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Adsorption and selectivity characteristics of several human serum proteins with immobilised hard Lewis metal ion–chelate adsorbents

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Abstract

In this investigation, human serum has been used as an example of a crude protein mixture to define the protein binding characteristics and selectivity of several immobilised hard Lewis metal ion affinity chromatographic (IMAC) adsorbents. Specifically, the binding properties of immobilised *O*-phosphoserine (*im*-OPS) and 8-hydroxyquinoline (*im*-8-HQ), with immobilised iminodiacetic acid as a control system, have been investigated in combination with the hard Lewis metal ions, Al^{3+}, Ca^{2+}, Fe^{3+}, Yb^{3+}, and the borderline metal ion, Cu^{2+}, over the pH range pH 5.5 to pH 8.0 with buffers of 0.5 *M* ionic strength. The same IMAC adsorbents were also investigated for their protein binding capabilities with buffers of an ionic strength of 0.06 *M* at pH 5.5 and pH 8.0. The binding behaviour of four "marker" proteins, namely transferrin, α_2-macroglobulin, gammaglobulin and human serum albumin have furthermore been employed to monitor the differences in protein selectivity exhibited by these IMAC systems. The experimental findings confirm that these hard Lewis metal ion IMAC adsorbents function in a "mixed" binding mode with both coordination and electrostatic characteristics evident, depending on the ionic strength and pH of the equilibration or elution buffers. Based on a screening protocol, several members of the *im*-M^{n+}–8-HQ and *im*-M^{n+}–OPS adsorbent series have been identified with high selectivity for transferrin and α_2-macroglobulin. These hard Lewis metal ion IMAC adsorbents thus provide attractive alternatives for selective fractionation of human serum proteins. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Adsorbents; Metal ion affinity chromatography; Proteins; Metal chelates

1. Introduction

Human serum consists of hundreds of proteins and other biological substances of varying molecular mass, shape, charge, isoelectric point, hydrophobicity and function. The structures and biological properties of many different serum proteins have been previously reviewed [1,2]. The protein components of human serum are important in the prevention of disease as well as in the transport of a variety of nutrients to cells and tissues. For over 50 years, the primary method for fractionation of the proteins in human plasma has been based on a series of precipitation methods pioneered by Cohn and co-workers [3,4]. Although the Cohn procedure for the fractionation of serum proteins is still widely used by industry, with the advent of improved methods of protein purification, there has been a continuous search during the past decades for more efficient protocols, particularly those employing novel chromatographic techniques. Immobilised metal ion af-

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finity chromatographic (IMAC) adsorbents, derived from tridentate ligands such as iminodiacetic acid (IDA) [5] or *cis*- and *trans*-carboxymethylproline [6], tetradentate ligands such as nitrilotriacetic acid (NTA) [7,8], or pentadentate ligands such as *N,N,N'*-tris(carboxymethyl)ethylene-diamine (TED) [9] exploit the propensity of different metal ions to form bipyrimidal coordination complexes with these chelates, thus providing an avenue to achieve differential selectivity and resolution with serum proteins [10–14].

As a complex mixture, human serum represents an ideal source of proteins to investigate the potential of other types of immobilised metal ion–chelate complexes (IMCCs) of similar or different coordination geometries, such as immobilised *O*-phosphoserine (*im*-OPS) [15] or immobilised 8-hydroxyquinoline (*im*-8-HQ) [16] (Fig. 1). Metal ion complexation with *im*-OPS or *im*-8-HQ can be used to exploit the properties of hard Lewis metal ions, defined according to the Pearson's classification [17], including the favourable stability constants for the metal ion–chelate complex formation [18,19]. Hard Lewis metal ion IMCC systems are, moreover, finding increasing application in protein fractionation studies [20–22]. Consequently, the purpose of the present investigation was to screen the protein binding characteristics of several new hard Lewis metal ion IMAC adsorbents, based on *im*-8-HQ and *im*-OPS in combination with the hard Lewis metal ions, Al^{3+}, Ca^{2+}, Fe^{3+}, Yb^{3+}, and the borderline metal ion, Cu^{2+}, over the range pH 5.5 to pH 8.0 at two different buffer ionic strengths, namely 0.06 *M* and 0.5 *M* using human serum as an example of a crude protein mixture. The potential for these hard Lewis metal ion IMAC adsorbents (*im*-M^{n+}–8-HQ and *im*-M^{n+}–OPS) to be used in the fractionation of human serum proteins of commercial importance was, in particular, assessed using four "marker" proteins, namely transferrin (Trf), α_2-macroglobulin (α_2-M), the gammaglobulin (IgG) fraction and human serum albumin (HSA). These proteins are commercially and therapeutically important components of human serum. HSA, for example, represented approximately 50% of all sales of therapeutic plasma protein products [23], and is widely used in the treatment of hypoproteinemia. The tetrameric protein, α_2-M, is a valuable protease inhibitor [24,25] in certain inflammatory and cardiovascular disorders. Purified IgG fractions are used for the treatment of immunodeficiency states, including the IgG deficiency known as Bruton's agammaglobulinemia [26]. The protein, Trf, is important for the transportation of metal ions, particularly Fe^{3+}

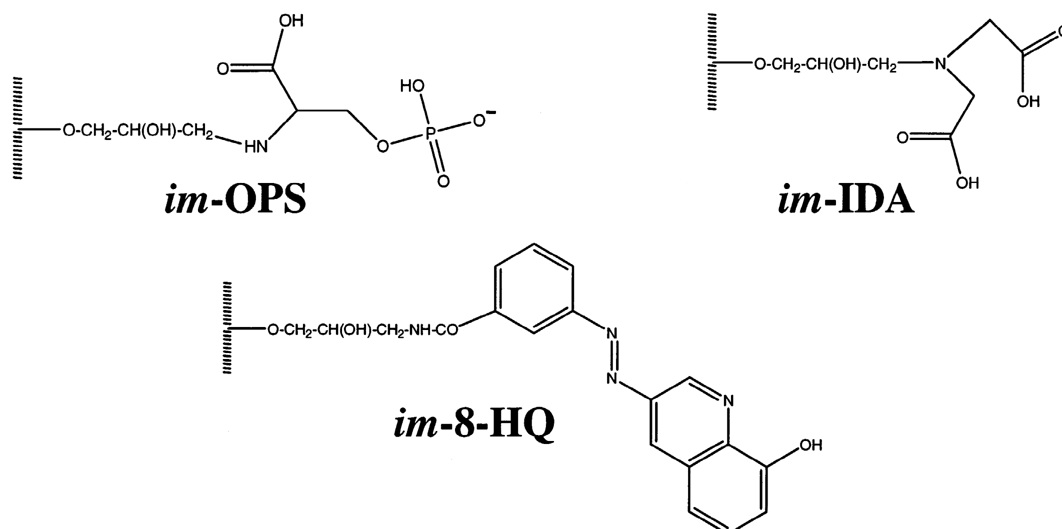

Fig. 1. Structures of the immobilised metal ion–chelate ligands: immobilised *O*-phosphoserine (*im*-OPS); immobilised 8-hydroxyquinoline (*im*-8-HQ) and immobilised iminodiacetic acid (*im*-IDA).

ions [27,28]. Decreases in the levels of Trf in human serum result in metal ion intolerance. In the present investigations with the hard Lewis metal ion IMAC systems based on *im*-M^{n+}–8-HQ and *im*-M^{n+}–OPS adsorbents, the results indicate that, depending on the choice of metal ion, chelating ligand and buffer condition, these new IMAC adsorbents exhibit high selectivity, particularly for Trf and α_2-M, when compared to the previously used Cu^{2+}– or Zn^{2+}–IDA adsorbents for serum protein fractionation.

2. Materials and methods

2.1. Chemicals

2-(*N*-Morpholino)ethanesulfonic acid (MES) in the free acid form, *N*-2-(hydroxyethyl)piperazine-*N′*-(2-ethanesulfonic acid) (HEPES) in the free acid form, imidazole, malonic acid in the free acid form, monopotassium glutamate and Brij-35 (30%, w/v, solution), were purchased from Sigma (St. Louis, MO, USA). Metal nitrate salts of Al^{3+}, Ca^{2+}, Cu^{2+}, Fe^{3+} and Yb^{3+} were purchased from Aldrich (St. Louis, MO, USA). Sepharose CL-4B was obtained from Amersham-Pharmacia (Uppsala, Sweden).

2.2. Preparation of IMAC adsorbents for protein binding and elution studies

The *im*-M^{n+}–8-HQ, *im*-M^{n+}–OPS and *im*-M^{n+}–IDA Sepharose CL-4B adsorbents were prepared based on methods previously employed in this laboratory [6,12,14–16] to prepare various types of IMAC adsorbents. In brief, Sepharose CL-4B was activated [20,21,29] with epichlorohydrin at pH 11.0, and washed with 20 volumes of Milli-Q water. An aliquot of the wet cake of the epoxy-activated gel (50 g) was suspended in 1.5 volumes of 25% (v/v) ammonia solution and gently shaken for 1.5 h at 40°C. The aminated Sepharose CL-4B gel was then washed with 10 volumes of Milli-Q water, before being passaged via acetone into a neat chloroform solvent using 20% (v/v) solvent composition changes. The aminated Sepharose CL-4B gel in neat chloroform was allowed to react with *p*-nitrobenzoyl chloride (0.1 g per g gel) in the presence of triethylamine (0.1 ml per g gel) for 48 h at 50°C with shaking. The derivatised Sepharose CL-4B gel was transferred from the chloroform suspension via acetone into Milli-Q water using five volumes of 20% (v/v) solvent composition changes. The derivatised *p*-nitrobenzoylated Sepharose CL-4B gel was reduced to the *p*-aminobenzoylated gel with 5% (w/v) sodium dithionite at 45°C for 15 h, followed by washing with 20 volumes of Milli-Q water. The *p*-aminobenzoylated Sepharose CL-4B gel was then suspended at 4°C for 30 min in 100 ml of a 2% (w/v) sodium nitrite–10 m*M* HCl solution with gentle stirring. The diazo-gel was then washed with 10 volumes of Milli-Q water, and passaged into neat ethanol using five volumes of 20% (v/v) increments of the solvent. The final mixture containing the diazo-gel and 2% (w/v) 8-hydroxyquinoline was gently stirred for 3 h at room temperature. The *im*-8-HQ-Sepharose CL-4B gel was recovered by suction filtration, suspended in water–ethanol (80:20, v/v) solution and stored at 4°C until required for the chromatographic binding experiments. Similarly, an aliquot of the wet cake of the epoxy-activated Sepharose CL-4B gel (50 g) was suspended in two volumes of a 0.2 *M* solution of *O*-phosphoserine in Milli-Q water at pH 10.5 (adjusted with KOH) for 4 h. The *im*-OPS-Sepharose CL-4B was then washed with 10 volumes of Milli-Q water, five volumes of 50 m*M* acetic acid, pH 4.0, and 10 volumes of Milli-Q water. The gel was suspended in water–ethanol (80:20, v/v), where it was stored at 4°C until required for the chromatographic binding experiments. The *im*-IDA-Sepharose CL-4B gel was prepared according to the method of Porath and coworkers [5,29]. Elemental nitrogen analysis of the various IMCC gels was carried out at the Dairy Technical Services Laboratory (Melbourne, Australia), revealing that the immobilised chelate densities were as follows: *im*-8-HQ, 280 μmol/g dry gel, *im*-OPS, 400 (μmol/g dry gel) and *im*-IDA, 660 μmol/g dry gel, respectively.

