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Isotopic variations of copper at the protein level in neuronal human cells exposed in vitro to uranium

Eduardo Paredes*¹, Véronique Malard^{2,3}, Claude Vidaud², Emilie Avazeri², Richard Ortega^{4,5}, Anthony Nonell¹, Hélène Isnard¹, Frédéric Chartier⁶, Carole Bresson¹

¹Den – Service d'Etudes Analytiques et de Réactivité des Surfaces (SEARS), CEA, Université Paris-Saclay, F-91191, Gif sur Yvette, France.

²CEA, DRF, Biosciences and biotechnologies institute (BIAM), Bagnols-sur-Cèze, France.

³Aix Marseille Univ, CEA, CNRS, BIAM, Protein-metal interaction (LIPM), Saint-Paul-lez-Durance, France F-13108.

⁴University of Bordeaux, CENBG, UMR 5797, F-33170 Gradignan, France

⁵CNRS, IN2P3, CENBG, UMR 5797, F-33170 Gradignan, France.

⁶Den – Département de Physico-Chimie (DPC), CEA, Université Paris-Saclay, F-91191, Gif sur Yvette, France.

Abstract

The study of isotopic variations of endogenous and toxic metals in fluids and tissues is a recent research topic with an outstanding potential in biomedical and toxicological investigations. Most of the analyses have been performed so far in bulk samples, which can make the interpretation of results entangled, since different sources of stress or the alteration of different metabolic processes can lead to similar variations in the isotopic compositions of the elements in bulk samples. The downscaling of the isotopic analysis of elements at the sub-cellular level, is considered as a more promising alternative. Here we present for the first time the accurate determination of Cu isotopic ratios in four main protein fractions from lysates of neuron-like human cells exposed *in vitro* to 10 μ M of natural uranium for seven days. These protein fractions were isolated by Size Exclusion Chromatography and analysed by Multi-Collector Inductively Coupled Plasma Mass Spectrometry to determine the Cu isotopic variations in each protein fraction with regard to the original cell lysate. Values obtained, expressed as $\delta^{65}\text{Cu}$, were $-0.03 \pm 0.14 \text{ ‰}$ (U_c , $k=2$), $-0.55 \pm 0.20 \text{ ‰}$ (U_c , $k=2$), $-0.32 \pm 0.21 \text{ ‰}$ (U_c , $k=2$) and $+0.84 \pm 0.21 \text{ ‰}$ (U_c , $k=2$) for the four fractions, satisfying the mass balance. The results obtained in this preliminary study pave the way for dedicated analytical developments to identify new specific disease biomarkers, to get insight into the knowledge of stress-induced altered metabolic processes, as well as to decipher metabolic pathways of toxic elements.

Introduction

The study of the isotopic signatures of endogenous elements, such as Cu, Zn, and Fe, in body fluids/ tissues and *in vitro* cultured human cell lines is a recent research topic which has shown an outstanding potential for biomedical investigations. (1,2,3) In particular, Cu and Zn isotopic signatures seem to be promising tools for the diagnosis of cancer (4,5,6,7) and neurodegenerative diseases, (8,9,10,11) as well as for the follow-up of patients. (12) *In vitro* cultured human cell lines are powerful tools to help identifying the altered metabolic processes leading to disease-induced isotopic variations, (13) as well as performing other metabolic or toxicological studies. (14,15,16,17) Isotopic fractionations may occur during the redistribution of an element among different chemical species, as predicted by *ab initio* calculations. (18) The stress-induced alteration of the metabolic processes involving any of these chemical species may lead to a modification of the element isotopic signatures. Since the altered metabolic processes may depend on the source of stress (disease, toxic element, etc), the chemical species undergoing isotopic signature variations may differ. Most of the studies performed so far have been based on the isotopic analysis of the element in the bulk sample, but two articles recently published (19,20) have demonstrated the potential of the determination of the isotopic signatures at the sub-cellular level.

