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Membrane-based receptor affinity chromatography

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ABSTRACT

Membrane-based receptor affinity chromatography (MRAC), which utilizes the molecular recognition between an immobilized receptor and its soluble protein ligand, has been developed for the purification of human interleukin-2 and related biomolecules. The multi-purpose affinity membrane used in this study consisted of a soluble form of interleukin-2 receptor (IL-2R) chemically bonded to hollow-fiber membranes in an oriented fashion. A model system involving anti-Tac-H (a humanized monoclonal antibody to IL-2R) was used to study the important factors influencing the performance of MRAC, including support morphology, mass transfer rate and adsorption kinetics. All three are shown to be highly efficient. MRAC has been successfully applied to the purification of anti-Tac-H, recombinant human interleukin-2 (rIL-2) and interleukin 2–*Pseudomonas* exotoxin fusion protein (IL2–PE40). Overall, MRAC was found to be a viable, scalable and extremely productive affinity purification method.

INTRODUCTION

The development of a novel affinity method, termed receptor affinity chromatography (RAC), for the purification of recombinant human interleukin-2 (rIL-2) and interleukin-2–*Pseudomonas* exotoxin fusion protein (IL2–PE40) has been reported previously [1,2]. Receptor affinity chromatography, which exploits the biochemical interactions between an immobilized receptor and its soluble protein ligand, is characterized by both its specificity and fast adsorption kinetics. However, only the former could be fully utilized in particle-based receptor affinity chromatography, resulting in low productivity. This has been attributed to the slow diffusion rate of the target protein into and out of the particle's interior where the immobilized adsorbent molecule is situated.

In order to overcome mass transfer resistances in bioaffinity purifications, flat-sheet and hollow-fiber membranes have been used as an alternative to diffusion-controlled particles [3–6] and there are several US and European patents on their use in analytical-scale protein purification. Conceptually, membrane-based purification systems have several advantages over conventional particle-based methods. The high porosity and minimal mass transfer resistance in membranes allow a high volumetric throughput, resulting in extremely short process times. The membrane structure is such that the adsorbent molecule is situated on the inner surfaces of the membrane pores, in effect along the flow path of the soluble target protein, thereby minimizing the diffusional and accessibility problems associated with gel beads used in packed column operations. The end result is maximum utilization of the immo-

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bilized adsorbent molecule. The high mass transfer capabilities of the membrane facilitate maximum utilization of the fast adsorption kinetics of affinity chromatography.

We explored the possibility of incorporating these useful characteristics of the membrane into receptor affinity chromatography. In this paper, we report a systematic approach to the design and development of membrane-based receptor affinity chromatography (MRAC) and its utility in the purification of rIL-2, and IL-2 linked immunotoxin (IL2–PE40) and a humanized monoclonal antibody to IL-2 receptor (anti-Tac-H). A model system involving a multi-purpose IL-2 receptor (IL-2R) affinity membrane and anti-Tac-H as the soluble protein ligand (adsorbate) was used to study the mass transfer and kinetic aspects of MRAC.

THEORY

Diffusion control

In order to achieve maximum efficiency in RAC, the rate at which the soluble protein ligand binds to the immobilized receptor must be maximized. The binding affinity between the ligand, L and the receptor, R, and the rate of diffusion of the soluble ligand into the receptor binding site determine the rate of ligand binding to the receptor affinity sorbent. As the receptor–ligand binding affinity is sufficiently high, this is rarely problem in RAC. Thus, binding rates are generally determined by diffusional limitations of the affinity support. The fast receptor–ligand adsorption kinetics can only be taken advantage of fully when the characteristic diffusion time, t_D (the time needed for the soluble ligand to reach the immobilized receptor), is much shorter than the ligand's residence time, t_R, in the affinity purification device, *i.e.*, $t_D \ll t_R$.

A smaller diffusional distance should yield a shorter diffusion time. In membranes, the bulk of mass transfer occurs via convection through the pores rather than by diffusion. Because of their short diffusional distance (< 1 μm), membranes afford a short diffusion time and, for all practical purposes, mass transfer resistances in membranes are negligible. These minimal mass transfer restrictions enable one to study the kinetic limitations of the affinity membrane.

Binding kinetics

Assuming that the interaction which takes place in affinity chromatography is analogous to the molecular recognition reaction in free solution, a simple theoretical analysis of the rate of receptor–ligand binding in RAC can be performed. The theoretical treatise of Nishikawa *et al.* [7] describing the equilibrium binding of an adsorbate to an affinity ligand, with Chase's [8] modification for antigen–antibody binding, is also applicable to receptor–ligand binding. The binding kinetics are described by the following rate expressions:

$$\mathrm{R} + \mathrm{L} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{R \cdot L} \tag{1}$$

$$\frac{\mathrm{d[R \cdot L]}}{\mathrm{d}t} = k_1[\mathrm{R}][\mathrm{L}] - k_{-1}[\mathrm{R \cdot L}] \tag{2}$$

$$K_{de} = \frac{k_{-1}}{k_1} = \frac{[\mathrm{R}][\mathrm{L}]}{[\mathrm{R \cdot L}]} = \frac{(q_m - q^*)C^*}{q^*} \tag{3}$$

where k_1 is the binding rate constant, k_{-1} is the rate constant for the desorption of ligand from the complex, [R] is the concentration of unoccupied receptor binding sites, [L] is the concentration of soluble ligand, [R · L] is the concentration of receptor–ligand complex, K_{de} is the effective dissociation constant for the binding reaction in the affinity system, q_m is the maximum experimentally determined binding capacity per unit volume of the affinity sorbent and q^* is the binding capacity per unit volume of the sorbent in equilibrium with C^* concentration of the adsorbate in solution. The effective dissociation constant, K_{de}, takes into account only the receptor which remains functionally effective after immobilization, and this may differ from the normal K_d in solution.

