Applications of a salt tolerant cation exchanger carrying sulfate groups

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Highlights

- Modeling of acid-base properties of sulfonic type and sulfate type ligands
- Difference in salt tolerance and hydrophobicity of sulfonium and sulfate cation-exchangers
- Separation of vitamin K dependent clotting factors from human plasma by use of salt-tolerant sulfate type cation-exchanger
- Isolation of active clotting factor IX and removal of harmful contaminants
- Immediate application of feedstock or eluate from previous isolation step to sulfate-based resin

Abstract

Difference between two strong cation-exchange resins, namely sulfonium type and sulfate type regarding both their salt tolerance and hydrophobicity were investigated. There is only tiny variation between sulfate and sulfonic group and at the first glance it seems unlikely that it could be the reason for changed selectivity and salt tolerance that was detected in our preliminary experiments. For that reason salt tolerance and hydrophobicity of both ligands was investigated by using two representative polymethacrylate-based ion exchangers as for the sulfonium type TOYOPEARL GigaCap S-650M and for the sulfate type TOYOPEARL Sulfate-650F. In addition some in-silico calculations were performed for model substances representing the sulfonium and sulfate group, and significant differences were calculated regarding their hydrophobicity. These experiments confirmed the working hypothesis that salt tolerance and higher affinity and selectivity for some human plasma derived vitamin K dependent clotting factors and inhibitors are interrelated and dependent from the presence of the sulfate group. The affinity for these proteins was experimentally verified by separation of clotting factor IX from the prothrombin complex concentrate. Presented results show that a simple and fast separation between clotting factor IX and other vitamin K dependent clotting factors II, VII and X is possible, only if the resin with the sulfate, and not with sulfonic acid ligand was applied. Consequently, an immediate application of undiluted feedstock or the eluate from previous isolation step to sulfate resin is possible, and a significant optimization of downstream process can be achieved.

Introduction

Capturing is the first step in chromatographic downstream processing, and there is still a need to avoid a conditioning step to apply the feedstock directly to the chromatographic resin. At this early stage of purification, ion exchange is still the most frequently used chromatographic step. Ion exchange based chromatographic supports are robust and chemically resistant, they have relatively high capacity and there are no problems with their regeneration and sanitation. However, salt concentrations of fermenter supernatants and/or clarified cell homogenates as well as other raw materials like human plasma are usually higher than physiological sodium chloride solution with a conductivity of about 10 mS/cm. In order to enable high capacity binding and optimal enrichment of target molecules, an expensive dilution or ultrafiltration/diafiltration step is frequently necessary. A consequence is that the production costs are high and even years after there is still to a need to design a process that enables direct application to the chromatographic resin without additional processing [1-3].

Ion exchange chromatography of proteins starts in diluted buffer solutions with low conductivity values (usually lower than 1 mS/cm). The electrostatic interactions between the target molecule and the resin are necessary in this chromatographic mode. In order to enable the optimal binding of the target substance to the resin, ion exchange chromatography starts with sample application in buffer solutions with low conductivity [2,3]. Elution of the bound molecules is usually accomplished by an increase in salt concentration. Consequently, it appears as a conflict to perform ion exchange with salt tolerance because the main operation parameter is drastically changed. In addition to above discussed topics, there are some additional problems that come along with the salt tolerance in ion exchange chromatography:

1. The binding capacity of biomolecules to chromatographic support could significantly decrease;
2. Salt gradient is not sufficient for elution;
3. pH gradients are necessary;
4. The product recovery and activity could be negatively influenced.

The problem to solve is now how to achieve salt tolerance for an ion exchange resin. Burton and Harding [4] present several opportunities of applications of higher salt concentrations in starting buffers in industrial chromatography. In general, salt tolerance in biochromatography is more the rule than an exception. Many modes like bioaffinity chromatography or pseudoaffinity chromatography (Protein A, Protein L, metal chelate chromatography, thiophilic adsorption) or hydrophobic interaction chromatography are highly efficient at higher salt concentrations [5-7]. Unfortunately, this is not the case for the ion exchange chromatography. A common

possibility to achieve salt tolerance is to use a combination of ionic with hydrophobic groups. There are two ways to get this kind of separation:

1. In a tailor-made ligand or

2. To combine a hydrophobic base matrix with a hydrophilic ligand [2].

The combination of hydrophobic motifs (alkyl-, aryl- or electronic donator acceptor groups) with weak cationic or anionic groups are usually described as mixed- mode adsorbents. Salt-independent adsorption was found for hydrophobic amine ligands as well as for alkyl carboxy matrices. Salt concentration had only a little effect on binding capacity of this kind of resins. Carboxylate and amine groups are predominately ionized at pH 7. They are only charged at pH values below 3 and above 10.5. For that reason in charge, weaker bases and acids, frequently pyridine, phenolic other modified aromatic groups are used. The adsorption is carried out at pH 7. The substance of interest is eluted in the next step by decreasing or increasing the pH value. This mode is well known as hydrophobic charge-induction chromatography, and a lot of chromatographic resins with the ionic-hydrophobic type of ligand are commercially available as so-called multimodal or mixed mode materials [4,8].

