Towards local electromechanical probing of cellular and biomolecular systems in a liquid environment

Sergei V Kalinin\textsuperscript{1,2,5}, Brian J Rodriguez\textsuperscript{1,2}, Stephen Jesse\textsuperscript{1}, Katyayani Seal\textsuperscript{2}, Roger Proksch\textsuperscript{3}, Sophia Hohlbauch\textsuperscript{3}, Irene Revenko\textsuperscript{3}, Gary Lee Thompson\textsuperscript{4} and Alexey A Vertegel\textsuperscript{4}

\textsuperscript{1} Materials Sciences and Technology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37931, USA
\textsuperscript{2} The Center for Nanophase Materials Sciences, Oak Ridge National Laboratory, Oak Ridge, TN 37931, USA
\textsuperscript{3} Asylum Research, Santa Barbara, CA 93117, USA
\textsuperscript{4} Department of Bioengineering, Clemson University, Clemson, SC 29634, USA

E-mail: sergei2@ornl.gov

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Abstract

Electromechanical coupling is ubiquitous in biological systems, with examples ranging from simple piezoelectricity in calcified and connective tissues to voltage-gated ion channels, energy storage in mitochondria, and electromechanical activity in cardiac myocytes and outer hair cell stereocilia. Piezoresponse force microscopy (PFM) originally emerged as a technique to study electromechanical phenomena in ferroelectric materials, and in recent years has been employed to study a broad range of non-ferroelectric polar materials, including piezoelectric biomaterials. At the same time, the technique has been extended from ambient to liquid imaging on model ferroelectric systems. Here, we present results on local electromechanical probing of several model cellular and biomolecular systems, including insulin and lysozyme amyloid fibrils, breast adenocarcinoma cells, and bacteriorhodopsin in a liquid environment. The specific features of PFM operation in liquid are delineated and bottlenecks on the route towards nanometre-resolution electromechanical imaging of biological systems are identified.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Coupling between electrical and mechanical phenomena is ubiquitous in biological systems. Multiple examples include phenomena such as voltage controlled muscular contractions [1], cell electromotility [2], and electromotor proteins [3]. In many cases, these couplings are the functional bases for processes such as transduction of acoustic signals into electrical pulses in the outer hair cell stereocilia, cardiac activity, energy storage in mitochondria, etc. On a more fundamental level, cellular membranes are flexoelectric, i.e. possess linear coupling between electric field and strain gradient (or local radius of curvature) [4]. Biopolymers forming calcified and connective tissues are often piezoelectric [5], i.e. exhibit linear coupling between electric field and strain. Significant effort has been directed towards understanding the origins of electromechanical activity of these types of biological systems and its relationship to functionality [6–15]. However, due to the complex spatial structure of biological systems, this task requires real-space probing of electromechanical coupling on the nanoscale.
2. Experiment

2.1. Principles of PFM

Piezoresponse force microscopy is based on the detection of the bias-induced piezoelectric surface deformation. The tip is brought into contact with the surface, and the piezoelectric response of the surface is detected as the first harmonic component, \( A_{1v} \), of the tip deflection, \( A = A_0 + A_{1v} \cos(\omega t + \varphi) \), induced by the application of the periodic bias, \( V_{pp} = V_{dc} + V_{ac} \cos(\omega t) \), to the tip. The phase of the electromechanical response of the surface, \( \varphi \), yields information on the sign of the piezoresponse.

PFM is implemented on commercial SPM systems (Veeco NS-IIIa MultiMode, NanoMan V, and Asylum Research MFP-3D) equipped with additional function generators and lock-in amplifiers (DS 345 and SRS 830, Stanford Research Instruments, and model 7280, Signal Recovery). Measurements in ambient conditions were performed using Pt and Au coated tips (NSC-12 C, Micromasch, and lock-in amplifiers (DS 345 and SRS 830, Stanford Research Instruments, and model 7280, Signal Recovery). Measurements in ambient conditions were performed using Pt and Au coated tips (NSC-12 C, Micromasch, l ≈ 130 μm, resonant frequency ∼150 kHz, spring constant \( k \sim 4.5 \text{ N m}^{-1} \)). Measurements in liquids were performed using a Cr–Au-coated CSC-38 cantilever with l ≈ 300 μm, resonant frequency ∼14 kHz, and spring constant \( k \sim 0.05 \text{ N m}^{-1} \). Vertical PFM (VPFM) measurements were performed at frequencies of 50 kHz–1 MHz, which minimizes the longitudinal contribution to the measured vertical signal and allows imaging close to the resonances [37].

