Anal. Chem. **2002,** *74,* 626−631

Use of Surface-Modified Capillaries in the Separation and Characterization of Metallothionein Isoforms by Capillary Electrophoresis Inductively Coupled Plasma Mass Spectrometry

Zhixin Wang and Andreas Prange*

GKSS Research Center, Institute for Coastal Research/Physical and Chemical Analysis, Max-Planck-Strasse, D-21502 Geesthacht, Germany

The separation of metallothionein (MT) isoforms using capillary electrophoresis (CE) has been improved by applying surface-modified capillaries, and the metal composition of MTs has been characterized by subsequent inductively coupled plasma sector field mass spectrometry (ICPSFMS). Nine MT complexes in a commercial preparation from rabbit liver were successfully separated on an anionic polymer-coated column, prepared by immobilizing poly(2-acrylamido-2-methyl-1-propanesulfonic acid) on the fused-silica surface via a linking agent. On uncoated capillaries or those coated dynamically with cationic materials, only three complexes could be separated. On-line isotope dilution analysis combined with CE/ICPSFMS indicated the stoichiometric molar metal contents in the MT complexes.

Metallothionein (MT)[1] is a class of low molecular mass (6−7 kDa) and cysteine-rich protein that is involved in heavy metal metabolism and detoxification in living organisms due to its high metal-binding ability. Being the product of genetic polymorphism, MT occurs as a mixture of different isoforms, each of which contains a certain number of subisoforms with minor differences in amino acid sequences or metal-binding properties.[2] To establish the function and expression of individual MT genes, a powerful analytical technique with high resolution to different MT isoforms and subisoforms as well as selectivity to metals is required.

The hyphenation of capillary electrophoresis (CE) with inductively coupled plasma mass spectrometry (ICPMS) appears to be competent to this challenge. CE has been widely used for the separation of MT in both free[3] and micellar solutions[4] due to its high separation efficiency and small sample requirements. Surface-modified capillaries, usually coated with polyamine or linear polyacrylamide,[5,6] provide more or less enhanced resolution compared to uncoated ones by either adjusting the electroosmotic flow (EOF) or minimizing wall adsorption. ICPMS[7] has been favored in speciation analysis not only because of its multielement character and low detection limits but also because of its ability to perform isotope ratio measurements, thus enabling the isotope dilution analysis.[8]

The application of CE/ICPMS has made dramatic progress in MT analysis in recent years.[9] The metal complexes of two major MT isoforms, i.e., MT-1 and MT-2, were separated and element selectively detected by CE/ICPMS coupled via various interface designs.[10−12] In recent work,[12] MT isoforms containing MT-3 in human brain cytosols were determined, which might be helpful for the diagnosis of Alzheimer's disease. The introduction of the on-line isotope dilution technique into CE/ICPMS permitted the quantification of separated MT isoforms and the characterization of their metal compositions.[13] However, further improvement in the separation followed by characterization of more MT complexes is still desired.

The present work is aimed at improving the CE selectivity to MT using surface-modified capillaries and determining the metal compositions of individual MT complexes by ICP-sector field MS (ICPSFMS). Three different types of coated capillaries were prepared and evaluated for their ability to resolve MT isoforms. An anionic polymer-coated capillary proved to be the most effective and, hence, was chosen for the isotope dilution analysis.

EXPERIMENTAL SECTION

Chemicals and Materials. All the preparations of rabbit liver MT (MT, Lot 19H7812; MT-1, Lot 127H7810; MT-2, Lot 49H7822) were purchased from Sigma (St. Louis, MO). Polyethyleneimine

* Corresponding author: (e-mail) andreas.prange@gkss.de; (fax) + 49−4152−871875.

