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Effect of feed flow-rate, antigen concentration and antibody density on immunoaffinity purification of coagulation factor IX

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ABSTRACT

A simple physical model of immunoaffinity chromatography (IAC) demonstrates that immobilized monoclonal antibody (MAb) capacity in IAC purification will be a function of many parameters, including feed flow-rate and antigen concentration, and MAb density (mg MAb immobilized/ml resin). We studied IAC of factor IX, and examined the effect of parameter variation on MAb capacity. MAb capacity (1) was not affected by feed flow-rate or antigen concentration, and (2) decreased as MAb density increased. (1) Suggested that diffusion of factor IX into the resin bead was not limiting. Characteristic diffusion, convection and reaction times were calculated and used in dimensional analysis to compare their relative magnitudes. If MAb was assumed to be localized to the outer 10% of the bead volume, this analysis concluded that diffusion was not limiting, consistent with the suggestions of our experimental data. (2) Suggests that high MAb densities make MAb less accessible.

INTRODUCTION

The isolation and purification of proteins from complex mixtures is a current focus in bioprocessing research. Proteins may be purified by several methods such as liquid chromatography [1,2], gel electrophoresis [3], fractional precipitation and liquid–liquid extraction [4]. One method for isolating a protein to near 100% homogeneity is immunoaffinity chromatography (IAC) using monoclonal antibodies (MAbs) [5] as the affinity ligand. IAC takes advantage of the specific and reversible interaction between the MAb and the desired protein. The MAb is generated following Kohler and Milstein [5], may be produced in large quantities [6], purified [7], and immobilized on a solid support [8] which is then usually packed into a chromatography column [9] for purification of the desired protein.

Factor IX (FIX) deficiency leads to Hemophilia B, a bleeding disorder which is treated by replacement therapy [10]. The American Red Cross has developed an IAC process for purifying FIX [11]. The FIX produced has been shown to be homogeneous [12] and the process has been scaled up to 10-l columns [13].

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In this paper, first, a simple physical model of a resin bead in IAC is developed to show the dependence of MAb capacity (mg antigen/mg immobilized MAb, or, more specifically, units FIX/mg MAb) for FIX on feed flow-rate and FIX concentration, and on MAb density. Second, we use simple dimensionless analysis [14] to develop model equations to compare the relative importance of diffusion, convection and reaction in a packed IAC column. Third, we report results of experiments where the effect of varying feed flow-rate, feed FIX concentration and MAb density on MAb capacity was studied.

We demonstrate that the MAb capacity for FIX is not affected by feed flow-rate or by feed FIX concentration, and show that MAb capacity decreases with increasing MAb density. Possible reasons for these observations are suggested.

MODEL DEVELOPMENT

In IAC, feed containing the protein to be purified is pumped into a packed column containing resin beads on which MAb against the desired protein (or antigen) has been immobilized. The antigen binds to the MAb while contaminating proteins are washed through. Column conditions are subsequently altered permitting the MAb to release the antigen, yielding a homogeneous protein product.

Studying an individual resin bead, as shown in Fig. 1, several variable groups that mediate MAb capacity for antigen, are evident. These are: (1) the bead, (2) the MAb, (3) the antigen (FIX, in this instance), and (4) kinetics, and are described in detail below.

(1) Bead: the radius of the particle has been used as a measure of a characteristic diffusion distance [14]. Bead porosity affects the movement of protein in the bead, but is not considered explicitly because it is fixed upon choice of a particular commercial resin.

(2) MAb (or other ligand): Eveleigh and Levy [15] have shown that decreasing ligand density increases the efficiency of antigen capture. With immobilized MAb (IMAb), orientation also affects the efficiency of antigen capture. Typically, a MAb is represented as a Y-shaped structure, with the two "arms" of the Y having the antigen-binding sites (the Fab regions) while the third "leg" is called the constant region (Fc) [16]. If the Fc portion of the molecule is bound to the bead, the MAb will, potentially, be 100% active. If the Fab regions attach to the bead, the MAb may be less active. In all our experiments the method of coupling was the same and thus orientation of MAb is not considered as an independent variable.

