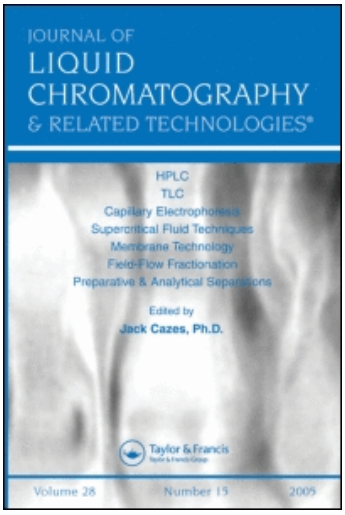

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Chromatographic Characterization of Immobilized Metal Ion Hollow-Fiber Affinity Membranes Obtained by Direct Grafting

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CHROMATOGRAPHIC CHARACTERIZATION OF IMMOBILIZED METAL ION HOLLOW-FIBER AFFINITY MEMBRANES OBTAINED BY DIRECT GRAFTING

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ABSTRACT

Iminodiacetic acid was immobilized onto membranes with different grafting degrees by reaction in phosphate buffer or water/dimethyl sulfoxide. Membranes subjected to conversion in water/dimethyl sulfoxide underwent greater conversion than those modified in phosphate buffer, despite their grafting degree.

Copper saturation capacity increased consistently with the grafting degree and histidine saturation capacity was approximately half than that of copper. When working with proteins, membrane behavior was related to the molecular weight of the protein tested. Accessible sites for lysozyme decreased with the increase in the grafting degree and the rise in the conversion of epoxy groups in iminodiacetic groups in water/dimethyl sulfoxide while they remained practically

unchanged when the conversion step was performed in phosphate buffer. When working with hemoglobin, this effect was the same but at lower capacities. For hollow fibres with 60 and 75% grafting, capacities were the same despite the conversion reaction solvent.

INTRODUCTION

Affinity chromatography is now a suitable technique for industrial protein purification processes, the pseudobiospecific ligands having a great influence on that success.[1] Textile dyes, immobilized metal ions, and amino acids are good examples of low cost and high chemical stability ligands for raw material protein purification.

Immobilized metal ion affinity chromatography (IMAC) is a protein purification method that takes full advantage of metal ion affinity of surface functional groups, mainly histidines.[2,3] IMAC is a good option in preparative protein purification processing taking into account its high yields and ligand economy and stability.[4]

Low flow rates, compressibility of gel beds, and high mass transfer resistance are inherent limitations of packed bed chromatography. Alternatively, microporous membranes with an attached ligand for affinity chromatography show a better performance because of their large surface area, reduced diffusion distance and low operating pressure[5-7] thus resulting in high volume throughput, high ligand utilization, and low cost.

A variety of module configurations (hollow fibres, spiral-wound cartridges, flat membrane sheets, etc.) are currently marketed.[8]

Saito et al.[9] have developed a new type of affinity hollow-fibre membrane by the preirradiation grafting technique. Grafting is a useful method to chemically modify existing polymers. In this way, a high degree of chemical modification of chromatographic supports can be obtained.[10]

The high energy radiation-induced graft polymerization process can be carried out in two steps (preirradiation process) or in only one step (simultaneous irradiation process). A hollow-fibre membrane is superior to a flat-sheet membrane because of its high surface area / volume ratio.[11]

When the grafting technique is applied to incorporate functionality into a porous membrane, grafted polymer branches are formed on the pore surface thus inducing a decrease in water permeability.

Yamagishi et al.[11] suggested a novel method to introduce the high density chelate-forming group into a commercial microfiltration membrane without lowering the water flux. By working with porous polyethylene commercial hollow fibres, the authors studied the behavior of membranes with a high degree of grafting, typically 30 to 300 %.

Grasselli et al.,[12] by using the mutual grafting technique on hydrophilized polyethylene hollow-fibre membranes, achieved reproducible grafting degrees at much lower values, the fluxes and ion-capacities being of the same order than those reported by Yamagishi et al.[11]

The aim of this paper is to find the best conditions for conversion of epoxy groups into IDA groups in hydrophilized polyethylene hollow fibres and to characterize the IDA membranes as regards different molecular weight adsorbates accessibility.

