Single-molecule detection of proteins and toxins in food using atomic force microscopy

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ABSTRACT

Background: Food safety is vital in everyday life. Food toxins are small peptides or proteins that can cause disease by disrupting biological macromolecules such as enzymes or cellular receptors. Toxins are introduced either externally or from internal, spoilage-related pathogens. To keep these unsafe foods off the market, detection techniques for various toxins have been devised. Although many techniques serve this purpose well, challenges remain regarding sensitivity, specificity, and cost, especially for trace amounts of highly lethal proteinaceous toxins.

Scope and approach: Atomic force microscopy (AFM) is a widely used nanotechnology for imaging and force measurement in biophysics and biology. Its high sensitivity in probing specific, single-molecule binding between molecules is applicable to food toxin detection. In this review, after a summary of the development of AFM, different detection operation modes are introduced along with examples of their applications.

Key findings and conclusions: Cantilever sensing and recognition imaging are found to be the appropriate AFM techniques for detection. Their shared functionalization approaches are outlined for two categories of surfaces: silicon and gold. Recent progress in AFM biosensors and their applications to food toxin detection are discussed. Single-molecule sensitivity and ease of designing sensing schemes make these AFM techniques excellent candidates for real-world application. Existing challenges in designing sensing molecules and preventing the food matrix from confounding signals are not only applicable to AFM techniques but to most current biosensors. Through the collaboration among materials science, chemistry, and molecular biology, solving these issues will promote significant advancement in AFM-based food toxin detection.

1. Introduction

Food safety is critically important to human health. The Centers for Disease Control and Prevention calculates that there are 9.4 million episodes of foodborne illness in the U.S. alone each year, resulting in tens of thousands of hospitalizations and over a thousand deaths (Scallan et al., 2011).

Food safety is compromised by either the introduction of external pathogens during cultivation, processing, or preparation; or by pathogens resulting from spoilage. Of key interest here is the contamination by proteinaceous toxins that are a byproduct of these pathogens and cause sickness at very low concentrations. These toxins include staphylococcal enterotoxin B (SEB), a commonly found cause of gastroenteritis producing 185,000 cases annually (Mead et al., 1999); Clostridium botulinum neurotoxins, a deadly toxin found in improperly canned foods and which is difficult but critical to quickly identify after symptom onset; saxitoxin, responsible for shellfish poisoning resulting from eating shellfish harvested from areas with algal blooms; and ricin, a byproduct of castor bean oil (Buehler, 2005). Rapid, cheap, and accurate testing of food for these toxins is an important element to ensuring safety of the food supply.

2. Current toxin detection methods

Plating and culture enrichment can be used to identify pathogens, but these methods are costly in terms of time and labor. Polymerase chain reaction (PCR) emerged to take a major role in testing, but inhibition of amplification by components of the food matrix can interfere with this method and thus necessitate several controls to ensure sufficient amplification of the correct targets (Hoorfar et al., 2003; Scheu, Berghof, & Stahl, 1998). Additionally, these methods are ill-suited to recognizing certain proteinaceous contaminants, which are able to cause illness at extremely low levels (Rasooly & Herold, 2006). Several advancements in biosensor research have resulted in a number of
methods for recognizing these, typically relying on an antibody or aptamer to specifically recognize the toxin of interest and then coupling that recognition element to a transduction scheme (Turner, 2013). Methods based on piezoelectric crystal sensors, electrochemical schemes (Patel, 2002), surface plasmon resonance (Homola et al., 2002), and ELISA have all been reported (Freed, Evenson, Reiser, & Bergdoll, 1982). ELISA is widely considered the industry standard with regards to toxin detection, making up for relatively costly sample preparation with flexibility and a sub-nanomolar detection limit.

A significant challenge for all techniques remains dealing with the non-toxic food matrix, which is highly heterogeneous, frequently viscous and unmanageable to instruments, and can nonspecifically interact with the recognition element to generate false positives. Advancements in sample preparation can largely be shared among the various biosensing schemes (Myszka, 1999), however, ultimately promoting those with lower detection limits and greater cost-effectiveness.

In a manner similar to the other biosensors, a recognition element can be coupled to an AFM to produce an instrument that is capable of providing extraordinarily high sensitivity, high selectivity, and a cost-effective path toward recognizing these contaminants and securing the safety of the food supply.

