Application of Atomic Force Microscopy on Rapid Determination of Microorganisms for Food Safety

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ABSTRACT: Rapid detection and quantification of microorganisms is important for food quality, safety, and security. In this field, nanotechnology appears to be promising in its ability to characterize an individual microorganism and detect heterogeneous distribution of microbes in food samples. In this study, atomic force microscopy (AFM), a nanotechnology tool, was used to investigate *Escherichia coli* (*E. coli*) qualitatively and quantitatively. *E. coli* strains B and K12 were used as surrogates to represent pathogenic strains, such as *E. coli* O157: H7. The results from AFM were compared with those from scanning/transmission electron microscopy (SEM/TEM). The qualitative determination was obtained using morphology and characteristic parameters from AFM images, and the quantitative determination was obtained by calculating the microorganisms in AFM images. The results show that AFM provides a new approach for rapid determination of microorganisms for food safety.

Keywords: atomic force microscopy (AFM), food safety, nanostructure, nanotechnology, rapid determination

Introduction

The presence of hazardous microorganisms can be introduced at any stage of food production and distribution including: preharvest, production, processing, transport, retailing, domestic storage, or meal preparation (Bergwerff and van Knapen 2006). Sometimes, highly complex environments make microorganisms in contaminated feed and food elude detection and inactivation.

There are many challenges for the detection of microorganisms in foods. The contamination level in foods is normally low and adequate sampling is difficult. The microbial background populations are generally high and affect the determination of pathogens. Most importantly, many foods have a short shelf life. Therefore, rapid detection and determination of pathogens with a high degree of specificity and sensitivity are critical for maintaining a safe and high quality food supply (Hanna and others 2005).

Although traditional culturing and biochemical assays have proven useful in quality and safety control for food, they cannot meet the testing requirements of the modern food industry. These methods require many hours to several days to obtain the results (Hanna and others 2005). Rapid detection and determination methods are needed for evaluating food safety decisions. The categories of hazards generally include: microorganisms and toxic products, chemicals, heavy metals and pesticide residues, and foreign materials (Dostálek and Brányik 2005). In the 1st category, food scientists have the greatest interest in 2 groups of microorganisms: pathogens and indicator organisms (Dostálek and Brányik 2005). The results of rapid detection should be faster than traditional culture methods of media and plates. Several technologies were developed in recent years including: bioluminescence (Dostálek and Brányik 2005), infrared spectroscopy (Lin and others 2005; Al-Holy and others 2006), nucleic acid sequence-based amplification (Duvall and others 2006; Rodríguez-Lázaro and others 2006), real-time and multiplex PCR (Rijpens and Herman 2002; Duvall and others 2006; Fricker and others 2007; Settanni and Corsetti 2007), biosensor (Bergwerff and others 2006; Rasooly and Herold 2006), and flow cytometry (Corry and others 2007). Fung (2002) estimated that about 30% of all microbiological testing in the food industry used rapid methods. In terms of pathogen tests, the number is about 50%; however, there are still many limitations when applying the aforementioned methods. For instance, the quenching of emitted light can adversely affect the microbial ATP bioluminescence method (Dostálek and others 2005). Also, variations in sample preparation of pathogens from food samples can affect the results by sensitive biosensors (Fung 2002). Most of these methods require cell manipulation before examination, which may greatly compromise the validity of analyses. The result is generally obtained from a group of microorganisms and not at the level of individual microorganisms. Therefore, it is important to find a new, nondestructive method that can detect and quantify microorganisms in a short time period for food safety decisions (Dufrêne 2002).

Recently, atomic force microscopy (AFM) has provided a way to investigate the food samples at nanoscale with high resolution. With minimal sample preparation, the technique generates 2- and 3-dimensional images of the surface ultrastructure in nearly real time. AFM is more than a surface-imaging tool; it can obtain many physical properties of the specimen including molecular interactions, cell growth and division, surface hydrophobicity and viscoelastic properties (Touhami and others 2003; Tang and others 2004; Touhami and others 2004). These measurements provide new insight into the structure-function relationships of food samples (Ahimou and others 2002; Dufrêne 2002, 2003; Yang and others 2007a). AFM has been successfully introduced into food science to characterize and manipulate the molecules of food polysaccharides (starch and peach pectins, for instance) and proteins (fish gelatin, for instance) (Yang and others 2005, 2006a, 2006b, 2007a,

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2007b, 2008; An and others 2008; Wang and others 2008), and investigate the delicate structure and physical properties of microbial surfaces and biofilms (van der Aa and others 2002; Brehm-Stecher and Johnson 2004; Sullivan and others 2005; McLandsborough and others 2006; Wright and Armstrong 2006). AFM has the capability to investigate microorganisms at a level as low as even single microbe.

