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Development and Characterization of Affinity Membranes for Immunoglobulin Purification

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Abstract: The purification of antibodies is conventionally performed using affinity chromatography columns, with Protein A as ligand. The development of valid alternatives to Protein A is one of the challenges of the research in downstream processing, which becomes more important as the production capability of the biopharmaceutical industry increases. The objective of this work is the characterization of affinity membranes derivatized with two different synthetic ligands that show high specificity for immunoglobulins. The affinity membranes have been prepared and characterized, in view of their application in the capture purification step.

Keywords: Affinity membranes, D-PAM, AZP, Immunoglobulins, IgG, capture

INTRODUCTION

Affinity chromatography is one of the most powerful techniques available for the purification of biomolecules in large scale productions, and is commonly applied in downstream processes. Its use, however, is associated to the availability of effective specific ligands for each target biomolecule. In view of their relevance, significant efforts have been devoted to the development of synthetic ligands for the purification of antibodies (1, 2). Affinity ligands naturally available, such as Protein A or Protein G for IgG purification, or lectins for IgA

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and IgM purification, suffer from several drawbacks, including high cost, their biological origin, the requirement of accurate analytical tests to assure the absence of contaminants, and the limited stability toward sanitizing agents (3).

Several studies describing the purification of immunoglobulins with synthetic ligands immobilized on chromatography beads have been reported in the literature (4–8). The immobilization of these ligands on polymeric membranes is a relatively new area of research and few works have appeared in the open literature (9, 10). About a decade ago there has been an intense research effort on affinity membranes with the development of Protein A membranes that became commercially available (11–13). However, due to the lower binding capacity of membranes with respect to chromatography beads and to the fact that the production of therapeutics based on monoclonal antibodies was only at a development stage, membrane adsorbers were never considered by the biotechnology industry as a possible alternative to conventional bead-based chromatography. The great number of monoclonal antibodies currently in the pipeline for approval, together with the higher titers achievable by fermentation technology, underlined the capacity limit of the downstream process (14, 15). In particular, suitable alternatives to Protein A chromatography are under investigation and membrane adsorbers are among the technologies considered (16, 17).

The present work was developed as part of the AIMs project, an Integrated Project funded by the European Union which focuses on the development of new materials and new processes for the purification of monoclonal antibodies, with the aim to prepare and characterize affinity membranes for the purification of immunoglobulins. To this purpose two different synthetic ligands have been immobilized on microporous membrane matrices made of regenerated cellulose. The affinity membranes obtained have been characterized in terms of static binding capacity, non-specific binding and selectivity, by using pure proteins solutions, artificial mixtures, as well as human serum.

MATERIALS AND METHODS

Reagents

Different immunoglobulins have been utilized: Gammanorm, from Octapharma Sweden, was chosen as a source for human IgG; it is a polyclonal antibody containing all the four subtypes of h-IgG and has an average molecular weight of 150 kDa. IgG1 Kappa from murine myeloma (Catalog n° M7894), $M_w = 160$ kDa, human IgM (Catalog n° I8260), $M_w = 900$ kDa, human serum (Catalog n° H4522) and all other proteins and chemicals were supplied by Sigma-Aldrich (Milan, Italy).

The BCA protein assay reagents were purchased from Pierce.

Molecular weight markers (Standards Broad Range), all reagents for SDS–PAGE electrophoresis and SEC-HPLC Standards were purchased from Bio-Rad Laboratories (Milan, Italy).

Membranes

Sartobind epoxy and Sartobind aldehyde activated membranes (Sartorius AG, Göettingen, Germany) were used as supports for ligand coupling. The membranes are made from reinforced stabilized cellulose, and have a total porosity of about 63%, a nominal pore size of 0.45 μm, an average thickness of 275 μm, and a permeability to water of 40–60 ml/(cm^2 bar min).

Ligands

Two different ligands, A2P and D-PAM, that exhibit affinity towards immunoglobulins have been coupled on the membrane supports considered.

D-PAM (Xeptagen S.p.A, Pozzuoli, Italy) is a tetrameric ligand, identified through the synthesis and screening of multimeric combinatorial peptide libraries, which is able to recognize the immunoglobulin Fc portion (18, 19). Lyophilized D-PAM (TG19320), Mw = 2141 g/mol, was kindly provided by Xeptagen SpA, (Pozzuoli, NA, Italy).

