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COMPARATIVE STUDIES ON THE METAL SORPTION CHARACTERISTICS OF CHELATING GELS FOR IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY

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ABSTRACT

A systematic investigation of the metal sorption and leaching characteristics of two chelating gels viz., iminodiacetic acid (IDA) and Tris(2-aminoethyl)amine bound to Sepharose 6B, under varying chemical conditions relevant for immobilized metal ion affinity chromatography (IMAC) of proteins (e.g., buffer salts, pH (4–9), ionic strength (0–3 *M* NaCl), feed metal concentration (0–50 *mM*)) has been undertaken. The studies were carried out for the two most frequently used metal ions namely, Cu(II) and Ni(II). Results indicated that the metal sorption capacities of these

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chelating gels show strong dependence on the changes in pH around them. However, the effect of ionic strength and feed metal concentration on metal sorption capacities is moderate. On the basis of these investigations, conditions for optimum sorption capacities were identified. Further, metal leaching studies were conducted for various solution conditions (pH, ionic strength) on these gels to assess their suitability for use in protein separations. These studies may provide an outset for preparing stable metal chelated gels for protein separations.

Key Words: Chelating gels; Iminodiacetic acid; Tris(2-aminoethyl)amine; Metal sorption; Metal leaching

INTRODUCTION

Chelating agents bound to solid supports are utilized widely for metal preconcentration, wastewater treatment, and purification of small organic molecules.[1–3] The application of chelating gels for fractionation and purification of proteins through recognition of surface exposed amino acids such as histidine, cysteine, and tryptophan was first reported by Porath et al. in 1975.[4] The chromatography of proteins based on the chemical affinity between chelated or immobilized metal ion and proteins was initially called "Metal Chelate Affinity Chromatography (MCAC)" and later renamed as "Immobilized Metal ion Affinity Chromatography (IMAC)" to encompass all modes of metal chelate interaction chromatography including ligand exchange.[5] Over the past two decades, IMAC has emerged as an essential laboratory tool for the isolation and purification of proteins especially the recombinants and is beginning to find industrial applications. The various fundamental aspects, latest developments and applications of IMAC for protein purification have extensively been reviewed.[6–15] However, there are still certain aspects that need closer examination.

A separation process based on IMAC involves three experimental steps: metal sorption, protein adsorption, and protein elution and all of the three steps need to be thoroughly investigated for achieving optimal performance of the gel. Though the last two steps are specific for a particular protein system, the first step i.e., metal sorption is common to all. The metal ion is responsible for the formation of metal chelate (or immobilized metal ion) in the presence of chelating gel and provides sites for protein adsorption. Quite a few studies have illustrated that the surface metal concentration of an IMA gel can be directly correlated with its retention and selectivity for various amino acids, peptides, or proteins.[11,12,16–18] Todd et al.[19] were indeed able to explain quantitatively the influence of copper loadings on

protein retention in their study on the equilibrium binding characteristics of engineered histidine-containing cytochrome *c* on TSK Guardgel Chelate-5PW. There was a substantial increase in the protein association constants with an increase in copper loading capacities. While the Langmuir type of isotherm was sufficient to explain the adsorption behavior of different cytochrome *c* histidine variants at low copper loadings, deviations were observed at higher copper loadings indicating the change in binding mode from single to multiple-site binding interactions. All these studies firmly indicate the direct influence of metal loading on protein sorption characteristics of metal chelated gels. Nevertheless, the nonavailability of relevant metal sorption data for most of the IMA gels renders the quantitative comparison of protein adsorption results from different investigations difficult.

Metal sorption capacity of a chelating gel is a complex function of a large number of variables including chelating agent, metal ion, and the solution environment (i.e., buffer salts, pH, ionic strength, and feed metal concentration) used for loading the chelating gel with metal. The dependence of metal sorption on solution environment is rather understood for metal chelate formation in solutions. Generally, metal chelates in solution have been found to be unstable under extremely acidic or basic conditions. Variation in ionic strength also affects the stability of metal chelates. Thus, by varying pH, ionic strength, and feed metal concentration, the sorption capacities and stability of these metal chelates can be altered. These variations can, however, be much different for chelating agents bound to stationary supports largely due to steric hindrance. Zachariou et al.[21] were indeed able to quantify these variations in terms of the acid–base dissociation constants and the metal ion stability constants for different immobilized OPS- and 8-HQ- systems. Their studies indicated that the stability constants for the immobilized metal ion-chelate systems were generally lower than those observed for the unbound metal ion-chelate complexes in solutions. These findings highlight the need for caution when extrapolating data obtained from solution studies with metal ion- chelate complexes to the chelating ligands immobilized to chromatographic supports.

The values of formation constants for some of metal chelates (1:1) in solution is available in literature.[20] However, these values are specific for particular solution conditions and, therefore, can only be useful in developing a fundamental understanding of the interaction between metal ion and chelating agent during complexation. This information, however, cannot be translated to all practical conditions due to its marked dependence on the surrounding chemical milieu. Nevertheless, studies by Zachariou et al.[21] have demonstrated that there exists an excellent correlation between the metal sorption capacities and stability constants for a gel determined under various sets of experimental conditions. It is, therefore, desirable to have a detailed knowledge of the influence of the mentioned variables on the metal sorption characteristics of chelating gels.

