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Evaluation of factors which affect column performance with immobilized monoclonal antibodies

Model studies with a lysozyme–antilysozyme system

MILTON T. W. HEARN* and JEFFREY R. DAVIES[a]
Monash University, Department of Biochemistry and Centre for Bioprocess Technology, Clayton, Victoria 3168 (Australia)

ABSTRACT

Methods are described for the automated evaluation of affinity columns by frontal boundary analysis. These methods were used to evaluate the performance of immunoaffinity columns based on antilysozyme monoclonal antibody–lysozyme immunoaffinity system. This model system enabled the effects of (i) matrix activation and (ii) the density of immobilized antibody on the change in specific activity of immobilized antibody to be quantitatively assessed. Experimental data were accumulated with carbonyldiimidazole-activated Fractogel HW65F and Trisacryl GF2000 resins and cyanogen bromide-activated Sepharose 4B. An increase in the molar ratio between the concentration of the active groups on the activated matrix and the concentration of immobilized antibody ligands did not result in significant change in the specific activity of the immobilized antibody in the immunochromatographic system. However, increased antibody density with the Fractogel HW65F resin resulted in an increase in the apparent heterogeneity of antibody binding sites for lysozyme and a significant decrease in the specific activity of the immobilized antibody. Furthermore, data from size-exclusion studies with these immunoaffinity matrices demonstrated that at high antibody densities, the accessibility of the immobilized antibody was further decreased due to steric resistance as the antigen size increased.

INTRODUCTION

Over the past several years monoclonal antibodies (MAbs) have found application in the purification of a variety of biologically important proteins, including various lymphokines[1–3] and blood coagulation or plasma proteins such as factor VIII[4,5], protein C[6], factor IX[7] and α-fetoprotein[8]. However, the expense of producing

[a] Present address: Commonwealth Serum Laboratories, Poplar Road, Parkville, Victoria 3052, Australia.

bulk (*i.e.* 10–100 g) quantities of MAbs of the correct epitopic specificity and affinity has largely limited their application to laboratory-scale purification studies. From a process automation point of view, one advantage of MAb systems is their potential to allow the development of rational sequential strategies by which large purification factors (*e.g.* > 50-fold) can be achieved. Examples where immunoaffinity chromatographic systems have already been applied at the process scale within biotechnological industries include the biorecovery of interferons[1,2] and the blood coagulation protein factor VIII[5].

Practical experience has demonstrated with both dynamic and static (batch) adsorption conditions that a high percentage of the immobilized antibodies may not bind specific antigens[9–19]. A number of hypotheses can be advanced for this phenomenon. For example, during the synthesis of the immunoaffinity support, the paratope (antigen binding) structure of the MAbs may be destroyed or modified by multisite immobilization due to the presence of high levels of clustered active groups at different patches on the matrix surface[14,19]. Furthermore, lack of control over the orientation of the MAb during immobilization could give rise to functionally inaccessible MAb molecules, *i.e.* the Fab regions are orientated toward the matrix polymer backbone and thus effectively become buried. Alternatively, antigenic binding in a stoichiometric sense may be restricted due to a high density and proximity of immobilized antibody molecules (steric crowding due to antibody clustering). A similar effect can also be expected to arise on steric grounds due to the size and hydrodynamic properties of the antigen (the steric crowding effect due to antigen size). Finally, the issue of ligand leakage will give rise to decreasing capacity with usage. Ligand leakage often necessitates the implementation of further purification stages with high-value therapeutic proteins. Adequate remedy of these current limitations requires quantitative evaluation of the characteristics and performance of the immunoaffinity matrix.

Important concepts in the design of immunoaffinity systems and approaches to optimising and predicting their performance on scale up have previously been described in the scientific literature; for a compendium of recent reviews see refs. 9, 10 and 15–19. In process-scale separations, column chromatographic systems have been preferred because of the ease of automation of these systems compared to batch methods. The capture of antigen by an immobilized antibody in such systems is dependent on a number of factors including: (i) concentration of antigen, (ii) volume of feedstock loaded, (iii) concentration of accessible and active immobilized antibody, (iv) the degree of contaminant fouling and (v) efficiency of regeneration following product desorption and sorbent washing. In order to ensure the greatest capture of antigen, the view has been held by some investigators that the concentration of active immobilized antibody should be as high as possible. However,the studies of Eveleigh and Levy[9] have shown that under such conditions with CNBr-activated Sepharose 4B and porous glass supports a substantial proportion of immobilized antibody will not be functional in immunoaffinity systems. In particular, these investigators have provided data, obtained with supports of equivalent activation levels but different antibody densities, which illustrated that the antibody density effects, rather than the activation levels, played the major role in decreasing the immunoadsorbent capacity with CNBr-activated Sepharose 4B systems. The impact of activation levels on column performace in process biospecific affinity chromatography is nevertheless an im-

portant aspect to study because it is well known that the stability of an immobilized ligand will decrease as the number of immobilized groups per ligand decreases[9,18,20]. Hence for stability and regeneration purposes, high activation levels are desirable. Therefore it is important to establish if the immobilization chemistry *per se* is responsible for decreased specific activity of an immobilized antibody or if the activation chemistry has changed the mass transfer properties of the matrix which in turn results in decreases in the specific activity of the immobilized antibody.

In the present and associated studies we have examined alternative ways to quantitatively evaluate the characteristics of biospecific affinity matrices, including the influence of surface accessibility, ligand density and ligand stability. This manuscript, in particular, examines the influence of matrix activation levels and immobilized antibody density on the specific activity of the immobilized antibody and the binding site heterogeneity, in a model immunoaffinity chromatographic system. A monoclonal immunoaffinity system was used in this study to eliminate polydispersity effects in terms of affinity constants which are an intrinsic feature of polyclonal antibody immunoaffinity systems. Three activated supports were evaluated [carbonyldiimidazole (CDI)-activated Fractogel HW65F and Trisacryl GF2000 and CNBr-activated Sepharose 4B] using a lysozyme–antilysozyme MAb system. The use of Fractogel or Trisacryl in immunoaffinity systems has some practical advantages for large-scale fractionation systems over traditional Sepharose-based supports in that these resins exhibit higher mechanical stability at high flow-rate.

