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High-performance liquid chromatography of amino acids, peptides and proteins

CXXXI☆. O-Phosphoserine as a new chelating ligand for use with hard Lewis metal ions in the immobilized-metal affinity chromatography of proteins

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ABSTRACT

Conditions for the immobilization of O-phosphoserine (OPS) to epoxy-activated Sepharose CL-4B are described. The binding behaviour of OPS and iminodiacetic acid (IDA) immobilized onto Sepharose CL-4B, toward the hard Lewis metal ions Al^{3+}, Fe^{3+}, Ca^{2+} and Yb^{3+}, and Cu^{2+} ion as a borderline metal ion control, over the pH range pH 4.0 to pH 8.0, was examined. Immobilized OPS shows a stronger affinity for Fe^{3+} and Al^{3+} ions but a lower affinity for Cu^{2+} and Yb^{3+} ions, compared to immobilized iminodiacetic acid (IDA), over the equilibrating range examined. Immobilized OPS-M^{n+} was screened for protein binding using as model proteins tuna heart cytochrome *c* (THCC), horse myoglobin (HMYO) and hen egg while lysozyme (HEWL) over the pH range 5.5 to 8.0. Immobilized OPS-Fe^{3+} bound THCC under all the examined equilibrating conditions, bound HMYO between pH 5.5 and pH 7.0 and did not bind HEWL under any condition examined. Immobilized OPS thus presents an additional mode of metal ion and protein selectivity in immobilized-metal affinity chromatography.

INTRODUCTION

The concept of covalently attaching a chelating agent to a support material was first suggested by Meinhardt in 1948 [1]. Sorbents containing phosphonic acid functional groups were amongst the first chelating resins to be described [2]. These sorbents were noted for their strong affinity for Fe^{3+} ions. Soon after, Helfferich [3] adapted the idea of immobilized metal–chelate complexes for use in ligand-exchange chromatography of polar compounds, predominantly amines and amino acids. Similar ligand-exchange chromatographic sorbents were later extended to other low-molecular-mass compounds such as nucleic acid components [4]. By the early 1960s, the ability of free metal ions to bind with high affinity to proteins was a well established observation [5]. In 1974, 8-hydroxyquinoline, which had been covalently immobilized to an agarose sorbent and chelated with Zn^{2+} ions, was used to isolate metalloproteins [6]. In 1975 Porath *et al.* [7], recognising the selectivity inherent to immobilized iminodiacetic acid (IDA), extended this chromatographic mode significantly to all protein classes and termed this technique immobilized-metal affinity chromatography (IMAC) [7]. Since then the studies of Porath and co-workers

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[8–10] as well as those of Sulkowski [11,12] and Andersson and co-workers [13–15] and many other investigators, have extensively documented the high selectivity which can be achieved with IDA-based sorbents, resulting in this mode of IMAC becoming a very popular approach for protein purification. The use of IMAC with proteins has been recently reviewed [16–18] whilst the more general use of chelating resins and their properties has been extensively reviewed by Sahni and Reedijk [19] and Davankov [20].

The metal ions used in IMAC separation of proteins have mainly been Cu^{2+}, Ni^{2+} and Zn^{2+}. These metal ions are known as borderline Lewis metal ions as defined by Pearson [21] and display preferential binding towards nitrogen or sulphur atoms contained in the side chains of the amino acid residues. Such a preference has resulted in the use of these borderline metal ions in IMAC separations with specificity toward polypeptides and proteins based on interaction with the indole group of tryptophan, the thiol group of cysteine or the imidazole group of histidine residues. Additionally, it is feasible to probe the surface characteristics of proteins on the basis of their histidine content with IMAC methods and soft metal ions [11,22,23].

Hard Lewis metal ions on the other hand, display preferential binding toward oxygen-containing side chains of amino acid residues and have only recently found favour amongst users of IMAC. Examples of these metal ions are found throughout the group II, group III, lanthanides and some transition metal ions such as Fe^{3+} and Mn^{2+}. Because of their properties Fe^{3+}, Al^{3+} and Ca^{2+} have been the most commonly used of the hard Lewis metal ions in IMAC. These ions can exhibit electrostatic binding characteristics but also manifest other forms of interaction not yet completely understood [12,24]. The preference of hard Lewis metal ions for oxygen containing ligands has been used to select proteins on the basis of their surface content of negatively charged amino acid residues [12,25] and has been shown to be independent of surface accessible histidine, tryptophan, cysteine, phosphate or carbohydrate residues [25]. Surface accessible aspartic and glutamic acids have been implicated in the binding of proteins to immobilized Fe^{3+} [25,26]. Glycoproteins [8] and phosphoproteins have also been shown to have a high affinity for immobilized hard Lewis metal ions such as Fe^{3+} and Al^{3+} [8,13,26]. Phosphoserine and phosphothreonine residues have been suggested to be responsible for the binding of phosphorylated proteins to immobilized IDA-Fe^{3+} [8,26]. Phosphoserine residues have been also attributed with the binding of metal ions to ovalbumin phosphoproteins [8]. Separation of phosphoproteins using immobilized Fe^{3+} or Al^{3+} is believed to occur predominantly through the oxygen groups on the phosphoserine and phosphothreonine moieties [26].

O-Phosphoserine (OPS) is found as a constituent of biological membranes and is believed to act as a mediator in ion transport action [27]. OPS is also found to be an important amino acid residue in caseins where it behaves as a metal ion chelator, in particular for Ca^{2+} ions [28–30]. However, it was not until 1957 that the metal ion binding behaviour of OPS was quantitatively assessed [31]. Binding of metal ions by free OPS is dependent on the ionization state of the OPS and can involve tridentate binding with metal ions through the oxygen atoms in the phosphate and the carboxyl groups, and the nitrogen of the amine [32]. As a bidentate chelating agent OPS binds metal ions through the carboxyl and the phosphate groups [33,34] or through the ionized oxygen atoms of the monoesterified phosphate group of OPS [35].

The use of sorbents containing immobilized phosphate is commonplace. For example, phosphonic acid sorbents and amino phosphonic acids have been extensively used for the separation of metal ions since 1956 [2,20]. Immobilized phosphonic acid ligands have also been used in the bioaffinity isolation of alkaline phosphatases [36], while phosphoadenosines and other phosphosubstrates are available commercially, for example, 2′,5′-ADP and 5′-AMP Sepharoses, for the affinity purification of dehydrogenase enzymes. Phosphate modified zirconia has been used for the separation of amines and ammonia [37] and recently been employed as a cation exchanger for the separation of proteins [38] and in biospecific affinity chromatography [39]. Without doubt, the most well known example of immobilized phosphate groups in biochromatog-

raphy involves phosphate groups complexed with calcium ions to form the sorbent known as hydroxyapatite which has been used for nearly 30 years for the isolation of proteins and nucleic acids [40–42]. Hydroxyapatite separation is predominantly an electrostatic mechanism for protein separation and is therefore different to IMAC.

