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Investigations of liquid–liquid partition chromatography of proteins

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ABSTRACT

In the liquid–liquid partition chromatography (LLPC) of proteins, the favourable properties of aqueous two-phase systems are combined with the advantages of column chromatography. An investigation of the basic properties shows that the efficiency of resolution is determined by the same parameters as for liquid chromatography and the same mathematical models can be applied. In affinity LLPC the high sensitivity of the elution volume to small changes of the partition coefficient in the range 0.2–1.5 may be exploited using biospecific ligands. This paper reports the purification of formate dehydrogenase and monoclonal antibody by affinity LLPC. As a result of biospecific interaction in a homogeneous phase, affinity LLPC gives a better utilisation of ligands than conventional affinity chromatography.

INTRODUCTION

Aqueous two-phase polymer systems have been used successfully for the separation of bioactive molecules and viable cells [1]. Phase diagrams for aqueous phase systems consisting of polyethylene glycol (PEG)–dextran (Dx) or PEG–salt have been established for use in partition experiments [1]. The partition of added components such as proteins in aqueous phase systems is mainly determined by size and surface properties, *e.g.* hydrophobicity and charge. The partition coefficient (K) can also be affected by pH, partitioning of the ionic species, and the molecular weight and concentration of the polymers used. To increase the selectivity, Flanagan and Barondes [2] added affinity ligands covalently bound to one of the phase-forming polymers to aqueous two-phase systems [2]. Affinity partition has been used succesfully for the purification of enzymes [3–9], nucleic acids and oligonucleotides [10,11] and for separating cells [12,13].

Müller [14] developed matrices for liquid–liquid partition chromatography using aqueous PEG–Dx phase systems. Müller [14] grafted linear polyacrylamide chains of 15–25 monomers to macroporous diol-substituted silica and synthetic polymers (TSK-HW gel), which resulted in a resin that is preferentially wetted by the dextran-rich phase as a result of the incompatibility of PEG and polyacrylamide. Liquid–liquid partition chromatography (LLPC) was performed using the PEG-rich phase as the mobile phase and the dextran-rich phase as the stationary phase [14].

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In this way the favourable properties of the aqueous phase systems could be combined with the advantages of column chromatography. This paper reports investigations of the basic chromatographic properties of the support materials and new applications in affinity LLPC.

EXPERIMENTAL

The support materials LiParGel 750 and 650, particle size 25–40 μm, were a gift of E. Merck (Darmstadt, Germany). The company also provided experimental samples of polyacrylated LiChrospher Diol, a porous spherical silica gel with 1000 Å pores and 10 μm particle size. All other materials and methods were as described by Walsdorf and co-workers [15,16]. Ascites fluid with monoclonal antibody (mAb) against horseradish peroxidase (POD) was kindly provided by E. Hadas (Department of Biotechnology, Tel Aviv University, Israel). The mAb was purified as described by Elling *et al.* [17].

RESULTS AND DISCUSSION

General investigations of liquid–liquid partition chromatography

Calibration of the column

According to Martin and Synge [10], the volume of the stationary phase (V_s), the volume of the mobile phase (V_m), the elution volume of component 1 and 2 (V_{el}) and the partition coefficient ($K_{(n)}$) of component n are related as follows:

$$V_s = (V_{el(2)} - V_{el(1)})/(1/K_{(2)} - 1/K_{(1)}) \quad (1)$$

$$V_m = V_{el(1)} - V_s(1/K_{(1)}) \quad (2)$$

$$V_{el} = V_s/K + V_m \quad (3)$$

$$K_{(n)} = V_s/(V_{el} - V_m) \quad (4)$$

To calculate V_{el} or K, the volumes of the mobile and stationary phases for a given column must be known. The data for V_{el} and K were obtained by independent LLPC and batch partition experiments, respectively, and were fitted to a hyperbolic function (eqn. 3), which yielded reliable values for V_m and V_s (Fig. 1).

The non-ideal behaviour of proteins in LLPC is related to electrostatic interactions with negatively charged groups on the matrix (*e.g.*, lysozyme in Fig. 1), and size exclusion effects, which limit the access of large proteins ($\geqslant$ 100 000 dalton, see ferritin in Fig. 1) to the stationary phase inside the LiParGel matrix [15]. Mass transfer effects by film diffusion are not responsible for the observed changes in available V_s for LiParGel 750. The elution volumes of various proteins remain constant over the usual range of linear flow velocities. Mass transport effects are evident in other parameters describing the chromatographic processes, as discussed later. Size exclusion was not observed on polyacrylated LiChrospher Diol columns for proteins at least up to 500 000 daton molecular weight [15].

