Pharmacokinetics of a sustained release formulation of PDGFβ-receptor directed carrier proteins to target the fibrotic liver

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ABSTRACT

Liver fibrogenesis is associated with excessive production of extracellular matrix by myofibroblasts that often leads to cirrhosis and consequently liver dysfunction and death. Novel protein-based antifibrotic drugs show high specificity and efficacy, but their use in the treatment of fibrosis causes a high burden for patients, since repetitive and long-term parenteral administration is required as most proteins and peptides are rapidly cleared from the circulation. Therefore, we developed biodegradable polymeric microspheres for the sustained release of proteinaceous drugs. We encapsulated the drug carrier pPB-HSA, which specifically binds to the PDGFβR that is highly upregulated on activated myofibroblasts, into microspheres composed of hydrophilic multi-block copolymers composed of poly(ε-caprolactone) and poly ethylene glycol/poly(ε-caprolactone), allowing diffusion-controlled release. Firstly, we estimated in mice with acute fibrogenesis induced by a single CCl\textsubscript{4} injection the half-life of \textsuperscript{125}I-labeled pPB-HSA at 40 min and confirmed the preferential accumulation in fibrotic tissue. Subsequently, we determined in the Mdr2 \textsuperscript{-/-} mouse model of advanced biliary liver fibrosis how the subcutaneously injected microspheres released pPB-HSA into both plasma and fibrotic liver at 24 h after injection, which was maintained for six days. Although the microspheres still contained protein at day seven, pPB-HSA plasma and liver concentrations were decreased. This reduction was associated with an antibody response against the human albumin-based carrier protein, which was prevented by using a mouse albumin-based equivalent (pPB-MSA). In conclusion, this study shows that our polymeric microspheres are suitable as sustained release formulation for targeted protein constructs such as pPB-HSA. These formulations could be applied for the long-term treatment of chronic diseases such as liver fibrosis.

1. Introduction

Sustained release drug delivery systems are increasingly used as a patient-friendly alternative to conventional dosage forms [1,2]. When controlling the release rate of a drug its therapeutic actions can be drastically improved by obtaining prolonged release associated with less fluctuations in plasma concentration. This avoids peak levels and reduces side effects. This way, the bioavailability, efficacy and safety of drugs can be significantly enhanced [2,3]. One such drug delivery system is microspheres, that allows flexible dosing of the drug [4]. This approach is particularly interesting for application of potent protein-based therapeutics, as the administration frequency can be largely reduced as compared to intravenous administration. Moreover, when encapsulated in polymeric microparticles, the biopharmaceutical can be effectively protected from degradation induced by biological conditions or enzymes [2,5].

In the treatment of chronic diseases such as fibrosis, the application of sustained release formulations like microspheres for antifibrotic drugs could significantly improve patient compliance and therapeutic efficacy, especially since the expected treatment would be long-term. Hepatic fibrosis is a progressive, pathological condition affecting millions of people worldwide [6]. Following chronic liver injury, inflammatory and bile ductular cells release a variety of mediators that provoke the activation of fibroblasts and hepatic stellate cells to
myofibroblasts, which start to produce extracellular matrix (ECM) components, especially fibrillar collagens in a chronic would healing reaction. In cirrhosis, which represents an advanced stage of fibrosis, the liver vascular architecture gets progressively distorted and functional parenchymal cells are ultimately replaced by abundant ECM, which causes liver failure, and finally decompensated cirrhosis [7–9].

For some patients with cirrhosis, liver transplantation or treatment with a new generation of highly effective antiviral agents against Hepatitis B and C may be a curative treatment, however there is still an urge for effective antifibrotic treatments to fulfill the needs of all patients [10–12]. Many promising new drugs are biological-based, such as growth factors, cytokines and monoclonal antibodies. A class of therapeutic proteins currently under development is the fusion proteins, including biologic-based drugs modified with targeting moieties [13,14]. Such proteins are particularly interesting as their therapeutic effects can be increased while avoiding side effects [15]. The platelet-derived growth factor beta receptor (PDGFβR) is abundantly expressed on myofibroblasts in fibrotic tissues with high fibrogenic activity [16,17], and therefore its expression was exploited as a potential target for the cell-specific delivery of antifibrotic drugs. A carrier protein with high affinity for the PDGFβR was designed, composed of an albumin core with multiple cyclic PDGFβR-recognizing peptides (pPB) binding the PDGFβR. Previous studies have shown that the carrier, referred to as pPB-HSA, selectively bound to the PDGFβR and accumulated in fibrogenic cells in the fibrotic liver which highly expressed the PDGFβR. The carrier itself did neither elicit an antifibrotic effect nor induced proliferation of fibroblasts [18].

