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Polymeric microspheres for the sustained release of a protein-based drug carrier targeting the PDGFβ-receptor in the fibrotic kidney

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ABSTRACT

Injectable sustained release drug delivery systems are an attractive alternative for the intravenous delivery of therapeutic proteins. In particular, for chronic diseases such as fibrosis, this approach could improve therapy by reducing the administration frequency while avoiding large variations in plasma levels. In fibrotic tissues the platelet-derived growth factor receptor beta (PDGFβR) is highly upregulated, which provides a target for site-specific delivery of drugs. Our aim was to develop an injectable sustained release formulation for the subcutaneous delivery of the PDGFβR-targeted drug carrier protein pPB-HSA, which is composed of multiple PDGFβR-recognizing moieties (pPB) attached to human serum albumin (HSA). We used blends of biodegradable multi-block copolymers with different swelling degree to optimize the release rate using the model protein HSA from microspheres produced via a water-in-oil-in-water double emulsion evaporation process. The optimized formulation containing pPB-HSA, showed complete release *in vitro* within 14 days. After subcutaneous administration to mice suffering from renal fibrosis pPB-HSA was released from the microspheres and distributed into plasma for at least 7 days after administration. Furthermore, we demonstrated an enhanced accumulation of pPB-HSA in the fibrotic kidney. Altogether, we show that subcutaneously administered polymeric microspheres present a suitable sustained release drug delivery system for the controlled systemic delivery for proteins such as pPB-HSA.

1. Introduction

Organ fibrosis is a progressive and chronic condition that is hallmarked by excessive deposition of extracellular matrix (ECM) proteins by myofibroblasts. Ultimately, functional cells are replaced by redundant ECM proteins, which causes scarring of the affected organ, leading to impaired organ function with a high mortality of up to 45% in the developed world (Mehal et al., 2011). Currently, the therapeutic options to treat advanced fibrosis are very limited, demonstrating the necessity for the development of innovative therapies to attenuate fibrosis.

A promising strategy in the development of a novel antifibrotic therapy is the targeting of therapeutic proteins to key pathogenic cells, mainly myofibroblasts. These cells highly and specifically express the platelet-derived growth factor beta receptor (PDGFβR) (Borkham-Kamphorst et al., 2008; Poosti et al., 2015), which makes it an excellent target for the delivery of potential antifibrotic compounds. The drug carrier protein pPB-HSA is composed of multiple PDGFβR-recognizing peptide moieties (pPB) coupled to human serum albumin (HSA) (Beljaars et al., 2003), and binds to the PDGFβR without activating the downstream intracellular signaling pathway. The potential of pPB-HSA as a carrier protein was demonstrated previously by the potent antifibrotic effect when small molecules such as doxorubicin or proteins like interferon gamma were coupled to it (Bansal et al., 2011; Beljaars

Abbreviations: ECM, extracellular matrix; EE, encapsulation efficiency; HSA, human serum albumin; MALDI-TOF MS, matrix-assisted laser desorption/ionization − time of flight mass spectrometry; MDSC, modulated differential scanning calorimetry; PDGFβR, platelet-derived growth factor β receptor; PCL, poly(ε-caprolactone); PEG, poly(ethylene glycol); PLGA, poly (lactic-co-glycolic acid); PLLA, poly(L-lactic acid); pPB, PDGFβR-recognizing peptide; pPB-HSA, drug carrier of HSA with pPB moieteies attached; UUO, unilateral ureteral obstruction; W/O/W, water-in-oil-in-water

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et al., 2003; Hagens et al., 2007; Prakash et al., 2010). It is hypothesized that after binding of the pPB-moiety to the PDGFβR, the whole construct is internalized and the antifibrotic compound is released from the construct after lysosomal degradation (Hagens et al., 2007; Prakash et al., 2010).

The most common route of administration for proteins such as pPB-HSA is an (intravenous) injection, as this provides the most efficient delivery by ensuring complete bioavailability. However, the fast elimination of proteins causes large variations in plasma levels. Moreover, such rapid elimination makes frequent injections necessary, which creates a high burden to the patient. To overcome these issues, subcutaneously or intramuscularly injectable sustained release drug delivery systems providing sustained release are increasingly used. Advantages of this type of drug delivery system are that the administration frequency is reduced and that a constant protein plasma level can be achieved. Biodegradable polymers are excellent matrices for such drug delivery systems; they offer a versatile platform for a multiplicity of release profiles and dosage forms (Prajapati et al., 2015; Wu and Jin, 2008).

A frequently applied and FDA approved biodegradable polymer for sustained release is poly (lactic-co-glycolic acid) (PLGA), which degrades in the body by hydrolysis (Houchin and Topp, 2008). However, the use of PLGA for protein delivery may lead to changes in protein structure and incomplete release related to, amongst others, its hydrophobicity and its acidic degradation products (Houchin and Topp, 2008; van de Weert et al., 2000). Moreover, the release from PLGA matrices is influenced by many factors, which results in unpredictable and often unfavorable, multi-phasic release profiles (Fredenberg et al., 2011). As an alternative, phase-separated multi-block copolymers composed of amorphous hydrophilic poly(ε-caprolactone) – poly (ethylene glycol) – poly(ε-caprolactone) (PCL-PEG-PCL) blocks combined with semi-crystalline poly(L-lactic acid) (PLLA) blocks can be used. In contrast to PLGA, the hydrophilic nature of these polymers allows continuous release by diffusion, caused by controlled swelling of PEG in the amorphous blocks by water uptake (Stanković et al., 2013). By varying the size of the PEG blocks, the ratio of the blocks within the copolymer or by blending different copolymers, the release of proteins can be customized to the desired characteristics needed for a specific protein (Stanković et al., 2015).

In this study, we used these [PCL-PEG-PCL]-*b*-[PLLA] multi-block copolymers to prepare microspheres, thereby aiming for the sustained release of the drug carrier pPB-HSA. As an *in vivo* proof of concept, we assessed the release of pPB-HSA from the microspheres up to 7 days after subcutaneous administration of pPB-HSA containing microspheres in mice suffering from kidney fibrosis.

