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Microfiltration membranes as pseudo-affinity adsorbents: modification and comparison with gel beads

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ABSTRACT

Microfiltration membranes were studied as alternative adsorbents to the usual spherical hydrogels beads for the purification of proteins. Assumption of monolayer adsorption onto the internal area allowed the capacity measured by adsorption of model proteins to be predicted. The membrane could be modified with triazine dyes to yield an affinity-like support. The capacity of the dyed membrane was similar to the capacity of dyed Sepharose and the membrane could be used repeatedly. Enzymes from a crude extract of baker's yeast were selectively adsorbed in both batch and continuous experiments, allowing purification factors of at least ten.

INTRODUCTION

Dye ligand chromatography has long been known as a versatile and robust tool for purifying proteins from the analytical to the preparative scale [1]. In order to shorten the process time, more efficient adsorbents have been sought. The application of beads with small diameters allows the restrictions set by diffusive transport of the proteins to the ligand to be reduced considerably [2]. However, high operating pressures are required. Alternatively, porous polymer matrices modified as ion exchangers (Memsep, Millipore; Zetaprep, Cuno) have been introduced recently because of their high surface area, convective solute transport and low pressure drops [3].

Microfiltration membranes are potentially attractive adsorbents because they offer the same advantages as the matrices mentioned above together with mechanical strength, a highly porous structure and a large range of configurations for operation. Moreover, membranes are capable of handling unclarified solutions. Capillary membrane modules modified to yield affinity supports allowed, for instance, antibodies to be isolated from a cell culture medium [4].

In this work, microfiltration membranes made of nylon were tested as affinity-like adsorbents. The available capacity of the basic material was evaluated in two ways: from measurements of the inner surface area available in principle for adsorption and from experiments involving adsorption of model proteins. Modification of a nylon membrane with various pseudo-affinity ligands (triazine dyes) was

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performed and the modified membranes were tested as adsorbents. A proprietary dye ligand membrane was used for enrichment of enzymes from a crude yeast extract. Batch and filtration mode experiments were compared with the different membranes and their reusability was assessed. The results were compared with those obtained with pseudo-affinity agarose gels.

EXPERIMENTAL

Materials

Microfiltration membranes (Ultipor) were obtained from Pall (Dreieich, Germany). According to technical information from the manufacturer, Ultipor are isotropic membranes made of nylon 66 with a high surface concentration of amine and carboxylate groups in a 1:1 ratio. Modified nylon membranes with a positively charged surface, Zetapor (0.2 μm) and Zetabind (0.45 μm), were acquired from Cuno Europe (Cergy-Pontoise, France). Zetabind is intended for DNA and protein blotting. Samples from a proprietary nylon-based membrane modified with triazine dyes were a gift from Sartorius (Göttingen, Germany).

Cibacron Blue F3G-A was purchased from Ciba Geigy (Basle, Switzerland), Procion dyes from ICI (Frankfurt, Germany), polyethylenimine (PEI) from Sigma Chemie (Deisenhofen, Germany) and glutaraldehyde from Fluka (Neu-Ulm, Germany). All other reagents were of analytical-reagent grade.

Bovine serum albumin (BSA), fraction V (Boehringer Mannheim, Tutzing, Germany) was dissolved in water and hen egg lysozyme (Fluka) in 1 m*M* potassium phosphate buffer (pH 7.0).

A crude enzyme extract was prepared from commercial baker's yeast (*Saccharomyces cerevisiae*). A 40% (w/v) suspension in 100 m*M* potassium phosphate buffer (pH 6.0)–1 m*M* EDTA–5 m*M* mercaptoethanol was disintegrated in a high-pressure homogenizer, centrifuged and filtered through a 0.2-μm filter. The extract was stored at −20°C. Before use, samples were again clarified by centrifugation and diluted 4-fold with a 1 m*M* EDTA solution.

Dye ligand immobilization

The dye ligands were coupled to Ultipor membranes (0.1-μm nominal pore size) either directly or using PEI as spacer. The latter was first bound to membranes previously activated by glutaraldehyde at pH 8.0 [5]. The dyeing was carried out in two steps: a 1-h dye adsorption at room temperature from 6% NaCl solution, followed by reaction for 1 h at 50°C after raising the pH to 10.8 with carbonate buffer. A large excess of reagents were used and the membranes were rinsed repeatedly after every reaction step. After a final rinse with water and methanol, the membranes were stored dry at room temperature. The amount of dye coupled to the membrane was determined spectrophotometrically after dissolution of samples in phenol–methanol. Dyeing of Sepharose CL-4B was performed as described for the membranes.

