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Journal of Pharmaceutical and Biomedical Analysis
24 (2000) 95–104

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Comparison of antibody binding to immobilized group specific affinity ligands in high performance monolith affinity chromatography

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Received 30 July 1999; received in revised form 6 June 2000; accepted 13 June 2000

Abstract

A novel biochromatographic principle is introduced taking the quantitative analysis of affinity interactions between antibodies and immobilized group specific ligands (protein A, G, and L) as example. The name high performance monolith affinity chromatography (HPMAC) is proposed for this technique. HPMAC uses rigid, macroporous monoliths, so-called convective interaction media (CIM™)-disks, as stationary phase. An optimized procedure is described for the covalent immobilization of the group specific affinity ligands to such disks. The binding of polyclonal bovine IgG and a recombinant human antibody (type IgG1-κ) to all affinity disks is discussed. An essential feature of HPMAC is its compatibility to unusually high mobile phase flow rates (>4 ml/min). Chromatographic experiments are thus completed within seconds without significant loss in binding capacity and retentive power. This makes HPMAC a promising tool for applications in fast process monitoring or screening. As an example for the former, the direct quantitative isolation of recombinant antibodies from serum-free culture supernatant is demonstrated.

Keywords: High performance monolith affinity chromatography; Antibodies; Immobilized group specific ligands

1. Introduction

The specific interaction between immunoglobulin A and G (IgA, IgG) subtypes with microbial surface receptors such as protein G and protein A is widely used in the purification of these substances by affinity chromatography. It is known that the Fc-fragments of the immunoglobulins bind to these fibrous receptors and that each receptor molecule bears several IgG interaction sites [1,2]. Another type of group specific ligand, namely protein L (*Peptostreptococcus magnus*), has been introduced more recently [3]. This ligand binds to an eventually present κ-light chains in the antibody molecule [4,5]. Protein L has, for example, been proposed for the purification of both whole antibodies and antibody fragments [6,7].

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0731-7085/00/$ - see front matter
PII: S0731-7085(00)00414-3

Recently, a novel type of biochromatography has been introduced [8], for which we propose the name high performance monolith chromatography (HPMC). A characteristic feature of HPMC is the type of stationary phase used. Typically these are flat, monolithic and macroporous disks, which are now commercially available under the name of convective interaction media (CIM™)-disks. The macroporous structure of the CIM™ material allows overcoming many of the disadvantages of conventional biochromatography. Most importantly, the mass transport is dramatically improved, since it occurs mainly by convection rather than by total or partial diffusion as in conventional chromatography [8]. While in the latter case, mass transfer steps may be limiting, in HPMC it is most likely to be the surface interaction, which determines the overall reaction rate. HPMC in general has been shown to be a useful technique for efficient and fast isolations of proteins from such complex sources as blood serum [9–11] or cell culture supernatants [12,13]. In general, HPMC combines high capacity and selectivity with low backpressure and short operation times.

In this paper, we describe and compare for the first time the use of CIM™ affinity disks bearing different group specific ligands such as protein A, G, and L. The corresponding affinity units were used for fast biospecific separation of different IgGs including recombinantly produced monoclonal antibodies (mAbs).

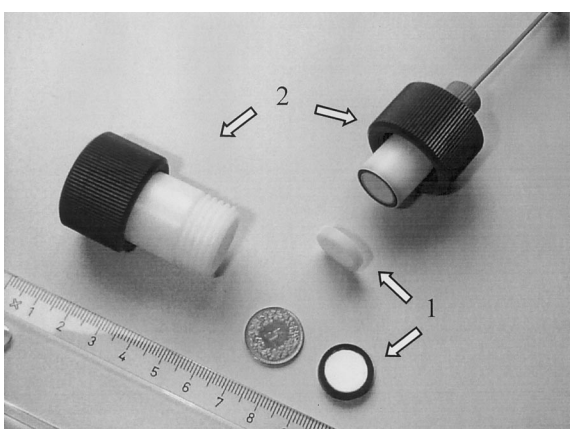

Fig. 1. CIM™-disk 12 × 3 mm (1) and cartridge (2).

