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Review

Polymer ligands for mild hydrophobic interaction chromatography —principles, achievements and future trends

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ABSTRACT

Mildly hydrophobic polymers, such as those generally used in the "partition between aqueous two-phase systems" technique, can be immobilized on inert supports to afford stationary phases which are less denaturing towards biomolecules than those involved in traditional hydrophobic chromatography. Various aspects of this technique are reviewed. Examples of successful fractionations are presented to illustrate its principles, and the main trends for future studies in this field are discussed.

CONTENTS

1. INTRODUCTION

Since the initial work of Er-el *et al.* [1], hydrophobic interaction chromatography (HIC) on agarose gels has become an important separation method [2–6]. Hydrophobic ligands, such as *n*-alkyl or phenyl groups, are chemically attached to the agarose matrix and the separations are based on differences in the surface hydrophobicity of proteins [7]. However, strong hydrophobic interactions sometimes result in almost irreversible adsorption [3,6,8] or denaturation during the elution with often harsh mobile phase conditions (*e.g.*, low pH, chaotropic agents, detergents, organic solvents, etc.).

Therefore, it can be envisaged that ligands with intermediate hydrophobic character would be of interest, as they would provide an adequate binding strength

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without the above drawbacks. "Partition between aqueous two-phase systems", a separation technique extensively described by Albertsson [9], exploits these mild hydrophobic interactions. The systems are composed of either two polymers, or a polymer and a salt. Poly(vinyl alcohol)–dextran, polyethylene glycol–dextran and polyethylene glycol-$MgSO_4$ are among the most widely used biphasic systems. However, although many successful purification experiments have been reported, it is clear that the high viscosity of the solutions affords one likely explanation, among others, for its limited practical use. Recent improvements might, however, result in some revival of this technique in the near future [10,11].

Its seems logical that the principles of the partition technique could be transposed to a chromatographic procedure if one of these polymers were immobilized on the chromatographic support. This idea is not totally new, as Morris [12] and more recently Anker [13] exploited this concept many years ago. However, probably owing to the difficulties caused by the fact that the aqueous polymer phase was merely soaked up by the support beads, the idea received no further attention for about 10 years.

It was only in the early 1980s that different groups revived this concept [14–19] and started to immobilize covalently various mildly hydrophobic polymers on chromatographic supports, to produce stationary phases less denaturing towards biomolecules than those used in traditional HIC.

This paper is intended to review our own work in this field over the past few years [20–24]. The synthesis and physico-chemical characterization of the stationary phases, fractionation achievements and future trends will be discussed.

2. STATIONARY PHASES: SYNTHESIS AND PHYSICO-CHEMICAL CHARACTERIZATION

Water-soluble polymers, such as those involved in aqueous two-phase systems, are generally regarded as hydrophilic species, obviously because of their solubility in this solvent. However, a closer look at thermodynamics-related parameters, such as surface tension, reveals the relative nature of the hydrophilicity–hydrophobicity concept. The surface tension of polyethylene glycols (PEGs) for instance, in the range $\gamma_{sv} \approx 42$–46 mJ/m^2 [25], is situated mid-way between those of water ($\gamma_{sv} \approx 70$ mJ/m^2) and of low-energy materials such as C_6–C_{16} alkanes ($\gamma_{sv} \approx 18$–28 mJ/m^2) [26]. PEGs are slightly more hydrophobic than various other water-soluble polymers such as poly(vinyl alcohol) (PVA), polyvinylpyrrolidone (PVP), polyacrylamide or dextran [27], but less hydrophobic than, for instance, polystyrene ($\gamma_{sv} \approx 32$ mJ/m^2) [28] and *a fortiori* than the C_6–C_{16} alkanes, which are the most commonly used ligands in traditional HIC.

From this point of view, it is clear that PEGs, and also the other polymers used in the partition technique, and which all fall within a narrow range on the hydrophobicity scale, may definitely be considered as hydrophobic materials. Conversely, when compared with alkyl chains, their designation as "mild hydrophobic ligands" [15] is perfectly justifiable (Fig. 1).

As many other groups, we selected the polyethers as ligands for mild hydrophobic chromatography studies. PVA is the only other polymer, used in a few instances in this field [15,19].

