# The Development of a Sustained and Controlled Release Device for Pharmaceutical Proteins based on Lipid Implants

**Dissertation**

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**Silke Mohl**
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## Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Herrn Prof. Dr. G. Winter betreut.

## Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig und ohne unerlaubte Hilfe angefertigt.

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**To my parents,**

**for their enduring love**

# List of Abbreviations

| | |
|---|---|
| BSA | bovine serum albumin |
| DOPC | 1,2-Dioleyl-sn-Glycero-3-Phosphocholine |
| DSC | differential scanning calorimetry |
| G-CSF | granulocyte colony stimulating factor |
| HLB | hydrophilic-lipophilic balance |
| HP-ß-CD | hydroxypropyl-ß-cyclodextrin |
| HPLC | high performance liquid chromatography |
| IFN $\alpha$-2a | interferon $\alpha$-2a |
| MW marker | molecular weight marker |
| NIRS | near infrared spectroscopy |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | pH 7.4 isotonic 0.01 M phosphate buffer |
| PBST | pH 7.4 isotonic 0.01 M phosphate buffer with 1 % polysorbate 80 |
| PCR | principle component regression |
| PEG | polyethylene glycol 6000 |
| PEGFs | polyglycerol ester of fatty acids |
| Ph.Eur. | European Pharmacopoeia |
| pI | isoelectric point |
| PLA | poly(D,L-lactide) |
| PLGA | copolymers of lactic and glycolic acid |
| PLSR | partial least square regression |
| RP-HPLC | reversed phase HPLC |
| rpm | rounds per minute |
| RT | room temperature |
| SD | standard deviation |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SE-HPLC | size exclusion HPLC |
| SEM | scanning electron microscopy |
| SEP | standard error of prediction |
| WAXS | wide-angle X-ray scattering |

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# Chapter I: Introduction

Over the last two decades peptide and protein pharmaceuticals gained significant importance in the treatment of several severe diseases including autoimmune diseases, memory impairment, hormonal disorders and different cancers. Major advantages of protein pharmaceuticals are both their extremely specific activity and their high tolerability. With the advance of recombinant DNA technology the large scale production of peptides and proteins for pharmaceutical purposes has become feasible, but the number of protein pharmaceuticals available on the market still lags behind this achievement.

The reasons for that are multiple: an inherent physical and chemical instability of most proteins, which is associated with relevant difficulties in purification, storage and delivery, and the problem of making protein drugs access their target sites at the right time and for adequate duration [213; 184].

Though a number of innovative oral protein delivery approaches have been developed – i.e. drug entrapment within liposomes and nanoparticles or the covalent coupling to carriers like vitamin $B_{12}$ - , one major technical challenge for the pharmaceutical scientist still is to overcome the enzymatic barrier of the gastrointestinal tract [118]. In other words, protein pharmaceuticals are administered traditionally through parenteral injection. Unfortunately, most of these proteins are only therapeutically useful when following a therapeutic regimen of frequent injections or intravenous infusions throughout a long period. This leads to poor patient compliance, side effects, and cost-consuming hospitalisation [184].

In this realm, non-invasive administration methods such as the pulmonal route [196] as well as the transdermal and the nasal application ways could provide major benefits [10]. Nevertheless, the development of injectable, sustained and controlled release systems can be regarded as the most promising strategy in protein delivery. By slowly releasing the protein, these systems virtually act infusion-like.

Thus, key advantages of those systems can be [150; 42]:

- reduced injection frequency, associated by an improved patient compliance
- increased efficiency due to long-term blood levels
- decreased adverse reactions and side effects
- passive and/ or active targeting
- cost savings

Injectable depot delivery systems can be divided into four major groups: implants, microspheres, nanospheres, and injectable depot solutions, many of them based on various matrix materials. The research on synthetic biodegradable polymers such as poly(D,L-lactide) (PLA), copolymers of lactide and glycolide (PLGA) and polyanhydrides led to FDA approval of several sustained release formulations for peptides [184]. Though, the delivery of proteins proves to be more difficult. Despite a considerable amount of research only one protein-containing polymeric device gained FDA approval in 1999. However, this year Genentech and Alkermes announced their decision to discontinue the commercialisation of Nutropin Depot$^{TM}$, which is composed of PLGA microparticles loaded with recombinant human growth hormone [99]. This limited success may be caused by drawbacks synthetic polymer-based systems can inhere. During manufacturing and after administration, parameters like shear forces, interface formation, acidification and protein-polymer interactions can result in protein denaturation and aggregation [130; 68; 176; 198]. Besides, the issue of residual organic solvents remains unsolved [219].

By these latest scientific findings, interest in the evaluation of biocompatible natural materials as alternative to synthetic polymers increased [104; 188; 211]. Nonetheless, the effort to establish lipids such as triglycerides and monoglycerides as controlled delivery systems for protein drugs has been neglected in the last years.

Lipid matrix materials exhibit several potential advantages in comparison to polymers, i.e. high biocompatibility, simple manufacture by compressing or moulding. Furthermore, the slower water uptake after administration may result in a less detrimental environment for incorporated proteins [153; 201]. Consequently, this somewhat shadowy existence should not persist any longer.

## 1. Fundamentals of protein stability

### 1.1 Protein structure

In spite of the enormous number of naturally occurring proteins, a mere of 20 amino acids construct proteins. The vast difference in the three-dimensional structure and, therefore, also in protein function originates solely from a different amino acid sequence, i.e. the unique structure of a protein is determined by the chemical and physical properties of the amino acids aligned within the protein sequence.

Protein structure can principally be described at four different levels. *The primary structure* refers to the linear arrangement of amino acid residues along a polypeptide chain and to the location of covalent bonds, such as disulfide bonds, between chains or within a chain. *The secondary structure* describes the folding of parts of these chains into regular, ordered structures like $\alpha$-helices and $\beta$-sheets. Furthermore, areas with increased flexibility – the so-called turns or loops – are to be subsumed in this level of protein structure. The domains of the secondary structure and all non-covalent interactions such as hydrogen bonds and hydrophobic, electrostatic, or van der Waals interactions generate the intrinsic, three-dimensional arrangement of a protein, *the tertiary structure* (Fig. 1). The association of secondary structures shield a substantial fraction of the non-polar amino acid residues from solvents due to an embedding within the protein molecule interior. Some proteins consist of several polypeptide chains. Finally, *the quaternary structure* characterises the non-covalent interactions binding these chains into a single protein molecule. For example, haemoglobin consists of four polypeptide chains, which are associated by one $Fe^{2+}$ ion.

The retention of the tertiary structure is deemed the primary requirement for the biological activity of protein molecules [41]. However, the biochemical and structural complexity of these molecules is the reason for proteins to react sensitive to even marginal changes in their natural environment.

## 1.2 Chemical instability

Some amino acid side chains are chemically reactive, whereas others are chemically inert. It has been demonstrated that “labile” amino acid residues are susceptible to covalent modifications via bond formation or cleavage through non-enzymatic reactions, including [119]

- hydrolysis
- deamidation
- oxidation
- racemisation
- ß-elimination
- disulfide exchange.

## 1.3 Physical instability

Mentioning physical instability of globular proteins denotes that these super structured molecules can undergo changes independent of any chemical modification. The loss of tertiary structure, and frequently also of secondary structure is generally referred to as denaturation of the protein.

### *1.3.1 Denaturation, aggregation and adsorption of proteins*

The folded state of proteins is connected to conformational stability, which is expressed as the free energy change △G during the unfolding/denaturation reaction under physiological conditions [142]. The higher the △G value, the greater the stability of the protein. However, the reported △G values for proteins of 45 +/- 15 kJ/mol [92] indicate that the folding state is only marginally more stable than the denatured state. As a matter of fact, the conformational stability of a protein in aqueous solution tallies with only a few H-bonds or ion pairs [213].
Simplified, protein denaturation can be described as:

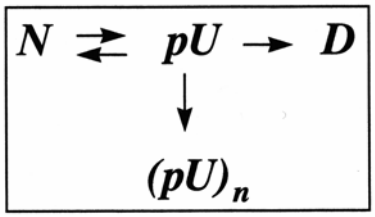


**Figure 1: Equation of the native and denatured state of a protein [176].**

The native state N exists in an equilibrium with a partially unfolded state pU. This unfolding of the native protein can be reversible, e.g. an increase of the temperature causes unfolding, which can be reversed by a subsequent temperature decrease [123]. Generally, the loss of the tertiary structure implies an increase in the protein molecule's reactivity. Hydrophobic regions, which were accumulated in the core of the folded protein, are then exposed to surrounding solvents. As a consequence, side reactions can now lead to an irreversible, denatured state D. Alternatively, partially unfolded proteins may encounter irreversible aggregation $(pU)_n$. Constantly elevated temperatures, extreme pHs, the formation of interfaces during shaking, shearing, adsorption to hydrophobic surfaces, high pressure, and denaturants such as urea and GdnHCl foster irreversible transitions [197; 91; 63; 122].

Protein aggregation is defined as the association of at least two denatured protein molecules. Non-covalent aggregation is caused by interactions between the exposed hydrophobic residues of denatured protein molecules. Initially, the formation of soluble aggregates occurs. With increasing numbers of molecules the solubility of these species will decrease, eventually resulting in the precipitation of the protein. Covalently linked protein aggregates are due to chemical reactions, e.g. ß-elimination, disulfide exchange or transamidation.

Protein aggregation is to be considered as major event of physical instability and consequently as major problem during the development of protein pharmaceuticals [213]. Several human diseases such as Parkinson's or Alzheimer's are linked to protein aggregation [1]. The presence of aggregates in therapeutic protein pharmaceuticals can cause adverse effects within patients, ranging from immune response to anaphylactic shock [20]. Since 1998, there has been a significant increase of the number of kidney patients who developed pure red cell aplasia during the course of epoetin $\alpha$ treatment (Eprex$^{TM}$/Erypo$^{TM}$), a version of human recombinant erythropoietin, due to neutralising antibodies. The increase in reported cases coincides with the removal of human serum albumin from the original formulation. It has been proposed that the new formulation is less stable, allowing aggregates of erythropoietin molecules to form, which increases the probability of antibody formation [33].

A further particularity playing a key role in the formulation of protein pharmaceuticals is the adsorption of proteins upon various surfaces and interfaces, e.g. container surfaces, membrane filters or air-water interfaces. Proteins are large amphiphilic molecules, meaning they are intrinsically surface-active. Once adsorbed, proteins can undergo different levels of orientation/conformation changes resulting in a protein loss and/or in unfolding and aggregation processes [4]. Here, one major parameter influencing the extent of adsorption seems to be the protein concentration, reaching a maximum above a certain concentration-surface ratio [80].

## 2. Polymeric systems

The first controlled-release technology was developed in 1962 and was based upon diffusion of small molecules (< 500 – 1000 Da) through the wall of silicone rubber tubing [65]. More than a decade later, Davis et al. reported the sustained

release of various peptides and proteins from polymeric devices. Crosslinked polyacrylamide and polyvinylpyrrolidone gels have been subcutaneously implanted into hamsters to deliver immunoglobulins, luteinizing hormone, bovine serum albumin, or insulin over a prolonged period. It was shown that the release rate declined with increasing polymer concentrations, enabling the sustained release of luteinizing hormone in vivo for over 50 days [52]. However, the use of those polymers was accompanied by a high inflammatory potential in animal tissues [114].

In 1976, Langer and Folkman published that proteins and other large molecules with molecular weights up to 2 x $10^6$ Da can be liberated from hydroxyethylmethacrylate (Hydron) and ethylene-vinyl acetate polymeric devices, respectively. The development of so-called “sandwich” pellets facilitated a three months steady release of soybean trypsin inhibitor, lysozyme and catalase. Furthermore, the biological activity of the delivered proteins was verified [114; 115; 66]. In the next years Langer and his co-workers improved the reproducibility of the protein delivery by new incorporation techniques, e.g. solvent casting or sintering. Furthermore, mathematical models were established in order to clarify the underlying release mechanisms [182; 88]. However, one major problem persisted: the polymer systems were non-biodegradable and required a surgical removal after drug delivery was complete. A non-removal often went along with toxicological problems.

As a consequence the search for appropriate materials in the past two decades was focused on biodegradable polymers. Biodegradable means that the polymer device degrades over time due to hydrolysis (non water-soluble polymers) or solubilisation (water-soluble gels). Concomitantly, the drug is released. Potential degradation products can be absorbed by the body, obviating the need for surgical removal.

In this respect, biodegradable matrices must meet the following characteristics: high tolerability by the body, no interaction with the incorporated protein, reproducible in vivo degradation, constant drug diffusion rates, no toxicity, and rapid metabolism of the degradation products [159]. Table 1 lists natural and synthetic polymers that have been investigated for the delivery of proteins and peptides.

| Material | Degradation mechanism |
|---|---|
| *Natural* | |
| Starch | Amylase |
| Alginate[b] | pH, enzymes |
| Collagen (gelatin) | Collagenase |
| Proteins (cross-linked albumin) | Enzymes |
| Tricalcium phosphate[c] or calcium carbonate (hydroxyapatite) | Dissolves over time |
| *Synthetic* | |
| Hydrogels | Chemical or enzymatic hydrolysis, solubilization in aqueous media |
| Polyanhydrides | Hydrolysis |
| Polyesters (polylactides) | Ester hydrolysis, esterases |
| Poly(ortho esters) | Ester hydrolysis, esterases |
| Polyiminocarbonates | Hydrolysis |
| Polycaprolactones | Hydrolysis |
| Polyamino acids | Enzymes |
| Polyphosphazenes[b] | Hydrolysis, dissolution |

**Table 1: Biodegradable materials for controlled delivery of proteins or peptides [42].**

Natural polymers like alginate materials are water-soluble, whereby the protein drugs can be embedded without the use of organic solvents or elevated temperatures. These mild conditions can reduce degradation or inactivation effects on protein molecules during manufacturing. However, the risks of immunogenicity and contamination often restricts a broad use of these materials as drug delivery matrices [42; 184].

Scanning the literature on the field of synthetic, biodegradable polymers used as controlled release systems reveals that in recent years research has been focused on polyanhydrides and especially on polyesters. In other words, polyesters, in particular esters prepared from poly(lactic acid) and/or poly(glycolic acid) – i.e. poly(D,L-lactide) **(PLA)**, poly(glycolic acid) **(PGA)** and copolymers of lactide and glycolide **(PLGA)** – are the biodegradable polymers investigated most widely for controlled drug delivery.

## 2.1 Controlled release systems based on PLGA

Polymer devices for controlled protein delivery can be configured in several forms, including small microspheres or implants of various shapes and sizes.

*Microparticles* are usually defined as spherical devices within a 1 - 1000 µm range. For parenteral use it is aimed for sizes ideally less then 125 µm. In a general sense, microparticles can be further divided into microcapsules, i.e. microparticles containing the drug in a central core, surrounded by a polymeric membrane, and microspheres, i.e. microparticles containing the drug in a polymeric matrix. Microparticles can be injected readily subcutaneously or into the target site. The standard encapsulation method for protein drugs is the double (multiple) emulsion technique including a subsequent evaporation or extraction step. Further established techniques are phase separation (coacervation), spray drying, or microparticle formation using supercritical fluids [93].

*Implants* are cylindrical, monolithic devices implanted by a minor surgical incision or injected through a large bore needle (trocar) into the subcutaneous tissue. Implants are commonly manufactured by extrusion or solvent casting techniques, generating implants with millimetre and centimetre dimensions. Implants cannot be applied in therapy regimen which require a weight-based dosing, e.g. x milligrams of drug per kilogram of body weight.

The family of homo- and copolymeric systems based on lactic and glycolic acid has gained considerable attention due to the ease of fabrication, the FDA approval for use in humans and due to the successful use for several decades in biodegradable sutures [78]. However, the availability of these polymeric delivery systems for protein drugs was not accompanied by a corresponding output – meaning the work invested does not mirror in a huge number of products marketed in pharmaceutics (Tab. 2). This allows the conclusion that the polymers available hitherto are deemed a restricted-use stock where the scientists can choose from when needing device materials for protein delivery.

In the past few years incomplete release of native protein as a result of protein instability phenomena was identified as a major problem. Considerable drawbacks synthetic polymer-based systems can inhere will be outlined in detail in the following parts.

| *Drug* | *Trade name* | *Company* | *Polymer* | *Route* | *Application* |
|---|---|---|---|---|---|
| buserelin acetate | Profact®Depot, Suprefact®Depot | Hoechst Marion Roussel | PLGA | s/c implant | prostate cancer |
| goserelin acetate | Zoladex®Depot | Astra Zeneca | PLGA | s/c implant | prostate cancer, endometrioses |
| leuprorelin acetate | Lupron® Depot, Enantone® Depot, Enantone® Gyn Depot<br>Trenantone® | Takeda-Abbott | PLGA<br>PLA | 3-month depot suspension, 1-month suspension<br>3-month suspension | prostate cancer, endometrioses |
| octreotide acetate | Sandostatin LAR®Depot | Novartis Pharma | PLGA | s/c suspension | GH suppression, anti cancer |
| triptorelin | Decapeptyl® Depot | Debiopharma | PLGA | s/c depot injection | LHRH agonist, prostate cancer |
| recombinant human growth hormone | Nutropin®Depot, [discontinued commercialisation since 06/2004] | Genentech-Alkermes | PLGA | monthly s/c injection | growth hormone deficiency |

**Table 2: Overview of peptide/protein controlled, release systems based on PLGA.**

### *2.1.1 PLGA microspheres: manufacture and protein stability issues*

#### *2.1.1.1 Double (multiple) emulsion technique*

During the past 25 years considerable work was spent on the investigation of microencapsulation of pharmaceutical compounds with PLGA via solvent evaporation technique (conventional O/W encapsulation). However, for water-soluble drugs like peptides and proteins this method appeared to be insufficient. In other words, the formation of an O/W emulsion leads to a rapid diffusion of the drug from the organic phase into the aqueous phase resulting in microspheres with low or even no drug loading at all [135]. In case the solvent evaporation technique is modified by the formation of multiple W/O/W emulsions (Fig. 4), the preparation of PLGA microspheres with higher drug loads and a more efficient encapsulation is rendered possible [85]. This was demonstrated for leuprorelin acetate - PLGA microspheres, where the application of the double emulsion method led to FDA approval in 1992; and Lupron® depot became the first peptide microparticulate depot product entering the U.S. market [137; 42].

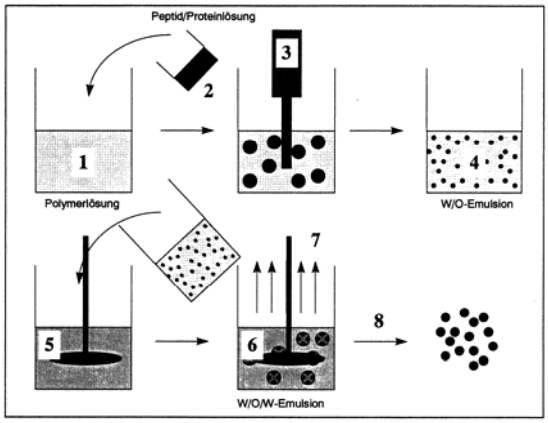


**Figure 2: Schematic illustration of the double (multiple) emulsion method, a technique widely used for the PLGA microspheres preparation.**

As illustrated in Fig. 2, an aqueous protein solution (2) is added to an organic polymer solution (1) – i.e. polymer dissolved in methylene chloride or ethyl acetate - followed by an emulsification step using either high speed homogenisers or sonicators (3). This primary W/O-emulsion (4) is then rapidly transferred in a second aqueous phase containing a stabiliser, usually polyvinylalcohol (5). The formation of the W/O/W emulsion is obtained by stirring or vortexing (6). To ensure the entrapment of the protein and to remove the organic solvent (7), additional water is added resulting in hardened microspheres (8). Another possibility for organic solvent removal is the extraction with a water-miscible solvent (solvent extraction). The washed and collected microparticles are dried by vacuum drying or lyophilisation. A modified technique implies the addition of the protein in solid form – then, the suspension is transformed to an S/O/W system.

At first glance this method appears simple, but scrutinising the technique clearly indicates that several complex procedures own potential to imperil protein stability during microparticles manufacture.

*Effect of the formation of large water/organic solvent interfaces*

As surface active molecule, a protein tends to adsorb at water/organic solvent interfaces. This adsorption step can induce protein unfolding, inactivation, and irreversible aggregation during the first emulsion step [165; 147; 49]. Morlock et al. demonstrated the formation of the primary emulsion being mainly responsible for the occurrence of erythropoietin (EPO) aggregates during manufacture. All the following

steps did not increase the total amount of detected EPO aggregates [130]. These findings were substantiated by van de Weert et al., revealing the non-covalent aggregation of lysozyme by means of Fourier-Transform Infrared Spectroscopy (FTIR), whereby the non-recovered lysozyme was found at the water/organic solvent interface as white precipitates [197]. The extent of protein loss due to interface adsorption and subsequent aggregation mainly depends on the protein per se, e.g. 38 % recovery was found for ovalbumin, 72 % for lysozyme, and 99 % for bovine serum albumin (BSA), applying identical emulsion conditions [166].
During the microparticle formation interfacial stress may be detrimental to protein molecules positioned at the surface of the growing particles [198]. In addition, a potential protein loss due to diffusion of protein from the inner aqueous phase into the outer aqueous medium can be observed [14].

*Effect of the emulsification process*

During the emulsification process protein molecules are exposed to shear and cavitation stress. The way how the primary emulsion is prepared – for instance via homogenisation or ultrasonication - can have a considerable impact on the protein denaturation rate. Morlock et al. showed the aggregation of erythropoietin being more pronounced when ultrasonication or vortexing is used for the emulsification. The ratio of EPO aggregates could be substantially reduced by the application of homogenisers [130]. Also, sonication may induce an increase in temperature (hot spot) and the formation of free radicals resulting in protein impairment [198; 108].
Generally, hydrophobic interactions between protein and polymer during emulsification may foster protein unfolding and aggregation [102].

*Effect of Organic solvents*

After the addition of the aqueous protein solution to the organic polymer phase, organic solvent molecules diffuse into the aqueous phase to a certain extent, e.g. 2 % of methylene chloride migrate into an aqueous phase. The organic solvent can bind directly to the protein by hydrophobic interactions or can alter the ionic strength conditions inside the aqueous medium, fostering the destabilisation of protein molecules [109]. Cleland et al. showed that the destabilising effect of the organic solvents mainly depends on two factors: (1) how is the protein incorporated

(aqueous phase or solid protein?) and (2) which organic solvent is chosen for the dissolution of the polymer. The recovery of human growth hormone was noticeably increased by using ethyl acetate rather than the commonly applied methylene chloride as dissolution medium or by adding solid protein to the methylene chloride phase. In solid form, the conformation of a protein is severely restricted, and the protein can be stabilised in a water-free organic solvent. However, even a small amount of water within the organic phase results in an increase of protein mobility, thereby inducing protein denaturation tendency. In other words, the detrimental effect of organic solvents on protein stability mainly depends on the amount of water present inside the organic medium [45; 109].

A further critical parameter to be outlined is the content of residual solvents within the microspheres after drying. Methylene chloride is classified by the international conference of harmonisation (ICH) as toxic organic solvent whose application should be constricted. The concentration in pharmaceutical products is limited to 600 ppm [219], and a pharmaceutical product can suffer FDA rejection due to an exceeding residual organic solvent content.

*2.1.1.2 The phase separation technique (coacervation)*

Generally, the protein is suspended into a dilute polymer solution, e.g. methylene chloride. Silicone oil is usually added to the suspension acting as phase inducer. As a result the polymer is subjected to phase separation, encapsulates the drug and `embryonic` microspheres are formed. This system is then transferred into a second non-solvent to harden and form the final microparticles. After repeated extraction steps, microparticles are collected and dried.

The major advantage of the phase separation technique is the avoidance of the contact between protein molecules and an aqueous phase. For instance, the non-aqueous microencapsulation procedure revealed a significant decrease in bovine serum albumin (BSA) covalent aggregation [31]. However, the additional solvents are often difficult to remove and their residue limits within pharmaceutical products are often exceeded.

*2.1.1.3 (Cryogenic) spray drying*

Spray drying is a rapid, convenient technique which can be conducted under aseptic conditions. First, a polymer – prevalently PLGA is applied - is dissolved in a volatile organic solvent such as methylene chloride or acetone. The protein is suspended as solid or emulsified as aqueous solution in this organic solution by homogenisation. After that, the resulting dispersion is atomised through a (heated) nozzle into a heated air flow. The organic solvent evaporates, thereby forming microparticles with dimensions of typically 1 – 100 µm. The microparticles are collected in a cyclone separator. For the complete removal of the organic solvent, a vacuum drying or lyophilisation step can follow downstream.

Morlock et al. demonstrated the successful use of a modified spray drying technique for the encapsulation of EPO within PLGA microspheres. By this, a reduced amount of EPO aggregates was detected when compared to EPO microspheres prepared by a standard W/O/W emulsion method. Moreover, the encapsulation efficiency rose due to obviating protein diffusion processes during emulsification [14].

One variation of the conventional spray drying method, which is subsumed to the cryogenic technique, uses very cold temperatures for freezing biologically active agents into polymeric microspheres (Fig. 3). A protein/PLGA organic solvent dispersion is atomised into a vessel containing a liquid non-solvent such as ethanol that is overlaid with a liquefied gas phase, usually nitrogen, at temperatures below the freezing point of the protein/polymer mixture. During evaporation of the liquid nitrogen, the non-solvent melts, followed by an extraction of the organic solvent from the formed microparticles [76; 75]. Besides the advantage of low temperature conditions, this method completely avoids the use of water during manufacture (non-aqueous encapsulation).

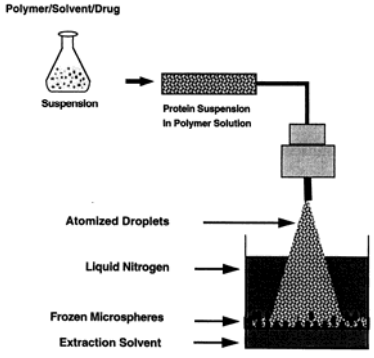


**Figure 3: Cryogenic spray drying process [195]**

This technology has been effectively applied to the encapsulation of zinc-complexed human growth hormone in PLGA microspheres, resulting in a one-month effect after one single injection [99; 100]. The investigations in that realm yielded FDA approval of one unique protein-containing PLGA formulation. However, the commercialisation of Nutropin® Depot is discontinued since June 2004.

*2.1.1.4 Techniques using supercritical fluids*

Generally, the application of supercritical (SC) fluids for the encapsulation of peptides and proteins has been fueled by the recognition that the established methods implicate some drawbacks. The application of supercritical fluids, especially of supercritical carbon dioxide, can minimise or even eliminate the use of organic solvents and renders work at moderate temperatures possible [155]. The term “supercritical” defines the area above the critical point, which specifies the final point of the liquid-gas phase transition curve. Beyond that critical point, isobar/isotherm alterations of pressure or temperature alter the density of the critical phase, but do not lead to a separation into two phases. A density change is directly associated with a change of the solvent power, thus the method features a high variability. Usually carbon dioxide is used as supercritical fluid due to its critical point ($T_c$ = 31.1 °C, $P_c$ = 73.8 bar), which can be easily reached. That allows a moderate working temperature and leaves no toxic residues since it returns to the gas phase at ambient conditions. Two SC $CO_2$ based processes have been reported for the preparation of drug-loaded polymeric microspheres: first, the rapid expansion from supercritical solutions (RESS) process, whereby a SC $CO_2$ solution of an active agent and a polymeric carrier is rapidly expanded. This quickly transforms the SC $CO_2$ into a liquid that is a much poorer solvent, thereby precipitating the active agent/carrier mixture as small particles [53; 222]. Second, the aerosol solvent extraction process, also referred to as antisolvent process. Here, a solution of the active agent and the polymeric carrier is sprayed into a chamber loaded with SC $CO_2$. The SC $CO_2$ extracts the solvent from the spray droplets, and induces co-precipitating of the active agent and the polymeric carrier in form of small, solvent-free particles [215; 15]. However, the use of organic solvents can not be avoided, which is to be deemed as a major disadvantage of both techniques.

In protein pharmaceutics, the antisolvent technique is predominantly applied for the preparation of microparticulate protein powders as an alternative to common drying processes. However, Winters et al. reported an increase of ß-sheet aggregates during the precipitation of lysozyme, trypsin and insulin as a consequence of stress parameters such as organic solvent, pressure and shear forces [218]. One reason why these methods were not credited as encapsulation techniques for protein within PLGA may be the tendency of several polymers to rapidly precipitate and agglomerate during the process [18].

### *2.1.2 PLGA implants: manufacture and protein stability issues*

While countless publications are describing the manufacture of microparticulate systems, literature dealing with PLGA implants delivering proteins is yet sparse.

With the solvent casting method, the polymer is dissolved in a volatile organic solvent, e.g. methylene chloride or acetone, and the solid protein is suspended within the solution by homogenisation. This mixture is poured into moulds with a definite size, and the organic solvent evaporates slowly. Garcia et al. reported the development of biodegradable laminar implants for recombinant human growth hormone (rhGH) using the solvent casting technique. Thereby, a PLGA solution in methylene chloride was poured onto the lyophilised cake of rhGH and methylene chloride evaporated slowly at 4 °C during 48 hrs. The resulting rhGH polymer-film was vacuum dried and then cut into discs of 6 mm in diameter [70].

A modified solvent casting technique was established by Zhu and Schwendeman, who filled a BSA/PLGA suspension in a syringe and extruded the mixture into a 0.8 mm silicone tubing at a rate of about 0.1 mL/min [226; 225].

An injection-molding process was exemplified by Rothen-Weinhold et al.. The peptide/polymer mixture was heated at 110 °C for plasticising, followed by injection at 100 °C and 130 bar pressure. The obtained implants revealed a diameter of 4.6 mm and a length of 2.8 cm [161].

Extrusion processes are the most convenient way for implant manufacturing. *Screw extruders* consist of single or twin helical rotating screws inside a stationary cylindrical barrel. By definition, the extruder is divided into three sections: feed zone, transition zone, and metering zone. Both, the polymer and solid protein are

transported to the transition zone where they are mixed, pressed, melted and eventually plasticised. In the metering zone the plasticised mixture is extruded through a die, and simultaneously the temperature decreases. By using *ram extrusion,* the protein/polymer mixture is filled into a barrel into which a piston rod is inserted and then moved further into the barrel with apt pressure. The extrusion temperature is usually set at 80 °C – 110 °C and the mixture is compressed through a die with diameters around 1.5 mm [190].

The manufacture of the goserelin acetate implant (Zoladex®), available on the market since 1987, is realised by an extrusion technique. The drug and the polymer material are dissolved in a capable solvent followed by a lyophilisation step. This protein/polymer cake is then formed into implants by extrusion and the implants are sterilised by γ-irradiation [89; 90].

Deghenghi et al. described the extrusion manufacture of polymeric implants containing bioactive peptides as follows: the sterilised and ground PLGA copolymer is wetted with a sterile aqueous slurry of peptide. Then, this composition is homogenised and dried at reduced pressures and at temperatures below 25 °C. Subsequently, the peptide/polymer mixture is extruded at temperatures between 70 and 110 °C and the obtained cylindrical rods are cut into the pharmaceutical implants [54]. A similar extrusion technique was shown by Marion et al.. The solid peptide and dry PLGA were mixed homogeneously and then granulated by adding a suitable liquid such as ethanol. This mixture was extruded at a temperature profile ranging from 30 °C at extruder entering up to 110 °C at extruder leaving. Under these conditions the PLGA melts, and forms a continuous matrix which coats the peptide particles [124].

*Effects of the manufacturing processes*

During implant preparation, peptides and proteins are exposed to various unfavourable conditions. The exposure to high temperatures, shear forces, or high pressures may cause protein unfolding and irreversible aggregation. Rothen-Weinhold et al. showed the purity of the somatostatin analogue vapreotide rapidly decreasing at high temperatures during ram extrusion. This effect is increased with process time [160]. In a later work, they demonstrated the main peptide impurity

being a lactoyl lactyl-vapreotide conjugate that originated due to a covalent bonding between free lactide and a phenylalanine residue [162].

### *2.1.3 Protein stability issues during release*

Two mechanisms control the release of proteins from biodegradable polymeric devices, e.g. PLGA. The first mechanism is the simple diffusion of the protein out of the polymer matrices. Initially, protein molecules located at or close to the surface of the matrices diffuse rapidly out of the matrices (responsible for the often cited “burst effect”), then a slower diffusion of the protein from the interior of the polymeric system through porous channels follows. The other mechanism is the erosion of the polymeric matrix which is initiated by the degradation of the polymer backbone (chain cleavage). As the drug surrounding polymer erodes, the drug escapes. An erosion/ diffusion controlled release mechanism represents a possible combination of both processes [129; 42; 78; 72].

#### *2.1.3.1 Surface erosion versus bulk erosion*

Polymer erosion is defined as the mass loss of the polymer from one initial level. The erosion of “erodible” polymers commences with the degradation of the polymer backbone due to hydrolysis of the ester bonds. Erosion is accompanied by a decrease of the average molecular weight and an increase of polymer porosity due to the release of the degradation products, i.e. oligomers and monomers [72]. Degrading polymers are classified into *surface-eroding* and *bulk-eroding* polymers (Fig. 4).

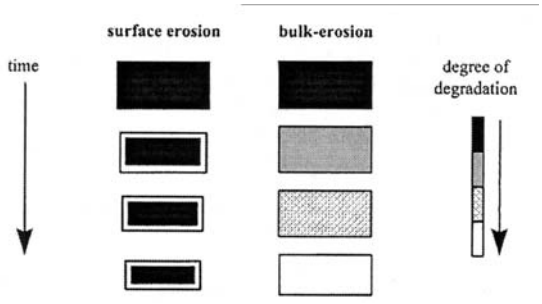


**Figure 4: Pattern of the changes a polymer matrix undergoes during surface and bulk erosion [204]**

Erosion kinetics depend on two major factors: the diffusion of the water into the polymer bulk and the degradation rate of the polymer backbone. If the degradation of

the polymer backbone is faster than the diffusion of water into the polymer, water will be consumed mainly on the surface by hydrolysis and will thus be prevented from diffusion into the matrix. This phenomenon is defined as *surface erosion*, though only fast degrading polymers such as polyanhydrides and poly(ortho)esters undergo surface erosion.

If the diffusion of water is faster than the degradation rate of the polymer backbone, the complete matrix is wetted. The degradation is then not confined to the polymer surface, and the matrix system undergoes *bulk erosion*. Usually, biodegradable polymers, especially PLGA matrices, underlie this bulk erosion phenomenon [204; 72].

### *2.1.3.2 Consequences of bulk erosion on protein stability*

The rapid water uptake of PLGA matrices results in a complete morphology change of the polymer from a glassy to a rubbery state by a lowered glass transition temperature [78]. In addition, the polymer matrix becomes more hydrophilic, and degradation products may alter the pH in the interior of the polymeric system. Thus, the incorporated protein is confronted to completely different environmental conditions, which may lead to conformational changes followed by irreversible denaturation or aggregation. An overview of potential detrimental consequences of the bulk erosion is shown in Fig. 5.

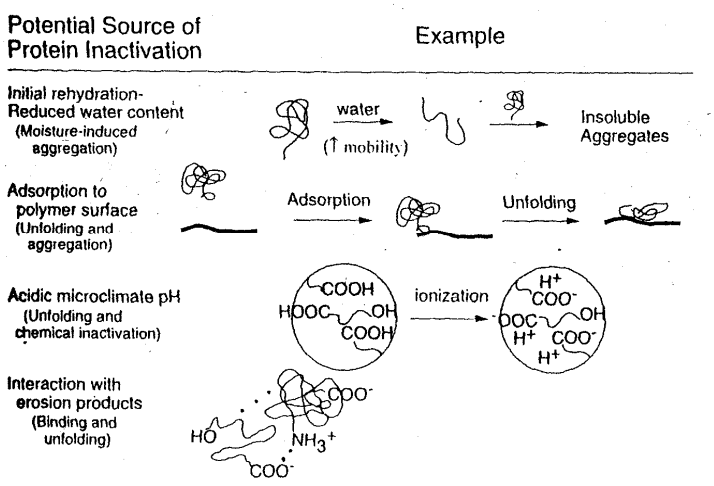


**Figure 5: Potential sources of protein inactivation after administration [176].**

*Protein rehydration – moisture-induced aggregation*

As the polymeric matrices become hydrated the solid protein is exposed to increasing moisture, which can lead to irreversible aggregation (moisture-induced aggregation). The presence of water increases protein mobility, possibly accelerating chemical reactions as well as physical instability events [79; 48; 121; 130].
The moisture-induced solid aggregation has been described as a result of thiol-disulfide interchange. This process preferentially proceeds under basic conditions [121], implying moisture-induced aggregation being impeded in the acidic environment of the hydrated polymer systems. However, the formation of non-covalent aggregates induced by moisture was documented in the case of insulin also under acidic conditions [48].

*Changes of pH and of osmotic pressure within the polymeric matrices*

The degradation products of PLGA are of acidic nature, which leads to a pH drop. If these degradation products can not freely diffuse out of the matrices, an acidic microclimate within the polymeric devices is generated. In addition, the accumulation of monomers and oligomers increases the osmotic pressure within the matrices. Both an acidic microclimate and a high osmotic pressure are potential sources for unfolding and aggregation of encapsulated proteins [22; 14; 130; 147].
The evidence of an acidic microclimate has been demonstrated by confocal laser microscopy measurements and by electron paramagnetic resonance analyses, respectively. pH values between 1.5 and 4.7 were reported [68; 22]. Zhu et al. published encapsulated BSA undergoing peptide bond hydrolysis and non-covalent aggregation during release due to an acidic environment (pH < 3) [225].

*Adsorption of proteins to polymers*

Non-covalent and ionic interactions between PLGA and encapsulated proteins were stated as a reason for an incomplete protein release by Park and co-workers [106; 50; 144]. Extraction of non-released protein was performed by separately adding 0.5 M sodium chloride, 5 M guanidine hydrochloride (GnHCl), and 5 mM sodium dodecyl sulphate (SDS) to the extraction medium. Sodium chloride extracts protein molecules which interacted electrostatically with PLGA. GnHCl dissociates non-covalent aggregates, and SDS extracts both non-covalent aggregates and

proteins adsorbed to PLGA. The difference in the amount of extracted protein allows to calculate the loss of protein due to non-specific adsorption to the polymer matrix. With this experimental set-up it could be proved that an incomplete release of BSA was mainly due to non-specific adsorption, as GnHCl could not extract this protein [50]. In comparison, recombinant human growth hormone is largely lost due to non-covalent aggregation - no higher amount of protein could be extracted when using SDS [106]. Lysozyme interacts electrostatically when encapsulated in uncapped PLGA microspheres, whereas the incorporation in hydrophobic PLGA results in non-covalent aggregation and hydrophobic adsorption [144].

### *2.1.4 Protein stabilisation upon encapsulation and release from PLGA polymers*

#### *2.1.4.1 Protein stabilisation during preparation*

During the last years, several straightforward strategies towards protein stabilisation upon encapsulation were presented. These approaches are summarised in the following table.

| Stress factor | Stabilisation approach | Mechanism | Literature |
|---|---|---|---|
| water/organic solvent interfaces | **increase of protein loading** | **reduction of protein/interface ratio** | [45; 148; 165] |
| | **addition of other proteins** | **competition at interfaces** | [197; 166; 130] |
| | **addition of sugars, polyols, PEG, salts** | **mechanism of preferential hydration [8] or protein hydrophilization [131]** | [130; 102; 149] |
| | **avoidance of emulsification, addition of the protein as a solid (non-aqueous process)** | **no interfaces, restricted protein mobility** | [31; 100; 109] |
| **shear forces** | **avoidance of sonication, use of other homogenisation methods** | **absence of cavitation stress, no risk of free radicals formation** | [130; 108] |
| **organic solvent** | **replacement of methylene chloride by ethyl acetate** | **increased protein stability in ethyl acetate, less toxicological problems** | [45; 219] |
| | **avoidance of the impact of water, non-aqueous process** | **restricted protein mobility, protein mobility depends on the presence of water** | [31; 45] |

**Table 3: Protein stabilisation approaches during manufacture; table was created in accordance to van de Weert et al. [198].**

The success of zinc-complexed rhGH in combination with cryogenic spray drying as preparation method (Nutropin® Depot) offered new potential for protein stabilisation during encapsulation. Lam et al. showed that the formation of an insoluble rhNGF-zinc complex prior to encapsulation into PLGA microspheres stabilised the protein during both encapsulation and release [112].

#### *2.1.4.2 Protein stabilisation during release*

In addition to general proposals to improve protein stability in aqueous formulations [213], specific stabilisation approaches are normally required when protein molecules are confronted with the environment of degrading PLGA matrices (Table 4).

| Stress factor | Stabilisation approach | Mechanism | Literature |
|---|---|---|---|
| **moisture-induced aggregation** | **general strategy: increase of matrix hydrophobicity** | **inhibition of the bulk erosion phenomenon, reduced water uptake** | **[147]** |
| **acidification** | **addition of basic salts**<br><br>**increase of the porosity of the polymer matrices** | **buffering**<br><br>**enabling acidic degradation products to diffuse out and buffer components permeate into the matrices** | **[225; 207; 179]**<br><br>**[98; 40]** |
| **protein/PLGA contacts** | **addition of other proteins** | **competition for PLGA** | **[36]** |

**Table 4: Protein stabilisation approaches during release; table was created in accordance to van de Weert et al. [198].**

## 2.2 In situ formed devices

The traditional methods of preparing PLGA microparticles suffer from drawbacks such as (i) the need of reconstitution before application, (ii) the hazards and the environmental concern using methylene chloride for preparation, and (iii) the residual organic solvents remaining in the final product. PLGA implants did not have much commercial success, primarily due to requirements of a surgical incision or of a special type of injector [93].

To improve patient compliance, Shah et al. and researchers from Atrix Laboratories described in the 90´s a novel implant system for the delivery of macromolecules, which is parenterally administered as a liquid and that subsequently solidifies into a gel (matrix) *in situ.* The drug is released in a controlled manner [178; 59]. The

method was first patented as Atrigel® system for low molecular weight drugs, and is carried out as follows: preferably PLA/PLGA copolymers are dissolved by heating in a water-miscible, biocompatible solvent (e.g. N-methyl-2-pyrrolidone NMP, dimethylsulfoxide DMSO or triacetin), which may also act as a plasticiser. After cooling, the biologically active agent is either dispersed by homogenisation or dissolved in a solvent (e.g. PEG 400) miscible with the polymer solution. This polymer-solvent-drug system is highly viscous but still syringeable by a convenient syringe and needle. When the liquid composition is injected via the intramuscular or subcutaneous route, the solution diffuses in the body. After contact with aqueous buffer (in-vitro) or with the physiological fluid (in-vivo) the polymer precipitates and a semi-solid implant is formed [96; 60]. While later patents mainly addressed the variation of the plasticiser solution, Jain et al. demonstrated the preparation of microglobules, which form microspheres at the injection site. Fig. 6 illustrates the modified process of the in situ production of various biodegradable PLGA devices.

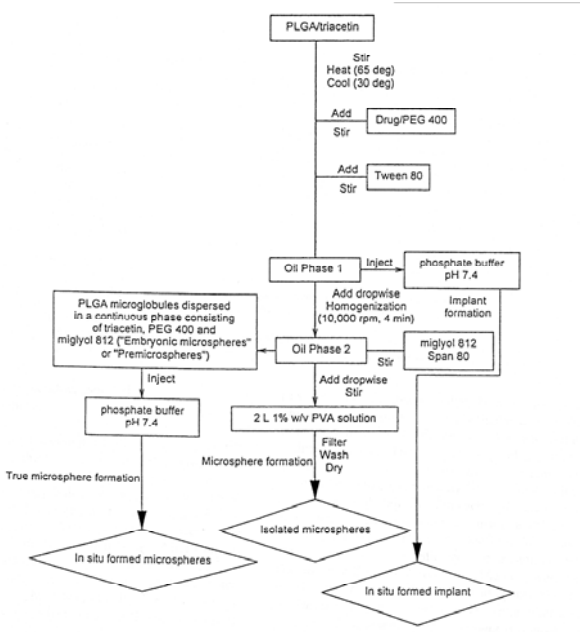


**Figure 6: Modified encapsulation process for various biodegradable PLGA devices in situ [96].**

The preparation of in situ microspheres exhibited a substantial increase in the encapsulation efficiency when compared to implant systems. Implants reveal a lag period between the injection of the liquid implant and the subsequent hardening, leading to a protein loss of about 50 % due to diffusion processes. In contrast, the

novel in situ microsphere process exhibited an encapsulation efficiency of 75 % and a controlled protein release for a 2 week period [94; 96; 95].

Using the Atrigel® technology resulted in the first drug approval a German biotechnology company achieved. Leuprogel®, marked by Medigene AG, combines standard hormone therapy with the patient-friendly and efficient Atrigel® depot system. The delivery device allows extended release of leuprorelin acetate for a period of one month [167; 146].

One other interesting possibility to apply an injectable protein delivery system in situ is the use of *sucrose acetate isobutyrate (SAIB).* Sucrose acetate isobutyrate is a highly lipophilic sugar derivative, which is currently used as stabiliser and emulsifying agent to human diets in the Food Industry. The so-called SABER™ technology was patented by Tipton and Richard (Southern Biosystems, Inc.) in 1995. The high viscosity of the liquid sucrose acetate isobutyrate carrier is lowered by the addition of a water soluble or miscible solvent such as ethanol or dimethylsulfoxide. After addition of the drug, the composition is injected and forms a highly viscous implant in situ, which releases the drug over time [194]. A major advantage in comparison to the in situ PLGA devices is the possibility to use ethanol as solvent, which is approved as co-solvent in parenteral products. In 2002/03, Cleland et al. at Genentech transferred the then established SABER™ technology to the field of sustained protein release. Zinc-complexed rhGH was suspended in SABER™ solutions, containing various portions of PLA and sucrose as additives. By varying the sucrose and PLA ratio, a sustained protein release could be achieved in vivo. However, histological examinations exhibited a local tissue response 7 days after administration which appeared to be very similar to that observed with PLA and PLGA systems [138; 46].

Other sustained release injectables formed in situ and based on poloxamer, glycerol monooleate or PEG-PLGA-PEG triblock polymers are currently under investigation. On the other hand, considerable efforts are made in developing marketable sustained-injectables formed in situ for the veterinary market [126].

## 2.3 Hydrogels

The first application of hydrogels in controlled delivery of macromolecules is ascribed to Davis et al. [52]. Hydrogels are three-dimensional, water-swollen, polymeric networks composed of hydrophilic homopolymers or copolymers such as hydroxyethyl methacrylate, methoxyethoxy methacrylate, vinylacetate and various PEG acrylates. Due to a multitude of chemical and physical crosslinks, these hydrogels are insoluble systems. Crosslinking provides the network structure and the physical integrity of these systems. Numerous biomedical and pharmaceutical applications are mainly due to their swollen and rubbery nature which resembles natural tissue more than any other class of synthetic biomaterials. In the last years, stimuli-sensitive swelling controlled release systems were developed. For example, pH-sensitive hydrogels contain either acid or basic pendant groups. In aqueous media with appropriate pH and ionic strength, the pendant groups can ionise, adopt fixed charges and result in an increased swelling. Temperature-sensitive hydrogels have gained in importance in the pharmaceutical field, e.g. for the application of biosensors due to their ability to swell or to contract, depending on temperature. A further advantage that has been recognised only recently is that hydrogels may protect peptides and proteins from the harsh environment in the vicinity of the release site. Thus, such carriers may be scrutinised on their applicability for oral protein delivery in the future. Furthermore, hydrogels appear to be applicable as targetable carriers or as bioadhesive devices for therapeutic agents [145; 116].

Recently, Hubbell et al. reported the development of a novel proteolytically sensitive, biologically active polyethylene glycol (PEG)-peptide hydrogel. This in situ formed device facilitates arterial healing, e.g. after balloon angioplasty by temporarily protecting the arterial injury from blood contact [177].

