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Elemental bioimaging of manganese uptake in *C. elegans*

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Abstract

A new method for elemental bioimaging with laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) was developed and applied to study the uptake of manganese (Mn) in *Caenorhabditis elegans* (*C. elegans*). *C. elegans* is a well-established model organism in neuroscience, genetics and genomics, which has been extensively studied to decipher mechanisms of heavy metal toxicity. Knowledge about the distribution of manganese (Mn) and other metals in this organism will be helpful in elucidating pathways and mechanisms of transport, distribution and excretion. The LA-ICP-MS method requires limited sample preparation and can be used rapidly and easily to visualize the Mn distribution in *C. elegans*. Due to thorough optimization of the analytical parameters, intense Mn signals in *C. elegans* wild-type (WT) and mutants were obtained at a spatial resolution as small as 4 μm , thus proving the suitability of LA-ICP-MS to study the uptake of metals in *C. elegans*.

Introduction

Caenorhabditis elegans (*C. elegans*) represents an excellent model organism in neuroscience as well as in genetics and genomics. Its small size (1 mm) and a short reproduction time of just 3 days enable studies with a relatively high sample throughput.^{1,2} *C. elegans* is less complex than the mammalian system, while still sharing high genetic homology (60–80%).^{3–5} In addition, the simple nervous system of this organism consists of just 302 neurons and approximately 5000 synapses, displaying all essential functions of the human nervous system.⁶ One scientific field, in which this organism has gained an important role, is the elucidation of mechanisms behind the toxicity of heavy metals.^{7–9} Proposed mechanisms include oxidative stress and metal accumulation in specific brain regions. In the past, studies related to these questions mostly focused on changes in survival or uptake rates after genetic manipulation.

Information concerning the distribution of these elements in various genetic backgrounds may afford important novel insights into gene $\times$ environment interactions, with characterization of metal distribution and the underlying mechanisms of their toxicity.

In earlier studies by Gao *et al.*, microbeam synchrotron radiation X-ray fluorescence was used to visualize the uptake of Cu nanoparticles.¹⁰ McColl *et al.* utilized this method to image the Mn distribution in this small organism.¹¹ Although this technique is able to provide highly resolved images of elemental distributions, it is expensive, time consuming and therefore not suitable for routine analysis of a larger number of samples. For this reason, laser ablation (LA) coupled to inductively coupled plasma mass spectrometry (ICP-MS) provides an attractive alternative for carrying out fast and easy analysis in the absence of complex and laborious sample preparation.

LA-ICP-MS has been employed in bioimaging of many different elements and samples. Examples include the imaging of Cu and Zn in sheep liver, Cu, Zn, P and S in brain sections, Pt in tumour tissue or Pt, Mn, Fe, Cu and Zn in mouse organs.¹²⁻¹⁷ So far, no investigations were performed using this technique to visualize the distribution of metals in *C. elegans*.

Although Mn is an essential trace element, concerns are rising about its neurotoxic effects upon exposure in humans. Chronic inhalation and oral overexposure have been shown to result in specific clinical central nervous system symptomatology, including tiredness, behavioural changes, and delayed neurological disturbances characterized by dystonia or kinesis, which highly resemble Parkinson's disease (PD). To date, modes of neurotoxic action of Mn are still poorly understood, but are thought to be strongly related to Mn accumulation in brain and subsequent oxidative stress.¹⁸⁻²¹

Additionally, the interaction of Mn and PD-associated genes is of significant research interest since only 10–20% of PD cases are documented as having genetic causes.²² This implies an environmental contribution to its pathogenesis. One of the genes imparting heightened risk for autosomal recessive, early-onset PD is DJ-1, which is involved in regulating oxidative stress pathways.²³ Therefore, the determination of Mn content in biological samples such as *C. elegans*, especially with the background of mutated DJ-1, may help to clarify the mechanisms of Mn-induced neurotoxicity.

