Modulation of growth factor functionality through immobilization in starPEG-heparin networks

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List of abbreviations

A(v) ATR-FTIR absorbance spectrum
Ac Acetyl group
Akt Protein kinase B
ANOVA Analysis of variance
ATR Attenuated total reflection
BSA Bovine serum albumin
CAM Chorioallantoic membrane
CLSM Confocal laser scanning microscopy
Col I Collagen I
CX Cytokines with a "cysteine-separating amino acid-cysteine" motif
DMSO Dimethyl sulfoxide
DPSS laser Diode-pumped solid-state laser
EC Endothelial cell
ECGM Endothelial cell growth medium
ECM Extracellular matrix
ED Embryonic day
EDC 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA Ethylenediaminetetraacetic acid
EGF Epidermal growth factor
ELISA Enzyme-linked immunosorbent assay
ERK Extracellular signal-regulated kinase
et al. et alii
FCS Fetal calf serum
FDA Fluorescein di-O-acetate
FGF-2 Fibroblast growth factor-2
FGFR Fibroblast growth factor receptor
Fmoc Fluorenylmethyloxycarbonyl
FTIR Fourier transform infrared spectroscopy
GDNF Glial-derived neurotrophic factor
GF Growth factor
HPLC High-performance liquid chromatography
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>HPSEC</td>
<td>High performance size exclusion chromatography</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>I</td>
<td>Intensity</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulinlike growth factor-1</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>Kd</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>MilliQ</td>
<td>deionized, decarbonized water</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metallo-proteinase</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyltetrazoliumbromide</td>
</tr>
<tr>
<td>NADH/NADPH</td>
<td>Nicotinamide adenine dinucleotide/Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Particle charge detector</td>
</tr>
<tr>
<td>PDADMAC</td>
<td>Poly(diallyldimethylammonium chloride</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIGF</td>
<td>Placenta growth factor</td>
</tr>
<tr>
<td>pK</td>
<td>logarithmic measure of the acid dissociation constant</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLG</td>
<td>Poly(lactide-co-glycolide)</td>
</tr>
<tr>
<td>Ras family</td>
<td>Rat sarcoma family of signaling transduction molecules</td>
</tr>
<tr>
<td>SF</td>
<td>Serum-free</td>
</tr>
<tr>
<td>s-NHS</td>
<td>N-hydroxysulfosuccinimide</td>
</tr>
<tr>
<td>Src</td>
<td>Sarcoma family of signaling transduction molecules</td>
</tr>
<tr>
<td>TAMRA</td>
<td>TetramethylRhodamine</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor-β1</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>U</td>
<td>Unit</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>Vis</td>
<td>Visible light</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
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<tr>
<td>w/w</td>
<td>weight/weight</td>
</tr>
<tr>
<td>ξ</td>
<td>Mesh size</td>
</tr>
<tr>
<td>γ</td>
<td>Molar ratio of starPEG to heparin</td>
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1 Introduction and aim of the thesis

Vascular disease generally refers to a pathological state affecting the circulatory system of the body. Main risk factors include physical inactivity, hypertension, smoking, obesity, dyslipidemia, and hormone status. As its prevalence increases with the rise of the average age, vascular disease represents the leading cause of death in industrialized nations. Apart from the loss of life years, a diminished quality of life as well as enormous direct and indirect medical costs have to be directly associated with the occurrence of vascular disease [Kim et al., 2011].

As a major cause of vascular disease, atherosclerosis defined as the narrowing of the arteries by deposition of lipids and fibrous elements on their walls, limits the blood flow [Lusis, 2000]. Subsequently, resulting ischemia can lead to substantial functional deficits and tissue damage most commonly affecting the heart, brain and lower limbs.

However, the formation of new blood vessels from pre-existing ones, a process designated as angiogenesis, only restrictively occurs in the body, so that a natural recovery of the ischemic site is rather unlikely. Therefore, as potential therapies, usually anti-thrombotic strategies as well as bypass grafting are pursued. However, autologous transplantation of vessels might often not be feasible due to damage and pre-existing disease. In contrast, the application of allogeneic or synthetic grafts can lead to immunologic and thrombotic problems. Consequently, there is an urgent need for appropriate arterial substitutes, tissue regeneration after ischemia, and the formation of mature blood vessel networks, which is very actively explored by researchers in the context of vascular tissue engineering [Zhang et al., 2007].

Tissue engineering, often interchangeably used with the term regenerative medicine [Lee et al., 2011], merges principles of life sciences and engineering towards the development of biological replacements that restore, maintain or improve tissue function. Besides vascular tissue engineering, which aims to create physiologically interactive substitutes for diseased or injured vascular tissue [Zhang et al., 2007], sufficient vascularization represents an absolute requirement for any other type of engineered tissue as well to ensure gas exchange and supply of essential nutrients, the removal of metabolites and the establishment of a biochemical communication within regenerative approaches [Ko et al., 2007]. Consequently, the development of strategies to support effective blood vessel formation is of outstanding general interest for almost every therapeutic tissue engineering concept. In this context, therapeutic angiogenesis attempts to apply and enforce the natural process of blood vessel formation by the provision of bioactive effectors [Zisch et al., 2003b; Zhang et al., 2007]
on the molecular (such as angiogenic growth factors) or cellular (such as vessel-
constituting endothelial cells) level [Lee et al., 2001].
Inspiration for regenerative approaches originates from the fact that in their natural
environment cells closely interact with the extracellular matrix (ECM), which represents
a modular, three-dimensional and intricate scaffold that governs tissue function and
organization by providing the cells with an adhesive mechanical support and specific
biomolecular cues such as growth factors [Kleinman et al., 2003]. Following nature’s
lead, strategies for vascular tissue engineering therefore generally rely on the use of
polymeric matrices to deliver angiogenic effectors [Zisch et al., 2003b; Zhang et al.,
2007]. As growth factors or cytokines are critically important for determining the cellular
behavior, many biomaterials were developed to allow for a controlled administration of
these proteins. However, besides the possibility to effectively store and sustainably
release the cytokines, for successfully promoting therapeutic angiogenesis, there are
several additional requirements to be fulfilled by a certain growth factor delivery system
[Lutolf et al., 2005]. As blood vessel formation is governed by both mechanical and
biomolecular cues provided by the ECM, scaffolds to be used in angiogenic tissue
engineering should offer similar, precisely adjustable structural and biological (including
bioadhesiveness, enzymatic degradability and cytokine-binding ability) recognition
properties. Moreover, since physiological angiogenesis is characterized by the complex
interplay of various growth factors, the potential for an independent and adaptable
parallel delivery of multiple cytokines defines another favorable characteristic of
angiogenesis-stimulating biomaterials. Finally, in order to specifically meet the intricate
demands of effective therapeutic angiogenesis, the growth factor provision should be
tunable in response to local environmental cues or externally applied triggers [Lee et
al., 2011]. Although several powerful cytokine delivery systems for angiogenic tissue
engineering were developed to closely mimic mechanical and biomolecular key
properties of the ECM [Zisch et al., 2003a], to allow for the combined administration of
several cytokines [Richardson et al., 2001] or to permit the precise triggering of the
growth factor release [Ehrbar et al., 2008], the integration of all these features into one
single material represents a challenging, but indispensable prerequisite to further
enhance therapeutic effectiveness.

Consequently, the aim of this work was to evaluate the potential of a modular biohybrid
hydrogel composed of star-shaped poly(ethylene glycol) (starPEG) and heparin as a
delivery system for two major angiogenic cytokines, fibroblast growth factor-2 (FGF-2)
and vascular endothelial growth factor (VEGF) [Klagsbrun et al., 1999]. For this system, effective administration of the cytokines was based on their reversible affinity-interaction with heparin, a highly charged glycosaminoglycan capable of complexing various different growth factors [Capila et al., 2002]. After the establishment of appropriate analytical methods, binding and release of both cytokines had to be investigated for different heparin-based hydrogel matrices of varied mechanical characteristics and degree of biofunctionalization (e.g. with adhesion ligands). Moreover, the possibility to deliver distinct concentrations of either single growth factors or combinations of FGF-2 and VEGF had to be explored. To evaluate the general suitability for pro-angiogenic stimulation, the provision of the cytokines from scaffolds differing in physicochemical characteristics and biofunctionalization had to be studied using human endothelial cells, the cell type that forms the inner layer of any blood vessel [Carmeliet, 2003], in culture. Based on these experiments, matrices most effectively stimulating pro-angiogenic cellular responses were to be selected for \textit{in vivo} experiments consisting of the incorporation into the chorioallantoic membrane (CAM) system of fertilized chicken eggs, an assay commonly used to quantify the vascularization potential of biomaterials \textit{in vivo} [Auerbach et al., 2003]. After evaluating the ability of a growth factor delivery by starPEG-heparin matrices to stimulate therapeutic angiogenesis, further options to specifically modulate the growth factor release had to be explored to adjust the system for maximal pro-angiogenic response. Taken together, this work aimed at investigating the possibility to control immobilization and subsequent release of FGF-2 and/or VEGF from starPEG-heparin hydrogels to promote angiogenesis in tissue engineering. Using a novel type of biohybrid matrix platform, this project set off to generate fundamental insights into the provision of functional signaling molecules from polymer scaffolds with far-reaching control over structural parameters like stiffness, degradability and adhesive properties.
2 Theoretical background

2.1 Physiological angiogenesis

2.1.1 The biological process of physiological angiogenesis

The establishment and maintenance of a vascular supply is an absolute requirement for the growth and survival of organs and tissues [Ko et al., 2007]. Depending on the size of a particular blood vessel, its composition may vary. While small vascular tubes consist only of endothelial cells, larger vessels are surrounded and stabilized by mural cells (pericytes in medium-sized and smooth muscle cells in large vessels) [Carmeliet, 2003].

Angiogenesis is defined as the formation of new blood capillaries from pre-existing vessels [Distler et al., 2003]. While abnormal development of vasculature is associated with various diseases such as cancer, arthritis, psoriasis, blindness, obesity, arteriosclerosis, asthma and some infections, physiological angiogenesis mainly occurs during embryonic development [Carmeliet, 2003]. In the adult organism, it is a very rare event usually restricted to wound healing and the female reproductive system [Klagsbrun et al., 1999; Liekens et al., 2001; Distler et al., 2003]

The biological process of angiogenesis (Fig. 1) involves several steps, which are controlled by the complex interplay of cells, soluble angiogenic or angiostatic effector molecules and the extracellular matrix (ECM) [Liekens et al., 2001]. Upon initiation, angiogenic factors such as fibroblast growth factor-2 (FGF-2) or vascular endothelial growth factor (VEGF) bind to their receptors on endothelial cells thereby activating different signal transduction pathways [Klagsbrun et al., 1999]. One of the first events that occur in the following is the vasodilation of the pre-existing blood vessel [Distler et al., 2003]. During this step, nitric oxide synthase in endothelial cells is activated by FGF-2 [Cuevas et al., 1996; Tiefenbacher et al., 1997] or VEGF [Murohara et al., 1998; Garcia-Cardena et al., 1998; Hood et al., 1998; Bouloumié et al., 1999]. In response to an increase in the intracellular levels of nitric oxide, guanylcyclase synthesizes cyclic guanosine monophosphate, which results in a relaxation of mural cells. Moreover, VEGF enhances the permeability of endothelial cells by initiating the formation of interconnected vesicles and vacuoles [Kohn et al., 1992]. Upon cluster formation, they are able to span the whole cytoplasm. As a result of the increased cell permeability mediated by these organelles and by inter-endothelial cell junctions [Dvorak et al., 1995], the transport of plasma proteins such as fibrinogen and plasminogen from the
blood stream into the surrounding tissue is highly facilitated. Within the next steps, these plasma proteins provide a scaffold for the migrating endothelial cells. However, in advance, the removal of mural cells, the disintegration of the endothelial cell basement membrane and the remodeling of the perivascular stroma has to occur [Klagsbrun et al., 1999; Liekens et al., 2001; Distler et al., 2003]. The key players involved in these processes are angiopoietin 2 [Maisonpierre et al., 1997] as well as different proteases including plasminogen activators and matrix metallo-proteinases (MMPs) [Mignatti et al., 1996]. After the vessel destabilization and matrix degradation, endothelial cells start to proliferate and to migrate in response to a gradient of chemotactic molecules through the degraded basement membrane into the remodeled and softened perivascular space [Klagsbrun et al., 1999; Liekens et al., 2001; Distler et al., 2003]. These processes are induced by a variety of different angiogenic effectors such as members of the VEGF- [Veikkola et al., 1999; Ferrara, 1999] and FGF-family [Bussolino et al., 1996; Presta et al., 2005], angiopoietin 1 [Koblizek et al., 1998; Hayes et al., 1999] and 2 [Mochizuki et al., 2002; Lobov et al., 2002], angiogenin [Badet, 1999], epidermal growth factor (EGF) [Schreiber et al., 1986; Sato et al., 1993; Bussolino et al., 1996], CXC-chemokines (containing a cysteine-separating amino acid-cysteine motif) [Keane et al., 1999] and insulinlike growth factor-1 (IGF-1) [Bar et al., 1988], while some of them are released by the degraded ECM or different cell types. After the endothelial cells have reached the site with decreased vessel density, they start assembling in a monolayer and finally build a capillary sprout, which is further propagated by proliferating endothelial cells until a lumen is formed [Klagsbrun et al., 1999; Liekens et al., 2001; Distler et al., 2003]. Among others, different VEGF isoforms [Veikkola et al., 1999; Ferrara, 1999] as well as adhesion molecules such as integrin αvβ3 [Eliceiri et al., 1998] or E-selectin [Nguyen et al., 1992] are essential for these processes. In the last step, the stabilization of the vascular tube by mural cells completes the formation of a new vessel [Klagsbrun et al., 1999; Liekens et al., 2001; Distler et al., 2003]. For this, mesenchymal cells of the surrounding tissue can be recruited in response to effectors such as the platelet-derived growth factor (PDGF) isoform PDGF-BB [Lindahl et al., 1997]. Upon these signals, they start to proliferate and to migrate to the abluminal surface of the premature vessels [Klagsbrun et al., 1999; Liekens et al., 2001; Distler et al., 2003]. Here, they either differentiate into pericytes, which are found within the basement membrane, or into vascular smooth muscle cells, which are located abluminal of the basement membrane [Kurz, 2000].
2.1.2 The angiogenic cytokines fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF)

The term growth factor or cytokine is broadly used to describe a naturally occurring, soluble-secreted signaling polypeptide that is able to stimulate a specific cell behavior such as survival, proliferation or differentiation in the context of a biological environment. After secretion from a producer cell, many growth factors can be stored via an interaction with ECM molecules. The function of these proteins is usually mediated by an interaction with surface receptors on the target cells which show an
intrinsic tyrosine kinase activity. Upon complex cascades involving phosphorylation of target proteins, ion fluxes, changes in metabolism, gene expression and protein synthesis, the signal created by the growth factor is translated into an integrated biological response (Fig. 2) [Lee et al., 2011].

In the context of angiogenesis, two of the most important regulators are the angiogenic cytokines fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) [Klagsbrun et al., 1999; Liekens et al., 2001; Distler et al., 2003].
FGF-2

FGF-2 belongs to the FGF family [Gospodarowicz, 1975], which contains more than 20 heparin-binding members [Presta et al., 2005]. It consists of twelve anti-parallel β-sheets that are organized into a tragonal pyramidal structure and has a hydrodynamic diameter of 3.4 to 4 nm [Eriksson et al., 1991; Nugent et al., 2000]. FGF-2 contains four cysteine residues with no intramolecular disulfide bonds and two sites (serine 64 and threonine 112) that can be phosphorylated. Moreover, a large number of basic residues (pI = 9.6) [Bikfalvi et al., 1997] mediate the highly affine (Kd = 23 nM) [Ashikari-Hada et al., 2004] electrostatic binding to the negatively charged sulfate groups of heparin. Here, two sites (aspartic acid 28, arginine 121, lysine 126 and glutamine 135 as well as lysine 27, asparagine 102 and lysine 136) [Faham et al., 1996] interact with the 2-O- and N-sulfate groups of the glycosaminoglycan [Habuchi et al., 1992; Turnbull et al., 1992; Faham et al., 1996], with affinity increasing with heparin chain length [Nugent et al., 2000]. Although the heparin 6-O-sulfate groups do not participate in FGF-2 binding, they are involved in the enhancement of the protein activity [Pye et al., 1998].

While larger isoforms (22, 22.5 or 24 kDa) resulting from alternative splicing are predominantly localized to the nucleus, the most prominent 18 kDa form of FGF-2 is mainly found outside of the cell [Florkiewicz et al., 1991]. As it contains no signal sequence for a cellular export, this protein does not progress via the regular secretory pathway [Mignatti et al., 1992]. Instead, FGF-2 could be released to the extracellular space via non-lethal membrane disruptions as well as cell damage and death [Nugent et al., 2000]. In the ECM, FGF-2 is stored upon binding to heparin-like molecules [Folkman et al., 1988]. While this interaction protects the protein from proteolytic cleavage and heat or acid denaturation, the dynamic FGF-2 binding and release by the glycosaminoglycans also controls its diffusional movement and distribution [Gospodarowicz et al., 1986a; Dowd et al., 1999]. Here, enzymatic degradation of the heparin-like molecules as well as the presence of competitive antagonists, soluble glycosaminoglycans, FGF-binding proteins and analogs influence the availability of FGF-2 from ECM sites [Nugent et al., 2000].

Upon release from the glycosaminoglycans, FGF-2 interacts with four different, structurally related tyrosine kinase cell surface receptors (FGFR1-4) and their splice variants [Zhang et al., 2006]. FGF-2 contains two separate receptor interaction sites, which allow a single FGF-2 to bind to two receptor molecules or to interact with one receptor molecule at two different sites [Kan et al., 1993]. Upon a binding to heparin or
heparan sulfate, the affinity of FGF-2 for its receptors is highly increased [Yayon et al., 1991], as, due to the presence of a heparin interaction site on FGFR1 [Kan et al., 1993], a ternary complex of FGF-2, the glycosaminoglycan and the receptor can be formed. This cluster might act as a bridge to facilitate FGFR dimerization, which results in an activation and the subsequent autophosphorylation of the cytoplasmatic tyrosine kinase domain [Nugent et al., 2000]. This event represents the starting point for several intracellular signaling cascades including the Ras (derived from “rat sarcoma”), Src (derived from “sarcoma”) family tyrosine kinases, phosphoinositide 3-kinase (PI3K) and the phospholipase C (PLC) pathway which all lead to a FGF-2 mediated cell response [Cross et al., 2001]. After that, FGF-2 is either taken up by the cell bound to the glycosaminoglycans in the context of their constitutive internalization and turnover or together in the ternary complex with heparin or heparan sulfate and FGFR in order to degrade the cytokine in the lysosomes [Nugent et al., 2000]. However, intracellular FGF-2 can also translocate to the cell nucleus and directly exert gene regulatory functions [Sperinde et al., 1998; Choi et al., 2000; Nugent et al., 2000].

As the expression of FGF-2 is nearly ubiquitous, it influences the survival, growth, differentiation and migration of various cell types being derived from the mesoderm or neuroectoderm [Gospodarowicz et al., 1986b] such as fibroblasts, myeloblasts, osteoblasts, neural or endothelial cells [Bikfalvi et al., 1997]. Nevertheless, disruption of the mouse FGF-2 gene results in mice with decreased vascular tone and low blood pressure, but which displayed relatively mild cardiovascular, skeletal, and neuronal phenotypes thereby indicating compensatory actions of other FGF family members [Dono et al., 1998; Cross et al., 2001]. In contrast, a transgenic over-expression of FGF-2 predominantly affects the development and mineralization of bone [Coffin et al., 1997]. In the context of angiogenesis, cell or ECM-released FGF-2 has been shown to stimulate the proliferation, migration and differentiation of endothelial cells both in vitro and in vivo [Presta et al., 2005]. Moreover, it is also directly expressed by endothelial cells [Schweigerer et al., 1987; Vlodavsky et al., 1987]. Here, FGF-2 induces the production of nitric oxide, proteases or adhesion molecules thereby contributing to the propagation of the angiogenic process at several levels [Presta et al., 2005]. Besides, FGF-2 demonstrates a strong synergistic action together with VEGF. Both in vitro and in vivo, this affects the induction of endothelial cell proliferation and migration as well as the formation of capillary tubes [Pepper et al., 1992; Goto et al., 1993; Asahara et al., 1995; Vernon et al., 1999; Yan et al., 2001; Kano et al., 2005].
VEGF

VEGF belongs to the VEGF family, which currently comprises the six members VEGF-A (which refers to the VEGF originally identified) [Senger et al., 1983; Ferrara et al., 1989], placenta growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D and the orf parapox virus VEGF, denoted as VEGF-E [Veikkola et al., 1999; Zachary et al., 2001; Robinson et al., 2001]. As a result of alternative splicing of the human gene encoding VEGF-A, at least six isoforms of 121, 145, 165, 183, 189 and 206 amino acids are generated [Robinson et al., 2001]. Out of these variants, VEGF-A165 (from here on denoted as VEGF) is the predominant and most biologically active form [Veikkola et al., 1999; Zachary et al., 2001]. This protein is glycosylated at asparagine 74 and it contains 16 cysteine residues that are involved in intra- and inter-chain disulfide bonds [Ferrara et al., 1991]. VEGF is typically expressed as a ~46 kDa disulfide-linked homodimer, while each monomer consists of a four-stranded β-sheet. The protein has a hydrodynamic diameter of ~6 nm [Muller et al., 1997] and it possesses several basic residues (pI = 8.5) [Ferrara et al., 1991]. Here, mainly one linear stretch consisting of the amino acids arginine 123, and 124, lysine 125 and 140, arginine 145, 149 and 156 with potential contributions from lysine 162, as well as arginine 164 and 165 [Fairbrother et al., 1998] mediates the electrostatic binding to heparin (Kd = 165 nM) [Ashikari-Hada et al., 2004]. While this interaction preferentially occurs on 6-O- and N-sulfate groups of the glycosaminoglycan, nevertheless also the 2-O-sulfate and the carboxylic acid residues of heparin contribute to the overall binding strength [Ono et al., 1999; Robinson et al., 2006].

VEGF is a diffusible protein that is secreted into the extracellular medium of the producer cell, where it can be associated to the ECM upon binding to heparin-like molecules [Houck et al., 1992]. Similar to FGF-2, this interaction protects the biological integrity of VEGF [Lee et al., 2005], controls its availability and mediates the action of the protein upon modulation of its receptor affinity [Gitay-Goren et al., 1992]. Here, the receptor tyrosine kinases VEGFR-1 and VEGFR-2 have been identified as high-affinity binding sites for VEGF on the vascular endothelium, while neuropilins modulate the VEGF interaction with these two main receptors [Zachary et al., 2001; Robinson et al., 2001]. Comparable to VEGF itself [Shweiki et al., 1992], the receptor expression is regulated by oxygen tension [Tuder et al., 1995; Li et al., 1996]. While VEGFR-1 might function as a negative regulator of VEGFR-2, the most important angiogenic effects of VEGF are mediated by VEGFR-2 [Zachary et al., 2001; Robinson et al., 2001]. Upon
ligand binding, VEGF triggers the receptor dimerization and subsequent auto-/trans-phosphorylation which finally results in a signal transduction to the nucleus thereby leading to a specific cell response. Here, VEGF mainly exert its action via the PLC, extracellular signal-regulated kinase (ERK), PI3K/Akt (protein kinase B) or Src family tyrosine kinases pathways [Zachary et al., 2001; Cross et al., 2001].

In normal tissues such as brain, kidney, liver or spleen [Veikkola et al., 1999], VEGF is expressed by most of the cell types such as monocytes, macrophages, hepatocytes, lymphocytes, keratinocytes, osteoblasts or smooth muscle cells. In contrast to FGF-2, VEGF is generally not secreted by its main targets, the endothelial cells [Maharaj et al., 2007]. Nevertheless, the importance of VEGF in vascular development is highlighted by the fact that the loss of only a single VEGF allele already leads to embryonic lethality [Carmeliet et al., 1996; Ferrara et al., 1996]. During angiogenesis, VEGF has been shown to stimulate the survival, proliferation, migration and differentiation of endothelial cells both in vitro and in vivo, while it is not mitogenic for most other cell types [Veikkola et al., 1999; Maharaj et al., 2007]. Moreover, it increases vascular permeability by stimulating the nitric oxide synthase and enhances basal membrane and ECM degradation by supporting the expression of proteases [Distler et al., 2003]. Due to their critical roles in the context of vascular development, both FGF-2 and VEGF are key players in the concept of therapeutic angiogenesis.

2.1.3 Angiogenesis as a therapeutic target

Although a strong clinical interest has emerged in developing anti-angiogenesis reagents for pathological phenomena associated with undesired or enhanced formation of new vessels, several strategies also aim to support angiogenesis for the treatment of ischemic diseases or for enabling a sufficient vascularization of an engineered implant. Ischemia is defined as hypoxia or necrosis of a tissue as a result of an obstruction of the arterial blood supply or an insufficient blood flow due to underlying metabolic diseases or trauma. Depending on the amount of viable cells remaining in the affected tissue, the body is able to spontaneously develop collateral blood vessels to re-supply the ischemic area thereby supporting a functional recovery. However, with a few exceptions, the body favors a physiological steady-state, in which angiogenesis is suppressed. The vascular endothelium therefore remains rather inactive and represents one of the tissues with the lowest mitotic rate [Zhang et al., 2007].
Consequently, in order to successfully treat ischemic diseases or to support an effective vascularization of an engineered construct for implantation, strategies to induce blood vessel formation are required. In this context, the concept of therapeutic angiogenesis tries to use and enhance the natural process of blood vessel formation upon the administration of bioactive factors. By increasing the blood vessel density, the tissue function close to the ischemic site should be preserved or restored [Zhang et al., 2007]. In contrast, upon the induction of vascularization of an implant, sufficient oxygen or nutrient supply, waste removal and the provision of a biochemical communication guide within the engineered construct are ensured [Ko et al., 2007].

Following the strategy of therapeutic angiogenesis, either whole cells or signaling molecules (including drugs, proteins or oligonucleotides) could be delivered as the angiogenic effectors (Fig. 3). The first concept could use matured endothelial cells [Lee et al. 2001], which can be obtained directly from the patient, after which they are expanded in vitro and administered to areas of ischemia tissue or to the site of an implant, where they should participate in blood vessel formation (Fig. 3, a-c). However, these implanted cells might suffer from the lower mitogenic and morphogenic stimuli provided by the natural host environment of elderly people thereby leading to extensive cell death after introduction to the body and thus leaving cell therapies rather insufficient [Uebersax et al., 2009]. In contrast to the first strategy, the application of signaling molecules (most commonly recombinant angiogenic growth factors) to the ischemic site or the implant area should result in a stimulation of the growth, migration and differentiation of cells involved in the process of angiogenesis (Fig. 3, d-f). Here, major problems arise from ineffective or undefined dosing and unresponsiveness to the angiogenic effectors [Zhang et al., 2007], which often show a short half-life and poor bioactivity due to rapid diffusion and denaturation or degradation. As many of these factors also act on different tissues, severe adverse effects could occur upon the transport of these signaling molecules to adjacent sites. Moreover, angiogenesis is tightly controlled by the concentration-dependent, spatially- and temporally-determinated action of multiple factors [Lee et al., 2011]. Consequently, the success of an undefined bolus injection of a single angiogenic signaling molecule might be limited, as already indicated by the rather disappointing results of clinical trials with infusions of FGF-2 [Simons et al., 2002] or VEGF [Henry et al., 2003]. To overcome these problems, more advanced strategies combining either several signaling molecules or both whole cells and molecular angiogenic effectors might be required [Zhang et al.,
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2007]. However, to address these challenges, precisely tunable delivery systems are needed.