MATERIALS

Hollow-fibre membranes (Plasmaflo OP 05) were obtained from Asahi Medical Co., Japan. Each fibre was 20 cm long, 12.2 μL in volume, and capillary internal and external diameters were 0.34 mm and 0.44 mm respectively. Pore size was 0.3 μm and porosity, 0.7.

Chicken-egg lysozyme and hemoglobin were from Sigma Chemical Co. (St Louis, MO, USA). L-histidine hydrochloride was from BDH Chemicals Ltd., England. All other reagents were AR grade.

METHODS

Histidine, Lysozyme, and Hemoglobin Concentration Measurements

Histidine, lysozyme, and hemoglobin solution concentration was determined by measuring their absorbance at 220 nm, 280 nm and 430 nm, respectively.

Chelating Hollow-Fibre Synthesis

Hydrophilized polyethylene hollow-fibre membranes were surface-modified, according to Grasselli et al.,[12] by direct grafting of glycidyl methacrylate using a ^{60}Co gamma-radiation source. The degree of grafting (percentage increase in weight of the fibres) was between 20 and 75%.

The amount of epoxy groups incorporated was calculated as the increase in weight of the fibres divided by 142, where the factor 142 corresponds to the glycidyl methacrylate molecular weight.

Iminodiacetic acid (IDA) was immobilized onto the membranes by suspending the fibres either in 1M IDA-2Na in a sodium phosphate buffer, pH 10.0[13] or in 1M IDA-2Na in dimethyl sulfoxide (DMSO)/water (1:1).[11] The reaction was performed at 80°C for 24 h. In order to hydrolyze the remaining epoxy groups, the fibres were then immersed in 0.5 M sulfuric acid for 2 h at 80°C. After being washed with water, the fibres were immersed in 0.5 M $CuSO_4$, and three hours later were washed once again.

Copper content was determined spectrophotometrically by soaking the fibres with 0.1 M EDTA, pH 7.5, for 3 h and comparing the absorbance of the supernatant at 715 nm with that of 0.1 M EDTA with Cu(II) at various concentrations.[14]

The conversion of the epoxy group into iminodiacetic acid was calculated as:

$$[(\text{mol copper / mL fibre}) / (\text{mol epoxy group / mL fibre})] \times 100$$

Adsorption Experiments

The adsorption of histidine, lysozyme, and hemoglobin to the IDA hollow-fibre membranes were studied and their capacities compared with that of copper.

A total volume of 62 μL of fibres was immersed for 24 h in an excess of each adsorbate in 20 mM sodium phosphate buffer, pH 7.0, 250 mM NaCl, in a final volume of 3 mL. After thrice washing with the above buffer (10 mL each), the elution of the adsorbed molecules was performed by treating the fibres with 3 mL of 100 mM sodium acetate, pH 3.0, 250 mM NaCl, for 24 h.

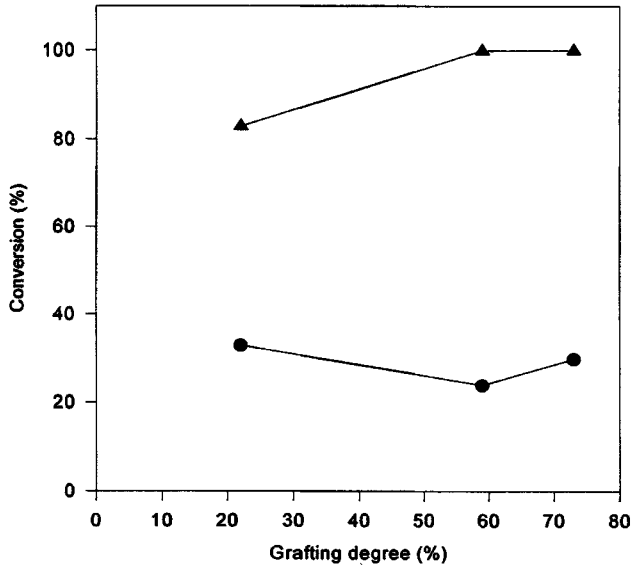

Figure 1. Influence of the grafting degree on the conversion of epoxy groups into IDA groups by using a sodium phosphate buffer, pH 10 (●), or water/DMSO (1:1) (▲) as the reaction solvent.

