Journal of Chromatography A, 1236 (2012) 90–96

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Purification of a PEGylated single chain Fv

Anna Moosmann*, Elke Gerlach, Robert Lindner, Heiner Böttinger

Institute of Cell Biology and Immunology, University of Stuttgart, Allmandring 31, Stuttgart 70569, Germany

ARTICLE INFO

Article history:
Received 9 November 2011
Received in revised form 28 February 2012
Accepted 1 March 2012
Available online 8 March 2012

Keywords:
PEGylation
Cation exchange chromatography
scFv
Hydrophobic interaction chromatography
Arginine chloride
N-terminal PEGylation
Bioactivity
Drug delivery

ABSTRACT

In this manuscript we describe the two-step purification of a mono-PEGylated anti-epidermal growth factor receptor (EGFR) single-chain Fv. A weak cation exchanger was used for capture. Elution using arginine suppressed protein aggregation and allowed a very good resolution with purity and product-recovery was above 90%. Free PEG was removed completely. The use of hydrophobic interaction chromatography (HIC) increased purity to 98%. Increasing the size of PEG from 5 to 30 kDa increased retention on HIC and reduced it on cation exchangers. Bioactivity of PEGylated scFv was confirmed by ^{125}I based cell tests. Proteins modified with 5 kDa PEG showed higher bioactivity than proteins modified with larger PEGs. The combination of cation exchange and HIC provides a rational and effective basis for PEGylated scFv purification.

1. Introduction

Targeted tumour therapies are one of the most recent fields of cancer research; therein the epidermal growth factor receptor (EGFR) is a very prominent target as it is overexpressed in many tumours such as breast, renal and colon carcinomas. The activation of EGFR leads to the initiation of cell proliferation and survival associated proteins [1,2]. An approach to overcome its proliferative function is to block the EGFR with an antagonistic molecule, such as an antibody. For example, cetuximab (Erbitux, ImClone Systems Inc., New York, USA) is an EGFR binding monoclonal antibody efficiently inhibiting downstream signalling [3–5].

Single-chain variable fragments (scFvs) are the smallest antigen-recognising proteins of about 25 kDa size [6,7]. This provides them with rapid distribution and potentially permits their administration by non-invasive means such as nasal mists or eye-drops. In addition, their small size makes them ideal candidates for large-scale bacterial production, however imposes the disadvantage of rapid renal clearance.

Among other methods PEGylation, the addition of a polyethylene glycol (PEG) to a protein, is an established method to enlarge the proteins' hydrodynamic size [8,9]. PEG is an uncharged, non-immunogenic and non-toxic polymer [10]. The pioneer works in PEGylation were made in the 1970s by Davis and Abuchowski [11,12]. Additionally, it was shown that protein PEGylation partly masks the protein surface, thereby reducing immunogenicity and degradation related protease targets [13–15]. However, PEGylation induced masking of active sites could affect bioactivity. Indeed, while some authors have reported full conservation of bioactivity [16], several others showed a significant reduction in bioactivity upon PEGylation [17–19]. The FDA already approved several PEGylated protein drugs such as PEGasys® (Hoffman-La Roche) and PEG Intron® (Schering-Plough/Enzon) [10,20], both containing α-interferon for hepatitis C treatment.

Several chromatography modes are usable for subsequent fractionation to obtain the most suitable conjugate composition, free from under- and over-modified forms. For example, size exclusion chromatography (SEC) is intuitively attractive because of its simplicity and size discrimination but suffers from low productivity [10]. Each PEG-molecule added removes a positive charge from a protein lysine residue, potentially reducing retention on anion exchangers (AEX) and increasing retention on cation exchangers (CEX), both of which have been applied successfully [18–21]. Finally, PEG by itself is hydrophobic, and augments the overall conjugate hydrophobicity, creating the basis for successful fractionation by hydrophobic interaction chromatography (HIC) [21,22].

The present study describes the separation of PEGylated scFv with preparative hydrophobic interaction and cation exchanger resins. Binding of scFv or PEGylated scFv to intact cells was confirmed using displacement of ^{125}I labelled EGF by scFv or PEGylated scFv.

* Corresponding author. Tel.: +49 71168560262; fax: +49 71168567484.
E-mail addresses: an-na-mo@gmx.de, anna.moosmann@izi.uni-stuttgart.de (A. Moosmann).

0021-9673/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2012.03.004

2. Materials and methods

2.1. Chemicals

Methoxy-PEG-aldehyde with an average molecular weight of 5 and 30 kDa was purchased from NOF Corp. (Grobbendonk, Belgium). All other chemicals were provided by Merck (Darmstadt, Germany).

2.2. Production and PEGylation of scFv

The *Escherichia coli* strain *BL21 DE3 rha*$^-$ was used to produce the scFv of 28 kDa using the rhamnose induction system [23,24]. An osmotic shock procedure modified according to Rathore [25] was used: the pellet was solubilised in 100 mM phosphate-buffer pH 7.0 containing 50% glucose followed by addition of pure water. The protein solution was furthermore purified using protein L-chromatography (GenScript, Piscataway, USA) [26]. A 20 mM sodium phosphate buffer at a pH of 7.0 was used for loading and a 100 mM sodium citrate/phosphate buffer at a pH of 2.5 for elution. Afterwards diafiltration and lyophilisation for long-term storage followed.

For compositional analysis the protein absorption at 280 nm was used. As PEG is UV inactive, it does not contribute to the UV 280 nm absorption and therefore percent information refers to total protein content.

