Reactive carriers for the immobilization of copper ions

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ABSTRACT

Macroporous functionalized gels based on copolymers of trimethylolpropane trimethacrylate and glycidyl methacrylate were prepared for the adsorption of copper ions. The gels were functionalized with iminodiacetate, tris(2-aminoethyl)amine, tetraethylenepentamine (TEPA) and ammonia. The most favourable conditions for obtaining a high yield of TEPA were a high concentration of TEPA in the reaction mixture (at least 3 mol of TEPA per mole of epoxy), toluene as solvent, elevated temperature (70°C) and at least 4 h of reaction.

INTRODUCTION

Ligand-exchange chromatography was introduced in 1975 [1] and was later renamed immobilized metal ion affinity chromatography (IMAC) [2]. Recently, chromatographic methods for the purification of proteins and peptides were reviewed by Janson and Rydén [3]. The IMAC method takes advantage of transition metal interactions with biological molecules such as peptides and proteins [2,4–6]. The hydrophilic supports used for this affinity chromatographic method are usually based on cross-linked dextran or agarose [2,7,8]. However, functionalization of these gels usually involves at least two steps, activation and functionalization, which might cause losses of epoxy groups due to side-reactions. Hence it would be advantageous to omit the activation step.

The monomer glycidyl methacrylate (GMA) contains an epoxy group that has been successfully copolymerized with, *e.g.*, ethylene dimethacrylate to produce hydrophilic gels [9–11]. The reaction of the epoxy groups with various reagents and the chelation of the derivatized polymers were studied by Kalál and co-workers [12–15].

New materials based on trimethylolpropane trimethacrylate (TRIM) have been prepared [16–22]. They are mechanically stable and 40 mol% GMA can be incorporated without seriously affecting their mechanical properties [20]. As copolymerization is a random or close to random process, the epoxy groups will be located in both dense and open regions of the gels. Hence steric hindrance can affect the accessibility of the epoxy groups to solutes, which manifests itself in yields of product ligands lower than theoretically expected. High degrees of substitution of the epoxy groups have been achieved with dialkylamines and diamines [19].

This paper describes the synthesis and basic properties of functionalized poly(TRIM-co-GMA) gels. The gels were functionalized with iminodiacetate (IDA), tris(2-aminoethyl)amine (TREN), tetraethylenepentamine (TEPA) and ammonia. The reaction conditions necessary to obtain high yields of TEPA and the capacity of the amines mentioned above to chelate copper ions in distilled water and in

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buffers were investigated. The aim was to prepare functional gels that could be converted into metal complexes for IMAC of proteins and peptides.

EXPERIMENTAL

All chemicals were of analytical-reagent grade and used as received unless stated otherwise. The chromatographic columns were ordinary Pasteur pipettes (short form, 150 mm total length × 5 mm I.D.).

Materials

Trimethylolpropane trimethacrylate (TRIM) and glycidyl methacrylate (GMA) (technical quality, Merck and Fluka, respectively) were separately filtered through basic aluminium oxide (Fluka) before use. Ethylhydroxyethylcellulose (Bermocoll E 411 G, Batch 3537) was supplied by Berol Nobel (Sweden). Tetraethylenepentamine (TEPA) of technical quality and disodium iminodiacetate monohydrate (98% pure) was obtained from Fluka and [2-(N-morpholino)ethanesulphonic acid] (MES) from Sigma.

Synthesis of poly(TRIM-co-GMA)

The poly(TRIM-co-GMA) matrices were prepared by suspension polymerization as described

TABLE I

GMA TO TRIM RATIO AND MONOMER TO TOLUENE RATIO OF THE REACTION MIXTURE FOR THE PREPARATION OF poly(TRIM-co-GMA) GELS

Sample	Content of total monomer (%, v/v)	GMA (%, w/w)
TG8	50	21.6
TG10	30	19.6
TG20	30	20.1

elsewhere [21]. Details of the composition of the matrices are given in Table I. The polymer particles were allowed to sediment in distilled water at 50°C. The supernatant was decanted four times. The slurry was dried at 60°C and sieved (aperture 38 μm). Most of the particles were 20–30 μm in diameter, as measured by a Malvern Mastersizer.

