https://doi.org/10.31925/farmacia.2026.3.10 **ORIGINAL ARTICLE**

USE OF A CU(II)-CHELATING RESIN FOR THE PARTIAL ENRICHMENT OF HUMAN SERUM ALBUMIN AND IMMUNOGLOBULIN G FROM HUMAN PLASMA BY IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY

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Abstract

In this proof-of-concept study, we investigated the use of Immobilized Metal Affinity Chromatography (IMAC) for the partial enrichment of Human Serum Albumin (HSA) and Immunoglobulin G (IgG) from human plasma. A functionalized chromatographic adsorbent, N, N-bis(salicylidenepropylenetriamine)-aminomethyl polystyrene, was synthesized and loaded with Cu(II) ions. The optimal conditions for adsorption and desorption of target proteins were evaluated using different buffer systems. Protein-containing fractions were analyzed by SDS-PAGE and quantified using an automated biochemical analyzer. HSA-enriched fractions were obtained using a buffer containing 5 mM Tris-HCl, 10 mM imidazole, 10 mM MOPS, and 35 mM NaCl, or a phosphate buffer (10 mM) supplemented with 35 mM NaCl and 5 mM imidazole. The recovery of HSA in eluted fractions was 19.5 - 28.6% of the theoretical HSA input; when including the non-adsorbed flow-through fraction, the total protein accounted for reached 38.6 - 67.8% of the theoretical input, excluding NaOH wash fractions. IgG-enriched fractions were recovered using low imidazole concentrations (5 - 10 mM) in a phosphate/MOPS buffer system, with a recovery of 24% of the theoretical IgG input in selectively eluted fractions. These results demonstrate for the first time the potential of this novel Schiff base-functionalized Cu(II)-chelating resin as a cost-effective and promising IMAC support for the partial enrichment of major plasma proteins under mild buffer conditions.

Rezumat

În acest studiu s-a analizat utilizarea cromatografiei de afinitate cu metale imobilizate (IMAC) pentru îmbogățirea parțială a albuminei serice umane (HSA) și a imunoglobulinei G (IgG) din plasma umană. A fost sintetizat un adsorbant cromatografic, N, N-bis(salicilidenpropilentriamin)-aminometil polistiren, care a fost încărcat cu ioni Cu(II). Condițiile optime pentru adsorbția și desorbția proteinelor țintă au fost evaluate folosind diferite sisteme tampon. Fracțiile conținând proteine au fost analizate prin SDS-PAGE și cuantificate folosind un analizor biochimic automat. Fracțiile îmbogățite cu HSA au fost obținute folosind un tampon conținând 5 mM Tris-HCl, 10 mM imidazol, 10 mM MOPS și 35 mM NaCl, sau un tampon fosfat (10 mM) suplimentat cu 35 mM NaCl și 5 mM imidazol. Recuperarea HSA în fracțiile eluate a fost de 19,5 - 28,6% din aportul teoretic de HSA; atunci când s-a inclus fracția de flux neadsorbită, proteina totală a reprezentat 38,6 - 67,8% din aportul teoretic, excluzând fracțiile de spălare cu NaOH. Fracțiile îmbogățite cu IgG au fost recuperate utilizând concentrații scăzute de imidazol (5 - 10 mM) într-un sistem tampon fosfat/MOPS, cu o recuperare de 24% din aportul teoretic de IgG în fracțiile eluate selectiv. Aceste rezultate demonstrează pentru prima dată potențialul acestei noi rășini de chelatare a Cu(II) funcționalizate cu bază Schiff ca suport IMAC rentabil și promițător pentru îmbogățirea parțială a principalelor proteine plasmatice în condiții tampon blânde.

Keywords: human serum albumin, Immunoglobulin G, Immobilized Metal Affinity Chromatography, Cu(II)-chelating resin, plasma fractionation

Introduction

Human plasma is a complex biological fluid containing more than 3,000 distinct proteins, spanning a concentration range of over ten orders of magnitude. Among these, Human Serum Albumin (HSA) and Immunoglobulin G (IgG) are the two most therapeutically relevant proteins. HSA, which constitutes approximately 55 - 60% of total plasma protein content (35 - 50 g/L), plays essential physiological roles including the maintenance of oncotic pressure, transport of fatty acids, hormones, and drugs, as well as antioxidant activity. IgG, representing the most abundant class of immunoglobulins (~ 70 - 75% of total immunoglobulins, 7 - 16 g/L in healthy adults), is central to humoral immunity and constitutes the active component of numerous immunotherapeutic and diagnostic preparations (1, 2).

The industrial purification of these proteins from human plasma is of major importance for the production of life-saving biologics, including albumin solutions used in hypovolemia and liver disease, intravenous

immunoglobulin (IVIG) preparations for immunodeficiencies, and various antibody-based diagnostic reagents. Conventional plasma fractionation methods, such as the cold ethanol precipitation process originally developed by Cohn and colleagues in the 1940s, remain widely used at industrial scale. However, these processes are multi-step, time-consuming, and often yield products of variable purity that require extensive downstream processing (3).

Chromatographic techniques have increasingly replaced or complemented precipitation-based methods due to their superior resolution, reproducibility, and scalability. Among these, affinity chromatography offers the highest selectivity, exploiting specific molecular interactions between a ligand immobilized on a solid support and a target protein in solution. Protein A and Protein G chromatography are well-established approaches for IgG purification, but their high cost, limited reusability, and potential for ligand leaching remain significant drawbacks for large-scale applications (4).

Immobilized Metal Affinity Chromatography (IMAC), first introduced by Porath and colleagues in 1975, represents an attractive alternative. IMAC exploits the coordinative interactions between transition metal ions, such as Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}, immobilized on a chelating support, and electron-donor groups present on the surface of proteins, particularly the imidazole ring of histidine, the thiol group of cysteine, and the indole group of tryptophan residues. The strength and selectivity of these interactions can be modulated by adjusting buffer composition, pH, ionic strength, and the nature and concentration of competing ligands such as imidazole (5, 6).

IMAC has been extensively applied to the purification of recombinant His-tagged proteins, but its application to native plasma proteins, which naturally expose metal-binding residues on their surface, remains comparatively less explored. HSA, for instance, contains a well-characterized copper-binding site at its N-terminus (ATCUN motif), while IgG molecules possess surface-accessible histidine residues that can interact with immobilized metal ions under appropriate conditions (7, 8). These properties make IMAC a potentially useful tool for the partial enrichment of both proteins from human plasma in a single chromatographic platform.

The chelating support used in the present study is a novel functionalized polymer, N, N-bis(salicyldenepropylenetriamine)-aminomethyl polystyrene, synthesized and characterized at the Applied Biochemistry Laboratory of Sétif University. This Schiff base-functionalized polystyrene resin exhibits high metal-chelating capacity and has demonstrated affinity for divalent metal ions, including Cu(II). Its use as an IMAC support for plasma protein partial enrichment has not been previously reported, and its physicochemical properties suggest potential advantages in terms of stability, reusability, and cost-effectiveness compared to commercial IMAC matrices (4,5).

The objective of this study was therefore to evaluate the performance of this Cu(II)-loaded resin for the adsorption and desorption of HSA and IgG from human plasma, and to identify the optimal buffer conditions enabling their chromatographic partial enrichment. The degree of enrichment of the recovered fractions was assessed by SDS-PAGE, and the recovered protein concentrations were determined using an automated biochemical analyser.

Materials and Methods

Resin and column specifications

The stationary phase used in this study consists of microbeads of N,N-bis(salicylidenepropylenetriamine)-aminomethyl polystyrene (Figure 1), synthesized by Charef and collaborators (2010) at the Applied Biochemistry Laboratory, University of Sétif, Algeria (4). The resin particle size is approximately 100 - 200 µm, as determined by optical microscopy. The metal loading capacity of the resin is 0.42 mmol Cu^{2+}/g dry resin, as determined by atomic absorption spectroscopy (AAS). The resin was packed into a glass column (1 cm internal diameter × 10 cm length; bed volume ≈ 7.85 mL). The chromatographic system operated at room temperature (25 ± 1°C) at a flow rate of 0.5 mL/min (linear velocity ≈ 6.4 cm/h), under gravity-driven or peristaltic pump-assisted flow. System back-pressure was monitored and maintained below 0.3 MPa throughout all experiments.

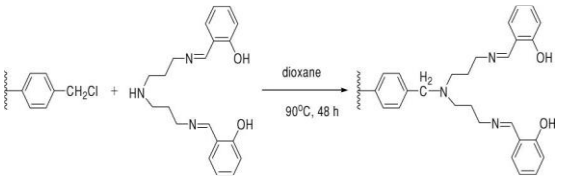

Figure 1.
Resin formation from polystyrene and Schiff base (4)

Chemicals

All reagents were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA) and Fluka (Buchs, Switzerland), unless otherwise stated. Buffers were prepared using ultrapure water (resistivity ≥ 18.2 MΩ·cm), filtered through 0.22 µm membranes (Millipore, USA), and degassed by vacuum before use. pH adjustments were performed at 25°C using a calibrated pH meter (HANNA Instruments, Romania). Ionic strength was calculated for each buffer system and is reported alongside the respective experiments.

Donor characteristics and sample preparation

Blood samples were collected from five healthy adult volunteer donors (3 male, 2 female; age range 25 - 45 years) at the University Hospital Centre (CHU) of Sétif, Algeria, with no history of chronic disease,

infection, or medication known to affect plasma protein levels. Blood samples were collected from five healthy adult volunteer donors; however, chromatographic experiments were performed using plasma from three of these donors, selected based on sample quality and volume availability. The remaining two samples were retained as backup. Blood was collected by venipuncture into Vacutainer tubes containing sodium citrate as anticoagulant (129 µL of 3.8% sodium citrate solution *per* tube, corresponding to a 1:9 anticoagulant-to-blood ratio). Samples were centrifuged at 3,000 rpm (≈ 1,000 × g) for 10 minutes at 4°C to obtain platelet-poor plasma. The resulting plasma was carefully aspirated, aliquoted into 1 mL fractions, and stored at -20°C until analysis. Plasma from individual donors was used separately and not pooled, unless otherwise stated.

