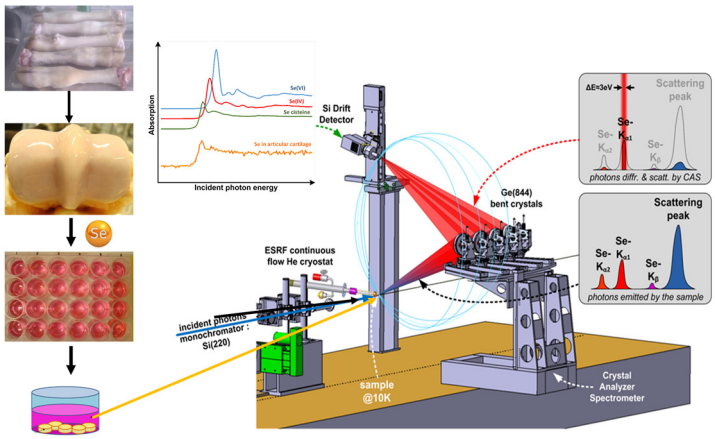
Sub-ppm level high energy resolution fluorescence detected X-ray absorption spectroscopy of selenium in articular cartilage

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The speciation of highly-diluted elements by X-ray absorption spectroscopy in a diverse range of materials is extremely challenging, especially in biological matrices such as articular cartilage. Here we show that using a high energy resolution fluorescence detected X-ray absorption spectroscopy (HERFD-XAS) technique coupled to an array of crystal analyzers, selenium speciation down to 400 ppb (µg/kg) within articular cartilage can be demonstrated. This is a major advance in the speciation of highly-diluted elements through X-ray absorption spectroscopy and opens new possibilities to study the metabolic role of selenium and other elements in biological samples.

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Introduction

Selenium (Se) is a dietary oligo-element essential for human health, with biological activities and toxic effects that are dependent on the total Se concentration and also on its chemical form[1]. Distinct chemical forms can be studied through X-ray absorption spectroscopy (XAS), an in-situ speciation method that can identify elemental species based on their redox state and metal-ligand information. Through XAS, the presence of elements such as Se in tissue can be fingerprinted in various organic or inorganic forms.[2] Importantly, the sample preparation for XAS is minimal, with samples being kept in a frozen hydrated state in an anoxic environment prior to analysis at cryogenic temperature allowing preservation of elemental species integrity, which is critical for redox sensitive elements such as Se. Using cryogenic approaches, samples can be investigated close to their natural, hydrated state, and radiation damage can be reduced. In this regard, XAS contrasts with hyphenated methods such as chromatography-mass spectrometry that are prone to speciation changes during multi-step sample preparation and analysis. However, using solid-state detector (SSD), XAS detection limits is only around ten parts per million (ppm; mg/kg),[3-5] which is higher than for hyphenated techniques, e.g., high performance liquid chromatography mass spectrometry, that has at least one order of magnitude lower detection limits, and allow high sensitivity detection (µg/kg or ppb range or lower) of specific molecules.[6] Because toxic elements including arsenic, cadmium, lead or mercury as well as elements of physiological importance such as manganese, copper or selenium typically occur in cell and tissues in the low ppb to few ppm concentration range, lowering the detection limits of XAS for elemental species to concentrations in the range of part per billion (ppb) is a major goal and challenge in analytical chemistry with implications in various fields such as biology. Our objective was to determine the major selenium species present in bovine articular cartilage during an accelerated maturation process,[7] in presence (in excess or not) of Se (Se supplemented as sodium selenite in

the culture media). Thus far, direct speciation and concentration of Se in cartilage have never been reported most probably due to a lack of in situ methods with the required sensitivity. Our interest in analyzing Se stems from its role in articular cartilage physiology, and in particular, the relationship between osteoarthritis and Se deficiency.[8] Selenium is incorporated in proteins through the amino acids selenocysteine (Sec, selenomethionine and methylselenocysteine). Selenocysteine has been shown to act as a co-factor in the reduction of antioxidant enzymes such as glutathione peroxidase and the redox-sensitive functions of selenoproteins during growth and differentiation.[9] Through a meta-analysis of studies covering five types of Se supplementation for treatment of Kashin-Beck disease (KBD) in children, Xie et al. demonstrated that this supplementation was beneficial in repairing metaphyseal lesions.[10] The ability to identify and quantify speciation of highly diluted element, i.e. much below ppm level, is of key importance to advance understanding of Se bio-transformation and incorporation in cartilage matrix.

Results and discussion

A set-up for High Energy Resolution Fluorescence Detected X-ray Absorption Spectroscopy (HERFD-XAS) technique has been recently developed at the CRG-FAME and FAME-UHD beamlines (BM30b & BM16) at the European Synchrotron Radiation Facility (ESRF, France). In our recent review,[11] we found that this method which was originally introduced by Hämäläinen et al.,[12] is available at different synchrotron sources but up to now it has mainly been used for high resolution studies in material science but not often to push the sensitivity of the method towards highly diluted elements. Although it has been used in the bio-environmental,[11,13] and bio-inorganic fields,[14] it was not used to our knowledge on elements of physiological importance within biological tissues such as cartilage.

