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Sialic acid-specific affinity chromatography for the separation of erythropoietin glycoforms using serotonin as a ligand

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ABSTRACT

Recombinant human erythropoietin (rhEPO) is an important CHO cell-derived glycoprotein and the degree of sialylation of this hormone is crucial for its in vivo bioactivity. In order to improve the purification process serotonin as a potential affinity ligand was tested for preparative chromatographic separation of rhEPO glycoforms into fractions of different degrees of sialylation. Therefore, two chromatographic matrices were prepared by immobilizing serotonin on CNBr- and NHS-Sepharose™. First it was shown both matrices bind rhEPO only in its sialylated form. Results indicate that binding is pH independent between pH 3.5 to 8 suggesting it is not only based on electrostatic interactions. Second, after optimal binding conditions were identified, semi-purified rhEPO was loaded onto both matrices and eluted using a stepwise elution gradient of sodium chloride. For comparison same affinity purification experiments were performed using wheat germ agglutinin-coupled agarose, a lectin known for its affinity towards sialylated glycoproteins. To monitor changes in *N*-glycan fingerprint, eluate fractions were analyzed by multiplexed capillary gel electrophoresis coupled to laser-induced fluorescence (xCGE-LIF). For the serotonin matrices an increasing degree of sialylation was observed from the first to the third elution fraction while purity of rhEPO could be increased at the same time. The late elution fractions of serotonin-coupled CNBr- and NHS-Sepharose™ also showed an overall sialylation degree exceeding that of the starting material. In contrast, for rhEPO bound to wheat germ agglutinin-coupled agarose, no distinct change in the degree of sialylation could be observed after elution. Overall, these encouraging results highlight the potential of serotonin as a chromatographic ligand for the improvement of pharmaceutical purification processes of rhEPO.

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1. Introduction

The recombinant human glycoprotein hormone erythropoietin (rhEPO) has one of the highest market shares among biopharmaceuticals worldwide. With increasing demands and companies facing competition by biosimilar manufacturers, process optimization plays a crucial role. In vivo rhEPO stimulates the differentiation of erythroid progenitor cells into mature erythrocytes, thus playing an important role in red blood cell production [1–4]. Patients suffering from anemia, i.e. due to renal failure, AIDS or cancer treatment, depend on external supply of rhEPO [5]. The 34 kDa glycoprotein holds three *N*-glycosylation sites (Asn-24, Asn-38, and Asn-83), and one *O*-glycosylation site (Ser-126) [6,7]. As for other glycoproteins,

Abbreviations: A, $_{280280}$UV adsorption units at 280 nm; $A_{280,max}$, UV maximum; AEPO, asialo erythropoietin; AU, adsorption units; be, Blue Sepharose™ eluate (intermediate purification step); CBQCA, 3-(4-carboxybenzoyl quinoline-2carboxaldehyde); xCGE-LIF, multiplexed capillary gel electrophoresis coupled to laser induced fluorescence detection; CHO, Chinese hamster ovary; DBC, dynamic binding capacity; ds, desialylated; E, elution; EB, elution buffer; EPO, erythropoietin; fb, final bulk; FT, flow through; GlcNAc, N-acetylglucosamine; MTU, migration time units; RB, running buffer; rh, recombinant human; RT, room temperature; S, stripping; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; V_R, retention volume; WGA, wheat germ agglutinin.

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rhEPO production is typically performed in Chinese hamster ovary (CHO) cells [8,9].

For rhEPO, as for many other glycoproteins, the degree of sialylation is essential for its in vivo bioactivity. While desialylated asialo EPO (AEPO) has a higher in vitro bioactivity, the in vivo half-life of AEPO is approximately twenty times lower because the sialic acid protects the hormone from being cleared from the blood by galactose binding proteins of hepatic cells [10,11]. In vivo bioactivity of rhEPO is also influenced by the branching of the glycan structures and the overall number sialic acids. For instance, it was shown that the tetrasialylated tetra-antennary *N*-glycan content is significantly and positively correlated with in vivo bioactivity [12–15]. In recombinant protein production it is well known that selection of culture conditions, cell line, media, and feeding strategy has an impact on the glycosylation of recombinant glycoproteins in upstream processing [13,16–18]. For key properties of biopharmaceuticals, i.e. bioactivity, a specific and consistent glycosylation pattern is required to guarantee product quality. However, batch-to-batch variability in terms of glycoforms is a challenging concern in biopharmaceutical production processes [19]. Therefore, it is important to control the composition of glycoforms of the end product during downstream processing of cell culture harvests.

Currently it is not possible to express rhEPO only in its natural highly sialylated form in cell culture. Hence, in order to preserve a high bioactivity, low sialylated glycoforms have to be removed during the purification process. Often, this is achieved in one of the last unit operations in the purification train by ion exchange chromatography (IEC) [6,20–24]. However, this separation method is based on the overall accessible charge of the glycoproteins and not particularly on the degree of sialylation depending on the number of negatively charged terminal sialic acids on the glycans. In addition, the overall charge of glycoproteins is also influenced by phosphorylation and sulfation, which may differ from batch to batch. As a result, separation of less sialylated but more strongly phosphorylated or sulfated proteins may occur [13,25–27]. An attractive alternative to IEC for separation of glycoforms would be the use of suitable sialic acid-specific ligands. In the past, lectins, natural proteins binding carbohydrates from plants or animals [28–30], have been used for glycoprotein purification [31–33]. However, lectins are frequently toxic and therefore do not comply with safety regulations for human pharmaceutical production processes [34–36]. In addition, similar to the use of antibodies, the high costs of lectins renders their use in large scale purification economically unattractive. A promising option, however, seems to be serotonin, a small and nontoxic molecule [37–39] whose biochemical and pharmacokinetic properties are well characterized [39]. and which is commercially available at a relatively low price. Affinity of serotonin towards sialylated glycoproteins was first reported by Ochoa and Bangham in 1976 [40]. Since then it was used successfully in several laboratory scale processes (liquid chromatography and solid phase extraction) for the analysis [41,42] and purification [43,44] of glycans, glycopeptides, and glycoproteins using serotonin-immobilized silica or Sepharose beads. However, while these studies showed the potential of serotonin for isolating sialylated glycoproteins, it has not been used for the preparative fractionation of glycoforms of a sialylated glycoprotein so far.

Here, we describe the development of a preparative affinity chromatographic process for the purification and fractionation of rhEPO glycoforms, based on the interaction of their sialylated glycomoieties with respective immobilized ligands. The evaluation of this process involved the immobilization of serotonin onto different chromatographic matrices to study its separation performance in small scale affinity chromatography. As a control matrix immobilized wheat germ agglutinin (WGA), a lectin with known binding properties and affinity towards sialyllactose [45,46], was used. For a better understanding of the fractionation efficiency of the process with respect to the glycosylation pattern, glycoprofiling was performed. Based on multiplexed capillary gel electrophoresis coupled to laser induced fluorescence detection (xCGE-LIF), detailed information about the glycosylation patterns of the distinct affinity chromatographic fractions could be gained [47–49].

2. Material and methods

2.1. Materials and reagents

Milli-Q advantage A10 system (Millipore, USA) to produce ultrapure water used for all experiments and solutions. Hydrochloric acid (HCL, #1003171000, Merck KGaA, Darmstadt, Germany) and sodium hydroxide (NaOH, #6771, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for setting the pH of buffers. Serotonin HCL (#B21263, Alfa Aesar GmbH & Co. KG, Karlsruhe, Germany). Sodium borate (borax, #S9640-500G), ethanolamine (#E9508-100ML), and sodium acetate (#71183), all from Sigma–Aldrich (Steinheim, Germany). Sodium hydrogen carbonate ($NaHCO_3$, #6885.2), Tris (#5429.1), ethanol (#5054.1), acetic acid (#7332.1), sodium chloride (NaCl, #P029.1), di-sodium hydrogen phosphate (#P030.1), and sodium di-hydrogen phosphate monohydrate (## K300.1), all from Carl Roth GmbH & Co., KG (Karlsruhe, Germany. Sodium phosphate buffer was prepared by mixing Sodium di-hydrogen phosphate solution with di-sodium hydrogen phosphate solution). CNBr-activated Sepharose™ 4B (CNBr-Sepharose™, #17-0430-01) and NHS-activated Sepharose™ 4 Fast Flow (NHS-Sepharose™, #17-0906-01), both from GE Healthcare Bio-Science AB (Uppsala, Sweden). Agarose wheat germ agglutinin (WGA-agarose, #AL-1023, Vector Laboratories Ltd., Peterborough, United Kingdom). Control agarose resin (#26150, Thermo Scientific, Rockford, Illinois, USA). Eppendorf Protein LoBind Tubes (Eppendorf AG, Hamburg, Germany) and low binding tips from Sorensen Bio Science, Inc. (Salt Lake City, Utha, USA) to reduce losses due to unspecific binding during assay quantification. Thermomixer comfort (Eppendorf AG, Hamburg, Germany) for incubation, agitation and temperature control. Tecan infinite M200 Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland) to read out microtiter plates.