2.2. Model systems

2.2.1. Lysozyme fibrils. Fibril formation and purification procedures were based on those reported by Vernaglia et al [38]. A 6 mg ml\(^{-1}\) chicken egg-white lysozyme (lysozyme, No L6876 from Sigma) stock solution was prepared in 20 mM potassium phosphate (KH\(_2\)PO\(_4\)), adjusted to pH 6.3 ± 0.1 with 1 M potassium hydroxide. Dilution with 4 M guanidine hydrochloride (GuHCl, No 105696 from MP Biomedicals, Inc.) in 20 mM KH\(_2\)PO\(_4\) yielded ∼1.8 mg ml\(^{-1}\) lysozyme in ∼2.8 M GuHCl and 20 mM KH\(_2\)PO\(_4\). Fibrillation was induced by incubating the solution for 24 h in a glass vial on a heated stir plate at 50 °C with mild agitation (∼100 rpm). The fibrils were centrifuged at 14,000 g for 10 min, the supernatant was discarded, and the pellet was washed with the same volume of HPLC-grade water (Fisher) using gentle shaking for 10 min. This step was repeated for a total of 15 washes. The fibril suspension was diluted with HPLC-grade water to 0.25 mg ml\(^{-1}\), and samples for PFM imaging were prepared by incubating 25 μl of the resulting suspension on freshly cleaved mica (No 71851-05 from Electron Microscopy Sciences) at room temperature for 2.5 min. Incubation was followed by 30 washes with 100 μl HPLC-grade water, and gentle drying with compressed nitrogen. Another 20 washes with 100 μl HPLC-grade water and finally gentle drying with compressed nitrogen completed the adsorption of lysozyme onto mica. Before imaging, 100 μl of HPLC-grade water was added onto the sample for solution PFM imaging.

2.2.2. Insulin amyloid fibrils. Insulin amyloid fibrils were formed and purified following the procedures reported by Guo and Akhremitchev [39]. Bovine insulin (No 15500 from...
Sigma-Aldrich) was reconstituted to 5 mg ml\(^{-1}\) in 10 mM hydrochloric acid (HCl, Fisher Chemical). This solution was incubated in a polypropylene microcentrifuge tube in a block heater at 80 °C for 48 h. The fibril suspension was diluted tenfold with 10 mM HCl to 0.5 mg ml\(^{-1}\) protein and was centrifuged at 3000 g for 1 min to remove smaller aggregates from the longer fibrils. The precipitates were collected and washed by gentle shaking for 5 min with the same volume of 10 mM HCl as had been removed. This was repeated twice for a total of three washes. The fibril suspension was diluted 1:1 with 10 mM HCl to 0.25 mg ml\(^{-1}\), and samples for PFM imaging were prepared by incubating 25 μl of the resulting suspension on freshly cleaved mica (No 71851-05 from Electron Microscopy Sciences) at room temperature for 1 min. Incubation was followed by five washes with 35 μl of 10 mM HCl and gentle drying with compressed nitrogen. The residual HCl was then removed by five additional washes with 35 μl of HPLC-grade water and finally gentle drying with compressed nitrogen. Before imaging, 100 μl of HPLC-grade water was added onto the sample for solution PFM imaging.

2.2.3. Breast adenocarcinoma cells. Breast adenocarcinoma cells (MCF7 cell line) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with glucose and L-glutamine (No D5523 from Sigma-Aldrich) supplemented with 10% FBS (No ES1055 from Biomedia), penicillin–streptomycin (No P0781 from Sigma-Aldrich), and non-essential amino acids (No M7145 from Sigma-Aldrich). The cells were plated onto pretreated Corning 60 mm polystyrene Petri dishes (No 430166 from Fisher Scientific) and were maintained in a CO\(_2\) incubator at 37 °C until imaging.

