Second harmonic atomic force microscopy of living *Staphylococcus aureus* bacteria

Robert D. Turner,[1,2] Jennifer Kirkham,[2] Deirdre Devine,[2] and Neil H. Thomson[1,2,a]

[1]*School of Physics and Astronomy and Astbury Centre for Structural Molecular Biology, University of Leeds, West Yorkshire LS2 9JT, United Kingdom*
[2]*Department of Oral Biology, Leeds Dental Institute, University of Leeds, West Yorkshire LS2 9LU, United Kingdom*

Monitoring higher harmonics of the drive frequency in amplitude-modulation atomic force microscopy can give extra information on local surface properties. The first to fourth harmonics inclusive were monitored on the surface of individual *Staphylococcus aureus* bacteria and on the polycarbonate filter in which they were trapped. The second harmonic response was sufficient to create the first higher harmonic images of the surfaces of living bacterial cells under aqueous buffer. Mapping the second harmonic signal onto the height (*Z* piezo-signal) shows that they are largely uncorrelated, suggesting that it measures local surface properties related to mechanics and/or chemical interactions. © *2009 American Institute of Physics*. [DOI: 10.1063/1.3073825]

Intermittent contact mode atomic force microscopy (AFM) under liquid[1] has proved to be a useful tool for studying bacteria.[2] In addition to high resolution imaging, measurement of local nanomechanical properties such as local effective elastic modulus or Hamaker constant would provide potentially important information for use in both applied and fundamental microbiological research. Techniques have been proposed to measure a number of different properties including force volume imaging[3] and phase imaging.[1] Recently developed techniques based on monitoring the motion of sinusoidally driven cantilevers under liquid, at higher harmonics than the fundamental as they interact with the surface, should prove to be a useful complementary approach as they offer higher speeds and pixel density than force volume and may be easier to interpret and quantify than fundamental phase mapping. These methods may involve driving the cantilever at more than one frequency[4] or driving at a single frequency and monitoring the response at its harmonics.[5] Similar techniques have been developed for use in air.[6–8]

An AFM cantilever in liquid can be modeled as a harmonic oscillator, the tip interacting with a surface via van der Waals forces and interactions taken from standard contact mechanics models.[9] Higher harmonic imaging was initially used in air on solid state surfaces and predictions were made that the higher frequency harmonics would be the most sensitive to the elastic modulus of the surface,[8] using the Euler–Bernoulli equation to describe the oscillator, with Derjaguin–Muller–Toporov (DMT) contact mechanics. A driven damped harmonic oscillator model with identical contact mechanics was used by Preiner *et al.*[5] to predict a relationship between elastic modulus and second harmonic amplitude for hard surfaces. According to these models, when a cantilever impacts on a surface, the energy of the collision is conserved but the motion of the cantilever is altered. The ability of the cantilever to transmit this signal depends on the quality factor (Q) of the lever, since it acts as a mechanical filter that does not permit large motions outside the normal modes of vibration. In liquid, the quality factors can be low, around unity, and therefore the frequency response is fairly flat, allowing excitation of the cantilever at multiple harmonics of the driving signal when intermittent contact is occurring.[5] We suggest that excitations are reduced on softer surfaces because the energy of the impact is dissipated into the sample and the energy remaining with the oscillator therefore decreases. The highly damped response of a soft cantilever ($k \sim 0.1$ N/m) in liquid allows higher harmonic imaging to be performed,[5] whereas amplitude modulation (AM) in air, where the Q is typically 500, may require higher vibrational modes to be specifically driven[4,6] but not always.[7,8]

Second or higher harmonic amplitude maps can be acquired under liquid at normal intermittent contact mode AFM scan rates, which are, in most cases, at least ten times faster than using force volume mapping (the closest comparable technique for working under liquid) and have typically about ten times the pixel density. This means that surface mechanical and adhesive property variations occurring at shorter time and length scales are accessible. However, as harmonic amplitudes are dependant on mean tip-sample separation as well as surface properties, convolution of topographic features with these properties will occur at a level determined by the efficiency of the feedback loop. Here, we report for the first time, higher harmonic AM imaging applied to individual living bacteria trapped in polycarbonate tracked etched (PCTE) filter pores under buffer conditions.

Staphylococcus aureus (NCTC 8532) were grown in Brain Heart Infusion broth overnight and then subcultured and grown to early exponential phase, before being concentrated by centrifugation and passed through a PCTE filter (Whatman, Nucleopore) with an appropriate pore size,[10] washed in phosphate buffered saline, *p*H 7.4 (PBS), and mounted under PBS in a multimode AFM (Nanoscope IV controller, Veeco). For imaging, the raw photodiode signal was captured via a Signal Access Module along with the drive frequency directly from the Nanoscope IV controller and fed into an external lock-in amplifier (Signal Recovery 7265), the output of which was connected to an auxiliary

