that extensive vascular surgery became available and was no longer the exclusive domain of a few [2]. From there on, the use of autologous vein grafts was a common procedure. During World War I and II and the Korean War there was significant improvement in the techniques used for vascular surgery and vessel replacement [3-4].

Ever since, vascular grafts have been extensively used and today coronary and peripheral vascular bypass grafting is performed on a daily basis in the United States and Europe [5]. Nevertheless, it is not without significant constraints and complications that these procedures are performed. Ever since the first bypass surgery the ultimate goal was to achieve complete revascularization with patency of the transplanted grafts for the duration of a patient's lifetime [6]. However, most attempts, especially in the field of small diameter vascular grafts (< 6 mm) and low blood flow locations have failed. These failures are due to the occurrence of adverse events, such as graft infection, thrombogenicity of the internal graft surface or growth of smooth muscle cells leading to intimal hyperplasia resulting in occlusion.

Therefore, there is a tremendous momentum in the vascular community to develop synthetic small-diameter grafts that have high long-term patency rates. Off-the-shelf availability in various diameters and lengths, uncomplicated storage or preparation requirements, and ease of handling are the main advantages of such grafts [7]. Several improvements to vascular grafts have been made in the laboratory setting, but failed to convince in the clinic, which is the reason that to this day the majority of surgeons continue to implant the well-established products of the past decades [8].

2 Vascular grafts

2.1 Materials used for vascular grafts

The first materials used for vessel replacement were made of metals, glass or ivory. However, the materials used in modern day vessel replacement have undergone a tremendous development, comparable to the surgical techniques employed to place them. In contemporary surgeries the use of autologous, homologous, heterogenous and alloplastic vessel prostheses is common practice. However, the most preferred material to date by surgeons is still the autologous vein graft such as the saphenous vein or, for coronary artery grafting, the internal mammary artery [9-10]. Yet, in at least 30% of the patients, these veins or others can not be used due to earlier removal or other pre-existing conditions. In the absence of suitable autologous veins, homologous and heterogenous alternatives can be considered as substitutes. The so called Dardik prostheses makes use of the human umbilical vein (homologous graft) in combination with a polyester mesh and has been used for replacements. However, its difficult preparation process, which includes intraoperative irrigation with large volumes of heparinized solutions and the difficulty of sewing make it an unattractive alternative [11]. Bovine and ovine arteries (heterogenous grafts) have also been used for vessel replacement. However, first promising results concerning their effectiveness had to be put in perspective, since long term follow up documented aneurysm formation and decreased resistance to infection [11].

The development of alloplastic alternatives started with the accidental observation of a pseudointima formation around a silk suture [12-13]. A continuous search for better, more blood-compatible materials and improved manufacturing processes resulted in prostheses of many different substances and fabrics in a short time. Prostheses were made of Nylon®, Teflon®, Orlon®, Dacron®, plastic and polyurethane [1]. Nylon was soon abandoned, due to the rapid degeneration after implantation, resulting in aneurysm formation [1, 14]. Teflon® (Polytetrafluoro-ethylene) and Dacron® (Polyethylene terephthalate) showed the most suitable and superior properties [14] and represent the gold standard of synthetic vascular grafts nowadays.

2.1.1 Dacron®

Dacron® is a form of multiple filaments either woven or knitted into vascular grafts (compare Figure 1-1). While woven grafts have smaller pores, knitted grafts formed by looping fibers together have larger pores which promote greater tissue ingrowth [15]. They are commonly used for larger vessel replacements in regions of high blood flow. Before application, porous alloplastic Dacron® grafts are impregnated with connective tissue proteins or preclotted with the patient`s blood in order to reduce blood loss, aid clotting, and stimulate tissue ingrowth [13, 15].

(a) (b)

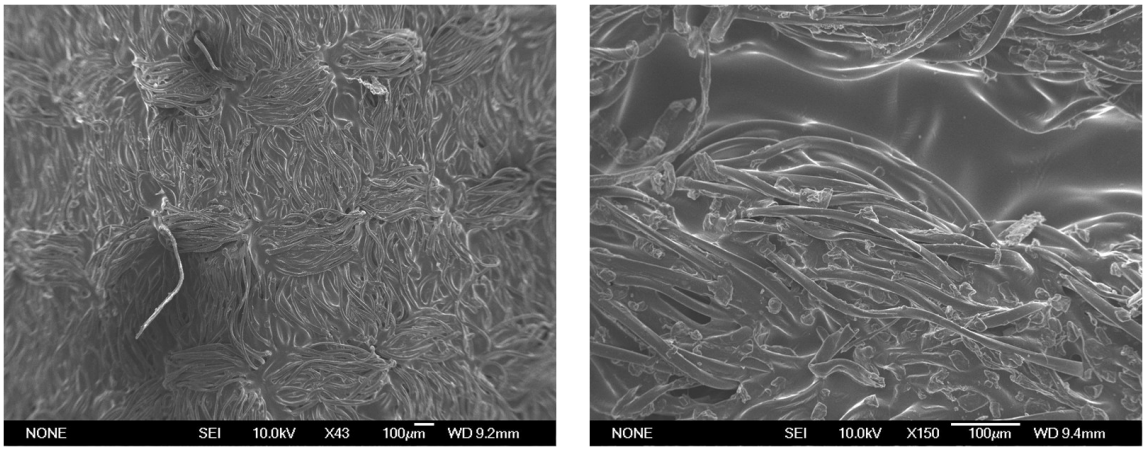


Figure 1-1: Scanning electron microscopical appearance of a Dacron® prosthesis with large folds of Dacron® material (a) and fiber bundles (b).

2.1.2 ePTFE

PTFE or Teflon® was first used for artificial heart valves and subsequently a more microporous material was developed by extrusion and sintering to form expanded PTFE (ePTFE) for vascular grafts [15]. It is characterized by circumferentially aligned, thin and irregular-shaped solid membranes, the so-called ‘‘nodes’’, and a dense meshwork of fine fibrils stretching between the nodes (Figure 1-2) [8]. The porosity of ePTFE grafts is defined by internodual distance (IND), therefore “low porosity” (30 µm IND) and “high porosity” (60 µm IND) grafts are available. Tissue ingrowth is only possible down to an IND of approximately 45 µm, which leaves “low

porosity" grafts impermeable for tissue ingrowth such as transmural capillarization [8].

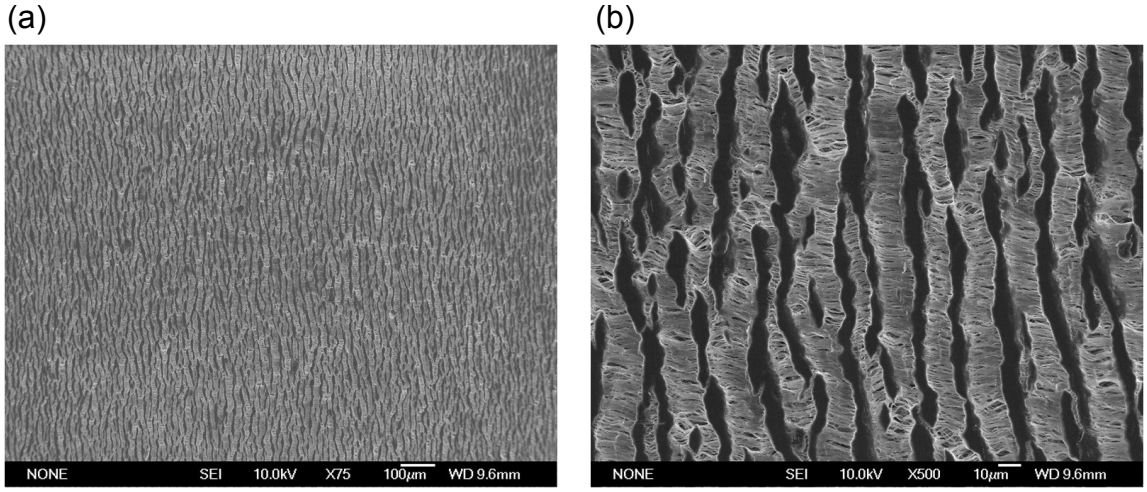


Figure 1-2: Expanded polytetrafluoroethylene (ePTFE) prosthesis as seen with scanning electron microscope of the regular pattern at the inner surface (a) and numerous and densely packed fine internodular fibrils (b).

2.2 Failure of vascular grafts

It is well known that autologous vein grafts are still the graft of choice by surgeons in peripheral arterial bypass procedures due to superior patency rates when compared to prosthetic grafts [16]. Most of the above described materials show good performances and patency in high blood flow regions and with large inner diameters (> 6 mm). However, when implanted in regions with low blood flow and smaller diameters, vascular grafts start failing. When looking at patency rates of vascular prosthesis, synthetic materials have yet to match those of autologous grafts [10]. Therefore, synthetic grafts are only used when the use of autologous material is contraindicated. Some of the reasons include compliance mismatch, thrombogenicity and poor haemodynamics. The main problems that occur during the use of artificial vein grafts that have been reported are graft infection [17], thrombogenicity of the internal graft surface [18], or growth of smooth muscle cells resulting in intimal hyperplasia leading to occlusion [19]. Graft infection is a devastating complication with incident rates of 1 % to 6 % [13]. Significant mortality and limb amputation rates have been reported for infections occurring in vessel

replacement therapy [17]. However, the main reasons for low patency rates are thrombogenicity and intimal hyperplasia, with thrombogenicitiy being responsible for early graft occlusion [20]. Upon implantation of a vascular graft, plasma proteins immediately adsorb to the wall. They present binding sites for integrin receptors, which are to be found on platelets and many other cells [21]. Adhesion to these, as well as, to the vessel wall itself may cause activation of the platelets [18], resulting in thrombosis. Intimal hyperplasia, the reason for late graft occlusion, which is associated with the abrupt change in distensibility between native vessel and stiff prosthesis at the anastomosis [22], is commonly caused by smooth muscle cell proliferation and extracellular matrix deposition [23]. Several of these causes have their origin in the lack of a functional endothelial cell lining on the inner surface of grafts, especially in mid graft sections after even long periods of implantation [8]. Despite the shortcomings of contemporary vascular prostheses, no alternative concept has yet emerged that promises to replace the current generation of synthetic grafts.

2.3 Improvement of vascular graft performance

Several approaches to overcome the limitations and complications connected with the use of small caliber vascular grafts and their endothelialization have been undertaken. Concerning vascular graft infection, it has been reported that even high local concentrations of antibiotics can not completely eradicate bacteria in established biofilms on vascular grafts [24-25], therefore avoiding the bacterial adhesion on vascular grafts is of high importance [13, 26]. In order to achieve this, the coating of vascular graft surfaces with antibiotics has been established. Several antimicrobial agents [27-28] have been employed, as well as, different ways of attachment on the graft surface. Common methods are the use of surfactant mediated agents or the incorporation of drugs in biodegradable polymer carriers [13, 29-30].

In order to reduce thrombogenicity and enhance the blood compatibility of vascular graft materials, and therefore one of the reasons for early graft occlusion [18], several approaches using Heparin have been undertaken [16, 31]. However,

different results were obtained in dependence of the method of bonding, with covalent bonding being responsible for a lasting reduction in thrombogenicity in vascular grafts made of PTFE [32] and Dacron® [33]. Yet, transient coatings did not result in an improvement of patency [18]. These findings have lead to the development of a commercially available vascular ePTFE graft (GORE-TEX PROPATEN® Vascular graft, W.L. Gore and Associates, Flagstaff, AZ) with long-term bonding of heparin accomplished by covalent linkage of the anticoagulant [16]. Another approach that has been utilized or the reduction of thrombogenicity of vascular grafts is the impregnation with carbon [34]. Due to its hydrophobic nature and negative charge these coatings lower platelet deposition. However, this approach has shown no real advantage in comparison to standard ePTFE grafts [35-36]. Other attempts to improve graft patencies have involved the coating with albumin, gelatin, and collagen [37-39]. Several other investigations aim at the establishing of a functional endothelial cell lining on the inner surface of the vascular graft material to mimic the natural conditions via endothelial cell seeding or the improvement of endothelial cell adhesion on the graft surface (compare 3.2).

3 Endothelial cells and their role in vessel replacement

Endothelial Cells (ECs) were once thought to be a monolayer of passive cells lining the vasculature [40]. Nowadays, it is known that this endothelial monolayer that lines the healthy blood vessel serves as regulator of cardiovascular physiology [5]. It provides structural integrity by forming a thromboresistant barrier between circulating blood and the arterial wall [41]. It controls blood flow and vessel tone [42], platelet activation, adhesion and aggregation, leukocyte adhesion [43] and smooth muscle cell (SMC) migration, and proliferation [5]. Therefore, the lack of a functional endothelial cell lining on the inner surface of vascular grafts is considered to be one of the main factors for small caliber vascular graft failure [5]. Consequently, ECs are interesting as coverage of inner prosthetic graft surfaces with the idea behind EC seeding or attraction to improve the patency of small diameter vascular grafts by establishing a functional biological lining on the luminal surface.

3.1 Endothelial cell related problems

Most of the problems occurring during the use of vascular grafts (compare 2.2) are related to the lack of an endothelial cell lining, independent of the origin of graft material. In case of autologous vein grafts, one would expect a functional endothelial cell lining to be present on the inner graft surface since these vessels were removed from other regions in a patient's body prior to their use and were functional until the time of removal. However, even in the case of autologous grafts, vascular endothelium related problems occur [10]. During the excision of autologous veins for use as vein grafts, in both the arterial and venous circulations, the endothelium of the vessel is thought to be traumatized with a resulting reduction in its functional capability [44], which causes the aforementioned complications.

In case of alloplastic vascular grafts, endothelialization mainly occurs at the anastomotic region of prosthetic grafts with a maximum depth of penetration of endothelial cells into the graft of approximately 10 - 20 mm, even after years of implantation [8] leaving the inner part of the grafts permanently without endothelium and its resulting complications. Another way of endothelialization of the vascular graft surface is transmural migration of endothelial cells in case of ePTFE grafts with high IND and Dacron® grafts with sufficient porosity. This effect is responsible for the majority of confluent endothelium forming in animal models [18], however only to a negligible extent in humans [8, 45]. A further mechanism that has gained interest in recent years is the endothelialization of vascular grafts by the transformation of endothelial progenitor stem cells (EPCs) with the identification and origins of EPCs to be defined [18, 46].

3.2 Approaches for endothelialization

A functioning endothelial cell lining on the inner graft surface is the prerequisite for long patency rates of vascular grafts [5]. Therefore, the establishing of a thin layer of endothelial cells on vascular grafts has been the interest of research groups for a long time.

3.2.1 In vitro endothelialization

Endothelial cell seeding seemed a promising approach and first successful approaches for seeding vascular grafts in dogs were reported by Herring et al. [47]. Several studies in dogs followed implementing different harvesting techniques [40]. Two principal methods for the harvest were described: mechanical and chemical. The first method was the scraping of endothelial cells from the intima, with the drawback of cell damage and possible contamination with smooth muscle cells, which could lead to intimal hyperplasia. Latter techniques involved the incubation of endothelium with collagenase to separate the cells from the extracellular matrix and basement membrane [40, 48]. First studies of seeded cells in humans were reported in 1984 [49] with several studies following, however the results were controversial [50-52].

In the following years, different seeding techniques were implemented with the two stage seeding technique that makes use of cultured endothelial cells to be seeded on grafts [40, 53] and the in vitro culturing of cells on the prosthetic graft. This way, the amount of cells and the resistance of cells to shear stress were achieved [54], which were thought to be the reason of failure for the previously described attempts to seed cells. Despite strong evidence that the effect of endothelial cell seeding [55-57] is beneficial for the performance of vascular grafts, a major drawback of all studies is the labor intensity. There is a 4 to 5 week delay between cell harvest and graft implantation, making this an unfeasible alternative for emergency applications. In addition, growth and infection problems can occur and the costs of cell culture under good manufacturing practice are substantial [40, 58]. In order to avoid these risks, a search for other cell types and for other EC sources has been started, with a focus on mesothelial cells, microvascular endothelial cells, and endothelial progenitor cells [40].

3.2.2 In vivo endothelialization

In order to overcome the limitations of in vitro endothelial cell seeding, as mentioned above, but still establish a functional endothelial cell lining on the inner surface of vascular grafts, in vivo endothelialization has been a focus for several years. The

goal is to increase the transanastomotic ingrowth of endothelial cells and to capture endothelial progenitor cells present in the blood stream and stimulate their growth and attachment. Several approaches to achieve this endeavor have been undertaken using a multitude of modifications of the graft surface. The modification of graft surfaces using plasma have been shown to enhance the cell compatibility properties of ePTFE grafts [59]. Another approach is the attachment of RGD peptide sequences to vascular graft surfaces, as they are widely acknowledged as cell-binding signals [60].

Several other approaches include the use of growth factors to induce endothelial cell growth and attraction. Among the growth factors investigated, the family of fibroblast growth factors, especially basic fibroblast growth factor (bFGF), has been investigated for the use to enhance endothelialization [61]. However bFGF has also been shown to have stimulatory effects on smooth muscle cell growth, which in turn can lead to intimal hyperplasia and vessel occlusion [62-63], making it a less ideal candidate. Another widely investigated growth factor is the Vascular Endothelial Growth Factor (VEGF). VEGF is a secreted protein ligand that activates transmembrane receptors on endothelial cells. It is a disulfide-linked homodimer and exists in several isoforms, four of which consist of 121, 165, 189 and 206 amino acids [64] and are produced from a single human gene as a result of alternate splicing [65]. This growth factor has been shown to have very high endothelial cell specificity without mitogenic activity for other cell types [66], making it an ideal candidate for the specific stimulation of endothelial cell growth in vascular graft applications. Multiple ways of presenting these growth factors have been investigated, including extracellular matrix coatings [67], electrostatic interaction [45], and fibrin matrices [65, 68], all of which have shown promising results.

The controlled release of therapeutic agents from the vascular graft surface constitutes a promising approach for the improvement of vascular graft patency and the establishing of a functioning endothelial cell lining. A coating with biocompatible and biodegradable polymers for this approach and the subsequent local drug delivery for modifying the response of the surrounding tissue could induce spontaneous endothelialization and/or inhibit smooth muscle cell proliferation and therefore overcome the limitations of small caliber vascular grafts.

4 Polymers for controlled release applications

As mentioned above (compare 3.2) the establishing of a functioning endothelial cell lining on the vascular graft surface is an important prerequisite for the long term patency of small caliber grafts. The controlled release of endothelial cell specific growth factors, such as VEGF, from film coatings on vascular grafts presents a promising approach to overcome the problems encountered in in vitro and in vivo endothelialization. This paragraph discusses commonly used biocompatible and biodegradable polymers for controlled release applications and their potential use for vascular graft coatings.

4.1 Natural polymers - collagen

The class of natural polymers can be divided in two main groups polysaccharide and protein based polymers. The polysaccharide based polymers comprise of chitosan, starch, alginate, hyaluronic acid and chondroitin sulphate [69-70] and can be obtained from different sources, such as microbial, animal, or vegetal and show good hemocompatibility and non-toxicity [71]. Protein based polymers include collagen, gelatin, fibrin, and albumin [69, 72]. The availability of large quantities and the good biocompatibility of these natural polymers makes them an attractive alternative for drug delivery devices [72]. However, due to their natural origin, batch to batch variations in their composition can occur [73].

A commonly used biopolymer in controlled release and biomaterial applications is collagen [74-79]. Collagen is the primary and major structural protein of vertebrates, representing almost 30% of total protein present in a body [80]. Due to its mechanical and biochemical properties, it can primarily be found in areas of high mechanical strain. Hence, 90% of extracellular protein in tendon and bone, and more than 50% in skin, consist of collagen [81]. Most of the structural support in mammals is achieved by collagen, its primary function in the extracellular matrix. Nevertheless, to date 27 collagen types have been isolated with a variety of functions [82]. However, collagen type I is the most investigated for biomedical applications.

4.1.1 Structure of collagen

Collagen is characterized by a very high mechanical strength and stability caused by the characteristic triple helical structure [83-84]. These helices are composed of three polypeptide α-chains each consisting of more than 1000 amino acids, which are arranged in a characteristic repetitive unit glycine-X-Y (Gly-X-Y) (Figure 1-3a). The absence of a side chain in glycine results in the formation of a typical left-handed triple helix as closest packing with glycine oriented in the core [72], representing the secondary structure of collagen (Figure 1-3b). The X- and Y-positions can be occupied by any amino acids, however about 35% of the non-glycine positions are dominated by proline in the X-position and 4-hydroxyproline in the Y-position [83]. Three triple helical polypeptides then form a rope-like right handed supercoil, also know as tropocollagen, with a length of approximately 300 nm and a diameter of 1.5 nm (Figure 1-3b). In addition, there are regions of about 9-26 amino acids at the amino and carboxyl terminal ends that are not incorporated in the helical structure, the so called telopeptides, which are the main molecular sites involved in collagen cross linking [85]. These tropocollagen molecules form longitudinal and bilateral microfibrils and further fibrils with distinct periodicity [79] (Figure 1-3c). These collagen fibrils organize into fibers which on their part can form even larger fiber bundles [83].

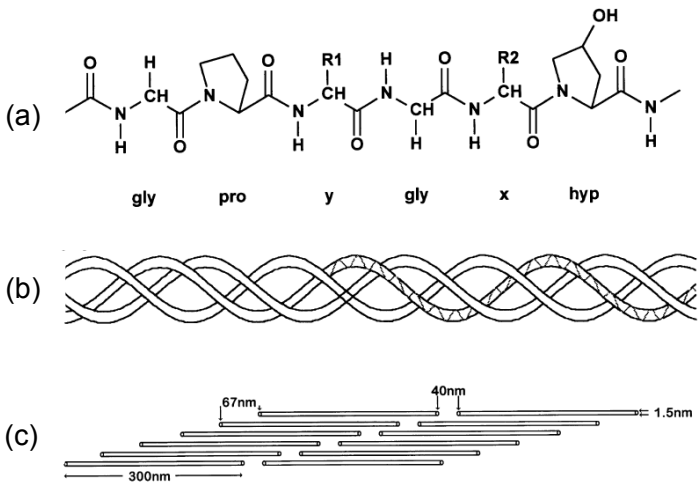


Figure 1-3: Chemical structure of collagen type I with primary amino acid sequence (a), secondary left handed helix and tertiary right handed triple-helix structure (b) and staggered quaternary structure (c) [80].

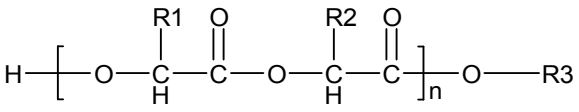
4.1.2 Crosslinking of collagen

Natural crosslinking gives high tensile strength and proteolytic resistance to collagen. Due to dissociation of crosslinks in the course of the isolation processes, reconstituted forms of collagen such as films, fibers, or sponges can lack sufficient strength and may disintegrate upon handling or collapse under the pressure from surrounding tissue in vivo [83]. In addition, the biodegradation and therefore the release of incorporated compounds may need to be customized, which can be achieved by crosslinking of collagen by means of chemical or physical methods. The amount of penetrating water responsible for the swelling of the collagen decreases in correlation to the increase of crosslinking degree [86], thus limiting the release of high molecular weight compounds due to the entrapment of such compounds in the crosslinked matrix in contrast to low molecular weight compounds [83]. Chemical crosslinking of collagen can be performed using a variety of reagents. Aldehydes (e.g. glutaraldehyde) have been extensively used due to their good efficiency, inexpensiveness, and short treatment times [83, 87]. However, crosslinking may sometimes be restricted to the surface and heterogenous crosslinking can occur. In addition, glutaraldehyd is incorporated in the new linkage raising the question of cytotoxicity and biocompatibility [87]. Carbodiimides (e.g. Ethyl(dimethylaminopropyl)-carbodiimide) and acyl azides represent zero-length crosslinking methods, where the chemical is not integrated in the newly formed bond, but acts as an initiator for the linkage of free carboxylic and amino groups to form amide bonds. Furthermore, crosslinking can be performed by the use of tannic acid [88], polyepoxy compounds [89], and hexamethylene-diisocyanate [90]. In addition to chemical crosslinking methods, physical procedures such as dehydrothermal treatment [79, 83, 91] and UV radiation have been reported [83, 92].

These methods of crosslinking can be applied in order to prevent rapid degradation of collagen based biomaterials during in vivo application, improve its mechanical stability [93] and reduce tissue response [94]. Crosslinking is especially useful when using collagen in controlled release applications. By means of crosslinking the release of compounds entrapped in the collagen matrix can be tailored to the specific needs of the application.

4.2 Synthetic polymers - PLGA

Synthetic polymers have been widely used in the field of controlled release applications and biomaterials [95-98]. They comprise of poly(amides), poly(amino acids) poly(alkyl-α-cyano acrylates), poly(acrylamides), and poly(esters) [99]. Amongst them, the aliphatic poly(esters) polylactides (PLA), polyglycolides (PGA), and especially PLGA have been of great interest due to their good biocompatibility and biodegradability [100-102]. They are the most widely known, studied, and used bioabsorbable synthetic polymers in medicine. Polyglycolide and polylactide (PLA) homopolymers and their copolymers (PLGA) are all poly (α-hydroxyacids). Poly (α-hydroxy acids) can be polymerized via condensation, although only low molecular weight polymers are produced [103]. In order to obtain a higher molecular weight and thus mechanical strength and longer degradation time, the polymers are polymerized from the cyclic dimers dilactide and diglycolide via ring-opening polymerization using appropriate initiators and co-initiators [104]. The general chemical structure is displayed in Figure 1-4.



polylactic acid (PLA):	R1, R2 = -CH_3
polyglycolic acid (PGA):	R1, R2 = H
poly(lactic-co-glycolic) acid (PLGA):	R1 = H, R2 = -CH_3
non end-capped versions:	R3 = H
end-capped versions:	R3 = -alkyl

Figure 1-4: General chemical structure of poly(α-hydroxyacids).

By modification of molecular weight [105], ratio of the used monomers, and degree of crystalllinity [106-107] the degradation and release properties of the copolymers can be tailored to the specific needs of the application. Furthermore, the release

properties can be altered by esterification of the carboxylic end groups of the polymer using long chain alcohols in order to obtain end capped polymers with a more hydrophobic character in contrast to the more hydrophilic properties of the non-end-capped varieties [108]. The degradation of these polymers is mainly driven by hydrolysis of the polymer chains in smaller fragments [109-110] down to lactic and glycolic acid [111] that can be eliminated by the kidneys or in form of carbon dioxide via the lungs. Enzymatic involvement in the degradation of the polymers is unclear [106]. The rate of hydrolysis and therefore the rate of degradation is mainly controlled by the amount of penetrating water [112-113]. It has been shown that this rate is decreased for pure crystalline poly-l-lactide and polyglycolide and that it increases with an increasing ratio of glyoclid in the copolymer and increasing hydrophilicity [114-115]. Therefore, a whole range of polymers with varying properties and applications is commercially available from Boehringer Ingelheim under the name of Resomer® [116-117]. However, PLGA copolymers as well as PLA and PGA are also available from other suppliers, such as Birmingham Polymers or Purac Biomaterials.

Despite the wide range of polymers available, their release rates are difficult to predict and dependent on the compounds incorporated and the geometry of the device [118]. In general, their release profiles are characterized by an initial burst release of drug that is deposited near the surface and is caused by its dissolution [119], followed by a sustained release phase characterized by zero order kinetics [120]. Consequently, release mechanisms with different stages are proposed for devices made of PLGA [121]. Other limitations of PLGA based release systems for protein delivery are the use of organic solvents during the manufacturing process and the decrease in pH caused by acidic erosion products that may cause harm to the incorporated protein [122]. Nevertheless, PLGA is considered the “gold standard” of biodegradable polymers [73].

The described polymers, collagen, and PLGA have been widely used in controlled release applications due to their good biocompatibility and biodegradability. Collagen in particular has been investigated for the release of growth factors for

vascular graft application [61] with promising results. Both polymers have been shown to be suitable for vascular applications [112, 123] and intravenous drug delivery. Therefore, these polymers present a promising tool for the controlled release of endothelial cell specific growth factors from film coatings on vascular grafts as a device to overcome the problems encountered in in vitro and in vivo endothelialization.

5 Conclusions

The use of alloplastic prothestic vascular grafts in vessel replacement therapy is inevitable and many approaches to overcome the limitations, especially in the field of small diameter grafts in low blood flow regions, have been undertaken. The lack of a functioning endothelial cell lining, being one of the main contributors to these limitations, has been addressed by a multitude of studies and several promising attempts have been carried out. Yet, some are inapplicable in emergency situations due to long preparation times and difficult preparation procedures. Therefore, despite all the research in this field no alternative concept has yet emerged that promises to replace the current generation of synthetic grafts, which enables off-the-shelf availability and supports complete endothelialization of the inner surface and complete healing of the vascular graft to ensure patency for a patient's lifetime.

6 Objectives of the Thesis

The goal of the thesis was the investigation of functional coatings for the improvement of vascular graft performances, especially in regards of endothelialization with the help of endothelial cell specific growth enhancers.

It was of interest to be able to apply these modifications on existing commercially available vascular grafts; therefore a suitable model system for the investigations needed to be developed. Due to the low adhesion of coatings on the model surface PTFE, it was necessary to enhance its coating accessibility. Therefore, the first objective was the investigation and implementation of the plasmabrush® for argon plasma treatment of the PTFE surfaces and the characterization thereof (Chapter 2).

For the presentation of cell growth enhancers to the endothelial cells two approaches were considered:

The first approach was the incorporation of the cell growth enhancer in matrices and the controlled release thereof within an appropriate period of time. Therefore, the second main objective of the thesis was the investigation of biodegradable film coatings for the controlled release of the growth enhancers. The polymers of choice were PLGA and collagen. The influences of several parameters on the release needed to be investigated, specifically in the case of collagen, the influence of crosslinking on the release rate. The coating cell compatibility and the cell growth enhancement of the released protein were to be studied and quantified (Chapter 3 and 4).

For the second approach, the presentation of a cell growth enhancer at the surface, the covalent linkage of an enhancer to modified films was investigated. Thus, the third objective was the chemical modification of PLGA and collagen using a functional polyethylene glycol (PEG) spacer and the covalent linkage of the enhancer to the surface of a film consisting thereof. Furthermore, detection methods for the surface bound protein needed to be evaluated and developed. After successful linkage, the influence of the covalently attached protein was to be

evaluated in a cell assay concerning its effect on endothelial and smooth muscle cells (Chapter 5 and 6).

Thus, in summary the main objectives of the thesis were:

1. Implementation of the plasmabrush® and establishing a method for surface modification of PTFE to enhance the coating accessibility, and understanding the change in physical properties induced by plasma modification by means of surface sensitive analytical techniques (Chapter 2).
2. Investigation and modification of PLGA and collagen films for the controlled release of the endothelial cell specific growth enhancer $VEGF_{165}$. Establishing an in vitro cell culture system and quantification of the stimulatory effect of the developed release systems (Chapter 3 and 4).
3. Covalent attachment of $VEGF_{165}$ to chemically modified PLGA and collagen films by the use of a bifunctional PEG-spacer and the identification of analytical methods to detect surface bound protein. Evaluation of developed systems concerning their cell growth enhancing properties (Chapter 5 and 6).

7 References

1. Hess, F., *History of (MICRO) vascular surgery and the development of small-caliber blood vessel prostheses (with some notes on patency rates and re-endothelialization).* Microsurgery, 1985. **6**(2): p. 59-69.
2. Carrel, A., *Ultimate Results of Aortic Transplantations.* J. Exp. Med., 1912. **15**(4): p. 389-392.
3. FISHER, G.W., *Acute Arterial Injuries Treated By the United States Army Medical Service in Vietnam, 1965-1966.* The Journal of Trauma, 1967. **7**(6): p. 844-855.
4. DeBakey, M.E. and F.A. Simeone, *Battle Injuries of the Arteries in World War II: An Analysis of 2,471 Cases.* Annals of Surgery, 1946. **123**(4): p. 534-579.
5. Seifalian Alexander, M., et al., *Improving the clinical patency of prosthetic vascular and coronary bypass grafts: the role of seeding and tissue engineering.* Artificial organs, 2002. **26**(4): p. 307-20.
6. Desai Nimesh, D., et al., *Impact of patient and target-vessel characteristics on arterial and venous bypass graft patency: insight from a randomized trial.* Circulation, 2007. **115**(6): p. 684-91.
7. Kakisis John, D., et al., *Artificial blood vessel: the Holy Grail of peripheral vascular surgery.* Journal of vascular surgery official publication, the Society for Vascular Surgery [and] International Society for Cardiovascular Surgery, North American Chapter, 2005. **41**(2): p. 349-54.
8. Zilla, P., D. Bezuidenhout, and P. Human, *Prosthetic vascular grafts: Wrong models, wrong questions and no healing.* Biomaterials, 2007. **28**(34): p. 5009-5027.
9. Greenwald, S.E. and C.L. Berry, *Improving vascular grafts: the importance of mechanical and haemodynamic properties.* The Journal of Pathology, 2000. **190**(3): p. 292-299.
10. Klinkert, P., et al., *Saphenous vein versus PTFE for above-knee femoropopliteal bypass. A review of the literature.* European journal of vascular and endovascular surgery the official journal of the European Society for Vascular Surgery, 2004. **27**(4): p. 357-62.
11. Esquivel, C.O. and F. William Blaisdell, *Why small caliber vascular grafts fail: A review of clinical and experimental experience and the significance of the interaction of blood at the interface.* Journal of Surgical Research, 1986. **41**(1): p. 1-15.
12. Arthur B. Voorhees, J., A.J. III, and A.H. Blakemore, *The use of tubes constructed from vinyon "N" cloth in bridging arterial defects.* Annals of Surgery, 1952. **135**(3): p. 332-336.
13. Matl, F., *Infected Biomaterials - New Strategies for local Anti-Infective Treatment*, in *Pharmaceutical Technology and Biopharmaceutics*. 2008, LMU: Munich.
14. Harrison, J.H., *Synthetic materials as vascular prostheses : I. A comparative study in small vessels of nylon, dacron, orlon, ivalon sponge and teflon.* The American Journal of Surgery, 1958. **95**(1): p. 3-15.
15. Kannan, R.Y., et al., *Current status of prosthetic bypass grafts: A review.* Journal of Biomedical Materials Research Part B: Applied Biomaterials, 2005. **74B**(1): p. 570-581.
16. Heyligers Jan, M.M., et al., *Heparin immobilization reduces thrombogenicity of small-caliber expanded polytetrafluoroethylene grafts.* Journal of vascular surgery official publication, the Society for Vascular Surgery [and] International Society for Cardiovascular Surgery, North American Chapter, 2006. **43**(3): p. 587-91.

17. Antonios, V.S., et al., *Prosthetic vascular graft infection: A risk factor analysis using a case-control study.* Journal of Infection, 2006. **53**(1): p. 49-55.
18. Sarkar, S., et al., *Addressing thrombogenicity in vascular graft construction.* Journal of Biomedical Materials Research Part B: Applied Biomaterials, 2007. **82B**(1): p. 100-108.
19. Landymore, R.W., C.E. Kinley, and C.A. Cameron, *Intimal hyperplasia in autogenous vein grafts used for arterial bypass: a canine model.* Cardiovasc Res, 1985. **19**(9): p. 589-592.
20. Walts, A., et al., *Ruptured atheromatous plaques in saphenous vein coronary artery bypass grafts: a mechanism of acute, thrombotic, late graft occlusion.* Circulation, 1982. **65**(1): p. 197-201.
21. Kidane, A.G., et al., *Anticoagulant and Antiplatelet Agents: Their Clinical and Device Application(s) Together with Usages to Engineer Surfaces.* Biomacromolecules, 2004. **5**(3): p. 798-813.
22. Tiwari, A., et al., *Improving the Patency of Vascular Bypass Grafts: the Role of Suture Materials and Surgical Techniques on Reducing Anastomotic Compliance Mismatch.* European Journal of Vascular and Endovascular Surgery, 2003. **25**(4): p. 287-295.
23. Wilson, N.V., J.R. Salisbury, and V.V. Kakkar, *The effect of low molecular weight heparin on intimal hyperplasia in vein grafts.* European Journal of Vascular Surgery, 1994. **8**(1): p. 60-64.
24. Darouiche, R.O., *Treatment of Infections Associated with Surgical Implants.* N Engl J Med, 2004. **350**(14): p. 1422-1429.
25. Dunne, W.M., Jr, E.O. Mason, Jr, and S.L. Kaplan, *Diffusion of rifampin and vancomycin through a Staphylococcus epidermidis biofilm.* Antimicrob. Agents Chemother., 1993. **37**(12): p. 2522-2526.
26. Darouiche, Rabih O., *Device-Associated Infections: A Macroproblem that Starts with Microadherence.* Clinical Infectious Diseases, 2001. **33**(9): p. 1567-1572.
27. Hernandez-Richter, T., et al., *The prevention and treatment of vascular graft infection with a Triclosan (Irgasan)-bonded Dacron graft: an experimental study in the pig.* European journal of vascular and endovascular surgery the official journal of the European Society for Vascular Surgery, 2000. **20**(5): p. 413-8.
28. Bergamini, T.M., et al., *Antibiotic Efficacy againstStaphylococcus epidermidisAdherent to Vascular Grafts.* Journal of Surgical Research, 1996. **60**(1): p. 3-6.
29. Harvey, R.A. and R.S. Greco, *The noncovalent bonding of antibiotics to a polytetrafluoroethylene-benzalkonium graft.* Annals of Surgery, 1981. **194**(5): p. 642-647.
30. Gollwitzer, H., et al., *Antibacterial poly(D,L-lactic acid) coating of medical implants using a biodegradable drug delivery technology.* J. Antimicrob. Chemother., 2003. **51**(3): p. 585-591.
31. Scharn, D.M., et al., *Thrombogenicity and Related Biological Properties of Heparin Bonded Collagen Coated Polyester and Human Umbilical Vein Prosthetic Vascular Grafts.* Journal of Surgical Research, 2006. **134**(2): p. 182-189.
32. Iwai, Y., *Development of a Thermal Cross-Linking Heparinization Method and Its Application to Small Caliber Vascular Prostheses.* ASAIO Journal, 1996. **42**(5): p. M693-697.
33. Mohamed, M.S., M. Mukherjee, and V.V. Kakkar, *Thrombogenicity of heparin and non-heparin bound arterial prostheses: an in vitro evaluation.* Journal of the Royal College of Surgeons of Edinburgh, 1998. **43**(3): p. 155-157.

34. Laredo, J., et al., *Silyl-heparin bonding improves the patency and in vivo thromboresistance of carbon-coated polytetrafluoroethylene vascular grafts.* Journal of Vascular Surgery, 2004. **39**(5): p. 1059-1065.
35. Kapfer, X., W. Meichelboeck, and F.M. Groegler, *Comparison of Carbon-impregnated and Standard ePTFE Prostheses in Extra-anatomical Anterior Tibial Artery Bypass: A Prospective Randomized Multicenter Study.* European Journal of Vascular and Endovascular Surgery, 2006. **32**(2): p. 155-168.
36. Bacourt, F., *Prospective randomized study of carbon-impregnated polytetrafluoroethylene grafts for below-knee popliteal and distal bypass: Results at 2 years.* Annals of vascular surgery, 1997. **11**(6): p. 596-603.
37. Marois, Y., et al., *Carbodiimide cross-linked gelatin: a new coating for porous polyester arterial prostheses.* Biomaterials, 1995. **16**(15): p. 1131-1139.
38. Patel, M., et al., *Comparative Evaluation of the Elasticity and Flexibility of Bioimpregnated Knitted Grafts.* Annals of vascular surgery, 1992. **6**(2): p. 127-133.
39. Guidoin, R., et al., *Can collagen impregnated polyester arterial prostheses be recommended as small diameter blood conduits?* Asaio J, 1996. **42**(6): p. 974-83.
40. Heyligers, J.M.M., et al., *Improving Small-Diameter Vascular Grafts: From the Application of an Endothelial Cell Lining to the Construction of a Tissue-Engineered Blood Vessel.* Annals of vascular surgery, 2005. **19**(3): p. 448-456.
41. Rubanyi, G.M., *The Role of Endothelium in Cardiovascular Homeostasis and Diseases.* Journal of Cardiovascular Pharmacology, 1993. **22**(Supplement 4): p. S1-14.
42. Furchgott, R.F. and J.V. Zawadzki, *The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine.* Nature, 1980. **288**(5789): p. 373-376.
43. Cybulsky, M.I. and M.A. Gimbrone Jr, *Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis.* Science, 1991. **251**(4995): p. 788.
44. Davies, M.G., et al., *The morphology of venovenous bypass graft endothelium.* Cardiovascular Surgery, 1997. **5**(1): p. 82-91.
45. Crombez, M., et al., *Improving arterial prosthesis neo-endothelialization: Application of a proactive VEGF construct onto PTFE surfaces.* Biomaterials, 2005. **26**(35): p. 7402-7409.
46. Jevon, M., A. Dorling, and P.I. Hornick, *Progenitor cells and vascular disease.* Cell Proliferation, 2008. **41**(s1): p. 146-164.
47. Herring, M., A. Gardner, and J. Glover, *A single-staged technique for seeding vascular graft with autogenous endothelium.* Surgery, 1978. **84**: p. 498-504.
48. Mosquera, D.A. and M. Goldman, *Endothelial cell seeding.* British Journal of Surgery, 1991. **78**(6): p. 656-660.
49. Herring, M., A. Gardner, and J. Glover, *Seeding human arterial prostheses with mechanically derived endothelium. The detrimental effect of smoking.* Journal of Vascular Surgery, 1984. **1**: p. 279-289.
50. Herring, M., et al., *Endothelial seeding of polytetrafluoroethylene femoral popliteal bypasses: The failure of low-density seeding to improve patency.* Journal of vascular surgery : official publication, the Society for Vascular Surgery [and] International Society for Cardiovascular Surgery, North American Chapter, 1994. **20**(4): p. 650-655.
51. Örtenwall, P., et al., *Endothelial cell seeding reduces thrombogenicity of Dacron grafts in humans.* Journal of vascular surgery : official publication, the Society for Vascular

Surgery [and] International Society for Cardiovascular Surgery, North American Chapter, 1990. **11**(3): p. 403-410.

52. Zilla, P., et al., *Endothelial cell seeding of polytetrafluoroethylene vascular grafts in humans: A preliminary report.* Journal of vascular surgery : official publication, the Society for Vascular Surgery [and] International Society for Cardiovascular Surgery, North American Chapter, 1987. **6**(6): p. 535-541.
53. Leseche, G., et al., *Above-Knee Femoropopliteal Bypass Grafting Using Endothelial Cell Seeded PTFE Grafts: Five-Year Clinical Experience.* Annals of vascular surgery, 1995. **9**(Supplement 1): p. S15-S23.
54. Andrews, K.D., et al., *Vascular Prostheses: Performance Related to Cell-Shear Responses.* J. Surg. Res., 2008. **149**(1): p. 39-46.
55. Deutsch, M., et al., *Clinical autologous in vitro endothelialization of infrainguinal ePTFE grafts in 100 patients: A 9-year experience.* Surgery, 1999. **126**(5): p. 847-855.
56. Meinhart, J.G., et al., *Clinical autologous in vitro endothelialization of 153 infrainguinal ePTFE grafts.* The Annals of Thoracic Surgery, 2001. **71**(5, Supplement 1): p. S327-S331.
57. Magometschnigg, H., et al., *Prospective clinical study with in vitro endothelial cell lining of expanded polytetrafluoroethylene grafts in crural repeat reconstruction.* Journal of vascular surgery : official publication, the Society for Vascular Surgery [and] International Society for Cardiovascular Surgery, North American Chapter, 1992. **15**(3): p. 527-535.
58. Zilla, P., et al., *Clinical in vitro endothelialization of femoropopliteal bypass grafts: An actuarial follow-up over three years.* Journal of vascular surgery : official publication, the Society for Vascular Surgery [and] International Society for Cardiovascular Surgery, North American Chapter, 1994. **19**(3): p. 540-548.
59. Jardine, S. and J.I.B. Wilson, *Plasma Surface Modification of ePTFE Vascular Grafts.* Plasma Processes and Polymers, 2005. **2**(4): p. 328-333.
60. Gauvreau, V. and G. Laroche, *Micropattern printing of adhesion, spreading, and migration peptides on poly(tetrafluoroethylene) films to promote endothelialization.* Bioconjugate Chemistry, 2005. **16**(5): p. 1088-1097.
61. Wissink, M.J.B., et al., *Improved endothelialization of vascular grafts by local release of growth factor from heparinized collagen matrices.* Journal of Controlled Release, 2000. **64**(1-3): p. 103-114.
62. Greisler, H.P., *Growth factor release from vascular grafts.* Journal of Controlled Release, 1996. **39**(2-3): p. 267-280.
63. Lindner, V., et al., *Role of Basic Fibroblast Growth Factor in Vascular Lesion Formation.* Circulation Research, 1991. **68**(1): p. 106-113.
64. Ferrara, N., et al., *The vascular endothelial growth factor family of polypeptides.* J. Cell. Biochem., 1991. **47**(3): p. 211-18.
65. Weatherford, D.A., et al., *Vascular endothelial growth factor and heparin in a biologic glue promotes human aortic endothelial cell proliferation with aortic smooth muscle cell inhibition.* Surgery, 1996. **120**(2): p. 433-439.
66. Ferrara, N. and T. Davis-Smyth, *The Biology of Vascular Endothelial Growth Factor.* Endocr Rev, 1997. **18**(1): p. 4-25.
67. Randone, B., et al., *Dual Role of VEGF in Pretreated Experimental ePTFE Arterial Grafts.* Journal of Surgical Research, 2005. **127**(2): p. 70-79.
68. Zisch, A.H., et al., *Covalently conjugated VEGF-fibrin matrices for endothelialization.* Journal of Controlled Release, 2001. **72**(1-3): p. 101-113.