## 2.4 Surface erosion polymers: polyanhydrides and poly(ortho esters)

The bulk erosion phenomenon of PLGA systems has been verified as one major problem concerning protein stability issues (chapter I, *2.1.3.2*). However, the erosion process per se, which is confined predominantly to the surface layers, inheres some well-known advantages. First, if the drug is well immobilised in the matrix, its release is solely controlled by the erosion rate of the matrix. Second, drug release and erosion take place concomitantly. As a consequence, no matrix remains

if the drug is completely liberated. And third, hydrolysis products, which are generated at the surface of the matrix, diffuse away from the devices and do not accumulate in the bulk material. Thus, the interior of the matrix does not turn acidic as observed in PLGA systems, and, as a consequence, acid sensitive drugs such as proteins can be liberated without activity loss. After considerable efforts in the development of surface erosion polymers in the last years, two polymer families emerged: polyanhydrides and poly(ortho esters) [82].

### *2.4.1 Polyanhydrides*

Medical applications of polyanhydridic compounds were investigated by Langer and his group in the 1980`s, and FDA approval was obtained for the treatment of brain tumour with the chemotherapeutic carmustine (BCNU), delivered by means of a polyanhydride (PCPP:SA) wafer carrier [110]. In that realm, the background of Gliadel® is to be framed: while numerous clinical applications of ethylene vinyl acetate (EVA) (refer to chapter I, 2) include insulin therapy, asthma treatment, and chemotherapy, the EVA polymer has never been approved for use in the brain. However, the new generation of PLGA systems (i.e. microparticles and implants) seemed not to be suitable for drug delivery of chemotherapeutic agents due to a possible sporadic drug dumping as a result of bulk erosion. In 1985, Langer and his group developed the polyanhydride poly[bis(p-carboxyphenoxy)] propane-sebacic acid (P(CPP:SA)), an extremely hydrophobic polymer with surface-controlled erosion. Gliadel® entered the U.S. market in 1996, and is today approved in several countries of the world [209].

Polyanhydrides were also investigated as controlled delivery systems for peptides and proteins. Principally, countless variations of polyanhydrides (e.g. aliphatic or aromatic polyanhydrides, poly(ester)- or poly(ether)-anhydrides, crosslinked polyanhydrides or amino acid based anhydrides) could be used for drug delivery, but the pharmaceutical research has been focused on polyanhydrides derived from sebacic acid (SA), 1,3-bis(p-carboxyphenoxy)propane (CPP) and fatty acid dimer (FDA) - (P(CPP:SA) or P(FAD:SA)) [158; 110]. Because the temperature required for the fabrication of polyanhydride devices is too high for sensitive drugs such as peptides and proteins, a solvent extraction technique derived from the standard W/O/W emulsion method for PLGA microspheres is used [81]. When lysozyme,

trypsin, ovalbumin, BSA or immunoglobulin were incorporated in 25/75 fatty acid/sebacic acid copolymers, a constant release could be achieved for about 2 weeks. The different proteins exhibited almost identical release rates over this period, suggesting the release mechanism being primarily erosion controlled. However, a notable drop in the molecular weight of the polymeric microspheres was also monitored within the first 90 hours, followed by a continuous erosion rate [191]. These results may indicate that bulk erosion can not be completely suppressed when using polyanhydride devices.

A second approach of Langer and co-workers was the development of biodegradable poly(anhydride-co-imides), in particular those containing L-tyrosine in their polymer backbone. Due to the incorporation of the immunological adjuvant L-tyrosine, these polymeric devices seemed to be very promising for the delivery of vaccines. From these systems a BSA release closely correlates to microsphere erosion - as indicated by polymer weight loss - proposing a release mechanism mainly controlled by polymer erosion [39].

In recent years, the interest in the development of controlled protein delivery systems based on polyanhydrides has been abated. One possible reason may be the difficult fabrication process, since both the hot melt encapsulation and the solvent evaporation method can lead to inactivation of proteins during manufacturing.

### *2.4.2 Poly(ortho esters)*

Poly(ortho esters) are under research since the 70`s and by that time, four families have been developed (Fig. 7):

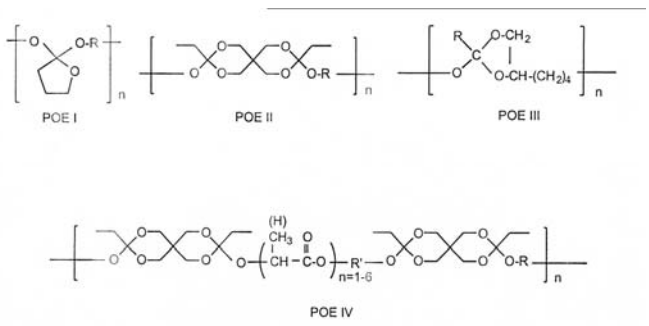


**Figure 7: Survey of the chemical structures of the poly(ortho ester) families: POE I- POE IV [84].**

Though extensive investigations have been carried out, the families of POE I, II and III did not lead to the success originally anticipated [84]. Therefore, only the

development and potential applications of the poly(ortho esters) IV will be outlined in detail in the course of this review. POE IV is a more hydrophilic modification of POE II, which has been realised by the incorporation of a short segment based on either glycolic acid or lactic acid in the polymer backbone. This very segment reveals a rapid hydrolyses after administration, what triggers in turn the catalysis of hydrolytic cleavage of the ortho ester linkages. Such polymers have been designated as auto-catalysed poly(ortho ester) variants [84].

Several studies demonstrated the variability in the polymer characteristics, evidenced the surface erosion mechanism and in consequence their high potential in controlled peptide and protein delivery [163]. The common group of poly(ortho esters) IV exhibited three major features of protein release kinetics: there is no burst, even with high protein loads, a notable lag time was monitored, and third, the rate of protein release is linear to polymer weight loss and protein release occuring concomitantly (surface erosion controlled protein release) [164, 38]. A reduced negative lag time can be achieved by simple admixing the monomethyl ether of polyethylene glycol to the polymer/protein mixture (AB block copolymers). These polymeric extrudates liberated the protein for a 2 months period following zero-order kinetics [83; 163].

One other variation is the development of semi-solid, auto-catalysed poly(ortho esters). These polymers feature the advantage of an easy manufacturing process: protein can be loaded into the POE matrix by mixing the two compounds without the need of organic solvents or elevated temperatures. Release studies from these systems also indicate the protein release to be linearly, however the release was much more faster terminating after 7 days [83; 199].

These studies using poly(ortho esters) for peptide and protein delivery are certainly very promising, but often protein stability issues are not reflected adequately in the literature. Due to the fact that bulk degradation cannot be completely inhibited [84], the question of what exactly happens in the interior of these systems is still to be addressed.

The suffered setbacks in the establishment of synthetic, biodegradable polymeric devices for controlled peptide and protein delivery, in particular PLGA matrices, and the loss of an ultimate solution for overcoming this dilemma, explain the mismatch between the number of peptide/protein containing polymeric systems

under preclinical and clinical investigation and the number of polymeric devices approved by the FDA. Especially in the light of the recent discussion around immunogenicity of even a small amount of denatured or otherwise altered protein for s.c. use (e.g. immunogenicity of Eprex® [33]), the distinctive rates of protein inactivation during manufacturing and after administration can be deemed the main problem of PLGA systems.

Therefore, the following part will specify the potential of lipophilic materials, such as fatty acids, glycerides, waxes and cholesterol for the application as controlled protein delivery systems.

## 3. Lipids as drug delivery systems

For a long time, lipids have been used for the encapsulation of drugs in the field of oral delivery systems. For example, in the product mucosolvan retard capsules® (Boehringer-Ingelheim Pharma KG), the drug is encapsulated in wax matrices in the form of lipid pellets resulting in an extended drug release. However, the use of lipid carriers for parenteral controlled drug delivery, in particular for the delivery of peptides and proteins, has been investigated only in a humble range over the last years.

Potential lipid-based carrier systems for a controlled peptide and protein release are:

- liposomes (including DepoFoam™ technology)
- solid lipid nanoparticles
- oily suspensions
- lipid microspheres
- lipid implants

### 3.1 Liposomes

Phospholipid vehicles as drug delivery systems were rediscovered as “liposomes” in 1965 by Bangham [11]. In the early 90`s, three products for intravenous injection entered the market: Ambisome® for the systemic fungal treatment, and two chemotherapeutic liposomal formulations (Doxil® and Daunosome®).

The use of liposomes as protein carriers was thought to improve their functioning as a circulating microreservoir for sustained release after intravenous administration.

For example, the encapsulation of vasopressin within long-circulating liposomes extended the bioactivity from 24 hours to 7 days, determined by diuresis measurements in rats. Vasopressin entrapped in PEGylated long-circulating liposomes even remained bioactive one month after intravenous injection. The application of liposomes containing human recombinant IL-2 inhibited the growth of intradermal tumours. However, the locoregional administration had to be performed three times per week [189].

A new approach, rather than using unilamellar or multilamellar liposomes, is based on the DepoFoam™ system. These multivesicular liposomes (1-100 µm) contain multiple non-concentric internal aqueous compartments and lead to an increase in the encapsulation efficiency. After subcutaneous injection, the release of encapsulated peptide and protein was shown to be prolonged up to 7 days for DepoInsulin and up to 3 weeks for the DepoLeuprolide® formulation [221].

Recently, the company Novosom AG patented a novel liposome-based depot system for proteins and peptides. The Cagicles® depots are produced by a two step method: first, proteins are dissolved in an aqueous medium and then added to solutions of membrane-forming substances, which are selected such that the resulting membrane enters into a reversible mutual reaction with the protein. This mild-condition process enables to increase the encapsulation rate over 30 % of incorporated protein. Furthermore, a one month sustained protein release was feasible after subcutaneous or intramuscular injection of the Cagicles® depots [143].

These studies have proven the basic applicability of liposomes for protein-based pharmaceutics in vitro and in vivo. Though, the time period for release out of liposomes usually is short, generally less than two weeks, whereas liposomal formulations are limited in their use as sustained, controlled protein release carriers. However, liposomal dosage forms may be revaluated in drug targeting by the possibility of positioning marker proteins such as antibodies or cytokines upon the surface of liposomes or due to the potential of being uses as vaccine formulations.

## 3.2 Solid lipid nanoparticles

The concept of solid lipid nanoparticles (SLNs) was coined in the beginning of the nineties by Müller et al. [132]. Solid lipid nanoparticles represent a colloidal carrier system mainly based on triglycerides for intravenous injection. SLNs are

produced by melt dispersion combined with high pressure homogenisation or by microemulsion techniques, resulting in particles with a size range of 50 – 1000 nm. Due to their hydrophobic nature and their small size, SLNs may be more appropriate for incorporation of lipophilic drugs, which can be easily dissolved in the melted mixture. For instance, only small quantities of lysozyme (50 - 500 µg protein/g lipid) can be incorporated into various lipids, even with the use of a solubilisation technique applying a surfactant [5]. A further problem using SLNs as drug carrier is the burst release observed for various active agents after intravenous administration [132]. In summary, it may be stated that solid lipid nanoparticles own potential for the encapsulation of drugs with a low solubility (e.g. paclitaxel), for the application of surface-modified SLNs in drug targeting, or maybe for the use as adjuvant for vaccines. Furthermore, it can be hypothesised that SLNs can be applied for oral drug delivery in the form of aqueous dispersions or that they can alternatively be used as additives in traditional dosage forms such as tablets, capsules or pellets.

### 3.3 Oil suspensions of peptides and proteins for a sustained release

Generally, the viscosity of oily media is considerably higher than the viscosity of an aqueous phase such as buffer. Therefore, drug release can be prolonged by implementing oil suspensions. In addition, the viscosity of the oily carrier can be further increased by the addition of gelling agents such as aluminium monostearate – thus enabling the control of process parameters like drug solubility and drug transfer rate.

A further important aspect using oils as drug carrier refers to the distribution coefficient of compounds in the oily medium and the surrounding tissue. A lipophilic drug with a high distribution coefficient will primarily accumulate in the oily medium resulting in further deceleration of effective drug actions.

For several years, various peptides and proteins have been dispersed in oils to engineer sustained-release formulations. Nestor et al. patented as early as 1979 the development of long-acting injectable depot formulations for super-agonist analogues of luteinizing hormone-releasing hormone (LH-RH), applying oils such as peanut oil or sesame oil and a gelling agent such as aluminium stearate [134].

The complexation of bovine somatotropin with zinc and the use of sesame oil and aluminium monostearate as suspension medium prepared the ground for FDA

approval of Posilac® (Monsanto agro) in 1993. Posilac® bovine somatotropin has become one of the leading dairy animal health products in the United States and many other countries, despite the general value is discussed controversially. Supplementing dairy cows subcutaneously with single-dosed bovine somatotropin enhances the milk production for a period of one month after drug administration [128].

Yu et al. reported the in vivo evaluation of an oil suspension of growth hormone releasing factor analogue for the enhancement of animal growth. The delivery of the growth hormone could be sustained for over 2 weeks. Furthermore, peptide stability was substantially increased within the oil solution when compared to the stability in aqueous media [223].

Shan et al. (Amgen Inc.) described protein/polyol/oil suspensions for the parenteral application to animals, realising the prolonged liberation of granulocyte colony stimulating factor over 2 week period [74].

Apparently oil suspensions have been under investigation mainly in the field of veterinary pharmaceutics, thereby revealing potential as drug carriers for peptides and proteins.

## 3.4 Lipid microparticles

Several techniques have been proposed for the manufacture of lipid microparticles. Primarily those methods are related to the techniques established for polymer microspheres [47]. For example, Reithmeier et al. encapsulated somatostatin, thymocarpin and insulin within glyceryl tripalmitate using a solvent evaporation technique. But the same drawbacks concerning encapsulation efficiency and protein stability, which has already been described in detail under *2.1.1.1*., were noted [153; 154].

However, lipophilic materials such as fats and waxes inhere one basic difference to polymers: the processability of low-viscosity melts, thus obviating the need for organic solvents.

### *3.4.1 Melt dispersion technique*

The melt dispersion technique for the preparation of wax microparticles as an alternative to polymeric systems was first described by Bodmeier et al. [16; 19]. In that realm, Domb et al. presented a melt dispersion method for the preparation of

lipospheres consisting of a solid-lipid core surrounded by a phospholipids layer [57]. By vigorous vortex-mixing or stirring the drug is incorporated into the melt as solid or an aqueous solution. The dispersion is transferred into an aqueous phase, which is tempered above the melting point of the lipid that normally contains also emulsifiers such as polyvinylalcohol or gelatine. The formulation is rapidly cooled to room temperature by immersing the formulation flask in a cool water bath without ongoing agitation. In this context it is to be noted that the term melt “dispersion” technique is not to be perceived in absolute sense, since adding protein solutions to lipid melt is defined to yield emulsions. Though, both dispersion and emulsion are to be subsumed under melt dispersion technique.

The application of the melt dispersion for the incorporation of somatostatin, thymocarpin and insulin led to essentially better encapsulation efficiencies when compared to the use of the emulsion methods illustrated above, This may be ascribed to the higher viscosity of the lipid melt [153; 154]. However, two factors take centre stage in the encapsulation of peptides and proteins in lipid drug carriers, especially when a hot melt dispersion technique is applied: one factor deals with polymorphic transformations of the lipid, which may occur during preparation and subsequent storage of the dosage form. Second, elevated temperatures the protein is exposed to during microparticles preparation can cause protein inactivation.

Polymorphic behaviour of lipids

It is common knowledge that long-chain compounds can show polymorphism, and this is the more valid for fats and lipids. For example, saturated triglycerides usually can form three polymorphs ($\alpha$-, $\beta$`- and $\beta$-form), that differ in molecular structure: disordered aliphatic chain conformation is observed in $\alpha$, intermediate packing in $\beta$`, and most dense packing in $\beta$-forms. This necessitates Gibbs free energy values to be highest in $\alpha$, intermediate in $\beta$`, and lowest in $\beta$. In other words, the $\alpha$-modification represents the most instable form, whereas the $\beta$-modification stands for the stable form of a triglyceride. Generally, polymorphism is influenced by several external factors such as temperature, pressure, solvent etc. [168]. As a consequence, polymorphic transformations may be encountered during the preparation of lipid microparticles. This may account for differences in solubility and

of melting points of active and excipients, and, moreover, such transformations could impact the release profile of incorporated drugs due to conversion after storage [62]. Reithmeier et al. reported that glyceryl tripalmitate microparticles - produced by melt dispersion - crystallised in the instable α-modification due to a fast congealing. On the contrary, microparticles prepared by solvent evaporation reveal the same thermal behaviour of the lipid bulk crystallising in the stable β-form. One explanation therefore may be the slower solidification of the microparticles within the lipid molecules arranging in the thermodynamically most stable β-modification [153]. Steber et al. found that microparticles prepared from mixtures of hard fats (e.g. tristearin) and liquid fats show an accelerated transition from the instable α-modification to the stable β-form within several hours or days when melt-blended and spray atomised. Quite the contrary, pure hard fats retained the α-modification for many months [185]. A further possibility to accelerate the transformation from α-form in the β-modification directly upon manufacturing is the application of a tempering process [168].

Influence of elevated temperatures during preparation of the dosage form on protein stability

Proteins unfold above certain temperatures. During thermal unfolding, the temperature at which 50 % of the protein molecules are unfolded is defined as the unfolding or melting temperature $T_m$ of a protein. The higher $T_m$, the greater is the thermal resistance of a protein [213]. For several proteins, an increase of the melting temperature $T_m$ was observed [107] as a result of the reduced flexibility of solid proteins in anhydrous, organic media [45; 109]. For example, the thermal unfolding temperature $T_m$ of bovine pancreatic ribonuclease suspended in the anhydrous alkane nonane is 124 °C, whereas that in water is only 61 °C [107]. However, it is at least questionable if an increase in the melting temperature $T_m$ within anhydrous solvents can be transferred to the situation of a protein within a lipid melt. Literature dealing with this question is not too extensive. Yamagata et al. demonstrated that the loss of interferon α-2a activity suspended in polyglycerol ester of fatty acids during heating up to 60 °C amounted only 5 %. The further exposure to this temperature over 5 hrs did not result in a further activity loss [220].

### *3.4.2 Cryogenic micronization*

An alternative to the standard procedures represents the cryogenic micronization described by Del Curto et al. [56]. The drug is either added to the lipid melt (co-melting) by stirring or the drug and the lipid are both dissolved in benzyl alcohol-ethanol mixture (solvent stripping) before being poured into a petri dish for solvent evaporation. The obtained lipid matrices are micronized in a milling apparatus with liquid nitrogen providing -80 °C conditions during the procedure. The incorporated GnRH antagonist reveals a continuous release over 1 month in vitro and in vivo [55]. It still needs to be shown if this method really is a match for melt dispersion or the emulsion technique.

### *3.4.3 Spray congealing*

Similarly to the preparation of polymeric devices, protein loaded lipid microparticles can also be produced by atomisation (or prilling) the molten protein/lipid suspension within a cold air stream [188; 27; 185; 125]. This technique led to encapsulation efficiencies for insulin and somatostatin of up to 100 %. Stability data during in vitro release proved that neither insulin nor somatostatin were influenced by the manufacturing process [125].

### *3.4.4 Application of supercritical carbon dioxide*

A novel supercritical fluid process for the coating of protein particles with lipids such as trimyristate or Gelucire® 50-02 was patented in 2000/01 by the group of Benoit [13; 58]. In contrast to the techniques described in *2.1.1.4*, this method completely avoids the use of organic solvents by solubilising the lipid carrier directly within SC $CO_2$ medium. Furthermore, the microcapsules produced are composed of a preformed core of active material surrounded by a shell of coating material. Coating material and protein particles are placed in an autoclave equipped with an impeller. The autoclave is heated and pressurised with carbon dioxide until the supercritical conditions (35-45 °C, 70-200 bar) are provided. Then, the system is equilibrated for 1 h in order to solubilise the coating material. After this, cooling of the autoclave induces a pressure decrease and the transformation of the SC $CO_2$ into the liquid phase, therefore insolubilising the coating material that consequently precipitates upon the insoluble protein crystals [193].

It was shown that bovine serum albumin was not affected by heat or pressure during the manufacturing process. The protein loss was found to be between 34 % and 87 % depending on the preparation conditions. Furthermore, the release of BSA from these microcapsules was sustained over 5 hrs for crystals coated with trimyristate and over 24 hrs for crystals coated with Gelucire® 50-02. Advantages of this method are the mild manufacturing conditions and the possibility to change the solvent power of SC $CO_2$ by varying the temperature/pressure profile. However, a sustained release of a protein over 24 hrs is not deemed sufficient in order to replace liquid pharmaceutical formulations [155; 157; 156].

### 3.5 Lipid implants

In contrast to polymeric systems, lipophilic materials such as fats and waxes inhere the competitive edge of their high compressibility. Since polymers are to be plasticised by extrusion and injection molding at elevated temperatures before implant preparation, lipid implants can easily be produced by compacting using a tablets press or a hydraulic press. This method provides mild conditions without the use of organic solvents and heat. As a consequence, peptides and proteins are not exposed to these detrimental effects during the manufacturing process, what may result in better protein stability. Due to the fact that most of the studies described in the literature prepared lipid implants by mixing and compressing processes, in the following these efforts will be outlined in chronological order.

One of the very first studies dealing with lipophilic materials - mainly with cholesterol - as implant matrices for sustained macromolecule release is addressed by the patent of Kent et al. from 1984. Thereby - referring to Langer and Folkman [182] – it was aimed for the development of a matrix, which functions by simple diffusion through a tortuous pore network [104]. In 1987, Wang et al. dispersed insulin in a pellet disk made by compressing a cholesterol mixture. The implantation of these pellets within rats reduced blood glucose levels for up to 24 days [210]. Another group around Opdebeek and Tucker worked with pure cholesterol and cholesterol/lecithin implants for use in veterinary vaccines, in particular for disease prophylaxis in livestock [141, 105; 206]. The implantation of the matrices (5.5 mm diameter, 1.8 mm thick) induced an antigen response within mice for 40 days, whereby pure cholesterol implants showed no erosion at all. When those cholesterol implants were enriched with

amphiphilic lecithin, the devices eroded over time due to a higher infiltration of water into the matrices [105].

As mentioned above, Wang et al. first used cholesterol as implant system for the insulin delivery. Later on, palmitinic acid was presented as promising excipient in sustained insulin release. These implants eroded slowly with time reducing blood glucose levels in diabetic rats for up to 40 days. In contrast, both lauric acid and myristic acid matrices eroded within 3 days causing blisters in the subcutaneous areas. Various triglycerides exhibited no apparent changes after 1 month incubation, whereas the applied monoglyceride monocaprin was completely adsorbed by the body within 20 hours resulting in hypoglycaemic shock of the test animals. These studies effectively evidence the influence of the erosion properties of the material - a broad variety of lipophilic substances was investigated – on protein release [211; 212].

A completely different work to previous studies was presented at the end of the 80`s and the beginning of the 90`s by Cady et al.. A series of $C_{10}$ - $C_{20}$ fatty acid salts of a synthetic growth hormone releasing hexapeptide (tri-fatty acid hexapeptide salt) was prepared and compressed into implants. The implants were partially coated with either biodegradable polymers such as poly(L-lactide/glycolide) copolymer or with non-biodegradable polymers such as silicone elastomer. The level of daily drug release could be varied with the proper selection of the fatty acid salt. The longer the fatty acid chain length, the less soluble the hexapeptide fatty acid salt, and thus the lower the daily rate of release. Furthermore, implants coated with the non-biodegradable polymer approached zero-order kinetics over a 42 days period [25; 26].

Steber et al. published in 1989 implant compositions based on triglycerides, aiming for the delivery of polypeptides and proteins - in particular for growth factors such as somatotropin. In comparison to the work of Wang, the implants contained up to 25 % of a salt and up to 15 % of a filler material. In order to achieve a better release control, implants were partially coated with methacrylate polymer acting as semipermeable membrane. Elevated blood levels of somatotropin in pigs could be reached for 1 month, whereby the long release duration from the triglyceride matrices can be attributed to the additional water-soluble salt within the matrices [186; 187].

Recent works dealing with triglycerides as matrix material have used gelatin in order to adjust release to different time periods by adding varying amounts of gelatin as release modifier [73; 202]. Bovine serum albumin was liberated over 20 days from matrices containing 1 % gelatin as porogen, and the lion's share of protein was delivered within 5 days. Using higher gelatin quantities led to more pronounced burst effects, liberating the protein within 24 hours. Release studies with hyaluronidase loaded implants have been cited as evidence that neither the lipid itself nor the manufacturing process induced protein instability [202].

In order to avoid variations of physico-chemical characteristics, which may occur when using natural materials, Yamagata et al. presented polyglycerol ester of fatty acids as novel sustained-release dosage form for interferon $\alpha$-2a. In contrast to the other work illustrated above, these implants were prepared by a *heat extrusion technique,* whereby protein activity was not reduced by extrudate manufacture. Interferon $\alpha$-2a could be liberated from these systems over 15 days, and the total amount of released protein was mainly depending on the degree of fatty acid esterification and the fatty acid chain length. The constant release rate matched well to the modified Jander´s equation indicating that the release process from these matrices being diffusion-controlled [220].

### 3.6 Lipophilic matrices after administration

As mentioned above, lipophilic matrices are not as susceptible to erosion phenomena as polymeric systems. Although Reithmeier et al. found a degradation of triglyceride microparticles after implantation into mice after 7 days [154], the erosion of lipophilic systems, and in particular of lipid implants - seems to be virtually nonexistent or, if any, very slow. Intensive studies on the water uptake and the erosion behaviour of triglyceride implants revealed pure triglyceride implants showing no obvious water uptake for more than 6 months in vitro. The constant mass of the matrices after drying indicates that no erosion took place throughout the entire observation period of 32 days. In vivo data additionally verified that the defined geometry of the implants did not change throughout a 15 days implantation time [201; 202]. Starting from an inert, water-insoluble matrix, drug liberation from lipophilic systems is assumed to be a diffusion-controlled process through pores. The

mathematical background of the diffusion process and the principles of controlled macromolecule release from these systems will be outlined in the next section.

### *3.6.1 Controlled release of macromolecules from lipophilic systems*

The diffusion process is commonly described by the Fick´s laws relating the particle current j to the concentration gradient ∇c as the driving force. The first Fick´s law (noted by Adolf Fick in 1855) is defined as

$$j = -D \cdot \frac{dc}{dx}$$

whereby D is the concentration-independent drug diffusion coefficient, j refers to the drug molar flux, and dc/dx is the concentration gradient.

Accordingly, release from an insoluble, inert matrix such as a wax matrix or a non-biodegradable polymer matrix can be described by Higuchi´s equation [174], provided diffusion being the rate-determining process:

$$Q = \sqrt{\frac{D \cdot \varepsilon}{\tau} \cdot (2A - \varepsilon C_s) \cdot C_s \cdot t}$$

with Q being the amount of drug released per area of the disk units exposed to the solvent, D being the diffusion coefficient of the drug in the solvent, ε the porosity of the matrix, τ the tortuosity of the matrix, A the concentration of solid drug in the matrix, $C_s$ the solubility of the drug in the solvent, and t representing the time.

Detailed theoretical studies of the controlled release mechanism of macromolecules dispersed in non-biodegradable devices – e.g. in ethylene vinyl acetate matrices - were performed by Siegel and Langer [182; 113; 12]. The release of macromolecules from these monolithic, polymeric systems was postulated to occur through a porous network created by the drug particles. Whereas a high drug loading creates a complete interconnected network leading to drug release due to drug dissolution followed by diffusion through this very network, the release from low loaded matrices is incomplete due to the interconnected network being incomplete. The retardation of peptide and protein release through the formed network has been attributed to a geometrical feature of the pore structure in the matrix (Fig. 8).

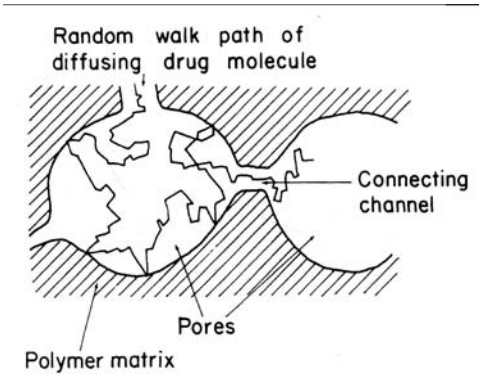


**Figure 8: Scheme of pores through which a diffusing drug molecule must pass [182].**

A protein molecule is to traverse through several pores, and it is a prerequisite that the pores are directly connected to each other, forming an intact channel network. Often, the channel radius is much smaller than the pore radius, and this represents the bottleneck of the diffusion-based release. A diffusing molecule is somewhat executing random walks, and, as a consequence, the molecule will traverse into the pore wall many times before exiting that pore. The confinement of the protein molecules in several pores due to these constrictions was hypothesised a reason for the decelerated release from polymer matrices [182].

Kaewvichit and Tucker confirmed that in fatty acid matrices obtained by compressing neighbouring drug particles form interconnecting networks similar to monolithic polymeric system (Fig. 9).

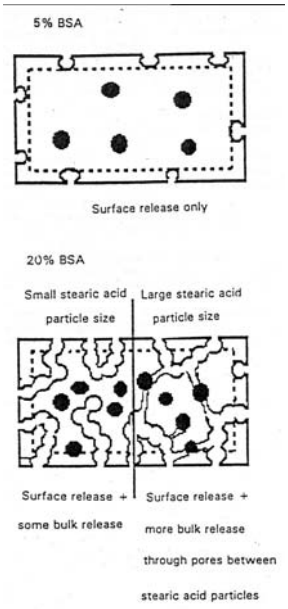


**Figure 9: Model of release from compressed matrices showing effects of BSA load and stearic acid particles size [101].**

This - as well as the appending protein release from fatty acid matrices – was found to be namely depended on the protein load and the fatty acid particle size. Protein particle size and void spaces between particles generated by the compressing process are other parameters that are to be discussed in terms of affecting the release profile [101].
The described studies can be used as theoretical model for the controlled release of macromolecules from lipophilic matrices. Though, it is to be clarified inasmuch the diffusion-controlled process is influenced or overlapped by other parameters, for example impact by slow matrix erosion.

### *3.6.2 Biocompatibility and protein stability issues after administration*

A comparative biocompatibility study was performed by Reithmeier et al., assessing the biocompatibility of lipid microparticles in comparison to polymeric matrices by implanting both forms subcutaneously into mice. The proliferation of a connective tissue combined with a slight infiltration of inflammatory cells was more pronounced in the polymer group as early as 2 days after implantation. After 7 days, the reaction of the connective tissue came to a halt in the lipid group, whereas in the polymer group the fibres of the connective tissue were still solidifying, and fibroblasts were visible. The lipid microparticles were concluded to be more biocompatible than the PLGA polymeric microparticles [154]. Various evaluations of the inflammatory potential of lipid matrices after implantation [105; 206; 211] appear to assert that controlled release devices which base on lipids as natural materials and which are derived from body tissue constituents exhibit a high biocompatibility after administration. This advantage may turn the balance for the application of lipophilic materials rather than synthetic polymers in the development of controlled protein release systems.
Matrix swelling and the degradation of synthetic polymers like PLGA as result of a substantial water uptake during release lead to an increased osmotic pressure and to an acidification within the matrices. Both effects often cause protein aggregation during in vitro release (see also *2.1.3.2* ) [14; 22; 130; 147; 225].
Generally, swelling and erosion occur only marginally in lipid implants, if at all [201; 211; 105]. In that realm, lipophilic matrices might provide a less detrimental environment for incorporated proteins after administration. This hypothesis was

verified by various research groups demonstrating that protein activity was not affected during in vitro release studies [220; 125; 55; 202].
In the consequence, this major advantage is giving lipids as encapsulation matrix for peptides and proteins a competitive edge when compared to the commonly used PLGA systems.

## 4. Short survey of the pharmaceutical proteins used in this thesis

### 4.1 Interferon α-2a (IFN α-2a)

Investigating basic mechanisms of viral interference in 1957, Isaacs and Lindemann exposed chorioallantoic membranes of embryonated eggs to temperature inactivated influenza virus. A few hours later, the supernatant fluid recovered and was found to prevent infection of other cells by active live virus. This factor transferring invulnerability was named “Interferon”. IFN was notified not directly anti-viral, not an antibody, and obviously to be a protein. Later, IFNs were defined as “proteins” which convey virus non-specific anti-viral activity - at least in homologous cells - via cellular metabolic processes involving the synthesis of both RNA and protein.
Interferon was the “prototype” member of a class of bioactive compounds which is called “cytokines”. Cytokines are proteins or glycoproteins usually smaller than 30 kDa. Principally, cytokine action is paracrine and/or autocrine and usually not endocrine – but high concentrations of IFN (and other cytokines) are found in plasma during systemic infections. Interferon can be produced by almost every kind of cells in response to most viruses and several non-viral inducers. However, Interferon is not a single substance; there are several IFNs which have different physico-chemical and biological properties and that can be distinguished by antibody assays. *IFN-α (produced by leucocytes)* and IFN-β *(produced by fibroblasts)* are known as type I IFN. Their production is induced by viruses. IFN-γ is known as type II IFN and is only produced by T-cells on mitogenic/antigenic stimulation.
*Interferon alpha-2a* is a 19,237 Da immunomodulating cytokine composed of 165 amino acids. cDNA cloning allows the production in Escherichia coli since the early 80`s. Recombinant human (Rh) IFN α-2a has shown antitumour and antiviral efficacy

and is clinically used in the treatment of patients with hair cell leukaemia, AIDS related Kaposi's sarcoma, and chronic hepatitis B and C.

### 4.2 Granulocyte colony stimulating factor (G-CSF)

In the mid 1960`s, scientists in Israel and Australia attempted to grow mouse leukaemia cells in nutrient agar plates. Although not succeeding, they observed the spectacular growth of healthy white blood cells in clusters of colonies around other tissue fragments in the agar. Number and size of these white blood cell colonies were dependent on adding other cells or tissue fragments to the cultures, giving rise to the speculation that some factor contained in this material was stimulating haematopoietic cells to divide and form these colonies. This still unidentified factor was dubbed a colony stimulating factor (CSF). Further detailed studies by an Australian group around Metcalf and by the scientists of the Sloan Kettering Center in New York led to the identification of several CSFs, including G-CSF.

Colony stimulating factors (growth factor cytokines) regulate the proliferation and differentiation of immature cells to white blood cells (leucocytes), red blood cells (erythrocytes) and platelets, which are all produced in the bone marrow.

A 19.6 kDa glycoprotein composed of 174 amino acids, granulocyte colony-stimulating factor (G-CSF) is responsible for the proliferation and differentiation of neutrophilic immature cells to neutrophilic granulocytes. Since the mid 80`s, recombinant G-CSF can be produced either in Escherichia coli (non-glycosilated G-CSF with an extra N-terminal methionine, Filgrastim) or in chinese hamster ovary cells (glycosilated G-CSF identical with the human G-CSF, Lenograstim).

G-CSF is clinically used for the regeneration of the immune system in parallel to a cytotoxic chemotherapy – treatment of neutropenia - or after bone marrow transplantation. The first product, Neupogen® - available since 1991 - is marketed by AMGEN. The tenor Jose Carreras, who came down with leukaemia at the end of the 80´s, was one of the first people to be treated with G-CSF. In 2003, sales totalled more than $2.6 billions, with an annual growth rate of 15 %.

## Chapter II: Aim of the thesis

The successful controlled release of proteins from biodegradable polymeric devices – in particular from PLGA systems – is deemed a daunting task. The most remarkable obstacle in the development of such polymeric systems for proteins has emerged as the instability of the protein during encapsulation and release [45; 130; 144; 225].

For lipophilic sources (e.g. fatty acids, glycerides and waxes) as alternative biomaterials used in controlled protein release systems, protein-compatible manufacture processes as well as less detrimental conditions for proteins during release potentially might cope with the setbacks polymeric systems generally suffered in protein stabilisation [202; 155; 55; 125; 105; 211; 188].

Despite showing promise, however, the efforts spent in this specific field – particularly approaches in the development of lipid delivery systems for pharmaceutically relevant proteins – were kept at low levels over the last years.

It was therefore the aim of this thesis to develop an implantable delivery system for a pharmaceutically relevant protein based on lipid materials.

Commercially available interferon $\alpha$-2a (IFN $\alpha$-2a) formulations (i.e. Roferon® A from Roche) which are clinically used in the treatment of patients with leukaemia, AIDS related Kaposi sarcoma and hepatitis B and C, require a therapeutic regimen of subcutaneous administration several times per week. This is why IFN $\alpha$-2a was deemed an interesting drug candidate for use in parenteral depot systems. Besides a second pharmaceutical protein (i.e. granulocyte colony stimulating factor) and one model protein compound (i.e. bovine serum albumin) were investigated.

Implants were chosen as delivery system because a reproducible geometry with a defined surface area will allow release studies in a more systematic way.

It was predominantly aimed for

- the establishment of a manufacturing process which retains protein integrity.
- the development of a lipid carrier system which allows continuous protein release over a 1-month period
- to achieve a complete and controlled protein liberation from the device, plus
- to maintain protein stability during the release process.

The applicability of various lipid materials such as fatty acids, triglycerides and monoglycerides as protein drug carriers as well as the incorporation of different release modifiers were to be evaluated. Moreover, specific evaluation work in order to assess and to retain protein stability during manufacturing and release were considered a cornerstone throughout all experimental studies.

# Chapter III: Materials and methods

## 1. Materials

### 1.1 Interferon alpha-2a (IFN α-2a)

Rh IFN α-2a bulk was provided by Roche Diagnostics, Penzberg, Germany. The protein was formulated in a 25 mM acetate buffer of pH 5.0 containing 120 mM sodium chloride. Protein concentration was 1.7 mg/mL.

In contrast to the native protein, rh-IFN α-2a remains non-glycosilated and contains an extra N-terminal methionine. The IFN α-2a molecule owes two disulfide bonds, one between amino acids 1 and 98, and the second between amino acids 29 and 138, whereby only the loss of the second disulfide bond induces an activity loss. IFN α-2a can be considered as “all-helical” protein with a total of seven α-helices arranging the tertiary structure. The isoelectric point of IFN α-2a is around a pH value of 6.

### 1.2 Granulocyte colony-stimulating factor (G-CSF)

Rh G-CSF bulk was provided by Roche Diagnostics, Penzberg, Germany. The protein (conc. 4.2 mg/mL) was formulated in a 20 mM pH 4.2 sodium phosphate buffer.

The G-CSF molecule features two disulfide bonds between amino acids 36 and 42 and between amino acids 64 and 74, respectively. G-CSF forms four α-helices in an “up-up-down-down” structure. A great part of the G-CSF molecule consists of hydrophobic regions resulting in a high aggregation/self-association tendency of the liquid formulations during storage. In addition, G-CSF is extremely sensitive to elevated temperatures, e.g. the exposure of G-CSF to a temperature of 85 °C led to a complete activity loss within 10 minutes [133; 86].

### 1.3 Bovine serum albumin (BSA)

Bovine serum albumin ($M_r$ 66,300 Da) is extracted by the Cohn fractionation process. The hydrophilic BSA molecule features 17 disulfide bonds, restricting the mobility of the molecule. Approximately 55 – 60 % of the BSA molecule are arranged as α-helices. The isoelectric point of bovine serum albumin is around 4.7 – 4.9. BSA

inheres a considerable amount of dimers (~ 9.2 %) and trimers or higher-ordered aggregates (~ 2.5 %) [120].
Lyophilised bovine serum albumin Fraction V was purchased from Sigma-Aldrich Chemie, Steinheim, Germany.

### 1.4 Triglycerides

Glyceride esters of saturated, pair and non-branched fatty acids (Dynasans®) were provided by Condea Chemie, Witten, Germany as microcrystalline products in stable β-modification with distinct melting points. Dynasans® are physiologically harmless.

| | Chemical structure | Acid value (mg KOH/g) | Hydroxyl number OHZ (mg KOH/g) | Saponification number VZ (mg KOH/g) | Iodine number IZ (g $I_2$/100g) | Melting area (°C) |
|---|---|---|---|---|---|---|
| **Dynasan 112** | **triglyceride of lauric acid** | **max. 3** | **max. 10** | **229-238** | **max. 1** | **47** |
| **Dynasan 118** | **triglyceride of stearic acid** | **max. 3** | **max. 10** | **186-192** | **max. 1** | **70-73 °C** |

**Table 5: Characterisation of Dynasan® products.**

### 1.5 Stearic acid

Stearic acid ($C_{18}H_{36}O_2$), a hard wax-like saturated acid with a molecular weight of 248.47 g/mol was provided by Condea Chemie, Witten, Germany. Stearic acid is almost water-insoluble, physiologically harmless, and a basic constituent of several animal and vegetable fats and oils.

### 1.6 Monoglycerides and mono-diglycerides

Palmitic acid monoglyceride (Myverol 18-04 K) and palmitic acid mono-diglyceride (Admul MG 40-04 K) were provided by Quest International, Netherlands. Monoglycerides and mono-diglycerides are the most widely used emulsifiers in the food industry.

| | Total monoglyceride (min. %) | Free glycerol (max. %) | Acid value (mg KOH/g) | Iodine number IZ (g $I_2$/100g) | Source |
|---|---|---|---|---|---|
| **Myverol 18-04 K** | **95** | **1** | **max. 3** | **max.3** | **palm** |
| **Admul MG 40-04 K** | **45** | **1.5** | **max. 3** | **max. 3** | **palm** |

**Table 6: Characterisation of monoglycerides and mono-diglycerides.**

## 1.7 Polyglycerol ester of fatty acids (PEGFs)

PEGFs (Admul PGE 1415 K) were provided by Quest International, Netherlands. PEGFs are obtained by polymerisation of glycerol and subsequent esterification with fatty acids of various chain lengths. The polyglycerol moiety is predominantly di-, tri- and tetraglycerol. PEGFs are widely used in the food industry.

| | Acid value (mg KOH/g) | Iodine number IZ (g $I_2$/100g) | Saponification number (mg KOH/g) | Source |
|---|---|---|---|---|
| **Admul PGE 1415 K** | **< 3** | **< 3** | **90-120** | **palm** |

**Table 7: Characterisation of polyglycerol ester of fatty acids.**

## 1.8 Chemicals and reagents

***Acetic acid (100%), p.A.,*** VWR International GmbH, Darmstadt, Germany

***Acetonitrile, gradient grade (LiChrosolv®),*** MerckKGaA, Darmstadt, Germany

***Bromphenol blue,*** VWR International GmbH, Darmstadt, Germany

***Dextran 60,*** VWR International GmbH, Darmstadt, Germany

***Dipotassium hydrogen phosphate, $K_2HPO_4$, p.A.,*** VWR International GmbH, Darmstadt, Germany

***1,2-Dioleyl-sn-Glycero-3-Phosphocholine, DOPC,*** Avanti Polar Lipids Inc., Alabaster, U.S.

***Disodium hydrogen phosphate dihydrate, $Na_2HPO_4 \times 2H_2O$, p.A.,*** VWR Int. GmbH, Germany

***Free fatty acids, Half-micro test,*** Roche Diagnostics, Mannheim, Germany

***Glycerin, p.A.,*** Merck KGaA, Darmstadt, Germany

***Guanidine hydrochloride, GnHCl, p.A.,*** VWR International GmbH, Darmstadt, Germany

***Hexane, p.A.,*** Merck KGaA, Darmstadt, Germany

***Hydranal®-Coulomat AG,*** Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany

***Hydroxypropyl-ß-cyclodextrin (HP-ß-CD),*** VWR International GmbH, Darmstadt, Germany

***Potassium chloride, KCl, p.A.,*** VWR International GmbH, Darmstadt, Germany

***Potassium dihydrogen phosphate, $KH_2PO_4$, p.A.,*** VWR International GmbH, Darmstadt, Germany

***Lowry protein assay, Bio-Rad $D_c$ Protein-Assay,*** Bio-Rad Laboratories, Hercules, Canada

***Methanol, p.A.,*** VWR International GmbH, Darmstadt, Germany

***Methylene blue, p.A.,*** VWR International GmbH, Darmstadt, Germany

***Methylene chloride, p.A.,*** Merck KGaA, Darmstadt, Germany

***Poloxamer® 188,*** VWR International GmbH, Darmstadt, Germany

***Polyethylene glycol 6000 (PEG),*** Clariant, Gendorf, Germany

***Sodium azide, $NaN_3$, p.A.,*** VWR International GmbH, Darmstadt, Germany

***Sodium chloride, NaCl, p.A.,*** VWR International GmbH, Darmstadt, Germany

***Sodium dihydrogen phosphate monohydrate, $NaH_2PO_4 \times H_2O$, p.A.,*** VWR Int. GmbH, Germany

***Sodium dodecyl sulphate, $C_{12}H_{25}NaO_4S$, p.A.,*** Merck-Schuchardt GmbH, Hohenbrunn, Germany

***Sodium hydroxide, NaOH, p.A.,*** VWR International GmbH, Darmstadt, Germany

***Sodium sulphate, $Na_2SO_4$, p.A.,*** VWR International GmbH, Darmstadt, Germany

***Trifluoroacetic acid, p.A.,*** Merck KGaA, Darmstadt, Germany

***Tris (hydroxymethyl) aminomethane, p.A.,*** VWR International GmbH, Darmstadt, Germany

***Triton X-100, $C_{33}H_{60}O_{10,5}$, p.A.,*** VWR International GmbH, Darmstadt, Germany

***α,α-Trehalose-dihydrate,*** British Sugar, Peterborough, UK

***Tween® 20 pure, polysorbate 20,*** Serva, Heidelberg, Germany

***Tween® 80 pure, polysorbate 80,*** Serva, Heidelberg, Germany

***Ultra pure water, taken from Purelab Plus®,*** USF GmbH, Ransbach-Baumbach, Germany

### 1.9 Packaging material

Borosilicate glass vials (2R), parenteral quality glass I, siliconised and not siliconised, respectively, as well as cycloolefin copolymer TopPac® vials (10R) were provided by Schott GmbH, Mainz, Germany. Stoppers were teflon covered chlorobutyl rubber stoppers.

## 2. Methods

### 2.1 Freeze-drying process

Freeze-drying was performed using a freeze-drying apparatus ε12G (Christ, Osterode, Germany). Sample volumes were always 1.0 mL; primary package materials were 2R glass vials. Samples were frozen to – 45 °C at a rate of 1.6 °C/min. This temperature was maintained for 90 min. Then, temperature was increased by 0.1 °C/min up to -20 °C, and the chamber pressure was reduced to $10^{-2}$ mbar. That primary drying step was applied for 30 hrs. For the secondary drying, the temperature was further increased by 0.1 °C/min up to 20 °C. With a chamber

pressure of $10^{-3}$ mbar, those conditions were maintained for 15 hrs. After chamber venting with nitrogen up to a pressure of 800 mbar, the samples were crimp-sealed and eventually stored at 4 °C.

### *2.1.1 Freeze-dried protein/sugar formulations*

#### *2.1.1.1 G-CSF*

G-CSF was lyophilised with trehalose in a protein/sugar ratio of 1:4. Each lyophilisate consisted of 4 mg protein and 16 mg trehalose.

#### *2.1.1.2 BSA*

BSA was lyophilised with trehalose in protein/sugar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5. Per each lyophilisate, the relevant BSA portions account for 4 mg of the freeze-dried cake.

#### *2.1.1.3 IFN α-2a*

- IFN α-2a was lyophilised with trehalose in protein/sugar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5. Per each lyophilisate, the relevant IFN α-2a portions account for 4 mg of the freeze-dried cake.
- IFN α-2a was lyophilised with trehalose, dextran 60 and hydroxylpropyl-ß-cyclodextrin, respectively, in a protein/excipient ratio of 1:3. Per each lyophilisate, the relevant IFN α-2a portions account for 4 mg out of the 16 mg cake mass. Before lyophilisation, the pH of the stock solutions was adjusted to 4.2.
- IFN α-2a was lyophilised with trehalose, dextran 60 and hydroxylpropyl-ß-cyclodextrin, respectively in a protein/excipient ratio of 1:3. Per each lyophilisate, the relevant IFN α-2a account for 16 mg out of the 64 mg cake mass. Before lyophilisation, the pH of the stock solutions was adjusted to 4.2.

## 2.2 Karl Fischer titration

Residual moisture in freeze-dried products was determined via coulometric Karl Fischer titration (KF 373, Metrohm GmbH &Co, Filderstadt, Germany), with generating the $I_2$ electrolytically to keep the Karl Fischer reaction ongoing. Samples were dissolved in 1.0 mL anhydrous methanol inside a nitrogen flooded glove box

followed by 5 min vortexing step. Per sample, 500 µl were injected into the coulometric cell filled with Hydranal® Coulomat AG. At the end of each experiment, residual moisture data was calculated as amount of water in %.
For later studies, an advanced coulometric Karl Fisher titrator with a Head-Space oven was used (Analytik Jena AG, Jena, Germany). Sealed freeze-dried samples were fixed in the oven chamber and heated up to 110 °C. The vaporised water was transported into the Karl Fisher cell via a needle system.