2. Experimental procedures

2.1. Materials

Poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) disks, dimensions of 12 × 3 mm (CIM™-disks) and specially designed disk cartridges were from BIA Separations Ltd. (Ljubljana, Slovenia), Fig. 1. Recombinant protein A was from Calbiochem–Novabiochem Corp. (La Jolla, CA, USA); recombinant protein G from Sigma Bioscience (St. Louis, MI, USA); recombinant protein L from ACTIgen Ltd. (Cambridge, UK). Bovine IgG was from Fluka AG (Buchs, Switzerland). Fine chemicals and bulk chemicals for buffer and eluent preparation were all of analytical grade and from Fluka, Sigma, Merck (Darmstadt, Germany) or Acros Organics (Geel, Belgium). UHP water was used throughout.

2.2. SDS-PAGE

The purity of the affinity isolated antibodies was verified by sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE). A Mini-Protean II System (Bio-Rad Laboratories, Hercules, CA, USA), Tris–HCl ready gels (7.5% resolving gel) and silver staining detection (Silver Stain Plus kit) were used (all Bio-Rad). Samples were loaded twice on the gels, once using standard concentration the other time overloaded.

2.3. Immobilization procedure

Immobilization of protein ligands to the disks took place via the epoxy groups found naturally in the polymer structure of the disk material and the ε-aminogroups of the proteins. After washing the disks with increasingly polar solvents (methanol, methanol:water 1:1 (v/v), water) the wet disks were immersed in a 100-mM carbonate buffer, pH 9.3, for 1–2 h before transfer to 1.0 ml of a 5.0 mg/ml solution of the affinity ligand (S1) in the same carbonate buffer. The immobilization reaction was allowed to proceed for 16 h at 30°C (water bath). Afterwards the solution was recovered (S2) and saved for quantification of residual ligand. A further 1.0-ml of carbonate buffer was

put onto the disk for a 1 h washing. This washing buffer (S3) was also collected for quantification of the affinity ligand. Remaining epoxy groups were subsequently blocked by exposure to 1.0 M ethanolamine at room temperature for 1 h. The disks were then washed with water and immersed overnight in 2–3 ml phosphate buffered saline solution (PBS, 50 mM phosphate — KH_2PO_4/Na_2HPO_4 — buffer containing 120 mM sodium chloride, pH 7.4) at 4°C.

2.4. Protein quantification

The amount of ligand bound to the disk was calculated indirectly from quantification of the protein concentration in the ligand solution prior and after the immobilization reaction and also in the washing solution (S1, S2, S3). Appropriately diluted samples were injected into an FPLC system (Amersham Pharmacia, Uppsala, Sweden) equipped with a weak anion exchanger (DEAE) disk (BIA Separations Ltd.), under non-binding conditions (mobile phase, Tris 20 mM + 1.0 M NaCl, pH 7.7, at 4 ml/min flow rate, detection: $\lambda = 280$ nm). The amount of ligand immobilized on the disk was calculated from the ratio between the peak areas taking dilution factors and exact sample volumes of S2 and S3 into account. Purified IgG concentrations were determined by absorption at 280 nm. Calculations were based on the assumption that a 1.0 mg/ml solution of IgG has an absorbency of 1.4.

2.5. Affinity chromatography

Affinity chromatography was performed as a step-wise adsorption–desorption process using a step gradient from 100% binding buffer to 100% elution buffer. Unless indicated otherwise, the binding buffer was 50 mM PBS + 120 mM NaCl, pH 7.4, and the elution buffer 10 mM HCl, pH 2.0. No special washing step after adsorption was used.