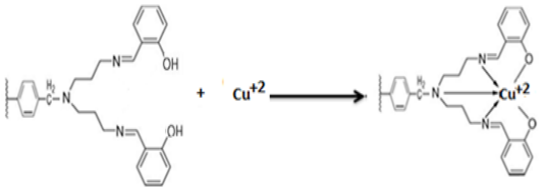

Figure 2.
Formation of the ion-saturated Cu(II) resin bivalent metals (4)

Preparation of the stationary phase

The Cu(II)-loading of the resin was performed using a batch equilibrium technique. Briefly, 0.5 g of dry resin *per* 100 mL of solution was suspended in a 10 mM copper(II) acetate trihydrate ($Cu(CH_3COO)_2 \cdot 3H_2O$) solution prepared in ultrapure water (pH unadjusted, approximately 5.5 - 6.0). The suspension was stirred continuously at 25°C for 6 hours. The metal-loaded resin was then recovered by filtration and washed extensively with ultrapure water (5 × 10 mL) to remove unbound Cu^{2+} ions (Figure 2). Metal loading was confirmed by visual inspection (characteristic blue coloration of Cu^{2+}-chelated resin) and quantified by measuring residual Cu^{2+} in the wash fractions using AAS. The Cu^{2+}-loaded resin was stored in equilibration buffer at 4°C until use.

Instrumentation

The equipment used in this study includes: a centrifuge (Sigma) 3K30 C, a pH meter (HANA), a spectrophotometer (TechomP, UV/VIS-8500), an electrophoresis apparatus (Pharmacia-LKB), the COBAS INTEGRA 400 PLUS automated analyser, and the IMAC chromatography apparatus.

Chromatography

The Cu(II)-loaded resin was packed into a column (1 cm × 10 cm) and equilibrated with the adsorption buffer (phosphate buffer (10 mM), pH 8.0). Gentle stirring was employed to avoid air bubble formation, which could interfere with detection (*e.g.*, by producing unwanted peaks). The resin was equilibrated by passing the adsorption buffer through the column 5 to 6 times its volume (≈ 40 - 50 mL) until a stable UV baseline at 280 nm was achieved. Before injection, plasma samples were thawed at 4°C overnight and centrifuged at 10,000 × g for 5 minutes at 4°C to remove any precipitates. After equilibration, 0.5 mL of plasma was diluted 1:5 (v/v) in the equilibration buffer (either 5 mM Tris-HCl, pH 8.0, or 10 mM phosphate buffer, pH 8.0, depending on the experiment), yielding a final volume of 2.5 mL. This diluted sample was pre-incubated for 10 minutes in a closed tube at room temperature (static batch incubation), prior to continuous loading onto the pre-equilibrated column at a flow rate of 0.5 mL/min. Following sample loading, the column was washed with 3 column volumes (CV, ~ 24 mL) of adsorption buffer to remove non-adsorbed proteins. Elution was then performed using different buffers (6). The complete composition, final pH, calculated ionic strength, and experimental use of all buffers employed in this study are summarised in Table I. Each elution step used approximately 2 CV (~ 16 mL) *per* buffer condition. After elution, the column was regenerated with 200 mM NaOH (pH ≈ 13.3) to remove strongly adsorbed proteins from the resin (5).

Table I
Complete summary of all buffer systems used in this study

No.	Buffer ID	Composition (all reagents at stated concentration)	Final pH	Ionic Strength (I, mM)*	Experimental Use (section)
A. Equilibration / Adsorption Buffers					
1	Equilibration A	Tris-HCl 5 mM	8.0	~ 5 mM	Column equilibration; plasma sample dilution.
2	Equilibration B	Phosphate 10 mM	8.0	~ 12 mM	Column equilibration; removal of non-adsorbed proteins.
B. Elution Buffers – Tris-HCl / NaCl / Imidazole series					
3	Elution T1	Tris-HCl 10 mM + imidazole 10 mM + NaCl 35 mM	8.0	~ 55 mM	HSA elution – optimal condition.
4	Elution T2	Tris-HCl 10 mM + imidazole 10 mM + NaCl 60 mM	8.0	~ 80 mM	Effect of NaCl concentration on BSA desorption.
5	Elution T3	Tris-HCl 10 mM + imidazole 10 mM + NaCl 100 mM	8.0	~ 120 Mm	Effect of NaCl concentration on BSA desorption.

6	Elution T4	Tris-HCl 10 mM + imidazole 10 mM + NaCl 200 mM	8.0	~ 220 mM	Effect of NaCl concentration on BSA desorption.
7	Elution T5	Tris-HCl 10 mM + imidazole 10 mM + NaCl 400 mM	8.0	~ 420 mM	Effect of NaCl concentration on BSA desorption.
8	Elution T6	Tris-HCl 10 mM + imidazole 10 mM + NaCl 600 mM	8.0	~ 620 mM	Effect of NaCl concentration on BSA desorption.
C. Elution Buffers – Tris-HCl / MOPS / NaCl series					
9	Elution M1	Tris-HCl 5 mM + imidazole 10 mM + MOPS 10 mM + NaCl 35 mM	8.0	~ 50 mM	HSA elution – optimal condition with MOPS.
10	Elution M2	Tris-HCl 10 mM + imidazole 10 mM + MOPS 10 mM + NaCl 35 mM	8.0	~ 55 mM	Elution of BSA-IgG mixture.
11	Elution M3	Tris-HCl 10 mM + imidazole 10 mM + MOPS 10 mM + NaCl 50 mM	8.0	~ 80 mM	Effect of NaCl with MOPS on plasma protein desorption.
12	Elution M4	Tris-HCl 10 mM + imidazole 10 mM + MOPS 10 mM + NaCl 100 mM	8.0	~ 130 mM	Effect of NaCl with MOPS on plasma protein desorption.
13	Elution M5	Tris-HCl 10 mM + imidazole 10 mM + MOPS 10 mM + NaCl 200 mM	8.0	~ 230 mM	Effect of NaCl with MOPS on plasma protein desorption.
D. Elution Buffers – Phosphate / Imidazole series					
14	Elution P1	Phosphate 30 mM + imidazole 5 mM	8.0	~41 mM	Co-elution of BSA and IgG.
15	Elution P2	Phosphate 30 mM + imidazole 10 mM	8.0	~ 47 mM	Partial selective elution of BSA.
16	Elution P3	Phosphate 30 mM + imidazole 15 mM	8.0	~ 51 mM	Imidazole gradient study.
17	Elution P4	Phosphate 30 mM + imidazole 20 mM	8.0	~ 56 mM	Imidazole gradient study.
18	Elution P5	Phosphate 30 mM + imidazole 25 mM	8.0	~ 61 mM	Imidazole gradient study.
E. Elution Buffers – Phosphate / NaCl series					
19	Elution PN1	Phosphate 30 mM + imidazole 5 mM + NaCl 35 mM	8.0	~ 76 mM	HSA partial enrichment – optimal phosphate/NaCl condition.
20	Elution PN2	Phosphate 30 mM + imidazole 5 mM + NaCl 60 mM	8.0	~ 101 mM	Effect of NaCl in phosphate system.
F. Elution Buffers – Phosphate / MOPS / Imidazole series					
21	Elution PM1	Phosphate 10 mM + MOPS 5 mM + imidazole 5 mM	8.0	~ 27 mM	IgG partial recovery – optimal condition
22	Elution PM2	Phosphate 10 mM + MOPS 5 mM + imidazole 10 mM	8.0	~ 32 mM	IgG partial recovery.
23	Elution PM3	Phosphate 10 mM + MOPS 5 mM + imidazole 15 mM	8.0	~ 37 mM	Imidazole gradient + MOPS study.
24	Elution PM4	Phosphate 10 mM + MOPS 5 mM + imidazole 20 mM	8.0	~ 42 mM	Imidazole gradient + MOPS study.
25	Elution PM5	Phosphate 10 mM + MOPS 5 mM + imidazole 25 mM	8.0	~ 47 mM	Imidazole gradient + MOPS study.
G. Washing / Regeneration Buffers					
26	Wash/Regen. 1	NaOH 200 mM	~ 13.3	~ 200 mM	Column regeneration/cleaning; elution of strongly bound proteins - not a product fraction (likely contains denatured and non-specifically bound proteins).
27	Regen. 2 (metal)	HCl 3 M	~ 0.5	~ 3000 mM	Removal of Cu^{2+} ions; full resin regeneration.

*Ionic strength calculated using $I = 0.5 \times \Sigma(c_i \times z_i^2)$, where ci is the molar concentration and zi the charge of each ionic species at the stated pH. Values are approximate (± 2 mM) due to partial ionisation of weak acids/bases.
Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MOPS, 3-(N-morpholino) propanesulfonic acid; NaCl, sodium chloride; NaOH, sodium hydroxide; HCl, hydrochloric acid. All buffers were prepared in ultrapure water (≥ 18.2 MΩ·cm), filtered through 0.22 µm membranes, and degassed. pH measured at 25°.

Fraction Collection

Fractions were collected in tightly sealed tubes and stored at 4°C for subsequent analyses. Flow-through fractions were also collected and analysed for protein content using the COBAS INTEGRA 400 PLUS automated analyser to confirm protein retention on the

resin. The detection limit of the UV monitor (280 nm) under the conditions used was approximately 0.05 AU (~ 0.05 mg/mL total protein). In all experiments, protein concentrations in flow-through fractions were below 0.10 g/L, consistent with retention of > 9 5% of the applied protein load.

Resin Regeneration

The matrix can be regenerated using 3 M HCl with agitation for 30 minutes, which effectively removes metal ions (Figure 3). The same resin batch was successfully employed throughout all experiments conducted in this study, suggesting satisfactory operational stability under the conditions used. Prior work by Charef *et al*. (2010) demonstrated that similar chelating resins can withstand more than 100 regeneration cycles while maintaining selectivity, capacity, and reproducibility (5), providing a strong literature basis for the reusability potential of this class of resin. A systematic evaluation of reuse performance over multiple cycles represents an important direction for future work.