The optimisation of PEGylation conditions has been carried out elsewhere [27]. In the present study, the recommendations of Moosmann et al. [27] were attended: scFv in a concentration of 1 g/L for 5 and 30 kDa PEG with a 5-fold molar PEG excess was dissolved in 20 mM sodium acetate buffer at a pH of 4.0 containing 20 mM $NaCNBH_3$ [8,21,27,28]. The reaction time was about 16 h at room temperature.

The scFv possesses 9 lysine residues and one N-terminal amino group. The random PEGylation reaction therefore can lead to more than one mono-PEGylated isoform and also different di-PEGylated forms as well as higher PEGylated species.

2.3. Analytical procedure

2.3.1. SDS-PAGE

According to Laemmli [29], SDS-PAGE under reducing conditions 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. Protein samples were solubilised in sample buffer and heated at 95 °C for 3 min. The gels were stained with Page-Blue Protein Staining solution (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instruction. To specifically stain PEGylated proteins a barium-iodine staining was carried out. The procedure was modified according to Kurfürst [30], following the instructions of Bailon et al. [31].

Purity and product-recovery percentage data were obtained from SDS-PAGE using ImageJ image process and analysis software for quantification (http://rsb.info.nih.gov/ij/) if not indicated otherwise in the text. PEGylation does not contribute to coomassie staining, hence only the protein portion in one lane was analysed and the percentage of each band was calculated. All experiments were performed at least two times.

2.3.2. Size-exclusion-chromatography

An analytical TSKgel G3000SW$_{XL}$ column (7.8 mm × 30 cm, Tosoh Bioscience GmbH) was used. To overcome the problem of column plugging caused by the sticky PEG [32], as mobile phase a 20 mM sodium-HEPES/HEPES buffer, pH 6.4, containing 50 mM NaCl was used as mobile phase. The SEC chromatography was performed either on a Thermo Separation HPLC SpectraSYSTEM (Thermo Fisher Scientific GmbH) with a sample amount of 10 μL and a flow rate of 1.2 mL/min, or on a Viscotek GPCmax with TDA305 (Malvern, Herrenberg, Germany) with a sample amount of 100 μL and a flow rate of 1.0 mL/min. The Thermo Separation HPLC SpectraSYSTEM contained a UV detector to monitor the experiment, whereas the Viscotek GPCmax with TDA305 was a multidetector system, containing a UV and a RI detector as well as a viscometer and a right angle light scattering unit. The TDA offers the opportunity to calculate the molecular mass directly via light scattering, therefore no calculation or extrapolation of standards needs to be used.

Purity determination was mostly done by SDS-PAGE, when indicated SEC was used. In SEC UV absorbance was used to determine protein concentration under the assumption of PEG being UV inactive. For percentage data the area under each peak was determined.

2.3.3. N-terminal sequencing

N-terminal sequencing using Edman degradation [33] was carried out from Toplab (Martinsried, Germany). PEGylated proteins were therefore sent to Toplab blotted on an iBlot® Transfer Stack, PVDF Regular-Membrane (Invitrogen), according to the manufacturer's procedure.

2.4. Preparative purification

For all preparative purifications an Äkta Explorer System (GE Healthcare, Uppsala, Sweden) was used. The Äkta UV unit at 280 nm was used to monitor the experiments. Omnifit glass columns (6 mm × 25 mm, Bio-Chem Fluidics, Cambridge, UK) with a flow rate of 1 mL/min (212 cm/h) were used for all experiments. To purify the PEGylated scFv a two-step purification, consisting of a HIC and a CEX step was established. For screening 1 mL PEGylation mix was used for loading, later the first purification step was carried out with 10 mL PEGylation mixture, corresponding to 14 mg protein per mL chromatography resin.

2.4.1. Hydrophobic interaction chromatography (HIC)

After solubility and binding studies with different salt systems, buffer B consisting of 2.6 M NaCl, 0.9 M $(NH_4)_2SO_4$ and 100 mM sodium phosphate buffer at a pH of 6.5 was chosen for loading and buffer A, a 20 mM sodium acetate buffer at pH 4.0, for elution. A Toyopearl PPG-650-M resin (Tosoh Bioscience GmbH, Stuttgart, Germany) was packed in an Omnifit glass column (6 mm × 25 mm, Bio-Chem Fluidics) for the HIC purification step. To achieve binding conditions, the sample was diluted 1:1.5 with loading buffer.

2.4.2. Cation-exchange-chromatography (CEX)

Buffer A consisted of 20 mM sodium acetate, pH 4.0. Elution buffer B was prepared by adding 1 M NaCl to a 20 mM sodium acetate buffer or by solubilising 1 M of L-arginine-monohydrochloride, the pH of both buffers was brought to 4.0 by adding acetic acid. Omnifit glass columns (6 mm × 25 mm, Bio-Chem Fluidics) were packed with Toyopearl GigaCap CM-650M or GigaCap S-650M (Tosoh Bioscience GmbH) for purification of PEGylated scFv.

When samples from HIC elution were applied, they were diafiltered against CEX loading buffer until conductivity below 10 mS/cm was achieved. Diafiltration of the sample was performed using a Vivaflow 50 membrane filter (Sartorius Stedim Biotech S.A., Aubagne, France) and a centrifuge (Heraeus Multifuge 3 L-R, Thermo Fisher Scientific GmbH).

