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Analytical and preparative separation of PEGylated lysozyme for the characterization of chromatography media

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ABSTRACT

The effect of PEGylation on cation exchange chromatography was studied with poly(ethylene glycol) of different chain lengths (5 kDa, 10 kDa and 30 kDa) using lysozyme as a model system. A stable binding via reduction of a Schiff base was formed during random PEGylation on lysine residues with methoxy-PEG-aldehyde. A purification method for PEGylated proteins using cation exchange chromatography was developed, and different isoforms of mono-PEGylated lysozyme were isolated. TSKgel SP-5PW and Toyopearl GigaCap S-650M showed the best performance of all tested cation exchange resins, and the separation of PEGylated lysozyme could be also scaled up to semi-preparative level. Size-exclusion chromatography, SDS-PAGE and MALDI-TOF mass spectrometry were used for analysis. Separated mono-PEGylated lysozyme of different sizes was used to determine dynamic binding capacities (DBC) and selectivity of cation exchange chromatography resins. An optimization of binding conditions resulted in a more than 20-fold increase of DBC for Toyopearl GigaCap S-650M with 30 kDa mono-PEGylated lysozyme.

1. Introduction

Chemical modification of therapeutic proteins in order to enhance their biological activity is of increasing interest. One of the most frequently used methods for protein modification is the covalent attachment of poly(ethylene glycol), which is also called PEGylation. Over the past years many PEGylated biopharmaceuticals were brought to the market, such as PEGasys® (Hoffman-La Roche) and PEG Intron® (Schering-Plough/Enzon) [1,2] which both contain α-interferon for hepatitis C treatment.

Since the first steps in PEGylation of proteins were made in the 1970s by Abuchowski [3,4], many problems were solved. PEGylation often influences enzymatic activity, receptor binding and antigen recognition of a protein. Conserving the biological function or even the improvement of its therapeutic activity is possible by now [5–7].

The aim of PEGylation is a prolonged in situ half-life which is caused by a masking effect of the PEG. This kind of chemical modification reduces protein immunogenicity, its sensitivity to proteolytic degradation, and the size of the protein molecule is increased, altogether resulting in a reduced renal filtration of the modified therapeutics [3,5,6,8]. Other physicochemical properties such as bio-distribution, thermal stability and solubility may be influenced positively, too. This leads to new possibilities of drug administration [9] but also influences the behaviour of PEGylated proteins during chromatographic separation [10,11] and therefore the whole purification process [1].

After PEGylation, the reaction mixture has to be purified in order to remove non-reacted protein and undesired reaction products. Chromatography is the most common purification technique. All chromatographic modes, such as ion-exchange chromatography (IEX) and hydrophobic interaction chromatography (HIC) depend basically on interactions between a stationary phase and sample components. These interactions are highly influenced by the physicochemical properties of the sample molecule such as charge or hydrophobicity, which may be changed by PEGylation because of masking and shielding effects. As a result all chromatographic modes used in down-stream processing show altered behaviour of the PEGylated proteins in comparison to non-modified ones [1,12]. An influence on chromatographic behaviour of proteins by PEG chains could be shown in size-exclusion chromatography (SEC), IEX and also in reversed-phase chromatography (RPC) [1,12].

This paper concentrates on the altered behaviour of PEGylated proteins in cation exchange chromatography [13,30]. Lysozyme, a standard protein which is well defined, good characterized and widely used in chromatography, was chosen as model protein.

The decision prior to the purification is not only which chromatographic mode will be used, but also the selection of a

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stationary phase. This requires several tests [14]. In a first step mono-PEGylated lysozyme was produced as a model protein in a reasonable amount for testing cation exchange chromatography bulk materials. The separation of different PEGylated lysozymes is shown, different cation exchange resins were tested and also the influence of the PEG chain length used for PEGylation was investigated [15].

The PEGylation reaction used in these investigations took place between the aldehyde group of methoxy-PEG-aldehyde and the free amino acid group (NH_2-group) of lysine residues within lysozyme. A Schiff base with a reactive double bound was formed during that reaction. Sodium cyanoborohydride was added to the reaction buffer [5] for covalent attachment.

The reaction was a random PEGylation, which leads not only to mono-PEGylated lysozyme. Also poly-PEGylated lysozyme was formed [6,15]. The different PEGylated lysozymes were subsequently analyzed by SEC, SDS-PAGE, and MALDI-TOF mass spectrometry. It could be shown that cation exchange chromatography is the method of choice for analytical and preparative scale separation of PEGylated lysozyme. In a second step different cation exchange resins were examined. Both, dynamic binding capacity and selectivity provided promising results for industrial implementation and further scientific studies.

