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X-Ray Fluorescence Microprobe Imaging in Biology and Medicine

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Abstract Characteristic X-ray fluorescence is a technique that can be used to establish elemental concentrations for a large number of different chemical elements simultaneously in different locations in cell and tissue samples. Exposing the samples to an X-ray beam is the basis of X-ray fluorescence microscopy (XFM). This technique provides the excellent trace element sensitivity; and, due to the large penetration depth of hard X-rays, an opportunity to image whole cells and quantify elements on a per cell basis. Moreover, because specimens prepared for XFM do not require sectioning, they can be investigated close to their natural, hydrated state with cryogenic approaches. Until several years ago, XFM was not widely available to bio-medical communities, and rarely offered resolution better then several microns. This has changed drastically with the development of third-generation synchrotrons. Recent examples of elemental imaging of cells and tissues show the maturation of XFM imaging technique into an elegant and informative way to gain insight into cellular processes. Future developments of XFM—building of new XFM facilities with higher resolution, higher sensitivity or higher throughput will further advance studies of native elemental makeup of cells and provide the biological community including the budding area of bionanotechnology with a tool perfectly suited to monitor the distribution of metals including nanovectors and measure the results of interactions between the nanovectors and living cells and tissues.
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The biology of the past decade has significantly changed scope, and large compendia of data have been accumulated to enable scientists to study biological "meta units" such as the genome, proteome, and transcriptome. At this time, the term "metalome" is still used only rarely, and the majority of scientists interested in establishing metalome databases undertook the daunting task of developing and using different dyes for different elements and their ions in order to determine elemental locations and concentrations in cells and tissues. The presence of metals and trace elements is essential for the existence of life as we know it. In any organism, there are very few intracellular processes that are not dependent on the presence of metals or other trace elements. In fact, it is estimated that one-third of all known proteins contain metal cofactors and the majority of these function as essential metalloenzymes. With current developments in genomics and proteomics, our knowledge of the enormous number of pathways in which metals and trace elements are necessary for life is ever increasing. 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, stress responses, etc. Likewise, our knowledge about the redistribution of metals and trace elements accompanying the development of different diseases is equally lacking. Elemental

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fluctuations in cellular homeostasis involve dynamic changes in the concentrations and localization of metals, and an ever increasing body of biological literature is showing the importance of metals in health and disease (see e.g., Finney and O'Halloran [2003]; Morel and Price [2003]; Oremland and Stolz [2003]; Thompson and Orvig [2003]). Metals are essential to most biological functions, their dysregulation contributes to many degenerative diseases, their uptake is important for many pathogenic diseases including cancer growth, and metals and metal chelators represent an increasingly important class of drugs used to treat a diverse variety of diseases [Gassen and Youdim, 1997; Guo and Sadler, 1999; Reedijk, 1999; Weinberg, 1999; Kicic et al., 2001; Mercer, 2001; Farrell, 2002; Buss et al., 2003; Desoize, 2003; Redman et al., 2003; Vartsky et al., 2003; Zhang and Lippard, 2003; Doraiswamy and Finefrock, 2004; Richardson, 2004; Walcourt et al., 2004; Zhao et al., 2004a; Cui et al., 2005].

In addition to studies of the metalome and determination of elemental concentrations in "natural" biological samples, there is also a growing interest in studies of elemental concentrations in samples obtained as a result of different medical or nutritional treatments. The distribution pattern of elements present in biological material also changes when their concentrations fall below or rise above normal physiological concentrations. Studies of the effects of metal chelators can also best be conducted at the level of determination of elemental distribution and concentration. Subcellular distribution of different drugs containing "non-natural" elements is often inferred from their activity rather than directly determined, for example, the subcellular localizations for many types of biomedical imaging reagents are largely unknown. Moreover, the rapidly developing new field of bionanotechnology critically depends on knowledge about tissue and cell specific and sub-cellular distribution of nanovectors [Ferrari, 2005]. Bionanotechnology is developing nanovectors to be targeted and to be able to overcome biological barriers, to provide true disease-tailored therapies. Nanomaterials—materials smaller than 100 nanometers, and characterized by novel physical and chemical properties—offer new methods for biomedical sensing, imaging, and therapy. Each nanovector—a unit of the nanomaterial with a biomedical mission—typically carries out several functions. For example, a nanovector developed for biomedical imaging should be able to find a cell of a certain phenotype and highlight it over the background of all other cells. In the majority of cases, the physical target of nanovectors used for bionanotechnology is the intracellular space, or cell and tissue features of subcellular size, just as it was for "small molecule therapies". However, nanovectors are closer in size to intracellular macromolecular assemblies; likewise, they need to be observed by high-resolution imaging approaches.

