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Optimization of Random PEGylation Reactions by Means of High Throughput Screening

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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.

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KEYWORDS: PEGylation; lysozyme; high throughput process development; high throughput experimentation; quality-by-design

Introduction

Nowadays, recombinant proteins and peptides represent a large share of successfully approved drugs, gaining an increasing importance as therapeutics (Leader et al., 2008). 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 (Basu et al., 2006; Chaffee et al., 1992; Lee et al., 2007; Palm et al., 2011). 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 (Fee and Van Alstine, 2006; Jevsevar et al., 2010; Pasut and Veronese, 2012).

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 (Sato, 2002; Sergi et al., 2009; Yang et al., 2003). 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

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of already approved PEGylated products, such as PEGylated interferon and epoetin (Pegasys® and Micera® by Hoffman-LaRoche, Basel, Switzerland 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 (Roberts et al., 2002; Veronese and Pasut, 2008). 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 (Bonora and Drioli, 2009; Kinstler et al., 1996; Roberts et al., 2002). 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 (Jevsevar et al., 2010).

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 Pharmaceuticals, Piscataway, NJ) between 2000 and 2003 due to deviations in activity (Gaberc-Porekar et al., 2008; Payne et al., 2011) 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, Thousand Oaks, CA) 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 (Hansen et al., 2012) 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. (2011). 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 (Bhambure et al., 2011). 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 (Maiser et al., 2012), 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.

Materials and Methods

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 (M_w 14.3 kDa, six lysine residues), 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). Cyclohexylaminobutansulfonic acid (CABS) was used for pH-gradient buffer preparation and was provided by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Lyophilized cells of *M. 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.

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+ multiangle light scattering device in combination with an Optilab rEx refractive index detector, both from Wyatt Technology (Santa Barbara, CA) were used. Thermal stability assays were realized using an DynaPro Plate Reader also from Wyatt Technology.

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). 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). 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.

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 molar 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 to 12 h were prepared for each buffer condition.

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. (2011). 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. (2011). 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.

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 (Roberts and Harris, 1998; Roberts et al., 2002), 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.

Analytical Procedure

PEGamer Separation

Employing the PEGylation degree analysis, the reaction preparations were analyzed with size exclusion chromatography (SEC) using a Zenix™ SEC 300 column from Sepax Technologies, Inc. (Newark, DE). 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 and 250 μL were chosen. The PEGylation degree was validated applying molecular weight determination with combined light scattering and refractive index analysis.

Isoform Separation

Isoform separation was conducted as previously reported on a MonoS 4.6/100 column from GE Healthcare (Maiser et al., 2012). Sample volumes between 100 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 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, resulting in an analysis time of 15 min per sample. 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.

Lysozyme Activity Assay

For lysozyme activity measurements a tubidometric assay based on a *M. 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 and Yang (2002), using UV-star flat bottom plates from Greiner Bio-One. The lysozyme assay was conducted with protein sample concentrations in the range of 1–5 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) 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 1 unit in accordance to Lee and Yang (2002) and Freitas and Abrahao-Neto (2010).

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). Subsequently, a temperature ramp from 25 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.

Results and Discussion

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.

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.

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. (2011). 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.

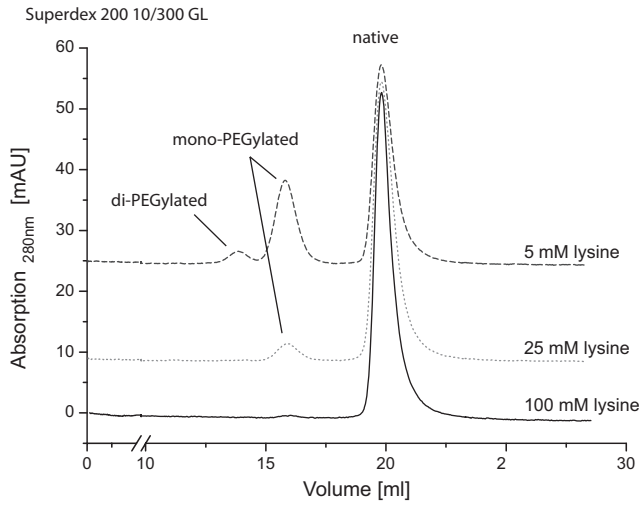

Figure 1. SEC chromatogramms of mPEG-aldehyde lysozyme reaction preparations with different lysine concentrations after 20 h reaction time. Reaction conditions were pH 6.2 and 0.15 protein to PEG ratio.

