Biomedical applications of the ESRF synchrotron-based microspectroscopy platform

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ABSTRACT

Very little is known about the sub-cellular distribution of metal ions in cells. Some metals such as zinc, copper and iron are essential and play an important role in the cell metabolism. Dysfunctions in this delicate housekeeping may be at the origin of major diseases. There is also a prevalent use of metals in a wide range of diagnostic agents and drugs for the diagnosis or treatment of a variety of disorders. This is becoming more and more of a concern in the field of nanomedicine with the increasing development and use of nanoparticles, which are suspected of causing adverse effects on cells and organ tissues. Synchrotron-based X-ray and Fourier-transformed infrared microspectroscopies are developing into well-suited sub-micrometer analytical tools for addressing new problems when studying the role of metals in biology. As a complementary tool to optical and electron microscopes, developments and studies have demonstrated the unique capabilities of multi-keV microscopy: namely, an ultra-low detection limit, large penetration depth, chemical sensitivity and three-dimensional imaging capabilities. More recently, the capabilities have been extended towards sub-100 nm lateral resolutions, thus enabling sub-cellular chemical imaging. Possibilities offered by these techniques in the biomedical field are described through examples of applications performed at the ESRF synchrotron-based microspectroscopy platform (ID21 and ID22 beamlines).

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1. Introduction

"New Truths become evident when new tools become available", a famous quote from Rosalyn Sussman Yalow (Nobel Prize in Physiology and Medicine, 1977), is exemplified today by the impressive number of techniques which have provided breakthroughs in cellular machinery, like far-field fluorescence nanoscopy (Hell, 2007) or electron tomography (Steven and Baumeister, 2008).

Extending those techniques to the X-ray domain offers unique opportunities to probe sub-cellular chemical processes. For example, soft X-ray microscopes have established capabilities in absorption contrast imaging of thick hydrated biological material in their near-native environments at spatial resolutions approaching 30 nm, well beyond those achievable with conventional visible light microscopy (Carrascosa et al., 2009; Jacobsen, 1999; McDermott et al., 2009; Schneider et al., 2010). In the past decade, there has been a strong tendency in X-ray microscopy to develop alternative contrast mechanisms and spectroscopic methods, which can provide both valuable complementary information on the sample nature and/or a reduction of the necessary radiation doses (Aitken et al., 2011; Andrews et al., 2011; Bacquart et al., 2007; Chapman, 2010; de Jonge and Vogt, 2010; de Jonge et al., 2008; Heine et al., 2011; Holzner et al., 2010; Jiang et al., 2010; Lombi and Susini, 2009; Schroer et al., 2010). Simultaneously, the development of high brilliance, high energy synchrotrons, coupled with advances in manufacturing technologies of focusing optics, has led to significant improvements in sub-micrometer probes for spectroscopy, diffraction and imaging applications in the

Abbreviations: EDX, X-ray energy-dispersive spectrometry; EELS, electron energy loss spectrometry; EFTEM, energy filtered transmission electron microscopy; FITR, Fourier transformed infrared spectroscopy; LA-ICP-MS, laser ablation inductively coupled plasma mass spectrometry; PXE, particle induced X-ray emission; SIMS, secondary ion mass spectrometry; SR-XANES, synchrotron X-ray absorption near-edge spectroscopy; STXM, scanning transmission X-ray microscopy; XANES, X-ray Absorption Near-Edge Spectroscopy; X-PEEM, X-ray photoelectron emission microscopy.

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multi-keV X-ray range (≥1 keV termed “hard” X-ray regime). Both by extrapolation of the experience gained in the soft X-ray regime and by the development of new techniques, “hard” X-ray microscopes now offer a unique analytical tool, which can contribute to a wide range of existing and new applications (Lombi and Susini, 2009; Ortega et al., 2009a; Paunesku et al., 2006).

Living systems, for survival, depend on their ability to accumulate, release and use certain elements, particularly metal ions, to define a certain composition that is held constant within a given homeostatic state. Several essential metal ions participate in the control of numerous metabolic and signaling pathways, but their rich coordination chemistry and redox properties confer them a propensity to randomly coordinate and catalytically react inside the cell with protein sites other than those tailored for that purpose. Indeed, about one third of all structurally characterized proteins are metalloproteins and bound metal ions or co-factors, which play a pivotal role in the structure–function relationship of proteins and other bio-molecules. In addition, all these cellular essential metals are also potentially toxic. Thus, a number of sophisticated networks of trafficking pathways are available to tightly regulate their uptake, intracellular transport and compartmentalization, and to avoid their toxic side effects. However, in spite of all the progress made, we are still merely on the brink of understanding these processes.