Multi-element SDD achieving high counting rate coupled to fast electronic is commonly used for XAS in fluorescence detection for speciation of diluted element. However, with such detectors the intrinsic detection limit comes mainly from the overlap of elastic and inelastic scattering events with the measured X-ray fluorescence signal.[15,16] Still the state of the art detection limit with an SDD remains around 10-100 ppm in weight or below 1 mmol/L, but XAS measurements below 1ppm has been already performed using HERFD-XAS.[11] A schematic of the HERFD-XAS experimental set-up previously installed on the CRG-FAME beamline (BM30b) at the European Synchrotron Radiation Facility (ESRF, France) is shown in Figure 1A. Fluorescence signals were measured using a crystal analyzer spectrometer (CAS) in the Johann geometry[5]. The spectrometer was equipped with 5 spherically bent Ge(844) crystals in a Rowland geometry[5]. Crystals have a 1m radius of curvature. The spectrometer was aligned at the maximum of the Se-Kα1 fluorescence line (11.222 keV, Bragg angle: 73.09°). A helium bag (not shown in Figure 1A) was used to limit the absorption of the fluorescence signal on the sample - crystals - detector path (~2m length). Photons diffracted by the spectrometer are focused on a Silicon Drift Detector (energy resolution: 250 eV). The narrow energy bandwidth of the CAS allows us to filter out all unwanted X-ray events (mainly scattering processes) from the Se fluorescence signal (diffracted by the crystals), improving the signal-to-background ratio. The total energy resolution of the setup including both the spectrometer, for the emission photons, and the monochromator, for the incident photons, was optimized to 3.0 eV as measured from the pseudo-elastic peak (Figure 1B). A comparison between conventional XAS measurement (obtained in the transmission mode) and HERFD XAS is given in Figure 1C. The first observation is that the spectral resolution of the HERFD-XAS spectra is slightly better than that observed with conventional-XAS, as can be seen by comparing the characteristic widths of the energy levels involved. This is an effect that has been extensively explained previously,[17,18] i.e., in conventional-XAS, the final state has a 1s core–hole with an energy width of 2.33 eV. In HERFD-XAS, the Kα1 fluorescence line corresponds to 2p3/2 to 1s transition (to fill the 1s core–hole created during the absorption process). The final core–hole is 2p3/2, the characteristic width is then 1.0 eV. The improvement in the spectral resolution is (i) a direct consequence of the difference between the final state widths and (ii) an effect of the energy resolution of the CAS which allows selection between each of the final state interactions. The difference is here small, the energy resolution of the CAS being of the same order of magnitude than the 1s core–hole width. The second observation is that the pre-edge of the raw HERFD-XAS spectra is close to zero (Figure 1C). The narrow energy resolution of the CAS allows

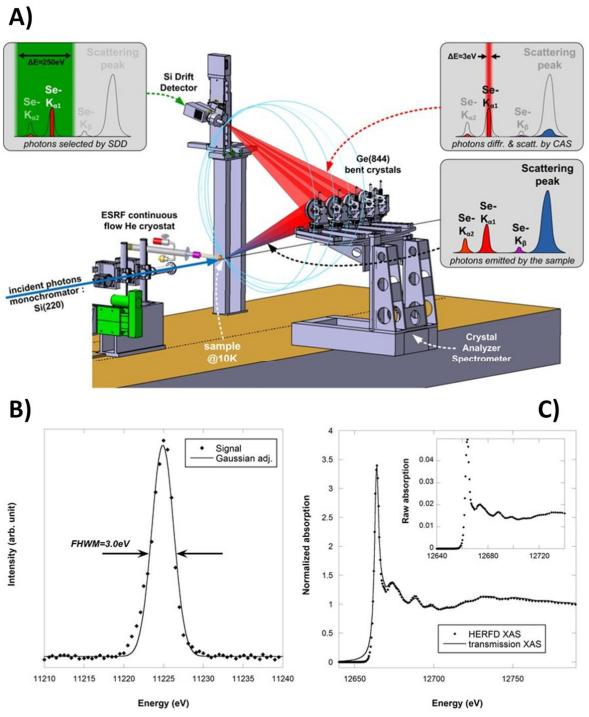


Figure 1. A) Schematic of the HERFD-XAS experimental set-up on CRG-FAME beamline (BM30B, ESRF, Grenoble, France). **B)** Pseudo-elastic peak used to measure the energy resolution of the CAS. **C)** Selenite (Sodium selenite solution, 1% Se in ultrapure degassed water and prepared under anoxic condition) spectra obtained in HERFD (dots) and transmission (line) modes. Insert: raw spectrum obtained in HERFD mode.

selection of only the photons with the appropriate energy, with almost no background. Even if the signal of interest is very small, which is the case when the probed element is highly diluted, the low background allows spectra with a good signal-to-background ratio to be obtained. The shape of the edge is sharper in HERFD-XAS mode than in classic CAS mode (Figure 1) which allows better extraction of the signal of interest from the pre-edge in the first mode.