2.3. Buffer equilibration of immobilised metal ion–chelate-Sepharose CL-4B adsorbents

The binding of the metal ions to the IMCC adsorbents was carried out as described elsewhere [18–21]. The IMCC adsorbents were incubated with either 10 m*M* $Fe(NO_3)_3$, 10 m*M* $Al(NO_3)_3$ or 50

mM $Ca(NO_3)_2$, 50 mM $Cu(NO_3)_2$ or 50 mM $Yb(NO_3)_3$ for 30 min at 25°C. The metal ion-containing IMAC adsorbents were then washed with 10 volumes of Milli-Q water, 10 volumes of 50 mM acetic acid containing 100 mM KNO_3, pH 4.0, and 10 volumes of Milli-Q water before being incubated in the different equilibration buffers described below for 30 min at 25°C in a ratio of 5 g (wet mass) of adsorbent to 50 ml of equilibration buffer. The compositions of the equilibration buffers used in these experiments were as follows:

(A) Equilibration buffers of 0.5 M ionic strength: for the protein binding studies carried out with buffers designed to have a constant ionic strength of 0.5 M low ionic strength (I), the following equilibration buffers were employed:

Buffer A: 30 mM MES, 30 mM imidazole, 0.44 M NaCl, 0.005% Brij-35, adjusted to pH 5.5 with 5 M HCl.

Buffer B: for Buffer B but adjusted to pH 6.0 with 5 M HCl;

Buffer C: for Buffer B but adjusted to pH 7.0 with 5 M NaOH;

Buffer D: 30 mM HEPES, 30 mM Imidazole, 0.44 M NaCl, 0.005% Brij-35, adjusted to pH 8.0 with 5 M NaOH.

(B) Equilibration buffers of 0.06 M ionic strength: for the protein and metal ion binding studies carried out at low ionic strength, the following equilibration buffers were employed:

Buffer E: 30 m M MES, 30 mM imidazole, 0.005% (v/v) Brij-35, adjusted to pH 5.5 with HCl.

Buffer F: 30 mM HEPES, 30 mM imidazole, 0.005% (v/v) Brij-35, adjusted to pH 8.0 with NaOH.

All buffers were filtered through a 0.22-μm Durapore membrane filter (Millipore Intertech, Milford, MA, USA) before use. The metal ion contents of the various IMAC adsorbents, prepared as described above, were determined [18,19] using atomic absorption spectrophotometry and flame emission spectroscopy with a Varian SpectrAA 200 spectrophotometer.

2.4. Preparation of human serum proteins

Plasma protein fraction I (Batch CA64), with the cryoprecipitant removed and depleted of fibrinogen, was a generous gift of the CSL, Blood Products Division (Melbourne, Australia). The plasma protein fraction was supplied in liquid form at a protein concentration of 50 mg/ml in 8% (v/v) ethanol, and stored frozen at −25°C in 5-ml ampoules until required. The ampoules were then thawed rapidly in lukewarm water prior to desalting the proteins into the appropriate equilibrating buffer. For this purpose, Sephadex PD-10 columns (10 cm×0.5 cm^2) (Amersham-Pharmacia) were equilibrated with 20 ml of the appropriate equilibrating buffer at room temperature. These desalting columns had a void volume of 3.0 ml as determined by blue dextran. A 2-ml aliquot of the plasma protein fraction I was applied to the pre-equilibrated Sephadex PD-10 column and allowed to elute under gravity with the appropriate equilibrating buffer. The first 3.0 ml collected from the desalting column was discarded. The next 2.5 to 3.0 ml was collected as the desalted protein fraction. The desalting column was then washed with a further 10 ml of equilibrating buffer for cleaning purposes. The desalted protein fraction was then diluted to a concentration of 16.7 mg/ml using the appropriate equilibrating buffer and stored on ice. The desalting and dilution procedures were carried out immediately prior to the protein binding studies.

2.5. Screening of the binding and elution behaviour of human serum proteins with different IMAC adsorbents

The different IMAC adsorbents prepared as described above were screened for their binding properties with serum proteins with buffers at I=0.5 M at pH 5.5, pH 6.0, pH 7.0 and pH 8.0 and at I=0.06 M at pH 5.5 and pH 8.0 using the equilibration buffers described above. In brief, the IMAC adsorbents, loaded with the different metal ions and equilibrated in the appropriate buffer, were packed in 10 ml Bio-Rad econocolumns (Bio-Rad, Richmond, CA, USA) to dimensions 2 cm×0.25 cm^2 (1.0 ml graduated mark on the column). A flow-rate of approximately 0.5 ml/min was maintained during all chromatographic steps. Aliquots of 300 μl of a desalted serum protein solution (16.7 mg/ml), representing 5 mg of serum proteins, were added to each of the columns. The column was then washed with a further 0.2 ml of the appropriate equilibration buffer before filling the column reservoir and eluting with 4.0 ml of the appropriate equilibrating buffer. The

4.5 ml collected was labelled the non-adsorbed fraction. The binding procedure was performed at room temperature (25°C). Bound proteins were eluted from the IMAC adsorbents using 200 m*M* EDTA (Ajax, Sydney, Australia) and 500 m*M* NaCl buffer, adjusted to pH 8.0 using NaOH, as described by Mantovaara et al. [30]. Briefly, 0.2 ml of the eluting buffer was initially applied to the column, followed by a further 3.0 ml of eluting buffer. The 3.2 ml collected was termed the eluted fraction. The recovered fractions were stored at −25°C until required for analysis.

2.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and staining procedures

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using a Pharmacia Phast System apparatus (Amersham-Pharmacia). Non-adsorbed, eluted and initial samples of serum proteins were analysed using 4–15% gradient SDS–PAGE gels and SDS buffer strips (Amersham-Pharmacia). The method described in the Pharmacia Electrophoresis Separations Manual for the electrophoretic separation of proteins on 4–15% SDS–PAGE gels was used, whilst the gels were stained using a silver staining method based on the procedure of Morrisey [31].

2.7. Ouchterlony immunodiffusion and protein dye binding assays

The Ouchterlony immunodiffusion assays were based on the methods described by Crowle [32], using rabbit polyclonal antisera specific for human α_2-macroglobulin, and goat polyclonal antisera specific for human transferrin and human IgG. The α_2-macroglobulin, transferrin and human IgG standards were obtained from CSL (Parkville, Australia) or Sigma. For estimation of protein concentrations, the dye binding method of Bradford [33] was used, with the protein dye kit purchased from Bio-Rad.

3. Results

In these investigations, the binding and step elution behaviour of human serum proteins with different hard Lewis metal ion IMAC adsorbents was examined by total protein assay and SDS–PAGE. During the initial screening studies, the binding properties of these new hard Lewis metal IMAC adsorbents with the serum proteins was determined, with several adsorbents exhibiting very high selectivity for the marker proteins. Such high selectivity could potentially result in substantial purification factors for these proteins in a single fractionation step, following scale-up. In addition, the compositions of the different equilibration and elution buffers were so chosen as to permit comparisons to be made between the binding affinities of the marker proteins and other serum proteins with the various IMAC adsorbents.

Prior to loading onto the different IMAC adsorbents, the human serum proteins were desalted in the appropriate adsorption buffer. The adsorption characteristics of the different IMAC adsorbents were studied at pH 5.5, pH 6.0, pH 7.0 and pH 8.0 at an ionic strength of 0.5 *M* and at pH 5.0 and pH 8.0 at an ionic strength of 0.06 *M*. Aliquots of a 16.7 mg/ml solution of serum protein mixture, in the appropriate buffer, were loaded onto the *im*-M^{n+}–8-HQ, *im*-M^{n+}–IDA or *im*-M^{n+}–OPS IMAC adsorbents, chelated with either Al^{3+}, Ca^{2+}, Fe^{3+}, Yb^{3+} or Cu^{2+}. The metal ion contents of the various IMAC adsorbents under equilibration buffer conditions, determined by atomic absorption spectrophotometry and flame emission spectroscopy, are presented in Table 1. As evident from these data, the immobilised metal ion contents for the Fe^{3+}- and Cu^{2+}-containing IMAC adsorbents reached a level corresponding to nearly quantitative saturation of the IMCC ligand binding sites, whilst in the case of the Al^{3+}, Ca^{2+} and Yb^{3+} ions a lower occupancy of the immobilised chelating ligand was observed. This metal ion-dependent behaviour, reflecting differences in the stability constants (log β) of these metal ions with these IMCC systems, has previously been observed with other types of IMAC adsorbents and can be attributed [9,18–22] to a combination of electronegativity differences, steri-molar crowding and entropic effects associated with the anisotropy of the IMCC ligand distribution on the surface of the support material. In this context, the metal ion free sites on these IMAC adsorbents could potentially result in the IMCC ligands acting as cation-exchange moieties under some buffer pH conditions. Such electrostatic effects became particularly apparent

Table 1
The immobilised metal ion contents of the different hard and borderline Lewis metal ion IMAC adsorbents, *im*-M^{n+}–IDA, *im*-M^{n+}–8-HQ and *im*-M^{n+}–OPS under the equilibration buffer binding conditions

Immobilised M^{n+}	Metal ion densities relative to chelating ligand density[a] (μmol immobilised M^{n+}/μmol immobilised chelating ligand)		
	IDA-Sepharose CL-4B	8-HQ-Sepharose CL-4B	OPS-Sepharose CL-4B
Al^{3+}	0.41±0.02	0.39±0.02	0.82±0.03
Ca^{2+}	0.43±0.05	0.50±0.02	0.48±0.01
Cu^{2+}	1.00±0.01	0.93±0.02	0.98±0.02
Fe^{3+}	1.10±0.02	0.87±0.02	1.12±0.01
Yb^{3+}	0.69±0.02	0.42±0.02	0.58±0.01

[a] The immobilised chelating ligand densities relative to the mass of the Sepharose CL-4B support material were as follows: *im*-IDA, 660 μmol/g dry gel; *im*-8-HQ, 280 μmol/g dry gel; and *im*-OPS, 400 μmol/g dry gel, respectively. All results represent the mean value (±S.E.M.) of the mol immobilised/mol immobilised chelating ligand. Metal ion content of the adsorbents was determined using atomic absorption spectrophotometry and flame emission spectroscopy using a Varian SpectrAA 200.

with some adsorbents when buffers of ionic strength of 0.06 *M* were employed. Differences in protein capture and selectivity with these different IMAC adsorbents were determined from the protein concentrations of the non-bound and bound fractions following elution with the equilibration buffer or 200 m*M* EDTA–500 m*M* NaCl, pH 8.0, respectively, as determined by the Bradford dye binding method and by SDS–PAGE procedures. Furthermore, relative differences in protein selectivity could be monitored for the non-adsorbed and eluted fractions by SDS–PAGE using several marker proteins. Since it was also essential to ascertain whether the bound serum proteins could be eluted in high yield from the IMAC adsorbents under conditions applicable for their use in larger scale chromatographic applications, a general non-specific elution buffer, 200 m*M* EDTA–500 m*M* NaCl, pH 8.0, was chosen for this purpose. Because of the magnitudes of the metal ion stability constants [18,19] for these hard Lewis metal ion IMAC adsorbents, it was anticipated that the inclusion of 200 m*M* EDTA in combination with 0.5 *M* NaCl would strip any chelated metal ion from the IMAC adsorbent and, as a result, permit any protein bound to the immobilised metal ion to also elute. With this elution buffer, it was observed that $>$90% of the bound proteins could be accounted for in the non-bound and eluted fractions. The $<$10% mass loss of the serum proteins, in some cases, could be attributed to the standard experimental errors associated with the protein assay measurements and losses of protein during dialysis prior to the protein assay.

