Aldehyde PEGylation kinetics: A standard protein versus a pharmaceutical relevant single chain variable fragment
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A standard protein versus a pharmaceutical relevant single chain variable fragment

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Keywords: PEG, PEGylation, lysozyme, scFv, single chain variable fragment, kinetics, poly(ethylene glycol), PEG-scFv, PEG-lysozyme

Abbreviations used: IEX, ion-exchange chromatography; mPEG-AL, Methoxy-PEG-aldehyde; PEG, poly(ethylene glycol); Rf, relative migration length; scFv, single chain variable fragment; SEC, size exclusion chromatography;
Abstract

The mPEG-aldehyde PEGylation with two different PEG sizes and two proteins was experimentally defined in case of yield, conversion and selectivity. The kinetic behaviour of these PEGylation reactions was simulated using a numerically solved set of differential equations. The assumption of an inactivation of mPEG-aldehyde was crucial for the simulation of the overall PEGylation, and the inactivation was found to be pretty pH dependent. It could be shown that ideal PEGylation parameters should be chosen carefully depending on the protein and PEG size in use. In terms of ideal selectivity and yield it could be shown, that the reaction should not reach the endpoint but be stopped slightly before. Also choosing room temperature and a slightly acidic pH about 6 is a good starting point. To optimize selectivity, a shorter reaction time can be chosen and the temperature can be humiliated.
Introduction

There are lots of drugs based on proteins of different classes, such as enzymes and antibodies both being of great potential in pharmaceutical use. But the usage of proteins in general is restricted by their biocompatibility and their in-situ half-life. Especially small molecules have got very short half-lives, this restricts the use of small proteins in drug design. For example, antibody fragments have many characteristics that would make them ideal drug candidates, such as easy and cheap production and high effectiveness, but they are small proteins and the blood and renal clearance is very high. For such proteins an effective way of prolonging the in-situ half-life is known. They can be polymer-modified, which artificially enlarges the hydrodynamic radius and leads to a masking effect. The renal clearance depends highly on the hydrodynamic radius and so will be overcome. The masking effect of a polymer modification will also decrease the target sites for proteases and thereby prolonging the half-life, too. One possible way of polymer modification is PEGylation. PEGylation denominates the chemical attachment of a poly(ethylene glycol)-chain to molecules such as proteins or peptides. Since the first steps in PEGylation have been made in the 1970s the method raised to a state-of-the-art technology. Eight PEGylated protein drugs are approved by the FDA by now, such as PEGasys® (Hoffman-La Roche) and PEG Intron® (Schering-Plough/ Enzon) which both contain α-Interferon for hepatitis C treatment. PEG is the polymer of choice because it is a neutral, non-immunogenic and inert polymer. PEG has to be functionalised with a reacting end group to get attached to another molecule. Each functionalization of PEG results in an altered PEGylation kinetic, but additionally the protein in use affects the kinetic characteristic of the PEGylation as well as the ambient conditions. One of the most frequently used functionalization is the aldehyde-modified PEG which reacts with free amino-groups of the protein (fig.1). This reaction is random, as all available
amino-groups can react. The pKa and the accessibility of each free amino-group define the probability for PEGylation. These characteristics can be influenced by the surrounding area. The pH is a critical factor in PEGylation characteristics, as well as temperature and concentrations of protein and PEG. PEGylation changes the physicochemical properties of a protein and the chromatographic behaviour of proteins is modified by PEGylation, too. The changed behaviour of PEGylated proteins in comparison with their non-PEGylated parental proteins could be shown for ion-exchange chromatography (IEX), hydrophobic interaction chromatography as well as for size exclusion chromatography (SEC). All modes are useful for purification but the present paper focused on IEX for a rapid separation of PEGylated and non-PEGylated proteins to follow the PEGylation reaction as fast as possible. To gather deeper insights in the reaction kinetic, a model was developed to replicate the reaction behaviour. Also the characteristics of the environment have been changed to judge their influence on the kinetics. The following study compares the PEGylation kinetics of two very different proteins, on the one hand a pharmaceutical relevant single-chain-variable-fragment which is produced in E.coli, on the other hand a commercially available standard protein, the enzyme lysozyme. Both proteins were PEGylated and the reaction was closely observed to get the critical data for kinetic analysis. Researches already exist for the standard protein lysozyme, regarding its behaviour in chromatography as well as detailed information about outstanding PEGylation sites for aldehyde chemistry, but by now no detailed research about the kinetics of the aldehyde PEGylation reaction itself exists.
Material and Methods

Chemicals

Methoxy-PEG-aldehyde (mPEG-AL) with an average molecular weight of 5 and 30 kDa were purchased from NOF Corp. (Grobbendonk, Belgium). Lysozyme (98% pure, chicken egg white) was provided by Sigma (St. Louis, USA). All other chemicals were provided by Merck (Darmstadt, Germany).

Production of scFv

The single-chain variable fragment used in this study was produced in *E.coli* BL21 DE3 rha \(^{21,22}\) using the rhamnose induction system. Following a modified osmotic shock procedure according to Rathore \(^{23}\), the pellet was solubilised in 100 mM phosphate-buffer containing 50% glucose followed by adding pure water. After centrifugation, the protein solution was purified using protein L-chromatography (GenScript, Piscataway, USA). The purified scFv was further diafiltered and lyophilized for long-term storage.

PEGylation of scFv and lysozyme

Protein and 5 or 30 kDa PEG of the desired concentration were dissolved in a 20 mM sodium phosphate buffer pH 6.0 or 7.0 or a 20 mM sodium acetate buffer pH 3.0, 4.0 or 5.0 containing 20 mM NaCNBH\(_3\) \(^{4,18,19}\). The PEGylation-reaction was performed in a final volume of 1 ml at different temperatures for up to 20 hours.