The experimental design was used in our studies based on frontal boundary analysis[21–23]. Experimental data were analyzed in terms of system capacities, binding constants and extent of ligand heterogeneity evaluated from Stewart–Petty (double reciprocal), Scatchard and Sips plots[24–27]. The equations describing these plots assume that near equilibrium conditions prevail for binding of the antigen to both high- and low-affinity binding sites. The Steward–Petty plot is evaluated from the dependency of the total bound antigen $[Ag]_b$ and the initial antigen concentration [Ag], the immobilized antibody concentration [Ab] and the dissociation constant, K_d, namely

$$\frac{1}{[Ag]_b} = \frac{K_d}{[Ag]2[Ab]} + \frac{1}{2[Ab]} \tag{1}$$

The Scatchard plot is given by

$$\frac{r}{[Ag]} = -rK + nK \tag{2}$$

where the ratio, r, corresponds to the number of moles of bound antigen to the total molar concentration of immobilized antibody and is a measure of the extent of saturation of the binding sites: K is the association constant and n is the apparent valency of the antibody. For ideal immunoglobulin G (IgG)–antigen interactions, $n = 2$.

Transformation of eqn. 2 yields

$$\frac{r}{n-r} = K\,[\mathrm{Ag}] \tag{3}$$

which on incorporation of the coefficient "a" (Sips heterogeneity index) yields the Sips plot given by

$$\frac{r}{2-r} = K\,[\mathrm{Ag}]^a \quad \text{or} \quad \log(r/2-r) = \log K + a\log[\mathrm{Ag}] \tag{4}$$

The Sips heterogeneity index is a measure of the non-ideality of the Ab–Ag interaction.

Previous work in this and other laboratories have shown that these equations can readily be adapted for analysis of immunoaffinity and other biospecific affinity systems using frontal boundary data[21–23]. Thus for frontal elution systems eqn. 1 takes the form

$$\frac{1}{[\mathrm{Ag}](V_e - V_{0,\mathrm{ni}})} = \frac{K_d}{[\mathrm{Ag}]2[\mathrm{Ab}]_a} + \frac{1}{2[\mathrm{Ab}]_a} \tag{5}$$

where V_e = elution volume of antigen; $V_{0,\mathrm{ni}}$ = elution volume of a non-interactive]species of similar hydrodynamic characteristics as the antigen and $[\mathrm{Ab}]_a$ = the concentration of accessible antibody assuming a valency of 2.

Similarly the frontal boundary data can also be analysed in terms of the Scatchard and Sips plots (eqns. 2 and 4). However, these numerical derivations require the determination of the variable r. Determination of the concentration of the total antibody immobilized, $[\mathrm{Ab}]_t$, can be achieved by direct measurement of antibody incorporation using ^{125}I labelled antibody, mass difference analysis or amino acid composition of the hydrolysed support. Determination of $[\mathrm{Ab}]_a$ can be achieved from the experimental data by plotting $1/[\mathrm{Ag}](V_e - V_{0,ni})$ *vs.* $1/[\mathrm{Ag}]$ using eqn. 5. Under ideal homogeneous binding conditions with both paratope binding sites of the MAb operating independently (*i.e.* when the association constants for antigen binding to each paratope are identical), the Steward–Petty plot will be linear with the value of the y axis intercept corresponding to $1/(2[\mathrm{Ab}]_a)$; the Scatchard plot will be linear with the numerical value of the x axis intercept being 2 by definition for binding interactions with an IgG class antibody, and the Sips plot will be linear with a slope of 1.

In immunoaffinity systems with MAbs of high ligand densities and close ligand proximity, changes in the binding affinity constants of antibodies may occur due to steric crowding of the immobilized ligand or chemical and physical inhomogeneities of the matrix surface. These effects will result in heterogeneous immunosorbents encompassing several classes of different binding sites with different affinities being generated. Non-ideal interactive behaviour will then be manifested as a boundary set of near equilibrium interactions between the paratope and the (monovalent) antigen such that each paratope, P_i, of the immobilized antibody in each different micro-environment (*e.g.* 1, 2, ...*i*...*n*) on the surface of the stationary phase will exhibit a different affinity constant for the antigen as described in the set of equations below.

$$P_1 + Ag \overset{K_1}{\rightleftharpoons} P_1Ag$$

$$P_2 + Ag \overset{K_2}{\rightleftharpoons} P_2Ag$$

$$\vdots$$

$$P_i + Ag \overset{K_i}{\rightleftharpoons} P_iAg$$

$$\vdots$$

$$P_n + Ag \overset{K_n}{\rightleftharpoons} P_nAg$$

The presence of multiple affinity sites will result in complex dependencies of K_i and the binding kinetics on the ligand density, $[Ab]_t$. In addition as the $[Ab]_t$ increases a proportional increase in the number of binding sites may not result. Such heterogeneity can also theoretically be anticipated to lead to apparent negative cooperativity in the MAb–Ag interaction and be reflected in non-linear Scatchard plots and Sips plots with gradients less than 1. Such interpretation of heterogeneity introduced by the matrix activation chemistry and ligand density effects has previously been complicated by the use of polyclonal antibodies. In order to examine these effects, immunoaffinity sorbents of different MAb ligand densities were prepared using antilysozyme MAbs and the experimental data evaluated in terms of a two component model of binding sites. We have not attempted in the present manuscript to quantify the contribution of third, fourth or higher classes of binding sites in these numerical analyses.

