Low-energy X-ray fluorescence microscopy opening new opportunities for bio-related research

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Biological systems are unique matter with very complex morphology and highly heterogeneous chemical composition dominated by light elements. Discriminating qualitatively at the sub-micrometer level the lateral distribution of constituent elements, and correlating it to the sub-cellular biological structure, continues to be a challenge. The low-energy X-ray fluorescence microspectroscopy, recently implemented in TwinMic scanning transmission mode, has opened up new opportunities for mapping the distribution of the light elements, complemented by morphology information provided by simultaneous acquisition of absorption and phase contrast images. The important new information that can be obtained in bio-related research domains is demonstrated by two pilot experiments with specimens of interest for marine biology and food science. They demonstrate the potential to yield important insights into the structural and compositional enrichment, distribution and correlation of essential trace elements in the lorica of *Tintinnopsis radix*, and the lateral distribution of trace nutrients in the seeds of wheat *Triticum aestivum*.

Keywords: X-ray spectromicroscopy; X-ray fluorescence; synchrotron radiation; marine biology; food science

1. INTRODUCTION

Determination of the distribution and chemical state of elemental constituents within biological systems at sub-cellular level down to trace level concentrations is of growing importance for gaining new insights about the highly complex functions of elements within the tissue or the cell. Different analytical techniques have been developed in the past, which are complementary in terms of lateral resolution, chemical sensitivity, quantitative analysis, depth profiling or bulk sensitivity and detection of elemental isotopes. Among those techniques, energy dispersive X-ray analysis (EDX) in a transmission electron microscope provides the highest lateral resolution (<10 nm) but a moderate chemical sensitivity (0.01–0.1 wt%) and requires the specimen to be analysed in vacuum and to be sectioned to thin slices. Secondary ion milling spectroscopy has very high chemical sensitivity (ppb–ppm) and high spatial resolution

(<100 nm), allowing the detection of elemental isotopes, but quantification of data is difficult. Electron energy-loss spectroscopy has very high resolution (<10 nm) and chemical sensitivity in the ppm range but requires sectioning of the specimen, and like secondary ion milling spectroscopy and EDX is only surface sensitive and does not provide bulk information (Mills *et al.* 2005). A distinct limitation of the electron-based techniques is that they require conducting surfaces and high vacuum environment. Particle-induced X-ray emission (PIXE), although achieving today's lateral resolution in the micron range, is not as sensitive for higher-Z elements and can impart stronger beam damage to the specimen than X-ray-induced methods (Janssens *et al.* 1996). X-ray spectrometric techniques by means of excitation of an inner shell electron and emission of a quantum of characteristic fluorescence X-ray was first proposed in 1928 (Glocker & Schreiber 1928) and was well established during the 20th century (Jenkins 1999). X-ray fluorescence (XRF) analysis observed its advent with the development of high brilliant and energy-tunable synchrotron radiation sources and the possibility of focusing such X-ray light down to sub-micron probes

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(Bertsch & Hunter 2001), and emerges as an important technique to complement characterization by traditional techniques as well as other emerging methods including Fourier-transform infrared (FTIR) and Raman spectroscopy. Laboratory and synchrotron-based XRF instrumentation typically uses hard X-rays and only a few soft X-ray XRF instruments have been reported (Flank *et al.* 2006). The use of soft X-rays for XRF analysis has mainly been limited by low fluorescence yield of low-*Z* elements and the unavailability of suited detectors and electronics. Such a low-energy XRF system operated in the soft X-ray regime is especially suited for bio-related research as it gives access to the elemental distribution of low-*Z* elements carbon, nitrogen, oxygen, fluorine, iron, zinc, magnesium and other elements with fundamental importance for metabolism in biological systems on the cellular or sub-cellular level. Here we report on a low-energy XRF setup with multiple silicon drift detectors (SDDs) for low-*Z* element detection coupled to a fast read-out electron multiplied charge-coupled device (CCD) camera that allows simultaneous collection of the XRF emission signal and the analysis of the specimen's morphology in brightfield, differential phase and darkfield contrast. We describe the low-energy X-ray fluorescence (LEXRF) setup, and demonstrate its potential using exemplary samples from marine biology, especially the analysis of the lorica of microzooplanktonic species *Tintinnopsis radix* as an important constituent of the marine food web, and the sub-cellular distribution of trace nutrients such as zinc in the seed of wheat *Triticum aestivum*.