Batch adsorption experiments

Samples of membrane were contacted with the solution of proteins in a shaken vessel for about 3 h. No significant change in protein concentration in the liquid occurred typically after 90 min up to 24 h. The amount adsorbed was calculated from

the mass balance. The experiments were performed in flasks for BSA and lysozyme and in micro test-tubes (Eppendorf) for yeast extract. The support volume was about 2% of the solution volume. Blanks without membranes were incubated in parallel to check that no deactivation or unexpected losses occurred. Before the adsorption experiments, the membranes were equilibrated with the corresponding solution without protein.

Adsorption and desorption in filtration mode

Membranes discs of 25 mm diameter were used; the effective filtration area was 3.3 cm^2.

To perform the adsorption experiments with lysozyme, filter holders (Sartorius, SM16517E) connected to a syringe were used. The following solutions (5 ml) were successively passed through the filter: buffer, 0.1 g/l lysozyme, 1 *M* sodium chloride and then 10 ml buffer. Residual liquids were flushed out of the holder with air. Lysozyme adsorption was measured by the decrease in concentration on filtration. The above cycle was performed three times.

For the experiments with the yeast extract, a filter holder with a minimum dead volume was constructed and inserted into an a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden). One to ten membranes could be fitted in the holder. Samples of 500 μl were injected. The membrane was washed with 10 ml of buffer. Unless stated otherwise, elution was carried out with a 10-ml linear gradient from 0 to 0.25 *M* KCl, a 5-ml step with 2 *M* KCl in buffer and a 5-ml step with starting buffer. Fractions of 5 ml were collected. Before the next run, a few milliliters of starting buffer were passed through the module without collecting the fractions.

Assays

Lysozyme concentrations were measured at 280 nm. BSA and yeast proteins were assayed with the Coomassie or with the micro-BCA test using kits purchased from Pierce (Oud Beijerland, The Netherlands). Malate dehydrogenase (MDH) and glucose-6-phosphate dehydrogenase (G6PDH) activities were measured according to Bergmeyer [6] and adenylate kinase according to Ito *et al.* [7].

RESULTS

Preliminary evaluation of membrane capacity for model proteins

The capacity of the membranes considered as adsorbents for proteins was evaluated in two ways: first by calculating the amount that could be adsorbed in a close-packed monolayer using literature data for the physical dimensions of model proteins and measuring the surface area available within the matrix; and second by measuring the adsorption of BSA under conditions favourable to adsorption.

The internal surface areas of the membranes were measured by nitrogen adsorption [8] using a Sorptomatic apparatus (Carlo Erba, Milan, Italy). The results for Ultipor membranes of various nominal pore sizes are shown in Fig. 1. Together with monolayer adsorption values calculated from the BSA and lysozyme dimensions (3.8 nm diameter × 15.0 nm length and 4.5 × 4.4 × 3.0 nm, respectively [9,10]), the overall surface would allow *ca.* 150 μg of lysozyme and 150–600 μg of BSA to bind to a 1-cm^2 piece of membrane with 0.1-μm nominal pore size. The two values for BSA

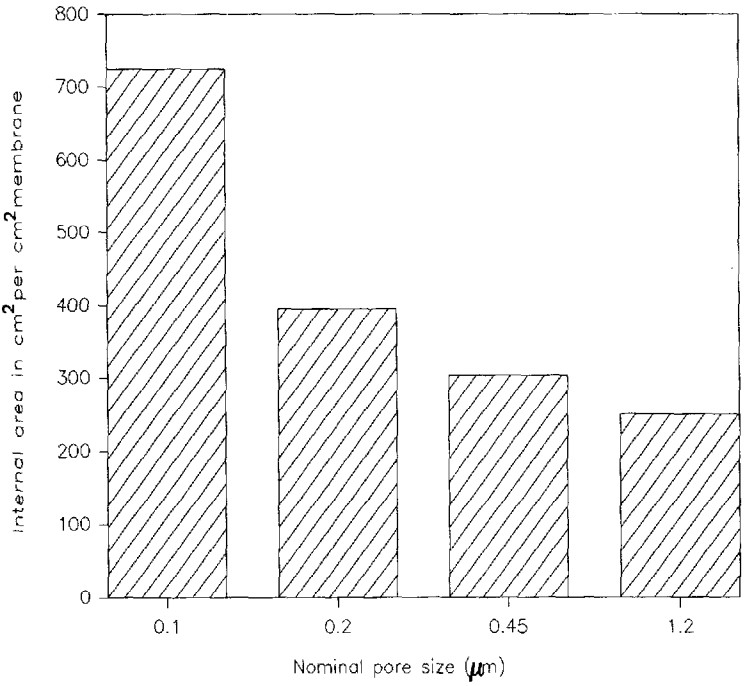

Fig. 1. Inner surface area of microfiltration membranes (Ultipor, Pall) with differents pore sizes, determined by nitrogen adsorption.

refer to adsorption of the protein molecule with its main axis parallel or perpendicular to the support surface.

