*Journal of Chromatography*, 396 (1987) 101–113
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 513

# AN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC MATRIX BASED ON AGAROSE CROSS-LINKED WITH DIVINYL SULPHONE

STELLAN HJERTÉN*, BO-LIANG WU* and JIA-LI LIAO*
*Institute of Biochemistry, University of Uppsala, Biomedical Center, P.O. Box 576, S-751 23 Uppsala (Sweden)*
(Received February 23rd, 1987)

## SUMMARY

By systematic investigation of some parameters [concentration of reducing agent (sodium borohydride), temperature, pH, reaction time, concentration of divinyl sulphone (DVS), solvent for removal of excess of DVS, agents for reaction with free unreacted vinyl groups] that affect the cross-linking of agarose with DVS, an highly reproducible method has been obtained for the preparation of a chromatographic medium exhibiting low adsorption and high rigidity to allow high flow-rates: a 300 mm × 6 mm bed of 12% cross-linked agarose beads with diameters around 17 μm permitted flow-rates up to 5 ml/min (40 bar). The cross-linking procedure was performed at pH 12.0–12.5 at room temperature in the presence of appropriate concentrations of sodium borohydride. Above and below these concentrations the flow-rate decreases and below the risk of adsorption increases. To reinforce the hydrophilic character of the cross-linked gel and to make it somewhat more rigid, OH-rich substances were coupled to the cross-linked agarose via its unreacted vinyl groups. By varying the molecular weight of these substances the pore size of the gel can be varied. No decrease in the rigidity of the beads was observed upon exposure to pH 13 during a test period of 15 days. Additional evidence for the chemical stability of the beads is that no impairment of the performance of a molecular sieve chromatography column could be detected (except for a decrease in flow-rate which, however, returned to normal after repacking) during a 4-year period of use, alternating in conventional buffer media and buffers containing sodium dodecyl sulphate or guanidine.

## INTRODUCTION

Agarose is the most universally utilized chromatographic gel matrix for the separation of biopolymers in such modes as molecular sieving (gel filtration), ion-exchange chromatography, affinity chromatography, dye-ligand chromatography, borate chromatography and hydrophobic interaction chromatography. The wide

* Permanent address: Department of Biology, Jinan University, Canton, China.

0021-9673/87/$03.50 

applicability of agarose is based on its inertness (neutrality, hydrophilicity), the ease with which it can be derivatized and cross-linked and its porosity and pH stability. Until recently, agarose has been used exclusively for conventional low-pressure chromatography. However, several years ago we showed that it can be used also for high-performance liquid chromatography (HPLC), which makes the application range still wider[1,2].

A decisive requirement of bed materials for HPLC is that they tolerate relatively high pressures without appreciable deformation; some compressibility may, however, be desirable since the void volume then decreases whereas the inner volume is almost unaffected, which can afford an increase in resolution[3]. We have shown that cross-linked agarose gels of high concentration (which are still quite permeable to proteins) fulfil this requirement[1–13]. Among the cross-linking agents we have tested, divinyl sulphone (DVS), introduced by Porath and co-workers[14,15] for cross-linking of agarose, has the great advantage of giving gels which tolerate the high pressures

