

Speciation of Human Serum Proteins Based on Trace Metal Mapping Analysis by CIM Monolithic Disk Column HPLC/ICP-MS in Complement with Off-Line MALDI-TOF-MS Analysis

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A multiply hyphenated HPLC/UV/ICP-MS system equipped with a fraction collection valve for subsequent MALDI-TOF-MS analysis was constructed, and its applicability to the speciation of metalloproteins in human serum was evaluated. A weak anion-exchange CIM® monolithic disk column was employed for protein separation, in which gradient elution of proteins with volatile salts was carried out while taking into account the compatibility with ICP-MS instrument. As a result of optimization, representative serum proteins, such as γ-globulin (γGb), albumin (Ab), ceruloplasmin (Cp), transferrin (Tf), and α_2-macroglobulin (α_2Mgb), were separated on the disk column with a linear concentration gradient of CH_3COONH_4 in 20 mM Tris-HCl buffer (pH 7.4). These proteins, which were primarily assigned by UV absorption at 280 nm, were clearly identified with on-line multielement detection using ICP-MS as well as with off-line MALDI-TOF-MS analysis of each successive fraction collected. It was concluded that the present multiply hyphenated system will be useful for identifying proteins in biological samples.

Trace elements are well known to be essential for regulating various physiological functions in animals including human beings.[1–6] For instance, syntheses and metabolisms of biological compounds cannot be performed without the aid of particular metalloenzymes, in which metal ions act as cofactors. Biological-functions induced by metal ions also include nutritional, structural, and homeostatic supports. Thus, elemental speciation analysis has attracted sustained interest over the last few decades.[7–11] Recently, Haraguchi has proposed "metallomics" as the integrated biometal science to promote the research on metal-induced biological and physiological functions.[12] Because of the importance of biometals in biological, pharmaceutical, and environmental systems, metallomics has steadily and rapidly gained acceptance as a complementary research field to genomics and proteomics.[13–17]

It is estimated that metalloproteins constitute as much as 40% of proteomes, and the speciation of metals bound to proteins is a major challenge in current metallomics research.[12] Thus, a variety of hyphenated systems that combine high-resolution separation techniques with highly sensitive and selective detection methods have been exploited.[18–21] However, it is still difficult to identify and differentiate individual metal-binding proteins, because of the complexity of the matrices of biological fluids and cells as well as the coexistence of many different proteins in them.

For the implementation of such a metallomics research, the construction of more sophisticated systems incorporating several "state-of-the-art" techniques is desirable.[22,23] In particular, the combination of elemental and molecular mass spectrometric detections for metalloproteins appears indispensable. In the present paper, a multiply hyphenated system involving HPLC/UV/ICP-MS with the aid of off-line MALDI-TOF-MS is presented. It is also desirable that the separation time is as short as possible to reduce the risk of desorption and/or exchange of metals from proteins during the separation.[24] For this purpose, a highly permeable and short monolithic column may be the best.[25–27] Thus, a 3 mm-long CIM® (convective interaction media) disk column was employed in the present study, and the applicability and usefulness of the multiply hyphenated system were evaluated through the analysis of human serum proteins.[28,29] It was found that the cooperative use of the metal-profiling analysis of human serum proteins by ICP-MS and molecular weight measurements by MALDI-TOF-MS was a powerful tool for the identification of metalloproteins.

Experimental

Apparatus. The chromatographic separation of human serum proteins was performed on a CIM monolithic disk column (BIA Separations, Ljubljana, Slovenia). In the present experiment, a weak anion-exchange CIM® disk monolith (12 mm i.d. × 3 mm long, methacrylate-based monolithic support with diethylamine (DEAE) groups) was used. The disk column was connected to a gradient elution system consisting of two HPLC pumps (Model LC-20AD, Shimadzu, Kyoto, Japan) equipped with a sample injector with a 20-μL sample loop (Model 7725, Rheodyne, Cotati, CA, U.S.A.). A schematic diagram of the present experimental system is shown in Fig. 1. Proteins separated were monitored on-

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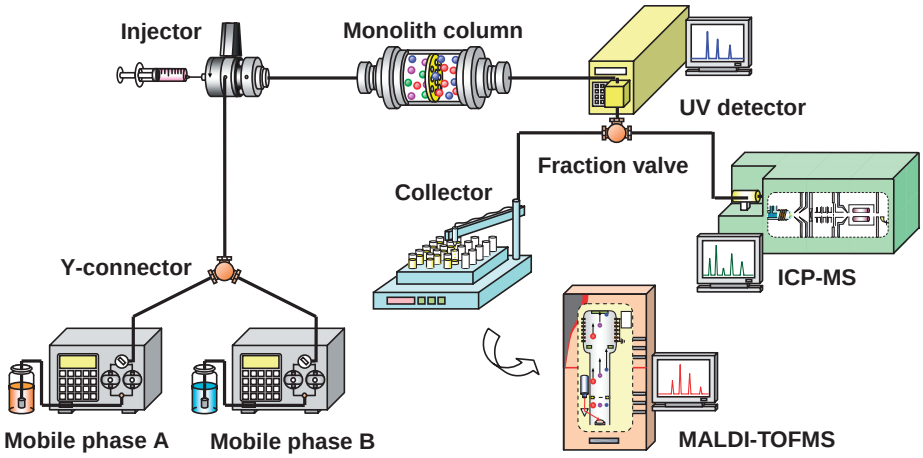