Mimetic A2P (Prometic Biosciences, Cambridge, UK), Mw = 296 g/mol, has been obtained by screening of combinatorial ligand libraries. It has been designed to mimic the structure of two key amino acid side chains of Protein A, Phe 132 and Tyr 133, (7, 20).

Equipment

A Shimadzu UV-1601 UV-Visible spectrophotometer has been used for protein concentration measurements, at 280 nm for pure protein solution and at 562 nm for the BCA colorimetric assay.

The purity of protein samples has been analyzed with gel electrophoresis and HPLC: SDS–PAGE analysis of the protein solutions was performed with Criterion electrophoresis system from Bio-Rad Laboratories using precast gels; a Waters Alliance 2695 separation module and a 2487 dual-wavelength absorbance detector were used for HPLC analysis, with a size exclusion column SEC BioSep-SEC-S 4000 from Phenomenex.

Ligand Immobilization

A2P ligand has been coupled onto Sartobind epoxy membranes by Prometic Biosciences, obtaining A2P-Sartoepoxy affinity membranes.

D-PAM has been coupled on both Sartobind epoxy membranes and Sartobind aldehyde membranes in our laboratories, obtaining D-PAM-Sartoepoxy and D-PAM-Sartoaldehyde affinity membranes, respectively.

Two different protocols have been used to couple D-PAM on the membrane matrices depending on the membrane pre-activation chemistry.

Coupling on Sartobind epoxy membranes was performed according to the following (5):

i. D-PAM was dissolved in a solution of sodium bicarbonate (0.1M $NaHCO_3$ and 0.5 M NaCl) pH 8.5 at a concentration of 1.75 mg/mL.
ii. The membranes were immersed in the coupling solution and the mixture was shaken for 48 hours at room temperature.
iii. The membranes were then extensively washed with deionized water:
iv. Neutralization of non-reacted epoxy groups was obtained with ethanolamine 0.42 g/L.
v. The system was shaken for 4 hours at ambient temperature and the membranes were thoroughly washed alternatively with deionized water and bicarbonate solution.
vi. The affinity membranes were stored in PBS with 0.02% sodium azide at 4°C.

Sartobind aldehyde membranes have been modified following a two-stage protocol assessed from the general indications for D-PAM immobilization given by the manufacturer:

i. The membranes were placed in a peptide solution prepared with 2 g/L of D-PAM, and 3.2 g/L of $NaCNBH_4$ as catalyst in phosphate buffer (0.1 M NaCl, 0.115 M NaH_2PO_4, pH 7) and kept under gentle agitation overnight at room temperature; the duration of this step was chosen after several trials over different time intervals.
ii. The membranes were then thoroughly washed with phosphate buffer.
iii. In order to block the remaining aldehyde active groups a cross-linking stage was carried out: the activated membranes were immersed in a solution of 0.42 g/L ethanolamine and 1.26 g/L of $NaCNBH_4$ for a period of 4 hours at room temperature.
iv. Finally, the membranes were extensively washed alternately with deionised water and coupling buffer and stored in 0.02% sodium azide at 4°C.

Ligand Density

Due to the peptidic nature of D-PAM it was possible to measure the amount of ligand immobilized on the membranes using a method based on the BCA protein assay (21). Since the coupling reaction was conducted with a strong excess of D-PAM, it was not possible to obtain an accurate measure of the decrease of ligand concentration in the coupling solution.

The aldehydic groups of the cellulose matrix do interfere with the BCA test, since they react with the cuprous cation present in the BCA reagent that is responsible for color formation. In the presence of $NaBH_4$ the aldehydic groups of cellulose are converted into hydroxyl groups, which do not interfere with the assay, thus a reduction of the membranes with a solution of 0.5% $NaBH_4$ in PBS for 15 minutes was introduced as a pre-treatment stage before the BCA test. The determination of the amount of immobilized D-PAM was performed using BCA assay with calibration curves prepared by using pure D-PAM solutions at known concentration values.

Experimental

Binding kinetics of different immunoglobulins has been determined for all the affinity membranes prepared through a series of experiments performed at different protein concentration in the feed solution. The membranes were immersed in a beaker containing the protein solution and kept under mechanical agitation by the action of an orbital shaker. The concentration of the protein in solution has been measured at regular intervals of time by reading the absorbance of the protein solution at 280 nm. The typical behavior is shown in Fig. 1. Adsorption has been interrupted once the steady state condition has been reached. Each experiment has been repeated at least twice in order to check reliability and reproducibility.