For the isolation of recombinant IgG from cell culture supernatant, the following buffers were used:

protein A high performance monolith affinity chromatography (HPMAC), binding PBS 50 mM + 120 mM NaCl pH 7.5; elution glycine, 100 mM pH 2.0;

protein G HPMAC, binding PBS 50 mM + 67 mM NaCl pH 7.4; elution HCl, 10 mM pH 2.0; and

protein L HPMAC, binding Tris 20 mM + 12% Na_2SO_4 anh. pH 9.0 [14]; elution glycine, 100 mM pH 2.0.

Bovine IgG and recombinant purified humanized r-IgG1-κ were dissolved/diluted in 50 mM PBS + 120 mM NaCl pH 7.4 buffer. Cell culture supernatants were diluted 5:3 in the binding buffer of the corresponding affinity disk type. The above-mentioned FPLC system was used for all affinity experiments.

2.6. Adsorption isotherms and determination of affinity parameters

Adsorption isotherms were constructed using the frontal analysis approach, as described previously [15]. For this purpose, ten solutions of increasing concentrations (from 2.5 to 180 mg/l) of the purified recombinant IgG were prepared in 50 mM PBS + 120 mM NaCl pH 7.4. Starting with the lowest concentration, these solutions were pumped through the disk at a given flow rate (usually 4 ml/min, in case of the protein L disk also 6 ml/min) until the disk was saturated (complete breakthrough of the antibody). The amount of antibody actually retained on the disk (q_1) was calculated from the breakthrough volume of the solution under investigation (V_c), the breakthrough volume of an unretained tracer (V_o), and the concentration of the solution under investigation (C_1), as follows to give the first C/q-point of the isotherm:

$$(V_c - V_o)C_1 = q_1 \quad (1a)$$

Afterwards the next higher concentration was applied directly to the disk in a similar manner. q_2 could then be calculated according to:

$$(V_c - V_o)C_2 + q_1 = q_2 \quad (1b)$$

Increasingly higher concentrations were used to calculate the subsequent points until the plateau of the isotherm was reached. The maximum binding capacity, q_m, and the complex dissociation

Table 1
Quantitative data on the repeated immobilization of protein ligands on epoxy CIM™-disks

	Molecular mass (kDa)	Amount of immobilized ligand	
		mg per disk	μmol per disk
Protein A	45	0.86 (±20%)	0.0191 (±20%)
Protein G	34	0.99 (±20%)	0.0293 (±20%)
Protein L	36	0.57 (±20%)	0.0159 (±20%)

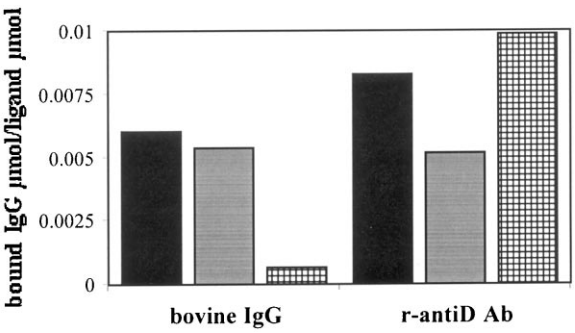

Fig. 2. Histogram of the results obtained by HPMAC in regard to the relative affinity retention of different types of antibody on the disks with different affinity ligands. ■, Protein A; ▒, protein G; ⊞, protein L. Conditions: CIM™-disks, 12 × 3 mm; flow rate, 4 ml/min; UV detection at 280 nm; adsorbing buffer was PBS pH 7.4; eluting buffer was 10 mM HCl pH 2.0; the amounts loaded were 25 μg of bovine IgG or recombinant IgG1-κ antibody in 100 μl adsorption buffer.

constant, K_d, were obtained graphically from the linearized isotherm, assuming the Langmuir equation to apply, see below.

3. Results

In this study, three microbial receptors, namely protein A, G and L, all of which were known to bind IgG in a highly specific manner, were investigated. The three ligands were repeatedly immobilized on the CIM™-disks using the standard protocol described in the experimental section. The amounts of ligand immobilized on the disks were similar in all the three cases with actual quantities ranging between 0.02 and 0.03 μmol per disk (Table 1). The immobilization reaction was typically completed after 15–20 h, since no further increase in the surface concentration could be observed for longer exposure times.