APPROACHES TO MONITOR METALS

Excitation and subsequent detection of characteristic X-ray fluorescence is a simple but accurate approach to determine elemental concentrations in whole cells and elemental distribution in cells and tissue sections. There are three approaches currently used to induce elemental X-ray fluorescence—exposing the samples to a particle beam, typically electrons (electron-probe X-ray microanalysis or EPXMA), to an X-ray beam (X-ray fluorescence microscopy; XFM), or to a proton beam (proton-induced X-ray emission or PIXE). In XFM, hard X-rays are focused onto a specimen to induce characteristic X-ray fluorescence in the samples. X-ray fluorescence microscopy, which is also occasionally called X-ray probe X-ray microanalysis (XPXMA) or synchrotron radiation-induced X-ray emission (SRIXE), has a very high sensitivity for most elements of biomedical interest, particularly due to a virtual absence of a bremsstrahlung background [Sparks, 1980]. This has been shown to result in improvement of the ability to detect elements of at least two orders of magnitude compared to EPXMA. Additionally, due to the large penetration depth of hard X-rays, specimens do not need to be sectioned, but can be investigated close to their natural, hydrated state with cryogenic approaches, without introduction of artificial dyes. Due to the large penetration depth of hard X-rays, X-ray fluorescence is induced throughout the thickness of a whole cell and can therefore be used to quantify elements on a per cell basis. Unless the incident energy is chosen to be very close to the elemental absorption edge, different chemical states of elements have no influence on the estimate of elemental quantity. By scanning the incident X-ray energy

across an absorption edge, the complementary technique of micro X-ray absorption near edge spectroscopy (μ-XANES) can allow for studies of elemental speciation. Such studies can be complemented by EPXMA, which currently can achieve higher spatial resolution than XFM, but then requires specimens to be sectioned (100 nm). PIXE has an elemental sensitivity intermediate between EPXMA and XFM and requires a vacuum environment for samples, similar to EPXMA. Radiation damage in EPXMA and PIXE far exceeds that of XFM studies, making it very difficult to study frozen-hydrated samples by characteristic fluorescence using any technique other than XFM.

At present, synchrotron-radiation based XFM is the only available technique for quantitative elemental imaging of whole cells with submicron-resolution [Dillon et al., 2003; Paunesku et al., 2003; Twining et al., 2003a; Kemner et al., 2004; Yang et al., 2005]. While many possible sources of X-rays exist, only third-generation synchrotron sources (such as exist in the USA [APS], France [ESRF], and Japan [SPring8]) provide the high brilliance at high-photon energies required to acquire elemental maps, at subcellular spatial resolution, with trace element sensitivity, of "soft" biological materials using hard X-ray fluorescence. Three recent workshops [Lai et al., 2002, 2004; Miller et al., 2005; Glesne et al., 2006; Hall et al., 2006; Liu et al., 2006; McRae et al., 2006; Palmer et al., 2006; Wagner et al., 2006] at the Advanced Photon Source confirmed the needs of the scientific community for biological hard X-ray microscopy and its significance for elemental mapping of cells and tissue sections.

Any element within a living organism can be found either as free or secreted, or bound to complex organic molecules. For example, zinc is one of the key elements maintaining the structure of proteins and is typically tightly bound, for example, in "zinc fingers" of many transcription factors. At the same time, free zinc can serve as an ionic signal and it is secreted from a number of cell types and tissues: submandibular salivary gland, pancreatic cells, prostate epithelial cells, Paneth cells, mast cells, three types of granulocytes, four types of pituitary cells, and three types of central nervous system neurons [Frederickson et al., 2005]. The concentration of zinc varies significantly from one tissue to another and from one subcellular compartment to another, for example, termini of neurons have a free zinc concentration of up to 300 μM. On the other hand, it is known that too low a Zn concentration leads to apoptosis, while too high Zn concentration leads to toxicity [Frederickson et al., 2005]. The total cellular Zn concentration in undifferentiated mammalian cells is 0.5 mM (O'Halloran, personal communication), while the free Zn concentration in the brain falls within the 1–10 nM range [Frederickson et al., 2005].

These types of studies of zinc concentrations were made possible by the fact that many different probes for it were developed over the years, such as the ion-specific fluorescent probe FluoZin-3 (Molecular Probes, Inc.) and the total zinc probe Zinbo 5 (developed by the O'Halloran laboratory). However, these dyes are limited by the fact that the zinc in the cell must be accessible to the probe, a fact which may not be true for all intracellular compartments. This and other factors such as background fluorescence make quantification difficult. More significantly, dyes for determination of elemental concentration probes are not available for most of the elements present in cells. In XFM, on the other hand, fluorescence signatures of most elements of biological interest are acquired simultaneously, that is, one is able to examine, for example, the Zn distribution inside of a cell in the context of the P, S, Cl, K, Ca, Fe, and Cu concentrations, with perfect registration.

XFM is also a method of choice for monitoring the intracellular distribution and stability of nanovectors. While there are other approaches to image subcellular details (e.g., EPXMA, etc.), the X-ray microprobe is the only instrument that can image whole, frozen-hydrated cells at very high-spatial resolution and provide correlated elemental maps in 2D and, at somewhat reduced spatial resolution, in 3D. The subcellular distributions in different intracellular organelles, can be monitored by either using X-ray differential phase contrast to visualize structures directly, or by employing immunolabeling to highlight specific subcellular structures (e.g., Fahrni's group used antibodies labeled with gold 1.4 nm nanoparticles to label mitochondria and Golgi apparatus [McRae et al., 2006]).

Areas of study opened by the use of XFM falls into two major categories—studies of "natural" metal and trace element distributions in health and disease and studies of distributions of

metals and trace elements deliberately or unintentionally introduced into organisms or cells. Both of these areas are of great biological and medical significance, and we will briefly review each of them from the standpoint of current and future research, and place them in context of XFM. Some of the examples will include XFM studies, others will highlight the potential to use XFM techniques.

