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Bifunctional etherification of a bead cellulose for ligand attachment with allyl bromide and allyl glycidyl ether

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Abstract

Efficient carboxymethylation of a bead cellulose was achieved with dimethyl sulphoxide–water mixtures but low activation levels were found with epoxidation reagents. The highest activation levels were obtained with allyl bromide and allyl glycidyl ether. This was attributed to the relative inertness of the allyl group towards water and cellulose, limiting hydrolysis and crosslinking. Advantages over epoxidation chemistry for bead cellulose were efficient reagent use, reproducible activation up to high levels and specificity of matrix modification. Predictable activation was obtained and facile titration methods were developed. Efficient activations of agarose and methacrylate matrices were also obtained with the allyl reagents.

Keywords: Bead cellulose; Stationary phases, LC; Etherification; Allyl bromide; Allyl glycidyl ether; Cellulose

1. Introduction

Attachment of ligands to chromatographic supports usually requires pre-activation of matrix functional groups (e.g., hydroxyl). Preferably this activation is (i) aqueous and inexpensive, (ii) allows attachment of a wide range of ligands, through bonds stable between pH 1 and 14 and (iii) allows the option of an uncharged and hydrophilic linkage. The activated group should be stable, yet be sufficiently reactive for ligand attachment. Chemical modification should not compromise preferred matrix properties [1,2]. Many activation methods have been reported [3–6], but all contain at least one major drawback, especially for large scale use. Several require anhydrous reaction conditions [7–12] use expensive reagents for activation [11,12] or ligand attachment [13], and/or produce weak linkages [14]. If the ligand and matrix are compatible with a pH>9, epichlorohydrin [15] and bisepoxide [16] chemistry have the best combination of aqueous activation, reagent cost and bond strength properties for general use.

Epoxidation has been used to immobilise a wide range of hydroxyl, thiol and amine containing ligands by strong ether, thioether and amine bonds [16–21]. Crosslinking and epoxide hydrolysis side reactions compete with activation, limiting the efficiency of reagent use and hence the activation level which can be obtained. Uncontrolled crosslinking may also have a deleterious effect on chromatographic properties e.g., steric restrictions and reduced porosity. High epoxidation levels reported for Sepharose [15,16,22], have not been reproduced with

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Perloza [23] or Divicell [24] bead celluloses. Perloza has good physical and chemical properties for low pressure chromatography [25–28] but has been limited by lack of efficient aqueous activation methods.

A preferred strategy for activation with bifunctional reagents would be to "block" one functional group for the activation step and subsequently remove the "blocking group", analogous to the amino acid coupling techniques of solid-phase peptide synthesis [29]. A similar effect could be obtained by using bifunctional reagents whose functional groups (and their reactivities) are substantially different. Ideally one functional group would react readily with the matrix, but the second would be relatively inert. The second group should nevertheless be reactive with a wide range of ligands with or without prior conversion to a more reactive form. An example of this is carboxymethylation with chloroacetic acid followed by a carbodiimide catalysed condensation with an amine ligand.

A preferred bifunctional reagent would activate the matrix by etherification, attaching a less reactive group by an hydrophilic spacer arm. The less reactive group would be used for subsequent attachment of ligands by O-, S- or N-alkylation. Such an activation has been reported for Sephadex, using epichlorohydrin and an acid catalyst [30]. The chlorohydroxypropyl ether formed was unreactive at the acidic activation pH but did react with nucleophilic ligands under alkaline conditions. This method has also been applied to Perloza bead cellulose [31]. However these activations were carried out in dry dichloromethane and a 25:1 mixture of dioxane and water, respectively. This requirement for organic solvents would contribute significantly to the cost and difficulty of these methods.

An alternative approach would be etherification of matrices with an allyl halide or allylglycidyl ether. At alkaline pH the halide or glycidyl group should be reactive but the allyl group is expected to be comparatively inert. After allylation matrices could be "activated" by aqueous bromination to form bromohydroxypropyl groups and then used to attach nucleophilic ligands. Activation of cellulose and agarose with allyl reagents and subsequent ligand substitution was described recently [32,33] but experimental details were limited.