All materials containing rhEPO were obtained from Merckle Biotec GmbH (TEVA, Ulm, Germany). This included partially purified rhEPO (rhEPObe, obtained after blue Sepharose™ chromatography), the purified final bulk (rhEPOfb, obtained at the end of the process train after IEC), and rhAEPO (enzymatically desialylated rhEPOfb) all expressed in CHO cells.

2.2. Preparation of serotonin matrices

Serotonin matrices were prepared by coupling serotonin to two different matrices, CNBr-Sepharose and NHS-Sepharose™. While the active group of the first material is directly linked to the Sepharose™ the NHS possesses a 10-atom spacer between the matrix and the active group. Serotonin was immobilized via its amine on CNBr-Sepharose™ (further referred to as serotonin-CNBr) by the cyanogen bromide method, following the manufacturer's instructions Briefly, 2 g of lyophilized CNBr-Sepharose™ were suspended in 1 mM HCl, pH 3.0 and subsequently washed with 1 mM HCl for 15 min on a sintered glass filter with a fiber glass filter on top. 40 mg serotonin-HCl were solved in 10 ml coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and added to the activated CNBr-Sepharose™ in 15 ml reaction tubes. The tubes were protected from light and then incubated for 2 h at room temperature (RT) by rotating the mixture with a Multi Bio RS-24 (Kisker Biotech GmbH & Co., KG, Steinfurt, Germany). After

incubation, excess ligand was washed away with 35 ml coupling buffer. Following, the suspension was incubated with 10 ml of 1 M ethanolamine, pH 8.0 for 1 h at RT in order to block any remaining active groups. After blocking, the material was washed with three cycles of alternating pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.0 and 0.1 M Tris–HCl, pH 8.0). The final product was then washed with 50 ml ultra-pure water followed by 50 ml of 20% (v/v) ethanol and stored in light protected reaction tubes at 4 °C.

Serotonin immobilization on NHS-Sepharose™ (further referred to as serotonin-NHS) was done via the *N*-hydroxysuccinimide method according to the manufacturer's instructions. The procedure differs from the procedure of CNBr-Sepharose™ coupling as follows. All solutions were cooled down to 4 °C. 5 ml drained NHS matrix as well as 296 mg serotonin, solved in 2.5 ml coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) have been used. During the coupling reaction pH had to be adjusted to pH 8.0. For blocking 0.5 M ethanolamine with 0.5 M NaCl, pH 8.3 was added.

Preparations of the negative control matrices ethanolamine-CNBr and ethanolamine-NHS were done analogous skipping the immobilization step of serotonin. After washing, all materials were kept light protected at 4 °C until use. Immobilization was evaluated by elementary analysis performed by Dr. Sabine Busse and Sabine Preiß from the Institute of Chemistry of the Otto-von-Guericke University (Magdeburg, Germany).

2.3. Dialysis

For dialysis cellulose ester Spectra/Por Biotech Dialysis Tubes with a molecular weight cut off of 3.5–5 kDa (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) were used. Dialysis ran over night with a buffer exchange after 4 h resulting in a sample to buffer ratio of at least 1:1,000.

2.4. Chromatography

All experiments were performed with an ÄKTA Explorer or ÄKTA Basic system and Tricorn™ 5/20High Performance columns with an inner diameter of 5 mm (all GE Healthcare Bio-Science, Uppsala, Sweden) and packed with the respective affinity matrix. Columns were kept at 4 °C during experiments using a Thermo Scientific water bath V15 (Thermo scientific, Germany). Unless otherwise stated the following steps and flow rates were used in chromatography: Equilibration with running buffer (composition of buffers below) (0.5 ml/min); sample injection (0.25 ml/min); washing with running buffer (0.25 ml/min); elution with elution buffer (0.5 ml/min); stripping with stripping buffer (1 ml/min); re-equilibration with running buffer (1 ml/min). 500 mM NaCl solved in running buffer was used as stripping buffer for all experiments. UV adsorption units at 280 nm (A_{280}), conductivity, and pH values were recorded online as required. Peaks of the chromatogram were characterized by maximum A_{280} ($A_{280,max}$) and retention volume (V_R). If necessary, fractions were collected for further analysis.

2.4.1. Optimization of buffer conditions and test of binding specificity

All columns were packed with approximately 300 μl of the respective matrix. Chromatograms were evaluated using blank runs (without rhEPOfb) to discriminate between effects of buffer composition and protein elution on the UV signal. In all experiments the columns were loaded with 100 μg of sample (rhEPOfb, rhEPObe or rhAEPO) 1:4 diluted in running buffer. The binding and elution behavior was evaluated on the basis of the UV-signal at 280 nm.

To investigate the influence of the pH value, a serotonin-CNBr column was used with 50 mM sodium phosphate, pH 6 as running buffer. After loading rhEPOfb and washing of the column, elution was performed with a 18 ml linear gradient from 0 to 100% of 50 mM sodium acetate, pH 3.5 (pH was adjusted with acetic acid) or 50 mM sodium phosphate, pH 8.0; the pH value was monitored online.

To test the effect of ionic strength on elution, a serotonin-CNBr column was used with 20 mM sodium phosphate, pH 6 as running buffer. After loading rhEPOfb and washing, the elution was done with a 9 ml linear gradient from 0 to 100% of 20 mM sodium acetate, pH 6.0 with 500 mM NaCl.

In addition, the binding specificity of the serotonin matrices to sialylated rhEPO was tested. Therefore, rhEPOfb or rhAEPO were diluted in running buffer (20 mM sodium phosphate, pH 6.0) and applied to serotonin-CNBr and ethanolamine-CNBr as well as to the negative controls serotonin-NHS and ethanolamine-NHS. After loading, the columns were washed and the bound material eluted using 500 mM NaCl in running buffer. To examine whether the running buffer has an impact on binding specificity, this test was repeated with three other running buffers (sodium acetate, ammonium acetate, and MES).

The elution gradients where optimized for serotonin-CNBr, serotonin-NHS and WGA-agarose. Step gradients (two different NaCl gradients of 10 steps; 4.5 ml per step) were applied to test the sodium chloride concentration, at which rhEPO can be eluted from the matrices. In all experiments rhEPObe was loaded. The first run was performed using a gradient of 0 mM–100 mM NaCl in running buffer (20 mM sodium phosphate, pH 6.0) and the second run using a gradient of 0 mM–300 mM NaCl in running buffer (for WGA 0 mM to 500 mM). NaCl concentrations at which a distinct UV-peak was visible were selected as elution steps for the fractionation experiments. The elution strategy for each material was tested before the fractionation experiments and adjusted if necessary.

2.4.2. Dynamic binding capacity estimation

Columns were packed with approximately 100 μl of respective matrix. rhEPOfb was dialyzed against running buffer (20 mM sodium phosphate, pH 6.0). The dialyzed sample was loaded onto the column until the UV signal exceeded 10% of UV maximum indicating breakthrough. Dynamic binding capacity (DBC) was calculated from the load concentration and the total load volume at 10% breakthrough.

2.4.3. Fractionation of partially purified rhEPObe

rhEPObe, dialyzed against running buffer (20 mM sodium phosphate, pH 6.0), was used as load sample. Columns were packed with approximately 500 μl of respective matrix and samples were loaded with about 20% of the respective DBC. After loading and washing the bound material was eluted with a 3-step gradient that was adapted to the elution behavior of the respective column (see 2.4.1.). 0.3 M NaCl in running buffer was used as elution buffer and fractions (1 ml/fraction) of the whole run were collected. Fractions of a single peak were pooled prior analysis. For separations using WGA-agarose, the chromatographic method was identical except that a 1 ml Tricorn column was applied, and 0.5 M and 1 M NaCl was used as buffer for elution and stripping, respectively.