Analytical procedures

An analytical TSKgel SP-NPR column (ID 4.6mm x L 35mm, Tosoh Bioscience GmbH, Stuttgart, Germany) was used to track the PEGylation reaction. For lysozyme the buffer consisted of 25 mM sodium phosphate buffer, pH 6.0. For elution 1 M NaCl was added. For
scFv the buffer consisted of 20 mM sodium acetate buffer, pH 4.5. For elution 1 M NaCl was added. The analytical IEX was carried out on a Thermo Separation HPLC SpectraSYSTEM (Thermo Fisher Scientific GmbH, Dreieich, Germany).

SDS-PAGE under reducing conditions was performed according to Laemmli. Protein samples were solubilised in sample buffer and heated at 95 °C for 3 min. SDS-PAGE was performed with precast NuPAGE® Novex® 10 % Bis-Tris Midi-gels (Invitrogen Corporation, Carlsbad, USA) in an XCell4 SureLock Midi-Cell (Invitrogen) according to the manufacturer’s procedure. The gels were stained with PageBlue Protein Staining solution (Fermentas, St. Leon-Rot, Germany) according to the manufacturer’s instruction. To stain specifically the PEGylated proteins a barium-iodine staining was carried out. The procedure was modified according to Kurfürst, following the instructions of Bailon et al.

SEC was carried out on an analytical TSKgel G3000SWXL column (7.8 mm x 30 cm, Tosoh Bioscience GmbH). As mobile phase a 100 mM sodium phosphate buffer, pH 6.7, containing 100 mM Na$_2$SO$_4$ and 0.05% NaN$_3$ was used. The SEC chromatography was performed on a Thermo Separation HPLC SpectraSYSTEM (Thermo Fisher Scientific GmbH).

**Mathematic modelling**

The set of differential equation describing the reaction kinetic (see Kinetic studies and mathematic modeling) can be solved numerically with the program MatLab (Version R2010a, MathWorks, Ismaning, Germany). The MatLab ode23s solver implemented in our case, uses the Runge-Kutta-method, which is an iterative method for the approximation of solutions of ordinary differential equations. Furthermore an optimization was carried out using the MatLab toolbox fminsearch to fit the reaction kinetic parameters to the measured data.
Calculation of conversion, yield and selectivity

The calculation of conversion, yield and selectivity followed the conventions 27.

Conversion X:

\[
X = \frac{c_E^0 - c_E}{c_E^0}
\]

- \( c_E \) = reactant concentration
- \( c_E^0 \) = reactant concentration at start of reaction

Yield Y:

\[
Y = \frac{c_P}{c_E^0}
\]

- \( c_P \) = product concentration, Mono-PEGylated protein for the present study

Selectivity S:

\[
S = \frac{Y}{X}
\]
Results and discussion

PEGylation of lysozyme

The PEGylation reaction was closely followed using an analytical SP-NPR column (Tosoh Bioscience GmbH) with a total analysis time of 6 minutes. Chromatograms were evaluated using the peak area as given by the program ChromQuest (Thermo Separations). Figure 2 shows typical chromatograms of lysozyme and PEGylated lysozyme before the start of the reaction and after a reaction time of 970 minutes with a 5 kDa PEG. Lysozyme remains in the mixture, as PEG does which is not visible in UV and thereby not shown. During the reaction two isoforms of Mono-PEGylated lysozyme are formed as well as a Di- and a Tri-PEGylated form. In a former study, fractions of the elution peaks shown in figure 2 were collected and sent to the Friedrich-Alexander University (Erlangen, Germany) to perform the MALDI-TOF analysis. The peaks were identified as labelled in figure 2. Analogue experiments were done for PEGylation with 30 kDa PEG and the results were comparable, but the retention times shortened slightly. As already shown in prior work the retention times in IEX decrease with increasing PEG length and increasing number of added PEG-chains. Additionally a SDS-PAGE with the reaction mixtures was carried out at the end of the reaction (fig. 3). The coomassie staining reveals the protein fractions in blue, whereas the barium-iodine staining shows PEG in brown. For the PEGylated proteins a mixture of both colours can be seen which results in green to brownish bands. It is well known that PEG in SDS-PAGE does migrate slower than proteins, so the protein standard used leads to higher molecular weights of PEG and PEGylated proteins. PEG of 5 kDa size is not visible in figure 3 because it migrated out of the gel during the washing and staining procedure, but it was visible in the very first barium-iodine-staining (results not shown) the apparent size in SDS-PAGE was estimated to be 8 kDa, for 30 kDa PEG the apparent size in SDS-PAGE is about 50 kDa. Taking these values into account a new, theoretical size in SDS-PAGE can be estimated (tab
1). This estimated size correlates well with the measured size gained with the help of the protein standard. The bigger the size of the PEGylated proteins get, the more imprecise the sizes do match but that can be a gel-specific problem and was not further investigated in this work. The SDS-results confirm the results from Maldi-TOF analysis and can alternatively be used as a tool for estimation of size for PEGylated proteins.

