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Elevated mercury bound to serum proteins in methylmercury poisoned rats after selenium treatment

Yunyun Li · Yuqin Fan · Jiating Zhao · Xiaohan Xu · Hui Jing · Lihai Shang · Yuxi Gao · Bai Li · Yu-Feng Li

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Abstract Methylmercury is a toxic pollutant and is generated by microbial methylation of elemental or inorganic mercury in the environment. Previous study found decreased hepatic MDA levels and urinary mercury levels in methylmercury poisoned rats after sodium selenite treatment. This study further found increased mercury levels in serum samples from methylmercury poisoned rats after selenium treatment. By using size exclusion chromatography coupled to inductively coupled plasma mass spectrometry, three Hg- binding protein fractions and two Se-binding protein fractions were identified with the molecular weight of approximately 21, 40, and 75 kDa and of 40 and 75 kDa, respectively. Elevated mercury level in the 75 kDa protein fraction was found binding with both Hg and Se, which may explain the decreased urinary Hg excretion in MeHg poisoned rats after Se treatment. MALDI-TOF-MS analysis of the serum found that the 75 kDa protein fractions were albumin binding with both Hg and Se and the 21 kDa fraction was Hg- binding metallothionein.

Keywords Methylmercury · Selenite · Albumin · SEC–ICP–MS · MALDI-TOF-MS · Rats

Introduction

Mercury (Hg) is a global pollutant, which is highly toxic to the human being and the environment. Among all the Hg species, methylmercury (MeHg) is one of the most toxic, which is generated by microbial

Yunyun Li and Yuqin Fan have contributed equally to this work.

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Y. Li · J. Zhao · X. Xu · H. Jing · Y. Gao · B. Li · Y.-F. Li (✉)
State Environmental Protection Engineering Center for Mercury Pollution Prevention and Control, and Laboratory of Metallomics and Nanometallomics, Institute of High Energy Physics, Chinese Academy of Sciences, Beijing 100049, China
e-mail: liyf@ihep.ac.cn

Y. Li · X. Xu
University of Chinese Academy of Sciences, Beijing 100049, China

Y. Fan
Shandong Maternity and Child Care Hospital, Jinan 250001, China

L. Shang (✉)
State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, Guiyang 550002, China
e-mail: shanglihai@vip.skleg.cn

methylation of elemental and inorganic mercury in the environment (Hu et al. 2013; Liu et al. 2014). MeHg can be biomagnified through the food chain and cross the placental and blood–brain barriers (Karagas et al. 2012; Li et al. 2015a). Recent studies have shown that the daily diet serves as the main source of MeHg exposure in human beings, among which fish and rice contributed above 83 % of daily dietary intake (Tang et al. 2015). A long-term feeding of Hg contaminated rice in rats significantly increased MeHg contents in their brains, livers and kidneys and disturbed oxidative balance (Cheng et al. 2009). Rats fed with MeHg contaminated fish led to behavioral differences and increased systolic blood pressure, lipid peroxidation and genotoxicity in blood cells(Bourdineaud et al. 2012; Grotto et al. 2011).

Selenium (Se) is an essential micronutrient and has been found to antagonize the toxicity of Hg when both were co-administered (Ganther et al. 1972; Marettova et al. 2003; Moreno et al. 2014; Ralston and Raymond 2010). For example, low-level addition of sodium selenite (Na_2SeO_3) to freshwater decreases MeHg in aquatic food chains and therefore the bioaccumulation in fish muscles (Mailman et al. 2014). Other study found that the absorption and transportation of Hg in garlic (*Allium sativm*) were significantly inhibited with Se addition in the culture medium (Zhao et al. 2013).

Although the antagonism of Hg and Se has long been recognized, people exposed to Hg are generally not exposed to Se simultaneously. Therefore, it is interesting to study if Se could be used as a therapeutic agent to detoxify Hg. A pilot Se supplementation trial in peoples exposed to MeHg through fish consumption found significantly reduced pubic hair Hg level, suggesting Se could reduce the body load of Hg in these exposed populations (Seppanen et al. 2000). Treatment with appropriate level of Se in rice planted in Hg contaminated farmland was found to reduce Hg accumulation in grains, thereafter to protect the health of the rice-consuming populations (Li et al. 2015b) Se supplementation was also found to decrease urinary malondialdehyde (MDA) and 8-hydroxy-2-deoxyguanosine levels in the Hg exposed residents due to Hg mining, thus promoted their health status (Li et al. 2012). Our recent animal study found decreased hepatic MDA levels and urinary Hg excretion in MeHg poisoned rats after Se treatment (Jing et al. 2014). The decreased hepatic MDA levels suggest the decreased oxidative damage after Se supplementation.