Because many therapeutic proteins have poor in vivo pharmacokinetic properties as reflected by relatively short in vivo plasma half-lives, we developed a microsphere formulation containing pPB-HSA that ensures gradual protein release over a period of 14 days, which could be suitable for therapeutic application of other large therapeutic proteins as well. A blend of two biodegradable semi-crystalline multi-block co-polymers, composed of crystalline blocks of poly(ε-caprolactone) (PCL) and amorphous blocks of poly ethylene glycol (PEG) and poly(ε-caprolactone) (PCL), was used as a matrix for these microspheres. Whilst mostly polymers are used that show degradation-controlled release [19], e.g. poly(lactic-co-glycolic acid), these particular polymers provided diffusion-controlled release of proteins, caused by swelling of the PEG blocks by water uptake [20]. Disadvantage of degradation-controlled release is that the degradation products of the polymer are usually incompatible with proteins [21].

We previously showed proof of concept of effective release of drug carriers from microspheres composed of these multi-block copolymers in the unilateral ureter obstruction model for kidney fibrosis. In that study, we demonstrated the release of pPB-HSA from subcutaneously injected microspheres into plasma and the subsequent localization of this drug carrier in the fibrotic kidney 7 days after administration of the microspheres [22]. Although we were able to demonstrate protein release after 7 days, the in vivo release characteristics and the correlation with the in vivo kinetics remained undefined.

In the present study, we therefore further explored the applicability of these microspheres as a sustained controlled release formulation for biologicals. For this, we examined the in vivo kinetic behavior of pPB-HSA in two different mouse models for liver fibrosis that display high and specific PDGFβR-expression, i.e. the acute CCl4 model and the Mdr2−/− model. In the acute CCl4 model, we used 125I-labeled pPB-HSA to determine pharmacokinetic parameters and tissue distribution of this carrier protein. Subsequently, the in vivo release profile of pPB-HSA from microspheres was determined in the chronic Mdr2−/− model.

2. Materials and methods

2.1. Pharmacokinetics of 125I-labeled pPB-HSA

The experimental protocols for animal studies with the CCl4-model were approved by the Animal Ethical Committee of the University of Groningen (The Netherlands). Male C57BL/6 mice (20–22 g) were obtained from Envigo (Horst, The Netherlands). Animals received ad libitum normal diet with a 12 h light/dark cycle. Mice (n = 12) received a single injection of CCl4 (Sigma Aldrich, Zwijndrecht, The Netherlands) diluted in olive oil (0.5 mg/kg) intraperitoneally. After 24 h, mice were intravenously injected with tracer amounts of 125I-labeled pPB-HSA (1 × 105–5 × 105 cpm per minute (CPM) in PBS) and sacrificed after 10, 30 or 60 min (n = 4 per time point), after which blood and all organs were collected to assess radioactivity [18]. pPB-HSA was labeled with 125I as described before [18].

2.2. Synthesis of polymers and proteins

The two multi-block co-polymers were synthesized as described before from the prepolymer PLLA and PCL-PEG-PCL [22,23]. In short, [PLLA] was chain-extended with [PCL-PEG1000-PCL or PCL-PEG3000- PCL] using 1,4-butanediisocyanate (Actu-all Chemicals B.V., Os, The Netherlands), to prepare the multi-block co-polymers [PCL-PEG1000- PCL]-[PLLA] (50/50 weight ratio) and [PCL-PEG3000-PCL]/[PLLA] (30/ 70 weight ratio). For this, [PLLA] and [PCL-PEG1000-PCL or PCL- PEG3000-PCL] were dissolved in dry 1,4-dioxiane (80 °C, 30 wt-% solution), 1,4-butane diisocyanate was added and the reaction mixture stirred for 20 h. The reaction mixture was frozen and freeze-dried at 30 °C shelf temperature to remove 1,4-dioxiane.