Fig. 3 Schematic of the two main approaches used in therapeutic angiogenesis; adapted from [Lee et al., 2001]. Blood vessel formation is either supported by a direct introduction of the target cells (e.g. endothelial cells; a) or by the provision of growth factors (d) to the site of interest. The transplanted cells form new blood vessels starting at the site of their introduction towards the host tissue, while they might also provide stimuli to the existing vessels to grow and sprout into their direction (b). In contrast, soluble cytokines promote the growth of existing blood vessels from the surrounding host tissue into the direction of their delivery (e). Finally, the combination of new vessels with existing ones creates functional vascularization capable of blood flow (c, f).

2.2 Angiogenic tissue engineering

2.2.1 General requirements of biomaterials for an application in angiogenic tissue engineering

Due to the problems associated with the systemic delivery of angiogenic effectors, in the context of therapeutic angiogenesis, there is a demand for scaffolds that provide the appropriate environment for both cells and signaling molecules [Zhang et al., 2007]. In the body, this function is fulfilled by the extracellular matrix (ECM) representing a complex, dynamic and critical component of all tissues (Fig. 4). It is composed of extracellular structural proteins (e.g. collagen or elastin), proteoglycans (e.g. perlecan or aggrecan), glycosaminoglycans (e.g. heparan sulfate or hyaluronic acid) and adhesion proteins (e.g. fibronectin or laminin), as well as growth factors (e.g. FGF-2 or VEGF), which form an intricate, three-dimensional meshwork [Frantz et al., 2010]. Here, the ECM acts as a scaffold for tissue morphogenesis, provides cues for cell
growth and differentiation, supports the maintenance of matured tissues and enhances the repair response after injury [Kleinman et al., 2003]. Based on these important roles of the ECM, the conceptual design of biomaterials to be used for effective tissue engineering approaches involves the mimicry of the two most important ECM attributes: its structural and its biological recognition (including bioadhesiveness, enzymatic degradability and growth factor-binding capacity) properties [Hubbell, 2003].
Structural requirements

The structural character of the ECM is determined by its fibrillar and viscoelastic properties. While the ECM represents a network consisting of tensile components such as fibrillar and amorphous structural proteins, it also contains compressive elements such as amorphous proteoglycans and glycosaminoglycans [Hubbell, 2003]. Together, this composition provides not only support and scaffolding for tissues and cells, but also offers an appropriate mechanical strength. Upon the interaction of a substrate with the cytoskeleton and cellular adhesion ligands, mechanical forces can be directly transduced into chemical signals thereby resulting in a certain cell response [Kleinman et al., 2003]. As it has been shown that substrate stiffness critically determines cellular behavior [Discher et al., 2005], the mechanical properties of biomaterials used for regenerative approaches have to resemble the natural environment of the tissue of interest. In the context of angiogenesis, it has been demonstrated that endothelial cell adhesion and proliferation is favored on stiff substrates, while due to the fact that their requirements for growth and adaptation of an elongated morphology differ, primarily differentiate when cultured on a soft matrix that allows for an retraction and reorientation of the cell shape [Ingber et al., 1989; Deroanne et al., 2001; Liu et al., 2005; Saunders et al, 2010]. As there might be an optimal range for balancing both parameters, the mechanical characteristics of an engineered matrix for therapeutic angiogenesis have to be adapted precisely. Here, mimicking the structural character of the natural ECM, biomaterials for tissue engineering are generally either designed as fibrillar matrices (e.g. by electrospinning; Fidrich et al., 2003) or, since the pioneering work of [Wichterle et al., 1960], most commonly, as viscoelastic hydrogels defined as hydrophilic polymer networks that may absorb up to thousands of times their dry weight in water. Hydrogels can be grouped into either physical gels, where the networks are held together by molecular entanglements, and/or secondary forces (including ionic, H-bonding or hydrophobic forces) or into chemical gels when they are covalently crosslinked. Here, physical hydrogels can be either formed upon change of temperature or pH or by simply combining oppositely charged components. In contrast, chemical crosslinking is usually performed via radiation, the addition of chemical crosslinkers or the mixing of multi-functional reactive compounds. Generally, in order to provide sufficient mechanical strength, hydrogels are often formed out of a diversity of synthetic polymers [Hoffman, 2002], as their mechanical properties can be easily manipulated at micro- and macroscopic levels by blending, copolymerization, or
crosslinking. As an advantage, these molecules can be easily synthesized in large amounts with well-defined characteristics such as molecular weight, molecular architecture, and microscopic morphology. Moreover, the introduction of various readily accessible functional groups at pre-determined polymer sites allows for a versatile macromer chemistry [Jia et al., 2008]. Examples of synthetic polymers used for the formation of tissue engineering constructs include poly(ethylene glycol) (PEG), poly(L-lactic acid), poly(glycolic acid), polycaprolactones, polyoxyesters, polyanhydrides and polycarbonates [Chan et al., 2008].

**Biofunctional requirements**

Although biomaterials based on synthetic polymers show physicochemical and mechanical characteristics similar to those of biological tissues, their biofunctionality is often insufficient, thereby limiting their potential. Consequently, numerous research groups have turned their attention to mimicking the biological recognition character of the ECM in terms of bioadhesiveness, enzymatic degradability and growth factor-binding capacity by designing materials based on naturally occurring polymers, which are analogously assembled into physical or chemically crosslinked gel matrices. These biopolymers are purified from the ECM, isolated from microbial cultures or produced in recombinant cells [Hubbell, 2003]. Although also non-ECM-derived proteins such as albumin or silk fibroin [Uebersax et al., 2009] and polysaccharides such as alginate, chitosan or agarose [Lee et al., 2001] represent important examples for building blocks used to create biofunctional systems, many materials are based directly on ECM components. Once again, both proteins such as collagen or fibrinogen/fibrin and polysaccharides such as the glycosaminoglycans heparin/heparin sulfate, hyaluronic acid or chondroitin sulfate could be elements of these engineered matrices [Hubbell, 2003; Uebersax et al., 2009]. To a certain extent, cells are able to interact with these naturally derived molecules resulting in increased adhesion to the particular biomaterial. Moreover, they may express specific degrading enzymes that permit the remodeling of such systems. Due to the interaction of growth factors with the numerous functional residues found in biopolymers, also the cytokine-binding ability of the ECM can be partially reconstructed. Therefore, to a certain extent, materials based on naturally derived polymers are able to mimic the *in vivo* situation [Lutolf et al., 2005]. However, despite their enhanced biofunctionality, there are also some drawbacks of these systems. For example, besides limitations concerning the material stiffness, the
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possibilities to tune and process these biopolymers are restricted compared to synthetic molecules [Jia et al., 2008].

As a possible solution to overcome these problems, recent approaches rely on the creation of biohybrid materials. These systems are composed of both synthetic and natural polymer constituents interconnected via chemical or physical means, thereby combining the advantages of each single class of polymers. Here, as a structural basis, which additionally provides appropriate mechanical properties, synthetic molecules are used. Using different chemical modifications, they can be combined with natural polymers. However, instead of whole molecules, often low molecular weight peptides, which may possess biological activity in certain applications, are incorporated. Most of these peptides are truncated versions of naturally occurring proteins, which are produced by solid phase synthesis. This results in defined peptides that are slightly less biologically active than the complete, native protein, but do not require a specific secondary structure to be functional. For example, as they were shown to enhance endothelial cell adhesion and proliferation [Wang et al., 2002; Chung et al., 2002], peptide sequences containing the arginine-glycine-aspartic acid (RGD) motif of integrin ligands such as fibronectin [Ruoslahti et al., 1987] or other ECM proteins are incorporated into biohybrid materials. Moreover, also the cell-mediated proteolytic degradation and matrix remodeling can be controlled upon addition of peptide sequences with the GCRDGPQ-GIWGQDRCG motif (in single-letter amino acid code) of common MMP substrates such as collagen, fibronectin or laminin in order to allow for endothelial cell migration and scaffold reorganisation [Seliktar et al., 2004; Kraehenbuehl et al., 2009]. Beside the support of bioadhesiveness and enzymatic degradability, similar to another important molecular recognition property of the ECM, biomaterials used for successful tissue engineering approaches have to be capable to deliver growth factors. Due to the focus of this work, this particular issue will be discussed more in detail.

2.2.2 Biomaterials for growth factor delivery in angiogenic tissue engineering

In order to control the spatio-temporal availability of bioactive growth factors, therapeutic concepts rely on the presentation of cytokines by polymeric biomaterials. For tissue engineering strategies, two different approaches are used to administer
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these effectors. Here, growth factors are either physically entrapped in the delivery matrix or chemically conjugated to the scaffold (Fig. 5).

Fig. 5 Schematic of the two approaches for growth factor presentation by polymeric biomaterials within the context of tissue engineering; adapted from [Lee et al., 2011]. (a) Physically entrapped cytokines can be released from the scaffold to target for example specific cells to migrate and direct tissue regeneration. (b) Alternatively, growth factors can be chemically bound to the delivery system, making them available to cells that infiltrate the material.

**Physical growth factor encapsulation**
The former concept of a physical encapsulation is based on the entrapment of growth factors during the fabrication of a biomaterial, where hydrophobic or hydrophilic interactions among the cytokines and the polymers might facilitate this process. Later on, the cytokine release is determined by diffusion out of the network and/or matrix degradation. Common processing methods for such delivery systems include solvent casting/particulate leaching, freeze drying, phase separation, melt moulding, phase emulsion, *in situ* polymerization and gas foaming, while both synthetic and natural polymers are used as building blocks. Materials composed of synthetic constituents
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could be based on poly(a-hydroxy acids), poly (orthoesters), poly(anhydrides), poly(amino acids), dextrin, poly(glycoside), poly(L-lactide) and their copolymers [Lee et al., 2011]. Using such systems, it was for instance shown that the sustained delivery of VEGF encapsulated in poly(lactide-co-glycolide) (PLG) microspheres can enhance vascular density \textit{in vivo} [Rocha et al., 2008]. Examples for natural polymers applied for growth factor encapsulation strategies include silk, keratin, starch, carrageenan, cellulose or alginate [Lee et al., 2011]. Here, using alginate, VEGF could be entrapped into spherical beads, while its release effectively stimulated endothelial cells \textit{in vitro} [Peters et al., 1998]. Moreover, FGF-2 as well as VEGF-loaded alginate gels promoted the formation of new blood vessels \textit{in vivo} [Lee et al., 2003]. While the growth factor delivery can be combined with other biomaterial variables, another advantage of physical cytokine encapsulation is the simplicity of the approach, as injectable or transplantable systems with relevant mechanical strength, porosity and degradation rates can be readily fabricated. However, as the growth factors have to be embedded during the fabrication of the materials, the processing conditions could negatively affect the bioactivity of the cytokines. Moreover, often additional effort has to be made in order to prevent a high initial burst release resulting from the lack of specific chemical interactions between the growth factor and the scaffold [Lee et al., 2011].

\textbf{Chemical growth factor conjugation}

In contrast to the concept of physical cytokine entrapment, the approach of chemical immobilization of cytokines into or onto a matrix involves covalent binding or affinity interaction between the growth factor and the particular biomaterial. Within the context of chemical growth factor conjugation to a certain biomaterial, the strategy of covalent immobilization has emerged as a generally important method for cytokine presentation. Using this concept, the growth factors may be already active in the bound state or could be activated as they are released from the matrix upon cleavage from the scaffold or network degradation [Lee et al., 2011]. Most commonly, the proteins are either introduced upon a coupling reaction (using water-soluble carbodiimide chemistry) or immobilized via UV light (using succinimidyl ester-phenyl azide or monoacrylated PEG-succinimidyl ester chemistry). Additionally, in a more advanced approach, genetic engineering can be used to generate growth factors with modified protein sequences that allow for a covalent coupling to the biomaterial. Applying these strategies, cytokines could be chemically linked to both synthetic and natural polymeric
matrices. Representatives for synthetic building blocks include PEG, poly(L-lactide) or PLG [Masters, 2011]. For example, with PEG hydrogels deployed in the context of tissue engineering for therapeutic angiogenesis, covalently immobilized FGF-2 was shown to stimulate the proliferation and migration of vascular smooth muscle cells [DeLong et al., 2005], while the covalent conjugation of VEGF promoted the tube formation of endothelial cells [Leslie-Barbick et al., 2009]. Natural polymeric biomaterials used with a covalent growth factor functionalization comprise among others chitosan, gelatin, collagen, agarose or fibrinogen/fibrin [Masters, 2011]. In angiogenic tissue engineering, for instance fibrin [Zisch et al., 2001], gelatin [Ito et al., 2005] or collagen [Chiu et al., 2010] matrices were applied for the covalent immobilization of VEGF. Performing cell culture experiments, these conjugated materials were found to support the proliferation [Zisch et al., 2001; Ito et al., 2005; Chiu et al., 2010], migration [Zisch et al., 2001] and tube formation [Chiu et al., 2010] of endothelial cells. Generally, a covalent growth factor immobilization to a certain biomaterial potentially allows for a more sustained release of the cytokines from the scaffolds. Moreover, matrix-conjugated growth factors could be capable to bind and activate their cellular receptors but, in parallel, might be subject to slower degradation and internalization, thereby prolonging their bioactivity [Lee et al., 2011; Masters, 2011]. However, there are also some disadvantages of this strategy. For some applications, higher initial release rates independent of matrix cleavage or degradation could be beneficial. In addition, it might be difficult to determine a specific coupling site on the immobilized protein, so that it can be bound in the correct orientation to allow for an effective interaction with its receptor. Moreover, the bioactivity of the growth factor could be decreased upon conjugation, as biologically relevant protein domains, amino acids or functional groups might be damaged or screened [Lee et al., 2011].

Another strategy to chemically immobilize cytokines on a certain biomaterial is the physical adsorption of the protein based on hydrogen bonding or electrostatic/hydrophobic forces between the growth factor and the matrix. This approach can be used to provide cytokines which are either directly accessible when interacting with the polymeric scaffold or which display their activity after release from the material upon diffusion, cleavage from the matrix or degradation of the network. As the physical adsorption to biomaterials is based on affinity interaction between the growth factor and the scaffolds, following the physiological concept of a reversible cytokine storage in the body, usually natural ECM components such as heparin-like
molecules, chondroitin sulfate, hyaluronic acid, fibronectin/fibrin, collagen or gelatin [Lee et al., 2011] as well as synthetic ECM analogues such as glycosaminoglycan mimicking oligosaccharides or protein mimicking oligopeptides [Uebersax et al., 2009] are used as building blocks for such polymeric systems. Here, among the earliest examples of such materials used in the context of therapeutic angiogenesis, fibrin was used as a delivery system for FGF-2 [Fasol et al., 1994; Albes et al., 1994] or VEGF [Weatherford et al., 1996] and was shown to effectively promote endothelial cell growth \textit{in vitro} [Fasol et al., 1994; Albes et al., 1994] and vascularization \textit{in vivo} [Weatherford et al., 1996]. In addition, beneficial effects on angiogenesis in animal models were observed for the usage of VEGF-functionalized collagen [Tabata et al., 2000] or FGF-2-modified gelatin [Iwakura et al., 2003] hydrogels. Moreover, due to its high affinity for angiogenic growth factors, biomaterials designed for the support of blood vessel formation often also include the glycosaminoglycan heparin. In several examples, the FGF-2 or VEGF delivery by such systems was found to promote pro-angiogenic cell behavior \textit{in vitro} and vascularization \textit{in vivo} [Wissink et al., 2000; Tanihara et al., 2001; Steffens et al., 2004; Yoon et al., 2007]. Overall, a physical growth factor adsorption to biomaterials based on secondary chemical interactions can be considered as a compromise between a non-specific entrapment within the matrix and a covalent conjugation to the scaffold. Therefore, depending on the particular strength of the cytokine affinity to the material, limitations of the concept are either related to a high burst release due to weak physisorption forces or to low overall delivery rates because of nearly irreversible interactions being almost as strong as in the case of a chemical bond. However, this strategy also combines the advantages of a growth factor encapsulation and a covalent immobilization approach. Consequently, by designing materials with a beneficial intermediate cytokine affinity, such systems might allow for optimal release properties thereby delivering the proteins right in the desired concentration regime. Moreover, some of the growth factors might exert their action already in the bound state, thereby prolonging their bioavailability and, as this interaction could protect the proteins from denaturation or degradation, also enhancing their bioactivity. Finally, due to the affinity of the cytokines to the biomaterial, it is possible to introduce the proteins by adsorption after scaffold processing. As this immobilization procedure can be performed under physiological conditions, the biofunctionality of the growth factors might be preserved more easily [Lee et al., 2011].
Additional scaffold variables
Besides all the progress in the field of polymeric cytokine delivery, there are also other factors influencing the potential success of a certain biomaterial for the use in therapeutic tissue engineering. As discussed earlier, following the example of the natural ECM (Fig. 6), the physicochemical and mechanical properties of the scaffold as well as the provision of insoluble cues mediating cell adhesion and permitting cellular matrix remodeling are key characteristics to govern effective tissue formation and regeneration [Hubbell et al., 2003; Lutolf et al., 2005]. Consequently, growth factor delivery systems designed for the support of therapeutic approaches should ideally integrate all of these demands. Among the first materials which were developed according to this requirement, a synthetic mimetic of collagenous ECM for the delivery of a bone morphogenic protein was produced. This scaffold was combined with

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Fig. 6 Illustration of the main ECM characteristics that have to be translated to biomaterials for successful tissue engineering; modified from [Mager et al., 2011]. While scaffolds have to provide appropriate viscoelastic properties, the presence of insoluble cues mediating cell adhesion and material remodeling as well as the supply of diffusible growth factors are unconditional requirements to effectively support regenerative approaches.
peptides controlling cell adhesion as well as substrates for cell responsive degradation and could be successfully applied to repair bone defects [Lutolf et al., 2003]. Based on this pioneer system, several biomaterials closely mimicking the key properties of the ECM were also used in the context of angiogenic tissue engineering [Zisch et al., 2003a; Leslie-Barbick et al., 2009; Phelps et al., 2010]. However, in order to develop matrices that effectively support angiogenesis, the influence of both the viscoelastic properties and molecular signals on the cellular behavior have to be explored separately. Here, none of these currently applied biomaterials allow for a far-going, systematic and independent variation of mechanical and biomolecular characteristics [Liu et al., 2005].

Beside the influence of the viscoelastic ECM properties and the presence of insoluble cues controlling cell adhesion and matrix remodeling, the complex process of angiogenesis involving cell migration, proliferation and differentiation is also governed by the temporally-, spatially- and concentration-dependent interplay of several growth factors [Zisch et al., 2003b]. In angiogenic tissue engineering, although the administration of either single FGF-2 or VEGF was able to support vascularization in animal models, problems associated with the vessel stability were observed [Bruick et al., 2001; Komori et al., 2005]. Moreover, as it was shown for FGF-2 and VEGF, several growth factors demonstrate considerable cooperative effects on endothelial cells in vitro and angiogenesis in vivo [Pepper et al., 1992; Goto et al., 1993; Asahara et al., 1995; Vernon et al., 1999; Yan et al., 2001; Kano et al., 2005]. Therefore, in addition to the delivery of a single cytokine, simultaneous or sequential provision of multiple growth factors was also exploited to enhance the therapeutic efficiency (Fig. 7). Here, the first single matrix system used to deliver multiple cytokines with distinct kinetics was applied for the release of VEGF and PDGF. In an in vivo model, the combined provision of both growth factors led to a dramatic increase in the number of matured blood vessels compared to the delivery of each single cytokine [Richardson et al., 2001]. Despite of this visionary work, only few studies were performed on the parallel administration of FGF-2 and VEGF by different biomaterials. Here, these systems either physically entrapped the growth factors into biodegradable microspheres [Larsen et al., 2010; De Laporte et al., 2011] or relied on the cytokine affinity of molecules such as fibrin [Wong et al., 2003; Wilcke et al., 2007; Losi et al., 2010], gelatin [Ribatti et al., 2001] or heparin [Nillesen et al., 2007] in order to control
the storage and release of both effectors. For all these materials, in order to precisely adjust the most beneficial conditions for therapeutic angiogenesis, an independent and specifically adaptable provision of FGF and VEGF would be advantageous.

Fig. 7 Schematic of a biomaterial capable to deliver multiple growth factors; inspired from [Lee et al., 2011]. A scaffold loaded with distinct quantities of two different cytokines can be applied to independently provide defined amounts of each single factor.

Biomaterials that effectively mimic the key characteristics of the ECM and which are able to provide combinations of several cytokines are appealing cytokine delivery systems for an application in therapeutic angiogenesis. However, there might be a need for a more far-going adaptation of the growth factor release profiles in order to specifically adjust for the versatile requirements of tissue engineering concepts. Consequently, recent research turned its attention to the development of materials that respond to local environmental signals or externally applied cues in order to control the release, so-called ‘release on demand’ systems (Fig. 8). In order to obtain scaffolds that are able to provide growth factors upon external triggering, stimuli-responsive components are introduced into the delivery matrix, which most commonly respond to changes in pH or temperature, the action of proteins such as enzymes releasing the cytokines upon cleavage or the application of drugs, ions, light, magnetic fields or ultrasound [Lee et al., 2011].
So far, many systems used for cytokine release on demand can be triggered by pH- or temperature change. Materials that are responsive to pH are characterized by the presence of acidic (e.g. carboxylic and sulfonic acids) or basic (e.g. ammonium salts) groups [Bawa et al., 2009], while examples for such pH-sensitive moieties include sulphamethazine oligomers, sulphonamide and methacrylic acid [Lee et al., 2011]. Upon environmental changes in pH, these groups are able to either accept or release protons, while an ionization causes electrostatic repulsive forces. This leads to conformational changes of the soluble polymers, resulting in differences in the swelling behavior of the hydrogels [Bawa et al., 2009]. By an increase in the mesh size of such networks during swelling, growth factors that have been tightly entrapped before could get released [Lee et al., 2011]. In contrast, thermo-responsive polymers contain moderately hydrophobic groups such as methyl, ethyl and propyl residues that could be mixed with hydrophilic elements. For most commonly applied systems such as the frequently used synthetic polymer poly(N-iso-propylacrylamide), at lower temperatures, hydrogen bonding between the hydrophilic segments and water leads to enhanced dissolution; however, as the temperature increases, the hydrophobic segments are strengthened, thus resulting in shrinking of the hydrogels due to inter-polymer chain associations [Bawa et al., 2009]. Once again, depending on the thermo-responsive change in the mesh size of such networks, growth factors can be retained or released by such matrices. As one example, by responding to local changes in pH- and temperature in an animal model of ischemia, a hydrogel system based on the pH- and temperature-sensitive poly(N-isopropylacrylamide-co-propylacrylic acid-co-butyl acrylate) provided a prolonged, local delivery of FGF-2, improved angiogenesis, and achieved therapeutic effects in regional blood flow and cardiac function [Garbern et al., 2011]. This study illustrates the advantage of such systems, as the pH and temperature conditions are often tissue-dependent, so that responsive materials might provide local specificity to the cytokine release. However, as the triggering parameters could only be varied within the physiological range, the degree of freedom in designing such materials is clearly restricted.

As another possibility to include stimuli-responsive components into growth factor delivery matrices, the incorporation of enzymatically degradable linkers not only allows for a cellular remodeling of the scaffolds as discussed before, but also permits the release of the immobilized cytokines upon cleavage. Here, the delivery of the growth factors can be modulated by modifying the chemical composition of the degradable
linker or by changing the scaffold characteristics in order to facilitate or restrict the enzymatic accessibility of the cleavable element [Lee et al., 2011]. Studies of [Seliktar et al., 2004] are one example for mediating the release of both covalently attached VEGF as well as physically entrapped transforming growth factor-β1 (TGF-β1) upon network degradation by activated endothelial cells. While the direct release of the cytokines in response to the matrix remodeling might be highly beneficial for promoting different cellular behavior, there could be also a few applications, where permanent matrices are needed. Moreover, in order to effectively release the growth factors upon network degradation, the expression of the cleaving enzymes in appropriate quantities by the cell types of interest must be guaranteed.

Depending on the material composition, upon the application of drugs [e.g. Ehrbar et al., 2008], ions [e.g. Ehrick et al., 2005], light [e.g. Kloxin et al., 2009], electric/magnetic fields [e.g. Jensen et al., 2002/Namdeo et al., 2009] or ultrasound [e.g. Epstein-Barash et al., 2010], generally the cytokine delivery can be tuned by a change in the scaffold properties (affecting for example the swelling/deswelling behavior or the network stability) or, in case of affinity-binding, by modulating the interaction of the growth factors and the matrix (thereby leading to a displacement of the proteins from the material). Although these systems offer exciting possibilities for precisely tuning the cytokine delivery, many of them to date are still prototypes [Lee et al., 2011]. Consequently, these materials were often only used to study the release of some model drugs and were not applied in any cell culture experiments yet. Examples of modular growth factor delivery systems already proven to have the potential for supporting therapeutic angiogenesis include hydrogels that release VEGF upon drug-mediated network dissociation [Ehrbar et al., 2008] or drug-induced volume changes due to conformational alterations of the building blocks [King et al., 2010]. With this concept, VEGF-induced endothelial cell proliferation could be triggered specifically [Ehrbar et al., 2008]. Generally, most advantageous, such smart materials allow for a precise and broad modulation of the growth factor release. However, sometimes it might be difficult to adapt these cytokine delivery concepts to the in vivo situation, as externally applied triggers could negatively affect or, in the case of unphysiological parameters, even harm the body. Consequently, there is a need for biomaterials that provide options for a precise and far-going modulation of the growth factor release under physiological conditions.
Theoretical background

Fig. 8 Illustration of biomaterials that are tailored to allow for a growth factor release on demand. Upon local environmental signals (such as a change in pH) or in response to externally applied triggers (such as the application of light), scaffold-immobilized cytokines are released into the environment due to changes in the network properties (e.g. upon an increased swelling of the pH-responsive polymeric system) or due to a modulation of the interaction between the growth factor and the scaffold (e.g. upon cleavage of the cytokine affinity sites within a photo-degradable material).

Taken together, by the usage of both synthetic and natural polymeric biomaterials, several different concepts to store and deliver cytokines can be developed. Nevertheless, every ideal release system has to balance between a sustained but still sufficient growth factor delivery. Moreover, the possibility to independently control the physicochemical and mechanical properties of the scaffold as well as the provision of insoluble cues mediating cell adhesion and permitting cellular matrix remodeling are unconditional parameters for the successful application of a certain biomaterial in the context of therapeutic angiogenesis. In addition to the delivery of a single cytokine, release systems should also permit an adjustable provision of multiple growth factors in order to improve the effectiveness of tissue engineering approaches. As a final requirement, for the precise adaptation to different needs in regenerative concepts, the
option to modulate the cytokine provision upon the response to local environmental cues or externally applied triggers represents a further benefit provided by a certain biomaterial. Conclusively, the optimal growth factor delivery system for an application in therapeutic angiogenesis should satisfy all of these demands.

2.2.3 Tunable biohybrid starPEG-heparin hydrogels as potential growth factor delivery systems

Biohybrid hydrogels composed of four-arm amino-end-functionalized, star-shaped poly(ethylene glycol) (starPEG) and heparin were originally developed to aid cell replacement therapies for neurodegenerative diseases [Freudenberg et al., 2009]. In this study, the starPEG-heparin matrices were shown to support the growth and differentiation of primary nerve cells and neural stem cells in vitro and demonstrated excellent shape stability together with good histocompatibility after intrastriatal transplantations in vivo. Due to these promising results, the application of the hydrogel system should be extended towards therapeutic angiogenesis. In this concept, after characterization, the biofunctionalized material ideally allows for systematic studies on the support of pro-angiogenic behavior of endothelial cells in vitro. Finally, bioactive hydrogels should be implanted to a potential target site in vivo and promote the effective formation of capillary networks by initiating the growth, migration and differentiation of endothelial cells.

Building blocks

Biohybrid starPEG-heparin hydrogels are formed using the synthetic polymer starPEG (Fig. 9, left) and the naturally occurring glycosaminoglycan heparin (Fig. 9, right) as building blocks. Generally, PEG represents one of the most common synthetic polymers used for the design of biomaterials. Beside its excellent biocompatibility, the hydrophilic and uncharged character of PEG effectively repels proteins. Moreover, as PEG represents a hydrolytically stable polymer with a good solubility in water and many different organic solvents, the possibility to easily modify its terminal functional groups opens up perspectives for a versatile PEG macromer chemistry [Tessmar et al., 2007].