Table 1 Total Hg and Se concentrations in serum samples (mean ± SD, n = 3)

	The control group	Se treatment group	
		30 days	90 days
Hg (μg/L)	85.1 ± 3.9	103.9 ± 5.8	112.2 ± 10.2
Se (μg/L)	409.7 ± 18.7	649.7 ± 15.8	676.8 ± 36.2

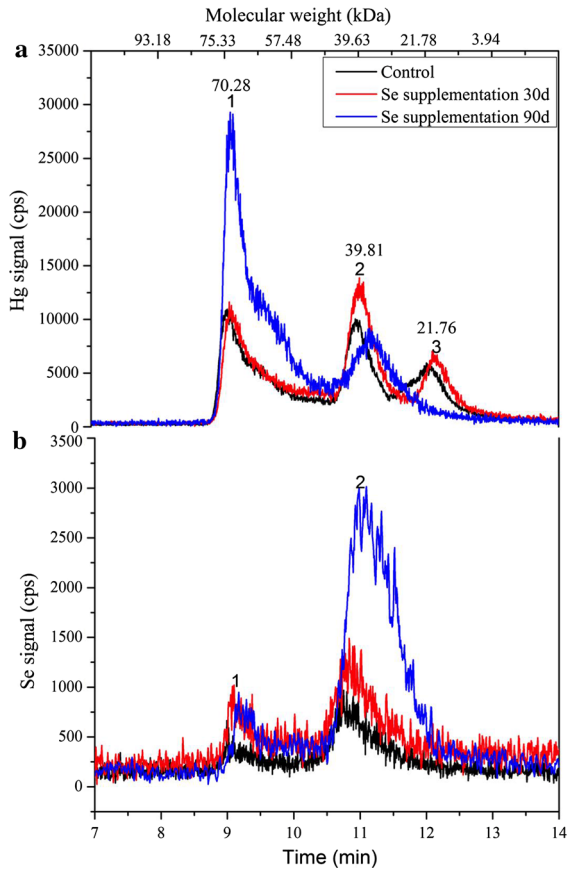


Fig. 1 SEC-ICP-MS chromatogram of serum samples at different groups. **a** Hg, **b** Se

However, the reason for the decreased urinary Hg excretion was not clear yet.

As is known, urine is formed through the extraction of the soluble wastes from the bloodstream by kidney (Yang et al. 2014). The decreased urinary Hg excretion may be ascribed to the decreased filtration of Hg from the bloodstream by the kidney after Se treatment. Therefore, this study investigated the mercury, selenium levels and Hg and/or Se binding proteins in

serum samples from the MeHg poisoned rats after selenite treatment.

Materials and methods

This study was approved by the Ethics Committee on Animal Care of Institute of High Energy Physics, Chinese Academy of Sciences.

The MeHg poisoned rats and selenite treatment

The SD rats were purchased from Beijing Vitalriver Company and were maintained in animal care facility with room temperature maintained at 26 °C, relative humility at 40–70 %, and a 12 h light and 12 h dark cycle. The rats were exposed to MeHg by gavage every other day and the dose of MeHg was 4 mg/kg diluted in normal saline. MeHg was of analytical grade (95 %, Alfa Aesar). After 4 weeks of gavage, behavioral symptoms of MeHg intoxication were shown in most rats, like the decreased mobility and hind-leg ataxia (Jing et al. 2014).

The MeHg poisoned rats were randomized into two groups (each group with 9 rats) as follows and treated through gavage every other day: (i) The control group with normal saline; (ii) The selenite treatment group with 300 μg/kg Na_2SeO_3 in normal saline. Na_2SeO_3 was of analytical grade (95 %, Beijing Chemical Plants, China).

Blood samples were collected through eyeball extirpating before Se treatment and after Se treatment for 30 days and 90 days. The serum samples were obtained by centrifugation of blood samples at 4000 rpm for 20 min and then were stored at −80 °C prior to further analysis.