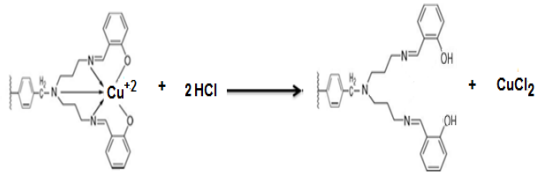

Figure 3.
Resin regeneration (5)

Dialysis

Collected fractions were pooled and transferred into dialysis tubing (molecular weight cut-off: 12 - 14 kDa; Sigma-Aldrich). Dialysis was performed against phosphate-buffered saline (PBS, pH 8.0, adjusted with NaOH) under continuous magnetic stirring at 4°C overnight (approximately 16 hours), with one buffer change after 8 hours. To concentrate the dialyzed protein fractions, the exterior of the dialysis tubing was covered with polyethylene glycol (PEG 20,000; Sigma-Aldrich) powder at 4°C for 2 - 4 hours, until the desired volume reduction was achieved (typically 5- to 10-fold). The concentrated samples were recovered and centrifuged at 5,000 × g for 10 minutes at 4°C to remove any PEG residues before further analysis.

Protein quantification

Total protein concentration in all fractions was determined using the COBAS INTEGRA 400 PLUS automated analyser (Roche Diagnostics, Switzerland), based on the bromocresol green (BCG) method for albumin and the immunoturbidimetric method for IgG. Calibration was performed using certified human serum calibrators supplied by the manufacturer. The linearity, precision, and accuracy of the assay were verified in the presence of the buffer components used in this study (Tris-HCl, phosphate buffer, imidazole, MOPS and NaCl), confirming the absence of significant matrix interference at the concentrations employed. Results are expressed in g/L.

SDS-PAGE analysis

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) (7). Resolving gels of 10% and 12% acrylamide were used to resolve proteins in the molecular weight range of 10 - 200 kDa. Stacking gels of 4% acrylamide were prepared in 0.5 M Tris-HCl buffer (pH 6.8). Samples were prepared by mixing equal volumes of protein fraction with 2× Laemmli sample buffer (containing 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue, and 0.125 M Tris-HCl, pH 6.8) and heated at 95°C for 5 minutes. A volume of 20 µL *per* well (corresponding to approximately 5 - 10 µg total protein) was loaded *per* lane. A commercial prestained molecular weight marker (PageRuler, Thermo Fisher Scientific, range 10 - 170 kDa) was included in each gel. Electrophoresis was conducted at 80 V through the stacking gel and 120 V through the resolving gel in Tris-glycine-SDS running buffer. Gels were stained with Coomassie Brilliant Blue R-250 (0.25% in methanol/acetic acid/water, 45:10:45, v/v/v) for 1 hour and destained in methanol/acetic acid/water (25:10:65, v/v/v) until background was clear. Gel images were acquired using a digital gel documentation system. Band intensity was assessed qualitatively by visual inspection of Coomassie-stained gels. Quantitative densitometric analysis was not performed in the present study and is identified as a priority for future work.

Statistical analysis

Chromatographic experiments were performed using plasma from three different donors, each constituting an independent biological replicate. Within each experiment, multiple fraction tubes were collected *per* elution step; the value "n" reported in the tables refers to the number of tubes collected *per* condition within a single run, not the number of independent donor replicates. Protein concentrations from individual tubes were averaged within each donor run, and the mean ± SD values reported in the tables reflect variability across the three independent donor replicates. Each buffer condition was tested with plasma from all three donors. Experiments were conducted in a fixed sequential order, as presented in the Results section, rather than in a randomised order, as the study was exploratory in design and aimed at progressively identifying optimal elution conditions. The absence of randomisation represents a limitation that will be addressed in future confirmatory studies.

Primary objective and outcome measures

The primary objective of this study was to evaluate the capacity of the Cu(II)-loaded Schiff base-functionalized resin to selectively adsorb and elute HSA and IgG from human plasma under various buffer

conditions. The primary outcome measures were: (i) total protein recovery in each eluted fraction (mg and % of theoretical input), as determined by automated biochemical analysis; and (ii) the degree of enrichment of HSA and IgG, as assessed by SDS-PAGE.

Results and Discussion

The chromatographic column was filled with Cu(II)-resin loaded with Cu^{2+} ions. A 0.5 mL sample of human plasma was diluted 1:5 in Tris-HCl buffer (5 mM, pH 8) and incubated for 10 minutes. Elution was performed using various buffers, and washing was carried out with NaOH (200 mwM, pH ≈ 13.3).

Effect of the (Tris-HCl, NaCl, imidazole) complex on BSA desorption

Based on the methods of Porath and his collaborators (1975) and Ayyar and his collaborators (2012), with modifications (8,9), a 0.5 mL BSA sample (5 mg/mL) was eluted with buffers at the same pH but with varying NaCl concentrations (35, 60, 100, 200, 400, 600 mM, and 1 M). The concentrations of Tris-HCl and imidazole were fixed at 10 mM each (as *per* previous results). The BSA fractionation results are shown in Figure 4.

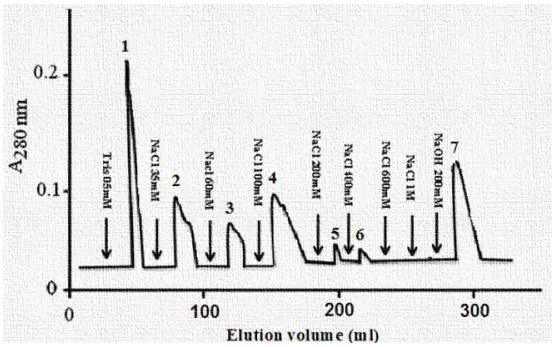

Figure 4.

Elution by varying NaCl concentration while keeping Tris-HCl and Imidazole fixed at 10 mM. Peak 1: Adsorption buffer Tris-HCl (5 mM). Peak 2: 35 mM NaCl. Peak 3: 60 mM NaCl. Peak 4: 100 mM NaCl. Peak 5: 200 mM NaCl. Peak 6: 400 mM NaCl. Peak 7: 200 mM NaOH

The first peak, obtained with the adsorption buffer (Tris-HCl, 5 mM), represents the unadsorbed BSA protein with a concentration of 0.894 g/L. As the NaCl concentration increases, the BSA concentration recovered is indicated by peaks 2, 3, and 4. However, beyond a NaCl concentration of 100 mM, BSA elution starts to decrease and eventually stops at 600 mM NaCl (Table II). Peak 7, obtained with 200 mM NaOH, represents the strongly adsorbed proteins on the resin, with an eluate concentration of 2.156 g/L. This fraction was used as a regeneration wash; the material eluted under these conditions likely represents a mixture of denatured and non-specifically bound proteins, and should not be considered as a biologically active product.

The selective retention of BSA on the Cu(II)-IMAC support is primarily governed by coordinative interactions between the immobilized Cu^{2+} ion and surface-accessible electron-donating residues, particularly histidine, but also cysteine and tryptophan (5, 6). BSA was used as a model protein for HSA in these experiments, given the well-documented structural and biochemical analogy between the two albumins, including the presence of a homologous N-terminal copper-binding ATCUN motif (Asp-Thr-His in BSA; Asp-Ala-His in HSA). The mechanistic interpretations derived from BSA experiments are therefore directly applicable to the expected behaviour of HSA on the Cu(II)-IMAC support (8).

A nuanced consideration concerns, however, the structural difference between the ATCUN motifs of BSA and HSA. Although both form square-planar Cu^{2+} complexes with high stability constants (log Kf ≈ 12 - 13) (10, 11), the presence of threonine rather than alanine at position 2 results in a measurable difference in Cu^{2+} binding affinity, with HSA exhibiting slightly tighter binding than BSA (10). This distinction is relevant when extrapolating mechanistic interpretations from BSA model experiments to the behaviour of native HSA in plasma. More significantly, it must be acknowledged that in native human plasma, a substantial fraction of HSA's N-terminal ATCUN site may already be occupied by Cu^{2+} or other transition metal ions, given that plasma Cu^{2+} concentrations are physiologically maintained near the dissociation constant of the HSA-Cu^{2+} interaction (12). If a significant proportion of HSA enters the column with its ATCUN site pre-saturated, the retention mechanism on the Cu(II)-IMAC support would need to rely on secondary interactions, surface-accessible histidine or cysteine residues, rather than exclusively on the N-terminal site. This could, in principle, reduce the selectivity of HSA capture relative to that predicted from the ATCUN affinity data alone, and may contribute to the incomplete recoveries observed in plasma experiments. Future experiments using metal-depleted plasma, prepared by dialysis against EDTA followed by extensive buffer exchange, would help to deconvolute the contributions of the N-terminal ATCUN site and secondary metal-binding residues to the overall retention behaviour of HSA on the Cu(II)-IMAC support.

Recent studies have further characterised the thermodynamic properties of the HSA-Cu^{2+} ATCUN interaction, reporting formation constants (log Kf ≈ 13), corresponding to femtomolar dissociation constants (Kd ~ 10^{-13} M), for the Asp-Ala-His motif at physiological pH (10), consistent with the strong and selective retention observed on the Cu(II)-IMAC support in the present study. The successful elution of HSA with relatively mild conditions (35 mM NaCl,

10 mM imidazole) is consistent with the competitive displacement of Cu^{2+} from the ATCUN motif by free imidazole acting as a competing ligand. The progressive decrease in BSA recovery above 100 mM NaCl suggests that, beyond an optimal ionic strength, high salt concentrations may destabilize the protein–metal coordination complex rather than simply reducing non-specific electrostatic interactions.

The use of Tris-HCl as a buffer component in IMAC experiments has been noted in the literature as potentially problematic, as Tris possesses a primary amine group capable of weakly coordinating transition metal ions, including Cu^{2+}, and could theoretically compete with protein binding sites or promote metal leaching (6, 13). However, several considerations justify its use in the present system. First, the Tris concentrations employed (5 - 10 mM) are substantially lower than those reported to cause significant metal displacement in conventional IDA-based IMAC supports (typically > 50 mM) (13). Second, the tridentate Schiff base chelating ligand of the present resin forms a more stable coordination complex with Cu^{2+} than standard IDA, which is a tridentate ligand of lower binding strength (4); the higher stability constant of the Schiff base–Cu^{2+} complex is therefore expected to confer greater resistance to competitive displacement by Tris. Third, the characteristic blue coloration of the Cu^{2+}-loaded resin was visually confirmed to be maintained throughout all chromatographic experiments with Tris-containing buffers, providing qualitative evidence of metal retention on the support. Quantitative determination of residual Cu^{2+} in product fractions by atomic absorption spectroscopy (AAS) or inductively coupled plasma mass spectrometry (ICP-MS) is planned as part of future process characterisation, in accordance with ICH Q3D elemental impurity guidelines. Notwithstanding these considerations, future experiments will employ MOPS or phosphate as the sole buffer components to eliminate any potential Tris-related interference.