2. Materials and methods

2.1. Polymer synthesis and characterization

The prepolymers PLLA and PCL-PEG-PCL were synthesized using procedures similar to described in (Stanković et al., 2013).

The PLLA prepolymer with a target molecular weight of 4000 g/mol was prepared of 1001 g (13.89 mol) anhydrous L-lactide (Corbion, Gorinchem, The Netherlands), using anhydrous 1,4-butanediol (22.7 g, 251.9 mmol, Thermo Fisher Scientific, Waltham, MA, USA) to initiate the ring-opening polymerization and stannous octoate (Sigma Aldrich, Zwijndrecht, The Netherlands) as a catalyst at a catalyst/monomer molar ratio of 5.38×10^{-5}/1. The mixture was magnetically stirred for 136 h at 140 °C and subsequently cooled to room temperature.

The [PCL-PEG_{1000}- PCL] prepolymer with a target molecular weight of 2000 g/mol and the [PCL-PEG_{3000}-PCL] prepolymer with a target molecular weight of 4000 g/mol were synthesized in a similar way using 250 g (2.19 mol) CL (Thermo Fisher Scientific), 250 g (250 mmol) PEG_{1000} (Thermo Fisher Scientific) and molar catalyst/monomer ratio of 7.94×10^{-5}/1 for [PCL-PEG_{1000}- PCL] and 69.6 g (0.61 mol) CL, 229 g (76.33 mmol) PEG_{3000} (Thermo Fisher Scientific) and molar catalyst/monomer ratio of 3.03×10^{-4}/1 for [PCL-PEG_{3000}-PCL]. The mixture was magnetically stirred at 160 °C for 24 h ([PCL-PEG_{1000}-PCL]) or 12 days ([PCL-PEG_{3000}-PCL]) and subsequently cooled to room temperature.

The [PLLA] was then chain-extended with [PCL-$PEG_{1000or3000}$-PCL] using 1,4-butanediisocyanate to prepare *x*[PCL-$PEG_{1000or3000}$-PCL]-*y* [PLLA] multi-block copolymer where *x*/*y* is the [PCL-$PEG_{1000or3000}$-PCL]/[PLLA] weight ratio, being 50/50 with PCL-PEG_{1000} (referred to as polymer A in this paper) or 30/70 with PCL-PEG_{3000} (referred to as polymer B in this paper) (Table S1). [PLLA] and [PCL-$PEG_{1000or3000}$-PCL] were dissolved in dry 1,4-dioxane (80 °C, 30 wt-% solution). 1,4-Butanediisocyanate (Actu-all Chemicals B.V., Oss, The Netherlands) was added and the reaction mixture was mechanically stirred for 20 h. Subsequently, the reaction mixture was frozen and freeze-dried at 30 °C shelf temperature to remove 1,4-dioxane.

The synthesized multi-block copolymers 50[PCL-PEG_{1000}-PCL]-50[PLLA]/polymer A and 30[PCL-PEG_{3000}-PCL]-70[PLLA]/polymer B were analyzed for chemical composition, intrinsic viscosity and residual 1,4-dioxane content (Table S2). The actual composition of the multi-block copolymers, as determined by ^{1}H NMR from LA/PEG and CL/PEG molar ratios, was in agreement with the target composition. The residual 1,4-dioxane contents were well below 600 ppm indicating effective removal of the solvent by freeze-drying. A schematic representation of composition of the multi-block copolymers is displayed in Fig. 1.

2.2. Synthesis of pPB-HSA

The cyclic peptide pPB was covalently coupled to HSA as described previously (Beljaars et al., 2000). In brief, 15 µmol N-γ-maleimidobutyryl-oxysuccinimide ester in dry dimethylformamide (DMF) was added to 0.75 µmol HSA (purified from Cealb®, Sanquin, Amsterdam, The Netherlands) in PBS (10 mM, pH 7.2 in all experiments). After dialysis for 2 d against PBS using a 10 kDa cut-off dialysis membrane (Thermo Fisher Scientific), 15 µmol of N-succinimidyl S-acetylthioacetate (SATA)-modified pPB (C*SRNLIDC*, 20 mg/mL in dry

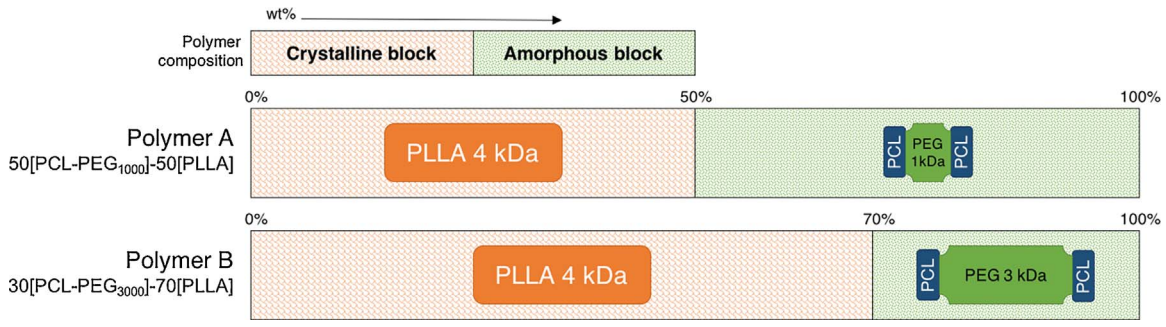


Fig. 1. Schematic representation of the composition of the two semi crystalline multi-block copolymers used in this study. The amorphous and crystalline blocks are randomly distributed. PLLA, poly(L-lactic acid); PCL, poly(ε-caprolactone); PEG, poly(ethylene glycol).

Table 1
Characteristics of HSA microspheres of different polymer ratios. X_{10}, X_{50} and X_{90} represent the volume percentages of particles (10%, 50% and 90% undersize, respectively).

Ratio polymer A: polymer B	Particle size (μm)[a]			Span	Encapsulation efficiency (%)
	X_{10}	X_{50}	X_{90}		
100:0	3.6	22.4	50.4	2.1	91
90:10	2.8	18.6	55.6	2.8	85
70:30	4.7	27.8	57.7	1.9	39
50:50	6.7	26.4	52.0	1.8	76
30:70	5.7	27.4	72.4	2.4	92
10:90	3.1	20.4	51.0	2.4	67
0:100	2.3	13.6	46.9	3.3	86

[a] For clarity reasons, individual standard deviations are not displayed. The maximum standard deviations are: 0.3 μm for X_{10}, 1.7 μm for X_{50} and 1.6 μm for X_{90}.