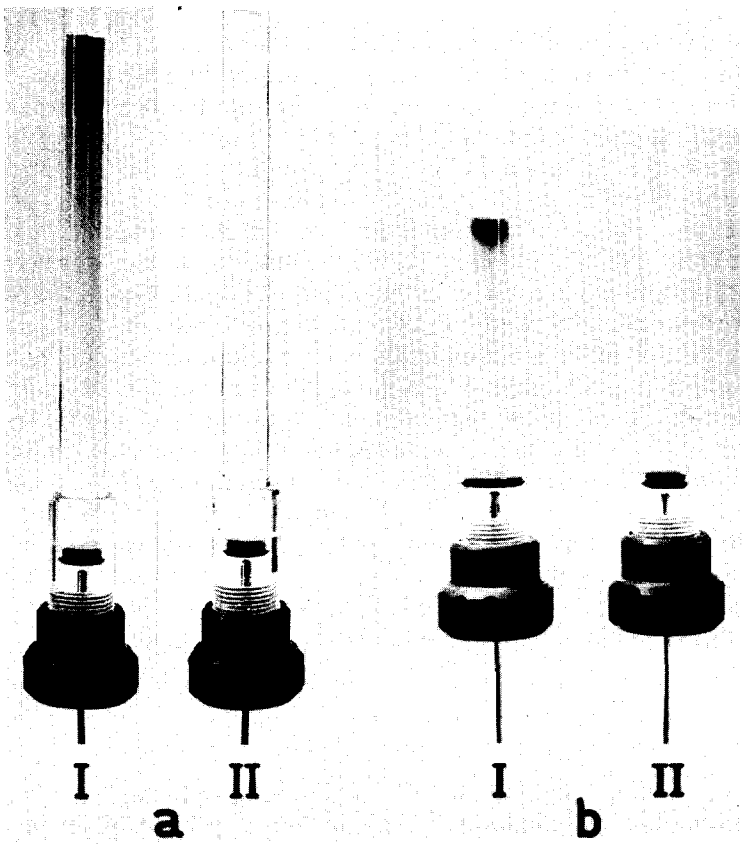



Fig. 1. Test for adsorption of Blue Dextran and haemoglobin to agarose cross-linked with DVS. The photographs illustrate that the adsorption of Blue Dextran (a) and haemoglobin (b) was strong in the absence of $NaBH_4$ (cross-linking method I, described in ref. 14), whereas very little adsorption was observed in the presence of $NaBH_4$ (method II described in this paper under the section First version). The experiment was performed in 0.01 *M* potassium phosphate buffer, pH 7.4.

and flow-rates required for HPLC. However, we soon found that agarose gels cross-linked with DVS in the manner described[14] had some undesirable properties which limited their usefulness in HPLC: (1) Blue Dextran used as a void-volume marker was irreversibly adsorbed (Fig. 1a, column I); (2) some model proteins were also adsorbed (haemoglobin irreversibly; Fig. 1b, column I); in the presence of 6 *M* guanidine the adsorption of albumin has been reported to be irreversible[16]; (3) the reproducibility of the cross-linking procedure was not satisfactory, since two consecutive cross-linking experiments often gave different maximum flow-rates when the same batch of agarose beads was used; (4) it has been stated[14,15] that DVS-cross-linked gels are not stable above pH 9.

The studies presented in this paper were aimed at the elimination of these drawbacks to make cross-linking with DVS more suitable for the preparation of rigid agarose beads for chromatography, particularly HPLC. Another objective was to investigate whether the conditions used earlier for cross-linking agarose with DVS[14] could be modified to give more rigid agarose beads allowing still higher flow-rates.

In ref. 14 the cross-linking of agarose gels of concentrations 2 and 4% was reported. Adsorption (points 1 and 2 above) to these low-concentration gels would be expected to be less than that to the 12% gels used in our investigation, and the pH stability (point 4) should be lower. These differences in gel concentration with attendant differences in gel properties should be recalled when the cross-linking method in ref. 14 is discussed. Besides, that method was developed for beads to be used for classical low-pressure chromatography where the requirements for high flow-rates are not so stringent as in HPLC. The need for optimization of the conditions to maximize the flow-rate was therefore not as urgent as in our HPLC studies.

## MATERIALS AND EQUIPMENT

Agarose was obtained from IBF (Villeneuve-La-Garenne, France), divinyl sulphone from Fluka (Buchs, Switzerland), sodium borohydride ($NaBH_4$) from Merck-Schuchardt (Hohenbrunn bei München, F.R.G.), cytochrome *c* and lysozyme from Sigma (St. Louis, MO, U.S.A.), ribonuclease A, ovalbumin, Blue Dextran, Dextran 10 and Dextran 500 from Pharmacia Fine Chemicals (Uppsala, Sweden). Human serum albumin was a gift from Dr. Lars-Olov Andersson, Kabi-Vitrum, Stockholm.

Agarose beads were prepared as described previously[17] and then subjected to elutriation in distilled water to attain a more uniform size distribution. The packing of the column was also performed in distilled water (at constant pressure). The home-made column tubes (350 mm × 6 mm) were of Plexiglas and were equipped with a movable upper plunger for adjustment to different heights of the agarose bed; the bed was supported by a 2-$\mu$m metal frit. Flow-rates as a function of pressure were determined with an HPLC pump (Model 750) from Micromeritics (GA, U.S.A.), since this pump can be operated at constant pressure.

For the experiment shown in Fig. 8 we used a Model 2150 HPLC pump from LKB (Bromma, Sweden) in combination with a Model 786 variable-wavelength detector from Micromeritics.

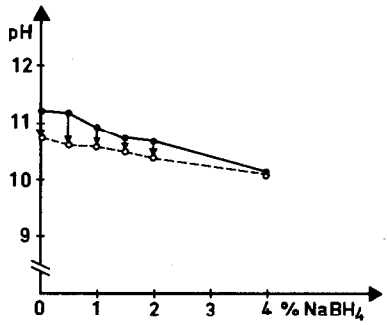



Fig. 2. The pH of a 0.5 *M* sodium carbonate solution containing different amounts of $NaBH_4$. The upper curve shows the pH at the start of the cross-linking and the lower broken curve the pH of the same solutions at the end.

