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Assay for determining the number of reactive groups on gels used in affinity chromatography and its application to the optimisation of the epichlorohydrin and divinylsulfone activation reactions

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Abstract

An assay has been developed for measuring the number of reactive groups on gels activated for use in affinity chromatography with epichlorohydrin or divinylsulfone. The activated gel is reacted with mercaptoacetic acid and the number of acidic groups introduced is determined by titration. The results obtained are in good agreement with those obtained by elemental analysis and are significantly higher than those obtained by reaction with sodium thiosulfate. Using this assay, the epichlorohydrin and divinylsulfone activation reactions were optimised so that, by varying the amount of activating reagent and time of the reaction, Sepharose CL-4B can be activated in a controlled manner, to between 2 and 34 μmol active groups per ml of gel with epichlorohydrin, and to between 2 and 55 μmol active groups per ml of gel with divinylsulfone. Activation at pH 12 was found to be optimal for divinylsulfone activation. The stability of these activated gels to incubation for 40 h over the pH range 2–14 was examined, as was the stability to long-term storage at 4°C.

Keywords: Affinity adsorbents; Epichlorohydrin; Divinylsulfone; Activated gels

1. Introduction

The ligand density is one of the most important parameters in affinity chromatography [1–4] and should be carefully controlled during the synthesis of an affinity adsorbent to ensure batch-to-batch reproducibility. The synthesis usually occurs as a two-step process, with the support being first activated, then reacted with the ligand. Since the optimal ligand density is rarely the highest obtainable [2,4] it is necessary to limit either the degree of activation or, more commonly, the coupling efficiency [1,5,6].

When a highly activated gel is reacted with a limiting amount of ligand, the excess active groups must be blocked prior to chromatography [6,7]. This is usually achieved by reaction with low molecular mass thiols or amines. With the blocking species often present at higher concentrations than that of the ligand, care must be taken to ensure that these agents do not affect protein binding. Amines introduce a positive charge at neutral pH, while 2-mercaptoethanol, the thiol commonly used, may itself act as an affinity ligand when attached to a divinylsulfone-activated support [8].

The problems associated with blocking the excess active groups may be avoided if the gels are instead limited in their degree of activation, and then reacted to completion with the ligand. This approach re-

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quires a method to activate gels to defined and predictable levels, which in turn requires methods for the analysis of activated gels. There must also be efficient methods for the coupling of the ligands and, ideally, for assessing the final ligand density.

In this work Sepharose CL-4B is activated with epichlorohydrin [9] or divinylsulfone [10]. Both reagents activate agarose gels under aqueous conditions and the activated gels react smoothly with amines and thiols. Divinylsulfone activation may be determined by elemental analysis of the sulfur introduced. However, with the possibility of hydrolysis of the active vinyl group or of reaction of the introduced vinyl group with a second hydroxyl group on the matrix, elemental analysis may seriously overestimate the concentration of active groups. The epichlorohydrin activation method does not lend itself to elemental analysis.

A better approach for analysing the activation density is based on the reactivity of the activating group, where the activated gel is reacted with a reagent that allows subsequent analysis. The reaction must be quantitative, there must be no side reactions occurring that would cause an over-estimation of the number of active groups present and there must be a means of accurately determining the amount that has reacted.

Most activated gels are reactive towards nucleophiles. One method for determining the degree of activation of vinylsulfone and epoxide activated gels exploits this, that is, with the reaction of the activated matrix with sodium thiosulfate [11]. This is followed either by titration of the hydroxyl ions released [6,11], or by reduction and analysis of thiols present by reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) [12] or 2,2′-dipyridyl disulfide [13].

In the current work, this reaction approach is examined with an alternative reagent, mercaptoacetic acid, which is expected to fulfil the requirements of this type of assay method. Since thiols are known to react readily with activated gels [1,8], such a low molecular mass thiol can be expected to react quantitatively. Secondly, this thiol is unlikely to react except with the introduced active groups on the gel. Finally, the introduced acidic groups provide a means for analysis by in situ titration. The method has been applied to both divinylsulfone- and epichlorohydrin-activated gels, but it should be applicable to all activated gels that react with nucleophiles. The results are compared with those obtained by analysis of the amount of sulfur introduced and with those obtained from the sodium thiosulfate assay, quantified by both the titration and the thiol analysis methods.