Fig. 1. Schematic illustration of a multiply hyphenated HPLC/UV/ICP-MS system with off-line MALDI-TOF-MS for inorganic and organic multi-measurements.

Table 1. Concentrations and Molecular Weights of Representative Proteins in Human Blood Serum

	Concentration in serum /μg mL^{-1}	Molecular weight /Da
Ab	40000	66000
IgG	12000	150000
α_2Mgb	4200	725000
α_1-Antitrypsin	4000	51000
Tf	3200	76000
Haptoglobin	3000	200000
IgA	2000	160000
IgM	1500	900000
Cp	600	132000

line with a UV absorption detector (Model 870-UV, Jasco, Tokyo, Japan) at 280 nm and an ICP-MS instrument (model HP-4500, Agilent Technologies, Yokogawa Analytical Systems, Tokyo, Japan). The operating conditions of the ICP-MS instrument were the same as described previously.[30] In the present system, a fraction collection valve was set after the UV absorption detector to continuously obtain the protein fractions (ca. 0.1 mL each) for molecular weight measurements by a MALDI-TOF-MS instrument (AXIMA-CFR plus, Shimadzu).

Reagents. Representative serum proteins, including γ-globulin (γGb), albumin (Ab), ceruloplasmin (Cp), transferrin (Tf), and α_2-macroglobulin (α_2Mgb), were purchased from Sigma (St. Louis, MO, U.S.A.). Concentrations and molecular weights of representative proteins in human blood serum are summarized in Table 1.[31,32] Sinapinic acid (SA) used as the matrix and trifluoroacetic acid (TFA) were obtained from Aldrich (St. Louis, MO, U.S.A.). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Merck (Darmstadt, Germany). All other reagents and solvents were of analytical-reagent grade or HPLC grade from Wako Pure Chemicals (Osaka, Japan) and were used as received. Throughout the present work, doubly de-ionized water (18.2 MΩ cm) obtained by a Milli-Q deionization system (model Element A-10, Nihon Millipore Kogyo, Tokyo, Japan) was used. The human serum certified reference material Seronorm Serum was purchased from Sero AS (Billingstad, Norway), and the serum sample was diluted 4-fold with 20 mM Tris-HCl (pH 7.4) before injection into the HPLC system.

Results and Discussion

Separation of Human Serum Proteins on CIM DEAE. A salt gradient elution with NaCl is commonly used for anion-exchange chromatographic separations of proteins, but NaCl has a deleterious effect on ICP-MS instrument due to clogging of the nebulizer. In order to prevent damage to the instrument, several volatile salts suitable for ICP-MS measurement, including NH_4Cl, CH_3COONH_4, and $HCOONH_4$, were tested as the alternative. A model mixture consisting of γGb, Ab, Cp, Tf, and α_2Mgb was prepared and used for optimizing the gradient elution conditions.

All the volatile salts tested (NH_4Cl, CH_3COONH_4, and $HCOONH_4$) and NaCl virtually provided quite similar separation performance to each other. The chromatogram for human serum obtained under the optimum elution conditions is shown in Fig. 2a. The chromatographic separation was performed with a linear gradient of CH_3COONH_4 in 20 mM Tris-HCl buffer (pH 7.4). The flow rate was set at a normal flow rate of 1.0 mL min^{-1}, and the pressure drop was as low as 0.4 MPa. The effluent was monitored with an UV absorption detector at 280 nm. As seen in Fig. 2a, five or more peaks with shoulders were observed. In general, the present CIM disk monolith has low flow resistance and shows efficient mass transfer, which makes it possible to use a high flow rate, resulting in rapid separation. Indeed, the disk column could be properly operated at a flow rate of up to 10 mL min^{-1} without significant loss of column efficiency.