In order to investigate species and sub-type depending differences in the IgG binding behavior of the three affinity ligands, the interaction of two sample molecules, a polyclonal bovine IgG obtained from Fluka and an affinity purified recombinant humanized antibody (IgG1-κ type) prepared in house, was compared. The histogram presented in Fig. 2 shows that protein G binds both the bovine and the recombinant human IgG1-κ with approximately the same capacity, whereas protein A has less affinity to bovine IgG than to human IgG1-κ. As expected, protein L hardly binds bovine IgG at all [14].

Additional experiments were carried out to optimize the adsorption/desorption conditions in order to find those, which could provide maximum affinity and adsorption capacity as well as suitable chromatographic properties (peak shape, reproducibility) during antibody elution. A set of adsorption/desorption conditions was investigated, which included conditions recently proposed by us for protein G [11,12] and those suggested by the distributor of protein L [14]. In spite of these recommendations, in all cases, the best results were obtained using PBS pH 7.4 during product binding (adsorption step) and HCl pH 2.0 for desorption. These conditions were, therefore, maintained during all experiments, safe for the recovery of antibody from cell culture supernatants.

Frontal analysis was used to obtain the isotherms and thereby the affinity constants of the involved interactions. Fig. 3 shows the adsorption isotherms of the recombinant antibody sample (humanized IgG1-κ) on disks with immobilized protein A, G and L, respectively, under condition of dynamic binding, i.e. at a flow rate of 4 ml/min. The recorded adsorption isotherm can be fitted to the Langmuir equation, i.e.:

$$q = \frac{q_m \cdot C}{K_d + C} \quad (2)$$

where K_d is the dissociation constant of the ligand-antibody pair, C is the antibody concentration in the solution, q is the amount of antibody adsorbed onto the disk, and q_m is the corresponding maximum adsorption capacity of a given affinity disk.

The linearization of this equation (Eq. (3)) allows the calculation of q_m and K_d, from the slope and the intercept, respectively (Table 2).

$$\frac{K_d}{q_m} + \frac{C}{q_m} = \frac{C}{q} \quad (3)$$

Taking the molecular masses of both the IgG and the investigated ligands into account, the molar ratio between affinant (antibody) and affinity ligand (immobilized proteins) were calculated (Table 2).

The influence of the flow rate on the antibody binding was exclusively studied for the protein L disks (Table 3). For this purpose, binding isotherms were recorded for the recombinant human IgG1-κ antibody for mobile phase flow rates of 4 and 6 ml/min, respectively, Fig. 4. Under these condition, a slightly higher K_d value was