(1) Nordberg, M. *Talanta* **1998**, *46*, 243−254.
(2) Stillman, M. J.; Shaw, C. F.; Suzuki, K. T. *Metallothioneins Synthesis, Structure and Properties of Metallothioneins, Phytochelatins and Metalthiolate Complexes*; VCH: New York, 1992.
(3) Virtanen, V.; Bordin, G. *Anal. Chim. Acta* **1999**, *402*, 59−66.
(4) Beattie, J. H.; Richards, M. P. *J. Chromatogr., A* **1995**, *700*, 95−103.
(5) Richards, M. P.; Beattie, J. H. *J. Chromatogr., B* **1995**, *669*, 27−37.
(6) Minami, T.; Matsubara, H.; O-higashi, M. *J. Chromatogr., B* **1996**, *685*, 353−359.
(7) Sutton, K.; Sutton, R. M. C.; Caruso, J. A. *J. Chromatogr., A* **1997**, *789*, 85−126.
(8) Rottmann, L.; Heumann, K. G. *Fresenius' J. Anal. Chem.* **1994**, *350*, 221−227.
(9) Lobinski, R.; Chassaigne, H.; Szpunar, J. *Talanta* **1998**, *46*, 271−289.
(10) Mounicou, S.; Poleć, K.; Chassaigne, H.; Potin-Gautier, M.; Lobinski, R. *J. Anal. At. Spectrom.* **2000**, *15*, 1−8.
(11) Taylor, K. A.; Sharp, B. L.; Lewis, D. J.; Crews, H. M. *J. Anal. At. Spectrom.* **1998**, *13*, 1095−1100.
(12) Prange, A.; Schaumlöffel, D.; Brätter, P.; Richarz, A.; Wolf, C. *Fresenius' J. Anal. Chem.* **2001**, *371*, 764−774.
(13) Schaumlöffel, D.; Prange, A.; Mark, G.; Heumann, K. G.; Brätter, P. *Anal. Bioanal. Chem.* **2002**, 372.

10.1021/ac010862y CCC: $22.00
Published on Web 12/22/2001

Table 1. Experimental Parameters for CE/ICPSFMS

	normal analysis	isotope dilution analysis
	CE	
buffer	20 mmol/L Tris, adjusted by HNO_3 to pH 7.4	20 mmol/L Tris, adjusted by HNO_3 to pH 7.4
temperature	15 °C	15 °C
voltage	30 kV	30 kV
MT concentration	0.1 g/L	10 g/L
injection	10 mbar for 2 s	10 mbar for 2 s
	CE/ICPMS Interface	
makeup liquid matrix	10 mmol/L NH_4NO_3	10 mmol/L NH_4NO_3
spike element	10 μg/L Rb or Ge	600 μg/L ^{33}S, 10 μg/L ^{65}Cu, 15 μg/L ^{70}Zn, 70 μg/L ^{106}Cd
makeup flow rate	8.0 μL/min	8.0 μL/min
	ICPSFMS	
cool gas flow rate	15 L/min	15 L/min
auxiliary gas flow rate	0.9 L/min	0.9 L/min
nebulizer gas flow rate	1.0−1.1 L/min	1.0−1.1 L/min
rf power	1075−1100 W	1075−1100 W
mass resolution	low (300)	medium (3000)
scan duration	600 ms	880 ms

(PEI, MW 750 000) and 2-acrylamido-2-methyl-1-propanesulfonic acid (AAMPS acid) were from Aldrich. Tetramethylenediamine (TEMED), ammonium persulfate (APS), and methacryloxypropyltrimethoxysilane (MAPS) were the products of Fluka AG (Buchs, Switzerland). Enriched isotopes of ^{33}S (abundance, 99.06%), ^{65}Cu (99.2%), ^{70}ZnO (95.43%), and ^{106}Cd (98.4%) were from JSC JV Isoflex (Moscow, Russia). Quaternarized piperazine, [(*N*-methyl, *N*-4-iodobutyl)-*N*'-methylpiperazine] (Qpip), was provided by courtesy of Dr. Sebastiano (Department of Chemistry, Politecnico of Milano, Italy). The chelating agent used for stabilizing the metal ions, 2,6-diacetylpyridinebis(*N*-methylenepyridiniohydrazone) dichloride, was synthesized according to the literature.[14] The other chemicals were of analytical grade from Merck (Darmstadt, Germany). All the solutions were prepared with ultrapure Milli-Q water (Millipore, Milford, MA).

MT preparations were dissolved in water and stored under an argon atmosphere at −18 °C to prevent oxidation. Running buffer was prepared by adjusting 20 mmol/L tris(hydroxymethyl)-aminomethane solution (Tris) with sub-boiled nitric acid to pH 7.40 and then removing metal contamination with Chelex 100 ion-exchange resin (Fluka). This buffer solution was also used for dissolving Qpip or PEI powder and diluting MT sample to the required concentrations.