Determination of Hg and Se in serum samples

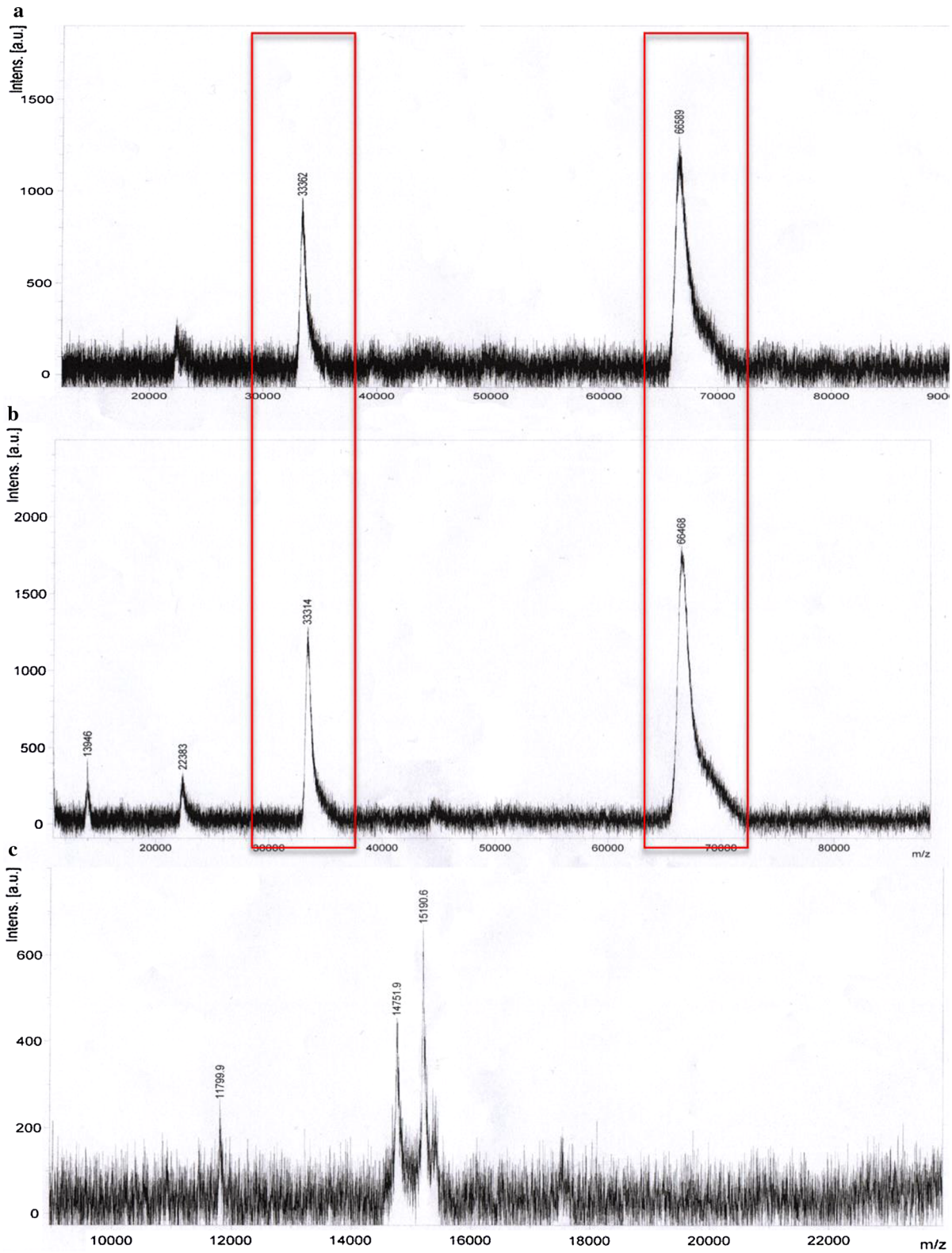
0.5 mL serum samples were mixed with 2 mL HNO_3 (BV-III grade) and 0.5 mL 30 % (V/V) H_2O_2 (MOS grade) in polytetrafluoroethylene (PTFE) digestion vessels. The vessels were heated at 160 °C for 5 h in sealed pots in an oven. The resulting solutions were volatilized to 0.5–1 mL at 90 °C, and then diluted to 5 mL with 2 % HNO_3 containing 0.1 % β-mercaptoethanol. Total Hg and Se concentration was determined by inductively coupled plasma mass spectrometry (ICP-MS, Thermo Elemental X7, USA) with indium (In) and bismuth (Bi) as the internal standards. A reference material Seronorm™ trace elements in serum (203105, L-2) was used for QA/QC with the recovery falls in the range of 90–110 %. The operating conditions of ICP-MS were shown in Table S1.

