

1. INTRODUCTION

1.1. Protein delivery

1.1.1. Proteins as pharmaceuticals

It was in 1922, when the potential of protein drugs for therapies of severe physiological disorders was indicated the first time. A bovine pancreatic extract containing insulin was able to successively treat a diabetic patient (Banting et al., 1922). Pancreatic extracts were already administered by other researchers before (Minkowski and von Mering in 1889, Blumenthal in 1898 and Zülzer in 1908). However, either the applied extraction procedures were unsuitable to produce active and non-immunogenic (antigen-free) formulations or oral administration resulted in inactivation of extracts.

Not before 1955, the chemical structure of insulin was characterized (Sanger et al., 1955). Zahn et al. demonstrated the feasibility to chemically synthesize insulin using a reaction pathway of more than 200 steps in 1963. This was not suitable to replace the extraction of insulin from animal pancreata. But a study of the insulin demand forecasted supply shortages of animal insulin for the year 1992 (Mohan, 2002). The breakthrough to overcome this limitation was the development of the genetic engineering of proteins, established by Goeddel and co-workers in 1979. This method allowed insulin and other protein drugs to be produced independently of limited natural resources. Because of this potential, human insulin was launched as the first bioengineered drug product in 1982.

Further advent of bioengineering and the advances in biotechnology, genomics and proteomics have increased the understanding of physiological control mechanism or led to improved methodologies to fabricate proteins of choice. As a result, protein-based drugs for various pharmaceutical treatments of human diseases were developed in recent years. Today, 121 marketed products with 88 genetically engineered drugs exist on the German market, representing 3.6% of all approved drug substances (Figure 1).

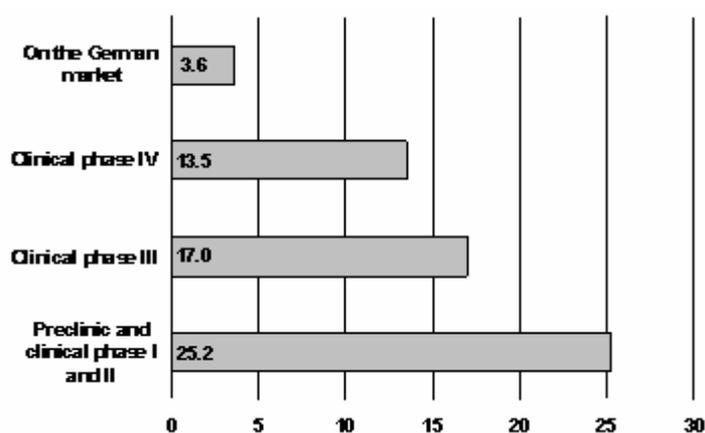


Fig. 1: Proportions of genetically engineered drugs on the German market or in development, % (INSIGHT Health, VFA – Brochure of the German Association of Research-based Pharmaceutical Companies, from January 2006)

Besides solely recombinant human proteins, other polypeptide drugs are monoclonal antibodies or viral or bacterial proteins. The importance and the remarkable scope of functions of proteins in the human or animal body are evident in their roles in catalysis of chemical reactions, transport and storage, in providing coordinated motion, mechanical support and immune protection, in generation and transmission of nerve impulses, and in control of growth and differentiation (Stryer, 1988). Protein drugs can be therefore found in various therapeutic areas like endocrinology (e.g. for diabetes mellitus), immunology (e.g. for rheumatoid arthritis or Crohn's disease), hematology (e.g. for anemia), infectiology (e.g. in vaccination), neurology (e.g. for Multiple sclerosis), oncology (e.g. for colon and breast cancer) or the treatment of genetic disorder (e.g. for cystic fibrosis). Although only 3.6% of all approved drug substances are bioengineered protein drugs, the research pipelines of pharmaceutical companies contain already up to 25% of these compounds, which reflects the increasing importance of protein drugs (Figure 1).

1.1.2. Challenges of protein delivery

Favorable characteristics, like broad applicability, high potency and specificity make proteins an attractive class of therapeutic agents. However, the delivery of protein drugs to the human or animal body is hampered due to their physicochemical properties.

1.1.2.1 Routes of administration of proteins - Bioavailability

Several routes to introduce protein drugs into the body have been investigated. They were administered to the mouth (oral, buccal, sublingual); placed into the nose (intranasal); inserted in the rectum (rectal); breathed into the lungs (pulmonary); given by injection (intravenous, intramuscularly or subcutaneously); instilled in the eye (by the ocular route); delivered through the skin (transdermal). Within these routes local (topical) drug administrations have to be differentiated from drug therapies affecting the entire body (systemic). Each route has specific purposes, advantages and disadvantages.

Localized delivery of drugs can be preferred when considering the use of highly potent drugs, e.g. bone morphogenetic proteins for the induction of bone and cartilage growth. The high systemic drug levels required to achieve an effect in the target tissues would cause unwanted systemic toxicity (Kirker-Head, 2000). Therefore, local delivery can improve the therapeutic potential of the protein drug. Side effects after bolus injections may also be a concern for systemically acting proteins. Cytokines such as interleukin-2, for example, cause flu-like symptoms at high peak serum levels. This may be avoided by the development of a delivery system that provides sustained low plasma levels (Cleland, 2001).

Another important factor, which guides the selection of the administration route, is how easily the drug can enter the body at the site of application. This will significantly affect the rate and extent a substance reaches the systemic circulation and is available at the site of action (bioavailability). The permeability is thereby a function of the tissue (Rojanasakul et al., 1992) and of the physicochemical properties of drug. Limited permeation of drugs through biological membranes is mainly due to their large molecular size and solubility characteristics. Good bioavailability can be obtained with molecular weights of less than 500–700 daltons (Da), whereas the bioavailability decreases sharply beyond masses of 500–700 Da (Donovan et al., 1990).

Proteins are biopolymers, which consist of monomers from a group of mainly twenty, so called “standard”, amino acids. They can contain as many as 4000 amino acid residues, whereas peptides contain less than 20–30 (Lodish et al., 2000). With a mean molecular weight

of the standard amino acids of 111 Da, even very small proteins would be far beyond the boundary for passive transport across biological membranes.

The standard amino acids can be categorized into three groups based on the nature of their side-chain: hydrophilic amino acids, with a polar side-chain (charged or uncharged); hydrophobic amino acids, with an aliphatic or bulky aromatic side-chain; and amino acids with a special group, as in cysteine, glycine and proline. Proteins usually contain amino acids of all three classes making the overall molecule more or less amphiphilic. Since hydrophobic molecules try to minimize exposure to an aqueous environment, hydrophobic amino acids reside primarily in the interior of proteins, whereas the hydrophilic residues are situated primarily on the exterior of the protein. Hence, proteins are usually hydrophilic, but for compounds lacking of a minimum degree of hydrophobicity, the passive absorption on the paracellular pathway is restricted to molecular sizes 100–300 Da (Chediack et al., 2003).

Oral delivery

The most convenient and preferred route of drug administration is the oral route. Additionally to the mentioned problem of proteins to enter the human or animal body across biological membranes, proteolytic degradation of proteins in the GI-tract limit the extent to which protein drugs become available in the bloodstream or at the target tissue after administration. Digestive enzymes secreted into the GI-tract (e.g. pepsin, trypsin, chymotrypsins, elastase and carboxypeptidase) cleave peptide bonds, which could result in inactive fragments of the protein drug. However, the proteolytic activity is highest in the stomach and duodenum, and is significantly reduced in the ileum and colon (Goldberg and Gomez-Orellana, 2003). Hence, digestion of protein drugs can be minimized with systems for colonic delivery, like enteric coatings or polymeric materials specifically degradable by the colonic microflora. Another possibility is the co-administration of enzyme inhibitors (Yamamoto et al., 1994).

Reasonable mucosal protein drug absorption appears to be inevitably linked to the utilization of absorption enhancers. Absorption enhancing substances can be categorized into five main groups: 1) chelators, 2) surfactants, 3) bile salts 4) fatty acids and 5) non-surfactants (Lee et al., 1991). The mechanism of action is mostly based on an affected integrity of the mucosal membrane, facilitating uptake by passive diffusion.

Among the pathways to passively cross the intestinal epithelium the paracellular is preferred over the transcellular route, due to the circumvention of proteolytic enzymes inside epithelial cells (Figure 2). Thus, an opening of the tight junctions between enterocytes is often targeted.

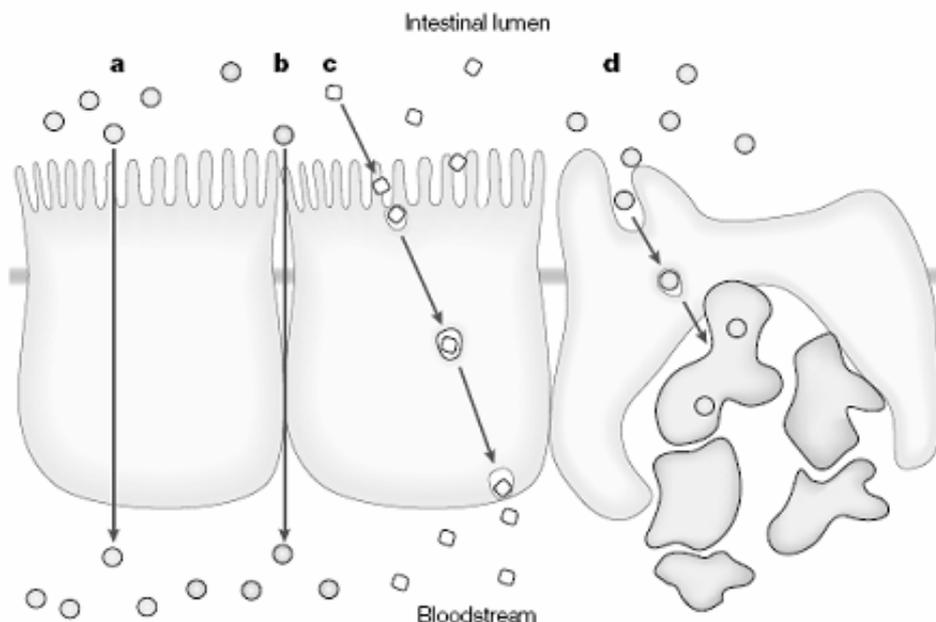


Fig. 2: Schematic depiction of the intestinal epithelium and the pathways available for drug absorption. a: transcellular pathway, b) paracellular pathway, c) transcytosis and receptor-mediated endocytosis and d) absorption into the lymphatic circulation via M-cells of Peyer's patches (Goldberg and Gomez-Orellana, 2003)

However, opening the tight junctions means to compromise the integrity of the epithelium and thus to increase the toxicity risks due to co-transported harmful compounds residing in the colon.

Certain carrier molecules were reported to enhance the permeability of large hydrophilic drugs without compromising the integrity of the epithelial barrier layer (Leone-Bay et al., 1995). Although reported oral bioavailabilities for some of the carrier-protein assemblies were less than 1% (Stoll et al., 2000), further clinical studies on three protein drugs (insulin and recombinant human growth hormone) delivered with one derived carrier molecule (Sodium N-(8-[2-hydroxybenzoyl]amino)caprylate, SNAC, Eligen, Emisphere Technology, Inc., USA) are currently undertaken. However, considering a low bioavailability, this approach will have to prove its suitability for oral protein delivery due to the high manufacturing costs of protein drugs.

Pinocytosis, a form of endocytosis, occurs in the M-cells of the Peyer's patches, which are lymphatic islands in the intestinal epithelium. Small particles (nanometer to a few

micrometers) could be systemically absorbed through transcytosis by both enterocytes and M-cells. However, poor particle absorption and thus, limited systemic bioavailability were observed. More success is expected for the local delivery of vaccine-loaded particles to the M-cells (Goldberg and Orellana, 2003).

Buccal / sublingual delivery

Although the buccal and sublingual routes face two major problems for the successful systemic delivery of protein drugs, a relatively thick multi-layered mucosae and a continuous production of saliva (Owens et al., 2003), a bioavailability of 7–8% (van de Weert et al., 2005) was reported for a liquid insulin aerosol formulation of mixed micelles sprayed into the mouth using a metered-dose valve system (Oral-lyn, Genex Biotechnology Corporation, Canada). This approach appears to become an alternative to the parenteral administration of insulin soon, due to the late phase III clinical trials conducted in the US and in Europe momentarily.

Intranasal delivery

The nasal route is probably the most popular alternative to the oral route, because self-administration is simple and convenient. The nasal mucosa provides a vascularised subepithelium and lymphatic system. Furthermore, the nasal cavity is an attractive administration site, since it provides a route to deliver drugs to the CNS under circumvention of the blood brain barrier (Illum, 2004). However, disadvantages for protein delivery include an efficient mucociliary clearance mechanism and an enzymatically active and fairly impermeable nasal epithelium. Local irritation and disruption or damage to the nasal mucosa and the ciliary function are other risks related to intranasal delivery. Clinical experiences for insulin showed that bioavailability rarely exceeds 20%. Hence, commercial development of the majority of nasal systems is challenging also due to concerns about their safety (Hinchcliff and Illum, 1999). Some preclinical and clinical data on an in situ gelling powder formulation (GelVac, Delsite Biotechnologies Inc, USA) showed promising results for vaccination via the intranasal route.

Rectal delivery

As with other mucosal routes (buccal, sublingual, nasal), rectally administered protein can enter the systemic circulation under circumvention of a fast hepatic clearance (first-pass

effect). However, the absorption from the rectum is poor. The bioavailabilities of insulin in humans, for example, were usually less than 10% (Owens et al., 2002).

Pulmonary delivery

The lungs have been extensively studied as a delivery route for proteins because of the large surface area and excellent vascularisation in the deep lung. However, following inhalation of a single dose of insulin, only 60% of the drug reaches the alveoli. The other 40% are deposited in the mouth or are lost in the upper airways. Depending on the potency of the drug the delivery of proteins to the lungs can require the application of large doses for systemic administration (>100 mg protein) (Owens et al., 2002). The total bioavailability of inhaled insulin in humans was in the range of 10–15% (Patton, 2000). However, marketed products of inhaled protein drugs for systemic as well as local treatments include insulin (Exubera, Pfizer Ltd., USA) for the treatment of diabetes and recombinant human DNase (Dornase alpha) for the treatment of cystic fibrosis (Pulmozyme, Hoffman-La Roche, Switzerland). The pulmonary route is thus the first available way to systemically deliver insulin non-invasively. However, annual costs per patient using inhaled insulin were estimated to be € 1600, without considering educational costs and pulmonary function tests (Bellary and Barnett, 2006). Comparable numbers for conventional subcutaneous insulin therapy were not published recently. Hence, inhaled insulin will have to prove its cost-effectiveness.

Transdermal

Another attractive site for drug delivery is the easily accessible skin. However, the transdermal pathway is naturally not suitable to deliver hydrophilic drugs systemically, due to the lipophilic character of the external 10-20 cell layers, the stratum corneum. And for reasons discussed above, the permeability of proteins through the skin is further hampered by the molecular dimensions of the macromolecules.

Several methods were employed to improve the systemic delivery of protein through the skin including iontophoresis (Langkjaer et al., 1998), low frequency ultrasound (Mitagotriet al., 1995) and phosphatidylcholine-based vesicles (Gupta et al., 2005). High costs for the necessary devices and handling issues related with the application of the first two methods favor the formulation approach. The vesicular carrier system was reported to enable transport of macromolecules up to 30,000 Daltons in size through tight junctions between skin cells.

The applicability of these vesicles for transdermal insulin delivery is currently evaluated (TPM-02, Phosphagenics Ltd., Australia).

Parenteral delivery

Although absorption-enhancing strategies developed during recent years improved the efficiency of protein transport across epithelial barriers of the various non-invasive administration routes, bioavailability remains fairly low to be of significant clinical and commercial value. It was expressed previously, that protein drug products with bioavailabilities of not more than 10–20% would have limited chance for success due to higher costs for drug manufacturing (Cleland et al., 2001).

