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Capture of a monoclonal antibody and prediction of separation conditions using a synthetic multimodal ligand attached on chips and beads

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Abstract

A synthetic ligand called 2-mercapto-5-benzimidazolesulfonic acid has been successfully used for the specific chromatographic capture of antibodies from a cell culture supernatant. Adsorption occurred at physiological ionic strength and pH range between 5.0 and 6.0, with some binding capacity variations within this pH range: antibody uptake increased when the pH decreased. With very dilute feedstocks, as was the case with the cell culture supernatant under investigation, it was found that the pH had to be slightly lowered to get a good antibody sorption capacity. To optimize separation conditions, a preliminary study was made using ProteinChip® Arrays that displayed the same chemical functionalities as the resin. Arrays were analyzed using SELDI–MS. By this mean, it was possible to cross-over simultaneously different pH conditions at the adsorption and the desorption steps. Best conditions were implemented for preparative separation using regular lab-scale columns. At pH 5.2, antibody adsorption was not complete, while at pH 5.0 the antibody was entirely captured. pH 9 was selected at elution, rather than pH 8.0 or 10.0, and resulted in a complete desorption of antibodies from the column. Benefits of the prediction of separation conditions of antibodies on MBI beads using SELDI–MS were a significant reduction in analysis time and in sample volume. This was possible because the separation of IgG on the chip surface did mimic very well the separation on beads.

Keywords: Antibodies; 2-Mercapto-5-benzimidazolesulfonic acid; Affinity chromatography; ProteinChips®; Surface enhanced laser desorption ionisation–time of flight–mass spectrometry (SELDI–TOF–MS)

1. Introduction

Antibodies for research, diagnostic and therapeutic applications represent a domain of large and fast growing interest. The common method for the selective capture of antibodies is affinity chromatography on Protein A sorbents. The high specificity of Protein A for the Fc antibody domain of IgG provides good chromatographic selectivity. However, serious complications are associated with the use of Protein A. Indeed, Protein A is subjected to degradation by proteases present in feedstocks. Such degradation results in Protein A fragments leaching in the eluate. Because of Protein A toxicity, this can represent a real problem for therapeutic applications. Equally important, the necessity to adopt acidic pHs for the elution of antibody can lead to aggregate formation or loss of activity. Moreover, Protein A sorbents cannot be subjected to cleaning-in-place procedures using sodium hydroxide solutions, preferred in process-scale applications. Consequently, compared to other current chromatographic media, the number of use-cycles that can be achieved is relatively limited. Finally, the cost of Protein A is relatively high compared to other sorbents. For all these reasons, alternatives to Protein A columns have been investigated during the last two decades [1,2].

Among the numerous proposed approaches, thiophilic chromatography and peptidomimetic affinity chromatography have been proposed [3–8]. These methods circum-

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vent several problems associated with Protein A chromatography.

However, some practical issues remain unresolved, such as the necessity to work in the presence of lyotropic salts with thiophilic ligands and the adjustment of both pH and ionic strength of feedstocks when using peptidomimetic ligands. More recently, hydrophobic charge induction chromatography has been proposed as an improvement towards approaching the ideal situation in the design of an antibody selective sorbent [9–11]. In fact, the adsorption process is performed in physiological conditions of pH and ionic strength and desorption is accomplished by mild acidification. An evolution of this approach has been recently published [12], where antibodies are desorbed by raising the pH instead of using acidic buffers. This undoubtedly represents a significant advantage for many antibodies that tend to precipitate at slightly acidic conditions, and therefore, create aggregates that must be removed, when dealing with therapeutic antibodies.

The structure of this ligand, called 2-mercapto-5-benzimidazole sulfonic acid is based on the presence of a heterocycle, a sulfur atom and an aromatic ring supporting a strong acidic group, which is negatively charged over the whole range of working pH. Antibodies are adsorbed in physiological ionic strength at a pH between 5.0 and 5.5. Elution is achieved by raising the pH to 8.5–10.0.

