Microscopy and other imaging techniques in food structure analysis

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Microscopy and imaging techniques are the most appropriate techniques for evaluating food structure because they are the only analytical methods that produce results in the form of images rather than numbers. However, images may now also be converted into numerical data to allow for statistical evaluation. Advances in microscopy and imaging techniques are made, for the most part, outside the field of food science, drawing from the fields of materials science, biology and medicine. Such techniques cannot, in most cases, be directly applied to study food structure. They must be adapted because the processing conditions that turn biological raw materials into food cause structural and textural changes which, in turn, change the innate properties and behaviour of the foods. This necessitates the development of appropriate methods and also the specialization of researchers. Future developments in this field can be divided into the use of new equipment developed for use in other fields, and the application of techniques modified to solve specific food science problems, such as the development of new foods with particular properties and texture or the detection of defects in foods.

Microscopy (optical or light, electron and atomic microscopy) and other imaging techniques (e.g. magnetic resonance imaging) generate data in the form of an image. They are an extension of the visual examination of foods that has been practised by consumers and food processors alike. Microscopy techniques vary in method of image production, resolution and type of signal detected, and give a particular type of structural information that is unique to the technique used[1].

Studies suggest that foods having similar structures can be loosely grouped together as foods that have similar textures. Most foods are of biological origin, but are processed to varying degrees, sometimes to such an extent that their biological origin is not readily apparent, for example grain versus bread, muscle versus salami or milk versus cheese. Visual changes due to processing (e.g. milling of grain, gelatinization of starch, comminution of meat, heat denaturation of proteins, gelation of milk and proteolysis of proteins) are the results of changes at the microscopic and molecular levels. Imaging techniques can be used to help evaluate such changes in terms of morphology and composition.

Optical microscopy was initially used in food science to detect the contamination or adulteration of foods, either accidental or deliberate. This was followed by interest in the microstructure of food itself, and how it related to other properties of a particular food. With the commercialization of food production, it has become necessary to understand the processes leading to the development of various structures (e.g. foams, emulsions, dispersions, extrudates and fibres), to produce such structures in newly designed foods and to avoid defects in the foods being manufactured.

The structural analysis of foods has advanced rapidly during the past 20 years. Aguilera and Stanley[2] have reviewed this progress and discussed the techniques, findings and interpretations. Electron microscopic techniques used in food science have also been reviewed[3]. Although optical and electron microscopy have been used in food analysis for many years, few university food science departments include food structure studies in their curricula. Special techniques (e.g. confocal laser-scanning microscopy[4] and atomic force microscopy[5]) and procedures such as elemental analysis (X-ray microanalysis[6] and electron energy loss spectrometry[7]) are used to study specific problems by relatively few food scientists on a routine basis. Other imaging techniques, including magnetic resonance imaging[8], acoustic microscopy[9] and ultrasoft X-ray microscopy[10] are, at present, used in food science either infrequently or not at all, although they are potentially very useful and their importance is expected to increase.

The visualization of true food structure is extremely difficult; each step of the preparation of a specimen for microscopy alters the food sample to some extent. Inadvertent or deliberate removal of water, lipid or other substances during preparation alters the sample, and changes the relationships that exist between its components. Such alterations must therefore be taken into consideration when drawing conclusions and generalizations from the analytical results. The best approach is to subject each food sample to several imaging techniques for comparison and confirmation of results. The power of imaging techniques is in their use as part of an integrated system, where changes observed at various levels of resolution are systematically evaluated.

Microscopy

Light microscopy

Bright-field, polarizing and fluorescence microscopy techniques are used most frequently. Although

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manufacturers regularly produce 'new and improved' microscopes, objectives and various bits of peripheral equipment, the basis of image formation in these techniques remains unchanged. In conventional bright-field microscopy, illumination is transmitted sequentially through a condenser, the specimen and the objective, producing a real image that is upside down and reversed, and magnified within the microscope tube. The real image is then magnified again by the ocular lens, which produces either a virtual image that appears to be ~25 cm from the eye, or a real image on photographic film (or video) placed above the microscope tube[11]. If the specimen is not highly coloured, contrast must be introduced to make it visible. This is commonly achieved by the use of dyes or stains of known specificity for different components of the specimen. The blue staining of starch with iodine is a long-established but nevertheless very useful reaction for the food microscopist. Fast Green or Acid Fuchsin are among the many stains that are useful for the localization of proteins. Toluidine Blue O (TBO) is a metachromatic dye that is especially useful in the examination of foods: pectin-containing plant cell walls (e.g. in fruit and vegetable tissues) stain pink to purple with TBO, whereas cell walls containing lignin (e.g. in vascular tissues) stain dark blue. In meat products stained with TBO, muscle tissue is pale pink, fibroblasts are bluish and elastin fibres are turquoise[12]. Lipid-soluble dyes such as Oil Red O are used for staining fats. Another method for introducing contrast is to alter the path of the light hitting the specimen, for example as in polarizing microscopy.