Separation of Hg-, and/or Se-containing proteins by SEC–ICP–MS and characterization by MALDI-TOF-MS

0.5 mL serum samples were diluted by adding 2 mL 150 mmol/L phosphate buffer solution (PBS, pH = 7.2) and then separated by size-exclusion chromatography (SEC, Zenix SEC-150) connected with ICP-MS. The separation conditions were shown in Table S2. The Hg- and/or Se- binding proteins monitored by both UV and ICP-MS were collected and concentrated using ultra centrifugal filter units (Amicon Ultra-4 Centrifugal filter Units, Millipore, USA) with a 3000 Da molecular weight cut-off. The concentrated samples were then analyzed with a matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS, Autoflex, Bruker).

The in vitro interaction of MeHg and/or Se with bovine serum albumin or metallothionein

Bovine serum albumin (BSA) and metallothionein of rabbit's liver (MT) (>99 %, lyophilized power) were purchased from Sigma (USA). BSA and MT solutions were prepared in a PBS buffer solution (0.01 mmol/L, pH = 7.2). The concentrations of BSA and MT were both 150 μmol/L. The protein solutions were designed as follows: (1) 150 μmol/L protein solutions (2) 150 μmol/L protein solutions + 5.4 mmol/L MeHgCl (3) 150 μmol/L protein solutions + 5.4 mmol/L MeHgCl + 5.4 mmol/L Na_2SeO_3. For the third group, MeHgCl was added into the protein solutions and then shaken for 1 h. Mercury ions were removed using ultra centrifugal filter units with a 3000 Da molecular weight cut-off. And then equimolar Se was added and then shaken for 1 h. Se ions was removed and proteins were preconcentrated by using ultra centrifugal filter units with 3000 Da cut-off. The protein concentrations in preconcentrated samples were diluted to 10 mg/mL for BSA and 1 mg/mL for MT. Then protein solutions were analyzed with MALDI-TOF-MS .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◀**Fig. 2** MALDI-TOF-MS analysis of Hg-containing fractions. **a** fraction 1, **b** fraction 2, **c** fraction 3 separated by SEC-ICP-MS as in Fig. 1

Results and discussion

Hg and Se levels in the serum samples

The Hg and Se levels in the serum samples are shown in Table 1. The concentration of Hg and Se in serum from the control group (MeHg poisoned rats) before Se treatment was 85.1 ± 3.9 μg/L and 409.7 ± 18.7 μg/L, respectively. After Se treatment, Hg contents increased to 103.9 ± 5.8 and 112.2 ± 10.2 μg/L while Se increased to 649.7 ± 18.7 and 676.8 ± 36.2 μg/L on the 30th and 90th day, respectively. It can be seen that both Hg and Se levels in serum increased significantly after Se treatment compared to the control. This is similar to the co-administration of both Hg and Se in mice. For example, it was reported that more Hg levels in serum were found in mice co-administered with both Hg and Se than those received Hg alone (García-Sevillano et al. 2015). In this study, the increased Hg level in serum should come from the release of Hg from the internal tissues to the bloodstream since Hg was stopped giving to the rats after Se treatment. The most possible release source organ should be kidney since it is the primary target organ after exposure to mercury (Zalups 2000). This was also supported by the finding that the Hg level in kidneys of these Se treated rats was lower than that in the control group (Jing et al. 2014). As is known, the binding affinities of Hg for Se (10^{45}) are up to a million times higher than that to sulfur (10^{39}) (Chen et al. 2006b; George et al. 2011; Li et al. 2014, 2015b) thus it is reasonable that the supplemented Se helps to release Hg which was bound to sulfur in enzymes and proteins from the internal tissues. This may explain the increased Hg levels in serum samples in MeHg poisoned rats after Se treatment.