Preparation of chelating polymers

Tetraethylenepentamine–poly(TRIM-co-GMA) gel. This ligand was chosen for reduced factorial design experiments as it was readily available and contained five nitrogens. The poly(TRIM-co-GMA) gel particles were washed with methanol and acetone

TABLE II

REACTION CONDITIONS FOR FUNCTIONALIZATION OF poly(TRIM-co-GMA) GEL PARTICLES (TG20) WITH TETRAETHYLENEPENTAMINE (TEPA)

The matrix for TEPA9 was TG8.

Sample	Monomer/toluene (%, v/v)	TEPA/epoxy (mol/mol)	Temperature (°C)[a]	Solvent	Time (h)
TEPA1	30	3.34	AT	Toluene	4
TEPA2a	30	6.63	AT	Toluene	8
TEPA2b	30	6.63	AT	Toluene	27
TEPA3	30	3.34	70	Toluene	8
TEPA4	30	6.63	70	Toluene	4
TEPA5	30	3.34	AT	Water	8
TEPA6a	30	6.63	AT	Water	4
TEPA6b	30	6.63	AT	Water	27
TEPA7	30	3.34	70	Water	4
TEPA8	30	6.63	70	Water	8
TEPA9	50	6.64	70	Toluene	4

[a] AT = Ambient temperature.

and 6.00 g of the suction-dried gel were weighed in (TG20 for samples TEPA1–TEPA8 and TG8 for TEPA9). Tetraethylenepentamine (TEPA) and 25 ml of distilled water or toluene were added. Details of the composition of the reaction mixtures are given in Table II. The Hypo-vials were sealed and placed in an agitating device (Tecam SB-4 shaking water-bath) at the desired temperature and time. Gels functionalized in distilled water (solvent) were washed with distilled water until pH 6 and then with methanol and acetone. Gels prepared in toluene were washed with methanol and mixtures of methanol and distilled water and of methanol and acetone. The levels of nitrogen atoms in the dry metal-free gels are given in Table III.

Three additional samples were prepared in a slightly different manner from that described above. A 3.85-g amount of ammonia (25% aqueous solution), 8.27 g of TREN and 10.88 g of TEPA (TEPA10) were separatively weighed into Hypo-vials together with 25 ml of toluene. Poly(TRIM-co-GMA) particles (6.00 g of TG20) were added to each Hypo-vial. The Hypo-vials were sealed and placed in the agitating device at 70°C. After 4 h, 5 ml of 50% acetone in distilled water were injected in each

Hypo-vial and after 1 h 5 ml of distilled water were added. The time of reaction was 8 h. The dry, metal-free gels contained 0.42, 3.75 and 4.01 wt.% N, respectively.

Iminodiacetate–poly(TRIM-co-GMA)gels. These were prepared according to a previously described procedure [2] with some minor modifications. A 200-ml volume of 2 M Na$_2$CO$_3$ solution and 20 g of disodium iminodiacetate monohydrate were added to 10 g of dry polymer (TG10, see Table I).

For IDA1, the suspension was kept at 60°C overnight with stirring. The gel was washed with distilled water until pH 6 was reached and then with ethanol and acetone. The dry gel contained 0.21% (w/w) N.

For IDA2, the suspension was stirred at 60°C for 40 h. Ethanol (5 ml) was added after 15 h of reaction. The washing procedure was performed as stated above. The dry gel contained 0.31% (w/w) N.

Hydrophilization of polymers. An amount of 4 mol of ethanolamine per mol of epoxy group in the poly(TRIM-co-GMA) gel and 9 ml of distilled water per gram of dry polymer were added to the dry polymer in a Hypo-vial (50 ml). The Hypo-vials were sealed and placed in the agitating device at

TABLE III

ELEMENTAL ANALYSIS OF NITROGEN IN poly(TRIM-co-GMA) GELS FUNCTIONALIZED WITH VARIOUS AMINES

The conversion of epoxy groups was calculated according to eqn. 1.