The synchrotron microspectroscopy techniques as developed today contribute to elucidating the distribution, concentration and chemical state of metals inside tissues and cells at the organelle level. This contribution is not only highly challenging but represents important objectives in modern analytical chemistry and an essential step towards the precise understanding of some cellular physiopathological or toxicological processes. As sketched in Fig. 1, multi-keV microspectroscopy can provide insights into the three key aspects when dealing with metallobiology. Several recent reviews have reported on biomedical applications of synchrotron-based microscopy techniques (McRae et al., 2009; Ortega et al., 2009a; Qin et al., 2011). The scope of this paper is to present the activity of the European Synchrotron Radiation Facility (ESRF) in the field of metallobiology, in particular at the two micro-spectroscopy beamlines, ID21 and ID22. The two instruments are dedicated to high spatial resolution quantitative and chemical imaging and cover a wide energy range from 2 to 70 keV providing access to almost all metal absorption edges. This X-ray microscopy platform includes the nano-imaging station ID22NI, the ID22 microprobe and the ID21 scanning X-ray microscope (SXM). It is completed by a synchrotron-based infrared microscopy end-station, located at ID21.

The details of the instruments will be presented and the sample preparations briefly discussed. This review provides some examples of application of the ID21 and ID22 X-ray microscopy platform in the biomedical field with an emphasis on physiological and toxicological aspects of metals in cells and tissues.

2. Materials and methods

2.1. ID21 scanning X-ray microscope

The ID21 beamline hosts two end-stations: (i) a scanning X-ray microscope, optimized for submicron X-ray fluorescence (XRF) imaging and X-ray Absorption Near-Edge Spectroscopy (micro-XANES) in the 2–9 keV range (P to Cu K-edges and L- or M-lines of heavier elements) (Cotte et al., 2007); (ii) a FTIR microscope exploiting the synchrotron emission in the mid-infrared region. The high spatial resolution and sensitivity of the SXM make it particularly suitable for the mapping and speciation of metals in cells and tissues, while the molecular information brought by the FTIR allows the study of organic matter (Walsh et al., 2008).

The SXM is located on a straight section equipped with two undulator plus one wiggler sources. A fixed exit double crystal monochromator offers an energy resolution down to $\Delta E / E = 1.5 \times 10^{-4}$ for spectroscopy. The microscope is located at 51 m from the source and housed in a chamber allowing operation in air or in vacuum ($10^{-5}$ mbar). A load-lock system allows a fast exchange of samples under vacuum, and greatly facilitates operation under cryogenic conditions. The microscope can host two different optical focusing configurations: either Fresnel zone plates or a Kirkpatrick–Baez (KB) mirror system, which achieve a typical spot size of 0.3 (V) $\times$ 0.7 (H) μm² with a flux of $10^9$–$10^{10}$ photons/s. The advantages of the mirror configuration are its higher efficiency and its achromaticity, which is preferable for micro-XANES.

The SXM can be equipped with a single or a 7-element HpGe fluorescence detector (Princeton Gamma-Tech, US), which offers an increased solid angle for an optimized collection of fluorescence photons, and a large area (80 mm²) XFlash 5100 Bruker Silicon Drift Diode (SDD). A compact X-ray wavelength dispersive spectrometer, achieving an energy resolution of a few tens of eV, has also been implemented for highly selective fluorescence detection. The sample stage can accommodate various sample environments depending on the nature of the samples (solid, liquid or frozen). A vibration-free cryo-stage, passively cooled by a LN$_2$ dewar, allows the analysis and preservation of hydrated samples, which is essential for biological applications, in particular for metal speciation in near native state. A visible light video-microscope allows visualization of the sample, even under vacuum, for precise alignment in the beam.

2.2. ID21 infrared microscope

This infrared end-station collects the infrared light produced by a bending magnet and a set of mirrors directs the beam toward a FT-IR Nexus Spectrometer coupled to a Nicolet Continuum microscope. When using the synchrotron source, the beamsize can be easily reduced to ~6 $\times$ 6 μm², without compromising too much the spectral quality and associated acquisition time. The detection is carried out in transmission or in reflection mode, using a 50 μm Mercury Cadmium Telluride detector, in the spectral range 4000–7000 cm$^{-1}$. Biological samples are usually prepared as thin sections deposited onto proper substrates (IR transparent windows for transmission, reflecting slides for double-transmission). By
choosing an adequate substrate, the same sample can be consecu-
tively studied with both the X-ray and the FTIR microscopes.

2.3. ID22EH1 hard X-ray microprobe end-station

The beamline ID22 is optimized for high energies and is
equipped with two undulators (a U23 in-vacuum undulator and a
U19/U32 revolver system) that enable energy tunability and high
flux in the 6–70 keV range. The beamline serves two end-stations:
the ID22EH1 microprobe and the ID22NI nano-imaging station.
The hard X-ray microprobe is located at 41 m from the source.
It delivers an X-ray focused beam down to 1 (V) × 3.5 (H) μm² with
a photon flux higher than 10¹⁹ ph/s in a monochromatic mode
(double Si (1 1 1) crystals monochromator, ΔE/E = 1.5 × 10⁻⁴). A
KB mirror system operating mostly in total reflection geometry is
used to focus the beam. This makes the end-station particularly
well adapted for X-ray Absorption Spectroscopy experiments
(micro-XANES, micro-EXAFS). Two silicon drift detectors (Vortex
EX-90, SII Nano-Technology USA Inc.) are used as main detectors
for XRF. This microscope can easily accommodate in situ environ-
ments like a cryo-stage as well as various combinations of detec-
tors for X-ray diffraction or X-ray Excited Optical Luminescence.
A parallel beam micro-tomography setup provides full-field 3D imaging as complementary imaging method.

