Protein delivery from polymeric matrices

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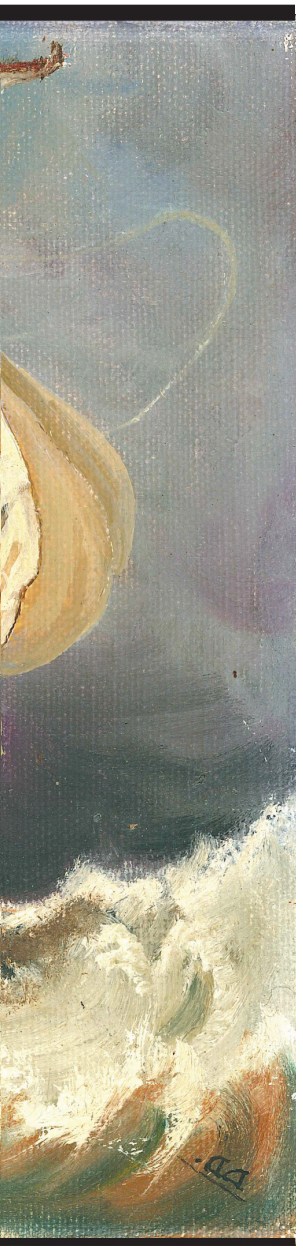

INTRODUCTION AND SCOPE OF THIS THESIS

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The therapeutic effects of insulin were first discovered almost a century ago in 1922. Although at that time insulin was an impure pancreatic extract rather than a pure therapeutic protein, the administration of this extract is widely recognized as the first application of a proteinaceous drug in human medicine.[1,2] In the following decades, more therapeutically active proteins were discovered, such as blood factors, for which the production was based on extraction of these proteins from animal (or even human) sources. Then, in the 1970's, genetic engineering and recombinant DNA technologies were developed enabling synthesis of recombinant (human) proteins. These developments lead to the next large breakthrough in medicine: the large scale production of recombinant therapeutic proteins.[3,4] Consequently, a plethora of recombinant vaccines, peptides and proteins (mainly antibodies) have been commercialized ever since, reaching 166 distinct active ingredients and 212 products in 2014,[5] and this novel class of drugs, the so-called biopharmaceuticals, have revolutionized the treatment of many diseases.[6]

Following its discovery, insulin was thoroughly characterized,[2] and during this process, the difficulty of delivering proteins to patients became apparent.[3] For example when administered via the conventional, oral route, proteins are denatured in the highly acidic environment of the stomach or rapidly degraded by proteolytic enzymes in the gastrointestinal tract (*e.g.* pepsin, trypsin, and chymotrypsin). Moreover, the size of proteins hampers passage through intestinal membranes and thereby the absorption into the systemic circulation. Naturally, researchers attempted to find the ideal route of administration and formulation for insulin, though their endeavors more often failed than succeeded.

Although the pulmonary administration of insulin was already described in 1924,[7] to date, biopharmaceuticals are almost exclusively administered using aqueous solutions which provide limited shelf life. Furthermore, protein-based therapy often involves frequent injections which impair both patient comfort and compliance. Many products suffer from stability issues due to the large and complex structure of proteins, which in turn may lead to safety issues (*i.e.* immunological reactions). Hence, stabilization and formulation strategies for proteins continue to be a subject of investigation.

One approach to relieve the patient's burden of frequent injections is to deliver biopharmaceuticals using systems for sustained and controlled release. In particular, biodegradable polymers are considered to be excellent matrices for such controlled delivery systems,[8,9] and release of proteins from such systems can range from hours

to months depending on the polymer type, size and composition.[9,10] These polymers can be processed into different formulation types such as microparticles, micro-sized implants and gels, which usually can be administrated subcutaneously thereby further reducing patient discomfort.

In these polymeric drug delivery systems, however, the stability of proteins is not guaranteed. Destabilization of proteins can occur during the production process, but also during storage or release which may be due to interactions with the polymer and its degradation products. In ***Chapter 2*** an overview of production methods is presented for polymeric micro- and nanoparticles containing proteins and peptides as well as an overview of potentially destabilizing conditions during the production of these particles and approaches to improve the stability of the encapsulated biomacromolecules.