Sample	Content of nitrogen (%, w/w)	Amine (mmol ligand/g dry matrix)	Ratio[a], mol epoxy/mol ligand
TEPA1	0.59	0.09	16.5
TEPA2a	0.93	0.14	10.4
TEPA2b	3.59	0.58	2.5
TEPA3	4.00	0.64	2.2
TEPA4	3.99	0.64	2.2
TEPA5	1.75	0.26	5.4
TEPA6a	1.65	0.25	5.7
TEPA6b	2.98	0.46	3.1
TEPA7	2.65	0.41	3.5
TEPA8	2.80	0.43	3.3
TEPA9	4.04	0.65	2.3
Ammonia	0.42	0.32	4.7
TREN	3.75	0.74	1.9
TEPA10	4.01	0.64	2.2
IDA1	0.21	0.15	9.0
IDA2	0.31	0.23	6.1

[a] The molar ratio of epoxy groups in the starting material to amino groups in the product.

70°C for *ca.* 5 h. The gels were washed with distilled water until pH 6 was reached.

Metal adsorption experiments

Method 1. A stock solution of 0.5 *M* CuCl₂ containing 0.2 mol/l of NaCl was prepared. The solution was diluted tenfold with distilled water before use. The suction-dried gels were packed in Pasteur pipettes in distilled water (0.6–1.2 ml of gel). The pipettes were loaded with *ca.* 0.5 ml of the diluted copper solution containing sodium ions and then with distilled water. The amount of copper ion bound by the ligand was approximated from the volume of the migrated coloured front as

$$\frac{\text{mmol Cu}^{2+}}{\text{ml gel}} = \frac{cV}{\pi r^2 L} \tag{1}$$

where c is the concentration of copper ions, V is the volume of added copper solution (containing sodium ions), r is the radius of the column (Pasteur pipette) and L is the length of the stabilized migrated coloured front.

Method 2. This method, described by Muszynska *et al.* [23], was used to determine the displacement of copper ions by some buffers as a function of the bed volume. The adsorbents, 0.6–1.2 ml, were packed in Pasteur pipettes in distilled water. Approximately 0.26 ml of the copper ion solution (A) per millilitre of bed volume was percolated through each pipette. The pipettes were thoroughly washed with 2–4 bed volumes of solutions B, C and D to remove unbound and loosely bound copper ions. Solutions E and F were intended for the elution of future proteins. The volume of the coloured front was calculated according to eqn. 1, subsequent to the percolation of each of the following solutions:

(A) 50 m*M* CuCl₂ in 0.1 *M* sodium acetate containing 1 *M* NaCl, pH 4;
(B) 0.1 *M* sodium acetate (pH 4) containing 1 *M* NaCl;
(C) 0.1 *M* sodium acetate, pH 4;
(D) 0.1 *M* glycine, pH 8;
(E) equilibration buffer, 50 m*M* MES, pH 6;
(F) 0.1 *M* imidazole in 50 m*M* MES, pH 6;
(G) 20 m*M* EDTA solution;
(H) 1 *M* NaCl, pH 7.2.

The copper ions were removed using solution G. Solutions H and B were subsequently used to condition the column for a re-run.

Characterization

Elemental analysis of nitrogen was performed on dried gel samples by the micro-Kjeldahl method with mercury as catalyst. The amine content was calculated as

$$\frac{\text{mmol ligand}}{\text{g dry matrix}} = \frac{p_N}{100 \cdot 14 n_N m_{\text{matrix}}} \tag{2}$$

where p_N is the percentage of nitrogen atoms in the dry gel, n_N is the total number of nitrogen atoms in the ligand and m_{matrix} is the weight of the dry poly(TRIM-co-GMA).