EXPERIMENTAL

Materials

Carbonyldiimidazole, ethanolamine, bovine serum albumin, horse heart cytochrome *c*, hen egg white lysozyme, ovalbumin and bovine γ-globulin, were purchased from Sigma, St. Louis, MO, U.S.A. Fractogel HW65F was purchased from Merck, Darmstadt, F.R.G., Trisacryl GF2000 was from Reactifs IBF, Pointet Girade, France. CNBr-activated Sepharose 4B and Blue Dextran 2000 were obtained from Pharmacia, Uppsala, Sweden. [^{14}C]Lysine and ^{125}Iodine were obtained from Amersham, U.K.

Methods

Purification of monoclonal antibody. Monoclonal antibody against lysozyme was purified from Balb/C mouse ascites fluid using an fast protein liquid chromatographic (FPLC) system with an HR16/10 Mono Q anion-exchange column (Pharmacia). Ascites fluid was diluted two-fold with 20 m*M* Tris · HCl pH 8.0 (buffer A) and loaded onto the column through pump A. The partially fractionated MAb was eluted with a 0–0.5 *M* sodium chloride gradient in buffer A. The flow-rate was 8 ml/min and the gradient time was 60 min. The IgG peak was diluted twofold with buffer A and rechromatographed as described above to ensure resolution of the specific MAb from contaminating proteins. The purified MAb was estimated to be >95% pure from

sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis. Purified IgG was labelled with ^{125}Iodine using a modified chloramine T method[28].

Matrix activation. Acetone was dried by distillation over potassium carbonate and stored under anhydrous conditions. Chromatographic supports were thoroughly washed with dried acetone and then activated with CDI according to the method of Hearn[21]. The concentration of CDI ranged from 0.6 to 4 mmol/g of acetone moist cake of chromatographic gel. Activation levels were assessed by previously described methods[21] and by the quantitative immobilization of [^{14}C]lysine coupled at pH 8.9 in 0.1 *M* sodium borate, 0.5 *M* sodium chloride. Following ligand coupling, the affinity resins were thoroughly washed with 0.1 *M* sodium borate, 0.5 *M* sodium chloride, pH 8.9 and 0.1 *M* sodium acetate, 0.5 *M* sodium chloride, pH 4.5. The amount of [^{14}C]lysine immobilized per ml of resin was determined by hydrolysis of a measured amount of [^{14}C]lysine resin with 6 *M* hydrochloric acid at 60°C for 90 min. The ^{14}C content of the hydrolysate was then determined directly by β-scintillation counting.

Using the above protocols lower levels of activation were achieved with Trisacryl GF2000 resin than found with Fractogel HW65F or agarose-based materials. In order to increase the activation levels, Trisacryl was washed in 100% acetic acid prior to the acetone wash. This treatment resulted in a doubling of the level of CDI-activation when using high ratios of CDI per g moist cake of resin. These results suggest that hydrogen bonding, occurring between hydroxyl groups in this support, plays a significant part in controlling the access of the CDI to the internal surfaces of the porous network and subsequent chemical activation of Trisacryls.

Antibody immobilization. Purified MAb was coupled to activated supports in 0.1 *M* sodium phosphate, 0.1*M* sodium chloride, pH 7.0 in amounts ranging from 1 to 20 mg of antibody per ml of activated resin. Samples were spiked with ^{125}I-labelled antibody with coupling time typically 48 h at 4°C. Following coupling the resins were washed with 0.1*M* sodium borate, 0.5 *M* sodium chloride, pH 8.9 and 0.1 *M* sodium acetate, 0.5 *M* sodium chloride, pH 4.5 then 0.1 *M* phosphate–ethanolamine pH 8.0. The gel was incubated in the phosphate–ethanolamine buffer for 16 h in order to block any unreacted sites. The amount of IgG immobilized was determined by direct measurement of [^{125}I]IgG incorporation.

Calculation of the specific activity of immobilized antibodies. Purified anti-lysozyme IgG was assumed to be 100% pure and specific activities for immobilized IgG systems were expressed as a percentage of the theoretical capacity determined from the amount of IgG immobilized according to the following formula:

$$\text{Specific activity} = 100 \cdot [\text{lysozyme}]_{\text{bound}}\ (M)/2 \cdot [\text{IgG}]_{\text{immobilized}}$$

where the molecular weight of lysozyme was assumed to be 14 300 and IgG 150 000.

Size-exclusion analysis of fractogel matrices. Size-exclusion analysis of Fractogel HW65F resins (native, CDI-activated and bovine IgG Fractogels) was carried out using 0.5-ml bed volume in glass columns (5 mm I.D.). The retention times of hen egg white lysozyme, ovalbumin, bovine serum albumin and bovine γ-globulin were determined from quadruplicate measurements in 8 *M* urea, 0.5 *M* sodium chloride at a flow-rate of 0.2 ml/min. Solute accessibility was determined using the following equation:

$$\text{Solute accessibility} = \frac{V_e - V_0}{V_t - V_0}$$

where V_e = elution volume of solute, V_0 = column void volume (measured using Blue Dextran 2000) and V_t = total column volume (measured using acetone).

Evaluation of regeneration protocols. Desorption and regeneration conditions for the lysozyme–antilysozyme system were determined using several different zonal elution protocols. Protein samples were sequentially injected onto the immunoaffinity supports until a constant peak height was observed for the breakthrough zone, (*i.e.* until the column was saturated with lysozyme). Different reagents were then tested for their ability to restore lysozyme binding capacity of the immunoaffinity support after injection of one column volume pulses of the reagent through the column.