Despite the obvious advantages of OPS for the chelation of hard Lewis metal ions and its specificity with high affinity in the free state for certain compounds such as viral RNA [43], we could find no published reports on the application of this ligand immobilized as a chromatographic sorbent for metal ion separation or for the IMAC separation of proteins. Most of the work in IMAC using borderline metal ions or hard Lewis metal ions has been carried out using immobilized IDA (Fig. 1). However, there have been numerous instances where other types of immobilized chelating agents have been used particularly with hard Lewis metal ions, in order to overcome problems of metal leakage which can occur with IDA systems [44] and to introduce alternative coordination geometries to IMAC for the separation of proteins [9,25,44]. The low binding affinity of Ca^{2+} for the tridentate chelator, IDA [45] moreover, can be remedied with the tetradentate chelating agent, carboxymethylated aspartic acid [46]. With this chelator, metal ion leakage is reduced but the tris(carboxymethyl)ethylenediamine (TED)-M^{n+} sorbents exhibit low protein capacities. The successful use of the bidentate hydroxamate type resins in IMAC for the immobilization of Fe^{3+} has been reported [24]. Recent studies with the bidentate chelating agent 8-hydroxyquinoline for the immobilization of Al^{3+}, Fe^{3+} or the lanthanide Yb^{3+}, have documented that this immobilized chelating agent when used in the IMAC separation of proteins can display different selectivities when compared to the corresponding IDA-M^{n+} sorbents [25].

The metal ion complexing behaviour of free OPS in solution has only briefly been examined in the literature. However, these studies provide a first indication of its potential as a ligand in chromatographic sorbents. For example, free OPS has a K_a smaller than either free IDA or 8-hydroxyquinoline for Fe^{3+} ions [45]. To further

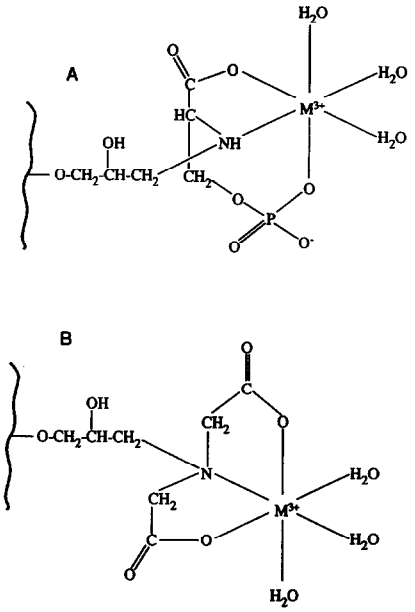

Fig. 1. Diagrammatic representation of the immobilized chelating agent OPS tentatively presented in tridentate coordination geometry with trivalent metal ion (M^{3+}) (A) and the immobilized tridentate chelating agent IDA (B), on epoxy-activated Sepharose CL-4B.

investigate the behaviour of OPS immobilized as a chromatographic ligand, conditions for immobilizing OPS onto Sepharose CL-4B are described. The metal ion binding capabilities of OPS-Sepharose CL-4B and IDA-Sepharose CL-4B, for the hard Lewis metal ions Al^{3+}, Ca^{2+}, Fe^{3+}, Yb^{3+} and Cu^{2+} as a borderline metal ion control, under different equilibrating conditions are also described. The binding behaviour of immobilized OPS-M^{n+} toward the model proteins tuna heart cytochrome *c* (THCC), horse myoglobin (HMYO) and hen egg white lysozyme (HEWL) was examined under equilibrating conditions that promote the interaction of surface exposed negatively charged amino acid residues on the proteins with immobilized hard Lewis metal ions.

EXPERIMENTAL

Materials

THCC, HEWL, HMYO, O-phospho-L-serine, metal nitrates (analytical-reagent grade) and iminodiacetic acid were purchased from Sigma–

Aldrich (St. Louis, MO, USA). Sepharose CL-4B was purchased from Kabi-Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade purity.

Immobilization of chelating agents to Sepharose CL-4B

The procedure of Porath and Olin [9] was used for the immobilization of IDA to Sepharose CL-4B.

Sepharose CL-4B was epoxy-activated by modifying the method of Porath and Olin [9]. Briefly, for every 10 g (wet mass) of Sepharose CL-4B, 10 ml of 2 *M* NaOH and 37.5 mg of sodium borohydride were used. This mixture was incubated for 2 h at room temperature before 12 ml of epichlorohydrin was added. The mixture was then gently stirred at room temperature for 15 h. The epoxy-activated Sepharose was recovered by vacuum filtration and then washed extensively with 20 volumes of Milli-Q water.

To a 0.2 *M* solution of OPS in distilled water were slowly added solid KOH pellets until the required pH at 4°C was reached. Aliquots of this solution (30 ml) were added to 15 g (wet mass) of the epoxy-activated Sepharose CL-4B and the suspension mixed on a rotary stirrer at the required temperature and for the required coupling time. The same method was used to study the effect of OPS concentration on ligand substitution over the range 0.2 *M* to 1.0 *M* OPS.

The OPS-Sepharose CL-4B was then washed with 10 volumes of Milli-Q water, 5 volumes of 50 m*M* acetic acid pH 4.0 and 10 volumes of Milli-Q water. The OPS-Sepharose CL-4B sorbents were analysed for their nitrogen and phosphorus content by Dairy Technical Services (Melbourne, Australia). Similar elemental analysis for nitrogen content with the IDA-Sepharose CL-4B was also carried out. The absolute error for the nitrogen analysis was 0.01% (w/w) whilst that for phosphorus was 0.05% (w/w). The chelating density of IDA-Sepharose CL-4B was $6.6 \cdot 10^{-4}$ mol IDA/g dry Sepharose CL-4B [0.92% (w/w) nitrogen]. The optimum conditions for immobilizing OPS onto Sepharose CL-4B were used to prepare the OPS-Sepharose CL-4B for the protein binding studies. The substitution level for the optimum OPS-Sepharose CL-4B preparation was $4.0 \cdot 10^{-4}$ mols OPS/g dry Sepharose CL-4B [0.56% (w/w) nitrogen and 1.24% (w/w) phosphorus].

