Hochschule für Angewandte Wissenschaften Hamburg
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Microencapsulation of mPEG-Modified Lysozyme in PLGA by Spray Drying

Master Thesis

Pharmaceutical Biotechnology

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Summary

Protein-based drugs hold great promise as new therapeutic agents because of their high specificity. Unfortunately, they are limited by their short half-life in the organism to be treated. This drawback is caused by premature proteolytic degradation, rapid renal clearance, and instabilities of the protein itself. In this thesis, methods to improve the applicability of protein based drugs were investigated. PEGylation and microencapsulation are two commonly used methods which already proved great potential in drug development processes. The combination of these methods is applied in this thesis using lysozyme as a model protein and mPEG-pNp and PLGA as polymers.

First, the PEGylation process was closely analyzed. The reaction showed a great dependence on the reaction time, the polymer-to-protein mass ratio, and the pH. PEGylated lysozyme acts like a molecule many times larger than its actual size. Hence, the rapid clearance from an organism will be prolonged. Mixtures containing PEGylated conjugates of more and higher degrees of modification show a decrease in their enzymatic activity.

The purification of the modified protein from the unreacted lysozyme and the remaining polymer was performed by a SEC. A complete separation of the single modification degrees was not possible, but mixtures containing predominantly higher or lower degrees of PEGylation were produced. These mixtures show an improved stability and an improved resistance to proteolysis, which reduces instability and premature degradation.

The microencapsulation in spherical particles with a rough surface was conducted using solvent evaporation and spray drying. Suitable particle sizes for injectable microspheres were produced by spray drying with an average yield of 40 %, but solvent evaporation achieved 20 % higher yields. The BCA assay does not appear to be an appropriate method for the determination of the protein release from PLGA microspheres.

The PEGylation and the microencapsulation of lysozyme in PLGA were successfully conducted. Positive effects of these formulation methods on proteins could be determined during this thesis, but further studies, especially on the release kinetics of the encapsulated proteins, are necessary.

1. Introduction

The formulation of drugs is a crucial part in drug development processes. Especially after the completion of the human genome project protein and peptide drugs shifted into the focus of researchers. Their suitability as possible new therapeutic agents is due to their high specificity and effectiveness [1, 2, 3]. Many of the newly approved drugs already are protein-based [4], including recombinant drugs (e.g. insulin and erythropoietin), monoclonal antibodies and viral or bacterial proteins used as vaccines [5]. However, severe problems such as a rapid renal clearance, a low solubility and enzymatic degradation come along with this kind of drugs. Furthermore, protein-based drugs often provoke immune responses. Physical and chemical instabilities such as deamidation, oxidation, aggregation and adsorption can cause problems, too [6]. Even slight changes in pH, ionic strength, or temperature can reduce e.g. the biological activity [7]. As a result, protein-based drugs exhibit a short half-life making high frequented drug injection necessary, sometimes even several times a day [8]. In addition, proteins are often unstable leading to storing problems.

Promising techniques to overcome these problems and thereby prolong the half-life of the therapeutic proteins exist and are already used in the formulation of drugs. Proteins can be modified by crosslinking [9], fusion to other proteins, glycosylation and other methods [10]. Currently, especially the modification of protein drugs by the attachment of polymers holds great promise. A formulation method without changing the drug itself is the usage of drug delivery systems such as polymer microspheres.

Therapeutic proteins covalently linked to chains of the polymer poly(ethylene glycol) (PEG) have shown enhancements in therapeutic and biotechnological potential [11,12]. PEG is the most commonly used polymer used to alternate protein-based drugs. It is EMA (European Medicines Agency) and FDA (Food and Drug Administration) approved and already used in certified protein-based drugs, e.g. PEG-erythropoietin (Mircera®) and PEG-uricase (Pegloticase; Krystexxa®) [13].

Polymers also serve as drug delivery systems. Here, they are not covalently linked but encapsulate the drug, forming microparticles protecting the therapeutic agent from premature degradation. Especially biodegradable polymers are favored because of their excellent biocompatibility and biodegradability. These release the encapsulated therapeutic agent in a controlled manner. The release rate is dependent on the physicochemical properties of the drug, the morphology and size of the microparticle as well as the kind of polymer used, since different kinds of polymers show different

degradation behaviors. A popular polymer used for the microencapsulation of drugs is poly(lactic-co-glycolic acid) (PLGA).

2. Theoretical Background

2.1 Pharmacokinetics and Pharmacodynamics

When dealing with drugs one will always come across the terms “pharmacokinetics” and “pharmacodynamics”. Despite their similar sound the meanings of these expressions differ widely. While pharmacokinetics describes how an organism responds to a drug, pharmacodynamics characterizes how a drug is affecting an organism [14]. When a drug is applied to the body it is immediately exposed to a flood of different conditions which influence the active substance and its therapeutic effect. In order to make a statement about a drug´s effect both pharmacokinetics and pharmacodynamics need to be taken into account.

Pharmacokinetics

Pharmacokinetics helps to understand how an organism reacts once a drug enters. This knowledge is very important in order to set dosages of drugs in a way in which they will not cause toxic effects, but will still be able to show its therapeutic effects. Additionally, pharmacokinetics deals with the duration time of drugs in the organism before they get cleared by its processes. Typically, the pharmacokinetic properties of a drug are studied along four distinct process steps [15]:

- Absorption of the drug
- Distribution of the drug molecules
- Metabolism of the parent drug (biotransformation)
- Excretion or elimination of the drug and its metabolites

The absorption is majorly dependent on the route of administration. For example, oral administration is very convenient and free of pain, but first pass destruction and other disadvantages may occur in contradiction to, for instance, rectal administration. Generally, a good blood flow, a great surface and a long endurance time in the area to be supplied improve the absorption of a drug. Furthermore, chemically stable and well soluble drugs show good absorption. The sites of action of some drugs are local while others show an effect throughout the whole body. For the latter the distribution into body fluids, the uptake in body tissues, the extent of plasma protein binding as well as the passage through possible barriers are of high interest. The process by which a parent drug is transformed into its metabolites is called biotransformation. Majorly, it occurs in the liver. This process step is essential as not only the parent drug, but also and

occasionally only the metabolites are therapeutically active. The speed of the metabolism defines the duration of action or impact of a drug, which in turn defines the required frequency of administration. There are different principles of excretion: renal clearance, fecal elimination, and enterohepatic recirculation. A too quick or early clearance of a drug from the body can render a drug ineffective by shortening the duration of the therapeutic effect, whereas delayed clearance of the drug and its metabolites can cause toxic effects.

Pharmacokinetics is studied in order to enhance efficacy and decrease toxicity in a patient´s drug therapy [16]. Mathematical models describe how the concentration of a drug changes over time. There are two general cases which are differentiated: First, the linear pharmacokinetic where the concentration decreases proportional to the time and second the non-linear pharmacokinetic where this is not the case [14].

Summing up, one can say that pharmacokinetics is used to monitor the time course of concentration changes of drugs in an organism after application [15]. The monitoring is conducted via detecting the drug´s concentration in blood, plasma, urine, saliva and other fluids [16].

Pharmacodynamics

Pharmacodynamics is used to monitor the biological effect of a drug in correlation to the concentration at the reaction site [16]. The key determinant of the biological effect of a drug is its concentration, but there are also other factors which need to be taken into account: The density of receptors on the cell surface, the mechanism by which a signal is transmitted or regulatory factors controlling gene translation and protein production [16]. The targets where the drugs attack can be enzymes, receptors, ion-channels and membrane transport proteins [15].

The so called EC_{50} (50 % effective concentration) is a value measured to compare the potency of drugs. It is defined as the drug concentration at which 50 % of the maximum effect is achieved. The drug with the lower EC_{50} value is considered more potent, but it does not take into account other important factors such as the duration of the effect in the body.

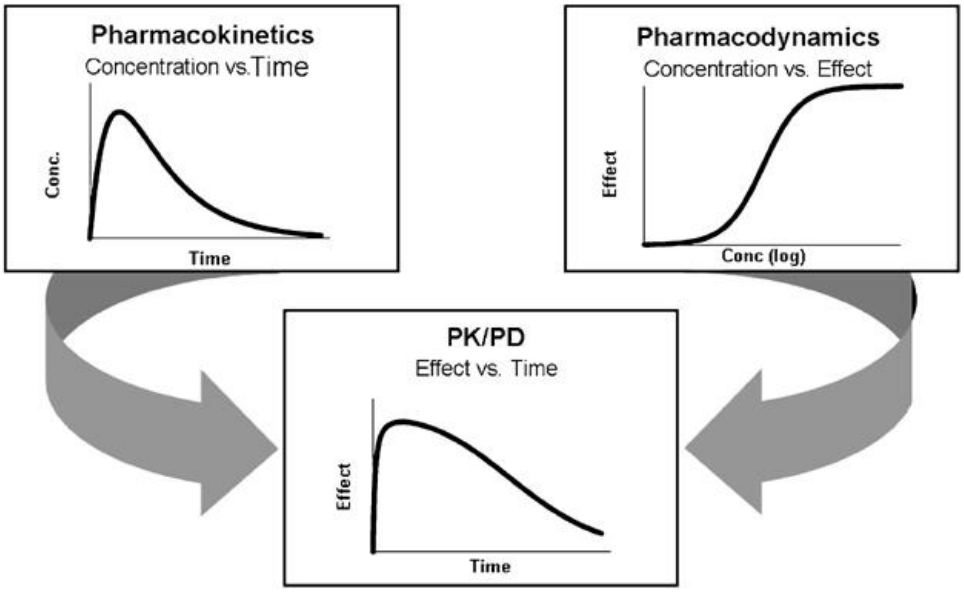

Figure 1: PK/PD Model - The amalgamation of a PK (conc. vs. time) and a PD model (effect vs. conc.) to form a PK/PD diagram describing the intensity of a drug´s effect vs. the time [17].

PK/PD models combine the PK (Pharmacokinetic) model, monitoring the time course of the drug concentration, and the PD (Pharmacodynamic) model, describing the correlation of the concentration vs. the effect of the drug. This combination leads to the PK/PD model showing the time course of the intensity of a drug´s effect [17].

2.2 PEG

2.2.1 Properties of Poly(ethylene glycol)

PEG is a polyether diol which is amphiphilic, highly soluble and not charged in solution. It occurs in either a zigzag, random coil structure (shorter chains) or a winding, helical structure (longer chains) [18]. Three water bridges are built per monomer unit due to the oxygen atoms in the polymer chain [19]. Thus, the polymer chains are highly hydrated and thereby inhibit the approach of other molecules. Currently, two different types of PEG are available, the linear and the branched form. At molecular weights below 1000 Da PEG is in a viscous liquid state, at molecular weights above 1000 Da PEG comes as a solid.

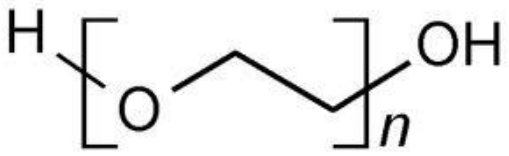

Figure 2: Chemical Structure of Poly(ethylene glycol) [20]

PEG is EMA and FDA approved for human administration by mouth, injection or dermal application. It is inert, non-toxic and non-immunogenic [21]. The clearance of the body takes place through renal clearance if the polymer is below 30k Da and through the liver or feces [22].

The production of PEG is conducted by anionic polymerization. Two OH-groups at both ends of the chain are formed. These OH groups can be activated for the purpose of chemical protein modifications [23]. PEG can be produced for a wide range of polymer length. The process can be controlled quite precisely, which enables narrow weight distributions [22, 24]. Nevertheless, the polydispersitivity always needs to be considered, specifically when working with low molecular weight proteins. In this case the molecular weight of the polymer represents a significant part of the PEG-protein complex.

2.2.2 PEGylation

The process of covalently attaching one or more PEG polymers to a protein is called PEGylation (Figure 3). Typically, an activated monofunctional PEG reacts with one or more lysine residues or the N-terminal amino group of a protein. However, other nucleophilic sites, for example, histidine or cysteine are also possible for the PEGylation process [25, 26, 27, 28]. Additionally, hydrophobic or hydrophilic co-solutes and buffer components need to be considered as they can alter solution properties such as ionic strength and viscosity [29]. The reaction usually is conducted in a solution where the polymer starts to bind at the most reactive binding site and afterwards binds at less reactive sites. As a result, several different isomers are formed which differ either in their degree of PEGylation, leading e.g. to mono-, di-, or triPEGylated conjugates, or in the site of PEGylation. The reaction parameters often can be optimized to achieve predominantly, but not entirely, the desired PEG conjugates [30]. Important factors which are influencing the reaction are the protein concentration, the PEG-to-protein ratio, the reaction pH and temperature as well as the reaction time and the characteristics of the protein to be modified [26].

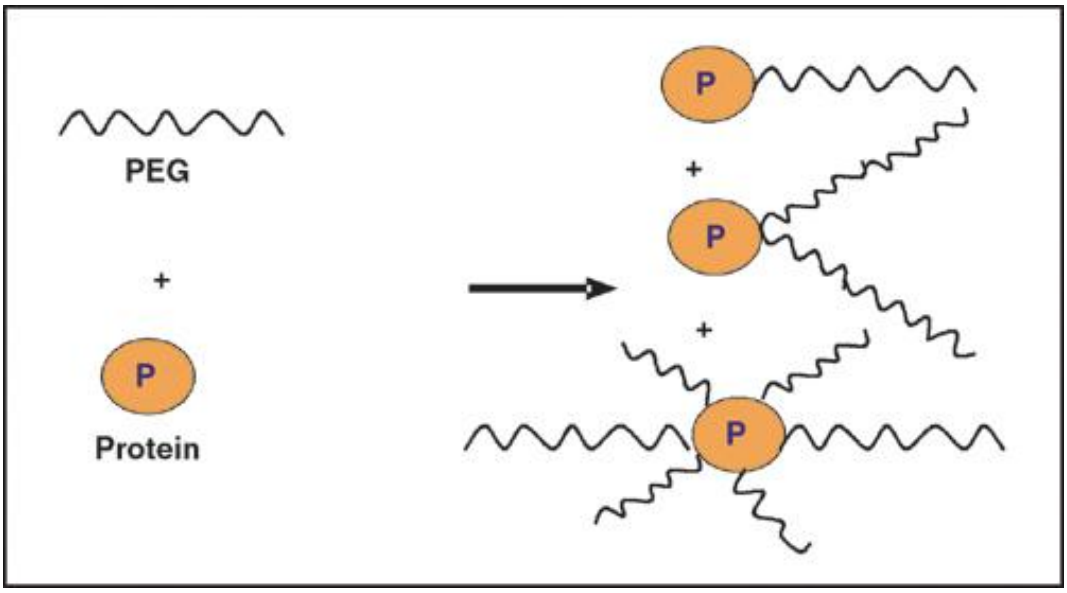

Figure 3: Scheme of the PEGylation Reaction – A protein is covalently binding to a PEG polymer chain. Depending on the reaction parameters the degree of PEGylation can differ [22].

Drug manufacturing processes are developed with respect to high product quality, process robustness and low cost [31]. In PEGylation processes the purification and characterization are challenging tasks. Due to the similar physicochemical characteristics of the different isomers it is complicated to separate the single isomers. To avoid a too wide distribution of isomers usually a control of the PEGylation reaction with simultaneous size-exclusion chromatography is combined [32, 33]. However, the FDA has approved mixtures of isomers in cases in which the reproducibility of the reaction was proven. At least this was the case for the first two PEG drugs on the market. Nowadays, the requirements are stricter and the characterization of each isomer is obligatory, if this is possible [24]. For industrial manufacturing primarily the size exclusion chromatography is troublesome, because of its low throughput, high cost and bad scale-up possibilities.

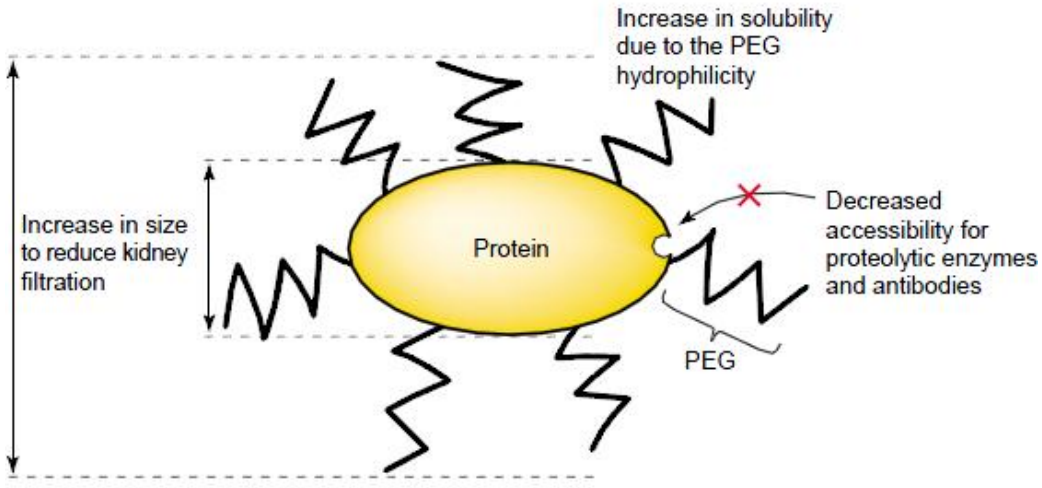

Figure 4: Improvements Caused by PEGylation of Proteins – This figure represents a protein covalently linked to PEG polymer chains. The polymer chains protect the protein from proteolytic enzymes and antibodies by steric hindrance. Furthermore, they increase the size of the protein conjugate and thereby reduce the kidney filtration. The solubility of the conjugate is also improved resulting from the PEG´s properties [24].

PEGylation was first described by Abuchowsky et al. in 1977 on modifications of catalase and albumin [34]. They found out that PEGylated proteins show alternated pharmacokinetics and pharmacodynamics in comparison to the unPEGylated drugs. Most improvements of the modified drugs are attributed to the polymer itself [35]. Modified proteins were found to be protected against degradation and their solubility in water was increased. Furthermore, the renal clearance could be reduced due to the bigger size of the PEGylated proteins and the toxicity could be limited [36]. The kidneys clear molecules according to their size, the smaller a molecule is the faster it gets

cleared. The immune response which some protein-based drugs provoke and physical and chemical instabilities can also be reduced by PEGylation. Resulting from these positive effects the half-life of PEGylated protein drugs is improved presenting the drug a longer time to perform its therapeutic effect. By an increasing number of conjugated polymers or also by increasing PEG polymer size the immunogenicity and antigenicity decreases [37].

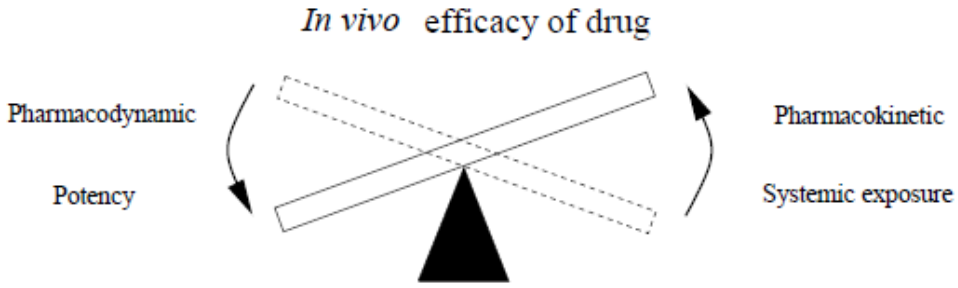

Figure 5: Alternation of the *in vivo* Efficiency of a Drug due to PEGylation – The balance between pharmacodynamic and pharmacokinetic is changed when modifying a protein by PEG. Pharmacokinetics are improved, e.g. due to prolonged half-lives, while pharmacodymics decrease, e.g. due to reduced binding affinities. Thus, the PK/PD profile is changed by PEGylation. However, the overall effect of therapeutic efficiency is improved [38].

However, the modification of protein drugs does not only have positive effects. Beside their polydispersitivity PEGylated proteins often exhibit reduced binding affinity to their reaction sites. These are evoked by the larger size of the modified protein which leads to a steric hindrance. Additionally, the biological activity can be negatively influenced by the PEGylation process. These negative factors seem severe, but they are often overcompensated by the positive effects mentioned above (Figure 5). Especially the slower clearance and thereby prolonged duration time make up for the reduced activity and binding affinity. The improved pharmacokinetics are not only due to an increased efficacy but also to reduced dosages or dosing frequencies. An illustrative example is the PEGylated α-interferon Pegasys®, which only retains 7 % of its original activity, but still shows an impressively improved performance because of its increased pharmacokinetics [39]. This example illustrates how difficult it is to extrapolate the *in vivo* effect from the results of the *in vitro* outcomes.

The pharmacokinetic and pharmacodynamic properties of PEGylated proteins are generally influenced by the site at which the PEG is attached, the polymer´s molecular weight, the number of attached PEG chains and the stability of the linkage between PEG and protein [40].