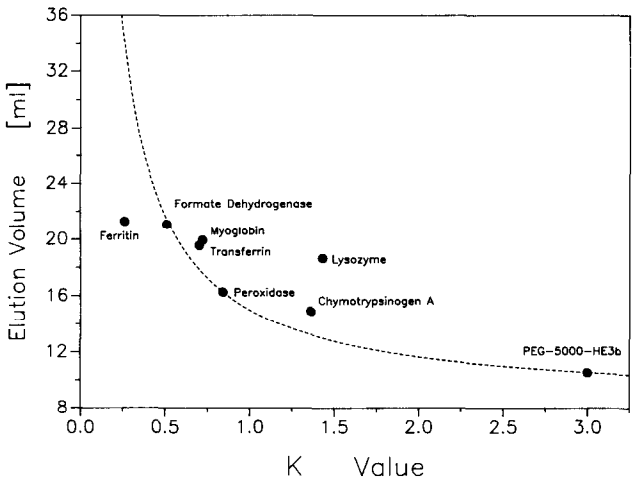


Fig. 1. Relationship between the elution volume and the partition coefficient (K) in liquid–liquid partition chromatography for LiParGel 750. Phase system: 2.7% PEG 20 000–4.5% Dx 500 in 10 mM potassium phosphate buffer (pH 7.0) with 75 mM potassium bromide. Flow-rate, 1 ml/min; column, 30 × 1 cm. ---- = Calculated elution volume; ● = measured elution volume.

Physical data for the support are available for LiChrospher Diol. From the bead dimensions, density (0.5 g/cm^3) and internal surface area (25–30 m^2/g), it can be calculated that the dextran-rich stationary phase layer has a thickness of approximately 18 nm, assuming an even distribution over the entire surface. Based on the same assumptions it was calculated that about 98% of the stationary phase is located inside the porous particles. With an average pore size of 1000 Å, the mobile phase is able to enter the beads. Mass transport by diffusion over a large interface of thin layers of the immiscible phases should be sufficiently fast to reach partition equilibrium; diffusion within the pores should therefore represent a major resistance. This resistance may be more pronounced than in adsorption chromatography as the mobile phase has a higher viscosity than aqueous buffers due to the presence of PEG.

Factors influencing resolution in liquid–liquid partition chromatography

The efficiency of LLPC columns, *i.e.*, the resolution of two peaks, can be defined by the column parameters used in liquid chromatography [18].

Influence of linear flow-rate on peak resolution and plate height. Fig. 2 shows the influence of linear flow-rate on the resolution (R_s value) and the plate height for a LiParGel 750 column. The resolution for POD-myoglobin increases with decreasing linear flow-rate until a maximum is reached at 0.02 cm/s. Linear flow-rates lower than 0.02 cm/s gave higher height equivalent to a theoretical plate (HETP) values and a lower resolution. The HETP is related to the molecular weight of the proteins and lower values are observed for myoglobin (molecular weight 17 600 daltons) than for peroxidase (molecular weight 40 000 daltons). This is due to the higer diffusion rate of lower-molecular-weight molecules, which leads to a higher mass transfer rate within the pores. The characteristic curve for the relative resolution of myoglobin and preoxidase can be explained by the Van Deemter equation [18], which described the relationship between the linear flow-rate and plate height.

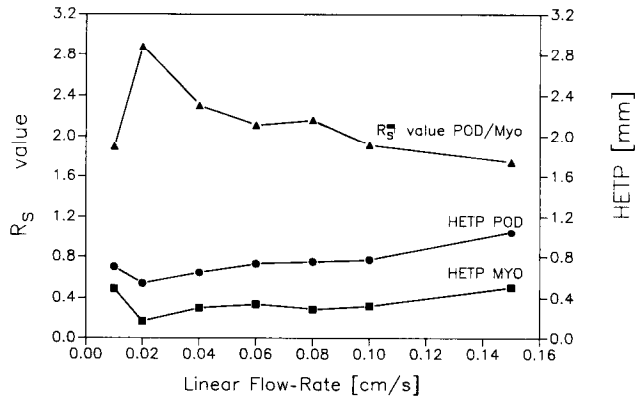


Fig. 2. Influence of the linear flow-rate on peak resolution (R_s) and the height of a theoretical plate (HETP). Support material: LiParGel 750. Phase system 5.4% PEG 6000–9.0% Dx 40 in 100 m*M* sodium chloride, 50 m*M* sodium phosphate buffer (pH 7.5). Column dimensions: 30 × 1 cm. The HETP standard deviation (n = 3) was 4.8 and 3.1% for myoglobin and peroxidase, respectively.