DMF, Ansynth Service B.V., Roosendaal, The Netherlands) and activation solution (1.0 mmol hydroxylamine and 12.7 μmol EDTA in PBS) were added. This mixture was allowed to react overnight at room temperature and was extensively dialyzed afterwards. Next, the product was purified using chromatographic methods and then freeze dried and stored at −20 °C. MALDI-TOF MS analysis showed an average molecular weight of 73.4 kDa, corresponding to 7 pPB units coupled per HSA molecule.

2.3. Microsphere production

Microspheres were produced using a water-in-oil-in-water double emulsion extraction/evaporation method. The polymers (1 g in total) were dissolved in dichloromethane (DCM) in the desired ratio (Table 1) to obtain a 15 wt-% solution, which was subsequently filtered (PTFE, 0.2 μm). Next, to make the primary emulsion, PBS (control) or a solution of 80 mg/mL protein (HSA or pPB-HSA and HSA in a 3:2 ratio) in PBS was added to the polymer solution to obtain 5 wt-% theoretical protein load and homogenized for 40 s at 13,500 rpm using a turrax mixer (Heidolph Diax 600, Salm & Kipp, Breukelen, The Netherlands). To make the secondary emulsion, the primary emulsion was added in 40 s to an aqueous solution containing 4 wt-% poly vinyl alcohol (M_w: 13–23 kDa, 87–89% hydrolyzed, Sigma Aldrich) and 5 wt-% NaCl solution (1:100 v/v ratio) under stirring (19,000 rpm) also using a turrax mixer. Consecutively, the secondary emulsion was mixed for an additional 20 s under these conditions and then stirred at 200 rpm with a magnetic stirrer for 3 h to evaporate DCM. Next, the hardened microspheres were collected by filtration and washed with 750 mL of 0.05% Tween 80 in Millipore water and 750 mL of Millipore water. Finally, the microspheres were freeze dried.

2.4. Microsphere size analysis

Laser diffraction (Helos/BF, Sympatec GmbH, Clausthal-Zellerfeld, Germany) was performed with a 100 mm lens (range: 0.5/0.9–175 μm). In brief, 12–15 mg of microspheres were dispersed in duplo in 1 mL of Millipore water by ultra-sonication. Next, 0.2 mL of the suspension was dispersed in 40 mL Millipore water in a 50 mL quartz cuvette. Three single measurements of 10 s were recorded with a 50 s pause in between. The last step was repeated with another 0.2 mL of suspension, resulting in a total of 12 single measurements per microsphere batch. The particle size distribution was calculated according to the Fraunhofer diffraction theory. The span of the particle size distribution was calculated using Eq. (1),

$$Span = \frac{X_{10} - X_{90}}{X_{50}} \quad (1)$$

where X_{10}, X_{50} and X_{90} represent the volume percentages of particles (10%, 50% and 90% undersize, respectively).

2.5. Protein content of microspheres

Microsphere samples of 5 mg were accurately weighed in triplicate in 4.0 mL glass vials. Next, 0.4 mL of dimethyl sulfoxide was added and the samples were placed at 37 °C. After 3 h, 3.6 mL 0.5 wt-% sodium dodecyl sulfate (SDS) in 0.05 N NaOH was added, and the polymer was allowed to dissolve overnight. Next, the protein content was determined with the bicinchoninic acid (BCA) assay. The BCA reagent mixture was prepared by mixing 4 wt-% aqueous copper (II) sulfate solution with BCA reagent A (Thermo Fisher Scientific) in a 1:50 vol ratio. Samples of 25 μL were added in triplicate to a 96-wells plate. After addition of 200 μL BCA reagent to the wells, the plate was incubated for 2 h at 37 °C. The absorbance was measured at 562 nm after the plate was cooled to room temperature (Synergy HT Microplate Reader, BioTek Instruments, Winooski, VT, USA). Protein concentrations were calculated using an 8-point calibration curve.

The protein content was used to calculate the encapsulation efficiency (EE) according to Eq. (2):

$$EE = \frac{Weight\ of\ encapsulated\ protein}{Weight\ of\ total\ protein\ used} \times 100\% \quad (2)$$

2.6. Scanning electron microscopy

Images were obtained using a JSM-6460 microscope (Jeol, Tokio, Japan) at an acceleration voltage of 10 kV. Samples were fixed on an aluminum sample holder using double sided adhesive carbon tape. Excessive microspheres were removed using pressurized air. The samples were sputter coated with 10 nm of gold.

2.7. in vitro release

The *in vitro* release was measured in triplicate by a sample-and-replace method. In brief, 10 mg of microspheres were accurately weighed in a 2.0 mL glass vial and suspended in 1.0 mL of release buffer (100 mM sodium phosphate, 9.1 mM NaCl, 0.01 wt-% Tween 80, 0.02 wt-% NaN_3, pH 7.4). The vials were placed in a 37 °C shaking water bath. Samples of 0.8 mL were taken at predetermined time points and replaced by fresh buffer. At the final time point, the whole volume of buffer was taken to facilitate facile drying of the remaining microspheres for scanning electron microscopy. Total protein concentration in the release medium was determined using the BCA assay (section 2.5). Protein concentrations were calculated using a 13-point calibration curve.