The mercaptoacetic acid assay of activation was found to be an improvement over the established thiosulfate method. It was then used to further study the epichlorohydrin- and divinylsulfone-activation reactions. The degree of activation was examined over time as a function of reagent amounts, and for the divinylsulfone reaction, as a function of the activation pH. The stability of the epoxy- and vinylsulfone-activated gels over a wide range of pH conditions was examined, as was the stability of the activated gels to long-term storage at 4°C.

2. Experimental

Sepharose CL-4B was purchased from Pharmacia (Sydney, Australia). Divinylsulfone, epichlorohydrin (1-chloro-2,3-epoxypropane) and mercaptoacetic acid were from Sigma Aldrich (Sydney, Australia). Tris (purity greater than 99.9%) was obtained from Boehringer Mannheim (Melbourne, Australia) and was used without further purification. Milli-Q water was freshly prepared, degassed under vacuum and used where specified. All other reagents were of analytical grade. Unless otherwise indicated, all procedures were performed at room temperature.

2.1. Epichlorohydrin activation of Sepharose CL-4B

The epichlorohydrin activation procedure [14] was modified, with sodium borohydride being omitted from the reaction mixture. Damp Sepharose CL-4B was washed with NaOH of the concentration under investigation, before being suspended in 1 ml/g of the same solution. Epichlorohydrin was added and the mixture was stirred for the time indicated, before being filtered and washed with copious quantities of deionised water. The gel used in the development of the assay method was reacted with 0.2 ml of epichlorohydrin per g of gel in 1 *M* NaOH at 20°C

for 3 h, with stirring. The effects of reaction time, amounts of epichlorohydrin, concentration of NaOH and temperature were then examined.

2.2. Divinylsulfone activation of Sepharose CL-4B

Divinylsulfone activation was carried out with modifications to the procedure of Ersson [10]. To damp Sepharose CL-4B was added 1 ml/g of gel of 0.5 *M* carbonate (0.25 *M* carbonate in the final suspension), adjusted to the appropriate pH with NaOH or HCl. Divinylsulfone was added and the mixture was stirred at 20°C for the time indicated before being filtered and washed with deionised water. The assay conditions were determined on gel activated at pH 11 with 50 μl of divinylsulfone per g of gel, with stirring for 1 h at 20°C. The effects of pH, amount of divinylsulfone and reaction time were then examined.

2.3. Mercaptoacetic acid coupling

Mercaptoacetic acid solutions were always freshly prepared, with 1 ml added per g of gel. Coupling proceeded with stirring at room temperature for the time indicated, before filtering and washing of the sample. The pH optimum for the coupling of mercaptoacetic acid was determined by reacting epoxy- and vinylsulfone-activated gels overnight with 0.2 *M* mercaptoacetic acid in a buffer consisting of 50 m*M* phosphate, 50 m*M* borate and 50 m*M* carbonate, adjusted to pH 8, 9, 10, 11 or 12. The thiol concentration and time required for maximum coupling was determined by varying the mercaptoacetic acid concentration from 0.1 to 2 *M* and following the time course of the reaction with both epichlorohydrin- and divinylsulfone-activated gels at pH 10.5. The conditions giving maximum coupling and used for the remainder of the experiments were reaction for 1 h with 1 *M* mercaptoacetic acid in 0.1 *M* carbonate at pH 10.5.