In affinity membranes, t_D is sufficiently small compared with t_R, and since receptor–ligand interactions involve a low dissociation constant, k_{-1} is also very small. Therefore, k_1, [R] and [L] are the primary factors which affect the adsorption rate in MRAC. Under such conditions, the adsorption kinetics approach homogeneous, non-diffusion-controlled reaction kinetics in solution, the difference being that the receptor is on a membrane surface. [R] is determined by the immobilization density, which is dependent on the membrane surface area and the efficiency of immobilization. [L]

is the feed stream ligand concentration and k_1 is determined by characteristics of the receptor–ligand interaction. With the aim of efficiently using the multi-purpose IL-2R affinity membrane in the purification of rIL-2, IL2–PE40 and anti-Tac-H, we studied the effects of [R], [L] and k_1 on the capture efficiency of a hollow-fiber membrane-based IL-2R affinity system.

The other aspect of affinity purification which must be considered is the desorption phase. Elution of the adsorbed ligand from the receptor sorbent requires complete dissociation of ligand from the receptor–ligand complex. Commonly used methods of elution involve non-specific eluents which alter the three-dimensional structure of either the ligand, the receptor or both, thereby reducing or completely eliminating the binding avidity. Applying the expression of Lowe and Dean [9] for the dissociation of an antigen–antibody complex to receptor–ligand dissociation, the elution kinetics can be described by eqn. 4.

$$\frac{d[R \cdot L]}{dt} = k_{-2}[R \cdot L] \quad (4)$$

where k_{-2} is the rate constant for dissociation. The fast and complete elution by non-specific eluents such as low pH buffers, chaotropes and denaturants provides evidence that these eluents actually promote breakdown of the complex rather than preventing rebinding of the dissociated ligand. Overall, the elution conditions are chosen based on protein stability, receptor–ligand binding strength and types of forces involved in the receptor–ligand binding, *e.g.*, electrostatic and hydrophobic interactions, van der Waals and London dispersion forces and hydrogen bonding.

EXPERIMENTAL

Materials

Interleukin-2 receptor and anti-Tac-H were prepared at Hoffmann-La Roche (Nutley, NJ, USA). SP2/0 cell culture supernatant (conditioned with 5–10% fetal calf serum) containing anti-Tac-H and *Escherichia coli* cells expressing rIL-2 and IL2–PE40 were obtained from the Bioprocess Development Department, Hoffmann-La Roche. NuGel-Hz and Affi-Gel-Hz were purchased from Separation Industries (Metuchen, NJ, USA) and Bio-Rad Labs. (Richmond, CA, USA), respectively. Superfine Sephadex G-25 was obtained from Pharmacia LKB Biotechnology (Piscataway, NJ, USA). Developmental and process-scale hydrazide hollow-fiber membranes (0.4- and 9.7-ml membrane volume, respectively) were purchased from Sepracor (Marlborough, MA, USA). A prototype automated Affinity-15 protein purification system was provided by Sepracor. Sodium metaperiodate, sodium cyanoborohydride and sodium azide were purchased from Sigma (St. Louis, MO, USA), and concentrated phosphte-buffered saline (10 × PBS) was purchased from Whittaker Bioproducts (Walkersville, MD, USA). All other chemicals were of analytical-reagent grade. All experiments were conducted at 4°C unless nioted otherwise.

Construction of receptor affinity sorbents

Oxidation of IL-2R [10]. Purified IL-2R at the desired concentration (2.5–10.0 mg/ml) was exchanged into the coupling buffer (0.1 *M* potassium phosphate buffer, pH 5.5) by dialysis. One tenth the volume of 0.1 *M* sodium metaperiodate in water was added to the protein solution. The mixture was shaken in the dark for 1 h at room temperature. The oxidized IL-2R was then desalted in a 21 × 2.2 cm I.D. superfine Sephadex G-25 column developed with the coupling buffer at a flow-rate of 1 ml/min. The column operations were monitored, and 3-min fractions were collected. The fractions containing oxidized IL-2R were pooled and the protein concentration was determined using the UV absorptivity of IL-2R (1.65 A_{280} for a 1 mg/ml solution). The pool was then diluted to the desired concentration.

Immobilization of IL-2R [11]. All of the hydrazide hollow-fiber membrane modules used for immobilization have a membrane volume of 0.4 ml. The membrane module was prewashed with the coupling buffer. A 6-ml volume of the oxidized IL-2R (0.65 mg/ml) was recirculated overnight through the module at a flow-rate of 2 ml/min using a peristaltic pump.

The immobilization of IL-2R on to NuGel-Hz and Affi-Gel-HZ (both contain hydrazide) was done according to the following procedure: 2 ml of each of the gel supports was packed into a G-10 Amicon column (10 × 1 cm I.D.) in the coupling buffer and equilibrated in the same buffer. A 5-ml volume of the

oxidized IL-2R (0.65 mg/ml) solution was recirculated overnight through the column under the same conditions as described for the membrane.