The depth and homogeneity of MAb coupling may also affect the efficiency of antigen capture. Lasch *et al.* [17] concluded that radial gradients of ligand are present which are symmetric around the center of the bead. Carleysmith *et al.* [18] found that the depth of immobilized protein penetration depended on initial protein concentration in the immobilization mixture as well as the contact time between the resin bead and the protein. At a bulk protein concentration of 2 mg/ml, they found that only 13% of the outer radial depth of the bead had been penetrated at saturation. This has implications for the assumed diffusion distances used in modelling. If the IMAb does not penetrate homogeneously throughout the bead, the actual diffusion distance may be the MAb penetration depth, which may be estimated if the immobilization conditions are known and following the empirical correlations that can be deduced

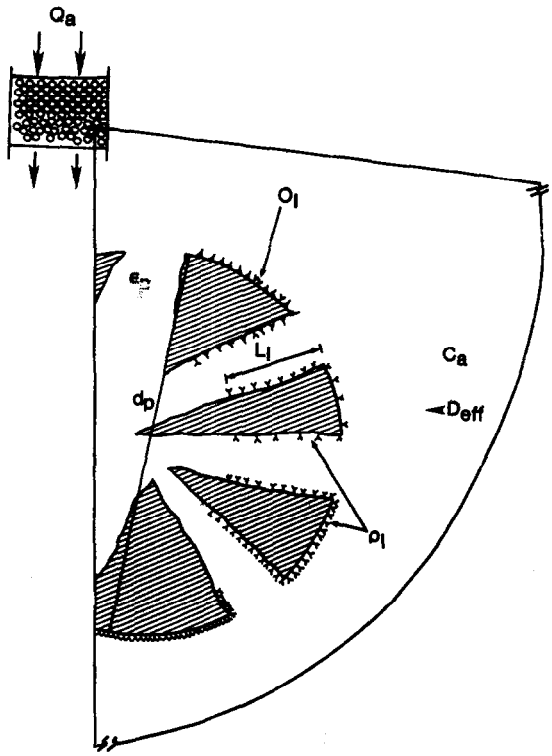

Fig. 1. Expanded view of a bead within an affinity column. C_a is the bulk concentration of FIX which flows past the beads at a flow-rate Q_a. The beads are of porosity ε_p, of diameter d_p (radius r_p), and have MAb (represented here as Y's) immobilized on them. The MAb is immobilized at a certain density, ρ_l, depth, L_l, and orientation, O_l.

from Carleysmith *et al.* [18]. In this work MAb penetration depth was not measured.

(3) FIX (or other antigen): FIX concentration and effective diffusion coefficient will affect the penetration of FIX into the bead. The diffusion coefficient will be fixed for a particular solute under set conditions and so $D_{eff,a}$ is not considered as a variable. Increasing the bulk FIX concentration may, however, enhance FIX flux into the bead. Also, slower bulk FIX feed flow-rates may offer longer times for antigen binding to MAb, which may be important if the kinetics of antigen–MAb binding are slow.

(4) Kinetics: the rate of FIX–MAb coupling in solution is the intrinsic association rate and will be a constant, as for any specific MAb–antigen pair at defined conditions. Upon immobilization, the rate of association is modified due to conformational changes in the IMAb as well as steric hindrances. Consequently, an adsorption rate constant must be measured with IMAb as was done here (see Table I). This parameter will be fixed for this MAb–FIX pair, and thus k_{ads} is considered constant in the equations.

Other factors may be involved such as the kinetics of cross reaction of the MAb with non-specific proteins. However, these interactions have been shown to be unimportant in this system [12] and have been excluded from the model development.

