ADVANCES OF RESEARCH ON STRUCTURAL CHARACTERIZATION OF AGRICULTURAL PRODUCTS USING ATOMIC FORCE MICROSCOPY

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ABSTRACT Atomic force microscopy (AFM) is a relatively novel form of microscopy which has many unique features: high magnification with high resolution; minimal sample preparation; the ability to obtain different views of the sample from a single data collection; acquiring 2D and 3D images at the same time; observing ongoing processes directly and so on. AFM, as a nanotechnology tool, has been used to investigate the nanostructure information of materials in many fields. This review focuses solely on its application to characterize the macromolecular nanostructure and surface topography of agricultural products. First, the fundamentals of AFM are briefly explained. And then the macromolecular nanostructure information of agricultural products from AFM images is introduced, exploring the structure-function relation in three aspects: agricultural products processing, agricultural products storage, and genetic and environmental factors. The surface topography characterization of agricultural products using AFM is also discussed. The results reveal that AFM could be a new nanotechnology tool providing a deeper understanding of the mechanisms of structure and quality variations of agricultural products, which could be instructive in improving the agricultural products processing and storage technologies.

Keywords: Atomic force microscopy (AFM), agricultural products, nanostructure, surface topography

INTRODUCTION In order to control the quality of agricultural products, which is one of the key factors for success in the commercialization of agricultural products, it is necessary to explore the structure-function relation during the process of processing and storage of agricultural products. A single agricultural product is a complex system, mainly composing by water, polysaccharides (starch, cellulose, pectin, etc.), proteins (soy proteins, zein, wheat proteins, etc.), lipids and other important ingredients. Small changes of these ingredients may have important effect on the texture of agricultural products. Different surface characteristics, including firmness, roughness, homogeneity, surface morphology also have great relation to the quality of agricultural products.
Many forms of microscopes have been used in investigating agricultural products. Some representative reports are light microscopy (LM) on apple tissue (Nieto et al., 2004), transmission/scanning electron microscopy (TEM) / (SEM) on prickly pear fruits (Habibi et al., 2009). These microscopes bring insightful information of agricultural products; however, there are some drawbacks that limit them from being widely used. For example, it is difficult to get high-resolution molecular structures from LM. Samples for SEM and TEM imaging should be pretreated: staining and vacuum (Yang et al., 2007a).

Atomic force microscopy (AFM) is a relatively novel form of microscopy, which was proposed in 1986. Unlike conventional microscopes the AFM generates images by feeling the surface of the sample: the microscopy senses the changes in force between the surface and a probe as the sample is scanned relative to the probe (Morris et al., 2001). In comparison with common forms of microscopes, AFM has many unique features: high magnification with high resolution; minimal sample preparation; the ability to obtain different views of the sample from a single data collection; acquiring 2D and 3D images at the same time; observing ongoing processes directly; the possibility of manipulating macromolecules; investigating the interaction between macromolecules and so on (Yang et al., 2007a). With these unique features, AFM is a powerful tool used in many fields involving biology, microbiology, chemistry, biochemistry, materials science, more recently, food science and technology. In these years, more and more researchers have started studying agricultural products using AFM: characterizing macromolecular nanostructures, imaging skins, investigating the nanostructure changes during the process of storage and processing of agricultural products, exploring the interaction between nanostructure and quality.

This review focuses solely on the fundamentals of AFM, its application to characterize the macromolecular nanostructure and surface topography of agricultural products.

**FUNDAMENTALS OF ATOMIC FORCE MICROSCOPY**

AFM images are obtained by measuring changes in the magnitude of the interaction between the tip and the sample surface (commonly van der Waal’s force) as the surface is scanned beneath the tip (Yang et al., 2007a). The schematic image of the AFM imaging process is showed in figure 1. The sample is mounted on a piezoelectric scanner, which ensures three-dimensional positioning with high resolution. The force is monitored by attaching the tip to a flexible cantilever and measuring the cantilever deflection. To measure the cantilever deflection with high resolution, a laser beam is usually focused on the free end of the cantilever and the position of the reflected beam is detected by a position-sensitive photodiode (Dufrene, 2003). As the tip moves in response to the sample topography during scanning, the angle of the reflected laser beam changes, and so the laser spot falling onto the photodiode moves, producing changes in intensity in each of its quadrants. Then the map of the surface topography from the measured cantilever deflection is generated by computer and is shown in a monitor and the control part is shown in another monitor (Morris et al., 1999).