Experimental

Chemicals

All chemicals were of the highest purity available. MnCl_2 (>99.995%) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and NaCl from Fisher Scientific (Loughborough, UK). For ICP-MS optimization, solutions were prepared using nitric acid (65% w/w), trace element grade from Fisher Scientific and ICP-MS single element standard solutions containing Mn or Rh, respectively (both 1 mgL^{-1} , SCP Science, Courtaboeuf, France).

C. elegans strains and handling

C. elegans strains were handled and maintained at 20 °C as described earlier.¹ The following strains were used: N2 (wildtype; WT) (Caenorhabditis Genetics Center, CGC), the *djr1.1*

(tm918) deletion mutant (CGC) and the djr1.1 (tm918), djr1.2 (tm1346) double deletion mutant. The double deletion mutants were obtained by crossing the deletion mutants djr1.1 (tm918) and djr1.2 (tm1346) provided by CGC.

Synchronous L1 populations were obtained by isolating embryos from gravid worms using a hypochlorite bleaching solution (1% NaOCl and 0.25 M NaOH) and separating eggs from worms and bacterial debris by flotation on a sucrose gradient, as previously described.²⁴

Acute Mn treatments and sample preparation

2 M MnCl₂ stock solutions were prepared in 85 mM NaCl and diluted down to 10 mM. MnCl₂ treatments were performed for 30 min using 50 000 synchronized L1 worms to ensure that all groups are incubated under the same conditions. Subsequently, worms were pelleted by centrifugation at 7000 rpm for 3 min, washed twice with 85 mM NaCl and twice with bidistilled water and diluted down to 1–2 worms per μ L. Finally, worms were prepared for LA-ICP-MS analysis by air-drying single, separated worms on microscopic slides (Superfrost, Thermo Fisher Scientific, Waltham, MA, USA). Prior to analysis, a light-microscopic image was obtained from each worm using a microscope (BZ 9000, Keyence Deutschland GmbH, Neu-Isenburg, Germany) using the bright-field option and CFI 60-optics (Nikon, Chiyoda, Tokyo, Japan).

Instrumental settings

Experiments were carried out using a LSX-213 G2⁺ LA system (Teledyne CETAC Technologies, Omaha, NE, USA) coupled to a quadrupole-based ICP-MS instrument (iCAP Qc, Thermo Fisher Scientific, Bremen, Germany), which is equipped with a flatapole collision/reaction cell (QCell). The ablation system included a low-volume HelEx cell as an ablation chamber and quintupled, Q-switched Nd:YAG laser (wavelength 213 nm), whose laser beam could be focused on a laser spot down to 4 μ m in diameter. The ablation of the samples was performed in a multiline scan using the parameters listed in Table 1. For analysis, the ablated material was transferred into the ICP using He as carrier gas. For improved transport efficiency, an additional Ar flow was mixed with the carrier gas downstream of the ablation cell. Between LA and ICP-MS instruments, a Peltier-cooled cyclonic spray chamber equipped with a μ FLOW ST nebulizer (Elemental Scientific, Omaha, NE, USA) was added in order to introduce an additional wet aerosol into the ICP system. This improves the stability of the plasma, and also continuously delivers Rh as internal standard (0.01 μ g L⁻¹ in 2% (v/v) HNO₃) to monitor and if required, compensate for drift effects during analysis.

The ICP-MS instrument was tuned for maximum sensitivity on the only naturally occurring isotope of Mn at m/z 55. Due to strong spectral, polyatomic interferences *e.g.* ⁴⁰Ar¹⁵N⁺, measurements were performed in the high sensitivity kinetic energy discrimination mode (KEDS) with He as cell gas. The tuning procedure is described below, while other important parameters are shown in Table 2.

To evaluate the performance of the instrument, limits of detection (LOD) were determined by the analysis of a Mn standard solution (1 μ g L⁻¹ in 2% (v/v) HNO₃) and dilute HNO₃ (2% (v/v)) as blank reference. Both solutions were analysed in triplicate with five main runs

and a dwell time of 0.1 s. Based on these data, LODs were calculated according to Boumans (3σ criterion).²⁵

Data processing

The obtained transient signals were converted into color-coded 2D distribution images using ImageJ (National Institute of Health, Bethesda, MD, USA) using the lookup table 'blue green red'.

