Analysis of Organic and Biological Surfaces

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PREFACE

A wide range of analytical techniques are now available for the characterization of inorganic, organic, and biological materials. Not all these methods are applicable to organic samples, either because of limitations due to specimen preparation and examination and/or the damaging effect some of the techniques can have on organic samples. This book collects together 24 analytical techniques and methods that can be used to give a total characterization of the surfaces of organic and biological samples. Each chapter is complete in itself in that it explains the basis of the analytical method, how it can be applied to organic material, and the precautions that must be observed during the experimental process and in the analysis of the results. No single method will give all the answers; a judicious combination of techniques will allow quantitative information to be obtained from organic samples at the atomic, molecular, and morphological level.

At first glance it would appear that some methods have been omitted from this book. This is quite deliberate, for some of the methods are now commonplace and already adequately described in the scientific literature. Other methods have not been included either because they are too destructive or simply because they are at present not applicable to organic samples. The 24 chapters in this book, each written by an acknowledged expert in the field, contain methods and techniques that allow a proper analysis of organic and biological surfaces. It is recommended that the introductory chapter is read first because it gives an overview of the whole subject. In addition, it mentions others methods which, although not at present being used to analyze organic surfaces, offer considerable potential for studying this type of specimen.

PATRICK ECHLIN

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INTRODUCTION

Although we live in a complex, three-dimensional world lit from above, our cognition of this environment takes place by a process of visual perception of interlocking two-dimensional surfaces. Through experience, we can recognize patterns and shapes, and because we possess the remarkable facility of binocular vision, we are able to synthesize a series of planar surfaces into familiar three-dimensional objects. In many instances, and in particular in microscopy, we are able to codify and recognize new images that we have never seen before. With a few exceptions we are unable to see below the surface of natural objects, yet our experience tells us that many of the subsurface features of an object are important in determining the surface characteristics that are presented to us. Organic surfaces, and in particular biological surfaces, are heterogeneous and generally more complex than inorganic surfaces. The surfaces of a block of metal, a crystal of an inorganic salt, or a piece of concrete appear the same from whichever direction they are viewed, although important submicroscopic and crystallographic differences do exist. If we section or fracture these homogeneous materials, the internal surfaces we reveal are, at a macroscopic level, identical to the external surface. This is not true of many organic materials, and much of their functional significance can be related to the internal surfaces that are normally hidden from our view.

An analysis of organic surfaces must necessarily look below the surface and it is important to consider some of the ways we can expose internal surfaces without introducing artifacts during the preparative procedures. These manipulations are particularly important in the examination of biological surfaces, and great care must be taken in converting or replicating the specimens into a form that is conducive to the alien environment of the system used to image and abstract the information.

It is convenient to consider the properties of surfaces from the point of view of their *morphology* and their *composition*. The morphological features of a surface may be divided into an examination and measurement of the microstructural features (topography) and a quantitation of their interrelationships (topology). This morphological information is essentially two-dimensional and is frequently represented as a series of picture points along the X and Y coordinates. The real surface, as seen by our eyes, and

images of the surface (photographs, drawings, maps, etc.) only become three-dimensional because our previous experience in pattern recognition tells us that the various surface features are separated along an axis normal to the mean depth of the surface. This recognition is usually quite easy in the real world but may become problematic when we examine images of unfamiliar objects, for here the third dimension may not be directly observed but only perceived. In these circumstances it is essential to subject the planar surface projections to stereopair analysis, which makes it easier to perceive the distances between points on a nonplanar surface. Stereogrammetric techniques are the only way in which accurate measurements may be made on nonplanar surfaces. The chapter by Howell and Boyde considers these matters in some detail.

The compositional analysis of a surface must necessarily delve below the surface, although it could well be argued that the wavelength contrast (color), albedo (solar reflectivity), and specular features (shininess) can give us compositional information about an object (the shiny yellow color of gold is a good example). The depth analysis may be confined to the first few atomic layers of the surface or penetrate several micrometers below the surface. The information gained can be related to the elemental, molecular, and even macromolecular composition of the surface.