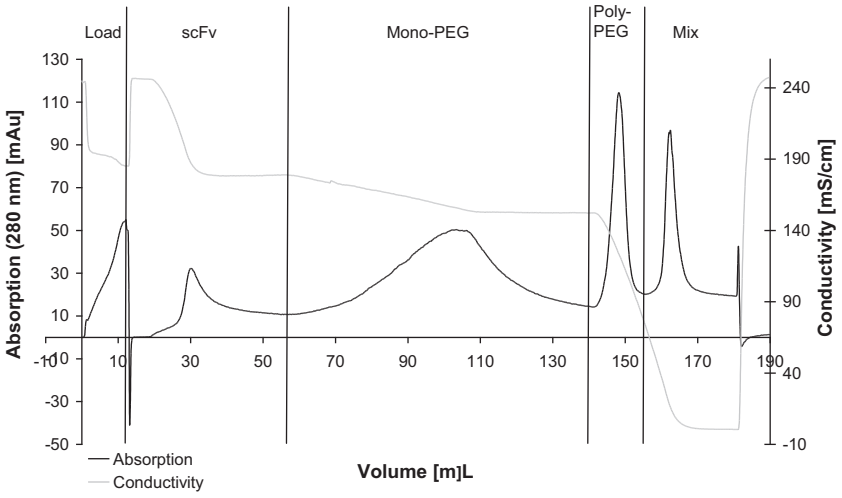

Fig. 1. Preparative purification of 5 kDa PEGylation mixture on the HIC resin Toyopearl PPG-650M. Absorption at 280 nm is shown in black, conductivity in grey. The elution of species is marked by black bars. scFv eluted first, PEGylated species (mono-PEG and poly-PEG) at lower salt concentrations. An unseparated mixture peak (Mix) eluted at a conductivity of 0.5 mS/cm.

2.5. Biological activity

The biological activity was determined *in vitro* using a cell-based system with the colon-cancer cell line A431. The A431 cell line over-expresses the EGF-receptor, which is recognised by the scFv used in this study. The scFv specifically binds to EGFR, thereby blocking binding of EGF to its receptor.

A431 cells were cultivated in RPMI 1640 (Invitrogen) medium containing 5% FCS (HyClone Laboratories, Logan, USA).

EGF (GenScript) was iodinated using the chloramine-T method [34] to a specific activity of about 170 MBq/mg. ^{125}I-labelled EGF was separated from free iodide using gelfiltration on Sephadex G-25 (GE Healthcare, Freiburg, Germany). Binding assays were performed as described elsewhere [35,36]. All binding assays were performed as triplicates with 1×10^6 A431 cells/tube and 1 ng ^{125}I-EGF/tube. Ligand or competitors were added to the given concentrations. The reaction was started by adding ^{125}I-EGF. Radioactivity specifically bound to the cells was detected using a LB211 gamma-counter (BERTHOLD TECHNOLOGIES GmbH & Co. KG, Bad Wildbad, Germany). Inhibitor constants were calculated according to Cheng and Prusoff [37].

3. Results

3.1. Production and PEGylation of scFv

The production and initial protein L-based purification yielded scFv with more than 90% purity, which was used for PEGylation. The 5 kDa as well as the 30 kDa PEGylation mixture consisted of about 60% mono-PEGylated-scFv after a reaction time of 16 h. About 17% and 25% non-modified scFv was left in the PEG 30 kDa mixture and the PEG 5 kDa mixture, respectively, as determined by SEC analysis (data not shown). Free PEG is not UV active and hence invisible in SEC, therefore it was not considered within the compositional analysis. Apparent sizes in SEC were 47 kDa and 300 kDa for mono-PEG-5-scFv and di-PEG-30-scFv, respectively.

3.2. Purification and identification of PEGylated proteins

PEGylation of scFv was followed by hydrophobic interaction chromatography (HIC). Mono-PEG-5-scFv was eluted at conductivity between 177 and 152 mS/cm (see Fig. 1).

The purity of the mono-PEG-5-scFv pool after diafiltration was about 80% (determined using SEC). The non-PEGylated scFv nearly completely vanished (data not shown). Most of it was already lost during load and did not bind to the column due to the salt concentration, which was not high enough for the least hydrophobic non-PEGylated scFv to bind quantitatively. The whereabouts of the free PEG were observed using SDS-PAGE and barium-iodine staining (data not shown). Most of the free 5 kDa PEG did not bind to the column and was also lost during load, as observed for the non-modified scFv. Only low amounts of both species were bound and coeluted at the beginning of the gradient at very high salt concentrations.

In Fig. 1, at a conductivity of 0.5 mS/cm an unseparated mixture peak eluted, containing small portions of all species.

First tests for CEX chromatography were conducted using 1 mL of the PEGylation mixture. Two preparative matrices, the strong cation exchanger Toyopearl GigaCap S-650M and the weak cation exchanger GigaCap CM-650M were used. For elution 1 M NaCl was used (see Fig. 2). For GigaCap S mono- as well as di-PEGylated scFv showed two peaks with scFv eluting in the middle. GigaCap CM showed the expected elution behaviour, the more PEG attached to the protein, the lower the elution salt concentration was. Hence, GigaCap CM was used for further purifications.

For injection on CEX after the HIC step, the eluted mono-PEG-5-scFv was pooled. Diafiltration of the mono-PEG-5-scFv pool against CEX loading buffer was conducted until conductivity below 10 mS/cm was reached. The sample volume used for the purification with the weak cation exchanger Toyopearl GigaCap CM-650M was about 10 mL. The mixture applied on the HIC column as well as the eluted mixture from the HIC column, which was also the CEX load, was analysed using SEC.

GigaCap CM using NaCl for elution showed a very poor resolution upon injection of 10 mL sample volume. Application of a step gradient did not further improve resolution (see Fig. 3, top). Annathur et al. [38] applied arginine chloride for elution and thereby enhanced CEX resolution. The use of amino acids to increase resolution and recovery was also investigated by the group of Arakawa [39].

A small scale test with a linear arginine chloride gradient was applied on GigaCap S-650M and CM-650M. The elution profiles were similar to those observed using NaCl for elution (data not shown). Again GigaCap S provided two peaks for mono- and di-PEGylated scFv with non-PEGylated scFv eluting between them.

