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Preparation and characterization of prototypes for multi-modal separation aimed for capture of positively charged biomolecules at high-salt conditions

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Abstract

Several prototypes of aromatic (Ar) and non-aromatic (NoAr) cation-exchange ligands suitable for capture of proteins from high conductivity (ca. 30 mS/cm) mobile phases were coupled to Sepharose™ 6 Fast Flow. These new prototypes of multi-modal cation-exchangers were found by screening a diverse library of multi-modal ligands and selecting cation-exchangers resulting in elution of test proteins at high ionic-strength. Candidates were then tested with respect to breakthrough capacity of bovine serum albumin (BSA), human IgG and lysozyme in buffers adjusted to a high conductivity. By applying a salt-step or a pH-step the recoveries were also tested. We have found that aromatic multi-modal cation-exchanger ligands based on carboxylic acids seem to be optimal for the capture of proteins at high-salt conditions. Experimental evidence on the importance of the relative position of the aromatic group in order to improve the breakthrough capacity at high-salt conditions has been found. It was also found that an amide group on the α-carbon was essential for capture of proteins at high-salt conditions. Compared to a strong cation-exchanger such as SP Sepharose™ Fast Flow the best new multi-modal weak cation-exchangers have breakthrough capacities of BSA, human IgG and lysozyme that are 10–30 times higher at high-salt conditions. The new multi-modal cation-exchangers can also be used at normal cation-exchange conditions and with either a salt-step or a pH-step (to pH-values where the proteins are negatively charged) to accomplish elution of proteins. In addition, the functional performance of the new cation-exchangers was found to be intact after treatment in 1.0 M sodium hydroxide solution for 10 days. For BSA it was also possible to design cation-exchangers based on non-aromatic carboxyl acid ligands with high capacities at high-salt conditions. A common feature of these ligands is that they contain hydrogen acceptor groups close to the carboxylic group. Furthermore, it was also possible to obtain high breakthrough capacities for lysozyme and BSA of a strong cation-exchanger (SP Sepharose™ Fast Flow) if phenyl groups were attached to the beads. Varying the ligand ratio (SP/Phenyl) could be used for optimizing the function of mixed-ligand ion-exchange media.

Keywords: Multi-modal separation media; High-salt conditions; Capture; Cation-exchange ligands; Library of chromatographic media

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1. Introduction

Ion-exchange chromatography (IEC) is an efficient separation method that has been widely employed for the purification of proteins and enzymes [1–3]. This technique is primarily based on interactions between charged amino acids on the protein surface and oppositely charged immobilized ligands [4–6]. However, protein retention on an ionic surface is a much more complex process compared to ion-exchange with inorganic ions or individual amino acids and cannot be satisfactorily explained by the p*I*-value of a protein. Factors such as intramolecular charge asymmetry in the protein, the nature of the support surfaces and the mobile phase displacing ions also have significant impact on the retention [7,8]. Furthermore, there are other interactions that may influence IEC such as hydrogen bonding, hydrophobic interactions and dispersive interactions. It is possible to take advantage of these factors to solve specific separation problems in IEC. Multi-modal chromatography is a general term that encompasses all forms of separations in which multiple chromatographic mechanisms are deliberately utilized [9]. This type of chromatography can, for example, be accomplished by attaching two or more chemically different ligands that interact with the sample molecules in different ways [10–12]. Multi-modal chromatography can also be achieved by utilizing one type of ligand able to interact with the target molecule through different intermolecular forces [9,13].

The use of classical ion-exchangers is not always optimal in the purification of proteins. To achieve an acceptable sample capacity of the ion-exchanger a dilution of broth is necessary due to high conductivity of the solution [14]. Dilution increases the volume of the feedstock and may not be realistic at manufacturing scale. Direct loading of a cell culture supernatant would substantially improve and simplify purification processes of recombinant biomolecules. In the current work, a diverse library of multi-modal cation-exchange prototypes has been prepared and tested in order to develop cation-exchangers that can adsorb proteins at high ionic-strengths. Recently, several prototypes of non-aromatic (NoAr) multi-modal ligands suitable for the capture of negatively charged proteins from high conductivity (28 mS/cm) mobile phases were coupled to Sepharose™ 6 Fast Flow [15]. These new prototypes of multi-modal anion-exchangers were found by screening a diverse library of multi-modal anion-exchange ligands. We observed that the best high-salt multi-modal anion-exchangers were based on non-aromatic weak amines as ligands. With this background, we decided to develop cation-exchange prototypes with high breakthrough capacities of proteins at high-salt conditions. The elution conductivity of ribonuclease, cytochrome *c* and lysozyme and the breakthrough capacity at high and low-salt conditions of bovine serum albumin (BSA), human IgG and lysozyme were tested on different prototypes of multi-modal cation-exchangers. The results of the new kind of "high-salt" cation-exchangers have been compared to the commercially available cation-exchangers SP Sepharose™ Fast Flow and CM Sepharose™ Fast Flow.

2. Experimental

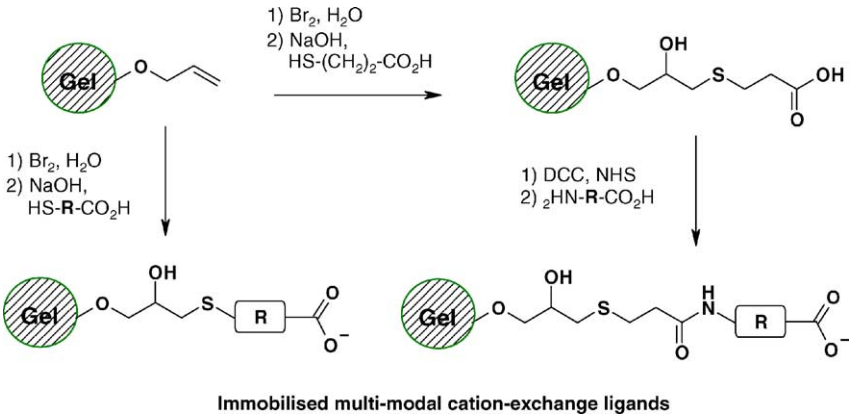
2.1. Media and reagents

The cation-exchange media SP Sepharose™ Fast Flow and CM Sepharose™ Fast Flow and the base matrix Sepharose™ 6 Fast Flow and the hydrophobic interaction media Phenyl Sepharose™ High Sub and Phenyl Sepharose™ Low Sub were obtained from Amersham Bioscienses (Uppsala, Sweden). All ligands used for synthesis of cation-exchange media were of p.a. quality and most of them were purchased from Aldrich.

2.2. Synthesis

The library of multi-modal media was mainly prepared by immobilization of carboxylic acid containing ligands to an activated Sepharose™ 6 Fast Flow. To simplify the analysis of the multi-mode of interaction the formation of zwiterions had to be avoided and since we also wanted to prepare media with good stability towards clean in place procedure at pH 14, the coupling chemistry was restricted to the formation of thio–ether bonds or amide functions.

