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Continuous superporous agarose beds for chromatography and electrophoresis[1]

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Abstract

Continuous agarose beds (monoliths) were prepared by casting agarose emulsions designed to generate superporous agarose. The gel structures obtained were transected by superpores (diameters could be varied in the range 20–200 μm) through which liquids could be pumped. The pore structure and the basic properties of the continuous gel were investigated by microscopy and size exclusion chromatography. The chromatographic behaviour was approximately the same as for beds packed with homogeneous agarose beads with a particle diameter equivalent to the distance between the superpores. In one application, the superporous continuous agarose bed was derivatized with a NAD^+ analogue and used in the affinity purification of bovine lactate dehydrogenase from a crude extract. In another application, a new superporous composite gel material was prepared by adding hydroxyapatite particles to the agarose phase. The composite bed was used to separate a protein mixture by hydroxyapatite chromatography. In a third application, the continuous superporous agarose material was used as an electrophoresis gel. Here, a water-immiscible organic liquid was pumped through the superpores to dissipate the joule heat evolved, thus allowing high current densities.

Keywords: Superporous agarose beds; Stationary phase, LC

1. Introduction

The chromatographic separation of biomolecules is a key step in the biotechnology industry. One factor to improve the cost-effectiveness of a chromatographic process is the reduction of media costs [1]. Most chromatography columns are made of a bed packed with spherical particles (beads) but their preparation is often time-consuming and expensive. The development of alternative chromatography support formats is therefore of considerable interest. One alternative is the use of membranes which have been derivatized with different ligands and used in affinity-, ion-exchange- and hydrophobic chromatography separation of biomolecules. These adsorptive membranes are commercially available from many suppliers and are found in various shapes (thin sheets, hollow fibers, rolled sheets, membrane stacks) with cellulose as the dominating base material [2]. Special membranes based on synthetic polymers (polymethacrylates) have also been developed [3,4]. These so-called macroporous discs are up to several mm in thickness and have been used for protein separations.

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Continuous beds (rods, monoliths) have been developed from different base materials [5–13]. These beds, with similar column dimensions as beds packed with particles, offer an interesting alternative since they can be prepared in one piece directly in the column, simplifying the preparation procedure. Hjertén and co-workers [5–7] prepared such beds by polymerization of different acrylamide–acrylate monomers in the presence of an initiator and a high salt concentration. The beds were subsequently compressed to create a hydrophilic and non-porous bed transected by flow pores (3–4 μm) and used for the separation of proteins and peptides in several chromatographic modes. Svec and Fréchet [8,9] and Wang et al. [10] polymerized different methacrylate and styrene monomers in the presence of a porogenic solvent directly in the column. The procedure created continuous rods, having both large flow pores (0.5–2 μm or 20 μm) and smaller diffusion pores (<0.5 μm). The rods were used for protein separations in various chromatographic modes. Recently, continuous silica rods have also been developed [11–13], mainly for reversed-phase chromatography separations of small molecules.

In previous reports [14–17] we have described a new type of support material in bead form, a so-called superporous agarose. These beads have large connecting flow pores, which carry part of the chromatographic flow [16]. The superporous agarose beads give an improved performance in ion-exchange, affinity and hydrophobic interaction chromatography separations of proteins, compared with homogeneous agarose beads of the same particle size [14,15,17]. In this paper we have used the superporous agarose material to prepare continuous beds. The basic properties of these monolithic beds were briefly characterized by size exclusion experiments and by microscopy. The monoliths were further used in chromatographic applications and a preliminary electrophoresis application.

2. Experimental

2.1. Materials

Agarose powder (Sepharose quality) was a gift from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Polyoxyethylenesorbitanmonooleate (Tween 80) and cyanogen bromide were obtained from Merck-Schuchardt (Munich, Germany). Cyclohexane (puriss.) was obtained from Merck (Darmstadt, Germany). The synthesis of N^6-[*N*-(2-aminoethyl)carbamoylmethyl]-NAD^+ [18] was similar to the synthesis of the corresponding 6-aminohexyl analogue [19]. The 6-aminohexyl analogue was synthesized according to a method which included the following steps: alkylation with iodoacetic acid to give 1-carboxymethyl-NAD^+, rearrangement to N^6-carboxymethyl-NAD^+ and finally condensation with 1,6-diaminohexane to give N^6-[*N*-(6-aminohexyl)carbamoylmethyl]-NAD^+. The 2-aminoethyl analogue, used in this paper, was synthesized in the same way, except for the last step, where 1,6-diaminohexane was replaced for 1,2-diaminoethane. The protein assay kit was obtained from Bio–Rad Labs. (Hercules, CA, USA). Hydroxyapatite (type 1), lysozyme from chicken egg white, cytochrome *c* from horse heart, carbonic anhydrase from bovine erythrocytes and β-lactoglobulin A, B from bovine milk were obtained from Sigma (St. Louis, MO, USA). Bovine serum albumin was purchased from Boehringer Mannheim GmbH (Mannheim, Germany).