Another interesting opportunity for salt tolerance was described in the paper from Johansson et al. [9]. This group has found that non-aromatic alkyl anion-exchange ligands based on primary or secondary amines (or both) are suitable for protein capturing at high salt concentrations. The binding mechanisms of these non-aromatic mixed mode ligands under given conditions are described as a combination of electrostatic interaction and hydrogen bonding. When these kinds of ligands were used, the recovery for bovine serum albumin was found to be superior compared to the aromatic type of ion exchangers. The elution of bound protein was effective by applying a combined pH and salt gradient. Yoshimoto et al. [3] investigated salt-tolerant of a weak anion-exchanger from alkyl amine type TOYOPEARL NH_2-750F for the removal of BSA aggregates. According to this study, salt tolerance and the so-called crowding effects of applied osmolytes arginine-HCl and polyethylene glycol [10,11] might be due to the combined effects of electrostatic and hydrophobic effects that influence retention of investigated proteins. Endres and Dabre [12] applied this salt-tolerant anion-exchange resin for continuous removal of antibody aggregates. The mechanism of salt tolerance for that resins is still unclear.

Most of the multi-modal or mixed mode cation-exchangers carry weak cationic groups. As described, the suppression of ionization as function of the pH value, is a reason of their multimodality.

Because of this a multi-modal kind of function, the use of strong cation exchanger seems to be not possible for this kind of chromatography. The reason is that the ionic group is charged over the entire pH range.

However, according to our preliminary investigations, a strong cation exchanger TOYOPEARL Sulfate-650F carrying sulfate acid group has a higher salt tolerance than for other commercially available sulfonium type strong cation exchangers tested in parallel experiments (https://www.separations.eu.tosohbioscience.com/solutions/process-media-products/by-mode/ion-exchange/cation-exchange/TOYOPEARL-sulfate-650f). Most of the applied cation exchangers are from the sulfonic acid type, and cation exchangers with sulfate groups are not frequently used. As shown in Figure 1, the sulfonic type ligand contains a covalent bond connection between the carbon atom from the resin matrix to the sulfur atom of the acidic group, and one oxygen atom from the original sulfate ion is replaced by the carbon group. At the other hand, the sulfate ester group contains a sulfur group that is bound to an oxygen atom and via this oxygen to a carbon atom of the resin (Figure 1).

Figure 1:
The difference between sulfonium type ion exchangers (left) and sulfate type ion exchanger (right side)

Sulfate type chromatographic resins are more often described for application in affinity chromatography. Dextran sulfate and heparin are polyelectrolyte polymers that contain the sulfate group bound to a polysaccharide backbone. Resins with immobilized sulfate-type polymers are commercially available like a crosslinked agarose with either immobilized dextran sulfate (Capto DeVir S, comp. Cytiva) or heparin ligand also covalently bound to crosslinked agarose (e.g. Heparin Sepharose Fast Flow 6B, Cytiva). Both resins are used for the purification of antithrombin III, blood coagulation factors like FIX, FVIII-von Willebrand complexes, lipoproteins, DNA binding proteins and various virus types [13-15]. Plasma-protein binding capacity and selectivity of antithrombin III, fibrinonectin, as well as vitamin K dependent clotting factors and inhibitors are similar for the heparin type resins as well for polysaccharide-based resins with immobilized sulfate groups. The specific binding of vitamin-K dependent proteins seems to be thus not dependent on the carbohydrate backbone of the polyectrolyte chain, but from something else which could not be identified. The only mutual similarities of all resins is: They all are containing a sulfate group. As a working hypothesis we assumed that salt tolerance

and higher affinity and for plasma proteins are interrelated and dependent from the presence of the sulfate group [13-18].

On the first glance it appears to be unlikely that the really tiny difference between sulfate group and sulfonic group could be the reason for the changed affinity and salt tolerance. But as described above is salt tolerance in some mixed mode ligands related to their hydrophobicity. For that reason we have experimentally investigated the salt tolerance and hydrophobicity of both ligands by using two representative ion exchangers as for the sulfonium type TOYOPEARL GigaCap S-650M and for the sulfate type TOYOPEARL Sulfate-650F. Despite the mean pore sizes of and the particle sizes are different are the base matrices as polymethacrylates almost the same. In addition some in-silico calculations were performed for model substances representing the sulfonium and sulfate group. The affinity for clotting factors was experimentally verified by the separation of highly pure FIX from the prothrombin complex concentrate [19]. Presented results show that a separation between clotting factor IX from other vitamin K dependent clotting factors II, VII and X is possible, only if the resin with the sulfate ligand was applied. Additionally, starting buffer with high salt concentration was applied, and the elution was performed by a change of salt concentration and pH. Consequently, an above discussed processing of the feedstock or the eluate from previous isolation step is not necessary.