Separation and identification of Hg and/or Se-containing proteins in serum

The size-exclusion column used in this study could separate compounds with molecular weight from 0.5 to 150 kDa. A group of standard proteins: 1.0 g/L transferrin, 1.0 g/L bovine serum albumin (BSA), 1.0 g/L ovalbumin albumin, 1.0 g/L myoglobin and 1.0 g/L metallothionein (MT) of rabbit's liver were used for calibration. The chromatogram presents a satisfactory separation of the standard proteins by this SEC-column (Fig. S1a). A satisfied correlation (R = 0.991) between the molecular weight and retention times was found (Fig. S1b).

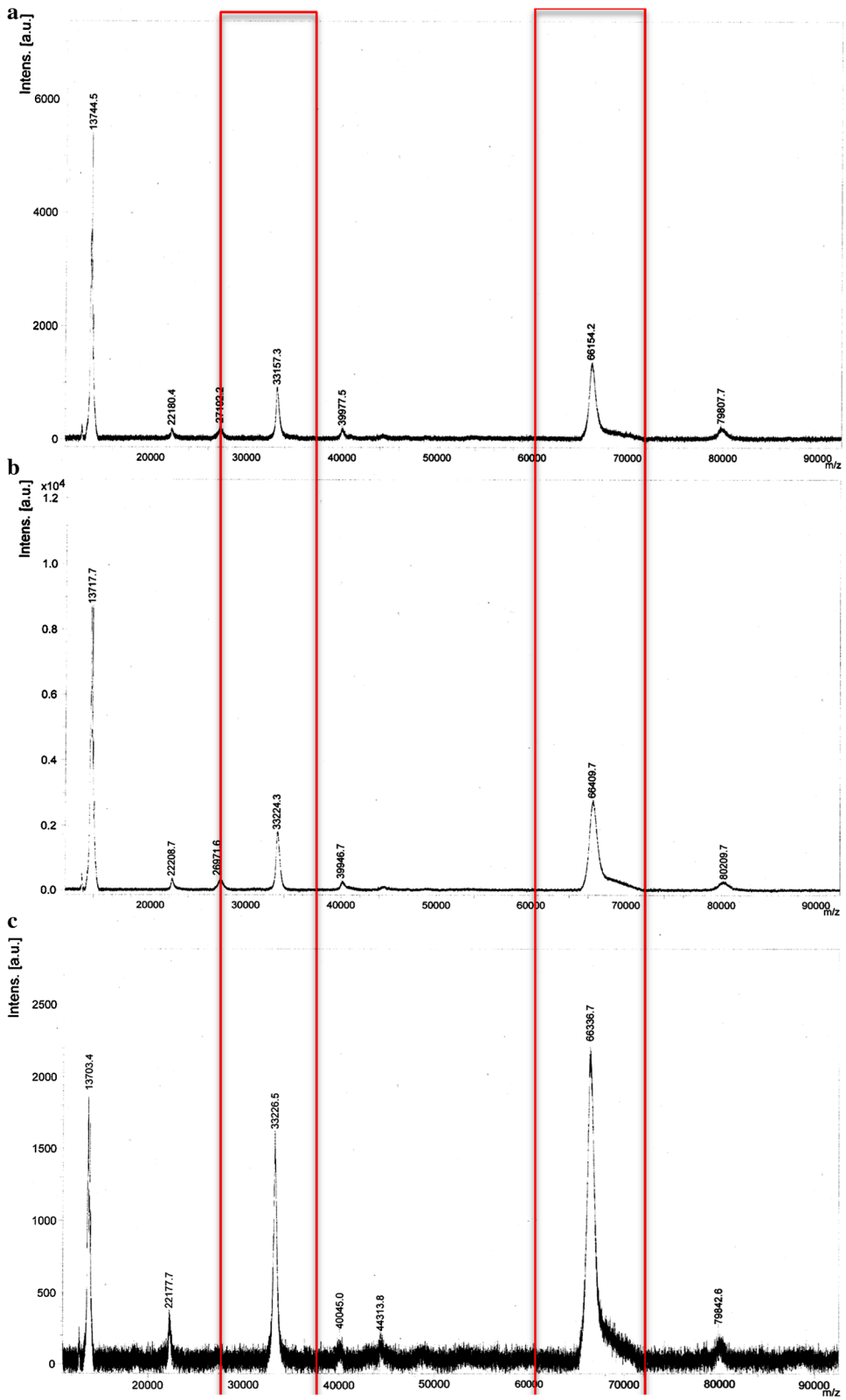
The elution curves detected by UV and ICP-MS for ^{202}Hg and ^{80}Se in the serum samples of different groups are shown in Fig. 1. Three Hg- containing protein fractions (fraction 1, 2 and 3) were eluted and identified by ICP-MS in serum samples in the control group (MeHg poisoned rats). Their molecular weights were calculated to be approximately 75, 40, and 21 kDa, respectively according to the calibration curve. Compared to the control group, Se supplementation for 30 days did not change the distribution pattern of Hg- containing protein fractions in serum samples. However, the 21 kDa Hg- containing protein fraction disappeared with Se treatment for 90 days.