2.5. Analysis

2.5.1. rhEPO ELISA

rhEPO concentration was determined with an rhEPO-specific enzyme linked immune sorbent assay (ELISA) kit (Merckle Biotec GmbH, Ulm, Germany). Analysis was done according to the manufacturer's instructions. Briefly, samples were diluted at least 1:100 in the assay buffer of the kit. 100 ml of each sample and standard (1 ng/ml to 20 ng/ml rhEPOfb), all in duplicates, were added to a pre-coated 96-well plate and incubated for 60 min in the thermo

mixer at 600 rpm and RT. Subsequently the plate was washed with 4 cycles of assay buffer. 100 μl of enzyme conjugate was added to each well followed by incubation and washing as before. Then 100 μl of substrate/chromogen solution was added to each well. Incubation was stopped after a light blue color had developed by adding 100 μl of stop solution. The plate was read out at 450 nm. The assay was validated in a range of 2 ng/ml to 20 ng/ml rhEPOfb and samples were measured in duplicates (relative SD of the method 1.98%). (*Note*: this assay does not enable differentiation of rhEPO obtained from individual purification steps, i.e. rhEPObe, rhEPOfb, and rhAEPO).

2.5.2. Bradford assay

Samples with an expected protein concentration above 5 μg/ml were analyzed using the Bradford assay as published previously [50]. As protein assay dye reagent Coomassi Brilliant Blue G-250 (Bio-Rad Laboratories, München, Germany) was used. The assay was calibrated according to an internal standard operating procedure using rhEPOfb in a range of 5–40 μg/ml. If necessary, standards and samples were diluted in phosphate buffered saline (PBS). All measurements were done in triplicates.

2.5.3. CBQCA (3-(4-carboxybenzoyl quinoline-2carboxaldehyde) assay

If protein concentration was below 5 μg/ml, it was measured by the CBQCA Protein Quantitation Kit (Invitrogen, Eugene, Oregon, USA). Detection is based on the ATTO-TAG CBQCA reagent that forms highly fluorescent derivatives by reacting with primary amines in the presence of cyanide (You et al., 1997). Samples and standards (rhEPOfb) were first diluted 1:2 with 100 mM borax buffer, pH 9.3. Further dilutions were done with working buffer (1:2 dilution of 100 mM borax buffer with respective sample buffer). The rest of the procedure followed the manufacturer's instructions. Briefly, 135 μl of each sample were transferred to a black fluorescence low binding 96 well microtiter plate (Greiner Bio-One GmbH, Frickenhausen, Germany). 5 μl of 20 mM potassium cyanate were added followed by 10 μl of 5 mM CBQCA reagent after quick agitation. The plate was protected from light and incubated for 1 h (1000 rpm, RT). The fluorescence signal was measured at an excitation and emission wavelength of 465 nm and 550 nm, respectively. The assay was validated in a range of 0.2 μg/ml–2.0 μg/ml (relative SD of the method 4.3%). Samples were measured in quintuplicates to identify outliers which occurred frequently.

2.5.4. Glycan analysis

N-glycosylation of rhEPO samples was analyzed by xCGE-LIF according to [47–49]. Briefly, samples were first enriched with 3 kDa MWCO Amicon Ultra-0.5 centrifugal filter units (#UFC500324, Merck-Millipore, Darmstadt, Germany) and separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Bands were excised from Coomassie Blue-stained SDS-PAGE gels between 28 kDa and 40 kDa, where rhEPO should accumulate. *N*-glycans were enzymatically released from rhEPO protein backbone by peptide-*N*-glycosidase F (#P7367, Sigma-Aldrich, Steinheim, Germany) treatment and subsequently fluorescently labeled with 8-aminopyrene-1,3,6-trisulfonic acid (APTS, #09341, Sigma–Aldrich, Steinheim, Germany). The APTS labeled *N*-glycans were purified by hydrophilic interaction chromatography (HILIC) in solid phase extraction mode, followed by xCGE-LIF measurement [51]. For analyzing xCGE-LIF generated electropherograms, glyXtool™ (glyXera, Magdeburg, Germany) was used, including migration time normalization to an internal standard from glyXera, generating so called *N*-glycan fingerprints [52]. As master profile standard the *N*-glycan fingerprint of CHO cell produced rhEPOfb was used (Fig. 1). Due to the highly reproducible normalized migration times a peak assignment with *N*-glycan structures to an in-house database could be performed. Furthermore, for quantitative comparison, the relative peak height (RPH: ratio of peak height to the total height of all peaks) of each peak within the *N*-glycan fingerprint was calculated representing the relative concentration of at least one respective *N*-glycan structure beyond the peaks.

3. Results and discussion

3.1. Ligand immobilization

Serotonin was coupled to CNBr-SepharoseTM and NHS-SepharoseTM. To evaluate the immobilization efficiency of serotonin to the respective matrix, an elementary analysis was performed. The change in nitrogen content was used to evaluate serotonin coupling. For serotonin-CNBr nitrogen content increased by 307 μmol/g dried material. Since the number of nitrogen atoms increases by 2 per coupled serotonin molecule, this corresponds to 153.5 μmol coupled serotonin per gram of dried material. For serotonin-NHS nitrogen content increased by 435 μmol/g of dried material. Since the number of nitrogen atoms increases by 1 per coupled serotonin molecule, this corresponds to 435 μmol coupled serotonin per gram of dried material. In case of NHS-Sepharose™ the maximum number of coupling sites is about 80 μmol/g dried material which is more than five times lower than the calculated amount of coupled serotonin. In case of CNBr-Sepharose™ no number of coupling sites is given by the manufacturer. Assuming the number is similar to NHS-Sepharose™ the calculated amount of coupled serotonin exceeds this number almost two times. It looks like in both cases nitrogen has not only been introduced by serotonin but also by unexpected side reactions. Clearly, elementary analysis is a method for proving qualitative success of ligand coupling rather than giving quantitative numbers. But from the high increase in nitrogen content for both materials full saturation is assumed.

3.2. Scouting experiments for determination of optimal binding conditions

Different binding and elution conditions were tested as well as negative controls to demonstrate principal functionality.

3.2.1. Buffer conditions

To investigate the effect of increasing and decreasing pH on elution rhEPOfb was loaded onto serotonin-CNBr matrix using a linear elution gradient (Fig. 2). Since only pure rhEPOfb was loaded the obtained UV-signal reflects changes in the rhEPOfb concentration. For both experiments (Fig. 2A and B) very low amounts of rhEPOfb were eluted shown by low UV signals during the elution step which are slightly above the signals of the blank runs (Fig. 2C and D). The high peak ($A_{280,max}$ = 78.4 mAU, V_R = 31.1 ml) during the stripping step after pH decrease from pH 6.0 to 3.5 (Fig. 2A) confirmed that most of the rhEPOfb was still bound. Even when the isoelectric point of rhEPOfb, which ranges between pH 4.2 and 4.6 [8] was exceeded, no elution occurred. When rising the pH from 6 to 8 (Fig. 2B) a smaller peak ($A_{280,max}$ = 40.3 mAU, V_R = 31.2 ml) during the stripping step suggested that either part of the bound rhEPOfb eluted before or that the binding strength was increased because of the increased sialic acid charge so that the ionic strength of the stripping buffer was not sufficient for full elution. Since no significant elution was observed while changing the pH the latter seems more reasonable. This results support the findings of Sturgeon and Sturgeon [43] who reported that ion exchange effects are negligible for the binding of sialic acid to serotonin. On the other hand it would be in contrast to the study of Ochoa and Bangham [40] who proposed that the interaction is mainly electrostatic.