The proteins pPB-HSA and pPB-MSA were synthesized as described before [24]. Briefly, N-(maleimidobutryl)-oxysuccinimide ester was added to either human (purified from Ceba®, Sanquin, Amsterdam, The Netherlands) or mouse serum albumin (Equitech-Bio inc., Kervil, TX, USA). Next, N-succinimidy 1-acetylthioacetate (SATA)-modified pPB (C®SRNLIDC®, Ansynth Service B.V., Roosendaal, The Netherlands) and activation solution (containing hydroxyxylamine and EDTA) were added. After extensive dialysis, the monomeric product was freeze-dried and stored at −20 °C.

2.3. Production and characterization of microspheres

Microspheres were produced using a similar water-in-oil-in-water double emulsification evaporation method as described previously [22]. In brief, the multi-block co-polymers were dissolved in dichloromethane in a 50:50 weight ratio. For the primary emulsion, PBS (control) or a solution of 80 mg/mL protein (HSA; or pPB-HSA and HSA, or the mouse equivalents, in a 3:2 ratio) was added to the filtered polymer solution to obtain 5 wt-% theoretical protein load and homogenized. For the secondary emulsion, the primary emulsion was added to 4 wt-% poly vinyl alcohol (Sigma Aldrich) + 5 wt-% NaCl solution in water under stirring. The hardened microspheres were collected by filtration, washed and freeze dried.

Microspheres were characterized for morphology by scanning electron microscopy, for particle size distribution by laser diffraction, and for protein content and in vitro release of HSA, pPB-HSA or pPB-MSA as described by [22]. Scanning electron microscopy imaging was performed at an acceleration voltage of 10 kV (JSM-6460 microscope, Jeol, Tokio, Japan). Samples were fixed on an aluminum sample holder using double sided adhesive carbon tape and sputter coated with 10 nm of gold. The particle size distributions of microspheres were determined with laser diffraction and subsequently the span of the particle size distribution was calculated using Eq. (1),

$$\text{Span} = \frac{X_{90} - X_{10}}{X_{50}}$$  

(1)
where $X_{10}$, $X_{50}$ and $X_{90}$ represent the volume percentages of particles (10%, 50% and 90% undersize, respectively). The in vitro release was measured in triplicate by a sample-and-replace method. Briefly, 10 mg of microspheres were suspended in 1.0 mL release buffer (100 mM sodium phosphate buffer, containing NaCl, Tween 80 and NaN3). Samples of 800 μL were taken at predetermined time points and replaced by fresh buffer. Protein concentrations were determined with BCA assay and an in-house developed ELISA for pPB-HSA (see section ELISA). The protein content of the remaining microspheres was determined using BCA assay as described before [22] and was used to calculate the encapsulation efficiency (EE), which is defined as the weight of encapsulated protein (i.e. HSA, pPB-HSA or pPB-MSA) divided by the weight of total protein used.

2.4. Pharmacokinetics of pPB-HSA from microspheres

Studies with the Mdr2−/− mouse model were approved by the Animal Ethical Committee of the State of Rhineland Palatinate. Female FVB mice (n = 8) were obtained from Jackson Laboratory (Jackson Laboratory, Bar Harbor, ME, USA) and FVB Mdr2−/− mice (n = 24) (20–28 g) were bred in hometozogy at the Institute of Translational Immunology at Mainz University Medical Center. Mdr2−/− mice aged 11–15 weeks display advanced liver fibrosis with a 4 to 5-fold increased liver collagen content [25]. Wildtype and Mdr2−/− mice were housed with a 12 h light/dark cycle with water and ad libium normal diet. At age 11–15 weeks Mdr2−/− mice were injected subcutaneously in the neck, a site with sufficient subcutaneous space, with a suspension of 12.6 wt-% microspheres in 500 μL 0.4% carboxymethyl cellulose (CMC, Aqualon high Mw, Ashland, pH 7.0–7.4). Microspheres contained either 5 wt-% HSA (n = 4), 3 wt-% pPB-HSA + 2 wt-% HSA (n = 16), 3 wt-% pPB-MSA + 2 wt-% MSA (n = 8), or were empty (polymer only) (n = 4). Groups of mice were sacrificed at 1, 3, 5 or 7 days for pPB-HSA microspheres (n = 4 at each time point) and all other groups at day 7 after microsphere administration. Blood, liver and off target organs were collected for further analysis.