2. METHODS

2.1. The TwinMic spectromicroscope at the Elettra synchrotron radiation facility

The TwinMic X-ray spectromicroscope at Elettra (Kaulich *et al.* 2006) was constructed by the concerted effort of eight European facilities and institutions and includes various imaging modes including full-field imaging (Niemann *et al.* 1976) and raster-scanning (Rarback *et al.* 1987), and different contrast techniques such as bright-field, darkfield, differential phase and interference contrast. Principles and applications of X-ray microscopy have recently been reviewed by Howells *et al.* (2007). The analytical potential of the instrument is based on X-ray near-edge absorption spectroscopy, across absorption edge imaging and recently implemented LEXRF. X-ray microprobes are formed by means of diffractive focusing optics and the lateral resolution achievable with this instrument is dependent on the imaging mode 0.05–0.5 μm . Operated in the 280–2200 eV photon energy range, TwinMic accesses the Wolter water window and major low-*Z* elements (K-edge: $Z = 5\text{--}9$, L-edge = 10–37) of interest for bio-related and other applications, as indicated in figure 1.

2.2. The scanning transmission X-ray microscope mode of the TwinMic station

Scanning transmission X-ray microscopy is the most versatile imaging mode as it accomplishes acquisition

Figure 1. Elements accessible by the TwinMic station.

of the transmission and emission signals and is therefore specially suited for simultaneous analysis of the morphology and chemistry of the specimen. In the TwinMic station a zone plate (ZP) forms a microprobe from the focused and monochromatized light (Bianco *et al.* 2005) of the hybrid short undulator X-ray source, and the specimen is raster-scanned across the microprobe as shown in figure 2. The transmission signal is acquired by means of a Andor Ixon fast read-out electron-multiplying CCD camera coupled to a phosphor screen-based visible light converting system, which allows simultaneous detection of brightfield or absorption, differential absorption and differential phase contrast signal (Gianoncelli *et al.* 2006; Morrison *et al.* 2006).

2.3. Simultaneous acquisition of the X-ray emission signal by a LEXRF detector system

The morphological analysis of the specimen on cellular or sub-cellular level is complemented by a LEXRF setup consisting of up to eight large-area SDDs in an annular back-scattering configuration around the specimen, and customized preamplifier and bias electronics and an ultra-fast data acquisition system (Niculae *et al.* 2006) provided by Politecnico Milano/INFN, Italy. The LEXRF setup has a full width at half maximum energy resolution of 69 eV at the C absorption edge, a high-energy resolution stability with a measured worsening of 3.5 eV at the manganese $K\alpha$ absorption edge and 150 kcps input photon rate, and very low peak shift (Alberti *et al.* 2009). The achieved lateral resolution is currently limited by the necessary signal-to-noise ratio of the acquisition. The system is operated from the boron to the phosphorus absorption edges with a measured detection limit of about 10 ppm at the fluorine K-absorption edge. Figure 3 shows a typical LEXRF spectrum acquired from a wheat specimen as described in §3.1.

3. RESULTS

The experiments reported here were performed using a LEXRF setup consisting of three SDD detectors

Figure 2. The raster-scanning mode of the TwinMic station: a diffractive focusing optic (ZP) in combination with an order selecting aperture (OSA) forms a microprobe and the specimen is scanned across the microprobe. The transmission signal is recorded by a fast read-out charge-coupled device camera (FRCCD).

Figure 3. Microspot LEXRF spectrum of a wheat specimen *Triticum aestivum*. Photon energy 1467 eV (elastic), dwell time 300 s, spot size 0.8 μm . The peaks of the spectrum were deconvoluted after constant baseline subtraction using the PyMCA software for the energy-dispersive X-ray fluorescence analysis (Sole *et al.* 2007).

produced by PNSensor, Germany. The X-ray microprobe was formed by a ZP in Au with a diameter of 250 μm and 80 nm outermost zone width, providing diffraction limited lateral resolution of 98 nm (Attwood 1999). The ZP was fabricated by TASC/INFM, Italy (Di Fabrizio *et al.* 2001). However, the effective lateral resolution depends on the X-ray source size and its geometrical demagnification, which determines the photon flux needed for measuring sufficiently high signal. Considering this compromise, imposed by the type of the samples under investigation, the reported measurements were performed with lateral resolution of the order of 0.8 μm .