Heparin and heparan sulfate are sulfated glycosaminoglycans. These linear polysaccharides are composed of repeating hexuronic acid-glucosamine disaccharide
Theoretical background

units, while a number of structural variations of this disaccharide lead to microheterogeneties of both glycosaminoglycans (as indicated by the major and minor disaccharide repeating units, Fig. 9, right). Generally, heparan sulfate is less sulfated but exhibits a higher molecular chain weight and structural variance than heparin. Moreover, heparan sulfate can be found attached to core proteins in proteoglycans on cell surfaces or within the ECM of nearly all mammalian cells and tissues, while heparin is mainly stored in the granules of certain mast cells and in some hematopoietic cells [Capila et al., 2002]. Upon release from these cells into the vasculature due to injury or immunological activation [Hiromatsu et al., 2003], heparin can either directly initiate endothelial cell proliferation and migration [Azizkhan et al., 1980] or release angiogenic growth factors from heparan sulfate storage depots in the ECM [Taipale et al., 1997], thereby contributing to angiogenesis. In addition, the anticoagulant properties of heparin prevent thrombus formation in the new vessels [Hiromatsu et al., 2003]. Although heparin is not considered to play a major role in local growth factor storage in the ECM, it can be mass produced and it is cheaper than heparan sulfate. Due to this fact, besides similar cytokine binding-properties at a smaller structural variance of its shorter chains, heparin rather than heparan sulfate is often used as building block for biomaterials [Uebersax et al., 2009].

Fig. 9 Building blocks of biohybrid starPEG-heparin hydrogel scaffolds. left: structure of four-arm amino end-functionalized synthetic starPEG. right: structure of natural heparin with its major and minor disaccharide repeating units (X=H or SO\(^3^-\), Y=Acetyl, SO\(^3^-\) or H); adapted from [Capila et al., 2002].
Network formation
In order to covalently combine both starPEG and heparin to generate biohybrid hydrogels, the two building blocks are connected by crosslinking of the amino end-functionalized starPEG with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysulfosuccinimide (EDC/s-NHS)-activated carboxylic acid groups of heparin. Upon the addition to heparin, EDC reacts with its carboxylic acid groups to form an amine-reactive O-acylisourea intermediate, which may then react with an amino group on the starPEG, thereby yielding a conjugate of the two polymers joined by a stable amide bond. However, at a neutral pH, the O-acylisourea intermediate is unstable and short-lived in aqueous solution, so that it may hydrolyze and regenerate the carboxylic acid group. Upon the addition of s-NHS, the EDC can be used to convert the carboxylic acid groups to amine-reactive s-NHS esters. As this compound is substantially more stable than the O-acylisourea intermediate, the effectiveness of the crosslinking reaction under physiological conditions is enhanced substantially [Staros et al., 1986].

Applying this coupling chemistry, biohybrid hydrogels composed of starPEG and heparin can be formed (Fig. 10). As already discussed, such materials combine the advantages of a structurally well-defined synthetic polymeric system with superior mechanical properties with the benefits of a naturally derived matrix providing enhanced biofunctionality. Here, by applying EDC/s-NHS chemistry, RGD adhesion ligands and growth factors can be covalently bound to the biohybrid hydrogel.
ligands can be covalently coupled to the carboxylic acid groups of heparin. Moreover, similar to the natural situation, heparin-binding growth factor such as the angiogenic cytokines FGF-2 or VEGF can be reversibly immobilized to the glycosaminoglycan upon electrostatic interaction of their basic residues with the sulfate groups of heparin. Due to these advantages, several biomaterials designed for the delivery of angiogenic growth factors are based on PEG and heparin [Benoit et al., 2005; Cai et al., 2005; Andreopoulos et al., 2006; Tae et al., 2006; Nie et al., 2007].

**Design concept**

Conceptually, the network composition of starPEG-heparin matrices goes beyond classical strategies since polymer-based hydrogels are often formed via interconnecting a polymeric building block with a short crosslinker. In contrast, the particular gel design follows a rational concept where starPEG functions as a flexible, structural building block, while heparin acts as a stiff, multifunctional crosslinker. The 14 kDa heparin used in this particular setting carries up to ~ 24 carboxylic acid moieties [Capila et al., 2002]. Consequently, by varying the molar ratio of starPEG to heparin ($\gamma$) from ~ 1.5 to 6, up to six four-arm starPEG molecules could be attached to form a dense meshwork. Applying a mean field approach, conditions could be identified, where the underlying expansion and retraction forces in the swollen hydrogels compensate each other in such a way that in a physiological situation, the concentration of the highly charged multifunctional crosslinker (i.e. heparin) stays nearly constant. This key property of the starPEG-heparin matrices was theoretically predicted and experimentally verified [Sommer et al., 2011]. As a consequence, upon the increase of the molar ratio of starPEG to heparin ($\gamma$) from 1.5 to 6 in the initial reaction mixture, gel types with an enhanced starPEG content (~ 8, 16.8 and 36 mg/ml gel for $\gamma = 1.5$, 3 and 6), but a constant heparin concentration (~ 8 mg/ml gel for $\gamma = 1.5$, 3 and 6) are obtained (Fig. 11, top left and Fig. 12, left), while the molar ratio of the building blocks defines the degree of crosslinking.

Moreover, as the synthetic building block represents the component which critically determines the viscoelastic properties of the hydrogel matrices, correlating with an increasing content of starPEG, less hydrated (volumetric swelling of ~ 53, 30 and 22 for $\gamma = 1.5$, 3 and 6) and stiffer (storage modulus of ~ 1000, 7300 and 14800 Pa for $\gamma = 1.5$, 3 and 6) scaffolds were produced (Fig. 11, top right and bottom left). This finding is related to a higher number of covalent crosslinks, therefore leading to the formation of a denser network,
which is more rigid (higher storage modulus) and exhibits restricted water uptake due to larger retraction forces caused by the higher number of covalent bridges within the gel (lower swelling). Despite of the overall high water content of the materials, varying between 95 % ($\gamma = 6$) and 98 % ($\gamma = 1.5$), all gel types revealed an excellent network stability. Rheological measurements demonstrated frequency-independent storage moduli in the range of 0.1-10 rad/s to be approximately two orders of magnitude higher than the loss moduli (data not shown), which is characteristic for an almost ideal elastic behavior of a stable covalently crosslinked material. Moreover, as an elastic hydrogel subjected to a small deformation of less than 20 % will completely recover its original dimension, the network structure could be analyzed according to the rubber-elasticity

Fig. 11 Gradual mechanical properties and network characteristics of different starPEG-heparin hydrogels determined by the varying content of synthetic starPEG. top left: varying starPEG and constant heparin concentration in swollen gels matrices prepared from different molar starPEG/heparin ratios. top right: gradual volumetric swelling characteristics of different hydrogel types depending on the starPEG content ($p < 0.05$ for any comparison of the data for $\gamma = 1.5$, 3 or 6; ANOVA). bottom left: gradual storage moduli of different hydrogel types depending on the starPEG content ($p < 0.05$ for any comparison of the data for $\gamma = 1.5$, 3 or 6; ANOVA). bottom right: gradual network mesh sizes of different hydrogel types depending on the starPEG content ($p < 0.05$ for any comparison of the data for $\gamma = 1.5$, 3 or 6; ANOVA). All data are presented as mean ± root mean square deviation from $n \geq 4$. 
Theoretical background

theory to estimate an average mesh size of the networks (for details about the theory see “3.3.2 Determination of rheological characteristics” or [Freudenberg et al., 2009]). Comparable to that of similar hydrogel systems [Raeber et al., 2005], the pore size of starPEG-heparin matrices was found to be in the nm scale and decreased from ~16 over 8.3 to 6.5 nm for $\gamma = 1.5, 3$ and 6 (Fig. 11, bottom right) upon increasing the starPEG content and therefore the crosslinking degree. Consequently, varying the starPEG to heparin ratio allows the production of different gel types with gradual mechanical properties.

However, independent of the different viscoelastic characteristics of the networks, the hydrogels contained large quantities of ~8 mg heparin per ml gel, which -as already discussed- remained approximately constant for scaffolds with different molar ratios of starPEG to heparin (Fig. 11, top left and Fig. 12, left). This rationally designed characteristic of the hydrogels seems crucial for control and modulation of subsequent biomolecular functionalization, as it should principally allow for a decoupling of the structural parameters and the biofunctionality. For instance, to mediate cell adhesive scaffold properties, integrin-binding cyclic RGDYK (in single-letter amino acid code) peptides (RGD-peptides, Fig. 10) were coupled to EDC/s-NHS activated heparin carboxylic acid groups by their lysine amino group (Fig. 12, right). Based on the constant heparin concentration in hydrogels with varying mechanical characteristics, similar amounts of RGD-peptides (~0.6 mol RGD/mol heparin) were attached to the different gel types as previously demonstrated by [Freudenberg et al., 2009].

![Graph](image)

**Fig. 12** Equal RGD concentration of different starPEG-heparin hydrogels determined by the constant content of natural heparin. **left**: varying starPEG and constant heparin concentration in swollen gel matrices prepared from different molar starPEG/heparin ratios. **right**: constant RGD functionalization of different hydrogel types depending on the heparin content ($p > 0.05$ for any comparison of the data for $\gamma = 1.5, 3$ or 6; ANOVA). All data are presented as mean ± root mean square deviation from $n \geq 3$. 
Consequently, since heparin also represents the basis for a subsequent growth factor functionalization of the scaffold, the matrices might represent a system, where the cytokine delivery could be adapted independently of structural and mechanical material properties.

Since angiogenesis is critically determined by both molecular signals and the viscoelastic characteristics of the surrounding matrix [Ingber et al., 1989; Deroanne et al., 2001; Liu et al., 2005; Saunders et al, 2010], this approach offers a way to separately explore the influence of both factors on cellular behavior, which might help to create optimized scaffolds for an application in angiogenic tissue engineering.

As a second advantage, compared to many similar systems, starPEG-heparin hydrogels are characterized by significantly higher heparin concentrations (up to 0.8% [w/w]) in the swollen matrices. Therefore, as the structural integrity of heparin could be preserved up to higher degrees of crosslinking, this attribute might also permit a rather unaffected interaction with several heparin-binding growth factors. Consequently, the starPEG-heparin hydrogels could represent a promising material for the independent and parallel delivery of multiple cytokines as it might be beneficial to aid complex regenerative processes such as therapeutic angiogenesis.

Finally, the design of the biohybrid starPEG-heparin matrices offers several possibilities to tune the release of reversibly immobilized growth factors. As it was already shown [Tsurkan et al., 2010], upon incorporation of cleavable peptide linkers, enzymatically degradable scaffolds can be developed. Besides the option of cellular matrix remodeling, this might also offer a way to modulate the delivery of soluble cytokines in response to cell-mediated proteolysis. Moreover, as the growth factor immobilization to starPEG-heparin hydrogels is based on affinity binding between the cytokines and heparin, this interaction might intrinsically represent a parameter that can be modulated under physiological conditions. For example, modifying the growth factor binding sites of heparin or applying heparin-affine competitor molecules could be ways to tune the cytokine release. By this, a precise and far-going modulation of the growth factor delivery might open up new perspectives to adapt the cytokine provision to the actual requirements of effective angiogenic tissue engineering.

Following these promising possibilities, in this work, the potential of starPEG-heparin hydrogels to provide the angiogenic cytokines FGF-2 and VEGF should be explored.
3 Materials and methods

3.1 Preparation of starPEG-heparin hydrogel networks

3.1.1 Modification of glass surfaces

According to their purpose, borosilicate glass surfaces (Carl-Roth GmbH & Co, Karlsruhe, Germany) were either functionalized with aminosilane or with hexamethyldisilazane. For covalent attachment of starPEG-heparin networks to glass cover slips, aminosilanization was used to generate surface-bound amino groups on the glass [Pompe et al., 2003] that functioned as anchoring points and enabled a stable linkage with the activated heparin carboxylic acid groups in the hydrogel. Alternatively, to distribute and cover the liquid starPEG-heparin mixture on plain surfaces, treatment of the cover slips with hexamethyldisilazane was used to introduce methyl groups resulting in hydrophobic surfaces that prevented sticking to the gel.

All cover slips were initially pre-cleaned via ultrasonication for 30 min in deionized, decarbonized water (MilliQ) followed by a subsequent 30 min ultrasonication step in 70 % ethanol (VWR International GmbH, Darmstadt, Germany) and rinsing in MilliQ. Next, the glass surfaces were freshly oxidized for 10 min at 70 °C in a mixture of 29 % aqueous solution of NH$_3$ (Acros Organics, Geel, Belgium), medical extra pure 35 % H$_2$O$_2$ (Merck Chemicals KGaA, Darmstadt, Germany) and MilliQ in a ratio of 1:1:5 and then two times rinsed in MilliQ.

Functionalization of glass surfaces was performed according to its purpose either from solution or from vapor phase. For aminofunctionalization from solution, samples were treated with a 20 mM 3-aminopropyl-triethoxysilane (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) solution in a mixture of isopropanol (Acros Organics) and MilliQ (9:1) for 2 h at room temperature. Next, the samples were washed with isopropanol and dried with nitrogen. In order to stabilize the coating, cover slips were subsequently incubated in the drying oven for 1 h at 120 °C. For hexamethyldisilazane (Fluka, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) treatment from vapor phase, cleaned cover slips were dried for 1 h at 120 °C and then placed in a Petri dish with a smaller one containing 100 µl hexamethyldisilazane in the centre for at least 3 h. Finally, they were incubated for 1 h at 120 °C.
3.1.2 Formation of starPEG-heparin hydrogels

StarPEG-heparin hydrogels were formed by crosslinking amino end-functionalized four-arm starPEG with EDC/s-NHS activated carboxylic acid groups of heparin [Freudenberg et al., 2009]. For this, a total polymer content of 11.6 % and a 2:1:1 ratio of EDC:s-NHS:NH$_2$-groups of starPEG [mol/mol] were used. The molar ratio of starPEG to heparin was varied from 1.5 to 6 (for exact composition of the different gel types see Tab. 1).

Heparin (14,000 g/mol; Calbiochem (Merck), Darmstadt, Germany) and starPEG (10,000 g/mol Polymer Source, Inc., Dorval, Canada) were each dissolved in one third of the total volume of ice-cold MilliQ by ultrasonication and afterwards kept on ice (~ 2-4 °C). Similarly, s-NHS (Fluka) and EDC (Sigma-Aldrich) were separately dissolved in the sixth part of the total volume of ice-cold MilliQ. Subsequently, s-NHS and EDC solutions were added to heparin, mixed well and incubated for 15 min on ice to activate heparin carboxylic acid groups. Finally, the star-PEG solution was added to the activated heparin and quickly mixed by vortexing (Minishaker MS2, IKA, Staufen, Germany).

For fluorescence microscopy, gels were prepared from heparin spiked with 0.5 % [w/w] of heparin labeled with Alexa 488 (Molecular Probes distributed by Invitrogen GmbH, Darmstadt, Germany; conjugate synthesized by M. Tsurkan, IPF Dresden; for details on preparation and characterization see appendix A). To modulate the interaction between the cytokines and the hydrogels, networks were produced using selectively desulfated, N-reacetylated heparin (N-; 2-O-; 6-O- + N- or completely desulfated heparin; synthesized by A. Röhrich, BCube Dresden; for details on preparation and characterization see appendix A). For the preparation of enzymatically degradable networks, the common starPEG was substituted by a starPEG-(GPQG↓↑IWGQ, in single-letter amino acid code) conjugate with a hydrolytically stable (succinimide-derivative) bond between the starPEG and the peptide sequence (synthesized by M. Tsurkan, IPF Dresden; for details on preparation, characterization and degradation studies see appendix A).

To allow for a practical performance of quantitative growth factor binding/release studies and for cell culture experiments, surface bound gels with a final thickness of ~ 50 µm were prepared. For this, 3.11 µl of the gel mixture per cm$^2$ were used. To obtain surface immobilized networks, the gel solution was placed on freshly aminofunctionalized glass cover slips or directly into aminofunctionalized glass bottom
24-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) to allow covalent attachment of heparin through its activated carboxylic acid groups. In order to spread the solution equally, the mixture on the glass slides was covered with a hydrophobic glass cover slip that has been treated with hexamethyldisilazane or by placing an ethylen-chlorotrifluorethylene-copolymer slide (Goodfellow, Cambridge, England) onto the gel solution in the glass bottom wells. For preparation of free-standing gel disks, 104.7 µl of the liquid gel mixture were placed onto a 1 cm² hydrophobic glass cover slip and covered with a second hydrophobic one. To allow for an introduction into an in vivo system, small hydrogel clots were prepared by pipetting 1.4 µl of the gel mixture on a hydrophobic glass cover slip.

After polymerization overnight at 22 °C in a humidified atmosphere, any hydrophobic cover slips were removed. Each gel sample was washed in phosphate buffered saline (PBS, Sigma-Aldrich) to remove s-NHS/EDC and any non-bound starPEG/heparin. PBS (pH 7.4) was exchanged five times, once per h, and once again after storage for 24 h. Subsequently, the swollen gels were immediately used for further experiments. For cell culture, sterilization was performed by UV-treatment for 30 min. For additional treatments, all solutions were sterile unless otherwise indicated.

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### 3.2 Biomodification of starPEG-heparin hydrogels

The biomodification of starPEG-heparin hydrogels with an adhesion ligand is performed by coupling the lysine amino groups of the cyclo(arginine-glycine-aspartic acid-D-tyrosine-lysine) (RGD) peptide (Peptides International, Louisville, USA) to EDC/s-NHS activated carboxylic acid groups of heparin. For this, surface-bound, swollen hydrogels were placed into 24-well plates (TPP, Trasadingen, Switzerland) and washed 3 times with ice-cold 1/15 M phosphate buffer (pH 5; 1/15 M Na₂HPO₄ and 1/15 M KH₂PO₄ in a 1:124 mixture; Merck Chemicals KGaA, Darmstadt, Germany).
This solution was then exchanged with a s-NHS/EDC mixture (25 mM s-NHS and 50 mM EDC in 1/15 M phosphate buffer (pH 5)) to activate the carboxylic acid groups of heparin. After incubation for 45 min, the scaffolds were washed 3 times in ice-cold 0.1 M borate buffer (pH 8; 55.85 ml 0.2 M borate stock solution made from boric acid (Sigma-Aldrich), 44.15 ml 0.1 M HCl (Acros Organics) and 100 ml MilliQ) to remove unbound s-NHS/EDC. Subsequently, the gels were incubated in RGD-solution (50 µg/ml; dissolved in borate buffer) for 2 h at room temperature. Finally, all samples were washed in PBS 3 times.

To electrostatically immobilize FGF-2 (Miltenyi Biotech, Bergisch Gladbach, Germany) or VEGF165 (PeproTech GmbH, Hamburg, Germany) to the starPEG-heparin networks, the respective protein was dissolved in PBS at the desired concentration. Unless otherwise indicated, PBS-swollen, pure or RGD-modified gels were placed into 24-well plates and immersed in the protein solution (200 µl per cm²) at room temperature. The cytokines were allowed to penetrate the networks for 24 h followed by rinsing twice with an excessive volume of PBS.

3.3 Analysis of starPEG-heparin scaffold properties

3.3.1 Determination of volumetric swelling degree

Swelling of hydrogels is based on incorporation of liquid into the pores of the gel network. Besides this, excessive washing is necessary to remove the activation chemicals s-NHS/EDC as well as any unbound starPEG and heparin.

The initial diameter of the free-standing disks (for preparation see 3.1.2) was measured with a digital vernier calliper (MMO, Börnicke, Germany), while the same procedure was repeated after swelling in PBS for 24 h at room temperature. Volumetric swelling degree Q (change in gel volume after swelling compared to the dry volume of the gels, $V_0$) was calculated by $Q = (d_t/d_{\text{react}})^3 \cdot V_{\text{react}}/V_0$, where $d_t$ is the diameter of the disk after the washing process, $d_{\text{react}}$ is the diameter of the unswollen gel disk (cured reaction mixture), $V_{\text{react}}$ the volume of the cured reaction mixture and $V_0 = n \cdot V_{\text{starPEG}} + n \cdot V_{\text{heparin}}$. The heparin and RGD content is expressed in relation to the final volume of the PBS swollen gel network.
3.3.2 Determination of rheological characteristics

Rheology is defined as the study of the change in form (deformation) and the flow of matter, embracing elasticity, viscosity, and plasticity of a material. Two important parameters that can be determined by this method are the storage (G’) and loss modulus (G’’). In viscoelastic solids they measure the stored energy, representing the elastic portion, and the energy dissipated as heat, representing the viscous portion.

Storage and loss modulus of free-standing starPEG-heparin hydrogel disks (n = 4) were determined using oscillating measurements on a rotational rheometer (Ares LN2, TA Instruments, Eschborn, Germany) with plate-plate geometry (plate diameter 25 mm, gap width 1.2-1.5 mm). Dynamic frequency sweep tests under strain control were carried out at 25 °C in a shear frequency range of $10^{-2}$-$10^{-1}$ rad/s. The strain amplitude was set to 3 % and storage and loss modulus were measured as a function of the shear frequency.

From the rheological characteristics, pore sizes of the network could be estimated according to the rubber-elasticity theory [Rubinstein et al., 2003; Freudenberg et al., 2009]. As a natural rubber, a hydrogel subjected to a relatively small deformation of less than 20 % (here: 3 %), will rapidly and fully recover to its original dimension. Following the assumptions that all chains of the polymer network contribute to the retraction force after deformation (affine deformation), end effects of single chains are neglected and that any influence of physical entanglements is excluded, the mesh size $\xi$ (distance between two entanglement points) can be calculated by $\xi = (G' * N_A / R * T)^{-1/3}$, where $G'$ is the storage modulus, $N_A$ is the Avogadro constant, $R$ is the molar gas constant, and $T$ is the temperature.

3.4 Characterization of the biomodification

3.4.1 Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) can be applied for acquiring high-resolution optical images with depth selectivity. This method is based on the application of pinholes to produce a point source of light thereby rejecting all scattered light except that emitted from the illuminated specimen. For the image acquisition, a galvanometric scanning mirror is used that moves laterally line by line over the samples. Moreover, also the z-direction can be adjusted by a z-motor replacement. Consequently, CLSM
Materials and methods

can be deployed to resolve the object of interest into confocal section plains for the three-dimensional reconstruction of the observed specimen (xyz-scans). In the context of this work, CLSM was used to study the uptake of fluorescently labeled molecules into starPEG-heparin hydrogels.

FGF-2 or VEGF were labeled with tetramethylrhodamine (TAMRA) according to the FluoReporter Tetramethylrhodamine Protein Labeling Kit manual (Molecular Probes, distributed by Invitrogen).

TAMRA-FGF-2 or -VEGF (5 µg/ml) were diluted in PBS. The corresponding solutions were added to starPEG-heparin gels (n = 2, 200 µl/cm²) that were directly immobilized in glass bottom 24-well plates. Fluorescence intensity was quantified using a Leica SP5 (Leica, Bensheim, Germany) confocal laser scanning microscope with a 40x magnification immersion objective (HCxPL APO, Leica) and aperture pinhole set at 68 µm. The argon-laser (excitation wavelength 488 nm, laser intensity 20 %) was used for exciting Alexa 488-labeled gels whereas the diode-pumped solid-state (DPSS) laser (excitation wavelength of 561 nm, intensity 20 %) was used for excitation of TAMRA labeled proteins. Alexa 488 and TAMRA emission were analyzed in the 500-550 nm or 570-630 nm range, respectively.

The time-dependent intensity of the TAMRA-FGF-2 or -VEGF was quantified for the solution (supernatant of the gel body) and for the gel body performing an XZ-scan at defined intervals. Intensity profiles (XZ-scan) at three different X-positions were evaluated for each time point.

3.4.2 Radiolabeling studies

Radiolabeling studies of FGF-2 and VEGF were performed to analyze the uptake and release of these proteins by starPEG-heparin hydrogels. For this, the cytokines were subjected to iodination, which involves the introduction of radioactive iodine-125 ($^{125}$I) into certain amino acids (usually tyrosines) in the proteins. Quantification of these labeled proteins is performed with the help of a gamma counter. As a scintillation detector, these counters use crystals with luminescent properties. Upon interaction with photons from the gamma rays, the crystals emit light. This light energy is then converted into electric energy by a photomultiplier in order to deliver a meaningful measure.
While $^{125}$I-labeled FGF-2 was purchased from Chelatec SAS (Nantes, France), VEGF was labeled with $^{125}$I using IodoBeads (Pierce, Rockford, USA). These polystyrene beads are coated with an oxidizing reagent which converts sodium iodide to its corresponding reactive iodine form that spontaneously incorporates into tyrosyl groups. For this, 1 mCi Na$^{125}$I (PerkinElmer Massachusetts, USA) dissolved in 100 µl of PBS (pH 7.4) was added to a single IodoBead that had been rinsed with PBS. After 5 min of incubation at room temperature, 200 µl VEGF stock (1 mg/ml) was added and allowed to react for 20 min. By size exclusion chromatography (NAP-5 column, GE Healthcare, Munich, Germany) using PBS as the eluent, unbound iodide was removed yielding iodinated protein with less than 2 % free $^{125}$I. The resulting protein concentration was determined by an UV/vis spectrometer (BioPhotometer Plus, Eppendorf, Hamburg, Germany) while the specific activity of the protein solution was analyzed via gamma counting (LB 123, Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany).

To perform protein binding and release studies, surface-bound gels ($n = 2-4$) were placed in custom-made incubation chambers that decreased the exposure of the protein to surfaces not originating from the hydrogels to a minimum. Native FGF-2 or VEGF protein solution was spiked with $^{125}$I-labeled FGF-2 or VEGF as a percentage of total protein (2.5-100 %). After incubation with mixtures containing 0.5, 1, 5 or 10 µg/ml FGF-2 or VEGF, radioactivity was measured twice per sample using gamma counting. Immobilized protein was quantified with the help of $^{125}$I-FGF-2 or -VEGF standards prepared by dropping defined aliquots of a known concentration on glass cover slips. After immobilization, FGF-2 or VEGF were allowed to release from these gels ($n = 2$) at 22 °C into 250 µl/cm² of serum-free (SF) endothelial cell growth medium (ECGM; Promocell GmbH, Heidelberg, Germany) supplemented with 0.02 % [w/v] sodium azide (Fluka). At defined time intervals, the medium was withdrawn and the remaining FGF-2 or VEGF bound to the gels was monitored twice via gamma counting. An equal volume of fresh medium was added back after each measurement.

### 3.4.3 Amino acid analysis via high performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is chromatographic technique used to separate compounds that are dissolved in solution. In this work, it has been applied to quantify the amount of RGD or FGF-2 and VEGF in starPEG-heparin hydrogels. For
that, peptide bonds were broken down by acidic hydrolysis so that peptides or proteins are decomposed into single amino acids. After derivatization with a fluorescent label, the different amino acids pass through a chromatographic column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase. Following the separation procedure, they can be detected by their fluorescent signal after excitation with a suitable wavelength.

Quantification of immobilized RGD-peptide (50 µg/ml; n = 4), FGF-2 (10, 25 or 50 µg/ml; n = 2), or VEGF (10, 25 or 50 µg/ml; n = 2) in the gels was performed by acidic hydrolysis and subsequent HPLC analysis as described elsewhere [Salchert et al., 2003]. Briefly, gel-coated substrates were subjected to vapor hydrolysis in vacuo using 6 M HCl at 110 °C for 24 h and subsequently neutralized. Extraction of amino acids from the samples was accomplished by repeated rinsing with a definite volume of 50 mM sodium acetate buffer at pH 6.8. The released amino acids were chromatographically separated after precolumn derivatization with ortho-phthalaldehyde (Sigma-Aldrich) on a Zorbax SBC18 column (4.6 × 150 mm, 3.5 µm, Agilent Technologies, Boeblingen, Germany) using an Agilent 1100 LC system (Agilent) with fluorescence detection (excitation wavelength of 335 nm, emission measured at 455 nm). Amino acids were quantified using external standards.