Results and discussion

Sample preparation

To improve the throughput of the method, a fast and easy sample preparation was needed. Accordingly, the L1 worms were washed after incubation, pelleted by centrifugation and suspended in water. This solution was dropped onto microscopic slides and air-dried in order to obtain single, separated worms. During the drying process, worms died. From this point, the distribution of Mn is not affected by metabolic functions of the organism. Slides were immediately placed in the ablation cell of the LA system for analysis. The small diameter of *ca.* 40 μm enabled a complete ablation of the sample in one ablation run. Prior to LA, a microscopic image was recorded to correlate the elemental distribution with the structure of the worm. The complete analytical process is presented in Fig. 1.

Improved sensitivity of LA-ICP-MS

The analysis of these small organisms required the use of a small laser spot diameter in order to obtain good spatial resolution. As the amount of ablated material, and thus the analytical signal correlates with the square of the lateral dimension per pixel, the development of a highly sensitive ICP-MS method was required to achieve the desired spatial resolution. Due to polyatomic interferences on m/z 55 including $^{40}\text{Ar}^{15}\text{N}^+$, $^{41}\text{K}^{14}\text{N}^+$ and $^{39}\text{K}^{16}\text{O}^+$, an interference suppression was required to improve sensitivity. For this purpose, a collision/reaction flatapole cell was applied to perform analyses in kinetic energy discrimination (KEDS) mode. This way, the kinetic energy of ions with larger collision cross sections, such as $^{40}\text{Ar}^{15}\text{N}^+$, is reduced by collision with helium as collision gas. As a result, these ions can be prevented from entering the quadrupole by an energy barrier between the flatapole cell and the quadrupole.^{26,27}

To find the most suitable instrumental settings, the cell gas flow was first optimised by scanning the He gas flow from 0 mL min^{-1} to 6 mL min^{-1} in steps of 0.2 mL min^{-1} while analyzing a solution of Mn (1 $\mu\text{g L}^{-1}$) and a solution of dilute nitric acid as blank. Nitric acid was selected as blank solution due to its high nitrogen and natural potassium content to generate at least one possible interfering ion during the tuning process to ensure sufficient suppression of interferences. The noted intensities were plotted against the gas flow. The quotient of the analyte and blank signals was calculated in order to obtain the signal-to-noise-ratio (S/N ratio). This value was plotted against the cell gas flow, and the S/N ratio showed a maximum at a gas flow rate of 4.6 mL min^{-1} . For this reason, this flow rate was selected for further optimization.

The optimal settings of the lenses after the collision reaction cell are strongly dependent on cell gas and cell gas flow due to the altered energies of the ions. Hence, they were tuned in an automated manner for maximum sensitivity in the following order: D1 lens (while D2 lens was set at a fixed potential of -157 V), focus lens and quadrupole entrance lens. The optimal parameters are shown in Table 2.

After this optimization, the background signal for m/z 55 was around 65 cps and an instrumental LOD of 36.7 ± 0.1 ng L⁻¹ was determined. This instrumental LOD obtained by analyses of fluid standards shows that the use of KEDS mode with the improved settings discriminates the polyatomic interferences very well but retains the analyte ions. It furthermore proves that sufficient sensitivity could be obtained to conduct LA-ICP-MS analyses with a small spot diameter of 4 μ m even in *C. elegans* at the L1 stage.

Throughout the analytical procedure, a Rh standard solution (1 μ g L⁻¹) was introduced into the ICP *via* a cyclonic spray chamber. The feasibility of delivering an additional solution *via* a nebulizer was previously shown by Pickhardt *et al.* In their work, this technique was applied to develop quantification strategies.²⁸ In our case, the delivery of Rh solution was successfully carried out to obtain more stable plasma conditions by introducing a fluid and to observe and compensate for drift effects. The intensity determined for Rh during the line scans of the imaging process proved to be stable.