Although, the mechanism of action is not yet completely elucidated, contributions for antibody adsorption come from the presence of imidazole heterocycle [13], the sulphur group [3,4,9] and the aromatic ring [14,15]. The sulfonate group is introduced to prevent the adsorption of acidic species that tend to co-adsorb on the resin by electrostatic repulsion, such as albumin.

In spite of significant advancements in the design of synthetic ligands and their capture performance, conditions of adsorption and desorption may have to be fine tuned according to the properties of antibodies, such as isoelectric point, hydrophobicity index, type and species. This situation induces an optimization step that may require a significant volume of sample and can be time consuming when a large number of experiments are operated using chromatographic columns.

It has been recently demonstrated that the use of surface enhanced laser desorption ionisation–mass spectrometry (SELDI–MS), where ProteinChip® Arrays carry the same chemical groups as the chromatographic resins, leading this way to the same interaction with the proteins of interest, is an efficient and rapid way to develop or to optimize process chromatography protocols [16–18].

In this paper, is described a method for the purification of monoclonal antibodies produced by recombinant cell culture using 2-mercapto-5-benzimidazolesulfonic acid as a capturing ligand. The procedure necessitating an optimization of standard conditions, chips of the same nature were used for the development of a more adapted protocol under conditions that use very small volumes of the feedstock and short experimental time.

2. Experimental

2.1. Chemicals, biologicals and equipment

MBI HyperCel™ beads supporting 2-mercapto-5-benzimidazolesulfonic acid as ligand were from BioSepra (Process Division of Ciphergen Biosystems, Cergy, France). MBI and NP20 ProteinChip® Arrays and sinapinic acid were from Ciphergen Biosystems Inc. (Fremont, CA, USA). The processed arrays were read by laser desorption ionisation–time-of-flight–mass spectrometry using a PBSIIc Reader also supplied by Ciphergen Biosystems Inc.

Protein-free cell culture supernatant containing mouse monoclonal IgG_1 was from Life Technologies (Paisley, UK). Pure polyclonal human antibodies were supplied by Intergen (MA, USA). Chromatography was performed on an AKTA Explorer 100 system from Amersham Biosciences (Uppsala, Sweden). Columns (3 mm i.d. × 100 mm height and 6.6 mm i.d. × 72 mm height) were from Omnifit (Cambridge, UK).

Pre-casted polyacrylamide gels and mini Protean II electrophoresis equipment came from BioRad Laboratories (Ivry sur Seine, France). Simply Blue™ SafeStain for gel staining was purchased from Invitrogen (CA, USA). Total protein content in chromatography fractions was evaluated using a BCA assay kit from Pierce Biotechnology (IL, USA). All chemicals used in this study were from Aldrich (Brussels, Belgium) and were of analytical grade.

All experiments are performed in a controlled and conditioned environment between 20 and 25 °C.

2.2. Determination of antibody binding capacity of as a function of pH

Binding capacity for antibodies was measured in a column of 6.6 mm i.d. × 72 mm height packed with MBI HyperCel™ sorbent. Solutions of pure human polyclonal antibodies of concentrations ranging from 50 μg/mL to 5 mg/mL were prepared in 50 mM sodium acetate buffers, containing 0.14 M sodium chloride, either of pH 5.0 or 5.5. The determination of binding capacity was made by titrating the column according to a frontal analysis method and calculations were made at 10% breakthrough. Linear flow rate was maintained constant at 100 cm/h, corresponding to a residence time of 4.3 min.

