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Analytical approaches to uptake and release of hydrogel-associated FGF-2

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Abstract Strategies to control the delivery of growth factors are critically important in the design of advanced biomaterials. In this study we investigated the binding and release of fibroblast growth factor 2 (FGF-2) to/from a biohybrid hydrogel matrix by four independent analytical methods: radioisotope and fluorescence labeling, amino acid analysis and Enzyme-Linked Immunosorbent Assays (ELISA). The compared analyses provided qualitatively similar uptake characteristics while the results of the FGF-2 quantification strongly depended on the particular experimental conditions. The release kinetics of FGF-2 from the gels could be monitored sensitively by ^{125}I labeling and by ELISA-techniques. The latter method was concluded to be advantageous since it permits the application of unmodified ("native") growth factors.

1 Introduction

Cellular fate decisions are determined by interactions with extracellular matrices (ECM). The ECM is an intricate network of collagens, proteoglycans and adhesion proteins interacting with soluble factors, such as growth factors and chemokines. In the formation, maintenance and repair of any tissue interactions with the ECM are crucial for survival, growth and differentiation of all cell types involved. Visco-elastic and structurally responsive characteristics, bioadhesive properties and the provision of soluble effectors such as growth factors are highly orchestrated aspects of any type of ECM and critically important for cellular responses [1]. Therefore, current approaches to the design of biomaterials for tissue engineering strategies aim to closely mimic these features of the ECM [2]. The delivery of growth factors is key to many strategies for enhanced wound healing and tissue regeneration. Fibroblast growth factor 2 (FGF-2, also known as basic fibroblast growth factor) is one of the most interesting proteins due to its effects on many cell types [3]. This 17-kDa polypeptide is a potent modulator of fibroblast, keratinocyte, chondrocyte, endothelial and smooth muscle cell proliferation, survival, motility and differentiation and consequently is involved in embryonic development [4], angiogenesis [5], osteogenesis [6], chondrogenesis [7] and wound repair [8]. However, degradation due to the rapid diffusion of soluble FGF-2 from the target site [9] makes it is necessary to stabilize the protein during the supply. In vivo, FGF-2 can be stored at various sites by interactions with glycosaminoglycans such as the anionic polyelectrolyte heparin [10]. Here, binding is mainly triggered by spatially matching electrostatic interactions between the negatively charged functional groups of heparin and the lysine and arginine residues of FGF-2 [11]. By binding to heparin, FGF-2 diffusion can be slowed down while simultaneously stabilizing the protein against loss of bioactivity [12, 13]. Several recent approaches take advantage of this feature by creating heparin-containing biomaterials [14–18] often also in combination with synthetic building blocks [19]. These hybrid matrices offer both the advantages of a defined functionality and biocompatibility (like the natural ECM) as well as a high variability concerning material and structure (like in a synthetic matrix) [19]. Due to its excellent biocompatibility, its

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hydrophilic and uncharged character as well as the possibility to easily modify its terminal end groups [20], poly(ethylene glycol) is frequently applied in such approaches [21–25]. Recently, a new biohybrid hydrogel consisting of heparin and star-shaped poly(ethylene–glycol) (starPEG) has been produced 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 (see Fig. 1) [26]. Two key features distinguish this system from previously reported materials: First, the hydrogels contain a significantly higher amount of heparin (up to 0.8% (w/w)). As a consequence, the structural integrity of heparin is preserved to higher degrees upon crosslinking, which allows for rather undisturbed interactions with heparin-binding growth factors. Second, the biohybrid hydrogel system can be gradually and independently varied in physical characteristics and biomolecular functionalization.

This approach offers exciting options to explore the interplay of both mechanical and biomolecular stimuli using a platform of well-defined ECM-inspired biomaterials. Studies with ^{125}I-FGF-2 proved the modular heparin–starPEG network to be suitable for the controlled uptake and release of this protein [26]. However, due to known stability issues of radiolabeled FGF-2 [27], we undertook additional efforts to characterize binding and sequestering of FGF-2 with a set of independent analytical methods. For that purpose, studies with radiolabeled FGF-2 were directly compared to FGF-2-Enzyme-Linked Immunosorbent Assays (ELISA), fluorescently labeled FGF-2 (detected by confocal Laser Scanning Microscopy, cLSM) and amino acid analysis of the hydrolyzed protein (using High Performance Liquid Chromatography, HPLC). The results obtained by the different methods are compared to provide a rational base for future analytical approaches to uptake and release of biomaterials-associated growth factors.