Following the immobilization step, one tenth of the volume of 0.5 *M* sodium cyanoborohydride was added to the coupling reaction mixture and recirculated through the various ffinity supports for another 2 h. Each of the affinity supports was then washed with *ca.* 30 ml of PBS (pH 7.4), followed by 5 ml of the elution buffer (0.2 *M* acetic acid containing 0.2 *M* sodium chloride, pH 2.8). The washings were pooled, saved and their volumes noted. The affinity supports were re-equilibrated with the PBS buffer.

The protein content of the washing (uncoupled IL-2R) was determined using the IL-2R UV absorptivity of 1.65. The difference between the starting amount of IL-2R and uncoupled IL-2R in the wash, divided by the membrane volume (0.4 ml) or the gel volume (2.0 ml), yielded the coupling density (mg/ml). The affinity supports were stored in PBS containing 0.02% of sodium azide.

Binding capacity determination

The IL-2R affinity sorbent ws equilibrated with PBS buffer and saturated with an excess of purified or crude anti-Tac-H (soluble protein ligand) at a flow-rate of 1 ml/min. The unadsorbed materials were then washed away with PBS followed by PBS containing 0.25 *M* sodium chloride at a flow-rate of 2 ml/min. The adsorbed anti-Tac-H was eluted at 1 ml/min with 0.2 *M* acetic acid containing 0.2 *M* sodium chloride. Two-minute fractions were collected and the effluents were monitored as described previously. The protein fractions were pooled and the protein content was determined using the A_{280} value of 1.4 for a 1 mg/ml anti-Tac-H solution in PBS. The coded molecular weight of $144 \cdot 10^3$ (relative molecular mass, M_r) for anti-Tac-H was used to determine the number of nanomoles of anti-Tac-H bound to the affinity sorbent. The observed (static) binding capacity was expressed as the number of nanomoles bound per unit volume of the affinity support. The theoretical or expected binding capacity of the affinity support was calculated from the IL-2R coupling density, taking into account the $25 \cdot 10^3$ M_r of IL-2R and assuming 1:1 binding between the receptor and ligand [12].

Mass transfer rate and adsorption kinetics

Mass transfer rate. A 139-ml volume of PBS solution containing 500 μg of anti-Tac-H (3.6 μg/ml) was loaded on to the affinity membrane module (0.73 mg of IL-2R immobilized) at flow-rates of 1, 5, 15, 25, 50 and 100 ml/min. In each of these experiments the binding capacity of the affinity membrane was determined as described previously. The residence time (t_R) at each of these flow-rates was calculated from the equation

$$t_R(s) = \frac{\text{membrane volume (ml)} \cdot 60}{\text{filtrate flow-rate (ml/min)}}$$

Feed stream ligand concentration. The affinity membrane module used in this study was the same as in the preceding experiment. The dynamic binding capacity of the affinity membrane was determined when the feed stream ligand concentrations were 1.0, 2.5, 5.0, 10.0 and 25.0 n*M*. In all experiments, a total of 0.5 mg of anti-Tac-H was used and the loading was carried out at a fixed flow-rate of 15 ml/min.

Binding sites concentration (coupling density). Five IL-2R affinity membrane modules with coupling density 0.55, 1.40, 1.82, 3.75 and 4.50 mg/ml were prepared by treating the hydrazide membranes with 6 ml of oxidized IL-2R at concentrations ranging from 0.1 to 2.0 mg/ml according to the procedure described earlier. The binding capacities and efficiencies of these membranes were determined and plotted against the respective coupling densities, expressed as molarities.

Determination of ligand breakthrough point. Three IL-2R affinity sorbents based on a hollow-fiber membrane (0.4 ml), NuGel and Affi-Gel (0.5 ml each) were prepared. These affinity supports contained 1.20, 1.04 and 2.56 mg of immobilized IL-2R and their binding capacities were 1.034, 0.80 and 0.70 mg of anti-Tac-H, respectively. A 100-ml volume of a 20 μg/ml anti-Tac-H solution in PBS was applied to each of the affinity supports at a flow-rate of 4 ml/min. During loading, 1-min fractions were collected for the affinity membrane, whereas 20-s fractions were collected for the affinity gels. The column effluents were monitored as described for the binding capacity determination. The UV absorbance of the flow-through material in each fraction was determined and expressed as a percentage of the original feed stream absorbance. These values, which represented the percentage of un-

bound ligand (anti-Tac-H) in the breakthrough material, were plotted against the amount of feed stream ligand which had passed through the affinity sorbent up to and including that fraction. The amount of ligand which had come into contact with each affinity support up until the breakthrough point was determined and then converted into a percentage of the static binding capacity of the affinity sorbent. The point at which an amount of ligand equal to the gel or membrane's predetermined binding capacity had passed through the affinity support was taken as the expected saturation point. The observed saturation point occurred when the ligand content (UV absorbance) of the breakthrough material reached that of the original feed stream.

Membrane-based affinity purification

A schematic illustration of membrane-based receptor affinity chromatography is shown in Fig. 1. A feed stream containing the soluble protein ligand is pumpted through the membrane lumen in a cross-flow mode, and a transmembrane pressure differential draws the fluid convectively through the walls of the fiber. This results in protein ligand binding to the immobilized receptor within the membrane walls. The ligand–depleted filtrate which is drawn through the membrane wall is discarded, while the feed stream still containing the soluble ligand is recirculated through the membrane. A wash buffer removes unbound contaminants, leaving behind bound ligand, which is then eluted with an elution buffer and collected. A regeneration wash prepares the system for the next cycle of operation.