The influence of the linear flow-rate on the resolution (R_s) for LiParGel 650 is illustrated in Fig. 3. For curves 1–3 in Fig. 3, $R_s > 1$, which means a good resolution of the proteins under investigation. Again, a decrease in the linear flow-rate improves the resolution; the limiting linear flow-rate could not be reached with the chromatographic equipment used. Peak resolution of the proteins in curves 4 and 5 is not reached ($R_s < 1$). Here the partition coefficient must be altered, *e.g.*, by changes in the phase composition, to improve the resolution. The curves presented may be used to assess the maximum flow-rate with good resolution for preparative separations.

Influence of the sample volume on the number of theoretical plates. Fig. 4 shows

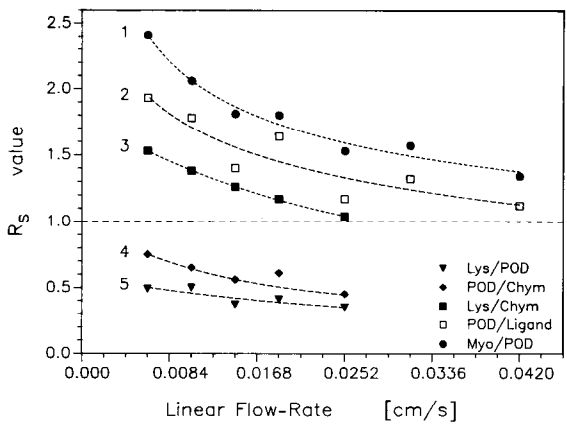


Fig. 3. Influence of the linear flow-rate on peak resolution of different proteins. Lys = lysozyme; POD = horseradish peroxidase; Chym = chymotrypsin; myo = myoglobin; ligand PEG-5000 HE3b. Support material: LiParGel 650. Phase system: 5.4% PEG 6000–9.0% Dx 40; column dimensions: 30 × 0.5 cm; sample volume, 100 µl. For conditions see Fig. 2.

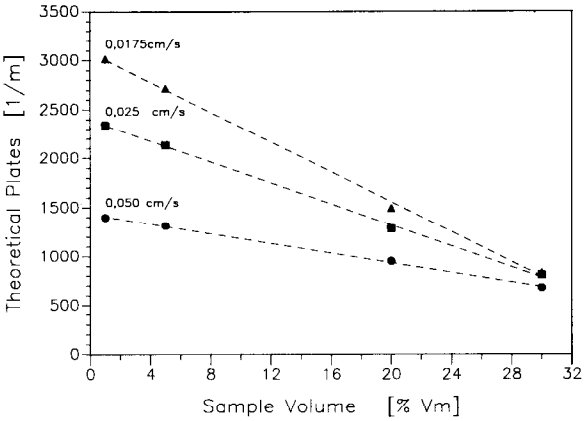


Fig. 4. Number of theoretical plates as a function of sample volume of myoglobin (17 600 dalton; 1 mg/ml). Support material: LiParGel 750; phase system, 2.7% PEG 20 000–4.5% Dx 500 in 12.6 m*M* potassium phosphate buffer (pH 7.0) with 50 m*M* sodium chlorate; column dimensions, 30 × 1 cm; V_m = 10.6 ml; V_s = 5.34 ml.

the plot of the number of theoretical plates (*N*) *versus* sample volume for LiParGel 750.