2 Materials and methods

2.1 Preparation of heparin–starPEG hydrogels

Heparin–starPEG hydrogels were formed by cross-linking of amino end-functionalized starPEG with EDC/s-NHS activated carboxylic acid groups of heparin [26]. For this, a total polymer content of 11.6% and a 2:1 ratio of EDC to s-NHS [mol/mol] were used while the calculation is based on a 1.75 molar excess of EDC for each NH_2-group of starPEG.

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 deionised, decarbonised water (MilliQ) by ultrasonication and afterwards kept on ice (approx. 2–4°C). Similarly, EDC (Sigma-Aldrich, Munich, Germany) and s-NHS (Fluka, Seelze, Germany) were separately dissolved in the sixth part of the total volume of ice-cold MilliQ. Subsequently, EDC and s-NHS solutions were added to heparin, mixed well and incubated for 15 min on ice to activate heparin carboxylic groups. After that, the starPEG solution was added to the activated heparin and mixed for 15 min at 8°C (at 900 rpm, Thermomixer Comfort, Eppendorf, Hamburg, Germany). For fluorescence microscopy, gels were prepared similarly from heparin spiked with 5% (w/w) of Alexa 488-labeled heparin (synthesized by M. Tsurkan, IPF Dresden).

To allow for a practical performance of FGF-2 binding and release studies, surface-bound gels with a final thickness of approx. 50 μm were prepared. For this 3.11 μl of the gel mixture per cm^2 were used, while all upcoming results are expressed for a scaffold prepared from 4.14 μl of the gel mixture. 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,

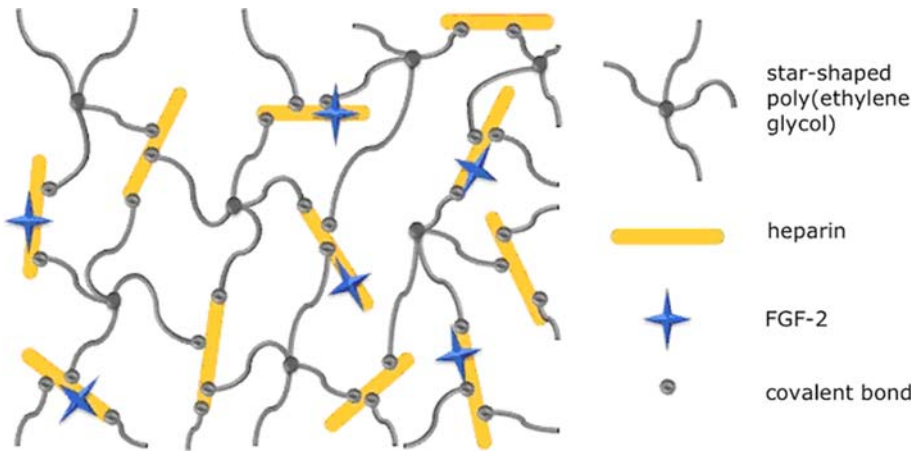

Fig. 1 Heparin–star-shaped poly(ethylene–glycol) (starPEG) networks are formed by cross-linking of the amino end-functionalized starPEG with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/*N*-hydroxysulfosuccinimid (EDC/s-NHS)-activated carboxylic acid groups of heparin

Germany) to allow covalent attachment of heparin via its activated carboxylic acid groups [28]. 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 (Fluka) from vapor phase or by placing an ethylen-chlortrifluorethylen-copolymer slide (Goodfellow, Cambridge, England) onto the gel solution in the glass bottom wells. After polymerization over night at 22°C, the cover slips were removed. Surface-bound gels were washed in phosphate buffered saline (PBS, Sigma-Aldrich) to remove EDC/s-NHS and any non-bound heparin/starPEG. PBS was exchanged five times every hour and once again after storage for 24 h. Subsequently, the swollen gels were immediately used for FGF-2 binding and release studies.

2.2 Characterization of heparin–starPEG hydrogels

Heparin–starPEG hydrogels were characterized as described elsewhere [26]. Briefly, the storage modulus of the final networks ($n = 4$) was determined using oscillating measurements on a rotational rheometer 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 in [26].