Chromatographic evaluation of MAb-based immunoaffinity gels. The various immunoaffinity chromatographic gels were evaluated using frontal boundary analysis as previously described[21–23], using column bed volumes between 0.2 and 0.5 ml (column internal diameter, 5 mm). The analysis was automated by use of Pharmacia FPLC system equipped with MV7 and MV8 motorized valves. The configuration of the FPLC system used for the frontal analysis is shown in Fig. 1. The system was

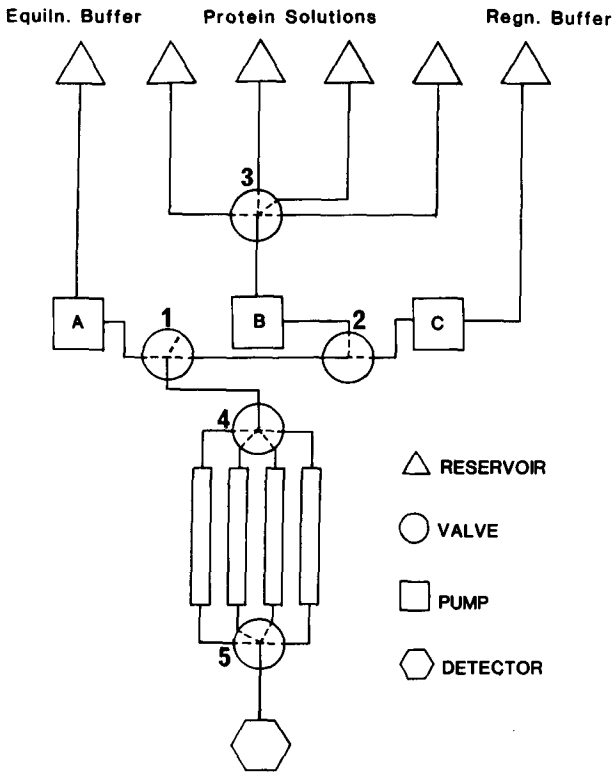

Fig. 1. Automated FPLC system for Frontal Analysis of Immunoaffinity supports. All pump valve and recorder switching was achieved using an LCC500 microprocessor. Sample was applied through pump B (P500) and the recorder (Shimadzu C-R3A Chromatopac) automatically activated when pump B started. Regeneration buffer was applied using pump C (P1 peristaltic) and re-equilibration buffer (20 m*M* sodium phosphate, 0.15 *M* sodium chloride) applied using pump A. Valve 1 was an MV7 valve with the outlet from pump A connected to port 1 and the tubing from valve 2 connected to port 2. All other valves were MV8. (The controlling program can be obtained if required by writing to the authors.)

controlled by a GP500 controller which allowed replicate analyses to be conducted for each measurement. Data were collected and reprocessed using a Shimadzu C-R3A recorder. This data handling feature was necessary for the automated system in order to generate normalised breakthrough curves when samples of different antigen concentrations were being analysed.

Frontal analysis was conducted at a flow-rate of 0.2 ml/min using a lysozyme concentration of 2–100 μg/ml. The $V_{0,ni}$ value used in eqn. 5 was determined using cytochrome *c* at concentrations from 2.5 to 100 μg/ml in 0.1 *M* sodium phosphate, 0.15 *M* sodium chloride, pH 7.0. Cytochrome *c* was chosen beause it has a similar hydrodynamic volume to lysozyme but did not interact with the immunoaffinity support. Measurement of V_e for lysozyme under the different experimental conditions was determined by measuring the volume of eluent corresponding to the $V_{0.5}$ of the breakthrough curve.

Six immunoaffinity chromatographic gels were evaluated. Two Fractogel resins with similar CDI activation but different ligand densities; two Trisacryl resins with different CDI activation levels and similar ligand densities and two CNBr-activated Sepharose derived supports with different ligand densities. With the exception of CNBr-activated resins capacities were determined by evaluation of frontal analysis data. For CNBr systems, biphasic breakthrough curves were obtained complicating interpretation of frontal analysis data. Experimental values of capacities derived from the analysis of the second component of the breakthrough curves exceed the maximum theoretical value. Hence, capacities determined for these experimental systems were obtained from frontal analysis data using 100 μg/ml solutions of lysozyme only and assuming the first front of the breakthrough curve represented saturation of immobilized antibody due to the biospecific interaction and not electrostatic interactions known for systems derived from CNBr activation[29–31].

RESULTS AND DISCUSSION

Influence of buffer conditions on immobilization efficiency

The influence of buffer effects on the efficiency of immobilization of bovine

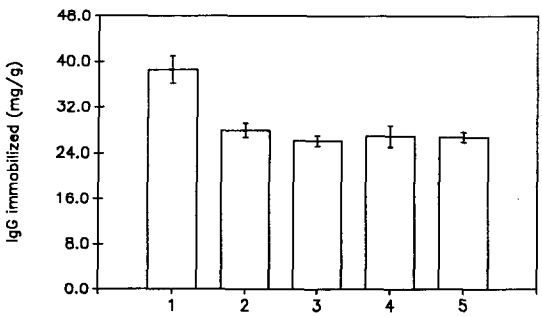

Fig. 2. pH dependence of γ-globulin coupling to CDI-activated Fractogel. The concentration IgG was 40 mg/g of activated Fractogel. All coupling were conducted in 0.5 *M* sodium chloride. IgG immobilizations were determined in triplicate. 1 = 0.1 *M* sodium phosphate, pH 7.0; 2 = 0.1 *M* Tris · HCl, pH 8.0; 3 = 0.1 *M* sodium borate, pH 8.9; 4 = 0.1 *M* Tris · HCl, pH 8.0, 0.2% NaN_3; 5 = 0.1 *M* Tris · HCl, pH 8.0, Brij-35.

polyclonal antibodies to preactivated supports are summarized in Fig. 2. The data show that maximum immobilization efficiency for bovine γ-globulin was achieved at the lowest pH tested, *i.e.* at pH 7.0 (0.1 *M* sodium phosphate) and not at pH 8.0 (0.1 *M* Tris · HCl) or pH 8.9 (0.1 *M* sodium borate). A similar pH effect has also been noted in our earlier investigations on the coupling of various ligands using CDI- and CNBr-activated supports[21,30]. Efficient immobilization of proteins to preactivated matrices could be achieved in the presence of Tris buffer. This result is consistent with the observation that the amino group in Tris is sterically hindered[32] and does not significantly compete with free side chain primary amino groups of proteins at the coupling stage. The presence of the additives NaN_3 (0.2%) and Brij-35 (0.01%), did not interfere with the antibody immobilization. Conditions for the MAb immobilization to preactivated gels were selected on the basis of these observations.