Intravenous administration (IV) results in 100% bioavailability due to an absence of the absorption phase. Intravenously administered solutions or nano-suspensions / -emulsions are injected or infused in critical patient care settings due to dosing precision and the rapid and complete bioavailability. The intramuscular (IM) and subcutaneous (SC) routes are less hazardous than the IV route and facilitate administration of aqueous or non-aqueous suspensions, emulsions or gels for sustained drug delivery without the risk of capillary blockage. Comparable to non-invasive routes, an absorption step is necessary for the drug to reach the blood circulation and absorption rates and extents also depend on the physicochemical properties of the drugs (Sund and Schou, 1964). However, subcutaneously applied recombinant human growth hormone, for example, showed a bioavailability of 70-90% (package insert of Saizen, Serono, Inc., USA) and 61-86% bioavailability were reported for subcutaneously applied human insulin (Nosadini et al., 1988). A further improvement of these numbers can usually be expected for an intramuscular administration (Chan et al., 2003).

Due to the relatively high manufacturing costs for protein drugs, it is hard to compete with parenteral delivery of proteins to the human body with regard to cost-effectiveness. Advances in the development of parenteral depot systems for protein drugs were made during recent years to overcome issues with patient acceptance and compliance related to multiple injections in the treatment of some chronic diseases. Furthermore, sustained or controlled parenteral delivery offers opportunities like long-lasting constant drug plasma levels and localized delivery in the body (Cleland et al., 2001). Even pulsatile release patterns appear to be feasible.

1.1.2.2. Protein stability

Protein stability refers to the ability to maintain its molecular structure and biological activity under various deleterious conditions. Poor stability during expression, purification, formulation, packaging and storage is often the biggest hurdle for the safe and effective delivery of protein drugs, due to their delicate molecular structure.

1.1.2.2.1. Protein structure

A protein consists of one or more polymer chains of amino acids (polypeptides) assembled in a specific three-dimensional structure. Thereby, four hierarchical levels of organization are differentiated.

The linear amino acid sequence of a protein and the location of disulfide bonds are referred to as primary structure. Elements of the secondary structure describe the three-dimensional form of local segments. Most prevalent elements are α -helix, β -strand, -sheet and -turn all of which are stabilized by hydrogen bonds between atoms of the peptide backbone. Predominantly hydrophobic interactions but also other non-covalent bonds and covalent disulfide bonds stabilize folding of secondary structure elements into a three-dimensional arrangement or conformation of the whole protein molecule, which is called the tertiary structure. Functionality of a protein is based on its native three-dimensional structure, which is defined by its primary structure (Lodish et al., 2000). A quaternary structure is not a mandatory feature of proteins. It is formed through non-covalent association of a certain number of protein subunits.

Proteins or domains of a protein can be divided into fibrous and globular molecules or regions. Fibrous proteins often have a structure giving or stabilizing function and are rarely water-soluble (e.g keratins). Folded globular proteins comprise a globelike shape, where the side chains of hydrophobic amino acid residues are generally buried inside the protein molecules, thus contributing to protein stability through hydrophobic interactions. With the hydrophobic side chains of amino acids in the core and the hydrophilic residues at the surface, globular proteins are mostly water-soluble.

Hen egg white lysozyme, a hydrolytic enzyme, is one example of a globular protein. Variants of lysozyme are widespread and occur in humans and in various other vertebrates, invertebrates as well as in bacteria, phages and plants (Jollès and Jollès, 1984). They play a role in the unspecific prevention of bacterial infections due to their catalyzing effect in the hydrolysis of glycosidic bonds between the alternating amino sugars N-acetylglucosamine and N-acetylmuramic acid in the bacterial peptidoglycan. Gram-positive cells are quite

susceptible to this hydrolysis since their cell walls have a high proportion of peptidoglycan. Among the Gram-positive bacteria, *Micrococcus lysodeikticus* and *luteus* are very easily destroyed by lysozyme, which is the reason for their utilization in turbidimetric assays of the biological activity of the enzyme (Shugar, 1952). Gram-negative bacteria are less susceptible due to the presence of an outer membrane and a lower proportion of peptidoglycan. Lysozyme from the hen egg white is a basic (isoelectric point 11), single chain polypeptide composed of 129 amino acids (Figure 3 A). The tertiary structure is stabilized with four disulfide bonds (Figure 3 A and B).

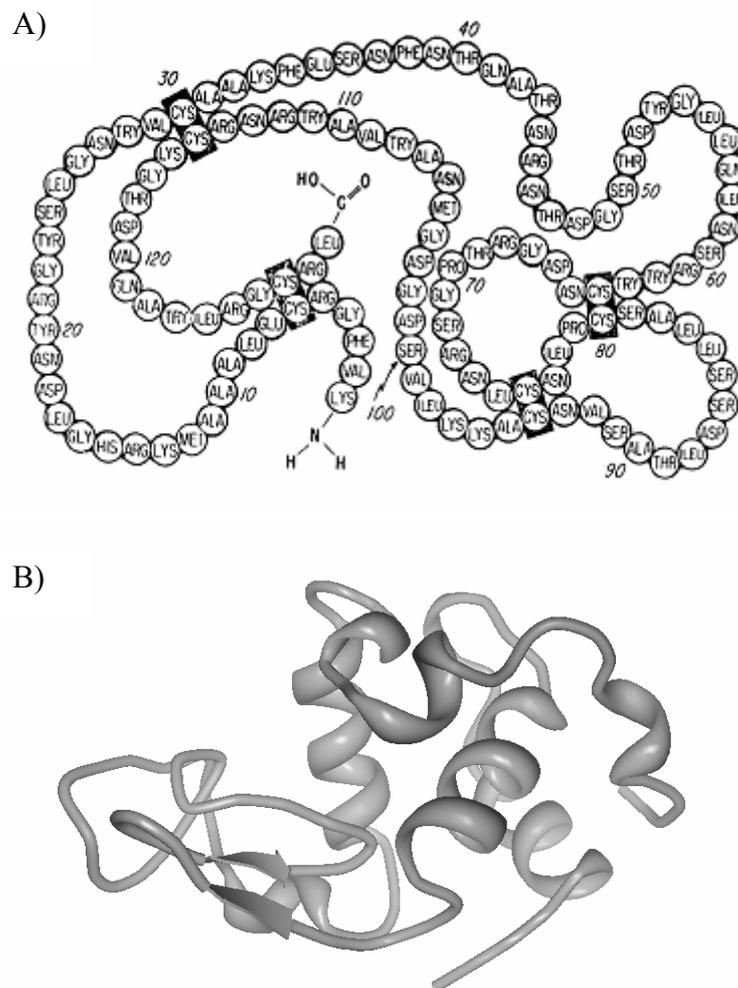


Fig. 3: A) Primary (Canfield and Liu, 1965) with stabilizing disulfide bonds and B) tertiary structure (ribbon diagram of 1GXV from RCSB Protein Data Bank) of hen egg white lysozyme

Lysozyme is used in the food industry to inhibit bacterial growth. One example is the control of *Clostridium tyrobutyricum* growth during the maturation of cheeses (Wasserfall and Teubner, 1979). In the pharmaceutical area hen egg white lysozyme is used in medicated toothpastes and, with a questionable efficacy, in tablets for the antibacterial peroral therapy of chronic sinusitis for example (Neuzym, Eisai Co., Ltd., Japan).

However, lysozyme was selected because of the broad existing knowledge with respect to structure, biological function and stability, which make it an attractive indicator for stresses occurring during manufacturing and characterization of protein containing parenteral pharmaceutical dosage forms (van de Weert, 2000a-c).

1.1.2.2. Reversible inactivation

Reversible inactivation of a protein can mean a loss of biological activity due to the formation of associates or due to alterations of the native conformation. An alteration of the three-dimensional structure with resulting loss of biological activity is called denaturation.

Denaturation

The conformational stability of a protein is determined by the extent to which the combination of numerous cooperative interactions exceeds the folding entropy of a protein. A typical net difference in the Gibbs free energy of folding is about 45 kJ per mole only (Jaenicke, 1999), reflecting the fragility of the three-dimensional structure. Folding of denatured or unfolding (U) of native proteins (N) is usually reversible and proceeds via more or less stable, partially denatured intermediate (I) conformations along folding (unfolding) pathways. These can be visualized using three-dimensional energy plots, so called folding funnels (Dinner et al., 2000), or can be expressed in a simplistic thermodynamic equation (Equation 1).



Denaturing conditions include temperatures above (Salvettiet et al., 2002) but also below physiological conditions (Privalov, 1990), which is due to lowered hydrophobic interactions at low temperature. The result is a parabolic shape of the free energy difference between denatured and native conformations of proteins versus temperature. Proteins denaturation also occurs due to extensive dehydration (Prestrelski et al., 1993a and Griebenow and Klibanov, 1995), for example during lyophilization, which is explained with the loss of the stabilizing

hydration shell (Nagendra et al., 1998). The conformation and activity can be also compromised by shear forces, for example during pumping, mixing, filtration or grinding (Wolf et al., 2003). The sensitivity of proteins to elevated temperatures and high shear forces often impede sterilization of such products with heat or filtration.

Many globular proteins are susceptible to the exposure to interfaces or surfaces. The amphiphilic molecules attach to interfaces / surfaces (e.g., air / water or immiscible solvent / water, vials, stoppers, tubes or excipients), through a directed exposure of hydrophobic residues to hydrophobic surfaces or air and hydrophilic residues to the aqueous phase (Lu and Thomas, 1998). The susceptibility to unfold at a surface (interface) depends on the protein (Lad et al., 2006). The absorption behavior depends on the protein, the surface / interface and the experimental conditions (Wang et al., 1999). Lysozyme was reported to preferentially absorb to hydrophilic surfaces (Lu and Thomas, 1998 and Voeroes, 2004).

Electrostatic interactions are contributing to the stability of the native conformation and are reflected by pKa values of dissociable residues. Unsuitable pH conditions can lead to distortions of the native conformation through protonation or deprotonation of functional groups, introducing additional or weakening attractive or repulsive forces. As a result, an optimal pH range for stability is usually obtained, which is around pH 4.5 for lysozyme (Claudy et al., 1992).

The type and the concentration of buffering agents, salts or other co-solutes can also affect the tertiary structure of proteins. The Hofmeister series (Hofmeister, 1888) divides ions into two groups of depending on their effects on proteins in solution. Proteins are salted out, i.e., stabilized, by kosmotropic ions and salted in, i.e., destabilized, by chaotropic ions (Herberhold et al., 2004). The mechanisms involved in destabilization of proteins by salts are complex and still not well understood. Direct effects (binding to, interacting with or screening of protein residues) can be differentiated from indirect effects (altering the solvent environment). Direct effects include alteration of protein net charge or weakening intramolecular interactions by screening or perturbing stabilizing non-covalent forces (Bostroem et al., 2003). Electrostatic screening of surface charges can also lead to aggregation. Indirect effects of co-solutes on protein conformation are related to the water structure. Chaotropic agents disrupt water structure and diminish hydrophobic stabilization of proteins (Bennion and Daggett, 2003).

Anionic and cationic surfactants are also able to denature proteins. Sodium dodecyl sulfate (SDS) addition to lysozyme solutions ($\text{pH} < \text{pI}$) led to protein precipitation. Quantitative precipitation was obtained at a molar ratio, where charge neutralization was reached. SDS

binding beyond neutralization led to redissolution and to loss of lysozyme activity (Glassman and Molnar, 1951). Investigations of the bovine serum albumin-SDS system revealed a necklace and bead type structure of denatured complexes in which unfolded protein wrapped around surfactant micelles (Narayanan and Deotare, 1999). Restoration of the biological activity of lysozyme from protein-surfactant associates requires complete removal of the surfactant anion (Dong et al., 1997). This was only achievable with complex protocols, which resulted in remarkable losses of lysozyme (Yoo et al., 2001).

Organic solvents were used as denaturants in numerous folding studies of proteins in aqueous solutions. The conformation of dissolved protein is compromised by organic solvent addition above a critical point but proteins suspended in organic solvents alone are kinetically trapped (Griebenow and Klibanov, 1996). A very few organic solvents are capable of dissolving reasonable amounts of proteins. One of these solvents is dimethyl sulfoxide (DMSO) (Chang et al., 1991, Chin et al., 1994 and Houen, 1996), which is suitable for parenteral administration. Lysozyme and other proteins lose their three-dimensional structure in DMSO (Jackson and Mantsch, 1991), which could be attributed to the loss of the essential water layer around a protein, but the conformation and biological activity could be restored upon dilution with aqueous buffer (Chang et al., 1991).

Aggregation

Association of proteins molecules is another reason for reversible inactivation. Associates of native proteins include oligomers, macromolecular complexes and protein fibers, as well as artificially produced protein crystals used for X-ray diffraction studies of their structures. Association of native protein and aggregation of unfolded protein are not anymore strictly differentiated, since protein-protein interactions and folding-unfolding-refolding are in characteristic balances with each other (Figure 4), which depend on the protein itself and the environmental conditions as described for the denaturation. These are competitive processes in the energy landscape of a polypeptide chain and were therefore co-incorporated into folding funnel diagrams, recently (Figure 5).

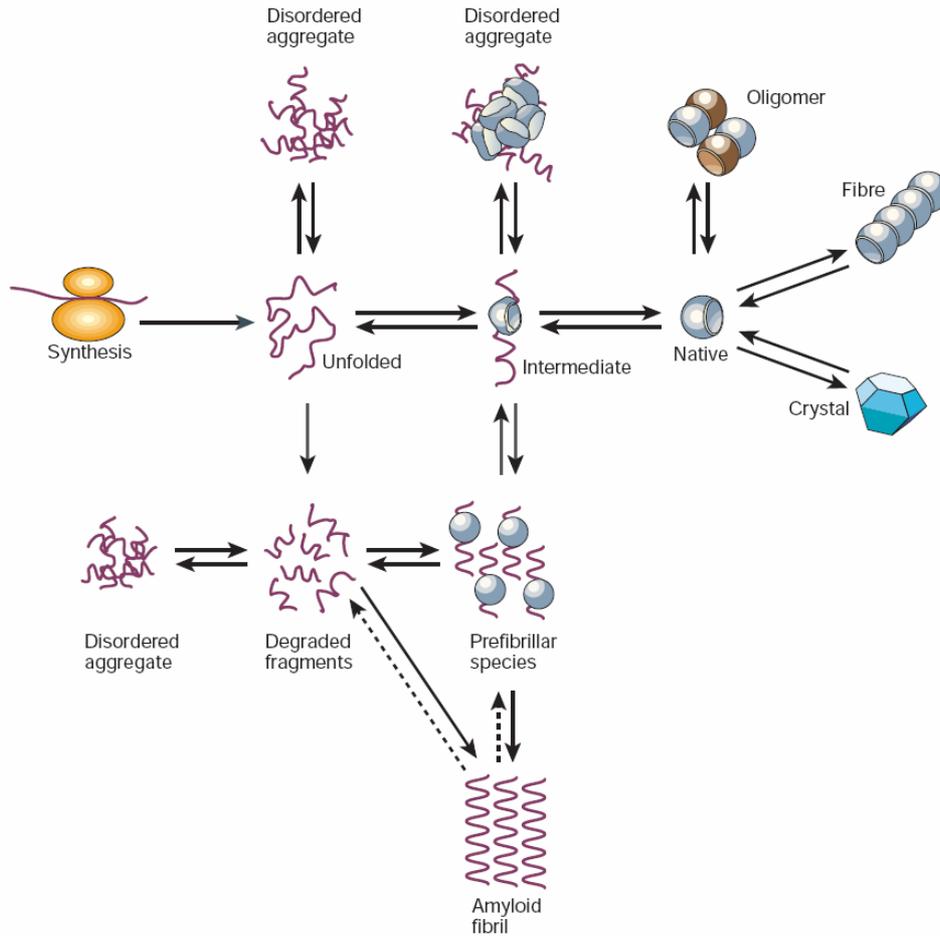


Fig. 4: Schematic of possible states of a polypeptide chain, including unfolded, intermediate and native monomers and various forms of aggregated species (Dobson, 2003)

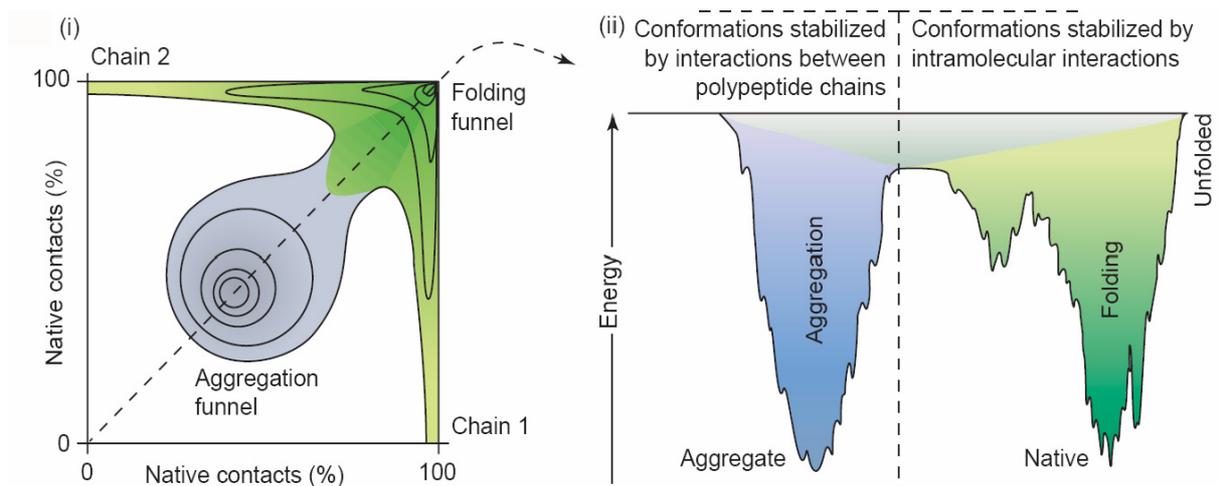


Fig. 5: Hypothetical pathway (broken line) of a protein through the energy landscape of aggregation and folding visualized by (i) a contour plot and (ii) the corresponding double funnel (Clark, 2004).