2.2.4. Bacteriorhodopsin. Bacteriorhodopsin from Halobacterium salinarium (No B0184 from Sigma-Aldrich) was diluted to a final imaging concentration of 50 μg ml\(^{-1}\) in 10 mM Tris, 150 mM KCl buffer, pH 8. To prepare the sample, 10 μl of the bacteriorhodopsin solution was added to 10 μl of buffer onto a freshly cleaved mica disc. The solution was incubated for 10 min then rinsed with 100 μl of buffer solution. Additional buffer was added for a final volume of 200 μl. Patches of bacteriorhodopsin were imaged with a metal coated tip in tapping mode with low drive and set-point amplitudes.

3. Results

3.1. Liquid PFM imaging of model ferroelectric systems

To illustrate the feasibility of electromechanical imaging in liquid environment, the PFM was performed in air and in DI water on a periodically poled lithium niobate (PPLN, Crystal Technology) as a model system. The electromechanical response of PPLN in the point contact geometry of a PFM experiment is well known both from calculations [40] and direct measurements [41] and 12 pm V\(^{-1}\). PPLN is thus a convenient model system for calibration of the system, i.e. determination of the conversion factor between the lock-in amplifier output and local electromechanical response. Furthermore, the noise level in the PFM measurements can be determined from the dispersion of the signal on a single domain. If the vertical sensitivity of the system is known independently, the electrical contact quality between the tip and the surface can be estimated. Note that both types of calibration are generally applicable only for frequencies well below the first resonance of the cantilever. Otherwise, cantilever dynamics, which is strongly dependent on the contact stiffness of the tip–surface junction, will provide a sample-dependent multiplicative factor to the signal [42].

The PFM amplitude and phase images taken from the PPLN in air and distilled water are shown in figure 1. Both images demonstrate clear domain contrast unrelated to topographic features in amplitude images. The phase
Figure 2. PFM images of a lysozyme fibril adsorbed onto mica. The scan size is 2.97 μm × 2.97 μm, with a scan rate of 1 Hz and 512 samples per line. Ac biases, 10, 5, and 2 V, as shown in the left, middle, and right rows, respectively, were applied with a frequency of 383 kHz, a 0° phase angle, and a lock-in bandwidth of 1 kHz. The first row shows topographic images, the second row piezoresponse phase images, and the third row piezoresponse amplitude images. Z scales are 50 nm, 360°, and 2.5 mV for the topographic, piezoresponse phase, and piezoresponse amplitude images, respectively. The height of the feature ranged from about 10 nm to a maximum of about 25 nm, consistent with the diameters of lysozyme fibrils reported by Vernaglia et al [38]. Note that the amplitude feature disappears with decrease in applied ac bias from 10 V (g) to 2 V (i) while the topographic and phase images reveal that the fibril bundle remains present.

contrast in both cases illustrates phase change by 180° between antiparallel domains. The noise level and effective domain wall width in the liquid image are higher, indicative of more significant spreading of the electric field anticipated in the high dielectric constant (ε = 80 in water versus ε = 1 in air). However, clear qualitative agreement between PFM images in both environments serves as a strong indicator of the electromechanical nature of the contrast.