Imidazole has a high affinity for borderline and soft Lewis metal ions and a low affinity for hard Lewis metal ions. Imidazole was therefore incorporated into all equilibrium buffers for the adsorption studies with the serum proteins to quench any affinity that histidine residues within individual proteins may have for the immobilised hard Lewis metal ions. This histidine-quenching effect then allowed the immobilised hard Lewis metal ions to select serum proteins primarily on the basis of their surface accessible aspartic and glutamic acid residues, thus offering the potential for an alternative mode of protein selectivity to that obtained with the commonly used borderline Lewis metal ion Cu^{2+} when chelated to *im*-IDA. This combination of alternative chelating adsorbents, alternative metal ions and non-traditional adsorption buffers was anticipated to provide different modes of selectivity when applied to the fractionation of serum proteins.

3.1. (A) binding behaviour of the serum proteins with the metal ion–chelate adsorbents

In preliminary studies, the protein content of the non-adsorbed and eluted fractions relative to the initial protein content loaded onto the hard Lewis metal ion IMAC adsorbents using different loading buffers of different ionic strengths (I=0.5 *M* and I=0.06 *M*) at different pH values were determined. These experiments provided an overall guide to the choice of binding and elution conditions of the serum proteins from the different IMAC adsorbents.

Table 2
Comparison of the total protein recovery from the adsorption of a solution of human serum proteins (5 mg) to various immobilised hard Lewis metal ion–chelate adsorbents, *im*-M^{n+}–8-HQ, *im*-M^{n+}–IDA and *im*-M^{n+}–OPS adsorbents in combination with the hard Lewis metal ions, Al^{3+}, Ca^{2+}, Fe^{3+}, Yb^{3+}, and the borderline metal ion, Cu^{2+}, as well as the metal ion free adsorbents (BL), with the equilibration buffers of 0.5 *M* ionic strength and different pH values, i.e., (from left to right in each panel) at pH 5.5, pH 6.0; pH 7.0 and pH 8.0[a]

	Al^{3+}				Ca^{2+}				Cu^{2+}				Fe^{3+}				Yb^{3+}				BL			
	pH 5.5	pH 6.0	pH 7.0	pH 8.0	pH 5.5	pH 6.0	pH 7.0	pH 8.0	pH 5.5	pH 6.0	pH 7.0	pH 8.0	pH 5.5	pH 6.0	pH 7.0	pH 8.0	pH 5.5	pH 6.0	pH 7.0	pH 8.0	pH 5.5	pH 6.0	pH 7.0	pH 8.0
im-8-HQ-Sepharose CL-4B																								
Non-bound	2.9	4.1	4.8	4.9	5.0	5.0	4.9	5.0	3.0	4.1	4.5	4.8	1.7	3.6	4.2	4.7	2.9	4.4	4.1	4.7	5.0	4.6	4.9	5.0
Bound	1.9	0.9	0.2	0.1	0.1	n.d.	0.2	n.d.	n.d.	2.0	0.9	0.5	0.2	3.3	1.4	0.8	0.3	1.9	0.6	1.0	0.3	n.d.	0.2	n.d.
im-IDA-Sepharose CL-4B																								
Non-bound	3.8	4.0	4.6	4.6	3.8	4.1	4.6	4.7	2.8	3.7	4.5	4.5	n.d.	0.1	3.0	4.6	3.8	3.9	4.4	4.4	4.8	4.1	4.7	4.4
Bound	0.1	0.1	0.2	0.1	0.2	0.2	0.3	0.2	1.6	0.3	0.1	0.1	5.0	4.5	1.5	0.1	0.2	0.3	0.4	0.1	0.2	0.2	0.3	0.2
im-OPS-Sepharose CL-4B																								
Non-bound	4.9	4.7	4.9	4.5	5.0	5.0	4.6	4.9	3.9	4.5	4.6	4.9	4.0	4.7	4.2	4.9	4.8	5.0	4.7	4.5	4.8	4.9	4.6	5.0
Bound	0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.0	0.3	n.d.	n.d.	1.0	0.3	0.8	0.1	0.1	n.d.	0.3	0.2	0.1	n.d.	n.d.	n.d.

[a] The cumulative protein amounts determined in the non-adsorbed fraction and the eluted fraction from the various adsorbents are presented as protein recovered (mg). n.d.: No protein detected. Other experimental details are given in the Materials and Methods section.

3.1.1. (i) Serum protein binding behaviour of metal ion–chelate adsorbents using 0.5 M ionic strength adsorption buffers at pH 5.5 to pH 8.0

The trends in the total recovery of serum protein in the non-adsorbed and eluted fractions from the *im*-M^{n+}–8-HQ, *im*-M^{n+}–IDA and *im*-M^{n+}–OPS adsorbents, as well as the metal ion free adsorbents, under the equilibration buffer conditions of pH 5.5 to pH 8.0 at 0.5 *M* ionic strength, are shown in Table 2. Several observations can be made from these data. Firstly, there was, in general, a decrease in the amount of bound protein as the pH was increased from pH 5.5 to pH 8.0. Secondly, with the exception of the *im*-Al^{3+}–IDA and *im*-Ca^{2+}–IDA adsorbents at pH 5.5, greater than 90±10% of the total protein could be recovered in the non-adsorbed and eluted fractions. Thirdly, the eluted fractions from the metal ion free (BL) adsorbents contained minimal amounts of protein with total recovery always in excess of 95±5%. These results confirmed that irreversible adsorption of serum protein onto most of these IMAC adsorbents or participation of strong electrostatic interactions of proteins with the support material or the immobilised ligand do not represent the dominant basis of the adsorption phenomena. Finally, with the exception of Ca^{2+}, all other metal ions when chelated to *im*-8-HQ, bound significant amounts of serum proteins at pH 5.5 or pH 6.0 with 0.5 *M* ionic strength buffers. Under these conditions, the hard metal ions, Al^{3+}, Ca^{2+}, Fe^{3+} or Yb^{3+}, as well as the borderline metal ion, Cu^{2+}, show greater affinity toward the proteins when bound to *im*-8-HQ than occurred for the naked *im*-8-HQ or *im*-Ca^{2+}–8-HQ adsorbent.

3.1.2. (ii) Serum protein binding behaviour of the metal ion–chelate adsorbents using 0.06 M ionic strength adsorption buffers at pH 5.5 and pH 8.0

The trends in the total recovery of serum protein in the non-adsorbed and eluted fractions from the *im*-M^{n+}–8-HQ, *im*-M^{n+}–IDA and *im*-M^{n+}–OPS adsorbents, as well as from the metal ion free adsorbents, under the equilibration conditions of pH 5.5 and pH 8.0 at 0.06 *M* ionic strength, are shown in Table 3. As evident from these results, all IMAC adsorbents bound protein to some extent at both pH 5.5 and pH 8.0. Firstly, with the exception of the *im*-Al^{3+}–OPS adsorbent, more protein was found in the eluted fractions when equilibration buffers of pH 5.5 rather than pH 8.0 were employed. Secondly, greater than 90±10% of the total protein was accounted for the combined values of the non-adsorbed and eluted fractions of all samples indicating again that minimal irreversible binding of proteins had occurred to these IMAC adsorbents. Finally, when low-ionic-strength equilibration buffers were

Table 3
Comparison of the total protein recovery from the adsorption of a solution of human serum proteins (5 mg) to various immobilised hard Lewis metal ion–chelate adsorbents, *im*-M^{n+}–8-HQ, *im*-M^{n+}–IDA and *im*-M^{n+}–OPS adsorbents in combination with the hard Lewis metal ions, Al^{3+}, Ca^{2+}, Fe^{3+}, Yb^{3+}, and the borderline metal ion, Cu^{2+}, as well as the metal ion free adsorbents (BL), with the equilibration buffers of 0.06 *M* ionic strength and at two different pH values, i.e., (from left to right in each panel) at pH 5.5 and pH 8.0[a]

	Al^{3+}		Ca^{2+}		Cu^{2+}		Fe^{3+}		Yb^{3+}		BL	
	pH 5.5	pH 8.0	pH 5.5	pH 8.0	pH 5.5	pH 8.0	pH 5.5	pH 8.0	pH 5.5	pH 8.0	pH 5.5	pH 8.0
im-8-HQ-Sepharose CL-4B												
Non-bound	n.d.	3.3	n.d.	3.1	n.d	3.6	n.d.	3.7	n.d.	3.4	n.d.	3.2
Bound	4.5	1.5	5.0	1.7	5.0	1.2	4.8	1.2	5.0	1.9	4.8	1.7
im-IDA-Sepharose CL-4B												
Non-bound	2.9	4.7	2.6	4.7	2.8	4.5	n.d.	4.6	2.9	4.4	2.9	4.7
Bound	2.1	0.3	2.4	0.4	2.2	0.5	4.80	0.4	2.1	0.5	2.1	0.3
im-OPS-Sepharose CL-4B												
Non-bound	4.2	3.9	3.2	4.0	2.1	3.8	1.1	3.8	3.3	3.7	3.3	4.0
Bound	0.8	1.0	1.8	0.8	2.8	1.1	3.9	1.2	1.8	1.0	1.7	0.8

[a] The cumulative protein amounts determined in the non-adsorbed fraction and the eluted fraction from the various adsorbents are presented as protein recovered (mg). n.d.: No protein detected. Other experimental details are given in the Materials and Methods section.

used, the eluted and non-adsorbed fractions from the metal ion free IMCC adsorbents and the metal ion-containing IMAC adsorbents contained, in most cases, similar amounts of protein. This trend was observed for all of the *im*-M^{n+}–8-HQ adsorbents, the *im*-M^{n+}–IDA adsorbents (with the exception of the *im*-Fe^{3+}–IDA adsorbent at pH 5.5) and the *im*-M^{n+}–OPS adsorbents with the exception of the *im*-Al^{3+}–OPS adsorbent at pH 8.0 or the *im*-Cu^{2+}–OPS adsorbent and the *im*-Fe^{3+}–OPS adsorbent at both pH 5.5 and pH 8.0.

3.2. (B) Selectivities of immobilised hard-Lewis metal ion–chelate complexes as applied to serum marker proteins

As apparent from the results shown in Tables 2 and 3, as well as from the SDS–PAGE analysis of the non-bound and bound fractions of the serum proteins separated with these IMAC adsorbents (cf. the representative results obtained for the *im*-M^{n+}–8-HQ and *im*-M^{n+}–OPS adsorbents shown as Figs. 2 and 3, respectively), significant differences exist, qualitatively and quantitatively, between protein binding and selectivity characteristics of these hard Lewis metal ion IMAC adsorbents. For example, when the hard Lewis metal ions Al^{3+} and Yb^{3+} were chelated with *im*-8-HQ, the derived IMAC adsorbents showed a greater affinity for serum proteins than did the corresponding *im*-M^{n+}–IDA or *im*-M^{n+}–OPS adsorbents, despite the lower metal ion content of the *im*-M^{n+}–8-HQ adsorbent. This behaviour occurred over the range pH 5.5 to pH 8.0 when the ionic strength of the adsorption buffer was either 0.5 *M* or 0.06 *M*. Furthermore, when Cu^{2+} and Fe^{3+} were chelated to *im*-8-HQ, the derived IMAC adsorbents bound more serum proteins at all tested pH values at 0.5 *M* ionic strength (with the exception of *im*-Fe^{3+}–IDA adsorbent) than the *im*-M^{n+}–OPS or *im*-M^{n+}–IDA adsorbent counterparts. This effect was also noted with all *im*-M^{n+}–8-HQ adsorbents when 0.06 *M* ionic strength conditions were used. It can therefore be concluded that serum proteins generally show a greater affinity for the *im*-M^{n+}–8-HQ adsorbents than for the *im*-M^{n+}–IDA or *im*-M^{n+}–OPS adsorbents. Two reasons can be advanced for these differences. Firstly, the higher capture may be a result of the additional coordination site available for protein–metal ion interactions when the metal ions are chelated to *im*-8-HQ rather than to *im*-IDA or *im*-OPS, thus increasing overall the favourable free energy of interaction. Secondly, the longer spacer arm of the *im*-8-HQ-based adsorbents (due to the chemistry employed for the immobilisation of this chelating ligand) will both increase the accessibility of the protein to the chelated metal ion and also enable hydrophobic effects to contribute to the overall interaction.