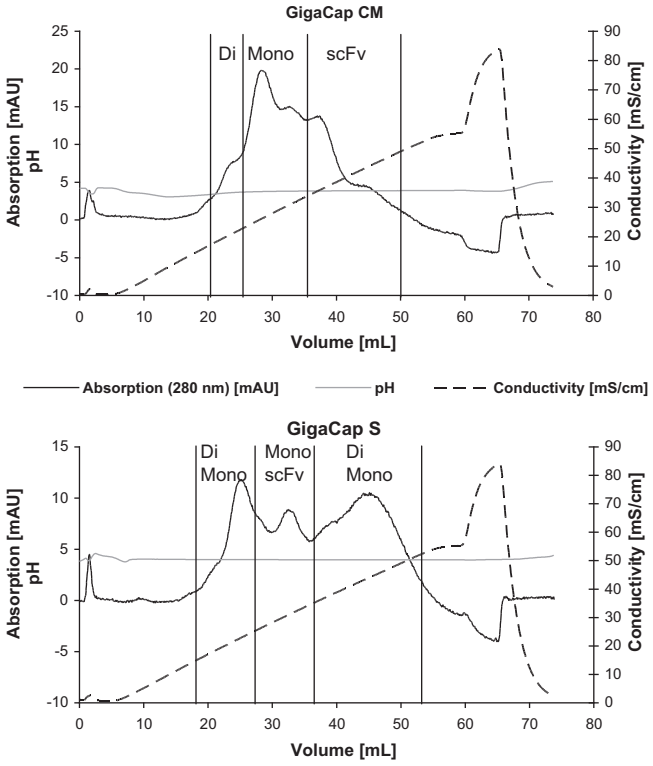

Fig. 2. Purification of 5 kDa PEGylation mixture on GigaCap CM-650M (top) and GigaCap S-650M (bottom) using 1 M NaCl as eluent in a linear gradient. Black line: absorption at 280 nm, grey line: pH, dashed line: conductivity. Black bars denote eluted species identified by SDS-PAGE. Di: di-PEGylated-5 kDa-scFv, mono: PEGylated-5 kDa-scFv, scFv: non-PEGylated scFv.

After step gradient optimisation, the use of arginine chloride increased resolution and reduced the number of peaks and shoulders on Toyopearl GigaCap CM, even for the application of a large sample volume (see Fig. 3, bottom). Using NaCl elution several partly separated peaks were visible. Hence, GigaCap CM with arginine chloride for elution was used for further purification steps. The poly-PEGylated scFv eluted first. Afterwards two mono-PEGylated-scFv peaks (mono-PEG-5-1-scFv and mono-PEG-5-2-scFv (see Fig. 3, bottom)) were found and confirmed as being mono-PEGylated scFv by SDS-PAGE (data not shown).

To further investigate these two peaks, SEC was carried out. In case of mono-PEG-5-1-scFv the SEC showed a di-PEG-5-scFv content of about 15% and in case of mono-PEG-5-2-scFv most of the purified protein had a retention volume close to di-PEG-5-scFv. However, di-PEG-5-scFv was not detectable in mono-PEG-2-scFv and only traces were detectable in mono-PEG-1-scFv using SDS-PAGE (data not shown).

The N-terminal Edman degradation of mono-PEG-5-1-scFv and mono-PEG-5-2-scFv, done by TopLab in Munich, resulted in identification of about 85–90% N-terminally PEGylated protein for both mono-PEGylated peaks. Another CEX preparation with a steeper gradient led to the elution of one mono-5-PEGylated peak. This peak was further investigated using the Viscotek GPCmax with multidetector array, which offered the possibility to investigate the molecular size as well as the protein content of a peak (see Fig. 4).

GPCmax separated two peaks with molecular masses of 35 and 70 kDa, calculated directly with the light scattering detector, matching perfectly the size of a monomer and a dimerised mono-PEG-scFv. Via UV absorption and concentration calculation done with the RI detector, a weight fraction of the protein of 0.8 for both peaks was calculated. This corresponds to a protein content of 80% and a PEG content of 20%. Taking into account the molecular mass of 35 kDa, the second peak corresponded to a PEG of 7 kDa and a protein of 28 kDa. The first peak with 70 kDa molecular mass consisted of a PEG of 14 kDa and a protein of 56 kDa corresponding exactly to dimerised mono-PEG-5-scFv.

SDS-PAGE was again used to reveal the whereabouts of free PEG (data not shown). PEG did not bind to the cation exchanger. A product-recovery of 80% for the HIC step could be calculated, but the cation exchanger product-recovery rate was only about 30%.

The initial aim for using HIC as first step was the resulting salt free product after the CEX step. On the other hand putting HIC second results in smaller loading volume and reduced amounts of salt to add for reaching loading conditions. The low product-recovery thus led to the reversion of the purification steps starting with CEX (see Fig. 5).

Putting CEX first and HIC second led to an overall product-recovery of 66% instead of 20% obtained with the original sequence. CEX as first step led to a purity of about 90% and a product-recovery of 94%. The second step, HIC, showed a product-recovery of 72%. However, impurities of di-PEGylated or non-PEGylated scFv were not visible in SDS-PAGE.

In case of the PEGylation with 30 kDa PEG purification with HIC as first step could not be established. The 30 kDa PEGylation mixture formed an aqueous two-phase system after adding the salt concentration necessary for HIC load. Unfortunately the aqueous two phase system did not fit into the purification scheme but rather decreased the protein concentration. Therefore the purification protocol for 30 kDa PEGylation was reversed as seen for 5 kDa PEGylation. CEX was used at first followed by HIC. Thus, the separation of two mono-30-PEG peaks was ruled out.