Table II

Quantification of BSA by varying NaCl concentration while keeping Tris-HCl and Imidazole concentrations fixed

Condition	Concentration (g/L)	Mass recovered (mg)	Recovery (%)
Adsorption buffer	0.894 ± 0.090	1.79 ± 0.18	8.9 ± 1.2
35 mM NaCl	0.989 ± 0.099	1.98 ± 0.20	9.9 ± 1.3
60 mM NaCl	0.231 ± 0.046	0.46 ± 0.09	2.3 ± 0.6
100 mM NaCl	0.459 ± 0.069	0.92 ± 0.14	4.6 ± 0.9
200 mM NaCl	≤ 0.093 ± 0.028	≤ 0.19 ± 0.06	≤ 0.9 ± 0.3
400 mM NaCl	≤ 0.078 ± 0.023	≤ 0.16 ± 0.05	≤ 0.8 ± 0.3
600 mM NaCl	0.0 ± 0.0	0.0 ± 0.0	00 ± 00
1 M NaCl	0.0 ± 0.0	0.0 ± 0.0	00 ± 00
200 mM NaOH	2.156 ± 0.216	4.31 ± 0.43	21.6 ± 2.5

Theoretical input: BSA = 2.5 mg (0.5 mL × 5 mg/mL). NaOH fractions are excluded from the yield calculation as they contain denatured proteins. Values are expressed as mean ± standard deviation (SD) for three independent donor replicates. "n" in the Condition column = number of fraction tubes *per* elution step within a single run.

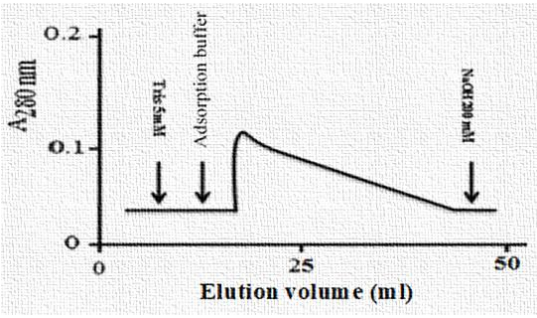

Figure 5.
Chromatography at Low BSA Concentration on Cu(II)-Resin. Peak was obtained using the elution buffer (5 mM Tris-HCl, 10 mM imidazole, 10 mM MOPS, 35 mM NaCl)

Effect of the complex with MOPS

BSA/ IgG Injection. Injecting 2 mg/mL of BSA did not yield a peak for unadsorbed proteins (Figure 5), as no UV-absorbing peak was detected in the flow-through fraction, suggesting that the applied protein was largely retained by the resin under the loading conditions used. Flow-through fractions were confirmed to contain less than 0.10 g/L total protein by automated biochemical analysis, consistent with > 95% retention. The peak obtained with the elution buffer (10 mM Tris-HCl, 35 mM NaCl, 10 mM MOPS, and 10 mM imidazole) allowed the recovery of two 20 mL tubes. After concentration using PEG, the average BSA concentration obtained was 0.962 mg/mL.

Applying the same buffer with the same concentration (2 mg/mL) for IgG resulted in a peak overlapping with that of Figure 5. The co-elution of both BSA and IgG under these conditions indicates that this buffer did not achieve selective separation of the two proteins, and further optimisation of buffer composition is required.

Injection of the BSA-IgG mixture. A mixture of 4 mg/mL (2 mg BSA and 2 mg IgG) was injected into the column. Elution was performed using the elution buffer (10 mM Tris-HCl, 35 mM NaCl, 10 mM MOPS, and 10 mM imidazole). The fractionation results are shown in Figure 6.

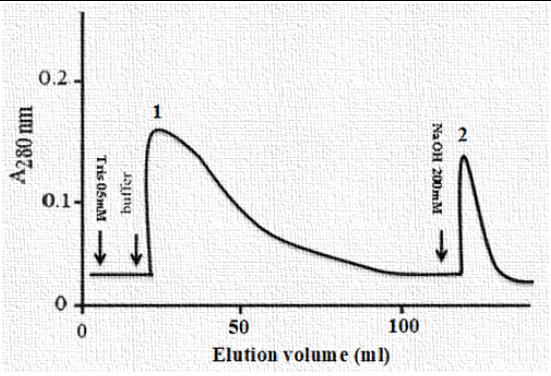

Figure 6.
Elution of the mixture (BSA-IgG) on the Cu(II) resin. Peak 1: elution buffer (5 mM Tris-HCl, 10 mM imidazole, 10 mM MOPS, 35 mM NaCl). Peak 2: 200 mM NaOH

The injection of 4 mg/mL of the mixture did not produce a detectable UV-absorbing peak in the flow-through fraction, suggesting that the applied proteins were largely retained by the resin. The peak obtained with the elution buffer contains the BSA eluted from the column, as confirmed by SDS-PAGE (Figure 7), with a concentration distributed over 4 fractions and an average of 0.486 mg/mL after concentration by PEG. Peak 2 represents IgG (Figure 7); the elution buffer could not desorb it, but the wash buffer (200 mM NaOH) allowed its desorption at a concentration of 1.904 g/L after concentration by PEG. It should be noted that NaOH (200 mM) was used as a regeneration wash, and the material eluted under these conditions likely represents a mixture of denatured and non-specifically bound proteins, which should not be considered as a biologically active product.

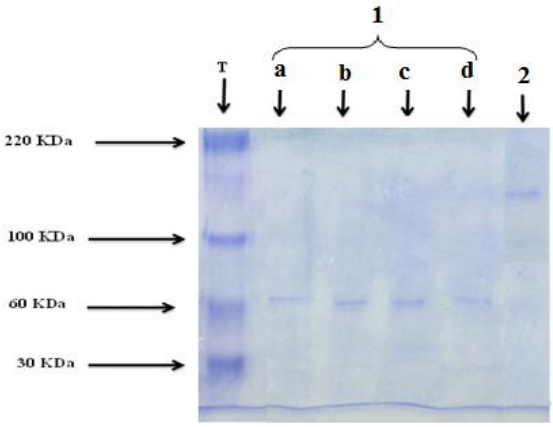

Figure 7.
Electrophoresis (SDS-PAGE) of the mixture (BSA-IgG). Well 1: Molecular weight standards. Wells 2, 3, 4, and 5: the four fractions (a, b, c, and d) corresponding to peak 1. Well 6: wash with 200 mM NaOH

The differential elution behaviour of BSA and IgG under these conditions reflects the fundamental difference in their metal-binding mechanisms. BSA interacts with Cu^{2+} primarily through its ATCUN motif, a specific and well-defined coordination site that can be competitively displaced by imidazole at moderate concentrations (8). IgG, by contrast, lacks an equivalent high-affinity motif; its interaction with the Cu(II) support is mediated by surface-accessible histidine residues distributed across the Fab and Fc regions, as well as by non-specific electrostatic and hydrophobic contributions (11). Consequently, IgG binds more tenaciously and requires harsher conditions for desorption. The addition of MOPS to the buffer appeared to modulate the ionic environment sufficiently to retain selective IgG adsorption while enabling BSA elution. Building on these model protein findings, the following sections investigate the chromatographic behaviour of the Cu(II)-resin with human plasma under the optimised buffer conditions identified above.

Effect of the buffer (complex-MOPS) on the desorption of plasma proteins

A 0.5 mL sample of human plasma was diluted 1/5 in Tris-HCl (5 mM, pH 8). Adsorption and equilibration were performed in Tris-HCl buffer (5 mM, pH 8), and elution was carried out with buffers at the same pH but with different NaCl concentrations (35, 50, 100, and 200 mM), while fixing the concentrations of Tris-HCl at 10 mM, imidazole at 10 mM, and MOPS at 10 mM. Washing was done with NaOH (200 mM, Ph ≈ 13.3). The results of human plasma fractionation on Cu(II) resin are shown in Figure 8.

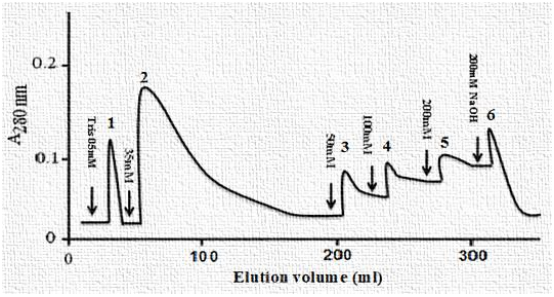

Figure 8.
Elution of human plasma on Cu(II) resin by varying NaCl concentration and fixing the concentrations of the other three components. Peak 1: obtained with the adsorption buffer Tris-HCl (5 mM). Peak 2: 35 mM. Peak 3: 50 mM. Peak 4: 100 mM. Peak 5: 200 mM. Peak 6: 200 mM NaOH

The first peak, obtained with the adsorption buffer (Tris-HCl 5 mM), represents non-adsorbed proteins with a concentration of 5.7 g/L, and the corresponding electrophoresis revealed two dense bands and two lighter bands (well 1 in Figure 9). The second peak represents a partially enriched albumin fraction (wells 2 - 5 in Figure 9), recovered in four 20 mL tubes. After concentration by PEG, the average obtained

is 0.962 g/L. No trace was obtained (wells 7, 8, and 9 in Figure 9) for the 50, 100, and 200 mM concentrations, as the protein concentration was below the detection threshold (Table III). The elution with the 200 mM NaOH wash solution represents proteins strongly bound to the resin that could not be desorbed by the various elution buffers; this fraction likely contains denatured and non-specifically bound proteins and should not be considered as a biologically active product. The concentration of this eluate is 4.86 g/L.