**PEGylation of scFv**

The PEGylation reaction was followed as done with lysozyme. The peaks were identified via SDS-PAGE (fig. 4) as in principle shown for lysozyme. The scFv monomer and dimer peak were recognized using SEC as they are not stable under the presence of SDS (data not shown). In the course of the PEGylation reaction the dimer of the scFv vanishes and several peaks of shorter retention times appear (fig. 5). This matches the chromatographic behaviour of PEGylated lysozyme. Retention times in IEX decrease the higher the PEGylation degree gets. The SDS-PAGE revealed bands matching the presumption as Mono-, Di- and Tri-PEGylated forms are visible. For the PEGylation with 30 kDa PEG an unexpected band appears above the Mono-PEGylated scFv which is light brown. The absence of blue colour is an evidence for the absence of a protein fraction. Also the calculated size of about 116 kDa confirms this assumption as a PEG 30 kDa dimer should migrate to about 100 kDa in SDS-PAGE. Another evidence is the slightly visible band in fig. 3 below the Di-PEG-30-lysozyme band, this confirms that it is not a protein specific phenomenon. To verify the assumption of having a dimer PEG 30 kDa, another SDS-PAGE was carried out with PEG of 5 and 30 kDa in PEGylation reaction buffer of pH 5 and NaCNBH₃ without the presence of protein (fig. 6, shown for pH 5). For 30 kDa PEG clearly a second band is visible, for 5 kDa PEG this band is barely visible and vanished very fast. This is an evidence for a certain dimerization of PEG under the present reaction conditions. With this information in mind, the peaks of the IEX chromatogram were labelled as shown in figure 4. For the PEGylated scFv only one Mono-
PEGylated isoform is visible. As for lysozyme the PEGylation with 30 kDA PEG shows analogue performance.

**Kinetic studies and mathematic modelling**

For the monitoring of the PEGylation reaction all Mono-PEGylated isoforms were summed up to Mono-PEG-protein and for scFv the monomer- and the dimer-peak were also summed up to unmodified protein. Peak areas were used to determine the concentrations of the PEGylated forms. For the unmodified proteins the concentration in the beginning of the reaction is known. For the PEGylated forms and the unmodified forms at all time points the concentration was estimated via the peak area, under the assumption of PEG being undetectable for UV absorbance. As PEG is undetectable no experimental data for the PEG concentration was utilizable, rather the PEG concentration was calculated from the protein concentration by the following equation:

\[ c(PEG) = c(PEG)^0 - c(mono-PEG-protein) - 2 \times c(di-PEG-protein) - 3 \times c(tri-PEG-protein) \]

In a first attempt the following reaction model was listed:

1: PEG + protein \(\rightarrow\) Mono-PEG-protein

2: Mono-PEG-protein + PEG \(\rightarrow\) Di-PEG-protein

3: Di-PEG-protein + PEG \(\rightarrow\) Tri-PEG-protein

The reaction rates “r” were thereby defined to:

\[ r1 = PEG \times protein \times k1 \]

\[ r2 = PEG \times Mono-PEG-protein \times k2 \]

\[ r3 = PEG \times Di-PEG-protein \times k3 \]
and the differential equations were specified to:

\[
\begin{align*}
\frac{d x(\text{PEG})}{d t} &= -r_1 - r_2 - r_3 \\
\frac{d x(\text{protein})}{d t} &= -r_1 \\
\frac{d x(\text{Mono-PEG-protein})}{d t} &= r_1 - r_2 \\
\frac{d x(\text{Di-PEG-protein})}{d t} &= r_2 - r_3 \\
\frac{d x(\text{Tri-PEG-protein})}{d t} &= r_3
\end{align*}
\]

With the program MatLab the differential equations were numerically solved and the experimental data was compared to the calculated results and an optimization was carried out as specified in *Mathematic modelling*. As a result Matlab showed the experimental data as crosses and the calculated reaction as lines (exemplarily shown in fig. 7 for four data sets). The first attempt of reaction modelling seemed to work well at a pH of 7 (fig. 7 right side). But the more acidic the pH got, the worse the data fitted the simulated reaction (fig. 7 left column). The real reaction worked faster on a short time scale and then reached a plateau.

Having in mind that dimerized PEG could be detected in SDS-PAGE (fig. 6) the next step was to include another reaction that inactivates PEG with the ability to form a PEG-molecule which can not react anymore. There are some possibilities for PEG to get inactivated, there can be some reaction with the NaCNBH₃ or an aldol-reaction could take place, this would lead to PEG-multimers. In SDS-PAGE dimers were detected, but they appeared at different pH values (see fig. 6 for pH 5). Contrariwise the kinetic simulation seems to favour a reaction that is pH dependent. In a second attempt an inactivation step for PEG was included in the reaction set as done by Buckley and co-workers for mPEG-succinimidyl propionate:

1: \( \text{PEG} + \text{protein} \rightarrow \text{Mono-PEG-protein} \)
2: Mono-PEG-protein + PEG → Di-PEG-protein
3: Di-PEG-protein + PEG → Tri-PEG-protein
4: PEG_{reacting} → PEG_{inactive}

The reaction rates \( r \) were thereby defined to:

\[
\begin{align*}
    r_1 &= PEG_{reacting} \times \text{protein} \times k_1 \\
    r_2 &= PEG_{reacting} \times \text{Mono-PEG-protein} \times k_2 \\
    r_3 &= PEG_{reacting} \times \text{Di-PEG-protein} \times k_3 \\
    r_4 &= PEG_{reacting} \times k_4
\end{align*}
\]

and the differential equations were specified to:

\[
\begin{align*}
    dx(PEG_{all}) &= -r_1 - r_2 - r_3 \\
    dx(\text{protein}) &= -r_1 \\
    dx(\text{Mono-PEG-protein}) &= r_1 - r_2 \\
    dx(\text{Di-PEG-protein}) &= r_2 - r_3 \\
    dx(\text{Tri-PEG-protein}) &= r_3 \\
    dx(PEG_{reacting}) &= -r_1 - r_2 - r_3 - r_4 \\
    dx(PEG_{inactive}) &= r_4
\end{align*}
\]