There are three primary imaging modes in AFM operating: contact mode, noncontact mode and tapping mode. The contact mode is the most direct AFM mode, where the tip is brought in contact with the surface at all times. This yields a topographic image, which gives calibrated height information about the sample. Because the tip is permanently in contact with the surface while scanning, a considerable shear force can be generated,
causing damaging to the sample, especially on very soft specimens like biomolecules or living cells. In noncontact mode, images are taken by keeping a constant frequency shift during scanning, and usually this is performed by monitoring the amplitude of the cantilever oscillation at a fixed frequency and feeding the corresponding value to the feedback loop exactly. The tip-sample interactions are very small in noncontact mode, and good vertical resolution can be achieved, whereas lateral resolution is lower than in contact or tapping modes. The greatest drawback is that it cannot be used in liquid environment, only on dry samples. Tapping mode is similar to that of noncontact mode, but in this case during oscillation the tip contacts the surface intermittently at the lowest point. Usually in tapping mode the oscillation amplitude of the cantilever is larger than the one used for noncontact. There are several advantages that have made this mode of operation quite popular. The vertical resolution is very good together with lateral resolution, there is less interaction with the sample compared with contact mode (especially lateral forces are greatly reduced), and it can be used in liquid environment (Braga et al., 2004). Different samples have different characteristics, such as aggregation, elasticity, firmness, roughness, and then it is necessary to select the appropriate tip and imaging mode for different samples.

CHARACTERIZING MACROMOLECULAR NANOSTRUCTURE OF AGRICULTURAL PRODUCTS Polysaccharides, proteins, lipids are the main types of building blocks of raw materials from which agricultural products are made. Their macromolecular architectures often determine the physical and mechanical properties of agricultural products. Though theoretically almost all the macromolecules can be imaged by AFM, but in practical researches, almost all reports are focused on polysaccharides and proteins (Yang et al., 2007a).

In earlier years, many studies had been made to image individual macromolecules and their intermolecular association. Round et al. (1997) used AFM to image pectic polysaccharides extracted from unripe tomato plant cell walls. The AFM images reveal for the first time a branched structure for tomato pectins that differs from that proposed for the neutral sugar side chains from enzymatic hydrolysis and sugar analysis. The branches are between 30 and 170nm long are relatively linear. After that, AFM was also used to investigate the nature of the long branches attached to these tomato pectins (Round et al., 2001). Analysis of the AFM images and comparison with neutral sugar and linkage analyses of the two pectin fractions suggest that the distribution and total amount of branches observed do not correspond with the pattern of neutral sugar distribution. The polysaccharide structure of alginic acid was examined as individual molecules and as dense gels using tapping-mode AFM by Decho (1999). The author found that dilute (picomolar) non-ionic solutions of polymer molecules exhibited frequent “kinks” or abrupt right-angle changes in orientation, while dense alginate gels exhibited a relative regular spacing of solvent cavities (namely, H2O) within the gel. Ridout et al. (2002, 2004) observed the AFM images of the ultra-structure of starch granules for starches from different botanical (maize, potato and pea). Under the correct conditions, “growth rings” and blocklet structures were observed in “near native” granules, which supported the blocklet model of starch structure (Gallant et al., 1997) consisting of amylpectin-based blocklets suspended in an amylase-based supporting matrix. Szymonska et al. (2003a, 2003b) investigated the influence of multiple freezing and thawing on the nanostructure of potato starch granules using a high resolution noncontact AFM. Their results also supported the blocklet model of starch granule structure.
In recent years, more and more researchers are focusing on using AFM to illustrate the mechanism of changes in macromolecular nanostructures during the process of processing and storage of agricultural products, and to investigate the influences on nanostructures caused by genetic and environmental factors.

**Agricultural products processing** During the process of agricultural products processing, some physical and chemical processes may have great effect on the properties of macromolecular nanostructures. Many researchers have been investigating these effects, which are instructive in the choice of processing methods and appropriate materials for food.

Tang et al. (2007) used AFM in tapping mode to investigate the formation of molecular networks by retrogradation of gelatinized starch. A conclusion drawn from their results is that amylose has a key role in the formation of molecular networks in retrograded starch. Liu et al. (2007) observed the changes of starch nano-unit chains during a gelatinization process using AFM with a view to pinpointing the process from the point that starch granules break down to the total vanishing of crystallinity in the gelatinization process. They put forward a model of the gelatinization process for starch nano-unit chains, completed the gelatinization model of starch, and proved that the nano-unit chains contain crystalline areas and amorphous areas. An et al. (2008) demonstrated the effects of heating modes and the kind of starch on the nanostructure of starch molecules using AFM. In their experiment, potato and corn starches were subjected to convective and microwave heating. AFM images showed that the uneven microwave heating caused potato starch to form networks. Both starches formed the capped chains structures under microwave heating. Heating modes influence the potato starch much more compared to its influence on corn starch. The results could be applied on material selection for microwaved food development.