2.2. Casting of continuous agarose rod for chromatography

A suspension of agarose in water (100 ml, 6% w/v) was heated to 95–100°C in a microwave oven (with occasional shaking to keep the agarose powder well suspended), and kept at that temperature for 1 min. The agarose solution was cooled to 60°C in a stirred glass reactor and a mixture of 100 ml cyclohexane and 6.0 ml Tween 80 (60°C) was added. The mixture was emulsified by stirring at 1000 rpm for 4 min. The emulsion was poured into glass columns, (16 mm I.D., fitted with rubber plugs at the bottom end, pre-warmed to 60°C in a water bath). The temperature of the water bath was then decreased by adding cold water (5°C) to the bottom of the water bath, after 5 min the temperature of the water bath was 20°C. The solidified continuous agarose rods thus obtained were trimmed to a length of 6 cm and positioned in glass columns, (16 mm I.D.), equipped with flow adapters. The organic

phase in the superpores was removed by pumping water, ethanol–water (50:50, v/v) and finally degassed water through the column.

2.3. CNBr activation of continuous agarose rods

The column containing the superporous continuous agarose rod was placed in a water bath (ice-cold) and 50 ml of an ice-cold solution of 1 *M* Na_2CO_3 was pumped through the column. Ice-cold CNBr solution (45 ml of a 56 mg ml^{-1} solution in 1 *M* Na_2CO_3–acetonitrile–water (2.5:1:1)) was pumped through the column, followed by a washing step with 90 ml of ice-cold 0.2 *M* phosphate buffer pH 8.5.

2.4. Coupling of N^6-[N-(2-aminoethyl)-carbamoylmethyl]-NAD^+ to continuous agarose rods

After the washing step a coupling solution containing 200 mg N^6-[*N*-(2-aminoethyl) carbamoylmethyl]-NAD^+ in 10 ml of water was recirculated through the column. After 5 min the pH of the coupling solution was adjusted to 8.5 and the recirculation continued for 1 h, before being adjusted to room temperature for an additional 15 h. The coupling was then terminated by pumping 0.2 *M* glycine–NaOH pH 8.65, water and finally 1 m*M* sodium acetate pH 5.0 through the column. The continuous, superporous NAD^+-agarose rod was stored at 4°C until further use.

2.5. Preparation of continuous agarose membranes

A membrane casting device was prepared from two thick glass plates (3×130×130 mm) and a specially cut piece of U-shaped Vyon rubber (5 mm thick), serving both as a spacer and a seal. The glass plate-Vyon rubber–glass plate sandwich was kept together by strong paper clamps, Fig. 1. The casting device was pre-warmed to 60°C (water bath) and a freshly prepared, agarose-cyclohexane emulsion (60°C; 20 ml, Section 2.2) poured between the plates. After 1 min at 60°C the casting device was transferred to a cold water bath (0–3°C) to induce the agarose phase to gel. The casting device was dismantled and the white membrane sheet obtained briefly washed with water, after which circular pieces of membrane were punched out from the sheet with a sharpened steel punch. The membranes were placed in a membrane holder and the organic solvent/detergent phase was removed from the superpores by washing with water, ethanol–water (50:50, v/v) and finally water. The superporous membranes (5×16 mm I.D.) obtained were almost as transparent as the corresponding homogeneous agarose gels.

2.6. Preparation of continuous agarose-hydroxyapatite composite gel

Hydroxyapatite particles (25 ml sedimented volume) in 1 m*M* sodium phosphate buffer pH 6.8 were subjected to a series of sedimentations to remove fines (in total 9 ml of sedimented hydroxyapatite were removed). Five ml of the remaining hydroxyapatite (sedimented volume) in 1 m*M* sodium phosphate buffer pH 6.8 was warmed to 60°C. Fifteen ml of an agarose solution (8% w/v, 95–100°C) was prepared as described in Section 2.2. The agarose solution was cooled to 60°C and directly added to the hydroxyapatite. The agarose-hydroxyapatite suspension was subsequently stirred at 1000 rpm in a water bath (60°C). After 5 min, a mixture of 0.75 ml Tween 80 and 10 ml cyclohexane (60°C) was added. The mixture was emulsified by stirring at 1000 rpm for 2 min. The emulsion was poured into glass columns, (16 mm I.D., fitted with rubber plugs at the bottom end, pre-warmed to 60°C in a water bath). After 30 s the glass columns were transferred to an ice water bath for cooling. The solidified continuous agarose-hydroxyapatite composites thus obtained were trimmed to a length of 1.4 cm and positioned in glass columns, (16 mm I.D.) equipped with flow adapters. The organic phase in the superpores was removed by pumping 1 m*M* sodium phosphate buffer pH 6.8, ethanol–1 m*M* sodium phosphate buffer pH 6.8 (50:50, v/v) and finally degassed 1 m*M* sodium phosphate buffer pH 6.8 through the columns. The superporous composite rods were stored at 4°C until further use.