1.1.2.2.3. Irreversible inactivation

Sources for irreversible inactivation are chemical reactions and aggregation / precipitation. Chemical reactions cause irreversible inactivation, since alterations in the primary structure of proteins cause most likely also changes in higher order structures. Although aggregation alone can lead to irrecoverable protein precipitates, both mechanisms can also coincide.

Aggregation

The formation of insoluble protein aggregates is involved in several human diseases, such as Alzheimer or Creutzfeldt-Jakob disease but aggregation also results in decreased drug potencies of protein pharmaceuticals and can cause immunogenicity (Rosenberg, 2006) or clogging of implantable delivery devices (Lougheed et al., 1980).

Irreversible binding of monomeric protein molecules often requires conformational transitions leading to the formation of intermolecular bonding, which means that conditions facilitating denaturation can also promote aggregation processes. However, since proteins can also be considered as colloidal particles, solution conditions favoring coagulation, such as protein concentration (Zettlmeissl et al., 1979) or osmolarity can lead to loose protein associates (Chi et al., 2003), which is in agreement with the existence of associates of native protein molecules (Figure 4).

Types of irreversible protein aggregates, containing unfolded or partially unfolded protein monomers, include disordered structures and amyloid fibrils (Figure 4). The latter are characterized by a highly organized and often very stable hydrogen-bonded structure capable of seeding subsequent conversions of additional quantities of protein into amyloid fibrils (Dobson, 2003). Fibril formation via hydrogen bond formation appears to be a common feature of polypeptides, which depends very much on the solution conditions (Cao et al., 2004) Lysozyme fibrils, for example, occurred in ethanol / water mixtures (Yonezawa et al., 2002) but also upon increasing the temperature of acidic solutions (pH 2) to about 50°C (Arnaudov and de Vries, 2005).

Recently, an interesting study revealed the occurrence of fibrils of the polypeptide bovine calcitonin upon exposure to phospholipid membranes (Wang et al., 2005). It was concluded, that the formation of insoluble aggregates can be initiated by conformational changes of polypeptides at surfaces and interfaces, which facilitate intermolecular interactions. The deleterious interactions can have a hydrophobic, like in the case of the phospholipid-bovine calcitonin system, or a hydrophilic nature. It was reported that electrostatic interactions, for

example, can significantly contribute to protein binding processes (Xu et al., 1997). However, adsorption induced aggregation does not strictly lead to an irreversible protein loss. Only adsorbed lysozyme in close contact with the hydrophilic surface of contact lenses was denatured and irreversibly lost (Castillo et al., 1985). Adjacently attached layers of protein molecules appeared to be less distorted and only reversibly adsorbed. Interestingly, the time-dependent adsorption process was characterized by a linear increase of adsorbed protein after a lag time when plotted against the logarithm of time.

In this respect it was further reported, the lysozyme adsorption layer formed on a hydrophilic surface was thick but loose, whereas a thinner but denser layer was found on a hydrophobic surface (Voeroes, 2004). The density of the adsorbed protein layer appears to correlate with the reversibility of adsorption (Wang, 1999). Accordingly, adsorption of dissolved lysozyme to hydrophobic surfaces led to irreversible inactivation (Colombiè et al., 2001 and Lad et al., 2006).

Irreversible losses of protein or decreases of biological activity are no unique features of aqueous but are also experienced with organic protein solutions (Moshashaèe et al., 2000).

Chemical inactivation

Chemical reactions affect the primary structure of a protein and are therefore likely affecting higher order structures and thus the biological activity of protein molecules. The susceptibility of amino acid residues can be increased for sites, which are exposed to the surface of the globular protein (Apenten, 1998). Most common degradation reactions of proteins are deamidation, racemization, isomerization, β -elimination, exchange of disulfide bonds and oxidation (Kameoka et al., 2003).

Irreversible degradation of lysozyme is mainly caused by alterations at asparagine (Asn) and aspartic acid residues (Tomizawa et al., 1994a and b). Deamidation of asparagine (Asn) and glutamine residues involves the formation of a cyclic imide at mildly acidic to basic conditions (Wang, 1999). A succinimide can be also formed by aspartic acid residues at low pH. The cyclic imides can show racemization and / or hydrolyze to protein isomers (Figure 6).

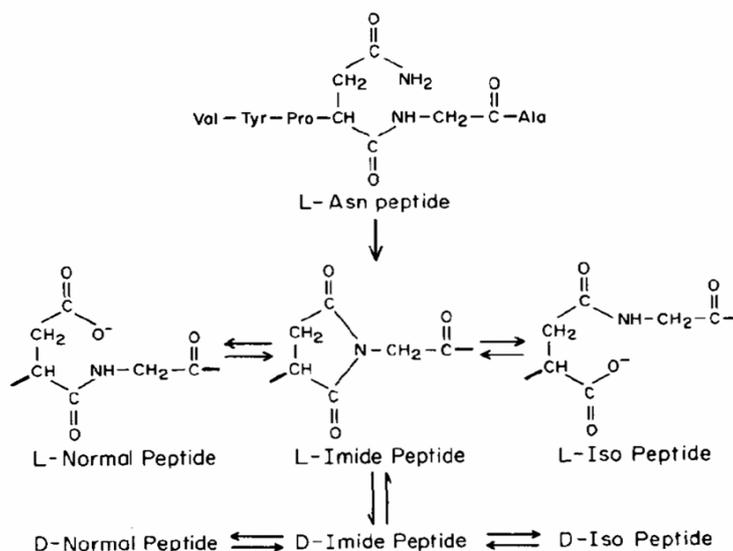


Fig. 6: Deamidation, isomerization and racemization of Asp and Asn residues in a model peptide (Geiger and Clarke, 1987).

Deamidation is promoted when the susceptible residue is followed by glycine (low steric hindrance), in presence of phosphate ions (Imoto et al., 1997), at high ionic strength and at basic pH (Kameoka et al., 2003).

In hen egg white lysozyme, Asn at position 103, which is surrounded by two glycines, is the most susceptible residue for deamidation (Robinson et al., 2001). However, mono-deamidated lysozyme derived from incubation at alkaline pH was still about 80% active. Aspartic acid (Asp) at position 101 can also undergo isomerization and racemization via the succinimide intermediate under mildly acidic conditions. The formation of cyclic imide increased with increasing acetate buffer concentration. Interestingly, the biological activities of the succinimide and the isomer were higher than for native lysozyme. Irreversible thermo-inactivation of lysozyme was a result of destabilization by chemical reactions, which caused permanent denaturation. The destabilizing reactions (isomerization, deamidation, racemization and peptide bond cleavage) mainly occurred at aspartic acid residues (Tomizawa et al., 1994b).

Peptide bond hydrolysis is another mechanism involved in irreversible protein inactivation. Bond cleavage also occurs at Asn, for example, if the β -amide nitrogen attacks the peptide bond carbonyl, which was described for Asn 103 of lysozyme (Geiger and Clarke, 1987). A C-terminal succinimide is formed, which can further hydrolyze to Asn or iso-Asn (Figure 7). The main chain of cleaved lysozyme was still 90 % active (Tomizawa et al., 1994b). At

elevated temperatures, peptide bond cleavage could also happen at Asp-X bonds of lysozyme (Ahern and Klibanov, 1985).

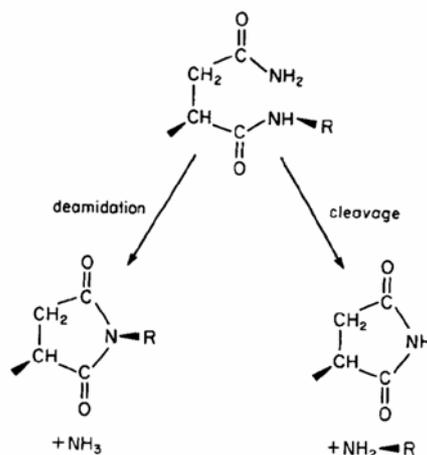


Fig. 7: Comparison of succinimide formation in deamidation and peptide bond cleavage reactions of asparaginyl residues (Geiger and Clarke, 1987).

The destruction of stabilizing disulfide bonds was reported to play also a significant role in the irreversible thermo-inactivation of lysozyme (Ahern and Klibanov, 1985). However, this could not be confirmed in another study (Tomizawa et al., 1994b).

Covalent cross-linkings are other chemical modifications potentially contributing to irreversible protein degradation by stabilization of physical aggregates (Gao and Mehta, 2001). Cross-linkings can consist of reducible and non-reducible bonds. The former typically involves cysteine residues and can result from thiol-disulfide exchange. A non-reducible bond could be formed after a β -elimination at a cysteine residue, which results in a thiocysteine and a dehydroalanine. The dehydroalanine can then react with the amino-group lysine to form lysinoalanine (Putney and Burke, 1998). A non-reducible dimer of lysozyme was previously detected in commercial samples. It showed a biological activity of only 34% (Snell et al., 2000).

Cysteine and methionine are most sensitive for oxidations among the susceptible amino acid residues (histidine, methionine, cysteine, tryptophane, and tyrosine). Oxidations preferentially occur at neutral to basic pH (Shacter, 2000). Traces of residual metal ions can catalyze oxidations at specific sites (Wang, 1999). Atmospheric oxygen, UV-light or oxidizing inactive formulation ingredients (e.g. peroxides formed during storage in Polysorbate 80, Ha et al., 2002) can cause oxidation of proteins.

1.1.2.2.4. Protein stabilization

A protein drug candidate could be covalently modified in order to increase the stability of the native / decrease stability of the denatured conformation. Possible ways include the exchange of susceptible residues or sequences, introduction of additional cross-links, glycosylation (Imoto et al., 1997) or pegylation (Diwan and Park, 2001 or Baker et al., 2006). In the pharmaceutical field, the latter two methods receive more and more attention and a number of approved drug products are already on the market (Davis and Robinson, 2002 and Greenwald et al., 2003).

Prior to formulating a pharmaceutical dosage form of a given protein drug, possible mechanisms of inactivation have to be identified to prevent or minimize irreversible losses. Thus, deleterious formulation and process variables can be avoided or favorable, stabilizing conditions created.

Removal of water and maintenance of low temperatures have been basic strategies for minimizing rates and extents of reactions but usually not the degradation pathways (Niu and Chiu, 1998). One interesting strategy to obtain storage stable, desolvated protein could be crystallization (Shenoy et al., 2001) but freeze drying is applied most often.

Freeze drying allows forming a protein cake with a high surface area, which can be quickly re-dissolved upon contact with water. However, protein dehydration, low temperature denaturation and absorption to glass walls during the process are frequently occurring problems and they need to be prevented before enhanced storage stability can be obtained. Thus, co-incorporation of stabilizing excipients, such as buffering and bulking agents, surface active substances, polymers or amino acids, is required to achieve a stable anhydrous protein formulation (Costantino et al., 1995 and Randolph, 1997).

The solution pH is important for conformational stability (Branchu et al., 1999) and chemical stability of a protein. A pH away from the isoelectric point (pI) can also stabilize a protein against aggregation due to increased electrostatic repulsion. Phosphate containing solutions of a physiological pH are often used as buffers (Pèrez et al., 2003) but in some instances, including lysozyme, a decrease of protein stability (Imoto et al., 1997) and a promotion of surface adsorption suggest the use of alternative buffers (Jiang et al., 2002).

Low molecular weight polyhydric alcohols (polyols), such as trehalose, sucrose, mannitol or maltose, are not only applied as bulking agents in freeze drying, they also act as stabilizers of the native protein state in solution. They are also called cryo- or lyoprotectants, due to their capability to prevent protein inactivation upon dehydration and rehydration during lyophilization (Allison et al., 1996). Polyols are preferentially excluded from the protein

surface, which stabilizes the folded state relative to the unfolded state because it exposes less surface area from which the co-solute must be expelled (Arakawa and Timasheff, 1982). Furthermore, cryoprotectants stabilize dehydrated proteins due to substitution of essential water molecules by formation of hydrogen bonds (Prestrelski et al., 1993a and b).

Amino acid addition can result in stabilization due to chelation of metal ions, antioxidant activity, aggregation inhibition or preferential exclusion (Shiraki et al., 2004 and Valente et al., 2005).

Divalent metal ions, such as calcium, were reported to stabilize certain enzymes. However, calcium and magnesium ions were reported to destabilize lysozyme (Wang, 1999). Some proteins can be stabilized by the addition of zinc ions, which was attributed to a decreased susceptibility to degradation due to a lowered solubility of so formed physically crosslinked protein oligo- or polymers (e.g., insulin, recombinant human growth hormone, recombinant human nerve growth factor and bovine serum albumin) (Johnson et al., 1997b, Costantino et al., 2000 and Fu et al., 2000a). The dynamics of the zinc-complexation are determined by the affinity of the protein for the metal, availability of metal coordination sites, protein and metal concentrations, and solution pH (Yang et al., 2000a). Consequently, the re-dissolution of the complexes can be sensitive to variations in the ionic environment (Prabhu et al., 2001).

Although lysozyme has the ability to bind 2 mol of zinc- or copper(II)-ions (Moreau 1995), no physical stabilization by such a electrostatic crosslinking was reported for both ions. Copper-ions contributed to an improved thermal stability of dissolved lysozyme due to a suppression of disulfide interchange (Tomizawa et al., 1995, Putney and Burke, 1998). However, metal ion binding could also affect the structural integrity negatively. Copper-binding, for example, was discussed to cause the irreversible denaturation of the native cellular prion protein into the insoluble and protease-resistant pathologic form in Creutzfeldt-Jakob disease (Quaglio et al., 2001).

Binding of substrates, inhibitors and cofactors could lead to a stabilization of proteins. Tri-N-acetylglucosamine, for example, increased the molecular rigidity of the protein conformation by binding at the active site of lysozyme (Pace and McGrath, 1980).

Surfactants are added to protein solutions in a concentration close to the critical micelle concentration to decrease the surface tension and compete with the protein for adsorption at the surface or interface (Wang, 1999). Non-ionic polysorbate and poloxamer 188 are often used but also anionic SDS can be found in a lyophilized drug product (Proleukin, Chiron, US). Surfactants also bind to proteins, which could lead to solubilization or destabilize the native state of the protein or to inhibition (paragraph 1.1.2.2.2.). Furthermore, it was reported

that a polysorbate, containing polyethylene glycol (PEG) chains, can form peroxides upon storage in the presence of oxygen (Ha et al., 2002), which could lead to oxidative degradation of proteins.