MONITORING FLUCTUATIONS IN LOCAL CONCENTRATIONS OF METAL AND TRACE ELEMENTS IN NORMAL PROCESSES ACCOMPANYING NATURAL CHANGES IN HOMEOSTASIS IN CELLS AND ORGANISMS

Cardiac Function

Using the X-ray fluorescence technique Palmer et al. [2006] studied Zn and Ca distribution in heart muscle cells. Maps of the intracellular Zn distribution in cardiomyocytes showed a high-Zn concentration in or near the sarcoplasmic reticulum, visible as distinct longitudinal striations. This group hypothesized that intracellular Zn facilitates cardiomyocyte relaxation by regulating calcium fluctuations and intend to use XFM for tease out the details of correlation between the two elements.

Differentiation

The process of differentiation of HL60 macrophages is dependent on Cu and Zn availability. Recent observations using existing XFM-based resources [Glesne et al., 2006] have demonstrated that translocations of zinc are required for successful treatment of hematopoietic malignancies with phorbol esters or retinoic acid derivatives. Further XFM experiments will lead to a more complete understanding of this process, and may also lead to the discovery of valuable additional targets (in addition to Zn and Cu) for differentiation therapy.

Bone Loss

Bone loss through osteoporosis and osteopetrosis are important contributors to morbidity in an aging human population, but the processes themselves are poorly understood. Because bone is composed predominantly of calcium and other hard structures, and thus hard to section, tomographic studies of bone microstructure can be used instead to understand the processes themselves. Elemental concentrations, particularly those for Ca and P are linked with bone growth and normal acquisition and loss of bone mass [Abrams, 2003; Shapiro and Heaney, 2003]. In addition, with the use of prostheses and implants, studies of the interactions at the interface of the bone-tissue and prostheses can be accomplished using X-ray microscopic approaches.

Cell Biology

General studies of organelle function in cells using tomographic imaging of organelles will contribute greatly to our understanding of basic cell biology. It is estimated that one-third of all known proteins contain metal cofactors and the majority of these function as essential metalloenzymes. Studies of their intracellular distribution at different times and following different stresses would contribute greatly to an understanding of the physiological processes themselves. In addition, using nanoparticles and quantum-dots to label different subcellular components or engineer overexpression of metal-containing proteins, scientists can probe a variety of cellular processes including transcription, translation, apoptosis, and others.

Mitochondrial Function

Mitochondria are intracellular organelles smaller than 2 μ in size that contain their own genetic material that is separate from that in the nucleus and that provide the energy for a cell to function. A recent finding suggests that in addition to the Golgi apparatus, the mitochondria are also a site of accumulation of labile Cu [Yang et al., 2005]. Recent studies have identified several diseases associated with mitochondria and have also pointed to mitochondrial defects in a variety of neurologic diseases. For example, copper imbalance was linked to mitochondrial dysfunction [Rossi et al., 2004]; as well as iron [Yoon et al., 2004]. Studies of mitochondrial metal-containing proteins are likely to provide the basis for understanding mitochondrial function since so many of the important mitochondrial proteins are metal containing. Among these are Mn-superoxide dismutase (important in handling oxidative damage to the mitochondrion), iron-sulfur protein and copper containing cytochromes (involved in energy production), and others involved in oxidative metabolism. Elemental

distribution patterns within mitochondria and other subcellular organelles will contribute to an understanding of the roles of these proteins in normal and disease states.

Proteomics

As progress is made in understanding the role trace metals play both in endogenous cellular processes and as a result of exposure to exogenous metals, many of the protein targets that interact with these metals will be identified. It is expected that protein–metal interactions will not be static and that interaction with a metal will influence the activity of a protein. The current inability to measure not only the level of expression of a protein but also its level of metal cofactor binding represents a gap in our understanding of functional genomics. For example, there are numerous cases in which the levels of a given protein's cognate mRNA transcript and the protein itself do not change, yet there are changes in the activity of the protein. Traditional nucleic acid based technologies (microarray, rtPCR, Northern blotting) and protein detection technologies (Western blotting, antibody-arrays, ELISA, protein arrays) cannot detect such metal-dependent activity changes. By combining XFM capabilities with traditional and newly developed high-throughput protein capture technologies, we will be able to fill this current gap in the translation of protein expression data into meaningful protein activity correlation matrices. At this moment there are already several avenues for combining XMF approaches with "classical" protein electrophoresis [Miller et al., 2003; Verbi et al., 2005], and it is easy to envision how the use of micro- and nano-arrays could further advance this nascent field.

Microbiology and Single Cell Organism Fate

Studies of (trace) metal distribution in microorganisms, including their subcellular localization, as well as studies of mechanisms of import of needed nutrients and clearance of excess (toxic) metals across membranes by active transporters [Ferguson and Deisenhofer, 2004], will give insight in the process of nutrient acquisition and detoxcification. The understanding of these processes is crucial for the field of microbial pathogenesis and bioremediation.