2.3. On-chip optimization of separation conditions

The cell culture supernatant was directly deposited on MBI ProteinChip® Arrays according to a previously described method [16]. Several runs were performed in parallel, in order to identify proper conditions of antibody adsorption and desorption. Four different adsorption buffers were used: 50 mM sodium acetate, pH 5.0, 5.2 or 5.5; or 50 mM sodium citrate, pH 6.0. Seven desorption buffers were used: 50 mM sodium acetate, pH 5.0, 5.2 or 5.5; 50 mM sodium citrate, pH 6.0; 50 mM Tris–HCl, pH 8.0 or 50 mM sodium bicarbonate

pH 9.0 or 10.0. All buffers contained 0.14 M sodium chloride. Each single spot was equilibrated three times with 200 μL of the investigated adsorption buffer for 5 min. Then, each spot surface was loaded with 50 μL of the sample previously adjusted to the corresponding pH. After an incubation period of 60 min under vigorous shaking, each spot was washed three times with 200 μL of the appropriate desorption buffer for 5 min to eliminate non-adsorbed proteins, followed by a quick rinse with deionized water.

All surfaces were dried and loaded twice with 1 μL of a saturated solution of sinapinic acid in two volumes of pure acetonitrile and one volume of 25% (v/v) formic acid and dried again. All arrays were then analyzed using a PBSIIc Reader in a positive ion mode, with a source voltage of 20 kV and a detector voltage of 2.8 kV. The molecular weight range investigated was from 0 to 200 kDa. Focus mass was set at 150 kDa. Laser intensity responsible for the desorption–ionization of proteins on the spot surface was set at 190 units and sensitivity of the detector at 10 units.

Adsorption of antibodies on the MBI array appeared as a signal at the appropriate molecular mass and absence of signal was interpreted as desorption of the antibody. All experiments were run in triplicates.

2.4. Column chromatography separations of antibodies

MBI HyperCel™ was packed in regular glass columns of 3 mm i.d. × 100 mm height. The bed was equilibrated with a 50 mM sodium acetate buffer, containing 0.14 M sodium chloride at a pH of either 5.0 or 5.2. The cell culture supernatant was adjusted to pH 5.0 or 5.2 according to the column equilibration conditions, and directly loaded onto the column. Non-adsorbed proteins were washed out by means of the equilibration buffer and elution was obtained using a 50 mM sodium bicarbonate buffer, containing 0.14 M sodium chloride, pH 9.0. Finally, the sorbent was cleaned by a wash with five column volumes of 1 M sodium hydroxide. Chromatography separations were accomplished at a linear flow rate of 150 cm/h, corresponding to a residence time of 4 min. Absorbance of column effluents was recorded at 280 nm. Flowthrough peaks were collected under two separated fractions and elution peaks were collected in a single fraction and, then, processed for analysis.

All experiments were performed in a controlled and conditioned environment between 20 and 25 °C.

2.5. Analysis of separated fractions

SELDI–MS analysis using NP20 ProteinChip® Arrays was similar to the above-described methodology except that 5 μL of the protein solution was deposited with no prior adjustment of pH (the chip surface adsorbs most proteins). Once the loading was accomplished, the spot was air-dried, then washed three times with 5 μL of deionized water and air-dried again. 0.8 μL of sinapinic acid was deposited and chip surfaces were processed as indicated by the supplier.

Electrophoresis of chromatography fractions was performed in classical conditions using 15-well pre-casted 12% polyacrylamide gel plates. Samples were diluted two-fold in Laemmli sample buffer. 12.5 μL samples were loaded per lane and electrophoretic migration was performed at 200 V for 45 min. Staining was achieved using a microwave procedure with Simply Blue SafeStain solution following Invitrogen's instructions. Destaining was performed using deionized water.

3. Results and discussion

Monoclonal antibodies are commonly purified using affinity chromatography on Protein A sorbents. Due to problems related to these sorbents, an alternative method using 2-mercapto-5-benzimidazolesulfonic acid as ligand has been recently described for the capture and separation of antibodies [12]. This study reported several comparative separation examples of antibodies from various biological fluids, such as rat hybridoma cell culture supernatant and human serum fractions. Antibody purity was in all cases at least as high as the one obtained with Protein A resin, moreover, the study demonstrated the ability of MBI resin to effectively capture rat IgG while Protein A was unable to interact with this class of antibody in physiological conditions [12].