## 2.3 Implant manufacturing

*Placebo implants* were prepared by using a hydraulic press (Maassen, Eningen, Germany). Lipid powder was ground and compressed to 5 mm diameter implants by applying a pressure of 2 tons over 30 s.
For reasons of comparison, p*lacebo implants* were prepared by melting the lipid, pouring the melt in 96-well plates and freezing the melt with liquid nitrogen.
*Methylene blue loaded implants and protein loaded implants* were prepared by using the hydraulic press. The components – lyophilised methylene blue or lyophilised protein, additive and lipid – were admixed stepwise in an agate mortar and the mixture was filled into a compacting tool with 5 mm in diameter (Fig. 10).
The powder was compressed with a pressure of 2 tons yielding implants with an average weight of 50 mg and with 2.3 mm in height. The drug load was calculated based on total implant weight (w/w).


**Figure 10: Compacting equipment for the preparation of implants with 5 mm in diameter.**

The powder was compressed with a pressure of 2 tons yielding implants with an average weight of 50 mg and with 2.3 mm in height. The drug load was calculated based on total implant weight (w/w).

## 2.4 Microscopic studies

Microscopic studies of methylene blue loaded tristearin matrices were carried out using a SMX-2T microscope (Nikon, Düsseldorf, Germany), equipped with an incident light source (Tungsten 6 V, 12 W) . Standard magnifications from 0.5 x to 2 x were applied in order to visualise methylene blue distribution on implants surfaces. Microscopic pictures were collected with a TK-C1380 colour video camera (camera digital – ½ inch CDD, JVC Professional Products GmbH, Friedberg, Germany) coupled with Lucia software.

## 2.5 Free fatty acids, half-micro test

The determination of free fatty acids – i.e. non-esterified fatty acids, NEFA - as a function of the erosion behaviour of triglyceride matrices was conducted by using an optimised enzymatic colourimetric assay (Roche Diagnostics GmbH, Mannheim, Germany). At the presence of the enzyme acyl-CoA synthetase, free fatty acids are converted by adenosine-5`-triphosphate (ATP) and coenzyme A (CoA) to acyl-coenzyme A (acyl-CoA) (addition of 50 µl sample to 1.0 mL reagent A followed by 10 min vortexing). Acyl-CoA reacts with oxygen in the presence of acyl-CoA oxidase to 2,3-enoyl-coenzyme A (enoyl-CoA). With peroxidase being present, the resulting peroxide converts 2,4,6-tribromo-3-hydroxyl-benzoic acid and 4-aminoantipyrine to a red dye (start of the reaction by the addition of 50 µl reagent B, followed by 15 min vortexing). The dye intensity was measured spectrophotometrically at 546 nm wavelength. Solutions of stearic acid (0.3 – 0.7 mM/L) were prepared by dissolving the fatty acid in ethanol and Triton X-100 for the generation of calibration curves.

## 2.6 Differential scanning calorimetry (DSC)

Placebo implants and protein loaded implants were ground. DSC measurements (DSC 204 Phoenix, Netzsch, Selb, Germany) were performed using 5 – 10 mg of the sample. Heating and cooling were conducted at a scan rate of 5 K/min within a 25 – 100 °C temperature range.

## 2.7 X-ray diffraction

Pure tristearin implants and protein loaded implants were ground. Wide-angle X-ray scattering (WAXS) was performed by an X-ray Diffractometer XRD 3000 TT (Seifert, Ahrensberg, Germany), equipped with a copper anode (40 kV, 30 mA,

wavelength 0.154178 nm). Experiments were conducted at 0.05 ° (2 theta) within a 5° - 40 ° range.

## 2.8 Extraction of protein from the lipid matrix

### *2.8.1 Method I*

The protein-loaded lipid matrix was dissolved in 5.0 mL organic solvent (i.e. methylene chloride or hexane). The organic phase was overlayed with 5.0 mL of a pH 7.4 isotonic 0.01 M phosphate buffer (PBS), and the protein was extracted by gentle agitation. Protein concentration was determined by using a Lowry protein assay (Bio-Rad $D_c$ Protein-Assay, Bio-Rad Laboratories, Hercules, Canada), and protein stability was evaluated by gel electrophoresis (SDS-PAGE).

### *2.8.2 Method II*

The protein-loaded matrix was ground in an agate mortar. 30 – 50 mg of the mixture were suspended in 2.0 mL of a pH 7.4 isotonic 0.01 M sodium phosphate buffer with 0.05 % (w/v) sodium azide and 1 % polysorbate 20 (PBST). After gentle agitation over various periods of time, the samples were filtrated or centrifuged at 5,000 g for 5 minutes. Protein concentration and stability were determined as described for Method I.

## 2.9 Lowry protein assay, Bio-Rad $D_C$ Protein Assay

The Bio-Rad $D_C$ Protein Assay is a colourimetric assay on protein concentration similar to the well-documented Lowry assay. The assay is based on two steps: (I) the reaction between protein and copper in an alkaline medium (addition of 500 µL reagent A to 100 µL protein samples followed by vortexing), and (II) the subsequent reduction of Folin`s reagent by the copper-treated protein (addition of 4.0 mL reagent B to the samples followed by vortexing) resulting in a characteristic blue colour within 15 min, with an absorbance maximum at 750 nm wavelength (U-1100 Spectrophotometer, Hitachi, Berkshire. U.K.). Colour development is primarily due to the amino acids tyrosine and tryptophane, and, to a lesser extent, cystine, cysteine, and histidine. In case of protein samples containing detergents, 20 µl of 10 % sodium dodecyl sulphate solution (reagent S) are added

per each mL of reagent A. Protein solutions of known concentrations (50.0 – 1000.0 µg/mL) were used to generate calibration curves.

## 2.10 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS-PAGE was conducted under non-reducing conditions using an XCell II Mini cell system (Novex, San Diego, CA, USA). Samples were diluted in a pH 6.8 Tris-buffer containing either 2 % or 4 % SDS and 2 % glycerin, were stored for 30 min at 90 °C, and subsequently loaded into the gel wells (NuPAGE® Novex 10 % Bis-Tris Pre-Cast Gel 1.0 mm from Invitrogen, Groningen, Netherlands). Electrophoresis was performed in a constant current mode of 30 mA in a Tris-glycine/SDS running buffer (MES running buffer from Invitrogen). After staining with either a coomassie blue kit or a silver staining kit (Colloidal Blue Staining Kit and SilverXpress® Stain Kit both from Invitrogen), the gels were dried using a DryEase® Gel Drying System (Invitrogen).

## 2.11 In vitro release studies

In vitro release studies were conducted by incubating the implants at 37 °C either in glass vials or in TopPac® vials (both from Schott GmbH, Mainz, Germany), each containing 2.0 mL isotonic 0.01 M phosphate buffer (pH 7.4 with 0.05 % (w/v) sodium azide; PBS). The samples were shaken at 40 rpm (Certomat® IS, Braun Biotech International, Melsungen, Germany). At predetermined time points, the release medium was completely removed and replaced with fresh buffer. The set-up of the in vitro release system was designed for comparative development purposes only, and not designed for mimicking the conditions at an s.c. injection site. Each experiment comprised analysis of three individual samples. Protein concentration and protein integrity were assessed either by reversed phase HPLC (IFN $\alpha$-2a and G-CSF) or by size exclusion HPLC (IFN $\alpha$-2a and BSA).

In order to further investigate the protein integrity within the matrices, protein was extracted from several implants (see chapter II, *2.7.2*) after incubation and analysed by SDS-PAGE. In addition to that, samples drawn at various times were also analysed by gel electrophoresis.

## 2.12 Reversed phase HPLC (RP-HPLC) of IFN α-2a

Protein concentration and the amount of chemically modified protein - e.g. oxidised species - can be assessed by RP-HPLC (Fig. 11), which was performed with a Jupiter 5u C18 300 A 250 x 4.60 mm column (Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of a 49:51 (v/v) acetonitrile/ultra purified water mixture, which was acidified with 1 mL trifluoroacetic acid per L of eluent. The flow rate was adjusted to 1 mL/min, UV-detection (UV 1000, Thermo Electron Cooperation, Dreieich, Germany) was performed at 215 nm wavelength. IFN α-2a solutions of known concentrations (37.5 – 600.0 µg/mL) were used calibration curve set-up.

## 2.13 Size exclusion HPLC (SE-HPLC) of IFN α-2a

SE-HPLC of IFN α-2a (Fig. 11) was conducted using a TSKgel G3000SWXL 7.8 mm x 30.0 cm column (Tosoh Biosep, Stuttgart, Germany). 120 mM disodium hydrogen phosphate dihydrate, 20 mM sodium dihydrogen phosphate and 4 g/L sodium chloride, adjusted with hydrochloric acid to a pH of 5.0 was used as mobile phase. The flow rate was set to 0.6 mL/min, UV detection (UV 1000, Thermo-Electron) was performed at 210 nm wavelength. IFN α-2a solutions of known concentrations (42.0 – 600.0 µg/mL) were used to generate calibration curves.

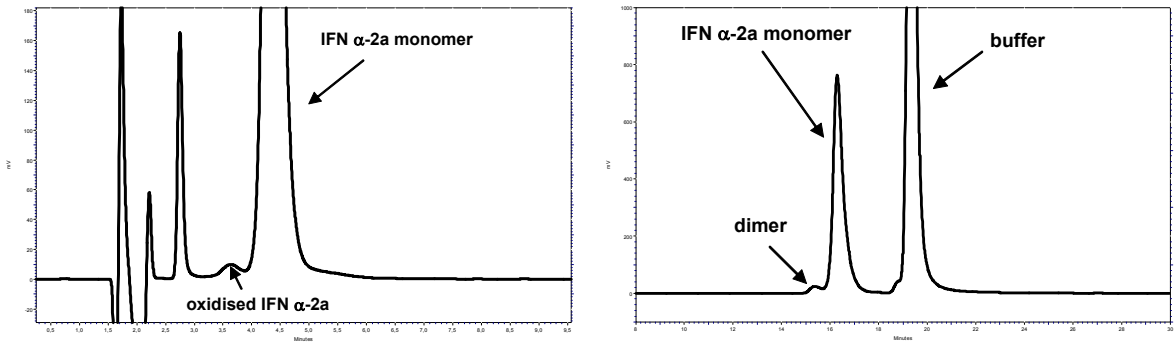


**Figure 11: Detection of oxidised and aggregated IFN α-2a specimen by RP-HPLC (left) and SE-HPLC (right), respectively.**

## 2.14 Reversed phase HPLC (RP-HPLC) of G-CSF

RP-HPLC of G-CSF was accomplished using a Jupiter 5u C18 300 A 250 x 4.60 mm column (Phenomenex). The mobile phase was made up of acetonitrile and ultra purified water 60:40 (v/v), acidified with 1 mL trifluoroacetic acid per L medium. The flow rate was set to 1 mL/min, UV detection (UV 1000) was performed at 220 nm wavelength. Calibration curves were generated by G-CSF solutions at a range of 21.0 – 420.0 µg/mL.

## 2.15 Size exclusion HPLC (SE-HPLC) of BSA

In order to assess protein concentration and protein stability, BSA was analysed via SE-HPLC (Fig. 12). A TSKgel G3000 SW 7.5 mm x 30 cm column (Tosoh Biosep) was used, with the mobile phase consisting of 250 mM sodium sulphate and 10 mM potassium dihydrogen phosphate, adjusted to a pH of 6.8. The flow rate was set to 0.6 mL/min, UV detection (UV 1000) was performed at 210 nm wavelength. BSA solutions of known concentrations (25.0 – 600 µg/mL) were used for instrument calibration.

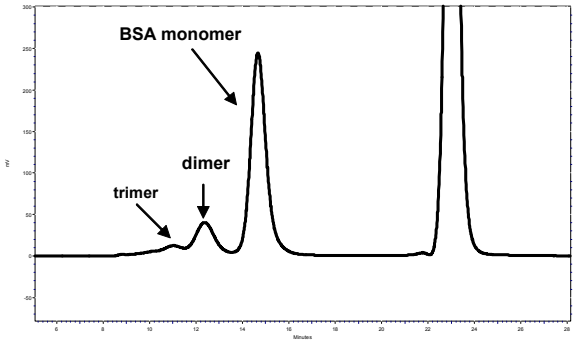


**Figure 12: Detection of BSA specimen by SE-HPLC.**

## 2.16 Light obscuration analysis

Light obscuration technique – often referred to as light blockage - is a state-of-the-art method for subvisible particle counting in liquid parenterals with a lower detection limit of ~1 µm. A proceeding aggregation process within the liquid formulation may result in insoluble aggregates/precipitates which can be analysed by this technique. Light obscuration measurements were carried out using a SVSS-C[40] apparatus (PAMAS GmbH, Rutesheim, Germany). Analytical sample volumes were

consistently 0.3 mL, and each protein sample was measured three times (n=3). Data of counted particles were correlated to a sample volume of 1.0 mL.

## 2.17 Scanning electron microscopy (SEM)

For SEM sample preparation, dry implants were coated with gold. Incubated implants were dried before coating in a VO 200 vacuum chamber (Memmert, Schwabach, Germany) for 48 hrs before coating. Surface morphology of the implants was analysed by a Field Emission Scanning Electron Microscope JSM-6500 F (Joel, Ebersberg, Germany).

## 2.18 Mercury porosimetry

Incubated implants were dried in a VO 200 vacuum chamber (Memmert) for 48 hrs. Pore size measurements were performed using a Auto Pore IV Porosimeter and a 5 bulb 0.38 stem solid penetrometer (both from Micromeritics, Moenchengladbach, Germany). The contact angle used was 141 °. The samples were evacuated for 5 min with an evacuation pressure of about 50 mg for low pressure runs. The mercury filling pressure was 0.0028 MPa. The equilibration time during low and high pressure measurements was set to 30 sec.

## Chapter IV: Tentative experiments

Lipophilic materials – e.g. fatty acids, triglycerides, and waxes - reveal a high compressibility. As a consequence, lipid implants for use in drug delivery are commonly prepared by means of standard compacting equipment like tablet presses or hydraulic presses, respectively [211; 25; 187]. In theory, the mild condition process is assumed to deliver implants with a reproducible geometry and a definite drug load, what should also be exploited in this thesis.

Thus, a standard hydraulic press in combination with a potassium bromide moulding pellet tool (d = 13 mm) was used in pilot surveys in order to verify the compressibility of the lipophilic materials. These investigations indicated the manufacture of lipid implants by compacting being possible, but due to the required pressure of some 12 tons as well as due to the fact that the implants stuck on the compacting tool the diameter of this tool had to be reduced. Furthermore, implants` dimensions should be kept in a feasible range with respect to the demands of parenteral application pathways.

As shown in chapter II, a purpose-built compacting tool (Maassen GmbH) enabled the preparation of implants with a diameter of 5 mm by applying a pressure of solely 2 tons for 30 seconds. Using this set-up yielded implants with weights ranging between 30 - 100 mg. Though for reasons of a higher reproducibility during future release studies matrices were prepared with weights between 45 – 55 mg and height dimensions of 2 - 2.5 mm (Fig. 13A).

The implants could easily be loosened from the punching tool without sticking. Furthermore, the obtained matrices exhibited a high physical stability (e.g. a 27 N breaking strength) and the surface appeared smooth with only low ruggedness which is ascribed to the compressing process per se (Fig 13B).

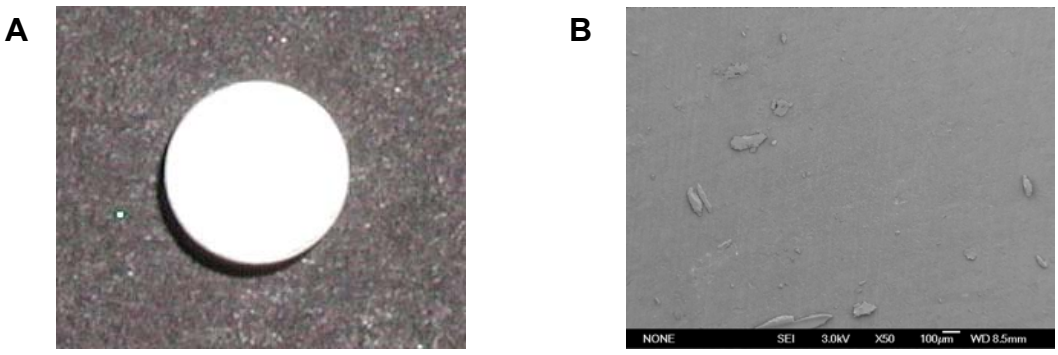


**Figure 13: (A) Triglyceride matrix, (B) surface zoom by SEM.**

Dissolving the implants manufacture related irritations was the first approach in the tentative experiments. Based on the first trouble shooting the next step was to characterise the pure lipid implants in terms of polymorphic behaviour, erosion properties and basic applicability as delivery systems for protein drugs.

## 1. Characterisation of lipid modification

Generally, polymorphism of long-chain compounds, in particular of fats and lipids, is influenced by several external factors such as temperature, solvents and pressure (refer to chapter I, 3.4.2). The transformation of the stable β-modification to the instable α-form due to melting has been rated a major disadvantage of the melt dispersion technique, which is commonly used for the preparation of triglyceride microparticles [153]. During storage, this transition can impact the release profile of incorporated drugs [62].

Therefore, the melting points of compressed tristearin -, trilaurin -, and stearic acid implants, respectively were investigated by differential scanning calorimetry (DSC) and compared to unprocessed bulk materials as well as to implants produced by melting and freezing. Data of the thermal behaviour of tristearin material are illustrated in Fig. 14.

The thermogram of tristearin bulk (A) revealed one single endothermic peak at 72.3 °C originating from the melting of the crystalline β-modification. Implants (B), ground prior to DSC measurements exhibited a peak maximum at 74.5 °C. This minor displacement of the peak maximum versus bulk lipid is considered an effect caused by different particle sizes of the ground implants [23]. However, pure tristearin implants obtained by melting and freezing (C) demonstrated modified thermograms, like two endothermic peaks. The lower peak ($T_m$ = 56.1 °C) represents the melting characteristic of a metastable α-fraction followed by an exothermic peak representing the recrystallisation. The higher temperature peak is assigned to the melting of the stable β-modification. As expected thermograms obtained from trilaurin samples featured identical results (data not shown).

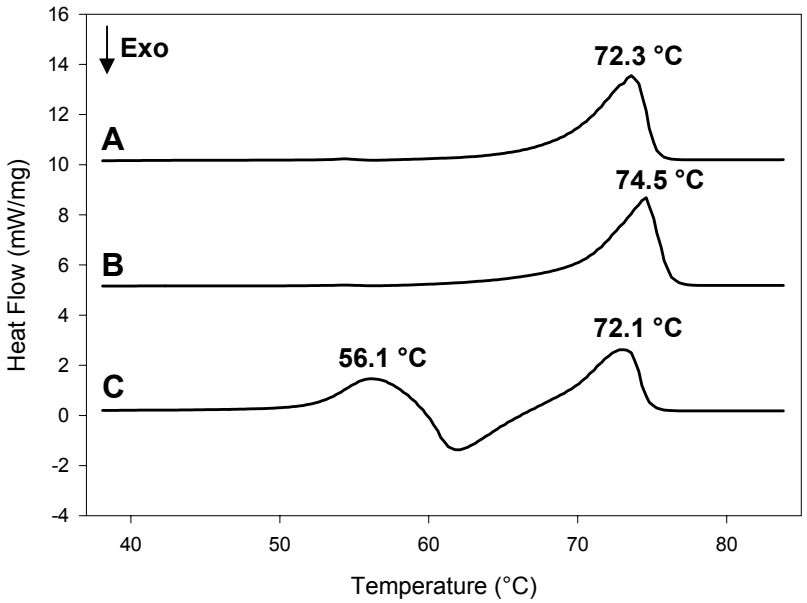


**Figure 14: DSC analysis of (A) tristearin bulk, (B) tristearin implants obtained by compressing exerting a pressure of 2 tons, and (C) tristearin implants obtained by melting and freezing with liquid nitrogen. (For better visualisation the plot baselines are displaced vertically).**

Crystal forms of even fatty acids are referred to as A, B and C. For stearic acid, the C variant is the most thermodynamically stable form ($T_m$ = 69.6 °C) prevalently obtained by the solidification of the melt. Crystallisation from a non-polar solvent usually yields either the A-form ($T_m$ = 64.0 °C) or a mixture of the B ($T_m$ = 54.0 °C) and C-forms. Though, the A-form preparation also was reported by pressing stearic acid upon a glass plate [69; 71; 136].

One single endothermic peak at 70.5 °C was detected in the thermogram of stearic acid bulk (Fig. 15 (A)), which is assigned to the melting of the stable C-polymorph. Implants obtained by compressing (B) feature a displaced peak maximum at 73.8 °C [23]. However, the visible shoulder (→) of this peak may indicate the existence of an A-polymorph fraction originating from the compression process [136]. In contrast, implants obtained by melting and freezing possessed one sharp endothermic peak. This implicates the melt solidification in the thermodynamically stable C-polymorph during implant preparation.

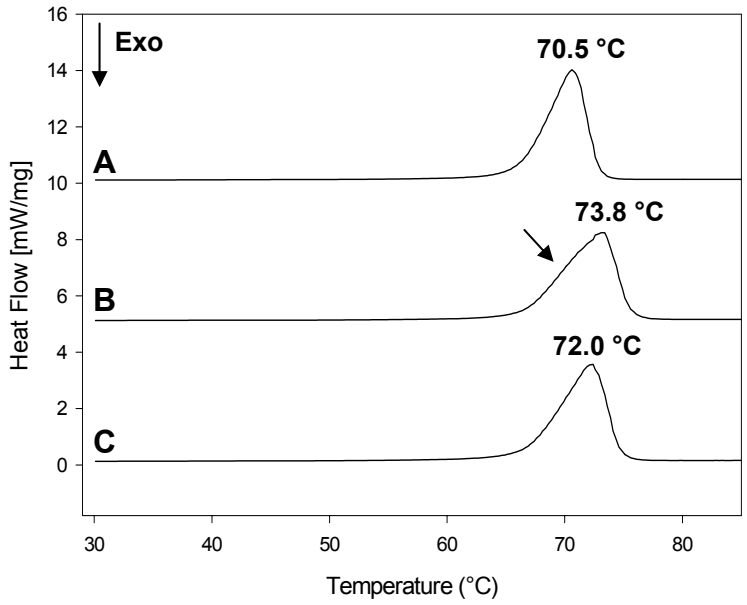


**Figure 15: DSC analysis of (A) stearic acid bulk, (B) stearic acid implants obtained by compressing exerting a pressure of 2 tons, and (C) stearic acid implants obtained by melting and freezing with liquid nitrogen. (For better visualisation the plot baselines are displaced vertically).**

DSC results pointed out the manufacturing process by compressing does not impact the lipid modification of triglycerides. Besides, it can be concluded that the implants consist mainly of stable β-modification material.

However, for stearic acid implants the compressing step did generate A-polymorph material. This instable form being now present within the matrices may result in polymorphic transformation during storage [61]. Therefore, the findings have to be taken into account with respect to the evaluation of the applicability of stearic acid material as delivery system for proteins.

## 2. Erosion behaviour of lipid implants

The main parameter influencing the stability of incorporated proteins is the erosion behaviour of the relevant controlled release systems. Completely changing the morphology of polymeric devices, a rapid water uptake also alters the microclimate. Given that conditions, the proteins are exposed to acidic degradation products within both the matrices and the release media [176; 78].

At the beginning of this work only few authors dealt with swelling and erosion phenomena of lipophilic matrices [211; 105]. These studies indicated that the water uptake and the erosion rate of lipophilic materials is substantially lowered in comparison to polymeric systems. However, a saponification of triglycerides was assumed to take place during drug release. This triglyceride degradation would liberate long-chain fatty acids, which may result in an alteration of the pH within the matrices or the release media. Moreover, it could be assumed that the exposure of stearic acid implants to aqueous media may result in an increased water uptake and eventually an accelerated degeneration rate due to the more hydrophilic nature of this material [211].

In a first approach, each pure tristearin, trilaurin and stearic acid implants were prepared and incubated at 37 °C each in 2.0 mL of a pH 7.4 isotonic 0.01 M phosphate buffer (PBS) over a time period of 4 weeks. PBS medium was moderately agitated via shaking (40 rpm, Certomat® IS, BraunBiotech International, Germany). The pH of the buffer media was measured after 2 weeks and after 4 weeks, respectively (Fig. 16).

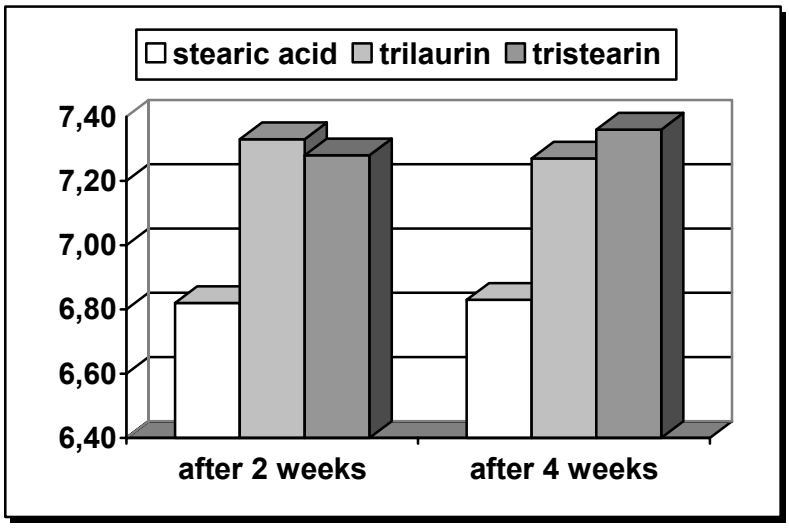


**Figure 16: pH alteration within the release medium (pH 7.4 isotonic 0.01 M phosphate buffer) due to interaction with added lipophilic matrices.**

In that experiment, triglyceride matrices featured no obvious erosion during 4 weeks of incubation and the implants maintained their original geometry in diameter and height. Also no mass loss was detectable. Furthermore, measuring constant pH values within the aqueous media did not indicate any liberation of free fatty acids In

contrary, stearic acid matrices underlie a complete degeneration within 2 weeks of incubation, associated with a pH drop of the buffer media down to values around 6.8 (Fig. 16).

In order to confirm the poor erosion rate of triglyceride matrices, a half-micro test for the determination of the free fatty acids content within aqueous media was set-up. For reasons of harmonisation with release studies planned later on, triglyceride implants with a 10 % or 20 % trehalose load were manufactured and incubated in PBS for a 8 week period (37 °C, 40 rpm).

Data on free fatty acids content in buffer media are listed below.

| Implant composition | Free fatty acids content after 3 weeks [mg/mL] | Free fatty acids content after 8 weeks [mg/mL] |
|---|---|---|
| 10 % trehalose/90 % trilaurin | 3.20 | 8.22 |
| 20 % trehalose/80 % trilaurin | 2.17 | 9.36 |
| 10 % trehalose/90 % tristearin | 3.13 | 6.90 |
| 20 % trehalose/80 % tristearin | 5.69 | 6.61 |

**Table 8: Free fatty acids content in buffer media as a consequence of the degradation of various triglyceride matrices. Lipid implants with an average weight of 50 mg were incubated in 2.0 mL of a pH 7.4 isotonic 0.01 M phosphate buffer (PBS).**

Results obtained by the free fatty acids test clearly proved the degradation of triglyceride matrices being negligible. Even for trilaurin implants which appear to degrade more during an 8 week incubation period, an average free fatty acid concentration of 8.96 mg/L did not indicate pronounced erosion behaviour. The detected free fatty acid content corresponded to 0.042 % of implant weights.

The evaluation of the erosion behaviour of various lipophilic matrices led to the following conclusion:

a) Triglyceride matrices reveal a slow water uptake and poor erosion. As a consequence, incorporated proteins may be deemed more stable. On the other hand, a chance to control protein release by the erosion process per se – similar to surface erosion polymers - has to be jettisoned when using triglyceride matrices as carrier material.

b) Stearic acid implants exhibit a rapid water uptake leading first to a swelling of the matrices, and secondly to a complete degradation of the implants within 2 weeks. This erosion behaviour may facilitate protein liberation, but constant protein stability could be jeopardised by interaction with eroded lipid material.

## 3. Evaluation of protein stability within isotonic buffer media

Due to the inherent chemical and physical instability, each protein usually requires a unique stabilisation approach. This also includes the adjustment of the formulation pH 213].

G-CSF and IFN $\alpha$-2a bulk solutions have been provided in slightly acidic buffer media because of an increased stability these proteins feature within acidic environments [224; 86]. However the optimisation of the buffer media on protein stability requirements is only effective in order to guarantee protein integrity within the dosage forms prior to administration.

After application, proteins are exposed to the pH 7.4 physiological buffer medium. Consequently, within controlled release systems proteins have to deal over an extended time period with the infiltration of this pH 7.4 medium into the release device and the incorporated proteins are expected to be stable under these conditions. Thus, a better correlation between in vitro release studies and in vivo conditions can be obtained when in vitro release buffer media mimic physiological conditions.

In accordance with the aspects outlined above, stability studies of BSA, IFN $\alpha$-2a and G-CSF were performed in solutions formulated either in pH 7.4 isotonic 0.01 M phosphate buffer (PBS) or in PBS with 0.05 % (w/v) sodium azide as preservative. In order to clarify the influence of protein concentration on aggregation and adsorption tendency [213; 80] stock solutions with protein concentrations of 0.05 mg/mL and 0.25 mg/mL were prepared and each 2.0 mL of solution were incubated at 37 °C over 7 days (40 rpm). At predetermined points of time the proteins were characterised in terms of concentration (Lowry assay) and integrity (PAGE), respectively.

BSA stock solutions exhibited constant protein concentrations in both buffer media (PBS and PBS with 0.05 % (w/v) sodium azide) over 7 days. Independently from the original protein concentration and from the addition of the $NaN_3$ preservative 99 % protein recovery was obtained (graph not shown). Also gel electrophoresis did not demonstrate BSA instability and consequently it was assumed BSA formulations being stable at pH 7.4 conditions.

Contrarily, with the same experimental parameters IFN $\alpha$-2a - formulated in pure PBS - showed a continuous decrease in protein concentration over the time. Adding preservatives resulted in constant protein concentrations for 7 days regardless of the

original IFN α-2a concentrations (Fig. 17A). These results suggested IFN α-2a denaturation due to bacterial growth within the non-preserved buffer medium during incubation at 37 °C.

PAGE was applied for further clarification of that finding (Fig. 17B): a pronounced destruction of IFN α-2a molecules was notified after 7 days incubation in non-preserved media. However, preserved media samples exhibited only a small amount of dimer specimen in comparison to IFN α-2a gel standard. For future in vitro release studies, conditions enabling a sufficient stability of IFN α-2a in PBS with 0.05 % (w/v) sodium azide were applied.

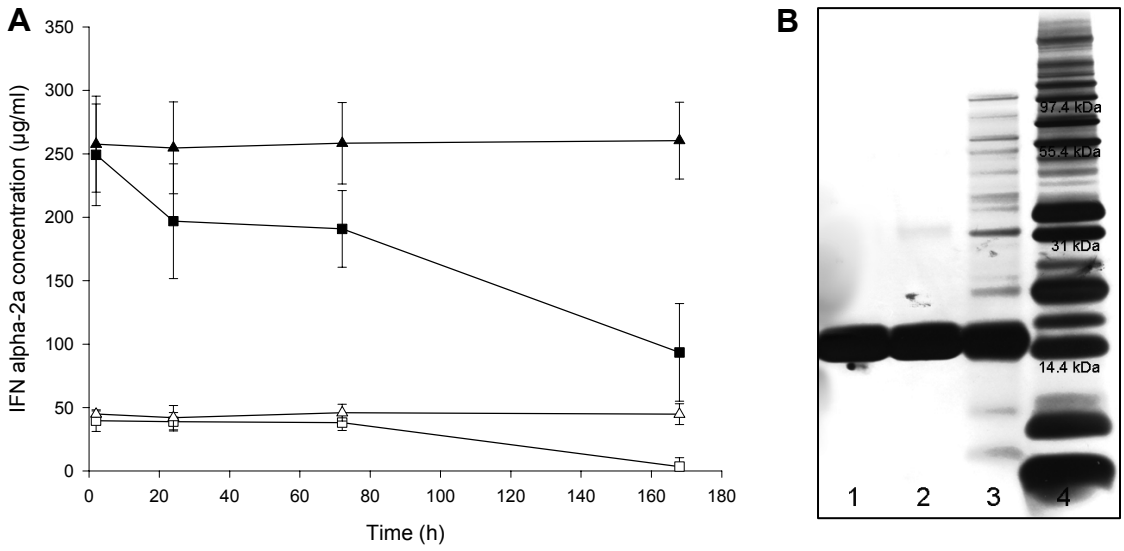


**Figure 17: Influence of the addition of a preservative (0.05 % (w/v) $NaN_3$) on IFN α-2a recovery and stability in pH 7.4 isotonic 0.01 M phosphate buffer (PBS). (A) Alteration of protein concentration over 7 days of incubation - 0.25 mg/mL (▲) and 0.05 mg/mL (△) IFN α-2a in PBS with $NaN_3$, 0.25 mg/mL (■) and 0.05 mg/mL (□) IFN α-2a in PBS; (average ± SD., n = 3). (B) IFN α-2a stability in buffer media after 7 days (0.25 mg/mL protein) - lane 1: protein standard, lane 2: IFN α-2a in PBS/0.05 % (w/v) $NaN_3$, lane 3: IFN α-2a in PBS, lane 4: MW marker.**

In order to investigate stability of G-CSF in solution, similar experiments were performed. In PBS, the samples featured a constant recovery over 3 days followed by a notable decrease in concentration with incubation continuing for up to 7 days. It is worth to be outlined that the addition of 0.05 % (w/v) $NaN_3$ to PBS does not alter the profile of the concentration data (Fig. 18).

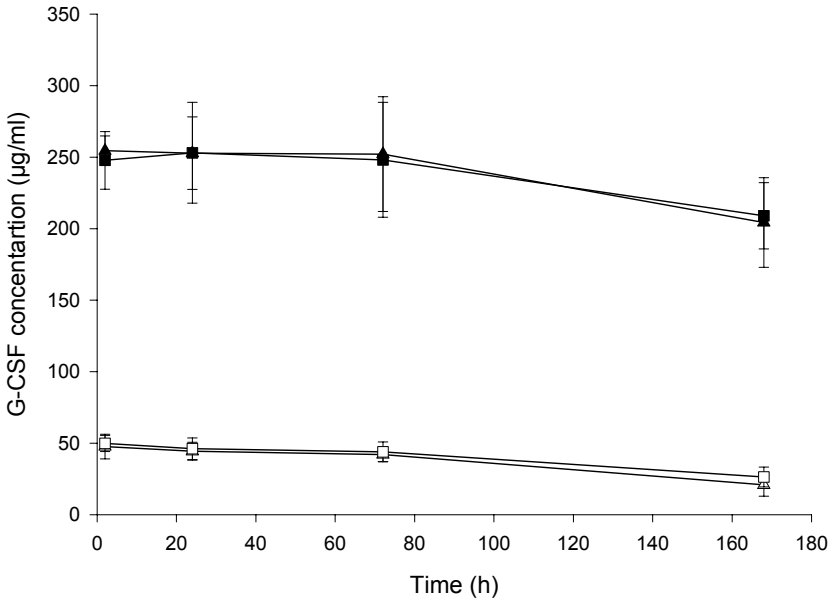


**Figure 18: Influence of the addition of a preservative (0.05 % (w/v) $NaN_3$) on G-CSF recovery and stability in pH 7.4 isotonic 0.01 M phosphate buffer (PBS). Alteration of protein concentration over 7 days of incubation - 0.25 mg/mL (■) and 0.05 mg/mL (□) G-CSF in PBS with $NaN_3$, 0.25 mg/mL (▲) and 0.05 mg/mL (△) G-CSF in PBS (average ± SD, n = 3).**

PAGE studies of G-CSF samples formulated in PBS with 0.05 % (w/v) $NaN_3$ reveal a solely marginal dimer fraction after 3 days of incubation. Aggregation unambiguously became more intense with continuing incubation time, mirrored in the development of dimer and trimer aggregates as well as of higher-order oligomer fractions (Fig. 19).

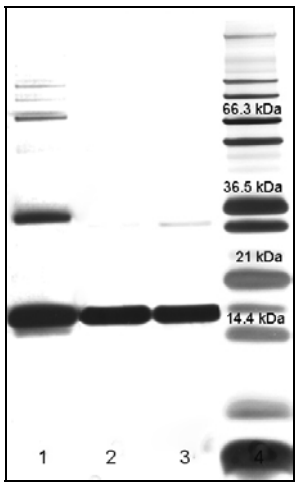


**Figure 19: G-CSF stability in PBS media after various time periods (0.25 mg/mL protein). Lane 1: G-CSF in PBS/0.05 % (w/v) $NaN_3$ after 7 d, lane 2: protein standard, lane 3: G-CSF in PBS/0.05 % (w/v) $NaN_3$ after 3 d, lane 4: MW marker.**

These results implicate the stability of G-CSF within neutral buffer media being abased over longer incubation [37].
In summary, those tentative stability studies suggested that future in vitro release studies could be conducted using pH 7.4 isotonic 0.01 M phosphate buffer (PBS) including 0.05 % (w/v) sodium azide, a buffer medium resembling physiological conditions. Here, not all proteins exhibited satisfying stability over a time range up to 7 days. As a consequence, it is outlined that in future studies release medium will be removed completely and replaced by fresh buffer in a three days interval.
Furthermore, stability appeared to be virtually independent from protein concentration - an important point considering that during in vitro release studies varying amounts of protein will be liberated from the matrices.

## 4. Evaluation of interactions between proteins and lipid matrices

The adsorption phenomenon of proteins to polymeric matrices after release from these systems has been described as a reason for triggering unfolding and aggregation. Similarly, direct binding between decomposition products and protein molecules can lead to protein inactivation after administration [176].
In order to evaluate the possibility of protein/ lipid interactions, pure lipid matrices - i.e. tristearin and stearic acid implants - were added to 2.0 mL protein stock solutions (0.25 mg/mL) formulated in PBS/0.05 % (w/v) sodium azide. The samples were incubated at 37 °C for 7 days (40 rpm). At predetermined points of time samples were drawn and assessed in terms of protein concentration and protein integrity.
Evaluating studies of IFN $\alpha$-2a featured no substantial difference in protein recovery between samples with and without pure tristearin matrices. Almost a 100 % IFN $\alpha$-2a recovery was detectable over 7 days (Fig. 20). Although the average value was 100 %, a 20 % standard deviation of the applied Lowry assay is to be highlighted. Matrices revealed no obvious change in appearance after incubation for one week demonstrating again the poor erosion behaviour of this very material. However, stearic acid implants revealed visible erosion already after 24 hrs of incubation. The erosion went along with a decrease in IFN $\alpha$-2a concentration over the time, yielding a mere 15 % IFN $\alpha$-2a recovery after 7 days (Fig. 20).

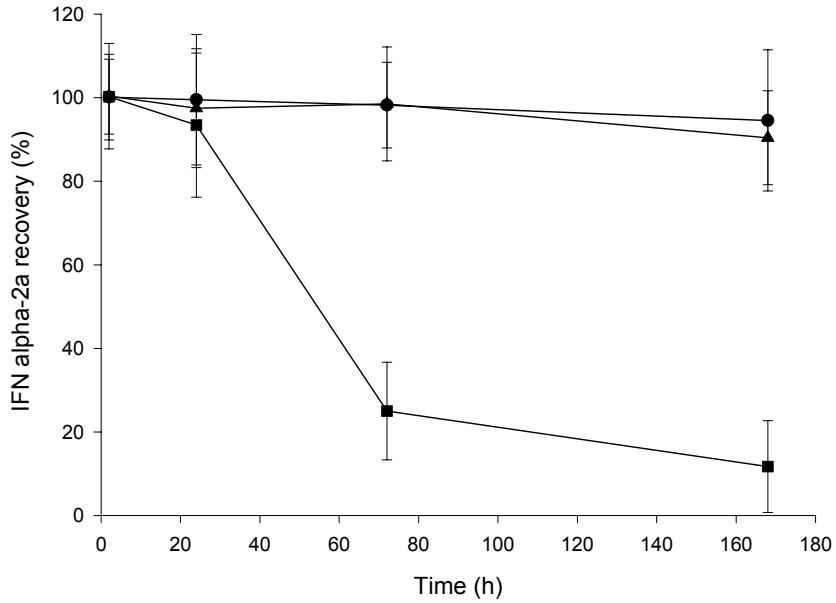


**Figure 20: IFN α-2a recovery in samples containing pure lipophilic matrices over 7 days of incubation (0.25 mg/mL protein). Matrix free samples (●), samples containing one tristearin matrix (▲), and samples containing one stearic acid matrix (■) (average ± SD, n = 3).**

For G-CSF experimental data were comparable (Fig. 21).

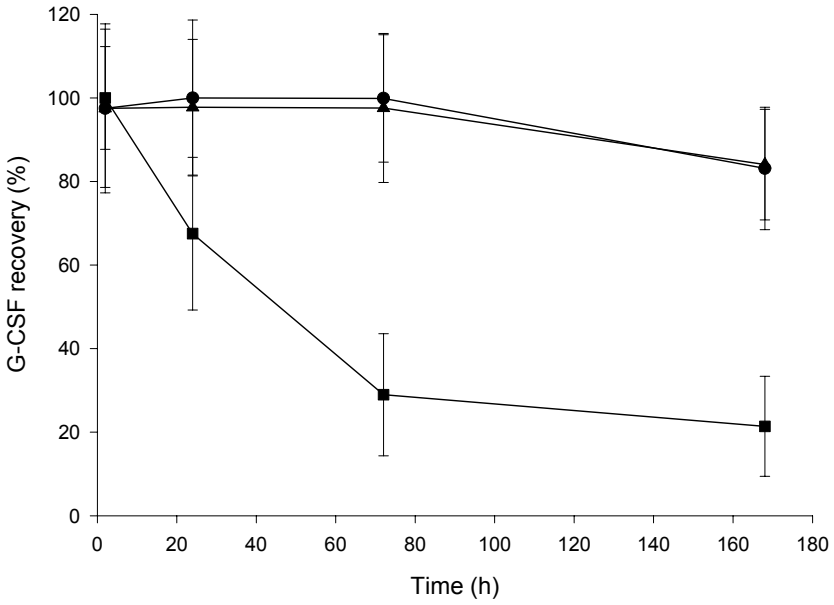


**Figure 21: G-CSF recovery in samples containing pure lipophilic matrices over 7 days of incubation (0.25 mg/mL protein). Matrix free samples (●), samples containing one tristearin matrix (▲), and samples containing one stearic acid matrix (■) (average ± SD., n = 3).**

Again, the erosion of stearic acid matrices was accompanied by a low protein recovery of 20 % after 7 days of incubation, and virtually no difference was noted between matrix free samples and samples containing tristearin matrices.

In the realm of protein integrity, PAGE analysis of samples containing a stearic acid matrix demonstrated extensive protein aggregation after already 3 days of incubation (Fig. 22). This suggested interactions between protein molecules and eroded stearic acid materials. For G-CSF, almost full protein degradation took place, evidenced by almost vanished monomer band and high-molecular weight aggregates, present even in the loading nests of the gel. For IFN $\alpha$-2a, intense aggregation bands were also detected, even though the protein degradation appears less pronounced compared to G-CSF.

In contrast, the presence of tristearin matrix material did not substantially impact protein integrity. Aggregated species were only humble detected in case of IFN $\alpha$-2a samples, and solely native monomer was detected in G-CSF samples (Fig. 22).

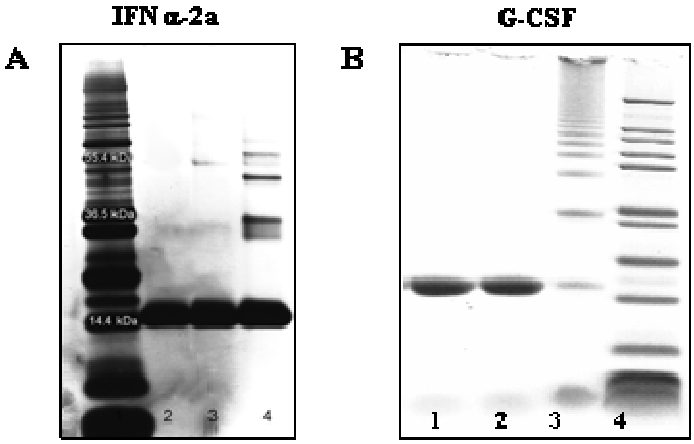


**Figure 22: Influence of tristearin and stearic acid matrices on protein stability after 3 days of incubation. (A) IFN $\alpha$-2a - lane 1: MW marker, lane 2: matrix-free sample, lane 3: sample containing one tristearin matrix, lane 4: sample containing one stearic acid matrix; (B) G-CSF - lane 1: matrix free sample, lane 2: sample containing one tristearin matrix, lane 3: sample containing one stearic acid matrix, lane 4: MW marker.**

Based on data and subsequent conclusion, stearic acid matrices appear to be inappropriate for further investigations. The existence of multiple polymorphic modifications within the implants (refer to chapter IV, 1) and the associated strong protein aggregation as a consequence of the rapid erosion rate featured this material not being useful in the development of controlled protein release devices.

## 5. Drug homogeneity within lipid mixtures and lipid implants

The preparation of protein/lipid compositions by mixing may not necessarily yield homogeneous mixtures. Consequently variations of implants drug load could be possible. Furthermore, it may be argued that a non-uniform drug distribution of the lipid implants can also be caused by the compressing forces exerted during the manufacture process potentially as a result of sintering processes.

Due to its colour enabling a convenient visual control, lyophilised methylene blue was used as model drug compound for homogeneity evaluations of the manufactured implants.

Mixtures containing 10 % lyophilised methylene blue/sugar mixtures and 90 % powdered tristearin were prepared by mixing the components in an agate mortar. The mixtures were divided into three identical portions and dissolved in 20.0 mL isopropyl alcohol. The solutions were further diluted with isopropyl alcohol for UV absorbance measurements at 663.5 nm wavelength. Methylene blue solutions with concentrations in a 0.2 – 3.7 mg/mL range were used to generate calibration curves for the calculation of methylene blue recoveries (% values, table 9).

| Mixture | Recovery [%] of | | | |
|---|---|---|---|---|
| | Portion 1 | Portion 2 | Portion 3 | SD [%] |
| I - methylene blue/trehalose lyophilisate A/ tristearin | 109.82 | 111.65 | 113.46 | 1.82 |
| II - methylene blue/trehalose lyophilisate B/ tristearin | 79.05 | 80.42 | 81.43 | 1.19 |
| III - methylene blue/HP-ß-CD lyophilisate/ tristearin | 80.20 | 82.46 | 83.77 | 1.81 |

**Table 9: Methylene blue recovery data from methylene blue/ lipid mixtures portioned into three subplots (SD = standard deviation).**

The results of absorbance measurements of mixture 1 led to a higher methylene blue recovery in all fractions when compared to the other mixtures. This is addressed to a systematic failure during preparation of the methylene blue stock solution prior to freeze-drying. However, comparing the standard deviation of recoveries from the three fractions of each mixture revealed a sufficient homogeneity of methylene blue/ lipid mixtures for subsequent implant manufacture (Table 9).

In order to evaluate the uniformity of drug distribution within lipid matrices, methylene blue loaded implants were prepared from mixtures as described above. Implants were portioned into three pieces, which were dissolved in 20.0 mL isopropyl alcohol

via 15 minutes ultrasonication. Absorbance measurements were conducted at a wavelength of 663.5 nm after further dilution steps.

