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SIMPLE METHOD TO PREPARE NON-CHARGED, AMPHIPHILIC AGAROSE DERIVATIVES, FOR INSTANCE FOR HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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SUMMARY

γ-Glycidoxypropyltrimethoxysilane is coupled in an aqueous medium to agarose via the trimethoxy groups. The epoxide ring can then be employed for attachment of, for instance, alcohols in solutions of dioxane or acetone and in the presence of boron trifluoride diethyl etherate as a catalyst. In the range pH 4–10 the stability of the linkage between the ligand and agarose is similar to that of ligands coupled to agarose via an ether bond. A method is also described where the epoxide reaction with alcohols is catalyzed by stannic chloride. Both catalysts can degrade cross-linked agarose. However, by using appropriate experimental conditions for the stannic chloride-catalyzed reaction the degradation is negligible, which is of importance when high flow-rates are desired as in high-performance liquid chromatography.

INTRODUCTION

We have previously described a method for the preparation of agarose derivatives by which glycidyl ethers of the components to be attached are allowed to react with the hydroxy groups in the agarose beads[1]. This technique was used particularly for the preparation of amphiphilic, neutral agarose beads for hydrophobic interaction chromatography. The first step in this coupling method involves the synthesis of the glycidyl ether from an alcohol and epichlorohydrin in the presence of boron trifluoride diethyl etherate as a catalyst[2]. We will now describe a coupling method in which this relatively time-consuming synthesis step is avoided. This simple immobilization method, which we have used for several years with good results, involves as a first step the preparation of an epoxide-containing agarose derivative. Compounds con-

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taining hydroxy groups, for instance alcohols, are then coupled to the agarose via the epoxide group with either boron trifluoride or stannic chloride as a catalyst.

MATERIALS

γ-Glycidoxypropyltrimethoxysilane (Silane Z-6040) was a gift from Dow Corning (Midland, MI, U.S.A.). Boron trifluoride diethyl etherate was obtained from Merck-Schuchardt (Darmstadt, F.R.G.) and stannic chloride from Mallinckrodt (St. Louis, MI, U.S.A.). Sepharose® 4B, Sepharose® CL-4B and Octyl-Sepharose® CL-4B were gifts from Pharmacia Fine Chemicals. Agarose (EEO, −0.17) was obtained from Réactifs IBF (Villeneuve, La Garenne, France).

Phycoerythrin and phycocyanin from *Ceramium rubrum* were prepared as described[3].

EXPERIMENTAL AND RESULTS

Synthesis of γ-glycidoxypropylsilylagarose

The reaction scheme is outlined in Fig. 1a. About 35 g of sedimented Sepharose 4B (agarose concentration: about 4%) was washed three or four times with 100-ml portions of water, and resuspended in 100 ml of water; 10 ml of γ-glycidoxypropyltrimethoxysilane were added dropwise from a pipette. The suspension was stirred at room temperature for 30 min. The pH was around 6; it was not adjusted to any particular value, since the reaction is not pH sensitive (see below). The gel beads were washed several times with dioxane (or acetone) and then suspended in 100 ml of dioxane (or acetone).

Coupling of alcohols to γ-glycidoxypropylsilylagarose with boron trifluoride as a catalyst (method A)

The principle of the reaction is shown in Fig. 1b. The coupling procedure will be described for octanol, although the same technique can be used for other alcohols (although the reaction times may be different).

A 5-ml volume of boron trifluoride diethyl etherate ($BF_3 \cdot Et_2O$) was added with stirring to the above suspension of γ-glycidoxypropylsilylagarose in dioxane (or
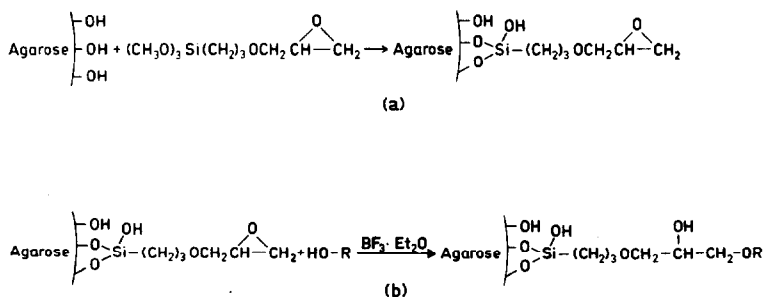