Static binding capacity was measured in batch adsorption experiments of pure protein solutions in PBS buffer pH 7.4. The membranes were immersed in a beaker containing a pure protein solution of known concentration and

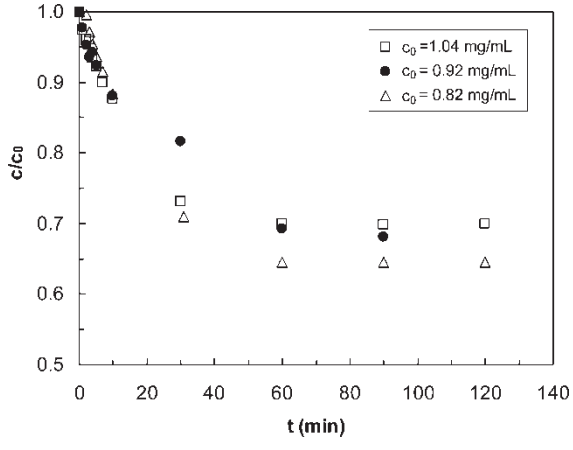

Figure 1. Binding kinetics of human IgG on A2P-Sartoepoxy, dimensionless protein concentration as a function of time with the following experimental conditions: membrane area 24.5 cm^2, feed volume 5 mL, T = 25°C.

gently agitated in an orbital shaker. The protein uptake at equilibrium was determined by measuring the protein concentration in solution. The duration of the adsorption step has been determined from the kinetic experiments described above. After adsorption, a washing step with buffer was performed to remove the proteins non-specifically adsorbed onto the membranes. To recover the protein selectively adsorbed on the affinity membranes, different elution buffers have been used depending on the protein adsorbed and on the membrane; in particular 0.1 M glycine pH 2.8, 50 mM citric acid pH 2.5 and 0.1 M acetic acid were mainly utilized.

Non-specific adsorption has been evaluated for the pre-activated membranes, without ligand, as well as for the affinity membranes obtained. Two different proteins, HSA with pI 4.9 and lysozyme with pI 10.7 have been chosen for the non-specific adsorption tests.

The affinity membranes have been tested for selectivity towards immunoglobulins using an artificial mixture prepared with 1 mg/mL murine IgG, 10 mg/mL HSA, and 2 mg/mL myoglobin in PBS buffer pH 7.4 and also using human serum.

Five membrane discs of 2.5 cm in diameter have been used with human serum, which was diluted 1:20 in PBS buffer pH 7.4. The dilution factor was chosen after several tests, to obtain a detectable amount of immunoglobulin bound on the membranes. The affinity membranes were immersed for 120 minutes in the diluted serum solution; two washing steps were then performed with PBS pH 7.4 and the adsorbed protein was eluted with 0.1 M glycine pH 2.8 for 120 minutes.

Membrane Regeneration

After use, the membranes were regenerated with 1 M NaOH, 2M NaCl in a 20% ethanol aqueous solution. The membranes were soaked for 20 minutes in the regenerating solution at room temperature, then they were washed with buffer and used for subsequent adsorption-elution cycles.

RESULTS AND DISCUSSION

Ligand Density

The values of ligand density obtained from BCA protein assay for D-PAM affinity membranes were 0.0013 μmol D-PAM/cm^2 of membrane for D-PAM-Sartoepoxy and 0.0026 μmol D-PAM/cm^2 of membrane for D-PAM-Sartoaldehyde, respectively.

Higher D-PAM densities were thus obtained on Sartobind aldehyde membranes indicating that the binding protocol used for this ligand is more efficient with the aldehyde activation chemistry.

The amount of A2P immobilized on Sartobind epoxy membranes has been quantified by Prometic Biosciences by following an indirect method, based on the titration of the active epoxy groups on the membrane matrix; by assuming 100% efficiency of the coupling reaction one thus estimates a ligand density of 1.4 μmol A2P/cm^2 of membrane.

It is worth pointing out that the immobilization estimated for A2P on Sartobind epoxy membranes gives a molar ligand density three orders of magnitude higher than the immobilization of D-PAM on the same membrane matrices. However, the two methods used for determining ligand density are sensibly different and not directly comparable.