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 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

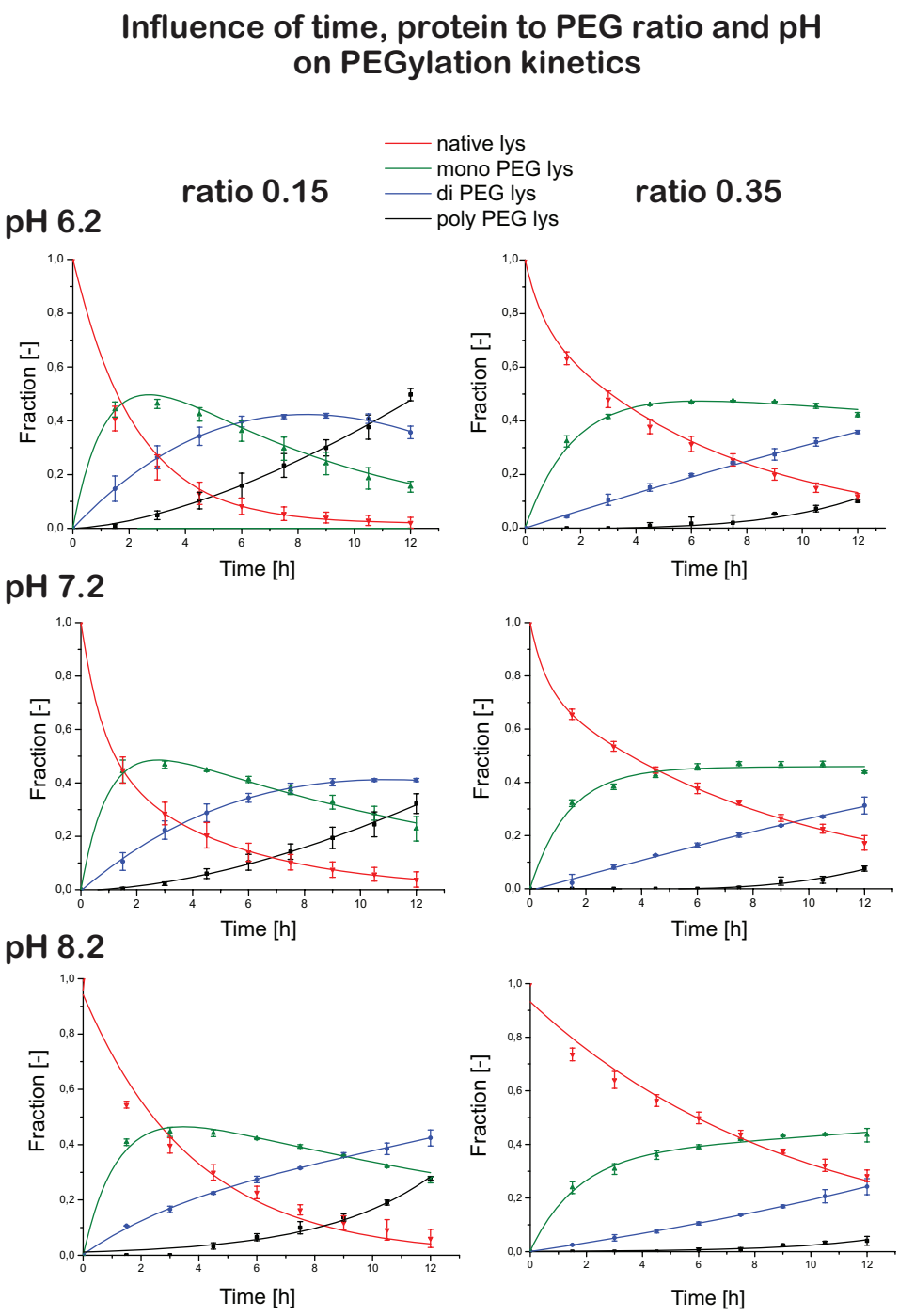

Figure 2. Native lysozyme and PEGamer distribution of different reaction conditions, based on SEC chromatograms.