The free PEG was lost completely during CEX load as indicated by SDS-PAGE (data not shown). The elution order resembled the 5 kDa PEGylation mix. PEG did not bind, poly-PEGylated scFv eluted next and mono-PEGylated scFv was the last to be eluted (Fig. 6, left side). The mono-PEGylated scFv was 1:1.5 diluted with HIC buffer and directly applied onto the HIC column (see Fig. 6, right side). scFv eluted first, followed by mono-PEG-30-scFv.

The product-recovery rate of 30 kDa mono-PEGylated scFv for the CEX step was 94% and for the HIC step 67%. The overall purity of the 30 kDa mono-PEGylated scFv was about 98% after the two step purification.

3.3. Biological activity

The determination of biological activity is based on the displacement of ^{125}iodine labelled EGF with unlabelled protein. The corresponding IC_{50} was calculated using a curve fit.

The IC_{50} of scFv was less than the one calculated for EGF (5 and 16 nM respectively). A 5 kDa PEGylation did not change the IC_{50} significantly (5.3 nM). The addition of a 30 kDa PEG did increase the IC_{50} value to 7.6 nM. No significant changes upon addition of a 5 kDa PEG were visible. The addition of a 30 kDa PEG led to a flattening of the displacement curve (see Fig. 7).

4. Discussion

4.1. Production and PEGylation of scFv

The 30 kDa PEGylation was more effective regarding the absolute amount of PEGylated scFv, however, the amount of mono-PEGylated scFv (60%) was identical for both PEG sizes.

A massive increase of hydrodynamic radius was observed upon PEGylation, which is a common observation already made by several groups [16,19,28,40].

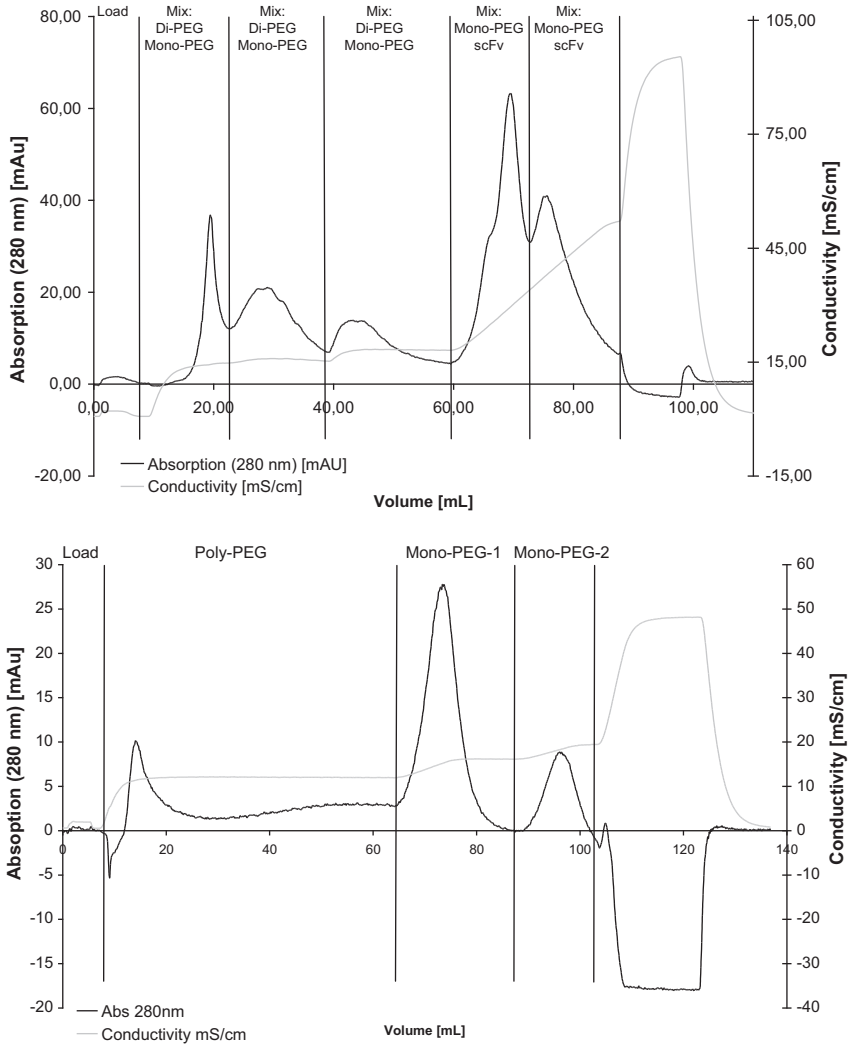

Fig. 3. Purification of mono-PEG-5-scFv pool eluted from HIC on GigaCap CM-650M using NaCl (top) and arginine-HCl (bottom) as eluent. Black line: absorption at 280 nm, grey line: conductivity. Black bars denote eluted species identified by SDS-PAGE.

4.2. Preparative purification

Hydrophobic interaction chromatography with a mixture of NaCl and $(NH_4)_2SO_4$ was used as first purification step for the 5 kDa PEGylation. A high purity after one purification step was achieved, scFv was mostly removed.

In a second purification step a weak cation exchanger was applied. Cation exchange is a common chromatography mode for the purification of PEGylated proteins and was already used by Seely and Richey and other working groups [14,22,41]. Most CEX processes use strong cation exchangers. The use of the uncommon weak cation exchanger was necessary here, because the resolution was much better than using the strong cation exchanger. Elution with NaCl did not work for large sample volumes whereas the use of arginine chloride increased the resolution dramatically, which was already investigated by the groups of Annathur [38] and Arakawa [39]. Arakawa et al. [39] justified the positive influence of arginine on its ability to suppress aggregation and interaction with the column. The group of Trout investigated the influence of arginine-HCl on protein stability and aggregation [42–44] and found arginine-HCl being an association suppressor. The assumption of an indifferent formation of dimers and polymers of scFv and PEGylated scFv arose as possible explanation for arginine chloride resolution enhancement.

