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Research paper

Long-term release and stability of pharmaceutical proteins delivered from solid lipid implants

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ABSTRACT

Solid lipid implants (SLIs) prepared by twin-screw (tsc) extrusion represent a promising technology platform for the sustained release of pharmaceutical proteins. In this work, we report on two aspects, long-term release and stability of released protein. First, SLIs were produced by tsc-extrusion containing the low melting triglyceride H12 and the high melting triglyceride Dynasan D118. Two different proteins available in a freeze-dried matrix containing hydroxypropyl-β-cyclodextrine (HP-β-CD) were incorporated into the lipid matrix: a monoclonal antibody (mAb) from the IgG_1 class and the f_{ab}-fragment Ranibizumab (Lucentis®). SLIs, composed of 10% protein lyophilizate and both triglycerides, were extruded at 35 °C and 40 rpm. Sustained release of both proteins was observed in a sustained manner for approximately 120 days. Protein load per implant was increased by three different approaches resulting in a protein load of 3.00 mg per implant without affecting the release profiles. The incubation medium containing the released protein was collected, concentrated and analyzed including liquid chromatography (SE-HPLC, IEX, HIC), electrophoresis (SDS-PAGE, on-chip gel electrophoresis) and FT-IR spectroscopy. The mAb showed a monomer loss of up to 7% (SE-HPLC) and IEX analysis revealed the formation of 16% acidic subspecies after 18 weeks. FT-IR spectra of mAb indicated the formation of random coil structures towards the end of the release study. Ranibizumab was mainly released in its monomeric form (>95%), and approximately 5% hydrophobic subspecies were formed after 18 weeks of release. FT-IR analysis revealed no changes in secondary structure. The release and stability profiles of both proteins underline the potential of SLIs as a delivery system. SLIs provide a promising platform for applications where really long-term release is needed, for example for intraocular delivery of anti-vascular endothelial growth factor (VEGF) drugs for age related macular degeneration (AMD).

1. Introduction

The controlled release of pharmaceutical protein drugs is a key strategy to reduce both the frequency of administration and side effects which are related to high drug concentrations [1]. However, developing protein delivery systems which ensure both long-term release profiles and at the same time maintain the stability of the protein drug remains a challenge [2]. The need for such devices can easily be illustrated using the example of intraocular treatment of AMD with anti-VEGF drugs such as Ranibizumab (Lucentis®) or Aflibercept (Eylea®). To date, anti-VEGF drugs are the standard treatment of AMD [3], but their frequent administration causes high costs in the health care system [4]. Besides the stress for the patient, the required monthly injections, for example of Lucentis®, into the vitreous causes injection-related adverse effects like endophthalmitis [5]. Hence, it would be preferable to prolong the period between two intravitreal injections by using sustained release devices to improve patient convenience, safety, and efficacy. As a model anti-VEGF drug, the f_{ab}-fragment Ranibizumab was chosen for this study.

Since the early 2000s, lipid based drug delivery systems have gained more and more interest as platform for sustained release of proteins [6–10]. Lipid implants have excellent properties for *in vivo* applications as demonstrated by good biodegradability and biocompatibility [11]. Moreover, long-term release of protein drugs was successfully demonstrated: interferon α-2a was delivered over more than 60 days [12], and sustained release of lysozyme was described for more than 200 days [13]. Several research groups are working on lipid based depots including implants for sustained erythropoietin release as described by Appel et al. [14] and Jensen et al. [15]. The intravitreal use of clindamycin phosphate loaded lipidic implants [16] or the use of solid lipid nanoparticles (SLNs) used by others for various application

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routes demonstrates the large variety of application possibilities [17–20]. Preserving the integrity of incorporated proteins such as interferon α-2a [12,21] or brain-derived neurotropic factor (BDNF) [22], is the most valuable benefit over the commonly used and well investigated poly-lactic acid (PLA) or poly-lactic-co-glycolic acid (PLGA) polymers. PLGA polymer erosion causes pH drop and an increase in osmotic pressure – particularly inside the matrix – which in turn leads to protein degradation caused by acylation, deamidation or aggregation [23–25] and incomplete release [26–29].

Within our group, Schulze et al. [12] introduced tsc-extrusion as manufacturing technique for lipid implants which meanwhile became the standard manufacturing technique replacing direct compression or casting methods [22,30,31]. Compared to release profiles of compressed implants, a more sustained release from extruded implants in addition to a more homogenous drug distribution was observed [32]. Tsc-extrusion was already used in several studies [13,33,34] allowing the manufacturing of triglyceride blends of a low melting and a high melting lipid at comparatively low temperatures of 42 °C [12]. For lipid based (mainly triglycerides) depots, first investigated in the early 2000s, research focused on release profiles and underlying release mechanisms [35–38], the solid-state behaviour [39,40], effect of release modifiers [30,41–43] or *in vivo-in vitro* correlations of various drugs [13,31,44,45], including pharmaceutical relevant proteins such as rh-interferon-α and interleukin-18 [7].

In addition to release data from lipid based systems, stability aspects of encapsulated and released protein have first been described in 2004 [6]. For example, the integrity of rh-interferon α-2a after incorporation into SLIs as well as after 28 days of *in vitro* release was measured by SDS-PAGE, showing no noticeable aggregation or fragmentation of the protein [12,21]. The released fractions where further analyzed by SE-HPLC for up to 60 days, confirming that the protein was mainly released in its monomeric form (>95%) [12]. Conversely, the SDS-PAGE analysis of extracted brain-derived neurotropic factor (BDNF) from a glyceryl tripalmitate matrix revealed the formation of dimers upon incubation for 1 month [22].