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 (Morar et al., 2006; Roberts and Harris, 1998) and can be described as follows:

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

Comparing the results with lysozyme reaction kinetics at pH 7 and a protein to PEG ratio of 0.5 published by Moosmann et al. (2011) 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 fraction of formed mono-PEG lysozyme over the reaction time. The contour plots were generated with a triangle based cubic interpolation using MATLAB, based on evaluated SEC chromatograms.

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 calculated. 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.

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. (2007) for PEGylated interferon using turbidimetric methods or by Kinstler et al. (1996) evaluating the aggregation level of

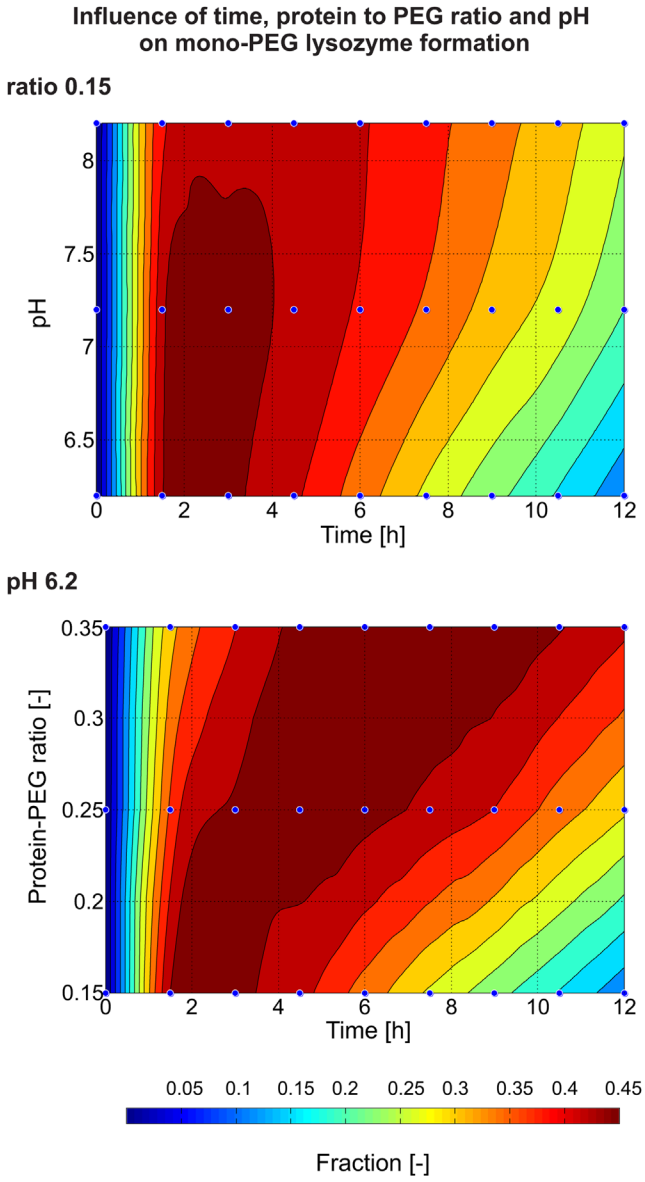

Figure 3. Contour plots with the fraction of mono-PEG-lysozyme, based on SEC chromatograms. Dots show reaction conditions evaluated.

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.

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. (2011) suggest a model where the attached PEG random coil is adjacent to the protein.

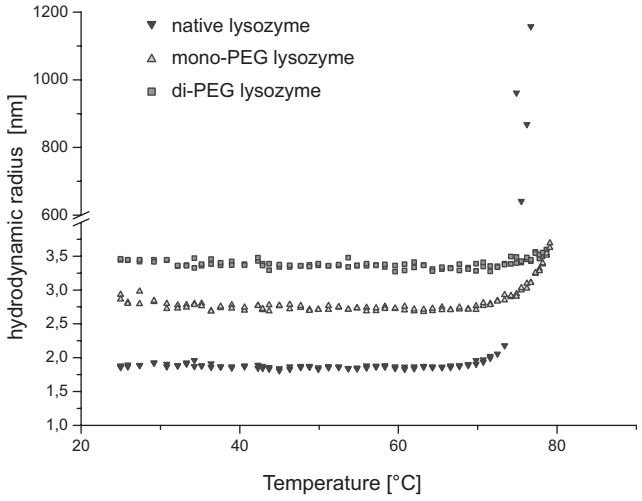

Figure 4. Hydrodynamic radii of native and PEGylated lysozyme as a function of temperature.