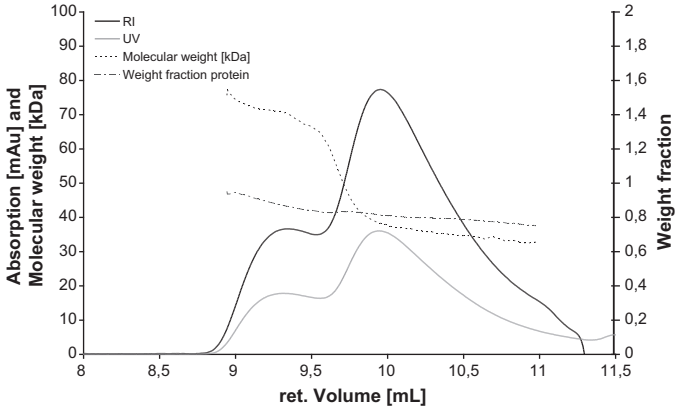

Fig. 4. Mono-5-PEG eluted as single peak in CEX, analysed with RI (black line) and UV (grey line). The molecular weight (dotted line) was calculated by Viscotek GPCmax using information from light scattering detector. The protein weight fraction (dash and dot line) was calculated by GPCmax using the quotient of RI and UV absorption under the presumption of PEG being UV inactive. Left peak: 35 kDa, protein weight fraction 0.8, right peak: 70 kDa, protein weight fraction 0.8.

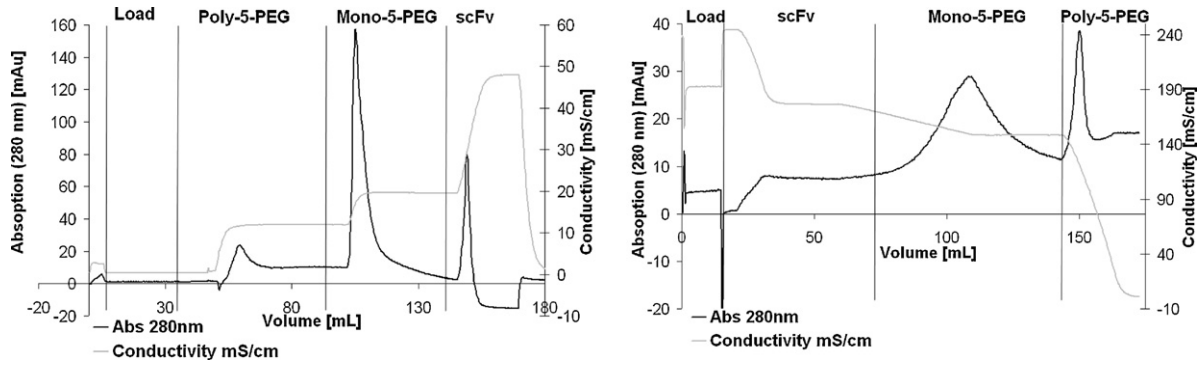

Fig. 5. Two step purification of 5 kDa PEGylated scFv from PEGylation mixture on GigaCap CM-650M using arginine-HCl as eluent (left, first step) and PPG-600M (right, second step). In the first step PEGylated protein eluted prior to scFv, in the second step scFv eluted at first. Black line: absorption at 280 nm, grey line: conductivity. Black bars denominate eluted species identified by SDS-PAGE.

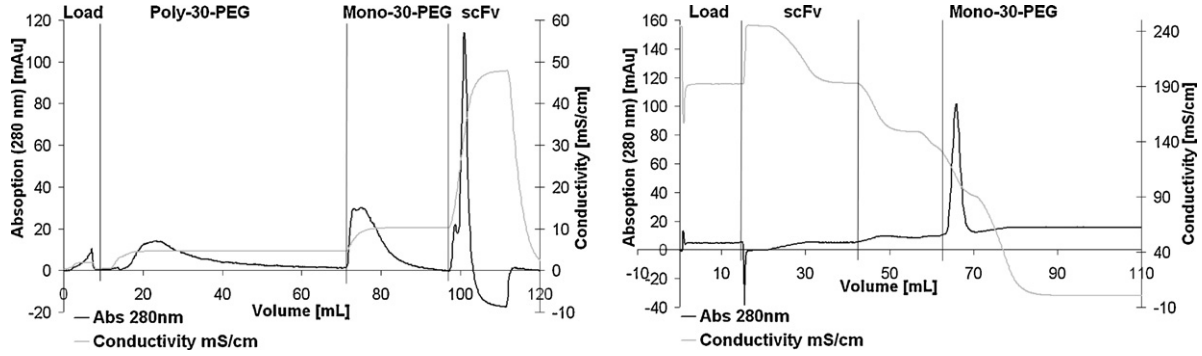

Fig. 6. Two step purification of 30 kDa PEGylated scFv. First step on GigaCap CM-650M using arginine-HCl as eluent (left figure). Second step: HIC on PPG-600M (right figure). In the first step PEGylated proteins eluted prior to scFv, in the second step scFv eluted at first. Black line: absorption at 280 nm, grey line: conductivity. Black bars denominate eluted species identified by SDS-PAGE.

The higher the protein fraction of the PEGylated protein in CEX, the more salt was needed for elution. PEG itself did not even bind to the column. This result is in line with previous findings of Pabst et al. [45]. Seely and Richey [13,14] explained this behaviour via a masking effect of uncharged PEG on charged proteins. The masking effect leads to a reduction of surface charges, and is stronger than the charge reduction gained by the removal of lysine during PEGylation reaction. Therefore a reduction of binding activity to the charged cation exchanger matrix was observed.

