Instrumentation developments in scanning soft x-ray microscopy at the NSLS (invited)

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(Presented on 18 July 1994)

The Scanning Transmission Soft X-ray Microscope at the NSLS has been instrumented for the following new forms of imaging: (1) XANES microscopy for the mapping of chemical constituents and for absorption spectroscopy of small specimen areas; (2) luminescence microscopy for locating visible light emitting labels at the resolution determined by the size of the x-ray microprobe; and (3) dichroism microscopy for mapping the alignment of molecules whose absorption spectra are polarization dependent. Since the instrument is used mostly for the imaging of biological and other radiation sensitive materials, a cryostage is being planned to accommodate frozen hydrated specimens. © 1995 American Institute of Physics.

I. THE SCANNING SOFT X-RAY MICROSCOPE AT THE NSLS

The Scanning Transmission Soft X-ray Microscope (STXM) has been operating on the NSLS X1A beamline since 1989, with zone plates fabricated by E. Anderson (Center for X-ray Optics, LBL). The X1 soft x-ray undulator serves as the bright tuneable source and the zone plate forms a microprobe that provides the 50 nm resolution of the instrument.1 (Fig. 1). In routine operation the specimen is mechanically scanned and the transmitted fraction of the incident x rays is used to form the image. With only an efficient x-ray detector following the specimen, the scanning microscope provides images which correspond to mapping specimen absorptivity with the lowest possible radiation dose.

Scanning microscopy is among the major beneficiaries of high brightness, in that the speed of image acquisition is proportional to the coherent flux, which in turn is proportional to the brightness. Coherent illumination is required to form the finest (diffraction limited) microprobe that the zone plate is capable of producing. At the NSLS the emittance of the electron beam is such that roughly 0.3% of the undulator output is coherent in the 20–40 nm wavelength range. The excess output is put to good use partly by splitting the radiation cone into two beamlines2 (and splitting off a third one is being planned), and partly to overfill the apertures and thereby improve the stability of the illumination.

Transmission images are taken in digital form, suitable for quantitative analysis. One study which used standard transmission imaging, undertaken in collaboration with J. Van t’Hof and S. Lamm, involved measurements on several hundred images of chromosomes from three plants and one mammal.3 Based on the data and independent information on the size of the genome in these species, the DNA fraction in the chromosomes was measured. In spite of the fourfold variation in genome size, the DNA fraction was found to be 39% within experimental error in all cases. This suggests a conservation of the DNA packaging mechanism.

II. RECENT INSTRUMENTATION DEVELOPMENTS

The following new instrumentation developments have added significantly to the capabilities of the microscope.

A. XANES microscopy

By scanning the monochromator with the x/y scan of the sample stopped, an absorption spectrum can be taken from a small (submicron) specimen area. Because the zone plate is highly chromatic (focal length proportional to photon energy), the focus must track the monochromator to keep the exposed spot in focus. Absorption spectra near elemental edges (XANES) often exhibit resonances or other features that are characteristic of the chemical environment of the absorbing atom. Based on the observed spectral structure one selects characteristic energies at which to image the specimen to map its chemical components. The technique was demonstrated by delineating the morphology of polymer blend.4 Applications to other polymeric systems, to biological specimens5,6 and to coal7 soon followed. Up to now we have been limited to the carbon K and the calcium L edges, but the obstacles to work at the nitrogen and oxygen edges (air, silicon nitride windows, and quartz mirrors) should be removed within the next year. To keep the beam focused on

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FIG. 1. Schematic diagram of the scanning soft x-ray microscope. The specimen is placed on an x/y scanning stage with coarse and fine motions driven by stepping motors and piezos, respectively. A zone plate (ZP) is used to focus the incident beam, and an order sorting aperture (OSA) absorbs all but the radiation in the first order focus. Vacuum window, zone plate substrate, specimen chamber windows, and proportional counter window are all 100-nm-thick silicon nitride membranes.

the same area as the photon energy is scanned, the focusing motion is being upgraded with a precise compensated flexure. This avoids the slight transverse wobble inherent in standard translation stages.