Various oligo- and polymers, such as cyclodextrins (Kang et al., 2002), albumin (Chen et al., 1997), polyvinyl alcohol (van de Weert et al., 2000c), polyethylene glycol (Cleland et al., 1992), gelatin (Lu et al., 1995), dextrans, polyvinyl pyrrolidone (Wang, 1999) and polyelectrolytes like polyamines (Kudou et al., 2003 and Powroznik et al., 2004) were applied to increase protein stability in solution or during drying. The suggested mechanisms behind the stabilization of proteins in aqueous solution are interface or surface competition, preferential exclusion, binding with steric hindrance of protein-protein interactions and / or increased viscosity.

Water does not need to be removed completely to slow down protein degradation. Substitution of aqueous by organic solvent fractions could decrease hydrolysis as well. Small amounts of water-miscible organic solvent could stabilize proteins (Tomizawa et al., 1995 and Wang, 1999). However, higher concentrations destabilize the proteins, by decreasing the conformational stability gained from the burial of hydrophobic residues in the core (Griebenow and Klibanov, 1996). However, the conformational stability of proteins in solvents can be as high as in the solid state when the protein is dispersed in a non-solvent under retention of its essential hydration shell (Laane et al., 1987) or through substitution of these water molecules by suitable additives (Dabulis and Klibanov, 1992). Protein losses at water / organic or organic / organic interfaces and other surfaces can be minimized or prevented with the drug being in the dispersed state during encapsulation procedures (Pérez et al., 2002b).

Water-miscible organic solvents such as DMSO, which were reported to reversibly denature proteins (Jackson and Mantsch, 1991, Chang et al., 1991), have the potential to be used as inert medium to store proteins over extended periods of time, since most serious degradation reactions are hydrolytic reactions (Stevenson, 2000).

1.2. Sustained and controlled protein delivery

Although significant opportunities exist for technology solutions that are noninvasive alternatives to injection, their capabilities to continuously deliver protein drugs over prolonged periods of time are limited. However, controlled protein delivery offers many advantages with regard to safety and efficacy of a drug therapy. A stable blood or tissue concentration of an active ingredient in the therapeutically effective range can be obtained by delivering a drug continuously to the body (systemic) or a target tissue (local), leading to an improved therapeutic effectiveness. The frequent administration of immediate release doses is usually not capable to achieve this. The safety of a drug therapy can be enhanced by preventing a patient from high drug plasma levels, what leads to a decreased risk for adverse effects. This is of special importance for highly potent therapeutic agents, such as protein drugs (Younes et al., 2002). Additionally, the usually short in vivo half lives of protein drugs make them an attractive target for the development of sustained or controlled release formulations.

Sustained and controlled release formulations can improve the patient acceptance for parenteral administrations due to the reduction in the frequency of dosing. Another benefit of a sustained or controlled drug delivery can be that once administered, the compliance to the drug therapy is guaranteed for the entire delivery period, which could enable the control over a treatment for some patients.

Approaches to achieve sustained protein delivery include means to alter the physicochemical properties of the drug. The decreased solubility of an insulin derivative for example (Lantus, Sanofi-Aventis, France) allows patients a once daily treatment with basal insulin (Gerich, 2004.). Another example is the pegylation of proteins, which can lead to a dosing frequency of once a week (PegIntron, Essex Pharma, Germany; Pegasys, Hoffman-La Roche, Switzerland; Adagen, Enzon Pharmaceuticals, Inc., US) or even once every two weeks (Oncaspar, Enzon Pharmaceuticals, Inc., US). However, decreased absorption and degradation rates obtained through pegylation do not prevent the patients from drug plasma level peaks and thus from severe side effects.

A sustained action of protein could also be achieved by non-covalent modification of the protein solubility, for example through complexation and crystallization with zinc or protamine as done for insulin. However, the drug absorption can be affected by the environmental conditions at the injection site. Physical exercises, for example, can increase insulin absorption from the site of injection and lead to hypoglycemic events.

A simple oily protein suspension allows controlling the drug release by several formulation variables and can decrease the sensitivity of the release to environmental conditions. In vivo delivery durations of one to two weeks were reported for suspensions of proteins in viscous oils (Yu et al., 1996). Heat stressed suspensions of relaxin in sesame oil containing aluminum stearate even showed elevated plasma levels for a 3 week period, which was surprising and could not be explained (Anschel, 1960.). At least one such product (Posilac, Monsanto, US) for dairy cows is approved by the FDA to increase milk production. Despite an increased stability of the protein within the oil (Yu et al., 1996), irreversible protein inactivation at the oil / water interface due to interactions with the gelling agent might occur and could lead to denaturation and unexpected immunological side effects due to the exposure of new epitopes (Cleland et al., 1993).

Water-in-oil emulsions could also provide controlled release of a drug, e.g. following intramuscular administration. However, stability issues are much more of a concern here due to the presence of water, surfactants and a high interfacial area, which increase the probability for chemical and physical degradation (Jorgensen et al., 2004) of proteins.

Proteins can be covalently or non-covalently attached to micelles to prolong the circulation time in the blood. But beside the potential of surfactants to denature proteins, the retardation potential of micelles is limited (Weissig et al., 1998). Liposomes as carriers for proteins can increase the residence time of protein in the compartment upon intravenous or subcutaneous administration. However, their capability to sustain the drug release is mostly limited to release durations of less than a week (Storm et al., 1995 and van Slooten et al., 2001). Furthermore, recent studies revealed a high risk for conformational changes of proteins upon attachment to phospholipid membranes (Wang et al., 2005 and Halter et al., 2005), which also challenges the suitability of related release technologies like Cagicles (Novosom, Germany) or DepoFoam (SkyePharma, UK) for protein delivery.

A large flexibility in the achievable drug release profiles and long delivery durations can be obtained with implantable systems. Probably the first successful attempt to deliver a protein in a controlled manner was reported by Davis in 1972. Subcutaneous implants based on cross-linked polyacrylamide were able to release insulin in vivo for about 3 weeks. Alternative polymers were also investigated as implantable matrices for proteins (Davis, 1974) and release periods of up to 100 days could be achieved (Langer and Folkman, 1976). Unfortunately, some of these materials showed insufficient biocompatibility.

In the year 2000, the FDA approved an implantable, non-degradable osmotic pump (DUROS, Alza, US), which is capable to systemically or locally deliver low molecular weight drugs for

up to one year. Momentarily, this technology is under evaluation for the delivery of proteins, such as interferon (Omega DUROS, Intarcia Therapeutics, Inc., US). Although non-degradable systems have the advantage to facilitate a premature termination of a long term-treatment by removal of the device, the inconvenient surgical removal of the implants after a normal regimen lead to research efforts focusing on materials, which degrade or slowly dissolve during or after the drug is released.

1.2.1. Biodegradable materials

Polymer degradation is a process, where chemical reactions result in a cleavage of main-chain bonds producing shorter oligomers, monomers, and / or other low molecular weight degradation products (Edlund and Albertsson, 2002). Biodegradable materials are characterized by a backbone that can be hydrolyzed under physiological conditions or under contribution of enzymes. Examples of functional groups with this characteristic are esters, anhydrides, amides, peptides and glycosides. The corresponding materials can be divided into natural compounds, such as proteins (e.g., albumin, collagen, fibrin, gelatin), polysaccharides (e.g., alginates, cellulose, chitosan, dextran, hyaluronic acid, and starch) or lipids and synthetic substances, such as poly(amides), poly(amino acids), poly(anhydrides), poly(cyanoacrylates), poly(dioxanone), polyester (e.g., poly(caprolactones), poly(carbonates), poly(glycolides) (PGA), poly(hydroxybutyrates), poly(lactides) (PLA), poly(tartrates)), poly(ortho ester), poly(phosphazenes) and various copolymers (Gombotz and Pettit, 1995, Winzenburg et al., 2004 and Park et al., 2005). Furthermore, a biodegradable polymer has to be biocompatible and thus would not show any sign of inflammation or other toxic responses after placing it into the body. Ideally, it would be fully metabolized upon in-vivo degradation (Edlund Albertsson, 2002).

Although some of the biodegradable materials have been approved for the use in medical devices (Middleton and Tipton, 1998), biodegradable polymers, used as drug release controlling matrices in approved parenteral products, only include poly(lactide) (PLA), poly(lactide-co-glycolide) (PLGA) and the polyanhydride polifeprosan 20 (Table 1 and Whittlesey and Shea, 2004).

Tab. 1: Pharmaceutical products containing biodegradable polymers for controlled drug release upon parenteral administration on the German market (Rote Liste, Jan. 2007; * not included in Rote Liste)

Product	Manufacturer	Dosage form	Polymer	Drug	Duration, month
Profact Depot	Aventis Pharma	Implant	PLGA	Buserelin acetate	2 and 3
Zoladex	AstraZeneca / Promed / pharma-stern	Implant	PLGA	Goserelin acetate	1 and 3
Gliadel*	MGI PHARMA	Implant	Polifeprosan 20	Carmustin	~ 1
Decapeptyl N and Gyn	Ferring Arzneimittel	Microparticles	PLGA	Triptorelin acetate	1
Enantone	Takeda	Microparticles	PLGA	Leuprorelin acetate	1
Trenantone	Takeda	Microparticles	PLA	Leuprorelin acetate	3
Pamorelin LA	IPSEN PHARMA	Microparticles	PLGA	Triptorelin acetate	1 and 3
Sandostatin LAR	Novartis Pharma	Microparticles	PLGA	Ocreotid acetate	1
Risperdal Consta	Janssen-Cilag	Microparticles	PLGA	Risperidon	0.5
Atridox	Atrix / curasan	In situ implant	PLA (NMP)	Doxycyclin	4
Eligard	Astellas Pharma	In situ implant	PLGA (NMP)	Leuprolide acetate	1 and 3

The history of the polyesters as biodegradable drug delivery matrices started in the early 1970's (Heller and Ng, 1999). But at that time, PLA, PLG and copolymers thereof have already been used as absorbable suture materials in the medical field for about ten years (Tice

2004). Thus, the polyesters provide a safety record of 40 years, which explains the presence of corresponding drug delivery products on the worldwide markets.

1.2.1.1. Biodegradable polyesters based on lactic and glycolic acid

1.2.1.1.1. Synthesis of PLGA

Polymers of lactic and glycolic acid may be prepared by direct polycondensation (Figure 8) or via ring-opening polymerization of cyclic diesters (Figure 9). Higher molecular weights (> 10,000 g/mol) can be achieved with the ring-opening polymerization of glycolide and / or lactide. Furthermore, this method allows a better control of the molecular weight distribution (polydispersity) and the endgroup functionality of the (co-)polymer (Jain, 2000a).

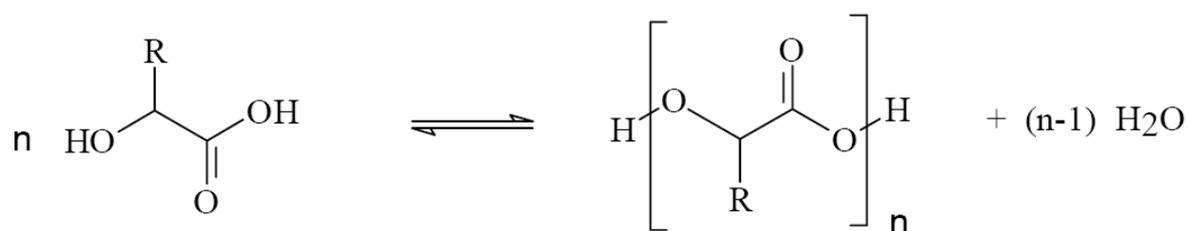


Fig. 8: Synthesis of poly(lactide) by direct polycondensation

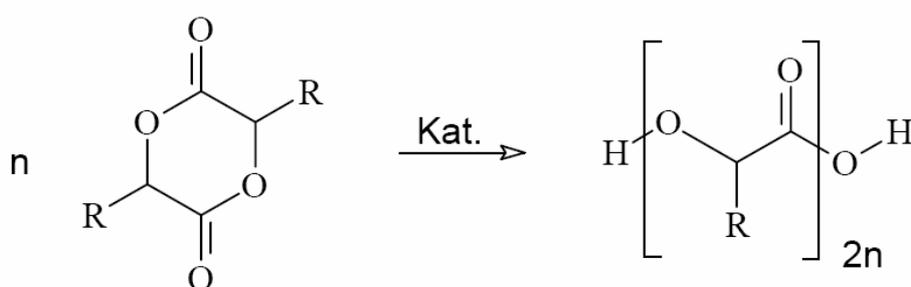


Fig. 9: Synthesis of poly(lactide) by ring-opening polymerization

The selection of the reactants and the synthesis conditions will determine the physicochemical properties of the resulting polyesters, such as hydrophilicity, mechanical strength, glass transition and crystallinity (Gilding and Reed, 1979 Omelcuk and McGinity, 1992 and Miller et al., 1977). The characteristics, which can be used to describe the final polymers include the weight or number averaged molecular weight, the polydispersity, the ratio of lactic and glycolic acid monomers, the ratio of D- and L-lactic acid monomers and the endgroup functionality. Although rarely specified, in random copolymers the segment length of

monomeric repeat units of a product is important since short block lengths avoid the formation of crystalline domains in the polymer, which provides homogeneous controlled release matrices (van Vaeck et al., 1999 and Shard et al., 2002).

1.2.1.1.2. Degradation of PLGA

Polyesters like PLA and PLGA degrade by hydrolysis of the functional groups in an aqueous environment, without significant contribution of enzymes (Bucholz, 1992), which necessitates the presence of water. Degradation of the polyesters leads to polymers of shorter chain length and below a critical molecular weight of about 1050-1150 Da (Park et al., 1994), the oligomers can dissolve in the aqueous surrounding and diffuse from the matrix.

If water diffusion through the polymer matrix is slow compared to the degradation process as it is with polyanhydrides and poly(ortho esters), surface erosion of the device would occur (Figure 10). In case of PLA and PLGA hydrolysis is slow compared to water diffusion and thus the entire polymer matrix is affected by degradation, which is called bulk erosion (Goepferich, 1997).

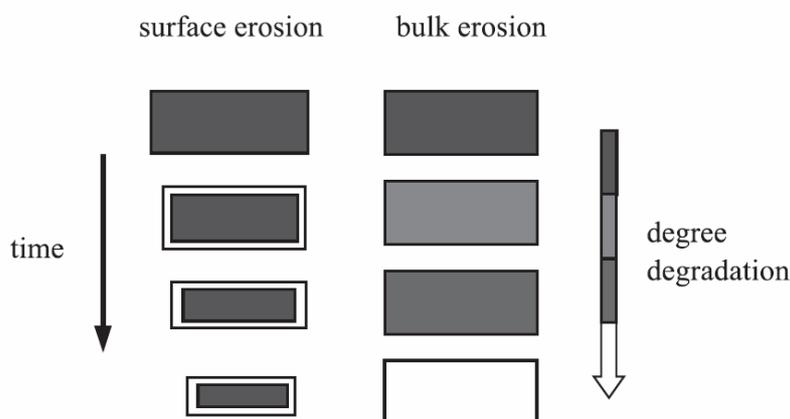


Fig. 10: Schematic illustration of polymer degradation mechanisms: surface vs. bulk erosion

The degradation behavior of the polyesters PLA, PLG and PLGA has been studied extensively and several factors material but also formulation properties can affect the degradation kinetics (Brannon-Peppas, 1997). The degradation rates of the polyester are influenced by parameters such as the initial weight average molecular weight, the hydrophobicity (lactic acid > glycolic acid monomers), the degree of crystallinity (e.g., increased in PGA and L-PLA) and the glass transition temperature of the amorphous phase (Figure 11).