Fourier transform infrared (FT-IR) spectra were recorded on a Perkin-Elmer Model 1720X spectrophotometer fitted with a diffuse reflectance device (DRIFT). The samples were dried under vacuum at 60°C for 24 h and then ground together with KBr (1:20, w/w) before analysis. The Kubelka–Munk equation [24] was applied to the data to obtain the spectra. The peak at 910 cm⁻¹ (epoxy group) was divided by the reference peak at 1733 cm⁻¹ (carbonyl group). The value thus obtained was used as an approximation of the yield of reacted ligand [21].

RESULTS AND DISCUSSION

Synthetic aspects

The optimum reaction conditions for obtaining 0.64 mmol of TEPA per gram of dry matrix were to add TEPA in excess (3 mol of TEPA per mol of epoxy group in the matrix), elevate the temperature (70°C), use toluene as solvent and let the reaction run for at least 4 h (Table III). It was important to have a good swelling agent for the matrix in combination with elevated temperature during the reaction. The yield of TEPA could be increased fourfold by prolonging the reaction time (27 h) if ambient temperature during the reaction was desirable (TEPA2a and TEPA2b in Table III). Distilled water was inferior as a solvent but the yield increased 1.8-fold after 27 h of reaction compared with 4 h of reaction at ambient temperature (TEPA6a and TEPA6b in Table III).

The ratio between the epoxy groups in the poly-(TRIM-co-GMA) gel and TEPA was *ca.* 2 mol/mol for gels containing *ca.* 0.6 mmol of TEPA per gram of dry matrix (Table III). It can be assumed that the two primary amino groups of TEPA had reacted and formed "bridges", giving a 1:1 ratio between epoxy groups and primary amino groups.

TABLE IV

RESULTS OBTAINED BY FT-IR FOR THE CONVERSION OF EPOXY GROUPS ACCORDING TO THE KUBELKA–MUNK EQUATION [24]

Hydrophilized samples are designated by "-OH".

Sample	$A(910\ cm^{-1})/A(1733\ cm^{-1})$	Conversion of epoxy groups (%)	
		FT-IR	Elemental analysis
TG10	0.0190	0	0
Ammonia-OH	0.0038	80.0 ± 0.0030	21[a]
TEPA10-OH	0.0022	88.0 ± 0.0018	45[a]
TREN-OH	0.0024	87.4 ± 0.0015	52[a]
IDA1	0.0092	51.6 ± 0.0055	11
IDA1-OH	0.0043	77.4 ± 0.0035	–

[a] Calculated for non-hydrophilized samples.

The amount of incorporated TEPA (sample TEPA10) did not increase when a mixture of distilled water and acetone was introduced into the Hypo-vials after a few hours of reaction (Table III). The number of amino groups seemed to be important for the incorporation of the amine. A higher yield (0.74 mmol of ligand per gram of dry matrix) was obtained for TREN, containing three primary amino groups, than for TEPA (0.64 mmol of ligand per gram of dry matrix), containing two primary amino groups. The yield of ammonia was low owing to the unfavourable reaction conditions.

An elemental analysis of IDA1 showed that 11% of the epoxy groups had reacted with IDA molecules (Table IV). However, FT-IR measurements on the same sample showed that about 51.6% of the epoxy groups had reacted. The discrepancy could be due to side-reactions that are still not fully understood. Another explanation could be the difficulty in measuring the peak at 910 cm^{-1} (epoxy group) owing to partial coverage of another peak [21] (Fig. 1). Hydrophilization of the IDA1 gel increased the amount of reacted epoxy groups to 77.4% (IDA1-OH).
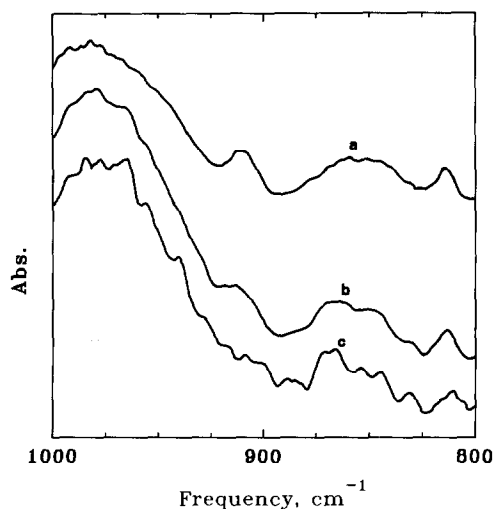

Fig. 1. FT-IR analyses of (a) poly(TRIM-co-GMA) gel, (b) poly(TRIM-co-GMA)–IDA gel (IDA1) and (c) hydrophilized poly(TRIM-co-GMA)–IDA gel (IDA1-OH). Abs. = Absorbance.