In this report, bifunctional etherification of Perloza bead cellulose is described, using conventional and new reagents, and aqueous or mixed organic–aqueous solvation. Optimised allyl activation chemistry and simple, reliable titration methods are reported. Activation of other matrices with these reagents is also reported. The advantage of allyl reagents, which contain functional groups of dissimilar reactivity, over epihalohydrins and bisepoxides is discussed.

2. Experimental

2.1. Reagents and equipment

Perloza MT (various grades) bead celluloses were from Tessek or ICS, both of Prague, Czech Republic; Sepharose 6B and CL6B were from Pharmacia, Uppsala, Sweden; Sepabeads FP-HG13 from Mitsubishi, Tokyo, Japan; butanediol diglycidyl ether (70%) and dimethyl sulphoxide (DMSO) from Sigma, St. Louis, MO, USA; allyl glycidyl ether, mercaptoacetic acid and mercaptopropionic acid from Janssen Chimica, Geel, Belgium; epichlorohydrin from Dow Chemical, Midland, MI, USA; propylene oxide and chloroacetic acid from J.T. Baker, Phillipsburg, NJ, USA; butadiene diepoxide and ethyleneglycol diglycidyl ether (50%) from Aldrich-Chemie, Steinheim, Germany; calcium hydroxide, allyl bromide and Convol NaOH and HCl were from BDH, Poole, UK; bromine from Hopkin and Williams, Essex, UK; barium hydroxide octahydrate from Riedel-de Haen, Selze, Germany; and sodium sulphite from May and Baker, Manchester, UK. Fractogel HW 55 was a gift from Industrial Research, Gracefield, Lower Hutt, New Zealand. Ethanol was technical grade and water Milli-Q grade. All other reagents were analytical grade. Epibromohydrin was prepared from glycerol by the methods of Braun [34]. Allyl bromide was distilled prior to use unless otherwise noted.

2.2. General reaction methods

Reactions at room temperature were mixed by rotation (Ballmill roller or Cole-Parmer Roto-Torque rotator) or shaking (Ika Vibra-mix), unless otherwise indicated. Reactions at elevated temperature were

incubated in a water bath (without mixing). Reactions were mixed in glass vials or jars (25–1250 ml capacity). Perloza 100 fine (80–100 μm beads) was used for activation reactions unless otherwise indicated. Perloza, solvated with water or DMSO–water mixtures, was prepared and suction-dried using a sintered glass funnel. Activation mixtures (epoxide and allyl) are identified by the volume of reagent used, expressed in ml/g suction-dried matrix or as a percentage. The units used are g for suction-dried and g dry for oven-dried matrix masses. Titration data are expressed accordingly in mmol/g or mmol/g dry.

2.3. Carboxymethylation of Perloza

The method of Peterson and Sober [35] was adapted, using aqueous solvated Perloza, chloroacetic acid and 30% NaOH in the ratio of 10 g:0.45 g:1.6 ml or 10 g:0.95 g:3.3 ml. Subsequent experiments used 20 g Perloza solvated with acetone (or ethanol)–water (75:25) or DMSO–water (80:20), prepared by a batch solvent exchange [7]. Chloroacetic acid (0.5 g for DMSO resins, 0.9 g for acetone and ethanol resins) was dissolved in 7.5 *M* NaOH (3.5 ml per g of chloroacetic acid) and mixed (30 s) with suction-dried Perloza, in a stainless-steel vessel. Propylene oxide (0.6–1 ml) and epichlorohydrin (0.12–0.2 ml) were included with the DMSO resins to control the swelling properties of the product [36]. The vessel was then sealed and immersed in a water bath (60°C), for 4 h.