Such a material is, therefore, of interest any time a crude feedstock from different origin containing antibodies is to be processed. In a previous published paper, it was indicated that this ligand is capable of adsorbing antibodies at a pH between 4.5 and 6.0 and reach a capture capacity ranging between 15 and 30 mg/mL of resin. The adsorption of antibody was not dependent on the presence of salt within a concentration range of 0.15–1 M sodium chloride. The mechanism

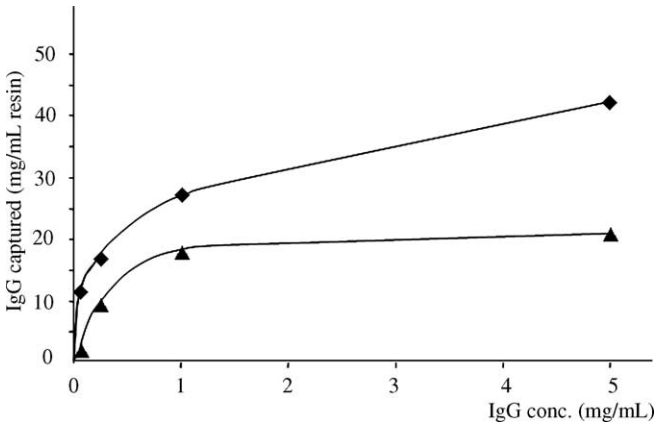

Fig. 1. Polyclonal human IgG binding capacity of MBI HyperCel™ as a function of IgG concentration at room temperature. Column, 6.6 mm i.d. × 72 mm height; sample, pure solution of polyclonal IgG at 50, 250, 1000 or 5000 μg/mL; linear flow rate, 100 cm/h (residence time, 4.3 min). Upper curve (♦) was obtained at a pH 5.0 using a 50 mM sodium acetate buffer containing 0.14 M sodium chloride. The lower curve (▲) was obtained using the same buffer but at pH 5.5. Binding capacity determinations were made by frontal analysis at 10% breakthrough.

of adsorption was described as being neither dependent on hydrophobic associations nor associated with ion exchange associations, but rather on a relatively complex mechanism where the heterocycle, the sulphur atom and the aromatic ring contributed to the interaction [12].

Within the frame of the present work, attempts were made to capture and purify a monoclonal IgG from a crude cell culture supernatant. A first set of experiments was devoted to the determination of pure polyclonal IgG binding capacity in different conditions. Experimental data demonstrated that binding capacity at a constant physiological ionic strength (0.14 M sodium chloride) and at a pH of 5.5 appeared significantly dependent on the antibody concentration (Fig. 1). Indeed, when the concentration of IgG dropped from 1000 to 250 and 250 to 50 μg/mL, binding capacity was decreased two-fold (18 to 9 mg/mL) and six-fold (9 to 1.5 mg/mL), respectively. A way to maintain a decent binding on the resin was to decrease the adsorption pH to 5.0 (Fig. 1). Indeed, when the pH was decreased from 5.5 to 5.0, the binding capacity was increased seven-fold (1.5 to 11 mg/mL) and two-fold (9 to 17 mg/mL), at 50 and 250 μg/mL, respectively. Therefore, when the concentration of expressed antibodies is low, a slight modification in the pH can accommodate acceptable binding capacities. One has to keep in mind that as reported [12], albumin is not or low co-adsorbed on this chromatographic material when the pH is 5.0 and above. Therefore, a pH decrease to 5.0 is acceptable even in the presence of albumin with a minimal risk of co-adsorption. This is due to the fact that, at this pH, the resin and albumin are both negatively charged. At pH below 5.0, albumin or other acidic species may co-adsorb. However, the presence of a relatively high ionic strength (0.14 M sodium chloride) minimizes the co-adsorption of a large number of species even when the pH is below 5.0. To conclude, it is of strong benefit to optimize binding conditions pH between 5.0 and 5.5, especially when the concentration of antibody is low (below 250 μg/mL).