2.7. Preparation of lactate dehydrogenase extract

A lactate dehydrogenase extract was prepared from bovine heart, essentially as described earlier [15,20]. The preparation of the extract included the

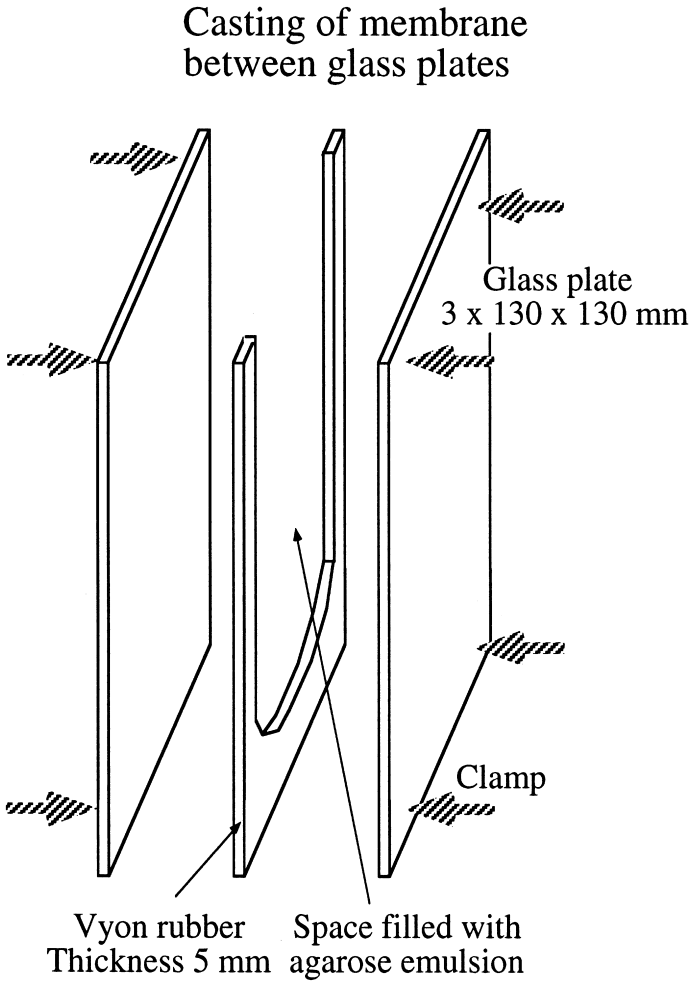

Fig. 1. Casting of superporous agarose membranes.

following steps: disintegration, centrifugation and ammonium sulphate fractionation (30%–60% saturation). The precipitated enzyme was dissolved in a small volume of 0.05 *M* phosphate buffer pH 7, giving an ammonium sulphate concentration of approximately 1 *M*. The extract had a protein content of 220 mg ml^{-1}, determined according to the method of Bradford [21] and an activity of 300 U ml^{-1} (see Section 2.9).

2.8. Purification of bovine lactate dehydrogenase

The continuous rod derivatized with the NAD^+ analogue was positioned in a 16-mm diameter column, equipped with flow adapters. The mobile phase was pumped with a positive displacement pump (Altex Model 110 A, Altex Sci., Berkeley, CA, USA) into the column via a 6-port valve (Valco, Houston, TX, USA) provided with a 50-ml superloop (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Part of the effluent from the column was diverted with a separate, peristaltic pump to a fraction collector (FC 203, Gilson Med. Electr., Middleton, WI, USA) for analysis in 96-well microtiter plates.

Bovine lactate dehydrogenase extract (25 ml), prepared as in Section 2.7, was mixed with 25 ml of the adsorption buffer and sodium oxalate was added to a final concentration of 25 m*M*. The sample was

injected via the superloop and the eluate was collected at 2-ml intervals by the fraction collector for subsequent protein and activity analysis (see Section 2.9). After the adsorption-wash step, elution was carried out by injecting 50 ml of the elution buffer containing 1 m*M* NADH. The adsorption-wash step and the elution step were carried out at the same flow-rate. All steps were carried out in the cold room (about 4°C).