In a conventional fashion, the activation was started by the introduction of allyl groups on the solid support. A degree of allylation of 0.3 mmol/ml gel was chosen to be able to obtain a final ionic capacity in the same



Scheme 1. Synthesis of a library of multi-modal cation-exchange media.

range as for the reference media (CM Sepharose™ Fast Flow and SP Sepharose™ Fast Flow). For thiol containing ligands the immobilisation was performed directly after activation via bromination, but for the amino acid containing ligands a three steps procedure had to be employed to avoid the formation of zwiterions (Scheme 1). Mercaptopropionic acid was first coupled to the brominated solid support in high yield, then the acidic function was activated with dicyclohexyl carbodiimide (DCC) and *N*-hydroxy succinimide (NHS) and the resulting NHS ester was finally reacted in a parallel format with the diverse amino acid containing ligands. In general, the last NHS coupling was rather efficient, but it is still important to notice that the formation of the NHS ester and the coupling of the ligand are not complete. Therefore, about a 1/1 mixture of mercaptopropionic acid and of the desired carboxylic acid is immobilized on Sepharose™ 6 Fast Flow.

By these two approaches a small library of about 30 multi-modal cation-exchanger media was obtained. The structures of all the ligands are shown in Fig. 3.

In a typical synthesis procedure Sepharose™ 6 Fast Flow was allylated with allyl glycidyl ether. Sepharose™ 6 Fast Flow (80 g drained gel) was mixed with $NaBH_4$ (0.5 g), Na_2SO_4 (13 g) and an aqueous solution of NaOH (40 ml of 50% NaOH solution (w/w)). The mixture was stirred for 1 h at 50 °C. After addition of allylglycidyl ether (100 ml) the suspension was left at 50 °C under vigorous stirring for an additional 18 h. After filtration of the mixture, the gel was washed successively with 500 ml distilled water, 500 ml ethanol, 200 ml distilled water, 200 ml of 0.2 M acetic acid, and finally with 500 ml distilled water. The allyl content of the beads was analyzed via bromination of the allyl groups and the resulted bromide ions were determined by titration with silver ions. The degree of substitution obtained was 0.3 mmol of allyl groups/ml gel.

The coupling of the cation-exchange ligands containing a thiol group (5, 9, 18 and 21) was started by adding bromine (until a persistent yellow color was obtained) to a stirred suspension of allyl activated Sepharose™ 6 Fast Flow (100 ml, 0.3 mmol allyl groups/ml drained gel), containing 4 g of sodium acetate and 100 ml distilled water. Sodium formate was then added until the suspension was fully decolorized. The reaction mixture was filtered and the gel washed with 500 ml distilled water. A suitable aliquot (ca. 5 ml) of the activated gel was then transferred to a reaction vessel and coupled with the appropriate cation-exchange ligands (ca. five times excess compared to the molar amount of activated allyl groups) at pH 11.5. The reaction was stirred for 18 h at 50 °C. The suspension was filtered and the gel was successively washed with 3 × 10 ml distilled water. The degree of substitution was determined by titration of the carboxylic groups. Under these conditions mercaptopropionic acid (21) was immobilized on a large scale (100 ml of gel) with a degree of substitution of 0.29 mmol of carboxylic groups/ml gel.

Coupling of ligands containing both amino and carboxyl functions was made possible via activation of

the carboxylic group of the gel obtained after immobilization of mercaptopropionic acid (21). Thus, mercaptopropionic acid derived medium (100 ml) was washed successively with 300 ml of 1 M NaCl, 500 ml of 0.1 M HCl, 500 ml of 50% (v/v) aqueous acetone and 500 ml acetone. The gel was allowed to settle and the supernatant siphoned off. The gel was then quantitatively transferred to a reaction vessel followed by a solution of *N*-hydroxy succinimide (15.2 g) in acetone (80 ml) and another solution of dicyclohexyl carbodiimide (29.9 g) in acetone (80 ml). The slurry was stirred for 18 h at 30 °C. The mixture was filtered and the gel was successively washed (by gravity flow) with 10 × 150 ml of isopropanol during a period of about 8 h. The degree of activation of the mercaptopropionic acid derived media was estimated by reaction with NH_4OH and measurement of the release of *N*-hydroxy succinimide by UV absorption as compared to a reference curve [16]. By this method, the activation was found to correspond to approximately 75% of the acid groups, giving a gel with 0.22 mmol of NHS-activated acid/ml gel.

Before coupling the ligands containing both amino and carboxyl functions, the NHS-activated mercaptopropionic acid Sepharose^{TM} 6 Fast Flow (25 ml) was washed quickly with 50 ml of ice-cold 1 mM solution of HCl on a sintered glass funnel. The gel was then transferred to an Erlenmeyer flask and a buffered solution of the ligand was added to it. The ligand solution was prepared by dissolving the ligand (about two times excess as compared to the molar amount of NHS ester) in 16 ml of 0.5 M $NaHCO_3$ adjusted to pH 8.5. In some cases, with poorly water-soluble ligands, 5–20 ml ethanol were as well added. The reaction mixture was then shaken for 18 h at room temperature. The reaction mixture was filtered and the gel was washed successively with 100 ml of distilled water, 50 ml of ethanol, 50 ml of 0.25 M aq. ethanolamine, 50 ml distilled water, 50 ml of 1 M NaCl, and finally with 50 ml distilled water.

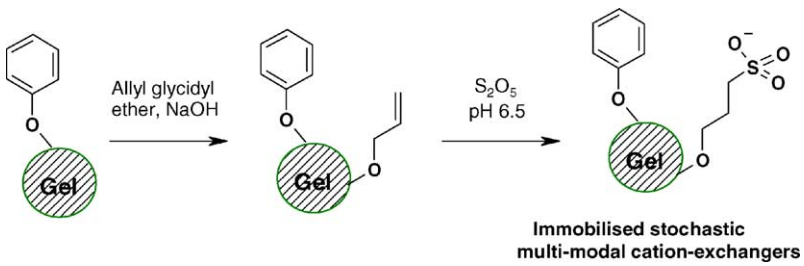
For thienyl serine (3) the efficiency of coupling was determined to be about 70% by elementary sulphur and nitrogen analysis which corresponds to a degree of substitution of 0.15 mmol of thienyl serine/ml of gel.

The concept of multi-modal interactions was further investigated by preparing stochastic media where chemically different ligands were introduced on the same beads separately. This approach is yielding media with no defined distance between the ligands, but it allows an access to some gels where the relative proportions of the different ligands can be easily modified. These stochastic multi-modal media can, for example, be easily prepared from commercially available Phenyl Sepharose^{TM} Fast Flow after treatment with allylglycidyl ether and introduction of the ionic group with sodium metabisulfite (Scheme 2).

In a typical procedure, 50 ml of drained Phenyl Sepharose^{TM} 6 Fast Flow Low Sub (20 μmol phenyl ligands/ml gel) was suspended in 10 ml of distilled water and mixed with 20 ml of a 50% (w/w) aqueous solution of NaOH, 0. 2 g of $NaBH_4$ and 6.5 g of Na_2SO_4. The mixture was stirred for 1 h at 50 °C. After addition of allylglycidyl ether (7 ml) the suspension was left at 50 °C under vigorous stirring for an additional 18 h. After filtration of the mixture, the gel was washed successively, with 5 × 50 ml distilled water, 5 × 50 ml ethanol, 2 × 50 ml distilled water, 2 × 50 ml of 0.2 M acetic acid and 5 × 50 ml distilled water.