We have previously developed a procedure for the accurate isotope ratio determination of U, Zn and Cu in bulk SH-SY5Y human neuroblastoma cell samples, after differentiation of the cells into neuron-like cells and exposure to low concentrations of natural U for seven days. (21,22) In the current study, isotopic analysis was downscaled at the protein level for the first time. To meet this aim, additional analytical efforts were required to isolate the protein fractions containing U, Zn and Cu, in combination with accurate isotope ratio measurements of the very small amounts of elements contained in these fractions. In these sense, we developed a method dedicated to the isotopic analysis of Cu, Zn and U in different protein fractions of cell lysates obtained by Size Exclusion Chromatography (SEC), after exposure of SH-SY5Y neuron-like cells to 10 μ M of natural U for seven days. Only Cu isotope ratio results are presented, since it

was not possible to accurately determine the isotope ratios of Zn and U in the protein fractions, as discussed in the following.

Experimental

Reagents and solutions

All the aqueous solutions were prepared using ultrapure water (resistivity > 18.2 MΩ cm at 25 °C) from a Milli-Q® system (Millipore). Eagle's minimum essential medium (EMEM, ATCC, 30-2003), F12 medium (Life Technologies, 21765-029), fetal bovine serum (FBS, ATCC, 30-2020) and penicillin/ streptomycin (Gibco-Thermo Fisher Scientific, 15070-063) solutions were used to prepare the culture medium for cell growing and exposure experiments. Retinoic Acid (RA, Sigma-Aldrich, R2625) and 12-O-tetradecanoylphorbol-13-acetate (TPA, Sigma Aldrich, P8139) were used for cell differentiation. TrypLE Express 1X/EDTA (Gibco-Thermo Fisher Scientific, 12605-010) was used for cell trypsinization. Phosphate buffer saline (PBS, pH 7.4) free of CaCl₂ and MgCl₂ (Gibco 10010-015) was used to wash the cells after trypsinization. A 100mM ammonium acetate (Normapur grade, VWR Prolabo) solution in ultrapure water was used as mobile phase for the SEC experiments.

Plasma Pure Plus 34-37% HCl and Plasma Pure Plus 67-70% HNO₃ ultrapure reagents (SCP Science) were used for the sample preparation steps and for the preparation of solutions analyzed by Multi-Collector Inductively Coupled Plasma Mass Spectrometry (MC-ICPMS). IRMM-3702 [$n(^{66}\text{Zn})/n(^{64}\text{Zn}) = 0.56397 \pm 0.00030$ (U_c , $k=2$)] and ERM®-AE633 [$n(^{65}\text{Cu})/n(^{63}\text{Cu}) = 0.44563 \pm 0.00042$ (U_c , $k=2$)] isotopic certified reference materials (i-CRM) traceable to SI units were purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). The ERM®-AE633 i-CRM was used to prepare a bracket solution analyzed just before and after the samples for Cu isotope ratio measurements. This bracket solution and the samples were spiked with the IRMM-3702 i-CRM, which was used as an internal standard for the correction of mass discrimination.

In-house natural uranium powder (U_3O_8) was dissolved in $0.5 \text{ mol L}^{-1} \text{ HNO}_3$ to prepare a natural uranium stock solution at 150 mM. An intermediate U solution ($\text{pH} = 8\text{-}8.5$) was then prepared by 1:5 v/v dilution of this solution in a buffer solution containing $0.1 \text{ mol L}^{-1} \text{ NaHCO}_3$ (analytical reagent, Normapur), $0.1 \text{ mol L}^{-1} \text{ Na}_2\text{CO}_3$ (99.95% extra pure, Acros Organics), $0.15 \text{ mol L}^{-1} \text{ NaCl}$ (puriss. p.a., Sigma-Aldrich), and $0.05 \text{ mol L}^{-1} \text{ TRIS}$ (ultrapure grade $\geq 99.9\%$, Sigma-Aldrich) in ultrapure water. This dilution was performed by dropwise addition of the U stock solution into the buffer solution to avoid U precipitation. Finally, the exposure solutions ($10 \text{ }\mu\text{M}$ of U) were prepared by diluting the intermediate U solution into an appropriate volume of the culture medium consisting in an equal mix of EMEM and F12 mediums supplemented with 10% FBS and 1% penicillin/ streptomycin.