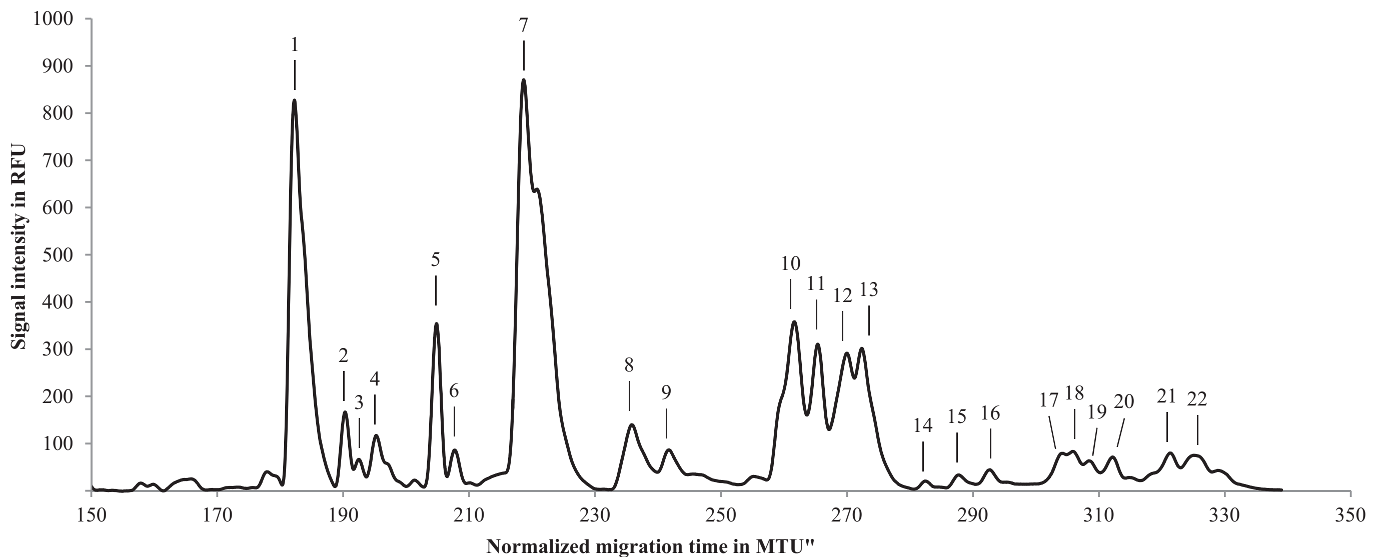

Fig. 1. *N*-glycan fingerprint of CHO cell-produced recombinant human erythropoietin final bulk (rhEPOfb). Measured by multiplexed capillary gel electrophoresis coupled to laser induced fluorescence detection. The black continuous line depicts the signal intensity in relative fluorescence units (RFU) over normalized migration time units (MTU"). Peaks assigned to one or more *N*-glycan structure(s) (listed in Table 1) are indicated by peak numbers.

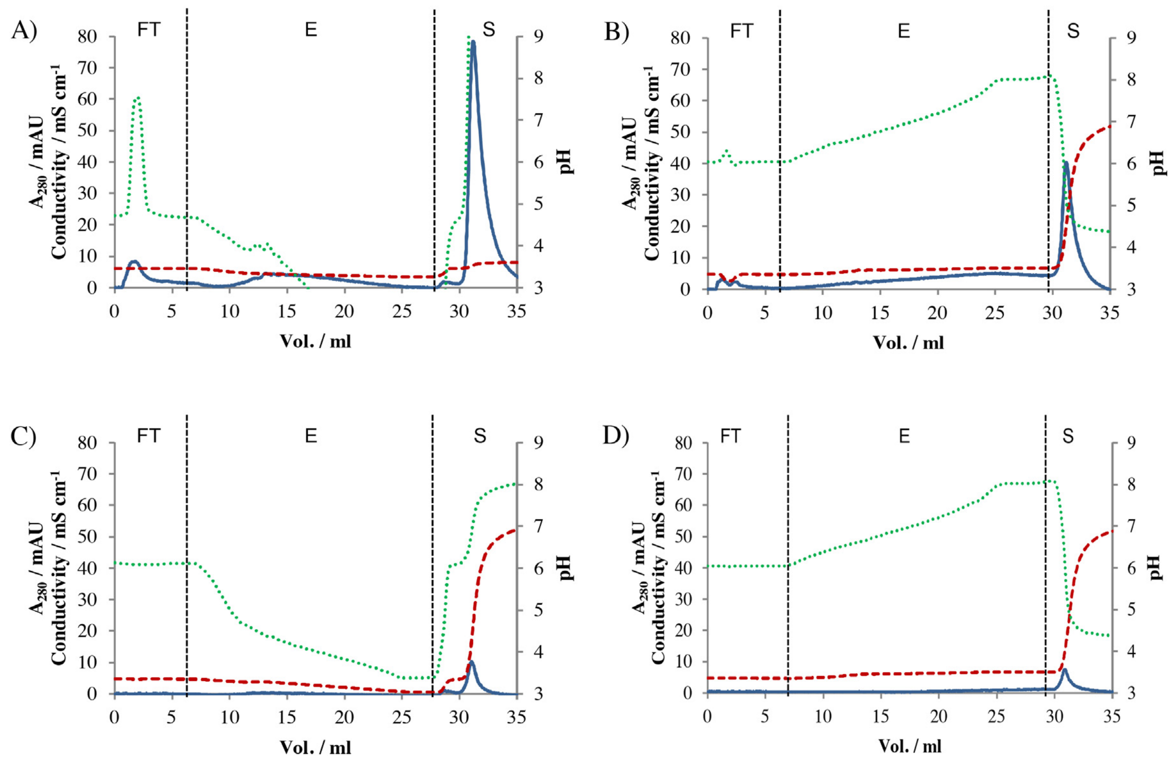

Fig. 2. Effect of pH value on elution. (A,B) recombinant human erythropoietin final bulk (rhEPOfb) was loaded (running buffer 50 mM sodium phosphate, pH 6.0) onto serotonin-CNBr (300 μl in a Tricorn 5/50 column). After sample injection a linear elution gradient from 0 to 100% of elution buffer (A,C) 50 mM sodium acetate, pH 3.5; (B,D) 50 mM sodium phosphate, pH 8.0) was applied. (C) and (D) are blank runs where running buffer was injected instead of rhEPOfb. UV-absorption at 280 nm (A_{280}) which reflects changes in the protein concentration (rhEPOfb or rhAEPO, respectively) is displayed as blue continuous line ( ), conductivity, which reflects the ionic strength in the buffer as red discontinuous line ( ), and pH as green dotted line ( ). Process steps: load/flow through (FT), elution (E), and stripping (S) are separated by a black dotted line (......).

Besides testing the influence of pH the effect of salt concentration on elution was tested as well (data not shown). It could be seen that binding of rhEPOfb is very sensitive to NaCl concentration which should not exceed 20 mM in the running buffer. In particular, 500 mM NaCl (1 M in case of WGA) in the running buffer was found to be sufficient as stripping buffer. This is not surprising since it has been frequently reported that interaction of carbohydrates with other materials is comparatively low and affinity constants can be found in the mM range [53–57].

Sodium phosphate, sodium acetate and ammonium acetate showed similar results while the use of MES buffer caused a shift in the UV-signal that could not be explained. 20 mM sodium phosphate (pH 6.0) was chosen as running buffer. Based on experimental

