Development of Optimized Reaction- and Purification Strategies for Polymer-Modified Proteins

zur Erlangung des akademischen Grades eines
Doktors der Ingenieurwissenschaften (Dr.-Ing.)

der Fakultät für Chemieingenieurwesen und Verfahrenstechnik des
Karlsruher Instituts für Technologie (KIT)

genehmigte
Dissertation

vorgelegt von
Dipl.-Ing. Benjamin Maiser
aus Landau in der Pfalz

Referent: Prof. Dr.-Ing. Jürgen Hubbuch
Koreferent: Prof. Dr.-Ing. Matthias Franzreb
Tag der mündlichen Prüfung: 23.09.2013

Danksagung

Es gibt eine Vielzahl an Menschen, die mich während der gesamten Zeit begleitet und unterstützt haben. Ein besonderer Dank geht hierbei an folgende Personen:

- Prof. Jürgen Hubbuch für die Aufnahme in seine Arbeitsgruppe und die fachliche Unterstützung. Seine stets freundliche und offene Art und die umfangreiche Laborausstattung des Lehrstuhls garantierten perfekte Arbeitsbedingungen.
- Prof. Matthias Franzreb für die Übernahme des Co-Referats.
- Dr. Florian Dismer für die fachlichen Diskussionen und die überaus angenehme Büroatmosphäre während der gesamten Zeit am Institut. Durch die gemeinsamen Rennradtouren im Karlsruher Süden war stets ein sportlicher Ausgleich geschaffen, wodurch einer kontinuierlichen Körpergewichtszunahme (auf beiden Seiten) Einhalt gewährt werden konnte.
- Susanne Haid und Marion Krenz für die Hilfe bei der Überwindung bürokratischer Hürden, den ständigen Überblick über die Ausgaben und die Bereitstellung von Verpflegung außerhalb der Mensaöffnungszeiten. Ohne eure kontinuierliche Arbeit im Hintergrund wäre ein funktionierender Lehrstuhl nicht möglich.
- Für die gemeinschaftliche Atmosphäre bei den Frühstücksrunden, bei der Laborarbeit oder über den Feierabend hinaus möchte ich mich bei der gesamten MAB Gruppe bedanken. Hierbei geht ein besonderer Dank an die Kollegen der ersten Stunde: Patrick Diederich, Sigrid Hansen, Jörg Kittelmann, Frieder Kröner, Natalie Rakel, Stefan Oelmeier und Anna Osberghaus.
- Michèle Delbé für die zahllosen gemeinsamen gelaufenen Kilometer im Karlsruher Hardtwald.
- Meine Studien-und Diplomarbeiter für die Unterstützung im Labor: Alexander Hanke, Johannes Kamm, Kai Baumgartner, Nicolas Lehle und Isabell Thanheiser.
- Meine Eltern Thomas und Karola für ihre finanzielle und moralische Unterstützung. Danke für die gewährten Freiheiten und die Bereitschaft auch dem vierten Kind eine anständige Ausbildung zu gewähren.
- Meine Frau Mareike, die trotz Wochenendarbeit im Labor oder Zuhause und zahlloser Bahnfahrten zwischen Karlsruhe und Stuttgart an meiner Seite blieb.

Abstract

This thesis deals with a polymer modification method for proteins, which is known as PEGylation. It represents the covalent attachment of polyethylene glycol (PEG) to a target molecule and is currently the state of the art technology to improve important characteristics of biopharmaceuticals. Even though this methodology was developed forty years ago, there still exist significant issues regarding process control and product purification. This work is focused on the optimization of aforementioned aspects and is divided in two parts:

- Development of fast and high sensitive analytics for PEG-protein conjugates.
- Optimization of PEGylation reactions by means of the established analytics and high throughput experimentation.

PEGylation reactions generally result in heterogeneous mixtures, if more than one accessible binding site is available. Besides unmodified protein, the product mixture consists of different PEG-protein conjugates varying in position and number of attached PEG molecules. However, each conjugate can offer different characteristics affecting relevant clinical aspects, including activity and stability. Consequently, characterization and purification of PEG-protein mixtures is of high interest and mandatory for regulatory approval. However, the separation especially of positional isoforms is still a challenging task.

As PEGylation target, lysozyme was used as model protein in this work, which consists of six lysine residues. If the PEGylation reaction is applied with amino coupling PEG reagents, such as PEG-aldehyde, multi-PEGylated conjugates and theoretically six isoforms of mono-PEGylated lysozyme are generated. To separate PEG-protein conjugates, ion exchange chromatography (IEC) can be applied, due to PEG binding and thus altered or shielded surface protein charge. However, different PEGylation studies with PEGylated lysozyme were not able to detect and separate more than three isoforms using IEC with a classical salt gradient elution.

In a first step, a linear pH gradient was applied in this work to separate purified mono-PEG lysozyme isoforms on a cation exchange column. In contrast to salt gradient chromatography runs, five isoform were separated, representing a significant increase in resolution. To identify the corresponding PEG attachment sites, the isoelectric point (pI) of each isoform was calculated based on structural data using an *in silico* approach. For this, the assumption that every PEG to amino residue conjugation neutralizes a positive charge was used. With a comparison of elution pH values and the calculated isoelectric points the identification of PEG attachment sites could be achieved. Based on UV areas it was possible to determine

corresponding reactivities, additionally. Accordingly, the N-terminal amine at lysine 1 (in the following labeled as PEG-lys_1) exhibited the highest reactivity under neutral PEGylation buffer pH values, followed by the lysine 33 residue. To verify the results, classical peptide mapping and MALDI-TOF MS analytics were applied. In addition, it could be shown that increased PEG molecular weight does not affect the isoform distribution, but results in lowered elution pH values of corresponding isoforms.

In the following, the development of fast PEGamer analytics was conducted. In contrast to PEG-conjugate isoforms, PEGamers vary in the number of attached PEG and can be easily separated using size exclusion chromatography (SEC), due to different hydrodynamic radii. However, using high throughput experimentation in modern downstream development, automation, miniaturization and parallelization enable the generation of a large number of samples. To guarantee a fast and efficient use of high throughput screenings, analytical speed is consequently an important aspect. An increased sample throughput of existing PEGamer analytics was achieved by a multivariate data analysis (MVDA). Based on the data of five chromatograms, including native lysozyme, PEGamer variants (mono-, di- and tri-PEGylated lysozyme) and a blank sample, different multivariate approaches were evaluated. The approaches were compared and multilinear regression (MLR) showed the most precise calibrations. Applying this method, a precise assay for PEGamer quantification was established with an analysis time of two minutes per sample.

The second part of this work covers the optimization of the PEGylation reaction in free solution and the so called solid-phase PEGylation. Due significant influence of various process parameters on PEGamer and isoform formation, high-throughput experimentation (HTE) was applied to optimize the PEGylation reaction in free solution. For this, small scale PEGylation experiments were conducted in 96-well microtiter plates with a total sample volume of 300 μL. The reactions were carried out in different reaction buffers, to identify the influence of different parameters such as buffer pH value and PEG excess. Applying an automated sample generation, the influence of time on the model reaction could be evaluated, by generating kinetics of mono-, di- and tri PEGylated species. With the established isoform analytics, a significant impact of the buffer pH value on the isoform distribution could be demonstrated. Accordingly, PEGylation occurs mainly at the N-terminal amino residue, if buffer pH values around pH 6 are used, while the most reactive site at pH 8 is lysine 33. This knowledge was used in the following to identify optimal process conditions to achieve a maximum concentration of each isoform. In further experiments, *Micrococcus lysodeikticus* based activity assays enabled the evaluation of the residual activity of resulting PEG-lysozyme isoforms. According to this, lysine 33 PEGylated lysozyme (PEG-lys_{33}) exhibited the highest residual activity, followed by PEG-lys_1. To show the potential for an industrial application, the established methods were combined and a control space for a PEGylation reaction was defined, which allows a constant and optimal volumetric activity of isoform mixtures.

Besides the optimization of PEGylation reactions in free solution, the aspect of solid-phase PEGylation was elucidated. This method represents the PEG modification of adsorber bound protein and can provide advantages with respect to process control, as two unit operations (PEG reaction and the first purification step) can be combined. Due to missing isoform analytics, the influence of this method regarding an altered isoform distribution could not be analyzed so far. Using automated sample generation with combined SEC and high sensitive isoform analytics, differences between adsorber based lysozyme PEGylation and the reaction in free solution regarding PEGamer kinetics and isoform formation were evaluated. As adsorber matrices, a conventional cation exchange resin (SP Sepharose FF) and a grafted resin type (SP Sepharose XL) were investigated. Lysozyme bound onto the adsorbent matrices showed generally a reduced PEGylation reaction compared to reactions in free solution, which was attributed to additional film- and pore diffusion. Additionally, an altered isoform distribution could be demonstrated. In contrast to predominant PEGylations at lysine 1 and lysine 33 of lysozyme in liquid phase at pH 6, a main modification of opposite-located lysine residues 97 and 116 was found for solid phase experiments. This result could be explained with binding orientations on corresponding adsorbent materials, as previous studies showed a main binding site onto both adsorbent matrices between lysine 1 and lysine 33. An increased activity of the isoform mixture due to a favorable isoform distribution using the solid-phase PEGylation could thus not be achieved, as PEG-lys_{33} was investigated in previous studies to be the most active isoform. However, the important influence on important aspects could be confirmed and has to be taken into account, if solid–phase PEGylation is applied. Additional experiments showed an influence of the initial adsorber surface coverage with bound lysozyme on the isoform distribution, which can be explained with sterically hindered attachment sites, due to neighboring bound lysozyme molecules.

Zusammenfassung

Die vorliegende Doktorarbeit befasst sich mit der als PEGylierung bezeichneten Methode, bei der es sich um die kovalente Bindung von Polyethylenglykol (PEG) an ein Zielmolekül handelt. Obwohl diese Art der Modifizierung bereits seit Jahrzehnten erfolgreich in der pharmazeutischen Industrie, vor allem bei therapeutischen Proteinen, angewendet wird, bestehen nach wie vor zahlreiche Probleme im Hinblick auf die Prozessführung der PEGylierungsreaktion und die vollständige Auftrennung der entstehenden Konjugate. Der Fokus der Arbeit liegt auf der Entwicklung von Lösungsansätzen bezüglich der angesprochenen Problematiken und ist in zwei Teilbereiche gegliedert:

- Etablierung von geeigneten hochauflösenden und hochdurchsatzfähigen chromatographischen Analytiken von PEG-Protein Konjugaten.
- Anwendung der entwickelten Analysemethoden zur Optimierung von PEGylierungsreaktionen mit Hilfe von Hochdurchsatzexperimenten und Untersuchung industrierelevanter Fragestellungen.

Bei der Reaktion von PEG mit einem Zielprotein entsteht bei der Verfügbarkeit mehrerer Bindestellen eine Mischung aus verschiedenen Produkten, die sich in der Anzahl, aber auch in der Position der gebundenen PEG Moleküle unterscheiden. Die Auftrennung der entstehenden Konjugate ist dabei ein wichtiger Bestandteil in der Herstellung und Charakterisierung PEGylierter Proteine, da sich durch die veränderte Bindung zwischen PEG und Protein entscheidende Änderungen in klinisch relevanten Eigenschaften, wie zum Beispiel der Aktivität, ergeben können. Die vollständige chromatographische Auftrennung von Positionsisoformen ist allerdings schwierig und konnte bei der gewählten Reaktion mit dem Modellprotein Lysozym bisher nur unzureichend erzielt werden. Bei der Modifizierung von Lysozym mit PEG-aldehyd können neben multi-PEGylierten Varianten insgesamt sechs Isoformen von mono-PEGyliertem Lysozym gebildet werden, die sich aus sechs Aminogruppen in Form von Oberflächen Lysingruppen bzw. dem N-Terminus ergeben.

Durch die Bindung von PEG erfolgt eine Veränderung bzw. eine Abschirmung der Oberflächenladungen des Proteins, so dass die einzelnen PEG-Protein Konjugate mit Ionenchromatographie (IEC) aufgetrennt werden können. Während in bisherigen Veröffentlichungen bei der Aufreinigung von mono-PEGyliertem Lysozym auf einem Kationentauscher lediglich drei Isoformen unter Verwendung eines Salzgradienten aufgetrennt werden konnten, wurde im ersten Teil der Arbeit die Elution mit einem pH Gradienten durchgeführt. Hierbei konnte eine deutliche Verbesserung der Auflösung im Vergleich zu Salzgradienten beobachtet werden und erstmals eine Auftrennung von fünf der insgesamt sechs möglichen Isoformen erfolgen.

Um eine Zuordnung der getrennten Isoformen zu den jeweiligen PEG-Lysozym Bindestellen zu erhalten, wurden anschließend auf Basis der Proteinstruktur die Berechnung der isoelektrischen Punkte (pI) aller möglichen Isoformen durchgeführt. Durch eine Korrelation der berechneten Werte mit den Elutions-pH-Werten konnte eine Zuordnung der Elutionspeaks mit den jeweiligen Bindestellen des Lysozyms erfolgen. Dabei konnte der N-Terminus von Lysozym bei einem neutralen pH-Wert des Reaktionspuffers als reaktivste Gruppe bei der PEGylierungsreaktion ermittelt werden. Dieses Ergebnis konnten mit tryptisch verdauten PEG-Lysozym Isoformen in Verbindung mit massenspektrometrischen Messungen (MALDI-TOF MS) verifiziert werden.

Im weiteren Verlauf der Arbeit stand die Entwicklung einer Analytik für sogenannte PEGamere im Vordergrund, die sich im Gegensatz zu Isoformen lediglich in der Anzahl der gebundenen PEG Moleküle unterscheiden. Durch die unterschiedlichen hydrodynamischen Radien der Konjugate kann die chromatographische Trennung der PEGamere in der Regel mit Hilfe von Größenauschlusschromatographie (SEC) erfolgen. Da durch die Anwendung von Hochdurchsatzexperimenten in der modernen Prozessentwicklung von pharmazeutischen Proteinen mit Hilfe von Techniken wie Automatisierung, Miniaturisierung und Parallelisierung eine große Anzahl an Proben generiert werden, wird der angewendeten Analytik ein hoher Stellenwert zuteil. Diese stellt allerdings häufig einen zeitlichen Engpass dar und limitiert somit die Anzahl der zu untersuchenden Proben. Um die PEGamer Analytik für effiziente Hochdurchsatzexperimente nutzen zu können, stand somit eine Reduzierung der benötigten Probenzeit im Vordergrund. Auf Basis der Daten von fünf Reinstoffchromatogrammen bestehend aus unmodifiziertem Lysozym, Mono-, Di- und Tri-PEG-Lysozym und einer Blindprobe, wurde eine Multivariate Daten Analyse (MVDA) durchgeführt. Hierbei kamen verschiedene Modelle zum Einsatz, die miteinander verglichen wurden. Dabei lieferte der Ansatz über Multilineare Regression (MLR) die besten Ergebnisse, so dass mit Hilfe dieser Methode ein präzises Assay entwickelt werden konnte und eine deutliche Reduzierung der Probenzeit auf unter zwei Minuten pro Probe erzielt wurde.

Der zweite Teil der vorliegenden Arbeit befasst sich mit der Verbesserung des Prozessverständnisses der vorliegenden PEGylierungsreaktion in freier Lösung und der Festphasen-PEGylierung unter Zuhilfenahme der zuvor entwickelten Analytiken.

Aufgrund des großen Einflusses verschiedener Prozessparameter bei der PEGylierungsreaktion im Hinblick auf die PEGamer Bildung und Isoformenverteilung, wurde eine Optimierung der Modellreaktion mit Hilfe eines Hochdurchsatzscreenings durchgeführt. Hierzu erfolgte zunächst eine Miniaturisierung der PEG-Lysozym Reaktion auf einen 96-well Mikrotiterplatten Maßstab mit einem Gesamtvolumen von 300 μL. Die PEGylierungsreaktion wurde in verschiedenen Reaktionspuffern durchgeführt, so dass der Einfluss der Reaktionsbedingungen wie pH-Wert und PEG-Überschuss untersucht werden konnte. Zudem konnten durch die automatisierte Probengenerierung Kinetiken bezüglich der Mono-, Di- und Tri-PEG Lysozym Bildung aufgestellt werden, um so den zeitlichen Einfluss auf die Reaktion zu beurteilen. Mit Hilfe der zuvor entwickelten Isoformenanalytik konnte des Weiteren

die Isoformenverteilung von mono-PEGylierten Lysozym in Abhängigkeit der untersuchten Prozessparameter analysiert werden und ein deutlicher Einfluss des pH-Wertes festgestellt werden. Demnach stellt der N-Terminus bei pH Werten von 6 die reaktivste Gruppe dar, wobei bei pH 8 hauptsächlich Lysin 33 PEGyliert wird. Damit konnten für jede Isoform ideale Prozessparameter identifiziert werden, die eine maximale Konzentration ergeben. Die Etablierung eines Aktivitätsassays basierend auf *Micrococcus lysodeikticus* ermöglichte es zudem die spezifische Aktivität der Isoformen zu ermitteln, wobei sich PEG-Lysozym$_{33}$, gefolgt von PEG-Lysozym$_1$ als am aktivsten erwiesen. Durch die Kombination der entwickelten Methoden konnte ein Bereich bestimmt werden, der eine konstante volumetrische Aktivität eines Gemisches von Isoformen gewährleistet und so eine optimale Prozessführung von nach wie vor zugelassenen PEG-Protein Isoformengemischen darstellt.

Neben der Optimierung der Reaktionen in freier Lösung sollte auch die sogenannte Festphasen-, bzw. on-column PEGylierung untersucht werden, die eine PEGylierung von bereits immobilisiertem Zielprotein auf Adsorbermaterial darstellt. Dieses Vorgehen kann Vorteile im Bezug auf die Prozessführung darstellen, da zwei Prozessschritte (Reaktion und erster Aufreinigungsschritt) kombiniert werden können. Aufgrund von fehlenden hochauflösenden Analytiken konnte allerdings bisher der Einfluss der solid-phase PEGylierung insbesondere auf die Isoformenverteilung nicht ausreichend untersucht werden. Wie bei der Reaktionen in freier Lösung wurden mit Hilfe einer Roboterstation miniaturisierte Reaktionsansätze hergestellt, bei denen Lysozym vor der PEGylierungsreaktion an verschiedene Adsorbertypen immobilisiert wurde. Neben SP Sepharose FF, einem konventionellen Kationentauscher, wurde mit SP Sepharose XL auch ein gegrafteter Adsorbertyp untersucht. Bei beiden Adsorbertypen konnte eine deutlich geringere Reaktionsgeschwindigkeit zu PEG-Protein Konjugaten ermittelt werden, die sich auf zusätzliche Film- und Porendiffussion bei den Adsorberpartikel zurückführen lässt. Des Weiteren konnte eine veränderte Isoformenverteilung im Gegensatz zu den PEGylierungsreaktionen in freier Lösung festgestellt werden, die sich mit der entwickelten Isoformenanalytik untersuchen ließ. Während der N-Terminus an Lysin 1 und Lysin 33 in freier Lösung die HauptPEGylierungsstellen und somit die reaktivsten Gruppen darstellen, wurden bei den adsorbergebundenen Reaktionen hauptsächlich die gegenüberliegenden Gruppen Lysin 97 und Lysin 116 PEGyliert. Dieses Ergebnis korreliert mit Studien über die Bindungsorientierung von Lysozym auf den entsprechenden Adsorbern, bei denen die Hauptbindestelle zwischen Lysin 33 und Lysin 1 ermittelt werden konnte, so dass es zu einer PEGylierung der gegenüberliegenden Lysin Reste kommt. Da in den zuvor durchgeführten Aktivitätsassays PEG-Lysozym$_{33}$ als aktivste Isoform ermittelt wurde, konnte mit Hilfe der Festphasen-PEGylierung somit keine höhere spezifische Aktivität des entstehenden Isoformengemisches erreicht werden. Allerdings konnte erstmals der Einfluss auf die Isoformen und somit eventuell auch auf klinisch relevante Eigenschaften nachgewiesen werden und die Bindungsorientierung von Lysozym auf den entsprechenden Adsorbern bestätigt werden. Zusätzliche Versuche mit variierender Oberflächenbeladung zeigten ebenfalls einen Einfluss auf die Isoformenverteilung und konnten mit sterischen Effekten durch benachbarte Lysozym Moleküle erklärt werden.

Contents

1 Introduction

1.1 Historical perspective of PEG-protein conjugation

Pioneering work in the field of protein modification with polyethylene glycol (PEG) was conducted in 1977 by the group of Abuchowski and Davis [Abuchowski et al., 1977]. Although PEG conjugation to proteins and surfaces were already done in the early 70s by numerous research groups, including Merrill and Sehon [Merrill, 1992, Sehon, 1992], the experiments by Abuchowski and Davis can be seen as a significant landmark.

As recombinant proteins were generally not available at this time, the initial intention of their experiments was to enable the benefits of non-human bioactive proteins to humans, without the severe immunological responses [Davis, 2002]. By injecting PEG-modified bovine serum albumin (BSA) into rabbits, a reduced immunogenicity and enhanced blood circulation half-life compared to the native form could be determined. The results were related to a reduced proteolytic digestion of resulting conjugates due to the protective effect of attached PEG and demonstrated the potential of this approach for pharmaceutical applications.

Although, other non-toxic polymers were also investigated in following years, PEG remained the main conjugation reagent, as it was already being used in the food and cosmetics industry. Additionally, PEG was designated by the FDA as "generally recognized as safe" (GRAS), due to its use in the pharmaceutical industry, for example as solubilizing agent in injectable therapeutics. The preparation of methoxy-PEG (mPEG) and thus the availability of monofunctional molecules with only one activated terminal group was another positive effect of PEG, reducing a diol formation and preventing a cross linkage between two proteins.

In 1990, Enzon Pharmaceuticals, which was founded by Abuchowski and Davis, achieved the approval of the first PEGylated therapeutic by the regulatory authorities. This can be seen as final breakthrough of the PEGylation technology, as it paved the way for subsequent PEGylated therapeutics, only thirteen years after the first experiments. The new drug was called Adagen®, representing PEG-modified adenosine-deaminase and treated a rare immunodeficiency disease (SCID) in children. In contrast to the unmodified form, the PEGylated molecule was not readily cleared by the kidneys, due to increased size and it was the first therapy for this disease.

Adagen®, as well as Oncaspar®, which was also a PEGylated enzyme and invented by Enzon, reached only a limited number of patients, due to rare therapeutic indications. However, with the advent of humanized recombinant proteins the improved residence time of PEGylated proteins became an interesting aspect for a large part of biopharmaceuticals. Consequently, following approved PEG-modified therapeutics mainly achieved blockbuster

Table 1.1: Approved PEGylated products in clinical practice. Data according to [Pasut and Veronese, 2012, Veronese et al., 2009, Kang et al., 2009]

Target & tradename	Size and quantity of PEG molecules	Binding site	Therapeutic indication	Year to market
Adenosine deaminase Adagen®	5 kDa 1	lysine *random*	SCID	1990
Asparaginase Oncaspar®	5 kDa 1	lysine *random*	Leucemia	1994
Interferon-α2b PEG-Intron®	12 kDa 1-2	lysine, histidine *random*	Hepatitis C	2000
Interferon-α2a Pegasys®	40 kDa 1	lysine *random*	Hepatitis C	2001
hGH antagonist Somavert®	5 kDa 4-6	N-terminus, lysine *random*	Acromegaly	2002
G-CSF Neulasta®	20 kDa 1	N-terminus *selective*	Neutropenia	2004
Anti-VEGF aptamer Macugen®	2x20 kDa 1	N-terminus *selective*	ADM	2004
Epoetin Mircera®	30 kDa 1	N-terminus, lysine *random*	Anemia	2007
Anti-TNF Fab Cimzia®	40 kDa 1	thiol residue *selective*	Rheumatoid arthritis	2008
Uricase Krystexxa®	10 kDa 36	lysine *random*	Chronic gout	2010

ADM: age-related macular degeneration; G-CSF: granulocyte-colony stimulating factor
SCID: severe combined immunodeficiency desease; TNF: tumor necrosis factor;
VEGF: vascular endothelial growth factor

status, representing sales over 1 billion US$ per year. Very successful PEG-protein conjugates include PEGylated interferon (Pegasys®, from Hoffman-LaRoche) for the treatment of hepatitis C and PEGylated granulocyte colony-stimulating factor (G-CSF, Neulasta® from Amgen) to treat leukemia. According to sales per year, both drugs were listed under the 100 most successful pharmaceuticals worldwide in 2010 [McGrath et al., 2010], indicating a well-established technology with significant therapeutic value. Up to now, ten PEGylated products are in clinical practice and are listed in Table 1.1. Except for Macugen®, representing a PEGylated RNA oligonucleotide, all of them are PEGylated proteins.

As can be seen, listed products vary in number and size of attached PEG, as well as the PEGylation reaction, indicating a broad spectrum of modification techniques. In addition to stated products, there are currently many different PEGylated molecules in various stages of development, which are especially small parenteral administered molecules such as hormones or antibody fragments [Fee and Van Alstine, 2011]. However, in spite of nearly forty years of development, there still exist problems regarding process control and purification procedures. The drawbacks comprise in particular preparative isoform separation and process

reproducibility in terms of Quality-by-Design (QbD) and are discussed in detail in following sections and manuscripts.

1.2 General benefits of PEGylated products

The elimination from blood of proteins with a molecular weight below the kidney threshold of about 70 kDa (M_w based on protein mass) occurs mainly via renal clearance. Consequently, small intravenously administered molecules of pharmaceutical interest generally offer short *in vivo* half-life and rapid clearance and thus show poor therapeutic effect. To overcome this drawback, the attachment of PEG can be applied, as already shown in the early experiments by Abuchowski and Davis, where this technique enhanced the residence time of BSA. This advantage can mostly be related to the increased hydrodynamic radius of formed conjugates, resulting in a reduced filtration through the renal glomeruli. Thus, PEGylation offers a possibility for small molecules to develop their therapeutic use, if an appropriate PEG size is applied.

Besides the benefits for small molecules, the increase in size can also have positive effects for larger molecules. In contrast to the native unmodified form, PEGylated species require a less frequent dosing due to an enhanced *in vivo* half-life. For example interferon (IFN) for the treatment of hepatitis C has to be injected three times a week to ensure a high drug concentration. In contrast, the PEGylated version Pegasys® has to be injected only once a week, representing a less stressful administration for patients.

In addition to these aspects, PEGylated proteins can exhibit a reduced immunogenicity and antigenicity due to suppressed proteolysis and recognition by the immune system. Although the conformation of PEG after protein attachment is not completely elucidated, it is assumed that a so called shielding effect prevents the drug from degradation by proteolytic enzymes, antibody recognition and uptake by phagocytes. This also results in an extended drug residence time and enables the administration of molecules with increased immunogenicity.

Another positive aspect of PEGylation can comprise a reduced aggregation propensity, which is explained with masked hydrophobic patches on the protein surface by attached hydrophilic PEG. This allows high concentrated protein solutions without aggregation. Studies by Chapman et al. [Chapman, 2002] observed for example a solubility of PEGylated antibody fragments up to 200 mg/mL, which represents an interesting option for the formulation of proteins. The hydrophilic characteristics of PEG can also enhance the solubility of small insoluble molecules including commonly used anticancer drugs such as docetaxel and paclitaxel [Kang et al., 2009]. Improved physical and thermal stability can be also detected for PEGylated proteins, which is an important factor for formulation and delivery aspects.

1.3 Basic characteristics of PEG

Polyethylene glycol (PEG) is a polymer, consisting of covalently coupled ethylene oxide subunits, each having a molecular weight of 44 Da. It is commercially available in linear, as

well as branched forms and offers in its inactivated status hydroxyl residues at each terminus. As these hydroxyl groups are less reactive, PEG can be functionalized, enabling different conjugation reactions. There exist a large number of different PEG reagents, varying in molecular weight and terminal residues at one or both ends. To prevent a cross linkage and reduce diol formation, methoxy-PEG (mPEG) is generally used for protein conjugation.

As already mentioned, PEG can be provided with different average molecular weight. However, as most synthetic polymers, PEG exhibits mainly a polydispersity, which can be analyzed with high sensitive analytics such as MALDI-MS. As illustrated in Figure 1.3 varying PEG masses can be detected, all differing in the monomer mass of 44 Da. In spite of this heterogeneity, PEG reagents up to 30 kDa with a polydisperse index of 1.05 are accepted for pharmaceutical purposes.

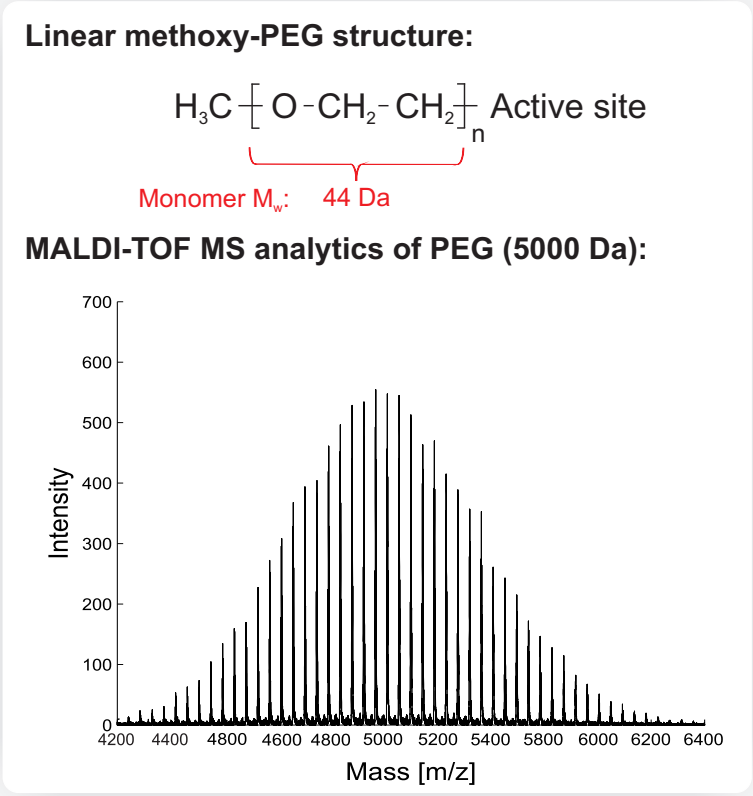


Figure 1.1: Illustration of a linear methoxy-PEG molecule (above) and a MALDI-TOF MS spectrum of PEG_{5000}-aldehyde, indicating the polydisperse nature of PEG (below).

While the ethylene moiety exhibits hydrophobicity, the oxygen allows a binding of about 6–7 water molecules, resulting in a good solubility in aqueous solutions, as well as many organic solvents. Due to its strong hydration and flexibility, a PEG polymer in solution offers a random coil structure. An improved synthesis by French et al. [French et al., 2009] resulting in a monodisperse PEG with 16 subunits enabled a first PEG crystallization and revealed secondary 3_{10} helical structures.

1.3.1 Toxicity and *in vivo* clearance

Although PEG is referred as non-biodegradable component, small PEG molecules can be degraded *in vivo* by cytochrom P450 and aldehyde dehydrogenase. PEGs with a molecular weight below 400 Da can be metabolized by alcohol dehydrogenase to toxic products. Larger PEG molecules are eliminated mainly via renal pathways and are thus excreted by the urine, if the molecular weight is below 40 kDa. Over the limit of the kidney threshold of about 60 kDa, longer circulation as well as liver uptake can be detected and should thus be avoided. Consequently, PEG reagents up to 40 kDa are currently used for protein conjugation, representing a tradeoff between easy kidney clearance of PEG and improved circulation time of the conjugate. Toxicity studies with high concentrated PEG solutions revealed a formation of vacuoles in renal tubules cells in rabbits [Fruijtier-Pölloth, 2005]. Although the amount of administered PEG in PEG-protein pharmaceuticals is generally below the concentrations applied in cited study, high dosages as a part of life long therapies might thus be problematic.

Additionally, recent studies with PEG-uricase showed an anti-PEG immune response. Numerous patients developed specific antibodies against PEG after the administration of the drug, which puts the claimed immunogenicity of PEG into question [Armstrong et al., 2007].

1.3.2 Hydrodynamic radius

As already mentioned, the size of PEG and corresponding conjugates plays an important role in drug clearing processes and consequently in the body residence time. To compare the size of different molecules, their hydrodynamic radius or Stokes radius (R_h) is commonly used, which represents the radius of a hypothetical hard sphere having the same diffusional characteristics as the described molecule. Due to the bound water molecules and resulting high flexibility, PEG offers an increased hydrodynamic radius compared to proteins with an equivalent molecular weight. Consequently, the impact on size of attached PEG molecules to proteins is higher, as might be expected from the PEGs nominal molecular weight.