A standard bright-field microscope can be easily converted for polarizing microscopy by the insertion of two polarizers in the light path: one between the light source and the specimen, and the other between the objective and the viewer. Plane-polarized light, which is produced by the first polarizer, has rays that vibrate only in one plane, perpendicular to the direction of travel. A common and inexpensive method of producing this effect is to use Polaroid™ film. If the second polarizer, the analyzer, is rotated such that the transmitted vibration is at right angles to the vibration of the incident light (crossed polars), amorphous regions within the specimen will appear dark, whereas crystalline or ordered regions will appear very bright against a dark background. The bright areas are the result of their components having two principal refractive indices: such substances are said to be birefringent[13]. A wide variety of food components are birefringent, including starch, fats, plant cell walls, muscle fibres as well as many flavour and seasoning ingredients.

In fluorescence microscopy, light of a specific wavelength is absorbed by specific molecules that are present in the specimen, and the energy is re-emitted as light of a longer wavelength and lower intensity (fluorescence). The most versatile fluorescence microscopes use incident light, or epi-illumination, rather than transmitted light. For epi-illumination, the objective performs as both the condenser lens and the objective lens simultaneously, eliminating the difficulty of aligning the two lenses. A chromatic beam splitter, or dichroic mirror, which is positioned between the light source and the objective, reflects light that is shorter than a particular wavelength, and transmits light of longer wavelengths. Thus, the shorter-wavelength-exciting illumination is reflected down onto the specimen, whereas the longer-wavelength fluorescence emitted from the specimen is transmitted to the eyepiece. In addition, extraneous shorter-wavelength light reflected back from the specimen or the optics is also reflected by the dichroic mirror, preventing it from reaching the eyepiece, so that the image seen is formed only by the emitted fluorescence[14].

Many food components of plant and animal origin exhibit inherent fluorescence (autofluorescence). In plants, these include pigments (e.g. chlorophyll and carotenoids), and both high and low molecular mass phenolic compounds (e.g. lignin and ferulic acid). In animal tissues, the main sources of autofluorescence are bone and cartilage, collagen, elastin and some fats. Other ingredients such as vitamins and flavourings or seasonings are also fluorescent. In addition to autofluorescence, there is an ever-increasing selection of fluorescent probes to choose from, designed to impart fluorescence to the component of interest. Some, like Calcofluor, a fluorescent brightener used to localize β(1→3),(1→4)-D-glucan in cereal grains, fluoresce both in solution and bound to the glucan. Others, like Nile Blue, which has a component that is soluble in lipids, require a specific environment to be fluorescent, like the nonpolar milieu inside a fat droplet. Still greater specificity is imparted by the use of antibodies and lectins labelled with fluorescent compounds: with these tools, specific proteins and saccharides rather than entire classes of compounds (e.g. proteins) can be labelled (Fig. 1).

Advances in instrumentation have been made in light microscopy, most notably in the development of confocal laser-scanning microscopy (CLSM). Although the confocal microscope was invented in 1957, it was not until the 1980s that innovations and combinations in technology made the commercial production of instruments, and hence the more widespread use of the technology, possible. The main difference between a confocal and a conventional microscope is in the placement of a pinhole at the focal plane of the image, which has the effect of removing out-of-focus light, thus producing a clearer image, as well as allowing optical sectioning of the specimen, that is focusing at predetermined levels beneath the surface. Unlike conventional light microscopy, where the entire specimen or field of view is illuminated uniformly, in confocal microscopy the specimen is illuminated and imaged one point at a time, through the pinhole (hence the appellation 'scanning' microscopy).

To date, the area of food analysis in which CLSM has proved most advantageous is the examination of high-fat foods, which are difficult to prepare for conventional microscopy without loss or migration of the fat. Using CLSM, larger samples of food can be sectioned optically, allowing the imaging of delicate relationships

within the sample, which are often distorted or destroyed by physical sectioning or smearing techniques. For example, CLSM has been used to characterize high-fat spreads such as butter and margarine, and lower-fat (40%) spreads such as 'Halvarine', using Nile Red to stain the lipids, and fluorescein isothiocyanate to stain the proteins[4,15]. Bulk specimens were used, so that the size and position of the fat globules within the spreads were undisturbed by specimen preparation. The same researchers also used CLSM to observe the development of the structure of wheat dough during proofing, and the localization of additives in the wheat gluten protein. Several different components can be identified and localized at once with CLSM by using specific fluorescent labels, depending upon the number of laser lines available on the instrument. As with conventional fluorescence microscopy, the use of antibodies and lectins greatly increases the number of very specific protein and saccharide components that can be studied.