2.5. ELISA

2.5.1. ELISA for pPB-HSA

pPB-HSA levels both in vitro (100 μL release buffer) and in vivo (100 μL plasma or 1.4 mg/100 μL protein of liver, as determined with Lowry assay) were assessed with our in-house developed sandwich ELISA [22]. In short, the capture antibody α-pPB was diluted in coating buffer (100 mM NaHCO3/33 mM Na2CO3 in water (pH 9.5) and incubated overnight (100 μL, 6.5 mg/L 1:1000 custom prepared by Charles Rivers, Den Bosch, The Netherlands) in a 96-well high protein binding plate (Corning, New York, NY, USA). The plate was washed extensively with PBS containing 0.5% Tween-20 (PBS-T) and blocked with 200 μL 5 wt-% nonfat dry milk in PBS-T for 1 h. After washing, 100 μL sample was incubated for 2 h. The plate was washed again, and the detection antibody goat α-HSA was applied (100 μL, 1:8000, ICN Biomedicals, Zoetermeer, The Netherlands) for 1 h. The appropriate HRP-conjugated secondary antibody (100 μL, 1:2000, DAKO, Santa Clara, CA, USA) was applied for 1 h, and the substrate tetramethyl benzidine (100 μL, R&D Systems, Minneapolis, MN, USA) was incubated for 20 min after washing with PBS-T. The absorbance was measured at 450 nm (THERMOMax microplate reader, Molecular Devices, Sunnyvale, CA, USA) after addition of 50 μL 2 N H2SO4.

2.5.2. ELISA for immunoglobulins

Plasma levels of immunoglobulins against pPB-HSA or pPB-MSA were measured with an in-house developed ELISA. Either pPB-HSA or pPB-MSA (100 μL, 10 μg/mL coating buffer) was incubated for 2 h at room temperature in a high protein binding 96-well plate (Corning). After extensive washing with PBS-T, non-specific binding sites were blocked with 200 μL 5 wt-% nonfat dry milk in PBS-T for 1 h. Plasma samples (100 μL plasma, diluted 1:100) were added for incubation of 1 h after washing with PBS-T. The plate was washed again and HRP-conjugated anti-mouse immunoglobulins diluted in blocking buffer (100 μL, 1:2000, DAKO) were incubated for 1 h. After washing with PBS-T, the substrate tetramethyl benzidine (100 μL, R&D Systems) was incubated for 20 min. The absorbance was measured at 450 nm after addition of 50 μL 2 N H2SO4.

2.6. Quantitative real-time PCR

Total RNA from was isolated from livers using a Maxwell® LEV simply RNA Cells/Tissue kit (Promega, Madison, WI, USA) according to manufacturer’s instructions. RNA concentrations were determined using NanoDrop ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The primers used include procollagen α1(I) forward: 5′-TGACTGGAGAGGCGGAGATG-3′; reverse: 3′-ATCCATC CGGCTATGCTCTCT-5′; PDGFβ-receptor forward: 5′-AACCCCCCTTAACGT GTCC-3′; reverse: 3′-TTCCCTCTATTGGCCATCTC-5′; β-actin forward: 5′-ATGGTGGTGACATCAAAAGA-3′; reverse: 3′-ATGCCAGAGTCTACCC TACC-5′ (all Sigma-Aldrich). Quantitative real-time PCR analysis was performed with 10 ng cDNA per sample according to manufacturer’s instructions (SensiMix™ SYBR kit, Bioline, Taunton, MA, USA) and was analyzed by the ABI7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). For each sample, the threshold cycles (Ct values) were calculated with the SDS 2.3 software program (Applied Biosystems) and mRNA expression was normalized for β-actin.