The peaks in Fig. 2a could to some extent be assigned on the basis of the elution volume and the abundance of the known human serum proteins; however, human serum contains a variety of proteins over a wide concentration range. Thus, the peaks for low abundance proteins may be hidden by the peaks of major serum proteins. In order to identify low abundance proteins, in the present work, the effluent was also monitored by ICP-MS. Selected element-selective chromatograms are shown in Figs. 2b–2d, which were measured with ICP-MS at m/z of 65, 66, and 57 for Cu, Zn, and Fe, respectively. The respective peaks corresponding to those in Fig. 2a were marked with the same numbers. As can be seen in Figs. 2b–2d, each metal-selective chromatogram provided several characteristic

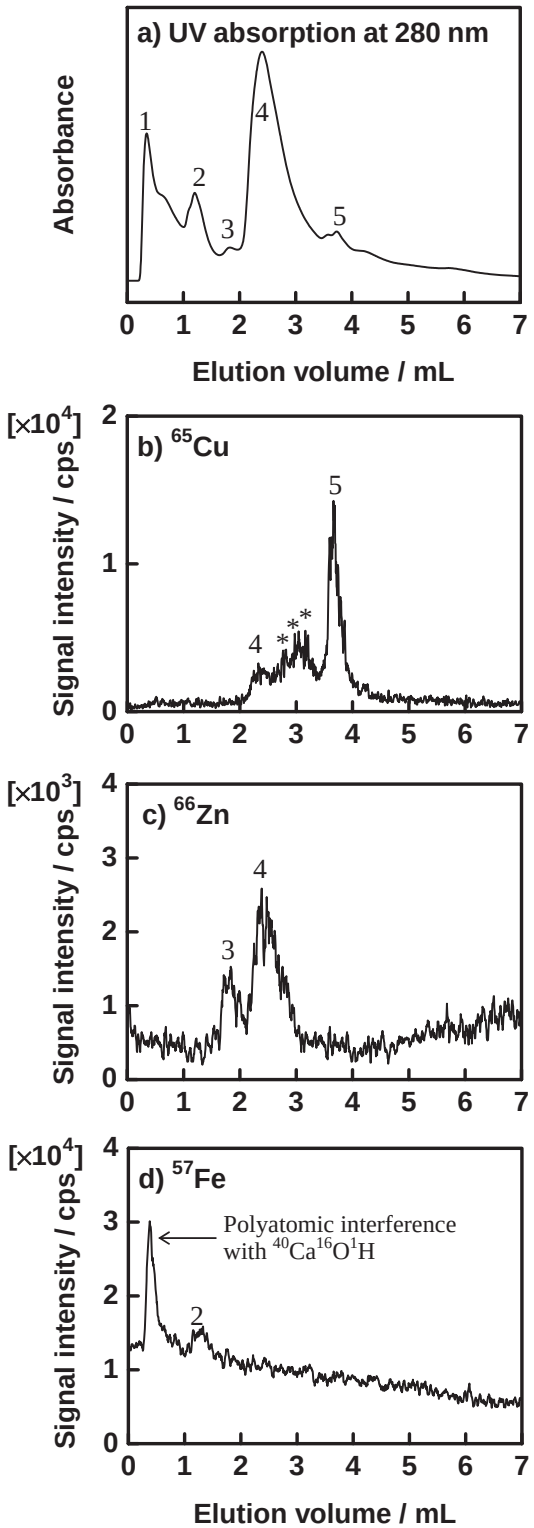

Fig. 2. Chromatograms of a human serum sample on 3 mm-long CIM DEAE monolithic disk column by a CH_3COONH_4 gradient elution. Column size: 3 mm long × 12 mm i.d.; mobile phase: solvent A (20 mM Tris-HCl, pH 7.4), solvent B (buffer A containing 1 M CH_3COONH_4); linear gradient: 0–70% B in 10 min; flow rate: 0.7 mL min^{-1}; Detection: (a) UV absorption at 280 nm, (b) ICP-MS detection at m/z of 65 for Cu, (c) m/z of 66 for Zn, and (c) m/z of 57 for Fe. Peaks: 1, γGb; 2, Tf; 3, α_2Mgb; 4, Ab; and 5, Cp.

peaks. For example, two peaks were observed in the Zn-selective chromatogram at elution volumes of 1.8 and 2.4 mL. With aid of the element-specific peaks, the former peak was confidently assigned to α_2Mgb (No. 3), while the latter peak was attributed to Ab-bound Zn (No. 4). The Cu-selective chromatogram had a large peak at an elution volume of 3.66 mL and broad split peaks in elution volume range of 2.2 to 3.4 mL. In a similar manner, the large Cu peak (No. 5) was assigned to Cp. The first peak (No. 4) of the split peaks was identified as Ab-bound Cu, because this peak corresponded to the No. 4 peak of Ab-bound Zn. The peaks marked with asterisk in Fig. 2b could not be assigned, but this result indicated the existence of several Cu-binding proteins. In the Fe-selective chromatogram, two peaks appeared at elution volumes of 0.45 and 1.25 mL. The latter peak (No. 2) was attributed to Tf, while the former peak was concluded to be from polyatomic interference ($^{40}Ca^{16}O^1H^+$) based on the result of Ca-selective chromatogram. From these results, it was confirmed that the present metal profiling analysis was valuable for making the element-specific fingerprintings for exploration of unknown proteins in future work.