The differential equations were solved as described above. The result of the kinetic simulation now did fit the experimental data very well. For lysozyme as well as for scFv a very good match at all pH values could be achieved (fig. 8). All experimental data sets were simulated with the above demonstrated set of differential equations. The rate constants \( k_1 \) and \( k_2 \) were determined and are listed for different temperatures in table 2 for lysozyme and table 3 for...
scFv. For lysozyme the rate constants for k1 are always higher than for k2, meaning that the Mono-PEGylation is much faster than the addition of a second PEG. For scFv this is not always the case. In an acidic pH and at very high protein concentrations the rate constant k2 exceeds k1 for the PEGylation with a 5 kDa PEG. Using the 30 kDa PEG leads to a more uniform behaviour. As seen for lysozyme, the rate constant k1 exceeds k2 (data not shown). Especially for different protein concentrations a wide difference in the rate constants was visible. The higher the protein concentration the faster the reaction gets. This behaviour could also be observed for a rising PEG-to-protein ratio, but the increase in rate constants was much higher for increasing protein concentrations (data not shown). The influence of temperature on reaction velocities is very high. At 30 °C the PEGylation reaction for lysozyme goes on fastest. And at a temperature of 10 °C it is slowest. For scFv this behaviour changes, the reaction at 10 °C was faster than at 20 °C. In theory a temperature rise of 10 ° should double the reaction velocity \(^{30}\). This matches perfectly for lysozyme with 5 and 30 kDa PEG. For 30 kDa PEG and lysozyme for example k1 for 10 °C is 0,0028 l/mol*min, for 21 °C it is 0,0063 l/mol*min and for 30°C 0,0124 l/mol*min. For scFv the 20 °C value is not twice the 10 °C value but even lower, whereas the 30 °C value is nearly four times the 10 °C value. There seems to be a problem in the mid temperature range. The reaction does not follow the usual parameters in this temperature range (tab. 3 and 4).

**Production parameters**

To decide which reaction conditions should be chosen for a scale-up, the conversion, yield and selectivity of each reaction was monitored after 5, 10 and 20 hours and plotted against different ambient conditions.

**Reaction time**

For all reactions the selectivity is slightly decreasing over the time, whereas the yield and conversion increase continuously (fig. 9).
pH

Yield increases with increasing pH for the 30 kDa version of scFv PEGylation, whereas the pH does not influence the lysozyme PEGylation (data not shown). Also the scFv PEGylation with 5 kDa PEG is not changed significantly by pH changes (fig. 10). The increase in yield with increasing pH is somewhat logical because of the observation made for mPEG-aldehyde, inactivating side reactions decrease the more neutral the pH gets. In selectivity no pH dependent trend was visible (data not shown).

Protein concentration

An increase in total lysozyme concentration with unchanged PEG to protein ratio leads to rising conversion. Yield also increases slightly whereas the selectivity decreases. A plateau is reached between 5 and 10 g/l protein (fig. 11). The increase in conversion was also visible for scFv PEGylation but a plateau was not reached for 30 kDa PEG. For scFv the yield decreases with increasing protein concentration whereas the conversion rises slightly for 5 kDa PEG and powerful for 30 kDa PEG (fig. 11). Selectivity decreases rapidly with increasing protein concentration for all proteins and PEGs. An explanation for the decrease in yield with rising scFv concentration is the massive decrease in selectivity leading logically to a reduced yield despite the rising conversion.

PEG to protein ratio

The PEG to protein ratio was studied only for scFv PEGylation. With increasing PEG excess the yield increased as did conversion. This can be nicely seen for 30 kDa PEG whereas for 5 kDa PEG a plateau in yield is reached between 5 and 10 fold PEG excess. The selectivity decreases with increasing PEG to protein ratio except for PEG 30 kDa where no difference between 1- and 5-fold excess of PEG could be found (fig. 12).
Temperature

The temperature rise for lysozyme PEGylation led to increased yield and conversion, a plateau is reached for PEG 5 kDa between 21 and 30 °C, but the selectivity decreases with increasing temperature. For scFv the selectivity also decreases, but a trend is not that clearly visible for all factors. The trend of the yield seems to decrease for 5 kDa PEG or remains unchanged for 30 kDa (fig. 13). An irregularity in the temperature range of 20 °C could also be shown for the reaction rates of the scFv PEGylation (tab. 4). It looks like an unknown mechanism influences the reaction. That could be because of some unexpected interaction between PEG and scFv. The temperature has no effect on the scFv PEGylation with 30 kDa PEG, whereas for 5 kDa PEG the rising temperature leads to a decrease of yield and selectivity. For lysozyme an increase in yield and a decrease in selectivity was visible regardless the PEG size.

Best conditions

For all reactions, increasing reaction time increased yield as well as conversion. Between 10 and 20 hours reaction time the selectivity was slightly reduced. The reaction time should be chosen before the endpoint of the reaction is reached, around 15 h to guarantee high yield and selectivity. For the pH no greater differences were visible but it could be shown (see mPEG-aldehyde inactivation) that mPEG-aldehyde gets inactivated at low pH values. The pH thereby should be chosen to be ideal for the solubility of the protein. For lysozyme with a pI of 11 the solubility in the studied region does not play an important role, but for scFv with a pI in the neutral range it does. ScFv is better soluble in more acidic surroundings and they are recommended for the PEGylation of the here studied scFv. It should be kept in mind that studies exist which found a pH dependence of the selectivity for certain PEGylation sites, especially the selectivity for the N-terminus was found to be increased with deceasing pH, but that was not part of the present study. A rising protein concentration leads to an increase
in conversion but also a decrease in selectivity. The ideal protein concentration depends on the used protein. For lysozyme a concentration of 5 g/l is a good compromise whereas for scFv the ideal protein concentration is in the lower range and depends on the used PEG size.