2.8. p PB-HSA ELISA

The concentration of pPB-HSA in the *in vitro* release medium samples was determined using an in-house developed sandwich ELISA. Briefly, the capture antibody rabbit α-pPB (100 μL, 1:1000, custom prepared by Charles Rivers, Den Bosch, The Netherlands) was incubated overnight in a high protein binding 96-well plate (Corning, New York, NY, USA). After extensive washing with PBS containing 0.5 wt-‰ Tween-20 (PBS-T), the plate was blocked with 5 wt-% nonfat dry milk in PBS-T (200 μL) for 1 h and washed again with PBS-T. Next, samples (100 μL) were incubated for 2 h. The plate was washed again, followed by the addition of the detection antibody goat α-HSA (100 μL, 1:8000, ICN Biomedicals, Zoetermeer, The Netherlands) for 1 h and subsequent washed once more. The appropriate HRP-conjugated secondary antibody was applied for 1 h, and after washing with PBS-T, the substrate tetramethyl benzidine (100 μL, R & D Systems, Minneapolis, MN, USA) was added. The absorbance was measured at 450 nm (THERMOmax microplate reader, Molecular Devices, Sunnyvale, CA, USA) after addition of 50 μL 2 N H_2SO_4.

This protocol was also used to determine the concentration of pPB-HSA in 50 μL mouse plasma samples.

2.9. Modulated differential scanning calorimetry (MDSC)

Samples of 6 to 7 mg of microspheres were analyzed in duplicate in open aluminum pans using a Q2000 MDSC (TA Instruments). The samples were cooled to −80 °C and kept isothermal for 5 min to equilibrate. Next, the sample was heated to 150 °C at 2 °C/min and a modulation amplitude of ± 0.212 °C every 40 s. Differences within duplicates were < 0.5 °C for melting and crystallization temperatures and < 1.5 J/g for enthalpies.

2.10. Animal experiments

All the experimental protocols for animal studies were approved by the Animal Ethical Committee of the University of Groningen (The Netherlands). Male C57BL/6 mice, aged 8–10 weeks, were obtained from Envigo (Horst, The Netherlands). Animals received ad libitum normal diet with a 12 h light/dark cycle. Mice (n = 6) were subjected to unilateral ureteral obstruction (UUO) by a double ligation of the left ureter proximal to the kidney (Poosti et al., 2015), and injected subcutaneously in the neck directly after surgery with 31.5 mg microspheres (dispersed in 500 µL 0.4 w/v% carboxymethyl cellulose (Aqualon high M_w, Ashland)) containing either 5 wt-% HSA (n = 3) or 3 wt-% pPB-HSA/2 wt-% HSA (n = 3). The total administered doses were 1.58 mg HSA for the microspheres containing 5 wt-% HSA and 0.95 mg pPB-HSA/0.63 mg HSA for the microspheres containing 3 wt-% pPB-HSA/2 wt-% HSA. Mice were sacrificed at day 7, after which blood and different organs were collected and processed for further analysis.

2.11. Histochemistry

Cryosections of neck skin tissue were cut at a thickness of 4 µm (CryoStar NX70 cryostat, Thermo Fisher Scientific), dried and stored at −20 °C until analysis. Paraffin sections for kidney were cut at a thickness of 4 µm (Leica Reichert-Jung 2040 microtome).

2.11.1. Haematoxylin and eosin staining

The cryosections were dried and fixed for 10 min in formalin-macrodex (6 wt-% dextran-70 in 0.9 wt-% NaCl containing 3.6 wt-% formaldehyde and 1 wt-% $CaCl_2$, pH 7.4). After extensive washing in water, the slides were incubated in haematoxylin solution (Clinipath Pathology, Osborne Park, Australia) for 15 min, washed in tap water, and incubated 1.5 min in eosin (Clinipath Pathology). Sections were embedded in DePeX mounting medium (VWR, Amsterdam, The Netherlands) after dehydration in ethanol.

2.11.2. Immunohistochemical stainings

Neck skin cryosections were dried and fixed with acetone. Kidney paraffin sections were deparaffinized in xylene and ethanol. Sections were then rehydrated in PBS and incubated 1 h at room temperature with a primary antibody (rabbit α-HSA (1:1500, ICN Biomedicals), rabbit α-pPB (1:1000, Charles Rivers), goat α-collagen I & III (both 1:200 + 5% normal mouse serum, Southern Biotech, Birmingham, AL, USA)) or boiled in 10 mM Tris/1 mM EDTA (pH 9.0) for 15 min prior to overnight incubation with a primary antibody at 4 °C (rabbit α-PDGFβR (1:50, Cell Signaling, Leiden, The Netherlands)). Subsequently, sections were incubated 30 min at room temperature with the appropriate HRP-conjugated secondary antibody. The HRP-conjugated antibodies were visualized with ImmPACT NovaRED kit (Vector, Burlingame, CA, USA). Hematoxylin counterstaining was performed. Digital photomicrographs were captured at 400 x magnification (Aperio, Burlingame, CA, USA). Microsphere sizes (median of 100) were determined using Cell D software (Olympus).

2.12. Western blot

Samples (100 µg protein, as determined using Lowry assay) were applied on a SDS polyacrylamide gel (10%) and transferred to a polyvinylidene difluoride membrane. Membranes were blocked for 1 h in 5 wt-% nonfat dry milk in tris-buffered saline/0.1% Tween-20 (ELK/TBS-T) at room temperature, followed by overnight incubation at 4 °C with the primary antibody in ELK/TBS-T. The primary antibodies used include goat α-HSA (1:1000, ICN Biomedicals) and mouse α-GAPDH (1:20000, Sigma-Aldrich). The membranes were extensively washed in TBS-T before application of the appropriate HRP-conjugated secondary antibodies in ELK/TBS-T for 2 h. After extensive washing of the membranes with subsequently TBS-T and TBS, bands were visualized with enhanced chemiluminescence and quantified with GeneSnap (Syngene, Synoptics, Cambridge, UK).

2.13. Statistical analyses

At least 3 individual experiments were performed to measure *in vitro* effects. All the data are represented as mean ± standard deviation (SD). *In vivo* data are presented as mean ± standard error of the mean (SEM). Differences between groups for the ELISA were assessed by Mann-Whitney *U* test. The differences between the groups for the western blot were assessed by Kruskal-Wallis test followed by Dunn's multiple comparison test. The graphs and statistical analyses were performed using Graphpad Prism version 6.0 (GraphPad Prism Software Inc., La Jolla, CA, USA).