Isotope-enriched products of ^{65}Cu, ^{70}ZnO, and ^{106}Cd were digested by nitric acid into ions and then diluted with water to a concentration of 1 g/L individually. A simple compound of ^{33}S was digested sequentially using a potassium hydroxide solution at a temperature of 100 °C, and then 5% H_2O_2, and was finally diluted with water to 1 g/L for stock.

A 10 mmol/L ammonia solution adjusted by sub-boiled nitric acid to pH 7.40 was employed as a matrix for the makeup liquid. For normal ICPMS analysis, 10 μg/L Rb or Ge was added to the matrix as a nebulization marker, whereas for isotope dilution analysis, 600 μg/L ^{33}S, 10 μg/L ^{65}Cu, 15 μg/L ^{70}Zn, and 70 μg/L ^{106}Cd, stabilized by 0.2 mmol/L chelating agent, were added as isotopic spikes. The standard solution used to calibrate the mass flow of the spike isotopes contained Cu, Zn, Cd, and S with natural isotope abundances at a concentration of 3, 1.5, 3, and 25 ng/L, respectively.

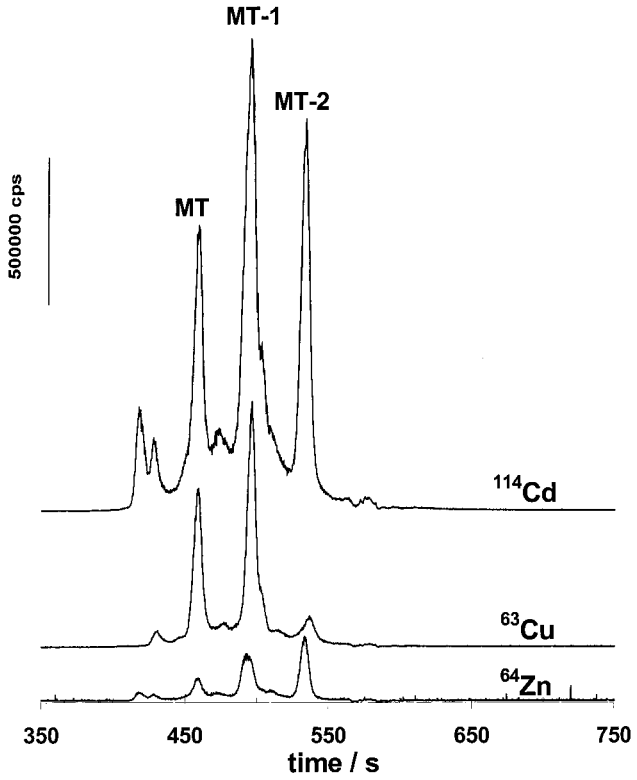

Figure 1. Normal analysis of MT preparation on uncoated capillary. Experimental conditions in Table 1. Capillary length, 70 cm; injection mass, 0.22 ng.

Instrumentation. Electrophoretic experiments were performed on an Agilent 3D CE system (Waldbronn, Germany). Fused-silica capillaries (Thermo Separation Products, Egelsbach, Germany) of 75-μm i.d. and varying lengths were used for all the tests. The interface (CEI-100, CETAC, Omaha, NE) as fully described in previous publications[15,16] was used for coupling the CE to the ICPSFMS (Element, Finnigan MAT, Bremen, Germany). More details of the instrumental parameters are listed in Table 1.

Throughout the experiments, running buffer was refreshed for each run, and the makeup liquid and the MT sample were renewed everyday.

Capillary Coating Procedure. (i) Qpip Dynamically Coated Capillary. Qpip[17] is a novel diamine agent which could be immobilized onto silica surface by flushing a new capillary with 1 mol/L KOH for 30 min, water for 15 min, and 5 mmol/L Qpip solution for 15 min sequentially. This Qpip coating can stick strongly on the silica surface and reverse the direction of EOF over a wide pH range. Prior to analysis, the column was washed with Qpip solution for 3 min and running buffer for 2 min each. The coating procedure should be repeated whenever necessary.

(ii) PEI Dynamically Coated Capillary. The deposition of the PEI coating was the same as that of the Qpip coating except

(14) Main, M. V.; Frity, J. S. *Anal. Chem.* **1989**, *61*, 1272−1275.