2.4. Titrations

Titrations were performed on 1 ml (drained volume) samples of the gel. This volume was obtained by pouring a suspension of slightly more than 1 ml of gel in water into a small chromatography column with the volume of 1 ml clearly marked. Water was allowed to drain away under gravity and the excess gel was removed so that exactly 1 ml of the drained gel remained in the column. Reproducibility was ensured by always using columns of the same dimensions (23.5×7.4 mm I.D.). The acidic groups on the gel were protonated by washing with 5 ml of 0.1 *M* HCl in Milli-Q water and the excess acid was removed by washing with 15 ml of Milli-Q water. Trials of the protonation procedure were performed by washing with 5 ml of 1 *M* HCl or 10 ml of 0.1 *M* HCl and by varying the water wash from 10–20 ml. The gel was transferred to a vial and 5 ml of 0.1 *M* NaCl in Milli-Q water that had been freshly degassed under vacuum was added. The gel was then titrated with a solution of Tris of known concentration, prepared in degassed Milli-Q water. Manual titrations were performed with the Tris added portionwise via a 100-μl glass syringe, and the pH was noted at each step. The pH was plotted as a function of the amount of Tris added and the end-point of the titration was determined from the inflection point of the plot, which occurred at pH 6.4, midway between the pK_a values of Tris and the coupled mercaptoacetic acid. Alternatively, titrations were performed automatically, with the Tris added via a Dosimat E535 (Metrohm Herisau, Switzerland) pump system and the pH was monitored using a Metrohm pH meter, E512. The pump and pH meter both had signal output and were connected to an Omniscribe (Activon, Sydney, Australia) dual-input chart recorder. To ensure that the Tris was added sufficiently slowly for the pH meter to attain a steady reading, the Dosimat system was set on the slowest pump speed (0.2 ml/min) and connected to a laboratory-made switch, so that the pump was only on for 1 s in 20. The amount of Tris added and the resulting pH were recorded using an Omniscribe dual input chart recorder. The end-point was determined to be the volume of Tris corresponding to the inflection point of the recording of pH against time.

2.5. Sulfur analyses

One ml portions (drained volume) of the gels were washed as described in Section 2.4 and were dried to constant weight at 40–50°C under vacuum. Sulfur analyses were performed on the dried samples by

NAL Laboratories (Notting Hill, Vic., Australia). The %S (w/w) values obtained were normalised using the mass per ml values determined above to μmol of S per ml of adsorbent.

2.6. Thiosulfate reaction

The degree of activation of 1 ml of drained gel was determined by reaction with thiosulfate [11] and titration of the hydroxyl groups released according to Pepper et al. [6]. The concentration of HCl was determined by titration with the same Tris solution used for the mercaptoacetic acid titration analyses.

2.7. Thiol analyses

After titration, the thiosulfate reacted gels (1 ml) were washed with deionised water and equilibrated with 50 m*M* potassium phosphate buffer, pH 8. The thiosulfate was reduced with 0.1 ml of 2-mercaptoethanol in 2 ml of the above solution. The reduced gels were then washed with degassed 50 m*M* His, pH 6 (6 ml) and reacted with 20 ml of 20 m*M* DTNB [11,12] in 50 m*M* potassium phosphate buffer, pH 7. The gels were washed with the pH 7 buffer and the amount of reacted DTNB was determined ($E_{410\ nm}$ 13 600 M^{-1} cm^{-1}) [12].

3. Results and discussion

3.1. Development of the mercaptoacetic acid assay

The number of acidic groups on the gel was determined by titration with a standard solution of 0.1 *M* Tris. Analyses were performed on a drained volume of 1 ml of gel, since it is the volume rather than the dry weight of an adsorbent that is the useful parameter in chromatography. Protonating the acidic groups on the gel was achieved by washing with 5 ml of 0.1 *M* HCl; increasing the amount or concentration of HCl did not result in an increase in the number of acidic groups detected (not shown). No difference in the number of acidic groups was observed on varying the water wash from 10–20 ml (not shown) so 15 ml was used in further work. Titrations were performed either manually or using an automated system, with the same results obtained

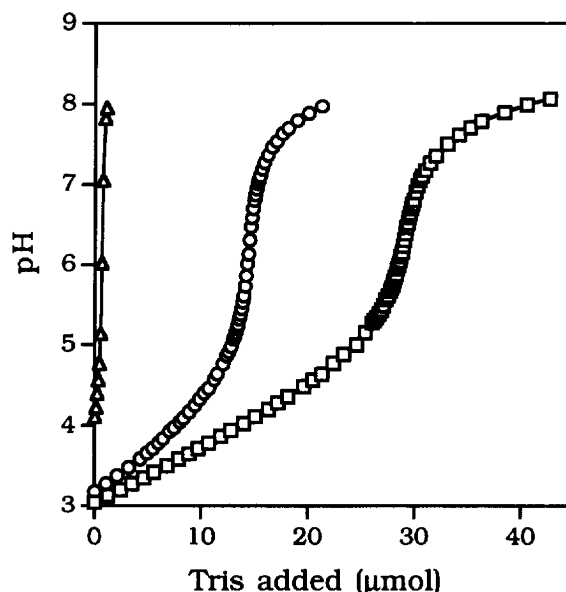


Fig. 1. Titration curve for mercaptoacetic acid-treated epichlorohydrin (□) and divinylsulfone (○) activated Sepharose CL-4B, and untreated non-activated Sepharose Cl-4B (△).

from both methods. Typical titration curves for the acidic group analyses are shown in Fig. 1. The end point is determined from the steepest part of the curve and the results for the gels used in developing the assay method are given in Table 1. The experiments were performed in quadruplicate and the range of the data is indicated by the error estimate.