This study focused on two aspects: the feasibility of tsc-extruded lipid implants for long-term release of pharmaceutical proteins and the stability profile of such protein drugs after release from the lipid matrix. First, we investigated the long-term release of a monoclonal antibody (mAb) from the IgG_1 class and the f_{ab}-fragment Ranibizumab (Lucentis®), two proteins which differ by structure and molecular weight. The lipid mixture – a binary mixture of the triglycerides H12 and Dynasan D118 – described within this study has already been used previously [12,13]. In contrast to previous publications, polyethylene glycol (PEG) as a pore forming [30,42] and precipitating agent [46] was eliminated due to its potential negative effect on protein integrity [47] and allergic potential [48–50]. Furthermore, two different approaches were followed to increase protein load per implant. Protein fractions were collected at predetermined time points, concentrated, and analyzed by applying SE-HPLC, IEX, HIC, SDS-PAGE, on-chip gel electrophoresis, and FT-IR.

2. Materials and methods

Triglycerides Dynasan D118 (100% tristearin; melting point 70.2 °C) and H12 (mixture of trilaurin, trimyristin and tripalmitin; melting point 42.9 °C) were kindly donated by Cremer Oleo (Hamburg, Germany). Hydroxypropyl-β-cyclodextrine (HP-β-CD) was a gift of Wacker Chemie AG (Burghausen, Germany). The mAb is a monoclonal IgG_1 antibody with a molecular weight of about 150 kDa. The f_{ab}-fragment Ranibizumab (Lucentis®) with a molecular weight of approximately 50 kDa was contributed by the University of Utah (Salt Lake City, Utah, USA) in the form of the marketed product (formulated with 10 mM histidine-HCl, 10% α, α-trehalose$*2H_2O$, 0.01% polysorbate 20, pH 5.5). All salts used within this study were purchased from Sigma Aldrich (Steinheim, Germany) and were of analytical grade.

2.1. Preparatory steps and preparation of SLIs

2.1.1. Dialysis and exchange of stabilizing excipients

Dialysis of the protein formulations was performed in order to substitute the different stabilizers comprised within the protein bulk material with HP-β-CD. HP-β-CD was used as lyoprotectant to assure protein stabilization during freeze drying and storage as reported previously for rh-interferon α-2a [21] and erythropoietin [26]. The proteins were dialyzed against 50 mM phosphate buffer pH 6.2 at 4 °C using CelluSep T1 dialysis tubes from Orange Scientific (Braine-l'Alleud, Belgium) with a molecular weight cut off of 3500 Da. After dialysis, the protein concentration was determined spectrophotometrically applying an UV–VIS spectrometer Agilent 8452 (Böblingen, Germany), protein concentration was set to 10 mg/ml and HP-β-CD was added to obtain a ratio of 1:1 [w/w] or 3:1 [w/w].

2.1.2. Lyophilization process

Aliquots (2.0 ml) of the formulations from 2.1.1 were filled into 10R vials and lyophilized using an Epsilon 2-6D freeze dryer from Martin Christ GmbH (Osterode, Germany). At a rate of 1 °C/min, the solution was frozen to −50 °C and the temperature was held for 30 min. Primary drying was performed at a shelf temperature of −10 °C and a pressure of 0.09 mbar for 24 h. Afterwards, temperature was increased to 25 °C for 4 h. Secondary drying was then performed at 25 °C for 7 h. After the cycle was finished, freeze dried samples were held at 5 °C at 0.09 mbar. Finally, the freeze dryer was vented with filtered (0.2 μm) nitrogen gas to approximately 800 mbar. Vials were stoppered and crimped after unloading.

2.1.3. Twin-screw extrusion of SLIs

Extrudates were prepared from a powder mixture as described previously [13]. The formulation consists of 10–20% protein lyophilizate; the lipid matrix comprised 50% of the low melting lipid H12 and 50% of the high melting lipid D118. H12, D118 and lyophilized protein were weighed into an agate mortar and admixed by hand to create a uniform powder mixture. Tsc-extrusion was performed using a mini-extruder ZE-5 from Three-Tec GmbH (Seon, Switzerland) comprising three heating zones. Approximately 1 g of the powder mixture was fed into the barrel of the mini-extruder and extrusion was performed at 35 °C. The rotation speed of the screws was set to 40 rpm. The implant diameters were adjusted by applying outlet plates with 1.5 mm and 1.7 mm diameter, respectively. Extruded implants were cut into a length of 15 mm resulting in an implant weight of around 30.7 mg ± 0.78 mg (1.5 mm diameter) and 39.3 mg ± 0.96 mg for the extrudates having 1.7 mm in diameter.

2.1.4. Protein release tests from extrudates

Extrudates (n = 4) were placed in 2.0 ml micro-centrifuge tubes (VWR, Radnor, PA, USA) and incubated at 37 °C in a Certomat IS (Sartorius BBI, Göttingen, Germany) horizontal shaker at 40 rpm in 1.0 ml PBS buffer pH 7.4. At predetermined time points, the release medium was exchanged completely. Protein concentration was analyzed spectrophotometrically at 280 nm applying an UV–VIS spectrometer (Agilent 8453, Böblingen, Germany).