Se-containing proteins were also investigated. Se was found in the two main protein fractions at 75 and 40 kDa in the control group, but not in the 21 kDa protein fraction. When treated with selenite, both fractions at 75 and 40 kDa are conserved.

Hg and Se in serum were found co-existed in 75 and 40 kDa protein fractions in serum samples in both the control group and the Se treatment group. After Se treatment, both Hg level in the 75 kDa protein fraction (fraction 1) and Se level in the 40 kDa protein fraction was found increased significantly. It can be seen that although Se treatment could release Hg from the internal tissues, the released Hg was bound to large molecular weight protein fraction. These mercury-binding proteins will not pass through the kidney filtration system and this may explain the observed decreased urinary Hg excretion in these rats.

Characterization of Hg- and/or Se- containing proteins in serum samples

MALDI-TOF-MS was used to analyze the Hg- and/or Se-containing proteins fractions. Since SEC-ICP-MS found identical protein fractions in both groups, only protein fractions (fraction 1, 2 and 3 in Fig. 1) in the control group were further analyzed by MALDI-TOF-MS. It can be seen from Fig. 2 that fraction 1 has two MS peaks at 33362 and 66589 Da. Fraction 2 has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◀**Fig. 3** MS analysis of BSA at different treatment groups. **a** BSA, **b** BSA + MeHgCl, **c** BSA + MeHgCl + Na_2SeO_3

similar MALDI-TOF-MS spectra as fraction 1. As is known, albumin is a protein abundant in plasma (40–60 % of total proteins in plasma) and an important circulating carrier of endogenous and exogenous compounds including Hg present in blood (Shcharbin et al. 2007). Considering the molecular weight and the abundance of proteins in serum, these two protein fractions (fraction 1 and 2) were assigned to albumin. This is also in agreement with other studies. For example, Lao and Yun et al. found that >90 % of the Hg was bound to albumin in human serum/plasma (Lau and Sarkar 1979; Yun et al. 2013). Our study further verified that Se was also bound to albumin. This is not strange since selenium can be nonspecifically incorporated into albumin as selenomethionine (Reyes et al. 2003). Besides, this study also found elevated Hg released from internal tissues after Se treatment was bound to albumin. Considering the molecular weight of fraction 1, another possible protein may be Selenoprotein P (SelP), which is a glycosylated plasma protein with a molecular weight of ~57 kDa and plays a definitive role in Hg detoxification (García-Sevillano et al. 2014; Suzuki et al. 1998; Yoneda and Suzuki 1997a; b). It was reported that Hg and Se could bound to SelP to form $[(HgSe)_{100}]_{35}$-SelP (Gailer et al. 2000; Suzuki et al. 1998). However, the molecular weight of $[(HgSe)_{100}]_{35}$-SelP will be >150 kDa, which is out of the detection range of the SEC column used in this study. Therefore, the Hg-, Se-containing proteins in fraction 1 were assigned to albumin but the possibility of the formation of Hg, Se-binding SelP could not be excluded from this study.