Binding Kinetics

The adsorption of human IgG on A2P-Sartoepoxy affinity membranes was completed in about 60 minutes, for all the IgG concentrations considered in the feed solution, up to 1.0 mg/mL, while for the adsorption of murine IgG on D-PAM-Sartoepoxy about 90 minutes were required to achieve equilibrium. Typical results of the binding kinetic experiments on A2P-Sartoepoxy and on D-PAM-Sartoepoxy are reported in Figs. 1 and 2, respectively.

Static Binding Capacity

The static binding capacity of human IgG onto A2P-Sartoepoxy affinity membranes is reported in Fig. 3 as a function of the protein concentration

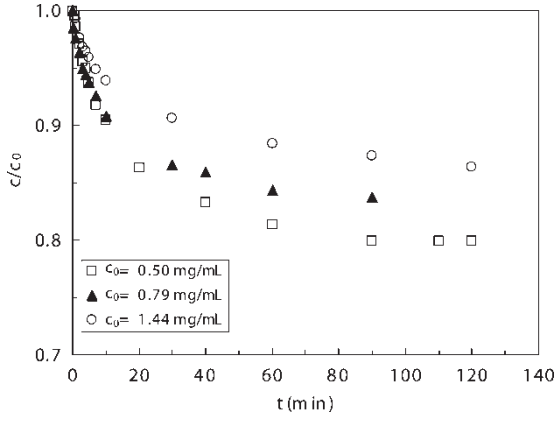

Figure 2. Binding kinetics of murine IgG on D-PAM-Sartoepoxy, dimensionless protein concentration as a function of time with the following experimental conditions: membrane area 19.6 cm^2, feed volume 4 mL, T = 25°C.

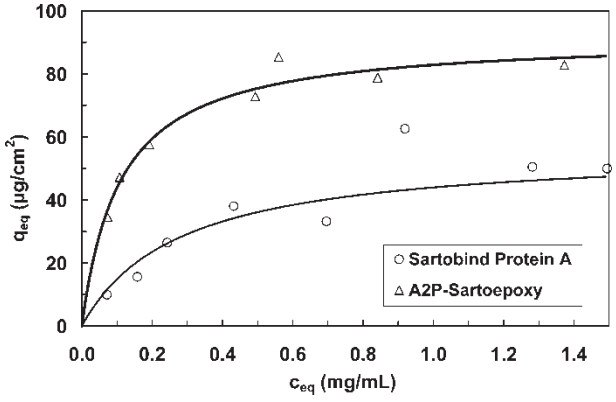

Figure 3. Equilibrium adsorption isotherms of human IgG on A2P-Sartoepoxy and on Sartobind Protein A affinity membranes.

in solution at equilibrium. As a comparison, the results of IgG adsorption on a benchmark membrane, Sartobind-Protein A, have also been reported in the same plot. The experimental results have been interpolated using a Langmuir adsorption isotherm (22, 23) and the corresponding parameters, maximum static binding capacity, q_{max}, and dissociation constant, K_d, are reported in Table 1. The static binding capacity of the membranes derivatized with A2P is about 63.5% higher than that of the commercially available Sartobind-Protein A.

The new membranes have then been used in subsequent adsorption-washing-elution cycles with no intermediate regeneration steps. The binding capacity at equilibrium for the first three cycles of adsorption together with the relevant isotherms are illustrated in Fig. 4, and the corresponding Langmuir parameters are reported in Table 2. The static binding capacity decreases from the first cycle to the third cycle, although the drop in capacity is more remarkable between the first and the second cycle for which a decrease of about 35 % in capacity has been observed.

The ageing effects due to subsequent affinity cycles in A2P Sartoepoxy membranes are also confirmed by the data reported in Fig. 5, in which the amount of protein recovered in the elution step has been reported as a function of the cycle number for duplicate experiments, with two different

Table 1. Langmuir parameters for human IgG of Sartobind Protein A and A2P-Sartoepoxy

	q_{max} (μg/cm^2)	K_d (mg/mL)
Sartobind Protein A	56.2	0.278
A2P-Sartoepoxy	91.9	0.109