Titration gave a degree of substitution of 0.13 mmol of allyl groups/ml gel.

Following the same procedure as above, but treatment with a larger amount of allylglycidyl ether



Scheme 2. Synthesis of multi-modal stochastic SP-exchange media.

(28 ml), Phenyl Sepharose™ 6 Fast Flow Low Sub gave a gel with a higher degree of substitution (0.22 mmol of allyl groups/ml gel).

Under similar conditions, Phenyl Sepharose™ 6 Fast Flow High Sub (40 μmol phenyl ligands/ml gel) gave with the two different amounts of allylglycidyl ether, media with 0.17 and 0.22 mmol of allyl groups/ml gel, respectively.

Introduction of $-SO_3^-$ groups on the four different allylated Phenyl Sepharose™ Fast Flow media was performed following conventional method. Accordingly, 9 g of sodium disulfite were added to a slurry (45 ml drained gel and 15 ml distilled water) of allyl Phenyl Sepharose™ 6 Fast Flow Low Sub (0.13 mmol allyl groups/ml gel). The pH was then adjusted to 6.5 by addition of a 50% (w/w) aqueous solution of NaOH. The reaction was left 18 h under stirring at room temperature with a slow air bubbling. After filtration of the mixture, the gel was washed successively, with 4 × 50 ml distilled water, 2 × 50 ml of 0.5 M HCl and, 3 × 50 ml of 1 mM HCl. Titration gave a degree of substitution of 0.12 mmol of sulfopropyl/ml of gel. Following the same protocol, allyl Phenyl Sepharose™ 6 Fast Flow Low Sub (0.22 mmol allyl groups/ml gel) gave a gel with a degree of substitution of 0.18 mmol of sulfopropyl/ml of gel. With the allyl Phenyl Sepharose™ 6 Fast Flow High Sub (0.17 mmol allyl groups/ml gel) a gel with a degree of substitution of 0.12 mmol of sulfopropyl/ml of gel was obtained. Finally, the allyl Phenyl Sepharose™ 6 Fast Flow High Sub (0.22 mmol allyl groups/ml gel) gave a gel with a degree of substitution of 0.15 mmol of sulfopropyl/ml gel.

2.3. Function test

All chromatographic experiments were carried out using Amersham Biosciences ÄKTA™ explorer 10 XT system. The UV monitor (path length: 10 mm) was adjusted to 280 nm. All sample solutions and buffers were carefully filtered through a 0.45 μm Millipore millex HA filter before use.

The multi-modal cation-exchangers were packed in 1.0 ml HR 5/5 columns and equilibrated with 20 column volumes of the A-buffer (20 mM phosphate buffer; pH 6.8). Fifty microliters of a protein mixture (6 mg/ml Ribonuclease, 2 mg/ml cytochrome *c* and 2 mg/ml lysozyme) was applied to the column and eluted with a linear gradient (gradient volume = 20 column volumes) to 100% of the B-buffer (A-buffer plus 2.0 M NaCl). The flow rate was adjusted to 0.3 ml/min (100 cm/h).

2.4. Breakthrough capacity (Q_b) at high-salt conditions

The breakthrough capacities of three different samples were tested, namely lysozyme, bovine serum albumin and human immunoglobuline (IgG, Gammanorm article number 008565 Pharmacia & Upjohn). The media to be investigated were packed in HR 5/5 columns and the sample solutions were:

S1: BSA (4 mg/ml) dissolved in 20 mM acetate buffer (pH 4.0) with 0.25 M NaCl added;
S2: IgG (4 mg/ml) dissolved in 20 mM acetate buffer (pH 4.5) with 0.25 M NaCl added or;
S3: lysozyme (4 mg/ml) dissolved in 20 mM phosphate buffer (pH 6.8) with 0.30 M NaCl added.

The conductivity of the sample solutions S1, S2 and S3 was 27.4, 29.3 and 32.7 mS/cm, respectively. The solutions were pumped at a flow rate of 1.0 ml/min through the column after equilibration with buffer solution. The breakthrough capacity was evaluated at 10% of the maximum UV detector signal (280 nm). The maximum UV signal was estimated by pumping the different test solutions directly into the detector. The breakthrough capacity at 10% of absorbance maximum (Q_b) was calculated according to the formula:

$$Q_b = (T_{R10\%} - T_{RD}) \times \frac{C}{V_c}$$

where $T_{R10\%}$ is the retention time (min) at 10% of absorbance maximum, T_{RD} the void volume time in the system (min), C the concentration of the sample and V_c the column volume (ml).

To investigate if the new multi-modal cation-exchange ligands also work properly at low-salt conditions the breakthrough capacity of lysozyme was investigated at such conditions (20 mM phosphate buffer, pH 6.8).

2.5. Recovery

To a column (HR 5/5) equilibrated with buffer solution (see conditions for determination of breakthrough

capacities of BSA, IgG and lysozyme) one of the sample solutions (S1, S2 or S3) corresponding to 30% of its breakthrough capacity was applied. The column was then washed with two bed volumes of the equilibration buffer and BSA or IgG were eluted with 100 mM phosphate buffer (pH 7.0) and in case of lysozyme the desorption buffer was 20 mM phosphate buffer (pH 6.8) with 2.0 M NaCl added. The column effluent at elution was collected (20 ml) and the recovery was determined spectrophotometrically at 280 nm (BSA and IgG) or 254 nm (lysozyme).

3. Results and discussion

While a number of cation-exchange methods currently are employed for the purification of biomolecules they frequently suffer from practical or economical problems. For example, adjustment of feedstock composition is usually necessary with most chromatographic techniques. The use of ion-exchange chromatography requires that the feedstock be diluted, diafiltered or dialyzed in order to decrease the ionic-strength and the pH-value must be adjusted to be compatible with the separation medium used [17]. Thus, one way of increasing the applicability of ion-exchange chromatography would be to design ion-exchange ligands with ability to adsorb proteins at high ionic-strengths. Previous work on high-salt anion-exchange prototypes [15] has shown that multi-modal ligands composed of primary or secondary amines (or a combination of both types of these amines) have high capacities of proteins at high ionic-strengths (ca. 28 mS/cm). Furthermore, the presence of hydrogen donor groups in the proximity of the amine groups seemed to be important. In order to find multi-modal cation-exchangers able to adsorb proteins at high-salt conditions this study has primarily focused on investigation of weak cation-exchange ligands with hydrogen acceptor groups attached to the ligand. In cation-exchange chromatography the biomolecules to be separated are positively charged which means that they can exhibit hydrogen donors properties. Thus, any functional group in the ligand, able to participate in hydrogen bond interaction as an acceptor, should be able to generate the extra mode of interaction required to obtain high breakthrough capacities at high ionic-strength conditions.