Metal binding studies

Metal nitrate salts were used in preference to the commonly used metal chloride salts in order to obviate the formation of strong chloride complexes with metal ions such as Fe^{3+} and Al^{3+} [47]. Metal ions were loaded onto the immobilized chelate Sepharose CL-4B as described earlier [25]. Briefly, the chelating sorbents were incubated with 10 m*M* $Fe(NO_3)_3$, and $Al(NO_3)_3$ or 50 m*M* $Ca(NO_3)_2$, $Cu(NO_3)_2$ or $Yb(NO_3)_3$ for 30 min at 25°C. The metal ion concentrations of Ca^{2+}, Cu^{2+} and Yb^{3+} were 20 times in excess of the immobilized ligand concentration whilst those for Al^{3+} and Fe^{3+} were 4 times in excess. The lower excess concentration was used for Al^{3+} and Fe^{3+} so as to minimize the formation of hydrolytic species which are known to readily form with these metal ions [48]. Similar ratios of metal ion to immobilized chelating agent have also been used previously [49]. The metal ion immobilized sorbents were then washed with 10 volumes of Milli-Q water. The Milli-Q water-washed metal ion immobilized sorbents were then incubated with 10 volumes of 50 m*M* acetic acid containing 0.1 *M* KNO_3, pH 4.0 (buffer A) for 10 min at 25°C to remove any loosely bound metal ions.

The metal ion chelate sorbents were then rinsed with 10 volumes of Milli-Q water before incubating the IMAC sorbents for 30 min at 25°C with the appropriate equilibration buffer. The equilibration buffers were: buffer B: 30 m*M* 2-(N-morpholino)ethanesulphonic acid (MES), 30 m*M* imidazole, 0.44 *M* NaCl, 0.005% Brij-35, adjusted to pH 5.5 with HCl; buffer C: as for Buffer B but adjusted to pH 6.0 with HCl; buffer D: as for Buffer B but adjusted to pH 7.0 with NaOH; buffer E: 30 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 30 m*M* imidazole, 0.44 *M* NaCl, 0.005% Brij-35, adjusted to pH 8.0 with NaOH.

These buffers were also used for the protein binding studies described subsequently and were designed to have a constant ionic strength of about 0.5 *M*. The following protocol was fol-

lowed for metal ion analysis at the various stages of washing. About 5 g (wet mass) of a sample of the immobilized metal–chelate sorbent or metal ion-naked Sepharose CL-4B, washed, suspended in 40 ml of Milli-Q water and centrifuged at about 650 *g* for 10 min at 4°C. The supernatant was discarded and the pelleted sorbent was placed into 40 ml of 50% acetone and mixed. The acetone–sorbent mixture was then centrifuged at about 650 *g* for 10 min at 4°C. This step was repeated twice before the sorbent was snap frozen in liquid nitrogen and freeze dried overnight. The method described by Failla and Santi [50] was modified and adapted for the dissolution of the chelating sorbents. Briefly, about 0.1 g of the dry gel was weighed accurately and incubated with 5 ml of 4 *M* HCl at 50°C for 4 h. After the sorbent had dissolved, 5 ml of Milli-Q water were added and the samples were analysed for metal ion content by atomic absorption spectroscopy (CSL, Parkville, Australia). The Yb^{3+} ion content was analysed by means of flame emission spectroscopy also by CSL. All metal ion binding studies were performed in duplicate.

To calculate the moles of immobilized metal ion per mole of immobilized ligand (*M*) the equation of $M = A/B$ was used where A = moles of immobilized metal ion/g dry Sepharose CL-4B and B = moles of immobilized chelator/g dry Sepharose CL-4B. The moles of immobilized IDA were determined from the nitrogen elemental analysis whilst the moles of nitrogen and phosphorus were determined to give the moles of immobilized OPS.

Chromatography

The equilibrated IMAC sorbents were packed to the 1-ml mark in 20-ml Bio-Rad econo-columns. An aliquot of 0.2 ml THCC, 0.23 ml HEWL or 0.28 ml HMYO, of a 1.0 mg/ml protein solution, corresponding to approximately 16.1 nmol of each of the different proteins, were loaded onto the individual columns which were then washed with 4 ml of the appropriate equilibrating buffer. The breakthrough/wash volume was collected and labelled the "non-adsorbed" fraction. This experimental design approximates batch adsorption methods, allowing bound and free protein concentrations to be readily determined and an assessment made of the relative affinities and capacities of the IDA-M^{n+} or OPS-M^{n+} sorbents for the different proteins. The ratio of IMAC sorbent to equilibrating buffer used for this column washing step was 1 ml of sorbent to 4 ml of buffer. This wash ratio was chosen to ensure that all protein not bound or weakly bound was collected as the non-adsorbed fraction. Similar ratios of the volume of sorbent to the volume of equilibrating buffer used for washing unbound proteins have previously been used to screen affinity sorbents for enzyme isolation [51]. The immobilized metal ion content on the IMAC sorbents was 70 to 1400 times greater in molar terms to the amount of the loaded protein so as to ensure that protein solutions did not overload the chelate sorbent.

Protein quantitation was achieved by analytical high-performance liquid chromatography, using J.T. Baker (Phillipsburg, NJ, USA) Baker-bound C_8 reversed-phase column attached to a Hewlett-Packard 1090 HPLC and 1040 diode array detector set at 215 nm and 400 nm wavelength, as described elsewhere [25]. All adsorption experiments were carried out in duplicate.

RESULTS

OPS Immobilization onto epoxy-activated Sepharose CL-4B

The effect of pH, temperature, coupling time and OPS concentration on the stability and substitution levels of OPS immobilized Sepharose CL-4B was studied. Experimental conditions were sought whereby the OPS could be immobilized directly and in the correct orientation, without the need for lengthy OPS protection/deprotection protocols. Furthermore, an efficient OPS immobilization protocol was sought whereby high levels of OPS substitution onto epoxy-activated Sepharose CL-4B could be achieved in the shortest period of time without the use of excessive amounts of OPS. The molar nitrogen to phosphorus ratio (N:P) of immobilized OPS was utilized as an informative guide for studying the stability of OPS during the im-

mobilization studies. An N:P ratio of 1.0 implies immobilization of intact OPS. The nitrogen content of naked Sepharose CL-4B was found to be less than 0.01% (w/w) whilst the phosphorus content was less than 0.05% (w/w).

Effect of reaction mixture pH on OPS immobilization onto epoxy-activated Sepharose CL-4B. The amount of OPS immobilized onto epoxy-activated Sepharose CL-4B after 4, 24 and 48 h, at 25°C, over the pH range 8.0 to 12.0 was investigated (Fig. 2). At pH 8.0 and for any of these coupling times, there was no detectable immobilization of OPS on Sepharose CL-4B. With the exception of the experiments involving the 48-h coupling time, as the pH and coupling times increased, the nitrogen to phosphorus ration also increased. This result implies that a hydrolytic loss of phosphate groups occurs from the OPS sorbent. The 48-h coupling trials, however, showed an overall decrease in the N:P ratio as the pH increases, even though at pH 9.0 and pH 10.5 the N:P ratio was greater than the respective N:P ratio for the 4- and 24-h coupling times.