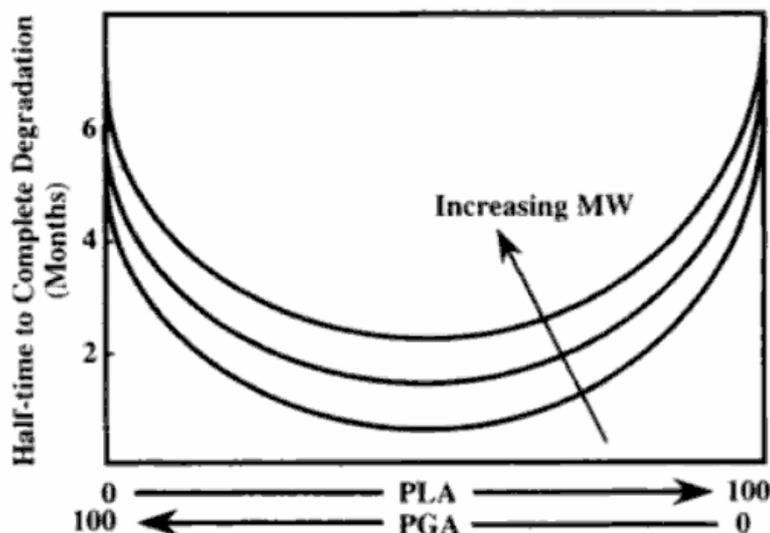


Fig. 11: Half-life of poly(lactide-co-glycolide) in rat tissue (Cleland, 1997d)

The hydrophobicity of capped PLGA, which is esterified with an alkyl alcohol, is higher than for PLGA with free carboxyl groups (uncapped PLGA). Accordingly, uncapped PLGA degrades faster than capped PLGA (Tracy et al., 1999). Another factor affecting the degradation of the polyesters is the enantiomeric composition of the polymer (de Jong et al., 2001). Two stereoisomeric forms of PLA are commercially available, optically active L-PLA and racemic D,L-PLA. L-PLA is a semicrystalline material (isotactic), whereas D,L-PLA is amorphous (Gilding and Reed, 1979). However, during hydrolysis degradants of D,L-PLA can crystallize and thus further degrade at a lower degradation rate (Li et al., 1990a).

Hydrolysis of the polymer backbone is the initiation of the erosion process, which is a series of events, including a decrease in the molecular weight, a loss of mechanical properties and finally, a loss of mass via the dissolution of small polymer fragments (Lyu et al., 2005). The decrease of the molecular weight of PLGA and PLA follows a pseudo-first order kinetic (Goepferich et al., 2002), which reflects a random chain scission process. As the degradation continues, some polymer fragments become small enough to be water-soluble, and a loss of mass begins. Thus, a typical lag time occurs until an onset of the mass loss of the matrices can be detected, which was previously interpreted as a retardation due to the presence of the slower degrading skin layer (Li et al., 1990a and b).

The pH has an important effect on the hydrolysis rate of polyesters. PLA and PLGA have a stability optimum at pH 4 - 5 and are hydrolyzed under acid and base catalysis (de Jong et al., 2001). The cleavage of an ester bond linkage yields a hydroxyl and a carboxyl group and the formation of carboxylic acids during degradation of the polyesters can accelerate the

hydrolysis of other ester bonds. This process is referred to as autocatalysis. Autocatalysis in polyester matrices is increased in the center of large specimen (Lu et al., 1999), which is often attributed to leaching of acidic degradation products from the exterior layer of the device (Vert et al., 1997). The acidification of the polymer matrix up to pH 2 during degradation (Shenderova et al., 1999, Fu et al., 2000b) is not solely responsible for autocatalysis since degradation in pH 2 medium also resulted in differences between the core and the skin of matrices (Goepferich, 1997). As consequence of autocatalysis, bimodal molecular weight distributions can be found in size exclusion chromatograms of samples from degradation studies.

In order to explore achievable release profiles with duration below or equal to one month, commercially available fast degrading short-chain poly(DL-lactide-co-glycolides) with a lactide to glycolide ratio of 50:50 were focused in this work.

1.2.2. PLGA-based delivery systems for controlled protein delivery

Biodegradable delivery systems based on PLGA can be in the form of solid implants, microparticles or delivery systems that form in situ.

The structures of polymeric controlled release devices can be divided into reservoir and matrix systems (Figure 12).

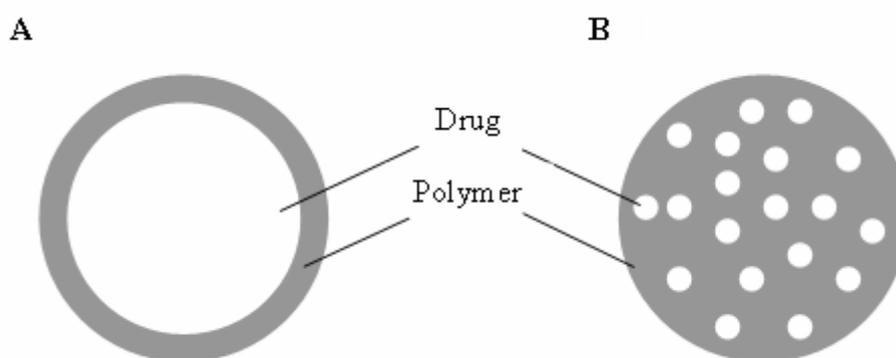


Fig.12: Cross-sections through reservoir- (A) and matrix-type (B) drug delivery systems.

In reservoir systems the drug depot is surrounded by the insoluble polymer, such as PLGA, which represents a barrier for drug diffusion. Reservoir systems offer the opportunities to obtain continuous zero-order releases and high final drug loadings. Despite these advantages, most commonly drugs are incorporated into polymer matrices, since matrices are usually easier to manufacture than reservoir-type systems and their risk for dose dumping is generally

lower (Huang and Brazel, 2001). Drug release from matrix devices is controlled by diffusion and erosion of the matrix.

The development of long-term controlled release systems for proteins involves the challenge to maintain protein stability. Optimization of fabrication and storage conditions alone is rarely sufficient to ensure protein stability in the device until administration. Improvements of the formulations through addition of stabilizing excipients are often required to counteract the various stress factors throughout the entire lifecycle of the dosage form, which also includes the *in vitro* / *in vivo* release phase. And even if stabilizers are present, full retention of the native protein structure remains challenging (van de Weert et al., 2000a).

1.2.2.1. Biodegradable implants

Two PLGA based implants delivering small peptides are available on the market (Table 1). The implants for controlled release of drugs are usually cylindrical polymer matrices (rods), which can be on the millimeter to centimeter scale, facilitating large loadings of active materials. More complicated three-dimensional implant structures, such as tubes, scaffolds or other structural supports, which are of special interest for tissue engineering applications, can be fabricated as well. Manufacturing techniques include solvent casting (Park et al., 1992), extrusion (Zhang et al., 1993), melt compression (Asano et al., 1985), injection molding (Sundback et al., 2003), compression molding (Schliecker et al., 2004) and freeze drying (Hsu et al., 1996). The methods differ in complexity and the applied conditions, but all include potentially deleterious process steps for proteins (e.g., presence of solvents, heat and pressure). The major disadvantage of these delivery systems is their limited patient acceptance, due to the required painful administration into the subcutaneous tissue by surgical intervention or insertion using large-bore needles (trocar).

Proteins are exposed to various deleterious conditions during implant preparation. The exposure to high temperatures, shear forces, or high pressures may cause protein unfolding, irreversible aggregation and can even lead to covalent modifications of proteins (Rothen-Weinhold et al., 2000).

1.2.2.2. Biodegradable microparticles

Microparticles are spherical, polymeric carrier particles of a size between 1 and 1000 μm , which contain drug either in form of a reservoir (microcapsules) or dissolved / dispersed in the polymer matrix (microspheres). Preferentially, microparticles have a size of less than 250 μm (Park et al., 2005), which allow injection through smaller needles after re-dispersing

them in a suitable aqueous medium. The applicability of smaller needles reduces pain during administration and thus improves the patient comfort. The more convenient administration compared to implants makes microparticles an attractive biodegradable drug delivery system. However, their manufacturing is technically challenging.

1.2.2.2.1. Microencapsulation of proteins

Microencapsulation processes include at least three steps before a free flowable microparticle powder is obtained: protein loading, microparticle formation and drying. Rarely, complete drug encapsulation is obtained. Beside losses during manufacturing, another drug fraction generally remains at or has direct access to the microparticle surface and is rapidly released, when the microparticles are placed into aqueous release medium (Sah et al., 1994, Yang et al., 1997, 2000b, 2001, Iguarta et al., 1998, Kim and Park, 1999, Johansen et al., 1999a and b, Lam et al., 2000, van de Weert et al., 200b, Rebeiro Dos Santos et al., 2002, Takada et al., 2003, Blanco-Prieto et al., 2004, Kim and Park, 2004 and Wei et al., 2004). This increased initial release, which is also called burst, is a problem, especially for highly potent drugs, since it increases the risk for serious side effects in vivo. After this initial phase, a diffusion-controlled release phase could be observed, which is attributed to pore-diffusion of protein. Protein that has been entrapped within the polymer matrix might be released in a phase controlled by polymer erosion. Therefore, protein release from PLGA microspheres is often characterized by a triphasic pattern (Zolnik et al., 2006).

Several techniques are available to prepare drug-loaded microparticles. The suitability of a particular method depends significantly on the physicochemical properties of a drug. Considerable work has been done on the incorporation of drugs into PLGA using the oil-in-water (o/w) emulsion solvent diffusion / evaporation method. There, the drug is dispersed or dissolved in a solution of the polymer in a water immiscible solvent (oily phase), which is emulsified in an aqueous continuous phase. Microparticle formation occurs upon solvent removal by diffusion / evaporation and polymer precipitation. The collected particles have to be washed and dried or most commonly freeze-dried to obtain a free-flowing powder. Several variables have been identified, which influence the properties of the resulting microparticles including the type (molecular weight, hydrophilicity) and concentration of the polymeric carrier, the solvent system, the composition and the volume of the continuous phase, the temperature, the drug loading and its solubilities (O'Donnell and McGinity, 1997). Adjustments of these parameters affect the polymer precipitation kinetics and the drug migration during the microparticle formation, which allows optimization of drug entrapment

and minimization of the burst release (Yeo et al., 2004a). The o/w emulsion solvent evaporation system has been successfully employed to encapsulate poorly water-soluble drugs. However, the technique cannot efficiently encapsulate hydrophilic substances like proteins due to rapid diffusion / partitioning of the drugs from the organic into the aqueous phase (O'Donnell and McGinity, 1997). The encapsulation efficiency was improved for hydrophilic drugs such as the nonapeptide leuprolide, by introducing an additional emulsification step (Ogawa et al., 1988). In the so called multiple (w/o)/w emulsion technique (Herrmann and Bodmeier, 1995), an aqueous solution of the hydrophilic drug was first emulsified into the polymer solution. This primary w/o emulsion is then incorporated into a second aqueous phase to remove the solvent and precipitate the polymer. Thus higher drug loadings could be achieved, which obviously outweighed the increase in complexity of the microencapsulation process, since Lupron Depot, a one-month formulation of the nonapeptide leuprolide acetate, was marketed in the US in 1989 (Okada, 1997).

However, the delivery of proteins with microparticles is not only challenging because of formulation and manufacturing issues, which seek to increase drug loading and encapsulation efficiency, to control size, morphology and initial burst or to overcome a certain release pattern. Incomplete release of native protein as a result of protein instability during manufacturing was recognized as a major problem. Certain process conditions, especially the generation of large interfaces between aqueous protein solutions and the organic polymer solutions, were found to be the main problems causing destabilization of various proteins during microencapsulation (Lu and Park, 1995, Ghaderi and Carlfors, 1997, Morlock et al., 1997, Kim and Park, 1999, Krishnamurty et al., 2000 and Pèrez et al., 2002a).

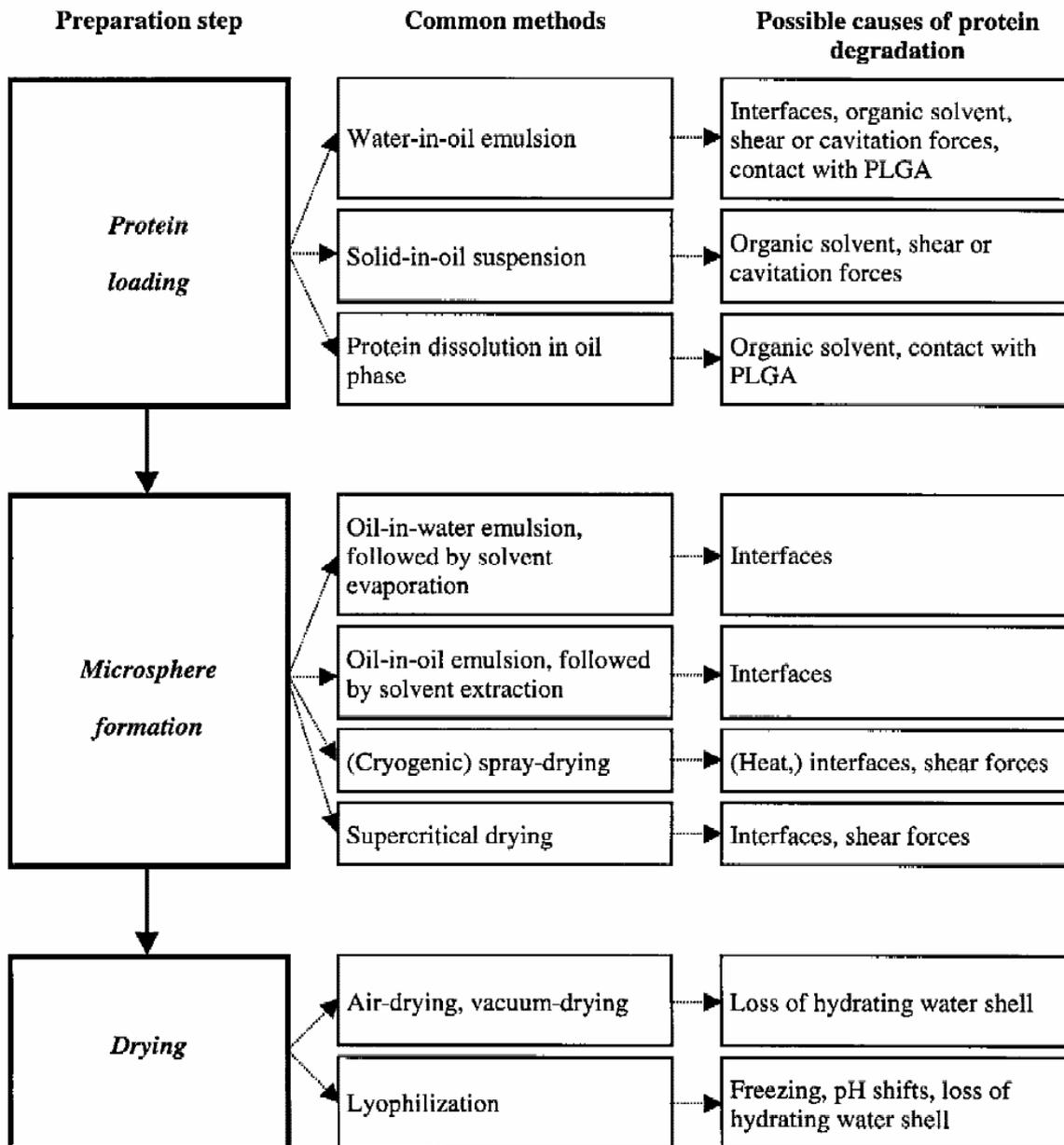


Fig. 13: Analysis of critical process steps in common microencapsulation techniques for proteins (van de Weert, 2000a).

Production procedure induced loss of soluble protein fraction can result in incomplete protein release and thus in a decreased bioavailable dose (Morlock et al., 1997, Kim and Park, 1999). This contributes to high material and manufacturing costs of sustained protein delivery formulations. But denatured or aggregated protein is not only inactive. Even low amounts of these species may already cause unpredictable effects, such as immunogenicity or toxicity (van de Weert 2000a).