2.4. Epihalohydrin and bisepoxide activations

The standard method used for epichlorohydrin (ECH) activation was adapted from that described by Matsumoto et al. [22] for agarose. Perloza (5 g) was mixed with ECH (0.75 ml) and 0.66 *M* NaOH (10 ml) for 9 h at room temperature (18°C). Epibromohydrin was used similarly to ECH except 3 ml DMSO was included after 3 h. Less water (80% DMSO solvated Perloza, 3.5 ml 2 *M* NaOH), higher temperature (37°C, 3 h reaction) and more reagent (2.5 ml ECH and 11 ml 2 *M* NaOH) were used in other ECH experiments. The method of Sundberg and Porath [16] was adapted for bisepoxide activations. Perloza 100 and 200 fine (10 g) were mixed with 5 ml of 1 *M* NaOH and bisepoxide (2.5 ml butadiene diepoxide, 3.75 ml BDE and 4.25 ml ethyleneglycol diglycidyl ether). Reactions were at room temperature and sodium borohydride was not included. In other reactions 75% DMSO solvated Perloza was used: 5 g cellulose was mixed with 0.5 ml butadiene diepoxide and 20 g cellulose reacted with 10 ml BDE (+0.5 and 2 ml of 1 *M* NaOH, respectively).

2.5. Allyl bromide activation

2.5.1. Initial conditions

Initial activations used 75% DMSO solvated Perloza (10 g) reacted at 60°C (4 h) with a 3% or 6% AB activation mixture, with 1 ml of 3.75 *M* NaOH included per 0.3 ml AB. The percentage refers to the volume of reagent (ml) per 100 g cellulose. A 6% reaction was also carried out for 1 h at room temperature. Subsequently a 24 h reaction at room temperature was standard.

2.5.2. Other base reagents

In some (6%) activations sodium hydroxide solutions were replaced by calcium hydroxide, barium hydroxide or potassium phosphate (0.5, 2 and 3 g, respectively) per ml AB used. Barium hydroxide was also used for a 10% activation of 75% DMSO solvated Perloza (10 g), 5–15% activations of water solvated Perloza (5 or 10 g, with 0.2 ml DMSO included per g) and a 25% activation of water solvated Perloza (10 g).

2.5.3. Solvent variations

The standard reaction (24 h) mixture was 10 g water solvated Perloza, 1.5 ml solvent, 0.7 ml AB and 2.4 ml of 3.75 *M* NaOH. Solvents used were: DMSO, dioxan, acetone, ethanol and water. DMSO solvation was also used for a 7 h reaction and another sample contained 1.4 g $Ba(OH)_2$ and 2 ml water instead of NaOH solution.

2.5.4. Optimised Perloza activation

Water solvated Perloza (10 g), with or without 1.5 ml DMSO, was mixed with 0.7 ml AB and 3 ml of 3 *M* NaOH, 4.5 ml of 2 *M* NaOH or 5 ml of 2 *M* NaOH. Subsequently DMSO use was abandoned. A stock 7% activated Perloza was prepared by reaction

of 100 g Perloza with 7 ml AB and 45 ml of 2 *M* NaOH. For other AB percentages, the volume of NaOH used was maintained between 0.4–0.45 ml of solution/g Perloza. However the NaOH molarity was varied such that 12.5–13 mmol of hydroxide were used per ml (approximately 12 mmol) of AB. For example a 2% AB activation of 10 g Perloza used 4.5 ml of 0.6 *M* NaOH and a 10% activation of Perloza (75 g) used 7.5 ml AB and 32 ml of 3 *M* NaOH.

2.5.5. Activation of other matrices

Sepharose 6B (10 g) was reacted with 0.5 ml AB, 0.1 ml ECH (crosslinker) and 6 ml of 1.7 *M* NaOH for 48 h at room temperature. Another 10 g sample of Sepharose 6B was reacted likewise, except that 1 ml AB and 6 ml of 2.5 *M* NaOH were used. Optimised 7% activations of Sepharose CL6B (12 g), Fractogel HW 55 (10g) and Sepabeads (8 g) and a 6% activation of Sepharose CL6B (10 g) were carried out according to the methods described in Section 2.5.4 for Perloza, except 5 ml of NaOH was used per 10 g matrix.

2.6. Allyl glycidyl ether activation

2.6.1. Initial methods

Perloza was washed with 0.3 *M* NaOH, suction-dried and 10 g samples mixed with 3 ml AGE (30% activation). Reaction mixtures were shaken manually and formed a "gel-like" suspension. These were incubated for up to 48 h at room temperature, 40°C or 60°C. Samples (1 g) were removed at various times for assay. The standard method adopted was 48 h at room temperature. Activation mixtures containing 10, 20 and 40% AGE were prepared and reacted in the same manner. The reaction mixtures used were not amenable to mechanical mixing although the "fluidity" of the 30% and 40% samples improved after 24 h of the reaction. These samples were mixed for the second half of the reaction by shaking.