2.9. Protein and activity analysis

The activity of lactate dehydrogenase was assayed by following the oxidation of NADH at 340 nm (room temperature), as described earlier [15]. Suitably diluted fractions (10 μl) were added to 96-well microtiter plates containing 190 μl of an assay solution (4 m*M* NADH, 1 m*M* pyruvate, 1 m*M* EDTA, 1 m*M* mercaptoethanol, 0.05 *M* sodium phosphate buffer pH 7). The decrease in the absorbance was measured using a plate reader (Multiskan MCC/340, Labsystem, Helsinki, Finland).

Protein was analyzed according to the method of Bradford [21]. Suitably diluted fractions (10 μl) were added to 190 μl of the protein assay solution in 96-well microtiter plates. After 4 min incubation at room temperature, the absorbance at 595 nm was measured using a plate reader.

2.10. Preparation of superporous continuous agarose composite cylinder for electrophoresis

A superporous continuous agarose rod (20×10 mm I.D.) was cast as described in Section 2.2. and placed in a glass column (16 mm I.D., fitted with a rubber plug at the bottom end, pre-warmed to 60°C in a water bath). The gel rod was carefully centred in the glass column leaving a 3 mm space between the bed and the glass wall. An agarose solution (6% w/v, 60°C) was prepared as in Section 2.2 and poured into the empty space. The glass column was then placed in a beaker of cold water (5°C) to solidify the agarose. The composite cylinder obtained was removed from the glass column and its ends trimmed with a razor blade. The composite cylinder was then positioned in a specially constructed electrophoresis cell (Fig. 5, Section 3.5) equipped with flow adapters.

2.11. Electrophoresis with internal cooling of the separation gel

A special electrophoresis cell was constructed to allow simultaneous electrophoresis and pumping of coolant through the flow pores of the superporous agarose (Fig. 5). The cell consisted of a glass column (16 mm I.D.) with two cylindrical holes (5 mm I.D.) each one connecting to a buffer reservoir. The agarose rod (Section 2.10) was positioned in the glass column. Standard chromatography flow adaptors were inserted in the glass column and attached to a peristaltic pump to deliver cooling liquid (heptane equilibrated to room temperature). The buffer reservoirs were provided with electrodes and connected to the power supply. An agarose plug containing carbonic anhydrase and β-lactoglobulin A, B (8 μg each) and tracking dye (bromophenol blue) was then placed close to the composite cylinder in the reservoir containing the positive electrode. The electrophoresis run was initiated by applying a voltage to the electrodes and simultaneously starting the circulation of cooling liquid through the superpores. The temperature inside the gel was measured by a thin thermocouple (Digitaltermometer GTH 1200, Tradoterm) introduced in the composite cylinder via the bottom flow adapter.

3. Results and discussion

3.1. Preparation and general properties of continuous agarose beds (rods)

One obvious advantage of cast continuous agarose rods, compared to packed beds based on agarose beads, lies in the manufacturing step-bead preparation and classification is omitted. Furthermore, while beads need to be properly packed, followed by a check for bed homogeneity, continuous agarose rods can easily be removed and re-inserted into the separation column without time-consuming packing procedures and with the same separation properties each time. The superpores in the continuous agarose rod are equivalent to the interstitial pores in a normal packed bed and can easily be tailor-made to better suit each application, in our experience the superpore

volume can be varied between 20–50% and the superpore diameter can be varied between 20–200 μm to give a small or a large superpore surface area or to give a small or a high interstitial volume.

Continuous superporous agarose beds may be prepared in a number of physical appearances, for example, as cast rods, as membranes and as fibers. Fig. 2 exemplifies this, showing several self-supported, superporous agarose structures. Fig. 2 also shows a micrograph of the pore structure of one of the membrane disks. Before taking the micrograph, the membrane was allowed to dry in air for about 5 min allowing the water in the superpores to slightly withdraw, resulting in better contrast.

When exploring various formats for continuous agarose beds it was noted that large diameter rods (>2.5 cm) were difficult to prepare with satisfactory separation properties by a direct casting procedure. Such columns showed an uneven pore size distribution, with larger pores at the centre of the column, leading to an uneven flow profile. The reason for the larger pores at the centre is probably that this part of the continuous rod is the last to solidify (cooling from the outside). The longer time before solidification leads to increased phase separation of the bi-continuous phase system which results in larger pores. A remedy for this situation would be the use of phase systems with higher stability. Peters et al., [22] recently described inhomogeneities in the porous structure of polymerized monoliths caused by the heat of polymerization. The proposed remedies e.g. gradual addition of the polymerization mixture to the reaction vessel and decreasing the rate of polymerization are obviously not applicable in our case.