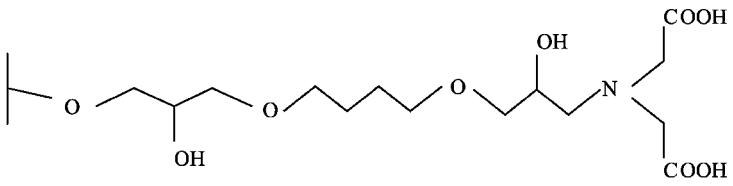
To this end, we have investigated the influence of different buffer salts, pH (4–9), ionic strength, (0–3 *M* NaCl) and feed metal concentration (0–50 *mM*) on

the metal sorption characteristics of two chelating gels, having iminodiacetic acid (IDA) and Tris(2-aminoethyl)amine (TREN) as the chelating agents bound to Sepharose 6B (Fig. 1). While the choice of IDA[4] was prompted by its wide applicability to resolve a variety of protein mixtures, TREN[22] was chosen due to its greater selectivity towards certain proteins.[23,24] The influence of solution environment on metal sorption characteristics of these gels remains hitherto unexplored. We conducted sorption studies for the two most frequently used metal ions namely, Cu(II) and Ni(II), and solution conditions for optimal metal sorption capacities were identified. Furthermore, leaching of the metal ions from the metal chelated gel was examined under various conditions of pH (3–9) and ionic strength (0–3 *M* NaCl) to assess the suitability of optimized conditions for use in protein fractionation. It is anticipated that these studies will be useful for preparing stable metal chelated gels for large scale protein separations, and aid in designing and analyzing them in quantitative manner.

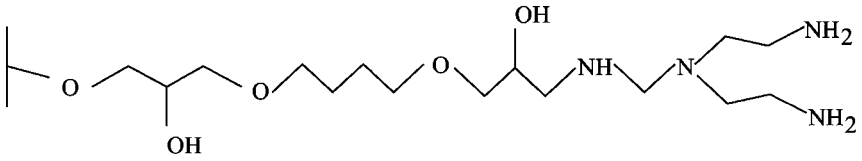
EXPERIMENTAL

Materials

Sepharose 6B was procured from Pharmacia-LKB Biotechnology (Uppsala, Sweden). Tris(2-aminoethyl)amine (22563-0) from Aldrich Chemical



IDA -Sepharose 6B



TREN-Sepharose 6B

Figure 1. Structures of IDA-Sepharose 6B and TREN-Sepharose 6B gels.

Co. (Milwaukee, WI; and IDA (I-5629) and 1,4-butanediol diglycidyl ether (B-7381) from Sigma Chemical Co. (St. Louis, MO). All other chemicals and buffer reagents were of analytical grade and procured locally. Sodium acetate, acetic acid, and ethylenediaminetetraacetic acid disodium salt (EDTA) were purchased from Qualigens, Tris(hydroxymethyl)aminomethane (Tris), sodium chloride, sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate, hydrochloric acid, copper sulfate, and nickel sulfate from E. Merck. All the buffers and solutions were prepared in ultrapure water (resistivity 18.2 Mohm-cm) obtained from a Milli-Q unit (Millipore Corporation, Bedford, MA) and prefiltered through a 0.22μM membrane filter just prior to removing any particulate or colloidal matter.

Preparation of Chelating Gels

The method published by Winzerling et al.[22] was used to prepare the two IMA gels, IDA -Sepharose 6B and TREN- Sepharose 6B with some modifications. Briefly, Sepharose 6B (supplied in 20% ethanol aqueous solution) was suction dried on glass filter and washed with ultrapure water several times to remove residual ethanol. After washing properly, the suction dried gel (200 g) was resuspended in 100 mL of 0.5 M sodium hydroxide. To this 100 mL of 1,4-butanediol diglycidyl ether, a bisoxirane, was added in a water bath for 24 hr at 25°C and 150 rpm. The activated gel was washed with ultrapure water until neutral pH is reached and then suction dried. Immediate use of the gel is preferred; however, activated gels can be stored for days in a refrigerator without extensive hydrolysis. The suction dried oxirane activated gel (50 g) (prepared previously) was then transferred to a 500 mL Erlenmeyer flask and suspended in a 50 mL of 0.5 M sodium bicarbonate ($NaHCO_3$), pH 10.0. The chelating agent (IDA (50 mmoles) or Tris (2-aminoethyl)amine (33 mmol ∽ 5 mL of the original chemical) was dissolved in 25 mL of Milli Q water and its pH is adjusted to 10.0 using NaOH pellets followed by addition of water to a final volume of 50 mL. This solution was mixed with the gel suspension and shaken in water bath at 25°C and 150 rpm for 48 hr. The gel was collected in a glass filter, washed thoroughly with ultrapure water to reach neutral pH and suction dried for storage.

Metal Loading of the Chelating Gel

Batch Method

A known volume of the metal salt solution (20 mM in the buffer of appropriate pH/ionic strength) was added to an equal volume of 1:5 (v/v) suspension of IMA

(IDA- or TREN- Sepharose 6B) gel in a flask which was continuously agitated in a shaking water bath (120 rpm) maintained at 25°C. Aliquots of 3 mL were withdrawn at different time intervals (15, 30, 45 sec, 1–15 min) filtered through 0.45 μm filter and analyzed for the metal ion concentration in the supernatant.