(a)

(b)

Fig. 1. (a) The reaction between agarose and γ-glycidoxypropyltrimethoxysilane. The number of bonds to the agarose (here assumed to be two) depends, among other things, on the number of sterically available hydroxy groups in the agarose. (b) The reaction between alcohols and γ-glycidoxypropylsilylagarose.

acetone) followed by 5 ml of *n*-octanol. After stirring for 45 min the gel was washed on a büchner funnel or by centrifugation at 1000 *g* according to the following procedure: dioxane, 100 ml; dioxane–acetone (1:1), 100 ml; acetone, 100 ml; five 100-ml portions of water.

Test for covalent attachment of octanol to agarose

Part of the Sepharose 4B gel, presumed to contain octyl groups, was packed into a Pasteur pipette and equilibrated with 0.01 *M* sodium phosphate (pH 6.8) containing 4 *M* sodium chloride. The red protein phycoerythrin was adsorbed strongly to the column in the latter buffer, but was eluted easily upon omission of the sodium chloride. In a control experiment, phycoerythrin was not adsorbed to γ-glycidoxypropylsilylagarose treated with boron trifluoride as described above but without addition of octanol. The adsorption of the protein was accordingly due to hydrophobic interaction with the octyl groups[4,5]. An indication that these groups were covalently coupled to agarose was the fact that the adsorption of phycoerythrin was not affected by extensive washings of the gel with various solvents: acetone–water (1:2, 2:1); acetone; ethylene chloride–acetone (2:1, 1:2); ethylene chloride; tripropylene glycol methyl ether–water (1:2, 2:1); tripropylene glycol methyl ether; dioxane–water (1:2, 2:1); dioxane; hexane–dioxane (1:2, 2:1); hexane; decanol–dioxane (1:2, 2:1); decanol. Additional evidence for covalent binding of the ligand was obtained as follows. The gel was first liquified by boiling. When the temperature decreased to room temperature a gel was formed again. The gel block was granulated by pressing it through a net[6]. The gel granules still retained the capacity to adsorb phycoerythrin at high (but not low) ionic strength.

The long-term pH stability of the amphiphilic agarose derivatives

It is of some importance to know the pH-stability of the bond between the ligand and agarose. To measure this we first tried to determine the amount of silicon in the gels, but could not obtain reproducible analyses. Therefore, we decided to measure the protein capacity of the gels, *i.e.*, the amount of phycoerythrin adsorbed to octylagarose treated with buffers of various pH; a decrease in adsorption indicates a breakage of the ligand bond. The experiment was performed as follows.