The same affinity membrane with 1.5 mg of IL-2R immobilized was used for all three affinity purifications described below.

Anti-Tac-H. The IL-2R affinity membrane was equilibrated with PBS (pH 7.4). A 1-l volume of crude anti-Tac-H (SP2/0 conditioned media) was filtered through a 0.8/0.2 μm filter (Sartorius, Yauco, Puerto Rico) and applied to the affinity membrane at a flow-rate of 20 ml/min. The unadsorbed materials were washed away with PBS containing 0.25 *M* sodium chloride. The antibody was then eluted with 0.2 *M* acetic acid containing 0.2 *M* sodium chloride at a flow-rate of 1 ml/min, and 2-min fractions were collected. The column effluents were monitored as described previously. The fractions containing the antibody were pooled and the pH was adjusted to 6.0. The pool was dialyzed against PBS and the protein concentration was determined from its A_{280} value using 1.4 as the UV absorptivity for a solution of 1 mg/ml anti-Tac-H. An enzyme-linked immunosorbent assay (ELISA) [13] specifically designed for detecting anti-Tac-H

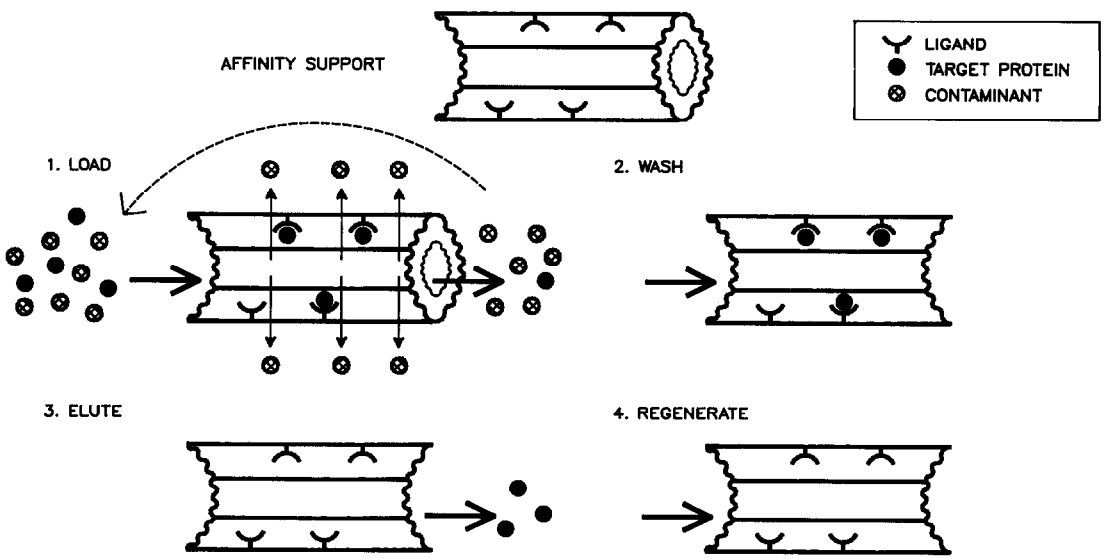

Fig. 1. Schematic illustration of membrane-based receptor affinity chromatography (MRAC). The receptor is chemically bonded to hollow-fiber membranes in an oriented fashion. A single hollow fiber is shown for clarity.

was used to determine the activity of the purified material. Purity was also determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [14].

Recombinant human interleukin-2. A 200-ml volume of the crude rIL-2 extract was prepared from *E. coli* cell paste containing recombinantly expressed IL-2, as described [1]. The experimental conditions were the same as in anti-Tac-H purification. The eluate fractions containing the rIL-2 were pooled and the protein content was determined using 0.665 as the UV absorptivity for 1 mg/ml rIL-2. The bioactivity of the purified material was determined by the IL-2-dependent CTLL proliferation assay [15]. The recovered protein was also subjected to SDS-PAGE.

Interleukin 2–Pseudomonas exotoxin fusion protein (IL2–PE40). The crude extract of this recombinantly produced fusion protein was prepared as described elsewhere [2]. The loading of the extract (400 ml) on to the affinity membrane and the washing were done as in the rIL-2 purification. The elution was carried out with 1.5 *M* potassium thiocyanate in 50 m*M* potassium phosphate (pH 6.0). The eluate was dialyzed against PBS, bioassayed [2] and subjected to SDS-PAGE.

Scale-up and automation

Process-scale IL-2R affinity membrane. An 80-ml volume of oxidized IL-2R (0.5 mg/ml), prepared according to the procedure described earlier, was recirculated overnight through a process-scale (9.7-ml membrane volume) hollow-fiber membrane module at a flow-rate of 10 ml/min using a peristaltic pump. An 8-ml volume of a 0.5 *M* sodium cyanoborohydride solution were then added to the reaction mixture and recirculated for an additional 2 h. The affinity membrane was washed with 300 ml of PBS followed by 50 ml of elution buffer. The washings were pooled and the final volume was noted. The uncoupled IL-2R in the washing and the coupling density of the affinity membrane were determined as described earlier, taking into account the 9.7-ml membrane volume of the process-scale module.

Large-scale purification. Anti-Tac-H, rIL-2 and IL2–PE40 were purified from their respective crude extracts using the process-scale IL-2R affinity membrane. All the purification steps were carried out using a Sepracor prototype Affinity-15 automated system.