Data of the methylene blue incorporated in the three respective pieces of one implant exhibited standard deviations in a range of 7 – 8.45 % (table 10), proving homogeneity of the methylene blue being unsatisfactorily.

| Implant | Recovery [%] of | | | |
|---|---|---|---|---|
| | Piece 1 | Piece 2 | Piece 3 | SD [%] |
| I - methylene blue/trehalose lyophilisate A/ tristearin | 105.79 | 100.12 | 116.75 | 8.45 |
| II - methylene blue/trehalose lyophilisate B/ tristearin | 88.35 | 76.40 | 75.38 | 7.21 |
| III - methylene blue/HP-ß-CD lyophilisate/ tristearin | 86.58 | 81.16 | 72.61 | 7.04 |

**Table 10: Recovery data of methylene blue from lipid implants, portioned into three pieces (SD = standard deviation).**

Microscopic studies (SMX-2T microscope, Nikon, Düsseldorf, Germany) of implant surfaces confirmed the non-uniformity of methylene blue distribution after compressing. Darker and brighter areas were visible in the different images, being a result of different methylene blue contents in these regions (Fig. 23).

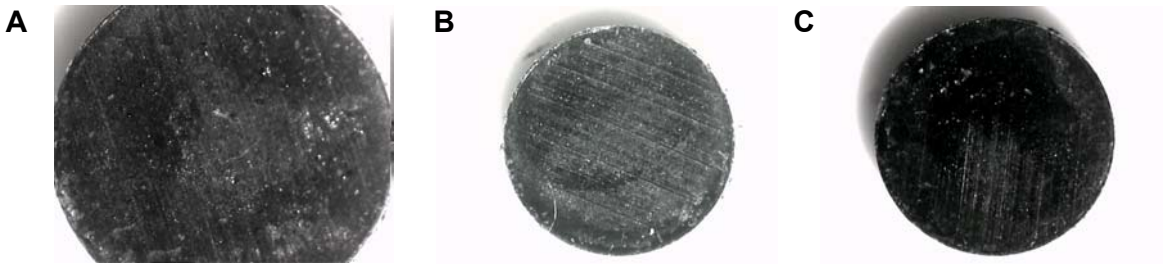


**Figure 23: Microscopic images of (A), (B) and (C) methylene blue/lipid implants I – III.**

Based on these study data it can be concluded that certain variability in release curves must be anticipated during release experiments. Despite same manufacturing process, implants may differ in (I) their actual drug load, (II) the protein distribution between surface and interior and (III) the size distribution of the pores formed during compressing.

## 6. Development of a protein extraction method from lipid implants

Generally, protein aggregation during dosage forms manufacture can be induced by a variety of parameters such as shear forces, elevated temperatures, organic solvents, pressure etc. [176; 14; 198; 109; 160]. The formation of protein aggregates may imperil the safety of the devices by inducing grave adverse effects in patients i.e. immune response and anaphylactic shock [33; 20]. Moreover, the release kinetics of implant systems may be influenced by a deceleration or even restriction of aggregated protein liberation due to a reduced solubility of aggregates or because of pore size limitations within the matrices [106; 216]. Therefore the successful development of a method for protein extraction was deemed crucial in order to clarify whether the manufacturing process by compressing influences protein stability.

Extraction of proteins from microparticulate systems is usually accomplished by dissolving the matrices in an organic solvent and yielding the protein by shaking out with buffer or by centrifugation [98; 154].

For the first approaches methods from the literature were adapted for protein extraction from lipid implants. Proteins were lyophilised with trehalose in a ratio of 1:4 (and with 0.05 % (w/v) polysorbate 80). Gel electrophoresis of reconstituted protein lyophilisates did not demonstrate any aggregated species potentially induced by the freeze drying process per se (residual moisture content ranging between 0.3 – 1.9 %). These data verify protein integrity prior to implant manufacture.

Protein loaded tristearin matrices were prepared with a 2.0 % protein load (i.e. 10% lyophilised protein) for all implants. Matrices were dissolved either in methylene chloride or in hexane followed by protein extraction with pH 7.4 isotonic 0.01 M phosphate buffer (PBS) and slight agitation. Each experiment comprised analysis of three individual samples (n=3). Results of recovered protein in the aqueous media after various time periods are shown in Fig. 11.

Dissolving the matrices in methylene chloride resulted in a 5 % average recovery of G-CSF, 10 % for IFN $\alpha$-2a and 50 % for BSA, respectively. In the case of G-CSF and IFN $\alpha$-2a maximal values were reached after an agitation period of 2 hrs, but recovery values were lower when agitation was applied longer than that (Fig. 24A).

Better results were yielded when hexane was used as solvent medium, i.e. values averaging 67 % for G-CSF and 47 % for IFN $\alpha$-2a were found in the buffer media

after 8 hrs of agitation. However, BSA recovery (28 %) was lower than in the experiment with methylene chloride (Fig. 24B).

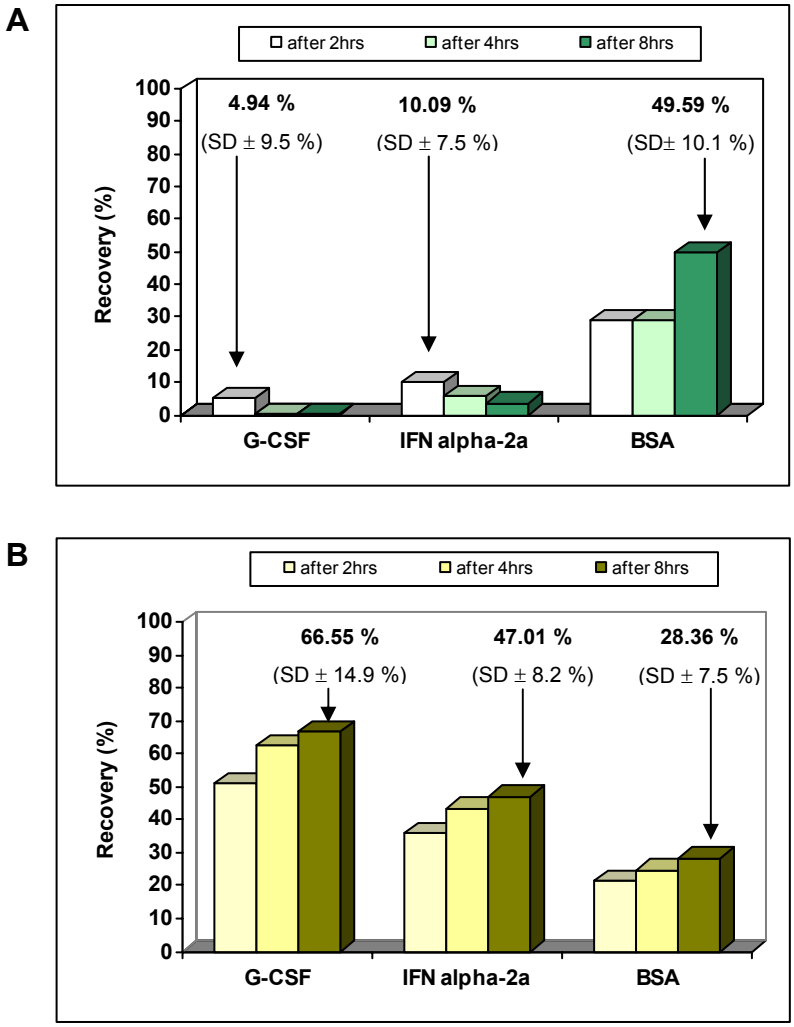


**Figure 24: Protein recovery after implant dissolving in (A) methylene chloride or in (B) hexane, resp., followed by extraction with buffer medium (average ± SD, n = 3).**

Protein integrity analysis unveiled that protein aggregation took place during the protein extraction by means of organic solvent/buffer (Fig. 25). For G-CSF, heavy destruction of protein molecules was observed when applying hexane, whereas apparently no G-CSF was extracted when methylene chloride was used as organic solvent.

In contrast, IFN $\alpha$-2a and BSA demonstrated to be extractable when methylene chloride was applied, though, the aggregation was more pronounced than for hexane/buffer extraction (Fig. 25).

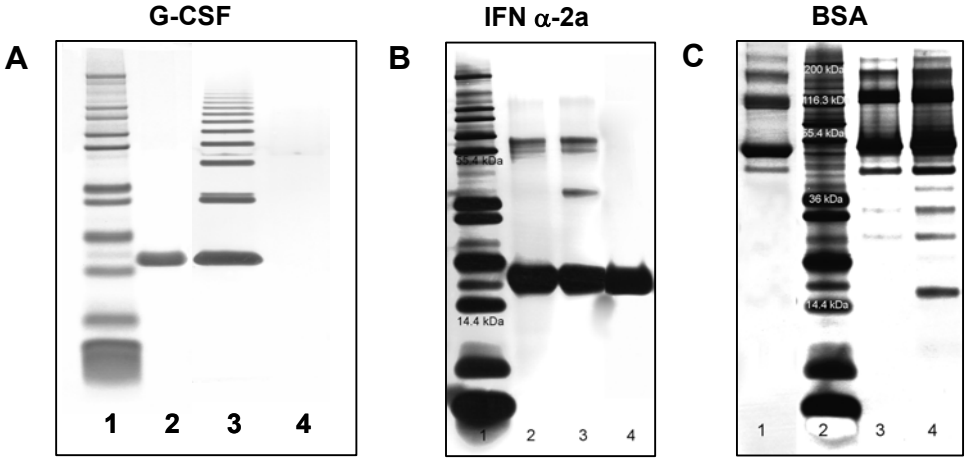


**Figure 25: Protein integrity after extraction from lipid implants via the organic solvent/buffer technique. (A) G-CSF – lane 1: MW marker, lane 2: protein standard, lane 3: extracted via hexane/buffer, lane 4: extracted via methylene chloride/buffer; (B) IFN α-2a – lane 1: MW marker, lane 2: extracted via hexane/buffer, lane 3: extracted via methylene chloride/buffer, lane 4: protein standard; (C) BSA – lane 1: protein standard, lane 2: MW marker, lane 3: extracted via hexane/buffer, lane 4: extracted via methylene chloride/buffer.**

Extracting proteins via organic solvent/buffer systems implies the formation of a water/organic solvent interface as well as diffusion processes of organic solvent and water molecules. In that respect, protein molecules can unfold and aggregate either due to adsorption to the water/organic solvent interface [165] or to destabilising effects of water-saturated organic solvent [109; 45]. Given the background of aggregated protein featuring an increased hydrophobicity, it may be argued that the extraction of protein - using an aqueous medium - may be somewhat incomplete.

Strong aggregation provided, insufficient solubility of more hydrophobic aggregates - and thus the extraction method per se - may be deemed the primary cause for low recovery rates rather than parameters associated with the manufacture of the implants.

Thoughts on an extraction technique avoiding the use of organic solvents led to the development of the extraction method II (refer to chapter III, 2.7.2). Here, the ground protein-loaded matrix mixtures were suspended in PBS. After gentle agitation, the lipid fraction was removed first by filtration.

Whereas the suspension in pure buffer medium resulted in no protein recovery at all, the addition of polysorbate 20 in low concentrations (0.05 – 0.2 % (w/v)) already enabled recovery values of up to 70 %. A further increase of the polysorbate 20

content revealed that increasing surfactant concentrations appear to induce even higher protein recoveries.

A non-ionic surfactant like polysorbate 80 features surface-activity and solubilising effects. Consequently, solid protein may be separated from lipid particles followed by protein dissolution in the buffer medium. However, non-ionic surfactants in higher concentration were described to inhere potential for protein destabilisation [103]. Though, the time the protein is exposed to the surfactant contact may play a key role in exerting detrimental effects on protein integrity. Protein recovery data in PBS with 1 % (w/v) polysorbate 20 are illustrated in Fig. 26. Each experiment comprised analyses of three individual samples. In order to avoid protein loss due to filtration lipid fractions were now removed by centrifugation at 5000 G for 5 minutes.

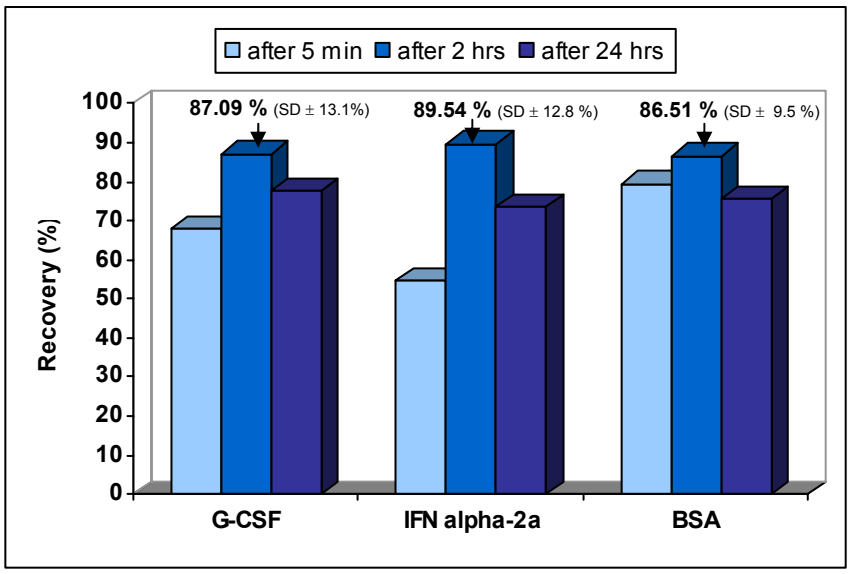


**Figure 26: Protein recovery within the buffer media applying an extraction method without using organic solvents (average ± SD, n = 3).**

In average, recovery values of approximately 90 % could be reached for all proteins when samples were agitated for 2 hrs. Longer agitation periods were accompanied with a decrease of protein detectable in the buffer. This indicates aggregation presumably induced by present surfactant. However, the results clearly attribute this developed extraction method a higher potential than the organic solvent/buffer technique.

Gel electrophoresis of extracted samples agitated over 2 hrs proved high protein purity (Fig. 27). For G-CSF, no bands of aggregated protein could be detected at all.

For IFN α-2a samples, marginal dimer and trimer specimen were identified. Generally, the quantification of the band intensities revealed that the aggregated fraction account for less then 1 % of overall band intensity.

Extracted BSA revealed more pronounced aggregated bands than G-CSF and IFN α-2a, but it is to be outlined that the BSA applied inhered substantial fraction of oligomers already in the standard (Fig. 27).

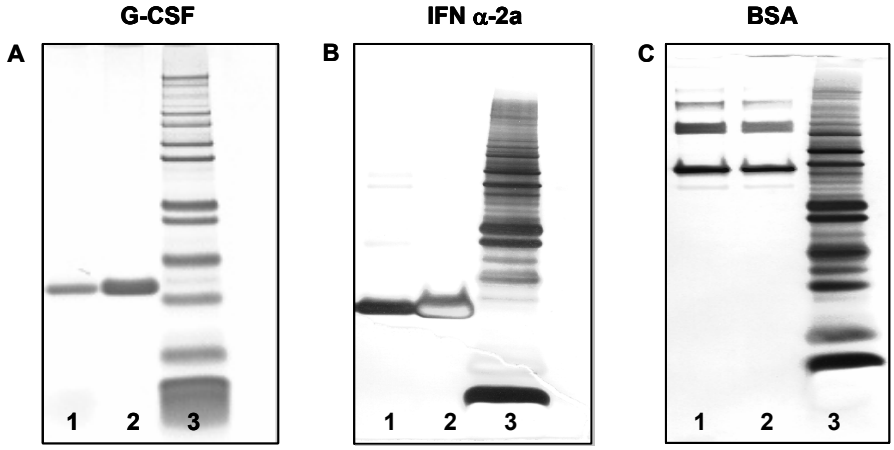


**Figure 27: Protein integrity after extraction from lipid implants without using organic solvents for (A) G-CSF, (B) IFN α-2a and (C) BSA. Lanes 1: extracted proteins, lanes 2: protein standards, lanes 3: MW marker.**

The results indicated that the newly developed extraction method inheres only low influence on protein stability, if any. The detected protein loss of approximately 10 % may be a result of either the grinding process or incomplete separation of protein and lipid particles. An IFN α-2a aggregate fraction of less then 1 % can be deemed as acceptable for investigative purposes. The more, as lyophilised IFN α products even available on the market contain up to 1 % aggregate [20].

Finally, it could be demonstrated that the manufacturing process by compressing did not induce aggregate formation. Consequently, lipid implants being loaded almost exclusively with native protein before release studies were initiated.

## 7. Summary and discussion

In the course of the pilot surveys described in this chapter, stearic acid was shown to reveal constraints in its applicability as carrier material for the controlled delivery of protein drugs. Despite the existence of multiple polymorphic forms could

have been overlooked in the first experiments, protein aggregation within a time range of 3 – 7 days induced by eroded stearic acid products was enough reason to abandon the effort of developing a delivery system based on this material.

Triglyceride systems featured only one polymorphic form after implant manufacture. However, the more important attainment of this pilot surveys was that protein integrity was not influenced by interactions with triglyceride material i.e. binding to erosion products or adsorption to lipophilic matrices. These findings were promising in terms of future protein release studies from these systems. The poor erosion behaviour of pure triglyceride systems namely excludes the control of protein liberation by the erosion process itself. However, a decelerated water uptake and the absence of degradation products may guarantee increased protein stability within triglyceride matrices during drug release.

Homogeneity studies using methylene blue as model drug featured non-uniform drug distribution within the matrices. As already discussed before, these findings have to be taken into account during release experiments because a non-uniform protein distribution can result in fluctuations of the liberation curves.

Moreover, the compressing process of protein/lipid mixtures was shown to work under relatively mild conditions. Protein integrity was retained during implant manufacture, what can be deemed as a major attainment - in contrast to results obtained with numerous PLGA devices [198; 184] – in terms of controlled protein delivery from lipophilic matrices.

At this stage of the thesis work, protein concentration was determined with Lowry assay. However, it was shown that the assay inhered high standard deviations throughout the experiments. As a consequence, chromatographic methods (SE-HPLC and RP-HPLC) were established for all further experiments for assessing protein concentration and stability.

## Chapter V: Protein delivery from lipid implants: Providing grounds…

The potential of triglycerides as compatible matrix materials for protein delivery, i.e. protein stability during manufacture, absence of degradation products influencing protein integrity during release etc. was framed in chapter IV. This chapter addresses basic approaches in the development of a protein delivery system which allows protein liberation over weeks in a linear mode.

Proteins were lyophilised with trehalose in a weight ratio of 1:4 (and with 0.05 % (w/v) polysorbate 80). The residual moisture of the freeze-dried products (table 11) ranged between 0.3 % for G-CSF products and 1.9 % for BSA products. Normally, it is aimed to reduce moisture contents in freeze-dried protein formulations to values below 2 %. This was also achieved for the lyophilised proteins, though IFN α-2a and BSA products featured borderline values.

| | G-CSF products | IFN α-2a products | BSA products |
|---|---|---|---|
| **Residual moisture content** | **0.3 ± 0.3 %** | **1.7 ± 0.5 %** | **1.9 ± 0.2 %** |

**Table 11: Residual moisture contents of protein formulations lyophilised with trehalose in 1:4 ratios and 0.05 % (w/v) polysorbate 20.**

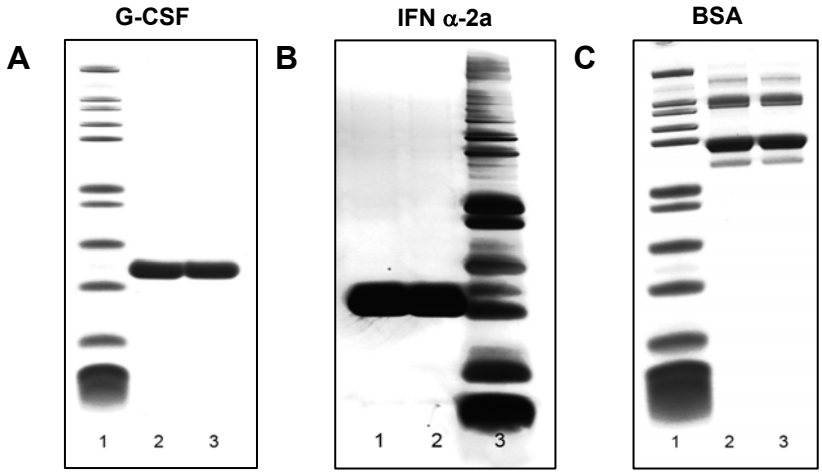


**Figure 28: Protein stability after reconstitution of the freeze-dried products. (A) G-CSF lyophilisate - lane 1: MW marker, lane 2: protein standard, lane 3: after reconstitution; (B) IFN α-2a lyophilisate - lane 1: after reconstitution, lane 2: protein standard, lane 3: MW marker; (C) BSA lyophilisate - lane 1: MW marker, lane 2: protein standard, lane 3: after reconstitution.**

PAGE analysis of reconstituted protein lyophilisates did not detect any aggregated species induced by the freeze-drying process (Fig. 28). These data verify protein integrity prior to implant preparation.

## 1. Influence of the drug load on protein release

The formation of an interconnected pore network during implant manufacture by compressing and the corresponding protein release kinetics from non-biodegradable matrices is reported to be mainly depending on the drug load [181; 101; 182].

Therefore, initial in vitro release studies were performed from triglyceride matrices loaded with up to 30 % lyophilised protein formulations consisting of a protein/sugar mix in a 1:4 ratio (table 12). Accordingly, the protein itself represents one fifth of the lyophilised formulation. After implant preparation, in vitro release studies were performed as described in chapter III. Each experiment comprised the analysis of three individual samples (n=3).

| Triglyceride | Lyoph. G-CSF formulation [%] | Lyoph. IFN α-2a formulation [%] | Lyoph. BSA formulation [%] |
|---|---|---|---|
| 90 % tristearin | 10 | 10 | 10 |
| 80 % tristearin | 20 | 20 | 20 |
| 70 % tristearin | 30 | 30 | 30 |
| 90 % trilaurin | 10 | 10 | 10 |
| 80 % trilaurin | 20 | 20 | 20 |
| 70 % trilaurin | 30 | 30 | 30 |

**Table 12: Triglyceride matrix formulations of various proteins with varying protein loads.**

When implants were loaded with 10 % lyophilised G-CSF, less than 10 % of the incorporated protein were liberated after 3 weeks of incubation. Higher protein loadings substantially increased the total releasable amount of protein up to 23 %. However, this was associated with higher burst release of G-CSF, followed by non-release and subsequent incomplete release after 1 month of incubation (Fig. 29).

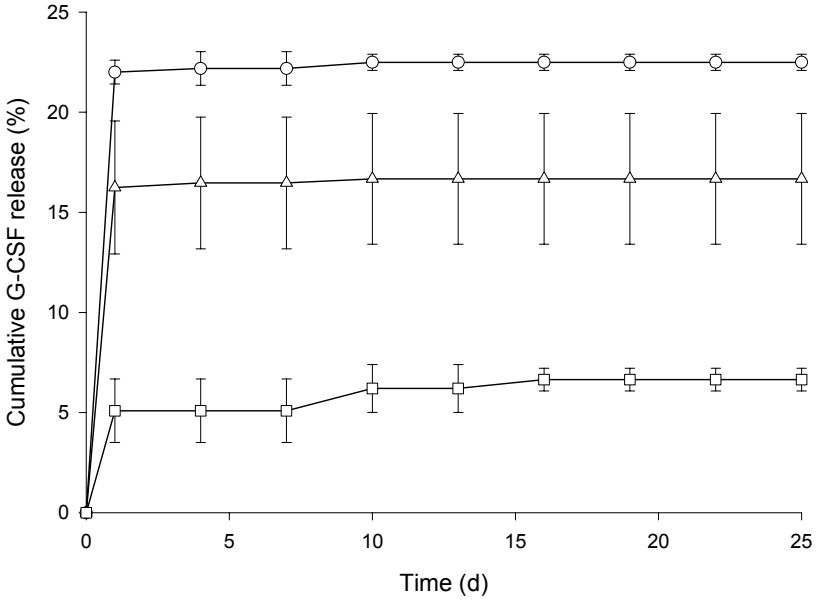


**Figure 29: Influence of the drug load on G-CSF release from tristearin matrices. Content of lyophilised G-CSF (with protein/sugar ratio being 1:4): 10 % (□), 20 % (△), 30 % (○) (average ± SD, n = 3).**

For IFN α-2a, experimental data were comparable (Fig. 30).

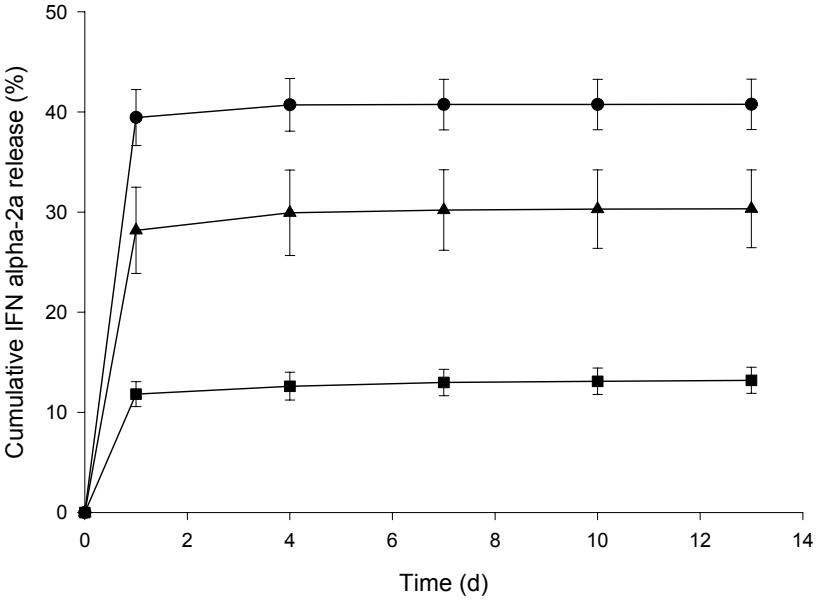


**Figure 30: Influence of the drug load on IFN α-2a release from tristearin matrices. Content of lyophilised IFN α-2a (with protein/sugar ratio being 1:4): 10 % (■), 20 % (▲), 30 % (●) (average ± SD, n = 3).**

Again, higher protein loads led to an increased burst release, but no extended protein liberation over the time could be achieved. Otherwise, 40 % of the incorporated IFN $\alpha$-2a were liberated.

For BSA matrices, in vitro release profiles are illustrated in Fig. 31. Whereas implants with a 30 % protein load caused a high burst release - e.g. 35 % BSA liberation within 24 hrs - the reduction of the drug load notably lowered BSA release rates. For example, devices with 10 % protein content came up with a sustained delivery over one month up to 40 % releasable protein.

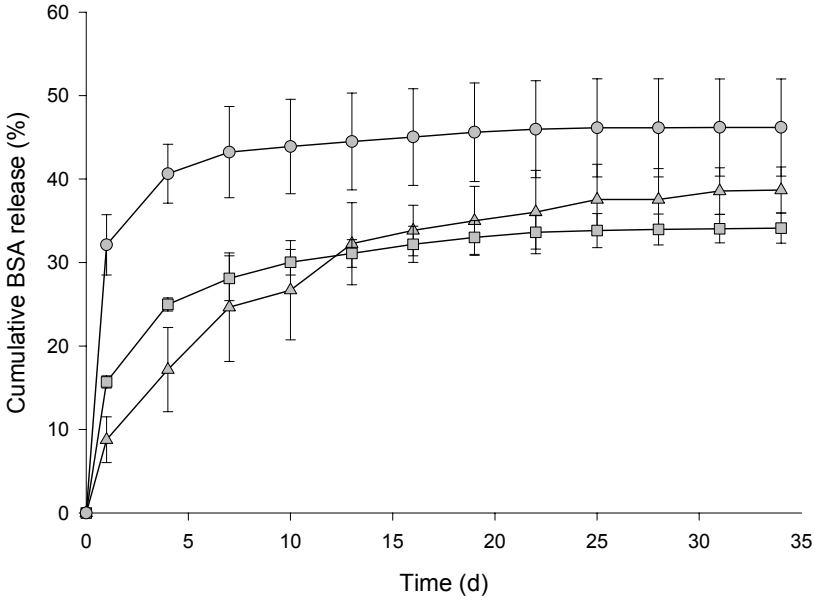


**Figure 31: Influence of the drug load on BSA release from tristearin matrices. Content of lyophilised BSA (with protein/sugar ratio being 1:4): 10 % (■), 20 % (▲), 30 % (●) (average ± SD., n = 3).**

Evaluating studies of trilaurin devices found no difference in protein release behaviour. Consequently, protein liberation was assumed to be independent from the fatty acid chain length of the triglycerides and thus from the lipophilic nature of the respective triglyceride.

However, the variation of the drug load will not result in absolute controlling the protein release. In particular for implants with a higher drug load high burst effects up to 40 % protein delivery were observed within the first hours. Extended liberation over time only could be obtained from BSA systems with 10 % drug load. However, incomplete protein release was also to be detected from these matrices.

## 2. Influence of the compression force on protein release

Packing arrangements of drug and matrix material particles can be affected by the compression force, which influences the pore characteristics and consequently protein release [101].

Exerting a pressure of 2 tons during implant production yields triglyceride matrices with high physical stability, mirrored by breaking strengths of ~27 N. Such compression forces configured a dense packaging arrangement wherein protein molecules were caught. Consequently, this may result in non-release or even in incomplete protein release.

Evaluation studies whether or not the compression force influences protein release were solely performed with BSA/tristearin devices. The actual BSA load represented 2 % (i.e. 10 % lyophilised protein formulation) of implant weight for all experiments. With a pressure below 1.2 tons no compact implants were obtained, i.e. the matrices broke apart when detached from the compacting tool. Therefore, implant manufacture was conducted by applying compression forces in a range from 1.2 – 2 tons over 30 sec.

Surprisingly, reduced compression forces (e.g. 1.2 and 1.5 tons) resulted in a lower protein release of about 20 % of the BSA initially incorporated (Fig. 32).

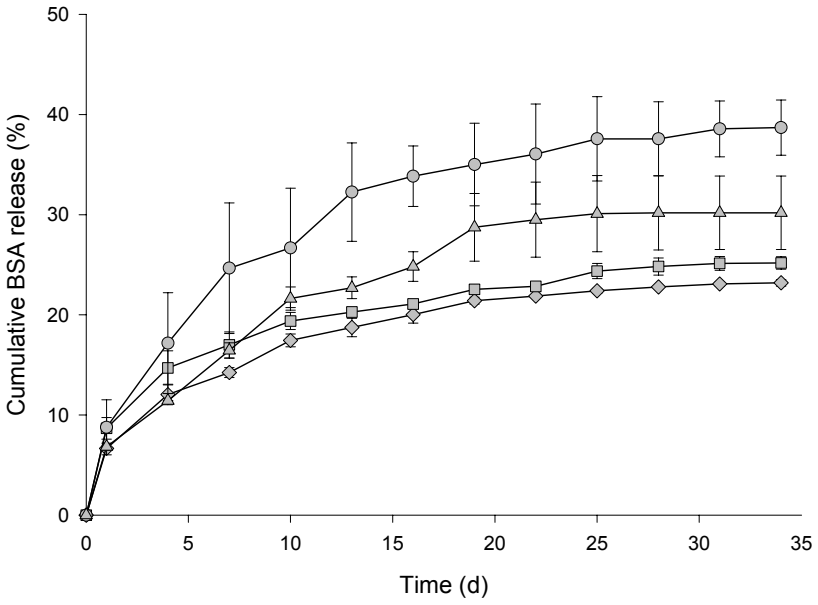


**Figure 32: Influence of the compression force during implant manufacture on BSA release. Compression force: 1.2 t (◆), 1.5 t (■), 1.7 t (▲), 2 t (●) (average ± SD, n = 3).**

When compared to matrices yielded with a pressure of 2 tons, the latter ones revealed release rates after 1 month of incubation of more than double that value.
Based on these data, it could not be substantiated that incomplete protein release is caused by a too tight packaging. In contrast, minimal compression forces appear to be required for a suitable particle arrangement which leads to the formation of pores and void spaces where the dissolved protein molecules can diffuse through.

## 3. Influence of various excipients on protein release

In order to enhance drug release from inert matrices, the incorporation of additives such as pore forming substances or emulsifying agents has been discussed [17; 187; 186; 98].

### 3.1 Incorporation of hydrophilic excipients - polyethylene glycol derivatives

Polyethylene glycol 6000 (PEG) and Poloxamer 188, an ABA block copolymer of ethylene glycol and propylene glycol, represent solid excipients easily miscible with the lipids in various ratios. Such additives are soluble in the release medium without inhering a swelling potential. Consequently, diffusion-driven protein release from triglyceride matrices can be increased by the formation of aqueous medium filled pores as a result of excipient dissolution [98]. In addition, these compounds feature a solubilising potential, and for PEG stabilising effects on proteins were noticed [43; 34].
Tristearin implants loaded with a varying amount of excipients were prepared (pressure = 2 tons). The actual protein load represented 2 % (i.e. 10 % lyophilised protein) of implant weight for all formulations (table 13).

| Formulation | Lyophilised protein [%] - G-CSF, IFN α-2a or BSA (protein/sugar ratio 1:4) | PEG 6000 [%] | Poloxamer 188 [%] | Tristearin [%] (Dynasan 118) |
|---|---|---|---|---|
| I | 10 | - | - | 90 |
| II | 10 | 2 | - | 88 |
| III | 10 | 5 | - | 85 |
| IV | 10 | - | 2 | 88 |

**Table 13: Tristearin matrix formulations with various excipients such as pore forming substances and emulsifiers with respect to their influence on protein release (n = 3).**

For G-CSF experiments, the incorporation of PEG lowered protein liberation in comparison to the protein amount delivered from pure tristearin implants (Fig. 33). Only 5 % protein were released from such devices.

When Poloxamer 188 was applied, more protein was liberated. However, still 88 % of the incorporated G-CSF retained unreleased after the incubation time (Fig. 33).

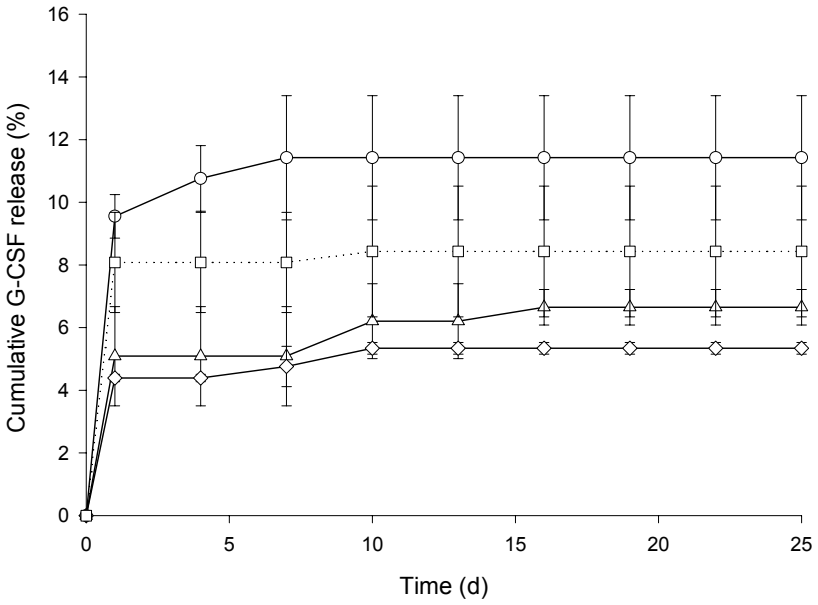


**Figure 33: Influence of pore forming substances on G-CSF release from tristearin implants. PEG content: 0 % (□), 2 % (△), 5 % (◇); Poloxamer 188 content: 2 % (○) (average ± SD, n = 3).**

Poloxamer 188 also increased the total amount of releasable IFN α-2a and BSA up to 20 % and 42 %, respectively, when compared to matrices without that excipient (Fig. 34 A/B). However, the non-delivered fractions remaining within the matrices after incubation were still dominant.

When PEG was applied, somewhat different effects for the particulate protein were observed (Fig. 34 A/B): compared to the excipient-free devices, IFN α-2a liberation slightly increased and was more sustained, but a lower total amount of BSA was delivered from such matrices.

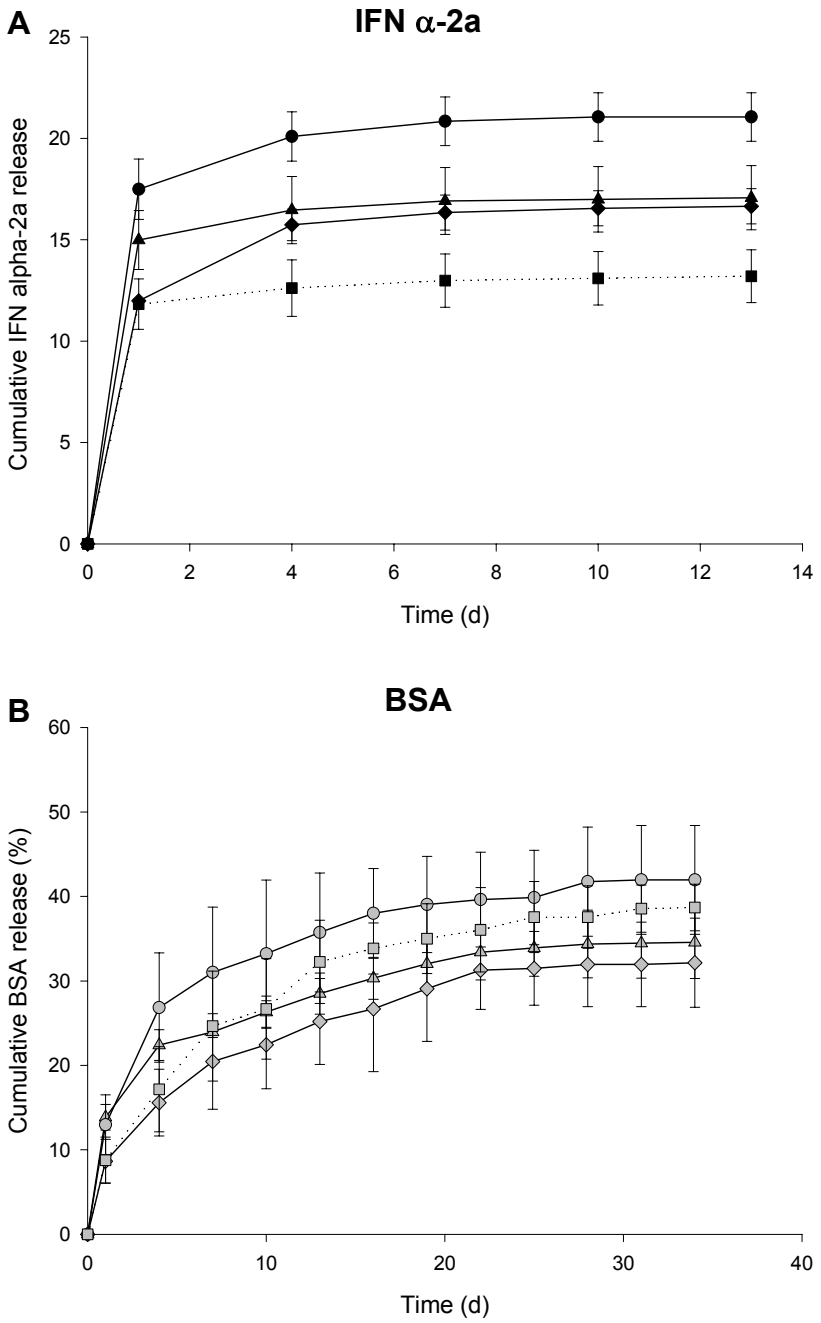


**Figure 34: Influence of pore forming substances on protein release from tristearin matrices. (A) IFN α-2a - PEG content: 0 % (■), 2 % (▲), 5 % (◆); Poloxamer 188 content: 2 % (●); (B) BSA – PEG content: 0 % (■), 2 % (▲), 5 % (◆); Poloxamer 188 content: 2 % (●) (average ± SD, n = 3).**

The addition of pore forming agents (e.g. PEG and Poloxamer 188) in contents of 2 - 5 % of the implant weight did not accomplish the results anticipated. Neither a substantial increase of releasable protein nor a delivery control could be reported.

## 3.2 Incorporation of 1,2-Dioleyl-sn-Glycero-3-Phosphocholine (DOPC)

Phospholipids like DOPC are commonly used as membrane constituents of liposomal delivery systems. While DOPC is insoluble in the release medium, it possesses an emulsifying and swelling potential. Thereby, the formation of a swollen structure with higher water content was expected during release which could result in increased protein liberation [105].

Tristearin implants with a protein load of 2 % (i.e. 10 % lyophilised protein formulation) and with a 5 % and 10 % DOPC content, respectively, were prepared. Thereby, a reduction in the compressibility of the mixture was notified. During incubation, matrices featured a distinctive water uptake followed by partial surface degradation of the implants.

In Fig. 35 release profiles of G-CSF implants are demonstrated. The visible surface degradation effects were assumed to facilitate G-CSF release. In contrast, decreased levels of G-CSF delivery were observed when compared to matrices without DOPC charge (Fig. 35).

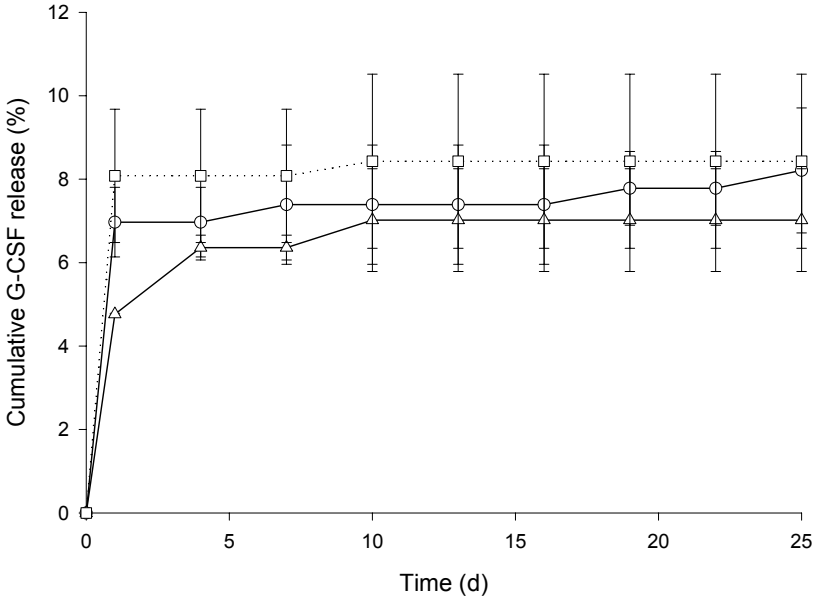


**Figure 35: Influence of an emulsifying phospholipid (DOPC) on G-CSF release from tristearin matrices. DOPC content: 0 % (□), 5 % (○), 10 % (△) (average ± SD, n = 3).**

For IFN α-2a samples, the release profiles indicated that the addition of DOPC to tristearin matrix material resulted in a substantial change in protein release kinetics (Fig. 36).

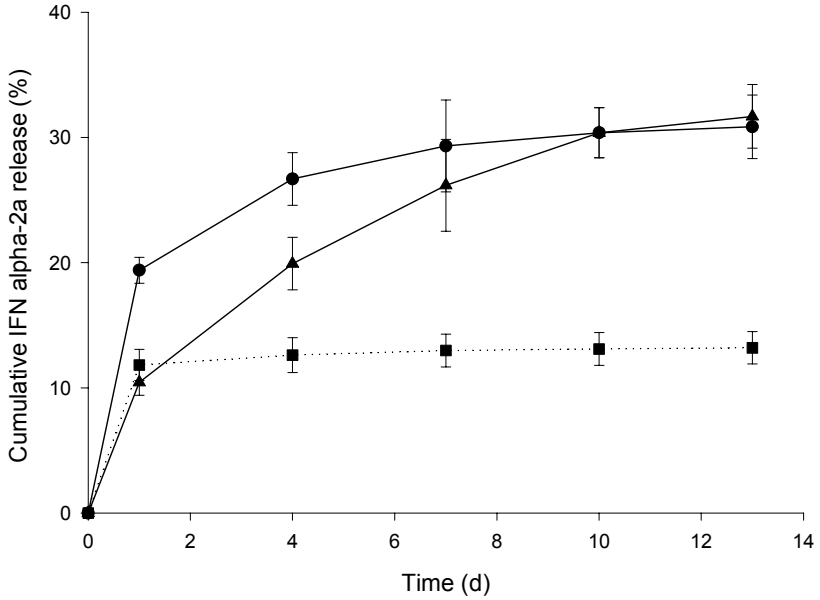


**Figure 36: Influence of an emulsifying phospholipid (DOPC) on IFN α-2a release from tristearin matrices. DOPC content: 0 % (■), 5 % (●), 10 % (▲) (average ± SD, n = 3).**

When compared to protein delivery rates from matrices without DOPC, implants containing such excipient featured extended protein releases. A 10 % DOPC load induced 30 % of the incorporated protein being continuously liberated for 12 days incubation.

The effect of DOPC on BSA release was not comparable the findings presented above (Fig. 37). Only a marginal enhancement in delivered protein quantities was notified, and the release profiles were less sustained compared to those obtained from excipient-free matrices.

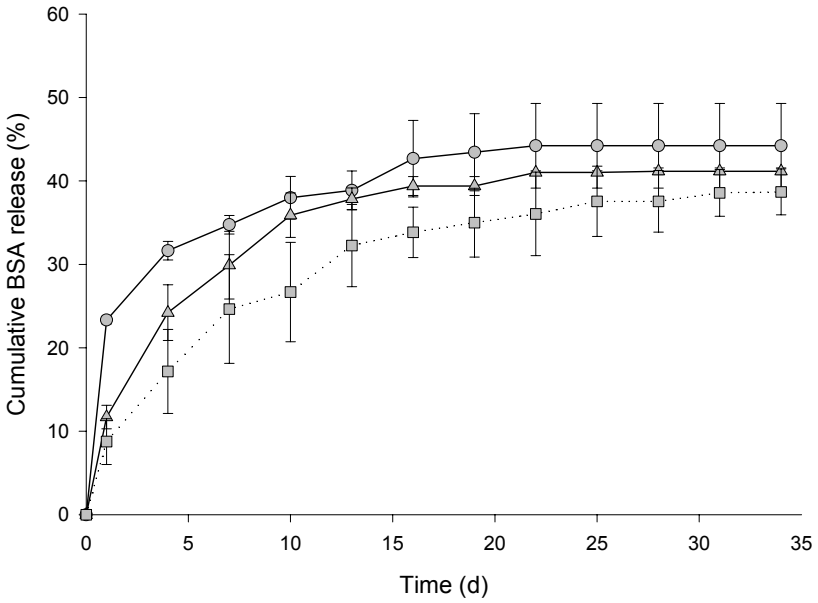


**Figure 37: Influence of an emulsifying phospholipid (DOPC) on BSA release from tristearin matrices. DOPC content: 0 % (■), 5 % (●), 10 % (▲) (average ± SD, n = 3).**

In principle, DOPC as emulsifying compound increased the water uptake. Furthermore, surface degradation of the matrices was notified during release studies. This erosion behaviour resulted in a promising change of IFN α-2a release kinetics and moreover, in a prolonged IFN α-2a liberation for up to 2 weeks.

## 4. Summary and discussion

Generally, these basic in-vitro release studies demonstrated the potential of protein delivery from lipophilic triglycerides matrices. However, the release kinetics achieved were inappropriate. High burst release followed by non-release and subsequent incomplete protein release was noticed as hampering parameters.

An extended liberation profile was neither obtained by drug load increase nor by the reduction of the compression forces. This results did neither overlap with what was expected, nor was the published information matched [117; 12; 101]. Langer et al. demonstrated BSA release from ethylene vinyl acetate matrices being proportional to the square root of time when very high protein loads (e.g. 30 – 50 %) were applied. With decreasing drug loads, the release curves were more concave and resulted in zero-order release at low protein load scenarios (e.g. 5 % - 10 % BSA load). The

change in the release kinetic was assumed to be the result of a considerable amount of entrapped protein at low drug loadings [181; 182].

Our results obtained from triglyceride matrices were not comparable to this theoretical model of macromolecule release from inert matrices. Higher protein loads resulted in higher burst release, however, protein liberation did not continue over time in the majority of cases. Only for BSA, a continuous liberation up to 1 month from tristearin matrices with a 10 % drug load could be obtained. Though, almost 60 % of the BSA remained within the implants after release experiments. A higher BSA load resulted in a higher burst release, but the total amount of releasable protein increased only slightly, if at all. Contrarily, the group of Benoit reported a 100 % BSA release from trimyristin matrices loaded with only 13 - 24 % protein [155].