For long PEG chains a higher protein concentration is recommended than for short PEG chains. For example 2 g/l for scFv PEGylation with 30 kDa PEG and only 1 g/l for PEGylation with 5 kDa PEG. For scFv PEGylation a lower PEG-to-protein ratio led to higher selectivity but lower yield and conversion, so a compromise has to be chosen. A relatively high PEG to protein ratio for example 5:1 would be our suggestion. Temperature should be a factor with constant effects on all proteins as the Arrhenius theory predicts, but that did not hold true here. For lysozyme increasing temperature increased the yield and decreased selectivity, so a temperature of 20 °C should be chosen for a good compromise. For scFv the influence is not clear. At 30 °C all parameters get worst, and the reaction is very fast. But between 10 and 20 °C no clear trend is visible. Our suggestion would be choosing the compromise again and use 15 °C.

The optimized conditions were tested for lysozyme at a pH of 6 and a temperature of 20 °C with a two-fold PEG excess and a lysozyme concentration of 5 g/l. This was chosen for both PEG sizes, only the reaction time varied. For PEGylation with 5 kDa 15 hours reaction time was chosen, whereas the PEGylation with 30 kDa had a duration of 20 hours. For both used PEG sizes yield, conversion and selectivity were very good, especially the yield was in the focus and could be improved to 0.54 for 5 kDa and 0.59 for 30 kDa PEG. Both values are the best ever tested and they were accompanied by a conversion of 0.7 and 0.6 respectively. Especially the combination of good values for all parameters is a very nice improvement which could also be approved for scFv PEGylation. Here also, the yield could be pushed up to 0.6 and 0.57 for 5 and 30 kDa PEG respectively, both in combination with good conversion and selectivity.
mPEG-aldehyde inactivation

The inactivation reaction for mPEG-AL seems to be essential for mathematical modelling (see Kinetic studies and mathematic modelling) even though mPEG-AL should not be susceptible to hydrolysis \(^{14}\). At a neutral pH no inactivation of PEG occurs, but at acidic pH values inactivation takes place. To find out more about the inactivation process, mPEG-AL was incubated over night in different buffers and the next day a PEGylation reaction was started. It could be shown that no reaction occurred with PEG pre-incubated in a NaCNBH\(_3\) containing buffer of pH 4 neither using lysozyme nor using scFv as PEGylation protein. Also the size of the used PEG molecule did not affect the inactivation reaction (fig. 14).

It was not further investigated if hydrolysis is the driving force or if other chemical compounds of the reaction mix play any role in the inactivation reaction. In the following experiments the inactivation reaction was observed as it took place in the PEGylation reactions without pre-incubation. All factors mentioned above were observed but especially temperature and pH had a significant influence. Increasing temperature leads to increased inactivation reaction rates, but the factor of temperature dependent changes seems to be also dependent on the protein. For lysozyme a change is visible in small temperature ranges. The reaction rate keeps constant between 21 and 30 °C. For the scFv PEGylation in the low temperature regions only small changes are visible but between 20 and 30 °C a considerable difference could be found (fig. 15). The temperature dependence is independent of the PEG-size (data not shown). But it can not be excluded that the pH is the factor influencing the absolute values of this experiment most as the PEGylation of lysozyme was carried out at a pH of 6 whereas for the scFv PEGylation a pH of 4 was used. The pH dependence is clearly visible, the inactivation is increased in acidic pH and decreases the more basic the ambient gets (fig. 16). This is visible for all proteins and all PEG sizes (fig. 15, shown exemplarily for scFv and 30 kDa PEG). The PEG concentration also influences the PEG inactivation, increasing PEG concentration leads to increasing inactivation rate constants (data not shown).
Inactivation reactions for PEG are not new at all, but it was not clear beforehand in what range inactivation would occur using mPEG-AL. Buckley and co-workers calculated the rate constant for mPEG-succinimidyl propionate inactivation to be 1.31 l/h, this inactivation rate is approximately twice the highest inactivation rate of mPEG-AL found in the present study at a PEG concentration of 1.8 mol/l and a pH of 4. This clearly shows the higher stability of mPEG-AL in comparison with mPEG-succinimidyl propionate.
Conclusions

Lysozyme and a scFv were PEGylated successfully via aldehyde chemistry. The PEGylation reaction was monitored and a mathematical model was established. An additional inactivation reaction for mPEG-AL was included and the reaction could be simulated very closely.

By analyzing the reaction parameters like reaction rates, conversion, yield and selectivity, lysozyme PEGylation turned out to be a real model reaction. All factors behaved as expected, but scFv PEGylation showed not such an exemplary characteristic. The calculated rate constants for the reaction of protein to Mono-PEGylated protein were in the range of 0.002 – 0.016 l/mol*min for lysozyme and 0.009- 0.07 l/mol*min for scFv. The rate constants of the reaction of Mono-PEGylated protein to Di-PEGylated protein were about half the above mentioned values, 0.001-0.009 l/mol*min for lysozyme and 0.001-0.04 l/mol*min for scFv.