2. Materials and methods

2.1. Chemicals

Methoxy-PEG-aldehyde with an average molecular weight of 5 kDa, 10 kDa and 30 kDa was purchased from NOF Corp. (Grobbendonk, Belgium). Lysozyme (98% pure, chicken egg white) was provided by Sigma (St. Louis, USA). Standard proteins for calibration of the SEC-column were purchased from BioRad (Munich, Germany). Standard polymers for ISEC (inverse size-exclusion chromatography) were provided by PSS (Mainz, Germany). All other chemicals were provided by Merck (Darmstadt, Germany).

2.2. PEGylation of lysozyme

5 g/l lysozyme and 4 g/l 5 kDa PEG, 8 g/l 10 kDa PEG or 24 g/l 30 kDa PEG, respectively were dissolved in a 25 mM sodium phosphate buffer pH 6.0, containing 20 mM $NaCNBH_3$. The reaction took place at 15 °C for 16 h. The reaction was stopped by separating the reaction mix on a chromatographic column.

2.3. Analytical procedure

The PEGylation reaction was tracked using SEC on an analytical TSKgel G3000SW_{XL} column (7.8 mm × 30 cm, Tosoh Bioscience GmbH, Stuttgart, Germany). As mobile phase a 100 mM sodium phosphate buffer, pH 6.7, containing 100 mM Na_2SO_4 and 0.05% NaN_3 was used. The SEC chromatography was performed on a Shimadzu HPLC System (Shimadzu, Duisburg, Germany).

An analytical TSKgel SP-5PW column (Omnifit, 6.6 mm × 50 mm, Resin: Tosoh Bioscience GmbH, Stuttgart, Germany) was also used to track the PEGylation reaction. Buffer A consisted of 25 mM sodium phosphate buffer, pH 6.0. For elution 0.5 M NaCl were added. The analytical IEX was carried out on a Dionex UltiMate® 3000 HPLC System (Dionex Corporation, Sunnyvale, USA).

MALDI-TOF MS analysis was used to determine the resulting products of the PEGylation reaction, separated by cation exchange chromatography. For sample preparation 1 μl of undiluted sample was mixed with 1 μl of a matrix (containing 10 mg/ml sinapic acid in 50% (V/V) acetonitrile and 0.1% TFA) and was applied and air dried on the plate. The measurement was performed with MALDI-TOF mass spectrometer AXIMA-CFR (Kratos Analytical, Manchester, UK) in a linear modus (lambda = 337 nm, pulse width 3 ns, pulse rate 5 Hz). The measured spectra are results of 1000 profiles with five laser shots (laser energy ~20 μJ). The MALDI-TOF experiments were performed at the Friedrich-Alexander University Erlangen-Nurnberg, Germany.

SDS-PAGE under reducing conditions was performed according to Laemmli [32]. Protein samples were solubilised in sample buffer and heated at 95 °C for 5 min. SDS-PAGE was performed with precast 4–15% Tris–HCl gels (8 cm × 10 cm, BioRad, München, Germany) in a Mini Protean 3 cell (BioRad) according to the manufacturer's procedure. The gels were stained with Silver Staining Kit for SDS-PAGE (SERVA, Heidelberg, Germany) according to the manufacturers' instruction.

2.4. Preparative purification

An Äkta Explorer System (GE Healthcare, Uppsala, Sweden) was used for all preparative purifications. The experiments were continuously monitored using the Äkta UV unit at 280 nm. Buffer A consisted of 25 mM sodium phosphate buffer, pH 6.0. Elution buffer B was prepared by adding 1 M of NaCl to buffer A. Omnifit glass columns (25 mm × 400 mm, Bio-Chem Fluidics, Cambridge, UK) were used for first tests. The cation exchange resins in test were Toyopearl SP-650S, Toyopearl CM-650S, Toyopearl GigaCap S-650M and TSKgel SP-5PW (all Tosoh Bioscience GmbH, Stuttgart, Germany).

For purification of 5 kDa PEGylated lysozyme TSKgel SP-5PW was used. In case of 10 kDa and 30 kDa PEGylated lysozyme Toyopearl GigaCap S-650M was used.

A gradient elution to buffer B was used for separation of non-reacted protein and differently PEGylated lysozyme variants. The sample amount was 150 ml. Desalting and concentration of the purified fractions was performed using a Vivaflow 50 membrane filter (Sartorius Stedim Biotech S.A., Aubagne, France). Finally the fractions were lyophilized for long-term storage with a Christ Alpha 1-2 freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany).

2.5. Selectivity comparison

The selectivity comparison was carried out on a Dionex UltiMate® 3000 HPLC System (Dionex Corporation, Sunnyvale, USA). Omnifit glass columns (6.6 mm × 50 mm) with the resins Toyopearl SP-650M, Toyopearl GigaCap S-650M and TSKgel SP-5PW were tested. Also a TSKgel SP-NPR column (4.6 mm × 35 mm) (all Tosoh Bioscience GmbH, Stuttgart, Germany) was tested.

For selectivity comparison buffer A and elution buffer B were used throughout.