## EXPERIMENTS AND RESULTS

*Preliminary experiments to eliminate the adsorption to agarose gels cross-linked with DVS, increase their rigidity and enhance the reproducibility of the cross-linking method*

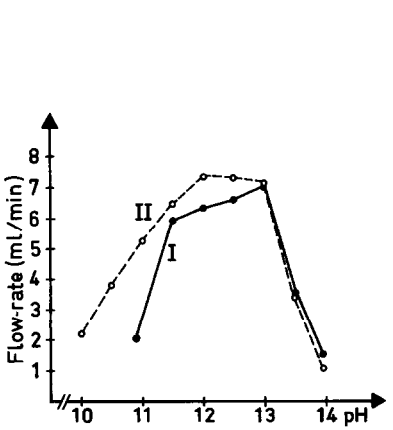
Initially the cross-linking with DVS was performed as described by Porath *et al.*[14]. However, as mentioned, we found that the resulting gels strongly adsorbed Blue Dextran and some of our model proteins (see Fig. 1). In an early attempt to suppress the adsorption, we added sodium borohydride to the reaction mixture in order to avoid destruction of agarose. No adsorption of Blue Dextran was then observed, but unfortunately the maximum flow-rate obtained was considerably lower than for gels cross-linked in the absence of sodium borohydride. Although it seemed unlikely, we would not completely exclude the possibility that this decrease in flow-rate (cross-linking) was due to saturation of one or both of the double bonds in part of the DVS molecules by the hydrogen liberated from the sodium borohydride. In another cross-linking experiment the borohydride was therefore replaced by a stream of nitrogen. The result was, however, unsatisfactory, since the adsorption of Blue Dextran was still strong. The problem of suppressing adsorption without decreasing the flow-rate was not solved until in some preliminary experiments we measured the flow-rate (at a constant pressure) as a function of the pH at which the cross-linking took place (the cross-linking experiment was performed in the presence of $NaBH_4$). It then appeared that a small decrease in pH below 11 diminished the flow-rate considerably. When we investigated whether the addition of sodium borohydride changed the pH of the 0.5 *M* sodium carbonate solution used as the reaction medium in ref. 14, we found that the pH dropped from 11.3 to 10.7 upon addition of sodium borohydride to a concentration of 2% (Fig. 2, upper curve). This decrease in pH was sufficient to give a lower degree of cross-linking and a lower flow-rate. During the cross-linking reaction the pH decreased further (Fig. 2, lower curve) and in a non-reproducible way. In addition, upon storage for some weeks the pH of a 0.5 *M* sodium carbonate solution decreased from 11.0 to 10.6. These observations gave us at least one explanation for the decrease in flow-rate observed when we performed the cross-linking in the presence of sodium borohydride (another possible explanation is given in Discussion). Therefore, we decided to repeat the above cross-linking

experiments with sodium borohydride as reducing agent (to suppress adsorption of Blue Dextran and haemoglobin), but at a pH > 11 to increase the degree of cross-linking, *i.e.*, the effective flow-rate. The results of these experiments were very satisfactory, as shown in Fig. 1 (the test substances no longer adsorbed) and Fig. 3 (high and reproducible flow-rates were obtained when the cross-linking was done in the interval pH 12.0–12.5).

The above observations, *i.e.*, the non-reproducible decreases in pH occurring during the cross-linking reaction and the finding that the pH of a 0.5 *M* sodium carbonate solution decreases upon storage, explain why the method described in ref. 14 for cross-linking with DVS may give variable and sometimes unexpectedly low flow-rates.

*Establishment of conditions (reducing agent, temperature, pH, reaction time, concentration of DVS, solvent for removal of excess DVS, agent for coupling to unreacted vinyl groups) for the preparation of rigid, low-adsorption, pH-stable agarose beads upon cross-linking with DVS*

The above considerations indicate that the cross-linking should be performed under reducing conditions to prevent or minimize adsorption of Blue Dextran and proteins. We have chosen sodium borohydride as the reducing agent since it has long been used to prevent degradation of agarose at high pH. The risk of destruction of agarose is, of course, more pronounced at elevated temperature. This probably explains why the conditions used in ref. 14 (pH 11, 22°C, 2 h + pH 11, 45°C, 2 h) give a somewhat discoloured agarose not only in the absence (as in ref. 14), but also in the presence of sodium borohydride. Therefore, we conduct the entire cross-linking procedure at room temperature, which gives a white product.



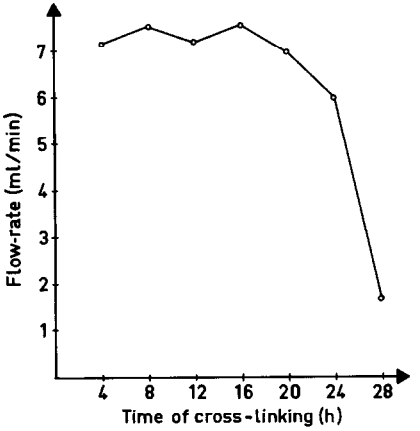




Fig. 3. The flow-rate as a function of the pH of the cross-linking procedure (in the presence of $NaBH_4$). Bed material: 12% agarose beads (5–40 μm). Column dimensions: 12 cm × 0.6 cm. Pressure: 30 bar. Curve I was obtained when the cross-linking was performed in a solution of 0.5 *M* sodium carbonate adjusted prior to the cross-linking to the pH indicated on the *X*-axis, and curve II when the cross-linking was performed in water where pH was occasionally adjusted to the pH indicated on the *X*-axis. For other experimental conditions see section First version.