Another advancement in light microscopy is the coupling of microscopy with various types of spectroscopy, so that specific chemical groups can be localized and mapped *in situ*. While much of the instrumentation is not particularly new, it is only with the addition of computers to control the process and analyze the data that wider applications have become possible. Spectral characteristics provide information about the chemical nature of an unstained specimen down to the level of a single cell or microstructural feature. For example, using UV absorption, it was possible to differentiate between samples of insoluble fibre manufactured for use in bakery products[16]. Fourier transform infrared microspectroscopy has been used to study bacterial infection in potato tubers[17], and also to characterize the chemical nature of components within cereals, oilseeds, spices and flavour compounds[18]. By scanning across a specimen at a single wavelength, the distribution of a particular component within that specimen can be mapped. For example, because of interest in the use of mixed-linkage β-glucan from oat as a dietary adjunct to help control serum cholesterol levels, fluorescence microspectrometry is being used to study the distribution of the polysaccharide in oat kernels, as a way of identifying suitable varieties for processing purposes[19] (Fig. 2).

Electron microscopy

Compared with light microscopy (LM), the resolution is considerably improved with electron microscopy (EM)[20]. Image formation in EM is similar to that in LM, but the illumination source is electrons focused with magnetic lenses rather than photons focused with glass lenses. As electrons are absorbed by air, EM is carried out *in vacuo*; thus, the sample must not release any volatile substances when placed in the microscope. This can be achieved by drying or freezing the sample before examination, or replicating it with platinum and carbon and examining the resultant platinum–carbon replica. There are two principal EM modes, which complement each other, namely scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The methods differ mainly in the method of image formation. The interpretation of the resulting images depends to a great extent on the experience of the food scientist or microscopist.

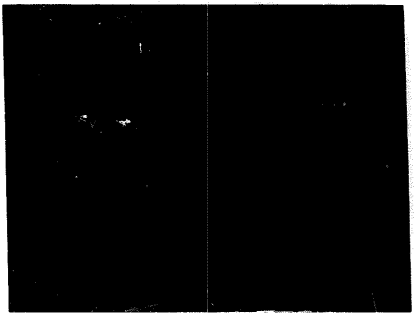

Fig. 1

Fluorescence micrograph showing binding of the fluorescein-labelled lectin *Ulex europeaus* agglutinin I (UEA I) in a section of the oilseed canola. UEA I (yellow) has an affinity for α-L-fucosyl groups, which are found in the fucoxyloglucan of the cell walls in the seed. The section was counterstained with Fast Green, causing the protein bodies to fluoresce red. Scale bar = 10 μm.
(S. Shea Miller, unpublished.)

Scanning electron microscopy

SEM is used to examine surfaces[21]. The sample is either dry (conventional SEM) or frozen below −80°C (cryo-SEM). A 5–20 nm-thick metal (gold) coating provides electric conductivity. The sample is scanned by a focused electron beam, and secondary or back-scattered electrons are processed to form its enlarged image. The absence of water in dried samples exposes their solid structures for examination. SEM images have a great depth of focus and are relatively easy to understand (Fig. 3a). Modern field-emission scanning electron microscopes offer a high resolution (Fig. 3b).

Hydrated food samples destined for cryo-SEM are first rapidly cryo-fixed to retain water and convert it into vitreous ice[22]. Only structures in the fracture plane are visualized in the presence of water. Optional 'freeze etching' (sublimation of a thin layer of the ice) exposes solid structural elements under the surface for examination. Cryo-SEM is very useful for observing high-fat samples and other food samples that are difficult to stabilize using conventional preparation methods[23]. Like other techniques, cryo-SEM is not free of artifacts. The formation of ice crystals, which can displace structural elements and destroy the initial structure, poses one of the greatest risks – one that may be reduced by proper processing (i.e. by using a small sample, a high freezing rate and a low temperature).

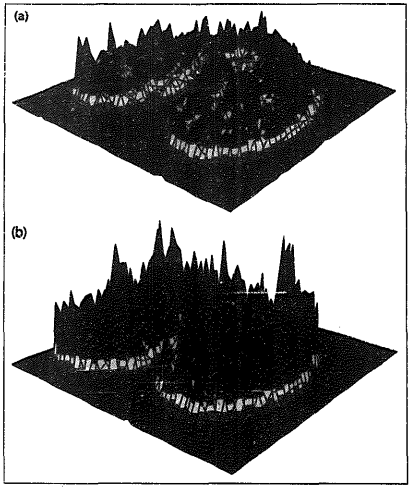

Fig. 2

Mapping of β-glucan distribution in a cross section of oat using scanning microspectrofluorometry of Calcofluor bound to β-glucan in the endosperm cell walls (excitation 365 nm; emission >418 nm). (a), Cultivar with low β-glucan (Cascade, 3.27%); (b), cultivar with high β-glucan content (Belmont, 5.54%). Relative fluorescence intensity, which increases from red (lowest) through yellow, green and blue to fuchsia (highest), varies with β-glucan concentration. (S. Shea Miller, unpublished.)

