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PREPARATION OF SILICA–AGAROSE BEADS FOR GEL CHROMATOGRAPHY

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SUMMARY

When agarose is mixed with colloidal silica, beads containing a large proportion of agarose and a minor amount of silica are formed. The beads are stabilized and remain spherical by the addition of polyethylene glycol and polyvinylpyrrolidone. They can be used for gel chromatography of proteins and polysaccharides.

INTRODUCTION

Agarose gels have been used widely for gel chromatography of proteins and other high molecular weight materials as well as matrices for affinity chromatography. The preparation of spherical gel beads usually includes dispersion of the agarose solution in an organic solvent[1–3]. We have found that spherical beads can be formed in aqueous solution by mixing the agarose with colloidal silica or aluminium oxide. Stabilization of the beads is effected by the addition of polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP).

The silica–agarose beads can be used for gel chromatography. High flow-rates are attainable owing to their mechanical stability also at high hydrostatic pressures.

MATERIALS AND METHODS

Materials

Agarose (ash content 3.9%) was kindly supplied by Pharmacia (Uppsala, Sweden). The silica sol (Ludox) was obtained from DuPont (Wilmington, Del., U.S.A.). The samples used were Ludox SM [36% (w/v), specific surface area 375–420 m^2/g SiO_2, average particle size 7–8 nm, pH 9.9]; Ludox HS [52% (w/v), 210–230 m^2/g, 13–14 nm, pH 9.7]; and Ludox TM [70% (w/v), 125–140 m^2/g, 22–25 nm, pH 8.9]. Aluminium oxide (Al_2O_3) was obtained from Merck (Darmstadt, G.F.R.). Polyethylene glycol (PEG) of MW 4000, 6000 and 20,000 daltons was obtained from Union Carbide (New York, N.Y., U.S.A.) and of MW 100,000 daltons (polyethylene oxide) from BDH (Poole, Great Britain). Polyvinylpyrrolidone (PVP) of MW 40,000 daltons was obtained from A. H. Thomas (Philadelphia, Pa., U.S.A.).

Proteins of MW 10^4–10^6 daltons were obtained from Kabi (Stockholm, Sweden), Miles-Seravac (Lausanne, Switzerland) and Sigma (St. Louis, Mo., U.S.A.); the last-named also supplied DNP-alanine. Dextran and Blue dextran, both of MW 2×10^6 daltons were obtained from Pharmacia. Well characterized glycosaminoglycan fractions were kindly supplied by Drs. Å. Wasteson and P.-H. Iverius of this Institute.

Preparation of silica–agarose beads

A colloidal solution of silica in water (300 ml) was added to 100 ml of boiling water containing 3 g of agarose. The temperature of the mixture fell to about 50° and it was then heated to 85° over a period of 7–8 min, during which time beads were formed; 5 ml of 50% (w/v) PEG (MW 4000 daltons) were added, and the mixture was allowed to cool to room temperature before adding 10 ml of 20% (w/v) PVP. A magnetic stirrer was used throughout the entire procedure. The mixture was allowed to stand without stirring for 30 min so as to allow phase separation between the silica–agarose beads and the remaining silica solution. The beads in the upper phase were washed several times with water in order to remove extraneous silica. Extreme sizes of the beads (about 10%) were removed according to the method of Vassilou and Kunin[4].

Preparation of aluminium oxide–agarose beads

Agarose (1.5 g) was dissolved in 100 ml of boiling water and 0.5 g of aluminium oxide powder and 10 ml of 30% (w/v) of PEG (MW 20,000) were then added. The mixture was heated, with constant stirring, at 80–90° so as to form the beads. Twenty millilitres of 20% (w/v) PVP were then added to the mixture at room temperature. After cooling to room temperature the beads were washed twice with 1-l portions of water.