69. Malafaya, P.B., G.A. Silva, and R.L. Reis, *Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications.* Advanced Drug Delivery Reviews, 2007. **59**(4-5): p. 207-233.
70. Nishinari, K. and R. Takahashi, *Interaction in polysaccharide solutions and gels.* Current Opinion in Colloid & Interface Science, 2003. **8**(4-5): p. 396-400.
71. Cascone, M.G., et al., *Bioartificial polymeric materials based on polysaccharides.* Journal of Biomaterials Science, Polymer Edition, 2001. **12**: p. 267-281.
72. Metzmacher, I., *Enzymatic degradation and drug release behavior of dense collagen implants*, in *Pharmaceutical Technology*. 2005, LMU: Munich. p. No pp given.
73. Pillai, O. and R. Panchagnula, *Polymers in drug delivery.* Current Opinion in Chemical Biology, 2001. **5**(4): p. 447-451.
74. Koch, S., et al., *Enhancing angiogenesis in collagen matrices by covalent incorporation of VEGF.* J. Mater. Sci. Mater. Med., 2006. **17**(8): p. 735-741.
75. Danielsson, C., et al., *Modified collagen fleece, a scaffold for transplantation of human bladder smooth muscle cells.* Biomaterials, 2006. **27**(7): p. 1054-1060.
76. Geiger, M., R.H. Li, and W. Friess, *Collagen sponges for bone regeneration with rhBMP-2.* Advanced Drug Delivery Reviews, 2003. **55**(12): p. 1613-1629.
77. Ruszczak, Z. and W. Friess, *Collagen as a carrier for on-site delivery of antibacterial drugs.* Advanced Drug Delivery Reviews, 2003. **55**(12): p. 1679-1698.
78. Dorotka, R., et al., *Marrow stimulation and chondrocyte transplantation using a collagen matrix for cartilage repair.* Osteoarthritis and Cartilage, 2005. **13**(8): p. 655-664.
79. Geiger, M., *Porous Collagen/Ceramic Composite Carriers for Bone Regenaration Using Recombinant Human Bone Morphogenetic Protein-2 (rhBMP-2).* PhD Thesis, FAU Erlangen, Germany, 2001.
80. Friess, W., *Collagen - biomaterial for drug delivery.* European Journal of Pharmaceutics and Biopharmaceutics, 1998. **45**(2): p. 113-136.
81. Lee, C.H., A. Singla, and Y. Lee, *Biomedical applications of collagen.* International Journal of Pharmaceutics, 2001. **221**(1-2): p. 1-22.
82. Brinckmann, J., et al., *Collagen: Primer in Structure, Processing and Assembly. [In: Top. Curr. Chem.; 2005, 247]*. 2005. 252 pp.
83. Friess, W., *Drug Delivery Systems Based on Collagen.* 1999.
84. Brodsky, B. and J.A.M. Ramshaw, *The collagen triple-helix structure.* Matrix Biology, 1997. **15**(8-9): p. 545-554.
85. Herrmann, M. and M. Seibel, *The amino- and carboxyterminal cross-linked telopeptides of collagen type I, NTX-I and CTX-I: A comparative review.* Clinica Chimica Acta, 2008. **393**(2): p. 57-75.
86. Sano, A., et al., *Atelocollagen for protein and gene delivery.* Advanced Drug Delivery Reviews, 2003. **55**(12): p. 1651-1677.
87. Jayakrishnan, A. and S.R. Jameela, *Glutaraldehyde as a fixative in bioprostheses and drug delivery matrices.* Biomaterials, 1996. **17**(5): p. 471-484.
88. Heijmen, F.H., et al., *Cross-linking of dermal sheep collagen with tannic acid.* Biomaterials, 1997. **18**(10): p. 749-754.
89. Zeeman, R., et al., *Crosslinking and modification of dermal sheep collagen using 1,4-butanediol diglycidyl ether.* Journal of biomedical materials research, 1999. **46**(3): p. 424-433.

90. Wachem, P.B.v., et al., *Biocompatibility and tissue regenerating capacity of crosslinked dermal sheep collagen.* Journal of biomedical materials research, 1994. **28**(3): p. 353-363.
91. Ming-Che, W., G.D. Pins, and F.H. Silver, *Collagen fibres with improved strength for the repair of soft tissue injuries.* Biomaterials, 1994. **15**(7): p. 507-512.
92. Weadock, K.S., et al., *Effect of physical crosslinking methods on collagen-fiber durability in proteolytic solutions.* Journal of biomedical materials research, 1996. **32**(2): p. 221-226.
93. Pieper, J.S., et al., *Preparation and characterization of porous crosslinked collagenous matrices containing bioavailable chondroitin sulphate.* Biomaterials, 1999. **20**(9): p. 847-858.
94. Wachem, P.B.v., et al., *In vivo biocompatibility of carbodiimide-crosslinked collagen matrices: Effects of crosslink density, heparin immobilization, and bFGF loading.* Journal of biomedical materials research, 2001. **55**(3): p. 368-378.
95. Friess, W. and M. Schlapp, *Release mechanisms from gentamicin loaded poly(lactic-co-glycolic acid) (PLGA) microparticles.* J. Pharm. Sci., 2002. **91**(3): p. 845-855.
96. Budhian, A., S.J. Siegel, and K.I. Winey, *Controlling the in vitro release profiles for a system of haloperidol-loaded PLGA nanoparticles.* International Journal of Pharmaceutics, 2008. **346**(1-2): p. 151-159.
97. Webber, W.L., et al., *Characterization of soluble, salt-loaded, degradable PLGA films and their release of tetracycline.* J. Biomed. Mater. Res., 1998. **41**(1): p. 18-29.
98. Boccaccini, A.R., et al., *Preparation and characterisation of poly(lactide-co-glycolide) (PLGA) and PLGA/Bioglass® composite tubular foam scaffolds for tissue engineering applications.* Materials Science and Engineering: C, 2005. **25**(1): p. 23-31.
99. Jain, R.A., *The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices.* Biomaterials, 2000. **21**(23): p. 2475-2490.
100. Gao, J., L. Niklason, and R. Langer, *Surface hydrolysis of poly(glycolic acid) meshes increases the seeding density of vascular smooth muscle cells.* Journal of biomedical materials research, 1998. **42**(3): p. 417-424.
101. Anderson, J.M. and M.S. Shive, *Biodegradation and biocompatibility of PLA and PLGA microspheres.* Advanced Drug Delivery Reviews, 1997. **28**(1): p. 5-24.
102. Oh, S.H., et al., *Fabrication and characterization of hydrophilic poly(lactic-co-glycolic acid)/poly(vinyl alcohol) blend cell scaffolds by melt-molding particulate-leaching method.* Biomaterials, 2003. **24**(22): p. 4011-4021.
103. Jain, R., et al., *Controlled Drug Delivery by Biodegradable Poly(Ester) Devices: Different Preparative Approaches.* Drug Development and Industrial Pharmacy, 1998. **24**(8): p. 703-727.
104. Dechy-Cabaret, O., B. Martin-Vaca, and D. Bourissou, *Controlled Ring-Opening Polymerization of Lactide and Glycolide.* Chemical Reviews, 2004. **104**(12): p. 6147-6176.
105. Jaraswekin, S., S. Prakongpan, and R. Bodmeier, *Effect of poly(lactide-co-glycolide) molecular weight on the release of dexamethasone sodium phosphate from microparticles.* Journal of Microencapsulation, 2007. **24**(2): p. 117-128.
106. Lewis, D.H., *Controlled release of bioactive agents from lactide/glycolide polymers.* Drugs Pharm. Sci., 1990. **45**(Biodegrad. Polym. Drug Delivery Syst.): p. 1-41.
107. Miyajima, M., et al., *Effect of polymer crystallinity on papaverine release from poly (-lactic acid) matrix.* Journal of Controlled Release, 1997. **49**(2-3): p. 207-215.

108. Luan, X. and R. Bodmeier, *Influence of the poly(lactide-co-glycolide) type on the leuprolide release from in situ forming microparticle systems.* Journal of Controlled Release, 2006. **110**(2): p. 266-272.
109. Kenley, R.A., et al., *Poly(lactide-co-glycolide) decomposition kinetics in vivo and in vitro.* Macromolecules, 1987. **20**(10): p. 2398-2403.
110. Li, S.M., H. Garreau, and M. Vert, *Structure-property relationships in the case of the degradation of massive aliphatic poly-(α-hydroxy acids) in aqueous media.* J. Mater. Sci. Mater. Med., 1990. **1**(3): p. 123-130.
111. Jalil, R. and J.R. Nixon, *Biodegradable poly(lactic acid) and poly(lactide-co-glycolide) microcapsules: problems associated with preparative techniques and release properties.* Journal of Microencapsulation, 1990. **7**(3): p. 297-325.
112. Wang, X., et al., *Controlled release of sirolimus from a multilayered PLGA stent matrix.* Biomaterials, 2006. **27**(32): p. 5588-5595.
113. Batycky, R.P., et al., *A theoretical model of erosion and macromolecular drug release from biodegrading microspheres.* J. Pharm. Sci., 1997. **86**(12): p. 1464-1477.
114. Witschi, C. and E. Doelker, *Influence of the microencapsulation method and peptide loading on poly(lactic acid) and poly(lactic-co-glycolic acid) degradation during in vitro testing.* Journal of Controlled Release, 1998. **51**(2-3): p. 327-341.
115. Park, T.G., *Degradation of poly(d,l-lactic acid) microspheres: effect of molecular weight.* Journal of Controlled Release, 1994. **30**(2): p. 161-173.
116. Ingelheim, B., *Technical Bulletin - Resomer® - Thermal Properties.* 2008.
117. Ingelheim, B., *Technical Bulletin - Resomer® Product Range.* 2009.
118. Klose, D., et al., *PLGA-based drug delivery systems: Importance of the type of drug and device geometry.* International Journal of Pharmaceutics, 2008. **354**(1-2): p. 95-103.
119. Dorta, M.J., et al., *Potential applications of PLGA film-implants in modulating in vitro drugs release.* International Journal of Pharmaceutics, 2002. **248**(1-2): p. 149-156.
120. Faisant, N., J. Siepmann, and J.P. Benoit, *PLGA-based microparticles: elucidation of mechanisms and a new, simple mathematical model quantifying drug release.* European Journal of Pharmaceutical Sciences, 2002. **15**(4): p. 355-366.
121. Sinha, V.R. and A. Trehan, *Biodegradable microspheres for protein delivery.* Journal of Controlled Release, 2003. **90**(3): p. 261-280.
122. van de Weert, M., W.E. Hennink, and W. Jiskoot, *Protein Instability in Poly(Lactic-co-Glycolic Acid) Microparticles.* Pharm. Res., 2000. **17**(10): p. 1159-1167.
123. Chen, M.-C., et al., *A novel drug-eluting stent spray-coated with multi-layers of collagen and sirolimus.* Journal of Controlled Release, 2005. **108**(1): p. 178-189.

Chapter 2

Modification of PTFE using atmospheric plasma and its characterization

Abstract

The implementation of the plasmabrush® (Reinhausen Plasma GmbH, Regensburg, Germany) and the optimization of the plasma treatment process for polytetrafluoro-ethylene (PTFE) to enhance coating adhesion was studied. Furthermore, the changes induced by the atmospheric argon plasma treatment process were characterized using surface sensitive techniques, such as scanning electron microscopy (SEM), Fourier transform infrared (FT-IR) spectroscopy, X-ray photoelectron spectroscopy (XPS), and surface energy determination. Scanning electron microscopy did not reveal any changes in surface morphology and FT-IR was not sensitive enough to detect alterations in surface chemistry. XPS revealed minor variations in the binding energy region of oxygen; however, a decrease was shown for plasma treated samples, which suggested a cleansing effect of the argon plasma treatment for the PTFE samples. A change in surface energy was detectable for the treated samples using test inks. In addition, it was shown that the treatment significantly increased coating adhesion of applied collagen and poly(lactic-co-glycolic acid) (PLGA) films, as well as, cell adhesion of human umbilical vein endothelial cells (HUVECs) in comparison to untreated PTFE.

Keywords: PTFE, atmospheric argon plasma, surface energy, endothelial cells

1 Introduction

Synthetic polymeric materials have been widely used in the field of biomaterial applications. Polymers such as polyurethane, poly(methylmethacrylate), ultrahigh molecular weight polyethylene, polyetheretherketone (PEEK), polyethylene terephthalate (PET), and PTFE have been used for bone plates, screws, artificial lenses, bone cements, abdominal wall prostheses, and vascular grafts [1-2]. However, most of these materials do not meet the demands for both their surface and bulk properties when used as biomaterials [3]; a very low surface energy and resulting poor adhesion being one crucial factor. Adhesion is generally considered to be a surface property, where only molecular layers at the surface of the material are responsible for the effect [4]. Several approaches have been undertaken to change the surface properties of polymeric surfaces, with the most obvious being a surface roughening by sand blasting or etching in order to increase the surface area for enhanced adhesion [5]. Other methods applied are oxidation [6], ion implantation [7], or graft polymerization [8]. In recent years, plasma treatment of polymer surfaces has been employed as an interesting alternative for surface modification [4]. Depending on the gas composition and plasma conditions, ions, electrons, fast neutrals, radicals, and UV radiation contribute to the polymer treatment, resulting in etching, activation, and/or cross-linking [9]. Since these changes generally occur within the top layers of the treated materials [10], plasma treatment is an interesting approach to modify the near surface region without modifying the bulk properties of the polymers.

Therefore, the goal of this study was the implementation of the atmospheric plasma treatment torch plasmabrush® for the modification of PTFE surfaces, and the enhancement of its coating accessibility and cell compatibility. For this purpose, changes induced by atmospheric plasma treatment were characterized by various surface sensitive techniques, such as SEM, FT-IR spectroscopy, XPS, and surface energy determination. To get insight in the cell compatibility and attachment, cell studies using human umbilical vein endothelial cells (HUVECs) were performed.

2 Materials and Methods

2.1 Materials

PTFE was obtained from GM GmbH (Freiham, Germany), argon process gas and nitrogen from Linde AG (Pullach, Germany), collagenase A from Roche (Penzberg, Germany), endothelial cell growth medium from Provitro (Berlin, Germany), heat inactivated fetal bovine serum from Biochrom (Berlin, Germany), buffered formaldehyde solution (4%) from Polysciences Inc. (Warrington, PA, USA), crystal violet and dichloromethane from Merck KGaA (Darmstadt, Germany), Resomer® (RG 502H, RG 503, RG 503H and RG 504H) from Boehringer Ingelheim (Ingelheim, Germany), and equine collagen type I derived from tendon from Innocoll GmbH (Saal/Donau, Germany). The pH of the solutions was adjusted using hydrochloric acid or sodium hydroxide from Merck KGaA (Darmstadt, Germany) and measured with a pH meter Inolab level 1 from WTW (Weilheim, Germany).

2.2 Methods

2.2.1 Plasma activation

PTFE-discs were activated with an atmospheric plasma jet (plasmabrush®, Reinhausen Plasma GmbH, Regensburg, Gemany) (see Figure 2-1) using argon with a purity of 5.0. Gas flow was adjusted to 10 l/min. The voltages for plasma discharge and treatment time were evaluated.

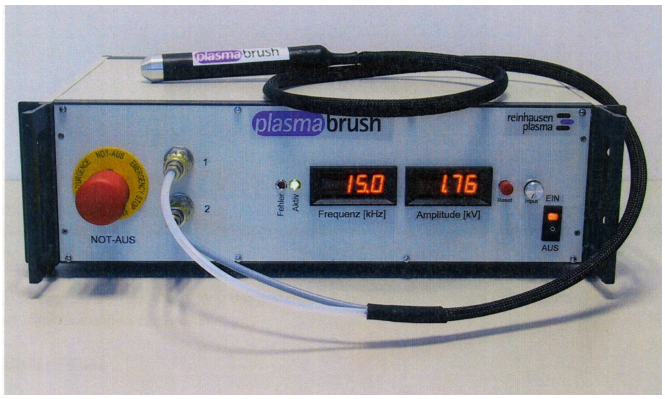


Figure 2-1: plasmabrush® atmospheric plasma jet (Reinhausen Plasma GmbH, Regensburg, Germany)

2.2.2 Characterization of surfaces after plasma modification

2.2.2.1 Scanning Electron Microscopy

Samples for SEM were fixed on specimen holders using conductive double sided tape before sputtering with a thin layer of carbon under vacuum. SEM images were obtained using a Philips XL Series XL20 (Philips, The Netherlands) at 4.0kV.

2.2.2.2 X-ray Photoelectron Spectroscopy (XPS)

XPS measurements were performed using a Vacuum Science Workshop (VSW) surface analysis chamber equipped with a VSW HA 100 hemispherical analyzer (Vacuum Science Workshop, United Kingdom). The analyzer was operated in Fixed Analyzer Transmission (FAT) mode with variable retarding potential of 22 eV. The base pressure during the XPS measurements was $<10^{-7}$ Pa.

2.2.2.3 Attenuated Total Reflection- FT-IR spectroscopy (ATR-FT-IR)

FT-IR measurements were performed on a Tensor 27 FT-IR spectrometer (Bruker Optics GmbH, Ettlingen, Germany) using the Miracle ATR unit. The recorded spectra were obtained from 4000 to 900 cm^{-1} wavenumbers, in attenuated total reflectance (ATR) mode at 20℃. Each measurement wa s an average of 240 scans. While data acquisition was performed, the optical bench was purged with dry nitrogen to reduce interference from water vapour IR absorption and each spectrum was corrected for the background.

2.2.2.4 Surface energy determination

The change in surface energy of plasma treated discs was evaluated using test inks for surface energy determination (Plasmatreat Testtinte, Plasmatreat GmbH, Steinhagen, Germany) in a range of 30 – 72 mN/m.

2.2.3 Cell compatibility

The cell compatibility of untreated and plasma treated PTFE-discs was evaluated using HUVECs. HUVECs were prepared by digestion of umbilical veins with 0.1 g/l collagenase A. Cells were cultured in endothelial cell growth medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere at 5% CO_2 and 37℃. Cells were used at passage no. 3. For visualization of cells on the different surfaces, cells were fixed with a buffered formaldehyde solution (4%) and nuclei were stained with crystal violet (0.5% in 20% methanol). Images were obtained with a SZX7 microscope and an ALTRA20 CMOS camera (Olympus, Hamburg, Germany).

2.2.4 Film adhesion

1% (w/v) Resomer® (RG 503 and RG 503H) solutions in dichloromethane and 0.5 and 1% (w/v) aqueous collagen dispersions were used to coat untreated and argon plasma treated PTFE-discs with a diameter of 15 mm. Therefore, the discs were placed in 24-well aluminum well plates and covered with the different coating solutions. The discs were air dried for one hour and subsequently removed from the wells, and dried under vacuum over night or until further use. For the investigation of film adhesion, the coated discs were examined concerning the ablation of PLGA and collagen films after drying by means of visual inspection.

3 Results and Discussion

3.1 Surface characterization

In order to gain insight in the changes of surface properties and composition of the atmospheric plasma treated samples, as well as, to obtain a better understanding of the plasma treatment in general, several methods to investigate the changes and the process were utilized. Therefore, the samples were plasma treated and subsequently analyzed by means of SEM, FT-IR spectroscopy, and XPS. In

addition, the change in surface energy was determined using commercially available test inks.

3.1.1 Surface morphology

One of the main effects reported for the treatment of polymer surfaces with plasma is the ablation or the etching of material of the surface, which can remove weak boundary layers and increase the surface area [4, 11]. Therefore, changes in surface morphology could potentially be visualized using scanning electron microscopy. For the investigations, untreated and plasma treated PTFE samples were prepared according to 2.2.1 and examined concerning their morphology.

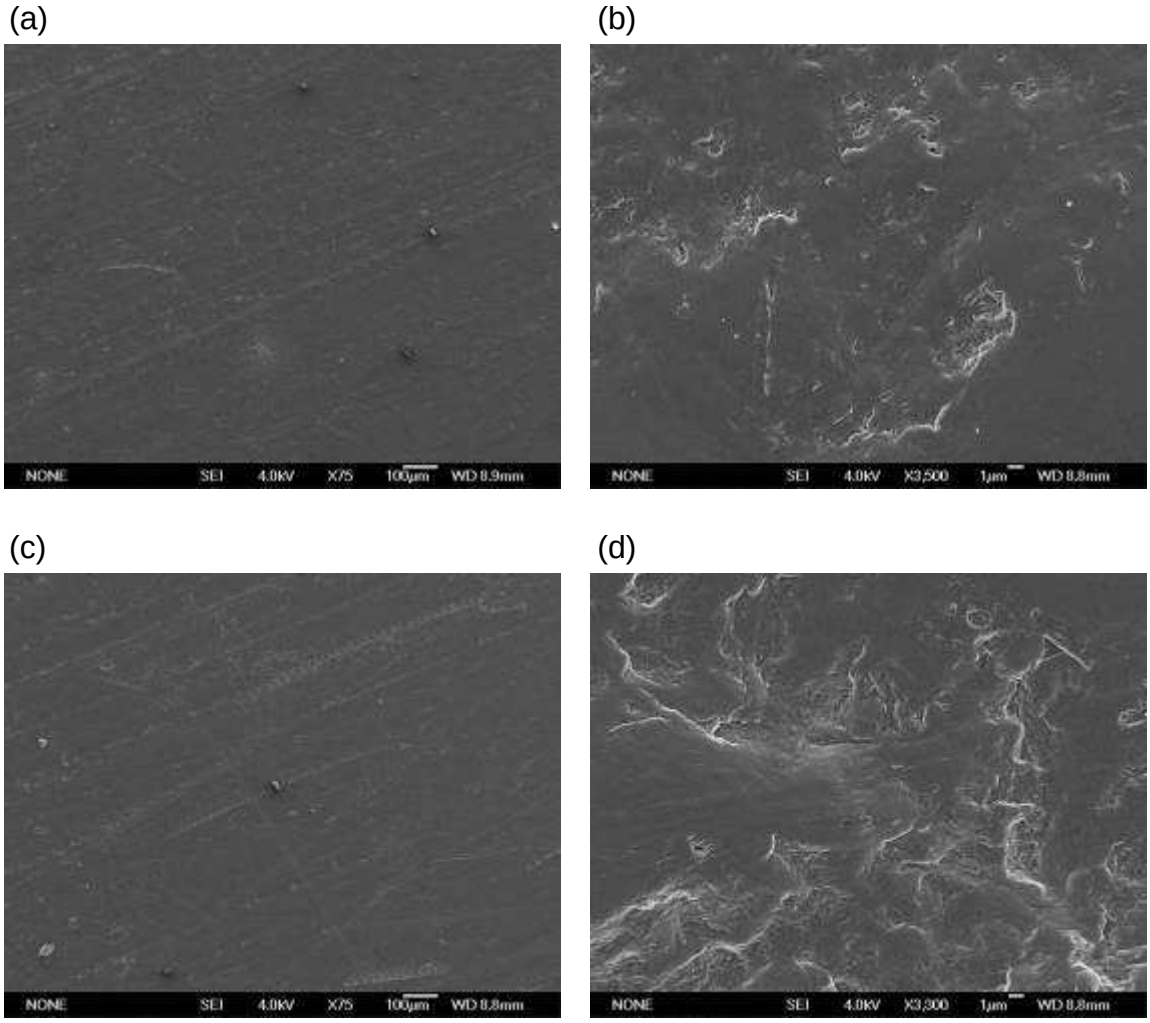


Figure 2-2: Scanning electron micrographs of untreated PTFE discs as overview (a) and magnification (b) and argon plasma treated PTFE discs as overview (c) and magnification (d).

Figure 2-2 shows the surface of plasma treated and untreated polymer by SEM. The overview micrographs of both samples (Figure 2-2a and c) showed similar scratch patterns on the surface that originated from the manufacturing process of the PTFE. Magnifications of both samples (Figure 2-2b and d) showed again no differences concerning the surface morphology, such as, smoothing of the surface due to etching or ablation in contrast to cases reported in literature for different polymers using various process gases [10, 12]. However, Park et al. reported similar findings when investigating the change in surface morphology of polymers under the influence of plasma treatment [13]. Therefore, it must be concluded that the short treatment times of the atmospheric argon plasma did not suffice to inflict changes on the surface morphology of the used PTFE samples.

3.1.2 Surface chemistry analysis by ATR-FTIR

In order to investigate the introduction of functional groups on the surfaces of plasma treated PTFE discs, FT-IR measurements were performed. For the measurements, PTFE-discs were treated with argon plasma according to 2.2.1 with a constant nozzle to surface distance of 10 mm, an excitation voltage of 4.5 kV, and a treatment time of 15 s. FT-IR spectra were recorded prior to treatment for pristine PTFE samples and immediately after plasma treatment.

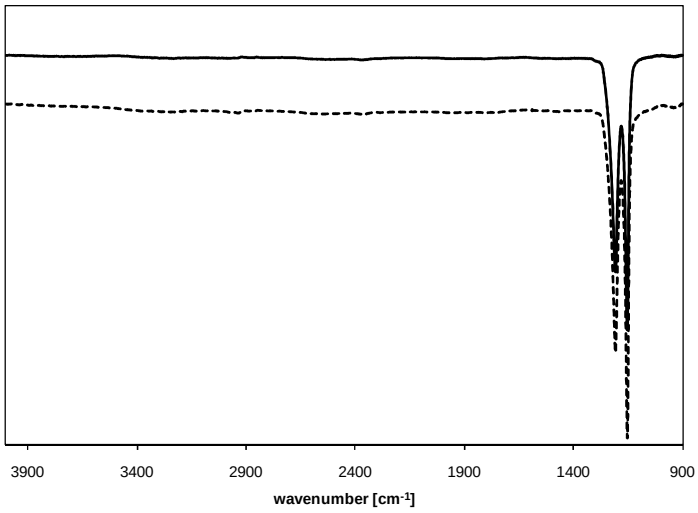


Figure 2-3: FT-IR spectra of untreated (——) and plasma treated (- - - -) PTFE discs using argon with adjusted intensity for better comparability.

Figure 2-3 shows the recorded ATR-FTIR spectra of untreated and argon plasma treated samples. No differences of the recorded spectra were observable over the range of wavenumbers investigated. It has been reported in literature that plasma treatment of polymer surfaces with various process gases results in the removal of hydrogen atoms especially for inert gas plasmas [14] or in the case of PTFE the removal of fluorine atoms from the polymer surface and the formation of carbon radicals. Most carbon radicals will then be oxidized into oxygen functional groups, when the polymer specimen are exposed to air in case of low pressure plasma or during the treatment process in case of atmospheric plasma treatment [8, 15-16]. The results demonstrate that in case of plasmabrush® treatment of PTFE, oxygen functional groups are not introduced due to the very high bond strength between carbon and fluorine atoms [17] or are introduced at a concentration which is below the limit of detection for FT-IR spectroscopy. The depth of penetration for FT-IR of several microns [18] might be responsible for a reduced sensitivity as argon plasma affects the 30 nm region of surfaces treated [10]. It has also been reported that the main effect of argon plasma on polymer surfaces is ablation and etching [10], but could not be confirmed using SEM.

3.1.3 Surface composition

XPS has been widely used in the investigation of the influence of plasma modification on polymer surfaces [19-21]. It is a method sensitive to minor changes in the surface composition of studied materials, which made it a promising tool for the investigation of the upper layers within the top 3-6 nm of the investigated polymer [22]. For the investigations, untreated and plasma treated PTFE samples were prepared according to 2.2.1 and were examined concerning their surface composition (compare 2.2.2.2).

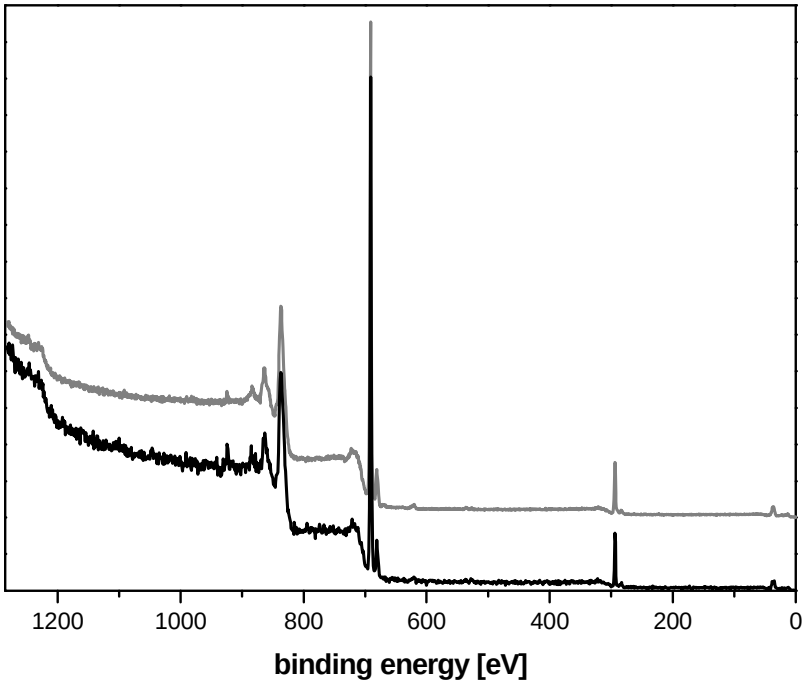


Figure 2-4: Overview XPS spectra of untreated (—) and argon plasma treated (—) PTFE discs with adjusted intensity for better comparability.

The overview XPS spectra recorded for the untreated and treated PTFE samples are shown in Figure 2-4. The peak that occured at 294 eV could be ascribed to the C1s spectrum attributed to the CF_2 species and was present in both untreated and argon plasma treated polymer species. The second characteristic peak at 691 eV could be ascribed to the F1s spectrum and was observed in both polymer species as well. In order to further investigate the influence of argon plasma on the chemical composition of the polymer surface, the areas of the XPS spectra for the peaks representing the C1s, F1s, O1s and N1s were analyzed at higher resolution. The later were investigated with the intention of studying the introduction of functional groups including oxygen and nitrogen.

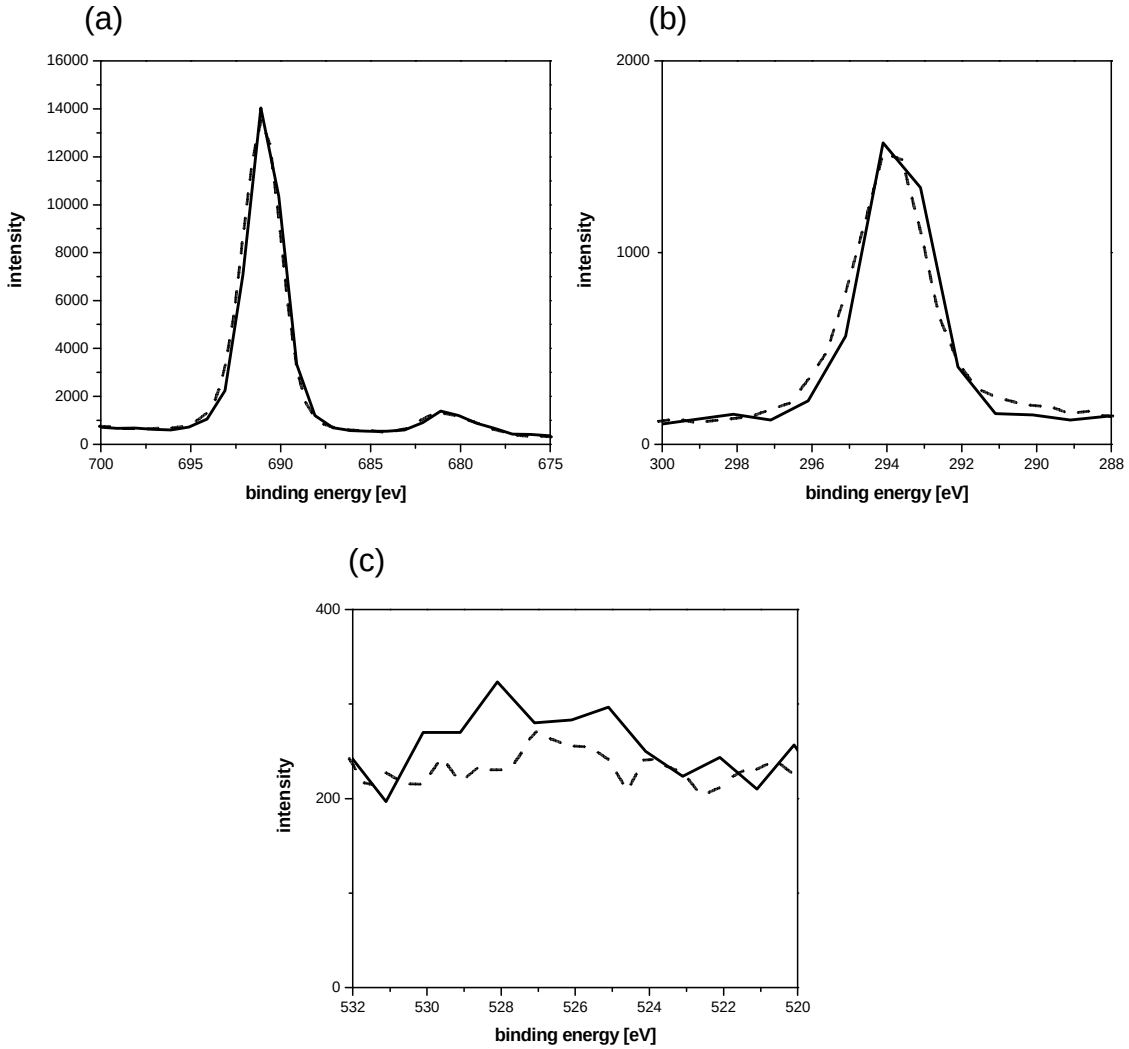


Figure 2-5: Magnification of XPS spectra of untreated (——) and argon plasma treated (— —) PTFE discs of binding energy regions of F1s (a), C1s (b) and O1s (c).

The magnification of the binding energy regions for the F1s spectrum (Figure 2-5a) revealed no differences for the untreated and plasma treated PTFE samples. The same result was obtained when investigating the magnification of the binding energy region of the C1s spectrum (Figure 2-5b) and the N1s region (data not shown). When studying the magnification of the O1s region of the binding energy minor differences were observable that were hardly delimitable from background noise. However, contrary to the expectations, a decrease of oxygen content was detected for the plasma treated samples in comparison to the unmodified PTFE (Figure 2-5c). The introduction of oxygen containing species by the formation of

radicals during the plasma treatment process and subsequent saturation by oxygen present during the plasma treatment process or post treatment with ambient air have been reported in literature [23-24]. A decrease in oxygen content for the plasma treated samples could be ascribed to cleansing of the surface by ablation of weak boundary layers [4] contaminated with oxygen. A removal of plasma activated layers and the exposure of untreated polymer as reported in literature [25] might have added to that effect.

3.1.4 Determination of surface energy - hydrophilicity

For the determination of surface energy of the untreated and argon plasma treated PTFE samples, commercially available test inks were used. It is a simple, quick, and easy to use method for measuring surface energy on materials such as plastic, metal, or glass. In order to determine the surface energy of samples, the test inks were applied to the surface. In case good wetting occurred after application, the surface energy of the material being tested was higher than the corresponding surface tension value of the ink applied. The test was repeated with the next higher test value until wetting failed to occur. Accordingly, the surface energy of the material corresponds to the value of the test ink that last wetted it for at least 2 seconds. Obtained values for surface energies are relative values and can not be directly compared to values acquired with other methods.

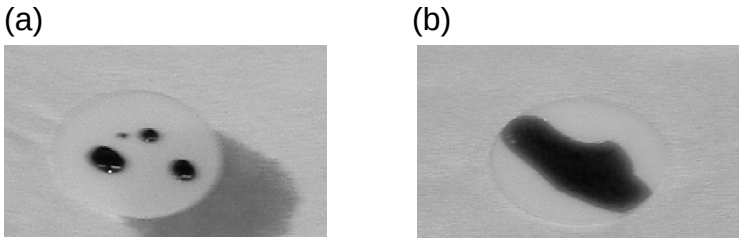


Figure 2-6: Determination of surface energy of PTFE discs using 30 mN/m test inks of a disc prior to treatment (a) and after argon plasma treatment (b).

Figure 2-6 shows the results obtained for an untreated and plasma treated sample. Prior to treatment PTFE, had a surface energy of below 30 mN/m (Figure 2-6a), the

lower limit of detection for the applied test inks, as could be seen by the bad wetting of the applied test ink. These results are in accordance to values reported in literature for pristine PTFE of approximately 15 – 20 mN/m [17, 26-27]. Following plasma treatment, the surface energy of the samples increased to values above 30 mN/m as could be seen by the good wetting of the applied test ink (Figure 2-6b). This increase in surface energy for plasma treated samples was in accordance to studies reported in literature [23, 28-29].

Thus, despite the fact that neither XPS, ATR-FTIR, nor SEM could prove chemical or surface morphology changes, the important parameter for coating [30] or cell adhesion [31], surface energy could be significantly increased by atmospheric plasma treatment.

3.2 Optimization of the plasma activation process using the plasmabrush®

In order to optimize the procedure for atmospheric plasma treatment using the plasmabrush®, the aforementioned method to determine changes in surface energy using test inks was employed to characterize the treated surfaces and to optimize the treatment parameters. The effect of changes in parameters, such as, ignition voltage of the plasma, treatment time, and aging of the treated surfaces were investigated.

3.2.1 Optimization of ignition voltage

For the optimization of the ignition voltage of the plasma, treatment time for all PTFE samples was kept constant at 10 s, as well as, the nozzle to surface distance, which was 10 mm. Samples were plasma treated using argon gas at a flow rate of 10 l/min with different ignition voltages ranging from 3.5 kV, the lowest voltage applicable due to non ignition of plasma at lower voltages, and 8.0 kV, the highest voltage applicable due to arc discharges occurring at higher voltages. Surface energy of the treated samples was measured immediately after the plasma treatment.

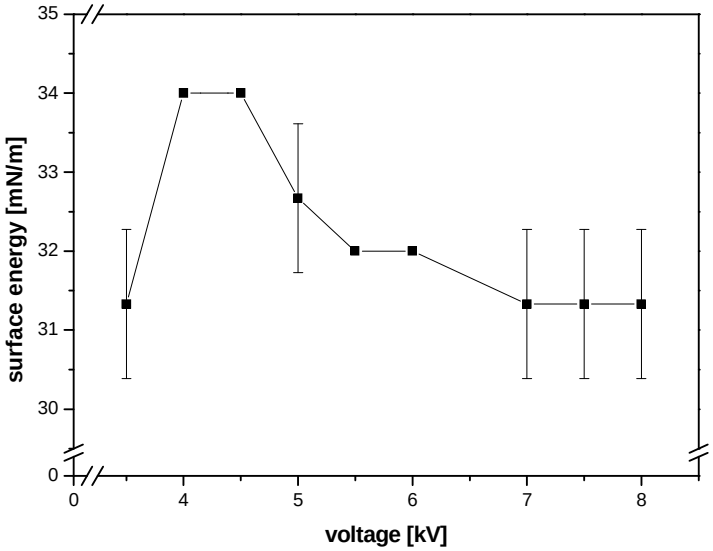


Figure 2-7: Development of surface energy in correlation to treatment voltage for argon plasma treated PTFE discs. (average ± SD; n=3)

Figure 2-7 shows the development of the surface energy of the plasma treated samples in accordance to the ignition voltage. The activation started at 3.5 kV with a surface energy of the treated samples of approximately 31 mN/m and reached a maximum of activation at 34 mN/m for the samples treated with 4.0 and 4.5 kV. Surface energies for the samples treated with voltages higher than 4.5 kV decreased to 32 mN/m for those treated with 5.5 and 6.0 kV. A further decrease to approximately 31.5 mN/m was experienced for the samples treated with a higher voltage than 6.0 kV up to 8.0 kV, the highest voltage applicable.

Contrary findings have been reported in literature. Water contact angles, the basis for the calculation of surface energy based on the method of Owens and Wendt [32], were measured in dependence of different acceleration voltages in a low pressure plasma setting [33]. Water contact angles increased with low voltages leading to lower surface energies and decreased with higher voltages, reflecting higher surface energies. Similar findings were also reported by Liu et al [34] for atmospheric plasma treatment.

In this case the ignition voltage of up to a 4.5 kV in combination with the short treatment time of 10 s might be responsible for a cleansing and modification of the

surface of the PTFE film leading to an increased surface energy. Further increase of the ignition voltage could then cause a removal of activated polymer layers and the exposure of untreated polymer, as reported in literature [25], resulting in a subsequent decrease of surface energy, as observed during the optimization process.

3.2.2 Optimization of treatment time

In order to optimize treatment times of the plasma treatment process nozzle to surface distance for all PTFE samples was kept constant at 10 mm and two ignition voltages 3.5 and 4.5 kV were investigated. Samples were plasma treated using argon gas at a flow rate of 10 l/min with different treatment times ranging from 5 s to 60 s. Surface energy of the treated samples was measured immediately after the plasma treatment.

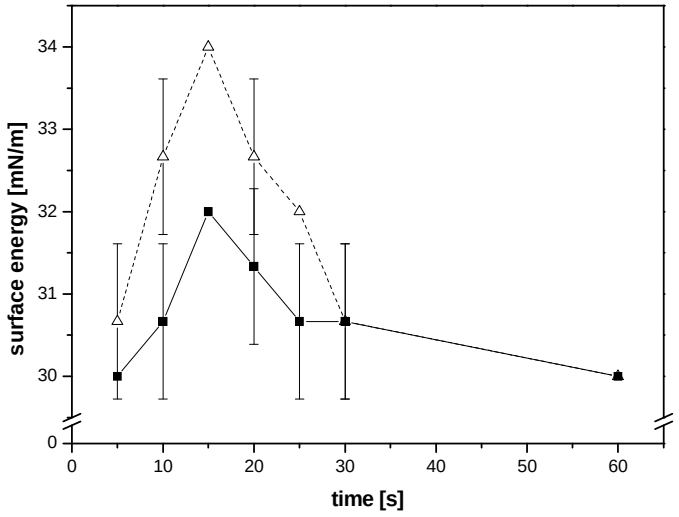


Figure 2-8: Development of surface energy in correlation to treatment time for 3.5 kV (—■—) and 4.5 kV (---△---). (average ± SD; n=3)

It was expected that increasing treatment times could result in increasing modification of the surface, in this case the surface energy, as reported in literature [17, 35]. For both excitation voltages, an increase in surface energy was detected in

the first 15 s of treatment time, with surface energies of 30.0 and 30.5 mN/m for 3.5 and 4.5 kV respectively after 5 s of treatment and a maximum of 32.0 and 34.0 mN/m after 15 s (Figure 2-8). Increasing the treatment times did not result in a further increase of surface energy, but in a decrease to 30.5 mN/m for both voltages after a treatment time of 30 s. A further reduction of surface energy to 30 mN/m after treatment times of 60 s, irrespective of the excitation voltage was observed. The decrease in surface energy might indicate that other processes, such as, molecular turn-over, weak boundary layers, and etching were dominating the treatment [36] and were therefore leading to the ablation of plasma activated layers and the exposure of untreated polymer [25].

3.2.3 Influence of aging time post treatment on surface energy

With the aim of bulk production of plasma treated samples, it was important to investigate the influence of aging time post treatment on plasma modified PTFE samples. Therefore, PTFE discs were treated with atmospheric plasma using argon gas as process gas at a flow rate of 10 l/min, a nozzle to surface distance of 10 mm, an excitation voltage of 4.5 kV, and a treatment time of 15 s. For the investigations sufficient, amounts of PTFE samples were plasma treated and stored at room temperature under a fume hood until measurement using the test inks. For each time point new samples were measured and discarded afterwards in order to eliminate the effect of the test ink on the surface.

The results for the effect of aging time on the surface energy of argon plasma treated samples are shown in Figure 2-9. A maximum of activation could be observed 60 min post treatment that decreased with increasing aging time and stabilized at a surface energy of 32 mN/m that was maintained for several weeks (data not shown). Similar results have been described using low pressure plasma. Koenig et al. reported that in dependency of storage conditions post treatment the changes in surface properties on PTFE samples were reversible [18]. The same phenomenon has also been reported for other polymers, for example polycarbonate [9].

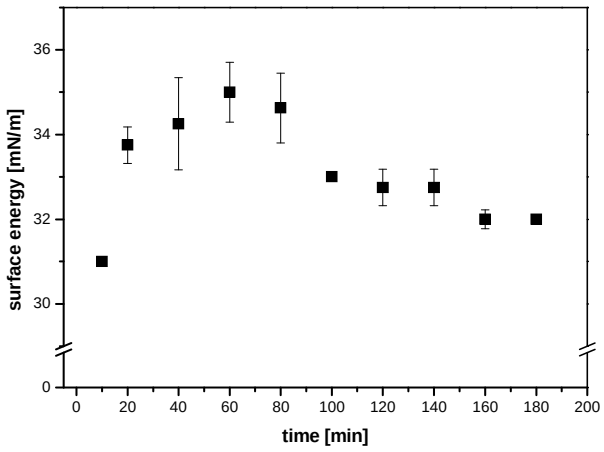


Figure 2-9: Development of surface energy of PTFE discs in correlation to aging time after argon plasma treatment. (average ± SD; n=4)

It has been shown that O_2 plasma treated surfaces generally show short aging times and recover hydrophobicity partially or even completely. The possible mechanisms for this are believed to be reorientation or migration of treated polymer chains from the surface to the bulk. The driving force is the minimization of interfacial energy, and this irreversible recovery is found in most plasma-treated polymer surfaces [37]. A similar process in case of the argon plasma treated samples could be responsible for the recovery of hydrophobicity over time.

However, the effect achieved by argon plasma treatment was stable throughout several weeks and a surface energy of 32 mN/m could be maintained during the investigated time period. In contrast to the initial surface energy of pristine PTFE of 15 – 20mN/m, this represents a significant increase in hydrophilicity by means of plasma treatment.

3.3 Cell compatibility of plasma treated surfaces

For the intended application of the plasma treatment process for the modification of vascular grafts in order to enhance adhesion of polymer film coatings for the controlled release of endothelial cell specific growth enhancers, an important

prerequisite is the cell compatibility of the plasma modified surfaces. Therefore, argon plasma activated PTFE samples were tested concerning their HUVEC compatibility in an in vitro setting. Unmodified and plasma modified PTFE samples were incubated with endothelial cells for 3 days and cells were subsequently visualized according to 2.2.3. In addition, control samples of HUVECs were grown on collagen coated well plates and treated accordingly. Cells were analyzed concerning cell morphology, cell viability, and proliferation on the disc.