We applied this highly performant method of Se speciation determination to an in vitro model of articular cartilage maturation for which we used cartilage explants from immature bovine metacarpophalangeal joints cultured for 21 days (Figure S1).[7] For analysis, explants were snap-frozen in precooled hexane and cryo-milled to obtain a homogeneous fine powder that was transferred under anoxic conditions to a He-cryostat for HERFD-XAS. Total Se concentrations were determined by Triple Quadrupole Inductively Coupled Plasma Mass Spectrometry (ICP-QQQ, Agilent 8800) using MS/MS mode with hydrogen (H2) as reaction gas to avoid spectral interferences on Se at m/z 78 (Table S1 and Figure S2 in supporting Information), following acid digestion of the powdered samples. The ICP-MS analysis of cartilage samples unexposed to Se (Tables 1 and S2-S3) shows a concentration range of 100-200 ppb for dried immature and mature samples, and below 60 ppb for cartilage sample in hydrated conditions. For such low Se concentration with the current experimental set-up, HERFD-XAS did not provide spectra with a quality good-enough due to insufficient signal-to-background ratios. Selenium concentration for cartilage cultured in standard serum-free medium classically supplemented with insulin-transferrin-selenium (ITS) containing 6.7 µg/L sodium selenite as the source of Se was in the range 400-500 ppb, HERFD-XAS measurements allow then the Se speciation.

Although Se concentrations were as low as 400 ppb, we obtained spectra with a good signal-to-background ratio with our experimental HERFD-XAS techniques, whereas standard XAS method was not effective.

Table 1. Total Se concentrations (Setot) of bovine articular cartilage explants in µg/kg (ppb) measured by ICP-QQQ analysis after an acid digestion procedure. Samples cultured in standard conditions with insulin-transferrin-selenium (ITS), in presence (ITS FT-treated) or absence (ITS-Control) of growth factors, or further supplemented with 50 µg/L sodium selenite, (N≥4). The native immature and matures explants were analyzed without any further culturing or treatments. The standard deviation represents the replicate variation (N=3) corresponding to the biological variation appearing in this culture model. The limit of quantification is found to be 0.020 µg/L and the sample detection limit is 0.09 µg/L, which corresponds to about 70 ppb µg/kg dry weight of Se in the samples.

Sample	Setot. (µg/kg, ppb)	Standard deviation σ (µg/kg, ppb)
Native Immature Articular Cartilage (N=5)	176	49
ITS control (N=6)	401	56
ITS FT-treated (N=7)	519	160
Se-Supplemented control (N=4)	2172	540
Se-Supplemented FT-treated (N=4)	2979	739
Native Mature Articular Cartilage (N=10)	159	83

Identification of the major Se species present in cartilage were performed by a least-squares fitting process of linear combinations (LCF) of edge spectra from a series of inorganic and organoselenium reference compounds measured in the same experimental conditions as the cartilage samples (Figures 2 and S3, Tables 2 and S4). The quality of this components determination depends firstly of the representativeness of the references set of spectra We choose the most relevant Se-biological forms that can be present within the cells and the tissue such as i) selenocysteine, ii) selenomethionine, iii) glutathionine peroxidase that were found present within the tissue by quantitative polymerase chain reaction analysis (data not shown), iv) sodium selenite that are used in cell culture and v) selenodiglutathione, the reductive metabolite of selenite. These references were analyzed pure on their solid form, but also in aqueous solutions to be as close as possible to the native state of the selenium in biological system. A second condition to fulfil in order to obtain valuable information with LCF is to have significant spectral differences between the model compounds. Almost all the selenium XANES from our references set of spectra present the same features (Figure S3): a well-defined peak after the edge, the so-called white-line (WL), and a well-marked oscillation 6-7eV after the WL. The two exceptions are the sodium selenide in the solid state (with a strong peak in the edge) and the glutathione peroxidase with a very large WL and no oscillation after. With an energy resolution around 0.6eV, the energy position of the WL (gathered in Table S4) allows to separate for example the contributions of Se(0) (WL at 12659.1 eV), Selenocysteine (12661.1 eV) and Se(IV) (12664 eV). However, it appears difficult to distinguish unambiguously several model compounds spectra, for example selenomethionine (R-Se-R, WL at 12661.3 eV) and selenocysteine (R-Se-H, 12661.1eV), the two compounds with Se-Se bounding, selenocystine (12660.0 eV) and selenocystamine dihydrochloride (12659.8 eV). Selenodiglutathione XANES present a WL at the same energy than selenocystine, but energy position and shape of the second oscillation allows to distinguish the two components.