Materials and Methods

Chemicals

Cation-exchange resins TOYOPEARL GigaCap S-650M (strong cation-exchanger sulfonic acid type, cf. Fig. 1), particle distribution 50-100 µm, ion exchange capacity : 0.1 -0.2 eq/L, protein binding capacity: 136-176 g/L (human IgG), TOYOPEARLSulfate-650F (strong cation exchanger with sulfate group as ligand, cf. Fig.1), particle size distribution 30-60 µm, ion exchange capacity : min 0.53 eq/L, protein binding capacity: min 114 g/L (human IgG), and TOYOPEARL Butyl-650M (40-90 µm) support for hydrophobic interaction chromatography were all from Tosoh Bioscience (Griesheim, Germany). Heparin Sepharose affinity chromatography resin and other Sepharose-based resins were from Cytiva (former GE, Freiburg/Br. Germany). All other chemicals were purchased were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (Darmstadt, Germany), or Fluka Honewell (Charlotthe, NC, USA). Water for chromatography was supplied from in-house deionization system. .

Octaplex, human prothrombin complex concentrate, containing 500 IU/vial clotting factor IX (FIX) and other vitamin K dependent clotting factors (FII, FVII and FX) and inhibitors (Protein C, Protein S and Protein Z) and Octanyne F (500 or 1000IU of

FIX/vial), clotting factor IX concentrate were from Octapharma Pharmazeutika Prod. Ges.mbH (Vienna, Austria).

Human plasma was purchased from Institute of Transfusion Medicine (Zagreb Croatia). Institutes Ethical Committee approved the use of human plasma for experimental purposes.

Chromatography

Experiments were performed by use Äkta Explorer chromatographic system (Cytiva) with a 2mL sample loop, and a 1mL SkillPak column (Tosoh Bioscience, Griesheim, Germany) containing different chromatographic supports was used. For optimized separation on TOYOPEARLSulfate-650F separation following buffers were used; Buffer A (starting buffer), 400mM NaCl, 20mM phosphate , pH 4.5, 39.1 mS/cm; Collected fractions were eluted with Fraction 1, Buffer 1: 300 mM NaCl, 20mM phosphate buffer pH 4.52, 30.7 mS/cm, Fraction 2, Buffer 2: 250Mm NaCl, 20mM phosphate buffer, pH 4.58, 27.2 mS/c, Fraction 3, Buffer 3: 250M NaCl, 20mM phosphate buffer, pH 6.07, 25.2 mS/cm, Fraction 4, Buffer 4: 250M NaCl, 20mM phosphate buffer, pH 7.19, 24.8 mS/cm, Fraction 5, Buffer 5: 50M NaCl, 20mM phosphate buffer, pH 7.2, 8.2 mS/cm, Fraction 6, Buffer 6: 20 mM NaCl, 20mM phosphate buffer, pH 7.18, 4.71 mS/cm, Fraction 7, Buffer 7: 10mM phosphate buffer, pH 8.06, 1.7 mS/cm. Other experimental conditions are listed in Figure Legends.

Determination of coagulation activity

For determination of activity of coagulation factors, a Stago coagulation analyzer was used (Diagnostica Stago S.A.S., Asnières sur Seine Cedex, France). Determination of the activity of vitamin K dependent coagulation factors (FII, FVII, FIX and FX) was performed according to the producer's protocol (see also Ref. [11] and [12]). Following reagents were purchased from Stago: STA-Deficient II, STA-Deficient VII, STA-Deficient IX , STA-Deficient X, STA-Owren-Koller, Neoplastine Cl, C.K. Prest. Rox Factor IX chromogenic kit was purchased from Rossix (Mölndal, Sweden).

SDS-PAGE and immunoblot

Determination of protein concentration by use of BCA protein assay kit (Thermo Scientific Pierce, Waltham, MA, USA), SDS-PAGE under reducing conditions and immunoblot were performed as described previously [20]. Shortly: the amount of protein loaded in each well of the gels for SDS-PAGE was 10 µg, proteins were separated and stained. For the immunoblot, proteins were transferred to a PVDF membrane (Serva, Heidelberg, Germany). Primary antibodies used were following:

monoclonal mouse anti-FII antibody (Santa Cruz Biotechnology, Heidelberg, Germany), monoclonal mouse anti-FVII antibody and monoclonal mouse anti-FIX antibody (both from Sigma-Aldrich), polyclonal rabbit anti FX antibody (Thermo Fisher, Rockford, IL). Both goat anti-rabbit and anti-mouse IgG, HRP conjugates were from Merck-Millipore (Burlington, MA, USA).

Hydrophobicity measurements
Two ml of sedimented resin were suspended in 200 ml of a 2 M ammonium sulfate solution with 20 mM phosphate buffer containing 1 mg/ml hen egg white lysozyme pH=7. The solution was shaken overnight. The concentration of the supernatant was determined at 280 nm. The bound concentration was calculated as the difference to a standard solution without resin. At the end the resin were washed with 2 M ammonium sulfate + 20 mM phosphate buffer pH 7 and the bound protein eluted with 20 mM phosphate puffer pH 7. The recovery was calculated as the difference between bound and eluted protein.

Calculation of the ligand hydrophobicity

An in-silico calculation of the pK_S value and the octanol-water distribution coefficient was executed by using the program package Cosmotherm (Cosmologic, Biovia Dassault Systems, Vélizy-Villacoublay, France). As the model substances methansulfonc acid and methylsulfate (both Sigma-Aldrich) were selected.