2.6.2. Organic and aqueous slurry activations

Perloza (10 g) was pretreated with 0.3 *M* NaOH or a 1:1 mixture of 0.3 *M* NaOH and DMSO and suction-dried. The DMSO reaction mixture had superior "fluid" properties to the aqueous mixture and was mixed by mechanical shaking. Reaction was for 72 h at room temperature. Slurry activation samples were prepared by mixing water solvated Perloza (8 g) with 2 ml of 1.5 *M* NaOH and 1.2 ml AGE. For a repeat activation, reagents and products of the first reaction were washed out (in a sintered glass funnel) with water before the second addition of reagent and NaOH.

2.7. Titrations

2.7.1. Titration of epoxide activated and carboxymethyl Perloza

A Radiometer ETS822 autotitrator was used for all acid/base resin titrations. Matrix epoxide groups were titrated by a proprietary method. Carboxymethyl matrices were washed extensively with water, followed by 4×10 bed volumes of 0.1 *M* HCl to convert to the protonated form. Excess acid was removed by washing with at least 5×10 bed volumes of water, until the wash pH was neutral. The sample was suction-dried, weighed (1 g) into a titration vessel and mixed with 5 ml of 1 *M* NaCl. The sample was titrated with Convol 0.1 *M* NaOH to pH 8 (values in mmol/g). Titrated samples were washed with water (50 volumes), oven-dried at 110°C for 1.5 h and weighed to obtain values in mmol/g dry.

2.7.2. Bromine water titration of matrix allyl groups

Bromine water titration was by incremental addition of calibrated (approximately 2%) bromine water to a 0.5–2 g sample of activated matrix. The smallest increments (50 ml) were used after about 75% of the total addition. The endpoint was determined visually. Titration was usually completed in 2–5 min. Bromine water was calibrated by two methods:

(I) Bromine water of 1.5–2.5% was prepared as accurately as possible by weighing bromine into a 100 ml volumetric flask and dissolution in water. The molarity of the bromine water was calculated assuming no bromine vapour loss.

(II) Bromine water of similar concentration range was prepared as a stock solution and assayed immediately before use. Bromine water (0.5 ml) was diluted with water to 25 ml and its absorbance measured at 410 nm. Bromine content was calculated

by comparison with the values of standards, freshly prepared by method I.

2.7.3. Mercaptoacid titration of allyl groups and sulphonation of bromohydrin groups

Activated matrix (1 g) was mixed with 100 μl of mercaptoacetic acid (MAA) or mercaptopropionic acid (MPA), and 1–5 ml water. The mixture was incubated for 4–16 h at 60°C or for 24–48 h at room temperature (MAA only). Samples were transferred to a sintered glass funnel and excess reagent washed out with 20 ml water, 20 ml 0.1 *M* NaOH and 10×20 ml water. Preparation and titration of carboxylic acid groups was otherwise the same as in Section 2.7.1. Brominated matrices (1–1.5 g) were reacted with a 3% sodium sulphite solution at 60°C for 16 h. Further preparation and titration was as described for carboxylic acid derivatives, except 1 *M* HCl was used instead of 0.1 *M* HCl to generate the protonated matrix.

3. Results and discussion

3.1. Carboxymethylation

The reaction of water swollen Perloza (20 g) with 0.9 g chloroacetic acid resulted in a low substitution level (0.4 mmol/g dry). This was approximately 0.45 g/g dry cellulose, similar to the amount used previously [35]. The substitution level remained low (0.37 mmol/g dry) using a much greater amount of chloroacetic acid (1.9 g). The low levels were attributed to a higher water content in the mixture and hence a greater proportion of reagent hydrolysis rather than cellulose carboxymethylation. Using 75% acetone solvated Perloza (20 g) and 0.9 g chloroacetic acid, a resin substitution of 1.6 mmol/g dry was obtained. By contrast, no increase over the aqueous reaction level was found using 75% ethanol solvation.