Transmission electron microscopy

TEM visualizes the internal structure of food samples. Thin (15–90 nm) sections of samples embedded in epoxy resin or platinum–carbon replicas of the sample are placed in the path of the electron beam, and the enlarged image is observed on a fluorescent screen or photographed on film. The electrons are transmitted through the sample with varying degrees of energy loss. Differences in the electron density of structures stained in the resin sections with heavy-metal salts (e.g. of uranium or lead) or differences in the thickness of the metal replica due to differences in the angles at which the metal is deposited on the fractured sample result in the formation of the image[20]. Images of replicas resemble those images obtained by cryo-SEM; replicas may be stored for future studies.

Negative staining and metal shadowing are other TEM techniques suitable for visualizing suspensions and emulsions[3].

Specimen preparation

The choice of the preparation techniques used is dictated both by the sample type and the information required from the sample. Chemical fixation methods used in biology are geared towards the stabilization of hydrated cell-based systems. These methods must be modified for use with food samples because the cells and their organelles present in the raw materials become disrupted during food processing such that the resulting food product becomes a new complex system in which the compartmentalization of the components is no longer cell-based. The conditions necessary to stabilize most foods differ from those used to stabilize the raw materials. A glutaraldehyde solution is used as a primary fixative to crosslink proteins, whereas an osmium-tetroxide (OsO_4) solution is used as a heavy-metal oxidative fixative to stabilize unsaturated lipids. Protein-based foods (e.g. meat and milk products) are relatively easy to fix, but high-fat foods pose problems: saturated fats cannot be chemically fixed. Unsaturated fats react with OsO_4 but then release OsO_3 and turn into diols[24]. However, the presence of imidazole stabilizes OsO_4-fixed fats[25].

Foods with high polysaccharide contents, particularly of gelatinized starch (e.g. puddings, pasta and bakery products), are also difficult to fix chemically. Cryo-fixation[26] is a better option. The de-oiling of fats such as butter, margarine, fat spreads and shortenings is used to visualize the crystalline fat network by SEM[27], whereas cryo-fixation followed by replication produces superior TEM images[28] (Fig. 4).

The removal of water, which routinely follows sample fixation, and the optional removal of fat deplete the food sample of important constituents as well as of any substances dissolved in them. Such removal procedures are often necessary to allow the visualization of matrices of either solid protein or fat crystals, and must be taken into account in the interpretation of the resulting images.

Using EM, it is possible to see that foods made from similar raw materials may have different structures depending on the other ingredients present and the manufacturing procedures used. For example, although yoghurt, full-fat cheese and low-fat cheese are all made from milk, yoghurt has an open (porous) protein matrix composed of interconnected chains and clusters, full-fat cheese has a compact protein matrix with fat globules and whey droplets interspersed in it, and low-fat cheese has the most compact matrix of all three products with very few fat globules. These differences in structure determine the manner in which the samples should be prepared for EM: fixation, dehydration and impregnation with a resin take more time with compact than with open samples of similar dimensions. Neglect of such considerations may lead to several artifacts, which may invalidate the results and conclusions.

Combination of methods

A combination of various EM techniques provides a more accurate view of food structure than any one technique. For example, void spaces revealed by cryo-SEM in yoghurt or cheese indicate air pockets. Subsequent freeze etching reveals the location of the aqueous phase

