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Dual independent delivery of pro-angiogenic growth factors from starPEG-heparin hydrogels

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ABSTRACT

Effective vascularization is a prerequisite for the success of various different tissue engineering concepts. While simultaneous administration of basic fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF) has been previously demonstrated to boost angiogenesis, the combined long-term delivery of both growth factors from biomaterials is still a major challenge. In this work, two important heparin binding cytokines were delivered in parallel from a modular starPEG (multi-armed polyethylene glycol) — heparin hydrogel system to human umbilical vein endothelial cells (HUVECs) grown in culture and in a chicken embryo chorioallantoic membrane (CAM) model. As the utilized gels contain high quantities of heparin, loading and subsequent release of both growth factors (as determined by radiolabeling studies and Enzyme-Linked Immunosorbent Assay [ELISA]) occurred independently from each other. The combined delivery of FGF-2 and VEGF through starPEG-heparin hydrogels resulted in pro-angiogenic effects *in vitro* (study of cell survival/proliferation, morphology and migration) and *in vivo* (quantification of CAM vascularization) being clearly superior over those of the administration of single factors. Consequently, the independent delivery of growth factor combinations by biohybrid starPEG-heparin matrices allows for the precise multifactorial control of cellular processes critically determining regeneration.

1. Introduction

Angiogenesis, the formation of new blood capillaries from pre-existing vessels, is a process that mainly occurs during embryonic development. Under physiological conditions in the adult organism, it is a very rare event usually restricted to wound healing and the female reproductive system [1,2]. Consequently, in order to obtain sufficient vascularization of an engineered construct, recent approaches in regenerative medicine rely on biomaterials able to induce a localized angiogenic response after implantation to the target site [3]. However, angiogenesis is a multifactorial process comprising basement membrane degradation, endothelial cell migration, capillary tube formation, and proliferation, and, as such, is controlled by the interplay of a variety of different effectors [1,4]. Two of the most extensively studied positive regulators of angiogenesis are basic fibroblast growth factor (FGF-2) and vascular endothelial growth factor A (VEGF) [5]. *In vitro* experiments demonstrated the induction of an angiogenic phenotype by promoting the proliferation/survival, migration and differentiation of endothelial cells [5–7]. Although the administration of either single FGF-2 or VEGF was able to support angiogenesis in animal models, problems associated with the vessel stability were observed [8,9]. As both cytokines demonstrated considerable cooperative effects on endothelial cells, *in vitro*, and angiogenesis, *in vivo*, a combined provision of the two growth factors seems advantageous [10–16]. Despite these findings, few studies have been 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 [17] or relied on the cytokine affinity of molecules such as fibrin [18–20], gelatin [21] or heparin [22] in order to control the storage and release of both effectors.

Recently, a biohybrid hydrogel has been developed composed of star-shaped poly(ethylene-glycol) (starPEG) and heparin, and prepared by cross-linking of the amino end-functionalized starPEG with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysulfosuccinimide (EDC/s-NHS)-activated carboxylic acid groups of heparin [23]. Besides the possibility of an independent variation in the physical characteristics and the biomolecular functionalization, this material is characterized by its high heparin content. Consequently, large amounts of FGF-2 and VEGF could be bound and released by the hydrogels [24], which makes them ideal candidates for application as multifactor delivery systems. Moreover, it was already shown that human umbilical vein endothelial cells (HUVECs) could be successfully cultured on starPEG-heparin hydrogels, while their behavior could be modulated through dependence on the physicochemical network structure and the biomolecular functionalization [24].

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In this study, the capability of starPEG-heparin networks to function as a storage and delivery matrix for combinations of FGF-2 and VEGF was investigated. In addition, the effect of the parallel cytokine administration was evaluated by analyzing differential HUVEC behavior, *in vitro*, as well as by monitoring angiogenesis in the chicken embryo chorioallantoic membrane (CAM) model, *in vivo*.

2. Materials and methods

2.1. Preparation of starPEG-heparin hydrogel networks

StarPEG-heparin hydrogels were formed by cross-linking amino end-functionalized four-arm starPEG with EDC/s-NHS-activated carboxylic acid groups of heparin [23,24], while 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. For an application in different experimental setups, gels were either formed as surface bound networks with a final thickness of approx. 50 μm (3.11 μl gel mixture/cm² used for quantitative FGF-2 or VEGF binding/release studies and *in vitro* cell culture experiments) or as free-floating gel disks (104.7 μl gel mixture/cm² used for characterization of starPEG-heparin network properties) as described in [24]. To allow for an introduction into an *in vivo* system, small hydrogel droplets were prepared by pipetting 1.4 μl of the gel mixture on a hydrophobic glass cover slip treated with hexamethyldisilazane (Fluka, Seelze, Germany). After polymerization overnight at 22 °C, each gel sample was washed in phosphate buffered saline (PBS, Sigma-Aldrich, München, Germany) to remove s-NHS/EDC and any non-bound starPEG/heparin [23]. For cell culture, sterilization was performed by UV-treatment for 30 min. For additional treatments, all solutions were sterile unless otherwise indicated.