A principal component analysis (PCA) has been performed in order to determine which and how many references are needed to perform the LCF on the samples. Because of the long acquisition time and the extremely low Se-concentration, the PCA composed a four elements collection, which can only provide an indication of the adequate references and the number of components to use: Se(IV) sodium selenite, glutathione peroxidase, selenocysteine-like compounds and selenodiglutathione. Experimental and LCF spectra results are shown on Figure 2, data are gathered on Table 2.

The LCF analysis of the HERFD-XAS spectra indicate that a major contributing species is in the form of a thioselenide compound such as selenodiglutathione (R-S-Se-S-R, Table S4). It is the major metabolite of selenite in mammalian tissues.[19] Selenite and reduced glutathione spontaneously react to form R-S-Se-S-R, and reduced glutathione concentrations are known to be high in nearly all tissues.[21]

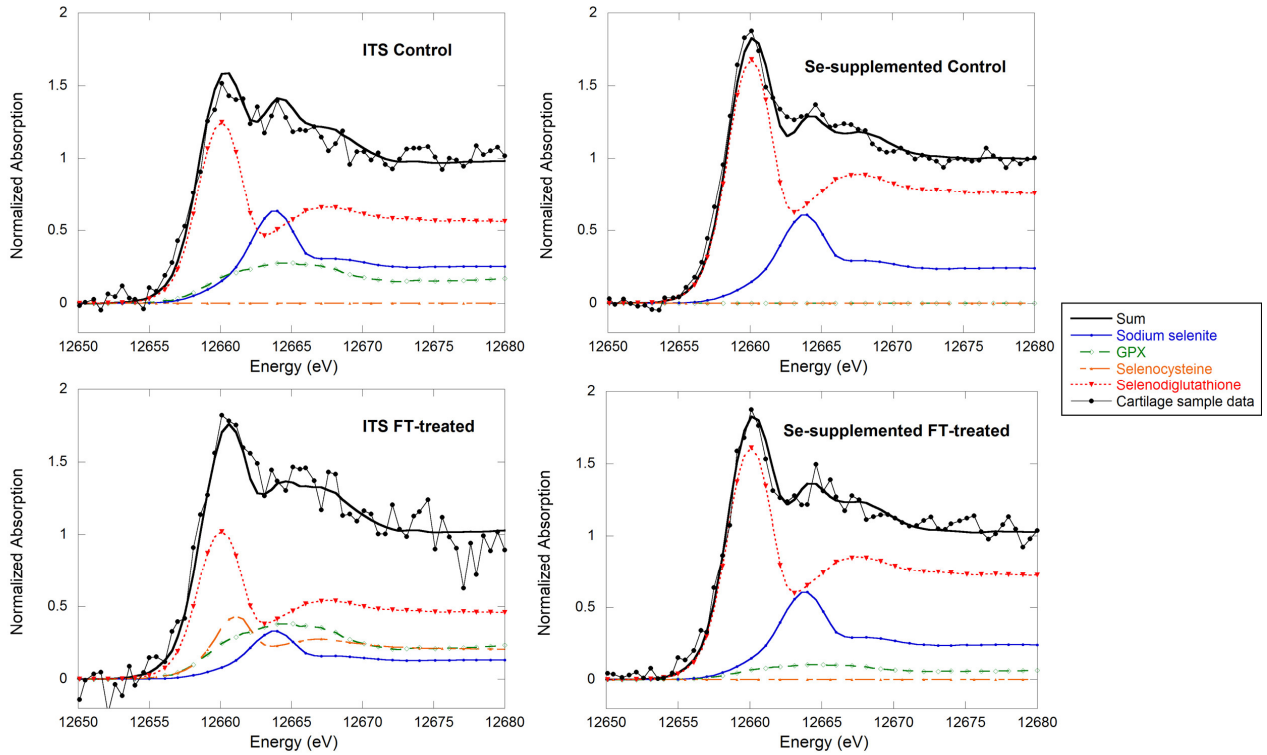
The Se HERFD-XAS spectra of ITS treated cartilage explants and those treated both with ITS and growth factors ("FT-treated") were found similar. Despite a highly accelerated maturational response for the later, , the LCF analysis of the HERFD-XAS spectra indicate that in both case alkyl selenide (R-Se-R) is the main contributing Se compounds (Selenocysteine-like and gluthatione peroxidase type of compounds) of the ITS and ITS FT-treated cartilage explants. This result indicate that we are able to detect in these cartilage explants chemical forms of Se typical of selenoproteins that are also of key interest for cellular metabolism.[19] Actually, the importance of monitoring Sec levels is highlighted by studies showing that deleting the Sec tRNA gene produced KBD-like phenotypes in mice. In humans the genetic polymorphisms in the glutathione peroxidase-1 gene has been reported to be associated to higher risk of developing KBD.[20]

Table 2. Relative abundance of Se species determined by linear combination fitting of experimental spectra using the reference selenium compounds spectra measured in the present work..