The amount of adsorbates bound was obtained by measuring them in the elution solution as indicated previously in this section. As sodium acetate interferes in the spectrophotometric determination of histidine at 220 nm, it was measured at 340 nm after reaction with trinitrobenzenesulfonic acid.[15]

RESULTS AND DISCUSSION

IDA was immobilized onto membranes with different grafting degrees by reaction in sodium phosphate buffer, pH 10[13] or in water/DMSO (1:1). The latter was used taking into account that Yamagishi et al.[11] had demonstrated that the addition of DMSO to the reaction solvent enhances the conversion of the epoxy group into an IDA group in grafted polyethylene hollow fibres. The amount of IDA introduced was determined from the measurement of the copper saturation capacity assuming an estequiometric ratio. Figure 1 shows that membranes subjected to reaction in water/DMSO (1:1) suffer a greater conversion than those modified in the absence of DMSO (80-100 % and 25-35 % respectively) despite the grafting degree. This effect becomes more evident

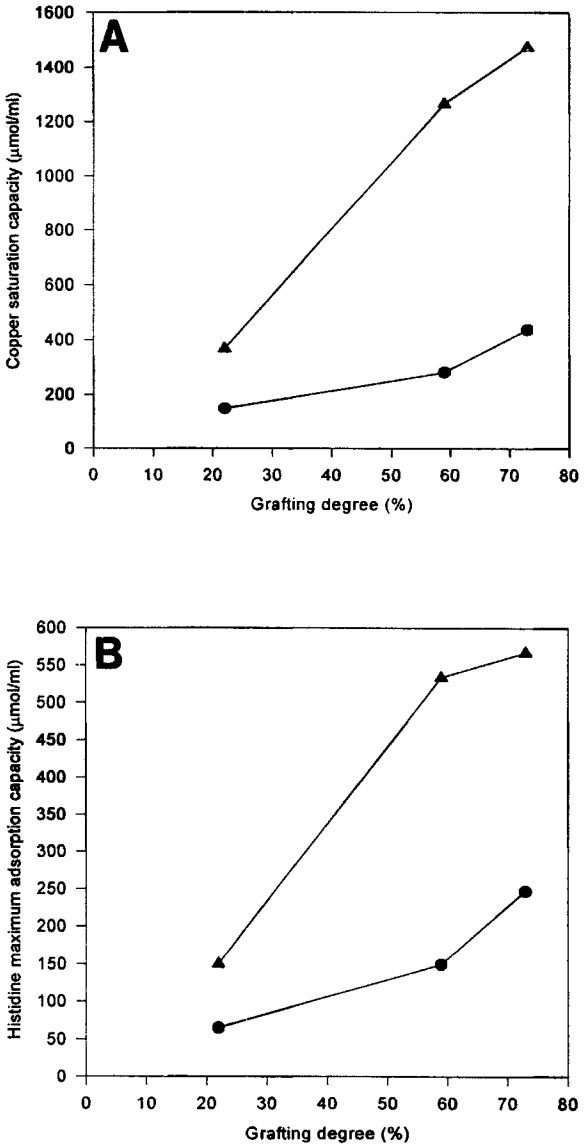

Figure 2. Influence of the grafting degree on the copper (A) and histidine (B) saturation capacity of membranes prepared by using a sodium phosphate buffer, pH 10 (●), or water/ DMSO (1:1) (▲) as the solvent of the conversion reaction.

in membranes with a high grafting degree. Yamagishi et al.[11] reported conversion degrees of 50-60 % for grafting degrees of 30-300 % by working with water/DMSO as the conversion reaction solvent in polyethylene hollow fibres.

Rodemann and Staude[16] introduced epoxy groups in polysulfone by metalation with butyllithium and reaction with glycidyl-4-oxohexylether and then obtained flat-sheet membranes with that material. The conversion of the epoxy group in an IDA group in such membranes was only 7 % by using the method of Smidl et al.[13] that had been successful for Separon HEMA conversion.