The hydrodynamic radii of PEG molecules, as well as from proteins can be calculated based on their molecular weight (M_w), given in daltons according to [Fee and Van Alstine, 2004] and [Hagel, 1998]. Assuming a globular structure of the protein the hydrodynamic radius $R_{h,protein}$ in $\AA$ is given with Equation 1.1.

$$R_{h,protein} = (0.82 \pm 0.02)\ {M_w}^{0.333} \tag{1.1}$$

The correlation between the molecular weight of random coiled PEG in solution with its hydrodynamic radius is given with Equation 1.2.

$$R_{h,PEG} = 0.1912\ {M_w}^{0.559} \tag{1.2}$$

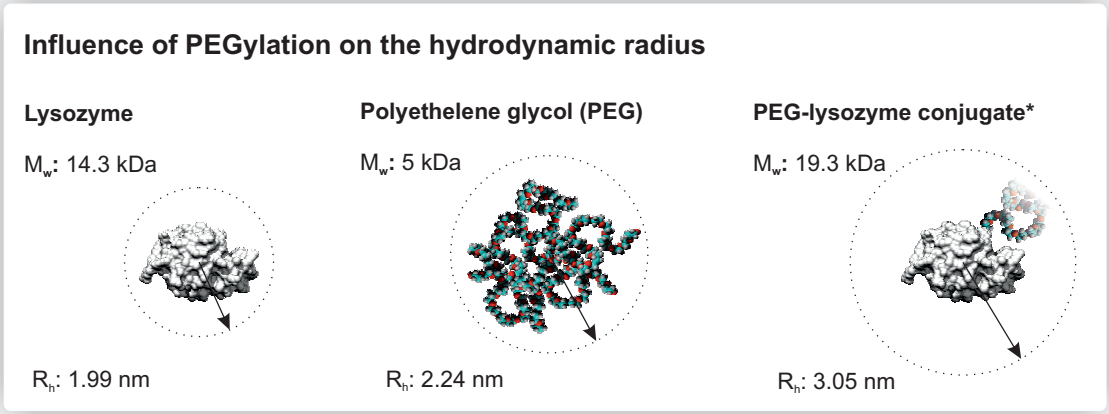


Figure 1.2: Schematically illustration of lysozyme, a random coiled PEG-molecule and resulting PEG-protein conjugate with corresponding hydrodynamic radii. Values were calculated according to equation 1.1 to 1.4. *PEG-protein confirmation is not completely elucidated.

With Equation 1.3 and 1.4, the Stokes radius of a PEGylated protein can be predicted, according to [Fee and Van Alstine, 2004].

$$R_{h,PEGprot} = \frac{A}{6} + \frac{2}{3A} {R_{h,PEG}}^2 + \frac{1}{3} R_{h,PEG} \tag{1.3}$$

with

$$A = \left[108 {R_{h,protein}}^3 + 8 {R_{h,protein}}^3 + 12 \left(81 {R_{h,protein}}^6 + 12 {R_{h,protein}}^3 {R_{h,PEG}}^3 \right)^{\frac{1}{2}} \right]^{\frac{1}{3}} \tag{1.4}$$

As the radius of the conjugate is independent of the number of attached PEG molecules, $R_{h,PEG}$ is calculated using the total PEG mass. To illustrate the size differences, Figure 1.2 shows the calculated hydrodynamic radii of lysozyme, a 5 kDa PEG molecule and resulting conjugate. With a radius of 3.05 nm, the size of a mono PEGylated PEG_{5000}-lysozyme conjugate corresponds thus to a protein weight of 51.5 kDa, illustrating the significant impact of PEGylation.

Up to now, the structural characteristics of PEG-proteins are not completely understood and different models are discussed. The PEG confirmation after protein attachment is commonly described with a shell structure, where the protein is completely surrounded by PEG chains. The PEG interacts with the protein via the hydrophobic ethylene units and corresponding patches on the protein surface [Morar et al., 2006]. Using this model, reduced recognition by the immune system and improved aggregation behavior of PEG-protein conjugates can be explained. Alternative models suggest a worm-like structure of PEG with

no PEG-protein interaction. Recent studies by Pai et al. [Pai et al., 2011] using small angle neutron scattering (SANS), suggests a conformation, where a random coiled PEG molecule is adjacent to the protein.

1.4 PEGylation strategies

The first PEG-protein reactions used by Abuchowski and Davis modified amino residues using chlorotriazine activated PEG molecules. By now, there exist a large number of PEGylation strategies, targeting different residues of surface amino acids. The most common PEG conjugation technologies are listed below and are illustrated in Figure 1.3.

- **Amino residue modification**
  The most commonly used PEGylation reactions for pharmaceutical therapeutics modify the amino residues of the target molecule including the α-amino residue (N-terminus) and the ϵ-amino residues, such as lysine. For amino residue modification, different PEG molecules can be applied, including PEG-aldehyde or N-hydroxysuccinimidyl (NHS) activated PEGs. In addition to lysine residue PEGylation, PEG-NHS is also known to react with histidine and tyrosine residues. Additionally, it is worth noting that acylating PEGs such as PEG-NHS can alter the charge of the target molecule by neutralizing the positive charge of a lysine residue due to amide or urethane formation. Alkylating PEGs including PEG-aldehyde maintain the charge. As lysine residues are generally well represented in proteins, these kind of reactions often result in multi-PEGylation, requiring additional purification. A positive aspect of PEG-aldehyde is given with the possibility of a selective N-terminal PEGylation. Using mild acidic pH values of about 5–6, the N-terminal amino group can be PEGylated selectively due to its lower pK_a value, compared to the ϵ-amino residues.

- **Enzyme mediated modification**
  Enzyme mediated PEGylation can be provided, targeting different surface amino acids. Two main conjugation methods are established and are partially used for products in clinical phases. To mimic natural glycosylation, so called GlycoPEGylation can be applied. For this, a two step conjugation is necessary, where the first step involves a glycosylation of serine and threonine residues (O-glycosylation) with N-acetylgalactosamine (GalNAc). The second step includes a subsequent PEGylation of glycosylated residues with sialyl-PEG, using sialyltransferase. This method was successfully applied for mono-PEGylated G-CSF and IFN, although both proteins offer numerous serine and threonine residues. A recent study by Ostergaard et al. [Ostergaard et al., 2011] demonstrated the PEGylation of already N-glycosylated proteins via asparagine residues.

  Another possibility of enzyme PEGylation enables a glutamine residue modification using transglutaminase (Tgase), which catalyzes the addition of a primary amine to an acyl residue. Consequently, amino- PEGs (PEG-NH_2) can be used as PEGylation reagents for this reaction. As Tgase only recognizes glutamine residues that are located

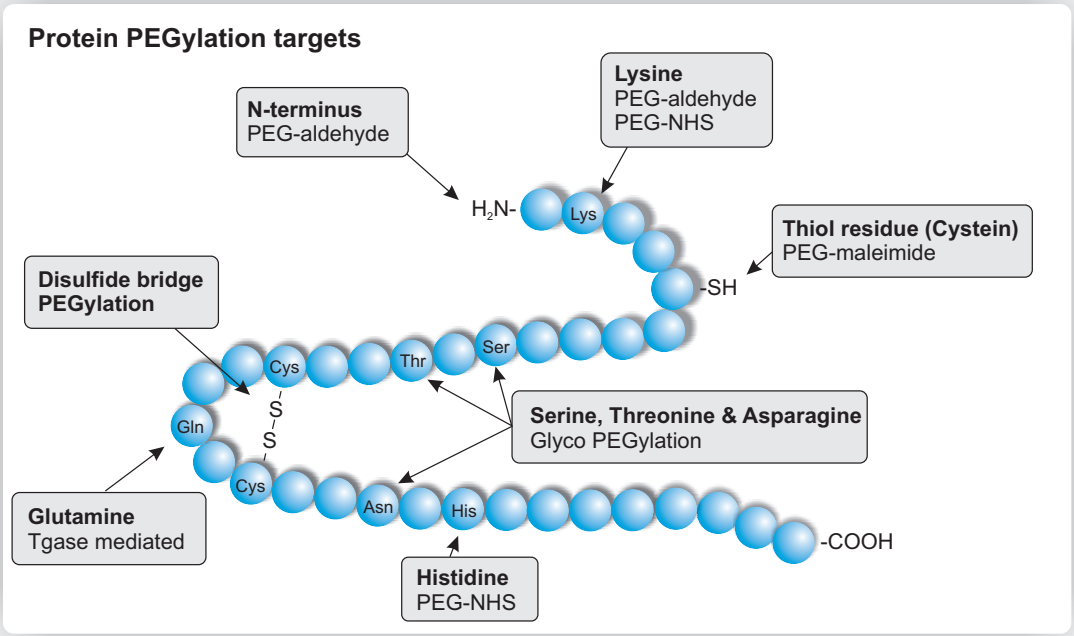


Figure 1.3: Different possibilities for protein PEG modification and corresponding PEG reagents. Illustration adapted from [Pasut and Veronese, 2012].

in exposed and flexible regions of the target molecule, this approach can thus be used to reduce the number of positional isoforms. Studies with G-CSF, consisting of 17 glutamine residues resulted in a site-specifically mono-PEGylation, by applying this method [Sato, 2002].

- **Thiol residue modification**
  PEGylation of unpaired cystein residues can be conducted with PEG-maleimide, which is already used in clinical practice for example to produce PEGylated anti-TNF antibody fragments (Cimzia ®). However, free cystein residues are generally not exposed in natural proteins, as they are often involved in disulfide bridges. Thus, reducing conditions are needed in case of antibody fragment PEGylation to prevent diol formation and to enable a PEG conjugation. The integration of free cystein residues into the amino acid sequence of a target molecule using genetic engineering, can provide the possibility of a site specific PEGylation. However, this approach often leads to low yields due to dimerization and incorrect protein folding [Basu et al., 2006].

- **Disulfide bridging**
  A novel approach providing a selective PEGylation of interferon α-2b via a disulfide bridge was first described by Shaunak et al. [Shaunak et al., 2006]. For this, a specific mono-sulfone PEG reagent was used to perform a site selective PEGylation of both cystein sulfur atoms via a so called three carbon PEGylation bridge. The disulfide bridge involved in the conjugation was preserved, resulting in a correct conformation

of the PEG-protein conjugate. An advantage of this approach is given with the small number of disulfide bridges that are present in proteins of pharmaceutical interest.

1.5 PEGylation challenges and purification issues

As all therapeutic drugs, PEGylated proteins have to meet strict regulatory criteria with respect to product homogeneity and activity. However, a large part of established PEGylation reactions target more than one PEG attachment site, which generally results in a mixture of conjugates, varying in number (PEGamers) and site (isoforms) of attached PEG chains. These types of reactions are typically designated as random, due to the great influence of process parameter on the product mixture. The quantity of PEGamers using random PEGylation reactions is given with the available binding sites of the protein. The potential number of positional isoforms (P) can be calculated according to the binominal coefficient as follows:

$$P = \binom{m}{k} = \frac{m!}{(m-k)!k!} \tag{1.5}$$

where m is the number of available binding sites and k the number of modified sites [Roberts et al., 2002]. For lysozyme as model protein in present work, amino coupling PEG reagents can modify six residues. Consequently, six isoforms can be formed for mono-PEG-lysozyme, and 15 for di-PEG-lysozyme, indicating a significant increase of isoforms with increasing number of attached PEG molecules.

The variation in number and PEG-attachment sites yield conjugates with different product characteristics, requiring purification after the PEG conjugation step. Additionally, the target product has to be separated from unreacted PEG and other low molecular reaction by-products. Due to difficulties in the chromatographic separation of positional isoforms, characterized isoform mixtures are still allowed by the FDA, although they can exhibit significant differences in important characteristics relevant to clinical effects.

An increase in product homogeneity can be provided by improved PEGylation reactions, offering an increased specificity towards a single site, as described in the PEGylation reactions section. However, the development of site-specific reactions is not trivial and fast approval by the regulatory authorities might be problematic, especially for genetically introduced binding sites. Another possibility includes reactions resulting in PEG-conjugate mixtures, followed by an isolation of the target molecule and an effective removal of unwanted species. To purify PEG modified proteins, differences in physicochemical properties such as size, hydrophobicity and electrostatic charge can be used similar to unPEGylated proteins and are discussed in the following.

- **Size based separation**

  Due to increased size of PEG, the separation of PEGylated proteins and the native

counterpart can be easily achieved via SEC and can be combined with a removal of potential by-products, such as NHS. However, a good separation between PEG-proteins with high PEG-extend becomes difficult with increasing number of attached PEG, due to reduced differences in size and depends on the molecular weight of involved components. Additionally, SEC is not suitable for the separation of PEG-protein isoforms due to the same size of positional isoforms.

- **Charge based separation**

  The shielding effect of PEG and thus blocked charge of the target molecule and increased distance to chromatographic media allows a separation of PEGamers using IEC. Multi-PEGylated species generally elute before di- and mono-PEGylated variants, followed by the native unPEGylated protein. As the position of attached PEG can have a great influence on the proteins charge distribution, shallow salt gradients can yield a separation of positional isoforms. Additionally IEC offers the advantage of a PEG flow through while column loading, enabling a fast and effective PEG removal. Due to the increased hydrodynamic radius of PEG and PEG-conjugates a drastic reduction in column loading, can be observed resulting in increased costs for column media in PEG-protein purification.

- **Separation based on hydrophobicity**

  As PEG interacts with HIC (hydrophobic interaction chromatography) media due to its hydrophobic nature, free PEG of crude PEGylation mixtures interfere with the separation, resulting generally in poor resolution performance. The elution order of native protein and PEGylated species is not fixed and can vary with the applied buffer salt [Müller et al., 2010].

Due to foaming and viscosity problems, a fast removal of free PEG is recommended for the purification of PEG-protein mixtures. Various studies suggest that SEC followed by IEC and HIC is a basis for a general PEG-purification process. Another possibility comprises the use of two consecutive IEC separations. [Jevsevar et al., 2010]. In the first step, resins with large particles and high porosity can be used to remove unreacted PEG, while the second step is performed to achieve PEG-protein separation.

1.6 Current research trends in protein modification

Several research trends regarding PEGylation and protein modification can be observed over the last few years and are briefly described in the following A large part of recently published manuscripts are focused on the development of site specific-PEGylation techniques. Due to separation and process challenges using random reactions, site-specific reactions are desirable, offering a single and well-defined product. As already shown in the PEGylation reactions section some promising reaction technologies are under development and are object of intense research.

Another topic includes releasable PEGylation especially for non-protein drugs enabling the removal of the attached PEG after the administration. This could allow a controlled release of the target drug without the disadvantage of reduced activity.

Due to PEG-antibody formation in some clinical studies, numerous conjugation alternatives have been also developed including HESylation which describes the attachment of hydroxyethyl starch or PASylation, representing the conjugation of proline, alanine and serine. However, it remains questionable if these alternatives can compete with PEGylation in terms of efficacy and acceptance.

2 Research Proposal

As already described the introduction, so called random PEGylation methods are widely used to overcome particular limitations of biopharmaceuticals. In contrast to difficult site-specific PEGylation methods, the random approach via surface amino acids provides a simple PEG attachment without the need of an additional and complex modification of the target protein. Due to accelerated approval procedures by the FDA, random PEGylation processes enable a short time to market and underline the established status of this method. However, as the term random already points out, this approach has distinct drawbacks. Although "random" indicates an uncontrollable modification of available binding sites, the name is rather derived from the significant influence of numerous process parameters on reaction behavior and the PEG attachment site. Consequently, variations in reaction conditions result in different heterogeneous product mixtures, with different isoform distributions, which affects important clinical aspects. The difficult isoform separation complicates the analysis of resulting mixtures, and thus the impact of corresponding parameters. This results in a lack of process knowledge, hindering the development of an ideal process. The missing process knowledge is also in contrast to regulatory guidelines, where well characterized processes and defined parameter control spaces are mandatory for a reproducible product profile. Possible disadvantages of random PEGylation processes consequently include the operation of the process far from the optimum and batch rejects of already approved processes due variations in product quality.

A possibility to improve such processes and to identify the influence of reaction parameters is given with the concept of high throughput screening (HTS), which is often applied in the downstream process development (DSP). Automated screening platforms enable an analysis of large parameter sets, which allows a systematic understanding of the impact on the corresponding process. Combined with miniaturized sample reactions and fast analytics, HTS can provide a fast optimization with minimal sample consumption. However, to achieve an optimization of random PEGylation reactions using HTS, the development of appropriate isoform analytics is a basic requirement.

At the beginning of this work, the main aim was consequently the development of high sensitive analytics to investigate isoform distributions in PEG-protein mixtures. Due to numerous studies with lysozyme and mPEG-aldehyde, this model reaction was chosen in this context. Another objective was the miniaturization of PEGylation samples and the automated sample preparation to provide sufficient data points for the screening of reaction conditions. The combination of high throughput experimentation (HTE) with isoform analytics should enable a deeper process understanding of the model reaction that results in a rather directed and precise PEGylation of available binding sites, than a "random"

modification. Another aspect in this work included the concept of on-column or solid-phase PEGylation which represents an alternative PEGylation method in contrast to commonly applied batch PEGylation reactions. With the obtained information about the impact of PEGylation conditions in free solution, the adsorber based PEGylation should be evaluated.

3 Publications and Manuscripts

1. Isoform separation and binding site determination of PEGylated lysozyme using pH chromatography

Benjamin Maiser, Frieder Kröner, Florian Dismer, Gerald-Brenner-Weiß, Jürgen Hubbuch

In this paper the chromatographic separation of purified mono-PEG-lysozyme isoforms with a pH gradient is described. Five of six possible isoforms were separated, which represents a significant increase in resolution in contrast to classical salt gradient runs. Applying a correlation between calculated isoelectric points (pI) of each isoform and their elution pH value, PEG binding sites were identified. A common peptide mapping approach with combined MALDI-MS analytics verified the results.

Published manuscript, Journal of Chromatography A, 1268, 102-108, 2012

2. Rapid quantification of protein-PEG conjugates by multivariate evaluation of chromatographic data

Sigrid K. Hansen, Benjamin Maiser, Jürgen Hubbuch

In this manuscript the development of a fast protein-PEG conjugate analysis is presented. An increase in sample throughput was achieved by using high flow rates and small column volumes. Multivariate calibration was applied to offset the reduction in resolution with analytical speed. Additionally, a dynamic calibration approach was developed to account for changes in column performance. Compared to different multivariate approaches multilinear regression was found to be the most suitable method.

Published manuscript, Journal of Chromatography A, 1257, 44-47, 2012

3. Optimization of random PEGylation reactions by means of high throughput screening

Benjamin Maiser, Florian Dismer, Jürgen Hubbuch

This publication describes the implementation of high throughput process development (HTPD) to the optimization of lysozyme PEGylation reactions. Applying PEGamer and

isoform analytics introduced earlier by the authors, the influence of different reaction parameters on kinetics and isoform distribution was investigated. It could be demonstrated, that different buffer pH values led to a main PEGylation of either lysine 33 or the N-terminus. To show the potential for an industrial application, the parameter control space for maximal volumetric activity was calculated with an additional enzyme activity assay.

submitted manuscript, Biotechnology and Bioengineering, 2013

4. Effect of lysozyme solid–phase PEGylation on reaction kinetics and isoform distribution

Benjamin Maiser, Florian Dismer, Jürgen Hubbuch

In this manuscript, the concept of solid–phase PEGylation was applied using small scale PEGylation experiments. It includes the influence of two different adsorber types on PEGamer reaction kinetics and the isoform distribution compared to reactions in free solution. PEGylation kinetics showed a significantly reduced reaction towards PEGylated species and the altered isoform distribution showed a good correlation to lysozyme binding orientations on corresponding resins.

Isoform Separation and Binding Site Determination of PEGylated Lysozyme using pH Gradient Chromatography

Benjamin Maiser[a], Frieder Kröner[a], Florian Dismer[a], Gerald Brenner-Weiß[b] and Jürgen Hubbuch[a]*

[a] *Institute of Engineering in Life Sciences, Biomolecular Separation Engineering Karlsruher Institute of Technology (KIT), Karlsruhe, Germnay*

[b] *Institute of Functional Interfaces, Karlsruher Institute of Technology (KIT), Karlsruhe, Germany*

*Corresponding author: Tel: +49 721 608-46263, Mail: juergen.hubbuch@kit.edu
Engler-Bunte-Ring 1, 76131 Karlsruhe

Journal of Chromatography A
Volume 1268, (2012), 102-108

Abstract

Covalent attachment of PEG to proteins, known as PEGylation, is currently one of the main approaches for improving the pharmacokinetics of biopharmaceuticals. However, the separation and characterization especially of positional isoforms of PEGylated proteins are still challenging tasks. A common purification strategy uses ion exchange chromatography with increasing ionic strength by shallow salt gradients. This paper presents a method which applies a linear pH gradient chromatography to separate five of six possible isoforms of mono PEGylated lysozyme, modified with 5 kDa and 10 kDa mPEG-aldehyde. To identify the corresponding PEGylation sites a comparison of elution pH values and calculated isoelectric points of each isoform, was used. The resulting correlation showed an $R^2 > 0.99$. Fractionation, tryptic digestion and subsequent MALDI-MS analysis of each peak, verified the predicted elution order. Based on UV areas the N-terminal amine at lysine 1 exhibited the highest reactivity, followed by the lysine 33 residue.

Keywords: PEGylation, lysozyme, isoform separation, pH-gradient

1 Introduction

Since the first PEGylation in 1977 by Abuchowski and Davis, polymer modification with poly(ethylene glycol) (PEG) has become an important method to enhance the pharmacological properties of therapeutic biopharmaceuticals [1]. The covalent attachment of PEG chains to a target molecule is well established and successfully used for numerous FDA approved proteins such as PEGylated interferon-α and erythropoietin (Pegasys® and Micera® from Hoffmann LaRoche, respectively).

Advantages of polymer modification generally include increased circulation half-life and a reduced immunogenicity of the conjugate compared to the unmodified form. Additional positive effects of PEGylation can be an increased thermal stability as well as a higher solubility which are also important for the therapeutics final formulation [2–4]. These changes in the pharmacological behavior can mostly be explained with the increased hydrodynamic radius of the conjugate and the resulting "shielding effect" of the attached PEG, which is reviewed in detail by many publications [5–7].

For PEG attachment, various activated PEG agents and coupling strategies are commercially available. Depending on the polymer modification, the attachment takes place at different surface residues of the target molecule. A common chemistry for PEGylation targets accessible amino residues such as lysine or the N-terminus. For this, a modification with succinimidyl activated PEGs (PEG-NHS) or PEG-aldehyde as shown in Figure 1, can be applied. While PEG-NHS is capable of binding also with histidine and tyrosine residues, the latter approach allows a main binding of PEG at the N-terminal α-amine at low pH values and was chosen by Kinstler et. al. [8, 9] to develop polymer modified granulocyte colony stimulating factor (G-CSF, Neulasta® from Amgen).

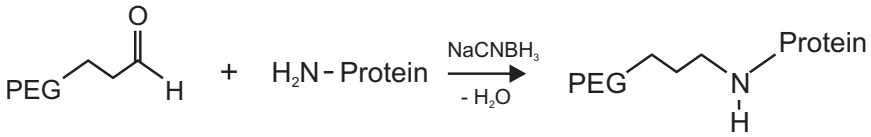


Figure 1: PEGylation reaction with PEG-aldehyde and $NaCNBH_3$ as reducing agent.

Reactions with target molecules that exhibit more than one accessible conjugation site, result in randomly attached PEGs and thus in heterogeneous mixtures. The resulting product consists of proteins with a different number of bound PEG and positional isoforms. Due to steric hindrance of the attached polymer, changes in PEGylation degree and binding site can have a major influence on biological activity of the different conjugates [10, 11]. With only 7% residual activity compared to the native protein, Pegasys®, a mixture of different PEG-interferon (IFN) isoforms, shows how drastic this effect can be [12]. Additional experiments with PEG-IFN showed a range of residual activity from 6% to 40% depending on the binding site of the attached PEG [13]. The loss in activity is generally compensated by the increased body residence time, but the high variance in activity underlines the influence of the PEGylation site. Consequently, mixture characterization and isoform identification is of

high interest and also needed for regulatory approval. Additionally, the selective PEGylation either of a single site or the screening of reaction conditions towards favorable PEGylation sites with high residual activity is preferred.

The separation of conjugate mixtures regarding the PEGylation degree can be achieved effortlessly with size exclusion chromatography (SEC), due to the increase in size. Thus, fast monitoring of crude PEG-protein mixtures combined with an improved data evaluation, such as multivariate data analysis (MVDA) can be achieved by size based separation and can be used in a first step to screen and optimize different PEGylation conditions [14]. A less systematic approach for a lysozyme PEGylation optimization without an isoform analytic was shown, for example, by Moosmann et al. [15].

However, the separation of isoforms with varying attachment site and the preparative purification is challenging. Many approaches showed that ion exchange chromatography (IEC) is an effective tool and currently the method of choice for conjugate and positional isoform separation, by means of shallow salt gradients [16–18]. The different behavior is based upon the shielding effect of attached polymer chains, and thus reduced interactions between chromatographic matrix and protein. Additionally a decreasing protein surface charge with an increasing number of bound PEG weakens the interaction with the oppositely charged resin, which was already shown by Fee in an *in silico* approach [19]. Applying classical salt gradient chromatography, different lysozyme PEGylation studies with PEG-NHS and PEG-aldehyde showed that it was not possible to separate more than three isoforms, although six isoforms are being formed during PEGylation. Even though some approaches showed promising results, only little attention was paid to alternative ion exchange chromatography with pH gradients [13, 20].

Besides the isoform separation, identification of PEG attachment sites is another challenge. Time consuming methods that are widely used comprise Edman degradation for small peptides and peptide mapping with combined MALDI-TOF analysis [21–23]. The mass spectrometric approach was applied by Lee and Park [21] for the characterization of PEGylated lysozyme. For lysine residue modification the authors used biotin-PEG-NHS, to separate PEG-peptide fragments from unmodified peptides, after tryptic digestion. Employing mass spectrometrical analysis of the peptide fragments, three positional isoforms with different reactivities were identified.

The presented work describes the separation of mono-PEGylated lysozyme isoforms with a linear pH gradient on a cation exchange column. In contrast to salt gradient chromatography runs, a significant increase in resolution could be achieved and five of six possible isoforms were separated. Based on the assumption that every PEG conjugation to amino residues neutralizes an effective charge of the protein, a fast *in silico* approach was used to calculate the isoelectric point of each isoform. The elution pH values were correlated with the calculated isoelectric points to identify the PEGylation sites and to determine the isoform reactivities. Peptide mapping and the common mass spectrometric approach were applied to verify the results.

2 Materials and methods

2.1 Chemicals

Sodium phosphate and sodium chloride for chromatography buffer and PEGylation buffer preparation were obtained from Merck (Darmstadt, Germany). Hen egg white lysozyme and sodium cyanoborohydride ($NaCNBH_3$) were provided from Sigma-Aldrich (St. Louis, MO, USA). Methoxy-PEG-propionaldehyde (mPEG-aldehyde) with an average molecular weight of 5 kDa and 10 kDa was provided by NOF Cooperation (Tokyo, Japan). CABS (cyclohexylamino-butansulfonic acid) with a pK_a value of 10.7 was provided from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and was used as buffer substance for pH-gradient chromatography. The calibration of the pH meter was performed with high precision calibration standards from Hanna instruments (Woonsocket, RI, USA). The reagents for the proteolytic digestion of the PEGylated protein, including ammoniumbicarbonate which was used as the buffering substance, as well as dithiothreitol and iodoacetamide which were used for the reduction and alkylation of the disulfide bonds, were obtained from Sigma-Aldrich. Proteolytic digestion was prepared with proteomics grade trypsin from Sigma-Aldrich and RapiGest surfactant (Waters Corporation, Milford, MA, USA). For the hydrolysis of the acid labile surfactant trifluoracetic acid (TFA) from Sigma-Aldrich was used. For MALDI matrix preparation α-cyano-4-hydroxycinnamic acid (CHCA) from Sigma-Aldrich and LC-MS grade acetonitrile from Merck (Darmstadt, Germany) was used. Ultrapure water was generated with the Arium pro water purification system from Sartorius Stedim (Goettingen, Germany). All solutions used for chromatography were filtered using 0.2 μm cellulose acetate filters from Sartorius Stedim (Goettingen, Germany) and degassed for 20 min in an ultrasonic bath.

2.2 Batch PEGylation reaction

Lysozyme (5 mg/mL) and mPEG-aldehyde with a molar polymer to protein ratio of 6:1 were dissolved in a 25 mM sodium phosphate buffer pH 7.2. containing 20 mM sodium cyanoborohydride as reducing agent. The reaction was carried out in a continuously shaken falcon tube at room temperature, for about 3.5 h. Monitoring of the PEGylation reaction was conducted with size exclusion chromatography (SEC) using a Superdex 200 GL10/300 column on a Äkta Ettan system (GE Healthcare, Uppsala, Sweden). For SEC 25 mM sodium phosphate pH 7.2 containing 150 mM NaCl as mobile phase was used.

2.3 Analytical protocol

2.3.1 Separation of PEGylation degree

After the PEGylation reaction an IEC was used as a first chromatographic step to separate the different PEGylation degrees and to stop the reaction. The reaction mixture was separated with a Toyopearl GigaCap S-650M resin (Tosoh Bioscience GmbH, Stuttgart, Germany) packed according to the manufacturer's protocol into an Omnifit glas column (25 mm x 400 mm, Diba Industries Ltd., Cambridge, UK). The resulting bed volume was 13.4 mL.

The sample was diluted with ultrapure water (1:1) to reduce ionic strength. After column equilibration with running buffer (25 mM sodium phosphate, pH 7.2), 50 mL sample mixture was loaded onto the column. For elution a gradient ranging from 0 to 40 % of the elution buffer (25 mM sodium phosphate, 500 mM sodium chloride, pH 7.2) was performed over 21 column volumes. The flow rate was maintained at 1 mL/min. To obtain mono-PEG lysozyme samples, the fractionation volume was set to 5 mL. Until further measurement, the fractions were pooled and stored at -32°C.

2.3.2 Molecular weight determination

Peak fractions from IEC were analyzed by SEC with a light scattering (LS) detector. This analysis was conducted using an Äkta Ettan system from GE Healthcare (Uppsala, Sweden) in combination with a Dawn Heleos 8+ multi-angle LS detector and an Optilap rEX refractive index (RI) detector, both from Wyatt Technology (Santa Barbara, USA). The LS detector was equipped with a fused silica cell and a laser with a wavelength of 658 nm. The LS and RI detectors were calibrated with toluene and NaCl, respectively. The LS detector was normalized using 2 mg/ml of bovine serum albumin (BSA) monomer from Sigma Aldrich, as reference. For SEC a Superdex 200 GL10/300 (GE Healthcare, Uppsala, Sweden) with a mobile phase of 25 mM sodium phosphate, pH 7.2, containing 150 mM NaCl was used. The flow rate was set to 0.8 mL/min. Injection volumes between 50 and 100 μL were chosen. After UV absorbance monitoring at 280 nm using the Äkta UV-900 monitor, each sample passed the LS and RI detectors. Correction of detector alignment and band broadening, as well as molecular weight (M_w) calculation were done by the ASTRA software (software version 5.3.4.18)

2.3.3 Separation of isoforms

Isoform separation was conducted on a MonoS 4.6/100 column from GE Healthcare (Uppsala, Sweden). Sample volumes between 100 μL and 150 μL of previously purified mono-PEG lysozyme (modified with 5 kDa and 10 kDa mPEG) were chosen. For pH-gradient elution 20 mM CABS was used as buffer component. Running buffer A and elution buffer B were titrated with 4 M NaOH to pH 10.5 and pH 11.5, respectively. The pH was measured with a pH meter from Hanna Instruments (Woonsocket, RI, USA), calibrated from pH 10 to 12 with high precision calibration standards. The elution was carried out with a linear gradient ranging from 0% to 100% buffer B over 12 column volumes. The flow rate was set to 1.5 mL/min. To obtain samples for MALDI-TOF analysis the resulting peaks from mono-$PEG_{5.000}$-lysozyme isoforms were fractionated with a constant volume of 250 μL. The fractions of multiple runs were pooled to reach sufficient amount of single isoforms for subsequent mass spectrometrical peptide mapping. The results of the pH gradient separation were additionally compared with a classical salt gradient based cation exchange chromatography. For salt gradient elution the same column, injection volume as well as gradient length were used. As buffer system 25 mM sodium phosphate pH 7.2 was used. The elution was carried out with a salt gradient reaching from 0 mM to 500 mM NaCl.