Differentiation of cells, exposure to natural uranium and cell lysis.

Human SH-SY5Y (ATCC, CRL-2266, Batch 59740436) cells were first grown and differentiated into neuron-like cells. Cells were grown in 175 cm^2 flasks at 37°C in 5% CO_2 for 10 days. The culture medium was replaced with fresh medium every 3-4 days. Afterwards, the cells were passaged by trypsinization using TrypLE Express 1X/EDTA and then seeded at $25000 \text{ cells cm}^{-2}$. The differentiation into neuron-like cells was performed according to the method developed by Presgraves *et al.* (23) For this, the culture medium was replaced with fresh medium containing $10 \text{ }\mu\text{M}$ RA, and cells were incubated for 3.5 days. This solution was then replaced with fresh medium containing 80 nM TPA and left for 3.5 days again. The effectiveness of the cell differentiation was assessed by phase contrast microscopy and western blot showing neurite outgrowths and tyrosine hydroxylase expression in differentiated cells. (24)

The differentiated cells were exposed to freshly-prepared $10 \text{ }\mu\text{M}$ U exposure solutions for 7 days, with exposure solution renewal once after 3 days. After exposure, cells were trypsinized, collected, washed twice with PBS, and finally counted using a Vi-CELL XR 2.04 cell viability analyzer (Beckman Coulter).

Cell proteins were extracted using a mechanical method. For this, cells were suspended at 30×10^6 cells per mL in 0.02 M TRIS $\text{pH} 7.5$ containing anti-proteases (Roche cocktail-EDTA

free) and 0.002 M spermine base (Sigma). The cells were disrupted by one shot at 1000 bars using a cell disruption system (Constant Systems) and then incubated for 30 min at 4 °C. The extracts were then ultra-centrifuged (1 h at 100,000 g) and the supernatants collected. Aliquots of the supernatants were stored at -20 °C until use.

Separation and detection of metal enriched protein fractions

A Smartline chromatographic system (KNAUER, Berlin, Germany) equipped with a dual Pump 1000 was used for the chromatographic separation of metal enriched protein fractions by SEC. The column was a 4.6 x 150 mm BEH 200 (Waters, Milford, USA) with 1.7 µm particle size and 200 Å pore size. The mobile phase consisted in a 100 mM ammonium acetate solution in ultrapure water, and the flow rate was 0.3 mL min⁻¹. Cell lysates (20 µL) were manually injected in the column with a Rheodyne injector valve (model 9725). The column was coupled to a UV detector (Smartline PDA detector 2800) connected in series to a quadrupole ICPMS (q-ICPMS, X7, Thermo) through a 1-meter length PEEK tubing (i.d: 125 µm). The monitoring of metal enriched protein fractions was performed by UV at $\lambda = 280$ nm, and by q-ICPMS by following the signal of the isotopes ⁶⁵Cu⁺, ⁶⁶Zn⁺ and ²³⁸U⁺. The integration time per chromatographic point in q-ICPMS was 10 ms, and approximately 5800 points per isotope were acquired for a run time of 10 min.

To estimate the molecular weight (MW) range of the protein fractions, the calibration of the column was performed by injection of 10 mg L⁻¹ standards of Aprotinin (6.5 kDa), Ovalbumin (43 kDa), Conalbumin (75 kDa), Monoclonal antibody (150 kDa), Ferritin (440 kDa) and Thyroglobulin (669 kDa) diluted in the mobile phase. The signal was monitored by UV at $\lambda = 280$ nm and the retention time (average of 3 replicates) was determined.