Copper saturation capacity increased consistently with the grafting degree (Figure 2A), and that of a 70/100 (grafting degree / conversion degree) fibre was 1500 μmol Cu/mL fibre, comparable to the commercial resins utilized for metal ion trapping. Histidine saturation capacity of the fibres was comparable to that of the copper (Figure 2B), but the molar ratio of histidine adsorbed to IDA-Cu was approximately 0.5 thus indicating that only 50% of the immobilized ligand was accessible to histidine. In both cases, the saturation capacity of the fibres modified in water/DMSO was higher than that of those modified in phosphate buffer.

Rodemann and Staude,[16] working with polysulfone IDA flat-sheet membranes in phosphate buffer, also found that approximately 50 % of the IDA groups were accessible to histidine.

When working with proteins, membrane behavior was different and related to the molecular weight of the protein tested. With lysozyme (14 kDa) the membranes with 20 % grafting showed an increase in adsorption capacity consistent with the increase of the conversion of epoxy into an IDA group by the use of water/DMSO as the reaction solvent. However, in membranes of a higher grafting degree, that increase in conversion caused a decrease in lysozyme capacity thus evidencing that the IDA incorporated is less accessible to the protein.

As shown in Figure 3, the accessible sites for lysozyme decrease with the increase in the grafting degree and with the rise in the conversion of epoxy groups into IDA groups in water/DMSO while they remained essentially constant when the conversion step was performed in phosphate buffer. When working with a protein of higher molecular weight like hemoglobin (68 kDa), this effect was the same (Figure 4) though at lower capacities. For hollow fibres with 60 and 75 % grafting, capacities were the same despite the conversion reaction solvent.

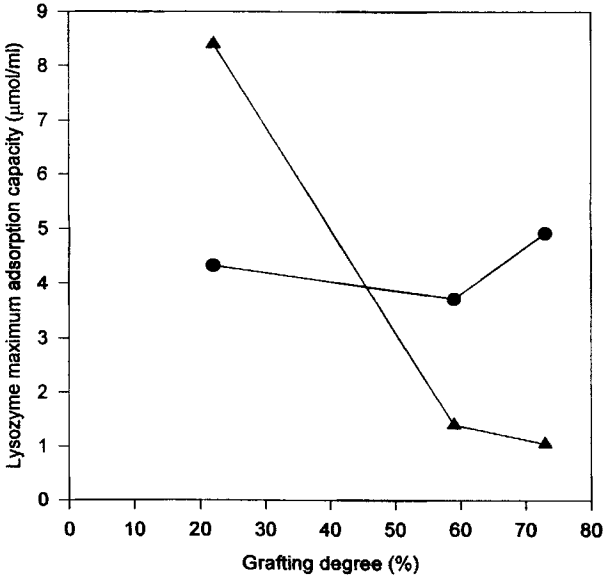

Figure 3. Influence of the grafting degree on lysozyme maximum adsorption capacity by using a sodium phosphate buffer, pH 10 (●), or water/DMSO (1:1) (□) as the reaction solvent of the conversion reaction.

The above results indicate that although the DMSO enhances the conversion of the epoxy group into an IDA group (Yamagishi *et al.*, 1991 and our results), the IDA incorporated is fully accessible only for small ions and molecules (copper and histidine) but partially accessible for proteins. Only with fibres of a low grafting degree (20 %), the enhanced incorporation of IDA groups due to the presence of DMSO is reflected by an increase in the protein maximum capacity. With fibres of a higher grafting degree, the increase in IDA groups did not increase, and in some cases decreased, the protein maximum capacity, this depending on the molecular weight of the protein. In this way, membranes with the highest copper capacity can be used as high-capacity adsorbents for small molecules and cations, e.g. removal of metal ions,[9] though they are not useful for protein purification.