In terms of the binding profile of the ‘‘marker’’ proteins, it was apparent from the SDS–PAGE analyses that significant selectivity differences existed between these IMAC adsorbents, e.g., the *im*-M^{n+}–OPS compared to the *im*-M^{n+}–IDA adsorbent, with the binding dependent inter alia upon the ionic strength and pH of the equilibration loading buffer. For example, the *im*-Yb^{3+}–OPS but not the *im*-Yb^{3+}–IDA adsorbent bound Trf (M_r 76 000) at pH 5.5 and 0.06 *M* ionic strength (data not shown), despite the fact that the *im*-Yb^{3+}–OPS and *im*-Yb^{3+}–IDA adsorbents had similar Yb^{3+} content and are both tridentate IMAC systems. Other differences in selectivity for serum proteins with these two types of IMCCs can be observed from the protein binding results shown in Tables 2 and 3 and Figs. 2 and 3. Both *im*-IDA and *im*-OPS bind metal ions via three coordination sites, but differ in their metal ion complex stereochemistry. The above results for the chelated Yb^{3+}-adsorbents, for example, can be rationalised in terms of differences in protein–metal ion–chelate coordination stereochemistries, since all other parameters such as ionic strength, pH, type of support material, activation chemistry and metal ion content were similar. The various IMAC adsorbents also exhibited different serum protein adsorption behaviour as a result of changes to the ionic strength. At 0.06 *M* ionic strength only a few adsorbents exhibited selectivities which were different to the metal ion free adsorbent, e.g., *im*-Fe^{3+}–IDA and *im*-Fe^{3+}–OPS adsorbents. Other examples included the *im*-Cu^{2+}–OPS adsorbent, which bound Trf at pH 8.0 whilst the *im*-OPS adsorbent did not (results not shown). Similarly, α_2-M (M_r for tetramer 720 000 and monomer 180 000) bound to the *im*-Al^{3+}–OPS, *im*-Cu^{2+}–OPS, *im*-Fe^{3+}–OPS and *im*-Yb^{3+}–OPS adsorbents to different extents, but not to the corresponding *im*-OPS adsorbent. Moreover, IgG (M_r

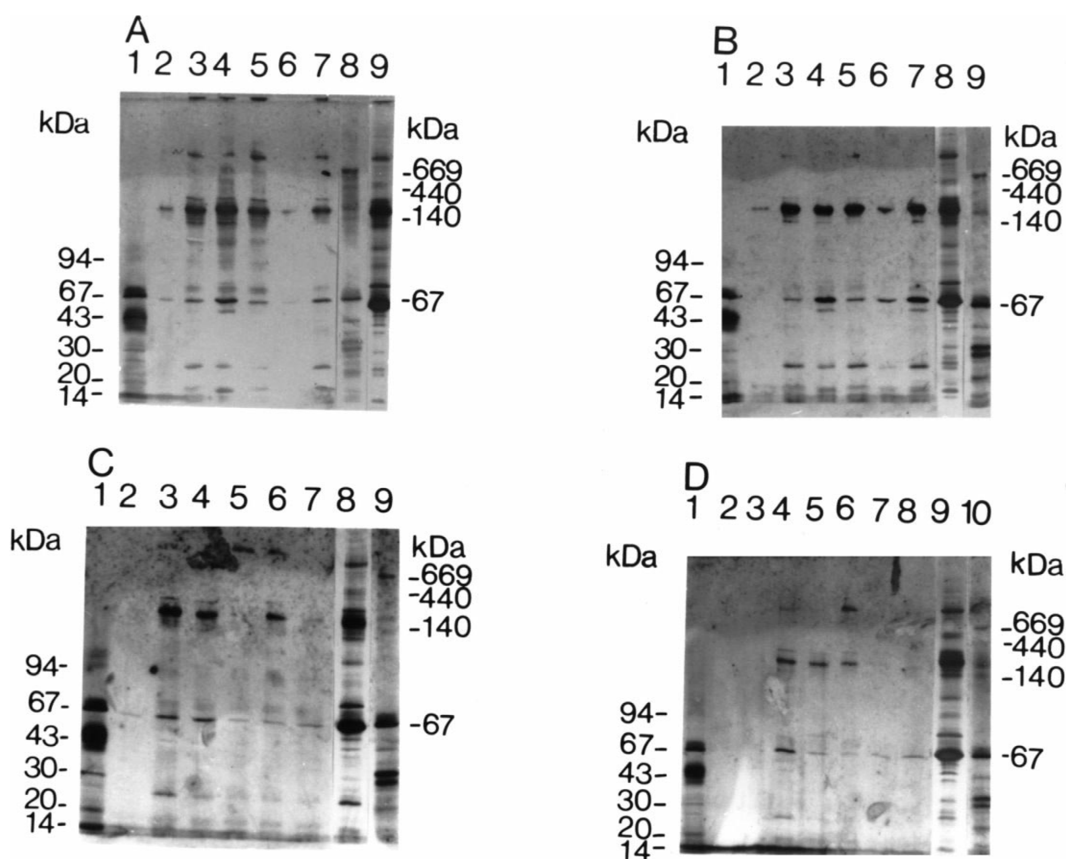

Fig. 2. SDS–PAGE profiles of the eluted fractions derived from the *im*-M^{n+}–8-HQ adsorbents after human serum proteins (5 mg) had been loaded onto a column (1 ml) in equilibration buffers of various pH values and 0.5 *M* ionic strength. Adsorption and elution of the human serum proteins from the *im*-M^{n+}–8-HQ adsorbents was carried out as described in the Materials and Methods section. The following pH conditions prevailed for the adsorption step: (A) at pH 5.5 (gel A); pH 6.0 (gel B); pH 7.0 (gel C); and pH 8.0 (gel D). The SDS–PAGE analysis was carried out with the eluted fractions of *im*-M^{n+}–8-HQ adsorbents using 4–15% gradient gels with the protein bands detected by a modified silver staining procedure adapted from the method of Morrisey [31]. The legend to the gel and lane codes are as follows: (A) SDS–PAGE gel A represents the eluted fractions from metal ion free *im*-8-HQ (lane 2), *im*-Yb^{3+}–8-HQ (lane 3), *im*-Fe^{3+}–8-HQ (lane 4); *im*-Cu^{2+}–8-HQ (lane 5); *im*-Ca^{2+}–8-HQ (lane 6), *im*-Al^{3+}–8-HQ (lane 7) and the initial human serum sample (lane 9). Lane 1 represents low-molecular-mass markers, phosphorylase B (M_r 94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000) and α-lactalbumin (14 400). Lane 8 represents the high-molecular-mass markers thyroglobulin (669 000), ferritin (440 000), lactate dehydrogenase (140 000) and bovine serum albumin (67 000). (B) SDS–PAGE gel B represents the eluted fractions from metal ion free *im*-8-HQ (lane 2), *im*-Yb^{3+}–8-HQ (lane 3), *im*-Fe^{3+}–8-HQ (lane 4); *im*-Cu^{2+}–8-HQ (lane 5); *im*-Ca^{2+}–8-HQ (lane 6), *im*-Al^{3+}–8-HQ (lane 7) and the initial human serum sample (lane 8). The low- and high-molecular-mass protein markers are the same as for gel A and are in lanes 1 and 9, respectively. (C) SDS–PAGE gel C represents the eluted fractions from metal ion free *im*-8-HQ (lane 2), *im*-Yb^{3+}–8-HQ (lane 3), *im*-Fe^{3+}–8-HQ (lane 4); *im*-Cu^{2+}–8-HQ (lane 5); *im*-Ca^{2+}–8-HQ (lane 6), *im*-Al^{3+}–8-HQ (lane 7) and the initial human serum sample (lane 8). The low- and high-molecular-mass markers are the same as for gel A and are in lanes 1 and 9, respectively. (D) SDS–PAGE gel D represents the eluted fractions from metal ion free *im*-8-HQ (lane 3), *im*-Yb^{3+}–8-HQ (lane 4), *im*-Fe^{3+}–8-HQ (lane 5); *im*-Cu^{2+}–8-HQ (lane 6); *im*-Ca^{2+}–8-HQ (lane 7), *im*-Al^{3+}–8-HQ (lane 8) and the initial human serum sample (lane 9). Lane 2 was not loaded with sample. The low- and high-molecular-mass markers are the same as for gel A and are in lanes 1 and 10, respectively. kDa=Kilodalton.

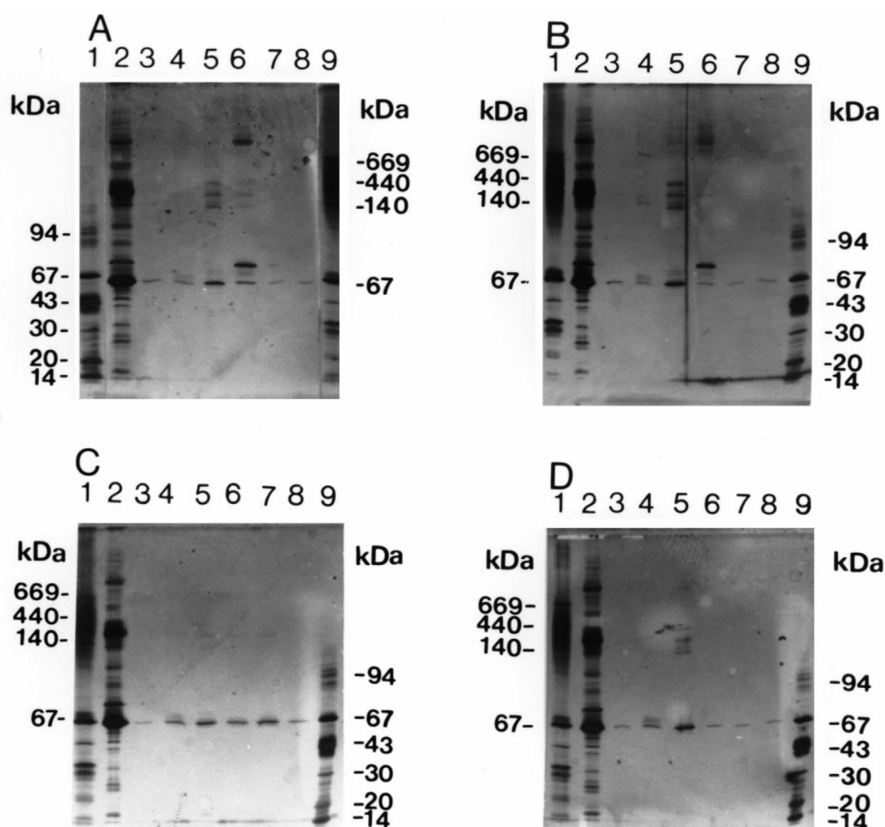

Fig. 3. SDS–PAGE profiles of the eluted fractions derived from the *im*-M^{n+}–OPS adsorbents after human serum proteins (5 mg) had been loaded onto a column (1 ml) in equilibration buffers of various pH values and at 0.5 *M* ionic strength. Adsorption and elution of the human serum proteins from the *im*-M^{n+}–OPS adsorbents was carried out as described in the Materials and Methods section. The following pH conditions prevailed for the adsorption step: (A) at pH 5.5 (gel A); pH 6.0 (gel B); pH 7.0 (gel C); and pH 8.0 (gel D). The SDS–PAGE analysis was carried out with the eluted fractions of *im*-M^{n+}–OPS adsorbents using 4–15% gradient gels with the protein bands detected by a modified silver staining procedure as described in the legend for Fig. 2. The legend to the SDS–PAGE gels A, B, C and D and the lane codes are as follows: initial human serum sample (lane 2); and the eluted fractions from metal ion free *im*-8-OPS (lane 3), *im*-Yb^{3+}–OPS (lane 4), *im*-Fe^{3+}–OPS (lane 5); *im*-Cu^{2+}–OPS (lane 6); *im*-Ca^{2+}–OPS (lane 7), *im*-Al^{3+}–OPS (lane 8). Lane 1 on gel A and lane 9 on gels B, C and D, represent low-molecular-mass markers, phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000) and α-lactalbumin (14 400). Lane 9 on gel A and lane 1 on gels B, C and D represent the high-molecular-mass markers thyroglobulin (669 000), ferritin (440 000), lactate dehydrogenase (140 000) and bovine serum albumin (67 000), respectively.