(15) Schaumlöffel, D.; Prange, A. *Fresenius' J. Anal. Chem.* **1999**, *364*, 452−456.

(16) Prange, A.; Schaumlöffel, D. *J. Anal. At. Spectrom.* **1999**, *14*, 1329−1332.

(17) Sebastiano, R.; Gelfi, C.; Righetti, P. G.; Citterio, A. *J. Chromatogr., A* **2000**, *894*, 53−61.

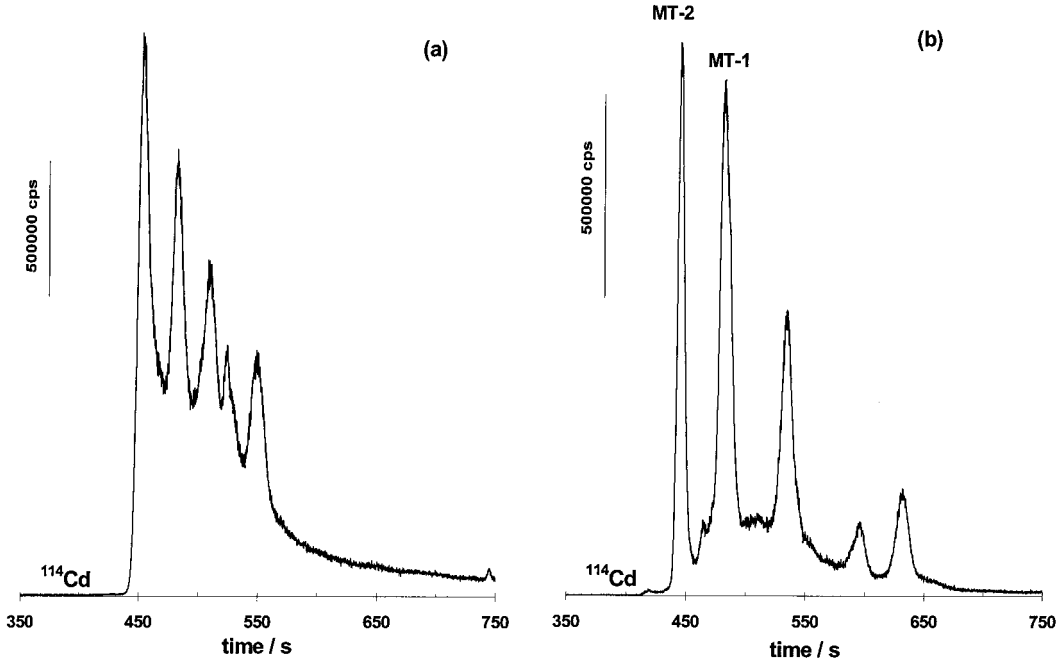

Figure 2. Normal analysis of MT preparation on (a) PEI- and (b) Qpip-coated capillaries under negative polarity. Experimental conditions in Table 1. Capillary length, 70 cm; injection mass, 0.22 ng.