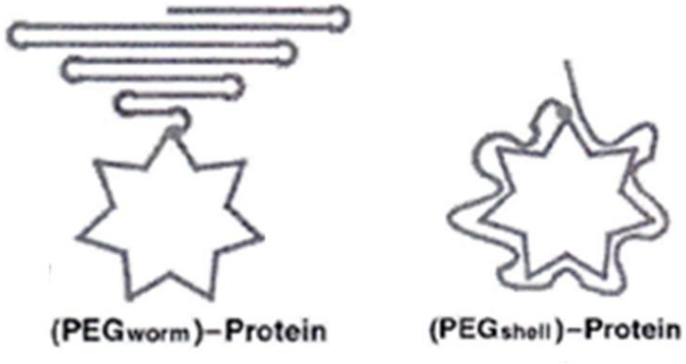

Figure 6: Structural Conformations of PEGylation Proteins – When covalently bound to a protein the PEG polymer chain can build different conformations. On the left side the worm-like structure is shown whereas the right picture shows the shell-like structure [modified after 22]

The attachment of PEG polymers to a protein leads to changes in size, conformation, and electrostatic properties in comparison to the unmodified protein. Protein-polymer conjugates can adopt different structures because PEG can exist in various conformations dependent on the solution conditions [18]. For example, a shell-like structure as shown in Figure 6 can be created that leads to a higher solubility and a reduction of the immunogenicity [7, 41]. This conformation provides an explanation of how PEG can mask the protein surface from proteolytic cleavage [22]. Another option without PEG-protein interaction is forming a worm-like helical structure (Figure 6) resulting in a reduced immunogenicity as well. Furthermore, Veronese *et al.* discovered that branched PEG chains were more effective than linear PEG chains in decreasing the immunogenicity of the drug (Figure 7). For example the binding of a branched 10 kDa PEG to asparaginase reduced the antigenic character of the protein about 10-fold compared to an asparaginase bound to a linear 5 kDa PEG [42]. Caliceti *et al.* found similar results when binding these two PEG chains to uricase [43]. Additionally, Monfardini *et al.* (1995) have shown that drugs bound to branched polymer PEG chains increase the pH and thermal stability of the drug and improve the resistance to proteolysis better than drugs modified with linear PEG chains [44]. The attachment of multiple PEG polymers to a drug is supposed to increase the possibility of steric hinderence on the active site of the drug, thereby reducing its activity [45]. Thus, branched polymer chains and high molecular weight polymers prevent the drug from anti-protein antibodies better than linear polymer chains and low molecular weight polymers. However, the biological activity is reduced significantly. Veronese *et al*. describe an enzyme activity of 32 % compared to the original uricase activity before PEGylation when

bound to branched PEG chains and an enzyme activity of only 2.5 % when bound to linear PEG chains [46].

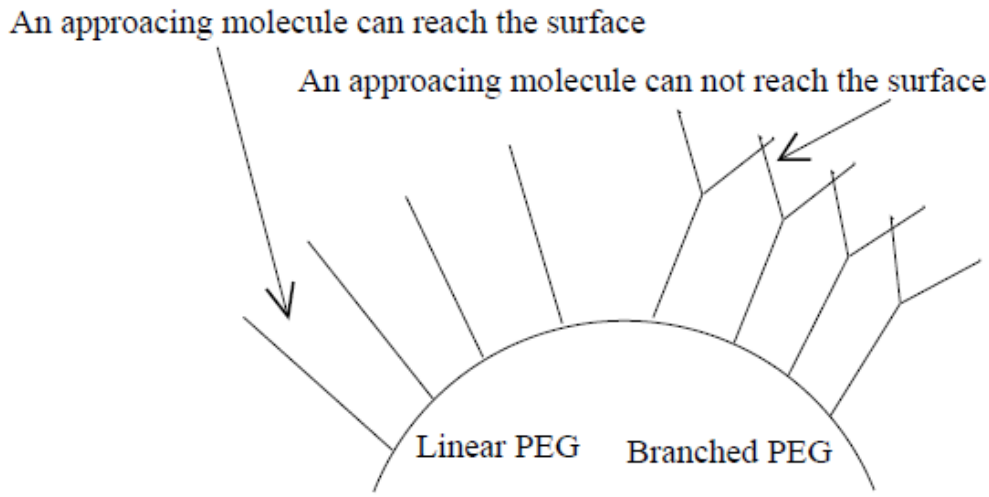

Figure 7: Comparison of the Effect of Linear and Branched PEG – Approaching molecules are more likely to reach the surface if a linear PEG is bound to the protein compared to the PEGylation with branched PEG [45, 27].

The most important limitation on the use of PEG is that once very large molecules can accumulate in the liver, resulting in a macromolecular syndrome. The exact size limit is complicated to determine, because due to the high water coordination of PEG the hydrodynamic volume of a PEGylated protein can be 3-5 times higher than of an unmodified globular protein of the same size [24]. Evidence suggests that a size of approximately 30 kDa is a reasonable threshold below which renal elimination can be expected [22].

2.2.3 Applications of PEG

Due to their significant and well-established positive effects PEGylated protein drugs already hold large shares of the newly approved drugs despite some manufacturing challenges. Table 1 shows a list of several PEGylated drugs which are already available on the market. But the field is still expanding. PEGylated drugs are already successfully used throughout many diverse fields such as enzymes replacement [47], blood substitute

[48], antibody fragments [49, 50, 51], cytokines [52], adenovirus [53] and protein and peptide anticancer drugs [54, 52].

Table 1: A selection of approved, PEGylated drugs already available on the market

Brand Name	Product	Indication	Year of approval	Manufacturer
Adagen ®	PEG-ademase bovine	Severe Combined Immunodeficiency Disease (SCID)	1990	Enzon
Oncaspar ®	PEG-aspargase	Leukemia	1994	Enzon
Pegasys ®	PEG-interferon α-2a	Hepatitis C, Hepatitis B	2000	Hoffmann – La Roche
Neulastra ®	PEG-filgrastim	Neutropenia	2002	Amgen
Somavert ®	PEG-visomant	Acromegaly	2002	Pfizer
Krystexxa ®	PEG-loticase	Gout	2010	Savient
Omontys ®	PEG-inesatide	Anemia associated with Kidney Disease	2012	Affymax/Takeda Pharmaceuticals

The PEGylation of drugs continous to be an important and current research topic. PEGylated antibodies [49] and enzymes [55, 56] have been tested to treat various types of cancer. First studies conducted with PEGylated antibodies have already shown enhanced tumor localization [49,56]. Also, the PEGylation of interferon, which is used to treat hepatitis C, has resulted in greatly increased circulation times. While the unPEGylated drug is cleared after 9 hours and has to be applied three times a week, the modified drug showed an elimination half-life of 77 hours and only needs to be applied once a week [57]. This is an impressive example that highlights the extraordinary potential of this modification method.

When applying drugs the application form needs to be decided. Parameters which should be considered in order to choose the correct administration route are e.g. the molecular weight of the PEG polymer and the degree of PEGylation since these are influencing the volume of distribution and the half-life of the drug [58]. Intramuscular as well as subcutaneous administration could slow down adsorption and diffusion preventing the drug from reaching the bloodstream. As a result the protein might act as a depot and the degradation could also be increased. Concluding the intravenous administration path is favored for PEGylated drugs [29].

2.3 Microparticles

Microparticles are defined by the International Union of Pure and Applied Chemistry (IUPAC) as particles with sizes ranging from 0.1 to 100 µm [59]. They are subdivided into two different categories: In polymer microspheres the drug is homogenously spread in the particle, whereas in microcapsules the drug forms a core which is surrounded by an outer layer of polymer (Figure 8) [60].

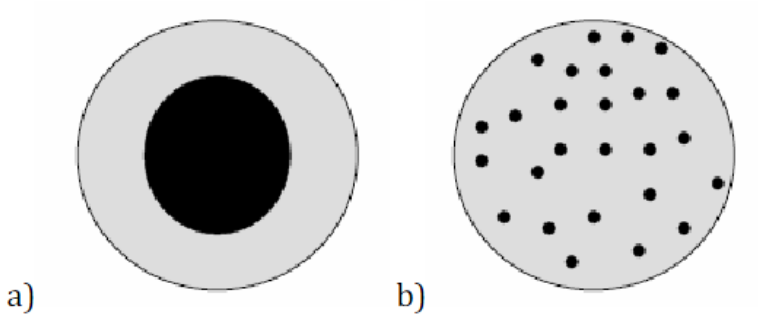

Figure 8: Dipersity of Drugs in Different Microparticles – a) in microcapsules the drug is forming a core surrounded by a polymer layer; b) in microspheres the drug is homogenously spread throughout the particle [60]

2.3.1 Biodegradable Microspheres as Drug Delivery Systems

Uncontrolled release rates are a great problem of conventional oral drug administration. Here, the drug concentration increases steeply after administration (initial burst), followed by a short time window in which the concentration stays within the therapeutic level, after which the concentration declines to ineffectiveness and finally complete clearance. To avoid toxic concentration levels in the initial burst, often high frequencies of re-administration of lower doses are required. This is rather ineffective because the time during which the concentration of the drug is within the therapeutic window is quite short. To overcome these limitations, researchers focus on polymer microspheres for drug delivery. Here, the drug is encapsulated in a slowly degrading matrix. This allows for a more controlled drug release over time, which in turn can avoid the initial burst and significantly increase the duration the drug concentration stays within the therapeutic window. In some cases even organ-targeted release is possible [61].

The ideal release profile is a constant release rate over a long time period. To achieve this, the different release mechanisms that affect the freeing of the drug have to be controlled. First, all surface and badly encapsulated drug molecules are released in the initial burst. Second, the drug diffuses through pores formed during sphere hardening. Third, the degradation of the polymer leads to the freeing of the drug. This mechanism is called bioerosion, which can occur as surface or as bulk-erosion [62]. These mechanisms occur in the mentioned order. Moreover, drug release is also affected by factors such as the molecular weight of the polymer, the size of the microspheres, its distribution and its morphology.

2.3.2 Preparation Methods

The preparation process for microspheres needs to meet at least four key criteria. First, the process conditions should maintain the chemical and physical stability of the bioactive compound as well as its biological activity. For example, the contact of the drug with hydrophobic, organic solvents or acidic/basic aqueous solutions should be avoided. Second, the encapsulation efficiency and the yield should be suitable for mass production. Third, the polydispersity should be in an acceptable range and the particle size needs to match the possible future use e.g. small enough for parenteral administration using a syringe needle. The release profile is supposed to be constant and the initial burst shall be kept low. Fourth, the process should produce the product in the form of free flowing powder that makes uniform suspensions easy to prepare [63]. A variety of preparation methods are available for microencapsulation. The choice of the best suited method is dependent on the type of polymer and drug, the site of drug action and the duration of the therapy [64, 65, 66]. In the following, the three most common methods for the production of microspheres are briefly described: Solvent evaporation/extraction, phase separation, spray drying.

Solvent Evaporation / Extraction

Solvent Evaporation is the most widely used method for the preparation of microspheres. It is used in two different forms. The single emulsion method, used for the encapsulation of hydrophobic drugs through oil-in-water (o/w) emulsification and the double emulsion method, used for water soluble drugs by a water-in-oil-in-water (w/o/w) system [63].

Using the single emulsion method the polymer is dissolved in an organic solvent and the drug is dissolved or suspended in this solution. This drug/polymer mixture is emulsified in a large amount of water [64, 67, 68]. The organic solvent is removed either by evaporation or by extraction in a large volume of water absorbing the solvent. The removal of the solvent depends on the temperature, the solubility characteristics of the polymer and the type of solvent [67, 68, 69]. The resulting microspheres can be harvested by centrifugation or filtration and are washed and dried before use [70].

In the double emulsion system the drug, being in an aqueous solution, is emulsified with the polymer, being dissolved in an organic solution, hereby forming a water-in-oil emulsion. This emulsion is transferred into a large amount of water containing an emulsifier under strong stirring resulting in a water-in-oil-in-water emulsion. Again, the solvent is removed by evaporation or extraction. This method yields high encapsulation efficiencies, which is the reason for its wide spread usage in preparing protein delivery systems [71, 72, 73]. The characteristics of the microspheres are dependent on the polymer properties, the polymer/drug ratio, the concentration and nature of the emulsifier, the temperature and the agitation speed during emulsification [63].

Phase Separation

Another method to produce microspheres is the phase separation method. Here, the solubility of the encapsulating polymer is decreased by adding a third component to the organic solvent the polymer is solved in [74, 75, 76]. The entire process consists of three steps: First, the phase separation of the coating polymer solution, second, the extraction of the polymer solvent which generates coacervate droplets containing the drug, and third the solidification of the microspheres. The two phases of this method are the coacervate phase, which contains the polymer and the supernatant phase depleted in the polymer. The drug is in the polymer phase coated by coacervate [77].

In detail, the polymer is dissolved in an organic solution and the drug particles, solved in an aqueous solution, are dispersed in the polymer solution. Thereby, a water-oil emulsion is formed. If drugs like steroids, which are hydrophobic, are to be encapsulated they need to be solubilized first, or the solid particles are dispersed in the polymer solution. Next, an organic nonsolvent (e.g. silicone oil, vegetable oil, light liquid paraffin) is added to the stirred system gradually extracting the polymer solvent. The polymer forms coacervate droplets which entrap the drug particles. Following, the system is transferred to a large volume of another organic nonsolvent (e.g. hexane, heptanes, petroleum ether) to harden the microdroplets. The final microspheres are washed,

filtrated or centrifuged and dried [75, 77]. The characteristics of the microspheres are determined by the molecular weight of the polymer, the viscosity of the nonsolvent and the polymer concentration [78, 79].

This method imposes less complicated requirements on the solvent than the solvent evaporation/extraction method discussed above, because the solvent does not need to be immiscible with water. Furthermore, the boiling point could be higher than the boiling point of water [75]. Yet, this method is not as widely used as the microspheres tend to form agglomerates. During the phase separation, there are problems in mass production, large quantities of organic solvents are required and it is difficult to remove residual solvents from the final microspheres [80].

Spray Drying

The microsphere preparation by spray drying [81] has been developed in order to improve the stability of labile biomolecules. The method of spray drying is very useful for the encapsulation of hydrophobic drugs and also very suitable for the up-scaling of the production processes of microspheres [82]. Furthermore, it is an extremely rapid method, very convenient, it involves mild conditions and it is less dependent on the solubility of the drug and the polymer [74, 80, 83]. Additionally, it shows a high reproducibility and allows the controlling of the particle size [63]. Nevertheless, spray drying leads to significant product losses due to the adhesion on the inner walls of the instrument and also tends to form agglomerates [80]. Moreover, spray drying is rarely used in early research stages, because usually only little amounts of the possible future drugs are available and thereby this method is an expensive method to use.

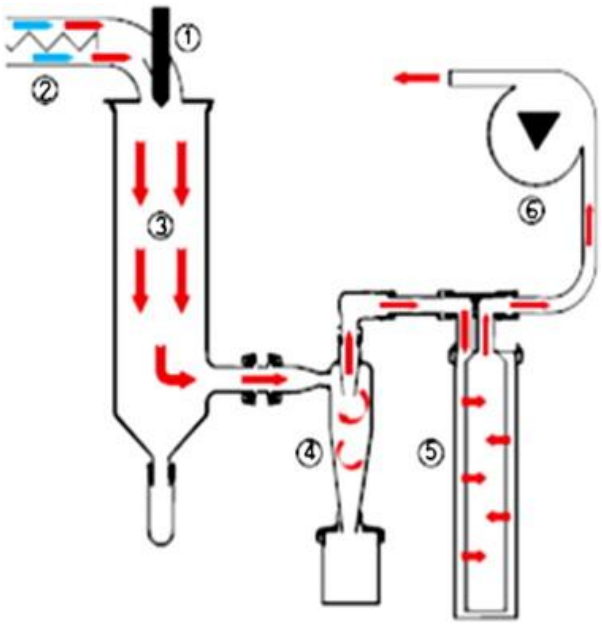

Figure 9: Assembly and Air Flow of a Spray Drying Instrument – 1) nozzle, 2) tube, 3) spray cylinder, 4) cyclone, 5) off gas filter, 6) aspirator [84]

In general, spray drying processes are used to produce dry solid particles out of a solution by removing all liquid parts. The polymer is dissolved in an organic solvent and

the drug particles are dissolved or dispersed in this solution. Figure 9 shows the assembly of a spray dryer and the air flow through the equipment which develops during the process. The instrument is composed of a nozzle (1) and a tube (2) which are located at the top of the spray cylinder (3). Below the spray cylinder is an exit which leads into the cyclone (4). At the bottom of the cyclone is a collecting vessel which collects the product at the end of the process. At the top of the cyclone is a connection to an off gas filter (5) which leads to the aspirator (6). During the spray drying process air is heated up and pushed through the system. The drug/polymer solution is pumped through the nozzle forming little droplets which are immediately surrounded by hot air. The size of the droplets is dependent on the pump rate and the nozzle diameter as well as the spray gas. The higher pump rate and spray gas flow and the smaller the nozzle diameter the smaller the diameter of the droplets created and vice versa. Due to the heat the solvent evaporates in the spray cylinder. The droplets containing the solvent are dried from their outside to their inside forming the microspheres. The resulting microspheres are moved by the hot air stream into the cyclone where they are separated from the air flow and collected in the vessel.

2.3.3 Biodegradable and Biocompatible Polymers

The polymers used as microspheres should be biodegradable to circumvent the need to remove them after the delivery of the drug. The first biodegradable polymer coating was reported by Mason *et al.* in 1976 [85]. Naturally, biodegradable polymers which are also biocompatible would be the best choice. There are synthetic polymers as well as natural polymers available which are biodegradable and biocompatible. These polymers are reduced into non-toxic components by hydrolysis. Therefore, they are suitable for implants which needed to be removed by surgery. The release kinetics of drugs can be controllably modified by the different characteristics of the polymers. A number of different factors can be used for this modification, such as the physicochemical properties [86, 87] of the polymers as well as the degradation kinetics of the polymer itself [88, 89, 90, 86]. Moreover, thermodynamic compatibility between polymer and drug [91] and the shape of the devices [92, 93, 94] are used to manipulate the release profiles. In the following, different groups of polymers are going to be introduced (Figure 10).

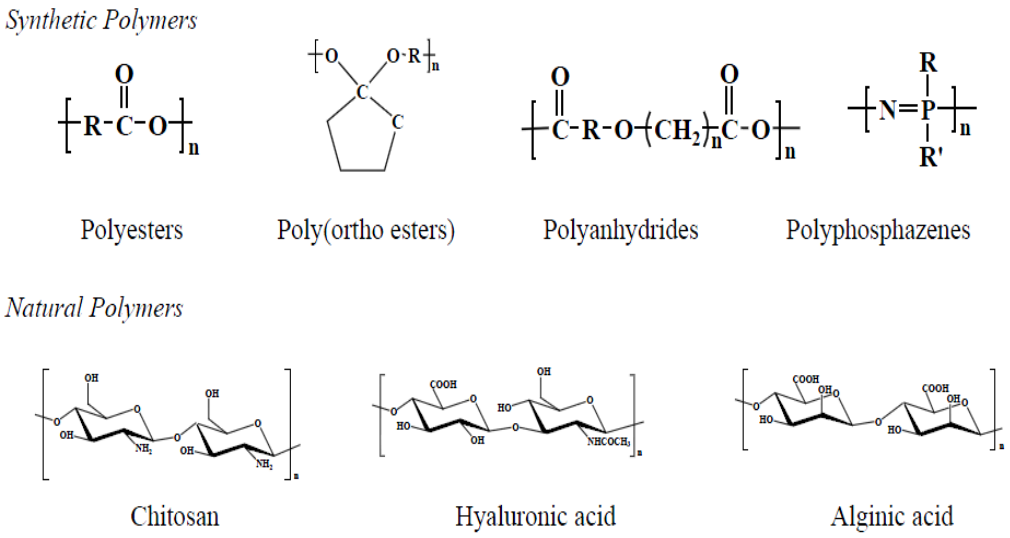

Figure 10: Chemical Structures of Different Biodegradable Polymers [63].

Polyesters

Polyesters show great biodegradability and biocompatibility [86, 64, 95]. The ester bonds in the backbones of polyesters degrade via hydrolytic cleavage. Some exemplary chemical structures are shown in Figure 11.

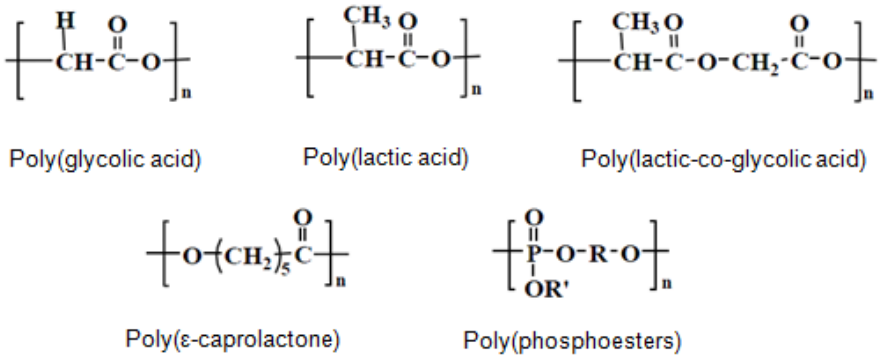

Figure 11: Chemical Structures of several Polyesters [modified after 63].