Guo et al. (2005) took advantage of the special properties of AFM to explore the nanostructure of zein from maize (*Zea mays*), focusing especially on its reticular structure. They found that in aqueous ethanol solution zein exists as small globules with diameters between 150 and 550 nm. The mechanism of zein film formation was also explored by the authors. The characteristic film structure consists of a meshwork composed of doughnut structures formed from asymmetric rods joined to each other. The authors thought that the structural properties of this meshwork give zein films high strength and low gas permeability together with high hydrophobicity. One of the most promising applications for zein appears to be in biodegradable films and plastics for use as packaging. The aggregation process of the proteins coagulated by glucono-δ-lactone (GDL) was monitored by using AFM (Tay et al., 2005). Solutions of glycinin (11S), β-conglycinin (7S) and 2S proteins at 100°C for 10min, were mixed with GDL and deposited onto the mica for 1, 2 and 4min. The authors found that the speed of aggregation was in the order of 11S > 2S > 7S. Their results were helpful to have a greater understanding of the interactions of the different soy protein fractions to shed more light on the factors that contribute to tofu texture. In addition to this, they found that 2S, despite having the least percentage of soy protein (as compared to 7S and 11S) plays a significant role during the gelation process. In order to develop a better understanding of the structure-function relationship of the 2S fraction of soy protein, Tay et al. (2006) done a continuous investigation. The results of the study did confirm the previous observations, showing that indeed 2S exhibits better foaming and emulsification
properties than the other two molecular fractions. Surprisingly, it was also found that in the initial stages of structure formation, 2S fared better than 7S, with 11S exhibiting the highest rates of aggregation; given time, 7S produced a firmer network with a better water-holding capacity than that of 2S.

Gelatin has a wide range of applications in the food, pharmaceutical, and photographic industries. Understanding and then improving the physical properties of gelatin is important for increasing their utilization (Yang et al. 2007b). Some researchers focus their studies on the gelatin from catfish (*Ictalurus punctatus*) and mammalian. Catfish gelatin at a nanoscale level for the first time was described by Yang et al. (2007b). The gelatin was extracted at an optimized acid concentration after alkaline processing. They obtained height mode with a 2-dimensional plane, 3-dimensional topographical images, and error signal mode images to analyze the structure of catfish gelatin particles. Both annular pores with diameters averaging 118 nm and spherical aggregates with an average diameter of 267 nm were seen in the AFM images of fish gelatin. From the AFM images, the authors proposed that the structures formed were determined by whether the solution penetrated into the gelatin molecules evenly or not during hydrolysis. They also proposed a scheme for the formation of annular pores and spherical aggregates. In their following up study, the authors illustrated the correlation between the physical properties and nanostructure of gelatins made of channel catfish skins (Yang et al., 2008). Four samples including water, 0.1M acid and 0.25 and 1.0M alkaline-pretreated groups’ nanostructures were studied. The AFM images showed that the acid-pretreated gelatin was composed of sponge-like aggregates, while the others showed separated individual aggregates. Annular pores were only found in the alkaline pretreatment group. There was no significant correlation between the diameters of the spherical aggregates and the physical properties. The results are helpful for illustrating the mechanism of the alkaline or acid pretreatment of the extraction technology. The results also suggest that AFM is a promising nanotechnology for obtaining a better understanding of gel nanostructure. Wang et al. (2008) not only got the similar results, but also developed imaging of a relatively high concentration of gelatin (0.5%) successfully.