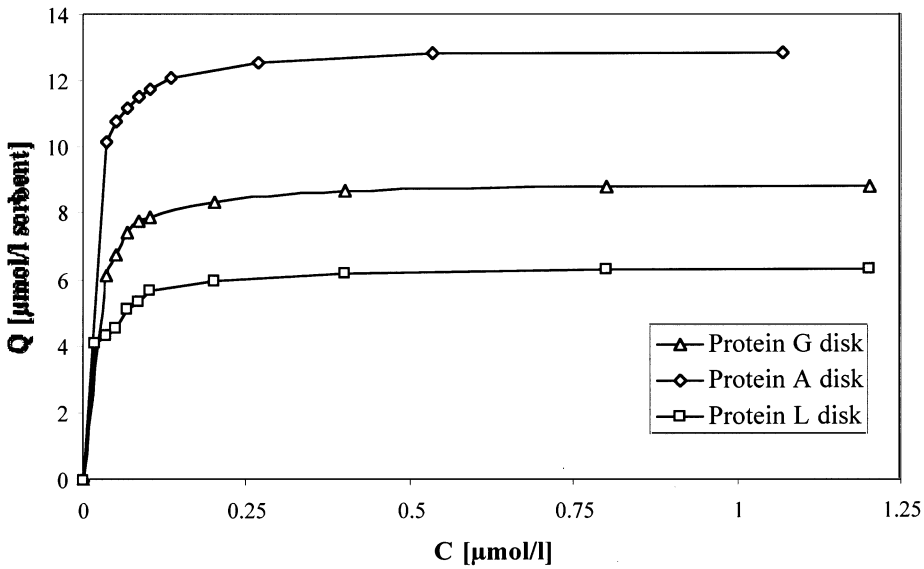

Fig. 3. Experimental adsorption isotherms obtained by frontal analysis for the recombinant IgG1-κ antibody binding to different protein ligands. Conditions: CIM™-disks, 12 × 3 mm; flow rate, 4 ml/min; UV detection at 280 nm; adsorbing buffer was PBS pH 7.4.

Table 2
Data from the linearized experimental adsorption isotherms[a]

	Protein A	Protein G	Protein L
Linearized equation	$C/q = C0.77 + 0.00075$	$C/q = C0.11 + 0.0015$	$C/q = C0.16 + 0.0024$
S.D. (r^2)	1.000	1.0000	0.9999
Number of points	9	9	10
q_m (mg/ml sorbent)	1.95	1.33	0.96
q_m (mg IgG per mg ligand)	0.47	0.46	0.57
q_m (mol IgG per mol ligand)	0.14	0.10	0.14
K_d (µmol/l)	0.0097	0.013	0.015

[a] The parameters of the linearized isotherm are given by the linear trendline (Excel) of C/q as a function of C; q_m (µmol/l sorbent) is calculated from 1/slope; K_d (µmol/l) is calculated from intercept/slope.

Table 3
Data obtained for Protein L activated CIM-Disks at a flow rate of 6 ml/min[a]

	Protein L
Linearized equation	$C/q = C0.19 + 0.0015$
Standard deviation (r^2)	0.9999
Number of points	10
q_m (mg/ml sorbent)	0.80
q_m (mg IgG per mg ligand)	0.47
q_m (mol IgG per mol ligand)	0.11
K_d (μmol/l)	0.0078

[a] The parameters of the linearized isotherm are given by the linear trendline (Excel) of C/q as a function of C; q_m (μmol/l sorbent) is calculated from 1/slope; K_d (μmol/l) is calculated from intercept/slope.

calculated (7.77×10^{-9} M at 6 ml/min in contrast to 1.52×10^{-8} for 4 ml/min), while the maximum of the adsorption capacity, q_m, was slightly lower than before (5.3 at 6 ml/min vs. 6.4 at 4 ml/min). Neither change was considered significant for the intended application.

The main goal of the research described here was to develop a fast and efficient bioanalytical system. In the context of bioreactor monitoring during the production of recombinant human IgG1-κ, it will, for example, be necessary to reliably determine even low product concentrations. It was, therefore, deemed necessary to investigate the effect of the sample concentration on the recorded signal (peak area). This investigation required a series of experiments during which samples containing the same absolute amounts of IgG albeit at different concentrations (volumes) were analyzed chromatographically. The ensuing chromatograms showed very similar peak areas, which corresponded in each case to the total amount of injected product (Table 4).

Taking into account the main goal of our research, in a final application it was attempted to recover recombinant human IgG1-κ from serum free CHO cell culture supernatants. All three ligand types were investigated. In all cases, quantitative recovery was possible, even of small amounts of product (μg/ml range) in the presence of some 'background' proteins in the supernatant.