Although it is convenient to consider morphology and composition separately, they are in reality very closely linked. This link sometimes puts very severe restraints on the effectiveness of the imaging systems, for while one particular technique may give accurate compositional information the very act of quantitation may destroy, and in most instances the process of preserving and subsequently observing may only be achieved at the expense of the natural composition of the object. This paradox, "the very act of observing introduces artifacts," is alas one of the problems we have to accept in the microscopy and analysis of organic materials.

The 24 chapters in this book are concerned with the in situ analysis of organic samples, as they usually present greater problems in preparation, examination, and analysis than do their inorganic counterparts. Biological specimens present the most severe challenge to the microscopist and analyst, because they are nearly always highly hydrated. The chapter by Robinson considers the procedures whereby hydrated specimens can be examined directly by the microscope. The presence of water is less of a problem in the other types of organic samples, such as natural and artificial polymers, and elastomers, and the low water content and structural characteristics of some of the more robust plant and animal products (chitin, wood, bone, teeth, etc.) are such that they require little preparation prior to examination in the microscope.

ANALYSIS OF SURFACE MORPHOLOGY

The structural features of the specimen surface may be readily localized using a beam of photons or electrons. Other imaging systems exist e.g., those based on sound waves, x rays, and ions, that give a different view of surfaces. *Ultrasoft* x-ray microscopy, although not a new technique, has recently become a subject of renewed interest (Parsons, [1]). The x-ray imaging depends on the differential absorption of x rays in the wavelength range 1–10 nm, by the various components in the specimen. Recent work by Rudolph et al. [2] has shown that it is possible to obtain high resolution (70 nm) images of organic material using zone plate optics in conjunction with a high x-ray flux. Alternatively, the image need not be viewed directly but a replica is formed on a photoresist by the x rays that pass through the specimen. In areas of high mass density the x rays are absorbed and these regions appear in high relief after the photoresist is chemically developed and examined in a scanning electron microscope. The spatial resolution varies but can be as good as 5 nm. The advantages of the system are the reduced specimen damage and the ability to examine hydrated samples. This procedure is quite distinct from *direct x-ray microscopy*, which although giving information from wet specimens up to 5 μm thick can only produce images up to a magnification of ×200.

The advantages of *acoustic microscopy* are discussed in the chapter by Wickramasinghe. Acoustic waves are reflected or deflected by variations in specimen density on unstained specimens immersed in water. The resolution is consequently no better than 2.0 μm. By using cryogenic liquids instead of water it has been possible to obtain a spatial resolution of about 100 nm.

The limited resolution of *light microscopes* is the only restraint that can be put on this form of imaging system. A wide range of light microscope systems is discussed in separate chapters by Robinson, Ploem, Wayne, Cohen, Brackenoff, and Johnson. If we use light in the visible range, we are able to obtain information about the surfaces of all organic materials (except those that are photosensitive) and of course living, physiologically intact specimens. The information is presented to the observer in both wavelength and amplitude contrast, albeit at somewhat limited spatial resolution (~100–200 nm). The process of information transfer does not appear to damage or unduly perturb the organic specimen and we assume we are observing the specimen close to its natural state.

To increase the spatial resolution of the imaging system, we must use an illuminating system of much reduced wavelength. High energy electrons are a convenient illuminating and imaging system and although the spatial resolution is decreased to a few tens of nanometers, the use of

electron beams creates serious problems for many organic specimens. The short mean free path of high energy electrons in air necessitates the use of a high vacuum inside the microscopes. Such low pressures put severe restraints on many organic samples, particularly those that contain volatile material and an appreciable amount of unbound water.

The beam of electrons may also cause thermal and radiation damage to specimens, and organic samples are more susceptible to these deleterious effects than are inorganic samples. A possible way of avoiding these radiation effects would be to use a *mirror electron microscope* (Gvosdover and Zel'dovich, [3]). The primary electron beam reverses its direction and is reflected by an equipotential very close to the specimen, which is at a slightly negative bias voltage. The spatial resolution is only 100–200 nm but it does permit surface inhomogeneities to be imaged. There is some evidence that ion currents may develop at the surface of the sample, which could damage organic specimens.

