Electronic Supplementary Material (ESI) for Analyst.

Supplementary Information

Sub-ppm level high energy resolution fluorescence detected X-ray absorption spectroscopy of selenium in articular cartilage

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Materials and Methods

A) Media Chemicals:

a) DMEM/F-12 (1:1) (1X) + GlutaMAXTM-I – Dulbecco's Modified Eagle Medium F-12 Nutriment Mixture (Ham) 500mL, Gibco by life technologiesTM, Ref 31331-028

b) L-Ascorbic Acid-2-Phosphate, sesquimagnesium salf hydrate ≥ 95%, Sigma-Aldrich, Ref A8960-5G

c) Gentamicin Reagent Solution, 50mg/ml, Gibco by life technologies, Cat. No 15750-060

d) HEPES, Sigma-Aldrich H-3375, special preparation, 1M, pH 7.5 filtered

e) ITS, Insulin-Transferrin-Selenium, Gibco by life technologies, Cat No. 51500-056

f) Sodium Selenite (powder), stock 10g, S5261, Sigma-Aldrich

B) ICP-MS References:

a) Single Cell Proteins (BCR 274) LGC Standards: certified Se concentration of 1.3 ± 0.05 µg/g,

b) Bovine Liver (SMR 1577b) LGC Standards: certified Se concentration of 0.73 ± 0.06 µg/g

c) Human Hair (BCR 397) LGC Standards: certified Se concentration of 2.0 ± 0.08 µg/g

d) NIST-Reference National Institute of Standards and Technology: Standard Reference Material (SRM) 1643f, NIST Office of Reference Materials, Gaithersburg, MD 20899, 18.08.2015

e) Single-element ICP-Standard solution Se 1000 mg/L ± 0.2%, ROTH, ROTI®-STAR

C) ICP-MS – Chemicals used for digestions:

a) Hydrogen peroxide (H2O2) 30% suprapure, MERCK

b) Nitric Acid (HNO3) JT Baker 65% "Baker Analyzed"; double distilled with picotrace system

D) Selenium Reference Compounds

a) SeVI (Sodium) selenate, from Sigma-Aldrich (Sodium selenate, ref. S8295-10G)

b) SeIV (Sodium) selenite, from Sigma-Aldrich (Sodium selenite, ref. S5261-10G)

c) Se-II (Sodium) selenide from Sigma-Aldrich (Sodium selenide, ref. 796948-100MG)

d) (Se-)glutathione peroxydase, from Sigma-Aldrich (Glutathione peroxidase from bovine erythrocytes, ref. G6137-100U)

e) Se-methionine, from Sigma-Aldrich (Seleno-DL-methionine, ref. S3875-25MG)

f) Se-cysteine, from Sigma-Aldrich (Se-(Methyl)selenocysteine (hydrochloride), ref. M6680-100MG)

g) Se-diglutathione – synthesis from L-glutathione and Sodium selenite (protocol see below)

h) Se-cystine, from Sigma-Aldrich (Seleno-DL-cystine, ref. S1650-25MG)

i) Se-cystamine dihydrochloride, from Sigma-Aldrich (Selenocystamine dihydrochloride S0520-25MG)

j) Se-urea, from Sigma-Aldrich (Selenourea, ref. 230499-1G)

k) (Elemental) Se0 red from bacterial production, kindly provided by Geraldine Sarret (1)

l) L-glutathione reduced, from Sigma-Aldrich (ref. G6013-5MG Sigma-Aldrich)

m) Fisher Chemicals Ultratrace Elemental Analysis Grade, Ultrapure Water, ref : W9-1

Experimental

A) Explant Culture

An in vitro culture of 4-mm explants excised under sterile conditions from the lateral aspect of the medial condyle of the metacarpophalangeal joints of immature male (7 days-old) bovine calves were used (Figure S2). A thin layer of the subchondral bone is present on the basal aspect of each explant. To induce maturation in cartilage explants, cartilage in serum-free medium (Dulbecco's modified Eagles medium, Insulin-transferrin-selenium (ITS), HEPES buffer (1M), and antibiotic (gentamicin, 50 μg/ml) and antimycotics were supplemented with 100 ng/mL fibroblast growth factor 2 (FGF-2) and 10 ng/mL transforming growth factor β1 (TGFβ1) (4). The combination of growth factors induces profound morphological changes in immature articular cartilage consistent with a highly accelerated maturational response within 21 days (4). Growth factor stimulation induced apoptosis and resorption from the basal aspect and cellular proliferation in surface chondrocytes (5).