3. Results

3.1. Properties of microspheres of different polymer ratios

Different blend ratios of polymer A and B were used to obtain a microsphere formulation with the desired release profile, *i.e.* sustained release with minimal burst and complete release within 14 days. In these screening experiments, the HSA target loading of the microspheres was 5%. This protein was later used as filler and untargeted equivalent to pPB-HSA, as the physicochemical properties of both proteins can be considered similar. The polymer blend ratios affected the microsphere size distribution and the span of the size distribution (Table 1), as determined with laser diffraction. In particular, microspheres consisting of only polymer B had a smaller median particle size and a larger size distribution span than microspheres of other compositions. All formulations had a broad particle size distribution, which is reflected in the relatively high span values. Such high polydispersity is common for microspheres produced by water-in-oil-in-water (W/O/W) emulsification by homogenization (Ye et al., 2010). The span could be decreased at the expense of the particle size, however this negatively affected the injectability of the microspheres. The encapsulation efficiency (EE) of the model protein HSA was consistently high (albeit one exception, for unknown reasons), and seemed to be unaffected by polymer blend composition and particle size.

Scanning electron microscopy images revealed the morphology of the microspheres prepared from all polymer blends as spherical particles with a smooth surface and few pores. A representative image of lyophilized microspheres composed of a 50:50 polymer blend is depicted in Fig. 2. The microspheres retained their spherical shape and smooth surface during 42 days of release *in vitro*, but additional pores were formed in time (Fig. S1).

3.2. Thermal properties of microspheres

The thermal characteristics of the polymer matrix of microspheres composed of different polymer blends without protein were investigated using MDSC (Fig. 3, Tables 2 and 3). Glass transitions were observed in the reversing heat flow signal at around −56 °C in microspheres of all polymer ratios except 10:90 and 0:100 (Fig. S2). The peak at 37 °C in the total heatflow thermograms (Fig. 3) can be ascribed to melting of crystalline PEG. With increasing content of polymer

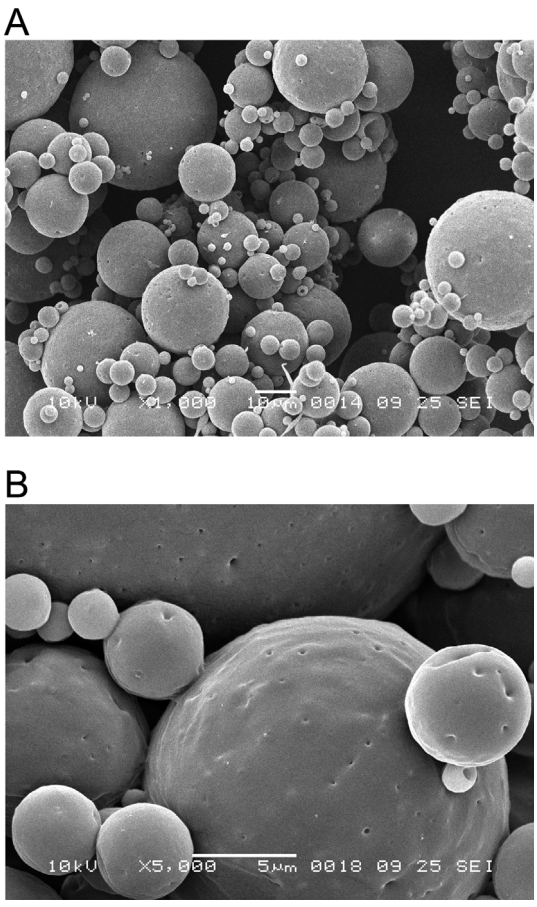


Fig. 2. Representative scanning electron microscopy image at a 1000× (panel A) and 5000× (panel B) magnification of lyophilized microspheres composed of a 50:50 polymer blend of polymer A and B.

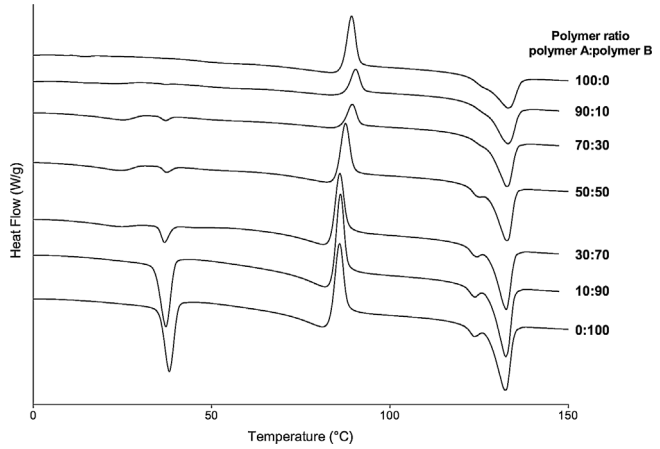


Fig. 3. Thermograms of microspheres of different polymer blends. On the y-axis, exothermic is up. The melting peak of PEG is observed at about 37 °C, the cold crystallization peak of PLLA at 85–90 °C and the melting peak of PLLA at 132–133 °C.

B, the enthalpy of the melting peak at 37 °C increased, even though the total PEG content is decreased (Table 2). This result indicates that PEG_{3000} is crystalline, but PEG_{1000} might be too small to crystallize. Furthermore, the cold crystallization temperature T_{cc} of PLLA shifted from around 90 °C for polymer blends with a high polymer A content to around 85 °C for polymer blends with a low polymer A content (Fig. 3, Table 3). Also, the melting temperature T_m of PLLA showed a minor decrease with decreasing polymer A content (Table 3). The origin of the shoulder in the PLLA melting peak is unknown, but such thermal events

Table 2
Theoretical PEG content and thermal characteristics of microspheres of different polymer blends as determined with MDSC. The relative enthalpy (ΔH_{rel}) was calculated by dividing the melting enthalpy (J/g) by the PEG fraction in the copolymer.

Polymer ratio	Total PEG (%)	PEG 1 kDa (%)	PEG 3 kDa (%)	T_m (°C)	ΔH_{rel}
100:0	25	25	0	N.A.	N.A.
90:10	24.75	22.5	2.25	37.1	0.37
70:30	24.25	17.5	6.75	37.2	2.04
50:50	23.75	12.5	11.25	37.6	3.04
30:70	23.25	7.5	15.75	37.0	9.12
10:90	22.75	2.5	20.25	37.4	40.97
0:100	22.5	0	22.5	37.7	43.18

Table 3
Theoretical PLLA content and thermal characteristics of microspheres of different polymer blends as determined by MDSC. The relative enthalpy (ΔH_{rel}) was calculated by dividing the melting enthalpy (J/g) minus the crystallization enthalpy (J/g) by the PLLA fraction in the copolymer.