TABLE V

ADSORPTION OF COPPER IONS ON FUNCTIONALIZED GELS IN DISTILLED WATER CONTAINING SODIUM IONS (pH 4) ACCORDING TO METHOD 1

Hydrophilized samples are designated by "-OH".

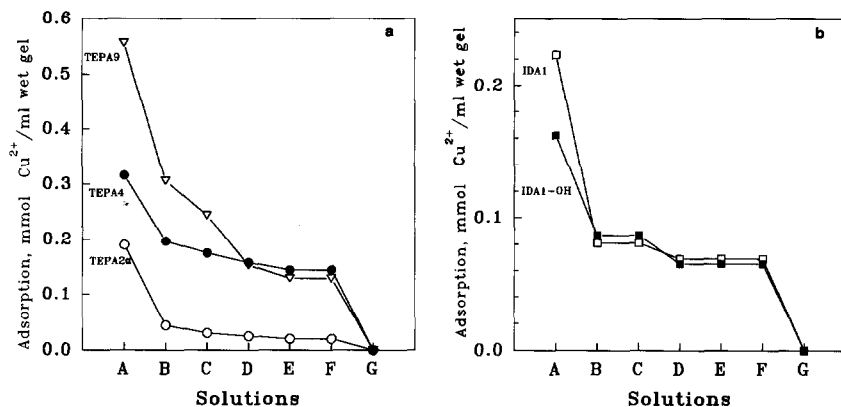| Polymer	Adsorption of copper ions (mmol Cu^{2+}/ml wet gel)
TG10	0
Ammonia-OH	0.02
TREN-OH	0.13
TEPA3-OH	0.14
TEPA10-OH	0.13
IDA1	0.08

Fig. 2. Adsorption capacity of copper ions after percolation of a series of buffers according to method 2 for (a) some of the poly(TRIM-co-GMA)–TEPA gels and (b) the poly(TRIM-co-GMA)–IDA gel and the hydrophilized IDA gel (IDA1-OH).

Chelating properties of functional poly(TRIM-co-GMA) gels

The adsorption of copper ions in distilled water, according to the first chromatographic method, depended on the ligand concentration in the gel (Tables III and V). The copper ion complexes were more stable for the TREN and TEPA gels than for the IDA1 and ammonia gels. The TEPA and TREN gels adsorbed *ca.* 0.13 mmol of copper ions per millilitre of wet gel, although the ligand concentration was higher in the TREN than in the TEPA gel (Table III).

The second chromatographic method showed that the metal leakage was reduced almost to zero after the percolation of 0.1 M glycine (pH 8) (Fig. 2a and b). Despite the displacement of copper ions, it was shown that the more TEPA that was immobilized, the higher the copper ion adsorption capacity (Fig. 2a). The hydrophilization of the IDA1 gel did not alter the amount of copper ions adsorbed (Fig. 2b).

CONCLUSIONS

Optimum reaction conditions for the incorporation of TEPA were obtained when TEPA was added in excess (at least 3 mol of TEPA per mol of epoxy groups in the matrix), the temperature was elevated (70°C), toluene was used as the solvent and the time for reaction was at least 4 h. The yield could be increased by prolonging the reaction time if ambient temperature and aqueous diluents, such as distilled water, were desirable reaction parameters.

The highest yield of incorporated amine was obtained with TREN. The lowest yield was obtained with ammonia owing to the unfavourable reaction conditions.

The adsorption of copper ions was proportional to the amount of attached chelating ligand. The hydrophilization of the IDA gel with ethanolamine did not change the adsorption capacity of copper ions in various buffer solutions.

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