Fig. 4. The relationship between the flow-rate and the time of the cross-linking reaction. The cross-linking was performed in 0.5 *M* sodium carbonate at an initial pH of 12.5. For data on material and column dimensions, see Fig. 3 and for experimental conditions see section First version.

Fig. 3 shows that cross-linking at pH 12.0–12.5 in the presence of sodium borohydride not only gives high, but also reproducible flow-rates, since the curves have a flat maximum in this pH region. Small variations in pH during the cross-linking can therefore be tolerated.

Fig. 4 shows that the flow-rate is independent of the time used for the cross-linking reaction in the interval 4–20 h, which also contributes to high reproducibility.

The amount of DVS required to give maximum rigidity and flow-rate varies with the agarose concentration. For a 12% agarose gel the appropriate amount is given in the next section. However, with one of the DVS solutions used (which had a slightly brown coloration) considerably more DVS was required. Therefore it may be advisable to do some preliminary experiments with any new batch of DVS to determine the amount of DVS required for the cross-linking.

The removal of the excess of DVS should not be performed with distilled water (as in ref. 14), but in water of pH 11.5–12.0 (adjusted with potassium hydroxide), because DVS is not very soluble in distilled water; the removal of DVS should be done in the presence of $NaBH_4$ to avoid degradation of agarose. After washing at this high pH the agarose is white. When the washing is done at neutral pH the gels are yellowish.

Glycine or mercaptoethanol has been recommended for deactivation of unreacted, free vinyl groups in DVS[14]. However, the use of glycine gives rise to a charged matrix at normal pH values and mercaptoethanol leads to the introduction of thioether groups, which (co-operatively with the sulphone groups) may cause complicated interactions with proteins[18]. For these reasons we avoid these deactivating compounds and use instead neutral and extremely hydrophilic substances such as galactose (one of the monomeric units of agarose), D-mannitol and OH-rich polymers, for instance dextran (see below). These compounds not only deactivate unreacted vinyl groups but also improve the chromatographic properties of the cross-linked gel for the following reasons. (1) They decrease the hydrophobicity of the matrix, since the reduction in OH groups in the agarose chains caused by the cross-linking procedure is counteracted (or even overcompensated) by the OH groups in galactose, mannitol or dextran. (2) They can form a cross-linking bridge between two unreacted vinyl groups, thereby increasing the rigidity of the agarose gel, *i.e.*, the flow-rate. Since the distances between these vinyl groups in the agarose are different, a compound containing many OH groups at different distances from each other has a good chance of giving a cross-link. From this point of view, mannitol and dextran might be preferable to galactose. (3) The increase in OH groups facilitates the attachment of different ligands to agarose, for instance, for ion-exchange and hydrophobic interaction chromatography.

*Detailed description of the method for cross-linking of agarose with DVS*

*First version.* Guided by the results described in the two preceding sections we devised the following procedure for cross-linking of agarose with DVS. The method is described for cross-linking of the relatively small amounts of agarose beads required for HPLC. There are, however, no obstacles to scaling up the method.

About 10 g of sedimented 12% agarose beads, prepared as described previously[17], was suspended in an Erlenmayer flask in 12 ml of 0.5 *M* dipotassium hydrogenphosphate adjusted to pH 12.0–12.5 with 4 *M* potassium hydroxide. [We

previously used 0.5 *M* sodium carbonate, but since its buffering capacity is virtually nil in this pH interval ($pK_a$ = 10.3) we now prefer dipotassium hydrogenphosphate ($pK_a$ = 12.3).] One can also perform the experiment in water if the pH is maintained at pH 12.0–12.5 by occasional addition of 4 *M* potassium hydroxide. About 0.06 g of $NaBH_4$ were added with stirring followed by 0.6 ml of DVS. The stirring was continued for 17 h at room temperature. The agarose suspension was then centrifuged at 500 *g* for 10 min, the supernatant was removed and the gel resuspended in about 12 ml of water of pH 11.5–12 (adjusted with 4 *M* potassium hydroxide) containing 0.06 g of $NaBH_4$. After stirring, the suspension was centrifuged again and the supernatant removed. Three additional washings removed most of the excess of DVS. For coupling to unreacted vinyl groups D-mannitol (2 g) and $NaBH_4$ (50 mg) were added to the beads suspended in 20 ml of water adjusted to pH 12.0 with 4 *M* potassium hydroxide. After stirring for 6 h, the gel beads were washed five times (or to neutrality) with water by centrifugation.