The cellulose volume was reduced to approximately 25% of original in the alkaline acetone medium and reswelled to original or greater volume when returned to water after the reaction. This extreme shrinkage and swelling could have a detrimental effect on the matrix properties. When Perloza was solvated in a more polar solvent (DMSO), there was no apparent change in matrix volume after addition of alkali. Consistent, high carboxymethylation levels (1.3–1.4 mmol/g dry) were obtained using 0.5 g chloroacetic acid per 20 g cellulose [37]. DMSO was therefore used as the organic solvent of choice for water replacement in matrix activation experiments.

3.2. Epoxidation with bifunctional reagents

ECH activation levels up to 0.8 mmol/g dry have been reported for Sepharose [22] but application of these methods to Perloza gave much poorer results (≤0.15 mmol/g dry). Activation at higher temperature or with epibromohydrin gave slightly lower results. The activation level was increased using either 80% DMSO solvated Perloza or more ECH (Table 1) but the maximum level obtained remained moderate. The latter product had a 20% higher dry mass than original Perloza, which could not be explained by mass increase due to epoxypropyl groups. The difference was attributed to extensive matrix modification by hydrolysis and crosslinking side reactions. The relatively low activation levels were consistent with other reports [23,24].

Aqueous bisepoxide activation of Perloza resulted in low activation levels (≤0.5 mmol/g dry), despite using large amounts of reagent (Table 1). Activation levels in 80% DMSO were even lower. The most effective bisepoxide (BD) is volatile, hazardous and expensive. It was assumed that side reactions were again limiting activation levels.

The activation levels obtained with ECH and bisepoxides were much lower than those reported for Sepharose. The activation levels were at best marginal for chromatography applications which require medium to high ligand density.

3.3. Allyl bromide activation and titration methods

3.3.1. Initial experiments

An initial allyl bromide reaction with 75% DMSO solvated Perloza produced a high titration level (Table 1), despite using a small ratio (3%) of reagent to cellulose. Activation with 0.6 ml AB ("6% AB" mixture) for 3 h, bromination and substitution with sodium sulphite resulted in a ligand density significantly higher than any obtained by ECH activation (Table 1). A high activation level (0.15 mmol/g

Table 1
Comparison of Perloza activation levels with various reagents

Epoxide reagent	Reagent % ml/100 g Perloza	Activation	
		mmol/g	mmol/g dry
ECH	15	0.017	0.15
ECH (37°C)	15	0.015	0.11
Epibromohydrin	15	0.015	0.11
ECH (DMSO)	15	0.042	0.36
ECH	50	0.058	0.39
ECH (repeat)	2×50	0.073	0.63
Butanediol diglycidyl ether	37.5	0.027	0.27
Ethyleneglycol diglycidyl ether	42.5	0.041	0.39
Butadiene diepoxide	25	0.059	0.5
Butadiene diepoxide (DMSO)	10	0.014	0.13
Butanediol diglycidyl ether (DMSO)	200	<0.01	<0.1
Allyl bromide (DMSO, 60°C)	3	0.15[a]	n.d.
Allyl bromide (DMSO, 60°C)	6	0.200[b]	1.22

Activations were aqueous solvated and at room temperature unless otherwise indicated. DMSO=75% DMSO solvated cellulose was used. Activation levels for epihalohydrin and bisepoxide activated cellulose were determined by a proprietary method. Allyl bromide levels were titrated with bromine water ([a]) or by bromination and sulphonation ([b]). n.d.=not determined.

by bromine water titration) was also obtained after a 1 h reaction at room temperature, using a 6% AB mixture. All subsequent allyl bromide activations were at room temperature but the reaction time was increased to 24 h.

The above activation conditions were chosen to minimise water content and provide a slight (1.05–1.1) molar excess of hydroxide over AB. A 10–15% drop in matrix swollen volume was found for highly activated samples and it was not restored by sulphite substitution. The reduced swollen volume might have been caused by extensive crosslinking or by hydrophobic effects of the allyl groups. AB should not cause crosslinking itself [33] but impurities might do so. The AB used was slightly brown coloured whereas the pure reagent is colourless. A colourless reagent, obtained by distillation (b.p. 70°C, middle cut), was used for subsequent experiments.