Analysis of silica–agarose beads

The beads were analysed for dry weight at 100° (over phosphorus pentoxide *in vacuo* to constant weight) and ash. Galactose was determined by the anthrone reaction[5]. The size and shape of the wet beads were examined by phase-contrast microscopy using a Leitz Ortho Lux microscope (E. Leitz, Wetzlar, G.F.R.) supplied with a Nikon ocular micrometer (Nippon Kogaku K.K., Tokyo, Japan). For scanning electron microscopy the beads were dehydrated in 95% ethanol and studied at low magnification in a Jeol JSM-U3 instrument (Japan Electron Optics Lab., Tokyo, Japan).

The density of the beads in the native wet state was determined by using an isopycnic centrifugation technique as described earlier[6]. The beads were centrifuged in linear pre-formed density gradients (1.00–1.10 g/ml) of dialyzed Ludox HS–PVP at 97,000 *g* for 30 min in a Beckman L4 ultracentrifuge (rotor SW 36; 30,000 rpm). The fractions collected were examined for density in organic density columns, and for number and size of beads by microscopy.

Removal of silica from the beads

The beads prepared as described above were treated with a 1% solution of hydrogen fluoride (Merck) in water for 20 h to remove all of the silica present. They were then neutralised by washing several times with water.

Chromatographic techniques

Chromatography was performed in glass columns of various diameters (Labassco AB, Gothenburg, Sweden), which were packed with silica–agarose beads prepared with Ludox SM under a hydrostatic pressure equal to or higher than that used in the subsequent elution. The columns were eluted with 0.5–1 *M* sodium chloride, either alone or 0.02 *M* in Tris–HCl buffer (pH 8). Columns packed with beads that had not been treated with hydrogen fluoride were washed with 1 % polyethylene oxide to prevent adsorption effects due to hydrogen bonding to silica[7]. The void volume (V_0) of the column was determined with dextran, and the total volume (V_t) by measuring the volume of water required to fill the glass column to the original level of the gel bed. The K_{av} value of a test substance was calculated from its elution volume (V_e) according to the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$[8].

Usually 0.5–2 mg of each sample component was applied to the column in a volume of 0.5 ml. Effluents were analysed for proteins, blue dextran, DNP-alanine and PEG by ultraviolet absorption at 280 nm, for dextran (glucose) by the anthrone reaction[5] and for glycosaminoglycans (uronic acids) by the carbazole reaction[9].

RESULTS AND DISCUSSION

The beads were presumably formed in the reaction mixture by adsorption of agarose, *e.g.*, through hydrogen bonding, on the silica or aluminium oxide surface, the complex acting as a nucleation centre for agarose bead formation. Another explanation could be phase separation when agarose and colloidal silica were mixed[10], as it was observed that high concentrations of silica were required for bead formation.

TABLE I

ANALYSES OF SILICA–AGAROSE BEADS

Silica solution used*	*Bead** diameter (μm)*	*Bead*** density (g/ml)*	*Chemical composition of bead (%)* §							
			Water		*Silica*		*Agarose*		*PEG+PVP*§§	
			w/w	*v/v*	*w/w*	*v/v*	*w/w*	*v/v*	*w/w*	*v/v*
Ludox SM (400 m^2/g)	100–200	1.038	91.0	94.5	3.2	1.5	5.0	3.2	0.8	0.8
Ludox HS (220 m^2/g)	25– 80	1.022	94.0	96.1	0.5	0.2	5.0	3.1	0.5	0.5
Ludox TM (130 m^2/g)	10– 20	1.01								

* Manufacturer's designation of colloidal silica and average specific surface area per g SiO_2. For preparation of beads see text.

** Measured by microscopy.

*** Measured by isopycnic centrifugation.

§ The beads were centrifuged for 30 min at 1000 *g* to obtain a pellet, which was analysed for water content, ash [*i.e.*, silica (SiO_2)] and agarose as described in the text. From the weight fractions (w/w) obtained, the volume fractions (v/v) were calculated assuming the following densities: silica, 2.2; agarose, 1.65; PEG and PVP 1.00 g/ml. The specific weights calculated from chemical analyses were in fair agreement with the observed densities. Owing to their small size and low density, the beads prepared from Ludox TM were difficult to analyse.

§§ PEG + PVP is the remaining dry weight when silica and agarose have been subtracted.