The chromatographic parameter *N* is inversely proportional to the sample volume. The sample volume is normalized as a percentage of the mobile phase (V_m) to facilitate comparisons between different column dimensions. The slopes obtained for different linear flow-rates indicate that the loss of theoretical plates is higher at lower linear flow-rates. For an increase in sample size (in this instance myoglobin) from 1 to 30% V_m, the loss of theoretical plates is 72 and 52% for linear flow-rates of 0.0175 and 0.05 cm/s, respectively. When the sample volume is increased to 60% V_m, a linear decrease of the number of theoretical plates is also observed [Fig. 5, with bovine

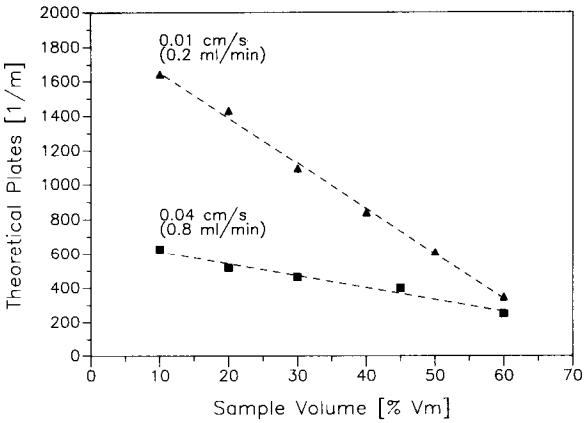


Fig. 5. Number of theoretical plates as a function of sample volume of bovine serum albumin (BSA 68 000 dalton, 1 mg/ml). For conditions see Fig. 4.

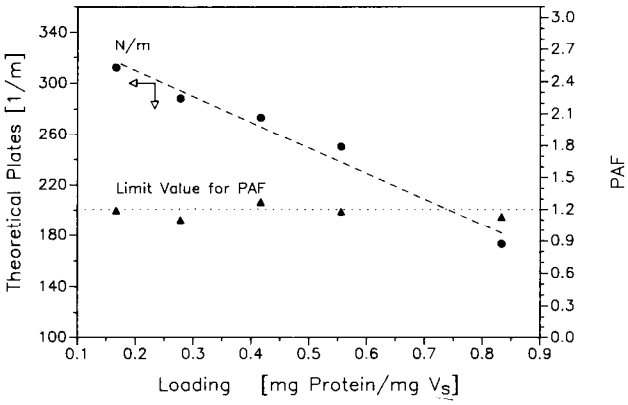


Fig. 6. Influence of column loading on the number of theoretical plates and the peak asymmetry factor (PAF). Support material: LiParGel 650; phase system, 5.4% PEG 6000–9.0% Dx 40; flow velocity, 0.0345 cm/s (0.15 ml/min); sample volume, 4.35% of V_m (100 µl); V_m = 2.3 ml; V_s = 1.2 ml. For conditions see Fig. 2. Density of V_s = 1.1 mg/ml.

serum albumin (BSA) as an example]. The peaks are still symmetrical under these conditions [peak asymmetry (PAF) $\leqslant$ 1.2] and the number of theoretical plates can be calculated from the peak width. Owing to the higher molecular weight of BSA and the lower number of available theoretical plates, the loss in N is not as pronounced as for myoglobin with increasing sample volume.

Influence of sample loading on the column resolution. The influence of increasing protein concentration in the sample on the number of theoretical plates and the PAF was investigated using BSA (Fig. 6).

The number of theoretical plates decreases with higher protein loadings, which are expressed as a percentage of the stationary phase. The symmetry of the peaks is not affected up to 0.85 mg BSA/mg V_s (PAF $\leqslant$ 1.2). Protein loadings of more than 0.85 mg/mg V_s resulted in asymmetric multiple peaks, as shown in Fig. 7. Experi-

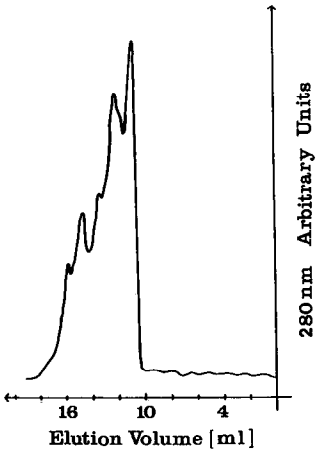


Fig. 7. Peak deformation as a consequence of column overloading (1.875 mg BSA per mg stationary phase). Flow velocity, 0.0345 cm/s (0.15 ml/min). For conditions see Fig. 6.

ments with larger diameter columns (1.6 and 2.6 cm) gave comparable results (data not shown). In a similar manner to liquid chromatography, the capacity of an LLPC column can be increased by increasing the diameter. A limiting factor remains the solubility of the product in the mobile phase, although this was not a problem with BSA.