As microscopists, we are familiar with the basic instrumentation, optical pathways, and mechanisms of image formation of microscope systems such as TEM, HVEM, SEM, and STEM. Suffice it to say that to obtain morphological information about surfaces it is necessary either to collect a signal reflected from a surface or one transmitted through a thin section of the surface. The familiar *scanning electron microscope* and its more recent derivative, the *low-loss electron microscope,* can provide a wealth of morphological detail at a resolution between 2 and 8 nm by collecting electrons reflected from the surface. The low-loss image is formed by collecting electrons that have lost only a small amount of energy (less than 0.1%) while being backscattered from a solid specimen. The primary electrons strike the specimen at oblique incidence and an energy filter is used to collect the electrons that have undergone the smallest deflection and the least energy loss. The microscope described by Wells et al. [4] and Broers [5] collects "low-loss" electrons that emerge from the bottom of the condenser-objective after they have been scattered from the surface of solid specimens. The specimens are placed approximately at the center of the lens gap, which means the lens operates at a focal length of 1 mm. The lens aperture is 30 μm in diameter, which yields a beam half-angle of 1.5×10^{-2} rad. The beam current is 20 pA and the beam diameter is 1 nm. The microscope can give very high resolution (~3 nm) images of solid surfaces. (A detailed chapter on scanning electron microscopy has deliberately not been included in this book because a number of good texts are available on the subject; see, for example, Goldstein et al., [6].) A related technique is *photoemission microscopy* discussed in the chapter by Griffith, where the sample itself is the source of electrons that are generated by bombarding the sample with UV photons. Small changes

of topographic detail can be readily discerned at a spatial resolution of between 5 and 20 nm. This technique can also give chemical information about surfaces.

The transmission electron microscope (TEM) and the scanning transmission electron microscope (STEM) reveal information contained within thin sections of surfaces, or in replicas derived from surfaces, at a spatial resolution of 0.2–3 nm. As the chapter by Pasquali-Ronchetti shows, with a conventional TEM operating in the 80–120 keV range it is necessary to limit section thickness to between 50 and 80 nm, and thinner still if high resolution is required. With a high voltage electron microscope, Carasso shows in her chapter on this technique that the thickness can be increased up to 1–3 μm but at the expense of resolution.

There is some doubt regarding the *amount* as distinct from the *quality* of information that can be obtained from a very thin section cut across an organic surface. A single section, although informative, is generally unrepresentative and it is necessary to reconstitute several hundred planar images into a three-dimensional model before true details of the surface become apparent. Complementary procedures may be found in morphometry and stereology in which the profiles and topological parameters of thin sections are reduced to mathematical terms that may be processed to give dimensions that may be related to the bulk material from which the section was cut. Quantitative relationships exist between the average dimensions of inclusions within a bulk sample and those of their profiles in sections. When certain conditions are met, the sum of profiles in a unit area of section may be quantitatively related to the sum of the inclusions contained in a unit volume of the solid. The chapter by Gundersen should be consulted for details of these procedures and for some of the ambiguities that may arise from three-dimensional interpretation of projections of thin sections. The process of image formation using a reflected signal and/or a transmitted signal is unaffected by the nature of the specimen, although the quality of the image, that is, the signal-to-noise ratio, can show considerable variation.

