Post-loading of proangiogenic growth factors in PLGA microspheres

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ABSTRACT

Active self-encapsulation (ASE) is a recently developed post-loading method based on absorption of (positively charged) proteins in microporous PLGA microspheres loaded with negatively charged polysaccharides (trapping agents). The aim of this study was to investigate ASE for simultaneous loading and controlled release of multiple growth factors. For this purpose, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and insulin-like growth factor (IGF) were loaded in microspheres containing high molecular weight dextran sulfate (HDS) as trapping agent; loading was performed in a concentrated growth factor solution of low ionic strength and of pH 5 under conditions at which the proteins are positively charged. Subsequent pore closure was induced by incubation of the growth factor-loaded microspheres at 42.5 °C, i.e. above the T_g of (hydrated) PLGA ($\sim$30 °C). A 1:1:1 combination of VEGF, FGF and IGF was loaded with high loading (4.3%) and loading efficiency (91%). The *in vitro* release kinetics and bioactivity of loaded growth factors were studied for 4 weeks using ELISA and an endothelial cell proliferation assay, respectively. While IGF was released quickly, VEGF and FGF were continuously released for 4 weeks in their bioactive form, whereby a growth factor combination had a synergistic angiogenic effect. Therefore, ASE is a suitable method for co-loading growth factors which can provide sustained release profiles of bioactive growth factors, which is attractive for vascularization of biomaterial implants.

1. Introduction

Recombinant growth factors have the potential to regulate and reprogram stem cells and immune cells [1–3]. Further, recombinant growth factors are promising biotherapeutics within the field of regenerative medicine for functional tissue-engineered constructs [4,5]. Tissue-engineered constructs are based on biomaterial scaffolds (implants) in which cells are incorporated [6,7]. Upon transplantation, the formation of blood vessels in the vicinity of the implant is crucial for facilitating the supply of transplanted cells with oxygen and nutrients and to ensure clearance of waste products [8]. Angiogenesis towards the implants can be stimulated and triggered by loading and release of proangiogenic growth factors into/from the implant, of which vascular endothelial growth factor (VEGF) is the most prominent growth factor [9–11]. Besides VEGF, also other growth factors play an important role

in angiogenesis, such as fibroblast growth factor (FGF) and insulin-like growth factor (IGF) [12–14]. Several studies in rodent models suggest that the formation of functional vessels encompassing the device is achieved upon approximately four weeks after implantation [15–17]. Although continuous growth factor levels in the vicinity of the implant are sometimes deemed desirable [18], their short half-life after systemic administration is a major limitation to achieve persistent tissue levels [19] and in fact can lead to undesired side effects [20]. Controlled drug delivery systems, such as polymeric microspheres, offer the possibility of local controlled release of growth factors in a desired time frame [4,11,21,22]. Commonly, proteins, such as growth factors, are formulated in microspheres based on poly(lactic-co-glycolic acid) (PLGA), a well-known and well-characterized biodegradable polymer, by a double emulsion method [23–26]. However, the applied methods to prepare protein-loaded microspheres (e.g. emulsion evaporation technologies)

expose proteins to shear stresses and organic solvents, which can adversely affect their structural integrity and biological activity [27–29].

As an alternative, post-loading of the protein into PLGA microspheres can circumvent the limitation described above, foremost by mitigating shear stress and the exposure to organic solvents. An important additional advantage of this method is the possibility to sterilize the microspheres prior to loading the desired drug e.g. a pharmaceutical protein, thereby avoiding exposure of the bioactive agent to sterilization methods, reducing production costs and increasing production efficiency [30]. The post-loading method active self-encapsulation (ASE) has been developed recently, whereby a negatively charged biopolymer ("trapping agent") loaded in porous PLGA microspheres is used to bind and trap (load) positively charged protein therapeutics through electrostatic interaction under mild conditions (room temperature, buffer of neutral pH and low ionic strength). Afterwards, the temperature is raised above the glass transition temperature of PLGA which leads to the closure of pores (Fig. 1) [31–33]. Upon pore closure, the loaded protein is entrapped (far right schematic microsphere, Fig. 1) and its release is governed by complex erosion of the polymeric matrix (influenced by co-encapsulated trapping agent and poorly water-soluble base), rather than by its diffusion through pores of the polymeric matrix (center schematic microsphere in Fig. 1). During degradation, acidic degradation products are formed, resulting in a decrease of the pH within the polymeric matrix [34,35], which in turn might result in loss in bioactivity of the loaded protein [36–38]. The incorporation of the poorly water-soluble basic additive $MgCO_3$ in the microspheres neutralizes the acid degradation products and facilitates mass transfer of both the encapsulated protein and the sequestered acids, and thus strongly inhibits the acidification of polymeric matrix during degradation [35,38–40].

ASE has been achieved with a high loading content (up to 4.5 wt%), high loading efficiency (up to 100%) in PLGA microspheres of the model protein lysozyme (a protein of 14 kDa and a pI of 11.4 [41]). Further, growth factors VEGF and FGF were loaded with high loading and loading efficiency in PLGA microspheres using high molecular weight dextran sulfate (HDS) as trapping agent and VEGF was slowly released *in vitro* with minimal loss of heparin-binding activity [32]. Although these studies have shown the potential to encapsulate growth factors into PLGA microspheres, a combination of several growth factors will have stronger proangiogenic effects and it therefore seems attractive to prepare slow-releasing particles which are co-loaded with an optimal blend of growth factors that can induce angiogenesis [42–44].

In this study, we investigated the loading and subsequent release of

VEGF in PLGA microspheres using three different trapping agents, i.e. the anionic polysaccharides dextran sulfate, hyaluronic acid and chondroitin sulfate. We also explored the possibility of loading individual growth factors (i.e. VEGF, FGF and IGF) as well as the combination of such growth factors in one microsphere formulation, studied the subsequent release of the loaded growth factors from the microspheres and assessed their *in vitro* bioactivity with an endothelial cell proliferation assay.

2. Materials and methods

2.1. Materials

PLGA (50:50, lauryl end-capped, i.v. 0.59 dL/g) was purchased from Lactel Durect Corporation (Birmingham, AL, USA). PVA (9,000 – 10,000 kDa, 80% hydrolyzed), D-(+)-trehalose dihydrate, high molecular weight dextran sulfate (~500 kDa), fluorescein isothiocyanate (FITC)- dextran sulfate sodium (~500 kDa), sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$), bovine serum albumin (BSA), magnesium carbonate ($MgCO_3$), sodium azide (NaN_3) and succinic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Tween 20 was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Cyanine 5 (Cy5)-labelled lysozyme was purchased from NanoCS (New York, NY, USA). Chondroitin sulfate sodium salt from shark cartilage (~63 kDa) and hyaluronic acid sodium salt from Streptococcus equi (50 – 70 kDa) were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Recombinant human vascular endothelial growth factor ($VEGF_{165}$, 38.2 kDa) was purchased from PeproTech Inc. (Rocky Hill, NJ, USA). Recombinant human insulin-like growth factor (IGF-1, 7.6 kDa) and recombinant human fibroblast growth factor (FGF2, basic FGF, 16 kDa) were purchased from R&D Systems (Abington, United Kingdom). Gibco® Dulbecco's Phosphate Buffered Saline (DPBS, 10x; when diluted to a 1X working concentration, PBS contains 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4) was purchased from Thermo Fisher Scientific (Amsterdam, the Netherlands). Collagen I (from rat tail) was obtained from Corning (Corning, NY, USA). All other reagents and solvents were obtained from Sigma Aldrich, if not specified differently.