2.4. ID22NI nano-imaging end-station

The nano-imaging end-station provides a high flux nanoprobe
for magnified phase contrast imaging and scanning XRF micro-
copy. It operates in an energy range from 17 to 29 keV and is lo-
cated at 64 m from the source. The X-ray spot is as small as
45 (V) × 60 (V) nm². The energy bandwidth of the incoming beam
can be tuned from ΔE/E = 1.6 × 10⁻² (flux of 10¹² ph/s) to
1.5 × 10⁻⁴ (flux of 10¹⁰ ph/s) to accommodate different techniques
(3D imaging and XRF and/or X-ray diffraction).

The X-rays are focused by a compact multilayer coated KB
focusing system. Two identical silicon drift detectors (Vortex
EX-90, SII Nano-Technology USA Inc.) are used to measure the
XRF signal. The end-station is optimized for scanning and full-field
microscopy with a high quality nano-spindle and precise scanning
stages.

2.5. Sample preparation

The large penetration depth of hard X-rays (ranging from a few
microns to a few mm for a biological matrix depending on the exci-
tation energy) offers possibilities for simple preparation proce-
dures and more versatile in situ observations in controlled envi-
ronments. With hard X-rays, whole cells can be studied with-
out sectioning nor using exogenous dyes; nor do they require high
vacuum conditions, and experiments can be performed on frozen-
hydrated samples, thus reducing radiation damage and optimizing
the preservation of cell structure. However, the ionizing radiation
prevents hydrated biological samples from being analyzed in their
natural state, except in some particular cases as shown for instance
in the study of vanadocytes from ascidians (Fayard et al., 2009).

Sample preparation is a key and often complex procedure in
general and more particularly for biological samples and it is far
from being a routine procedure. Samples can be prepared either
by chemical fixation methods or by fast cryofixation. Until now,
cells are cultured directly onto a plastic target covered with a poly-
mer film a few microns thick, such as polycarbonate or onto silicon
nitride membranes a few hundred nanometers thick. The protocol
must be optimized for every step of the preparation and for each
cell line. Indeed, some cells grow and differentiate preferentially
when specific adhesion molecules are used as coating on the
surface of the target. The culture medium, and any potentially
added exogenous compounds, should be removed using an appro-
priate buffer of similar pH and osmolality to preserve cellular
integrity. Generally, polyphosphate buffered saline (PBS) or ammo-
nium acetate buffer solution are used. Whenever possible, the cells
are rinsed quickly with ultrapure water to remove all extracellular
salts that would appear as hot spots in the fluorescence maps and
that can saturate the energy dispersive detector. Immediately after
rinsing, the target is carefully blotted and plunged into liquid nitro-
gen-chilled isopentane (−160 °C) or ethane (−180 °C). It can then be
stored in a liquid nitrogen dewar prior to analysis under cryo-
genic temperatures (frozen hydrated). However, it is often most
practical to prepare dried samples using a freeze-drying procedure
under vacuum at low temperature (−60 to −100 °C). Chemically
fixed cells can also be analyzed although the process can induce
some chemical modifications such as the removal of intracellular
diffusible ions, leaching or redistribution of some labile metal ions
and trace metals contamination if high-grade chemical fixation
solution is not used. (Hackett et al., 2011) Infrared and Raman
microspectroscopies have shown that formaldehyde fixation is
the best at preserving the lipids, phosphates and protein compo-
nants of cells compared to other chemical fixation (Gazi et al.,
2005; Meade et al., 2011). Thus, in some particular cases, such
preparation can be useful but should be avoided as much as possible
when X-ray fluorescence mapping or X-ray absorption micro-
spectroscopy are used due to the risk of elemental contamination
and chemical state modification. In the case of tissues, X-ray ana-
ytical techniques allow the investigation of conventional sections
prepared for histology or cryosections from, respectively, chemi-
cally fixed or cryofixed tissue biopsies. In some particular cases,
it has been demonstrated that combined chemical fixation and
high-pressure freezing can be of much interest for the ultrastruc-
tural preservation of brain tissues (Sosinsky et al., 2008). Impor-
tantly, most of the time, human tissue collections rely on
archived tissue samples that are chemically fixed. Although the ef-
fects of chemical fixation and long-term fixed tissue storage are
still under debate (Chwiej et al., 2005; Hackett et al., 2011; James
et al., 2011; Schrag et al., 2010), this can be used when tissues pre-
pared and stored in the same way are compared. The use of cryo-
imobilized cells through fast freezing methods appears as a
method of choice with the development of X-ray nanochemical
imaging techniques. The vitrification of a piece of biological mate-
rial allows to analyze the cells or the tissues in their near-native
hydrated state and provides a high-quality preservation of the
intracellular structures. This process is also known to better pre-
serve diffusible or displaceable ions. Also, free radicals, bond rup-
tured or small molecular fragments produced by ionization tend
to remain immobilized at low temperature (Meents et al., 2010).
It is very likely that X-ray nanoprobe applications in biology will
require cryo-environment below 100 K and that biological samples
will need to be processed as it is routinely done for cryoelectron
microscopy (Al-Amoudi et al., 2004; Vanhecke et al., 2011).