2.3.4 Tryptic digestion of PEGylated lysozyme

After the chromatographic runs the collected fractions were transferred to VivaSpin 20 ultrafilters (Sartorius Stedim, Germany) with a molecular weight cutoff of 5 kDa and were diafiltrated into 50 mM ammoniumbicarbonate buffer with pH 8.0 including 0.1 % (v/v) RapiGest. Additionally the fractions were concentrated to the maximum degree and the protein concentration was determined via UV 280 nm absorption measurement on a Infinite M200 plate reader (Tecan, Maennedorf, Switzerland). The samples were then chemically reduced by the addition of dithiotreitol up to a final concentration of 20 mM and denatured for 30 minutes at 60°C on a thermoshaker (Eppendorf, Hamburg, Germany). Alkylation was carried out by adding iodoacetamide with a final concentration of 60 mM and mixing the samples for 45 min on a shaker under exclusion of light. The prepared samples were then digested with a trypsin to protein ratio of 1/2 - 1/10, depending on the sample protein concentrations. The digestion was carried out at 37°C over night. After the digestion, TFA was added to a final concentration of 0.5 % (v/v) and the samples were incubated for another 45 minutes and then centrifuged to remove the RapiGest surfactant from the solution. The samples were stored at -32°C until measurement.

2.3.5 MALDI-MS based PEGylation site identification

To prepare samples for the final MALDI-MS measurement, they were processed with C18 ZipTips (Millipore Corporation, Billerica, MA, USA). The samples were bound to preconditioned C18 ZipTips by pipetting and then eluted with the matrix solution, 10 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in 70% (v/v) ACN and directly spotted on a MALDI stainless steel target. After drying the sample spots, MS analytics were analyzed in a MALDI TOF/TOF 4800 analyzer (Applied Biosystems, Framingham, MA, USA). The mass spectrometer was run in positive, reflectron mode. Optimized device settings for the matrix were used. The measured data was analyzed and exported using Data Explorer Software 4.0 (Applied Biosystems, Framingham, MA, USA). To identify the PEGylation site the masses of the expected peptides were predicted with the PeptideCutter tool (Expasy - SIB Bioinformatics Resource Portal), modified by the mass of the carbamidomethylation and compared to the results of the single measurements. The PEGylated peptide fragment is heavier by the mass of the PEG molecule (5008 Da) and could therefore be identified. Due to the polydispersity of the PEG molecule the average mass of the measured fragment was determined by "gauss-fitting" the resulting signal and determining the maxima of the fitted curve with MATLAB (MathWorks, Natick, MA, USA).

2.4 pI calculation

Isoelectric point calculation of native lysozyme and PEGylated lysozyme isoforms were conducted with the freely accessible web tool 'protein continuum electrostatics' (PCE). This tool calculates pK_a values of titratable groups in proteins solving the Poisson–Boltzmann equation based on the MEAD (macroscopic electrostatics with atomic detail) program, devel-

oped by Bashford [24, 25]. For pI calculation, the structural information of native lysozyme (PDB-ID: 132L) obtained from the RSCB protein data bank was used. With resulting pK_a values, pI calculation was done applying the Henderson–Hasselbalch equation with Microsoft Excel (Microsoft, Redmond, MA, USA). The pI value of each PEG-isoform was calculated with neutralized lysine residues, involved in the conjugation.

3 Results and discussions

3.1 Lysozyme PEGylation degree

As PEGylation target the model protein lysozyme from chicken egg was used in this work. According to the manufacturers datasheet it has a molecular weight of 14.3 kDa and an isoelectric point of 11.3. Possible binding sites for the PEG-aldehyde reaction are six lysine residues and the N-terminal amino group. The three-dimensional model, shown in Figure 2, illustrates a lysozyme molecule and depicts that all lysine residues as well as the N-terminus are located at the surface of the molecule, which can be explained with their hydrophilic character. With the additional N-terminal amino group, lysine 1 contains two binding sites. Consequently, PEGylation reactions with amino coupling PEG agents can yield six mono-PEG lysozyme isoforms. However, different PEGylation studies with PEG-NHS and PEG-aldehyde showed, that it was not possible to detect and separate more than three isoforms [18].

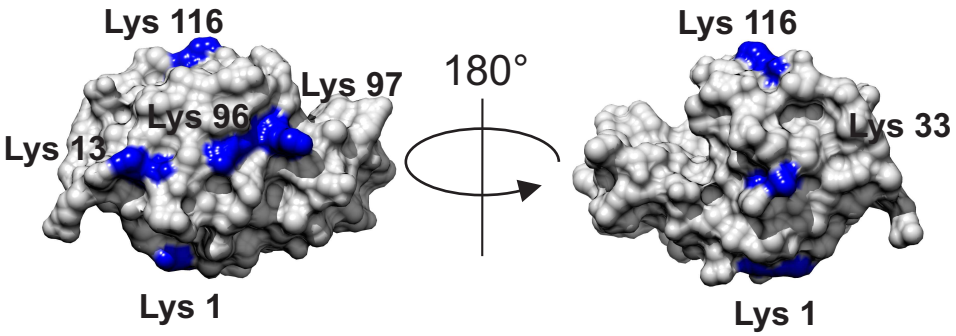


Figure 2: Two orientations of the three dimensional structure of lysozyme with labeled surface lysine residues.

The PEGylation reaction was monitored using SEC, to optimize the reaction time towards a high mono-PEG-lysozyme yield. In Figure 3 the SEC chromatograms of PEG-lysozyme mixtures modified with 5 kDa and 10 kDa PEG-aldehyde, after 3.5 hours reaction time are shown. The different elution volumes in each mixture result from the increase in size by PEG attachment and indicates different PEGylation degree. As can be seen, di-$PEG_{5.000}$- and mono-$PEG_{10.000}$-lysozyme conjugates have the same retention time in SEC, resulting from the same hydrodynamic radius. This illustrates that the increase in size is only dependent of the molecular weight and is regardless of the number of bound PEG, which was already shown by Fee and Van Alstine [26]. As PEG-aldehyde is a non UV active component,

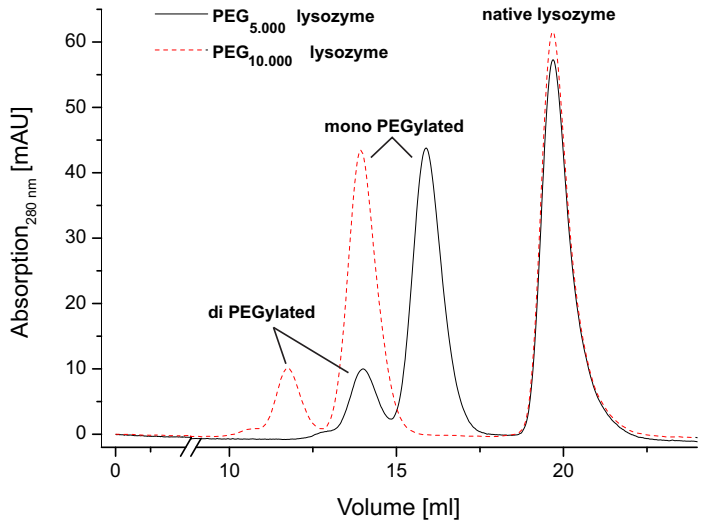


Figure 3: SEC chromatograms of 5 kDa and 10 kDa PEGylated lysozyme mixtures after 3.5 h with a Superdex 200 10/300 GL column and following elution order: di-PEG-lysozyme, mono-PEG-lysozym and native lysozyme.

unreacted PEG was not detectable in the 280 nm UV trace.

For mono-PEG-lysozyme sample preparation, preparative purification of PEG-lysozyme mixtures with ion exchange chromatography was applied, according to Moosmann et al. [27]. In addition to PEG-lysozyme conjugate separation, this method provides a removal of unreacted native protein as well as a flow through of unreacted PEG. Figure 4 depicts a resulting chromatogram with a 5 kDa PEG-lysozyme mixture and shows a comparable elution behavior to SEC (see Figure 3). After peak fractionation, SEC with combined light scattering was conducted with mono-PEG-lysozyme samples to verify purity and PEGylation degree. The overall molecular mass of pooled mono-$PEG_{5.000}$-lysozyme samples were calculated to 19.2 kDa with a protein fraction of 14.1 kDa. Calculated values of mono-$PEG_{10.000}$-lysozyme samples provided masses of 24.2 kDa and 13.9 kDa, respectively. SEC chromatograms of purified mono-PEG-lysozyme samples showed no impurities of other PEGylation degrees (data not shown).

3.2 Isoform separation

The isoform separation was conducted with purified mono-PEGylated-lysozyme samples, modified with 5 kDa and 10 kDa PEG-aldehyde. In Figure 5 the resulting chromatograms with salt gradient and pH gradient elution are shown. Five peaks could be detected in the 280 nm trace in the pH elution chromatogram, while salt gradient elution resulted in a separation of only three peaks. The different elution volumes of each peak can be linked to altered surface charge due to different PEGylation sites. As only pure mono-PEG lysozyme samples were injected, each peak represents lysozyme with one bound PEG chain attached to a different amino residue. Even though lysozyme consists of six possible conjugation sites, as mentioned before, only five isoforms were detected. The separation of a 10 kDa PEG

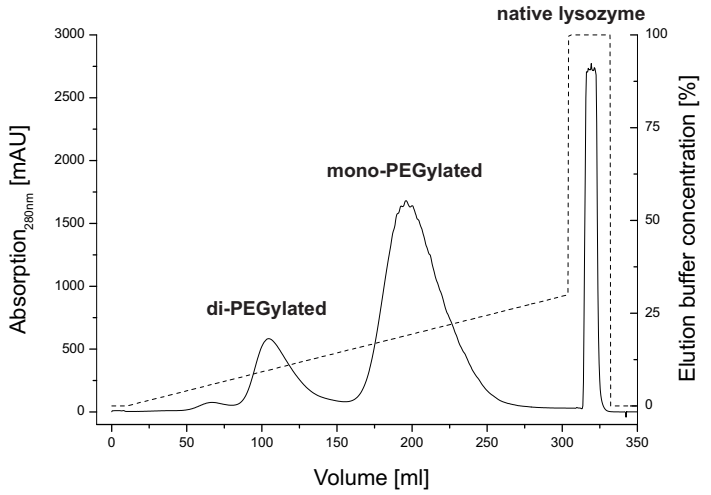


Figure 4: Chromatogram of preparative purification of 5 kDa PEG-lysozyme mixture, using a GigaCap 650S ion exchange adsorber. PEGylation degree was validated with SEC-LS.

modified lysozyme sample showed lower elution pH values, but the same elution pattern compared to mono-$PEG_{5.000}$-lysozyme.

3.3 Isoform identification

In pH gradient chromatography the elution of proteins is based on a decreasing surface charge, which results in a decreasing strength of interaction between the adsorbed proteins and the oppositely charged adsorber. Thus, resulting elution pH values correlate sometimes with the proteins isoelectric point [28]. In classical salt gradient chromatography the proteins are eluted due to an ion exchange process. This difference leads to salt and pH gradient elution strategies, which can result in a different separation performance. To indicate the elution order of the separated isoforms, pI calculations were performed with the PCE tool from Miteva et al. [29]. Applying the described method, the pI for native lysozyme was calculated as 11.28, with a good correlation to already published values by Ahamed et al. [28].

The altered charge of the lysine residues caused by attaching a PEG molecule was taken as a basis for the isoform pI calculations. Assuming that every PEG conjugation to the primary amine of a lysine residue neutralizes the positive charge, surface net charge, isoelectric point and also elution pH are reduced compared to native lysozyme. For isoform pI calculation of PEG-lysozyme, lysine pK_a values involved in the conjugation were ignored. Titration curve calculations, using the Henderson-Hasselbalch equation to determine the proteins pI, were applied regardless of the attached PEG molecular weight. Comparing the elution pH values with the calculated isoelectric points, a peak identification to corresponding binding sites as shown in Table 1 was conducted. The isoform with PEG binding site lysine 33 (in the following labeled as PEG-lys_{33}) exhibited the lowest calculated pI and was assigned to the peak with the lowest elution pH. As lysine 97 and 116 exhibited the same calculated pI, these isoforms were assumed to elute in one peak. The isoform elution sequence obtained from pI

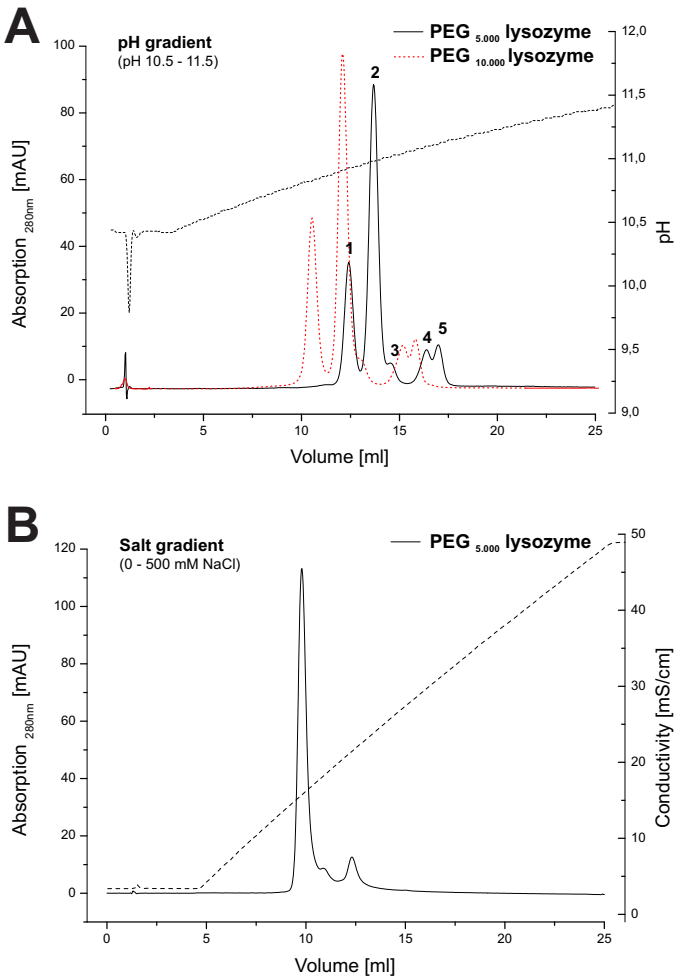


Figure 5: Chromatogragraphic separation of PEG-lysozyme isoforms using a pH gradient with a pH rage from 10.5 to 11.5 (**A**) and a salt gradient from 0 mM to 500 mM NaCl in 25 mM phosphate buffer, pH 7.2 (**B**) on a Mono S 4.6/100 column. The peak labels correspond to different positional isoforms.

calculation results, starting with the lowest elution pH, was as follows: PEG-lys$_{33}$, PEG-lys$_{1}$, PEG-lys$_{96}$, followed by PEG-lys$_{97}$ and PEG-lys$_{116}$ eluting in one peak and PEG-lys$_{13}$ eluting last.

Figure 6 depicts the obtained results and shows that native lysozyme eluted roughly at its isoelectric point. PEG-isoforms eluted earlier and at lower pH values than native lysozyme, as expected. Additionally the elution volume of both PEG species correlated with an $R^2 > 0.99$ to the calculated pI values. However, the elution pH of 10 kDa-PEG isoforms and 5 kDa-PEG isoforms were shifted to lower pH values, indicating a lower interaction to the adsorber matrix with increasing PEG weight.

In addition to reduced net charge, attached PEG chains might weaken the interaction of the isoforms to the adsorber matrix, due to steric hindrance. This effect is probably dependent on the attached molecular PEG weight. An increased protein-resin distance, might thus explain the differences in the elution behavior of mono-PEG$_{5.000}$- and mono-PEG$_{10.000}$-lysozyme isoforms. A study by Abe et al. [10] investigated this effect for ion exchange chromatography and can be used for a detailed view of PEG-protein binding mechanisms. By comparing the

Table 1: Elution pH values of separated mono-PEG-lysozyme isoforms and corresponding calculated isoelectric points. Calculation of isoelectric points were applied with pK_a values based from PCE-tool.

Binding site	Calculated pI	Elution pH 5 kDa	Elution pH 10 kDa
lys 33	11.07	10.94	10.88
lys 1	11.12	10.99	10.92
lys 96	11.18	11.03	10.98
lys 97 lys 116	11.27	11.11	11.05
lys 13	11.28	11.13	11.08
native lysozyme	11.28	11.23	

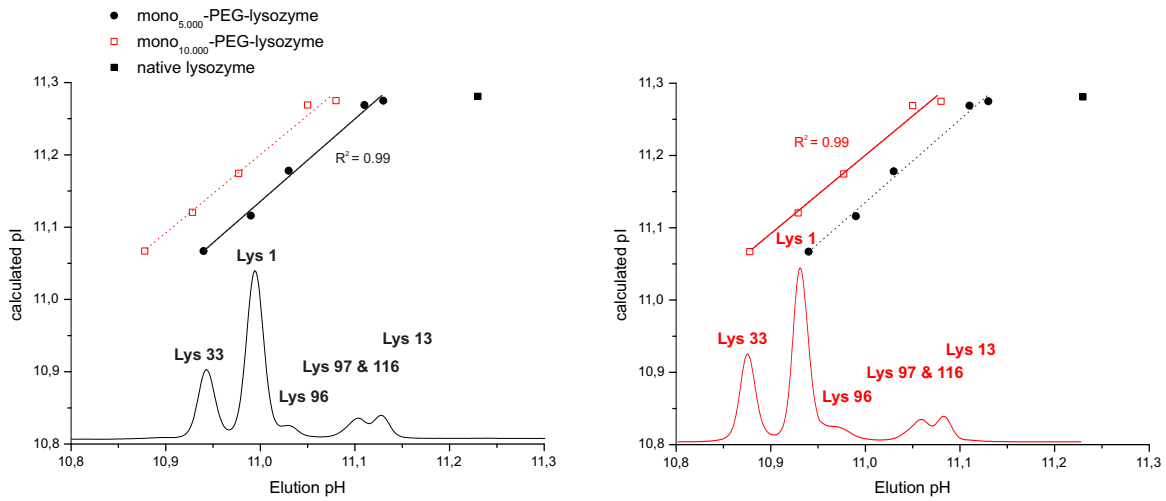


Figure 6: Plotted elution pH values of mono-PEG-lysozyme isoforms (left: modified with 5 kDa, right: modified with 10 kDa) and native lysozyme versus calculated isoelectric points.

UV areas of each isoform, the reactivity of the different binding sites was evaluated. Thus, PEGylation with 5 kDa and 10 kDa PEG occurs preferentially at lys 1, followed by lys 33 using the described reaction conditions. Only small reactivities were found for lysine 97, 116 and 96.

3.4 MALDI-MS analysis

To verify the conducted binding site identification, peptide mapping in combination with mass spectrometric analysis was applied. The common procedure involves a tryptic digestion of the isoform mixture and the comparison of the resulting peptides with the peptide pattern of a digested native protein solution. Considering trypsin is sterically blocked by attached PEG molecules, missing peptides refer to PEG-conjugation sites.

Instead of analyzing the peptide pattern, we decided to investigate the mass of the PEG-peptide fragments. Lysozyme peptides resulting from tryptic digestion, hydrolyzed at lys 1 or lys 97 consist of only one amino acid and are consequently difficult to detect within the signals of the used MALDI matrix. With the analysis of the heavier PEG-peptide fragments, this problem was avoided. Analyzing unbound PEG-aldehyde samples with the described

Table 2: PEG binding site, resulting mass of peptide-fragments and measured mass of PEG-peptide fragments of $mono_{5000}$-PEG-lysozyme isoforms. Theoretical mass of each PEG-peptide-fragment was calculated with a PEG weigth of 4990 kDa and corresponding peptides.

Peak number	**PEGylation site**	**Peptide mass [m/z]**			**PEG-peptide fragment mass [m/z]**	
					expected	measured
1	**Lys 33**	22-**33**	34-35			
		1325.63	1428.65		7744	7697
2	**Lys 1**	**1**	2-5			
		147.11	478.28		5615	5619
3	**Lys 96**	74-**96**	97			
		2508	147.11		7645	n/A
4	**Lys 116**	115-**116**	117-125			
		307.14	1045.54		6343	6352
	Lys 97	**97**	98-112			
		147.11	1675.80		6813	n/A
5	**Lys 13**	6-**13**	14	15-21		
		893.42	175.12	874.42	6933	6854

MALDI-TOF analytics the polydisperse character of the polymer was obvious, which is caused by the production process. Pure PEG samples showed a normally distributed signal which was composed of single mass peaks. Each peak showed differences in weight of 44 Da, indicating PEG chains with a different number of monomer units. Fitting the PEG sample peaks using a gaussian fit function in MATLAB®, the calculated average molecular mass was 5031 Da. The quality analysis by the PEG supplier provided an average weight of 5008 Da for the same charge, confirming the used fitting method.

Four PEG-peptide fragments were detected in the separated and fractionated PEG-lysozyme isoform samples. Mass differences between the mass peaks in each detected PEG-peptide signal were 44 Da and refer consequently to PEG. The PEG-peptide fragments, detected in isoform fractions PEG-lys_{33} and PEG-lys_1 are shown in Figure 7, with the corresponding calculated gaussian fits and peak maxima. Weight differences between the two fragment signals can be noticed, which are caused by different peptide masses due to varying PEG binding sites.

Calculated theoretical PEG-peptide fragments and evaluated average masses of analyzed PEG-peptide signals are listed in Table 2 and assigned to their corresponding binding sites. For calculating the theoretical total PEG-peptide mass, the PEG suppliers weight specification was used and the mass of one water molecule was subtracted. A good agreement of theoretical and the measured masses of the first two peaks, labeled as PEG-lys_{33} and PEG-lys_1 can be noticed. Considering baseline separation was provided between these peaks the proposed peak identification was validated.

Peak 3, labeled as PEG-lys_{96} contained no detectable PEG mass peaks. Due to small sample concentrations, a detection of the PEG-peptide signal was not possible. In addition,

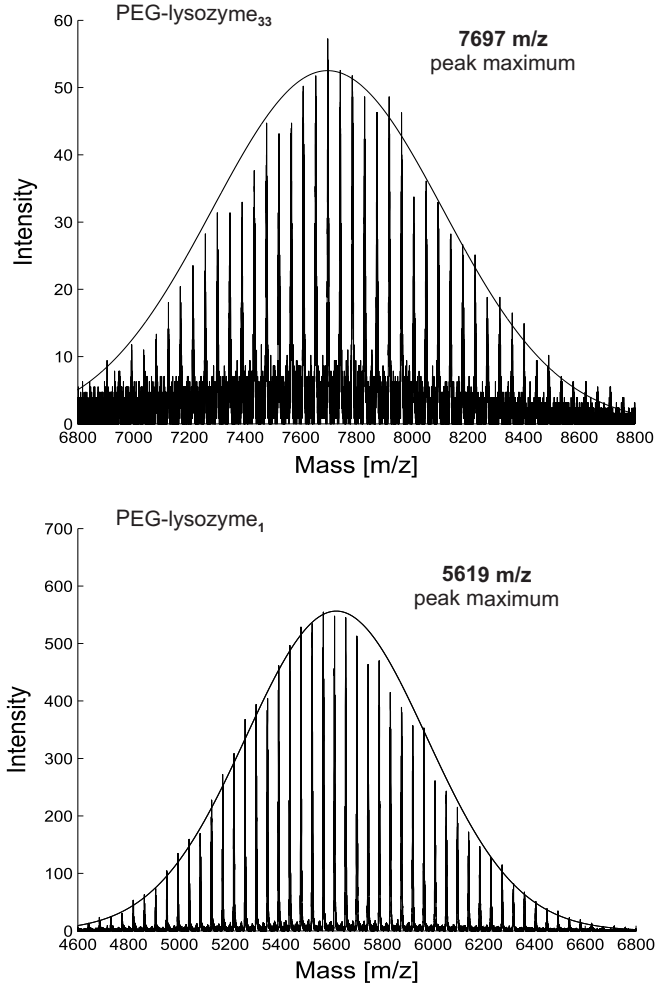


Figure 7: MALDI-TOF spectra with PEG-peptide fragments of two 5 kDa PEG-lysozyme isoforms. Spectra correspond to lysozyme with bound PEG at lysine 33 (PEG-lysozyme$_{33}$) and lysine 1 (PEG-lysozyme$_1$), respectively

.

the small resolution to peak 2, hindered the fractionation of sufficient sample volume.

Instead of two PEG signals in peak 4, only one PEG-peptide fragment was found. With an average weight of 6352 m/z the measured signal corresponds well to the theoretical PEG-peptide mass of 6343 m/z of the proposed lys 116 binding site.

In peak 5, labeled as PEG-lys$_{13}$ a theoretical PEG-peptide weight consisting of two peptides and attached PEG could not be found. Thus, the corresponding PEG-peptide mass was corrected assuming a sterical hindrance of PEG blocking the tryptic digestion between lys 13 and arg 14 additionally. The resulting expected fragment weight of 6933 m/z agreed then with the measured average weight of 6854 m/z. Comparing the mass peaks of the last isoform peak, it can be noticed that the measured mass of 6854 m/z is between the expected weight of binding site lys 97 and lys 13. Therefore, the proposed lys 13 binding site of peak 5 could not be validated by MS analytics.

A comparison of the findings using MS analytics with the alternative *in silico* binding

site identification showed a good correlation and demonstrates the applicability of this fast method. Lysozyme PEG binding site identifications were already conducted by numerous authors including Lee et al. and Tilton et al. [21, 30], using MS analytics. Studies by Lee et al. suggest lysine 33, lysine 97 and lysine 116 as major PEG binding sites, using a PEG-NHS modification. Tilton applied a PEG-aldehyde reaction and proposed a predominant N-terminal (lysine 1) modification of mono-PEG-lysozyme, with slight modifications at lysine residues 33 and 97. This suggests a major influence of the coupling reaction to the resulting PEG conjugation site. By comparing the results made in this study with results published by Tilton with the same PEG reaction, a good agreement regarding the lysine reactivities can be detected. In addition both results corresponds with the fact that main attachment of PEG-aldehyde primarily occurs at the N-terminal amino group at low buffer pH values.

4 Conclusion and outlook

In this paper the separation of five positional isoforms of mono-PEGylated lysozyme is shown, and thus represents an increase in resolution compared to published results. By applying a pH gradient elution, we were able to achieve a superior resolution in contrast to classical salt gradient runs, which underlines the high separation performance of pH gradients regarding charge variant separation. An isoform elution order and the resulting PEG conjugation sites were identified, using a fast in silico approach. The resulting reactivities of the identified lysine residues were evaluated, and showed a good agreement to comparable PEG reactions [30]. To validate the proposed binding site results, common MS analytics were applied. The usability of the *in silico* binding site identification application was only shown for PEG-lysozyme. With the presented chromatographic method, the evaluation of lysozyme isoform reaction kinetics are possible and are already under investigation. In addition, on-column PEGylation experiments can now be conducted and analyzed for changes in lysozyme binding orientations.

5 Acknowlegments

We gratefully acknowledge financial support by the Federal Ministry of Education and Research (BMBF). In addition we thank Gerald Brenner-Weiss and Boris Kühl from the Institute of Functional Interfaces, Karlsruhe Institute of Technology (KIT) for supporting the MALDI-TOF analysis.

References

[1] A. Abuchowski, T. Vanes, N. C. Palczuk, and F. F. Davis. Alteration of immunological properties of bovine-serum-albumin by covalent attachment of polyethylene-glycol. *J. Biol. Chem.*, 252(111):3578–3581, 1977.

[2] Y. Nodake and N. Yamasaki. Some properties of a macromelecular conjugate of lysozyme

prepared by modification with a monomethoxypolyethylene glycol derivative. *Biosci. Biotechnol. Biochem.*, 64(4):767–774, 2000.

[3] B. K. Lee, J. S. Kwon, H. J. Kim, S. Yamamoto, and E. K. Lee. Solid-phase pegylation of recombinant interferon alpha-2a for site-specific modification: Process performance, characterization, and in vitro bioactivity. *Bioconjugate Chem.*, 18(6):1728–1734, 2007.

[4] R.l S. Rajan, T. Li, M. Aras, C. Sloey, W. Sutherland, H. Arai, R.. Briddell, O. Kinstler, A. M. K. Lueras, Y. Zhang, and et al. Modulation of protein aggregation by polyethylene glycol conjugation : Gcsf as a case study. *Protein Sci.*, 15(5):1063–1075, 2006.

[5] F. M. Veronese and G. Pasut. Pegylation, successful approach to drug delivery. *Drug Discov.Today*, 10(21):1451–1458, 2005.

[6] J. M. Harris, N. E. Martin, and M. Modi. Pegylation - a novel process for modifying pharmacokinetics. *Clin.Pharmacokinet.*, 40(7):539–551, 2001.

[7] Vladka Gaberc-Porekar, Irena Zore, Barbara Podobnik, and Viktor Menart. Obstacles and pitfalls in the pegylation of therapeutic proteins. *Curr. Opin. Drug Di. De.*, 11(2):242–50, Mar 2008.

[8] O. Kinstler, G. Molineux, M. Treuheit, D. Ladd, and C. Gegg. Mono-n-terminal poly(ethylene glycol)-protein conjugates. *Adv. Drug Deliver Rev.*, 54(4):477–485, 2002.

[9] O. B. Kinstler, D. N. Brems, S. L. Lauren, A. G. Paige, J. B. Hamburger, and M. J. Treuheit. Characterization and stability of n-terminally pegylated rhg-csf. *Pharm. Res.*, 13(7):996–1002, 1996.

[10] M. Abe, P. Akbarzaderaleh, M. Hamachi, N. Yoshimoto, and S. Yamamoto. Interaction mechanism of mono-pegylated proteins in electrostatic interaction chromatography. *Biotechnology journal*, 5(5):477–83, May 2010.

[11] G. Pasut. Pegylated alpha-interferons: two different strategies to achieve increased efiicacy. In F. M. Veronese, editor, *PEGylated Protein Drugs: Basic Science and Clinical Applications*, pages 205–216. Birkhäuser Verlag, 2009.

[12] F. M. Veronese and G. Pasut. Pegylation: Posttranslational bioengineering of protein biotherapeutics. *Drug Discov. Today*, 5(2-3):e57–e64, Sep 2008.

[13] S. P. Monkarsh, Y. M. Ma, A. Aglione, P. Bailon, D. Ciolek, B. DeBarbieri, M. C. Graves, K. Hollfelder, H. Michel, A. Palleroni, and et al. Positional isomers of monopegylated interferon alpha-2a: Isolation, characterization, and biological activity. *Anal. Biochem.*, 247(2):434–440, 1997.

[14] Sigrid K. Hansen, Benjamin Maiser, and JÃ$\frac{1}{4}$rgen Hubbuch. Rapid quantification of protein-peg conjugates by multivariate evaluation of chromatographic data. *J. Chrom. A*, Aug 2012. in press.

[15] A. Moosmann, J. Blath, R. Lindner, E. Müller, and H. Böttinger. Aldehyde pegylation kinetics: a standard protein versus a pharmaceutically relevant single chain variable fragment. *Bioconjugate Chem.*, 22(8):1545–58, Aug 2011.

[16] S. Foser, A. Schacher, K. A. Weyer, D. Brugger, E. Dietel, S. Marti, and T. Schreitmüller. Isolation, structural characterization, and antiviral activity of positional isomers of monopegylated interferon alpha-2a (pegasys). *Protein Expres. Purif.*, 30(1):78–87, Jul 2003.

[17] C. J. Fee and J. A. Van Alstine. Peg-proteins: Reaction engineering and separation issues. *Chem. Eng. Sci.*, 61(3):924–939, Feb 2006.

[18] S. Yamamoto, S. Fujii, N. Yoshimoto, and P. Akbarzadehlaleh. Effects of protein conformational changes on separation performance in electrostatic interaction chromatography: unfolded proteins and pegylated proteins. *J. Biotechnol.*, 132(2):196–201, 2007.

[19] C. J. Fee. Protein conjugates purification and characterization. In F. M. Veronese, editor, *PEGylated Protein Drugs: Basic Science and Clinical Applications*, pages 113–125. Birkhäuser Verlag, 2009.