In order to maintain the protein integrity during the (w/o)/w preparation method, several typical stabilization strategies (paragraph 1.1.2.2.4.) were applied without or with limited success (Lu and Park, 1995, Cleland and Jones, 1996, Chen et al., 1997, Uchida et al., 1998, Pèan et al., 1999, Sah, 1999, Li et al., 2000, Meinel et al., 2001, Kang et al., 2002, van de Weert et al., 2000c and Pèrez et al., 2003). The degree of protein loss at a water-organic solvent (especially during the primary emulsion step) was protein dependent (Sah, 1999, Kim and Park, 2001) and could be influenced by the nature of the organic solvent (Cleland and Jones, 1996, Sanchez et al., 1999). As a consequence of the finding that proteins can be kinetically trapped as suspension in organic solvents (Griebenow and Klibanov, 1996), the problems associated with the (w/o)/w double emulsion technique were tried to be circumvented by the encapsulation of solid protein.

The exposure of dissolved protein to water / organic solvent interfaces could be reduced using a solid-in-oil-in-water (s/o/w) technique (Wang et al., 1991, Cleland and Jones 1996, Castellanos et al., 2001). However, only totally anhydrous methods can completely avoid this stress factor.

Therefore, a solid-in-oil-in-oil (s/o/o) phase separation (coacervation) process, which was used to prepare peptide-loaded microspheres before (Ruiz et al., 1989), was successfully applied to encapsulate bovine serum albumin protein under anhydrous conditions (Wang et al., 1991). Phase separation around the dispersed protein was thereby induced by the addition of silicone oil (polymer non-solvent) to the drug / polymer solution phase (s/o). The silicone oil had to be washed-off with petrol ether or heptane, which was removed under vacuum, thereafter. Another study confirmed the retention of the structural integrity of solid protein during encapsulation under anhydrous conditions (Carrasquillo et al., 2001). The challenge with the complete removal of all potentially toxic solvents (coacervating and hardening agents) is another shortcoming of the coacervation method (Thomasin et al., 1998).

Spray drying, for example, could be a simple and rapid technique, which offers the chance for an economical continuous processing. A PLGA solution containing dispersed protein is atomized in a hot air stream, which evaporates the volatile organic solvent (methylene chloride, ethyl acetate or acetone) and thus, leads to drug encapsulation. The particles, which can have a size between 1-100 μm , are collected with filters or cyclones. The high temperatures required for spray-drying could affect the protein integrity although the duration of heat exposure is usually very short (Johnson, 1997a). Spray drying of solid-in-oil dispersions of insulin was characterized by low process yield and ineffective entrapment,

which often results in a high drug burst (Johansen et al., 1999, Quaglia et al., 2003). However, the influence of the protein particle size was not investigated.

Originally developed to circumvent heat exposure of proteins during spray drying, a complex, discontinuous spray-freeze-drying process was developed (Gombotz et al., 1991, Prolease technology, Alkermes, US) and applied to encapsulate proteins such as human growth hormone and bovine serum albumin into biodegradable microparticles (Johnson et al., 1996, 1997b, Costantino et al., 2000, 2002). As an example, PLGA solutions in methylene chloride containing solid, zinc-complexed protein were atomized into liquid nitrogen, where frozen ethanol was placed underneath. Methylene chloride in the frozen particles is then extracted into the liquefied polymer non-solvent ethanol upon gentle warming. This highly sophisticated method led to the one and only FDA approved controlled release product of a protein in 1999 (Nutropin Depot, Genentech, Inc., US). However, the commercialization of Nutropin Depot discontinued in 2004 for economical reasons, which can be imagined considering that a second spray freeze drying step was necessary to produce the protein powder.

All anhydrous encapsulation methods require solid protein particles of a suitable size for a successful micro-encapsulation (Costantino et al., 2000, 2002, 2004, Morita et al., 2001). The production of such protein particles further complicates the multi-step manufacturing processes. Methods for protein particle size reduction include aqueous precipitation, conventional milling (Herceg et al., 2005), spray drying (Costantino et al., 1995), freeze drying (Morita et al., 2000, 2001), spray freeze drying (Costantino et al., 2000, 2002, 2004) and techniques utilizing supercritical fluids (Winters et al., 1996, Thiering et al., 2000, Moshashaèe et al., 2000).

Milling is an unfavorable technique because it exposes proteins to high shear, which can result in structural perturbations and loss of biological activity (Platz et al., 1994, Cleland and Jones, 1996, Wolf et al., 2003). Aqueous precipitation (e. g. non-solvent induced) is a common technique in protein purification, however, the used additives have to be removed and the harvested protein particles dried or usually freeze dried before incorporation into PLGA solutions can be conducted. Spray drying exerts thermal and shear stress as well as a water-air interface (Figure 13) and is characterized by low process yields (Prinn et al., 2001). Spray freeze drying is a complicated discontinuous method and, similar to freeze drying, is an energy consumptive and expensive technique. In most cases, stabilizers (e.g., polyols) are required to protect proteins from dehydration induced denaturation during the preparation of solid protein particles. These substances are often hydrophilic and have low-molecular

weights (paragraph 1.1.2.2.4.), which are highly water-soluble and hence cause high initial releases (Sanchez et al., 1999, Carrasquillo et al., 2001). Additionally, spray drying, spray freeze drying and freeze drying require stabilizers to protect the protein against dehydration induced structural changes (Prestrelski et al., 1993a, b). Those stabilizers are often hydrophilic in nature (e. g. polyols) and can therefore act as pore-forming materials, affecting the initial drug release of microparticles (Jaganathan et al., 2005). The impact on the initial burst can be avoided applying an additional downstream purification step to remove the stabilizing additive (Maa and Hsu, 1997), which complicates the process further. However, storage stability might be decreased after removal of the stabilizing excipient. Another complex and costly method to form protein particles involves the utilization of supercritical carbon dioxide. Due to the limited solubility of CO₂ in water, proteins were precipitated from organic protein solutions in dimethyl sulfoxide. But proteins dissolved in DMSO are usually denatured (Jackson and Mantsch, 1991), which could make them prone for aggregation and precipitation. Other stress factors like dehydration / desolvation, elevated pressure and temperature could also explain the reduced yields and compromised biological activities of lysozyme precipitated under various conditions (Winters et al., 1996, Thiering et al., 2000). Correspondingly, a method was developed, where lysozyme dissolved together with PLGA in dimethyl sulfoxide and methylene chloride was emulsified into an aqueous phase to prepare microparticles (Park et al., 1998). It was not elucidated whether the incomplete lysozyme release was a result of the intimate contact of lysozyme and PLGA during the preparation method or whether it was caused during the release phase.

1.2.2.2.2. Storage

Protein encapsulated in microparticles is in the solid state and thus reaction rates and extents can be minimized (Niu and Chiu, 1998) but several chemical reactions, such as the formation of amides between carbonic acid (van de Weert et al., 2000a) or amide (D'Souza et al., 2003) groups of polymers and primary amines of proteins could still occur (Lai and Topp, 1999). Dehydration and rehydration induced denaturation of protein formulations is usually avoided by the addition of lyoprotectants and by controlling the storage conditions, especially with respect to humidity. Low residual moisture in the formulation levels have to be maintained to decrease the polymer chain mobility and prevent PLGA from premature polymer hydrolysis, which can lead to accelerated protein degradation by deamidation and covalent aggregate formation for example (Shao and Bailey, 2000). An acidic environment

also facilitates the hydrolysis of non-reducing sugars commonly used as lyoprotectants. The resulting reducing sugars can lead to the Maillard-reaction with proteins.

1.2.2.2.3. Protein release

The release of drugs from biodegradable microparticles and also implants based on PLGA is controlled by a series of chemical and physical events. Water penetration into the microparticles leads to drug dissolution or detachment from the surface. The initial release is thereby a result of protein, which is situated at or has access to the microparticle surface. The polymer hydrolysis starts and drug and polymer degradation products can be released by diffusion. In the case of hydrophilic macromolecules, like proteins, with very low diffusivities in polymeric compounds (Pitt, 1990), the diffusional release depends on the access to water-filled pores. Polymer degradation leads to the erosion of the matrix, which can control drug release (Siepmann et al., 2002). Bi- and triphasic drug-release behavior are usually observed with biodegradable microspheres (Hora et al., 1990, Kim and Park, 2001), although attempts to overcome the discontinuous release patterns were suggested recently (Luan et al., 2006a). The complex release patterns, however, are not yet well described by mathematical models, which often have only an empirical character and are rarely based on physical and / or chemical mechanisms (Batycky et al., 1997, Faisant et al., 2002, Siepmann et al., 2002). The mathematical interpretation of the release patterns is even more challenging, considering issues with the protein stability (Johansen et al., 1998a).

Besides protein losses during manufacturing and storage, incomplete release is often attributed to protein instability within a PLGA device during release (Wang et al., 1991, Lu and Park, 1995, Crotts and Park, 1997, Morlock et al., 1997, Cleland et al., 1997a, Park et al., 1998, Iguarta et al., 1998, Kim and Park, 1999, Shao and Bailey, 2000, Nam et al., 2000, Schrier and DeLuca, 2001, Jiang and Schwendemann, 2001b, Shi et al., 2001, Panyam et al., 2003, Yeo and Park, 2004c, Jaganathan et al., 2005). Protein is not only irrecoverably lost. It can also be released with a decreased biological activity (Park et al., 1995b, Johansen et al., 1998b, c, Sanchez et al., 1999).

Three mayor factors exist, which are responsible for irreversible inactivation of proteins encapsulated into PLGA (Figure 14): contact of dehydrated proteins with moisture, the acidic microclimate induced by the acidic degradation products of PLGA and adsorption of the protein to the polymer surface.

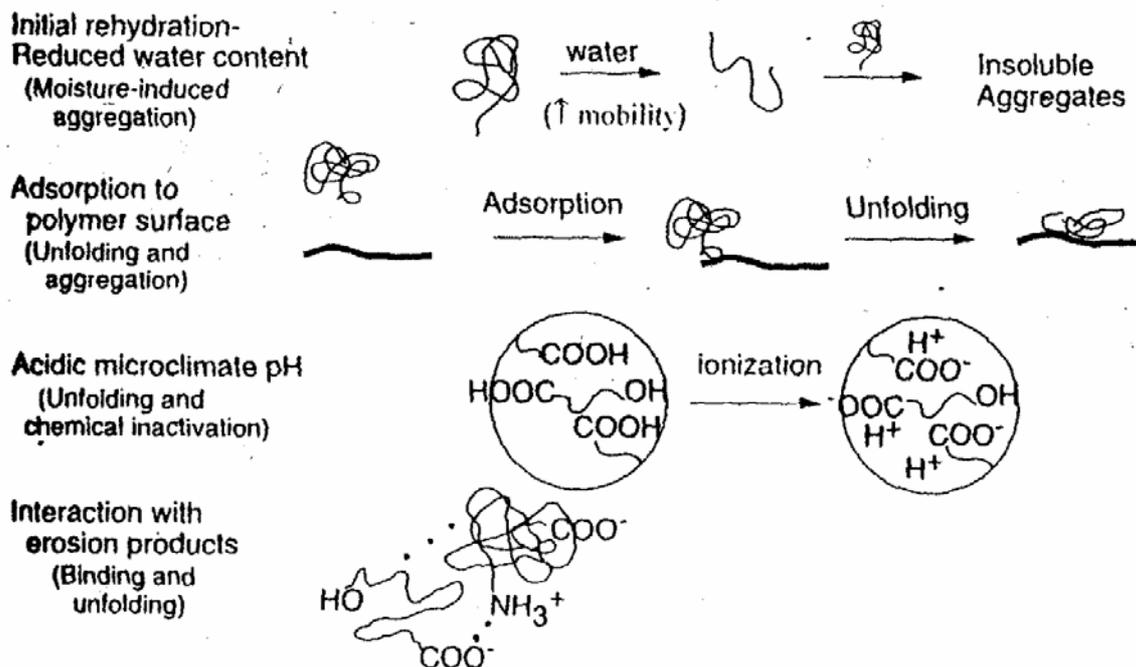


Fig. 14: Potential sources of protein inactivation during drug release from biodegradable devices based on PLGA (Schwendeman et al., 1996)

Release studies with PLGA devices showing complete protein recovery are rare (Lam et al., 2000, Kim and Park, 2004 and the Griebenow group: Castellanos et al., 2001, Carrasquillo et al., 2001, Al-Azzam et al., 2002). The results have to be interpreted with some caution, due to their dependence on the quality of the applied analytical methodologies. Complete release describes the matching of the cumulative protein release and the drug loading (100 % value). Thus, an overestimation of the release of bioactive protein or an underestimation of the drug loading could mask an incomplete protein release.

An overestimation of the release of biologically active protein occurs, if the main degradation pathway of the protein remains unconsidered in the choice of the analytical quantification assay. It had been described for bovine serum albumin (BSA), for example, that it undergoes hydrolysis during release from PLGA microparticles (Iguarta et al., 1998). Nevertheless, Griebenow and his group determined the total release of soluble protein material by UV, leaving potential changes of the extinction coefficients soluble fragments of BSA unconsidered. The complete release patterns would imply, that no aggregation occurred, which is at least questionable considering other reports (Zhu and Schwendeman, 2000, Estey et al., 2006). Another risk is the utilization of blank microparticles in order to correct the dissolution data for excipient-related extinctions (Castellanos et al., 2001, Al-Azzam et al.,

2002), since polymer degradation might be affected by the presence of drugs, including BSA (Catiker et al., 2000).

An underestimation of total protein loading occurs for example, if the extraction method is not capable to recover the drug completely. This could be simply due to losses of native protein but also due to denaturation / aggregation / adsorption during the separation process, since proteins are often exposed to potentially deleterious conditions like organic solvent interfaces, surfactants or extremes of pH, during extraction (Crotts and Park, 1997, Crotts et al., 1997, Sah, 1997, Lam et al., 2000 and Wolf et al., 2003). Another problem occurs, if insoluble protein aggregates were already formed during manufacturing of the dosage form (paragraph 1.2.2.2.1.). More complex analytical methods, which involve a complete degradation of protein and carrier into soluble monomers, such as amino acid analysis, can help to determine all protein-related derivatives in a formulation and can facilitate an impartial evaluation of classical extraction methods. Thus, the unsuitability of protein separation in presence of aqueous / organic interfaces was shown (Sharif and O'Hagan, 1995).

Finally, the choice of an analytical methodology to determine the total amount, the release or the unreleased fraction of an encapsulated protein has also to consider the biological activity. This can only be characterized, if a protein is stable enough to retain its native conformation during the extraction procedure or can restore it thereafter (Dong et al., 1997, Wolf et al., 2003).

Rehydration induced protein inactivation

In order to prevent protein inactivation during release from controlled release devices based on PLGA (Figure 14) several formulation additives were investigated. Initial rehydration of dehydrated solid protein can lead to irreversible aggregation (moisture-induced aggregation). Upon rehydration of an unfolded protein, kinetic competition between intramolecular refolding interactions and intermolecular aggregate-forming interactions occurs (Prestrelski et al., 1993a, Allison et al., 1996). The initial hydration process upon water imbibition into PLGA matrices often leads to high local protein concentrations, which favor aggregation over refolding (Estey et al., 2006). Besides physical interactions, moisture-induced aggregation can also involve covalent bond formation, as described for bovine serum albumin (Liu et al., 1991). Strategies to circumvent rehydration induced protein inactivation are the same as used to stabilize proteins against stress due to dehydration (paragraph 1.1.2.2.4.). Hydrophilic cryoprotectants could be used, which likely increase the initial burst (Carasquillo et al., 2001, Al-Azzam et al., 2002). Another approach could be the decrease of

the protein solubility by zinc-complexation, for example (paragraph 1.1.2.2.4.). Thus, high local protein concentrations upon rehydration of the PLGA matrices could be avoided. Another alternative to circumvent aggregation by favoring redissolution of aggregates might be pegylation of proteins, as suggested elsewhere (Diwan et al., 2001).

Protein inactivation during polymer degradation

The protein microenvironment changes significantly upon polymer degradation and the occurrence of acidic oligomers. The lowered pH and increased osmolality (paragraph 1.2.1.1.2.) can promote protein denaturation, aggregation and to chemical degradation (paragraph 1.1.2.2.). Among the chemical degradation pathways of proteins, especially peptide bond hydrolysis (Takahata et al., 1998, Shao and Bailey, 1999, Zhu et al., 2000a, Jiang and Schwendeman, 2001, Sandor et al., 2002, Estey et al., 2006) but also deamidation (Shao and Bailey, 2000) occur at these conditions. But lactic and glycolic acid oligomers can also react with amino acid residues. The primary amine groups at the N-terminus and at lysine residues and the hydroxyl groups of tyrosine or serine residues could be potential reaction sites (Lucke et al., 2002, Lucke and Goepferich, 2003). However, this was obtained with small peptides and appeared to be less of a problem for insulin (Ibrahim et al., 2005).