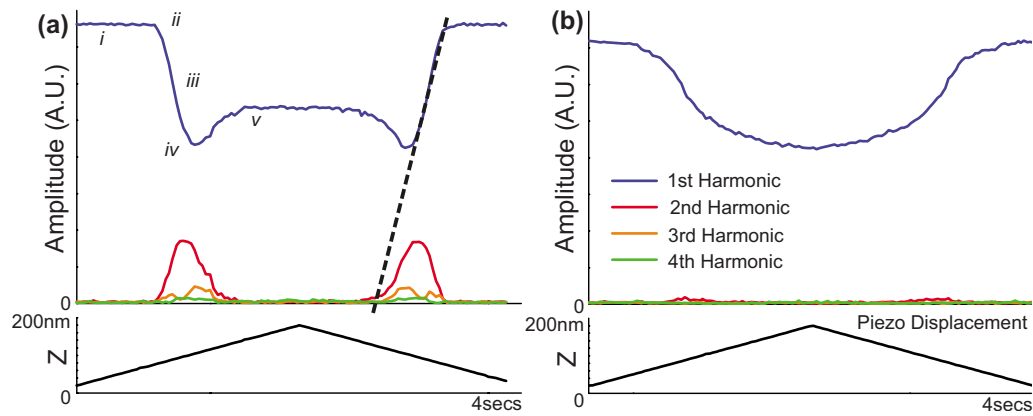a)Electronic mail: n.h.thomson@leeds.ac.uk.

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FIG. 1. (Color online) Amplitude and piezo displacement (*Z*) vs time measurements for the first to fourth harmonics on (a) the PCTE filter membrane and (b) on the surface of a *S. aureus* bacterium. The amplitudes are the heights of the peaks in a Fourier transform of the photodiode signal. The amplitude scale is arbitrary but the signals between harmonics are comparable. The amplitude of the cantilever varies as it moves toward then away from the surface. (i) The cantilever is "free" i.e., it is not influenced by surface forces, (ii) the tip comes into contact with the sample at the lowest point of its oscillation, (iii) the amplitude of the first harmonic decreases with the mean tip-sample separation, (iv) an inflection point where the first harmonic amplitude begins to increase and second harmonic amplitude peaks, and (v) amplitudes stabilize.

input on the controller. For amplitude traces, the raw photodiode signal was obtained in the same way, along with the low voltage *Z* piezodrive signal and fed into a data capture card (PXI-6120) for processing in LABVIEW 7. The photodiode signal periodically underwent a Fourier transform and the heights of the peaks were measured. Veeco NP-S cantilevers with a nominal spring constant of 0.12 N/m and resonant frequency of 14–26 kHz were driven at a frequency of ~9.4 kHz resulting in a peak-to-peak amplitude of oscillation ~10 nm.

Amplitude and piezo-extension were measured against time over a 4 s cycle during which the piezo moved the sample 200 nm toward and then away from the tip at a point on the polycarbonate filter and another close to the center of the *S. aureus* cell (Fig. 1), yielding qualitatively similar results over multiple cells, cantilevers, and sample preparations.[11]

On the relatively hard filter surface, as the first harmonic amplitude begins to drop, all of the higher harmonics show an increase in amplitude, with the second harmonic showing the highest response [Fig. 1(a)] in good qualitative agreement with the results of Preiner *et al.*[5] On the softer surface of a living *S. aureus* bacterium, higher harmonics are at much lower amplitudes [Fig. 1(b)], although the second harmonic gives a measurable signal, which can be used for imaging (Figs. 2 and 3). The decrease in the first harmonic amplitude on first intermittent contact with a bacterial surface does not have a well-defined start, due to the soft contact on this surface. This amplitude decreases in a nonlinear way to the expected point of permanent contact (as compared

with the filter) and beyond. This is symptomatic of the soft polymer mesh of peptidoglycan that forms the cell wall of bacteria.[12] High magnification images (Fig. 3) are similar to those published by Touhami *et al.*[13] indicating that the bacterial strain used has no capsule, fibrils, or fimbrae.

This is the first time that higher harmonic measurements have been taken under liquid using an indirect method such as a piezo to drive the cantilever. Using this indirect drive technique, the amplitude of the first harmonic does not fall to zero as the tip contacts the surface but increases to another plateau value on further shortening of the distance between the cantilever holder and the sample surface [Fig. 1(a)]. The minimum amplitude of the first harmonic is usually about 50% of the free value on the hard filter surface.[11] This is the point at which the sample position in the vertical direction is at the equilibrium position of the resting cantilever. The additional amplitude upon bringing the cantilever and sample closer together may be due to a recoil effect when the tip-sample distance is less than half the cantilever amplitude, which is a consequence of the indirect excitation method moving the support chip relative to the sample. Different experiments yield slightly different final amplitude values but they are in the range of 65% to 85% of the free amplitude.[11] The second harmonic signal begins as intermittent contact starts and peaks at the cantilever equilibrium point and then decreases with a similar rate of change. Extrapolation of the linear part of the first harmonic amplitude to zero coincides with the decrease in the second harmonic amplitude to zero [see Fig. 1(a)]. This is the point of permanent contact of the tip to the surface, where the end of the
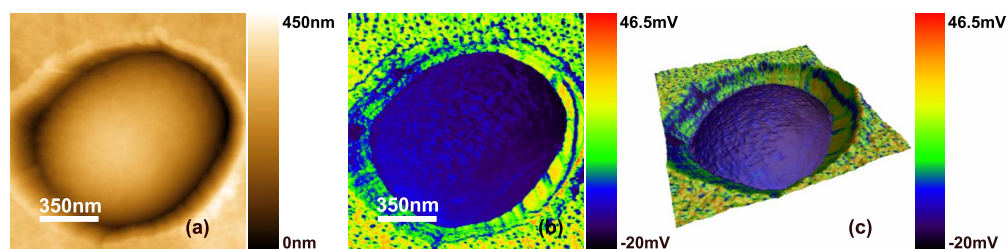