3. Atomic force microscopy

Developed in 1986, an AFM consists of a flexible, sharp (tens of nanometers) probe mounted on the end of a cantilever. A laser is reflected off the backside of the cantilever and reflected onto a position sensitive photodetector, allowing for the detection of the cantilever to be measured. The tip can be raster-scanned across a surface to generate a three-dimensional image of the surface morphology (Binnig, Quate, & Gerber, 1986), with the laser deflection used as the input of a feedback control system that allows the probe to follow the surface topography (Meyer & Amer, 1988) (Fig. 1).

The high lateral forces exerted on the substrate as the tip is scanned can across damage of softer biological samples. Amplitude-modulated AFM (AM-AFM), commonly known as tapping mode, was developed to circumvent this problem. In AM-AFM, the cantilever is oscillated by an external driving force, where it acts as a harmonic oscillator. Thus, at some specific frequency, it will oscillate with a set free amplitude and can then be lowered into contact with the surface, reducing the oscillation amplitude until it reaches a set fraction of the free amplitude, at which the set point of the feedback system is established and maintained for the duration of the scan (Fig. 1B). Because the tip only intermittently contacts the surface and only applies a predominantly normal force, lateral forces are effectively eliminated (Zhang, Inniss, Kjoller, & Elings, 1993). This technique was coupled to AFMs amenability in liquid environments (Drake et al., 1989), establishing AFM as a capable tool for probing a broad range of biological samples in physiologically-relevant conditions (Müller & Dufrene, 2008).

Another mode resulting from the amplitude modulation is phase imaging. Dissipative interaction from inelastic soft or viscous samples causes a phase shift to develop between the sinusoidal signal driving the cantilever oscillation and the recorded output signal. This phase lag, when mapped to each point scanned during the image, shows changes in the mechanical properties of the surface that are otherwise topographically homogeneous (Magonov, Elings, & Whangbo, 1997).

4. Achieving chemical specificity with AFM

4.1. Probe functionalisation

An AFM observes only the sample-tip forces as a reflection or amplitude signal and that all other information must be interpreted from this information. Collecting chemical information in addition to the standard morphological information requires that a chemically functional molecule be attached to the probe tip and used to scan a surface modified with the cognate molecule of the tip-bound recognition element.

A variety of chemical functionalization schemes have been developed for this purpose with the two most common methods being silanization and exploitation of simple thiol-gold bonding on gold-coated surfaces.

4.1.1. Silanisation of silicon and silicon nitride probe tips

AFM tips are typically made from silicon or silicon nitride, which can be modified by organosilanes, forming self-assembled monolayers with pendant functional groups. (3-Aminopropyl)trimethoxysilane (APTES) is a common organosilane used for this and exposes a readily-modified amine group (Fig. 2A). The main challenge with this method is the hydrolytic instability of APTES and similar compounds. More recently, 1-(3-aminopropyl)disiloxane (APS) has been demonstrated as a more hydrolytically stable chemical for silanization with otherwise similar performance (Shylakhtenko et al., 2003).

The density of the pendant functional can be controlled by combining the reaction solution with another organosilane without an available functional group. This is the strategy used by Li and coworkers, wherein triethoxy(ethyl)silane (TEES) is added to APTES in a ratio of 4:1 (v/v) to reduce the density of the pendant amino group (Li, Michaelis, Wei, & Colombi Ciacchi, 2015).

A bifunctional PEG linker can be coupled to the pendant amine, permitting further functionalization. PEG linkers provide additional advantages: preventing non-specific tip-sample interactions and providing conformational flexibility to the bound recognition element, improving its binding (Hinterdorfer et al., 2000).

4.1.2. Gold-coated probe tips

Gold surfaces are biologically inert and easily modified with simple thiol chemistries. It is thus the preferred choice for biological detection with AFM. A thin layer of gold (on the order of 10s of nanometers) can be sputtered onto a standard silicon AFM probe. The sensing element can then be directly bound if it features an exposed, suitable thiol or disulfide. If this is not feasible, the tip can then be incubated with bifunctional polyethylene glycol (PEG) linker with at least one thiol end. Again, a long PEG linker reduces non-specific tip-sample interactions and allows the bound recognition element the conformational flexibility needed bind its cognate ligand (Hinterdorfer et al., 2000). The thiolated end will react with the gold tip surface and expose the other functional group, allowing further modification in a variety of directions (Barattin & Voyer, 2008; Ebner, Hinterdorfer, & Gruber, 2007) (Fig. 2B). Commonly, carboxylate groups are used, which can be
4.2. Substrate modification