The polyether class includes the following: polyethylene glycols (PEGs), $HO(CH_2CH_2O)_nH$; polypropylene glycols (PPGs), $HO[CH_2(CH_3)CHO]_nH$; poly-

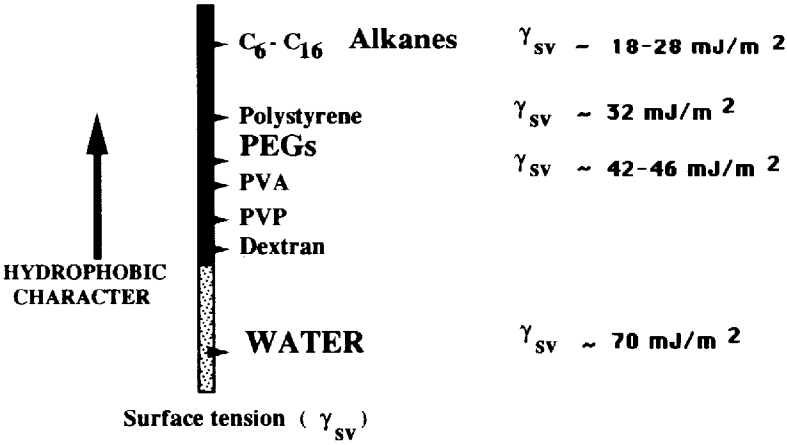

Fig. 1. Schematic hydrophobicity scale based on surface tension of various chemicals.

tetramethylene glycols (PTMGs), $HO(CH_2CH_2CH_2CH_2O)_nH$; and various PEG–PPG copolymers which are also commercially available as Pluronics (Serva) or Jeffamines (Texaco).

The hydrophobic character of these polymers increases with increasing number of carbon atoms (2–4) in the repeating unit: PEGs < PEG–PPG copolymers < PPGs < PTMGs. Further, as all these polymers are available with a large range of molecular masses, the polyethers thus form a family of wide potential.

Covalent immobilization of polyethers on Sepharose requires a preliminary activation of either the matrix or the polymer. As we are dealing with mild hydrophobic interactions, much care should obviously be taken not to introduce strongly interacting spacer arms on the gels, as they would lead to erroneous interpretations of the phenomena observed.

This was effectively experienced [20,21] when performing the activation reaction with certain commonly used bisepoxides such as 1,4-butanediol diglycidyl ether or ethylene glycol diglycidyl ether (Fig. 2). In fact, these activation groups remained in large amounts on the gel after the coupling step and, owing to the presence of $(CH_2)_n$ units in their structure ($n = 2$ or 4), acted as hydrophobic ligands, to afford strongly adsorbing stationary phases, even in the absence of any immobilized polyether. No

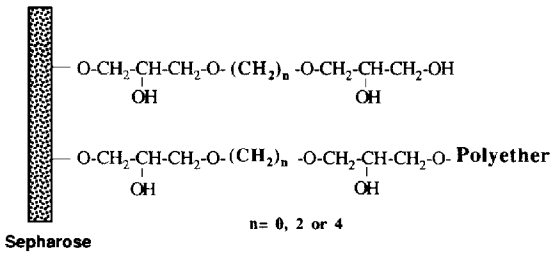

Fig. 2. Schematic representation of the stationary phases obtained by coupling a polyether to Sepharose previously activated by various bisepoxides.

such undesirable side-effect was observed when (±)-1,3-butadiene diepoxide was used ($n = 0$) (Fig. 2).

Unambiguous immobilization of polyethers on chromatographic supports, by means of spacer arms not liable to interfere in the hydrophobic interaction, can be obtained using carbonyldiimidazole (CDI) or *p*-nitrophenyl chloroformate as activation reagents. However, the reaction of the matrix thus activated, with polyethers, directly via their terminal hydroxyl groups, would result in both instances in carbonate spacer arms which are sensitive to hydrolysis. More stable immobilization is afforded by the urethane group, formed when the coupling of the activated matrix is carried out with the polyethers, after prior transformation into their corresponding amino derivatives [29].

3. QUANTIFICATION OF IMMOBILIZED POLYETHERS

Obviously, the quantification of polyethers immobilized on inorganic supports such as siliceous materials presents no major difficulties, as their high content of carbon atoms allows their direct measurement by elemental analysis. Conversely, with polysaccharide supports such as agarose, no such distinctive feature can be exploited, and one must therefore turn to more sophisticated methods.

The agarose thermoreversible gel results from the rigid helical conformation adopted by the molecule when the solution conditions (temperature, ionic strength, solvent, etc.) are no longer favourable to the coil state [30]. A direct non-destructive NMR analysis in solvents [dimethyl sulphoxide (DMSO) or hot water] preventing the formation of the ordered conformation may, therefore, be considered, provided that immobilized polyethers contain distinctive CH_3 or CH_2 groups, resulting in signals sufficiently resolved from the peaks corresponding to the saccharide backbone. All polyethers, except PEGs, fullfil this condition.