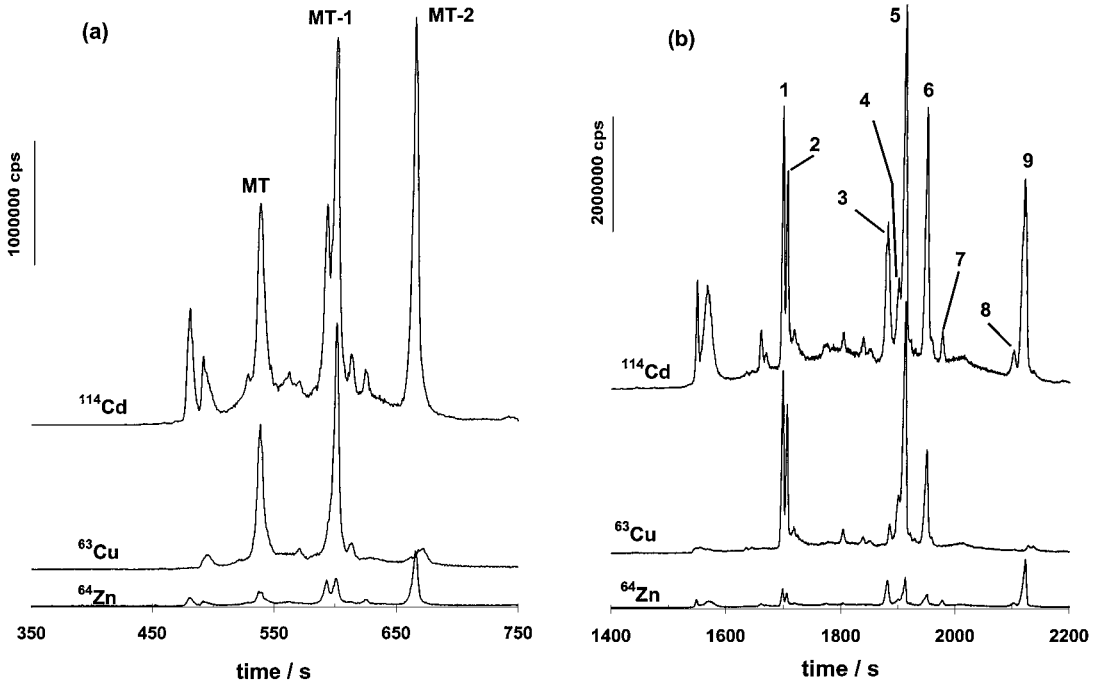

Figure 3. Normal analysis of MT preparation on AAMPS-coated capillary. Experimental conditions in Table 1. (a) Capillary length, 70 cm; injection mass, 0.22 ng. (b) Capillary length, 120 cm; injection mass, 0.13 ng.

that Qpip solution was replaced by 10% PEI solution. Before injection, the capillary was flushed with running buffer for 3 min. The coating was refreshed by flushing the capillary with 10% PEI solution after several runs.

(iii) AAMPS Polymer Permanently Coated Capillary. The polyAAMPS-coated column was prepared in a manner analogous to that introduced by Hjertén.[18] A clean and dried capillary was first filled with a dichloromethane solution containing 1% v/v MAPS and 0.5% v/v acetic acid and left for 4 h to achieve silanization. After washed with methanol and water for 3−5 min respectively, this capillary was then filled with a mixture of 1 mL of 0.5 mol/L AAMPS acid adjusted to pH 7.0 by KOH, 5 μL of TEMED, and 5 μL of 0.2 g/mL APS and allowed to polymerize at room temperature for 12 h. The column was finally washed with water for 5 min and dried with nitrogen overnight. Before a run, the capillary was flushed with water and running buffer for 2 min each. When it was not going to be used for several hours, the capillary was cleaned with water and dried by airflow.

(18) Hjertén, S. *J. Chromatogr.* **1985**, *347*, 191−198.