2.6. Breakthrough experiments and ISEC

Breakthrough experiments were carried out on the Äkta Explorer system (GE Healthcare, Uppsala, Sweden) with Omnifit glass columns. The experiment was monitored via the UV unit of the Äkta Explorer system at 280 nm. For first tests buffer A was used with the resins Toyopearl SP-650M, Toyopearl GigaCap S-650M, TSKgel SP-5PW, Toyopearl SP-550C and Toyopearl SP-550EC. Two resins, Toyopearl SP-650M and Toyopearl GigaCap S-650M were used for optimization. In an optimization procedure the buffer was varied between 5 and 100 mM sodium phosphate and pH-values of 6.0, 7.0 and 8.0. Simultaneously a sodium acetate buffer, varying between 5 mM and 100 mM, pH 4.0 and 5.0, was tested. Dynamic binding capacity was measured at 10% of the maximum $UV_{280\,nm}$ absorbance signal. A flow rate of 150 cm/h was used and an analyte concentration of 1 g/l.

Table 1
Chromatographic ion-exchange media and characteristics.

Chromatographic media	Particle size [μm]	Pore size [Å]	Application
TSKgel SP-NPR	2.5	0	Analytics
TSKgel SP-5PW	20	1000	Polishing
Toyopearl SP-650S	35	1000	Intermediate purification
Toyopearl SP-650M	65	1000	Intermediate purification
Toyopearl CM-650S	35	1000	Intermediate purification
Toyopearl SP-550C	100	500	High throughput capture
Toyopearl SP-550EC	200	500	High throughput capture
Toyopearl GigaCap S-650M	75	1000	High throughput capture

Neutral PEG-Polymers (PSS, Mainz, Germany) of different chain lengths (0.4–511 kDa,) were used for ISEC. The experiments were monitored via the RI unit of the GPC system HLC-8220GPC (Tosoh Bioscience GmbH, Stuttgart, Germany).

2.7. Chromatographic media for IEX

A survey of the investigated IEX resins with selected properties and their suggested applications are shown in Table 1.

2.8. Instrumentation

The Dionex UltiMate® 3000 HPLC system (Dionex Corporation, Sunnyvale, USA) consisted of a pump module P580 Pump, an UltiMate® 3000 Autosampler, UVD170S UV-detector module and a Degasys DG-1210 degasser.

The Shimadzu HPLC system (Shimadzu, Duisburg, Germany) contains a DGU-20A3 degasser, a SIL-20AC prominence auto sampler, two pumps (LC-20AD liquid chromatograph), a CTO-20AC column oven, one CBM-20A communication bus module, a RID-10A refraction index detector and one SPD-M20A diode array detector, and a FRC-10A fraction collector.

The ÄKTA Explore FPLC system (GE Healthcare, Uppsala, Sweden) is composed of a pH/C-900 pH-meter module, a UV-900 UV-detector module, one P-900 pump module and a FRAC-950 fraction collector.

A GPC system HLC-8220GPC (Tosoh Bioscience GmbH, Stuttgart, Germany) is equipped with UV and RI detector unit and a pump module.

3. Results

3.1. PEGylation of lysozyme

Fig. 1 shows typical chromatographic profiles of a reaction mix of PEGylated lysozyme separated on TSKgel SP-5PW. PEG chain lengths of 5 kDa, and 30 kDa are shown from left to right. The profiles indicate a similar reaction characteristic. Non-reacted

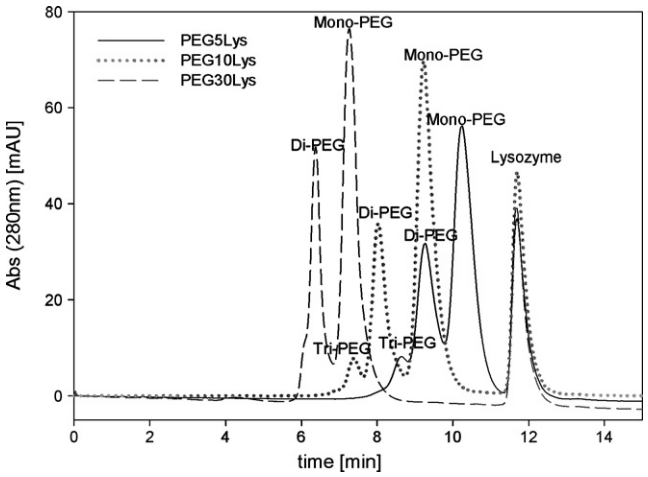

Fig. 2. SEC analysis of reaction mixes performed with a G3000SW$_{XL}$ column. Lysozyme and PEGylated lysozyme derivates for all tested sizes are shown. Continuous line: PEG5Lys, dotted line: PEG10Lys, dashed line: PEG30Lys. For chromatographic conditions see Section 2.

lysozyme remained in the reaction mixture, mono-PEGylated lysozyme as well as poly-PEGylated lysozyme were formed during the reaction [5,15].