An N:P ratio very close to 1.0, indicative that the OPS had been predominantly immobilized intact onto epoxy-activated Sepharose CL-4B, was observed when the coupling was carried out at pH 9.0 for 4 h at 25°C and also at pH 10.5 for 4 h at 25°C.

Effect of temperature on OPS immobilization onto epoxy-activated Sepharose CL-4B. The effect of temperature on OPS immobilization onto epoxy-activated Sepharose CL-4B was studied at 25, 40 and 60°C, at pH 9.0 and 4, 24 and 48 h coupling times (Fig. 3.). As the temperature increased the N:P ratio of OPS immobilized onto epoxy-activated Sepharose CL-4B also increased. This trend was consistent for all three coupling times. This result implies an increasing loss of phosphate groups from the immobilized OPS at elevated temperatures.

Effect of OPS concentration on OPS substitution levels with epoxy-activated Sepharose CL-4B. The optimum conditions for studying the effect of OPS concentration on the immobilization of OPS onto epoxy-activated Sepharose CL-4B were pH 10.5 incubated at 25°C with a coupling time of 4 h. The conditions were chosen on the basis that the best stability of immobilized OPS and the greatest amount of intact OPS was immobilized with epoxy-activated Sepharose CL-4B, in the shortest time.

The OPS concentration range studied was 0.2 to 1.0 *M*. Fig. 4 shows the amount of OPS immobilized rises linearly as the free OPS concentration was increased up to 0.72 *M* but thereafter approaches a plateau. A similar pat-

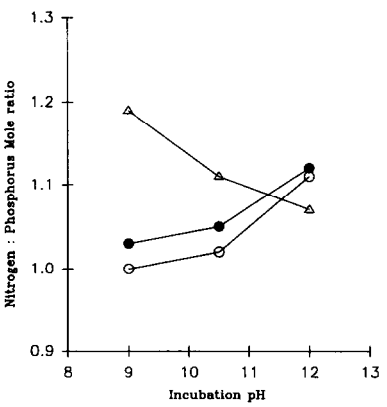

Fig. 2. Effect of the reaction mixture pH and coupling times on the immobilized OPS nitrogen:phosphorus mole ratio following the immobilization of OPS onto Sepharose CL-4B at 25°C. Epoxy-activated Sepharose CL-4B was coupled with free OPS at pH 8.0, 9.0, 10.5 and 12.0 for 4 (○), 24 (●) and 48 h (△). Nitrogen and phosphorus content were determined as described in the Experimental section. At pH 8.0 there was no detectable level of immobilized OPS and thus is not represented in this figure.

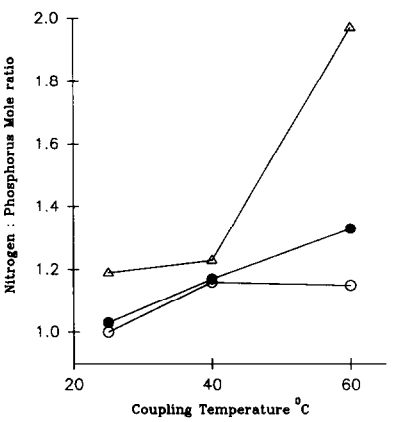

Fig. 3. Effect of coupling temperature and coupling times on the immobilized OPS nitrogen:phosphorus mole ratio following the immobilization of OPS onto Sepharose CL-4B at pH 9.0. Epoxy-activated Sepharose CL-4B was coupled with free OPS at 25, 40 and 60°C for 4 (○), 24 (●) and 48 h (△) as described in the Experimental section.

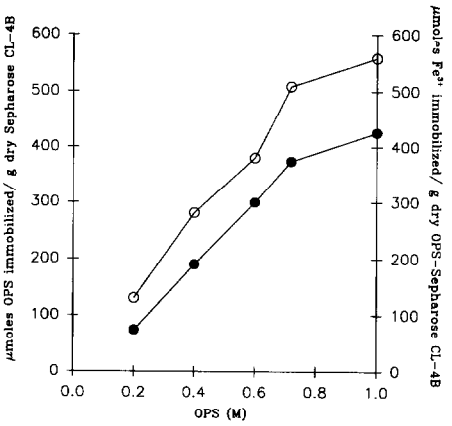

Fig. 4. OPS immobilization levels with epoxy-activated Sepharose CL-4B and Fe^{3+} chelating capabilities of immobilized OPS-Sepharose CL-4B as a function of free OPS concentration. Epoxy-activated Sepharose CL-4B was coupled with varying concentrations of free OPS at pH 10.5, 25°C and 4 h as described in the Experimental section. The amount of OPS covalently immobilized onto Sepharose CL-4B (●) and the amounts of Fe^{3+} ion bound (○) at each OPS substitution level are presented.

tern is observed with the amount of Fe^{3+} ion immobilized to the OPS-Sepharose CL-4B indicating that Fe^{3+} ions are binding specifically to the immobilized OPS. Hence, the concentration of OPS which would be the most efficient to use under these immobilization conditions is about 0.72 *M*, even though slightly more OPS can be immobilized at 1.0 *M* OPS concentration. Interestingly, the most effective chelator concentration for the synthesis of IDA-Sepharose CL-4B, prepared using the optimized method of Porath and Olin [9] requires 0.7 *M* IDA. Using the same batch of epoxy activated Sepharose CL-4B, a comparison of the substitution levels which can be achieved when IDA or OPS are immobilized onto epoxy activated Sepharose CL-4B was carried out. This study revealed $6.6 \cdot 10^{-4}$ mol of IDA/g dry gel and compared to $4.0 \cdot 10^{-4}$ mol OPS/g dry gel when the concentration of free chelating agent was 0.70 *M* OPS was used.

Metal ion binding to IDA-Sepharose CL-4B and OPS-Sepharose CL-4B

The binding capacity of IDA-Sepharose CL-4B and OPS-Sepharose CL-4B for Al^{3+}, Ca^{2+}, Cu^{2+}, Fe^{3+} and Yb^{3+} was investigated with a range of different equilibrating conditions which would be used later for protein binding studies with these sorbents. Naked Sepharose CL-4B was also examined for metal ion binding characteristics as described in the Experimental section. Naked Sepharose CL-4B bound less than 20 μmol of M^{n+}/g dry Sepharose CL-4B for all metal ions. The immobilized metal ion-chelate complexes were first washed with Milli-Q water and then buffer A so as to remove any loosely bound metal ions, as described in the Experimental section. The washed M^{n+}-chelate sorbents were then further washed as independent experiments with the appropriate equilibrating buffers, over the pH range 5.5 to 8.0 (buffers B, C, D, E) as described in the Experimental section.