2.3 Investigation of FGF-2 uptake and release

2.3.1 Detection of radiolabeled FGF-2

Surface-bound gels ($n = 4$) were placed in custom-made incubation chambers that decreased the exposure of the protein to surfaces not originating from the hydrogels to a minimum (Fig. 1). Native FGF-2 protein solution (Miltenyi Biotech, Bergisch Gladbach, Germany) was spiked with ^{125}I-labeled FGF-2 (Chelatec SAS, Nantes, France) as a percentage of total protein. This mixture containing 0.5, 1 or 5 μg/ml FGF-2 in PBS was added to surface-bound hydrogels (200 μl per cm^2) 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 (LB 123, Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Immobilized protein was quantified using ^{125}I-FGF-2 standards.

After immobilization using 1 μg/ml protein, FGF-2 was allowed to release from these gels ($n = 2$) at 22°C into 250 μl/cm^2 of endothelial cell growth medium (Promocell GmbH, Heidelberg, Germany). At defined time intervals, the medium was withdrawn and the remaining FGF-2 bound to the gels was monitored twice via gamma counting. An equal volume of fresh medium was added back after each measurement.

2.3.2 Detection of fluorescently labeled FGF-2

FGF-2 was labeled with tetramethylrhodamine (TAMRA) according to the FluoReporter Tetramethylrhodamine Protein Labeling Kit manual (Molecular Probes, distributed by Invitrogen, Netherlands). TAMRA-FGF-2 was dissolved in PBS (5 μg/ml) and added to heparin–starPEG gels ($n = 2$, 200 μl/cm^2) 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 40× 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 DPSS laser (excitation wavelength of 561 nm, intensity 20%) was used for excitation of TAMRA-labeled FGF-2. 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 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.

2.3.3 Protein quantification by amino acid analysis

Surface-bound gels ($n = 2$) were put into 24-well plates TPP (Trasadingen, Switzerland). 200 μl of FGF-2 solution (25 μg/ml) were added per cm^2. The protein was allowed to adsorb over night at 22°C followed by washing in PBS (1 min) twice.

Quantification of immobilized FGF-2 was performed by acidic hydrolysis, HPLC separation and subsequent amino acid detection as described elsewhere [29]. Briefly, gel-coated substrates or volume samples (FGF-2 standards) 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. FGF-2 amount was quantified using external standards. Each sample was analyzed in duplicates.

2.3.4 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 (Fig. 2). 200 µl of FGF-2 solution (0.5, 1 or 5 µg/ml) were added per cm^2. Immobilization was performed over night at 22°C. The FGF-2 solution was taken out followed by washing with PBS twice. Each of these solutions was collected and assayed in duplicates using an ELISA Quantikine kit (R&D Systems, Minneapolis, USA). After immobilization using 1 µg/ml protein, FGF-2 was allowed to release from these gels at 22°C into 250 µl/cm^2 of endothelial cell growth medium. Samples taken at intervals were stored at −80°C until analyzed by ELISA. An equal volume of fresh medium was added back at each time point.

2.4 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

3.1 Network characteristics

Heparin–starPEG hydrogel scaffolds were prepared by cross-linking amino end-functionalized starPEG with EDC/s-NHS-activated carboxylic acid groups of heparin. As shown in Table 1, the final network is characterized by a high content of heparin and water. Nevertheless, varying the starPEG to heparin ratio allows control over the storage modulus and mesh size of the gels independently of the heparin content.

3.2 Analysis of FGF-2 binding and release

3.2.1 Experimental parameters

In order to analyze FGF-2 uptake and release by heparin–starPEG hydrogels, detection of radioisotope and fluorescently labeled FGF-2, amino acid analysis and ELISA were performed. Since all of these approaches are based on a distinct detection mechanism, experimental parameters had to be adjusted to the requirements of the particular method (Table 2). However, the combination of all four methods allows for analysis of FGF-2 binding and release over a wide range of concentrations.