2.2. Biomodification of starPEG-heparin hydrogels

Biomodification with cyclo(Arg-Gly-Asp-D-Tyr-Lys) (RGD) peptide (Peptides International, Louisville, KY, USA) was performed as described in [23]. Briefly, heparin carboxylic acid groups of swollen hydrogels were activated with s-NHS/EDC solution (25 mM s-NHS, 50 mM EDC in 1/15 M phosphate buffer (pH 5)) for 45 min at 4 °C and subsequently incubated with 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 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 (1 or 5 μg/ml). PBS-swollen, pure or RGD-modified gels were immersed in this solution at room temperature for 24 h followed by rinsing twice with PBS.

2.3. Analysis of starPEG-heparin hydrogel properties

StarPEG-heparin hydrogels were characterized as described elsewhere [23]. Briefly, the storage modulus of the final networks ($n=4$) was 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 this, pore sizes of the network could be estimated according to the rubber-elasticity theory as described elsewhere [23]. Volumetric swelling degree v_t was calculated by $v_t=(d_t/d_0)^3$, where d_0 is the diameter of a non-swollen gel disk and d_t is the diameter of the disk after the washing process in PBS for 24 h. The heparin and RGD content is expressed in relation to the final volume of the PBS-swollen gel network.

2.4. Characterization of the biomodification

2.4.1. Amino acid analysis via high performance liquid chromatography (HPLC)

Quantification of RGD-peptide (50 μg/ml; $n=4$) in the gels was performed by acidic hydrolysis and subsequent high performance liquid chromatography (HPLC) analysis as described elsewhere [25]. 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 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. Amino acids were quantified using external standards.

2.4.2. Enzyme-Linked Immunosorbent Assay (ELISA)

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. 200 μl of FGF-2 (1 or 5 μg/ml) and/or VEGF (1 or 5 μg/ml) solution were added per cm². Immobilization was performed over night at 22 °C. The FGF-2 and/or VEGF solution was removed followed by two washes with PBS. Each of these solutions was collected and assayed in duplicate using an ELISA Quantikine kit (R&D Systems, Minneapolis, USA). After immobilization, FGF-2 and/or VEGF were allowed to release from these gels at 22 °C into 250 μl/cm² of serum-free (SF) endothelial cell growth medium (ECGM; Promocell GmbH, Heidelberg, Germany) supplemented with 0.02% sodium azide (Fluka) ± 1 mg/ml bovine serum albumin (BSA, Sigma-Aldrich). 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 protein release (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.

2.4.3. Radiolabeling studies

^{125}I-labeled FGF-2 was purchased from Chelatec SAS (Nantes, France), VEGF was labeled with ^{125}I as described in [24]. Briefly, the protein was treated with IodoBeads (Pierce, Rockford, USA) and subsequently purified by size exclusion chromatography (NAP-5 column, GE Healthcare, Munich, Germany) yielding iodinated VEGF 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 and/or VEGF protein solution was spiked with either ^{125}I-labeled FGF-2 or VEGF as a percentage of total protein (2.5–100%). This mixture containing 1 or 5 μg/ml FGF-2 and/or VEGF in PBS, respectively, was added to surface-bound hydrogels (200 μl per cm²) and the protein was adsorbed over night at 22 °C. After the incubation period, gels were rinsed two times with an excess volume of PBS. Radioactivity was measured twice per sample using gamma counting. Immobilized protein was quantified using ^{125}I-FGF-2 or -VEGF standards.

2.5. In vitro endothelial cell culture experiments

2.5.1. Cultivation of HUVECs

Human endothelial cells from the umbilical cord vein (HUVECs) were collected according to the procedure suggested by [26] and grown to

confluence. After one to four passages, ~11300 cells per cm^2 surface were seeded on starPEG-heparin networks, 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% CO_2 on RGD-treated starPEG-heparin hydrogels. These networks 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.

2.5.2. Survival studies

Analysis of cell survival was performed via Live/Dead staining [27] as described by [24] after 3 days of culture on the different substrates (n = 2–4). Briefly, a solution containing 0.1 μg/ml fluorescein di-O-acetate (FDA; Fluka) and 2 μg/ml propidium iodide (PI; Fluka) dissolved in PBS were added to each sample. After incubation for 2 min at 22 °C, the cells were visualized by fluorescence microscopy (DMIRE2, Leica) using a 10× dry objective (HC PL Fluotar 10×0.30, Leica).