2.7. Immunohistochemistry

Cryosections of neck skin tissue were cut with a thickness of 4 μm (Cryostar NX70 cryostat, Thermo Fisher Scientific), dried and fixed with acetone. Paraaffin sections of livers were cut with a Leica Reichert Jung 2040 microtome (Leica Microsystems, Nussloch, Germany) with a thickness of 4 μm. The sections were deparaffinized in xylene and ethanol. All sections were rehydrated and PBS was incubated for 1 h with the primary antibody (goat anti-collagen I&III (both 1:200 ± 5% normal mouse serum (Southern Biotech, Birmingham, AL, USA)) or rabbit anti-HSA (1:1500 (ICN Biomedicals)) at room temperature or boiled in 10 mM Tris/1 mM EDTA (pH 9.0) for 15 min prior to overnight incubation with rabbit anti-PDGFβ-receptor (1:50, Cell Signaling) at 4 °C. Next, sections were incubated with the appropriate HRP-conjugated secondary antibody (1:100, DAKO, Santa Clara, CA, USA) for 30 min at room temperature, which were visualized with ImmPACT NovaRED (both Vector, Burlingame, CA, USA). Hematoxylin counterstaining was performed. Digital photomicrographs were captured at 400 × magnification (Aperio, Burlingame, CA, USA).

2.8. Statistical analyses

At least 3 individual experiments were performed for the in vitro microsphere characterization and these data are represented as mean ± SD. All other data are represented as mean ± SEM. The graphs and statistical analyses were performed with Graphpad Prism version 6.0 (GraphPad Prism Software, Inc., La Jolla, CA, USA). The differences between the groups were assessed by ordinary one-way ANOVA followed by Bonferroni’s multiple comparison test unless stated otherwise. Basic pharmacokinetic modeling was performed with the computer program Multifit for non-linear curve-fitting [26].

3. Results

3.1. In vivo pharmacokinetics of pPB-HSA

To determine the in vivo kinetics and tissue distribution of pPB-HSA, all organs of mice with CCL4-induced acute liver fibrogenesis were collected and examined after a single intravenous injection with 131I. 
labeled pPB-HSA. Ten minutes after injection, high amounts (65 ± 6% of the dose) accumulated in the liver, expressing the PDGFβR [15], whereas 15 ± 2% was still found in the plasma. A minor amount of the remaining fraction was detected in the kidneys (2.6 ± 0.5%), spleen (2.1 ± 1.0%), lungs (1.4 ± 0.5%), heart (0.9 ± 0.3%) and brains (0.2 ± 0.1%). The active targeting of pPB-HSA to myofibroblasts in the fibrotic liver has been shown in previous studies [18], and we herewith confirmed the steering of pPB-HSA to the organ with the highest PDGFβR-expression (Fig. 1). Based on the plasma concentration per milliliter at 10, 30 and 60 min after injection, the in vivo plasma half-life was calculated to be approximately 40 min. Basic kinetic modeling assuming 1-compartment kinetics further yielded a rough estimate for the clearance of 58 μL/min and for the volume of distribution of 3 mL (Fig. S1 and Table S1).

3.2. Microsphere characterization

Microspheres containing pPB-HSA that ensure gradual and prolonged release into plasma for at least 7 days were developed. All microspheres were spherically shaped and had a smooth surface with little to no pores, as shown with scanning electron microscopy (Fig. 2A, Fig. S2). Laser diffraction analysis revealed that the two batches of protein-loaded microspheres had similar particle size distributions with a median particle size of around 25 μm, while polymer-only control microspheres were slightly smaller at all volume percentages (Table 1). The polydispersity is expressed in the span (Eq. (1)), which is comparable to values found in earlier studies applying the same production process [22]. Both protein-loaded microsphere formulations yielded high encapsulation efficiencies of protein (Table 1) and showed low burst release and sustained release in vitro for at least 14 days (Fig. 2B), with a cumulative release after 14 days of 52% for pPB-HSA and 55% for HSA. The slightly higher molecular weight of pPB-HSA (~74 kDa) than HSA (67 kDa) did not affect the release rate, with average values of 4.5 ± 0.65%/day and 5.0 ± 0.29%/day, respectively.