An alternative way of preparing large diameter columns was briefly investigated. Superporous agarose membrane sheets (5 mm thickness) were cut into circular membranes and packed in columns to a suitable height. The chromatographic beds thus obtained gave an even flow profile, indirectly proven by size exclusion experiments (see below), resulting in low HETP-values. Although this method of column ‘packing’ is hardly suitable for columns with substantial heights, we found it quite convenient for those with shallow beds.

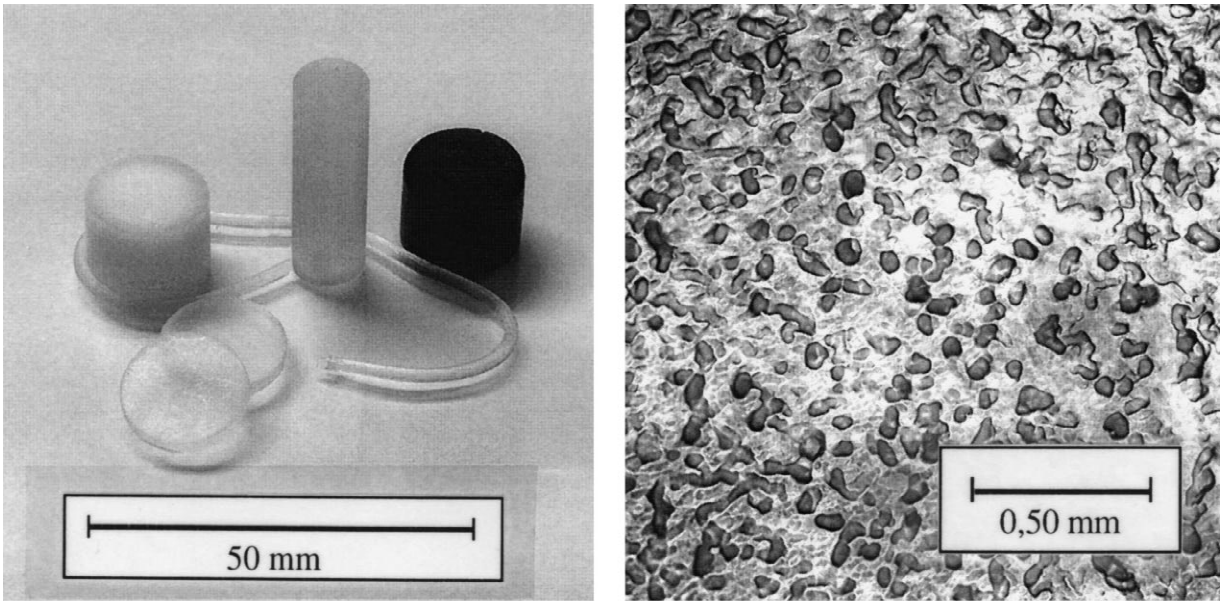

Fig. 2. Self-supported superporous agarose structures. From left to right, superporous agarose-hydroxyapatite composite, superporous agarose membranes, superporous agarose rod, superporous agarose rod derivatized with cibacrone blue. Also shown is a superporous agarose fibre. The right panel shows a micrograph of the surface of one of the membranes shown in the left panel.

3.2. Characterization by size exclusion experiments

The performance of continuous agarose rods was compared in size exclusion chromatography experiments with columns packed with normal agarose beads. The experiments showed that the continuous agarose rod behaved chromatographically as a normal bed packed with agarose beads, having a particle size of 75–106 μm. The column size for both type of packings was 30×16 mm I.D. and gave a HETP-value for BSA of 1.7 mm for both type of packings, at a superficial flow velocity of 0.12 cm min^{-1}. These results seem reasonable, since the diffusion distances between the superpores in this continuous agarose rod were approximately 100 μm, i.e. about the same distance as the bead diameter in the reference column, (as measured by microscopy on thin slices of the continuous agarose rod). In other experiments it was noted that continuous superporous rods behaved similarly to packed bed columns where the particles had a diameter equivalent to the distance between the superpores.