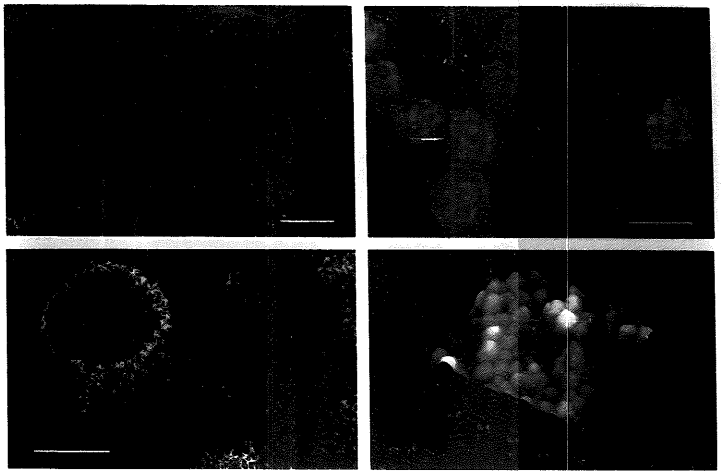

Fig. 3

(a), Conventional scanning electron microscopy (SEM) of roller-dried milk powder, showing the convoluted (fractal) particle surfaces. Scale bar = 200 μm. (M. Kalab, unpublished.) (b), High-resolution SEM of casein micelles in milk. Scale bar = 0.25 μm. (Courtesy of W.R. McManus and D.J. McMahon, Western Center for Protein Research and Technology, Utah State University, Logan, UT, USA.) (c), An example of the use of electron energy loss spectrometry (EELS) in food science: computer-enhanced transmission electron micrograph showing calcium distribution in a casein micelle in milk. The colour changes from yellow to dark blue as the calcium concentration increases. Scale bar = 0.25 μm. (Courtesy of W.R. McManus and D.J. McMahon, Western Center for Protein Research and Technology, Utah State University, Logan, UT, USA.) (d), Casein micelles in condensed skim milk as imaged by atomic force microscopy (AFM). A drop of condensed milk was placed on a glass cover slip and stored for 15 min at room temperature. A gold-coated silicon nitride tip was used to image the casein micelles with a Digital Nanoscope III AFM. The sample was scanned in air under atmospheric conditions at a scan rate of five lines per second in a constant-force mode at 3 nN. The image was obtained by scanning the casein micelles from above and represents the surface above the coverslip. Scale: 1 division = 0.2 μm on the x, y and z axes. (Image courtesy of S. Chastain and N. Desai, NSC Technologies, Mount Prospect, IL, USA.)

(as ice), which gradually vanishes as sublimation progresses. Chemical fixation of proteins using glutaraldehyde alone makes it possible to extract lipids and focus on the void spaces remaining in the protein matrix, whereas post-fixation of parallel samples with imidazole-buffered OsO_4 preserves the lipid droplets for examination. A network shown by TEM to contain a high proportion of single particles in a thin section may subsequently be shown by SEM to consist of interconnected chains or fibres. Thus, a conclusion based on counting the single particles would be erroneous as these represent cross sections of the chains or fibres.

Exceptions to all of the rules mentioned in this article exist with specific procedures; for example, hydrated samples may be examined in an 'environmental' SEM at a very low accelerating voltage[29] of several hundred to a few thousand volts without a metal coating, or sections as thick as 1 mm may be examined by TEM in special microscopes.

Localization techniques

Conventional microscopy (LM, TEM and SEM) has been used in food structure studies to obtain a qualitative description of the structure of samples. Localization techniques can be used in conjunction with the conventional microscopies, to determine the distribution of structural elements, macromolecules and elements of interest in the samples. Such techniques form the basis of what is generally called analytical microscopy, and fall into two broad categories: those in which the localization probes are applied during specimen preparation (such as immunolocalization), and those that depend on the detection of other signals resulting from electron beam interaction with the sample, using additional,

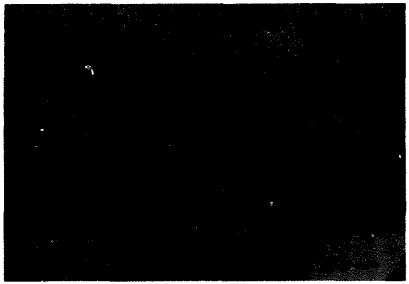

Fig. 4

'Freeze fracturing' of process cheese followed by replication with platinum and carbon shows a homogeneous distribution of fat globules (F) in the protein (P) matrix. The crystalline character of the fat globules is clearly visible. The process cheese contained 60% fat in the dry matter. Scale bar = 1 μm. (Courtesy of W. Buchheim[28] and Marcel Dekker, Inc.)

specialized equipment attached to the microscopes (e.g. elemental analysis).

Immunolocalization of specific proteins and polysaccharides by LM and EM

Many of the stains available for both LM and EM identify classes of compounds such as proteins and carbohydrates. The specific identification of many individual proteins, glycoproteins and polysaccharides is possible by using appropriately labelled antibodies and lectins. In LM, this is most effectively achieved with fluorescent labels such as fluorescein or rhodamine; the labelled probes can be used with either conventional fluorescence microscopy or CSLM. For EM, colloidal gold[30] has now surpassed ferritin or diaminobenzidine as an electron-dense marker for immunolabelling. Gold particles can be manufactured in very precise size ranges, making it possible to label two or more different components within the same section[31].