(a) (b)

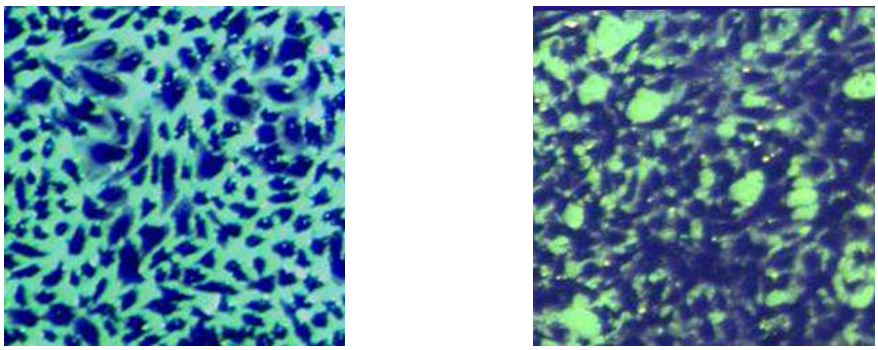


Figure 2-10: HUVEC compatibility and growth on collagen coated well plate (a) as control and argon plasma treated PTFE disc (b).

Figure 2-10 shows pictures of the stained cells after a three day incubation period. A confluent monolayer of endothelial cells grown on the argon plasma treated PTFE discs was found. Cell viability and proliferation were comparable to cells grown on collagen coated control wells. Unmodified PTFE discs did not support endothelial cell attachment and cell growth by any means (data not shown). These findings are in accordance to studies reported in literature that demonstrated the non adherence of endothelial cells with increasing hydrophobicity of the polymer [38], and an increase in cell growth and attachment could be shown for plasma modified polymer surfaces [39].

3.4 Film adhesion

In order to investigate the film adhesion of collagen and PLGA films on plasma treated and untreated PTFE discs, films were prepared according to 2.2.4. After

coating of the surfaces, the film adhesion was investigated by visual inspection and rated according to the following scaling: Films that exhibited very good adhesion of close to 100% of the film on the PTFE surface were rated as “++”. In case minor ablation of the films occurred around the edges of the PTFE discs, the film adhesion was considered good and rated as “+”. In case of poor film adhesion with more than just the edges of the film detaching from the surface, the adhesion was considered poor and was rated as “-“. In case more than 50% of the film did not adhere to the surface, the adhesion was considered very poor and was rated as “--“.

Table 2-1: Evaluation of PLGA and collagen film adhesion on plasma treated and untreated PTFE discs. (++ = very good adhesion; + = good adhesion; - = poor adhesion and -- = very poor adhesion).

	plasma treated PTFE	untreated PTFE
RG 503	++	--
RG 503H	++	--
0.5% collagen	+	--
1% collagen	+	--

Table 2-1 provides an overview of the results obtained for the investigated collagen and PLGA materials on PTFE discs. Both PLGA materials exhibited very poor adhesion on the untreated PTFE surfaces, in both cases showing ablation of more than 50% of the film applied on the surface. However, after plasma treatment, both PLGA materials exhibited very good adhesion to the plasma modified polymer surface. Collagen exhibited very poor adhesion, irrespective of the dispersion concentration on untreated PTFE surfaces with more than 50% of the film detaching after drying, and in some cases showing complete ablation of the film. After plasma treatment of the PTFE surface collagen materials exhibited a good adhesion with only minor ablation visible at the edges of the coated discs.

These results demonstrate the increased coating adhesion of PLGA and collagen films on plasma modified PTFE surfaces. Plasma treatment of polymer surfaces, in

this case PTFE, has been proven to be an easy and cost effective approach to enhance the adhesion of coatings on modified surfaces in a lab setting.

4 Conclusions

The aim of this study was the implementation of the plasmabrush® for plasma modification of PTFE substrates. The main goal was the investigation of a fast and straightforward method to modify the polymer with the aim of enabling coating accessibility. It was of interest to characterize the changes induced by the atmospheric plasma treatment by means of SEM, ATR-FTIR, XPS, and surface energy determination. In the process of this study, a plasma treatment process for making PTFE more hydrophilic and accessible for coating was successfully developed. The effect characterized by surface energy determination was stable for several weeks. It was possible to increase the surface energy of PTFE samples by 10-15 mN/m depending on process conditions. The characterization of changes induced by atmospheric argon plasma, by means of the other methods than surface energy determination, did not show any variations. The improved hydrophilicity and wettability of the polymer was unlikely to result from surface roughening, since SEM revealed no differences in surface morphology for investigated samples. Furthermore, the introduction of functional groups containing oxygen or nitrogen species by the treatment process could not be confirmed using ATR-FTIR as well as XPS, which did not reveal any chemical alteration on the outer surface. These methods, however, might have not been sensitive enough to detect the changes induced by the atmospheric plasma treatment.

The established plasma activation process significantly increased the coating adhesion of PLGA and collagen films applied on the modified surfaces in contrast to the non adhesion of these films on unmodified PTFE surfaces. In addition, it was shown that the plasma modification greatly improved the adherence of endothelial cells on the plasma treated surfaces. Therefore, the atmospheric plasmabrush® was shown to be an easy and efficient approach to modify surfaces for coating in a lab setting.

5 References

1. Ramakrishna, S., et al., *Biomedical applications of polymer-composite materials: a review.* Composites Science and Technology, 2001. **61**(9): p. 1189-1224.
2. Ratner, B.D., *Biomaterials Science: Overview and Opportunities with Special Reference to Organic and Polymeric Glow Discharge Plasma Treatments*, in *Plasma Processing of Polymers*, R. d'Agostino, P. Favia, and F. Fracassi, Editors. 1997, Kluwer Academic Publishers. p. 453-464.
3. Ikada, Y., *Surface modification of polymers for medical applications.* Biomaterials, 1994. **15**(10): p. 725-736.
4. Liston, E.M., L. Martinu, and M.R. Wertheimer, *Plasma surface modification of polymers for improved adhesion: a critical review*, in *Plasma Surface Modification of Polymers: Relevance to Adhesion*, M. Strobel, C. Lyons, and K.L. Mittal, Editors. 1993, VSP: Utrecht, The Netherlands. p. 3-39.
5. Dayss, E., G. Leps, and J. Meinhardt, *Surface modification for improved adhesion of a polymer-metal compound.* Surface and Coatings Technology, 1999. **116-119**: p. 986-990.
6. Mathieson, I. and R.H. Bradley, *Improved adhesion to polymers by UV/ozone surface oxidation.* International Journal of Adhesion and Adhesives, 1996. **16**(1): p. 29-31.
7. Manso, M., et al., *Tailoring surface properties of biomedical polymers by implantation of Ar and He ions.* Acta Biomaterialia, 2005. **1**(4): p. 431-440.
8. Wang, C. and J.-R. Chen, *Studies on surface graft polymerization of acrylic acid onto PTFE film by remote argon plasma initiation.* Applied Surface Science, 2007. **253**(10): p. 4599-4606.
9. Hegemann, D., H. Brunner, and C. Oehr, *Plasma treatment of polymers for surface and adhesion improvement.* Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms, 2003. **208**: p. 281-286.
10. Gomathi, N. and S. Neogi, *Surface modification of polypropylene using argon plasma: Statistical optimization of the process variables.* Applied Surface Science, 2009. **255**(17): p. 7590-7600.
11. Shenton, M.J. and G.C. Stevens, *Surface modification of polymer surfaces: atmospheric plasma versus vacuum plasma treatments.* Journal of Physics D: Applied Physics, 2001. **34**(18): p. 2761-2768.
12. Huang, C.-Y. and C.-L. Chen, *The effect of plasma surface modification from a rotary plasma reactor on the styrene grafting onto a polypropylene surface.* Surface and Coatings Technology, 2002. **153**(2-3): p. 194-202.
13. Park, J.-k., et al., *Pre-treatments of polymers by atmospheric pressure ejected plasma for adhesion improvement.* Surface and Coatings Technology, 2003. **174-175**: p. 547-552.
14. Clouet, F. and M.K. Shi, *Interactions of polymer model surfaces with cold plasmas: Hexatriacontane as a model molecule of high-density polyethylene and octadecyl octadecanoate as a model of polyester. I. Degradation rate versus time and power.* Journal of Applied Polymer Science, 1992. **46**(11): p. 1955-1966.
15. Inagaki, N., K. Narushima, and T. Yamamoto, *Surface modification of tetrafluoroethylene-perfluoroalkyl vinylether copolymer (PFA) by plasmas for copper metallization.* Journal of Applied Polymer Science, 2002. **85**(5): p. 1087-1097.
16. Momose, Y., et al., *Chemical reactivity between Teflon surfaces subjected to argon plasma treatment and atmospheric oxygen.* Journal of Vacuum Science & Technology A: Vacuum, Surfaces, and Films, 1992. **10**(1): p. 229-238.

17. Gesche, R., R. Kovacs, and J. Scherer, *Mobile plasma activation of polymers using the plasma gun.* Surface and Coatings Technology, 2005. **200**(1-4): p. 544-547.
18. König, U., et al., *Stability and ageing of plasma treated poly(tetrafluoroethylene) surfaces.* Colloids and Surfaces B: Biointerfaces, 2002. **25**(4): p. 313-324.
19. Vandencasteele, N. and F. Reniers, *Plasma modified polymer surfaces: characterization using XPS.* Journal of Electron Spectroscopy and Related Phenomena, 2009. **In Press, Accepted Manuscript**.
20. Junkar, I., et al., *Influence of oxygen and nitrogen plasma treatment on polyethylene terephthalate (PET) polymers.* Vacuum, 2009. **84**(1): p. 83-85.
21. Seidel, C., et al., *Ar plasma treated and Al metallised polycarbonate: a XPS, mass spectroscopy and SFM study.* Applied Surface Science, 1999. **150**(1-4): p. 19-33.
22. Paynter, R.W., *Angle-resolved XPS study of the effect of x-radiation on the aging of polystyrene exposed to an oxygen/argon plasma.* Surface and Interface Analysis, 2002. **33**(1): p. 14-22.
23. Noeske, M., et al., *Plasma jet treatment of five polymers at atmospheric pressure: surface modifications and the relevance for adhesion.* International Journal of Adhesion and Adhesives, 2004. **24**(2): p. 171-177.
24. Shenton, M.J., et al., *Chemical-surface modification of polymers using atmospheric pressure nonequilibrium plasmas and comparisons with vacuum plasmas.* Journal of Polymer Science Part A: Polymer Chemistry, 2002. **40**(1): p. 95-109.
25. Bismarck, A., et al., *Effects of surface plasma treatment on tribology of thermoplastic polymers.* Polymer Engineering & Science, 2008. **48**(10): p. 1971-1976.
26. Fang, Z., et al., *Polytetrafluoroethylene surface modification by filamentary and homogeneous dielectric barrier discharges in air.* Applied Surface Science, 2009. **255**(16): p. 7279-7285.
27. Liu, H., H. Zhang, and J. Chen, *Surface analysis of long-distance oxygen plasma sterilized PTFE film.* Applied Surface Science, 2009. **255**(18): p. 8115-8121.
28. Pascu, M., C. Vasile, and M. Gheorghiu, *Modification of polymer blend properties by argon plasma/electron beam treatment: surface properties.* Materials Chemistry and Physics, 2003. **80**(2): p. 548-554.
29. Vallon, S., et al., *Improvement of the adhesion of silica layers to polypropylene induced by nitrogen plasma treatment.* Thin Solid Films, 1996. **290-291**: p. 68-73.
30. Lugscheider, E. and K. Bobzin, *The influence on surface free energy of PVD-coatings.* Surface and Coatings Technology, 2001. **142-144**: p. 755-760.
31. Valk, P.v.d., et al., *Interaction of fibroblasts and polymer surfaces: relationship between surface free energy and fibroblast spreading.* Journal of biomedical materials research, 1983. **17**(5): p. 807-817.
32. Owens, D.K. and R.C. Wendt, *Estimation of the surface free energy of polymers.* Journal of Applied Polymer Science, 1969. **13**(8): p. 1741-1747.
33. Kereszturi, K., et al., *Nitrogen plasma-based ion implantation of poly(tetrafluoroethylene): Effect of the main parameters on the surface properties.* Applied Surface Science. **In Press, Corrected Proof**.
34. Liu, C., et al., *Effects of DBD plasma operating parameters on the polymer surface modification.* Surface and Coatings Technology, 2004. **185**(2-3): p. 311-320.
35. Tu, C.-Y., et al., *Expanded poly(tetrafluoroethylene) membrane surface modification using acetylene/nitrogen plasma treatment.* European Polymer Journal, 2005. **41**(10): p. 2343-2353.

36. Shenton, M.J., M.C. Lovell-Hoare, and G.C. Stevens, *Adhesion enhancement of polymer surfaces by atmospheric plasma treatment.* Journal of Physics D: Applied Physics, 2001. **34**: p. 2754-2760.
37. Hyun, J., et al., *Effect of Ar^+ ion beam in the process of plasma surface modification of PET films.* Journal of Applied Polymer Science, 2000. **77**(8): p. 1679-1683.
38. Wachem, P.B.v., et al., *The influence of protein adsorption on interactions of cultured human endothelial cells with polymers.* Journal of biomedical materials research, 1987. **21**(6): p. 701-718.
39. Pu, F.R., et al., *Effects of plasma treated PET and PTFE on expression of adhesion molecules by human endothelial cells in vitro.* Biomaterials, 2002. **23**(11): p. 2411-2428.

Chapter 3

Investigation of PLGA films for controlled release applications

Abstract

The use of poly(lactic-co-glycolic acid) (PLGA) for protein drug carrying film coatings of vascular grafts to enhance endothelialization was investigated. The in-vitro release of Fluorescein isothiocyanat (FITC) dextran as a model compound from PLGA coated discs was evaluated and the influence of the properties of a group of commercially available polymers was studied. Investigations revealed the dependency of the release rate on molecular weight and esterification of carboxylic end groups, as previously described. In addition, the independency of the release rate from the particle size of model compound suspended in the coating solution, especially in regards to the burst release, could be shown.

Furthermore, the cell compatibility of PLGA coatings was shown using human umbilical vein endothelial cells (HUVECs) and cell quantification methods were evaluated. Fluorescence Activated Cell Sorting (FACS) and Propidium Iodide staining were found unsuitable to reproducibly detect cell growth differences in the desired range. Cell Titer-Blue®, a commercially available cell viability test, was found suitable to successfully detect differences.

Keywords: PLGA, FITC-dextran, vascular graft, endothelial cell, Cell Titer-Blue®

1 Introduction

Synthetic polymers are widely utilized in the fields of controlled release applications and biomaterials. Commonly used polymers include poly(amides), poly(amino acids), poly(alkyl-α-cyanoacrylates), poly(acrylamides), and poly(esters) [1]. Amongst them, the aliphatic poly(esters) polylactid (PLA), polyglycolid (PGA), and especially PLGA are of great interest due to their good biocompatibility and biodegradability [2-4]. These polymers have been used in a multitude of applications for controlled release of active ingredients, in form of micro- and nanoparticles [5-6], film coatings [7-8], and scaffolds [9]. PLGA, PLA and PGA were first used as absorbable sutures, clamps, and meshes [10] before their relevance for controlled release applications was discovered. This type of polymers are synthesized via condensation from lactic acid and glycolic acid for lower molecular weight polymers and ring opening polymerization of dilactide and diglycolide for the higher molecular weight species [11].

The swelling, the water uptake, the degradation, and therefore, the release properties can be tailored to the needs of its application. The mechanism of degradation of these polymers is based on the hydrolysis of the polymer chains into smaller fragments that can be eliminated via the kidneys in case of D-lactic acid, or can be metabolized via the citric acid cycle in case of L-lactic acid and glycolic acid, and can be exhaled via the lungs in form of carbon dioxide [12]. The rate of hydrolytic degradation can be controlled by altering the physical properties, such as molecular weight, degree of crystallinity, or glass transition temperature (T_g) [13]. In addition, the carboxylic end groups of the polymer can be esterified using long chain alcohols in order to obtain end capped polymers with a more hydrophobic character, in contrast to the more hydrophilic properties of the non-end-capped varieties, which then leads to changed release properties [14]. In general, all these modifications have an influence on the rate and extent of water penetration into the polymer structures, which are critical for the rate of degradation [8, 15], and the diffusion or erosion controlled release of incorporated compounds. It has been shown that e.g. the rates are decreased for pure crystalline poly-L-lactide and polyglycolide, and

that the process accelerates with an increasing ratio of glycolide to lactide in the copolymer and increasing hydrophilicity [16-17].

In this study, the influence of polymer molecular weight and the esterification of carboxylic functional groups on the release rates of a model compound (FITC-dextran) from PLGA films reflecting potential coatings on vascular grafts, were investigated. Release rates of incorporated compound of 10 - 14 days were desired to enhance endothelialization in the early stages after implantation to overcome complications, such as intimal hyperplasia. Cell compatibility of the established PLGA coatings is required to be highly beneficial for cell growth and was evaluated. Detection methods for the quantification of endothelial cell growth, such as FACS, propidium iodide staining, and the use of a cell viability assay, Cell Titer-Blue®, were established and applied.

2 Materials and Methods

2.1 Materials

FITC-dextran with a molecular weight of 40 kDa was purchased from Sigma Aldrich (Steinheim, Germany), dichloromethane and cover glasses from VWR (Darmstadt; Germany), Resomer® (RG 502H, RG 503, RG 503H and RG 504H) from Boehringer Ingelheim (Ingelheim, Germany), PTFE from GM GmbH (Freiham, Germany), Falcon tubes from Greiner (Frickenhausen, Germany), disposable Plastibrand® PMMA plastic cuvettes from Brand (Wertheim, Germany), collagenase A from Roche (Penzberg, Germany), endothelial cell growth medium from Provitro (Berlin, Germany), heat-inactivated fetal bovine serum from Biochrom (Berlin, Germany), buffered formaldehyde solution (4%) from Polysciences, Inc. (Warrington, PA, USA), propidium iodide from Fluka (Steinheim, Germany), 0.1 M sodium hydroxide solution from Merck KGaA (Darmstadt, Germany), Trypsin from PAN-Systems GmbH (Aidenbach, Germany), ethylenediaminetetraacetic acid (EDTA) from Carl Roth GmbH & CO. KG (Karlsruhe Germany), and Cell Titer-Blue® from Promega (Madison, WI, USA).

The pH of the solutions was adjusted using hydrochloric acid or sodium hydroxide from Merck KGaA (Darmstadt, Germany) and measured with a pH meter Inolab level 1 from WTW (Weilheim, Germany).

2.2 Methods

2.2.1 FITC-dextran size reduction

2.2.1.1 Swing mill

FITC-dextran with a molecular weight of 40 kDa was milled under the exclusion of light in a MM200 swing mill (Retsch GmbH, Hahn, Germany) in dichloromethane (VWR, Darmstadt; Germany) with a frequency of 30 s^{-1}. The resulting FITC-dextran suspension was used for size analysis or coating and release experiments.

2.2.1.2 Spray drying

FITC-dextran 40 kDa was dissolved in water at a concentration of 1 mg/ml. The solution was spray dried using a Büchi Nano Spray Dryer B-90 (Büchi Labortechnik AG, Flawil, Switzerland). Spray dried FITC-dextran was stored in a desiccator under the exclusion of light until analysis or further use.

2.2.2 Morphological analysis

2.2.2.1 Scanning electron microscopy

Samples for scanning electron microscopy (SEM) were fixed on specimen holders using conductive double sided tape before sputtering with a thin layer of carbon under vacuum. SEM images were obtained using a Philips XL Series XL20 (Philips, The Netherlands).

2.2.2.2 Laser diffraction

Particle sizes were determined using a Horiba Laser Diffraction Particle Size Distribution Analyzer LA-950 (Retsch Technology GmbH, Hahn, Germany). Therefore samples were dispersed in dichloromethane.

2.2.3 PLGA coating procedure

Different species of Resomer® (RG 502H, RG 503, RG 503H and RG 504H) were used to coat PTFE-discs, argon plasma activated with a plasmabrush® (Reinhausen Plasma GmbH, Regensburg, Germany), or cover glasses, both with a diameter of 15 mm. Therefore, the discs were placed in a 24-well aluminum well plate and covered with different concentrations of the PLGA-species dissolved in dichloromethane. The discs were air dried for one hour and subsequently removed from the wells and dried under vacuum over night or until further use. For the release studies, 0.01% (w/w) FITC-dextran as a model compound was suspended in the PLGA solutions. For the cell culture studies all these procedures were performed under a laminar flow workbench (Thermo, Langenselbold, Germany).

2.2.4 In-vitro release studies

For the release analysis, triplicates of coated discs were incubated in 10.0 ml phosphate buffered saline (PBS) pH 7.4 in 50 ml Falcon tubes in a water bath (Haake SWB25, Haake, Karlsruhe, Germany) at 37°C and at 25 rpm horizontal shaking under exclusion of light. 2 ml samples were drawn and replaced with fresh PBS buffer at several time points. Released FITC-dextran was quantified using fluorescence spectroscopy, which was performed using a Varian Cary Eclipse fluorescence spectrometer (Varian GmbH, Darmstadt, Germany). Therefore, samples were measured in disposable Plastibrand® PMMA plastic cuvettes with an excitation wavelength of 495 nm, an emission wavelength of 517 nm, and were adjusted with a PBS buffer blank. Samples were diluted using PBS buffer, if necessary. The amount of released FITC-dextran was calculated using a calibration curve.

2.2.5 Cell compatibility testing

The cell compatibility of PLGA coated discs was evaluated using HUVECs. HUVECs were prepared by digestion of umbilical veins with 0.1 g/l collagenase A. Cells were cultured in endothelial cell growth medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere at 5% CO_2 and 37°C. Cells were used at passage no. 3. For visualization of cells on the surfaces, cells were fixed with a buffered formaldehyde solution (4%) and were stained with propidium iodide in PBS buffer with a final concentration of 50 µg/ml. Images were obtained with a Zeiss LSM 510 confocal laser scanning microscope (CLSM) (Zeiss, Oberkochen, Germany).

2.2.6 Evaluation of cell quantification methods

2.2.6.1 Propidium iodide dye

For quantification of cells on the surfaces, cells were fixed with a buffered formaldehyde solution (4%) and were stained with propidium iodide in PBS buffer. After rinsing, propidium iodide was quantified via fluorescence spectroscopy using a Spectrafluor plus plate reader (Tecan, Crailsheim, Germany) with an excitation wavelength of 530 nm and emission recording at 635 nm. The dye was quantified in its bound state on the vacuum dried discs or an aliquot of the eluted dye after incubation with 0.1 M sodium hydroxide solution (NaOH) at 2-8°C overnight.

2.2.6.2 Fluorescence activated cell sorting (FACS)

For quantification of cells, incubated discs with cells were washed three times with PBS buffer pH 7.4. Subsequently, cells were covered with 300 µl of Trypsin/EDTA in PBS buffer, containing 0.05% Trypsin and EDTA. Discs were then incubated in a water bath at 37°C for 8 min. 250 µl of the cell suspension were added to 150 µl of a buffered formaldehyde solution (4%). 120 µl of this cell suspension were quantified using the BD FACSCanto II in High Throughput Screening (HTS) mode (Becton Dickinson GmbH, Heidelberg, Germany).

2.2.6.3 Cell Titer-Blue®

Cells on the discs were quantified using Cell Titer-Blue®. Therefore, discs were incubated with Cell Titer-Blue® and an aliquot of the supernatant was analyzed using a Spectrafluor plus plate reader (Tecan, Crailsheim, Germany) with an excitation wavelength of 550 nm and emission recording at 595 nm.

3 Results and Discussion

3.1 FITC-dextran size reduction

All PLGA coatings needed to be prepared in organic solvents, due to the non-solubility of PLGA in aqueous solutions. Suitable solvents for PLGA are, amongst others, N-methyl-2-pyrrolidinone, ethyl acetate, acetone, dimethyl sulfoxide, and dichloromethane. The solvent of choice for all investigations within these studies was determined to be dichloromethane, due to the good solubility of PLGA in this solvent and the high vapor pressure of dichloromethane to enable fast and efficient drying. However, the model substrate used for the release investigations, FITC-dextran, is insoluble in dichloromethane or other organic solvents suitable for the preparation of PLGA solutions. Therefore, the preparation of FITC-dextran suspensions in PLGA solutions in dichloromethane for the coating of PTFE discs was required. In order to guarantee reproducible results for the coatings, homogenous size distributions of the FITC-dextran particle was required. Therefore, different techniques for particle size reduction of FITC-dextran were investigated.

3.1.1 Size reduction by milling

One suitable option for FITC-dextran particle preparation in dichloromethane is milling. The influence of milling time on the size reduction of FITC-dextran was investigated. Therefore, FITC-dextran in dichloromethane was milled for a period of 180 min and the size of the particles (d^{90}) was analyzed at several time points using laser light diffraction in order to determine a suitable milling time to achieve small

and homogenous particle sizes. The results shown in Figure 3-1 indicated a correlation between milling time and average particle size.

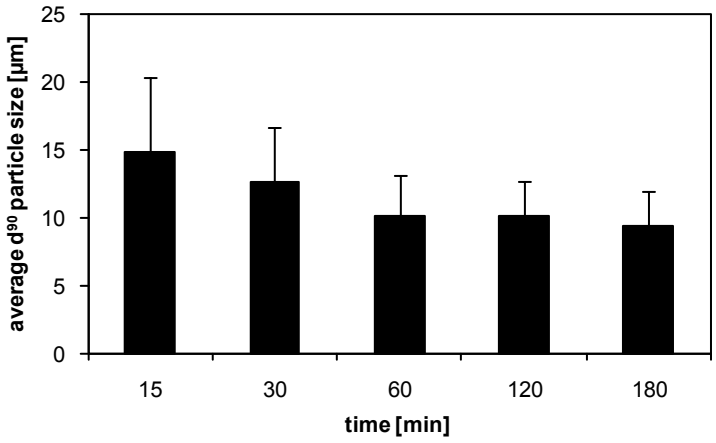


Figure 3-1: FITC-dextran particle size (d^{90}) in correlation to milling time. (average ± SD; n=3)

The particle size determined for FITC-dextran decreases with increasing milling time. After 15 min of milling, a particle size of approximately 15 µm was determined that decreased to 12 µm, and 10 µm after milling for 30 min and 60 min, respectively. A further increase in milling time only resulted in little further size reduction. Therefore, the standard milling time for the preparation of further milled FITC-dextran particles was set to 60 min in order to lower the temperature stress on the samples and the risk of degradation due to accidental exposure to light of the model compound.

3.1.2 Size reduction using spray drying

In order to evaluate the influence of FITC-dextran particle size on the release rates from PLGA films, it was necessary to obtain a second set of FITC-dextran particles with a smaller particle size as achieved by milling. Therefore, a solution of FITC-dextran in water with a concentration of 1 mg/ml was spray dried using the Büchi Nano Spray Dryer B-90. Spray-drying was conducted under the exclusion of light in order to guarantee the stability of FITC-dextran. However, the increase in

temperature during the spray drying process, in addition to some light exposure during the preparation and collection of spray dried particles, might have affected the stability of FTIC-dextran. Therefore, samples of spray dried FITC-dextran were analyzed using fluorescence spectroscopy, according to the procedures described in 2.2.4, to investigate any changes in excitation or emission wavelength, as well as, decreases in fluorescence intensity compared to standard samples that did not undergo spray drying. Findings (data not shown) confirmed that the spray drying process, as well as, accidental exposure to light did not have a negative effect on the stability and fluorescence properties of the fluorophore of FITC-dextran.

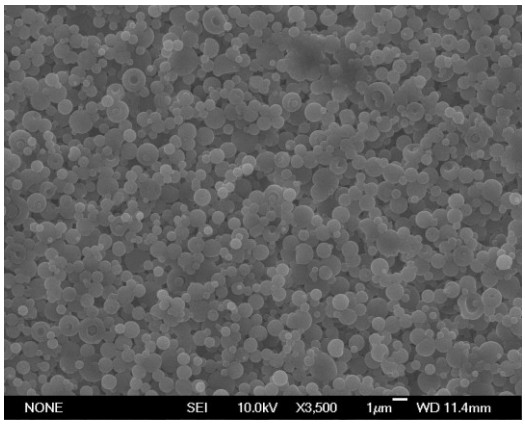


Figure 3-2: Scanning electron micrograph of spray dried FITC-dextran particles.

Samples were analyzed using SEM to investigate the size distribution and particle shape of the FITC-dextran particles. Figure 3-2 shows a representative particle population for a spray dried sample. A homogenous particle distribution in a size range of 1 µm could be detected. The majority of particles were of spherical shape, but some doughnut shaped particles could be observed. Therefore, spray drying can be considered a suitable method to generate FITC-dextran particles with size distributions in the low µm-range.

3.2 In vitro release studies

The release rates of model compound from the generated PLGA films are highly dependant on the polymer used for their production and its characteristic physical

and chemical properties, such as molecular weight, modification of the terminal carboxylic group of the polymer, crystallinity, and the lactide:glycolide ratio [18]. In order to investigate their effect on the release rates of formed films, several polymers varying in molecular weight and esterified end groups were used for the release investigations. Polymers with a low molecular weight were the focus of these investigations in order to guarantee a release of model compound in a one to two week time period.

3.2.1 Influence of PLGA molecular weight on release rates

The influence of molecular weight on the release of the model compound FITC-dextran was investigated using the PLGAs Resomer® RG 502H, RG 503H, and RG 504H. The molecular weights, as well as, the inherent viscosities of these PLGAs can be seen in Table 3-1. In general, release profiles from biodegradable monolithic systems are a combination of several phases depending on the dominating process. Predominantly, biphasic release patterns can be observed, which are characterized by a strong burst release of drug or model compound that are deposited near the film or microparticle surface and are caused by their dissolution [19]. It is followed by the second phase of release which is usually diffusion controlled and is characterized by sustained release that can be described by zero order kinetics [20].

Table 3-1: Overview of molecular weight, inherent viscosity and thermal properties of PLGA variants used during release investigations [21-23].

Polymer	M_w [Da]	Inherent viscosity [dl/g]	T_g [°C]
Resomer® RG 502H	13,500	0.16 – 0.24	42 – 46
Resomer® RG 503H	36,000	0.32 – 0.44	44 – 48
Resomer® RG 503	36,000	0.32 – 0.44	44 – 48
Resomer® RG 504H	48,000	0.45 – 0.60	46 – 50

The release profiles for the investigated films in PBS buffer pH 7.4 at 37°C can be seen in Figure 3-3. Films made from Resomer® RG 502H were characterized by an initial burst release of approximately 20% (compare Figure 3-3a), followed by a continuous release of 100% FITC-dextran up to 14 days. Films made with Resomer® RG 503H (Figure 3-3b) exhibited the same level of burst release, however, the following sustained release was characterized by a slower release rate and complete release of almost 100% model compound took place within 21 days. Resomer® RG 504H films showed a higher burst release compared to the lower molecular weight PLGAs of approximately 40%, followed by a continuous release of model compound that was investigated for 4.5 weeks and afterwards aborted, due to the unsuitability of the release profile for the intended application with a release of model compound within a period of one to two weeks. It was hypothesized that the higher inherent viscosity of the higher molecular weight species of PLGA was responsible for a lower sedimentation rate of the FITC-dextran particles within the coating solution, which resulted in higher amounts of model compound close to the film surface, leading to higher initial burst release rates.

To further elucidate this phenomenon, investigations using different FITC-dextran particle sizes in the coating solution were performed to be able to clarify the hypothesis of lower sedimentation rates in the higher viscous coating solutions (compare 3.2.3). It was shown, that the viscosity of both RG 503H and RG 502H were not high enough to circumvent sedimentation of FITC-dextran, which led to a depletion of model compound on the surface and resulted in lower burst release.

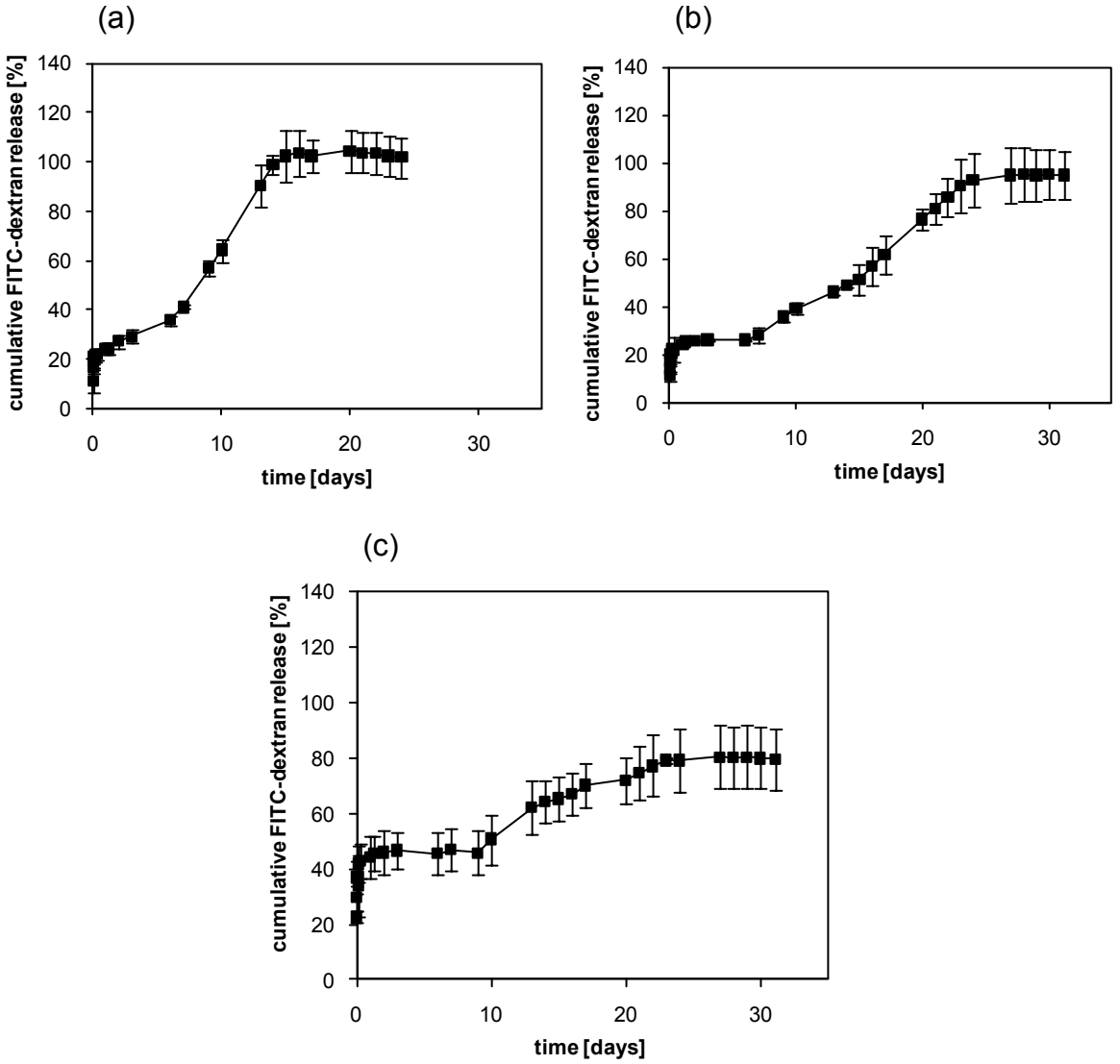


Figure 3-3: : Cumulative in-vitro FITC-dextran release from PLGA coated discs RG 502H (a), RG 503H (b) and RG 504H (c). (average ± SD; n=3)

The observed release profiles were in accordance with reported biphasic release profiles for PLGA based drug delivery systems [20, 24]. Water penetration into the system leads to release by dissolution of surface bound compound and diffusion of molecules through water filled pores [19]. Specifically for lower molecular weight PLGA species, release based on diffusion takes place much faster in comparison to high molecular weight PLGAs [25]. Correspondingly, a decrease in continuous release was observed in dependency of the increase of molecular weight. In addition to the lower rate of diffusion, this is due to the water penetration into the

PLGA films that causes the hydrolysis of the polymer. Hydrolysis causes the polymer to be degraded to smaller molecular weight species which upon reaching a certain level of molecular weight become water soluble and erosion of the matrix takes place, thereby releasing incorporated compounds [18], in this case FITC-dextran. Due to the difference in molecular weight and consequently longer polymer chain units for the higher molecular weight PLGAs, the degradation of these units in water soluble PLGA fragments is more time consuming compared to the low molecular weight PLGA Resomer® RG 502H. This effect caused the observed slower release rates for the continuous sustained release phase of the higher molecular weight PLGAs which, in turn, lead to longer total release times. Therefore, only the PLGA Resomer® RG 502H films, with a total release within the first 14 days of incubation, were within the desired period of release of one to two weeks.

3.2.2 Influence of esterification on release rates

In addition to the investigations concerning the influence of molecular weight differences of the applied PLGAs on the release rates, the influence of end capping of the carboxylic group of Resomer® RG 503 / 503H was studied. Therefore, PTFE-discs were coated with solutions of each polymer containing 0.01% (w/w) FITC-dextran and investigated concerning the release in vitro in PBS buffer pH 7.4 at 37°C. The release profiles for the investigated films can be seen in Figure 3-4. Both PLGA species exhibit a burst release of approximately 25% of the loaded FITC-dextran, followed by a continuous sustained release for Resomer® RG 503H up to day 21 when 100% of the model compound was liberated. However, Resomer® RG 503 exhibited a lag phase of release after the initial burst for approximately 20 days followed by a fast release of another 50% of FITC-dextran.

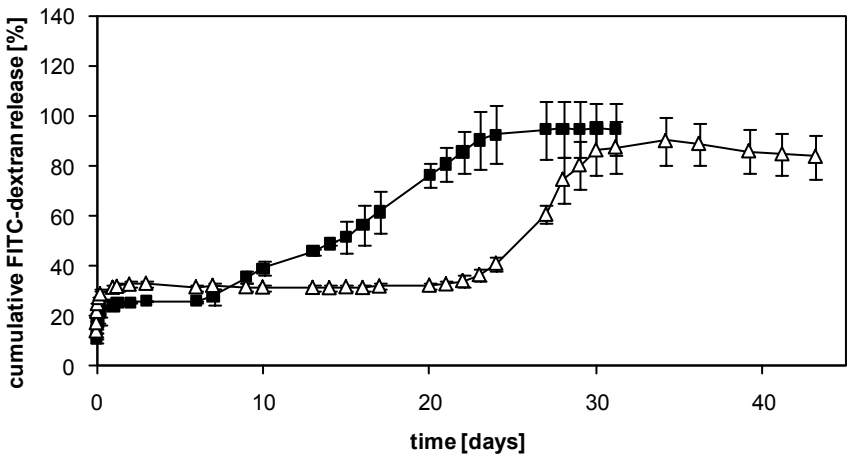


Figure 3-4: Cumulative in-vitro FITC-dextran release from RG503 coated discs with endcapped carboxylic group (—△—) and free carboxylic group (—■—). (average ± SD; n=3).

These findings are in accordance with results reported in literature for end capped polymers and polymers with free carboxylic groups [5]. The sigmoidal release profile for the films made of Resomer® RG 503 indicates bulk erosion of the polymer [26], as well as, diffusion controlled release of the model compound. As mentioned before (see 3.2.1), the penetration of water into the polymer is responsible for the hydrolysis of the polymer into smaller fragments that are required for achieving water solubility [27-28]. However, this penetration of water into the more hydrophobic polymer RG 503 is strongly reduced compared to the more hydrophilic polymer RG 503H due to the lack of hydroxyl and carboxylic groups that are present in RG 503H. This reduction of penetrating water leads to a significant decrease of hydrolysis of the polymer, which leads to slower erosion and in turn a decreased release of compound. In general, it can be stated that the water uptake of polymer increases with the hydrophilicity of the polymer, which enables more drug to be released and the polymer to be degraded faster [6]. Therefore, the release rate from the more hydrophic species of PLGA are characterized by a distinctive lag phase after the initial burst release as observed in the case of RG 503 and RG 503H.

3.2.3 Influence of FITC-dextran particle size on release rates

The results obtained when investigating the influence of molecular weight on the release rate of PLGA films (compare 3.2.1) led to the hypothesis of an influence of FITC-dextran particle size present in the coating suspension on the release rate. The initial burst release for the higher molecular weight PLGA Resomer® RG 504H was more pronounced in comparison to the two lower molecular weight species RG 502H and RG 503H potentially due to sedimentation of FTIC-dextran particles in the lower molecular weight PLGA solutions. According to Stokes, the rate of sedimentation in a suspension is dependant on the square of the particle diameter. Therefore, film coatings made of PLGA with FITC-dextran of two different size distributions were investigated. Resomer® RG 503 was used for these investigations with an inherent viscosity between RG 502H and RG 504H.

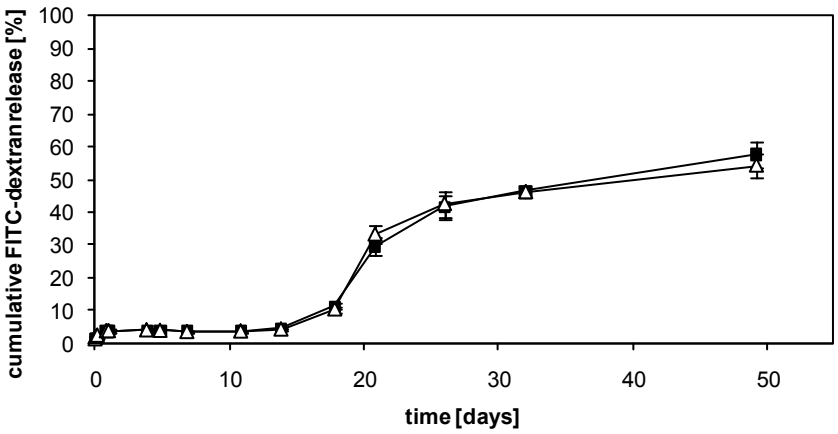


Figure 3-5: Cumulative in-vitro FITC-dextran release from PLGA RG 503 coated discs with FITC-dextran particle size of 1µm (—■—) and 10µm (—△—). (average ± SD; n=3).

The release rates shown in Figure 3-5 were identical for both sets of PLGA coatings with particles of 1 and 10 µm. Burst release was low with approximately 5% FITC-dextran released within the first 24 hours. After a pronounced lag phase characteristic for PLGAs with end capped carboxylic groups, release continued with a fast release of approximately 40%, followed by slow continuous release until the end of the investigation period. The findings did not support the hypothesis of a

reduced exposure of FITC-dextran particles at the surface of the coatings of 502H and 503H due to sedimentation.

A possible other explanation could be the difference in T_g of the polymers. The T_g for RG 502H and RG 503H is lower than that of RG 504H (compare Table 3-1). It is reported in literature that incubation of PLGA devices at temperatures above T_g leads to increased mobility of compounds, and diffusivity of both penetrating water and drug are higher [5, 17]. Consequently, faster hydrolysis and drug release are observed [29-30], which can be seen by the higher release rates after initial burst release for the lower molecular weight PLGAs. In addition, once T_g is exceeded, the initial glassy device converts to a rubbery state with hydration which makes polymer chain segments more mobile [17]. This higher polymer mobility might cause a sealing of the film surface leading to a reduced initial burst release in case of RG 502H and RG 503H upon exposure to the incubation solution at 37°C. In case of RG 504H, the incubation temperature is below the T_g and therefore the film remains in its glassy state leading to a higher initial burst release, because the surface sealing does not take place, followed by the continuous release controlled by diffusion and erosion.

3.3 Cell compatibility of PLGA coatings

In addition to their release characteristics, the cell compatibility of the different PLGA species was investigated. Cell compatibility is of great importance, especially for the intended use as biomaterial substrate to potentially enhance attachment, attraction, and endothelial cell growth, either by itself due to its properties, but also in its function as matrix for the release of for example endothelial cell specific growth enhancers, such as Vascular Endothelial Growth Factor (VEGF). PLGA constructs have been reported to be biocompatible, as well as, their degradation products lactic and glycolic acid [2, 4]. Therefore, it has been widely used in controlled release applications such as micro- and nanoparticles [5-6], film coatings [7-8], and scaffolds [9]. Two PLGA species, RG 503H representing the polymers of higher hydrophilicity due to its free carboxylic group and RG 503 a more hyrdrophobic polymer due to its esterified carboxylic group, were investigated

concerning their cell compatibility using HUVECs. PTFE discs were coated with either polymer, subsequently seeded with HUVECs and cells were allowed to attach and grow on the polymers and were stained using propidium iodide after 3 days. The stained cells were analyzed using CLSM concerning the cell morphology, cell viability, and proliferation on the discs.

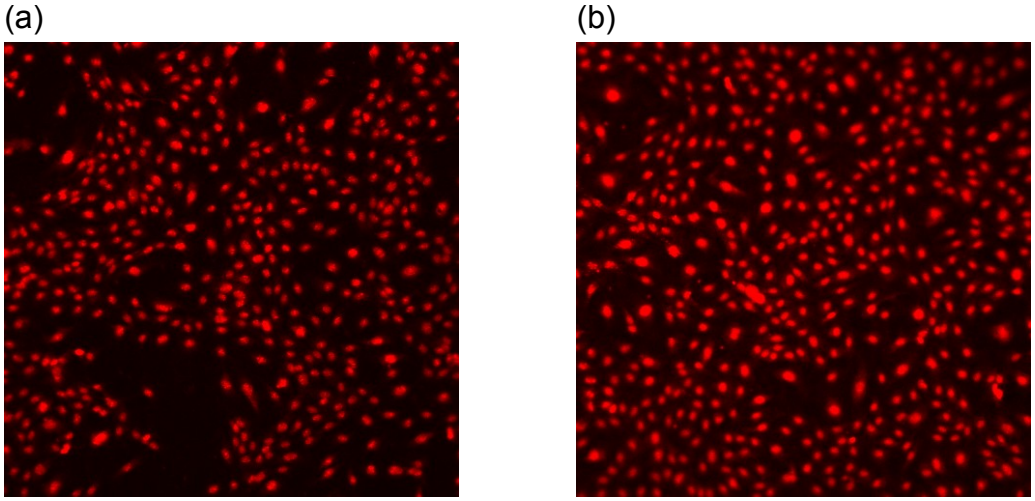


Figure 3-6: Propidium iodide stained cells on PLGA coated PTFE discs after incubation for 3 days on RG 503 (a) and RG 503H (b)

Figure 3-6 shows the acquired pictures for the stained cells after a three day incubation period. All pictures showed a confluent monolayer of endothelial cells grown on the PLGA films with a morphology attributed to HUVECs. Therefore, it can be concluded that the applied polymers, as well as the polymers used for other studies (RG 502H and RG 504H) had good cell compatibility, which was in accordance to prior findings.