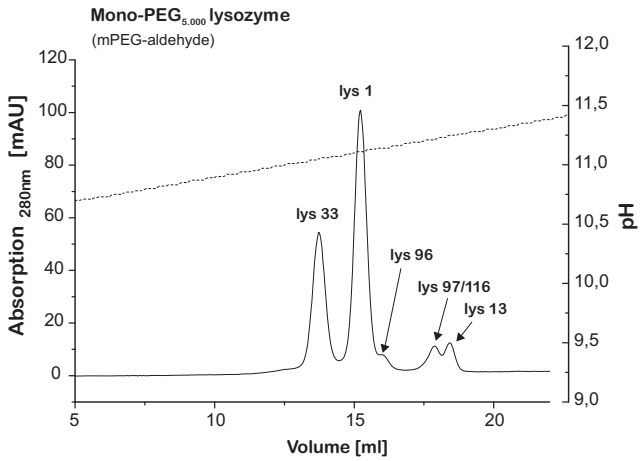

Figure 5. Chromatogragraphic separation of mono-$PEG_{aldehyde}$-lysozyme isoforms. Peak labels correspond to PEG binding sites, according to Maiser et al. (2012).

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. The corresponding PEGylation sites were identified via an *in silico* approach and MALDI-MS analytics. In addition, lysine 1 was identified to be the most reactive PEG conjugation site, followed by lysine 33 using reaction buffer pH values of 7.2 (Maiser et al., 2012). 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. (1996), 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 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. Using a low buffer pH, 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 would generate a relative increase of lysine 1 mono-PEGylated variants. Another possibility is of course an increased reaction rate to lysine 1 PEGylated lysozyme.

The combination of mono-PEG-lysozyme concentration and the measured isoform fractions allows the determination

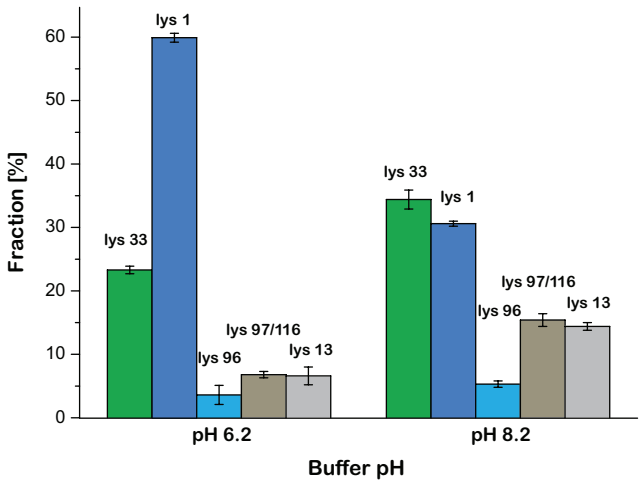

Figure 6. Distribution of mono-PEGylated lysozyme isoforms in dependency of the buffer pH. Both reactions after 12 h reaction time and a protein to PEG ratio of 0.15.

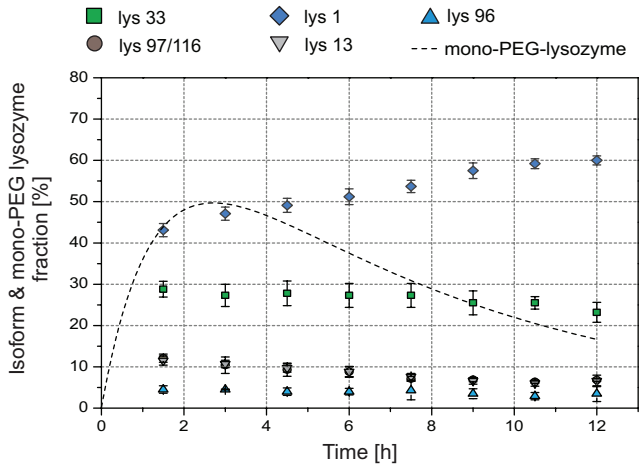

Figure 7. Distribution of mono-PEGylated lysozyme isoforms in dependency of the buffer pH. Both reactions after 12 h reaction time and a protein to PEG ratio of 0.15.