Column Method

All the experiments were conducted at room temperature (25°C). The glass column (10 cm × 10 mm ID, packing height = 2 cm, packed bed volume = 1.57 mL) pre-equilibrated with 10 *mM* buffer of appropriate pH and ionic strength (molar concentration of NaCl) was charged with metal by passing 16 column volumes of the solution of copper or nickel sulfate (0–50 *mM*) followed by washing with 20 column volumes of the same buffer. The column was, then, equilibrated with 6–7 column volumes of 20 *mM* sodium phosphate buffer (1 *M* NaCl, pH7.0) followed by elution with 50 *mM* EDTA in the same buffer. After thorough washing with Milli Q water, the column was ready for next experiment. The buffers used for this investigation were: sodium acetate–acetic acid, Tris–HCl, and Tris–acetate. Sodium phosphate buffer was not used for metal loading due to the precipitation of metal salts for the investigated solution conditions. All of the experiments (except the ones that involved the effect of linear flow velocities) were performed in duplicate at a linear velocity of 2.123×10^{-4} m sec^{-1} (volumetric flow rate = 1 mL min^{-1}). The range of investigated linear flow velocities was: $1.062 \times 10^{-4} - 3.185 \times 10^{-4}$ m sec^{-1}. The flow was always in an upward direction.

Determination of the Amount of Chelated Metal(II) Ions

Frontal Method

The pre-equilibrated column was loaded with metal under desired conditions. The loosely bound and excess metal ions in the column were removed by thorough washing with the same buffer. The amount of the sorbed metal was, then, determined by applying a mass balance across the column. The absorbance was measured using a Kontron UV-visible spectrophotometer (Kontron Instruments, UK). The selected λ_{max} for Cu(II) and Ni(II) were 775 and 395 nm, respectively.

Direct Method

The metal loaded column was equilibrated with 6–7 column volumes of 20 *mM* sodium phophate buffer (1 *M* NaCl, pH 7.0) followed by elution with

50 *mM* EDTA in the same buffer. The total amount of M(II) ions in the eluate was determined by Atomic Absorption Spectrophotometry (AAS) (Hitachi Ltd., Tokyo, Japan; Model # Z-8100) after proper dilution using EDTA as blank. The selected λ_{max} for Cu(II) and Ni(II) were 324.8 and 232 nm, respectively.

Metal Leaching Studies

The buffer solution of appropriate pH/ionic strength (NaCl concentration) (in which metal leaching is to be investigated) was passed through the metal (Cu(II)/Ni(II)) preloaded IMAC column (10 cm × 10 mm ID, packing height = 2 cm, packed bed volume = 1.57 mL) at a volumetric flow rate of 1 mL min^{-1}. The flow was always in an upward direction. All the fractions (up to 20 CV of buffer) were collected and analyzed using Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (Perkin-Elmer Plasma 40, 1989 model) for the presence of metal ions. The λ_{max} used for Cu(II) and Ni(II) were 324 and 221 nm, respectively.

RESULTS

Metal Sorption Studies

Screening of Buffers

In order to select an appropriate buffer for preparing solutions for metal loading a preliminary screening of the three aqueous media viz. sodium acetate–acetic acid (10 *mM*), Tris–HCl (10 *mM*), and Tris–acetate (10 *mM*) was carried out. The suitability of the buffer medium was assessed on the basis of the solubility of the metal salt and metal sorption capacity of the gel in the selected medium.

The affinity of the metal ion for hydroxide ion may restrict the choice of metal ion under some pH conditions. All the buffer solutions were, therefore, screened for the solubility of sulfates of copper and nickel under varying conditions of pH. Nickel was found to be soluble throughout the pH range of 4–9 (the proposed range for Sepharose gels) in all the selected buffer media. However, the solubility of copper varied in different aqueous media under different conditions of pH (Table 1). Copper ions precipitated in Tris–acetate and sodium acetate–acetic acid medium beyond pH 5.0. However, it was soluble in Tris–HCl medium throughout the specified range.

Copper sorption capacities of the gels were examined in the three aqueous media at pH 5.0 (Table 2). Binding capacity was highest in sodium acetate–

Table 1. Screening of Buffers for Solubility of Copper

	pH					
Buffer	4	5	6	7	8	9
Sodium acetate–acetic acid	+	+	±	−	−	−
Tris–acetic acid	+	+	−	−	−	−
Tris–HCl	+	+	+	+	+	+

+ : soluble; − : not soluble; ± : precipitation within 10 min of solution preparation.

acetic acid medium. Lower sorption capacities in Tris based buffers are probably due to the competition between the chelating agents, IDA (with one amino and two carboxylic groups) or TREN (which has four- one tertiary and three primary-amino groups) and Tris(hydroxymethyl)aminomethane (Tris), a primary amine, constituting the buffer. Binding capacities obtained using both the methods, Frontal as well as Direct (using atomic absorption spectophotometry), matched fairly well in sodium acetate–acetic acid medium. The difference in the mean values ($n = 4$, $p < 0.008$ at 95% confidence level) obtained by the two methods and hence, percent variation was quite significant for Tris–HCl buffer (13.2% for IDA and 12.5% for TREN) (Table 2). This may be due to the slight leakage of metal ions during equilibration with phosphate buffer prior to elution with EDTA. This observation together with lower binding capacities in Tris–HCl buffer point at the weaker metal binding to the gels in the presence of Tris–HCl buffer.