In yield, Mono-PEG-lysozyme ranged from 0.27 to 0.54 whereas Mono-PEGylated-scFv achieved values between 0.11 and 0.79. The conversion for lysozyme ranged from 0.39 to 0.79 and for scFv from 0.1 up to a complete conversion of 1. Unfortunately the best selectivity was not attended in line with the highest conversion. The highest conversion was obtained at 30 °C, but the selectivity was not good at such a high temperature. The reaction time is also a very interesting point. The longer the reaction time, the better yield and conversion get, but selectivity decreases. After about 20 hours the PEGylation reaction reaches an endpoint. In points of good selectivity and yield it is favourable to stop the reaction before it reaches the end, after about 15 hours. To test the ideal pH is described as a crucial point but that could not be confirmed here. For lysozyme the pH was not a benchmark for a good working PEGylation. Only for scFv a higher pH correlated with a better yield, but unfortunately the solubility of the used scFv is restricted to acidic pH values. The inactivation of mPEG-AL was found to be pH dependent and could be the driving force here. In acidic pHs the PEG inactivation is accelerated and thereby the pH should be chosen in the neutral or
slightly acidic range, depending on the proteins solubility behaviour. Also prior work\textsuperscript{13,15} described the isoform formation being pH dependent. This should be investigated in later studies. The PEG:protein ratio was also determined. The lower it gets, the more selective the reaction is. To get a good compromise between selectivity and yield the PEG excess should be approximately 2 to 5-fold. An ideal protein concentration of about 5 g/l for lysozyme was found, but scFv showed another behaviour. For the PEGylation with 5 kDa PEG an increase in protein concentration led to a decrease in yield. This can be explained by a lower solubility of scFv. But somewhat the addition of the longer PEG 30 kDa led to a reduction of this phenomenon. Possibly PEG with long chains increases the solubility of scFv. For scFv the temperature dependent behaviour was also not as expected. For lysozyme an increase of the reaction rate with increasing temperature was found as predicted by Arrhenius\textsuperscript{27}. That was also expected for scFv, but was not the case. For 30 °C yield and reaction rate were highest, but the 10 °C values are higher than the ones for 20 °C which does not meet the theory of van’t Hoff and Arrhenius\textsuperscript{27}. Another factor seems to manipulate the reaction mechanism. Some interaction between PEG and protein could explain this protein concentration and temperature dependent behaviour. A hydrophobic-force driven interaction between PEG and protein could easily be temperature dependent, leading to a higher interaction at lower temperature, also leading to a better reactivity at 10 °C because of a higher possibility of PEG and protein to meet each other. At 30 °C this behaviour could reach an endpoint and the Brownian motion breaks the interaction and a normal reaction progression is reached again. Apparently this interaction would also influence the dependence of the reaction on the protein concentration which was also unusual. A final experiment was conducted with the results seen so far. This led to a combination of high yield, conversion and selectivity. The chosen conditions for lysozyme were 5 g/l protein concentration, 20 °C, 2-fold PEG excess and a reaction time of 15 h for 5 kDa PEG and 20 h for 30 kDa PEG at a pH of 6. For scFv PEGylation a pH of 4 was chosen in regards of best protein solubility, 15 °C, 15 h and 5-fold
PEG excess was used for both PEG sizes. With 5 kDa PEG a protein concentration of only 1 g/l was favourable whereas for 30 kDa PEG 2 g/l was found to be the ideal concentration. The inactivation of mPEG-AL was found to be dependent on the temperature and most of all on pH. Small inactivation rates can be induced by avoiding acidic pH values and high temperatures. An inactivation rate for m-PEG-AL could be calculated between 0.01 1/h and 0.6 1/h depending on ambient conditions. This is about half the value another group found for mPEG-succinimidyl propionate inactivation 14 with 1.31 1/h. Hu et al. already found the PEGylation rate to be dependent on the PEG functionalization even when the same PEGylation chemistry was used 28 that could also be an effect of the different PEG inactivation rates for differently functionalized PEGs. MPEG-AL could be determined to be a lot more stable than mPEG-succinimidyl propionate in the present study. The conducted experiments led to the conclusion that the PEGylation reaction parameters need to be chosen and tested carefully for each protein and every PEG size. Only minimal general predictions can be made, such as prefer avoiding high temperatures and acidic pH values.
Acknowledgements

We thank the Bundesministerium fuer Bildung und Forschung (BMBF), Germany for supporting this work.
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preparative separation of PEGylated lysozyme for the characterization of chromatography


Tab. 1: Relative migration length (\(R_f\) 1. column), theoretical sizes (3. column) and calculated sizes (4. column calculated with the protein marker only, 5. column calculated theoretical apparent size taking into account the calculated size of PEG (column 4) as it appears in SDS-PAGE) for analysed samples of PEGylated lysozyme.

<table>
<thead>
<tr>
<th>Rf</th>
<th>Sample</th>
<th>Theoretical size [kDa]</th>
<th>Calculated size [kDa]</th>
<th>Theoretical apparent size of PEGylated protein in SDS-PAGE [kDa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.55</td>
<td>Poly-PEG-30-Lys</td>
<td>134.4</td>
<td>588.45</td>
<td>214.4</td>
</tr>
<tr>
<td>0.9</td>
<td>Tri-PEG-30-Lys</td>
<td>104.4</td>
<td>258.07</td>
<td>164.4</td>
</tr>
<tr>
<td>1.35</td>
<td>Di-PEG-30-Lys</td>
<td>74.4</td>
<td>130.92</td>
<td>114.4</td>
</tr>
<tr>
<td>2</td>
<td>Mono-PEG-30-Lys</td>
<td>44.4</td>
<td>67.81</td>
<td>64.4</td>
</tr>
<tr>
<td>2.4</td>
<td>PEG 30 kDa</td>
<td>30</td>
<td>49.98</td>
<td>50</td>
</tr>
<tr>
<td>2.8</td>
<td>Tri-PEG-5-Lys</td>
<td>29.4</td>
<td>38.61</td>
<td>38.4</td>
</tr>
<tr>
<td>3.3</td>
<td>Di-PEG-5-Lys</td>
<td>24.4</td>
<td>29.33</td>
<td>30.4</td>
</tr>
<tr>
<td>4</td>
<td>PEG-5-Lys</td>
<td>19.4</td>
<td>21.26</td>
<td>22.4</td>
</tr>
<tr>
<td>4.95</td>
<td>Lysozym</td>
<td>14.4</td>
<td>14.88</td>
<td>14.4</td>
</tr>
</tbody>
</table>