Table 1
Ligands used in the synthesis of different multi-modal cation-exchangers and the immobilised ligand codes (ILC)

ILC	Ligand name
1	4-Pyridylethylcysteine
2	3-Phenylserine
3	2-Thienylserine
4	Phenylalanine
5	4-Mercaptohydrocinnamic acid
6	2-Thienylalanine
7	Tyrosinethreonine
8	Dihydroxy-phenylserine
9	5-Mercapto-1,3,4-thiadiazol-2-yl thioacetic acid
10	3-(2-Furyl) serine
11	5-Aminosalicylic acid
12	Serinemethionine
13	3-Amino-4,4,4-trifluorobutyric acid
14	3-Amino-1-propanesulfonic acid
15	3-Amino-4(propylsulfonyl)thiophene-2-carboxylic acid
16	*iso*-Serine
17	5-Amino-2-nitrobenzoic acid
18	*N*-(2-Mercaptopropionyl)-glycine
19	Sulfopropyl[a]
20	Carboxymethyl[b]
21	Mercaptopropionic acid
22	*N*-Acetyl-cysteine
23	GlyAbu
24	Homoserine
25	Serine
26	Glycineserine
27	2-Amino-2-deoxy gluconic acid
28	Aspartic acid
29	Glutamic acid

[a] SP Sepharose™ Fast Flow.
[b] CM Sepharose™ Fast Flow. These two media are used as reference media as they are commercially available.

3.1. Characterization of multi-modal cation-exchangers aimed for the capture of proteins at high-salt conditions

A small library of multi-modal cation-exchangers was prepared by coupling different ligands to an activated form of Sepharose™ 6 Fast Flow (Scheme 1). The cation-exchange ligands tested are presented in Table 1. All ligands contain, in addition to the cation-exchange group, an amine or a mercapto group used for attaching the ligand to the activated matrix. The ligands are either aromatic (Ar) or non-aromatic ligands. An amide is formed upon coupling

amine-containing ligands to the NHS-activated Sepharose™ 6 Fast Flow (Table 1). The amide is not taking part in the ion-exchange process, but can still contribute to secondary interactions by building hydrogen bonds. One of the ligands results in a strong cation-exchanger (ILC 14 in Table 1) and the rest result in weak cation-exchangers. The strategy in the search for "high-salt" cation-exchange ligand candidates has been to screen for cation-exchangers exhibiting high elution conductivities and breakthrough capacities for a number of test proteins and conditions. To verify that the weak cation-exchangers are negatively charged at low pH-values the capacity of BSA was determined at pH 4.0. In addition, two commercially available cation-exchangers SP Sepharose™ Fast Flow (ILC 19) and CM Sepharose™ Fast Flow (ILC 20) not optimized for high-salt conditions have also been characterized. Table 2 shows that all proteins could be eluted from all tested cation-exchangers. Only three cation-exchangers resulted in low breakthrough capacity (<6 mg/l) of all three tested biomolecules (Table 2). The two investigated strong cation-exchangers were among these three (ILC 14 and 19, Table 2). To evaluate the data in Table 2 principal component analysis (PCA bi-plot) was used [18]. A bi-plot is a two-dimensional

Table 2
Elution conductivity of ribonuclease (Rib), cytochrome *c* (Cyt *c*) and lysozyme (Lys) at pH 6.8 and breakthrough capacity of lysozyme, BSA and IgG at high-salt conditions on different immobilised multi-modal cation-exchange ligands

ILC	Elution conductivity (mS/cm)			Q_bLys[a] (mg/ml)	Q_bBSA[b] (mg/ml)	Q_bIgG[c] (mg/ml)
	Rib	Cyt *c*	Lys			
1	29	35	143	66	2	4
2	42	43	89	65	50	22
3	46	48	98	61	49	6
4	47	53	120	61	43	14
5	66	78	150	60	28	17
6	40	45	97	51	44	20
7	34	42	71	46	49	6
8	37	37	87	43	44	24
9	29	36	109	42	25	7
10	32	39	62	38	42	27
11	32	38	65	32	40	23
12	26	35	53	20	57	10
13	24	33	47	7	37	7
14	20	30	39	6	4	2
15	26	34	56	5	56	33
16	23	29	37	5	19	2
17	26	33	51	5	41	27
18	23	31	39	5	51	4
19	19	32	32	4	3	1
20	17	26	30	4	15	2
21	25	33	44	4	51	4
22	19	29	34	4	20	2
23	21	29	36	3	46	3
24	20	28	38	3	17	2
25	20	29	33	3	14	2
26	20	31	31	3	20	2
27	20	27	33	3	4	1
28	28	33	33	2	17	2
29	24	32	32	2	12	2

[a] Q_bLys was determined by using 4 mg lysozyme/ml in 20 mM phosphate buffer (pH 6.8) as sample solution.
[b] Q_bBSA was determined by using 4 mg BSA/ml in 20 mM acetate buffer (pH 4.0) as sample solution.
[c] Q_bIgG was determined by using 4 mg IgG/ml in 20 mM acetate buffer (pH 4.5) as sample solution.