TABLE I

VARIABLES USED IN ANALYSIS

Parameter group	Parameter	Symbol	Value	Reference
Bead	Radius	r_p (= $d_p/2$)	80 μm	27
Ligand	Density	ρ_l	0.5–10 mg MAb/ml resin	12[a]
	Depth of penetration	L_l	8–80 μm	18
	Orientation	O_l[b]	–	–
Antigen (FIX)	Bulk concentration	C_a	7–115 units/ml	12[a]
	Effective diffusion coefficient	$D_{eff,a}$	$6 \cdot 10^{-7}$ cm^2/s	26
	Flow-rate	Q_a	0.003–0.02 cm^3/s	12[a]
Kinetics	Adsorption constant	k_{ads}	2 units FIX/(ml $\cdot$ s^{-1})	[c]

[a] Value from this study.

[b] A numerical value for O_l is difficult to establish. It may, for example, range from 0.0, completely inactive MAb, to 1.0, where the MAb may be coupled to the resin by the Fc region and be 100% active; the symbol is provided to show that this is a parameter that may affect MAb capacity.

[c] Calculated from a batch kinetic experiment: MAb resin was mixed in a plastic beaker with feed material. Samples were removed at short time intervals through a syringe filter and the FIX activity was assayed. Plotting the decrease in supernatant FIX concentration as a function of time yielded a preliminary value for an adsorption rate constant. On desorption, the FIX eluted at an equal rate; data not shown.

Table I lists the variables. Defining the capacity of the MAb as moles of FIX bound per mole of MAb, the following functional relationship may be written:

$$\begin{aligned} \text{Capacity} &= (\text{moles FIX/mole MAb or units of FIX/mg MAb}) \\ &= f(r_p, \rho_l, L_l, C_a, Q_a, D_{eff,a}, k_{ads}, \ldots) \end{aligned} \tag{1}$$

Eqn. 1 may be simplified by eliminating the parameters considered as fixed. It then becomes:

$$\text{Capacity} = f(\rho_l, C_a, Q_a) \tag{2}$$

Following this equation, we designed our experiments to examine the effect on MAb capacity of variation of feed flow-rate and FIX concentration, and the MAb density.

It is also useful to estimate the relative importance of convection, diffusion and reaction. We may use the parameters enumerated above to estimate relative diffusion, convection and reaction times. We can utilize C_a, Q_a, $D_{eff,a}$ and r_p to define a Peclet and a Damkohler number [19]:

$$\text{Peclet number} = Pe = \frac{\text{Characteristic diffusion time}}{\text{Characteristic convection time}} \tag{3a}$$

$$= \frac{[(r_p)^2]/D_{eff,a}}{(V_o/Q_a)} \tag{3b}$$

and

$$\text{Damkohler number} = Da = \frac{\text{Characteristic diffusion time}}{\text{Characteristic reaction time}} \tag{4a}$$

$$= \frac{[(r_p)^2]/D_{\text{eff,a}}}{(C_{\text{o,a}}/k_{\text{ads}})} \tag{4b}$$

Using typical values for the parameters as shown in Table I, *Pe* and *Da* for each experiment may be calculated. If a system is not limited by diffusion, *Pe* should be much less than one [14]. A large *Da* would imply that the diffusion time is very large or the antibody–antigen kinetics are slow. Slow binding kinetics may be possible for ligands other than MAbs. *Da* should be less than or equal to one, which implies that the rate of antigen binding to the IMAb is equal to or greater than the rate of antigen diffusion to the IMAb.

MATERIALS AND METHODS

Monoclonal antibody and resins

A monoclonal antibody to FIX that binds FIX in the presence of divalent cations was obtained from ascites fluid [20], purified [12], and coupled at a resin–MAb density of 1.6 mg MAb/ml resin to cyanogen bromide-activated Sepharose CL2B (Pharmacia) following the protocol of March *et al.* [21]. In this method, purified MAb solution was mixed with cyanogen bromide-activated Sepharose CL2B resin beads overnite in the cold. The resin was then washed to remove all non- or loosely bound MAb and then blocked. The amount of IMAb was determined by measuring the total protein remaining in the supernatant of the coupling mixture after MAb immobilization, as well as the protein in the resin washes, and then subtracting this sum from the total protein that was in the initial coupling mixture. Dividing this by the total volume of resin provided the amount of MAb immobilized per unit volume of resin. The amount of MAb on the resin bead was also varied to yield IAC resins with MAb densities of 0.5, 1.0, 2.96, 8.12 and 9.67 mg MAb/ml resin, respectively.