In the present case, the feedstock contained antibodies at a concentration of 150 μg/mL and, thus, an optimization of adsorption conditions was necessary for a proper separation process on the selected MBI HyperCel™ resin. This optimiza-

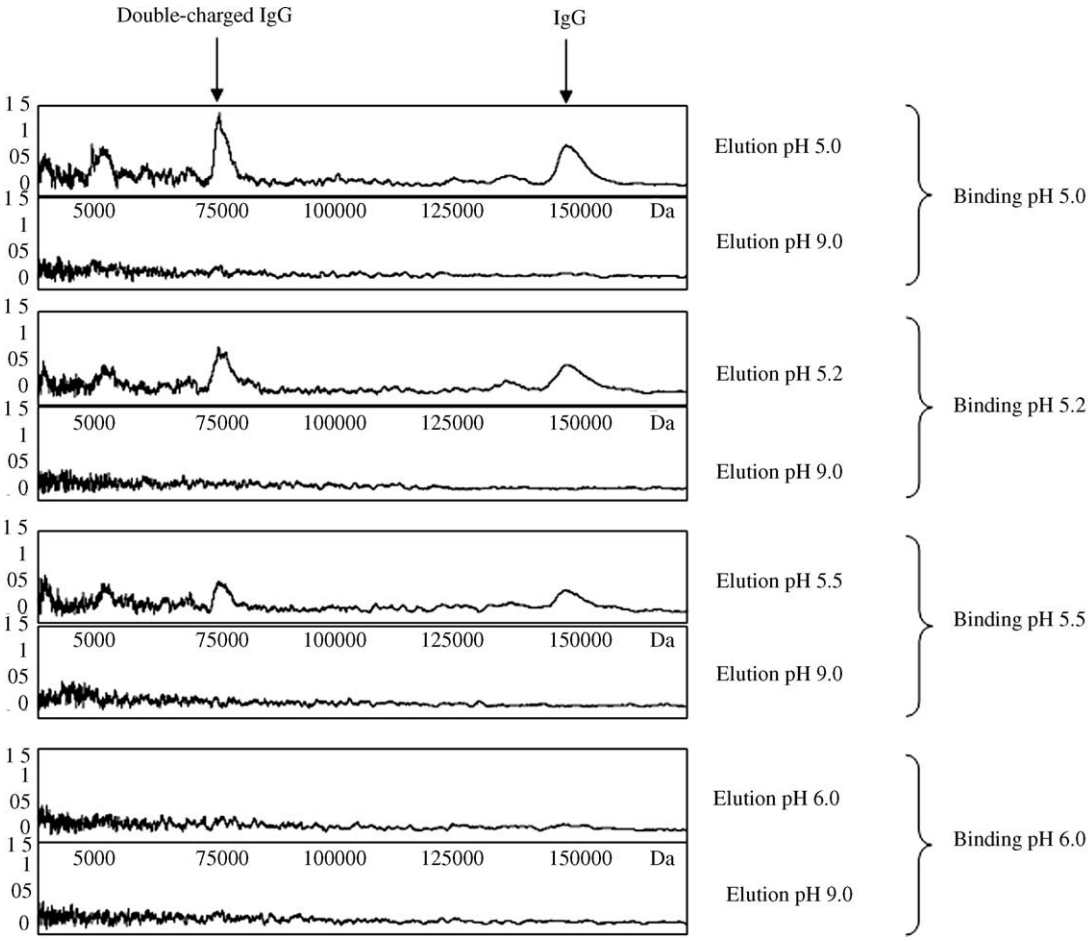

Fig. 2. SELDI–MS results using MBI ProteinChip® Arrays for the determination of separation conditions of a mouse monoclonal IgG_1 from a cell culture supernatant. Chip surface supported 2-mercapto-5-benzimidazolesulfonic acid ligand. Different conditions of loading were tried (pH 5.0, 5.2, 5.5 and 6.0). Desorption of antibodies was performed at pH 5.0, 5.2, 5.5, 6.0 or 9.0. Sample, cell culture supernatant, containing IgG_1 at a concentration of 150 μg/mL and after adjustment of the pH to the value used for loading. Arrows indicate the positioning of IgG: IgG represents the single-charged species of the protein and doubled-charged IgG is the double charged species of the protein.