Responses of trace element concentrations in natural algae and protozoan cells suggests strong linkages between the elements. For example, when Fe is a limiting nutrient, cellular concentrations of Mn and Zn are also low and when cellular Fe increases, cellular concentrations of the other metals also increase. One hypothesis is that Fe limitation causes a physiological cascade that affects all metals involved in cell metabolism and reproduction. Likewise, low availability of Fe limits the assimilation of nitrate by diatoms [Milligan and Harrison, 2000], as well as carbon fixation [Boyd et al., 2004]. Nanoscale maps of metal distribution within the cell coupled with other spectroscopic and microscopic techniques would allow us to evaluate these hypotheses.

Marine Biology

Single-celled photosynthetic and non-photosynthetic aquatic protists often require transition elements in order to support normal metabolic function. As a consequence, these organisms form the basis of trace element cycles in the oceans. Twining et al. [2003a,b], are using XFM to quantify the elemental contents of plankton cells. Production of organic matter of the oceans is limited by the supply of trace elements (particularly Fe) to the surface waters of oceans; this impacts the ocean nutrient cycles and determines the amount of carbon stored at depth [Morel and Price, 2003]. Several groups have suggested that bioengineering atmospheric carbon dioxide through iron fertilization of the ocean could be linked with the ocean nutrient cycle. High-spatial resolution X-ray (0.3–1.0 μm) fluorescence microprobes have enabled the determination of trace element concentrations in single cells of algae and protozoa >2 μm in size [Twining et al., 2003a,b]. These results have predicted the amount of atmospheric carbon that will be stored in surface waters after iron fertilization and the effects of such fertilization on the geochemical cycling of other elements [Twining and Fisher, 2004].

Exobiology

Several projects are currently underway to bring extraterrestrial samples back from the outer reaches of our solar system. Using XFM, investigators are trying to identify signatures of life in these samples. Most of the extraterrestrial samples are quite small and at the same time are not abundant. Only a few analyses will

be possible with each sample, making it essential to maximize the information that can be gained. Such studies are already planned with XFM at lower resolution [Schwartz et al., 1995; Mautner et al., 1997], but improved resolution and sensitivity will enhance these studies and provide a better basis for establishing whether life signatures are present in the samples.

STUDIES OF METAL AND TRACE ELEMENT ABUNDANCE REQUIRED FOR THE DEVELOPMENT OR INHIBITION OF DISEASES; ANALYSIS OF ACCUMULATION OF METALS OR TRACE ELEMENTS AS A CAUSE OR CONSEQUENCE OF THE DEVELOPMENT OF A PATHOLOGIC PROCESS

Neuropathology and Neurodevelopmental Abnormalities

Studies of metal accumulation in brains of patients with Alzheimer disease (AD), Parkinson disease, and others and spinal cords of Lou Gehrig's disease (amyotrophic lateral sclerosis) patients and others have already demonstrated that some metals (Al, Mn, Fe, Cu, Zn) are associated with disease progression and may be associated with disease onset [Campbell et al., 2001; Zatta et al., 2003; Doraiswamy and Finefrock, 2004; House et al., 2004; Zecca et al., 2004]. Some authors emphasize that it is the fact that these are redox metals that contributes the most to the pathology of these diseases [Huang et al., 2004; Todorich and Connor, 2004]. Nevertheless, the precise cellular and subcellular locations, and in some cases even the regional brain locations, of these metals are not known. For instance, defining the precise locations of copper or zinc in plaques, tangles, non-tangle bearing neurons, and synapses in AD brain tissue by imaging with the hard X-ray BioNanoprobe would be an important contribution to an understanding of the pathogenesis of the disease. At the same time, such studies could lead to the development of new diagnostic procedures. A 10 s of nm scale resolution seems likely with future hard X-ray microprobes will be particularly useful for investigating changes at the synapse level, since synapses are less than 1 μ in size. While it has been known for some time that the decrease in synapses correlates the best with the cognitive decline observed in AD [Terry et al., 1991], the sequence of events occurring to cause this decline remains unclear. Lately, an increasing number of metal chelators (including metal chelating nanoparticles [Cui et al., 2005]) is being tested for treatment of CNS disorders [Gassen and Youdim, 1997; Doraiswamy and Finefrock, 2004; Richardson, 2004].

Recent studies have suggested a role for abnormal metal accumulation in patients with autism [Davidson et al., 2004]. This disease is poorly understood at all levels, and a better understanding of the metal accumulation component of the disease is likely to contribute to improved and perhaps targeted therapies in the future.

Menkes and Wilson diseases have both been shown to have errors in expression and/or function of Cu-transport proteins [Strausak et al., 2001]; understanding how normal Cu-transport proteins function will provide a basis for understanding how Cu is handled by the human body but also how the Cu-transport defect contributes to the pathogenesis of these diseases.

Cu topography in cells was studied by XFM by the Fahrni group [Yang et al., 2005] and nanogold-labeled antibodies were used as fiducial markers to label cellular organelles [McRae et al., 2006]. This was done in order to establish an approach to conduct a study to see if mitochondria and Golgi compartments might function as storage places of labile copper pools as suggested in the literature [Cobine et al., 2004; Yang et al., 2005].

Autoimmune Disease

Metals may activate a T-cell autoimmune response [Fournie et al., 2001; Martin, 2004] and metal accumulations have been found in immunodeposits in affected tissues of many tissue-specific autoimmune diseases including systemic lupus erythematosis (kidney deposits), rheumatoid arthritis (joint deposits), Grave's disease (thyroid deposits), and others; studies of these metal accumulations by XFM would provide important clues as to the pathology of the deposits, and may lead to an improved understanding of how these deposits are formed and how they can be prevented.