Anti-Tac-H. A 9-l volume of SP2/0 media containing *ca.* 4 μg/ml of anti-Tac-H was recirculated through the IL-2R affinity membrane at a loading flow-rate of 2 l/min and a filtrate flow-rate of 240 ml/min. Washing and elution were carried out at a flow-rate of 200 ml/min using the same buffers as in the small-scale purification. The time needed to complete one cycle of operation was 45 min. The purified material was bioassayed and subjected to SDS-PAGE.

rIL-2. A 500-ml volume of *E. coli* extract containing 4–5 μg/ml of rIL-2 was recirculated through the IL-2R affinity membrane at a loading flow-rate of 1 l/min and at a filtrate flow-rate of 140 ml/min; washing and elution were carried out as in the anti-Tac-H purification. The process time was 5 min. Purified rIL-2 was bioassayed and analyzed by SDS-PAGE.

IL2–PE40. A 500-ml volume of *E. coli* extract containing *ca.* 10 μg/ml of IL-2–PE40 was used in each purification cycle. The experimental conditions were the same as in rIL-2 purification, except that elution was carried out with 1.5 *M* potassium thiocyanate in 50 m*M* potassium phosphate (pH 6.0). The purified material was dialyzed against the phosphate buffer, bioassayed and subjected to SDS-PAGE. One purification cycle was completed in 5 min.

RESULTS

Definitions

Receptor coupling density is defined as the absolute amount of receptor coupled per milliliter of support. For membranes, the volume includes the pore space within the membranes but excludes the dead space in the membrane lumen and in the device housing. With gels, the support volume is taken as the bed volune of the column, and includes the interstitial space between the beads. The static ligand binding capacity is taken as the experimentally determined number of nanomoles of ligand bound per unit volume of the affinity sorbent whereas the dynamic capacity refers to the amount of ligand which binds under the experimental conditions. The binding efficiency is the percentage of receptor

TABLE I

BINDING CAPACITIES OF IL-2R AFFINITY SORBENTS

IL-2R was coupled to hydrazide derivatives of hollow-fiber membranes, Nu-Gel and Affi-Gel according to the procedures described under Experimental. Binding capacities were calculated taking into account the M_r of IL-2R and anti-Tac-H (soluble protein ligand) as $25 \cdot 10^3$ and $144 \cdot 10^3$, respectively. The binding stoichiometry was taken as 1:1. Binding efficiency is the observed binding capacity expressed as a percentage of the expected binding capacity.

Affinity sorbent	Coupling density (mg/ml)	Binding capacity (nmol/ml)		Binding efficiency (%)
		Expected	Observed	
Hollow-fiber membrane	0.55	22	16	73
	1.82	73	23	31
	3.75	150	47	31
Nu-Gel	1.40	56	12	21
Affi-Gel	1.50	62	18	29

effective in capturing the ligand relative to the theoretically expected values based on the amount of receptor immobilized, assuming 1:1 binding stoichiometry. The ligand capture efficiency is the dynamic binding capacity expressed as a percentage of the static binding capacity.

IL-2R affinity sorbents

Oxidized IL-2R was immobilized to commercially available hydrazide derivatives of hollow-fiber membrane, NuGel and Affi-Gel. The hollow-fiber membrane has a surface area of 3.7 m^2/ml, with a pore size of 0.4 μm and a hydrazide content of 2–5 μmol/ml membrane volume (manufacturer's specifications). For membranes, the coupling efficiency was in the range 30–40% and the coupling densities ranged from 0.55 to 3.75 mg/ml. For NuGel and Affi-Gel the hydrazide contents were 8–10 μmol/ml gel. When the reaction mixture contained 2 ml of each of the gel and 3.25 mg of IL-2R at a concentration of 0.65 mg/ml, the coupling efficiencies were *ca.* 80% and the coupling densities were 1.4 and 1.5 mg/ml, respectively. The coupling densities, binding capacities and binding efficiencies of three IL-2R affinity membranes along with those for NuGel and Affi-Gel-based receptor sorbents are listed in Table I.

TABLE II

EFFECT OF FLOW-RATE ON CAPTURE EFFICIENCY

The dynamic binding capacity of an IL-2R affinity membrane (0.73 mg of IL-2R immobilized) was determined at various flow-rates, using 500 μg of anti-Tac-H (soluble protein ligand). The capture efficiency corresponding to each fluid residence time is the dynamic binding capacity expressed as a percentage of the amount of anti-Tac-H (500 μg) applied to the affinity membrane.

Flow-rate (ml/min)	Residence time (s)	Capture efficiency (%)
1.0	24.00	99
5.0	4.80	96
15.0	1.60	94
25.0	0.96	84
50.0	0.48	73
100.0	0.24	47

Effect of flow-rate

Table II shows the ligand capture efficiency of an IL-2R affinity membrane at increasing flow-rates or decreasing fluid residence times. At fluid residence times of 24, 4.8, 1.6, 0.96, 0.48 and 0.24 s, the capture efficiencies were 99, 96, 94, 84, 73 and 47%, respectively. The diffusion time, t_D, in the hollow-fiber membrane is estimated to be 16 ms ($t_D = l^2/D$, diffusional distance $l = 4.0 \cdot 10^{-5}$ cm and diffusion coefficient $D = 1.0 \cdot 10^{-7}$ cm^2 s^{-1} for an average molecular size protein).