A total of 3.90 mg of partially enriched albumin fraction was recovered in eluted fractions (35 - 200 mM NaCl steps) from human plasma using the elution buffer (10 mM Tris-HCl, 10 mM imidazole, 10 mM MOPS, 35 mM NaCl), representing 19.5% of the theoretical HSA input (20 mg, based on 0.5 mL plasma containing approximately 40 g/L albumin). When including the non-adsorbed flow-through fraction (11.40 mg), the total protein accounted for was 7.71 mg (38.6%) of the theoretical input, excluding NaOH wash fractions. Similar results were obtained using IDA-Cu(II) resin, as reported by König and Skerra (1998) and Kovac and collaborators (2008) (14, 15), confirming that the binding mechanism is conserved across different chelating matrices. However, it must be acknowledged that the SDS-PAGE profiles of fractions eluted at higher NaCl concentrations revealed multiple protein bands, indicating incomplete selectivity under these conditions.

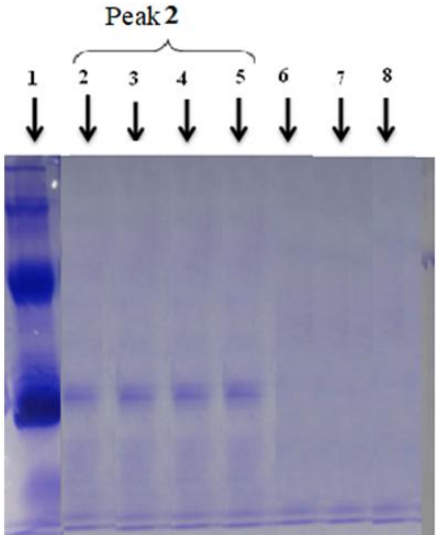

Figure 9.
Electrophoresis (SDS-PAGE) of enriched fraction of human plasma by varying NaCl concentration**.** Well 1: fraction of non-adsorbed proteins. Wells 2, 3, 4, and 5: the four fractions corresponding to peak 2. Well 6: fraction from 50 mM NaCl. Well 7: fraction from 100 mM. Well 8: fraction from 200 mM

Table III

Results of plasma protein quantification by varying NaCl concentration and fixing the concentrations of the other three components

Condition	Concentration (g/L)	Mass recovered (mg)	Recovery (%)
Adsorption buffer	5.70 ± 0.29	11.40 ± 0.58	—
35 mM NaCl (n = 4)	0.96 ± 0.26	1.92 ± 0.52	9.6 ± 2.6
50 mM NaCl	0.392 ± 0.039	0.78 ± 0.08	3.9 ± 0.6
100 mM NaCl	0.371 ± 0.037	0.74 ± 0.07	3.7 ± 0.6
200 mM NaCl	0.228 ± 0.023	0.46 ± 0.05	2.3 ± 0.4
200 mM NaOH	4.86 ± 0.24	9.72 ± 0.48	—
Total HSA (eluted fractions only)	—	**3.90 mg**	**19.5%**

Mass recovered (mg) = Concentration (g/L) × 0.002 L × 1000. Theoretical input: HSA = 20 mg (0.5 mL plasma × 40 g/L); IgG = 10 mg (0.5 mL plasma × 10 g/L). NaOH fractions are excluded from the yield calculation. Values are expressed as mean ± SD for n = 3 independent donor replicates. "n" in the Condition column = number of fraction tubes *per* elution step within a single run.

Based on the experimental data, the apparent binding capacity of the Cu(II)-resin can be estimated. A maximum of approximately 13.57 mg of total protein was recovered *per* run from a 7.85 mL bed volume, yielding a minimum apparent capacity of approximately 1.7 mg protein/mL resin under the conditions used. However, the dynamic binding capacity at the operating flow rate of 0.5 mL/min (linear velocity ~ 6.4 cm/h) is expected to be lower and was not directly determined in the present study. Future work will include frontal analysis experiments to establish both static and dynamic binding capacities under the optimised buffer conditions identified here.

Effect of imidazole with phosphate buffer

BSA/IgG Injection. According to Gaberc-Porekar and Menart (2001) (6) as well as Prasanna and Vijayalakshmi (2010) (16), with modifications, was performed with 30 mM phosphate buffer and 5 mM imidazole. Washing was carried out with a 200 mM NaOH buffer (Figure 10). The result from the wash with the adsorption buffer (10 mM phosphate buffer) shows that no UV-absorbing peak was detected in the flow-through fraction, suggesting that the applied BSA was largely retained by the resin. The peak obtained with the elution buffer (30 mM phosphate buffer and 5 mM imidazole) allowed recovery of BSA in 2 tubes of 20 mL each, with an average of 0.97 mg/mL after concentration by PEG.

Applying the same buffer at the same concentration (2 mg/mL) for IgG also yielded a peak overlap. Two tubes of 20 mL each were recovered, with an average of 0.9501 mg/mL after concentration by PEG. The

co-elution of both BSA and IgG under these conditions indicates insufficient selectivity, and further buffer optimisation is required to achieve separation of the two proteins.

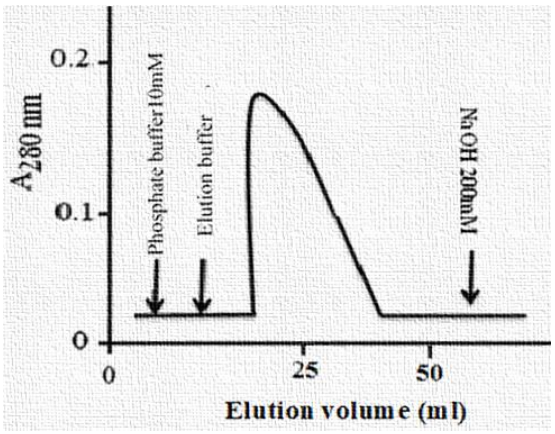

Figure 10.
BSA Elution. Peak 1: obtained with the elution buffer (30 mM phosphate buffer, 5 mM imidazole)

Injection of the mixture (BSA-IgG). A mixture of 4 mg/ml (2 mg BSA and 2 mg IgG) was injected into the column. The elution was initially carried out with the elution buffer (30 mM phosphate buffer and 5 mM imidazole), followed by varying the concentration of imidazole (Figure 11).

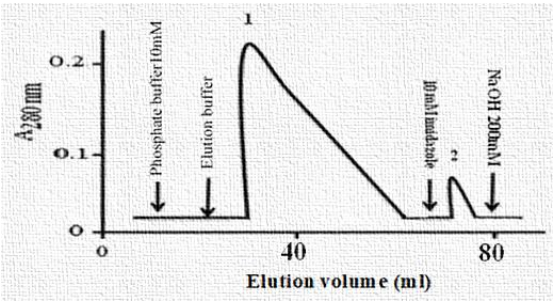

Figure 11.
Elution of the BSA-IgG mixture on Cu(II) resin by varying the imidazole concentration. Peak 1: elution buffer fraction (30 mM phosphate buffer and 5 mM imidazole). Peak 2: 10 mM imidazole

The wash with the adsorption buffer (10 mM phosphate buffer) showed that no UV-absorbing peak was detected in the flow-through fraction, suggesting that the BSA-IgG mixture was largely retained by the resin. The peak obtained with the elution buffer (30 mM phosphate buffer and 5 mM imidazole) eluted both proteins simultaneously (Figure 12), with an average eluate concentration of 1.08 g/L over 3 fractions after concentration by PEG. The eluate from 30 mM phosphate and 10 mM imidazole contained 0.725 g/L of BSA alone (Figure 12), demonstrating that a step-elution gradient based on imidazole concentration can achieve partial separation of BSA from IgG.

The competitive displacement of Cu^{2+} coordination by increasing imidazole concentrations provides a mechanistic explanation for these observations. At 5 mM imidazole, both BSA and IgG are co-eluted. At 10 mM imidazole, the increased competitive pressure selectively displaces the remaining BSA-Cu^{2+} interactions. This sequential elution confirms the utility of imidazole gradient strategies for partial fractionation (17), though complete separation of the two proteins under these conditions was not achieved.

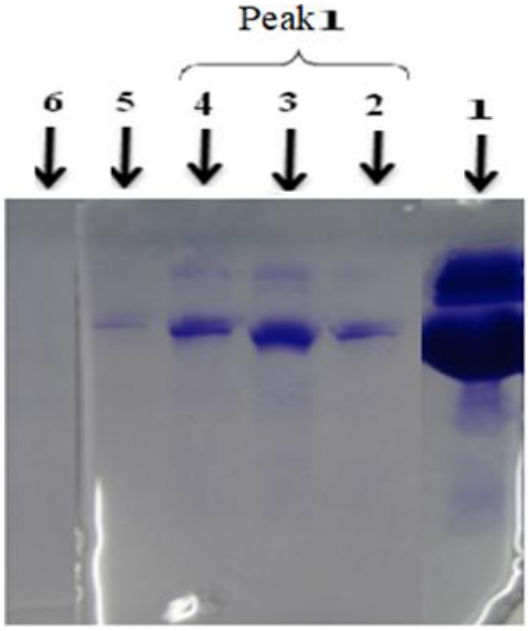

Figure 12.
Electrophoresis (SDS-PAGE) of the BSA-IgG mixture. Well 1: BSA and IgG. Wells 2, 3, and 4: the three recovered fractions corresponding to peak 1. Well 5: the 10 mM imidazole fraction. Well 6: the 200 mM NaOH fraction

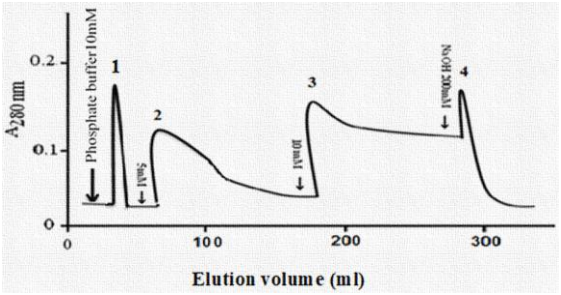

Figure 13.
Elution of human plasma on Cu(II) resin by varying the imidazole concentration while keeping the phosphate buffer concentration fixed at 30 mM. Peak 1: obtained with the adsorption buffer (10 mM phosphate buffer). Peak 2: 5 mM imidazole. Peak 3: 10 mM imidazole. Peak 4: 200 mM NaOH.