Fractions of the elution peaks shown in Fig. 1 were collected and sent to the Friedrich-Alexander University to perform the MALDI-TOF analysis [5]. The peaks were identified as labelled in Fig. 1.

SEC was performed as shown in Fig. 2. By the use of retention volumes from SEC analysis, the viscosity radius of PEGylated lysozyme was determined under the assumption of being a globular protein.

The formula from Fee and Van Alstine [1] provided the possibility to use the molecular weight predicted from the SEC retention volume, using the molecular size rather than the molecular weight for the calibration curve. Using Eq. (3) the theoretical viscosity radius of PEGylated proteins was predicted as shown in Table 2

$$R_{\mathrm{h,prot}} \approx (0.82 \pm 0.02)\sqrt[3]{M_{\mathrm{Prot}}} \tag{1}$$

$$R_{\mathrm{h,PEG}} = 0.1912 M_{\mathrm{PEG}}^{0.559} \tag{2}$$

$$R_{\mathrm{h,PEGprot}} = \frac{1}{6}\left[108R_{\mathrm{h,prot}}^3 + 8R_{\mathrm{h,PEG}}^3 + 12(81R_{\mathrm{h,prot}}^6 + 12R_{\mathrm{h,prot}}^3 R_{\mathrm{h,PEG}}^3)^{1/2}\right]^{1/3} + \frac{2}{3}\left\{\frac{R_{\mathrm{h,PEG}}^2}{[108R_{\mathrm{h,prot}}^3 + 8R_{\mathrm{h,PEG}}^3 + 12(81R_{\mathrm{h,prot}}^3 + 12R_{\mathrm{h,prot}}^3 R_{\mathrm{h,PEG}}^3)^{1/2}]^{1/3}}\right\} + \frac{1}{3}R_{\mathrm{h,PEG}} \tag{3}$$

According to the SEC behaviour the mono-PEG5Lys was equivalent to a 50 kDa globular protein, the mono-PEG10Lys to a 110 kDa

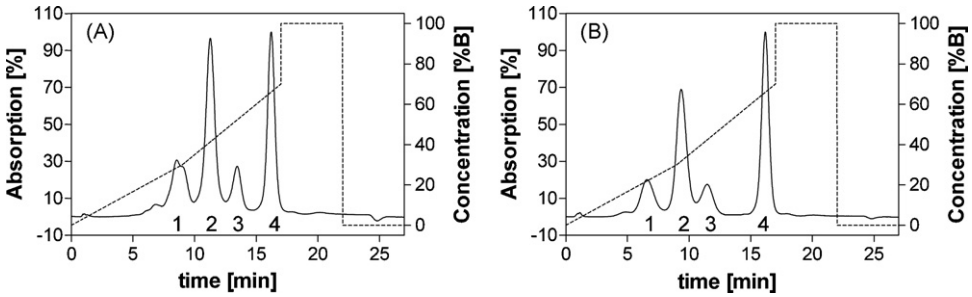

Fig. 1. Separation of PEGylated lysozymes on an analytical TSKgel SP-5PW column. Gradient elution to 0.5 M NaCl in buffer A. Continuouse line: absorption, dashed line: elution profile. Lysozyme PEGylated with (A) 5 kDa PEG, (B) 30 kDa PEG. Peak contents were identified via MALDI-TOF analysis, identical sizes were numbered consecutively. (1) Di-PEGylated lysozyme, (2) 1-mono-PEGylated lysozyme, (3) 3-mono-PEGylated lysozyme, and (4) lysozyme.

Table 2
Retention volume from SEC and therewith calculated molecular weight and viscosity radius of all tested proteins. Theoretical molecular weight is calculated by addition of the molecular weights of lysozyme and PEG.

Species	V_R (measured) from SEC [ml]	M_W calculated from V_R [kDa]	Theoretical M_W [kDa]	$R_{h,PEGprot}$ predicted with Eq. (3) [Å]
Lysozyme	10.5	18	14.7	19.9
Mono-PEG5Lys	9.2	50	19.7	30.7
Poly-PEG5Lys	8.3	100	24.7	38.3
Mono-PEG10Lys	8.2	110	24.7	38.3
Poly-PEG10Lys	7.1	270	34.7	51.5
Mono-PEG30Lys	6.4	450	44.7	62.8
Poly-PEG30Lys	5.7	780	74.7	90.6

globular protein and the mono-PEG30Lys to a 450 kDa globular protein [16]. A linear correlation between the molecular weight determined via SEC and the theoretical molecular weight of the PEGylated proteins was found ($R^2 = 0.99$) [13].