2.2. Methods

2.2.1. Preparation of microspheres

Trapping agent-loaded PLGA microspheres were prepared by a double emulsion solvent evaporation method similarly as described previously [32]. The trapping agents used in this study were high molecular weight dextran sulfate (HDS), FITC-labelled high molecular weight dextran sulfate (FITC-HDS), chondroitin sulfate (CS) and hyaluronic acid (HA); their chemical structures are shown in Fig. 2.

A solution of 250 mg/ml PLGA in 1 ml DCM supplemented with 3 wt % $MgCO_3$ (particle size $\leq$ 90 μm, wt% referring to PLGA) was emulsified with 200 μl of a trapping agent solution in water (4 wt% target loading, supplemented with 3 wt% trehalose) using a Virtis Tempest I.Q.[2] Sentry Microprocessor homogenizer (Triad Scientific, Manasquan, NJ, USA; settings: 18,000 rpm, 60 s) in an ice bath. Next, 2 ml of a 5% PVA solution was added to the primary emulsion and vortexed for 60 s. The resulting double emulsion was added to 100 ml of 0.5% PVA solution in water and stirred for 3 h for DCM evaporation. The hardened microspheres were sieved (63 μm-mesh and 20 μm-mesh sieves, Newark Wire Cloth Company, Newark, NJ, USA) to collect the microspheres fraction of 20 to 63 μm. Next, the microspheres were washed with double-distilled water and freeze-dried overnight. For microspheres prepared without trapping agents ("none"), a 1 ml solution of 250 mg/ml PLGA in DCM supplemented with 3 wt% $MgCO_3$ was emulsified with 200 μl of water, followed by the preparation steps described above. The size distribution of the microspheres was determined by optical particle sizing (Accusizer 780, Santa Barbara, California, USA).
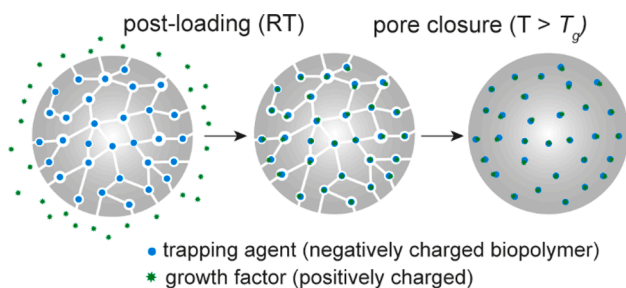

Fig. 1. Schematic image of the active self-microencapsulation post-loading method.[28] First, negatively charged trapping agent-containing porous PLGA microspheres are incubated at room temperature with a growth factor in a buffer of low ionic strength at which the protein carries a positive charge, whereby the positively charged growth factor (green star) is loaded into the porous microspheres through electrostatic interaction with the negatively charged biopolymer (trapping agent, in blue). Subsequently, the pores of the microspheres are closed at a temperature above the glass transition temperature of PLGA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
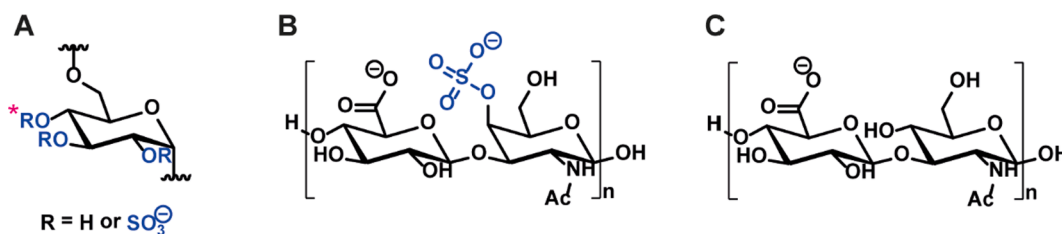
Fig. 2. Chemical structures of trapping agents loaded in the PLGA microspheres. A:) dextran sulfate, *site of FITC-labelling, degree of substitution 0.001 – 0.006 mol FITC / mol glucose (as stated by the supplier), B: chondroitin sulfate, C: hyaluronic acid.

2.2.2. Elemental analysis of trapping agents

The sulfur content of trapping agents dextran sulfate and chondroitin sulfate was determined by elemental analysis. Accurately weighed samples of ~ 5 mg were analyzed on a Vario Micro Cube elemental analyzer in CHNS mode (Elementar GmbH, Langenselbold, Germany) according to supplier's recommended equipment settings.

2.2.3. Active self-encapsulation of growth factors in microspheres

Growth factor solutions (1.0 mg/ml of VEGF, FGF, IGF or a 0.33/0.33/0.33 mg/ml combination of VEGF, FGF and IGF) were prepared by reconstituting lyophilized growth factors (as received by the supplier, molecular weights and isoelectric points stated in Table 1) in ASE loading buffer, consisting of 5 mM succinate, 275 mM trehalose (pH 5). Next, 20 mg of freeze-dried PLGA microspheres with and without loaded trapping agent were suspended in 1 ml of growth factor solution and incubated for 48 h at room temperature (~21 °C) under mild agitation. Then, the sample was incubated for 42 h at 42.5 °C under mild agitation to induce pore closure. Afterwards, the sample was centrifuged at 8000 rpm ($5724 \times g$, Sigma 1–16 K, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) for 5 min and the supernatant was collected for determination of the fraction non-loaded growth factors (see Section 2.2.4). The microspheres were washed twice with 1 ml of deionized water and freeze-dried overnight.

2.2.4. Determination of growth factor loading by Coomassie plus assay

The growth factor content of the microspheres was determined indirectly by quantifying the protein content in supernatants collected after ASE by Coomassie Plus protein assay (Thermo Scientific, Amsterdam, the Netherlands). In short, 10 µl of growth factor standards or supernatant samples were pipetted into the wells of a 96-well plate. Next, 200 µl of Coomassie Plus reagent was added to the wells. The plate was incubated for 10 min at room temperature after which the absorbances of the wells at 595 nm was recorded using a SPECTROstar Nano plate reader (BMG Labtech, Ortenberg, Germany).

2.2.5. Scanning electron microscopy

The morphology of freeze-dried microspheres was analyzed by scanning electron microscopy (SEM; Phenom, FEI Company, Eindhoven, the Netherlands). Lyophilized microspheres were transferred onto 12-mm diameter aluminum specimen stubs (Agar Scientific Ltd., Essex, United Kingdom) using double-sided adhesive tape. Next, the samples were frozen in liquid nitrogen and cut with a razor. Prior to analysis, the microspheres were coated with platinum using an ion coater under vacuum.