This article proposes the use of AFM for rapid determination of bacteria qualitatively and quantitatively. The instrumentation and methodologies are presented along with advantages and limitations that may be encountered with the use of this technique.

Materials and Methods

Principle of AFM

AFM-imaging is obtained by sensing the force (commonly van der Waal's force) between a very sharp probe and the sample on a very smooth surface (mica, for instance) (Figure 1). A laser beam from a laser diode is adjusted to the end of the cantilever. The laser beam is then reflected by a mirror onto a position-sensitive photodetector. During scanning, the probe tip moves in response to the sample topography. The angle of the reflected laser beam changes causing the laser point mirrored onto the photodetector to move. This beam movement produces changes in the intensity, and this generates an electrical signal which quantifies the motion of the tip. The topography of the sample surface causes the cantilever to deflect as the force between the tip and sample changes. The surface topography and control apparatus are generated by computer software and displayed on different monitors (Yang and others 2007a). This process is different from the other microscopies that images are generated by means of an incident beam. In summary, an AFM image is generated by recording the force changes as a probe scanning in the *X* and *Y* directions, and the sample height information as well as other surface information is obtained (Dufrêne 2002).

Typically, AFM cantilevers and probes are made of silicon or silicon nitride. Generally, there are 3 primary imaging modes in AFM operation: contact mode, noncontact mode, and tapping mode; however, for different companies, the name of the modes may be slightly different due to intellectual copyright. For instance, the noncontact mode of AFM from Pacific Nanotechnology Inc., Calif., U.S.A., is similar to the Tapping mode of AFM from Digital Instruments, Calif., U.S.A. Tapping mode is one of the most widely used modes for biological or other soft sample testing. In tapping mode, the tip is attached to the end of an oscillating cantilever that intermittently contacts the surface at the lowest point in the oscillation near the resonance frequency of the cantilever. Imaging microorganisms with this mode is promising (Dufrêne 2002; Yang and others 2007a).

AFM-imaging of microbes

Two nonpathogenic strains of *E. coli* (K12 and B) were graciously provided by Dr. Tung-Shi Huang (food safety lab) at Auburn Univ. These strains were used as surrogates for pathogenic strains (O157:H7, for instance). Each single colony was picked from an overnight-grown Luria-Bertani (LB) plate (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar), and bacteria were grown overnight from picked colonies in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37 °C with shaking at 250 rpm. Bacteria were then diluted 10-fold with LB and grow further at 37 °C with shaking at 250 rpm. The density of bacteria cultures was monitored using optical density at 600 nm (OD. 600) with LB medium as blank. When the OD_{600} reached 0.6 to 0.9, the cultures were in log phase and were then harvested by centrifugation (6000 × *g* for 10 min). The bacteria were washed with phosphate-buffered saline. *E. coli* K12 and B were serially diluted, and these diluted solutions were disrupted by a Vortex mixer (Fisher Scientific, Pittsburgh, Pa., U.S.A.) for even distribution. The solutions then were deposited onto freshly cleaved mica sheets (about 1.5 × 1.5 cm^2) (Muscovite Mica; Electron Microscopy Sciences, Hatfield, Pa., U.S.A.). For each sample, about 20 μL of the solution were deposited onto the mica surface using a pipette. The mica surface was naturally air-dried at room temperature before AFM-imaging. The mica with sample was attached to a specimen disc (TED Pella Inc., Redding, Calif., U.S.A.) using double-sided tabs. Then, the disc was mounted magnetically onto the sample stage. Afterward, the samples were imaged by a Nano-R2™ AFM (Pacific Nanotechnology Inc., Santa Clara, Calif., U.S.A.) in noncontact mode. The microscope was equipped with a Z scanner operating at ambient temperature. The NSC 11/no Al (Mikro-Masch, Wilsonville, Oreg., U.S.A.) tip with a resonance frequency of