FIG. 2. (Color online) Intermittent contact mode imaging of a *S. aureus* bacterium trapped in a PCTE filter showing; (a) height (*Z*-piezo signal), (b) amplitude shift in the second harmonic relative to the amplitude on engagement with the surface and (c) 3D representation of height colored with second harmonic amplitude.
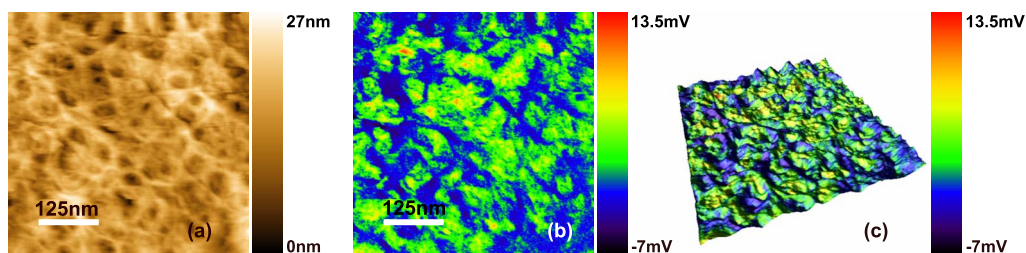
FIG. 3. (Color online) Intermittent contact mode imaging of the surface of *S. aureus* trapped in a PCTE filter showing (a) height (*Z*-piezo signal), second order flattened, (b) amplitude shift in the second harmonic relative to the amplitude on engagement with the surface, and (c) 3D representation of height colored with second harmonic amplitude.

cantilever is effectively clamped against the surface.

While the third and fourth harmonic amplitude signals get progressively smaller, they are measurable on the hard filter surface and for the third show two maxima and a minimum at the point of inflection of the first harmonic amplitude. The origins of these signals need further investigation but they may be caused by different flexural modes of the cantilever at increasing frequencies. In spite of the first harmonic amplitude not being suppressed on permanent contact of the tip to the sample, we do not anticipate that this is a major problem during imaging applications, since they are performed when the first harmonic amplitude is usually 70% or more of the free amplitude.

Images on individual *S. aureus* bacteria were taken at the second harmonic, as this has the strongest response above the first, and compared with the height (*Z*-piezo) signal (Fig. 2). Figures 2(c) and 3(c) were rendered using WSXM 4 (Ref. 14) using height to generate a three-dimensional (3D) relief upon which the second harmonic image was mapped. There is no apparent correlation between the height signal and the second harmonic suggesting that topographical coupling is minimal, although there are correlations between the first and second harmonics at large changes in topography, for instance at the edge of the pore between the bacterium and the top of the filter membrane [see Figs. S3 and S5 (Ref. 11)].

Mapping the second harmonic image onto the height signal is useful to compare how the topography influences the second harmonic signal [Fig. 2(c)]. Higher magnification images of the bacterial cell surface [Fig. 3(c)] reveal that the second harmonic signal at short length scales, where the topographical difference is low (~20 nm), appears uncorrelated with the height. Cross-section analysis indicates that the second harmonic is measuring properties of the surface that are not due to topography [see Figs. S4 and S6 (Ref. 11)]. According to the recent model of Preiner *et al.*,[5] the second harmonic amplitude would increase as the elastic modulus decreases. It is clear from this study that this model and others[8] are insufficient to explain these data, since on the softer bacterial surface the second harmonic signal is less pronounced. On complex biological surfaces, there are other interactions between the tip and surface that could be influencing the second harmonic signal. These might include adhesive interactions and viscoelastic responses of the bacterial cell surfaces. As pointed out by Stark and Heckl[8] previously, DMT contact mechanics models are only suitable when energy dissipation is not present.

In summary, we have monitored the amplitude of the first to the fourth harmonics of piezo driven oscillations of an AFM cantilever as it was brought into and out of contact with polycarbonate and with *S. aureus* bacteria. It was found that harmonics are at higher amplitudes on harder polycarbonate than on softer bacterial cells. This is likely to be largely due to more energy being dissipated in deforming the surface in the interaction between the tip and the bacteria than between the tip and the polycarbonate. The second harmonic amplitude was mapped simultaneously with topography at low and high magnifications on a cell trapped in a polycarbonate membrane. These images confirmed that there is a significant drop in the amplitude of the second harmonic as the tip moves from polycarbonate onto a bacterium and show that there is measurable variation in second harmonic amplitude across the bacterial cell. This variation is largely uncorrelated with height and is attributable to, as yet, unknown surface properties.

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