Figure S1. A) Metacarpophalangeal joints of immature male (7 days-old) bovine calves. B) 4mm-explants from metacarpophalangeal joints of immature calves.

Cartilage explants were cultured supplemented with ITS (containing 6.7 ng/ml of sodium selenite in the culture medium) and medium with excess Se-supplementation where sodium selenite solution was added at 50 ng/ml, (Se-supplemented). For each group, explants are then placed in two sets: one control and another called “FT-treated” where explants are treated with the growth factors cocktail described above. There was a minimum sample number of 4, and cartilage explants in each group were taken from separate joints. After 3 weeks in culture, explants were cryo-milled and the resultant powder was freeze-dried at -40°C under vacuum (0.150 mbar) during 12 hours and stored at 4°C in a sealed box

B) Cartilage preparation for total Se analyses

To determine total selenium concentrations (Setot) in the tissues, digests of the pre-processed material were performed. After 3-weeks of in vitro culture of cartilage explants, the samples were snap-frozen in precooled hexane and underwent an ultra-thin cryo-milling in order to obtain a highly homogeneous fine powder. The powdered samples and the reference materials (between 10 to 30 mg) were weighted into acid washed (HNO_3 and HCl) Teflon beakers (Savillex©). The digestion protocol used here was selected based on a literature review (6-8). The digestion was performed in 3 mL of 14 M HNO_3 at 100 °C for 24 h followed by the addition of 1 mL of H_2O_2. After H_2O_2 attack during 12 h at room temperature, the solutions were heated at 100 °C for 72 h to accelerate the decomposition of the organic matrix. After evaporation at 100 °C for 6 h, residues were dissolved in 5 mL of 1% HNO_3 solution, heated at 100 °C for 10 min and sonicated (ultrasonic cleaner Branson200) for 5 min to guarantee complete dissolution. Finally, these homogenized samples were used for analyses.

C) ICP-QQQ: parameters and calibrations

1- Machine parameters:

Total Se concentration within these tissues were determined by Triple Quadrupole ICP-MS (ICP-QQQ; Agilent 8800). Hydrogen (H_2, 5.0, PanGas) was used as reaction cell gas to avoid interference on m/z 78. The detection was performed in MS/MS mode (m/z 78/78). Indium (In) was used as internal standard (In, 5 ppb). Further experimental details on the conditions of the Agilent 8800 are given in Table S1.

Table S1. Agilent 8800 experimental set-up for analysis of 1% HNO_3 solutions of digested articular cartilage explants: acquisition method and parameters.

Parameters	Details
Plasma conditions	General purpose
Scan type ISTD In (5ppb)	MS/MS 115 -> 115
CRC conditions	H_2 gas flow rate 7.0 mL/min
Acquisition parameters	Spectrum
Q2 peak pattern	1 point
Replicates Stabilisation time	3 10 sec
Acquisition time	0.8 min
Integration time	0.3 s (Se and In)

2- Experimental calibrations

The matrix based (1% HNO3) calibration (Figure S2) was performed in a calibration range of from 0.005 to 50µg/L Se (1 µg/L = 1 ppb). The detection limit (DL) calculated by MassHunter (based on the background signal for Se and according to definition 3 * BEC) equals to 0.007 µg/L (Figure S3). This DL was calculated using 10 blank solutions at 1% HNO3. Five digestion blanks were analyzed and the obtained value for these blanks was 0.020 µg/L. According to these values, the quantification limit of the samples was 0.09 µg/L, corresponding to 70 µg/kg (ppb) dry weight of the Se in samples. Each sample is analysed three times by ICP-QQQ. The machine standard deviation is the standard deviation of these three measured values. Machine Standard Deviation – RSD: (Variance/Average)*100.