3.2. Liquid PFM imaging of amyloid fibrils

After verification on the ferroelectric system with known electromechanical response, PFM was applied to a variety of biological systems. Figure 2 shows topographic ((a)–(c)) and PFM images of a lysozyme fibril as a function of imaging bias, V_{ac}. PFM phase images are shown in figures 2(d)–(f) and PFM amplitude images are shown in figures 2(g)–(i) for 10, 5, and 2 V, respectively. The piezoresponse amplitude linearly depends on the applied bias, and the phase response is stronger for higher bias. The fibril does not appear to degrade with repeated scanning or application of ac bias. Some internal structure is discernible in PFM amplitude images, while the phase response appears to be uniform within a fibril. Similar behaviour is observed for the insulin fibril in figure 3, shown for two driving voltages. The strong dependence of the phase contrast on driving amplitude and small phase shifts (≪180°) between dissimilar regions suggests significant contribution of the electrostatic forces (modified by the presence of liquid) to the PFM signal. Based on the visual inspection of the topographic, PFM phase and PFM amplitude data it is obvious that (1) the apparent spatial resolution in the PFM amplitude image is higher compared to topography and (2) phase images show clear contrast on the parts of the fibril in which topographic height is at the detection limit. Below, we explain these observations by suggesting that the primary origin of the PFM signal is the variability of the local elastic properties between the substrate and the fibril. This elastic contrast is convoluted with electromechanical and electrostatic responses, precluding unambiguous determination of the latter. However, the increased resolution and detection limit clearly illustrate the usefulness of this detection method.
Figure 3. PFM images of an insulin fibril adsorbed onto mica. The scan size is 603 nm × 603 nm, with a scan rate of 1 Hz and 512 samples per line. Ac biases, 10 Vac and 2 Vac, as shown in the top and bottom rows, respectively, were applied with a frequency of 437.8 kHz, a 0° phase angle, and a lock-in bandwidth of 1 kHz. The first column shows topographic images, the second column piezoresponse phase images, and the third column piezoresponse amplitude images. Z scales are 25 nm, 180°, and 2.0 mV for the topographic, piezoresponse phase and piezoresponse amplitude images, respectively. The height of the feature ranged from 5 to 10 nm, which is consistent with the range of insulin fibril heights reported by Guo and Akhremetchev [39]. Note that the amplitude feature decreases and fades with decrease in applied ac bias from 10 Vac (e) to 2 Vac (f), while the topographic and phase images reveal that the fibril bundle remains present.

Figure 4. Live breast adenocarcinoma cells (MCF7 cell line) imaged in DMEM culture medium. (a) Topography (180 nm z-scale), (b) PFM amplitude, and (c) PFM phase images. Images were acquired in contact mode using the MFP-3D.

3.3. Imaging of cellular systems

The extension of liquid PFM imaging to viable cells is illustrated in figure 4, showing topography, PFM amplitude, and PFM phase of living breast adenocarcinoma cells. Only weak contrast between different cells or at cell boundaries is observed in the PFM amplitude image. However, the cells with larger height profiles appear to have a slightly different PFM phase response than the surrounding cells. In addition, cell boundaries are resolved in the PFM phase image. Similarly to the amyloid and insulin fibrils, these observations suggest the possibility of a strong elastic contribution to the PFM signal. The reason for the relatively poor quality of cell PFM images is the presence of electrolytes in the cell culture media because high conductivity of the solution results in the uniform biasing of the liquid. To avoid this problem, the imaging should be performed using insulated or shielded probes, in which the central part of the tip is insulated from the solution except for the region near the probe apex.

3.4. Electromechanical imaging of bacteriorhodopsin and PFM resolution

The general analysis of PFM image formation suggests that the electromechanical contribution to the signal is favoured by high effective stiffness of the system. This is due to the fact that the electromechanical contribution is independent of spring constant, and electrostatic contribution scales reciprocally with effective spring constant [42]. Furthermore, optimal imaging conditions with minimal topographic cross-talk correspond to flat surfaces. Hence, we have chosen the bacteriorhodopsin (BR) purple membrane as a relatively stiff and topographically
The experiments with BR also establish the potential for molecular-level spatial resolution. The operation of PFM requires reliable control of both dc and ac tip bias in the 1 kHz–1 MHz range. Operation in liquid environment brings additional limitations on the conductance of the tip coating, which should be greater than that of the liquid to stray current paths through the solution. This necessitates the use of metallic or metal coated tips, which typically have significantly higher radii of curvature compared to Si or Si₃N₄ probes. Figure 6 illustrates that the use of intermittent contact mode with a metal coated tip in liquid allows molecular resolution to be achieved.