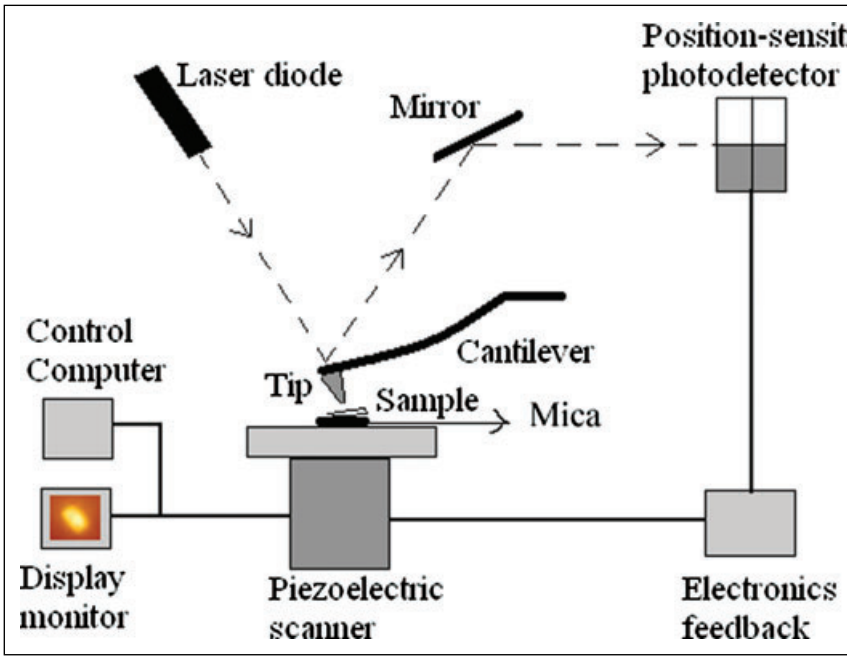

Figure 1 — Schematic illustration of the AFM-imaging process (based on Yang and others 2007a, 2007b)

330 KHz and force constant of 48 N/m was applied with the scan speed set at a range of 0.5 to 2 Hz.

The AFM images were analyzed by the software provided by the company. The bright and dark colors in the images corresponded to high and low parts in the *Z*-axis direction, respectively. Both height and error-signal mode images were obtained simultaneously (Yang and others 2007b). The qualitative and quantitative information of the microbes can be obtained using the AFM software. To improve the quality of image, a function in software called "leveling" was applied to reduce the electronic noise in the raw data; however, the quantitative parameters were determined based on the unprocessed images to maintain maximum accuracy.

Statistical analysis

Twenty to 40 parallel samples were examined for each specimen to obtain statistically reliable and valid results. The AFM data of the microorganism dimensions were from our experiments, while the data of scanning/transmission electron microscopy (SEM/TEM) for comparison purpose were from images reported by others (Burdett and Murray 1974a, 1974b; Lüdi and others 2006; Ugarte-Remoro and others 2006; Yu and others 2006). The dimensions were reported as means ± standard deviations. Statistical analyses using analysis of variance (ANOVA) ($P < 0.05$) and Duncan's multiple range test for differences in the dimensions of the *E. coli* strains were performed using SAS software (Version 9.1.3; Statistical Analysis Systems, Cary, N.C., U.S.A.). Comparisons that yielded P values < 0.05 were considered significant.