In addition to functionalization of the AFM probe, the analyte under study is usually fixed to a substrate (this is not always the case, as will be discussed in 5. Cantilever sensors). To achieve high resolution and high sensitivity of detection, the surface must exhibit sub-nanometer flatness, which are hydrophilic and possess a negative surface charge at neutral pH. Silanization is again one of the more common methods for functionalizing AFM tips. Reprinted with permission from (Bain et al., 2009). Copyright 2009 American Chemical Society. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

activated to an N-hydroxysuccinimidy (NHS) ester and then be non-specifically linked to a protein’s or peptide’s primary amines.

These methods largely bind elements together with uncontrollable geometry. When this is a problem, it is also possible to bind sensing elements using copper-free click chemistry methods (Chen, Ning, Park, Boons, & Xu, 2009), wherein an alkyne- or azide-modified silane or thiol is first reacted to the silicon or gold (respectively) tip, followed by reaction with an azide- or alkyne-modified recognition element (respectively) (Fig. 2C). These functional groups are not naturally featured in proteins and thus provide a singular point for binding, controlling the orientation of the recognition element with respect to the probe tip.

Overall, there is considerable flexibility in functionalization schemes, lending a high degree of flexibility to the method dependent on the nature of the sensing element—for example, a protein exhibiting a binding site rich in lysine residues may not be suitable for NHS coupling and should instead be engineered with a specific free thiol or azide.

5. Cantilever sensors

Cantilever sensors, or micromechanosensors, constitute one direction for applying AFM to food toxin detection without the need for a modified substrate. In it, a cantilever bearing a recognition element is oscillated at its resonant frequency and amplitude, establishing a baseline, and is then exposed to a solution bearing the sensing molecule’s cognate analyte. The mass of bound analyte affecting the resonance frequency (Xu, 2012). This change can be calibrated to provide quantification, with dynamic range limited by the number of recognition sites able to fit on the cantilever. This method has already been applied to the detection of SEBs (Maraldo & Mutharasan, 2007), achieving a detection limit of 2.5 fg/mL in apple juice and 25 fg/mL in milk within excellent alternative, but it must be synthesized as it is not yet widely commercially available. Both expose a pendant amine that is readily converted to an amide bond with molecules through NHS coupling.

Gold(111) surfaces are another common choice, exhibiting ultra-flatness and readily produced by thermally evaporating gold onto cleaved muscovite mica. As with tip functionalization, gold substrates are ideal for biological AFM study due to negligible toxicity to biological samples and ready modification with thiolated or disulfide-containing biomolecules or with linkers for subsequent cross-linking (Bain & Whitesides, 1989).

Like mica, highly-oriented pyrolytic graphite (HOPG) easily cleaves, exposing an ultraflat surface. Unlike mica, however, it is hydrophobic and conductive, and while popular for imaging applications, the hydrophobicity has been reported to denature proteins (Muller & Engel, 2008), reducing its biological uses. Both HOPG and mica have proven to be suitable supports for lipid bilayers (Egawa & Furusawa, 1999; Muller & Engel, 2008; Tanaka & Sackmann, 2005), permitting membranes, their embedded proteins, and other associated biological macromolecules to be probed with AFM in highly relevant biological conditions, while still maintaining tight control over surface morphology.
Fig. 3. Depiction of the cantilever sensing scheme for detection of botulinum toxin. (A) The syntaxin 1A (green) on the functionalized tip interacts with the synaptobrevin 2 (red), attaching the bead to the tip as it oscillates. Exposure of botulinum neurotoxin B (BoNT-B) cleaves the bead-bound synaptobrevin 2, releasing the bead. (B) An overview of the components of the cantilever sensor. The tip oscillation is driven by a function generator coupled to a piezoelectric element mounted to the tip. (C) An increase in the resonance frequency of the cantilever is detected upon the release of the bead from (C) to (D). Reprinted with permission from (Liu et al., 2003). Copyright 2003 National Academy of Sciences. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Finally, an antibody-functionalized cantilever sensor was used to detect the antigens on the cell membrane of the food- and waterborne pathogen cholera in a dilute buffer solution, capable of a detection limit of ~1000 CFU/mL, low enough to preclude the need for enrichment (Sungkanak, Sappat, Wisitsoraat, Promptmas, & Tuantranont, 2010).