3.4 Evaluation of cell quantification methods

For the intended application of the PLGA films as potential coating for vascular grafts to enhance endothelialization through the incorporation of cell growth specific growth enhancers, such as $VEGF_{165}$ [31], it was of importance to qualify methods of

quantification for the initiated cell growth on the PLGA film surfaces. Therefore, PTFE and glass discs were coated using PLGA, incubated with HUVECs, and quantified using several methods for cell quantification.

3.4.1 Cell quantification using FACS

For the cell quantification using FACS, different amounts of cells ranging from 100,000 to 120,000 cells per well were cultured on PLGA RG 502H coated discs and were allowed to adhere for 24 hours. Subsequently, cells were harvested using Trypsin/EDTA incubation for 8 min at 37°C. After the addition of buffered formaldehyde solution (4%), cells were quantified using the BD FACSCanto II in High Throughput Screening (HTS) mode. Cell numbers present in the measured aliquots were then calculated back to the numbers of cells harvested per well.

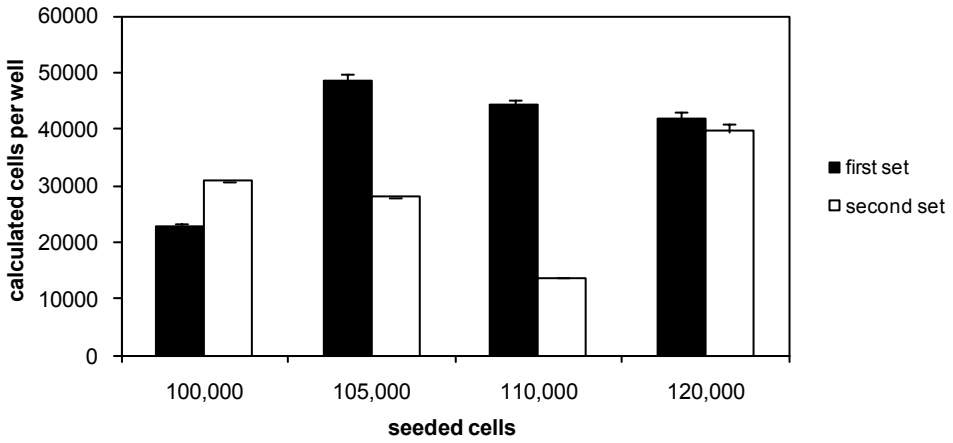


Figure 3-7: Calculated cells per well after adhesion on PLGA RG 502H coated discs and subsequent detachment using Trypsin / EDTA and FACS analysis. (average ± SD; n=2).

Figure 3-7 shows the calculated values of recovered cells after the harvesting process using Trypsin/EDTA and the FACS analysis for two sets of identically treated PLGA coated discs. Calculated values for one set of discs seeded with equal amounts of HUVECs showed a very low standard deviation indicating good reproducibility within one set of discs in one experiment. However, there was no correlation between the amount of seeded cells and the amount of recovered cells

for a concentration series investigated. For the first set, cell counts increased significantly from the discs incubated with 100,000 to 105,000 cells, however the recovered cell amounts for 110,000 and 120,000 seeded cells declined from thereon. In comparison, the second set of experiments showed different results, with a decrease of recovered cells after 100,000 seeded cells and a significant increase in recovered cell amounts for the discs seeded with 120,000 cells. Since no correlation between the amounts of cells seeded and the detected cell number was found, and even worse non-reproducibility between two sets of experiments was shown, this method of cell quantification was unsuitable for the quantification of HUVECs after growth on PLGA films. A possible cytotoxic effect of the coatings on HUVECs can be ruled out, since cell compatibility of the coatings was shown (compare 3.3). Low recovery and varying amounts of recovered cells might be explained by non adherence of cells during the incubation period resulting in a loss of cells during the washing procedure. In addition, an insufficient detachment of cells during the Trypsin/EDTA incubation time might have added to the low recovery of cells seeded. The incubation time was not further increased due to concerns of digestion of HUVECs by Trypsin.

3.4.2 Cell quantification using propidium iodide dye

Propidium iodide has already been shown to be a suitable detection method for the evaluation of cell compatibility of PLGA coatings (compare 3.3). Due to its fluorescence properties, it might also represent a possible alternative for cell quantification. Therefore, sets of 100,000 and 200,000 HUVECs were seeded on PLGA coated discs, incubated for 24 hours, and subsequently stained using propidium iodide. The amount of bound dye on the dried PLGA coated discs was then quantified using fluorescence spectroscopy. In addition, the dye was eluted using sodium hydroxid solution and was again measured using fluorescence spectroscopy.

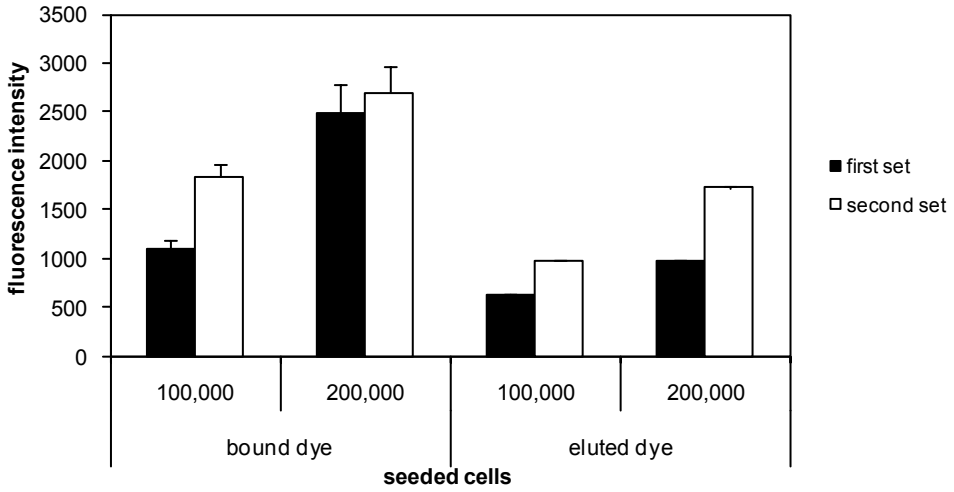


Figure 3-8: Fluorescence intensity of propidium iodide stained cells in bound state and after elution using 0.1 M sodium hydroxide solution. (average ± SD; n=2).

The results of the fluorescence measurements for both sets of investigations are shown in Figure 3-8. A fluorescence intensity difference of propidium iodide on discs incubated with 100,000 and 200,000 cells can be detected for both bound and eluted dye. A significant decrease in fluorescence was observed for the eluted dye in comparison to bound dye due to dilution and insufficient elution during the incubation procedure. However, reproducibility of these measurements with a second set of PLGA coated discs under the same conditions was not given as can be seen in Figure 3-8 (second set). Fluorescence intensity for all samples increased for all measured samples, making this an unsuitable method for cell quantification on PLGA surfaces. Again, low recovery and varying amounts of fluorescence might be explained by non adherence of cells during the incubation period resulting in a loss of cells during the washing procedure and hence, resulting in varying fluorescence intensities.

3.4.3 Cell quantification using a cell counting chamber

Since propidium iodide staining with subsequent fluorescence measurements failed to provide reproducible results for cell quantification on PLGA coated discs,

however, provided good properties for cell visualization on the surfaces, cell quantification using a cell counting chamber was evaluated. Therefore, cells were grown on PLGA RG 502H covered discs with varying cell amounts seeded per well. Cells were then subsequently stained using propidium iodide and evaluated using CLSM.

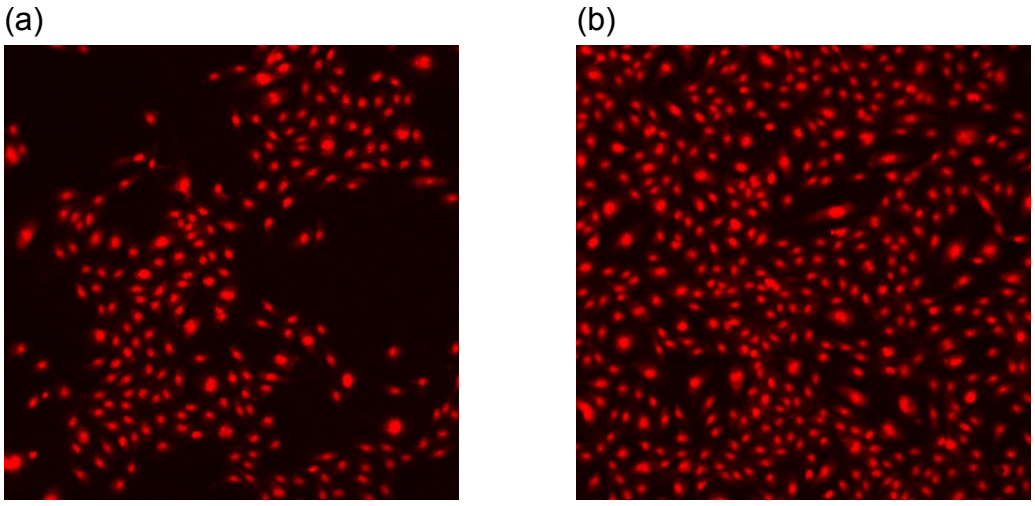


Figure 3-9: Propidium iodide stained cells on a PLGA RG 502H coated PTFE discs seeded with 100,000 cells after incubation for 3 days at center of disc (a) and border of disc (b).

Figure 3-9 shows representative exemplary CLSM pictures of PLGA coated discs seeded with 100,000 cells stained with propidium iodide. Similar results were obtained for all other discs incubated with varying amounts of cells. Figure 3-9a shows the center of the disc investigated with non confluent amounts of cells detected. In contrast, Figure 3-9b shows the border of the identical disc with high amounts of endothelial cells present. This inhomogeneity made it impossible to find representative areas on discs suitable for cell quantification by the use of a cell counting chamber. The patches of no-cell growth in the center of the disc substantiate the prior findings using other techniques for cell quantification. The non reproducibility of cell quantification was most likely due to the loss of cells grown on the PLGA surface, which detached after hydrolysis of the PLGA. Cells and PLGA fragments were then lost in the necessary washing steps that were performed for the different quantification methods, leading to non reproducible and unreliable results as seen above.

3.4.4 Cell quantification using Cell Titer-Blue®

In order to quantify cell growth on PLGA surfaces, a suitable method needed to be implemented that did not require washing steps after the incubation. Therefore, Cell Titer-Blue® a commercially available cell viability assay was evaluated. Cell Titer-Blue® is based on the metabolic capacity of cells an indicator of their viability.

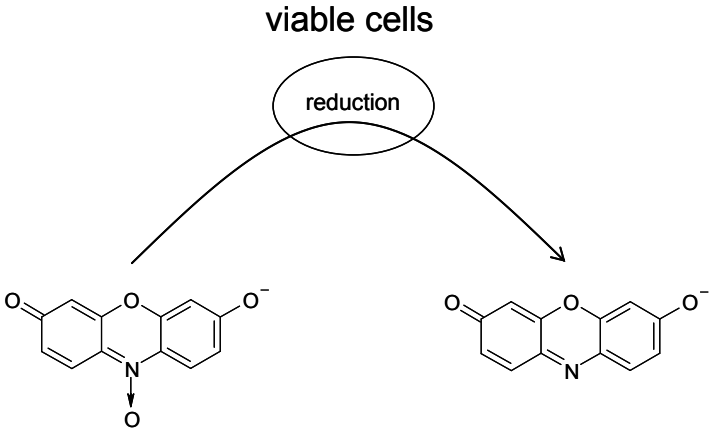


Figure 3-10: Conversion of resazurin to resorufin by metabolically active cells adapted from Promega [32]

The indicator dye resazurin is used to measure the metabolic capacity of cells. Cells reduce resazurin into resorufin, which is highly fluorescent (Figure 3-10) and can be detected at an excitation wavelength of 550 nm and an emission wavelength of 595 nm. Nonviable cells rapidly lose metabolic capacity, and therefore do not reduce the indicator dye, and thus do not generate a fluorescence signal.

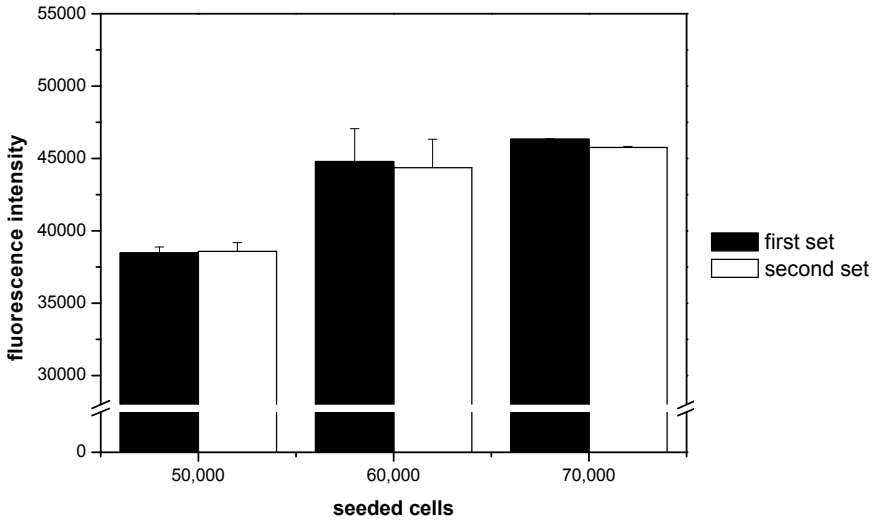


Figure 3-11: Fluorescence intensity of Cell Titer-Blue® after incubation for 6 hours on endothelial cell seeded PLGA RG 502H coated discs – broad cell number screening. (average ± SD; n=2).

In a first suitability screening, PLGA RG 502H coated discs were seeded with 50,000 to 70,000 cells per well and were cultured for 24 hours. After the incubation time Cell Titer-Blue® was added to the wells and further incubated for 6 hours, sufficient time for the cells to reduce the indicator dye. Subsequently, aliquots of the incubation solution were measured using fluorescence spectroscopy. In order to investigate reproducibility of this method, a second set of PLGA coated discs were treated accordingly. The results represented in Figure 3-11 showed an increase in fluorescence with an increasing amount of cells seeded on the discs with low standard deviations. However, the increase in fluorescence was not in correlation to the increase in seeded cells. Therefore, an absolute quantitative measurement did not seem possible, however, a relative quantification appeared possible. The second set of experiments confirmed the reproducibility of the experiment, making the quantification with Cell Titer-Blue® the method of choice for cell quantification. Nevertheless, since the increase or difference in cell growth expected for discs coated with PLGA and cell growth stimulant were anticipated to be in a range of 20%, further investigations were necessary to elucidate on the possibility of quantifying smaller amounts and differences in cell growth.

Therefore, another set of experiments was performed with a narrower distribution in cell numbers. PLGA coated discs were seeded with cells in a range of 20,000 to 30,000 cells and treated according to the above described procedure. Again, two sets of experiments were performed in order to gain information about the reproducibility of the quantification method.

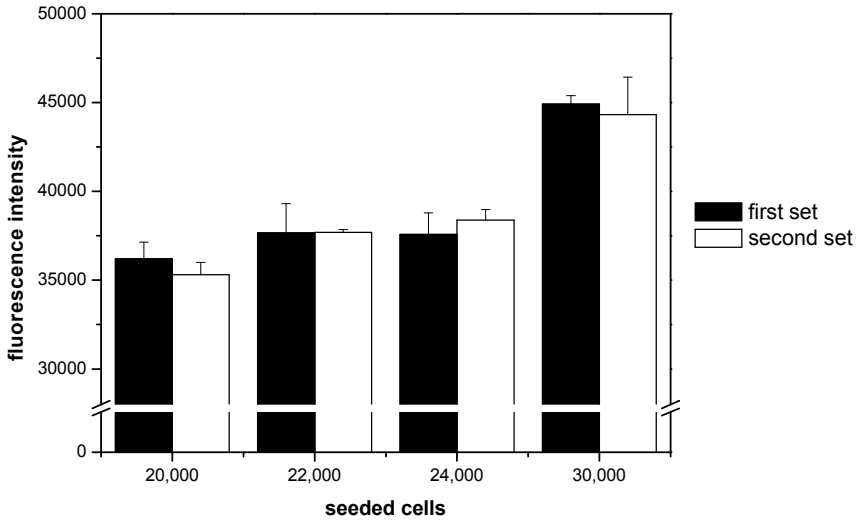


Figure 3-12: Fluorescence intensity of Cell Titer-Blue® after incubation for 6 hours on endothelial cell seeded PLGA RG 502H coated discs – narrow cell number screening. (average ± SD; n=2).

Employing the quantification method using Cell Titer-Blue® it was possible to differentiate between small amounts of cell growth differences with weaknesses in the range of 22,000 to 24,000 cells. Fluorescence of the incubated discs increased in accordance to the seeded cells (Figure 3-12). However, the correlation was not linear, making it a relative quantification method as described above. The reproducibility could be confirmed using the second set of experiments which showed results of similar fluorescence for the cell seeded incubated discs.

4 Conclusions

PLGA films were investigated for potential coating of vascular grafts to improve endothelialization. The incorporation of endothelial cell specific growth enhancers, such as $VEGF_{165}$, and its subsequent release from the matrix could be used to enhance the cell retention and cell growth on vascular grafts. Therefore, different PLGA species were investigated concerning their release properties using a model compound, FITC-dextran, with a molecular weight comparable to $VEGF_{165}$. The influence of the polymer molecular weight on the release rates was shown. Resomer® RG 502H, a low molecular weight species of a commercially available polymer, was shown to be the only suitable polymer in the range of polymers investigated. Its release rate suited the intended purpose and the desired release time period of 1 to 2 weeks. In addition, it was shown that the influence of esterification of free carboxylic groups of the used polymers did not have a beneficial effect on the release rates of the coatings. The influence of the suspended model compound particles on the coating process and the subsequent release was investigated and was shown to have no influence on the outcome of release investigations, especially in respect of burst release from such coatings in contrary to previously reported findings.

In addition, the cell compatibility of the applied PLGA coatings was shown using HUVECs. Detection methods to quantify the increase in cell growth on such coatings were investigated towards their ability to reveal small differences in cell growth. FACS analysis, propidium iodide staining in combination with fluorescence measurements, and using a counting chamber were shown to be unsuitable for this application. However, Cell Titer-Blue®, a commercially available cell viability assay, was found to be appropriate to detect minor changes in cell growth increase.

5 References

1. Jain, R.A., *The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices.* Biomaterials, 2000. **21**(23): p. 2475-2490.
2. Gao, J., L. Niklason, and R. Langer, *Surface hydrolysis of poly(glycolic acid) meshes increases the seeding density of vascular smooth muscle cells.* Journal of biomedical materials research, 1998. **42**(3): p. 417-424.
3. Anderson, J.M. and M.S. Shive, *Biodegradation and biocompatibility of PLA and PLGA microspheres.* Advanced Drug Delivery Reviews, 1997. **28**(1): p. 5-24.
4. Oh, S.H., et al., *Fabrication and characterization of hydrophilic poly(lactic-co-glycolic acid)/poly(vinyl alcohol) blend cell scaffolds by melt-molding particulate-leaching method.* Biomaterials, 2003. **24**(22): p. 4011-4021.
5. Friess, W. and M. Schlapp, *Release mechanisms from gentamicin loaded poly(lactic-co-glycolic acid) (PLGA) microparticles.* J. Pharm. Sci., 2002. **91**(3): p. 845-855.
6. Budhian, A., S.J. Siegel, and K.I. Winey, *Controlling the in vitro release profiles for a system of haloperidol-loaded PLGA nanoparticles.* International Journal of Pharmaceutics, 2008. **346**(1-2): p. 151-159.
7. Webber, W.L., et al., *Characterization of soluble, salt-loaded, degradable PLGA films and their release of tetracycline.* J. Biomed. Mater. Res., 1998. **41**(1): p. 18-29.
8. Wang, X., et al., *Controlled release of sirolimus from a multilayered PLGA stent matrix.* Biomaterials, 2006. **27**(32): p. 5588-5595.
9. Boccaccini, A.R., et al., *Preparation and characterisation of poly(lactide-co-glycolide) (PLGA) and PLGA/Bioglass® composite tubular foam scaffolds for tissue engineering applications.* Materials Science and Engineering: C, 2005. **25**(1): p. 23-31.
10. Eilert, J.B., et al., *Polyglycolic acid synthetic absorbable sutures.* The American Journal of Surgery, 1971. **121**(5): p. 561-565.
11. Dechy-Cabaret, O., B. Martin-Vaca, and D. Bourissou, *Controlled Ring-Opening Polymerization of Lactide and Glycolide.* Chemical Reviews, 2004. **104**(12): p. 6147-6176.
12. Brady, J.M., et al., *Resorption rate, route of elimination, and ultrastructure of the implant site of polylactic acid in the abdominal wall of the rat.* Journal of biomedical materials research, 1973. **7**(2): p. 155-166.
13. Lewis, D.H., *Controlled release of bioactive agents from lactide/glycolide polymers.* Drugs Pharm. Sci., 1990. **45**(Biodegrad. Polym. Drug Delivery Syst.): p. 1-41.
14. Luan, X. and R. Bodmeier, *Influence of the poly(lactide-co-glycolide) type on the leuprolide release from in situ forming microparticle systems.* Journal of Controlled Release, 2006. **110**(2): p. 266-272.
15. Batycky, R.P., et al., *A theoretical model of erosion and macromolecular drug release from biodegrading microspheres.* J. Pharm. Sci., 1997. **86**(12): p. 1464-1477.
16. Witschi, C. and E. Doelker, *Influence of the microencapsulation method and peptide loading on poly(lactic acid) and poly(lactic-co-glycolic acid) degradation during in vitro testing.* Journal of Controlled Release, 1998. **51**(2-3): p. 327-341.
17. Park, T.G., *Degradation of poly(d,l-lactic acid) microspheres: effect of molecular weight.* Journal of Controlled Release, 1994. **30**(2): p. 161-173.
18. Paul McGee, J., S.S. Davis, and D.T. O'Hagan, *Zero order release of protein from poly(-lactide-co-glycolide) microparticles prepared using a modified phase separation technique.* Journal of Controlled Release, 1995. **34**(2): p. 77-86.

19. Dorta, M.J., et al., *Potential applications of PLGA film-implants in modulating in vitro drugs release.* International Journal of Pharmaceutics, 2002. **248**(1-2): p. 149-156.
20. Faisant, N., J. Siepmann, and J.P. Benoit, *PLGA-based microparticles: elucidation of mechanisms and a new, simple mathematical model quantifying drug release.* European Journal of Pharmaceutical Sciences, 2002. **15**(4): p. 355-366.
21. Benelli, P., et al., *Clonazepam microencapsulation in poly-D,L-lactide-coglycolide microspheres.* Journal of Microencapsulation, 1998. **15**(4): p. 431-443.
22. Ingelheim, B., *Technical Bulletin - Resomer® Product Range.* 2009.
23. Ingelheim, B., *Technical Bulletin - Resomer® - Thermal Properties.* 2008.
24. Lam, X.M., et al., *Sustained release of recombinant human insulin-like growth factor-I for treatment of diabetes.* Journal of Controlled Release, 2000. **67**(2-3): p. 281-292.
25. Raman, C., et al., *Modeling small-molecule release from PLG microspheres: effects of polymer degradation and nonuniform drug distribution.* Journal of Controlled Release, 2005. **103**(1): p. 149-158.
26. O'Donnell, P.B. and J.W. McGinity, *Preparation of microspheres by the solvent evaporation technique.* Advanced Drug Delivery Reviews, 1997. **28**(1): p. 25-42.
27. Bodmeier, R. and J.W. McGinity, *Polylactic acid microspheres containing quinidine base and quinidine sulphate prepared by the solvent evaporation method. III. Morphology of the microspheres during dissolution studies.* Journal of Microencapsulation, 1988. **5**(4): p. 325-330.
28. Fukuzaki, H., et al., *In vivo characteristics of high, molecular weight copoly(l-lactide/glycolide) with S-type degradation pattern for application in drug delivery systems.* Biomaterials, 1991. **12**(4): p. 433-437.
29. Omelczuk, M.O. and J.W. McGinity, *The Influence of Polymer Glass Transition Temperature and Molecular Weight on Drug Release from Tablets Containing Poly(DL-lactic Acid).* Pharm. Res., 1992. **9**(1): p. 26-32.
30. Aso, Y., et al., *Effect of temperature on mechanisms of drug release and matrix degradation of poly(d,l-lactide) microspheres.* Journal of Controlled Release, 1994. **31**(1): p. 33-39.
31. Ferrara, N., et al., *The vascular endothelial growth factor family of polypeptides.* J. Cell. Biochem., 1991. **47**(3): p. 211-18.
32. Promega, *Technical Bulleting - Cell Titer-Blue® Cell Viability Assay.* 2009.

Chapter 4

Investigation of collagen films for controlled release of $VEGF_{165}$

Abstract

The influence of Vascular Endothelial Growth Factor ($VEGF_{165}$) incorporated into collagen films for the coating of vascular grafts to enhance their endothelialization was investigated. Therefore, the release behavior of collagen films was initially investigated using Fluoresceinisothyocyanat (FITC) dextran as a model compound. In order to minimize initial burst release that was observed for unmodified collagen films, collagen was cross-linked using Ethyl(dimethyl-aminopropyl)-carbodiimide (EDC). Release rates for crosslinked collagen films showed a decrease in initial burst release in correlation to the crosslinking degree and increasing amounts of entrapped FITC-dextran in the collagen matrix. The modified release parameters were confirmed using $VEGF_{165}$ that was incorporated in the collagen films. The biological activity of the incorporated $VEGF_{165}$ in modified and unmodified collagen films was investigated using human umbilical vein endothelial cells (HUVECs) and rat aortic smooth muscle cells (SMCs). An increase in endothelial cell growth was observed in dependency of the concentration of incorporated $VEGF_{165}$. No effect of VEGF on SMCs could be detected. The stability and biological activity of $VEGF_{165}$ was maintained throughout the production process and incubation of collagen films contrary to the generally low stability of this protein in vitro and in vivo, making this an interesting approach for vascular grafts to enhance their performance.

Keywords: Collagen, crosslinking, VEGF, endothelial cell, controlled release

1 Introduction

The controlled release of therapeutic agents from the vascular graft surface constitutes a promising approach for the improvement of vascular graft patency and the establishing of a functioning endothelial cell lining. A coating with biocompatible and biodegradable polymers for this approach and the subsequent local drug delivery for modifying the response of the surrounding tissue could induce spontaneous endothelialization and / or inhibit smooth muscle cell proliferation, and therefore overcome the limitations of small caliber vascular grafts.

Collagen is the primary and major structural protein of vertebrates, representing almost 30% of total protein present in a body [1]. It has been widely used in the field of controlled release applications and biomaterials [2-4] due to its biocompatibility and biodegradability. Especially in the field of biomaterials, one of its advantages is the ease of production in aqueous media without the application of high temperatures, which still results in a variety of forms and applications, such as coatings, fibers, films, implants, injectable solutions, membranes, sheets, and sponges. Collagen crosslinking using physical and / or chemical methods can be applied to prevent rapid degradation of such collagen based biomaterials during in vivo application, improve its mechanical stability [5], and reduce tissue response [6]. Crosslinking is also useful when using collagen in controlled release applications.

Several approaches using collagen for the improvement of vascular grafts have been undertaken, among others, collagen in combination with growth factors [7], heparin [8], or a combination thereof [6]. Among the growth factors investigated, basic fibroblast growth factors (bFGF) [7, 9-10] and VEGF [11-13] have been widely investigated. Yet, bFGF has been shown to have stimulatory effects on smooth muscle cell growth, which can lead to intimal hyperplasia and vessel occlusion [14-15]. VEGF, on the other hand, has been shown to have very high endothelial cell specificity without mitogenic activity for other cell types [16].

The aim of this study was to prove a successful incorporation of VEGF into collagen matrices, while maintaining its biological activity in order to stimulate endothelial cell growth without stimulating other cells after being released. Therefore, the first studies focused on the investigation of release behavior of FITC-dextran 40 kDa

from collagen matrices and the chemical modification of collagen via crosslinking in order to adjust the release profiles. The characterization of changes induced by crosslinking was important, in order to understand the effect of the treatment. DSC measurements and cell compatibility investigations were conducted for that purpose. Based on the results from the release experiments, two suitable collagen materials for the release of $VEGF_{165}$ were to be selected and investigated using an enzyme linked immunosorbent assay (ELISA) to confirm the release behavior. In order to prove the preservation of biological activity and to get further insight into the cell growth enhancing properties, the influence of $VEGF_{165}$ incorporated into collagen films towards its effect on HUVECs and SMCs was investigated.

2 Materials and Methods

2.1 Materials

Equine collagen type I derived from tendon was provided by Innocoll GmbH (Saal/Donau, Germany) as lyophilized material, VEGF was kindly donated by Genentech (San Francisco, CA, USA), and rat aortic smooth muscle cells (SMCs) were provided by PD Dr. Wolgang Erl (Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, Ludwig-Maximilians-Universität München, Munich, Germany). EDC, Na_2HPO_4, N-Hydroxysuccinimide (NHS), 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris), and FITC-dextran with a molecular weight of 40 kDa was purchased from Sigma Aldrich (Seelze, Germany), 1 M HCl, 1 M NaOH and glacial acetic acid from Merck KGaA (Darmstadt, Germany), 50 ml Falcon tubes from Greiner Bio-One GmbH (Frickenhausen, Germany), plastic Petri dishes (diameter: 5.5 cm) and cover glasses from VWR (Darmstadt, Germany), Polytetrafluoroethylene (PTFE) from GM GmbH (Freiham, Germany), 24-well plates from Corning (Amsterdam, The Netherlands) and TPP Techno Plastic Products (Trasadingen, Switzerland), collagenase A from Roche (Penzberg, Germany), endothelial cell growth medium from Provitro (Berlin, Germany), heat-inactivated fetal bovine serum from Biochrom (Berlin, Germany), M199 from PAA (Pasching, Austria), buffered formaldehyde solution (4%) from Polysciences, Inc. (Warrington, PA, USA), propidium iodide from Fluka (Steinheim, Germany), disposable Plastibrand® PMMA plastic cuvettes from

Brand (Wertheim, Germany), Human VEGF ELISA from Ray Bio® from Ray Biotech Inc. (Norcross, GA, USA), smooth muscle cell growth medium DMEM /F12 from PAA (Pasching, Austria), and Cell Titer-Blue® from Promega (Madison, WI, USA)

The pH of the solutions was adjusted using hydrochloric acid or sodium hydroxide from Merck KGaA (Darmstadt, Germany) and measured with a pH meter Inolab level 1 from WTW (Weilheim, Germany).

2.2 Methods

2.2.1 Collagen crosslinking

2.2.1.1 Crosslinking procedure

Crosslinking of collagen with EDC was performed according to a protocol from Metzmacher [17]. Therefore, 1% (w/w) collagen in water was adjusted to pH 3.5 with 1 M HCl and pre-swollen for 1 hour. After dispersion for 10 minutes with an Ultraturrax® (IKA-Werke GmbH Co. KG, Staufen, Germany), the pH was adjusted to pH 5.1 with 1 M Na_2HPO_4 and 10% stock solutions of EDC and NHS (molar ratio 5:2) were added. Three different collagen / EDC-ratios were used: 1 g / 16.9 mg, 1 g / 67.7 mg and 1 g / 203.1 mg. The dispersion was homogenized and the reaction was performed for 2 hours at room temperature under pH-control. Subsequently, the pH was adjusted to 9.1 by adding 1 M Na_2HPO_4 and 1 M NaOH. After another 2 hours of stirring, the pH was adjusted to 6.5 using 1 M HCl. Subsequently, each collagen dispersion was transferred into 50 ml Falcon tubes and centrifuged at 5,100 rpm for 20 minutes using a Sigma 4 K15 lab centrifuge (Sigma, Osterode, Germany). Each residue was washed using 50 ml of Milli-Q water with centrifugation at 5,100 rpm after each washing step to collect the residues. After 10 washing steps, residues were lyophilized (see 2.2.1.2).

2.2.1.2 Lyophilization

Lyophilization was performed in a ε2-6D special freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) with EDC crosslinked

collagen dispersions prepared according to 2.2.1.1. Collageneous materials were cast in plastic Petri dishes (diameter: 5.5 cm) to a height of about 10 mm. Samples were lyophilized according to the following program:

Table 4-1: Lyophilization protocol for the freeze-drying of EDC crosslinked collagen material.

Step	Time [hh:mm]	Temperature [°C]	Vaccum [mbar]
Start	00:00	20	---
Ramp	02:00	- 20	---
Freezing	02:00	- 20	---
Ramp	00:05	- 20	0.1
Ramp	03:00	15	0.1
Primary Drying	25:00	15	0.1
Ramp	01:00	25	0.1
Ramp	00:03	25	0.045
Secondary Drying	15:00	25	0.045

2.2.1.3 Differential Scanning Calorimetry

Samples were analyzed with a Mettler Toledo DSC 821^e machine (Mettler-Toledo GmbH, Giessen, Germany). Samples of approximately 10 mg were incubated in 1 ml 0.05 M Tris buffer pH 7.5 at room temperature for at least 2 hours. Subsequently, samples were transferred in aluminum pans (ME 26763 AL-Crucibles 40 µl without pin, Mettler-Toledo GmbH, Giessen, Germany) and containers were sealed. Samples were heated from 20 to 90°C at 5 K/min. Analyses were performed in triplicates against an empty reference pan.

2.2.1.4 Karl-Fischer titration

Residual moisture of samples was determined by Karl-Fischer titration using an Aqua 40.00 titrator with Head-Space oven (Analytik Jena AG, Jena, Germany).

Sealed samples were fixed in the oven chamber and heated to 60°C for 30 minutes. Vaporized water was transported into the Karl-Fischer cell, determined coulometrically, and calculated as water amount in % (w/w). Measurements were carried out in triplicate.

2.2.2 Collagen coating procedure

2.2.2.1 Preparation of collagen dispersion

Collagen dispersions were prepared by redispersion of collagen material in water at 1% and 3% (w/w). The pH was adjusted to 3.5 using glacial acetic acid. Swelling was performed at room temperature for 4 hours with dispersion for 60 s using an Ultraturrax® (IKA-Werke GmbH Co. KG, Staufen, Germany) every 30 minutes at 10,000 rpm and an additional dispersion step at the end of the swelling period of 3 minutes at 10,000 rpm. The dispersion was then centrifuged at 5,000 rpm at 10°C for 10 minutes using a Sigma 4 K15 lab centrifuge (Sigma, Osterode, Germany) to remove air bubbles from the dispersion. Thereafter, the dispersion was used for the coating (compare 2.2.2.2 and 2.2.2.3) or stored at 2-8°C until further use.

2.2.2.2 Coating procedure – cover coating

Collagen dispersions (compare 2.2.2.1) were used to coat Polytetrafluoroethylene (PTFE) -discs, argon plasma activated with a plasmabrush® (Reinhausen Plasma GmbH, Regensburg, Germany) or cover glasses, both with a diameter of 15 mm. Therefore, the discs were placed in 24-well plates and covered with the collagen dispersions. The discs were air dried for 1 hour and subsequently dried under vacuum over night or until further use. For cell culture studies all these procedures were performed under a laminar flow workbench (Thermo, Langenselbold, Germany).

2.2.2.3 Coating procedure - squeegee

The higher concentrated collagen dispersion (compare 2.2.2.1) was used to coat PTFE-discs, argon plasma activated with a plasmabrush® or cover glasses, both with a diameter of 15 mm. Therefore, the discs were covered with the collagen dispersion using a squeegee with a defined height of 1 mm (Figure 4-1). The discs were subsequently placed in 55 mm petri dishes, air dried for 1 hour, and subsequently dried under vacuum over night or until further use. For the cell culture studies, all these procedures were performed under a laminar flow workbench.

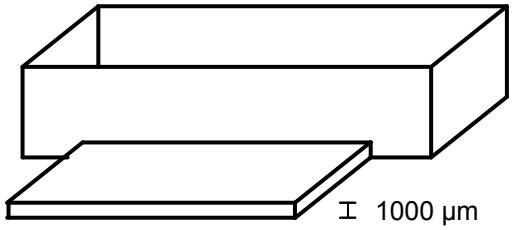


Figure 4-1: Schematic of collagen coating using a squeegee.

2.2.3 Cell compatibility

The cell compatibility of collagen coated discs was evaluated using Human Umbilical Vein Endothelial Cells (HUVECs). HUVECs were prepared by digestion of umbilical veins with 0.1 g/l collagenase A. Cells were cultured in endothelial cell growth medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere at 5% CO_2 and 37°C. Cells were used at passage no. 3. For visualization of cells on the different surfaces, cells were fixed with a buffered formaldehyde solution (4%) and were stained with propidium iodide in phosphate buffered saline (PBS) buffer with a final concentration of 50 µg/ml. Images were obtained with a Zeiss LSM 510 confocal laser scanning microscope (CLSM) (Zeiss, Oberkochen, Germany).

2.2.4 In-vitro release studies

2.2.4.1 Release study of FITC-dextran from collagen matrices

For the release study, plasma activated PTFE-discs were coated with collagen dispersions containing 0.1% (w/w) of FITC-dextran with a molecular weight of 40 kDa based on the dry weight of collagen in accordance to 2.2.2.2 and 2.2.2.3. After coating, discs were left to dry at room temperature for 1 hour and subsequently under vacuum for several hours or until further use. Drying was performed under the exclusion of light, due to the light sensitivity of FITC-dextran. For the release analysis, triplicates of the coated discs were incubated in 10.0 ml PBS pH 7.4 in 50 ml Falcon tubes in a water bath (Haake SWB25, Haake, Karlsruhe, Germany) at 37°C and at 25 rpm horizontal shaking under exclusion of light. 2 ml samples were drawn and replaced with fresh PBS buffer at several time points. Released FITC-dextran was quantified using fluorescence spectroscopy, which was performed using a Varian Cary Eclipse fluorescence spectrometer (Varian GmbH, Darmstadt, Germany). Therefore, samples were measured in disposable Plastibrand® PMMA plastic cuvettes with an excitation wavelength of 495 nm, an emission wavelength of 517 nm, and adjusted with a PBS buffer blank. Samples were diluted using PBS buffer, if necessary. The amount of released FITC-dextran was calculated using a calibration curve.

2.2.4.2 Release study of $VEGF_{165}$ from collagen matrices

For the release study, glass-discs were coated with collagen dispersions containing $VEGF_{165}$ in accordance to 2.2.2.3. The amount of $VEGF_{165}$ incorporated per disc was 1 µg, unmodified and crosslinked collagens were used in this study. After coating, discs were left to dry at room temperature for 1 hour and subsequently under vacuum for several hours or until further use. For the release analysis, triplicates of the coated discs were in incubated in 10.0 ml phosphate buffered saline (PBS) pH 7.4 in 50 ml Falcon tubes in a water bath (Haake SWB25, Haake, Karlsruhe, Germany) at 37°C and at 25 rpm horizontal shaking. In order to investigate the stability of $VEGF_{165}$ in buffer, 1 µg $VEGF_{165}$ was incubated in 10.0 ml PBS buffer pH 7.4 in 50 ml Falcon tubes, as well. These investigations were

performed in duplicate. 1 ml samples were drawn and replaced with fresh PBS buffer at several time points. Samples were frozen at -80°C until measurement. Released amounts of $VEGF_{165}$ were quantified with a sandwich enzyme-linked immunosorbent assay (ELISA), using a commercially available kit for human VEGF from Ray Bio®. The ELISA was performed according to protocol; samples were measured at 450 nm using a Spectrafluor Plus plate reader (Tecan, Crailsheim, Germany). The amount of released $VEGF_{165}$ was then calculated using a calibration curve.

2.2.5 Influence on cell growth by incorporated $VEGF_{165}$

2.2.5.1 Rat aortic smooth muscle cells

The influence of discs on the cell growth of smooth muscle cells was evaluated using rat aortic smooth muscle cells (SMCs). Cells were cultured in smooth muscle cell growth medium DMEM /F12 supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere at 5% CO_2 and 37°C.

2.2.5.2 Cell growth study

For the study VEGF containing collagen coated discs holding varying amounts of VEGF were prepared. Therefore, collagen dispersions were prepared according to 2.2.2.1 with VEGF added to the collagen dispersions prior to coating according to 2.2.2.3. Control samples not carrying VEGF were treated accordingly. The discs were then placed on the bottom of 24-well plates and subsequently covered with 500 µl of starvation medium: in case of HUVECs, containing 80% of M199 and 20% of endothelial cell growth medium, in case of SMCs, 100% of DMEM /F12. 20,000 viable HUVECs (prepared according to 2.2.3) or SMCs (prepared according to 2.2.5.1) were seeded per well. Thereafter, cells were cultured in a humidified atmosphere at 5% CO_2 and 37°C. Cells on the discs were quantified at day 3 using Cell Titer-Blue®. Therefore, cells were incubated with Cell Titer-Blue® for 6 hours and an aliquot of the supernatant was analyzed using a Spectrafluor plus plate reader (Tecan, Crailsheim, Germany) with an excitation wavelength of 550 nm and

emission recording at 595 nm. The increase in cell growth was calculated as % increase in relation to cells grown on collagen coated discs without $VEGF_{165}$.

3 Results and Discussion

3.1 Characterization of crosslinked collagen

For a complete understanding of the properties of the collagen materials and the release behavior, it was of importance to characterize physical or chemical changes induced by the crosslinking process. Therefore, DSC and Karl-Fischer measurements of the different collagen materials were performed to determine the melting temperature and the residual moisture levels after drying. These two critical parameters are indicative for, on the one hand, a successful crosslinking in case of DSC [17], and on the other hand, can be a first indicator for the swelling properties and the release characteristics in case of residual moisture determination [18-20].

3.1.1 Differential Scanning Calorimetry

Collagen variants were analyzed using DSC in order to determine the midpoint of transition, also known as melting temperature (T_m). It is of critical interest to evaluate this characteristic value since it gives insight in the physicochemical changes that can occur during in vitro and in vivo tests or be an indicator for variations that can occur during storage. The heating of collagen results in a loss of structural properties of the collagen, especially the helical structure and is therefore a valuable tool to determine the degree of crosslinking of swollen collagen samples [21-22]. Stronger interactions between the collagen fibers that result from crosslinking are responsible for an increase in melting temperature and this increase can be used to indirectly identify the degree of crosslinking of the analyzed samples [17]. Impartial to the degree of crosslinking, thermal denaturation occurs at temperatures above 100°C for collagen in a dried state [1], therefore indicating that a storage of dried collagen at room temperature is possible without risking temperature induced changes.

For the collagen material investigated in this study, the non crosslinked equine collagen raw material showed a denaturation temperature of 53°C (Figure 4-2). During subsequent cooling and a second heating step of the sample, no peaks were detectable. This was an indicator for the complete and irreversible denaturation of the collagen material, which was in accordance with literature [21]. EDC crosslinking resulted in an increase in melting temperatures of the collagen samples in correlation to the employed EDC : collagen ratios. For the collagen samples 1:16.9 (g collagen : mg EDC), a melting temperature of 54°C was detected, which increased for the samples 1:67.7 to 59°C and a further increase to 65°C was detectable for the samples with a ratio of 1:203.1 (Figure 4-2).

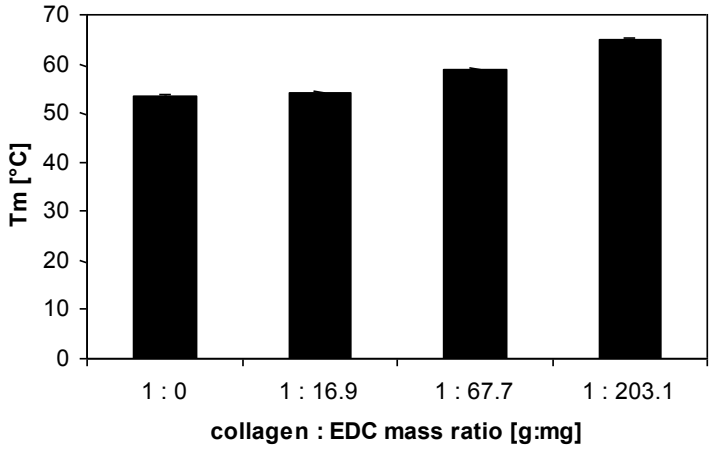


Figure 4-2: Melting temperature of equine collagen raw material crosslinked with different EDC ratios determined by DSC (average ± SD; n=3).

These results indicated a stronger interaction of the collagen fibers, which resulted in an increase in denaturation temperature for the EDC crosslinked collagen samples. Crosslinking collagen is a useful tool to control the properties of collagen and is often applied to prevent a rapid degradation of collagen-based biomaterials during in vivo application, in order to suppress its antigenicity and to improve mechanical properties [5, 17]. Overall, this data is consistent with data presented by Metzmacher, who showed an increase in T_m for EDC crosslinked equine collagen in a range of 54°C and 65°C [17].

3.1.2 Residual moisture of crosslinked collagen

The residual moisture of EDC crosslinked collagen after freeze-drying was determined using Karl-Fischer titration in order to guarantee low moisture levels in the collagen material to ensure stability during storage. The untreated non crosslinked equine collagen starting material showed a residual moisture level of 8.0% ± 0.16. In comparison, all EDC crosslinked materials showed lower levels in residual moisture decreasing with increasing collagen : EDC ratios. The collagen with the lowest degree of cross-linking exhibited residual moisture levels of 7.4% ± 0.15, medium crosslinked material showed 7.1% ± 0.10, and highest crosslinked collagen 7.0% ± 0.23 residual moisture.

These lower numbers for residual moisture of crosslinked collagen material could be associated with the swelling behavior of crosslinked collagen that was reported in literature. In studies, decreases in swelling were shown for crosslinked collagen matrices, which were caused by a decrease in the amounts of penetrating solvents [18-20].

3.2 Cell compatibility of collagen variants

In addition to collagen melting temperature and residual moisture, the different crosslinked collagen species were analyzed concerning their cell compatibility. Cell compatibility is of great importance, especially for the intended use as biomaterial substrate to enhance attachment, attraction, and endothelial cell growth, either by itself due to its properties as a natural collagenous tissue, and also in its function as matrix for the release of endothelial cell specific growth enhancers, such as $VEGF_{165}$. Studies of collagen in a similar application have been performed before, but only for native collagen with human origin [23] or with cells of different origin than endothelial cells [24]. Thus, it was of interest how collagen of different origins and after chemical crosslinking would perform. Therefore, the collagen materials were used to coat glass discs, which were subsequently seeded with HUVECs. Cells were allowed to attach and grow on the modified and unmodified materials and were stained using propidium iodide after 3 days. The stained cells were

analyzed using CLSM concerning the cell morphology, cell viability, and proliferation on the discs.