The most popular polyesters are poly(lactic-co-glycolic acid) (PLGA) copolymers, because their degradation rate and mechanical properties can be precisely controlled by varying the ratio of lactic and glycolic acids and the alternation of its molecular weights

[63]. If the polymer is produced with a high rate of glycolic acid (up to 70 %) it degrades faster. The degradation of PLGA is also accelerated with a decreasing molecular weight, because the higher content of carboxylic groups at the end of the polymer chain increases the acid-catalyzed degradation. PLGA undergoes bulk erosion during degradation. However, PLGA significantly affects the stability and activity of bioactive compounds due to its hydrophobicity and its acidic degradation products [63].

Poly(ortho esters)

Four different groups of poly(ortho esters) as biodegradable polymers exist: POE I, POE II, POE III, POE IV (Figure 12) [96, 97, 98]. In contrast to the homogenous degradation of polyesters Poly(ortho esters) undergo surface erosion due to their high hydrophobicity and water impermeability. Surface erosion is a desired property to attain near zero-order drug release profiles [99]. Thus, Poly(ortho esters) are predestined for constant release rates with low initial burst behavior [100, 101, 97].

The ortho ester bonds of POE I are highly susceptible to acids and thus need to be stabilized with a base to prevent an uncontrolled hydrolysis reaction. As a result, POE I is very limited in biomedical applications. The synthesis of POE II is simple and highly reproducible, but extremely hydrophobic limiting the access of water to the hydrolytically labile ortho ester linkages. In order to increase the erosion rate the incorporation of acidic excipients into the polymer matrix is necessary. Hence, it is difficult to design surface eroding devices. On the one hand POE III enables the preparation of injectable drug delivery systems without the need of using organic solvents or elevated temperatures. No autocatalysis occurs and the ortho ester linkages are only sensitive to the acidic products. On the other hand difficulties in the synthesis and poor reproducibilities of the synthesized polymers limit its biomedical application [98]. The erosion rate of POE IV can be precisely controlled due to modification in the backbone of the polymer thereby allowing the manipulation of the release profile of a drug. Furthermore, the acidic environments in the bulk of the microspheres are prevented [102].

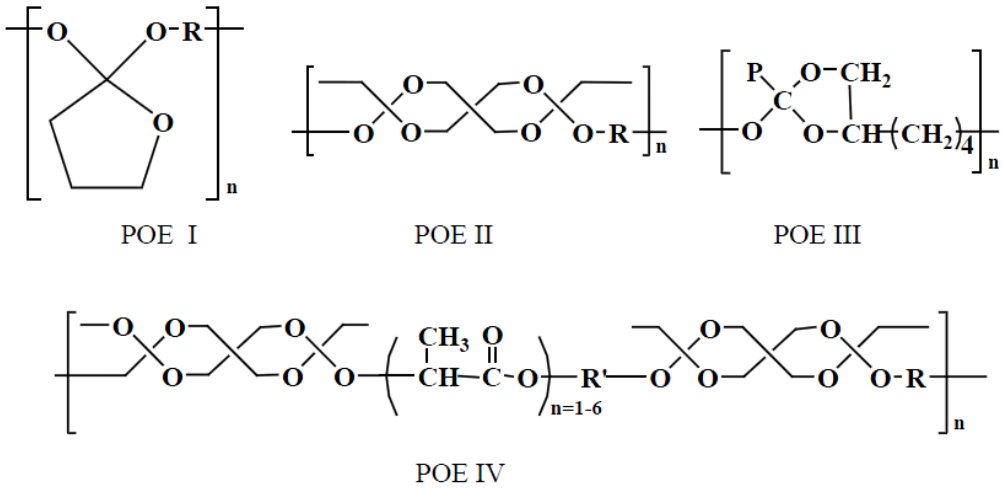

Figure 12: Chemical Structures of the Four Different Groups of Poly(ortho esters) [63].

Polyanhydrides

Polyanhydrides show a rapid degradation *in vivo* and have limited mechanical properties, which makes them suitable for short-term controlled delivery of drugs [103, 104, 105]. Due to various available diacids the polymer can be modified in its composition in order to receive the desired physicochemical properties. They undergo surface erosion, show minimal inflammatory reaction and their degradation product are non-mutagenic and non-cytotoxic [106, 107]. Polyanhydrides have been used in FDA approved drugs, e.g. as carriers of antitumor agents [108]. Their main disadvantage is, that they usually have to be stored at frozen state under anhydrous conditions because of their hydrolitic instability.

Polyphosphazenes

Polyphosphazenes are rapidly delevoping as biomedical polymers [109, 101]. Various substituents can be introduced into the polymer´s backbone hence, making polyphosphazenes very versatile. In aqueous solutions they are cleaved into nontoxic, low molecular weight products. The type of erosion can differ; bulk erosion as well as surface erosion is possible. This is depending on the lability of the bond and on the hydrophobicity of the polymer [109].

Natural Polymers

Although many promising synthetic polymers exist, natural polymers remain attractive for biomedical applications due to their outstanding biocompatibility and the fact that they can be easily modified by simple chemistry. The most common natural polymers used in drug delivery systems are proteins, especially collagen, gelatin and albumin, and polysaccharides, namely starch, dextran, hyaluronic acid and chitosan. The use of proteins is significantly limited by their poor mechanical properties, their immunogenicity, and their high costs [71]. On the contrary, polysaccharides are commercially available at low costs and they provide a broad range of different physicochemical properties. Especially chitosan and its derivatives display convincing results. They show excellent biocompatibility, precisely controlled biodegradability, low immunogenicity and biological activity [110, 111].

2.3.4 Applications

Starting in the late 1930s, microspheres were merely used as a protection for vitamins against oxidation [112]. The interest in their use in the formulation of drugs and in tissue engineering arose several decades later. Nowadays, synthetic microspheres are frequently used in clinical practice. They are applied as fillers and bulking agents, embolic particles, and as drug delivery vehicles [113]. Sometimes they are just used in order to mask the bad taste or odor of some bioactive compounds or to improve the flow of powders.

Fillers and bulking agents are used to replace tissue lost due to a disease, an injury, or simply due to aging. Thus, the areas for application could not be more diverse. Wrinkles are treated with these agents, but also lipotrophy of HIV patients as well as stress-urinary-incontinence (SUI) [114, 115, 116, 117]. The injected fillers and bulking agents differ significantly in their properties and behavior. Some are designed to replace volume lost by aging at minimal response of the surrounding tissue. In order to avoid complications with granuloma formation, the material should be degraded within a certain time-span [118, 119, 120]. Reapplications are necessary to maintain the effect. Other materials aim at stimulating the tissue to repair itself. They are also biodegradable, but they interact with the surrounding tissue to enhance cell growth. The last category of materials aims for a permanent filling effect. They are non-biodegradable and remain at the site of injection for the patient´s lifetime. Their mechanical properties need to suit the intended application and need to ensure its functionality. When working with non-

biodegradable polymers it is important to ensure that the material stays in place. Migration could lead to serious complications such as a pulmonary embolism or even a stroke [120, 121, 122]. Therefore, the material needs to be detectable after application in order to rule out a migration.

Microspheres are also used in embolic therapy to close a target artery by acting as a thrombotic emboli leading to an obstruction of arterial blood flow [123, 124, 125, 126, 127]. This is done in order to starve downstream target tissues from oxygen and nutrients. This method is used for a variety of treatments such as tumors, hemorrhages and vascular anomalies like venous and lymphatic malformations. Inoperable tumors, especially tumors in the brain, are often targets of embolotherapy, although they are often palliative. Benign tumors are also treated with this method since it is a minimal invasive alternative to surgery. Furthermore, embolotherapy targets severe bleedings in order to stop lethal blood loss [128].

Especially the use of microspheres composed of FDA approved PLGA as drug delivery systems is widely spread. These systems combine the degradation of the polymer with the delivery of the drug [82]. Several design parameters of these microspheres have great influence on the drug release profile. This complexity complicates the usage in clinical use, because the exact control of all the parameters is necessary. However, the precise control over these parameters allows the realization of a vast potential of microspheres for the use as drug delivery systems.

2.4 Aim of this Thesis

Protein-based drugs hold great promise as therapeutic agents, but many complications have to be overcome before these kinds of drugs can be used. Especially the short half-life and the immunogenicity of several protein-based drugs complicate the medical application of these drugs [8]. The modification by polymers and the microencapsulation of the bioactive protein by polymers have shown great improvements regarding the half-life and the immunogenicity of protein-based drugs.

In this thesis, the PEGylation and the microencapsulation drug formulation methods are combined and analyzed. First, the success of the PEGylation reaction is tested under varying process parameters. Second, the PEGylated protein conjugates, which usually have very similar properties, are purified and separated. The third aim of this thesis is to investigate the quality of the microspheres produced by solvent evaporation and spray drying.

Lysozyme was chosen as a model protein, representing a protein-based drug. In principle, other proteins would be possible, too. Lysozyme is a protein known for its stability and an established activity assay is available. The model protein is modified by the commonly used polymer PEG, which is FDA and EMA approved and already used in a lot of commercially available drugs (Table 1). The PEGylation process is conducted using an activated PEG called methoxypoly(ethylene glycol) p-nitrophenyl carbonate (mPEG-pNp) (Figure 13) which provides the advantage of cross-linkage prevention. PEGylation is closely analyzed regarding the process parameters, the time course, the polymer/drug ratio impact, and the influence on the biological activity of lysozyme.

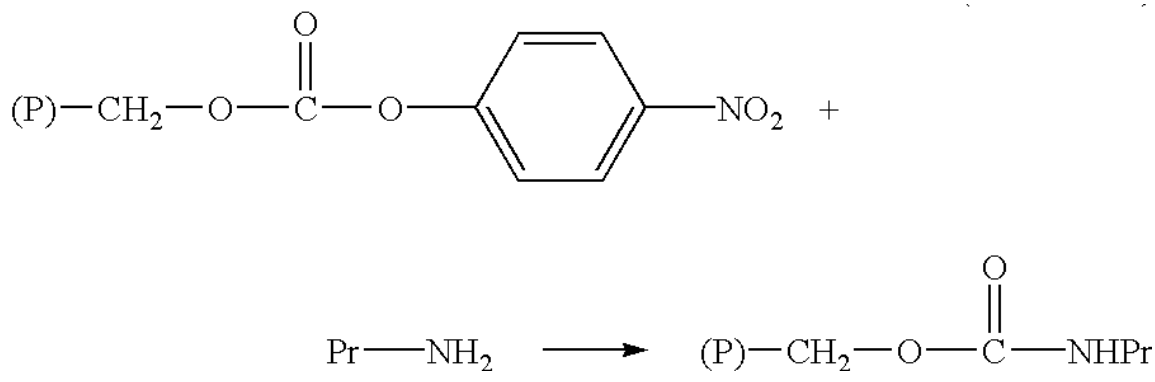

Figure 13: Chemical Reaction of Methoxypoly(ethylene glycol) p-Nitrophenyl Carbonate with a Protein

Due to the seven binding sites of lysozyme for mPEG-pNp [129, 33] different types of protein conjugates are expected after the PEGylation process. These conjugates are purified and separated using chromatographic methods and varying column materials. The purified and separated conjugates are characterized as precisely as possible with regards to their activity, their stability, and their resistance to proteolysis.

After purification and separation, the conjugates are further improved by microencapsulation in PLGA. The polymer PLGA is chosen, because the degradation rate and the mechanical properties can be controlled precisely by variations of the ratio of lactic and glycolic acids and the variation of its molecular weight [63]. Moreover, it is FDA approved. The encapsulation methods solvent evaporation and spray drying are compared. Solvent evaporation promises high encapsulation efficiencies [71, 72, 73], but its solvent requirements are complicated and it is not very suitable for scale-up. Spray drying provides a rapid process with mild conditions and great preconditions for scale up, but shows problems regarding the yield [82, 80]. Following the encapsulation, the produced microspheres are investigated with respect to release profiles, yields, encapsulation efficiencies, and particles sizes.

3. Material and Methods

3.1 Model Protein Lysozyme

Lysozyme is an enzyme discovered in 1922 by Alexander Fleming [130]. It is found in great concentrations in blood, saliva, tears, and milk, where it prevents bacterial growth. The enzyme is isolated from chicken egg white and has a molecular weight of 14.3 kDa [131] and an isoelectric point of 11.35 [132]. It consists of 129 amino acids and forms a single chain polypeptide [133]. Lysozyme hydrolyses the (1-4)-glycosidic linkages between N-acetylmuraminic acid and N-acetyl-D-glucosamine residues in peptidoglycans [134]. The optimal pH for the activity of lysozyme lies between 6 and 9 [135]. Lysozyme can be inhibited by indole derivatives and surface-active agents such as dodecyl alcohol [136, 137]. Applications of Lysozyme can be found in the food industry as preservatives [138] and in biology in order to lyse bacteria via hydrolysis of their peptidoglycan cell walls.

The structure of lysozyme (Figure 14) shows two major domains: The alpha helices in magenta and the beta sheets colored in yellow. The active site of lysoyme is located within the cleft between these domains. Ten active site residues are located at this pit with great affinity for the substrate. The residues form numerous hydrogen bonds to grant the enzymatic reaction [139]. The active site residues Asn46 and Asp53 are located in the beta domain, while Glu35 and Ala108 are in the alpha domain.

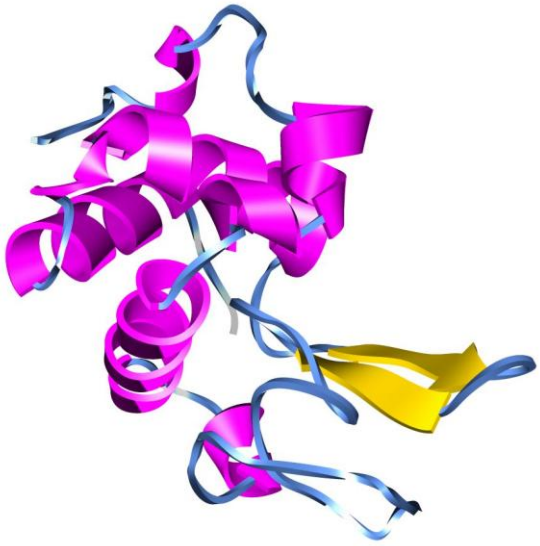

Figure 14: Structure of Lysozyme – In magenta the alpha helices of the enzymes are shown and yellow represents the beta sheets [140].

Furthermore, Asp102, Trp64, Tyr63, Asp49, Asn60, and Gln104 are involved in the hydrogen bonding network forming the enzyme-substrate complex. Glu35 and Asp53 are the acidic amino acids actually involved in the hydrolysis reaction [140].

3.2 Material

Table 2: Chemicals

Substance	Manufacturer
Lysozyme from chicken egg white	Fluka Analytical, Sigma-Aldrich
Potassium dihydrogen phosphate	Carl Roth GmbH +Co KG
Micrococcus lysodeikticus	Sigma –Aldrich Chemie GmbH
Methoxypoly(ethylene glycol) p-nitrophenyl carbonate (MW 5000Da)	Sigma –Aldrich Chemie GmbH
poly(ethylene glycol) (MW 2000Da)	Carl Roth GmbH +Co KG
Poly(lactid-co-glycolic acid) 50:50	Sigma –Aldrich Chemie GmbH
Protein Marker Roti Mark Standard	Carl Roth GmbH +Co KG
Cotton Seed Oil	Sigma –Aldrich Chemie GmbH
Lecithin	Carl Roth GmbH +Co KG
Acrylamide	Carl Roth GmbH +Co KG
Tris	Carl Roth GmbH +Co KG
Sodium dodecylsulfate (SDS)	Carl Roth GmbH +Co KG
Temed	Carl Roth GmbH +Co KG
Ammonium persulfate	Carl Roth GmbH +Co KG
100% Ethanol	Carl Roth GmbH +Co KG
BCA Reagent 1	Carl Roth GmbH +Co KG
BCA Reagent 2	Carl Roth GmbH +Co KG
Proteinase K	Sigma –Aldrich Chemie GmbH
Methylene chloride	Carl Roth GmbH +Co KG
Petroleum ether	Carl Roth GmbH +Co KG
Acetonitrile	Carl Roth GmbH +Co KG
Dimethylsulfoxide (DMSO)	Carl Roth GmbH +Co KG
Phosphate buffered saline (PBS)	Biochrom AG

Table 3: Chromatographic Columns and Media

Column/Medium	Manufacturer
HiLoad Sephacryl S-200 Hiprep 16/60	GE Healthcare Life Sciences
HiLoad Superdex 75 rep grade 26/60	GE Healthcare Life Sciences
HiTrap CM FF 1ml	GE Healthcare Life Sciences
Sepharose 4B	Sigma-Aldrich

Table 4: Devices

Devices	Manufacturer
Spray Dryer	Büchi, B-290
ÄKTA purifier 100 chromatographic system	GE Healthcare Life Sciences
Laser Diffraction Particle Size Analyzer	Beckamn Coulter LS 13 320
Photometer Ultrospec 2100 pro	Amersham Biosciences
Dry freezer	Christ alpha 1-4 LSC
Phase Contrast Microscope	Olympus
Nanodrop 1000 Spectrophotometer	Thermo Scientific
Fraction Collector	Bio Rad
Centrifuge	Eppendorf
Scale	Sartorius
pH-Meter 766 Calimatic	Knick
Vacuum Pump	Leybold, Divac 2.4 L
Magnetic Stirrer	Heidolph, MR 2002
Incubator	Binder
Pipettes	Brand
Refrigerator	Liebherr
Thermomixer	Eppendorf
Vortexer	Heidolph
Power supply	Biorad

Table 5: Software

Software	Manufacturer
Microsoft Word 2007	Microsoft
Microsoft Excel 2007	Microsoft
Adobe Photoshop CS4	Adobe
Unicorn 5.31	GE Healthcare Life Sciences
Silver Fast Ai	Laser Imaging AG

3.3 Methods

3.3.1 Lysozyme Activity

The activity of lysozyme and its conjugates was measured by monitoring the turbidity changes in a *micrococcus lysodeikticus* suspension (0.2 mg/ml in 66 mM KH_2PO_4, pH 6.2). The assay relies on the fact that lysozyme hydrolyzes the (1-4)-glycosidic linkages between N-acetylmuraminic acid and N-acetyl-D-glucosamine residues in peptidoglycans of gram positive bacteria. Due to the lysozyme induced lysis of the bacteria the turbidity of the suspension decreases and hence can be monitored by photometric analysis. 10 µl of the protein solution to be measured were added to 990 µl of the bacteria suspension. After shortly mixing the sample the extinction was measured at 450 nm every minute for a time interval of 5 minutes. At the end of this time interval the extinction values are expected to be linear. A decrease in absorbance of 0.001 is defined as 1 unit of lysozyme activity. The enzyme activity could be calculated from the measured values using the formula [141]:

$$activity(U) = \frac{adsorption\ at\ 450nm/min}{0.001} * dilution\ factor$$

3.3.2 SDS-PAGE

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the PEGylated lysozyme. The gel was prepared with 12 % acrylamide and stained with Coomassie® Blue, their exact compositions are shown in Table 6 and 7:

Table 6: Composition Separation Gel

Separation gel (12 %):	
30 % acrylamide solution	2 ml
3 M Tris-HCl, pH 8.8	625 µl
10 % SDS	50 µl
ddH_2O	1.7 ml
Temed	2.5 µl
10 % ammoniumpersulfate	32.5 µl

Table 7: Composition Collection Gel

Collection gel (5 %):	
30 % acrylamide solution	850 µl
3 M Tris-HCl, pH 6.8	625 µl
10 % SDS	50 µl
ddH_2O	2.85 ml
Temed	5 µl
10 % ammonium persulfate	32.5 µl

3.3.3 PEGylation of Lysozyme – Time Course

The PEGylation reaction of lysozyme was observed after different time periods. 300 µl of a 10 mg/ml lysozyme solution in 100 mM KH_2PO_4 buffer, pH 8 were added to 200 µl of a 10 mg/ml mPEG solution in the same buffer. The mixture was incubated in a shaking thermoblock at 35 °C and 500 rpm. Samples (20 µl) were withdrawn from the reaction mixture after 5, 15, 30, 60,120,180 and 240 min. After the withdrawal the samples were mixed with 20µl sample buffer, immediately heated up to 95 °C for 5 min, rested on ice, and analyzed by a SDS-PAGE [modified after 142].

3.3.4 PEGylation of Lysozyme – Mass Ratio

Different mass ratios of mPEG-pNp (MW 5000 Da) and PEG (MW 2000 Da) to lysozyme were analyzed in the PEGylation reaction. A 10 mg/ml lysozyme and a 10 mg/ml mPEG solution in 100 mM KH2PO4 buffer, pH 8 was prepared. Then the mass ratios, 0.1, 0.25, 0.5, 1, 2 and 3 were produced by different mixtures of the stock solutions. The mixtures were incubated in a thermomixer at 35 °C and 500 rpm. Samples (20 µl) were withdrawn after 2 hours from the reaction mixture. These samples were mixed with 20 µl sample buffer, heated up to 95 °C for 5 min and analyzed by SDS-PAGE. Moreover, the enzymatic activity was determined [modified after 142].