	Reference compounds				
	Seleno-diglutathione	Seleno-cysteine	Glutathione peroxydase	SeIV Sodium selenite	Rfactor (%)
Se-Supplemented Control	74 %	-	-	22 %	0.9
Se-Supplemented FT-treated	71 %	-	-	22 %	1.3
ITS Control	55%	0 %	16%	23 %	2.3
ITS FT-treated	45 %	20 %	22 %	12 %	3.9

Furthermore, when the ITS and ITS FT-treated explants are exposed to additional sodium selenite (Na_2SeO_3), LCF of the HERFD-XAS spectra indicate a limited number of contributing species, selenodiglutathione and sodium selenite, components representing 93% of the experimental spectra recovery. Selenite is known to react during its absorption with thiol compounds such as glutathione to form selenodigluthatione following a non-enzymatic pathway. Although, this interesting result deserve further studies, it can be speculated that this Se metabolite is mainly delivered into the cartilage explant tissues facilitating the selenite absorption to such significant proportion. Of note, R-S-Se-S-R is a highly reactive substrate for the thioredoxin system, and recently it has been reported that thioredoxin reductase-2 deficiency induced impaired proliferation and chondrocyte cell death. This pathological mechanism has been suggested to be another cause for osteoarthropathy, occurring as a result of Se deficiency [22]. Dietary supplementation with sodium selenite offers a potential treatment for KBD in children [23]. We noted that Se concentrations rose 5.5-fold in cartilage cultured explants following a 7.5-fold increase in sodium selenite supplementation (50 µg/L) in the culture medium when compared to normal culture conditions (ITS only), as quantified by ICP-QQQ analysis. This is in line with the Se-Kα X-ray fluorescence counts obtained in HERFD-XAS measurements ~ 5 times higher for Se-supplemented cartilage explant compare to cartilage explant obtain in normal culture conditions (data not shown). This observation was made for cartilage explants independently of the treatment i.e. with or without the addition of growth factors. These results suggest that, under supplementation with sodium selenite, Se incorporation into tissues of articular cartilage explants is limited independently of the growth factor treatment known to regulate chondrocyte metabolism [24]. The main contributing Se model compounds indicated through the LCF analysis was selenodiglutathione (R-S-Se-S-R) an intermediate chemical species produced by a non-enzymatic reaction between selenite and reduced gluthatione[19]. Selenodiglutathione is known to be metabolized further to hydrogen selenide (HSe−) via selenopersulfide (R-S-Se-S-H) formation, two volatile and highly reactive compounds, by glutaredoxin systems in the presence of excess amounts of GSH, which leads to spontaneous oxidation of HSe− to elemental Se, Se(0). These later species were not detected in our samples and HERFD-XAS spectra suggest that when our model of articular cartilage accelerated maturation is exposed to elevated levels of exogenous sodium selenite, Se in the form of R-S-Se-S-R is preferentially found in the cartilage matrix. It would be of interest to obtain a spatially resolved distribution of the Se species identified above and to explore the nature of Se bonding within chondrocytes or within the protein fraction of the articular cartilage matrix, however, this is beyond the scope of the present work. Se-XAS mapping could be achieved at sub-cellular level using state-of-the-art synchrotron micro-spectroscopy beamlines [25]. Still, we have demonstrated that up to now the limit of speciation at a sub-cellular level using such high flux micro-spectroscopy beamline was 13 ppm for arsenic [3], an element with a K-edge close to that of Se. This is still around 2 order magnitude higher than the Se concentration found in our cartilage samples. In order to bring the spatially resolved speciation method to the attractive range for biology of sub-ppm elemental concentration, the proposed HERDF XAS method could be coupled to synchrotron X-ray microprobe setup that uses Kirkpatrick-Baez mirror system as focusing optics opening a new era in the field of analytical chemistry.

Figure 2. Selenium K-edge High energy resolution fluorescence detected X-ray absorption spectra of model Se compounds. Selenium spectra of "maturated" cultured cartilage explants, which have matured in vitro during 3 weeks in medium supplemented with Insulin -Transferrin-Selenium (ITS) (which intrinsically contains 6.7 µg/L sodium selenite) (left figures) or with both ITS and sodium selenite for a total Se-concentration of 50 µg/kg (right figures), in absence (control) or presence or of growth factor (FT-treated). Energy calibration was achieved by setting the first inflection point of a Se metal foil K-edge spectrum at 12658 eV. The scans were recorded in a step-by-step mode in the range of 12600–12800 eV and with a counting time of 15 s for each measured point. For each sample, between 2 (reference compounds) and 90 (diluted samples) scans were necessary to obtain an acceptable signal -to-background ratio. For each experimental cartilage sample data, a spectral decomposition with the four major species is presented with the sum spectrum (≥93%) attesting of a good recovery.