The adsorption measured with BSA in aqueous solution at concentrations of 0.25 and 0.5 g/l on two positively charged membranes (Zetapor, Zetabind) and an amphoteric membrane (Ultipor) are shown in Table I. At the end of the experiment, an excess of BSA remained in solution. On doubling the initial concentration, the increase in the amount adsorbed was small relative to the concentration left in solution for Ultipor and Zetapor, therefore indicating saturation.

Immobilization of dye ligands to Ultipor membrane

The amounts of crude dye immobilized on the Ultipor membrane in two batches were in good agreement (Table II). A five to ten times higher dye concentration was bound when PEI was used as spacer. Dye concentrations in the same range were obtained with mono- (Cibacron, Procion H) and dichlorotriazinyl (Procion MX) dyes.

Adsorption of lysozyme to dyed Ultipor membrane

Batch adsorption was performed in duplicate. Adsorption in the filtration mode was repeated three times with every membrane specimen, the membrane being washed with sodium chloride solution and buffer between runs. As the amounts adsorbed did not show any trend, the averages from three data are reported. The amounts of

TABLE I

AMOUNTS OF BSA ADSORBED IN BATCH FROM 0.25 AND 0.5 g/l SOLUTIONS IN WATER ON CATIONIC (ZETAPOR, 0.2 μm; ZETABIND, 0.45 μm) AND AMPHOTERIC (ULTIPOR, 0.1 μm) NYLON MEMBRANES

Membrane	Introduced ($\mu g/cm^2$)	Adsorbed ($\mu g/cm^2$)	Remaining in solution ($\mu g/ml$)
Ultipor	159	80	126
	320	108	337
Zetapor	158	144	22
	320	176	264
Zetabind	132	47	161
	264	106	307

lysozyme introduced in the experiment were similar in the batch and filtration modes, namely 126 and 152 μg per square centimetre of filter, although the initial concentration was 2.5 times higher in the batch experiment. Table III shows that the amounts of lysozyme adsorbed per square centimetre of filter were very similar in the batch and filtration modes. Adsorption was systematically higher on the dyed membrane. The highest adsorption was observed with the blue membrane modified first with PEI.

Adsorption of yeast proteins to dyed membranes or Sepharose CL-4B

The proprietary membrane from Sartorius and Sepharose CL4-B carrying the blue (Cibacron F3G-A) or the yellow ligand (Procion Yellow HE3-G) were compared in batch experiments. A large excess of bulk protein was incubated in contact with the support in order to test the selectivity of the binding for the target enzymes. About 1000 times more protein than the expected capacity was used. As a consequence, the concentration change in solution was negligible and a mass balance would not allow the amount of proteins adsorbed to be measured. Also, an overestimation of protein

TABLE II

AMOUNTS OF DYE LIGANDS COUPLED TO ULTIPOR MEMBRANES (0.1 μm) IN μg OF CRUDE DYE PER mg OF DRY MEMBRANE

Ligands were coupled directly or using polyethylenimine as a spacer (denoted PEI-).

Dye	Dye coupled (μg/mg membrane)	
	Batch 1	Batch 2
PEI-Cibacron Blue F3G-A	21.4	15.5
Cibacron Blue F3G-A	2.71	2.57
Procion Blue MX-R	6.65	6.6
Procion Red MX-5B	4.04	3.38
Procion Red HE3-B	4.14	5.4
Procion Yellow HE3-G	0.56	1.1

TABLE III

AMOUNTS OF LYSOZYME ADSORBED ON DYED MEMBRANES FROM A BUFFER SOLUTION (POTASSIUM PHOSPHATE, pH 7.0, 1 m*M*) IN BATCH AND FLOW-THROUGH MODES

The amounts introduced were 126 and 152 $\mu g/cm^2$ membrane, respectively. Membrane sample: Ultipor (0.1 μm). Average and standard deviation are from duplicate and triplicate experiments, respectively.