Fraction Collection and Off-Line MALDI-TOF-MS Measurements. Information about molecular weight often provides decisive evidence for the identification of chemical species. In the present work, the effluent of the human blood serum was successively fractionated through a collection valve, and each fraction was subjected to MALDI-TOF-MS analysis. An aliquot of each fraction was mixed with a SA solution and loaded onto a MALDI substrate according to the manufacturer's instructions. The MALDI-TOF mass spectra of several representative fractions are presented in Figs. 3a–3f. As expected, major serum proteins, i.e., γGb, Ab, Cp, and Tf, were observed in the corresponding fractions. In the MALDI-TOF mass spectra, most proteins were detected as singly and doubly charged protonated molecular ions. In addition to the molecular ions, Ab was detected as a dimer. Unfortunately, α_2Mgb with high molecular weight was not detected under the present experimental conditions, probably due to its low ionization efficiency. It should be noted that several peaks were observed at m/z of 45000, 50000, 100000, 117000, 200000, etc. in the mass spectra. In order to explore these unknown peaks, mass spectrometric analysis and/or peptide mapping after enzymatic digestion are required, and such work is now in progress.

Conclusion

A multiply hyphenated system combining HPLC, UV, ICP-MS, and MALDI-TOF-MS was constructed and used to identify human serum proteins. It was found that representative serum proteins, such as γGb, Ab, Cp, Tf, and α_2Mgb, were roughly separated on a CIM DEAE monolithic disk column with a CH_3COONH_4 gradient elution. Owing to the sensitive and element-specific detection capability of ICP-MS, some of serum proteins could be identified. Also, the existence of several unknown Cu-binding proteins was suggested from the element-specific chromatograms. In other words, it was demonstrated that metal profiling analysis of proteins by ICP-MS and molecular weight assignment by MALDI-TOF-MS may be helpful to identify proteins in biological samples.

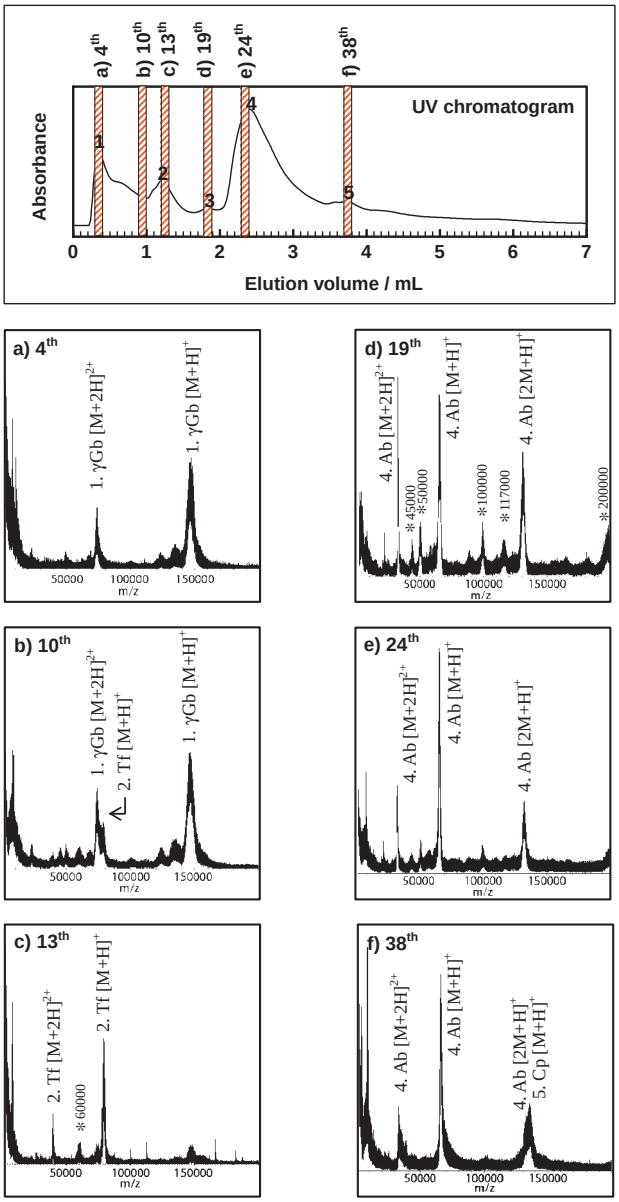

Fig. 3. MALDI-TOF mass spectra of fractions collected. A total of 70 fractions of ca 0.1 mL each were collected. An aliquot of each fraction was mixed with a SA solution and loaded on the substrate for MALDI-TOF-MS measurements. The mass spectra for the fractions of a) 4th, b) 10th, c) 13th, d) 19th, e) 24th, and f) 38th are shown.

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