Plasma proteins

A 0.5 mL sample of human plasma was diluted 1:5 in 10 mM phosphate buffer. Elution was carried out

with buffers containing different concentrations of imidazole (5 and 10 mM), while keeping the phosphate buffer concentration at 30 mM. The wash was performed with 200 mM NaOH (Figure 13).

Peak 1, obtained with the adsorption buffer (10 mM phosphate buffer), represents non-adsorbed proteins with a concentration of 4.81 g/L. Peak 2 yielded five tubes of 20 mL each, with an average protein concentration of 1.265 g/L *per* tube after concentration by PEG (Table IV). According to the electrophoresis traces, the partially enriched protein fractions are consistent with the expected molecular weights of albumin (~ 67 kDa) and IgG heavy chain (~ 50 kDa) (wells 1 - 5 in Figure 14). A dense albumin band and a very faint band consistent with IgG (well 6) were obtained with 10 mM imidazole. A total of 3.58 mg of partially enriched protein fractions (HSA and IgG) was recovered in eluted fractions (5 and 10 mM imidazole steps), representing 11.9% of the combined theoretical input (30 mg: 20 mg HSA + 10 mg IgG). When including the non-adsorbed flow-through fraction (9.62 mg), the total protein accounted for was 13.20 mg (44%) of the theoretical input, excluding NaOH wash fractions. The previously reported value of 79% was arithmetically inconsistent and has been corrected.

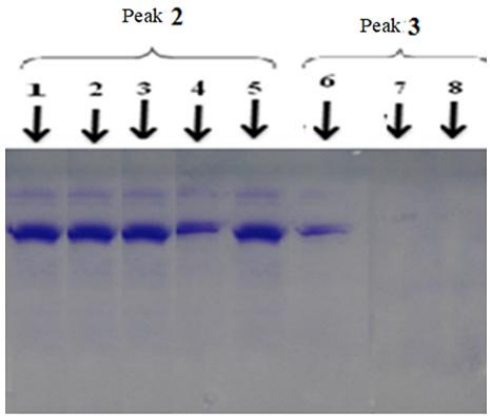

Figure 14.
Electrophoresis (SDS-PAGE) of protein fractions isolated from human plasma by variation in the concentration of imidazole. The 5 fractions recovered from peak 2 are presented in wells 1, 2, 3, 4, and 5. The fractions from peak 3 are presented in wells 6, 7, and 8

Table IV

Results of the determination of protein fractions isolated from human plasma by variation in the concentration of imidazole, with the phosphate buffer concentration fixed

Condition	Concentration (g/L)	Mass recovered (mg)	Recovery (%)
Adsorption buffer	4.810 ± 0.241	9.62 ± 0.48	—
5 mM (n = 5)	1.266 ± 0.131	2.53 ± 0.26	12.66 ± 1.31
10 mM (n = 3)	0.525 ± 0.223	1.05 ± 0.45	5.25 ± 2.23
200 mM NaOH	2.304 ± 0.115	4.61 ± 0.23	—
Total HAS + IgG (eluted fractions only)	—	3.58 mg	11.9% of 30mg
Total incl. flow-through (excl. NaOH)	—	13.20mg	44% of 30mg

Mass recovered (mg) = Concentration (g/L) × 0.002 L × 1000. Theoretical input: HSA = 20 mg (0.5 mL plasma × 40 g/L); IgG = 10 mg (0.5 mL plasma × 10 g/L). NaOH fractions are excluded from the yield calculation. Values are expressed as mean ± SD for n = 3 independent donor replicates. "n" in the Condition column = number of fraction tubes *per* elution step within a single run.

The mild elution conditions employed in this study: low imidazole concentrations (5 - 10 mM), physiological pH (8.0), and moderate ionic strength, are generally considered compatible with the structural integrity of plasma proteins, as documented across multiple IMAC studies using similar buffer systems (5, 6, 17). Nevertheless, a systematic assessment of HSA ligand-binding capacity and IgG antigen-binding activity following IMAC processing would represent a valuable addition to the characterisation of the enriched fractions, particularly in view of potential therapeutic or diagnostic applications, and is planned for future work.

A legitimate concern in any Cu(II)-based chromatographic system is the potential for copper-induced protein aggregation, particularly through oxidative cross-linking of cysteine residues or copper-catalysed oxidation of histidine and tryptophan side chains (18). However, several features of the present experimental design minimize this risk. First, all chromatographic steps were conducted at pH 8.0, a condition that does not favour free radical generation *via* Fenton-type chemistry, which is maximal at acidic pH (18). Second, the processing temperature was maintained at 25 ± 1°C throughout, well below the thermal unfolding temperatures of both HSA (Tm ≈ 56 - 67°C) (19) and IgG (Tm ≈ 71°C for the Fc domain) (20). Third, the buffer systems employed are devoid of reducing agents or oxidants that could catalyse metal-mediated protein modification. Finally, the tridentate coordination geometry of the Schiff base chelating ligand is expected to maintain Cu^{2+} firmly immobilized on the resin surface, limiting the availability of free Cu^{2+} ions in solution, a prerequisite for copper-catalysed oxidative damage. While a formal aggregation assay (*e.g.*, dynamic light scattering or analytical size-exclusion chromatography) was not performed in this proof-of-concept study, the mild and controlled conditions described above are consistent with protocols widely reported to preserve protein structural integrity in Cu(II)-IMAC applications (13, 21).

Effect of the (complex-NaCl) buffer on the desorption of plasma proteins

Elution was performed with buffers containing different NaCl concentrations (35 and 60 mM), while keeping the phosphate buffer concentration at 30 mM and imidazole at 5 mM (22, 23). The results of the fractionation of human plasma are presented in Figure 15.

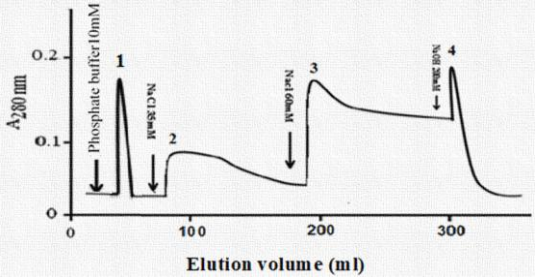

Figure 15.

Influence of NaCl concentration on the desorption of plasma proteins. Peak 1: obtained with the adsorption buffer (10 mM phosphate buffer). Peak 2: 35 mM NaCl. Peak 3: 60 mM NaCl. Peak 4: 200 mM NaOH

Peak 1, obtained with the adsorption buffer (10 mM phosphate buffer), represents non-adsorbed proteins with a concentration of 4.390 g/L. According to electrophoresis, three dense bands and three faint bands can be observed (Figure 16). The second peak represents a partially enriched albumin fraction (wells 2 - 4 in Figure 16), recovered in three fractions with an average albumin concentration of 1.083 g/L *per* tube after concentration by PEG (Table V). The two fractions with 60 mM NaCl concentration showed several bands of different proteins (wells 6 and 7), including a band consistent with the expected molecular weight of IgG.

A critical consideration in IMAC-based plasma protein enrichment is the distinction between specific metal-coordinative interactions and non-specific binding arising from hydrophobic, electrostatic, or ionic exchange mechanisms (15, 17). Human plasma contains hundreds of proteins, many of which possess surface-exposed histidine, cysteine, or tryptophan residues capable of interacting with immobilised Cu^{2+} (5, 6). The high total protein load of plasma (60 - 80 g/L) inevitably leads to competitive binding at the resin surface, and the selectivity of any given elution condition reflects a complex balance between these competing interactions (15, 24). The addition of NaCl served the dual purpose of reducing non-specific electrostatic interactions by increasing ionic strength and potentially modulating the hydration shell of both the protein surface and the metal coordination sphere (22, 23). Nevertheless, the SDS-PAGE profiles of fractions eluted at 60 mM NaCl revealed multiple protein bands, indicating incomplete selectivity under these conditions, consistent with the well-documented challenge of achieving selective protein enrichment from complex biological matrices by IMAC (17).

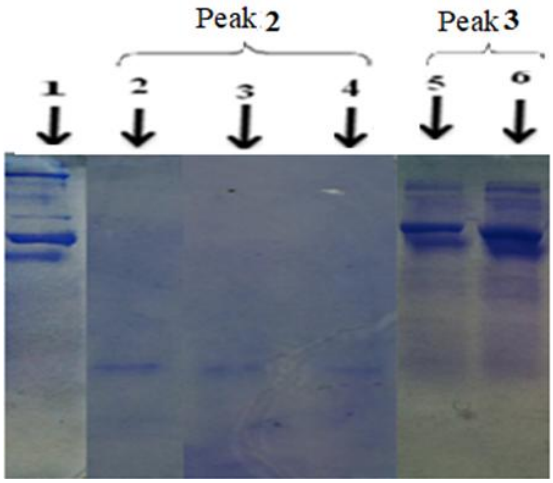

Figure 16.

Electrophoresis (SDS-PAGE) of protein fractions isolated from human plasma by varying the NaCl concentration, while keeping the imidazole and phosphate buffer concentrations fixed. The fraction from the adsorption buffer is presented in well 1. The fractions from peak 2 are presented in wells 2, 3, and 4. The two fractions from peak 3 are presented in wells 5 and 6

It must be acknowledged that SDS-PAGE, while an essential and widely accepted first-line analytical method for protein characterisation, does not by itself confirm the mechanism responsible for selective protein retention. In particular, SDS-PAGE cannot distinguish between histidine-driven metal coordinative interactions and non-specific electrostatic contributions arising from the ionic character of the resin surface at the low ionic strengths employed in this study (I = 5 - 55 mM for several buffer systems). At these ionic strengths, electrostatic interactions between the positively charged Cu^{2+}-bearing resin and negatively charged protein surfaces, both HSA and IgG, have isoelectric points below pH 8.0, may contribute meaningfully to protein retention (6, 24). The increasing NaCl concentration used as an elution modifier in several experiments was intended to progressively reduce these non-specific electrostatic contributions, but does not permit their complete decoupling from specific coordinative interactions. To rigorously assign the observed retention behaviour to histidine-mediated Cu^{2+} coordination, complementary biophysical experiments are warranted. Isothermal titration calorimetry (ITC) would directly quantify the thermodynamic parameters (ΔH, ΔS, Kd) of the HSA–Cu^{2+} and IgG–Cu^{2+} interactions in solution, providing binding constants and stoichiometry consistent or not with specific coordinative binding (11). Surface plasmon resonance (SPR) would offer real-time kinetic characterisation of the protein–metal

interaction at the solid phase surface (21). These experiments are identified as high-priority future work and will allow a mechanistically grounded interpretation of the selectivity patterns observed in the present study.