Interestingly two mono-PEG peaks were found in CEX. Because of the random PEGylation an isoform formation was supposed first, as already seen for lysozyme [28,46] and for α-interferon [20]. However, other explanations are possible, for example a dimer formation of mono-PEGylated-scFv. Combining the SEC and SDS-PAGE results, it seemed to be likely that a dimer formation was investigated here. This was confirmed using Edman digestion. An analysis using a multidetector GPC system confirmed the assumptions showing two peaks with different molecular sizes but identical protein weight fractions. This directly proved that both peaks consisted of mono-PEGylated-scFv. A steeper gradient led to a single mono-PEGylated peak with a purity of about 90–95%.

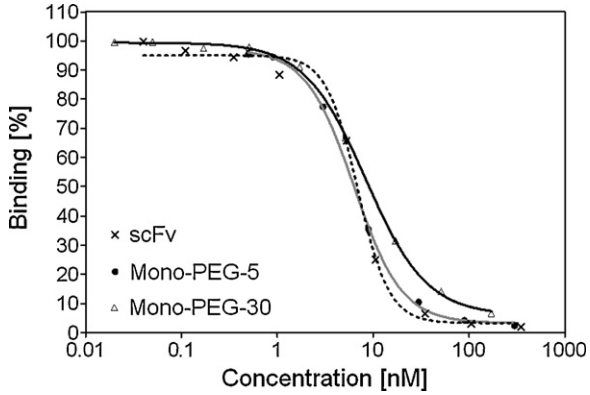

Fig. 7. ^{125}Iodine based activity determination of scFv (dotted line, crosses), mono-PEG-5-scFv (grey line, dots) and mono-PEG-30-scFv (black line, triangles).

The unsatisfying overall product-recovery of the two step purification with just about 20% led to a repetition of the purification. By reversing the purification steps the overall product-recovery was improved to 66%. Even the use of single-step purification can be considered, as the first CEX step led to a purity of 90% with a product-recovery of 94%. The HIC step reduced product-recovery but massively increased purity to a virtually non-detectable amount of poly- or non-PEGylated protein.

The purification of the 30 kDa PEGylation mix had to be done with CEX as first step. The elution order was identical to 5 kDa PEGylation, but the conductivity needed for elution was lower. That was already expected as longer PEG chains lead to lower salt concentrations for elution [13,14,45]. The first CEX step led to a purity of about 95% and a product-recovery of 95%. HIC used in a second step enhanced purity to about 98% but showed a product-recovery below 70%.

The already very high purity after the first CEX step thus implies that single-step purification could be sufficient.

4.3. Biological activity

Significant changes in binding activities were not discovered upon PEGylation with a 5 kDa PEG. Furthermore, it could be

demonstrated that the addition of PEG up to a size of about 30 kDa did not significantly affect binding activity. Consequently, the PEGylation up to a size of about 30 kDa seemed to be uncritical for the biological function of the scFv. Nevertheless, these *in vitro* experiments should be verified by *in vivo* experiments.

5. Conclusions

The PEGylation of an anti-EGFR scFv at a pH of 4.0 resulted in 85% N-terminal PEGylation.

The use of arginine-HCl as aggregation suppressor enabled a very good separation on a weak cation exchanger without changing the elution order found for NaCl. The unconventional elution order on a strong cation exchanger should be investigated in further studies.

A two step purification employing a HIC and a CEX step resulted in the separation of two mono-PEGylated peaks. Both consisted of mono-PEGylated scFv, no apparent difference in biological activity was detected. The peaks seemed to represent mono-PEGylated-scFv and dimerised mono-PEGylated-scFv whereas different mono-PEGylated isoforms could not be found.

To increase product-recovery, the purification protocol was repeated in reversed order. This led to a product-recovery of 66% and purity beyond 98%. A single-step purification using the weak cation exchanger was possible, resulting in a product-recovery around 95% and a purity of about 90%.

In case of 30 kDa PEGylation HIC could not be used as first step because of the formation of an aqueous two-phase system. Therefore, CEX was used as first step. The overall product-recovery was about 50% and the purity reached 98%. Again a single-step purification using CEX led to a higher product-recovery of 95% and a purity of 95%. Therefore, the single-step purification could be used for both PEG sizes.

An *in vitro* assay to determine the biological activity revealed no significant changes in biological activity by adding one 5 kDa PEG. The addition of a 30 kDa PEG induced a slight decline in binding activity.

Acknowledgements

We thank the Bundesministerium fuer Bildung und Forschung (BMBF), Germany, for supporting this work and Dr. Angelika Hausser for proof-reading.