Portions (2 ml) of a suspension of a uniform slurry containing 1 g of sedimented octylagarose, prepared as described above, were pipetted into each of 55 Pasteur pipettes equipped at the bottom with a plug of glass wool. These columns were divided into eleven groups (1–11), each group containing five columns (I–V). The columns I–V in each group were equilibrated with 0.1 *M* sodium citrate–phosphate buffers (the molarity refers to the phosphate ions) of pH 4.0, 5.6, 7.0 and 0.1 *M* glycine–sodium hydroxide buffers of pH 8.6 and 9.8, respectively; 5.0 ml of each of these five buffers were used for equilibration, which proceeded for about 2 h. The columns in group 1 were then equilibrated with 5.0 ml of 0.01 *M* sodium phosphate buffer, pH 7, containing 4 *M* sodium chloride. A 2-ml volume of phycoerythrin in this buffer ($A_{500}^{1 cm} \approx 0.35$) was applied to each of these five columns. After washing with 1 ml of the same buffer, the absorbance, A_e, at 500 nm of the 3 ml of eluate was measured. The absorption, A_s, of 2 ml of the sample solution of phycoerythrin diluted with 1 ml of the buffer was also determined. The amount of phycoerythrin adsorbed is proportional to $A_s - A_e$.
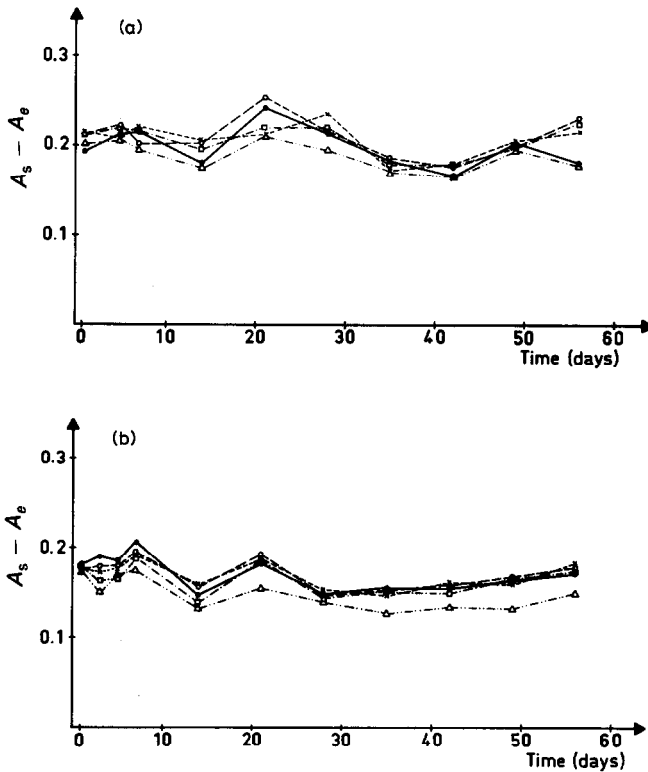
Fig. 2. A test of the pH-stability of octylagarose prepared by method A (a) and of commercial octyl-Sepharose CL-4B (b). $A_s - A_e$ is a measure of the protein capacity of the column. The capacity (which is related to the ligand density) was measured after exposure of the gels to different pH values during 8 weeks. As seen, the stability is good in the range pH 4–10. Octyl-Sepharose CL-4B is cross-linked, whereas octylagarose, prepared as described above, is not. pH values: 4.0 ($\triangle$— $\cdot$ $\cdot$ —$\triangle$); 5.6 ($\bullet$—$\bullet$); 7.0 ($\bigcirc$----$\bigcirc$); 8.6 ($\times$---$\times$); 9.8 ($\square$— $\cdot$ —$\square$).

The columns in group 2 were left to stand at the five different pH values for 3 days and were then equilibrated with the sodium chloride-containing phosphate buffer of pH 7. Phycoerythrin solution was added and the amount adsorbed was calculated from absorption measurements as above. The same procedure was then repeated for the columns in groups 3–11 after 5, 7, 14, 21, 28, 35, 42, 49 and 56 days, respectively; to increase the accuracy the experiments were done in duplicate, 110 columns being used in all. The results are shown in Fig. 2a.

For comparison the same experiments were performed on columns packed with the commercial Octyl-Sepharose CL-4B (Fig. 2b). Sepharose CL-4B (without octyl ligands) showed no adsorption of phycoerythrin in the 0.01 M phosphate buffer containing 4 M sodium chloride. Fig. 2a and b shows that the non-cross-linked octylagarose prepared by the present method has about the same stability in the range pH 4–10 as the commercial cross-linked product prepared by reaction with octyl glycidyl ether[1], which gives a stable ether bond.

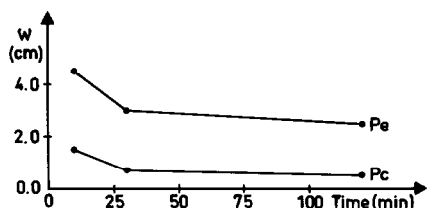Fig. 3. Effect of the reaction time on the synthesis of γ-glycidoxypropylsilylagarose. w = Width of the phycoerythrin (Pe) and the phycocyanin (Pc) zones.