It is not possible to demonstrate that a reagent reacts to completion with the active groups without an independent measurement of the degree of activation. For the purposes of this assay it was deemed sufficient to find conditions where maximum reaction of the mercaptoacetic acid with the activated gel occurs. The pH optimum was determined by overnight reaction of both the epoxy- and vinylsulfone-activated gels with 0.2 *M* mercaptoacetic acid over the pH range 8–12. There was found to be little variation in the amount coupled over the range pH 8–11 for the vinylsulfone gel and over the pH range of 9–12 for the epoxy gel (data not shown), and pH 10.5 was chosen for the coupling. The time course of the reaction was followed over the concentration range 0.1–2 *M* thiol. The reaction was rapid, complete after 1 h for concentrations of thiol greater than 50 m*M*. The amount of mercaptoacetic acid coupled

Table 1
The dry weight, concentration of acidic groups and concentration of sulfur for non-activated and for epichlorohydrin- and divinylsulfone-activated Sepharose CL-4B, and for these gels after treatment with mercaptoacetic acid

Sample	Dry weight (mg/ml)	Acidic groups (μmol/ml)	Sulfur content (μmol/ml)
Sepharose CL-4B	29.5±0.2	0.6±0.2	0.9±0.5
Sepharose CL-4 treated with mercaptoacetic acid	29.3±0.3	0.5±0.1	1.8±0.5
Epichlorohydrin-activated Sepharose CL-4B	32.6±0.3	0.6±0.1	1.0±0.5
Epichlorohydrin-activated Sepharose CL-4B reacted with mercaptoacetic acid	35.9±0.4	28.8±0.2	28.0±0.6
Divinylsulfone-activated Sepharose CL-4B	30.5±0.1	0.6±0.2	15.2±0.5
Divinylsulfone-activated Sepharose CL-4B reacted with mercaptoacetic acid	33.2±0.6	14.6±0.1	32.2±0.5

increased with the concentration of the thiol for both activated gels, up to a thiol concentration of 0.8 *M*, with no increase in the amount coupled on increasing the concentration to 2 *M*. After mercaptoacetic acid coupling using the conditions chosen (reaction with 1 *M* mercaptoacetic acid at pH 10.5 for 1 h), the reacted epichlorohydrin-activated gel was found to have 28.8 μmol/ml acidic groups and the reacted divinylsulfone-activated gel contained 14.6 μmol/ml (Table 1).

By treating non-activated gel with mercaptoacetic acid under the same conditions, it was shown that there is no background reaction of mercaptoacetic acid with the gel. The concentration of acidic groups present on the non-activated Sepharose CL-4B (0.6 μmol/ml) is the same as is found on the mercaptoacetic acid-reacted Sepharose, and on the unreacted epichlorohydrin- and divinylsulfone-activated gels (Table 1). Agarose is known to contain acidic groups, predominantly as sulfate and pyruvate groups [15], and although the agarose is extensively desulfated under alkaline reducing conditions [16] in the preparation of Sepharose CL-4B, it is perhaps not surprising that a small concentration of acidic groups still remain.

The degree of activation of the epoxy- and vinylsulfone-activated gels was obtained by subtracting the background concentration of acidic groups from the concentration of the mercaptoacetic acid-coupled activated gels, giving 28.2 μmol/ml for the epoxy-activated Sepharose and 14.0 μmol/ml for the divinylsulfone-activated gel (Table 2).

An alternative way to measure the amount of mercaptoacetic acid coupled and hence the number of active groups is by elemental analysis, determining the amount of sulfur introduced. Sulfur analyses were performed on gels dried to constant weight and the results were converted to μmol of S per ml of gel by determining the dry weight of 1 ml of each gel sample (Table 1). It is interesting to note that the dry weight of the sample increases with substitution of the agarose hydroxyl groups with ether-linked activating groups, and again as the ligand is coupled (Table 1). Often when gel samples are analysed, the results are presented as dry weight of gel, which clearly cannot be converted to a volume amount without measuring the relationship between these parameters for that particular gel.