150 000) did not bind to the *im*-Yb^{3+}–IDA adsorbent but did bind weakly to the metal ion free *im*-IDA adsorbent under these low ionic strength conditions.

Further illustrative of the selectivity differences between these different IMAC adsorbents were also the results noted for α_2-M under the various adsorption conditions. For example, all of the *im*-M^{n+}–8-HQ adsorbents bound this protein at pH 5.5 and an ionic strength of 0.06 *M*, whilst only the *im*-Cu^{2+}–IDA, *im*-Ca^{2+}–IDA, *im*-IDA, *im*-Al^{3+}–OPS, *im*-Cu^{2+}–OPS, *im*-Fe^{3+}–OPS and *im*-Yb^{3+}–OPS adsorbents bound α_2-M under these conditions. At 0.5 *M* ionic strength, Cu^{2+} when immobilised to all chelating adsorbents bound α_2-M, whilst Yb^{3+}, Al^{3+} and Fe^{3+} only bound this protein when immobilised to *im*-8-HQ. Increasing the pH from pH 5.5 to pH 8.0 resulted in weaker binding of α_2-M to these

IMAC adsorbents in all cases. Increasing the ionic strength from 0.06 *M* to 0.5 *M* at pH 8.0 also resulted in fewer adsorbents binding α_2-M. This effect, however, was not always the case as found at pH 5.5, with the higher binding of α_2-M to the *im*-Al^{3+}–8-HQ, *im*-Cu^{2+}–8-HQ, *im*-Fe^{3+}–8-HQ, *im*-Yb^{3+}–8-HQ, *im*-Cu^{2+}–IDA or *im*-Cu^{2+}–OPS adsorbents. In these cases, a composite binding effect appears to have lead to reinforcement of the coordination interactions with increasingly dominant electrostatic contributions occurring at lower ionic strength values.

Selectivity differences detected for IgG between the various IMAC adsorbents were particularly notable when the adsorption ionic strength was 0.5 *M*. All the *im*-M^{n+}–8-HQ adsorbents bound IgG when the adsorption buffer was 0.5 *M* ionic strength and the pH was either pH 5.5 or pH 6.0. When Al^{3+} was, however, chelated to *im*-IDA or *im*-OPS, these IMAC adsorbents did not show any significant affinity for the IgG fraction despite these adsorbents having a higher Al^{3+} content than the *im*-Al^{3+}–8-HQ adsorbent. Similarly, Yb^{3+} when chelated to *im*-OPS did not bind IgG, but did so when chelated with *im*-8-HQ. As the pH became more alkaline, less IgG bound to all of these IMACs. Increasing the ionic strength to 0.5 *M* also generally decreased the binding of IgG to the IMAC adsorbent. The cases where increasing the ionic strength did not have any significant affect on IgG binding occurred at pH 5.5. Again, the mode of interaction of IgG with the metal ion adsorbents appears to be mediated through a combination of electrostatic and coordination effects.

HSA (M_r 67 000) showed affinity for most IMAC adsorbents under all conditions of pH and ionic strength. This protein has an isoelectric point (p*I*) of 4.9 [2] and at the adsorption pH used in these studies would be negatively charged. However, HSA was observed to bind to most IMAC adsorbents despite increases in the ionic strength or pH of the adsorption conditions. With the exception of the metal ion free *im*-8-HQ adsorbent at 0.5 *M* ionic strength and over the range pH 6.0 to pH 8.0, HSA also bound to some extent to the metal ion free adsorbents. Only the *im*-Fe^{3+}–IDA, *im*-Fe^{3+}–OPS and *im*-Cu^{2+}–OPS adsorbents bound more HSA than did their metal ion free counterparts as judged by SDS–PAGE and protein assay methods.

3.3. (C) Selection of immobilised hard Lewis metal ion–chelate adsorbents suitable for use in the purification of the marker proteins

During the binding and SDS–PAGE analysis experiments described above, it became obvious that in several cases the marker proteins IgG, Trf and α_2-M could be substantially fractionated from other serum proteins with high recoveries. These cases were repeated at larger scale under the same adsorption and elution conditions and then further investigated by examining their eluted fractions by SDS–PAGE. The identity of the marker proteins was confirmed by molecular size and immunochemically using Ouchterlony immunodiffusion analysis. On the basis of these results, the following IMAC systems were examined with a specific protein, IMCC system, adsorption and elution buffer combinations in mind; e.g., (i) for the IgG fractionation, *im*-M^{n+}–8-HQ adsorbents at pH 7.0 and 0.5 *M* ionic strength; (ii) for purification of Trf, *im*-M^{n+}–OPS adsorbents at pH 5.5 and 0.5 *M* ionic strength; (iii) for purification of α_2-M, *im*-M^{n+}–8-HQ adsorbents, at pH 5.5 and 0.5 *M* ionic strength and *im*-M^{n+}–OPS adsorbents at pH 5.5 and ionic strengths of 0.5 *M* and 0.06 *M*.

3.3.1. (A) Purification of the IgG fraction

(i) Using the *im*-Yb^{3+}–8-HQ or *im*-Al^{3+}–8-HQ adsorbents, the fractionation of IgGs from other serum proteins was carried out at pH 7.0 and 0.5 *M* ionic strength. SDS–PAGE analyses of the eluted fractions collected from the *im*-Yb^{3+}–8-HQ or *im*-Al^{3+}–8-HQ adsorbent indicated (Fig. 3, gel E and F, lane 4) that under the adsorption conditions of pH 7.0 and 0.5 *M* ionic strength, the eluted fraction contained a major band (M_r 150 000) with significantly reduced levels of the M_r 67 000 (HSA) and 76 000 (Trf) proteins compared to the initial mixture. The composition of this major band was confirmed to be IgG by Ouchterlony immunodiffusion procedures (Fig. 4). Very low levels of IgG were detected in the non-adsorbed fraction by Ouchterlony immunodiffusion and SDS–PAGE methods. Recovery of the IgGs in the eluted fraction from the *im*-Yb^{3+}–8-HQ adsorbent was >90%.

(ii) Using the *im*-Fe^{3+}–8-HQ adsorbent, the IgG

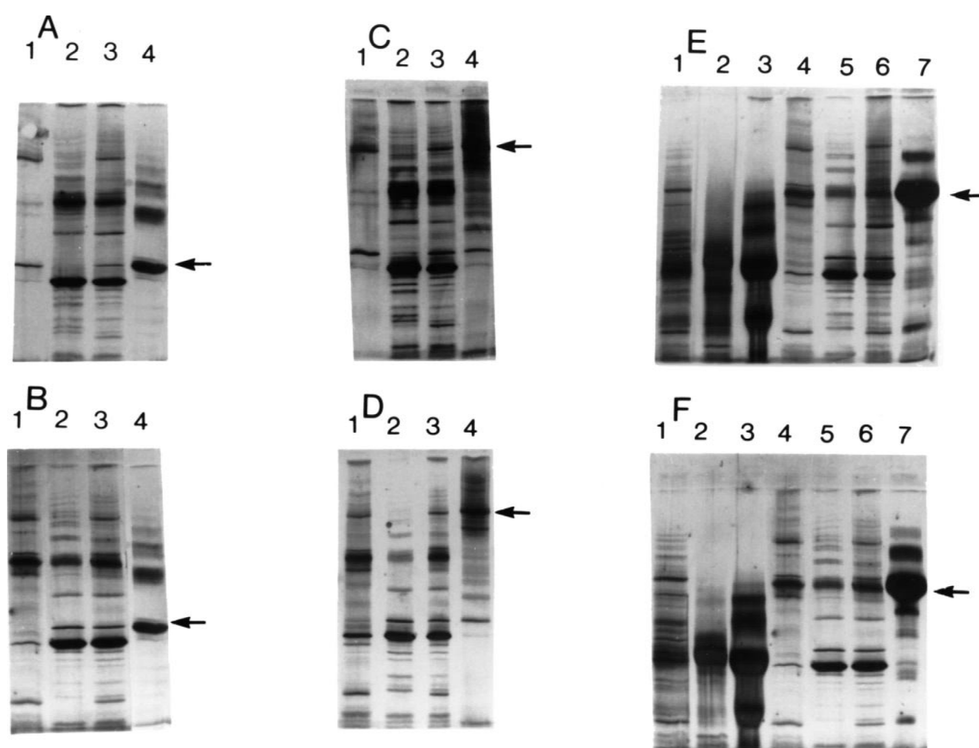

Fig. 4. SDS–PAGE profiles of the purification of transferrin, α_2-macroglobulin and the IgG fraction, of human serum using IMAC adsorbents. The SDS–PAGE analysis was carried out on 4–15% gradient gels with silver staining as described above in the legend to Fig. 2. The adsorption of serum proteins onto the adsorbents was carried out at pH 5.5 and an ionic strength of 0.5 *M*, whilst the elution was carried out using 200 m*M* EDTA–500 m*M* NaCl, pH 8.0, as described in the Materials and Methods section. The legend to the SDS–PAGE gels and the lane codes are as follows: SDS–PAGE gels A and B correspond to the fractionation of transferrin from human serum achieved using the *im*-Cu^{2+}–OPS and *im*-Al^{3+}–8-HQ adsorbents, respectively. For SDS–PAGE gels A and B, lane 1 represents the eluted fraction; lane 2, the non-adsorbed fractions; lane 3, the initial crude human serum samples; and lane 4, the ovotransferrin standard. SDS–PAGE gels C and D correspond to the fractionation of α_2-macroglobulin from human serum achieved using the *im*-Cu^{2+}–OPS and *im*-Al^{3+}–8-HQ adsorbents, respectively. For SDS–PAGE gels C and D, lane 1 represents the eluted fraction; lane 2, the non-adsorbed fraction; lane 3, the initial crude human serum sample; and lane 4, the human α_2-macroglobulin standard. SDS–PAGE gels E and F correspond to the fractionation of the IgG fraction from human serum achieved using the *im*-Yb^{3+}–8-HQ and *im*-Al^{3+}–8-HQ adsorbents, respectively. For SDS–PAGE gels E and F, lane 1 represents the β-mercaptoethanol reduced sample of the eluted fraction; lane 2, the reduced sample of the non-adsorbed fraction; lane 3, the reduced sample of standard human IgG; lane 4, the non-reduced eluted fraction; lane 5, the non-reduced non-adsorbed fraction; lane 6, the non-reduced initial crude human serum sample; and lane 7, the non-reduced human IgG standard.

fraction of human serum could also be isolated as the major protein component (Fig. 2, gel C lane 4). When a loading buffer of pH 7.0 and 0.5 *M* ionic strength was employed, the SDS–PAGE profile of the eluted fraction indicated that the IgG (M_r 150 000) was the predominant band, and substantially free of the M_r 76 000 band (Trf). The eluted fraction also had considerably reduced levels of the M_r 67 000 band (HSA). The SDS–PAGE and protein concentration determination, however, indicated that the recovery on elution of the bound IgG fraction from the *im*-Fe^{3+}–8-HQ adsorbent, although high, was lower than that obtained for *im*-Yb^{3+}–8-HQ adsorbent. No IgG was detected by SDS–PAGE in the non-bound fraction. This was further confirmed by the inability to detect any IgG in the non-adsorbed fraction using Ouchterlony immunodiffusion.