Effects of reaction time and pH on the synthesis of γ-glycidoxypropylsilylagarose

One batch of γ-glycidoxypropylsilylagarose prepared exactly as described above was compared with two others prepared in the same way except that the reaction times were 10 and 120 min, respectively. After coupling of octanol by the procedure described (using $BF_3 \cdot Et_2O$ as a catalyst) the three batches were packed into Pasteur pipettes and equilibrated with 0.01 M sodium phosphate buffer, pH 7.0, containing 4 M sodium chloride. A sample of 50 μl of the coloured proteins phycoerythrin and phycocyanin was applied to each column followed by 5 ml of the buffer. The widths of the two protein zones were measured. These are related to the protein capacity, *i.e.*, the number of available octyl groups of the columns: the smaller the width the larger is the capacity. The results are presented in Fig. 3, which shows that the reaction between γ-glycidoxypropyltrimethoxysilane and agarose is complete within 30 min.

The effect of pH on this reaction was studied by a similar technique: four batches of γ-glycidoxypropylsilylagarose were prepared, at initial pH values of 4.0, 6.5, 9.0 and 11, respectively (adjusted by addition of hydrochloric acid or potassium hydroxide). After 5 min the pH values were 4.0, 6.2, 7.7 and 8.5 and after 30 min they were 3.9, 6.0, 7.0 and 8.1 respectively. After attachment of octanol the relative capacities of these batches for phycoerythrin was estimated by packing them into Pasteur pipettes and measuring the width of the protein zone as described. These experiments indicated that the capacity, and therefore the synthesis of γ-glycidoxypropylsilylagarose, is independent of the pH at which the synthesis is performed (at least in the interval pH 4–11).

Coupling of alcohols to γ-glycidoxypropylsilylagarose with stannic chloride as a catalyst (method B)

The use of this catalyst will also be described in detail because it has some advantages over boron trifluoride (see Discussion). The dioxane (or acetone) in the suspension of γ-glycidoxypropylsilylagarose was exchanged for the alcohol to be coupled by the following washing steps: dioxane–water (1:1), 100 ml; dioxane, 100 ml; dioxane–the alcohol to be coupled (1:1), 100 ml; the alcohol, 100 ml.

The beads (cross-linked by a method to be described elsewhere) were then suspended in 100 ml of the alcohol and the catalyst stannic chloride ($SnCl_4 \cdot 5H_2O$) was added to a concentration of 0.4% (w/v) with stirring. The coupling was allowed to proceed with stirring for 1 h at room temperature. The beads were washed and transferred to water as described above for procedure A. The stannic chloride-cata-

TABLE I

CAPACITY FACTOR, k', FOR TRANSFERRIN AND THE RIGIDITY OF THE AGAROSE BEADS (EXPRESSED AS MAXIMUM FLOW-RATE) FOR VARIOUS CATALYST CONCENTRATIONS AND REACTION TIMES

$SnCl_4 \cdot 5H_2O$ (%, w/v)	Reaction time (h)	k'	$V_{max\neq}$ (relative values)
0.1	0.5	1.3	—
0.1	1	1.8	4
0.1	2	3.0	3
0.1	3	3.5	3
0.1	4	∞	1
0.2	1	∞	5
0.4	1	∞	5

lyzed reaction has been used for coupling of pentanol and octanol. Other alcohols may require other reaction times and catalyst concentrations.

The reaction time (1 h) and the amount of stannic chloride (0.4%) in the above protocol were selected from the data in Table I, which shows the capacity factor, k' (which is related to the ligand density), and the relative rigidity of the beads (expressed as the maximum flow-rate obtained for a bed packed with the beads) for different reaction times and catalyst concentrations. The experiments were performed in 0.05 M sodium phosphate (pH 7) containing 1.3 M ammonium sulphate. Table I shows that when the reaction time is 1 h the catalyst concentration should be 0.2–0.4% for strong adsorption of transferrin and for a high flow-rate. The low flow-rates obtained at long reaction times indicate that some bonds in the cross-linked agarose are broken by stannic chloride, as is the case with boron trifluoride (see Discussion).