**Agricultural products storage** The texture of fruit and vegetables and their properties are related to the structural integrity of their constituent pectin, and the functional properties of pectin are closely related to its chemical and physical structures. The AFM affords an opportunity to directly image individual pectin molecules and polymers (Yang et al., 2005a). Trying to explore the relation between the texture of the peach and storage conditions, Yang et al. (2005a, 2006a, and 2006b) made a set of study on the characteristics of pectin chain widths of yellow peaches during storage. The probability of small-width water-soluble pectin (WSP), chelate-soluble pectin (CSP) or sodium carbonate-soluble pectin (SSP) increased with time in controlled and regular atmosphere storage groups, but the probability was larger in the regular atmosphere group, indicating that controlled atmosphere conditions could inhibit the degradation of pectin molecules. Widths of pectin chain were all composed of four basic units, and linkages or intertwists between the basic units were fundamental structural conformations for pectin molecules. These results prove that pectin plays an important role in the qualities of peaches, and controlled atmosphere conditions are better than regular atmosphere for the qualities of peaches. The degradation mode of structural polysaccharides from quantitative AFM analysis could be correlated with other physicochemical analysis and has been used to illustrate the roles of these structural polysaccharides (Yang et al., 2007a). Kirby et al.
used AFM to image the structure of pectin molecules isolated from unripe tomato and sugar beet tissue. The authors’ studies confirmed that pectin molecules extracted from unripe tomato cell walls were not purely linear structures. Imaging of the extracted pectin revealed largely un-aggregated chains: a small fraction (33%) of which were extended stiff polysaccharide chains and a major fraction (67%) of which were of polysaccharide-protein complexes containing a single protein molecule attached to one end of the polysaccharide chains (‘tadpoles’). In addition, the sample contained a small number of aggregated structures. The effects of calcium and storage time on physicochemical properties and nanostructure of chelate-soluble pectin (CSP) of apricots (Prunus armeniaca L.) at 0°C were investigated by Liu et al. (2009). In the study, the morphology changes of CSP caused by calcium treatment were characterized by AFM. Treatment with 1% calcium retarded the changes of physicochemical properties and the depolymerization of CSP during storage. The results could help to illustrate the fundamental of changes of apricot fruits during storage. To investigate the fundamental of firmness changes of crisp peaches, Zhang et al. (2010) studied firmness and pectin contents of two peach (Prunus persica L. Batsch) cultivars (‘Cangfangzaosheng’ and ‘Songsenzaosheng’) stored at 2°C, 8°C and 15°C. The widths of the peach SSPs were very consistent. The SSP chain widths of both peach cultivars were similar and were composed of several basic units. Based on the results of chain widths, they proposed schematic models of the changes of SSP chains and concluded that the firmness of peach was closely related with the contents and nanostructure of SSP. In conclusion, all of these studies reveal the changes of macromolecular nanostructures under different storage conditions, which may be helpful in improving agricultural products storage technology.

Genetic and environmental factors AFM also has great application potential in studying the genetic and environmental influences on the biosynthesis that lead to considerable variation in macromolecules between and within plant species. These variations may be in the size and shape of starch granules, amylase, and amyllopectin content, and in the branching pattern of amyllopectin, all of which can affect the functional and nutritional properties of the starch (Tang et al., 2007). Ridout et al. (2003, 2006) analyzed AFM images of wild-type pea starch and a set of isogenic pea mutants (r, rb, rrb, rug3-a, rug4b, rug5-a, lam-c) to examine how specific mutations in the starch biosynthetic pathway influence granule structure and functionality. They found that mutations that lead to substantial changes in the amylase-amyllopectin ratio lead to substantial changes in granule architecture and function. A mutation at the rb locus did not lead to significant changes in granule architecture. However, a mutation at the r locus led to loss of growth rings and changes blocklet structure. The double mutant rrb is observed to show intermediate behaviour between that observed for the r and rb single mutants. A mutation at the locus rug4 was found to have little effect on the granule ultrastructure. However, mutations at rug3 and lam, which give rise to low-amylose starches, led to granules that showed banding (growth rings) in which individual blocklets could not easily be seen. High-amplose (rug5) mutants formed granules ranging in shape from simple ellipsoids through to quite complex, convoluted structures.

The variations may also be in the texture of agricultural products which are in different cultivars or at different stages of ripeness. Zhang et al. (2008) first investigated firmness and physicochemical properties of 2 Chinese cherry (Prunus pseudocerasus L.) cultivars (soft cultivar “Caode” and crisp cultivar “Bende”) at unripe and ripe stages, and used AFM to determine the qualitative and quantitative information about SSP nanostructures.
The lengths and widths of the cherry SSPs were very regular: almost all of the widths and lengths of SSP single molecules were composed of several basic units. The widths of the SSP chains were 37, 47, 55, and 61 nm, and the lengths were 123, 202, and 380 nm in both cultivars. The results show that the firmer cherry groups (crisp fruit) contain more percentages of wide and short SSP chains than soft fruit, and the unripe groups contain more percentages of wide and long SSP chains than corresponding ripe groups. The results also indicate that those nanostructural characteristics of SSP are closely related with firmness of the Chinese cherries in each cultivar. In their following up study (Chen et al. 2009), AFM was used to describe and measure the nanostructure of hemicellulose (HC) extracted from two cultivars of Chinese cherry, “Caode” (soft) and “Bende” (crisp) at different stages of ripeness. The widths of the HC molecules and aggregates were consistent with little difference between the two cultivars. The results show that crisp fruit contain a higher percentage of thicker HC chains than soft fruit, suggesting that the thickness of the HC chains may be related to the textural difference observed in the cultivars of the Chinese cherries. Yang et al. (2009) compared the nanostructures of three kinds of pectins in two peach (Prunus persica L. Batsch) cultivars (soft and crisp) when they investigated the reasons for firmness differences between soft and crisp fruit cultivars. WSP, CSP and SSP were extracted and nanostructures were conducted and analyzed using AFM. It was found that a large difference existed in the lengths of SSP chains between the two cultivars, while the WSP and CSP chain lengths were not much different. There were no statistical differences for chain heights and widths in the three kinds of pectins between fruit of the two cultivars. The results demonstrate that neutral sugar-rich pectins from the primary cell wall of peach flesh may be the source of the main differences in pectins and firmness between the two cultivars. All of these researches expand our knowledge on detailed structures affected by genetic and environmental factors.