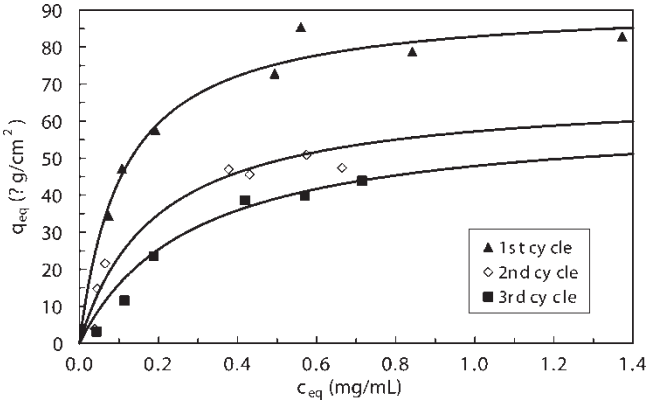

Figure 4. Equilibrium adsorption isotherms of human IgG on A2P-Sartoepoxy affinity membranes in three subsequent cycles.

elution buffers. In this case the amount of recovered protein decreases up to the third cycle, then it levels off and remains almost constant for the three further cycles. The amount of protein recovered is the same for the two different elution buffers considered, 0.1 M glycine pH 2.8 and 50 mM citric acid pH 2.5, indicating that both buffers can be equivalently utilized for the elution step. The decrease of membrane capacity with use is ascribable to an incomplete protein recovery with the elution step that is mainly due to the non-specific adsorption observed for these membranes. After the first two cycles the non-specific adsorption sites have been saturated with protein and the amount of IgG that is bound in the following cycles is completely recovered with elution (24).

After the six consecutive adsorption, washing and elution cycles the membranes have been regenerated and used for six further cycles. After regeneration the binding capacity of the membranes is not completely restored to the initial value, but a significant increase of the amount of protein recovered is nonetheless achieved. This is illustrated in Fig. 6 in which the amount of protein recovered is reported as a function of cycle number for

Table 2. Langmuir parameters for human IgG on A2P-Sartoepoxy for three adsorption cycles

	A2P-Sartoepoxy	
Cycle number	q_{max} (μg/cm^2)	K_d (mg/mL)
1st cycle	91.9	0.109
2nd cycle	68.1	0.191
3rd cycle	61.5	0.285

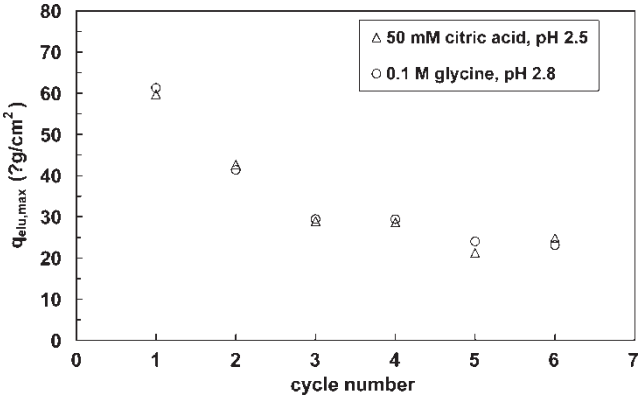

Figure 5. Maximum elution in subsequent cycles of human IgG from A2P-Sartobind Epoxy affinity membranes, after adsorption from a $c_0 =$ 1.1 mg/mL protein solution; comparison of two different elution buffers.

the twelve subsequent cycles performed with regeneration after the sixth cycle. However, after the third cycle the amount of protein recovered is constant and no differences can be noticed between the behaviours of the membranes before and after regeneration.

For the D-PAM derivatised membranes the adsorption properties have been determined for several immunoglobulins, since this ligand exhibits affinity for different immunoglobulins. Adsorption equilibrium data of human IgG, murine IgG and human IgM have been obtained for both

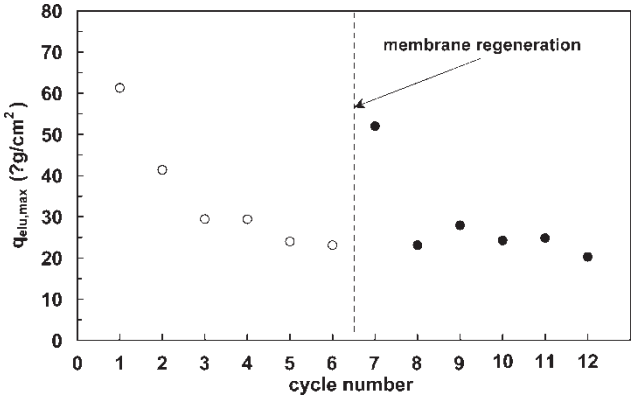

Figure 6. Maximum elution in subsequent cycles of human IgG from A2P-Sarto-epoxy affinity membranes, after adsorption from a $c_0 =$ 1.1 mg/mL protein solution; elution buffer 0.1 M glycine, pH 2.8. Difference between new (open symbols) and regenerated membranes (closed symbols).