Example of LA-ICP-MS application

To demonstrate the capability of the developed method, several L1 worm strains were analysed, whereas only exemplary results are shown in Fig. 2. The first image (A) shows the Mn distribution in a L1 worm treated with MnCl₂ (10 mM, 30 min). The physiological range of Mn concentrations without symptoms is approximately 75 to 100 μ M, and clinical signs increase in frequency and severity above this level, suggesting that this concentration is near the threshold of toxicity.²⁹ Since the *C. elegans* cuticle is a barrier to permeation, the doses in worm exposures are generally higher compared to those used in mammals.³⁰ For example, in the worm, it is necessary to use 50 mM 6-OHDA (*vs.* 50 μ M in mammalian studies) in order to cause *in vivo* DAergic neurodegeneration.³¹ For this reason, the worms were incubated with a Mn solution of 10 mM concentration.

The signal intensities were in the range of 500 cps to 800 cps, whereas the element showed an equal distribution over the whole worm. In comparison, the untreated worm shown in Fig. 2B exhibited much lower intensities. This confirms that Mn is taken up by worms during treatment and that the obtained intensities are not the result of contamination or matrix effects like spectral interferences. Regarding Mn uptake, several mechanisms and transporters are discussed including the divalent metal transporter 1 (DMT1) (summarized by A. B. Bowman *et al.*).³² In recent studies, Au *et al.* identified and cloned three functional *C. elegans* DMT1 orthologues SMF-1, SMF-2 and SMF-3. While the three SMFs differ in localization and function, SMF-3 has to be shown as the major Mn transporter. In SMF-1 and SMF-3 deletion mutants, an increased Mn tolerance could be observed, whereas the deletion of SMF-2 increases Mn sensitivity indicating the protective role of SMF-2 against Mn.³³

Fig. 3A and B show the Mn distribution in Mn treated worms with distinct gene mutations. These mutations are concerning homologues of the PD associated gene DJ 1 which is related to oxidative stress regulation.²³ The model organism *C. elegans* contains two different homologues of this gene: djr1.1 is localized in the nucleus and djr1.2 in the cytosol of head neurons.³⁴

The first image (Fig. 3A) shows Mn levels in djr1.1 deletion mutants. Mn uptake in this mutant is significantly higher compared to WT worms. This is indicated by higher signal intensities, while the internal Rh standard remained at a constant value. Over the complete worm, intensities were in the range of 800 cps or higher. The highest intensities can be observed in the centre of the worm. This indicates that Mn is really taken up and not only sticks to the outer surface of the worm. If this was the case, the intensities in the outer parts would be higher than in the centre due to the higher amount of cuticle that is ablated in this area. All this establishes that a qualitative evaluation of Mn uptake is possible by comparing the elemental mappings of the distribution.

The second image (Fig. 3B) depicts Mn distribution in djr1.1, djr1.2 double deletion mutants. Mn uptake is significantly higher compared to the non-incubated WT-control group, but in contrast to the WT and the djr1.1 mutants, the Mn is not evenly distributed over the organism. In the rostral part of the worm, obtained intensities were around 200 cps and up to only 350 cps. In the caudal part, intensities were up to 800 cps and higher. This unequal distribution indicates that Mn only reaches the gastrointestinal tract.

Thus, these experiments provide evidence that DJ 1 homologues can influence Mn uptake in *C. elegans*. For example, Mn uptake was increased due to deletion of djr 1.1, whereas the elemental distribution of Mn was altered significantly in the djr 1.1, djr 1.2 double deletion mutants.