[20] P. K. Ng, J. He, and M. Snyder. Separation of protein mixtures using ph-gradient cation-exchange chromatography. *J. Chrom. A*, 1216(9):1372–6, Feb 2009.

[21] H. Lee and T. G. Park. A novel method for identifying pegylation sites of protein using biotinylated peg derivatives. *J. Pharm. Sci.*, 92(1):97–103, Jan 2003.

[22] O. B. Kinstler, D. N. Brems, S. L. Lauren, A. G. Paige, J. B. Hamburger, and M. J. Treuheit. Characterization and stability of n-terminally pegylated rhg-csf. *Pharm. Res.*, 13(7):996–1002, 1996.

[23] J. Roberts Michael and J. Milton Harris. Attachment of degradable poly(ethylene glycol) to proteins has the potential to increase therapeutic efficacy. *J. Pharm. Sci.*, 87(11):1440–1445, 1998.

[24] D. Bashford and K. Gerwert. Electrostatic calculations of the pka values of ionizable groups in bacteriorhodopsin. *J. Mol. Bio.*, 224(2):473–86, Mar 1992.

[25] D. Bashford. *An object-oriented programming suite for electrostatic effects in biological molecules An experience report on the MEAD project*, pages 233–240. Springer, 1997.

[26] C. J. Fee and J. M. Van Alstine. Prediction of the viscosity radius and the size exclusion chromatography behavior of PEGylated proteins. *Bioconjugate Chem.*, 15(6):1304–1313, 2004.

[27] A. Moosmann, J. Christel, H. Böttinger, and E. Müller. Analytical and preparative separation of pegylated lysozyme for the characterization of chromatography media. *J. Chrom. A*, 1217(2):209–215, 2010.

[28] T. Ahamed, B. K. Nfor, P. Verhaert, G. van Dedem, L. van der Wielen, M. Eppink, E. van de Sandt, and M. Ottens. ph-gradient ion-exchange chromatography: an analytical tool for design and optimization of protein separations. *J. Chrom. A*, 1164(1-2):181–8, Sep 2007.

[29] M. A. Miteva, P. Tuffery, and B. O. Villoutreix. Pce: web tools to compute protein continuum electrostatics. *Nucleic Acids Res.*, 33(Web Server issue):W372–5, Jul 2005.

[30] S. M. Daly, T. M. Przybycien, and R. D. Tilton. Adsorption of poly(ethylene glycol)-modified lysozyme to silica. *Langmuir*, 21(4):1328–1337, Feb 2005.

Rapid Quantification of Protein-PEG Conjugates by Multivariate Evaluation of Chromatographic Data

Sigrid K. Hansen, Benjamin Maiser and Jürgen Hubbuch*

Institute of Engineering in Life Sciences, Biomolecular Separation Engineering
Karlsruher Institute of Technology (KIT), Karlsruhe, Germnay

*Corresponding author: Tel: +49 721 608-46263, Mail: juergen.hubbuch@kit.edu
Engler-Bunte-Ring 1, 76131 Karlsruhe

Journal of Chromatography A
Volume 1257, (2012), 41-47

Abstract

Size exclusion chromatography (SEC) is often applied for characterization protein-polyethylene glycol (PEG) conjugates regarding the number of attached PEG chains (PEGamers). SEC analysis is advantageous as it is precise, robust, and straightforward to establish. However, most SEC based assays have a maximal throughput of a few samples per hour. We present a strategy to increase analytical throughput based on combining a short column with a fast flow rate, and finally multivariate calibration in order to compensate for the resolution lost in the trade off for speed. Different multivariate approaches were compared and multilinear regression was shown to result in the most precise calibrations. Further, a dynamic calibration approach was developed in order to account for changes in column performance over time. In this way, it was possible to establish a highly precise assay for protein PEGamer quantification with a throughput of 30 samples per hour.

Keywords: multivariate calibration; high throughput analytics; high throughput experimentation; PEGamer quantification; protein PEGylation; high throughput process development

1 Introduction

Attaching polyethylene glycol (PEG) polymer chains to proteins (PEGylation) has been shown to improve their pharmacokinetics. The increase in size reduces renal clearance of the therapeutic and there is proof of immunogenicity and antigenicity being reduced by PEGylation [1, 2]. Further, solubility of hydrophobic proteins can be increased by PEGylation [3]. The PEGylation process determines the number (PEGamers) and positions(isoforms) of the PEG molecules attached to the protein. If random PEGylation is performed, the product of the reaction will be very heterogeneous. This poses a problem in a regulative environment which demands extremely high homogeneity of well defined products. In general, there are two ways to reach a homogeneously PEGylated product. Either the product mixture of the random PEGylation can be purified to contain only the desired PEGylated species or site specific PEGylation can be performed.

In our lab, small scale PEGylation experiments performed automated on liquid handling stations have been established. This enables high throughput screenings of PEGylation process parameters and is a part of a general trend where automated high throughput experimentation (HTE) is applied for high throughput process development (HTPD) of biologicals [4]. For the evaluation of the performed experiments to stand in relation to experimental speed when performing HTE, the analytical throughput must match the experimental throughput. In some cases this comes easily, for instance if the evaluation of screenings performed in HTE mode can be based merely on univariate spectroscopic measurements such as total protein quantification via UV absorption measurements [5–7]. If selective or specific quantification is necessary, other methods have to be considered. In the case of quantitative separation of protein PEGamers, size exclusion chromatography (SEC) is widely applied. However, for SEC based assays to match the speed of HTE, the analysis time per sample must be reduced to merely a few minutes.

SEC is a standard method for selective and specific quantification of proteins. If exact quantification is the primary objective, most analysts will seek to achieve high resolution ($R > 1.5$) of the components. If a short analysis time is the primary objective, faster flow rates and/or shorter columns can be applied, however, at the expense of resolution. One approach to increase analytical speed without losing resolution is the interlacing of injections and/or parallel operation of two columns on a chromatographic system [8, 9]. When interlaced injections are performed in SEC analysis, the result is elimination of initial lag time between sample injection and start of elution. If interlaced injections are combined with parallel operation of two columns, the waiting time post elution of the smallest sample molecule of interest can be reduced or even eliminated. Thus, in favorable situations, analysis time per sample can be reduced to the time span in which the molecules of interest elute.

The separation of protein PEGamers using SEC poses a very challenging task, as the relative increase in molecular size decreases for each additional PEG molecule attached to the protein [10]. A size based chromatographic separation of native protein and mono-PEGylated species might be achieved effortlessly, however a resolution of the higher protein PEGamers will become increasingly difficult.

Our proposition is that for analytical purposes the degree of separation necessary for correct quantification can be decreased significantly by applying alternative methods for the evaluation of chromatographic data. This idea is based on the assumption that overlapping elution of different species will result in a chromatogram which is a linear superposition of the signals of each single analyte. Therefore, a linear multivariate correlation between elution profile (chromatogram) and amounts of the different components in the respective samples can be expected. This, of course, presumes that the amount of the analytes does not influence the elution profile. In analytical chromatography, which is mostly performed in the linear range of adsorption, this assumption would apply. Further, the separation mechanism of SEC does not depend on direct interaction with the column material and thus it is not expected that different load concentrations should cause a non-linear change of the elution profile. If the linear correlation between analyte concentration and elution profile is given, it should be possible to calibrate a multivariate regression model based solely on chromatograms of each pure component. Such a multivariate regression model could then give precise determinations of sample composition despite low chromatographic resolution of the sample components.

The presented work describes how multivariate calibration can be used to gain quantitative results of high quality from low resolution chromatograms. By doing so, faster analysis times are achievable through application of short columns and high flow rates that would otherwise be avoided due to inferior separation performance.

In general, a balanced ratio of analytical and experimental time allows a more efficient use of the HTE platform. The goal was to achieve an assay time of maximum two minutes. This would facilitate an 24 hour experiment-analysis cycle for PEGylation screenings in 96 well HTE format, where screening experiments which are performed during day time can be analyzed over night. Further, fast assays can be useful for PEGylation reaction monitoring and subsequent purification process monitoring.

2 Methods and materials

2.1 Chemicals

Potassium phosphate, potassium chloride and analytical grade ethanol for SEC buffer preparation were purchased from Merck (Darmstadt, Germany). Lysozyme was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methoxy-PEG aldehyde with an average molecular weight of 5 kDa was provided from NOF Cooperation (Tokyo, Japan). Sodium phosphate, sodium chloride and sodium cyanoborohydride ($NaCNBH_3$) for PEGylation buffer and IEC buffer preparation were purchased from Merck (Darmstadt, Germany).

2.2 PEGylated lysozyme

2.2.1 PEGylation Reaction

Lysozyme (5 g/L) and PEG were dissolved in a 25 mM sodium phosphate buffer pH 7.2, containing 20 mM sodium cyanoborohydride. The molar polymer to protein ratio was set to 6:1. The reaction was carried out in a continuously shaken falcon tube at room temperature, for 10 h.

2.2.2 Preparative separation of lysozyme PEGamers

Single lysozyme PEGamers were purified using cation exchange chromatography. Toyopearl GigaCap S-650M resin (Tosoh Biosience GmbH, Stuttgart, Germany) was packed in an Omnifit glass column (25 mm x 400 mm, Diba Industries Ltd., Cambrigde, UK) according to the manufacturer's protocol. The resulting bed volume was 13.4 mL. A gradient elution was performed at a flow rate of 0.7 mL/min with 25 mM sodium phosphate buffer pH 7.2 as mobile phase. After column equilibration, 50 mL sample mixture was loaded onto the column. Elution was performed with a gradient from 0 to 200 mM sodium chloride over 21 column volumes. The fractionation volume was set to 5 mL.

2.2.3 Molecular weight determination

Resulting peak fractions were analyzed with respect to lysozyme PEGamer sizes using combined size exclusion chromatography (SEC) and light scattering (LS). This analysis was conducted using an ÄKTA Ettan system from GE Healthcare (Uppsala, Sweden) in combination with a Dawn Heleos 8+ multi-angle light scattering detector and an Optilap rEX refractive index (RI) detector, both from Wyatt Technology (Santa Barbara, USA). For SEC a Superdex 200 GL10/300 (GE Healthcare, Uppsala, Sweden) with a mobile phase of 25 mM sodium phosphate, pH 7.0, containing 150 mM NaCl was used. The flow rate was set to 0.8 mL/min. Injection volumes between 50 and 100 μL were chosen. After UV absorbance monitoring at 280 nm, each sample was measured by LS and RI detection. Molecular weight (M_w) and hydrodynamic radius (R_h) calculation were calculated using the ASTRA software (v.5.3.4.18).

2.3 Chromatography system setup

An UltiMate3000 RSLC x2 Dual system from Dionex (Sunnyvale, CA, USA) was used for UHPLC analysis. The system was composed of two HPG-3400RS pumps, a WPS-3000TFC-analytical autosampler and a DAD3000RS detector. The autosampler was equipped with a 5 μL sample loop. The volume of the injection needle was 15 μL and the syringe size was 250 μL. For control of the UHPLC equipment and for data evaluation the Chromeleon software (6.80 SR10) was used. The software was extended by an additional time base. This enables a virtual separation of the LC system in two parts which can then be controlled separately. Such a setup is necessary in order to facilitate separate data recording

of each analyzed sample when performing SEC in interlaced mode. A thorough description of this setup and the performance of interlaced chromatography has been described by Farnen et al. [9] and Diederich et al. [8].

2.4 Size exclusion chromatography

SEC columns (Zenix SEC-300) were purchased from Sepax Technologies (Newark, DE, USA). The Zenix SEC-300 phase is a silica based material with a hydrophilic coating. The 3 μm sized particles have a nominal pore size of 300 Å. Columns of the dimensions 4.6x150 mm and 4.6x300 mm were used. The short colum was operated with a flow rate of 0.6 mL/min and the long column with a flow rate of 0.4 mL/min. The columns were mounted with 0.2 μm Opti-Solv®EXP™ inlet filters (Optimize Technologies, Oregon City, OR, USA). For analysis, 5 μl sample was injected via full loop injections and a 250 mM potassium phosphate buffer at pH 6.8 with 200 mM potassium chloride was used as running buffer. To prevent fouling of the columns due to PEG binding, 10 % (V/V) analytical grade ethanol was added to the buffer. Interlaced injection mode was performed in order to eliminate lag time between injection and elution of the first sample components.

2.5 Multivariate calibration

Where nothing else is stated, the multivariate regression models were calibrated with six chromatograms: single component chromatograms of each purified lysozyme PEGamer (mono-, di-, and triPEG) and native lysozyme, one chromatogram of all components in mass equivalent ratio with respect to lysozyme, and one chromatogram of a blank injection. Chromatograms of the pure components and the mixture of all four components are shown in Fig. 1A. The samples of pure components all had a concentration of 1 g/L where as each component in the mixed sample had a concentration of 0.5 g/L. All mentioned concentration are with respect to lysozyme.

Different multivariate regression types were applied: multilinear regression (MLR), partial least squares (PLS) regression, and multivariate curve resolution (MCR). All data processing for multivariate calibration was performed with MATLAB. For multivariate regession with MLR and PLS, the chromatographic data was preprocessed by mean centering. All PLS based regressions were based on 4 latent variables. MCR was performed with the MCR-ALS algotithm. After the MCR model was calibratied with the above mentioned six chromatograms, it was able to deconvolute overlapping chromatograms unknown to the model. This resulted in values corresponding to the integrated aera of each pure component present in each of the validation samples. From the initial MCR model calibration, the relation between area and concentration was known and based hereon, the concentration of each component in each validation sample was calculated. Hence, the MCR procedure is a combination of a multivariate approach for chromatogram deconvolution combined with an univariate calibration of the determined area and related concentration.
For multivariate calibrations based on integrated peak areas, calibration samples were generated according to a three layer onion design generated with MODDE (Umetrics, Sweden)

which consisted of 32 combinations of the four components.

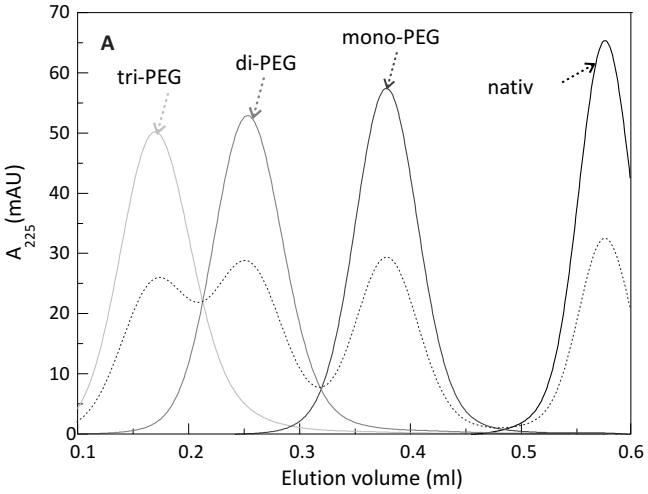


Figure 1: Artificial chromatograms based on superimpositions of pure component chromatograms. These were generated to calibrate multivariate regression models. A: overlay of single component chromatograms of native lysozyme and three lysozyme PEGamers (mono-, di- and tri-PEGylated). All single component samples had a concentration of 1.0 g/L with respect to lysozyme. B–F: examples of single component chromatogram superpositions according to the concentrations listed in the figures.

2.5.1 Chromatographic data

Each multivariate calibrations was based either on full chromatograms or four defined points within the chromatogram. The four points were either defined as the UV signal at the elution volume corresponding to the peak maximum of each pure component or as the UV signal at actual peak maxima of the recorded chromatogram (see Fig. 2, left). For the purpose of comparison, calibrations based on integrated peak areas were also performed. Here, vertical peak limits as well as exponential rider skimming were applied to determine peak areas (see Fig. 2, right). Data from full chromatograms consisted of the UV the signal recorded with a frequency of 10 Hz for the short column and 5 Hz for the long column in a defined elution range. This range was defined as 1.4 – 1.9 mL for the short column and 2.8 – 3.75 mL for the long column with respect to chromatograms based on conventional injection mode as shown in Fig. 3. A full chromatogram consisted of 712 data points when generated with the long column and 500 data points when generated with the short column.

2.5.2 Determination of calibration precision

The calibrated concentration range for native lysozyme and each PEGamer was 0 – 1 g/L with respect to lysozyme. To determine the precision of this suggested approach for evaluation of chromatograms featuring low resolution, validation samples of defined concentrations were analyzed. The validation samples were prepared according to a three layer onion design (designed by MODDE) which resulted in 32 samples with seven different concentration levels. These samples were each injected three times and before each injection of a new sample,

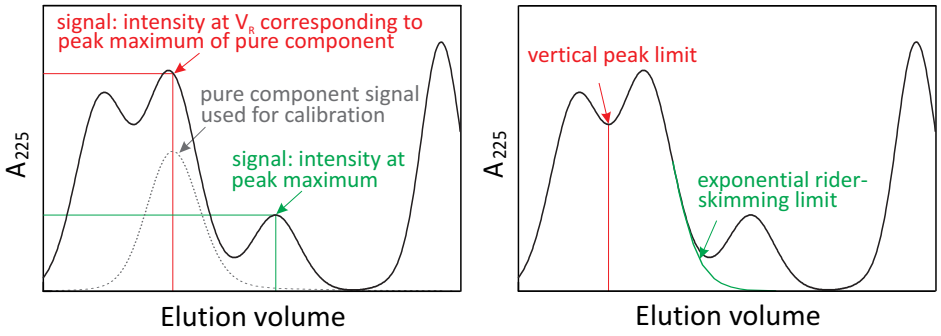


Figure 2: Schematic illustration of the different chromatographic data used for calibration. Left figure: definition of peak maxima for calibrations based on single chromatogram data points. Right figure: definition of peak limit for calibration based on area integration.

a blank run was performed. Based on the obtained chromatographic data, the calibrated multivariate models were used to predict the content of each lysozyme PEGamer and native lysozyme in the validation samples. For each model and each component the 95 % confidence intervals were calculated based on the total of 96 analyzed samples (threefold injections of 32 validation samples). First the MATLAB 'poly1' fit function was used to fit a linear function to the relation between the concentration predicted by the multivariate model and the nominal concentraion in the validation samples. After the fitting procedure, MATLAB was programmed to return the upper and lower confidence bounds for each linear fit. Finally, the confidence interval for each calibration and component was calculated by adding the upper and lower confidence bound. It should be noticed that the confidence interval does not to give excact information on the precision for different concentration levels within the calibration. Hence, the confidence intervals were only used for comparison of the different calibrations. To obtain more detailed information on presicion, the relative standard devaition (RDS%) was calculated for each concentration interval (0, 0.25, 0.33, 0.5, 0.67, 0.75, and 1.0 g/L) and presented for a selection of the most precise calibrations.

2.5.3 Dynamic and static calibration

Two modes of multivariate calibration were used. The simplest type was a static mode where one set of pure component chromatograms recorded immediately before analysis of the validation samples was used for calibration. However, to be able to account for systematic changes in column performance over time, a dynamic approach was developed. In order to do so, calibration samples were injected both before and after the analysis of the validation samples. The regression model was then recalibrated for each validation sample based on a linear interpolation between the chromatograms recorded before and after analysis of the validation samples. The interpolation calculated according to equation 1:

$$v_{i,j} = \frac{n-j}{n} \cdot v_{i,pre} + \frac{j}{n} \cdot v_{i,post} \tag{1}$$

where v_i is a vector containing the recorded chromatogram of calibration sample$_i$, n is the total number of measured samples, and j is the number of the sample in the sample sequence

for which the model is recalibrated. The indices *pre* and *post* indicates whether a calibration sample was measured before or after the validation samples.

3 Results and discussion

The aim was to establish a fast SEC assay ($\sim$ two minutes) for the quantification of lysozyme PEGamers and native lysozyme. Hence, a type of SEC columns which allows the use of rather high flow rates up to 3 cm/min was applied. In order to eliminate the lag time inherent in SEC analysis, injections were performed in interlaced mode. Finally, the narrow diameter of the chosen column type allowed for a sample size of 5 μL which minimizes the time necessary for sample injection preparation by the autosampler. All these measures resulted in an assay time of five and two minutes for the long and the short column, respectively. These measures, however, also caused insufficient resolution for precise quantification of all the lysozyme PEGamers when based on determined peak areas. Therefore, multivariate calibration was applied to achieve accurate and precise quantification despite the low resolution.

3.1 Assay time and chromatographic resolution

The resulting separation of lysozyme PEGamers using two different column lengths (150 and 300 mm) is shown in Fig. 3. The presented chromatograms result from samples injected in traditional sequential mode and, as can be seen, less that 50 % of the recorded signal contains relevant information. Hence, interlaced injection mode was applied to reduce the analysis time significantly without decreasing the resolution further. The final assay for the long column was performed with an elution volume of 2.0 mL per injected sample at a flowrate of 0.4 mL/min. This resulted in an assay time of five minutes. The final assay for the short column was performed with an elution volume of 1.2 mL per injected sample at a flowrate of 0.6 mL/min. This resulted in an assay time of two minutes.

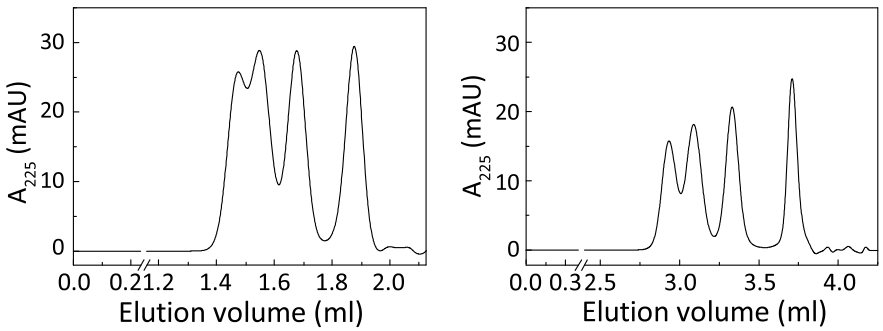


Figure 3: Separation of native lysozyme, mono-, di- and, tri-PEGylated lysozyme with two columns of different lengths. The samples injected on the two columns were identical and contained equal amounts of each component with respect to lysozyme. Left: separation performed on a 150 mm column. Right: separation performed on a 300 mm column.

The determined chromatographic resolutions generated by both columns of lysozyme PEGamers and native lysozyme are listed in table 1. These results are based on injection of equal

amounts of the two components in question. A resolution of $R \gtrsim 1.5$ is in general sufficient for correct quantification based on integration of peak areas. The short column only generated sufficient resolution for native and mono-PEGylated lysozyme whereas the long column generated sufficient resolution for all components except the tri-PEGylated lysozyme. These results accentuate the challenging task of separating higher PEGylation forms using SEC to a degree sufficient for quantification based on area integration. If sufficient resolution is to be achieved for the higher PEGylation forms, a great amount of dispensable resolution will be generated for native and lower PEGylation forms. Therefore, in order to save time, multivariate calibration was applied in the attempt to achieve precise and accurate quantification despite low resolution.

Table 1: Chromatographic resolution listed for each column. Each resolution was determined by injecting equal amounts of the two components in question.

column length	chromatographic resolution			
	native	mono-PEG	di-PEG	tri-PEG
300 mm	2.72	1.47	0.79	
150 mm	1.66	0.98	0.35	

3.2 Calibration precision

Multivariate models were calibrated based on multilinear regression (MLR), partial least squares (PLS) regression, and multivariate curve resolution (MCR). The results of the MCR were very similar to those of MLR and are therefore omitted. Further, traditional quantification based univariate calibration of determined peak areas was performed in order to compare multivariate calibration with the traditional approach. Finally, MLR calibration based on peak areas and peak heights was applied to investigate whether low peak resolution could be compensated by multivariate calibration of these parameters.

Confidence intervals were used to compare the precision of the different calibration approaches, both with regard to regression type and the data used for calibration (i.e. full chromatograms, peak maxima, peak areas). For this purpose, the mean confidence interval of the two components with the lowest chromatographic resolution was determined, in this case the di- and tri-PEGamers. The results are listed in table 2 and are presented and discussed in the following sections.

3.2.1 Static calibration

In general, a clear increase in precision was achieved by applying static multivariate calibration of chromatographic data. For the long column, the precision increased from 0.0476 g/L to 0.0155 g/L by applying MLR to full chromatogram data instead of univariate calibration of peak areas. Based on equivalent calibration for the short column, an increase in precision from 0.0580 g/L to 0.0247 g/L was achieved. For the stronger overlapping peaks generated

Table 2: Calibration results presented in order of precision. Different regression types were used to calibrate multivariate models based on different chromatogram data for evaluation of analytical chromatograms. The resulting confidence intervals are listed for each of the different regression types along with model mode and the applied chromatogram data. Further, results based on traditional area integration and univariate calibration are listed. See Fig. 2 for a schematic illustration of the different applied chromatogram data.

regression type	model mode	data range	confidence interval
300 mm column			
MLR	static	full chromatogram	0.0155
MLR	static	peak maxima[1)]	0.0164
MCR	static	full	0.0167
PLS	static	full chromatogram	0.0186
MLR	static	peak maxima[2)]	0.0206
MLR	static	peak area[3)]	0.0394
Univ.	static	peak area[3)]	0.0476
Univ.	static	peak area[4)]	0.0768
150 mm column			
MLR	static	peak maxima[1)]	0.0226
MCR	static	full	0.0226
MLR	static	full chromatogram	0.0247
PLS	static	full chromatogram	0.0347
MLR	static	peak area[3)]	0.0453
MLR	static	peak maxima[2)]	0.0491
Univ.	static	peak area[3)]	0.0580
MLR	dynamic	peak maxima[1)]	0.0164
MLR	dynamic	full chromatogram	0.0167
MCR	dynamic	full	0.0173
PLS	dynamic	full chromatogram	0.0191

1) signal height to base line at retention volumes corresponding to peak maxima of pure components

2) signal height to baseline at peak maximum

3) vertical peak limits

4) exponential rider skimming

by the short column, MLR calibration based on only four points in the chromatogram (obtained at V_R of the pure components) resulted in a more precise calibration with a confidence interval of 0.0226 g/L. MLR gave more precise results than PLS both for the long and the short column. Further, MLR calibration based on peak areas was more precise than univariate calibration of peak areas, however much less precise than calibration based on full chromatogram data. MLR calibration based on peak heights at peak maxima was more precise than MLR calibrations based on peak areas for the long column, however the opposite was the result for the short column. The elution profiles of the pure components (see Fig. 4) shows that severe peak overlapping was limited to approximately 50 % for the long

column. Therefore the position of peak maxima was less influenced by the presence of other components when comparing the long column with the short column.

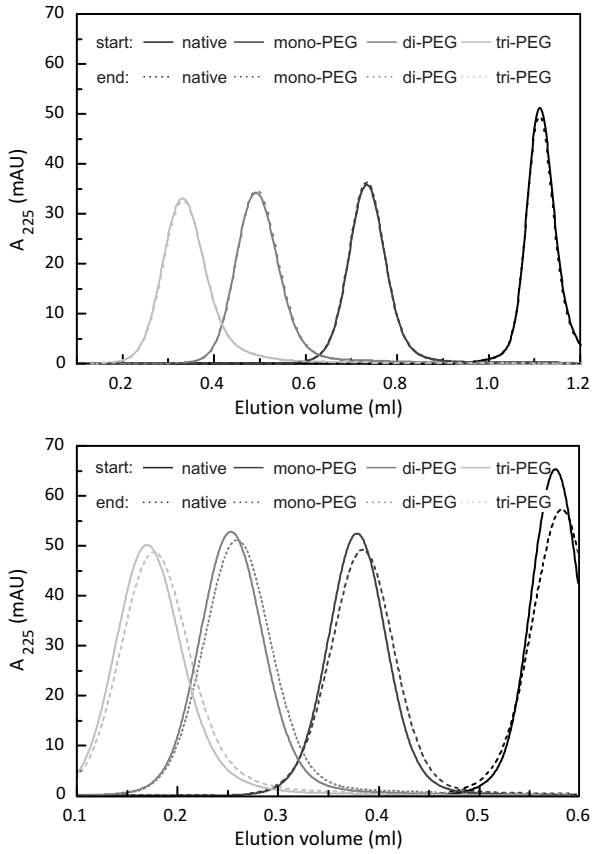


Figure 4: Overlay of single component chromatograms before and after the analysis of 140 test samples. Top: 300 mm column. Bottom: 150 mm column.

3.2.2 Dynamic calibration

The precision of the univariate peak area based calibration decreased by $\sim 25\,\%$ when comparing the results obtained by the long column to those obtained by the short column. In comparison, the precision of the multivariate calibration based on MLR and full chromatograms decreased by $\sim 70\,\%$. Even though the absolute value of the determined confidence interval was still several times lower for the multivariate calibration compared to univariate calibration(0.0247 *vs.* 0.0580 g/L) when using the short column, the relatively high decrease in precision of the multivariate calibration when switching from the longer to the shorter column (*e.g.* 0.0155 *vs.* 0.0247 g/L) was not expected. In Fig. 4A an overlay of pure component chromatograms recorded before and after analysis of the 140 samples on the long column is shown. The data shows stable column performance without a systematic change in peak height or retention volume over time. Fig. 4B displays the equivalent data for the short column and here a shift in retention time for all components along with peak broadening can be observed. To overcome the inaccuracy caused by the change in column performance, a

dynamic calibration approach was used. This calibration was based on linear interpolation of calibration samples measured immediately before and after the analysis of the validation samples. It was chosen to apply linear interpolation as the observed change in retention time was linear. The use of dynamic calibration for the short column resulted in an assay of similar precision compared to the static calibration of the long column (0.0167 *vs.* 0.0155 g/L).

In Fig. 5 the chromatogram residuals of the validation sample chromatograms are displayed. The shown residuals are derived from both the dynamic and the static PLS calibration for the short column based on the full chromatograms. The variation of the chromatogram residuals remains constant over the period of analysis for the dynamic calibration, where as the variation increases throughout the period of analysis for the static calibration. This supports the decision to use linear interpolation for the dynamic calibration.

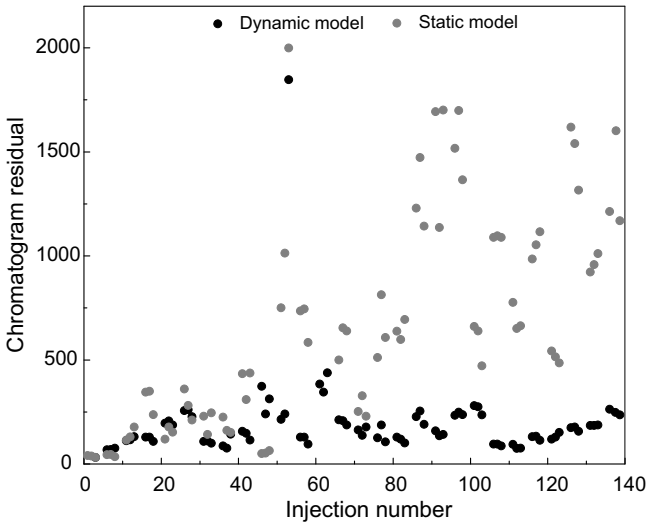


Figure 5: Residuals of the 140 test sample chromatograms as a function of injection order. Each data point represents one sample either evaluated with a static or a dynamic PLS based calibration model.

In order to visualize the effect of dynamic calibration, three artificial chromatograms were created and displayed along with a true chromatogram of a validation sample (Fig. 6). Two of the artificial chromatograms were based on the calibration samples either analyzed before or after the analysis of the validation samples. Neither of these chromatograms is of good resemblance to the validation sample chromatogram. The third artificial chromatogram was based on the linear interpolated chromatograms of the calibration samples which were created for the dynamic calibrations. This chromatogram was built to match the point in time where the displayed true chromatogram was recorded and exhibits a high resemblance to the true validation sample chromatogram. For the right peak (monoPEG-protein conjugate), the resemblance is high both for peak intensity and peak shift. The fact that the left peak of the sample chromatogram deviates from the dynamic calibration chromatogram in peak intensity is most likely due to a pipetting error during preparation of the validation samples.