2.5.3. 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. Light microscopy images were then taken (Olympus IX50, Olympus, Hamburg, Germany) at 10× 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 days 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.

2.5.4. Proliferation assay

Cell proliferation was studied after 3 days of culture with the help of a 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma-Aldrich) proliferation assay [28] as described by [24]. Briefly, a 1/5 mixture of MTT (5 mg/ml) and SF ECGM was added to each sample and incubated for 5 h at 37 °C. After removing the supernatant from the substrates, dimethyl sulfoxide (DMSO; Fluka) was added and samples were incubated for 20 min at 37 °C. Absorption of this solution was subsequently measured in a plate reader (Genios, TECAN, Crailsheim, Germany) at 540 nm. Experiments were performed for at least three samples.

2.5.5. Investigation of cell migration

To study HUVECs migration, the MilliCell modified Boyden chamber (8 μm pores; Millipore, Bedford, USA) migration assay was performed [29]. Chambers (n = 4–6 for each condition) were prepared by pre-coating the upper surface of the polycarbonate membrane with 100 μl 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 before. As a control, the filters were also introduced to wells without any gel networks. All substrates were then coated with 600 μl 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 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 of 70% ethanol for 1 h at room temperature. After rinsing the chamber with PBS, cells were stained using 300 μl of 25% Crystal Violet (Sigma-Aldrich) diluted in MilliQ water. Cell migration values were determined by elution of the Crystal Violet stain in 400 μl of 10% acetic acid (20 min, room temperature) and measuring absorbance at 590 nm.

2.6. In vivo experiments using the chicken chorioallantoic membrane (CAM) assay

Experiments were performed on chicken embryos grown by the shell-free culture method [30]. 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 days during which blood vessels of CAM vascular system developed. The RGD-functionalized starPEG-heparin hydrogels described earlier 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. The quantitative results were expressed as a ratio of untreated sample.

2.7. Data analysis

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

3. Results and discussion

3.1. Network characteristics

Formation of starPEG-heparin hydrogel networks was performed by a cross-linking reaction of amino end-functionalized starPEG with s-NHS/EDC-activated carboxylic acid groups of heparin. As demonstrated in Table 1, the key characteristics of the resulting scaffolds are the high content of heparin and water. Nevertheless, through variation of the starPEG to heparin ratio, physico-chemical material parameters such as storage modulus and mesh size of the gels can be adapted independently of the heparin content and, therefore, also the subsequent biofunctionalization such as the amount of immobilized RGD [23].

3.2. Functionalization with FGF-2 and/or VEGF

Due to their high heparin content, starPEG-heparin hydrogels were shown to bind and stabilize numerous cytokines [24] and might be promising candidates for an application as multifactor delivery matrix. Therefore, the binding and subsequent release characteristics of FGF-2 and VEGF, introduced either as single components (1 μg/ml or 5 μg/ml) or different ratio combinations (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) to starPEG-heparin hydrogels, were analyzed.

Protein binding studies were performed using ELISA (Fig. 1A, top panel) and further validated by radiolabeling studies (Fig. 1A, bottom panel). While quantitative differences determined with the two

Table 1
Key characteristics of the starPEG-heparin network.

starPEG/heparin ratio [mol/mol]	Heparin content [μg/μl]	RGD/heparin [mol/mol]	Water content [%]	Storage modulus [kPa]	Pore size [nm]
3	7.8	0.6	97	7.3	11

methods might be due to experimental conditions as systematically investigated in [31], these techniques were selected because both provide qualitatively similar results.

A linear correlation was observed between the concentration of the incubation solution and the amount of immobilized cytokine within the gel (~199 or 90 ng/cm² scaffold area immobilized from a solution of 1 μg/ml and ~995 or 443 ng/ cm² scaffold area immobilized from a solution of 5 μg/ml as detected by ELISA or radiolabeling studies; Fig. 1A top or bottom, single factors). These findings are in line with published studies demonstrating that binding of either FGF-2 or VEGF to the starPEG-heparin networks did not reach saturation up to a protein concentration of 50 μg/ml, where the molar excess of heparin to protein was still 26:1 for FGF-2 and 62:1 for VEGF as quantified by amino acid analysis via HPLC [24]. Additionally, as reported for FGF-2 [32], 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 here, while due to the small size of FGF-2 (~3 nm; 17.2 kDa) [33] and VEGF (~6 nm; 38.2 kDa) [34] also no spatial restrictions within the gel network should limit the uptake of the proteins. Consequently, different combinations of FGF-2 and VEGF (Fig. 1A, combinations) can be immobilized to the networks with the same efficiency observed 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). Additionally, the immobilized quantities of combinations of FGF-2 and VEGF at a defined concentration were found to be similar for both proteins, indicating that the two cytokines do not affect each other during interaction with the gel network. This finding is most probably related to the large excess of heparin in the scaffolds, which allows for an undisturbed co-immobilization of FGF-2 and VEGF, making the starPEG-heparin hydrogel system a highly efficient storage matrix for angiogenic cytokines.