For the collection of the main protein fractions, 100 injections of cell lysates (20 µL) were performed, and the 100 eluate volumes corresponding to the same Cu protein fraction (F1, F2, F3 and F4) were pooled before Cu isotopic analysis. For comparison, 500 µL of cell lysates were subjected to isotopic analysis without protein separation step. For matrix matching between the original lysate and the Cu protein fractions, the original lysate was diluted in the appropriate volume of the mobile phase before sample preparation for isotopic analysis.

Furthermore, procedural blanks were prepared by subjecting the same volume of the mobile phase to the analytical procedure.

Sample preparation for isotopic measurements

Sample preparation and analysis were performed at the *Laboratoire de développement Analytique, Nucléaire, Isotopique et Élémentaire* (LANIE, CEA). The labware used for sample preparation previous to isotopic measurements was systematically pre-washed with acid solutions in accordance with clean lab practices. A 120 x 52 x 70 cm glovebox (Ateliers de Technochimie, Ivry sur Seine, France) was purposely designed to perform all sample preparation steps in order to protect samples from atmospheric contamination. This glovebox was made of polyvinyl chloride (PVC) and was equipped with High Efficiency Particulate Air (HEPA) filters to avoid contamination by dust particles.

The sample preparation for the procedural blanks (mobile phase) and the samples (protein fractions and cell lysate diluted in the mobile phase) was similar to that published elsewhere. (21) First, the samples/ procedural blanks were evaporated at 80 °C on a heating block. The residues were then acid digested in 15 mL closed Savillex vessels at 85 °C for 2 h after the addition of 1 mL of 67-70% HNO₃. The acid was then evaporated at 85 °C until dryness and the residue was re-dissolved in 1 mL of 3 M HNO₃ for the purification of U, Cu and Zn according to a protocol described elsewhere. (21)

Isotope ratio measurements

All isotope ratio measurements were performed with a Neptune Plus MC-ICPMS (Thermo Fisher Scientific, Darmstadt, Germany) equipped with 9 Faraday detectors fitted to 10¹¹ Ω resistors. The sample introduction system consisted of an OpalMist nebulizer at around 10 μL min⁻¹ (Glass Expansion, Melbourne, Australia) coupled to an Apex HF desolvation system (Elemental Scientific, Omaha, USA). ‘Jet’ sampler and X-type skimmer cones adapted to dry plasma conditions (25) were employed. The cup configuration used can be found elsewhere. (21)

Preamp gain calibration was performed daily. The Zn hydride formation rates were measured by monitoring the signal at m/z 69 (⁶⁸Zn¹H⁺) at the beginning of each measurement session. ZnH⁺

formation rates were 0.005-0.008% and the interference of $^{64}\text{Zn}^1\text{H}^+$ on $^{65}\text{Cu}^+$ was systematically corrected. A background correction was performed by running a 2% HNO_3 solution before the samples, procedural blanks and bracketing solutions. Three procedural blanks were run at the beginning of the session and the average signal measured for the procedural blanks was subtracted to the signal of the samples.

The so-called modified sample-standard bracketing (m-SSB) approach, (21,26) which combines the classical sample-standard bracketing and the inter-element correction approaches, was used to determine the $\delta^{65}\text{Cu}$ values. For this, the samples were bracketed by ERM[®]-AE633 solutions and the Cu concentrations in the samples and the bracketing solutions were matched to less than 50 % difference. Both the samples and the bracketing solutions were spiked with IRMM-3702 at the same Zn (internal standard) concentration. For the determination of $\delta^{65}\text{Cu}$ in a sample, $(\delta^{65}\text{Cu})_{\text{analyte}}$ and $(\delta^{66}\text{Zn})_{\text{spike}}$ were calculated using a classical sample-standard bracketing as shown in equations 1 and 2:

$$(\delta^{65}\text{Cu})_{\text{analyte}} = \left(\frac{(r_{65/63})_{\text{sample}}}{(r_{65/63})_{\text{bracketing}}} - 1 \right) \times 1000 \quad (1)$$

$$(\delta^{66}\text{Zn})_{\text{spike}} = \left(\frac{(r_{66/64})_{\text{sample}}}{(r_{66/64})_{\text{bracketing}}} - 1 \right) \times 1000 \quad (2)$$