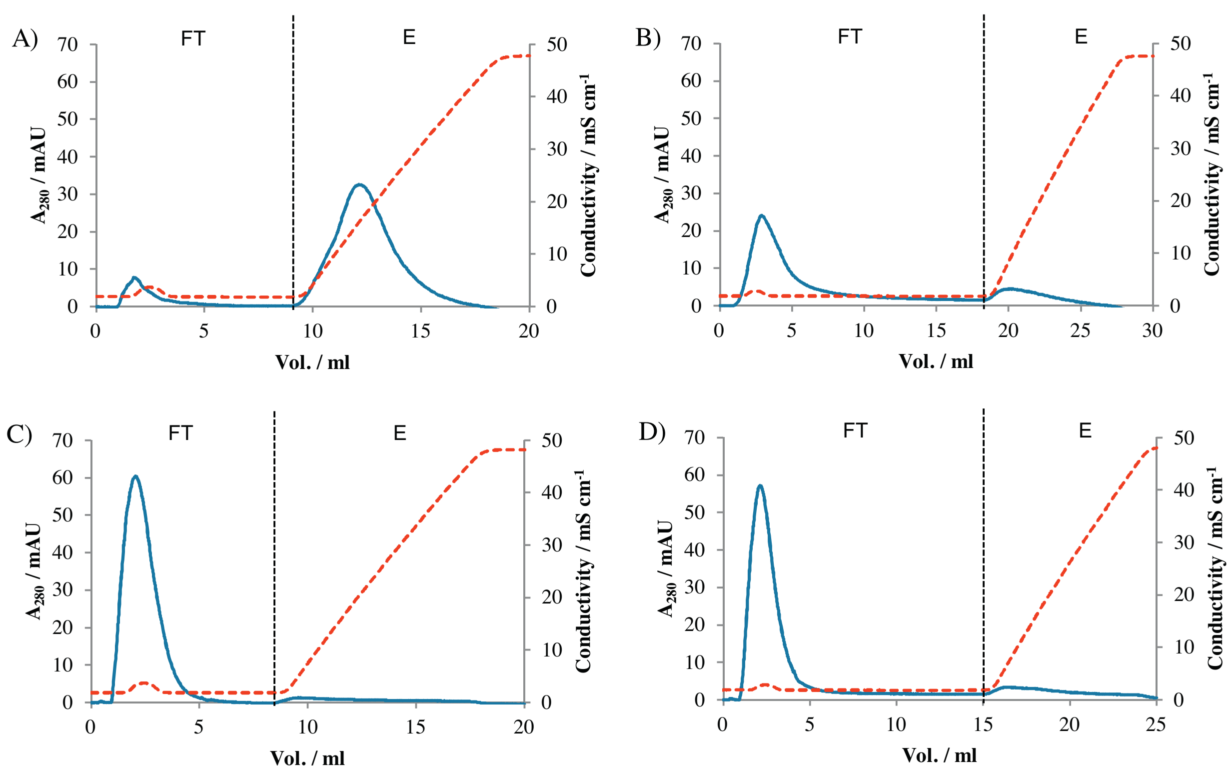

Fig. 3. Test of basic functionality of serotonin-CNBr. Binding of recombinant human erythropoietin final bulk (rhEPOfb) and asialo rhEPOfb (rhAEPO) to serotonin-CNBr (A and B) and ethanolamine-CNBr (C and D) (running buffer (RB) 20 mM sodium phosphate buffer, pH 6.0). Bound rhEPOfb or rhAEPO was eluted with 500 mM NaCl in RB. UV-absorption at 280 nm (A_{280}) which reflects changes in the protein concentration (rhEPOfb or rhAEPO, respectively) is displayed as blue continuous line ( ), and conductivity, which reflects the ionic strength in the buffer as red discontinuous line ( ). Process steps: load/flow through (FT) and elution (E) are separated by a black dotted line (......).

setups reported elsewhere [58] these binding conditions were assumed to be suitable for WGA-agarose as well.

After the optimization of the elution conditions, sodium chloride concentrations were identified at which EPO fractions do elute (data not shown). For serotonin-CNBr and for serotonin-NHS three elution fractions were obtained, for WGA-agarose only two. To test the suitability of NaCl as elution buffer for WGA-agarose, a column was loaded with pure rhEPOfb until saturation and eluted with 1 M NaCl in running buffer (as described in the method for dynamic binding capacity testing). The amount of rhEPO in the elution fraction was comparable to the calculated dynamic binding capacity for WGA-agarose. Also, the addition of *N*-acetyl-D-glucosamine (GlcNAc) to the elution buffer was tested for WGA-agarose. However, its use was found not to be practical in this study. When using GlcNAc it did not result in a better fractionation compared to NaCl. Furthermore, the use of GlcNAc as a component of the elution buffer should be avoided due to possible interference of glycans in the subsequent glycan analysis. Overall, it can be assumed that NaCl can be used for elution at sufficient concentrations and that highly sialylated EPO will bind stronger to WGA and therefore eluate at higher ionic strength of the elution buffer.

3.2.2. Test of specific binding

In order to demonstrate the basic functionality of both serotonin matrices several positive and negative controls were performed. Therefore, rhEPOfb and rhAEPO were loaded onto serotonin-CNBr, serotonin-NHS, and the negative controls ethanolamine-CNBr and ethanolamine-NHS (Fig. 3 and Fig. 4). After loading rhEPOfb onto the serotonin-CNBr column a small UV-peak ($A_{280,max}$ = 7.8 mAU, V_R = 1.7 ml) appeared during flow through and a large peak ($A_{280,max}$ = 32.3 mAU, V_R = 12.2 ml) during elution showing that most of the loaded rhEPOfb has bound to the serotonin-CNBr column (Fig. 3A). The flow through peak indicates that low quantities of rhEPOfb did not bind. This could not be verified by later experiments where the flow through fraction was analyzed by the rhEPO ELISA where no rhEPO could be detected. Since loaded samples were not dialyzed against running buffer but diluted 1:8 with running buffer the flow through peak could also be due to changes in sample buffer composition. Loading of rhAEPO resulted in a larger peak ($A_{280,max}$ = 23.6 mAU, V_R = 2.8 ml) during flow through and a much smaller peak ($A_{280,max}$ = 4.5 mAU, V_R = 20.1 ml) during elution (Fig. 3B). This strongly suggests that serotonin-CNBr does not bind rhAEPO. Nevertheless, low amounts of the rhAEPO sample might still be available for binding to serotonin-CNBr because rhAEPO is not totally free of sialic acid after enzymatic digestion (information by MERCKLE BIOTEC). The negative control, using ethanolamine-CNBr did neither bind rhEPOfb nor rhAEPO (Fig. 3C and D). Accordingly, rhEPOfb as well as rhAEPO was found mainly in the flow through as indicated by the large UV-peaks ($A_{280,max}$ = 59.8 mAU, V_R = 2.0 ml; $A_{280,max}$ = 56.5 mAU, V_R = 2.0 ml) during loading. Overall, results clearly demonstrated that serotonin is necessary for the binding of rhEPOfb and that rhAEPO or low sialylated rhEPO is not binding to the ethanolamine-CNBr matrix used.

Additional experiments were performed using serotonin- and ethanolamine-NHS. When loading rhEPOfb it bound almost completely to the matrix (Fig. 4A). In contrast, two UV-peaks ($A_{280,max}$ = 56.7 mAU, V_R = 11.5 ml; $A_{280,max}$ = 38.8 mAU, V_R = 13.9 ml) were visible during elution. Interestingly, analysis by Bradford assay and rhEPO ELISA revealed that the second peak neither contained rhEPOfb nor protein. As the focus of this study was on protein purification, this peak was not considered further. Again, similar to ethanolamine-CNBr, low amounts of rhAEPO seemed to bind to ethanolamine-NHS. This is also indicated by