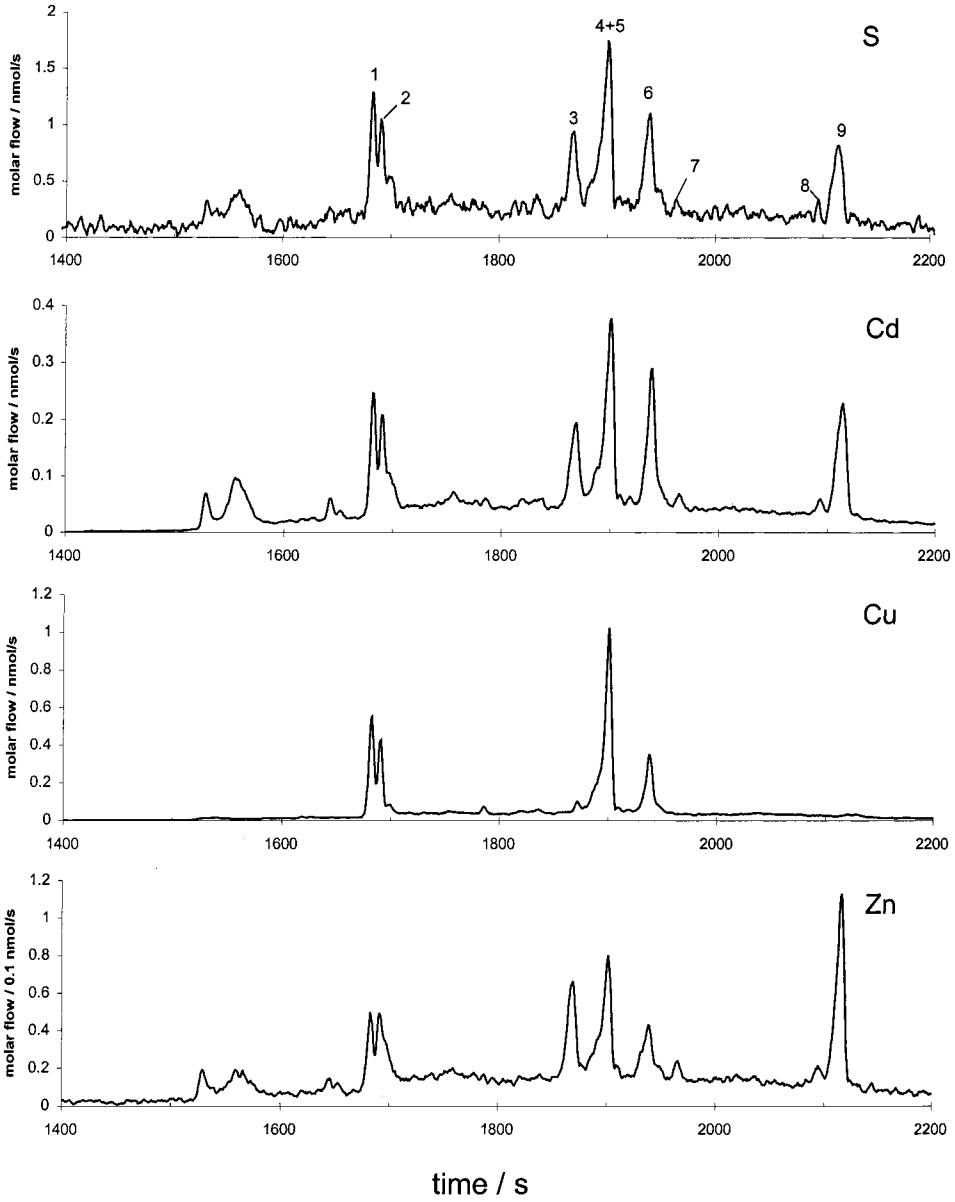

Figure 4. Derived molar flow electropherograms of MT preparation from isotope dilution analysis on AAMPS-coated capillary. Experimental conditions in Table 1. Capillary length, 120 cm; injection mass, 13 ng.

Isotope Dilution Analysis. The on-line isotope dilution technique was carried out by continuous introduction of a species-unspecific spike solution enriched in isotopes as makeup liquid. The principle of the characterization is based on the 21 sulfur atoms in MT, which can be used to normalize the atomic numbers of cadmium, copper, and zinc by measuring their molar ratios to sulfur. There is a detailed description of this topic in another report.[13] In brief, the analytical procedure included three steps: the calibration of the spike-isotopic mass flows with a standard solution, the derivation of MT molar flow electropherograms from the isotope ratios, and the calculation of the peak height or area from molar flow electropherograms in terms of the individual elements.

RESULTS AND DISCUSSION

To evaluate the influence of the capillary coatings on MT separation, the results from the uncoated capillary were checked first. Figure 1 shows the separation of MT preparation from rabbit liver on an uncoated capillary under the optimized experimental conditions demonstrated in Table 1. The two major classes of MT isoforms, MT-1 and MT-2, identified by comparison with the migration times of individual preparations, were well separated in Tris buffer at the natural cytosol pH. We assume that the first two peaks in the electropherograms represent the degraded products of MT, whereas the peak marked "MT" has not yet been identified and characterized. However, more isoforms or subisoforms widely known to be present in MT remain still unresolved.

Capillaries Coated with Cationic Materials with Reversed EOF. Both Qpip[17,19] and PEI[20] have been reported to be efficient coating agents for reversing the direction of EOF and therefore improving the separation of some compounds. The results in this work are exemplified by the cadmium electropherograms in Figure 2. On both columns, MT migrated toward the anode near

(19) Olivieri, E.; Sebastiano, R.; Citterio, A.; Gelfi, C.; Righetti, P. G. *J. Chromatogr., A* **2000**, *894*, 273−280.
(20) Córdova, E.; Gao, J.; Whitesides, G. M. *Anal. Chem.* **1997**, *69*, 1370−1379.