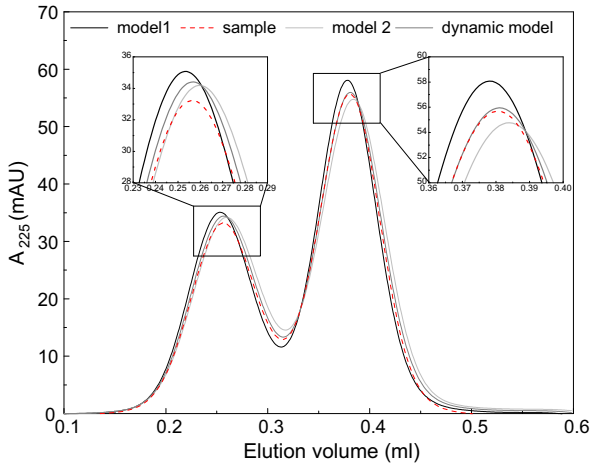


Figure 6: Visual comparison of the effect of dynamic calibration. One true chromatogram and three artificial chromatogram based on linear combinations of pure component chromatograms are displayed. Two of the artificial chromatograms were built from the pure component chromatograms recorded either before (artificial 1) or after (artificial 2) analysis of the validation samples. The third artificial chromatogram (artificial D) was built from the linear interpolations used in the dynamic calibrations. The linear interpolation was built to correspond to the point in time where the true samples was analyzed.

3.2.3 Concentration related precision and sensitivity

The concentration related precision was determined for the dynamic and static MLR calibrations based on the short column and the MLR calibration based on the long column. The relative standard deviation (RSD%) for each concentration is displayed in Fig. 7. The displayed RSD% values are mean values of the RSD% for di- and tri-PEGylated lysozyme. For all three calibrations, the precision increased from the first to the second concentration level (0.25 – 0.33 g/L). For all concentration levels, the precision of the static MLR calibration based on the 150 mm column was lowest (RSD $\sim$ 3 – 1.5%). The precision for all concentration levels was similar for the dynamic MLR calibration based on the 150 mm column and the static MLR calibration based on the 300 mm column. This is in accordance with the finding that the overall precision of these calibrations were similar (95% confidence interval: 0.0167 g/L and 0.0155 g/L, see Table 2). This means that both the overall precision and the specific precision achieved for the long column can be maintained for the short column by applying multivariate calibration. For further comparison and characterization of the calibrations, the limit of quantification (LOQ) was determined. LOQ was determined as six times the standard deviation of all zero predictions in the calibration samples for each component. Again, the displayed LOQ values are mean values of the determined LOQ value for di- and tri-PEGylated lysozyme. With regard to the LOQ value, the dynamic approach does increase the sensitivity of the method, however not to the level of the MLR calibration based on the 300 mm column.

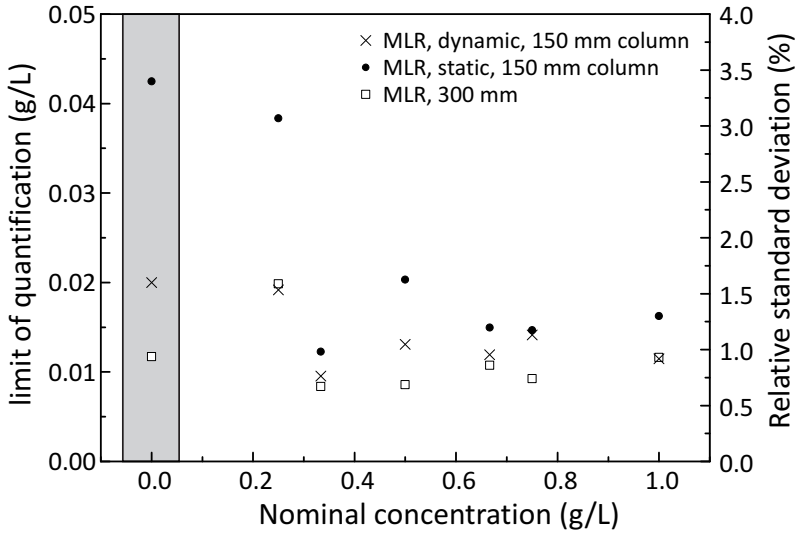


Figure 7: RSD % values for the concentration levels included in the validation sample design. Displayed are mean values of the determined RDS % for di- and tri-PEGylated lysozyme. The value dispayed for the concentration zero (grey bar) are LOQ values. Also the LOQ values are mean values of the determined LOQ for di- and tri-PEGylated lysozyme.

4 Conclusion and outlook

The aim of establishing an SEC assay for the quantification of lysozyme PEGamers with an analysis time of two minutes was achieved. The presented work clearly demonstrates that by applying multivariate calibration for the quantitative evaluation of badly resolved chromatograms the precision can be enhanced significantly when compared to traditional univariate calibration. Hence, fast chromatographic assays can easily be achieved by applying short columns and fast flow rates. The tested chromatographic assays included resolutions down to 0.35 demonstrating how little resolution is actually sufficient to achieve a highly precise chromatographic assay. The lower limit of resolution necessary for precise calibration is still to be determined. Further, the results demonstrated that a change in column performance over time can be handled without difficulty by using a dynamic calibration approach.

In the presented work, pure samples were used for calibration. If pure material is at hand or easily achievable, this is the most straightforward approach. If pure samples are difficult or impossible to obtain, mixed samples of defined composition can also be used. This then requires an alternative assay or analytics to define the mixed samples and which must contain sufficient variation.

The use of multivariate calibration is of course not limited to SEC. Any robust chromatographic assay established for defined samples can be evaluated by the presented approach. The approach cannot be applied to complex samples in which unknown peaks occur in the area used for calibration and also chromatographic assays based on non-linear chromatography will demand more sophisticated multivariate calibration of non-linear nature. This might increase the calibration complexity to a level which is no longer leveraged by the gained increase in assay speed. However, this is still to be investigated.

In working environments with limited time for assay development and data evaluation, it might be an unmanageable task to perform data evaluation not inherent in the chromatography system software. Hence, we propose the integration of multivariate calibration directly in the commercial software supplied with the chromatography systems.

References

[1] M. J. Knauf, D. P. Bell, P. Hirtzer, Z. P. Luo, J. D. Young, and N. V. Katre. Relationship of effective molecular size to systemic clearance in rats of recombinant interleukin-2 chemically modified with water-soluble polymers. *J. Biol. Chem.*, 263(29):15064–70, 1988.

[2] F. Fuertges and A. Abuchowski. The clinical efficacy of poly(ethylene glycol)-modified proteins. *J. Controlled Release*, 11:139–148, 1990.

[3] N. V. Katre, M. J. Knauf, and W. J. Laird. Chemical modification of recombinant interleukin 2 by polyethylene glycol increases its potency in the murine Meth A sarcoma model. *Proc. Natl. Acad. Sci. U.S.A.*, 84(6):1487–91, 1987.

[4] R. Bhambure, K. Kumar, and A. S. Rathore. High-throughput process development for biopharmaceutical drug substances. *Trends Biotechnol.*, 29(3):127–35, 2011.

[5] S. Oelmeier, F. Dismer, and J. Hubbuch. Application of an Aqueous Two-Phase Systems High-Throughput Screening Method to Evaluate mAb HCP Separation. *Biotechnol. Bioeng.*, 108(1):69–81, January 2011.

[6] S. Chhatre, R. Francis, D. G. Bracewell, and N. J. Titchener-Hooker. An automated packed protein G micro-pipette tip assay for rapid quantification of polyclonal antibodies in ovine serum. *J. Chromatogr. B*, 878(30):3067–75, 2010.

[7] M. Bensch, P. S. Wierling, E. von Lieres, and J. Hubbuch. High Throughput Screening of Chromatographic Phases for Rapid Process Development. *Chem. Eng. Technol.*, 28(11):1274–1284, 2005.

[8] P. Diederich, S. K. Hansen, S. A. Oelmeier, B. Stolzenberger, and J. Hubbuch. A sub-two minutes method for monoclonal antibody-aggregate quantification using parallel interlaced size exclusion high performance liquid chromatography. *J. Chromatogr. A*, 1218(50):9010–9018, 2011.

[9] D. Farnan, G. T. Moreno, J. Stults, A. Becker, G. Tremintin, and M. van Gils. Interlaced size exclusion liquid chromatography of monoclonal antibodies. *J. Chrom. A*, 1216(51):8904–9, 2009.

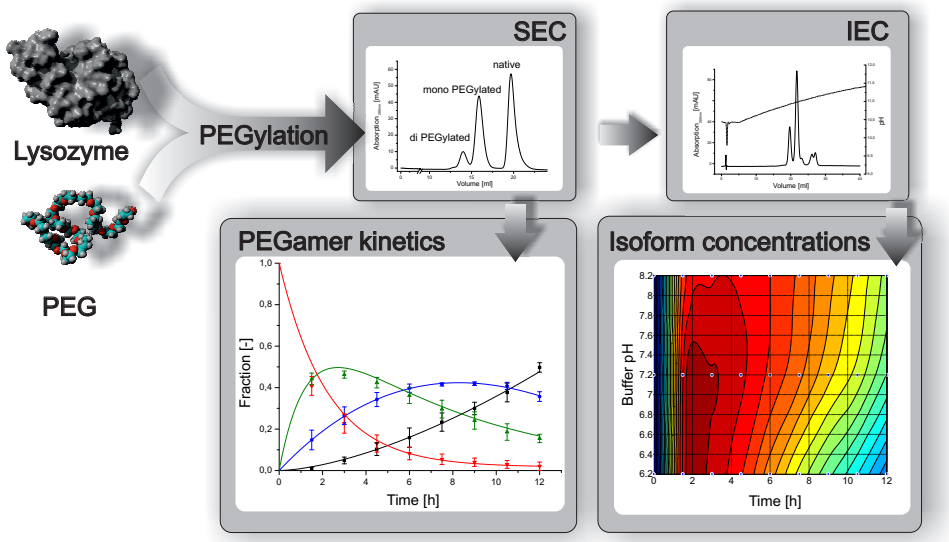
[10] C. J. Fee and J. M. Van Alstine. PEG-proteins: Reaction engineering and separation issues. *Chem. Eng. Sci.*, 61(3):924–939, 2006.

Optimization of Random PEGylation Reactions by means of High Throughput Screening

Benjamin Maiser, Florian Dismer and Jürgen Hubbuch*

Institute of Engineering in Life Sciences, Biomolecular Separation Engineering
Karlsruher Institute of Technology (KIT), Karlsruhe, Germnay

*Corresponding author: Tel: +49 721 608-46263, Mail: juergen.hubbuch@kit.edu
Engler-Bunte-Ring 1, 76131 Karlsruhe



submitted to
Biotechnology and Bioengineering (2013)

Abstract

Since the first FDA approval of a PEGylated product in 1990, so called random PEGylation reactions are still used to increase the efficacy of biopharmaceuticals and represent the major technology of all approved PEG-modified drugs. However, the great influence of process parameters on PEGylation degree and the PEG-binding site results in a lack of reaction specificity which can have severe impact on the product profile. Consequently, reproducible and well characterized processes are essential to meet increasing regulative requirements resulting from the Quality-by-Design (QbD) initiative, especially for this kind of modification type. In this study we present a general approach which combines the simple chemistry of random PEGylation reactions with high throughput experimentation (HTE) to achieve a well-defined process. Robotic based batch experiments have been established in a 96-well plate format and were analyzed to investigate the influence of different PEGylation conditions for lysozyme as model protein. With common SEC analytics highly reproducible reaction kinetics were measured and a significant influence of PEG-excess, buffer pH and reaction time could be investigated. Additional mono-PEG-lysozyme analytics showed the impact of varying buffer pH on the isoform distribution, which allowed us to identify optimal process parameters to get a maximum concentration of each isoform. Employing *Micrococcus lysodeikticus* based activity assays, PEG-lysozyme$_{33}$ was identified to be the isoform with the highest residual activity, followed by PEG-lysozyme$_1$. Based on these results, a control space for a PEGylation reaction was defined with respect to an optimal overall volumetric activity of mono-PEG-lysozyme isoform mixtures.

Keywords: PEGylation, lysozyme, high throughput process development, high throughput experimentation, Quality-by-Design

1 Introduction

Nowadays, recombinant proteins and peptides represent a large share of successfully approved drugs, gaining an increasing importance as therapeutics [1]. However, limitations such as rapid body clearance, aggregation and enzymatic degradation are still major drawbacks in the development of new biopharmaceuticals. PEGylation, the covalent attachment of polyethylene glycol (PEG) to biopharmaceuticals has been shown to overcome such obstacles and is widely used for improving therapeutics efficacy, especially for small, parenteral administered proteins. Reasons for the great success are the numerous positive effects accompanied by polymer modification, which mostly include improved solubility, enhanced thermal and proteolytic stability as well as reduced immunogenicity [2–5]. One of the major advantages of PEGylated products comprises the reduced renal clearance and thus an increased body residence time, resulting in a reduced dose administration. The altered physicochemical characteristics can mostly be attributed to an increased hydrodynamic radius and the protective effect of the attached polymer chains, and were object of intense research in the last four decades [6–8].

With respect to increasing regulative requirements in modern pharmaceutical production by means of reproducibility and product consistency, site-specific PEGylation is of growing interest. Proteins with a single PEG attached to a specific site are easy to purify and represent well defined products, offering a defined activity profile. Approaches for specific modification include for example enzymatic techniques such as glycoPEGylation and transglutaminase mediated modification or an attachment via introduced binding sites [9–11]. However, such techniques are non-trivial and demand for a time consuming development. In addition all approaches are still in development or clinical phase and a fast FDA approval especially for genetically introduced binding sites might be problematic.

In contrast to the site specific approach, so called random PEGylation reactions were successfully used in most instances of already approved PEGylated products, such as PEGylated interferon and epoetin (Pegasys® and Micera® by Hoffman-LaRoche, respectively). Acylating derivatives such as N-hydroxysuccinimidyl activated PEGs (PEG-NHS) target amino and hydroxyl residues of surface amino acids, including lysine, histidine or serine [12, 13]. Yielding amide or urethane bonds, this reaction type reduces the protein charge by the number of attached PEG chains. Alkylating reactions such as PEG-aldehyde exclusively reacts with the ϵ-amino side chains of lysine or the N-terminal α-amino group, leading to secondary amines, preserving the protein charge [13–15]. A major advantage of random reactions comprises the simple chemistry and the availability of binding sites to provide PEG attachment, without the need of an additional modification of the target molecule. Due to the established status, so called fast track designations by the FDA can reduce the time to market of new PEGylated products, if the non-PEGylated counterpart has already been approved [6].

However, as lysine residues are generally well represented in proteins, PEG-aldehyde and PEG-NHS reactions mostly result in complex mixtures of different conjugates, varying in number (PEGamers) and binding site (isoforms) of attached PEG. Additionally, all formed conjugates mostly offer a reduced activity compared to the native protein, depending on

number, size and even modification site of attached PEG, due to steric hindrance by the attached polymer chains. Thus, each type of conjugate can offer a different specific activity, which still provides the basis of the therapeutic efficacy. As the rate of residue modification can be influenced by the exposure and the nucleophilicity of the binding site, reaction conditions such as buffer pH and PEG excess can have a major influence on reaction velocity, PEGamer formation and isoform distribution. Consequently, batch-to-batch variations in the PEGylation reaction regarding important parameters result in deviations concerning PEGamer and isoform formation and thus affecting the overall activity of the product. The recall of five batches of PEG-asparaginase (Oncaspar®, Enzon) between 2000 and 2003 due to deviations in activity [16, 17] underlines the issues of random PEGylation reactions in a QbD driven environment.

To overcome such drawbacks, HTE combined with high sensitive isoform analytics can be applied and to gain a deeper process understanding and thus to increase reproducibility. Additionally, adjusting process parameters can help to improve the process towards a favorable product. Applying a low buffer pH value at PEG-aldehyde reactions, for example, the isoform distribution can be shifted to a predominant N-terminal modification, due to the difference of the pK_a values of the ϵ- amino groups compared to the N-terminal α-amino residue. This was successfully applied in the development of a PEG-modified G-CSF, (Neulasta®, Amgen) resulting in the first FDA approved mono-PEGylated protein on market. However, an N-terminal modification does not necessarily result in an isoform with the highest residual activity. Another approach would consequently include the screening of PEGylation conditions towards maximal activity of the product, which might consist of multiple isoforms. For a fast monitoring and screening of PEGylation reactions, size based separation with an improved data evaluation using multivariate data analysis is available [18] and can be used to optimize reaction conditions in a first step. A screening approach regarding the modification of lysozyme with PEG-aldehyde was shown by Moosmann et al. [19]. However this work is only focused on PEGylation degree because of missing isoform analytics and did not use modern methods of experimental design.

The presented study describes a systematic screening method to optimize random PEGylation reactions towards a maximum volumetric activity and to increase batch-to batch reproducibility using a high throughput platform and follows a general trend where HTS is implemented in process development [20]. Varying process parameter such as pH and protein to PEG ratio, lysozyme PEGylation kinetics with PEG-aldehyde concerning different PEGylation degrees were generated. Using isoform analytics of mono-PEG-lysozyme published recently [21], concentrations of each isoform were determined and combined with a *Micrococcus lysodeikticus* based activity assay. According to that, isoforms PEGylated at lysine 33 were found to exhibit the highest activity, followed by lysine 1 modified isoforms. The results were then used to define a parameter control space for the maximal volumetric activity of mono-PEG-lysozyme isoform mixtures, to show the potential for an industrial application.

2 Materials & methods

2.1 Chemicals

Methoxy-PEG-propionaldehyde (mPEG-aldehyde) and methoxy-PEG-succinimidyl carboxymethyl (mPEG-NHS) both with an average molecular weight of 5 kDa were provided by NOF Cooperation (Tokyo, Japan). Hen egg white lysozyme, L-lysine as well as buffer components including sodium phosphate, sodium chloride, potassium phosphate, potassium chloride and sodium cyanoborohydride ($NaCNBH_3$) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Cyclohexylaminobutansulfonic acid (CABS) was used for pH-gradient buffer preparation and was provided by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Lyophilized cells of *Micrococcus lysodeikticus* for lysozyme activity measurements were provided by Sigma–Aldrich. All solutions were prepared with ultrapure water provided by an Arium pro water purification system from Sartorius Stedim (Goettingen, Germany). For chromatography runs, buffer solutions were additionally filtered using 0.2 μm cellulose acetat membrane filters from Sartorius Stedim.

2.2 Apparatus

The automated PEGylation screenings were performed on a Freedom EVO® 200 liquid handling station from Tecan (Crailsheim, Germany). For pipetting, the system is equipped with eight fixed tips and a 96-channel liquid handling arm. The system is additionally outfitted with an automated plate handling arm and an integrated rotational shaker (Te-shake, Tecan). The activity assay as well as protein concentration measurements were conducted with an Infinite M200 plate reader from Tecan. All chromatography steps were conducted on an Äkta Ettan system from GE Healthcare (Uppsala, Sweden). For molecular weight determination a Dawn Heleos 8+ multi angle light scattering device in combination with an Optilab rEx refractive index detector, both from Wyatt Technology (Santa Barbara, CA, USA) were used. Thermal stability assays were realized using an DynaPro Plate Reader also from Wyatt Technology.

2.3 Software

The robotic station was operated using the Evoware 2.0 SP1 standard software from Tecan. The import of pipetting volumes was handled via Excel (Microsoft, Redmont, WA, USA). Controlling of the chromatography system as well as the determination of UV areas was done using the Unicorn 5.11 software package from GE Healthcare. All calculations and visualizations were realized with Matlab R2011a (The Mathworks, Natick, ME, USA). The Infinte spectrophotometer was controlled using the i-control 1.9 software from Tecan. The ASTRA Software (version 5.3.4.18) was used for molecular weigth determination, DLS experimets were evaluated with the Dynamics software (version 7.1.5) both from Wyatt Technology.

2.4 PEGylation conditions

Lysozyme modifications with mPEG-aldehyde and mPEG-NHS were conducted under various reaction conditions. For all PEGylation reactions, the lysozyme concentration was kept constant at 4 mg/mL. All buffer systems were composed of a 25 mM sodium phosphate buffer system with 150 mM sodium chloride. For mPEG-aldehyde reactions, 20 mM $NaCNBH_3$ was added as reducing agent additionally. Buffer pH values were varied in three steps ranging from 6.2 to 8.2. In addition three different protein to PEG ratios were screened including 0.15, 0.25 and 0.35, resulting in nine different buffer conditions in total. To obtain kinetic data for mPEG-aldehyde reactions, eight samples with reaction times from 1.5 h to 12 h were prepared for each buffer condition.

2.5 Automated PEGylation screening

The mPEG-aldehyde PEGylation screenings were performed on the robotic workstation and were conducted in 96-well polypropylene microtiter plates (MTP) with a total well volume of 360 μL from Greiner Bio-One (Kremsmünster, Austria). Correct liquid handling was guaranteed by using different liquid classes for buffer, PEG and protein solutions, respectively. Liquid class calibration was done by pipetting onto an analytical balance according to Oelmeier et al.[22]. All reactions were carried out with 300 μL sample volume and varying reaction conditions as described in the PEGylation conditions section. Buffer stock solutions were pipetted in each well of a plate row and were mixed with a corresponding volume of mPEG-aldehyde and lysozyme stock solutions. After 1.5 h of incubation on the rotational mixer, the same reaction preparations were placed in the row below. This procedure was repeated eight times, to obtain kinetic data. During incubation, the plates were covered to avoid evaporation. To prevent a further reaction of the samples while PEGylation degree analysis, all mPEG-aldehyde reaction preparations were terminated with lysine according to Ottow et al. [23]. For this, a 200 mM lysine solution was added (1:1), using the 96-channel liquid handling arm. Resulting protein concentration in all preparations were thus 2 mg/ml, containing 100 mM lysine.

2.6 Manual PEGylation screening

Lysozyme PEGylation samples using mPEG-NHS were prepared manually in 15 ml standard tubes, with a total sample volume of 5 ml. To account for fast hydrolysis of mPEG-NHS in aqueous solutions [13, 24], PEG was directly diluted in corresponding lysozyme buffer solutions, as described in the PEGylation conditions section. Due to fast reaction behaviour, kinetic data was not evaluated for this modification type.

2.7 Analytical procedure

2.7.1 PEGamer separation

Employing the PEGylation degree analysis, the reaction preparations were analyzed with size exclusion chromatography (SEC) using a Zenix^{TM} SEC 300 column from Sepax Technologies

Inc. (Newark, DE, USA). As running buffer a 250 mM potassium phosphate with 200 mM potassium chloride, pH 6.8 containing 10 % ethanol was used. The flow rate was set to 0.4 mL/min and an injection volume of 5 μL was applied. For mono-PEG lysozyme sample fractionation a Superdex 200 GL10/300 (GE Helathcare, Uppsala, Sweden) with a mobile phase of 25 mM sodium phosphate, pH 7.2, containing 150 mM NaCl was used. The flow rate was maintained at 1.0 mL/min and injection volumes between 100 μL and 250 μL were chosen. The PEGylation degree was validated applying molecular weight determination with combined light scattering and refractive index analysis.

2.7.2 Isoform separation

Isoform separation was conducted as previously reported on a MonoS 4.6/100 column from GE Healthcare [21]. Sample volumes between 100 μL and 150 μL of purified mono-PEG lysozyme were injected. For pH-gradient elution 20 mM CABS was used as buffer component. Running buffer A and elution buffer B were titrated with 4 M NaOH to pH 10.6 and pH 11.6, respectively. The elution was applied with a linear gradient ranging from 0% to 100% buffer B over 12 column volumes (CV). The flow rate was set to 1.5 mL/min. To obtain samples for the activity assay, resulting peaks from mono-PEG$_{aldehyde}$-lysozyme isoforms were fractionated with a constant volume of 250 μL. The fractions of multiple runs were pooled to reach sufficient amount of single isoforms.

2.7.3 Lysozyme activity assay

For lysozyme activity measurements a tubidometric assay based on a *Micrococcus lysodeikticus* suspension was used. For determination of protein concentrations UV absorption at 280 nm was measured in standard UV cuvettes. Activity measurements were performed in a 96-well microplate format according to Lee et. al [25], using UV-star flat bottom plates from Greiner Bio-One (Kremsmünster, Austria). The lysozyme assay was conducted with protein sample concentrations in the range of 1 μg/mL to 5 μg/mL to ensure linear decrease in turbidity. To provide PEG-lysozyme isoform samples, pooled fractions collected with pH gradient chromatography were transferred to VivaSpin 20 ultrafiltration units (Sartorius Stedim, Gerrmany) with a molecular weight cutoff of 5 kDa. Each sample was diafiltrated into 25 mM sodium phospahte buffer with pH 7.2. *Micrococcus* cells were suspended in 25 mM sodium phosphate buffer, pH 7.2 in a concentration of 0.3 mg/mL. Protein solutions, including native lysozyme and PEG-lysozyme isoform samples (50 μL) were pipetted in microplate wells and were mixed with 200 μL cell suspension. Absorption of light at 450 nm wavelength was measured in 1 min intervals for 7 min (native lysozyme) and 20 min (PEG-lysozyme isoforms), respectively. To prevent cell settling, the plate was shaken between the measurements. The lytic activity was evaluated, calculating the negative slope of the UV$_{450}$ signal. A decrease in absorbance of 0.001/min was defined as one unit in accordance to Lee et al. and Freitas et al. [25, 26].

2.7.4 Thermal stability studies

Dynamic light scattering (DLS) experiments were conducted to evaluate the thermal stability of PEGamer variants, modified with mPEG-aldehyde. Fractionated and pooled samples of mono- and di-PEG-lysozyme were diafiltrated in 25 mM sodium phosphate buffer, pH 7.2, and concentrated to 1.5 mg/mL. Sample volumes of 30 μL covered with 10 μL paraffin, were placed in black 384-well plates from Corning Inc. (Corning, NY, USA). Subsequently, a temperature ramp from 25 °C to 80 °C was carried out with a rate of 0.4°C/min. The maximal temperature was limited to 80 °C by the instrument.

3 Results and discussions

Optimization of random PEGylation processes is still a challenging task, due to missing isoform analytics and the manifold influence of numerous reaction parameters. Thus, the overall aim in this study was to achieve a better process understanding applying robotic based screening of PEGylation conditions in combination with a high sensitive isoform analytics. As model protein lysozyme was used, providing six lysine residues and thus allows PEGamer and isoform formation with amino coupling PEG reagents.

3.1 mPEG-aldehyde

In total 72 samples were prepared for mPEG-aldehyde PEGylation, varying in buffer pH, protein to PEG ratio and reaction time. All samples were prepared at least in triplicates.

3.1.1 Reaction termination

In order to achieve reproducible kinetic data, all mPEG-aldehyde reactions were terminated after sample preparation to inhibit further reaction while PEGylation degree analysis and mono-PEG lysozyme fractionation. For this, a lysine solution was used, as described by Ottow et al. [23]. To investigate a suitable concentration, capable of stopping the PEGylation, different amounts of lysine were added to a reaction preparation. As worst case scenario, a reaction directly after PEG addition, with the highest PEG excess (0.15 protein to PEG ratio) and a buffer pH of 6.2 was chosen. After a reaction time of 20 h, the formation of PEG-lysozyme conjugates was evaluated, applying SEC. Figure 1 shows the impact of varying lysine concentrations on the PEG-conjugate formation.

With a 5 mM lysine concentration, mono- and di-PEG-lysozyme conjugates were still observable in the chromatogram. A concentration of 100 mM lysine completely stopped the formation of mono-PEG lysozyme and was thus used in further work to terminate the reactions.

3.1.2 PEGamer kinetics

After reaction termination, PEGamer analysis was conducted by SEC, measuring UV_{280} areas of native, mono-, di- and poly-PEGylated lysozyme. As mPEG-aldehyde is non–UV

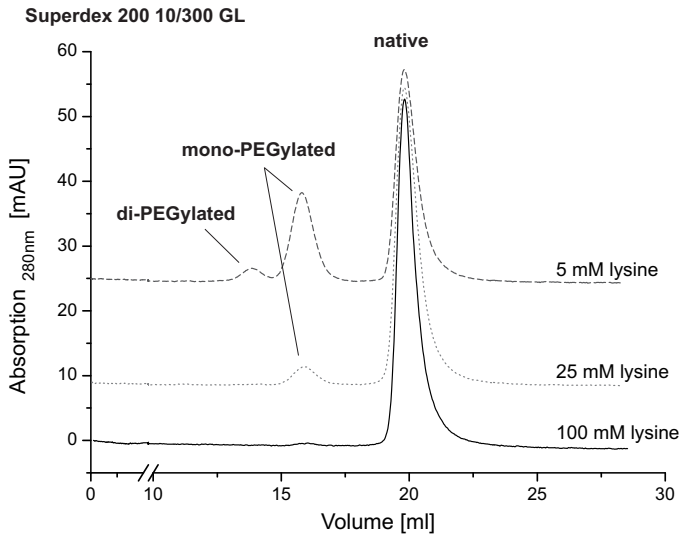


Figure 1: SEC chromatogramms of mPEG-aldehyde lysozyme reaction preparations with different lysine concentrations after 20 h reaction time. Reaction conditions were pH 6.2 and 0.15 protein to PEG ratio.

active, unreacted PEG could not be evaluated directly. However, the degree of PEG conversion could be calculated via initial PEG concentration and formed PEG-protein conjugates. For each buffer condition, fractions of reaction products were analyzed and obtained data were plotted over reaction time, using exponetial fit functions as illustrated in Figure 2.

All standard deviations were below 5% and mass balances were consistently between 88% and 100% in all sample preparations, indicating precise liquid handling and reproducibility of the automated PEGylation. As can be seen, a constant decrease of native lysozyme is connected with a simultaneous accumulation of different PEG-protein conjugates. Looking at buffer preparations containing a high PEG excess, maximal fractions of mono-PEG-lysozmye can be detected after approximately 2.5 h, followed by decrease of the formed conjugate. This reaction behavior is in concert with publications suggesting a consecutive pseudo-first order reaction behavior of random PEGylation reactions under conditions of a high PEG excess [24, 27] and can be described as follows:

$$\begin{aligned} PEG + protein &\rightarrow \text{mono-PEG-protein} \\ \text{mono-PEG-protein} + PEG &\rightarrow \text{di-PEG-protein} \\ &\vdots \end{aligned} \tag{1}$$

Comparing the results with lysozyme reaction kinetics at pH 7 and a protein to PEG ratio of 0.5 published by Moosmann et al. [19] a similar reaction behavior can be identified, while the differences in reaction velocity might be explained with additional mixing and increased PEG excess, made in this study.

To illustrate the influence of PEG excess and buffer pH, Figure 3 depicts the results for mono-PEG lysozyme, exclusively. The contour plot was generated using a triangle based

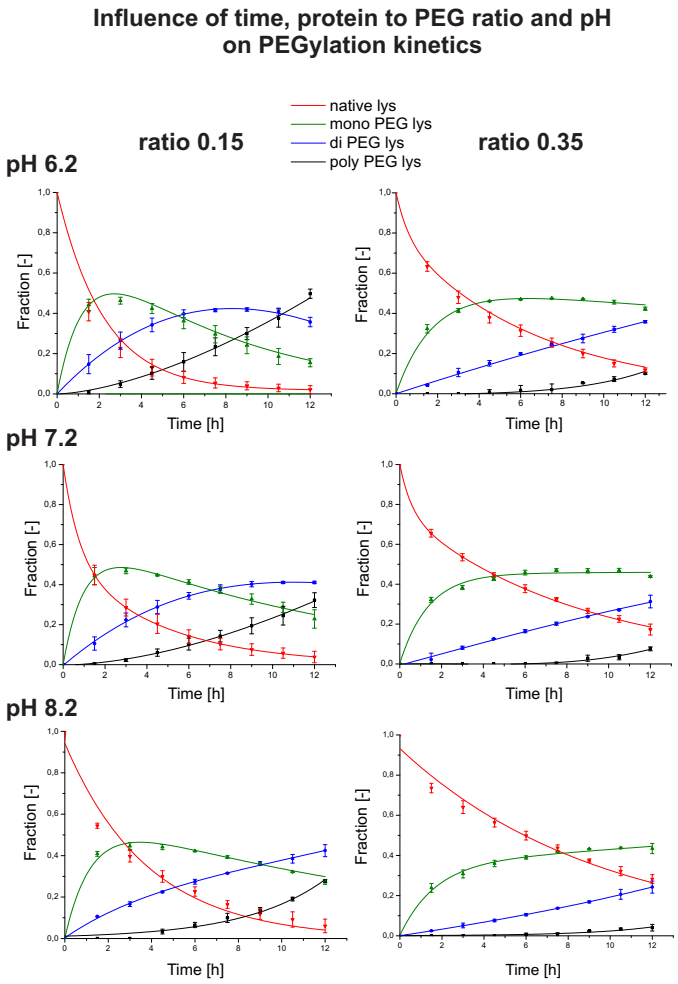


Figure 2: Native lysozyme and PEGamer distribution of different reaction conditions, based on SEC chromatograms.

cubic interpolation.