2.2.6. Confocal fluorescence microscopy

FITC-HDS loaded PLGA microspheres were prepared as described in Section 2.2.1. The microspheres were loaded with cyanine-5-labelled lysozyme (Cy5-LYZ) at 1 mg/ml concentration in 10 mM phosphate ($NaH_2PO_4 \cdot H_2O$) buffer (pH 7.0) as described in Section 2.2.3. The microspheres were washed with water before freeze-drying to remove traces of buffer salts. Freeze-dried Cy5-LYZ-loaded microspheres were fixed with ProLong Gold antifade reagent (Invitrogen, Thermo Fisher Scientific, Eugene, USA) on a glass slide for 30 min. FITC-HDS and Cy5-LYZ in microspheres were imaged using a Leica SP8 confocal fluorescence microscope (Leica, Buffalo Grove, USA; FITC-HDS (green channel) at λ_{ex} 492 nm and λ_{em} 518 nm; Cy5 (red channel) at λ_{ex} 650 nm and λ_{em} 670 nm).

2.2.7. Formulations for investigating the effect of ionic strength and pore closure on VEGF loading

Experiments were performed to study the effect of ionic strength of the buffer on the loading of VEGF in microspheres containing HDS as trapping agent, without subsequent pore closure. HDS-containing microspheres prepared as described in Section 2.2.1. were incubated with 1 mg/ml VEGF in either ASE buffer (5 mM succinate, 275 mM trehalose (pH 5)), 10 mM HEPES pH 7.4 or Dulbecco's PBS pH 7.4 (composition stated in Materials Section 2.1) for 48 h at room temperature under mild agitation. Afterwards, the suspension was centrifuged at 8000 rpm for 5 min ($5724 \times g$, Sigma 1–16 K, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), the supernatant was collected and the microspheres were washed twice with 1 ml of deionized water. The protein concentration in the supernatants was determined by the Coomassie Plus assay (see Section 2.2.4).

To study possible adsorption of VEGF on the surface of empty microspheres, the pores of HDS-containing microspheres were closed in a two-step process, similar to the ASE process described in Section 2.2.2. HDS-containing microspheres were incubated in 1 ml of ASE buffer (5 mM succinate, 275 mM trehalose (pH 5)) for 48 h at room temperature, followed by incubation at 42.5 °C for 42 h. Then, the suspension was centrifuged at 8000 rpm for 5 min and the microspheres were subsequently washed twice with 1 ml of deionized water and freeze-dried overnight. Next, the freeze-dried microspheres were incubated with 1 ml of a 1 mg/ml VEGF solution in ASE buffer (5 mM succinate, 275 mM trehalose (pH 5.0)) for 48 h at room temperature under mild agitation. The suspension was then centrifuged and washed as described above, after which the unbound VEGF content was determined in supernatant as described in Section 2.2.4.

2.2.8. In vitro release characteristics of VEGF-loaded microspheres

Five mg microspheres were accurately weighed and suspended in 1 ml of *in vitro* release buffer ("IVR buffer"), which consisted of Dulbecco's PBS pH 7.4, supplemented with 10 mg/ml BSA, 0.02% Tween 80 and 0.02% NaN_3.The samples were incubated at 37 °C under gentle agitation. At different time points, the samples were centrifuged at 8000 rpm for 5 min ($5724 \times g$, Sigma 1–16 K, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The supernatant was removed and

Table 1
Molecular weights and isoelectric points of proteins used in this study.

Protein	Molecular weight [kDa] [a]	Isoelectric point (*pI*)
lysozyme (LYZ)	14	11.4 [38]
vascular endothelial growth factor (VEGF)	38.2	8.5 [42]
fibroblast growth factor (FGF)	16	9.6 [43]
insulin-like growth factor 1 (IGF)	7.6	8.5 [44]

[a] : as stated by supplier.

replaced by fresh release buffer. The concentration of growth factor in the release samples was quantified with ELISA (Section 2.2.9.1).

2.2.9. Bioactivity of released growth factors

Samples of released growth factors were obtained by incubating 1 mg of accurately weighted microspheres in 1 ml of "bioactivity IVR buffer" (consisting of Dulbecco's PBS pH 7.4, supplemented with 0.5% BSA, 30 µg/ml gentamicin and 15 ng/ml amphotericin, 0.2 µm filtered) at 37 °C under gentle agitation. At appropriate time points, the samples were centrifuged at 8000 rpm for 5 min (5724 × g, Sigma 1–16 K, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The supernatant was removed and the release medium replaced by fresh buffer. The collected supernatants were analyzed for growth factor concentration using an ELISA (as described in Section 2.2.9.1) and used for bioactivity assays (as described in Section 2.2.11 and 2.2.12).

2.2.10. Quantification of released growth factors by ELISA

IVR samples were analyzed by a sandwich ELISA (Human VEGF DuoSet ELISA, Human FGF basic/FGF2 DuoSet ELISA, Human IGF-I/IGF-1 DuoSet ELISA, all R&D Systems, Abingdon, United Kingdom) according to the manufacturer's protocol. The stock solutions of the ELISA kits were used for calibration in the concentration range of 31 – 2000 pg/ml for VEGF and IGF, or 15.6 – 1000 pg/ml for FGF. Release samples were diluted with reagent diluent to fall within the working range of the assay and measured in duplicate. ELISA plates were read at 450 nm using a SPECTROstar Nano plate reader (BMG Labtech, Ortenberg, Germany).

2.2.11. Bioactivity of released growth factors by endothelial cell proliferation assay

The bioactivity of released growth factors from microspheres was analyzed by Alamar Blue proliferation assay with human umbilical vein endothelial cells (HUVECs), as described previously [45]. The assay was performed in growth factor-poor medium ("bioactivity medium"), consisting of M199 medium supplemented with 2.5% fetal bovine serum (FBS). In brief, HUVECs were seeded at 4000 cells/well in collagen-coated 96 well plates. Release samples and standards, diluted in bioactivity medium, were added to the wells and the plate was incubated for 96 h at 37 °C / 5% CO_2. The fluorescence, caused by the proliferating cells after addition of Alamar Blue, was measured with a microplate reader (λ_{ex} 530 nm and λ_{em} 600 nm; Berthold Mithras LB 940, Germany).

Release samples were diluted in bioactivity medium to fall within the responsive range of unformulated VEGF, i.e. corresponding to a concentration of 1–10 ng/ml VEGF based on loading data. A calibration curve with non-formulated VEGF was fitted with by a four parameter logistic regression model using GraphPad prism 7, and used to calculate the concentrations of VEGF in the release samples which were subsequently used to determine the cumulative release of bioactive VEGF normalized to the total release of bioactive VEGF at the end of the IVR study (time point 28 days).

For testing the bioactivity of the released growth factors VEGF, FGF and IGF, release samples were diluted in bioactivity medium to fall within the responsive range of VEGF, FGF and IGF combined in a 1:1:1 wt ratio, corresponding to a relative cell proliferation of 1 – 9.