*Second version.* When we observed that an agarose gel cross-linked as described was more stable at high pH than we had expected (see Fig. 6), the coupling between the free vinyl groups and mannitol (galactose, dextran, etc.) was further enhanced by increasing the pH from 12 to 13.7 *i.e.*, that obtained after suspending the washed, centrifuged agarose beads (10 g) in 10 ml of 1 *M* sodium hydroxide. This modification of the cross-linking procedure makes the agarose gel more hydrophilic, as evidenced by molecular sieve chromatography with human transferrin in a medium of high ionic strength to test for hydrophobic interaction (0.05 *M* sodium phosphate, pH 7, containing 1.3 *M* ammonium sulphate): the transferrin adsorbed when 2 g of mannitol was treated with the free vinyl groups at pH 12, whereas no adsorption was observed when the reaction proceeded at pH 13.7. However, the cross-linked gel treated with mannitol at pH 13.7 was still somewhat hydrophobic, since lysozyme was strongly retarded, although it migrated as a narrow well defined zone in the ammonium sulphate-containing buffer (the elution volume was five times greater than the total volume). When the mannitol was exchanged for 2 g of Dextran T 10 (mol. wt. 10000) the elution volume was considerably reduced. A large hydrophilic molecule (such as dextran) thus seems to shield the hydrophobic patches of the cross-linked gels better than a smaller one (such as mannitol). We want to stress, however, that in a "normal" buffer, such as 0.05 *M* sodium phosphate, pH 7 (in the absence of ammonium sulphate) lysozyme has the same elution volume (close to the total volume) whether the treatment of the agarose is done with mannitol, Dextran 10 or Dextran 500. It is accordingly only at extremely high ionic strengths that the hydrophobic interaction becomes significant. The lower limit of the separation range of these three differently treated agarose gels is around 15000 daltons (see Table I), since on these gels not only lysozyme (mol.wt. 12000) but also cytochrome *c* (12400) and ribonuclease (13700) have an elution volume in 0.05 sodium phosphate (pH 7) close to the total volume (determined by potassium chromate).

As shown in the next section and in Table I, the pore size of the agarose gel is affected when dextran is coupled to the gel: the higher the molecular weight of the dextran, the smaller is the effective pore size. By this simple technique the exclusion limit of a gel can easily be adjusted to the desired value.

It is interesting that we have observed a 10–30% increase in flow-rate when the reaction with mannitol is done at pH 13.7 instead of 12.0 (an example is shown

TABLE I

SEPARATION RANGES OF DVS-CROSS-LINKED 12% AGAROSE GELS

The free vinyl groups had been reacted with OH-containing substances of different molecular weights.

| *Substance* | *Molecular weight (daltons)* | *Separation range* | |
|---|---|---|---|
| | | *Exclusion limit (daltons)* | *Lower limit (daltons)* |
| D-Mannitol | 182 | 450 000 | 15 000 |
| Dextran T 10 | 10 000 | 200 000 | 15 000 |
| Dextran T 500 | 500 000 | 45 000 | 15 000 |

in Fig. 5, curves 1 and 2), probably reflecting the statement in point 2 on p. 106 (the reaction proceeds faster at the higher pH). The flow-rate could, however, be increased further by cross-linking with a five-fold amount of DVS (3 ml) and in the presence of 240 mg of $NaBH_4$ (Fig. 5, curve 3). A 30 cm × 0.6 cm I.D. bed of 17-$\mu$m, 12% agarose beads cross-linked with this larger amount of DVS (and 240 mg $NaBH_4$) permitted a maximum flow-rate of 5 ml/min (40 bar).

The risk of adsorption of Blue Dextran (and proteins) diminishes with increasing concentration of sodium borohydride. However, the experiments presented in Fig. 7 show that one should not exceed 240 mg of sodium borohydride per 10 g of sedimented 12% agarose beads when high flow-rates are required.

In later experiments we have removed the excess of DVS following the cross-linking step by washing with distilled water without adjusting the pH to 11.5–12 as in the first version of the cross-linking method. The reason is that in these later experiments the unreacted vinyl groups of DVS molecules immobilized to agarose

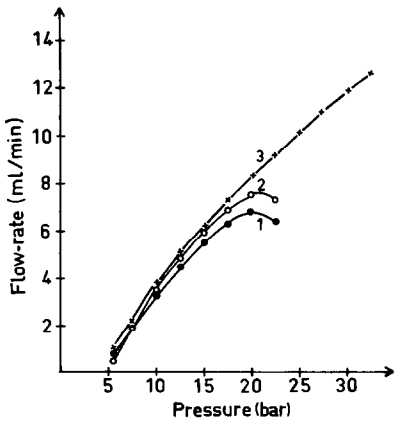



Fig. 5. Flow-rate as a function of pressure for cross-linked 12% agarose beads prepared under different conditions. Curves 1 and 3 correspond to the First and Second version, respectively of the cross-linking method described. Curve 2 was obtained when the experiment corresponding to curve 1 was repeated with the difference that the pH for the treatment with mannitol was 13.7 instead of 12. Bead diameter: 10–40 $\mu$m. Bed dimensions: 6 cm × 0.6 cm I.D.