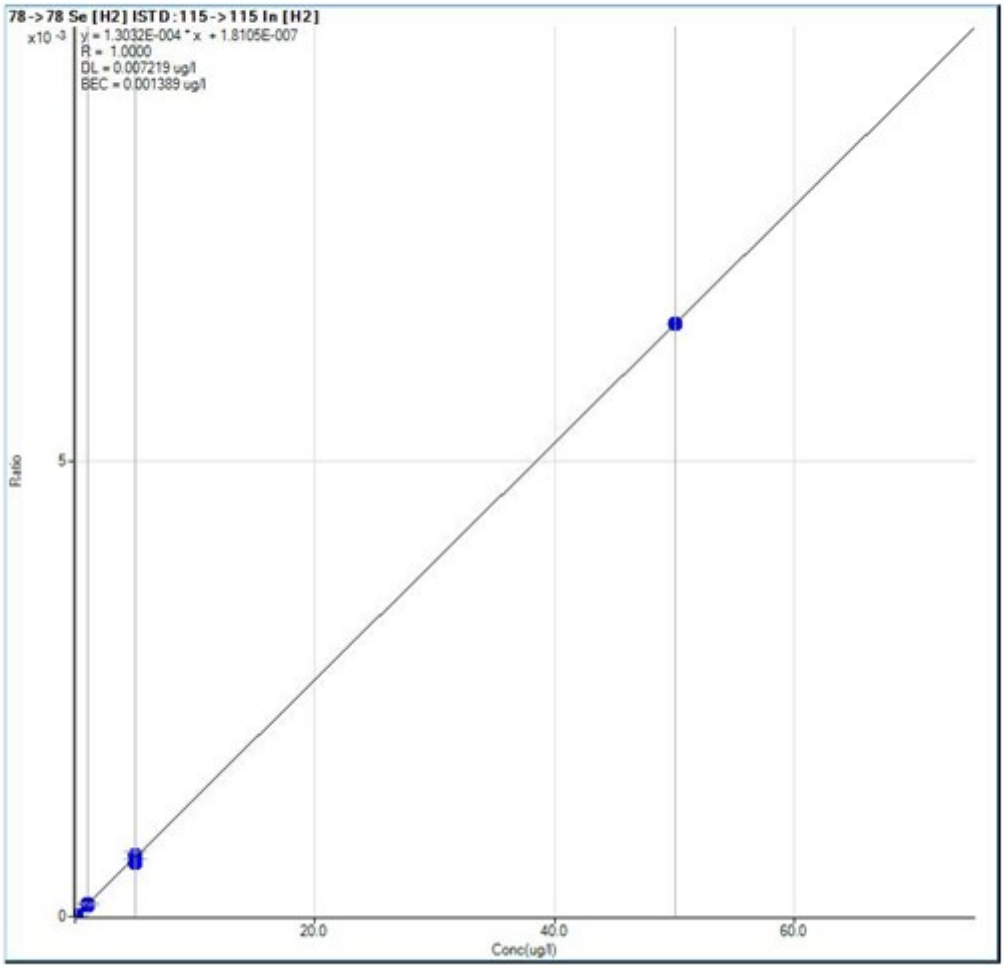
3- Experimental controls

Quality control of the digestions was done by co-digesting certified organic reference materials (Single Cell Proteins (BCR 274), Setot content 1.3±0.05 µg/g; Bovine Liver (SMR 1577b), Setot content 0.73±0.06 µg/g; and Human Hair (BCR 397), Setot content 2.0±0.08 µg/g) (Table S2) and yielded recoveries for Se of 97%, 89%, and 74%, respectively. An additional reference material (NIST 1643f "Trace Elements in Water", Setot content 11.7±0.08 µg/L) was analyzed as a routine instrument performance test (Table S3) with a recovery of 102%. As all samples had concentrations exceeding at least 10 times the quantification limit, the results of all samples can be taken into account. Concentrations of total Se in the articular cartilage tissue (independently of the applied treatment) was present in a range of 117 to 3360 ppb (Tables 1(main article) and S4).

Table S2. Quality control measurements for total Se analyses (Se_{tot}) of the digested reference materials (BCR 274, SMR 1577b, BCR 397 from LGC Standards) and the NIST reference material in a 1% HNO3 matrix. The table lists the analyzed Se contents, the SD of duplicate and /or triplicate measurements and their recoveries. *value expressed in µg/L.

Standard References (N replicates)	Reference Values ± SD (µg/g)/ *(µg/L)	Measured concentration (µg/g)/ *(µg/L)	Standard deviation (%)	Recovery (%)	Average ± SD (µg/g)/ *(µg/L)
BCR 274 Single Cell Proteins N=3	1.03 ±0.05	0.95	11	97	0.98± 0.09
		0.91	6	93	
		1.08	8	100	
BCR 397 Human Hair N=2	2.0 ± 0.08	1.28	11	67	1.41 ± 0.18
		1.54	-	80	
SRM 1577b Bovine Liver N=2	0.73 ± 0.06	0.56	13	84	0.59 ± 0.04
		0.62	9	93	
NIST-1643f liquid	11.7 ± 0.08 *	11.91 *	-	102	

Figure S2. Calibration used for Se total analyses in a range of 0.005-50 µg/L (ppb) in a 1% HNO3-matrix. The X-axis represents the standard concentration (ug/L). The Y-axis represents the ratio of the standard`s counts per second (CPS) at the respective level divided by the ratio of counts per concentration of the internal standard at the respective level (Agilent Technologies, Inc. (2015): MassHunter 4.2 Workstation Software for 8800 ICP-QQQ, G7201C, Manual).