Effect of ligand concentration

The capture efficiencies of an IL-2R affinity membrane when [L] = 1, 2.5, 5.0, 10 and 25 nM are

TABLE III

EFFECT OF FEED STREAM [L] ON CAPTURE EFFICIENCY

The IL-2R affinity membrane used in this study was the same as in Table II. The dynamic binding capacity of the affinity membrane was determined at a fixed flow-rate of 15 ml/min, at various feed stream [L] as described in the text. The respective capture efficiencies were calculated as in Table II.

Feed stream ligand concentration		Capture efficiency (%)
n*M*	μg/ml	
1.0	0.144	75
2.5	0.360	82
5.0	0.720	82
10.0	1.444	82
25.0	3.600	86

listed in Table III and were 72, 82, 82, 82 and 86%, respectively. The experimentally determined K_{de} of this IL-2R membrane was $2.1 \cdot 10^{-9}$ *M* (data not shown), whereas in solution $K_d = 3.3 \cdot 10^{-10}$ *M* [16].

Effect of receptor binding sites concentration

The ligand-binding capacities and efficiencies of five IL-2R affinity membranes in relation to [R] are shown in Fig. 2. The experimentally determined binding capacities were compared with the calculated values based on coupling densities and the 1:1 stoichiometry of the binding reaction. At the lowest coupling density of 0.55 mg/ml ($2.2 \cdot 10^{-5}$ *M*) the binding efficiency was 73% and at coupling densities >0.55 mg/ml the binding efficiency dropped to 31%.

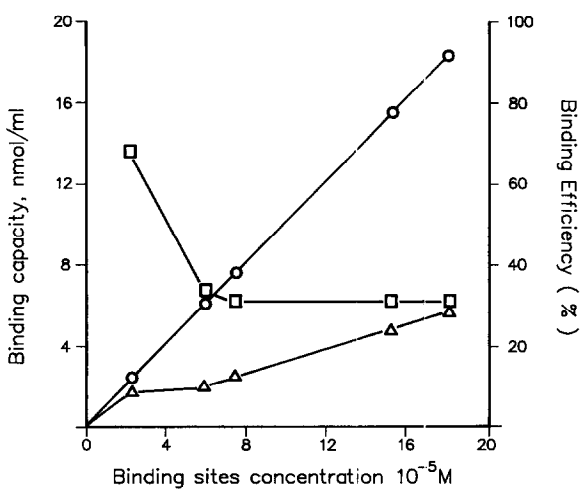

Fig. 2. Effect of binding sites concentration [R]. Five IL-2R affinity membranes were prepared with various coupling densities. Their (○) calculated and (△) observed binding capacities and (□) binding efficiencies were determined as described in the text. These values were plotted against the coupling densities expressed in molarity.

Ligand breakthrough point

The ligand breakthrough curves for hollow-fiber membrane, Nu-Gel and Affi-Gel-based IL-2R affinity sorbents are shown in Fig. 3. In the affinity membrane, the ligand breakthrough occurred very near to its static binding capacity (80%), whereas in gels the breakthrough occurred almost immediately following the start of the loading step. For Nu-Gel and Affi-Gel the breakthrough occurred at *ca.* 3% of their respective static binding capacities. It should be noted that the static binding capacities of the affinity sorbents were not identical. However, as the breakthrough points were calculated based on the individual binding capacities of each affinity sorbent, the differences in static binding capacities had no bearing on the corresponding breakthrough points.

Membrane-based receptor affinity purifications

Humanized-anti-Tac. In this chimeric monoclonal antibody, selected amino acids from within the variable region of a murine monoclonal antibody to the low affinity p55 subunit of the IL-2R (T-cell activating antigen or TAC) are molecularly engineered into a human IgG sequence [17].

Approximately 2.7 mg of anti-Tac-H were recovered from 1 l of SP2/0 medium (titre, 2–4 μg/ml), conditioned with 10% calf serum. The recovered material was >95% pure as indicated by SDS-PAGE (Fig. 4, lane 6) and by the bioassay results. The SDS-PAGE profile under reducing conditions demonstrated the presence of two bands corresponding to the heavy and light chains of the IgG molecule. The purified material was also albumin free, which is usually not the case when conventional methods are used (unpublished observation).

Recombinant human interleukin-2. Interleukin-2, previously known as T-cell growth factor [18], is a lymphokine whose interaction with its high-affinity receptor is essential for initiation and maintenance of a normal immune response. From 200 ml of