Experimental protocol for purification of FIX

The coupled resin was packed in an Amicon G10X150 Column, forming a bed of volume 7.5 cm^3. The column was equilibrated with five column-volumes of 10 m*M* magnesium chloride, 100 m*M* sodium chloride, 20 m*M* Tris, pH 6.8 buffer at a flow-rate of 1.5 ml/min. The FIX source to be used as the load material was an eluate from DEAE Sephadex adsorption of cryo-poor plasma [22]. Magnesium chloride at 1 *M* was added to this material to bring the final magnesium ion concentration to 40 m*M*. The load material was then pumped onto the column. Subsequently, the column was washed with 10 m*M* magnesium chloride, 1 *M* sodium chloride, 20 m*M* Tris, pH 6.8 buffer until the absorbance at 280 nm was below 0.09. At this point the buffer was changed to 20 m*M* sodium citrate, 110 m*M* sodium chloride, pH 6.8 which yielded one eluate peak. Column effluent pools of load, unadsorbed, wash and eluate were assayed for protein and FIX clotting activity. The affinity columns were regenerated by washing with 2 *M* sodium chloride and were re-used a minimum of ten

times during the course of this study and process development (over a year) with no noticeable decrease in either purity of the FIX obtained or in MAb capacity. All chemicals were reagent grade from Sigma.

In the first series of experiments the column volume was kept at 7.5 cm^3 and the flow-rates were varied from 0.19 to 1.4 ml/min. The MAb density was 1.6 mg MAb/ml resin and inlet FIX concentration was 50 units/ml for all the experiments. These flow-rates gave mean residence times in the column of 5.4, 12.5, 21 and 39 min, respectively.

Second, the inlet FIX concentration was varied from 7 to 115 units/ml. The flow-rate was kept constant at 0.6 ml/min and the column volume was 7.5 cm^3. The experiments were performed with resin that had a MAb density of 1.6 mg MAb/ml resin.

In the third series of experiments, MAb density was varied. MAb densities of 0.5, 1.0, 1.6, 2.96, 8.12 and 9.67 mg MAb/ml resin were obtained by using different initial concentrations of MAb in the immobilization mixture. The feed FIX concentration was 50 units/ml, mean residence time was 5 min, and the column volume was 7.5 cm^3.

The columns used in all experiments were identical. In all experiments, the amount of FIX activity bound was determined by subtracting the unadsorbed FIX activity from the total FIX activity loaded. A constant ratio of total FIX loaded per mg coupled MAb was maintained for all experiments. The experiments were repeated a minimum of three times and the data is presented as the mean $\pm$ one standard deviation.

Protein assays

Absorbance at 280 nm was used to measure protein content. Protein mass was estimated using a FIX extinction coefficient of 13.3 [23] for a 1% solution.

Coagulation assays

FIX activity was measured by a standard one-stage coagulation assay as described by Biggs [24] using FIX-deficient plasma (George King). Samples were pre-diluted in a buffer containing bovine albumin and Tween 20, as described by Miekka [25], to approximately one unit FIX/ml. Pooled fresh frozen plasma was used as the standard. The FIX activities were calculated from a semi-log plot. Further details of the assay method are given in Tharakan *et al.* [12].

RESULTS AND DISCUSSION

Fig. 2 shows the capacity of MAb for FIX at varying feed FIX concentrations. The MAb capacity remained similar when the bulk FIX concentration was changed from 7 to 115 units/ml. FIX diffusive flux into the bead is proportional to its bulk phase concentration. The relative invariance of MAb capacity with FIX concentration suggests that FIX diffusion into the bead is not limiting in this concentration range. Since the capacity of the MAb does not increase with increasing bulk phase concentration, and thus probable increasing penetration of FIX into the bead, there may not be MAb molecules immobilized deeper in the resin bead; instead, MAb may be localized near the surface of the bead. In this event, diffusion distances cannot be assumed to be the radius of the bead but are probably smaller and of the order of the depth of penetration of the MAb into the bead.