Elution of bound components and column regeneration

For regeneration of the immunochromatographic systems the most effective reagent tested was 2.5 *M* KSCN with complete elution being affected by a single column volume. Glycine 0.1 *M* (pH 2.8)–0.5 *M* sodium chloride was also effective but required 3–4 column volumes to regenerate the immunoaffinity support to equivalent capacity. Column capacity, irrespective of the support matrix, was invariably reduced after the first chromatographic run and thereafter the capacity stabilized. This finding is consistent with previous observations with polyclonal systems[9]. Other reagents tested were 2 *M* solutions of NaCl, $MgCl_2$ and LiCl but these conditions were found to be ineffective in desorbing bound antigen and/or regenerating the column to high capacity. With the KSCN elution system, although the lysozyme zone eluted in a sharp peak, extensive washing was required to remove all KSCN from the column on reequilibration. If this was not affected, earlier breakthrough and decreased capacity was noted with some KSCN-conditioned columns.

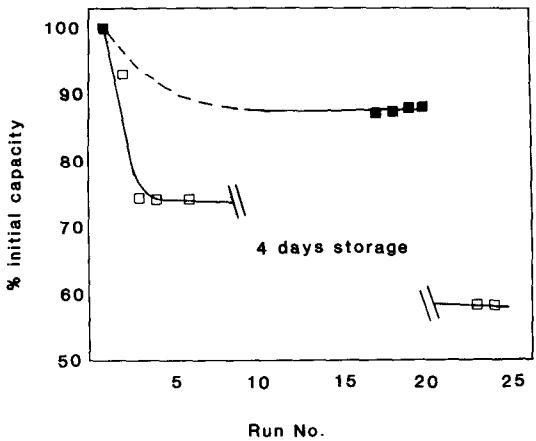

Fig. 3. Comparison of the effect of KSCN and low pH glycine–sodium chloride regeneration systems on antilysozyme–Fractogel columns. Capacities were determined by frontal analysis using a 10-μg/ml solution of lysozyme. The activation level and immobilized antibody concentration was: CDI level = 25 μmol/ml Fractogel; antibody density = 1 mg/ml Fractogel. Regeration with: ■ = 2.5 *M* KSCN, □ = 0.1 *M* glycine–0.5 *M* sodium chloride, pH 2.8.

A comparison of binding capacities (determined by frontal analysis) and the dependence of capacity on the number of runs of two antilysozyme Fractogel HW65F columns regenerated with the glycine and KSCN desorption systems, respectively, is shown in Fig. 3. In both cases a rapid drop in capacity was observed between the first and second runs but in subsequent runs the capacity remained constant with the KSCN elution system providing higher capacities (88% of the initial value) than the glycine system (74%). After 4 days storage at 4°C in 100 m*M* sodium phosphate, 0.1% NaN_3 and 25 adsorption/desorption cycles, the immunoaffinity system regenerated using the glycine system showed a further 20% loss in capacity (Fig. 3). In comparison, analysis of an antilysozyme–Trisacryl GF2000 column (MAb density 1.0 mg of antibody immobilized per ml of resin) regenerated using the KSCN system showed a 45% loss in capacity after 3 months storage at 4°C in 100 m*M* sodium phosphate, 0.1% NaN_3 and 56 cycles of usage.

The influence of immobilized MAb density on lysozyme binding

The influence of the density of immobilized MAb on antigen binding is shown in Table I. A 13-fold increase in MAb density on Fractogel HW65F resulted in only an 8.5-fold increase in the binding capacity. This represents, a decrease of 33% in the specific activity of the immobilized antibody with the high antibody density support. Although frontal breakthrough profiles of immunoaffinity columns with MAb immobilized to CDI-activated gels showed regular asymptotic Langmurian-like isotherm behaviour characteristic of a common retention mechanism, for immunoaffinity gels derived using CNBr-activated Sepharose 4B multimodal adsorption behaviour was evident. This observation is consistent with the well known non-biospecific, coulombic adsorptive properties of the charged isourea linkage generated from the immobilization of ligands onto CNBr-activated matrices[29–31].

Nonetheless with a 10-fold increase in ligand density CNBr-activated Sepharose 4B resulted in an approximately 11-fold increase in antigen binding capacity. Thus

TABLE I

THE EFFECT OF IMMOBILIZED MAb DENSITY ON COLUMN CAPACITIES

The theoretical capacity was determined as 2 · [antibody] (M) immobilized. The molecular weight assummed for antibody was 150 000 and for lysozyme 14 300. The specific activity of immobilized antibody was

$$\frac{100 \cdot \text{experimentally determined capacity}}{\text{theoretical capacity}}$$

Matrix	*Antibody density (mg/ml)*	*Theoretical capacity (μg/ml)*	*Observed capacity (μg/ml)*	*Specific activity of immobilized antibody (%)*
Fractogel	1.0	191	41	21
Fractogel	13.4	2554	350	14
Trisacryl	0.9	172	66	38
Trisacryl	1.2	228	71	31
CNBr-Sepharose	0.73	139	40	29
CNBr-Sepharose	7.3	1391	459	33