In addition to the ability to act as a reservoir for cellular effectors, biomaterials must be capable of releasing the cytokines in an adjustable manner. To investigate these properties, studies on the diffusion-based release of FGF-2 and/or VEGF from starPEG-heparin hydrogels were performed via ELISA.

Fig. 1B illustrates the cumulative release of either FGF-2 (top) or VEGF (bottom) 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 four days. Irrespective of the immobilized concentration or the particular factor considered, the release curves show a typical burst within the first 24 h followed by a continuous release over time. Here, a linear correlation between the amount of gel-bound growth factors and the quantities being released (~1 ng/cm² scaffold area released for 1 μg/ml and ~6 ng/cm² scaffold area released for 5 μg/ml; Fig. 1B, dashed lines) was observed for single FGF-2 or VEGF. Additionally, different combinations of FGF-2 and VEGF (Fig. 1B, continuous lines) could be released by the networks 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). Once again, the large excess of heparin appears to prevent any interference between the cytokines during their combined application. An additional advantage of these starPEG-heparin hydrogels is the comparable release of either cytokine at the particular loading quantities.

For evaluating the potential of cytokine delivery systems to promote a certain cell behavior *in vitro*, the absence of serum in the cell culture medium might be beneficial, as possible interferences of growth factors

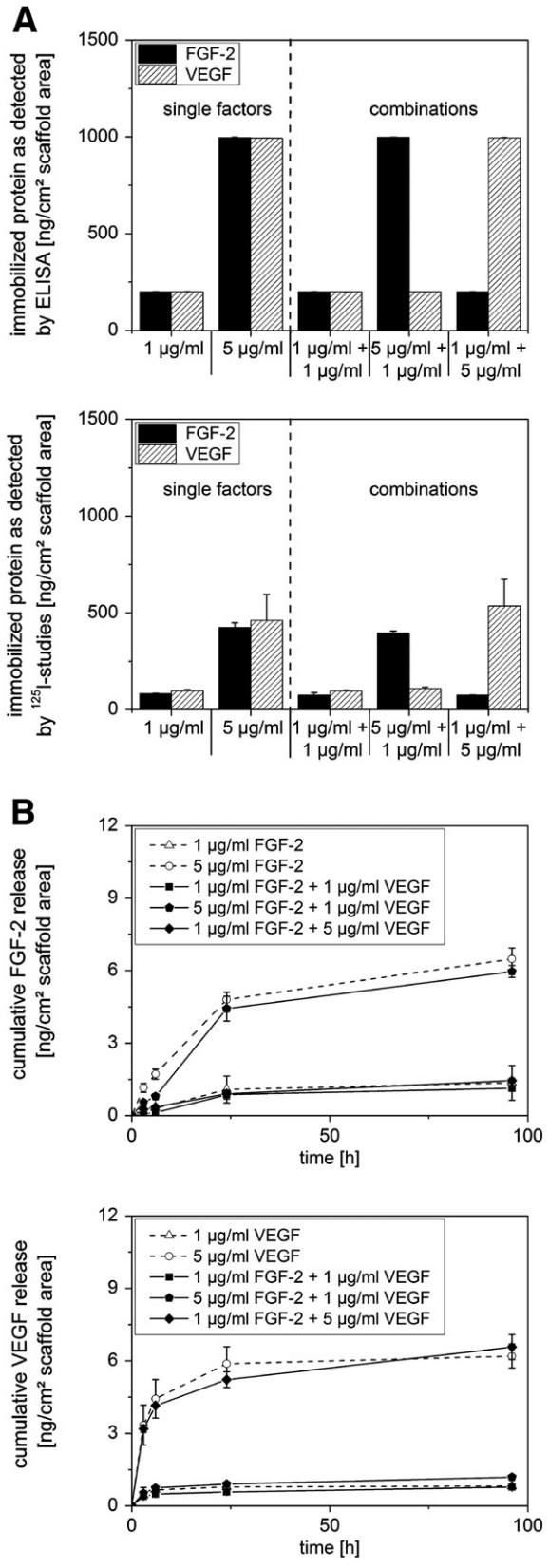

Fig. 1. FGF-2 and/or VEGF uptake (A) and release (B) experiments. 1A (top): amount of electrostatically bound FGF-2 and/or VEGF per cm² scaffold surface ($p>0.05$ for the immobilized amount of single FGF-2 or VEGF and the corresponding concentration used in the combination; ANOVA) as determined by ELISA. 1A (bottom): amount of electrostatically bound FGF-2 and/or VEGF per cm² scaffold surface ($p>0.05$ for the immobilized amount of single FGF-2 or VEGF and the corresponding concentration used in the combination; ANOVA) as determined by radiolabeling studies. 1B: cumulative amount of electrostatically bound FGF-2 (top) or VEGF (bottom) 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 $n=2$–4 (radiolabeling studies) or $n=3$ (ELISA).

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 *in vitro* HUVEC culture settings used in this study. However, this environment is significantly different from the situation that occurs *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. 2 shows the cumulative release of electrostatically bound FGF-2 (top) or VEGF (bottom) 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 also Fig. 1A, top) being released by the starPEG-heparin scaffolds.