For immunolocalization, primary mono- or polyclonal antibodies bind to specific antigenic sites on the sample. Colloidal-gold probes coated with a secondary antibody, or with staphylococcal protein A, then interact with the bound primary antibodies to label the component of interest. Individual primary antibodies can be labelled directly, but it is more economical to have a 'universal' secondary label that can be applied to a variety of primary antibodies. The localization of whey proteins (β-lactoglobulin) in a meat product may be used as an example of immunolabelling (Fig. 5). Antibodies are most commonly raised against protein antigens, although polysaccharides of sufficient size (e.g. β-glucans[33] and arabinoxylans[34]), and oligosaccharides or other small molecules conjugated to a sizeable protein will also elicit an antigenic response. Anti-polysaccharide antibodies are used primarily in biological

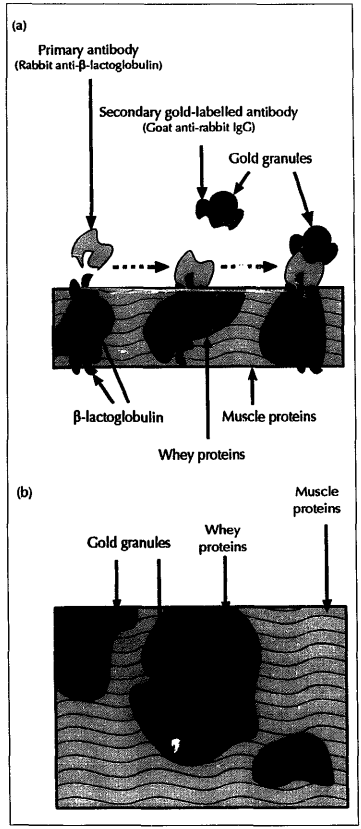

Fig. 5

(a), Primary, specific antibodies against β-lactoglobulin obtained from rabbit are applied to a thin section of the meat product (which contains whey proteins) embedded in a resin, where they bind to any exposed β-lactoglobulin molecules. Secondary, non-specific, gold-labelled goat anti-rabbit antibodies are then applied to mark the β-lactoglobulin sites with gold[32]. (b), Schematic diagram of a micrograph with the distribution of gold granules (which indicate the presence of β-lactoglobulin) and muscle as well as non-muscle proteins. (M. Kaláb, unpublished.)

applications as yet, but their use in foods will undoubtedly increase.

Immunochemical techniques are being used more frequently in food analysis[35], and therefore an increasing number and variety of antibodies are being produced. Immunogold labels may also be used for SEM samples. They have also been used as cell-surface markers in atomic force microscopy (AFM)[36], although not yet in a food context.

Lectins, which are proteins or glycoproteins of mostly plant origin, may be used in a similar fashion to antibodies. Lectins can be produced with either fluorescent or colloidal-gold labels; alternatively, the binding of lectins to samples may be detected using labelled antilectin antibodies. Polysaccharides may be localized by the use of polysaccharide-degrading enzymes bound to colloidal gold (e.g. xylans in cell walls[37] may be visualized by xylanase–gold complexes). Alternatively, antibodies to the enzymes can be used to visualize the enzymes bound to their substrate in the sample. The preferential binding sites of cellobiohydrolase and endogluconase on a cellulose microfibril were demonstrated using this technique, the two enzymes being labelled with different sizes of gold particles[38].

Elemental analysis

Electron beam interaction with a sample produces electrons that have many different characteristics. As mentioned earlier, EM images are formed by the detection of certain types of electrons that have reacted with the sample. In addition, the signals produced can be analyzed using special detectors, to provide the atomic composition of the samples, which may be mapped or quantified. This can be accomplished in a number of different ways, depending on the type of detector selected.

X-ray microanalysis is based on the emission of X-rays from areas bombarded by electrons in the microscope. The X-ray emission, as discrete energy levels, is characteristic of the element that generates it, and is detected as a unique series of peaks. The emission spectrum can be used to identify the element, making it possible to carry out qualitative as well as quantitative microanalysis of selected areas for their elemental composition or to map the distribution of particular elements for the entire area under study[6]. Elements of low atomic number such as boron, nitrogen or fluorine are more difficult to detect than heavier elements, but may be detected using specialized techniques such as electron energy loss spectrometry (EELS).

Types of X-ray detectors: energy-dispersive and wavelength-dispersive systems

The energy-dispersive (EDX) detection system, which has the ability to display the entire elemental spectrum at once with high sensitivity, is the system that is most used in both biology and food science. The EDX system has been used in food analysis in many instances; for example, to explain the crystallization of calcium phosphate on Camembert cheese as a result of changes in pH induced locally by decaying *Penicillium camemberti* hyphae[39], to distinguish lactose crystals from particles containing mineral constituents in spray-dried permeate obtained by the ultrafiltration of milk[40], or to study the distribution of titanium in wheat and spinach plants treated with soluble titanium substances to increase yield[41]. Although the wavelength-dispersive (WDX) system has some advantages over the EDX system (including the ability to discriminate closely spaced X-ray energy peaks), it is not widely used in either food science or biology. Improvements in instrumentation have made it possible to combine both the EDX and WDX systems in one microscope accessory, and to extend detection in the light-element range to carbon[29].

Electron energy loss spectrometry and electron spectroscopic imaging

The energy loss realized by the passage of inelastically scattered, transmitted electrons through a sample may be separated on the basis of their energy levels, using an electromagnetic spectrometer[20]. Like the EDX and WDX systems, EELS displays the spectrum of electron energies, which is characteristic of the elements responsible for the loss of energy in the beam electrons. The resolution of EELS is comparable to that of the EDX system and offers a more efficient detection level, especially with elements of low atomic number (Fig. 3c).