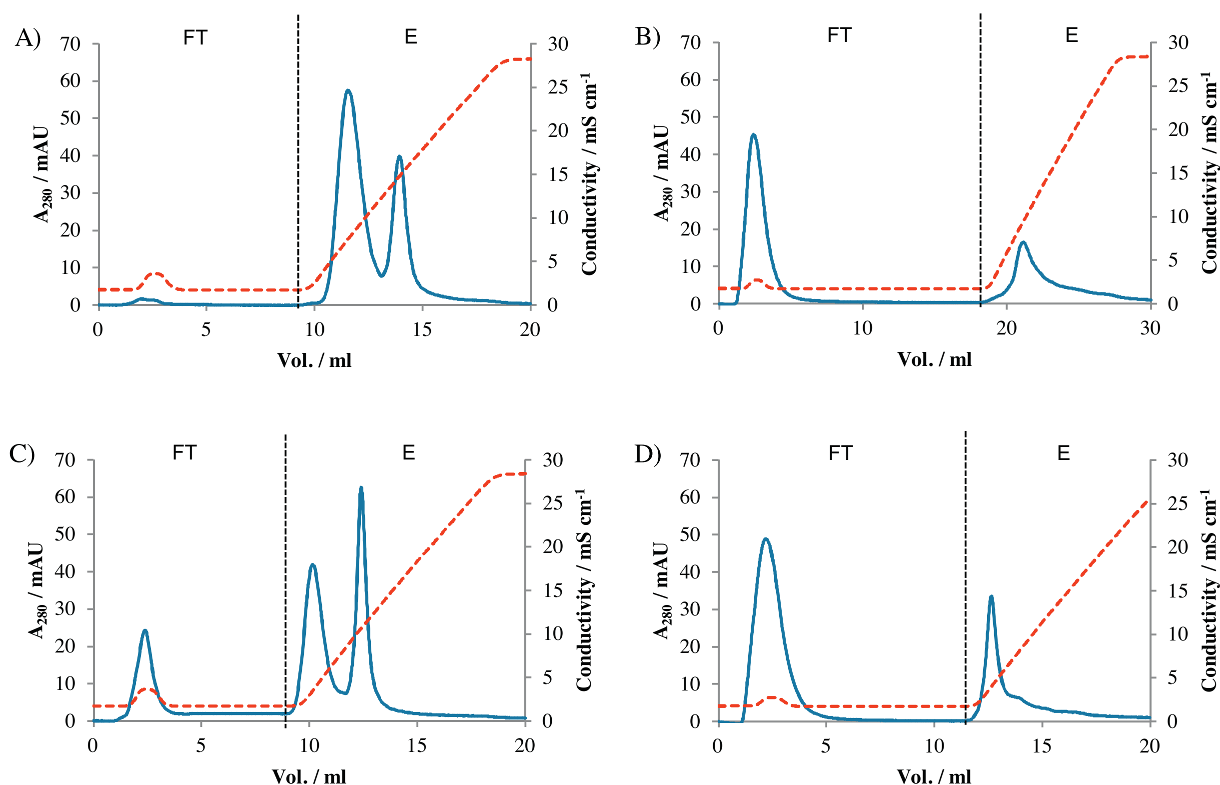

Fig. 4. Test of basic functionality of serotonin-NHS. Binding of recombinant human erythropoietin final bulk (rhEPOfb) and asialo rhEPOfb (rhAEPO) to serotonin-NHS (A and B) and to ethanolamine-NHS (C and D) (running buffer (RB) 20 mM sodium phosphate buffer, pH 6.0). Bound rhEPOfb or rhAEPO was eluted with 500 mM NaCl in RB. UV-absorption at 280 nm (A_{280}) which reflects changes in the protein concentration (rhEPOfb or rhAEPO, respectively) is displayed as blue continuous line (▬), and conductivity, which reflects the ionic strength in the buffer as red discontinuous line (▬ ▬). Process steps: load/flow through (FT) and elution (E) are separated by a black dotted line (……).

the small peak ($A_{280,max}$ = 16.1 mAU, V_R = 21.0 ml) during elution (Fig. 4B). Contrary to expectations, rhEPOfb was also bound by ethanolamine-NHS shown by the double peak ($A_{280,max}$ = 40.9 mAU, V_R = 10.1 ml; $A_{280,max}$ = 61.6 mAU, V_R = 12.6 m) during elution (the second peak again did neither contain rhEPO nor protein). This clearly suggests unspecific binding of rhEPOfb to NHS-Sepharose™ (Fig. 4C). Finally, Fig. 4D demonstrates low binding of rhAEPO to ethanolamine-NHS. Concluding from these results CNBr seems to be preferable over NHS as matrix for serotonin because of the unspecific binding of rhEPOfb to the negative control ethanolamine-NHS.

3.3. Dynamic binding capacity

For all materials including WGA-agarose and the negative controls of both serotonin matrices the DBC for rhEPOfb was measured (Fig. 5). Serotonin-CNBr as well as serotonin-NHS showed the highest DBC, i.e. compared to WGA-agarose. Unmodified control agarose resin did not bind any rhEPOfb while ethanolamine-CNBr bound small amounts of rhEPOfb. Furthermore, there was also a relatively high DBC of ethanolamine-NHS indicating unspecific binding of rhEPOfb. Although the comparison of the DBCs is difficult due to the different characteristics of the matrices and differences in ligand density, it still could be demonstrated that the prepared serotonin materials possess a DBC higher than that of WGA-agarose. Interestingly, both serotonin materials have a comparable DBC of about 7 mg/ml_{matrix} showing that the spacer did not improve the capacity. In contrast, the spacer of the ethanolamine-NHS matrix could even be related to the unspecific binding since it is the only structural difference between the two matrices.

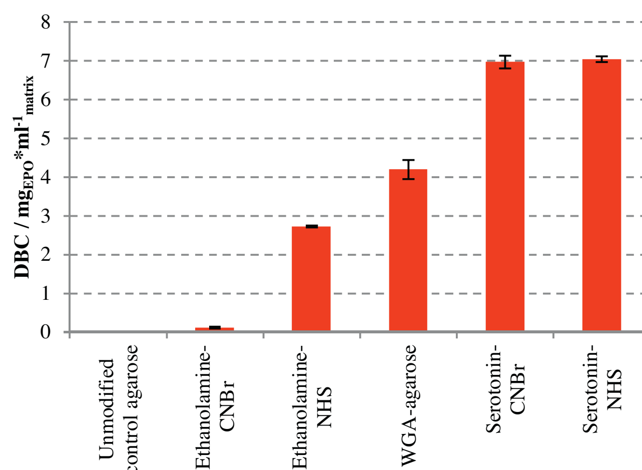

Fig. 5. Dynamic binding capacities (DBC). Recombinant human erythropoietin final bulk rhEPOfb (dialyzed against running buffer 20 mM sodium phosphate, pH 6.0) was loaded onto the respective column (approx. 100 μl of matrix) until the UV signal exceeded 10% of UV maximum indicating breakthrough. Dynamic binding capacity (DBC) was calculated from the load concentration and the total load volume at 10% breakthrough. Error bars are the SD of three runs.

3.4. Fractionation of partially purified rhEPObe

In a next step, serotonin-CNBr, serotonin-NHS and WGA-agarose were used for fractionation of rhEPObe. Using serotonin-CNBr and -NHS three elution steps were performed, and for WGA-agarose only two since no further fraction could be collected. As can be seen from Table 2, recovery of total protein and rhEPO

is comparable for both, serotonin-CNBr (protein: 35.9% and rhEPO: 62.3%) and -NHS (protein: 47.0% and rhEPO: 65.7%) while it is much lower for WGA-agarose (protein: 19.4% and rhEPO: 26.8%). In the study of Yodoshi et al. [44] recoveries of glycopeptides of about 80% were achieved but elution conditions were much harsher using 30% methanol, which is not applicable for a preparative chromatography of pharmaceutical purposes. The reason for low recovery in the case of WGA-agarose might be that elution conditions were not optimal even though 1 M of NaCl was used for stripping. Other research groups suggested the use of a competing glycan for elution like GlcNAc or sialyllactose. In particular, it was reported that a GlcNAc concentration in the range of 15–500 mM was needed for full elution [45,46]. However, as addressed above, the addition of GlcNAc in the elution buffer did not result in a better separation and is problematic regarding the subsequent glycan analysis performed in our study (see 3.2.1). Nevertheless, for other studies, and in the case that no detailed glycan analysis is required, it might be useful to include GlcNAc to improve elution and recovery of rhEPO from WGA. In general rhEPO recovery is higher than total protein recovery in all cases. One reason is probably a strong unspecific binding of some proteins to the matrix leading to a general loss of proteins.

Using the serotonin-CNBr matrix rhEPO was measured in all of the three elution fractions but mainly in the second and third (E1: 4.1%, E2: 44.7% and E3: 13.5%). When applying serotonin-NHS, rhEPO was only detected in the first two elution fractions (E1: 34.4% and E2: 31.3%) suggesting that binding of rhEPObe to this matrix is weaker compared to serotonin-CNBr. In case of serotonin-CNBr and -NHS, rhEPO was only detected in the elution fractions whereas in the case of WGA-agarose a relatively big amount was found in both, the elution fractions and the stripping fraction (E1: 4.1%, E2: 44.7% and S: 13.5%), showing the strong binding of rhEPO to WGA.

The purity of rhEPO in the elution fractions of the serotonin material was between 60% and 80% compared to about 39% in the starting material rhEPObe showing that rhEPO was, besides the fractionation, also separated from other proteins. Regarding the serotonin materials it seems that purity decreased at high salt concentration. A reason might be that other proteins bound to the column even stronger than rhEPO and are eluted at higher salt concentration. This would also explain the elution of proteins during stripping while no rhEPO was detected in these fractions. Therefore it would be interesting to figure out the nature of these proteins in future studies i.e. by the use of mass spectrometry (MS). Others have already shown that serotonin binds albumin [43]. an unglycosylated protein, as well as hydrophobic amino acids [44].