3.1. Sub-cellular analysis of aleurone layer in wheat *Triticum aestivum*

In edible plants like wheat *T. aestivum*, a worldwide cultivated staple food, which is the second most-produced

cereal crop after maize, elements and nutrients are not distributed evenly, but are present in different concentrations through the different parts of the plant. Despite the importance of wheat in traditional food products, there are only a few reports on the composition (major, minor and trace elements) and nutritional values are typically performed by bulk analysis rather than on cellular or sub-cellular level. Resolving the distribution and the concentration within different morphological structures of a specific tissue is essential for understanding the mechanisms involved in the element's regulation, allocation, absorption, transport, accumulation, functionality and bioavailability. Information on the elemental chemistry related to inherent structures is essential for plant-breeding programmes for selecting a superior variety of wheat fostering food and feed purposes, predicting their nutritive value and providing models to modern agro-industry. The assessment of the nutritional availability for a number of inorganic nutrients, such as zinc, involves the direct analysis of the nutrient itself, its repartition within and between different plant parts, as well its concentration and competition on cellular or sub-cellular levels. For example both selenium and zinc possess some anti-oxidative properties (Barker & Pilbeam 2006). Although the analysis of the organic composition of wheat by laboratory or synchrotron-based FTIR microscopy is reported in a variety of publications (e.g. Jamme *et al.* 2008), there are only a few analyses of the elemental composition on cellular or sub-cellular level (Mills *et al.* 2005). Here, LEXRF is used to better understand the role of zinc and its distribution in aleurone and sub-aleurone layers of *T. aestivum*.

Grains of the wheat were cryo-sectioned at -23°C to 10 μm thin slices using disposable stainless steel cryo-microtome blades. Flatness of the sections was ensured by covering the sections with pre-cooled filter paper. The sections were freeze-dried at -25°C and 10^{-5} bar. Freeze-dried specimens were mounted onto Au folding grids for analysis in the TwinMic spectromicroscope. Figure 4 shows simultaneously acquired transmission images in (a) brightfield and (b) differential phase contrast and LEXRF emission maps of (c) carbon, (d) oxygen, (e) the ratio of oxygen and carbon, (f) magnesium and (g) zinc. Obviously, the transmission images provide the necessary information about the morphology of the specimen, which is essential for correlating the LEXRF maps on sub-cellular level. The maps clearly illustrate that zinc is unevenly distributed in the wheat grain, localized in the aleurone cells and strongly correlated to the magnesium distribution. This was neither known nor has been demonstrated yet with imagery. The oxygen to carbon ratio indicates that zinc and magnesium are bound to the organic matter with lower oxygen content. Zinc is an essential trace element frequently limiting the nutritional value of plant materials for human consumption. Localization within the wheat has an impact on its solubility and bioavailability, both for the needs of the seed during germination as well as for the digestibility of trace element containing substances in case of ingestion as food.

Further analysis of the correlation of zinc to other elements, e.g. phosphorus, as well as complementary

Figure 4. Simultaneously acquired transmission and LEXRF $80 \times 80 \mu\text{m}^2$ maps of an aleurone layer and sub-aleurone structures of *T. aestivum*, illustrating the morphology and the corresponding elemental distribution. (a) Bright-field (absorption) image; (b) differential phase contrast image; (c) carbon; (d) oxygen; (e) oxygen to carbon intensity ratio; (f) magnesium; and (g) zinc. Photon energy: 1467 eV. Dwell time per pixel (80×80 pixel) 3 s. Qualitative measurement. Scales are in arbitrary units.

analysis with FTIR microscopy are currently ongoing and will be a subject of future publications.

3.2. Analysis of the morphology and elemental distribution of lorica of tintinnids:

Tintinnopsis radix

Tintinnid ciliates are unicellular marine organisms and form a major component of the microzooplankton, an important group of the marine food web (Fonda-Umani & Beran 2003). Tintinnids have been known to marine biologists since the 1880s. Their biogeographic distribution in relation to hydrography as well as indicators for upwelling has been investigated (Pierce & Turner 1993). Tintinnids are choreotrichous ciliates that are characterized by the lorica, a kind of

shell or exoskeleton. Taxonomy of tintinnids is based on their infraciliature and on the shape and consistence of their lorica. The lorica is composed of a complex organic matrix, which is supposed to be made of polysaccharides and/or proteins (Lynn & Small 2000). There are basically two types of loricae: hyaline loricae and agglutinated loricae. An agglomerated lorica differs from the hyaline type in the amount of biogenic material. In the case of an agglutinated lorica, organic and mineral particles are embedded in the organic matrix. This particulate matter could be absorbed casually from the environment or the organic matrix could be selective for elements, essential for the metabolism of the cell: for example iron, which is usually a growth-limiting factor in the sea. EDX analysis has revealed high content of sodium, magnesium,