According to that, higher PEG excess as well as lower buffer pH yields a faster PEGylation reaction. In addition, an influence of the pH on the maximal fraction of formed mono-PEG-lysozyme was observed. However, the pH independency of lysozyme PEGylation reactions proposed by Moosmann et al. could not be verified, as a clear influence of the buffer pH regarding reaction velocity and maximal mono-PEG-lysozyme accumulation was found. This underlines the importance of a systematic screening and the evaluation of sufficient data points, as Moosmann et al. investigated the influence of different process parameters only with 'one factor at a time' (OFAT) experiments for optimization purposes.

With the determination of the reaction kinetics, important process parameters such as the PEG conversion and the selectivity for a specific conjugate were also investigated. The highest selectivity for mono-PEG lysozyme (93 %) was found at pH 7.2 after 1.5 h with a protein to PEG ratio of 0.35. Maximal PEG conversion of 50 % was achieved with pH 6.2, and a protein to PEG ratio of 0.35, after 12 h.

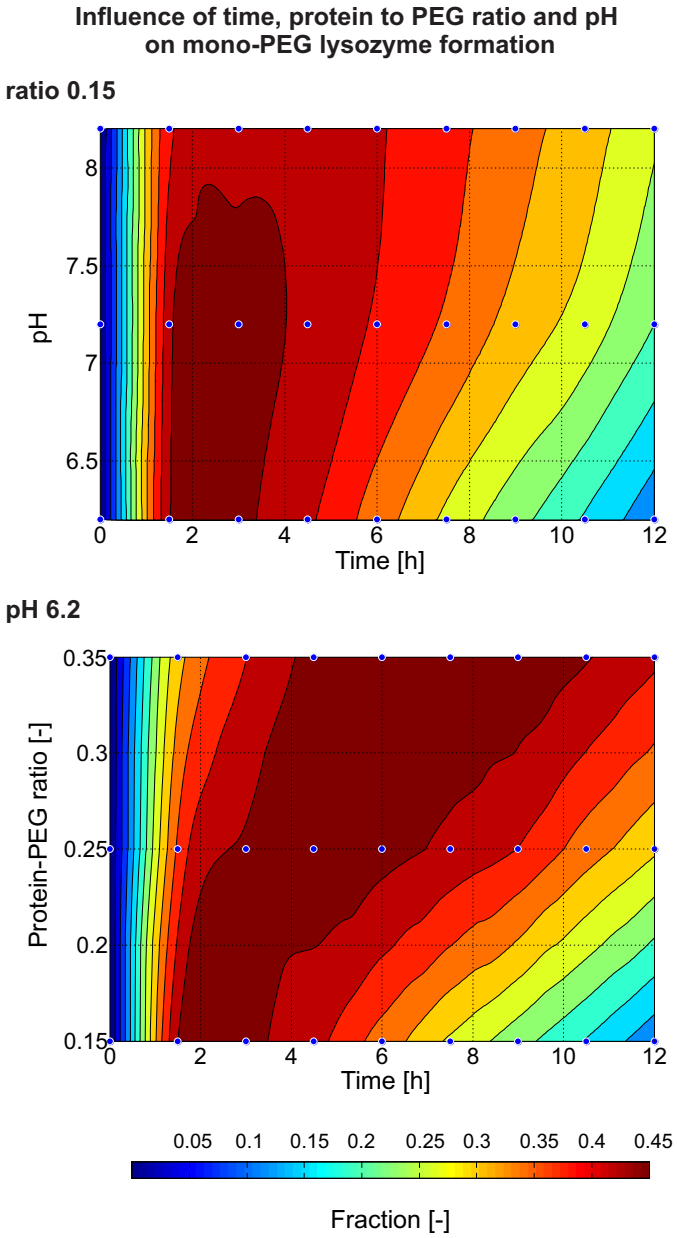


Figure 3: Contour plots with the fraction of mono-PEG-lysozyme, based on SEC chromatograms. Dots show reaction conditions evaluated.

3.1.3 PEGamer stability

Protein PEGylation has been shown in numerous studies to enhance thermal stability, that can result in improved storage behavior and extended shelf-life. An increased thermal stability was, for example, investigated by Lee et al. [2] for PEGylated interferon using turbidimetric methods or by Kinstler et al. [14] evaluating the aggregation level of PEG-G-CSF over time with SEC. Measuring the hydrodynamic radius (R_h) with DLS as a function of increasing temperature, the aggregation of PEGylated and native lysozyme was investigated, in this study. Figure 4 presents the obtained results and illustrates that aggregation of PEG-modified species occurred at higher temperatures compared to native lysozyme.

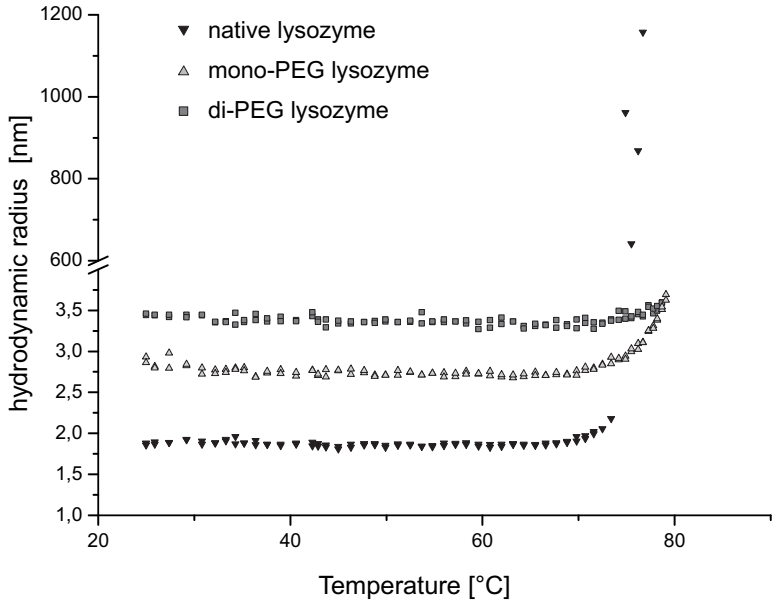


Figure 4: Hydrodynamic radii of native and PEGylated lysozyme as a function of temperature

The improved aggregation behavior of PEGylated species can be connected with the shielding effect of attached PEG molecules which prevents the interaction of hydrophobic patches. This might imply a complete entanglement of PEG around the protein. However, recent studies by Pai et al. [28] suggest a model where the attached PEG random coil is adjacent to the protein.

3.1.4 Isoform distribution

All 72 prepared PEGylation samples were analyzed for their mono-PEG lysozyme isoform distribution. For this, a recently published analysis using pH chromatography was used, which allows an isoform separation and the identification of the corresponding PEGylation sites [21]. Lysine 1 was identified to be the most reactive PEG conjugation site, followed by lysine 33 using reaction buffer pH values of 7.2. A chromatogram of purified mono-PEGylated lysozyme with the proposed elution order is shown in Figure 5.

As mono- and di-PEG conjugates co-elute using this analytics, an evaluation of crude PEGylation mixture was not possible (data not shown). Thus, a two-step analysis was chosen for the screenings, consisting of a mono-PEG-lysozyme fractionation using SEC, followed by a pH gradient chromatography. Employing the described analytics, an evaluation of the influence of buffer pH and reaction time on the isoform distribution could be achieved. Figure 6 depicts schematically the fractions of each isoform after a reaction time of 12 h.

As can be seen, lysozyme PEGylated at lysine 1 (in the following labeld as PEG-lys_1) represents the predominant isoform at pH 6.2, while the most reactive site at pH 8.2 is lysine 33. A correlation to this can be found at Kinstler et al. [14], where a decreasing buffer pH led to a PEG attachment at the N-terminus. As mentioned in the introduction, this behavior can be explained with the lower pK_a value of the the α-amino residue compared

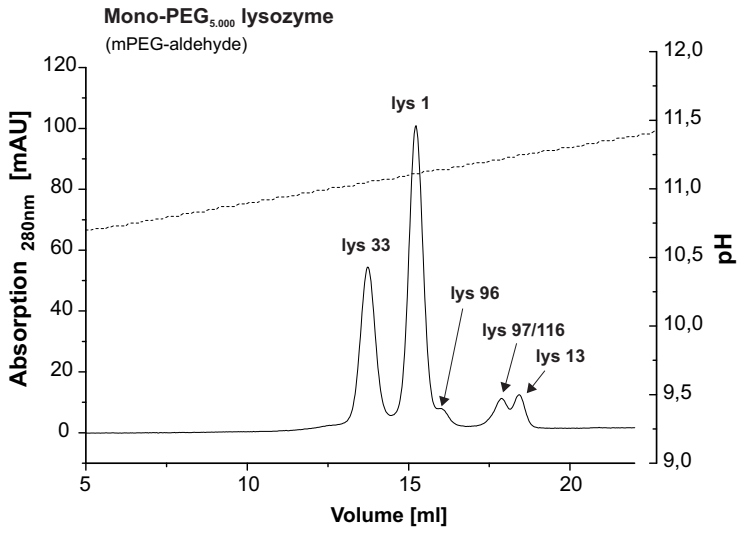


Figure 5: Chromatogragraphic separation of mono-PEG$_{aldehyde}$-lysozyme isoforms. Peak labels correspond to PEG binding sites, according to Maiser et al. [21]

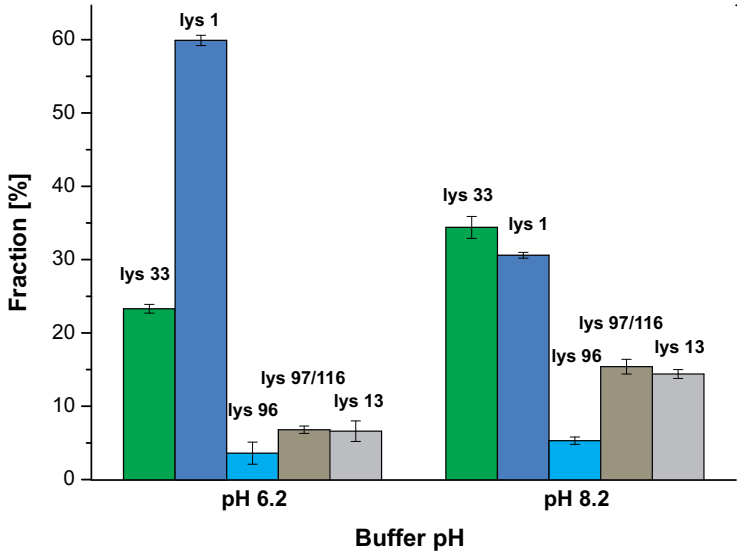


Figure 6: Distribution of mono-PEGylated lysozyme isoforms in dependency of the buffer pH. Both reactions after 12 h reaction time and a protein to PEG ratio of 0.15.

to the ϵ-amino residue. In addition, for reaction mixtures at pH 6.2, the reaction time had a significant impact on the isoform formation, which is illustrated in Figure 7.

The relative increase of PEG-lys$_1$ over time is probably connected to the different isoform reactivities and the formation of di-PEG-lysozyme. In this case mono-PEG-lysozyme conjugates are mostly formed by the attachment of PEG to lysine 1. If a mono-PEG-lysozyme is not PEGylated at lysine 1, the most reactive site is still free for a PEG conjugation, and reacts at first to di-PEG-lysozyme. Assuming the reaction rate from native to mono-PEG-protein is lower than from mono- to di-PEG protein, this results in a relative increase of lysine 1 mono-PEGylated variants.

The combination of mono-PEG-lysozyme concentration and the measured isoform frac-

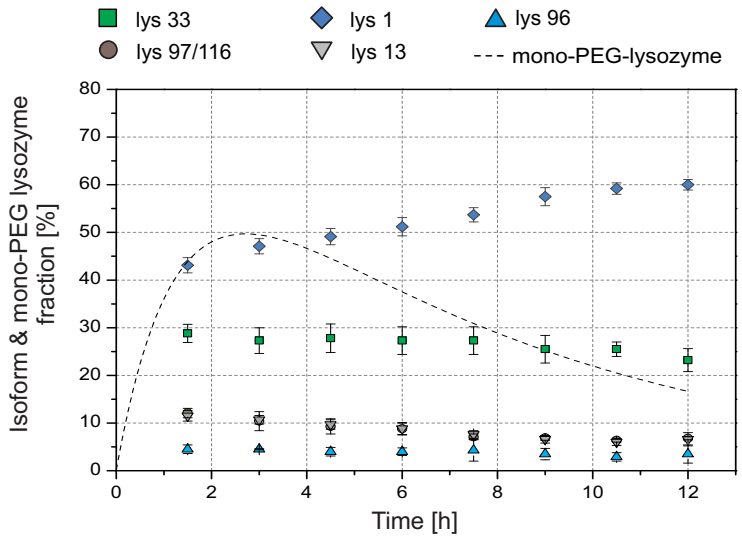


Figure 7: Isoform distribution as a function of reaction time. Reaction parameter: pH 6.2, 0.15 protein to PEG ratio

tions allows the determination of process parameters to achieve the maximal concentration of each isoform. This might be interesting for a pharmaceutical application, if a certain isoform provides improved characteristics compared to other isoforms, including increased stability or higher residual activity.

3.1.5 Lysozyme activity

First described in 1952 by Shugar [29], the lytic activity of lysozyme can be determined by measuring the changes in turbidity of a *Micrococcus lysodeikticus* suspension. Figure 8 A shows the normalized linear decrease in the UV 450 nm signal of analyzed isoforms and native lysozyme. Relative and specific activities of the evaluated samples are illustrated in Figure 8 B and Table 1, respectively.

Table 1: Specific and normalized activity of native and PEGylated lysozyme isoforms.

	Specific activity [U/mg]	normalized activity [%]
native lysozyme	22.271	100
Lys 33	7.681	35
Lys 1	5.287	24
Lys 96	3.163	14
Lys 97 & 116	3.824	17
Lys 13	3.196	14

All isoforms showed a reduced specific activity compared to native lysozyme, as expected. Lysozyme PEGylated at position lysine 33 provided with 35% the highest residual activity, followed by PEG-lys$_1$ with 24%. Studies by Abe et al. [30] suggest a residual activity of about 30% for 5 kDa mono-PEG lysozyme isoform mixtures, which is thus in the line

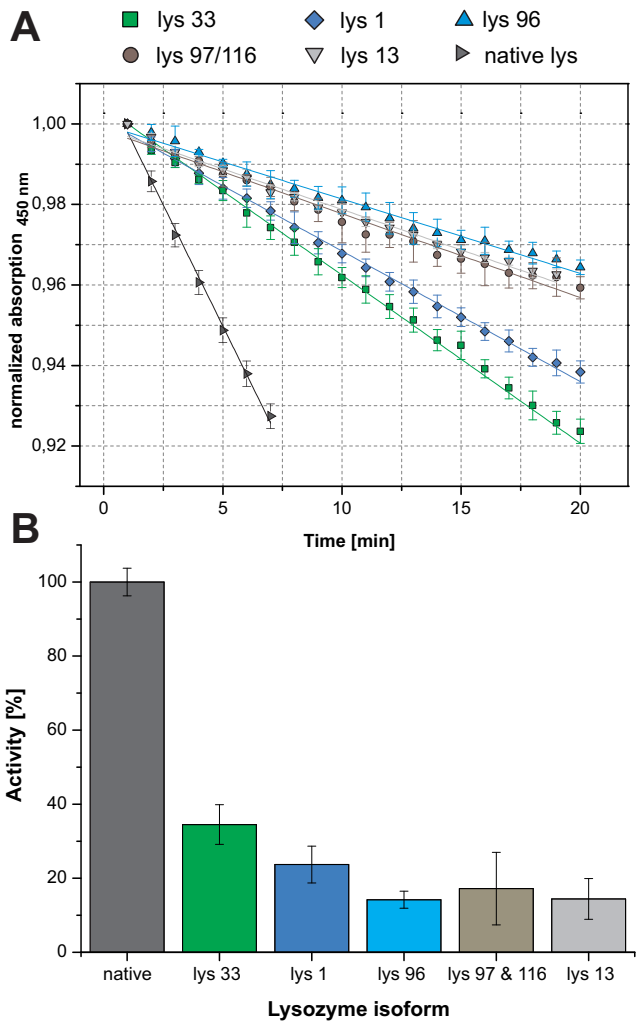


Figure 8: **A**: normalized linear decrease in the 450 nm signal of each sample over time. **B**: resulting realtive activity of each isoform, compared to native lysozyme.

with the results made in this study. As can be seen, the PEG conjugation site has an influence on the residual activity, which corresponds to Monkarsh et al. [31] where different activities in the range of 6% and 40% of mono-PEGylated interferon were found. Looking at the three-dimensional structure of lysozyme, the high residual activity of PEG-lys$_{33}$ cannot be explained with steric effects of PEG, as lysine 33 is close the catalytic residues Glu 35 and Asp 52. However, the PEG structure after protein conjugation is still not completely understood as already mentioned in the PEGamer stability section. In addition, differences in isoform activity might be explained with altered charge distributions on the protein surface, which plays an important role in enzyme-substrate binding mechanisms.

3.2 PEGylation conditions for a QbD approach: two case studies

In the development of pharmaceutical applications, reproducible processes and the delivery of a product with a constant activity profile represents an important aspect. The definition of control and design spaces can help to achieve this aim and is mandatory for regulatory

approval since the QbD initiative by the FDA and the International Conference on Harmonisation (ICH), respectively. In spite of possible variations in protein characteristics due to different PEG attachment sites, isoform mixtures are still allowed and represent the major product of all approved PEGylated pharmaceuticals so far. Applying a corresponding QbD approach to the present PEGylation reaction, isoform concentrations of mono-PEGylated lysozyme and the specific activities of each isoform were combined to calculate the volumetric activity of isoform mixtures. The obtained results were plotted as a function of buffer pH and reaction time, as shown in Figure 9A. Accordingly, maximal volumetric activities can be generated with reaction times of about 2 h and buffer pH values between pH 6.2 and pH 7.2, using a protein to PEG ratio of 0.15.

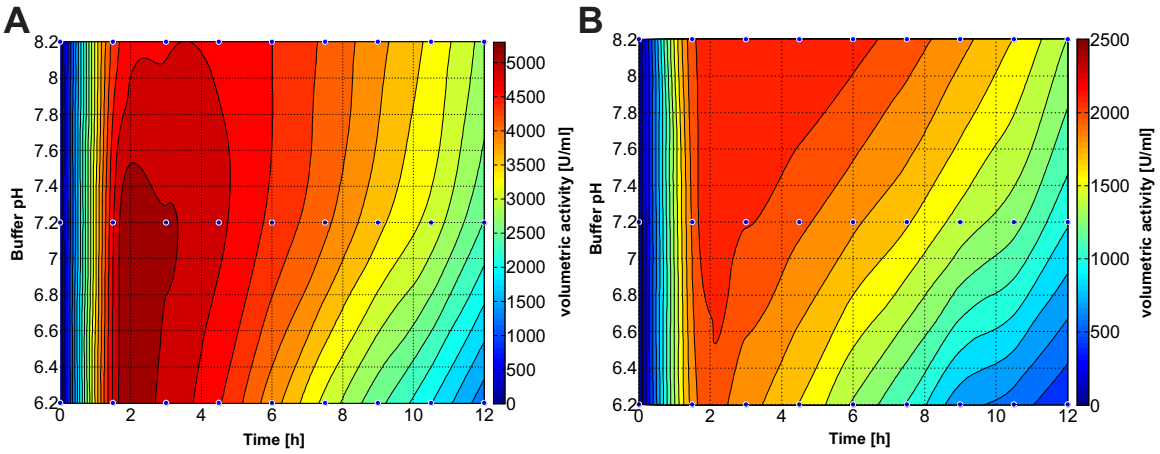


Figure 9: Calculated volumetric activity of mono-PEG lysozyme isofom mixtures (**A**) and PEG-lys$_{33}$ (**B**) as a function of reation time and buffer pH. Protein to PEG ratio was 0.15.

However, products with only one isoform might be required in future for a successful approval, due to improved site–specific PEGylation approaches or better preparative isoform purification techniques. To meet these possible requirements, an isolation of PEG-lys$_1$ or PEG-lys$_{33}$ isoforms would appear reasonable in the present case. The corresponding design space for PEG-lys$_{33}$ is illustrated in Figure 9B, indicating different process parameter to gain maximal volumetric activity. A further optimization of the product and the calculation of a sweet spot could be achieved by combining the existing activity results with data from additional analytics. This could include for example isoform stability studies with the described thermal stability analysis.

3.3 mPEG-NHS

In order to evaluate the influence of the PEG reagent regarding PEGamer and isoform formation, modifications with mPEG-NHS were carried out additionally. SEC chromatograms of the nine prepared samples showed constant PEGamer distributions after 30 min, which implies a fast hydrolysis of the used PEG (data not shown). Figure 10 A illustrates the obtained chromatograms of PEGylation mixtures, conducted with a protein to PEG ratio of 0.35 and varying buffer pH values.

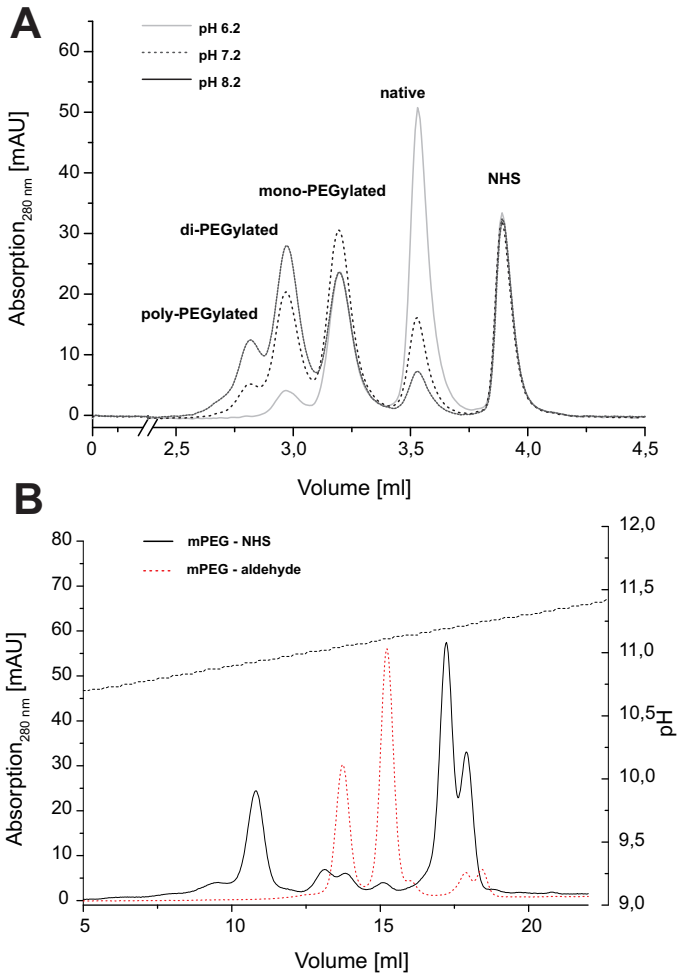


Figure 10: A: SEC chromatograms of mPEG-NHS modified lysozyme reaction mixtures with a Sepax Zenix™ SEC 300 column, **B:** Comparison of the isoform elution profiles of mono-PEG-lysozyme. Reaction buffer pH was 6.2

In contrast to mPEG-aldehyde reactions, an increased buffer pH value yields in an increased formation of PEGylated species. Thus, an accelerated hydrolysis of the NHS group or a reduced PEGylation reaction velocity can be assumed using low buffer pH values. In addition, larger PEG excess resulted in an increased accumulation of PEGylated species, which is comparable to mPEG-aldehyde reactions (data not shown). By comparing the retention volumes of mono-PEG lysozyme isoforms modified with mPEG-aldehyde and mPEG-NHS, shown in Figure10 B, the latter shows a small shift to lower elution pH values. This could be explained with the different chemistries between acylating and alkylating PEG derivatives, where mPEG-NHS removes and mPEG-aldehyde preserves the charge of the amino group. Looking at the isoforms being formed with mPEG-NHS, at least seven peaks can be detected, indicating more binding sites in contrast to mPEG-aldehyde modifications. This is probably connected with an additional PEG conjugation via histidine, serine or tyrosine residues, which is possible using mPEG-NHS, as reported by Veronese et al. [12]. However, a final identification of mPEG-NHS isoform binding sites was not conducted, as this would exceed the scope of this manuscript. Further, an influence on the isoform distribution with

varying pH values was not observed for the mPEG-NHS modifications (data not shown).

The additional mPEG-NHS modifications suggest a major influence of the PEG reagent on reaction behavior and resulting PEGamer and isoforms distribution. Additional differences between both described PEGylation methods, for example in stability or isoform activity were not evaluated but should be taken into account when choosing the type of PEGylation.

4 Conclusion and outlook

In the present study it was shown how a classical PEGylation reaction can be optimized with respect to maximal isoform concentration and maximal volumetric activity using high throughput methods. The method described herein, showed that so called random PEGylation reactions combined with a systematic screening approach and high sensitive analytics can result in a controlled PEGylation of a target molecule and thus can meet the FDA guidelines regarding process understanding and reproducibility. Additional experiments with mPEG-NHS illustrated the severe impact of a different PEG reagent on formed isoforms and reaction behavior. Future work might focus on the mathematic modeling of described reactions and the influence of different reaction parameters such as reaction temperature and PEG molecular weight.

5 Acknowledgments

We gratefully acknowledge financial support by the Federal Ministry of Education and Research (BMBF). In addition the authors would like to thank Isabell Thanheiser for conducting parts of the lab work.

References

[1] B. Leader, Q. J. Baca, and D. E. Golan. Protein therapeutics: a summary and pharmacological classification. *Nat. Rev. Drug Discov.*, 7(1):21–39, Jan 2008.

[2] B. K. Lee, J. S. Kwon, H. J. Kim, S.i Yamamoto, and E. K. Lee. Solid-phase PEGylation of recombinant interferon alpha-2a for site-specific modification: Process performance, characterization, and in vitro bioactivity. *Bioconjugate Chem.*, 18(6):1728–1734, 2007.

[3] A. Basu, K. Yang, and M. Wang. Structure-function engineering of interferon-beta-1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation. *Bioconjugate Chem.*, 17(3):618–630, 2006.

[4] T. Palm, R. Esfandiary, and R. Gandhi. The effect of PEGylation on the stability of small therapeutic proteins. *Pharm. Dev. Technol.*, pages 441–448, 2011.

[5] S. Chaffee, A. Mary, E. R. Stiehm, D. Girault, A. Fischer, and M. S. Hershfield. Igg antibody response to polyethylene glycol-modified adenosine deaminase in patients with adenosine deaminase deficiency. *J. Clin. Invest.*, 89(5):1643–51, May 1992.

[6] S. Jevsevar, M. Kunstelj, and V. Gaberc-Porekar. Pegylation of therapeutic proteins. *Biotechnol. J.*, 5(1):113–28, 2010.

[7] G. Pasut and F. M. Veronese. State of the art in PEGylation: The great versatility achieved after forty years of research. *J. Control. Release*, 161(2):461–72, Jul 2012.

[8] C. J. Fee and J. A. Van Alstine. Peg-proteins: Reaction engineering and separation issues. *Chem. Eng. Sci.*, 61(3):924–939, Feb 2006.

[9] M. Sergi, F. Caboi, C. Maullu, G. Orsini, and G. Tonon. Enzymatic techniques for PEGylation of biopharmaceuticals. In F. M. Veronese, editor, *PEGylated Protein Drugs: Basic Science and Clinical Applications*, pages 75–88. Birkhäuser Verlag, 2009.

[10] K. Yang, A. Basu, M. Wang, R. Chintala, M.-C. Hsieh, S. Liu, J. Hua, Z. Zhang, J. Zhou, M. Li, and et al. Tailoring structure-function and pharmacokinetic properties of single-chain fv proteins by site-specific pegylation. *Protein Engineering Design and Selection*, 16(10):761–770, Oct 2003.

[11] H. Sato. Enzymatic procedure for site-specific pegylation of proteins. *Adv. Drug. Deliv. Rev.*, 54(4):487–504, Jun 2002.

[12] F. M. Veronese and G. Pasut. PEGylation: Posttranslational bioengineering of protein biotherapeutics. *Drug Discov. Today*, 5(2-3):e57–e64, Sep 2008.

[13] M. J. Roberts, M. D. Bentley, and J. M. Harris. Chemistry for peptide and protein PEGylation. *Advanced Drug Delivery Reviews*, 54(4):459–476, 2002.

[14] O. B. Kinstler, D. N. Brems, S. L. Lauren, A. G. Paige, J. B. Hamburger, and M. J. Treuheit. Characterization and stability of N-terminally PEGylated rhG-CSF. *Pharm. Res.*, 13(7):996–1002, 1996.

[15] G. M. Bonora and S. Drioli. Reactive PEGs for protein conjugation. In F. M. Veronese, editor, *PEGylated Protein Drugs: Basic Science and Clinical Applications*, pages 33–45. Birkhäuser Verlag, 2009.

[16] R.W. Payne, B.M. Murphy, and M.C. Manning. Product development issues for PEGylated proteins. *Pharm. Dev. Technol.*, 16(5):423–40, Oct 2011.

[17] V. Gaberc-Porekar, I. Zore, B. Podobnik, and V. Menart. Obstacles and pitfalls in the PEGylation of therapeutic proteins. *Curr. Opin. Drug Di. De.*, 11(2):242–50, Mar 2008.

[18] S. K. Hansen, B. Maiser, and J. J. Hubbuch. Rapid quantification of protein pegamers by multivariate evaluation of chromatographic data. *J. Chrom. A.*, 1257:41–47, 2012.

[19] A. Moosmann, J. Blath, R. Lindner, E. Müller, and H. Böttinger. Aldehyde PEGylation kinetics: a standard protein versus a pharmaceutically relevant single chain variable fragment. *Bioconjugate Chem.*, 22(8):1545–58, Aug 2011.

[20] R. Bhambure, K. Kumar, and A. S. Rathore. High-throughput process development for biopharmaceutical drug substances. *Trends in biotechnol.*, 29(3):127–35, Mar 2011.

[21] B. Maiser, F. Kröner, F. Dismer, G. Brenner-Weiß, and J. J. Hubbuch. Isoform separation and binding site determination of mono-PEGylated lysozyme with ph gradient chromatography. *J. Chrom. A.*, 1268:102–108, 2012.

[22] S. Oelmeier, F. Dismer, and J. Hubbuch. Application of an aqueous two-phase systems high-throughput screening method to evaluate mAb HCP separation. *Biotechnol. Bioeng.*, 108(1):69–81, Jan 2011.

[23] K. E. Ottow, T. Lund-Olesen, T. L. Maury, M. F. Hansen, and T. J. Hobley. A magnetic adsorbent-based process for semi-continuous PEGylation of proteins. *Biotechnol. J.*, 6(4):396–409, Apr 2011.

[24] M. J. Roberts and J. M. Harris. Attachment of degradable poly(ethylene glycol) to proteins has the potential to increase therapeutic efficacy. *J. Pharm. Sci.*, 87(11):1440–1445, 1998.

[25] Y. C. Lee and D. Yang. Determination of lysozyme activities in a microplate format. *Anal. Biochem.*, 310(2):223–4, Nov 2002.

[26] D. Freitas and J. Abrahao-Neto. Biochemical and biophysical characterization of lysozyme modified by PEGylation. *Int. J. Pharm.*, 392(1-2):111–7, Jun 2010.

[27] A. S. Morar, J. L. Schrimsher, and M. D. Chavez. PEGylation of proteins: A structural approach. *Biopharm Int.*, 19(4), 2006.

[28] S. Pai, B. Hammouda, K. Hong, D. C. Pozzo, T. M. Przybycien, and R. D. Tilton. The conformation of the poly(ethylene glycol) chain in mono-pegylated lysozyme and mono-pegylated human growth hormone. *Bioconjugate Chem.*, 22(11):2317–2323, Nov 2011.

[29] D. Shugar. The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. *Biochem. Biophys. Acta*, 8(3):302–9, Mar 1952.

[30] M. Abe, P. Akbarzaderaleh, M. Hamachi, N. Yoshimoto, and S. Yamamoto. Interaction mechanism of mono-PEGylated proteins in electrostatic interaction chromatography. *Biotechnol. J.*, 5(5):477–83, May 2010.