Where $(r_{65/63})_{\text{sample}}$ and $(r_{66/64})_{\text{sample}}$ are the measured $^{65}\text{Cu}^+/^{63}\text{Cu}^+$ and $^{66}\text{Zn}^+/^{64}\text{Zn}^+$ ratios in the sample, whereas $(r_{65/63})_{\text{bracketing}}$ and $(r_{66/64})_{\text{bracketing}}$ are the average of the measured $^{65}\text{Cu}^+/^{63}\text{Cu}^+$ and $^{66}\text{Zn}^+/^{64}\text{Zn}^+$ in the two bracketing solutions run just before and after the sample. The final $\delta^{65}\text{Cu}$ in the sample (protein fractions or original lysate) with regard to the ERM[®]-AE633 solution was obtained by subtracting $(\delta^{66}\text{Zn})_{\text{spike}}$ to $(\delta^{65}\text{Cu})_{\text{analyte}}$. With these results, the $n(^{65}\text{Cu})/n(^{63}\text{Cu})$ isotope ratio in the samples was calculated. Finally, results were expressed as $(\delta^{65}\text{Cu})_{\text{Fi}}$, corresponding to the relative difference in parts per mil between the $n(^{65}\text{Cu})/n(^{63}\text{Cu})$ isotope ratio in the protein fraction i and the $n(^{65}\text{Cu})/n(^{63}\text{Cu})$ isotope ratio in the original lysate:

$$(\delta^{65}\text{Cu})_{\text{Fi}} = \left(\frac{(r_{65/63})_{\text{fraction i}}}{(r_{65/63})_{\text{original lysate}}} - 1 \right) \times 1000 \quad (3)$$

Where $(r_{65/63})_{fraction i}$ is the $n(^{65}\text{Cu})/n(^{63}\text{Cu})$ isotope ratio determined in the protein fraction i

and $(r_{65/63})_{original lysate}$ is the $n(^{65}\text{Cu})/n(^{63}\text{Cu})$ isotope ratio determined in the original lysate.

The methods used for U and Zn isotope ratio measurements can be found elsewhere (21) and are not described in this article, since only Cu isotope ratios could be accurately determined in the protein fractions.

Uncertainty estimation

The expanded uncertainty (U_c , k=2) of δ values was estimated by quadratic propagation of two sources of uncertainty (see ref. 21 for details): the within-day measurement reproducibility (SD_M) and the reproducibility associated to procedural blank correction (SD_{PB}), as shown in the following equation:

$$U_c = 2\sqrt{SD_M^2 + SD_{PB}^2} \quad (4)$$

Results and discussion

Under the experimental conditions used, the protein fractions from lysate injections eluted in less than 8 min, as shown in the UV profile of Figure 1. The column was previously calibrated with UV monitoring of standard proteins, which made it possible to assign the theoretical masses of the Cu, Zn and U protein fractions from their retention times (peak maxima). The different metal-containing protein fractions observed in the q-ICPMS chromatograms (Figure 1) corresponded to theoretical masses of approximately > 600, 110, 32 and 6 kDa for Cu, > 600, 33 and 6 kDa for Zn, and > 600, 70 and 3-6 kDa for U, respectively. We estimated the relative proportions of metal in each one of the peaks by comparing the peak areas corresponding to the main Cu, Zn and U peaks from 14 SEC-q-ICPMS chromatograms. These relative Cu proportions for the peaks corresponding to > 600, 110, 32 and 6 kDa were 38 ± 5 % (SD, n=14), 20 ± 4 % (SD, n=14), 22 ± 3 % (SD, n=14) and 21 ± 4 % (SD, n=14), respectively. The relative Zn proportions for the peaks corresponding to > 600, 33 and 6 kDa were 39 ± 5 % (SD, n=14), 42 ± 5 % (SD, n=14) and 18 ± 6 % (SD, n=14), respectively. A larger variability was found for the U peaks, being the relative U proportions for the peaks corresponding to > 600, 70 and 3-6

kDa of 50 ± 13 % (SD, n=14), 36 ± 16 % (SD, n=14) and 13 ± 4 % (SD, n=14), respectively.