As demonstrated in Fig. 2, 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 [35]. However, 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 [36,37]. The most likely reason for this effect could be that BSA might displace the cytokines from heparin by reducing their interaction [36,37]. 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.

In summary, the starPEG-heparin hydrogels could be deployed as highly potent storage systems and tunable delivery matrices for angiogenic cytokines. This material permits large quantities of several growth factors to be administered independently as deemed essential for the promotion of effective vascularization in tissue engineering concepts [38]. Consequently, this approach could provide realistic perspectives for application in this field.

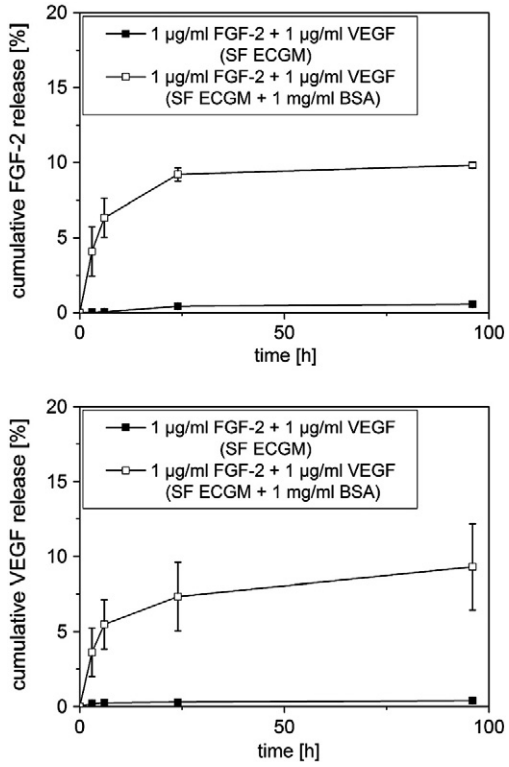

Fig. 2. FGF-2 (top) and VEGF (bottom) release experiments in dependence on the release medium. Plots show the cumulative percentage of electrostatically bound FGF-2 (left) or VEGF (right) released into SF ECGM ± 1 mg/ml BSA by gels which were loaded with a combination of 1 μg/ml FGF-2 + 1 μg/ml VEGF ($p<0.05$ 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.

3.3. In vitro HUVEC response to hydrogel released FGF-2 and/or VEGF

The presence of angiogenic growth factors such as 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 [8,9], only a few studies have investigated the effect of a combined provision of both FGF-2 and VEGF by a particular biomaterial [17–22].

3.3.1. Cell survival/proliferation and morphology

Scaffolds suitable for application in tissue engineering should be able to promote cell survival and proliferation over prolonged time periods. Moreover, materials have to support HUVEC differentiation into less circular and more elongated cells in order to form tubular structures for the stimulation of angiogenesis. To fulfill these requirements, the delivery of cytokines is a crucial material parameter [38–40]. Therefore the effects of FGF-2 and/or VEGF provision by starPEG-heparin hydrogels on HUVEC proliferation/survival and differentiation were investigated using live/dead staining (Fig. 3A, green/red cells) and a MTT assay (Fig. 3B) performed after three days of culture on the different substrates. Furthermore, cell morphology was investigated as assessed by HUVECs circularity (Fig. 3C).