2.2.12. Bioactivity of released VEGF by bioluminescent receptor-based cell assay

The bioactivity of released VEGF was additionally analyzed by a bioluminescent receptor-based cell assay (VEGF Bioassay, Promega, Madison, WI, USA) according to the manufacturer's instructions. This assay relies on the binding of VEGF to the VEGFR-2 receptor (also known as kinase insert domain receptor (KDR)) on KDR/NFAT-RE HEK 293 cells, which induces a luminescent response via intracellular NFAT-RE signaling [46]. In brief, KDR/NFAT-RE HEK 293 cells were thawed, reconstituted and seeded in 96-well plates in assay medium (DMEM with 10% FBS, according to the manufacturer's protocol). Non-formulated

VEGF was added in concentrations 0 – 1000 ng/ml, as well as IVR supernatants diluted 1200 – 6000-fold in assay buffer. Reporter cells were incubated with calibration or IVR samples for 6 h at 37 °C before adding Bio-GloTM reagent. After incubation at room temperature for 5–10 min, luminescence was measured on a microplate reader (Mithras LB 940, Berthold, Bad Wildbad, Germany) using an integration time of 0.25 s/well. Calibration and IVR samples were measured in triplicate. Results are shown as relative proliferative signals defined by the luminescence signal normalized by the luminescence signal of cells incubated without VEGF. A calibration line with non-formulated VEGF was fitted by a 4PL curve-fit with GraphPad prism 7, and used to determine the cumulative release of bioreceptor-assay active VEGF normalized to the total release of bioreceptor-assay active VEGF at the end of the IVR study (time point 28 days).

2.2.13. Statistical analysis

Data are presented as average with SD. Statistical analysis was performed with GraphPad Prism7 using the Holm-Sidak multicomparison test. Differences between the analyzed groups were considered significant if $p < 0.05$.

3. Results and discussion

3.1. Loading of VEGF in trapping agent-containing microspheres

The aim of this study was to load proangiogenic growth factors by active self-encapsulation (ASE) in PLGA microspheres using the negatively charged biopolymers dextran sulfate (HDS), chondroitin sulfate (CS) and hyaluronic acid (HA) as trapping agents, and to demonstrate co-encapsulation/delivery of the growth factors by this approach. These polysaccharides were selected for their binding capacities with positively charged proteins, such as the proteins used in this study, VEGF, FGF, IGF and lysozyme (LYZ) (*pI* reported in Table 1). Porosity of PLGA microspheres was introduced through the addition of sugar-leaching agent trehalose [31], which also served as lyoprotectant [47]. Magnesium carbonate ($MgCO_3$) was added as acid-neutralizing agent. Of the three trapping agents used in this study, dextran sulfate had the highest molecular weight (500 kDa) and highest number of sulfate groups (1.75 per glycopyranose unit, sulfur content (14%)), as shown in Table 2. Chondroitin sulfate and hyaluronic acid had lower average molecular weights, both ~60 kDa, and lower or none sulfate content, i.e. 5% (for CS; corresponding to 0.7 sulfate group per disaccharide unit) and 0% (for HA).

Table 2

Characteristics of biopolymers high molecular weight dextran sulfate (HDS), chondroitin sulfate (CS) and hyaluronic acid (HA) used as trapping agents for microspheres (average molecular weight and sulfur content) and VEGF loading and loading efficiency in microspheres containing either trapping agents HDS, CS, HA or no trapping agent. Average ± SD of n = 3 loading experiments.

trapping agent	molecular weight [kDa]	sulfur content [%] [a]	MSPs size [average ± SD] [b]	VEGF loading content [wt %] [c]	VEGF loading efficiency [%]
HDS	500	14	72 ± 23 µm	4.5 ± 0.0	98 ± 0
CS	~63	5	70 ± 22 µm	1.3 ± 0.6	28 ± 13
HA	50–70	0	81 ± 23 µm	0	0
none	n.a.*	n.a.*	67 ± 32 µm	0	0

[a] : determined by elemental analysis.
[b] : volume-weight size distribution.
[c] : indirect determination by protein quantification of the supernatant by Coomassie Plus assay.
* : not applicable. MSPs: microspheres.

Lyophilized trapping agent-containing microspheres had comparable size and polydispersity, as reported in Table 2. In the first step of the active self-encapsulation method, microspheres were incubated in a concentrated growth factor solution of pH 5, at which the loaded proteins have a positive charge. The growth factor solution had a low ionic strength and also contained trehalose as lyoprotectant. Subsequently, the pores of the PLGA microparticle matrix were closed by incubating the microsphere dispersions at temperature of 42.5 °C which is above the glass transition temperature (T_g) of the polymer (i.e. $\sim$ 45 °C for the dry polymer but $\sim$ 30 °C for the hydrated polymer [48,49]).

The ASE method of loading biomolecules in polymeric microspheres has been reported primarily for PLGA microspheres [50], but this concept is not necessarily limited to PLGA only. Microspheres prepared with polymers with T_g at reasonable temperatures (i.e. 20–40 °C) will show similar self-healing behavior under conditions that are considered mild for protein biopharmaceuticals.

The highest VEGF loading (4.5 wt% loading) and nearly 100% loading efficiency, was obtained for the HDS-containing microspheres. A lower VEGF loading and loading efficiency, i.e. 1.3 wt% loading and 28% loading efficiency, was observed for CS-containing microspheres. No VEGF was loaded in HA-containing microspheres as well as placebo microspheres without trapping agent.

Microsphere morphologies before and after ASE, in the legend described as before and after pore closure, are shown as SEM images in Fig. 3. Before ASE all microsphere formulations showed a porous morphology, both on the surface and inside of the microspheres (Fig. 3, left column). After the pore closure step, the surfaces of all microsphere formulations were smooth (Fig. 3, right column). Cross-sections of microspheres after pore closure revealed a less porous network, i.e. showing fewer small pores in the core of the particles, as compared to cross-sections of microspheres before pore closure.

Several aspects play a role in the differences in VEGF loading efficiency found for the different trapping agents. An important aspect is the density of sulfate groups of the trapping agents, as VEGF binding increases with increasing degrees of sulfation [51]. In our study, HDS contained a higher number of sulfate groups per monomer unit than CS whereas HA does not contain sulfate groups but has only carboxylate groups which apparently did not facilitate VEGF trapping. Our results hence show a clear correlation between sulfate density of the trapping agent and ASE loading of VEGF in line with a previous study [51].

Another possible explanation for the lower VEGF loading efficiencies in CS- and HA-microspheres could be due to differences in loading efficiencies of the trapping agents themselves, which may correlate to their respective molecular weights.

Unfortunately, our attempts to quantify the loading efficiencies of the trapping agent were not successful. Among others, we have tested the dissolution and destruction of microspheres in a high pH buffer or organic solvent, followed by GPC analysis. However, no peaks for the trapping agents were observed, not even for HDS- and CS-loaded microspheres which displayed good ASE properties and hence provided indirect evidence of the successful entrapment of these trapping agents.