The purity of all IgG preparations isolated by HPMAC was investigated by SDS-PAGE. According to the results demonstrated in Fig. 5, in all cases the purity of the isolated antibody was high and fully comparable to that of the elabo-

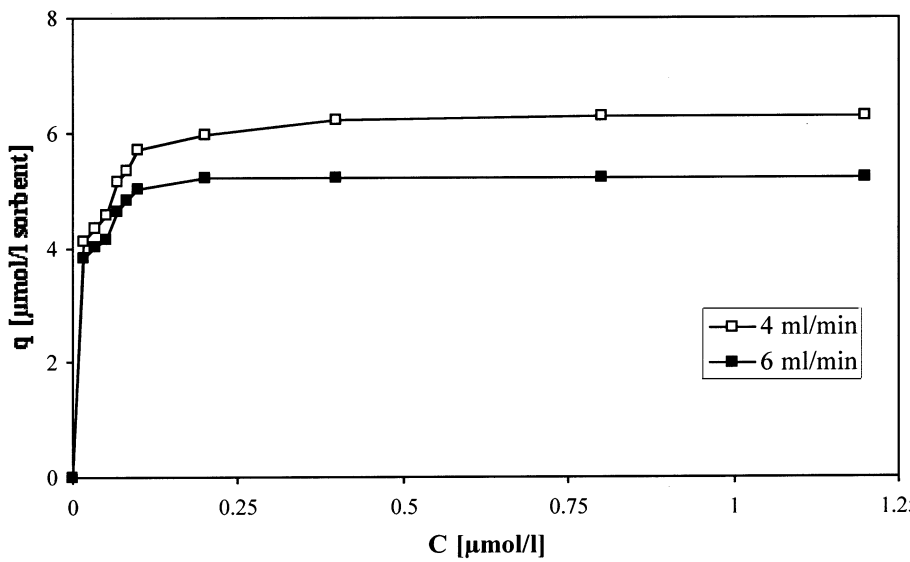

Fig. 4. Comparison of dynamic affinity interactions at different flow rates, adsorption isotherms obtained for the recombinant IgG1-κ antibody binding to a protein L activated CIM™-disk. Conditions: CIM™-disks, 12 × 3 mm; UV detection at 280 nm; adsorbing buffer was PBS pH 7.4.

Table 4
Recovery of IgG (peak areas) after loading solutions of different concentrations and volumes

	IgG (μg)[a]	Peak area (a.u.)	Sample volume (μl)
Protein A HPMAC	12	177.9	25
	12	170.8	50
	24	331.8	50
	24	341.8	100
Protein G HPMAC	12	183.5	25
	12	167.7	50
	24	331.7	50
	24	342.7	100
Protein L HPMAC	12	170.1	25
	12	164.0	50
	24	324.8	50
	24	321.7	100

[a] Absolute amount of IgG found in the sample of a given volume.

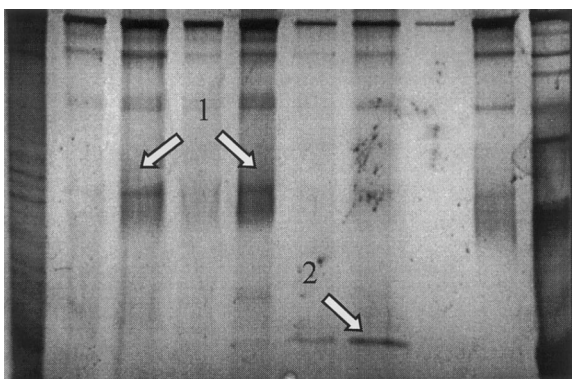

Fig. 5. SDS-PAGE analysis of fractions of the recombinant IgG1-κ antibody obtained after HPMAC purification. Lanes (from left to right): 1st, cell culture supernatant; 2nd and 3rd, IgG1-κ antibody obtained from protein A disk, normal and overloaded; 4th and 5th, IgG1-κ antibody from protein G Disk, normal and overloaded; 6th and 7th, IgG1-κ antibody from protein L disk, normal and overloaded; 8th and 9th, high purity recombinant IgG1-κ standard; 10th, molecular mass markers. Arrows indicate: 1, IgG heavy chain; and 2, IgG light chain.

rately purified antibody standard (lanes 8 and 9). In the case of the antibody purified using protein A (lanes 2 and 3) and protein G (lanes 3 and 4) disks, a band corresponding to the mass of the heavy chain (50 kD) is also present (arrow 1), while a band corresponding to the mass of the light chain (25 kD, arrow 2) is present in the antibody sample from the protein L disk (lanes 5 and 6).