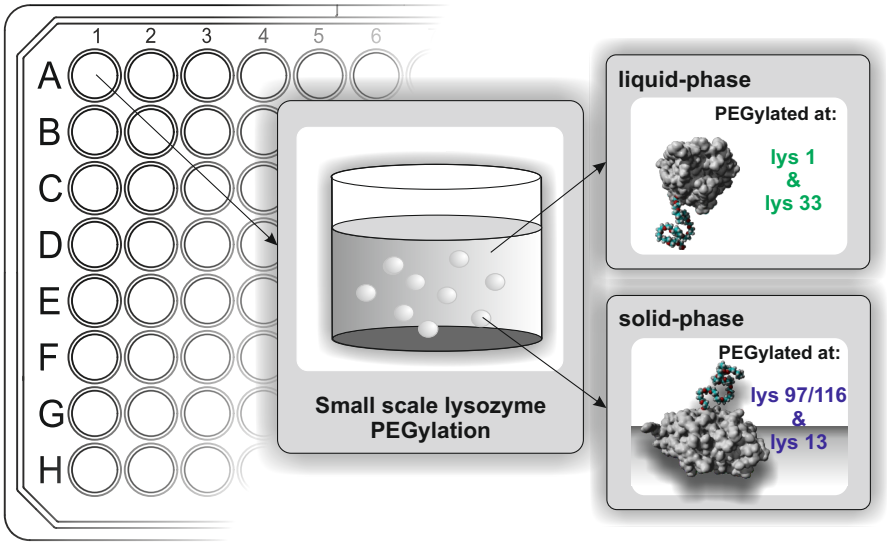
[31] S. P. Monkarsh, Y. M. Ma, A. Aglione, P. Bailon, D. Ciolek, B. DeBarbieri, M. C. Graves, K. Hollfelder, H. Michel, A. Palleroni, and et al. Positional isomers of monopegylated interferon alpha-2a: Isolation, characterization, and biological activity. *Anal.l Biochem.*, 247(2):434–440, 1997.

Effect of Lysozyme Solid-Phase PEGylation on Reaction Kinetics and Isoform Distribution

Benjamin Maiser, Kai Baumgartner, Florian Dismer and Jürgen Hubbuch*

Institute of Engineering in Life Sciences, Biomolecular Separation Engineering
Karlsruher Institute of Technology (KIT), Karlsruhe, Germnay

*Corresponding author: Tel: +49 721 608-46263, Mail: juergen.hubbuch@kit.edu
Engler-Bunte-Ring 1, 76131 Karlsruhe



Abstract

The combination of PEG-protein conjugation and a chromatographic separation is generally known as solid-phase or on-column PEGylation and can provide advantages compared to commonly applied batch PEGylation. Even though the concept was already applied by several authors, changes in the isoform distribution compared to liquid phase PEGylation, due to sterically hindered PEGylation sites could not be confirmed. In this manuscript, a method for solid-phase PEGylation experiments in a 96-well plate format, using an automated liquid handling station is described. Applying size exclusion chromatography (SEC) and high sensitive isoform analytics for mono-PEGylated lysozyme, we were able to investigate the differences in reaction kinetics and isoform distribution between adsorber based PEGylation and modifications in free solution. Accordingly, solid-phase PEGylation with SP Sepharose FF and XL showed generally a reduced PEGylation reaction. In contrast to the predominant N-terminal PEGylation of lysozyme in liquid phase, a main modification of lys 97 and lys 116 was found for solid phase experiments, which could be explained with binding orientations on corresponding adsorbent materials. Further experiments with varying amount of bound protein showed an influence on the isoform distribution of mono-PEGylated lysozyme additionally.

Keywords: PEGylation, lysozyme, high throughput process development, high throughput experimentation, Quality by Design

1 Introduction

Since four decades, attaching polyethylene glycol (PEG) to a target molecule is widely used to modify pharmacological properties of proteins, peptides or polynucleotides and is still the method of choice to improve the efficacy of biopharmaceuticals. Up to now, ten FDA approved products have been entered the market [1], confirming safety and therapeutic value of the applied method. Reported advantages of PEGylated proteins compared to unmodified species include enhanced circulation half-life, improved stability, increased solubility and reduced immunogenicity [2–4]. The altered characteristics can mostly be attributed to a shielding effect of attached PEG chains resulting in a delayed renal clearance, due to reduced proteolytic degradation and increased hydrodynamic radius [5–8]. However, these benefits are generally attended by a reduced specific activity, which is also induced by the protective effect of attached PEG molecules.

For protein PEGylation, so called random reactions are commonly used, targeting over-represented amino acids such as lysine or the N-Terminus. These reactions consequently yield complex product mixtures consisting of molecules with a different number of bound PEG (PEGamers) and positional isoforms. However, each PEG-protein conjugate can offer different properties with severe impact on important clinical aspects such as the final specific activity. Significant influence of the PEG attachment site on the activity was shown for example by Monkarsh et al. [9] where residual activities between 40% and 6% of mono-PEGylated interferon (IFN) were analyzed. Despite of such differences, characterized isoform mixtures are still allowed by the FDA, and provide the major product of all approved PEG-proteins. Due to maximal residual activity and minimal number of positional isoforms, proteins with a single attached PEG chain are thus the preferred modification type for pharmaceutical applications. However, as the PEGamer and isoform distribution can be influenced by process parameters such as pH and PEG-excess additionally, process control and reproducibility of the developed PEGylation reaction is essential to avoid variations in product configuration and thus in resulting product profile.

The standard PEGylation procedure uses batch modification of already purified and pure protein in free solution, followed by a chromatographic separation of resulting mixture [10]. The combination of both unit operations is known as solid phase or on-column PEGylation. It offers advantages with respect to process control, as PEGylation reaction, removal of unwanted variants and the isolation of the target conjugate can be realized in one step. This technique has been investigated by several authors and can be conducted in two different ways. One approach includes the immobilization of reactive PEG molecules on adsorbent matrices and the start of the reaction by adding proteins. Shang et al. [11] for example adsorb PEG-aldehyde on HIC membranes in a high salt environment. After a flow-through of lysozyme, the authors eluted mono- and di-PEGylated variants and proposed an increased selectivity towards mono-PEG lysozyme compared to equivalent experiments in free solution.

Another approach describes the second possibility, were the target molecule is immobilized to the adsorbent matrix and PEGylated by a flow-through of activated PEG. Lee et al. [4] and Suo et al. [12] describe this technique for the PEGylation of interferon-α (IFN) and

bovine hemoglobin, respectively. Both authors determined reduced multi-PEGylation compared to liquid phase PEGylation, which was explained with steric effects, such as blocked PEGylation sites.

In addition to reduced unit operations and suppressed multi-PEGylation using solid-phase PEGylation, changes in the isoform distribution resulting from an oriented binding behavior of proteins on the chromatographic media were also discussed, due to its important impact on the product profile. Monkarsh et al. [13] analyzed liquid- and solid-phase PEGylated IFN using an isoform analysis for mono-PEGylated variants. However, differences in the distribution of formed isoforms by comparing both elution profiles could not be detected. Studies by Baran et al. [14] reported higher residual activities of on-column PEGylated asparaginase and catalase using dye chromatography with Red Sepharose^{TM}in comparison to species being PEGylated in free solution. This implies a protection of the active site due to the on-column PEGylation and thus a different isoform composition. However, the authors did not validate this by own experiments but compared the obtained specific activities with liquid phase experiments provided by literature. As these reference reactions were partially conducted with different PEGylation reactions, the improved activity profile might also be related to the different chemistry. Thus a change in the isoform distribution could not be confirmed finally.

In recently published manuscripts, the separation and binding site identification of mono-PEGylated lysozyme isoforms has been discussed and the influence of buffer conditions on the isoform distribution has been investigated [15, 16]. Based on this background knowledge, solid-phase PEGylation of lysozyme was conducted in this study, employing automated small scale PEGylation reactions in 96-well plates. For this, lysozyme was initially bound onto SP Sepharose FF and XL and was subsequently incubated with mPEG-aldehyde. After elution, reaction mixtures were analyzed for PEGamer and isoform distribution of mono-PEG lysozyme using SEC and high sensitive pH gradient chromatography, respectively. Obtained results showed a significant influence of the resin type on reaction kinetics. Additionally, isoform changes compared to liquid-phase PEGylation were detected, demonstrating the applicability of used method to screen for favorable isoform compositions.

2 Material and methods

2.1 Chemicals

Components for buffer preparation including sodium phosphate and sodium chloride as well as hen egg white lysozyme were purchased from Sigma Aldrich (St. Louis, MO, USA). As PEG reagent, methoxy-PEG-aldehyde (mPEG-aldehyde) with an average molecular weight of 5 kDa from NOF Cooperation (Tokyo, Japan) was used. Sodium cyanoborohydride ($NaCNBH_3$) was used as reducing agent for PEGylation reactions and were obtained from Sigma-Aldrich. For isoform separation cyclohexylaminobutansulfonic acid (CABS) was used as buffer component and was provided by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Water was provided by an Arium pro water purification system from Sartorius Ste-

dim (Goettingen, Germany). Additional filtration with 0.2 μm cellulose acetat membrane filters was conducted for all buffer solutions. For solid–phase PEGylation experiments the conventional cation exchange resin SP Sepharose FF, as well as the tentacle-type resin SP Sepharose XL were used (both from GE Healthcare, Uppsala, Sweden).

2.2 Apparatus

PEGylation experiments and isotherm binding studies were performed on a Freedom EVO® 200 liquid handling station from Tecan (Crailsheim, Germany). The system is equipped with eight fixed tips, a 96-channel liquid handling arm, an automated plate handling arm and a rotational shaker (TeShake, Tecan). In addition, a centrifuge (Rotana 46RSC, Hettich, Tuttlingen, Germany) and an Infinite M200 UV spectrometer from Tecan are integrated to the system. To operate the robotic system, the Evoware 2.1 software from Tecan was used. Data handling and import of pipetting volumes was realized using Microsoft Excel (Redmond, WA, USA). All analytical chromatography steps were conducted on Äkta Purifer and Äkta Ettan systems from GE Healthcare using the Unicorn 5.1 software package for system control and data analysis of UV signals.

2.3 Binding isotherms

In order to evaluate the capacity of used adsorbent material, adsorption isotherms were determined. All isotherms were conducted using 25 mM sodium phosphate buffer, pH 7.2 and were performed at least in triplicates. Automated pipetting, mixing and centrifugation was carried out on the robotic liquid handling station from Tecan. Adsorbent samples (20.8 μL) were produced with a plaque device from Atoll (Weingarten, Germany) and were placed in 2.2 ml deep well plates from ABgene (Epsom, UK). Resin incubation with different concentrations of lysozyme was conducted using the rotational shaker for 2 h. After centrifugation with 1000 rpm for 10 min, the supernatant was transferred into 96-well UV star plates from Greiner (Frickenhausen, Germany) and UV_{280} was measured with the integrated spectrophotometer.

2.4 PEGylation procedure

Solid–phase PEGylation experiments including protein binding, PEG incubation and elution were carried out on the liquid handling station. PEGylation experiments in free solution were also conducted automatically as previously reported [16].

2.4.1 Protein binding

Protein loading of adsorbent material for solid–phase PEGylation experiments was conducted as described in the binding isotherms section. The sample volume was set to 1 ml. After incubation and centrifugation, the supernatant was analyzed, measuring UV 280 nm to evaluate the amount of bound protein. To remove unbound protein, two washing steps with 500 μL washing buffer (25 mM sodium phosphate buffer) were conducted, including flushing,

centrifugation and removal of supernatant. To investigate the influence of protein density on the adsorbent surface, the surface coverage, representing the percentage of maximal binding capacity, was varied. Different surface coverage from 50% to 100% was realized by changing the protein concentration in the adsorbent incubation step.

2.4.2 PEG incubation

After the protein binding, loaded adsorbent particles were suspended in PEGylation buffer (25 mM sodium phosphate buffer, containing 20 mM $NaCNBH_3$ and mPEG-aldehyde.) and incubated using the integrated shaker (1000 rpm). The PEG excess was kept constant by calculating the amount of bound lysozyme and adjusting the concentration of added PEG. The protein to PEG ratio was set to 0.15, which corresponds to a 6.6 PEG excess. To obtain kinetic data, PEG solution was added well-by well. The PEGylation reaction was stopped with a centrifugation step and a subsequent washing procedure using the 96-channel liquid arm.

2.4.3 Elution

The elution of bound protein was carried out using a 25 mM sodium phosphate buffer, with 1 M NaCl. For this, adsorbent plaques were repeatedly incubated with 200 μL elution buffer for 30 min. and supernatant was collected. 600 μL eluate was pooled for analytical purposes.

2.5 Analytical procedure

2.5.1 PEGamer separation

Pooled elution samples from solid- and liquid–phase experiments were analyzed for their PEGamer distribution with size exclusion chromatography (SEC) using a Superdex 200 GL10/300 from GE Healthcare. As mobile phase, a 25 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl was chosen and the flow rate was set to 1.0 mL/min. To obtain samples for the isoform analysis, mono-PEG lysozyme was fractionated with a constant volume of 250 μL. The PEGylation degree was previously validated applying molecular weight determination with combined light scattering and refractive index analysis [16].

2.5.2 Isoform separation

Isoform separation was conducted on a MonoS 4.6/100 column from GE Healthcare as reported in recent publication [16]. Sample volumes between 100 μL and 150 μL of purified mono-PEG lysozyme were injected. For pH-gradient elution 20 mM CABS was used as buffer component. Running buffer A and elution buffer B were titrated with 4 M NaOH to pH 10.6 and pH 11.6, respectively. The elution was applied with a linear gradient ranging from 0% to 100% buffer B over 12 column volumes. The flow rate was set to 1.5 mL/min.

3 Results and discussions

The aim in this study was primarily to elucidate the influence of solid-phase PEGylation on the isoform distribution and thus the possible impact on important product properties. A second aspect was to establish a robotic based batch PEGylation method, enabling the generation of reaction kinetics to determine the differences between solid- and liquid-phase PEGylation. Due to numerous PEGylation studies in free solution and the knowledge about binding orientations on different cation-exchange resins, lysozyme was used as model protein [17–20].

3.1 Isotherms

To consider the amount of bound protein on the adsorbent surface, isotherms for both investigated cation exchange resins were generated. The obtained isotherm data were fitted with the Langmuir model according to Equation 1 and maximal binding capacities Q_{max} were calculated as illustrated in Figure 1. Thus a capacity of 95.7 mg/mL was evaluated for SP Sepharose FF and 157.0 mg/mL for Sepharose XL, respectively.

$$Q^* = Q_{max} \frac{K_d c^*}{1 + K_d c^*} \tag{1}$$

With:
Q_{max}: maximal binding capacity
Q^*: protein concentration in stationary phase
c^*: protein concentration in liquid phase
K_d: equilibrium constant

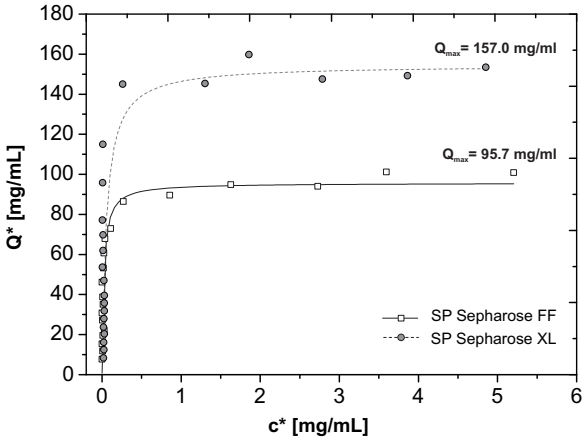


Figure 1: Isotherms for SP Sepharose FF and XL, both in 25 mM sodium phosphate buffer, pH 7.2. Maximal binding capacities were calculated using the Langmuir model.

.

Both investigated adsorbent materials offer a dextran based matrix carrying sulfopropyl (SP) ligands for cation exchange processes. Considering the flexible chains of tentacle type adsorbers the increased capacity of SP Sepharose XL can be connected with an increased accessibility of the ligands compared to conventional ion exchange resins. To compare solid–phase PEGylation reactions in the study, surface coverage was calculated and adjusted, which represents the percentage of the maximal binding capacity.

3.2 PEGylation kinetics

If proteins with multiple attachment sites are PEGylated in free solution and the presence of a PEG excess, native protein is modified via the most reactive site to a mono-PEG-protein conjugate, which reacts to di- and poly-PEG-protein in further reaction process. This can be described as a pseudo first order consecutive reaction [21, 22] and was shown for present lysozyme reaction in a study, published recently [16]. The comparison between a reaction in free solution and the solid-phase PEGylation using SP Sepharose FF and XL with respect to PEGamer formation is illustrated in Figure 2. All reactions were conducted under equal conditions, using a buffer pH of 7.2 and a protein to PEG ratio of 0.15, representing a PEG excess of 6.6. For solid-phase PEGylation, the lysozyme surface coverage of the resin particles was 60% and the recovery, determined by SEC, was consistently between 86% and 96%.

As can be seen, PEGylation of adsorbed lysozyme on SP Sepharose FF illustrated in Figure 2 A shows a maximal fraction of 10% for mono-PEGylated variants after 12 h, compared to a maximal value of 47% in liquid phase, after 3 h. Additionally, a formation of di-PEGylated variants could not be observed, indicating a significant reduction in reaction velocity for solid-phase experiments. These characteristics can be connected to different reasons. Considering lysozyme is immobilized on the resin surface, reduced amount of appropriate attachment sites due to steric hindrance by the resin surface might be possible and is discussed in the next section. In contrast to convection driven process in free solution, the reaction for solid-phase experiments is diffusion limited, given by film- and pore diffusion of PEG molecules, additionally. PEGamer kinetics for solid-phase PEGylation on Sepharose FF and XL over a reaction time of 60 h is shown in Figure 2 B and demonstrates a further reduction in reaction kinetics for the grafted resin type compared to the conventional ion exchange particles, which is probably based on increased protein density on the surface, and thus reduced protein accessibility. An influence of the reaction pH on the kinetics is shown in 3 and corresponds to former results for liquid-phase PEGylation, where decreasing buffer pH values led to an increased reaction velocity [16]. This effect could be observed both for SP Sepharose FF and XL (data not shown).

Comparing the solid-phase coupling kinetics made in this study with results provided by literature, a low reaction rate towards PEG-protein conjugates could generally be noticed, as a maximal conversion of only 37% for native lysozyme was detected after 60 h (see Figure 3). On-column experiments by Lee et al. [4] with packed CM-Sepharose particles and PEG-aldehyde resulted in an overall PEGylation of native IFN of 68%, after 120 min. However,

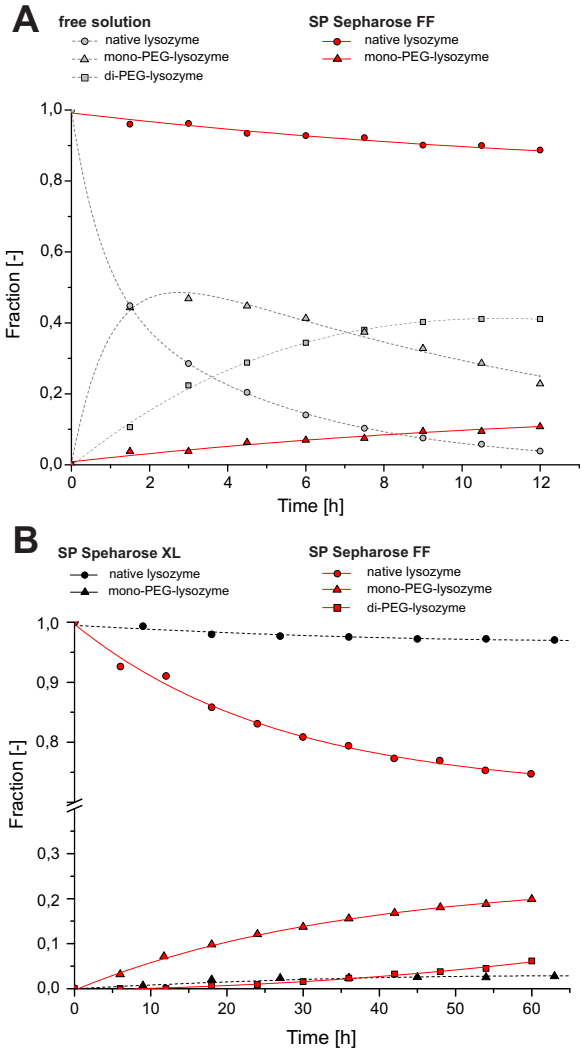


Figure 2: PEGamer kinetics based on SEC chromatograms and plotted using exponential fit functions. **A:** Comparison of liquid- and solid-phase PEGylation using SP Sepharose FF. **B:** Solid-phase PEGylation with SP Sepharose FF and XL. Reaction conditions: pH 7.2, 0.15 protein to PEG ratio, 60% surface coverage.

.

the authors used PEG concentrations up to a 40-fold excess. This consequently increases reaction velocity, but also results in an enormous consumption of PEG. An increased reaction rate might also be achieved by using a different PEGylation chemistry, as shown by Suo et al. [12], where PEG-NHS was used as PEG reagent. A possibility to overcome diffusion limitations might be the usage of monolithic columns or membranes, providing separation performance due to porous channels rather than chromatographic particles. As opposed to the experiments by Lee et al., a multi-PEGylation could not be avoided in this study, as di-PEGylated variants were formed using SP Sepharose FF. To reduce the fraction of multi-PEGylated variants the usage of PEG with an increased molecular weight and thus increased steric hindrance should be taken into consideration.

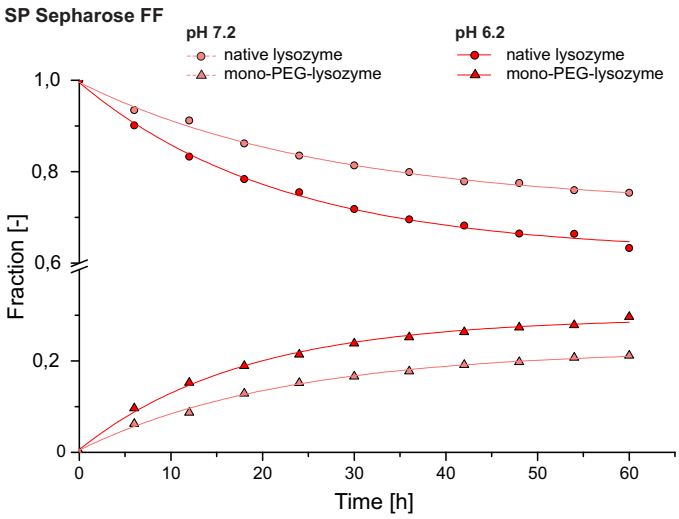


Figure 3: Solid-phase PEGylation kinetics on SP Sepharose FF for native and mono-PEGylated lysozyme using reaction buffers with pH 6.2 and 7.2. Protein to PEG ratio was 0.15.

.

3.3 Isoform distribution

Lysozyme consists of seven amino residues, including six lysine residues and the N-terminus. As lysine 1 exhibits both the α- and an ϵ-amino group, six mono-PEGylated isoforms can be formed, if mPEG-aldehyde is used. Lysozyme PEGylation studies, introduced by the authors recently [15] showed a separation and identification of five isoforms, using pH gradient chromatography. By comparing the UV areas, the N-terminus was evaluated to be the predominant PEG-attachment site, followed by lysine 33 using buffer pH values of 7.2. Only small reactivates were found for lys 13, lys 96 and lys 97/116. Employed solid-phase experiments were analyzed according to their isoform distribution and were compared to liquid-phase reactions. Chromatograms of purified mono-PEG-lysozyme, PEGylated on SP-Sepharose FF and in free solution are shown in Figure 4.

For lysozyme immobilized during the reaction, the illustrated data suggests a main modification of two peaks, representing lysine residues lys 13, lys 97 and lys 116, even though these residues comprise the lowest reactivity in free solution. In addition to pore and film diffusion, the modification of low reactive PEGylation sites can thus be seen as another aspect of reduced reaction velocity in the solid-phase mode. N-terminally PEGylated lysozyme (labeled in the following as PEG-lys$_1$) and PEG-lys$_{33}$ are also formed in minimal extend, but their fractions did not increase over time compared to the main isoforms. To explain the changes in the isoform composition, structural considerations and lysozyme binding orientations on ion exchange resins made by Dismer et al. [19] were used. Accordingly, main interaction sites of lysozyme on SP Sepharose FF particles were proposed between lys 1 & lys 33, and lys 116 & lys 33, respectively. Figure 5 illustrates a three-dimensional model of lysozyme and depicts the possible PEGylation positions and interaction sites with the adsorbent material.

As can be seen, a resin–binding region between lys 1 and lys 33 agrees well with the results

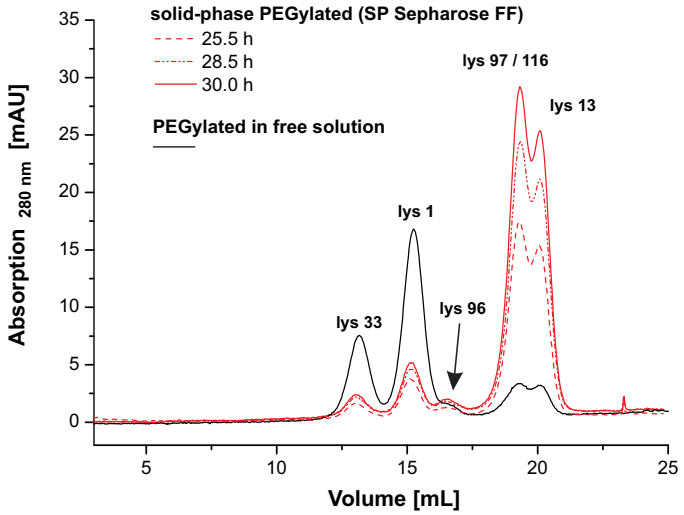


Figure 4: Isoform analytics of mono-PEG-lysozyme using liquid- and solid-phase reactions. Reaction conditions: pH 7.2, SP Sepharose FF with 60% surface coverage.

.

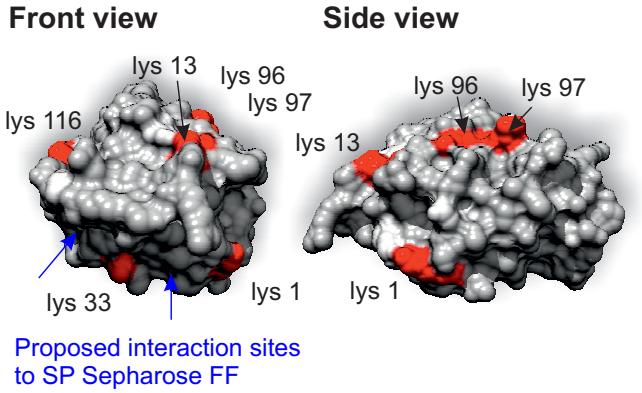


Figure 5: Three–dimensional model of lysozyme with labeled lysine residues. Resin interaction sites on SP Sepharose FF according to Dismer et al. [19] are labeled with blue arrows.

.

made in this study, allowing a PEGylation of opposite-located lysine residues lys 13 and lys 97. Additionally the reduced formation of PEG-lys$_1$ and PEG-lys$_{33}$ can thus be explained with a sterically hindrance of corresponding lysine residues. Even though lysine 96 is exposed to the mobile phase and thus well accessible according to the proposed binding orientation, an increase in corresponding isoform could not be observed. A possible explanation for this can be the higher reactivity of lys 97 compared to lys 96 in free solution, resulting in a blocking of attached PEG at lys 97 towards lys 96.

The constant amount of PEG-lys$_1$ and PEG-lys$_{33}$ over reaction time, shown in Figure 4, implies a formation of these isoforms at the beginning of the PEGylation and is in striking contrast to the increase of mainly formed isoforms PEG-lys$_{13}$ and PEG-lys$_{97/116}$ in the further reaction. This result can also be linked to the different lysine reactivities and accessibilities. All available, but limited lys 33 and N-terminal amino groups are modified at first, due to

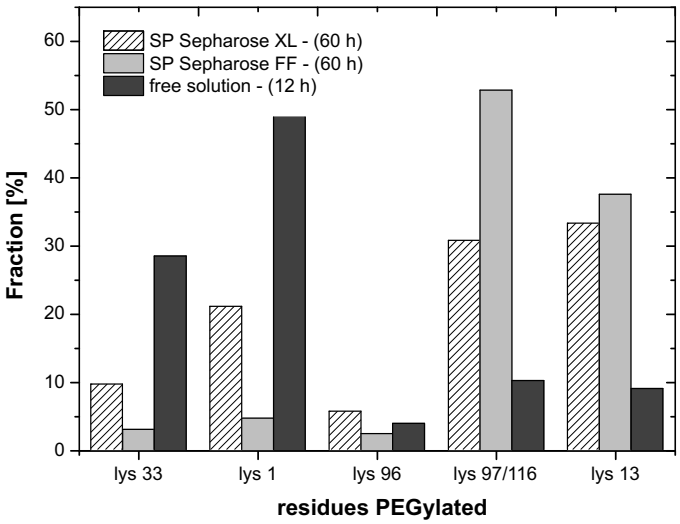


Figure 6: Isoform distributions of solid- and liquid-phase experiments at pH 7.2 with a protein to PEG ratio of 0.15. Solid-phase experiments show results with 60% surface coverage and 60 h reaction time

.

their increased reactivity. Further PEGylation occurs then at the residual PEGylation sites, even though they exhibit a reduced reactivity.

Comparing solid-phase PEGylated lysozyme on SP Sepharose XL with the non-grafted adsorbent, a similar isoform distribution with PEG-lys_{13} and PEG-$lys_{97/116}$ as main isoforms can be observed, as illustrated in Figure 6. Solid–phase experiments show results after a reaction time of 60 h, while liquid phase results represent the isoform distribution after 12 h.

The different isoform distribution using SP Sepharose XL in contrast to liquid–phase PEGylation is in the line with previous studies [20], where a multipoint adsorption of lysozyme via lys 116, lys 33 and lys 1, enabled by the flexible spacer chains, was suggested. However, the changed isoform conformation is not as clear as the results conducted with SP Sepharose FF. This aspect might be explained with the reduced formation of mono-PEG lysozyme, as only 3% of bound protein was modified after 60 h (see Figure 2B).

Besides the advantages in process control due to a reduction of unit operations, a possible change in the isoform composition, enabled by on-resin or solid-phase PEGylation, were discussed by numerous authors and could be verified in this study. However, activity measurements for the present model protein based on *Micrococcus lysodeikticus* cells and conducted by the authors previously [16], showed that PEG-lys_{33} and PEG-lys_1 exhibit the highest residual activity. Consequently, an increase of volumetric activity for eluted isoform mixtures of mono-PEG-lysozyme could probably not be achieved with the used resin material, due to its unfavorable isoform composition.

3.4 Influence of surface coverage

To investigate the influence of protein surface density on the isoform formation, varying surface coverage in the range of 50% to 100% was evaluated. Due to limited mono-PEG-

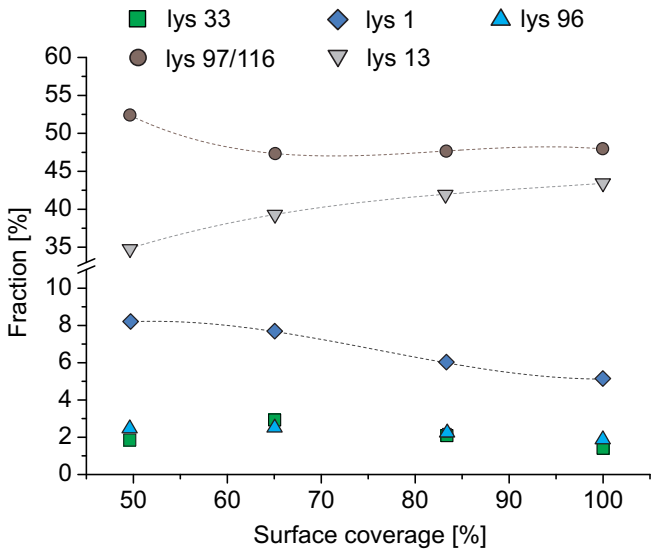


Figure 7: Changes in isoform distribution with respect to varying surface coverage on SP Sepharose FF. Reaction conditions: pH 7.2, 0.15 protein to PEG ratio, 42 h reaction time

.

lysozyme formation using SP Sepharose XL as adsorbent material, only SP Sepharose FF was used. In Figure 7 a significant increase of PEG-lys_{13} isoforms from 35% to 43% with increasing surface coverage is apparent.