Dye	Amount of lysozyme adsorbed ($\mu g/cm^2$ membrane)	
	Batch	Flow-through
PEI-Cibacron Blue F3G-A	122 ± 4	110 ± 18
Cibacron Blue F3G-A	78 ± 2	83 ± 4
Procion Blue MX-R	90 ± 22	92 ± 11
Procion Red MX-5B	85 ± 15	81 ± 38
Procion Red HE3-B	94 ± 21	102 ± 2
Procion Yellow HE3-G	87 ± 4	87 ± 8
Not dyed	40 ± 13	62 ± 6

desorption values (about 50%) was caused by the transfer of a significant amount of bulk proteins with interstitial liquids in the supports. Desorption was attempted by resuspending the drained support in buffer containing 0.5 *M* KCl. The amount of proteins and enzyme activity adsorbed or desorbed were related to the unit volume of support derived from the membrane dimensions or from weighing the drained gel and assuming a density of unity, respectively.

TABLE IV

COMPARISON OF PROPRIETARY DYE LIGAND MEMBRANES WITH SEPHAROSE CL-4B AS CARRIERS WITH BLUE OR YELLOW LIGANDS, FOR THE ADSORPTION OF ENZYMES FROM A CLARIFIED YEAST HOMOGENATE

Amounts of MDH, adenylate kinase, G6PDH activities and protein adsorbed and desorbed per millilitre of support. Variations are standard deviations.

Support	MDH (U/ml)	Adenylate kinase (U/ml)	G6PDH (U/ml)	Proteins (mg/ml)
Adsorption				
Sartorius blue	800 ± 100	350 ± 60	120 ± 10	nd[a]
Sartorius yellow	990 ± 120	260 ± 70	70 ± 10	nd
Sepharose blue	250 ± 50	430 ± 30	20 ± 10	nd
Sepharose yellow	780 ± 140	100 ± 70	20 ± 10	nd
Desorption				
Sartorius blue	200 ± 30	50 ± 1	5 ± 0.5	10.7 ± 0.5
Sartorius yellow	610 ± 30	50 ± 10	10 ± 1	12.0 ± 0.9
Sepharose blue	240 ± 10	210 ± 10	2 ± 0.4	13.4 ± 1.3
Sepharose yellow	620 ± 10	140 ± 10	4 ± 0.3	14.1 ± 1.0

[a] nd = Not determined.

The results in Table IV show that the amounts of proteins or individual enzymes involved in adsorption or desorption were of the same order regardless of the support material. The ligand had no general effect on the binding. However, more MDH was bound to the yellow ligand on both supports and more adenylate kinase to the blue gel.

On desorption, a low recovery (below 20%) was found for G6PDH on all the supports and for adenylate kinase on the membranes. The MDH recovery was higher than that of the other enzymes, and better with the gel supports.

The comparison of the specific activities in the original extract and the corresponding values in the desorption solutions (data not shown) revealed that significant enrichments occurred for MDH irrespective of the support, with a factor of 5 on the blue ligand and 12–14 on the yellow ligand. Enrichment factors for adenylate kinase were about 3 with membranes and 6 9 with gels, as a result of the better recovery on the latter. No significant enrichment of G6PDH could be measured.

Binding of yeast proteins to dyed membranes in filtration mode

The reusability of the dyed membrane was tested by repeated loading and elution (0 to 0.25 *M* KCl gradient and then a step with 2 *M* KCl) of proteins from the yeast