Experimental

Experiments were performed on CRG-FAME beamline (cartilage samples and solid references) and on CRG-FAME-UHD beamline (liquid references). On both beamlines the X-rays produced by the 6 GeV ESRF storage ring, operated with a maximum current of 200 mA, were tuned through a double crystal monochromator surrounded by 2 mirrors. The main optical characteristics of the beamline are identical. The size of the monochromatic beam on the sample was around 200x100 µm² (Full Width Half Maximum, Hor. x Vert.). The first crystal of the monochromator was cooled with liquid nitrogen to optimize both the flux and the energy resolution of the incident beam[26]. Energy resolution is then close to the intrinsic resolution of the Si(220) reflection, i.e. around 0.65eV at the Se K-edge. The two mirrors at 3mrad (Si mirrors covered by an Rh layer) allow to suppress higher harmonics. The incident and transmitted beam intensities were measured with Si diodes. The sample was transferred under anoxic conditions to a He-cryostat for HERFD-XAS. A continuous flow helium cryostat was used to cool down the samples at 10K to further limit potent X-ray radiation damage and photo-reduction[27]. No beam effects were observed on selenium compounds measured previously at 300K on the same beamline, i.e. with the same photons flux on the sample[2], and therefore, we assumed that the spectra we obtained at 10K are free from any radiation damages. Only between 4 to 8 spectral acquisitions were performed at a same location on the sample. Whenever necessary, subsequent acquisition were performed on another non-irradiated region of the sample by shifting the sample vertically with steps of 150 µm (beam height was 100 µm) thus further limiting any potential beam-induced photoreduction effects. HERFD signals were measured using a crystal analyzer spectrometer (CAS) in the Johann geometry[5,15]. The set of crystals were provided by XRS TECH LLC Company (Freehold, NJ, USA) and Saint-Gobain Crystals (Nemours, France). An average spectrum of each sample was composed of up to hundred scans to improve the signal-to-background ratio. Data reduction of the XANES spectra involved energy calibration and normalization.

Conclusions

Selenium is one of the highly-diluted element found in some biological matrices and speciation at sub-ppm level is an extremely challenging area that require sensitive analytical techniques. The present work demonstrated selenium speciation down to 400 ppb (µg/kg) within cartilage explant cultured in vitro in presence or absence of sodium selenite supplementation. The LCF analysis of the HERFD-XAS spectra provide indication on the main form of selenium found in the articular cartilage samples, i.e. in the form of a R-S-Se-S-R-type complex, most probably selenodiglutathione. The present study provides a step further in the speciation of highly-diluted elements such as selenium using synchrotron high energy resolution fluorescence detected X-ray absorption spectroscopy (HERFD-XAS) coupled to an array of crystal analyzers. This open new possibilities toward a better understanding of Se metabolism in articular cartilage and degenerative joint disease

Keywords

High energy resolution fluorescence detected X-ray absorption spectroscopy • selenium • cartilage • selenoproteins• synchrotron•

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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 (H_2O_2) 30% suprapure, MERCK

b) Nitric Acid (HNO_3) JT Baker 65% "Baker Analyzed"; double distilled with picotrace system

D) Selenium Reference Compounds:

a) Se^{VI} (Sodium) selenate, from Sigma-Aldrich (Sodium selenate, ref. S8295-10G)

b) Se^{IV} (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) Se^0 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

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 g/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).

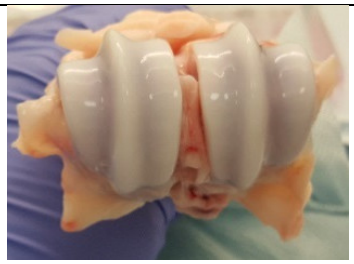




Figure S1. *Left*: Metacarpophalangeal joints of immature male (7 days-old) bovine calves. *Right* : 4 mm-explants from metacarpophalangeal joints of immature calves

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.

Cartilage preparation for total Se analyses

To determine total selenium concentrations (Se_{tot}) 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.

ICP-QQQ: parameters and calibrations

A) 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 S2.

B) Experimental calibrations:

The matrix based (1% HNO_3) calibration (Figure S3) 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% HNO_3. 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.