In contrast, for WGA-agarose the purity increased from 37% in elution fraction 1–72% in elution fraction 2. In particular, the stripping fraction seems to contain mainly rhEPO albeit in low concentration (5.2 μg/ml). Together with the recovery results this confirms that rhEPO is bound specifically and stronger than other proteins to WGA.

3.5. Glycoanalysis

After affinity chromatographic fractionation glycoanalysis of rhEPO-containing fractions was performed by xCGE-LIF to characterize rhEPO *N*-glycosylation and to determine its degree of sialylation. Only fractions which contained rhEPO where analyzed (see Table 2). For serotonin-CNBr, rhEPO was found in elution fractions 1–3. For serotonin-NHS, rhEPO was only found in elution fraction 1 and 2. When using WGA only two elution fractions where obtained, which both contained rhEPO. In contrast to the other materials, rhEPO was also found in the stripping fraction. Prior to xCGE-LIF measurements rhEPO was isolated from other proteins by SDS-PAGE. The distinct band at the molecular weight of rhEPO was excised from the gel and used for further analysis. For semi-quantitative evaluation and comparison of rhEPO glycosylation of the different chromatographic fractions, RPHs from xCGE-LIF derived *N*-glycan fingerprints were calculated. RPHs are the ratio of peak height to the total height of all peaks within the *N*-glycan fingerprint and correlate with the relative amounts of the respective glycans in a sample. Based on our database (Table 1) major peaks of the rhEPO *N*-glycan fingerprint (Fig. 1) could be assigned to one or more glycan structures.

Fig. 6 shows the RPHs of each glycan peak in all elution fractions (that contained rhEPO) for every resin. For all rhEPO containing samples, a high abundance of sialylated *N*-glycan structures was found (represented by the RPHs of xCGE-LIF peaks 1–13), while the non-sialylated *N*-glycan structures beyond peak 13, showed only low abundance (not shown in the figure). Even though all analyzed samples contained rhEPO with mainly sialylated glycan structures and the xCGE-LIF electropherograms of the analyzed samples (data not shown) had a similar *N*-glycan fingerprint like pure rhEPO (Fig. 1), it cannot be excluded completely, however, that some glycans derive from proteins with a similar molecular weight as rhEPO that were not separated by SDS-PAGE prior to xCGE-LIF. This influence, if there is any, is not believed to be significant but will be evaluated in future experiments by MS-based proteomic analysis of individual gel-bands.

After purification, main changes in the RPHs can be seen for peaks 1, 5 and 7 for all matrices. While peak 1 and 7 are assigned to glycan structures with 3 and/or 4 sialic acids, peak 5 is assigned to a glycan structure with only two sialic acids. Therefore, these peaks are indicators for an increase or decrease in the degree of sialylation. The standard deviations of peaks 5 and 7 are rather low so that changes in RPHs shown later are believed to be significant. In case of peak 1, which consists of two glycan structures which are slightly separated leading to varying peak heights, the standard deviation is higher and differences in RPHs are not significant but still follow expected trends. It also has to be considered, that standard deviation of the rhEPObe RPHs shown in Fig. 6 (white bars) result from three separate measurements, meaning that materials like SDS-PAGE gel, chemicals and enzymes were prepared separately for each measurement. In contrast glycan analysis of the chromatographic fractions was performed in one run, meaning they were all put on one SDS-PAGE gel and treated with the same batch of prepared chemicals and enzymes. Thus, it is believed that differences in RPHs among the chromatographic fractions are more significant than those between the chromatographic fractions and the starting material rhEPObe, especially for peak 1.

When using serotonin-CNBr for rhEPO fractionation, the starting material rhEPObe was clearly separated into 3 fractions with a different degree of sialylation (Fig. 6A). This can be seen, for instance, by an increase in the RPHs of peak 1 and 7 while RPH of peak 5 is decreasing. Compared to the starting material rhEPObe (white bars), Fig. 6A indicates for elution fraction 1 a decreased degree of sialylation, for elution fraction 2 sialylation is similar and for elution fraction 3 the degree of sialylation increases.

The same is more or less true for fractions obtained from serotonin-NHS (Fig. 6B). However, rhEPO was not found any more in elution fraction 3 and the RPHs of elution fraction 1 and 2 seem to correspond in heights to RPHs found in elution fraction 2 and 3 of serotonin-CNBr. Elution fraction 2 of serotonin-NHS shows even a higher degree of sialylation compared to elution fraction 3 of serotonin-CNBr. This shows that rhEPO, with a high degree of sialylation, elutes earlier from serotonin-NHS compared to serotonin-CNBr, again indicating that rhEPO binds less strongly to the serotonin-NHS matrix. However, also when using serotonin-NHS, the starting material rhEPObe could be separated into fractions of different degree of sialylation and taking into account recoveries (Table 2), the highest amount of rhEPO with a sialylation degree exceeding that of the starting material could be recovered in elution fraction 2 of the serotonin-NHS affinity matrix.

Table 1
In house data base. *N*-Glycan structures assigned to dominant peaks of the recombinant human erythropoietin final bulk *N*-glycan fingerprint. Peak number refers to the indicated peaks of the fingerprint (Fig. 1). Column "*N*-glycan structure" shows structures, which are identified to be beyond the indicated peaks. Braces indicate that the exact position of the sialic acids is not known. Symbols: (▼) Fucose; (■) *N*-acetylglucosamine; (●) Mannose; (○) Galactose; (◆) Sialic acid.

Peak number	N-glycan structure	No. of sialic acids
1		3 or 4
5		2
7		3 or 4
10		1 or 3
11		1 or 3
12		2 or 3
13		2
21		0
22		0

Table 2
Concentration, recovery and purity of all fractions collected from serotonin-CNBr, serotonin-NHS, and WGA-agarose. Total protein (measured by CBQCA) and recombinant human erythropoietin (rhEPO) (measured by rhEPO specific ELISA) concentration of all fractions of the separation runs. Recovery based on loaded material (partially purified rhEPObe); purity is the percentage ratio of rhEPO to total protein. Partially purified recombinant human erythropoietin (rhEPObe) (dialyzed against running buffer (RB) 20 mM sodium phosphate, pH 6.0) was loaded (20% of dynamic binding capacity) onto the respective column (purity of rhEPObe was 39.2 ±5.6%). Running buffer (RB) was 20 mM sodium phosphate, pH 6.0, and elution buffer (EB) was RB plus 0.3 M NaCl (0.5 M for WGA). Elution steps for serotonin-CNBr and -NHS were 10, 30 and 100% EB, and for WGA-agarose 25% and 100% EB.

Fraction[a]	Protein-CBQCA				rhEPO-ELISA				Purity	
	Concentration ± SD (*n* = 3) /μg ml^{-1}		Recovery ± SD (*n* = 3) /%		Concentration ± SD (*n* = 2) /μg ml^{-1}		Recovery ± SD (*n* = 2) /%		EPO of protein ± error[b] /%	
CNBr										
FT	2.1	±0.2	2.3	±0.2	ND		ND		ND	
E1	2.5	±0.8	2.2	±0.7	1.8	±0.9	4.1	±2.0	71.6	±56.7
E2	25.1	±2.7	22.5	±2.4	19.5	±1.6	44.7	±3.7	77.5	±14.5
E3	9.2	±1.2	8.2	±1.1	5.9	±0.5	13.5	±1.0	63.8	±13.5
S	1.5	±0.4	0.7	±0.2	ND		ND		ND	
Sum			**35.9**	**±4.6**			**62.3**	**±6.7**		
NHS										
FT	2.4	±0.4	3.7	±0.7	ND		ND		ND	
E1	14.0	±0.2	17.1	±0.2	11.0	±1.8	34.4	±5.6	78.5	±13.9
E2	15.5	±2.2	19.0	±2.7	10.0	±1.1	31.3	±3.6	64.3	±16.5
E3	5.1	±0.5	6.2	±0.7	ND		ND		ND	
S	1.6	±0.3	1.0	±0.2	ND		ND		ND	
Sum			**47.0**	**±4.5**			**65.7**	**±9.2**		
WGA										
FT	3.2	±1.1	4.4	±1.5	ND		ND		ND	
E1	4.7	±1.2	5.3	±1.4	1.8	[c]	5.0	[c]	37.0	±9.6
E2	7.0	±2.5	7.7	±2.8	5.1	[c]	14.4	[c]	72.6	±26.2
S	3.6	±1.0	2.0	±0.6	5.2	[c]	7.4	[c]	146.7	±43.1
Sum			**19.4**	**±6.3**			**26.8**	[c]		

ND, not detected.