Furthermore, it was demonstrated that protein release was almost independent of the used triglyceride itself. The fatty acid chain length of the triglyceride and consequently the lipophilic nature of the triglyceride did not impact protein release kinetics. That is consistent to erosion studies featuring no considerably increased water uptake of trilaurin devices in comparison to tristearin products.

Incorporating hydrophilic release modifiers such as PEG and Poloxamer 188 featured a moderate outcome with regards to an increased protein release. The additional excipients enhanced the amount of total releasable protein from tristearin implants marginally, if at all. For polyethylene glycol, G-CSF and BSA liberation rates decreased slightly when compared to the protein delivery from tristearin implants without any excipients added.

Those side-effects of the hydrophilic compounds could be explained by excipient contents remaining under a certain effective concentration threshold. That means, the addition of 2 % pore forming excipient was too low in order to achieve a substantial influence on the formation of an interconnected pore network and, consequently, on the protein release. Jiang and Schwendeman reported that less than 45 % of BSA were released from PLGA microspheres when the PEG content was less than 10 % of the polymer weight. When the PEG content was raised to 20 %, the amount of total protein released notably increased up to 75 % [98].

Solid release modifiers like PEG may be involved in the formation of an interconnected pore network during implant manufacture. When the amount added is too low, the resulting network is incomplete, particularly when the drug load accounts

for only 10 % of implant weight. Consequently, both protein and excipient particles can be entrapped by lipophilic tristearin material.

The addition of the amphiphilic 1,2-Dioleyl-sn-Glycero-3-Phosphocholine (DOPC) resulted in visible swelling and degradation effects, predominantly at the surface of the tristearin implants. Based on this surface erosion, the release kinetics of IFN $\alpha$-2a changed substantially, as a continuous protein liberation for up to 2 weeks was achieved by adding 10 % DOPC. However, the amount of total releasable proteins was deemed still insufficient.

Eroded material may be assumed to cause protein aggregation during release studies, but this hypothesis was not confirmed by gel electrophoresis.

A further reason for incomplete release is that effective DOPC levels could not be reached. In the literature it is reported that only 40 % of the encapsulated BSA were liberated from cholesterol implants with a 30 % lecithin load. The raise in lecithin content up to 60 % was shown to enforce erosion rate of cholesterol/lecithin implants, and resulted in an increased protein release up to 100 % [105].

However, it was not rendered possible to produce tristearin implants with higher DOPC contents (e.g. 30 – 50 %). The lubricious consistence of the mixtures caused a substantial decrease of material compressibility and resulted in the production of unformed, sticky implants.

For G-CSF, no release higher than 23 % in relation to the incorporated protein content was achieved during all experiments. A great part of the G-CSF molecule consists of hydrophobic regions [133; 86], and consequently it was assumed G-CSF accumulating within the hydrophobic tristearin environment due to adsorption processes throughout the release studies. This effect may enormously hamper the development of a controlled G-CSF release system based on triglyceride material.

Overall, new formulation strategies (e.g. variation of the lipid matrix) with respect to a sustained, controlled protein delivery from lipid implants were reconsidered so far. Furthermore, detailed analytical studies - applying gel electrophoresis or conducting long-term protein stability monitoring - appeared necessary to exclude protein instability as possible reason for incomplete drug release.

The following chapters will outline how improvements in enhancing and controlling protein delivery from lipid implants have been realised. Thereby chapter VI addresses investigations performed with BSA as a model protein compound. In

chapters VII and VIII, the development of a controlled release system for IFN $\alpha$-2a as pharmaceutically relevant protein is outlined. As a result of the basic investigations showed in this chapter, experimental work on G-CSF as protein compound was finalised at that state.

# Chapter VI: Quickening the paces: BSA as model protein

## 1. Alternative lipophilic matrix materials

Tristearin implants were demonstrated to be very lipophilic and thus water repellent drug delivery systems. The extremely low water uptake into the matrices during release experiments potentially inhibits dissolution and subsequent diffusion processes of encapsulated proteins, what results in incomplete protein release. Therefore, mono-/diglycerides and polyglycerol esters, which inhere a less hydrophobic nature than triglyceride material, were investigated in terms of their applicability as sustained, controlled protein release devices.

### 1.1 Mono– and diglyceride implants

Sustained release features of monoglycerides for amphiphilic low molecular weight drugs are described in literature. Thereby, drug release followed the square-root of time relationship during the initial release phase [35].

Monoglycerides and diglycerides have an emulsifying potential. For example, glycerol monostearate with a hydrophilic-lipophilic balance (HLB) value of about 3.8 is commonly used as lipophilic emulsifier in several cosmetic products.

Thereby, water uptake and swelling power of such implant systems may be substantially increased in comparison to tristearin devices. Consequently, diffusion-driven protein liberation could be facilitated.

Monoglycerides (Myverol 18-04 K) and a composition of mono- and diglycerides (Admul MG 40-04 K) were investigated on both matrix material use and, in a 1:1 combination excipient use with tristearin matrix material. All implants were loaded with 10 % lyophilised BSA (protein/sugar ratio 1:4), which corresponds to an actual protein load of 2 % of implant weight.

The experimental results made obvious that monoglycerides as well as the composition of mono- and diglycerides are not feasible as matrix materials for sustained BSA release devices. Both formulations exhibited high burst release, with up to 55 – 65 % of the incorporated protein being delivered from the implants within 4 days incubation (Fig. 38).

As an example for excipient use, the 1:1 combination of monoglycerides and tristearin material is shown (Fig. 38). However, this formulation was also found to

feature an enormous burst, whereby a total of 45 % BSA were released within 4 days.

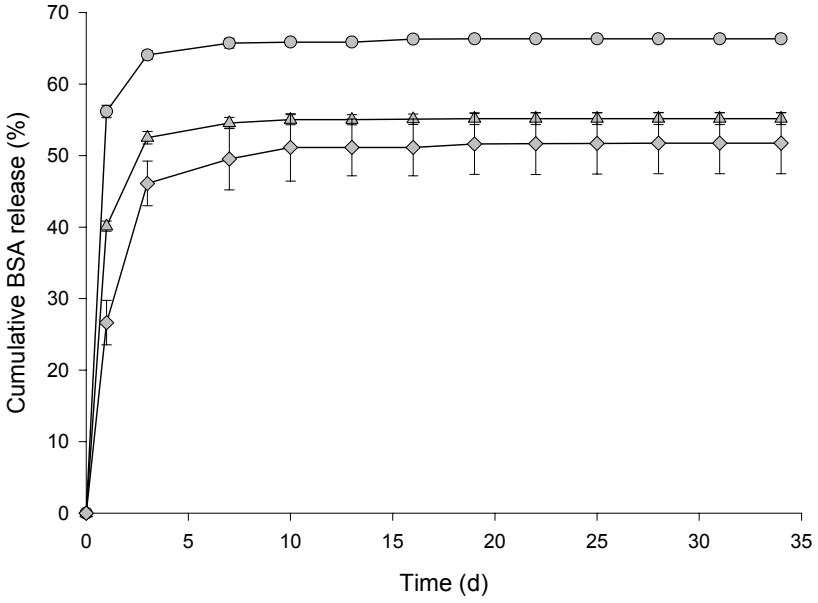


**Figure 38: BSA release from mono- and diglycerides devices. Matrix material: monoglycerides (●), composition of mono- and diglycerides (▲), 1:1 combination of monoglycerides and tristearin material (◆) (average ± SD, n = 3).**

Swelling and erosion of the matrices were not visible, therefore it is difficult to explain the increased but also accelerated BSA release from these systems in comparison to release profiles obtained when pure tristearin material was used (refer to chapter V). Nevertheless, the increased hydrophilic nature of mono- and diglyceride materials appears to be casting for this change, because an obvious reduction in total released protein was already notable when - instead of pure monoglycerides - the less hydrophilic mono-/diglyceride composition was applied.

### 1.2 Implants prepared with polyglycerol ester of fatty acids (PGEFs)

The use of polyglycerol ester of fatty acids as sustained release dosage forms for proteins were first reported by Yamagata et al. [220]. In accordance to previous work with low molecular weight drugs such as theophylline, protein release was shown being a diffusion-controlled process dependent on the degree of fatty acid esterification and the chain length of the fatty acids [3; 220].

Pilot studies with protein free devices featured compressed polyglycerol ester matrices swelling within hours. After a few days, this swelling was followed by a complete degradation of the implants during a 3 weeks incubation interval (Fig. 39).

**Figure 39: Swelling behaviour of polyglycerol ester implants during in vitro release studies. Left: implant immediately after incubation initiation, right: implant after 4 days incubation.**

That supposed both the diffusion-controlled process and the erosion to potentially contribute to optimisation of protein release.

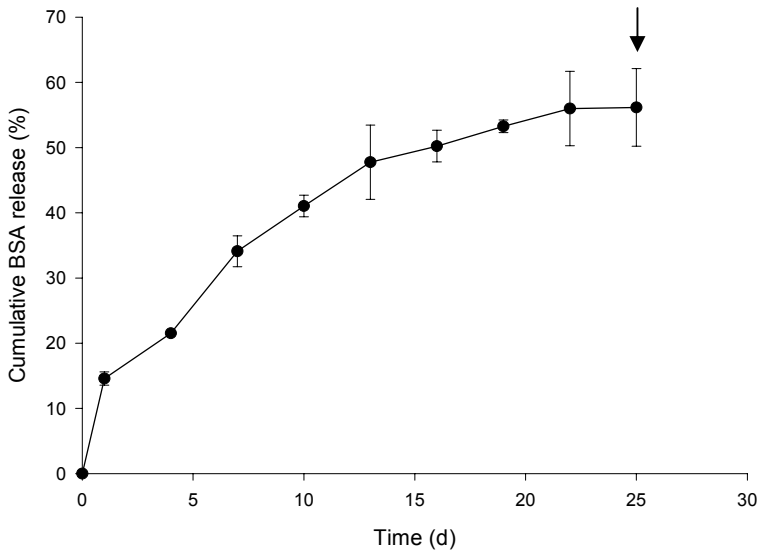


**Figure 40: BSA release profile from polyglycerol ester implants. Matrices were completely eroded after 25 days of incubation (↓) (average ± SD, n = 3).**

Actually, polyglycerol ester matrices loaded with 10 % lyophilised BSA (protein/sugar ratio 1:4) showed a continuous BSA delivery for up to 25 days, whereby the burst release was reduced to 15 %.

However, despite a complete degradation of the matrices within the monitored time period, only 55 % of the incorporated protein were detected in the release media by SE-HPLC (Fig. 40).

SE-HPLC revealed a substantial drop in BSA monomer content down to 65 % after 4 days of incubation (Fig. 41). At this time an additional peak was notified suggesting the occurrence of higher-order aggregate species. After 13 days of incubation this aggregate fraction already represented 20 % of total BSA content. In addition to that, BSA aggregation appeared to be linked with matrix swelling and erosion.

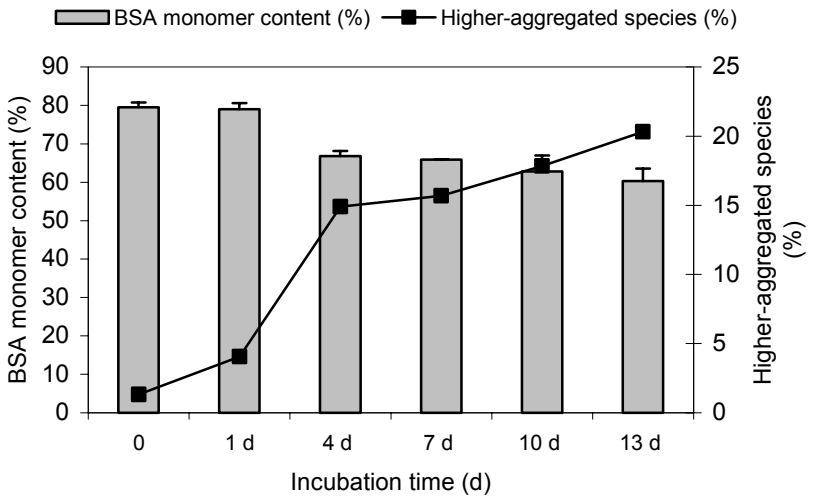


**Figure 41: BSA monomer (%) and higher-order aggregate content (%) detected within the release media at different points of time during in vitro release studies (average ± SD, n = 3).**

As the matrices become completely hydrated the solid protein is exposed to increasing moisture. That results in an increase of protein mobility, possibly accelerating chemical reactions as well as protein denaturation and moisture-induced aggregation [48; 121].

Therefore, it was assumed that the enormous water uptake of polyglycerol ester matrices increased the BSA aggregation tendency within the implant systems. Moreover, soluble aggregate species detected by SE-HPLC indicated also the potential formation of insoluble aggregate species during release experiments. Due to filtration of the release media prior to SE-HPLC investigations, these insoluble protein fractions, if present, were separated from the buffer samples and retained upon the filter material.

Besides, non-covalent and covalent interactions between eroded matrix material and protein molecules – as often described for PLGA devices [106; 50] – are assumed and such protein/lipid fractions also stuck upon the used filters.

In order to specify the formation of insoluble aggregates as well as protein interactions with the lipid material, filter components used for sample preparation prior to SE-HPLC analysis were collected and incubated in each 2.0 mL SDS solution (2 % SDS (w/v)) at 37 °C over 24 hrs. High-concentrated SDS solutions enable the disruption of ionic interactions and non-specific protein adsorption. Furthermore, insoluble non-covalent aggregates can be dissociated by SDS [197; 144].

Thus, protein species potentially removed by filtration may be dissolved in the SDS solution by incubating the filter material and consequently be available for analytical investigations (Fig. 42).

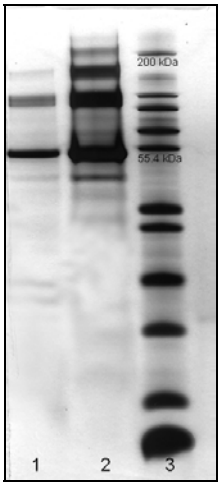


**Figure 42: BSA recovery in SDS solution after incubation of the filter material for 24hrs. Lane 1: BSA detected in the SDS solution, lane 2: protein standard, lane 3: MW marker.**

PAGE demonstrated BSA being present in the analysed supernatant solutions (Fig. 42). The bands were less pronounced, but virtually similar to those in the standard preparation.

Based on these data it was confirmed that polyglycerol ester matrices exhibited a detrimental effect on protein stability during in vitro release studies. This led to the formation of insoluble aggregates and/or to direct interactions between protein molecules with eroded lipid material. Consequently, the detected 45 % protein loss can be attributed to those BSA species.

### 1.3 Conclusion

It was demonstrated that the use of mono-/diglycerides and polyglycerol ester of fatty acids (PEGFs) as alternative matrix systems to tristearin implants did not show the data expected. The total amount of releasable BSA was increased when mono- and diglycerides were applied, but the observed release patterns, featuring a high burst release of up to 65 %, followed by non-release and incomplete release, were insufficient.

For PEGFs, the assessed BSA release was ongoing in a continuous manner over 25 days, what initially appeared very promising. But then protein instability as a result of implant swelling and degradation was noticed - a hampering issue. Not only this resulted in a 45 % protein loss during in vitro release, but the liberation of higher-aggregate species is intolerable in the light of potential immunogenical response of patients [41; 33].

## 2. BSA release from tristearin matrices with higher amounts of PEG

As already discussed, the use of polyethylene glycol as effective protein release modifier requires the incorporation of PEG in contents of at least 10 % of implant weight [98].

In a second survey, PEG contents in a range of 10 - 30 % were investigated in their influence on BSA release from tristearin matrices. Protein load was set to 10 % lyophilised BSA formulation (with protein/sugar ratio 1:4).

Raising the PEG content led to a substantial enhancement in overall protein liberation. For example, by increasing the PEG amount from 5 % up to 10 % (Fig 43), approximately twice as much BSA was released from implant systems. The release kinetic indicated a sustained mode, featuring a continuous protein delivery for up to 1 month.

In contrast, when the PEG content was increased to 20 % or 30 %, respectively, an accelerated protein release was notable, resulting in high bursts of up to 70 % BSA liberation within 4 days (Fig. 43).

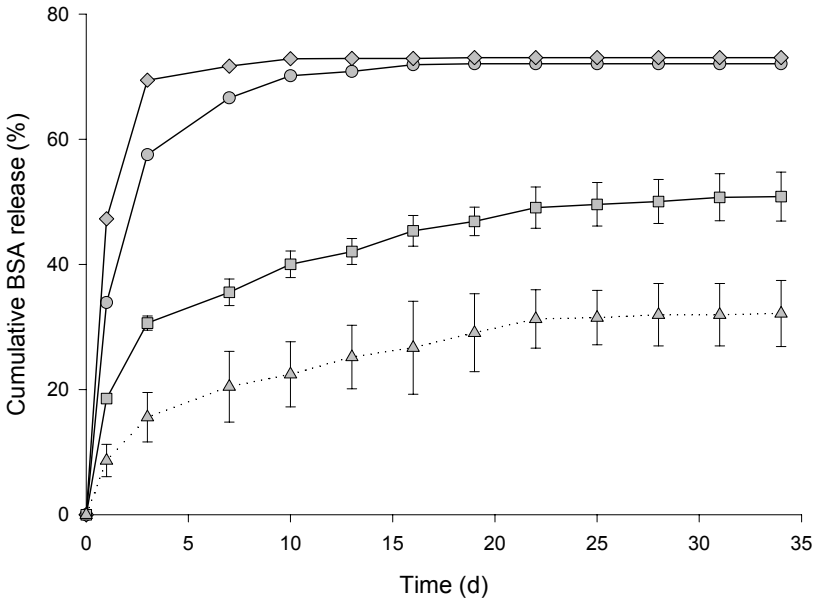


**Figure 43: Effect of higher PEG contents on BSA release from tristearin matrices. PEG content: 5% (▲), 10 % (■), 20 % (●), 30 % (◆) (average ± SD, n = 3).**

However, despite of an increased PEG content of up to 30 % implant weight, the BSA release obtained was still incomplete. Maximally, 70 % of the originally incorporated protein were liberated from the devices. SE-HPLC of the samples did not feature - as they did for polyglycerol ester matrices - higher-order aggregate species being evident in the release media.

However, polyglycerol ester matrices eroded completely over time, whereby even higher-order aggregates were liberated. In contrast, from non-biodegradable implant systems, the release of aggregates and especially precipitates could be decelerated or even restricted due to the reduced solubility of aggregates or because of the pore sizes within the matrices [106; 216].

In order to elucidate processes occurring within the matrices during release studies, the BSA portion, which remained in the devices after the 4 weeks release period was extracted and analysed by PAGE.

BSA recovered from tristearin formulations containing 30 % PEG showed a band very similar to the standard BSA band. The quantification of the band intensities revealed for both the extracted protein and the standard about 79 % monomer, 17 % dimer, and 4 % trimer species (Fig. 44), what is comparable to the standard BSA.

However, it has to be mentioned that even if these results refer to high protein stability within the tristearin matrices during release, the formation of non-covalent aggregate species and potential insoluble aggregates can not excluded by these SDS-PAGE data.

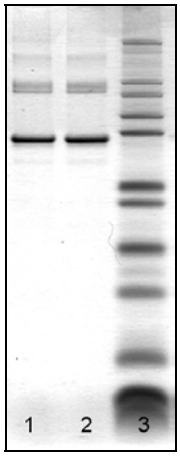


**Figure 44: BSA stability within the matrices during in vitro release. Lane 1: BSA extracted from tristearin implants with a 30 % PEG content after incubation, lane 2: protein standard, lane 3: MW marker.**

The use of polyethylene glycol 6000 (PEG) as pore forming agent was demonstrated to be very promising: (I) the amount of releasable BSA was increased in comparison to PEG free implants; (II) BSA was delivered continuously from tristearin implants with a 10 % PEG content for 1 month, and (III) high protein stability within the matrices can be assumed.

Nevertheless, a final conclusion explaining the incomplete protein release could not be drawn from these experiment data.

As a consequence, it was tested whether a change of the freeze-dried formulation would enable a 100 % BSA delivery from tristearin implants containing PEG.

## 3. Optimisation of the freeze-dried BSA formulation

### 3.1 Stabilisation of proteins during freeze-drying

Many proteins show conformational changes induced by the freeze-drying process [6]. Possible parameters affecting protein stability can be the formation of ice-water interfaces, freeze concentration of solutes and proteins, low temperature stress and potential pH changes due to selective precipitation of buffer components.

During the drying step, the loss of the protein hydration shell can induce protein instability [1]. To enhance the stability of freeze-dried products, usually excipients are added to the protein solution. Three mechanisms of protein stabilisation by additives during freeze-drying are discussed: (I) the Timasheff mechanism [8; 7], (II) the "glassy state" theory [67] and (III) the water replacement theory [51; 29; 28; 7].
Several excipients - e.g. sugars, amino acids, salts, polyols or polyethylene glycol - providing protein stability as cryoprotectants during the freezing step have been established in the formulation of freeze-dried protein products [214]. But only a few sugars such as sucrose and trehalose appear to be effective in protecting proteins from inactivation during the drying step [7; 30].
Furthermore, non-ionic surfactants such as polysorbates in low concentrations of 0.01 – 0.1 % are commonly added to protein formulations, in particular to inhibit protein unfolding as a result of ice/water interface adsorption during the freezing step and, secondly, to reduce protein aggregation tendency during later reconstitution [9].

## 3.2 Optimisation of the freeze-dried BSA formulation

At the beginning of the thesis work, BSA (conc. 20 mg/mL in ultrapurified water) was lyophilised with trehalose in a ratio of 1:4 (and with 0.05 % polysorbate 80). The analysis of the reconstituted samples by SE-HPLC did not indicate the freeze-drying process inducing BSA aggregation (table 14). Moisture content was found to be 1.9 %, achieving the maximal 2 % threshold of freeze-dried products commonly aimed for, and PAGE also did not exhibit further aggregated species (refer to chapter V). Though, the obtained lyo-cakes showed collapsed structures.

| | SE-HPLC | | | | Water content [%] |
|---|---|---|---|---|---|
| | Recovery [%] | Monomer [%] | Dimer [%] | Trimer [%] | |
| **BSA raw material** | - | 78.07 ± 1.31 | 16.60 ± 0.56 | 5.65 ± 0.89 | - |
| **Lyophilised BSA after reconstitution (BSA/sugar ratio 1:4)** | 99.13 ± 1.1 | 78.23 ± 0.45 | 16.05 ± 1.20 | 4.83 ± 1.52 | 1.9 ± 0.2 |

**Table 14: BSA integrity of freeze-dried products after reconstitution and determined water contents of the lyophilised products (average ± SD, n=3).**

Collapse is not necessarily detrimental to the long-term stability of freeze-dried proteins. For example, collapsed cake matrices of recombinant Factor VIII showed no difference in biological activity during storage at either 5 °C or ambient

temperature when compared to pharmaceutically acceptable products. At 40 °C storage, the stability of the collapsed products even appeared to be better than that of freeze-dried products with no collapse [208].

However, from a pharmaceutical point of view, collapsed freeze-dried formulations are unacceptable. Therefore new batches of BSA solutions with various levels of trehalose (and with 0.05 % polysorbate 80) were prepared. Conducting the freeze-drying process resulted in collapsed structures for BSA samples lyophilised in protein/sugar ratios of 1:4 and 1:5, respectively, whereas formulations with 1:1, 1:2 or 1:3 protein/sugar mixtures revealed amorphous cake structures (Fig. 45).


**Figure 45: Images of BSA freeze-dried with trehalose in various ratios. Left: 1:4 protein/sugar formulation resulting in collapsed freeze-dried products; right: 1:3 protein/sugar formulation resulting in amorphous freeze-dried products.**

A higher BSA content improved drying speed, and at the end of the primary drying such formulations were already ice-free. In contrast, the 1:4 and 1:5 mixtures contained residual ice at this state of the process and collapsed when shelf temperature was increased for secondary drying [97].

After reconstitution, BSA integrity in the amorphous products - i.e. 1:1, 1:2 and 1:3 protein/sugar mixtures - was confirmed by SE-HPLC, revealing 79 % monomer, 16 % dimer and 5 % trimer in average. The residual moisture contents of non-collapsed lyophilisates were substantially lower when compared to the collapsed samples, ranging between 0.3 – 0.7 %. Generally, a moisture content of trehalose formulations below 1 % implicates $T_g$ values well above room temperature, an important point with respect to long-term storage of freeze-dried pharmaceutics [1].

### 3.3 Influence of the freeze-dried formulation on BSA release from tristearin implants

The most promising matrix composition for controlled BSA release during the release studies conducted hitherto were tristearin implants containing PEG in a range of 10 - 30 %. Therefore, these matrix compositions were investigated again, employing non-collapsed freeze-dried BSA products.

The sugar itself also can affect protein release from lipid implants by the formation of pores as a result of sugar dissolution in the release medium. Consequently, BSA samples lyophilised with trehalose in a ratio of 1:3 were chosen from the reservoir of new formulations in order not to substantially change the protein/sugar ratio within the implants when compared to previous matrix compositions.

In Fig. 46, BSA release profiles of non-collapsed freeze-dried products (A) in comparison to profiles obtained from the collapsed products (B) are illustrated. Obviously, the freeze-dried formulation had a substantial impact on the release kinetics and on the amount of total releasable protein. Independent from the added PEG amount, more than 90 % of the incorporated BSA were delivered from tristearin implants over 12 days of incubation. Thereby, high burst release was observed, as 70 – 80 % of BSA were liberated within 24 hours (Fig. 46A).

Contrarily, when collapsed protein/sugar formulations were encapsulated, a continuous BSA liberation from tristearin matrices with a 10 % PEG content was achieved for up to one month (Fig. 46 B). However, the protein could not be released completely from the implants, even with higher PEG contents. After 1 month of incubation 30 - 50 % of the originally incorporated BSA retained within the matrices.

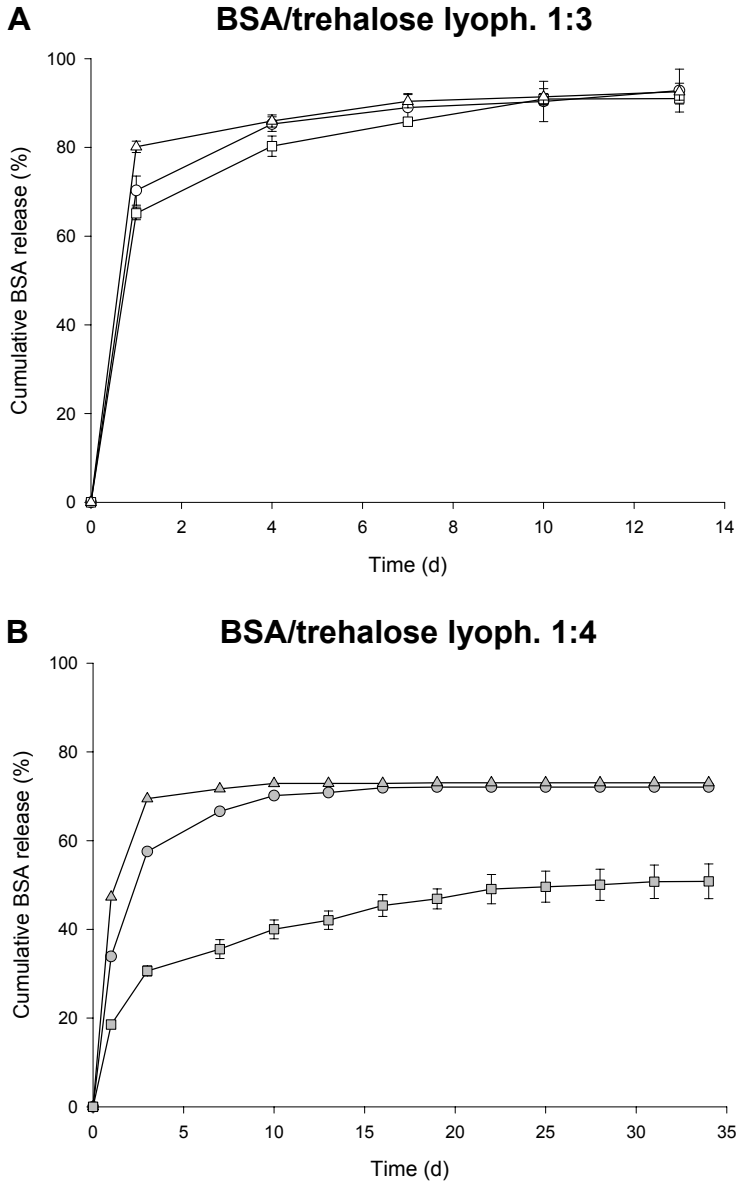


**Figure 46: Influence of the freeze-dried BSA formulation on protein release from tristearin matrices containing PEG as additive. (A) Non-collapsed BSA/trehalose lyophilisates (ratio 1:3) - PEG content: 10 % (□), 20 % (○), 30 % (△); (B) collapsed BSA/trehalose formulation (ratio 1:4) - PEG content: 10 % (■), 20 % (●), 30 % (▲) (average ± SD, n = 3).**

## 4. Summary and discussion

The hampering issue of incomplete BSA release was reported in previous release studies. This phenomenon was independent from matrix composition, drug load and the addition of various excipients. Based on the investigations outlined in this chapter, the problem could be deemed resolved.

The use of collapsed freeze-dried formulations was responsible for remaining protein fractions within implant systems during release experiments. For this, two potential explanations can be stated:

(I) Collapsed freeze-dried BSA formulations inhere an increased instability potential what leads to aggregation proceeding in the implant interior when water is present during release [32; 226]. Here, the formation of insoluble aggregates is predominantly suggested, since soluble aggregates have been detected by SE-HPLC and PAGE studies described in the upper part.

Insoluble aggregates per definition exhibit no solubility resulting in a restricted release of these species. In addition to that, pores and in particular the pore connecting channels throughout protein molecules must diffuse in order to be liberated from the inert matrices are known to be very narrow [106; 216]. Consequently, higher-order aggregate species may be inhibited from diffusion processes by getting stuck in the tortuous pore and channel net.

(II) Collapse of freeze-dried formulations can reduce the solubility of the obtained products [2]. Consequently, encapsulating collapsed freeze-dried protein formulations within lipid implants may lead to slower dissolution and subsequent diffusion of protein molecules, and thus to the retardation of protein release from these matrices.

Changing the freeze-dried formulation allowed the protein to be liberated almost completely from tristearin matrices charged with PEG as additive – more than 90 % of the originally incorporated BSA were detected in the release media. Being as important, the protein was found to be released in its native form.

The formulation change also involved a substantial impact on the release kinetics. High burst release of up to 80 % BSA was observed within the first day and, moreover, the effect was independent from the PEG content. Nevertheless, the systems enabled protein release ongoing for several days.

A high burst release of BSA encapsulated within implant systems was reported from several groups [98; 101; 202]. For example, trimyristate coated BSA microspheres liberated 80 % of the incorporated protein within 5 hrs, this with only a 15 % BSA load [156]. Vogelhuber et al. demonstrated trimyristate matrices containing gelatin as porogen being inadequate to control BSA burst release. Already the addition of

gelatin in the range of 1 – 5 % (w/w) led to pronounced BSA liberation of up to 60 % within the first day during release experiments [202].
One reason for rapid BSA release from inert matrices may be the very high solubility BSA inheres within aqueous media (i.e. over 300 mg/mL). In the case of such highly water-soluble proteins it was demonstrated that all of the drug in the matrix pore spaces dissolves rapidly and protein release occurs by transient diffusion processes through the broad faces of matrix systems [182].
Furthermore, Zhu and Schwendeman reported 15 % protein loading being sufficient for BSA to percolate effectively throughout PLGA implants. That results in a rapid protein release in particular with BSA loadings of beyond 15 % [226].
Given that the incorporated polyethylene glycol may be involved in the formation of an interconnected network during the manufacture of the tristearin implants, it can be assumed that both the very high solubility of BSA and the complete network have an influence on the rapid BSA liberation observed from the tristearin matrices containing PEG as additive.

From the very first, bovine serum albumin (BSA) was deemed to serve as model protein compound, being available easily. Furthermore BSA inheres a higher physical stability than many other proteins (particularly hydrophobic proteins). Thereby, challenges in the development of compatible dosage forms are often reduced.
BSA is not relevant as pharmaceutical drug and, consequently, it was not high priority to further optimise the results achieved, e.g. by spending efforts on burst release reduction. However, the gained know-how was important since it was used as platform for the development of a controlled release lipid implant system for the pharmaceutically relevant interferon $\alpha$-2a.

## Chapter VII: Development of a sustained release device for IFN alpha-2a

In the early stages of this work, liberation profiles achieved from interferon α-2a (IFN α-2a) implant systems which based on tristearin matrix material were insufficient and somewhat inappropriate (refer to chapter IV). In most cases, those profiles were characterised by initial burst effects followed by phases of non-release and eventually an incomplete IFN α-2a release. Solely the incorporation of the phospholipid DOPC in a 5 - 10 % content range resulted in continuous IFN α-2a delivery for up to 12 days, releasing 30 % of the encapsulated IFN α-2a.

Though, this approach was not to be proceeded: as mentioned before, the compressibility of the mixtures substantially decreased with higher DOPC contents - i.e. 20 % and 30 % DOPC -, what consequently resulted in an impractical implant preparation via compressing.

However, based on knowledge gained for BSA systems, new aspects in terms of the development of a controlled and sustained IFN α-2a delivery system were provided.

### 1. Optimisation of the freeze-dried IFN α-2a formulation – first approach

Freeze-dried IFN α-2a products (protein/sugar ratio 1:4) used in prior release studies featured collapsed structures in almost identical manner as demonstrated for the lyophilised BSA formulation. Based on data and conclusions derived from the BSA experiments (refer to chapter VI), these collapsed products were assumed the reason for the observed incomplete IFN α-2a release from tristearin implants, even though stability data of the collapsed formulations did not indicate a loss in protein integrity due to the freeze-drying process per se.

Freeze-drying experiments were performed with IFN α-2a formulated in various trehalose ratios (plus 0.05 % (w/v) polysorbate 80). In doing so, lyophilised products with a protein/sugar ratio of 1:3 yielded stable, amorphous cakes - an obvious quality improvement when compared to the collapsed 1:4 formulations. After reconstitution of these 1:3 products, almost 100 % of IFN α-2a were recovered in monomeric form. The residual moisture contents ranged between 0.3 – 0.5 %, being distinctively lower than those determined in the collapsed formulations with moisture contents of 1.7 % ± 0.5 %. Consequently, long-term storage stability of the 1:3 lyophilisates may be improved [1].

Tristearin implants loaded with 10 % lyophilisate were prepared from both non-collapsed products and collapsed formulations. This results in an actual IFN α-2a load of 2.5 % for the non-collapsed and a 2 % load for collapsed formulations, respectively.

During release experiments, the quality improvement of the freeze-dried formulation demonstrated a considerable impact on IFN α-2a release behaviour (Fig. 47). Whereas IFN α-2a release from implants containing collapsed products remained to show a 10 % burst level throughout the total timeframe monitored, the use of the non-collapsed formulation raised the liberation rate up to 40 % of total releasable IFN α-2a.

Furthermore, the release profiles approached to mirror a curve shape aimed for: to overcome the burst effect and to realise an ongoing protein liberation over 3 weeks of incubation.

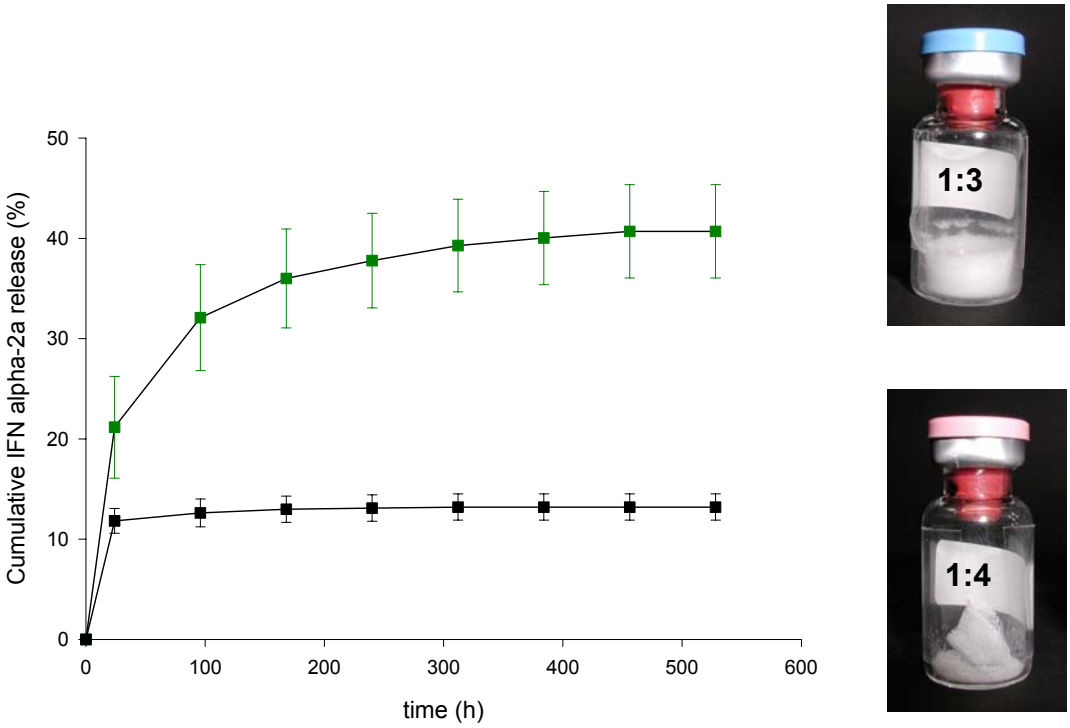


**Figure 47: Influence of the lyophilisate formulation on IFN α-2a release from tristearin implants. Implants consisted of 10 % lyophilised protein and 90 % tristearin (average ± SD, n = 3).**

Besides the substantial enhancement in IFN α-2a being releasable totally, protein burst release was kept in a feasible range. That was very important as a starting basis with respect to the development of a controlled drug delivery device.

For various protein devices, high burst release phenomena were almost impossible to control [106; 202; 156], and in theory it may be deemed more difficult to reduce protein release than to increase the liberation rate.

In contrast, starting from a 40 % IFN α-2a liberation level - with burst release encompassing about 20 % - will open the door to release optimisation from lipid implants, e.g. by variation of the matrix composition and by application of a wide range of apt additives.

## 2. Variation of matrix composition

### 2.1 Application of monoglycerides

Despite the use of monoglycerides revealed a high burst release of the model protein compound BSA (refer to chapter VI), this does not mean these compositions are inappropriate for utilisation in protein drug delivery.

Past experiments indicate the release behaviour of IFN α-2a being completely different from BSA release profiles. For example, the addition of the amphiphilic phospholipid DOPC notably impacted IFN α-2a liberation from tristearin matrices, whereas BSA release was almost not affected (refer to chapter V).

Consequently, it was conceivable that the release of the more hydrophobic IFN α-2a can to be improved by the application of monoglyceridic compounds, also from the point of view that these compounds reveal similar physical properties as DOPC does (e.g. emulsifying potential).

Evaluating studies of monoglycerides are listed in table 15. The IFN α-2a load of 2.5 % of implant weight - i.e. using 10 % lyophilised IFN α-2a formulation - was constant for all implant formulations.

| Formulation | Lyophilised IFN α-2a [%] (protein/sugar ratio 1:3) | Monoglycerides [%] (Myverol 18-04 K) | Tristearin [%] (Dynasan 118) |
|---|---|---|---|
| I | 10 | 90 | - |
| II | 10 | 70 | 20 |
| III | 10 | 30 | 60 |
| IV | 10 | 10 | 80 |
| V | 10 | - | 90 |

**Table 15: Matrix formulations investigating monoglycerides as both matrix material and excipient (n=3).**

The obtained release profiles indicated monoglycerides not to have a large potential as matrix compounds for sustained IFN α-2a delivery.

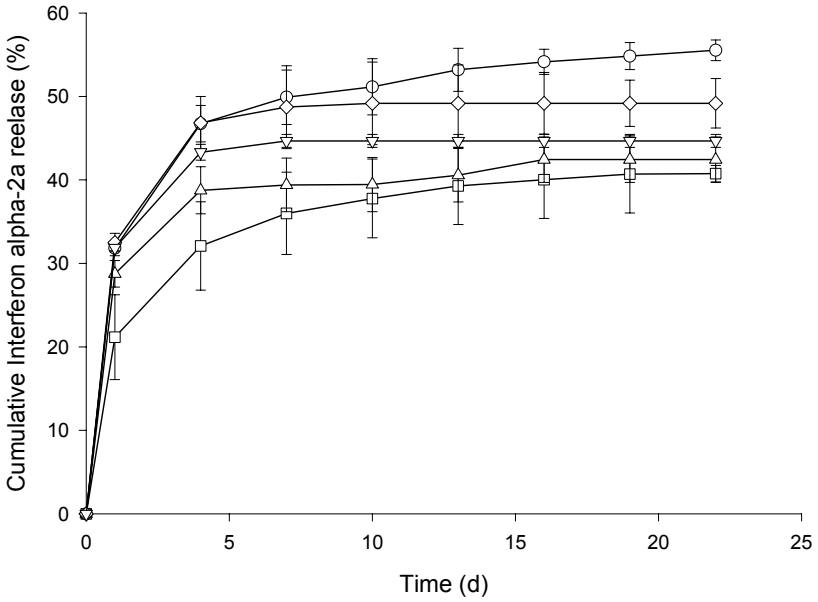


**Figure 48: IFN α-2a release from implants containing monoglycerides and tristearin material. Monoglyceride content: 0 % (□), 10 % (○), 30 % (◇), 70 % (▽), 90 % (△) (average ± SD, n = 3).**

For all formulations, within 7 days a high burst up to 50 % was observed. Furthermore, this burst release was followed by phases of almost non-release and consequently incomplete IFN α-2a release after a total 3 weeks incubation time (Fig. 48).

In addition to that, protein release decreased with higher monoglyceride content, leading to almost the same release profiles from pure monoglyceride matrices and pure tristearin matrices. Both implant systems liberated approximately 40 % of the incorporated protein within 3 weeks (Fig. 48).

Contrarily, a 10 % monoglyceride content featured 55 % of protein being releasable and, furthermore, the release was more sustained when compared to all other formulations (Fig. 48).

However, by applying monoglycerides an IFN α-2a release in linear mode during weeks could not be realised. High burst releases are to be considered a hampering issue.

## 2.2 Implants prepared with polyglycerol ester of fatty acids (PGEFs)

As already discussed, polyglycerol ester matrices for sustained protein release were demonstrated as apt form allowing the incorporated protein to liberate in a diffusion-controlled manner. In the work of Yamagata et al., lyophilised IFN α-2a was encapsulated in PGEFs matrices by a heat extrusion technique. The bioavailability of IFN α-2a after subcutaneous implantation of the various matrix formulations was almost equivalent to that after injection of IFN α-2a solution. Therefore, IFN α-2a was concluded IFN α-2a to remain stable during the 12 days release period [220].

However, these results were in contrary to our results from previous release studies where BSA was used as protein compound (refer to chapter VI). Here, 45 % of the incorporated protein were lost in the course of the experiments. Moreover, it could be concluded that this loss has to be ascribed to extensive protein aggregation induced by the eroded matrix material.

In awareness of this contradiction, polyglycerol ester material was re-investigated, but now with respect to its compatibility to IFN α-2a. Therefore, matrices with a 2.5 % IFN α-2a load (i.e. 10 % lyophilised IFN α-2a formulation) were prepared.

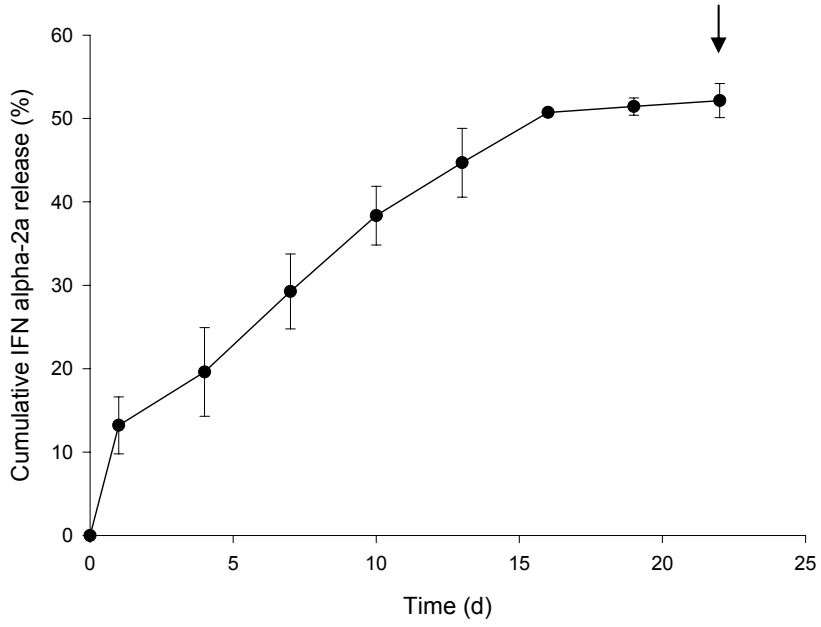


**Figure 49: IFN α-2a release from polyglycerol ester implants. Matrices were completely eroded after 22 days of incubation (↓) (average ± SD, n = 3).**

IFN α-2a release was almost the same as observed for the BSA devices. The protein was liberated in a continuous manner over 16 days, delivering 50 % of the incorporated IFN α-2a (Fig. 49). Again, despite the fact that the matrices were completely eroded after 3 weeks of incubation, the remaining IFN α-2a fraction was not detectable in the release medium.

PAGE analysis of the release media demonstrated covalent bounded aggregates being existent (Fig. 50). The major amount of such aggregated species originated from IFN α-2a dimer material. This dimer fraction was already detected after 24 hrs of incubation and became more pronounced with continuing incubation.

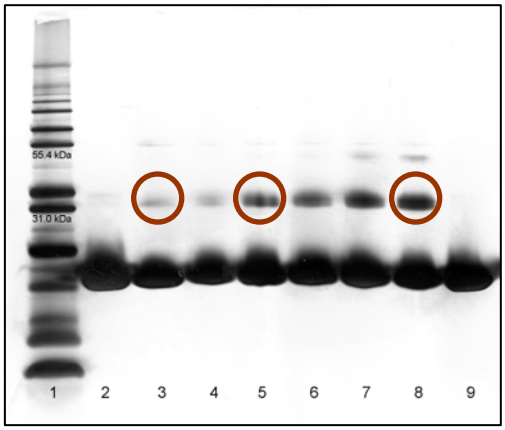


**Figure 50: IFN α-2a stability released from polyglycerol ester matrices. Lane 1: MW marker, lane 2: protein standard, lane 3: IFN α-2a released after 24 hrs, lane 4: IFN α-2a after 4 d, lane 5: IFN α-2a released after 7 d, lane 6: IFN α-2a released after 10 d, lane 7: IFN α-2a released after 13 d, lane 8: IFN α-2a released after 16 d, lane 9: protein standard, ○ IFN α-2a dimer material.**

Generally, quantification of the band intensities revealed dimer aggregates accounting for more than 35 % of overall band intensity after 16 days of incubation. Later on, a further protein band was detected, which is due to the formation of IFN α-2a trimer. This fraction represented another 4 % of overall band intensity in the monitored time period (Fig. 50).

The occurrence of IFN α-2a aggregates was associated with the hydration of the PGEFs matrices. Consequently, the extensive water uptake of these matrices can be stated as reason for accelerated IFN α-2a aggregation proceeding within the matrices. Furthermore, interactions between protein molecules and the eroded material may be involved in the promotion of IFN α-2a aggregation.

The aggregates analysed are species which are soluble in the release media. Thus, these fractions did not account for the 50 % protein loss observed during release studies. However, their occurrence indicated that also the formation of insoluble aggregates as well as protein/lipid products can be assumed. Due to filtration of the release media prior to both SE-HPLC and PAGE studies, such fractions were separated and retained on the filter materials. These filters were collected and incubated in 2.0 mL SDS solution each (conc.: 2 % SDS (w/v)) at 37 °C over 24 hrs. SDS enables the dissociation of non-covalently bound insoluble protein aggregates and protein-polymer interactions to be disrupted.