One of the polymeric systems where the nanoanalytical capabilities of the XI-STXM should prove illuminating are polyurethanes. For a number of years, phase segregation in flexible polyurethane foams has been a topic of considerable study by several techniques due to the detrimental influence of the precipitates on the foam properties. The chemistry of polyurethanes is complex and depends on numerous variables. Segregated phases formed are usually small and not completely isolated from the surrounding matrix. We have begun studies of a class of polyurethane foams based on methylene-diphenyl-disocyanate (MDI), as precipitates are large and reasonably well segregated from the matrix. The phase morphology of a typical phase segregated MDI-based foam as acquired with the X1-STXM is shown in Fig. 2. It clearly shows the presence of a bimodal distribution of precipitate size. The larger precipitates are about 5 μm in size, while the smaller precipitates are less than 0.5 μm in this particular foam. Since Fig. 2 was acquired at a photon energy of 285.5 eV, which is strongly absorbed by aromatic groups, the precipitates in the MDI foam have a high aromatic content, while the matrix has a relatively high polyl functionality (aliphatic, R-O-R'). Figure 3 shows point spectra from the large precipitates and the matrix. The spectra confirm the assessment made from the image that the precipitate has significantly higher aromatic functionality. One aspect that was somewhat surprising is the fact that there is relatively little difference in spectral intensity in the 289–295 eV energy range. The intensity in this energy range is mostly due to the carbonyl π* transition and the C-H σ* transition. Our tentative interpretation is that the increased carbonyl intensity expected for the precipitates (carbonyl groups are expected to correlate with aromatic groups) is offset by the increase in C-H intensity in the more aliphatic matrix. Unambiguous results concerning the carbonyl could be obtained by recording oxygen near edge spectra. Subsequent studies will therefore be aimed at clarifying the role and distribution of carbonyl groups and at elucidating the chemical difference between the small and large precipitates.

Previous studies on model MDI polyurethanes were carried out with electron energy loss spectroscopy (EELS) techniques with parallel detection. For high spatial resolution imaging and microanalysis, radiation damage, however, becomes an important issue for radiation sensitive materials such as polymers and particularly polyurethanes. It is estimated that XANES microscopy can be performed at lower radiation damage, up to three orders of magnitude lower than comparable electron microscopy. If radiation damage is the limiting factor of data acquisition it will thus be possible to...
characterize significantly smaller precipitates with x rays than with EELS.

DNA and protein are two important biological molecules with similar elemental components. Their different chemical compositions, especially the different concentration and environment in C–C, C–O, and C–N bonds, give rise to distinguishable features in their XANES spectra, as shown in Fig. 4. These features have been used to map DNA and protein in biological samples. The mix of DNA and protein in the sample makes the direct quantitative mapping of these two molecules by imaging at just two or three absorption peaks difficult. Instead, we have imaged the samples at six different energies, as indicated in the spectra, and separate the distribution through image analysis. Figure 5 shows the DNA and protein map in bull sperm. DNA is mainly concentrated in the sperm head, while there is rich protein content in the tail too, as expected. It also shows that the equatorial segment, an important structure in bull sperm, is protein rich.

Using these maps, we also get protein to DNA ratio in sperm, which is expected to shed light on DNA packing inside the sperm head, and provide useful information on fertility.

B. Scanning luminescence microscopy

If the x-ray probe is used to excite labels that emit visible light, the location of some labels can be determined with the resolution of STXM, which is about five times better than the resolution of the confocal microscope. This offers a potential route to high resolution immunolabeling. Initial experiments using inorganic phosphors (Fig. 6) and organic dyes (Fig. 7) showed promising results. More recently, Moronne et al. have shown that terbium chelates have superior resistance to radiation-bleaching compared to all-organic labels.

Luminescence microscopy requires efficient detection of the visible light emitted from the x-ray focal spot. At present, we use a 0.7 NA long-working-distance microscope objective to image the luminescent signal onto a cooled avalanche photodiode. This is incorporated into the optical microscope system used for sample alignment. A stereo luminescence detection system is planned which should allow us to exploit the three-dimensional (3D) imaging possibilities offered by luminescence microscopy.
vides clear indication of the molecular orientation within the
specimen. A small area spectrum of a Kevlar 49 thin film
peaks are associated with aromatic (285.5 and 286.3 eV) and
carbonyl (288.3 eV) groups. While these energies are charac-
teristic, the intensities depend on the orientation of the
excited bond relative to the linearly polarized x-ray beam.
Butterfly-like patterns characteristic of radially symmetric
structures have been observed in all three Kevlar fibers when
cut at 45°. The contrast of this pattern was reversed as the
sample was rotated by 90° about the optical axis. Micro-
graphs of Kevlar 149 are shown in Fig. 9 as an example. Our
observations are consistent with the average aromatic ring
plane pointing radially outwards.