In another arrangement, in which the spectrometer is placed between the objective and the intermediate lenses, the spectrometer functions as an energy filter, eliminating electrons of particular energies. Only those electrons that pass through the filter are used to form the image. Superior imaging is obtained even with thicker samples. Electron spectroscopic imaging (ESI), as this type of detection is known, has also been used for mapping various elements, including carbon, oxygen and nitrogen, in unstained sections only 30 nm thick[7].

Evaluation and quantification of images

Digital image analysis

The image produced by any microscopy technique is the starting point for digital image analysis, which is used to quantify various features of the image. Images may be obtained in digital form directly in the microscope; alternatively, negative film or prints of images can easily be digitized ('scanned') to prepare them for mathematical analysis. The quantification of images can indicate structural changes due to processing on any resolution scale, from macrostructure to microstructure. Such structural changes in a food may help to predict its texture, which is an important quality consideration for the consumer.

Sight is used to evaluate raw materials as well as finished food products for quality. The judgement is based on a view of their macrostructure as seen by a naked eye and also on structural details as visualized by microscopy. Our brain, which receives visual input from our eyes, enables us to draw conclusions quickly, even in complicated situations[42].

In scientific studies, it is important to describe images in quantitative terms. Computerized image analysis

serves this purpose[43]. Russ *et al.*[44] characterized feature-specific and global measurements as simple applications. Feature-specific measurements are parameters related to object dimensions (such as area, length, width, perimeter, volume, surface area and grey level) and shapes (such as form factor, aspect ratio, fractal dimension, number of holes, convexity and solidity). Global measurements concern the overall image and include area fraction, length and curvature of lines, and various kinds of gradients and orientation parameters. For example, in an evaluation of blisters that develop in cheese on pizza, the diameters, areas and grey levels would be feature-specific data whereas their numbers (counts), their distribution (distances to their nearest neighbours) and the cheese area covered by all of the blisters combined would be global measurement data. This information is important to adjust cheesemaking to maximize consumer acceptance of the product, and for obtaining statistics to determine correlations and to predict product behaviour. Such information could be programmed into quality-control robots for objective acceptance or rejection of products coming off a production line.

Fractal analysis

Fractal analysis is receiving a great deal of attention in economic, technical and scientific disciplines[45] because it can characterize complex curves, shapes and objects. In classic (Euclidean) geometry, a point, a line, an area and a volume have 0, 1, 2 and 3 dimensions, respectively. Smooth lines (curves) can be used to draw the images of many foods only approximately. In reality, the perimeters of most foods, particularly when viewed with a microscope, are jagged or convoluted. If a line is extremely jagged so that it almost fills an area, its 'fractal' dimension is close to two, that is the dimension number of that area. The length obtained by measuring convoluted curves (called 'fractal curves') increases as the length of the measuring ruler (caliper) is decreased. If the perimeter of a section through an object is a fractal curve, then the object is a 'fractal'. It has a detailed structure irrespective of how great the magnification is at which it is examined. The 'fractal dimension' of an object is a measure of its degree of irregularity.

As a form of image analysis, fractal analysis allows the estimation of the degree of irregularity of structure, and also the examination of how structure develops and its irregularity changes during food processing, for example agglomeration and crystallization. Fractal analysis might also help to understand the porosity and roughness of foods. Micrographs, particularly those obtained by CSLM or AFM, may be evaluated for fractal dimensions once they are digitized and various algorithms have been applied.

Fractal analysis is already used in other disciplines, and its use in food science[46] is also rapidly expanding. Particles of instant spray-dried milk powder, freeze-dried coffee or fruit juices, cereal flours and extrudates are examples of fractal objects, although they do not necessarily meet the requirement that the macrostructure would repeat itself infinitely on the micro- and sub-micro-scales. Foods produced by extrusion, spray drying, roller drying, freeze drying, milling, microparticulation and other processes have complex shapes and surfaces as shown by SEM (Fig. 3a). Various examples, including simulated 'eye' patterns in cheeses or fractal geometry of broccoli, have been presented in a review on fractals in foods by Peleg[47]. The author hypothesizes on relationships between the degree of ruggedness of agglomerated coffee particles and their tendency to disaggregate, to erode or to dissolve. The degree of ruggedness may be evaluated by measuring the fractal dimensions of the particle silhouettes. There are several published examples of fractal analysis in food science, for example the development of milk gels composed of casein micelles[48] or the measurement of the fractal dimensions of popcorn[44].

Although used rarely in food structure studies, fractal analysis methods have great potential and will undoubtedly be used frequently in food research to evaluate relationships between fractal dimensions and functional properties of foods.