4. Discussion

A large number of coupling chemistries have been proposed in affinity chromatography for linking proteinous affinity ligands to solid supports. The coupling reaction is known to affect the orientation of the polypeptide at the surface and hence can influence the interactions with the target molecule.

CIM™-disks allow very straightforward coupling of proteins to their surface. The polymer backbone of the macroporous disks contains highly active epoxy groups (surface concentration (3–5 mmol/g)) as a result of the chemistry chosen for synthesis of the polymer disks [16]. Together with the open channel-like morphology of the CIM™-disks, this allows carrying out the immobilization in a single step and under mild conditions. On the side of the affinity ligand some ε-amino groups are required to react with the disks' epoxygroups to form a covalent linkage.

The surface concentrations measured here for the final immobilized ligand, correspond to a situation, where the entire CIM™-surface is covered by active ligands. Moreover, contrary to the situation observed, for example, in some membrane chromatography applications [17], the simple and efficient epoxy-coupling technique apparently yields highly active disks even in the absence of spacer groups.

As already pointed out above, protein A, G, and L differ in their antibody binding mechanism. Protein A and G interact with the Fc-fragment of the antibody whereas protein L binds to antibody-κ light chains [5,6]. However, all three ligands show high affinities for the target molecules with the expected species and subtype peculiarities. The molar ratios calculated for the IgG/ligand pairs compiled in Table 3 demonstrate practically identical binding properties for all protein ligands investigated. While this is less

surprising in the case of protein A and G, which both bind to the Fc-part of IgG, a somewhat different adsorption capacity was expected in the case of the κ-light chain binding protein L. This is apparently not the case.

In our study we assumed that the Langmuir model could be fitted to the experimental isotherms. It should be noted, however, that the initial slope of the isotherms is steep and we already reach the maximum binding capacity for comparatively low concentrations, C, i.e. in most cases for 0.125 μmol/l (Fig. 3). From then on the adsorbed amount, q, becomes independent of C (plateau region of the isotherm).

The experiments showed that it was possible to extend the analytical range of the system by simply adjusting the sample volume. In this case, a 10-ml sample containing 0.1 mg/ml of antibody, a 1-ml sample containing 1 mg/ml of antibody, and a 100-μl sample containing 10 mg/ml of antibody gave exactly the same readout. HPMAC as introduced here will allow a reliable and reproducible quantification in all three cases, as confirmed by the data compiled in Table 4. Only if the sample concentration was very low, this assumption was no longer valid.

Speed is of essence in analytical biotechnology, be it for fast on line monitoring or high throughput screening. In this context, HPMC and more specifically HPMAC, are attractive options, since flow rates of more than one order of magnitude above those used in conventional column based chromatography can often be applied. The dissociation constants measured here, are orders of magnitude lower than those reported previously for conventional affinity chromatography [18], therefore binding should be much stronger to disks than to the conventional affinity gels. In fact the K_d-values measured by us are in the same order of magnitude as those expected for the affinity pair in free solution. Concomitantly, in the case of HPMAC these efficient binding conditions were compatible to extremely high flow rates and thus to fast isolation, respectively, analytical protocols.

The IgG-capacities in terms of mg/l of stationary phase are somewhat lower for the affinity disks than those reported for many of the conventional affinity stationary phases. However, since we already immobilized enough ligands to fully cover the entire disk surface, it was difficult to imagine how this could be improved. Clearly, the disk have advantages over conventional materials in terms of speed but will perhaps not be as suited for preparative and semipreparative applications, where capacity is of major importance.