Comparison of salt tolerances between sulfonic and sulfate type cation exchangers

The dynamic protein binding capacities were measured with 1 ml pre-packed TOYOPEARL GigaCap S-650M and TOYOPEARL Sulfate-650F SkillPak columns from Tosoh Bioscience . The capacity was determined with an eluent of 1 mg/ml hen egg white lysozyme in 20 mM phosphate or acetate buffer and addition of sodium chloride. The pH was changed from 5 to 7 in 0.5 steps units. The sodium chloride concentration was varied from 0 to 500 mM with 50 mM steps. The protein solution was loaded with 100 cm/h to the column until 10 % breakthrough.
The heat plots were obtained by using SigmaPlot 13 software [21].

Results and discussion

Modelling of acid-base properties and hydrohobicity of sulfonic type and sulfate type ligands

As the model substances methylsulfonate and methylsulfate were selected. These substances are not fully representative for a resin bound ligand, but they contain the essential features:
- hydrophobic part;
- methyl group, representing the resin backbone and
- ionic part by the sulfonic and sulfate group.

An in-silico calculation of the pK_S value and the octanol-water distribution coefficient was executed by using the program package Cosmotherm. Results are presented in Table 1.

Model Substance	$\log (C_{Octanol}/C_{water})$	pK_a
Methanesulfonic acid	-1.13	-2.8
Methylsulfate	0.66	-4.4

Table 1: Calculated $\log(C_{octanol}/C_{water})$ and pK_a values of methanesulfonic acid and methylsulfate .

According to Table 1, both substances are strong acids and fully dissociated over the entire pH range. However, according to the calculation presented in this Table, methylsulfate is hydrophobic ($\log (C_{Octanol}/C_{water})$ has a negative value) while methanesulfonic acid is hydrophilic (with a positive $\log (C_{Octanol}/C_{water})$ value). The difference in hydrophobicity of both groups should be measurable using hydrophobic interaction chromatography conditions.

Hydrophobicity measurement

The hydrophobicity of the sulfonium type strong cation exchanger TOYOPEARL GigaCap S-650M and the sulfate type cation exchanger TOYOPEARL Sulfate-650F at hydrophobic interaction conditions was measured by the determination of the lysozyme binding capacity.
Strong cation exchangers that are intended to be used for separations of proteins are usually low in hydrophobicity. Other nonspecific interactions are also suppressed in order to maintain high recoveries of proteins of interest. The applications of high concentrations of cosmotropic salts are thus not common. However, in presented experiment 2 M ammonium sulfate was used to enhance the hydrophobic character as loading condition in order to test both ion exchange resins. Lysozyme binding capacity of each investigated resin was compared to the binding capacity of TOYOPEARL Butyl-650M, a resin that has been applied for hydrophobic interaction chromatography [19]. Results of static binding capacities of investigated resins are shown in Figure 2 and Table 2.
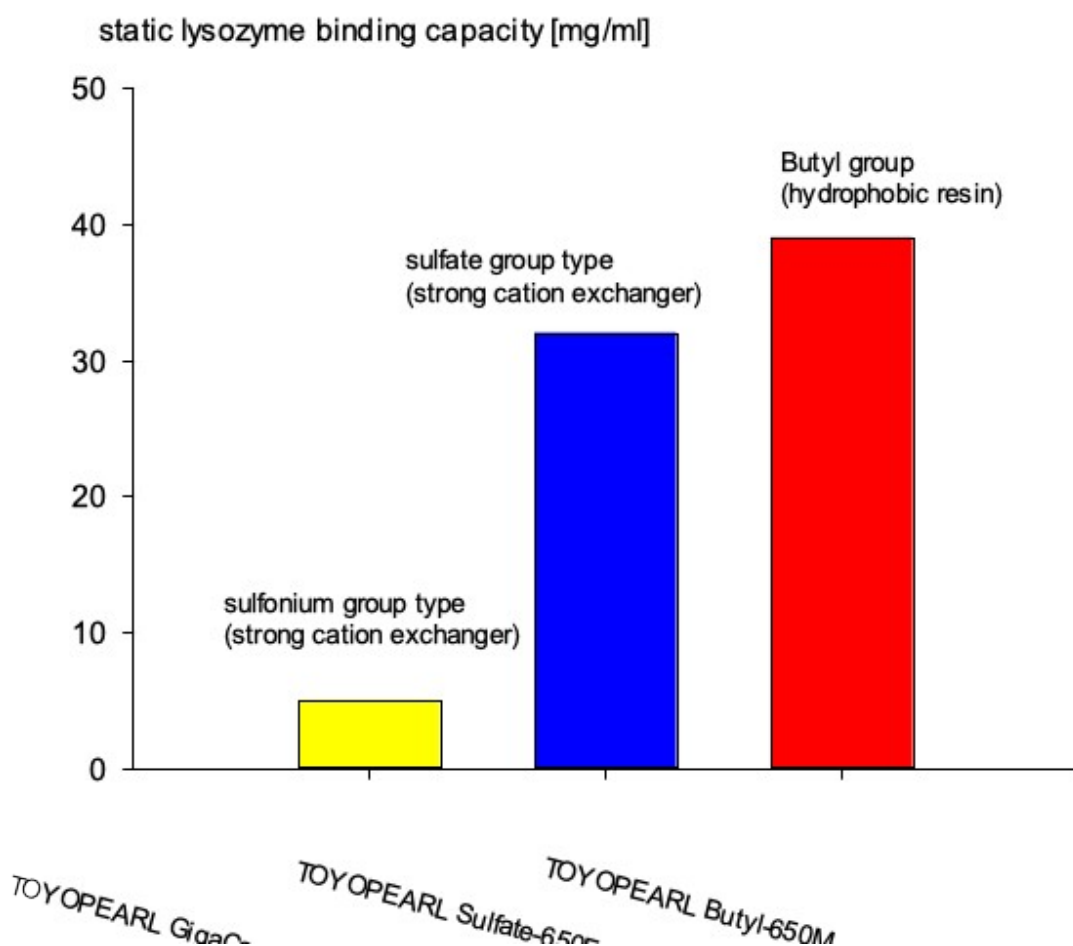
Figure 2:
Lysozyme static binding capacity of TOYOPEARL GigaCap S-650M (sulfonium type strong cation exchanger), TOYOPEARL Sulfate-650F (sulfate type strong cation exchanger) and TOYOPEARL Butyl-650M in 2 M ammonium sulfate buffer pH 7.