C) 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 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	MS/MS
ISTD In (5 ppb)	115 -> 115
CRC conditions	H_2 gas flow rate 7.0 mL/min,
Acquisition Parameters	Spectrum
Q2 Peak Pattern	1 point
Replicates	3
Stabilisation Time	10 sec
Acquisition Time	0.8 min
Integration Time	0.3 s (Se and In)

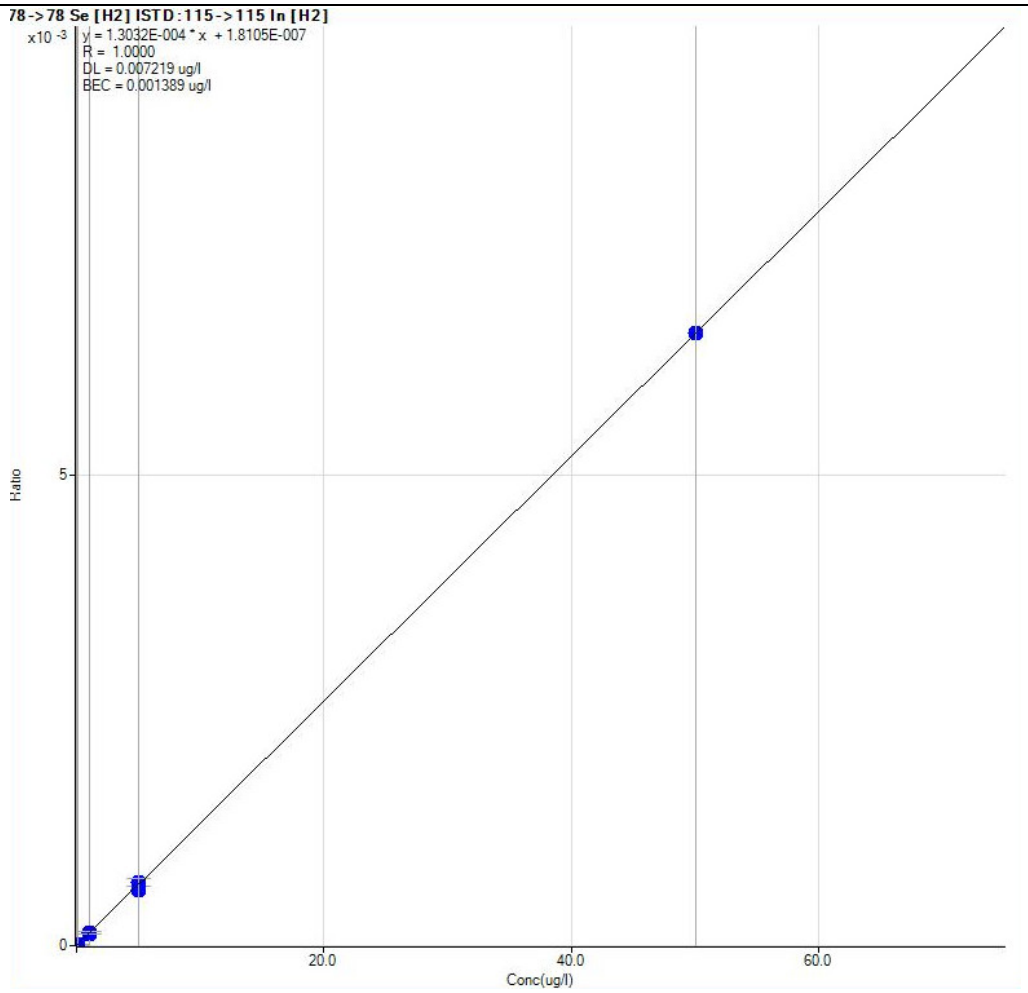


Figure S2: Calibration used for Se total analyses in a range of 0.005-50 µg/L (ppb) in a 1% HNO_3-matrix. The X-axis represents the standard concentration (ug/L). The Y-axis represents the ratio of the standard`s counts per second (CPS) at the respective level divided by the ratio of counts per concentration of the internal standard at the respective level (Agilent Technologies, Inc. (2015): MassHunter 4.2 Workstation Software for 8800 ICP-QQQ, G7201C, Manual).

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% HNO_3 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	

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

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 state as close as possible to the native conditions.

Fig. 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 S5.