6. Recognition AFM: imaging and dynamic force spectroscopy

6.1. Recognition imaging

In toposcopy and recognition imaging (TREC), a magnetic cantilever is oscillated in liquid by an external alternating magnetic field (Han, Lindsay, & Jing, 1996). Because of the hydrodynamic drag on the cantilever, the quality factor of the oscillation is significantly reduced, as energy is lost from the downswing to the upswing and back. In this overdamped state, the energy for oscillation is largely derived from the forcing of the magnetic field on the cantilever. Consequently, the maximum amplitude and the minimum amplitude are decoupled (Stroh et al., 2004). TREC requires that a recognition element—usually an antibody or aptamer—is tethered to the tip with a bifunctional PEG linker. The PEG linker must be long enough both to ensure the tip avoids nonspecific interaction with the surface and to lend flexibility to the recognition element such that its active binding site is capable of interacting with its ligand, but the linker must also be kept short as feasible to maintain high spatial resolution (Ebner et al., 2005). In practice, linkers anywhere from 5 to 20 nm are commonly used. The functionalized tip's interaction with raised features on the surface reduces the amplitude on the downswing but this is not carried over to the upswing as the magnetic actuation restores the amplitude. Conversely, recognition events reduce the upswing but not the downswing. The upper and lower extrema of the resulting signal versus time plot can be separated and each converted into an image, the bottom part showing topography and the upper part showing recognition sites as dark features that correspond in the xy-dimension to the topographical image (Fig. 4).

Validation of the specificity of recognition occurs via a series of controls (Stroh et al., 2004): 1) where the tip's recognition element is blocked by a free cognate ligand leading to an image with normal topography but no recognition, 2) one in which the surface ligand is blocked by free recognition elements, leading to possible topographical

Fig. 4. Cartoon depiction of TREC imaging. A) The Pico TREC box splits the top and bottom halves of the amplitude signal and converts them to an image. A structural feature on the surface will reduce the bottom half's amplitude (seen as a bump in the heavy blue line after the signal is split) whereas a recognition event will reduce the top half's amplitude (seen as a dip in the heavy red line after the signal is split). B) A representative TREC image. The topographical image is shown on the left shows bright spots where avidin molecules are bound to the surface. The corresponding recognition image (right) shows that the tip, biotinylated tip interacts with the bound avidin molecules, generating a recognition signal. Reprinted with permission from (Ebner et al., 2005). Copyright 2005 John Wiley & Sons, Inc.
identification of the now surface-bound recognition elements but again no recognition image, and 3) one in which a blank tip is scanned to ensure that stickier domains on the surface are not responsible for any apparent recognition.

As previously indicated, the PEG linker can reduce spatial resolution, especially for the recognition image, but the problem is limited because the binding event leading to recognition is a probabilistic event, and as the tip is scanned over and around a recognition site, the tethered recognition element will display some tolerance in its position, but that will be normally distributed over the recognition site, preserving high resolution (Senapati & Lindsay, 2016).

### 6.2. Dynamic force spectroscopy

Additional quantitative information can be achieved from these functionalized AFM probes through the use of single-molecule dynamic force spectroscopy (DFS). DFS interrogates a ligand-receptor pair by repeatedly lowering the functionalized tip’s ligand into contact with the surface-bound receptor, allowing a binding event to occur. The tip is then retracted, and the force of the interaction causes the cantilever to bend until the bond breaks and the cantilever snaps back to its equilibrium position. This entire cycle is recorded by the PSD as a force-distance curve (Florin, Moy, & Gaub, 1994) (Fig. 5). The maximum stretching distance and the force of the bond (on the order of pN) is measured. A collection of unbinding forces from these curves can be compiled into a histogram, to which a Gaussian function may be fit to establish the most probable unbinding force, $F^*$. The loading rate (pN/s) of the AFM cantilever can be varied, influencing the binding energy landscape, as higher loading rates study a far-from-equilibrium regime, which causes energy barriers to diminish and some binding states to become inaccessible, while lower rates can approach a near-equilibrium regime. $F^*$ collected over a wide range of loading rates (a few orders of magnitude) allows the plotting of $F^*$ as a function of log(loading rate). This analysis, developed by Evans, permits the derivation of the dissociation rates (Evans, 2001) and through further theoretical development by Jarzynski on the relationship between non-equilibrium work and free energy (Jarzynski, 1997), has led to the development of formalisms for extracting free energy profiles from DFS spectra (Hummer & Szabo, 2001; Liphardt, Dumont, Smith, Tinoco, & Bustamante, 2002).