Polymer ratio	Total PLLA (%)	PLLA polymer A (%)	PLLA polymer B (%)	T_{cc} (°C)	T_m (°C)	ΔH_{rel}
100:0	50	50	0	89.2	133.2	5.0
90:10	52	45	7	90.3	133.1	13.7
70:30	56	35	21	89.4	132.7	15.0
50:50	60	25	35	87.5	132.9	8.5
30:70	64	15	49	85.9	132.6	7.9
10:90	68	5	63	86.0	132.5	6.9
0:100	70	0	70	85.9	132.3	5.4

have been observed before in microspheres prepared from polymers of the same platform technology (Ramazani et al., 2015).

3.3. Release of HSA from microspheres of different polymer ratios

The release of HSA from microspheres containing HSA only and composed of different polymer ratios during the first 14 days is presented in Fig. 4. This is the relevant timeframe for the subsequent *in vivo* study. Protein release is assumed to be diffusion controlled, as was found previously using similar phase-separated multi-block copolymers (Stanković et al., 2014).

Only a minimal burst release of less than 10% in 3 h was observed in all formulations. The release profiles of the microspheres composed of the different polymer blends can roughly be categorized in three sets. Firstly, microspheres with a high content of polymer A (90 or 100%), showed almost no release of HSA apart from a small burst. Secondly, microspheres containing 0, 10, 30 and 70% of polymer A showed an intermediate release rate, with a cumulative release of 40% to 60% after 14 days. Thirdly, the fastest release rate was observed from the

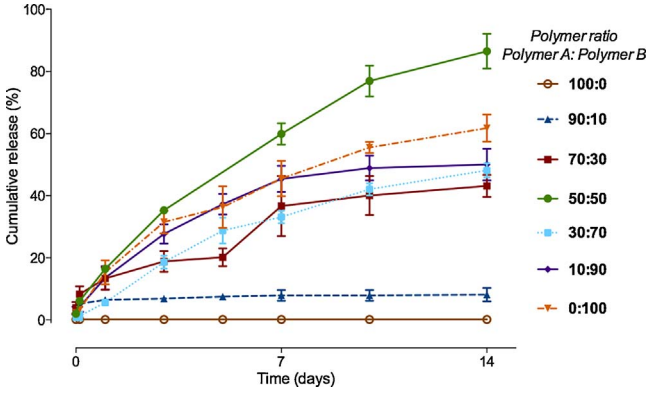


Fig. 4. Release of HSA from 5 wt-% HSA microspheres with different polymer ratios.

50:50 polymer ratio with a cumulative release of 87% after 14 days. Clearly, the release rates of HSA did not follow the polymer blend composition linearly.

3.4. Reproducibility of the production process

The reproducibility of the production process was assessed using 6 batches of HSA microspheres with a 50:50 polymer ratio, produced using identical process conditions. The average median particle size of these batches was 23.4 ± 5.3 μm (span: 2.0) and the average EE was 82 ± 7%. The normalized release showed the same release rate for all batches with a cumulative HSA release after 14 days of 88 ± 8% (Table S3, Fig. S3).

3.5. Production and characterization of pPB-HSA microspheres

For PDGFβR targeted HSA, a protein load of 3 wt-% was considered to be sufficient for cell specific delivery. Therefore, the required protein load of pPB-HSA was complemented with HSA to a total of 5 wt-% protein load to replicate the release rate and EE from the optimized 5 wt-% HSA microspheres with a 50:50 polymer ratio.

The morphology of these pPB-HSA microspheres was comparable with the morphology of HSA microspheres (Fig. 5A). The median particle size of 24.7 μm was similar to the particle size of HSA microspheres, while the span of the particle size distribution was slightly lower at a value of 1.6 (Fig. 5B). The *in vitro* release rate of pPB-HSA from pPB-HSA microspheres (EE: 83%) showed a similar release profile as HSA from HSA microspheres (Fig. 5C) and had a cumulative release of 103 ± 5% after 14 days. Evidently, the size difference between HSA and pPB-HSA (~7 kDa) did not affect the release rate.

3.6. Microspheres in vivo

The microspheres composed of the 50:50 polymer blend containing either pPB-HSA or its untargeted equivalent (HSA) demonstrated an optimal *in vitro* release profile, and were selected to be tested *in vivo*. Before this, we confirmed both *in vitro* in myofibroblasts and *ex vivo* in mouse kidney slices that the microspheres itself did not show any toxicity, as reflected by the unaffected viability in the presence of microspheres in different dilutions (Fig. S4). We injected the microspheres subcutaneously in the neck of mice suffering from fibrosis in their left kidney induced by unilateral ureter obstruction (UUO). Seven days after microsphere administration, we dissected the skin at the site of injection and performed a haematoxylin and eosin staining and confirmed the presence of microspheres. The microspheres had a mean diameter of 25.9 μm (Fig. 6A), which is in accordance with the size found *in vitro* by laser diffraction. Immunohistochemical staining for both HSA and pPB revealed that the subcutaneously located microspheres still contained HSA and/or pPB-HSA (Fig. 6B).

Sustained release into the systemic circulation of intact pPB-HSA from the microsphere depot was demonstrated. Plasma concentrations of 15.7 ± 4.0 ng pPB-HSA/ml were measured at day 7 after injection as determined by ELISA (Fig. 7B). In addition, the presence of pPB-HSA specifically in the fibrotic kidney, which showed high expression of collagens I & III and the PDGFβR (Fig. 7A), was demonstrated with western blot analysis for HSA (Fig. 7C). Clearly, pPB-HSA accumulated to a higher extent in the fibrotic kidney as compared to the healthy kidney. Inherent to the surgical procedure, some untargeted HSA was trapped in the fibrotic kidney as well, albeit to a lesser extent.