3. Results

3.1. Metals and cellular physiopathology

Redox and non-redox metals participate in the generation of
toxic reactive oxygen species under either metal ions overload or
a perturbation of the metal homeostasis as a result of some genetic
or environmental causes. Such imbalance will influence the
networks of trafficking pathways through which elements are reg-
ulated. Their uptake, intracellular transport and compartmentali-
ization can be disrupted leading to various diseases. Indeed, there
is evidence that metals influence the misfolding of proteins leading
to toxic aggregates involved in pathologies such as some neurodegenerative disorders: Parkinson's disease (PD) and Alzheimer's (AD). One approach to get a more comprehensive view of the role of metals in the cellular physiopathology is to study the heterogeneous distribution of metals quantitatively and their chemical local environment from tissular to sub-cellular scale ("mesoscale"). This knowledge, however, is largely "static" as we still do not have appropriate, sensitive approaches to follow fluctuations in normal metal homeostasis that accompany processes of development, differentiation, senescence and stress responses, etc.

Studies performed at the ESRF on beamlines ID21 and ID22 provide insight into the role of metals in some neurodegenerative diseases at cellular or tissular level (Bohic et al., 2008; Carmona et al., 2008; Chwiej et al., 2008; Ortega et al., 2007; Szczerbowska-Boruchowska, 2008) and fully exploit the multimodal capabilities offered by this X-ray microspectroscopy platform. For example, manganese (Mn) is known to be neurotoxic at high concentrations. It has been shown that a combined high intake of iron (Fe) and Mn may be linked to an increased risk of developing Parkinson's disease (Powers et al., 2003). Mn intoxication in rhesus monkeys results in Parkinsonian syndrome not responding to L-DOPA therapy (Olanow et al., 1996 #64174). Recently Mn inhalation tests on mice was proposed to obtain a novel animal model of Parkinson's disease (Ordonez-Librado et al., 2010). The ID22NI nanoprobe provided insight into the intracellular target of Mn (Carmona et al., 2010) in a dopaminergic cell line. The concomitant use of a fluorescent organelle specific dyes and XRF mapping at 200 nm spatial resolution suggested a preferential accumulation of Mn within the Golgi apparatus (Fig. 2). This specificity is further assessed with the use of a drug, Brefeldin A, known to cause the collapse of the Golgi apparatus, resulting in the complete redistribution of Mn throughout the cell (Fig. 2).

It is not only the spatial distribution of the concentration of elements that matters, their chemical form is also important when studying the role of metals in biological systems. Micro-XANES is a rather unique technique since it associates sub-micron spatial resolution with chemical selectivity and sensitivity to local atomic arrangement. Indeed, the transition energies and the shape of the absorption spectrum contain specific information about the chemical bonds. As an example, a study carried out by Szczerbowska-Boruchowska at ID21 reported preliminary results on sulfur in brain tumor tissues suggesting higher accumulation of sulfide ($S^{2-}$) in high-grade (IV) glioma compared to controls or grade II neoplasm (Szczerbowska-Boruchowska, 2008). In a previous work...
we evaluated the proportions and types of various sulfur species in the intraneuronal pigment neuromelanin (NM) during its different phases of development (Bohic et al., 2008). Similarly, we recently explored the sulfur chemical environment of NM in neurodegenerative disorders, particularly Parkinson’s disease. Sulfhydryl compounds of NM and alteration of the NM-biosynthetic pathway were suggested to play a role in the selective degeneration of melaninised neuron from the substantia nigra (Spencer et al., 1998). The typical sulfur K-edge l\textsubscript{3}-XANES spectra obtained on the intracellular pigment found in surviving pigmented dopaminergic neurons from sections of human substantia nigra (SN) are shown in Fig. 3. The spectral signatures for control, PD, AD and incidental Lewy Body disease (ILBD) cases were very similar. Three energies of maximum absorption were observed at 2473.6, 2476.4 and 2481.4 eV that can be assigned to different types of S functional groups. The least-square linear combination fits of normalized standard spectra of model S compounds shows that the white line (i.e. main absorption peak at 2473.6 eV) has three overlapping components consistent with the presence of thiol, organic monosulfide and organic disulfide. The other main peaks at 2476.4 and 2481.4 eV were assigned to the presence of sulfoxide and sulfonate. The model fits with the minimal set of predictor variables included S-methyl-\text{L-cysteine}, L-methionine, glutathione oxidized, methionine sulfoxide and anthraquinone sulfonic acid as model compounds and results in the best match of the experimental data. In dopaminergic neurons of control SN, a proportion of 64% organic monosulfide, 14% thiol, 13.5% disulfide, 6.4% sulfoxide and 2.1% sulfonate is found. Similar proportions are found for NM of surviving neurons within the substantia nigra of PD, ILBD and AD cases. Similar work that combined the different spectroscopic approaches (various high spatial resolution X-ray imaging modalities and FT-IR microscopy) has been recently conducted by Ducic et al. on myelinated sciatic neurons isolated from wild-type mice (Ducic et al., 2011). Such work using multi-modal X-ray imaging approaches will foster future studies in understanding morphological and chemical alterations in myelin that parallel some pathological processes involved in myelin related neurodegenerative diseases.