There is a low background level of sulfur in the untreated Sepharose CL-4B, as well as in the mercaptoacetic acid-treated and in the epoxy-activated gels, indicating that some sulfur remains after the desulfating procedures [16]. The number of active groups in the epichlorohydrin-activated gel can be obtained by subtracting this background level from the amount of sulfur in the mercaptoacetic acid-reacted gel. This gives 27.1 μmol of epoxy groups per ml of gel, which is in good agreement with the results obtained from the titration method (Table 2).

Activation with divinylsulfone introduces one

Table 2
Comparison of assay methods for determining the number of active groups per ml of gel for epichlorohydrin- and divinylsulfone-activated Sepharose CL-4B

Assay method	Epichlorohydrin-activated Sepharose CL-4B (μmol/ml)	Divinylsulfone-activated Sepharose CL-4B (μmol/ml)
Mercaptoacetic acid reaction, titration of acidic groups	28.2±0.4	14.0±0.3
Mercaptoacetic acid reaction, analysis of sulfur	27.1±0.6	17.0±0.5 14.3±0.5
Thiosulfate reaction, titration of released hydroxyl ions	23.8±0.5	4±1
Thiosulfate reaction, analysis of thiol by reaction with DTNB	24.2±0.4	5.8±0.8

sulfur atom per active group to the gel, and a second is introduced on reaction of the activated gel with mercaptoacetic acid, giving two possible measurements of the number of active groups. The degree of divinylsulfone activation may be obtained by subtracting the amount of sulfur in the untreated Sepharose from the amount in the activated gel, giving a value of 14.3 μmol/ml. Were hydrolysis of the vinyl group to occur, this value would be an overestimation of the number of active groups present. Alternatively, the number of active groups can be obtained from the amount of sulfur introduced on reaction of the activated gel with mercaptoacetic acid, giving the value of 17.0 μmol/ml. If the coupling reaction did not go to completion, so that not all of the vinyl groups introduced reacted with the mercaptoacetic acid, this second value would be an underestimation of the number of active groups on the divinylsulfone gel. Given that there is no background reaction of the mercaptoacetic acid with the matrix, there cannot be more sulfur introduced on coupling the thiol to the vinylsulfone-activated gel than was introduced on activation, and so there must be a slight error in the sulfur analyses. In any case, the discrepancy between these data and the results from the acidic group titration is not large (Table 2).

In order to compare the reaction with mercaptoacetic acid as a method of analysis with the established thiosulfate method, the epichlorohydrin- and divinylsulfone-activated gels were reacted with 1 *M* sodium thiosulfate and the released hydroxyl ions were titrated with 0.1 *M* HCl [6,11]. The degree of activation obtained and the spread of data for four analyses is shown in Table 2. The degree of epichlorohydrin activation as determined by this method is about 15% lower than that determined by the mercaptoacetic acid method, while the amount determined for the divinylsulfone activation is 75% lower. It would seem that the thiosulfate method provides a serious underestimation of the degree of activation, especially for vinylsulfone gels.

An alternative method for determining the amount of thiosulfate coupled to the gel is by reduction of the thiosulfate groups and analysis of the amount of thiol groups attached to the matrix [11,17] via reaction with DTNB [12]. These analyses were performed on the same thiosulfate samples used in the experiment above and the results and spread of data obtained are given in Table 2. The results obtained by this method are in agreement with the results obtained from titration of the hydroxyl ions released, indicating that the reaction of these gels with thiosulfate was not quantitative.

3.2. Optimisation of the epichlorohydrin activation of Sepharose CL-4B

The degree of epoxy activation as a function of time and concentration of epichlorohydrin was determined at 20°C and in 1 *M* NaOH (Fig. 2A). Borohydride was included in the original procedure [9] to prevent alkaline peeling [18], but it was not necessary for Sepharose CL-4B, which is treated with a reducing agent in the manufacturing process.