3.3.5 Packing of a Sepharose 4B Column

The Sepharose 4B media was supplied in 20 % ethanol. A slurry was prepared and degassed with a ratio of 75 % settled gel to 25 % eluent buffer. Using a packing connector the packing reservoir was mounted on top of the column. The bottom of the column got closed after the end piece was flushed with buffer in order to make sure that

no air is remaining in the column. Now the slurry got poured in the column in a single operation, the reservoir cap was closed, the bottom outlet was opened and the pump was started until the bed reached a constant height. The bottom outlet got closed again and the packing connector and reservoir got removed. The rest of the column was filled carefully with buffer to form an upward meniscus and the top adaptor was inserted at a 45 ° angle to prevent air from entering the system. The plunger was moved slowly down the column to the gel surface and a constant pressure was applied. When the bed height did not move further down the plunger was pushed another 3-4 mm downwards.

3.3.6 Concentration by Ultrafiltration

After the PEGylation of lysozyme at a mass ratio of 1 in a 150 mM phosphate buffer pH 8 for 4 hours the resulting conjugate mixture was concentrated by ultrafiltration. An Amicon stirred cell model was used with a total volume of 50 ml. A pressure of 3 bar was applied on the cell using N_2. The mixture was under constant mild agitation while pushed through an ultrafiltration membrane with a molecular weight cut off (MWCO) of 10 kDa. Thus, both the mPEG-lysozyme conjugates and the unmodified remaining parts of lysozyme and mPEG were held back in the cell. They were taken up in 2.5 ml of 100 mM KH2PO4 buffer, pH 8. The protein concentration was measured before and after the ultrafiltration to monitor possible product losses.

3.3.7 Size Exclusion Chromatography

In order to purify and separate the mPEG-lysozyme conjugates a SEC was performed using the system ÄKTA^{TM} purifier UPC 100 (GE Healthcare). After equilibrating the columns (Sepharose 4B (10/33), Superdex 75 (26/60), Sephacryl S-200 (16/60)) with 2 column volumes (CV) of 150 mM KH_2PO_4 pH 8 buffer 2 ml of the concentrated sample were injected onto the column. The flow rate was kept constant at 0.5 ml/min throughout the whole process. The fraction volume was set to 2 ml and the elution was conducted over about 4 CV. The fractions below the peaks were analyzed by SDS-PAGE and concentrated by ultrafiltration as described above; except that the conjugates of the pooled peak fractions were taken up in 5 mM KH_2PO_4 pH 8 buffer. Furthermore, their activity was measured at different pH values and the concentrated conjugates were lyophilized.

3.3.8 Enzymatic Stability of mPEG-Lysozyme Conjugates

The enzymatic stability of the mPEG-lysozyme conjugates was determined after incubation for one hour at 50 °C in 100 mM KH_2PO_4 buffers with differing pH values of 5, 8 and 11. Following this incubation, the enzymatic activity was determined as described above [modified after 142].

3.3.9 Resistance to Proteolysis

The resistance of the native lysozyme and the mPEG-lysozyme conjugates to proteolysis was measured by adding 20 IU of proteinase K to 600 IU of lysozyme or mPEG-lysozyme conjugate in a total volume of 1 ml 100 mM KH_2PO_4 buffer, pH 7. The mixture was incubated at 37 °C for 2 h. Samples (30 µl) were taken at 0, 20, 45, 75 and 120 min for immediate determination of the remaining enzymatic activity [modified after 142].

3.3.10 Spray Drying

Three different batches of PLGA microspheres were prepared using a Büchi B-290 spray dryer. 727 mg of PLGA were dissolved in methylene chloride. 23 mg of either native lysozyme or one of the two mPEG-lysozyme conjugates, resulting from the size-exclusion-chromatography, were dispersed in the polymer solution. The suspension was mixed using a magnetic stirrer at 1400 rpm and afterwards pumped into the system under constant stirring at 700 rpm. The operating conditions were as follows:

Nozzle diameter:	0.7 mm
Nozzle cleaning:	3
Spray gas:	30 mm
Pump rate:	15 %
Aspiration rate:	100 %
Inlet temperature:	45 °C
Outlet temperature:	32 °C

3.3.11 Solvent Evaporation

Using the method of solvent evaporation, lysozyme and the mPEG-lysozyme conjugates were microencapsulated. 72.7 mg of PLGA were dissolved in 10 ml 10 % aqueous acetonitrile. 57.5 µl of an aqueous 40 mg/ml lysozyme or mPEG-lysozyme conjugate solution were transferred into the polymer solution. Furthermore, 0.05 g of the emulsifier lecithin were added to 100 ml of cotton seed oil. Under constant agitation at 700rpm the protein/polymer mixture was transferred dropwise into the oil-phase. The temperature of the system was set to 40-45 °C to evaporate the organic solvent and the agitation was decreased to 400-500 rpm. After 4 h the microspheres were harvested by vacuum filtration through a 0.8 µm membrane. During the filtration process the membrane was washed frequently with petroleum ether. As a result, the washed and dried microspheres remained on the membrane [143]. The dry particles, which remained on the membrane, were gently scraped off by a plastic spatula.

3.3.12 Encapsulation Efficiency

To measure the encapsulation efficiency of the microencapsulation methods the procedure of Sah et al. [144] was used. 10 mg of accurately weighed microspheres were dissolved in 2 ml DMSO. After an incubation of 1 h at room temperature under occasional shaking 10 ml of 0.05 M NaOH containing 0.5 % SDS was added and gently mixed. Afterwards, another hour of incubation at room temperature followed before the protein concentration was determined by a BCA assay.

3.3.13 Particle Size of Microspheres

The particle size measurement was conducted working with a laser diffraction particle size analyzer. Due to the principle of dynamic light scattering the particle size distribution of particles between 0.04 to 2000 µm are determined. The microspheres were suspended in 70 % ethanol and applied to the instrument. The necessary amount of microspheres was indicated by the instrument during application.

3.3.14 Release Kinetics

10 mg of each microsphere type were dispersed in 1 ml PBS buffer, pH 7.4 and incubated at 37 °C under mild agitation. Samples were taken by centrifuging the reaction tubes at 5000 rpm in order to settle the microspheres. The supernatant was removed and analyzed for protein concentration by a BCA assay and for enzymatic activity as described above. The buffer was replaced after sampling.

4. Results and Discussion

4.1 PEGylation Reaction

Time course

The PEGylation of lysozyme was performed using a PEG activated with the functional group p-nitrophenyl carbonate. This functional group is suitable for undergoing reactions with lysine, the typical receptor amino acid of lysozyme [26]. The time course of this reaction was observed by SDS-PAGE.

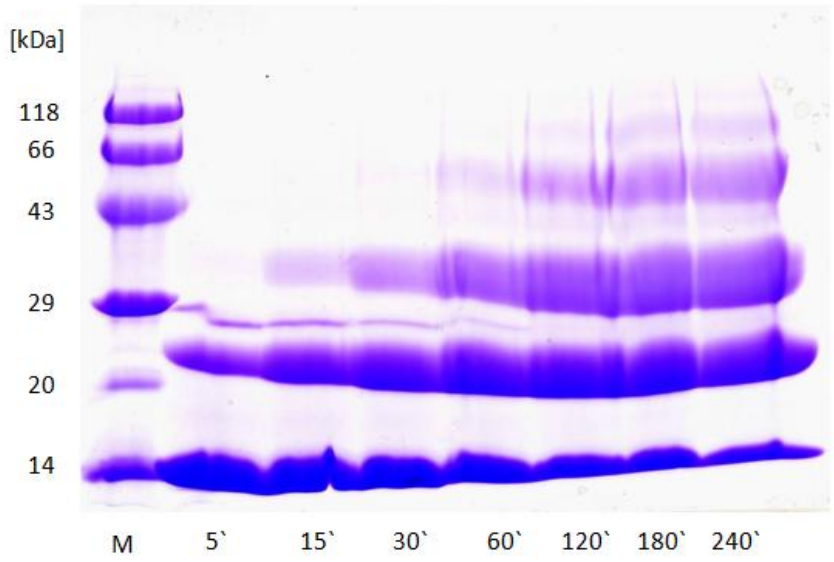

Figure 15: SDS-PAGE of the PEGylation Time Course – M: Protein marker; 5, 15, 30, 60, 120, 180 and 240 describe the time intervals in minutes between the sampling.

Clearly, a successful PEGylation reaction can be observed in Figure 15. The degree of modification is increasing with extended reaction time. The bottom band at about 14 kDa indicates the native lysozyme. It is decreasing with the time since more and more protein is reacting with the polymer. Correspondingly to the decrease of the lysozyme band´s intensity, the bands of the PEGylated lysozyme conjugates get broader with longer reaction time. Already after 5 minutes, larger molecules can be detected at about 24 and 28 kDa. At the end of the experiment, after 4 hours, 4 different degrees of modification can be observed in addition to the remaining part of unreacted lysozyme. The reaction times are quite exact, because the samples rested on ice after heating them up to 95 °C immediately after the sampling. Another method to stop the reaction would be to add an excessive amount of lysine to the samples [142]. The band at 28 kDa is

disappears after about one hour. Due to its size it might be a dimer of lysozyme disbanding after a certain reaction time. Compared to the gel electrophoresis of da Silva Freitas and Abrahao-Neto the polymer is reacting faster with the protein [142]. The ratio of protein and polymer is the same, but the buffer conditions differ slightly. Da Silva Freitas and Abrahao-Neto worked with Hepes buffer at a pH value of 7.5 whereas a KH_2PO_4 buffer pH 8 was used in this thesis. This higher pH value might have accelerated the reaction.

The polymer has an average size of 5 kDa. The apparent molecular weights of the modified lysozyme conjugates seem higher than they should be when linking 5 kDa polymers to a 14 kDa protein. But as already mentioned in section 2.2.2, the high water coordination of PEG makes PEGylated conjugates function as though they are much larger than a corresponding soluble protein of the same mass [36]. This has already been confirmed by SEC and gel electrophoresis [145]. Furthermore, PEGylated proteins differ in their running profiles in SDS-PAGE analysis compared to globular proteins. They show a lower mobility during gel-electrophoresis due to the PEG´s long, chain-like structure [146]. McGoff *et al.* suggest that the entanglement of PEG chains is not the only mechanism for the observed low mobility, but additionally the hydrophilic PEG evokes a charge shielding effect by its shell-like coverage of the protein [147]. Kurfürst proposes a calibration of the gel using PEGs of different molecular weights, since he showed a linear mobility of PEG molecules on 8-25 % polyacrylamide gels. Moreover, he determined a method which enables a very specific distinction between PEGylated proteins, non-PEGylated proteins and protein aggregates by a staining procedure after the SDS-PAGE. This specific staining is based on the formation of a barium iodide complex with the PEG molecule [148, 149, 150]. Cross-linkages between the PEGylated lysozyme conjugates are very unlikely since a methoxyPEG is used preventing the development of cross-linkages.

Lysozyme is a small protein with few attachment possibilities for PEG. It has seven binding sites to which mPEG-pNp can covalently attach; 1 α-amino group of the N-terminal and 6 ε-amino groups of the lysine residues K1, K13, K33, K96, K97 and K116 [129, 33]. In the conducted gel electrophoresis only four different PEG-lysozyme conjugates can be detected. Hence, it can be concluded that not all degrees of modification were produced at the end of this process. It is difficult to tell which degrees of modification were formed, since the molecular weight indicated by the SDS gel is not correctly displaying the molecular weight as discussed above. Nevertheless, it is likely that the lower degrees of modifications are produced here, because the reaction was monitored almost from the beginning and covalently bound polymers hinder other

polymers sterically due their conformations (see section 2.2.2). The first linked polymers might prevent other polymers from attaching to the remaining binding sites. The reactivity of the binding sites was determined to be K33>K97>K116 [151, 152, 33, 153]. Thus, the first band obtained after 5 minutes is likely to describe the PEGylation at the most reactive lysine site K33. The next two bands occurring one after another with an increasing reaction time are likely to be linkages to the reactive lysines K97 and K116. The N-terminal amino acid is lying on the inside of the protein, which makes it difficultfor the polymer to reach this reactive site [154].

The broad bands and their poor resolution are most likely due to the polydispersity of the polymer. The polymer size of 5 kDa is an average value. The polymerization reactions can be controlled quite well, but there are always deviations. These deviations need to be considered. The polydispersity should be kept as low as possible in order to be approved by the FDA.

The functional group p-nitrophenyl carbonate is known for a slow reaction, e.g. compared to succinimidyl succinate [24]. Thus, the reaction can be controlled more easily. The reaction could be stopped, for example by adding an excessive amount of lysine to the reaction mixture [142], when the desired degree of modification is reached.

Process Parameter pH Value

Important factors which influence the PEGylation reaction are the protein concentration, the PEG-to-protein ratio, the reaction pH and temperature as well as the reaction time and the characteristics of the protein to be modified [26]. Considering the deviation from the PEGylation reaction in this thesis to the one conducted by da Silva Freitas and Abrahao-Neto, the influence of the pH value during the process was examined. PH values between 6 and 8 were tested since lysozyme shows its activity optimum at pH 6 [142].

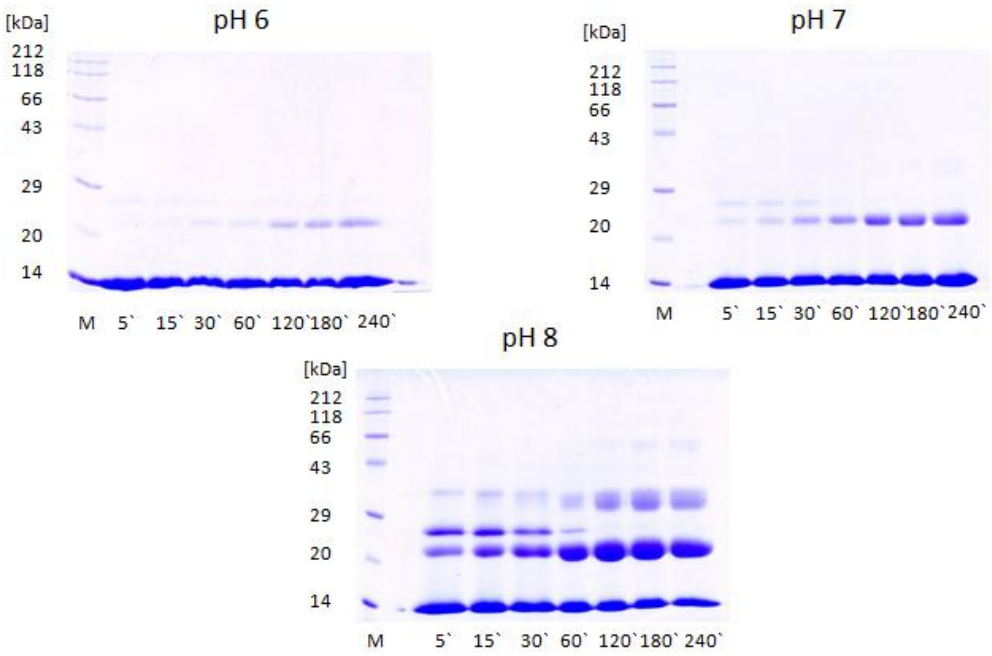

Figure 16: SDS-PAGE of the PEGylation at different pH-values – M: Protein marker; 5, 15, 30, 60, 120, 180 and 240 describe the time intervals in minutes between the sampling.

In order to determine the effect of the pH value present during the reaction three different pH values were tested and analyzed by SDS-PAGE. Figure 16 shows an increasing PEGylation reaction with an increasing pH value. Lysozyme is apparently reacting much slower in a phosphate buffer pH 6 than in the same buffer at pH 8. At pH 6 only two bands are detectable, one of which might be a lysozyme dimer as it is disappearing after about an hour reaction time. Three bands can be detected at the end of the reaction at pH 7, but the fourth band can only be detected at pH 8. This explains the difference in produced modification degrees, comparing the PEGylation time course experiments of this thesis and of da Silva Freitas and Abrahao-Neto´s in 2010. The experiment in this thesis was conducted at pH 8 whereas da Silva Freitas and Abrahao-Neto conducted their experiment at pH 7.5. Despite this only slight difference, the results presented in Figure 16 suggest that the pH value is a very important factor with a high influence on the PEGylation reaction. The pI of lysozyme lies at about 11, meaning that lysozyme has a greater positive charge the more acidic the buffer conditions get. The positive charge of the lysine attachment sites disappear more and more as the pH increases. Thus, more amine instead of ammonium ions are present in the lysine residues. Only the amines react with the polymer which leads to a faster reaction at higher pH values. Thus, these results also confirm the claim, described by Roberts *et al.* in 2002, that the pH value is an important process parameter.

Mass Ratio

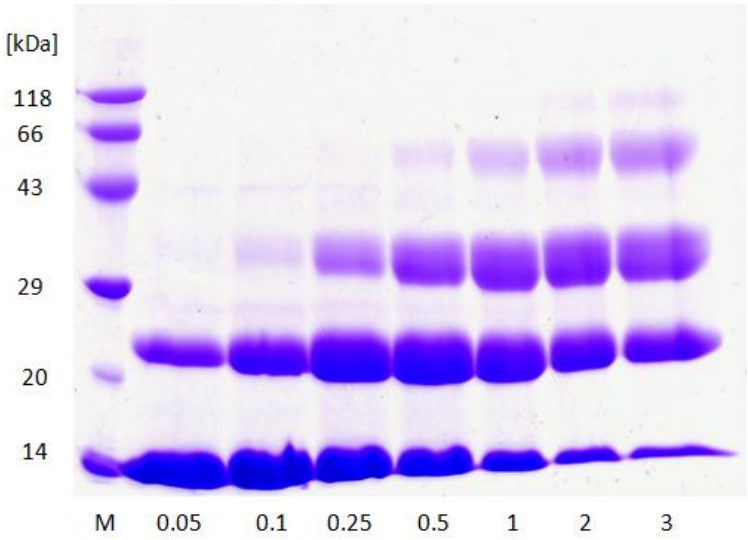

Figure 17: SDS-PAGE of the PEGylation reaction at different mass ratios – M: Protein Marker; 0.05, 0.1, 0.25, 0.5, 1, 2 and 3 describe the mass ratio polymer/protein. The reaction was conducted at 35 °C and 500 rpm for 4 h.

To assess the impact of the ratio of polymer to protein on the PEGylation reaction, the PEGylation results were studied for different ratios: Polymer/protein mass ratios of 0.05 to 3 were analyzed by SDS-PAGE after a reaction time of two hours. At a ratio of 0.05 only one modification can be monitored at the end of the reaction (Figure 17). The degrees of modification increase with a higher mass ratio. Hence, the desired degree of modification can be set by choosing the respective mass ratio. A big advantage of only one modification, as resulting from a mass ratio of 0.05, is that the purification of the modified conjugate is much simpler compared to a mixture of conjugates. The results of the time ratio experiment which was conducted with a mass ratio of 1 are a good match when looking at the reaction after 2 h.

Summing up, it can be said, that PEGylation is controlled by the reaction time, the pH value and the polymer/protein mass ratio. The degree of protein modification is increased and the PEGylation reaction is fastened by an increase of these process parameters. Thus, in order to control the reaction these factors and their influencing effects should be precisely analyzed and optimized when PEGylating proteins. The optimal parameters depend on the specific desires of the manufacturer. According to Caliceti and Veronese [155] an increasing degree of modification decreases the immunogenicity and antigenicity. Furthermore, the bigger the size of the modified proteins the longer the circulation time in the organism, since the kidneys are clearing molecules according to their size [29]. Hence, the conjugates with a higher degree of modification could be the

more effective ones. But, molecules which are too big can cause accumulations in the liver [24]. So, if a big protein is supposed to be PEGylated the degree of modification might need to be lower than when PEGylating a small molecule. However, the higher degrees of modification are often produced in mixtures, thereby greatly increasing the purification and separation effort. This leads to high production costs. To circumvent the production of these mixtures a stepwise PEGylation could be performed. When the ratio is kept small, monoPEGylated lysozyme can be produced (Figure 17). If this product would be purified from unreacted lysozyme and remaining polymer, the monoPEGylated lysozyme could be added to the polymer at a small ratio on more time. As a result, higher degrees of modification could be produced which are not mixed with other modification degrees. Thereby, the separation, which is particularly complicated, would not be necessary.

Enzymatic activity of the PEGylated lysozyme

PEGylation processes are proven to have great effects on the biological activity of the modified protein. The influences of the different degrees of mPEG-pNp attachment on the activity of lysozyme were determined using the substrate *M. lysodeikticus*.