The study of the role of metals in the cellular physiopathology is not limited to brain studies, but can also be applied to metallobiology in cancer (Collery et al., 2004). Indeed, Farquharson et al. showed a positive correlation between higher zinc levels and estrogen receptor positive tumor cells region in breast cancer tissues as compared to surrounding regions (Farquharson et al., 2009). This work highlights the importance of synchrotron XRF in studying the relationships between such biological variables and the changes in concentration and/or spatial distribution of metals in cells and tissues. Also, one of the well accepted hallmarks of cancer is angiogenesis (Hanahan and Weinberg, 2011) and the study of Finney et al. using an XRF nanoprobe provided some insight into the role of copper regulating angiogenesis (Finney et al., 2007).

Another example of trace element mapping in cells deals with studies into the role of trace metals in single spermatozoa developments, which are scarce (Zhang et al., 1996; Kehr et al., 2009). There exists a huge variation in the size of sperm across the animal kingdom. Human sperm cells consist of a head 5 μm and a tail 50 μm long. Their development from stem cells into mature spermatozoa is the result of the spermatogenesis. Trace elements play an important role within this process. The trace elements selenium and zinc are important for sperm motility and therefore are often associated with infertility. We used the high spatial resolution and the high flux offered by the ID22NI X-ray nanoprobe to demonstrate the capabilities of the technique in this domain. This is illustrated in Fig. 4 where the distribution of phosphorus, iron and zinc are well resolved. It clearly demonstrates a compartmentalization of these elements in different parts of the cell (head
where the nucleus with highly condensed DNA is located, the mid-piece and the tail). This clearly provides exciting perspectives to better understand how infertility could be linked to certain trace elements, infertility being classified by the World Health Organization as major health concern.

3.2. Tracking of metal-based compounds and metals-related toxicology in mammalian cells

Understanding the metabolism of exogenous metals in human cells has always been a key topic relevant to a wide range of health issues. It is a necessary step for improving the efficiency and targeting of metal-based drugs, some medical imaging contrast agents and for evaluating the toxicological effects of metal-based nanoconstructs and environmental metallic complexes. Along with the recent development of nanotechnologies for nanomedicine or for industrial and engineering purposes, growing concerns have arisen about their unintentional health and environmental impact. This has brought forward the need for new analytical tools able to characterize their biodistribution at the sub-cellular scale, an issue for which the emerging X-ray nanoprobes are highly competitive. Although, up to now a very large population of cells cannot be screened by any existing micro/nanoprobe, recent work shows the importance of getting simultaneous mapping of endogenous elements (P, S, Ca, K, Cl) as well as exogenous metals in cells.

A direct application of X-ray micro/nano-probes is the mapping of the distribution of metal-based drugs and nano-vectors in cells and tissues to track their fate. The XRF signal of metals and inorganic components forming these nano-materials can be exploited to localize them. We can cite as an example the mapping of the intracellular distribution of single wall carbon nano-tubes (CNT) in macrophage cells (Bussy et al., 2008). In this case, the XRF signal of the catalyst iron content of these CNT nano-materials was used as a tracking marker in micro-XRF to localize them. It was also evidenced that the presence of the nano-tubes modifies the intracellular concentration and distribution of endogenous elements, in particular calcium. It was associated to an increase in cellular calcium uptake that could be linked to oxidative stress, inflammation or cytotoxicity. Such quantitative micro-XRF measurements thus also provide valuable information about the interaction of exogenous metals with cell homeostasis. Comparable methodology was used at the ID22 nanoprobe to screen the intracellular localization of several series of newly developed lanthanide nanoparticles used as potential platforms for bio-imaging and bio-delivery applications (Lewis et al., 2010). Recently, the ID21 SXM was also used to study the uptake and interaction of cobalt ferrite (CoFe₂O₄) nanoparticles (NPs) in Balb/3T3 mouse fibroblast cells exposed to different concentrations of NPs (Fig. 5) (Marmorato et al., 2011). Functionalized quantum dots can be used to label cancer markers or specific proteins. CdSe/ZnS quantum dots are suitable probes for nano-XRF through the XRF signal of their intrinsic Se atoms. This labeling approach is very effective at colocalizing specific intracellular targets with elements naturally present in the cell and fosters new possibilities for correlative imaging between confocal fluorescence microscopy and elemental mapping (Corezzi et al., 2009; Lewis et al., 2010). Such atomic signature is also useful when studying the toxicity of imaging contrast agents for Magnetic Resonance Imaging (MRI). Here the XRF signal of the gadolinium (Gd) atoms contained in these contrast agents is exploited. Recently Gd-based contrast agents have come under scrutiny by the Food and Drug Administration due to gadolinium side effects such as nephrogenic...
systemic fibrosis. The X-ray fluorescent Gd L-line (6.05 keV) can be detected using the ID21 SXM and the results obtained by Altissimo et al. on cultured Chang liver cells assessed the intracellular presence of Gd and highlighted a peri-nuclear distribution inside the cells (Fig. 6) (Altissimo et al., 2011). X-ray differential phase contrast imaging was performed in parallel and provided a depiction of the cell morphology allowing correlation of the Gd distribution with subcellular structures. Endogenous elements such as phosphorus, sulfur, calcium and potassium were also mapped and the variation in their concentration and distribution in exposed and control cells could be compared.