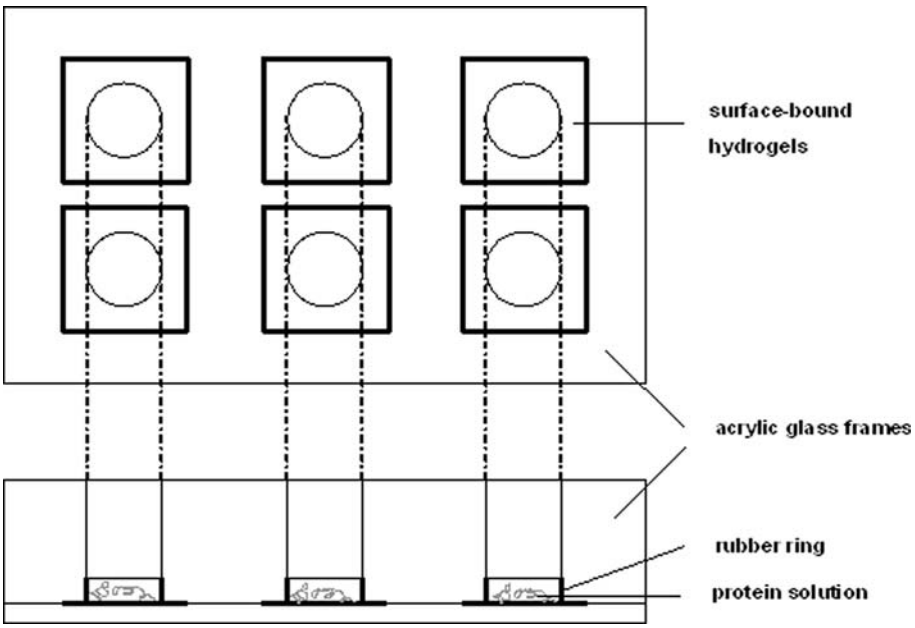

Fig. 2 Design of the custom-made immobilization chamber

Table 1 Key characteristics of the heparin–starPEG hydrogel network

Heparin/starPEG ratio (µmol/µmol)	Heparin content (µmol/scaffold)	Water content (%)	Storage modulus (kPa)	Mesh size (nm)
1/3	0.0125	97	2.57	11.7

Table 2 Experimental parameters used for FGF-2 binding and release studies with heparin–starPEG hydrogels (due to technical reasons, FGF-2 release experiments were only performed via radiolabeling studies and ELISA)

	Radiolabeling (^{125}I-) studies	cLSM	HPLC	ELISA
Performance	Immobilization chamber	Well plate	Well plate	Immobilization chamber
Analysis of FGF-2	In gel	In gel and supernatant	In gel	In supernatant
Protein labeled	Yes	Yes	No	No

3.2.2 FGF-2 binding studies

To characterize the uptake and distribution of FGF-2 within the heparin–starPEG hydrogels representative gel samples were analyzed using cLSM and fluorescently labeled FGF-2. As illustrated in Fig. 3, a homogeneous fluorescence intensity of TAMRA-FGF-2 within the hydrogel could be observed immediately after applying the protein to the network. TAMRA-FGF-2 was distributed equally throughout the entire gel. There was no increase in the relative TAMRA-FGF-2 fluorescence intensity inside the network ($\sim$60%, $P > 0.05$) and no corresponding decrease in the supernatant ($\sim$40%, $P > 0.05$) within the next hours (Fig. 4).

Quantification of FGF-2 binding to heparin–starPEG networks was performed by four different methods (for experimental parameters see Table 2). Due to the detection limits of the different approaches, FGF-2 concentrations used for loading had to be varied. However, in order to compare the different methods used here directly, the heparin–starPEG gel has to have the capacity to take up all the FGF-2 that is applied during the different experimental approaches. Figure 5 illustrates the results of these initial tests exemplarily for radiolabeling studies (^{125}I-studies, respectively) and ELISA. Similar percentages of the protein were immobilized (29.7%, 38% and 34.5%; $P > 0.05$ for radiolabeling studies; 98.2%, 98.6% and 98.4%; $P > 0.05$ for ELISA, respectively) independent from the FGF-2 concentration of the applied solution. This indicates that no saturation of the gel was reached within the concentration range monitored. The same tendency was observed when analyzing FGF-2 immobilization with concentrations up to 50 μg/ml via amino acid analysis (data not shown). Despite the fact that results obtained by the different methods used to investigate FGF-2 binding to heparin–starPEG hydrogels lead to the same qualitative conclusions, Fig. 5 clearly demonstrates that there were major discrepancies in FGF-2 quantities measured with the different approaches. This becomes even more obvious by considering all four methods applied for analysis of FGF-2 binding to heparin–starPEG networks.