Conclusions

In this work, for the first time, we demonstrate that LA-ICP-MS imaging is a powerful tool to examine the uptake of Mn in *C. elegans* L1 larvae. An exquisitely sensitive ICP-MS method enabled imaging of Mn distribution even in juvenile forms of this small organism, with an excellent spatial resolution of 4 mm. In addition, the developed fast and easy sample preparation allowed a relatively high throughput processing of the animals. In future studies, this method may be applied to other larval stages of *C. elegans* or to adult worms to analyse metal uptake and distribution thereby facilitating the understanding of metal toxicity. Furthermore, the development of a quantification method would give helpful additional information about concentration levels inside the worm. In addition, the method can be easily applied for analysis of other toxicologically relevant trace elements.

However, to state the influence of DJ 1 homologues more precisely, it is necessary to evaluate larger numbers of samples and to vary the genetic modifications.

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References

1. Brenner S, Genetics, 1974, 77, 71. [PubMed: 4366476]
2. Kaletta T and Hengartner MO, Nat. Rev. Drug Discovery, 2006, 5, 387. [PubMed: 16672925]
3. Harris TW, Chen N, Cunningham F, Tello-Ruiz M, Antoshechkin I, Bastiani C, Bieri T, Blasiar D, Bradnam K, Chan J, Chen CK, Chen WJ, Davis P, Kenny E, Kishore R, Lawson D, Lee RMuller HM, Nakamura C, Ozersky P, Petcherski A, Rogers A, Sabo A, Schwarz EM, Van Auken K, Wang Q, Durbin R, Spieth J, Sternberg PW and Stein LD, Nucleic Acids Res, 2004, 32, D411. [PubMed: 14681445]
4. Kuwabara PE and O'Neil N, J. Inherited Metab. Dis, 2001, 24, 127. [PubMed: 11405335]
5. Lai C-H, Chou C-Y, Ch'ang L-Y, Liu C-S and Lin W-C, Genome Res, 2000, 10, 703. [PubMed: 10810093]
6. White JG, Southgate E, Thomson JN and Brenner S, Philos. Trans. R. Soc., B, 1986, 314, 1.
7. Aschner M, Chen P, Martinez-Finley EJ, Bornhorst J and Chakraborty S, Front. Aging Neurosci, 2013, 5, 18. [PubMed: 23730287]
8. VanDuyn N, Settivari R, Wong G and Nass R, Toxicol. Sci, 118, 613. [PubMed: 20855423]
9. Martinez-Finley EJ and Aschner M, J. Toxicol, 2011, 2011, 895236.
10. Gao Y, Liu N, Chen C, Luo Y, Li Y, Zhang Z, Zhao Y, Zhao B, Iida A and Chai Z, J. Anal. At. Spectrom, 2008, 23, 1121.
11. McColl G, James SA, Mayo S, Howard DL, Ryan CG, Kirkham R, Moorhead GF, Paterson D, de Jonge MD and Bush AI, PLoS One, 7, e32685.
12. Hare D, Reedy B, Grimm R, Wilkins S, Volitakis I, George JL, Cherny RA, Bush AI, Finkelstein DI and Doble P, Metallomics, 2009, 1, 53.
13. Reifschneider O, Wehe CA, Raj I, Ehmccke J, Ciarimboli G, Sperling M and Karst U, Metallomics, 2013, 5, 1440. [PubMed: 23934158]
14. Becker JS, Zoriy MV, Dehnhardt M, Pickhardt C and Zilles K, J. Anal. At. Spectrom, 2005, 20, 912.
15. Kindness A, Sekaran CN and Feldmann J, Clin. Chem, 2003, 49, 1916. [PubMed: 14578324]
16. Reifschneider O, Wehe CA, Diebold K, Becker C, Sperling M and Karst U, J. Anal. At. Spectrom, 2013, 28, 989.
17. Jackson B, Harper S, Smith L and Flinn J, Anal. Bioanal. Chem, 2006, 384, 951. [PubMed: 16402172]
18. Flynn MR and Susi P, Int. J. Hyg. Environ. Health, 2009, 212, 459. [PubMed: 19181573]
19. Aschner M, Guilarte TR, Schneider JS and Zheng W, Toxicol. Appl. Pharmacol, 2007, 221, 131. [PubMed: 17466353]
20. Santamaria AB and Sulsky SI, J. Toxicol. Environ. Health, Part A, 2010, 73, 128. [PubMed: 20077284]
21. Crossgrove J and Zheng W, NMR Biomed, 2004, 17, 544. [PubMed: 15617053]
22. Dawson TM, Ko HS and Dawson VL, Neuron, 2010, 66, 646. [PubMed: 20547124]
23. Trempe JF and Fon EA, Front. Neurol, 2013, 4, 38. [PubMed: 23626584]
24. Stiernagle T, Maintenance of C elegans, in C. elegans: a practical approach, ed. Hope IA, Oxford University Press, 1999, pp. 51–67.
25. Boumans PWJM, McKenna RJ and Bosveld M, Spectrochim. Acta, Part B, 1981, 36, 1031.
26. Tanner SD, Baranov VI and Bandura DR, Spectrochim. Acta, Part B, 2002, 57, 1361.
27. Wehe CA, Bornhorst J, Holtkamp M, Sperling M, Galla H-J, Schwerdtle T and Karst U, Metallomics, 2011, 3, 1291. [PubMed: 22101428]