4. Towards electromechanical imaging in liquids

The results summarized in section 3 illustrate that electromechanical imaging in a liquid environment produces measurable contrast on a variety of biological systems. The presence of the contrast per se evidences measurable electromechanical and electrostatic interactions between the probe and the surface in liquid. In many (albeit not in all) cases, the contrast is visually distinct from surface topography or its first and second derivatives, suggesting that it is unrelated or only partially related to topographic cross-talk. Furthermore, contrast scales linearly with probing voltage, consistent with its (linear) electromechanical (e.g. piezoelectric or flexoelectric) origins. Based on these observations, we analyse the possible origins of observed signal and summarize the requirements for quantitative studies of electromechanical activity in liquids.

Local quantitative mapping of electromechanical activity in liquid environment requires us to

1. concentrate the probing ac field,
2. concentrate and/or control local electrochemical potential through the dc field,
3. detect local surface displacement induced by ac potential (electromechanical response),
4. separate electromechanical response from electrostatic capacitive interactions, and
5. avoid or separate topographic and elastic cross-talk that can contribute to the measured signal.

The theory of PFM, including electrostatic and strain field structure induced by the probe, resolution theory, local and non-local contributions to signal, surface–tip signal transfer and its frequency dispersion, are well studied in the context of ambient PFM [44]. Below, we briefly discuss principles of PFM imaging in ambient environment, and analyse some of the factors that can affect imaging in liquids.

4.1. Force interactions in liquid and air

The key element of any scanning probe microscopy (SPM) method is the mechanism of tip–surface interactions that determine the strength of the force signal detected by the cantilever, the locality of the imaging, and the
primary response mechanism. In the context of PFM, primary interactions include electrostatic interactions and electromechanical coupling in the material and electric double layers. The experimentally measured piezoresponse amplitude is \( A = A_{el} + A_{piezo} + A_{amb} \), where \( A_{el} \) is the electrostatic contribution, \( A_{piezo} \) is the electromechanical contribution and \( A_{amb} \) is the non-local contribution due to capacitive cantilever–surface interactions [45, 46]. Quantitative PFM imaging requires \( A_{piezo} \) to be maximized to achieve predominantly electroelastic contrast. The origins of electromechanical contrast and its relationship to local materials properties for piezoelectric materials have been extensively studied [47, 48]. Piezoelectric coupling is specific to materials, and thus is not affected by the environment. At the same time, electrostatic force interactions are strongly mediated by the medium.

In ambient environment or in vacuum, the tip interacts with the surface through long-range electrostatic forces, \( F_{el} = C_{\text{surface}}(V_1 - V_2)^2 \), where \( V_1 \) is the tip potential, \( V_2 \) is the surface potential, and \( C_{\text{surface}} \) is the tip–surface capacitance gradient. In the low frequency limit, the piezoresponse (PR) signal, i.e. the first harmonic component of tip oscillations induced by periodic bias applied to the tip, is

\[
P_R = a_n(h) \left( \frac{k_1}{k_1 + k} \right) + \left( C_{\text{cone}} + \frac{C_{\text{surface}}}{k_1 + k} \right)(V_{dc} - V_s),
\]

where \( a_n(h) \) is the effective electromechanical response of the material, \( a_n(h) = \frac{V_1}{\lambda_D} \), is the ratio of the ac tip potential to the ac surface potential (i.e. the potential drop in the tip–surface gap), \( k_1 \) is the spring constant of the tip–surface junction, \( k \) is the spring constant of the cantilever, and \( C_{\text{cone}}, C_{\text{surface}}, C_{\text{cone}} \) are capacitance gradients due to the spherical and conical parts of the tip and the cantilever. \( V_{dc} \) is the dc potential offset of the tip bias, and \( h \) is the tip–surface separation. In ambient conditions \( a_n(h) \) is for \( h < 0 \) (contact), i.e., the response is independent of penetration depth, and \( a_n(h) \) is for \( h > 0 \) (non-contact), giving rise to well known limits of PFM and Kelvin probe microscopy.