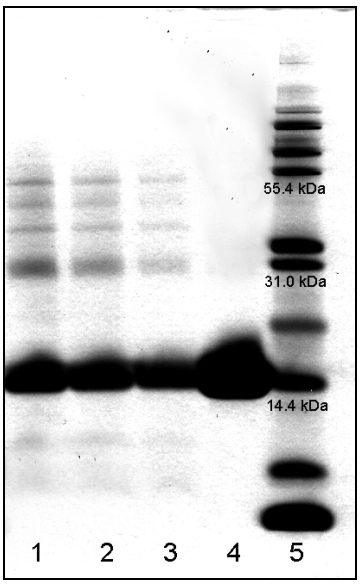


**Figure 51: IFN α-2a recovered in SDS solution after incubating used filters at 37 °C over 24 hrs. Lanes 1 – 3: IFN α-2a detected in SDS solution, lane 4: protein standard, lane 5: MW marker.**

PAGE studies of the supernatant solutions exhibited a major amount of IFN α-2a being present in the solution (Fig. 51). Furthermore, the protein was considerably destructed, mirrored by the development of dimer and trimer material and further altered protein bands.

These findings proved the extensive water uptake of PGEFs matrices as well as their erosion to cause protein instability during release studies. Here, the formation of insoluble aggregates and protein adsorption onto lipid erosion products can be assumed, leading to a 50 % IFN α-2a loss in the course of the experiments.

The results were contrarily to data reported by Yamagata et al.. There, however, no definite statement was provided addressing the erosion behaviour of the matrices

presented, and maybe this is the lynchpin for protein being destabilised or not. This means, if the matrices demonstrated inhere a slower swelling and erosion – for example due to a different product quality -, IFN α-2a is thought to be more stable within these very devices versus within implants described in this thesis [220].
For the polyglycerol ester material available, protein instability was noticed as impeding fact. Already a small amount of aggregated species was discussed as major reason for immunogenic reactions in mice after subcutaneous injection of IFN α-2a formulations [20]. Consequently, PGEFs materials were considered to be inappropriate for further investigations.

## 3. IFN α-2a release from tristearin implants containing PEG

### 3.1 In vitro release studies

When added in a content range between 10 – 30 %, polyethylene glycol 6000 (PEG) was demonstrated as an effective excipient for BSA delivery from tristearin matrices. BSA was liberated completely from these devices, whereby a high burst release has to be notified (refer to chapter VI).
However, Siegel et al. demonstrated that the high solubility of BSA (i.e. 300 mg/mL) within aqueous media can be deemed a reason for the rapid release from inert matrices leading to transient diffusion processes. In contrast, proteins with lower solubilities such as IFN α-2a (solubility in a range of 25 mg/mL) are suggested to be liberated from such matrices according to the Higuchi equation [182; 174].
In awareness of this expected difference in the release kinetics of IFN α-2a compared to BSA, tristearin matrices containing a varying PEG ratio were evaluated in their applicability as delivery devices for a controlled IFN α-2a liberation.
In table 16, the formulations investigated are illustrated. The IFN α-2a load represented 2.5 % of implant weight (i.e. 10 % lyophilised IFN α-2a formulation) in all experiments.

| Formulation | Lyophilised IFN α-2a [%] (protein/sugar ratio 1:3) | PEG [%] | Tristearin [%] (Dynasan 118) |
|---|---|---|---|
| I | 10 | - | 90 |
| II | 10 | 5 | 85 |
| III | 10 | 10 | 80 |
| IV | 10 | 12 | 78 |
| V | 10 | 14 | 76 |
| VI | 10 | 16 | 74 |
| VII | 10 | 18 | 72 |
| VIII | 10 | 20 | 70 |
| IX | 10 | 30 | 60 |

**Table 16: Tristearin matrix formulations with varying contents of polyethylene glycol 6000 (PEG) as additive (n=3).**

In the first instance, formulations with 5 %, 10 %, 20 %, and 30 % PEG contents were evaluated (Fig. 52). The release profiles indicated that the addition of PEG resulted in two different features: in a substantial enhancement of IFN α-2a liberation, and secondly in an obvious change of the release kinetics. PEG-free matrices liberated 40 % of the incorporated protein over the monitored timeframe. However, the initial drug release accounted for almost 30 %, what led to a parabolic release profile (Fig. 52).

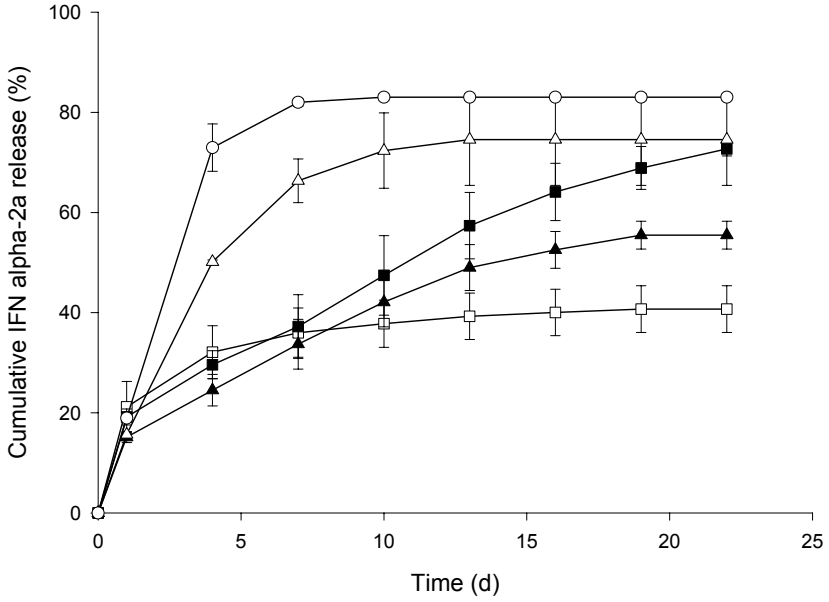


**Figure 52: The effect of the PEG content on the release kinetics of IFN α-2a from tristearin implants. PEG content: 0 % (□), 5 % (▲), 10 % (■), 20 % (△), 30 % (○) (average ± SD, n = 3).**

A protein release of up to 50 % in a steady manner over 17 days was achieved from implants containing 5 % PEG. Otherwise, implants containing 10 % PEG revealed a constant IFN α-2a liberation even over 3 weeks. After an initial drug release of about 20 %, in the ongoing state another 50 % of protein were released. Thereby zero-order kinetics were approached. Increasing the PEG content further on, e.g. 20 % and 30 %, resulted in a surge of IFN α-2a delivery of up to 80 %, yet the liberation succeeded faster and terminated after 10 days (Fig. 52).
In further release studies, a content range of 10 – 20 % PEG was specified (Fig. 53).

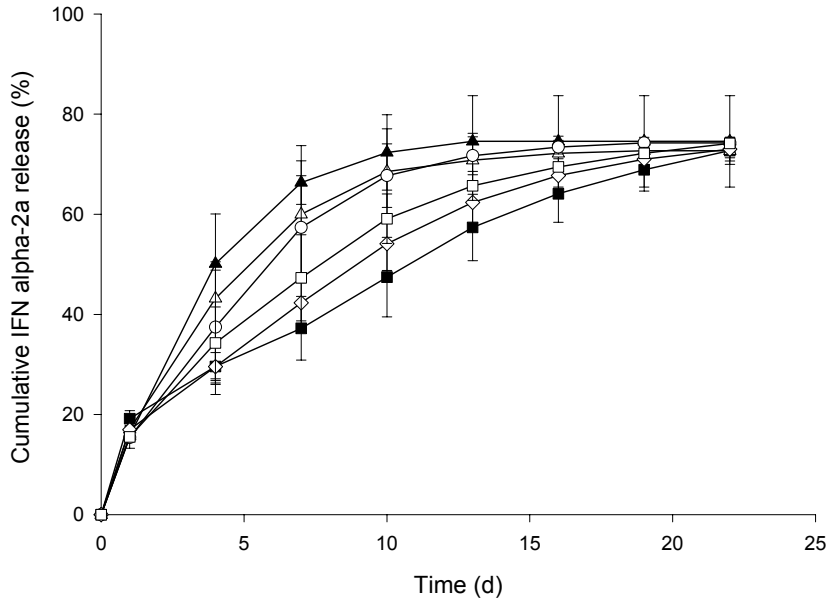


**Figure 53: The effect of the PEG content on the release kinetics of IFN α-2a from tristearin implants. PEG content: 10 % (■), 12 % (◇), 14 % (□), 16 % (○), 18 % (△), 20 % (▲) (average ± SD, n = 3).**

The release profiles demonstrated that a stepwise raise of the PEG content did not result in gradually enhancing the total protein release. However, the profiles indicated that incorporated PEG contents higher than 14 % accelerated protein liberation. The addition of 12 % and 14 %, respectively, exhibited linear profiles for 3 weeks in accordance to the 10 % PEG formulation. In contrast, a higher PEG content - i.e. 16 % and 18 % - approached non-linear profiles comparable to the 20 % PEG formulation (Fig. 53).

Based on this data set, it can be concluded that the additional incorporation of a solid, water soluble excipient contributes to the formation of an interconnected network [101].

Implants with a 10 % protein load and without PEG revealed only a slight increase in IFN α-2a liberation after initial burst effects. This suggests that most IFN α-2a particles are trapped within the matrix. Only the particles located directly at the surface or even close to it could be liberated.

The release kinetics and the total amount of releasable protein changed substantially by adding 5 % PEG, even more distinct by 10 % PEG as solid excipient (Fig. 52). The continuous delivery after initial burst release up to 70 % IFN α-2a liberated after 3 weeks of incubation supposed that dissolution and diffusion of non-surface IFN α-2a molecules through pores and interconnected channels occurred. The comparison of implants containing 5 % PEG with 10 % PEG matrices confirmed this suggestion: by the addition of 5 % PEG, more protein particles could be trapped within the matrix because an incomplete network may be formed during compressing. This resulted in solely 50 % IFN α-2a delivery over time. An increase in the PEG content (e.g. 20 % or 30 % PEG) may yield in reduced tortuosity and in a greater porosity enabling diffusion, noticeable by the fast IFN α-2a from these devices.

### 3.2 Protein stability during release

A negligible water uptake of matrices made alone of triglycerides was demonstrated (refer to chapter IV). However, the formation of an interconnected network – as described in the preceding section – enables water uptake into the pores leading to a high concentrated protein solution. The hydration of the solid protein leads to a greater conformational flexibility of protein molecules and to a higher mobility of protein species, whereby protein aggregation in a highly concentrated solution may be preferred [48; 79; 121].

Consequently IFN α-2a aggregation proceeding within the matrices during release studies could be one possible reason for some protein loss. The extraction of the remaining IFN α-2a part from incubated implants with a 5 % and a 10 % PEG content, respectively, and following analysis of the samples by gel electrophoresis backed this suggestion. After three weeks of incubation, dimer and trimer fractions were estimated in dimensions of 3.5 % of total protein extracted from implants

containing 5 % PEG (Fig. 54). IFN α-2a extracted from incubated matrices with a 10 % PEG content featured about 4 % higher-ordered aggregates, additionally to the dimer and trimer species.

The inflow of the release medium caused proceeding aggregation of IFN α-2a within the matrices. One reason for the more distinct IFN α-2a aggregation extracted from implants containing 10 % PEG may be that the dissolution of PEG led to more porous matrices in the course of the release process. Concomitantly, the water content within the matrices increased, facilitating protein aggregation.

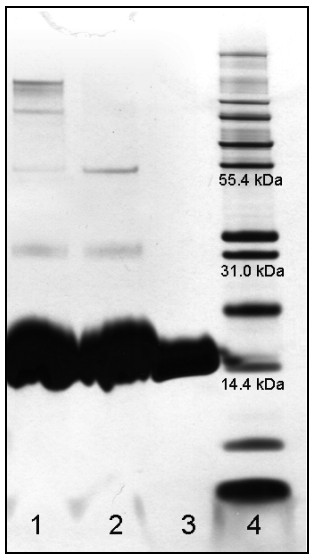


**Figure 54: IFN α-2a stability during in vitro release within tristearin matrices containing various ratios of PEG. Lane 1: IFN α-2a extracted from implants with a initial PEG content of 10 % after 1 month of incubation, lane 2: IFN α-2a extracted from implants with a initial PEG content of 5 % after 1 month of incubation, lane 3: protein standard, lane 4: MW marker.**

One further important question was whether aggregated species were liberated during release studies or not. For example, Morlock et al. published that erythropoietin (EPO) encapsulated in PLGA microspheres underwent moisture-induced aggregation and that EPO aggregates did not release from the microspheres [129].

Reversed phase HPLC did not indicate the existence of aggregated protein material in the release media, since 98.5 % of the detected IFN α-2a could be attributed to monomeric protein, even at stages of the release. The altered fraction can be ascribed to oxidised IFN α-2a species, whereby an average content of less then 2 % was present already in the bulk material.

In parallel to the assessment of protein concentration, the samples of all formulations were analysed by gel electrophoresis. The results for IFN α-2a released from implants containing 5 % PEG and 10 % PEG, respectively, are shown in Fig. 55 and Fig. 56 A/B.

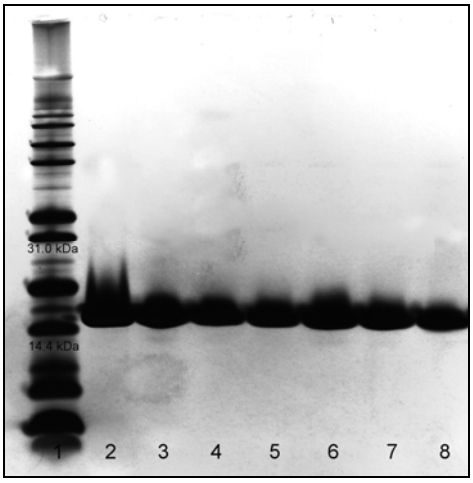


**Figure 55: IFN α-2a released from tristearin matrices with an initial PEG content of 5 %. Lane 1: MW marker, lane 2: protein standard, lane 3: IFN α-2a released after 24 hrs, lane 4: IFN α-2a released after 4 d, lane 5: IFN α-2a released after 7 d, lane 6: IFN α-2a released after 10 d, lane 7: IFN α-2a released after 13 d, lane 8: IFN α-2a released after 16 d.**

Generally, no dimer/trimer and higher aggregated species of IFN α-2a were assessed all along in the release media, neither when released from the 5 % PEG implants (Fig. 55) nor from the 10 % PEG matrices (Fig. 56 A/B). The comparison to IFN α-2a gel standard demonstrated identical bands of the samples and the standard.

Consequently, the released IFN α-2a was rated to almost exclusively consist of pure, native monomer.

Higher-ordered IFN α-2a could not liberate because a passive diffusion of aggregated protein species through the tortuous pores and channel network may be limited by the size of the pores [216]. Additionally, a higher molecular weight is associated with a smaller diffusion coefficient, thus also impeding liberation.

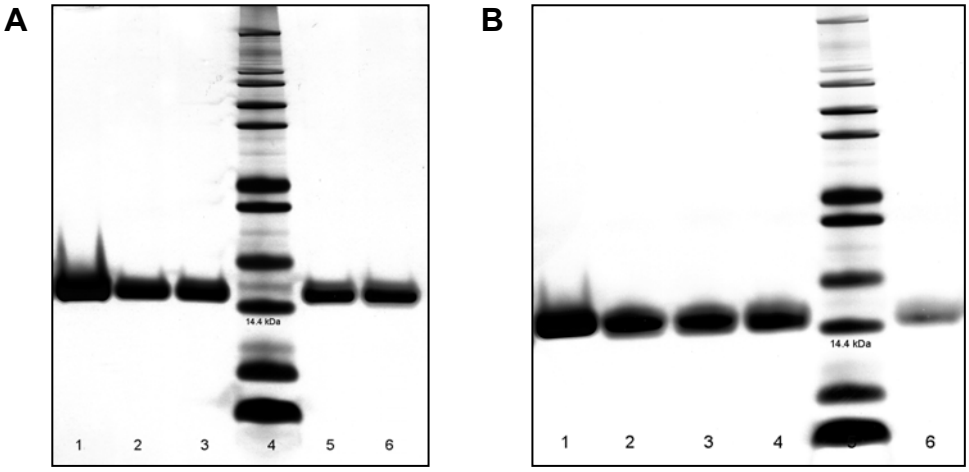


**Figure 56: IFN α-2a released from tristearin matrices with an initial PEG content of 10 %. (A) Lane 1: protein standard, lane 2: IFN α-2a released after 24 hrs, lane 3: IFN α-2a released after 4 d, lane 4: MW marker, lane 5: IFN α-2a released after 7 d, lane 6: IFN α-2a released after 10 d; (B) lane 1: protein standard, lane 2: IFN α-2a released after 13 d, lane 3: IFN α-2a released after 16 d, lane 4: IFN α-2a released after 19 d, lane 5: MW marker, lane 6: IFN α-2a released after 22 d.**

## 3.3 Summary and discussion

Tristearin implants containing polyethylene glycol 6000 (PEG) as non-toxic, hydrophilic excipient enabled the development of a delivery system allowing the protein to be continuously released over weeks. For instance, with a 10 % PEG content, up to 70 % of the incorporated protein could be delivered in a linear manner over a 1 month period. Moreover, released protein consisted almost exclusively of monomeric IFN α-2a.

It was further shown that varying the amount of PEG allows the control of protein liberation: whereas the addition of 20 % exhibited a 70 % IFN α-2a release within 10 days in non-linear mode, implants containing 10 % also released 70 % of the incorporated protein, but in a linear way within three weeks.

However, even with a 30 % PEG content, only 85 % of the incorporated protein could be liberated from these devices. That incomplete IFN α-2a liberation was not to be expected because the percolation threshold, i.e. the drug loading enabling the network to be coherent, was claimed for a 33.8 % loading [101]. Furthermore, a 15 % protein load was demonstrated to achieve a complete percolation of BSA when encapsulated in PLGA microspheres [226].

The extraction of the remaining IFN α-2a part exhibited some protein being existent as covalent aggregated species. However, non-covalently bound aggregates within the extracted samples may be dissociated upon dilution with SDS buffer medium and subsequent heating prior to PAGE analysis.
Therefore, it was the objective of further investigations to specify potential reasons for the incomplete protein release and, moreover, to minimise or even eliminate these parameters in order to achieve a complete protein liberation from the tristearin devices containing PEG.

## 4. Potential parameters causing incomplete IFN α-2a release

### 4.1 Studies on IFN α-2a stability in aqueous solution

Since it has been shown in tentative experiments (refer to chapter IV) that Lowry assay data reveal considerable variability, some of the stability experiments were repeated as far as IFN α-2a data are pertained. In doing so, assay data are substantiated by using chromatographic methods.

#### *4.1.1 IFN α-2a formulated in pH 7.4 isotonic 0.01 M phosphate buffer (PBS)*

In the previous chapter protein release was discussed as the result of dissolution and diffusion processes through pores and channels. Thus, the incorporated protein is exposed to the infiltration of PBS medium over a 1 month incubation period.
In order to evaluate whether the buffer environment itself or the elevated temperature of 37 °C promoted protein aggregation as well as chemical degradation pathways such as oxidisation throughout incubation, long-term stability studies of IFN α-2a were performed. IFN α-2a solutions (0.2 mg/mL) formulated in PBS with 0.05 % (w/v) $NaN_3$ were prepared and 2.0 mL each were incubated at 37 °C over time periods up to 2 months (stirring 40 rpm). At predetermined points of time protein concentration as well as protein integrity were characterised by SE-HPLC and RP-HPLC (Fig. 57 A/B).

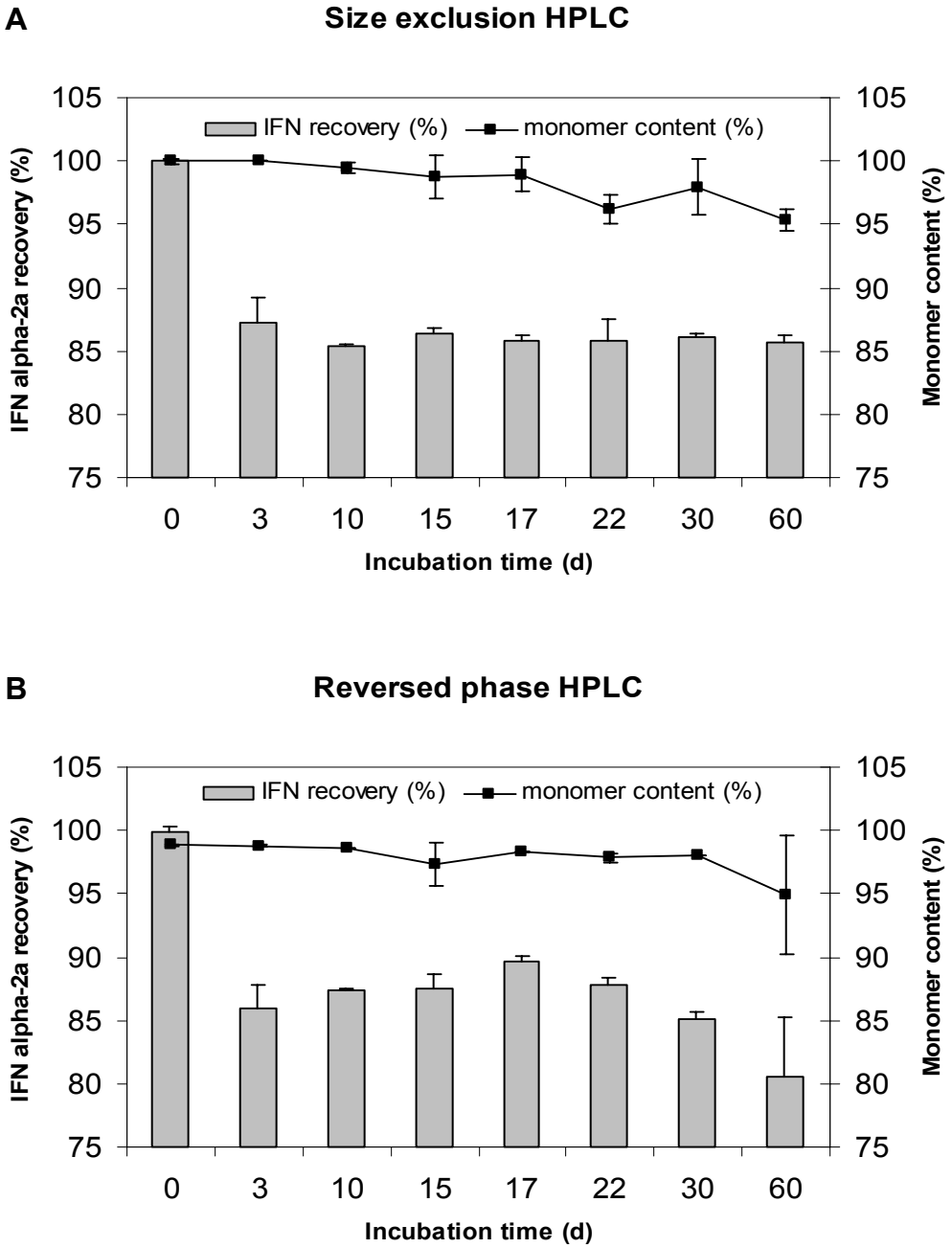


**Figure 57: Alteration of IFN α-2a concentration and stability in PBS/0.05 % (w/v) $NaN_3$ over 2 months of incubation. (A) Results of SE-HPLC; (B) results of RP-HPLC (average ± SD, n=3).**

Chromatographic analysis featured a substantial decrease in IFN α-2a concentration after 3 days of incubation. The protein loss ranged between 10 - 20 % detected by both SE-HPLC and RP-HPLC. However, for SE-HPLC studies the monomer content remained almost constant on a 100 % level over 10 days, following by a slight

decrease with incubation continuing and finally resulting in 95 % monomeric IFN α-2a after 2 months. The monomer loss could be attributed to dimer and trimer species which were detectable in the solutions after 2 weeks of incubation (Fig. 57 A).
Otherwise, chemical protein degradation was not observed over 1 month of incubation. RP-HPLC analysis demonstrated a constant 98 % monomer level. The remaining 2 % protein portion originated from oxidised IFN α-2a already existent in the protein bulk solution. Within the following month of incubation, the oxidative modified protein amount increased up to 5 % (Fig. 57 B).
In order to exclude the formation of insoluble aggregates causing the detected 15 % protein loss, these data were backed by light obscuration measurements (table 17). The 10 days values were not to be generated because of device-related problems.

| IFN α-2a formulated in PBS with 0.05 % (w/v) $NaN_3$ | Numbers of particles per mL with µm dimensions of | Before incubation | Incubation time [d] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 3 | 15 | 17 | 22 | 30 | 60 |
| | > 1 | 865 | 1,510 | 3,329 | 3,341 | 3,400 | 1,514 | 7,646 |
| | > 4.1 | 107 | 363 | 512 | 903 | 671 | 242 | 1,429 |
| | > 10 | 13 | 60 | 147 | 78 | 23 | 40 | 270 |
| | > 25 | 0 | 0 | 0 | 0 | 0 | 2 | 4 |

**Table 17: Light obscuration measurements of IFN α-2a solutions incubated over 2 months (n=3, data in cumulative presentation).**

According to the European Pharmacopoeia, particle requirements for parenteral liquid dosage forms (Ph.Eur. 2.9.19) of not more than 6,000 particles ≥ 10 µm with a spherical diameter and/or 600 particles with diameters ≥ 25 µm were not exceeded in IFN α-2a solutions, nor were they after 2 months of incubation. An incipient formation of insoluble aggregates may cause the increased particle numbers in the solutions incubated for 2 months. However, based on this data it can be excluded that the formation of insoluble aggregates accounted for decreasing protein recoveries detectable in the solutions after 3 days of incubation.

### *4.1.2 Influence of PEG on IFN α-2a stability formulated in PBS*

Polyethylene glycol 6000 (PEG) is soluble in aqueous release media. Consequently, this incorporated excipient will be dissolved during release experiments when buffer penetrates into the lipid devices. Thus, both solved protein

and PEG components will coexist in the interior of the matrices. Moreover, PEG diffusion processes throughout the matrix interior will occur simultaneously with protein diffusion.

For polyethylene glycols, stabilising effects on proteins in aqueous media have been reported [43; 44]. However, stabilisation effects seem to be dependent on protein type and PEG sizes. Human serum albumin (HSA) was stabilised in the presence of PEG 8000 and PEG 10000, respectively, showing negative preferential interaction. On the other hand, PEGs with lower molecular weight (e.g. $M_w$ 1000 and 4000) interacted favourable with the hydrophobic side chains when the protein was unfolded and eventually led to a stabilisation of the unfolded state [64].

In order to characterise potential IFN $\alpha$-2a-PEG interactions, tristearin implants with a 10 % PEG content were prepared. Stability studies were conducted by incubating the implants in 2.0 mL of a IFN $\alpha$-2a solution (0.2 mg/mL) formulated in PBS/0.05 % (w/v) $NaN_3$ at 37 °C over 2 months. The samples were agitated at 40 rpm. Protein concentration and protein integrity were assessed by SE-HPLC and RP-HPLC at predetermined points of time. In order to examine insoluble aggregates, samples were analysed by light obscuration. Fig. 14 illustrates determined protein recoveries and IFN $\alpha$-2a monomer contents versus time.

The results mirrored almost the same effects as faced in IFN $\alpha$-2a stability studies without any PEG-lipid implants. Within the first days, a considerable decrease in the protein concentration detectable was notified, ending up in protein recovery rates of approximately 87 %. However, no further substantial protein loss was detected over the monitored incubation time (Fig. 58).

Again, IFN $\alpha$-2a monomer content remained on a high constant level for 1 month, i.e. a 97 % monomer fraction was detected by SE-HPLC, resembling the 98 % monomer content assessed via RP-HPLC. These data indicated that neither a pronounced formation of soluble aggregated species nor of oxidised IFN $\alpha$-2a caused the detected protein loss.

Furthermore, it can be concluded that PEG released from the lipid implants is not compromising protein stability. Though in contact with the hydrophilic additive, high IFN $\alpha$-2a stability was maintained throughout 2 months of incubation.

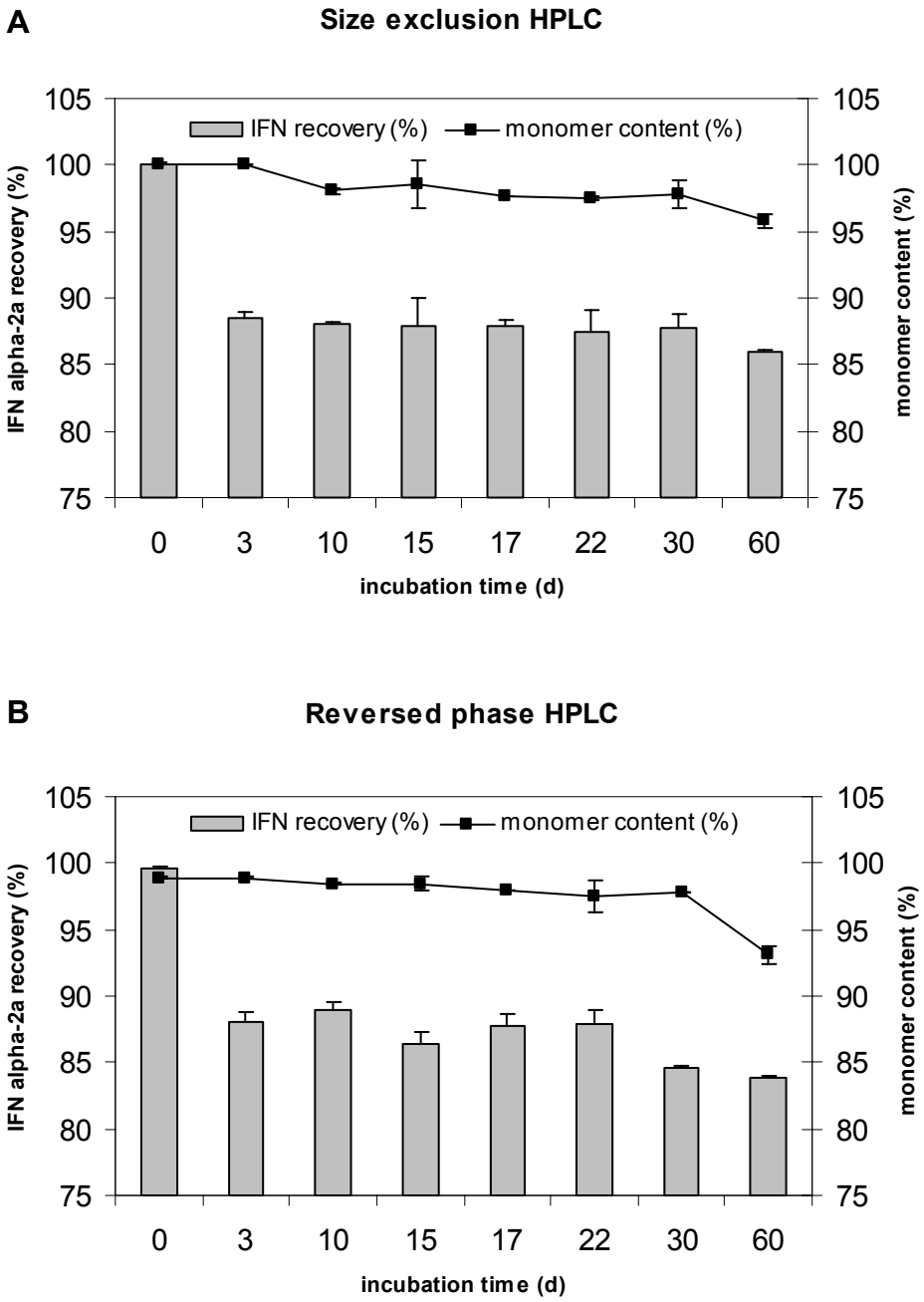


**Figure 58: Influence of polyethylene glycol 6000 (PEG) on IFN α-2a stability formulated in PBS/ 0.05 % (w/v) $NaN_3$ over 2 months of incubation. (A) Results of SE-HPLC; (B) results of RP-HPLC (average ± SD, n=3).**

Light obscuration measurements confirmed these data by excluding the formation of insoluble aggregates in IFN α-2a solutions during this 2 months period. Detectable particle numbers in the solutions were distinctively below the Ph.Eur. requirements (table 18) and showed no trending towards increasing particles counts over time.

| IFN α-2a formulated in PBS plus PEG-tristearin implant | Numbers of particles per mL with µm dimensions of | Before incubation | Incubation time [d] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 3 | 15 | 17 | 22 | 30 | 60 |
| | > 1 | 627 | 3,150 | 5,925 | 1,804 | 1,679 | 1,252 | 1,643 |
| | > 4.1 | 90 | 363 | 678 | 337 | 248 | 210 | 240 |
| | > 10 | 27 | 94 | 181 | 30 | 27 | 32 | 40 |
| | > 25 | 1 | 0 | 5 | 2 | 0 | 2 | 7 |

**Table 18: Light obscuration measurements of IFN α-2a solutions in PBS/0.05 % (w/v) $NaN_3$ containing one PEG-lipid matrix. Samples were incubated over 2 months (n=3, data in cumulative presentation).**

## 4.2 Protein adsorption upon packaging material

Adsorption of protein upon glass surfaces is a well-known and extensively described phenomenon. Surface adsorption may result in protein loss and/or destabilisation of proteins. For example, at 1 µg/mL conditions the adsorption of acidic fibroblast growth factor on surfaces of various container materials (untreated glass, siliconised glass, sulphur-treated glass, Purcoat glass, polyester, polypropylene and nylon) was accounting for a 20 – 40 % loss [203]. Protein surface adsorption is usually dependent on protein concentration and may reach a maximum above a certain concentration-surface ratio [24]. Furthermore, the type of the container material mainly influences protein adsorption to its surface [77]. Schwarzenbach et al. measured IFN α-2a interactions with glass vial surfaces by atomic force microscopy. It was shown that the adhesion force on coated borosilicate glass (Schott Fiolax Type I plus®) was reduced down to 40 % of the total adhesion force measured on standard type I borosilicate glass [175].

In order to specify a potential protein adsorption upon the borosilicate glass vials, which were used for preceding in vitro release studies, the recovery of IFN α-2a bulk solutions (protein concentration 0.2 mg/mL and 1.0 mg/mL, resp.) formulated in PBS with 0.05 % (w/v) $NaN_3$ was evaluated by spectroscopic measurements. Furthermore, potential adsorption upon the lipid implants was investigated by adding one pure tristearin matrix to IFN α-2a solutions. Besides these evaluations with untreated glass, experiments using siliconised glass (2R) and TopPac® vials (10R), made of a cycloolefin copolymer, were performed. The sample volumes were 2.0 mL consistently, and each experiment comprised analysis of three individual samples.

Samples were incubated at 37 °C and agitated with 40 rpm. Protein concentration was determined at 280 nm wavelength after 24 hrs and 3 days, respectively. IFN α-2a solutions with known concentrations (0.1 mg/mL – 1.0 mg/mL) were used to generate calibration curves.

Fig. 59 A/B illustrates the amount of IFN α-2a recovered, depending on the used package material, the initial protein concentration, and the incubation duration. Generally, not more than 86 % of the initial IFN α-2a concentration was measured in the solutions incubated in untreated, borosilicate glass. The notable protein loss was determined after 24 hrs, followed by a slight decrease, if any, within the next two days. Furthermore, the higher concentrated IFN α-2a solution exhibited a lower protein recovery (Fig. 59 A/B).

Based on comparable IFN α-2a recoveries in the pure protein solutions and in solutions containing additionally one tristearin matrix (Fig. 59 A/B), it could be concluded that the protein did not adsorb on the surface of the lipid implants. This was an important attainment of the lipid systems, since for various polymeric devices a substantial protein loss as a result of surface adsorption after protein release has been discussed [198].

When using siliconised glass vials as package material, higher protein concentrations were detected in the media. This advancement was more pronounced for the higher concentrated IFN α-2a solutions, and the protein loss occurred again within 24 hrs. But, as a matter of fact, a complete inhibition of protein adsorption processes could not be realised (Fig. 59 A/B).

From the cycloolefin copolymer vials (TopPac®), approximately 98 % of IFN α-2a were recovered and that result was independent from the initial protein concentration and the incubation period. Consequently, the plastic materials featured a high potential as package material for future in vitro release studies (Fig. 59 A/B).

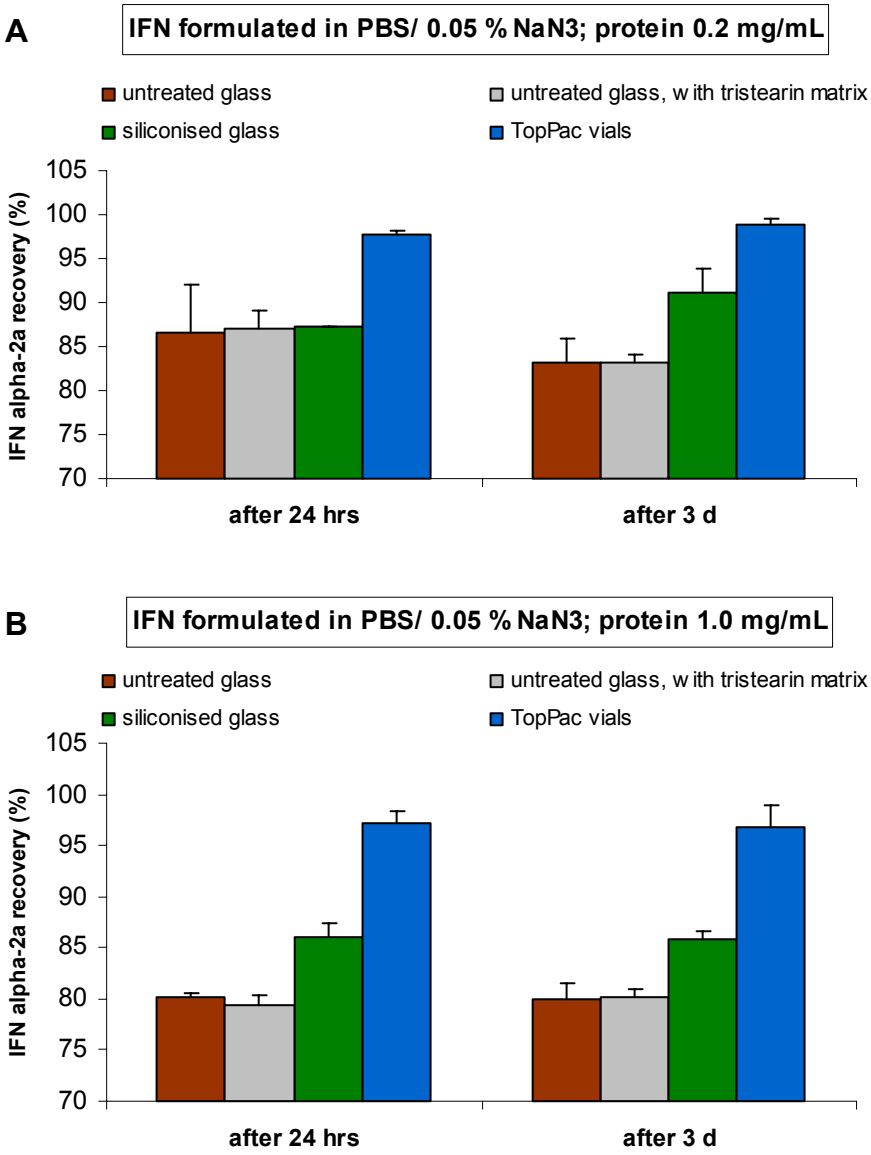


**Figure 59: Protein adsorption upon package material. IFN α-2a stock solutions were incubated in various containers at 37 °C over 3 days (average ± SD, n=3).**

In order to confirm the protein loss as a consequence of adsorption upon the package material, protein had to be removed from the surfaces. Therefore, the vials were rinsed with water after 3 days of incubation. Then, 2% (w/v) SDS-solution – with SDS enabling the disruption of non-specific adsorption - was filled into the vials. After incubation at 37 °C for 24 hrs, the supernatant solutions were analysed by PAGE (Fig. 60).


**Figure 60: IFN α-2a removed from the surfaces of various containers by supernatant SDS at 37 °C after 24 hrs. Lane 1: protein removed from untreated glass vials, lane 2: MW marker, lane 3: protein removed from siliconised glass vials, lane 4: protein removed from TopPac® vials, lane 5: protein standard.**

Monomeric IFN α-2a was detected in the supernatant solutions incubated in untreated glass vials and in siliconised glass (Fig. 60). Thereby, the protein band intensity was more pronounced in samples from untreated glass. This implicates that more protein was removed from these glass surfaces, in accordance with the increased protein loss of the samples, as determined by spectroscopic measurements.

From the surfaces of the cycloolefin copolymer vials, no protein was removed at all by the supernatant SDS substantiating the approximately 98 % IFN α-2a recovery value detected by UV.

The quantification of the removed protein by spectrophotometric measurements revealed recoveries corresponding to only 5 – 7 % of the initial protein concentration, what can be attributed to an incomplete removal of the protein by the supernatant SDS [205].

## 4.3 Optimisation of the lyophilised protein formulation – second approach

As outlined above, the quality improvement of the freeze-dried IFN α-2a formulation can be deemed as crux of the matter in protein delivery from lipid implants, and the first approach done in this field was proven by a considerable increase in total releasable IFN α-2a. Moreover, this optimisation enabled the

development of an IFN α-2a delivery system based on tristearin implants containing PEG, which allows a 70 % continuous protein release over a 1-month period.

Hence, one alternative optimisation approach by varying the applied cryo- and lyoprotective excipients, the cake masses, and the formulation pH was considered to further optimise the quality of the freeze-dried formulation - and consequently to improve the protein release behaviour (refer to chapter III, 2.1.1.3 B/C).

Besides the commonly used trehalose, the polymers dextran 60 and hydroxypropyl-ß-cyclodextrin (HP-ß-CD) were investigated on their efficiency in protein stabilisation during freeze-drying and subsequent solubilisation.

After reconstitution, hitherto used lyophilised IFN α-2a formulations had a pH of 5.6, which is a pH close to the isoelectric point of IFN α-2a. This may implicate an increased sensitivity of this formulation against freezing and subsequent dehydration stress, what could result in an accelerated aggregation tendency after protein resolvation [6; 226]. Generally, protein solubility within aqueous media is lowest at the isoelectric point. Here, it was assumed that portions of the protein could have remained unsolved within the lipid implants during release experiments.

Table 19 summarises the experimental design of these investigations, the pH of the formulations after reconstitution, and the determined residual moisture contents in the freeze-dried products.

After the freeze-drying process, all formulations showed elegant amorphous cake structures, and no obvious difference was visible among the various products. The determined water contents were below 1 %, ranging from 0.3 – 0.9 %. Here, the increased solid content of the formulation was accompanied by a decrease of the residual moisture contents (table 19).

Protein solutions adjusted to a pH of 4.2 exhibited slightly increased pH values in a range of 4.3 - 4.8 after the freeze-drying process. However, this formulation pH can be considered to guarantee a high physical and chemical IFN α-2a stability during storage and upon resolubilisation [224; 214]. In contrast, solutions of pH 7 resulted in a pH drop to values of 6.0 - 6.2 after lyophilisation - a pH close to the isoelectric point (table 19).

| Formulation | Excipient | Mass of the freeze-dried cake, protein sugar ratio of 1:3 [mg] | Adjusted pH | pH after reconstitution | Water content [%] |
|---|---|---|---|---|---|
| I (hitherto used) | trehalose | 16 | - | 5.53 | 0.81 |
| II | trehalose | 16 | 4.2 | 4.80 | 0.76 |
| III | trehalose | 16 | 7.0 | 6.14 | 0.78 |
| IV | trehalose | 64 | 4.2 | 4.54 | 0.39 |
| V | trehalose | 64 | 7.0 | 6.08 | 0.57 |
| VI | dextran 60 | 16 | - | 5.19 | 0.91 |
| VII | dextran 60 | 16 | 4.2 | 4.80 | 0.70 |
| VIII | dextran 60 | 16 | 7.0 | 6.00 | 0.70 |
| IX | dextran 60 | 64 | 4.2 | 4.33 | 0.41 |
| X | dextran 60 | 64 | 7.0 | 6.12 | 0.47 |
| XI | HP-ß-CD | 16 | - | 5.31 | 0.79 |
| XII | HP-ß-CD | 16 | 4.2 | 4.64 | 0.75 |
| XIII | HP-ß-CD | 16 | 7.0 | 6.16 | 0.81 |
| XIV | HP-ß-CD | 64 | 4.2 | 4.31 | 0.27 |
| XV | HP-ß-CD | 64 | 7.0 | 6.10 | 0.54 |

**Table 19: Evaluating studies on the stability of IFN α-2a freeze-dried formulations. Listed are various formulations, pH values (with 2 N acetic acid or 0.05 N NaOH, resp.) before lyophilisation, and pH values after reconstitution as well as determined residual moisture contents.**

After reconstitution, IFN α-2a stability was monitored for 7 days. The reconstituted formulations were incubated at 37 °C, stirred with 40 rpm and finally analysed by SE-HPLC after 2 days and 7 days of incubation, respectively.

Figs 61 – 63 illustrate the detected protein recoveries with regards to formulation pH, the employed excipient, and the incubation time.

Using trehalose as stabilisation excipient in combination with a formulation pH of 4.2 assured the protein to be stable throughout incubation. Approximately 98 % of IFN α-2a were recovered after 7 days (Fig. 61), and only a marginal dimer fraction accounting for less than 1 % of overall protein concentration was detected.

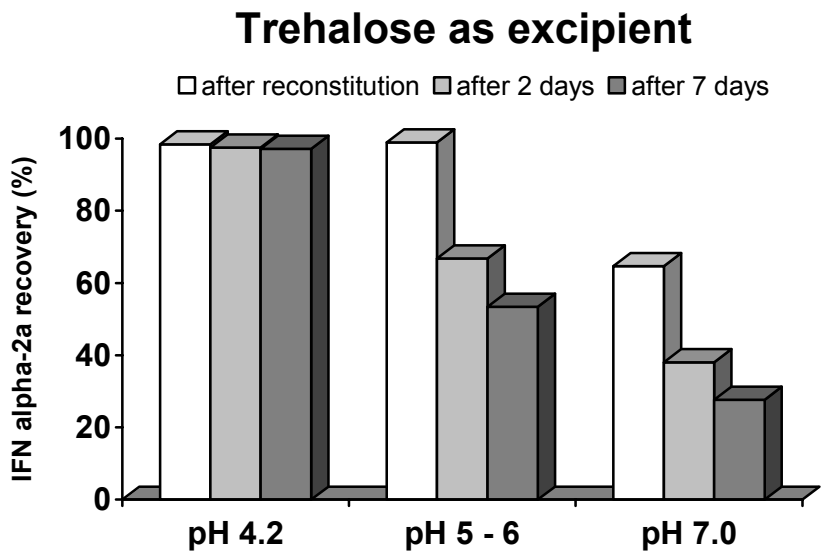


**Figure 61: IFN α-2a stability of trehalose freeze-dried formulations with different pH values after reconstitution and incubation at 37 °C (40 rpm).**

In contrast, formulations used hitherto with a pH of 5.6 featured almost 100 % recovery directly after reconstitution, but after 2 days only 60 % of the initial protein was found. This drop in protein recovery was associated by noticeable turbidity of the solutions what indicated protein aggregation and precipitation proceeding. Consequently, it was assumed that the lyophilisation process itself exerted more stress on IFN α-2a when formulated close to its isoelectric point around pH 6 than formulated at a pH of 4.2 (Fig. 61).

For the pH 7 formulation, protein precipitation was already visible during sample reconstitution, and that mirrored in only 60 % IFN α-2a recovery. The detectable protein amount further decreased to 30 % over the 7 days incubation period (Fig. 61). As demonstrated above, the pH of this formulation decreased to a pH of 6.1 during freeze-drying which may exhibit a destabilising impact on the protein structure and, consequently, resulted in a high degree of aggregate formation after reconstitution. Because of a pH at the isoelectric point, a reduced solubility of these products can be considered as further parameter leading to protein precipitation after reconstitution.