Resin	Lysozyme recovery [%]
TOYOPEARL GigaCap S-650M	98
TOYOPEARL Sulfate-650F	96
TOYOPEARL Butyl-650M	73

Table 2: Lysozyme recovery after loading at 2 M ammonium sulfate, pH 7

The base material of all resins is the polymethacrylate TOYOPEARL gel. Consequently, the observed difference is mainly the result of the surface modification. A big difference was found between the binding capacities of TOYOPEARL GigaCap S-650M with lysozyme static binding capacity of 5 mg/ml and TOYOPEARL Sulfate-650F with 32 mg/ml. As shown in Figure 2, the binding capacity of the investigated sulfate resin is closed to the value obtained for routinely used hydrophobic interaction resin TOYOPEARL Butyl-650M with 39 mg/ml [22]. These results confirm our above discussed hypothesis and results presented in Table 1 that the sulfate is much more hydrophobic than the sulfonium group at high ammonium sulfate concentration. Interestingly, the recovery of proteins is not different, and sulfate has only a slightly lower recovery than the sulfonium group (cf. Table 2). In addition to the hydrophobicity the salt tolerance of both resin was further investigated.

Comparision of salt tolerances between sulfonic and sulfate type cation exchangers

We have investigated the salt tolerance of both ligands by using two representative ion exchangers as for the sulfonium type TOYOPEARL GigaCap S-650M and for the sulfate type TOYOPEARL Sulfate-650F.
Heat plots obtained by using SigmaPlot 13 are shown in Figures 3 and 4. To directly compare the salt tolerance of both resins their capacities were normalized to the maximum measured binding capacity.
The normalized plots show that salt tolerance of the sulfonium type resin (TOYOPEARL GigaCap S-650M) is at 100 mM sodium chloride level. The salt tolerance of the sulfate type resin (TOYOPEARL Sulfate-650F) is in much higher range of 300 mM sodium chloride. Consequently, in addition to the increased hydrophobicity the sulfate resin is much more salt tolerant.
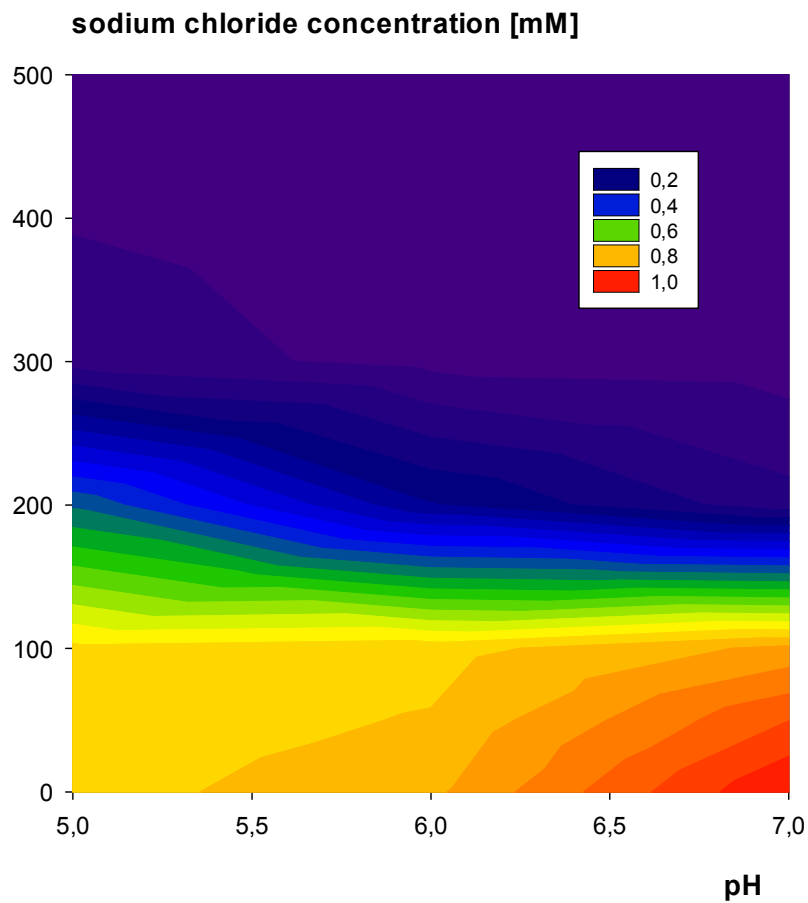
sodium chloride concentration [mM]