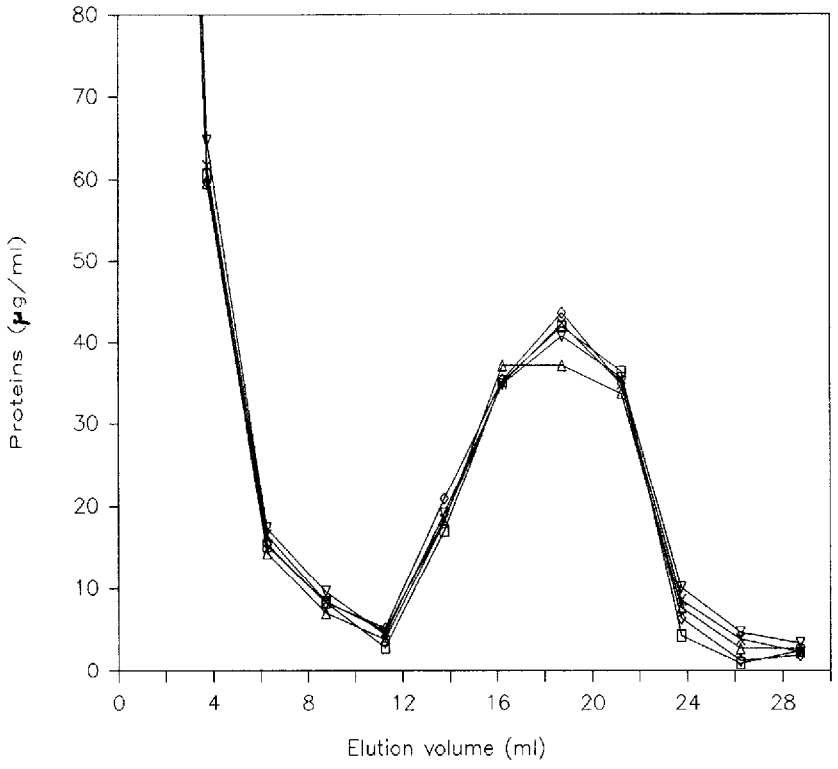

Fig. 2. Repeated use of a stack of ten membranes as stationary phase for binding proteins from the yeast extract. A 500-μl aliquot of the extract was loaded on the membrane, previously equilibrated with starting buffer; the membrane was washed with 10 ml of starting buffer followed by a 10-ml KCl gradient up to 0.25 *M*, a 5-ml step with 2 *M* KCl and a 5-ml step with starting buffer. Linear velocity, 0.3 cm/min. Succession of runs: □, ◇, △, ×, ▽.

TABLE V

REUSABILITY OF A MEMBRANE SPECIMEN (3.3 cm^2) FOR BINDING ENZYMES FROM THE YEAST EXTRACT

A 500-μl aliquot of the extract was loaded on the membrane previously equilibrated with starting buffer; the membrane was washed with 20 ml of starting buffer followed by a 40-ml KCl gradient up to 2 *M*. Proteins, G6PDH, MDH and adenylate kinase recovered in the eluted peak.

Run No.	Proteins (μg)	G6PDH (U)	MDH (U)	Adenylate kinase (U)
1	308	0.21	16.36	1.89
2	319	0.21	12.24	1.69
3	294	0.28	12.24	0.05

crude extract. A stack of ten membranes was used in the FPLC system. The elution profiles observed in five successive runs were highly reproducible (Fig. 2). The amount of proteins recovered was constant within 3% error. However, the elution peak was spread over about 7.5 ml.

The same kind of experiment was repeated with only one disc of membrane in the holder and using 20 ml of starting buffer to wash unbound proteins and a 40-ml linear gradient up to 2 *M* for elution. A frontal application of the sample was used to overload the support capacity as in the batch adsorption experiments. The flow-rate for elution was 1.0 ml/min in the first two runs and 0.05 ml/min in the third. MDH, G6PDH, adenylate kinase and the bulk of the eluted proteins were recovered in a typical broad peak without apparent separation from each other. The amount of proteins and enzymes found in the gradient was stable, in general (Table V). However, the recovery of adenylate kinase was lower in the third run, possibly because of deactivation over several hours.

The data in Table V, when related to the support volume, yield 3.6 U/ml G6PDH, 205 U/ml MDH and 27 U/ml adenylate kinase (first two runs only), in good agreement with the desorption from the blue membrane in the batch mode (Table IV). The desorption of protein, yielding 4.65 mg/ml, was notably lower than in the batch mode, but the latter value was biased, as mentioned above. In consequence, the enrichment factors were found to be about twice as high as in the batch mode.

DISCUSSION

Preliminary evaluation of the capacity of microfiltration membranes as adsorbents for proteins showed that experimental values obtained with BSA were in good agreement with the calculated value of 150 $\mu g/cm^2$ membrane, based on the actual inner area of the support and the assumption of adsorption in a monolayer of BSA on the surface. Under the conditions chosen, namely a low ionic strength, electrostatic attraction of protein to the support with the cationic membranes was expected to yield a high affinity of BSA for the support (Table I). The positively charged membrane Zetapor showed prompt saturation, as a very small increase in adsorption was observed while the remaining concentration in solution differed by a factor of about 10. On the other hand, the small residual concentration left in