Based on atomic absorption and flame emission spectroscopic analysis of the M^{n+} content of the IMAC sorbents, as well as nitrogen and phosphorus elemental analysis, comparisons of M^{n+} binding to IDA-Sepharose CL-4B and OPS-Sepharose CL-4B were made from the ratio mol immobilized M^{n+}/mol immobilized chelating agent thus taking into account any subtle differences in the amounts of immobilized chelating agent that may have occurred during these experiments. Table I shows the moles of immobilized metal ion/moles immobilized chelating agent after being washed only with Milli-Q water. Only Cu^{2+} and Fe^{3+} ions saturate the OPS-Sepharose CL-4B and IDA-Sepharose CL-4B, whilst Al^{3+}, Ca^{2+} and Yb^{3+} ions only partially saturate between 40 to 80% of the immobilized chelating agents.

Binding of Al^{3+} ions to IDA-Sepharose CL-4B and OPS-Sepharose CL-4B. Panel A of Fig. 5 indicates that about 40% of the IDA-Sepharose CL-4B has bound Al^{3+} ions. Furthermore, there is no significant change in the immobilized Al^{3+} ion content on IDA-Sepharose CL-4B as the pH was made more alkaline from pH 5.5 to 8.0. Similarly, Al^{3+} ion binding to OPS-Sepharose CL-4B also shows no significant change as the pH becomes more alkaline. However, the OPS-Sepharose CL-4B shows almost twice the capacity for Al^{3+} ions over the pH range 4.0 to 8.0 than does IDA-Sepharose CL-4B. Almost 80% of the immobilized OPS has bound Al^{3+} ions, when the Al^{3+}-IMAC sorbent is washed with buffers A to E.

TABLE I

BINDING OF METAL IONS TO IMMOBILIZED CHELATING AGENTS

The chelating sorbents were incubated with metal ions as described in the Experimental section. The M^{n+}-chelate sorbents were then washed with Milli-Q water and analysed for their metal ion content as described in the Experimental section. All results represent the mean value of mol immobilized M^{n+}/mol immobilized chelating ligand from duplicate experiments with the standard error from the mean.

Immobilized M^{n+}	Immobilized chelating ligand (mol immobilized M^{n+}/mol immobilized chelating ligand)	
	OPS-Sepharose CL-4B	IDA-Sepharose CL-4B
Al^{3+}	0.82 ± 0.03	0.41 ± 0.02
Ca^{2+}	0.48 ± 0.01	0.43 ± 0.05
Cu^{2+}	0.98 ± 0.02	1.00 ± 0.01
Fe^{3+}	1.12 ± 0.01	1.10 ± 0.02
Yb^{3+}	0.58 ± 0.01	0.69 ± 0.02

Binding of Fe^{3+} ions to IDA-Sepharose CL-4B and OPS-Sepharose CL-4B. Panel B of Fig. 5 indicates that Fe^{3+} ion binding capacities of IDA-Sepharose CL-4B and OPS-Sepharose CL-4B. The data indicate that all the chelating sites on the IDA-Sepharose CL-4B remain saturated with Fe^{3+} ions after washing this sorbent with Milli-Q water (Table I) and Buffer A (Fig. 5). However, as the pH becomes alkaline up to a 10% loss of Fe^{3+} ions from this IMAC sorbent occurs. On the other hand, the OPS-Sepharose CL-4B sorbent binds Fe^{3+} ions with a mole ratio of immobilized Fe^{3+} to immobilized OPS which is greater than 1.0, indicating that more than one Fe^{3+} ion can bind per immobilized OPS ligand under some circumstances. This was also apparent during the OPS immobilization studies (Fig. 3). Furthermore, the results indicate that there is no leakage of Fe^{3+} ions under any of the equilibrating buffer conditions used.

Binding of Cu^{2+} ions to IDA-Sepharose CL-4B and OPS-Sepharose CL-4B. Panel C of Fig. 5 shows the Cu^{2+} binding capacities of IDA-Sepharose CL-4B and OPS-Sepharose CL-4B under the equilibrating conditions mentioned in the Experimental section. The experimental results indicate that Cu^{2+} ions saturated all chelating sites on the immobilized IDA and that little loss of Cu^{2+} ions occurred after the IDA-Cu^{2+} sorbents were washed with the various equilibrating buffers. Conversely, the immobilized OPS, although initially saturating with Cu^{2+} ions (Table I), exhibits a decrease of 30% in Cu^{2+} ion content after being washed with Buffer A. A further decrease in Cu^{2+} ion content of up to 10% occurs after washing with Buffers B, C, D, or E.

Binding of Ca^{2+} ions to IDA-Sepharose CL-4B and OPS-Sepharose CL-4B. Panel D of Fig. 5 shows that Ca^{2+} ions bind weakly to both immobilized IDA and immobilized OPS. Both immobilized chelators were only about 50% saturated with Ca^{2+} ions prior to the wash with buffer A. However, the Ca^{2+} must be weakly bound to the immobilized chelators because after the buffer A wash only 5 to 10% of the immobilized chelators are complexed with Ca^{2+} ions. Washing with the pH 5.5 equilibrating buffer (buffer B) decreases the Ca^{2+} ion content of these sorbents further (Table I). However, as the pH becomes more alkaline there is a small but significant retention of immobilized Ca^{2+} ions (about 10%) with both the immobilized IDA and immobilized OPS chelators.

Binding of Yb^{3+} ions to IDA-Sepharose CL-4B and OPS-Sepharose CL-4B. Panel E of Fig. 5 show the binding capacities of IDA-Sepharose CL-4B and OPS-Sepharose CL-4B for Yb^{3+} ions. The experimental data show that about 50% of immobilized IDA sites were saturated