D) High energy resolution fluorescence detected X-ray absorption spectroscopy of selenium: sample preparation

All the references have been prepared in anoxic conditions, in glove box or in a Schlenk line, to avoid any potential oxido-reductive reactions. All the references are prepared with degasified ultrapure water in order to obtain liquid (aqueous) Se-form to stay as close as possible to the native conditions.

Table S3. List of all analyzed samples with their respective intrinsic Se-concentrations including certified references materials (BRC 274, BCR 397, SMR1577b) and cartilage samples determined through ICP-QQQ analysis.

Analysed Samples	Se-concentration (ppb, µg/kg)	Standard Deviation (%)
BCR 274 Single Cell Proteins A	948.98	11
BCR 274 Single Cell Proteins B	909.56	6
BCR 274 Single Cell Proteins C	1080.38	8
BCR 397 Human Hair A	1280.71	11
BCR 397 Human Hair B	1543.62	-
SRM 1577b Bovine Liver A	562.41	13
SRM 1577b Bovine Liver B	616.45	9
Immature A	266.55	3
Immature B	177.90	11
Immature C	167.49	5
Immature D	125	-
Immature E	144	-
ITS Control A	341.80	-
ITS Control B	355.90	10
ITS Control C	500.09	9
ITS Control D	428.40	1
ITS Control E	354	-
ITS Control F	427	-
ITS FT-treated A	401.94	1
ITS FT-treated B	475.04	12
ITS FT-treated C	691.02	11
ITS FT-treated D	403.50	7
ITS FT-treated E	828.75	10
ITS FT-treated F	457	-
ITS FT-treated G	377	-
Supplemented Se Control A	1432.36	16
Supplemented Se Control B	1903.61	8
Supplemented Se Control C	2812	-
Supplemented Se Control D	2541	-
Supplemented Se FT-treated A	3017.10	3
Supplemented Se FT-treated B	1781.20	13
Supplemented Se FT-treated C	3758	-
Supplemented Se FT-treated D	3358	-
Mature A	128.00	16
Mature B	126.46	9
Mature C	124.15	3
Mature D	117.32	41
Mature E	129.23	15
Mature F	118.99	22
Mature G	163.03	9
Mature H	230.18	7
Mature I	382.21	-
Mature J	75.24	29

Figure S3. Selenium K-edge high-energy resolution fluorescence detected X-ray absorption spectra of model Se compounds (all in solution, except Se-cysteine, in solid state). The position of the maximum of the white line is indicated in table S4.