The fidelity of detail and quality of information we can obtain from organic surfaces are dependent on the way the specimen has been prepared prior to examination. Organic samples are usually composed of low atomic number polymers that are thermolabile and radiation sensitive, frequently contain substances with a high vapor pressure, and, in the case of biological material, contain a substantial amount of water. In addition, one of the many special features of living material is that it has extensive internal surfaces that are quite different in structure and function from the external surface that forms an interface with the environment. Much effort has been put into discovering ways to preserve these internal surfaces

so that they may be examined and analyzed by different forms of electron beam instrumentation. A multitude of papers exists giving details of specimen preparation for both biological and organic samples. A comprehensive discussion of these techniques may be found in the recent book by Goldstein et al. [6]. Briefly, the preservation involves chemical stabilization of the macromolecular architecture, careful removal or immobilization of any water and, under some circumstances, the introduction of high atomic weight elements to specific sites in the sample. The perfect preparative technique should aim to transform the thermodynamically unstable organic matrix into a heat- and ionizing-radiation-resistant inorganic replica. For scanning microscopy one should aim to preserve the spatial relationship of the organic matrix and then proceed to cover the surface with a thin layer of metal in order to increase the electron emissivity of the sample.

Surprisingly enough, these purely chemical procedures result in the preservation of organic structures of consummate functional excellence and beauty. However, in the case of living material, this preservation is only achieved at the expense of up to 99% of the contents of the cells being lost during the process. This loss is of great concern where the chemical (elemental and molecular) composition is the main objective of the analytical procedures. Following preservation, the internal surfaces of the organic material may be exposed by a number of means, but only after the mechanical strength and stability of the sample has been enhanced. This may be achieved either by replacing any fluids by liquid resins that are then polymerized to a solid or by converting the low viscosity phases of the sample to a high viscosity form (solid) by quench cooling. The strengthened material may now be sectioned, fractured, or replicated to reveal the internal surfaces.

It should be apparent that the validity and usefulness of information obtained from organic material examined and analyzed by any form of high energy beam instrumentation is vitally dependent on the preparative procedures. Of all the methods that have been devised, low temperature techniques show the greatest promise, particularly where an accurate chemical characterization is to be made of the sample. Cryotechniques, if properly and conscientiously applied, result in minimal disruption of organic and biological material, leaving the soluble constituents of the sample more or less in their natural location. Low temperature methods are an easy and convenient way of increasing the mechanical strength of many plastics and elastomers. Rubber, for example, can be easily thin-sectioned at 133 K and polyethylene all too readily fractures when cooled to 77 K. For the biologist, low temperature methods have been used to produce high resolution images of macromolecular assemblages within

and on cell membranes, by making a replica of the rapidly frozen sample. This freeze-fracture technique only allows an examination of a surface replica, whereas it is now possible to examine *and analyze* the frozen surface directly by maintaining it at a low temperature inside the microscope. The chapter by Echlin discusses these procedures in some detail.

It is convenient to record all the morphological information we see in microscopes in the form of photographic images. We do this as a consequence of our fallibility as we can more readily convince our colleagues about the existence of a new surface feature if we can show them a picture, photograph, or map.

It is opportune to consider briefly here the effectiveness of the ways by which the observer conveys information seen in the microscope to other observers. There are two methods by which this information transfer can take place: the verbal or literal and the visual. With little conscious effort, we are able to recognize patterns and an immense amount of scientific information has been gathered by the direct visual recognition of similarities or analogues between pictorial patterns. This process of intersubjective pattern recognition is, as Polanyi [7] points out, a fundamental element in the creation of all scientific knowledge. But how true is the maxim "seeing is believing." This initial part of the information transfer process must be determined to a large extent by the primary observer, who is responsible for distinguishing fact from preparation-induced artifact and datum points from instrumental malfunction. Having, to the best of his or her ability, validated the acuity of the visual observation, the microscopist frequently takes a representative photograph of the observed surface. A micrograph is an important item of scientific observation, as it accurately locates observed details, as well as accentuating the consensual* elements in the image. But the micrograph, although undeniably an accurate representation of the surface image, is of limited usefulness. It may be limited by transient accidents of illumination, by absorbance and reflectivity, and by spatial effects of color. Although representative of a small area of the surface at a particular instant in time, it may well not be representative of the whole surface. This is a particularly acute problem with biological material, which is immensely variable, and a better graphic representation of a surface would be achieved by means of a drawing or a map. A drawing is able to condense information that may be read at normal optical resolution and is an expression of structural understanding. A drawing arises from a unique combination of