These preliminary investigations suggested the sodium acetate–acetic acid aqueous buffer to be the most appropriate one for metal sorption studies. A further screening of the sodium acetate–acetic acid buffers of two strengths (10 and 100 *mM*) for metal loading capacity of the gels indicated buffer medium of lower strength to be more appropriate as metal sorption capacities were nearly the same in both of them (data not shown).

Equilibration Time and Linear Flow Velocities

Preliminary studies of IDA and TREN gel with Cu(II) and Ni(II) indicated that more than 90% of the saturation capacity is achieved within 1 min of equilibration time. Thus, the rates of IMA gel–metal ion interaction are sufficiently rapid for preparing metal chelated gels for protein separations. In order to obtain an optimum chromatographic condition, sorption behavior of the metal(II) ions was examined at different linear flow velocities (1.062×10^{-4}–3.185×10^{-4} m sec^{-1}). Metal sorption was found to be independent of linear

Table 2. Copper Sorption Capacities of IDA- and TREN-Sepharose 6B Gels in Different Buffers at pH 5.0

	Copper Sorption Capacities[a] (μmol mL^{-1} gel)					
	IDA			TREN		
Buffers	Frontal	AAS	% V[b]	Frontal	AAS	% V[b]
Sodium acetate–acetic acid	30.9 ± 0.08	31.1 ± 0.09	0.65	33.3 ± 0.10	32.9 ± 0.08	1.21
Tris–acetic acid	20.1 ± 0.10	20.9 ± 0.19	3.90	22.5 ± 0.12	21.6 ± 0.22	4.08
Tris–HCl	10.5 ± 0.22	9.2 ± 0.38	13.20	6.8 ± 0.19	6.0 ± 0.35	12.50

[a] Reported values are mean ± standard deviation ($n = 4$) ($p < 0.008$ at 95% confidence level).
[b] %V: percentage variation in the mean values obtained by the two methods ($V = 2|m_1 - m_2|/(m_1 + m_2)$) where m_1 and m_2 are the mean values obtained using two methods namely, Frontal and AAS.

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flow velocities in the range investigated. Higher values of linear flow velocities were avoided as they may cause compression of Sepharose gel matrices.

Effect of pH

Effect of pH on the copper sorption characteristics of the two gels is shown in Fig. 2(a). Copper sorption experiments could not be conducted beyond pH 6.0 due to the immediate precipitation of the copper(II) as its hydroxide. The copper loading capacity attained a maximum value at pH 4.0 (45.7 μmol mL^{-1} gel) for IDA and at pH 5.0 (32.9 μmol mL^{-1} gel) for TREN. IDA being weakly acidic in nature has greater capacity and stability at lower pH values in comparison to the TREN gel, which is a weak base. Lower values for TREN are supposed to be

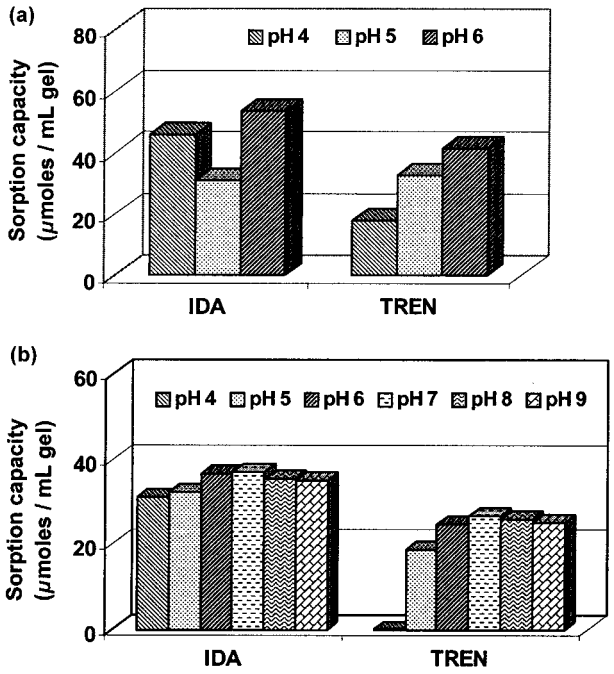

Figure 2. Effect of pH on metal sorption characteristics of IDA-Sepharose 6B and TREN-Sepharose 6B gels (10 *mM* sodium acetate; pH 4–9). (a) Copper sorption; abnormally high values at pH 6.0 for copper are due to precipitation of copper as its hydroxide and its deposition near the filter of the column; copper sorption studies could not be carried out beyond pH 6.0. (b) Nickel sorption.