3.4.4 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is a biochemical technique used to detect the presence of a specific antigen in a sample. In a sandwich ELISA, an unknown amount of antigen is affixed to a plate coated with a capture antibody. In the next step, a specific detecting antibody is applied over the surface so that it can bind to the antigen. After addition of an enzyme-linked secondary antibody that binds to the detecting antibody, a chemical substrate is applied which can be enzymatically converted to a detectable form (most commonly associated with a color change). By comparison to a standard of a defined concentration, the antigen in the sample can be quantified. In the context of this work, ELISA was used to analyze the uptake and release of FGF-2 or VEGF by starPEG-heparin hydrogels.

Surface-bound gels (n = 3) were placed in custom-made incubation chambers that allowed only minimal interaction of the protein solution with areas not originating from the hydrogel. Following the incubation with FGF-2 (0.5, 1, 5 or 50 µg/ml) or VEGF (0.5,
Materials and methods

1, 5, 10 or 25 µg/ml), the immobilization and washing solutions were collected and assayed in duplicates using an ELISA Quantikine kit (R&D Systems, Minneapolis, USA). After immobilization, FGF-2 or VEGF were allowed to release from these gels at 22 °C into 250 µl/cm² of SF ECGM with 0.02 % sodium azide ± 0.1 % bovine serum albumin (BSA; Sigma-Aldrich). For modulation of the growth factor delivery, the standard release medium containing 0.1 % BSA was either supplemented with 1 U/ml collagenase (Biochrom AG, Berlin, Germany) or 5 mg/ml chitosan (molecular weight ≈ 10-50 kDa; Chitosan 70/5, Heppe Medical Chitosan GmbH, Halle, Germany). As previous studies showed that due to the high heparin excess presumably resulting in a dynamic release/re-binding of the growth factors within the gel, there was no influence of changing the frequency of medium replacement on the overall amount of protein being released (data not shown), samples were always taken at the same intervals (after 3, 6, 24 and 96 h) and stored at -80 °C until analyzed by ELISA. An equal volume of fresh medium was added back at each time point.

3.5  In vitro endothelial cell culture experiments

3.5.1 Isolation and cultivation of human umbilical vein endothelial cells (HUVECs)

Human endothelial cells were isolated from the umbilical cord vein (human umbilical vein endothelial cells - HUVECs) according to the procedure suggested by [Weis et al., 1991]. Briefly, the vein of umbilical cords that were not older than 20 h was rinsed with PBS and filled with sterile collagenase (446 U/ml) in order to proteolytically release the cells. After incubation for 20 min at 37 °C, the reaction was stopped by adding PBS supplemented with 10 % fetal calf serum (FCS, Biochrom). The collagenase solution containing the detached endothelial cells was filled into a sterile tube and centrifuged (Labofuge 400 R, Heraeus, Berlin, Germany) for 5 min at 1500 rpm. Next, cells were resuspended in ECGM and seeded into a tissue culture flask (plastic cell culture flask, TPP) coated with 50 µg/ml fibronectin (Roche Diagnostics GmbH, Mannheim, Germany). HUVECs were grown to confluence at 37 °C and 5 % CO₂, while the medium was changed every second day.

After reaching confluence, a new passage of cells had to be prepared. For this, the medium was removed and the culture was rinsed with PBS twice in order to remove dead cells, cell debris as well as remnants of old medium. 1 ml of trypsin-EDTA
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(trypsin-ethylenediaminetetraacetic acid, Sigma-Aldrich) was added per 25 cm² of the culture flask for proteolytically detaching the cells during an incubation step for 2 min at 37 °C. The reaction was stopped by applying 5 ml PBS supplemented with 10 % FCS. HUVECs were transferred into a sterile tube and centrifuged for 5 min at 1500 rpm. Following resuspension in the corresponding medium, cells were either distributed to several culture flasks or directly used for experiments.

For culture on starPEG-heparin matrices, after one to four passages, ~ 11300 cells per cm² surface area were seeded onto the hydrogels, which were pre-equilibrated with SF ECGM for 30 min at 37 °C. HUVECs culture was performed for three days at 37 °C and 5 % CO₂ on either pure or RGD-treated starPEG-heparin hydrogels, while the RGD concentration used for functionalization (50 µg/ml) was selected due to its ability for inducing optimized HUVEC growth as determined in pre-experiments (data not shown). Such gel matrices were either used without any additional modification or loaded with either 1 or 5 µg/ml single FGF-2 or VEGF or with a combination of 1 µg/ml FGF-2 + 1 µg/ml VEGF.

3.5.2 Analysis of cell morphology after adhesion and subsequent culture

For characterizing cell adhesion, cells were allowed to adhere to the different surfaces for 2 h at 37 °C and 5 % CO₂. Light microscopy images were then taken (Olympus IX50, Olympus, Hamburg, Germany) at 10x magnification. Resulting cell shapes dependent on the culture conditions were analyzed with the help of ImageJ 1.41o (developed by W. Rasband, National Institutes of Health, Bethesda, USA) by tracing cell boundaries manually. After 3 d of culture, cell morphology was similarly analyzed using the circularity calculation within ImageJ. Here, a circularity of ‘1’ corresponds to a fully circular object, while a value of ‘0’ represents a straight line. For each condition, depending on the cell survival, between ~ 30 and 200 cells were analyzed for up to 13 different substrates.

3.5.3 Survival studies

Analysis of cell survival was performed via Live/Dead staining as described by [Jones et al., 1985] This technique is based on the capability of viable cell to incorporate the nonpolar and nonfluorescent compound fluorescein di-O-acetate (FDA). By using their
acetyl esterase activity, cells rapidly hydrolyze it to fluorescein, a polar and fluorescent compound which is retained within the cell. Nonviable cells no longer have such esterase activity and will not be fluorescently stained. However, dead cells are susceptible to DNA intercalation of compounds such as ethidium bromide or propidium iodide (PI), and can therefore be easily counterstained to differentiate them from viable cells in a fluorometric assay.

After 3 d of culture on the different substrates (n = 2-4), 1.13 ml/cm² of a solution containing 0.1 µg/ml FDA (Fluka) and 2 µg/ml PI (Fluka) dissolved in PBS were added to each sample and incubated for 2 min at 22 °C. The cells were then immediately visualized by fluorescence microscopy (DMIRE2, Leica) using a 10x dry objective (HCxPL Fluotar 10x 0.30, Leica). FDA fluorescence was monitored by excitation with an argon laser (excitation wavelength 492 nm, emission wavelength 520 nm) whereas PI positive samples were excited with a helium-neon laser (excitation wavelength 537 nm, emission wavelength 566 nm). Both images were combined to generate an overlay picture.

3.5.4 Proliferation assay

Cell proliferation was studied with the help of a 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma-Aldrich) proliferation assay as described by [Supino, 1995]. The MTT test is a colorimetric assay based on the reduction of the pale yellow substrate 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) to the purple dye formazan by the nucleotide cofactors nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NADH/NADPH) and is only catalyzed by viable cells. Dissolving the resulting crystalline formazan with a solubilization buffer permits the convenient quantification of product formation. The intensity of the product color, measured at 540 nm, is directly proportional to the number of living cells in the culture.

After 3 d of culture, 283 µl/cm² of a 1/5 mixture of MTT (5 mg/ml in PBS) and SF ECGM were added to each sample (n ≥ 3) and incubated for 5 h at 37 °C. Next, the supernatant was completely removed from the substrates and 170 µl/cm² dimethyl sulfoxide (DMSO; Fluka) was added. The samples were incubated for 20 min at 37 °C and 200 µl of the solution were then transferred into a 96-well plate. Absorption was subsequently measured in a plate reader (Genios, TECAN, Crailsheim, Germany) at
540 nm. Quantification was based on a standard curve from a similar MTT test of defined cell numbers seeded on well plates coated with 50 µg/ml fibronectin.

3.5.5 Investigation of cell migration

To study HUVEC migration, the MilliCell modified Boyden chamber (8 µm pores; Millipore, Bedford, USA) migration assay was performed [Boyden et al., 1962]. This method is based on a chamber of two medium-filled compartments separated by a cell permeable membrane, which is generally coated with some ECM component (e.g. fibronectin) to facilitate both cell adherence and migration. During this assay, cells are placed in the upper compartment and are allowed to migrate through the pores of the filter into the lower compartment, in which chemotactic agents are present. After an appropriate incubation period, the membrane between the two compartments is fixed and stained, and the number of cells that have migrated to the lower side of the filter is determined.

Migration chambers (n = 4-6 for each condition) were prepared by pre-coating the upper surface of the polycarbonate membrane with 100 µl/filter fibronectin (50 µg/ml in PBS) at room temperature overnight followed by air drying. They were then applied to 24-well plates containing gel scaffolds either untreated or loaded with 1 or 5 µg/ml single FGF-2 or VEGF or a combination of 1 µg/ml FGF-2 + 1 µg/ml VEGF as described above. As a control, the filters were also introduced to wells without any gel networks. All substrates were then coated with 600 µl/filter SF ECGM. To initiate the migration assay, HUVECs were added to the upper chamber (20,000 cells in 200 µl SF ECGM). After 20 h at 37 °C, the medium was removed from the upper chamber and non-adherent cells were washed off using 100 µl/filter PBS. HUVECs still adherent on the upper surface of the filter were removed by a cotton tip applicator and the migratory cells on the lower membrane surface were fixed by treatment with 600 µl/filter of 70 % ethanol for 1 h at room temperature. After rinsing the chamber with PBS, cells were stained using 300 µl/filter of 25 % Crystal Violet (Sigma-Aldrich) diluted in MilliQ. Cell migration values were determined by elution of the Crystal Violet stain in 400 µl/filter of 10 % acetic acid (Sigma-Aldrich) for 20 min at room temperature and measuring the absorbance at 590 nm.
3.6 In vivo experiments using the chicken chorioallantoic membrane (CAM) assay

To assess whether biomodified starPEG-heparin hydrogels could initiate an angiogenic response in vivo, experiments using the chicken chorioallantoic membrane (CAM) assay were performed. The CAM is a vascular membrane found in eggs of some amniotes, such as birds and reptiles. It is formed by the fusion of the chorion and the adjacent wall of the allantois. In the shell-free CAM assay, chicken embryos are cultured in Petri dishes so that the material of interest can be easily transplanted on the membrane. In case of an angiogenic response, the material causes a typical radial rearrangement of vessels towards and a clear increase of vessels around the graft approximately 1-4 d after onplantation. After this incubation period, angiogenesis can be quantified via image analysis or colorimetric detection methods.

Experiments were performed on chicken embryos grown by the shell-free culture method [Auerbach et al., 1974]. Fertile, specific pathogen-free chicken eggs (Erzeugergemeinschaft Pharmo-Ei GmbH, Mockrena, Germany) were obtained on embryonic day (ED) 0 and, following sterilization with ethanol, incubated under conditions of constant humidity (60 %) at 37 °C. On ED 3, the eggs were carefully cracked open and their contents transferred into sterile weighting boats. Subsequently, they were incubated for a further 5 d during which blood vessels of CAM vascular system developed. The RGD-functionalized starPEG-heparin hydrogels described above were either loaded with single 5 µg/ml FGF-2 or VEGF, with a combination of 5 µg/ml FGF-2 + 5 µg/ml VEGF or were not modified with any cytokines. Each network was then placed on the CAM surface at ED 8 and the embryos were returned to the incubator (n = 5-16). The untreated CAM served as a control. Analysis of the angiogenic response was performed during ED 12. Following Indian ink injection, the CAM vasculature was observed under a stereomicroscope (Leica S8AP0) and digital micrographs were taken. Quantification was performed by evaluating the amount of vessels surrounding the onplant in the proximity of 1 mm from its edge. Results were expressed as a ratio of untreated sample.

3.7 Data analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) and post-hoc Tukey-Kramer multiple comparison test. P values < 0.05 were considered statistically significant. All data are presented as mean ± standard deviation.
4 Results and discussion

4.1 Method establishment for analyzing the interaction of growth factors with starPEG-heparin hydrogels

StarPEG-heparin hydrogels closely mimic the characteristics of the ECM by containing large quantities of heparin, which electrostatically bind and stabilize numerous growth factors (see Fig. 10). To evaluate the potential of this heparin-rich system, the binding and release of FGF-2 and VEGF, two cytokines that are crucial for the process of angiogenesis, should be investigated. However, in order to thoroughly characterize the interaction of the growth factors with the material, several analytical methods have to be applied and compared. For this, detection of fluorescently or radioisotope labeled protein, amino acid analysis and ELISA were performed (for details on the methodological principles see chapter 3). As all of these approaches are based on a distinct detection mechanism, experimental parameters had to be adjusted to the requirements of the particular method (Tab. 2). Nevertheless, the combination of all four analytical approaches should allow for the characterization of FGF-2 or VEGF binding and release over a wide range of concentrations.

Tab. 2 Experimental parameters used for FGF-2 or VEGF binding and release studies with heparin-starPEG hydrogels (due to technical reasons, FGF-2 or VEGF release experiments were only performed via radiolabeling studies and ELISA); adapted from [Zieris et al., 2010a].

<table>
<thead>
<tr>
<th></th>
<th>CLSM</th>
<th>radiolabeling ((^{125}\text{I}-) ) studies</th>
<th>HPLC</th>
<th>ELISA</th>
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<tr>
<td>performance</td>
<td>well plate</td>
<td>immobilization chamber</td>
<td>well plate</td>
<td>immobilization chamber</td>
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<tr>
<td>analysis of protein</td>
<td>in gel and supernatant</td>
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<td>in supernatant</td>
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<tr>
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<td>yes</td>
<td>no</td>
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The different methods were analyzed with respect to their ability to accurately and reliably quantify cytokine binding and release (see appendix B, supplementary results and discussion for chapter 4.1) by starPEG-heparin hydrogels. It was found that only ELISA experiments could be performed using non-labeled FGF-2 or VEGF under conditions minimizing the contact area for non-specific protein interactions with ‘foreign’ glass or plastic surfaces. Therefore, this approach was concluded to be advantageous...
for analyzing growth factor binding and release. For a more detailed discussion of the methodological optimization the reader is referred to appendix B.

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4.2 Growth factor binding and release by starPEG-heparin scaffolds

As a main objective of this work, it should be elucidated if starPEG-heparin hydrogels could be used as a delivery matrix for the angiogenic cytokines FGF-2 and VEGF. Therefore, after evaluation and optimization of different analytical approaches (see chapter 4.1), the interaction of FGF-2 or VEGF with distinct starPEG-heparin hydrogels differing in their network characteristics ($\gamma = 1.5, 3$ and $6$) was investigated. Moreover, the binding and release of varying concentrations of FGF-2 and VEGF applied as single factors (0.5-50 µg/ml) or as different combinations (variation of the FGF-2 to VEGF ratio from 0.2-5) were investigated.

Quantitative protein binding studies were performed using ELISA, radiolabeling studies and amino acid analysis via HPLC, while experiments on the release of the cytokines were performed via ELISA and exemplarily confirmed by radiolabeling studies. As already discussed, quantitative differences determined with these methods might be due to experimental conditions. Nevertheless, these techniques were applied because all of them delivered the same qualitative results.

4.2.1 Uptake and release of FGF-2 or VEGF depending on the physicochemical network properties

First, the influence of the starPEG-heparin network structure on the binding and release of FGF-2 or VEGF was investigated for hydrogel types differing in their crosslinking degree ($\gamma = 1.5, 3$ and $6$) and therefore in their mechanical properties (soft, intermediate and stiff networks, see Fig. 11). For that, a protein concentration of 1 µg/ml was used for immobilization. Via ELISA (Fig. 13, top), radiolabeling studies and amino acid analysis via HPLC (Fig. B4, left and right, appendix B), it could be shown
that similar quantities of both FGF-2 or VEGF were immobilized for each scaffold independently of the gel type (for gels with molar ratios starPEG to heparin of γ = 1.5; 3 or 6 ~ 199 ng/cm² FGF-2 and ~ 197 ng/cm² VEGF as detected by ELISA; p > 0.05).

In addition to the binding experiments, the FGF-2 or VEGF sequestering depending on the meshsize of the particular network was determined for the three different gel types over the course of 96 h via ELISA (Fig. 13, bottom left and right) and radiolabeling studies (Fig. B5, left and right, appendix B). Both proteins showed an initial burst release within the first 6 h. Such burst characteristics are often attributed to surface effects [Huang et al., 2001] and could be caused by a FGF-2 or VEGF fraction entrapped in the meshwork but not bound specifically to heparin. However, after 24 h, the release continued slowly over the course of the entire time period that was investigated, indicating the potential of the material for applications with a need for long-term delivery profiles of growth factors. Moreover, comparable sequestered quantities of FGF-2 or VEGF were found for each scaffold independently of the gel type (for gels with molar ratios starPEG to heparin of γ = 1.5, 3, and 6 after four days ~ 1 ng/cm² FGF-2 or VEGF released as quantified via ELISA; p > 0.05 for the comparison of the different gel types).

Based on these findings, results obtained demonstrate that the binding and release of the two proteins is independent of the mechanical hydrogel properties. Considering the mesh sizes of the matrices with large pores in the range of ~ 16-7 nm for γ = 1.5-6 (see Fig. 11, bottom left) and the observations of the qualitative uptake experiments for FGF-2 (diameter of ~ 3 nm [Eriksson et al., 1991]) and VEGF (diameter of ~ 6 nm [Muller et al., 1997]) performed via CLSM showing no restrictions for penetration of both proteins (for details see appendix B), it becomes obvious that the diffusion of the cytokines is not affected by differences in the network structure (pore size, hydration etc.). Consequently, the FGF-2 and VEGF immobilization and delivery correlates only with the constant heparin concentration of the different scaffolds.
Fig. 13 Amount of hydrogel-immobilized or -released FGF-2 or VEGF determined for different gel types as quantified via ELISA; adapted from [Zieris et al., 2010b]. top: amount of electrostatically bound FGF-2 or VEGF per cm² scaffold area for the different gel types \( \gamma = 1.5; 3 \) or 6 (low, intermediate and high crosslinking degree, \( p > 0.05 \); ANOVA). bottom: cumulative amount of electrostatically bound FGF-2 (left) or VEGF (right) released by the different gel matrices \( \gamma = 1.5; 3 \) or 6 (low, intermediate and high crosslinking degree, \( p > 0.05 \); ANOVA). All data are presented as mean ± root mean square deviation from \( n = 3 \).

Taken together, the starPEG-heparin hydrogels might be used as FGF-2 or VEGF storage systems, which can present the growth factors independently of the particular structural and mechanical properties of the different scaffolds.

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4.2.2 Uptake and release of FGF-2 and/or VEGF depending on the protein concentration

Due to their high heparin content, starPEG-heparin hydrogels were shown to bind and stabilize both FGF-2 and VEGF. Consequently, they might be promising candidates for an application as a delivery system for large cytokine quantities or for the parallel provision of several growth factors. Thus, after evaluating the binding potential for high concentrations of single cytokines (from 0.5-50 µg/ml), the immobilization and subsequent release of FGF-2 and VEGF, introduced to the matrices either as single components (1 µg/ml or 5 µg/ml) or as combinations in different ratios (1 µg/ml FGF-2 + 1 µg/ml VEGF, 5 µg/ml FGF-2 + 1 µg/ml VEGF or 1 µg/ml FGF-2 + 5 µg/ml VEGF), were analyzed.

First, to evaluate whether the starPEG-heparin hydrogels could be used as efficient FGF-2 and VEGF storage system, the capacity of the matrices to take up various amounts of the growth factors was investigated for the gel with the intermediate crosslinking degree, $\gamma = 3$. By analysis via ELISA (Fig. 14, top left), radiolabeling studies and amino acid analysis via HPLC (Fig. B6, left and right, appendix B), the immobilized quantities at a defined concentration were found to be similar for both proteins. Moreover, a linear correlation between the concentration of the incubation solution and the amount of immobilized FGF-2 or VEGF within the gel could be found, which indicates that no saturation of binding was reached within the concentration range monitored. This result correlates well with estimations concerning the maximal storage capacity of the applied hydrogel system on the basis of the calculated heparin concentration within the swollen network and HPLC-based analysis of immobilization experiments with high concentrations of growth factors. Here, even after incubation with 50 µg/ml protein the molar ratio of heparin to growth factor was still 26:1 for FGF-2 and 62:1 for VEGF, respectively. Moreover, as reported for FGF-2 [Arakawa et al., 1994], each heparin molecule is able to interact with several cytokine molecules, so that a saturation of binding will occur only at concentrations much higher than used in this study, while due to the small size of FGF-2 and VEGF also no spatial restrictions within the gel network should limit the uptake of the proteins.

While a linear correlation was observed between the concentration of the incubation solution and the amount of immobilized cytokine within the gel (~ 199 ng/cm² immobilized from a solution of 1 µg/ml and ~ 995 ng/cm² immobilized from a solution of 5 µg/ml as detected by ELISA), it could be additionally shown that different combinations of FGF-2 and VEGF
(Fig. 14, top right and Fig. B7, appendix B) can be bound to the matrices with the same efficiency as determined for the individual factors \((p > 0.05\) for the immobilized amount of single FGF-2 or VEGF and the corresponding concentration used in the combination). Furthermore, the immobilized quantities of combinations of FGF-2 and VEGF at a defined concentration were found to be similar for both proteins.

Besides an evaluation of the starPEG-heparin hydrogel binding ability for FGF-2 and VEGF, experiments on the release of each single protein as well as of different combinations from the matrices were performed via ELISA and exemplarily confirmed by radiolabeling studies. Fig. 14 illustrates the cumulative release of either FGF-2 (bottom left) or VEGF (bottom right) alone (1 or 5 µg/ml) and of different combinations of both proteins (1 µg/ml FGF-2 + 1 µg/ml VEGF, 5 µg/ml FGF-2 + 1 µg/ml VEGF or 1 µg/ml FGF-2 + 5 µg/ml VEGF) measured over 96 h. Irrespective of the immobilized concentration or the particular factor considered, the release curves show once again the typical burst within the first 6 h followed by a continuous release over time. Similar to the trends observed for FGF-2 and/or VEGF immobilization, by ELISA analysis (Fig. 14, dashed lines), a linear correlation between the amount of gel-bound growth factors and the quantities being released was observed for single FGF-2 or VEGF \((\sim 1 \, \text{ng/cm}^2 \text{ released for 1 µg/ml and } \sim 6 \, \text{ng/cm}^2 \text{ released for 5 µg/ml})\), which was exemplarily confirmed via radiolabeling studies (single factors, Fig. B8, left and right, appendix B). Additionally, different combinations of FGF-2 and VEGF (Fig. 14, continuous lines) could be released by the matrices with the same efficiency as for the individual factors \((p > 0.05\) for the released amount of single FGF-2 or VEGF and the corresponding concentration used in the combination).

As an explanation, the large excess of heparin appears to prevent any interference between the growth factors during their combined application. Moreover, an additional advantage of these starPEG-heparin hydrogels is the comparable release of either cytokine at a particular loading quantity. Given this finding, the FGF-2 and/or VEGF release characteristics can be adjusted by the initial amount of protein loaded, which in turn can be tuned over a wide range of concentrations.
Fig. 14 Amount of hydrogel-immobilized or -released FGF-2 and/or VEGF determined for different protein concentrations as quantified via ELISA; adapted from [Zieris et al., 2010b and 2011]. top (left): uptake of single FGF-2 or VEGF in dependence on the protein concentration (0.5-50 µg/ml) in the immobilization medium; linear regression, $R^2$ (FGF-2) = 0.99999; $R^2$ (VEGF) = 0.99999. top (right): amount of electrostatically bound FGF-2 and/or VEGF per cm² scaffold area for different protein concentrations (p > 0.05 for the immobilized amount of single FGF-2 or VEGF and the corresponding concentration used in the combination; ANOVA). bottom: cumulative amount of electrostatically bound FGF-2 (left) or VEGF (right) released by gels which were loaded with either single cytokines (dashed lines) or different combinations of FGF-2 and VEGF (continuous lines) (p > 0.05 for the released amount of single FGF-2 or VEGF and the corresponding concentration used in the combination; ANOVA). All data are presented as mean ± root mean square deviation from n = 3 (ELISA).

In summary, results demonstrate that starPEG-heparin hydrogels could be utilized for an independent and modular delivery of both FGF-2 and VEGF over a broad range of concentrations.
For evaluating the potential of cytokine delivery systems to promote a certain cell behavior \textit{in vitro}, the absence of serum in the cell culture medium might be beneficial, as possible interferences of growth factors potentially provided by the serum itself are avoided. Consequently, FGF-2 and/or VEGF release experiments performed under serum-free conditions are representative for the \textit{in vitro} HUVEC culture settings investigated in this work (see chapter 4.3). However, this environment is significantly different from the situation that occurs \textit{in vivo}. Therefore, the FGF-2 and VEGF release from starPEG-heparin hydrogels was additionally analyzed in the presence of the serum protein BSA, thereby better corresponding to physiological conditions. Exemplarily, Fig. 15 shows the cumulative release of electrostatically bound FGF-2 (left) or VEGF (right) into SF ECGM ± 1 mg/ml BSA by gels which were loaded with a combination of 1 µg/ml FGF-2 + 1 µg/ml VEGF. For a more comprehensive comparison of the efficiencies, the data are expressed as percentage of initially bound growth factor (see Fig. 14, top right) being released by the starPEG-heparin scaffolds. As demonstrated in Fig. 14, both FGF-2 and VEGF release was found to be low in the absence of serum proteins in the environment (~ 0.5 %). This effect is most probably attributed to the high excess of heparin in the hydrogels, presumably resulting in a dynamic release/-re-binding of the growth factors within the scaffold. Nevertheless, the amount of cytokines delivered might be sufficient to promote a certain cell response, as growth factors already elicit their biological function when present at pico- or nanomolar concentrations [Flaumenhaft et al., 1992]. However, despite of similar delivery kinetics, compared to the FGF-2 and VEGF release in a serum-free environment, the efficiency was increased by magnitudes in the presence of proteins in the medium (~ 9.5 %; \( p < 0.05 \) for comparing released amounts of growth factors into SF ECGM or SF ECGM + 1 mg/ml BSA) as also observed by [Gu et al., 2004; Lee et al., 2007]. One reason for this effect could be that BSA might displace the cytokines from heparin by reducing their interaction. Moreover, BSA might also be able to stabilize FGF-2 and VEGF after they get released into the medium, thereby preventing physical loss of cytokine or its activity.
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Fig. 15 FGF-2 and VEGF release efficiency in dependence on the release medium as obtained by analysis via ELISA; adapted from [Zieris et al., 2011]. Plots show the cumulative percentage of electrostatically bound FGF-2 (left) or VEGF (right) released into SF ECGM ± 1 mg/ml BSA by gels which were loaded with a combination of 1 µg/ml FGF-2 + 1 µg/ml VEGF (p < 0.05 for the released amount of growth factors into SF ECGM or SF ECGM + 1 mg/ml BSA; ANOVA). All data are presented as mean ± root mean square deviation from n = 3.

Taken together, despite the dependence of the release efficiency on the environmental conditions, a combination of both FGF-2 and VEGF could be delivered by starPEG-heparin hydrogels in similar amounts for several experimental settings that are relevant for specific applications.

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4.3 *In vitro* HUVEC response to differently biomodified starPEG-heparin scaffolds

After it has been demonstrated that starPEG-heparin hydrogels could be used as efficient storage systems for several heparin-binding growth factors going along with well adjustable cytokine release characteristics, the potential of starPEG-heparin hydrogels for an application as a substrate for *in vitro* culture of human umbilical vein endothelial cells (HUVECs) was investigated.

For a long time it is known that the process of angiogenesis is tightly controlled by both chemical and mechanical cues. Consequently, to gain insight into the complex mechanism of its regulation there is a need for *in vitro* model systems that allow for an
independent investigation of both parameters. Therefore, in this work, endothelial cells were cultured on top of hydrogel types that differ in terms of stiffness along with varying degrees of biofunctionality. In order to ensure that any effects on HUVECs behavior is initiated by these biomodified scaffold rather than by cytokines possibly provided by serum-containing cell culture medium, all experiments were performed under serum-free conditions.