3.3. Pharmacokinetic profile of pPB-HSA released from microspheres in vivo

The pPB-HSA microspheres were subcutaneously injected in the neck of Mdr2−/− mice with advanced biliary liver fibrosis. These mice exhibit a profound increase in procollagen α1(I) and PDGFβR gene expression as compared to normal mice (Fig. 3A, D), which is a hallmark of fibrosis [25]. The livers of these Mdr2−/− mice showed characteristic deposition of collagen types I & III in the portal areas particularly around the bile ducts (Fig. 3B) as a consequence of the toxicity of accumulated of phosphatidylcholine in the hepatocytes, due to the knock out of the transporter gene. Collagen deposition extended into advanced portal to portal bridge formation in the parenchyma (Fig. 3C). Accordingly, myofibroblasts lining the fibrotic bile ducts and myofibroblasts in the parenchyma expressed the PDGFβR (Fig. 3E, F).

The microsphere injection site was inspected for presence and appearance of microspheres that resided for 7 days at the injection site. Clearly, during these 7 days the microspheres remained subcutaneous (Fig. 4A) and the staining for HSA demonstrated that the microspheres in vivo still contained protein after 7 days (HSA and pPB-HSA) (Fig. 4B, C), as was expected from the in vitro release studies.

The pharmacokinetic profile of the released pPB-HSA was determined at 1, 3, 5 and 7 days after injection. We confirmed sustained release of pPB-HSA from the microspheres into the plasma up to 7 days after injection reaching a steady state concentration of 44.9 ± 4.7 ng/mL, which equals 2.4 ± 10^{-3} ± 0.3 ± 10^{-3}%/mL based on the total pPB-HSA content of the microspheres, within 1 day after injection and remained constant for 5 days (Fig. 5A). The infusion rate of pPB-HSA from the microspheres into the circulation was determined by multiplying this value with the clearance as estimated in the acute CCL4-model, yielding an infusion rate of 0.2% per day. Interestingly, the steady state concentration in plasma was 78.5 ± 10.9% lower at 7 days after injection than at day 5 (p < 0.0001, unpaired student t-test).

In line with the PDGFβ-receptor expression, pPB-HSA was present in the fibrotic livers at all time points, reflecting a similar pattern as seen in plasma, reaching a steady state concentration of 121 ± 28.3 ng/
liver within 1 day after injection (Fig. 5B). Similar to the observations in plasma, a decline of 75.4 ± 5.3% in the pPB-HSA concentration was detected in the livers after 7 days as compared to day 5 (p = 0.002, unpaired student t-test). As expected, for both plasma and livers, the control group with microspheres containing HSA only did not show any presence of pPB-HSA.

The in vivo reduction in pPB-HSA levels 7 days after injection as compared to earlier time-points was rather unexpected, as protein was still present in the subcutaneous microspheres after 7 days (Fig. 4B and C) and the in vitro data showed minimal burst release of pPB-HSA followed by pseudo-zero order sustained release kinetics for 10 to 14 days (Fig. 2B). To further investigate this phenomenon, plasma samples were analyzed for the presence of antibodies against the albumin-based carrier as this is not a mouse based protein, which might interfere with our results. Indeed, the decline in pPB-HSA concentration at day 7 coincided with the induction of an antibody response against pPB-HSA as compared to the empty microspheres as control group. Mice that received microspheres containing HSA only also showed an induction of the immune response to the same extent (Fig. 6A). This immunological reaction in these mice to the drug carrier was absent when microspheres containing pPB-MSA, in which human serum albumin was replaced with the mouse equivalent (Fig. 6B), as the response was not exceeding background levels. The pPB-MSA microspheres displayed similar size distribution (median 22 μm) and morphology, and showed slightly faster in vitro release characteristics than the microspheres containing the human albumin-based carrier (Fig. S3, Table S2).