TABLE II

FLOW-RATE AND VOLUME CHANGES OF SILICA–AGAROSE GEL AT DIFFERENT OPERATING PRESSURES

Operating pressure (cm water) *	*Column diameter 1.5 cm***			*Column diameter 2.5 cm***			*Column diameter 5.0 cm***		
	Flow-rate		*Decrease in volume (%)*	*Flow-rate*		*Decrease in volume (%)*	*Flow-rate*		*Decrease in volume (%)*
	ml/min	*ml/cm²·h*		*ml/min*	*ml/cm²·h*		*ml/min*	*ml/cm²·h*	
50	0.4	13.4	2.5	0.7	8.5	2.5	2.3	6.9	2.5
150	1.0	34.0	6.5	2.2	26.5	6.5	8.0	24.0	6.5
250	1.6	53.0	8.5	3.2	38.5	8.5	15.0	45.0	8.5

* Height of buffer surface in reservoir bottle over capillary tip at bottom of the column.

** The columns were packed by allowing a suspension of silica–agarose beads (prepared from Ludox SM) in 0.15 *M* NaCl that was 0.02 *M* in Tris–HCl buffer (pH 8) to settle to a bed height of 19 cm. Flow-rates and volume changes (*i.e.*, column shortening) of the gel at different operating pressures were measured, using the same elution medium. The column changes were entirely reversible on releasing the pressure.