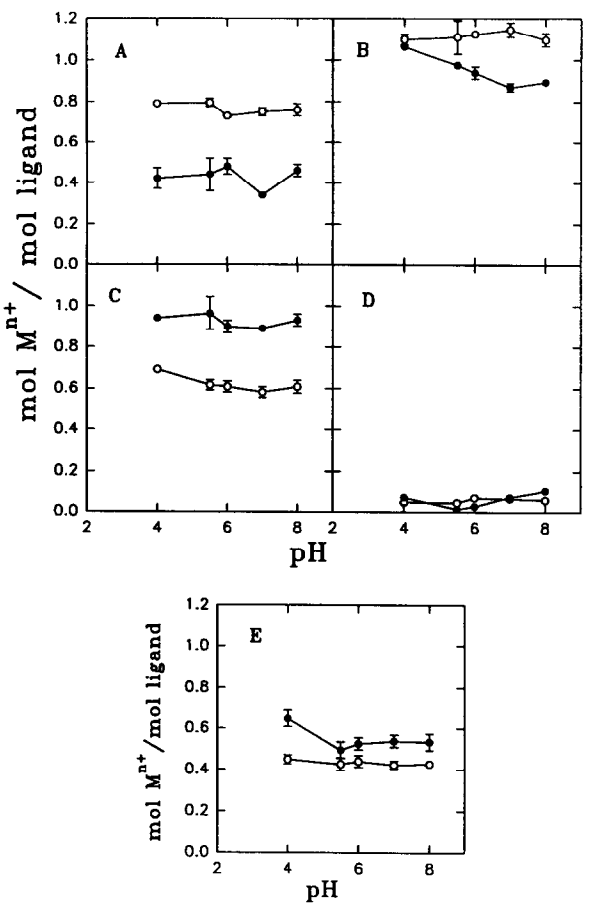

Fig. 5. Metal ion binding properties of OPS (○) and IDA (●), immobilized onto Sepharose CL-4B. The binding of Al^{3+} (A), Fe^{3+} (B), Cu^{2+} (C), Ca^{2+} (D) and Yb^{3+} (E), by OPS-Sepharose CL-4B after being incubated with various equilibrating buffers, were determined as described in the Experimental section. All results represent the mean value of mol M^{n+}/mol ligand from duplicate experiments with the standard error from the mean.

with Yb^{3+} ions after washing with buffer A after which there was no significant loss of this metal ion under any other buffer equilibrating condition. The OPS-Sepharose CL-4B was 40% saturated with Yb^{3+} ions and again there was no significant loss of this metal ion under any other buffer equilibrating condition.

Based on these experiments and the data shown in Fig. 5, the following metal ion affinity order can be derived for IDA-Sepharose CL-4B and OPS-Sepharose CL-4B over the equilibrating conditions of pH 4.0 to 8.0:

OPS: $Fe^{3+} > Al^{3+} > Cu^{2+} > Yb^{3+} >> Ca^{2+}$

IDA: $Fe^{3+} \sim Cu^{2+} > Yb^{3+} > Al^{3+} >> Ca^{2+}$

In terms of affinity for Al^{3+} ions, OPS-Sepharose CL-4B is a better sorbent than IDA-Sepharose CL-4B. Furthermore, OPS-Sepharose CL-4B binds Fe^{3+} ions stronger than IDA-Sepharose CL-4B whilst for Cu^{2+} and Yb^{3+} ions, however, OPS-Sepharose CL-4B has a lower affinity than IDA-Sepharose CL-4B. For Ca^{2+} ions OPS-Sepharose CL-4B and IDA-Sepharose CL-4B sorbents show no significant differences in affinity or capacity.

Binding of proteins to immobilized OPS-M^{n+}

The constituents of the equilibrating buffers the Good's buffers MES and HEPES, used for preparing the chelate sorbents for metal ion and protein binding studies were carefully selected for their low affinities for hard Lewis metal ions [52]. Such buffers are thus suitable for studying protein interactions with immobilized hard Lewis metal ions since they would minimize metal ion leakage during equilibration and would also be easily displaced by proteins if the buffer species was complexed with the immobilized metal. Imidazole also has low affinity for hard Lewis metal ions, but was included so as to quench any affinity that the immobilized hard Lewis metal ions or the Cu^{2+} ion had for any surface histidines or secondary affinity for exposed amine side chains existing on the protein surface. This type of interaction could occur for HMYO which has five surface histidine residues but would be less important for THCC which has no surface exposed histidines.

The metal ions Al^{3+}, Ca^{2+}, Cu^{2+}, Fe^{3+}, and Yb^{3+} were immobilized to OPS-Sepharose CL-4B and these sorbents screened for their protein binding ability with THCC, HMYO and HEWL over the pH range 5.5 to 8.0 as indicated in the Experimental section (Table II).

HMYO showed significant binding over the pH range 5.5 to 6.0, to immobilized OPS-Fe^{3+}. About 4 nmol of HMYO per ml of sorbent bound at pH 5.5. The amount of bound HMYO progressively decreased as the pH became more

TABLE II

BINDING OF PROTEINS AT DIFFERENT pH VALUES TO IMMOBILIZED OPS-M^{n+} SORBENTS

OPS-Sepharose CL-4B was loaded with metal ions as described in the Experimental section. Metal ion loaded columns (1 ml) were equilibrated with equilibrating buffers A, B, C or D as described in the Experimental section. An aliquot of 0.20, 0.28 and 0.23 ml of protein solution containing 16.1 nmol of either cytochrome *c* (THCC), myoglobin (HMYO) or lysozyme (HEWL), respectively, prepared in the appropriate equilibrating buffers was loaded, and the eluents were analyzed as described in the Experimental section. All results represent the mean % of protein detected in the non-adsorbed fractions from duplicate experiments with the standard error from the mean, relative to 16.1 nmol of protein originally loaded.

OPS-M^{n+}	% THCC in non-adsorbed fraction				% HMYO in non-adsorbed fraction				% HEWL in non-adsorbed fraction			
	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
OPS-Al^{3+}	82 ± 4	90 ± 5	83 ± 2	85 ± 3	93 ± 6	102 ± 2	101 ± 0	102 ± 2	99 ± 5	104 ± 5	96 ± 6	104 ± 3
OPS-Ca^{2+}	86 ± 5	92 ± 2	86 ± 1	86 ± 1	96 ± 6	101 ± 1	102 ± 1	98 ± 8	95 ± 5	100 ± 6	94 ± 5	103 ± 4
OPS-Cu^{2+}	86 ± 5	100 ± 6	79 ± 2	81 ± 2	90 ± 6	99 ± 1	101 ± 1	100 ± 4	98 ± 0	105 ± 9	100 ± 1	102 ± 3
OPS-Fe^{3+}	59 ± 4	70 ± 0	75 ± 1	75 ± 3	71 ± 3	83 ± 2	93 ± 3	95 ± 1	96 ± 3	104 ± 3	96 ± 2	103 ± 2
OPS-Yb^{3+}	86 ± 6	91 ± 6	86 ± 1	86 ± 3	90 ± 0	101 ± 0	100 ± 1	100 ± 2	105 ± 6	103 ± 5	98 ± 1	104 ± 4
OPS Blank	84 ± 6	90 ± 4	85 ± 4	89 ± 5	95 ± 5	97 ± 0	100 ± 1	100 ± 2	90 ± 1	100 ± 2	100 ± 2	104 ± 2

alkaline, reaching the level of about 2.2 nmoles HMYO per ml of sorbent at pH 6.0. There was no significant binding of HMYO to immobilized OPS-Fe^{3+} at pH 7.0 or 8.0 or any significant binding to the other immobilized OPS-M^{n+} or to the metal-free OPS-Sepharose CL-4B.