TABLE II

ANTIBODY DENSITY EFFECTS ON BINDING SITE HETEROGENEITY

Matrix	*Antibody density (mg/ml)*	*Heterogeneity*				
		Scatchard plot				*Sips plot a*
		n (%)	*K (M^{-1}) (10^6)*	*n (%)*	*K (M^{-1}) (10^6)*	
Fractogel	1.0	22	4.2	78	0.36	0.81
Fractogel	13.4	9	11.8	91	0.07	–
Trisacryl	0.9	100	8.4	–	–	0.92
Trisacryl	1.2	100	14.2	–	–	1.04

steric hinderance effects were not apparent for the Sepharose-based matrix consistent with a more open pore structure network for Sepharose compared to the Fractogel matrix. The result with CNBr-activated Sepharose 4B can be compared with the previous study of Eveleigh and Levy[9]. In their study a decrease in the specific activity from 21% to 18% was observed when the polyclonal anti HSA antibody density increased from 0.95 to 8.71 mg immobilized antibody per ml of resin. HSA is significantly larger than lysozyme (molecular weights 67 000 *vs.* 14 300) and hence more susceptible to steric hinderance effects.

The influence of ligand density on binding site heterogeneity

The influence of ligand density on binding site heterogeneity is summarized in Table II. The multi-compartmental influence of ligand density on the performance of antilysozyme–Fractogel HW65F support, was apparent when the extent of heterogeneity of the binding sites was quantitatively examined for the MAb-antigen interaction. In particular, a decrease in the proportion of high-affinity binding sites involved in the interaction was observed with immunoaffinity gels of higher antibody densities. Extrapolation of Steward–Petty and Scatchard plots were used to estimate the magnitude and proportion of high- and low-affinity sites over the investigated regions of the isotherms. These data indicated with an antibody density of 1 mg of immobilized antibody/ml of gel, 22% of binding sites could be classified as "high affinity". However, with an antibody density of 13.4 mg of immobilized antibody/ml of gel, only 9% of the total number of binding sites were of high affinity. This pattern can be clearly seen in Fig. 4A when the Scatchard plots for the Fractogel HW65F matrices are compared. In absolute terms, the total number of high-affinity binding sites of larger association constant(s) is greater with immunoaffinity matrices of higher antibody densities. In relative terms the proportion of high-affinity sites compared to low-affinity sites however become less abundant. Despite the relative change in the ratio of "high"- and "low"-affinity sites, the trend was evident that as the ligand density increased, the respective affinity constants of the high-affinity sites also increased. For example, almost a three-fold increase in the apparent affinity constant was observed for the MAb-based immunoaffinity system with the higher ligand density conditions on Fractogel HW65F. Furthermore, the increase in negative

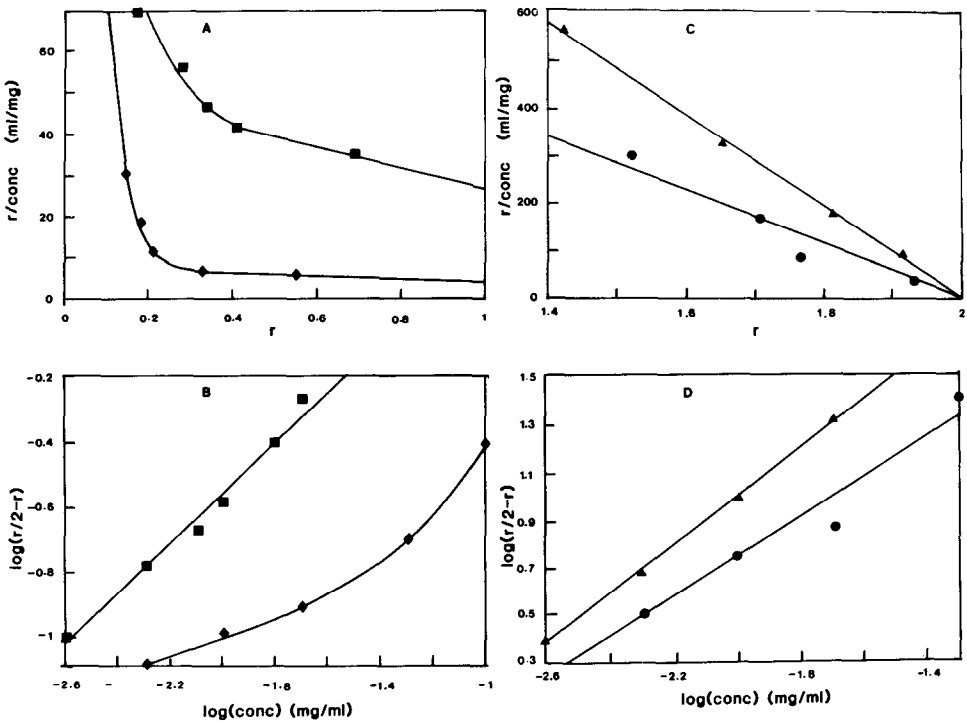

Fig. 4. Scatchard and Sips plots derived from the frontal analysis of antilysozyme immunoaffinity columns. (a) Antilysozyme–Fractogel columns. Scatchard and Sips plots are shown in Panels A and B, respectively. The activation level and immobilized monoclonal antibody concentrations were: ■: CDI per ml of Fractogel = 26 μmol; IgG per ml of Fractogel = 1.0 mg; ◆ = CDI per ml of Fractogel = 22 μmol; IgG per ml of Fractogel = 13.4 mg. (b) Antilysozyme–Trisacryl columns. Scatchard and Sips plots are shown in Panels C and D, respectively. ●: CDI per ml of Trisacryl = 2.0 μmol; IgG per ml of Trisacryl = 0.9 mg; ▲: CDI per ml of Trisacryl = 5.3 μmol; IgG per ml of Trisacryl = 1.2 mg.

cooperativity with the immunoaffinity gel of higher ligand density is clearly demonstrated by the Sips plot (Fig. 4B). With the immunoaffinity gel of low ligand densities (*e.g.* 1.0 mg of immobilized antibody/ml gel), the Sips plot showed slight curvature with the line of best fit having a gradient of *ca.* 0.8. At high ligand density (*e.g.* 13.4 mg immobilized MAb/ml gel) the Sips plot appeared to be curved with gradients ranging from 0.3 to 0.5 over the lysozyme concentration range of 10–50 μg/ml used for the adsorption studies.