Tab. 2: Relative migration length (1. column, Rf), theoretical sizes (3. column) and calculated sizes (4. column calculated with the protein marker only, 5. column calculated theoretical apparent size, tacking into account the calculated size of PEG as it appears in SDS-PAGE) for analysed samples of PEGylated scFv.

<table>
<thead>
<tr>
<th>Rf</th>
<th>Sample</th>
<th>Theoretical size [kDa]</th>
<th>Calculated size [kDa]</th>
<th>Theoretical apparent size of PEGylated protein in SDS-PAGE [kDa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.75</td>
<td>Di-PEG-30-scFv</td>
<td>88.5</td>
<td>156.82</td>
<td>128.5</td>
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<tr>
<td>2.1</td>
<td>PEG 30 kDa Dimer</td>
<td>60</td>
<td>116.01</td>
<td>100</td>
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<tr>
<td>2.6</td>
<td>Mono PEG-30-scFv</td>
<td>58.5</td>
<td>81.50</td>
<td>78.5</td>
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<tr>
<td>3.5</td>
<td>PEG 30 kDa</td>
<td>30</td>
<td>49.86</td>
<td>50</td>
</tr>
<tr>
<td>Temp [°C]</td>
<td>PEG [kDa]</td>
<td>k1 [l/mol*min]</td>
<td>k2 [l/mol*min]</td>
<td>Yield</td>
</tr>
<tr>
<td>-----------</td>
<td>------------</td>
<td>----------------</td>
<td>----------------</td>
<td>-------</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>0.0037</td>
<td>0.0017</td>
<td>0.34</td>
</tr>
<tr>
<td>21</td>
<td>5</td>
<td>0.0125</td>
<td>0.0061</td>
<td>0.5</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>0.0165</td>
<td>0.0089</td>
<td>0.49</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>0.0028</td>
<td>0.0013</td>
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<tr>
<td>21</td>
<td>30</td>
<td>0.0075</td>
<td>0.003</td>
<td>0.43</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
<td>0.0124</td>
<td>0.0022</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Tab. 3: PEGylation of lysozyme at different temperatures and the resulting rate constants \( k_1, k_2 \) and yield, conversion and selectivity after 20 h reaction time.

<table>
<thead>
<tr>
<th>Temp [°C]</th>
<th>PEG [kDa]</th>
<th>k1 [l/mol*min]</th>
<th>k2 [l/mol*min]</th>
<th>Yield</th>
<th>Conversion</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5</td>
<td>0.0199</td>
<td>0.0101</td>
<td>0.56</td>
<td>0.73</td>
<td>0.77</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>0.0144</td>
<td>0.0086</td>
<td>0.48</td>
<td>0.61</td>
<td>0.79</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>0.0727</td>
<td>0.0424</td>
<td>0.43</td>
<td>0.87</td>
<td>0.50</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>0.0153</td>
<td>0.0071</td>
<td>0.52</td>
<td>0.67</td>
<td>0.78</td>
</tr>
<tr>
<td>20</td>
<td>30</td>
<td>0.0115</td>
<td>0.0011</td>
<td>0.45</td>
<td>0.50</td>
<td>0.91</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
<td>0.0657</td>
<td>0.0312</td>
<td>0.53</td>
<td>0.86</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Tab. 4: PEGylation of scFv at different temperatures and the resulting rate constants \( k_1, k_2 \) and yield, conversion and selectivity after 20 h reaction time.
Figure 1:

Fig. 1: Methoxy-PEG-aldehyde reaction with the ε-amino-group of a proteins lysine residue.

A: Methoxy-PEG-aldehyde and protein with lysine residue, B: in the first reaction step a
instable Schiff base is formed, C: in the presence of NaCNBH$_3$ the Schiff base is reduced to a
stable secondary amine.
Figure 2:

Fig. 2: PEGylation reaction of lysozyme and 5 kDa PEG at the beginning and at the end of the reaction as analyzed via cation-exchanger and labelled as results of MALDI-TOF and SDS-PAGE indicated. Left chromatogram: reaction time 0 min, right chromatogram: reaction time 970 min. For chromatographic conditions see Analytical procedures.

Figure 3:
Fig. 3: SDS-PAGE of a lysozyme PEGylation with 5 kDa PEG (left) and 30 kDa PEG (right) at different pH values after a reaction time of approximately 1200 minutes. Blue: coomassie stained proteins, brown: barium-iodine stained PEG. Mixed colours represent PEGylated proteins. The marker proteins are on the left side.

Figure 4:

Fig. 4: SDS-PAGE of a scFv PEGylation with 5 kDa PEG (left) and 30 kDa PEG (right) at different PEG-to-protein ratios after a reaction time of approximately 1200 minutes. blue: coomassie stained proteins, brown: barium-iodine stained PEG. Mixed colours represent PEGylated proteins. The marker proteins are on the left side.

Figure 5:
Fig. 5: PEGylation reaction of scFv and 5 kDa PEG at the beginning and at the end of the reaction as monitored via cation-exchanger and labelled as results of SDS-PAGE indicated. Left chromatogram: reaction time 0 min, right chromatogram: reaction time 960 min. For chromatographic conditions see Analytical procedures.