Figure 5. Simultaneously acquired transmission and LEXRF $80 \times 80 \mu\text{m}^2$ maps of the tail of a lorica of the tintinnid ciliate *T. radix*, illustrating the morphology and the corresponding elemental distribution. (a) Brightfield (absorption) image; (b) differential phase contrast image; (c) oxygen to carbon intensity ratio; (d) iron; (e) aluminium; and (f) magnesium. Excitation photon energy 1686 eV. Dwell time per pixel (80×80 pixel) 11 s.

phosphorus, sulphur, chlorine and calcium, and in some cases aluminium and iron. Unexpectedly high concentrations of cadmium, manganese, lead, copper and zinc were measured by atomic absorption spectroscopy in the lorica of *Eufolliculina*, a spirotrich ciliate (Mulisch *et al.* 1982). Tintinnids are difficult to obtain and typically the small amount available is inadequate for conventional chemical analysis. The chemical sensitivity and the penetration power of X-ray transmission and emission spectromicroscopy turned out to be a suitable tool to reveal the morphology and elemental distribution in the loricae of tintinnid ciliates.

Specimens of tintinnid *T. radix*, which has an agglomerated lorica, were isolated directly from net

samples taken in the Gulf of Trieste, transferred into seawater filtered on $0.22 \mu\text{m}$ filters (Millipore, Durapore GV) without the addition of food. Under these conditions the ciliates leave the lorica after about 3 days. Empty loricae were washed three times in ultrapure water by transferring them through a micropipette, and dehydrated and fixed in a series of 30 per cent, 50 per cent, 70 per cent ethanol, 10 min each. For observation, single specimens were pipetted in 80 per cent ethanol and then transferred and dried on silicon nitride windows to be able to analyse them *in vacuo*. Figure 5 shows transmission images and LEXRF maps of the tail of the lorica of *T. radix*. The brightfield (a) and differential phase contrast (b) images show highly absorbing

clusters. These clusters, considering the soft X-ray 1686 eV energy, do not contain only organic matter. This supposition is supported by the O/C map (c), which does not correlate with the distribution of the clusters. Surprisingly, the oxygen content is higher in the tail (upper right) than in the main body (lower left) of the lorica, indicating a different composition of the organic matrix. Although LEXRF can give some hints of the organic distribution, a better understanding of the organic structure will require complementary FTIR analysis. The maps (d–f) show that the clusters in the main body of the lorica consist of iron, aluminium and magnesium. The presence of iron, which is rare in marine environments, has been observed for the first time. The fact that Fe is concentrated in the vicinity of the main body close to the living cell and not in the tail is a further indication of its importance for the physiology of the ciliate. Since the clusters in the tail do not show the presence of the low-Z elements, they should contain heavier elements to be confirmed by analysis using hard XRF or particle-induced X-ray emission (PIXE). Extended LEXRF analysis and correlated FTIR and PIXE studies are ongoing and are the subject of future communications.

4. SUMMARY AND CONCLUSION

The sub-micrometer lateral resolution, simultaneous morphology and chemical sensitivity (currently in 10 ppm range) and penetration power have made the transmission and emission soft X-ray microscopy a very powerful analytical tool for the analysis of biological specimens, complementing the other analytical techniques such as charged-particle microscopies, FTIR and Raman spectromicroscopy, hard X-ray microfluorescence and visible light microscopy. The combination of transmission imaging for the morphological analysis and X-ray emission microscopy for the elemental analysis allows immediate correlation of the elemental distribution to the morphology, which is of fundamental importance for understanding the processes occurring on the cellular and/or sub-cellular level. In addition, the simultaneous acquisition of absorption and differential phase contrast images is very helpful for defining in more detail the morphology when exploring low-absorbing organic matter. The performance of the LEXRF set-up is going to be improved by upgrading to eight detectors and implementing an adapted multilayer monochromator to increase the photon flux. These will reduce the acquisition time significantly and overcome the limits to the lateral resolution, imposed by the presently low fluorescence signal. Expectations for the near future are to reach the diffraction-limited resolution of the focusing optics and/or increase the chemical sensitivity to the ppb range with full quantitative analysis. This will make the instrument uniquely suited for bio-related research providing a powerful approach to assess quantitatively the composition and morphology and shed light on important biological processes. The particular field that should be explored is the intracellular localization of nano-objects in order to shed light on the possible health hazard when they are used

for medical purposes or are present in the polluted environment.

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