As a natural extension to XRF, micro-XANES is extremely powerful for studying the biotransformation of the metals entering biological systems. For example, Ortega et al. collected information on the carcinogenic mechanism of Cr by imaging the intracellular distribution and speciation of Cr in cells exposed to chromate compounds. Such a study was performed on cells exposed to soluble and low-solubility hexavalent chromium compounds (Ortega et al., 2005), which are carcinogens for the respiratory tract in humans. It was shown that soluble Cr(VI) compounds were fully reduced to Cr(III) in all cell compartments while low-solubility chromate compounds were partially reduced to Cr(III) and could coexist in the cell environment, as particles in perinuclear structures (Fig. 7). The stronger carcinogenicity of low solubility chromate with respect to soluble chromate compounds could thus originate from a long term exposure to a strong oxidant, Cr(VI) combined with direct genotoxic effects of intracellular Cr(III).

### 3.3. Metals in ancient medical practices

As discussed above, questions relating to the beneficial use and, conversely, to the potential danger of nanoparticulate materials seem to appear only at the end of the 20th Century. However, such materials have been in daily use since Antiquity, for example in inks, paintings and cosmetics (Murr, 2009). For instance, 2000 year-old Greco-Roman recipes for dying hair were found to rely on the synthesis of black PbS nano-crystals by reaction of lead compounds on sulfur contained in keratin (Walter et al., 2006). In the past, lead was frequently used in cosmetics and pharmaceutical products, which nowadays seems astonishing considering its known toxicity. Indeed, there is an apparent contradiction concerning the reputation of lead in ancient and modern times. A decade ago, an extensive research programme was initiated to reveal ancient cosmetic and pharmaceutical practices in Mediterranean countries. It was based on the analysis of ancient cosmetic powders preserved in their original containers over many centuries, some of

![Fig. 6.](image1.png)

Uptake of Gd in Chang liver cell exposed to 500 μM of Gd containing contrast agent for 20 h. Cells were fixed and imaged using the ID21 SXM. (a) Visible light image (b) differential phase contrast image (c) micro-XRF elemental maps of phosphorus (in red) and gadolinium (in green). Field of view 36 × 40 μm, 0.5 μm step size, 5 s dwell time, photon energy 7.3 keV (Altissimo et al., 2010).

![Fig. 7.](image2.png)

(a) Reference XANES spectra for Cr(VI) as PbCrO₄ and Cr(III) as CrCl₃, at the Cr K-edge, showing the characteristic pre-edge peak of Cr(VI) at 5.9935 keV. Micrograph (b) and XRF mapping of potassium (c), total chromium (d) and hexavalent chromium (e) of IGR-OV1 cells exposed for 24 h to PbCrO₄ at a concentration of 1 μg/cm². Maps (c and d) were acquired with an exciting X-ray energy of 6.030 keV while map (e) was acquired with an exciting X-ray energy of 5.9935 keV corresponding to Cr(VI) pre-edge peak: only Cr(VI) forms of chromium are detected. Pixel size is 1 μm × 1 μm. Scale bar is 10 μm.
which are now exhibited in the Louvre museum in Paris. Synchrotron-based X-ray diffraction was used for the identification of exotic inorganic lead hydroxy or carbonate chloride, which were proved to be synthetic products obtained by ‘wet’ chemistry (Walter et al., 1999). Synchrotron-based FTIR microscopy was employed to identify hybrid compounds, namely lead soaps, obtained when such inorganic compounds were mixed with fat (Cotte et al., 2005). Furthermore, the potential toxicity of such lead-based compounds was investigated. Generally, lead transdermal penetration is assessed by indirect measurements, such as quantification of lead concentration in blood, sweat or urine. Alternatively, synchrotron-based micro-analytical techniques were used for the direct observation of transdermal lead diffusion into transversal cuts in the skin. Model lead plasters were synthesised according to ancient recipes (Cotte et al., 2006) and applied to pig skin, for 24 h, in Franz-cell transdermal diffusion system. After dismounting, skin transversal sections were obtained and chemical and atomic distributions of drug penetration were imaged by synchrotron-based infrared micro-spectroscopy and micro-XRF.

Elemental mappings simultaneously reveal distributions of both endogenous elements (sulfur and potassium are good markers of the stratum corneum while phosphorus is more abundant in the cells of the epidermis, the corneocytes) and exogenous lead. In the present example (lead introduced as lead palmitate in propylene glycol), the diffusion of lead is stopped in the stratum corneum (Fig. 8). FTIR chemical mappings were carried out on the same skin sections, in particular to compare the penetration of an acid-based chemical, with its corresponding lead carbonate, as well as to estimate the effect of the chain length of fatty acids on their transdermal penetration (Cotte et al., 2004). Chemical mappings reveal the distribution of endogenous components (lipids, proteins) as well as exogenous fatty acids (applied as perdeuterated palmitic acid/propylene glycol for 1 day). This study shows the potential of synchrotron-based micro-analytical techniques to follow drugs and in particular penetration of metals into the skin. The combination of micro-XRF with micro-FTIR offers complementary information, based on atomic and molecular probes, respectively.