In conclusion, the results demonstrate that the efficiency of LLPC columns is determined by the same parameters as for liquid chromatography and the same mathematical models can be applied.

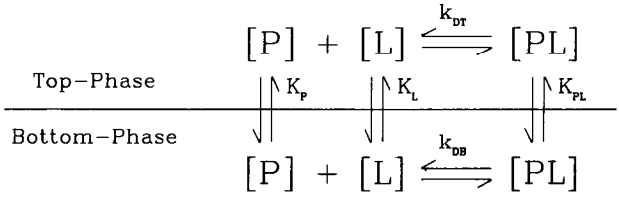
Affinity liquid–liquid partition chromatography

In Fig. 1 the relationship between elution volume V_{el}, and partition coefficient, K, is shown for a given column. The conditions that increase the partition coefficient in the range of 0.2–1.5 for the product of interest will lead to a faster elution. Biospecific interactions can be utilized to influence selectively the partition coefficient.

Fig. 8 illustrates the linked equilibria of partition and protein–ligand (PL) association for a 1:1 complex. The yield of PL is determined by the partition coefficient of the protein–ligand complex (K_{PL}), the dissociation constant in the top and bottom phase (K_{DT}, K_{DB}) and the ligand concentration [L]. A ligand with a high K_L value will selectively shift the protein–ligand complex to the top phase. High K_L values can be ensured by covalent binding to PEG.

Affinity liquid–liquid partition chromatography of formate dehydrogenase from Candida boidinii

As an example, the interaction of formate dehydrogenase (FDH) with Procion Red HE3b was studied [16]. Fig. 9 shows that increasing the ligand concentration in the mobile phase rapidly decreases the elution volume of FDH. Higher ligand concentrations cause only a minor further decrease of the elution volume as the binding sites of the enzyme are saturated and V_{el} is approaching the void volume for large K.



[P] = Conc. of Protein
[L] = Conc. of Ligand
[PL] = Conc. of Protein/Ligand Complex
k_{DT} = Equilibrium Constant of the Complex in the Top–Phase
k_{DB} = Equilibrium Constant of the Complex in the Bottom–Phase
K_P = Partition Coefficient of the Protein
K_L = Partition Coefficient of the Ligand
K_{PL} = Partition Coefficient of the Complex

Fig. 8. Linked equilibria in affinity partitioning.

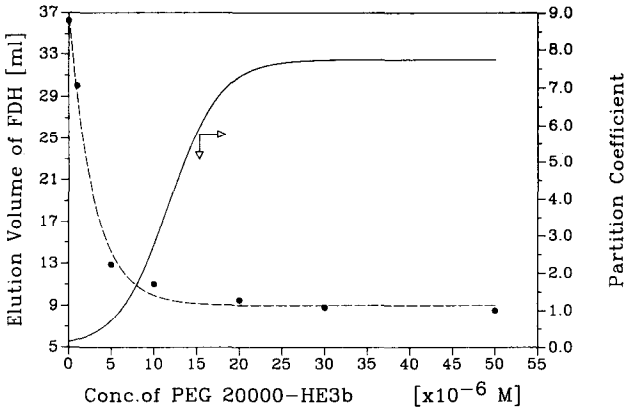


Fig. 9. Elution volume of formate dehydrogenase as a function of ligand concentration (PEG 20 000-HE3b) in the mobile phase. Support material, LiParGel 750; phase system, 4.5% PEG 20 000–6.9% Dx 500; buffer, 5 m*M* ammonium sulphate in 6.3 m*M* potassium phosphate (pH 7.0); column dimensions, 30 × 1 cm.

With $5 \cdot 10^{-5}$ *M* PEG–Procion Red HE3b in the mobile phase, FDH from a heat conditioned crude extract (10 min at 55°C to denature other dehydrogenases, 2.5 mg/ml protein) could be purified by a factor of 5.8 with 83% activity yield. The concentration of ligand required for purification is lowered by about two orders of magnitude compared to affinity partitioning of FDH ion batch systems [19].