solution with the smallest amount of BSA introduced showed that the affinity was very high. In fact, the concentration in the support matrix was about 470 times higher than that in the solution. The Ultipor membrane showed saturation to a lesser extent, but the amphoteric nature of the membrane made a net electrostatic attraction less evident as the unadjusted pH of the aqueous solution was close to the isoelectric point of the membrane, reported as 6.5 [11]. The Zetabind membrane surprisingly showed no saturation at all, despite the excess of BSA remaining in the solution. The latter shows that the affinity for BSA was low in comparison with the other two membranes. Gershoni and Palade [12] found a steady increase in BSA binding up to 500 $\mu g/cm^2$ when a large excess (up to 37 mg/cm^2) was contacted with a Zetabind membrane, which was then rinsed before counting the I^{125}-labelled BSA. The requirement for such a high concentration to achieve high adsorption values and the resistance to rinsing are contradictory if one assumes an equilibrium between adsorbed and soluble BSA. The binding mechanism on that membrane could therefore be particular, *i.e.*, a form of low affinity but irreversible insolubilization on the surface. Our experimental set-up based on mass balance to determine adsorption did not allow that range of concentrations to be investigated. Nevertheless, the high concentrations of BSA remaining in solution (Table I) indicated that no binding sites with high affinity were left on any of the membranes.

The lysozyme capacity on dyed membranes (Table III) was consistent with the monolayer capacity calculated for the Ultipor membrane. Moreover, the monolayer values predicted well the capacity measured on the proprietary ligand membrane (see Table V, values for 3.3 cm^2 sample area) with a protein mixture.

Fig. 1 suggests that the capacity of Ultipor membranes with larger pore sizes up to 1.2 μm would correspond to only a three times reduction in capacity.

The modification of nylon membranes with triazine dyes is reproducible, regardless of the dye reactivity (Table II). Concentrations of 0.5–3.6 $\mu mol/ml$ membrane can be calculated for Cibacron Blue without and with PEI as spacer. The values reported for agarose gels were in the range 1–10 $\mu mol/ml$ [1].

The adsorption of lysozyme under conditions favourable to electrostatic attraction showed the positive effect of the dye ligand on the membrane capacity (Table III) as a 2-fold adsorption was observed compared with the parent membrane. The value measured on the latter cannot be considered as a background for dyed membranes as the original binding sites could be masked by dye ligands. The maximum values in Table III can be converted to a concentration of 8.6 mg/ml support, which corresponds to about 60% of the maximum concentration reported on blue Sepharose CL-6B [13].

The good correlation between batch and flow-through data for lysozyme adsorption (Table III) indicated two important properties of the membranes as dye ligand adsorbents: first, no irreversible binding occurred, as washing of the support with 1 *M* NaCl restored the initial capacity; second, flow-through conditions allowed the adsorption to be achieved in a few minutes, even with a more dilute solution. The first point was confirmed with the proprietary dye ligand membrane and yeast proteins and enzymes (Fig. 2, Table V).

Batch and flow-through experiments with the latter membrane showed the applicability of the support to the isolation of specific enzymes from crude mixtures. The amounts of enzymes recovered were similar in both procedures (Tables IV and V).

Enrichment factors in the region of 10 would be very attractive in early separations as a large proportion of the bulk proteins would be discarded.

The volumetric capacity of the membranes was found to be comparable to that of the corresponding Sepharose gels, at least as measured by enzyme adsorption or protein desorption (Table IV).

The method adopted here to desorb enzymes showed a poor performance; in batch adsorption the recovery was not complete and notably inferior on membranes than on gels; in the flow-through mode, extensive peak spreading occurred. The latter may reflect sequential desorption of different proteins or axial dispersion in the holder or the lines. However, individual enzyme peaks were fairly broad also, covering at least 5 ml for the data in Table IV, and axial dispersion would account only for peaks of 2.5 ml duration as measured in complementary experiments (data not shown). In fact, no optimization of the desorption step has been attempted so far. Several possibilities exist for improving the desorption behaviour: optimization of the buffer composition and pH for a particular enzyme or eluting specifically with substrate or cofactor.

This paper demonstrates that the inner surface of microfiltration membranes made of nylon could be modified with dye ligands and used for adsorption of proteins, yielding volumetric capacities close to those of the corresponding agarose support. The binding capacity could be used repeatedly and no irreversible fouling was apparent. However, desorption using high salt concentrations was not satisfactory and notably worse than with dye ligand gels. Optimizing the desorption step is still possible.

Interestingly, the available area was not dramatically reduced when membranes with larger nominal pore sizes were considered. This might yield a very favourable opportunity to optimize the space–time yield of membrane adsorbents, as membranes with high permeabilities could be chosen without affecting the capacity.

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