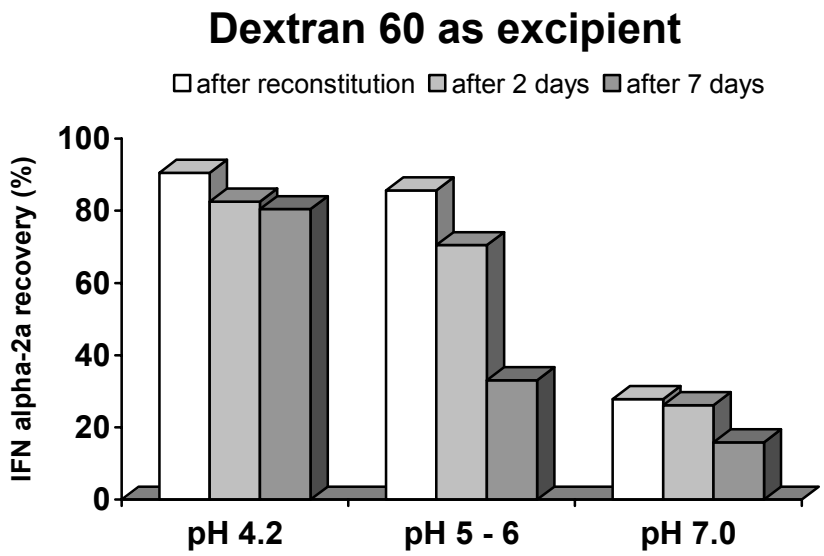


**Figure 62: IFN α-2a stability of dextran 60 freeze-dried formulations with different pH values after reconstitution and incubation at 37 °C (40 rpm).**

Employing dextran 60 as stabilising excipient revealed a similar dependency of protein stability on the formulation pH before freeze-drying. Only the formulation adjusted to a pH of 4.2 exhibited a high IFN α-2a stability throughout incubation (Fig. 62).

However, in comparison to the trehalose formulation, IFN α-2a recovery was obviously lower already after reconstitution. Only 90 % of the initial protein amount were detected, which was 9 % protein less than determined in trehalose products. Moreover, aggregated protein species were notified as early as after reconstitution. These experimental data confirmed the discussion around dextrans´ efficiency in protein lyoprotection, during which dextrans were demonstrated to form elegant amorphous cakes, but fail in protein protection due to steric hindrance [6; 1].

In contrast to dextran, the polymer hydroxypropyl-ß-cyclodextrin (HP-ß-CD) featured a potential in IFN α-2a lyoprotection comparable to trehalose. When formulated at pH 4.2, approximately 100 % protein were recovered in the reconstituted formulation throughout the 7 days incubation. However, the stabilisation potential was also limited to the acidic formulations. All other freeze-dried HP-ß-CD products exhibited decreasing protein recoveries over time, in particular the pH 7 formulations (Fig. 63).

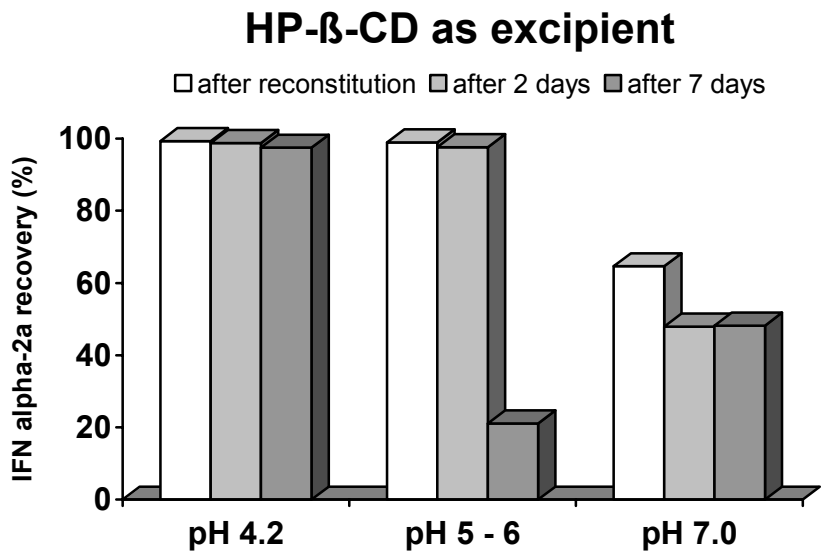


**Figure 63: IFN α-2a stability of HP-ß-CD freeze-dried formulations with different pH values after reconstitution and incubation at 37 °C (40 rpm).**

## 4.4 Summary and discussion

Based on stability data, high IFN α-2a stability in formulations of pH 7.4 isotonic 0.01 M phosphate buffer (PBS) was verified. After 2 months of incubation, 95 % of IFN α-2a almost exclusively consisted of native monomer. Moreover, this high protein stability was also provided by the addition of a PEG-lipid matrix substantiating that polyethylene glycol 6000 (PEG) exhibited no destabilising effects on IFN α-2a. For future release studies that means PEG as release modifier is not anticipated to detract from IFN α-2a stability within the tristearin matrices.

The detected protein loss during stability experiments could be explained by protein adsorption upon the used package materials. It was demonstrated that a major amount of IFN α-2a – up to 15 % of the initial protein dose – has been adsorbed to the borosilicate glass vials during previous stability experiments and as a conclusion during previous release studies. Hence, this protein dose was liberated from the lipid implants, but could not be detected in the release media.

However, it could also be demonstrated that this problem can be solved by the use of cycloolefin copolymer vials. This package material enabled an almost complete inhibition of IFN α-2a adsorption, what can be ascribed to a different surface integrity in comparison to glass materials. Consequently, it was assumed that by changing the

package material during future release studies the total release may be in the ballpark of 85 % values for IFN α-2a implants containing 10 % PEG.

Stability studies of freeze-dried protein formulations showed that the hitherto used pH 5.6 IFN α-2a/trehalose formulations still lack protein stability when reconstituted and incubated at 37 °C. It can be suggested that the freeze-drying process detracts from IFN α-2a stability leading to an accelerated protein aggregation/precipitation tendency when these products were exposed to rehydration stress and/or elevated temperature during incubation.

Freeze-dried IFN α-2a/trehalose products adjusted to a pH of 4.2 before lyophilisation revealed high protein stability. Approximately 100 % of the initial protein were recovered in the reconstituted formulations after 7 days of incubation. Neither the freeze-drying process per se nor subsequent active stress parameters (e.g. rehydration and elevated temperature) compromised IFN α-2a stability. Consequently, it was suggested that this highly stable lyophilised protein will be released to a greater amount from the lipid implants than a protein that already has experienced considerable stress. For future release experiments, that means previously incomplete protein release can potentially be reduced or eliminated by using the pH 4.2 lyophilised IFN α-2a/trehalose formulations – i.e. with cake masses of 16 mg and 64 mg, respectively.

Moreover, HP-ß-CD used as lyoprotective excipient inhered a high efficiency in protein stabilisation during freeze-drying. After reconstitution, approximately 100 % of the initial IFN α-2a were recovered in the acidic formulations after 7 days of incubation. The lyoprotective effect of HP-ß-CD is addressed to protein complexing, thereby shielding the protein in its native form [87]. For future release experiments the applicability of HP-ß-CD formulations should be exploited. Therefore, the pH 4.2 formulation with the higher cake mass of 64 mg was chosen.

## Chapter VIII: Continuous release of IFN alpha-2a from a lipid implant system

After extensively discussing the potential parameters being accountable for incomplete IFN α-2a release from tristearin matrices containing PEG as additive, the following chapter will relate to improvements attained in protein liberation by using new freeze-dried formulations and, moreover, by changing the repositories during release experiments. Detailed information concerning lipid modification, protein stability issues, and implant morphology throughout release studies are shown. As mentioned before, the pH 4.2 trehalose lyophilisates featured the same protein/sugar ratio, but differ in the mass of the freeze-dried cakes – i.e. 16 mg and 64 mg cake mass, respectively. In the following, these lyophilisates will be referred to as trehalose lyophilisates A (mass of 16 mg) and B (64 mg). For HP-ß-CD, lyophilisates with a higher mass (64 mg) were chosen. The investigated matrix formulations are listed below (table 20).

| Formulation | IFN α-2a/ trehalose lyoph. A [%] | IFN α-2a/ trehalose lyoph. B [%] | IFN α-2a/ HP-ß-CD lyoph. [%] | PEG [%] | Tristearin [%] |
|---|---|---|---|---|---|
| 1 | 10 | - | - | 0 | 90 |
| 2 | 10 | - | - | 5 | 85 |
| 3 | 10 | - | - | 10 | 80 |
| 4 | 10 | - | - | 12 | 78 |
| 5 | 10 | - | - | 14 | 76 |
| 6 | 10 | - | - | 16 | 74 |
| 7 | 10 | - | - | 18 | 72 |
| 8 | 10 | - | - | 20 | 70 |
| 9 | - | 10 | - | 0 | 90 |
| 10 | - | 10 | - | 5 | 85 |
| 11 | - | 10 | - | 10 | 80 |
| 12 | - | 10 | - | 12 | 78 |
| 13 | - | 10 | - | 14 | 76 |
| 14 | - | 10 | - | 16 | 74 |
| 15 | - | 10 | - | 18 | 72 |
| 16 | - | 10 | - | 20 | 70 |
| 17 | - | - | 10 | 0 | 90 |
| 18 | - | - | 10 | 5 | 85 |
| 19 | - | - | 10 | 10 | 80 |
| 20 | - | - | 10 | 14 | 76 |
| 21 | - | - | 10 | 16 | 74 |
| 22 | - | - | 10 | 18 | 72 |
| 23 | - | - | 10 | 20 | 70 |

**Table 20: Design of various matrix formulations; for each formulation, three implants were investigated (n=3). The drug load was calculated from implant weight, whereby constantly 2.5 % of implant weight accounted for the IFN α-2a dose.**

## 1. Characterisation of lipid modifications

Tentative experiments featured that lipid modifications are not influenced by implant compression (refer to chapter IV). However, these data only mirrored cases of pure tristearin implants. In order to clarify whether the lyophilised protein per se induced modification changes, previous differential scanning calorimetric data (DSC) were completed by investigations on that. Therefore, implants with a 10 % drug load were prepared and analysed in comparison to unprocessed tristearin bulk as well as to pure tristearin implants (Fig. 64).

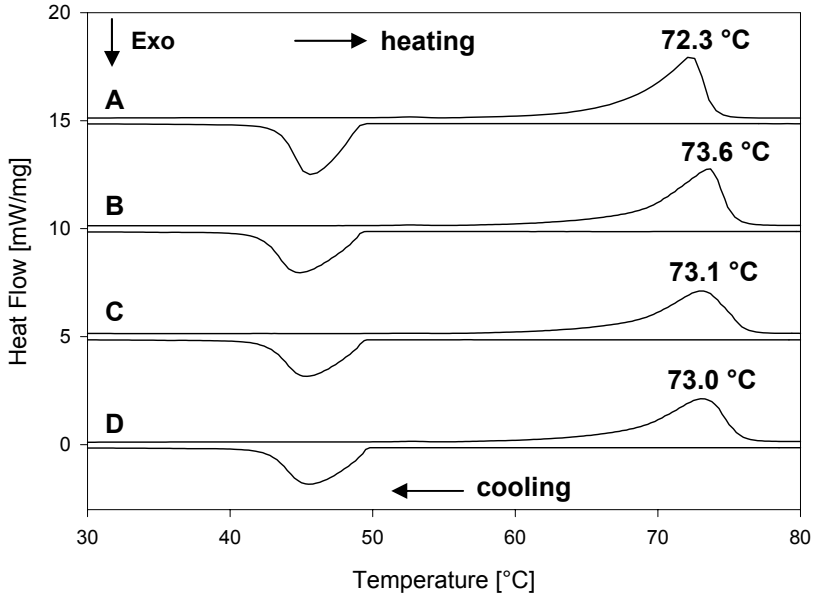


**Figure 64: DSC analysis of (A) tristearin bulk, (B) pure tristearin implants by compressing, (C) tristearin implants containing 10 % IFN α-2a/trehalose lyophilisate A, and (D) tristearin implants containing IFN α-2a/HP-ß-CD lyophilisate (the plots are displaced vertically for better visualisation).**

Unprocessed tristearin bulk (A) demonstrated one single endothermic peak at 72.3 °C, which is assigned to the melting of the stable ß-modification. The thermogram of both tristearin implants (B) and implants containing 10 % IFN α-2a/ trehalose lyophilisate A (C) illustrate similar data, as one endothermic DSC peak was detected at 73.6 °C and 73.1 °C, respectively. As already discussed, the displacement of the peak maxima towards lipid bulk is considered as an effect caused by different particle sizes of the ground implants [23]. In addition, implants

with a 10 % IFN α-2a/HP-ß-CD lyophilisate charge (D) showed also identical results compared to bulk material (one single endothermic peak at 73.0 °C).
In order to verify DSC data, wide-angle X-ray scattering (WAXS) was performed (Fig. 65).

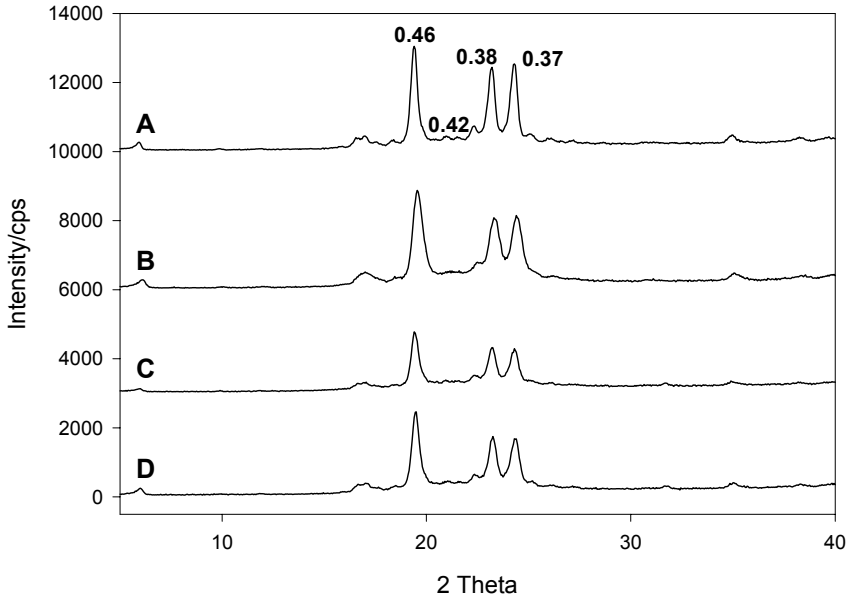


**Figure 65: X-ray diffraction patterns of (A) tristearin bulk, (B) pure tristearin implants, (C) tristearin implants containing 10 % IFN α-2a/trehalose lyophilisate A and (D) tristearin implants with 10 % IFN α-2a/HP-ß-CD lyophilisate. The numbers correspond to the short spacings in nm of the peak maxima (the plots are displaced vertically for better visualisation).**

The diffraction pattern of both implants comprising pure tristearin material (B) and implants containing 10 % lyophilised protein – i.e. 10 % IFN α-2a/trehalose lyophilisate A (C) and 10 % IFN α-2a/HP-ß-CD lyophilisate (D), respectively - resembled that of the bulk material (A). The visible short spacings at 0.46, 0.38 and 0.37 nm, respectively, correspond to short spacings typical for the stable ß-modification [217]. Only very weak reflections were detected at 0.42 nm (ß` polymorph: short spacings at 0.38 and 0.42 nm).
DSC and WAXS results suggested that both the amount of protein load and the manufacturing process per se have no direct impact on lipid modification. Moreover, lipid implants are consisting mainly of stable ß-modification after preparation.

## 2. Influence of the manufacturing process on the protein stability

Protein stability was assessed after implant manufacture applying the extraction technique without using organic solvents (refer to chapter IV). In each case, two implants consisting of 10 % IFN α-2a/trehalose lyophilisates (lyophilisate A and B) and 10 % IFN α-2a/HP-ß-CD lyophilisate, respectively, and 90 % tristearin were compressed. After extraction, the buffer media – containing the protein drug – were analysed by gel electrophoresis. In comparison, lyophilised IFN α-2a samples were reconstituted with PBS/0.05 % (w/v) $NaN_3$ containing 1 % (w/v) polysorbate 20 (PBST) and used as IFN α-2a standard (Fig. 66).

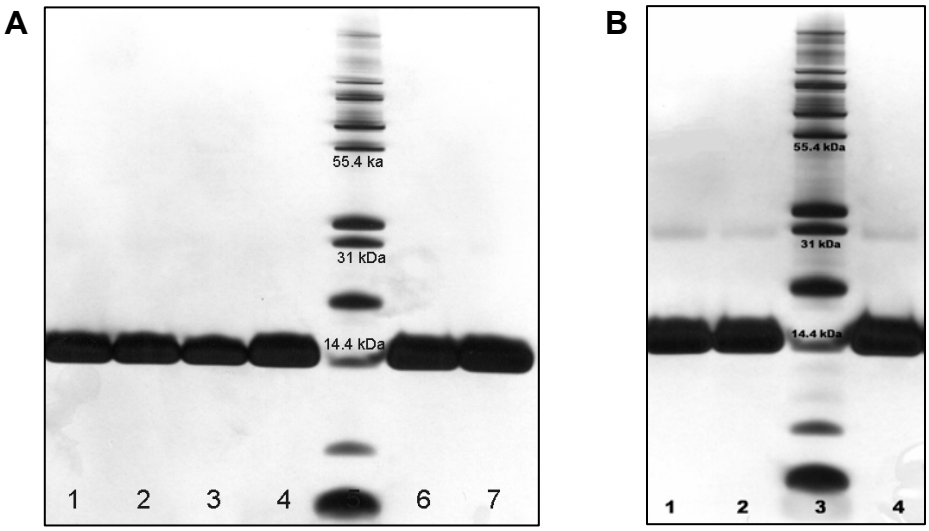


**Figure 66: Influence of the manufacturing process on protein stability. IFN α-2a was extracted from the implants subsequent to compressing. (A) Implants performed with IFN α-2a/trehalose lyophilisates A and B - lanes 1, 2: extracted samples (lyophilisate B), lane 3: lyophilisate A reconstituted in PBST, lanes 4, 6: extracted samples (lyophilisate A), lanes 5: MW marker, lane 7: lyophilisate B reconstituted in PBST; (B) implants performed with IFN α-2a/HP-ß-CD lyophilisates, lane 1: lyophilisate reconstituted in PBST, lanes 2, 4: extracted samples, lane 3: MW marker.**

The results confirmed that the compression process of protein/lipid mixtures did not detract from protein stability, thus works under mild conditions. Implants prepared from IFN α-2a/trehalose lyophilisates (Fig. 66 A) did not exhibit any IFN α-2a aggregates. Samples derived from IFN α-2a/HP-ß-CD lyophilisates were shown to marginally contain aggregated protein (Fig. 66 B). However, the identified dimer

species were also notified when analysing the reconstituted lyophilisates, and thus are not be attributed to the manufacture process.

Generally, such dimer fractions accounted less then 2 % of overall protein. Such an approach can be deemed acceptable for investigative purposes. As earlier discussed, even lyophilised IFN α-2a products available on the market contain up to 1 % aggregates [20].

As a conclusion, for assessing data of release studies described below, one can assume that the lipid matrices were loaded almost exclusively with native IFN α-2a before release studies were initiated.

## 3. In vitro release studies

Initially, IFN α-2a release from tristearin matrices without additives was investigated. As shown in Fig. 67 A, implants loaded with 10 % IFN α-2a/trehalose lyophilisate A liberated 84 % of the incorporated protein. However, the initial drug release accounted for almost 45 % of the total protein load, and after 4 days as much as 70 % of IFN α-2a were liberated.

Evaluation studies on implants prepared with 10 % IFN α-2a/trehalose lyophilisate B (Fig. 67 B) featured comparable release profiles. Overall, obtained liberation rates were lower for this formulation, only 60 % of the encapsulated IFN α-2a were released.

In order to maximise IFN α-2a release and to control the release profile, varying amounts of tristearin were replaced by PEG (table 20). Selected liberation curves are illustrated in Fig. 67 A/B.

As demonstrated by the release profiles the addition of PEG 6000 caused a considerable alteration of the release kinetics. This was in accordance with previous release experiments when PEG was used as release modifier (refer to chapter VII). Moreover, a notable decrease of about 25 % in the initial burst release was shown for the PEG containing formulations.

For IFN α-2a/trehalose lyophilisates A (Fig. 67 A), implants containing 5 % PEG and 10 % PEG, respectively, revealed a continuous and sustained protein liberation for up to 1 month. The total amount of released IFN α-2a did not change substantially in comparison to the formulations without PEG, i.e. 83 % and 89 % of the incorporated

protein, respectively, were liberated. Increasing the PEG content further on, e.g. 16 % and 20 %, resulted in liberation profiles approaching a 95 % protein release level. However, the liberation succeeded faster and terminated after 2 weeks of incubation.

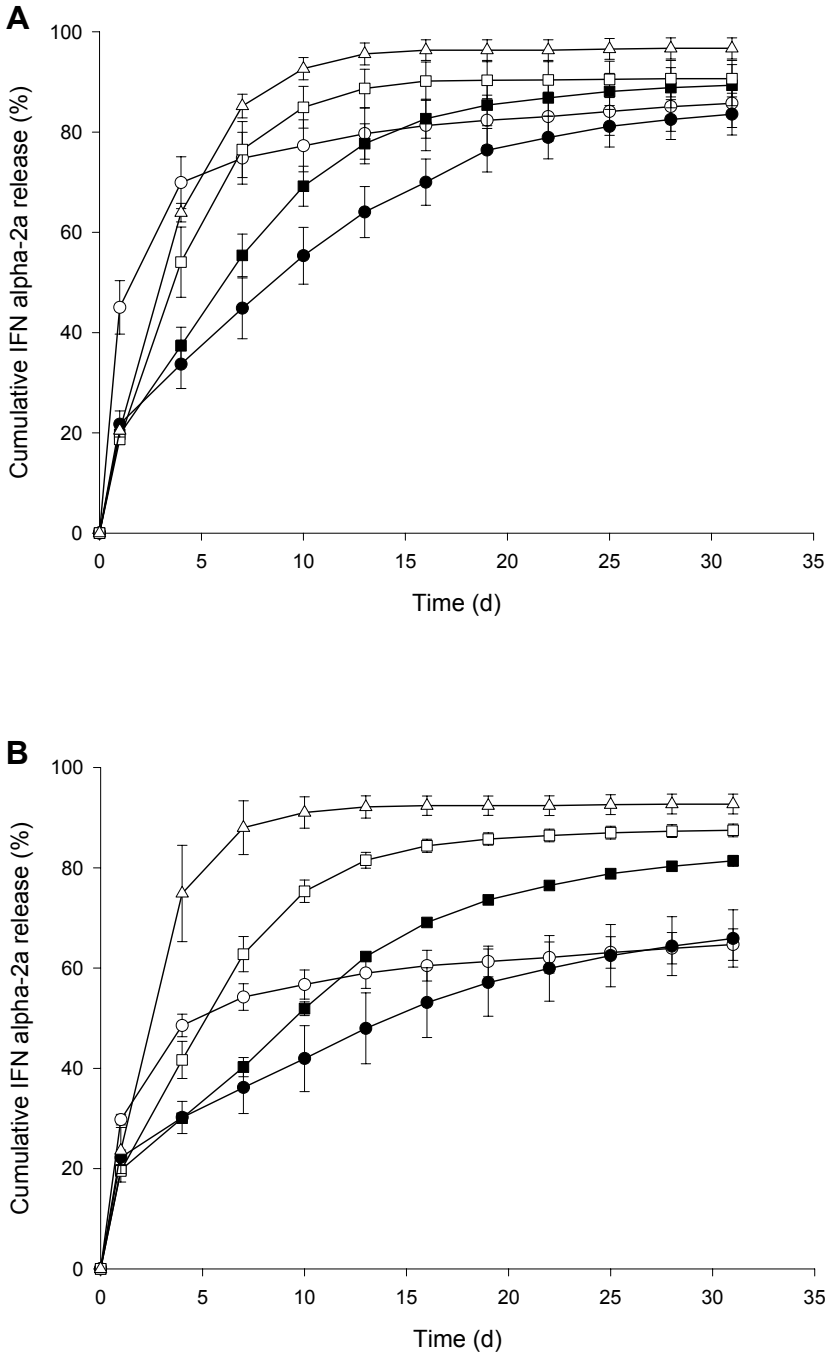


**Figure 67: Effects of lyophilisate formulation and PEG content on release kinetic of IFN α-2a. (A) Release profiles of implants performed with IFN α-2a/trehalose lyophilisates A - PEG content: 0 % (○), 5 % (●), 10 % (■), 16 % (□), 20 % (△); (B) Release profiles of implants performed with IFN α-2a/trehalose lyophilisates B - PEG content: 0 % (○), 5 % (●), 10 % (■), 16 % (□), 20 % (△) (average ± SD, n=3).**

Implants manufactured with IFN α-2a/trehalose lyophilisates B and a variation of PEG contents (Fig. 67 B) led to lower protein releases compared to implants containing the lyophilisate A formulations. But, in general, the profiles obtained were identical, meaning low PEG contents achieved a continuous IFN α-2a release for up to 1 month. With higher amounts of PEG an enhanced but also accelerated protein liberation occurred.

In Fig. 68, the release profiles of matrices formulated with IFN α-2a/HP-ß-CD lyophilisates are illustrated. The addition of PEG to these implants caused an enhancement of total released IFN α-2a up to 97 %, facing a 75 % release from matrices without any PEG. Moreover, with PEG the release behaviour appeared to be more sustained, and again burst effects were lowered. An optimal content of 10 % PEG was assessed. By that, 95 % of the initial incorporated protein were delivered continuously over 1 month, approaching zero-order kinetic within the very first 2 weeks.

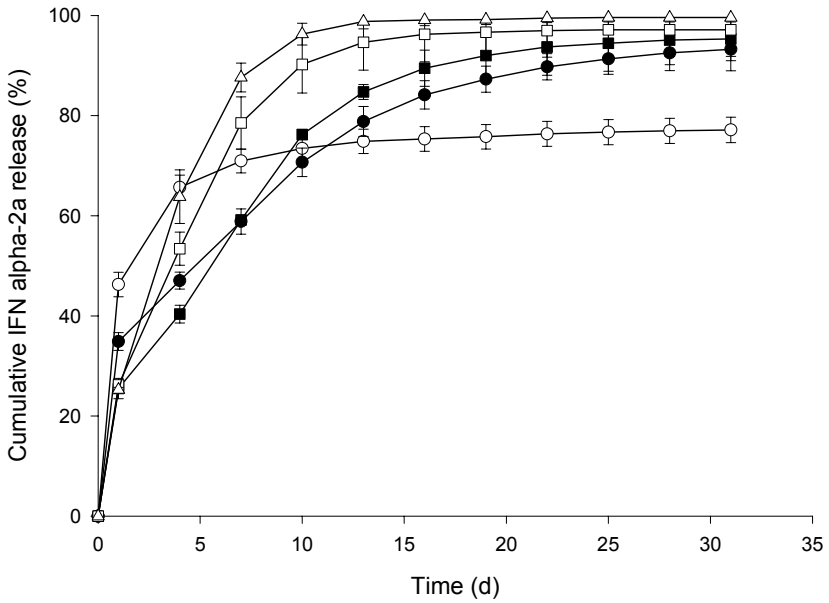


**Figure 68: Effect of lyophilisate formulation and PEG content on the release kinetic of IFN α-2a. Release profiles of implants performed with IFN α-2a/HP-ß-CD lyophilisates - PEG content: 0 % (○), 5 % (●), 10 % (■), 16 % (□), 20 % (△) (average ± SD, n=3).**

It can be concluded that both the lyophilisate formulation per se and the addition of PEG impacted the liberation profile, the total drug release, and the release mechanism.

The continuous drug release over a period of 1 month suggested that dissolution and diffusion of IFN α-2a occurs through pores and interconnected channels, formed by PEG and protein particles during the manufacturing process. Here, the raise in PEG content from 10 % up to 20 % did not result in a considerable enhancement of total drug delivery, but the liberation periods were considerably reduced from implants with higher PEG contents. It can be assumed that the increase in the PEG content – i.e. 16 % up to 20 % - led to easier accessible pathways (reduced tortuosity) and to a greater porosity within the interior of the lipid implants. Consequently, protein release was accelerated from such matrices.

Generally, IFN α-2a release was faster and more complete from implants formulated with IFN α-2a/HP-ß-CD lyophilisates, what can be ascribed to the solubilising and stabilising potential of HP-ß-CD for proteins as well as by drug complexing features [21; 192].

## 4. Protein stability during release

IFN α-2a aggregation proceeding within the matrices can be deemed a possible reason for residual protein remaining in the matrix after incubation (refer to chapter VII).

Fig. 69 A/B shows the result of IFN α-2a extraction from incubated implants produced with IFN α-2a/trehalose lyophilisates. In comparison to the samples extracted directly after compressing, higher-ordered aggregates were now detected in all samples.

For the IFN α-2a/trehalose lyophilisates A (Fig. 69 A), aggregation occurred more pronounced within implants containing a higher amount of PEG: the aggregate fraction increased from approximately 1 % (for PEG free matrices) to 7 % (for a 10 % PEG content). Here, a difference in the protein aggregates detected in the two samples extracted from 10 % PEG implants was to be noted (Fig. 69 A, lane 1 and 2). The stated value of 7 % of aggregated species matches to the sample showing a more pronounced aggregation (Fig. 69 A, lane 2). Considering the initial incorporated

IFN α-2a, 0.7 % - 1.4 % of the protein can be suggested being existent within the implants as higher-ordered aggregates after 1 month of incubation.

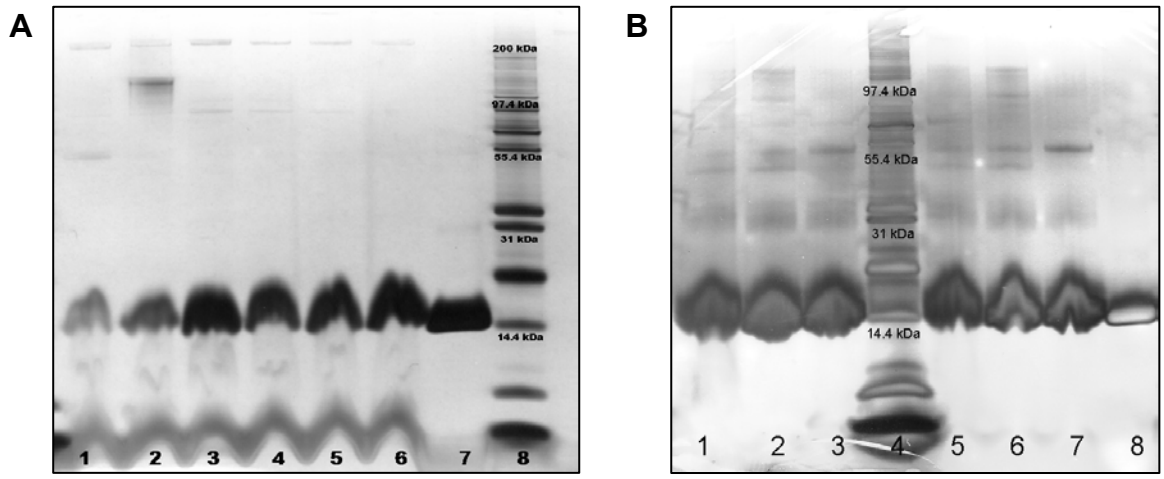


**Figure 69: IFN α-2a stability within the matrices during in vitro release, effect of the lyophilisate formulation on protein stability during release. (A) Extracted from IFN α-2a/trehalose lyophilisate A implants after 1 month of incubation - lanes 1, 2: 10 % initial PEG content, lanes 3, 4: 5 % initial PEG content, lanes 5, 6: 0 % initial PEG content, lane 7: protein standard, lane 8: MW marker. (B) Extracted from IFN α-2a/trehalose lyophilisate B implants after 1month - lanes 1, 2: 10 % initial PEG content, lanes 3, 5: 5 % initial PEG content, lane 4: MW marker, lanes 6, 7: 0 % initial PEG content, lane 8: protein standard.**

PAGE studies of IFN α-2a samples extracted from IFN α-2a/trehalose lyophilisate B implants (Fig. 69 B) after 1 month of incubation featured more intense protein aggregation than samples extracted from IFN α-2a/trehalose lyophilisate A matrices. Besides higher-order oligomer fractions, a considerable amount of dimer and trimer species was detected in the buffer media containing the extracted protein drug. The quantification revealed protein aggregated species accounting for more than 30 % of overall band intensity. This event was independent from the initial PEG content of the implants. Based on these PAGE data the extensive protein aggregation in the matrices during release experiments could be shown to reason the reduced and incomplete protein liberation from such IFN α-2a/trehalose lyophilisate B implants.

As visualised in Fig. 70, samples extracted from IFN α-2a/HP-ß-CD lyophilisate matrices also contained higher-order aggregates, yet the total amount of aggregates (≤ 3 %) was considerably lower compared to samples extracted from IFN α-2a/trehalose implants. Moreover, it can be claimed that only 0.15 % - 0.3 % of the

initial incorporated protein are existent as higher-order IFN α-2a species within the implants after 1 month of incubation.

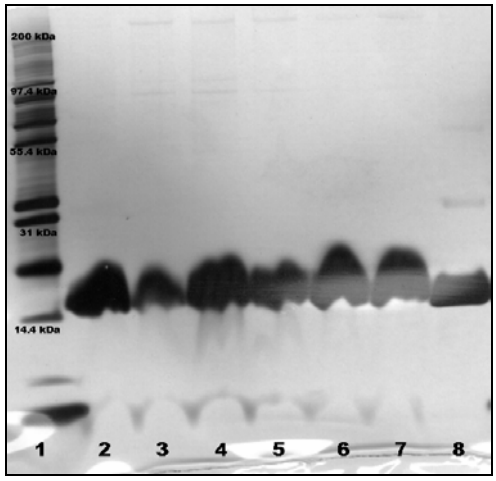


**Figure 70: IFN α-2a stability within the matrices during in vitro release, effect of the lyophilisate formulation on protein stability during release. IFN α-2a extracted from IFN α-2a/HP-ß-CD implants after 1 month of incubation - lane 1: MW marker, lanes 2, 3: 0 % initial PEG content, lanes 4, 5: 5 % initial PEG content, lanes 6, 7: 10 % initial PEG content, lane 8: protein standard.**

As presented before, HP-ß-CD demonstrates a stabilising effect on various peptides and proteins [192; 21]. Consequently, it can be assumed that IFN α-2a can be protected by HP-ß-CD within lipid matrices during the release process by minimizing aggregation phenomena.

Contrarily, the released IFN α-2a appears almost exclusively in monomeric form. SE-HPLC marginally revealed aggregated protein with less than < 1 % within the release media over all the incubation period, and RP-HPLC demonstrated a constant 1.5 % level of oxidised IFN α-2a species.

PAGE studies of samples drawn from implant studies conducted with IFN α-2a/trehalose lyophilisates A and a 10 % PEG content are illustrated in Fig. 71 A/B.

Only a minor amount of trimer specimen notified after 16 days of incubation mirrored protein aggregation. Data obtained from implants using IFN α-2a/trehalose lyophilisates B or IFN α-2a/HP-ß-CD lyophilisates showed comparable results (data not shown).

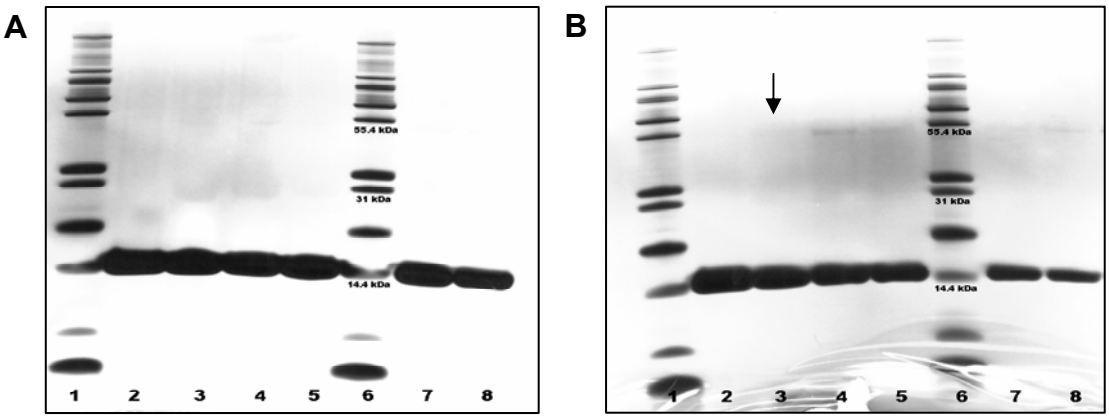


**Figure 71: Stability of IFN α-2a released from the matrices. (A, B) IFN α-2a liberated from implants using IFN α-2a/trehalose lyophilisates A with a 10 % PEG content - (A) lanes 1, 6: MW marker, lane 2: protein standard, lane 3: IFN α-2a released after 24 hrs, lane 4: IFN α-2a released after 4 d, lane 5: IFN α-2a released after 7 d, lane 7: IFN α-2a released after 10 d, lane 8: IFN α-2a released after 13 d; (B) lanes 1, 6: MW marker, lane 2: protein standard, lane 3: IFN α-2a released after 16 d, lane 4: IFN α-2a released after 19 d, lane 5: IFN α-2a released after 22 d, lane 7: IFN α-2a released after 25 d, lane 8: IFN α-2a released after 28 d, (↓) Occurrence of trimer species material.**

In accordance to chapter VII, these data confirmed the liberation of higher-order aggregate species being limited from the lipid implants because of their lower diffusion coefficients or due to the pore sizes within the implants. Moreover, it can be concluded 85 % - 95 % of IFN α-2a to be released in form of native monomer as well as aggregated dimer and trimer species to be liberated only marginally.

## 5. Implant morphology

Even with higher PEG contents, tristearin implants revealed a high physical stability during release. No erosion was visible after 1 month of incubation (Fig. 72).

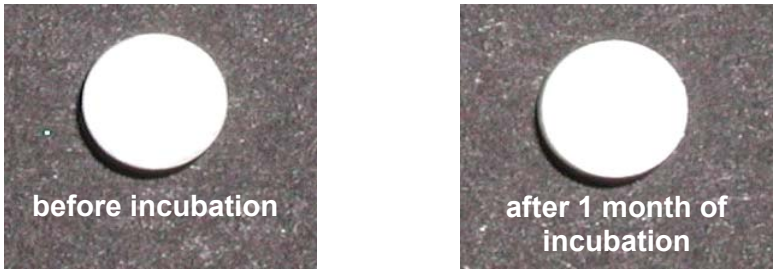


**Figure 72: Implants consisting of 10 % IFN α-2a/trehalose lyophilisate A, 10 % PEG and 80 % tristearin before and after 1 month of incubation.**

Due to this physical stability and the lack of erosion phenomena, the protein was assumed to be liberated through pores. After preparation of matrices containing 10 % lyophilisate, 10 % PEG and 80 % tristearin, implant surfaces appeared smooth and with only low ruggedness (Fig. 73 A/B).

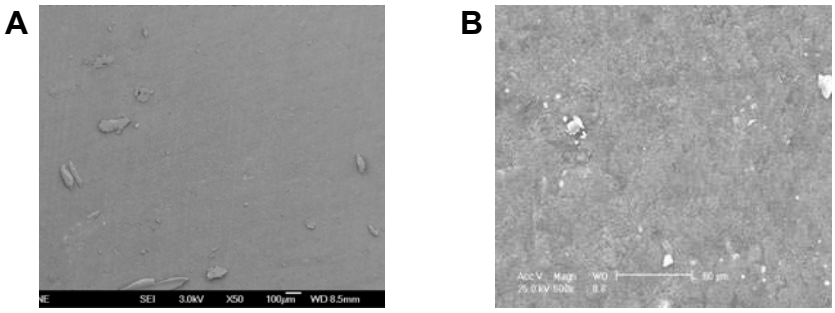


**Figure 73: SEM images of (A) implant from 10 % IFN α-2a/trehalose lyophilisate A/10% PEG/80% tristearin and (B) implant from IFN α-2a/HP-ß-CD lyophilisate/10% PEG/80% tristearin – both before incubation.**

Fig. 74 A - C illustrates SEM images of implants manufactured with 10 % IFN α-2a/trehalose lyophilisate A and a different amount of PEG after 1 month incubation.
A small number of pores were formed on the surface of implants containing 5 % PEG as additive (Fig. 74 A). With a higher PEG amount, the pore number and the pore sizes increased (Fig. 74 B/C). Pores with up to 100 µm in diameter were visible on such implant surfaces. Data obtained from IFN α-2a/trehalose lyophilisate B implants were comparable in data.

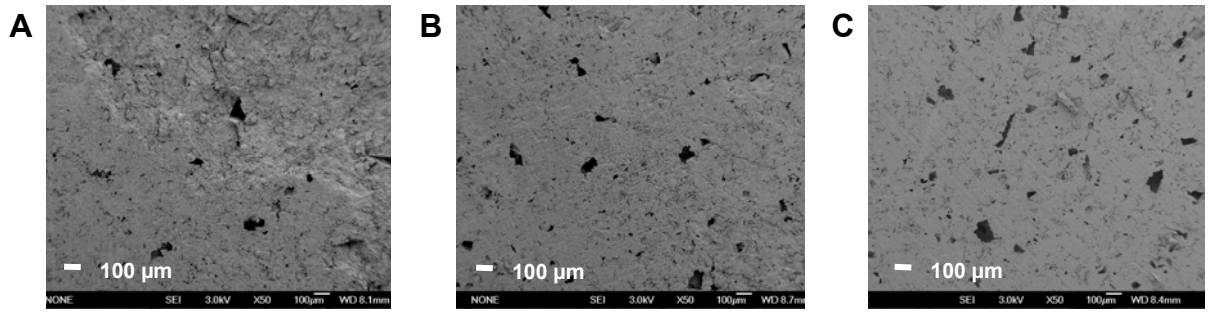


**Figure 74: SEM images of implants consisting of 10 % IFN α-2a/trehalose lyophilisate A and different levels of PEG after 1 month of incubation. PEG content: 5 % (A), 10 % (B) and 20 % (C) (images with 50x magnification).**

As visualised in Fig. 75 A – C, implants performed with IFN α-2a/HP-ß-CD lyophilisate and different PEG levels also exhibited pore formation after 1 month of

incubation. The diameters visible at up to 100 µm were comparable to those of the IFN α-2a/trehalose matrices. However, a dependency of both pore number and pore sizes on the initial PEG amount could not be asserted for the IFN α-2a/HP-ß-CD implants.

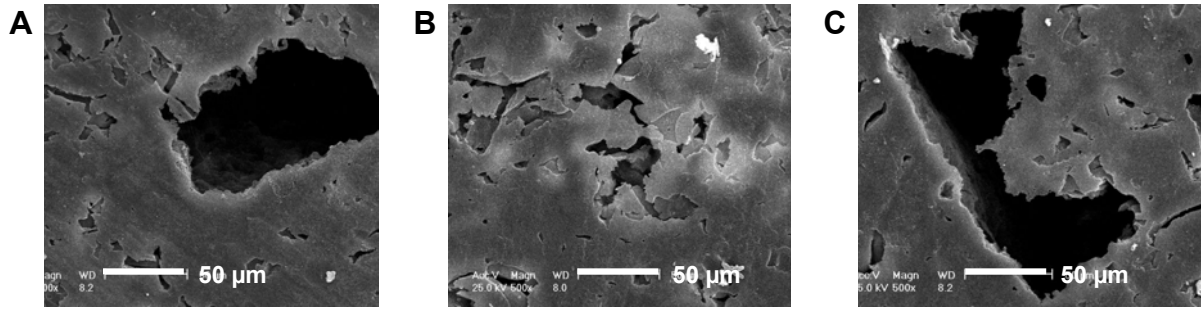


**Figure 75: SEM images of implants consisting of 10 % IFN α-2a/HP-ß-CD lyophilisate and different levels of PEG after 1 month of incubation. PEG content: 5 % (A), 10 % (B) and 20 % (C) (images with 500x magnification).**

SEM results were backed by mercurial porosimetry measurements in order to evaluate the inner surface of the implants after 1 month of incubation. The determined total intrusion volumes and calculated porosities of incubated implants are listed as a function of the initial PEG content in table 21. Dry implants revealed a total intrusion volume of 0.0446 mL/g. That can be attributed to a small number of pores located on matrix surfaces as a result of the compressing process.

| 10 % IFN α-2a/trehalose lyophilisate A implants | Initial PEG content [%] | | | | | |
|---|---|---|---|---|---|---|
| | 5 | 10 | 14 | 16 | 18 | 20 |
| Total intrusion volume (mL/g) | 0.1387 | 0.1651 | 0.2469 | 0.2662 | 0.3453 | 0.3650 |
| Porosity (%) | 9.68 | 11.02 | 17.70 | 18.78 | 23.04 | 22.74 |

| 10 % IFN α-2a/HP-ß-CD lyophilisate B implants | Initial PEG content [%] | | | | |
|---|---|---|---|---|---|
| | 10 | 14 | 16 | 18 | 20 |
| Total intrusion volume (mL/g) | 0.1605 | 0.2685 | 0.3246 | 0.3743 | 0.3919 |
| Porosity (%) | 11.13 | 17.05 | 21.42 | 22.33 | 24.76 |

**Table 21: Results of mercury porosimetry measurements. Implants were analysed after 1 month of incubation.**

Porosimetry data demonstrated a considerable enhancement in the final pore volume of 1 month incubated implants, when compared to dry matrices. Moreover, with higher levels of initial incorporated PEG these final pore volumes increased, e.g. a

rise from 0.1387 mL/g for IFN α-2a/trehalose lyophilisate A implants and a 5 % PEG content up to 0.3650 mL/g for a 20 % PEG load could be determined.

Such an effect was also mirrored by the calculated porosities. IFN α-2a/trehalose lyophilisate A implants with a 5 % PEG charge resulted in porosity values well below 10 %. Increasing the PEG content, e.g. up to 18 % and 20 %, led to a surge of implants porosity up to double values, what means that porous structures such as pores and channels account for over 20 % of the implant volume after 1 month of incubation.

Incubated matrices manufactured with IFN α-2a/HP-ß-CD lyophilisates and different PEG contents featured the same dependency of implant porosity on the initial PEG amount: higher PEG levels generated more porous structures during release experiments. For 10 % PEG matrices, porosity was determined being around 11 %, increasing constantly up to 25 % for matrices with a 20 % PEG content.

The data verified the formation of water-filled pores and channels within the lipid implants during release experiments. Given the background that only 10 % of implant weight is due to lyophilised protein and that tristearin matrices themselves do not erode at all, it can be assumed that this effect was the result of both PEG and IFN α-2a dissolution and diffusion. Consequently, protein liberation from the lipid implants appeared to be a result of pore diffusion and, moreover, was influenced by the initial incorporated PEG amount.

## 6. IFN α-2a storage stability within lipid implants containing PEG

Depending on the lyophilisate formulation, up to 95 % of the incorporated IFN α-2a could be liberated continuously over a 1 month period from tristearin implants with a 10 % PEG content. Moreover, the protein was released almost exclusively in its monomeric form.

However, the storage stability of drug dosage forms can be influenced by environmental conditions such as temperature, oxygen, light, and humidity. In this relation, the term stability refers to the time frame from initial preparation and packaging during which the dosage form continues to comply with quality and purity requirements.

As a result of the marginal storage stability of many proteins, long-term storage of protein dosage forms often goes along with conformational changes of the protein drugs incorporated and, consequently, purity requirements can not be fulfilled any longer [6].

In order to evaluate storage stability of IFN α-2a within lipid implants, tristearin matrices with 10 % lyophilised protein and a PEG content of 10 % were prepared. Subsequently, implants were deposited into borosilicate glass vials and overlayed with nitrogen before sealing in order to reduce potential detrimental effects of atmospheric oxygen. Samples were stored at 4 °C and room temperature (with 33 % relative humidity) over 3 months and 6 months, respectively. After the expiration of these terms, implants were transferred into TopPac® vials and release studies were conducted at 37 °C (40 rpm) over a 1 month incubation period.