### 4.3.1 Influence of the physicochemical network characteristics

To analyze the influence of the physicochemical starPEG-heparin network characteristics on the behavior of endothelial cells, HUVECs were cultured under serum-free conditions on top of hydrogels that differ in terms of their physicochemical characteristics ($\gamma = 1.5$, 3 and 6; gels with low, intermediate and high crosslinking degree and therefore low, intermediate and high stiffness and meshsize, respectively) in the presence or absence of the adhesion ligand RGD. For each hydrogel type, the amount of introduced adhesion molecules was constant (Fig. 12, right). Concerning every culture substrate, differential cellular responses in terms of adhesion, proliferation/survival and cell morphology were analyzed.

#### Cell adhesion

To allow for cell growth on a particular substrate, the initial process of adhesion to the surface is one of the most critical steps. In this study, HUVEC attachment and spreading depending on the biomolecular functionalization with adhesion ligands and the mechanical properties of the particular matrices was investigated by analyzing the cell circularity 2 h after plating them on the hydrogels (Fig. 16). Here, non-adherent cells remained circular, (as indicated by a number close to “1”), whereas the circularity decreased upon attachment due to spreading.

Despite their high content of the naturally occurring molecule heparin, poor adhesion was observed for all unmodified starPEG-heparin gel types $\gamma = 1.5$, 3 and 6 as cells cultured on these matrices possessed an almost fully circular morphology. By contrast, the different gel scaffolds modified with RGD were able to induce HUVEC attachment as indicated by the more elongated morphology ($p < 0.05$ for the comparison of pure gels or gels with RGD for every $\gamma$). Consequently, the presence of an adhesion ligand as provided
by the introduction of the RGD sequence to the gels was found to be absolutely essential to initiate attachment and spreading. Beside the major effect of the biomodification, an additional influence on HUVEC adhesion could be observed in terms of the substrate stiffness. Due to the poor overall attachment to pure starPEG-heparin scaffolds, no significant differences were found among the hydrogel types for these particular conditions (cell circularity of 0.988, 0.997 and 0.998 for γ = 1.5, 3 and 6; p > 0.05 for comparing the different gel types). However, for gel substrates treated with RGD, a correlation between the mechanical properties of the scaffold and the adhesion of HUVECs was observed (cell circularity of 0.672, 0.634 and 0.604 for γ = 1.5, 3 and 6; p < 0.05 for the comparison of γ = 1.5 and 6). For matrices with higher degrees of crosslinking, cells showed an increased tendency to adhere to these substrates as assessed via decreasing cell circularity. This finding is in line with results reported in literature, where stiffer, more rigid culture substrates were described to promote endothelial cell adhesion [Yeung et al., 2005].

Conclusively, significant differences in cell adhesion with respect to the gel type and the bioadhesiveness could be observed. While the presence of an adhesion ligand was found to be most critical for promotion of an effective cell attachment, biomaterials offering a higher stiffness seemed to be more advantageous to support the process of HUVEC adhesion.
**Cell survival/proliferation and morphology**

After successful initial attachment, scaffolds suitable for an application in tissue engineering should be able to promote cell survival and proliferation over prolonged time periods. Consequently, a live/dead (Fig. 17A) and a MTT (Fig. 17B, left) assay were performed after 3 d of culture on starPEG-heparin hydrogels with different physicochemical properties in the presence or absence of the adhesion ligand RGD. Moreover, materials have to support HUVEC differentiation into less circular and more elongated cells in order to form tubular structures during angiogenesis. Therefore, HUVEC circularity as it was dependent on the presence of RGD and the structural characteristics of the substrate was analyzed after 3 d of culture (Fig. 17B, right).

For the starPEG-heparin hydrogels it could be shown that coinciding with an advancing degree of biofunctionalization, also the HUVEC proliferation/survival rate increased. Very low survival with many dead cells in the surrounding medium could be observed on pure gels, while the few cells being still viable showed a round shape. This might be due to the mainly non-adhesive character of the starPEG therefore leading to the detachment of most cells. The very small number of HUVECs that could be found on the gel surface was most likely only weakly attached, so that cells were consequently not able to proliferate.

By introducing the adhesion peptide RGD into the starPEG-heparin hydrogels, the HUVEC survival rate could be increased significantly \((p < 0.05\) for the comparison of pure gels or gels with RGD for every \(\gamma\)\). Under these culture conditions, only very few dead cells were found in the medium while the viable HUVECs adapted also a more elongated morphology \((p < 0.05\) for the comparison of pure gels or gels with RGD for every \(\gamma\)\). The most important parameter for that might be the fact that these substrates were able to mediate effective initial cell adhesion. Therefore, HUVECs could successfully spread on these scaffolds so that a high number of the primarily plated cells survived. Since these results were observed on the RGD-modified starPEG-heparin hydrogels even under serum-free culture conditions, these data indicate that the scaffolds do not exhibit any toxic effects on the cells and might therefore be generally well suited to support growth of HUVECs.

In addition to the biomolecular functionalization, mechanical matrix parameters of engineered materials are important to promote a desired cellular response [Liu et al., 2005]. For pure gels, where survival due to lacking adhesion ligands was generally hardly possible, a change in the mechanical properties did not substantially affect
neither HUVEC proliferation/survival (~430, 190 and 340 cells/cm² scaffold area for γ = 1.5, 3 and 6) nor cell morphology (0.996, 0.991 and 0.997 for γ = 1.5, 3 and 6).

In contrast, for substrates functionalized with RGD, increasing cell numbers were found on the gels with a low or intermediate crosslinking degree, indicating that such network structures are most beneficial to promote HUVEC proliferation/survival (~6200, 5300 and 3100 cells/cm² scaffold area for γ = 1.5, 3 and 6; p < 0.05 for the comparison of γ = 1.5 or 3 to γ = 6).

Fig. 17 Interaction of different hydrogel types (γ = 1.5; 3 or 6; ± RGD) with HUVECs after 3 days of culture; modified from [Zieris et al., 2010b]. 17A: representative fluorescence microscopy images after live/dead staining of HUVECs (viable cells = green; dead cells = red) on the different substrates (scale bar 130 µm). 17B (left): HUVEC proliferation/survival as accessed via cell numbers on the different networks quantified by an MTT assay. All data are presented as mean ± root mean square deviation from n = 3-5. For statistics see supplementary data. 17B (right): HUVEC morphology as accessed via cell circularity on the different networks quantified by the circularity calculation within ImageJ 1.41o. All data are presented as mean ± root mean square deviation from n ≈ 30-140 cells quantified on up to 4 different substrates. * indicates statistically significant differences (p < 0.05; ANOVA). For statistical comparisons apart from the illustrated significant differences, see text.
terms of the effect on cell morphology, in the case of networks modified with RGD, HUVECs cultured on the intermediately crosslinked gel type $\gamma = 3$ showed a slightly more elongated shape ($0.502, 0.416$ and $0.575$ for $\gamma = 1.5, 3$ and $6$; $p < 0.05$ for the comparison of $\gamma = 1.5$ or $6$ to $\gamma = 3$). Although it has been described that endothelial cells preferentially grow on stiff substrates and, due to the fact that their requirements for proliferation and adaptation of an elongated morphology differ, primarily differentiate when cultured on a soft matrix that allows for a retraction and reorientation of the cell shape, there might be an optimal range for balancing both parameters [Ingber et al., 1989; Deroanne et al., 2001; Liu et al., 2005; Saunders et al., 2010]. Results obtained in this study demonstrate that with a HUVEC culture on the hydrogel $\gamma = 3$ offering an intermediate starPEG to heparin ratio going along with a storage modulus in the range of $\sim 7000$ Pa, rather high cell numbers possessing an elongated morphology could be observed.

Overall, starPEG-heparin hydrogels functionalized with the adhesion ligand RGD are suitable substrates for the culture of HUVECs. It was found that intermediate starPEG to heparin ratios showed beneficial effects on proliferation/survival while in parallel also HUVEC differentiation into tube-like structures could be promoted.

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4.3.2 Influence of the FGF-2 and/or VEGF delivery

Besides the influence of the physico-chemical properties of the cellular environment, also the presence of soluble molecular effectors such as the angiogenic growth factors FGF-2 or VEGF is an essential parameter for controlling endothelial cell behavior in tissue engineering. Although the administration of one cytokine is not sufficient to create well-developed mature blood vessels [Bruick et al., 2001; Komori et al., 2005], only a few studies have analyzed the effect of a combined provision of both FGF-2 and VEGF by a particular biomaterial [Ribatti et al., 2001; Wong et al., 2003; Nillesen et al., 2007; Wilcke et al., 2007; Briganti et al., 2010; Larsen et al., 2010]. Based on its overall beneficial effects on HUVEC adhesion, proliferation/survival and differentiation, for investigating the influence of the growth factor delivery by starPEG-
heparin matrices on the endothelial cell behavior, the intermediately crosslinked hydrogel type ($\gamma = 3$) was used in this study, while HUVECs were cultured under serum-free conditions on top of matrices in the presence or absence of the adhesion ligand RGD. These scaffolds were either used without any additional modification or loaded with either 1 or 5 $\mu$g/ml single FGF-2 or VEGF or with a combination of 1 $\mu$g/ml FGF-2 + 1 $\mu$g/ml VEGF. For every culture substrate, differential cellular responses in terms of adhesion, proliferation/survival, cell morphology and migration were analyzed.

**Cell adhesion**

As both angiogenic growth factors FGF-2 and VEGF are known to promote endothelial cell adhesion [Baird et al., 1988; Hutchings et al., 2003], studies were performed to examine cell attachment and spreading on starPEG-heparin hydrogels in the presence and absence of these cytokines (applied as single proteins or as a combination) and the presence and absence of an additional adhesion ligand (RGD). Once again, cell adhesion was assessed by analyzing cell shape (in terms of cell circularity) 2 h after plating HUVECs on the hydrogels (Fig. 18).

As demonstrated in appendix B, the covalent RGD attachment to the heparin carboxylic acid moieties does not influence the electrostatic binding (Fig. B9, top, appendix B) and release (Fig. B9, bottom, appendix B) of FGF-2 and VEGF. This effect is most probably related to the high heparin concentration within the material carrying various carboxylic acid groups as potential binding sites for the small RGD ligand, while the sulfate groups as main interaction sites for FGF-2 and VEGF are unaffected.

For analysis of HUVEC attachment, despite the high content of heparin, poor adhesion was observed for both pure starPEG-heparin matrices and for gels treated with 1 $\mu$g/ml or 5 $\mu$g/ml FGF-2, as assessed by the high degree of circularity (cell circularity of 0.997, 0.993 and 0.943, respectively; $p > 0.05$). In contrast, the HUVEC circularity decreased on scaffolds modified with 1 $\mu$g/ml or 5 $\mu$g/ml VEGF and the combination of both growth factors (cell circularity of 0.824, 0.882 and 0.861; $p < 0.05$ when comparing gels + 1 $\mu$g/ml VEGF to pure gels or gels + FGF-2). Here, as VEGF is involved in focal adhesion integrity [Abedi et al., 1997; Hutchings et al., 2003] it is likely that it might exert a slight beneficial effect on HUVEC attachment to starPEG-heparin hydrogels, even in the absence of an adhesion ligand. Nevertheless, the observation of lower cell circularity on starPEG-heparin gels modified with RGD (cell circularity of 0.637) alone or in combination with 1 $\mu$g/ml or 5 $\mu$g/ml FGF-2
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(cell circularity of 0.639 and 0.558), 1 µg/ml or 5 µg/ml VEGF (cell circularity of 0.635 and 0.665) and with FGF-2/VEGF (cell circularity of 0.587) demonstrated that HUVEC adhesion could be still enhanced \((p < 0.05 \text{ for comparing gels with or without RGD for each condition})\). This indicates that despite the supporting effect of VEGF, the presence of an adhesion ligand, such as the RGD sequence, is essential for maximal cell attachment and spreading. Besides the incorporation of this adhesion peptide, almost no additional influence of growth factor modification could be observed \((p > 0.05 \text{ when comparing gels with RGD between the different conditions})\), which is in line with findings of [Wong et al., 2003]. Here, the strong adhesive effect of RGD most probably overrides the cytokine impact on HUVEC attachment. However, as an advantage of the RGD decoration, all of the samples monitored for their impact on long-term cell culture started with HUVECs that similarly adhered to the gel surface.

As a conclusion, effective HUVEC adhesion could be observed depending on the modification of starPEG-heparin hydrogels. Although VEGF was found to influence cell attachment positively, introduction of the adhesion ligand RGD was still necessary to achieve a maximal effect.
**Cell survival/proliferation and morphology**

As the delivery of cytokines is a crucial material parameter to support endothelial cell proliferation/survival and differentiation [Fischbach et al., 2006; Zhang et al., 2007; Uebersax et al., 2009], also the effects of FGF-2 and/or VEGF provision by starPEG-heparin hydrogels on these parameters were investigated using live/dead staining (Fig. 19A, green/red cells) and a MTT assay (Fig. 19B, left) performed after 3 d of culture on the different matrices. Furthermore, cell morphology was analyzed as assessed by HUVEC circularity (Fig. 19B, right). As the functionalization of starPEG-heparin hydrogels with cytokines alone was not sufficient to support effective cell attachment and spreading (Fig. 18), which would be crucial for long term cell culture (Fig. 17), all scaffolds monitored for their effect on HUVEC survival/proliferation and morphology were additionally modified with the adhesion ligand RGD.

After 3 d of culture, substantial HUVEC survival (Fig. 19B, left) was observed for RGD-functionalized hydrogels (~ 2700 cells/cm² scaffold area) and for RGD-modified matrices loaded with 1 µg/ml of single FGF-2 or VEGF (~ 12100 or 10500 cells/cm² scaffold area), 5 µg/ml of single FGF-2 or VEGF (~ 12200 or 11000 cells/cm² scaffold area) or with a combination of 1 µg/ml FGF-2 + 1 µg/ml VEGF (~ 15600 cells/cm² scaffold area). The very small number of dead cells in the medium and the typical spindle-shape-like morphology of cells grown on the scaffolds (Fig. 19A) indicated once again that the introduction of RGD to starPEG-heparin matrices could generate successful HUVEC culture substrates. Moreover, in the presence of FGF-2 and/or VEGF in RGD-modified hydrogels, cell numbers (Fig. 19B, left) could be further increased (*p* < 0.05 when comparing gels + RGD to scaffolds modified with RGD and cytokines). Despite the positive effect of RGD + VEGF on HUVEC survival, after 3 d of culture the presence of RGD + FGF-2 yielded an even higher cell number than initially applied. However, maximal proliferation rates (Fig. 19B, left) were observed when starPEG-heparin hydrogels were used for the combined provision of FGF-2 and VEGF (*p* < 0.05 for comparing gels + RGD and one single growth factor to scaffolds + RGD and both FGF-2 + VEGF). Interestingly, increasing amounts of single FGF-2 or VEGF released from the matrices (loaded with 5 µg/ml, respectively) did not significantly change the HUVEC survival when compared to the treatment with lower concentrations (*p* > 0.05 for the comparison of 1 µg/ml and 5 µg/ml cytokine). Nevertheless, the combination of both factors (1 µg/ml FGF-2 + 1 µg/ml VEGF) significantly increased cell growth even though this provided a lower total amount of cytokine. Thus, the increased proliferation induced by hydrogels modified...
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with FGF-2 + VEGF clearly did not arise from the presence of higher growth factor concentrations, but rather seemed to result from a combined action of both cytokines as already described by [Goto et al., 1993].

In addition to the beneficial effect on cell proliferation/survival, culture on RGD-modified starPEG-heparin hydrogels also led to the formation of the typical spindle-shaped HUVEC morphology. Here (Fig. 19B, right), the lowest cell circularity representing HUVECs with the most elongated shape was observed for starPEG-heparin matrices treated with RGD + 1 µg/ml or 5 µg/ml VEGF (cell circularity of 0.34 or 0.35, respectively; \( p < 0.05 \) when comparing gels with RGD + VEGF to gels with RGD ± FGF²2), while there were no significant differences between RGD-functionalized scaffolds with 1 µg/ml or 5 µg/ml FGF-2 (cell circularity of 0.43 or 0.45, respectively) or without any growth factor (cell circularity of 0.43). However, consistent to the supporting effect of FGF-2/VEGF combinations on \textit{in vitro} tube formation [Pepper et al., 1992; Goto et al., 1993; Sun et al., 2004], also in the case of gels treated with the cytokine combination (RGD and FGF-2 + VEGF), HUVECs exhibited the tendency to differentiate into more stretched cells (cell circularity of 0.36; \( p < 0.05 \) when comparing scaffolds with RGD + FGF-2/VEGF with gels containing RGD ± 5 µg/ml FGF-2; \( p > 0.05 \) for the comparison with networks with RGD + 1 µg/ml FGF-2 or 1 or 5 µg/ml VEGF).

Given the fact that high cell numbers were observed with the parallel delivery of both FGF-2 and VEGF, the provision of the cytokine combination by starPEG-heparin hydrogels promoted both HUVEC proliferation and differentiation.
In summary, upon the introduction of the RGD adhesion ligand, a FGF-2 provision by starPEG-heparin hydrogels was able to stimulate HUVEC proliferation/survival, while VEGF seemed to promote cell differentiation. However, using starPEG-heparin hydrogels as a delivery system for the parallel administration of both cytokines, their beneficial effects could be combined to obtain high numbers of HUVECs undergoing differentiation.
Cell migration

The biological process of angiogenesis involves the migration of endothelial cells to the site where new vessel formation is needed. Therefore, the ability of biofunctionalized starPEG-heparin hydrogels to induce directional HUVEC motility was evaluated. In this setting, the hydrogels were used as a growth factor delivery matrix to initiate cell migration through a fibronectin-coated Boyden filter (upper chamber) towards the site of cytokine provision in the lower chamber (Fig. 20A). Results (Fig. 20C) are expressed as the relative cell migration compared to wells filled with untreated endothelial cell growth medium (ECGM) without any scaffold in the lower chamber (Fig. 20B).

While unmodified starPEG-heparin matrices were hardly able to support HUVEC migration (1.5 % increase compared to wells with untreated ECGM in the lower chamber), hydrogels loaded with 1 µg/ml of single FGF-2 or VEGF (11 or 9 % increase in comparison to wells with untreated ECGM in the lower chamber, respectively), 5 µg/ml of single FGF-2 or VEGF (15 or 18 % increase in comparison to wells with untreated ECGM in the lower chamber, respectively) or a combination of 1 µg/ml FGF-2 + 1 µg/ml VEGF (28 % increase in comparison to wells with untreated ECGM in the lower chamber; p < 0.05 for comparing pure gels to scaffolds modified with cytokines) significantly increased cell motility (Fig. 20C). Interestingly, in contrast to the other in vitro assays, where cells were directly seeded on the cytokine-loaded scaffolds, in this setting, the unaffected release and diffusion of growth factors into the medium over a larger distance is required to initiate an effect on HUVEC behavior. Consequently, the results obtained indicate the suitability of starPEG-heparin hydrogels to function as a cytokine delivery matrix, where the bioactivity of growth factors is preserved even after the release from the scaffolds.

When comparing the migratory cell response to FGF-2 or VEGF at one particular concentration, no significant differences were found (p > 0.05 for the comparison of 1 µg/ml FGF-2 to 1 µg/ml VEGF or 5 µg/ml FGF-2 to 5 µg/ml VEGF). Although several authors presented inconsistent results as whether FGF-2 [Sakomoto et al., 1995; Donohue et al., 2003] or VEGF [Yoshida et al., 1996; Castellon et al, 2002] is the most potent initiator of endothelial cell motility, these data might suggest that both factors are able to induce HUVEC migration in a similar manner. However, compared to the influence on cell adhesion, proliferation and morphology, there was clearly a stronger effect of the particular cytokine concentration on migration as HUVEC motility generally increased with larger growth factor quantities being released by the matrices (p < 0.05 for 1 µg/ml FGF-2 versus 5 µg/ml VEGF; 1 µg/ml VEGF versus 5 µg/ml FGF-2 or versus 5 µg/ml VEGF; p > 0.05 for
1 µg/ml versus 5 µg/ml FGF-2). These data are consistent with results described in literature [Yoshida et al., 1996], where a positive correlation between the endothelial migratory response and an increasing concentration of up to 10 ng/ml soluble FGF-2 or VEGF was found. However, despite the effect of the cytokine concentration, the combination of 1 µg/ml FGF-2 + 1 µg/ml VEGF showed the most beneficial influence on cell migration (p < 0.05 for comparing the migration towards networks modified with both FGF-2/VEGF to all other conditions). Although some authors describe the absence of such an effect on endothelial cell motility [Yoshida et al., 1996], in this system FGF-2 and VEGF seemed

Fig. 20 HUVEC migration in response to differently biomodified hydrogels (with or without 1 or 5 µg/ml of single FGF-2 or VEGF as well as a combination of 1 µg/ml FGF-2 + 1 µg/ml VEGF) as analyzed by a modified Boyden chamber assay; adapted from [Zieris et al., 2011]. 20A and B: representative images of HUVECs located on the lower site of the Boyden filter after migration through the membrane towards differently biomodified gel matrices (A) or towards the untreated ECMG which served as a control (B) (scale bar 100 µm). 20C: quantification of the relative HUVEC migration in relation to differently biomodified hydrogels. Data are presented as mean ± root mean square deviation from n = 4-6. * indicates statistically significant differences (p < 0.05; ANOVA). For statistical comparisons apart from the illustrated significant differences, see text.
to promote migration in a synergistic way as also observed by [Vernon et al., 1999; Yan et al., 2001]. However, as the delivery of both growth factors also significantly increased HUVEC numbers after 3 d of culture (Fig. 19B, left), when considering the cellular process of migration over 20 h, possible interferences of proliferation should be discussed. Since, with regard to a time course of 24 h, no increase in cell numbers occurred in standard HUVEC growth curves [Hoshi et al., 1984; Weiss et al., 1990], while also the presence of rather high concentrations of FGF-2, VEGF or FGF-2 + VEGF had no significant influence on endothelial cell numbers for such short periods of culture [Sakomoto et al., 1995], effects observed in this study might be indeed attributed to an impact of the growth factors on HUVEC migration rather than proliferation.

In total, cytokine-functionalized starPEG-heparin matrices could be applied as a **growth factor delivery matrix** in order to induce HUVEC directional migration. While **FGF-2 and VEGF supported cell motility** to a **similar** extent, their **combined action** was found to exert the **strongest effect on HUVEC migration**.

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### 4.4 *In vivo* CAM response to FGF-2 and/or VEGF provision via starPEG-heparin hydrogels

Although *in vitro* assays using endothelial cells can provide essential information on the general suitability of a certain biomaterial for an application in angiogenic tissue engineering, the final evaluation of factors which influence angiogenesis is best performed by *in vivo* experiments [Auerbach et al, 2003]. Therefore, to analyze whether the provision of FGF-2 or VEGF as single cytokines or in combination by starPEG-heparin hydrogels could initiate an angiogenic response *in vivo*, the effects of biofunctionalized hydrogel onplants were studied in a CAM assay (Fig. 21). Based on the data reported in literature [Wilting et al., 1991 & 1993; Nico et al., 2001], an intermediate concentration of either single (5 µg/ml FGF-2 or VEGF) or combined growth factors (5 µg/ml FGF-2 + 5 µg/ml VEGF) resulting in ~ 2 µg of every cytokine immobilized per scaffold (see Fig. 21) was used, while starPEG-heparin matrices
(γ = 3) were generated as clot-like grafts. As the importance of effective cell attachment was demonstrated in in vitro cell experiments, all networks were modified with the RGD adhesion ligand. After subsequent loading with cytokines, the starPEG-heparin hydrogels (Fig. 21A and C) were placed onto the developing CAM at embryonic day 8 (ED8) until ED12. The untreated CAM (Fig. 21B) served as a reference system.

As visualized in Fig. 21B, the untreated CAM shows a normal pattern of vascularization. The vessels are arranged in an organized manner with regular branches of the larger primary blood vessels into secondary vessels and tertiary capillaries. This pattern was hardly altered in samples containing onplants without any growth factors. However, in the presence of hydrogels loaded with cytokines, the onplants were surrounded by an increased number of allantoic vessels that looped towards the gel.

Quantification of any angiogenic response was performed by counting the vessels within the site of gel transplantation or the control area of the untreated CAM, respectively (Fig. 21C). Here, the starPEG-heparin onplants lacking any growth factor only led to a minimal increase in the relative vascularization compared to the untreated CAM (~ 4 %). This slightly improved vessel formation might result from the high heparin content of the matrices, as this molecule has been shown to induce a moderate angiogenic response in the CAM assay [Pacini et al., 2002]. In contrast, a significantly enhanced increase in vascularization could be observed in the presence of either single FGF-2 or VEGF or with a combination of both cytokines (~ 20 %, 35 % and 40 %, respectively; p < 0.05 for comparing gels without any growth factors to cytokine-modified scaffolds). Here, similar to the results of [Nico et al., 2001], the administration of single VEGF induced a stronger angiogenic effect than that of single FGF-2 (p < 0.05 for the comparison of gels modified with VEGF to FGF-2-functionalized scaffolds), while an increased vascularization compared to the provision of FGF-2 was also found when both cytokines were applied in combination (p < 0.05 for the comparison of hydrogels treated with FGF-2 + VEGF to FGF-2-modified hydrogels). Although the differences were not statistically significant compared to the delivery of single VEGF (p > 0.05 for comparing these two conditions), the parallel administration of FGF-2 + VEGF showed the best results on blood vessel formation. Thus, it could be concluded that the positive effects observed for the FGF-2 + VEGF combination in vitro are similar to the in vivo situation [Chow et al., 2011].
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Fig. 21 Chicken embryo CAM vascularization in response to differently biomodified hydrogels (with or without 1 or 5 µg/ml of single FGF-2 or VEGF as well as a combination of 1 µg/ml FGF-2 + 1 µg/ml VEGF); adapted from [Zieris et al., 2011]. 21A and B: representative images of the CAM vascularization in relation to differently biomodified gel matrices (A) or photograph of the untreated CAM which served as a control (B) (scale bar 1 mm). 21C: quantification of the relative CAM vascularization in response to differently biomodified hydrogels. Data are presented as mean ± root mean square deviation from n = 5-16. * indicates statistically significant differences (p < 0.05; ANOVA). For statistical comparisons apart from the illustrated significant differences, see text.

Taken together, the **administration of FGF-2 and/or VEGF** by starPEG-heparin hydrogels **induced** a substantial **angiogenic response** within the **CAM system**, while the **combination of both cytokines** tends to **increase vascularization most effectively**.

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4.5 Modulation of FGF-2 or VEGF release from starPEG-heparin hydrogels

Although the starPEG-heparin scaffolds with their intrinsic growth factor binding/release characteristics could be applied to promote pro-angiogenic effects both *in vitro* and *in vivo*, a more far-going modulation of the cytokine release profiles would be desirable to adapt to the requirements of approaches where complex and temporally adjustable release profiles are necessary [Uebersax et al., 2009; Lee et al., 2011].

Therefore, the provision of multiple growth factors by starPEG-heparin hydrogels should be easily adaptable and controllable via external triggers. For this, possible strategies could be the implementation of cleavable peptide sequences to the hydrogel matrices, the use of selectively desulfated heparin as a network building block as well as the modulation of the heparin/growth factor interaction via the addition of competing highly heparin-affine molecules.

In order to perform this study under physiological conditions which are relevant for *in vitro* and *in vivo* applications, all experiments were carried out with SF ECGM + 1 mg/ml BSA as a release medium rather than in an artificial serum-free environment. Since it has been shown that the mechanical properties did not influence the binding and release of the growth factors, as a standardized system, gels with an intermediate starPEG to heparin ratio were prepared for this study and loaded with 1 µg/ml FGF-2 or VEGF. Subsequently, the cytokine binding and release were investigated in dependence on the different strategies that could be beneficial to modulate the delivery of these growth factors (i.e. modification of the standard hydrogel system with cleavable crosslinks or desulfated heparin as a building block as well as the addition of competing heparin-affine molecules). As a final goal, the set of available materials should be extended towards customized systems capable to provide cytokines “on demand”.