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caused by the increased protonation of the nitrogen atoms at low pH values resulting in decreased donor capability. A significant reduction in copper sorption capacity was observed at pH 5.0 for IDA (31.1 μmol mL^{-1} gel) and pH 4.0 (17.9 μmol mL^{-1} gel) for TREN. Zachariou et al.[21] also noticed a decline in copper loading capacities for IDA with increasing pH with a maximum at pH 4.0. They compared the acid–base dissociation constants and stability constants for the three IMA gels, IDA-Sepharose CL-4B, OPS-Sepharose CL-4B, and 8-HQ-Sepharose CL-4B, determined with the optimized potentiometric titration procedure to the metal binding capacities for the same gels as determined using AAS. They found that the relative orders for metal ion content show good correlation with the stability constants determined using the potentiometric methods. Their observation clearly indicates that the experimentally determined metal sorption capacities of a chelating gel are sufficient enough to account for its stability in case the stability constants are not available at the specified solution conditions. The abnormally high values at 6.0 for copper for both the gels arise as a result of precipitation of copper in the loading solution within 10 min of solution preparation and its deposition near the filter of the column during copper loading.

Nickel binding was found to increase with pH in the acidic range with an optimum at pH 7.0 (36.8 μmol mL^{-1} gel for IDA and 26.6 μmol mL^{-1} gel for TREN) (Fig. 2(b)). The competition between the hydrogen ion and metal ion in the acidic range appears to be responsible for lower binding capacities in low pH range. The variation in nickel sorption capacities beyond pH 7.0 was marginal for both the gels. While the sorption capacities were nearly the same at pH 6.0 and 7.0 for IDA, the reduction was quite significant at pH 4.0 (15.8%) and 5.0 (12.2%) for TREN. The influence of pH on nickel loading capacities was much more pronounced for TREN in the acidic range, which may be due to the protonation of the four N- donor atoms of TREN ligand. As IDA has only one nitrogen donor atom, effect of pH on nickel loading is relatively less pronounced. The decrease in nickel sorption capacity of TREN gel was substantial at pH 5.0 (28.9%) and 6.0 (6.8%). The binding capacity was negligibly small at pH 4.0 (0.021 μmol mL^{-1} gel, not visible in the Fig. 2(b)).

Effect of Ionic Strength

In general, a decrease in metal loading capacities was noticed with increasing ionic strengths. This may be a result of the suppressed coordination interactions between the transition metal (Cu(II) or Ni(II)) and the chelator due to the presence of large amounts of other ions. However, this reduction was more pronounced in the lower range of ionic strengths (0–0.5 *M* NaCl). Beyond 0.5 *M* NaCl concentration, the variation was marginal for all the cases except TREN-

Cu(II) where the copper sorption capacities were found to be abnormally high. Precipitation of copper as its hydroxide and its deposition at the filter of the column within 10 min of solution preparation and loading under these conditions is supposed to be responsible for this anomaly. Maximum metal sorption capacities were obtained in the absence of ionic strength for all the metal chelated gels investigated (Table 3).

Effect of Feed Metal Concentration

In order to examine the effect of feed metal concentration on metal sorption capacity and prepare sorption isotherms, metal solutions of various concentrations (0–50 *mM*; 0 *M* NaCl) were loaded to the column using frontal method under optimal conditions of pH and ionic strength. The sorption isotherms for all the cases were nearly rectangular (Fig. 3). This indicates that the interaction between the immobilized chelating agent and metal ion is nearly irreversible. This behavior is similar to that shown by numerous chelating ion-exchangers. Studies by El Rassi and Horvath[12] have also indicated that a metal chelated sorbent can behave as a strong cation exchanger for acidic and basic proteins lacking exposed amino acids for specific metal interaction under low ionic strength conditions. Quite a few other studies have also confirmed weak ion-exchange properties of metal chelated gels for IMAC under low ionic strength conditions.[25,26] Dowex A-1

Table 3. Effect of Ionic Strength on the Metal Sorption Capacities of IDA- and TREN-Sepharose 6B (10 *mM* Sodium Acetate; pH 7.0; 0–3 *M* NaCl)

Ionic Strength (*M* NaCl)	Metal Sorption Capacities (μmol mL^{-1} gel)[a]			
	IDA		TREN	
	Copper	Nickel	Copper	Nickel
0	45.7 ± 0.09	36.8 ± 0.10	32.9 ± 0.08	26.6 ± 0.12
0.15	41.7 ± 0.15	34.7 ± 0.11	31.6 ± 0.09	25.5 ± 0.10
0.5	42.1 ± 0.16	33.2 ± 0.12	29.5 ± 0.13	23.0 ± 0.11
1.0	38.2 ± 0.12	32.2 ± 0.15	35.5[b] ± 0.15	17.6 ± 0.13
2.0	40.7 ± 0.15	32.1 ± 0.14	58.2[b] ± 0.18	18.8 ± 0.15
3.0	41.4 ± 0.18	32.4 ± 0.17	59.6[b] ± 0.22	19.2 ± 0.19

[a] Analysis using AAS. Reported values are mean ± standard deviation ($n = 4$).
[b] Abnormally high values due to precipitation of copper as its hydroxide and its deposition near the filter of the column.