In order to demonstrate the distribution of trapping agent and loaded proteins within the microspheres, microspheres loaded with fluorescently labelled HDS were prepared. The distributions of FITC-HDS and Cy5-labelled LYZ are shown in Fig. 4, demonstrating that both components (protein and trapping agent) were evenly distributed in the microsphere matrix. Microspheres used for this experiment were comparable to the microspheres used for VEGF loading.

3.2. Effect of ionic strength and pore closure on VEGF loading in microspheres

The effect of the ionic strength of the loading buffer used on the VEGF loading efficiency (without pore closure) is shown in table 3. The highest VEGF loading efficiency (92%) was found with ASE loading buffer (5 mM succinate, 275 mM trehalose, pH 5). A slightly lower VEGF
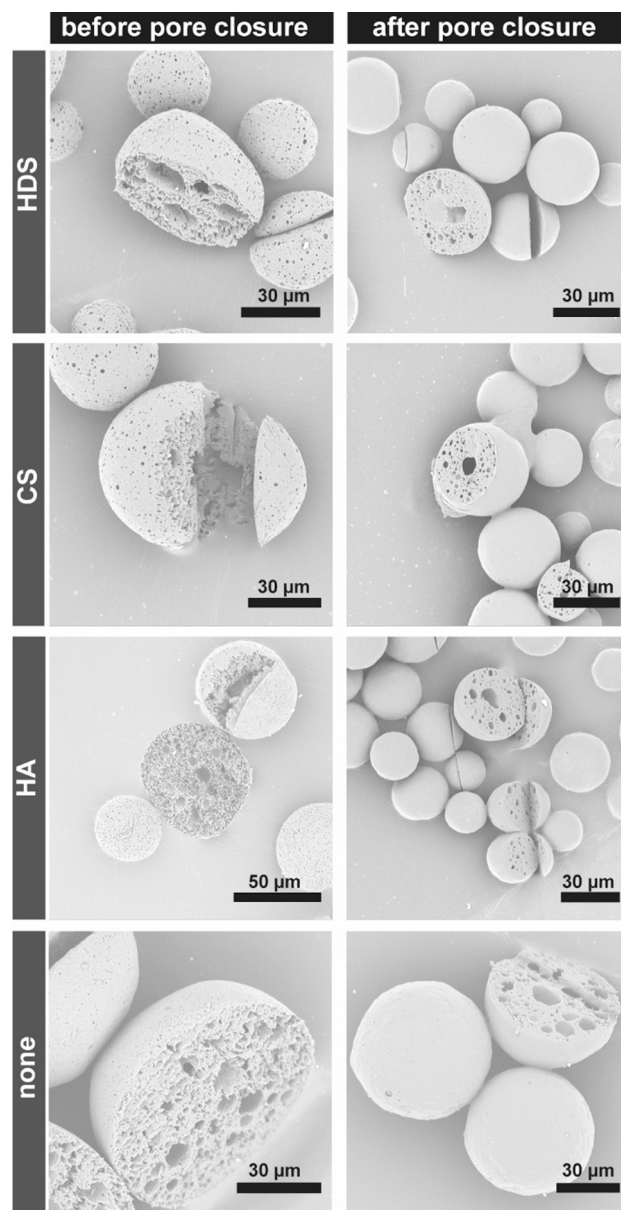

Fig. 3. SEM images of PLGA microspheres containing trapping agents HDS, CS, HA or no trapping agent ("none") before and after pore closure (loading of VEGF). The trapping agent per microsphere formulation is stated on the left column (HDS: high molecular weight dextran sulfate; CS: chondroitin sulfate; HA: hyaluronic acid; none: no trapping agent used).

loading efficiency (60%) was obtained with a loading solution at higher pH (pH 7.4) at similar ionic strength (10 mM HEPES). Due to VEGF's isoelectric point (8.5), the protein exhibits a higher positive charge at pH 5 than at pH 7.4 and therefore interacts stronger with HDS than at pH 5, leading to a higher loading efficiency. No VEGF was loaded when PBS pH 7.4 (with 150 mM ionic strength) was used as loading buffer. Most likely, the higher salt concentration shields the charges of both the trapping agent and the protein, thereby preventing ionic interaction between the protein and the trapping agent [52].

Further, HDS-containing microspheres after pore closure showed extremely low VEGF loading efficiency of 2% upon incubation of VEGF in ASE buffer (5 mM succinate, 275 mM trehalose, pH 5) (Table 3). This result confirmed that incubation of microspheres above the T_g of the polymer closes the pores and as a consequence changes the accessibility of the trapping agent, leaving only a small amount of surface-accessible binding sites for VEGF. Collectively, these data show that interaction of
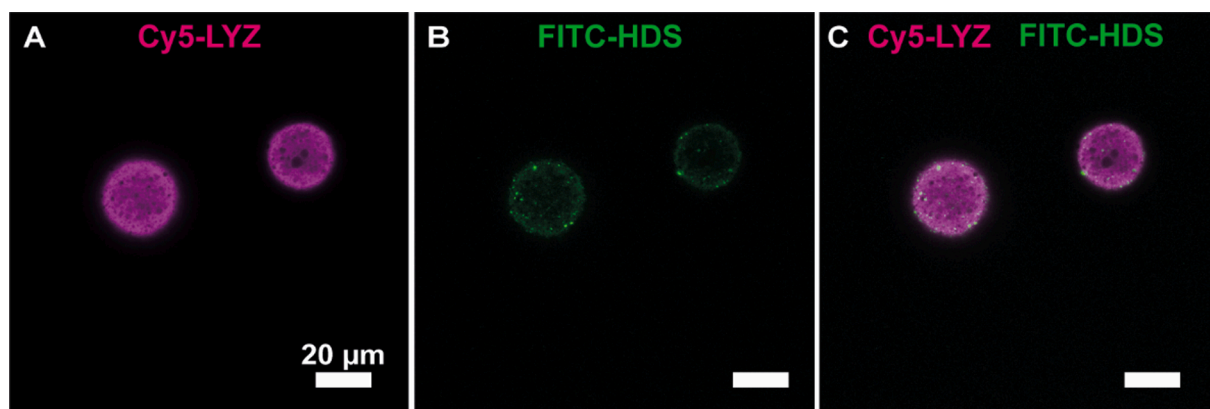
Fig. 4. Confocal fluorescence microscopy of Cy5-LYZ-loaded PLGA microspheres prepared with the trapping agent FITC-HDS. A: red channel shows Cy5-LYZ in microspheres, B: green channel shows FITC-HDS in microspheres, C: merge of red and green channels showing Cy5-LYZ and FITC-HDS. White scale bar represents 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Loading of VEGF in HDS-containing microspheres with varied ionic strength buffer and after previous microsphere pore closure. Average $\pm$ SD (n = 2).

Loading conditions	Loading buffer	VEGF loading efficiency [%]
incubation 48 h at RT, no pore closure	5 mM succinate, 275 mM trehalose, pH 5 (ASE buffer)	92 $\pm$ 6
	10 mM HEPES, pH 7.4	60 $\pm$ 4
	Dulbecco's PBS, pH 7.4 (containing 137 mM NaCl)	0 $\pm$ 0
pore closure, followed by incubation for 48 h at RT	5 mM succinate, 275 mM trehalose, pH 5 (ASE buffer)	2 $\pm$ 0

RT: room temperature.