3.3. Purification of bovine lactate dehydrogenase with continuous superporous NAD^+ agarose gel

Fig. 3 shows the affinity chromatography purification of lactate dehydrogenase by a continuous agarose rod. The continuous agarose rod was derivatized with a NAD^+ analogue which has affinity for many dehydrogenases. An extremely strong binding is formed between NAD^+ and lactate dehydrogenase in the presence of oxalate (ternary complex), even in the presence of high concentrations of salts [23]. No dialysis of the enzyme preparation was therefore necessary before application on to the affinity column. The flow-rate was 2 ml min^{-1}, corresponding to 60 cm h^{-1} superficial flow velocity. The continuous agarose bed used had a superpore/interstitial porosity of 50% (defined by the method of preparation and verified by size exclusion chromatography with 0.5 μm latex particles). Average superpore/interstitial pore diameter of the continuous agarose bed was 25 μm (measured by observations under microscopy on thin slices of the continuous agarose bed, as described earlier [14]). The amount loaded on the chromatographic run (7500 U with a specific activity of 1.4 U mg^{-1} protein) corresponded to approximately one sixth of the maximal apparent static binding capacity, about 45 000 U (as measured on small, crushed, continuous bed fragments in the presence of an excess volume of lactate dehydrogenase at a concentration of 90 U ml^{-1} ; equilibration time 2 h). The specific activity after the purification was 18.1 U mg^{-1} protein giving a purification factor of 13 with a 70% activity yield.

3.4. Continuous superporous composite beds-hydroxyapatite chromatography

The ease by which the superporous continuous agarose beds can be prepared make them suitable for combination with other materials to form composite beds. The percent of filling material that can be put in the agarose phase is dependent of the properties of the filling material such as particle size and hydrophobicity. We have found that up to 50% of the agarose phase can be exchanged for other materials and still give a satisfactory continuous bed. Examples of included materials, with relevance for separation purposes tested by us, are active carbon, ion-exchange materials, affinity materials (data not shown) and hydroxyapatite.

Fig. 4 shows the separation of three model proteins with our continuous agarose rod containing hydroxyapatite at three different superficial flow velocities (15 cm h^{-1}, 30 cm h^{-1} and 60 cm h^{-1}). The composite bed had a superpore/interstitial porosity of 33% (defined by the method of preparation) and an average superpore/interstitial pore diameter of 30 μm (measured by observations under microscopy).

It should be mentioned that a hydroxyapatite–agarose composite in bead form is available under the trade name HA Ultrogel [24]. These beads are claimed to solve the problem of column clogging often encountered in HA-chromatography. Our composite rod should serve the same purpose.

3.5. Superporous continuous agarose composite gels in electrophoresis

Electrophoretic separation offers very high resolution in analytical applications involving protein mixtures. However, the use of electrophoretic sepa-