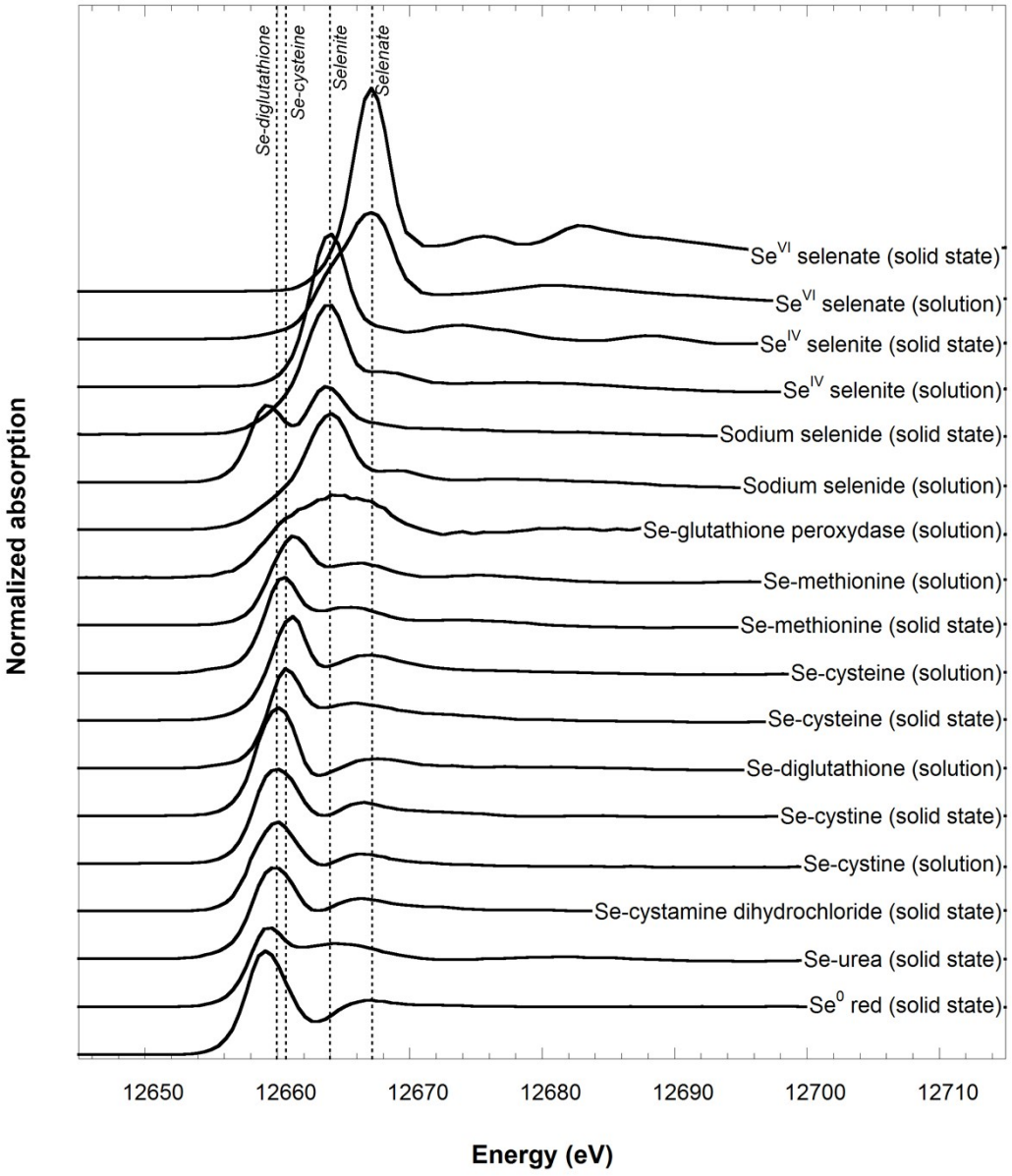
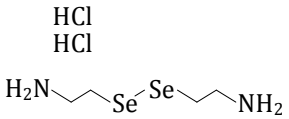


Table S4. Table of the reference selenium compounds ((organic and inorganic) spectra measured in the present work. Selenium K-edge High energy resolution fluorescence detected X-ray absorption spectra of model Se compounds have been performed in solution and/or solid state. All references have been prepared in anoxic conditions. The references are presented with their oxidation state and their chemical representation. The position of the maximum of the white line is indicated in the WL-position column.

Reference		State	Chemical Representation	WL position (eV)
(a)	SeVI (sodium) selenate	Solid state	$\begin{matrix} NaO \\ C \end{matrix} > Se = O$	12667.1
		Solution		12667.1
(b)	SeIV (sodium) selenite	Solid state	$\begin{matrix} NaO \\ NaO \end{matrix} > Se = O$	12664.0
		Solution		12664.0
(c)	Se-II (sodium) selenide	Solid state	Na_2Se (cubic)	12659.2 12663.7
		Solution	$\begin{matrix} Na \\ Na \end{matrix} > Se$	12664.0
(d)	Se-glutathione peroxydase	Solution	-	12664.8
(e)	Se-methionine	Solution	$R - Se - R$	12661.3
		Solid state		12660.6
(f)	Se-cysteine	Solution	$R - Se - H$	12661.1
		Solid state		12660.7
(g)	Se-diglutathione	Solution	$R - S - Se - S - R$	12660.0
(h)	Se-cystine	Solid state	$R - Se - Se - R$	12660.0
		Solution		12660.0
(i)	Se-cystamine dihydrochloride	Solid state		12659.8
(j)	Se-urea	Solid state	$Se = \begin{matrix} \vert \\ C \\ \vert \end{matrix}$	12659.4
(k)	Se0 red	Solid state	Elemental Se (monoclinic)	12659.1

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