Results and Discussion

Screening the AFM-imaging and analysis parameters

Microorganisms are delicate and soft biological samples. For many nanotechnology characterization techniques, sample preparations often change the status of the microorganisms. Therefore, it is still difficult to study the morphology and physical properties at the nanoscale level (Dufrêne 2002). Currently to our best knowledge, AFM is the only technique that can image the surface of a live microorganism at high resolution and in nearly real time with the sample's maximum native status. In such, it is complementary to SEM/TEM, for which vacuum manipulations are required and real-time analysis is not possible (Dufrêne 2002; Yang and others 2007a). Our previous study in the application of AFM to macromolecular characterization and the published data about characterization of microbiology using AFM led us to propose the idea of rapid determination of microbiology using AFM for food safety (Sullivan and others 2005). Since microorganisms are soft and fragile samples, tapping mode is more suitable for AFM scanning. Using tapping mode, probe-sample lateral forces were greatly reduced, which greatly reduced the deformation of the scanned microorganisms. Therefore, high-resolution AFM images of microorganisms were obtained (Dufrêne 2002). It was reported that microorganisms imaged in water showed hydrated and had some elongated dimensions (Sullivan and others 2005). We conducted AFM experiments in air to avoid hydration. Figure 2 shows the AFM images of *E. coli* K12 on mica. After leveling, the height information of microbes was clearer (Figure 2C) than unprocessed images (Figure 2A). It should be noted that the scales and the resolutions were different between the lateral (X, Y) and vertical (Z) axes. The AFM results show that the height and width of *E. coli* K12 were 3.97 ± 0.67 and 2.61 ± 0.55 μm, respectively (Table 1). AFM images can further supply the height dimension other than the traditional used length and width (Figure 2F). The height of the *E. coli* K12 was 381 ± 58 nm (Table 1). Compared with height mode (Figure 2A, 2C), the error signal mode was very useful for relatively flat samples and provided images that slow variation in topography, and it highlighted the edges of the microorganisms in the images (Figure 2B, 2D). Enlarged images provided further information of the microbes (Figure 2E). Figure 3 shows the AFM images of *E. coli* B. Images with leveling showed a smoother mica surface and provided high visual quality (Figure 3B). Two- and 3-dimensional height mode images (Figure 3A, 3C) were obtained simultaneously.

Qualitative determination of microorganisms

For practical qualitative determination of microorganisms, the morphology of the microorganisms should be characterized. Information concerning different surface and morphological characteristics of the 2 strains were obtained and compared. The qualitative and general structure information is easily viewed on AFM images. Aside from imaging a microorganism, another advantage of AFM is its ability to provide characteristic parameters such as length, width, and height of the microorganism to further help distinguish it. For obtaining characteristic parameters of the morphology of the microorganisms, "Line analysis" was applied to determine the featured structures (Figure 3F). Although there were almost no differences of the dimensions between the unprocessed and processed (leveling) images analyzed by the software, data from unprocessed images was used for comparing the strains of *E. coli* to maintain maximum accuracy. The structural characteristic parameters of the 2 strains from AFM results are listed and compared with the results of SEM/TEM from other references (Table 1). It should be noted that all the microorganisms studied were in exponential stages for both AFM and SEM/TEM. Besides traditionally used length and width information, AFM could provide height information (Z) of the microorganisms, which was not applicable for SEM/TEM. Table 1 shows that the dimensions of the microorganisms measured by AFM and SEM/TEM were different. Generally, for both strains, the length (L) and width (W) measured by AFM were larger than those by SEM/TEM. For instance, the average length of B was 3.23 μm by AFM, while the average length of B strain by TEM was only 1.49 μm. The lower values from SEM/TEM probably result from the manipulation of sample preparations; however, the ratio of L/W was stable in these 2 groups (2.23 for AFM and 2.22 for TEM results). Even for the same strain (such as K12) with the same measuring technology (SEM/TEM), the results varied among different research reports (Table 1). Possible reasons for this observation may attribute to slightly different morphology statuses of *E. coli* K12 caused by different sample preparations of SEM/TEM. In this study, we selected 2 strains of the same species because we believe that if AFM can distinguish 2 similar strains, it can distinguish 2 microorganisms belonging to 2 different genera (*E. coli* and *Salmonella*, for instance) considering the large difference of their morphology. The height and width of *Salmonella* Typhimurium determined by AFM was 185 ± 15 nm and 0.79 ± 0.07 μm, respectively (Handa and others 2008)—while the height and width were 309 ± 101 nm and 1.51 ± 0.58 μm for *E. coli* B and 381 ± 58 nm and 2.61 ± 0.55 μm for *E. coli* K12, respectively (Table 1). The different dimensions between the strains may provide a way to use characteristic parameters of the structure for discriminating strains.