Intuitively, one strategy to overcome acidification in PLGA matrices is the co-incorporation of basic additives or buffer agents (Johansen et al., 1998b, Shao and Bailey, 1999, Marinina et al., 2000, Zhu and Schwendeman, 2000, Jiang and Schwendeman, 2001a, b). The ideal additive would be able to maintain the pH of choice throughout the whole release interval, without creating pH values, which could denature the encapsulated protein (Sandor et al., 2002). Therefore, slightly soluble bases, like magnesium hydroxide, were applied to stabilize proteins, which are sensitive to hydrolytic degradation (Zhu et al., 2000). The advantage of such a substance is that its dissolution is enhanced when the pH starts to decrease. A fair evaluation of the stabilization approach needs to consider a retarding effect of basic additives on the degradation behavior of PLGA (Zhang et al., 1997). Otherwise, a sustained onset of PLGA degradation would be misinterpreted as a stabilizing effect, when protein extraction from stabilized and unstabilized specimen is conducted at the same time-point. Thus, the reported stabilization effects by basic additives on unreleased BSA (Zhu and Schwendeman, 2000 and Zhu et al., 2000) could just be artifacts. An indication is given by the reported molecular weights at the point of extraction. The “stabilized” samples had a significantly higher molecular weight after the incubation period, indicating that there might have been no soluble polymer degradants formed yet in these matrices. The described stabilizing effect

might therefore be ascribed to an assistance of the protein re-hydration process, due to the enhanced water uptake by the excipient containing specimen, which typically leads to increased initial releases.

Macromolecules like proteins are usually diffusing through water filled pores and not through the PLGA bulk. Thus, the drug fraction without access to the microparticles surface is dependent on matrix erosion. There the concentration of soluble acidic degradants is expected to be elevated (Cho et al., 2001). Thus, for acid-labile proteins it could be beneficial, if a neutral pH could be maintained throughout the erosion phase and not during the “lag phase” of mass loss.

But even in this case, the problem with the chemical stability of proteins during release from PLGA matrices is not necessarily solved. Moreover, it would probably only shift the main degradation pathway from peptide hydrolysis to oxidation or β -elimination and thus to covalent aggregation (paragraph 1.1.2.2.). Accordingly, a dependence of covalent aggregate formation on the amount of co-encapsulated basic additive was determined (Zhu and Schwendeman, 2000).

Alternative to the addition of basic excipients, an increase of the porosity of PLGA matrices was suggested to prevent the interior of microparticles from acidification through an enhancement of the out-diffusion of the degradation products (Cho et al., 2001). However, increasing the matrix porosity also increases the initial and the diffusional release of proteins (Jiang and Schwendeman, 2001b). This could be advantageous, since this protein would not be exposed to the acidic microclimate (Kim and Park, 2004). However, any unreleased protein would likely be degraded during the erosion phase (Sanchez et al., 1999).

Adsorption induced protein inactivation

Interactions between proteins and polymers are an important reason for incomplete release from PLGA matrices (Figure 14). Protein and polypeptide adsorption to PLGA can be due to hydrophobic contacts (Makino et al., 1986, Zambaux et al., 1999, Lu and Park, 1995, Tsai et al., 1996, Crotts et al., 1997 and Butler et al., 1999) and / or electrostatic interactions (Blanco and Alonso, 1997, Gaspar et al., 1998, Park et al., 1998, Nam et al., 2000). The susceptibility is dependent on the drug but the PLGA type also influences polymer-protein interactions due to different hydrophobicities of capped carboxyl end groups and uncapped carboxyl end groups (Lam et al., 2000). It was suggested that extraction with different media could reveal the mechanisms behind incomplete release of proteins. Incomplete release of carboxymethylated BSA and BSA was obviously due to adsorption, since lost protein could

not be recovered from PLGA-microparticles upon denaturant addition (guanidinium chloride), whereas the addition of an anionic surfactant (sodium dodecyl sulfate) redissolved larger fractions of adsorbed protein (Crotts and Park, 1997). Protein recovery upon salt (sodium chloride), denaturant or surfactant addition indicates the mechanisms behind incomplete release. Added salts can compete with the electrostatically bound protein for negative charges at carboxylate groups of PLGA. The denaturant would dissolve non-covalent aggregates and the surfactant would re-dissolve both, non-covalent aggregates and protein adsorbed to PLGA by hydrophobic interactions. The study of positively charged lysozyme (pI 11) suggested that non-covalent aggregation was probably initiated by electrostatic interactions between protein and free carboxyl end groups of PLGA, since once adsorbed, lysozyme could not be detached by addition of salt. Hydrophobic PLGA-protein contacts also contributed to the incomplete release (Park et al., 1998). Despite addition of additives for protein redissolution, only 70–90 % of total protein was recovered.

One of the strategies to prevent protein adsorption to surfaces is the addition of competing substances (paragraph 1.1.2.2.4.). Ideally, the excipient should show a higher affinity to the surface than the protein, without affecting the integrity of the drug in a negative way. Additionally, it needs to be present in a sufficient amount throughout the release period (Morlock et al., 1997), which leads to a preference of higher molecular weight additives.

Other proteins, such as albumins or gelatins, are often co-incorporated as stabilizers into PLGA microparticles (Lu and Park, 1995, Chen et al., 1997, Morlock et al., 1997, Meinel et al., 2001). The effects of co-incorporated competitor substances cannot strictly be attributed to a reduced adsorption to PLGA during the release phase, because stabilization against manufacturing- and rehydration-induced stress might also occur. Prevented adsorption of an active protein by causing aggregation of an auxiliary protein might not necessarily reduce the risk for an immunologic response. Thus, there might be concerns about the co-incorporation of such stabilizing proteins from the perspective of the regulatory authorities (Bilati et al., 2005a). However, tetanus toxoid adsorption to PLGA microspheres was significantly reduced in presence of bovine serum albumin, which indicated the potential of additive incorporation (Johansen et al., 1998a).

The stabilizing effect of non-ionic polyethylene glycols was attributed to competition for hydrophobic interactions between PLGA particles and human serum albumin (Quellec et al., 1999). Despite solving potential protein stability issues, the drawback with the co-incorporation of hydrophilic molecules like polyethylene glycols (PEG) is the resulting increase of the initial release (Al-Azzam et al., 2002). The negative effect of PEG on the burst

release was reported to even necessitate its removal, when it was used in higher amounts as cryoprotectant during protein micronization (Morita et al., 2000).

The co-incorporation of non-ionic surfactants (poloxamer 188 and 331) was reported to reduce interactions of bovine serum albumin with PLGA (Blanco and Alonso, 1997, 1998). Besides competition for surfaces, surfactants also denature proteins after binding to the macromolecule (paragraph 1.1.2.2.2.). Thus, negative effects on protein integrity need to be excluded.

Irreversible adsorption of proteins to PLGA might also be reduced by the addition of negatively charged low-molecular weight substances, which can compete with PLGA for binding to the protein. Basic fibroblast growth factor (bFGF), for example, could be stabilized by co-encapsulation of heparin (Zhu et al., 2000). As discussed, the risk for protein destabilization is elevated with additives, which directly bind to the macromolecules, due to the lack of suitable binding sites (van de Weert et al., 2004).

Buffers including non-ionic surfactants, such as Tweens, are commonly utilized for in-vitro drug release studies with microparticles (Morlock et al., 1997, Sanchez et al., 1999, Marinina et al., 2000, Zhu and Schwendeman, 2000, Jiang and Schwendeman, 2001a, b, Kim and Park, 2001, 2004, Pistel et al., 2001, Jaganathan et al., 2005, Estey et al., 2006) in order to prevent microparticles from clumping during release (Sanchez et al., 1996). However, surface-active agents generally compete with proteins for electrostatic and / or hydrophobic interactions with the PLGA surface. Thus, Tween addition to the release medium needs to be considered as in vitro protein stabilization approach (Cleland et al., 1997a, Blanco and Alonso, 1997, 1998). Surfactants like the anionic sodium dodecyl sulfate are even capable to redissolve already adsorbed protein molecules from PLGA, which increases protein recovery (Crotts and Park, 1997, Park et al., 1998). An increased protein recovery was also seen when bovine serum albumin was added to the release medium (Johansen et al., 1998a). This study opened up the question whether adsorption of protein to the PLGA surface would be relevant under in vivo conditions (van de Weert et al., 2000a). Increasing the ionic strength of the release medium could also lead to an increased recovery in cases of an electrostatically driven adsorption (Park et al., 1998). However, a salting-out of the protein molecule needs to be prevented (Tsai et al., 1996). Finally, a medium pH far from the isoelectric point could also minimize protein adsorption as previously shown with peptides (Tsai et al., 1996, Kostanski and DeLuca, 2000).

However, formulation and release conditions, which lead to an increased aggregation stability, would always need to be evaluated for negative effects on the physical and chemical

stability of the proteins (paragraphs 1.1.2.2.2. and 1.1.2.2.3.). This requires increased analytical effort and a differentiated view on the common terminology of “stability” and “recovery”.

Thus, the safest way to prevent adsorption induced protein inactivation appears to be the improvement of the thermodynamic stability of a protein in the aqueous state (paragraph 1.1.2.2.4.) since surface binding-induced denaturation appears to be a prerequisite for protein aggregation (Wang, 1999). Thus, stabilization of the native state might prevent proteins from denaturation, adsorption and aggregation. Frequent buffer replacement or the use of a dialysis bag, for example, could prevent the acidification of the release medium during PLGA degradation and thus reduce protein degradation (Park et al., 1995, Takahata et al., 1998). It is unsure, however, whether a constant neutral pH reflects the in-vivo situation, where PLGA devices are surrounded by a fibrous capsule that may hinder the efflux of acidic degradation products (van de Weert et al., 2000a). Furthermore, all adjustments of the release conditions, which affect the release behavior through excipient effects on the stability of a protein, need to exclude or understand potential effects on the delivery system.

Although the release conditions should ideally be close to the physiological conditions at the administration site (D’Souza and DeLuca, 2006), apparently unphysiological conditions, such as lower pH-values or higher buffer strengths, might be required to build meaningful in-vitro-in-vivo correlations (Cleland et al., 1997a, Tracy et al., 1999, Jiang et al., 2002).

1.2.2.3. Biodegradable in situ forming systems

In contrast to solid microparticles, in situ formulations are liquid formulations of drugs and carriers. Carrier solidification upon subcutaneous or intramuscular injection leads to drug encapsulation and thus to the formation of solid drug depots in situ, from which the drug is released in a controlled manner.

Over the last decade, a number of novel in situ forming devices have been developed, in order to overcome the manufacturing-related shortcomings of conventional microparticles, like utilization of potentially toxic solvents, complex and costly multi-step processes, difficult scale-up and low encapsulation efficiencies (Packhaeuser et al., 2004). Protein containing microparticles would need to be produced under aseptic conditions, since gamma-irradiation might harm these sensitive drugs (Shacter, 2000), whereas in situ formulations could potentially be subjected to sterile filtration. Besides the easy and cost-saving manufacturing, the liquid in situ forming systems offer an easier administration without an additional

reconstitution step prior administration and a patient-friendlier application through higher gauge needles.

In situ forming systems can be classified into in situ forming implant and in situ forming microparticle formulations.

1.2.2.3.1. In situ forming implants

In situ forming implants are in situ systems, which form a single unit depot after administration into the human or animal body. The in situ solidification of the carrier around the dispersed or dissolved drug can occur by chemical or physical means. According to the mechanisms involved in the depot formation, a categorization of the in situ systems into six classes was suggested. The following systems were differentiated: thermoplastic pastes, in situ cross-linked polymers, thermally- or pH-induced gelling systems, in situ cubosomes, in situ solvent removal (Chitkara et al., 2006).

Thermoplastic pastes

Thermoplastic pastes are heated polymer systems, which increase their viscosity and form a semi-solid mass upon cooling to body temperature. A polymer like low-molecular weight poly(D,L-lactide) requires a temperature of around 65°C to be applicable. Such temperatures, however, can be very painful upon injection and increase the chance of necrosis and scar formation at the site of injection (Hatefi and Amsden, 2002). Furthermore, the utilization of elevated temperatures in the preparation and administration process of protein delivery systems is counterintuitive due to their fragility. Low molecular weight poly(ortho ester)s appear to be very interesting in this respect. They possess relatively low softening temperatures (35–45°C) and they are surface eroding polymers. Although protein adsorption and aggregation during release could still occur (Weert et al., 2002b), poly(ortho ester) prevent encapsulated proteins from contact with water and hence, could prevent them from hydrolytic degradation during release. Unfortunately, poly(ortho esters) are not accepted by the regulatory authorities for parenteral application.

In situ cross-linked implants

Polymeric or gel depots can be formed by in situ crosslinking of polymers. Methods for in situ crosslinked systems include free radical reactions usually initiated by heat or absorption of photons, or ionic interactions between cations and polymer anions.

Thermoset polymers can flow and be administered when initially constituted, but heating initiates covalent reactions between polymer chains and the systems set into a final shape. The exothermic nature of the cross-linking reaction as well as the high reactivity of used radical forming initiators are major drawback with regard to toxicity (Hatefi and Amsden, 2002) and the stability of sensitive drugs such as proteins.

Photo-crosslinked gels can be used in place of chemically initiated thermoset systems. The photon-initiated reaction is performed in situ by using fiber optic cables immediately after introduction of the polymer to the desired site by injection (Chitkara et al., 2006). The release of proteins with molecular weight equal or below 60 kDa was controlled by diffusion and delivery periods up to 5 days were achieved. Larger molecules were not able to diffuse. Their release would depend on the degradation / erosion of the hydrogel (Hubbel, 1996). However covalent cross-linkings have the drawbacks that potentially toxic cross-linking agents are utilized and the risk for chemical reactions between functional excipients and incorporated proteins, which might damage proteins (de Jong et al., 2001).

Therefore, physical hydrogels formed by synthetic copolymers have attracted some attention. Physical cross-linking can form polymer networks suitable for protein delivery. Ion-mediated gelation, for example, has been reported for a number of polymer / ion systems, e.g. alginates / calcium or chitosan / phosphate. The concentrations of suitable counter ions are, however, insufficient to facilitate cross-linking in situ (Packhaeuser et al., 2004) and alginates were reported to be immunogenic (Kulseng et al., 1999). As another opportunity pre-prepared thixotropic hydrogels consisting of supramolecular complexes of poly(ethylene oxide)s and α -cyclodextrin were suggested to be applicable for the delivery of macromolecules. The hydrogels form physical cross-linkings upon standing and liquefy upon agitation. Time periods of more than 15 hours were reported to restore the initial viscosities of the gel after agitation. This leads to an increased chance for a high initial drug burst, considering a release period of the ideal (non-agitated) formulation of about 90 hours (Li et al., 2003).

ABA triblock copolymers of PLA or PLGA (A) and PEG (B) can be designed to exhibit rapid swelling upon contact with water, forming a physically cross-linked, biodegradable hydrogel. They were reported to be biocompatible (Kissel et al., 2002) but are not approved for parenteral applications.

Thermally-induced gelling systems

Thermally-induced gelling systems base on polymer systems, which show thermo-reversible sol / gel transitions. As an example, the sol-gel transition behavior of block

copolymers of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (poloxamers) has been studied for the delivery of various drugs including proteins. Interleukin-2 release of about 10 hours was obtained with aqueous 30% poloxamer 407 containing gels (Johnston, et al., 1992). The majority of the studies use poloxamers, since they represent the only thermoviscosifying materials approved by the FDA. However, poloxamer 188 is the only polymer, which has been approved by the FDA for parenteral administration. It is used as solubilizing agent for drugs upon intravenous injections in concentrations of not more than 0.6 %, which would be unsuitable for gel formation. Higher concentration of poloxamers showed unacceptable toxicity (Hatefi and Amsden, 2002).