Oncological Pathology

Different metals have been implicated in carcinogenesis [Leonard et al., 2004], and the role of Cd, Cr, As, Ni, V, Fe, Cu, and Mn in carcinogenesis is being scrutinized by many

investigators [Desoize, 2003; Waisberg et al., 2003]. Studies of metal accumulation in tumors, for example, found higher metal levels in smokers, which may be related to trace levels of metals found in cigarette smoke (such as Cd, Ni, and As). In other tumor systems, there is evidence of a high content of specific metals such as Fe or Ni, although little is known about the mechanism by which they act. In prostate cancer, the presence of Zn in addition to expression of the prostate-specific antigen (PSA) is considered a valuable diagnostic marker. For breast cancer, studies have suggested increased iron accumulation in the tumor due to increased expression of transferrin receptors on the cell surface, but for other tumor systems, mechanisms of metal accumulation have not been postulated. Imaging of metal-containing foci, determination of subcellular distribution patterns, and ultra-fine resolution elemental maps to visualize metal-containing complexes will help to determine these events. Different metal chelators are also being used experimentally for cancer control [Weinberg, 1999; Kicic et al., 2001; Richardson, 2002; Buss et al., 2003]. Recently, low-resolution high-throughput XFM was used to screen prospectively elemental distribution in esophageal squamous cell carcinomas and controls [Abnet et al., 2005]; an inverse correlation between Zn content in tissues and cancer risk was found.

Bacterial Pathogens

Many factors regulate interactions between host and carriers of infectious diseases including pH, osmolarity, concentration of reactive O and N compounds and inorganic ion concentrations. Determination of the concentrations of ions such as Fe, Cu, Zn, and their correlation with virulence factors, is important in understanding the pathogenicity of these agents, especially since it is established that trace elements are co-factors of crucial enzymes and regulators of virulence gene expression. For example, it is known that pathogenic mycobacteria (e.g., those that cause tuberculosis or disseminated *Mycobacterium avium* infections) accumulate iron in the phagosomes when ingested by macrophages [Maser et al., 2003; Wagner and Young, 2004; Wagner et al., 2005, 2006]. The pathogenicity of *Pseudomonas aeroginosa* depends on its ability to utilize a variety of heterologous siderophores. In *Salmonella typhimurium* (causing enteritis) recent mutation studies have suggested that one or more of its Mn^{2+}-dependent enzymes are essential for pathogenesis [Kehres and Maguire, 2003]. *Staphylococcus aureus* (a pathogen causing severe disseminated infections, endocarditis, and foreign body infections) heme iron scavenging is required for staphylococcal pathogenesis in animal hosts [Skaar et al., 2004]. These examples together with the growing resistance to commonly used antibiotics for these pathogens underscore the need to better understand the host–pathogen interaction at the molecular level. Changes in metal concentrations as a function of time after infection, and associating these metal changes with other parameters such as the presence or absence of particular virulence genes, host cell predispositions, and others will allow for a better understanding of the virulence of infectious agents and ultimately may allow the development of specific drugs to combat these diseases. Metal chelator drugs are considered by some investigators to be of potentially great benefit in this field as well [Weinberg, 1999; Walcourt et al., 2004].

Antiparasitic Drugs

Drug-resistant malaria and leishmaniasis are spreading in Africa, India, and South America; this has increased the search for new anti-parasitic therapeutics [Croft and Coombs, 2003; Harpstrite et al., 2003]. For leishmaniasis in these countries toxic but inexpensive antimony-containing drugs over 50 years old are often used. Information about mechanisms of resistance to metalloids and cross-resistance to other drugs is not available. The ability to measure the subcellular distributions of metals and their relationship to drug resistance would enhance our understanding of these mechanisms.

Biodistribution and Biotransformations of Metal-Containing Anti-Cancer, Anti-Inflammatory, and Anti-Diabetic Drugs

Platinum anti-cancer drugs are one of the main classes of drugs used in the treatment of cancer, and a range of other drugs with metals such as Ru, Ti, and Mo are currently undergoing clinical trials [Guo and Sadler, 1999; Reedijk, 1999; Farrell, 2002; Thompson and Orvig, 2003; Zhang and Lippard, 2003]. In order to improve the design of these drugs and to overcome drug resistance, it is essential to study the intracellular distributions and biotransformations of

these drugs in cultured cancer cell lines and in grafted tumors in animals treated with the drugs. Similar studies are also required on metal-based anti-inflammatory drugs that are currently used as veterinary drugs [Weder et al., 2001; Dillon et al., 2004] and are likely to enter human clinical trials, and vanadium and chromium complexes that are in clinical trials for the treatment of diabetes [Levina et al., 2003; Mulyani et al., 2004]. Such hard X-ray microbeam studies are already being conducted at a lower resolution and are feeding into drug design [Hall et al., 2003a,b], but higher resolution is required in order to understand in more detail how these drugs interact with organelles in the target cells. For example, the Hambley group has shown that μ-XANES can be used to determine the oxidation states of platinum in solution, in human ovarian cancer cells and in tumor spheroids [Hambley et al., 2002; Hall et al., 2003c, 2006]; and shown that XFM can be used to determine the distributions of platinum in cells, tumor spheroids, and tumors [Hall et al., 2003b]. Shimura et al. [2005] have compared the intracellular elemental content of cells sensitive or resistant to treatment with cis-diamminedichloro-platinum(II) (CDDP), a platinum-based anticancer agent, and show that a combined treatment of CDDP and a Zn(II) chelator results in increased CDDP uptake.