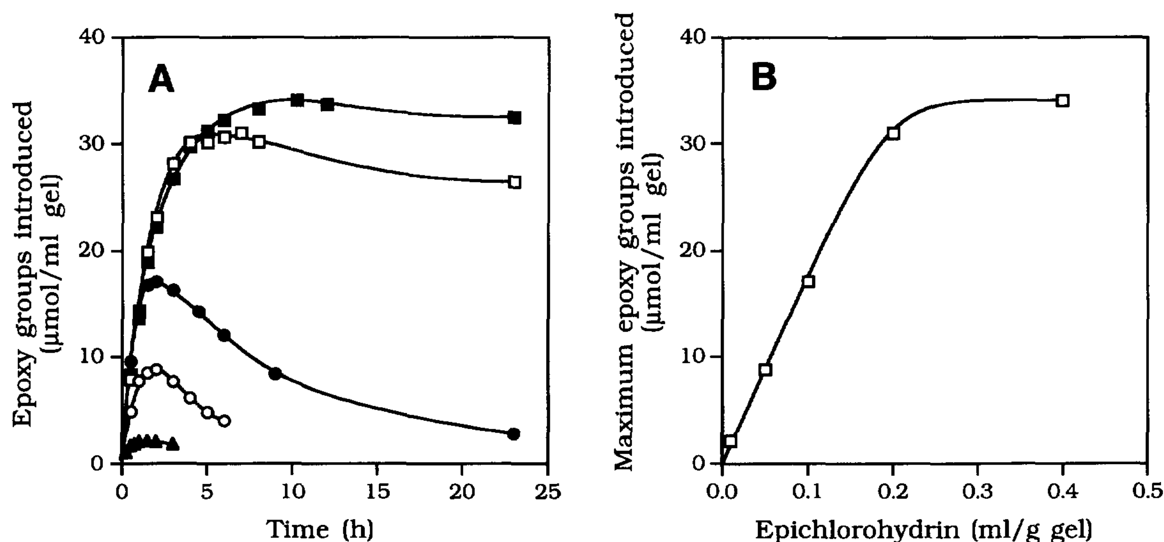


Fig. 2. (A) Degree of epoxide activation over time as determined using the mercaptoacetic acid assay for Sepharose CL-4B activated with (▲) 0.01, (○) 0.05, (●) 0.1, (□) 0.2 and (■) 0.4 ml of epichlorohydrin/g of gel in 1 *M* NaOH at 20°C. (B) The maximal level of activation as a function of the amount of activating agent.

For each amount of epichlorohydrin, the degree of activation increases with time up to a maximal value, and then decreases slowly, suggesting that the activated gel is either being hydrolysed or is able to react with other hydroxyl groups in the Sepharose matrix, forming cross-links. In a separate experiment, the loss of epoxy groups on incubation in 1 *M* NaOH was determined to follow pseudo first-order kinetics with a rate of 0.4 h^{-1} (not shown). Under activation conditions, both the activation and hydrolysis or cross-linking reactions occur simultaneously. For the higher amounts of epichlorohydrin, the loss of epoxy groups at later times is less pronounced; this is presumably due to there being sufficient reagent to contribute to the activation reaction even after several hours, rather than due to a decrease in the hydrolysis reactions (Fig. 2A). The maximal degree of activation increases with the amount of reagent up to 31 μmol/ml active groups with 0.2 ml of epichlorohydrin per g of gel, but at higher levels of reagent there was not much increase in the activation, with only 34 μmol/ml active groups obtained from 0.4 ml of epichlorohydrin/g of Sepharose (Fig. 2B).

The activation reaction was also examined at 60°C, with 0.1 ml of epichlorohydrin per g of Sepharose, in both 1 and 0.1 *M* NaOH. In 1 *M* NaOH, the hydrolysis or cross-linking reactions were so fast that the maximum activation occurred at 10 min and was less than the maximum observed for this amount of epichlorohydrin at room temperature. Reducing the concentration of NaOH to 0.1 *M* decreased the rate of hydrolysis or cross-linking, but also decreased the rate of activation, so that again the maximum activation obtained was less than for 1 *M* NaOH at 20°C (data not shown).

Epichlorohydrin activation may be conveniently carried out at room temperature in 1 *M* NaOH, with the amount of reagent appropriate to the desired level of activation of Sepharose CL-4B determined from Fig. 2B.