As shown in Fig. 6, except for the comparison of cLSM and HPLC ($P > 0.05$), there were significant differences between the results obtained with different approaches

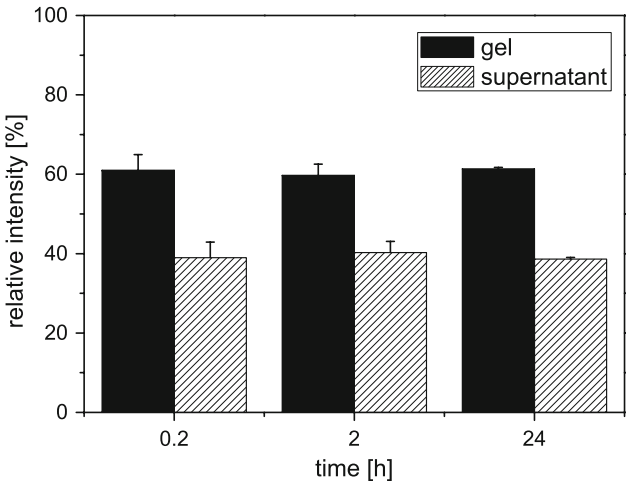

Fig. 4 Average fluorescence intensity of TAMRA-labeled FGF-2 in the supernatant (solution) and in the gel body (average over three Z-lines for each gel). Measurements were performed by using cLSM

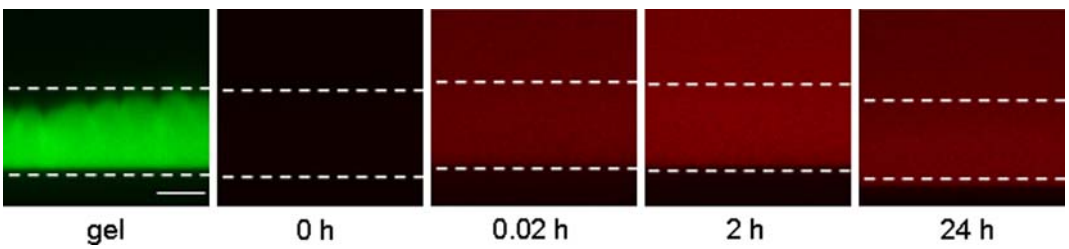

Fig. 3 Qualitative FGF-2 uptake experiments: Alexa 488-labeled surface-bound gel material (green, left) was incubated with TAMRA-labeled FGF-2 (red), pictures show the X-Z-cLSM scan of the gel body at four time points 0 h, 0.02 h, 2 h and 24 h. White dotted lines show the upper and lower gel boundary. Fluorescence intensity was quantified as the average over three Z-lines for each gel in the supernatant and in the gel body. Scale bar: 10 μm. (Color figure online)

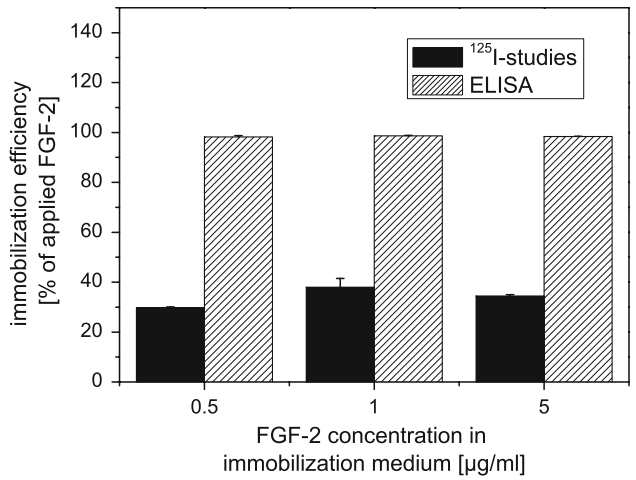

Fig. 5 FGF-2 immobilization efficiency in dependence on the protein concentration obtained by analysis with different methods. Values are expressed as percentage of FGF-2 bound to the heparin–starPEG hydrogels based on the initially applied protein amount

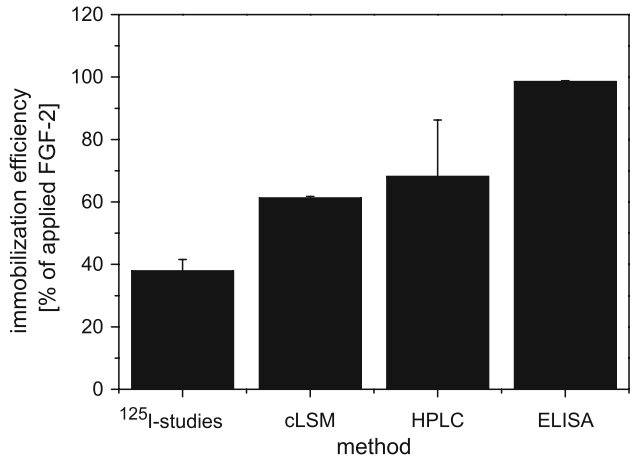

Fig. 6 FGF-2 immobilization efficiency obtained by analysis with different methods. Values are expressed as percentage of FGF-2 bound to the heparin–starPEG hydrogels based on the initially applied protein amount