## 6.1 Release studies after 3 months and 6 months of implant storage

As visualised in Fig. 76 A, matrices formulated with IFN α-2a/trehalose lyophilisates A and a 10 % PEG content yielded a decrease of total drug liberation after 3 month storage. When compared to release studies performed immediately after implant preparation, only 80 % of the incorporated protein were liberated from the implants – in contrast to a 90 % protein liberation rate of unstored implants. However, the release kinetics did not notably change, since IFN α-2a still was liberated continuously over 1 month. For implants stored at 4 °C reduced liberation rates were demonstrated in early release phases, but after incubation for 1 month the same total drug release was achieved as it was with implants stored at room temperature.

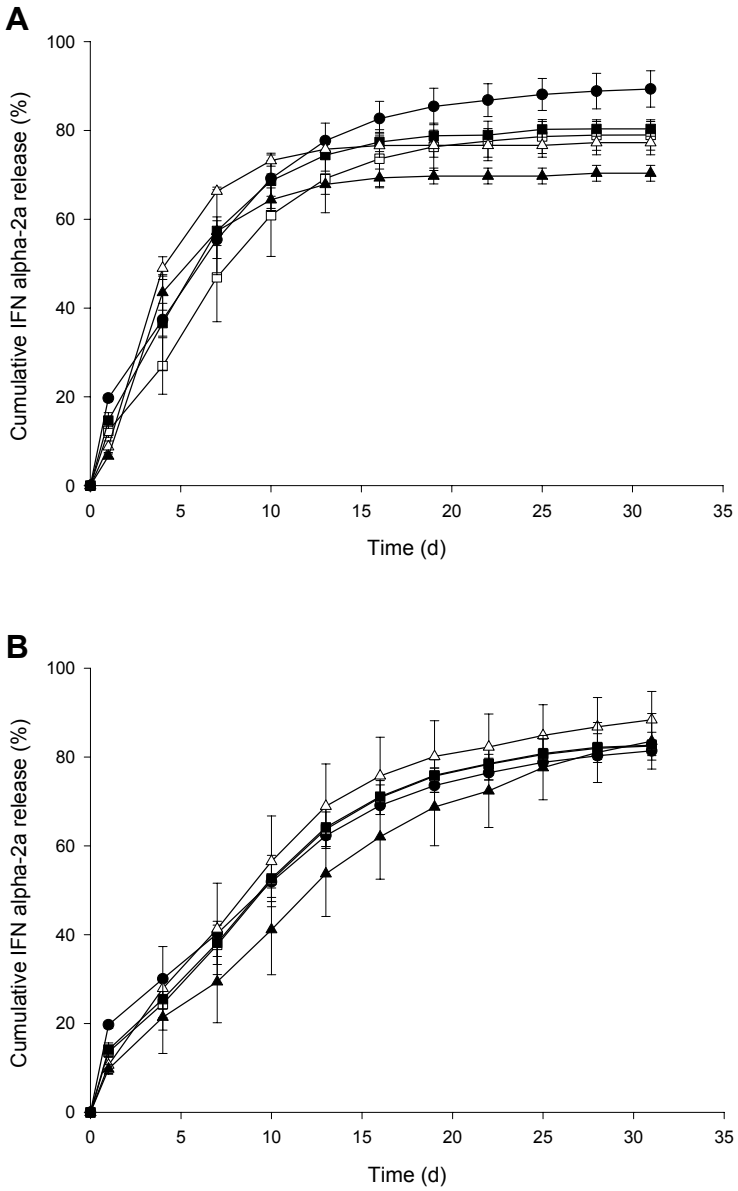


**Figure 76: Influence of storage conditions on IFN α-2a release from tristearin matrices formulated with trehalose lyophilisates and with 10 % PEG. Implants were stored over 3 months and 6 months at 4 °C and room temperature (33 % rel. humidity), respectively, before release studies were initiated. (A) Implants performed with IFN α-2a/trehalose lyophilisates A – before storage (●), stored for 3 months at 4 °C (□) and RT (■), stored for 6 months at 4 °C (△) and RT (▲); (B) Implants performed with IFN α-2a/trehalose lyophilisates B - before storage (●), stored for 3 months at 4 °C (□) and RT (■), stored for 6 months at 4 °C (△) and RT (▲) (average ± SD, n=3).**

The extension of the storage period up to 6 months led to a further reduction of total drug liberation. Only 70 % of the incorporated protein could be liberated from

implants stored at room temperature. Moreover, protein delivery in early release stages appeared to be accelerated in comparison to IFN α-2a liberation from implants before storage as well as after 3 month storage. Consequently, release profiles approached parabolic forms.

For implants performed with IFN α-2a/trehalose lyophilisates B and with PEG, no correlation of protein release with storage conditions was notified (Fig. 76 B). After a 3 month and 6 month storage, the attained liberation levels of up to 85 % protein were identical to that of matrices before storage. Also, the protein was delivered from stored implants in a virtually linear manner during a 1 month incubation period.

Fig. 77 illustrates IFN α-2a liberation profiles from implants formulated with IFN α-2a/ HP-ß-CD lyophilisates and with 10 % PEG.

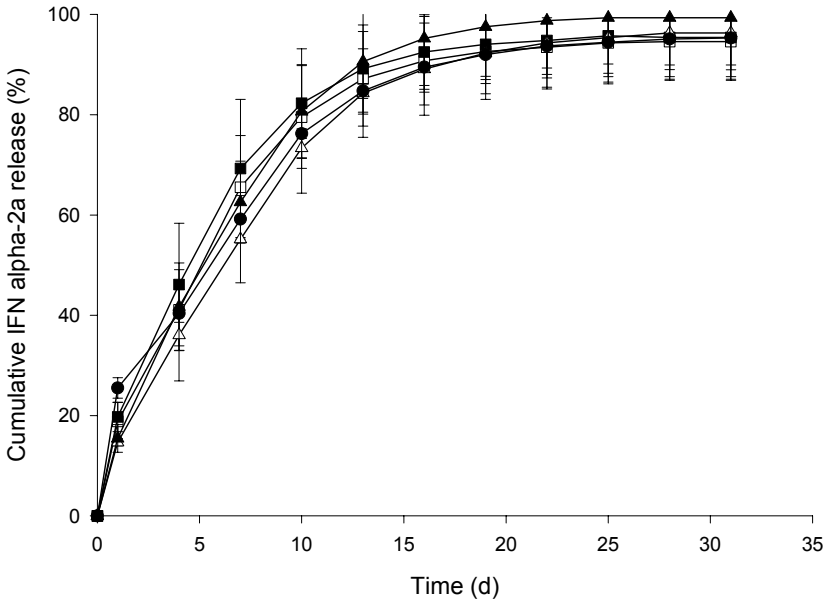


**Figure 77: Influence of storage conditions on IFN α-2a release from tristearin matrices formulated with HP-ß-CD lyophilisates and with 10 % PEG. Implants were stored over 3 months and 6 months at 4 °C and room temperature (33 % rel. humidity), respectively, before release studies were conducted - before storage (●), stored for 3 months at 4 °C (□) and RT (■), stored for 6 months at 4 °C (△) and RT (▲) (average ± SD, n=3).**

Generally, more than 95 % of the incorporated protein could be liberated from the matrices even after 6 month storage. This indicated protein stability within such implants not to be affected during storage. Moreover, the kinetic of release was

identical to that before storage, once more revealing a faster IFN α-2a release than out of implants comprising trehalose lyophilisates (Fig. 77).

## 6.2 Protein stability after 3 months and 6 months of implant storage

In Fig. 78, IFN α-2a dimer and trimer fractions of total released protein (release studies of implants stored at 4 °C over 3 months) are exemplified versus the parameter incubation time. Apparently, IFN α-2a liberated from implants containing IFN α-2a/trehalose lyophilisates A and PEG revealed a considerable amount of dimer and trimer species (> 3 %) as early as after 10 days of incubation. Aggregation unambiguously became more extensive with continuing incubation, since the aggregate fraction accounted for more than 8 % after 19 days of incubation.

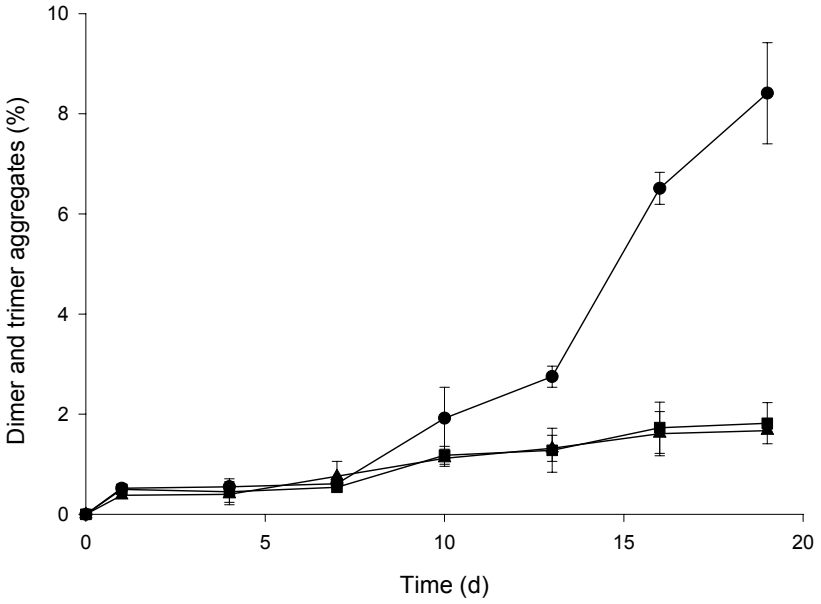


**Figure 78: IFN α-2a dimer and trimer species detected in the release media during in vitro release studies; implants were stored at 4 °C over 3 months before release studies were conducted. IFN α-2a released from implants performed with (●) IFN α-2a/trehalose lyophilisates A and 10 % PEG, (▲) IFN α-2a/trehalose lyophilisates B and 10 % PEG, (■) IFN α-2a/HP-ß-CD lyophilisates and 10 % PEG (average ± SD, n=3).**

In contrast, protein released from implants manufactured with IFN α-2a/trehalose lyophilisates B and IFN α-2a/HP-ß-CD lyophilisates also comprised dimer and trimer species, yet the total amount of aggregates remained below 2 % throughout the incubation period.

Furthermore, RP-HPLC analysis of the different samples exhibited a slightly increased amount of oxidised IFN α-2a species when the protein was released from the trehalose lyophilisate implants, e.g. in a range of 4 – 6 % in comparison to standard material (where oxidised protein was around 2 %). Contrarily, IFN α-2a liberated from HP-ß-CD lyophilisate matrices demonstrated levels of oxidised protein below 2 %.

After 6 month storage, approximately 15 % of total IFN α-2a released from implants manufactured with IFN α-2a/trehalose lyophilisates A and PEG had to be ascribed to dimer and trimer protein (Fig. 79).

The mass of the species released from IFN α-2a/trehalose lyophilisate B implants and from IFN α-2a/ HP-ß-CD lyophilisate matrices, respectively, remained constantly below 2 % total released protein over the timeframe monitored (Fig. 79).

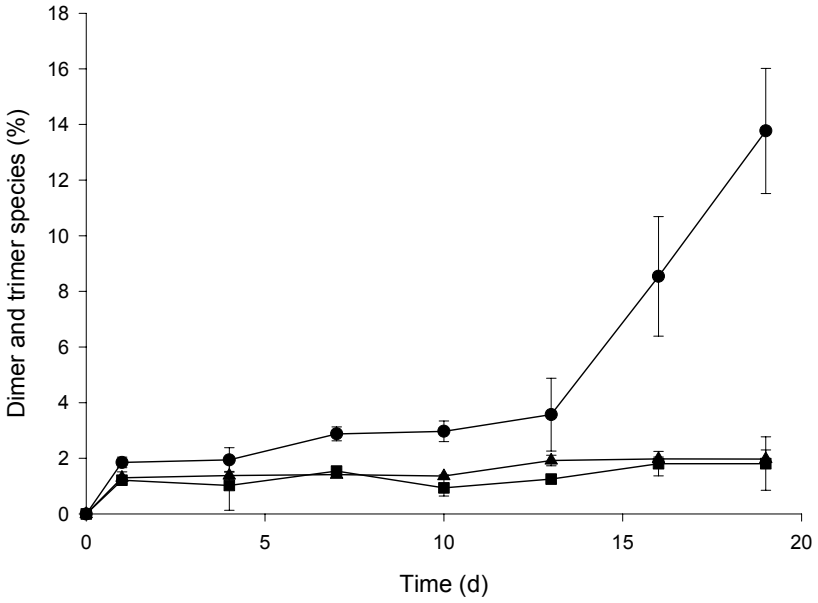


**Figure 79: IFN α-2a dimer and trimer species detected in the release media during in vitro release studies. Implants were stored at 4 °C over 6 months before release studies were conducted. IFN α-2a released from implants performed with (●) IFN α-2a/trehalose lyophilisates A and 10 % PEG, (▲) IFN α-2a/trehalose lyophilisates B and 10 % PEG, (■) IFN α-2a/HP-ß-CD lyophilisates and 10 % PEG (average ± SD, n=3).**

When IFN α-2a was liberated from implants processed with trehalose lyophilisates and PEG, oxidised protein was detected to an extent of 10 – 15 % in the release media after 6 month storage. In contrast, protein released from the HP-ß-CD

matrices remained at a 2 - 3 % level of chemically altered species throughout incubation.

Based on these storage stability data it can be concluded that the high amount of dimer and trimer aggregates released from the IFN α-2a/trehalose lyophilisate A implants refers to protein instability effects proceeding within the matrices during storage. Consequently, higher-order aggregates may also be assumed in the implant interior. These species may cause the notable decrease in total protein release compared to drug release from the same implants before storage.

Release experiments of implants formulated with IFN α-2a/trehalose lyophilisates B and with 10 % PEG exhibited an incomplete protein liberation of 80 % total releasable IFN α-2a before storage. This incomplete liberation was ascribed to aggregation proceeding within the matrices during release (refer to this chapter, paragraph 4). However, neither total drug liberation nor the release kinetic changed when implants were stored over 3 months and 6 months, respectively, prior to release experiment initiation. Moreover, the released dimer and trimer species remained below 2 % throughout the incubation time. On the other hand, an increased amount of oxidised protein species was notable.

Total protein liberation from implants formulated with IFN α-2a/HP-ß-CD lyophilisates and 10 % PEG remained on a 95 % release level also after 6 month implant storage. Moreover, the protein was released almost completely in its monomeric form. Here, protein liberation generally occurred faster than from implants manufactured with trehalose lyophilisates. These data verified HP-ß-CD inhering a solubilising and particularly stabilising potential for proteins encapsulated within lipid matrices during both storage and release process.

## 7. Summary and discussion

In chapter VII, parameters potentially causing an incomplete IFN α-2a release from lipid implants containing PEG as additive were discussed. Based on the resulting conclusions and consequences thereof - i.e. the change of the lyophilised protein formulation, and the quality improvement associated herewith, as well as the change of the containers used during release experiments - the development of a

delivery system which allows an almost complete and sustained protein release was realised.

Initially, tristearin implants composed of IFN α-2a/trehalose lyophilisates A and 10 % PEG exhibited a high potential as compatible protein delivery devices. Up to 90 % of the incorporated protein were released as native monomer in a steady mode over 1 month. However, long-term storage of such implants appeared to be detrimental for the stability of the incorporated protein, apparent by both the reduction of the achieved total drug release and the liberation of a high amount of dimer and trimer species. Moreover, chemically altered protein molecules were detected. It can be supposed that the mixture of protein, trehalose, PEG and tristearin material per se somewhat fosters IFN α-2a instability within the matrices during storage and subsequent release. That means lipid-protein interactions being possible especially during an extended time of storage, and trehalose lyophilisates A appear not to be effective to provide a full protein protection. Consequently, this may lead to both protein aggregation as well as to an increase in overall tendency for IFN α-2a to remain within the lipid matrix due to non-covalent interactions.

Not more than 83 % of the incorporated protein could be liberated from implants manufactured with IFN α-2a/trehalose lyophilisate B matrices and with 10 % PEG during all release experiments. This incomplete release may also implicate that trehalose did not protect the incorporated IFN α-2a sufficiently from aggregation and/or from lipid-protein interactions.

Otherwise, the total amount of releasable protein and the release kinetics did not alter due to the precedent long-term storage. Moreover, the amount of released dimer and trimer species remained below 2 % throughout incubation. Thus, it can be suggested that the protein/trehalose lyophilised B formulation appears to be more effective in IFN α-2a stabilisation within the tristearin implants over time than the lyophilisate A formulation.

In contrast, 95 % of the initially incorporated IFN α-2a could be liberated continuously during a 1 month period from tristearin implants manufactured with IFN α-2a/HP-ß-CD lyophilisates and PEG, even after 6 month storage of the implants. In addition to that, protein was released from such implants almost in its native monomeric form, whereas aggregated species were liberated only marginally.

This obviously indicates the high potential of HP-ß-CD in protein stabilisation and solubilisation within the matrix during both storage and release processes. It can be hypothesised that HP-ß-CD reduces the hydrophobic interactions of protein and lipid matrix material by complexing the protein in its native or potentially in its (partly) denatured form. Thereby, the overall tendency for IFN $\alpha$-2a to remain in the lipid matrix due to non-covalent interactions may be substantially lowered.

Moreover, it was shown that polyethylene glycol 6000 (PEG) enabled the control of IFN $\alpha$-2a release from such implants. Whereas implants without PEG showed a fast and high protein delivery during early release phases, the addition of PEG reduced the burst below 25 % and protein liberation lasted over 1 month, with release proceeding in almost linear manner within the very first two weeks. By increasing the PEG levels (> 14 %), protein liberation succeeded faster and terminated after 2 weeks.

Based on Fick`s laws and the Higuchi equation, the release of macromolecules from inert matrices (e.g. wax matrices or non-biodegradable polymeric matrices) is usually discussed being a diffusion-controlled process through water-filled pores and channels. That means diffusion to be the rate limiting factor in drug release, even when specific models are normally necessary to take factors into account such as drug solubility, the dependence of the diffusion coefficient on drug concentration and the mass transfer per se [12; 182; 113].

As a result of the release data presented in this chapter, it can be reported that IFN $\alpha$-2a liberation from tristearin matrices without PEG can also be described as diffusion-controlled process. The obtained release profiles matched well the mathematical models for diffusion-controlled drug release from cylindrical dosage forms [183].

However, the addition of PEG appears to inhere an obvious influence on the release mechanism from the tristearin implants. At this state of the thesis, a pure diffusion-controlled process could be excluded, since the mathematical model describing this procedure failed, even with a low amount of PEG having been added. On the other hand, SEM and mercurial porosimetry evidenced porous structures within the matrices, thus confirming dissolution and diffusion progression of PEG and protein particles to happen during release experiments.

PEG featured a distinctive effect on the arrangement of particles while compressing. This was apparent by the more compact matrices as a result of a denser packaging. The density of the implants increased step by step with higher amounts of PEG – i.e. a rise of 1.35 % in density for a 5 % PEG content compared to implants without PEG and up to 4.26 % for a 20 % PEG content was determined. Consequently, the denser packaging of PEG containing matrices can be asserted as one explanation for the sustained IFN $\alpha$-2a liberation in early release phases.

A further explanation for the altered release mechanism with PEG can be given when assuming reversible IFN $\alpha$-2a precipitation caused by PEG within the matrices during release. Sharma and Kalonia demonstrated that the solubility of IFN $\alpha$-2a decreased with an increase in PEG concentration and, also, with an increase of the PEG molecular weight. For a 5 % (w/v) PEG 1450 solution IFN $\alpha$-2a solubility at 25 °C, pH 6.5, was 0.8 mg/mL. Employing a 5 % (w/v) PEG 4600 solution led to a decrease in protein solubility below 0.4 mg/mL and with 10 % PEG solubility values around 0.05 mg/mL were determined. It was also shown that the occurring precipitation was reversible and affected the tertiary structure of the protein only marginally [180].

Of course, these literature data can not be transferred principally to the processes occurring within the lipid implant systems during release, but it is noticeable that a protein precipitation process can be suggested to explain the altered release mechanism and the sustained IFN $\alpha$-2a liberation from tristearin matrices with PEG. Specified investigations on that are in progress in order to further elucidate the different scenarios.

## Chapter IX: Final summary, conclusions, and prospective

The development of sustained and controlled injectable systems can be considered as the most promising strategy in protein parenteral delivery. However, over the last few years it has become obvious that the encapsulation of proteins in polymeric devices – in particular in PLGA systems – is hampered by substantial protein degradation occurring during both manufacturing and release [45; 130; 144; 225].

In the search for alternative biomaterials for use in controlled protein release systems natural lipophilic products such as fatty acids, glycerides and waxes appeared on the radar screen. Unambiguously inhering high potential in protein delivery, these materials took a back seat over the last years, mainly because it was focused on veterinary applications [211; 154; 188; 105]. It was the aim of this thesis to develop a protein delivery system based on lipid implants for human and animal use which allows the protein to be continuously released in its native form.

In the theoretical section **(Chapter I)**, protein release devices in general are introduced. Backgrounds of degradation of proteins encapsulated in polymeric biodegradable devices are presented as well as basic improvements and prosperities when using those systems. Furthermore, it is dealt with the development of protein release devices based on lipophilic materials. Various manufacturing techniques, principles of sustained and controlled release from such systems and several promising features already attained in the use of lipid materials are presented. A summary of the theoretical principles and the aim of this thesis are outlined in **Chapter II. Chapter III** lists the materials and methods used in this work, where three different protein drugs were employed – granulocyte colony stimulating factor (G-CSF), interferon $\alpha$-2a (IFN $\alpha$-2a) and bovine serum albumin (BSA).

**Chapter IV** addresses tentative investigations for the characterisation of pure lipid implants in terms of their polymorphic behaviour, erosion properties, and basic applicability as delivery systems for protein drugs. Here, stearic acid was tested and constraints in its applicability were revealed: (I) A notable polymorphic transition of stable stearic acid material to instable forms during implant manufacturing could potentially influence the stability of the dosage forms, and (II) proteins were

demonstrated to interact with erosion products of stearic acid material what results in denaturation and intense aggregation within a few days.
In contrast, triglyceride materials could be illustrated to reveal a high potential as compatible delivery systems for protein drugs. These products remained in stable ß-modification during implant compressing and, moreover, protein integrity was not affected by the triglyceride implant systems themselves.
In order to render investigations on the influence of the manufacturing process on protein stability possible, a novel method for protein extraction was developed. Without using organic solvents during extraction, this method avoids the detraction from protein stability due to extraction process per se.
Implant manufacture by compressing was demonstrated to work under mild conditions, retaining protein integrity during the preparation of the lipid devices. As a consequence, lipid matrices were loaded almost exclusively with native protein – thus, assessment of data of subsequent release studies could be performed on a meaningful basis and in an accurate way.

In **Chapter V**, grounds were provided for protein delivery from tristearin implants. Parameters such as drug load and compression force, which can potentially influence protein release, were scrutinised and optimised from a feasibility perspective.
In general, release profiles achieved during these first in vitro release experiments were characterised by initial burst effects followed by a phase of non-release and eventually incomplete protein release from the lipid implants. Only for BSA, sustained protein liberation could be realised - i.e. 40 % of the incorporated BSA were continuously released over 1 month from tristearin matrices with a 10 % drug load.
The application of various excipients as release modifiers was examined. Polyethylene glycol 6000 (2 % - 5 % range) and Poloxamer 188 (2 % content) have not shown any effects what was attributed to insufficient levels of the added components.
The phospholipid 1,2-Dioleyl-sn-Glycero-3-Phosphocholine (DOPC), on the other hand, was demonstrated to inhere a high potential in particular for a sustained IFN $\alpha$-2a release – when 10 % DOPC were added. A continuous liberation of up to 30 % protein was achieved over 12 days. But mixtures with higher DOPC levels, e.g. 20 %

and 30 %, suffered from their substantially reduced compressibility, thus implants could not be prepared by compressing.
Alternatively, throughout all experiments G-CSF delivery could not be enhanced above a total of 23 % releasable protein. Based on these study data, investigations on G-CSF as a model drug were abandoned up at this state.
For BSA and IFN $\alpha$-2a, special attempts in terms of enhancing and controlling protein delivery from lipid implants are outlined in **Chapter VI and VII**.

For BSA **(Chapter VI)**, employing monoglyceride and diglyceride material in protein drug delivery resulted in high burst effects of up to 60 % BSA release within 4 days. This burst phase was followed by almost non-release and thus incomplete BSA release from the devices.
It was further demonstrated that polyglycerol ester matrices allow a sustained protein delivery approaching zero-order kinetics, but intense aggregation as a result of matrix swelling and erosion was to be noted as drawback.
The addition of 10 % or more PEG to tristearin material overcame previous problems of low total drug release and protein instability. Nevertheless, complete BSA liberation could not be achieved. As a consequence, it was tested whether a change in the freeze-dried formulation would enable a 100 % BSA delivery from tristearin implants containing PEG. By this, a full release of BSA was allowed within 4 days.

This improvement by solely the lyophilised formulations alone was deemed as crux of the matter in protein delivery from lipid implants and this was proven by a substantial increase in total releasable IFN $\alpha$-2a **(Chapter VII)**.
Protein release from devices containing monoglycerides and/or diglycerides were studied, but found to perform badly due to high initial burst effects.
Polyglycerol ester matrices resulted in intense IFN $\alpha$-2a aggregation because of implant swelling and erosion.
On the other hand, tristearin implants containing PEG 6000 enabled the development of a delivery system allowing the protein to be continuously released over weeks. For instance, a PEG content of 10 % achieved an almost linear IFN $\alpha$-2a liberation up to 70 %, delivered over a 1 month period. Moreover, liberated protein consisted virtually

completely of monomeric protein. Higher PEG amounts accelerated protein liberation but did not offer a positive influence on the total amount of releasable IFN α-2a.
A high IFN α-2a adsorption upon the used glass repositories during release experiments was assessed, leading to the conclusion that actually more protein was released from the implants than detected in the buffer media. This problem was solved by using cycloolefin containers (TopPac®) in later experiments.
Moreover, specified stability studies showed that the IFN α-2a/trehalose lyophilised formulation used hitherto was still suboptimal for protein stabilisation, when rehydrated and incubated at 37 °C. As a consequence, the formulation stability was further improved by (I) changing the trehalose formulation pH prior to freeze-drying and (II) replacing trehalose by hydroxypropyl-ß-cyclodextrin (HP-ß-CD) as cryo- and lyoprotectant, with concomitant pH adjustment.

The experience gained so far was finally used for the successful development of an IFN α-2a delivery system based on tristearin material **(Chapter VIII)**. This system allows 95 % protein liberation in a continuous manner over a 1 month period, whereby a control of protein release was achieved by the addition of polyethylene glycol 6000 as pore forming agent. Initial burst release was reduced to values below 25 % - and low burst effects are considered a cachet in this field. Moreover, IFN α-2a was liberated almost exclusively in its native monomeric form.
A substantial impact of the applied lyophilised protein formulation on the stability of the dosage form during long-term storage has been notified. Trehalose featured to be less effective in the protection of encapsulated IFN α-2a, what resulted in reduced total drug release rates as well as in the liberation of aggregated protein species (i.e. dimer and trimer protein) from such lipid matrices after 6 month storage.
In contrast, HP-ß-CD can be considered a very valuable excipient providing IFN α-2a protection within the lipid implants during storage and during the release process. Even after an extended storage period of the dosage forms, the incorporated IFN α-2a was liberated in its native form in a sustained way over 1 month from IFN α-2a/HP-ß-CD lyophilisate tristearin devices containing PEG.

**Conclusions and prospective:**

The use of synthetic, biodegradable polymers is prevalently considered state-of-the-art in the development of controlled release systems for protein drugs. However, unveiled risks of protein inactivation during manufacturing and release represent a bottleneck in the final success of these systems over the last years [197; 176].

In this thesis, a sustained release implant device based on lipid materials for interferon α-2a (IFN α-2a) was developed, which provides high protein stability during implant preparation, storage, and drug release. Thus, the known problems of immune response associated with higher-order aggregate formation in proteinic drugs can be overcome. Adjustment of the lyophilisate formulation as well as of PEG and lipid qualities and quantities allow to control the release rate in order to realise the dosing schedule aimed for. Consequently, this device can be used as a very promising platform to deliver large pharmaceutical proteins for periods up to 1 month and even beyond.

Several studies addressed the poor erosion phenomena of triglyceride implants in vivo what can be attributed to the size, the compact geometry, and the reduced specific surface of implant devices [125; 141; 211]. Based on these literature data and on the notable lack of in vitro erosion properties, the option of fostering in vivo biodegradation by lipases might questionable for the developed lipid implants.

For human use, non-biodegradability would limit the application of this delivery device. However, in vivo studies monitored a timeframe of maximal one month after administration [211]. Consequently, it is conceivable that triglyceride implants undergo in vivo biodegradation, but with a substantial gap between the drug release period and the erosion process. Even in case the lipid matrices would degrade in vivo as late as after half a year, grave side effects are not to be expected due the high biocompatibility of these materials inhere [211]. Moreover, the option to isolate the release process from matrix degradation can be deemed advantageous in terms of protein liberation control. Therefore, it has to be the objective of further investigations to evaluate the in vivo biodegradation of the developed lipid implants and to especially clarify the role of lipases thereby.

Otherwise, the influence of the geometry - i.e. the use of smaller implants and microparticles - is to be illuminated with respect to a potential biodegradability and, furthermore, the chance of an improved patient compliance.
The in vivo degradation of triglyceride microparticles within 7 days was already demonstrated [154], and solid lipid nanoparticles (SLNs) were shown to erode in vitro by adding various quantities of a lipase/colipase assay [139]. These studies indicate that the effect of lipases seems to be impacted by the surface area the delivery system provides.
Additionally, the SLN degradation studies revealed matrices with lower crystallinity to exhibit a faster degradation rate than devices processing highly crystalline products [140]. Consequently, it can be assumed that the use of mixed-acid triglycerides, which inhere lower crystalline properties, may improve the biodegradability of the lipid implants [169; 127].
One further interesting approach may be the direct incorporation of lipase/colipase into the lipid implants before drug release already at the stage of manufacture. It is possible that the implant erosion might be triggered from the interior after administration.

For the veterinary market, the protein-lipid dosage form developed in this work can be applied without any doubt, since comparable lipid implant systems exhibited a high biocompatibility in vivo and did not cause severe side-effects or immune response due to implant size or the non-biodegradability [105; 141; 211].

# Chapter X: Near-Infrared Spectroscopy (NIRS) as non-destructive analytical tool for protein quantification within lipid implants

## 1. Background

Currently, great interest is put on the encapsulation of biologically active proteins in sustained release devices such as PLGA particles or, as focused on in this thesis as lipid implant carriers. In order to determine the actual drug load and drug distribution after encapsulation, for most analytical methods the protein first is to be extracted from the matrices. That implicates a potential protein loss or, in case of extraction with organic solvents, protein aggregation, which is often induced by such methodical procedures (refer to Chapter IV). It is to be noted, that during release studies protein quantification measurements are generally indirect. That means the liberated protein quantity is detected in the release medium, for example by chromatographic techniques. Consequently, the protein fraction remaining in the devices is calculated by subtraction.

Therefore, it would be advantageous to have a method available for performing such analysis directly within the controlled release device and thus without destructing the carrier system. In this respect, the successful use of (Fourier-transform) spectroscopy in the determination of protein conformation within PLGA microparticles was reported by van de Weert et al. [200].

One other technique amenable to non-destructive analysis of such systems is Near-Infrared Spectroscopy (NIRS). An introduction in NIRS technique, measurements and spectra interpretation is provided by Schoenbrodt et al. [170; 171]. Although NIRS has been used for many decades, it never was established as a common analytical and physical tool as so many other spectroscopic techniques [111]. With the development of novel imaging systems (e.g. focal plane array detection technology), it is now possible to rapidly collect high contrast images of large samples. That suggests considerable value of NIRS for the chemical and functional characterisation of complex solid pharmaceutical dosage forms including sustained protein release devices [152].

For example, Reich et al. demonstrated non-destructive NIRS monitoring of matrix hydration, polymer hydrolysis and free carboxylic end group ionisation of PLGA microparticles. Furthermore, drug-polymer interactions and drug release kinetics

were visualised [151]. In a later work, chemical mapping of the distribution and relative abundance of a model protein in polymer matrix tablets - immediately after processing and during the release phase - was shown [152].
That promising results obtained from polymeric devices exhibited the potential application of NIRS measurements for protein content determination within lipid implants.

## 2. The potential of NIRS for protein quantification in lipid matrices

The following investigations base upon a close collaboration with the Institute of Pharmaceutical Technology in Heidelberg, Germany, where NIRS measurements were conducted. For all experiments, a NIRTab® spectrometer (Büchi AG, Flawil, Switzerland) was used. Spectra analysis was performed with NIRCal® software package version 3.0 and 4.21 (Büchi AG, Flawil, Switzerland).

Initially, the general ability of NIRS for dosage forms based on lipid material was evaluated. In this realm, investigations were performed which should clarify to what extent the implant shaping potentially can influence the optical properties of the matrices and, consequently, the NIR spectra.
The first set of lipid implants was prepared with varying levels of lyophilised G-CSF (protein/sugar ratio of 1:4) and with tristearin material. This implant batch was characterised by diameters of 13 mm, but matrices varied in weights between 82 – 196 mg with height dimensions of 0.6 – 1.5 mm.
The spectra showed enormous scattering in transmittance intensity, what was ascribed to the sample geometry. Generally, implants were very flat with low optical density. The heterogeneity in implant dimensions resulted in different optical properties of the matrices. As a consequence, a quantitative calibration of transmittance spectra was not to be rendered possible. On the other hand, spectra measured in diffuse reflectance mode allowed good quantitative calibrations using principle component regression (PCR) and partial least squares regression (PLSR). Regression coefficients ranged between 0.994 – 0.997 [173; 172].
As a result, these first experiments showed the basic ability of NIRS to determine the protein content in lipid matrices. Due to the scattering in transmittance intensity, the

sample geometry was modified for further investigations, and implants with a diameter of 5 mm and height dimensions of about 3 mm were prepared.

The second approach should demonstrate that only the protein and not the sugar component of the incorporated freeze-dried formulation was spectroscopically detected. Therefore, two implant sets were prepared with BSA as protein drug compound: (I) BSA was lyophilised in different ratios with trehalose (protein/sugar ratios of 1:1 –1:5). However, the drug load of the implants was kept constant at 10 % (w/w) for all formulations. This resulted in actual BSA charges in a range of 1.66 - 5 % of implant weight. (II) Implants were prepared with pure BSA (lyophilised without additives) in content ranges between 2 - 10 % (w/w).

Analysis of transmittance spectra obtained from the first BSA implant set (I) yielded a calibration regression coefficient of 0.992. The standard value of prediction (SEP – appreciation value for the quality of the predicted calibration model) was 0.16 mg and BIAS failure, as parameter for the mean deviation of two data sets, was – 0.04 mg. Given the narrow calibration area as well as the low number of implant samples, the obtained calibration data were acceptable. Moreover, the calibration obviously was based on spectral information derived from the protein, due to the fact that the implants contained varying contents of protein and sugar [173; 172].

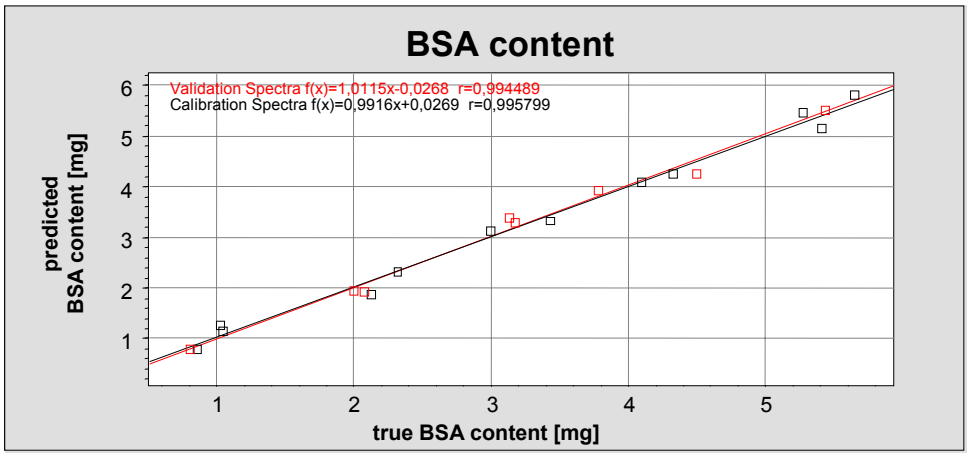


**Figure 80: BSA content in lipid implants. PLSR of transmittance spectra of implants containing pure BSA in various contents, 4 calibration factors, second derivative, 6000 – 11520 $cm^{-1}$ (n=24) [172].**

As visualised in Fig. 80, transmittance measurements of implants loaded with different levels of pure BSA exhibited good quantitative calibrations with regression coefficient of 0.994 (validation) and 0.996 (calibration), respectively. The SEP value

once showed 0.16 mg, but the BIAS failure substantially decreased to – 0.009 mg, what proved calibration to be of good acceptance [173; 172].
Based on these two experimental results, it could be demonstrated that calibration models really based on spectral features of the incorporated protein and not on the sugar component [173; 172].

In further investigations, the promising results obtained for BSA lipid implants should be transferred on IFN α-2a, a pharmaceutically relevant protein. Since the developed IFN α-2a delivery device based on tristearin matrices and contained PEG as release modifier (refer to chapter VII), the influence of PEG on NIRS calibration was to be specified. Two implant sets containing IFN α-2a lyophilised with trehalose in a ratio of 1:3 were prepared: (I) the drug load was varied between 10 - 30 % lyophilised protein and, (II) in addition to the different drug load the second implant batch contained 10 % PEG as additive. This experimental design resulted in IFN α-2a loads in a range of 1070 – 3700 µg protein per implant.
Fig. 81 illustrates the calibration of the transmittance spectra obtained from both IFN α-2a implants without PEG and matrices containing 10 % PEG.

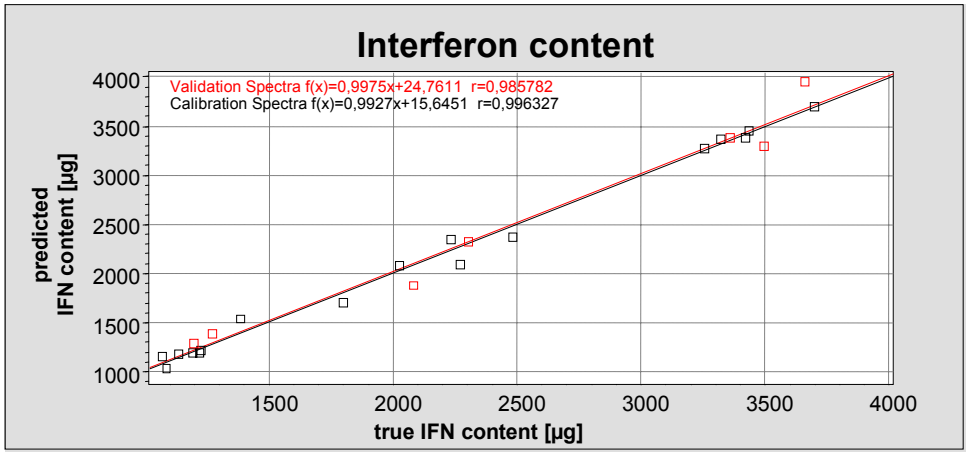


**Figure 81: IFN α-2a content in tristearin implants with and without PEG as additive. Transmittance spectra, PLSR, 7 calibration factors, first derivative and normalisation, 6000 – 11520 $cm^{-1}$ (n=24) [172].**

The calibration model apparently demonstrated NIRS enabling also the determination of the IFN α-2a load within lipid implants. The standard value of prediction was 176

µg and acceptable calibration coefficients (Fig. 81) were reached. Furthermore, calibration and validation curves were almost identical [172].

Moreover, these results suggested that the addition of PEG had no impact on the calibration at all, since both implants with and without PEG were included in this model system.

On the other hand, cluster analysis revealed qualitative spectral differences between implant systems with and without PEG (Fig. 82). However, the principle component analysis (PCA) mirrored a factor 4, what indicated the influence of PEG on the spectra data to be considered as marginally [172].


**Figure 82: Cluster analysis of IFN α-2a/tristearin implants with (□) and without (□) PEG. Transmittance spectra, cluster, first derivative, 7000 – 8000 & 9000 – 10000 $cm^{-1}$ [172].**

It can be concluded that protein quantification will not be affected by the amount of PEG. This was important with respect to the application of NIRS as tool for analysis of protein liberation during release experiments, because the PEG amount can change within the matrices during release.

Based on these encouraging data, the aptitude of NIRS to determine the protein content in the lipid implants during release was evaluated. Tristearin matrices loaded with 10 % lyophilised IFN α-2a (protein/sugar ratio of 1:3) and PEG contents of 5 % and 10 %, respectively, were prepared. In addition, implants with a protein load of 20 % and 10 % PEG were used as delivery systems, potentially allowing an accelerated and more complete protein release. Before this release studies, the actual protein load ranged between 915 – 1270 µg for implants loaded with 10 % lyophilised protein, and between 1600 – 2095 µg for the 20 % IFN α-2a/trehalose lyophilisate

matrices. Implants were incubated in 2.0 mL PBS (pH 7.4 with 0.05 % $NaN_3$) over various time periods (i.e. 1, 3, 5, 7, 9, 12, 15, 18 and 21 days).

IFN α-2a was continuously liberated from matrices with a 5 % and a 10 % PEG content. This resulted in remaining IFN α-2a fractions in a range of 200 – 1020 µg per implant after incubation. Implants loaded with 20 % lyophilised protein and with 10 % PEG led to an accelerated IFN α-2a release, delivering approximately up to 90 % protein over 6 days. Consequently, the remaining protein amount within the implants was between 70 – 380 µg. After the respective incubation time, implants were removed from the buffer media and were dried at room temperature as far as a constant weight was measurable. Then, transmittance spectra were taken.

As visualised in Fig. 4, NIRS allowed the prediction of the IFN α-2a content within the lipid implants, independent from the protein load before incubation. A calibration with a standard of prediction value (SEP) of 57 µg and a calibration regression coefficient of 0.987 was achieved. Moreover, even low protein contents of 100 – 200 µg could be addressed [172].

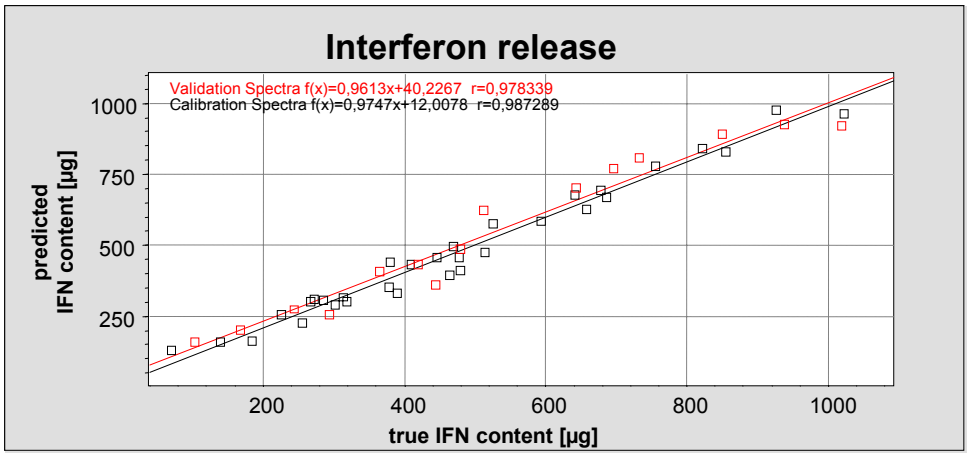


**Figure 83: IFN α-2a release from tristearin implants containing PEG. Before incubation, implants were loaded with 10 % and 20 % lyophilised protein, respectively. Implants were incubated over various time periods, dried and subjected before NIRS measurements. Transmittance spectra, PLSR, 5 calibration factors, MCS & second derivative, 6000 – 9000 $cm^{-1}$, (n=47) [172].**

These study data can be deemed a major attainment considering the complexity of the investigated delivery system. Despite the presence of lipid, sugar and PEG material, plus the considerable alteration of matrix composition and porosity during

release, NIRS still allows the determination of the protein content within the implant systems.

## 3. Summary

The ability of Near Infrared spectroscopy as non-destructive analytical tool for protein quantification in lipid implants was investigated.

Tentative experiments featured diffuse reflectance being useful for the determination of the drug load in very flat implants (height: 0.6 – 1.5 mm). In contrast, for protein determination in implants with base heights greater than 1.5 mm – as presented in this thesis work – transmittance measurements can be considered appropriate. Based on this, a good calibration model was achieved.

It was demonstrated that the protein per se and not the incorporated sugar accounted for the spectral information. BSA content measurements in both implants with varying protein/sugar ratios and implants loaded with different levels of pure BSA demonstrated this set-up to be a good calibration model.

In further investigations, the determination of the IFN $\alpha$-2a content in tristearin matrices containing polyethylene glycol (PEG) as additive was evaluated. It could be shown that NIRS can be used as a tool for content determination of various proteins, since transmittance spectra proved the good calibration also for IFN $\alpha$-2a measurements. Moreover, the influence of PEG was shown being negligible for IFN $\alpha$-2a quantification by NIRS.

During release experiments, NIRS allows a good prediction of the IFN $\alpha$-2a content remaining in the lipid implants. This results in a calibration regression coefficient of 0.987. In addition to that, minor protein amounts could also be determined demonstrating a low detection limit of NIRS.

Generally, calibration models for protein quantification mainly based on vibrations of the N-H bands in protein molecules [172].

In summary, these study data demonstrated NIRS to be a promising technique for the quantification of the protein content in sustained delivery systems such as lipid implants. As a non-destructive method, NIR enables to determine the protein load of implants without complicated protein extraction steps, subsequent to implant manufacture, and also during release experiments.

## Chapter XI: References


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## Publications and presentations associated with this work

### Original Research Articles

- Mohl, S.; Winter, G., Continuous release of rh-interferon $\alpha$-2a from triglyceride matrices, J. Contr. Rel. 97 (2004) 67 – 78.

### Poster presentations

- Mohl, S.; Reithmeier, H.; Winter, G., Towards controlled protein release from lipid implants, protein stability assessment during manufacturing and incubation, 4$^{th}$ World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Florence, Italy (2002).
- Mohl, S.; Reithmeier, H.; Winter, G., Sustained release of BSA from implant systems using physiological lipids, Colorado Protein Stability Conference, Breckenridge, USA (2002).
- Mohl, S.; Reithmeier, H.; Winter, G., Lipid implants as promising alternative to polymeric devices for the controlled release of pharmaceutical proteins, Meeting of the American Association of Pharmaceutical Scientists, AAPS, Toronto, Canada (2002).
- Schönbrodt, T.; Mohl, S.; Winter, G.; Reich, G., Nondestructive quantification of lyophilised protein drugs in lipid matrices, Meeting of the American Association of Pharmaceutical Scientists, AAPS, Toronto, Canada (2002).

# Curriculum vitae

## Silke Mohl

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|---|---|
| Date of birth | May 10, 1975 |
| Place of birth | Reutlingen, Germany |
| Marital status | unmarried |

## Education

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|---|---|
| 1981 – 1985 | Elementary school, Kirchentellinsfurt, Germany |
| 1985 – 1994 | Comprehensive secondary school, Tübingen, Germany |
| 1994 | General qualification for university entrance |
| 04/1995 – 04/1999 | Study at Tübingen University, School of Pharmacy, Tübingen, Germany |
| 05/1999 – 10/1999 | Practical education in pharmacy (retail), Rathaus-Apotheke, Reutlingen, Germany |
| 11/1999 – 04/2000 | Practical education in pharmacy (industry), Department of Pharmaceutical Development, Hexal AG, Holzkirchen, Germany |
| 06/2000 | Approbation as pharmacist |
| 07/2000 – 09/2000 | Scientific freelance, Department of Pharmaceutics, School of Pharmacy, University of London, London, UK<br>Subject of the work: “Pellet formulations containing different kinds of surfactants – evaluation of the process and the product characteristics” – supervised by Prof. em. Dr. J. M. Newton |
| 10/2000 to present | Ph.D. thesis; Department of Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-University, Munich, Germany<br>Supervisor: Prof. Dr. G. Winter |