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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MACROMOLECULES ON AGAROSE AND ITS DERIVATIVES

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## SUMMARY

The porosity of agarose gels decreases as the concentration of agarose increases. However, even gels of agarose concentrations as high as 15% are permeable to macromolecules such as proteins. Spheres prepared from such high-concentration agarose gels are very rigid. Therefore, columns packed with these gel spheres permit relatively high flow-rates (particularly after cross-linking) even if the diameter of the spheres is made small in order to increase the resolution. Due to the high porosity of the gel spheres they can be used with advantage for molecular sieving of high-molecular-weight substances, for instance, biopolymers, and as high-capacity matrixes for the fractionation of macromolecules by ion-exchange, affinity and hydrophobic interaction chromatography. The cost of preparing these high-concentration (cross-linked) agarose gel spheres is comparatively low, which is of particular importance when used in preparative high- or intermediate-performance liquid chromatography. The diameter of the gel spheres has been varied from 2 to 100 $\mu$m.

## INTRODUCTION

Twenty years ago Hjertén and co-workers introduced agarose gels into the fields of electrophoresis[1], immunoelectrophoresis[2] and chromatography[3]. Since then these gels have been widely used in these areas and the advantages over many other gel materials are well established and utilized. However, one property of agarose which renders it very suitable as chromatographic bed material has not been fully recognized, namely its unique ability to "give gels which are only slightly compressible. Therefore, relatively small gel spheres can be used without leading to low flow-rates. This is of great importance, as a decrease in the diameter of the gel spheres has the effect of increasing the resolution". This was written in 1966[4], long before the term high-performance liquid chromatography (HPLC) was coined, but very few seem to have observed it. The same paper included a molecular-sieve chromatography experiment which showed that agarose gels of concentrations even as high as 10% have a sufficiently open structure to afford a good fractionation of serum proteins. Such porous high-concentration gels are very rigid and therefore permit high flow-rates, even when the diameter of the gel spheres is small. Since these unique properties of agarose gels have not been generally recognized it seems justifiable to

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direct attention toward them by further examples and to discuss the great potentialities of porous high-concentration (cross-linked) agarose gels for different chromatographic methods. An obvious application of agarose gels is preparative high- (or intermediate-)performance liquid chromatography of macromolecules in both aqueous buffers and organic solvents.

## MATERIALS AND METHODS

### *Preparation of agarose*

When there is no need for a high-quality agarose we use method I b in ref. 5 for the preparation of agarose, since this is a very simple method involving only the washing of agar with phosphate buffer to remove most of the agaropectin. To avoid possible electrostatic interactions between proteins and gel spheres prepared from this agarose, relatively high salt concentrations should be used in the eluents. When a higher quality is required, the extraction of agaropectin with phosphate buffer is followed by an adsorption of agaropectin to a DEAE ion exchanger (method III b in ref. 5).

### *Preparation of agarose gel spheres*

Preparation of agarose gel spheres followed a suspension-gelation procedure described previously[6]. A narrow size distribution of the gel spheres was obtained by elutriation in water.

### *Column tubes*

Column tubes were made from Plexiglass with a metal frit at the bottom or a porous polyethylene disk covered with a low porosity filter paper or a stainless-steel mesh sheet.

### *Packing procedure*

A gel slurry was poured into the column tube and the flow-rate was adjusted with a peristaltic or high-pressure pump so that a sharp ascending boundary was formed between the gel bed and the slurry (increasing the pressure above a certain value did not give a higher flow-rate since the bed then became compressed).

### *Chromatographic equipment*

A Varian 5000 liquid chromatograph was used for fractionations by hydrophobic interaction chromatography. For the ion-exchange and the molecular-sieve chromatography runs an ordinary peristaltic pump afforded the flow-rates required. The effluent was monitored by absorption or fluorescence measurements with a Spectroflow Monitor SF 770 and an FS 970 fluorometer, respectively, from Schoeffel (Westwood, NJ, U.S.A.); 280 nm was selected as the wavelength for absorption and excitation.

## EXPERIMENTS AND RESULTS

### *Molecular-sieve chromatography*

The porosity of a 12% agarose gel is still so high that it can be used for the

fractionation of very-high-molecular-weight proteins. This is evident from the calibration diagram[7] in Fig. 1.

Fractionation of a crude extract of phycoerythrin and phycocyanin by molecular-sieve chromatography on a column of 12% agarose is shown in Fig. 2.

We have also successfully separated monomers, dimers and trimers of albumin on a 340 × 6 mm I.D. column, packed with 2–10 μm beads of cross-linked 12% agarose (flow-rate: 0.12 ml/min); the result was similar to that obtained with a TSK 3000SW column.