Kinetic effects should be considered in their own right, especially when high throughput is the goal or when the reaction is used for the fast on-line production monitoring needed by modern biotechnology [19,20]. In this context it is important to note that in the investigated case, the higher flow rate, i.e. 6 ml/min instead of 4 ml/min, did not in any way hinder the affinity reaction in the case of HPMAC on CIM™-disks. The affinity constants stay approximately the same. The q_m did decrease a little at the higher flow rate, however, this is to be expected and well within the acceptable range. The use of flow rates below 4 ml/min does not result in any improvement of either parameter, but does increase the time per analysis. Flow rates above 6 ml/min were not included into the investigation, due to the limitations of the chromatographic system.

Since mass transfer in general is less limiting in HPMC than in conventional chromatography, the highest flow rate still compatible with the kinetics of the affinity interaction can often be used without adverse effect on the recovery. In our case, the HPMAC separation took less than one minute (Fig. 6) to complete and presumably could be accelerated even more by further increasing the flow rate. Once more, the chromatographic system rather than the binding kinetics posed the limits in this case.

5. Conclusions

High performance monolith affinity chromatography emerges from this paper as multipurpose tool for analytical biotechnology and biochemistry. Its main feature is its compatibility to high flow rates. Contrary to conventional column based affinity chromatography, high flow rates do not impede the separation by affinity interaction

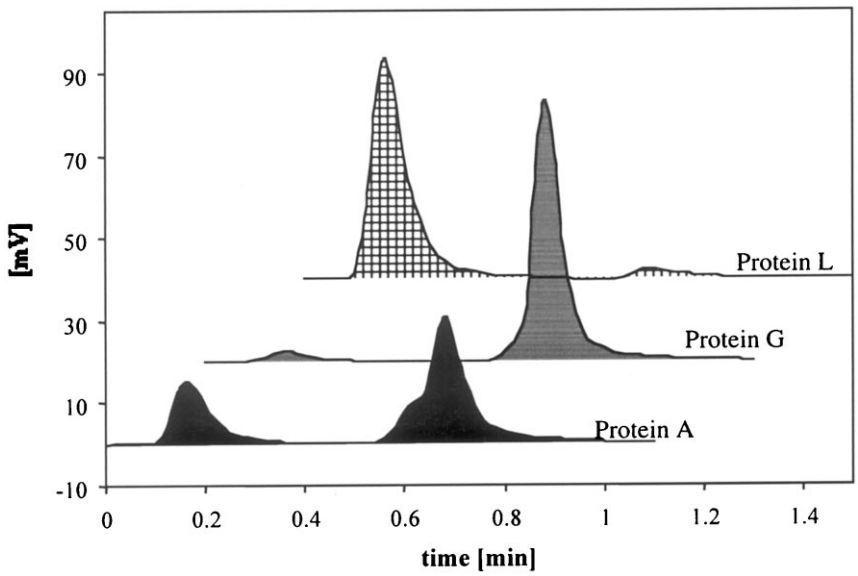

Fig. 6. Comparison of the HPMA chromatograms of bovine IgG using disks with different affinity ligands. Conditions: CIM™-disks, 12 × 3 mm; flow rate, 4 ml/min; UV detection at 280 nm; adsorbing buffer was PBS pH 7.4, eluting buffer was 10 mM HCl pH 2.0; amounts loaded were 25 μg of bovine IgG in 100 μl buffer A. Chromatography: step gradient; 0–0.5 min 100% A, 0.5–0.51 min from 100% A to 100% B, 0.51–1.1 min 100% B, 1.1–1.11 min from 100% B to 100% A and 1.11–1.3 min 100% A.

in the case of monolithic disk based chromatography. Possible areas of application of HPM(A)C include fast bioprocess monitoring (within seconds) or high throughput screening of binding partners and conditions. It also becomes possible to quickly study affinity characteristics in general, thus perhaps speeding up general method development in the various areas of application.

Acknowledgements

This work was supported by the Swiss National Foundation for Scientific Research within the Swiss Priority Project (SPP) Module 1. We wish to thank all members of the team involved in the project, and E. Kragten and M. De Jesus in particular for providing samples of purified material and cell culture supernatants. T. Tennikova is grateful for a personal visiting professors grant from the Swiss Federal Institute of Technology in Lausanne.

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