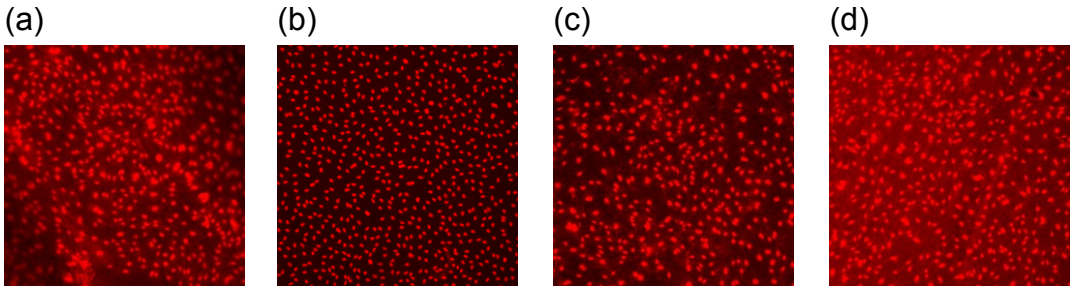


Figure 4-3: CLSM images of propidium iodide stained collagen coated discs after incubation with endothelial cells on native collagen (a), crosslinked collagen 1:16.9 (b), crosslinked collagen 1:67.7 (c) and crosslinked collagen 1:203.1 (d).

Figure 4-3 shows the acquired pictures for the stained cells after a 3 day incubation period. All pictures showed a confluent monolayer of endothelial cells grown on the collagen films with a morphology attributed to HUVECs. The different intensities originated from different levels of background staining of collagen. Crosslinking of the collagen material did not influence the growth behavior of endothelial cells; hence a treatment with EDC and NHS did not leave behind any cytotoxic residues and thereby did not affect cyctotoxicity of the collagen material. However, the issue of blood compatibility, especially thrombogenicity could not be elucidated by these investigations. This is one of the main concerns that arise when working in the field of vascular graft applications, which can be seen by the multitude of studies performed to overcome this problem [25-28]. Collagen itself has a thrombogenic potential [29], and therefore, several groups have investigated this effect and have come to the conclusion that collagen in combination with Heparin can lower this thrombogenic potential [8, 30]. In addition, it has been reported that crosslinking of collagen could lower the tissue reaction of surrounding tissue [6], thus being a first indicator that crosslinking can be beneficial in itself to lower thrombogenicity.

3.3 In vitro release studies

In the previous section, the collagen raw and crosslinked material was characterized with respect to physical behavior and cell compatibility. Consequently, the aspect of release behavior of higher molecular weight compound from collagen film coatings had to be elucidated. In our case, collagen was used to establish thin films on vascular graft raw materials, such as PTFE and later glass as model substrate. For first release investigations, FITC-dextran 40 kDa was incorporated in the films and its release from films investigated. Subsequently, the model compound was substituted by the endothelial cell specific growth enhancer $VEGF_{165}$ [31] with a molecular weight of 42 kDa and its release was studied.

Collagen devices exhibit strong swelling behavior in contact with water depending on their crosslinking degree [18]. Sano et al. observed that penetrating solvent dissolved water soluble drugs, which were incorporated into a matrix and that the release of the dissolved compound was controlled by the swelling behavior of the collagen matrix rather than the speed of dissolution [32]. These findings were confirmed by Maeda et al. who additionally suggested that the release occurs through water filled pores, and therefore an increasing release rate can be observed for porous structures [33]. In general, collagen devices, specifically collagen films, exhibit fast release of incorporated compounds [3].

3.3.1 Influence of coating procedure on FITC-dextran release profile

During preliminary FITC-dextran release investigations, highly variable release profiles were observed for drip coating collagen coated discs, with up to 60% difference in the totally released percentages. Release of model compound during these investigations was completed within 2 hours, which was in accordance with findings reported in literature [1, 3, 34]. Therefore, a 24 hour time period was chosen for the investigation of further release experiments. It was postulated that the coating procedure applied had a strong influence on drug liberation. Consequently, this influence was investigated by applying a different coating technique in comparison to the applied drip coating method.

Drip coating was performed by covering discs that were placed in a well plate with collagen dispersion. The discs were subsequently air dried for several hours before being dried under vacuum over night. Thereafter, discs were removed from the well plates, which lead to the loss of some of the coating material at the disc-well plate interface due to strong adherence of the collagen coating. The alternative coating approach was performed using a squeegee that enabled the application of higher concentrated collagen dispersions with a defined height on the discs. Since coating was performed outside of well plates, a loss of collagen after drying was circumvented. This coating procedure posed several advantages over the previously applied drip coating procedure. Due to the application of collagen dispersion with a defined height, a more homogenous coating with a constant thickness could be applied that might result in better drying of the coating, as well as faster drying due to the higher concentrations of collagen applied. In addition, the increase in concentration should lead to a slower diffusion of the incorporated compound, resulting in a more consistent distribution within the matrix, since the first dry spots are formed next to still high concentration collagen gel areas.

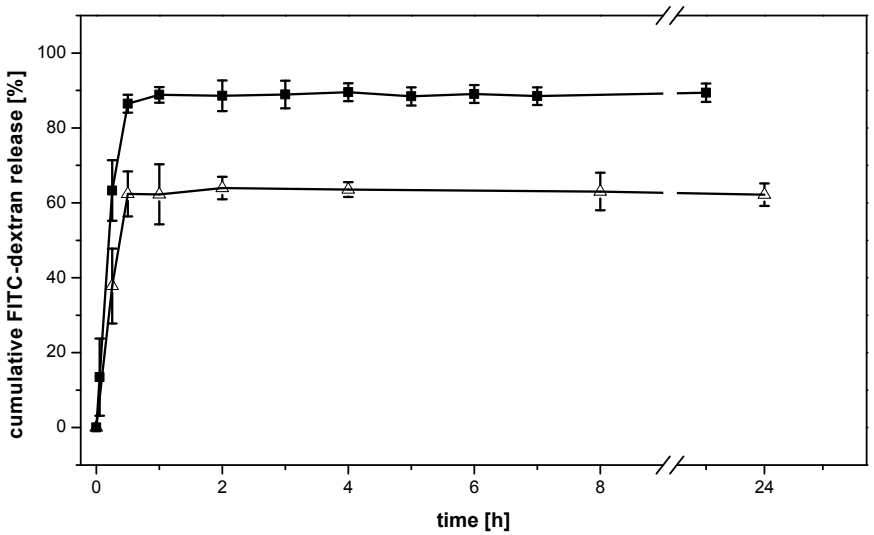


Figure 4-4: Cumulative in-vitro FITC-dextran release from drip coating collagen coated discs (—△—) and squeegee coating collagen coated discs (—■—). (average ± SD; n=3)

Figure 4-4 shows the cumulative release of the incorporated FITC-dextran. Collagen coated discs manufactured by drip coating exhibited an initial burst release of approximately 60% within the first hour which stayed constant throughout the entire investigated 24 hour time period. In contrast, the collagen coated discs manufactured by squeegee coating exhibited an almost complete release of FITC-dextran within the first hour that was constant throughout the investigated time period. It was apparent that the standard coating procedure resulted in a lower total release after the incubation period, indicating a loss of FITC-dextran, whereas the squeegee coated discs exhibited a close to 100% release of the model compound. These findings suggested a loss of FITC-dextran during the coating procedure or the subsequent retrieval of samples. However, the described loss of collagen coating after drying could not account for the high loss of incorporated model compound, assuming its even distribution in the film. Maeda et al. had observed cluster formation of their model compound during drying and hypothesized that this phenomenon occurred due to phase separation. Since their model compound did not exhibit significant affinity with collagen, a cluster formation was therefore possible [33]. This cluster formation and phase separation phenomenon was presumed to be the reason for the significant loss of FITC-dextran. Evidently, the phase separation caused an accumulation of FITC-dextran in the peripheral region of the discs and the well plate, thus leading to a substantially higher loss of FITC-dextran when amounts of collagen coating were lost during sample retrieval. These results indicated a superiority of the squeegee method over the standard coating method to coat discs for release investigations.

3.3.2 Influence of crosslinking on FITC-dextran release profile

An almost complete release of FITC-dextran from unmodified collagen films, as seen in 3.3.1, took place within the first hour after incubation. For an application of controlled release in the field of vascular grafts to stimulate endothelialization, a constant release over a prolonged period of time is desirable. Therefore, the influence of crosslinking on the release rate of the model compound was investigated in order to minimize the initial burst release and establish a steady

release to stimulate endothelial cells. In accordance to Sano et al. [32], it was expected that the higher collagen crosslinking degree would result in lower swelling of the established films and therefore would reduce the initial release and allow for a more constant release profile.

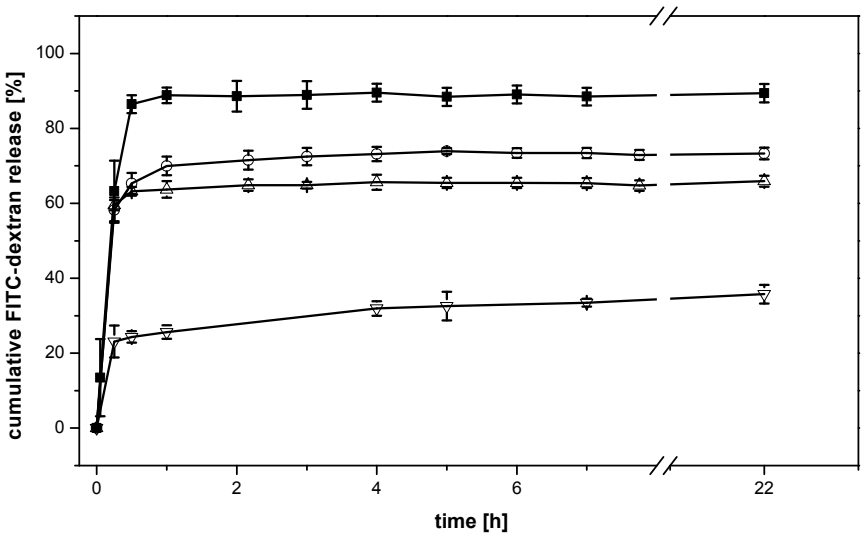


Figure 4-5: Cumulative in-vitro FITC-dextran release from native collagen coated discs (—■—), crosslinked collagen 1:16.9 (—○—), crosslinked collagen 1:67.7 (—△—) and crosslinked collagen 1:203.1 (—▽—) coated discs. (average ± SD; n=3)

The acquired release profiles can be seen in Figure 4-5. All systems investigated exhibited a strong burst release within the first 30 minutes of incubation. A complete release for the non-crosslinked collagen occurred within 1 hour. Lower total amounts of FITC-dextran were released from all other samples in correlation to their crosslinking degree. The samples with a collagen : EDC ratio of 1:16.9 showed a total cumulative release of 70%, followed by the samples with a ratio of 1:67.7 with a release of 60%, and the samples with a ratio of 1:203.1, which showed a release of 30%. Burst release rate for the unmodified samples, as well as, the two collagen varieties with the lower crosslinking degree were similar, however, the highest crosslinked collagen's burst release rate was lowered. It has been reported in literature that, especially for high molecular weight compounds, the release rates decrease with an increase in crosslinking degree. This phenomenon was observed independent of the method applied for crosslinking [1]. In contrast, low molecular weight substrates showed no differences in release rates in accordance to the

crosslinking degree [1]. The initial release can be explained by the presence of model compound that was released through the porous structure of the film, immediately after incubation. The remaining fraction of FITC-dextran was mechanically fixed within the collagen after drying and was released to a lesser degree due to lower swelling resulting from crosslinking [1, 35-36]. This mechanical fixation, however, can be beneficial for the intended application of such coatings on vascular grafts to enhance endothelialization. Collagenolytic enzymes are expressed in endothelial cells [37], therefore the trapped compounds can be released upon digestion of the matrix by the endothelial cells and become available to attract further cells and stimulate their proliferation.

3.3.3 Release of $VEGF_{165}$ from collagen matrices

After having successfully characterized the in vitro release profiles for the model compound FITC-dextran from non-modified and modified collagen films, the in vitro release profile of $VEGF_{165}$ incorporated in collagen films was to be tested. Non-modified and crosslinked collagen with a collagen : EDC ratio of 1:67.7 were used to coat glass discs carrying amounts of $VEGF_{165}$. In parallel to the release study, the stability of $VEGF_{165}$ under release conditions was analyzed and the equivalent amount of $VEGF_{165}$ incorporated in the collagen films was incubated in PBS buffer alone.

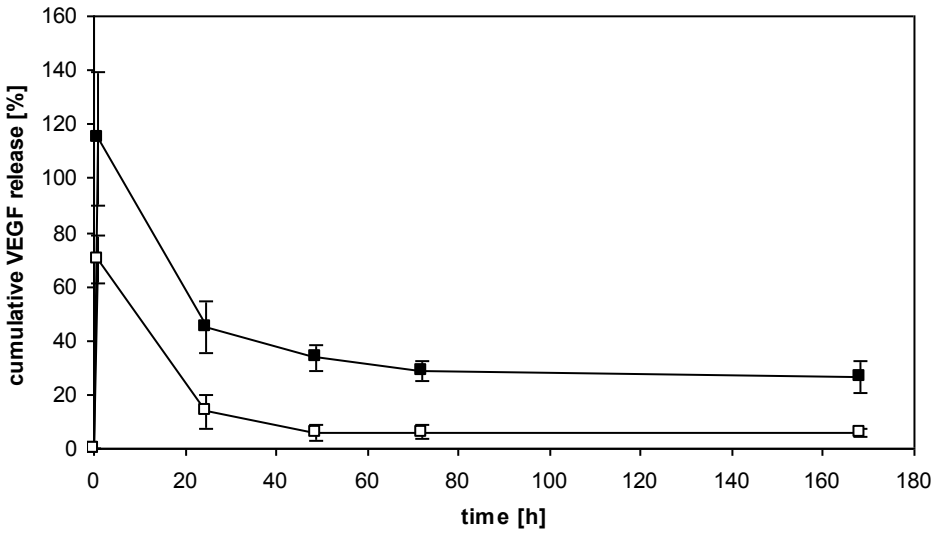


Figure 4-6: Cumulative in-vitro $VEGF_{165}$ release from native collagen coated discs (—■—) and crosslinked collagen coated discs (—□—). (average ± SD; n=3)

The release profiles for $VEGF_{165}$ from native and crosslinked collagen are presented in Figure 4-6. Burst release was seen for $VEGF_{165}$ for both versions of collagen films. The initial release for the protein embedded in the unmodified collagen film was complete within the first hour of incubation. The corresponding release from the crosslinked collagen was finished in the same period of time. However, the amount of total released protein was approximately 40% lower than observed for the umodified collagen. This was in accordance to the findings for FITC-dextran. These results also corresponded with findings in literature, as mentioned before, that the release of higher molecular weight compounds decreases with increasing crosslinking degree [1]. This was anticipated since the molecular weights of the model compound FITC-dextran and $VEGF_{165}$ are in a similar range of 40 kDa. However, after the initial release of $VEGF_{165}$, the amount present in the release medium drastically decreased within the following 48 hours and stabilized at a level of 30% for the unmodified collagen and 5% for the crosslinked collagen. A similar instability was observed for the $VEGF_{165}$ incubated in PBS buffer at 37°C over a time period of 7 days (Figure 4-7). After 24 hours only 5% of the initially present VEGF could be detected.

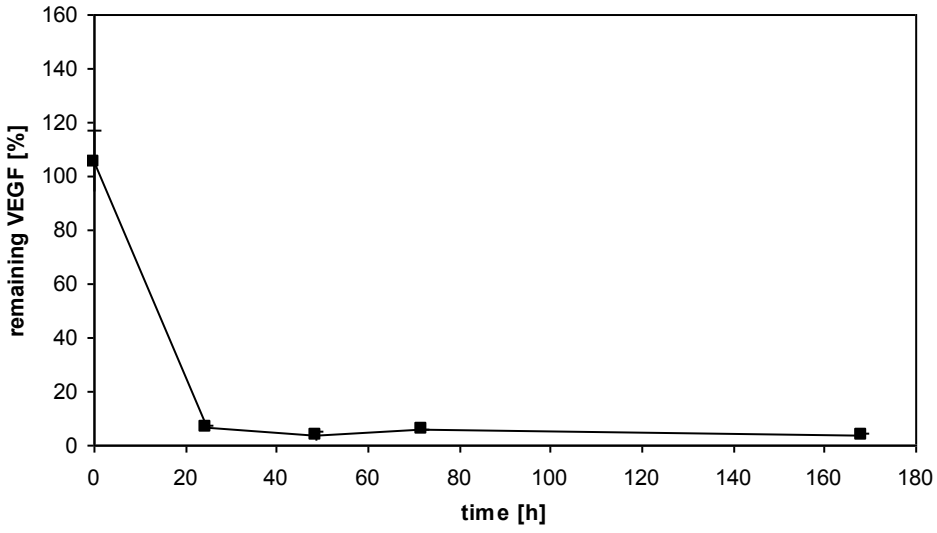


Figure 4-7: $VEGF_{165}$ stability in PBS. (average ± SD; n=2)

Contradictory findings about the stability of $VEGF_{165}$ in buffer have been reported in literature [38-39]. The present findings suggested a fast degradation and low recovery of $VEGF_{165}$ in solution at 37°C, which was in accordance to other in vitro findings [39]. However, it has been reported in literature that the addition of BSA to formulations containing VEGF can have a beneficial effect on VEGF recovery [38, 40]. Therefore, the low recovery of VEGF in these investigations determined by ELISA might be due to the adsorption of VEGF to container surfaces. Other possible explanations for the low recovery could be the loss of affinity of VEGF towards the antibody used during the ELISA measurements resulting in low recovery, but not reflecting loss of biological activity. Thus, more important than the amount of recovered $VEGF_{165}$ in the stability investigations is the availability and biological activity of released $VEGF_{165}$ in a cell growth setting (compare 3.4).

In conclusion, it can be said that native and crosslinked collagen showed comparable release profiles for VEGF as previously determined using FITC-dextran. Native collagen stabilized $VEGF_{165}$ in solution, as seen by higher levels of cytokine present in solution, in comparison to crosslinked collagen and VEGF in buffer alone over a time period of 170 hours. The biological activity of entrapped $VEGF_{165}$ in the crosslinked collagen matrix could not be investigated in this setting, however, it is suggested that due to the limited swelling of this collagen and therefore lower amounts of penetrating solvent, the biological activity of VEGF in the matrix was conserved. Furthermore, the high concentration of the obviously stabilizing agent collagen in the environment added to the preservation of biological activity. However, the cell growth experiments on pre-incubated samples should give further insight into the stability of the remaining VEGF (compare 3.4).

3.4 Influence of incorporation of $VEGF_{165}$ in collagen matrices on cell growth

The influence of incorporated $VEGF_{165}$ and its release on cells is a very important prerequisite for the application of collagen films for the intended purpose of vascular graft coating to improve the cell adhesion and growth of endothelial cells. $VEGF_{165}$ has been widely used in the field of vascular graft applications to enhance the

proliferation of endothelial cells in order to improve the performance of grafts [11-13]. Therefore, the goal of this study was to investigate the different effects of incorporated $VEGF_{165}$ on endothelial and smooth muscle cells in an in vitro setting. Additionally, it was crucial for the success of the application of collagen coatings for vascular grafts to examine the stability and biological activity of VEGF throughout the coating process, the incorporation into the collagen, and drying of the collagen films.

Therefore, glass discs were coated with non-modified and crosslinked collagen with a collagen : EDC ratio of 1:67.7 and varying $VEGF_{165}$ concentrations. The influence on the cell growth of SMCs and HUVECs was investigated in comparison to control samples not carrying VEGF. In order to identify a suitable concentration for cell growth stimulation, a wide range of VEGF concentrations of 1, 10 and 100 µg per disc, in case of HUVECs, were tested in a cell growth assay (compare 2.2.5.2). In order to investigate the stability of VEGF remaining in wet collagen films after initial burst release and its effect on SMCs and HUVECs, discs coated with both collagen carrying 10 µg $VEGF_{165}$ were incubated over night in PBS buffer, subsequently rinsed to ensure removal of adsorbed VEGF to the collagen surface and then transferred to fresh well plates to examine them in the same cell growth setting.

3.4.1 Smooth muscle cells

Contradictory findings concerning the effect of $VEGF_{165}$ on the proliferation and migration of SMCs are reported in literature. It has been reported, that $VEGF_{165}$ can have a positive effect on SMC growth and migration induced through various pathways [41-42]. However, other groups have identified $VEGF_{165}$ to have no stimulating effect on SMCs [11, 43]. In our case, no differences for the SMCs grown on the $VEGF_{165}$ collagen matrices were observed independent of the amount of $VEGF_{165}$ incorporated (Figure 4-8).

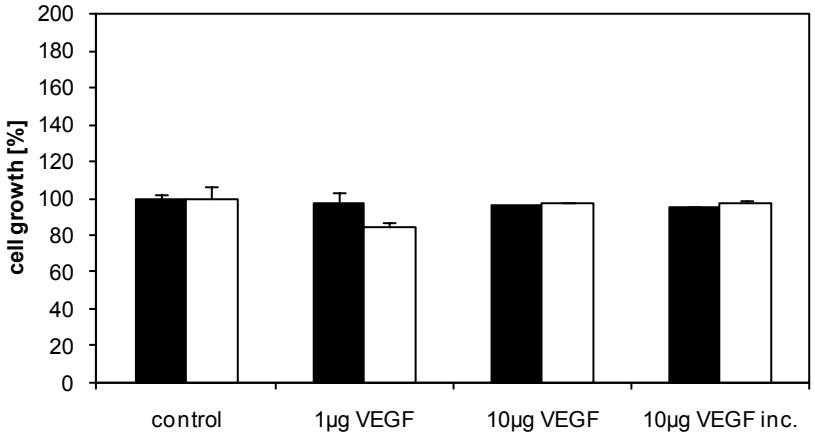


Figure 4-8: Smooth muscle cell growth on collagen (■) and crosslinked collagen (▭) coated discs with 1 or 10 µg incorporated $VEGF_{165}$ and after prior 24h incubation. (average ± SD; n=3)

All samples except the crosslinked collagen sample carrying 1 µg VEGF showed a SMC growth comparable to the control samples without VEGF. The crosslinked collagen matrix carrying 1 µg VEGF showed a decrease in cell growth of 15% compared to all other samples. This decrease could not be attributed to the effect of crosslinked collagen on SMCs, since all other crosslinked samples carrying higher amounts of VEGF did not show a reduction of cell growth. Pre-incubation of collagen samples with 10 µg VEGF showed neither a beneficial, nor a detrimental effect on SMC growth.

3.4.2 Vascular endothelial cells

VEGF is a widely investigated growth factor. It is a secreted protein ligand that activates transmembrane receptors on endothelial cells and it consists of a disulfide-linked homodimer. It exists in several isoforms, four of which consist of 121, 165, 189 and 206 amino acids [31] and are produced from a single human gene as a result of alternate splicing [11]. This growth factor has been shown to have very high endothelial cell specificity without mitogenic activity for other cell types [16], making it an ideal candidate for the specific stimulation of endothelial cell

growth in vascular graft applications. Multiple ways of presenting these growth factors have been investigated, including extracellular matrix coatings [13], electrostatic interaction [12], and fibrin matrices [11, 44] that have shown promising results.

The stability and remaining biological activity of VEGF within dried collagen matrices and after release from the coating is of great importance for the application of collagen coatings for the controlled release of VEGF to enhance endothelialization of vascular grafts. Therefore, the goal of these investigations was to show the influence of $VEGF_{165}$, which had been incorporated and dried in collagen matrices, on endothelial cells.

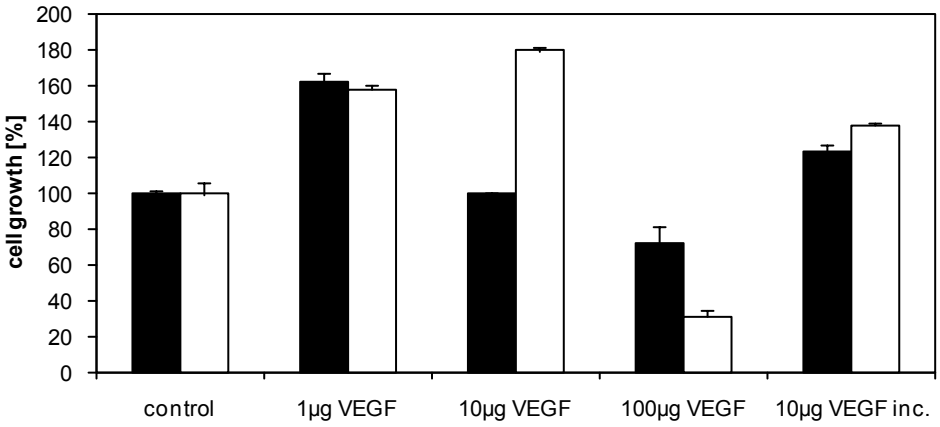


Figure 4-9: Endothelial cell growth on collagen (▬) and crosslinked collagen (▭) coated discs with incorporated amounts of $VEGF_{165}$ and after prior 24h incubation. (average ± SD; n=3)

The results of the cell growth investigations are presented in Figure 4-9. Endothelial cells grown on both collagen variations, the non-modified and crosslinked collagen films, exhibited good growth when grown on control samples without VEGF, ensuring the cell compatibility and non toxicity of the materials as shown before (see 3.2). Endothelial cell growth on films carrying 1 µg $VEGF_{165}$ increased by almost 60%, when grown on either collagen variety. No differences could be observed for the different collagen samples. When the film load increased to 10 µg $VEGF_{165}$ per film endothelial cell growth on non-modified collagen dropped to 100%,

comparable to the cells grown on control samples without VEGF. However, cells grown on crosslinked collagen films containing 10 µg VEGF showed comparable growth to those containing 1 µg. For the samples carrying films with 100 µg $VEGF_{165}$, the cell growth decreased to 70% for samples coated with non-modified collagen and 30% for those coated with crosslinked collagen. This decrease, as well as the decrease for the collagen coated samples containing 10 µg VEGF, could be ascribed to the immediate release of high amounts of $VEGF_{165}$, which led to an over stimulating effect of the cytokine on endothelial cells, which slowed proliferation. The preservation of cell growth increase observed for the samples coated with crosslinked collagen and 10 µg VEGF can be attributed to the lower immediate release during incubation, as also seen for FITC-dextran (compare 3.3.2), which resulted in a prolonged stimulatory effect of the cytokine on endothelial cells.

For the samples that were incubated in buffer prior to cell seeding to eliminate the effect of burst released VEGF and to investigate on the stability of the cytokine in swollen collagen matrices, an increase of 25% for the non-modified collagen and a 40% increase for crosslinked collagen could be observed. This is in agreement with findings from prior in vitro release experiments (compare 3.3) where varying amounts of model substrate and $VEGF_{165}$ remained in the collagen film depending on the crosslinking degree. Under in vitro conditions, the remaining substrate could not be released through further incubation for several hours, due to the higher molecular weight of the compounds and the entrapment in the pores of the collagen film that results from it [1]. However, in the case of the growth of endothelial cells, a stimulation of cells after incubation and rinsing was observed, which leads to the conclusion that the collagenolytic activity of the endothelial cells [45] was responsible for degradation of the collagen film and a further release of $VEGF_{165}$, which stimulated further cell growth. These findings show a sustained biological activity of $VEGF_{165}$ in the collagen film, despite the short half life and in vitro recovery as seen before (compare 3.3.3). Therefore, it can be concluded that the incorporation of $VEGF_{165}$ into collagen films stabilized the protein and its biological activity was sustained as shown by cell growth stimulation of collagen coated samples incubated for more than 24 hours.

4 Conclusions

The goal of the study was to show the cell growth stimulatory effect of $VEGF_{165}$ released from collagen films as potential coatings for vascular grafts to enhance their in vivo endothelialization.

Early results for the release of FITC-dextran showed fast release rates from collagen films within the first 2 hours. In order to modify these release characteristics, collagen crosslinking was performed using carbodiimide chemistry. Characterization of the crosslinked material was performed using DSC measurements to confirm the efficiency of the procedure and the successful crosslinking. In addition, the postulated low toxicity of carbodiimide crosslinked collagen was successfully confirmed using endothelial cells. Release studies, using FITC-dextran 40 kDa, showed modified release rates for the crosslinked collagen materials. It was shown that with increasing crosslinking degree, the initial release of model compound was reduced in accordance to the crosslinking degree. Therefore, crosslinking of collagen was shown to be an effective tool to modify release properties of collagen without risking the good biocompatibility of this material. Furthermore, the results obtained for FITC-dextran formed the basis for the investigation of $VEGF_{165}$ containing films concerning their release behavior. The predetermined release properties could be successfully confirmed for the endothelial cell specific growth factor. Therefore, the modified collagen materials constitute a suitable carrier for controlled release of VEGF.

Furthermore, it was of central interest to investigate the biological activity of the incorporated VEGF, after processing of the collagen films and subsequent release on cells. Therefore, VEGF incorporated into collagen films was investigated towards its effect on endothelial and smooth muscle cells. No stimulatory effect on SMCs could be shown. This is an important prerequisite for the application in vascular grafts, since an excessive growth could lead to vessel occlusion, the reason for the unsuitability of i.e. basic fibroblast growth factor. Furthermore, and more importantly, an increase in endothelial cell growth by VEGF released from collagen films was shown. Thus, it was possible to maintain the biological activity of a sufficient amount

of VEGF throughout the manufacturing and drying process of collagen films and the subsequent release to stimulate endothelial cell growth.

Therefore, Collagen film coatings carrying $VEGF_{165}$ constitute an interesting alternative for the modification of vascular grafts to enhance their endothelialization, and thereby their long term performance.

5 References

1. Friess, W., *Drug Delivery Systems Based on Collagen.* 1999.
2. Chen, M.-C., et al., *A novel drug-eluting stent spray-coated with multi-layers of collagen and sirolimus.* Journal of Controlled Release, 2005. **108**(1): p. 178-189.
3. Fujioka, K., et al., *Protein release from collagen matrixes.* Advanced Drug Delivery Reviews, 1998. **31**(3): p. 247-266.
4. Geiger, M., R.H. Li, and W. Friess, *Collagen sponges for bone regeneration with rhBMP-2.* Advanced Drug Delivery Reviews, 2003. **55**(12): p. 1613-1629.
5. Pieper, J.S., et al., *Preparation and characterization of porous crosslinked collagenous matrices containing bioavailable chondroitin sulphate.* Biomaterials, 1999. **20**(9): p. 847-858.
6. Wachem, P.B.v., et al., *In vivo biocompatibility of carbodiimide-crosslinked collagen matrices: Effects of crosslink density, heparin immobilization, and bFGF loading.* Journal of biomedical materials research, 2001. **55**(3): p. 368-378.
7. Wissink, M.J.B., et al., *Improved endothelialization of vascular grafts by local release of growth factor from heparinized collagen matrices.* Journal of Controlled Release, 2000. **64**(1-3): p. 103-114.
8. Scharn, D.M., et al., *Thrombogenicity and Related Biological Properties of Heparin Bonded Collagen Coated Polyester and Human Umbilical Vein Prosthetic Vascular Grafts.* Journal of Surgical Research, 2006. **134**(2): p. 182-189.
9. Lepidi, S., et al., *bFGF release is dependent on flow conditions in experimental vein grafts.* European Journal of Vascular and Endovascular Surgery, 1995. **10**(4): p. 450-458.
10. Wissink, M.J.B., et al., *Endothelial cell seeding of (heparinized) collagen matrices: effects of bFGF pre-loading on proliferation (after low density seeding) and pro-coagulant factors.* Journal of Controlled Release, 2000. **67**(2-3): p. 141-155.
11. Weatherford, D.A., et al., *Vascular endothelial growth factor and heparin in a biologic glue promotes human aortic endothelial cell proliferation with aortic smooth muscle cell inhibition.* Surgery, 1996. **120**(2): p. 433-439.
12. Crombez, M., et al., *Improving arterial prosthesis neo-endothelialization: Application of a proactive VEGF construct onto PTFE surfaces.* Biomaterials, 2005. **26**(35): p. 7402-7409.
13. Randone, B., et al., *Dual Role of VEGF in Pretreated Experimental ePTFE Arterial Grafts.* Journal of Surgical Research, 2005. **127**(2): p. 70-79.
14. Greisler, H.P., *Growth factor release from vascular grafts.* Journal of Controlled Release, 1996. **39**(2-3): p. 267-280.
15. Lindner, V., et al., *Role of Basic Fibroblast Growth Factor in Vascular Lesion Formation.* Circulation Research, 1991. **68**(1): p. 106-113.
16. Ferrara, N. and T. Davis-Smyth, *The Biology of Vascular Endothelial Growth Factor.* Endocr Rev, 1997. **18**(1): p. 4-25.
17. Metzmacher, I., *Enzymatic degradation and drug release behavior of dense collagen implants*, in *Pharmaceutical Technology*. 2005, LMU: Munich. p. No pp given.
18. Charulatha, V. and A. Rajaram, *Influence of different crosslinking treatments on the physical properties of collagen membranes.* Biomaterials, 2003. **24**(5): p. 759-767.
19. Radu, F.A., et al., *Modeling of drug release from collagen matrices.* J. Pharm. Sci., 2002. **91**(4): p. 964-972.

20. Reháková, M., et al., *Properties of collagen and hyaluronic acid composite materials and their modification by chemical crosslinking.* Journal of biomedical materials research, 1996. **30**(3): p. 369-372.
21. Friess, W. and G. Lee, *Basic thermoanalytical studies of insoluble collagen matrices.* Biomaterials, 1996. **17**(23): p. 2289-2294.
22. Geiger, M., *Porous Collagen/Ceramic Composite Carriers for Bone Regenaration Using Recombinant Human Bone Morphogenetic Protein-2 (rhBMP-2).* PhD Thesis, FAU Erlangen, Germany, 2001.
23. Williams, S.K., et al., *Adult human endothelial cell compatibility with prosthetic graft material.* Journal of Surgical Research, 1985. **38**(6): p. 618-629.
24. Wang, X.H., et al., *Crosslinked collagen/chitosan matrix for artificial livers.* Biomaterials, 2003. **24**(19): p. 3213-3220.
25. Amiji, M. and K. Park, *Surface modification of polymeric biomaterials with poly(ethylene oxide), albumin, and heparin for reduced thrombogenicity.* Journal of biomaterials science. Polymer edition, 1993. **4**(3): p. 217-34.
26. Heyligers Jan, M.M., et al., *Heparin immobilization reduces thrombogenicity of small-caliber expanded polytetrafluoroethylene grafts.* Journal of vascular surgery official publication, the Society for Vascular Surgery [and] International Society for Cardiovascular Surgery, North American Chapter, 2006. **43**(3): p. 587-91.
27. Llanos, G.R. and M.V. Sefton, *Immobilization of poly(ethylene glycol) onto a poly(vinyl alcohol) hydrogel: 2. Evaluation of thrombogenicity.* Journal of biomedical materials research, 1993. **27**(11): p. 1383-91.
28. Pärsson, H., et al., *Acute thrombogenicity and 4 weeks healing properties of a new stretch-ePTFE graft.* European Journal of Vascular Surgery, 1993. **7**(1): p. 63-70.
29. Guidoin, R., et al., *Can collagen impregnated polyester arterial prostheses be recommended as small diameter blood conduits?* Asaio J, 1996. **42**(6): p. 974-83.
30. Keuren, J.F.W., et al., *Covalently-Bound Heparin Makes Collagen Thromboresistant.* Arterioscler Thromb Vasc Biol, 2004. **24**(3): p. 613-617.
31. Ferrara, N., et al., *The vascular endothelial growth factor family of polypeptides.* J. Cell. Biochem., 1991. **47**(3): p. 211-18.
32. Sano, A., et al., *Atelocollagen for protein and gene delivery.* Advanced Drug Delivery Reviews, 2003. **55**(12): p. 1651-1677.
33. Maeda, M., et al., *Microstructure and release characteristics of the minipellet, a collagen-based drug delivery system for controlled release of protein drugs.* Journal of Controlled Release, 1999. **62**(3): p. 313-324.
34. Ruszczak, Z. and W. Friess, *Collagen as a carrier for on-site delivery of antibacterial drugs.* Advanced Drug Delivery Reviews, 2003. **55**(12): p. 1679-1698.
35. Rosenblatt, J., W. Rhee, and D. Wallace, *The effect of collagen fiber size distribution on the release rate of proteins from collagen matrices by diffusion.* Journal of Controlled Release, 1989. **9**(3): p. 195-203.
36. Metzmacher, I., et al., *A model describing the effect of enzymatic degradation on drug release from collagen minirods.* European Journal of Pharmaceutics and Biopharmaceutics, 2007. **67**(2): p. 349-360.
37. Moscatelli, D., E. Jaffe, and D.B. Rifkin, *Tetradecanoyl phorbol acetate stimulates latent collagenase production by cultured human endothelial cells.* Cell, 1980. **20**(2): p. 343-351.
38. Koch, S., et al., *Enhancing angiogenesis in collagen matrices by covalent incorporation of VEGF.* J. Mater. Sci. Mater. Med., 2006. **17**(8): p. 735-741.

39. Fischer, D., *Degradationsuntersuchung eines Komplexes aus VEGF165 und Kollagen Typ I in vitro im Kreislaufsimulationsmodell: Analyse der Freisetzungskinetik des VEGF165 und der licht- und elektronenmikroskopischen Morphologie des Komplexes*, in *Medizinische Fakultät*. 2006, Westfälische Wilhelms-Universität Münster: Münster. p. No pp given.
40. Gu, F., R. Neufeld, and B. Amsden, *Maintenance of vascular endothelial growth factor and potentially other therapeutic proteins bioactivity during a photo-initiated free radical cross-linking reaction forming biodegradable elastomers.* European Journal of Pharmaceutics and Biopharmaceutics, 2007. **66**(1): p. 21-27.
41. Banerjee, S., et al., *VEGF-A165 Induces Human Aortic Smooth Muscle Cell Migration by Activating Neuropilin-1-VEGFR1-PI3K Axis†.* Biochemistry, 2008. **47**(11): p. 3345-3351.
42. Li, D., et al., *VEGF regulates FGF-2 and TGF-[beta]1 expression in injury endothelial cells and mediates smooth muscle cells proliferation and migration.* Microvascular Research, 2009. **77**(2): p. 134-142.
43. Dorafshar, A.H., et al., *Vascular endothelial growth factor inhibits mitogen-induced vascular smooth muscle cell proliferation.* J. Surg. Res., 2003. **114**(2): p. 179-186.
44. Zisch, A.H., et al., *Covalently conjugated VEGF-fibrin matrices for endothelialization.* Journal of Controlled Release, 2001. **72**(1-3): p. 101-113.
45. Gross, J., et al., *Plasminogen activator and collagenase production by cultured capillary endothelial cells.* J. Cell Biol., 1982. **95**(3): p. 974-981.

Chapter 5

Cell growth stimulation by $VEGF_{165}$ covalently linked to a modified PLGA-matrix

Abstract

It was the goal to investigate the effect of Vascular Endothelial Growth Factor (VEGF) irreversibly linked to a modified poly(lactic-co-glycolic acid) (PLGA) on the growth of endothelial and smooth muscle cells (SMCs). In doing so, it was to be shown whether the irreversible linkage interfered with the mechanism of action of VEGF and its receptor internalization. Commercially available PLGA was modified using a homobifunctional polyethylene glycol (PEG) spacer for protein attachment. $VEGF_{165}$ was covalently attached via succinimidyl ester to discs coated with the modified polymer. Successful binding, potentially maintained VEGF integrity, and optimization of coating and coupling procedures could be shown by employing an antibody staining method, whereas FTIR and other adapted staining methods could not provide further insight.

The preservation of the biological activity of $VEGF_{165}$ was shown by a substantial growth increase of endothelial cells. Furthermore, PEG-PLGA could be shown to have a limiting effect on the proliferation of SMCs, due to its cell-adhesion-resistant properties, in comparison to unmodified PLGA matrices. Furthermore, it was shown that irreversibly linked $VEGF_{165}$ was still able to interact with its receptor and to stimulate endothelial cells.

Keywords: PLGA, VEGF, vascular graft, endothelial cell, covalent linkage, PEG

1 Introduction

After implantation of biomaterial surfaces in a body, the body generally identifies these surfaces as foreign objects resulting in a repair response rather than a regenerative response [1]. These repair responses lead to unwanted side effects which, in the case of vascular grafts for vessel replacement, can lead to complications [2-4]. Local treatment with bioactive molecules constitutes an interesting approach to overcome these problems. The easiest approach for site specific delivery of bioactive molecules to an implantation site is the pre-incubation of the implantable biomaterial in a solution containing the active ingredient, a method commonly used to treat vascular graft infection [5]. However, this method provides little, if any, control over the local delivery which is strongly influenced by the environment of the site of implantation and the patient's condition. A more controlled approach for delivery is the use of matrices or scaffolds that incorporate biomolecules that are released after implantation [6-7]. One big advantage is the controlled release of these molecules over an extended period of time. However, it also poses the risk of dose dumping and systemic side effects once released into the blood in case of vascular grafts.

Another alternative is the chemical attachment of these biomolecules to surfaces to enable a direct interaction of cells of interest with growth enhancers and provide a more controlled approach over cell – biomaterial interactions. However, the question arises whether the chemical attachment of such molecules interferes with their mechanism of action, especially in regards of binding sites being shielded due to linkage, sterical hindrance, conformational changes [8], or loss of receptor internalization [9-11] due to irreversible linkage. Several promising attempts have been carried out to immobilize a variety of proteins on biomaterial surfaces [12-13]. It has also been shown that the introduction of spacer molecules between the substrate surface and the attached biomolecule can be beneficial for the maintenance of the biological activity [14-15]. Furthermore, the use of PEG as a spacer might have the advantage of increasing the half life of used molecules [16-17], resulting in a longer activity, which is a common approach in pegylation of therapeutic proteins [18-20]. Approaches for the chemical attachment of proteins have made use of functional groups present within the sequence of proteins, such

as amino, carboxyl [21], and aldehyd groups [15]. $VEGF_{165}$, the predominant isoform from the VEGF family [22], carries these functional groups within its sequence and makes it accessible for covalent attachment to biomaterials. $VEGF_{165}$, a 42 kDa protein, has been widely investigated for the controlled release and use in artificial graft applications [13, 23-24]. VEGF is involved in several endothelial cell specific activities, such as proliferation and migration [25], at the same time limiting the mitogen-induced vascular SMC proliferation [26]. Therefore, it seems a very promising approach for the improvement of vascular grafts by controlled presentation of $VEGF_{165}$ at the graft surface by chemically attaching the protein to a modified matrix.

The objective of this study was to show a successful covalent linkage of $VEGF_{165}$ to modified PLGA, a commonly used polymer in controlled release applications with biodegradable and biocompatible attributions [27-28], while maintaining the biological activity. The polymer was to be chemically altered using a homobifunctional PEG spacer carrying succinimidyl ester groups to attach the PEG structur to amino moieties within the protein sequence.

The first focus was the evaluation of the linkage of VEGF to the matrix. Therefore, a suitable detection technique using infrared spectroscopy and several staining methods had to be identified. A suitable antibody staining method to determine bound protein needed to be developed and was used to optimize linkage conditions and examine the unspecific adsorption of VEGF on matrices. More importantly, the cell compatibility of the modified PLGA material and the preservation of the biological activity of the attached VEGF were evaluated. Therefore, their effect on HUVECs and SMCs was investigated

2 Materials and Methods

2.1 Materials

Resomer® (RG 502H and RG 504H) was provided by Boehringer Ingelheim (Ingelheim, Germany), $VEGF_{165}$ was kindly donated by Genentech (San Francisco, CA, USA), and rat aortic smooth muscle cells were provided by PD Dr. Wolgang Erl (Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, Ludwig-Maximilians-Universität München, Munich, Germany). *O*,*O'*-Bis(2-amino-propyl) polyethylene glycol ($PEG(NH_2)_2$) with a molecular weight of 500 Da, succinic anhydride, N-Hydroxysuccinimide (NHS), N,N'-Dicyclohexylcarbodiimide (DCCI), dimethyl sulfoxide (DMSO), 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS), Bovine Serum Albumin (BSA), and Reversible Protein Detection Kit were purchased from Sigma (Steinheim, Germany), toluene, and diethyl ether from Merck KGaA (Darmstadt, Germany), triethylamine, dichloromethane, ethylenediamine, ethanolamine, cover glasses, plastic petri dishes from VWR (Darmstadt, Germany), polytetrafluoroethylene (PTFE) from GM GmbH (Freiham, Germany), SilverXPress® Silver Staining Kit, and goat-anti-rabbit antibody Alexa Fluor 488 from Invitrogen (Karlsruhe, Germany), Deep Purple™ Total Protein Stain from GE Healthcare (Freiburg, Germany), rabbit Anti-Human VEGF antibody from Pepro Tech GmbH (Hamburg, Germany), collagenase A from Roche (Penzberg, Germany), endothelial cell growth medium from Provitro (Berlin, Germany), heat-inactivated fetal bovine serum from Biochrom (Berlin, Germany), smooth muscle cell growth medium DMEM /F12 and M199 from PAA (Pasching, Austria), 24-well plates from TPP Techno Plastic Products (Trasadingen, Switzerland), and Cell Titer-Blue® from Promega (Madison, WI, USA).

The pH of the solutions was adjusted using hydrochloric acid or sodium hydroxide from Merck KGaA (Darmstadt, Germany) and measured with a pH meter Inolab level 1 from WTW (Weilheim, Germany).

2.2 Methods

2.2.1 PLGA modification

2.2.1.1 Synthesis of PEG-spacer

A bifunctional PEG-spacer was synthesized using *O*,*O'*-Bis(2-aminopropyl) polyethylene glycol ($PEG(NH_2)_2$) with a molecular weight of 500 Da as starting material. In a first reaction step (Figure 5-1), the amine groups of the PEG were modified into carboxylic groups using succinic anhydride. The reaction was carried out in anhydrous toluene with the addition of triethylamine at room temperature over night.