4. Discussion

In the arsenal of available analytical tools for the study of metals in biology, spatially resolved techniques are scarce and mostly rely on highly sophisticated and complex instrumentation. Table 1 compiles the key performance indicators of the most commonly used metal imaging techniques. All these techniques are complementary and should be selected depending upon the research objectives, type of sample, spatial resolution and sensitivity, required throughput and even the availability of the technique. Scanning transmission electron microscopy with energy-dispersive X-ray analysis (STEM–EDX) or energy-filtered transmission electron microscopy (EFTEM) usually require ultrathin sample sections (30 nm range) which is also a prerequisite to surface sensitive techniques such as X-ray photoelectron emission microscopy (X-PEEM) and nanoSIMS. Electron microscopy has unsurpassed spatial resolution but the sensitivity, radiation damage and the restricted field of view can be limiting factors. NanoSIMS is fully complementary to electron microscopy and provides isotopic analysis in addition to the atomic composition. However, quantification is difficult due to matrix effects that influence the secondary ion yield depending on the chemical environment of the surface being sampled. The technique allows in-depth analysis but is destructive with only a limited number of elemental images available, up to seven using recently developed multicollectors.

Other alternative techniques, such as the following, provide access to much in-depth analysis. Particle-induced X-ray emission (PIXE) technique has a good sensitivity at the micron scale and even at sub-micron resolution, as recently demonstrated (Barberet et al., 2011). It is a fully quantitative method when coupled to Rutherford backscattering spectrometry but suffers from radiation-induced effects and a limited sensitivity compared to synchrotron XRF microscopy. Laser-ablation is a highly efficient, sensitive technique at the microscale. Although destructive and with limited...
spatial resolution, high quality quantitative images of large histological sections can be obtained (Becker, 2010; Hare et al., 2010).

Micro-XRF and micro-XANES offer quantitative and chemical information with intermediate spatial resolution in constant improvement towards the nanoscale. The access to K-absorption edges and XRF emission lines of most elements, the higher penetration depths compared to soft X-rays, ions or electrons allowing imaging of thicker samples or in situ experiments, the favorable wavelengths for coupling with X-ray diffraction studies and the relatively long focal lengths and depths of focus which are advantageous for the use of specific sample environments are unique attributes to X-ray microspectroscopy (Lombi and Susini, 2009).

The full control of both energy tunability and spectral bandwidth of the incoming monochromatic radiation minimizes the radiation damage without compromising the signal-to-noise and allows the concentration of the elements to be imaged. The radiation damages become an issue even if the sample is cryopreserved. To overcome this drawback, much can be gained from the improvement of the detection efficiency of the fluorescence emitted by the sample. Indeed, X-ray fluorescence is emitted over a solid angle of the detection efficiency of the fluorescence emitted by the sample. The time for the analysis of a cell (1 h) and less than few minutes for X-ray absorption spectra. The large penetration depth of X-rays allows probing sample thickness of tens of microns and the other hand X-ray spot of few tens of nanometer started to be routinely produced making 2D scanning challenging to probe the 3D heterogeneity of biological systems where subcellular compartments may overlap. However, it is ideally suited for tomographic X-ray microspectroscopy acquisition with the unique perspective to provide chemical images at the ultrastructural scale on whole cells (de Jonge and Vogt, 2010) and avoid the use of thin sectioning.

An important limitation will be the required analytical time to produce a full 3D image of a sample like a cell. Up to now, few tens of hours are required depending on the sample size and mostly on the concentration of the elements to be imaged. The radiation damages become an issue even if the sample is cryopreserved. To overcome this drawback, much can be gained from the improvement of the detection efficiency of the fluorescence emitted by the sample. Indeed, X-ray fluorescence is emitted over a solid angle of 4π while the actual detectors only cover a few % of it. The advent of new large area and fast detectors such as the MAIA allow to gain a factor 10 in efficiency (Lombi et al., 2011). This will provide a breakthrough in the biomedical application of X-ray nanoprobe to allow fast 2D/3D images of the chemical composition and changes within cellular and sub-cellular structures.

5. Conclusion and outlook

We have recalled the substantial scientific progress being made in metallobiology studies utilizing multi-keV micro-spectroscopy techniques. The number of applications is growing rapidly and the availability improves as more synchrotron radiation facilities.