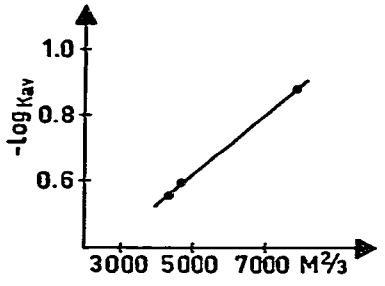


Fig. 1. A plot of $-\log K_{av}$ against $M^{2/3}$ for a 12% agarose column[7]. $K_{av}$ = distribution coefficient; $M$ = molecular weight of the proteins: thyroglobulin (700,000), ferritin (450,000) and phycoerythrin (290,000). Column dimensions: 400 × 3 mm I.D. Flow-rate: 0.02 ml/min. Diameter of the gel beads: 20–40 μm.

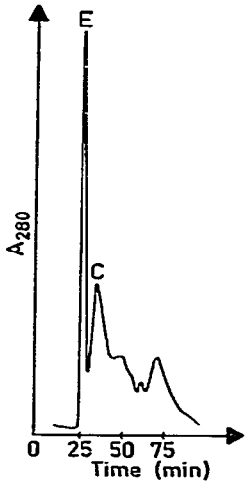


Fig. 2. Molecular-sieve chromatography of a crude extract of phycoerythrin (E; molecular weight: 290,000) and phycocyanin (C; molecular weight: 135,000) on a 12% agarose column. Column dimensions: 380 × 6 mm I.D. Flow-rate: 0.2 ml/min. Diameter of the gel beads: 90 μm. A similar chromatogram was obtained on a 140 × 6 mm I.D. column, packed with 10–20 μm cross-linked 12% agarose beads.

*Hydrophobic interaction chromatography*

About 10 μl of undialyzed normal human serum was applied on a 400 × 6 mm I.D. column packed with 12% agarose beads (about 100 μm in size) to which octyl groups had been coupled (Fig. 3). The coupling procedure was different from that

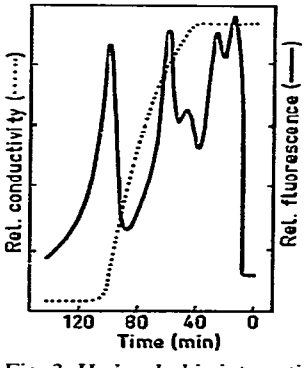


Fig. 3. Hydrophobic interaction chromatograpny of human serum. Bed material: 12% agarose. Ligand: octyl groups. Flow-rate: 0.5 ml/min. Diameter of the gel beads: 100 μm. For details, see text.

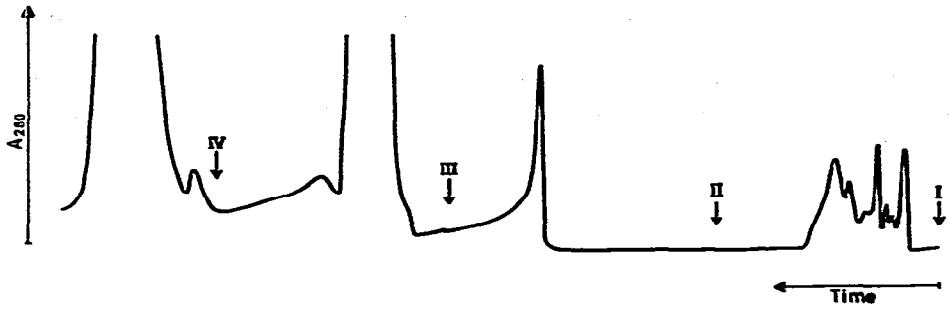


Fig. 4. Ion-exchange chromatography of serum on a 9% agar column. Column dimensions: 400 × 6 mm I.D. Flow-rate: 0.3 ml/min. Diameter of the gel beads: 80 μm.

described in ref. 8 and will be published elsewhere. The column was equilibrated with 0.02 *M* sodium phosphate buffer, pH 6.8, containing 1.3 *M* $(NH_4)_2SO_4$, and eluted for 10 min with the same buffer at a flow-rate of 0.5 ml/min. A linear gradient formed by this buffer and 0.02 *M* sodium phosphate, pH 6.8, was then used for elution during the next 60 min. (Since small ions such as $NH_4^+$ and $SO_4^{2-}$ are retarded on the agarose column due to its molecular-sieving properties, the adsorbed material that first elutes from the column emerges at the total bed volume.) For further details see ref. 9.