VEGF with available HDS/trapping agents in the porous polymer matrix is essential for VEGF loading.

3.3. VEGF release from microspheres containing CS or HDS as trapping agent

The *in vitro* release of VEGF from CS- or HDS-containing PLGA microspheres was studied for 28 days which time frame has been shown to be advantageous for growth factor-induced vascularization of tissue-engineered constructs [15–17]. The cumulative release, as measured by ELISA, is shown in Fig. 5a. As previous studies with the VEGF ELISA demonstrated issues with quantitative recovery of the loaded VEGF [45], we normalized the release curves to the final cumulative release observed at the end of the incubation period (i.e. day 28). Based on ELISA detection, 10 and 40% of the loaded VEGF was recovered at day 28 for HDS- and CS-containing microspheres, respectively. This is equivalent to an absolute release of 4.4 µg VEGF/mg HDS-containing microspheres, and 1.5 µg VEGF/mg CS-containing microspheres. This low recovery might be ascribed to an underestimation of the protein concentrations in the release sample as determined by ELISA [45,53] and that part of the protein molecules is still entrapped in the microspheres at day 28. We do not except significant degradation of the encapsulated proteins, as the protein was neither exposed to shear stress nor organic solvents, and the basic additive ($MgCO_3$) neutralizes acidic degradation products of PLGA. VEGF remaining in the microspheres will be released when the PLGA microspheres are fully degraded (2–3 months) [50,54,55].

VEGF was released continuously from both CS- and HDS-containing microspheres in the time frame of 28 days (Fig. 5a, release curves were fitted with the Korsmeyer-Peppas model [56], as an indicator for diffusion-controlled release). The high initial release during the first day of incubation is likely caused by superficially encapsulated VEGF [57].

3.4. Bioactivity of released VEGF

The bioactivity of released VEGF was studied using IVR samples that were diluted and tested in two different VEGF activity assays of which the results are shown in Fig. 5c and d. We did not incubate microspheres directly with cells since the timeframe of release (several weeks) is quite different from the culture conditions of the cells (i.e. few days). Moreover, we did not incubate the microspheres in endothelial cell culture media in view of the instability of serum components upon long-term incubation at 37 °C (i.e. IVR conditions) which in turn would affect the outcome of bioactivity assays.

We used a proliferation assay in which endothelial cells were incubated in growth-factor deprived medium spiked with aliquots of the release samples. After dilution of the supernatants towards the proliferation range of 0–10 ng/ml of non-formulated VEGF, all release samples showed 2 to 4-fold induced proliferation (Fig. 5c), as compared to HUVEC cultured in control medium without added VEGF. Since each of the diluted release samples gave proliferative responses in the expected concentration range after dilution, we concluded that bioactivity of released VEGF was retained. We reconstructed cumulative release curves of bioactive VEGF (Fig. 5d), assuming that the specific bioactivity of VEGF (i.e. 2–4-fold proliferative response for 3–5 ng/ml VEGF) of formulated and reference VEGF was similar. Both CS- and HDS-microspheres showed continuous release of bioactive VEGF during the complete 28-day time frame of the experiment, with relative lower initial release (~30%) as compared to the ELISA detection of Fig. 5a. The release profiles showed no clear differences between HDS- and CS-microspheres.

The bioactivity of VEGF was further determined by a bioluminescence VEGF reporter assay using HEK 293 cells transfected with the KDR/VEGFR2 receptor. Fig. 5b shows the cumulative release curves of bioreceptor-active VEGF, which were reconstructed using the bioluminescence dose–response curve of unformulated VEGF (Fig. S1). The observed release profiles measured by the bioreceptor assay correspond to the release profile measured by ELISA shown in Fig. 5a, with relative high initial release followed by a sustained release phase up to 28 days.

The VEGF recoveries (total protein released at day 28) based on the bioassay were approximately double of those found with ELISA, i.e. 25% and 70% versus the loading content of VEGF of HDS and CS microspheres, respectively. This is equivalent to an absolute release of 11 µg VEGF/mg HDS- microspheres and 2.5 µg/mg CS-containing microspheres. This result may indicate an underestimation of the amount of released VEGF by ELISA, which can be ascribed to the loss of immuno-detectable epitopes in VEGF. While such VEGF molecules may not be detected by ELISA, the bioactivity of released VEGF was, within the experimental error, preserved. Such observations, i.e. low growth factor
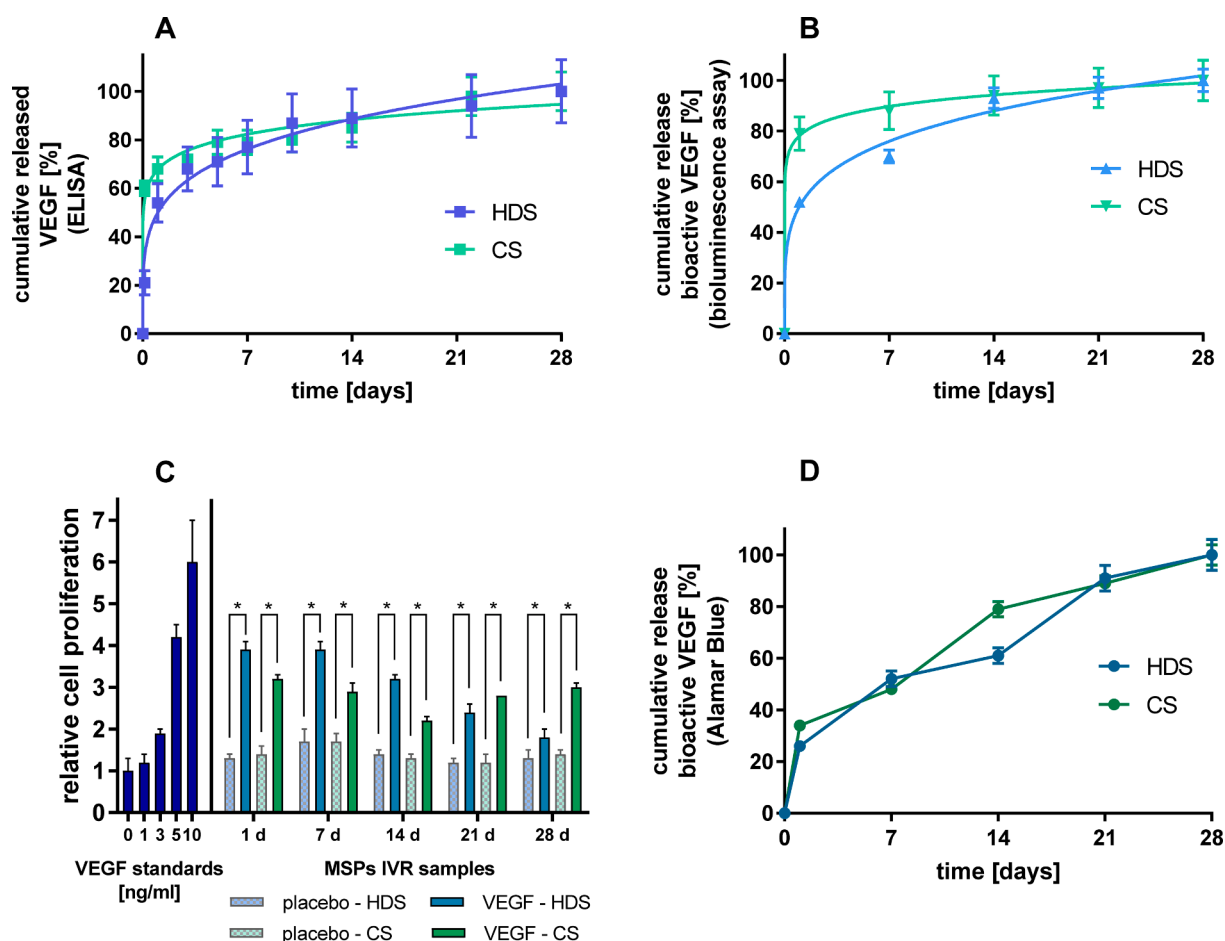
Fig. 5. A: Cumulative release of VEGF from PLGA microspheres prepared with trapping agents HDS or CS. *In vitro* release studies were performed at 37 °C in Dulbecco's PBS pH 7.4, supplemented with 10 mg/ml BSA, 0.02% Tween 80 and 0.02% NaN$_3$ under gentle agitation. The VEGF concentration in the release samples was quantified by ELISA. The cumulative release of VEGF was normalized to the cumulative amount of protein released at day 28. B: Cumulative release of bioactive VEGF from HDS- or CS-microspheres, as measured by the bioluminescence VEGF bioreceptor assay as described in Section 2.2.12. The release was normalized by the total amount of released VEGF after 28 days. C: Bioactivity of released VEGF released from microspheres prepared with trapping agents HDS or CS. Relative cell proliferation induced by VEGF standards 0 – 10 ng/ml (blue bars) and release samples ("MSPs IVR samples") from placebo or VEGF-loaded microspheres prepared with trapping agents HDS or CS. Bars represent average ± SD (n = 3). *p < 0.05. D: Cumulative release of bioactive VEGF (determined by HUVEC Alamar Blue proliferation assay), normalized to the total amount of bioactive VEGF after 28 days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