TRACKING OF METALS OR TRACE ELEMENTS DELIBERATELY INTRODUCED IN ORGANISMS, EITHER THROUGH THE ENVIRONMENT OR THROUGH CELLULAR MANIPULATION APPROACHES

Nanobiotechnology

Potential uses of nanotechnology in biology and medicine are overwhelming, as reviewed in the recent literature [Brannon-Peppas and Blanchette, 2004; Ferrari, 2005; Jain, 2005, 2006]. A few examples that illustrate the wide variety of some of the recently developed nanoparticles include bioconjugated silica particles for detection of bacteria [Zhao et al., 2004b] and metal chelator nanoparticles investigated for use against Alzheimer's disease [Cui et al., 2005]. Bionanotechnology strives to overcome limitations of both non-specific therapies (e.g., classical chemotherapeutic treatment) and so-far established specific therapies. For example, radioimmunotherapy targets specific cells using antibodies [Mather and Britton, 2004], but only 1–10 parts of 100,000 antibodies delivered intravenously reach their target in vivo [Ferrari, 2005]. Bionanotechnology is developing nanovectors to be targeted (for certain cell types or certain subcellular structures) and to be able to overcome biological barriers. In order to fulfill all these roles nanovectors are most often multipartite nanoparticles, synthesized as combination of organic and inorganic components.

Most nanovectors contain inorganic molecules which are essential to their function. With the exception of quantum dots and ultra-small gold nanoparticles, most inorganic nanoparticles are not fluorescent in the optical regime, and cannot be followed by light microscopy; therefore, use of X-ray fluorescence for mapping is the logical path to investigation of nanovectors. Moreover, XFM can provide means to monitor down to a single nanoparticle, dependent on its number of atoms.

The Woloschak group is developing for intracellular use a new type of bionanocomposite that has new functional properties inside cells and in vitro. These nanocomposites are composed of metal oxide (TiO_2) nanoparticles (4.5 nm average size) and DNA oligonucleotides bound via dopamine to the nanoparticles. Within the nanocomposites DNA oligonucleotides retain base-pairing specificity, hence combining several DNA oligonucleotides with a single nanoparticle provides both extended retention of the nanocomposite in cells and leads to signal amplification. TiO_2-oligonucleotide nanocomposites developed by our group [Paunesku et al., 2003] are an attempt to develop a universal tool for sequence-specific targeting of DNA. Our work so far has demonstrated that nanoparticles are localized within the cell and within nuclei [Paunesku et al., 2003].

In one of the recent experiments we electroporated human breast cancer cell line MCF7 with nanocomposites made with oligonucleotide sequences homologous to nucleolar ribosomal DNA, and were able to demonstrate that the nanocomposites localize to cellular structures in a pattern consistent with their target, the nucleolei (Fig. 1). Nanocomposite distribution pattern showed two nucleoli in two of three cells (Fig. 1a); similar picture can be obtained by electron microscopy (Fig. 1b). Figure 1c shows a typical spectra obtained from a cell transfected with nanocomposites. As example, elemental