4. Discussion

Protein-based therapeutics including fusion proteins, such as protein-based drugs modified with targeting moieties, drastically increased the treatment perspective for a wide variety of diseases [13,14,27]. In the past decades, we developed various compounds enriched with PDGFβ-receptor recognizing peptides (pPB) including the myofibroblast-selective drug carrier PDGFβR-targeted human serum albumin (pPB-HSA) [18]. We aim to apply this PDGFβR-targeted drug carrier, when coupled to an effective antifibrotic compound, for the treatment of chronic fibrotic diseases. However, delivery of protein-based compounds involves parenteral administration with consequent short-lasting peak-concentrations and the need for frequent application, posing a high burden to the potential patient. For effective treatment of such diseases the sustained release of therapeutics is essential. We therefore developed a patient-friendly formulation providing sustained release of protein therapeutics, which can be applied for protein constructs like pPB-HSA [22]. In the present study, we showed that pPB-HSA was released from the subcutaneous polymeric microspheres into the plasma and reached easily detectable levels in the fibrotic liver, where a steady state concentration was achieved within 1 day after microsphere injection, which lasted for 5 days. A sudden decrease of

<table>
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<th>Table 1</th>
<th>Characteristics of microspheres with different content as used in vivo in the Mdr2 −/− model.</th>
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<tr>
<td>Formulation</td>
<td>Protein load</td>
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<tr>
<td>pPB-HSA</td>
<td>3% pPB-HSA/2% HSA</td>
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<tr>
<td>HSA</td>
<td>5% HSA</td>
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Fig. 3. (pro-) Collagen and PDGFβ-receptor expressions at mRNA (A and D, respectively) and protein level (B, C, E, F) in livers of Mdr2 −/− mice. Of note, the staining (in red) is observed in particular around the bile ducts (BD) (B and E, respectively) and in the parenchyma (C and F, respectively). Differences between groups were assessed by unpaired student t-test. Arrows indicate fibrous collagen bands.
this concentration at day 7 was associated with antibody formation, while antibody formation was prevented with the mouse albumin-based carrier.

In an attempt to correlate the in vitro release of pPB-HSA from microspheres and the estimated in vivo infusion-rate, based on the steady state concentration in plasma and the plasma clearance, we calculated release rates of 4.5%/day and 0.2%/day, respectively. This yields approximately a 20-fold lower value in the in vivo situation. According to Cardot and Tomic a linear in vitro - in vivo correlation only holds true when either drug dissolution or release from the formulation are the sole limiting factors for the systemic absorption [28]. In our studies, multiple factors might have contributed to the limitation in the in vivo infusion-rate of pPB-HSA, which will be discussed in more detail below.

The release of any drug from a subcutaneous depot occurs, when dissolved, within the subcutaneous tissue (or hypodermis) predominantly by diffusion or convection. The anatomy and microenvironment of the hypodermis can largely affect the release and transport of the drug [29]. Firstly, components of the subcutaneous extracellular matrix including collagens, noncollagenous glycoproteins, hyaluronic acid and chondroitin, heparan or dermatan sulfates can interfere with locally dissolved drugs and impede their passage to the circulation [30]. Such interference can happen in several ways, for example by electrostatic or (non-) specific interactions with the released protein drug. This ultimately leads to the formation of charged and/or large complexes that are unable to diffuse through the tissue and consequently accumulate subcutaneously instead [31,32]. Also, steric exclusion of the drug by the ECM can play a role, causing not only limited diffusion, but might also influence the solubility of the protein drug [33]. Secondly, other microenvironmental parameters including the ionic composition and the pH of the injection site could limit protein absorption and stability [30,34]. Lastly, the uptake of drug in the systemic circulation can be reduced due to degradation of the protein at the injection site by the presence of proteases in the interstitial fluid [35].

After diffusion through the hypodermis, the released drug can reach the systemic circulation via local semipermeable capillaries or by lymphatic vessels, depending on the size of the protein. When the sustained release formulation is subcutaneously injected, the pressure in the interstitial fluid is increased. As compensatory mechanism, the local lymphatic flow is stimulated in order to transport excess of fluid into the lymphatic system. Consequently, more macromolecules with a diameter of up to 100 nm can be taken up, including pPB-HSA (which we assume to have a diameter of < 25 nm [36]), and eventually these molecules will be delivered to the bloodstream [37,38]. However, in mice Wu et al. demonstrated a limited uptake of a similar protein, i.e. bovine serum albumin, into the lymphatics following subcutaneous injection, thereby causing lower drug plasma concentrations [39].

Another reason could be pre-systemic metabolism of the drug which may occur in the draining lymphatics before reaching the circulation [35]. Such parameters cannot be easily mimicked in vitro. In summary, the in vitro studies are oversimplified as compared to the in vivo circumstances, which is clearly illustrated by our data. Recently, Kinnunen et al. made the first step towards an in vitro model that provides a better representation of subcutaneous conditions [40] and this model potentially yields better correlations with in vivo data in future studies.