In liquid, the PFM contrast is strongly mediated by the presence of mobile ions that screen electrostatic tip–surface interactions [36]. For the sphere–plane system [49],

\[
F_{el}(z) = \frac{\varepsilon_0 R 2V_1 V_2 \exp(h/\lambda_D) - (V_1^2 + V_2^2)}{\exp(2h/\lambda_D) - 1}.
\]

Electrostatic interaction in liquid is short range and decays exponentially for \( h > \lambda_D \). Thus, the PFM signal in liquid (for \( h > 0 \)) is

\[
P_{R_{el}} = \alpha(h) d_{el} \left( \frac{k_1}{k_1 + k} + \frac{\varepsilon_0 R 2V_1 \exp(h/\lambda_D)}{\lambda_D \exp(2h/\lambda_D) - 1} \right),
\]

where the first term is the piezoelectric response, \( k_1 \) is the tip–surface spring constant in liquid, and the second term is the response due to the electrostatic coupling in the double layer. The contribution from the conical part of the tip and cantilever are absent for \( R \gg \lambda_D \). The screening coefficient \( \alpha(h) = 1 \) for \( h < \lambda_D \), i.e. when the tip touches the surface, and \( \alpha(h) = 0 \) if the electric double layers around the tip and the surface do not overlap. Thus, the electromechanical response in solution gradually decays at distances of the order of the Debye length of the solution, and the electrostatic contribution is significantly minimized compared to ambient or vacuum imaging.

### 4.2. Ac and dc field concentration

The second key component of the PFM imaging is the concentration of the ac and dc field in the tip–surface junction and detection of local response. Notably, simple observation of high-resolution contrast is not enough to demonstrate the high localization of an ac electric field. Rather, the locality of the strain transfer through the mechanical tip–surface contact is a sufficient explanation. In the absence of additional information, the relative size of electrical (ac field region) and mechanical contact cannot be established and the spatial resolution provides only an estimate of the lower of the two.

The distribution of the dc field however can be probed directly by the polarization switching experiments in the liquid environment. A transition of ferroelectric switching behaviour from localized to uniform switching depending on the choice of the solvent established that imaging is possible at conductivities far larger than allowed for localized switching [50]. Furthermore, these results illustrated the degree to which the spatial extent of a dc field can be controlled in solution. Application of local dc pulses using conventional metal coated cantilevers is possible only in less conductive non-aqueous solvents such as isopropanol or methanol.

### 4.3. Contact mechanics

The third element determining PFM response is the signal transfer from material to the tip, determined by the cantilever spring constant, \( k \), and tip–surface spring constant, \( k_1 \). The importance of variations of effective spring constant on signal directly follows from equation (1), in which variations in local stiffness couple to electromechanical signal through the capacitive forces. This coupling will be the origin of both topographic and elastic cross-talks through variations in tip–surface contact stiffness.

These effects can be analysed using a simple Hertzian model for the tip–surface contact. The relationship between the indentation depth, \( h \), tip radius of curvature, \( R_0 \), and load, \( P \), is [51]

\[
h = \left( \frac{3P}{4E^*} \right)^{1/3} 2R_0^{1/3},
\]

where \( E^* \) is the effective Young’s modulus of the tip–surface system. The contact radius, \( a \), is related to the indentation depth as \( a = \sqrt{\pi} R_0 \). The contact stiffness is \( k_1 = (\partial h/\partial P)^{-1} \), and from equation (42) \( k_1 = 2aE^* \), or

\[
k_1 = 2E^* \sqrt{h R_0} = (6P R_0^2)\frac{1}{4}.
\]

Shown in figure 7(a) is the distance dependence of the electrostatic and electromechanical contributions to the PFM signal calculated for \( R = 50 \) nm, \( V_{dc} = 0.1 \) V, \( E^* = 100 \) GPA, \( d_1 = 50 \) pm V^{-1}, and \( k = 1 \) and 40 N m^{-1}. The distance dependence of the capacitive tip–surface forces and tip-induced surface potential was calculated neglecting the changes in the sphere area due to contact. The electrostatic
Figure 7. (a) Indentation depth dependence for electromechanical (blue) and electrostatic (red) contributions for a cantilever with $k = 1 \text{ N m}^{-1}$ (solid) and $k = 40 \text{ N m}^{-1}$ (dashed). (b) Indentation depth dependence as a fraction of the electromechanical contribution.