Table V

Results of the protein fractions isolated from human plasma assay by varying the NaCl concentration, while keeping the phosphate buffer and imidazole concentrations fixed

Condition	Concentration (g/L)	Mass recovered (mg)	Recovery (%)
Adsorption buffer	4.390 ± 0.220	8.78 ± 0.44	—
35 mM NaCl (n = 3)	1.083 ± 0.263	2.17 ± 0.53	10.9 ± 2.6
60 mM NaCl (n = 2)	1.768 ± 0.146	3.54 ± 0.29	17.7 ± 1.4
200 mM NaOH	2.734 ± 0.137	5.47 ± 0.27	—
Total HSA		13.57 mg	67.8%

Mass recovered (mg) = Concentration (g/L) × 0.002 L × 1000. Theoretical input: HSA = 20 mg (0.5 mL plasma × 40 g/L); IgG = 10 mg (0.5 mL plasma × 10 g/L). NaOH fractions are excluded from the yield calculation. Values are expressed as mean ± SD for n = 3 independent donor replicates. "n" in the Condition column = number of fraction tubes *per* elution step within a single run.

A total of 5.71 mg of partially enriched albumin fraction was recovered in eluted fractions (35 and 60 mM NaCl steps) from human plasma using the elution buffer (30 mM phosphate buffer, 35 mM NaCl, and 5 mM imidazole), representing 28.6% of the theoretical HSA input (20 mg, based on 0.5 mL plasma containing approximately 40 g/L albumin). When including the non-adsorbed flow-through fraction (8.78 mg), the total protein accounted for was 13.57 mg (67.8%) of the theoretical input, excluding NaOH wash fractions. Similar results have been obtained using Agarose-TREN gel (25) and Ni(II)-TRENPEVA (26), confirming the generalisability of the IMAC approach for HSA partial enrichment across different chelating matrices and metal ions. These results are consistent with those reported by König and Skerra (1998) (14) and Kovac and collaborators (2008) (15) using IDA-Cu(II) supports, and suggest that the interaction of HSA with Cu(II)-IMAC supports is attributable to the conserved ATCUN motif rather than to the specific architecture of the chelating ligand. A comprehensive review of HSA purification methods by Raoufinia *et al*. (2017) (27) confirmed that IMAC-based approaches represent a cost-effective alternative to ion exchange and affinity chromatography for HSA enrichment from plasma, particularly at the laboratory scale.

The moderate yields obtained in this study reflect a deliberate trade-off between operational simplicity and recovery, consistent with the "selective capture" paradigm increasingly advocated in the plasma fractionation literature (27, 28). The primary value proposition of the present Cu(II)-IMAC system lies in its capacity to achieve partial selective capture of HSA and IgG from a complex biological matrix in a single chromatographic step under mild conditions, using a cost-effective and chemically stable support. In research settings where moderate yield is an acceptable trade-off for operational simplicity, for instance, in biomarker studies, small-scale diagnostics development, or as a first capture step before downstream polishing, the recoveries reported here represent a practically useful outcome. Capture efficiency and selectivity at moderate purity are deliberately prioritised over maximal yield, and achieving higher purity suitable for clinical-grade applications would require additional polishing steps, which is acknowledged as a planned extension of this work.

The tridentate coordination geometry of the Schiff base ligand is expected to confer strong Cu^{2+} chelation stability, thereby minimising metal ion leaching into product fractions under the mild elution conditions employed in this study. This represents a potential advantage of the present resin architecture compared with conventional IDA-based supports, which are known to exhibit higher metal leaching due to their weaker tridentate coordination (5, 13). Nevertheless, quantitative determination of residual Cu^{2+} in product fractions by AAS or ICP-MS is planned as part of future process characterisation work, in accordance with the elemental impurity limits defined by regulatory guidelines (ICH Q3D) for parenteral products.

Effect of the (complex 2-MOPS) buffer on the desorption of plasma proteins

Elution was performed with buffers containing different imidazole concentrations (5, 10, 15, 20, and 25 mM), while keeping the phosphate buffer concentration at 30 mM and the MOPS concentration at 5 mM (29). The results of the fractionation of human plasma are presented in Figure 17.

The first peak is obtained with the adsorption buffer (10 mM phosphate buffer), containing non-adsorbed proteins with a concentration of 4.598 g/L. According to their traces in electrophoresis, four dense bands and three faint bands can be observed (Figure 18). The second and third peaks (5 and 10 mM imidazole) represent a partially enriched IgG fraction (wells 2 - 6 in Figure 18), recovered in five 20 mL tubes. After concentration by PEG, the average concentration recovered in each tube is 0.6 g/L (Table VI). The 15, 20, and 25 mM imidazole concentrations did not yield a fraction consistent with a single protein; their electrophoresis profiles show several bands of different molecular weights. The NaOH (200 mM) wash fraction, with a concentration of 2.946 g/L, likely represents denatured and non-specifically bound proteins

and should not be considered as a biologically active product.

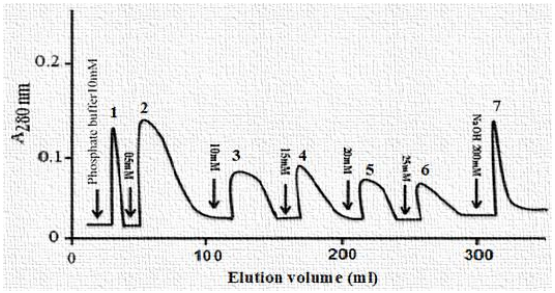

Figure 17.

The influence of imidazole concentration on the desorption of plasma proteins by adding MOPS (5 mM). Peak 1: obtained with the adsorption buffer (10 mM phosphate buffer). Peak 2: 5 mM. Peak 3: 10 mM. Peak 4: 15 mM. Peak 5: 20 mM. Peak 6: 25 mM. Peak 7: 200 mM NaOH

At 5 - 10 mM imidazole, the competitive displacement of IgG-Cu^{2+} coordinative bonds, primarily mediated by histidine residues exposed on the antibody surface, is favoured over the displacement of BSA-Cu^{2+} interactions, which are dominated by the high-affinity N-terminal ATCUN motif (Amino Terminal Cu(II)- and Ni(II)-binding motif) (30). The ATCUN motif forms square-planar complexes with Cu^{2+}, exhibiting significantly higher stability constants than typical histidine-mediated coordination, thereby requiring stronger competitive agents or higher imidazole concentrations for displacement (31).

This interpretation is consistent with the observation that serum albumins, such as HSA and BSA, exhibit stronger binding to immobilized Cu^{2+} due to their high-affinity N-terminal metal-binding site, which has been extensively characterized and shown to dominate metal affinity under physiological conditions (11, 12). Consequently, proteins lacking such high-affinity motifs, such as IgG, are more readily displaced under mild competitive conditions. Similar trends have been observed in immobilized metal affinity chromatography (IMAC), where proteins with surface-exposed histidine clusters elute at lower imidazole concentrations compared to those with multiple high-affinity coordination sites (31, 32).

A total of 2.40 mg of partially enriched IgG fraction was recovered in selectively eluted fractions (5 and 10 mM imidazole steps) from human plasma using the elution buffer (10 mM phosphate buffer, 5 mM MOPS, 5 or 10 mM imidazole), representing 24% of the theoretical IgG input (10 mg, based on 0.5 mL plasma containing approximately 10 g/L IgG). Fractions eluted at higher imidazole concentrations (15 - 25 mM) were excluded from this calculation as their SDS-PAGE profiles revealed multiple protein bands, indicating loss of selectivity and co-elution of non-IgG proteins.

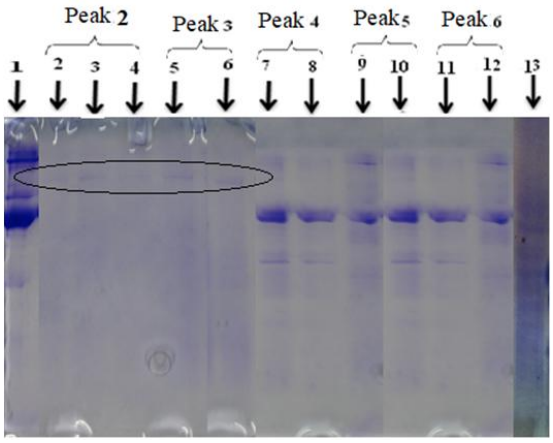

Figure 18.

Electrophoresis (SDS-PAGE) of protein fractions isolated from human plasma by varying the imidazole concentration, while keeping the MOPS and phosphate buffer concentrations fixed. The fraction from the adsorption buffer is presented in well 1. The fractions from peak 2 are presented in wells 2, 3, and 4. The fractions from peak 3 are presented in wells 5 and 6. The fractions from peak 4 are presented in wells 7 and 8. The fractions from peak 5 are presented in wells 9 and 10. The fractions from peak 6 are presented in wells 11 and 12. Well 13: fraction from the 200 mM NaOH

Table VI

Results of the protein fractions isolated from human plasma assay by varying the imidazole concentration, while keeping the phosphate buffer and MOPS concentrations fixed

Condition	Concentration (g/L)	Mass recovered (mg)	Recovery (%)
Adsorption buffer	4.598 ± 0.230	9.20 ± 0.46	—
5 mM (n = 3)	0.597 ± 0.022	1.19 ± 0.04	11.9 ± 0.4
10 mM (n = 2)	0.604 ± 0.020	1.21 ± 0.04	12.1 ± 0.4
15 mM (n = 2)	1.565 ± 0.142	3.13 ± 0.28	31.3 ± 2.8
20 mM (n = 2)	1.221 ± 0.010	2.44 ± 0.02	24.4 ± 0.2
25 mM (n = 2)	1.136 ± 0.027	2.27 ± 0.05	22.7 ± 0.5
200 mM NaOH	2.946 ± 0.147	5.89 ± 0.29	—
Total IgG (5 + 10 mM fractions)	—	2.40 mg	24% of 10 mg

Theoretical input: IgG = 10 mg (0.5 mL × 10 g/L). NaOH fractions are excluded from the yield calculation. Values are expressed as mean ± SD for n = 3 independent donor replicates. "n" in the Condition column = number of fraction tubes *per* elution step within a single run.