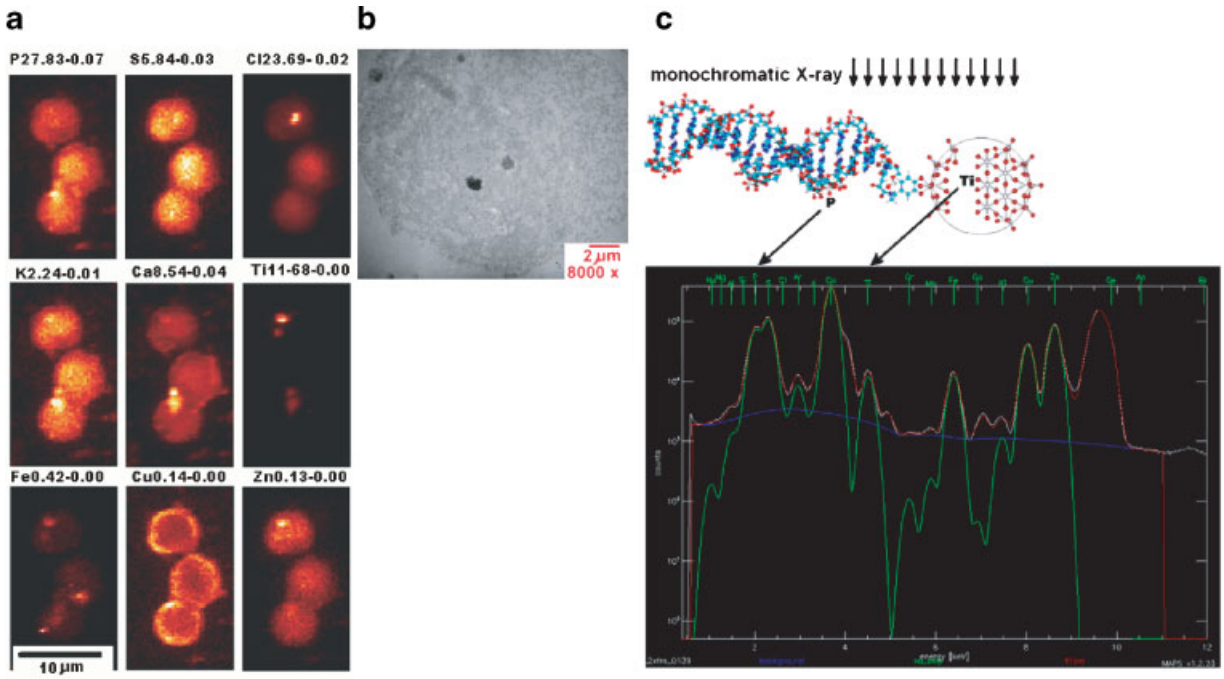

Fig. 1. **a**: Elemental maps in ''red temperature'' color scale of P, S, Cl, K, Ca, Ti, Mn, Fe, Cu, and Zn in three MCF7 cells transfected by electroporation with nanocomposites specific to ribosomal DNA. Cells were fixed by glutaraldehyde, washed with ethanol, and air-dried. Elements and the range of their concentration in the sample from highest (white) to lowest signal (black) are labeled above each map. In the Ti map two strong Ti signal dots each in two of three cells can be associated with nucleus rather than cytoplasmic region, consistent with the localization of nanoparticles to the nucleolei. **b**: Transmission electron micrograph of a cell from the same batch as cells in (a); however, cells visualized by TEM were embedded in epon and sectioned as 100-nm slices. **c**: Elemental spectra of the cells transfected with TiO2-DNA nanocomposites, showing a Ti-specific peak. Following microprobe calibration and signal de-convolution analysis this spectra provides information on the exact quantity of Ti in these cells.

signatures of phosphorus and titanium are indicated.

Molecular Imaging

Current applications of molecular imaging at the whole organism level depend upon using agents that are capable of detecting single molecules in a living organism. Many of these imaging applications include the use of tracers such as Gd, Fe, and others that are designed for in vivo imaging by magnetic resonance imaging, positron emission tomography and others, but all of them can also be detected by X-ray fluorescence imaging. For example, super-paramagnetic iron oxide nanoparticles (SPIO) were used recently for xenograft tumor imaging by magnetic resonance using the presence of folate receptors to provide the SPIO targeting [Choi et al., 2004]. Studies of these imaging compounds prior to their application in humans will include intracellular distribution studies, clearance studies, etc., that could best be done by highly sensitive X-ray fluorescence approaches. Q-dot approaches are another class of important and widely used molecular imaging tool that can be readily detected by X-ray fluorescence which can provide information complementary to optical fluorescence approaches. Two exciting recently developed nanoparticles are geared towards imaging: a gadolinium-rhodamine nanoparticles for in vivo imaging [Vuu et al., 2005] and protease-activated Au-nanoparticle-quantum dot nanoparticle [Chang et al., 2005]. More imaging nanoparticles were listed in recent reviews [Sullivan and Ferrari, 2004, 2005].

Stem Cell Imaging

The development of stem cell approaches depends largely on thorough initial experimentation in animal systems. Proofs of distribution patterns of newly seeded stem cells are hard to achieve (largely depending on fluorescent protein tagging of the would-be stem cells, which requires genetic engineering of the stem cells prior to use). An additional attractive way to image stem cells in whole animals is by MRI

approaches that depend on introduction of MRI contrast reagents (Gd, Fe) into cells. For example, neural stem cells could be followed due to the initial labeling with SPIO [Magnitsky et al., 2005] or because of the presence of Gd compounds [Modo et al., 2004]. For detailed ex vivo studies, using X-ray fluorescence for following of stem cell distribution can be accomplished easily if prior to release into the target tissue/organ stem cells are exposed to metals (Gd) or metal nanoparticles that remain captured inside the cells. Some approaches involve the use of cell lines engineered to express high levels of specific metal-binding proteins. These can be investigated prior to their use in MRI systems by XFM to establish their spatial distribution at the subcellular level, in order to fully understand their behavior.

DISCOVERY OF ACCIDENTAL OR OTHERWISE UNINTENTIONAL INTRODUCTION AND LOCAL ACCUMULATION OF METALS OR TRACE ELEMENTS IN CELLS OR SUBCELLULAR COMPARTMENTS, TISSUES, AND ORGANISMS

Implants

Metal accumulation is becoming a relevant problem in patients with metal implants (Ti in dental implants, medical implants, and dental prosthesis); studies of the actual tissue distributions of metals from the implants and generation of toxicity in animal models could have important implications for the clinic; studies of metal distribution in autopsy samples will also be important. Many studies have suggested "leaching" of metals from the implant to the surrounding tissues. This is very difficult to study by existing approaches because of the lack of sensitivity and resolution of the existing approaches. XFM studies on the animal models can provide information to assess whether toxicity is a significant concern in specific implant situations. For example, cell death can be induced through early mitotic arrest, apoptosis, and necrosis by the presence of metal ions [Cortizo and Fernandez Lorenzo de Mele, 2004]. Dental amalgam as a potential source of mercury is a very controversial topic and views on Hg release from amalgam vary from the notion that there is no effect at all [Brownawell et al., 2005] to hypothesis that exposure to mercury from this source plays a role in the pathogenesis of the Alzheimer's disease [Mutter et al., 2004].