3.2. Preparative purification

For preparative purification, three different cation exchange resins were tested with a reaction mixture of PEG5Lys. Toyopearl CM-650S as a weak cation exchanger, Toyopearl SP-650M and Toyopearl GigaCap S-650M as strong cation exchangers and TSKgel SP-5PW as a strong cation exchanger with smaller particle size. Applying a linear gradient resulted in separation of components for all resins, but only TSKgel SP-5PW clearly separated more than three components (data not shown). Toyopearl GigaCap S-650M and Toyopearl SP-650M both separated unPEGylated, mono- and poly-PEGylated lysozyme.

In following experiments, preparative purification was performed on TSKgel SP-5PW (Fig. 3) and a yield of about 40% for mono-PEGylated lysozyme was achieved throughout the whole process, including chemical reaction and chromatographic purification. As seen in Fig. 3 (continuous line, peak No. 3–5) the purification of PEG5Lys showed different isoforms of mono-PEGylated lysozyme. With increasing PEG size the selectivity for the mono-PEGylated lysozyme isoforms got lost. For PEG10Lys only the 1-mono-PEG10Lys isoform can be seen (peak No. 2, dashed line, Fig. 3), the other isoforms were not separated any more (dashed line, PEG10Lys, in Fig. 3 does not include peaks Nos. 4 and 5 of the continuous line, PEG5Lys).

In consequence Toyopearl GigaCap S-650M was used for preparative separations of PEG10Lys and PEG30Lys. As shown in Fig. 4, there was no separation of mono-PEGylated isoforms, but the separation of lysozyme, mono- and poly-PEGylated lysozyme derivates

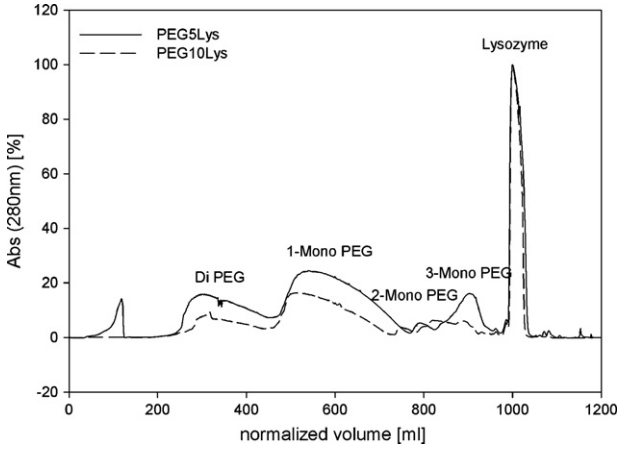

Fig. 3. Preparative purification of PEG5Lys (continuous line) and PEG10Lys (dashed line) on TSKgel SP-5PW. Linear gradient elution to 1 M NaCl. For chromatographic conditions see Section 2.

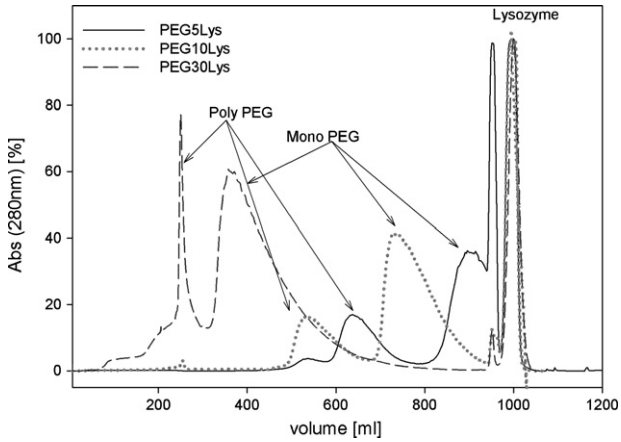

Fig. 4. Preparative purification of PEGylated lysozyme on Toyopearl GigaCap S-650M. Continuous line, PEG5Lys; dotted line, PEG10Lys; dashed line, PEG30Lys. For chromatographic conditions see Section 2.

was acceptable. The achieved yield was about 40% for mono-PEGylated lysozyme for the whole process and thereby equal to the purification with TSKgel SP-5PW.

After desalting and concentration on a Vivaflow 50 system the samples were freeze dried and stored at −20 °C. A purity of 95% was determined via SDS gel-electrophoresis (Fig. 5).

3.3. Selectivity comparison

The analytical selectivity of different media was tested (Fig. 6). Besides TSKgel SP-NPR all media was tested in the same column (Omnifit, 6.6 mm × 50 mm) with the same elution profile. TSKgel SP-NPR was only available in prepacked columns