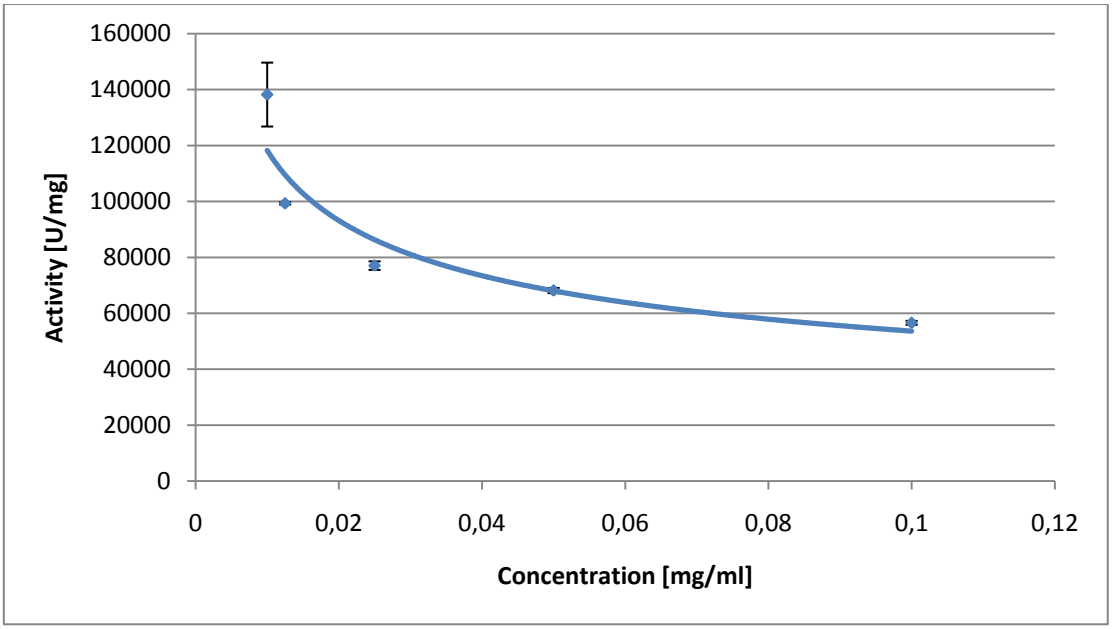

Figure 18: Enzymatic Activity at Different Concentrations of Lysozyme – The activity was determined using the substrate *M. lysodeikticus*.

Due to some inconsistencies regarding the activity assay, the assay protocol was closely investigated in a separate experiment. The activity of a dilution series of lysozyme was measured. When displaying the specific activity (U/mg) a straight line parallel to the x-axis would be expected at different concentrations. But the results presented in Figure 18 obviously show an increasing activity with a decreasing enzyme concentration. The standard deviations are very small, speaking for good reproducibilities at each concentration. In order to circumvent this problem henceforth all activity measurements were conducted at a concentration of 0.05 mg/ml.

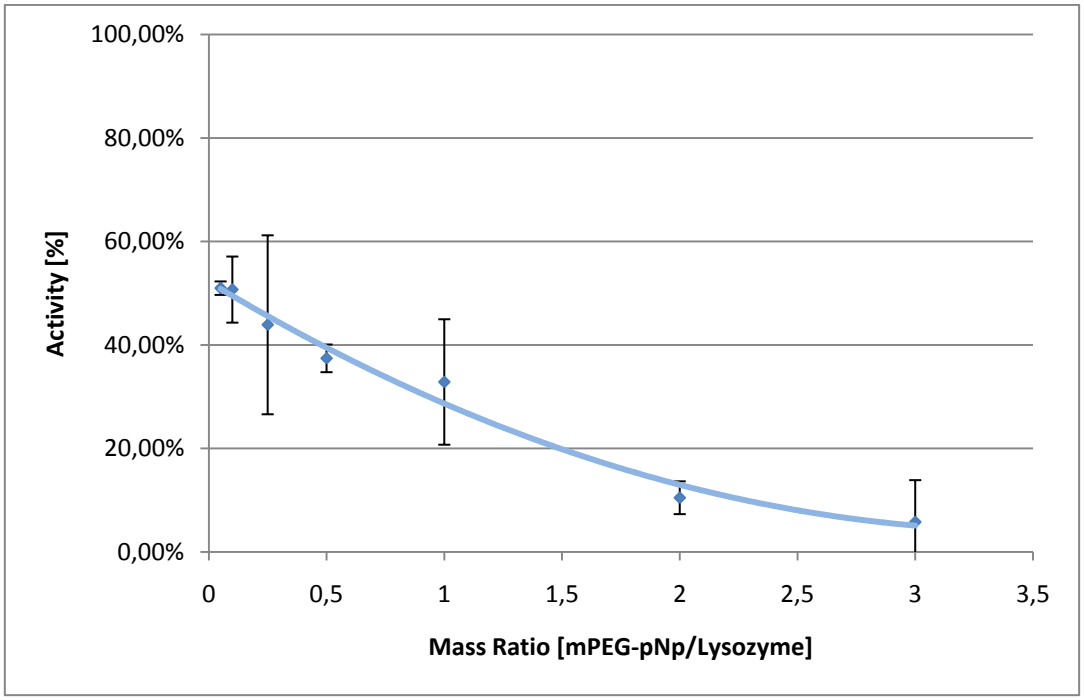

Figure 19: Remaining Enzymatic Activity at Different Polymer/Protein Mass Ratios – The residual activity after the PEGylation process was determined using the substrate *M. lysodeikticus*. The PEGylation was conducted at mPEG-pNp/lysozyme mass ratios of 0.05 to 3.

Figure 19 shows the relative activities of the different mass ratio products after the PEGylation process. As already known from Figure 17, the more polymer is present during the reaction the higher the degree of modification. Also, larger quantities of highly modified conjugates are produced with an increasing polymer/protein ratio. The activities are displayed relative to the activity of an unmodified lysozyme, which was treated exactly like the PEGylated lysozymes. Decreasing activities are observable as the degree of lysozyme PEGylation is increasing. Since no attachment sites for PEG are located in the active site of lysozyme the functional conformation of lysozyme´s active site is not

expected to be influenced. The attached mPEG-pNp on the reactive sites of the lysozyme cause steric hindrance and thereby prevent the enzyme-substrate complex formation. This is a general problem for proteins acting on macromolecular substrates [155, 156, 23, 129].

When applying a low mass ratio of 0.05, 51 % of the original activity is retained whereas a high mass ratio of 3 retained only 6 %. Figure 17 shows a single modification at the lowest mass ratio. Thus, it can be said that an attachment of one mPEG-pNp to lysozyme reduces its activity to about 50 %. High activity losses are well known for PEGylated proteins [39, 46]. But there have been studies leading to conjugates which obtain about 75 % of the original activity [129, 142]. Nevetheless, the PEGylation of proteins can still have greatly improved therapeutic effects despite their enormous loss of activity [39].

Da Silva Freitas and Abrahao-Neto (2010) also tested their conjugates with the much smaller substrate glycol chitosan. Here, the activity was not influenced by the PEGylation of lysozyme. Thus, the active site of lysozyme was not affected by the PEGylation process.

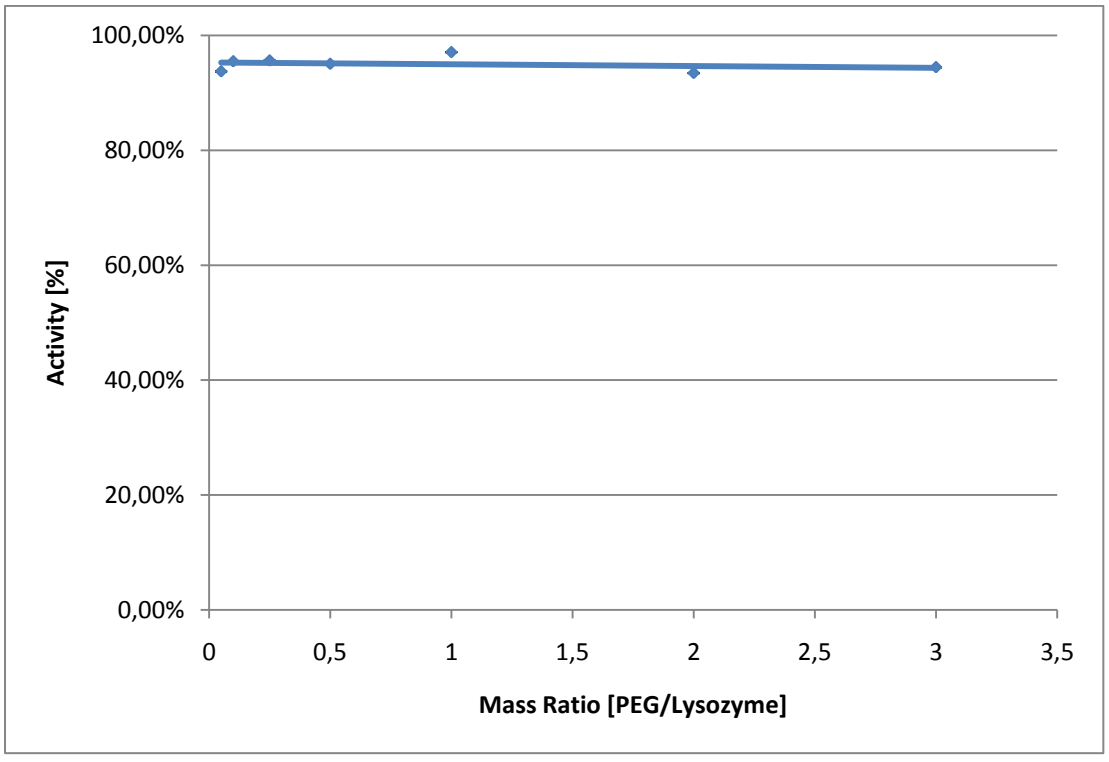

Figure 20: Remaining Enzymatic Activity at Different Polymer/Protein Mass Ratios – The acitivity of lysozyme was determined in the presence of increasing the ratio of PEG to lysozyme in the reaction mixture after incubating for 2 hours at 35 °C and 500 rpm. *M. lysodeikticus* was used as a substrate.

To verify that the activity loss, when working with the substrate *M. lysodeikticus*, is actually due to the steric hindrance of the PEGylated lysozyme conjugates another experiment was conducted. The mass ratio experiment was also executed with a non-activated PEG. This PEG will not bind to the protein, but might have an effect due to the presence of the long polymer chains in the reaction mixture. Figure 20 shows the results of this experiment. The retained activities are independent from the mass ratios and lie between 94-100 % of the original activity. Hence, the activity loss when working with a macromolecular substrate is due to the PEGylated conjugates and not observable when lysozyme is just blended with a non-activated PEG.

4.2 Purification and Separation of mPEG-Modified Lysozyme

After the analysis of the PEGylation reaction the process parameters for further experiments needed to be chosen. Since the separation process and the properties of the different kinds of conjugates should be investigated, process parameters were selected which result in various modification degrees. All following PEGylation processes were conducted with a polymer/protein mass ratio of 1 in a 150 mM phosphate buffer pH 8 for 4 hours.

Concentration

The separation and purification were carried out by SEC. In order to keep the sample volume small the PEGylated lysozyme mixture was concentrated. First, an ion exchange chromatography (IEC) was tested. Unfortunately, the mixture was not able to bind onto the column under the present conditions. Most likely, the phosphate concentration of 150 mM was already too high to allow a bond to the column material. Therefore, the IEC is not a suitable method for the concentration of the PEGylated lysozyme mixture at this phosphate concentration. Second, an ultrafiltration was performed in a stirred cell. The mixture was pushed through a membrane by a pressure of 3 bar. Due to the MWCO of 10 kDa the PEGylated conjugates as well as the unreacted lysozyme and the remaining polymer were held back in the cell and picked up in a small buffer volume.

Purification and Separation

The purification and separation are two of the main challenges in the production of PEGylated proteins. The unreacted protein and the remaining polymer need to be removed. Furthermore, the resulted conjugates shall be separated. The physicochemical properties of the different conjugates formed by the PEGylation reaction are very similar. Thus, the separation process is difficult to design. Chromatographic methods are commonly used for the separation and purification of PEGylated proteins. They depend on interactions between the sample and the stationary phase which are influenced by the properties of the sample molecule such as charge or hydrophobicity. These properties may be changed by the PEGylation process because of masking and shielding effects [159]. In consequence, chromatographic methods like SEC, IEC and RPC (reversed phase chromatography) show altered behavior on PEGylated proteins compared to unmodified proteins [160, 161].

For separation and purification in this thesis the commonly used SEC was performed. The concentrated sample was injected onto different kinds of columns (Sepharose 4B (10/33), Superdex 75 (26/60), Sephacryl S-200 (16/60)). Despite different column materials with varying separation ranges a complete segregation of the different kinds of conjugates was not possible. However, the purification of the conjugate mixture from the unreacted protein and polymer was successful using a Sephacryl S-200 column. Using the Sepharose 4B column the mPEG-lysozyme conjugates could not be purified from the unreacted lysozyme. No unreacted lysozyme could be detected using the Superdex 75 column, which is very unlikely since a complete reaction of all lysozyme molecules never happened. Thus, the Sephacryl S-200 column was chosen. Nevertheless, despite the mentioned improbable result, the Superdex 75 column showed the best separation of the different degrees of modification (data shown in annex). Thus, further studies using this column would also be very interesting.

Figure 21 shows the chromatogram of a SEC using a Sephacryl S-200 16/60 column. The chromatogram displays 3 peaks: The first one after about 70 min, the second one after about 180 min, and the third one after about 480 min. All three peaks were analyzed by SDS-PAGE. The first peak contains all mPEG-pNp-lysozyme conjugates (Figure 22), the second peak contains the unreacted lysozyme, and the third peak indicates the unreacted polymer (data not shown). Although the first peak contains all four degrees of modification, the SDS-PAGE of the single fractions below the peak shows an elution according to the PEGylation degree (Figure 22). The highest degrees of modification and thereby the biggest molecules are eluted before the lower degrees of modification.

In conclusion, the purification from unreacted protein and polymer was successful, but a complete separation of the different degrees of modification could not be achieved. However, fractions containing predominantly one degree of modification were produced. In order to analyze the impact of the different degrees of modification the first peak was divided into two different fraction pools. The red line in Figure 22 indicates the dividing line of two fraction pools. Henceforth, the fractions located at the left side of the red line are referred to as “Conjugate 1” and the fractions on the right side as “Conjugate 2”. Hence, conjugate 1 represents the higher degrees of PEGylation and conjugate 2 the lower degrees of PEGylation.

Moosmann *et al.* performed a SEC with a G3000SW$_{XL}$ column on PEGlated lysozyme [159]. Polymers of different sizes (5 kDa, 10 kDa, 30 kDa) were used in the prior PEGylation process. The modified lysozyme conjugates led to different elution profiles. The higher the polymer size and the higher the degree of modification the earlier the elution was observed. Comparable to this thesis, a purification from unreacted protein and remaining polymer was successful, but a complete separation of the different degrees of modification could not be achieved. Moosmann *et al.* showed that a mono-PEGylated lysozyme using a 5kDa polymer acts equivalent to a 50 kDa globular protein during the SEC procedure. A mono-PEGylated lysozyme using a 30 kDa polymer even acts as a 450 kDa globular protein [159]. This confirms the influence of PEG on the hydrodynamic volume of a protein one more time [24]. Kurfürst described a linear correlation between the molecular weight indicated by the gel and the theoretical molecular weight [146]. Correspondingly, a linear correlation could also be found between the observed and the theoretical SEC behavior [162].

A successful purification and separation of the different degrees of PEGylated lysozyme was achieved by Moosmann *et al.* using a cation exchange chromatography [159]. TSKgel SP-5PW was used when the prior PEGylation reaction was conducted with a 5 kDa polymer. The sample was applied at a low salt concentration of 25 mM and the elution was conducted by adding 1 M NaCl to the original running buffer. As a result, the higher the degree of PEGylation the earlier the conjugates were eluted. This earlier elution of the highly PEGylated proteins is the result of the masking effect of PEG [163,164]. Thus, the interaction of mPEG-lysozyme to the column material is not as strong in comparison to unmodified protein. Pabst found that the salt concentration decreases with an increasing PEG chain length [160]. The ion exchange chromatography conducted in this thesis in order to concentrate the PEGylated lysozyme mixture was unsuccessful. The strong influence of the ionic strength was underestimated.

Promising results for the separation and purification could also be achieved by an ultrafiltration membrane technology. Here, composite regenerated cellulose membranes with varying MWCO were used one after another, starting with the highest MWCO [165].

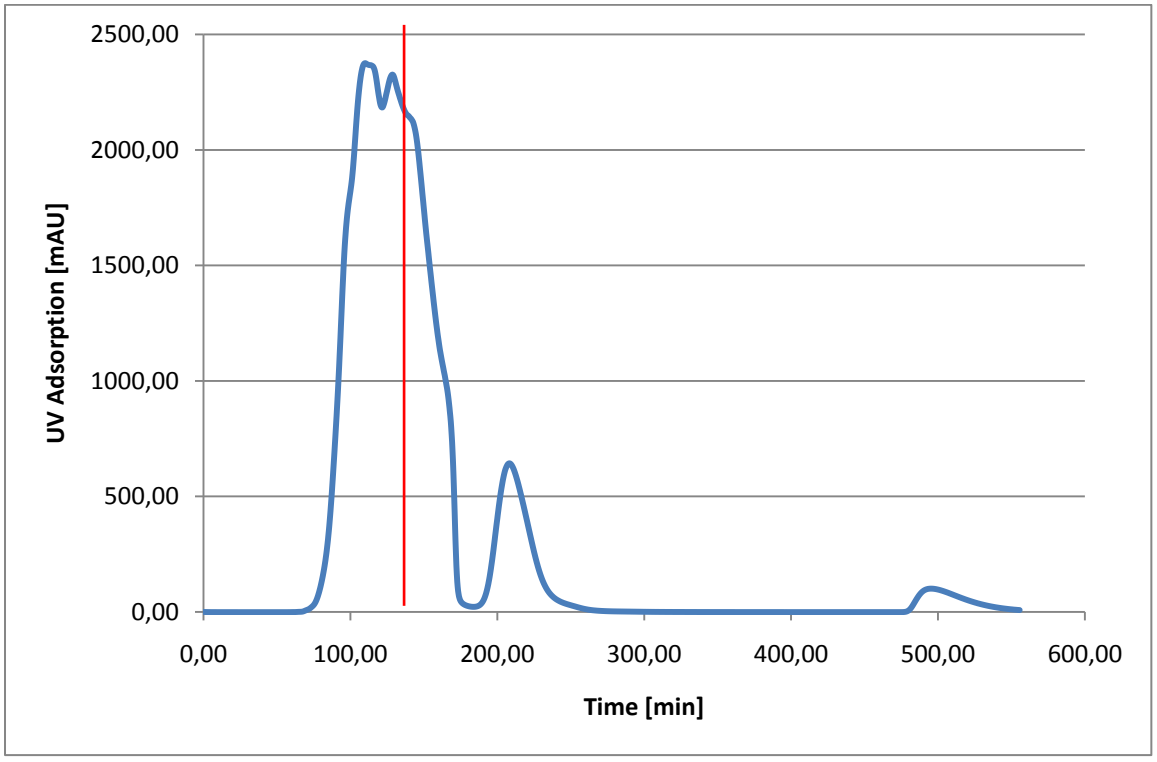

Figure 21: SEC Chromatogram of a PEGylated Lysozyme Mixture – A Sephacryl S-200 column was used in this chromatography. The blue line represents the UV adosprtion at 280 nm [mAU] and every red line indicates one 2 ml-fraction.

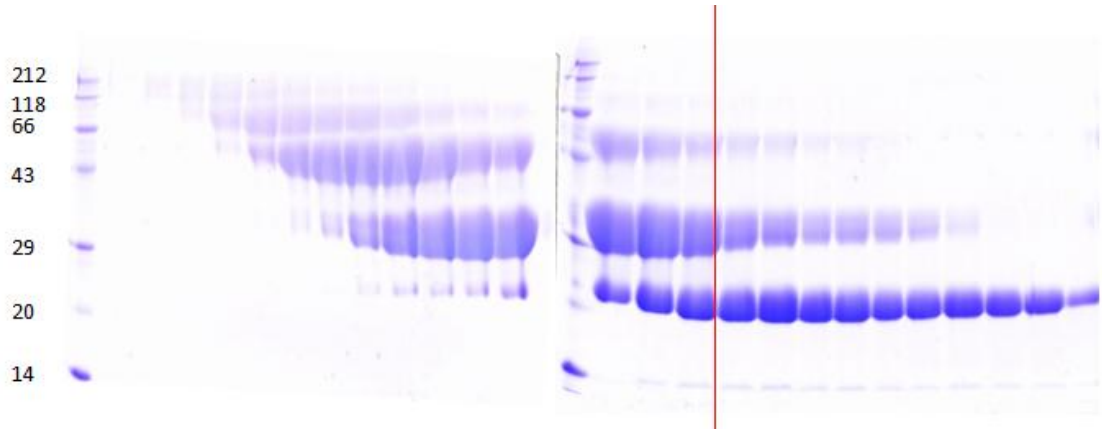

Figure 22: SDS PAGE Analysis of Peak 1 in Figure 21 – The single fractions located under the first peak of Figure 21 were applied in elution order.

Activity of Purified and Partially Separated Conjugates

The PEGylated conjugates 1 and 2, the unreacted lysozyme, and native lysozyme were tested for their biological activity at different pH values. Figure 23 shows that the optimal activity of native lysozyme at pH 7 decreases with the shift of the pH in the acidic or basic direction. The unreacted lysozyme of the PEGylation reaction shows a very similar course to the native lysozyme at an overall lower activity level. Concluding, the PEGylation, as mentioned above, and the following separation/purification procedure have a negative effect on the biological activity of the PEGylated and unreacted proteins. This effect is also described in other publications [39, 46].