Affinity liquid–liquid partition chromatography of monoclonal antibody

If the antigen (POD, $K = 1.0$) and the mAb ($K = 0.2$) prefer different phases in a PEG–Dx aqueous two-phase system, the characteristic separation profile of the LLPC column (Fig. 1) can be utilized to shift the elution position of mAb with the antigen as the affinity ligand, without prior PEG modification of the ligand. Different concentration ratios of ligand/mAb were prepared for incubation and were subsequently injected onto a small LLPC column (30 × 0.5 cm) with or without antigen as a ligand in the mobile phase ($V_s = 2.09$ ml, $V_m = 2.42$ ml). Fig. 10 demonstrates that the elution volume of the mAb can be decreased from 12 ml down to 9.0 ml (increase of K from 0.22 to 0.32). Owing to the dissociation of the mAb–antigen complex (1:1 complex), at least a ten fold higher ligand concentration is needed in the absence of ligand in the mobile phase (Fig. 10a) to reach the same K values shown in Fig. 10b. When antigen is present in the mobile phase the maximum decrease of the elution volume is reached at POD/mAb molar concentration ratios where the mAb concentration is even higher than the ligand concentration.

The partition equilibria in affinity partitioning (Fig. 8) are reached very fast. The dissociation constants of the immunocomplex are the limiting factors for separation assuming that they are equal in the top and bottom phases. With the ligand in the mobile phase (Fig. 10b), dissociation is depressed. Owing to the mode of separation, that is a mobile PEG-rich phase moving along a stationary Dx-rich phase with equal concentrations of ligand ($K_{POD} = 1.0$), a decrease of 2 ml in the elution volume is reached at molar concentration ratios of ligand/mAb < 1.

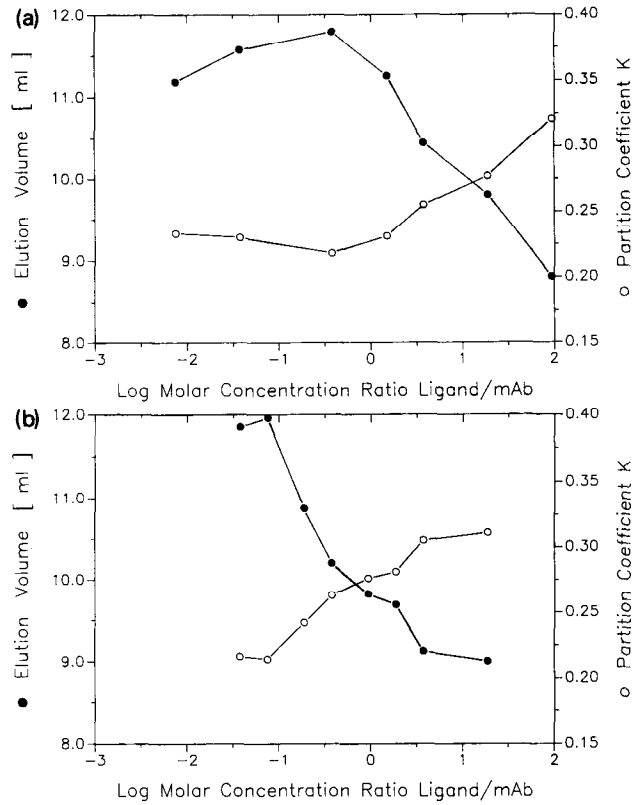


Fig. 10. Elution volume and partition coefficient K of monoclonal antibody as a function of the log of the molar concentration ratio of ligand (horseradish peroxidase) and mAb. (a) Without ligand in the mobile phase; the ligand was only present in the incubation mixture prior to separation. (b) With ligand in the mobile phase. Column: LiChrospher Diol 1000/10 (30 × 0.5 cm); phase system, 5.4% PEG 6000–9.0% Dx 70 in 50 mM potassium phosphate buffer (pH 7.4) with 150 mM sodium chloride; V_m = 2.42 ml; V_s = 2.09 ml.

CONCLUSIONS

In conclusion, affinity LLPC is a versatile technique for the purification of proteins. As a result of the high sensitivity of LLPC to small changes of the partition coefficient, affinity based purifications can be achieved as demonstrated for formate dehydrogenase at relatively low concentrations of PEG-bound triazine dye. This sensitivity can also be exploited when the unmodified ligand has a partition coefficient sufficiently different from the molecule (*e.g.* POD–mAb). In addition, size exclusion effects shown with LiParGel 750 (Fig. 1) may be utilized to improve the separation of free antigen and the corresponding immunocomplex. As the interaction of ligand and target molecule takes place in a homogeneous phase involving only liquid–liquid equilibria, but no adsorption or binding on the solid matrix, the utilisation of ligands is much improved compared to affinity chromatography and activity yields are expected to be high.

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