Experimental data indicative of homogeneous binding of lysozyme was achieved using the Trisacryl matrix with low ligand density (*ca.* 1.2 and 0.9 mg of immobilized antibody/ml of gel). Scatchard and Sips plots for these low density immunoaffinity matrices are show in Fig. 4C and D. The results achieved using this Trisacryl immunoaffinity gel demonstrate that the negative cooperativity observed for the Fractogel system is not inherent to the interaction of lysozyme with the monoclonal antibody, *i.e.* steric hindrance and inhibition of binding of the lysozyme antigen to one paratope, caused by the prior binding of lysozyme to the other paratope, does not normally occur when the surface is designed to allow adequate spatial distribution of

TABLE III

THE INFLUENCE OF ACTIVATION LEVELS ON ANTIBODY ACCESSIBILITY

Matrix	*Molar excess of CDI to immobilized antibody*[a]	*Ligand density (mg/ml)*	*Specific activity (%)*
Fractogel	3900	1.0	21
Fractogel	250	13.4	14
Trisacryl	330	0.9	38
Trisacryl	660	1.2	31
	Relative excess[b] *of CNBr to immobilized antibody*		
CNBr-Sepharose	10	0.73	29
CNBr-Sepharose	1	7.3	33

[a] Activation levels were measured using ^{14}C-lysine and MAb immobilization was measured by determining incorporation of ^{125}I-MAb as described in the methods section.

[b] CNBr-activated Sepharose 4B was a commercial preparation (Pharmacia). The activation level of this resin was not determined. The difference in the ratio of CNBr groups to immobilized antibody was due to the difference in ligand density.

the immobilized MAb ligands and appropriate volume occupancy of the bound antigen.

The influence of activation levels on the specific activity of immobilized MAbs

The determination of activation levels and immobilization densities allows the calculation of the molar ratio (R) of CDI groups–immobilized antibody where:

$$R = \frac{\text{molar concentration of active CDI groups per ml of gel}}{\text{molar concentration of MAb immobilized per ml of gel}}$$

The influence of matrix activation levels on the subsequent specific activity of the immobilized MAbs is shown in Table III. The results show that for the Fractogel HW65F systems activated with very high levels of CDI prior to immobilization of the antibody (*i.e.* molar excess of CDI groups:immobilized MAb of 3900:1), the amount of antibody subsequently available to bind lysozyme was 21% of the total immobilized antibody concentration compared to 14% when the molar excess of CDI groups to immobilized MAb was 250:1. Data derived from the analysis of Trisacryl GF2000 systems showed that a doubling of the ratio of the concentration of CDI groups available for coupling to the concentration of immobilized antibody resulted in a change in the specific activity of immobilized antibody from 38% to 31%. With the CNBr-activated supports a 10-fold increase in the ratio of the concentration of CNBr activation groups to the concentration of immobilized MAb resulted in a decrease in antibody accessibility from 33% to 29%. Data from the analysis of CNBr-activated Sepharose and CDI-activated Fractogel HW65F resins shows that multisite attachment *per se* plays an insignificant role in the relative decline of the antigen binding capacity as the activation and immobilized antibody concentrations were increased.

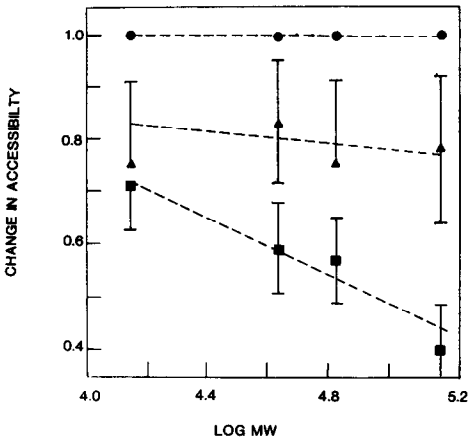

Fig. 5. The influence of CDI activation and IgG immobilization on the size-exclusion properties of Fractogel HW65F. Accessibility (A) was determined using the following formula:

$$A = \frac{V_e - V_0}{V_t - V_0}$$

Change in accessibility (A) was determined by dividing A values by the A value determined for native Fractogel. Analysis was carried out in 0.5 M sodium chloride, 8 M urea. Measurements were done using lysozyme (molecular weight, MW = 14 300), ovalbumin (43 000), bovine serum albumin (67 500) and bovine γ-globulin (150 000). ● = native Fractogel; ▲ = CDI-activated Fractogel (25 μmol/ml resin); ■ = IgG–Fractogel (13 mg/ml resin).

Consequently, other explanations involving MAb orientation at the surface, restricted access of the antigen to the immobilized MAbs due to steric compression or as a result of a decrease in pore volume following the activation or immobilization procedures, must also be considered.