The selective recovery of IgG at low imidazole concentrations (5 - 10 mM) in the presence of MOPS is one of the most significant findings of this study. As shown in Table VI and Figure 18, the fractions eluted at 5 and 10 mM imidazole yielded consistent protein concentrations (0.575 - 0.632 g/L), with SDS-PAGE profiles consistent with the expected molecular weight of IgG heavy chain (~ 50 kDa). By contrast, fractions eluted at higher imidazole concentrations (15 - 25 mM) showed multiple bands of different molecular weights, indicating that increasing imidazole concentration progressively disrupts the selectivity of the system by competitively displacing a broader range of metal-bound proteins. This observation is consistent with the well-established role of imidazole as a competitive ligand for Cu^{2+} coordination sites in IMAC systems (5, 6).

The role of MOPS in this buffer system appears to be critical. As a zwitterionic buffer with low metal-chelating capacity, MOPS modulates the ionic environment of the resin surface without directly competing for Cu^{2+} coordination sites. This selective modulation reduces non-specific electrostatic and hydrophobic interactions, shifting the balance towards specific coordinative binding (16, 24). As a result, at 5 - 10 mM imidazole, the competitive displacement of IgG-Cu^{2+} coordinative bonds, mediated by surface-accessible histidine residues in the Fab and Fc regions (26, 33), is favoured over the displacement of HSA-Cu^{2+} bonds, which are mediated by the higher-affinity ATCUN motif. This mechanistic interpretation is consistent with the SDS-PAGE profiles observed (Figure 18), which show a predominant band consistent with IgG in fractions eluted at 5 and 10 mM imidazole.

The multiple protein bands observed in SDS-PAGE profiles of eluted fractions are consistent with the well-documented behaviour of Cu(II)-IMAC systems when applied to complex biological matrices such as human plasma, which contains hundreds of proteins bearing surface-accessible histidine, cysteine, or tryptophan residues capable of interacting with immobilised Cu^{2+} (5, 6). Based on published proteomic characterisations of Cu(II)-IMAC plasma fractions (15), proteins such as haptoglobin, ceruloplasmin, and selected acute-phase proteins may be present in minor amounts in the eluted fractions alongside HSA and IgG. This is an inherent characteristic of IMAC-based enrichment from plasma and has been reported for all Cu(II)-IMAC matrices described in the literature (15, 17). Importantly, for the intended application of this resin, namely, partial enrichment as a first capture step before further purification, the presence of minor co-enriched proteins does not preclude its utility, as downstream polishing steps (*e.g.*, ion-exchange or size-exclusion chromatography) would be expected to achieve the required purity. Full characterisation of fraction composition by mass spectrometry-based proteomics is planned as a priority for future work and will allow a precise quantitative assessment of enrichment selectivity.

Inter-donor variability is an important parameter for any plasma-based purification process. In the present study, the SD values reported across three donors (Table III and Table VI) indicate moderate variability in protein recovery, with relative standard deviations typically in the range of 10 - 25%. This variability likely reflects differences in baseline plasma protein concentrations between donors, as well as minor differences in sample handling and freeze-thaw history. A systematic evaluation of inter-donor robustness using a larger and more demographically diverse donor cohort, with standardisation of protein input by pre-assay quantification, is planned for future work.

These results are consistent with those reported using IDA-Cu(II) (13, 26), TREN-Novarose and aminohexyl-agarose (28), and CIM-IDA-Cu(II) (15) supports, as well as with more recent studies using Cu(II)-chelated polymer membranes (33) and Cu(II)-PHEMA cryogels (30) for IgG recovery from human plasma, collectively confirming the feasibility of Cu(II)-IMAC-based IgG partial enrichment across a range of matrix architectures.

To properly contextualise the novelty of the present resin, a direct comparison with IDA-based Cu(II)-IMAC systems incorporating MOPS as a buffer component is warranted. König and Skerra (1998) (14) demonstrated selective IgG purification using IDA-Cu(II)-Sepharose with MOPS-containing buffers, reporting recoveries of approximately 30 - 40% with moderate purity. Kovac *et al*. (2008) (15) using CIM-IDA-Cu(II) monolithic supports also employed MOPS in elution buffers to modulate non-specific interactions, achieving IgG-enriched fractions from plasma at comparable purity levels. The present results for IgG partial recovery (24% of theoretical input at 5 - 10 mM imidazole in a phosphate/MOPS system) are therefore broadly consistent with these literature reports, confirming that the MOPS-mediated ionic modulation strategy is effective across different chelating matrix architectures. However, several features distinguish the present Schiff base-functionalized resin from these IDA-based systems. First, the tridentate coordination geometry of the N,N-bis(salicylidenepropylenetriamine) ligand is expected to provide significantly higher Cu^{2+} chelation stability than IDA, which is known for its tendency to leach metal ions under elution conditions (4, 13). Second, the polystyrene backbone of the present resin confers greater chemical resistance than agarose-based supports, particularly at extreme pH values used during regeneration (5). Third, the resin is prepared from relatively low-cost starting materials through a straightforward synthesis procedure, offering potential cost advantages for scale-up. While the present proof-of-

concept study does not include a head-to-head comparison with commercial IDA-Cu(II) matrices under identical conditions, an acknowledged limitation that will be addressed in future work, the consistency of the present results with the existing IDA-MOPS literature supports the conclusion that the novel Schiff base resin represents a viable and potentially advantageous alternative for Cu(II)-IMAC-based plasma protein enrichment.

The present Cu(II)-IMAC strategy based on a novel Schiff base-functionalized polystyrene resin offers several noteworthy characteristics compared with conventional approaches, such as cold ethanol (Cohn) fractionation and Protein A affinity chromatography (2, 3), including lower ligand cost, greater chemical stability, and simpler regeneration procedures (26, 28).

A brief comparison with established methods provides useful context. Cold ethanol (Cohn) fractionation typically yields HSA at 85 - 95% purity, but requires multi-step processing at subzero temperatures, generates fractions of variable quality, and involves extensive downstream polishing. Protein A affinity chromatography achieves IgG purity exceeding 95% in a single step, but involves high ligand costs, limited reusability, and risk of immunogenic ligand leaching. Ion-exchange chromatography offers a lower-cost alternative but requires extensive buffer optimisation and multiple steps to achieve comparable purity. The Cu(II)-IMAC approach presented here yields partially enriched HSA fractions (19.5 - 28.6% of theoretical input in eluted fractions, or 38.6 - 67.8% when including the flow-through fraction) and IgG fractions (24% of theoretical input in selectively eluted fractions) from plasma in a single chromatographic step under mild conditions, at significantly lower reagent cost. Its primary advantage lies in operational simplicity and cost-effectiveness; however, the current method yields partially enriched rather than highly pure fractions and would require additional polishing steps for clinical-grade applications.

Conclusions

This proof-of-concept study evaluated a novel Cu(II)-loaded Schiff base-functionalized polystyrene resin, N,N-bis(salicylidenepropylenetriamine)-aminomethyl polystyrene, as a chromatographic support for the partial enrichment of Human Serum Albumin (HSA) and Immunoglobulin G (IgG) from human plasma by Immobilized Metal Affinity Chromatography (IMAC). Partially enriched HSA fractions were obtained using buffer systems containing Tris-HCl or phosphate buffer supplemented with low concentrations of imidazole and NaCl, with a recovery of 19.5 - 28.6% of the theoretical HSA input in eluted fractions, or 38.6 - 67.8% when including the non-adsorbed flow-through fraction in the total protein accounted for, excluding NaOH wash fractions. Partially enriched IgG fractions were recovered using low imidazole concentrations (5 - 10 mM) in a phosphate/MOPS buffer system, with a recovery of 24% of the theoretical IgG input in selectively eluted fractions. Fractions eluted at higher imidazole concentrations (15 - 25 mM) were excluded from the IgG yield calculation due to loss of selectivity as evidenced by SDS-PAGE.

As is inherent to any proof-of-concept investigation, the present study was designed to establish feasibility rather than to provide exhaustive process characterisation, and the findings reported here represent a first and necessary step toward the development of a validated enrichment platform. Protein identity was assessed by SDS-PAGE, a well-established and widely accepted method for preliminary characterisation; confirmatory identification by Western blotting or mass spectrometry will further strengthen these findings. The functional activity of the recovered HSA and IgG fractions was not assessed in the present study, as the primary objective was chromatographic feasibility; activity assays are planned as part of the next phase of characterisation. Quantitative purity assessment by densitometry, residual Cu^{2+} quantification by AAS or ICP-MS, systematic multi-cycle reuse evaluation, and direct determination of dynamic binding capacity represent natural extensions of this work that will consolidate the process understanding established here. Finally, while the use of three independent donor replicates is appropriate for an exploratory study of this nature, expansion to a larger and more diverse donor cohort will be addressed in future confirmatory studies. Collectively, these planned investigations will build upon the solid proof-of-concept foundation established in the present work.

Overall, the present findings demonstrate for the first time the potential of this novel Schiff base-functionalized Cu(II)-chelating resin as an IMAC support for the partial enrichment of HSA and IgG from human plasma under mild buffer conditions, and define a clear roadmap for future investigations.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Ethics approval and consent to participate

This study was conducted in accordance with the ethical

principles of the Declaration of Helsinki and the ethical guidelines of the University Hospital Centre (CHU) of Sétif, Algeria. Blood samples were collected at the CHU of Sétif. Informed consent was obtained from all five healthy adult volunteers prior to blood collection. No personal identifiers are reported in this manuscript.

AI use disclosure

During the preparation of this manuscript, the authors used DeepL for translation from French to English and Claude (Anthropic) for improving spelling, grammar, word choice, readability, and clarity. All outputs generated by these tools were carefully reviewed and revised by the authors, who take full responsibility for the accuracy, integrity, and final content of the work.

Conflict of interest

The authors declare no conflict of interest.

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