The proteomic analysis of these protein fractions was carried out with the aim of identifying the U-target proteins, and the results are reported elsewhere. (27)

Four protein fractions F1, F2, F3 and F4 (dotted rectangles, Figure 1) corresponding to the main Cu protein fractions were collected. For Zn and U isotope ratio measurements, F2 and F3 were mixed after the element purification, since they corresponded to the same Zn and U peaks.

However, the accurate determination of the U isotope ratios was not possible because the recovered U amounts were too small. In the case of Zn, the amounts in the protein fractions were high enough for precise isotope ratio measurements. However, the Zn isotope ratios could not be accurately determined because of the high procedural blanks obtained (from 200 to 350 ng), corresponding to over 50% of the total Zn amounts in the samples. These high procedural blanks cannot be attributed to the sample preparation procedure for isotope ratio measurements, which were found to be 35 ± 19 ng, (21) and probably came from the mobile phase reagents and/or the stationary phase. Indeed, high base lines were observed for Zn in the q-ICPMS chromatograms (Figure 1).

On the contrary, accurate results could be obtained for the Cu isotope ratios. The Cu amounts were high enough for the measurement of isotope ratios with external precisions better than 0.15‰, and the uncertainty associated to the procedural blank correction ranged from 0.08 to 0.17‰ (2SD, n = 6, see ref. 21 for details on the calculation method). Figure 2 shows the $(\delta^{65}\text{Cu})_{Fi}$ in the 4 protein fractions, reflecting the isotopic signature of Cu in each of the fractions relative to the original lysate. Fraction 1 showed a similar isotopic signature as the original lysate with $(\delta^{65}\text{Cu})_{F1} = -0.03 \pm 0.14$ ‰ (U_c , k=2), whereas fractions 2 and 3 were depleted in ^{65}Cu with $(\delta^{65}\text{Cu})_{F2} = -0.55 \pm 0.20$ ‰ (U_c , k=2) and $(\delta^{65}\text{Cu})_{F3} = -0.32 \pm 0.21$ ‰ (U_c , k=2), respectively. Finally, fraction 4 was enriched in ^{65}Cu with $(\delta^{65}\text{Cu})_{F4} = +0.84 \pm 0.21$ ‰ (U_c , k=2). The mass balance was verified from these results using equation 5:

$$(\delta^{65}\text{Cu})_{\text{original lysate}} = \frac{f_1(\delta^{65}\text{Cu})_{F1} + f_2(\delta^{65}\text{Cu})_{F2} + f_3(\delta^{65}\text{Cu})_{F3} + f_4(\delta^{65}\text{Cu})_{F4}}{100} \quad (5)$$

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273 Where $(\delta^{65}\text{Cu})_{F1}$, $(\delta^{65}\text{Cu})_{F2}$, $(\delta^{65}\text{Cu})_{F3}$ and $(\delta^{65}\text{Cu})_{F4}$ are the $\delta^{65}\text{Cu}$ in the protein fractions
274 1 to 4 with regard to the original lysate, and f_1 , f_2 , f_3 and f_4 are the percentages of Cu in the
275 protein fractions 1 to 4 (see above). The $(\delta^{65}\text{Cu})_{\text{original lysate}}$ calculated when applying
276 Equation 5 was $-0.02 \pm 0.12 \text{ ‰}$, which is not significantly different from the expected value of 0
277 ‰.