4. Discussion

In this study, we developed sustained release microspheres containing pPB-HSA, a proteinaceous construct targeted to the PDGFβR expressed in fibrotic tissues including the kidneys. The release rate was controlled by blending two semi-crystalline multi-block copolymers yielding a 14-day sustained release formulation. Microspheres composed of a 50:50 polymer blend containing pPB-HSA or HSA were subcutaneously administered to mice suffering from kidney fibrosis. After 7 days *in vivo*, the microspheres still released pPB-HSA and the construct was localized in the fibrotic kidney, thereby showing the therapeutic potential of these microspheres.

We expected the release rate of proteins from polymer blends to follow a linear trend, *i.e.* an increasing release rate with increasing content of the more swellable polymer in the blend. Surprisingly, this was not the case for the two semi-crystalline multi-block copolymers we used in this study. Unfortunately, the thermal properties of the 50:50 polymer blend microspheres did not display a distinguished profile that could provide a definitive answer to the unconventional trend in release rate. Therefore, to explain the anomalous release from the 50:50 polymer blend, we hypothesize that the release is governed by the size and content of the PEG blocks, which will be further described below.

The release from the two semi-crystalline multi-block copolymers was diffusion controlled, as in the timeframe of the experiments no extensive degradation of the microspheres will have occurred (Stanković et al., 2014). Furthermore, the release rate of proteins from this type of polymers is usually governed by the content of the PEG blocks which swell by water uptake (Stanković et al., 2015, 2014). A higher PEG content results in an increased degree of swelling, which causes the release rate of proteins to increase (Stanković et al., 2014). Although differences were small, based on the total PEG content in the polymer blends in this study, we could expect the highest release rate from microspheres prepared from 100% polymer A (Table 1). However, properties regarding release rate possibly changed because of the difference in PEG block length between polymer A and B. Stankovic et al. showed that diffusion controlled release of high molecular weight proteins such as albumin is not possible in polymers with PEG block lengths of 1 and 1.5 kDa, even at very high contents (Stanković et al., 2015, 2014), as the release of albumin was limited to a burst release. Furthermore, Tran et al. showed that an increase of PEG domain size also accelerates release of proteins (Tran et al., 2012). Thus, the low release rate from blends with high polymer A content was probably

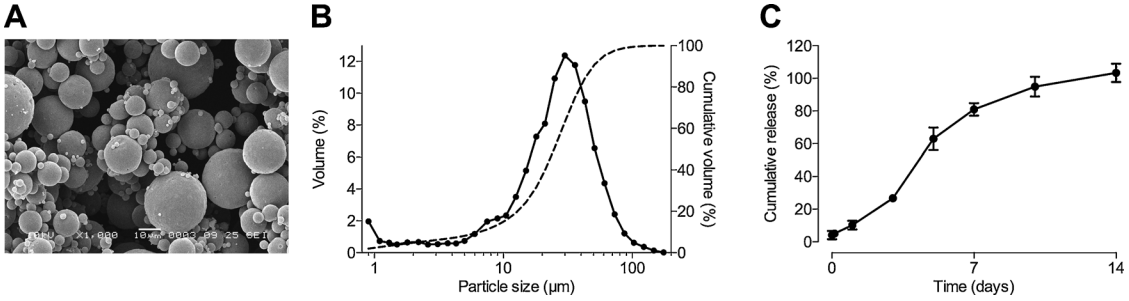
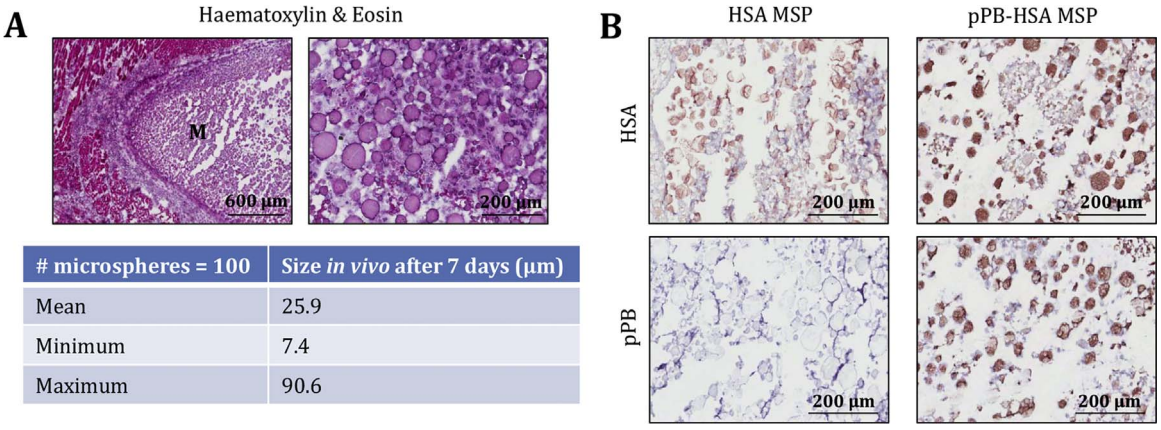


Fig. 5. Morphology, particle size distribution, and *in vitro* release of pPB-HSA microspheres. (A) Representative scanning electron microscopy image of pPB-HSA microspheres after freeze drying, 1000× magnification. (B) Particle size distribution of microspheres with volume percentage on left y-axis (solid line) and cumulative volume percentage on right y-axis (dashed line). (C) Cumulative *in vitro* release of pPB-HSA from pPB-HSA microspheres during 14 days, determined using ELISA and corrected for EE.



# microspheres = 100	Size *in vivo* after 7 days (µm)
Mean	25.9
Minimum	7.4
Maximum	90.6

Fig. 6. Subcutaneous localization of microspheres *in vivo*. (A) Haematoxylin and eosin staining of mouse neck skin at the site of microsphere injection, and quantification of the size of the microspheres. M denotes microspheres. (B) Immunohistochemical stainings for HSA and pPB of the skin of mice that received microspheres containing HSA or pPB-HSA.