Immobilized OPS-Cu^{2+} did not bind any HMYO at pH 8.0 despite the presence of eleven histidine residues in HMYO, five of which are on the surface of this protein. This result implies that the 30 m*M* imidazole present in the equilibrating buffers is sufficient to quench any affinity that immobilized OPS-Cu^{2+} may have for the surface accessible histidines of HMYO. To validate this assumption and to examine the behaviour of immobilized OPS-Cu^{2+} with different equilibrating conditions, imidazole was omitted from the pH 8.0 equilibrating buffer and the selectivity of immobilized OPS-Cu^{2+} for HMYO re-examined under these conditions. It was observed that all the HMYO that was loaded on to OPS-Cu^{2+} at pH 8.0, in the absence of imidazole in the equilibrating buffer, bound to the OPS-Cu^{2+}. Thus, it can be concluded that immobilized OPS-Cu^{2+} exhibits protein binding behaviour for HMYO, which is dependant on the amount of imidazole in the equilibrating buffer.

Immobilized OPS-Fe^{3+} bound THCC but the binding behaviour differed to that observed for HMYO with immobilized OPS-Fe^{3+}. Similar amounts of THCC bound to immobilized OPS-Fe^{3+} as did HMYO with the equilibrating buffer in the pH range of 5.5 to 7.0. However, THCC (but not HMYO) also bound at pH 8.0 to OPS-Fe^{3+}. The amount of THCC that bound to OPS-Fe^{3+} at pH 6.0 was the same as pH 7.0. THCC did not bind to any other metal ion immobilized onto OPS-Sepharose CL-4B. The metal-free OPS-Sepharose CL-4B did, however, bind up to 2.4 nmol of THCC per ml of sorbent despite an ionic strength of 0.5 *M* in the equilibrating buffers.

The immobilized OPS-M^{n+} sorbents showed no binding capacity for HEWL under any of the equilibrating conditions used. Furthermore, HEWL did not bind to metal ion-free OPS-Sepharose CL-4B.

Regeneration of OPS-Sepharose CL-4B

Regeneration of used OPS-M^{n+}-Sepharose CL-4B was achieved by incubating the sorbent with 0.2 *M* EDTA/0.5 *M* KH_2PO_4, pH 4.2, overnight at 25°C. This condition was not only sufficient to strip any metal ions bound to the immobilized OPS but also the high ionic strength of this buffer was sufficient to remove non-speci-

fically bound proteins. The same batch of OPS-Sepharose CL-4B was used for the protein binding studies and subsequently regenerated using the regeneration buffer each time, for a total of six cycles. From nitrogen and phosphorus analysis no significant change in the OPS density was evident, that is, the initial and final nitrogen or phosphorus content for this batch of OPS-Sepharose CL-4B was the same after six repetitive uses. This result indicates that immobilized OPS is not degraded during use in the IMAC protocol or that any protein has irreversibly bound to the OPS-Sepharose CL-4B.

DISCUSSION

This investigation describes a method to immobilize OPS on Sepharose CL-4B. The optimum condition for immobilizing OPS with epoxy-activated Sepharose CL-4B was determined to be pH 10.5 for 4 h at 25°C. Under these conditions the OPS was immobilized intact. With coupling times longer than 4 h at pH 10.5 or with temperatures greater than 25°C at pH 10.5, a N:P ratio greater than 1.0 was observed indicating that phosphate loss had occurred from the immobilized OPS. The β-elimination of electronegative substituents from α-amino acids, such as the phosphate from OPS, is well known [53]. It can also occur by enzymatic catalysis, for example, by phosphatases [53], non-enzymatically in the presence of pyridoxal and divalent metal ions or as in this case under alkaline conditions [43].

Immobilization of OPS with the epoxy-activated agarose via its α-amino group is assumed to have occurred. This conclusion is supported by the fact that amines are much stronger nucleophiles than phosphates or carboxyl groups [54] and is experimentally supported by the lack of OPS immobilization at pH 8.0 where two of the three ionizable oxygens on the phosphate moiety of OPS are fully deprotonated as is also the oxygen on the carboxyl group of OPS. The possibility that phosphate can behave as a nucleophile [54–56] may explain the decrease in the N:P ratio, to values close to 1.0, when the incubation time was 48 h and the pH was becoming increasingly alkaline. In the case of the pH 12.0 experiment, hydrolysis of the phosphate from OPS would have occurred with the free phosphate now at a sufficient concentration and ionized state to possibly act as a nucleophile and bind independently of the intact OPS to the epoxy-activated Sepharose CL-4B, thus showing an N:P ratio close to 1.0.

During the studies of Sundberg and Porath [57] on determining optimum conditions for activation of agarose supports with bisoxirane and the subsequent immobilization of proteins and small ligands the three reaction mixture parameters examined were pH, temperature and incubation time. Similar parameters and number of experimental points were examined during the determination of the optimum conditions for the immobilization of OPS to epoxy-activated Sepharose CL-4B.

Under the coupling conditions used, lower substitution levels of OPS with epoxy-activated Sepharose CL-4B were achieved compared to IDA mainly because of the phosphate hydrolysis. This result suggests that higher substitution levels may be attainable if the immobilization protocol is modified to include phosphate protection steps.

The Good buffers used to prepare the metal–chelate sorbents for protein binding did not strip significant levels of metal ion. This result is consistent with the low affinity the equilibrating buffer constituents have for hard Lewis metal ions [52] and highlights the importance of choosing the appropriate buffer where the K_a of immobilized ligand for metal ion is smaller than the K_a of the immobilized metal ion for the buffering species.

From comparisons of the metal ion binding data for OPS-Sepharose CL-4B and IDA-Sepharose CL-4B, the immobilized OPS appeared to favour binding with the hard Lewis metal ions instead of the borderline metal ion Cu^{2+}. The stability of Fe^{3+} on immobilized OPS was greater than that on immobilized IDA over all pH conditions examined. The high stability of Fe^{3+} ions on phosphonic acid sorbents compared to other metal ions has previously been reported [2]. Furthermore, free OPS is known to have a high affinity for immobilized Fe^{3+} [8]. Qualitatively, this preference of Fe^{3+} ions for im-

mobilized OPS over immobilized IDA mimics the behaviour of Fe^{3+} ions in solution when chelated with free IDA and free OPS. Interestingly, Fe^{3+} ions appear to bind to immobilized OPS at a ratio of greater than 1:1. It is not yet clear whether this is a result of Fe^{3+} ion binding non-specifically to the Sepharose CL-4B. Alternatively, it is possible that immobilized OPS can bind more than a single Fe^{3+} ion per chelating ligand, where the phosphate group of OPS is involved in the binding of two Fe^{3+} ions, one through a single oxygen and the other through its other ionized oxygen and the carboxyl group. The possibility that the Fe^{3+} ion is binding to the glycidyl spacer arm on the Sepharose CL-4B is unlikely since the pK_a of such hydroxy groups will be very alkaline [45].