($P < 0.01$). While ELISA data showed almost no protein remaining in the supernatant (immobilization efficiency of 98.6%), only about 38% of the deployed FGF-2 was detected in the network by radiolabeling studies. Immobilization efficiencies obtained by fluorescence labeling and amino acid analysis were 61.4% and 68.4%, respectively.

3.2.3 FGF-2 release studies

After analyzing the FGF-2 uptake into heparin–starPEG hydrogels, experiments on the release of the protein were performed. Due to methodological problems related to the lower sensitivity of cLSM and amino acid analysis, results

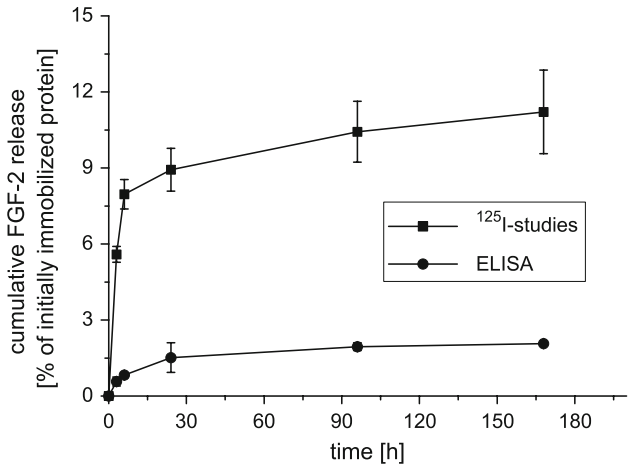

Fig. 7 Cumulative FGF-2 release obtained by analysis with different methods. Values are expressed as percentage of FGF-2 released from the heparin–starPEG hydrogels based on the initially immobilized protein amount

were only obtained from ^{125}I-studies and ELISA. Figure 7 illustrates the cumulative release of the protein measured for a time period of 7 days. FGF-2 release from the network showed an initial burst over the first 6 h (8% for radiolabeling studies; 0.8% for ELISA), followed by a rather slow release over the course of 1 week (11.2% for radiolabeling studies; 2.1% for ELISA). Similar qualitative conclusions concerning the release kinetics could be drawn from results obtained by both methods. However, once again there were significant differences in the quantities of FGF-2 release determined by the two different approaches ($P < 0.01$).

4 Discussion

The results presented in this study help to clarify the advantages and drawbacks of currently used techniques for the characterization of uptake and release of signaling molecules to/from hydrogel materials. Since growth factors exert their function already at concentrations within the nanogram and picogram range [3, 13], quantities supplied by the carrier scaffold have to be analyzed thoroughly. In this study, radiolabeling experiments, ELISA, fluorescence labeling and amino acid analysis were utilized for the detection of biomaterials-associated FGF-2. The application of all four approaches made it possible to follow the FGF-2 binding to heparin–starPEG networks over a wide range of concentrations.

Heparin–starPEG hydrogels were formed via reaction of amino end-functionalized starPEG with EDC/s-NHS-activated carboxylic acid groups of heparin as previously reported elsewhere [26]. The gel networks closely mimic

the characteristics of the ECM by containing large quantities of heparin which bind and stabilize numerous growth factors. The mesh sizes of the gel system were adjusted to allow for diffusion-controlled binding and release of smaller proteins such as FGF-2 (diameter of ~3–4 nm, [30]) while bigger proteins are excluded.

In this study, this fact was confirmed by detection of fluorescently (TAMRA) labeled FGF-2 within the gels using cLSM. After applying the protein to the gel a homogenous distribution of the molecules within the network was observed already after an incubation time of only 1 min and was maintained as time passed. These findings demonstrate that there were no significant structural heterogeneities in the scaffold and that the mesh sizes of the hydrogel did not restrict diffusion of this small molecule. Moreover, cLSM seems to be an efficient qualitative method to follow uptake of the FGF-2 in situ.