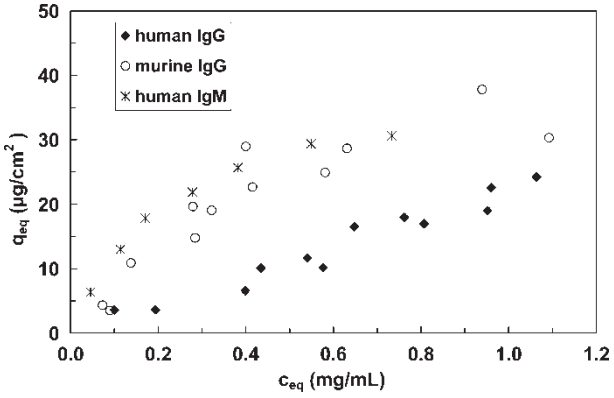

Figure 7. Equilibrium adsorption isotherms of human IgG, murine IgG and human IgM on D-PAM-Sartoepoxy affinity membranes.

D-PAM-Sartoepoxy and D-PAM-Sartoaldehyde and the results are reported in Figs. 7 and 8, respectively. The static binding capacity for the different proteins has been calculated by interpolating the experimental data with the Langmuir isotherm and the resulting parameters are reported in Tables 3 and 4, respectively. The adsorption behaviors of the two affinity membranes are similar to one another, with higher binding capacity for murine IgG and human IgM and lower capacity for human IgG. The D-PAM-Sartoaldehyde affinity membranes have a static binding capacity higher than the D-PAM Sartoepoxy membranes, for all the immunoglobulin investigated, indicating that the efficiency of the same ligand is affected by the chemical nature of the active sites of the preactivated membranes.

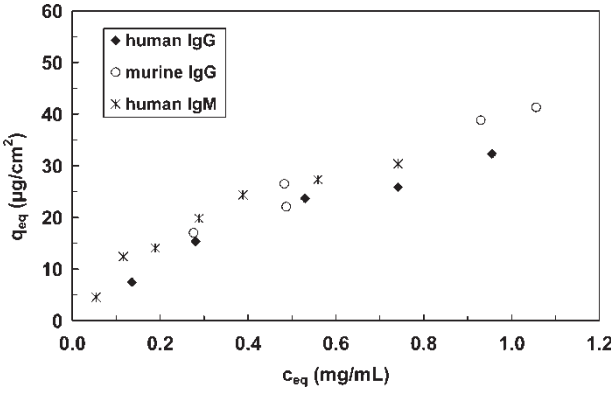

Figure 8. Equilibrium adsorption isotherms of human IgG, murine IgG and human IgM on D-PAM-Sartoaldehyde affinity membranes.

Table 3. Langmuir parameters for different immunoglobulins on D-PAM-Sartoepoxy membranes

	D-PAM-Sartoepoxy	
Protein	q_{max} ($\mu g/cm^2$)	K_d (mg/mL)
Human IgG	38.0	0.954
Murine IgG	47.6	0.488
Human IgM	41.2	0.238

Adsorption from Complex Mixtures

An artificial mixture containing human IgG, HSA and myoglobin (1/10/2 mg/mL) in PBS pH 7.4 was adsorbed in batch on A2P-Sartoepoxy membranes. The results of SEC HPLC analysis on the eluate gave a concentration ratio of IgG to HSA varying between 2.1 ÷ 7.1, which is 20 ÷ 70 times larger than the concentration ratio IgG/HSA of 0.1 used in the feed, indicating a separation factor towards IgG in excess of 200. However, a high non-specific binding towards HSA, of about 30 $\mu g/cm^2$, was detected, indicating that the operating conditions need to be optimised in order to decrease the non-specific binding.

Tests performed with the activated membranes without ligand using IgG, HSA and lysozyme did not show any binding indicating that the non-specific adsorption detected for the A2P affinity membranes is due either to the ligand or to the spacer arm.