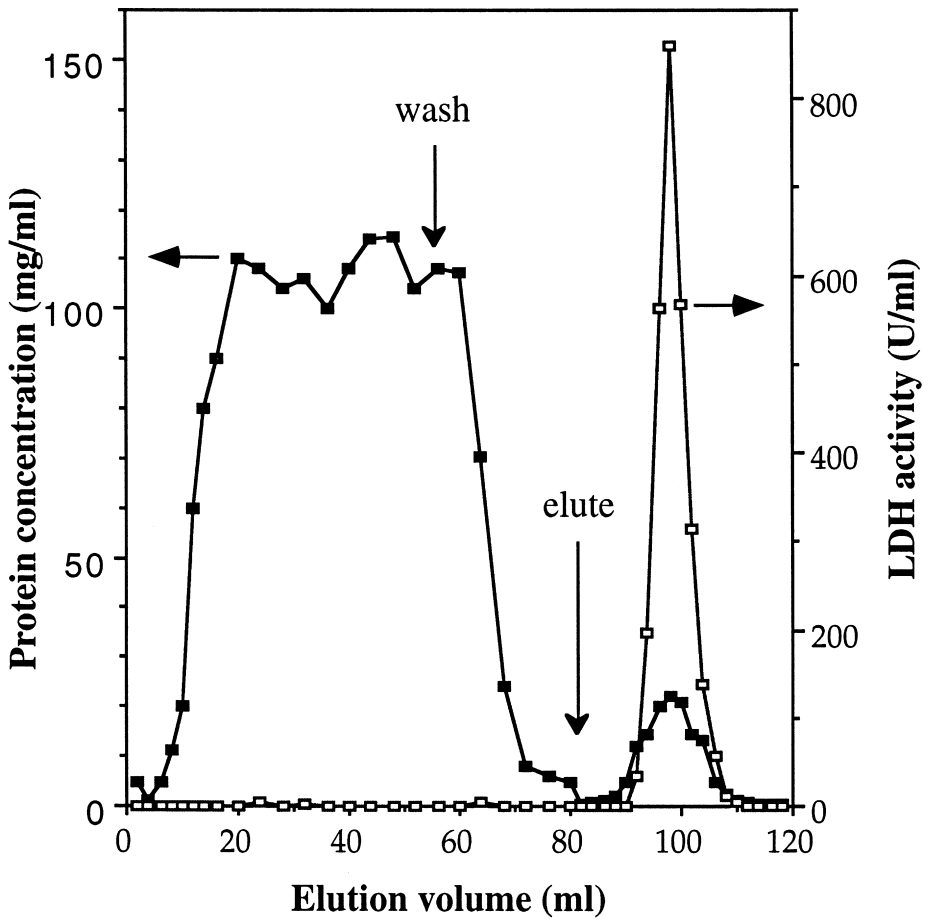

Fig. 3. Affinity chromatography purification of lactate dehydrogenase on a superporous continuous NAD^+-agarose rod. Column size: 60×16 mm (I.D.). Superficial flow velocity: 60 cm h^{-1}. Sample: 50 ml of a crude bovine lactate dehydrogenase extract. Adsorption–wash buffer: 0.05 *M* sodium phosphate buffer, pH 7 containing 1 m*M* EDTA, 1 m*M* mercaptoethanol and 25 m*M* sodium oxalate. Elution buffer: 0.05 *M* sodium phosphate buffer pH 7 containing 1 m*M* EDTA, 1 m*M* mercaptoethanol and 1 m*M* NADH. Further details in Section 2.8.

rations on a preparative scale is limited due to the problems of dissipating the heat generated during the electrophoretic runs [25–27]. Heat leads to temperature gradients in the separation gel deteriorating the resolution. A solution to this problem would be to use separation gels with improved thermal properties.

To this end we briefly investigated the idea of using the flow pores in superporous agarose as internal cooling channels during electrophoretic runs. An electrophoretic cell was constructed (Fig. 5) which allowed the pumping of a water immiscible coolant (heptane) through the gel structure during the electrophoretic run.

The experiments conducted showed that the flow of organic solvent through the superpores in the composite cylinder removed a substantial part of the joule heat produced during the runs. For example, at an applied voltage of 250 V and a current of 31 mA, the measured temperature in the composite cylinder was 44°C (at an organic solvent flow-rate of 1 ml min^{-1}) while in the reference run with an homogeneous agarose bed the temperature was considerably higher (60°C). These preliminary experi-

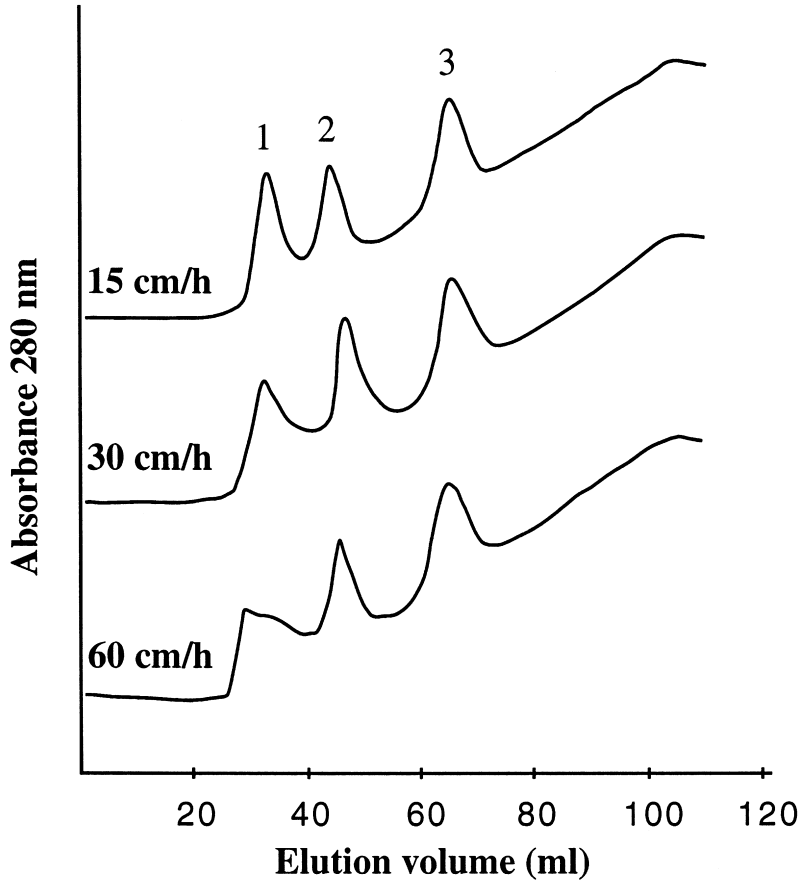

Fig. 4. Hydroxyapatite chromatography separation using a continuous agarose–hydroxyapatite composite rod. Sample: 100 μl protein mixture containing 0.10 mg bovine serum albumin (1), 0.017 mg lysozyme (2) and 0.033 mg cytochrome c (3) in starting buffer. Column size: 14×16 mm (I.D.). Starting buffer: 1 m*M* sodium phosphate, pH 6.8. End buffer: 300 m*M* sodium phosphate, pH 6.8. The increasing phosphate gradient started after 20 ml elution volume and ended after 100 ml elution volume. Protein was detected at 280 nm.