Quantitative determination of microorganisms

For quantitative analysis of microorganisms, media containing serial dilutions of bacteria with a reasonable range and interval were prepared according to information regarding the source of microbes and our previous experience on AFM imaging. A test image containing a known amount of solution with a known concentration was conducted. If the AFM image was

fully occupied with the microorganisms a reduced level medium would be imaged. If the AFM image was too empty, a raised level medium would be tested until a reasonable concentration was obtained when the individual microorganisms were separated and easily counted in the AFM image. Then the microorganisms in the AFM images were counted by the software or by hand (Figure 2E and 3C). The whole number of the microbes can be easily calculated based on the volume, concentration, and the

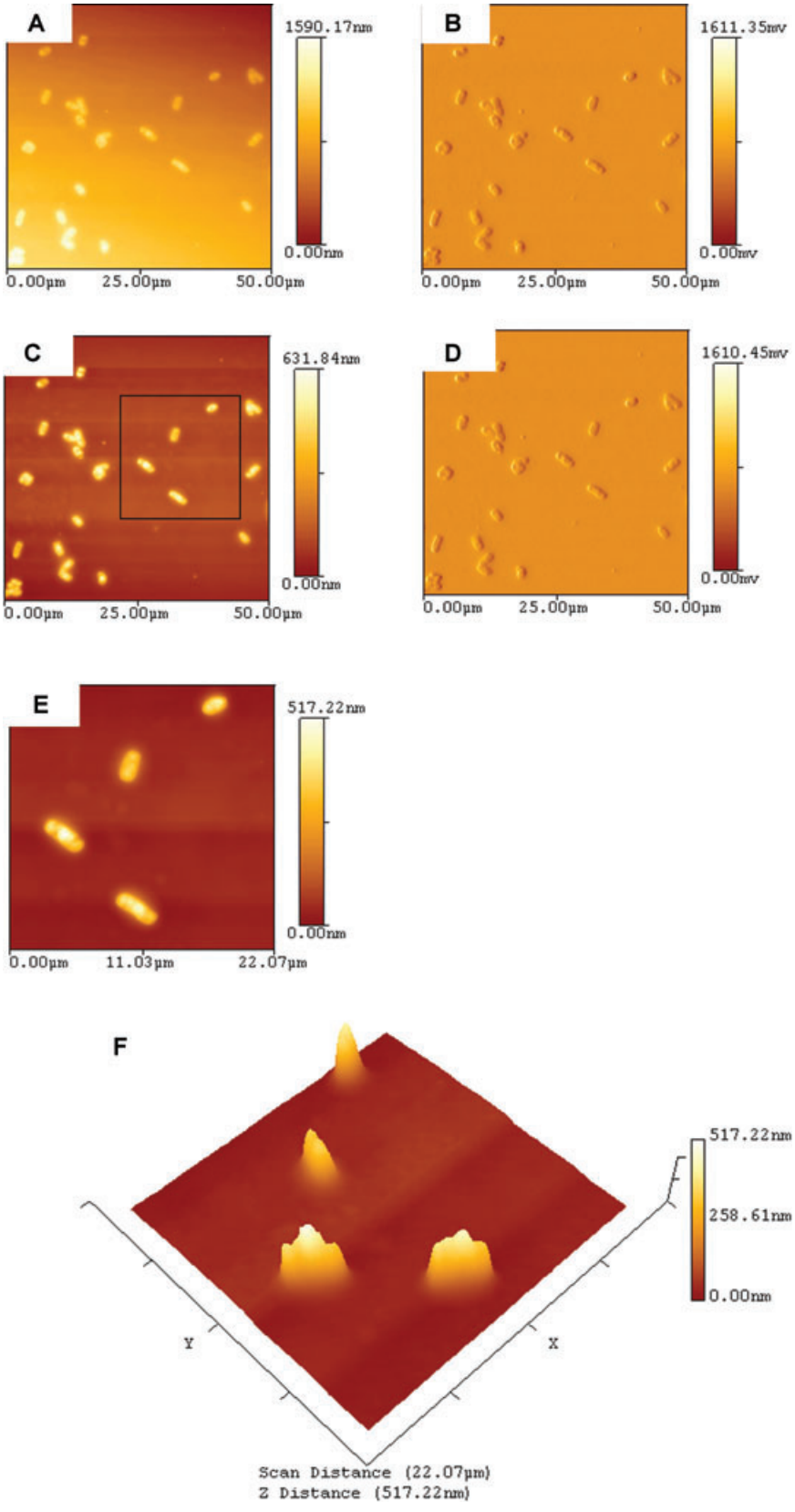

Figure 2—AFM images of K12 strain of *E. coli*: (A) unprocessed height image; (B) corresponding error-signal mode image of A; (C) height image after leveling; (D) corresponding error-signal mode image of C; (E) enlarged height images of C; (F) corresponding 3D images of E.