[a] Pooled fractions of each process step (FT = flow through fraction; E1 = elution fraction 1; E2 = elution fraction 2; E3 = elution fraction 3; S = stripping fraction).

[b] Based on error propagation from SD of c_{prot} and c_{rhEPO}.

[c] Not enough sample for duplicates.

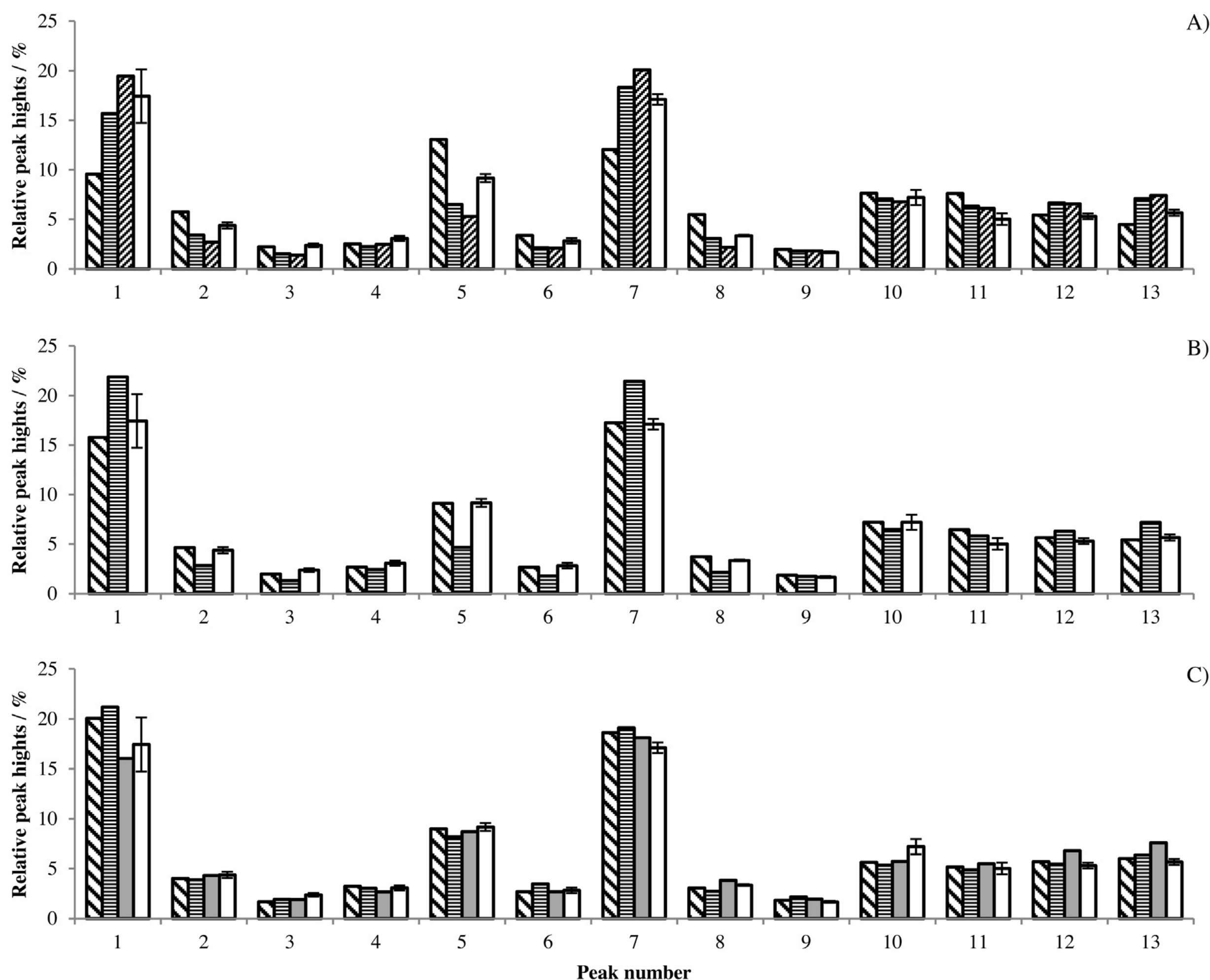

Fig. 6. Relative peak heights of partially purified recombinant human erythropoietin (rhEPObe)-containing fractions of the runs with (A) serotonin-CNBr and (B) serotonin-NHS and (C) WGA-agarose (Elution fraction 1 ( ); elution fraction 2 ( ); elution fraction 3 ( ); stripping fraction ( ). The white bars (□) refer to the starting material rhEPObe and the error bars present SD of three CGE-LIF measurements (error bars omitted for missing fractions of the respective matrix or for fractions not containing rhEPO). The peak numbers refer to the peak numbers of *N*-glycan fingerprints (see Fig. 1) and thus to the glycan structures assigned to the peaks (see Table 1).

In case of WGA-agarose (Fig. 6C) no distinct change in the RPHs within the *N*-glycan fingerprints can be seen. Only the stripping fraction shows a decrease in the RPH of peak 1 suggesting, contrary to expectations, that rhEPO found here was less sialylated. WGA is often described in literature as an affinity ligand for N-glycosylated proteins. On the other hand the binding of glycans to WGA is described as complex and is not fully understood yet. Thus it is difficult to identify an optimal elution strategy. Other studies have shown that even the use of buffers containing up to 1 M of GlcNAc only resulted in a recovery of 52% when the glycoprotein glycophorin was bound to WGA-Sepharose [46]. Therefore, it is possible that rhEPO with a higher degree of sialylation cannot be eluted sufficiently from WGA-agarose. However, even in the case that elution conditions can be further optimized, the use of lectins for purification of biopharmaceuticals is problematic as it involves the risk of negative side reactions [34–36]. Therefore, lectins should be considered only for use as a positive control rather than ligands for downstream processing of recombinant proteins.

From these results it can be concluded that both, serotonin-CNBr and -NHS, can be used for the separation of rhEPObe into fractions containing rhEPO with different degrees of sialylation. In comparison of the glycosylation pattern of the starting material it seems that fractions with an increased degree of sialylation could be accumulated. With respect to the standard deviation, especially of peak 1, that difference might however not be significant.

For the last rhEPO elution fractions, purified by the serotonin matrices, the glycans assigned to peak 1 and 7 of the *N*-glycan fingerprint make about 40% of all glycans. That is in good agreement with previous studies where it has been shown that rhEPO expressed in CHO cells contains about 40% of tetrasialylated tetraantennary *N*-glycans [13,59]. It was also stated that those glycans are the main determinant for in vivo bioactivity, while trisialylated tetraantennary and triantennary *N*-glycans have no correlation with in vivo bioactivity and the rest is negatively correlated [13].

Other studies have already shown that serotonin derivatized matrices can well separate glycans with a different content of sialic acid as well as sialylated from non-sialylated proteins [41–44]. While most of the studies focused on the separation of glycans released from proteins [42–44] in this study the separation of glycoforms of a single glycoprotein was investigated. Paskhughes [41] could separate sialylated from desialylated human chorionic gonadotrophin by serotonin derivatized LiChrosorb Diol HPLC but no further fractionation of sialylated protein was achieved. To our knowledge, this is the first study in which glycoforms of a single protein were separated in terms of sialic acid content by preparative affinity chromatography.

4. Conclusions

In this study it was shown that the use of serotonin-coupled matrices in downstream processing offers options for improving the product quality of rhEPO by increasing the ratio of tetrasialylated tetraantennary *N*-glycans in the end product. In particular, it enables separation of low sialylated rhEPO glycoforms from highly sialylated ones as demonstrated by xCGE-LIF analysis. Both, use of serotonin affinity chromatography to isolate rhEPO with a high degree of sialylation and use of xCGE-LIF for at-line monitoring could be very useful for design and optimization of pharmaceutical processes for production of rhEPO. In addition, this study can be considered a basis for further investigations towards glycoprotein glycoform separation and for testing of new ligands by linking chromatographic processes with a powerful glycoanalysis method that leads to an improved understanding of purification trains.

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