4.5.1 Incorporation of cleavable peptide linkers

The enzymatic degradability of a certain biomaterial represents a critical prerequisite for effectively mimicking the dynamic natural environment of cells which could be rearranged, as it is for instance necessary in the process of wound healing or tissue regeneration. However, the incorporation of protease-sensitive building blocks also extends the potential of starPEG-heparin hydrogels to function as a growth factor
delivery matrix, because the cytokine release might be tunable by the scaffold erosion. In this study, enzyme-dependent degradation of the gels was achieved by introduction of the MMP-responsive peptide crosslinking unit (GPQG↓↑IWGQ, in single-letter amino acid code), which is recognized and cleaved by several proteases, such as MMP1, MMP2, MMP3, MMP7, MMP8 and MMP9 [Nagase et al., 1996]. The peptide was conjugated to the arms of the starPEG building block by formation of a hydrolytically stable amide bond between the maleimide-functionalized starPEG and the thiol group of the cysteine within the MMP-cleavable peptide. The gel crosslinking reaction was performed analogously to the non-cleavable starPEG-heparin matrices involving the N-terminus of the PEG-peptide-conjugate and the s-NHS/EDC activated carboxylic acid groups of the heparin.

**Gel degradation**

In non-cleavable starPEG-heparin hydrogels, the release of the growth factors is only based on diffusion. In order to interpret how the network erosion might contribute to the overall growth factor release in the case of enzymatically cleavable scaffolds, the degradation of the hydrogels was analyzed (Fig. 22). For this, the gels were either incubated in pure PBS or in PBS containing 1 U/ml of bacterial collagenase IV. This enzyme has been shown to function as a reliable model of MMP activity [Seliktar et al., 2004]. While the MMP-cleavable gel demonstrated very low degradation in PBS (~ 15% after 96 h), in the presence of the collagenase the main fraction of the network was

![Fig. 22 Decomposition kinetics of the MMP-cleavable hydrogels ± collagenase as monitored by UV-spectroscopy of the released peptide; modified from [Chwalek et al., 2011].](image-url)
decomposed within 24 h of incubation (~85%). After 96 h, the scaffold was cleaved completely. Based on these degradation kinetics, a significant contribution of the enzymatically-triggered hydrogel erosion to the overall growth factor release from MMP-cleavable starPEG-heparin networks can be expected.

**Cytokine binding**

As with the presence of the MMP-cleavable peptide sequence in the starPEG-heparin hydrogels the network composition is changed, it has to be evaluated whether there is an influence of this modification on the binding of FGF-2 or VEGF. By ELISA analysis (Fig. 23), it could be shown that there was no significant difference in the amount of immobilized FGF-2 or VEGF by non-cleavable or MMP-cleavable hydrogels (~195 ng/cm² scaffold area as determined via ELISA), which could be further validated via radiolabeling studies (Fig. B10, appendix B). This finding might be related to the fact that both types of scaffolds contain similar amounts of heparin. Moreover, the sulfate groups as main interaction sites for FGF-2 and VEGF are unaffected by the introduction of the peptide sequence into the network. Consequently, MMP-cleavable starPEG-heparin hydrogels could be functionalized with growth factors similarly to the standard non-degradable matrices.

![Fig. 23 FGF-2 or VEGF uptake experiments in dependence on the degradability of starPEG-heparin hydrogels as quantified via ELISA. Plotted is the amount of electrostatically bound FGF-2 and VEGF per cm² scaffold surface for non-cleavable or MMP-sensitive networks (p > 0.05 for the immobilized amount of growth factors in non-degradable or MMP-cleavable gels; ANOVA). All data are presented as mean ± root mean square deviation from n = 3.](image-url)
Cytokine release

Starting from similar amounts of cytokines immobilized to non-degradable or enzymatically cleavable starPEG-heparin hydrogels, the influence of the network degradability on the release of the growth factors was analyzed. While Fig. 24 (top) shows the kinetics of the absolute release of FGF-2 (left) or VEGF (right), in the bottom part the release efficiency expressed as percentage of initial loading (left) and the relative change in the absolute release of the growth factors by cleavable networks normalized to non-degradable scaffolds (right) are plotted.

Similar growth factor release kinetics (Fig. 24, top; release efficiency depicted in Fig. 24, bottom left) were observed for non-cleavable or enzymatically degradable scaffolds in the absence of collagenase. However, a slightly elevated but not significantly enhanced release ($p > 0.05$; ANOVA) found for the MMP-sensitive hydrogels might be attributed to the higher non-specific degradation of $\sim 15\%$ in the absence of collagenase (see Fig. 22) of the MMP-sensitive scaffolds in PBS (non-cleavable scaffolds: 20.5 ng/cm² or of 10.3 % for FGF-2 and 22.3 ng/cm² or 11.2 % for VEGF were released after 96 h; MMP-degradable gels: 24.9 ng/cm² or 12.9 % for FGF-2 and 30.6 ng/cm² or 16.1 % for VEGF were released after 96 h).

The addition of collagenase led to a stronger increase of both FGF-2 (37.1 ng/cm² or 19.2 %) and VEGF (63.1 ng/cm² or 33.2 %) release from the enzymatically degradable starPEG-heparin hydrogels ($p < 0.05$ for FGF-2 or VEGF release from non-cleavable or enzymatically degradable gels under enzyme-free conditions to that of the MMP-cleavable gels upon addition of collagenase; ANOVA). As a reason, besides the diffusion-based release of the cytokines from the scaffolds, the cleavage of the protease-sensitive peptide crosslinks results in the decomposition of the network and, consequently, in an enhanced delivery of the growth factors into the medium.

However, although a significantly higher amount of both FGF-2 and VEGF were released upon the addition of collagenase to MMP-cleavable scaffolds, not the entire fraction of initially loaded growth factors could be detected by ELISA as expected from the complete degradation of the hydrogels after 96 h. The finding is most probably related to limitations of the ELISA-technique interfering with high heparin levels. In additional experiments, it was shown, that for defined FGF-2- or VEGF-levels in medium containing similar concentration of heparin as found in the hydrogels, only $\sim 20$-$35\%$ of the original cytokine concentration could be quantified by ELISA analysis (data not shown). Consequently, the presence of such high amounts of the glycosaminoglycan could disturb the detection of the growth factors by potentially
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masking their binding sites to the antibody that is used to immobilize FGF-2 or VEGF to the ELISA plates. Furthermore, heparin could also directly interfere with the antibody or simply sterically hinder its efficient interaction with the cytokines. Additionally, during earlier time points, a partial degradation of the networks might also lead to the appearance of some gel microclusters in the medium, in which the cytokines could be entrapped thereby making them inaccessible for detection via ELISA. While all these effects might disturb an efficient quantification of the cytokines, nevertheless FGF-2 and VEGF released by a proteolytic cleavage of the hydrogels could be preferentially available for interaction with cells, as it has been shown that their diffusion is decelerated [Taipal et al., 1997], they are stabilized [Saksela et al., 1988; Lee et al., 2005] and their receptor affinity is even potentiated when they are bound to heparin [Roghani et al., 1994; Gitay-Goren et al., 1992].

Interestingly, besides this possible methodological issue of a potentially restricted detection efficiency via ELISA, also a significantly lower release of FGF-2 from enzymatically degradable hydrogels was determined by comparison to VEGF delivery \( p < 0.05; \text{ANOVA} \). One explanation for this might be that the heparin binding affinity of FGF-2 \( (K_d = 23 \text{ nM}) \) is higher than that of VEGF \( (K_d = 165 \text{ nM}) \) [Ashikari-Hada et al., 2004], while its diameter is even smaller \( (~ 3 \text{ nm for FGF-2 and ~ 6 nm for VEGF}) \) [Eriksson et al., 1991, Muller et al., 1997]. Consequently, a greater fraction of FGF-2 might be strongly attached to the heparin in the release medium or the antibody binding sites of such a small cytokine could be shielded more effectively upon this interaction. Both effects could thereby disturb the detection via ELISA. However, another possible reason for the lower FGF-2 release might be that the protein could get cleaved by collagenase [Whitelock et al., 1996], while VEGF does not represent a substrate for this enzyme [Keyt et al., 1996]. Although the presence of heparin might protect FGF-2 from proteolysis [Coltrini et al., 1993] thereby preventing a complete cleavage of this cytokine, the smaller release rates determined for FGF-2 could nevertheless result from the degradation of a certain protein fraction.

Overall, there was a significant increase in the release of the growth factors from MMP-cleavable hydrogels upon addition of the enzyme compared to non-degradable scaffolds \( (~ 80 \% \text{ increase for FGF-2 or 180 \% increase for VEGF; } p < 0.05 \text{ for the comparison of FGF-2 or VEGF release from non-cleavable or proteolytically degradable gels under enzyme-free conditions to that of the MMP-cleavable gels upon addition of collagenase; ANOVA, Fig. 24, bottom right}) \). As expected, in the absence of collagenase differences in the cytokine release between
enzymatically cleavable and non-degradable gels were not significant (~ 22% increase for FGF or 38% increase for VEGF; p > 0.05 for the comparison of FGF-2 or VEGF release from non-cleavable or degradable gels to that of MMP-sensitive networks without addition of collagenase; ANOVA).

Fig. 24 FGF-2 or VEGF release experiments in dependence on the degradability of starPEG-heparin networks as quantified via ELISA. top: cumulative amount of FGF-2 (left) or VEGF (right) released by non-cleavable hydrogels or MMP-degradable scaffolds ± collagenase. bottom (left): cumulative percentage of initially bound FGF-2 or VEGF released by non-cleavable hydrogels or MMP-degradable scaffolds ± collagenase after 96 h (p < 0.05 for the comparison of FGF-2 or VEGF released by MMP-cleavable gels with addition of collagenase; ANOVA). bottom (right): relative change in FGF-2 or VEGF release form starPEG-heparin networks functionalized with MMP-cleavable peptide sequences with or without addition of collagenase in the medium. Plotted is the percentage increase of the cumulative absolute amount of FGF-2 or VEGF released after 96 h normalized to non-cleavable hydrogels (p < 0.05 for the comparison of FGF-2 or VEGF released by MMP-cleavable gels with addition of collagenase; ANOVA). All data are presented as mean ± root mean square deviation from n = 3. * indicates statistically significant differences (p < 0.05; ANOVA). For statistical comparisons apart from the illustrated significant differences, see text.
Conclusively, compared to the standard non-cleavable matrices, for 
**MMP-sensitive starPEG-heparin hydrogels** similar quantities of 
FGF-2 or VEGF could be **bound** to the network while the 
**delivered amounts significantly increased** as the **scaffold degrades**. 
Thus, an incorporation of cleavable peptide linkers does not 
only allow for the cellular remodeling of the gel matrix but also extends 
the potential of the matrices to function as an **efficient growth factor delivery system**.

### 4.5.2 Selective desulfation of heparin binding sites

As the starPEG-heparin matrices contain large quantities of the cytokine-binding glycosaminoglycan and both FGF-2 and VEGF bind to heparin by electrostatic interactions of their basic lysine and arginine residues with the negatively charged sulfate groups of this molecule, one way to trigger their potential as a delivery matrix for growth factors is the removal of sulfate groups in order to decrease the heparin affinity of the cytokines. Although FGF-2 interacts with short sequences that are rich in N- and 2-O-sulfate [Habuchi et al., 1992; Turnbull et al., 1992; Faham et al., 1996], the presence of heparin 6-O-sulfate groups is required for the activation of bound FGF-2 [Pye et al., 1998]. In contrast, while carboxylic acid groups and 2-O-sulfation also contribute to the overall binding strength of VEGF, this protein particularly interacts with N- and 6-O-sulfate groups of heparin [Ono et al., 1999; Robinson et al., 2006].

Upon desulfation of heparin, not only the overall amount of hydrogel-released cytokine should be raised as it could be already achieved by the incorporation of enzymatically cleavable peptide sequences into the network. Instead, a selective removal of one or more groups involved in the FGF-2 or VEGF binding should also allow for a gradual modulation of the growth factor delivery. Therefore, in order to obtain heparins with a varying degree of sulfation, a N-, 2-O-, 6-O- + N- as well as a complete desulfation treatment was performed, while these modified molecules were used as a building block for the formation of starPEG-heparin hydrogels.

**Desulfation and gel formation**

Tab. 3 summarizes the sulfate composition of the differently treated heparins. A high conversion efficiency was achieved for the N-, 6-O- + N- as well as the complete desulfation as determined via FTIR and PCD analysis. In contrast, the yield of the 2-O-desulfation was rather low. This might be attributed to problems occurring during the
lyophilisation process which led to a partial thawing and re-freezing of this particular sample thereby maybe affecting its integrity. As the 2-O-desulfated heparin represented the starting material for the preparation of completely desulfated heparin, although the exact conversion of each single group cannot be determined, it is very likely that most of the remaining sulfate can be found at the 2-O-position of the completely desulfated glycosaminoglycan. However, except for the 2-O-sulfate, an efficient desulfation could be obtained for all other groups of interest, which offers the possibility to use these heparins for the formation of hydrogels with varying degrees of sulfation.

Tab. B1 (see appendix B) summarizes the mechanical properties of gels that were prepared out of these differently desulfated heparins. For almost all modifications, severe differences resulting in either increased or decreased network stiffness and swelling were determined for the gels composed of desulfated heparin when compared to those formed out of the standard heparin. Here, a different swelling behavior due to a decreased charge density could partially contribute to the observed changes in the storage moduli of these hydrogels, but, more likely, altered properties of the desulfated heparin could also effect the network formation itself. Referring to this, the desulfation of distinct sulfate groups in close proximity to the carboxylic acid moieties relevant for crosslinking could lead to an altered ionization (local pK-shift) thereby affecting the reactivity [Park et al., 1978]. Besides a direct influence on the ionization of the carboxyl groups, conformational changes of the desulfated heparin and therefore altered (spatial) accessibility of the carboxylic acid moieties could be a further reason for the changed overall reactivity. Depending on the particular sugar ring and the specific position relative to the carboxyl groups as well as on the nature of the remaining residue, a certain desulfation could therefore either enhance or weaken a reaction of the heparin carboxylic acid groups with the amino groups of starPEG thereby leading to a higher or to a less efficient crosslinking reaction.

However, as it has been shown that the mechanical properties of the networks had no influence on the growth factor delivery and due to the fact, that the hydrogels contain a huge excess of heparin compared to the amount of immobilized cytokines, no effect of these differences in the crosslinking degree on either FGF-2 or VEGF binding or release are expected. Consequently, any change in the growth factor delivery capacity of modified starPEG-heparin hydrogels might be directly related to the modulation of the sulfation degree and, therefore, of the ‘primary binding sites’ of the two cytokines.
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Tab. 3 Comparison of the sulfate pattern depending on the heparin modification.

<table>
<thead>
<tr>
<th>heparin desulfation</th>
<th>theoretical sulfate content [%]</th>
<th>sulfate content determined via FTIR and PCD analysis [%]</th>
<th>efficiency of conversion [%]</th>
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<td>100</td>
<td>-</td>
</tr>
<tr>
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<td>73</td>
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<td>41</td>
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<tr>
<td>6-O- + N-</td>
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<tr>
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<td>0</td>
<td>18</td>
<td>82</td>
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### Cytokine binding and release

 Due to the lacking stabilization possibility among potentially restricted interaction of soluble cytokines with the desulfated starPEG-heparin hydrogels during the immobilization procedure, the indirect determination of the growth factor binding via detection of remaining protein in the solution via ELISA might be inappropriate for analysis of the FGF-2 or VEGF-binding. Consequently, here, the uptake of the cytokines was determined performing radiolabeling studies. Generally, due to the methodological issues with the lower cytokine binding efficiency detected by radiolabeling experiments (see appendix B, supplementary results and discussion for chapter 4.1), all immobilization data (Fig. 25) were only expressed as the relative percentage of the binding with respect to the standard non-desulfated scaffolds (set to 100 %) which were analyzed under similar conditions.

After it was evaluated whether a selective desulfation of heparin could decrease the affinity of FGF-2 or VEGF to starPEG-heparin hydrogels during immobilization studies, the influence of the variation in the sulfation degree on the release of the cytokines was determined by ELISA. Consequently, Fig. 26 (top) illustrates the kinetics of the absolute release of FGF-2 (left) or VEGF (right). For a comparison of the efficiency, in the bottom part the release from the scaffolds was plotted as percentage of initial loading (left), which was calculated based on the immobilization data shown in Fig. 25. Finally, the relative change in the absolute release of the growth factors by desulfated hydrogels normalized to non-desulfated matrices (right) are plotted.

Concerning the effect of a selective desulfation of heparin on the binding of FGF-2 or VEGF to starPEG-heparin hydrogels (Fig. 25), no significant change could be demonstrated for the interaction of FGF-2 or VEGF with matrices formed out of
2-O-desulfated heparin (91.7 % binding for FGF-2 or 100 % binding for VEGF compared to non-desulfated hydrogels; p > 0.05; ANOVA). Similarly to the uptake experiments, considering the absolute (Fig. 26, top) or relative (Fig. 26, bottom left) amounts of FGF-2 (20.5 ng/cm² or 10.3 %) or VEGF (22.3 ng/cm² or 11.2 %) released by non-desulfated scaffolds after 96 h, for the 2-O-desulfated hydrogels, analogously no substantial increase in cytokine release could be determined (24.1 ng/cm² or 13.1 % for FGF-2 or 22.7 ng/cm² or 11.4 % for VEGF; p > 0.05 for comparing the protein release from non-desulfated networks to that from 2-O-desulfated scaffolds; ANOVA). Since the 2-O-sulfate plays a major role in mediating the interaction of heparin with FGF-2, this finding might be related to the low efficiency of the 2-O-desulfation procedure (see Tab. 3). As the biohybrid hydrogels contain huge quantities of heparin, a sufficiently large fraction of 2-O-sulfate groups might be still present which allows for an efficient binding of the cytokines.

In contrast, a significant reduction of the FGF-2 or VEGF immobilization to N-desulfated heparin-containing matrices was observed (69.4 % binding for FGF-2 and 86.1 % binding for VEGF compared to non-desulfated hydrogels; p < 0.05 for comparing the FGF-2 or VEGF binding to non-desulfated hydrogels to the binding to N-desulfated matrices; ANOVA). Similarly to the trends determined during the growth factor immobilization studies, a moderate increase in the FGF-2 (35.7 ng/cm² or 25.7 %) or VEGF (29.8 ng/cm² or 17.5 %) release was found for the N-desulfation of the hydrogels (p < 0.05 for comparing the cytokine release from N-desulfated heparin-containing matrices to that observed for any other type of scaffold; ANOVA).

The strongest reduction of the FGF-2 or VEGF immobilization could be demonstrated for 6-O- + N- (35.9 % binding for FGF-2 and 62.5 % binding for VEGF compared to non-desulfated hydrogels) as well as for the completely (33.8 % binding for FGF-2 and 68.6 % binding for VEGF compared to non-desulfated hydrogels) desulfated heparin-containing hydrogels (p < 0.05 for comparing the FGF-2 or VEGF binding to non-desulfated hydrogels to the binding to 6-O- + N- or completely desulfated heparin-containing scaffolds; ANOVA). Consequently, with the usage of 6-O- + N- (60.7 ng/cm² or 84.4 % for FGF-2 or 40.9 ng/cm² or 33 % for VEGF) or completely desulfated scaffolds (50.8 ng/cm² or 75.3 % for FGF-2 or 42.2 ng/cm² or 31 % for VEGF), also the quantities of delivered protein could be even further elevated (p < 0.05 for the comparison of the growth factor release from 6-O- + N- or completely desulfated matrices to that observed for any other type of hydrogels; ANOVA).

As this finding was expected for VEGF, which mainly binds to 6-O- and N-sulfate groups of heparin, the further reduction of the FGF-2 affinity to 6-O- + N-desulfated matrices compared to only N-desulfated hydrogels is particularly interesting as the heparin 6-O-sulfate is not described to represent a key interaction site for FGF-2
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[Habuchi et al., 1992; Turnbull et al., 1992; Faham et al., 1996]. However, [Ishai-Michaeli et al., 1992] found that an over-sulfation of heparin groups originally not acting as major FGF-2 binding sites led to a substantial compensation of the lacking main interaction group and therefore to a significant restoration of the protein binding capacity of this particular heparin. In line with this effect, especially in the heparin-rich biohybrid hydrogels other groups than the primary important 2-O sulfate residues could enhance the intrinsic FGF-2 binding capacity. Therefore, the heparin interaction with the cytokines might rather correlate with the overall sulfate content of the scaffolds than with the presence of particular binding sites.

Besides this finding, with regard to Fig. 25 and 26, it is also obvious that no significant differences in the reduction of the FGF-2 or VEGF affinity to 6-O- + N- desulfated compared to the completely desulfated scaffolds were observed (p > 0.05 for comparing the FGF-2 or VEGF binding/release to/from 6-O- + N-desulfated networks with that to/from completely desulfated hydrogels; ANOVA). This might be due to the fact, that because of the low conversion efficiency observed for the 2-O-desulfation, for the completely desulfated heparin probably most of the remaining sulfate can be found at the 2-O-position of the glycosaminoglycan. Consequently, the variation in the overall sulfation pattern of 6-O- + N- desulfated heparin-containing matrices compared to completely desulfated hydrogels might be small, which could explain the similar data obtained for these two gel types. Moreover, the remaining sulfate content of ~ 20 % for scaffolds, which were composed of the heparin that had undergone each of the desulfation procedures for the N-, 2-O- and 6-O-sulfate groups, might be the reason for the fact that still a certain fraction of FGF-2 and VEGF could interact with matrices composed out of such heparin.

Generally, despite the same tendencies were observed for FGF-2 or VEGF binding and release to the starPEG-heparin hydrogels with different degrees of sulfation, for FGF-2, a significantly stronger reduction in the immobilization and a substantially higher increase in the release was determined for N-, 6-O- + N- or completely desulfated scaffolds when compared to the interaction of VEGF with these gel types (p < 0.05 for comparing the FGF-2 binding/release to/from N-, 6-O- + N- or to/from completely desulfated hydrogels to that of VEGF; ANOVA). The reason for this effect might be that, beside the main interaction sites at the N- and 6-O-position, the 2-O-sulfation and even the carboxylic acid groups of heparin contribute to the overall binding strength of VEGF [Robinson et al., 2006], so that more functional groups of heparin mediate its affinity to VEGF than in the case of
FGF-2. Consequently, there might be a stronger compensatory effect for the removal of key binding sites of VEGF. Moreover, the larger diameter of this protein (~ 6 nm) [Muller et al., 1997] compared to that of FGF-2 (~ 3 nm) [Eriksson et al., 1991] could result in a more efficient sterical entrapment within the hydrogel networks thereby supporting an immobilization even in the case of a reduced specific heparin affinity.

After discussing the effect of a selective heparin desulfation on the FGF-2 or VEGF binding and release separately for each desulfated group, for the general release kinetics (Fig. 26., top), it could be determined that, upon desulfation of heparin, no substantial change in the general appearance of the FGF-2 and VEGF release profile was observed. Here, also for the desulfated hydrogels more loosely entrapped protein showing no specific heparin interaction got released during an initial burst. Due to the removal of the growth factor binding sites, this effect is a bit more pronounced in the case of the desulfated matrices as indicated by the initial steep incline of the corresponding curves. After the burst release, a slow delivery of the growth factor fraction that showed a rather strong interaction with the remaining binding sites of heparin within the matrices was determined over the course of 96 h.

Considering these data, the selective desulfation of heparin turned out to be an appropriate tool to modulate the cytokine binding and release from starPEG-heparin hydrogels. Except for the 2-O-desulfated heparin suffering from low turnover rates (~17 % increase for FGF-2 or 2 % for VEGF p > 0.05 for the comparison of FGF-2 or VEGF release from non-desulfated or from 2-O-desulfated hydrogels; ANOVA), significantly higher absolute growth factor quantities (Fig. 26, bottom right) could be gradually delivered by selectively desulfated heparin-containing scaffolds (N-desulfation: ~ 74 % increase for FGF-2 or 34 % increase for VEGF; 6-O- + N-desulfation: ~ 196 % increase for FGF-2 or 84 % for VEGF; complete desulfation: ~148 % for FGF-2 or 90 % increase for VEGF; p < 0.05 for the comparison of FGF-2 or VEGF release from non-desulfated or from N-, 6-O + N- or completely desulfated hydrogels; ANOVA). This effect still holds true, if the overall lower amount of FGF-2 or VEGF that could be immobilized to desulfated starPEG-heparin hydrogels is considered.

As a reason, upon removal of the ‘specific’ growth factor interaction sites on heparin, the equilibrium might be shifted rather from the protein binding towards the release. This weakens the ability of the cytokines to re-bind to heparin so that they might be preferentially available in the surrounding environment, as it is required for many in vitro and in vivo cell applications. Another main advantage of using desulfated heparin
to form customized starPEG heparin matrices is that cell-secreted soluble effectors would remain accessible in the medium rather than that they might get entrapped by the gels upon interaction with heparin. Moreover, as the growth factor release could be gradually modulated by changing the overall sulfate content of the networks, a specific desulfation degree can be chosen in dependence on the particular requirements of a certain application. Consequently, as a large fraction of the immobilized cytokine was released within 96 h, the use of highly desulfated starPEG-heparin hydrogels could be interesting for any application, where a boosted release of growth factors is beneficial towards the therapeutical approach. In contrast, in need of long-term protein delivery profiles, non- or low desulfated heparin-containing scaffolds should be deployed. These matrices efficiently store and protect growth factors via heparin-effector interactions, so that loading-related quantities of FGF-2 or VEGF could be released constantly over the course of several weeks (tested for up to four weeks, data not shown).

Fig. 25 Relative FGF-2 or VEGF binding experiments in dependence on the desulfation of starPEG-heparin hydrogels as quantified via radiolabeling studies. Plotted is the percentage of electrostatically bound FGF-2 or VEGF with respect to the immobilization to scaffolds prepared out of non-desulfated heparin which was set to 100 % (p < 0.05 for the comparison of FGF-2 or VEGF released by N-, 6-O- + N-or completely desulfated networks; ANOVA). All data are presented as mean ± root mean square deviation from n = 3. * indicates statistically significant differences (p < 0.05; ANOVA). For statistical comparisons apart from the illustrated significant differences, see text.
Fig. 26 FGF-2 or VEGF release experiments in dependence on the desulfation of starPEG-heparin networks as quantified via ELISA. top: cumulative amount of FGF-2 (left) or VEGF (right) released by hydrogels prepared out of non-desulfated or selectively desulfated heparin. bottom (left): cumulative percentage of initially bound FGF-2 or VEGF released hydrogels composed out of non-desulfated or selectively desulfated heparin after 96 h (p < 0.05 for the comparison of FGF-2 or VEGF released by N-, 6-O- + N- or completely desulfated networks; ANOVA). bottom (right): relative change in FGF-2 or VEGF release form starPEG-heparin networks prepared out of selectively desulfated heparin. Plotted is the percentaged increase of the cumulative absolute amount of FGF-2 or VEGF released after 96 h normalized to hydrogels composed of non-desulfated heparin (p < 0.05 for the comparison of FGF-2 or VEGF released by N-, 2-O-, 6-O- + N- or completely desulfated networks; ANOVA). All data are presented as mean ± root mean square deviation from n = 3. * indicates statistically significant differences (p < 0.05; ANOVA). For statistical comparisons apart from the illustrated significant differences, see text.