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.

Lysozyme Activity

First described in 1952 by Shugar (1952), the lytic activity of lysozyme can be determined by measuring the changes in turbidity of a *M. 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 8B and Table I, respectively.

All is oforms 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. (2010) suggest a residual activity of about 30% for 5 kDa mono-PEG lysozyme isoform mixtures, which is thus in the line 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. (1997) 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.

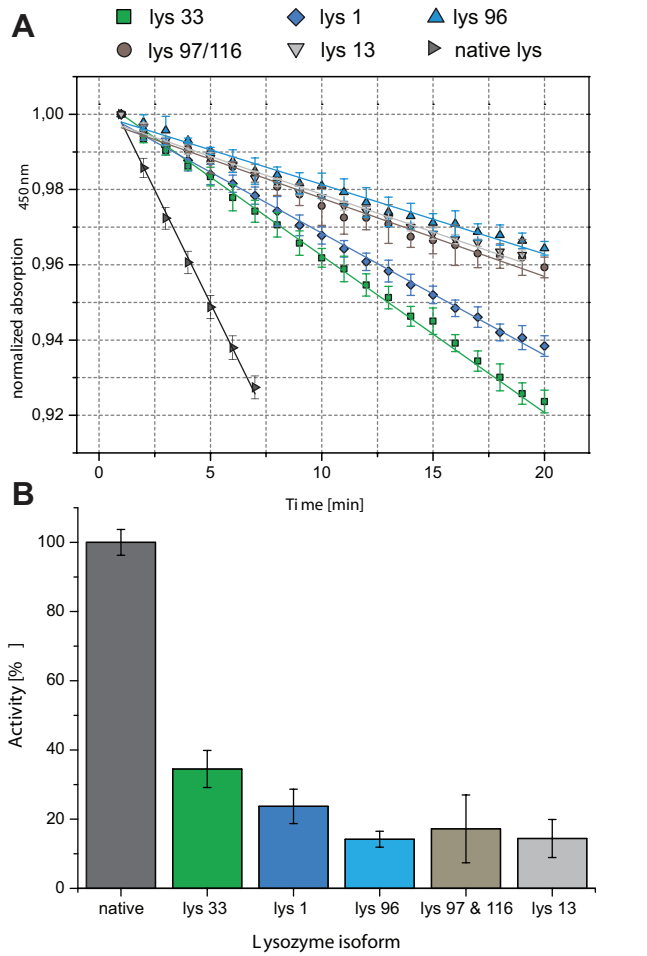

Figure 8. **A**: normalized linear decrease in the 450 nm signal of each sample over time. **B**: resulting realtive activity of each isoform, compared to native lysozyme.

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 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

Table I. 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 and 116	3.824	17
Lys 13	3.196	14

values between pH 6.2 and 7.2, using a protein to PEG ratio of 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 optimum process window 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.

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 10A illustrates the obtained chromatograms of PEGylation mixtures, conducted with a protein to PEG ratio of 0.35 and varying buffer pH values.

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 Figure 10B, 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 PEG-NHS, as reported by Veronese and Pasut (2008). 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