This approach affords perfect calibration graphs when artificial mixtures of Sepharose and various amounts of polyethers are analysed. Unfortunately, all the Sepharose–polyether stationary phases obtained are no longer soluble, either in hot water or in DMSO. This phenomenon can be explained either by a profound modification of the physico-chemical properties of the Sepharose (water solubility cannot be re-established even on addition of strong aggregation-disrupting agents such as urea or NaSCN [31]) or by a cross-linking phenomenon, due to the bifunctional character of the polyethers and despite their large excess used during the coupling step. The synthesis of a stationary phase starting from strictly monofunctional polyethers (such as monomethoxy PEGs) would allow one to distinguish between these two hypotheses. However, the commercially available "monofunctional compounds" have been proved to be contaminated by various amounts of difunctional species, sometimes as high as 20% [32].

As ether bonds are sensitive to acidic cleavage, many degradation techniques can be considered. We attempted to hydrolyse our stationary phases with formic or hydrochloric acid treatment and subsequently analyse the resulting mixture by ^1H or ^{13}C NMR, according to the procedure described by Shibusawa *et al.* [18]. The construction of a calibration graph is a prerequisite for this approach. It permits a comparison of the integral of the distinctive CH_3 (or CH_2) signal of the polyether in the hydrolysed stationary phase spectra with those obtained with artificial mixtures

containing Sepharose and known amounts of polyether. In our own experience, reproducible linear correlations are difficult to obtain for the calibration graph itself. This unreliability is due, in our opinion, to the experimental conditions under which the crude hydrolysis mixture is prepared for the NMR sample. It seems likely that some of the hydrolysis fragments are sufficiently volatile to be eliminated along with the solvent during its removal at 70°C under reduced pressure. Clearly, a far better approach consists in performing the hydrolysis experiment in dilute $^2H_2SO_4$, according to the procedure described by Andrade [33]. In fact, in this instance, the hydrolysis mixture can be directly analysed by NMR, without further work-up liable to produce undesirable effects.

In order to determine the PEG content of Sepharose–PEG samples, we tested another method, based on degradation by boron tribromide (BBr_3), proposed by Drevin and Johansson [34]. The cleavage fragments (*e.g.*, for PEGs, 2-bromoethanol and 1,2-dibromoethane) are subsequently analysed by gas chromatography (GC). Satisfactory results were reproducibly obtained with our own Sepharose–PEG samples with this method. Its ability to afford reliable results with more complex mixtures resulting from BBr_3 cleavage of PPGs (three types of fragments) or PEG PPG copolymers (five types of fragments) is currently under investigation.

4. FRACTIONATION ACHIEVEMENTS

Up to now, most of the work devoted to mild HIC by other groups has been carried out on standard protein mixtures or simple extracts, and has been mainly intended for physico-chemical or thermodynamic investigations. Obviously, this approach is of great value for a better understanding of the principles governing this new technique.

We started with a different strategy, attempting to investigate the ability of this non-specific technique to cope with actual fractionation problems with complex mixtures, and to afford high purification ratios together with a high recovery of enzymatic activities. Fractionation challenges concerning enzymes acting on hydrophobic substrates were selected in the steroid and lipid fields.

A crude extract of *Pseudomonas testosteroni*, containing $\Delta_{5\to4}$ 3-oxosteroid isomerase (isomerase), was chromatographed on a Sepharose–PEG stationary phase with various concentrations of dextran T70 in the mobile phase. The chromatographic behaviour of this enzyme, and that of contaminants, may be correlated with batch partition studies in PEG–dextran two-phase systems. Earlier experiments [35] showed that contaminants favoured the dextran-rich bottom phase (partition coefficient $K_{\text{contaminants}} \approx 0.4$), whereas isomerase established preferential interactions with the PEG-rich top phase ($K_{\text{isomrase}} \approx 4$). The chromatographic elution profile was in agreement with this partition result.

An increased dextran concentration in the mobile phase resulted in an enhanced separation of the peaks corresponding to isomerase and contaminants. Complete resolution could even be obtained with high concentrations of dextran in the mobile phase (Fig. 3).