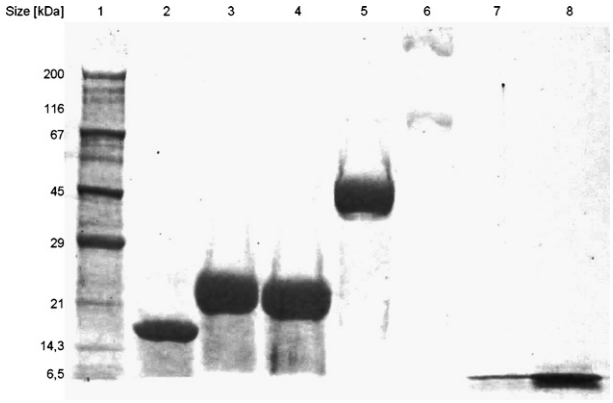

Fig. 5. Separation of PEGylated lysozyme by SDS-PAGE. Silver stained gel, no impurities are visible. Lane 1, marker; Lane 2, PEG5Lys; Lanes 3 and 4, PEG10Lys; Lane 5, PEG30Lys; Lane 6, poly-PEG30Lys; Lane 7, purified lysozyme; Lane 8, lysozyme. For separation conditions see Section 2.

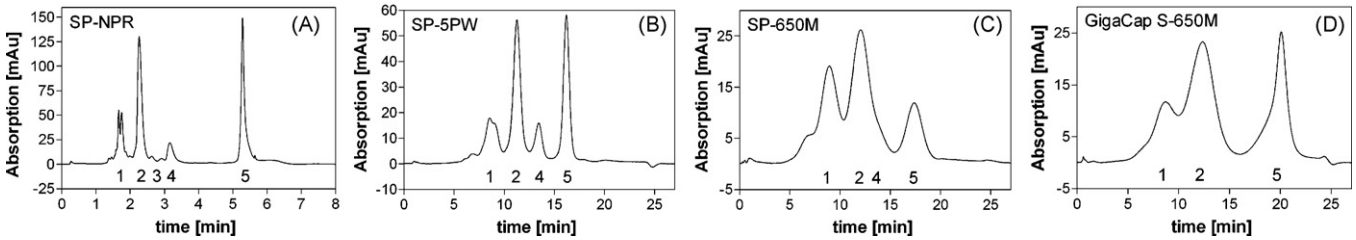

Fig. 6. Resolution dependency on particle size shown with 5 kDa PEGylated lysozyme reaction mix (column dimensions: A, 4.6 mm × 35 mm; B–D, 6.6 mm × 20 mm). (A) SP-NPR, (B) SP-5PW, (C) SP-650M, (D) GigaCap S-650M, (1) poly-PEG5Lys, (2) 1-mono-PEG5Lys, (3) 2-mono-PEG5Lys, (4) 3-mono-PEG5Lys, and (5) lysozyme. For chromatographic conditions see Section 2.

(4.6 mm × 35 mm). If this difference is left apart the chromatography of all media is comparable.

The particle size (shown in Table 1) was of great importance for the selectivity. Especially the non-porous particle TSKgel SP-NPR showed a very fine resolution, with a number of mono-PEGylated isoforms while two isoforms were visible for the di-PEGylated lysozyme. TSKgel SP-5PW is a polishing particle and about 10 times bigger than TSKgel SP-NPR. The resolution decreased, but two mono-PEGylated isoforms still remained visible (Fig. 6A and B). The difference between media for high throughput capture and polishing can also be seen (Fig. 6C and D). The selectivity for the particles TSKgel SP-5PW and Toyopearl SP-650M is in the same range but the particle size is changed. The loss in resolution for Toyopearl SP-650M in comparison to TSKgel SP-5PW was substantial, there was no baseline separation between di-PEGylated and mono-PEGylated lysozyme. The mono-PEGylated isoforms nearly vanished.

Toyopearl GigaCap S-650M showed no isoform separation and the separation between di- and mono-PEGylated lysozyme was worse than for Toyopearl SP-650M. Toyopearl GigaCap S-650M is a high throughput capturing particle, but the particle size is even bigger than for Toyopearl SP-650M.

3.4. Breakthrough experiments

Breakthrough experiments were performed at pH 6.0 in 25 mM phosphate buffer. The results for the DBC of lysozyme and mono-PEG5Lys are listed in Table 3. All concentrations are given on the basis of protein or PEG-protein, respectively. For some resins of interest the DBC with mono-PEG30Lys was also determined.

The DBC decreased with increasing PEG size. This binding weakening effect of PEG was expected because lysozyme is the charged part of the construct [10,12]. The uncharged PEG chain is covalently linked to lysozyme and covers a part of the protein molecule. Even if a flexible nature of the PEG-chain is assumed at least the covalently linked part of the PEG-chain will stay in place and cover a part of the lysozyme so the overall charge of the molecule is reduced. The covered part of lysozyme is inhibited in its binding affinity to the chromatographic matrix. The resulting reduction of DBC was observed for all tested materials. The largest drop in DBC was observed for Toyopearl GigaCap S-650M (Table 3).