Quantitative analysis of FGF-2 immobilization within heparin–starPEG hydrogels was performed by detection of radiolabeled and fluorescently labeled FGF-2, ELISA and amino acid analysis. Results obtained by the different methods showed a linear correlation between the immobilized FGF-2 amount and the applied protein solution concentration during loading (up to 50 μg/ml FGF-2). Due to the high content of heparin in the heparin–starPEG networks, after incubation with 50 μg/ml protein the molar ratio of heparin to FGF-2 was still 26:1. Since each heparin molecule can bind several molecules of FGF-2 [31], saturation of binding will occur only at concentrations much higher than used here, demonstrating that FGF-2 immobilization can be tuned over a broad range. Qualitatively, this conclusion could be drawn from results obtained by all three methods applied. However, when comparing the immobilized FGF-2 amount determined by radioisotope- or fluorescence labeling, ELISA and amino acid analysis significant differences were found.

Due to of the different principles of protein quantification in the gel body or the supernatant, all of these approaches require specific experimental conditions. To detect FGF-2 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 already during bacterial synthesis [32]), all of these treatments, as well as the presence of the label itself increase the probability of structural alterations of the growth factor [27]. This might of course influence the interaction with heparin during the immobilization procedure. Moreover, weakly attached label may become released from the protein [27] during uptake and release studies and thus influence the result 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, the high sensitivity and the possibility to detect the protein of interest 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 [17]. Any relative quantification of FGF-2 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 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 in contact with 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 advantage of quantifying 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 solution. Since after hydrolysis the analyzed peaks (HPLC) originate from the whole sample surface (loaded and non-loaded regions of the gel), signals coming from unloaded gel disturb the quantification of the protein. Due to these restrictions, alternative setups had to be used for FGF-2 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 immobilization efficiency determined by detection of fluorescently labeled FGF-2 and amino acid analysis after hydrolysis as it was already identified as the major source of physical FGF-2 loss in the studies of [17].

ELISA experiments could be performed using non-labeled FGF-2 under conditions minimizing the contact area for non-specific protein interactions with ‘foreign’ glass or plastic surfaces. Since almost no protein was detected in the supernatant of the incubation medium, the

majority of it seemed to be bound by the heparin–starPEG hydrogels. This clearly illustrates the suitability of the gel system for the efficient storage of FGF-2 within a broad range of protein concentrations.

Additionally, the potential of the different methods to follow the release of FGF-2 was investigated via ^{125}I-experiments and ELISA. 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 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 [29]. Consequently, it could not be applied for analysis of FGF-2 release within the scope of concentrations used for immobilization to heparin–starPEG hydrogels in this study.

However, although FGF-2 sequestering was only investigated by ^{125}I-studies and ELISA similar qualitative results could be obtained.

FGF-2 release from the network showed an initial burst for the first 6 h. Such burst characteristics are often attributed to surface effects [33] and could be caused by a FGF-2 fraction entrapped in the meshwork but not bound specifically to heparin. After that FGF-2 release continued slowly over the course of 1 week indicating the potential of the material for applications with need for long-term release profiles of growth factors. Once again differences concerning the quantities of FGF-2 released from the networks were determined by both 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 were detected via radiolabeling studies. One explanation for that could be that the presence of the label might weaken the interaction of FGF-2 and heparin, thereby leading to a faster release compared to the native protein which was used for ELISA studies. However, despite the fact that quantities of released FGF-2 detected via both methods differed, in any case the determined overall release was low. This is assumed to be caused by the huge molar excess of heparin compared to FGF-2, which led to strong interaction with the protein. Nevertheless, given this finding and the fact that the heparin–starPEG hydrogels were far from any saturation of binding FGF-2, obviously release characteristics can be adjusted by the initial amount of protein loaded which can be tuned over a wide range of concentrations.

5 Conclusion

In this study, FGF-2 binding and release to/from heparin–starPEG hydrogels was monitored by different analytical methods. Results show that all methods can be applied to analyze the binding of FGF-2 to heparin–starPEG matrices, while the release dynamics was possible to follow by radiolabeling and ELISA-techniques only. ELISA based detection turned out to be the best way to precisely characterize binding and release of the protein to/from hydrogel matrices since the method uses unlabeled growth factor and permits to minimize artifacts. In summary, our study demonstrates that a careful choice of the experimental parameters is key to a meaningful analysis of growth factor uptake and release to/from biomaterials.

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