As the functionalization of starPEG-heparin hydrogels with cytokines alone was not sufficient to support effective cell attachment and spreading (Fig. S2, Supplementary data), all scaffolds monitored for their effect on HUVEC survival/proliferation and morphology were

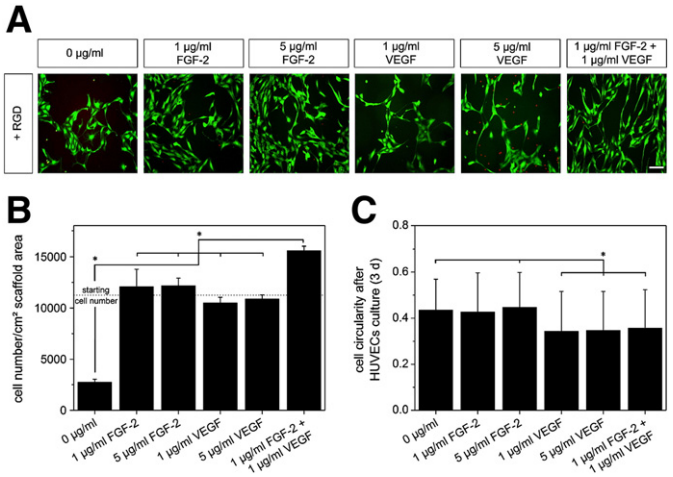

Fig. 3. Interactions of differently biomodified hydrogels (+ RGD; with or without 1 or 5 μg/ml of single FGF-2 or VEGF as well as a combination of 1 μg/ml FGF-2 + 1 μg/ml VEGF) with HUVECs after 3 days of culture. 3A: representative fluorescence microscopy images after live/dead staining of HUVECs (viable cells = green; dead cells = red) on the different substrates (scale bar 130 μm). 3B: HUVECs 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 = 3–4 (* indicates $p<0.05$; ANOVA). 3C: HUVECs 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 ≈ 40–200 cells quantified on up to 13 different substrates (* indicates $p<0.05$; ANOVA). For statistical comparisons apart from the illustrated significant differences, see text.

additionally modified with the adhesion ligand RGD. As demonstrated in the Supplementary material, the covalent RGD attachment to the heparin carboxylic acid moieties does not influence the electrostatic binding (Fig. S1A, Supplementary data) and release (Fig. S1B, Supplementary data) of FGF-2 and VEGF. This effect could be most probably attributed 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. Concerning the effects on cell adhesion, with the RGD incorporation, no additional influence of growth factor modification could be observed on HUVEC attachment (Fig. S2, Supplementary data). This indicates that 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.

After 3 days of culture, substantial HUVEC survival (Fig. 3B) was observed for RGD-modified hydrogels (~2700 cells/cm² scaffold area) and for RGD-modified hydrogels loaded with 1 μg/ml of single FGF-2 or VEGF (~12,100 or 10,500 cells/cm² scaffold area), 5 μg/ml of single FGF-2 or VEGF (~12,200 or 11,000 cells/cm² scaffold area) or with a combination of 1 μg/ml FGF-2 + 1 μg/ml VEGF (~15,600). The very small number of dead cells in the medium and the typical spindle-shape-like morphology of cells grown on the scaffolds (Fig. 3A) indicated that the introduction of RGD to starPEG-heparin networks could generate successful HUVEC culture substrates. Moreover, in the presence of FGF-2 and/or VEGF in RGD-modified hydrogels, cell numbers could be increased further (Fig. 3B; $p<0.05$ when comparing gels + RGD to scaffolds modified with RGD and cytokines). Despite the positive effect of RGD + VEGF on HUVEC survival, the presence of RGD + FGF-2 yielded an even higher cell number than initially applied as determined after 3 days of culture. However, maximal proliferation rates were observed when starPEG-heparin hydrogels were used for the combined provision of FGF-2 and VEGF (Fig. 2B; $p<0.05$ for comparing gels + RGD and one single growth factor to scaffolds + RGD and both FGF-2 + VEGF) as also described by [11]. Interestingly, increasing amounts of FGF-2 or VEGF released from the starPEG-heparin hydrogels (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). The combination of both factors (1 μg/ml FGF-2 + 1 μg/ml VEGF) significantly increased the cell survival even though this provided a lower total amount of cytokine. Thus, the enhanced proliferation induced by hydrogels modified with FGF-2 + VEGF clearly did not arise from the presence of higher cytokine concentrations, but rather seemed to result from a combined action of both growth factors as already described by [11].

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. 3C), the lowest cell circularity representing HUVECs with the most elongated morphology was observed for starPEG-heparin gels 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 ± FGF-2), while there were no significant differences between RGD-modified scaffolds with 1 μg/ml or 5 μg/ml FGF-2 (cell circularity of 0.43 or 45, respectively) or without any growth factor (cell circularity of 0.43). However, consistent with the supporting effect of FGF-2/VEGF combinations on *in vitro* tube formation [10–12], 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.