Solutions of potassium phosphate [20], a salt known to produce two-phase systems with PEG, may advantageously be used as the mobile phase instead of the viscous dextran solutions. In this instance, total retention of isomerase was obtained

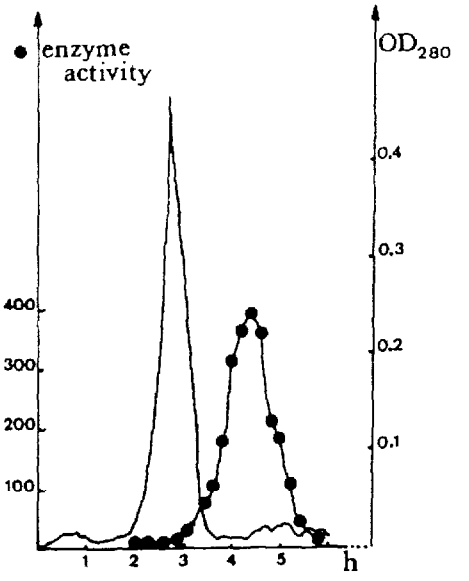

Fig. 3. Extraction of isomerase from a *Pseudomonas testosteroni* crude extract on Sepharose–PEG, with 12% (w/v) dextran T70 in the mobile phase. See ref. 20 for more detailed experimental conditions.

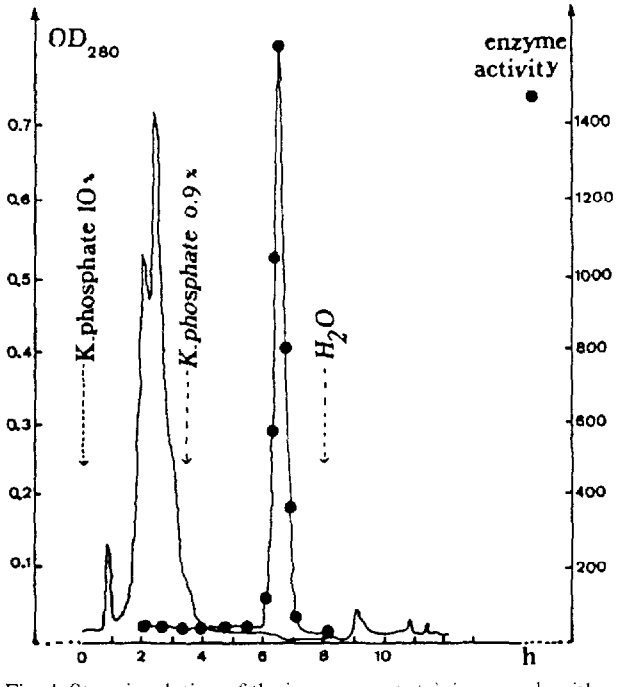

Fig. 4. Stepwise elution of the isomerase-containing sample with various potassium phosphate concentrations on Sepharose–PEG stationary phase. See ref. 20 for more detailed experimental conditions.

(potassium phosphate *ca.* 10%), whereas this enzyme was only retarded in the previous experiment with dextran. Stepwise elution with a decreased potassium phosphate concentration (*ca.* 1%) resulted in the recovery of isomerase in good yield (*ca.* 60%) and the removal of most of contaminants (*ca.* 7 μg compared with *ca.* 5 mg in the injected sample) (Fig. 4).

Similar purification results were obtained for an esterase from *Bacillus pumilus* (86% yield, 140-fold purification) on a Sepharose–Pluronic stationary phase [22].

Further, more sophisticated fractionations were attempted on Sepharose–PEG and Sepharose–Pluronic with three different enzymes [isomerase, 3α-hydroxysteroid dehydrogenase (α-HSD) and 3β,17β-hydroxysteroid dehydrogenase (β-HSD)] contained in the same *Pseudomonas testosteroni* crude extract. According to the hydrophobicity scale proposed by Bigelow [36], the purification of these three enzymes represents a real challenge [37].

Their chromatographic behaviours were predicted using "partition between aqueous two-phase systems" experiments [24]. These two-phase systems were prepared by mixing potassium phosphate (final concentration 10, 15 or 20%, w/w) with PEG and Pluronic mixtures of various compositions. The resulting systems are thus composed of a phosphate-rich bottom phase and a polyether-rich top phase, of increasing hydrophobicity with its Pluronic content. The partition coefficient of any considered enzyme therefore gives a direct estimation of its ability to establish preferential interactions with the polyethers. Consequently, when chromatography is carried out using Sepharose–PEG or Sepharose–Pluronic stationary phases and a potassium phosphate mobile phase, high partition coefficients ($K > 1$) should correspond to retention or retardation. Conversely, biomolecules with low partition coefficients ($K < 1$) favour the phosphate-rich bottom phase and should be eluted with no retardation.