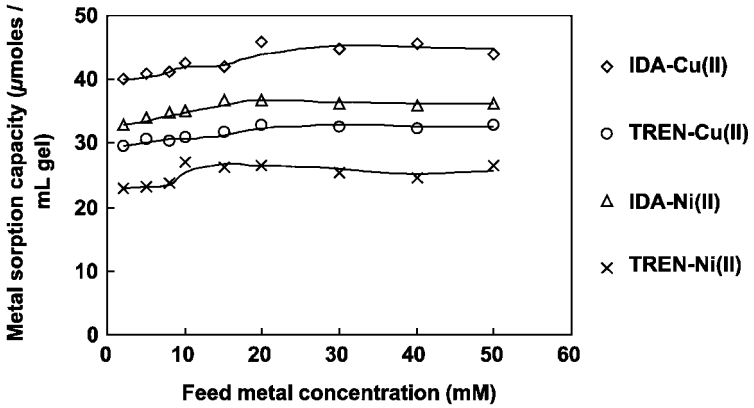

Figure 3. Metal sorption isotherms of IDA-Sepharose 6B and TREN-Sepharose 6B gels (10 *mM* sodium acetate; pH 7.0).

and Chelex-100 are the two chelating ion-exchangers based on IDA (see Figs. 4a and 4b) (which is the most popular chelating agent for IMAC as well) that are widely used for metal preconcentration and wastewater treatment.[3] Furthermore, the attainment of saturation capacity even at sufficiently low feed metal concentration justifies the use of these gels in batch mode and hence its combination with fast separation techniques such as membrane filtration. The optimal metal binding capacities were obtained at 20 *mM* feed concentration for both nickel and copper.

Metal Leaching Studies

The investigations on the metal sorption characteristics of IDA- and TREN-Sepharose 6B indicated that the metal sorption capacities are strongly influenced by pH and ionic strength and have optimal values at solution conditions specific for a particular chelating gel-metal system. In addition, there are certain conditions of pH and ionic strength, which are not suitable for direct metal loading (for instance, pH 6.0–9.0 and ionic strength $>0.5\,M$ for copper loading). Nevertheless, these solution conditions are often used during equilibration, protein adsorption, and elution in IMAC. Therefore, a priori knowledge about metal leaching under these conditions can be beneficial in designing protein separations using IMAC. In order to conduct metal leaching studies, different chelating gels were loaded at optimal conditions identified earlier.

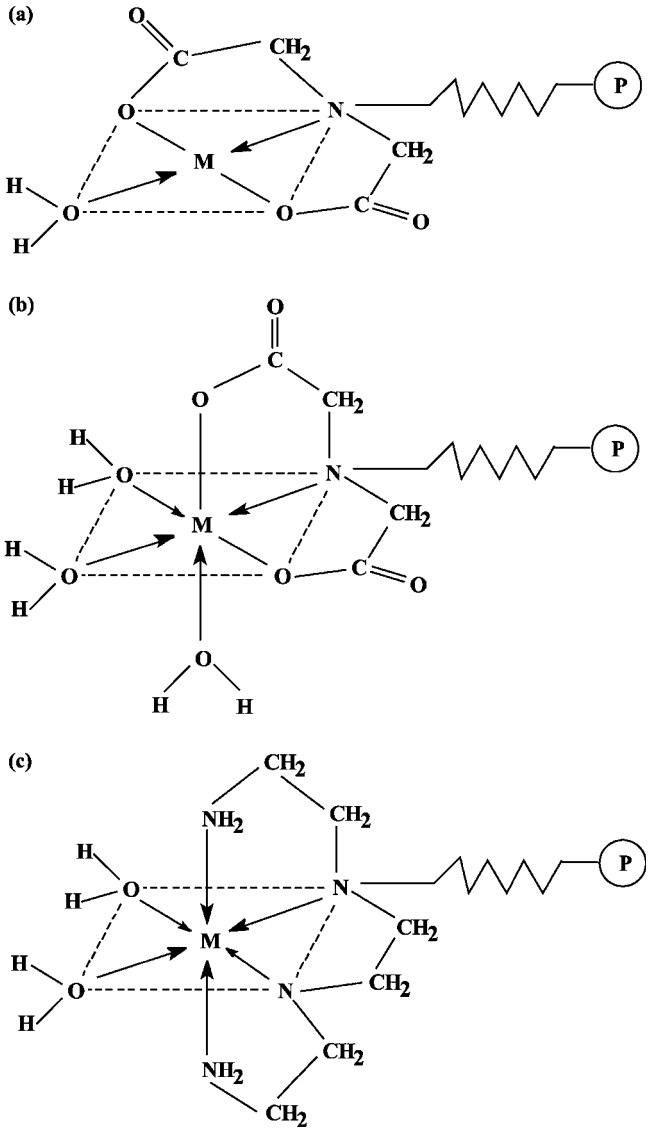

Figure 4. Proposed structures for metal chelated gels. (a) Metal chelated IDA, square planar, M(II) = Cu(II); (b) metal chelated IDA, octrahedral, M(II) = Cu(II), Ni(II); and (c) metal chelated TREN, octrahedral, M(II) = Cu(II), Ni(II).

Effect of pH

For IDA-Cu(II) only traces of copper (total amount $< 0.03\ \mu mol\ mL^{-1}$ gel) were noticed in the pH range 4.0–9.0. This indicates that IDA-Cu(II) is extremely stable in the recommended pH range for IMAC. This information is noteworthy in the wake of the fact that copper loading could not be studied accurately beyond pH 5.0 due to its precipitation as hydroxide. Copper leaching was negligible in the pH range of 5–9 ($< 0.1\%$) for TREN-Cu(II) system as well. Not even a single trace of copper was observed after the first fraction (i.e., after passing three column volumes of buffer). The extreme stability of copper loaded IDA and TREN gels under pH conditions of 5.0–9.0 ensure their repeated use for many runs without recharging the column after each application. However, copper leaching was 3.63% ($1.19\ \mu mol\ mL^{-1}$ gel) of the total sorption capacity %) for TREN-Cu(II) after passing 20 column volumes (CV) of the elution buffer at pH 4.0, indicating the low stability of TREN-Cu(II) chelate at this pH.