Taken together, the introduction of the RGD adhesion ligand yielded starPEG-heparin networks that could be successfully applied as substrates for HUVEC culture. While the presence of FGF-2 was able to stimulate HUVEC proliferation/survival, VEGF seemed to promote cell differentiation. However, using starPEG-heparin hydrogels as a delivery matrix for the independent administration of both cytokines, their beneficial effects could be combined to obtain high numbers of HUVECs undergoing differentiation.

3.3.2. 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. The networks 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. 4A). Quantitative results (Fig. 4C) 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. 4B).

While unmodified starPEG-heparin networks 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. 4C). Interestingly, the unaffected release and diffusion of growth factors into the medium over a larger distance is required to initiate an effect on the HUVECs in contrast to the other *in vitro* assays, where cells were directly seeded on the cytokine-loaded scaffolds. Consequently, the results obtained

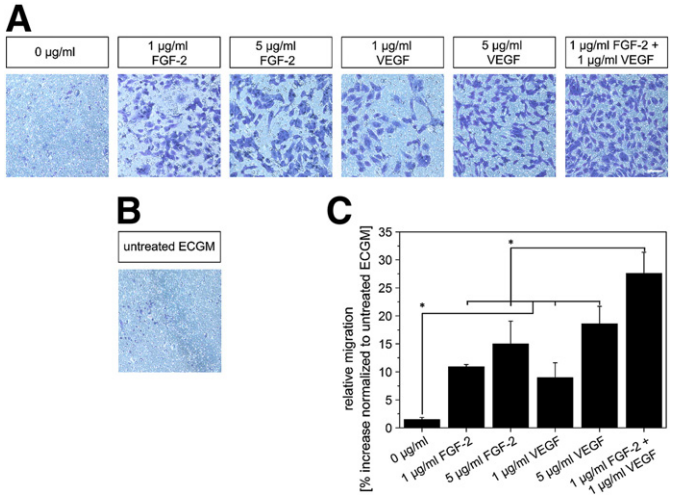

Fig. 4. HUVEC migration as analyzed by a modified Boyden chamber assay. 4A and B: representative images of HUVECs located on the lower site of the Boyden filter after migration through the membrane in response to starPEG-heparin hydrogels with or without 1 or 5 μg/ml of single FGF-2 or VEGF as well as a combination of 1 μg/ml FGF-2 + 1 μg/ml VEGF (A) or to the untreated ECGM which served as a control (B) (scale bar 100 μm). 4C: quantification of the relative HUVEC migration towards starPEG-heparin 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. Data are presented as mean ± root mean square deviation from n = 4–6 (* indicates $p<0.05$; ANOVA). For statistical comparisons apart from the illustrated significant differences, see text.

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 where 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 different authors presented inconsistent results as whether FGF-2 [41,42] or VEGF [43,44] 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. Compared with 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 networks ($p<0.05$ for 1 μg/ml FGF-2 vs. 5 μg/ml VEGF; 1 μg/ml VEGF vs. 5 μg/ml FGF-2 or vs. 5 μg/ml VEGF; $p>0.05$ for 1 vs. 5 μg/ml FGF-2). These data are consistent with results described in literature [43], 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 influence of the cytokine concentration, the combination of 1 μg/ml FGF-2 + 1 μg/ml VEGF showed the most beneficial effect 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 [43], in this system FGF-2 and VEGF seemed to promote migration in a synergistic way as also observed by [13,14]. However, whereas both growth factors also increased HUVEC numbers after three days of culture (Fig. 3B), when considering the cellular process of migration over 20 h, possible interferences of proliferation should be discussed. As, with regard to a time course of 24 h, no increase in cell numbers was observed in standard HUVEC growth curves [45,46], 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 [41], effects observed in this study might be indeed attributed to an impact of the growth factors on HUVEC migration rather than proliferation.

In summary, cytokine-functionalized starPEG-heparin networks could be successfully 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.

3.4. *In vivo CAM response to FGF-2 and/or VEGF delivery by 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 [47]. Therefore, to analyze whether the provision of FGF-2 or VEGF as single cytokines or growth factor combinations by starPEG-heparin hydrogels could initiate an angiogenic response *in vivo*, the effects of biofunctionalized hydrogel onplants were studied in a CAM assay (Fig. 5A, B and C). Based on the data reported in literature [48–50], 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 was used, while starPEG-heparin scaffolds were generated as droplet-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. 5A and C) were placed onto the developing CAM at embryonic day 8 (ED8) until ED12. The untreated CAM (Fig. 5B) served as a reference system.