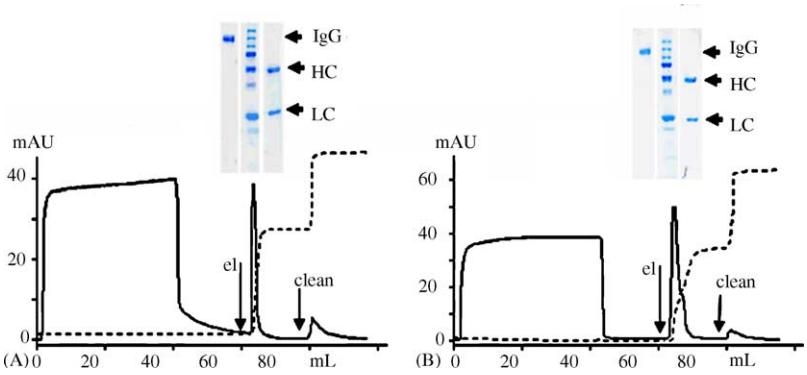

Fig. 3. Chromatographic separation profile of monoclonal IgG_1 from a cell culture supernatant using a MBI HyperCel™ column. The separation of monoclonal antibodies was performed at two different pHs: 5.2 (A) and 5.0 (B). Column, 3 mm i.d. × 100 mm height (0.7 mL resin); equilibration buffer, 50 mM sodium acetate, NaCl 0.14 M (pH 5.2 for A and 5.0 for B); sample, cell culture supernatant containing IgG_1 at a concentration of 150 μg/mL and after adjustment of the pH to the value used for loading; sample volume injected, 40 mL; linear flow rate, 150 cm/h (residence time, 4 min); elution of antibodies, 50 mM sodium bicarbonate buffer, 0.14 M NaCl, pH 9.0; el, elution; clean, regeneration in 1 M NaOH. Inserts, SDS polyacrylamide gel electrophoresis in non-reducing (left lanes) and reducing (right lanes) conditions. Middle lanes are molecular weight standards. HC, heavy chain; LC, light chain.

tion of separation conditions is very common when dealing with crude feedstocks. In the present case, the optimization was to be done on both adsorption and desorption stages in order to get, on one hand, a high binding capacity, and on the other hand, a good desorption for an optimized recovery. For this reason, a series of capture and elution trials were made using MBI ProteinChip® Arrays. These arrays are small surfaces coated with a thin layer of polymer similar to chromatographic resins. When components of a protein mixture are deposited on the functionalized surface of an array, an interaction occurs between the surface and solubilized proteins, resulting in adsorption of certain species. Equal distribution of proteins on the functionalized surface of ProteinChip® Arrays allows the signal intensities to correspond very well to the concentration of the proteins, assuring the ability to quantify individual protein levels in a given sample [19]. Previous work showed that the application of gradient wash conditions on ion exchange ProteinChip® arrays produces a step-wise elution of retained components that mimics elution on liquid chromatography columns packed with matching ion exchange sorbents [16]. Here, the surface was coated with a polysaccharide where 2-mercapto-5-benzimidazolesulfonic acid was coupled following the same chemistry used for the preparation of MBI chromatographic sorbent. These surfaces were, then, used as probes for SELDI–MS analysis. Results of this investigation indicated that the antibody was well adsorbed at pH 5.0 and 5.2, but was poorly adsorbed at pH 5.5 and not retained at pH 6.0 (Fig. 2).