recovery by ELISA but unaffected bioactivity, have also been made by Bock *et al.*[53].

Another explanation may be more technical, and relates to the sequential dilution steps that are made to the IVR supernatant towards the working range of the ELISA and the bioluminescence assay. These dilution steps might result in loss of protein due to adsorption on vials and pipette points.

3.5. Loading of a combination of proangiogenic growth factors in microspheres

In view of the more sustained release profile of HDS-containing microspheres, we further explored this type of microspheres for the simultaneous loading of VEGF combined with fibroblast growth factor (FGF) and insulin-like growth factor (IGF-1), all potent angiogenic growth factors [12,13] with high isoelectric points (Table 1). As angiogenesis *in vivo* is an interplay between many growth factors, delivering a combination of growth factors for therapeutic angiogenesis applications, such as vascularization of a biomaterial implant, is advantageous [5,11].

Table 4 shows the loading contents and loading efficiencies of FGF and IGF and the simultaneous encapsulation of VEGF, FGF and IGF by

Table 4
Loading content and loading efficiencies of a combination of VEGF, FGF and IGF, as well as of FGF and IGF separately in microspheres prepared with trapping agent HDS.

loaded growth factor(s)	loading content [wt %] [b]	loading efficiency [%]
VEGF + FGF + IGF (1:1:1[a])	4.3 ± 0.1	91 ± 3
FGF	4.6 ± 0.0	97 ± 0
IGF	3.6 ± 0.2	75 ± 3

[a] : weight ratios.
[b] : indirect determination by Coomassie Plus assay.

ASE loading in HDS microspheres. A total growth factor loading of 4.3 wt%, corresponding to a loading efficiency of 91%, was achieved for the co-loaded microsphere formulation. SE-UPLC measurements of the post-loading supernatants of the growth factor combination showed a remaining peak at the retention times of IGF, indicating that the non-loaded growth factor fraction consisted primarily of IGF (Fig. S2). FGF and IGF were also loaded separately into HDS-containing microspheres. FGF loaded microspheres had a loading of 4.6 wt% and 97% loading efficiency, while IGF loading in HDS-containing microspheres resulted

in a 3.6 wt% loading and 75% loading efficiency. Although IGF has approximately the same isoelectric point as VEGF, its ASE entrapment in the microspheres is less efficient. Plausibly, this may be ascribed to its small size (7.6 kDa) and thus lower net number of positive charges per protein molecule as compared to VEGF (38.2 kDa).

The release of simultaneously-loaded growth factors from microspheres was determined using growth factor-specific ELISAs for VEGF, FGF and IGF, respectively (Fig. 6a). VEGF was gradually released over the complete 28-day incubation time frame. In contrast, ~10% of FGF was released during the first week, followed by a sustained release during each consecutive week. IGF was nearly fully released within the first week (~90%).

3.6. Bioactivity of released growth factors

The bioactivity of the combination of proangiogenic growth factors was studied using the endothelial cell Alamar Blue proliferation assay (described in the Materials and Methods Section 2.2.11), as this assay detects a general bioactivity in endothelial cells, while the KDR-bioluminescence assay only detects VEGF activity. Unformulated growth factors and their combination were tested in the concentration range of 0 – 20 ng/ml. Growth factor combinations achieved higher relative proliferations (up to 9-fold) compared to separate growth

factors (up to 6-fold for FGF and 4-fold for VEGF), as shown in Fig. 6b. This result shows a strong proliferative effect of FGF and synergistic effect when VEGF and FGF were combined, in line with other studies [42,44]. FGF showed a higher cell proliferation response than VEGF, also in line with previous studies [58,59]. IGF (in yellow bars) did not induce endothelial cell proliferation in the concentrations tested, however, VEGF combined with IGF, or FGF combined with IGF showed increased cell proliferation demonstrating the bioactivity of IGF in our experiments (Fig. S3). A possible explanation for the absence of bioactivity of IGF is that the *in vitro* assay used is not suitable for determining IGF bioactivity, as recent studies demonstrated IGF's mitogenic properties by determining DNA levels in (tumor) cells [60,61].