scatter plot or map of scores for two principal components with the loadings displayed on the same plot. This makes it possible to interpret object properties (scores) and variable (loadings) relationships simultaneously. The closer two objects are in the plot, the more similar they are with respect to the two principal components concerned. Consequently, objects far away from each other are different from each other. Variables and objects situated far away from the center and in the same direction mean that the objects have a large value for these variables. In this interpretation the type of ligand is considered the objects and the elution conductivity and the breakthrough capacity of proteins are considered the variables (Table 2). The bi-plot in Fig. 1A, which explains 85% of the total variation in the data set, shows that the elution conductivity for Lys, Rib, Cyt *c* and the breakthrough capacity of lyzosyme (Q_bLy) covariate. Furthermore, the breakthrough values Q_bBSA and Q_bIgG also covariate and these variables are not correlated to the other variables (Lys7, Rib7, Cyt7, Q_bLy). Since Q_bBSA and Q_bIgG are determined at pH 4 and 4.5, respectively, and Lys7, Rib7, Cyt7 and Q_bLy at pH 6.8 the difference in pH of the mobile phase can probably explain the results. For example, secondary interactions (co-operative to the ion-exchange process) such as hydrogen donor–acceptor interactions, which some of these ligands are designed to take advantage of, are greater at acidic conditions. Therefore, it is logical that there are differences between the results at pH 4.5 and 6.8. It can also be noted that these two variable sets are placed orthogonally to each other, illustrating that the sets are independent of each other. In other words, it shows, for example, that a high Q_bLys does not necessarily imply that Q_bBSA and Q_bIgG must be high and vice versa. To simplify the interpretation of the bi-plot the different cation-exchange ligands have been coded according to if non-aromatic or aromatic. From Fig. 1B it can be observed that all aromatic ligands are placed in the first and fourth quadrants and the non-aromatic ligands are placed in the second and third quadrants. This grouping indicates that aromatic cation-exchanger are to be preferred in order to attract proteins at high ionic-strength. To interpret this more closely the different multi-modal cation-exchange ligands have been coded according to their Q_b-values of lysozyme, BSA or IgG (Fig. 2A–C, respectively). These figures clearly show that most of the good (high Q_b-values) cation-exchange ligands are situated in quadrant one and four. According to Fig. 1B these ligands correspond to aromatic multi-modal cation-exchangers. However, five non-aromatic cation-exchange ligands (ILC 12, 13, 18, 21 and 23) gave high (>37 mg/ml) Q_b-values of BSA (Figs. 1A and 2B). A common feature of these non-aromatic multi-modal cation-exchange ligands is that they contain hydrogen acceptor groups close to the carboxylic group (Fig. 3). CM Sepharose™ Fast Flow has no such acceptor groups which explains the much lower Q_bBSA-value obtained for this medium. It can also be noted that no good non-aromatic cation-exchange ligands were obtained for IgG and lysozyme. However, five aromatic cation-exchange ligands (ILC 2, 6, 8, 10, and 11) were found that gave high breakthrough capacities (Q_bLys >30 mg/ml, Q_bBSA >40 mg/ml and Q_bIgG>20 mg/ml) of all three test samples (Table 2 and Fig. 2). These aromatic cation-exchangers are placed to the right in the first quadrant (Fig. 1A) and the ligand structures are presented in Fig. 3. A common feature of these ligands is that an amine group (which becomes an amide group after attachment to the NHS-activated Sepharose™ 6 Fast Flow) is substituted on the carbon next to the carboxylic acid group (α-carbon). Furthermore, all these five multi-modal cation-exchange ligands have an aromatic group attached to the β-carbon. No ligands with only one of these two features have been found to have high Q_b-values for all three test samples investigated. From Table 1 and Fig. 2 two aromatic cation-exchange ligands (ILC 3 and 7) that have high values of Q_bLys (>45 mg/ml) and Q_bBSA (>45 mg/ml) and low values of Q_bIgG (<7 mg/ml) can also be found. Compared to ILC 6 that have high capacities for all test samples ILC 3 differs only in having a hydroxyl group at the β-carbon. Two aromatic multi-modal ligands (ILC 15 and 17) with high breakthrough capacities of BSA (>40 mg/ml) and IgG (>25 mg/ml) and low for lysozyme (<6 mg/ml) have also been found (Table 2 and Fig. 2). ILC 15 has a sulfone group and ILC 17 a nitro group that may be essential for high IgG capacity. Furthermore, one aromatic cation-exchange ligand (ILC 1) with high breakthrough capacity of lysozyme and low Q_b-values of the other test samples (Table 2) was discovered. This ligand is based on a pyridine structure (Table 1 and Fig. 3) which means that the nitrogen in the

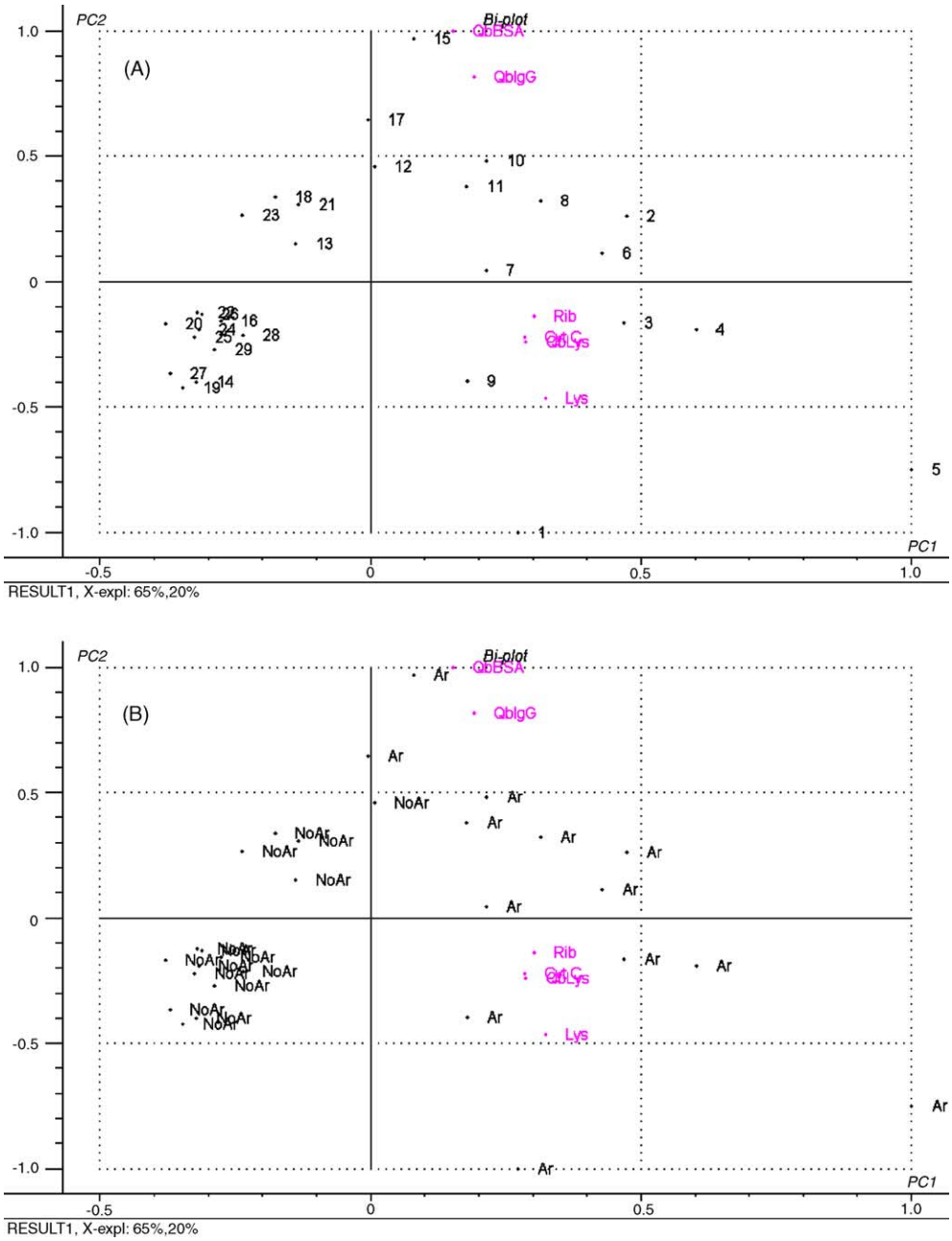


Fig. 1. Bi-plots (scores and loadings together) of the data in Table 2. The objects are coded as ligands according to Table 1 (A) or as an aromatic (Ar) or a non-aromatic (NoAr) cation-exchange group (B). The variables Lys, Rib and Cyt *c* correspond to the elution conductivity and Q_bBSA, Q_bIgG and Q_bLys to the breakthrough capacity of the proteins.