One widely applied strategy to improve the stability of proteins is drying them in the presence of sugars. Upon drying, a sugar glass is formed which stabilizes the protein by vitrification (*i.e.* immobilization; which reduces molecular mobility and thereby degradation reactions) and replacement of water in the hydration shell of proteins (*i.e.* the water molecules surrounding proteins by which they maintain their tertiary structure) by forming hydrogen bonds with functional groups at the protein's surface.[11,12] Disaccharides such as trehalose are known to be excellent stabilizers due to their ability to create a tight packing around the irregularly shaped surface of the protein. However, disaccharides are also prone to crystallization when temperature and humidity rise, and these sugars thereby lose their stabilizing properties. The onset of this crystallization process is partly determined by the glass transition temperature (T_g) of the sugar, which is lowered in the presence of plasticizers, such as water. Polysaccharides have a higher T_g due to their larger size, and corresponding sugar glasses are more resistant to temperature and humidity, though generally lack the ability to tightly surround the protein. In ***Chapter 3***, the advantages of disaccharides and polysaccharides are combined to optimize protein stabilization at non-ideal, yet clinically relevant conditions: temperatures above room temperature and high relative humidity. Several blends of trehalose and the polysaccharide pullulan are freeze-dried to form binary glasses and are evaluated based on their T_g at different relative humidities and their protein stabilizing abilities upon storage. In this work, we are the first to extensively investigate the polysaccharide pullulan for its stabilizing properties.

An alternative to binary glasses is the application of a flexible oligosaccharide such as inulin. Because of its flexible backbone, inulin can provide a tight packing around proteins while its larger molecular mass ensures resistance to higher temperatures.[13,14] This sugar is applied in ***Chapter 4*** where we investigate whether proteins can be successfully incorporated in polymeric implants produced by hot melt extrusion (HME). Because of the high temperatures that are usually required for the HME production process, proteins are inclined to denature. Therefore, HME is generally not considered to be a suitable production process for protein-containing formulations. Moreover, many biodegradable polymers have a hydrophobic nature which might induce unfolding of proteins and thereby destabilize them. The use of engineered phase-separated, multi-block copolymers which contain hydrophilic polyethylene glycol (PEG) blocks may reduce protein denaturation. In ***Chapter 4*** we hypothesize that several approaches could improve the stability of proteins during HME, namely lowering the extrusion temperature, the use of inulin pre-stabilized protein, and the use of hydrophilic polymers. This study was performed using two model proteins and six (co)polymers with different properties.

Research and development of formulations containing proteins is often performed using model proteins due to practical or financial reasons. Such model proteins are usually therapeutically inactive proteins, and are typically similar to the active protein in size or charge. Admittedly, it is unlikely that such a model is a perfect predictor for the behavior of a specific therapeutically active protein. Therefore, progress in formulation development of protein drugs might benefit from already using the active protein in early stages of development. In ***Chapters 5 and 6*** we put this thought into practice and developed a polymeric microsphere formulation for a protein drug carrier targeted to fibrotic tissue. Fibrosis is a progressive disease that can develop in various organs. Currently, no effective treatment is available for this disease15 which is partly due to the systemic side effects of many potential antifibrotic drugs. Thus, specific delivery of drugs to the diseased tissue – targeting – would enhance drug efficacy and may decrease side effects. As a target for our local drug delivery efforts, the platelet-derived growth factor β receptor (PDGFβR) was selected because this receptor is specifically upregulated in fibrotic tissue. Previously, a cyclic peptide referred to as pPB has been developed to target this receptor.16 This peptide binds to the PDGFβR without activating the intracellular downstream pathway. Furthermore, pPB was coupled to human serum albumin (HSA) to prolong its half-life.17 In ***Chapter 5***, we aimed to develop a controlled sustained release formulation for the carrier pPB-HSA.

To obtain the desired release profile, two phase-separated, multi-block copolymers were mixed in different ratios. In addition, differential scanning calorimetry was performed to gain more insight in the release mechanisms of pPB-HSA from our polymeric microspheres. The production process was optimized ensuring maximum robustness, and the resulting formulation was used for a proof of concept in vivo study using a mouse model for renal fibrosis.

In continuation of the proof of concept *in vivo* study, the *in vivo* pharmacokinetics of pPB-HSA were investigated in ***Chapter 6*** in two murine models for fibrosis: the CCl_4 model of acute fibrosis, as well as in the Mdr2 knockout model for advanced biliary (liver) fibrosis, using the same formulation as in the previous chapter.

The administration of proteins from different species, in this case human serum albumin to mice, can potentially induce an immunological response and thereby impair the efficacy of the delivery and targeting. Therefore, we also aimed to improve the delivery of the PDGFβR-targeted construct by using microspheres containing pPB coupled to mouse serum albumin (pPB-MSA). In addition, this chapter describes the extension of our protein targeting and delivery endeavors to a therapeutically active protein by coupling the antifibrotic rho-kinase inhibitor Y27632 to pPB-MSA and subsequent evaluation of this construct in Mdr2 knockout mice.

Chapter 7 gives an overview of all findings in this thesis, as well as some final remarks on how to get controlled release protein therapeutics to the clinic, how to apply the sugar glass technology in polymeric formulations and the potential of delivering targeted proteins from controlled release formulations.

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