Aqueous solutions of ABA triblock-copolymers of PLA or PLGA (A) and PEG (B) also offer thermo-gelling properties. For example, poly[(D,L-lactide-co-glycolide)-b-ethylene glycol-b-(D,L-lactide-co-glycolide)] forms thermosensitive gels with water and is biodegradable (ReGel, Protherics PLC, UK). These formulations are a sol below 15°C and are injectable through a small 25-gauge needle. Upon injection as a liquid, the product quickly becomes a gel at body temperature, forming a depot that slowly degrades over a period of four to six weeks. The solution of the triblock copolymer is and forms a highly viscous gel at body temperature. Based on this technology, a formulation of the anticancer drug paclitaxel is currently in clinical Phase II (OncoGel, Protherics PLC, UK).

The applicability for protein delivery has to be further investigated. The fairly stable, small protein insulin showed incomplete release due to aggregation, even when formulated in the zinc-stabilized form (Kim et al., 2001). The same was observed for zinc-stabilized porcine growth hormone and granulocyte stimulating factor (Zentner et al., 2001). Despite the potential benefit to circumvent organic solvents, aggregation-prone proteins might not necessarily benefit from these novel polymers.

pH-induced gelling systems

A sol / gel transition of an aqueous polymer system could also be induced by changes in the environmental pH. Acidic solutions of the basic polymer chitosan, a deacetylated derivative of naturally occurring chitin, form a viscous gel in situ through deprotonization of primary amino groups in neutral body fluids. However, the release of low molecular weight drugs was rapid without further chemical cross-linking (Ganguly and Dash, 2004). Furthermore, chitosan is not approved by the FDA, yet.

In situ forming cubosomes

Cubosomes are liquid crystalline phases, which assemble spontaneously upon contact of water-insoluble amphiphilic glycerides, such as glycerol monooleate or glycerol monolinoleate, with water. A three-dimensional bicontinuous network of lipid bilayers and water channels is thus formed. This liquid crystalline structure is gel-like and highly viscous (Hatefi and Amsden, 2002). Releases of dextran molecules of varying molecular weights from a glycerol monooleate cubic phase resulted in a decreasing dextran recovery above a critical molecular weight. More than half of the dextran loading was released, if the molecular weight was below 20 kDa (Drummond and Fong, 2000). Insulin (6 kDa) release from a cubic phase of glycerol monooleate lasted for up to 4 days. However, about 70 % of the protein was released within the first day (Sadhale and Shah, 1999).

In situ solvent removal

The most successful strategy to form drug depots in situ is the polymer solidification by solvent removal. These in situ forming implants are based on biodegradable carriers, which are dissolved in or diluted with biocompatible solvents for providing syringeable solutions. Drugs can be either dispersed or dissolved in the carrier solutions. Once the formulation is placed into the body the solvent diffuses away, leaving the system to coagulate, increase its viscosity or solidify, which leads to entrapment or encapsulation of drug within the carrier matrix.

Two implants formed by in situ solvent removal are based on biodegradable non-polymeric carriers. Sucrose acetate isobutyrate is a water-insoluble, viscous compound, which can be diluted with various solvents in order to decrease the viscosity and improve the syringeability (SABER, Durect Corporation, US). So far, sucrose acetate isobutyrate is only approved as a food additive but one potential product for the controlled release of bupivacaine is in clinical phase II (POSIDUR). The second non-polymeric approach utilizes amphiphilic derivatives of L-alanine, which can self-assemble and thus form organogels when admixed with vegetable oils and low amounts of organic solvents (Motulsky et al., 2005). Loaded with leuprolide, the gels showed prolonged release in-vitro and in-vivo (Plourde et al., 2005). However, suitability for proteins delivery has not been evaluated yet. Although a good biocompatibility of the systems was reported, the novel organogelators are not approved for parenteral use.

Two technologies are based on solutions of polymeric carriers, such as PLGA and PLA. They differ in the water-miscibility of the polymer solvent, basically.

Atrigel technology

The Atrigel technology is based on biodegradable polymeric carriers, which are dissolved in organic solvents with a water miscibility of more than 7% (Dunn et al., 1994, 1998). The concept was conceived by Dunn and co-workers (Dunn et al., 1990) at US-based Atrix Laboratories Inc. (now QLT Inc., Canada) and has already yielded in one commercial product for the controlled parenteral delivery of the nonapeptide leuprolide (luteinizing hormone releasing hormone agonist) (Ravivarapu et al., 2000a, b) and another one for the local administration of doxycycline into periodontal pockets (table 1). The success can be attributed to the utilization of ingredients, which are approved for parenteral use (Dunn et al., 1997).

Among the water-miscible polymer solvents used to formulate in situ implant systems N-methyl-2-pyrrolidone (NMP) and dimethyl sulfoxide (DMSO) are preferred due to their pharmaceutical precedence (Hatefi and Amsden, 2002). A guidance summarizes the acceptable limits for exposure of humans to residual organic solvents (Q3C Impurities: Residual Solvents), which was developed by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). They divided organic solvents according to their toxicity into three classes. Class 1 consists of toxic solvents, which should be avoided. Class 2 solvents are still somewhat toxic and their use should be limited. Whereas class 3 solvents have a low toxicity and their permitted daily exposure (PDE) exceeds 50 mg/day. Although NMP is classified as an ICH-class 2 and DMSO as an ICH-class 3 solvent, their PDEs are almost similar (48.4 and 50 mg/day). Only mild tissue irritations were seen upon exposure of monkeys to NMP- and DMSO-based in situ implants (Royals et al., 1999). The tissue response was similar to solid PLGA delivery devices.

Besides NMP and DMSO, other solvents with solubility parameters of about $9-13 \text{ (cal/cm}^3)^{1/2}$ can also be used to formulate in situ implants (Shiveley et al., 1995). Thus, acetone, 2-pyrrolidone, propylene carbonate (Dunn et al., 1992), ethyl acetate (Lambert and Peck, 1995), glycerol formal (Chern and Zingerman, 1999), glycofurool (Eliaz and Kost, 1997) or low-molecular weight polyethylene glycol (Su et al., 2004) have been suggested as interesting alternatives. A problem with in situ implants based on water-miscible solvents is the generally high initial drug release after contact with aqueous medium. This high initial release can be attributed to large finger-like pores formed because of a fast phase-inversion of polymer solutions, where the polymer solvent has a high affinity to the precipitant. An increase of the

polymer concentration can retard the phase inversion and thus lead to a more uniform, sponge-like structure (Strathmann et al., 1975 and Strathmann and Kock, 1977).

Although the basic principles of the phase-inversion dynamics had been investigated for decades, it was not before 1999 that the group of McHugh investigated the coagulation and solidification behavior of more complicated drug-loaded PLGA solutions. The effect of incorporation of the model protein lysozyme and other additives, such as polyvinyl pyrrolidone and poloxamers were investigated and related to the drug release upon contact with aqueous medium (Graham, et al., 1999, Brodbeck et al., 1999a, DesNoyer and McHugh, 2001, 2003). Interestingly, the released drug fraction from PLGA-containing systems was never more than 75%. Almost complete release (~95%) of the bovine serum albumin fraction was obtained with in situ formulations based on glycofurol (Eliaz et al., 2000, Eliaz and Kost, 2000). However, only 75% of co-incorporated tumor necrosis factor-receptor was released from the same formulation. Higher recoveries of both proteins were achieved only with formulations showing unacceptably high initial releases of 20–40% (Eliaz and Kost, 2000). Plasmid DNA was completely released from the in situ implants in-vitro and led to a sustained gene expression in-vivo (Eliaz and Szoka, 2002).

The high polymer concentrations needed to avoid increased initial releases (Lambert and Peck, 1995) result in high viscosities of the in situ implants and thus complicate their administration through hypodermic needles. Reproducibility of release pattern could be another challenge, since there is only insufficient control of the shape of this single unit system after administration. Different shapes lead to variable surface areas, which can finally affect the release pattern.

Alzamer technology

The Alzamer technology (ALZA Corporation, US) utilizes organic solvents with a water miscibility smaller than 7%, in contrast to Atrigel. Potential solvents include preferentially benzyl benzoate and ethyl benzoate but also other alkyl- or aralkyl ester of benzoic acid (Brodbeck et al., 2000), triacetin, triethyl citrate (Shah et al., 1993) or benzyl alcohol (Prabhu et al., 2005) could be used. Solvents with a low affinity to water significantly slow down the phase inversion rate and lead to dense, sponge-like drug matrices and a low drug burst upon contact with aqueous medium. The onset of PLGA degradation was retarded compared to solvents with higher affinity to water due to the slower dissipation of the polymer solvent (Brodbeck et al., 1999a, b). However, low amounts of solvents added to PLA or PLGA act as plasticizer and can decrease the glass transition temperature in a way that the

degradation rate might be increased (Kranz et al., 2000). Zinc-stabilized human growth hormone could be delivered for up to 28 days in vivo. However, protein stability during release was not investigated (Brodbeck et al., 1999b).

The release of cytochrom C and myoglobin, which were incorporated into triacetin- and triethyl citrate-based formulations, showed only drug release within the first 5 to 10 days in most cases (Shah et al., 1993). The cumulative amounts released were between 20–70%, while the highest release was obtained in release medium containing human serum albumin and sodium chloride, which points to protein adsorption as a reason for protein losses.

Complete release of lysozyme was obtained with in situ implants consisting of low molecular weight PLA in triacetin (Al-Tahami et al., 2006). Only the 10% polymer concentration resulted in complete lysozyme release over 7 days, whereas the release was slower and increasingly incomplete at higher polymer concentrations (20 and 30%). This could be due to an increased entrapment of lysozyme into the solidified polymer matrix. The release of the unrecovered amount of lysozyme would be expected to occur later, upon degradation of the slow-degrading polymer matrix (Kim et al., 2004).

The storage stability of the polypeptide bovine calcitonin was investigated in in situ implants based on benzyl benzoate and benzyl alcohol. There, the peptide was storage stable at 4°C (Prabhu et al., 2005) but it lost biological activity at higher storage temperatures.

1.2.2.3.2. In situ forming microparticles

In situ forming microparticles (ISM) were developed to overcome the problems associated with conventional microparticles and in situ forming implants. The complex manufacturing processes necessary to produce conventional biodegradable microparticles are not required. The formation of microparticles occurs after subcutaneous or intramuscular administration of liquid or semi-solid dispersions of polymer solutions and a second phase, e.g. oil for injection or water. Depending on the character of the second phase, in situ microparticle systems are distinguished into oil in oil (o/o) and oil in water (o/w) ISM systems. Microparticles are formed in situ due to polymer precipitation initiated by out-diffusion of solvent from or influx of aqueous body fluids into the polymer solution droplets. Thereby, drugs dispersed or dissolved in the polymer solutions will be entrapped into the in situ microparticles and can be released from the hardened particles in a controlled manner (Bodmeier, 1997, 2000).

An ISM formulation is a multiple unit dosage form and thus has potential advantages with regard to reproducibility of the release patterns over in situ forming implants. The shape of

the in situ implants might be difficult to control upon administration and could depend on the injection conditions, e.g. speed, as well as on the site of injection. The implant shape, thus its surface area, could affect the in-vivo release pattern. This might result in a lower reproducibility of the formulations.

High polymer concentrations are usually necessary to obtain prolonged release of drugs without an increased initial drug burst (Lambert and Peck, 1995). Increasing the polymer concentration of in situ implants increases the formulation viscosity, which hampers administration through hypodermic needles. The syringeability could be improved in case of the ISM formulations, due to a lower viscosity of the second, continuous phase of the dispersion. This could facilitate to apply higher polymer concentrations and thus, less organic solvent, which could decrease a potentially tissue irritation by the solvent.

The in situ forming microparticle dispersions can be stabilized with suitable excipients, which could save an additional re-dispersion step, as necessary for conventional microparticles prior to administration. Jain et al. described the preparation of microglobules or pre-microspheres containing the model proteins cytochrome c and myoglobin (Jain et al., 2000b, c). A dispersion of protein in PEG 400 was incorporated into a solution of PLGA in triacetin. Tween 80 addition completed the preparation of oil phase 1. This phase was added dropwise to a Span 80 containing Miglyol 812 solution (oil phase 2) under continuous homogenization. Thereby, phase separation (coacervation) of PLGA was induced and pre-microspheres, ready for injection, were formed (Jain et al., 2000a, b, c). After an initial burst of about 30-40%, the proteins appeared to be completely released in a continuous manner (Jain et al., 2000b, c). The complete release of these fragile proteins (Shikama, and Sugawara, 1978) has to be questioned due to the application of an unsuitable extraction method (methylene chloride / water interface), which is known to lead to underestimations of the drug loading and has a high risk to affect the structural integrity of proteins (paragraphs 1.1.2.2.2. and 1.1.2.2.3.). Therefore, further investigations about the suitability of in situ forming microparticle systems were initiated.

Another approach is to emulsify the drug containing polymer solution phase with a separately stored second (oily or aqueous) phase directly prior to injection (Bodmeier, 1997, 2000, Kranz et al., 2001). In case of o/w ISM, this avoids the contact of the polymeric carrier with water, which prevents the biodegradable polyester PLA and PLGA from degradation. The emulsification prior administration can be achieved by blending the contents of two coupled syringes, which comprise the individual phases.

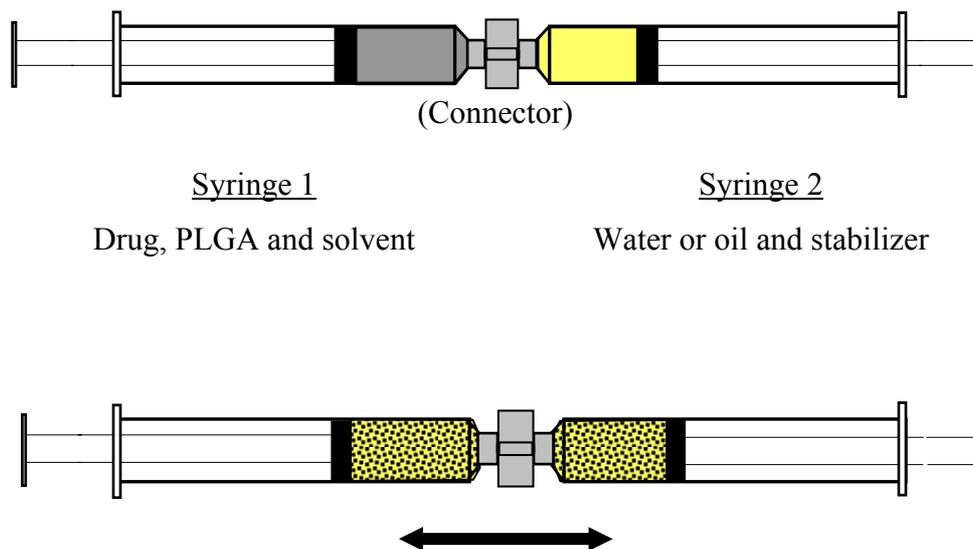


Fig. 15: Two-syringe system for the preparation of ISM-emulsion prior administration

Thus, dispersions of moderate stability can be prepared and administered without stability concerns. Stability issues with drugs in contact with PLGA or the solvent could be overcome by a separation of polymer, drug and solvent. Drug and polymer, for example, could be stored separately from the solvent in form of lyophilized sponges (Dong et al., 2006). The sponge could be dissolved with the polymer solvent prior to the emulsification step with the second phase. ISM formulations already showed their potential in the delivery of small peptides such as leuprolide and busserelin (Luan and Bodmeier, 2006b, c, Kranz and Bodmeier, 2007).

1.3. Objectives

The objective of this work was to evaluate the suitability of in situ forming biodegradable microparticles for the controlled delivery of proteins. Particular goals were:

- a) to evaluate the flexibility of the release patterns of in situ implant and in situ microparticle formulations using hen egg white lysozyme as model protein;
- b) to characterize, if and how the integrity of the model protein is affected by formulation ingredients, the manufacturing process, storage of the ISM and the release from the formulations;
- c) to optimize protein delivery from in situ forming implant and microparticle systems, with regard to shape and completeness of the release;
- d) to find / validate reliable methods allowing to extract and thus to characterize the model protein in biodegradable in situ formulations.