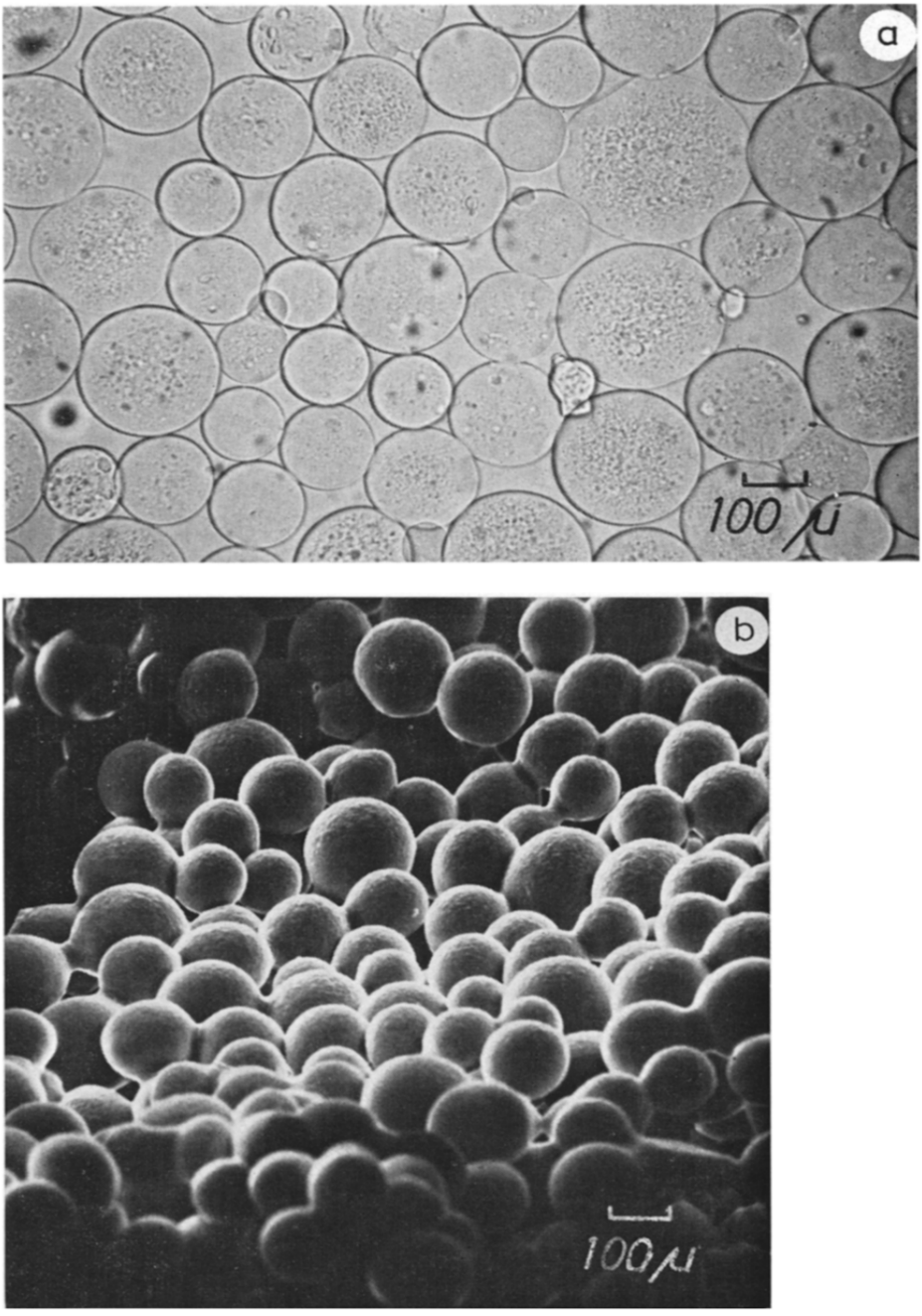

Fig. 1. Phase-contrast microscopic picture (a) and scanning electron micrograph (b) of silica–agarose beads prepared from colloidal silica (Ludox SM), agarose, PEG and PVP.

Analysis with anthrone showed that 90–95% of all agarose becomes included in the beads while only a small fraction of the original silica is involved in bead formation. PEG and PVP, which are known to adsorb on the surface of colloidal silica[6,11], were added to stop the reaction between silica and agarose. This effect was verified when PEG added to silica before the agarose prevented bead formation. If silica and agarose were mixed without PEG, the beads formed were unstable and dissolved to form a continous gel. When the reaction was stopped by the addition of PEG alone, stable beads were formed, but they tended to be oval shaped. Spherical beads were formed by the addition of both PEG and PVP. PVP without PEG resulted in very small but stable beads with diameters of 10–20 μm. Compared with earlier methods for preparing spherical gel beads[1–3], this method is different in principle as the whole reaction sequence is carried out in aqueous solution.

Analytical data on silica–agarose beads are presented in Table I. The agarose content is 5% (w/w). Beads prepared from small colloidal particles of silica (Ludox SM) appear larger, contain more silica, and thus have a higher density than beads prepared from larger silica particles (Ludox HS and TM). The particles were spherical and fairly homogeneous (Fig. 1). Beads prepared from aluminium oxide and agarose had a diameter of 50–150 μm, but no systematic analyses were made. Treatment of the beads with hydrogen fluoride completely removed the silica without destroying the beads.

The beads obtained with Ludox SM were tested for use as chromatographic material. Table II demonstrates the flow-rate and the volume changes of the gel bed at different pressures. Obviously the gel has flow properties similar to those of conventional agarose gels. The chromatography of some compounds on silica–agarose is shown in Fig. 2. The separation obtained is comparable with that given by Sephadex G-200[8]. The recovery of the components was 85% or more.