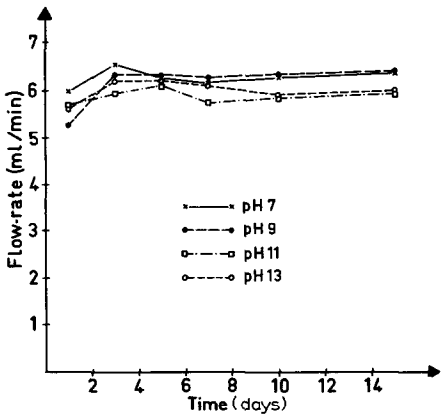



Fig. 6. The pH stability of agarose beads cross-linked with DVS as described in this paper. The cross-linking was performed in water the pH of which was occasionally adjusted to pH 12.5. The flow-rate was measured at a pressure of 35 bar for gel beads treated at different pH values during a period of 15 days. For data on the bed material and column dimensions, see Fig. 3. The pH stability of the cross-link is quite satisfactory, since no significant decrease in flow-rate was observed. (×) pH 7; (●) pH 9; (□) pH 11; (○) pH 13.

were blocked by addition of mannitol at pH 13.7, at which pH the reaction is much faster than at pH 11.5–12 (the pH we previously used); the excess of free DVS molecules will also react readily with mannitol at this higher pH and form a complex which is water-soluble and therefore easily removed in the subsequent washings with water.

To summarize, about 10 g of sedimented 12% agarose beads are suspended in 10 ml of 0.5 *M* sodium phosphate buffer, pH 12.5. Sodium borohydride (0.20–0.24 g) is immediately added with stirring followed by 3 ml of DVS. The stirring is continued for 16 h at room temperature. The agarose beads are then washed several times with distilled water by centrifugation at 500 *g* for 10 min until the supernatant has a pH of 7–8. The sedimented beads are then suspended in 10 ml of 1 *M* sodium hydroxide containing 0.1 g of $NaBH_4$ and 2 g of D-mannitol (or other OH-rich compounds). After stirring for 6 h the gel beads are washed with water by centrifugation until the pH of the gel suspension is about 7.

*The pH stability of the DVS-cross-linked agarose*

Since the DVS treatment of agarose as described in the literature has been reported to give a product which is not stable above pH 9[14,15,19], it was interesting to determine whether the cross-linked agarose prepared by our method had the same weakness (the first version of the cross-linking method was used in this experiment). Since the purpose of the cross-linking is to increase the allowed flow-rate it was important to see how this parameter was affected by exposure of the cross-linked gel to solutions of different pH for different periods of time.

The experiment, performed at room temperature in 0.05 *M* solutions of sodium phosphate of pH 7.0, 9.0, 11.0 and 13.0, gave the result shown in Fig. 6, indicating that the cross-linking was stable even at pH 13 during the whole test period (15 days). The stability is thus excellent. A separate experiment indicated that the cross-linking

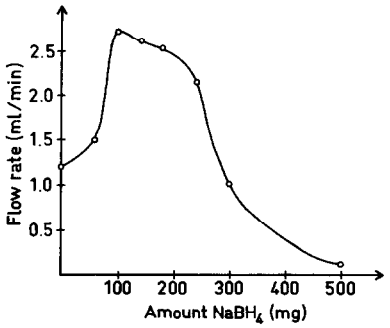



Fig. 7. Flow-rate as a function of the amount of $NaBH_4$ used in the cross-linking reaction. Bed dimensions: 5 cm × 0.6 cm. Diameter of the 12% agarose beads: 15–20 μm. The flow-rate was measured at a pressure of 5 bar.

was not even affected by treatment with 1 *M* sodium hydroxide (pH 14) for 15 h. This means, for instance, that columns of DEAE agarose cross-linked with DVS can be regenerated at a high pH in the conventional way without any decrease in flow-rate[11].

*A simple method to decrease the exclusion limit of the cross-linked agarose gels*

The reaction with free, unreacted vinyl groups was performed as described above under Second version with 2 g of mannitol (mol. wt. 182) or 2 g of Dextran T 10 (mol. wt. 10000) or T 500 (mol. wt. 500000). As expected, the exclusion limits in molecular sieve chromatography [determined with Blue Dextran (mol. wt. $2 \cdot 10^6$), dimer (132000) and monomer (66000) of human serum albumin, ovalbumin (43000) and chymotrypsinogen A (25000) in 0.05 *M* sodium phosphate pH 7.0] decreased with increasing molecular weight of the carbohydrate quencher (Table I).