3.3.2. (B) Purification of transferrin

Using the *im*-Cu^{2+}–OPS adsorbent, substantial purification of Trf from other serum proteins was achieved in a single step by carrying out the ad-

sorption at pH 5.5 and 0.5 *M* ionic strength. Two major bands were evident by SDS–PAGE analysis of the eluted fraction (Fig. 3, gel A, lane 1) with the *im*-Cu^{2+}–OPS adsorbent, namely, the M_r 76 000 and the 720 000 band corresponding to Trf and α_2-M, respectively. In comparison, the *im*-Al^{3+}–8-HQ adsorbent showed little affinity for transferrin as evident from the SDS–PAGE analysis of the eluted

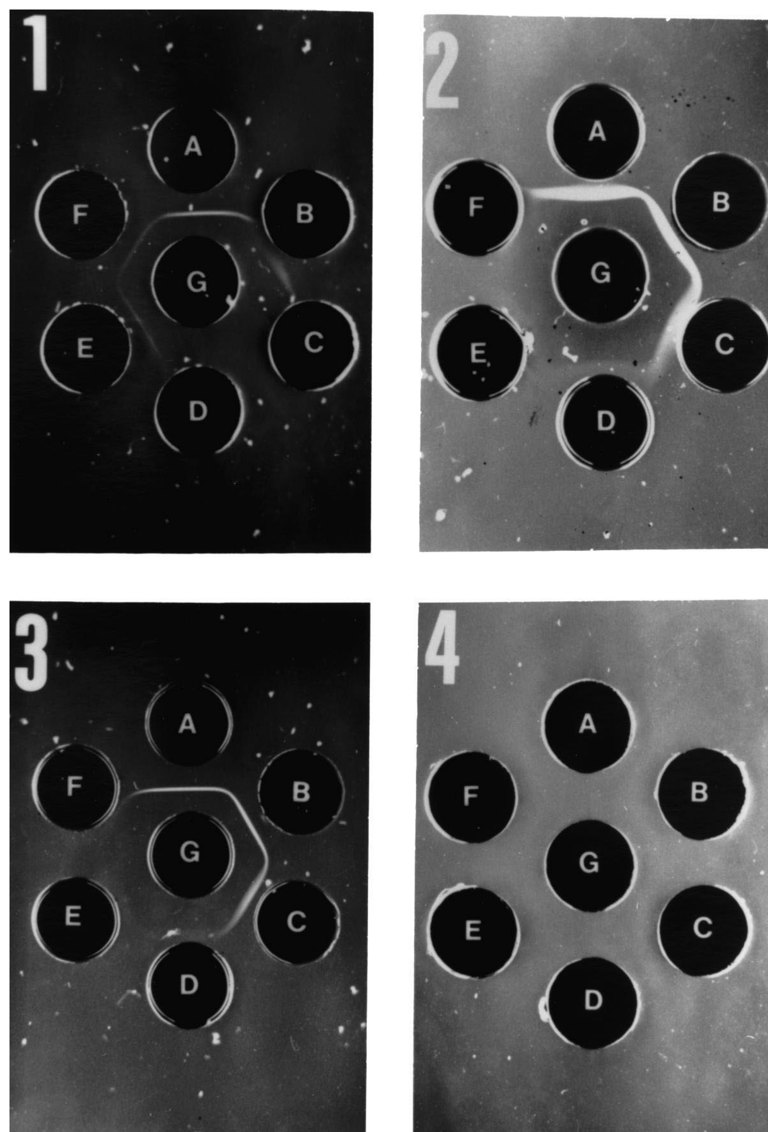

Fig. 5. Ouchterlony immunodiffusion identification of the IgG fraction in the eluted fraction recovered from the *im*-Yb^{3+}–8-HQ adsorbent following loading of human serum proteins in an adsorption buffer at pH 5.5 and 0.5 *M* ionic strength. The code for the plates is as follows: plate 1 (well G) represents standard IgG (100 μg); plate 2 (well G) represents the IgG fraction present in the initial serum protein mixture (diluted 1 in 5); plate 3 (well G) represents a 1 in 100 dilution of the of the eluted fraction from the *im*-Yb^{3+}–8-HQ column; plate 4 (well G) represents a 1 in 100 dilution of the non-adsorbed fraction from the *im*-Yb^{3+}–8-HQ column (a range of dilutions from undiluted to 1 in 1000 dilution were also negative for the IgG fraction in this fraction). Various dilutions of the IgG fraction antisera are presented in well A at a 1:5 dilution; well B at a 1:10 dilution; well C at a 1:20 dilution; well D at a 1:40 dilution; well E at a 1:60 dilution; and well F at a 1:80 dilution.

fraction (Fig. 3, gel B, lane 1) but a high affinity for IgG. The eluted fraction from the *im*-Cu^{2+}–OPS adsorbent had considerably reduced levels of IgG (M_r 150 000) and HSA (M_r 67 000) as indicated by SDS–PAGE. Moreover, Trf was identified in the eluted fraction from the *im*-Cu^{2+}–OPS adsorbent by Ouchterlony immunodiffusion (Fig. 5), and was not detectable in the non-adsorbed fraction. High re-

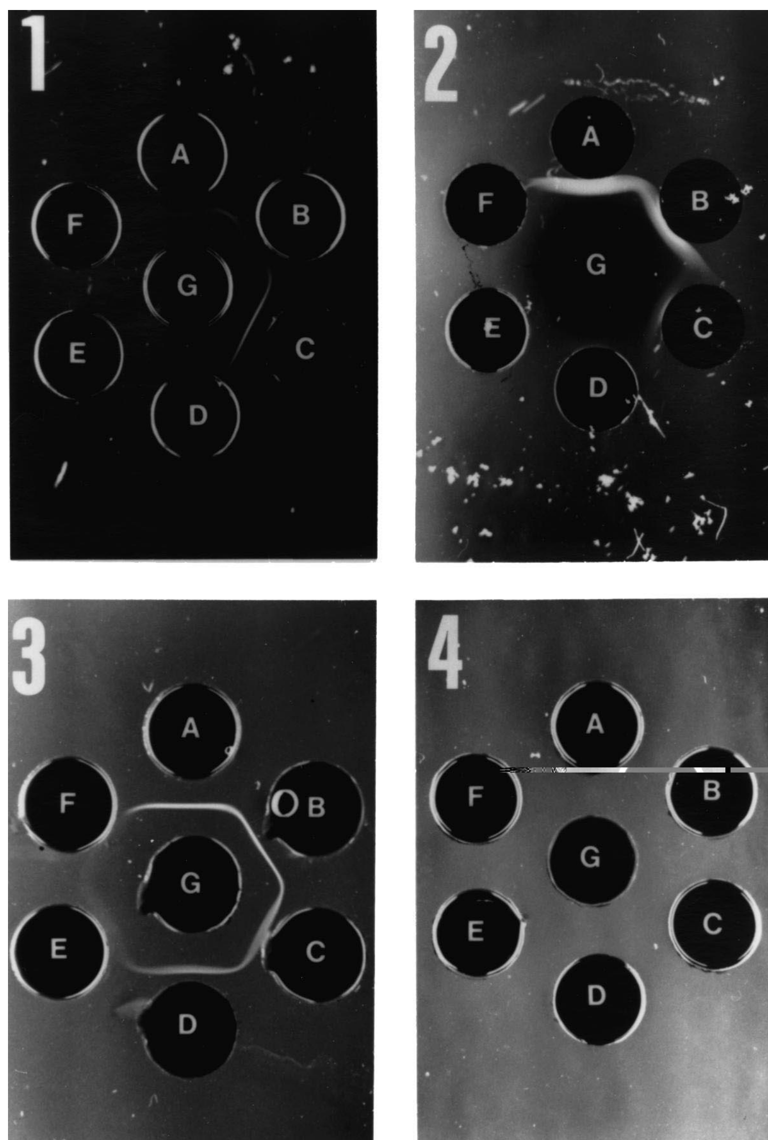

Fig. 6. Ouchterlony immunodiffusion identification of transferrin in the eluted fraction recovered from the *im*-Cu^{2+}–OPS adsorbent following loading of human serum proteins in an adsorption buffer at pH 5.5 and 0.5 *M* ionic strength. The code for the plates is as follows: plate 1 (well G) represents standard transferrin (100 μg), plate 2 (well G) represents the transferrin present in the initial serum protein mixture (undiluted); plate 3 (well G) represents a 1 in 2 dilution of the eluted fraction from the *im*-Cu^{2+}–OPS column; plate 4 (well G) represents a 1 in 2 dilution of the non-adsorbed fraction from the *im*-Cu^{2+}–OPS column (a range of dilutions from undiluted to 1 in 1000 dilution were also negative for transferrin in this fraction). Various dilutions of the transferrin antisera were presented in well A at a 1:5 dilution; well B at a 1:10 dilution; well C at a 1:20 dilution; well D at a 1:40 dilution; well E at a 1:60 dilution, and well F at a 1:80 dilution.

covery (>90%) of transferrin was achieved based on the comparative immunodiffusion, SDS–PAGE and protein concentration results for the eluted fraction and the unfractionated sample.