Figure 3:   Heat Plot of pH and Salt Concentration Dependence for normalized binding capacity of Lysozyme for TOYOPEARL GigaCap S-650M
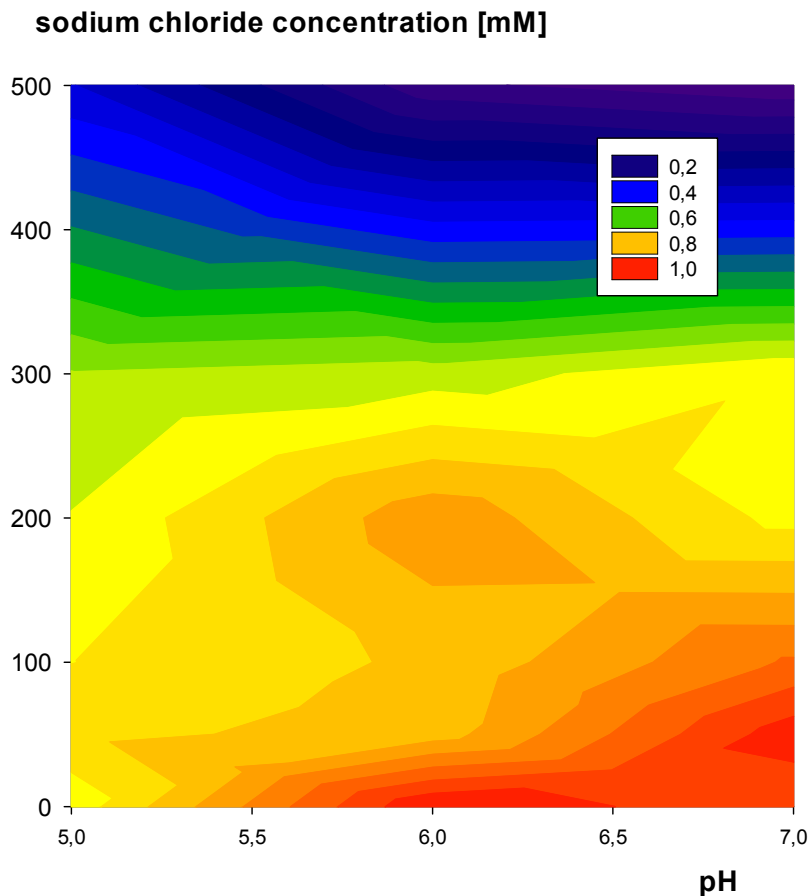
sodium chloride concentration [mM]

Figure 4:   Heat Plot of pH and Salt Concentration Dependence for normalized binding capacity of Lysozyme for TOYOPEARL Sulfate -650F

It could be argued that the change from sulfonic to sulfate group increases the overall hydrophobicity. The sulfate group is thus finally a very small mixed mode ligand. To find evidence for this hypothesis, we went first in the literature to check about already obtained experimental results for the difference of the both ligands. A lot of information is available about acid-base properties of the sulfate groups but only very limited information about their hydrophobicity.

Abd ur Rahman [23] investigated the difference in water hydration of sulfate and sulfonic type ligand by dielectric spectroscopy [24] using sodium methylsulfonate and sodium methylsulfate as model substances. A higher hydration number at infinite dilution was found for methylsulfate compared to methylsulfonate. At higher concentrations the hydration numbers of methylsulfate and methylsulfonate are different but the hydration numbers from the methylsulfate and the sulfate ion

in solution are identical. Analysis of these results leads to hypothesis that in the case of higher ligand density of the immobilized sulfate it can be considered as an cosmotropic type of cation exchanger ligand, and it behaves like the sulfate ion in water solution.

However, the higher hydration number alone does not allow drawing a conclusion about changed hydrophobicity of the sulfate ligand. More experimental confirmations are still necessary, but following application documents substantial difference between two investigated ligands regarding their protein binding and selectivity.

Isolation of physiologically active clotting factor IX from human plasma and removal of other vitamin K dependent clotting factors

The intention in modern process development is to design a downstream purification process that avoids conditioning steps like ultrafiltration/diafiltration, and that capturing by use of a tailored resin is the first step in downstream process. Exactly this step is frequently critical further movement in direction of continuous purification. As mentioned above, ion-exchange chromatography is the method that is still most frequently applied in this early stage of purification process, but problems of low salt resistance of still remains [1,2,25]. In order overcome these problems, mixed-mode resins were developed. These supports are modified with multimodal ligands that enable both selectivity and salt tolerant adsorption of product of interest, and modern downstream process can be reduced to only 2-3 separation steps. However, with reduction of purification steps the risk of remaining contaminants is increasing and developing of optimized chromatographic supports is necessary [26]. Affinity and pseudo-affinity chromatography, e.g. with polysaccharide based gels, frequently with different sulfate-based ligands could be ideal solution of this problem. Additional advantage of these materials is their ability to bind and remove viral contaminants [13]. However, there are frequent problems with chemical stability and virus safety of these supports, especially during the necessary sanitation procedure, mostly by use of concentrated sodium hydroxide solutions [15,19].