The main information obtained from batch partition analysis was as follows: (1) $K_{\text{isomerase}} > K_{\beta\text{-HSD}} \gg K_{\alpha\text{-HSD}}$ in all experiments; (2) the possibility of shifting the partition coefficient of α-HSD above 1 by increasing the phosphate concentration or (and) the hydrophobic character (*i.e.*, the Pluronic content) of the top phase; and (3) enhanced ΔK ($K_{\text{isomerase}} - K_{\beta\text{-HSD}}$) as the hydrophobic character of the polyether-rich top phase is increased. This perfectly paralleled the chromatographic experiments, *i.e.*, (1) elution in the order α-HSD, β-HSD, then isomerase; (2) retention or not of α-HSD, according to the ionic strength in the mobile phase and the nature of the stationary phase (PEG or Pluronic); and (3) better separation of β-HSD from isomerase on Sepharose–Pluronic than on Sepharose–PEG.

This preliminary information allowed us to develop a strategy for optimum chromatographic resolution (Fig. 5). Good enzymatic activity recovery (α-HSD, 60%; β-HSD, 40%; isomerase, 65%) together with almost quantitative removal of contaminants in each fraction could thus be obtained (residual contaminants expressed as a percentage of total protein in the starting crude extract: α-HSD, 1.5%; β-HSD, 2%; isomerase, not detectable).

5. FUTURE TRENDS

As mild HIC has already proved to be very efficient for sophisticated fractionation problems with complex mixtures, our future concern in this field ought

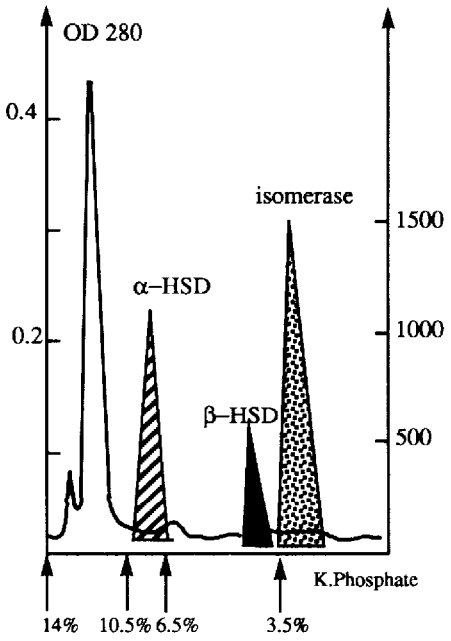

Fig. 5. Stepwise elution of the *Pseudomonas testosteroni* crude extract on Sepharose–Pluronic with various potassium phosphate concentrations. See ref. 24 for more detailed experimental conditions.

to turn to physico-chemical characterizations, rather than to additional biological studies. However, other polyethers such as PPGs or PTMGs have been immobilized on Sepharose and the resulting stationary phases should be tested, in order to obtain a complete study through the whole polyether family.

Further, the determination of immobilized polymers remains an important question, as the ligand concentration on the gel plays a prominent role in the interaction process. The solutions already exist but, in our opinion, should be more thoroughly investigated.

Ultimately, it would be of interest to quantify the hydrophobic character of the stationary phases. If the hydrophobicity of the polyethers is an important parameter, the interaction of the solute with the stationary phase is probably a global process, in which not only the nature of the immobilized polyether, but also the support itself and the amount of hydrophobic ligands, also play a role.

Surface tension is one of the most indicative measure of this hydrophobic character. Whereas the measurement of surface tension of liquids in straightforward, that of solid surface and especially of beads is far more complicated. However, certain methods already exist, such as contact angle measurements [38], reverse GC [39] and the sedimentation volume technique [40,41], and we are currently working on their application to the problems posed by our stationary phases.

This determination of the surface tension of the stationary phase (γ_{sv}) could be the first step in characterizing, in terms of thermodynamics, a three-component (sorbent–mobile phase–protein) system. In fact, the adsorption of a protein on

a sorbent being comparable to an adhesion process, the free energy of association may be given by the expression [42]

$$\Delta F_{smp} = \gamma_{sp} - \gamma_{sm} - \gamma_{mp}$$

where γ is the interfacial tension and the subscripts s, m and p represent sorbent, mobile phase and protein, respectively. All these interfacial tensions may be calculated through an equation of state [43] from the measurable individual surface tensions of the stationary phase (γ_{sv}), the protein (γ_{pv}) and the mobile phase (γ_{mv}). This makes possible explicit thermodynamic predictions of the relative extent of adhesion of the considered biomolecule (p) to a given sorbent (s), under various mobile phase (m) conditions.

In addition, as the measurement of chromatographic parameters such as retention volumes and capacity factors also leads to the determination of the association constant or the free energy of association [19,44], a second approach is therefore available for characterizing the three-component system in terms of thermodynamics.

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