The influence of CDI activation and antibody immobilization on size-exclusion properties of Fractogel HW65F

The above data suggest that significant steric hindrance between the antibody and the antigen occurs with immunoaffinity gels based on Fractogel HW65F and the other gels when antibodies are immobilized to high ligand densities. In order to determine the influence of CDI activation and antibody immobilization on solute accessibility, the size-exclusion properties of native Fractogel HW65F, CDI-activated Fractogel HW65F (25 μmol/ml) and antibody (bovine, 13 mg/ml of gel)-immobilized Fractogel HW65F were compared. Fig. 5 shows that solute accessibility is decreased by CDI activation. This result is in accord with other observations since CDI, and other chemical activation methods, are known to cause gel shrinkage associated with the cross linkage of the polymer chains of the matrix and reduction in pore volume[21]. Solute accessibility to the pores of CDI-activated Fractogel HW65F decreased by about 20% and showed little difference for solute molecules ranging in molecular mass from 14 000–150 000 dalton. A much greater decrease in solute accessibility within the pores was caused following antibody immobilization (at the level of 13 mg MAb/ml

gel) to the hydrophilic porous gels. Furthermore, solute accessibility decreased significantly as solute size increased with the antibody-Fractogel HW65F supports. The decrease in pore accessibility ranged from 30% for solute molecules of 14 000 dalton to between 50 and 60% with 150 000 dalton macromolecules.

SUMMARY AND CONCLUSIONS

The systems and protocols described in this manuscript permit rapid evaluation of the suitability of monoclonal antibodies in immunoaffinity chromatographic systems. Using the approaches which we have outlined, a large amount of experimental information can be derived about a particular immunoaffinity system under evaluation, and criteria for an optimal system determined. The use of mini columns permits pilot evaluations to be carried out with small amounts of valuable antibody and antigen, prior to the final decision being made on the preferred scale-up route. In practice the utility of this approach will depend on the ability to evaluate the concentration of the desired component in the column eluent. This can be achieved using radioactively labelled tracers, on-line assay systems or collection and assay of fractions over the breakthrough regions[10].

The efficiency with CDI-activated Fractogel HW65F of the monoclonal immunoaffinity systems described above, was significantly reduced by high ligand densities. The data indicate that increased ligand density resulted in an increase in the heterogeneity of the immunoaffinity system, and a decrease in the relative proportion of the immobilized antibody available to bind lysozyme. Size-exclusion data, demonstrated that high levels of CDI activation of Fractogel HW65F reduced solute accessibilities and therefore contributed to steric hinderance effects observed at high ligand densities. These data also illustrated that these effects are significantly magnified as the antigen size increases when using this matrix.

A further observation can be made relevant to the high-affinity sites of the two Fractogel supports, namely these sites were present in a lower proportion but exhibited higher affinities in the high-density gel than the low-density support over the same antigen concentration range. The opposite trend was evident observed for the low-affinity sites.

There are at least two explanations for these results both of which may contribute to the observed phenomena.

(i) The Fractogel matrix itself or multiple-site immobilization adversely influences lysozyme binding to the antibody. At higher ligand density externally immobilized antibodies are shielded from these effects by neighboring antibodies. However, within the matrix, crowding and matrix effects serve to lower antibody affinity constants.

(ii) At low ligand density the antibodies will be more evenly distributed throughout the outer region(s) of the matrix and will tend to be immobilized at or near the external surface of the gel. At high ligand densities antibodies are probably immobilized as a decreasing concentration gradient from the outer surface to the inner core of each particle. The theoretical treatments used above, were based on determination of the maximum capacities of the immunoaffinity gels and assumed even distribution of antibodies. If the antibody concentrations were locally higher (or lower) than determined then this would have contributed in the system as apparent heterogeneity of the affinity sites.

Differences in affinity constants for the Trisacryl resins could be measured despite the fact that these resins exhibited no or little apparent heterogeneity as assessed from the linearity of Sips and Scatchard plots. The immunoaffinity gel derived from the procedures using a higher ratio (R) of CDI to antibody immobilized exhibited the higher affinity constant. A possible explanation for this result is that immobilization of the MAb on the more highly activated support resulted in a greater proportion of antibody being externally immobilized. The data also demonstrate that immobilization involving the use of preactivated gels with high proportions of active groups to immobilized antibody will not necessarily lead to a decrease in the proportion of the immobilized antibody available to bind antigen. This finding is consistent with the observations of Eveleigh and Levy[9] for CNBr-activated Sepharose 4B–HSA polyclonal immunoaffinity system. The significance of these results is that highly activated resins (provided the activation has not severely compromised mass transfer properties of the gel as illustrated for Fractogel HW65F) may be suitable for the immobilization of antibodies and indeed may be preferred to low activation levels as increased stability of immobilized antibody can be anticipated using such supports. Under such conditions, a statistically high percentage of immobilized antibody molecules will be immobilized on the exterior surfaces of the support thus generating improved kinetics for antigen adsorption/desorption. The use of highly activated supports should also ensure a more complete reaction of antibody at during the immobilization step.

The experimental results also demonstrate there is little to be gained by attempting to immobilize saturation levels of antibody onto a porous matrix such as Fractogel (*i.e.* to make very high-density immunoaffinity supports), to maximise the use of the active groups and thus reduce the frequency of immobilization sites per MAb molecule, during the coupling step. When antigens with medium to high molecular weights are to be isolated (*e.g.* proteins with molecular mass between 20 000 and 250 000 dalton) such practices are likely to significantly decrease the accessibility of immobilized MAb in the immunoaffinity system. Reduced accessibility to ligands will result in wastage of valuable antibody, and decreased separation performance in terms of resolution and system productivity. The above conclusion is in concordance with previous studies using polyclonal immunoaffinity systems with Sepharose 4B and porous glass supports[9,21] and suggest the need for immunoaffinity systems based on mechanically stable non-porous or pellicular chromatographic medium. Such systems are likely to be of great benefit in the preparation of immunoaffinity supports for large proteins (*e.g.* factor VIII). They should also permit the synthesis of relatively homogeneous systems. This in turn should greatly enhance the predictability of these systems on scale up.

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