Both conjugates indicate high activities from pH 4 to 6. PH values in the neutral or basic range show decreased activities for conjugate 2. Conjugate 1 clearly shows the lowest overall activity level, but also the least variation. The activity of conjugate 1 drops noticeably only at neutral pH values, whereas acidic and basic values show similar activities. Hence, it is likely that the activity decreases with an increasing degree of modification, but higher degrees of modifications reduce the affectability to pH changes. As already described, the active site of lysozyme has no attachment sites for PEG [140]. Thus, the active site has not been altered due to the PEGylation reaction. The increased steric hindrance due to increased molecule sizes, as mentioned above, causes activity losses when using an assay working with a macromolecular substrate [155, 156, 23, 129]. This explains the overall lower activity levels of highly modified lysozyme, but the shift of the activity optimum cannot be explained by steric reasons. Maybe, this shift could be due to the change in protein charge by PEGylation. Charges which exist in native lysozyme could be reduced by the modification with PEG and thereby altering the pI.

The results described in this thesis are very different to the ones reported by da Silva Freitas and Abrahao-Neto [142]. They measured the activity of a purified mono-PEGylated lysozyme conjugate in comparison to native lysozyme. The course of the native lysozyme in this thesis was comparable to the one of da Silva Freitas and Abrahao-Neto. But the mono-PEGylated conjugate remained active in a much broader pH range than conjugate 2, although conjugate 2 is also predominantly composed of mono-PEGylated lysozyme.

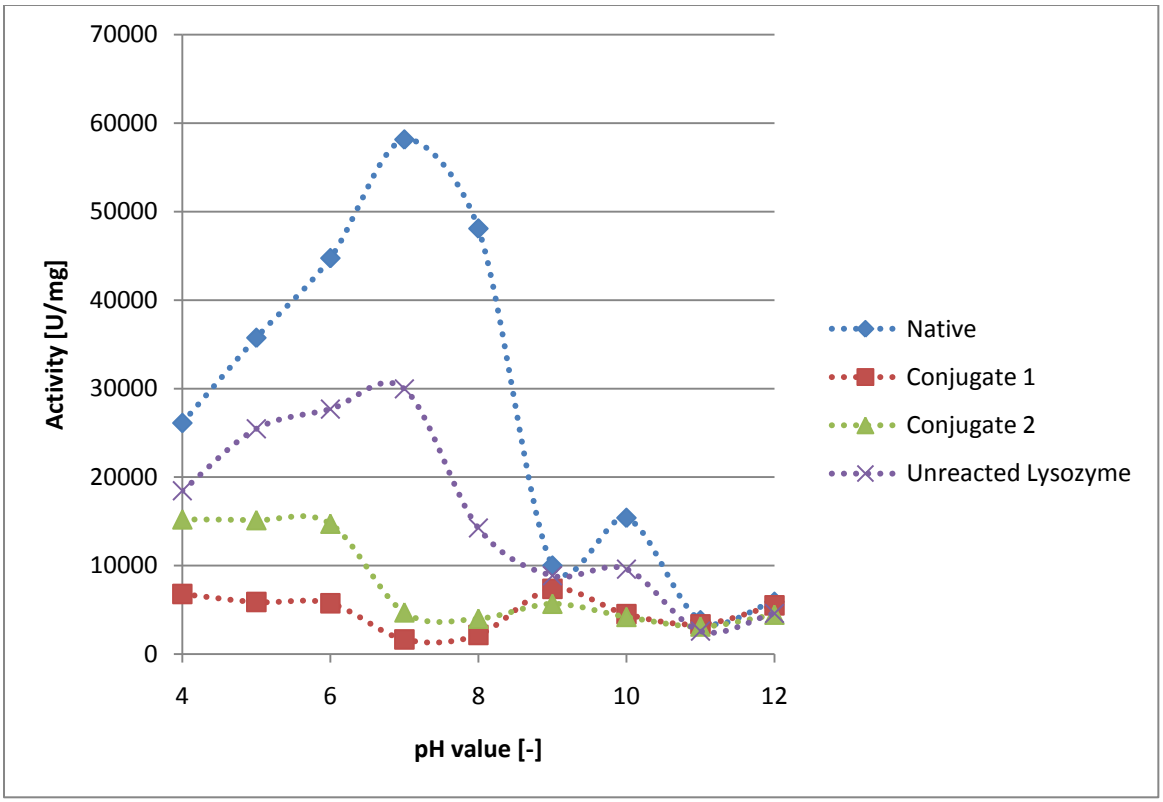

Figure 23: Activity Measurement of Modified and Unmodified Lysozyme at Different pH Values – The blue marks represent the activity of native lysozyme; conjugate 1 is shown in red; green indicates conjugate 2 and the unreacted lysozyme has purple marks.

The PEGylated conjugates were concentrated and dialyzed by ultrafiltration. The conjugates were taken up in 2 ml 5 mM KH_2PO_4 buffer and afterwards they were lyophilized. To monitor possible product losses during this procedure, the concentrations before and after the ultrafiltration were measured. The results, presented in Table 8, indicate no significant product losses.

Table 8: Protein Concentrations of Conjugates 1 and 2 Before and After Ultrafiltration

Before ultrafiltration:		
Conjugate	**Concentration [mg/ml]**	**Total Mass [mg]**
1	2.254	61.25
2	2.035	35.42
After ultrafiltration:		
Conjugate	**Concentration [mg/ml]**	**Total Mass [mg]**
1	30.5	61
2	17.36	34.72

Stability of PEGylated Conjugates

Figure 24 describes the stability of the conjugates 1 and 2. The experiment was conducted at 50 °C and the samples endured this temperature as well as different pH values for one hour. The highest activity of native lysozyme was measured at pH 8; at pH 5 almost no activity can be measured and at pH 11 no enzymatic activity remains. Both conjugates also show no remaining activity at pH 11. Thus, the PEGylation of lysozyme does not increase the stability at highly basic pH values. But they do increase the stability in the acidic range; at pH 5 the activities are greatly improved in both conjugates. At neutral pH conjugate 1 shows a much lower activity than at pH 5, but conjugate 2 shows an even higher activity than the native lysozyme. These tendencies partially confirm the outcomes of the previous experiment, although the absolute activities differ a lot. The only exception is the high activity of conjugate 2 at pH 7. This might be an outlier since the results were not confirmed in multiple experiments. The stability improvement of PEGylated proteins in the acidic range, which allows exposures at lower pH, could be due to increased hydrophobic interactions. When comparing the activity of native lysozyme at pH 8 in this experiment to the activity measurement before, no activity losses can be observed. Thus, it seems that the treatment at 50 °C in this experiment does not affect the activity. In contradiction, the activities of mPEGylated lysozyme differ significantly. The mPEGylated lysozyme conjugates show a much higher activity when incubated for one hour at 50 °C. These inconsistent results might be due to slight changes in concentration resulting from the dilution procedure. As described above, the concentration has a great impact on the outcomes of the activity assay. Further experiments will be necessary in order to classify these results.

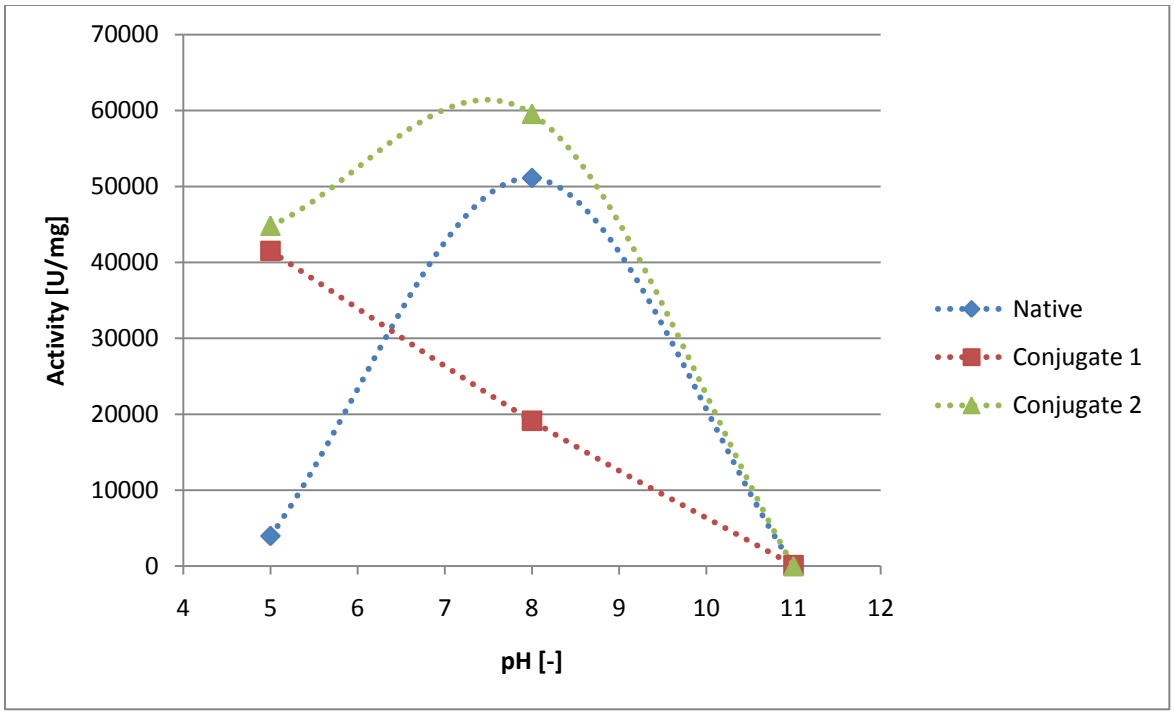

Figure 24: Stability Determination of Modified and Unmodified Lysozyme – The conjugates 1, 2, and native lysozyme were incubated at 50 °C for 1 h while the buffer conditions differed in pH (5, 8, 11). The determined enzymatic activity is shown in blue for native, in red for conjugate 1, and in green for conjugate 2.

Resistance to Proteolytic Degradation

Native lysozyme is known for its resistance to proteolytic degradation [157,158]. In order to analyze if a PEGylation of the already resistant lysozyme can be further improved, proteinase K was added to native lysozyme as well as the two conjugates. Samples were taken at different incubation times and the enzymatic activity was measured immediately. Resulting from the proteolytic degradation the biological activities of the native lysozyme and the two conjugates decrease (Figure 25). Variations in resistance referring to the PEGylation of the protein can be observed. The activity of native lysozyme after 2 hours of proteinase K exposure decreased 31 % while the activity of conjugate 2 decreased 26 % and the activity of conjugate 1 decreased only 20 %. Thus, it seems that a higher degree of PEGylation correlates with a higher resistance to proteolysis. This statement is reaffirmed by the observed masking and shielding effects of PEGylated proteins [159]. MonoPEGylated lysozyme was already tested regarding its resistance to proteolytic degradation and showed an improved resistance [142]. The monoPEGylated lysozyme, examined in the work of da Silva Freitas and Abrahao-Neto, showed a much stronger resistance of approximately 30 %, related to native lysozyme, compared to the results of this thesis. This difference might be due to the different

proteases which were used. While in this thesis proteinase k was used, da Silva Freitas and Abrahao tested the resistance to Proteomix, consisting of trypsin and chemotrypsin, Protex 6L, which is a bacterial protease, and a fungal protease.

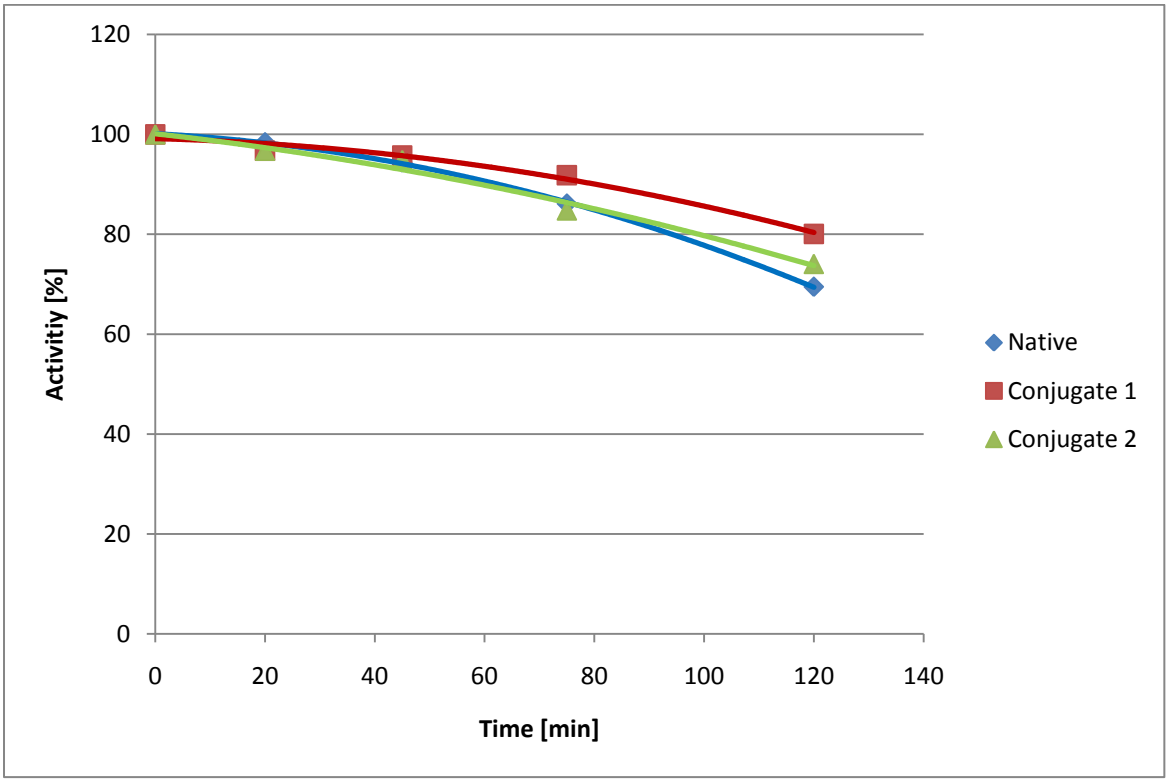

Figure 25: Resistance to Proteinase K at Different Incubation Times – Conjugates 1, 2, and native lysozyme were incubated with an excessive amount of proteinase K. The enzymatic activity was measured at increasing reaction time.

4.3 Microencapsulation

Proteins are usually administered by injections, because of their poor oral bioavailabilities [166, 167]. Drug delivery systems, such as microspheres, provide the potential of decreasing the injection frequencies and dosages. Therefore, the patient´s comfort as well as the therapeutic effect can be improved [168]. After the lyophilization the mPEG-pNp-lysozyme conjugates as well as the native lysozyme were encapsulated by solvent evaporation and spray drying. While solvent evaporation is known for its high encapsulation efficiency and yield [71, 72, 73], the benefits of spray drying are its rapid performance and its suitability for scale-up [80, 82].

Yield

Preparation processes need to be kept at a low cost level, which is the reason for the high importance of the product yield. The yield of every encapsulation process was determined by the measurement of the initial mass and the available product mass after the encapsulation procedure.

Table 9 displays the mass yields of the different encapsulation processes. While the solvent evaporation method achieves an average mass yield of approximately 59 %, the encapsulation by spray drying only shows an average yield of about 39 %. Most of the lost product in the spray drying process was found attached to the inner glass walls of the cyclone. This effect could be due to semi wet or sticky particles, caused by the product´s high affinity to the glass walls and polymer properties such as the glass transition temperature [169]. Spray drying is known for its high product losses [80]. The values achieved in this thesis are very well comparable to the results of other studies: 46 % [169], 37-49 % [170], 40 % [171], 30-40 % [172]. Comparing the yields of solvent evaporation in this thesis to the literature, the achieved yields of about 59 % were lower: 72 % [173], 68-82 % [174]. The product losses using solvent evaporation might be due to the harvest method. In this thesis, the dry particles stuck to the filtration membrane and were gently scraped off by a plastic spatula. During this procedure some of the product might have gone lost.

When examining the preparation methods, solvent evaporation on the one hand shows significantly higher yields of about 20 % in comparison to spray drying. On the other hand, spray drying provides many advantages such as a simple, rapid process

which is easy to scale-up and allows mild temperature conditions. Furthermore, it is less dependent on drug and polymer properties such as solubility [169].

Motlekar and Youan encapsulated low molecular weight heparin in Eudragit® S-100 by spray drying [175]. Eudragit® S-100 is a polymer which enables pH-dependent drug release [176]. The influences of different spray drying parameters were analyzed by design of experiments. According to their results, the yield is majorly dependent on the inlet temperature and the polymer concentration. They were able to reach yields of approximately 60 % by using high polymer concentrations (9 %) and high inlet temperatures (100 °C). An increase of the inlet temperature needs to be well considered, since higher inlet temperatures are known to cause altered morphologies. While microspheres produced at an inlet temperature of 55 °C lead to smooth microsphere surfaces, higher temperatures lead to shriveled microspheres with small craters and collapses [169]. An increase of the polymer concentration could be examined in further experiments.

Another method in order to increase the yield was described by Takada *et al.*. They supposed a double-nozzle spray-drying technique using mannitol as an anti-adherent. The PLGA/drug dispersion was sprayed through one nozzle while simultaneously a mannitol solution was sprayed through another. Thereby, the PLGA microspheres were coated with mannitol which resulted in higher yields and, in addition, decreased agglomeration [80]. Perhaps, the yields in spray drying processes could also be improved by the application of higher amounts of particles. If positively charged lysozyme would cover the inner glass walls of the spray drying instrument, following particles passing these walls would be repelled. Maybe the instrument could even be coated with lysozyme before the actual encapsulation process.

Table 9: Mass Yield Determination of the Preparation Methods Spray Drying and Solvent Evaporation

Sample	Preparation Method	Mass Yield [%]	Mean [%]
Native lysozyme	Solvent evaporation	66,13	58,50
Conjugate 1	Solvent evaporation	46,59	
Conjugate 2	Solvent evaporation	62,77	
Native lysozyme	Spray drying	40,12	39,20
Conjugate 1	Spray drying	35,20	
Conjugate 2	Spray drying	42,27	

Encapsulation Efficiency

The encapsulation efficiencies of the two different preparation methods were determined. First, the microspheres were incubated in DMSO and then another incubation with 0.05 M NaOH, containing 0.5 % SDS, followed before the protein concentration was measured. High encapsulation efficiencies up to 100 % are reported for both encapsulation methods [71, 72, 73, 83, 170, 172]. The results of the loading efficiency experiment are summarized in Table 10. The efficiencies are all above 100% which indicates a problem with the measurement method. The protein concentrations were measured using a BCA assay. Yang and Cleland also described problems measuring the protein release concentrations from PLGA microspheres with a BCA assay [177]. Nevertheless, they observed lower concentrations than expected. Maybe, the lactic acid which is formed during the degradation of the polymer or the hereby induced pH shift in the acidic direction interferes with the assay and leads to false results. If the lactic acid would assist the reduction of Cu^{2+} to Cu^{1+}, as it is known for reducing sugars, artifactually high concentrations would be measured [178]. However, the lactic acid should also interfere in Yang and Cleland´s work, who observed lower concentration than expected. Thus, the mentioned assisting effect is unlikely. A possible reason for the too high concentrations measured in this thesis could be the very low protein amounts and the high dilution during the experiment. Therefore, inaccuracies in weighing and dilution procedures might occur. But in order to investigate this problem, further studies need to be performed. Another protein concentration assay, e.g. a Bradford assay, should be conducted parallel to the BCA assay. Moreover, a BCA assay only containing PEG or lactic acid should be performed to rule out any interferences due to these substances. Despite the problems described, the BCA assay is used in many studies which examine the protein release from PLGA microspheres [179, 180]. In general, lower ratios of drug to polymer have been described to result in higher encapsulation efficiencies [181].

Table 10: Encapsulation Efficiency of the Preparation Methods Spray Drying and Solvent Evaporation

Sample	Preparation Method	Efficiency [%]	Mean [%]
Native lysozyme	Solvent evaporation	136,18	117,98
Conjugate 1	Solvent evaporation	204,28	
Conjugate 2	Solvent evaporation	113,49	
Native lysozyme	Spray drying	215,63	170,23
Conjugate 1	Spray drying	113,49	
Conjugate 2	Spray drying	181,58	

Release Kinetics

Often, protein drugs are limited in their biomedical applications due to their short half-life in the organism [8]. Drug delivery systems, like microspheres, encapsulate the drug in order to protect it e.g. from rapid degradation. The experimental observation of the *in vitro* release profiles are studied to find the optimal drug formulation for a controlled drug release over the desired period of time.

Figure 26 shows the release profile of the native lysozyme and the PEGylated lysozyme conjugates. No drug release from the microspheres prepared by solvent evaporation was detectable after a first low amount of protein released already after one hour. Since the encapsulation efficiency experiment did not provide trustworthy results the initial amount of protein present in the microspheres is unknown. The microspheres produced by spray drying showed a release of a major amount of lysozyme during the first hour. Thus, an initial burst could not be prevented. The following release appears to be rather constant. The encapsulated conjugates could not be detected any more after two days. The longest release was detected by the encapsulated native lysozyme. But as described above the BCA assay used to determine the protein concentration might have led to inaccurate results.

Several authors have observed an incomplete release from the microspheres caused by protein adsorption to PLGA [15,182, 183]. Especially positively charged proteins at neutral pH, such as lysozyme, are affected by these interactions [1,184]. However, it is not possible to determine whether protein adsorption is a problem in the present investigation, because the initial protein concentrations are uncertain. Thereby, the released protein concentrations, relative to the initial protein concentration, are not known either.