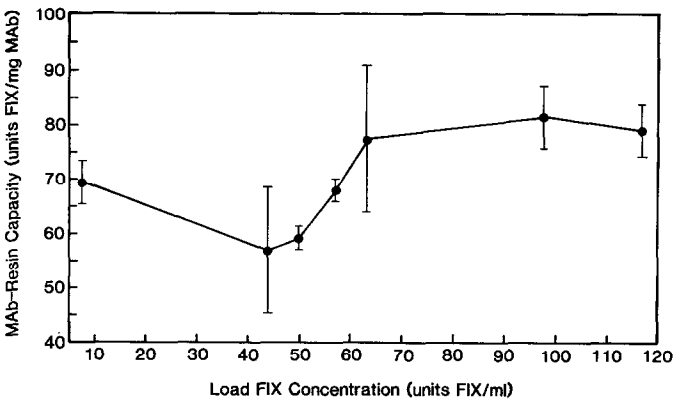

Fig. 2. Effect of feed factor IX concentration on the capacity of the MAb.

The effect of varying mean residence time on MAb capacity is shown in Fig. 3. Although the mean residence time was varied from 5 to 39 min, MAb capacity remained similar. In a diffusion limited system, increasing the mean residence time should increase the capacity of the MAb as the antigen has more time to diffuse into the bead and bind to any MAb that may be immobilized deeper in the bead. The lack of significant variation in MAb capacity with mean residence time suggests that the system is not diffusion limited. If the antibody were localized to the outer bead radius, the diffusion distances would be small, and increasing the contact time between FIX and the bead would not increase access to the MAb.

Fig. 4 compares the capacity of the MAb for different MAb densities and shows that the capacity of the IMAb increases as the MAb density decreases. For a density of 1.6 mg MAb/ml resin, the average capacity was 80 units of FIX/mg of MAb. If one assumes a molar ratio of 2:1 for FIX–MAb coupling, and a molecular weight ratio of 1:3 for FIX:MAb [10,13], the theoretical upper MAb capacity limit would be

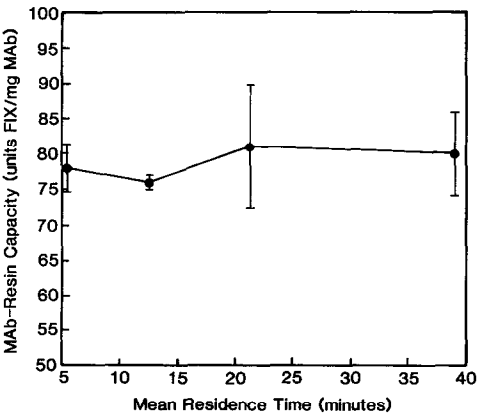

Fig. 3. Effect of mean residence time (column volume divided by bulk antigen flow-rate) on the capacity of the MAb.

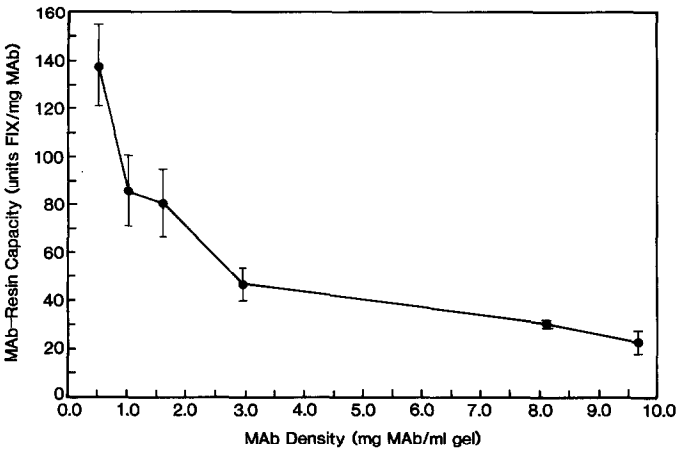

Fig. 4. Effect of resin MAb density on the capacity of the MAb.

