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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.

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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.

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