contribution decreases rapidly with penetration depth due to changes in the tip–surface stiffness constant. In comparison, shown in figure 7(b) is the relative electromechanical contribution depending on penetration depth. Note that in the Hertzian model the electromechanical signal dominates for a penetration depth of $\sim 1 \text{ Å}$, corresponding to a contact radius of the order of $\sim 2$ nm for $R = 50$ nm, imposing a limit on the spatial resolution of the technique.

The analysis becomes more complicated if adhesive effects are taken into account. In this case, the contact mechanics are described by the Johnson–Kendall–Roberts (JKR) model, applicable for ambient conditions. In the JKR model, the contact radius is

$$a^3 = \frac{R_0}{E^*}(P + 3\sigma \pi R_0 + \sqrt{6\sigma \pi R_0 P + (3\sigma \pi R_0)^2}), \quad (6)$$

where $\sigma$ is the work of adhesion, $P$ is the load and the indentation depth is

$$h = \frac{a^2}{R_0} \left[ 1 - \frac{2}{3} \left( \frac{r_0}{a} \right)^{3/2} \right]. \quad (7)$$

where $r_0^3 = 6\sigma \pi R^2 E^*$ is the contact radius at zero force. Shown in figure 8(a) are force versus indentation depth curves calculated for $\sigma = 0$ (Hertzian), $10^{-3}$, $10^{-2}$, $10^{-1}$, and
1 J m$^{-2}$. Shown in figures 8(b) and (c) are corresponding contact stiffnesses. Note that adhesive contact results in a rapid change of contact stiffness from zero to the value corresponding to contact, resulting in a well defined boundary between free and bound cantilevers. Finally, shown in figure 8(d) is the force dependence of the contact area. Even for a small work of adhesion, the contact radii at zero force are relatively large, of the order of nanometres. Corresponding contact stiffnesses are of the order of 100–1000 N m$^{-1}$, well above the typical spring constant of the cantilever.

Thus, for most cantilevers (0.01–50 N m$^{-1}$), jump-to-contact instability in air distinguishes the regions with $k_1 \ll k$ (free air, $k_1 = 0$) and $k_1 \gg k$ (in contact $k_1 \sim 1000$ N m$^{-1}$). These regions correspond to the purely electrostatic interaction in Kelvin probe force microscopy (KPFM), and predominantly electromechanical contrast in PFM. Conversely, in liquid the lack of capillary forces and reduced magnitude of van der Waals (vdW) forces suggests that $k_1$ can vary continuously when approaching the surface. Hence, there is no well defined boundary between predominantly electromechanical and purely electrostatic contrasts, and hence PFM and KPFM regimes are not well defined. Notably, similar behaviour is anticipated for the non-contact AFM and KPFM in ultrahigh vacuum.

In both Hertzian and JKR models, the transition to the predominantly electromechanical contrast occurs for contact areas larger than a certain critical value. From equation (1), when non-local electrostatic components are negligible, this condition can be generalized for materials with arbitrary properties as

$$a > a^* = \frac{C_{\text{sphere}}(V_{dc} - V_s)}{2\alpha(h) d_3 E^*},$$

where $a^*$ is the critical contact radius corresponding to equality of the electrostatic and electromechanical contributions to the signal. For classical ferroelectric materials (100 GPa, 50 pm V$^{-1}$), this condition becomes $a > a^* = 5(V_{dc} - V_s)$ A V$^{-1}$. For soft materials (10 GPa, 5 pm V$^{-1}$), $a > a^* = 50(V_{dc} - V_s)$ nm V$^{-1}$.

From this simple estimate, the resolution of PFM on hard materials can potentially achieve sub-nanometre resolution provided that the electrostatic contribution to the signal is minimized. For soft systems with small response coefficients ($\sim 1–100$ pm V$^{-1}$), the signal is likely to represent the convolution of electrostatic and electromechanical contributions. At the same time, for systems with large electromechanical responses (i.e. many cells have $\sim 10^3$ higher electromechanical coupling than inorganic piezo-and ferroelectrics), electromechanical response can be expected to be observable directly.