On the other hand, the membranes with a low grafting degree (i.e. 20 %) have a higher pure water flux[12] and show adsorption maximum capacities for hemoglobin and lysozyme similar to those of commercially available chelating gels (6.8 to 7.5 and 3.8 to 4.1 µmol of lysozyme per mL of Chelating Sepharose

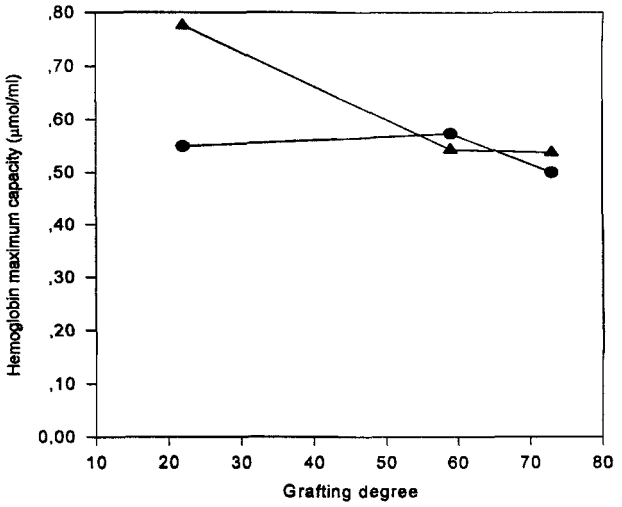

Figure 4. Influence of the grafting degree on hemoglobin maximum adsorption capacity by using a sodium phosphate buffer, pH 10 (●), or water/DMSO (1:1) (▲) as the reaction solvent of the conversion reaction.

Table 1

IDA to Protein Molar Ratio for Hollow Fibres of Various Grafting Degrees Converted in Sodium Phosphate Buffer, pH 10, or in Water/DMSO (1:1)

Protein/ Conversion Solvent	IDA to Protein Molar Ratio Grafting Degree (%) 20	60	75
Lysozyme/ sodium phosphate buffer, pH 10	34.9	65.8	91.8
Lysozyme/ water/DMSO (1:1)	44.7	867	1450
Hemoglobin/ sodium phosphate buffer, pH 10	278	431	900
Hemoglobin/ water/DMSO (1:1)	487	2453	2788

Fast Flow and TSK Gel Chelate, respectively). Table 1 shows the IDA to protein molar ratio for hollow fibres of a different grafting degree converted in a sodium phosphate buffer, pH 10, or in water/DMSO (1:1). It can be seen that, for lysozyme as well as for hemoglobin, IDA utilization is more effective when conversion has been performed in a phosphate buffer.

Increase in the grafting degree and greater conversion due to DMSO decreased the accessibility of the ligand immobilized onto the membrane for interaction with proteins. Similar results were obtained when working with gels of a different porosity.[17]

The adsorption capacity of the agarose-based adsorbent was higher than that of the TSK-based adsorbents. This was explained as the consequence of the differences in porosity between both gels which subsequently determines the volume of the stationary phase available for each protein.

This effect increased with the rise in the size of the protein which was a result of the molecular-sifting properties of the gels. Similarly, the increase in immobilized ligands due to the high grafting degree or increase in the conversion degree could diminish the membrane volume available for each protein , this volume being smaller for high molecular weight proteins.

The possibility that protein binding blocked the access to multiple metal ion sites at the surface of the chromatographic support, which explains the decrease in protein binding capacity with the increase in the molecular weight of a protein, was studied by Belew et al.,[17] Hutchens et al.,[18] and Todd et al.[19] This effect not only depends on the protein molecular weight but also on the ligand density.

When the Cu^{2+}-IDA complex was densely packed at the surface of the matrix, the IDA to protein molar ratio increased due to the blockage of copper ions. At low densities of immobilized ligand, the IDA to protein molar ratio decreased. In the same way, the adsorption of proteins to IDA membranes could block the access to other copper sites thus explaining why the IDA to protein molar ratio increases with the protein molecular weight and with the rise in ligand density.

Summing up, the increase of the grafting degree and conversion of epoxy groups into IDA groups in the membranes could diminish the volume of the stationary phase available for each protein and the increase in ligand density could emphasize the blocking of ligands brought about by the protein binding.

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