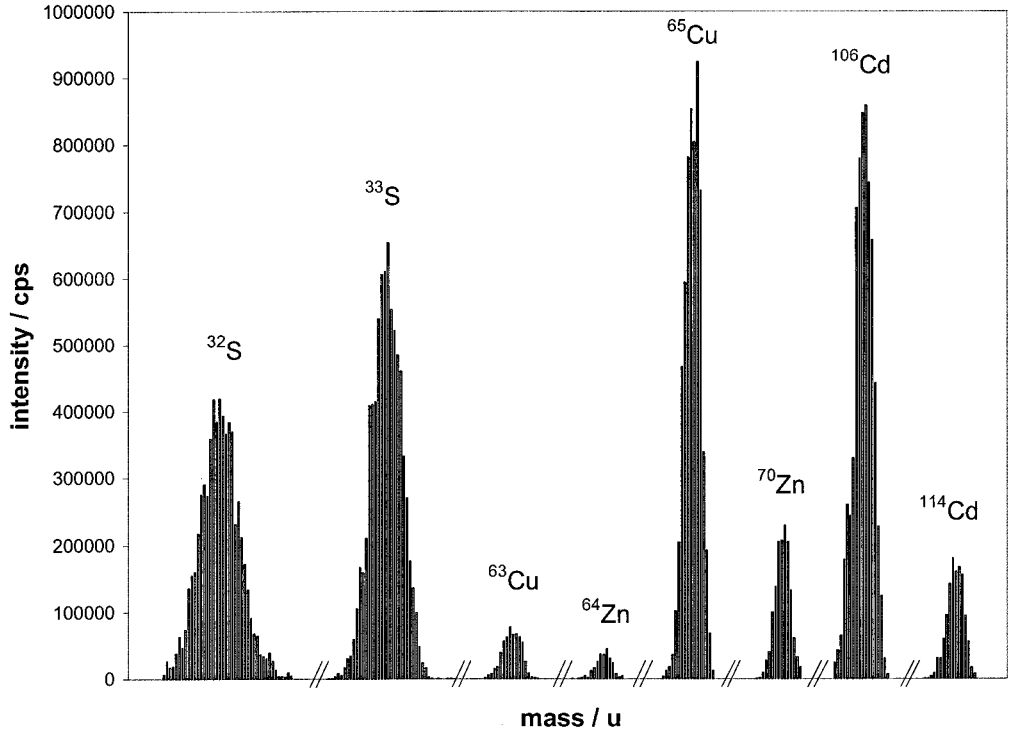

Figure 5. ICP mass spectrum taken in the apex of peak 9 in Figure 4. Data points of each element collected in one scan duration: ^{32}S, 42; ^{33}S, 42; ^{63}Cu, 18; ^{64}Zn, 18; ^{65}Cu, 18; ^{70}Zn, 18; ^{106}Cd, 18; ^{114}Cd, 18.

to the detector under negative voltage. The Qpip-coated capillary offered much better performance with respect to resolution and peak shape than the PEI-coated one, even though neither showed any additional selectivity to MT compared to the uncoated tube. Tests on MT-1 and MT-2 standards indicated an inverted elution order on this Qpip coating. Such a Qpip-coated capillary could be likely applied to the determination of MT-3, whose migration time is far longer than the other two isoforms in the normal electrophoretic direction.[12] Further investigations on this topic are underway.

Anionic Polymer-Coated Capillary. AAMPS is one of the most effective anionic coating agents used in CE separation. The polyAAMPS-coated capillary has strongly acidic groups on its inner surface and therefore can stabilize the EOF from the violent effect of buffer pH. By mixing an amount of acrylamide with AAMPS in the polymer solution, even capillaries with a particular EOF rate could be prepared.[21] Since the separation of MT was carried out under neutral pH conditions, the pure polyAAMPS-coated capillary was used in this case to ensure enough EOF. Figure 3a demonstrates that MT appears to have undergone some superior separations on the polyAAMPS coating. This is most likely due to the decreased EOF and the different interactions between the surface and MT complexes on the coated tube. Furthermore, the electrophoretic reproducibility of such a modified column was surprisingly good compared to that of uncoated tubes. Even after two months of storage, the capillary could still produce almost a copy of the previous electropherograms.