In summary, the interaction of FGF-2 or VEGF with starPEG-heparin hydrogels could be significantly weakened upon desulfation of heparin. Although this resulted in a reduction of the protein binding to the scaffolds, nevertheless higher absolute amounts of both cytokines could be released. As this effect is gradually dependent on the overall sulfate content of the modified hydrogels, the growth factor delivery system can be adapted to applications that require materials with different sets of protein release characteristics.
4.5.3 Application of competing highly heparin-affine polyelectrolytes

Upon variation of the network degradability or the heparin sulfation, it was shown that the overall amount of starPEG-heparin hydrogel-delivered FGF-2 and VEGF could be gradually increased in a defined manner. However, for different therapeutic approaches in tissue engineering, not only the quantities of growth factors provided by a certain biomaterial should be adjustable but also the cytokine release profiles have to be adaptable to the particular requirements [Uebersax et al., 2009; Lee et al., 2011]. As an optimum, a growth factor delivery system should be selectively tuneable, so that the soluble effectors could be provided at different, well-defined time points. Therefore, it was evaluated whether the release kinetics of starPEG-heparin-associated FGF-2 and VEGF could be modulated. In these studies, the temporarily defined addition of highly heparin-affine molecules should allow to competitively displace the growth factors from their binding sites, thereby resulting in a “triggered” cytokine release from the matrices. Conceptually, such modulator molecules can be directly used upon addition to any in vitro setting or upon injection close to the implanted starPEG-heparin scaffold in particular in vivo applications. As a more advanced approach, entrapment within carrier nanoparticles with defined decomposition kinetics could allow for a direct integration into the hydrogel matrix thereby permitting a combined administration. However, before the mode of application for such modulator molecules could be optimized, this work should deliver more general conceptual insights into the possibility to specifically trigger the FGF-2 and VEGF release from starPEG-heparin matrices.

Chitosan as heparin-affine molecule

For realizing the approach of a competitive growth factor displacement after interaction with heparin, the polysaccharide chitosan was chosen as the modulator molecule. Chitosan represents a copolymer of glucosamine and N-acetylglucosamine. It is usually derived upon partial deacetylation of chitin from crustacean shells and may differ in its degree of deacetylation (40-98 %), its viscosity (1 % chitosan in 1 % acetic acid < 2000 mPas) and its molecular weight (up to 2000 kDa) [Illum, 1998]. In order to modulate the growth factor release from starPEG-heparin hydrogels, this molecule fulfils several important requirements. As biocompatibility is an indispensable prerequisite for a latter in vitro or in vivo application, its very safe toxicity profile already resulting in an extensive usage of chitosan as an excipient in the pharmaceutical industry [Illum, 1998] constitutes a great advantage. Moreover, at neutral and acidic
conditions, the amine groups of the deacetylated polysaccharide are protonated. Accordingly, the polymer is positively charged in solution, which represents the basis for a sufficient affinity to the negatively charged heparin. As another requirement, although the molecular weight of the modulator should not restrict its network penetration, the dimensions of the molecule should be at least comparable to those of the immobilized cytokines to allow for an effective spatial displacement of these proteins. While chitosan is commercially available within a large range of molecular weights (from enzymatically derived oligomers with ~2 kDa up to naturally occurring polymers with ~2000 kDa), for this study a polysaccharide of an intermediate size (~10-50 kDa) was chosen. Together with a moderate degree of deacetylation (70%) resulting in a more compact, coiled conformation [Illum, 1998], this molecular weight goes along with a rather low viscosity (~7 mPas) which ensured a good solubility of this chitosan up to a pH of ~6.7 being close to the physiological pH. Consequently, this molecule represents an appropriate candidate to modulate the growth factor release from starPEG-heparin hydrogels.

As the matrices were loaded with FGF-2 or VEGF according to the standard procedure, they displayed the well known immobilization efficiencies for both cytokines (see chapter 4.2). For a modulation of the release, the chitosan was added later upon a medium exchange at different time points. For all these studies, a molar ratio of 0.19 mol/mol of applied chitosan to the heparin content in the gel was used. Considering the larger dimensions of this polysaccharide compared to heparin, this corresponds to an effective molecular weight ratio of 0.53 kDa/kDa. These chitosan quantities were chosen, because in pre-experiments, upon continuous concentration increase in the release medium, they represented the lowest amount of the polysaccharide introduced which could induce a significant enhancement of the FGF-2 or VEGF release from starPEG-heparin hydrogels (data not shown).

Upon an application of chitosan, the release kinetics should be modulated in a way that either a more constant delivery profile – via elongation of the initial burst release by chitosan addition right after this period (3 h, modulation type A) – or a belated, boosted release – via chitosan addition at a later time point (24 h, modulation type B) – could be obtained. Additionally, these two temporally different ways of modulating the kinetics of the FGF-2 and VEGF delivery by starPEG-heparin hydrogels should be compared with a continuous application of the polysaccharide over the whole course of the release experiment (addition already after 0 h, modulation type C).
Cytokine release

As upon a temporally-defined chitosan addition especially the growth factor release profiles should be modulated, for a clear illustration, in this study, the non-cumulative FGF-2 or VEGF release efficiencies are plotted in Fig. 27 (middle) separately for each particular time point. For reasons of transparency, the absolute quantities of FGF-2 or VEGF released under standard conditions in the absence of chitosan are plotted in a separate graph (top). For the standard cumulative illustration of the cytokine delivery, the reader is referred to (Fig. B11, appendix B). Moreover, any relative change in the overall protein amount that is delivered by the scaffolds upon chitosan addition compared to the application of standard release medium is illustrated in Fig. 27 (bottom).

Due to the different way of data presentation, the FGF-2 and VEGF release kinetics will be discussed additionally for the standard release medium without any application of chitosan (Fig. 27, top). Here, the curves for both proteins show a steep incline for the first 3 h clearly attributed to the burst release of the loosely entrapped factors. The slightly higher dimensions of this peak for FGF-2 might result from its smaller diameter thereby initially leading to a more rapid diffusion out of the network (for details see appendix B, supplementary results and discussion for chapter 4.1). This initial period is followed by the release of the cytokine portion which interacts with heparin. Thus, a tremendously decreased release of both growth factors was observed as represented by the distinct decline of the curves within the overall time frame of 6 h. The still higher VEGF release, as indicated by the more moderate slope of the curve compared to the FGF-2 release profile, might be caused by the lower heparin affinity of this cytokine (Kd =165 nM) compared to that of FGF-2 (Kd = 23 nM) [Ashikari-Hada et al., 2004].

After the initial burst release, the amounts of FGF-2 or VEGF delivered by starPEG-heparin hydrogels remain rather constant for the residual time course of the experiment. However, as indicated by the small decline of the FGF-2 release curve, slightly higher quantities of VEGF were released after 96 h in contrast to FGF-2 most probably attributed to the already mentioned differences in heparin affinity. Considering the overall experimental time course of 96 h, the total amounts of both proteins are equalized again (see Fig. B11, appendix B).

Modulation type B: For an application of chitosan-containing medium (Fig. 27, middle) after 24 h, a strong incline of the release curves was observed for FGF-2 (left) and VEGF (right). This increase could be attributed to a certain protein fraction being
actively displaced by competing chitosan molecules. Interestingly, compared to FGF-2, the VEGF release in response to the application of this heparin-affine polyelectrolyte was enhanced more effectively as indicated by the steeper rise of the graph. Most probably, due to its lower heparin affinity (see discussion above), VEGF could be displaced more easily by an interaction of heparin with chitosan than FGF-2. Consequently, under this condition, overall also higher cumulative amounts (Fig. 27, bottom) of VEGF were delivered when compared to FGF-2 (chitosan application after 24 h: 42.3 % increase for FGF-2 or 116.6 % increase for VEGF; p < 0.05; ANOVA).

Modulation type A: Addition of chitosan-containing medium after 3 h resulted in a continuous increase for both FGF-2 and VEGF release (as indicated by the steady rise of the graphs up to 24 h), therefore preventing the transition from the burst to the slower release as observed in the absence of chitosan. Again, the slightly steeper increase—and therefore stronger release—was found for VEGF, encouraging once again the discussion about the differences in heparin affinity as a reason for this behavior.

Nevertheless, results show that with an addition of chitosan-containing medium, both growth factors could be delivered in a more continuous manner within the time frame of 24 h. In the following, although still substantial amounts of both FGF-2 and VEGF were delivered by the hydrogels, the cytokine release slightly drops. A reason for this effect could be that upon addition of chitosan a fraction of protein bound in close proximity to the starPEG-heparin hydrogel surface was released nearly completely. After this portion is gone, the protein molecules that are bound in layers distant from the surface of the matrices have to be liberated and to diffuse through the network to the surface. Due to the high heparin content of the scaffolds, this diffusion process is most probably superimposed by a dynamic re-binding and release of the cytokines to adjacent heparin molecules within the scaffold. Consequently, the delivery of this fraction might be slower and could therefore explain the decrease in the FGF-2 and VEGF release curves. Cumulatively, with a provision of more chitosan at further time points, the release of both proteins could be additionally enhanced (chitosan application after 3 h: 202.4 % increase for FGF-2 or 164.3 % increase for VEGF). As an explanation, with the introduction of higher total quantities of the heparin-affine polyelectrolyte to the system, more cytokine molecules could be displaced from their heparin binding sites in the gel network.
Modulation type C: Upon a continuous application of chitosan over the whole course of the experiment starting at 0 h, the shape of the FGF-2 and VEGF release graphs resemble that ones determined in the complete absence of the polyelectrolyte. In detail, an initial burst effect, a smaller continuous release up to 24 h and a following decrease in the cytokine quantities being delivered by starPEG-heparin hydrogels, were observed. Nevertheless, cumulatively, highly elevated FGF-2 or VEGF quantities could be delivered (continuous chitosan application: 334.4 % increase for FGF-2 or 168.4 % increase for VEGF; \( p < 0.05 \); ANOVA).

Although VEGF could be displaced from its heparin binding sites more easily (see discussion above), the maximum improvement of the growth factor delivery by starPEG-heparin hydrogels was observed for FGF-2 rather than for VEGF. The reason for this finding could be that chitosan was found to effectively protect FGF-2 from inactivation or degradation via heat, proteolysis or acid [Masuoka et al., 2005; results of own stability tests, data not shown] via complex formation between negatively charged sites of the protein and positively charged amino groups of the chitosan. As such effect was not determined for VEGF, upon a continuous presence of the polyelectrolyte in the medium, an increased FGF-2 stability could superimpose the stronger ability of chitosan to displace VEGF.
Results and discussion

Fig. 27 FGF-2 or VEGF release experiments in dependence on the presence of chitosan in the release medium at different time points as quantified via ELISA. Plots show the non-cumulative percentage of electrostatically bound protein released under standard conditions (top) or FGF-2 (middle, left) or VEGF (middle, right) delivered upon the application of chitosan to the medium at different time points. The red symbol indicates the first addition of chitosan (rhomb: application after 0 h; square: application after 3 h; pentagon: application after 24 h). At the bottom, the relative change in FGF-2 or VEGF release form starPEG-heparin networks in dependence on the addition of chitosan in the medium at different time points is depicted. Plotted is the percentaged increase of the cumulative absolute amount of FGF-2 or VEGF released after 96 h normalized to the standard release medium without any chitosan (p < 0.05 for the comparison of FGF-2 or VEGF released upon the continuous application of chitosan or an addition after 24 h; ANOVA). All data are presented as mean ± root mean square deviation from n = 3. * indicates statistically significant differences (p < 0.05; ANOVA).
In conclusion, with this study it was shown that the release kinetics of FGF-2 or VEGF from starPEG-heparin hydrogels could be modulated in direct response to addition of chitosan-containing medium at different time points, while in parallel also the overall delivery effectiveness for both cytokines could be increased.

In detail, upon an early chitosan addition (e.g. after 3 h), prolonged constant release rates of both growth factors were achieved which could be beneficial towards applications where high initial doses are required.

In contrast, a chitosan application at later time points (e.g. after 24 h) created a peak-like upregulation of the cytokine release. Therefore, besides the fact that the overall cumulative growth factor delivery was increased, the commonly occurring flattening of the FGF-2 or VEGF release profiles determined in the absence of chitosan (see plateau range in Fig. 27, top) could be prevented. Thus, a later chitosan application allows for the gentle upregulation of the cytokine delivery over extended time periods and for temporally higher release upon request.

Finally, exploiting the FGF-2 or VEGF release upon continuous chitosan addition could be advantageous for applications where at a given growth factor functionalization of the hydrogel matrices the delivery of both proteins should be maximised while in parallel release profiles similar to the standard curves would be beneficial.
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5.1 The potential of starPEG-heparin hydrogels as a growth factor delivery matrix

Provision of soluble pro-angiogenic effectors is one of the key requirements for biomaterials in tissue engineering. Therefore, the aim of this work was to investigate the potential of starPEG-heparin hydrogels to deliver the angiogenic growth factors FGF-2 and/or VEGF, while heparin was utilized for a reversible, electrostatically controlled conjugation of both proteins.

By using optimized detection methods, the binding and release of the growth factors was analyzed depending on the physico-chemical network characteristics of gels with different crosslinking densities, the applied cytokine concentration and on the presence of cytokine mixtures to be delivered by the material. Fig. 28 summarizes the results of these studies.

The variation of the network properties (Fig. 28, left) did not influence the efficiency of FGF-2 or VEGF delivery. As a reason, by changing the starPEG to heparin ratio, the hydrogels can be modified in their mechanical characteristics while the heparin concentration of scaffolds differing in their density of crosslinks remains constant. Since heparin is the base for any subsequent biomodification, this key property of the hybrid material also allows for similar functionalization with various effectors. This fact and the significant dimensional difference between FGF-2 or VEGF and the pore size of the hydrogel network explains that both proteins could be equally bound and released by hydrogels with different crosslinking density.

Besides this finding, the applied concentration of FGF-2 or VEGF did not affect the growth factor delivery by starPEG-heparin matrices (Fig. 28, middle). Due to the fact that the hydrogels are characterized by a high heparin content and, furthermore, one heparin molecule has the ability to interact with several cytokine molecules, there is an excess of available binding sites within the network capable of immobilizing huge amounts of growth factor. Consequently, different concentrations of FGF-2 or VEGF can be bound and released with the same efficiency, so that the delivered cytokine quantities can be adapted upon a variation of the initial protein amounts applied during the immobilization procedure.

As another advantage, an introduction of FGF-2 and VEGF in parallel showed also no influence on the binding or release efficiency of either protein (Fig. 28, right). Here,
once again both the large excess of heparin as well as the sufficiently large mesh size of the network appears to prevent any interference between the cytokines during their combined delivery by the hydrogels. Thus, with an alteration of the growth factor ratio or concentration used for immobilization, FGF-2 and VEGF can be released in any constellation of quantities.

Taken together, the usage of starPEG-heparin hydrogels as a delivery matrix for the angiogenic cytokines FGF-2 and VEGF allows for the parallel provision of both effectors in any desired concentration independent of the physical network properties. These results open up new prospects for their application in the context of angiogenic tissue engineering, as demonstrated by cell culture experiments using the matrices as a growth factor delivery system.

![Diagram](image)

**Fig. 28** Key characteristics of starPEG-heparin hydrogels allowing for effective growth factor delivery.

However, despite of all these advantages, there are also restrictions of the starPEG-heparin hydrogel system in its function as a cytokine delivery matrix. First, the high content of heparin permits a very effective immobilization of huge growth factor
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quantities, but at the same time it represents also the reason for the limited overall release efficiency which is presumably mainly influenced by a dynamic protein release/re-binding from/to heparin. Besides that, the release of the cytokines is only based on diffusion, so that it always follows the same pre-determined kinetics. Consequently, new strategies were developed and applied to control both the overall amounts of soluble growth factors available upon release into the medium as well as the kinetics of the protein delivery by starPEG-heparin hydrogels. For this, either the network design was changed to modulate the heparin-protein interactions (incorporation of cleavable peptide sequences or of selectively desulfated heparin) or the release of heparin-conjugated growth factor molecules was stimulated via competitive interactions of highly heparin-affine molecules. For each concept, Fig. 29 depicts the main impact on the cytokine release and illustrates exemplarily the potential effect on a specific cell behavior.
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Fig. 29 Modulation of cytokine delivery from starPEG-heparin hydrogel system and its effects on the growth factor release and the cell behavior.
Upon an introduction of cleavable peptide linkers to the starPEG-heparin networks (Fig. 29, left), due to the fact that the heparin composition of the hydrogels was not affected, no change in the FGF-2 or VEGF binding efficiency was observed. However, with an addition of protease, the scaffolds were degraded going along with an increased cytokine release as the network gets fragmented and heparin is released. The advantage of this cleavable hydrogel system is that high quantities of growth factors can be delivered in direct response to the cellular remodeling of the matrix. While the cytokines might be still attached to the released heparin, this could represent an additional benefit, as growth factors that are bound to this glycosaminoglycan were found to have an increased bioactivity [Roghani et al., 1994; Gitay-Goren et al., 1992].

Minimizing the available cytokine binding sites within starPEG-heparin hydrogels utilizing selectively desulfated heparin for gel formation (Fig. 29, middle), the immobilization efficiency of the growth factors was reduced gradually. Here, due to the high heparin content of the scaffolds, potentially non-specific FGF-2 or VEGF binding regions could exert compensatory effects on the removal of the actual interaction sites. Consequently, the reduced cytokine immobilization efficiency rather correlated with the remaining overall sulfate content of the hydrogels. Interestingly, although lower amounts of FGF-2 or VEGF were immobilized when the scaffolds were composed out of desulfated heparin, nevertheless even the absolute quantities of growth factors being released could be gradually enhanced correlating with a decreasing degree of heparin sulfation. Consequently, the quantities of cytokines available for a delivery after immobilization to the desulfated hydrogels can be adapted to the particular requirements of a certain application and a more efficient cytokine administration can be achieved. Moreover, a further advantage of this system is that both the growth factors being released as well as medium/body fluid components and cell-secreted soluble effectors might be preferentially accessible for an interaction with cells in their direct surrounding, because they have a reduced opportunity to (re-)bind to the heparin sulfate groups.

Another possibility to decrease the FGF-2 or VEGF affinity to starPEG-heparin hydrogels is to apply competitive, highly-heparin affine molecules as the polyelectrolyte chitosan (Fig. 29, right). Upon addition with the release medium, this polysaccharide was shown to displace the growth factors from their heparin binding sites thereby resulting in an increased protein delivery. As chitosan could be introduced at different
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time points, also the growth factor release kinetics were found to be tunable, so that distinct delivery profiles could be adapted to a certain application. Therefore, in response to the temporally defined addition of chitosan, the boost in the cytokine delivery might be beneficial to trigger a certain cell behavior just at a desired time point.

Taken together, due to the opportunity to adapt the growth factor binding and release by starPEG-heparin hydrogels to almost any specific requirement being crucial for a certain application, this material represents a unique cytokine delivery system. In particular, the capability to adjust complex, time-resolved multi-factor release profiles from matrices with precisely and independently adaptable physical and biomolecular composition is not only expected to support therapeutic angiogenesis but similarly to promote a wide variety of advanced tissue engineering concepts.

5.2 The potential of biofunctionalized starPEG-heparin hydrogels to support pro-angiogenic effects *in vitro* and *in vivo*

Although starPEG-heparin hydrogels were found to represent effective and tunable growth factor delivery systems, it still had to be demonstrated whether they could be applied in the context of angiogenic tissue engineering. Therefore, scaffolds that differed in their physicochemical characteristics and their degree of biofunctionalization were used to study the behavior of endothelial cells *in vitro*. Based on these experiments, the matrices that were most effective in inducing pro-angiogenic cell responses were selected for further investigation in *in vivo* experiments by introducing them to the CAM of fertilized chicken eggs.

For the pre-selective *in vitro* cell culture experiments, the endothelial cell behavior was compared in terms of adhesion, survival/proliferation, differentiation and migration depending on the characteristics of differently biomodified starPEG-heparin hydrogel networks. As the starPEG-heparin hydrogel system allows for the decoupling of its mechanical properties from the biomolecular functionalization, the influences of the viscoelastic characteristics and the complexity of the biomodification on the endothelial cell response were investigated independently. For this, different types of soft, intermediate and stiff gel matrices were either used without any modification or were functionalized with the RGD adhesion ligand. Moreover, different FGF-2 or VEGF
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quantities as well as a combination out of both cytokines were provided via the hydrogels.

In terms of endothelial cell adhesion, starPEG-heparin hydrogels with a higher stiffness are more beneficial for effective cell attachment. While the presence of VEGF positively affects cell adhesion, the introduction of the adhesion ligand RGD is the most critical material parameter for supporting successful cell attachment.

Based on the indispensable requirement of the RGD peptide for effective initial cell adhesion, the functionalization of starPEG-heparin hydrogels with this ligand represents also an inevitable prerequisite for mediating endothelial cell growth and differentiation. Besides that, matrices offering an intermediate network stiffness with a storage modulus of ~ 7000 Pa provide the optimal mechanical characteristics to balance cellular demands for both survival/proliferation and adaptation of the pre-tubular, elongated morphology. In addition, combining the impact of FGF-2 on cell growth and the influence of VEGF on cell differentiation, the parallel delivery of both growth factors results in increased numbers of endothelial cells adapting a highly elongated shape.

The evaluation of endothelial cell migration in response to gel type- and RGD-independent cytokine release from starPEG-heparin hydrogels revealed that FGF-2 and VEGF similarly promote cell motility, but both growth factors act synergistically to further enhance migratory behavior.

Over all the parameters monitored, gels with an intermediate stiffness functionalized with the adhesion ligand RGD and a combination of the growth factors FGF-2 and VEGF were found to induce the most effective pro-angiogenic endothelial cell behavior and therefore represented promising candidates for studying the effect on vascularization in vivo. Here, in the CAM system of fertilized chicken eggs the provision of single FGF-2 or VEGF by intermediately stiff, RGD-decorated starPEG-heparin hydrogels induced a substantial angiogenic response. Also, a tendency towards further enhanced blood vessel formation was observed in these experiments upon the administration of the cytokine combination. Conclusively, these results demonstrate the suitability of biofunctionalized starPEG-heparin hydrogels as growth factor delivery systems for the support of angiogenesis in vivo.

In summary, starPEG-heparin hydrogels providing controlled release of pro-angiogenic cytokines can be successfully applied in angiogenic tissue engineering. For that, the
possibility to decouple their mechanical properties from the biofunctionalization is advantageous to precisely adjust various different material parameters, with the option to deliver several growth factors in parallel being of particular benefit for therapeutic angiogenesis.

### 5.3 Outlook

While growth factor delivery from starPEG-heparin hydrogels was shown to offer unprecedented opportunities for regenerative therapies, additional studies may further extend their applicability.

To deepen the understanding of starPEG-heparin hydrogels as a growth factor delivery matrix, the distribution of cytokines within the gel matrices due to the dynamic release from and re-binding to heparin within the network should be further investigated. Here, a microscopic analysis of the protein diffusion through macroscopic channels filled with the gel is ongoing. As this particular setting allows the examination of the growth factor distribution at a substantially larger scale (mm instead of µm dimensions), the information obtained would be of general significance for drawing conclusions about the cytokine distribution within bulk gel matrices to be used for an implantation into living systems.

Moreover, besides FGF-2 or VEGF, the possibility to immobilize and release also other angiogenic effectors should be analyzed. In this context, experiments with molecules lacking specific heparin binding sites would additionally increase the knowledge about the mechanisms of protein-network interactions and could help to extend the options for effectively governing therapeutic angiogenesis via the provision of further supportive factors.

While the possibility to tune the FGF-2 or VEGF release from starPEG-heparin hydrogels was already successfully demonstrated, *in vitro* and *in vivo* cell studies concerning the effect of a triggered growth factor delivery have to be performed in order to explore the suitability of a modulated cytokine provision to induce pro-angiogenic cellular effects and to broaden the information about the most beneficial parameters to stimulate therapeutic angiogenesis. Based on these results, also several ways to trigger the FGF-2 and/or VEGF delivery from starPEG-heparin hydrogels could be combined to adjust the system to the particular requirements of angiogenic tissue
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Moreover, using these options to extend the set of precisely tunable material characteristics and based on the fact that also many other cytokines possess heparin-binding sites which allow for an interaction with the gel scaffolds, starPEG-heparin matrices might not only be applicable for angiogenic tissue engineering but could represent a promising candidate to support a number of different therapeutic strategies. Here, one potential application might be the usage of the gel system in the treatment of neurodegenerative disorders such as Parkinson's disease, which is characterized by a continuous and selective loss of dopaminergic neurons in the brain region substantia nigra with a subsequent reduction of neurotransmitter release mainly in the striatum [Meyer et al., 2010]. In this context, the \textit{in vitro} expansion and differentiation of neural stem cells into dopaminergic neurons as a target cell type for direct transplantation into the brain could be supported by starPEG-heparin matrices with optimized mechanical properties, adhesive characteristics and functionalization with relevant cytokines such as FGF-2 and glial-derived neurotrophic factor (GDNF). As a more advanced approach, by bridging the gap between the substantia nigra and the striatum with biomodified hydrogels directly in the brain, a cytokine release from the matrices could also promote the proliferation and oriented outgrowth of dopaminergic extensions/axons from the substantia nigra towards the striatum [Meyer et al., 2010].

Other examples being currently explored include the application of starPEG-heparin hydrogel scaffolds as a growth factor delivery system to stimulate progenitor cells in kidney or cardiac regeneration.

Besides that, the growth factor delivery should be similarly analyzed for starPEG-heparin matrices produced by different gel formation schemes. Here, by applying conditions, which permit the introduction of micro-dimensional pores into the matrices upon cryogelation, interconnecting structures should facilitate a scaffold penetration by diffusional processes, thereby leading to an enhanced and accelerated provision of different factors via starPEG-heparin hydrogels. In addition, also increased network accessibility for molecular components or even for whole cells could be enabled by such porous matrices. Furthermore, using different crosslinking mechanisms which allow for gelation under physiological conditions, the starPEG-heparin hydrogels should be producible \textit{in situ} for the entrapment of both growth factors and cells. In the context of \textit{in vitro} and \textit{in vivo} studies, with the application of the porous scaffolds or the \textit{in situ} forming hydrogels, the ECM as the natural, three-dimensional cellular environment
could be mimicked even more closely. Moreover, apart from the provision of molecular effectors, the possibility to deliver whole cells via starPEG-heparin hydrogels might open up new perspectives for the usage of this material in versatile approaches for regenerative therapies.

Finally, besides an optimization of the crosslinking mechanism for preparing starPEG-heparin hydrogels, further options to precisely trigger the delivery of different factors under physiological conditions could be established. For example, photo-degradable matrices that can be cleaved upon the illumination by light within the harmless infrared range should be developed. In potential therapeutic concepts, such scaffolds could release immobilized effectors in response to this external trigger that can be directly applied to the body just within the desired local and temporal context.

Conclusively, together with the presented data and the already established starPEG-heparin hydrogel as an efficient growth factor delivery system, these further experiments and additional optimization procedures could lead to an increased general knowledge about the provision of therapeutic effectors via polymeric biomaterials and pave the way for the widespread medical application of such modular and thoroughly tunable materials to rekindle regeneration processes in diseased or injured tissues and organs of the mammalian organism.
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Appendix A – Supplementary materials and methods

Synthesis of Alexa conjugates
0.3 mg EDC (3.5-fold molar excess) and 0.17 mg s-NHS (2-fold molar excess) were added to a solution of 5.4 mg heparin (3.85 x 10^{-4} mmol) in 0.1 M borate buffer (pH 8). The reaction mixture was kept for 15 min at 5 °C. Next, 0.25 mg Alexa 488 (3.9 x 10^{-4} mmol) in 0.1 M borate buffer (pH 8) was added. The total volume of the reaction mixture was 50 µl. The reaction was run overnight at room temperature.

The Alexa 488 product was purified by dialysis (membrane with 1 kDa molecular weight cut, Spectra/Por, Spectrum Laboratories, Rancho Dominguez, USA) against 800 ml of 1 M sodium chloride (Sigma-Aldrich) in order to remove any unreacted Alexa dye, followed by dialysis against 800 ml of water three times. As a quality control, high performance size exclusion chromatography (HPSEC) was performed using a combination of a BioSep 2000 (Phenomenex, Torrance, USA) and a PolySep 3000 (Phenomenex) column with 0.1 M NH₄OAc (Sigma-Aldrich) as a mobile phase (runtime 1 h at a rate of 0.5 ml/min). At an absorption of 220 nm, HPSEC chromatogram of the purified product showed only one main peak at 17 min which is characteristic for heparin. The products displayed a strong absorption at 488 and emission at 530 which clearly indicated the presence of Alexa 488 within its structure.