Other imaging techniques

Other imaging methods have been developed which, unlike LM and EM, require little or no specimen preparation. These include the scanning probe microscopy (SPM) technique AFM, and also magnetic resonance imaging (MRI) and acoustic microscopy.

Atomic force microscopy

AFM is a form of SPM, developed in the materials sciences in the early 1980s. This imaging technique has many applications[49], and has been used in various fields, including food structure, to study surface roughness in air, liquid or *in vacuo*. The resolution spans LM and EM ranges.

In AFM (as in SPM), a sharp tip, located at the free end of a 100–200 mm-long cantilever, probes the sample surface. Forces between the tip and sample surface cause the cantilever to bend or deflect; in AFM, the main force contributing to the cantilever action is the van der Waals force. As the tip is scanned over the sample, or vice versa, the deflection of the cantilever is measured by a detector. A map of surface topography is then computer generated from the detected cantilever deflections.

AFM can be carried out in three modes: contact, non-contact and tapping mode, each having specific uses and advantages. Constant-force contact AFM for soft samples has been used by N. Desai (pers. commun. and in Ref. 50) to visualize various dairy products (Fig. 3d).

Because of the many advantages of using AFM and other types of SPM, these techniques will probably be increasingly used to image fresh food samples.

Magnetic resonance imaging

MRI was initially developed as a non-destructive analytical method for use in medical research and diagnosis, and has recently been introduced into food science.

An excellent review of MRI methods for food systems[51], a book[8] and a short review[52] in this journal have been published recently. MRI is suitable for real-time dynamic studies of food structure to follow processes such as frying, foam drainage or fat crystallization. MRI, which is based on nuclear magnetic resonance spectroscopy (NMR), may be used to detect concentrations of ^{1}H, ^{31}P, ^{15}N and ^{23}Na nuclei, and is particularly suited to study the distribution of water and lipids in agricultural products including foods. Two-dimensional (2-D) images show the distribution of hydrogen nuclei and, thus, water or lipids, in a plane running through the food being examined, for example an onion[51], as shown in Fig. 6. A series of 2-D images may be stacked up to produce an image of the 3-D structure. Rosenberg *et al.*[53] showed that MRI could be used to save Swiss cheese manufacturers the expenses they incur by ripening faulty cheese loaves: an early detection of the defects would allow another use (e.g. processing) to be found for the loaves and the space to be made free in the ripening room. Other uses for MRI have also been discussed by McCarthy[8].

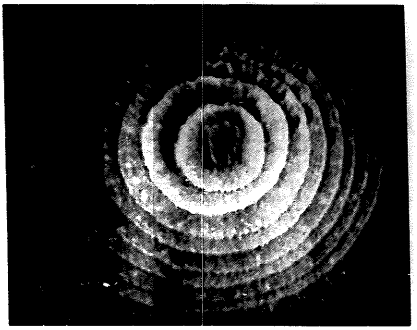

Fig. 6
Magnetic resonance imaging (MRI) of an onion, showing its rings and areas of internal bruises. (Courtesy of M.J. McCarthy[51], University of California, Davis, CA, USA, and Elsevier.)

Conclusions and future trends

Developments in the imaging of food structure are expected to parallel those in biology, medicine and materials science. Trends in biology are geared towards an increased range of applications, with the ultimate goal of improving the resolution and contrast of images, extending the range of specimen types that can be imaged, and reducing the possibility of false conclusions being drawn from the images by improving image analysis techniques.

In the study of foods, conventional EM can be used to disclose their ultrastructure in a pictorial form. The structure of some foods (such as milk, meat and cereal products and legumes) has been studied in great detail, but there are many other foods whose structures have not yet been examined. The trend will be to fill this gap. Structure is being studied in relation to the origin of the raw materials (e.g. bakery products made from various grains, cheeses made from goat's, sheep's or cow's milk, and the structure of roast pork from animals fed various diets[54]). The effects of processing such as milling, comminuting, heating and extruding will continue to be studied. Modern electron microscopes have the ability to record images on photographic material as well as magnetic media, and many laboratories are switching to a 'paperless' format with the addition of image archiving and the electronic transmission of images. It is no longer sufficient to characterize a food using so-called representative micrographs (which are usually the best micrographs obtained) and comparing them with other micrographs by qualitative visual evaluation. The trend is towards objectivization of microscopy and conversion of images into numerical data. Images in digital form will probably be routinely subjected to image analysis and fractal analysis.

It is anticipated that structural studies will play a more important role in elucidating the relationships between sensory attributes of foods, including texture, and food structure, which will have an increasing impact on designing new foods. Structural studies will probably also become an important component of biotechnology studies, being used to observe structural changes in foods that have been produced by genetic manipulation. Finally, it is anticipated that image analysis will become important to the food industry, in quality control, and as a robot – part of the production line.

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