Despite the expected discrepancy between the in vitro and in vivo

Fig. 4. Immunohistochemical staining for HSA of skin tissue samples at 7 days after injection. These samples were obtained at the subcutaneous injection site of the microspheres (MSP). MSP contained either (A) no protein, (B) HSA or (C) pPB-HSA.

Fig. 5. Concentration of pPB-HSA in (A) plasma and (B) livers of Mdr2−/− mice that received microspheres (MSP) subcutaneously for either 1, 3, 5 or 7 days containing pPB-HSA or HSA (only after 7 days) as determined with ELISA.
release, we were able to show protein release and measured pPB-HSA at a plasma steady state concentration of approximately 45 ng/mL during 5 days after injection (Fig. 5A), a sound result in view of the extremely low in vivo half-life of 40 min. It is noteworthy that the calculated half-life might be an overestimation, as the PDGFβR is most likely expressed at a lower level in the acute CCL4-model than in the more clinical relevant Mdr2−/− model, mimicking many disease features seen in human cirrhotic patients as well. Furthermore, a comparable concentration profile was demonstrated in the fibrotic liver as our target organ, in which the PDGFβ-receptor was highly expressed, with a steady state concentration of 121 ± 28.3 ng/liver within 1 day after injection. Whether this concentration is sufficient to reach therapeutic levels would depend on the potency of the attached antifibrotic compound.

Several studies showed the receptor selectivity of PDGFβR-targeted constructs in different animal models [18,41,42]. With the use of 1125-labeled pPB-HSA we confirmed the specific accumulation of this particular construct in mice suffering from acute liver fibrogenesis (Fig. 1). Because of the confirmation of the specific accumulation and the limited accumulation found in other organs (between 0.2 and 2.6%), we did not consider any accumulation in off-target organs in the pharmacokinetic study in the Mdr2−/− model.

The rapid decline in plasma concentration after 7 days was not anticipated, based on the pseudo zero-order in vitro release kinetics (Fig. 2B) and the presence of protein in the microspheres in vivo 7 days after injection (Fig. 4). We therefore hypothesized that this decline might be induced by antibody formation in mice against the human albumin-based carrier. Indeed, we demonstrated low antibody levels during the first 5 days, drastically increasing at day 7, which paralleled the decline in plasma and liver levels of pPB-HSA (Figs. 5, 6A). As part of the adaptive immune defense, B cells produce immunoglobulin antibodies against an antigen, i.e. a foreign protein like pPB-HSA or HSA in mice, upon prolonged exposure. The induced antibodies form complexes with pPB-HSA or HSA that are ultimately eliminated by the complement system [43].

Eventually, the induction of the immune response was successfully circumvented by replacing human albumin with mouse albumin (Fig. 6B), thereby creating a non-immunogenic drug carrier pPB-MSA. This alternative approach proved that the antibody formation was directed against the albumin part of pPB-HSA, and not against the pPB targeting moieties or the crosslinking domain. We now constructed a drug carrier that is compatible with our polymeric microsphere formulation and applicable for chronic administration of proteins.

In conclusion, we demonstrated that PDGFβR-targeted albumin was released in vivo from the subcutaneously injected polymeric microspheres using hydrophilic multi-block copolymers composed of poly(ε-caprolactone) and polyethylene glycol/poly(ε-caprolactone) blocks. pPB-HSA was detectable in the plasma and in the fibrotic liver up to 7 days after injection. This implies that sustained release microsphere formulations composed of these polymers are suitable for the parenteral delivery of therapeutic proteins and might constitute a realistic option for future application in chronic diseases such as liver fibrosis, bearing in mind that the origin of the potential therapeutic protein should be complementary to the species used as model. Future research should focus on pharmacodynamic aspects of the drug formulation, by incorporating the PDGFβR-targeted albumin-based drug carrier coupled to an anti-fibrotic drug, thereby directing towards the clinical application of this combined delivery system ensuring sustained release of targeted therapeutic proteins.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2017.11.029.

References