Some studies already achieved a quasi zero-order release profile, e.g. for growth factors over four weeks using a 10 % w/w PLGA-PEG-PLGA blockpolymer with 50:50 PLGA, which is also used in this thesis [185]. The release profile can be altered by selection of the used copolymer [83]. A complete and sustained release was observed over ten days using a 30 % w/w PLGA-PEG-PLGA blockpolymer with 85:15 PLGA and over four days using 30 % w/w PLGA-PEG-PLGA with 50:50 PLGA [185]. Furthermore, PEGylated insulin encapsulated in PLGA showed a very low initial release over one day and a near zero-order release profile after a lag of 3-4 days.

Different spray drying parameters have been found to affect the drug release from the microspheres formed during the process. High inlet temperatures lead to dense polymer matrixes and thereby result in low drug release rates. Low release rates are also caused

by low polymer concentrations which lead to a reduced porosity and high air flows which lead to small particle sizes [169]. In contradiction to this suggestion other authors describe higher release rates resulting from small particle sizes. [186, 187, 188]. Since the smaller particles have an increased surface, a higher release rate at smaller particles is more likely.

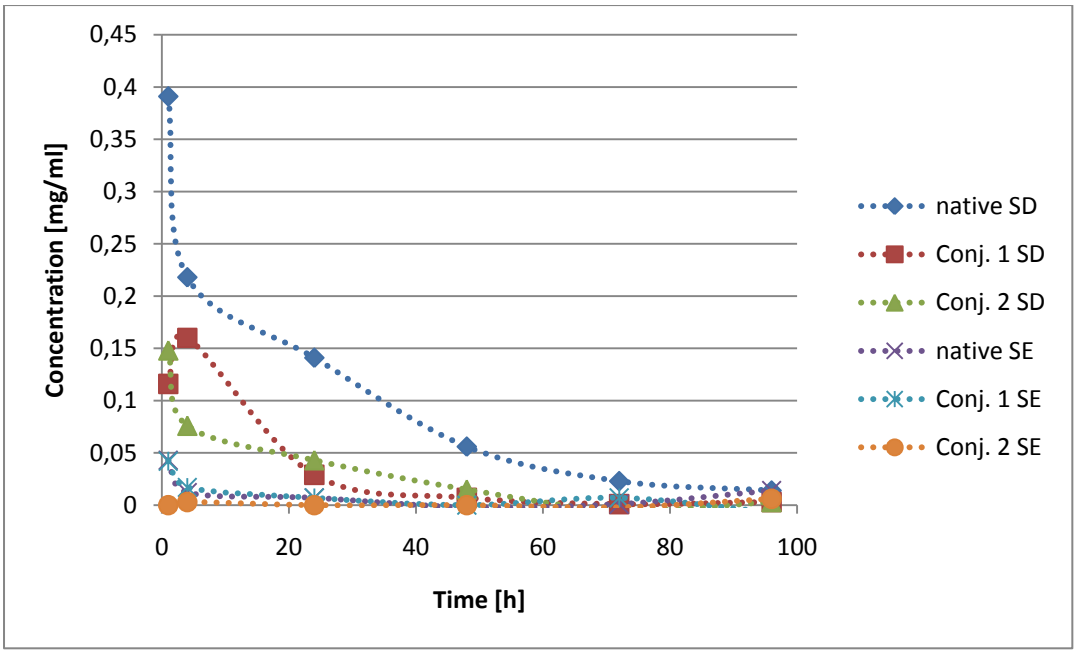

Figure 26: Release Kinetics of Encapsulated Modified and Unmodified Lysozyme – The protein release from the different kinds of microspheres was determined at different timepoints. SD stands for microspheres prepared by spray drying and SE stands for microspheres prepared by solvent evaporation.

Particle Size

Typically, particle sizes in the range of 20-100 µm are desired for injectable microspheres as drug-delivery depots [82]. In pulmonary applications, e.g. inhalation therapies, particle sizes have to be smaller than 5.8 µm in aerodynamic diameter [189]. Therefore, particle sizes are an important factor to consider in microsphere preparation.

Figure 27 describes the particle size deviations and Table 11 shows the mean and the mode of the produced types of microspheres. The microspheres prepared by spray drying indicate different deviations when comparing the encapsulated native lysozyme and the encapsulated conjugates. The conjugates show a very similar deviation range of about 1 to 100 µm with means of 21 µm and 25 µm and modes of 14 µm and 16 µm. In comparison, e.g. the encapsulation of PEGylated insulin in PLGA resulted in

microspheres with a diameter of 65 µm with a deviation range of 35-90 µm [190]. Native lysozyme on the contrary shows a much broader deviation and a much greater mean and mode of 128 µm. But, especially the encapsulated native lysozyme showed a strong tendency to agglomerate. As a result, these big agglomerates are measured by the instrument as one microsphere which causes the broad deviation and the higher values of the mean and mode. This corresponds with the described interactions between PLGA and lysozyme above [1,184]. Maybe, lysozyme builds bridges which support the agglomeration of the microspheres. Since the conjugates showed less agglomeration, it can be suggested that the modification of lysozyme by mPEG reduces these interactions. However, this does not match the results of the microspheres produced by solvent evaporation.

The drawback of agglomeration was successfully reduced by a mannitol coating of the microspheres produced by a method which uses two nozzles as described before [80]. Generally, when microspheres are produced by spray drying higher pump rates reduce the mean droplet size and increased air flows reduce the particle size. For example, the reduction of the particle size from 8 µm to 5 µm was reported due to an increased air flow of 400-800 l/h [169]. Decreased particle sizes often result in higher release rates [188, 189, 190]. Since a constant pump rate of 15 % and an air flow of 500 l/h were obtained in this experiment, these statements cannot be evaluated. Further studies with varying process parameters will be necessary. Maybe an increase of the air flow and a higher pump rate would also result in smaller microspheres and thereby increased release rates, corresponding to results mentioned above.

The particle sizes resulting from the encapsulation by solvent evaporation generally are larger than the ones produced by spray drying. Furthermore, the particle size deviation is extremely broad. Especially conjugate 2 shows predominantly very large particles with a mean of 400 µm and a mode of 623 µm. The large deviations might be due to an irregular droplet size created during the preparation. In contradiction to the automatic droplet formation in spray drying processes, the droplets in solvent evaporation processes are produced manually by a pipette.

Schubert and Geppert also determined the particle size of PLGA microspheres produced by solvent evaporation and spray drying (Figures 28 and 29). In contradiction to this thesis, their results show very similar deviations between 1-100 µm in both preparation methods [191]. These deviation curves are comparable to the ones conducted with the conjugates encapsulated by spray drying in this thesis.

In conclusion, the produced microspheres in this thesis show broad deviations especially using solvent evaporation. The microspheres produced by spray drying appear to hold potential as injectable microspheres, because of their suitable size. But the problem of microsphere agglomeration is severe. The agglomeration tendency of the microspheres was already observed and supposed to be due to the adsorption force of the small particles, being a sign of a high surface energy [169]. Further studies are necessary in order to investigate the effect of process parameters such as the air flow rate and the pump rate, and to examine the problem of agglomeration. Maybe a mannitol coating could be produced in two steps: First the microsphere production and in a second step the coating with mannitol in a separate spray drying process. This would not improve the yield losses occurring during the microencapsulation, but it could help to reduce the agglomeration during the particle size measurement.

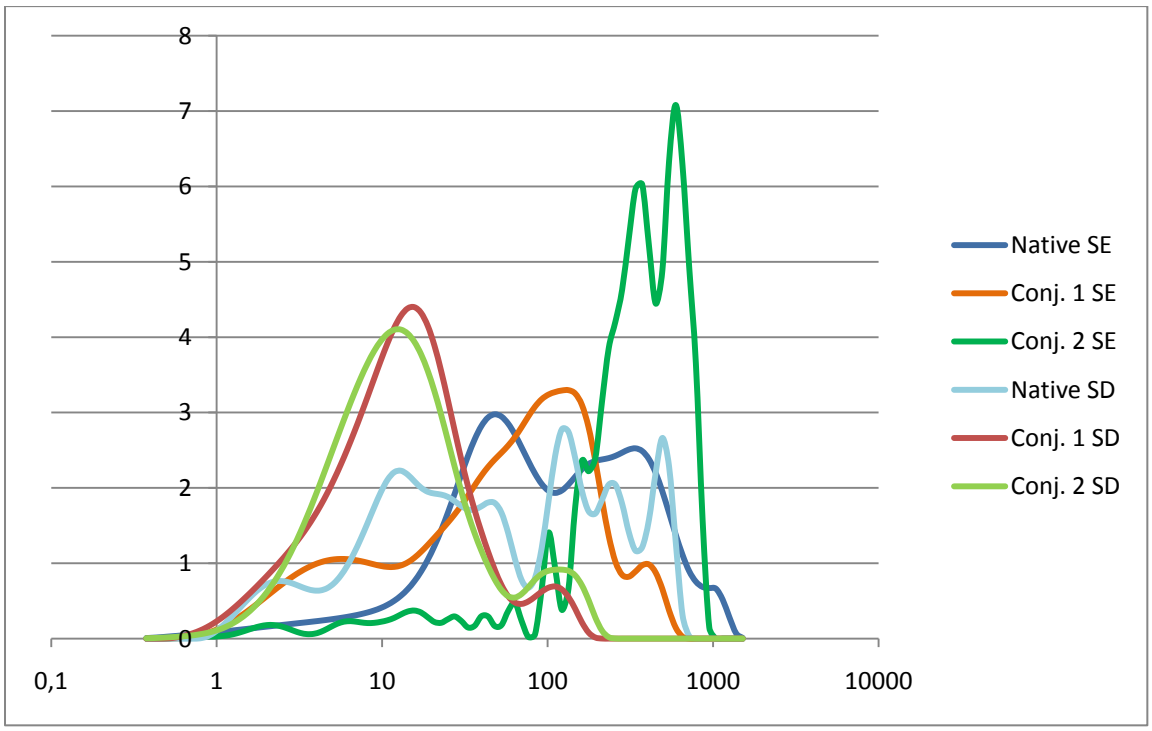

Figure 27: Particle Size Deviation of the Different Kinds of Microspheres - SD stands for microspheres prepared by spray drying and SE stands for microspheres prepared by solvent evaporation.

Table 11: Mean and Mode of the Microsphere Particle Sizes Encapsulated by Solvent Evaporation and Spray Drying

Sample	Preparation Method	Mean [µm]	Mode [µm]
Native lysozyme	Solvent evaporation	200	50
Conjugate 1	Solvent evaporation	97	140
Conjugate 2	Solvent evaporation	400	623
Native lysozyme	Spray drying	128	128
Conjugate 1	Spray drying	21	16
Conjugate 2	Spray drying	25	14

1

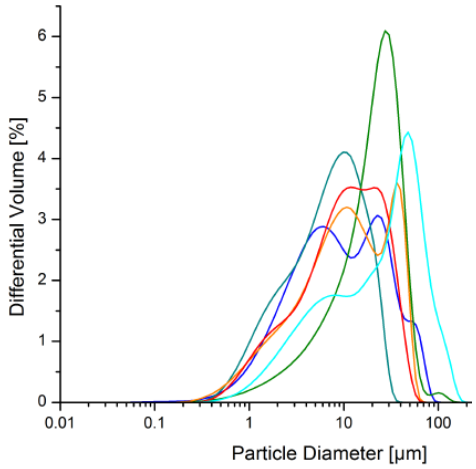

Figure 28: Particle Size Deviations of Microspheres Prepared by Solvent Evaporation[1]- Different stirrers were used in this experiment. Red and orange indicate microspheres prepared by a magnetic stirrer, blue and light blue were prepared using an ultra Turrax, and the greenish lines were prepared by a turbine stirrer.

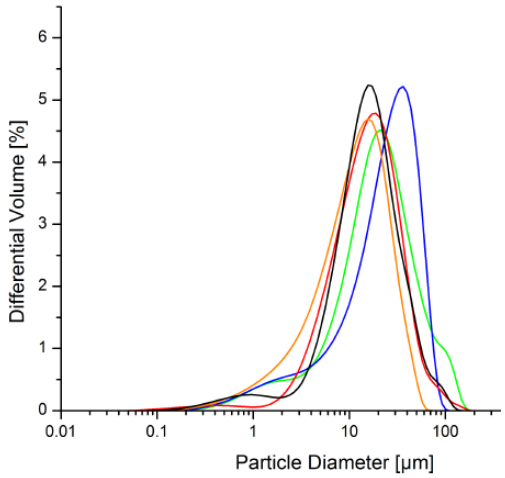

Figure 29: Particle Size Deviations of Microspheres Prepared by Spray Drying[1]- Different PLGA/protein ratios were used in this experiment. Red indicates 0.1 % PLGA/protein, orange 0.5 % PLGA/protein, green 1 % PLGA/protein (25% protein load), blue 2 % PLGA/protein and black 1 % PLGA/protein (10% protein load)

Morphology of Microspheres

In order to depict the morphology of the microspheres, pictures were taken using a phase contrast microscope. The broad particle size deviation observed before and the tendency to build agglomerates is confirmed by the impression during the examination of the morphology.

[1] With kind permission of Sebastian Schubert and Benedikt Geppert

In general, PLGA microspheres appear spherical with a smooth surface (Figure 34) [169]. The microspheres pictured in this thesis also look spherical, but with a rough surface displaying small craters (Figures 30,31, and 32). Arpagaus and Schafroth analyzed the microspheres by SEM (scanning electron microscopy) which enables a much higher resolution compared to the pictures possible with a phase contrast microscope. Furthermore, a phase contrast microscope is limited for the examination of solid particles because of possible side effects at curved surfaces. In addition, it has no depth of focus. SEM on the contrary even allows a sight into the pores of microspheres up to a certain depth.

Having a closer look at Figure 30, the native lysozyme encapsulated by spray drying shows similar microsphere morphologies to the ones encapsulated by solvent evaporation. But the craters on the surface of the microspheres encapsulated by solvent evaporation appear slightly larger. Due to the small particle sizes of the shown microspheres this difference is not very striking. The microspheres containing conjugate 1 are presented in Figure 31. Varying particle sizes could be observed in both preparation methods. This reflects the deviation range of 1 to 100 µm determined in the previous experiment. Again, differences in the surface character were observed looking at the 1000fold enlargements. The microspheres prepared by solvent evaporation seem to have more and larger craters on their surface. The preparation temperatures during encapsulation were similar, but in the solvent evaporation method this temperature is applied for 4 h while the microspheres prepared by spray drying only need to endure this temperature for approximately one second. The polymer PLGA is known to develop craters with increasing temperatures [169]. This might be the reason for the altered microsphere surface, although the temperatures in this thesis were set below the critical temperature of 65 °C noted by Arpagaus and Schafroth. Figure 32 shows the microspheres encapsulating conjugate 2. Again, the surface of the microspheres prepared by spray drying seem smoother than the ones prepared by solvent evaporation. But here, another difference is observable. The microspheres prepared by solvent evaporation do not appear spherical in contrast to the ones prepared by spray drying.

To put it in a nutshell, in all types of microspheres variations in the morphologies of the microspheres could be observed. The craters on the surface appeared larger when prepared by solvent evaporation. The encapsulated conjugate 2 also showed irregular shapes using this method.

High molecular weights of PLGA are supposed to cause irregular and incompletely formed particles and big agglomerates [185]. In this thesis, agglomerates were observed despite the low polymer size of only 5 kDa. As already mentioned, a correlation between

the temperature and the microsphere morphology was observed in spray drying processes [169]. PLGA microspheres produced at an inlet temperature of 55 °C appeared smooth and spherical, whereas the surface of the microspheres which were produced at higher inlet temperatures appear shriveled with small craters and collapses. In this thesis small craters were observed at inlet temperatures of 45 °C. Thus, the results described above were not confirmed since the surface did not appear smooth even though the inlet temperature was set below 55 °C.

Rough surfaces as observed in this thesis, are not automatically equal to high porosities. They can result either by certain process parameters or by decompositions inside the particle, which would result in porous structures. Only very little amounts of the mPEGylated lysozyme in comparison to PLGA were applied during the encapsulation processes, making a decomposition unlikely. Although, the initial burst and the fast release rates of lysozyme observed in Figure 26 would support the suggestion of porous microspheres. In general, large inner surfaces which are increased by high porosities potentially accelerate the drug diffusion through the microspheres due to the increase uptake of release medium [82].

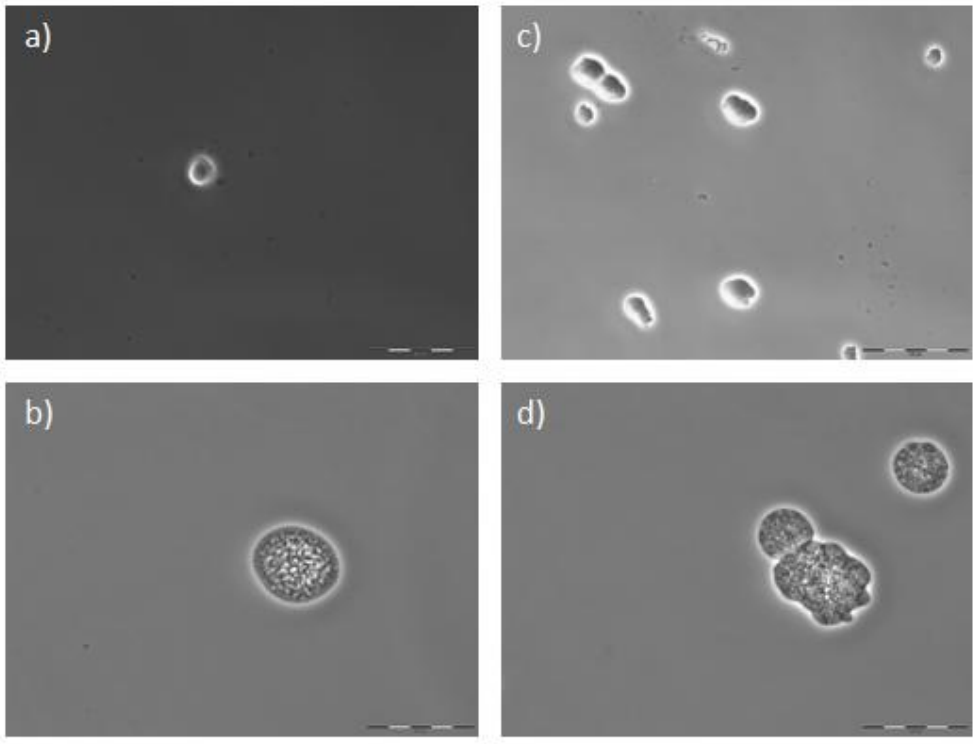

Figure 30: Microspheres Encapsulating Native Lysozyme – a) native lysozyme encapsulated by solvent evaporation (400x enlargement), b) native lysozyme encapsulated by solvent evaporation (1000x enlargement), c) native lysozyme encapsulated by spray drying (400x enlargement), d) native lysozyme encapsulated by spray drying (1000x enlargement)

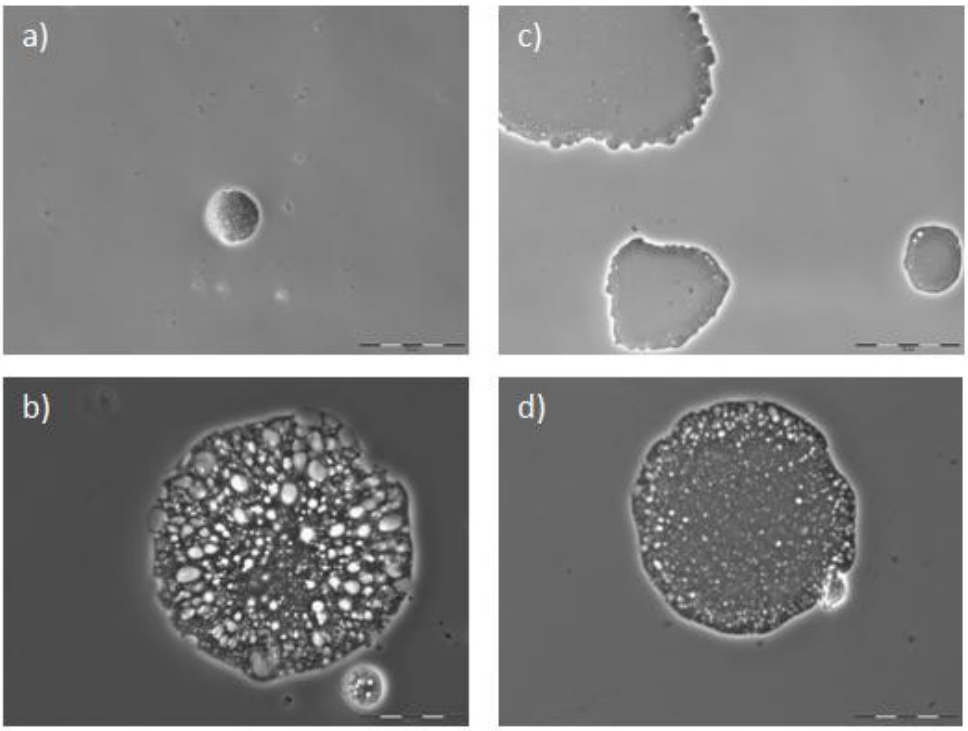

Figure 31: Microspheres Encapsulating Conjugate 1 – a) conjugate 1 encapsulated by solvent evaporation (400x enlargement), b) conjugate 1 encapsulated by solvent evaporation (1000x enlargement), c) conjugate 1 encapsulated by spray drying (400x enlargement), d) conjugate 1 encapsulated by spray drying (1000x enlargement)