3.3.2. Bromine water titration

The initial assays of activation level were hampered by variability, especially if bromine water was not freshly prepared. Quantification was improved using a spectrophotometric assay of the bromine water immediately before titration. A reasonably linear increase in absorbance at 410 nm was found up to 0.5, for a series of dilutions of stock bromine water [37]. Therefore measurements were confined to this range using the appropriate dilution. The absorbance of freshly prepared bromine water solutions correlated to: absorbance×dilution×0.12= bromine water%. This conversion was used for subsequent "quantitative bromine water titration". This bromine water titration was still less than ideal for a routine laboratory assay because of accuracy limitations and bromine fumes.

3.3.3. Alternatives to sodium hydroxide

The initial pH of reaction mixtures described above would be very high (estimated to be >14). Because lower reaction pH might reduce the rate of allyl bromide hydrolysis relative to the desired etherification, NaOH solutions were replaced by less soluble or weaker base salts and 2 ml water. A very high activation level was obtained using $Ba(OH)_2$ (0.46 mmol/g) but lower levels were found using $Ca(OH)_2$ and K_3PO_4 (both 0.1 mmol/g), consistent with the expected lower reaction pH of the latter two [38]. The results suggested that a reaction pH of 13–14 was optimal. However, reduction of matrix swollen volumes was confined to the highly activated samples produced with barium or sodium hydroxides.

Very high activation levels were obtained when higher AB percentages were used (0.71 mmol/g). Although reagent consumption was high, a 25% AB reaction with water solvated Perloza demonstrated that a high activation level (0.43 mmol/g) could be obtained in aqueous media. Therefore water solvated Perloza was used subsequently, although 10–20% DMSO was included to improve AB mixing.

3.3.4. Mercaptoacid titration at varying activation levels

The relationship between AB proportion and activation level was determined using three different titration methods. Correlation between bromine water and mercaptopropionic acid (MPA) titration values (Table 2) was reasonable and the variability due to visual assessment of titration endpoint and falling bromine content over time were avoided with the latter method. The relationship between reagent and activation level was roughly linear (Table 2), with no indication of reduced reactivity of cellulose at increasing levels of activation. A significant change in matrix swollen volume was not found at these activation levels. By contrast sulphonate titration values gave a low estimate of activation level, especially at high AB percentages.

Subsequently, slightly higher results were found when MPA was replaced by mercaptoacetic acid (MAA) and the latter was adopted for routine assay of activation levels. Completeness of reaction was assumed because titration values matched or exceeded those with other ligands and were repeatable. However bromine water could not be used to titrate any unreacted allyl groups because it is also decolorised by thioether bonds, which link the mercaptoacid groups to the matrix [39].

Table 2
Relationship between AB% and activation level: comparison of titration methods

AB (%)	Titration (mmol/g)		
	Bromine	MPA	Sulphonate
5	0.12	0.116	0.111
10	0.18	0.174	0.166
15	0.30	0.273	0.172
10 (no DMSO)	0.10	0.093	0.096

DMSO (2 ml/10 g cellulose) was included in reaction mixtures unless otherwise indicated. Bromine titration was by the quantitative method described in Section 2.7.2. Sulphonate derivatives were prepared by sulphite substitution of the brominated matrix.

3.3.5. Solvent and base effects

AB (10%) activation of water solvated Perloza was much more efficient if DMSO was included (Table 2) but no increase was found above 20% of the reaction volume [37]. With this lower proportion of DMSO, NaOH could be used without the initial pH exceeding 14. Indeed, replacement of $Ba(OH)_2$ with NaOH resulted in a small increase in activation level (Table 3). Variation of solvent type, using NaOH, showed that ethanol was a poor solvent but that replacement of DMSO with water had a relatively small effect (Table 3). This contrasted with the significant DMSO effect found with mixtures containing $Ba(OH)_2$.