*Ion-exchange chromatography*

The sample consisted of 0.5 ml of normal human serum, dialyzed against 0.01 *M* sodium acetate, pH 5.5 (buffer I). The same buffer was used for equilibration of the column, which was made up of beads of a 9% agar gel. In this experiment (Fig. 4) we utilized the cation-exchange properties of the agar (the agaropectin in native agar contains sulphate (and carboxylic) groups, see ref. 10). The elution was performed with buffer I, buffer II (0.01 *M* sodium phosphate buffer, pH 6.0), buffer III (0.01 *M* sodium phosphate, pH 7.0) and buffer IV (0.01 *M* glycine–NaOH, pH 9.8). Observe the many narrow peaks eluted with the starting buffer and the high capacity of the column (about 25 mg of protein was applied).

TABLE I

THE FLOW-RATE AS A FUNCTION OF AGAROSE CONCENTRATION AND DIAMETER OF THE GEL SPHERES

Column dimensions: 100 × 6 mm I.D.

| *Agarose concentration (%)* | *Diameter of the spheres (μm)* | *Flow-rate (ml/min)* | *Pressure (atm)* |
|---|---|---|---|
| 9 | 50 | 0.1 | 1 |
| 12 | 20–40 | 0.1 | 4 |
| 12 | 30–50 | 0.1 | <1 |
| 12 | 30–50 | 0.2 | <1 |
| 12 | 30–50 | 0.3 | <1 |
| 12 | 30–50 | 0.4 | 1 |
| 12 (cross-linked) | 30–50 | >10 | 10 |
| 12 (cross-linked) | 10–20 | 1 | 6 |
| 12 (cross-linked) | 2–10 | 0.6 | 10 |

*Flow-rate*

The maximum flow-rates vary with the agarose concentration and the diameter of the gel particles. Some values are given in Table I. As shown, higher flow-rates can be obtained by cross-linking[11].

## DISCUSSION

It has been pointed out that gels of dextran, polyacrylamide and agarose are too soft to be used in HPLC. As stressed in the Introduction, agarose gels are considerably more rigid than the other two gel materials, which is further substantiated by the experiments presented in this paper. If one wants still more rigid gels, the agarose can easily be cross-linked[11] (see Table I).

Silica beds have the advantage that they can afford higher flow-rates than beds of agarose gels at a given diameter of the bed material. The high flow-rates obtainable cannot, however, be utilized for the fractionation of biopolymers, owing to their slow diffusion rates, if optimum resolving power is desired. For instance, in the brochures of the commercial TSK SW columns (600 × 7 mm) a flow-rate of about 50 μl/min is recommended, although by increasing the pressure these columns can easily be operated at 2 ml/min (50 μl/min corresponds to elution times from 5 to 40 h; see brochures from LKB, Stockholm, Sweden).

In an excellent review of HPLC Regnier and Gooding[12] state, in a section on future trends, "The growth of protein fractionation by HPLC may be expected to be very rapid in the next 5 years. Several factors that will influence the growth of this technique are the cost of preparative columns, the existence of alternative analytical methodology and the ease of automation. For example, the current cost of analytical HPLC columns (US$ 300–400) is expensive but tolerable. However, most protein fractionations carried out today are with columns of 1 to 4 cm in diameter where the separation is preparative in nature. The cost of columns of this diameter packed with 10-μm high-performance support would range from US$ 2000 to 4000 apiece. It is questionable whether many laboratories can justify expenditures of this magnitude. For this reason, we believe that a new generation of intermediate or medium-performance liquid chromatography (mplc) supports must be developed. These materials will be of larger particle size (37–74 μm) and easier to pack. Although separation times will be in the range of 30 min to 3 h, their substantially lower cost and ease of packing will make them more cost effective." We believe that agarose gels of a high concentration (and cross-linked) will meet many of these requirements. These gels can often be operated at low pressures. Simple and relatively inexpensive pumps can therefore be employed.

Advantages of agarose in comparison with silica are its stability at pH values above 8 and the ease with which it can be derivatized with different ligands to prepare gels suitable also for ion-exchange, hydrophobic interaction and affinity chromatography. The disturbing non-specific adsorption (caused, for instance, by electrostatic and hydrophobic interactions[14]) that is characteristic of silica spheres is negligible in most experiments with agarose gels. Due to their more open structure these gels have a higher capacity and higher exclusion limits, the TSK SW gels being exceptions[13]. Furthermore, when silica spheres derivatized with alkyl groups (for instance octyl or octadecyl) are used for reversed-phase chromatography of proteins one is forced to

use organic solvents for desorption (and often low pH), *i.e.*, conditions which may cause denaturation of proteins[15]. When similar experiments are performed on agarose columns (hydrophobic interaction chromatography, see ref. 16) ordinary non-denaturing aqueous buffers can be employed.

Agarose gels are swellable also in organic solvents and should therefore be useful also for the separation of hydrophobic substances (high-performance gel permeation chromatography).

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