In the following experiments the DBC for mono-PEG30Lys was enhanced. Toyopearl GigaCap S-650M and Toyopearl SP-650M were chosen for optimization of binding buffer characteristics. The pH and buffer concentrations were changed. To achieve more acidic pH-values acetate buffer was also used. Fig. 7 shows the change of DBC for mono-PEG30Lys, mono-PEG5Lys and lysozyme between the starting conditions and the optimized buffer for Toyopearl SP-650M and Toyopearl GigaCap S-650M.

Both chromatographic media showed a similar dependence, the DBC was highest in acidic pH with low buffer concentrations (see Fig. 8 for GigaCap S-650M). Interestingly, Toyopearl SP-650M, which showed a lower decrease of DBC for mono-PEG30Lys had a very small increase in DBC depending upon the buffer system. On the other hand Toyopearl GigaCap S-650M had a very low DBC for mono-PEG30Lys, but the increase during the optimization process was high (Fig. 8), and a 20-fold increase of DBC for mono-PEG30Lys was achieved. The increase in DBC for lysozyme was small. Only for basic conditions with high buffer concentrations an apparent decrease of lysozyme DBC was detectable (data not shown).

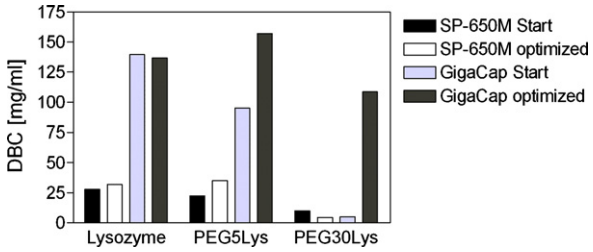

Fig. 7. Optimization of buffer concentration and pH for DBC of lysozyme, mono-PEG5Lys and mono-PEG30Lys on Toyopearl GigaCap S-650M and Toyopearl SP-650M media. Start buffer: 25 mM sodium phosphate buffer pH 6.0; optimized buffer: 5 mM sodium acetate buffer pH 4.0.

Overall the dependency on the buffer system was very strong for PEGylated lysozyme in contrast to the native lysozyme, where DBC did not change very much (see Fig. 8). This behaviour was also observed for the Toyoperal SP-650M resin. Again the DBC for lysozyme did not change very much whereas the DBC for mono-PEG5Lys was dependent on the buffer system.

To explain this behaviour, which is strongest for Toyopearl GigaCap S-650M, an ISEC was carried out to determine the pore size distribution as described by DePhillips and Lenhoff [17–19]. ISEC was carried out at pH 3.0 and 6.0 with buffer concentrations of 5 mM and 25 mM, respectively. There were no differences in the pore size distribution visible (data not shown). Therefore, pore size effects cannot be the reason for the changes seen in DBC.

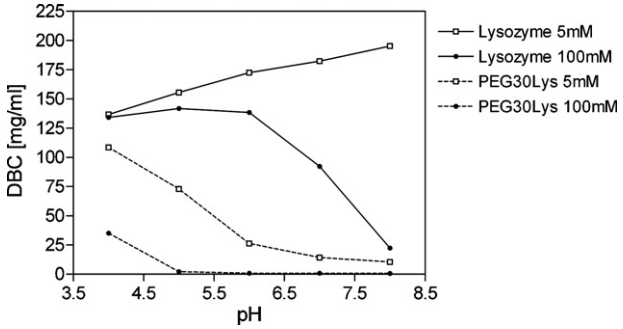

Fig. 8. Optimization of pH and buffer concentration for lysozyme and mono-PEG30Lys on Toyopearl GigaCap S-650M resins. Straight line, lysozyme; dashed line, PEG30Lys; square, buffer concentration 5 mM; circle, buffer concentration 100 mM. Buffer at pH 4.0 and 5.0: sodium acetate, buffer at pH 6.0, 7.0 and 8.0 sodium phosphate.

Table 3
DBC of different IEX materials for lysozyme and mono-PEG5Lys. Mono-PEG30Lys was tested for selected resins. Buffer: 25 mM sodium phosphate buffer, pH 6.0. Column size: 1 ml.

Material	DBC [mg/ml] for lysozyme	DBC [mg/ml] for mono-PEG5Lys	DBC [mg/ml] for mono-PEG30Lys
TSKgel SP-5PW	33	27.6	15
Toyopearl SP-650M	27.9	22.5	9.8
Toyopearl SP-550C	69	53	–
Toyopearl SP-550EC	51	28	–
Toyopearl GigaCap S-650M	139.5	95	4.9

4. Discussion

4.1. PEGylation of lysozyme

In the present work lysozyme as a model protein was PEGylated to examine the behaviour of PEGylated proteins in cation exchange chromatography. A random PEGylation of lysozyme using methoxy-PEG-aldehyde of the sizes 5 kDa, 10 kDa and 30 kDa was performed.

In size-exclusion chromatography a massive increase of size by PEGylation was observed. The SEC elution behaviour of lysozyme modified with a 30 kDa PEG was equal to a 450 kDa globular protein. There was a linear correlation between the theoretical molecular weight of the PEGylated protein and the molecular weight calculated via SEC [20]. This result illustrates the influence of PEG on the hydrodynamic radius of a PEGylated protein [5,6,21,22,29].