133.3 units FIX per mg MAb, assuming a specific activity for FIX of 200 units/mg [20]. With this theoretical maximum, at 1.6 mg MAb/ml resin, 61% of MAb is active in binding FIX, while only 17% of MAb is active with resin at 9.67 mg MAb/ml resin.

Eveleigh and Levy [15] observed that increasing antibody density on an affinity resin decreases the capacity of the antibody for antigen. They noted that although a low antibody density yielded a matrix with low binding capacity per unit volume, binding capacity per unit of antibody was high. Chase [9] concluded that low binding per unit volume of resin was desirable for efficient adsorption operations. These observations and our results suggest that optimum MAb use will obtain at lower resin MAb densities. This has important implications for IAC process development, since MAb cost is a crucial process viability determinant.

Brandt *et al.* [14] analysed resin and membrane based IAC and suggested that $Pe \ll 1$ (of order 0.001) for non-diffusion limited IAC. They based their analysis on the assumption that a characteristic diffusion distance was bead radius. The range of *Pe* (eqn. 3b) in our experiments where the flow-rates were varied is 0.04 to 0.33, consistent with apparent diffusion limitation. Additionally, if the reaction time is equal to or smaller than the diffusion time, FIX will bind as soon as it diffuses to the immobilized MAb. For the experiments where the inlet concentration of FIX was varied, *Da* (eqn. 4b) ranges from 2 to 31, which is also suggestive of diffusion limitation. The IAC of FIX appears, however, to be non-diffusion limited within the parameter ranges of concentration and flow examined here.

This apparent contradiction may result from the assumption that MAb is homogeneously distributed throughout the bead. Lasch *et al.* [17] have shown that this is usually not the case. Also, following Carleysmith *et al.* [18], protein immobilized on a resin bead only penetrates a certain radial depth into the bead, depending on protein concentration in the initial immobilization mixture and the length of immobilization. Considering the conditions of immobilization employed here and generalizing the results of Carleysmith to our MAb, the MAb may only be immobilized to the outer 10% of the bead. If this is the case, we may consider the actual diffusion distance to be

10% of r_p or about 8 μm. Recalculating *Pe* (eqn. 3b) and *Da* (eqn. 4b) with $r_p = 8$ μm, the ranges of *Pe* and *Da* are $0.0004 < Pe < 0.0033$ and $0.2 < Da < 3$, respectively. These values suggest the system is not diffusion limited and are consistent with the suggestions of the data.

CONCLUSION

The use of immunoaffinity chromatography as a tool for generating very pure preparations of a single protein is becoming increasingly common in the biotechnology industry. Understanding the fundamental transport and kinetic phenomena that occur is essential for efficient process design and optimum operation. This paper provides an empirical approach to understanding the role that the various transport and kinetic phenomena play in the affinity chromatographic purification of Factor IX. Utilizing the methods in this study, a simple set of experiments that evaluates the effects of feed antigen concentration, mean antigen residence time and support matrix MAb density can be performed to provide information that will help optimize IAC process design.

SYMBOLS

C_a	Antigen (FIX) concentration, units/ml
C_o	Feed antigen concentration, units/ml
$D_{eff,a}$	Effective antigen diffusion coefficient, cm^2/s
Da	Damkohler number, eqn. 4
d_p	Resin bead diameter, cm
k_{ads}	Antigen adsorption rate constant, units FIX/ml $\cdot$ s^{-1}
L_l	Depth of penetration, μm
O_l	MAb orientation
Pe	Peclet number, eqn. 3
Q_a	Antigen flow-rate, cm^3/s
r_p ($= d_p/2$)	Resin bead radius, μm
V_o	Column volume, cm^3
ε_p	Resin bead porosity
ρ_l	IMAb density, mg MAb/ml resin

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