In last twenty years the downstream processing of biologically active biopolymers (and nanoparticles) is focused on purification of fermentation supernatants or cell lysates [1,2,23]. However, human plasma still remains an important raw material for production of therapeutic proteins like human IgG concentrates as well as concentrates of clotting factors and inhibitors [27]. Chromatography is the method of choice in plasma fractionation, and there is still a need for optimization of production processes in direction of process economy and safety [28]. Biopolymers

with sulfate-based ligands, mostly Heparin Sepharose, are used in polishing steps, especially for production of both recombinant and plasma derived clotting factor IX, and possible introduction of more stable and virus-safe substitutions for this resin was frequently discussed [15,16,27,29].

Cryopoor human plasma, it means human plasma after removal of cryoglobulins by precipitation at 4°C, is the starting material for isolation of vitamin K dependent clotting factors and inhibitors. The first, capture step is performed by so-called solid-phase extraction with an anion-exchange resin, mostly DEAE or Q Sephadex. Next step is elution of vitamin K-dependent clotting factors and inhibitors by a buffer that contains more than 0.3 M NaCl [27]. Subsequent use of salt-tolerant affinity or pseudo-affinity resin as next purification step for a target protein, in this case clotting factor IX is an important step in direction of process optimization [29]. Few years ago was demonstrated that polymethacrylate base strong cation-exchange resin TOYOPEARL Sulfate-650F with immobilized sulfate groups (cf. Figure 1) binds human immunoglobulin G (IgG), and that this binding is salt-tolerant. The elution of target protein can be performed by a change of both NaCl-concentration and pH [27].

We used same resin for separation of vitamin K dependent clotting factors, FII, FVII, FIX and FX. The starting material is prothrombin complex concentrate that is a mixture of plasma proteins enriched with above mentioned clotting factors and vitamin K dependent clotting inhibitors protein C, protein S and protein Z (15,27,29]. To demonstrate the salt-tolerance of the resin, we used a starting buffer with high NaCl concentration [cf. Figure 5]. As shown in Figures 5 and 6, separation of clotting factors FII, FVII and FX was achieved, and a fraction with highly enriched, single, active clotting FIX was isolated, and residual FX activity in FIX-containing fraction is completely removed. It is one of main criteria for quality of FIX clotting concentrate [14,15,27]. These results are comparable with the results of the FIX purification step by use of Heparin Sepharose (seeRef. [27,29]). However, a dilution or ultrafiltration/diafiltration step is necessary before the affinity chromatography [27], and this step can be avoided, when sulfate resin TOYOPEARL Sulfate-650F was used. High salt tolerance of this resin was already demonstrated for isolation of monoclonal antibodies and removal of aggregates, as well as isolation of polyclonal IgG [30]. To reach conditions similar to them in the production facility, step gradient for elution was applied. This gradient can be further simplified, but presented experiments were performed in order to demonstrate selectivity of investigated resin and its capability to separate closely related proteins that are very similar in their structure and function. This kind of selectivity can be usually achieved only by use highly specialized affinity or pseudo-affinity resin [15,18,27,29]. Interestingly, the sulfonate type resin TOYOPEARL GigaCap S-650M showed different behavior, and separation between clotting factors FIX and X could not be achieved (cf. Fig. S1,

S2A and S2B in the Supplement). Above presented differences between two investigated resins (cf. Figures 1-4 and Tables 1-2.) can explain different behavior regarding their salt tolerance and selectivity.
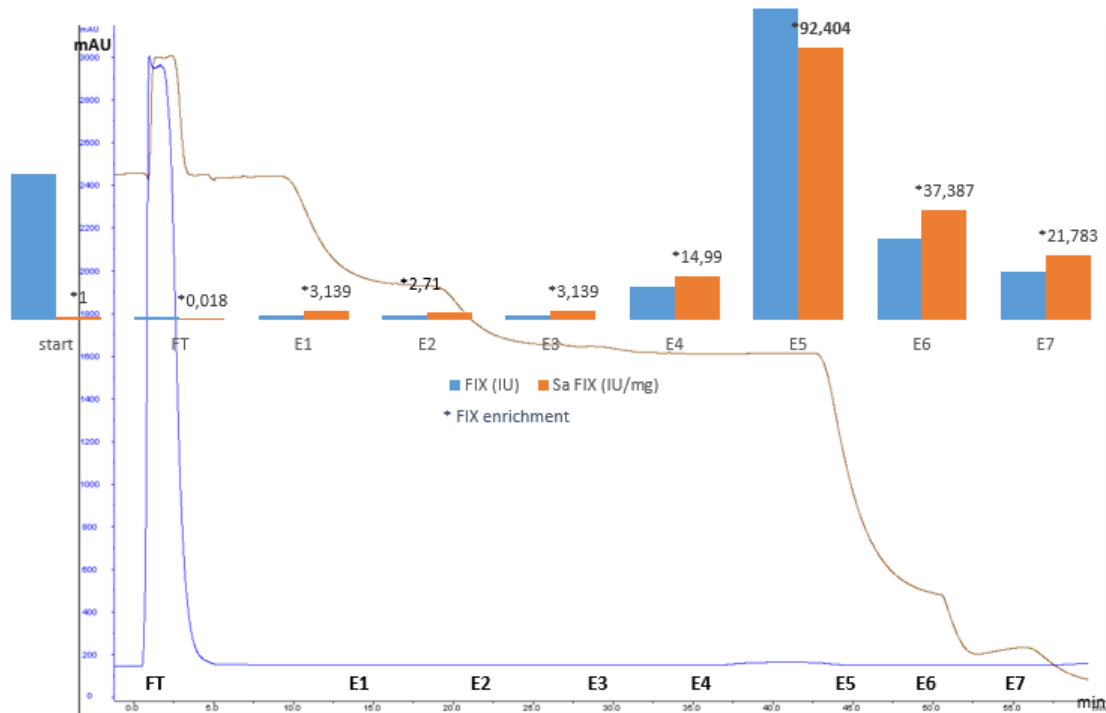