3.3.3. (C) Purification of α_2-macroglobulin

With the *im*-Cu^{2+}–OPS adsorbent at pH 6.5 and 0.06 *M* as well as 0.5 *M* ionic strength, most of the α_2-M bound to the IMAC adsorbent as indicated by

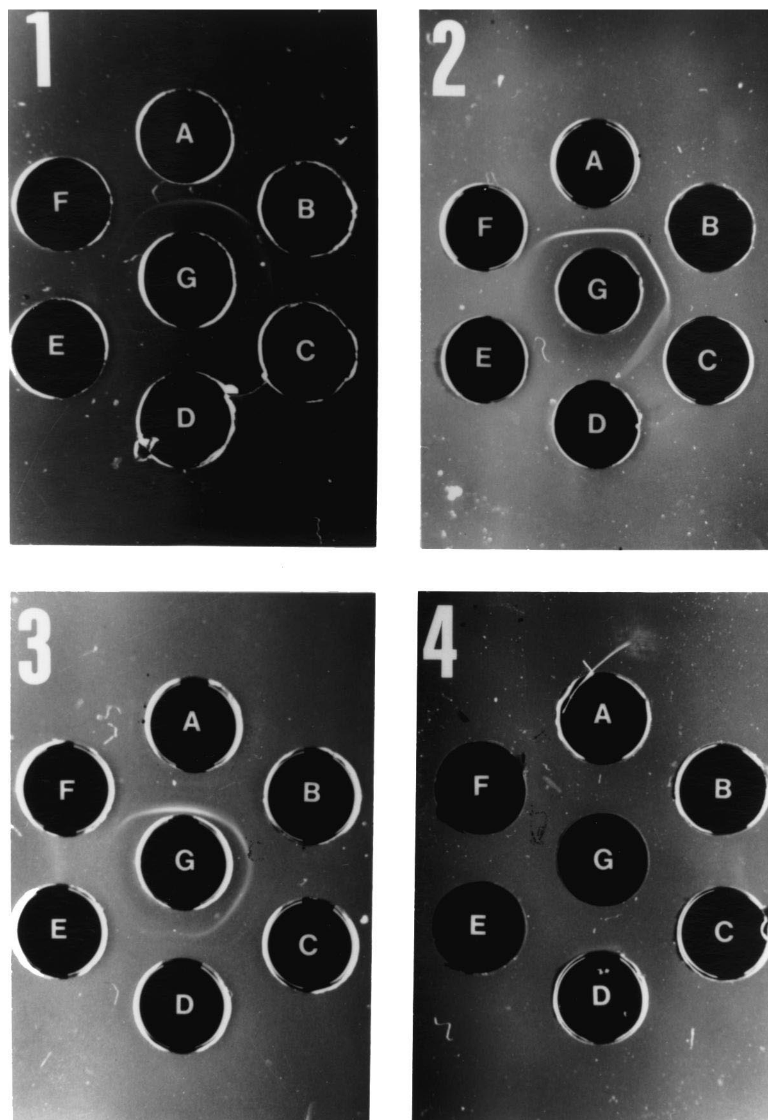

Fig. 7. Ouchterlony immunodiffusion identification of human α_2-macroglobulin in the eluted fraction recovered from the *im*-Yb^{3+}–8-HQ adsorbent following loading of human serum proteins in an adsorption buffer at pH 5.5 and 0.5 *M* ionic strength. The code for the plates is as follows: plate 1 (well G) represents standard human α_2-macroglobulin (100 μg); plate 2 (well G) represents the human α_2-macroglobulin present in the initial serum protein mixture (undiluted); plate 3 (well G) represents a 1 in 100 dilution of the eluted fraction from the *im*-Yb^{3+}–8-HQ column; plate 4 (well G) represents a 1 in 100 dilution of the non-adsorbed fraction from the *im*-Yb^{3+}–8-HQ column (a range of dilutions from undiluted to 1 in 1000 dilution were also negative for human α_2-macroglobulin in this fraction). Various dilutions of the human α_2-macroglobulin antisera are presented in well A at a 1:5 dilution; well B at a 1:10 dilution; well C at a 1:20 dilution; well D at a 1:40 dilution; well E at a 1:60 dilution; and well F at a 1:80 dilution.

the absence of this protein in the non-adsorbed fraction and presence as a M_r 720 000 protein in the eluted fraction as determined by SDS–PAGE and Ouchterlony immunodiffusion analysis (Fig. 3, gel A, lane 1, and Fig. 6). The SDS–PAGE analysis also indicated that the eluted fraction from the *im*-Cu^{2+}–OPS adsorbent contained Trf. No detectable HSA or IgG fraction was present in the eluted α_2-M sample from the *im*-Cu^{2+}–OPS adsorbent with the level of other contaminants low as indicated by SDS–PAGE and Ouchterlony immunodiffusion analysis. Compared to the eluted fraction obtained from the *im*-Al^{3+}–8-HQ adsorbent (Fig. 3, gel D, lane 1, and Fig. 7), for example, significantly higher selectivity was achieved with the *im*-Cu^{2+}–OPS adsorbent for α_2-M under these conditions.

4. Discussion

The primary purpose behind the development of most chromatographic adsorbents is to enable the purification of certain target molecules from a crude mixture. Thus, in the development of new adsorbents intended for use in protein purification, such as the IMCC/IMAC systems studied here, the ability of the adsorbents to selectively capture and separate different target proteins from a crude protein mixture needs to be documented, often via screening techniques. Furthermore, if a particular IMAC adsorbent is to find a useful application, it should manifest an alternative mode(s) of protein selectivity to those offered by other chromatographic systems. It was therefore the aim of this study to screen several new types of IMAC adsorbents for their potential in the fractionation of a crude mixture of proteins, with a number of target proteins in mind. To this end, the selectivity features of the IMAC adsorbents – *im*-M^{n+}–8-HQ, *im*-M^{n+}–OPS and *im*-M^{n+}–IDA (where M^{n+}=Al^{3+}, Ca^{2+}, Cu^{2+}, Fe^{3+} and Yb^{3+}) – have been compared to the commonly available *im*-Cu^{2+}–IDA adsorbent, employing serum proteins as an example of a crude protein mixture.

The selectivity and binding differences of these IMAC adsorbents were determined by SDS–PAGE of the non-bound and eluted protein fractions, together with the results obtained from the total protein assays. These preliminary results indicated that with incubation/equilibration buffers of 0.5 *M* or 0.06 *M* ionic strength, as the pH became increasingly alkaline, fewer proteins bound to these hard Lewis metal ion IMAC adsorbents. This pH-dependent protein binding characteristic, observed for all the hard Lewis metal ion IMAC adsorbents, is opposite to what is usually found with proteins with the borderline Lewis metal ion IMAC systems. This type of pH-dependent effect with these hard Lewis metal ion IMAC adsorbents is however consistent with the behaviour found previously with *im*-Fe^{3+}–hydroxamate, *im*-Fe^{3+}–TED or *im*-Fe^{3+}–IDA adsorbents during the fractionation of serum proteins [11,30,34,35]. The results of the present study thus extend these previous findings with *im*-Fe^{3+}–chelating ligand systems, and also demonstrate that hard Lewis metal ions such as Yb^{3+} and Al^{3+} also show a similar pH dependence when employed in IMAC systems. Furthermore, this study indicates that such effects are not dictated solely by the type of immobilised chelating ligand or metal ion per se. For example, the *im*-Fe^{3+}–OPS adsorbent showed affinity for only the IgG fraction at pH 5.5 and pH 6.0 with 0.5 *M* ionic strength buffers and affinity for HSA at all pH values, whilst the *im*-Fe^{3+}–8-HQ adsorbents, despite having a lower metal ion content, also showed affinity toward Trf and α_2-M at pH 5.5.

As indicated by the total protein assays and SDS–PAGE results, higher levels of serum proteins bound to these hard Lewis metal ion IMAC adsorbents and their metal ion free counterparts over the range of pH 5.5–8.0 when the ionic strength of the equilibration buffer was 0.06 *M* compared to 0.5 *M*. Decreases in the amount of bound proteins, resulting from increases in ionic strength, are usually observed with ion-exchange adsorbents. Cation exchangers are also characterised by a decrease in protein binding as the pH of the buffer becomes higher. When these observations on the binding behaviour of these hard Lewis metal ion IMAC adsorbents towards serum proteins are combined with data on the metal ion contents, the conclusion can be drawn that both electrostatic and coordination interactions occur under some buffer conditions. The fact that significant protein binding occurred in a low ionic strength milieu but not with a high-ionic-strength buffer suggests that for several of the partially metal ion clad systems, i.e., the *im*-Al^{3+}–IDA or *im*-Ca^{2+}–8-HQ adsorbents, the chromatographic surface acts as a bimodal binding environment with both coordina-

tion and electrostatic sites possibly interacting with the protein(s) in an independent fashion. Clearly, further detailed investigations are required to delineate the precise mechanism of interaction of these new classes of adsorbents with proteins. With the Fe^{3+}- and Cu^{2+}-based systems on the other hand, where nearly quantitative saturation of the immobilised chelating ligand by the metal ion has occurred, other explanations for the ability of these hard Lewis metal ion–chelate adsorbents to behave as pseudo cation exchangers under low-ionic-strength buffer conditions must be sought. In the case of the *im*-Fe^{3+}–OPS adsorbent, the binding behaviour is consistent with this hard Lewis metal ion IMAC system becoming more negatively charged at higher pH values. It is well known [18,19] that Fe^{3+} ions can form hydroxyl group coordination sphere complexes of the type *im*-$[Fe^{3+}(OH^-)_3L]$ or *im*-$[Fe^{3+}(OH^-)_2L]$ rather than aquo complexes, i.e., *im*-$[Fe^{3+}(H_2O)_3L]$ or *im*-$[Fe^{3+}(H_2O)_2L]$, when bound to chelating ligands at higher pH values, a characteristic also shown by other hard Lewis metal ions. Such hydroxyl complexes will tend to assume a pseudo-cation-exchange character with proteins, with the binding process manifesting a pH-dependent responsiveness to ionic strength effects. Such pseudo-cation-exchange behaviour has also been observed by Ramadan and Porath [36] with *im*-Fe^{3+} hydroxamate adsorbents with serum proteins. The findings of the present study support these results for Fe^{3+} ions complexed with immobilised bi- and tridentating chelating ligands. Furthermore these studies extend this behaviour to include several other hard Lewis metal ions, and indicate that this pseudo-cation behaviour evident with Fe^{3+} ions is independent of the type of immobilised chelating agent used. Interestingly, potentiometric measurements have not detected[18.19] similar *im*-$[Cu^{2+}(OH^-)L]$ complexes with *im*-Cu^{2+}–IDA adsorbents, although they have been proposed to exist [37] under alkaline buffer conditions.

Electrostatic interactions, however, do not constitute the only mode of interaction that occurs in the binding of serum proteins to these hard Lewis metal ion IMAC adsorbents. These cases are exemplified by adsorbents that do not exhibit a decrease in protein binding as a result of an increase in the ionic strength of the adsorption buffer. For example, identical binding results were obtained with the *im*-Fe^{3+}–IDA adsorbent when the serum proteins were loaded at pH 5.5 and 0.5 *M* ionic strength or at pH 5.5 and 0.06 *M* ionic strength. In these cases, composite coordination and electrostatic interactions appear to occur. As the pH becomes more alkaline, electrostatic interactions may start to dominate the mode of interaction with a specific protein, because of the increasing overall negatively charged character of the *im*-Fe^{3+}–IDA adsorbent, due to the progressive formation of the *im*-$[Fe^{3+}(OH^-)_nL]$ complexes (when $n=1–3$). Depending on whether the protein prefers to form a coordination or as an ionic interaction complex with the *im*-Fe^{3+}–IMCCs under these higher pH conditions will determine the magnitude of the equilibrium association constant and the mode of elution.

The high recovery of the bound protein achieved with 200 m*M* EDTA–500 m*M* NaCl, pH 8.0, eluent suggests that interactions other than electrostatic or coordination interactions do not significantly contribute to the protein adsorption/desorption process with these hard Lewis metal ion IMAC adsorbents. Hydrophobic interactions, for example, would be expected to be promoted by increased ionic strength. As such, an increase in the ionic strength of the equilibration buffer from 0.06 *M* to 0.5 *M* should result in reinforcement of the binding interactions between the protein and the IMCC, if the hydrophobic effect made a significantly contribution to the interaction. Furthermore, protein binding at an ionic strength of 0.5 *M* specifically occurred to the *im*-M^{n+}–IMCCs, since the metal ion free adsorbents typically exhibited insignificant binding of serum proteins at this ionic strength.