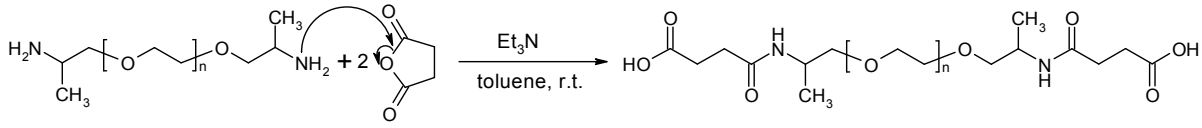


Figure 5-1: Reaction mechanism for the synthesis of PEG-$(COOH)_2$.

The resulting functionalized PEG carrying two carboxylic groups was subsequently activated (Figure 5-2) using N-Hydroxysuccinimide (NHS) and N,N'-Dicyclohexylcarbodiimide (DCCI) to carry two succinimidyl ester. The reaction was carried out in anhydrous dichloromethane at 0°C for 3 hours and subsequently at room temperature over night. The product was recovered and purified by precipitation in cold diethyl ether. The final compound was desiccated under vacuum for several hours and the activity was determined by a TNBS assay (compare 2.2.1.3).

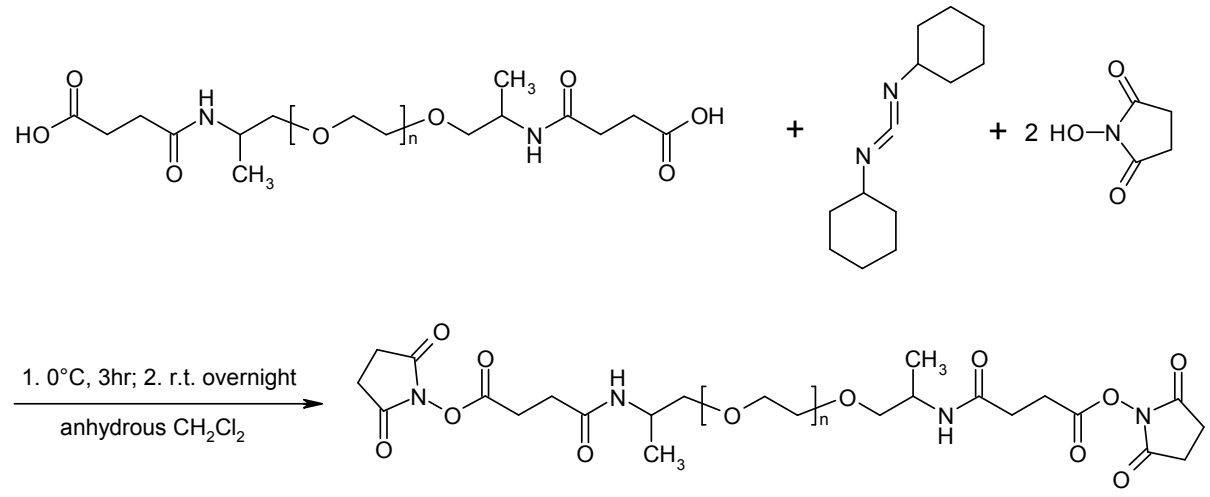


Figure 5-2: Reaction mechanism for the synthesis of PEG-$(NHS)_2$.

2.2.1.2 Synthesis of PEG-PLGA

The free carboxylic groups of two variants of non end-capped PLGA (RG 502H and RG 504H) were activated (Figure 5-3) in a first step using NHS and DCCI. The reaction was carried out in anhydrous dichloromethane at 0°C for 3 hours and subsequently at room temperature over night.

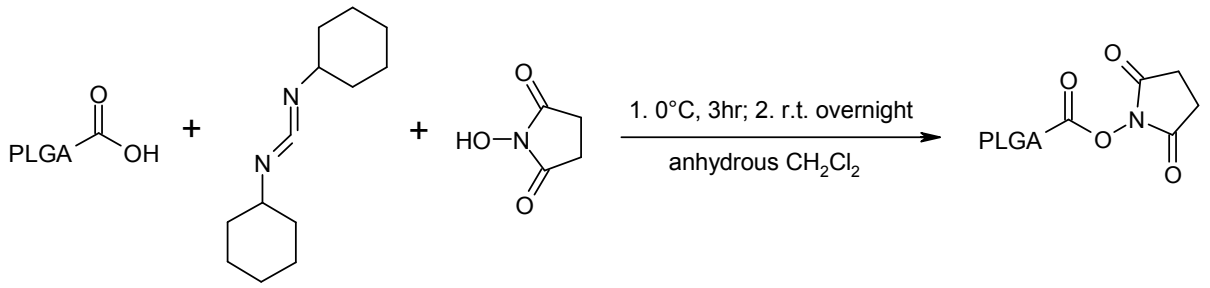


Figure 5-3: Reaction mechanism for the synthesis of activated PLGA.

The resulting activated PLGA carrying a succinimidyl ester was subsequently coupled to ethylenediamine in anhydrous dichloromethane at room temperature over night (Figure 5-4).

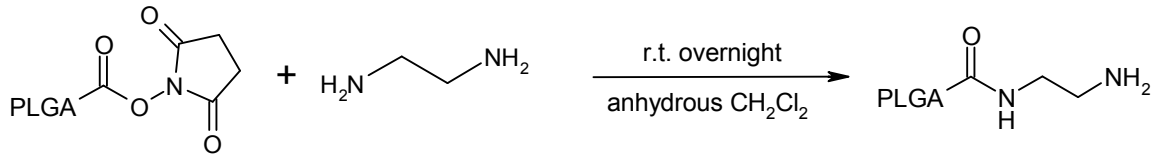


Figure 5-4: Reaction mechanism for the synthesis of PLGA-Ethylenediamine.

In a last step, the PLGA carrying the amine group was coupled to the PEG-spacer in anhydrous dichloromethane at room temperature over night (Figure 5-5).

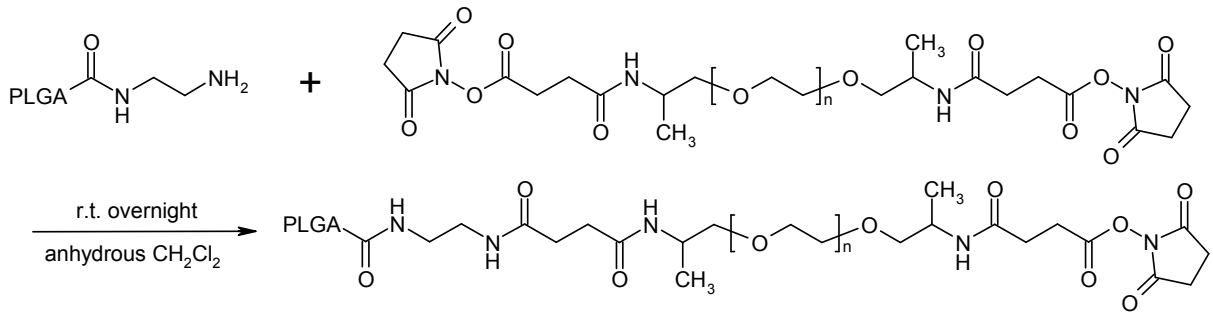


Figure 5-5: Reaction mechanism for the synthesis of PEG-PLGA.

The product was recovered and purified by precipitation in boiling diethyl ether. The final compound was desiccated under vacuum for several hours and the activity was determined by a TNBS assay (compare 2.2.1.3).

2.2.1.3 TNBS assay

The degree of ability of the activated species of PLGA and PEG to couple with primary amines within protein sequences was determined using a modified assay according to Snyder et al [29]. Therefore, the activated species were dissolved in anhydrous DMSO and were coupled with the primary amine of ethanolamine (Figure 5-6).

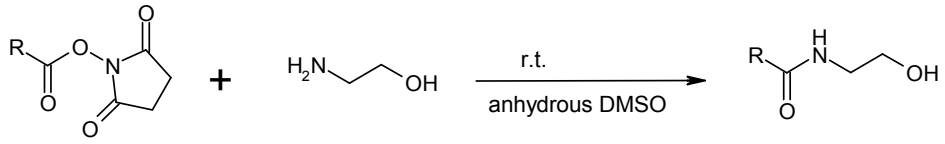


Figure 5-6: Conjugation reaction of activated species with ethanolamine.

In a next step, the free ethanolamine was quantified using TNBS in a 0.1 molar borate buffer pH 8.3. The yellow conjugate (Figure 5-7) formed was quantified via UV-spectroscopy at 420 nm using the Agilent 8453 (Agilent Technologies, Böblingen, Germany). The degree of activation was calculated as % of coupled ethanolamine compared to reference samples containing no activated species.

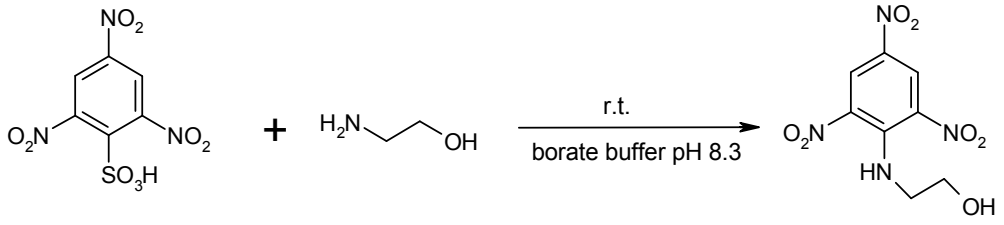


Figure 5-7: Quantification reaction for free ethanolamine.

2.2.2 Coating procedure for PEG-PLGA

Different species of PLGA (RG 504H, activated PLGA and PEG-PLGA) were used to coat PTFE-discs, argon plasma activated with a plasmabrush® (Reinhausen Plasma GmbH, Regensburg, Germany) or cover glasses, both with a diameter of 15 mm. Therefore, the discs were placed in a 24-well aluminum well plate and covered with different concentrations of the synthesized PLGA-species dissolved in anhydrous dichloromethane. The discs were air dried for one hour and subsequently removed from the wells and dried under vacuum over night or until further use. For the cell culture studies all these procedures were performed under a laminar flow workbench (Thermo, Langenselbold, Germany).

2.2.3 $VEGF_{165}$ linkage to modified PLGA matrix

For the linkage of $VEGF_{165}$, PLGA, and PEG-PLGA, coated discs were transferred to plastic petri dishes and covered with different concentrations of $VEGF_{165}$ in phosphate buffered saline (PBS) buffer pH 7.4 for 30 minutes. Reference samples not carrying $VEGF_{165}$ were treated with either PBS buffer alone or with PBS containing 1 mg/ml BSA to block unspecific binding. After incubation, the discs were intensely washed in Milli-Q water and stored in PBS buffer for immediate use. For the cell culture studies all these procedures were performed under a laminar flow workbench. The approach for protein attachment is shown in Figure 5-8.

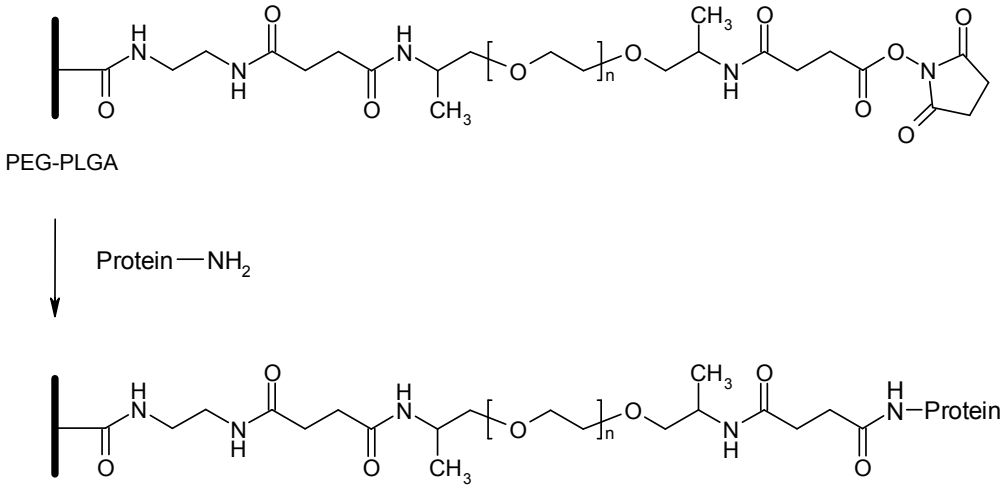


Figure 5-8: Idealized scheme for protein attachment to PEG-PLGA matrix.

2.2.4 Evaluation of detection methods for surface bound $VEGF_{165}$

2.2.4.1 Attenuated Total Reflection- FT-IR Spectroscopy (ATR-FTIR)

FT-IR measurements were performed on a Tensor 27 FT-IR spectrometer (Bruker Optics GmbH, Ettlingen, Germany) using the Miracle ATR unit. In addition, measurements were also performed using a Hyperion 3000 FT-IR microscope (Bruker Optics GmbH, Ettlingen, Germany) using the ATR object lense. The recorded spectra were obtained from 4000 to 900 cm^{-1} wavenumbers in attenuated total reflectance mode at 20°C. Each measurement was the average of 240 scans for the Miracle ATR unit and 64 scans in case of the ATR FT-IR microscope. While

data acquisition was performed, the optical bench was purged with dry nitrogen to reduce interference from water vapour IR absorption and each spectrum was corrected for the background.

2.2.4.2 $VEGF_{165}$ detection using staining methods

For the detection of surface bound $VEGF_{165}$, several staining techniques were evaluated. Silver staining of the discs was performed using a SilverXPress® Silver Staining Kit, Reversible Protein Detection Kit, and Deep Purple™ Total Protein Stain. The stained discs were air dried and the coating was analyzed using an Axiovert 25 microscope (Zeiss, Göttingen, Germany) using a 200fold magnification in case of silver staining and the Reversible Protein Detection Kit. In case of Deep Purple™ Total Protein Stain, stained samples were analyzed using the Zeiss LSM 510 confocal laser scanning microscope (CLSM) (Zeiss, Oberkochen, Germany).

2.2.4.3 Antibody detection

For $VEGF_{165}$ detection, discs were incubated with a rabbit Anti-Human VEGF antibody in PBS buffer pH 7.4 at 2-8°C over night. Thereafter, discs were rinsed with PBS buffer three times to eliminate unbound primary antibody. In a second step, samples were incubated with a secondary goat-anti-rabbit antibody Alexa Fluor 488 for 2 hours at room temperature under exclusion of light. Samples were again rinsed with PBS and subsequently analyzed using the Zeiss LSM 510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

2.2.5 Influence on cell growth by covalent linkage of $VEGF_{165}$

2.2.5.1 Vascular endothelial cells

The influence of discs on the cell growth of vascular endothelial cells was evaluated using Human Umbilical Vein Endothelial Cells (HUVECs). HUVECs were prepared by digestion of umbilical veins with 0.1 g/l collagenase A. Cells were cultured in endothelial cell growth medium supplemented with 10% heat-inactivated fetal

bovine serum in a humidified atmosphere at 5% CO_2 and 37°C. Cells were used at passage no. 3.

2.2.5.2 Rat aortic smooth muscle cells

The influence of discs on the cell growth of SMCs was evaluated using rat aortic SMCs. Cells were cultured in smooth muscle cell growth medium DMEM /F12 supplemented with 1% heat-inactivated fetal bovine serum in a humidified atmosphere at 5% CO_2 and 37°C.

2.2.5.3 Cell growth study

For the study, discs were placed on the bottom of 24-well plates and subsequently covered with a layer of starvation medium in case of HUVECs containing 80% of M199 and 20% of endothelial cell growth medium, in case of SMCs 90% of DMEM /F12 and 10% heat-inactivated fetal bovine serum. 20,000 viable HUVECs or SMCs were seeded per well. Thereafter, cells were cultured in a humidified atmosphere at 5% CO_2 and 37°C. Cells on the discs were quantified at day 3 using Cell Titer-Blue®. Therefore, cells were incubated with Cell Titer-Blue® for 6 hours and an aliquot of the supernatant was analyzed using a Spectrafluor plus plate reader (Tecan, Crailsheim, Germany) with an excitation wavelength of 550 nm and emission recording at 595 nm. The increase in cell growth was calculated as % increase in relation to cells grown on coated discs without $VEGF_{165}$.

3 Results and Discussion

3.1 PLGA modification

The goal of the PLGA modification was the introduction of a functionalized PEG-spacer into the PLGA matrix in order to covalently bind $VEGF_{165}$. It has been shown in literature that the use of spacer molecules between the substrate and the biomolecule to be attached can be beneficial for the maintenance of the biological activity [14-15]. Furthermore, the use of PEG as a spacer might have the advantage of increasing the half life of $VEGF_{165}$ in solution, as seen for other proteins reported in literature [16-17] and results in a longer activity, which is a common approach in pegylation of therapeutic proteins [18-20].

In a first attempt to chemically modify PLGA, RG 502H with a molar ratio of 50:50 of D,L-lactide to glycolide and an approximate molecular weight of 15,000 Dalton was used in combination with a homobifunctional PEG-spacer carrying succinimidyl ester for chemical linkage of amine groups that had a molecular weight of approximately 2,000 Dalton. The activity of all intermediates carrying succinimidyl ester was determined using a modified TNBS assay, and a binding activity of more than 90 % was assured before proceeding with synthesis of the following intermediates or the final product. For the last reaction step to form functionalized PEG-PLGA, the PLGA carrying amine groups was combined with the homobifunctional PEG-spacer. The final product was felled in boiling diethyl ether. However, the recovery of a solid precipitate was not possible. The product that was obtained was a viscous material that did not solidify after desiccation under vacuum for several hours and further additional felling steps to ensure the removal of any byproducts of the synthesis.

PEG has been reported to have a plasticizing effect on polymers in the case of PLGA:PEG di-block copolymers by lowering their glass transition temperature [30]. The plasticizing effect of PEG is based on the reduction of the attractive forces among the polymer chains, which leads to a decrease in the attractive forces leading to an increase in the mobility of the macrochains, resulting in the decrease of the glass transition temperature [31]. Furthermore, studies have shown that in dependency of the molecular weight of the PEG and PLGA employed the glass

transition temperature of resulting conjugates was drastically decreased [30-32]. In one reported case, the combination of PLGA RG 502 with PEG 2000 and 5000 formed compolymers with glass transition temperatures of 1.2°C and -29.9°C respectively [30]. Accordingly, in the case of the combination of the homobifunctional PEG-spacer with RG 502H, the plasticizing effect of PEG resulted in a decrease of the glass transistion temperature, leading to the viscous product.

Consequently, for the second approach to synthesize a functional PEG-PLGA material, the molecular weight of the PEG used for the synthesis of the PEG-spacer was reduced to 600 Dalton. Furthermore, the higher molecular weight PLGA variety RG 504H, with a molecular weight of approximately 50,000 Dalton, was used. Again, a binding activity of above 90 % was assured before proceeding with synthesis of the following intermediates or the final product. The final product was successfully felled in boiling diethyl ether and dried under vacuum for several hours. The product was a solid, white cluster that could be transformed into a white powder. Therefore, it was concluded that the adjustment of molecular weight for PEG and PLGA had lowered the plasticizing effect of PEG leading to solid product. The final product showed a succinimidyl ester activity of 94.4 % and was stored in a desiccator under the exclusion of moisture, due to the moisture sensitivity of the functional group.

Thus, an active PEG-PLGA material was successfully synthesized, but further experiments were necessary to show that the activity of the material was maintained throughout the coating process. Furthermore, the ability to covalently attach $VEGF_{165}$, while maintaining its biological activity, to the PEG spacer needed to be investigated.

3.2 Covalent linkage of $VEGF_{165}$ to modified PLGA matrices

3.2.1 Comparison of $VEGF_{165}$ detection methods

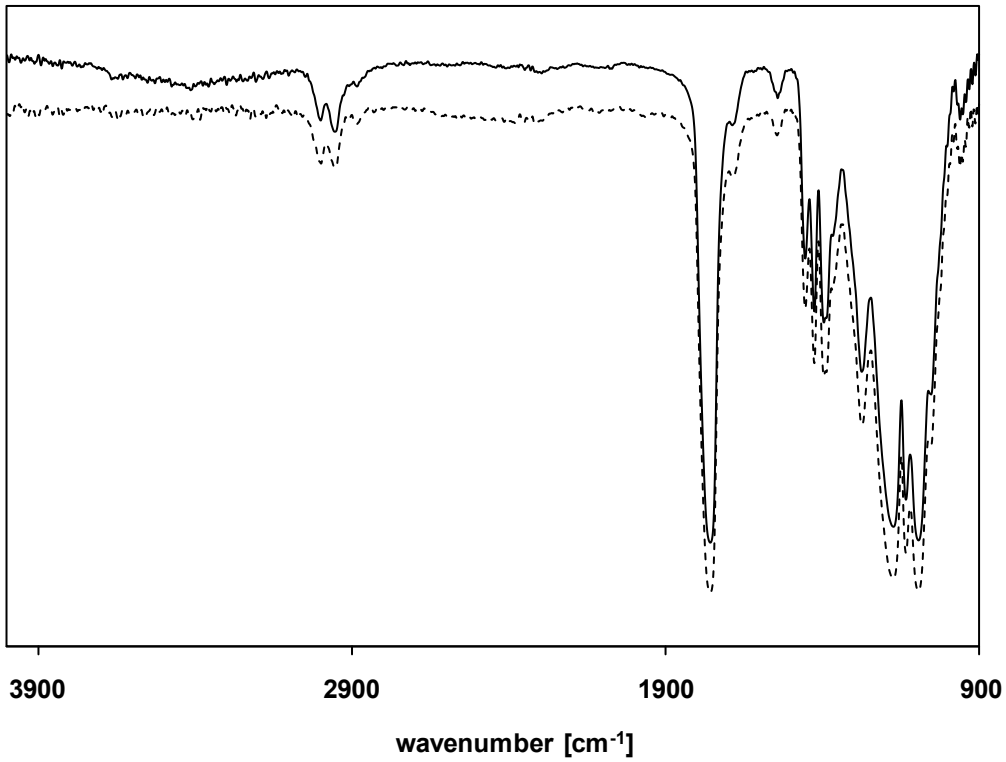
The successful linkage of $VEGF_{165}$ was an important prerequisite for further cell studies and a reliable method of detection therefore a crucial requirement. In order to determine a suitable detection method, commonly used methods for protein

detection, such as gel staining methods [33-34], FT-IR spectroscopy for insight into secondary structural changes [35], and antibody staining were adapted for the use on PLGA matrices and the evaluation of VEGF linkage.

3.2.1.1 FT-IR Spectroscopy

FT-IR spectroscopy is a commonly used method for the investigation of protein secondary structure [35]. This method was anticipated to not only provide information about the presence of $VEGF_{165}$, but could have also been used to characterize the protein's conformational status. Fu et al. were able to demonstrate that in case of PLGA microsphere formulations the C=O stretching vibration occurring at approximately 1750cm^{-1} of the PLGA polymer was well separated from the typical amid I vibration of proteins occurring at 1600 – 1700 cm^{-1} [36]. However, in our case, no differences between the recorded FT-IR spectra of surface bound $VEGF_{165}$ carrying samples and control samples could be detected (Figure 5-9). This was true for either discs coated with activated PLGA as well as PEG-PLGA. In general, spectra recorded for PLGA were consistent with literature [37].

(a)



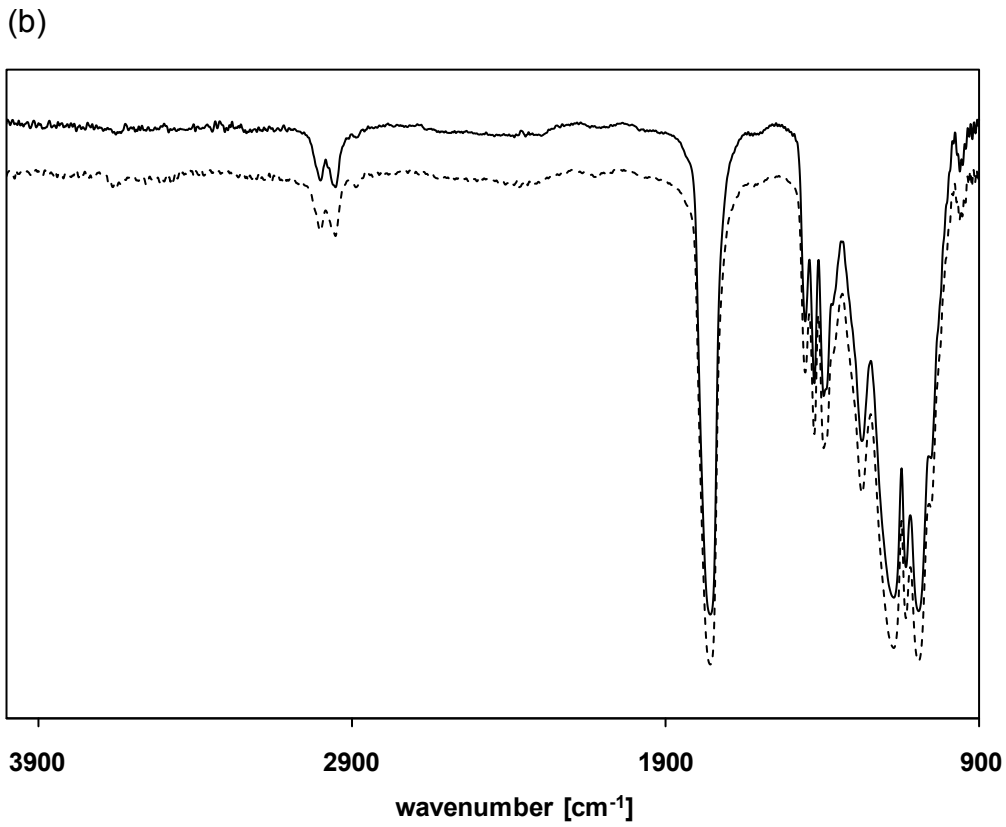


Figure 5-9: FT-IR spectra of PEG-PLGA (a) and activated PLGA (b), both with (----) and without (——) $VEGF_{165}$ with adjusted intensity for better comparability.

Spectra recorded using the more site specific and sensitive FT-IR microscope, confirmed the previous findings. No differences between blanks and the samples carrying $VEGF_{165}$ could be detected. Figure 5-10 shows a close-up of the spectra obtained for PEG-PLGA within the region of 1900 to 1400 cm^{-1}, the region commonly allotted to protein secondary structure, especially at 1650 cm^{-1} assigned to α-helical segments and at 1620 cm^{-1} assigned to intermolecular ß-sheet structures [38] were of interest. However, no changes were observable in this region, nor throughout the whole spectra.

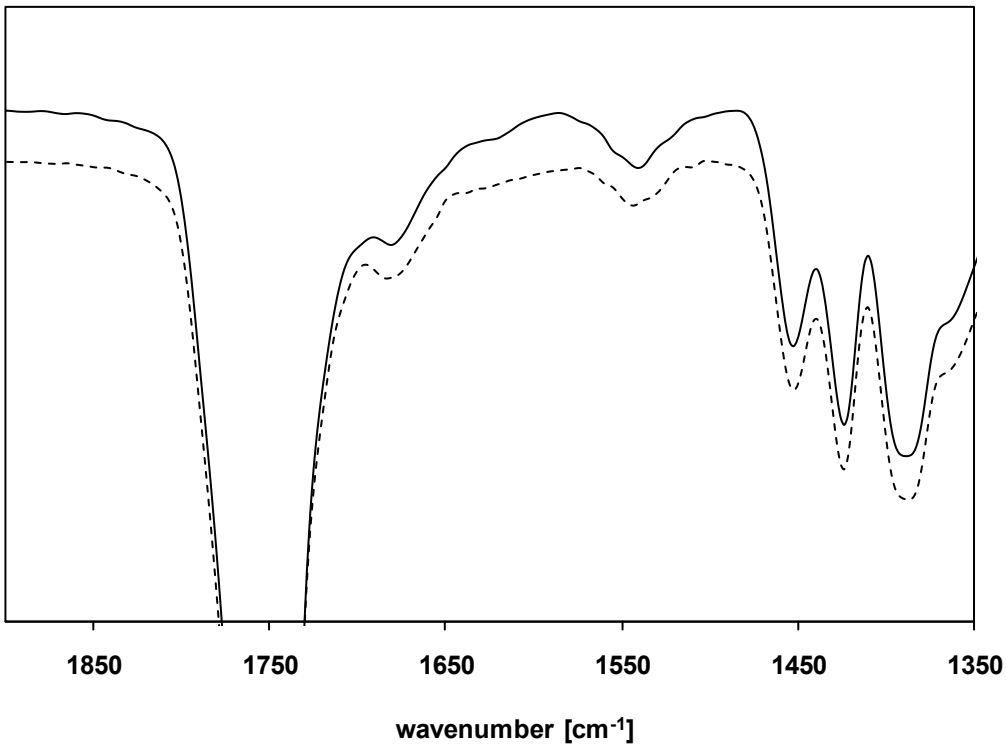


Figure 5-10: FT-IR microscopy spectra of PEG-PLGA with (- - - -) and without (——) $VEGF_{165}$.

The main reason for the unsuccessful detection of $VEGF_{165}$ using FT-IR spectroscopy is most likely the very low concentrations of protein (10 µg/ml) applied on the surfaces, resulting in even lower concentrations bound on the surface. Even with the more site specific FT-IR microscopy the sensitivity for this method could not be increased to a sufficient level to detect $VEGF_{165}$.

3.2.1.2 Reversible Protein Detection Kit

The subsequently applied staining methods were adapted from either gel staining or filter staining procedures, more sensitive methods in comparison to FT-IR spectroscopy. The Reversible Protein Detection Kit is commonly used to detect protein on filter membranes. However, when trying to determine whether the binding procedure for $VEGF_{165}$ on activated PLGA and PEG-PLGA was successful no staining could be detected for the coupled discs and the reference material. Thus, the covalent conjugation of $VEGF_{165}$ to the modified PLGA matrices could not be

determined. A reason for this phenomenon could be the fact that according to Li et al. this rather simple staining method is specific to insoluble protein aggregates on membranes and therefore, no single molecules in native state bound on a surface could be detected [33].

3.2.1.3 Silver staining of covalently linked $VEGF_{165}$

Silver staining is commonly used for protein detection on SDS-PAGE gels when a very high sensitivity is necessary and smallest amounts of protein need to be detected, therefore, silver staining could be useful to determine the covalent binding of $VEGF_{165}$ on discs. The staining was carried out on discs coated with PLGA and PEG-PLGA both coupled with $VEGF_{165}$ and placebo, as negative control. The microscopic images of the discs carrying $VEGF_{165}$ (Figure 5-11c and d) showed areas of black staining compared to the negative controls (Figure 5-11a and b), therefore indicating the presence of protein on the surface of the coated discs. Moreover, a difference in intensity can be seen for the samples carrying PEG-PLGA and $VEGF_{165}$ in comparison to those being coated with activated PLGA and carrying the protein.

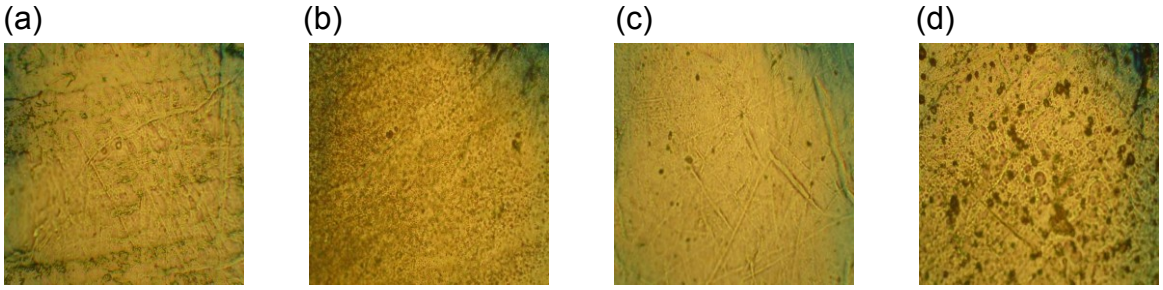


Figure 5-11: Microscopic images of silver stained discs coated with activated PLGA without VEGF as control (a), PEG-PLGA without VEGF as control (b), activated PLGA incubated with 10 µg/ml VEGF (c), and PEG-PLGA incubated with 10 µg/ml VEGF (d).

This was a first indicator for the hypothesis that the PEG-spacer is necessary to ensure accessibility of the linker presented at the surface to the binding sites within the protein structure. Prior findings in literature investigating the influence of spacer length on the effect of surface immobilized VEGF on endothelial cells [15], as well

as other studies investigating the increase in effectiveness by introduction of spacers [39-40] further support this hypothesis. Overall, silver staining provides a useful tool to characterize coated discs and to determine the successful linkage of protein. However, the detection method is very unspecific. An interference of the immobilized PEG-spacer could be ruled out due to previously reported studies that employed silver staining to detect protein in PEG environments [41].

3.2.1.4 Deep Purple™ Total Protein Stain for the detection of $VEGF_{165}$

As an alternative, Deep Purple™ Total Protein Stain was used to stain PEG-PLGA coated discs incubated with buffer and $VEGF_{165}$ (c = 10 µg/ml), in order to determine the successful linkage of the cytokine to the modified PEG-PLGA matrix. Deep Purple™ is a naturally occurring compound, epicocconone, extracted from the fungal species Epicoccum nigrum. Epicocconone reacts reversibly with primary amines in proteins and the result is a highly fluorescent enamine [34]. The stain is commonly used for gel staining and has been shown to be more sensitive towards proteins in comparison to other commercially available gel stains.

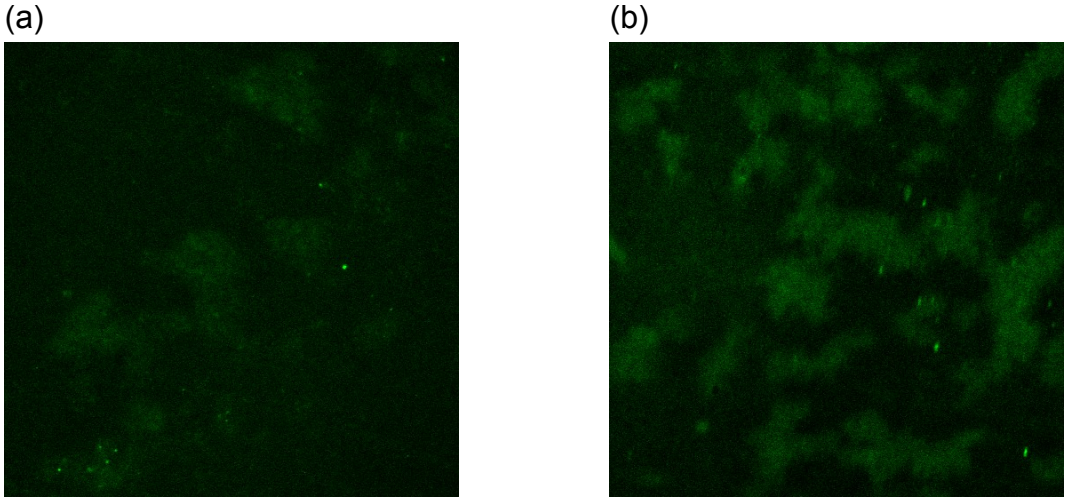


Figure 5-12: CLSM images of Deep Purple™ stained discs coated with PEG-PLGA without VEGF as control (a) and PEG-PLGA incubated with 10 µg/ml VEGF (b).

Figure 5-12 shows the CLSM images for the samples incubated with buffer as control and $VEGF_{165}$. For the samples incubated with the cytokine, a stronger

fluorescence could be detected, however a slight fluorescence was also observed for the control samples. Since the Deep Purple™ stain is a rather unspecific stain interacting with primary amines an unspecific interaction with other adsorbed proteins or unmodified primary amines, in the PEG-PLGA backbone that might have resulted from incomplete modification can not be ruled out. Therefore, the Deep Purple™ Total Protein Stain was another indicator for the linkage of $VEGF_{165}$, but could not provide final proof.

3.2.1.5 Antibody detection of covalently linked $VEGF_{165}$

In order to further characterize and to provide final proof of linkage of VEGF to the coated discs, a more specific approach was required. Therefore, a staining procedure utilizing a VEGF-specific antibody was established. Preliminary results (Figure 5-13) with the antibody detection performed on PEG-PLGA coated discs coupled with and without $VEGF_{165}$ using the primary and secondary antibody indicated a good specificity of the employed method towards the coated and coupled discs and gave first evidence of the sustained integrity of the attached protein.

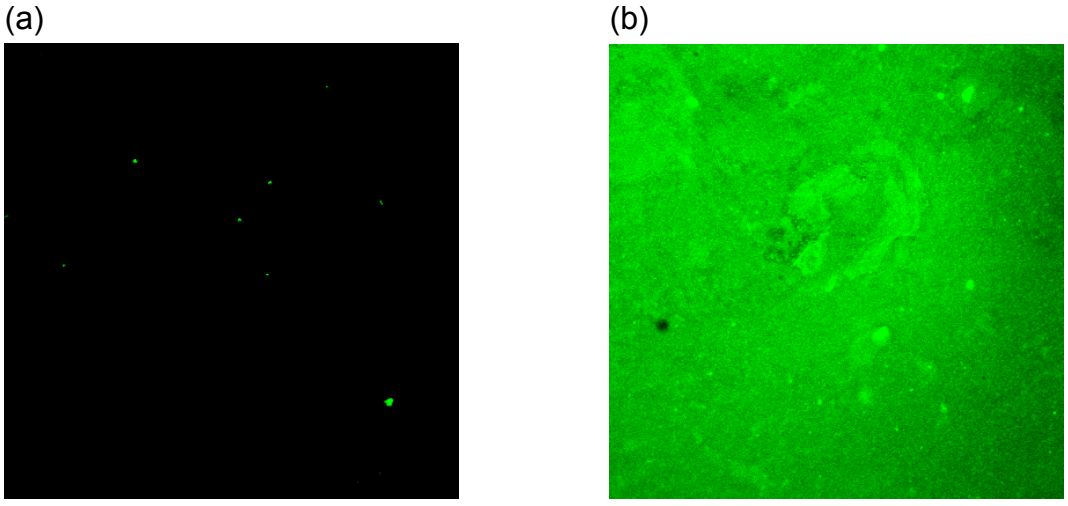


Figure 5-13: CLSM images of antibody stained discs coated with PEG-PLGA incubated without VEGF as control (a) and PEG-PLGA incubated with 10 µg/ml VEGF (b).

However, further investigations, including control experiments with unmodified PLGA coated discs (Figure 5-14), suggested unspecific protein adsorption. Samples coated with PLGA and incubation with $VEGF_{165}$ and samples coated with PLGA and incubation with PBS buffer, stained with both antibodies, showed fluorescence in CLSM (Figure 5-14 a and b). However, samples coated with PLGA, incubation with $VEGF_{165}$, and detection with solely the secondary antibody did not show fluorescence (Figure 5-14 c). These findings support the theory of unspecific protein adsorption of either $VEGF_{165}$ or the primary anti-human-VEGF antibody on the surface of the unmodified PLGA, since there seemed to be no unspecific interaction of the secondary antibody.

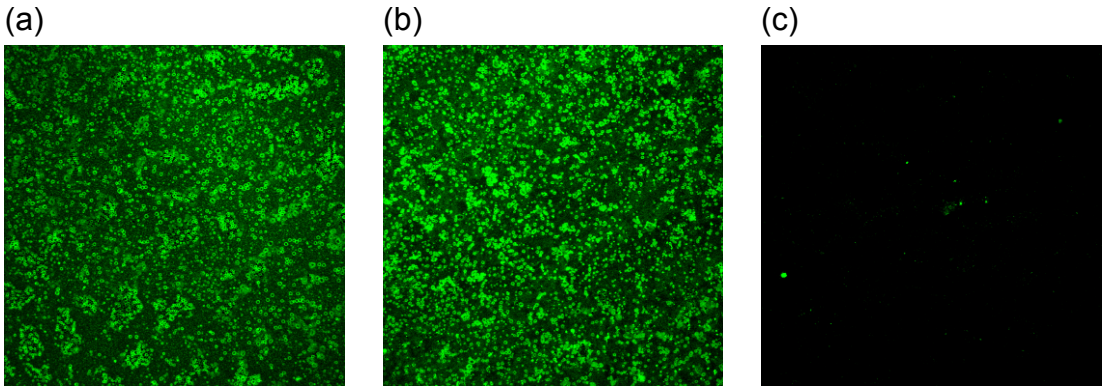


Figure 5-14: CLSM images of antibody stained discs coated with PLGA without VEGF as control (a), PLGA incubated with 10 µg/ml VEGF (b), and PLGA incubated with 10 µg/ml VEGF as control using only the secondary antibody (c).

To further identify and investigate these findings, control experiments using BSA solutions to block unspecific adsorption of $VEGF_{165}$ or the detection antibodies were performed. Therefore, the following incubations (Table 5-1) and analysis were performed and evaluated using CLSM.

Table 5-1: Overview of incubation and detection procedures for the evaluation of unspecific adsorption on PEG-PLGA and PLGA coated discs and order of incubation for PLGA-04 and PLGA-05, as well as PEG-PGLA-04 and PEG-PLGA-05.

	incubation solution			detection antibodies	
Sample	**PBS**	**$VEGF_{165}$ [20 µg/ml]**	**BSA [1 mg/ml]**	**anti-VEGF**	**goat-anti-rabbit**
PLGA-01		X		X	X
PLGA-02	X			X	X
PLGA-03		X			X
PLGA -04		1.) X	2.) X	X	X
PLGA-05		2.) X	1.) X	X	X
PLGA-06			X	X	X
PLGA-07			X		X
PEG-PLGA-01		X		X	X
PEG-PLGA-02	X			X	X
PEG-PLGA-03		X			X
PEG-PLGA-04		1.) X	2.) X	X	X
PEG-PLGA-05		2.) X	1.) X	X	X
PEG-PLGA-06			X	X	X
PEG-PLGA-08			X		X

PLGA coated samples showed a strong fluorescence for several of the incubated samples. A very high fluorescence could be seen for the samples incubated with $VEGF_{165}$, PBS buffer, and those incubated with BSA solutions and $VEGF_{165}$ subsequently (Figure 5-15 a, b and e). A slightly lower fluorescence could be detected for the samples incubated with $VEGF_{165}$ and subsequently BSA (Figure 5-15 d). The sample incubated with BSA, on the other hand, showed an irregular pattern of fluorescence, displaying areas of no intensity (Figure 5-15 g) or areas of spotted intensity (Figure 5-15 f). The control samples incubated with $VEGF_{165}$ and BSA (Figure 5-15 c and g), for which only the interaction of the secondary antibody was evaluated, showed no significant fluorescence.

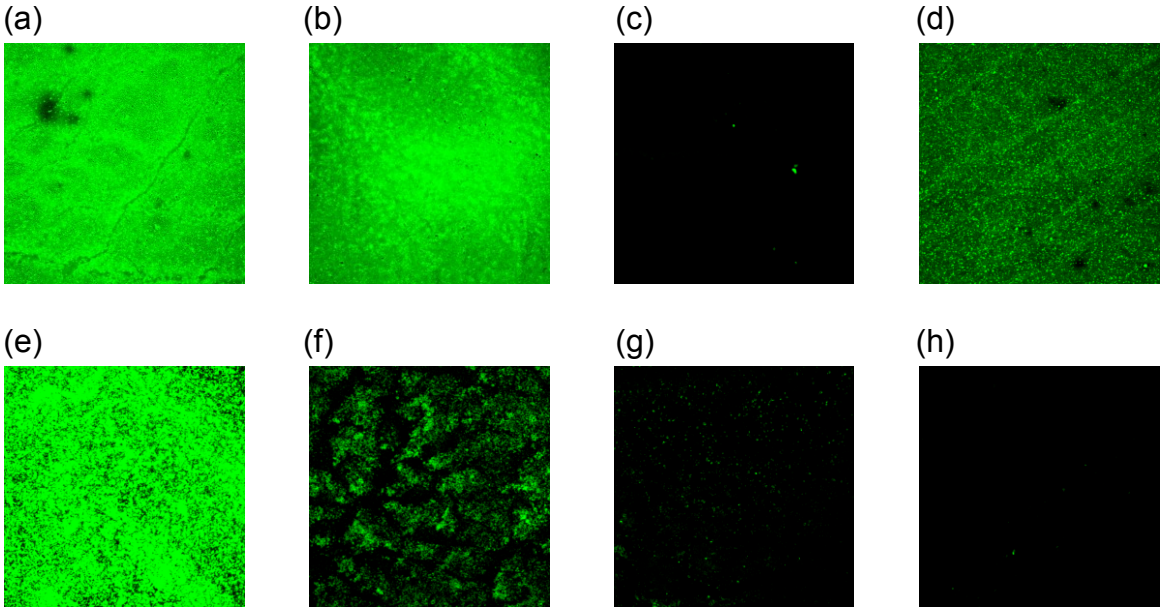


Figure 5-15: CLSM images of antibody stained discs PLGA-01 (a), PLGA-02 (b), PLGA-03 (c), PLGA-04 (d), PLGA-05 (e), PLGA-06.1 (f), PLGA-06.2 (g), and PLGA-07 (h).

Therefore, it can be concluded that there was no unspecific interaction of the secondary goat-anti-rabbit antibody with the surfaces, since no fluorescence could be observed for the samples treated solely therewith. However, there was an interaction observed for the anti-VEGF-antibody with the surface which can be clearly seen by the high fluorescence of the sample being incubated with buffer alone and to a lesser degree the sample being incubated with BSA. The interaction of $VEGF_{165}$ with the unmodified PLGA matrix could not be assessed. The unspecific interaction of the primary anti-VEGF-antibody made it impossible to determine the origin of the fluorescence, whether it was due to the antibody interacting with $VEGF_{165}$ or solely with the surface. The sample incubated with BSA and subsequently with $VEGF_{165}$ could be seen as a first indicator for the interaction of $VEGF_{165}$ with the surface. The increased fluorescence compared to the sample solely incubated with PBS buffer must have originated from $VEGF_{165}$ adsorbed onto the PLGA surface since an interaction of the primary antibody with the surface can be eliminated due to the prior incubation with BSA. The incubation with BSA could be shown to lower the unspecific interaction of the primary antibody. However, it was not possible to eliminate the interaction entirely.