3.3.6. Optimisation of aqueous activation of Perloza

Aqueous solvation was the standard adopted for allyl bromide activations. A free flowing slurry, suitable for optimal mixing, required 4 to 4.5 ml of NaOH solution per 10 g cellulose. Therefore the molarity rather than volume of NaOH solution was varied for different AB proportions. A small increase in the proportion of base resulted in a much lower activation level and slightly higher levels were found

Table 3
Effects of solvent variations on 7% AB activation level

Cosolvent	Bromine titration (ml/g)	Titration (mmol/g)		
		MPA	MAA	Sulphonate
Ethanol	1.0	0.121	0.125	0.104
Water	1.5	0.175	n.d.	0.156
Dioxan	1.6	0.190	0.195	0.156
Acetone	1.7	0.204	n.d.	0.157
DMSO	1.7	0.202	0.220	0.158
DMSO, 7 h	1.2	0.145	n.d.	0.124
DMSO, $Ba(OH)_2$	1.6	0.183	n.d.	0.139

Uncalibrated bromine water, approximately 0.1 mmol/ml was used. Good correlation was again found between bromine water and mercaptopropionic acid, whereas the sulphonate results were consistently lower. There appeared to be an upper limit to the sulphonate level of approximately 0.16 mmol/g. MAA titrations were carried out after storage of the water solvated allyl matrix for 15 months, predominantly at room temperature.

Table 4
Titration values for various AB%, scales and matrices

Reaction mixture	AB (%)	MAA titration	
NaOH, solvent		(mmol/g)	(mmol/g dry)
4.5 ml, 0.6 *M*	2	0.051	n.d.
20 ml, 2 *M* (50 g)	6	0.139	n.d.
4.5 ml, 2 *M*	7	0.169	1.22
5 ml, 2 *M*	7	0.111	0.84
45 ml, 2 *M* (100 g)	7	0.181	1.36
180 ml, 2.1 *M* (400 g)	7.5	0.188	1.46
35 ml, 2.3 *M* (80 g)	8	0.202	1.51
20.5 ml, 3 *M* (50 g)	10	0.252	1.67
300 ml, 2.2 *M* (700 g)	7.5	0.260	1.46
6 ml, 1.7 *M* (Sepharose 6B)	5	0.088	1.60
6 ml, 2.5 *M* (Sepharose 6B)	10	0.139	1.97
5.4 ml, 2 *M* (Sepharose CL6B)	6	0.099	1.42
5.4 ml, 2.2 *M* (Sepharose CL6B)	7	0.118	1.64
4.5 ml, 2.2 *M* (Fractogel)	7	0.41	1.49
3.6 ml, 2.2 *M* (Sepabeads)	7	0.19	0.48

Unless otherwise noted, 10 g Perloza fine was used. Medium Perloza was used for the 700 g reaction. 8 g Sepabeads, 10 g Sepharose 6B and Fractogel and 12 g Sepharose CL6B were used.

at larger scales. Otherwise good correlation was found between activation values and reagent level (Table 4).

3.3.7. Activation of other matrices

Reaction of Sepharose and synthetic polymers, Fractogel HW 55 and Sepabeads with 5–10% AB resulted in high titration values (Table 4). ECH (included to crosslink Sepharose 6B) was not expected to affect the titration level significantly because its proportion was low and it should react to completion with the base excess used. These results suggested that this chemistry could be applied readily to a range of polyhydroxylate matrices.

3.4. Allyl glycidyl ether (AGE) activation

3.4.1. Initial reaction conditions

AGE was reacted with Perloza solvated with 0.3 *M* NaOH, by analogy to the optimal hydroxide concentration reported for bisepoxide activation [16]. Reaction mixtures were prepared as thick suspensions, to minimise water content, by mixing AGE with suction dried, base washed cellulose. The AGE:cellulose proportion was 30%. Bromine water titration levels between 0.15 and 0.18 mmol/g were obtained by variation between 60°C and room temperature. Activation level was higher at room temperature but time for completion increased from 5 to 48 h. An identical titration value was obtained after sulphonation of the latter brominated matrix. This suggested that bromination and substitution reactions of the AGE matrix were more efficient than those of AB Perloza (Tables 2 and 3). The matrix swollen volume was not significantly altered by activation.

3.4.2. Reagent proportion and solvation effects

A comparison of activation level for 10–40% AGE mixtures demonstrated a lower efficiency of reagent use as the percentage was increased (Table 5). A very high activation level was obtained after a repeat 30% AGE reaction (0.32 mmol/g). Reaction efficiency was significantly increased by solvation of Perloza with DMSO–0.3 *M* NaOH (50:50) mixture, attributed to a lower rate of hydrolysis and superior mixing.