3.3. Optimisation of divinylsulfone activation of Sepharose CL-4B

The pH optimum for divinylsulfone activation was determined by following, over time, the degree of activation over the pH range 10–13 (Fig. 3). In each case, 0.5 *M* carbonate was adjusted to the appropriate pH using a pH meter. Activation at pH 13 proved to be inconveniently fast, with the maximum level of activation observed at 5 min. The activated

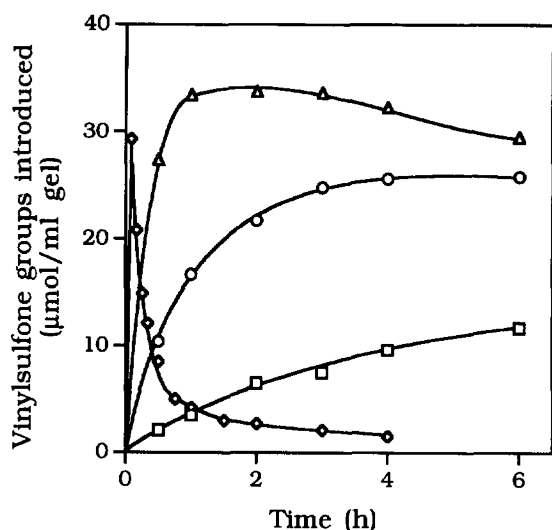
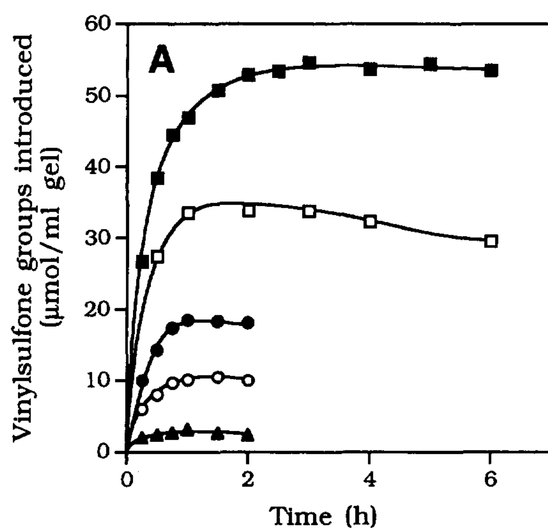


Fig. 3. Vinylsulfone activation of Sepharose CL-4B over time as determined using the mercaptoacetic acid assay for gel activated with 50 μl of divinylsulfone/g of Sepharose in 0.25 M carbonate at (□) pH 10, (○) pH 11, (△) pH 12 and (◊) pH 13.

gel was incubated at pH 13 and the loss of active groups, either by hydrolysis of the vinyl group or by cross-links being formed with other hydroxyls on the matrix, was rapid, occurring with a pseudo-first order rate constant of 9 h^{-1} (data not shown). At pH 12 the activation was more convenient, with a maximum level observed at 1 h, followed by only a slow decrease in the number of active groups, due to activation and hydrolysis or cross-linking reactions occurring simultaneously. In another experiment, the loss of active vinyl groups at pH 12 was determined to follow pseudo first-order kinetics with a rate of 0.4 h^{-1}. Divinylsulfone activation at pH 10 or 11 was slower, with the number of active groups still increasing after 6 h. The original method for the vinylsulfone activation of Sepharose 6B was at pH 11, with activation for only 70 min [10], giving lower levels of activation per ml of reagent than was potentially obtainable. Although vinylsulfone activation at pH 10 and 11 had not reached the maxima within the 6 h of the experiment, and so could perhaps reach levels as high as, or higher than for pH 12, activation at pH 12 was chosen for the remainder of the work.

Activation with divinylsulfone at pH 12 was examined over time with varying amounts of reagent (Fig. 4A). The maximum degree of activation was plotted against the amount of reagent (Fig. 4B). In this case, the levels of activation continued to increase with the amount of reagent; 0.1 ml of divinylsulfone per g of Sepharose was the highest amount studied, giving 55 μmol active groups per