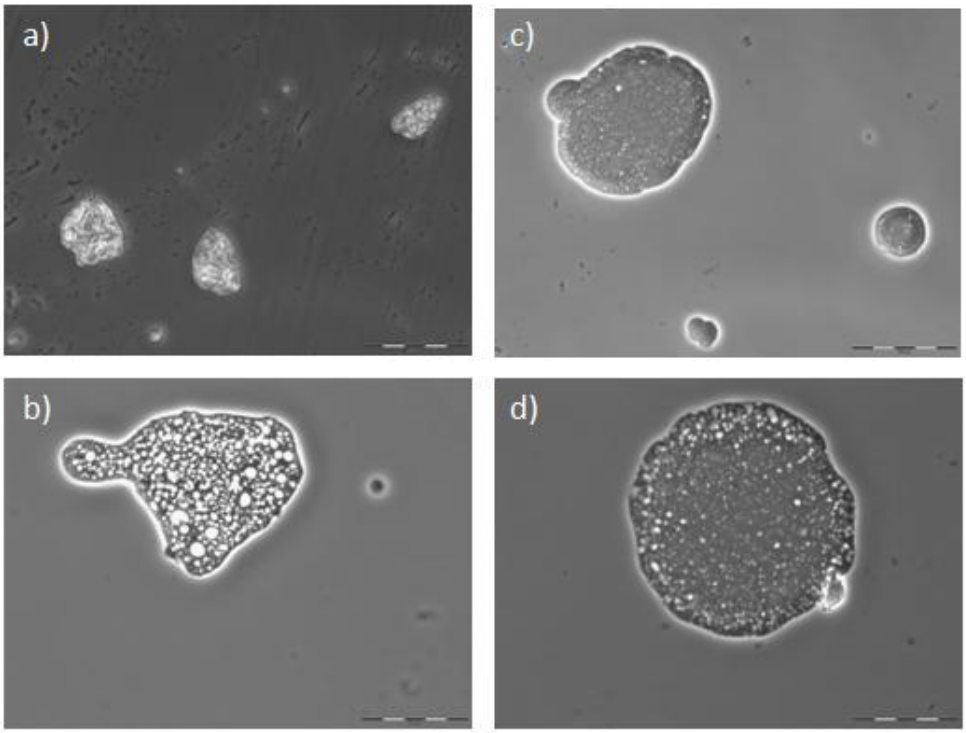

Figure 32: Microspheres Encapsulating Conjugate 2 – a) conjugate 2 encapsulated by solvent evaporation (400x enlargement), b) conjugate 2 encapsulated by solvent evaporation (1000x enlargement), c) conjugate 2 encapsulated by spray drying (400x enlargement), d) conjugate 2 encapsulated by spray drying (1000x enlargement)

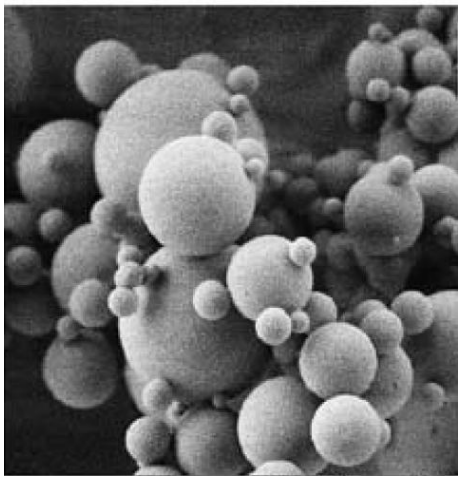

Figure 33: SEM Picture of Microspheres – The microspheres were prepared by spray drying in 50:50 PLGA [167]

5. Conclusion

A successful PEGylation reaction of the model protein lysozyme and the polymer mPEG-pNp was performed in this thesis. This reaction is strongly dependent on the reaction time, the polymer-to-protein mass ratio, and the reaction pH. Due to the strong influence of these parameters, the reaction needs to be narrowly controlled. The optimization of these paramenters can yield fractions that contain predominantly, but not entirely, the desired product. Already slight changes can cause highly differing products which contain different degrees of modification. The chosen functional group, p-nitrophenyl carbonate, is a good choice for PEGylation reactions which need to realize precise product requirements since it reacts slowly. Faster reactions are more complicated to control.

The PEGylated lysozyme acts as if it were a much bigger molecule than it actually is. Thus, the rapid clearance of protein-based drugs by an organism can be reduced. The enzymatic activity of the PEGylated lysozyme decreases with an increasing degree of modification. *In vivo* studies will need to determine whether the prolonged half-life is capable of compensating the activity losses.

The produced mixture containing four different degrees of modification was concentrated by ultrafiltration without significant losses. It was successfully purified from all unreacted lysozyme and remaining polymer by a SEC. This purification is essential to achieve a FDA approval. Furthermore, the separation of the different degrees of PEGylation is important because the various degrees of modification exhibit different properties. However, a separation of the single modification degrees by SEC was not possible, but due to the slight variations in their elution behavior, mixtures predominantly containing higher or lower modification degrees could be produced. In further studies a separation by cation exchange chromatography as described by Moosmann *et al.* should be tested [158]. In general, chromatographic methods are limited, because they are slow and difficult to scale up. But, alternative methods have not been developed except for an ultrafiltration membrane technology where composite regenerated cellulose membranes with varying MWCO are used [165]. The activity after the purification and separation procedure decreases. Furthermore, the previous result, describing the decrease of activity with an increasing degree of modification, is confirmed. Nevertheless, the resistance to proteolysis and the stability is increasing as the degree of modification increases. Thereby, the PEGylated lysozyme conjugates prolong the half-life. Again, *in vivo* studies are necessary in order to classify the quality of these results regarding the pharmacokinetics.

The measured encapsulation of PEGylated lysozyme in PLGA successfully resulted in microspheres independent of the preparation method. The encapsulation efficiencies as well as the release kinetics were inaccurate, because of measurement problems with the used BCA assay. As a result, a statement on the influence due to the combination of PEGylation and microsphere preparation is complicated. Pictures of the microspheres mostly presented spherical particles with a rough surface showing craters. It appears that the microspheres prepared by solvent evaporation show larger craters on the surface than the microspheres prepared by spray drying. In addition, by solvent evaporation encapsulated conjugate 2 did not result in spherical particles. This effect could be due to the long heat exposure during the preparation procedure.

Dependent on the desired route of administration microspheres need to fulfill certain requirements regarding the particle size. Especially, particles prepared by spray drying showed great potential as injectable drug delivery systems. But, the achieved yields by solvent evaporation were about 20 % higher than by spray drying. High yields are important in order to keep the production costs low. These low yields of spray drying processes are their greatest limitation. Apart from this drawback, all factors recommend spray drying as a very promising method in microsphere production. It is a one step, rapid method which is easy to scale up.

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7. Annex

7.1 List of Abbreviations

BCA	bicinchoninic acid
CV	column volume
EMA	European Medicines Agency
FDA	Food and Drug Administration
IEC	ion exchange chromatography
IUPAC	International Union of Pure and Applied Chemistry
MWCO	molecular weight cut off
mPEG-pNp	methoxypoly(ethylene glycol) p-nitrophenyl
PEG	poly(ethylene glycol)
PLGA	poly(lactide-co-glycolide)
RPC	reversed phase chromatography
SEC	size exclusion chromatography
SEM	scanning electron microscopy

7.2 List of Tables

7.3 List of Figures

7.4 Additional Data

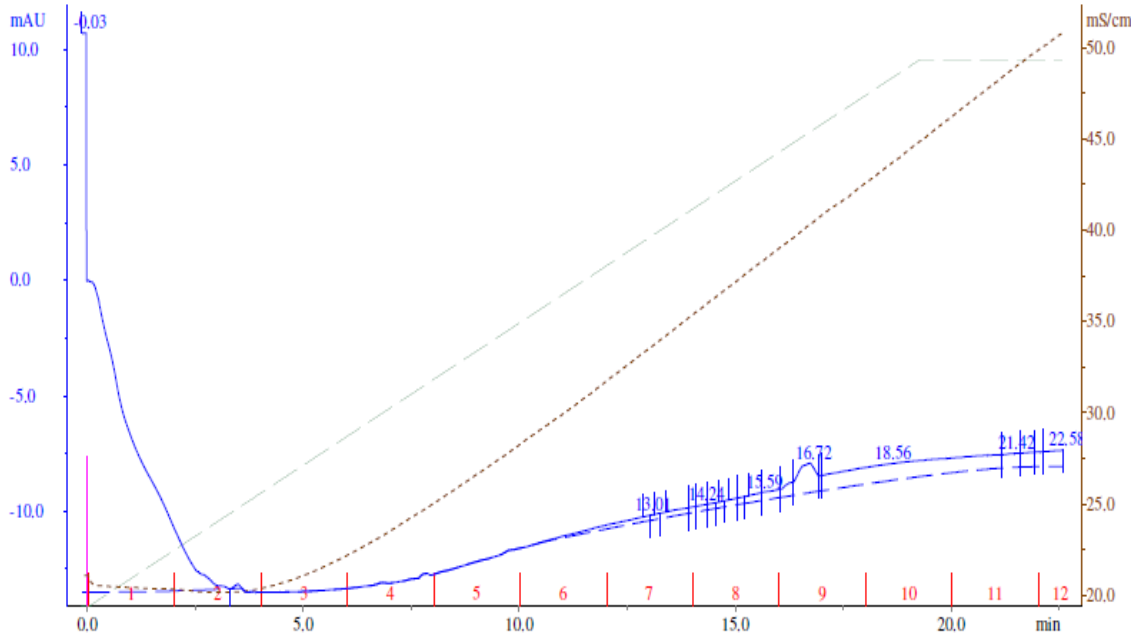

Figure 34: Original Chromatogram of IEC – PEGylated lysozyme was applied on a HiTrap CM FF (1 ml) column. The chromatogram starts at the elution, but the sample did not bind to the column at all. The UV adorption is shown in blue [mAU], the conductivity in brown [mS/cm], and the fractions in red.

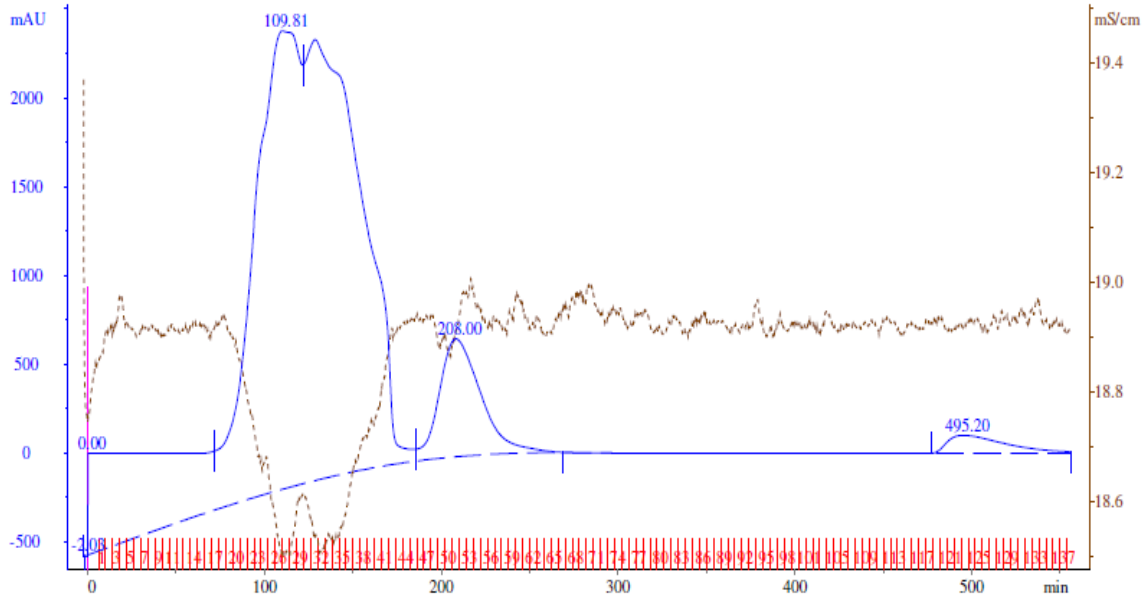

Figure 35: Original Chromatogram of the SEC of the PEGylated Lysozyme Mixture Using a Sephacryl S-200 Column –The UV adorption is shown in blue [mAU], the conductivity in brown [mS/cm], and the fractions in red.

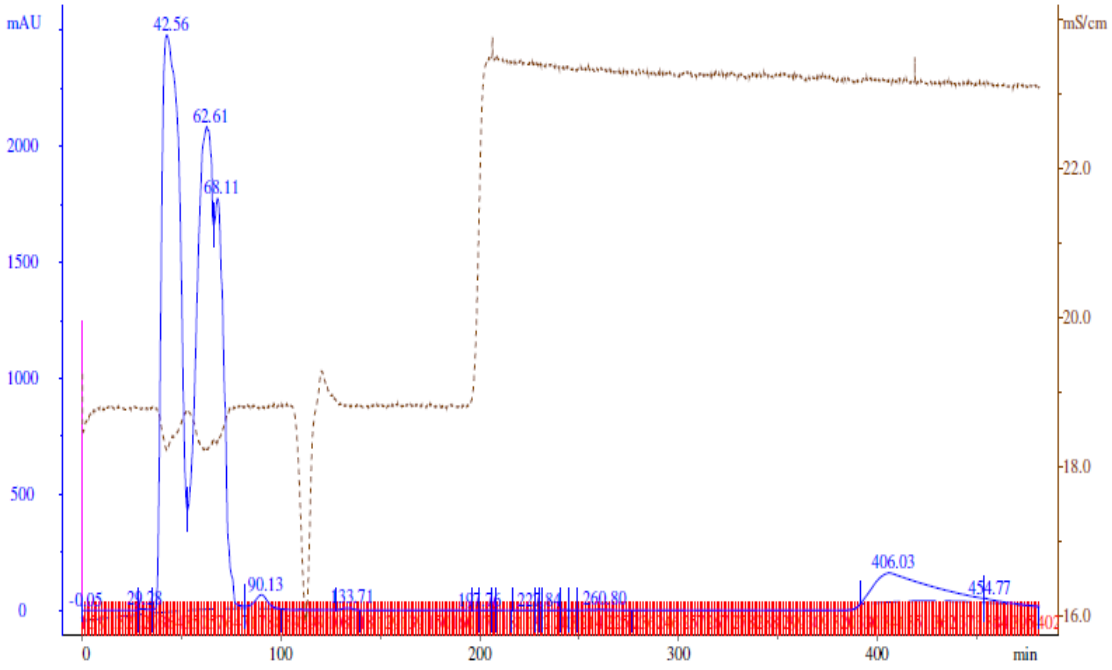

Figure 36: Original Chromatogram of the SEC of the PEGylated Lysozyme Mixture Using a Superdex 75 Column –The UV adorption is shown in blue [mAU], the conductivity in brown [mS/cm], and the fractions in red.

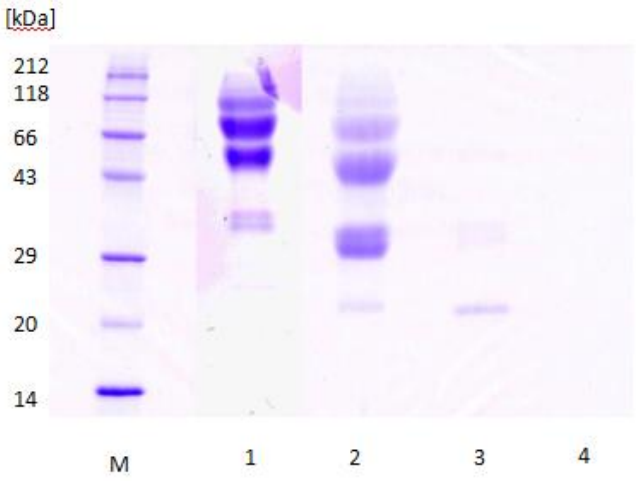

Figure 37: SDS PAGE of the SEC Using a Superdex 75 Column – The lanes 1, 2, and 3 show the Peaks 42.56, 62.61, and 90.13 of the chromatogram in Figure 36. In all lines PEGylated lysozyme conjugates were detected. Lane 3 shows a weak band indicating monoPEGylated lysozyme. Lane 4 shows no result which indicates the remaining PEG. Unreacted lysozyme is not detected.

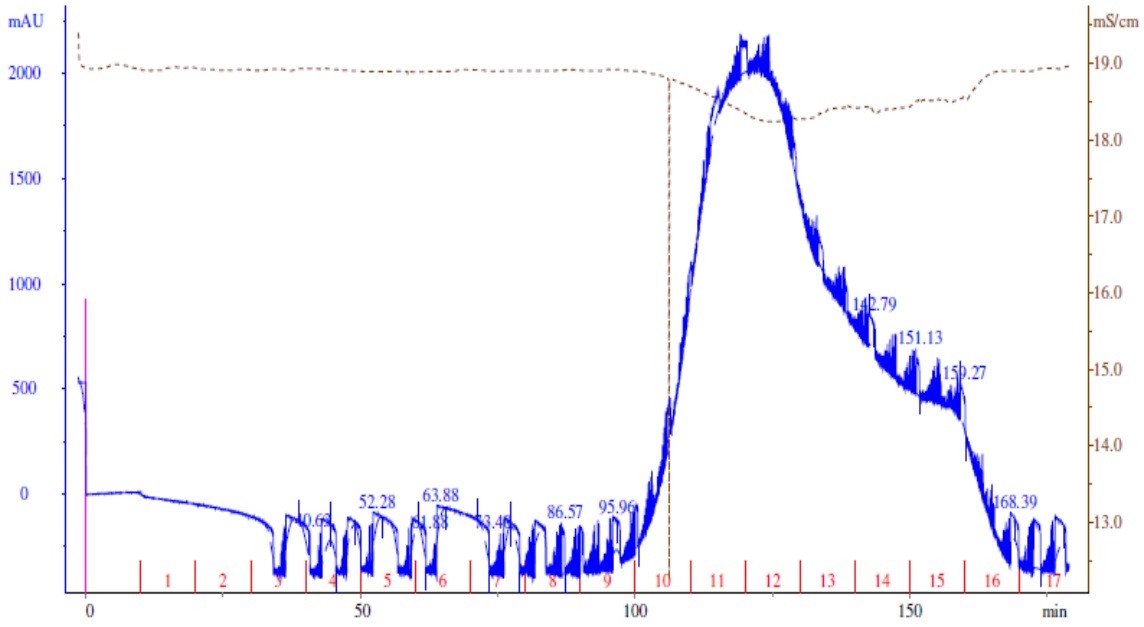

Figure 38: Original Chromatogram of the SEC of the PEGylated Lysozyme Mixture Using a Sepharose 4B Column –The UV adorption is shown in blue [mAU], the conductivity in brown [mS/cm], and the fractions in red. The various small "peaks" are probably due to air which entered the column during the process. Since the fractions volumes appeared normal these "air-peaks" most likely only affected the detector.

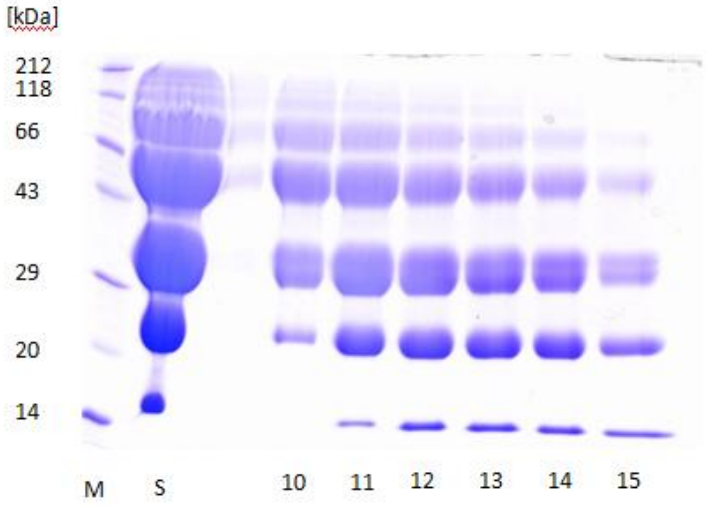

Figure 39: SDS PAGE of the SEC Using a Sepharose 4B Column – The lanes represent the fractions below the peak. A purification as well as a separation of the sample was not possible. The separation range (60-20.000 kDa) of this column material is much higher than the separation ranges of Sephacryl S-200 (5-250 kDa) and Superdex 75 (3-70 kDa). Lysozyme (14 kDa) could not be separated of the PEGylated lysozymes because the separation range of Sepharose 4B was too high.

Statutory Declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

Hamburg, 6th May 2014 _____

Katharina Rützel