This is attended with a decrease of isoforms modified at lys 1 and lys 97/116, respectively. Considering a mono-layer adsorption of lysozyme on SP Sepharose FF with the proposed binding site between lys 1 and lys 33, this result can be concluded of a reduced accessibility of side-located lys 116 and lys 1 by neighboring bound lysozyme molecules.

4 Conclusion and outlook

The presented work demonstrates the impact of adsorbent based PEGylation on the PEGamer kinetics and the isoform distribution. In contrast to reactions in free solution a significantly reduced reaction rate to PEGylated species was observed. Additionally, a changed isoform distribution from a predominant N-terminal PEGylation to a distribution dominated by PEG-$lys_{97/116}$ and PEG-lys_{13} was observed. These findings suggest a main binding of lysozyme on the adsorbent surface close to lys 1 and lys 33 for both investigated resins, which is in the line with studies by Dismer et al. [19, 20].

Using lysozyme as model protein, an increase in specific activity of the isoform mixture due to a favorable distribution could probably not be achieved, as PEG-lys_1 and PEG-lys_{33} were investigated in former studies to be the most active isoforms [16].

However, the influence of solid-phase PEGylation on important aspects could be confirmed in this study and are in contrast to studies by Monkarsh et al. [13] where a change in the isoform distribution applying on-column PEGylation of IFN could not be demonstrated. Consequently, different binding behavior of protein on adsorbent materials and thus changes in the isoform distribution should be taken into account if on-column or solid phase

PEGylation is applied.

The increase of reaction rates towards PEGylated species by applying a high PEG excess is only an option if PEG can be recycled due to high material costs. Avoiding diffusion limitations of chromatographic particles, future work will focus on monolithic columns or membranes as adsorbent matrix.

5 Acknowledgments

We gratefully acknowledge financial support by the Federal Ministry of Education and Research (BMBF).

References

[1] G. Pasut and F. M. Veronese. State of the art in PEGylation: The great versatility achieved after forty years of research. *J. Control. Release*, 161(2):461–72, Jul 2012.

[2] S. Chaffee, A. Mary, E. R. Stiehm, D. Girault, A. Fischer, and M. S. Hershfield. Igg antibody response to polyethylene glycol-modified adenosine deaminase in patients with adenosine deaminase deficiency. *J. Clin. Invest.*, 89(5):1643–51, May 1992.

[3] Y. Nodake and N. Yamasaki. Some properties of a macromelecular conjugate of lysozyme prepared by modification with a monomethoxypolyethylene glycol derivative. *Biosci. Biotechnol. Biochem.*, 64(4):767–774, 2000.

[4] B. K. Lee, J. S. Kwon, H. J. Kim, S.i Yamamoto, and E. K. Lee. Solid-phase PEGylation of recombinant interferon alpha-2a for site-specific modification: Process performance, characterization, and in vitro bioactivity. *Bioconjugate Chem.*, 18(6):1728–1734, 2007.

[5] J. M. Harris, N. E. Martin, and M. Modi. Pegylation - a novel process for modifying pharmacokinetics. *Clin.Pharmacokinet.*, 40(7):539–551, 2001.

[6] F. M. Veronese and G. Pasut. Pegylation, successful approach to drug delivery. *Drug Discov.Today*, 10(21):1451–1458, 2005.

[7] V. Gaberc-Porekar, I. Zore, B. Podobnik, and V. Menart. Obstacles and pitfalls in the pegylation of therapeutic proteins. *Curr. Opin. Drug Di. De.*, 11(2):242–50, Mar 2008.

[8] L. H. Brinckerhoff, V. V. Kalashnikov, L. W. Thompson, G. V. Yamshchikov, R. Pierce, H. S. Galavotti, V. H. Engelhard, and C. L. Slingluff. Terminal modifications inhibit proteolytic degradation of an immunogenic MART-1(27-35) peptide. *Int. J. Cancer*, 83(3):326–34, Oct 1999.

[9] S. P. Monkarsh, Y. M. Ma, A. Aglione, P. Bailon, D. Ciolek, B. DeBarbieri, M. C. Graves, K. Hollfelder, H. Michel, A. Palleroni, and et al. Positional isomers of monopegylated interferon alpha-2a: Isolation, characterization, and biological activity. *Anal. Biochem.*, 247(2):434–440, 1997.

[10] C. J. Fee and J. A. Van Alstine. Peg-proteins: Reaction engineering and separation issues. *Chem. Eng. Sci.*, 61(3):924–939, Feb 2006.

[11] X. Shang, D. Yu, and R. Ghosh. Integrated solid-phase synthesis and purification of PEGylated protein. *Biomacromolecules*, 12(7):2772–9, Jul 2011.

[12] X. Suo, C. Zheng, P. Yu, X. Lu, G. Ma, and Z. Su. Solid phase pegylation of hemoglobin. *Artif. Cell. Blood Sub.*, 37(4):147–155, Jan 2009.

[13] S. P. Monkarsh and P. Bailon. Isolation of positional isomers of mono -poly(ethylene glycol)ylated interferon/alpha 2a and the determination of their biochemical and biological characteristics. In J. M. Harris and S. Zalipsky, editors, *Poly (ethylene glycol): Chemistry and Biological Applications (ACS Symposium)*, pages 207–216. American Chemical Society, 1997.

[14] E. T. Baran, N. Ozer, and V. Hasirci. Solid-phase enzyme modification via affinity chromatography. *J. Chromatogr. B.*, 794(2):311–22, Sep 2003.

[15] B. Maiser, F. Kröner, F. Dismer, G. Brenner-Weiß, and J. J. Hubbuch. Isoform separation and binding site determination of mono-PEGylated lysozyme with pH gradient chromatography. *J.Chrom. A.*, 1268:102–108, 2012.

[16] B. Maiser, F. Dismer, and J. J. Hubbuch. Optimization of random PEGylation reactions by means of high throughput screening. *Bioconj. Chemistry.*, 2012. submitted.

[17] M. Abe, P. Akbarzaderaleh, M. Hamachi, N. Yoshimoto, and S. Yamamoto. Interaction mechanism of mono-PEGylated proteins in electrostatic interaction chromatography. *Biotechnol. J.*, 5(5):477–83, May 2010.

[18] A. Moosmann, J. Blath, R. Lindner, E. Müller, and H. Böttinger. Aldehyde PEGylation kinetics: a standard protein versus a pharmaceutically relevant single chain variable fragment. *Bioconjugate Chem.*, 22(8):1545–58, Aug 2011.

[19] F. Dismer and J. Hubbuch. A novel approach to characterize the binding orientation of lysozyme on ion-exchange resins. *J. Chrom. A.*, 1149(2):312–20, 2007.

[20] F. Dismer, M. Petzold, and J. Hubbuch. Effects of ionic strength and mobile phase ph on the binding orientation of lysozyme on different ion-exchange adsorbents. *J. Chrom. A.*, 1194(1):11–21, 2008.

[21] A. S. Morar, J. L. Schrimsher, and M. D. Chavez. PEGylation of proteins: A structural approach. *Biopharm Int.*, 19(4), 2006.

[22] M. J. Roberts and J. M. Harris. Attachment of degradable poly(ethylene glycol) to proteins has the potential to increase therapeutic efficacy. *J. Pharm. Sci.*, 87(11):1440–1445, 1998.

4 Conclusion and Outlook

This work is focused on a detailed view of random PEGylation reactions and includes three main aspects.

- Improved isoform separation, enabling a fast isoform identification.
- High throughput screening approach to achieve ideal reaction conditions, both for isoform mixtures and single isoforms.
- Influence of solid-phase PEGylation on process performance and product characteristics.

The essential work of this thesis was done with the development of high-sensitive isoform analytics using pH gradient chromatography. First of all, it could be shown that the applied model PEGylation reaction with lysozyme and PEG-aldehyde results in more than three isoforms of mono-PEGylated lysozyme, which is contrary to numerous published studies. Further, the correlation between calculated isoelectric points and elution pH values of separated isoforms allowed a first guess on the PEG-attachment site, which could be verified later on by standard methods. The usability of this fast *in silico* method was shown in this work only for PEG-lysozyme isoforms. Thus, the overall applicability for other PEG-protein conjugates needs to be demonstrated in future.

The developed chromatographic isoform separation method was used in further work as a basis to show the feasibility of HTPD with respect to an optimized lysozyme PEGylation. Combining the improved isoform analytics and a standard lysozyme activity assay with automated and miniaturized PEGylation sample preparation, an ideal lysozyme PEGylation process regarding maximal volumetric activity could be generated. It could be shown, that PEG excess, buffer pH value and the process time are significant parameters, affecting the isoform distribution and reaction velocity of PEG-aldehyde modifications. Controlling these parameters, a reproducible process with defined product mixtures can be generated. Consequently, this approach demonstrated a targeted PEG-modification of available binding sites using high throughput techniques rather than a randomized and uncontrollable PEG-attachment. Although the approval of PEG-protein mixtures might become more difficult in future due to improved isoform separation techniques, well characterized “random” PEGylation reactions with a subsequent isoform separation will still represent a simple and interesting PEG modification method in contrast to difficult site-specific PEGylations.

The last aspect of this work contributes to a question, which is discussed for a long time in the community and includes the influence of solid-phase PEGylation on the isoform distribution. Small scale solid-phase PEGylation experiments were conducted and showed

generally a reduced reaction towards PEGylated species. Additionally, an altered isoform distribution in contrast to reactions in free solution could be determined. In case of the applied model reaction, this change was connected with a reduced residual activity. However, the effect of a changed isoform distribution using solid-phase PEGylation might be interesting for other PEG-proteins, if an advantageous isoform distribution with improved characteristics in contrast to liquid phase PEGylation can be achieved. The first results of the solid-phase PEGylation reactions that are presented in this thesis were used in further experiments to establish a semi continuous on-column PEGylation process, were immobilized lysozyme was PEGylated by a flow-through of activated PEG.

5 Abbreviations and Symbols

Abreviations

ADM	age related macular degeneration
BSA	bovine serum albumin
CV	column volume
DLS	dynamic light scattering
Fab	fragment antigene binding
FDA	Food and Drug Administration
GalNAc	N-Acetylgalactosamine
G-CSF	granulocyte colony-stimulating factor
GRAS	generally recognized as safe
hGH	human growth hormone
HIC	hydrophobic interaction chromatography
HTE	high throughput experimentation
ICH	International Conference on Harmonisation
IEC	ion exchange chromatography
IFN	interferon
LS	light scattering
MALDI	matrix assisted laser desorption ionization
MALS	multi angle light scattering
MCR	multivariate curve resolution
MLR	multilinear regression
MVDA	multivariate data analysis
N	nitrogen
NHS	N-hydroxysuccinimidyl
pI	isoelectric point
PEG	polyethylene glycol
PLS	partial least squares
QbD	Quality-by-Design
RNA	ribonucleic acid
RSD	relative standard deviation
RI	refractive index
SANS	small angle neutron scattering
SCID	severe combined immunodeficiency desease
SEC	size exclusion chromatography
Tgase	transglutaminase
TNF	tumor necrosis factor
UV	ultraviolet
VEGF	vascular endothelial growth factor

Symbols

A	variable	$[nm]$
c	protein concentration	$[mg/mL]$
k	number of attached PEG molecules	[-]
K_d	equilibrium constant	$[mg/mL]$
M_w	molecular weight	$[Da]$
m	available binding sites	[-]
n	total number of measured samples	[-]
P	number of possible isoforms	[-]
Q	binding capacity	$[mg/mL]$
R	resolution	[-]
R_h	hydrodynamic radius	$[nm]$
V_R	retention volume	$[mL]$

Greek symbols

α	indicates the N-terminus
ϵ	indicates a lysine residue

Indices

i	calibration sample
j	sample number within the sample sequence
n	number of samples
PEG	PEG
PEGprot	PEG-protein conjugate
pre	indicates calibration before the validation samples
prot	protein
post	indicates calibration after the validation samples

Bibliography

[Abe et al., 2010] Abe, M., Akbarzaderaleh, P., Hamachi, M., Yoshimoto, N., and Yamamoto, S. (2010). Interaction mechanism of mono-PEGylated proteins in electrostatic interaction chromatography. *Biotechnol. J.*, 5(5):477–83.

[Abuchowski et al., 1977] Abuchowski, A., Vanes, T., Palczuk, N. C., and Davis, F. F. (1977). Alteration of immunological properties of bovine-serum-albumin by covalent attachment of polyethylene-glycol. *J. Biol. Chem.*, 252(111):3578–3581.

[Ahamed et al., 2007] Ahamed, T., Nfor, B. K., Verhaert, P., van Dedem, G., van der Wielen, L., Eppink, M., van de Sandt, E., and Ottens, M. (2007). ph-gradient ion-exchange chromatography: an analytical tool for design and optimization of protein separations. *J. Chrom. A*, 1164(1-2):181–8.

[Armstrong et al., 2007] Armstrong, J. K., Hempel, G., Koling, S., Chan, L. S., Fisher, T., Meiselman, H. J., and Garratty, G. (2007). Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer*, 110(1):103–11.

[Baran et al., 2003] Baran, E. T., Ozer, N., and Hasirci, V. (2003). Solid-phase enzyme modification via affinity chromatography. *J. Chromatogr. B.*, 794(2):311–22.

[Bashford, 1997] Bashford, D. (1997). *An object-oriented programming suite for electrostatic effects in biological molecules An experience report on the MEAD project*, pages 233–240. Springer.

[Bashford and Gerwert, 1992] Bashford, D. and Gerwert, K. (1992). Electrostatic calculations of the pka values of ionizable groups in bacteriorhodopsin. *J. Mol. Bio.*, 224(2):473–86.

[Basu et al., 2006] Basu, A., Yang, K., and Wang, M. (2006). Structure-function engineering of interferon-beta-1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation. *Bioconjugate Chem.*, 17(3):618–630.

[Bensch et al., 2005] Bensch, M., Wierling, P. S., von Lieres, E., and Hubbuch, J. (2005). High Throughput Screening of Chromatographic Phases for Rapid Process Development. *Chem. Eng. Technol.*, 28(11):1274–1284.

[Bhambure et al., 2011] Bhambure, R., Kumar, K., and Rathore, A. S. (2011). High-throughput process development for biopharmaceutical drug substances. *Trends Biotechnol.*, 29(3):127–35.

[Bonora and Drioli, 2009] Bonora, G. M. and Drioli, S. (2009). Reactive PEGs for protein conjugation. In Veronese, F. M., editor, *PEGylated Protein Drugs: Basic Science and Clinical Applications*, pages 33–45. Birkhäuser Verlag.

[Brinckerhoff et al., 1999] Brinckerhoff, L. H., Kalashnikov, V. V., Thompson, L. W., Yamshchikov, G. V., Pierce, R., Galavotti, H. S., Engelhard, V. H., and Slingluff, C. L. (1999). Terminal modifications inhibit proteolytic degradation of an immunogenic MART-1(27-35) peptide. *Int. J. Cancer*, 83(3):326–34.

[Chapman, 2002] Chapman, A. P. (2002). PEGylated antibodies and antibody fragments for improved therapy: a review. *Adv. Drug Deliv. Rev.*, 54(4):531–545.

[Chhatre et al., 2010] Chhatre, S., Francis, R., Bracewell, D. G., and Titchener-Hooker, N. J. (2010). An automated packed protein G micro-pipette tip assay for rapid quantification of polyclonal antibodies in ovine serum. *J. Chromatogr. B*, 878(30):3067–75.

[Daly et al., 2005] Daly, S. M., Przybycien, T. M., and Tilton, R. D. (2005). Adsorption of poly(ethylene glycol)-modified lysozyme to silica. *Langmuir*, 21(4):1328–1337.

[Davis, 2002] Davis, F. (2002). The origin of pegnology. *Adv. Drug Deliv. Rev*, 54(4):457–458.

[Diederich et al., 2011] Diederich, P., Hansen, S. K., Oelmeier, S. A., Stolzenberger, B., and Hubbuch, J. (2011). A sub-two minutes method for monoclonal antibody-aggregate quantification using parallel interlaced size exclusion high performance liquid chromatography. *J. Chromatogr. A*, 1218(50):9010–9018.

[Dismer and Hubbuch, 2007] Dismer, F. and Hubbuch, J. (2007). A novel approach to characterize the binding orientation of lysozyme on ion-exchange resins. *J. Chrom. A.*, 1149(2):312–20.

[Dismer et al., 2008] Dismer, F., Petzold, M., and Hubbuch, J. (2008). Effects of ionic strength and mobile phase ph on the binding orientation of lysozyme on different ion-exchange adsorbents. *J. Chrom. A.*, 1194(1):11–21.

[Farnan et al., 2009] Farnan, D., Moreno, G. T., Stults, J., Becker, A., Tremintin, G., and van Gils, M. (2009). Interlaced size exclusion liquid chromatography of monoclonal antibodies. *J. Chrom. A*, 1216(51):8904–9.

[Fee and Van Alstine, 2011] Fee, C. and Van Alstine, J. M. (2011). Purification of PEGylated proteins. In Janson, J. C., editor, *Protein Purification: Principles, High-Resolution Methods and Applications*, pages 339–362. John Wiley & Sons., Inc.

[Fee, 2009] Fee, C. J. (2009). Protein conjugates purification and characterization. In Veronese, F. M., editor, *PEGylated Protein Drugs: Basic Science and Clinical Applications*, pages 113–125. Birkhäuser Verlag.

[Fee and Van Alstine, 2004] Fee, C. J. and Van Alstine, J. M. (2004). Prediction of the viscosity radius and the size exclusion chromatography behavior of PEGylated proteins. *Bioconjugate Chem.*, 15(6):1304–1313.

[Foser et al., 2003] Foser, S., Schacher, A., Weyer, K. A., Brugger, D., Dietel, E., Marti, S., and Schreitmüller, T. (2003). Isolation, structural characterization, and antiviral activity of positional isomers of monopegylated interferon alpha-2a (pegasys). *Protein Expres. Purif.*, 30(1):78–87.

[Freitas and Abrahao-Neto, 2010] Freitas, D. and Abrahao-Neto, J. (2010). Biochemical and biophysical characterization of lysozyme modified by PEGylation. *Int. J. Pharm.*, 392(1-2):111–7.

[French et al., 2009] French, A. C., Thompson, A. L., and Davis, B. G. (2009). High-purity discrete PEG-oligomer crystals allow structural insight. *Angewandte Chemie (International ed. in English)*, 48(7):1248–52.

[Fruijtier-Pölloth, 2005] Fruijtier-Pölloth, C. (2005). Safety assessment on polyethylene glycols (PEGs) and their derivatives as used in cosmetic products. *Toxicology*, 214(1-2):1–38.

[Fuertges and Abuchowski, 1990] Fuertges, F. and Abuchowski, A. (1990). The clinical efficacy of poly(ethylene glycol)-modified proteins. *J. Controlled Release*, 11:139–148.

[Gaberc-Porekar et al., 2008] Gaberc-Porekar, V., Zore, I., Podobnik, B., and Menart, V. (2008). Obstacles and pitfalls in the pegylation of therapeutic proteins. *Curr. Opin. Drug Di. De.*, 11(2):242–50.

[Hagel, 1998] Hagel, L. (1998). Gel filtration. In Janson, J. C., editor, *Protein Purification.* John Wiley & Sons., Inc.

[Hansen et al., 2012] Hansen, S. K., Maiser, B., and Hubbuch, J. (2012). Rapid quantification of protein-peg conjugates by multivariate evaluation of chromatographic data. *J. Chrom. A*, 1257:41–47.

[Harris et al., 2001] Harris, J. M., Martin, N. E., and Modi, M. (2001). Pegylation - a novel process for modifying pharmacokinetics. *Clin.Pharmacokinet.*, 40(7):539–551.

[Jevsevar et al., 2010] Jevsevar, S., Kunstelj, M., and Porekar, V. (2010). PEGylation of therapeutic proteins. *Biotechnol. J.*, 5(1):113–28.

[Kang et al., 2009] Kang, J. S., Deluca, P. P., and Lee, K. C. (2009). Emerging PEGylated drugs. *Expert Opin. Emerg. Dr.*, 14(2):363–80.

[Katre et al., 1987] Katre, N. V., Knauf, M. J., and Laird, W. J. (1987). Chemical modification of recombinant interleukin 2 by polyethylene glycol increases its potency in the murine Meth A sarcoma model. *Proc. Natl. Acad. Sci. U.S.A.*, 84(6):1487–91.

[Kinstler et al., 2002] Kinstler, O., Molineux, G., Treuheit, M., Ladd, D., and Gegg, C. (2002). Mono-n-terminal poly(ethylene glycol)-protein conjugates. *Adv. Drug Deliver Rev.*, 54(4):477–485.

[Kinstler et al., 1996] Kinstler, O. B., Brems, D. N., Lauren, S. L., Paige, A. G., Hamburger, J. B., and Treuheit, M. J. (1996). Characterization and stability of N-terminally pegylated rhG-CSF. *Pharm. Res.*, 13(7):996–1002.

[Knauf et al., 1988] Knauf, M. J., Bell, D. P., Hirtzer, P., Luo, Z. P., Young, J. D., and Katre, N. V. (1988). Relationship of effective molecular size to systemic clearance in rats of recombinant interleukin-2 chemically modified with water-soluble polymers. *J. Biol. Chem.*, 263(29):15064–70.

[Leader et al., 2008] Leader, B., Baca, Q. J., and Golan, D. E. (2008). Protein therapeutics: a summary and pharmacological classification. *Nat. Rev. Drug Discov.*, 7(1):21–39.

[Lee et al., 2007] Lee, B. K., Kwon, J. S., Kim, H. J., Yamamoto, S., and Lee, E. K. (2007). Solid-phase PEGylation of recombinant interferon alpha-2a for site-specific modification: Process performance, characterization, and in vitro bioactivity. *Bioconjugate Chem.*, 18(6):1728–1734.

[Lee and Park, 2003] Lee, H. and Park, T. G. (2003). A novel method for identifying pegylation sites of protein using biotinylated peg derivatives. *J. Pharm. Sci.*, 92(1):97–103.

[Lee and Yang, 2002] Lee, Y. C. and Yang, D. (2002). Determination of lysozyme activities in a microplate format. *Anal. Biochem.*, 310(2):223–4.

[Maiser et al., 2012a] Maiser, B., Dismer, F., and Hubbuch, J. J. (2012a). Optimization of random PEGylation reactions by means of high throughput screening. *Bioconj. Chemistry.* submitted.

[Maiser et al., 2012b] Maiser, B., Kröner, F., Dismer, F., Brenner-Weiß, G., and Hubbuch, J. J. (2012b). Isoform separation and binding site determination of mono-PEGylated lysozyme with pH gradient chromatography. *J. Chromatogr. A.*, 1268:102–108.

[McGrath et al., 2010] McGrath, N., Brichacek, M., and J., N. (2010). A graphical journey of innovative organic architectures that have improved our lives. *J. Chem. Edu.*, 87:1348–1349.

[Merrill, 1992] Merrill, E. W. (1992). Poly(ethylene oxide) and blood contact. In Harris, J. M. and Zalipsky, S., editors, *Poly (ethylene glycol): Chemistry and Biological Applications (ACS Symposium)*, pages 199–220. American Chemical Society.

[Michael and Harris, 1998] Michael, J. R. and Harris, J. M. (1998). Attachment of degradable poly(ethylene glycol) to proteins has the potential to increase therapeutic efficacy. *J. Pharm. Sci.*, 87(11):1440–1445.

[Miteva et al., 2005] Miteva, M. A., Tuffery, P., and Villoutreix, B. O. (2005). Pce: web tools to compute protein continuum electrostatics. *Nucleic Acids Res.*, 33(Web Server issue):W372–5.

[Monkarsh and Bailon, 1997] Monkarsh, S. P. and Bailon, P. (1997). Isolation of positional isomers of mono -poly(ethylene glycol)ylated interferon/alpha 2a and the determination of their biochemical and biological characteristics. In Harris, J. M. and Zalipsky, S., editors, *Poly (ethylene glycol): Chemistry and Biological Applications (ACS Symposium)*, pages 207–216. American Chemical Society.

[Monkarsh et al., 1997] Monkarsh, S. P., Ma, Y. M., Aglione, A., Bailon, P., Ciolek, D., DeBarbieri, B., Graves, M. C., Hollfelder, K., Michel, H., Palleroni, A., and et al. (1997). Positional isomers of monopegylated interferon alpha-2a: Isolation, characterization, and biological activity. *Anal. Biochem.*, 247(2):434–440.

[Moosmann et al., 2011] Moosmann, A., Blath, J., Lindner, R., Müller, E., and Böttinger, H. (2011). Aldehyde PEGylation kinetics: a standard protein versus a pharmaceutically relevant single chain variable fragment. *Bioconjugate Chem.*, 22(8):1545–58.

[Moosmann et al., 2010] Moosmann, A., Christel, J., Böttinger, H., and Müller, E. (2010). Analytical and preparative separation of pegylated lysozyme for the characterization of chromatography media. *J. Chrom. A*, 1217(2):209–215.

[Morar et al., 2006] Morar, A. S., Schrimsher, J. L., and Chavez, M. D. (2006). PEGylation of proteins: A structural approach. *Biopharm Int.*, 19(4).

[Müller et al., 2010] Müller, E., Josic, D., Schröder, T., and Moosmann, A. (2010). Solubility and binding properties of PEGylated lysozyme derivatives with increasing molecular weight on hydrophobic-interaction chromatographic resins. *J. Chromatogr. A*.

[Ng et al., 2009] Ng, P. K., He, J., and Snyder, M. (2009). Separation of protein mixtures using ph-gradient cation-exchange chromatography. *J. Chrom. A*, 1216(9):1372–6.

[Nodake and Yamasaki, 2000] Nodake, Y. and Yamasaki, N. (2000). Some properties of a macromelecular conjugate of lysozyme prepared by modification with a monomethoxypolyethylene glycol derivative. *Biosci. Biotechnol. Biochem.*, 64(4):767–774.

[Oelmeier et al., 2011] Oelmeier, S., Dismer, F., and Hubbuch, J. (2011). Application of an aqueous two-phase systems high-throughput screening method to evaluate mAb HCP separation. *Biotechnol. Bioeng.*, 108(1):69–81.

[Ostergaard et al., 2011] Ostergaard, H., Bjelke, J. R., Hansen, L., Petersen, L. C., Pedersen, A., and Elm, T. (2011). Prolonged half-life and preserved enzymatic properties of factor IX

selectively PEGylated on native N-glycans in the activation peptide. *Blood*, 118(8):2333–41.

[Ottow et al., 2011] Ottow, K. E., Lund-Olesen, T., Maury, T. L., Hansen, M. F., and Hobley, T. J. (2011). A magnetic adsorbent-based process for semi-continuous PEGylation of proteins. *Biotechnol. J.*, 6(4):396–409.

[Pai et al., 2011] Pai, S., Hammouda, B., Hong, K., Pozzo, D. C., Przybycien, T. M., and Tilton, R. D. (2011). The conformation of the poly(ethylene glycol) chain in mono-pegylated lysozyme and mono-pegylated human growth hormone. *Bioconjugate Chem.*, 22(11):2317–2323.

[Palm et al., 2011] Palm, T., Esfandiary, R., and Gandhi, R. (2011). The effect of PEGylation on the stability of small therapeutic proteins. *Pharm. Dev. Technol.*, pages 441–448.

[Park and Na, 2010] Park, E. J. and Na, D. H. (2010). Microchip electrophoresis for monitoring covalent attachment of poly(ethylene glycol) to proteins. *Analytical biochemistry*, 400(2):304–6.

[Pasut, 2009] Pasut, G. (2009). Pegylated alpha-interferons: two different strategies to achieve increased efiicacy. In Veronese, F. M., editor, *PEGylated Protein Drugs: Basic Science and Clinical Applications*, pages 205–216. Birkhäuser Verlag.

[Pasut and Veronese, 2012] Pasut, G. and Veronese, F. M. (2012). State of the art in PEGylation: The great versatility achieved after forty years of research. *J. Control. Release*, 161(2):461–72.

[Payne et al., 2011] Payne, R., Murphy, B., and Manning, M. (2011). Product development issues for PEGylated proteins. *Pharm. Dev. Technol.*, 16(5):423–40.

[Rajan et al., 2006] Rajan, R. S., Li, T., Aras, M., Sloey, C., Sutherland, W., Arai, H., Briddell, R., Kinstler, O., Lueras, A. M. K., Zhang, Y., and et al. (2006). Modulation of protein aggregation by polyethylene glycol conjugation : Gcsf as a case study. *Protein Sci.*, 15(5):1063–1075.

[Roberts et al., 2002] Roberts, M. J., Bentley, M. D., and Harris, J. M. (2002). Chemistry for peptide and protein PEGylation. *Advanced Drug Delivery Reviews*, 54(4):459–476.

[Roberts and Harris, 1998] Roberts, M. J. and Harris, J. M. (1998). Attachment of degradable poly(ethylene glycol) to proteins has the potential to increase therapeutic efficacy. *J. Pharm. Sci.*, 87(11):1440–1445.

[Sato, 2002] Sato, H. (2002). Enzymatic procedure for site-specific pegylation of proteins. *Adv. Drug. Deliv. Rev.*, 54(4):487–504.

[Sehon, 1992] Sehon, A. H. (1992). Supression of antibody responses by conjugates of antigens and monomethoxypoly(ethyle glycol). In Harris, J. M. and Zalipsky, S., editors,

Poly (ethylene glycol): Chemistry and Biological Applications (ACS Symposium), pages 139–151. American Chemical Society.

[Sergi et al., 2009] Sergi, M., Caboi, F., Maullu, C., Orsini, G., and Tonon, G. (2009). Enzymatic techniques for PEGylation of biopharmaceuticals. In Veronese, F. M., editor, *PEGylated Protein Drugs: Basic Science and Clinical Applications*, pages 75–88. Birkhäuser Verlag.

[Shang et al., 2011] Shang, X., Yu, D., and Ghosh, R. (2011). Integrated solid-phase synthesis and purification of PEGylated protein. *Biomacromolecules*, 12(7):2772–9.

[Shaunak et al., 2006] Shaunak, S., Godwin, A., Choi, J., Balan, S., Pedone, E., Vijayarangam, D., Heidelberger, S., Teo, I., Zloh, M., and Brocchini, S. (2006). Site-specific PEGylation of native disulfide bonds in therapeutic proteins. *Nature chemical biology*, 2(6):312–313.

[Shugar, 1952] Shugar, D. (1952). The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. *Biochem. Biophys. Acta*, 8(3):302–9.

[Suo et al., 2009] Suo, X., Zheng, C., Yu, P., Lu, X., Ma, G., and Su, Z. (2009). Solid phase pegylation of hemoglobin. *Artif. Cell. Blood Sub.*, 37(4):147–155.

[Teske et al., 2007] Teske, C. A., Simon, R., and Niebisch, A. (2007). Changes in retention behavior of fluorescently labeled proteins during ion-exchange chromatography caused by different protein surface labeling positions. *Biotechnol.Eng.*, 98(1):193–200.

[Veronese et al., 2009] Veronese, F. M., Mero, A., and Pasut, P. (2009). Protein PEGylation, basic science and biological applications. In Veronese, F. M., editor, *PEGylated Protein Drugs: Basic Science and Clinical Applications*, pages 11–31. Birkhäuser Verlag.

[Veronese and Pasut, 2005] Veronese, F. M. and Pasut, G. (2005). Pegylation, successful approach to drug delivery. *Drug Discov.Today*, 10(21):1451–1458.

[Veronese and Pasut, 2008] Veronese, F. M. and Pasut, G. (2008). PEGylation: Posttranslational bioengineering of protein biotherapeutics. *Drug Discov. Today*, 5(2-3):e57–e64.

[Yamamoto et al., 2007] Yamamoto, S., Fujii, S., Yoshimoto, N., and Akbarzadehlaleh, P. (2007). Effects of protein conformational changes on separation performance in electrostatic interaction chromatography: unfolded proteins and pegylated proteins. *J. Biotechnol.*, 132(2):196–201.

[Yang et al., 2003] Yang, K., Basu, A., Wang, M., Chintala, R., Hsieh, M.-C., Liu, S., Hua, J., Zhang, Z., Zhou, J., Li, M., and et al. (2003). Tailoring structure-function and pharmacokinetic properties of single-chain fv proteins by site-specific pegylation. *Protein Engineering Design and Selection*, 16(10):761–770.