When the antibody was well adsorbed (namely, at pH 5.0 and 5.2), desorption was optimized by raising the pH to 8.0, 9.0 or 10.0. Results indicated that desorption was complete when the pH was at least 9.0 (data not shown). Then, these data were translated to chromatographic separation.

Taking into consideration that the adsorption pH could be a trade off between the binding capacity (Fig. 1), the recovery and the purity, two column chromatography trials were performed. As represented on Fig. 3, separation profiles appeared relatively similar. However, the chromatographic profile of the separation operated at pH 5.2 showed a flowthrough peak with a visible tail (Fig. 3). Analysis of recovered fractions either by SDS–PAGE (data not shown) or SELDI–MS (Fig. 4) showed that there was a slight loss of IgG in the flowthrough fractions when equilibration was done at pH 5.2. When the separation was made at pH 5.0, all antibodies were adsorbed on the resin and, then, desorbed at pH 9.0. Comparison of numerical data (Table 1) showed that the final purity

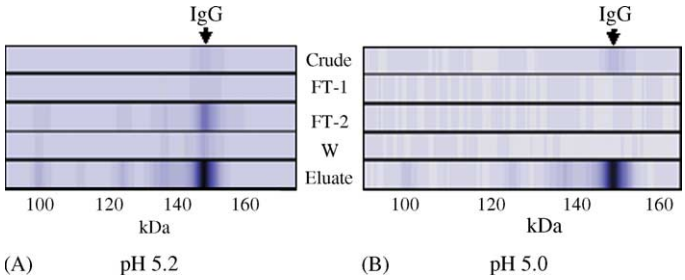

Fig. 4. Gel-view of a SELDI–MS analysis of fractions from chromatographic separations (Fig. 3) deposited on NP20 ProteinChip® Arrays. In (A) is represented analysis of the separation made at pH 5.2 and in (B) at pH 5.0. Dilutions of analyzed solutions were done in order to get an appropriate signal. Crude signifies the initial material prior fractionation. FT-1 and FT-2 are column flowthroughs (first part and second part of the peak); W is the wash fraction; eluate is the desorbed peak using the buffer at pH 9.0.

Table 1
Fractionation of cell culture supernatant at different pH using MBI HyperCel™ sorbent

	Volume (mL)	Total protein concentration (mg/mL)	Total protein content (mg)	IgG content (mg)	Recovery (%)	Purity (%)
pH 5.0						
Crude feedstock	40	0.47	18.8	6	100	32
Flowthrough	45	0.27	12.0	2.5	42	21
Wash	16.5	0	0	0	0	0
Eluate	10	0.35	3.5	3.5	58	>99
pH 5.2						
Crude feedstock	40	0.47	18.8	6	100	32
Flowthrough	45	0.33	14.9	3.4	57	23
Wash	15.5	0.1	1.8	0.4	6	22
Eluate	10	0.22	2.2	2.2	37	>99

was similar at pH 5.0 and 5.2. SDS–PAGE and SELDI–MS analyses demonstrated, as expected, a good final purity of the antibody. However, at pH 5.0, 3.5 g of IgG is recovered during elution, instead of 2.2 g at pH 5.2. Therefore, a decrease in 0.2 pH units of the equilibration buffer, lead to a gain of 37% of the amount of purified material.

4. Conclusion

This study confirmed the capability of the described chromatographic material to capture and separate antibodies. The adsorption, however, had to be optimized due to the low concentration of the antibody in the feedstock. This was possible by a limited set of experiments performed using SELDI–MS arrays coated with the same chemistry as the affinity resin. This approach allowed a reduction in analysis time and in biological material while crossing over a large number of separation conditions. SELDI–MS detection appeared also useful in the fact that it delivered, not only the information of the presence or the absence of adsorbed proteins, but also importantly, the number of species adsorbed and their respective molecular weight. This information is of paramount importance when conditions of adsorption are a compromise between the binding capacity, the purity and the recovery.

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