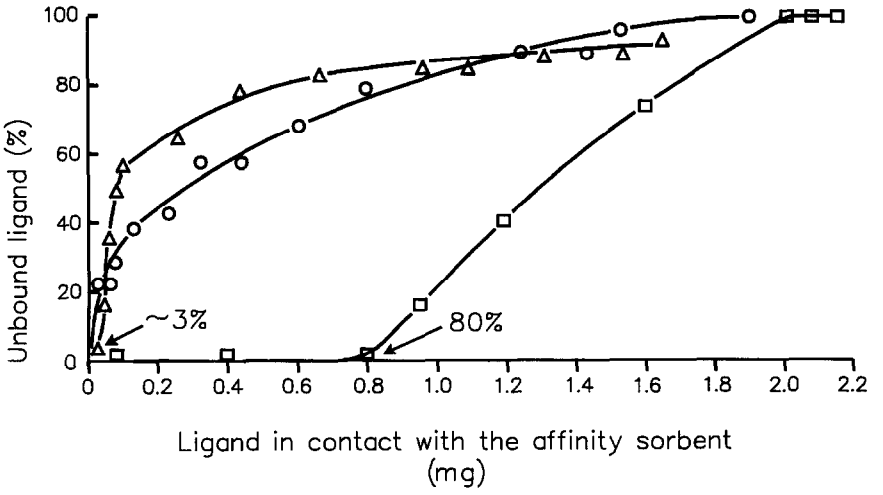

Fig. 3. Ligand (anti-Tac-H) breakthrough curves for (□) hollow-fiber membrane, (○) NuGel and (△) Affigel based IL-2R affinity sorbents. The ordinate represents the percentage of unbound ligand in breakthrough material. Points on the abscissa correspond to the amount of ligand that has passed through the support up to that point. Ligand breakthrough points of the various affinity sorbents occurred at (○,△) *ca.* 3% and (□) 80% of their static binding capacities.

extract, equivalent to 1.25 g of *E. coli* cells, 273 μg of rIL-2 were recovered. It should be noted that the predetermined binding capacity of the IL-2R affinity membrane was 280 μg of rIL-2. The high purity (>95%) of recovered rIL-2 was confirmed by SDS-PAGE (Fig. 4, lane 2) and by bioassay.

Interleukin 2–Pseudomonas exotoxin fusion protein. IL-2–PE40 is a $54.4 \cdot 10^3$ M_r fusion protein in

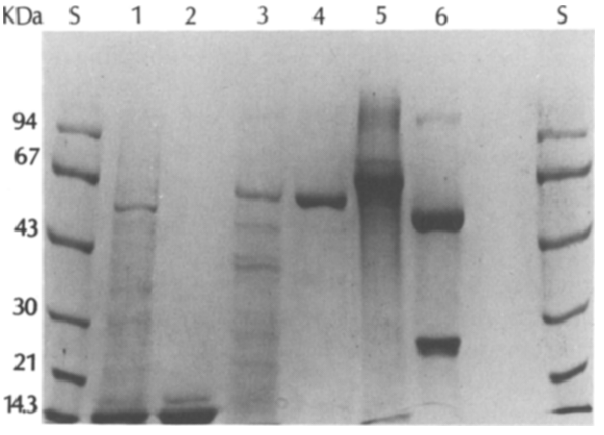

Fig. 4. SDS-PAGE of MRAC-purified biomolecules. Lanes: S = standard molecular mass marker proteins ($M_r \cdot 10^3$ indicated on the left); 1 and 2 = crude and purified rIL-2; 3 and 4 = crude and purified IL2–PE40; 5 and 6 = crude and purified anti-Tac-H.

which the cell recognition domain of *Pseudomonas* exotoxin is replaced with IL-2 [19]. IL-2–PE40 is a potential cytotoxic agent for IL-2R-bearing cells.

From 400 ml of extract, equivalent to 1 g of *E. coli* cells, *ca.* 200 μg of IL2–PE40 were recovered. The purified material, as expected, showed a major band around $(54–55) \cdot 10^3$ M_r when subjected to SDS-PAGE (Fig. 4, lane 4) and its purity of >95% was confirmed by the bioactivity.

Scale-up and automation. IL-2R (15.5 mg) was immobilized to a process-scale membrane module, which was then used in the fully automated, continuous purification of anti-Tac-H (conditioned media), rIL-2 and IL2–PE40 (*E. coli* extracts). A 30-mg amount of anti-Tac-H, 2 mg of rIL-2 and 5 mg of IL2–PE40 were processed in 45, 5 and 5 min, respectively. These recoveries correspond to a daily processing capability of 960 mg of anti-Tac-H, 576 mg of rIL-2 and 1440 mg of IL2–PE40. The quality of proteins purified by the automated system was comparable to that purified using the research-scale affinity modules, as indicated by SDS-PAGE and bioassays (data not shown).

Stability during long-term use. The affinity membrane was stable during long-term use. No impairment in functionality was observed after 50 cycles of operation. A regeneration step involving a wash with 20% ethylene glycol minimizes the chances of

membrane fouling. IL-2R leaching during the purification steps was monitored by a very sensitive bimolecular binding assay [20]. The IL-2R affinity membrane was subjected to washing and mock elution with the respective buffers. The effluents were collected and assayed for their IL-2R content. The observed leaching was in the range 7.5–12.5 pg/ml.

DISCUSSION

The hydrazide membranes used in the construction of IL-2R affinity membranes are generally inert, hydrophilic and biocompatible. The soluble IL-2R [21] used for immobilization was chosen on the basis of its ability to form a reversible receptor–ligand complex in addition to having a high enough affinity to bind ligand from even dilute solutions, yet low enough to permit dissociation under relatively mild conditions. The immobilization via the sugar moieties of the IL-2R was specifically chosen so that oriented coupling could be achieved.

Although the gels have a larger surface area and a greater number of hydrazide groups available for protein coupling than do membranes, a useful amount of receptor can still be coupled to the membranes, as indicated by the coupling densities listed in Table I. The results also show that the binding efficiency of the hollow-fiber membrane was comparable to that of the gels. However, the observed binding efficiencies in general are low for all three supports, especially considering that the receptor was immobilized to the support via oriented coupling. The loss in binding efficiency is probably due to a loss of receptor binding sites during oxidation and immobilization because of inter- or intramolecular cross-linking.