In addition, the stability of both, IDA-Cu(II) and TREN-Cu(II), was extremely low at pH 3.0. A continuous and abrupt leakage of metal was observed on passing the buffer of pH 3.0 through these gels. This change was visible as the colors (light blue for IDA-Cu(II) and dark blue for TREN-Cu(II)) of the columns were fading continuously with each volume of the buffer passed. Thus, pH 3.0 is not a proper condition for eluting a bioproduct from these gels.

The effect of pH on metal leaching was more pronounced in case of nickel, thus, indicating the lower stability of nickel chelated gels as compared to copper chelated gels. More amount of the nickel leached from IDA-Ni(II) as compared to TREN-Ni(II) in the basic pH range (i.e., pH 8 and 9) (Fig. 5). Percent nickel

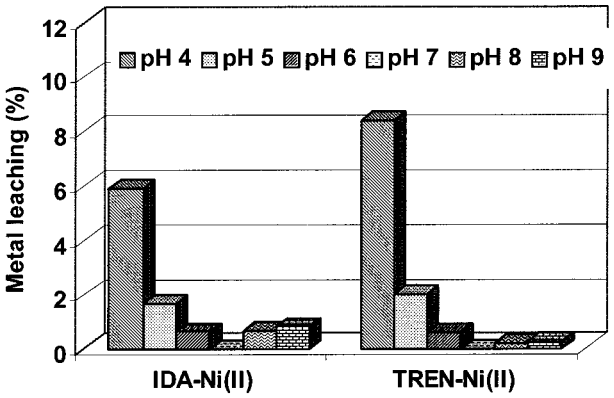

Figure 5. Effect of pH on nickel leaching from IDA-Ni(II) and TREN-Ni(II) gels (10 *mM* sodium acetate; pH 4–9).

leaching was nearly the same (~0.6%) for both the gels at pH 6.0. Also, nickel leaching occurred in significant amount at pH 4.0 and 5.0 for both the gels. It was more with TREN-Ni(II) (8.37% at pH 4.0 and 2.0% at pH 5.0) as compared to 5.89% at pH 4.0 and 1.67% at pH 5.0 for IDA-Ni(II).

As observed earlier for copper loaded gels, the stability of both the nickel-loaded gels was extremely low at pH 3.0. There was continuous and abrupt leaching of nickel from the columns as evidenced by their fading colors (pale green for IDA-Ni(II) and mauve for TREN-Ni(II)). Therefore, pH 3.0 condition should never be employed for conducting a separation on these metal chelated gels.

Effect of Ionic Strength

The metal leaching from different metal chelated supports was examined and quantified under various ionic strength conditions (0.15, 0.5, 1.0, 2.0, 3.0 *M* NaCl). There was no leaching of metal from any of metal chelated gels at low ionic strength values (0.15 and 0.5 *M* NaCl). Copper leaching for IDA-Cu(II) (0.61% (1 *M*) → 0.55% (2 *M*) → 0.41%(3 *M*)) and TREN-Cu(II) (0.51% (1 *M*) → 0.37% (2 *M*)) decreased a little with increasing concentration of NaCl in the buffer (Fig 6). This variation being too small can be considered as negligible. The very low leakage of copper at high ionic strength conditions ensures the utility of these gels for IMAC without any contamination with metal as most of the weakly bound metal ions are removed during pre-equilibration with the loading buffers.

The effect of ionic strength on metal leaching was more pronounced for nickel. Nickel leaching increased with increasing ionic strength for both the gels (Fig. 6b). The variation was quite large for IDA-Ni(II) and leaching became 2.83% of the total sorptivity at 3 *M* NaCl. It was much less for TREN-Ni(II) (<0.6% of the total sorptivity) under the same conditions. These observations indicate the greater stability of TREN-Ni(II) as compared to IDA-Ni(II) under high ionic strength conditions.

DISCUSSION

On the basis of the thorough analysis of the influence of buffer salts, pH, ionic strength, and feed metal concentration, conditions for maximum sorption capacity for all the four combination of metal chelated gels viz., IDA-Cu(II), IDA-Ni(II), TREN-Cu(II), and TREN-Ni(II) have been identified (Table 4). We observe that copper binding capacities are more than nickel binding capacities for both the gels. This is in agreement with Irwing–William's rule according to