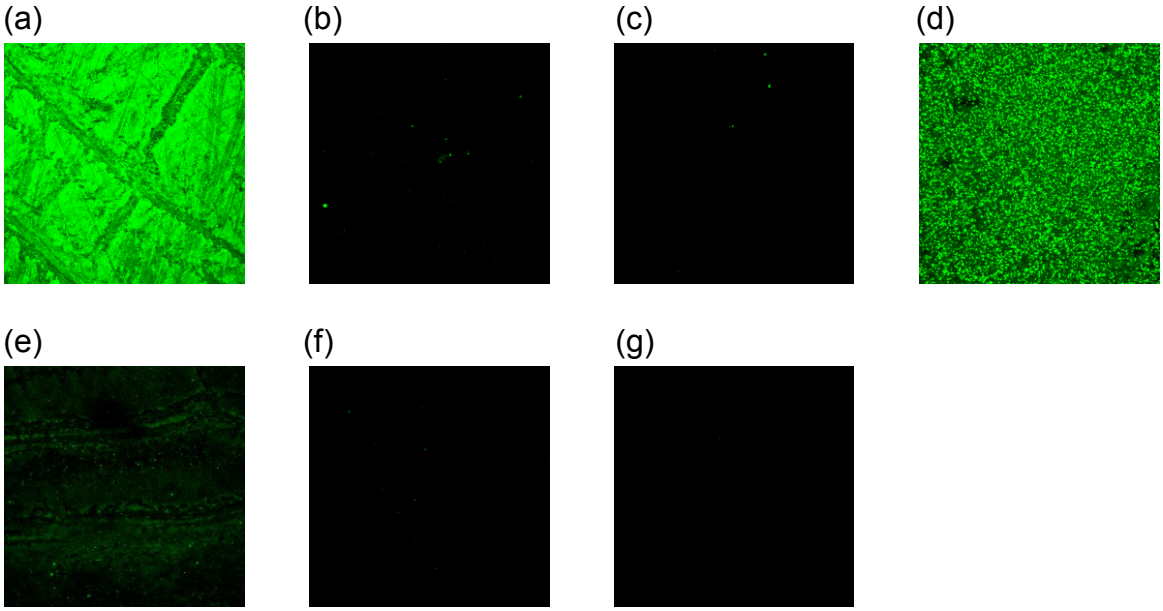


Figure 5-16: CLSM images of antibody stained discs PEG-PGLA-01 (a), PEG-PLGA-02 (b), PEG-PLGA-03 (c), PEG-PLGA-04 (d), PEG-PLGA-05 (e), PEG-PLGA-06 (f), and PEG-PLGA-07 (g).

Images obtained using CLSM for the samples coated with PEG-PLGA, on the other hand, were in accordance with previous findings and could not support the theory of unspecific protein binding on the surface of the coatings in contrast to the results obtained for PLGA matrices. The control samples using only the secondary detection antibody (Figure 5-16 c and g) exhibited no fluorescence, as well as the samples incubated with BSA solutions and PBS buffer detected with both antibodies (Figure 5-16 b and f). These findings are in accordance with literature, where the influence of PEG to modify the cell and protein adhesive properties has been investigated. It has been shown that the incorporation of PEG into polymer and biomaterial surfaces can decrease the unspecific adhesion of proteins, but allowed a controlled attachment of cells [42-44]. The samples incubated with BSA solution and subsequently $VEGF_{165}$ showed slight fluorescence, indicating the existence of still active functional groups that are able to couple $VEGF_{165}$ even after the incubation with BSA, in contrary to literature, where half lives of succinimidyl esters of less than 30 minutes are documented [45]. Samples incubated with $VEGF_{165}$ and those additionally treated with BSA demonstrated a strong fluorescence (Figure 5-16 a and d).

Therefore, it can be concluded, that a successful linkage of $VEGF_{165}$ to the modified PLGA-matrix was accomplished. Furthermore, unspecific adsorption of VEGF on the PEG-PLGA matrix could be ruled out and more importantly, the integrity of $VEGF_{165}$ throughout the preparation process was conserved, as seen by successful binding of the anti-VEGF antibody.

3.2.2 Influence of PLGA coating concentration on $VEGF_{165}$ linkage

Since the main objective of the present study was to investigate the effect of covalently attached VEGF on endothelial cells and smooth muscle cells, rather than to examine the dose-dependent effects of immobilized VEGF, the goal was to bind a maximal amount of VEGF on the surface. Therefore, in order to elucidate the effect of coating thickness or ablation of PEG-PLGA on the effectiveness of VEGF linkage, as well as to identify the conditions for maximum VEGF attachment, PTFE discs were coated with varying concentrations of PEG-PLGA. The coating solutions had a concentration of 0.1, 0.5, 1.0 and 3.0 % of PEG-PLGA in dichloromethane. Subsequently, all discs were incubated with $VEGF_{165}$ solution (20 µg/ml) or PBS-buffer. The bound $VEGF_{165}$ was detected with the antibody detection method as described above (compare 2.2.4.3).

The images that were obtained using CLSM (Figure 5-17) showed an increase in fluorescence with increasing concentration of PEG-PLGA that was used to coat the samples up to 1.0 %. The fluorescence reached a maximum for the samples coated with 1.0 % PEG-PLGA that was not further increased when increasing the concentration to 3.0 %.

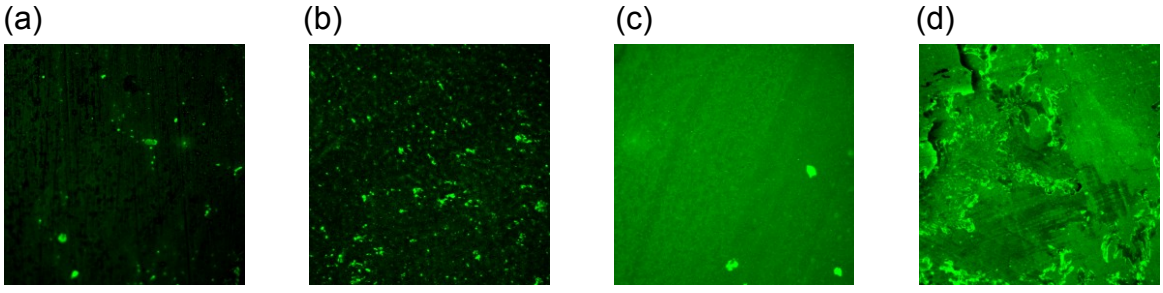


Figure 5-17: CLSM images of antibody stained discs coated with 0.1% (a), 0.5% (b), 1.0% (c), and 3.0% (d) PEG-PLGA and subsequent incubation with 20.0 µg/ml $VEGF_{165}$.

An increase in fluorescence observed in CLSM has a direct correlation with the amount of VEGF bound on the surface of the matrices. Since a maximum coverage of the surface with VEGF seems favorable to ensure a lasting effect of the cytokine during incubation and cell growth experiments, the coatings with the highest fluorescence observed appeared advantageous over the one's with lower fluorescence. Furthermore, in order to eliminate the risk of ablation of coating due to increased coating thickness, it was decided to proceed with the 1 % PEG-PLGA concentration for the further studies.

3.2.3 Influence of $VEGF_{165}$ concentration on the linkage procedure

A second parameter investigated was the effect of $VEGF_{165}$ concentration in the incubation solutions on VEGF binding. Again, the goal was to achieve good linkage efficiency with maximum coverage of the PEG-PLGA surface with VEGF. Therefore, discs were coated with PEG-PLGA in dichloromethane (compare 2.2.2) with a concentration of 1 % (w/v), and incubated for 30 minutes with varying concentrations of 2.0, 10.0, and 20.0 µg/ml $VEGF_{165}$ and analyzed using the antibody detection method.

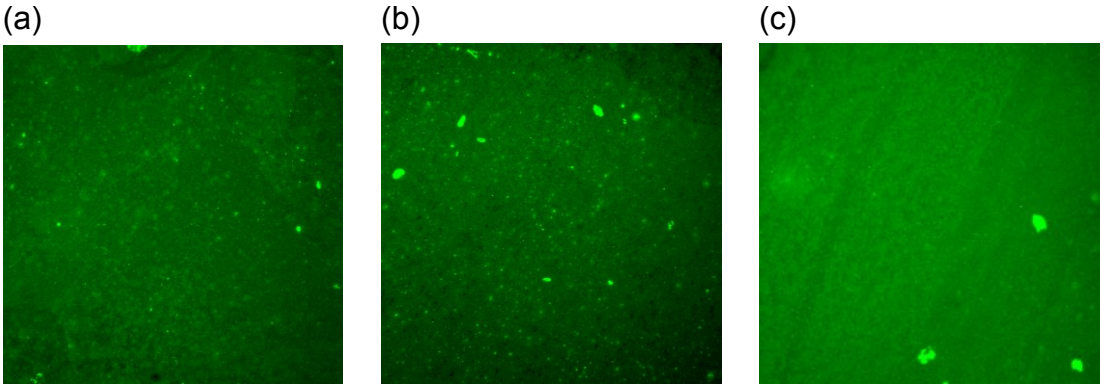


Figure 5-18: CLSM images of antibody stained PEG-PLGA discs incubated with 2.0 µg/ml (a), 10.0 µg/ml (b), and 20 µg/ml (c).

No clear difference in fluorescence between the samples incubated with 2.0 and 10.0 µg/ml were detectable (Figure 5-18 a and b), but the fluorescence seemed to intensify for the samples incubated with 20 µg/ml $VEGF_{165}$ (Figure 5-18 c)

suggesting better linkage efficiency. Consequently, since the goal was to bind a maximal amount of VEGF on the PEG-PLGA surfaces, rather than to examine the dose-dependent effects of immobilized VEGF, it was decided to perform all further linkage experiments with a concentration of $VEGF_{165}$ in the incubation solution of 20 µg/ml.

3.3 Influence of covalently linked $VEGF_{165}$ on cell growth

3.3.1 Vascular endothelial cells

In order to evaluate the activity of the covalently linked $VEGF_{165}$ and therewith the accessibility of the VEGF surface receptor (VEGFR-1) [15, 22] on the endothelial cells towards the covalently linked protein, cell growth studies were performed. In addition, these experiments were also meant to give further insight into the necessity of internalization of the receptor complex in order for the cytokine to stimulate proliferation of the cells, as described before in literature [9-11]. Additionally, several control experiments were performed to elucidate the effect of loosely adsorbed protein on the surface of unmodified coated discs. Therefore, PLGA and PEG-PLGA coated discs were treated with different incubation solutions in accordance to Table 5-2 and immediately thereafter rinsed and incubated with endothelial cells. After a 3 day incubation period, cells were quantified using Cell Titer-Blue®. The increase in cell growth was calculated as % increase in relation to the amount of cells grown on the PLGA and PEG-PLGA coated discs incubated with PBS buffer.

Table 5-2: Overview of incubation solutions for the evaluation of endothelial cell growth on PEG-PLGA and PLGA coated discs and order of incubation solutions applied.

	incubation solution		
Sample	**PBS buffer**	**$VEGF_{165}$ [20 µg/ml]**	**BSA [1 mg/ml]**
PLGA (control)	X		
PLGA (VEGF)		X	
PLGA (BSA)			X
PLGA (BSA + VEGF)		2.) X	1.) X
PLGA (VEGF + BSA))		1.) X	2.) X
PEG-PLGA (control)	X		
PEG-PLGA (VEGF)		X	
PEG-PLGA (BSA)			X
PEG-PLGA (BSA + VEGF)		2.) X	1.) X
PEG-PLGA (VEGF + BSA))		1.) X	2.) X

The results showed an increase in cell growth for PLGA coated discs incubated with $VEGF_{165}$ of around 30% compared to the discs incubated with buffer alone (Figure 5-19). The same increase in cell growth was detected for PLGA coated discs incubated with BSA and $VEGF_{165}$ subsequently. This leads to the conclusion that BSA could not be used to block all unspecific adsorption of $VEGF_{165}$ on the PLGA surface. PLGA discs incubated with BSA alone showed an extent of cell growth comparable to the discs incubated with buffer solely, therefore, eliminating an interference of BSA with the cell growth study. Investigations reported in literature for hydrophobic and hydrophilic surfaces came to the conclusion that the incubation procedure for BSA, especially such parameters as concentration and incubation time, had a strong influence on the blocking efficiency [46-47], which leads to the conclusion that the incubation conditions chosen for this setting needed further optimization. Furthermore, it has been reported that incubation of BSA only blocked 50 % of unspecific binding sites on hydrophobic surfaces [48] under various incubation conditions. Discs coated with PLGA incubated with $VEGF_{165}$ and subsequently with BSA showed an increase in cell growth comparable to the aforementioned discs. Therefore, all variations of incubation solutions containing $VEGF_{165}$ on PLGA coated discs showed comparable cell growth increase for

endothelial cells. Therefore, these results suggested an insufficient blockage of unspecific binding sites by BSA, which might have caused unspecific interaction of the cytokine $VEGF_{165}$ with the unmodified surface, leading to the increase in cell growth.

The modified PEG-PLGA discs that were incubated with $VEGF_{165}$ also showed an increase in cell growth. The effect was more pronounced as compared to the unmodified PLGA discs incubated with the same solution. $VEGF_{165}$ coupled PEG-PLGA discs exhibited a cell growth increase of approximately 50%, 20% more than the unmodified species. The same holds true for the PEG-PLGA discs incubated with $VEGF_{165}$ and subsequently BSA and vice versa. No differences could be observed for the order of incubation for $VEGF_{165}$ and BSA. It was expected to see a lower cell growth increase for the samples first incubated with BSA, due to inactivation of the active binding site for $VEGF_{165}$. Yet, this observation was not made, which leads to the proposition that the incubation time with BSA was not sufficient to inactivate the PEG-PLGA coating, which was therefore still carrying functional groups able to bind to $VEGF_{165}$. This was already observed in previous experiments when determining the linking sufficiency by antibody detection (compare 3.2.1.5). The surface charge of the different reaction partners under the chosen conditions might have influenced the efficiency of the linkage. BSA exhibits a negative surface charge [49], in contrast to VEGF, which has a positive charge at the chosen pH [50]. Taking into consideration that PLGA is reported to have a negative overall charge due to its carboxylic groups [51] at physiological pH, the repulsion between the also negatively charged BSA might have led to a decrease in linking efficiency and thus leading to residual active functional groups able to bind $VEGF_{165}$. Furthermore, PEG-PLGA coated discs incubated with BSA alone exhibited a cell growth increase of approximately 20 % compared to the control group grown on PEG-PLGA. This difference might be attributed to the cell repellent properties of PEG [52-53] present on the control group. These repellent properties might have been compensated by the covalent attachment of BSA leading to an increased cell growth.

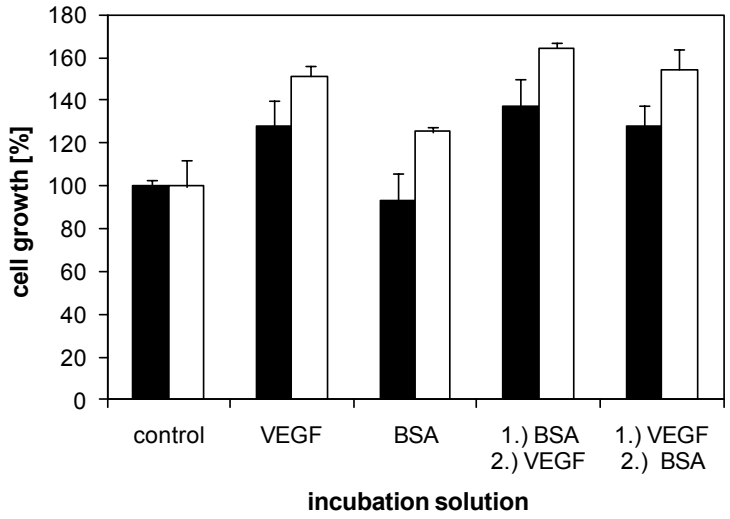


Figure 5-19: Endothelial cell growth on PLGA (■) and PEG-PLGA (▭) coated discs after treatment with different incubation solutions. (average ± SD; n=3)

These results indicate an unspecific interaction of the cytokine $VEGF_{165}$ with unmodified PLGA matrices and an adjunctive increase in cell growth. In addition, it was shown that the incubation with BSA was not able to block unspecific adsorption on unmodified PLGA, as well as linkage of $VEGF_{165}$ on PEG-PLGA, as was already seen for earlier investigations using the antibody staining method (compare 3.2.1.5) However, the linkage of $VEGF_{165}$ to modified PLGA matrices shows superiority over this unspecific effect, which can be seen by an increased cell growth. Covalent attachment of $VEGF_{165}$ did not interfere with the mechanism of action, as seen by the stimulation of endothelial cell growth increase, which was in accordance with findings reported in literature [15]. $VEGF_{165}$ was still capable to interact with the endothelial cell receptor and stimulate proliferation. Yet, it is still unclear whether $VEGF_{165}$ had to be separated from the PEG-PLGA back bone through degradation or digestion to be internalized to show an effect as reported in literature [9-11], or was able to activate the proliferation while still being attached to the surface [15].

3.3.2 Cell growth comparison study for smooth muscle and endothelial cells

In order to further elucidate the influence of the coupled $VEGF_{165}$ on other cells, a further study was initiated to investigate the effect on SMCs in comparison to endothelial cells. Therefore, discs were coated with PLGA and PEG-PLGA and subsequently incubated in buffer, BSA or $VEGF_{165}$. The samples were intensely rinsed after incubation and consequently incubated with endothelial cells and smooth muscle cells according to 2.2.5.3. After a 3 day incubation period, cells were quantified using Cell Titer-Blue®. The increase in cell growth was calculated as % increase in relation to the amount of cells grown on the PLGA and PEG-PLGA coated discs incubated with PBS buffer.

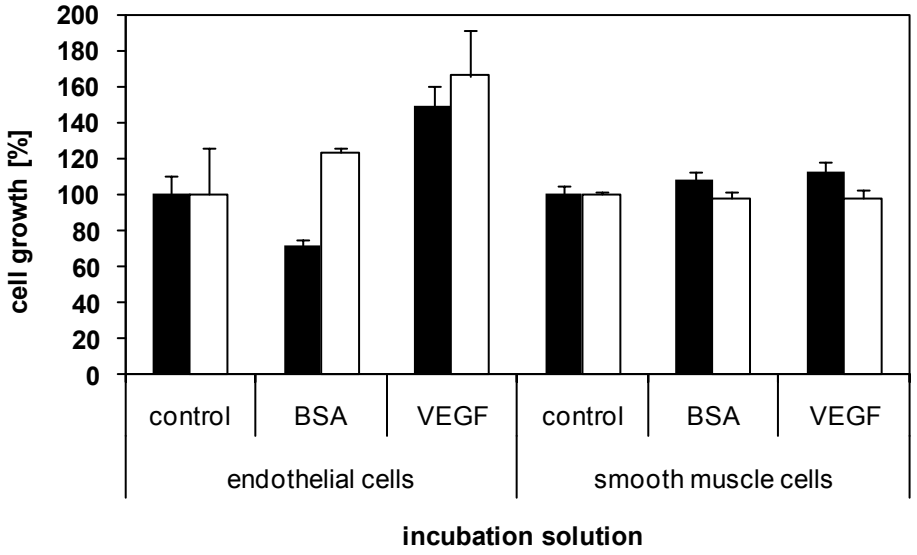


Figure 5-20: Endothelial and smooth muscle cell growth on PLGA (■) and PEG-PLGA (▭) coated discs after treatment with different incubation solutions. (average ± SD; n=3)

Figure 5-20 shows the effect on endothelial and smooth muscle cell growth for the PLGA and PEG-PLGA samples incubated with buffer, BSA, and $VEGF_{165}$. As described before (compare 3.3.1), an increase in cell growth could be detected for the samples incubated with $VEGF_{165}$. The increase for PLGA after incubation with $VEGF_{165}$ was in accordance with previous findings (compare 3.3.1), most likely originating from unspecific interaction of VEGF with the PLGA matrix. Furthermore,

cell growth on discs incubated with BSA alone was also in accordance with previous findings (compare 3.3.1).

The cell growth for the SMCs on the other hand showed no increase for all PEG-PLGA discs independent of the incubation solution and an increase of 10 % for PLGA discs incubated with BSA and $VEGF_{165}$. The superiority of the PEG-PLGA material in contrast to the PLGA matrices towards the reduction of SMC growth might be attributed to the cell-adhesion-resistant properties of PEG [54-55]. In case of endothelial cells, these adhesion-resistant-properties might have been overcome by the stimulating effect of the coupled $VEGF_{165}$ [25]. Thus, the cell growth comparison study successfully demonstrated the beneficial effect of $VEGF_{165}$ coupled PLGA matrices, PEG-PLGA matrices in particular, for the stimulation of endothelial cell growth. In addition, the slight decrease of SMC growth for the PEG-modified PLGA matrices provides an additional benefit of the PEG-PLGA matrices for the coating of vascular grafts to specifically enhance endothelial cell growth without stimulating SMC growth that is commonly attributed with vascular graft complications, such as neointimal hyperplasia [4, 56].

4 Conclusions

A functionalized PLGA polymer carrying a PEG-spacer with an activated succinimidyl ester was successfully synthesized. It was shown that this functionalized PEG-PLGA was able to covalently bind $VEGF_{165}$ using several staining techniques, such as silver staining and Deep Purple™ Total Protein Stain. Other techniques, for example FT-IR spectroscopy, which could have provided additional information about conformational changes of the protein in its bound state if successful, could not determine the presence of bound cytokine due to the low concentrations applied on the surface. However, another VEGF specific method, an antibody staining, could be developed to determine the presence of $VEGF_{165}$ on the modified surface and thereby confirming successful linkage of the cytokine to the effectively modified PLGA matrix. The interaction of the bound $VEGF_{165}$ with the antibody was a first indicator of the sustained functionality of the cytokine.

The modified PLGA matrix was used to coat model substrates, was subsequently coupled with $VEGF_{165}$, and was tested in several cell assays to investigate its effect on endothelial cells and smooth muscle cells. Covalently attached VEGF had a positive effect on the growth of endothelial cells. The effect was superior to effects for loosely adsorbed $VEGF_{165}$ on PLGA matrices that were observed during the investigations. Furthermore, PEG-PLGA could be shown to have a limiting effect on the proliferation of SMCs, due to its cell-adhesion-resistant properties, in comparison to unmodified PLGA, therefore, adding to the beneficial properties of the modified PLGA material. Thus, successful attachment of $VEGF_{165}$ could be shown whilst preserving the biological activity and endothelial cell specific growth stimulation. Moreover, it was shown, that the irreversible linkage of $VEGF_{165}$ still allowed for interaction of $VEGF_{165}$ with its receptor.

5 References

1. Anderson, J.M., A. Rodriguez, and D.T. Chang, *Foreign body reaction to biomaterials.* Seminars in Immunology, 2008. **20**(2): p. 86-100.
2. Antonios, V.S., et al., *Prosthetic vascular graft infection: A risk factor analysis using a case-control study.* Journal of Infection, 2006. **53**(1): p. 49-55.
3. Pärsson, H., et al., *Acute thrombogenicity and 4 weeks healing properties of a new stretch-ePTFE graft.* European Journal of Vascular Surgery, 1993. **7**(1): p. 63-70.
4. Landymore, R.W., C.E. Kinley, and C.A. Cameron, *Intimal hyperplasia in autogenous vein grafts used for arterial bypass: a canine model.* Cardiovasc Res, 1985. **19**(9): p. 589-592.
5. Torsello, G. and W. Sandmann, *Use of antibiotic-bonded grafts in vascular graft infection.* European Journal of Vascular and Endovascular Surgery, 1997. **14**(Supplement 1): p. 84-87.
6. Greisler, H.P., *Growth factor release from vascular grafts.* Journal of Controlled Release, 1996. **39**(2-3): p. 267-280.
7. Tigli, R.S., et al., *In vitro release of dexamethasone or bFGF from chitosan/hydroxyapatite scaffolds.* Journal of Biomaterials Science, Polymer Edition, 2009. **20**(13): p. 1899-1914.
8. Rao, S.V., K.W. Anderson, and L.G. Bachas, *Oriented immobilization of proteins.* Microchimica Acta, 1998. **128**(3): p. 127-143.
9. Santos, S.C.R., et al., *VEGF and VEGFR-2 (KDR) internalization is required for endothelial recovery during wound healing.* Exp. Cell Res., 2007. **313**(8): p. 1561-1574.
10. Li, W. and G. Keller, *VEGF nuclear accumulation correlates with phenotypical changes in endothelial cells.* J Cell Sci, 2000. **113**(9): p. 1525-1534.
11. Bikfalvi, A., et al., *Interaction of vasculotropin/vascular endothelial cell growth factor with human umbilical vein endothelial cells: Binding, internalization, degradation, and biological effects.* Journal of Cellular Physiology, 1991. **149**(1): p. 50-59.
12. Massia, S.P. and J.A. Hubbell, *Human endothelial cell interactions with surface-coupled adhesion peptides on a nonadhesive glass substrate and two polymeric biomaterials.* J. Biomed. Mater. Res., 1991. **25**(2): p. 223-42.
13. Crombez, M., et al., *Improving arterial prosthesis neo-endothelialization: Application of a proactive VEGF construct onto PTFE surfaces.* Biomaterials, 2005. **26**(35): p. 7402-7409.
14. Ikada, Y., *Surface modification of polymers for medical applications.* Biomaterials, 1994. **15**(10): p. 725-736.
15. Sharon, J.L. and D.A. Puleo, *Immobilization of glycoproteins, such as VEGF, on biodegradable substrates.* Acta Biomaterialia, 2008. **4**(4): p. 1016-1023.
16. Abuchowski, A., et al., *Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol.* Journal of Biological Chemistry, 1977. **252**(11): p. 3578-3581.
17. Abuchowski, A., et al., *Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase.* Journal of Biological Chemistry, 1977. **252**(11): p. 3582-3586.
18. Fontana, A., et al., *Site-specific modification and PEGylation of pharmaceutical proteins mediated by transglutaminase.* Advanced Drug Delivery Reviews, 2008. **60**(1): p. 13-28.

19. Brocchini, S., et al., *Disulfide bridge based PEGylation of proteins.* Advanced Drug Delivery Reviews, 2008. **60**(1): p. 3-12.
20. Caliceti, P. and F.M. Veronese, *Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates.* Advanced Drug Delivery Reviews, 2003. **55**(10): p. 1261-1277.
21. Sano, S., K. Kato, and Y. Ikada, *Introduction of functional groups onto the surface of polyethylene for protein immobilization.* Biomaterials, 1993. **14**(11): p. 817-822.
22. Otrock, Z.K., J.A. Makarem, and A.I. Shamseddine, *Vascular endothelial growth factor family of ligands and receptors: Review.* Blood Cells, Molecules, and Diseases, 2007. **38**(3): p. 258-268.
23. Zisch, A.H., et al., *Covalently conjugated VEGF-fibrin matrices for endothelialization.* Journal of Controlled Release, 2001. **72**(1-3): p. 101-113.
24. Tabata, Y., et al., *Controlled release of vascular endothelial growth factor by use of collagen hydrogels.* Journal of Biomaterials Science, Polymer Edition, 2000. **11**(9): p. 915-930.
25. Ferrara, N., et al., *The vascular endothelial growth factor family of polypeptides.* J. Cell. Biochem., 1991. **47**(3): p. 211-18.
26. Dorafshar, A.H., et al., *Vascular endothelial growth factor inhibits mitogen-induced vascular smooth muscle cell proliferation.* J. Surg. Res., 2003. **114**(2): p. 179-186.
27. Anderson, J.M. and M.S. Shive, *Biodegradation and biocompatibility of PLA and PLGA microspheres.* Advanced Drug Delivery Reviews, 1997. **28**(1): p. 5-24.
28. Middleton, J.C. and A.J. Tipton, *Synthetic biodegradable polymers as orthopedic devices.* Biomaterials, 2000. **21**(23): p. 2335-2346.
29. Snyder, S.L. and P.Z. Sobocinski, *Improved 2,4,6-trinitrobenzenesulfonic acid method for the determination of amines.* Analytical Biochemistry, 1975. **64**(1): p. 284-8.
30. Jeong, J.H., et al., *Synthesis, characterization and protein adsorption behaviors of PLGA/PEG di-block co-polymer blend films.* Colloids and Surfaces B: Biointerfaces, 2000. **18**(3-4): p. 371-379.
31. Fernández-Carballido, A., et al., *PLGA/PEG-derivative polymeric matrix for drug delivery system applications: Characterization and cell viability studies.* International Journal of Pharmaceutics, 2008. **352**(1-2): p. 50-57.
32. Mallardé, D., et al., *PLGA-PEG microspheres of teverelix: influence of polymer type on microsphere characteristics and on teverelix in vitro release.* International Journal of Pharmaceutics, 2003. **261**(1-2): p. 69-80.
33. Li, B., J. Flores, and V. Corvari, *A simple method for the detection of insoluble aggregates in protein formulations.* J. Pharm. Sci., 2007. **96**(7): p. 1840-1843.
34. Svensson, E., et al., *Fluorescent in-gel protein detection by regulating the pH during staining.* Analytical Biochemistry, 2006. **355**(2): p. 304-306.
35. Matheus, S., W. Friess, and H.-C. Mahler, *FTIR and nDSC as Analytical Tools for High-Concentration Protein Formulations.* Pharm. Res., 2006. **23**(6): p. 1350-1363.
36. Fu, K., et al., *FTIR characterization of the secondary structure of proteins encapsulated within PLGA microspheres.* Journal of Controlled Release, 1999. **58**(3): p. 357-366.
37. Paragkumar N, T., D. Edith, and J.-L. Six, *Surface characteristics of PLA and PLGA films.* Applied Surface Science, 2006. **253**(5): p. 2758-2764.
38. Pelton, J.T. and L.R. McLean, *Spectroscopic Methods for Analysis of Protein Secondary Structure.* Analytical Biochemistry, 2000. **277**(2): p. 167-176.

39. Ito, Y., et al., *Cell growth on immobilized cell growth factor. 6. Enhancement of fibroblast cell growth by immobilized insulin and/or fibronectin.* Journal of biomedical materials research, 1993. **27**(7): p. 901-907.
40. Goddard, J.M. and J.H. Hotchkiss, *Polymer surface modification for the attachment of bioactive compounds.* Progress in Polymer Science, 2007. **32**(7): p. 698-725.
41. DeLong, S.A., J.J. Moon, and J.L. West, *Covalently immobilized gradients of bFGF on hydrogel scaffolds for directed cell migration.* Biomaterials, 2005. **26**(16): p. 3227-3234.
42. Göpferich, A., et al., *Modulation of marrow stromal cell function using poly(D,L-lactic acid)-block-poly(ethylene glycol)-monomethyl ether surfaces.* Journal of biomedical materials research, 1999. **46**(3): p. 390-398.
43. Lieb, E., et al., *Poly(D,L-lactic acid)-Poly(ethylene glycol)-Monomethyl Ether Diblock Copolymers Control Adhesion and Osteoblastic Differentiation of Marrow Stromal Cells.* Tissue Engineering, 2003. **9**(1): p. 71-84.
44. Tziampazis, E., J. Kohn, and P.V. Moghe, *PEG-variant biomaterials as selectively adhesive protein templates: model surfaces for controlled cell adhesion and migration.* Biomaterials, 2000. **21**(5): p. 511-520.
45. Roberts, M.J., M.D. Bentley, and J.M. Harris, *Chemistry for peptide and protein PEGylation.* Advanced Drug Delivery Reviews, 2002. **54**(4): p. 459-476.
46. Jeyachandran, Y.L., et al., *Efficiency of blocking of non-specific interaction of different proteins by BSA adsorbed on hydrophobic and hydrophilic surfaces.* Journal of Colloid and Interface Science, 2009. **In Press, Corrected Proof**.
47. Kurrat, R., J.E. Prenosil, and J.J. Ramsden, *Kinetics of Human and Bovine Serum Albumin Adsorption at Silica-Titania Surfaces.* Journal of Colloid and Interface Science, 1997. **185**(1): p. 1-8.
48. Jeyachandran, Y.L., et al., *Quantitative and Qualitative Evaluation of Adsorption/Desorption of Bovine Serum Albumin on Hydrophilic and Hydrophobic Surfaces.* Langmuir, 2009. **25**(19): p. 11614-11620.
49. Böhme, U. and U. Scheler, *Effective charge of bovine serum albumin determined by electrophoresis NMR.* Chemical Physics Letters, 2007. **435**(4-6): p. 342-345.
50. Keshaw, H., A. Forbes, and R.M. Day, *Release of angiogenic growth factors from cells encapsulated in alginate beads with bioactive glass.* Biomaterials, 2005. **26**(19): p. 4171-4179.
51. Klose, D., et al., *PLGA-based drug delivery systems: Importance of the type of drug and device geometry.* International Journal of Pharmaceutics, 2008. **354**(1-2): p. 95-103.
52. Langer, R. and D.A. Tirrell, *Designing materials for biology and medicine.* Nature, 2004. **428**(6982): p. 487-492.
53. Blümmel, J., et al., *Protein repellent properties of covalently attached PEG coatings on nanostructured SiO2-based interfaces.* Biomaterials, 2007. **28**(32): p. 4739-4747.
54. Groll, J., et al., *A novel star PEG-derived surface coating for specific cell adhesion.* Journal of Biomedical Materials Research, Part A, 2005. **74A**(4): p. 607-617.
55. Llanos, G.R. and M.V. Sefton, *Immobilization of poly(ethylene glycol) onto a poly(vinyl alcohol) hydrogel: 2. Evaluation of thrombogenicity.* Journal of biomedical materials research, 1993. **27**(11): p. 1383-91.
56. Davies, M.G. and P.-O. Hagen, *Pathophysiology of vein graft failure: A review.* European Journal of Vascular and Endovascular Surgery, 1995. **9**(1): p. 7-18.

Chapter 6

Cell growth stimulation by covalent linkage of $VEGF_{165}$ to a modified collagen-matrix

Abstract

The effect of covalently attached Vascular Endothelial Cell Growth Factor ($VEGF_{165}$) to a modified collagen matrix on the growth of endothelial and smooth muscle cells (SMCs) was investigated. Therefore, collagen coated substrates were modified using a homobifunctional polyethylene glycol (PEG) spacer and $VEGF_{165}$ was covalently attached. A method for the detection of small amounts of attached protein was established, using an anti-human VEGF antibody. Successful binding of the protein and optimization of coating and coupling procedures could be shown.

In addition, in vitro cell growth experiments were performed to determine the effect of bound $VEGF_{165}$ on the cell growth of endothelial cells and smooth muscle cells. The biological activity of $VEGF_{165}$ was sustained, which was shown by the substantial growth increase of endothelial cells in comparison to control samples. Furthermore, the effect on SMCs was investigated and an unchanged growth behavior of SMCs was observed when grown on VEGF coupled matrices. It was also shown that the linkage and functionality of $VEGF_{165}$ to the collagen matrix was sustained, even after incubation in buffer medium for more than 24 hours, indicating a prolonged stability of the attached protein in vitro. Therefore, successful attachment of $VEGF_{165}$ was shown while preserving biological activity and endothelial cell specific growth stimulation.

Keywords: Collagen, VEGF, vascular graft, endothelial cell, covalent linkage, PEG

1 Introduction

The main objective of tissue engineering is the regeneration or replacement of cells or biological material and therewith the function formerly performed by the tissue [1]. However, after implantation of biomaterial surfaces in a body, the body generally identifies these surfaces as foreign objects resulting in a repair response rather than a regenerative response [2], which causes severe problems especially in case of vascular grafts [3-5]. A manifold of approaches to overcome these problems have been investigated, among others, the chemical attachment of proteins to biomaterial surfaces [6-8]. For the chemical attachment, functional groups present within the sequence of proteins, such as amino, carboxyl [9], and aldehyd groups [7] are commonly used. $VEGF_{165,}$ the predominant isoform from the VEGF family [10], carries these functional groups within its sequence and makes it accessible for covalent attachment to biomaterials. $VEGF_{165}$, a 42 kDa protein, has been widely investigated for the controlled release and use in artificial graft applications [6, 8, 11]. VEGF is involved in several endothelial cell specific activities, such as proliferation and migration [12], at the same time limiting the mitogen-induced vascular SMC proliferation [13]. Therefore, it seems a very promising approach for the improvement of vascular grafts by controlled presentation of $VEGF_{165}$ at the graft surface by chemically attaching the protein to a modified matrix.

Collagen has been widely used in biomaterial applications [14-16] due to its biocompatibility and degradation products that can be metabolized and excreted [17]. It is a well tolerated substrate for endothelial cell growth, which can be seen by its previous use in vascular graft applications [18-20]. In contrast to PLGA investigated in Chapter 5, collagen represents a naturally occurring polymer in mammals representing almost 30% of total protein present in a body [21]. Therefore, collagen constitutes an interesting and promising approach for the chemical modification and the covalent attachment of $VEGF_{165}$.

The main goal of this study was to prove a successful covalent linkage of $VEGF_{165}$ on collagen matrices while maintaining its biological activity in order to specifically stimulate endothelial cell growth without stimulation of other cells. For the attachment, the use of a homobifunctional PEG-spacer was investigated. Therefore,

first studies focused on the evaluation of an antibody staining method previously developed to detect VEGF attached to PLGA matrices (compare Chapter 5) to determine the successful linkage, an important precondition for the cell growth investigations. Furthermore, the results were to be used to optimize linkage conditions. More importantly, the cell compatibility of the modified collagen material and the preservation of the biological activity of the attached VEGF were evaluated. Therefore, their effect on HUVECs and SMCs was investigated

2 Materials and Methods

2.1 Materials

$VEGF_{165}$ was kindly donated by Genentech (San Francisco, CA, USA), rat aortic smooth muscle cells were kindly gifted by PD Dr. Wolgang Erl (Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, Ludwig-Maximilians-Universität München, Munich, Germany), and equine collagen type I derived from tendon provided as lyophilized material was provided by Innocoll GmbH (Saal/Donau, Germany). *O*,*O'*-Bis(2-amino-propyl) polyethylene glycol ($PEG(NH_2)_2$) with a molecular weight of 500 Da, succinic anhydride, N-Hydroxysuccinimide (NHS), N,N'-Dicyclohexyl-carbodiimide (DCCI), dimethyl sulfoxide, 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS), and Bovine Serum Albumin (BSA) were purchased from Sigma (Steinheim, Germany), toluene, diethyl ether, and glacial acetic acid from Merck KGaA (Darmstadt, Germany), triethylamine, dichloromethane, ethanolamine, cover glasses, plastic petri dishes from VWR (Darmstadt, Germany), polytetrafluoro-ethylene (PTFE) from GM GmbH (Freiham, Germany), goat-anti-rabbit antibody Alexa Fluor 488 from Invitrogen (Karlsruhe, Germany), rabbit Anti-Human VEGF antibody from Pepro Tech GmbH (Hamburg, Germany), collagenase A from Roche (Penzberg, Germany), endothelial cell growth medium from Provitro (Berlin, Germany), heat-inactivated fetal bovine serum from Biochrom (Berlin, Germany), smooth muscle cell growth medium DMEM /F12 and M199 from PAA (Pasching, Austria), 24-well plates from TPP Techno Plastic Products (Trasadingen, Switzerland), and Cell Titer-Blue® from Promega (Madison, WI, USA).

The pH of the solutions was adjusted using hydrochloric acid or sodium hydroxide from Merck KGaA (Darmstadt, Germany) and measured with a pH meter Inolab level 1 from WTW (Weilheim, Germany).

2.2 Methods

2.2.1 Synthesis of PEG-spacer

A bifunctional PEG-spacer was synthesized using *O*,*O'*-Bis(2-aminopropyl) polyethylene glycol ($PEG(NH_2)_2$) with a molecular weight of 500 Da as starting material. In a first reaction step (Figure 6-1), the amine groups of the PEG were modified into carboxylic groups using succinic anhydride. The reaction was carried out in anhydrous toluene with the addition of triethylamine at room temperature over night.

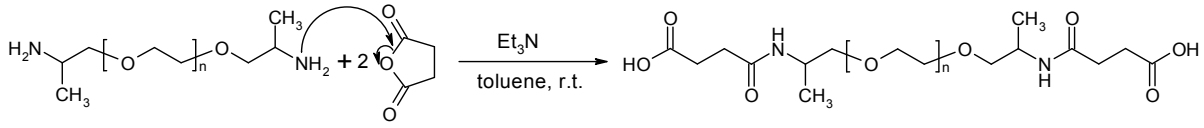


Figure 6-1: Reaction mechanism for the synthesis of PEG-$(COOH)_2$.

The resulting functionalized PEG carrying two carboxylic groups was subsequently activated (Figure 6-2) using NHS and DCCI. The reaction was carried out in anhydrous dichloromethane at 0°C for 3 hours and subsequently at room temperature over night. The product was recovered and purified by precipitation in cold diethyl ether. The final compound was desiccated under vacuum for several hours and the activity was determined by a TNBS assay (compare 2.2.2).

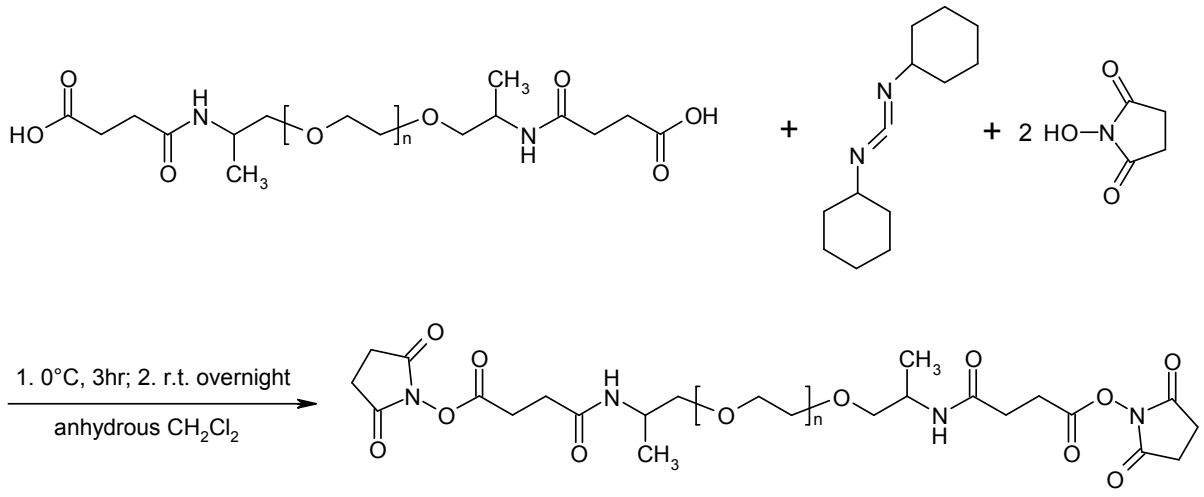


Figure 6-2: Reaction mechanism for the synthesis of PEG-$(NHS)_2$.

2.2.2 TNBS assay

The degree of ability of the activated species of the PEG-spacer to couple with primary amines within protein sequences was determined using a modified assay according to Snyder et al [22]. Therefore, the activated species were dissolved in anhydrous DMSO and were coupled with the primary amine of ethanolamine (Figure 6-3).

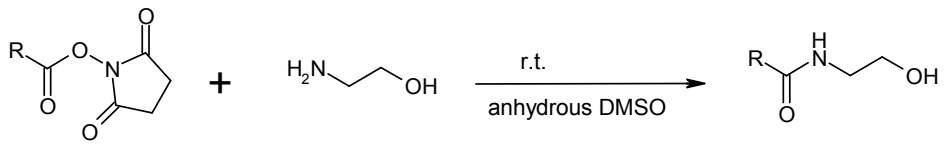


Figure 6-3: Conjugation reaction of activated species with ethanolamine.

In a next step, the free ethanolamine was quantified using TNBS in a 0.1 M borate buffer pH 8.3. The yellow conjugate formed (Figure 6-4) was quantified via UV-spectroscopy at 420 nm using the Agilent 8453 (Agilent Technologies, Böblingen, Germany). The degree of activation was calculated as percentage of coupled ethanolamine compared to reference samples containing no activated species.

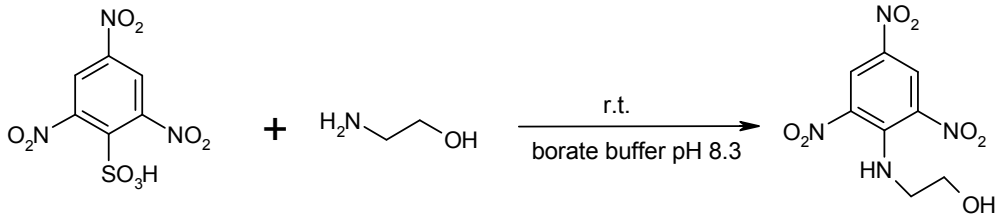


Figure 6-4: Quantification reaction for free ethanolamine.

2.2.3 Collagen coating procedure

2.2.3.1 Preparation of collagen dispersion

The collagen dispersion was prepared by redispersion of equine collagen type I derived from tendon in water at 1% (w/w). The pH was adjusted to 3.5 using glacial acetic acid. Swelling was performed at room temperature for 4 hours with dispersion for 60 s using an Ultraturrax® (IKA®-Werke GmbH Co. KG, Staufen, Germany) every 30 minutes at 10,000 rpm and an additional dispersion step at the end of the swelling period of 3 minutes at 10,000 rpm. The dispersion was then centrifuged at 5,000 rpm at 10°C for 10 minutes using a Sigma 4 K15 lab centrifuge (Sigma, Osterode, Germany) to remove air bubbles from the dispersion. Thereafter, the dispersion was used for the coating (compare 2.2.3.2) or stored at 2-8°C until further use.

2.2.3.2 Coating procedure

The collagen dispersion (compare 2.2.3.1) was used to coat PTFE-discs, argon plasma activated with a plasmabrush® (Reinhausen Plasma GmbH, Regensburg, Germany) or cover glasses, both with a diameter of 15 mm. Therefore, the discs were placed in 55 mm petri dishes and covered with 150 µl of collagen dispersion. The discs were air dried for one hour and subsequently dried under vacuum over night or until further use. For the cell culture studies, all these procedures were performed under a laminar flow workbench (Thermo, Langenselbold, Germany).