Table 1—Comparison of the dimensions of 2 *E. coli* strains using AFM and EM.

Dimension	*E. coli* B		*E. coli* K12			
	AFM ($n = 22$)	TEM[A] ($n = 9$)	AFM ($n = 32$)	SEM[B] ($n = 1$)	SEM[C] ($n = 7$)	TEM[D] ($n = 3$)
Length (L/μm)	$3.23 \pm 1.05^{a,b}$	1.49 ± 0.35^{c}	3.97 ± 0.67^{a}	1.50^{c}	0.85 ± 0.21^{c}	$2.18 \pm 0.12^{b,c}$
Width (W/μm)	1.51 ± 0.58^{b}	$0.67 \pm 0.07^{b,c}$	2.61 ± 0.55^{a}	$0.64^{b,c}$	0.18 ± 0.04^{c}	$0.79 \pm 0.09^{b,c}$
Height (Z/nm)	309 ± 101^{b}	–	381 ± 58^{a}	–	–	–
(L/W)	2.23 ± 0.58^{b}	2.22 ± 0.49^{b}	1.57 ± 0.38^{b}	2.33^{b}	5.64 ± 1.86^{a}	2.76 ± 0.17^{b}

Note: The AFM results were from this research. Superscripts with EM measurements shows that the results were calculated from other research groups (A. Burdett and Murray 1974a, 1974b; B. Ugarte-Romero and others 2006; C. Yu and others 2006, and D. Lüdi and others 2006). Values in the same row with different superscript letters indicate significant differences by the Duncan's multiple range test ($P < 0.05$).