*The flow-rate as a function of the sodium borohydride concentration used in the cross-linking*

We have pointed out above that the addition of sodium borohydride to the reaction mixture lowers its pH (Fig. 2), which explains why an initial pH of 11 (literature value) does not give optimum cross-linking. However, in some later experiments where the sodium borohydride concentration was considerably higher than that normally used, we found that gels of low rigidity were obtained, even when the pH during the cross-linking was kept at 12–13. This observation prompted us to investigate in more detail how the flow-rate depends on the concentration of sodium borohydride used in the cross-linking procedure. A series of experiments were therefore performed as described under Second version, with the exception that the amount of $NaBH_4$ was varied. The result is presented in Fig. 7, which shows that an optimum flow-rate was obtained when 100–200 mg of sodium borohydride were used per 10 g of sedimented 12% agarose beads (the optimum amount of sodium borohydride varies with the experimental conditions used).

## APPLICATIONS

The first version of our cross-linking method (see above) has been used in a

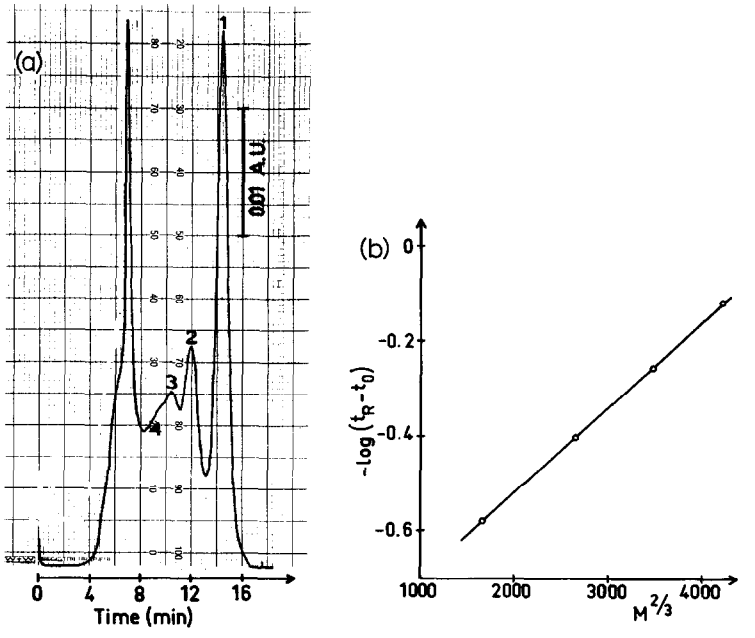



Fig. 8. (a) High-performance molecular sieve chromatography of human serum albumin on 12% cross-linked agarose. The cross-linked agarose was prepared as described under Second version. Bead size: 5–10 μm. Bed dimensions: 33 cm × 0.6 cm I.D. Sample volume: 5 μl. Eluent: 0.05 sodium phosphate, pH 6.8. Flow-rate: 0.4 ml/min. Pressure: 45 bar. Peaks: 1 = monomer; 2 = dimer; 3 = trimer; 4 = tetramer. (b) A plot of $-\log (t_R - t_0)$ against $M^{2/3}$ ($t_R$ = retention time; $t_0$ = retention time of a large molecule migrating with the void volume; $M$ = the molecular weight).

series of experiments described, for instance, in refs. 3–11. An application showing the performance of beads cross-linked according to the improved second version is presented in Fig. 8. It is noteworthy that the column has such high resolution that it can be used to reveal whether an albumin monomer is contaminated not only by dimer, but also by trimer and tetramer, particularly after a plot of $-\log (t_R - t_0)$ against $M^{2/3}$ ($t_R$ = retention time; $t_0$ = retention time of a totally excluded molecule; $M$ = molecular weight) since the points should fall on a straight line if they correspond to these forms of albumin (see Fig. 8b). This plotting technique has the advantage that it does not require any assumptions regarding the mechanism of molecular sieve chromatography (gel filtration), because it is based on thermodynamic considerations[20].

## DISCUSSION

The reaction between a cross-linker and a matrix can proceed such that either one or both of the functional groups of the cross-linker react with the matrix. In the former case the unreacted group is free to be used for different reactions, provided that no side reactions have converted it into an unreactive group. By coupling an OH-rich compound to these residual unreacted groups of a bifunctional cross-linker, the hydrophilicity and the rigidity of a chromatographic bed can be improved, as

shown in this paper for the reaction between mannitol (galactose, dextran) and free vinyl groups in agarose-linked DVS (*cf.*, ref. 21). The method has, of course, general applicability and can be used for other types of gels, cross-linkers and OH-containing compounds. The method can also be used to derivatize a matrix. The cross-linking and the derivatization are thus done as two consecutive steps in the same procedure. We have utilized this straightforward method to synthesize an agarose-based high-performance ion exchanger by coupling diethylaminoethanol to the unreacted vinyl groups in agarose-linked DVS[11].