pyridine ring will be positively charged at pH-values lower than about 5. Consequently, ILC 1 will not work as a cation-exchanger in mobile phases used for BSA and IgG capture. The presented results indicate that there are large variations in the breakthrough capacities of the different multi-modal cation-exchangers. However, a ligand that has high capacity of IgG and low of BSA and lysozyme at used conditions has not been found. To obtain high capacities of IgG it is also highly important that the pore sizes of the base matrix are large enough not to restrict the diffusion of the IgG-molecules. To illustrate the effect of the pore size 3-amino-4,4,4-trifluorobutyric acid was also immobilized on Sepharose^{TM} 4 Fast Flow which is an agarose matrix with larger pore sizes compared to Sepharose^{TM} 6 Fast Flow [19]. The Q_bIgG

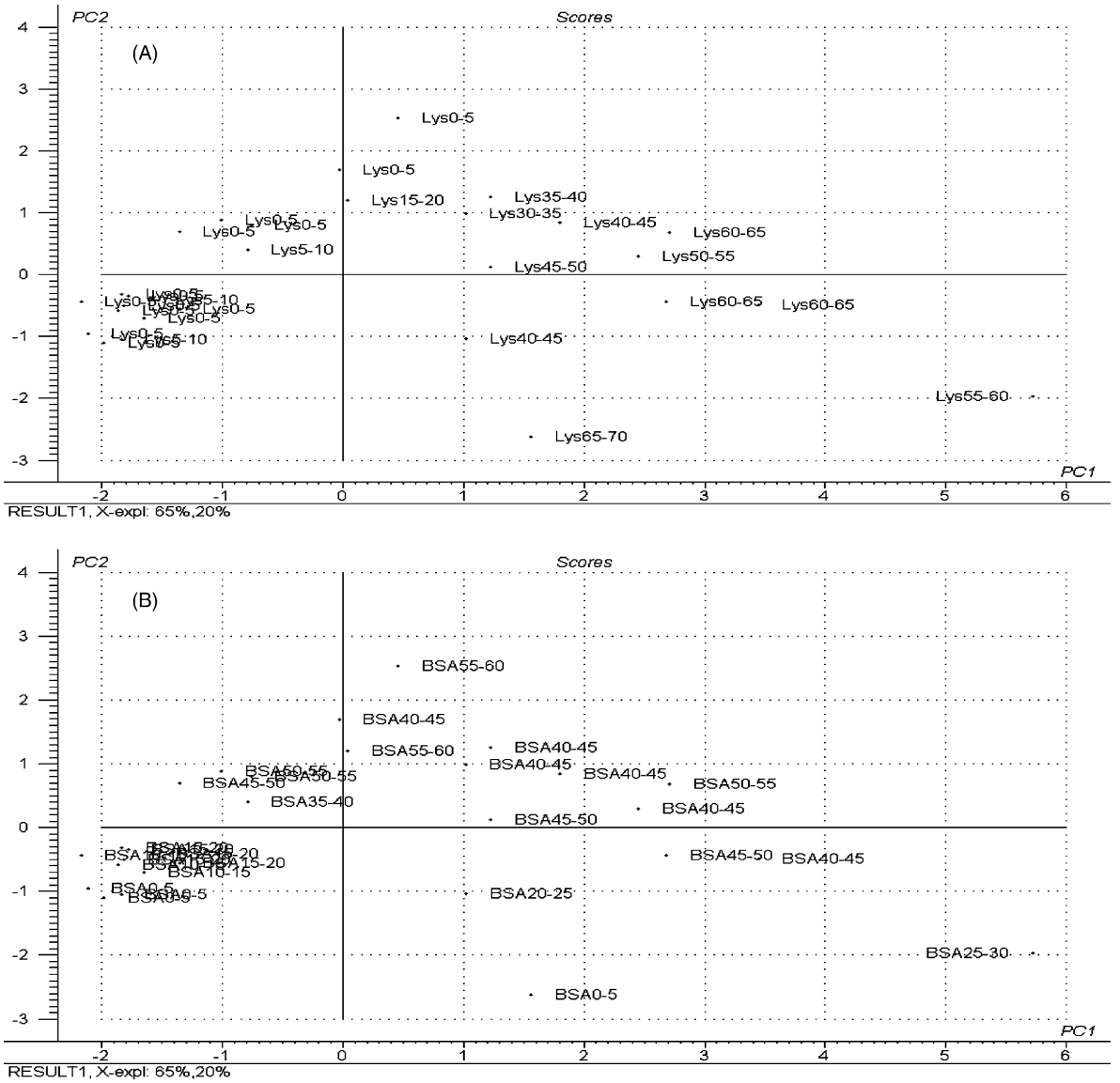


Fig. 2. Score-plots of data in Table 2. The objects are coded as breakthrough capacity (mg/ml) of lysozyme (A), BSA (B) and IgG (C). The two principal components describe 85% of the total variation.

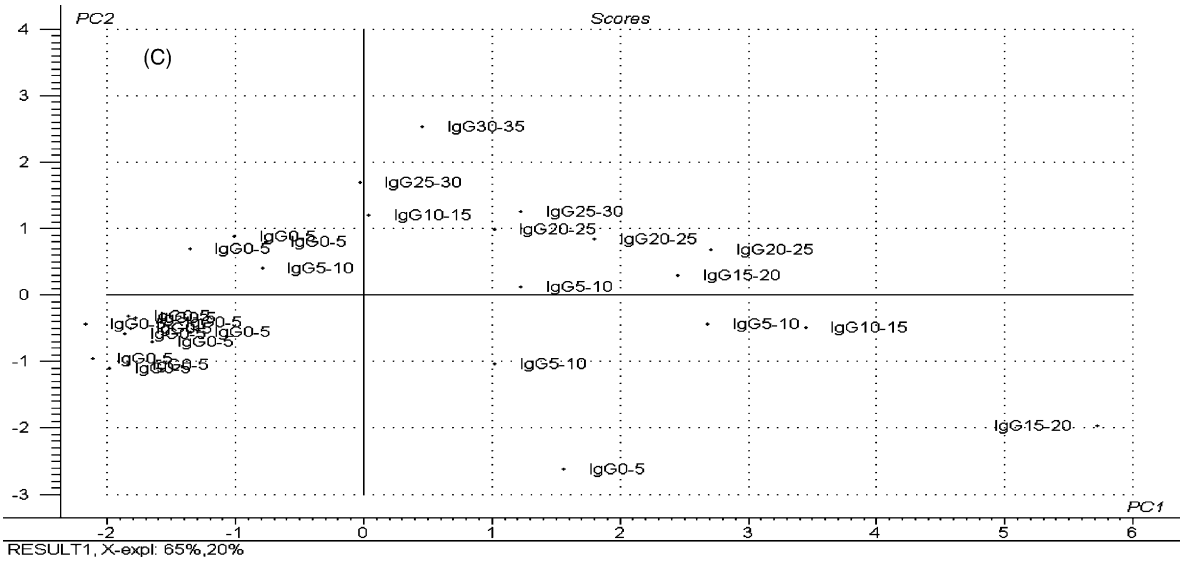


Fig. 2. (*Continued*).

increased from 7 mg/ml (ILC 13 in Tables 1 and 2) to 21 mg/ml when 3-amino-4,4,4-trifluorobutyric acid was immobilized on Sepharose^{TM} 4 Fast Flow. The breakthrough capacity of lysozyme was not affected since this protein is much smaller (molecular weight: 14 000 g/mol). However, Q_bBSA increased from 37 mg/ml (ILC 13 in Table 2) to 47 mg/ml. BSA is a larger protein (molecular weight: 67 000 g/mol) than lysozyme but smaller compared to IgG (molecular weight of ca. 160 000 g/mol). These results clearly indicate that in order to obtain high breakthrough capacity optimization of the pore size distribution is highly important for large biomolecules.