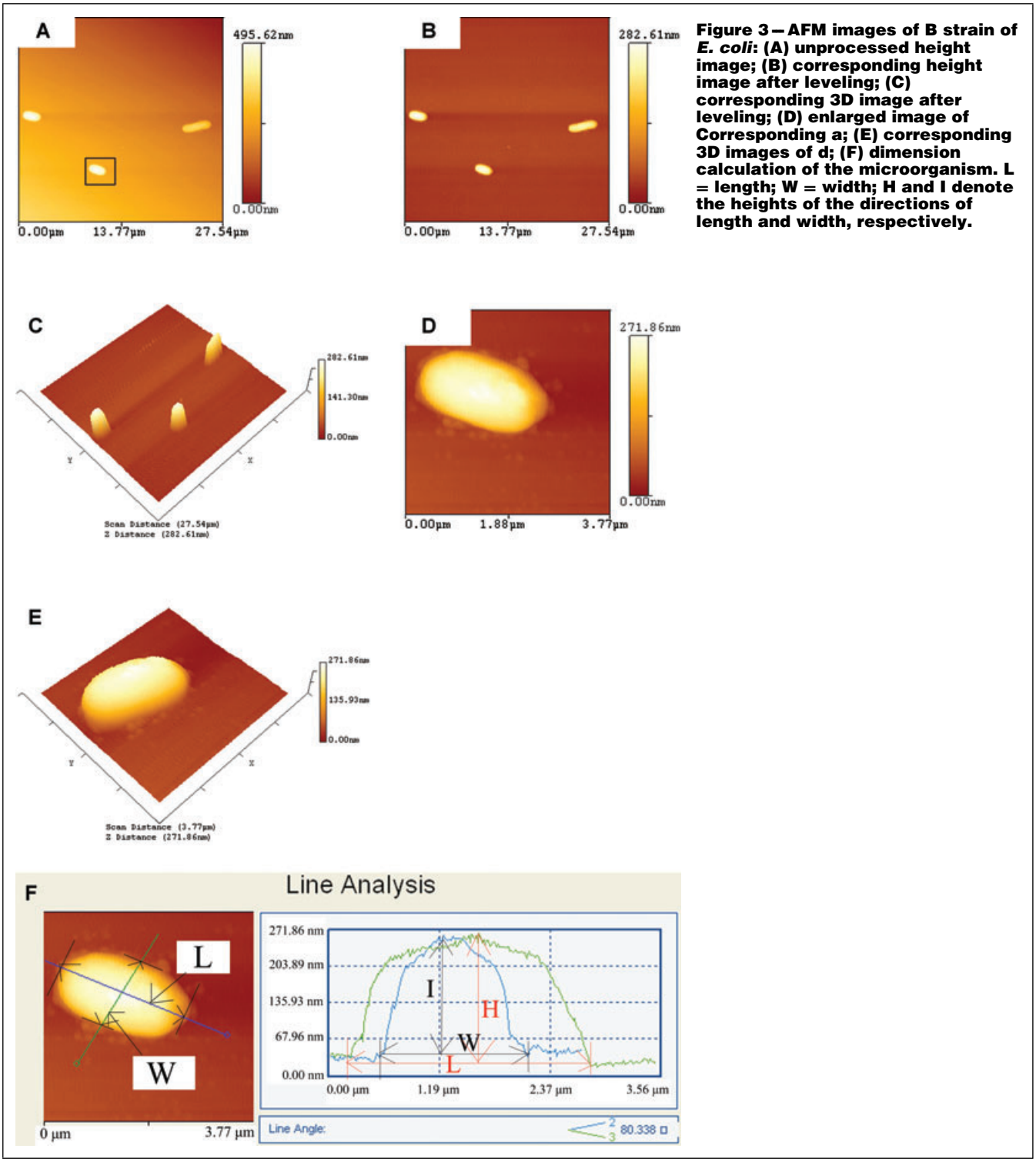

Figure 3—AFM images of B strain of *E. coli*: (A) unprocessed height image; (B) corresponding height image after leveling; (C) corresponding 3D image after leveling; (D) enlarged image of Corresponding a; (E) corresponding 3D images of d; (F) dimension calculation of the microorganism. L = length; W = width; H and I denote the heights of the directions of length and width, respectively.

number of microorganisms in the image. Compared with light microscopy, AFM has a reasonable magnification with high resolution, and it can detect small microorganisms. In addition, little or no sample preparation makes AFM possible to rapidly counting microorganisms in a short time when compared with SEM/TEM (Yang and others 2007a). Therefore, the concept of rapid determination of microorganisms for food safety using AFM is technically feasible.

However, quantitative determination of microorganisms on the mica for AFM-imaging may not be very accurate due to the possibility of uneven distribution of the microorganisms during the drying of the solutions on the mica before AFM-imaging. To obtain accurate results, we proposed a possible approach as follows: first, the sample could be diluted to a calculable and reasonable concentration. After estimating the number of microorganisms in the diluted solutions, a solution with a corresponding concentration is prepared. Second, a certain volume of the solution can be applied onto an apparatus similar to a counting chamber (hemocytometer, for example) (Figure 4). The apparatus is necessary for preventing mistakes occurring in the aggregates and an uneven distribution of the microbes, which is inevitable if the solutions are deposited directly onto mica surface without the counting chamber. Third, the counting chamber with solution is air dried by a forced clean air, or in a chamber with a suitable high temperature and low relative humility for evaporating the water. Fourth, the counting chamber with the sample (dried from the solution) can be scanned by AFM. It should be noted that the scan size of AFM should be larger than the reading zone of the counting chamber, and then the number of the microorganisms in the reading zone can be read completely through AFM images, which allows several reading zones that can be imaged for obtaining statistical results. If the number of the microorganisms in the reading zone is too large or too small, another concentration of the solutions can be prepared until a reasonable number of microorganisms are visualized from the AFM images. Finally, the quantitative data of microorganisms can be obtained by multiplying the number of microorganisms in the diluted solution with the dilution times in a second.

Currently, most of the counting area of commercial counting chambers (hemocytometers) was 1/25 mm^2, the area of the reading zone was about 200 × 200 μm, and the depth was about 100 μm (Figure 4); however, our AFM scanner had the maximum scan area of 75 × 75 μm, and the Z-axes was only 4 μm. Since the contamination level of foods was generally low, we modified both the counting chamber and the scanner to testify the hypothesis of the idea. Fortunately, a scanner with maximum $X(Y)$ range of 150 μm and Z range of 15 μm was developed and currently available on the market (http://www.nanotechnology.net/index.aspx?ID=69913). Therefore, we proposed a reasonable dimension of the counting chamber that the area of reading zone amounts 120 × 120 μm (number in the red square of Figure 4), the depth of the chamber was 15 μm, then the volume of the reading zone (Figure 4) was 0.216 mL. For example, if the solution had 50000 microbes per milliliter, the average number of microbes in the reading zone was 10.8. That meant about 10 to 11 microorganisms would be viewed in the reading zone. At this condition, the microbes of the counting chamber would be successfully determined by AFM. This hypothesis, if it worked, would provide an alternative rapid approach for the determination of microorganisms for food safety.