* Ziman [8] distinguishes between *consensible* messages, which are unambiguous statements describing an event and which may contribute to the general body of scientific knowledge, and *consensual* statements that have been fully tested and are universally agreed.

the precision of the hand, eye, and mind and unlike the photograph is timeless. In this way it should be possible to summarize the information gained by examining the images of many surfaces of the same object and give a representative picture. Alternatively, the information in the photographs could be codified either as a series of gray levels delineating areas of interest or could be transformed into a wider range of wavelength contrast levels. Alternatively, a series of measurements may be taken from stereopair micrographs to emphasize spatial characteristics such as shape connection and intersections. This information could be stored and added to, in this way giving some measure of the variability one might expect to see in a single micrograph.

The use of computers in microscopy is now becoming more popular particularly where the image is produced as a result of digital scanning methods. Simple analogue processing techniques such as black level subtraction, Y modulation, signal differentiation, artificial color coding, and contrast variation enable the operator to enhance the information content of the image but are limited when compared to digital processing. Provided one has a sufficiently high storage capacity (~1 megabyte per micrograph [Smith [9]), the photographic process can be eliminated, and the image stored on a magnetic disk. This stored image may be modified in light of information obtained from subsequent images and a hard copy representative image produced. This image could be in the form of a graph, a map, instructions for a three-dimensional model, or a photograph.

Although our consciousness is dominated by visual images, we tend to communicate scientific information by linguistic means. This requires a very precise use of language but can in some circumstances be a more accurate way of communicating information than a purely visual image.

A single photograph can do no more than convey basic information from observers to their colleagues. Hanson [10] states that "a picture is worth a thousand words." Most people forget the rest of the quotation, which continues "a picture is a thousand times less specific than a short sharp statement. A statement can supply a focus for the attention that is different in type from anything generated by confrontation with a picture." We need to combine the visual and the literal. How might we best convey the images we see in microscopes? The photograph is a convenient, high density information storage medium and relatively free of bias but with the limitation of only recording an unrepresentative two-dimensional planar projection of a surface that invariably has a third dimension. The photograph is an intermediary in the process of information transfer; it is not the end product. We should therefore use micrographs, together with the precise verbal information we record when examining the image

of the real object to construct the permanent baseline information either as a model or a map, and use this to convey new information to our colleagues or to store in the scientific archives.

ANALYSIS OF SURFACE COMPOSITION

The total characterization of a surface involves more than just obtaining high resolution topographical and topological information along the *X* and *Y* coordinates and the chemical identity of the surface layer of atoms. It should also include a coordinated in-depth analysis along the *Z* axis at as high a spatial resolution as may be obtained with a particular form of instrumentation. A surprisingly large number of techniques, based on exciting the sample with photons, electrons, or ions, are available for the analysis of organic surfaces.

TRANSMISSION ELECTRON MICROSCOPY

As Pasquali-Ronchetti shows, the TEM may be used to give analytical information by virtue of the increased electron scattering from high atomic number inclusions within the low atomic number matrix that characterizes most organic material. Unlike visible photon radiation, which is sensitive only to chemical bond structures, electron radiation is sensitive only to elemental atomic number. This seriously limits the ability of TEM to localize directly the molecular and macromolecular species that are the principal constituents of organic material and also makes it virtually impossible to localize directly the elements (C, H, O, N. S, P) that form the bulk of organic compounds. The direct localization of molecular species and chemical bonds can be carried out albeit at reduced resolution using the light microscope by virtue of natural pigments, that is, chlorophyll in leaves, vital and nonvital dyes, and the natural fluorescence of some molecules when irradiated with UV light. The chapters by Ploem, Wayne, and Cohen provide details of such methods.

Occasionally, high atomic number material, usually as heavy metals, may be present as a natural deposit in organic samples. Under these circumstances the TEM can only indicate that a high atomic weight particle is present; it cannot give information on the precise nature of the inclusion. If the inclusion is in a crystalline form, it may be identified by means of its characteristic *electron diffraction pattern* using the selected area diffraction device fitted to most modern transmission microscopes.