Bioanalytical systems designed with DFS do not necessarily need to directly measure the analyte. Systems have been designed that record Fig. 5. An overview of dynamic force spectroscopy.

Top pane: The tip is lowered into contact with the surface (B), where the ligand-receptor pair binds (between B and C). The tip is then raised until the ligand-receptor bond breaks at (E), yielding the unbinding force. Reprinted with permission from (Bizzarri & Cannistraro, 2010). Copyright 2010 The Royal Society of Chemistry.

Bottom pane: (A) Force-distance curves collected on the aptamer-ricin system at varying loading rates. For each loading rate, three curves are overlaid and shown. (B) A 3D histogram showing the frequencies of unbinding forces at each loading rate. (C) $F^*$ vs. log(loading rate), as discussed in 6.2. Linear fitting is for both aptamer (red) and antibody (blue). Reprinted with permission from (Wang et al., 2011). Copyright 2011 The Royal Society of Chemistry. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
the change in interaction force upon addition of the analyte, such as systems relying on the modulation of aptamer-surface interactions by a protein analyte (Wei, Steckbeck, Köppen, & Colombi Ciacchi, 2013). DFS’s flexibility in experimental designs has led to its use in measuring a variety of analytes, as reviewed by Li and coworkers (Li et al., 2016).

7. AFM applications toward food toxin detection

Recognition AFM has proven to be a capable tool for interrogating various biological systems, owing to its non-destructive nature and its high-resolution in biologically-relevant conditions. It has been successfully applied to the detection of DNA, individual proteins, and proteins on living cell membranes (Lin et al., 2009; Müller & Dufrêne, 2011; Stroh et al., 2004; Wang, Park, Xu, & Kwon, 2017). In 2009, Chen et al. used the method to detect the toxin ricin bound to a gold surface with an antibody-functionalized tip (Chen, Zhou, Park, & Xu, 2009), achieving single molecule resolution (Fig. 6), corresponding to a sub-molecular detection limit. The bonding between AFM probe tip and anti-ricin antibody was achieved using a click chemistry scheme (Fig. 2C), ensuring a specific recognition element (Chen et al., 2009). DFS was used to confirm the single-molecule nature of the antibody-ricin interaction, determining its binding strength to be 64.89 ± 1.67 pN. Additional work carried out by Wang et al. found that an RNA aptamer worked slightly better (as measured by binding interaction strength and k_{off}) than the antibody as a sensing molecule and was even able to distinguish between ricin and the corresponding recognition image. Free ricin was then used to block the antibody on the tip, leading to a control (c) recognition image (Fig. 6). Recognition image. Note that the topography changes little but that recognition is significantly decreased. Source: (Chen et al., 2009).

Panel B. (a) Topographic and (b) recognition images of ricin with an aptamer-functionalized tip. (c) Ricin molecules I-IV from the topographical image are selected and zoomed in to identify the specific conformation ricin has bound to the surface. These are each compared to a simulated structure of ricin, with good agreement. Reprinted with permission from (Wang et al., 2011). Copyright 2011 The Royal Society of Chemistry. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Panel C. (A) Topographic and (B) recognition images of ricin with an aptamer-modified tip. (C) Ricin molecules I-IV from the topographical image are selected and zoomed in to identify the specific conformation ricin has bound to the surface. These are each compared to a simulated structure of ricin, with good agreement. Reprinted with permission from (Wang et al., 2011). Copyright 2011 The Royal Society of Chemistry. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

8. Conclusion

Because of its perpetual importance, ensuring that foods are safe and free of proteinaceous toxins will remain important. AFM will be a capable tool toward securing food safety. Its biosensing capabilities have proven to be highly sensitive and specific, it can operate in all
ambient environments, and it exhibits a wide and flexible variety of functionalization schemes enabling it to be applied to virtually any food toxin or pathogenic protein (see Table 1). Work remains to be done in developing methods for sample preparation to better remove the confounding effects of the non-toxic food matrix, but the high degree of customizability in aptamers as recognition elements and work on anti-fouling coatings provide a path forward.

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**References**