Challenges of AFM-imaging microorganisms

Although AFM can be applied in characterizing microorganisms, it should be noted that there are still some limitations and challenges. Sample preparation, for instance, is one of the critical problems for imaging using AFM. During scanning, a force is exerted by the probe and the sample should attach the substrate well enough to withstand the force. Generally, considering the counting chamber is a flat substrate, we can just add the solution to the counting chamber to air-dry, or a chemical treat (for instance, glass slides can be modified with aminosilane molecules, and the silanized slides are then reacted with a dropped bacterial solution) to help the microorganism attach to the substrate of counting chamber. Then the sample can be imaged; however, for imaging some delicate live microorganisms, the solution cannot just be dried or chemically treated before imaging because the microorganisms may die or become injured during drying. Therefore, appropriate procedures for attaching the microorganisms to substrates should be developed;

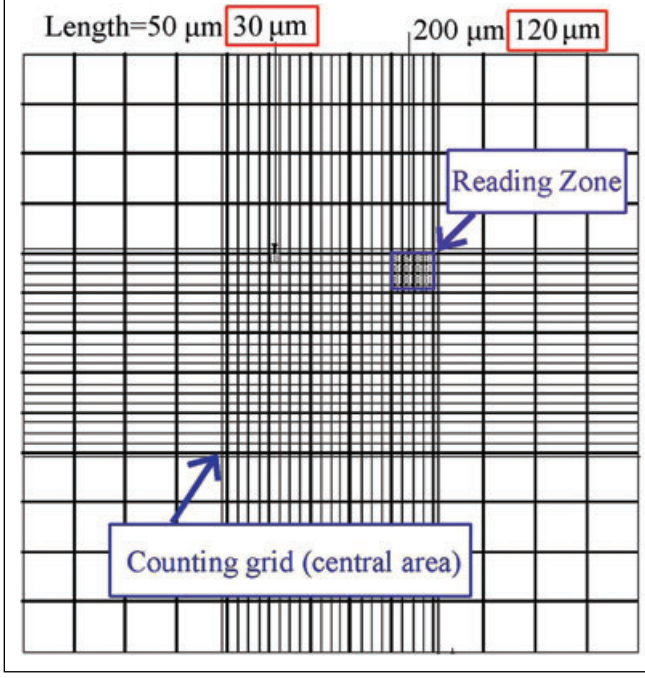

Figure 4—Counting chamber for quantitative determination of food microorganisms. The 1st dimensions are currently used in market and the dimensions in the red square are ideal values for AFM determination (modified from http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html).

for example, porous membranes can be used to immobilize the microorganisms for imaging live microorganisms (Dufrêne 2002).

Imaging force is another problem for imaging live or delicate microorganisms. When imaging in air, the interaction force between the tip and the sample is large, and it sometimes breaks the sample molecules. In addition, the tip will be contaminated and affect the next scanning (Yang and others 2005), which will result in multiple probe effects. Therefore, we must control the imaging force to obtain reliable and high-resolution images. The size, shape, and composition of the AFM probe are also important for obtaining high-quality images (Dufrêne 2002).

It is still not easy to obtain AFM images of microorganisms without breaking the microorganism surface. Sometimes, the AFM force measurements that can be measured without damaging the surface is dependent on the microbial sample (Wright and others 2006). Fortunately, the force has little influence on the general morphology of the microorganisms and there is no problem with counting microbes using AFM. Therefore, the rapid detection and determination of microorganisms for food safety using AFM is very promising.

Conclusions

AFM was proposed and applied to investigate microorganisms qualitatively and quantitatively. In this experiment, *E. coli* B and K12 were used as surrogates in place of pathogenic strains of *E. coli*. The morphology and characteristic parameters of the 2 strains from AFM were compared with those from SEM/TEM. The results show that AFM is very promising for rapid determination of bacteria for safety purposes.

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