4.4. Topography effect (cross-talk)

During imaging of multiphase materials, or objects with high aspect ratios, care must be taken to minimize topographic and elastic cross-talk with the electromechanical response through electrostatic interactions and variations in contact stiffness. These effects are expected to be particularly important when imaging at frequencies close to contact resonances of the tip. In this case, even minute changes in local properties and topography can drive the system through the resonance, with associated 180° change in phase response and spurious changes in the amplitude. From the structure of equation (1), the following contributions to the cross-talk signal in PFM can be differentiated:

- variations of long-range electrostatic interactions due to capacitive terms dependence on topography: $C_{\text{sphere}} + C_{\text{cone}}$ and local work functions, $V_s$;
- variations of effective electromechanical response with topography, $d_{33}$;
- variation of electrical contact quality, $\alpha(h)$;
- variations of local contact stiffness, $k_1$.

Topographic effects on scanning Kelvin probe microscopy, a non-contact (weakly dependent on interaction area), lift-mode technique, have revealed that forces are larger within topographic depressions, but that these relatively weak long-range effects often show up as offsets in the signal [52]. Therefore, capacitive terms are unlikely to produce sharp cross-talk contrast on the 1–100 nm length scale.

The detailed studies of the voltage-dependent contact mechanics of piezoelectric materials, as well as simple dimensionality arguments, suggest that electromechanical response is only weakly dependent on surface topography. The electrical contact quality is expected to be only weakly dependent on contact area for a good contact (fringing field in capacitor), but is highly sensitive to surface contamination. However, contamination is expected to be detectable directly from the apparent topographic image and, in the absence of such information, its presence cannot be established independently.

Finally, the most significant contribution to PFM cross-talk is the effect of topography on contact stiffness, resulting in strong topography-related signal in the resonance-enhanced PFM. This will result in the fine structure on the image, since the resolution is determined by the tip–surface contact area and large-scale variations in topography do not contribute to signal. The magnitude of this effect is studied in detail elsewhere, and it is shown that due to the specificity of the PFM driving mechanism, the stable imaging in the vicinity of the resonance can be achieved only using fast sweeps, sampling a segment of the amplitude–frequency curve in the vicinity of the resonance [53]. This limitation is especially pronounced for weakly piezoelectric materials such as calcified and connective tissues with small (1–5 pm V$^{-1}$) coupling coefficients. The detailed analysis of PFM cross-talk and optimal operation regimes will be reported later.

5. Summary

We have studied in liquid the electromechanical properties of a variety of biological systems, including amyloid insulin fibrils, carcinoma cells, and purple membranes, as well as a reference ferroelectric surface. The model PPLN system illustrates high veracity electromechanical contrast in ambient and in liquid, clearly associated with ferroelectric domains and observable even for relatively small ($\sim 12$ pm V$^{-1}$) electromechanical response. For all biological systems, strong amplitude and phase contrast are observed in the PFM image. These data typically illustrate a level of detail exceeding the topographic
resolution, and capacity for detection of signal even from small objects invisible on the topographic image. The PFM contrast can originate from a variety of sources, including local electromechanical response, variations in contact stiffness due to topography and elasticity, and electrostatic forces. In many systems, these sources of contrast cannot be distinguished unambiguously. However, studies on the purple membranes clearly demonstrate the difference in electromechanical response between the cytoplasmic and extracellular sides. This behaviour is anticipated for the membrane with built-in dipole moment. Furthermore, these results suggest that imaging with molecular resolution in biological systems is feasible with metal-coated probes.

The significant bottleneck for future electromechanical studies in liquid is the need to concentrate both ac and dc electric field produced by the probe. This is required both to improve the resolution and to minimize the stray currents in liquid and electrochemical reactions. This necessitates the development of shielded AFM probes, as suggested by a number of groups [54–56].

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