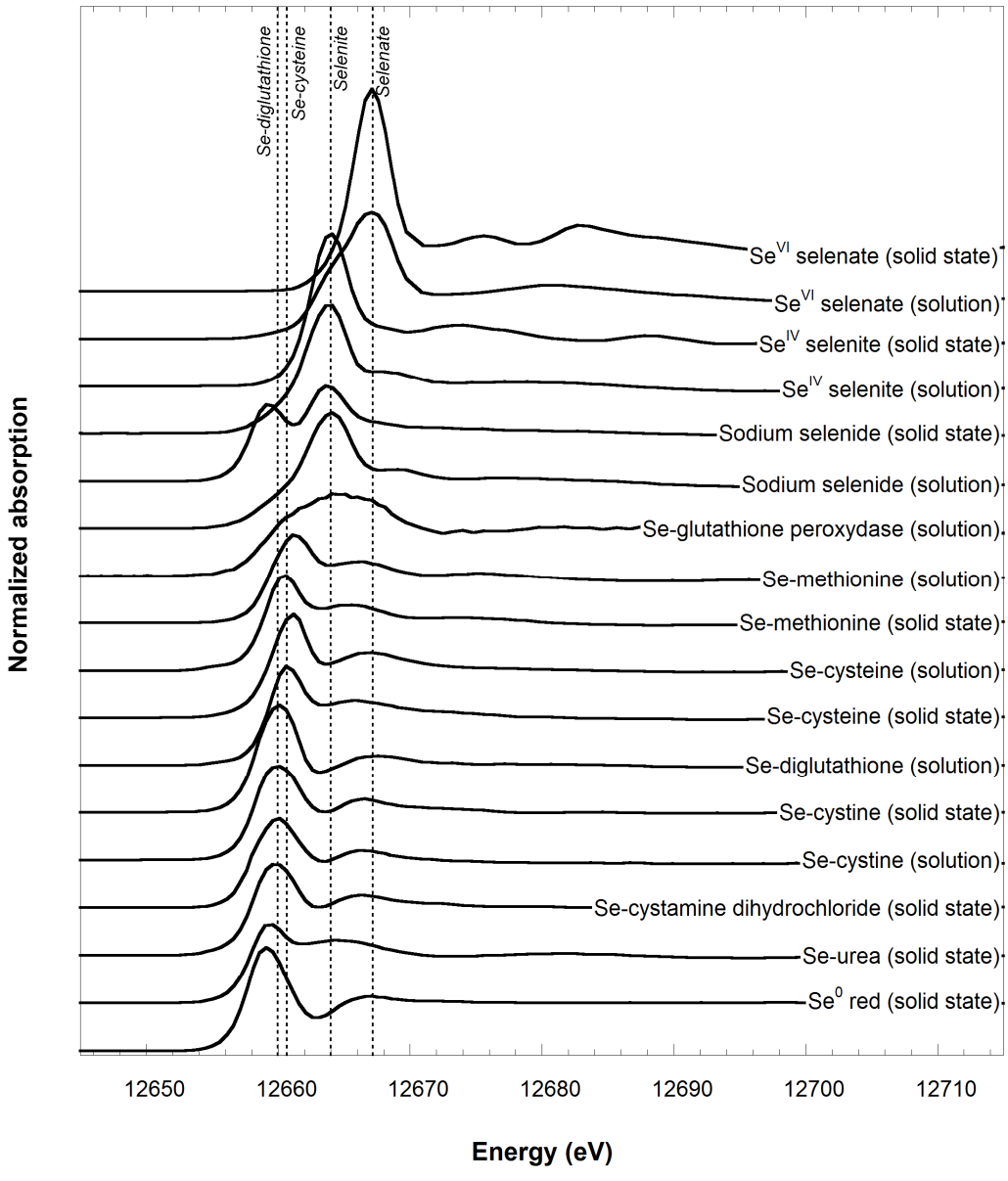
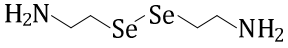


Table S4: Table of the database library of selenium compounds (organic and inorganic) analysed. Selenium K-edge High energy resolution fluorescence detected X-ray absorption spectra of model Se compounds have been performed in solution and/or solid state. All references have been prepared in anoxic conditions. 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)	Se^{VI} (sodium) selenate	Solid state	$\begin{matrix}\mathrm{NaO}\\ \mathrm{C}\end{matrix} > Se = O$	12667.1
		Solution		12667.1
(b)	Se^{IV} (sodium) selenite	Solid state	$\begin{matrix}\mathrm{NaO}\\ \mathrm{NaO}\end{matrix} > Se = O$	12664.0
		Solution		12664.0
(c)	Se^{-II} (sodium) selenide	Solid state	$\mathrm{Na_2Se}$ (cubic)	12659.2 12663.7
		Solution	$\begin{matrix}\mathrm{Na}\\ \mathrm{Na}\end{matrix} > Se$	12664.0
(d)	Se-glutathione peroxydase	Solution	-	12664.8
(e)	Se-methionine	Solution	$\mathrm{R-Se-R}$	12661.3
		Solid state		12660.6
(f)	Se-cysteine	Solution	$\mathrm{R-Se-H}$	12661.1
		Solid state		12660.7
(g)	Se-diglutathione	Solution	$\mathrm{R-S-Se-S-R}$	12660.0
(h)	Se-cystine	Solid state	$\mathrm{R-Se-Se-R}$	12660.0
		Solution		12660.0
(i)	Se-cystamine dihydrochloride	Solid state	$\begin{matrix}\mathrm{HCl}\\ \mathrm{HCl}\end{matrix}$ 	12659.8
(j)	Se-urea	Solid state	$\begin{matrix}\\ \mathrm{Se=C}\\ \\ \end{matrix}$	12659.4
(k)	Se^0 red	Solid state	Elemental Se (monoclinic)	12659.1

References:

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