3.2. Recovery of lysozyme, BSA and IgG

Elution of adsorbed biomolecules from multi-modal cation-exchangers cannot always be accomplished by increasing the salt content in the mobile phase. This is due to the fact that secondary interactions between sample and ligand increase with ionic-strength. Therefore, to accomplish elution of adsorbed proteins a pH-step may be necessary to achieve high recoveries when multi-modal ion-exchange ligands are used. The recovery of lysozyme and BSA from 12 different multi-modal cation-exchangers is presented in Table 3. Lysozyme was eluted with a salt-step and BSA with a pH-step (elution buffer: 100 mM phosphate buffer adjusted to pH 7.0). Recoveries of more than 90% were mostly observed for both proteins. However, the ligand (ILC 5) that gave the highest elution conductivities (Table 2) resulted in recoveries of only ca. 50%. On the other hand, no correlation between Q_b-values and recoveries was found. It can be noted that BSA cannot be eluted with high recovery with a salt gradient at pH 4.0 and this behavior was also observed for IgG. The recovery of IgG at a pH-step elution (elution buffer: 100 mM phosphate buffer adjusted to pH 7.0) was tested for four multi-modal cation-exchangers (ILC 2, 10, 11 and 15) and all recoveries were higher than 75%. The most optimal IgG multi-modal cation-exchanger ILC 15 with the highest breakthrough capacity (33 mg/ml, Table 2) had a recovery of 88%.

3.3. Breakthrough capacity of lysozyme at low ionic-strength

It is well known that commercially available cation-exchangers based on ion-exchange groups such as carboxylate (CM Sepharose^{TM} Fast Flow) and sulfonate (SP Sepharose^{TM} Fast Flow) have high capacities of proteins when the sample is dissolved in buffers with low-salt content [20]. Therefore, it is interest-

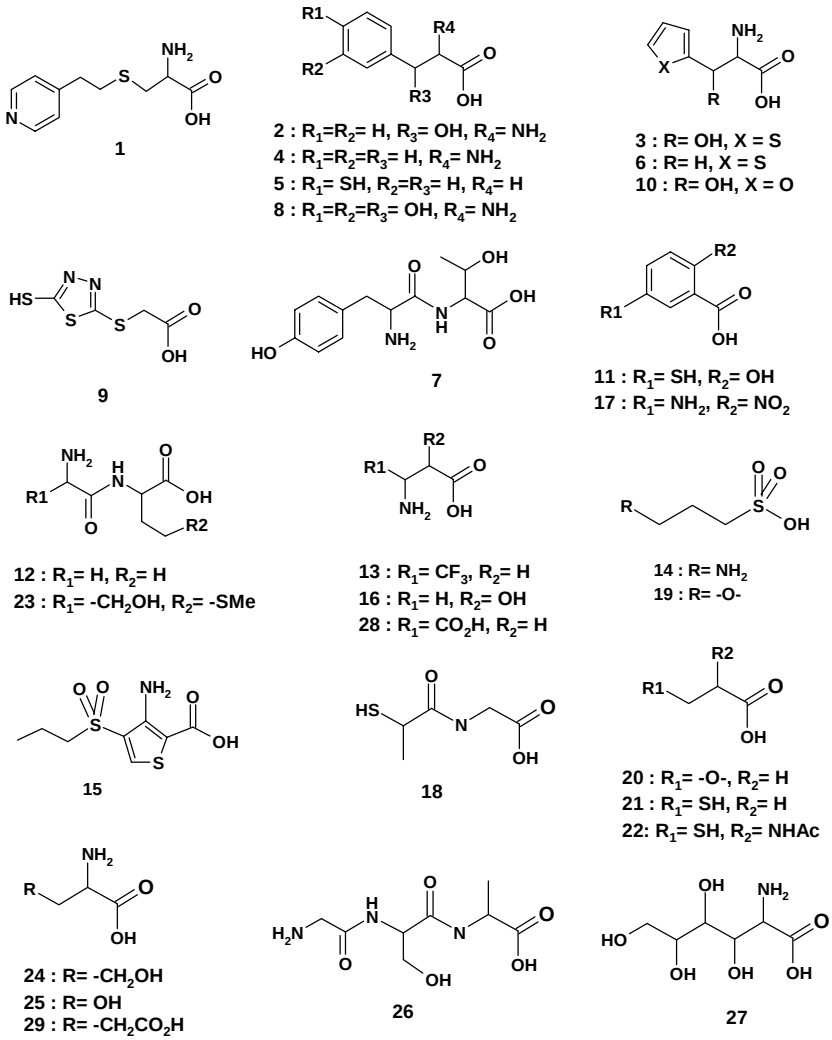


Fig. 3. Structures of cation-exchange ligands presented in Table 1.

ing to compare the breakthrough capacity of the new multi-modal weak cation-exchangers with commercially available strong and weak cation-exchangers at conditions where no extra salt has been added to the adsorption buffer. According to Fig. 4 CM Sepharose™ Fast Flow and SP Sepharose™ Fast Flow exhibited breakthrough capacities of 84 and 94 mg of lysozyme/ml, respectively, when a 20 mM phosphate buffer (pH 6.8) was used. The best multi-modal cation-exchangers (ILC 2 and 6) resulted in a Q_bLys of 96 and 94 mg/ml, respectively. These results indicate that the new multi-modal cation-exchangers can also efficiently capture proteins from mobile phases with low ionic-strength. When 0.30 M NaCl was added to the adsorption buffer (20 mM phosphate buffer, pH 6.8) the breakthrough capacity of lysozyme decreased to 4 mg/ml for SP and CM Sepharose™ Fast Flow. The same change in mobile phase composition when used with the two multi-modal cation-exchangers ILC 2 and 6 did only result in a decrease of Q_bLys to 65 and 51 mg/ml, respectively (Fig. 4). It can also be seen from Fig. 4 that an even less decrease of Q_bLys was observed for the two other multi-modal cation-exchangers tested

Table 3
Recovery of lysozyme and BSA for some of the most promising high-salt cation-exchange ligands

Ligand name (ILC[a])	Recovery lysozme (%)	Recovery BSA (%)
4-Pyridylethylcysteine (1)	84	nd[b]
3-Phenylserine (2)	97	93
2-Thienylserine (3)	87	93
Phenylalanine (4)	91	79
2-Thienylalanine (6)	102	86
Tyrosinethreonine (7)	95	92
Dihydroxy-phenylserine (8)	93	91
3-(2-Furyl) serine (10)	101	93
Serinemethionine (12)	78	93
3-Amino-4(propylsulfonyl)thiophene-2-carboxylic acid (15)	nd	82
5-Amino-2-nitrobenzoic acid (17)	nd	93
N-(2-Mercaptopropionyl)-glycine (18)	nd	92

The amount of lysozyme or BSA applied corresponds to 30% of the breakthrough capacity (see Section 2 for details).
[a] Immobilised ligand code.
[b] nd: not determined.