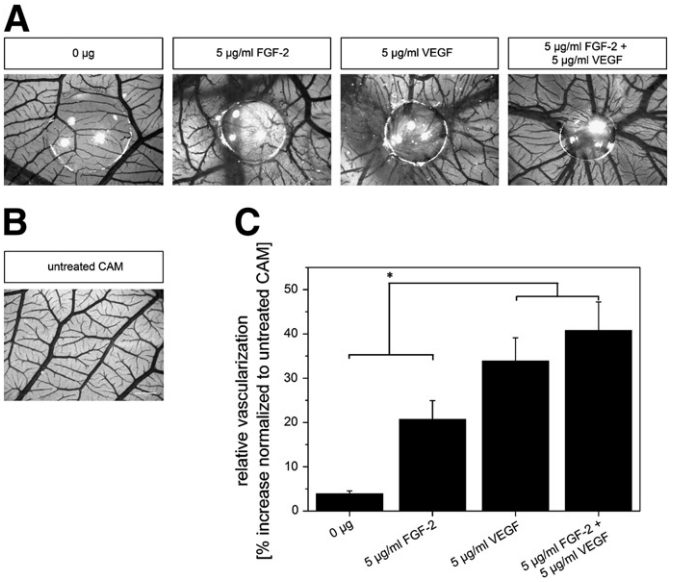

Fig. 5. Effects of growth factor provision by starPEG-heparin scaffolds on vascularization in the chicken embryo CAM assay. 5A and B: representative images of the CAM vascularization in response to starPEG-heparin hydrogels with or without 5 μg/ml of single FGF-2 or VEGF as well as a combination of 5 μg/ml FGF-2 + 5 μg/ml VEGF (A) or photograph of the untreated CAM which served as a control (B) (scale bar 1 mm). 5C: quantification of the relative CAM vascularization in relation to starPEG-heparin hydrogels with or without 5 μg/ml of single FGF-2 or VEGF as well as a combination of 5 μg/ml FGF-2 + 5 μg/ml VEGF. Data are presented as mean ± root mean square deviation from n = 5–16 (* indicates $p<0.05$; ANOVA). For statistical comparisons apart from the illustrated significant differences, see text.

As visualized in Fig. 5B, 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. 5C). 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 enhanced vessel formation might result from the high heparin content of the networks, as this molecule has been shown to induce a moderate angiogenic response in the CAM assay [51]. A significant, stronger increase in vascularization could be found 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 [50], the administration of single VEGF induced a stronger angiogenic response than FGF-2 alone ($p<0.05$ for the comparison of gels modified with VEGF to FGF-2-functionalized scaffolds), which could also be observed for the combination of FGF-2 + VEGF ($p<0.05$ for the comparison of hydrogels treated with FGF-2 + VEGF to FGF-2-modified hydrogels). Although the combined delivery of FGF-2 + VEGF showed the best effect on vascularization, this result was not statistically significant compared to VEGF-delivery alone ($p>0.05$ for comparing these two conditions). Thus, we conclude that the positive effects (proliferation, migration) for the FGF-2 + VEGF combination observed *in vitro* are similar to the *in vivo* situation [52].

Taken together, the administration of FGF-2 and/or VEGF by starPEG-heparin hydrogels induced a substantial angiogenic response within the CAM system.

4. Conclusions

This study demonstrated that starPEG-heparin hydrogels could be successfully utilized for the combined immobilization of large quantities of FGF-2 and VEGF and permitted an independent, tunable delivery of both growth factors. In HUVEC culture experiments and in the *in vivo* CAM assay, the simultaneous release of FGF-2 and VEGF exerted superior effects on cell behavior and the angiogenic response when compared with the provision of single cytokines. As such, the starPEG-heparin hydrogels performed outstandingly as an effective cytokine delivery matrix, allowing for the application in multi-factor settings essential for effective regenerative processes. These results are in line with previously reported *in vivo* data stating a synergistic effect of the parallel delivery of the two cytokines [15,16,22].

Additionally, further possibilities to tune the cytokine release profiles are currently being explored by modifying the scaffolds either with cleavable peptides [53,54], selectively desulfated heparin, or by modulating the heparin/growth factor interaction via the application of competing highly heparin-affine molecules. These avenues of investigation should yield a set of available materials that extend towards customized release systems with greater application in a broader variety of therapeutic options. To conclude, these results open up new prospects for the application of starPEG-heparin hydrogels in the context of angiogenic tissue engineering.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jconrel.2011.06.042.

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