2.2.4 $VEGF_{165}$ linkage

2.2.4.1 PEG-spacer coupling

In order to attach the bifunctional PEG-spacer to the free amino groups on the collagen matrix, the dried collagen covered discs were placed in an aluminum 24-well plate and covered with a 1% (w/v) solution of the PEG-spacer in anhydrous dichloromethane for 30 minutes at room temperature under gentle shaking. The derivatization reaction is shown in Figure 6-5.

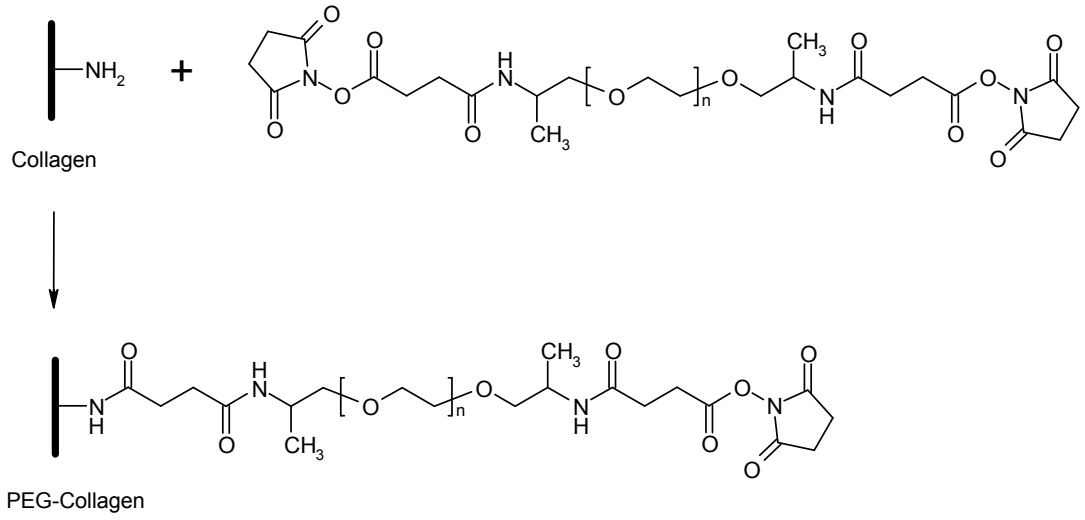


Figure 6-5: Idealized scheme for PEG-spacer attachment to collagen matrix.

The evaporated dichloromethane was replaced with fresh solvent if drying of the discs was anticipated. After 30 minutes the dichloromethane was removed by aspiration and the discs were washed with fresh dichloromethane in order to remove unbound PEG-spacer. Thereafter, discs were dried under vacuum for several hours. For the cell culture studies, all these procedures were performed under a laminar flow workbench.

2.2.4.2 VEGF$_{165}$ linkage procedure

For the linkage of VEGF$_{165}$, collagen and PEG-collagen coated discs were transferred to plastic petri dishes and covered with VEGF$_{165}$ in phosphate buffered saline (PBS) buffer pH 7.4 for 30 minutes. Reference samples not carrying VEGF$_{165}$ were treated with either PBS buffer alone or with PBS containing 1 mg/ml BSA to block unspecific binding. After the incubation, the discs were intensely washed in Milli-Q water and stored in PBS buffer for immediate use. For the cell culture studies, all these procedures were performed under a laminar flow workbench. The approach for protein attachment is shown in Figure 6-6.

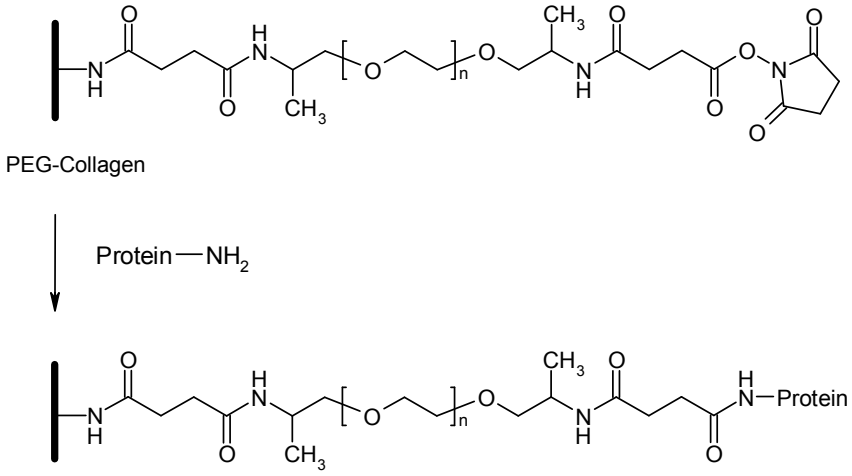


Figure 6-6: Idealized scheme for protein attachment to PEG-collagen matrix.

2.2.5 Antibody detection of surface bound VEGF$_{165}$

For VEGF$_{165}$ detection discs were incubated with a rabbit Anti-Human VEGF antibody in PBS buffer pH 7.4 at 2-8°C over night. Thereafter, discs were rinsed with PBS buffer three times to eliminate unbound primary antibody. In a second step, samples were incubated with a secondary goat-anti-rabbit antibody Alexa Fluor 488 for 2 hours at room temperature under exclusion of light. Samples were again rinsed with PBS and subsequently analyzed using the Zeiss LSM 510 confocal laser scanning microscope (CLSM) (Zeiss, Oberkochen, Germany).

2.2.6 Influence of covalently linked $VEGF_{165}$ on cell growth

2.2.6.1 Vascular endothelial cells

The influence of discs on the cell growth of vascular endothelial cells was evaluated using Human Umbilical Vein Endothelial Cells (HUVECs). HUVECs were prepared by digestion of umbilical veins with 0.1 g / l collagenase A. Cells were cultured in endothelial cell growth medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere at 5% CO_2 and 37°C. Cells were used at passage no. 3.

2.2.6.2 Aortic smooth muscle cells

The influence of discs on the cell growth of SMCs was evaluated using rat aortic smooth muscle cells. Cells were cultured in smooth muscle cell growth medium DMEM /F12 supplemented with 1% heat-inactivated fetal bovine serum in a humidified atmosphere at 5% CO_2 and 37°C.

2.2.6.3 Cell growth comparison study

For the study discs were placed on the bottom of 24-well plates and subsequently covered with a layer of starvation medium in case of HUVECs containing 80% of M199 and 20% of endothelial cell growth medium, in case of SMCs 90% of DMEM /F12 and 10% heat-inactivated fetal bovine serum. 20,000 viable HUVECs or SMCs were seeded per well. Thereafter, cells were cultured in a humidified atmosphere at 5% CO_2 and 37°C. Cells on the discs were quantified at day 3 using Cell Titer-Blue®. Therefore, cells were incubated with Cell Titer-Blue® for 6 hours and an aliquot of the supernatant was analyzed using a Spectrafluor plus plate reader (Tecan, Crailsheim, Germany) with an excitation wavelength of 550 nm and emission recording at 595 nm.

3 Results and Discussion

3.1 Covalent $VEGF_{165}$ linkage to modified collagen matrices

In order to evaluate the effect of modified collagen matrices on cell growth, it was important to evaluate the successful linkage of $VEGF_{165.}$ Therefore, a previously identified and optimized antibody staining method (compare Chapter 5) was first evaluated towards its suitability to detect surface bound $VEGF_{165}$ on collagen matrices. Furthermore, it was used to evaluate the coating procedure and to rule out unspecific adsorption of VEGF.

3.1.1 Evaluation of antibody staining method

For the evaluation of the antibody staining method, PEG-Collagen coated discs were incubated with buffer as control sample and $VEGF_{165}$ solution with a concentration of 20 µg / ml for 30 minutes. The discs were subsequently stained and investigated using CLSM.

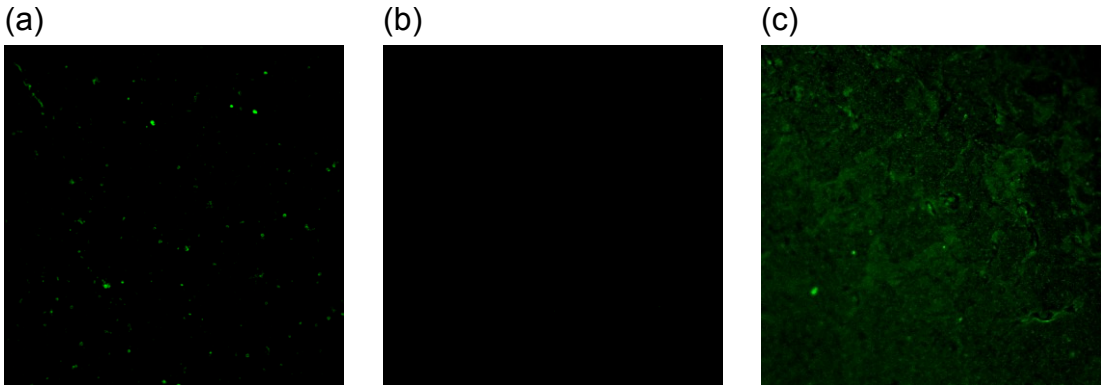


Figure 6-7: CLSM images of antibody stained collagen coated discs for PEG-Collagen control (0 µg/ml VEGF) (a), PEG-Collagen VEGF (20 µg/ml) secondary antibody only (b), and PEG-Collagen VEGF (20 µg/ml) (c).

The obtained images shown in Figure 6-7 indicated successful linkage of $VEGF_{165}$ to the PEG-Collagen matrix, which can be seen by the fluorescence of the

secondary antibody in Figure 6-7c compared to the considerably lower fluorescence of the control sample seen in Figure 6-7a. In addition, the interaction of the secondary antibody with either $VEGF_{165}$ or the PEG-Collagen matrix could be excluded, as seen by the lack of fluorescence for the sample only incubated with this antibody (Figure 6-7b). Therefore, it could be concluded that the previously established antibody staining method was also suitable to detect VEGF on collagen matrices. It also indicated the successful preservation of biological activity of VEGF, covalently attached to the collagen surface, throughout the preparation procedure.

3.1.2 Analysis of the linkage procedure of VEGF to PEG-Collagen

In order to further evaluate the suitability of the antibody detection method to identify bound $VEGF_{165}$ on the surface of the collagen matrix, a set of control experiments was performed. In addition, these experiments were performed to optimize the linkage conditions and procedure, as well as, to understand the process of VEGF coupling. Furthermore, it was the aim to rule out the unspecific adsorption of $VEGF_{165}$ on the modified collagen matrix. Thus, unmodified and modified collagen matrices were incubated with BSA, $VEGF_{165}$, or buffer. VEGF was detected with varying combinations of detection antibodies (compare Table 6-1).

Table 6-1: Overview of incubation and detection procedures for the evaluation of unspecific adsorption on PEG-PLGA and PLGA coated discs and order of incubation for Collagen-04 and Collagen-05, as well as PEG-Collagen-04 and PEG-Collagen-05.

	incubation solution			detection antibodies	
Sample	**PBS**	**$VEGF_{165}$ [20 µg/ml]**	**BSA [1 mg/ml]**	**Anti-VEGF**	**goat-anti-rabbit**
Collagen-01		X		X	X
Collagen-02	X			X	X
Collagen-03		X			X
Collagen-04		1.) X	2.) X	X	X
Collagen-05		2.) X	1.) X	X	X
Collagen-06			X	X	X
Collagen-07			X		X
PEG-Collagen-01		X		X	X
PEG-Collagen-02	X			X	X
PEG-Collagen-03		X			X
PEG-Collagen-04		1.) X	2.) X	X	X
PEG-Collagen-05		2.) X	1.) X	X	X
PEG-Collagen-06			X	X	X
PEG-Collagen-07			X		X

The results obtained for the unmodified collagen coated discs can be seen in Figure 6-8. All samples, independent of their incubation solution, showed very little to no fluorescence in the pictures obtained using CLSM. Only the samples incubated with $VEGF_{165}$ and BSA (Figure 6-8 d and e) exhibited slight fluorescence to the same extent that is in no correlation to the order of incubation solutions. The increased fluorescence in comparison to the other samples might be attributed to the increased background fluorescence when using a fluorescently labeled secondary antibody for detection and to the increased incubation time, which was double the time for the samples Collagen-04 and -05.

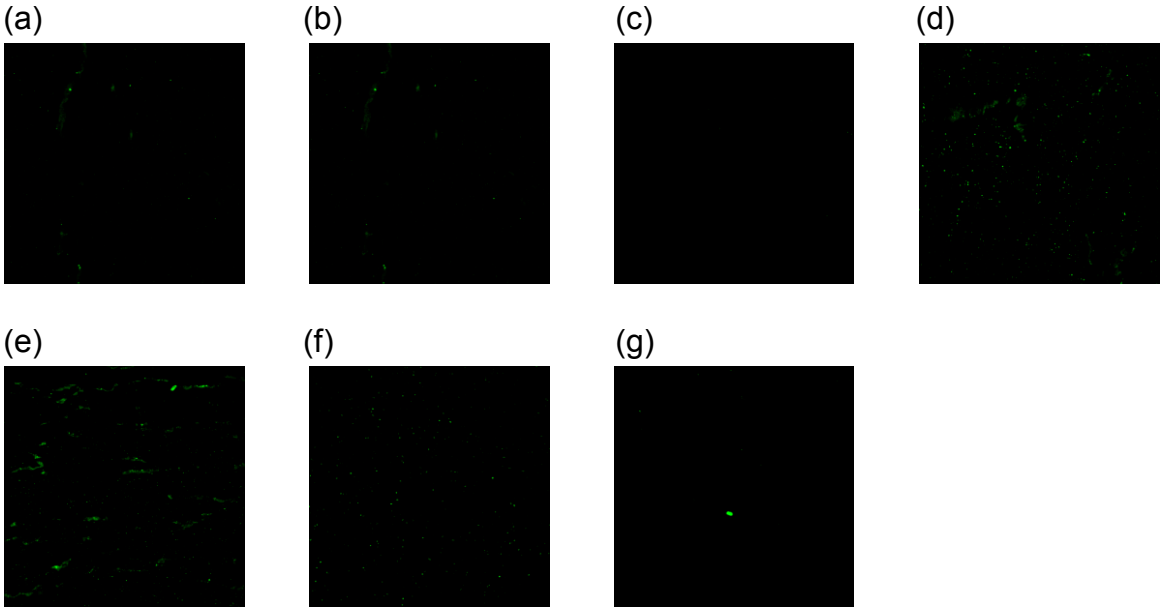


Figure 6-8: CLSM images of antibody stained discs Collagen-01 (a), Collagen-02 (b), Collagen-03 (c), Collagen-04 (d), Collagen-05 (e), Collagen-06 (f), and Collagen-07 (g)

The results obtained from the control experiments, with non modified collagen matrices, showed that there was no unspecific interaction or adsorption of $VEGF_{165}$ that was detectable using this antibody staining method.

The aim of the second set of experiments was to further investigate the binding efficiency and conditions of incubation for PEG-collagen matrices. Therefore the same set of incubations (compare Table 6-1) as for the non-modified collagen matrices were performed on PEG-collagen coated discs. The results obtained for these experiments can be seen in Figure 6-9. Figure 6-9a and b show the pictures of the samples incubated with $VEGF_{165}$ and buffer respectively. It can be seen that there was a strong fluorescence for the $VEGF_{165}$ incubated samples in contrast to the samples incubated with buffer alone, which indicated that the binding of $VEGF_{165}$ antibody is specific for covalently attached VEGF and shows no interaction with the PEG-collagen matrix. The control samples incubated with $VEGF_{165}$ and the fluorescently labeled secondary antibody showed no fluorescence (compare Figure 6-9c), proving that there is no unspecific interaction or attachment of the secondary antibody with the attached $VEGF_{165}$ and the PEG-collagen matrix. For the samples incubated with $VEGF_{165}$ and BSA solution (Figure 6-9d) a slight decrease in

fluorescence could be observed in comparison to the samples only incubated with $VEGF_{165}$. This decrease can be attributed to the increased incubation time of 60 minutes compared to 30 minutes and a therewith associated further rinsing step. In addition to the increased incubation time, the blocking of binding sites due to the BSA incubation can also account for the decrease in fluorescence.

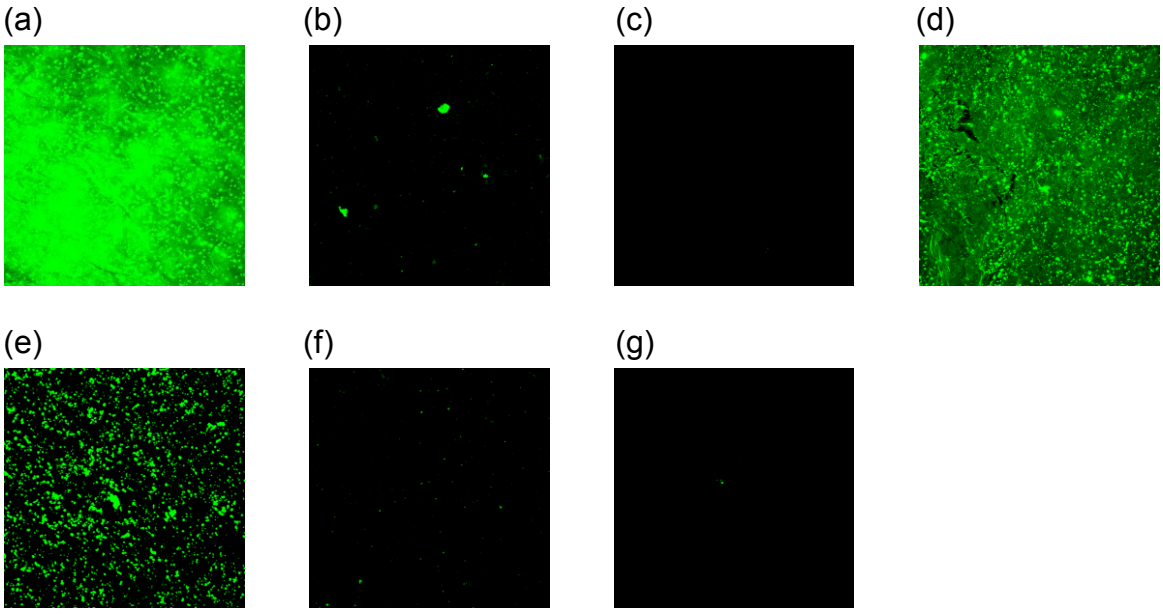


Figure 6-9: CLSM images of antibody stained discs PEG-Collagen-01 (a), PEG-Collagen-02 (b), PEG-Collagen-03 (c), PEG-Collagen-04 (d), PEG-Collagen-05 (e), PEG-Collagen-06 (f), and PEG-Collagen-07 (g)

Interestingly, the sample first incubated with BSA solution and subsequent incubation with $VEGF_{165}$ showed fluorescence (Figure 6-9e). It was expected that BSA would block all the active functional groups and hamper $VEGF_{165}$ attachment. In addition, it is reported in literature that the half life of activated succinimidyl ester is around 30 minutes [23], and therefore, a subsequent attachment of $VEGF_{165}$ was not expected. However, the fluorescence indicated successful linkage of the protein, which leads to the conclusion that the functional group was still active after BSA incubation. An interaction of BSA with the primary $VEGF_{165}$ antibody or the fluorescently labeled secondary antibody could be excluded, since both control samples with BSA and incubation with either both antibodies or the secondary antibody only showed no fluorescence (Figure 6-9f and g).

These experiments proved that an unspecific adsorption of $VEGF_{165}$ or the secondary fluorescently labeled antibody to the modified collagen matrix can be ruled out, as seen by the various control experiments using unmodified and modified collagen. Furthermore and more importantly, the successful linkage of $VEGF_{165}$ to the modified collagen matrix was shown.

3.2 Influence of $VEGF_{165}$ covalently linked to PEG-Collagen on cell growth

3.2.1 Vascular endothelial cells

In order to evaluate the activity of the covalently linked $VEGF_{165}$, and therewith the accessibility of the VEGF surface receptor (VEGFR-1) [10] on the endothelial cells towards the covalently linked protein, cell growth studies were performed. Additionally, several control experiments were performed to investigate the effect of loosely adsorbed protein on the surface of unmodified collagen coated discs. Therefore, collagen and PEG-collagen coated discs, respectively, were treated with different incubation solutions in accordance to Table 6-2 and immediately thereafter rinsed and incubated with endothelial cells. After a 3 day incubation period, cells were quantified using Cell Titer-Blue®. The results were calculated as % increase in relation to the amount of cells that had grown on the collagen and PEG-collagen coated discs incubated with PBS buffer.

Table 6-2: Overview of incubation procedures for the evaluation of cell growth on PEG-collagen and collagen coated discs and order of incubation.

	incubation solution		
Sample	**PBS buffer**	**$VEGF_{165}$ [20 µg/ml]**	**BSA [1 mg/ml]**
Collagen (control)	X		
Collagen (VEGF)		X	
Collagen (BSA)			X
Collagen (BSA + VEGF)		2.) X	1.) X
Collagen (VEGF + BSA))		1.) X	2.) X
PEG-Collagen (control)	X		
PEG-Collagen (VEGF)		X	
PEG-Collagen (BSA)			X
PEG-Collagen (BSA + VEGF)		2.) X	1.) X
PEG-Collagen (VEGF + BSA))		1.) X	2.) X

The results presented in Figure 6-10 showed no significant increase in cell growth for the collagen coated discs incubated with VEGF, BSA, or combinations thereof compared to the control group incubated with buffer irrespective of the incubation solution used. Only a decrease in cell growth of approximately 20 % for the cells grown on discs incubated with BSA solution was observed. However, the discs coated with the modified PEG-collagen material exhibited different growth behavior for the endothelial cells, depending on the incubation solution. Discs incubated with $VEGF_{165}$ alone showed an increase in cell growth compared to the buffer incubated samples of approximately 40%. An increase in cell growth was also observed for the samples incubated with $VEGF_{165}$ and BSA. Those samples first incubated with $VEGF_{165}$ and subsequent incubation with BSA showed an increase of approximately 35% in contrast to the samples incubated with BSA first and subsequent incubation with $VEGF_{165}$, which showed an increase of around 20%. This difference in increase can be explained by the lower amount of VEGF attached to the modified collagen surface, as observed before during antibody detection (compare 3.1.2). The increase in cell growth for these samples can not be attributed to the influence of BSA, since control samples incubated with BSA solely showed an extent of endothelial cell growth comparable to the control samples incubated with buffer.

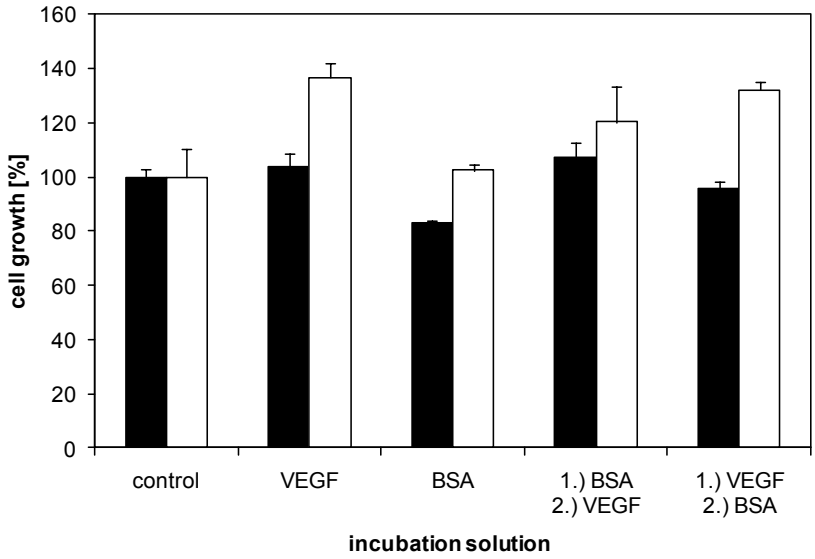


Figure 6-10: Endothelial cell growth on Collagen (▬) and PEG-Collagen (▭) coated discs after treatment with different incubation solutions.

Therefore, it can be concluded, that $VEGF_{165}$ was successfully attached to the modified PEG-collagen matrix without losing its biological activity. No unspecific interaction of VEGF with the unmodified collagen matrix could be observed in the cell growth study. In addition, it can be deducted that the attached VEGF on the PEG-collagen matrix was capable to stimulate the proliferation of endothelial cells.

3.2.2 Cell growth comparison study

In a second cell growth study, the influence of the covalently attached $VEGF_{165}$ on endothelial cells in addition to SMCs was investigated. Therefore collagen and PEG-collagen coated discs were incubated with buffer, BSA, and $VEGF_{165}$. The discs were subsequently rinsed using Milli-Q water and incubated with endothelial cells or smooth muscle cells for 3 days (compare 2.2.6.3). After the 3 day incubation period, cells were quantified using Cell Titer-Blue®. The results were calculated as % cell growth in relation to the amount of cells present on the collagen and PEG-collagen coated discs incubated with PBS buffer. In addition, one set of $VEGF_{165}$ exposed discs was incubated over night in PBS buffer and thereafter rinsed, placed

in a fresh 24-well plate, and treated accordingly. The additional experiment was performed to elucidate the stability of covalently attached VEGF. It was of interest whether the biological activity and the effect on endothelial cells could be sustained for an extended period of time during incubation in solution.

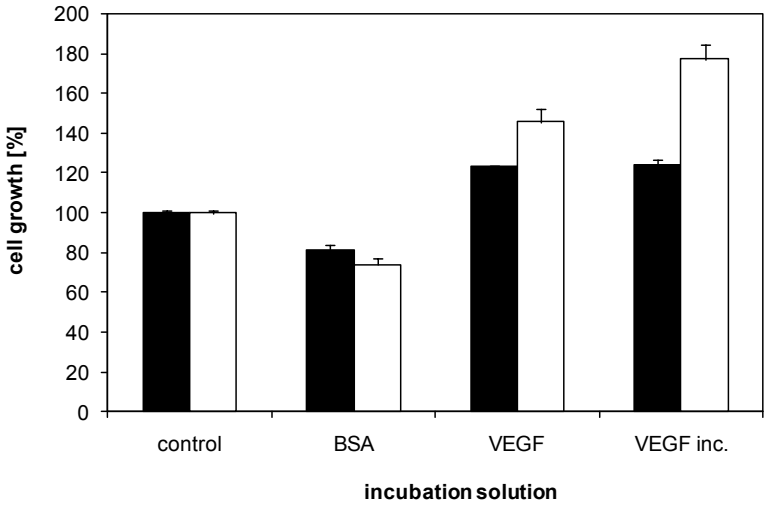


Figure 6-11: Endothelial cell growth on Collagen (▬) and PEG-Collagen (▭) coated discs after treatment with different incubation solutions.

The findings for the endothelial cells can be seen in Figure 6-11. Again, it was observed that BSA incubated discs demonstrated a decrease in cell growth compared to the buffer incubated samples after 3 days incubation, which was in accordance to the findings in previous endothelial cell growth studies (compare 3.2.1). These results were in contrast to previously determined endothelial cell growth on modified PLGA matrices carrying VEGF covalently attached (see Chapter 5). It has been reported in literature that positively charged surfaces are beneficial for endothelial cell adhesion [24-25]. At physiological conditions, BSA exhibits a negative surface charge [26] in contrast to VEGF that displays a positive charge under those conditions [27], which could explain the decrease in endothelial cell growth on the PEG-Collagen material incubated with BSA. In addition, a denser PEG coverage on the collagen modified material resulting from a higher density of modifiable amine groups in contrast to carboxylic groups on PLGA might have

caused the differences in cell growth observed for these two materials, since PEG is known to have protein repellent and cell adhesion resistant properties [28-29].

In contrary to prior findings, VEGF incubated collagen discs exhibited an increase in cell growth of approximately 20%, which was not observed before. Previous incubation of the samples over night in PBS buffer did not have an effect on the extent of cell growth increase in the case of collagen coated discs. However, PEG-collagen coated discs that were treated with $VEGF_{165}$ and discs that were subsequently incubated with buffer over night showed an increase in endothelial cell growth of approximately 45% and 80%, respectively. Therefore, the resulting difference in growth increase between VEGF incubated collagen and PEG-collagen discs was in a range of 25% and above, which was in accordance with prior findings (compare 3.2.1) where a growth increase for PEG-collagen discs with $VEGF_{165}$ in a range of 20 – 35% was achieved.

The considerable increase in cell growth of the $VEGF_{165}$ coupled PEG-collagen discs that were incubated prior to cell seeding showed the sustainable effect of the VEGF attachment to the surface. $VEGF_{165}$ was not lost during incubation or immediately eliminated from the surface, but showed an extended effect. It is a first indicator for a presentation possibility of $VEGF_{165}$ at the matrix surface enabling a prolonged interaction and stimulation of endothelial cells. In contrast, contradictory findings about the stability of free $VEGF_{165}$ in buffer have been reported in literature [30-31]. In this case, in accordance to findings reported in Chapter 4, the biological activity of $VEGF_{165}$ and its endothelial cell specific growth stimulation could be maintained in solution for more than 24 hours incubation. Similar results have been reported by Sharon et al. for the immobilization of VEGF using dihydrazide spacers [7].

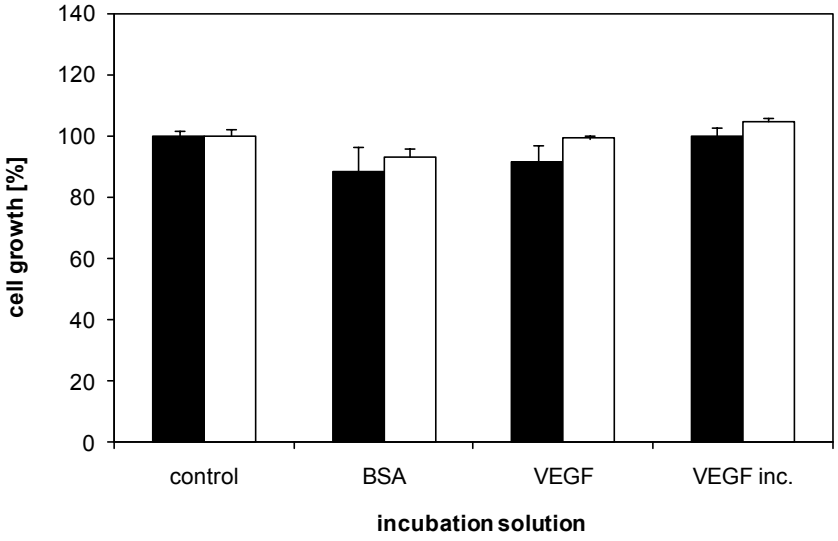


Figure 6-12: Smooth muscle cell growth on Collagen (▬) and PEG-Collagen (▭) coated discs after treatment with different incubation solutions.

In contrast to the findings observed for endothelial cells presented to $VEGF_{165}$ coupled discs, SMCs exhibited a different growth rate effect when grown on collagen and PEG-collagen coated discs (Figure 6-12). The growth of SMCs was not stimulated when presented with collagen coated discs irrespective of their incubation medium. A slight decrease was observed for cells grown on BSA incubated discs, as also seen for endothelial cells. The covalent attachment of $VEGF_{165}$ to the PEG-collagen matrix did not affect this observed pattern. All discs showed a cell growth comparable to the buffer incubated samples.

Therefore, it could be concluded that $VEGF_{165}$ had no stimulatory effect on SMCs, however increased the growth of endothelial cells. This is in accordance with previous findings documented in literature [8, 12, 13, 32, 33]. Thus, the cell growth comparison study successfully showed a beneficial effect of $VEGF_{165}$ coupled to PEG collagen matrices for the stimulation of endothelial cell growth. In addition, these experiments also gave further insight into the ability of internalization of the receptor complex in order for the cytokine to stimulate proliferation of the cells, as described before in literature [34-36]. Covalent attachment of $VEGF_{165}$ did not interfere with the mechanism of action as seen by the stimulation of endothelial cell growth increase, which was in accordance with findings reported in literature [7].

Furthermore, the matrices showed no stimulatory effect on SMC, which provides an additional benefit of the PEG-collagen matrices for the coating of vascular grafts to specifically enhance endothelial cell growth without increasing smooth muscle cell growth and therefore raising the risk of neointimal hyperplasia [5].

4 Conclusions

A functionalized collagen coating was developed for the covalent linkage and immobilization of VEGF to surfaces. Therefore, collagen, a naturally occurring polymer was used to coat PTFE and glass disc as model substrates for vascular prosthesis surfaces. The collagen matrix was successfully functionalized using a homobifunctional PEG-spacer carrying succinimidyl ester to covalently bind to amino moieties within protein sequences. In our case, $VEGF_{165}$, an endothelial cell specific growth stimulator, was used to be covalently attached to the modified polymer surface. The successful linkage of this protein was shown using an antibody staining method. The specificity and integrity of the method towards the target protein was shown using several control experiments.

In addition, the effect of covalently immobilized VEGF on endothelial cells was shown in cell growth experiments using endothelial cells and smooth muscle cells. The effect of the attached $VEGF_{165}$ to PEG-collagen matrices was superior to effects seen for loosely adsorbed protein on collagen matrices. In addition, the sustainable effect on endothelial cells of the attached protein was shown, proving the effective linkage. This is an advantage in regards to loosely adsorbed or in matrices incorporated protein, which can be washed away after release or during incubation. It is an important aspect especially with regards to vascular grafts that are used for vessel replacement and are placed in regions of varying flow conditions. Furthermore, it was shown, that the covalently attached VEGF did not have an effect on smooth muscle cells. Therefore, this approach is an interesting alternative for the modification of vascular grafts that can be used to improve the functionality of existing grafts. The next step to ensure its applicability to modify vascular grafts is the investigation of the performance of these coatings under flow conditions.

5 References

1. Baldwin, S.P. and W. Mark Saltzman, *Materials for protein delivery in tissue engineering.* Advanced Drug Delivery Reviews, 1998. **33**(1-2): p. 71-86.
2. Anderson, J.M., A. Rodriguez, and D.T. Chang, *Foreign body reaction to biomaterials.* Seminars in Immunology, 2008. **20**(2): p. 86-100.
3. Antonios, V.S., et al., *Prosthetic vascular graft infection: A risk factor analysis using a case-control study.* Journal of Infection, 2006. **53**(1): p. 49-55.
4. Sarkar, S., et al., *Addressing thrombogenicity in vascular graft construction.* Journal of Biomedical Materials Research Part B: Applied Biomaterials, 2007. **82B**(1): p. 100-108.
5. Landymore, R.W., C.E. Kinley, and C.A. Cameron, *Intimal hyperplasia in autogenous vein grafts used for arterial bypass: a canine model.* Cardiovasc Res, 1985. **19**(9): p. 589-592.
6. Crombez, M., et al., *Improving arterial prosthesis neo-endothelialization: Application of a proactive VEGF construct onto PTFE surfaces.* Biomaterials, 2005. **26**(35): p. 7402-7409.
7. Sharon, J.L. and D.A. Puleo, *Immobilization of glycoproteins, such as VEGF, on biodegradable substrates.* Acta Biomaterialia, 2008. **4**(4): p. 1016-1023.
8. Zisch, A.H., et al., *Covalently conjugated VEGF-fibrin matrices for endothelialization.* Journal of Controlled Release, 2001. **72**(1-3): p. 101-113.
9. Sano, S., K. Kato, and Y. Ikada, *Introduction of functional groups onto the surface of polyethylene for protein immobilization.* Biomaterials, 1993. **14**(11): p. 817-822.
10. Otrock, Z.K., J.A. Makarem, and A.I. Shamseddine, *Vascular endothelial growth factor family of ligands and receptors: Review.* Blood Cells, Molecules, and Diseases, 2007. **38**(3): p. 258-268.
11. Tabata, Y., et al., *Controlled release of vascular endothelial growth factor by use of collagen hydrogels.* Journal of Biomaterials Science, Polymer Edition, 2000. **11**(9): p. 915-930.
12. Ferrara, N., et al., *The vascular endothelial growth factor family of polypeptides.* J. Cell. Biochem., 1991. **47**(3): p. 211-18.
13. Dorafshar, A.H., et al., *Vascular endothelial growth factor inhibits mitogen-induced vascular smooth muscle cell proliferation.* J. Surg. Res., 2003. **114**(2): p. 179-186.
14. Geiger, M., R.H. Li, and W. Friess, *Collagen sponges for bone regeneration with rhBMP-2.* Advanced Drug Delivery Reviews, 2003. **55**(12): p. 1613-1629.
15. Ruszczak, Z. and W. Friess, *Collagen as a carrier for on-site delivery of antibacterial drugs.* Advanced Drug Delivery Reviews, 2003. **55**(12): p. 1679-1698.
16. Wallace, D.G. and J. Rosenblatt, *Collagen gel systems for sustained delivery and tissue engineering.* Advanced Drug Delivery Reviews, 2003. **55**(12): p. 1631-1649.
17. Friess, W., *Collagen - biomaterial for drug delivery.* European Journal of Pharmaceutics and Biopharmaceutics, 1998. **45**(2): p. 113-136.
18. Wissink, M.J.B., et al., *Improved endothelialization of vascular grafts by local release of growth factor from heparinized collagen matrices.* Journal of Controlled Release, 2000. **64**(1-3): p. 103-114.
19. Scharn, D.M., et al., *Thrombogenicity and Related Biological Properties of Heparin Bonded Collagen Coated Polyester and Human Umbilical Vein Prosthetic Vascular Grafts.* Journal of Surgical Research, 2006. **134**(2): p. 182-189.

20. Wachem, P.B.v., et al., *In vivo biocompatibility of carbodiimide-crosslinked collagen matrices: Effects of crosslink density, heparin immobilization, and bFGF loading.* Journal of biomedical materials research, 2001. **55**(3): p. 368-378.
21. Friess, W., *Drug Delivery Systems Based on Collagen.* 1999.
22. Snyder, S.L. and P.Z. Sobocinski, *Improved 2,4,6-trinitrobenzenesulfonic acid method for the determination of amines.* Analytical Biochemistry, 1975. **64**(1): p. 284-8.
23. Roberts, M.J., M.D. Bentley, and J.M. Harris, *Chemistry for peptide and protein PEGylation.* Advanced Drug Delivery Reviews, 2002. **54**(4): p. 459-476.
24. van Wachem, P.B., et al., *Adhesion of cultured human endothelial cells onto methacrylate polymers with varying surface wettability and charge.* Biomaterials, 1987. **8**(5): p. 323-328.
25. Bowlin, G.L. and S.E. Rittgers, *Electrostatic endothelial cell transplantation within smalldiameter (<6 mm) vascular prostheses: A prototype apparatus and procedure.* Cell Transplantation, 1997. **6**(6): p. 631-637.
26. Böhme, U. and U. Scheler, *Effective charge of bovine serum albumin determined by electrophoresis NMR.* Chemical Physics Letters, 2007. **435**(4-6): p. 342-345.
27. Keshaw, H., A. Forbes, and R.M. Day, *Release of angiogenic growth factors from cells encapsulated in alginate beads with bioactive glass.* Biomaterials, 2005. **26**(19): p. 4171-4179.
28. Langer, R. and D.A. Tirrell, *Designing materials for biology and medicine.* Nature, 2004. **428**(6982): p. 487-492.
29. Blümmel, J., et al., *Protein repellent properties of covalently attached PEG coatings on nanostructured SiO2-based interfaces.* Biomaterials, 2007. **28**(32): p. 4739-4747.
30. Koch, S., et al., *Enhancing angiogenesis in collagen matrices by covalent incorporation of VEGF.* J. Mater. Sci. Mater. Med., 2006. **17**(8): p. 735-741.
31. Fischer, D., *Degradationsuntersuchung eines Komplexes aus VEGF165 und Kollagen Typ I in vitro im Kreislaufsimulationsmodell: Analyse der Freisetzungskinetik des VEGF165 und der licht- und elektronenmikroskopischen Morphologie des Komplexes*, in *Medizinische Fakultät*. 2006, Westfälische Wilhelms-Universität Münster: Münster. p. No pp given.
32. Weatherford, D.A., et al., *Vascular endothelial growth factor and heparin in a biologic glue promotes human aortic endothelial cell proliferation with aortic smooth muscle cell inhibition.* Surgery, 1996. **120**(2): p. 433-439.
33. Shireman, P.K. and H.P. Greisler, *Mitogenicity and release of vascular endothelial growth factor with and without heparin from fibrin glue.* Journal of Vascular Surgery, 2000. **31**(5): p. 936-943.
34. Santos, S.C.R., et al., *VEGF and VEGFR-2 (KDR) internalization is required for endothelial recovery during wound healing.* Exp. Cell Res., 2007. **313**(8): p. 1561-1574.
35. Bikfalvi, A., et al., *Interaction of vasculotropin/vascular endothelial cell growth factor with human umbilical vein endothelial cells: Binding, internalization, degradation, and biological effects.* Journal of Cellular Physiology, 1991. **149**(1): p. 50-59.
36. Li, W. and G. Keller, *VEGF nuclear accumulation correlates with phenotypical changes in endothelial cells.* J Cell Sci, 2000. **113**(9): p. 1525-1534.

Chapter 7

Summary of the Thesis

The goal of this thesis was the improvement of vascular grafts to enhance endothelialization by applying surface coatings on existing commercially available grafts. Polytetrafluoroethylene (PTFE), chosen for its similar composition compared to expanded PTFE, which is used for vascular grafts, and glass, as models, were utilized as substrates for the coating. The general introduction gave an overview of frequently encountered problems when using vascular grafts in vessel replacement, especially for those employed in regions of low blood flow and of small diameter.

In order to establish functional coatings on the surface of PTFE, atmospheric plasma treatment with a plasmabrush® using argon gas was performed. Changes induced by the plasma treatment process were evaluated using several methods to detect and characterize the alterations in surface morphology and composition, such as Fourier transform infrared (FT-IR) spectroscopy, scanning electron microscopy, X-ray photoelectron spectroscopy and surface energy determination. Effects were successfully characterized by surface energy determination and were stable for several weeks. After optimization of the process conditions, treatment time and ignition voltage it was possible to increase the surface energy of PTFE samples by 10-15 mN/m. The characterization of modifications, by means of other methods than surface energy determination, did not reveal radical structural or chemical alterations. However, the methods might have not been sensitive enough to detect the effects induced by the atmospheric plasma treatment. The established plasma activation process significantly increased the coating adhesion to PLGA and collagen films applied on the modified surfaces, in contrast to the non adhesion of these films on unmodified PTFE surfaces. In addition, it was shown that the plasma modification greatly improved the adherence of endothelial cells on the plasma treated surfaces.

For the controlled presentation of $VEGF_{165}$, an endothelial cell specific growth enhancer, two approaches, matrix embedding combined with sustained release and

covalent attachment, were pursued. For the matrix release approach, PLGA was the first polymer used due to the experience with this polymer in biomaterial applications. The polymers investigated in the process of this study were Resomer® RG 502H, RG 503, RG 503H, and RG 504H. The influence of molecular weight on the release of FITC-dextran as model compound was shown. Resomer® RG 502H, the lowest molecular weight species investigated, was shown to be the most suitable in the range of polymers examined. Its release rate suited the intended purpose and the desired release time period of 1 to 2 weeks. In addition, it was shown that the influence of esterification of free carboxylic groups of the used polymers did not have a beneficial effect on the release rates of coatings. The influence of the suspended model compound particles on the coating process and the subsequent release was investigated and was shown to have no influence on the outcome of release investigations, especially in respect of burst release from such coatings in contrary to previously reported findings. In addition, the cell compatibility of the applied PLGA coatings was shown using human umbilical cord endothelial cells. Fluorescent activated cell sorting analysis, propidium iodide staining in combination with fluorescence measurements, and using a counting chamber were shown to be unsuitable to reveal small differences in cell growth. However, minor changes in cell growth increase were successfully detected using Cell Titer-Blue®, a commercially available cell viability assay.

The second polymer investigated for the matrix release approach was collagen, chosen for its ease of production in aqueous media and good cell compatibility. Crosslinking of collagen was performed and it was shown to be an effective tool to modify release properties of collagen without risking the good biocompatibility of this material. It was shown that in accordance with increasing crosslinking degree, the initial release of model compound was reduced and higher amounts, of up 70 % for the highest crosslinked material, were entrapped in the matrix. The predetermined release properties for the model compound, FITC-dextran, could be successfully confirmed for $VEGF_{165}$. VEGF incorporated into collagen films was investigated towards its effect on endothelial and smooth muscle cells and no stimulatory effect on smooth muscle cells could be shown. More importantly, an increase in endothelial cell growth by VEGF released from collagen films was demonstrated.

Therefore, biological activity of a sufficient amount of VEGF was maintained throughout the manufacturing and drying process of collagen films and the subsequent release to stimulate endothelial cell growth.

For the second approach, the influence of $VEGF_{165}$ covalently attached to modified PLGA and collagen matrices on endothelial cells and smooth muscle cells was investigated. The modification was accomplished using a homobifunctional polyethylene glycol (PEG) spacer carrying succinimidyl ester groups to bind to amine groups within the protein sequence, the collagen, and the modified PLGA.

For PLGA, it was shown that the functionalized PEG-PLGA was able to covalently bind $VEGF_{165}$ using several staining techniques, such as silver staining and Deep Purple™ Total Protein Stain. Other techniques, such as FT-IR spectroscopy, were not sensitive enough to detect VEGF. An antibody staining method was developed to determine the presence of $VEGF_{165}$ on the modified PLGA and collagen surfaces, and thereby confirming successful linkage of the cytokine to the effectively modified polymers. The interaction of the bound $VEGF_{165}$ with the antibody was a first indicator of the sustained functionality of the cytokine. In cell assays, covalently attached VEGF could be shown to have a positive effect on the cell growth of endothelial cells. Furthermore, PEG-PLGA exhibited a limiting effect on the proliferation of smooth muscle cells, due to its cell-adhesion-resistant properties. PEG-collagen matrices did not exhibit any effect on smooth muscle cell growth. The successful attachment of $VEGF_{165}$ could be shown while preserving the biological activity and endothelial cell specific growth stimulation for an extended period of time in solution. Moreover, it was proven, that the irreversibly linked $VEGF_{165}$ was still able to interact with its receptor and to stimulate endothelial cells.

Within the scope of this thesis, several approaches for the improvement of vascular grafts using an endothelial cell specific growth stimulant were successfully developed and could be shown to have a positive effect on the stimulation of endothelial cells without stimulating the growth of smooth muscle cells. Especially the covalent linkage of active $VEGF_{165}$ to a modified collagen or PLGA matrix appeared to be a promising tool to improve the performance of vascular grafts.

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