Fraction 3 shows two major MS peaks at 14752 and 15191 Da as shown in Fig. 2c. As is known, MTs are important cysteine-rich and low molecular weight (6–7 kDa) intracellular proteins with high affinity for many metals (Sakulsak 2012). The binding of metals to the MTs are the key defense mechanism against metal toxicity (Siscar et al. 2014). Hg is well documented to induce MTs synthesis(Yasutake and Nakamura 2011) and it has been reported that MTs were generally eluted as a dimmer in SEC (Chen et al. 2006a). Therefore, fraction 3 could be regarded as the dimmer of MTs containing Hg. Chen et al. reported that around 30 % of total cytosolic Hg was bound to MTs (Chen et al. 2006a). Hg load was further excreted by urine as Hg-MTs, which constitutes a vital detoxification mechanism for Hg (Garcia-Sevillano et al. 2013). Therefore, the detoxification process of Hg in rats could be attributed not only to the formation of Hg, Se-binding albumin, but also the Hg binding MTs in serum. In this study, the binding number of Hg to MTs was roughly calculated to be over 10 and this is in agreement with other studies (Jiang et al. 2002). The presence of Se seems to inhibit MTs production (García-Sevillano et al. 2015). This may explain the disappearance of Hg binding MTs with Se supplementation for 90 days in Fig. 1.

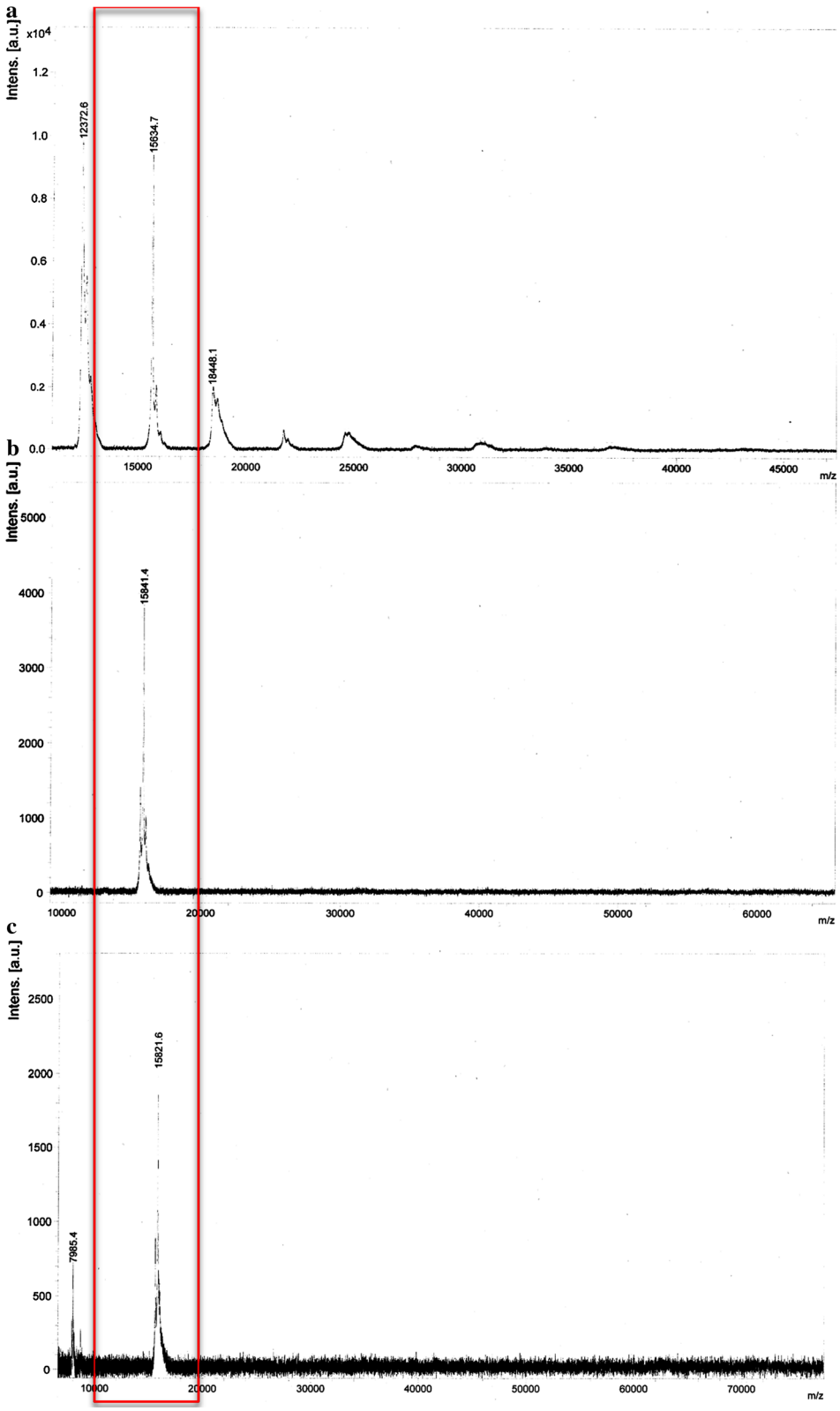
The in vitro interaction of MeHg, Se and BSA or MT