III. PLANS FOR FUTURE DEVELOPMENTS

The microscope is used mostly to examine biological
specimens, often in a wet state. Radiolytical etching and ra-
diation damage sets a severe limitation to multiple imaging
of such specimens. We are therefore developing instrumenta-
tion to adapt the microscope to handle frozen hydrated
(cryo) specimens. Model calculations\(^\text{16}\) suggest that working
with rapidly frozen samples at a temperature below -140 °C
provide an increase in the radiation dose tolerated by the
sample by a factor 10\(^3\)-10\(^4\) before image degradation is ob-
served (in comparison with unfixed wet biological objects at
room temperature). This is in good agreement with experi-
mental evidence from protein crystallography and electron
microscopy. Cryomicroscopy will make it possible to gener-
ate multiple imaging for chemical mapping in wet speci-
cimens, and tomographic data sets for 3D imaging. It will also
make it possible to image radiation sensitive specimens with-
out chemical fixative at the best instrumental resolution of
the microscope, which is expected to improve to the 20-30
nm level over the next few years. The cryomicroscope will
operate in good vacuum to minimize ice buildup on the
specimen.

IV. CONCLUSIONS

The scanning transmission soft x-ray microscope has
provided users with routine operation for high-resolution im-
aging of biological specimens. It has been used to study cell
cultures, myofibrils, chromosomes, secretion granules, ma-
laria infected red blood cells, and other types of specimens
by collaborators and users from several institutions. In the
recent past its capabilities have expanded to include XANES
mapping, luminescence, and dichroism microscopy. We are
planning further significant expansion of the instrumentation
in the near future by adding the capability to examine frozen
hydrated samples.

ACKNOWLEDGMENTS

We are grateful to the many people who contributed to
the development of the scanning soft x-ray microscope. Erik
Anderson of the LBL Center for X-Ray Optics made the
microprobe forming plates in Dieter Kern’s laboratory at the
IBM Yorktown Heights Research Center. Vivian Oehler,
Jenny Fu, and Angelika Osanna contributed to the imaging
experiments, while Tony Leonard and Steve Wang made sig-
ificant improvements to the hardware. This work was sup-
pported in part by the DOE Office of Health and Environmen-
tal Research under grant No. 89ER60858, and in part by the

FIG. 8. XANES spectrum of 0.1 \(\mu\)m² area of 0.1 \(\mu\)m-thick section of Kev-
lar 49 fiber cut at 45° as acquired with the X1-STXM. The prominent spec-
tral features at 285.5, 286.7, and 288.3 eV are \(\sigma^\pi\) resonances associated with
unsaturated bonding in the aromatic and carbonyl groups, respectively. The
energies are characteristic and provide for bond selective imaging, whereas
the intensity depends on polarization and the geometrical orientation of the
bonds. The broader feature at 293 eV is a \(\sigma^\pi\) resonance of the \(\text{C}==\text{C}\) bonds.

C. Dichroism microscopy

The undulator output is linearly polarized. To the extent
that the absorptivity of a component of the specimen depends
on its orientation relative to the plane of polarization (dichro-
ism), the image will change if the relative orientation
changes. Ade and Hsiao have recently imaged sections of
Kevlar fiber, rotating the specimen in the plane perpendicular
to the beam between images. The pattern of absorptivity pro-
vides clear indication of the molecular orientation within the
specimen. Three types of Kevlar fibers were investigated:
Kevlar 29, 49, and 149, which differ from each other in the
degree of crystallinity (Kevlar 149 is the most and Kevlar 29
the least crystalline) and the crystalline orientation along the
fiber axis. A small area spectrum of a Kevlar 49 thin film
(0.1 \(\mu\)m thick) cut at 45° relative to the fiber axis as acquired
with the X1-STXM is shown in Fig. 8. The most prominent
features at 285.5, 286.3, and 288.3 eV are \(\sigma^\pi\) resonances associated with
unsaturated bonding in the aromatic and carbonyl groups, respectively. The
energies are characteristic and provide for bond selective imaging, whereas
the intensity depends on polarization and the geometrical orientation of the
bonds. The broader feature at 293 eV is a \(\sigma^\pi\) resonance of the \(\text{C}==\text{C}\) bonds.

FIG. 9. Micrographs of 200-nm-thick Kevlar 149 fiber section (cut at 45°
relative to fiber axis) imaged with the electric field vector in the left-right
direction (A) and in the up-down (B) direction at a photon energy of 285.5
eV. This energy selects the aromatic group of the fiber polymer and the
butterfly patterns are due to the radial symmetry and orientational order of
the fiber. The observed linear dichroism in these images is confirmed by the
contrast changes between the two images.

NSF under grant No. BIR-9316594. It was carried out mostly at the NSLS which is supported by the Department of Energy Office of Basic Energy Sciences.


16 G. Schneider, in X-Ray Microscopy IV, edited by A. I. Erko and V. V. Aristov (Bogorodski Pechatnik, Chernogolovka, Moscow Region, 1994).