28. Pickhardt C, Becker JS and Dietze HJ, Fresenius' J. Anal. Chem, 2000, 368, 173. [PubMed: 11220576]
29. Stanwood GD, Leitch DB, Savchenko V, Wu J, Fitsanakis VA, Anderson DJ, Stankowski JN, Aschner M and McLaughlin B, J. Neurochem, 2009, 110, 378. [PubMed: 19457100]
30. Benedetto A, Au C, Avila DS, Milatovic D and Aschner M, PLoS Genet, 6, e1001084.
31. Nass R, Hall DH, Miller DM and Blakely RD, Proc. Natl. Acad. Sci. U. S. A, 2002, 99, 3264. [PubMed: 11867711]
32. Bowman AB, Kwakye GF, Herrero Hernandez E and Aschner M, J.Trace Elem. Med. Biol, 25, 191. [PubMed: 21963226]
33. Au C, Benedetto A, Anderson J, Labrousse A, Erikson K, Ewbank JJ and Aschner M, PLoS One, 2009, 4, e7792. [PubMed: 19924247]
34. Lee J-Y, Song J, Kwon K, Jang S, Kim C, Baek K, Kim J and Park C, Hum. Mol. Genet, 2012, 21, 3215. [PubMed: 22523093]

Fig. 1.
Overview of the workflow of the sample preparation and analysis process to visualize the distribution of Mn in *C. elegans*.

Fig. 2. Mn distribution in wildtype L1 worms; (A) after MnCl₂ incubation (10 mM, 30 min) and (B) the control group without Mn incubation.

Fig. 3. Mn distribution in different L1 worms with distinct gene manipulation after MnCl_2 incubation (10 mM, 30 min) (A) *djr1.1* deletion mutants and (B) *djr1.1*, *djr1.2* double deletion mutants.

Table 1

Conditions for the LA system used throughout the studies

Repetition frequency	20 Hz
Spot diameter	4 μm
Scan rate	4 $\mu\text{m s}^{-1}$
He flow 1	500 mL min^{-1}
He flow 2	300 mL min^{-1}
Additional Ar flow	400 mL min^{-1}

Table 2

Conditions for the ICP-MS system used throughout the studies

Power	1550 W
Nebulizer gas flow	0.78 L min ⁻¹
Cool gas flow	14.0 L min ⁻¹
Auxiliary gas flow	0.50 L min ⁻¹
Sampling depth	7 cm
Extraction lens 1	−217 V
Extraction lens 2	−183 V
D2 lens	−157 V
D1 lens	−349 V
CCT focus lens	1.6 V
Measurement mode	KEDS
Cell gas flow (He, 99.999%)	4.6 mL min ⁻¹
Bias potential	3V