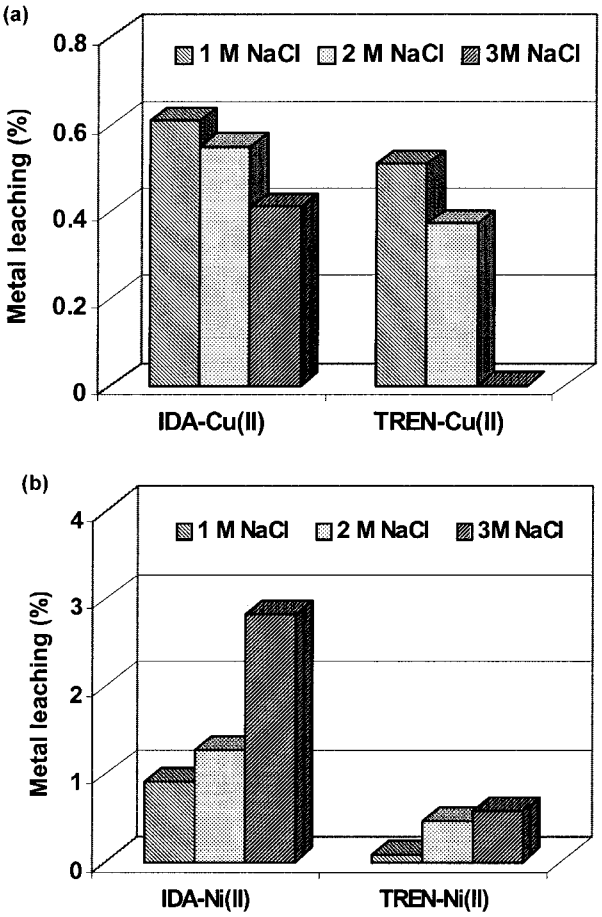

Figure 6. Effect of ionic strength on metal leaching from IDA-M(II) and TREN-M(II) gels (10 *mM* sodium acetate; pH 7.0; 1–3 *M* NaCl). (a) Copper leaching; (b) nickel leaching.

which the following order of coordination strength for amino and imino nitrogen is followed: $Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$. However, the metal sorption capacities of IDA-Ni(II) and TREN-Cu(II) cannot be compared directly as the stability and selectivity of a chelating gel depends both upon the nature of chelating agent as well as the metal ion. The IDA which is weakly acidic in nature forms a double five-membered ring chelate with tetra- and hexa-coordinate metal ions (Fig. 4a, b). Theoretically, TREN is clearly tetradentate chelator with four nitrogen atoms, three of which are primary in nature and the fourth one is tertiary.

Table 4. Optimized Conditions of Metal Sorption for IDA- and TREN-Sepharose 6B Gel in 10 *mM* Sodium Acetate-Acetic Acid Buffer

IMA gel	Metal	pH	Ionic Strength (*M* NaCl)	Feed Metal conc. (*mM*)	Sorption Capacity[a] (μmol mL^{-1} gel)
IDA	Copper	4.0	0.0	20	45.7 ± 0.09
	Nickel	7.0	0.0	20	36.8 ± 0.10
TREN	Copper	5.0	0.0	20	32.9 ± 0.08
	Nickel	7.0	0.0	20	26.6 ± 0.12

[a] Analysis using AAS. Reported value are mean ± standard deviation ($n = 4$).

However, whether it is a tri-or tetradentate is an open question.[15] Therefore, it is expected to form three five-membered rings (Fig. 4c). More is the number of rings formed; greater is the stability of metal chelate. On this basis, it can be inferred that TREN gels should be more stable as compared to IDA gels. However, this cannot be true for all the conditions. This is because of the fact that the nature of the donor atoms in the chelating ligand also plays a very important role in deciding the stability of the metal chelates especially in the varying solution environment. Greater stability of IDA-Ni(II) as compared to TREN-Ni(II) can be explained on the basis of the different natures of the two ligands. The IDA has two oxygen and one nitrogen while TREN has four nitrogen as donor atoms. At low pH values, protonation of the nitrogen moiety makes the lone pair on it less available for coordination while the coordination ability of oxygen is less affected. This makes TREN-Ni(II) more susceptible to changes in pH and comparatively less stable at low pH values.

CONCLUSIONS

In this research effort, we have investigated and analyzed the metal sorption characteristics of chelating gels under different experimental conditions. The metal sorption studies were conducted for two chelated gels viz., Iminodiacetate (IDA) and TREN, bound to Sepharose 6B under varying chemical environment (buffer salts, pH, ionic strength, and feed metal concentration) for Cu(II) and Ni(II) ions.

The effect of pH on sorption capacity was found to be much more pronounced for all the systems investigated as compared to the effect of ionic strength and feed metal concentration. The buffers without ionic strength (i.e., sodium chloride concentration) gave maximum sorption capacities at the optimum pH specific for a particular metal chelated gel. The effect of feed metal concentration was marginal on metal sorption capacities. The sorption isotherms

for all the cases were nearly rectangular indicating the near irreversible nature of the interaction between the immobilized chelating agent and metal ionlike numerous chelating ion-exchangers. Moreover, the saturation capacity of the gel was achieved even at sufficiently low feed metal concentration. On the basis of these investigations, conditions for maximum sorption capacity were identified for all of the chelating gel–metal systems. Furthermore, metal leaching studies were conducted in order to evaluate the suitability of these optimized solution conditions for use in protein fractionation processes. These studies may provide starting guidelines for designing quantitative IMAC separations on large scale.

ABBREVIATIONS

AAS	atomic absorption spectrophotometry
8-HQ	8-hydroxyquinoline
IDA	iminodiacetate
IMA	immobilized metal ion affinity
IMAC	immobilized metal ion affinity chromatography
MCAC	metal chelate affinity chromatography
OPS	*o*-phosphoserine
TREN	tris(2-aminoethyl)amine

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