D-PAM affinity membranes have been tested with human serum, using the same experimental conditions for both D-PAM-Sartoepoxy and D-PAM-Sartoaldehyde. SEC HPLC analysis of the different solutions gave the typical trend reported in Fig. 9 for D-PAM-Sartoaldehyde, in which all the chromatograms of the initial feed solution, of the solution at the end of the adsorption step and of the washing step show an HSA peak predominant with respect to IgG. However, the chromatograms of the eluted fractions, reported in Fig. 10, show the presence of IgM and IgG only, indicating a

Table 4. Langmuir parameters for different immunoglobulins on D-PAM-Sartoaldehyde membranes

	D-PAM-Sartoaldehyde	
Protein	q_{max} ($\mu g/cm^2$)	K_d (mg/mL)
Human IgG	41.5	0.400
Murine IgG	75.8	0.946
Human IgM	46.3	0.382

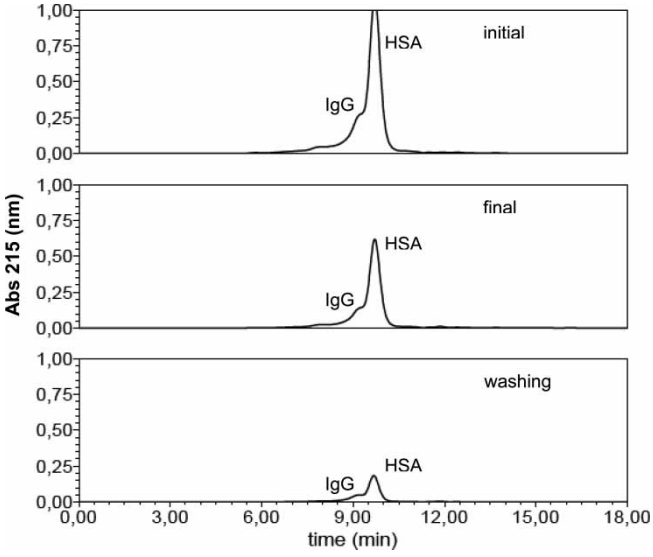

Figure 9. HPLC SEC chromatograms of the initial, final and washing stages of human serum on D-PAM-Sartoaldehyde membranes.

very good selectivity of these membranes for the immunoglobulins considered. These results confirmed the low non-specific adsorption of D-PAM affinity membranes that was always below 5 $\mu g/cm^2$ in all the cases investigated.

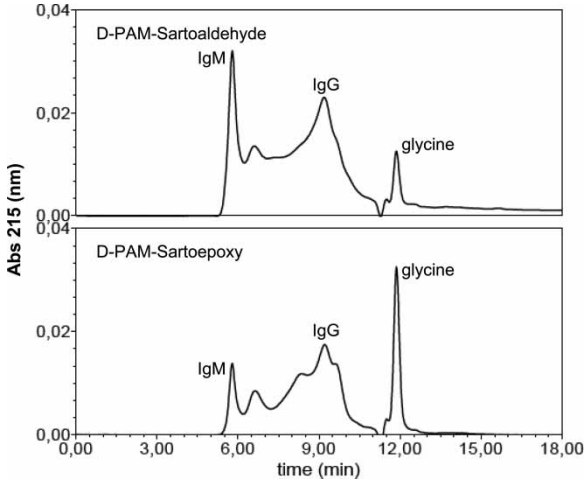

Figure 10. Comparison of the eluted fractions chromatograms obtained from adsorption of human serum on D-PAM-Sartoepoxy and D-PAM-Sartoaldehyde affinity membranes.

CONCLUSIONS

A2P-Sartoepoxy has a higher binding capacity than Sartobind Protein A for human IgG. After the first two cycles, the static binding capacity decreases by about 35%, and then remains constant for the subsequent further utilizations. The original binding capacity is re-gained with membrane regeneration, indicating that the protocol used is effective and it should be used after every complete adsorption-washing-elution cycle. This membrane presents a separation factor of IgG versus HSA in excess of 200, even though there is also a high non-specific binding towards human serum albumin.

D-PAM has been successfully immobilised onto two different preactivated cellulose membranes. The resulting affinity membranes have been tested for adsorption of different immunoglobulins. The highest binding capacity was observed for murine IgG and the lowest binding capacity was detected for human IgG for both D-PAM-Sartoepoxy and D-PAM-Sartoaldehyde. In all cases, D-PAM-Sartoaldehyde has higher capacity than D-PAM-Sartoepoxy, reflecting the more effective ligand immobilization.

Experiments with human serum demonstrate that these membranes have a very good selectivity towards immunoglobulins and in particular for human IgM.

Future work will be devoted to further inspect the effects of membrane matrix, immobilization chemistry, and spacer arm length on the separation performances.

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