**SURFACE TOPOGRAPHY** AFM is a powerful instrument for characterizing the surface topography of agricultural products. Many researchers used AFM to measure surface characteristics, including roughness, surface morphology, fractal analysis, and so on. Hershko et al. (1998) applied AFM to investigate the fine structural analysis of the onion skin and to compare the roughness of onion skin and other fruits and vegetables. The roughness of the onion skin was found to be ~4.9 to 6.0 μm Ra. Onion was smoother than garlic, as were mushrooms; green pepper had about the same roughness as garlic; apples were smoother than onion. To check the contribution of the natural wax to the skin’s roughness, the authors compared the skin’s roughness before and after removing the natural wax. The roughness of the skin was found larger than that before removing the natural wax, evidencing the wax’s very important role in skin roughness. Yang et al. (2005b) used AFM to examine the changes of peach skin characteristics with storage time. They analyzed arithmetic roughness (Ra) and root mean square roughness (Rq) of ‘Jinxiu’ yellow peach (Prunus persica L. Batsch.), finding that the Ra and Rq values increased with storage time in both controlled atmosphere (CA) and regular air (RA) storage, and the values of CA group were smaller than that of RA group. There was a linear correlation between Ra and Rq. They also gained the three-dimensional profiles of the peach skin. The results indicate that the roughness values increase with the storage time, and the roughness of CA group increases slower than that of RA group. Their work is helpful selecting storage conditions of the peach. Cardona et al. (2008) did some work about the epicarp characterization of coffee fruits by AFM. The objective of their study was to characterize the epicarp of coffee fruit, analyzing the changes that occur in the
roughness, in the mature and green states, and to determine the dimensions and density of the cells of epicarp by the AFM technique. The $R_s$ range of the mature fruit was found to be between 0.03 and 0.19μm, and for the green fruit it was between 0.23 and 0.38μm. The $R_q$ range of the mature fruit was found to be between 0.05 and 0.24μm, and for the green fruit it was between 0.29 and 0.49μm. It was determined that the cells have ellipsoidal form with an average of 194.62μm$^2$, and the density average is 4.206 cell/mm$^2$.

The results of their study could be used in later researches for the development of technology to separate immature fruits of coffee in the green state that are frequency found in the manual or mechanical gathering process.

CONCLUSION This review focuses solely on the application of AFM to characterize the macromolecular nanostructure and surface topography of agricultural products. The fundamentals of AFM are also briefly explained. The macromolecular nanostructure information of agricultural products from AFM images is introduced for exploring the structure-function relation in three aspects: agricultural products processing, agricultural products storage, and genetic and environmental factors. In some study, AFM has brought in much original knowledge on agricultural products properties, which could be used to direct agricultural products processing and storage. For example, An et al. (2008) investigated the effects of heating modes and sources on nanostructure of gelatinized starch molecules, and the results could be applied on material selection for microwaved food development. Yang et al. (2005a, 2005b, 2006a, 2006b, 2006c) used AFM to look into the changes of pectin at the molecular level induced by different storage conditions. The results could be applied to direct the peach storage, as well as other agricultural products’ storage. These studies proved that AFM is a powerful analytical tool to answer questions related to the macromolecular nanostructure and surface topography of the agricultural products.

The future will very likely bring us many more studies using AFM, and looking at the basic components of agricultural products. Imaging the other important components (i.e. cellulose, lipids, enzyme, etc.) by AFM to explore their roles on agricultural products may be a direction. AFM could also be applied to determine the characteristic parameters of some other kind of agricultural products (i.e. cereals, animal products, marine product, etc.). In addition to these, combining AFM and other technique tools may widen scope of the study.

REFERENCES


APPENDIX A

Figure 1. Schematic image of the AFM imaging process (based on Braga et al., 2004)