References

[1] R. Roskoski, Biochem. Biophys. Res. Commun. 319 (2004) 1.
[2] B. Vincenzi, G. Schiavon, M. Silletta, D. Santini, G. Tonini, Crit. Rev. Oncol. Hematol. 68 (2008) 93.
[3] S.-F. Wong, Clin. Ther. 27 (2005) 684.
[4] J. Baselga, Eur. J. Cancer (Oxford, England: 1990) 37 (Suppl. 4) (2001) S16.
[5] S. Li, K.R. Schmitz, P.D. Jeffrey, J.J.W. Wiltzius, P. Kussie, K.M. Ferguson, Cancer Cell 7 (2005) 301.
[6] P.S. Chowdhury, H. Wu, Methods 36 (2005) 11.
[7] N.E. Weisser, J.C. Hall, Biotechnol. Adv. 27 (2009) 502.
[8] F.M. Veronese, Biomaterials 22 (2001) 405.
[9] G. Molineux, Cancer Treat. Rev. 28 (2002) 13.
[10] C. Fee, J. Van Alstine, Chem. Eng. Sci. 61 (2006) 924.
[11] A. Abuchowski, J.R. McCoy, N.C. Palczuk, T. van Es, F.F. Davis, J. Biol. Chem. 252 (1977) 3582.
[12] A. Abuchowski, T. van Es, N.C. Palczuk, F.F. Davis, J. Biol. Chem. 252 (1977) 3578.
[13] J.E. Seely, C.W. Richey, J. Chromatogr. A 908 (2001) 235.
[14] J.E. Seely, S.D. Buckel, P.D. Green, C.W. Richey, BioPharm Int. (2005) 1.
[15] M. Azarkan, M. Nijs, N. Smolders, J. Guermant, J. Vlncentelli, Y. Looze, Appl. Biochem. Biotechnol. 60 (1996) 167.
[16] C. Delgado, R.B. Pedley, A. Herraez, R. Boden, J. Boden, P. Keep, K. Chester, D. Fisher, R.H. Begent, G.E. Francis, Br. J. Cancer 73 (1996) 175.
[17] K. Kitamura, T. Takahashi, T. Yamaguchi, A. Noguchi, A. Noguchi, K.-i. Takashina, H. Tsurumi, M. Inagake, T. Toyokuni, S.-i. Hakomori, Cancer Res. 51 (1991) 4310.
[18] S. Kubetzko, E. Balic, R. Waibel, U. Zangemeister-Wittke, A. Plückthun, J. Biol. Chem. 281 (2006) 35186.
[19] R. Clark, K. Olson, G. Fuh, M. Marian, D. Mortensen, G. Teshima, S. Chang, H. Chu, V. Mukku, E. Canova-Davis, T. Somers, M. Cronin, M. Winkler, J.A. Wells, J. Biol. Chem. 271 (1996) 21969.
[20] Y.-S. Wang, S. Youngster, M. Grace, J. Bausch, R. Bordens, D.F. Wyss, Adv. Drug Deliv. Rev. 54 (2002) 547.
[21] E. Müller, D. Josic, T. Schröder, A. Moosmann, J. Chromatogr. A 1217 (2010) 4696.
[22] S. Caserman, M. Kusterle, M. Kunstelj, T. Milunović, M. Schiefermeier, S. Jevsevar, V.G. Porekar, Anal. Biochem. 389 (2009) 27.
[23] T. Stumpp, B. Wilms, J. Altenbuchner, Biospektrum 1 (2000) 33.
[24] U.T. Bornscheuer, J. Altenbuchner, H.H. Meyer, Bioorg. Med. Chem. 7 (1999) 2169.
[25] A.S. Rathore, R.E. Bilbrey, D.E. Steinmeyer, Biotechnol. Prog. 19 (2003) 1541.
[26] K.R. Clark, S.T.R. Walsh, Protein Sci. 18 (2009) 2429.
[27] A. Moosmann, J. Blath, R. Lindner, E. Müller, H. Böttinger, Bioconjugate Chem. 22 (2011) 1545.
[28] A. Moosmann, J. Christel, H. Boettinger, E. Mueller, J. Chromatogr. A 1217 (2010) 209.
[29] U.K. Laemmli, Nature 227 (1970) 680.
[30] M.M. Kurfürst, Anal. Biochem. 200 (1992) 244.
[31] P. Bailon, A. Palleroni, C. Schaffer, C.L. Spence, W.J. Fung, J.E. Porter, G.K. Ehrlich, W. Pan, Z.X. Xu, M.W. Modi, A. Farid, W. Berthold, M. Graves, Bioconjugate Chem. 12 (2001) 195.
[32] N. Li, D. Ziegemeier, L. Bass, W. Wang, J. Pharm. Biomed. Anal. 48 (2008) 1332.
[33] P. Edman, Acta Chem. Scand. 4 (1950) 283.
[34] F.C. Greenwood, W.M. Hunter, J.S. Glover, Biochem. J. 89 (1963) 114.
[35] T. Fischer, K. Wiegmann, H. Böttinger, K. Morens, G. Burmester, K. Pfizenmaier, J. Immunol. 145 (1990) 2914.
[36] H. Böttinger, L. Béress, E. Habermann, Biochim. Biophys. Acta 861 (1986) 165.
[37] Y.C. Cheng, W.H. Prusoff, Biochem. Pharmacol. 22 (1973) 3099.
[38] G.V. Annathur, J.J. Buckley, K. Muthurania, N. Ramasubramanyan, J. Chromatogr. A 1217 (2010) 3783.
[39] T. Arakawa, K. Tsumoto, Y. Kita, B. Chang, D. Ejima, Amino Acids 33 (2007) 587.
[40] P. Yu, G. Zhang, J. Bi, X. Lu, Y. Wang, Z. Su, Process Biochem. 42 (2007) 971.
[41] S. Yamamoto, S. Fujii, N. Yoshimoto, P. Akbarzadehlaleh, J. Biotechnol. 132 (2007) 196.
[42] N. Chennamsetty, B. Helk, V. Voynov, V. Kayser, B.L. Trout, J. Mol. Biol. 391 (2009) 404.
[43] D. Shukla, C.P. Schneider, B.L. Trout, Adv. Drug Deliv. Rev. 63 (2011) 1074.
[44] B.M. Baynes, B.L. Trout, Biophys. J. 87 (2004) 1631.
[45] T.M. Pabst, J.J. Buckley, N. Ramasubramanyan, A.K. Hunter, J. Chromatogr. A 1147 (2007) 172.
[46] H. Lee, T.G. Park, J. Pharm. Sci. 92 (2003) 97.