The experimental results show that immobilized OPS has a high affinity for Fe^{3+} ions. Moreover, immobilized OPS-Fe^{3+} binds THCC and HMYO over the pH range 5.5 to 7.0 and binds THCC also at pH 8.0. This sorbent did not bind HEWL at any pH. Thus, the protein selectivity of immobilized OPS-Fe^{3+} is different to that of immobilized IDA-Fe^{3+} which will bind HEWL at pH 5.5 [58]. Furthermore, the selectivity at pH 8.0 by immobilized OPS-Fe^{3+} for THCC is also different to the behaviour observed for immobilized IDA-Fe^{3+} with this protein [58]. The latter immobilized ligand binds THCC at pH 5.5 only.

Immobilized OPS-Fe^{3+} binds THCC and HMYO with similar capacities at pH 5.5 and pH 6.0. This result is unexpected since HMYO has 10 more negatively charged amino acid residues that are surface accessible than THCC and should thus be expected to have a greater affinity for immobilized Fe^{3+} than THCC [58]. Furthermore, HEWL has similar numbers of negatively charged surface residues as THCC but did not bind. It can therefore be concluded that immobilized OPS-Fe^{3+} may present a different coordination geometry to proteins than does immobilized IDA-Fe^{3+}, despite the fact that both function as tridentate ligands.

The data also show that the hard Lewis metal ion Al^{3+} has a higher affinity for immobilized OPS than for immobilized IDA and is consistent with the high affinity that was shown by immobilized Al^{3+} for OPS [13]. This result is also in accord with the observation that, generally, hard Lewis metal ions have a higher affinity for oxygen atoms such as the two in the anionic forms of the phosphate and carboxyl groups of OPS. Conversely, with the immobilized IDA, there are fewer oxygen atoms available for coordination of Al^{3+} ions, resulting in a lower affinity for this metal ion. It has been established that free IDA coordinates to Al^{3+} ions in a tridentate manner [59] and could possibly do so in the immobilized form. There is no information currently available on the coordination geometry of free OPS-Al^{3+} complex.

Immobilized IDA-Al^{3+} has been shown to bind phosphoproteins [8,26] and also to some serum proteins [10]. However, in the present study the OPS-Al^{3+} IMAC sorbent did not bind any of the proteins tested under the equilibrating conditions examined. The same proteins have been shown also not to bind to immobilized IDA-Al^{3+} under these same equilibrating conditions [58]. In other studies, immobilized OPS-Al^{3+}, however, has been shown to have high selectivity for some rat liver proteins [60].

Solution studies have indicated that the stability constants for IDA-Cu^{2+} complexes are higher than those of free IDA-Al^{3+} even though free IDA coordinates both metal ions in a tridentate manner. This trend also appears to be followed with immobilized IDA-Cu^{2+} and -Al^{3+} complexes. However, the reverse order of stability occurs when comparing Cu^{2+} ions and Al^{3+} ions that are coordinated to immobilized OPS sorbents.

Free OPS binds Cu^{2+} ions in a bidentate manner at acidic pH values [33,34], binding through the phosphate and carboxyl groups. Free OPS becomes tridentate for Cu^{2+} ions at more alkaline pH where the amino group of OPS can also coordinate to the Cu^{2+} ion. When OPS is immobilized onto Sepharose CL-4B through the amino group, the flexibility of OPS, which exists in the unbound form, may be constrained. The Cu^{2+} ion, a borderline type metal ion which has a preference for amines, may thus be confined to coordinate to the immobilized OPS in a bidentate manner. The shorter amine-carboxyl length in IDA would still allow three coordination sites to be taken up around the Cu^{2+} ion

and form a stable square planar geometry [11] with the immobilized IDA which is bound through its amino group to Sepharose CL-4B. Immobilized OPS-Cu^{2+} did not bind THCC or HEWL. It has previously been found that immobilized IDA-Cu^{2+} bound HMYO at pH 5.5 [58]. Thus, the behaviour of HMYO with immobilized IDA and immobilized OPS sorbents is clearly different and as is the case of immobilized OPS-Fe^{3+} and immobilized OPS-Al^{3+}, immobilized OPS-Cu^{2+} appears to present a different coordination geometry to immobilized IDA-Cu^{2+}.

Although OPS-Sepharose CL-4B was originally designed for use with hard Lewis metal ions in IMAC, immobilized OPS-Cu^{2+} did exhibit affinity toward HMYO at pH 8.0 in the absence of any imidazole in the equilibrating buffer. Immobilized OPS has the potential to bind to Cu^{2+} in a bidentate manner at pH 5.5 and pH 6.0. Since some surface accessible histidine residues in proteins have a pK_a close to 5.0, due to local microenvironmental ionization effects, the imidazole ring of these amino acids residues will thus be able to donate electrons and bind to immobilized bidentate Cu^{2+} ions. This behaviour would imply that immobilized OPS-Cu^{2+} has a capacity and selectivity advantage at acidic pH values for proteins compared with the tridentate immobilized IDA.

The binding characteristics of Ca^{2+} ions to immobilized IDA and immobilized OPS were typical of weak coordination. The binding of Ca^{2+} ions to immobilized IDA or immobilized OPS was very close to that of the naked Sepharose CL-4B. However, the results support the argument that Ca^{2+} ions have bound through the immobilized chelating agents since after the Milli-Q water wash, of Ca^{2+}-naked Sepharose CL-4B and Ca^{2+}-immobilized chelating sorbent, the amount of Ca^{2+} ions left on the naked Sepharose CL-4B is much less than that found on the chelate sorbents [60]. It has previously been shown that Ca^{2+} ions had very poor binding characteristics for immobilized IDA. For example, in order to load Ca^{2+} ions onto the IDA-Sepharose CL-4B sorbent, a boric acid buffer pH 9.0 was required followed by a series of neutral pH washes so as to maximize the Ca^{2+} ion content and minimize leakage of this ion from the sorbent [46]! This metal ion as well as immobilized OPS-Yb^{3+} ion showed no selectivity for proteins, although Yb^{3+} ions immobilized to 8-hydroxyquinoline shows selectivity for THCC at pH 7.0 [25].

The immobilization of OPS onto Sepharose CL-4B for use in IMAC results in greater stability of the hard Lewis metal ions Al^{3+} and Fe^{3+} complexes compared to those obtained with conventionally used immobilized IDA. The results also suggest that immobilized OPS-M^{n+} presents an alternative geometry to proteins than was previously available in IMAC. Given that OPS plays a significant role in membrane biology and other facets of surface biorecognition with proteins, the use of immobilized OPS without a complexed metal ion present, may also have a place in affinity chromatography. Further work is being carried out to elucidate the coordination geometry of free and immobilized OPS complexed with metal ions, using Fourier transform infrared spectroscopy and potentiometric techniques and these results will be reported subsequently.

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