The typically low concentrations of the target protein in a crude feed stream necessitate processing large volumes at high flow-rates with short fluid residence times. If the receptor–ligand interaction is kinetically limited within the short residence times of interest, the mass transfer advantages conferred by membranes are of limited use. For this reason, we studied the effect of adsorption flow-rate on the affinity membrane's ligand capture efficiency. The results in Table II show that for all practical purposes, the affinity membrane's capture efficiency remained unchanged when the fluid residence time ranged from 24 to 1.6 s. However, when the fluid residence time falls below 1 s, its negative effect on adsorption kinetics becomes apprent. A two-fold reduction in capture efficiency (from 99 to 47%) was observed when the fluid residence time was reduced 100-fold (from 24 to 0.24 s). The smallest fluid residence time of 0.24 s used in this study is still much greater than the hollow-fiber membrane's t_D of *ca.* 16 ms, that is, $t_D \ll t_R$, which favors effective binding. These results indicate that although the adsorption kinetics in MRAC are highly efficient, it is adsorption kinetics rather than mass transfer which will first become limiting.

As seen in Table III, no significant differences in capture efficiencies were observed when [L] $> K_{de}$ (2.1 nM). Even when [L] was only 1 nM ($< K_{de}$) the capture efficiency was only slightly affected. This is a clear indication that the adsorption kinetics in MRAC are not seriously affected by the feed stream ligand concentrations, and that even at very low concentrations there is no need to slow the flow-rate during adsorption to allow longer receptor–ligand interaction. However, in order to achieve the maximum binding capacity of the affinity membrane, [L] $\gg K_{de}$. It should be pointed out that the recombinant protein concentration in a crude feed stream is usually >25 nM.

The increased adsorption kinetics and binding capacities predicted by eqn. 2 at increased receptor coupling densities [R] are limited by the molecular size of the molecules involved. The "crowding" effect or steric hindrance, which causes significant losses in binding efficiency at higher coupling densities, is well illustrated in Fig. 2. As expected, the highest binding efficiency was observed for the lowest coupling density and at higher coupling densities significant losses in binding efficiencies were observed. At high coupling densities, the receptors may be envisioned as bing too close together, resulting in restricted access to the binding sites, especially when the soluble protein is a large molecule as in the case of anti-Tac-H (*ca.* $144 \cdot 10^3$ M_r).

Even for a large ligand molecule such as anti-Tac-H and at a relatively short residence time of 6 s, ligand breakthrough in the membrane occurred very near to its static binding capacity, which illustrates the affinity membrane's better ligand capture efficiency. The latter can be attributed to its extremely

short diffusional distance (< 1 μm) and easily accessible receptor. Therefore, it is reasonable to conclude that the immobilized adsorbent molecule in a membrane is readily accessible to the soluble target protein, irrespective of the latter's molecular size. The diffusional limitation in the membrane is minimal, and breakthrough occurs primarily owing to the progressive decrease in available binding sites as more and more ligand binds to the receptor. At high levels of receptor saturation, ligand capture is proportionately reduced owing to the decreased receptor concentration, [R]. The rate of ligand capture is the product of k_1 and [R], ([L] remains steady) and hence a decrease in [R] could result in ligand breakthrough. Once the breakthrough has occurred, in order to achieve maximum (static) binding capacity, a considerable amount of feed stream has to pass through the affinity membrane, during which time the adsorbate molecule is not fully retained.

In gel beads, the relatively large particle size contributes to their greater diffusional distance and ligand accessibility problems. With particle sizes of 40–50 μm for Nu-Gel and 40–200 μm for Affi-Gel, and with necessary diffusion into narrow pores and channels, a significant amount of the feed stream ligand flows around the beads without ever making contact with the receptor immobilized within the bead. The broadening of the breakthrough curve for the beads is attributed to diffusion or mass transfer resistances, in addition to the depletion of available receptor sites.

In addition, ligand capture could be affected by the increased molecular size of the receptor–ligand complex formed and the resulting steric hindrance. The net result is a reduced ligand capture rate and subsequent increase in ligand breakthrough. The latter can also be affected by uneven fluid flow; however, in the membrane we are assuming uniform pore sizes resulting in fluid flow with minimal dispersion. Ligand breakthrough can probably be delayed by increasing the residence time, but this would be at the expense of higher productivity.

We have demonstrated that a multi-purpose IL-2R affinity membrane is capable of purifying anti-Tac-H, rIL-2 and IL2–PE40 from dilute feed streams at residence times of the order of seconds, with ligand breakthrough occurring near the static binding capacity of the affinity membrane. The extremely high mass transfer rates and resulting short residence times have little adverse effect on the capture efficiency of the affinity membrane, irrespective of the molecular size of the protein to be purified. Owing to the minimal diffusional resistance in membranes, the adsorption kinetics are very efficient. However, maximum utilization of the membrane's mass transfer capabilities is dependent on a sufficiently high receptor–ligand binding affinity.

It should be pointed out that the membrane-based affinity systems also have some technical disadvantages. In general, today's commercially available membranes have a relatively small surface area per unit membrane volume, which limits the amount of adsorbent molcule that can be immobilized. Therefore, the static binding capacity in membranes is relatively small compared with that in gels.

In conclusion, we have shown that MRAC is both a practical and highly efficient purification method for three closely related recombinant molecules, offering many advantages. Membrane-based purification systems are ideally suited for the industrial-scale production of biotherapeutics. The process time is shortened considerably in such systems, which permits faster purification, using less adsorbent.

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