A number of proteins and polysaccharides of different molecular weights were

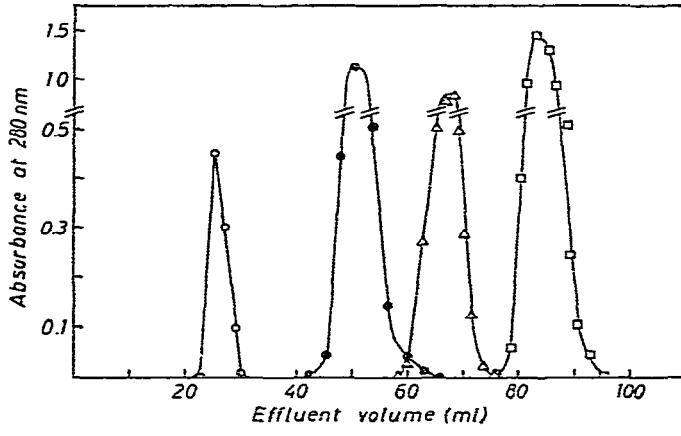

Fig. 2. Chromatograms (superimposed) obtained with a 2.0 × 26.2 cm silica–agarose column. Operating pressure 110 cm water, flow-rate 1 ml/min (19 ml/cm²·h) and elution medium 1.0 *M* NaCl that was 0.02 *M* in Tris–HCl (pH 8). ○ = Blue dextran (1 mg); ● = human serum albumin (12.5 mg); △, horse myoglobin (2.5 mg); □, DNP-alanine (0.5 mg). Fractions (2.5 ml) were collected and the absorption at 280 nm was measured.

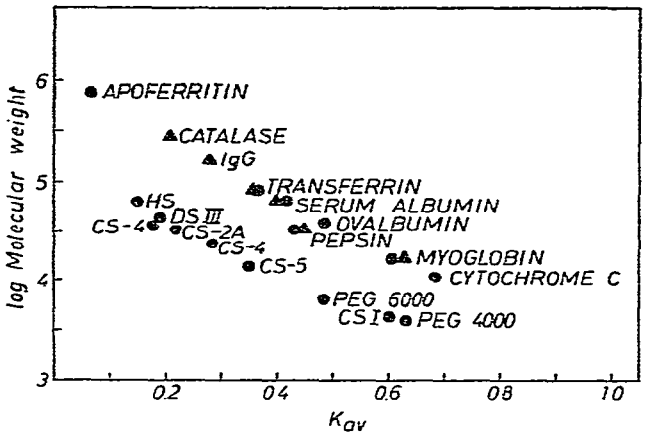

Fig. 3. Gel chromatography of various compounds on silica–agarose gels. ▲, represents samples run on a 2.0 × 26.2 cm column at a flow-rate of 19 ml/cm²·h as described in the legend to Fig. 2. ●, represents samples run on a 1.5 × 35.0 cm column at a flow-rate of 2.7 ml/cm²·h. Log MW of the test substances plotted *versus* their K_{av} values. HS = Heparan sulphate; CS = chondroitin sulphate; DS = dermatan sulphate. These polysaccharide fractions have been described by Iverius[12] and Wasteson[13].

chromatographed on a silica–agarose column at different flow-rates and the results are presented in Fig. 3. The K_{av} value[8] was independent of flow-rate within the range studied. There is a good correlation between the K_{av} value and molecular size (Stokes' radius[8,13]) for both types of compounds, thus showing that the silica–agarose beads have gel chromatographic properties.

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REFERENCES

1 S. Hjertén, *Biochim. Biophys. Acta*, 79 (1964) 393.
2 S. Bengtsson and L. Philipson, *Biochim. Biophys. Acta*, 79 (1964) 399.
3 J. Porath, J.-C. Janson and T. Låås, *J. Chromatogr.*, 60 (1971) 167.
4 B. Vassilou and R. Kunin, *Anal. Chem.*, 35 (1963) 1328.
5 T. A. Scott and E. H. Melvin, *Anal. Chem.*, 25 (1953) 1656.
6 D. A. Wolff and H. Pertoft, *J. Cell Biol.*, 55 (1972) 579.
7 C. W. Hiatt, A. Shelokov, E. J. Rosenthal and J. M. Galimore, *J. Chromatogr.*, 56 (1971) 362.
8 T. C. Laurent and J. Killander, *J. Chromatogr.*, 14 (1964) 317.
9 T. Bitter and H. M. Muir, *Anal. Biochem.*, 4 (1962) 330.
10 P. Å. Albertsson, *Partition of Cell Particles and Macromolecules*, Almqvist & Wiksell, Stockholm, 1960.
11 H. Pertoft, *Exp. Cell Res.*, 57 (1969) 338.
12 P.-H. Iverius, *Biochem. J.*, 124 (1971) 677.
13 Å. Wasteson, *Biochem. J.*, 122 (1971) 477.