Figure 5.

Purification of clotting factor IX and removal of other vitamin K-dependent clotting factors (FII, FVII and FX) on sulfate resin TOYOPEARL Sulfate-650F. Conditions: Äkta Explorer chromatographic system (Cytiva) with a 2mL sample loop, and a 1mL column, flow 2mL/min, room temperature. Buffer A (starting buffer), 400mM NaCl, 20mM phosphate , pH 4.5, 39.1 mS/cm; Collected fractions were eluted with Buffers 1-7 (cf. Materials and methods). Blue - Factor IX activity (IU/mL) and enrichment (red) were presented. The activity of FX was detected only in the flow-through fraction, and no FX clotting activity was detected in the eluate.
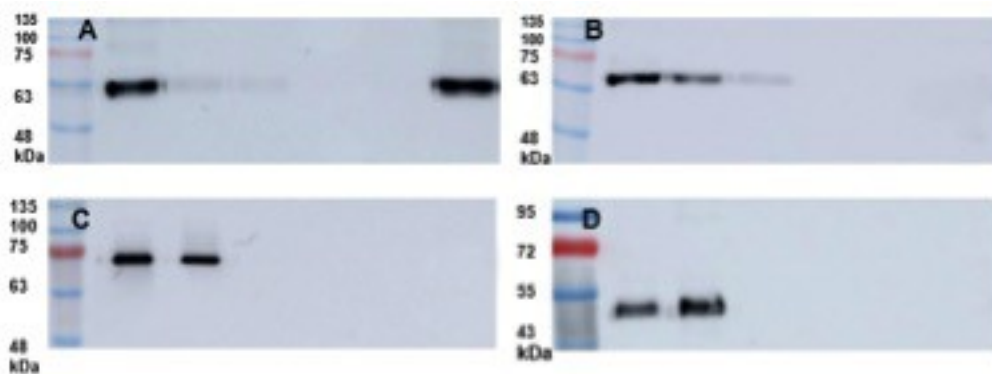
Figure 6.
SDS-PAGE and immunoblots of FIX (A), FX (B), FVII (C) and FII (D). Under optimized conditions, only FIX binds to the resin, and other vitamin K dependent clotting factors are separated.

Most investigations of salt-tolerance are performed for ion-exchange resins carry weak anion-and cation groups. There are less applications of strong ion exchanger resins.

Except the preliminary experiments with immunoglobulins [30], there are no comparable investigations about differences between salt tolerance and selectivity between sulfonate- and sulfate-based cation-exchange resins. Presented results are first step in the way to explain these differences. They also open new perspectives for use of salt-tolerant and pH stable sulfate resin for effective down-stream processing of biologically active plasma proteins.

Conclusions

- As a working hypothesis we assumed that salt tolerance and higher affinity and for plasma proteins are interrelated and dependent from the presence of the sulfate group.
- For this sake, the difference between two representative polymethacrylate-based strong cation-exchange resins, namely sulfonium type (TOYOPEARL GigaCap S-650M) and for the sulfate type (TOYOPEARL Sulfate-650F) were investigated regarding their salt tolerance and hydrophobicity.
- In addition some in-silico calculations were performed for model substances representing the sulfonium and sulfate group, and significant differences were calculated regarding their hydrophobicity.
- These experiments confirmed that the salt tolerance and higher affinity for some proteins are dependent from the presence of sulfate group.

- The affinity for human plasma-derived clotting factors and inhibitors was experimentally verified by the separation of clotting factor IX from the prothrombin complex concentrate. Presented results show that a simple and fast, salt-independent separation of clotting factor IX from other vitamin K dependent clotting factors II, VII and X is possible only when the resin with the sulfate, and not sulfonic acid ligand was applied.
- Consequently, an immediate application of undiluted feedstock from fermentation process, or other eluates with higher ionic strength to sulfate resin is possible, and a significant optimization of downstream processing in direction simplicity and continuous purification can be achieved.

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