3.4.3. Aqueous slurry activation

The use of DMSO improved the "fluidity" of the reaction mixture allowing efficient mixing by rotation or shaking. Extra 0.3 *M* NaOH (2 ml per 8 g of Perloza) was required for similar mixing of 100% aqueous samples. A 48 h activation of Perloza 100 medium by this method with 15% AGE resulted in

Table 5
AGE % versus activation level of bead cellulose

Reaction type	AGE (%)	MPA titration (mmol/g)
Suspension	10	0.078
Suspension	15	0.109
Suspension in 50% DMSO	15	0.194
Suspension	20	0.133
Slurry	27	0.153
Suspension	30	0.172
Suspension	40	0.168

an activation level of 0.113 mmol/g. Stock 27% AGE (Table 5) was prepared likewise, using 15% AGE and a second activation with 12% AGE.

3.5. Comparison of AB and AGE with other activation methods

The efficiency of AB activation in aqueous media was 20–25% using optimised reaction conditions. High activation levels were obtained using small amounts of this cheap reagent. Only tosyl chloride and ECH of the commonly used reagents are of comparable cost. The former reagent requires anhydrous solvation while activation of Perloza by the latter was shown to be inefficient and limited.

Activation was analysed simply by bromine water titration (semi-quantitative) or by mercaptoacid addition and subsequent titration of carboxyl groups. Although swollen volume reduction was found initially for very highly activated resins, this effect was not observed once activation methods were optimised. Consistent results were obtained when the preferred reaction volume and base excess were adhered to. This represents a more precise method of matrix modification than epoxide chemistry and has been successfully applied to a range of matrices. Allyl matrices can be stored for long periods in aqueous media without apparent reduction of activation level (Table 3).

Although Perloza activation with AGE was less efficient than with AB, especially at high levels, it was still a great improvement over conventional methods such as epoxidation. High activation levels could still be obtained by use of large amounts of AGE, partial organic solvation or repeat activation. Activation was therefore more expensive than for AB, yet still cheaper than most published methods. Activation levels up to 0.1 mmol/g, suitable for most applications, were obtained simply and cheaply. Favourable attributes of AB activation (aqueous conditions, stability of the activated matrix and precision of matrix modification) were maintained. Advantages over AB were the lower toxicity and flammability of AGE, greater substitution efficiency of brominated AGE Perloza and a 7 atom, hydrophilic spacer arm, considered advantageous for protein chromatography.

3.6. Ligand attachment to allyl Perloza

The product of aqueous bromination of allyl groups is bromohydroxypropyl Perloza. This undergoes intramolecular etherification at alkaline pH forming epoxide groups which can be used for attachment of ligands by nucleophilic substitution [14,15]. This usually requires the reaction to be carried out at pH≥10 or pre-incubation at pH 10–12 prior to ligand attachment. Alternatively, some reactions may be carried out at lower pH with suitable ligands (e.g., sodium sulphite). Optimisation of bromination and ligand substitution of allyl matrices and alternative reactions will be described in subsequent reports.

4. Summary

Methods of ligand attachment to Perloza using stable ether, thioether and amine linkages were studied. Aqueous reaction of Perloza with chloroacetic acid, epichlorohydrin and bisepoxides produced low carboxymethylation and epoxidation levels. Use of mixed aqueous and DMSO solvation resulted in higher carboxymethylation levels but had much less effect on epoxidation. Efficient activation of Perloza required a method which produced less side reactions. This was obtained by activation with allyl bromide or allyl glycidyl ether. The allyl groups are relatively inert under activation conditions, limiting side reactions. Nevertheless allyl groups can be used for facile ligand attachment. Very high activation levels were obtained using aqueous or aqueous–organic solvation and modest amounts of reagent. A roughly linear dependence was found between reagent amount and activation level for allyl bromide.

Simple, reproducible titration methods for allyl matrices were developed. If ligand attachment is efficient this chemistry provides a desirable method of resin preparation at both laboratory and industrial scales.

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