One of the aims of this study was to compare the protein selectivity of the hard Lewis metal ions with the behaviour found for the more commonly used borderline metal ion, Cu^{2+}, as the *im*-M^{n+}–8-HQ, *im*-M^{n+}–OPS and *im*-M^{n+}–IDA adsorbents. In general, the behaviour of the borderline metal ion *im*-Cu^{2+}–8-HQ and *im*-Cu^{2+}–OPS adsorbents was very similar to the hard Lewis metal ion IMAC adsorbents, with a decrease in protein adsorption as the pH became alkaline and as the ionic strength of the adsorption buffers was increased. Such an observation is contrary to that observed when *im*-Cu^{2+}–IDA adsorbents have previously been used for the frac-

tionation of serum proteins [8,38], where the capacity for serum proteins has been found to increase as the buffer pH became alkaline. This effect can be exploited with *im*-Cu^{2+}–IDA adsorbents through the use of amine-based buffers at the elution stage, in agreement with the concept that *im*-Cu^{2+} ions bind preferentially to amino groups (predominantly histidine residues) that are accessible on the surface of the proteins. This outcome also is in accord with the concept that borderline Lewis metal ions such as Cu^{2+} have a preference for nitrogen containing compounds [17] and consistently demonstrated findings for the binding behaviour of histidine-containing proteins using Cu^{2+} ions in the IMAC mode [9,39]. In the present work, the adsorption buffers also included imidazole, thus minimising the opportunity for histidine residues to contribute to the interaction with the IMAC adsorbent. It is thus of interest to note that strong binding was still observed for serum proteins with these hard Lewis metal ion IMAC adsorbents at pH 5.5 and pH 6.0, where surface histidine residues would most likely not be able to undergo any significant $n \rightarrow d$ orbital electron donation, via the imidazole moiety, with the *im*-M^{n+}–IMCCs. As a result, any proteins that bound must have done so via contributing side chain groups other than surface accessible amino- or imidazoyl moieties, such as aspartic and glutamic acid residues, where the preference of hard Lewis metal ions for oxygen-rich compounds can be exploited. The behaviour of the serum proteins with the hard Lewis metal ion and Cu^{2+}–IMAC adsorbents under the selected loading buffer conditions is thus consistent with that observed in previous work using cytochrome *c* and other model proteins [12,15,16,20,21]. It can be postulated that under the adsorption conditions chosen in this study, serum proteins thus bind to the immobilised hard Lewis metal ions, Al^{3+}, Ca^{2+}, Cu^{2+}, Fe^{3+} and Yb^{3+}, through aspartic and glutamic acid residues. Since the behaviour of the Cu^{2+}–IMAC adsorbents was similar to that observed for the other immobilised hard Lewis metal ion adsorbents, it is possible that immobilised Cu^{2+} ions also exhibit mixed mode (coordination and electrostatic) interactive behaviour under these adsorption conditions. If this is the case, then these results suggest that Cu^{2+} ions can also be included with the hard Lewis metal ions as exhibiting protein selectivity based on coordination interactions via O-donors, depending on the nature of the immobilised chelating ligand and buffer condition. Similar behaviour has already been delineated with other Cu^{2+}–IMCC systems based on the immobilised macrocycle 1,4,7-triazacyclonane, *im*-tacn, and its bis derivatives [40,41].

During the screening experiments, which addressed the protein selectivity features of the different adsorbents, it became apparent that in some cases substantial purification of several of the marker proteins had occurred in a single chromatographic step. For example, α_2-M was substantially resolved from other serum proteins with the *im*-Cu^{2+}–OPS adsorbent at pH 5.5 and when the adsorption buffer had an ionic strength of 0.5 *M*. Most of the bound α_2-M was recovered in the eluted fraction, with transferrin the only significant contaminant. The purification and recovery of this protein with these hard Lewis metal ion IMAC adsorbents is remarkable since an elution protocol was employed which aimed to elute simultaneously all bound proteins from the IMAC adsorbent. Importantly, the large molecular mass difference between α_2-M (M_r 720 000) and Trf (M_r 76 000) can be exploited by concentration and dialysis/ultrafiltration of the eluted fraction using a M_r 100 000 cut-off membrane to separate these two proteins. Alternatively, the concentrated sample can be resolved [42] by size-exclusion chromatography using Sephadex G-200 and 50 m*M* NaH_2PO_4–110 m*M* NaCl, pH 6.5 as the elution buffer, if further purification was required.

The partial fractionation of α_2-M from serum proteins has been previously described [10] using an *im*-Cd^{2+}–IDA adsorbent, but the extent of HSA contamination or the overall purity was not reported. Purification of α_2-M with an *im*-Cd^{2+}–IDA adsorbent, moreover, is not practical for the production of a therapeutical grade protein because of the potentially dangerous and toxic nature of Cd^{2+} ions. Alternative methods based on *im*-Cu^{2+}–IDA or *im*-Zn^{2+}–IDA adsorbents for the purification of α_2-M result in strong protein binding, requiring high concentrations of a chaotropic salt (sodium cacodylate) to be employed at the elution stage [10,24,25,43]. Similar conditions were required for the elution of α_2-M with the *im*-Cu^{2+}–IDA adsorbent used in this present work. In comparison, the

purity and level of recovery achieved for α_2-M using the milder elution protocol as a single chromatographic step with the *im*-Cu^{2+}–OPS adsorbent surpassed that achieved in this and previous studies with *im*-Cu^{2+}–IDA or *im*-Zn–IDA adsorbents. These results highlight the selective nature of *im*-M^{n+}–OPS adsorbent systems. An alternative procedure for the purification of α_2-M from serum can be based on the use of the *im*-Yb^{3+}–8-HQ adsorbent, at pH 5.5 and an ionic strength of 0.5 *M* (see Fig. 7). This IMAC adsorbent also bound α_2-M strongly but did not offer the same level of control over the purification as observed with *im*-Cu^{2+}–OPS adsorbent. However, *im*-Yb^{3+}–8-HQ, at pH 5.5 and an ionic strength of 0.5 *M* does not bind Trf, the major contaminant of the α_2-M preparation obtained with the *im*-Cu^{2+}–OPS adsorbent. As discussed elsewhere [42], high purity preparations of α_2-M can be obtained with good recoveries through the use of a tandem chromatographic approach, employing the eluted human serum protein fraction obtained from the *im*-Yb^{3+}–8-HQ adsorbent and loaded (appropriately dialyzed) onto the *im*-Cu^{2+}–OPS adsorbent, with the re-bound proteins then eluted with a 125 m*M* malonic acid–500 m*M* NaCl gradient.

Additionally, the *im*-Cu^{2+}–OPS adsorbent at pH 5.5 and 0.5 *M* ionic strength can also be used for the partial purification of Trf with good recoveries. If further removal of the α_2-M contamination from the Trf is required, again the use of the *im*-Yb^{3+}–8-HQ adsorbent as a second chromatographic step at pH 5.5 and an ionic strength of 0.5 *M*, whereby the Trf does not bind under these conditions, can be implemented. This tandem-column approach [42], therefore, represents another example of the high degree of flexibility that can be achieved through the use of these immobilised hard Lewis metal ion IMAC adsorbents relative to the more commonly used *im*-Cu^{2+}–IDA or other *im*-borderline Lewis metal ions–IDA adsorbents. Similarly, in previous studies using *im*-Fe^{3+}–hydroxamate and *im*-Ni^{2+}–IDA adsorbents the fractionation of serum has not resulted in a homogeneous preparation of Trf [9–11]. In these cases, Trf was found to be substantially contaminated with HSA and α_2-M, along with other contaminants despite the use of refined elution protocols such as salt gradients and alterations in the salt types being used. Methods that do not use IMAC for the purification of Trf from serum are available, but are time-consuming even though they offer in some cases high recoveries [1]. These earlier methods are based on a series of precipitation steps with Rivanol, ammonium sulfate or ethanol, followed by anion-exchange chromatography to yield the purified Trf. The IMAC procedures arising from the present study thus provide an alternative strategy for the fractionation of transferrin with improved efficiency over these existing methods in terms by minimising the number of steps.

Based on a similar strategy, the IgG fraction can also be substantially purified using the *im*-Al^{3+}–8-HQ, *im*-Yb^{3+}–8-HQ or *im*-Fe^{3+}–8-HQ adsorbents at pH 5.5, pH 6.0 or pH 7.0 and 0.5 *M* ionic strength. Elution of the proteins from these IMAC adsorbents indicated that most of the IgG was recovered, with the IgG representing the major band on SDS–PAGE, largely free of Trf and the major serum protein constituent, HSA. Some α_2-M was, however, present in the eluted fraction from these IMAC adsorbents under these binding conditions. Further purification of IgG may be achieved by increasing the pH of the adsorption to pH 6.0 but this results in some losses of IgG, although most of the α_2-M and the traces of Trf, however, were removed. In this respect, a substantial purification of IgG was achieved in a single IMAC step. These hard Lewis metal ion–chelate adsorbents offer a significant improvement over the level of fractionation provided by the *im*-Cu^{2+}–IDA adsorbent, which yielded a IgG fraction heavily contaminated with all of the marker proteins as well as by other minor protein contaminants under the similar conditions or even other conditions such as lower ionic strength. By incorporating a *im*-Cu^{2+}–OPS column prior to the *im*-Al^{3+}–8-HQ or *im*-Yb^{3+}–8-HQ columns equilibrated in tandem fashion at pH 5.5 and an ionic strength of 0.5 *M*, further purification of IgG can be achieved. For example, as shown in this study, removal of Trf and α_2-M present in serum can be achieved by the *im*-Cu^{2+}–OPS adsorbent, which does not bind most of the IgG fraction. The IgG fraction can then directly delivered onto the *im*-Al^{3+}–8-HQ or *im*-Yb^{3+}–8-HQ adsorbent, free of these major contaminants.

HSA has previously been effectively purified using *im*-IDA–borderline Lewis metal ions in good recovery, yielding an electrophoretically homoge-

neous preparation [29,44], i.e., in 99.9% purity and 65% recovery. Using the adsorption and elution methods described in the present study in combination with the hard Lewis metal ion IMAC systems, HSA was not recovered either in high yield or as a homogeneous product. This result, however, again highlights the distinctive selectivity characteristics of these hard Lewis metal ion IMAC adsorbents compared to those involving borderline Lewis metal ions.

The work arising from these investigations indicate that hard Lewis metal ion IMAC systems can be applied effectively to the separation of crude mixtures of proteins such as those present in human serum. The *im*-M^{n+}–8-HQ and *im*-M^{n+}–OPS adsorbents offer alternative modes of selectivity for protein fractionation to that provided by the more commonly used *im*-Cu^{2+}–IDA or related IMAC adsorbents generated with borderline metal ions, e.g., *im*-Cu^{2+}–TED or *im*-Ni^{2+}–NTA systems. These alternative hard Lewis metal ion IMAC systems can strongly interact with proteins at low ionic strength and acidic pH. Increasing the ionic strength and/or the pH results in changes to the protein selectivity capabilities of these adsorbents. Because of these characteristics, some of these hard Lewis metal ion IMAC systems can be employed as alternative purification procedures for important serum proteins such as Trf, α_2-M and IgG. Further refinements in selection of conditions for adsorption and, in particular, at the elution stage should facilitate greater control over the selectivity and result in even higher purity for these and some of the other serum proteins compared to the more commonly used *im*-Cu^{2+}–IDA or related *im*-M^{n+}–IMCC systems. Moreover, the results arising from this study indicate that a novel combination of coordination effects as well as electrostatic and hydrophobic interactions participate in the interactions between proteins and these hard metal ion IMAC adsorbents. Previously, we have documented that hydroxide ion–coordination complexes can form between the metal ion and hydroxide ions under some pH and buffer conditions. The formation of these hydroxide ion–coordination complexes results in the selectivity of these hard metal ion IMAC adsorbents switching from pseudo-cation-exchange behaviour under low ionic strength conditions to a more dominant coordination interaction as the ionic strength increases. At this stage of their development, the precise mechanism of interaction of hard metal ion IMAC adsorbents with proteins and peptides remains to be elucidated. Investigations addressing these issues are current underway in this laboratory. Based on the present study, and associated investigations, it can however be concluded that these hard Lewis metal ion IMAC adsorbents now provide an interesting set of alternatives for use in the fractionation of crude protein mixtures.

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