(ILC 3 and 4) when 0.3 M NaCl was added to the mobile phase.

3.4. Functional stability after treatment in 1.0 M sodium hydroxide solution

It is important to remember that ion-exchange media aimed for large-scale production of biomolecules require regular maintenance for economic and safety reasons. Cleaning conditions in the production of pure biological materials require effective procedures of removing contaminants such as fatty acids, endotoxins and macromolecular aggregates. Furthermore, inactivation of living organisms, such as bacteria, yeasts and viruses is of utmost importance. Treatment of used media with 1 M NaOH solution can in many cases solve these contamination problems. Therefore, it is desirable that cation-exchangers can sustain treatment with high pH solutions for extended time without any decline in chromatographic performance [21]. To test the functional stability at alkaline conditions, four multi-modal cation-exchangers were incubated for 10 days in 1.0 M NaOH solution. Table 4 shows that, according to the breakthrough results, the investigated cation-exchangers were not significantly influenced.

Table 4
The change of breakthrough capacity (Q_b) on four different aromatic multi-modal cation-exchange ligands for lyzosyme after 10 days of incubation in 1 M sodium hydroxide

Ligand name (ILC)[a]	Q_b of lyzosyme before incubation (mg/ml)	Q_b of lyzosyme after incubation in 1.0 M NaOH (mg/ml)
4-Pyridylethylcysteine (1)	66	66
3-Phenylserine (2)	65	64
2-Thienylserine (3)	61	61
2-Thienylalanine 6)	51	52

[a] Immobilised ligand code.

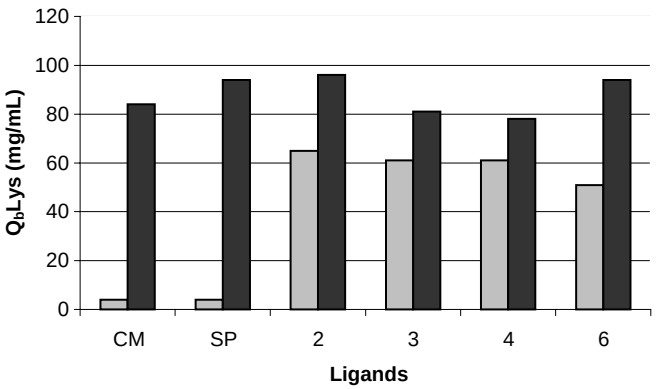


Fig. 4. Breakthrough capacity (Q_b) of lysozyme for SP Sepharose™ Fast Flow, CM Sepharose™ Fast Flow and four new cation-exchangers at high-salt conditions (light coloured bars) and low-salt conditions (dark coloured bars). See Section 2 for more details and Table 1 and Fig. 3 for ligand structures.

Table 5
Elution conductivity at pH 6.8 for three proteins and breakthrough capacity of lysozyme (pH 6.8 and 0.3 M NaCl), BSA (pH 4.0 and 0.25 M NaCl) and IgG (pH 4.5 and 0.25 M NaCl) on different mixed-ligand cation-exchangers based on Phenyl Sepharose™ media

Medium[a]	SP-content (mmol/ml)	Ligand ratio (SP/Phenyl)	Elution conductivity (mS/cm)			Q_bLys (mg/ml)	Q_bBSA (mg/ml)	Q_bIgG (mg/ml)
			Rib	Cyt *c*	Lys			
SP	0.20	na	19	32	32	4.3	2.6	0.8
SP/Phenyl LS	0.18	9	19	28	37	4.2	18	1.7
SP/Phenyl LS	0.12	6	18	22	43	7.2	41	2.5
SP/Phenyl HS	0.15	3.8	20	27	41	4.9	37	2.6
SP/Phenyl HS	0.12	3	23	29	54	20	41	4.0

na: not applicable.
[a] SP means SP Sepharose™ Fast Flow; SP/Phenyl LS means that sulfopropyl groups have been coupled to Phenyl Sepharose™ Low Sub (20 μmol phenyl groups/ml gel); SP/Phenyl HS means that sulfopropyl groups have been coupled to Phenyl Sepharose™ High Sub (40 μmol phenyl groups/ml gel). See Section 2 for details.

3.5. Mixed chemical bonded ligands

To investigate the possibility of improving the performance of strong cation-exchangers at high-salt conditions four media with a mixture of different ligands were tested. This was accomplished by attaching sulphonyl groups (SP) to Phenyl Sepharose™ Fast Flow HS or LS (Table 5). The combination of one aromatic ligand and one strong cation-exchange group was chosen since the best multi-modal high-salt cation-exchangers are based on aromatic ligands. According to Table 5 all four mixed-ligand (SP/Phenyl) media have a higher breakthrough capacity of lysozyme, BSA and IgG compared to SP Sepharose™ Fast Flow. It also seems as the breakthrough capacity increases with decreasing ligand ratio (SP/Phenyl) for all three test samples but to a different extent. Mixed-ligand ion-exchange media, containing both ionic and hydrophobic ligands, have been studied earlier and large effects in selectivity have been observed [22–24]. Mixed mode media may yield systems with unique separation features. This is also illustrated in Table 5 where the elution conductivities of the four SP/Phenyl media are compared to the results from SP Sepharose™ Fast Flow. The elution conductivity of ribonuclease and lysozyme increases for the mixed media with the lowest ligand ratio (SP/Phenyl). On the other hand, the elution conductivity decreases for cytochrome *c*. In other words, cytochrome *c* and lysozyme coelute on SP Sepharose™ Fast Flow but are well separated on the SP/Phenyl medium (ligand ratio of 3 according to Table 5). On SP/Phenyl media cytochrome *c* instead elutes close to ribonuclease.

4. Conclusion

Multi-modal cation-exchange prototypes have been synthesized and optimized to adsorb proteins from solutions of high ionic-strengths. The best cation-exchangers tested were based on aromatic carboxylic acids. Apart from the importance of the aromatic carboxylic functionality, it was discovered that it is crucial that the aromatic group is attached to the β-carbon and an amide group is attached to the α-carbon. It can also be noted that small changes in the ligand structure can affect the behavior to large extent. However, the number of multi-modal ligands tested is too small for more specific conclusions about the relationship between structure and function to be made.

Non-aromatic carboxylic acids can also be used as ligands for high-salt cation-exchangers if they contain hydrogen acceptors close to the carboxylic group. However, so far no non-aromatic cation-exchange ligand has been found to exhibit high breakthrough capacity for all investigated samples.

The captured proteins can easily be desorbed with high recoveries by increasing the ionic-strength or by raising pH to conditions where both protein and ligands are negatively charged. Multi-modal aromatic cation-exchangers may provide additional flexibility in the design of new purification processes, and the possibility to capture proteins directly from different feedstocks will be investigated in the near future. It should also be pointed out that these new ligands are weak ion-exchange ligands and therefore cannot be used at pH-values lower than about 3.

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