ments indicate that internal cooling might be a useful way of carrying out preparative electrophoresis in thick gels at high current densities. Clearly, a number of difficulties must be handled, difficulties that were not properly addressed during the present preliminary runs. It was noted, for example, that protein precipitation sometimes occurred at the superpore walls, which was evident after staining thin slices of the composite cylinder after the electrophoresis run. The protein precipitation (or adsorption) might be due to interactions with the organic phase at the superpore–agarose interface.

However, we think that the results exemplify a new electrophoretic principle, that might lead to high capacity electrophoretic separation units once properly developed.

4. Conclusion

We have described the preparation of cast, continuous superporous agarose beds and their use in several areas, particularly in chromatography, e.g. affinity purification of lactate dehydrogenase. The easy preparation of these monolithic beds make them a viable alternative to traditional beds.

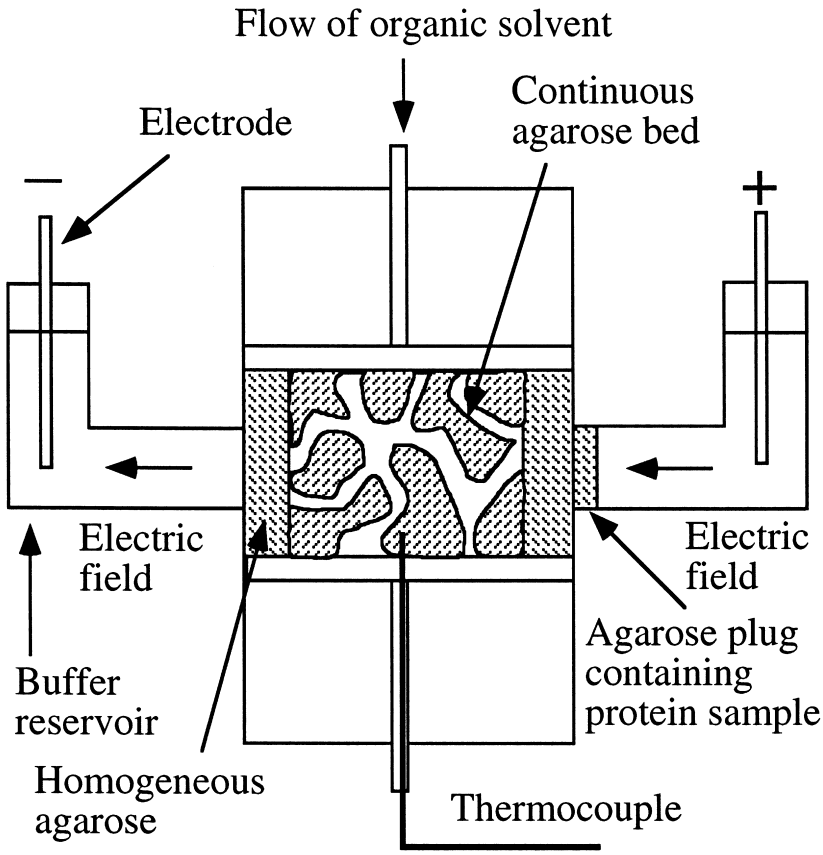

Fig. 5. Electrophoresis with an internally cooled separation gel. The protein sample migrated in the electric field from right to left. The cooling liquid (heptane) was pumped through the superpores from top to bottom. Further details in Section 2.11.

A special composite material based on continuous superporous agarose was prepared by partly substituting the agarose phase for hydroxyapatite particles. The composite bed retained the basic separation properties of hydroxyapatite in column chromatography applications, while offering potential benefits of having the active ingredient encapsulated in an agarose matrix, thereby preventing it's direct contact with the flowing medium.

In a third application of continuous cast beds, superporous agarose gels were used in electrophoresis. A cooling liquid was pumped through the gel during the electrophoretic run, dissipating the joule heat produced, allowing much higher current densities to be used than standard gels.

Acknowledgements

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