Although endothelial cell proliferation is an important aspect of angiogenesis, also other processes such as endothelial cell migration and tube formation play important roles in angiogenesis and can be evaluated with *in vitro* assays [62]. A recent study in which VEGF was co-encapsulated with granulocyte-colony stimulating factor (G-CSF) and erythropoietin (EPO) in dextran-PLGA microspheres showed significant enhancement of neovascularization in rodent models of ischemia [63]. The development of appropriate *in vitro* assays which capture the bioactivity of potent bioactive growth factors is certainly warranted to establish the contribution of each component of mixtures of bioactive proteins.
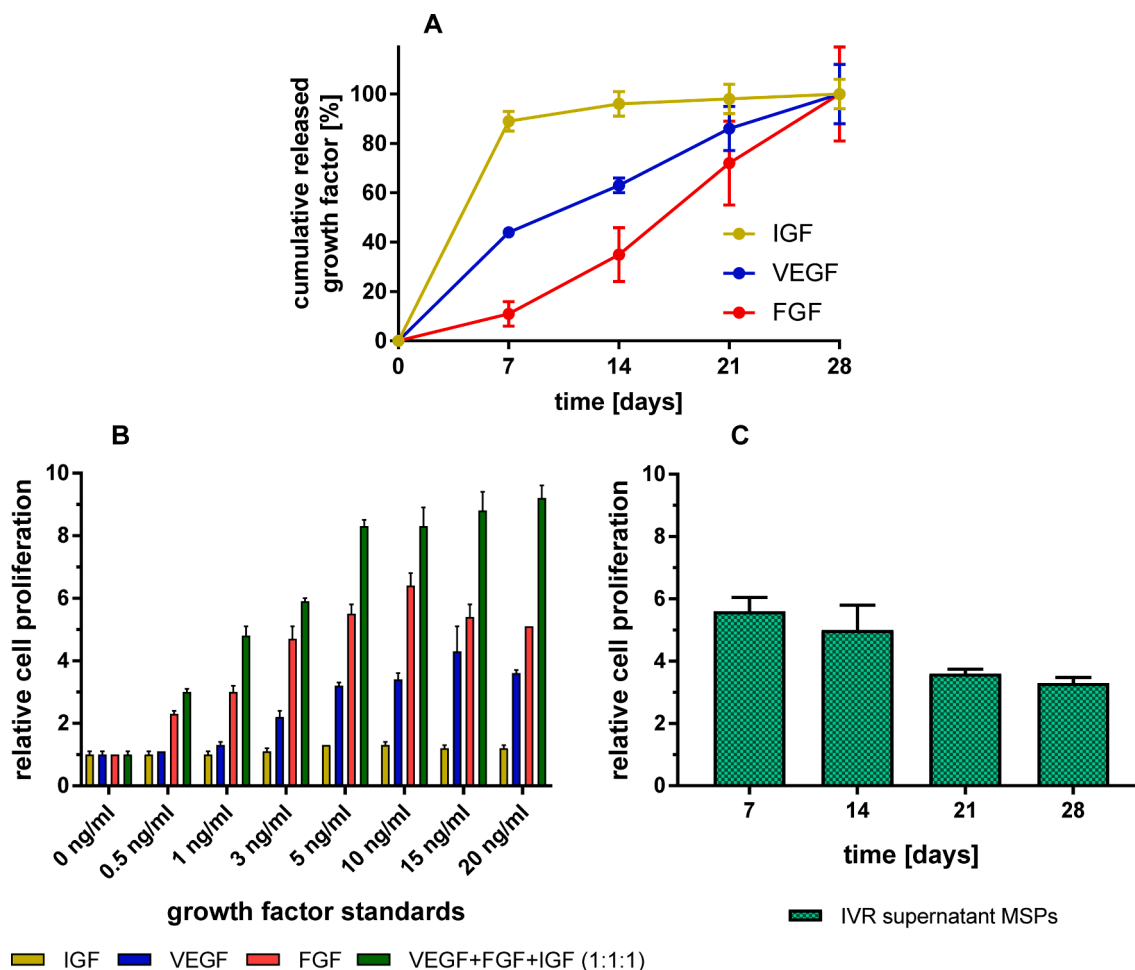

Fig. 6. A: Cumulative release [%] of IGF, VEGF and FGF from microspheres co-loaded with these growth factors. The release was performed at 37 °C in bioactivity IVR buffer consisting of Dulbecco's PBS pH 7.4, 0.2 μm filtered, 0.5% BSA, 30 μg/ml gentamicin and 15 ng/ml amphotericin. Released VEGF, FGF or IGF was quantified by specific ELISAs for each protein and normalized to the total cumulative release of each growth factor at day 28. B and C: Relative cell proliferation of growth factor standards and released growth factors was measured by Alamar Blue HUVEC proliferation assay. B: Relative cell proliferation of IGF (yellow bars), VEGF (blue bars), FGF (red bars) or a 1:1:1 combination of VEGF, FGF and IGF (green bars) in concentrations 0 – 20 ng/ml. C: Relative cell proliferation of release media collected from 1 mg microspheres loaded with VEGF, FGF and IGF using trapping agent HDS. IVR samples were diluted 500x. Bars represent average ± SD (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The relative cell proliferation of release samples of the microspheres loaded with VEGF, FGF and IGF is shown in Fig. 6c. At all tested time points, endothelial cell proliferation was significantly enhanced, ranging from 5-fold to 3-fold increased proliferation.

The results of Fig. 6 demonstrate that in a time frame of 28 days, bioactive growth factors were continuously released from microspheres, in profiles and quantities that are optimal for a vascularization strategy. To explain, current literature suggests that an average release rate of 150 ng VEGF/day for four weeks is sufficient for vascularization of a 0.1 ml biomaterial implant in rodents [17,18]. Based on the loading content of growth factors in microspheres (Table 4), an estimate of ~ 100 µg microspheres co-loaded with VEGF, FGF and IGF will provide sufficient growth factor release (see equations S1 and S2), considering the strong proliferative effect of FGF, the synergistic effect of VEGF and FGF, and IGF's reported mitogenic properties. Ultimately, an in vivo study investigating the microspheres developed in this study could give insight into the vascularizing capability.

Taken together, the ASE method is an attractive method for the sustained growth factor delivery as shown in this study. Although our study was focused on investigating ASE for co-encapsulation of growth factors that promote angiogenesis, i.e. VEGF, FGF and IGF, such an approach seems also feasible for other proteins containing heparin-binding domains or cationic properties [64]. Further, the ASE method is not only limited to the sustained release of growth factors, but has also been demonstrated for sustained vaccine delivery [65].

Compared to conventional double-emulsion methods for encapsulating growth factors, the release profiles shown in our study are similar to those reported previously of VEGF release from PLGA microspheres [21,22,26]. Importantly, the absence of exposure to organic solvent and shear stresses, as well as the addition of a basic additive, will likely have a favorable effect of the ASE method on protein stability. One important finding of Ennett et al. and Formiga et al. was an enhanced effect on vascularization in vivo due to the sustained release of VEGF (compared to unformulated VEGF)[21,22], which is of great importance for the in vivo applicability of the microspheres developed in this study.

4. Conclusions

In this study, the suitability of active self-encapsulation (ASE) for loading a combination of growth factors in PLGA microspheres for sustained release was demonstrated. With this method, high loading efficiencies of VEGF, FGF and IGF were achieved using dextran sulfate as trapping agent. Bioactive growth factors were released continuously over a release period of four weeks, whereby a combination of growth factors had a stronger and complementary proliferative response than solely VEGF. Therefore, self-encapsulating microspheres offer sustained release of proangiogenic growth factors in their bioactive form in a time frame that is attractive for incorporation in biomaterial implants to achieve its functional vascularization.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2020.10.022.

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