Unexpectedly, the resolution of MT isoforms on the polyAAMPS-coated capillary was remarkably enhanced with the increment of capillary length, while the resolution on either the uncoated or the cationic material-coated capillaries stayed nearly the same. A coated capillary of 120-cm length was selected as the optimum by compromising the resolution and the analysis time. As indicated in Figure 3b, the unknown MT isoform was resolved into two subisoforms (peaks 1 and 2). Five subisoforms (peaks 3−7) were detectable in MT-1, and MT-2 contained at least two subisoforms (peaks 8 and 9). It is still uncertain whether these separated subisoforms differ at the amino acid sequences or not. However, their different affinities to the metals could be expected from the electropherograms, which would be quantified by isotope dilution analysis in the next step. Moreover, the change of intensity ratios of different peaks on 70- and 120-cm capillaries might be caused by different degradation behaviors of the MT isoforms, since all the samples were prepared at the same time.

Characterization of MT Metal Complexes by Isotope Dilution Analysis. Since isotope dilution analysis must be performed in medium resolution to avoid the interference of O−O to ^{32}S, highly concentrated MT samples were required to meet the demands of ICPSFMS detection. Figure 4 shows the derived molar flow electropherograms from isotope ratios on the anionic polymer-coated capillary. In this case, the shoulder peak 4 merged with peak 5 due to the higher amount of MT than that loaded in low MS resolution. A typical mass spectrum taken in the apex of peak 9 (Figure 5) demonstrates that enough data points were collected in one scan duration of 880 ms by ICPSFMS, ensuring sufficient counting statistics of each element.

In the calculation of the molar ratios of Cd, Cu, and Zn to S, peak heights were measured because of the difficulties in the integration of nonbaseline-resolved peaks. The stoichiometric estimation was based on three independent measurements of MT each with two standard calibrations. Here, only the peaks having more than 10 times the intensity of noise were taken into account.

(21) Sun, P.; Landman, A.; Barker, G. E.; Hartwick, R. A. *J. Chromatogr., A* **1994**, *685*, 303−312.

Table 2. Molar Ratios of Sulfur to Metals in MT Complexes and the Suggested Formulas

peak no.	molar ratio S:Cu:Zn:Cd	suggested formulas
1	21:7.1:0.9:3.4	MT ($Cu_7Zn_1Cd_3$)
2	21:7.2:0.8:3.4	MT ($Cu_7Zn_1Cd_3$)
3	21:1.2:1.9:5.3	MT-1 ($Cu_1Zn_2Cd_5$)
4 + 5	21:8.2:0.9:3.3	MT-1 ($Cu_8Zn_1Cd_3$)
6	21:5.2:0.8:4.4	MT-1 ($Cu_5Zn_1Cd_4$)
9	21:0:3.2:4.6	MT-2 (Zn_3Cd_4)

The average molar metal contents and the suggested formulas of MT complexes are given in Table 2. According to the coordination chemistry of MT,[22] when monovalent-coordinated copper is present, the metal−thiolate cluster of MT can involve more than seven metals, which is the only binding possibility for mixed or signal cadmium and zinc. Therefore, even though the calculated cadmium content in peak 9 was 4.6 atoms per molecule, a composition of MT-2 (Zn_3Cd_4) was suggested. In addition, since the classification of subisoforms is still obscure, we prefer to give the formulas as MT-1 ($Cu_xZn_yCd_z$), but not MT-1a ($Cu_xZn_yCd_z$). Further determination of amino acid sequence of each complex with ESI-MS/MS is being planned.

(22) Stillman, M. J. *Coordination Chem. Rev.* **1995**, *144*, 461−511.

CONCLUSIONS

MT isoforms from rabbit liver were analyzed in a natural cytosol pH environment by using surface-modified capillaries with CE/ICPMS. Among three different types of coated capillaries, anionic polymer (polyAAMPS)-coated columns offered unique improvements in the separation of MT isoforms compared to the uncoated ones. Maximum resolution could be achieved with an optimized capillary length of 120 cm. The separated MT isoforms were subsequently analyzed using on-line isotope dilution technique with ICPSFMS. The stoichiometric formulas of MT metal complexes were suggested in the light of the normalized molar ratios of cadmium, copper, and zinc to 21 sulfur atoms.

Received for review July 31, 2001. Accepted November 2, 2001.

AC010862Y