278 The results obtained in this work clearly demonstrate a different isotopic distribution of Cu
279 among the four protein fractions. Recently, two papers have demonstrated that different Cu
280 pools of biological samples may show different isotopic signatures. In one of these articles, the
281 Cu isotope ratios were determined in the bulk serum of healthy individuals and alcoholic
282 cirrhosis patients, as well as in the exchangeable + ultrafiltrable (EXCH + UF) Cu fraction of
283 the serum, representing the labile Cu pool, and the non-exchangeable + non-ultrafiltrable
284 (NEXCH + NUF) Cu fraction containing the Cu bound to ceruloplasmin. (19) The results
285 showed a heavier Cu isotope ratio in the EXCH + UF fraction compared to the bulk serum and
286 the NEXCH + NUF fraction of healthy individuals, whereas this difference was not found in the
287 patients, potentially reflecting a labile Cu deregulation linked to the disease. (19) In another
288 article, the Cu isotope ratios were determined in sub-cellular fractions of the SH-SY5Y human
289 neuroblastoma cell line, corresponding to the mitochondria and the rest of the cell lysate,
290 demonstrating different isotopic signatures. (20) The present work can be considered as a step
291 forward in the downscaling of Cu isotope ratio determinations at sub-cellular level, since we
292 performed accurate Cu isotope ratio determinations at the protein level. These three studies open
293 new perspectives on the identification of new disease-specific biomarkers. In previous
294 publications, the study of the isotopic signature of the element as prognostic biomarker was
295 performed on bulk samples. The main drawback of such approach is that similar isotopic
296 variations in bulk samples can be induced by different types of diseases. For instance, similar
297 depletions in the heavier ^{65}Cu isotope have been found in the serum of cancer, (7) cirrhosis (28)
298 or Wilson disease (8) patients. Furthermore, in some cases, the isotopic variations observed

between the healthy and cancerous tissues do not lead to changes in the blood isotopic signatures, as observed for Zn isotope ratios in breast cancer patients. (5)

Conclusions

For the first time, the accurate determination of Cu isotope ratios was performed in intracellular protein fractions isolated by SEC, which represents a significant progress in the downscaling of isotope ratio determinations from the cellular level to the protein level. We evidenced differences in the Cu isotopic signatures in the four main protein fractions from SH-SY5Y neuron-like cell lysates. Since the Cu isotopic signatures in the proteins might be modified by stress sources altering the Cu metabolism, this work paves the way for the identification of new disease biomarkers as well as for the development of new strategies to get insight into the knowledge of stress-induced alteration of Cu metabolic processes, which can be critical in the development of diagnostic tools. However in this study, Zn and U isotope ratios could not be accurately determined in the protein fractions, and dedicated analytical developments are required to extend this approach to other elements than Cu, as well as different types of biological samples.

The upcoming major step is to perform isotopic analysis at the molecular level, through the study of isotopic fractionations of elements involved in different chemical species, in combination with *ab initio* theoretical calculations of these isotopic fractionations among these species. (18) This is key information for deeper investigation of stress-induced alteration of metabolic processes at the molecular level, as well as to aid deciphering the metabolic routes of toxic elements in a more specific manner.

Conflicts of interest

There are no conflicts to declare.

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Figure captions

Figure 1. SEC chromatograms of cell lysates of the SH-SY5Y human neuroblastoma cell line differentiated into neuron-like cells and exposed to 10 μM of natural U for seven days. The elution profiles correspond to the UV signal monitored at 280 nm and the q-ICPMS signals of $^{65}\text{Cu}^+$, $^{66}\text{Zn}^+$ and $^{238}\text{U}^+$ isotopes. For each q-ICPMS chromatogram, the signal relative to the maximum signal measured for the isotope throughout the chromatogram is plotted. The dotted rectangles show the 4 protein fractions collected for Cu isotopic analysis.

Figure 2. $(\delta^{65}\text{Cu})_{Fi}$ values for the 4 Cu protein fractions with regard to the original lysate. The vertical bars correspond to the expanded uncertainty of the results ($k=2$). The plain and dotted red lines indicate the $\delta^{65}\text{Cu}$ value of the original lysate, $(\delta^{65}\text{Cu})_{\text{original lysate}}$, defined as 0 ‰, and its expanded uncertainty.

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