To confirm the above findings, an in vitro investigation of MeHg and Se with serum albumin or MTs was conducted. Here BSA was used instead of rat serum albumin since the former are much easier to be obtained and both own structural homology (Wang et al. 2006). MALDI-TOF-MS was used to study the molecular weight (MW) of the MeHg and/or Se binding BSA/MTs (Figs. 3, 4).

The molecular weight of BSA is 66154 Da (Fig. 3a) while the molecular weight is 66410 Da when BSA reacted with MeHg (Fig. 3b), therefore, it can be calculated through the molecular weight difference (66410–66154 = 256 Da) is roughly the molecular weight of one molecule of MeHg (MW 215), that means the complex ratio between BSA and MeHg is roughly 1:1, and this is in agreement with the fact that albumin possesses one single free sulfhydryl group with which mercury can bind (Zalups and Barfuss 1993).

When BSA was reacted with both MeHg and Se, the molecular weight is 66337 Da (Fig. 3c). The molecular weight difference between this complex and BSA is 183 Da, which is lower than that of MeHg, and is roughly the MW of two Se atoms (MW 79), suggesting Se replaced MeHg to bind with BSA.

The in vitro study further proves that Hg was bound to albumin since the molecule weight of protein fractions in Fig. 2a, b and Fig. 3a–c are all in the range of 66 kDa. The small difference among them may be due to the different numbers of Hg and/or Se binding to albumin. If we also take 66154 Da in Fig. 3a as the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◀ **Fig. 4** MS analysis of MT at different treatment groups. **a** MT, **b** MT + MeHgCl, **c** MT + MeHgCl + Na_2SeO_3

MW of rats serum albumin, then MW difference could also be calculated. The MW difference between fraction 1 (Fig. 2a) and BSA was 435 Da while for fraction 2 (Fig. 2b) it is 314 Da. The MW difference of 435 Da may be consider as two molecules of MeHg ($2 \times 215 = 430$) while the MW difference of 314 Da may be considered one molecule of MeHg plus one molecule of Se ($215 + 79 = 294$). Therefore, the protein fraction 1 in Fig. 2a may be expressed as MeHg-albumin while the protein fraction 2 in Fig. 2b may be expressed as MeHg-Se-albumin. However, the exact structure of these complexes need more direct evidence like the data from single crystal structure analysis. It is a pity that no such data is available yet, to the best of our knowledge.

The molecular weight of rabbit MTs is 15635 Da as shown in Fig. 4a, which also suggests that dimmers of MTs are formed. The molecular weight for MTs binding with MeHg is 15842 Da (Fig. 4b) and 15822 Da with both Hg and Se (Fig. 4c). Through calculation based on the MW difference, it seems one molecule of MeHg was bound to the dimmer of MTs, i.e. in the form of MT_2-Hg. The further addition of Se did not change the MW of complexes as shown in Fig. 4c, suggesting that Se did not replace MeHg. This is also in agreement with the observation of fraction 3 in Fig. 1, which shows no Se was bound in that protein fraction.

Conclusions

Se treatment in MeHg poisoned rats increased Hg concentration in their serum and promotes the formation of Hg-, and Se-containing proteins. Elevated Hg was released after Se treatment and the Hg was bound to high molecule weight protein fractions in serum, which leads to the decreased Hg excretion in urine. MALDI-TOF-MS analysis suggested that Hg was bound to albumin and MTs in serum. in vitro experiment further showed that the complex ratio between BSA and MeHg was 1:1 while Se, MeHg and BSA form MeHg-Se-BSA. Although MeHg could bind to MT to form MT_2-MeHg, Se could not replace MeHg to bind with MTs. The results suggest that Se treatment could decrease mercury load in the internal tissues in MeHg poisoned rats and form Hg, Se-containing proteins in serums.

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