Toxicology

Studies of metal distribution in metal toxicity diseases such as those caused by Cd, As, Cr, Hg, and Pb and other metal accumulation disorders is becoming more important as contamination in food, drinking supplies, metal piping, and other sources becomes more apparent in human populations. Clarkson believes that three major mercury sources in the environment are fish, amalgam dental fillings, and ethyl-mercury (a vaccine antiseptic) [Clarkson, 2002]. The mechanisms of the toxicities are often not understood, though it seems apparent that elements such as Cd, Cr, As, Ni, Vd, Fe, Cu, Mn have both mutagen and carcinogen properties [Desoize, 2003; Waisberg et al., 2003]. The tissue "affinities" of these metals and their exact threshold quantities causing adverse effects are not well known and need to be studied in more detail; moreover, both distribution and biotransformation of these elements need to be studied [Harris et al., 2005]. Continued detailed studies of metal accumulation disorders will require high-resolution approaches to map the metals on the subcellular level, which is possible only through nm-scale resolution. In addition, some metals have known toxicities even when given deliberately to a patient. For example, carboplatin is used as an important form of chemotherapy, yet the drug also shows significant toxicities as a standard side-effect; understanding these consequences of drug treatment is an important part of chemotherapy. Similarly, large amounts of Cr dietary supplements are taken as slimming and muscle-building agents and for their potential to prevent and treat diabetes, but there are increasing concerns about their cancer risks and, hence, it is essential to learn more about how they interact with cells. Hard X-ray microprobe studies have already been used to study the intracellular distributions and biotransformations of Cr carcinogens [Dillon et al., 2002] and this is being extended to many of the other metals; it seems possible to gain much more information at the organelle level from higher resolution studies that should be possible in future hard X-ray microprobes both in construction or in planning. In many cases low-resolution XFM is already used for detection of heavy metals in tissues of people who were exposed to metals

(e.g., Pb in smelter workers [Schutz et al., 2005]), or in the tissues of experimental model animals (e.g., studies of stress caused by Pb, Cd, and As in experimental rats [Fowler et al., 2004]).

An additional area of investigation that has gained more interest recently with concerns about "dirty bombs" is following the presence and speciation of radioactive heavy metals in living organisms. Most studies on lanthanides were conducted before the 1970's; at that time our ability to detect subcellular distribution and biotransformations of these elements was much less than today. However, even a few of the more modern examples of these studies (e.g., uptake of Pu of different oxidation states in mice [Fouillit et al., 2004] and in the cells in culture [Planasbohne and Duffield, 1988]) give no detailed account of the exact intracellular localization and speciation.

Environmental Biology

In many cases, most of the body burden of toxic trace element contaminants in organisms comes from their diet. The efficiency by which these elements are absorbed from food depends on their distribution in food particles, and the chemical form of the element. The fraction that is contained in the cytoplasm is generally well absorbed, while that adsorbed to the surface is not [Reinfelder and Fisher, 1991]. The processes governing the distribution of elements between cytoplasm and cell surfaces have been studied in vitro. However, in situ studies are needed to test the findings of these lab studies and to evaluate the influence of those characteristics of the natural environment that are hard to reproduce. Such studies could be accomplished by mapping metal distributions in individual cells exposed to natural conditions. By localizing metals within cells, XFM may also suggest mechanisms of toxicity by determining the organelle target of a specific toxic element.

At the same time, in recent years there are many efforts to perform phytoremediation of polluted soils. Studies of such toxin accumulating plants are of great interest for the human population as a whole. Different organisms are used for phytoremediation, from higher plants to algae (e.g., Cr accumulation subterranean clover [Howe et al., 2003], or As accumulating cattail [Blute et al., 2004]). XFM enables quantitative studies of the cellular and intracellular distributions of metal contaminants, as well as their respective, local, oxidation state.

CONCLUSIONS

The development of third generation synchrotrons and X-ray optics is rapidly revolutionizing XFM into a technique that is pivotal for analysis, quantification, and mapping of elemental content in cells and tissues. Systematic studies of the metalome coupled with the inevitable build-up of a bioinformatics base will depend on XFM more universally than on any other imaging approach. The advancement of bionanotechnology will demand a tool as sensitive and multi-versatile as only XFM can be. Visible light and optical fluorescence microscopy, electron microscopy, X-ray diffraction, two-photon excitation microscopy, secondary ion mass spectrometry, scanning electrochemical microscopy, and atomic force microscopy are all very important tools of bionanotechnology; nevertheless XFM will be a critically important technique in nanovector development due to possibility to study whole cells, to image subcellular features in cells and tissues, to observe the cells in nearly native freeze-hydrated state, all accompanied by high-sensitivity and low-background signals. Observing nanovectors "in action" at the site of their activity in cells is possible using flash-frozen samples treated by nanocomposites. Such studies will yield critical data that can be used to streamline and guide nanovector design.

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