Lysozyme is one of the very strongly hydrophobic model proteins used in methodological studies of hydrophobic interaction chromatography (HIC). It is therefore a very suitable protein to test a matrix for its degree of hydrophobicity, particularly when the experiments are done at high ionic strengths (around 4 in the studies presented). This test revealed that the mannitol-treated cross-linked agarose still had some hydrophobic patches which, however, could be efficiently shielded by attachment of high-molecular-weight dextran. In conventional buffers, where the ionic strength is much lower, the hydrophobic interaction is considerably reduced and for most proteins is quite negligible. This is evident from Fig. 8b, since all points fall on a straight line, indicating that all interactions, including electrostatic and hydrophobic ones, are negligible (note that albumin is a rather strongly retarded protein in HIC[13]). We have, however, developed a new cross-linking method that gives an agarose matrix that is so hydrophilic that lysozyme shows no hydrophobic interaction, not even in a buffer containing 1.3 *M* ammonium sulphate. This method will be discussed elsewhere.

Fig. 7 shows a plot of flow-rate (which is a measure of the rigidity of the beads) against the amount of sodium borohydride used in the cross-linking reaction. It is interesting that the curve has a maximum. The low flow-rates obtained in the absence of sodium borohydride or at low concentrations are probably due to degradation of agarose caused by the high pH. The finding that high sodium borohydride concentrations also give rise to a decrease in rigidity may perhaps be explained by the observation that this reducing agent can react with double bonds and hydroxyl groups[22–24] and therefore possibly with the vinyl group in DVS or/and OH groups in agarose.

ACKNOWLEDGEMENTS

The authors are much indebted to Dr. Zhao-qian Liu for many stimulating discussions. This work was supported by the Swedish Natural Science Research Council and the Alice and Knut Wallenberg and Carl Trygger Foundations.

REFERENCES

1 S. Hjertén and K. Yao, *J. Chromatogr.*, 215 (1981) 317–332.
2 S. Hjertén, *Acta Chem. Scand., Ser. B*, 36 (1982) 203–204.
3 S. Hjertén, Z.-q. Liu and D. Yang, *J. Chromatogr.*, 296 (1984) 115–120.
4 S. Hjertén, *Protides Biol. Fluids*, 30 (1983) 7–17.
5 S. Hjertén, *Trends Anal. Chem.*, 3 (1984) 87–90.
6 S. Hjertén and K.-O. Eriksson, *Anal. Biochem.*, 137 (1984) 313–317.
7 S. Hjertén and D. Yang, *J. Chromatogr.*, 316 (1984) 301–309.

8 L.-G. Öfverstedt and K.-O. Eriksson, *Anal. Biochem.*, 137 (1984) 318–323.
9 K.-O. Eriksson, *J. Biochem. Biophys. Methods*, 11 (1985) 145–152.
10 K.-O. Eriksson and S. Hjertén, *J. Pharm. Biomed. Anal.*, 4 (1985) 63–68.
11 S. Hjertén and B.-l. Wu, *J. Chromatogr.*, 341 (1985) 295–304.
12 S. Hjertén, K. Yao, Z.-q. Liu, D. Yang and B.-l. Wu, *J. Chromatogr.*, 354 (1986) 203–210.
13 S. Hjertén, K. Yao, K.-O. Eriksson and B. Johansson, *J. Chromatogr.*, 359 (1986) 99–109.
14 J. Porath, T. Låås and J.-C. Janson, *J. Chromatogr.*, 103 (1975) 49–62.
15 T. Låås, *Protides Biol. Fluids*, 23 (1976) 495–503.
16 D. Eaker, personal communication.
17 S. Hjertén, *Biochim. Biophys. Acta*, 79 (1964) 393–398.
18 J. Porath, F. Maisano and M. Belew, *FEBS Lett.*, 185 (1985) 306–310.
19 J.-C. Janson, *Biochem. Soc. Trans.*, 13, No. 6 (1986) 1049–1052.
20 S. Hjertén, *J. Chromatogr.*, 50 (1970) 189–208.
21 J. Porath and L. Sundberg, *Nature (London)*, 238 (1972) 261–262.
22 C. F. Lane, *Chem. Rev.*, 76 (1976) 773–799.
23 H. C. Brown and S. Krishnamurthy, *Tetrahedron*, 35 (1979) 567–607.
24 M. Konieczny, D. Charytonowicz and A. D. Inglot, *Arch. Immunol. Ther. Exp.*, 30 (1982) 1–9.