4.2. Preparative purification

The reaction mixture was purified using cation exchange chromatography [11,13,23]. PEGylated lysozyme was eluted before native lysozyme [11,23]. This effect was explained via the masking effect of PEG as done by Seely and Richey [10,23,28,31].

Non-reacted, native lysozyme and poly-PEGylated lysozyme were removed by cation exchange chromatography. The yield of mono-PEGylated lysozyme was about 40% throughout the whole process. The salt concentration decreased with increasing PEG chain length as seen by Pabst for BSA [12].

4.3. Selectivity comparison

Cation exchange chromatography was capable to resolve the PEGylated isomers which are product of the random PEGylation [15,22,23]. The use of non-porous SP-NPR polishing resin leads to the best resolution. This is due to the better mass transfer kinetics for large molecules on small, non-porous particles [22].

Despite the loss in resolution it was useful to use a porous capturing resin with larger particle size for the first chromatographic step because of the higher capacity and better pressure-flow characteristics.

4.4. Breakthrough experiments

The DBC decreased for PEGylated lysozyme in comparison to native lysozyme [12] and it also decreased with the length of the PEG chains attached to the native molecule. This decrease was due to the increased size and the accompanying increase of mass transfer resistance and the modified electrostatic characteristic of lysozyme because of the covalently linked neutral PEG [13,16,24].

A dependency of DBC on buffer concentration and pH-value was found. This dependency varied for lysozyme and PEGylated lysozyme. PEGylated lysozyme showed an increase in DBC for acidic pH and low buffer concentration [24]. The dependency on buffer concentration and pH was stronger for PEG30Lys than for PEG5Lys. Toyopearl SP-650M had a low DBC for PEG30Lys with all tested buffers. In acidic buffers, the DBC of the Toyopearl GigaCap S-650M resin for PEG30Lys could be enhanced to more than 100 mg/ml. This shows the importance of resin selection and especially of careful choice of buffer concentration and pH-value.

ISEC experiments showed that changes in the pore size distribution are not the explanation for the enhanced DBC of Toyopearl GigaCap S-650M. The reason for the enhanced DBC cannot be an effect of the matrix itself. Consequently, the protein-matrix interactions play an important role in this process.

For the Toyopearl SP-650M resin, the pH and ionic strength effects are very low. For Toyopearl GigaCap S-650M on the other hand the increase in DBC was high. This can be explained if mass transfer in GigaCap S-650M is not only a diffusive process but electrostatic effects have a certain impact [25,26].

The protein charge increases at low pH, so the DBC should be higher at acidic pH-values, which is true for both tested resins, but Toyopearl GigaCap S-650M has a stronger response on the change of pH. That is an evidence for an electrostatic impact on mass transfer as described by Stone and Carta [25,26]. An even stronger evidence for this theory is the dependence on the size of the probe molecules. The size is a strong hindrance for diffusion while the electrostatic properties of the PEGylated lysozyme do not change much since lysozyme is the mediator of charge [12]. Especially for the very big PEG30Lys the difference between Toyopearl SP-650M and Toyopearl GigaCap S-650M is visible. The very small DBC of Toyopearl SP-650M is strong evidence that the transport is ruled mainly by diffusion. In Toyopearl GigaCap S-650M on the contrary the DBC is high which leads to the conclusion that transport in this particle must have another reason which is especially visible for long PEG chains. This reason can be electrostatic effects as described above [25,27].

5. Conclusions

The selectivity and dynamic binding capacities of various cation exchanger resins were evaluated with random PEGylated lysozyme (chicken egg white). The selectivity for separation of PEGamers was shown to be dependent on the particle size. All PEGamers could be resolved on a TSKgel SP-NPR column with a particle size of 2.5 μm and on a TSKgel SP-5PW column with a particle size of 20 μm. Further increase of the particle size leads to loss of resolution.

The dynamic binding capacity for PEGylated lysozyme is a function of the PEG molecular weight, binding conditions and resin type. For Toyopearl SP-650M binding capacities of 30–40 mg/ml for the 5 kDa and 30 kDa PEGylated lysozyme were achieved and these values were not affected by changing the pH and salt concentrations. In contrast to this, binding capacities for the 5 kDa PEG lysozyme of more then 100 mg/ml were found for Toyopearl GigaCap S-650M. The binding capacity for the PEG30Lys was initially low at neutral pH but could be also optimized to be higher then 100 mg/ml by changing to acidic pH-value and lower conductivity. The mass transfer in both resins could be assumed to be different. Whereas the mass transfer in Toyoperal SP-650M is mainly governed by simple pore diffusion the mass transfer in Toyopearl GigaCap S-650M seems to be supported by electrostatic interactions.

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