<table>
<thead>
<tr>
<th>Selectivity</th>
<th>STEM-EDX</th>
<th>STEM-EELS/EFTEM</th>
<th>Dynamic SIMS</th>
<th>X-PEEM</th>
<th>Soft X-ray STXM</th>
<th>SXRF (micro-XANES)</th>
<th>PIXE</th>
<th>LA-ICP-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spatial resolution (nm)</td>
<td>1</td>
<td>1</td>
<td>50–100</td>
<td>20</td>
<td>25–50</td>
<td>50–100 (150–1000)</td>
<td>500–1000</td>
<td>5000–20,000</td>
</tr>
<tr>
<td>Analytical depth (μm)</td>
<td>0.1–1</td>
<td>&lt;0.1 at 200 keV/0.5 at 100 keV</td>
<td>&lt;0.1</td>
<td>&lt;0.05</td>
<td>&lt;10</td>
<td>10–1000</td>
<td>100–1000</td>
<td>1000</td>
</tr>
<tr>
<td>Field of view</td>
<td>&lt;10 μm²</td>
<td>&lt;10 μm²</td>
<td>&lt;0.1 cm²</td>
<td>10–100 μm²</td>
<td>10 μm²</td>
<td>Sub mm² to cm²</td>
<td>Sub mm²</td>
<td>Sub cm² to 200 μm²</td>
</tr>
<tr>
<td>Detection limit (mg/Kg)</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>0.1</td>
<td>100–1000</td>
<td>&gt;1000</td>
<td>1000</td>
<td>0.1 (10–100)</td>
<td>1–10</td>
</tr>
<tr>
<td>Quantification</td>
<td>Semi-quantitative</td>
<td>Semi-quantitative</td>
<td>Semi-quantitative</td>
<td>Semi-quantitative</td>
<td>Semi-quantitative</td>
<td>Quantitative (poor)</td>
<td>Quantitative</td>
<td>Quantitative</td>
</tr>
<tr>
<td>Samples</td>
<td>Thin section (resin embedded material, frozen-hydrated)</td>
<td>Thin section (resin embedded material, frozen-hydrated)</td>
<td>Thin section or thin section of resin embedded material; cryofixed and freeze-dried</td>
<td>Thin section of resin embedded material; dried or embedded material</td>
<td>Frozen hydrated, dried or embedded material</td>
<td>Frozen hydrated; dried or embedded material</td>
<td>Freeze-dried samples/ embedded material</td>
<td>10–40 μm native cryosections</td>
</tr>
<tr>
<td>Environment</td>
<td>High-vacuum</td>
<td>High-vacuum</td>
<td>Ultra high-vacuum</td>
<td>Ultra high-vacuum</td>
<td>High-vacuum</td>
<td>High-vacuum</td>
<td>In air or high vacuum</td>
<td>High-vacuum</td>
</tr>
</tbody>
</table>

Table 1: Major spatially resolved methods for imaging metals in biology.

Fig. 9. The timeline of the X-ray focus size below and above 2 keV during the last 40 years.
appear around the world. Over the past three decades, the interest in X-ray microscopy has been revived, fostered by a number of major advances in X-ray sources, X-ray optics and X-ray detector technology. X-ray scanning microscopy techniques largely benefit from the high brilliance of X-ray beams produced by third-generation synchrotron sources. Driven by these unprecedented properties of X-ray beams, concomitant progress has been made in X-ray optics (Fig. 9). Above all, recent advances in manufacturing techniques have enlarged the accessible energy range of nano-focusing optics and offer new applications. Hard and soft X-ray focusing optics are now reaching similar levels of performance, with focused beam sizes below 20 nm (Chao et al., 2005, 2009; Kang et al., 2008; Mimura et al., 2010). However, these remarkable achievements remain at the demonstration stage and are still far from common practice. Furthermore, they are yet to be fully integrated into stable and reliable scanning X-ray microscopes. Such implementations will require not only outstanding quality optics, but also ultimate control of all experimental and environmental parameters. As part of the ESRF Upgrade Programme, the implementation of a new beamline for Nano-Imaging and Nano-Analysis (NINA) is underway (see http://www.esrf.fr/AboutUs/Upgrade/future-beamline-portfolio). It will operate in parallel two distinct branches optimized respectively for X-ray ultra-microscopy and nano-spectroscopy. This facility will replace the existing ID22 beamline from early 2014.

The natural evolution for XRF elemental mapping is its extension towards three dimensional (Bleuet et al., 2010) information. The knowledge of the elemental variation along the third dimension intrinsically improves the data quality and therefore its interpretation. Compared to the standard absorption X-ray computed tomography, XRF computed tomography is more challenging due to the long acquisition times associated with the scanning procedure. Recent works have demonstrated the potential of fluorescence tomography to map 3D elemental distributions in unstained and unfixed biological specimens (de Jonge and Vogt, 2010; Jackson et al., 2009; Ortega et al., 2009b).

Another important evolution is the combination of micro-spectroscopy methods with other imaging methods. This provides complementary information on the structure of the specimen and allows for a better quantification through mass normalization. It is readily available at the ID22NI end-station that combines in the same setup scanning XRF microscopy and full-field projection microscopy (see Kosior et al. in this issue). Further improvements are possible through the advances of lens-less X-ray diffraction microscopy (Chapman, 2010; Dierolf et al., 2010; Takahashi et al., 2011).

Assuming the synchrotron-based X-ray microscopy techniques are readily adopted, they are likely to evolve in two complementary directions. On one hand, a growing number of new methods exploiting the X-ray beam coherence will require brighter X-ray sources (Chapman, 2010), and on the other hand a significant effort to master technology for table-top instruments which aim to make X-ray microscopy more accessible for routine measurements (Sandberg et al., 2009; Takman et al., 2007).

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References