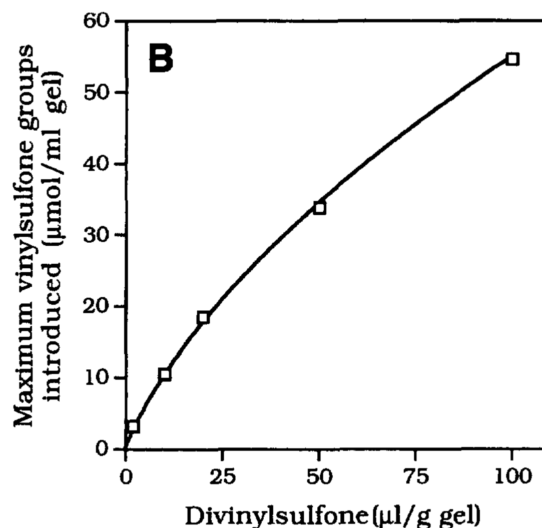


Fig. 4. (A) Vinylsulfone activation of Sepharose CL-4B as determined using the mercaptoacetic acid assay over time at pH 12 and with (▲) 2 μl, (○) 10 μl, (●) 20 μl, (□) 50 μl and (■) 100 μl of divinylsulfone/g of gel in 0.25 M carbonate at pH 12 at 20°C. (B) The maximal level of activation as a function of the amount of reagent.

ml of gel. Using these activation conditions, the amount of divinylsulfone required to achieve the desired level of activation may be obtained from Fig. 4B.

3.4. Stability of epichlorohydrin- and divinylsulfone-activated gels

The stability of epoxy and vinylsulfone groups to a range of pH conditions was examined. To achieve the highest possible coupling efficiency, it is important that the active groups are not hydrolysed at the coupling pH over the necessary time period. The epoxy-activated gel was found to be stable over a wide pH range, with more than 90% of the active groups remaining after 18 h incubation over the pH range 3–12 (Fig. 5). It was found to be less stable in acidic conditions, with more than 75% of the active groups being hydrolysed following an 18-h incubation in 10 m*M* $HClO_3$. Acid hydrolysis could provide a useful alternative to blocking excess epoxy groups when cost or solubility considerations mean that only low concentrations of the ligand can be used, leaving unreacted groups.

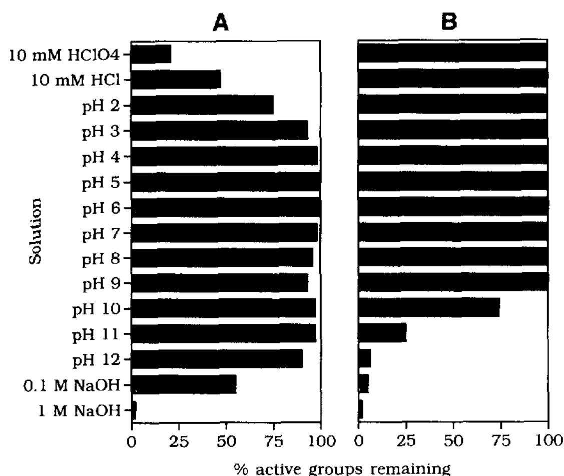


Fig. 5. The proportion of (A) epoxy groups and (B) vinylsulfone groups remaining after incubation for 18 h (total bars) or 40 h (light shaded bars) in 3 ml/g gel of the following solutions: 10 m*M* perchloric acid; 10 m*M* hydrochloric acid; 0.1 *M* phosphate buffer pH 2; 0.1 *M* citrate adjusted to pH 3, 4, 5 or 6; 0.1 *M* phosphate with 0.1 *M* borate at pH 7, 8, 9, 10, 11 or 12; 0.1 *M* NaOH and 1 *M* NaOH. The initial levels of activation were 22.0 μmol/ml for the epoxy gel and 20.2 μmol/ml for the vinylsulfone gel. The number of epoxy or vinylsulfone groups were determined by the mercaptoacetic acid titration method.

The vinylsulfone-activated gel was very stable to incubation under acidic or neutral conditions, with no loss of the vinylsulfone groups on incubation for 40 h from 10 m*M* $HClO_3$ to buffer at pH 8. There was some loss of active groups as the pH increased; for this gel, coupling at high pH would not be recommended.

The stability of the epoxy and vinylsulfone groups to storage at 4°C in 20% ethanol in water (pH 7.2) was also examined, with almost 80% of the epoxy groups and 95% of the vinylsulfone groups remaining after twelve weeks (data not shown).

4. Conclusion

Reaction of activated gels with mercaptoacetic acid followed by titration of the introduced acidic groups provides a convenient method for analysing the degree of activation of gels that are reactive towards nucleophiles. The method was applied to the optimisation of the epichlorohydrin and divinylsulfone activation methods, so that by varying the amount of reagent the degree of activation could be controlled. It is expected that the assay would be applicable to determining the degree of activation using other activation methods.

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