Figure 6:

![SDS-PAGE of 30 (left) and 5 kDa PEG (right) solubilised in reaction buffer at pH 5. Brown: barium-iodine stained PEG.](image)
Fig. 7: PEGylation reaction for lysozyme and 5 kDa PEG at a pH of 4 (top left), at a pH of 7 (top right), scFv and 5 kDa PEG at pH 4 (bottom left) and 7 (bottom right). Experimental data shown as crosses, simulated data shown as straight lines. Green: unmodified protein, red: Mono-PEG-protein, blue: Di-PEG-protein, yellow: Tri-PEG-protein.
Fig. 8: PEGylation reaction for lysozyme and 5 kDa PEG at a pH of 4 (top left) and at a pH of 7 (top right), scFv and 5 kDa PEG at pH 4 (bottom left) and 7 (bottom right). Experimental data shown as crosses, simulated data shown as straight lines. Green: unmodified protein, red: Mono-PEG-protein, blue: Di-PEG-protein, yellow: Tri-PEG-protein.
Figure 9:

![Conversion, yield and selectivity over the reaction time. Shown exemplarily for lysozyme PEGylation with 5 kDa PEG at 21 °C and a pH of 6. Grey bars: yield, black bars: conversion, white bars: selectivity.](image)

Fig. 9: Conversion, yield and selectivity over the reaction time. Shown exemplarily for lysozyme PEGylation with 5 kDa PEG at 21 °C and a pH of 6. Grey bars: yield, black bars: conversion, white bars: selectivity.

Figure 10:

![Yield over pH. Shown exemplarily for scFv PEGylation with 5 kDa PEG (left) and 30 kDa (right) at 21 °C after 20 h. Grey bars: yield.](image)

Fig. 10: Yield over pH. Shown exemplarily for scFv PEGylation with 5 kDa PEG (left) and 30 kDa (right) at 21 °C after 20 h. Grey bars: yield.
Figure 11:

![Conversion, yield and selectivity over the total protein concentration for scFv and 5 kDa PEG (left), 30 kDa PEG (mid left) and lysozyme with 5 kDa PEG (mid right) and 30 kDa PEG (right). Grey bars: yield, black bars: conversion, white bars: selectivity. The reactions were done at 21 °C, for lysozyme at a pH of 6 and for scFv at pH 4. The values were taken after approximately 20 h of reaction.](image-url)
Figure 12:

![Graph showing conversion yield and selectivity over the PEG-excess at constant 1 g/l scFv. For 5 kDa PEG (left), 30 kDa PEG (right). Grey bars: yield, black bars: conversion, white bars: selectivity. The reactions were done at 21 °C at a pH of 4. The samples were taken after approximately 20 h of reaction.](image)

Fig. 12: Conversion yield and selectivity over the PEG-excess at constant 1 g/l scFv. For 5 kDa PEG (left), 30 kDa PEG (right). Grey bars: yield, black bars: conversion, white bars: selectivity. The reactions were done at 21 °C at a pH of 4. The samples were taken after approximately 20 h of reaction.

Figure 13:

![Graph showing conversion, yield, and selectivity over the temperature at constant 1 g/l protein. For scFv and 5 kDa PEG (left), 30 kDa PEG (mid left). For lysozyme and 5 kDa PEG (mid right) and 30 kDa PEG (right). Grey bars: yield, black bars: conversion, white bars: selectivity. The reaction pH was 4 for scFv and 6 for lysozyme. The samples were taken after approximately 20 h of reaction.](image)

Fig. 13: Conversion, yield, and selectivity over the temperature at constant 1 g/l protein. For scFv and 5 kDa PEG (left), 30 kDa PEG (mid left). For lysozyme and 5 kDa PEG (mid right) and 30 kDa PEG (right). Grey bars: yield, black bars: conversion, white bars: selectivity. The reaction pH was 4 for scFv and 6 for lysozyme. The samples were taken after approximately 20 h of reaction.
Figure 14:

Figure 14: PEGylation reaction of lysozyme (left) and scFv (right), exemplarily shown with a 5 kDa PEG (right) and a 30 kDa PEG (left). PEG was pre-incubated in reaction buffer containing 20 mM NaCNBH$_3$ at a pH of 4. Continuous line: 0 min reaction time, dashed line: 4 h reaction time. No reaction occurred regardless the protein or PEG-size.

Figure 15:

Inactivation constant

<table>
<thead>
<tr>
<th>Temperature [°C]</th>
<th>lysozyme</th>
<th>scFv</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.05</td>
<td>0.15</td>
</tr>
<tr>
<td>20</td>
<td>0.15</td>
<td>0.2</td>
</tr>
<tr>
<td>30</td>
<td>0.25</td>
<td>0.35</td>
</tr>
</tbody>
</table>

PEG-concentration: 0.07 [mol/l] for lysozyme and 0.18 [mol/l] for scFv.
Fig. 15: Inactivation reaction rate constant \( k_4 \) (grey bars) over the temperature, shown exemplarily for 5 kDa PEG with lysozyme and a PEG concentration of 0.07 mol/l at a pH of 6 (left) and scFv with a PEG concentration of 0.18 mol/l at a pH of 4 (right).

Figure 16:

![Graph showing inactivation reaction rate constant \( k_4 \) over pH](image)

Fig. 16: Inactivation reaction rate constant \( k_4 \) (grey bars) over the pH, shown for scFv and 30 kDa PEG at 21 °C at a constant PEG concentration of 0.18 mol/l.