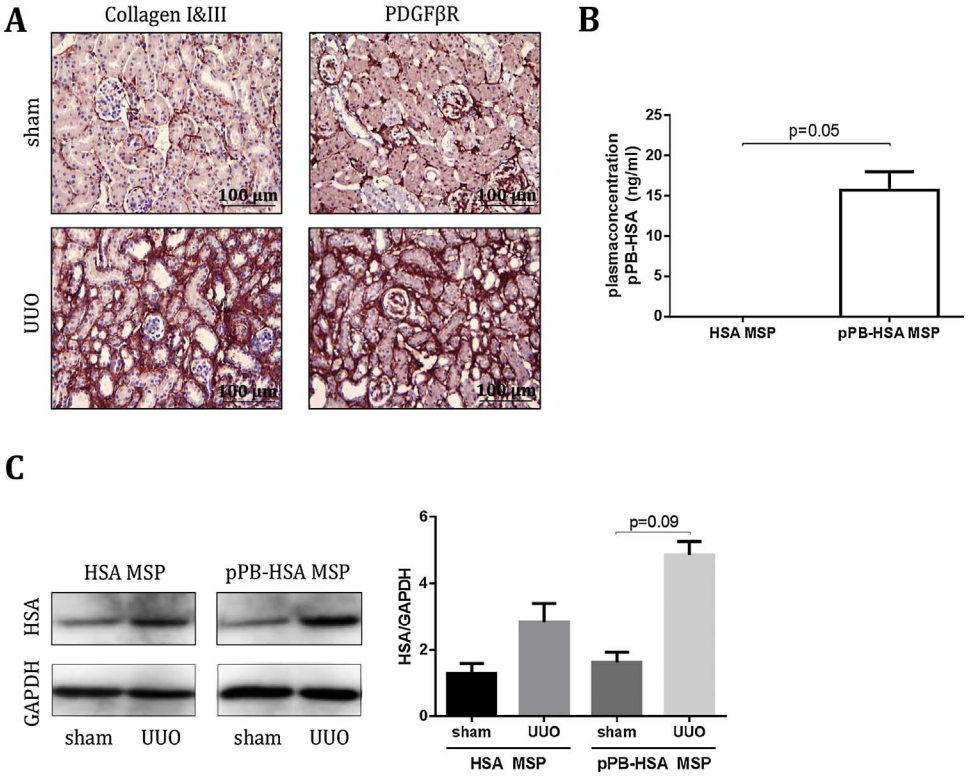


Fig. 7. *In vivo* release of pPB-HSA from microspheres, and localization of pPB-HSA in the UUO model. (A) Immunohistochemical stainings of sham and UUO-operated kidneys for collagen I & III and PDGFβR. (B) ELISA analysis of the plasma levels of pPB-HSA 7 days after subcutaneous injection with microspheres containing HSA or pPB-HSA. (C) Representative bands and quantitative analysis of western blot for HSA on kidney homogenates of mice 7 days after microsphere injection.

caused by the small PEG block (1 kDa). It is possible that the size of these PEG blocks was not sufficient to create a mesh size in the swollen PEG regions that was large enough for diffusion of HSA through the matrix.

Continuing this hypothesis, one would assume that increasing the size of the PEG block to 3 kDa as in polymer B would facilitate the release of large proteins. Indeed, the release rate of HSA increased when the content polymer B exceeded 30%, but decreased again at very high polymer B contents. However, as stated above, the total PEG content also contributes to the release rate. Thus, our hypothesis for the fast release rate from the 50:50 blend is that for HSA to be released, 3 kDa PEG blocks are necessary to create pores large enough for diffusion, but also a certain total PEG content is required to form a hydrated network for diffusion.

It is known that the production of protein-loaded microspheres by the W/O/W method may cause protein denaturation or aggregation due to shear and interfacial stresses (Thomas and Geer, 2011). Although the stability of the encapsulated proteins was not tested specifically, the sandwich ELISA for pPB-HSA required two epitopes for response, *i.e.* pPB and HSA, and could therefore provide an indication on preservation of structural integrity. Therefore, the fact that pPB-HSA could be recovered in release medium and plasma during 14 days *in vitro* and in plasma after 7 days *in vivo* release, indicates that the construct was still intact after microsphere production and release.

The development of PDGFβR-directed protein constructs is currently advancing towards clinical application of PDGFβR-directed antifibrotic compounds (van Dijk et al., 2015). These constructs are cell selective and very potent (van Dijk et al., 2015), and therefore perfectly suitable for (patient friendly) sustained release formulations. As a proof of concept for the sustained release of such a PDGFβR-directed protein construct, we assessed the protein release and targeting from pPB-HSA/HSA microspheres in mice suffering from renal fibrosis. Hereby, we aimed to bring the clinical application of PDGFβR-directed antifibrotic compounds one step closer.

It became apparent from the neck skin stainings (Fig. 6B) that not all protein was released 7 days after administration, which is in accordance

with the release profile found *in vitro* which showed a release for 14 days. Moreover, since pPB-HSA has a plasma half-life of approximately 45 min (data not shown), the manifestation of a plasma concentration of pPB-HSA suggests that release from the microspheres was still ongoing and that at least part of the pPB-HSA reached the circulation intact. The specific targeting of pPB-HSA in PDGFβR-rich areas after IV administration has been demonstrated before (Beljaars et al., 2003; Poosti et al., 2015). Kidney tissue stainings on pPB were performed in this study, although the concentration of protein construct was most probably too low to confirm the presence of pPB by immunohistochemical staining. Nonetheless, our western blot results show that specific localization to fibrotic tissue also occurs when pPB-HSA is administered as a polymeric sustained release formulation. Altogether, these results show that subcutaneously administered microspheres composed of biodegradable hydrophilic multi-block copolymers are suitable sustained release formulations for the systemic delivery of pPB-HSA. Future research could include pharmacokinetic studies on the release of pPB-HSA from microspheres and the delivery of an antifibrotic drug coupled to pPB-HSA by a sustained release formulation.

Author contributions

NT and FvD designed and performed experiments, analyzed data and prepared the manuscript. AB, ME and EP performed experiments. JZ designed experiments and prepared part of the manuscript. RS, KP, LB, WLJH and PO designed experiments. JLH prepared experimental protocols for the animal study. All authors contributed to critical review and approval of the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijpharm.2017.09.072.

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