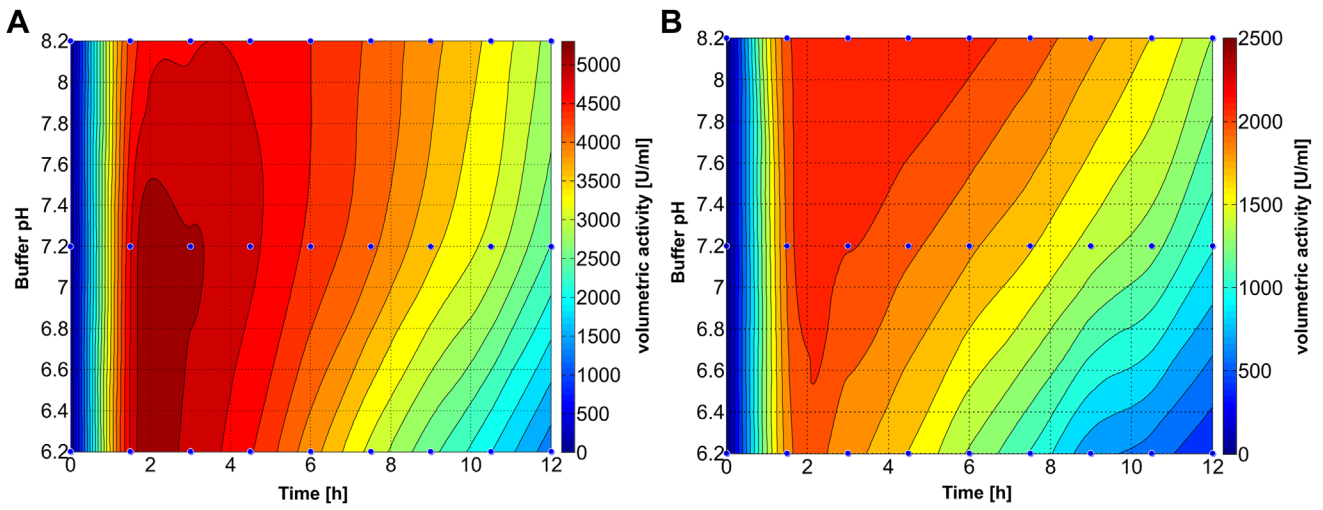

Figure 9. Calculated volumetric activity of mono-PEG lysozyme isofom mixtures (**A**) and PEG-lys_{33} (**B**) as a function of reation time and buffer pH. Protein to PEG ratio was 0.15.

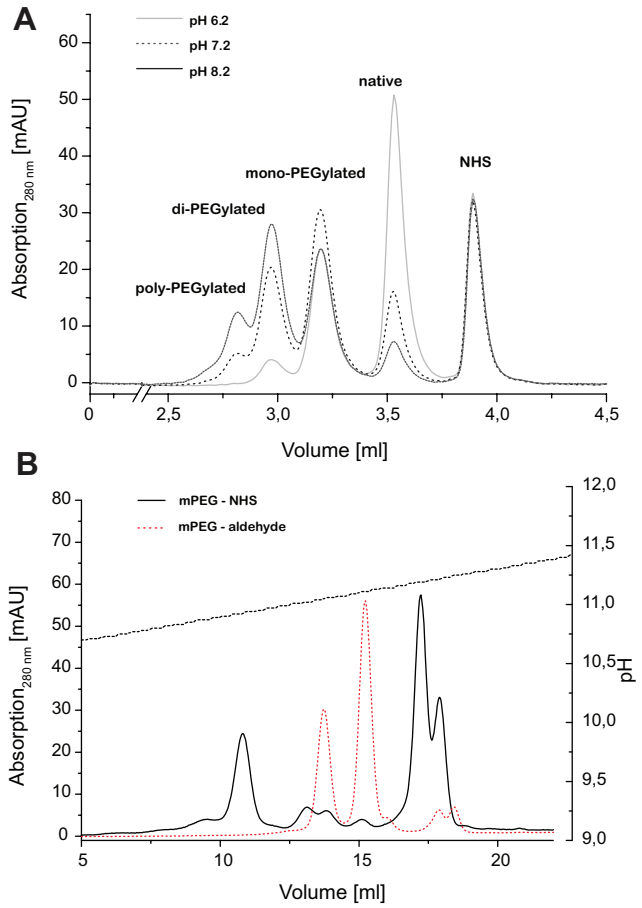

Figure 10. **A**: SEC chromatograms of mPEG-NHS modified lysozyme reaction mixtures with a Sepax Zenix™ SEC 300 column, **B**: Comparison of the isoform elution profiles of mono-PEG-lysozyme. Reaction buffer pH was 6.2.

example in stability or isoform activity were not evaluated but should be taken into account when choosing the type of PEGylation.

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.

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