Synthesis of enzymatically cleavable starPEG conjugates
The MMP cleavable sequence GPQG↑↓IWGQ was included as a bioactive module into the peptide NH₂-GGPQGIWGQGCG-CONH₂ (in single-letter amino acid code) which was synthesized using the solid-phase approach on an Activo P11 (Activotec, Cambridge, UK) peptide synthesizer by standard fluorenylmethyloxycarbonyl (Fmoc) chemistry. The 0.25 mmol scale protocol with a C-terminal capping protection strategy by amide was used. Activation was achieved by O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (Sigma-Aldrich), and 1-hydroxybenzotriazole (Sigma-Aldrich) in dimethylformamide (Sigma-Aldrich). Deprotection of the amino acid side chains and cleavage from the resin was performed by reaction with a mixture of trifluoroacetic acid (85 % [v/v]; Sigma-Aldrich), phenol (5% [v/v]; Sigma-Aldrich), dithiothreitol (2.5 % [v/v]; Sigma-Aldrich), triisopropylsilane (2.5 % [v/v]; Sigma-Aldrich) and water (5 % [v/v]) for 2.5 h at room temperature. The crude peptide was then precipitated in cold anhydrous diethyl ether (Sigma-Aldrich), collected by vacuum filtration and dried under vacuum. Final purification was achieved by preparative
reversed-phase HPLC using a XBridge Prep C18 column (10 µm particle size, 19 × 250 mm, Waters GmbH, Eschborn, Germany). A linear gradient of water/acetonitrile (Sigma-Aldrich) containing 0.1 % [v/v] trifluoroacetic acid was used as the mobile phase. The HPLC runs were performed over 30 min using a flow rate of 10 ml/min for the preparative columns. For HPLC separations, the monitoring wavelengths were set to a wavelength range of 210-278 nm. A two-pump system (Agilent Technologies 1200 Series) equipped with an UV/Vis diode array detector/spectrophotometer having a 1-cm path length cell was used. The collected peptides were lyophilized (LYOVAC GT2, GEA Lyophil GmbH, Hürth, Germany) and the purity of the collected peptide was verified analogously by analytical HPLC using a XBridge BEH300 C18 column (5 µm particle size, 2.1 × 250 mm, Waters GmbH).

For the preparation of maleimide terminated starPEG, 0.1 g (1 x 10^{-5} mol) of amino terminated starPEG was dissolved in 0.5 ml of CH₂Cl₂, (Sigma-Aldrich). Next, 112 mg (4.2 x 10^{-5} mol) of 3-Maleimidopropionic acid N-hydroxysuccinimide ester (Sigma-Aldrich) was added. The reaction was stirred overnight under N₂, following double precipitation from 150 ml of diethyl ether. The collected white precipitate was dried under vacuum overnight and kept under -20 °C, while the success of the functionalization was verified using 1H nuclear magnetic resonance (NMR; resolution of 500 Mz, Bruker BioSpin, Rheinstetten, Germany) spectroscopy with deuterated chloroform as a solvent. Via NMR, the conversion rate of PEG terminal amino groups could be calculated from the ratio between the signals of maleimide group residue and PEG core (100 % corresponds to the ratio 1:2; for detailed parameters of NMR analysis see [Chwalek et al., 2011]).

The maleimide-functionalized starPEG was converted into the starPEG-MMP conjugates by simple mixing of the stoichiometrical amounts of the peptide and functionalized starPEG (10 % [w/v]) in phosphate buffer pH 7. A small amount of tris(2-carboxyethyl)phosphine chloride (Sigma-Aldrich) was added to the reaction mixture in order to prevent oxidation of the cysteine residue. The reaction mixture was stirred overnight and purified by dialysis (molecular weight cut of 2 kDa, Spectra/Por, Spectrum) against water. White fluffy remnants were collected after lyophilizing the dialyzed solution. The purity of the formed starPEG-MMP conjugates was evaluated by single peak analysis in analytical reverse phase HPLC as it was explained in detail by [Tsurkan et al., 2010].
Desulfation of heparin

Preparation of pyridinium salt of heparin [Inoue et al., 1976; Ayotte et al., 1986; Baumann et al., 1996]: 35 g of Amberlite IR-120 H+ ion exchange resin (Rohm and Haas, Philadelphia, USA) were filled in a glass column and washed with deionized water, regenerated for 30 minutes with 10 % aqueous HCl, and washed with water until neutral pH. A solution of 1 g heparin (sodium salt) in 45 ml deionized water was applied. The combined filtrate and washings were adjusted to pH 6.0 with pyridine (Sigma-Aldrich). After solvent removal by evaporation, the product was lyophilized (1240 mg). Subsequently, the pyridinium salt of heparin served as the starting compound for the N- and 6-O-desulfation procedure.

N-desulfated, N-acetylated heparin [Inoue et al., 1976; Ishihara et al., 1997]: 25 ml DMSO containing 5 % water were added to the pyridinium salt of heparin and the mixture was stirred for 1.5 h at 50 °C. After dilution with 25 ml water, the pH was adjusted to pH 9.5 with 1 N aqueous NaOH (Sigma-Aldrich). The mixture was dialyzed (molecular weight cut of 8 kDa, Spectra/Por, Spectrum) against deionized water for 3 d. 20 ml 10 % methanol (Acros Organics) containing 50 mM Na2CO3 (Sigma-Aldrich) were added to the reaction mixture. While stirring, 200 µl acetic anhydride (Sigma-Aldrich) were added at 0°C. The pH was adjusted to 7-8 with saturated Na2CO3 solution. Every 30 min, 200 µl acetic anhydride were added for 3 h. The reaction mixture was dialyzed (molecular weight cut of 8 kDa) against deionized water for 3 d, concentrated and lyophilized.

2-O-desulfation procedure [Ishihara et al., 1997]: 500 mg heparin (sodium salt) were dissolved in 10 ml 0.4 M aqueous NaOH. The solution was frozen and lyophilized over night. Dissolving and lyophilizing was done twice. The pH was adjusted to 9.0 with 20 % acetic acid. After dialysis (molecular weight cut of 8 kDa) against deionized water for 3 d, the reaction mixture was concentrated and lyophilized.

6-O-desulfation procedure [Baumann et al., 1996]: 420 mg of the pyridinium salt of heparin were dissolved in 50 ml N-methyl-2-pyrrolidone (Sigma-Aldrich) containing 10 % water. The reaction was carried out for 24 h at 90 °C. After cooling to room temperature, 50 ml deionized water were added and the pH was adjusted to 9.0 by...
addition of 1 M aqueous NaOH. The reaction mixture was dialyzed (molecular weight cut of 8 kDa) against deionized water for 3 d, concentrated and lyophilized.

**Degradation studies of enzymatically cleavable hydrogels**
5 µl gel droplets were used for degradation experiments. After gelation overnight, the hydrogel drops were washed 3 times with PBS and swollen in PBS overnight. To determine the kinetics of degradation, gel drops were placed in plastic UV cuvettes (PlastiBrand, Brand GmbH + Co KG, Wertheim, Germany) with 2 ml of PBS ± 1 U/ml collagenase. The UV absorption was determined at 278 nm using a spectrophotometer (DU800, Beckman Coulter, Brea, USA) and was recorded for all samples every 15 min for a time period of 2500 min in total.

**Characterization of the sulfate content after heparin desulfation procedures**

*Polyelectrolyte titration [Müller et al., 2005]:* The anionic charge of the different heparin samples (non-desulfated, N-, 2-O-, 6-O- + N- and completely desulfated heparin; 1 mg/ml in MilliQ, pH adjusted to 10) was determined by the Particle Charge Detector (PCD, Mütek GmbH, Herrsching, Germany) via titration with PDADMAC (poly(diallyldimethylammonium chloride), respectively. Based on the exact charge compensation of the polyelectrolyte heparin by dropwise added PDADMAC solution (as indicated by streaming potential mesurements), the titration of the heparin samples reveals a quantitative and reproducible estimation of its charges. To determine the heparin sulfate content, all the data were corrected for the charge contribution of the carboxylic acid group by subtracting one quarter of the overall charge of the non-desulfated heparin from all measured values (considering only the major sequence of heparin, see Fig. 9). Finally, the decrease of the anionic charge for the different desulfated heparins indicated the sulfate removal so that the remaining sulfate content could be expressed with respect to the non-desulfated heparin, which served as the control sample (set to a sulfate content of 100 %).
**Infrared (IR) spectroscopy [Müller et al., 2005]:** IR measurements on the different heparin samples (non-desulfated, N-, 2-O-, 6-O- + N- and completely desulfated heparin; 1 mg/ml in MilliQ) were performed on an IFS 55 (EQUINOX, Bruker-Optics GmbH, Ettlingen, Germany) Fourier transform IR (FTIR) spectrometer in the attenuated total reflection (ATR) mode. The ATR-FTIR spectra were recorded on a special mirror setup using the “single-beam sample reference” concept. For that, the particular heparin solution was spread on the upper half (sample) of the silicon internal reflection element (50 x 20 x 2 mm³) and the lower uncoated half was used as the reference. Shuttling the two halves repeatedly in the IR beam, recording the respective intensity spectra \( I_{\text{Reference}}(\nu) \) and \( I_{\text{Sample}}(\nu) \), and computing \( A(\nu) = -\log(I_{\text{Sample}}(\nu)/I_{\text{Reference}}(\nu)) \) resulted in well-compensated ATR-FTIR absorbance spectra \( A(\nu) \). For determination of the heparin sulfate content, the intensity peaks of the sulfate and the carboxylic acid group were recorded and their ratio was calculated. The decrease of this ratio for the different desulfated heparins indicated the sulfate removal, so that the remaining sulfate content could be determined with respect to the non-desulfated heparin, which served as the control sample (set to a sulfate content of 100%).
Appendix B – Supplementary results and discussion

Supplementary results and discussion for chapter 4.1:  
“Method establishment for analyzing the interaction of growth factors with starPEG-heparin hydrogels”

Growth factor binding studies
For a first qualitative analysis of the protein uptake and distribution within the starPEG-heparin hydrogels, representative gel samples were analyzed using CLSM and fluorescently labeled FGF-2 or VEGF (Fig. B1A and B). Generally, it could be demonstrated that both proteins were able to diffuse into the networks. A homogeneous fluorescence intensity of TAMRA-FGF-2 (Fig. B1A, top and B1B, left) within the hydrogel could be observed immediately (~ 1 min) after applying the protein. In contrast, for TAMRA-VEGF (Fig. B1A, bottom and B1B, right) a complete penetration could be observed only after 30 min (~ 65 % for 0.02 h and 80 % for 0.5 h; p < 0.05 when comparing the different time points). This fact could be explained by the larger diameter of VEGF (~ 6 nm; 38.2 kDa) [Muller et al., 1997] compared to that of FGF-2 (~ 3 nm; 17.2 kDa) [Eriksson et al., 1991], which might result in a slower diffusion of this cytokine through the gel pores. After initial penetration, neither protein showed an increase in the relative fluorescence intensity inside the gel networks (after 24 h ~ 60 % for FGF-2 and ~ 90 % for VEGF) and no corresponding decrease in the supernatants (after 24 h ~ 40 % for FGF-2 and ~ 10 % for VEGF) over the course of the experiment. The lower fluorescence intensity of TAMRA-FGF-2 in the hydrogel might result from an increased tendency of this protein to attach to non-specific surfaces not originating from the starPEG-heparin networks or from a decreased heparin binding affinity due to interferences of the attached label with the particular FGF-2 molecular structure as it will be discussed later.

After penetration, both proteins showed a homogenous distribution throughout the entire scaffold (Fig. B1A). These findings demonstrate that there were no significant structural heterogeneities in the network and that the mesh sizes of the hydrogel did not prevent penetration of the rather small FGF-2 and VEGF molecules. In contrast to that, proteins with dimensions larger than the pore sizes of the gels could be excluded efficiently as shown by [Freudenberg et al., 2009]. This offers the advantage that,
besides the stabilizing effect that heparin exerts on FGF-2 and VEGF and the low tendency of starPEG to allow for unspecific protein adsorption, the penetration of some proteases known to degrade FGF-2 or VEGF such as neprilysin (~ 86 kDa, degradation of FGF-2) [Goodman et al., 2006], MMP3 (~ 54 kDa, degradation of VEGF) [Lee et al., 2005] or human plasmin 1 (~ 91 kDa, degradation of FGF-2 and VEGF) [Saksela et al., 1988; Keyt et al., 1996] is prevented by the particular gel structure. In summary, these findings show that the matrices could act as supportive carriers maintaining the biological activity of the bound cytokines. Moreover, CLSM seems to be an efficient qualitative method to follow uptake of FGF-2 or VEGF in situ.

Fig. B1 Qualitative FGF-2 or VEGF uptake experiments performed by CLSM studies. B1A: representative fluorescence microscopy images of FGF-2 (top) or VEGF (bottom) uptake into starPEG-heparin hydrogels; adapted from [Zieris et al., 2010a]. Alexa 488-labeled gel scaffolds (green) were incubated with TAMRA-labeled FGF-2 or VEGF (red). White dashed lines indicate the upper and lower boundary of the gel network (bar: 10 µm). X-Z-confocal laser scanning of the gel networks was performed at different time points after immobilization. B1B: average fluorescence intensity of TAMRA-labeled FGF-2 (left) or VEGF (right) in the gel and in the corresponding supernatant at different time point. Measurements were performed using confocal laser scanning microscopy. All data are presented as average over three X-lines from at least two different gel samples ± root mean square deviation. * indicates statistically significant differences (p < 0.05; ANOVA).
The quantitative analysis of FGF-2 or VEGF binding to starPEG-heparin scaffolds was performed utilizing four different methods (for experimental parameters see Tab. 2, chapter 4.1). Due to the detection limits of these approaches, protein concentrations used for loading had to be varied. However, in order to compare the different methods used here directly, the starPEG-heparin gels have to have the capacity to take up all the FGF-2 or VEGF that is applied during these experimental approaches, which was proven by radiolabeling studies (\(^{125}\text{I}\)-studies, respectively), amino acid analysis via HPLC and ELISA (see chapter 4.2.2) within a protein concentration regime between 0.5 to 50 µg/ml. Consequently, within the range of growth factor concentration applied in this study, the data generated by the four different methods used to quantify FGF-2 or VEGF binding to starPEG-heparin hydrogels can be compared directly.

After ensuring that the growth factor binding capacity of starPEG-heparin hydrogels does not limit the methodological evaluation, the FGF-2 or VEGF immobilization to the matrices was compared among detection via radiolabeling studies, CLSM, amino acid analysis via HPLC and ELISA. As shown in Fig. B2, for analysis of FGF-2, except for the comparison of CLSM experiments and amino acid analysis via HPLC (\(p > 0.05\)), there were significant differences between the results obtained with the different approaches (\(p < 0.05\)). While ELISA data showed almost no protein remaining in the...
supernatant (immobilization efficiency of ~ 99 %), only about 38 % of the deployed FGF-2 was detected in the scaffold by radiolabeling studies. Immobilization efficiencies obtained by fluorescence labeling and amino acid analysis were ~ 61 % and 68 %, respectively. In contrast, when considering results determined for VEGF immobilization, significant differences between the data generated by all the different methods (p < 0.05) were found except for comparing CLSM studies and ELISA (p > 0.05). Here, immobilization efficiencies were ~ 91 % (CLSM), 46 % (radiolabeling studies), 60 % (amino acid analysis via HPLC) and 99 % (ELISA), respectively.

As an explanation, due to the different principles of protein quantification in the gel body or the supernatant, all of these four approaches require specific experimental conditions. To detect FGF-2 or VEGF by radiolabel and fluorescence sensitive methods, the protein has to be converted before the immobilization process. The disadvantage of this approach is that the growth factor has to undergo a labeling procedure which may alter its characteristics. Although a certain fraction of the protein might be already unable to bind to heparin before the labeling procedure (e.g. due to structural changes during bacterial synthesis [Linemeyer et al., 1987]), all of these treatments, as well as the presence of the label itself increase the probability of structural alterations of the growth factor [Bos et al., 1999]. This might of course affect the interaction with heparin during the immobilization procedure. Moreover, weakly attached label may become released from the protein [Bos et al., 1999] during uptake and release studies and thus influence the results of the analytical experiments. Given these drawbacks of methods that require a labeled protein, one could explain the low FGF-2 immobilization efficiency observed in both radioisotope and fluorescence based detection. However, as the VEGF binding efficiency was slightly increased for radiolabeling studies and significantly higher for analysis by CLSM (p < 0.05 for comparing the immobilization efficiency for FGF²2 and VEGF), this protein seems to be less affected by any labeling procedure or the modification itself. Consequently, depending on the particular characteristics of the protein of interest, the high sensitivity and the possibility to detect the labeled molecule in presence of multicomponent biofluids make these approaches nevertheless attractive.

A second issue that becomes important during binding and release studies with both labeled and native protein is the problem of non-specific adsorption to surfaces not originating from the actual material that is being analyzed [Edelman et al., 1991]. Any relative quantification of FGF-2 or VEGF either in the gel body or in the supernatant
would be negatively affected by a certain protein fraction that is simply inaccessible to detection. In order to decrease the contact of FGF-2 or VEGF with such areas significantly, custom-made incubation chambers were used in the present study for performing radiolabeling experiments and ELISA. With this arrangement, the protein solution is almost exclusively applied to the material of interest and additionally contacts a small rubber ring only that separates the walls of the chamber and prevents leaking of the solution. Consequently, any interaction with the bottom of the glass cover slip used to prepare surface-bound hydrogels or with the plastic walls of the incubation system is avoided.

Unfortunately, these chambers cannot be used for fluorescence studies as the dimensions of the chamber do not allow for any usage within a microscopic setup. For amino acid analysis, which offers the possibility to quantify high concentrations of non-labeled protein, the problem arises that in the immobilization chambers a defined volume of the gel body outside of the rubber ring cannot be exposed to the FGF-2 or VEGF solution. As after hydrolysis the analyzed HPLC peaks originate from the whole sample surface (loaded and non-loaded regions of the scaffold), signals coming from unloaded gel disturb the quantification of the protein. Due to these restrictions, in the case of CLSM- and HPLC-based detection, alternative setups had to be used for FGF-2 or VEGF immobilization. Within these configurations, the protein could stick to large areas originating from the bottom of the glass cover slip used to prepare surface-bound hydrogels or to the plastic walls of the incubation system. Such unspecific protein adsorption on ‘foreign’ materials could therefore particularly account for the low FGF-2 immobilization efficiency determined by detection of fluorescently labeled protein and amino acid analysis after hydrolysis as it was already identified as the major source of physical FGF-2 loss in the studies of [Edelman et al., 1991]. However, since CLSM experiments performed under similar conditions, but not the amino acid quantification via HPLC, delivered high VEGF immobilization rates, loss of protein due to unspecific adsorption might not be the only effect influencing the determined binding efficiency. As one indication, the high standard deviation obtained by amino acid analysis by HPLC might point to an error that could be rather associated with the method used for quantification than with the experimental setup during protein immobilization. Although the analysis of bound growth factor was based on corresponding FGF-2 and VEGF standards similarly subjected to hydrolysis, both proteins could behave differently during this treatment in the presence of the gel components. This could result in
incomplete cleavage and, therefore, determination of immobilized protein amounts that were too low. Moreover, the presence of peaks originating from hydrolysis of heparin or starPEG which partially overlaid the signals coming from the amino acids might have also led to inaccuracies in growth factor quantification.

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**Growth factor release studies**

After analyzing the FGF-2 or VEGF uptake into starPEG-heparin hydrogels, experiments on the release of the proteins were performed. For these studies, CLSM and amino acid analysis could not be used since these methods were not sensitive enough to detect the small protein quantities in the pico- to nanogram range that were sequestered by the gels. For CLSM, the low decrease in the fluorescence intensity during release of TAMRA-FGF-2 or -VEGF from the gel body was hard to quantify precisely due to interfering processes like photobleaching or -degradation. In the case of amino acid analysis via HPLC, depending on the particular molecular composition, the method is not sufficient for the quantification of protein amounts below 0.8-0.2 µg [Salchert et al., 2003]. Consequently, it could not be applied for analysis of FGF-2 or VEGF release within the scope of concentrations used for immobilization to starPEG-heparin hydrogels in this study and results were only obtained from $^{125}$I- studies and ELISA.

![Fig. B3 Cumulative amount of hydrogel-released FGF-2 or VEGF as determined by analysis via radiolabeling studies or ELISA. Plotted are the cumulative amounts of FGF-2 (left) or VEGF (right) released per cm$^2$ scaffold area as quantified by radiolabeling studies or ELISA (p < 0.05 for the determination of the protein release by the different methods; ANOVA). All data are presented as mean ± root mean square deviation from n = 2-4.](image-url)
Fig. B3 illustrates the cumulative release of FGF-2 or VEGF measured within a time period of 96 h for gel matrices loaded with a protein concentration of 1 µg/ml. Similar qualitative conclusions concerning the release kinetics of both proteins could be drawn from results obtained by both methods. However, once again there were significant differences in the quantities of FGF-2 or VEGF delivery determined by the two different approaches (26.9 or 31.5 ng as determined by radiolabeling studies for FGF-2 or VEGF; 1.4 or 0.82 ng as determined by ELISA for FGF-2 or VEGF; p < 0.05 for the comparison of the two methods). Although in any case experiments were performed under conditions minimizing the contact area for non-specific protein interactions with glass or plastic surfaces, higher amounts of released FGF-2 or VEGF were detected via radiolabeling studies. One explanation for that could be that the presence of the label might weaken the interaction of FGF-2 or VEGF with heparin, thereby leading to a faster release compared to the native protein which was used for ELISA studies.

Supplementary results for chapter 4.2.1:

“Uptake and release of FGF-2 or VEGF depending on the physico-chemical network properties” (for discussion see particular chapter)

Fig. B4 Amount of hydrogel-immobilized FGF-2 or VEGF determined gel types as quantified via radiolabeling studies or amino acid analysis via HPLC; adapted from [Zieris et al., 2010b]. Plotted is the amount of electrostatically bound FGF-2 or VEGF per cm² scaffold area for the different gel types $\gamma = 1.5; 3$ or $6$ (low, intermediate and high crosslinking degree, $p > 0.05$; ANOVA) determined by radiolabeling studies (left) or amino acid analysis via HPLC (right). All data are presented as mean ± root mean square deviation from $n = 2$ (HPLC) or $n = 2-4$ (radiolabeling studies).
Fig. B5 Cumulative amount of hydrogel-released FGF-2 or VEGF determined for different gel types as quantified via radiolabeling studies. Plotted is the cumulative amount of electrostatically bound FGF-2 (left) or VEGF (right) released by the different gel matrices $\gamma = 1.5$; 3 or 6 (low, intermediate and high crosslinking degree, $p > 0.05$; ANOVA). All data are presented as mean ± root mean square deviation from $n = 2$.

Supplementary results for chapter 4.2.2:

“Uptake and release of FGF-2 and/or VEGF depending on the protein concentration” (for discussion see particular chapter)

Fig. B6 Amount of hydrogel-immobilized FGF-2 or VEGF determined for different protein concentrations as quantified via radiolabeling studies or amino acid analysis via HPLC; adapted from [Zieris et al., 2010b]. Plotted is the uptake of FGF-2 or VEGF in dependence on the protein concentration in the immobilization medium determined by radiolabeling studies (left) or amino acid analysis via HPLC (right); linear regression, $R^2$ (FGF-2; radiolabeling studies) = 0.99973; $R^2$ (VEGF; radiolabeling studies) = 0.99941; $R^2$ (FGF-2; HPLC) = not definable (immobilization with only 10 µg/ml FGF-2 could not be resolved by HPLC); $R^2$ (VEGF; HPLC) = 0.99991. All data are presented as mean ± root mean square deviation from $n = 2$ (HPLC) or $n = 2-4$ (radiolabeling studies).
Fig. B7 Amount of hydrogel-immobilized FGF-2 and/or VEGF as quantified via radiolabeling studies; adapted from [Zieris et al., 2011]. Plotted is the amount of electrostatically bound FGF-2 and/or VEGF per cm² scaffold area (p > 0.05 for the immobilized amount of single FGF-2 or VEGF and the corresponding concentration used in the combination; ANOVA). All data are presented as mean ± root mean square deviation from n = 2-4.

Fig. B8 Cumulative amount of hydrogel-released FGF-2 or VEGF determined for different protein concentrations types as quantified via radiolabeling studies. Plotted is the cumulative release of FGF-2 (left) or VEGF (right) in dependence on the protein concentration used for immobilization. All data are presented as mean ± root mean square deviation from n = 2.
Supplementary results for chapter 4.3.2:  
“Influence of the FGF-2 and/or VEGF delivery” (for discussion see particular chapter)

Fig. B9 FGF-2 and VEGF uptake and release experiments in dependence on the RGD functionalization of starPEG-heparin hydrogels as quantified via ELISA; adapted from [Zieris et al., 2011]. top: amount of electrostatically bound FGF-2 and VEGF per cm² scaffold surface (p > 0.05 for the immobilized amount of growth factors in pure or RGD-functionalized gels; ANOVA). bottom: cumulative percentage of electrostatically bound FGF-2 (left) or VEGF (right) released into SF ECGM + 1 mg/ml BSA by gels which were loaded with a combination of 1 µg/ml FGF-2 + 1 µg/ml VEGF (p > 0.05 for the released amount of growth factors by pure or RGD-functionalized gels; ANOVA). All data are presented as mean ± root mean square deviation from \( n = 3 \).
Supplementary results for chapter 4.5.1:
“Incorporation of cleavable peptide linkers” (for discussion see particular chapter)

![Graph showing FGF-2 or VEGF uptake experiments in dependence on the functionalization of starPEG-heparin hydrogels with MMP-cleavable peptide sequences as quantified via radiolabeling studies. Plotted is the amount of electrostatically bound FGF-2 and VEGF per cm² scaffold surface (p > 0.05 for the immobilized amount of growth factors in non-cleavable or MMP-cleavable gels; ANOVA). All data are presented as mean ± root mean square deviation from n = 2.]

Supplementary results for chapter 4.5.2:
“Selective desulfation of heparin binding sites” (for discussion see particular chapter)

Tab. B1 Physico-chemical properties of hydrogels prepared from heparin with different degrees of sulfation.

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Supplementary results for chapter 4.5.3:

“Application of competing highly heparin-affine polyelectrolytes”
(for discussion see particular chapter)

Fig. B11 Cumulative amount of hydrogel-released FGF-2 or VEGF in dependence on the presence of chitosan in the release medium at different time points as quantified via ELISA. Plotted is the cumulative release of FGF-2 (left) or VEGF (right) in dependence on the temporally defined addition of chitosan. The red symbol indicates the first addition of chitosan (rhomb: application after 0 h; square: application after 3 h; pentagon: application after 24 h). All data are presented as mean ± root mean square deviation from n = 3.
Publications and conference contributions

Papers


Book chapters

 Talks


Posters

Zieris A, Welzel PB, Panyanuwat W, Grimmer M, Tsurkan M, Freudenberg U, Werner C. Biofunctional modification of heparin-starPEG networks. 2nd CRTD Summer Conference on Regenerative Medicine, 6 June 2008, Dresden (Germany)


Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Die vorliegende Arbeit wurde am Max Bergmann Zentrum für Biomaterialien des Leibniz-Instituts für Polymerforschung Dresden e.V. in der Zeit von November 2007 bis Oktober 2011 unter der wissenschaftlichen Betreuung von Herrn Prof. Dr. rer. nat. Carsten Werner angefertigt.

Ich erkenne die Promotionsordnung der Fakultät Mathematik und Naturwissenschaften der Technischen Universität Dresden in der Fassung vom 23. Februar 2011 an.

Bisherige erfolglose Promotionsverfahren haben nicht stattgefunden.

Dresden, 1. November 2011

Andrea Zieris