The coupling conditions for methods A and B above refer to 4 and 12% agarose beads respectively. Some changes may be required when agarose beads of other concentrations are used.
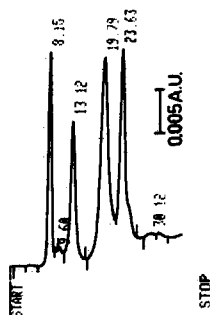

Fig. 4. High-performance hydrophobic interaction chromatography on octylagarose. The octyl groups were attached to 12% agarose by method B. Bead size: 5–7 μm. The sample consisted of 20 μl of a mixture of cytochrome c (retention time, t_R = 8.15), ribonuclease (t_R = 13.12), transferrin (t_R = 19.79) and lysozyme (t_R = 23.63). The elution was performed at a flow-rate of 0.2 ml/min on a 63 × 6 mm I.D. column. Desorption was achieved by isocratic elution during 5 min in 0.05 M sodium phosphate (pH 7.0) containing 1.0 M ammonium sulphate and then during 25 min by a negative linear salt gradient formed from this solution and 0.05 M sodium phosphate (pH 7.0).

Applications

We have recently shown that agarose beads prepared under special conditions can be used with advantage for high-performance liquid chromatography (HPLC) of biopolymers[7–16]. We have attached octyl groups to these beads by the techniques described in this paper. Fig. 7 in ref. 10 shows an example of an experiment done on an octylagarose column 1 year after the preparation of the beads, indicating that the columns can be employed over a long period of time (the coupling was by method A). During the intervening year the column was kept at pH 7 and was used occasionally. The use of method B for the preparation of amphiphilic agarose columns for high-performance hydrophobic interaction chromatography is illustrated in Fig. 4 and in ref. 17. The agarose beads were prepared as described in ref. 18 (the agarose concentration was 12%).

DISCUSSION

The method described herein for the coupling of alcohols to agarose with boron trifluoride as a catalyst is similar to that used by Chang *et al.*[19] for attachment of various ligands to silica beads used in HPLC. However, in sharp contrast to the amphiphilic derivatives of silica, those of agarose are stable even above pH 8 (Fig. 2). This is of importance for optimum utilization of a chromatographic bed material. In hydrophobic interaction chromatography, for instance, the relative retention times for the proteins to be separated, and thereby the resolution, change with pH, particularly above pH 8[17] where the silica columns unfortunately are not stable.

The γ-glycidoxypropylsilylagarose is prepared from a trimethoxysilane and can therefore probably be considered as a "bulk-modified material" with cross-linkings between the silane molecules, which decreases the risk of ligand leakage[20,21]. This risk is further reduced if the silicon atom can have two (as outlined in Fig. 1b) or three bonds to the agarose matrix. Three bonds can occur only (and two bonds perhaps more readily) if hydroxy groups from more than one agarose chain are involved. The stability of γ-glycidoxypropylsilylagarose is evident not only from Fig. 2a, but also from the finding that octylagarose prepared as described herein retained its amphiphilic character upon storage for 1 year[10].

The catalyst stannic chloride may not have been employed previously for the derivatization of chromatographic bed materials, although it was mentioned as early as 1956 as a very efficient catalyst for the reaction between epoxides and alcohols[22]. We noticed that boron trifluoride catalyzes cleavage of ether bonds when applying the above coupling method A on beads of agarose cross-linked by epichlorohydrin, since the flow-rate, and thereby the rigidity of the beads, decreased after the coupling procedure. (In order to avoid the cleavage of ether bonds, a smaller amount of boron trifluoride was used for attachment of ligand; however, the ligand density also decreased since proteins were only slightly adsorbed in a buffer containing 1.3 M ammonium sulphate.) Since stannic chloride at the concentration and with the reaction time employed in method B does not catalyze observable cleavage of any bonds (to judge from the finding that the flow-rate did not decrease upon coupling) we use this method for coupling ligands to agarose matrices designed for HPLC experiments where high flow-rates are essential. However, an increase in reaction time from 1 to 4 h (at constant catalyst concentration) increases the capacity factor, *i.e.*, the ligand

density (Table I). At the same time, the flow-rate decreases (Table I), indicating that stannic chloride can also catalyze degradation of cross-linked agarose. In this respect it resembles boron trifluoride (see above) and tribromide[23].

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