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Kelvin probe force microscopy in application to biomolecular films: Frequency modulation, amplitude modulation, and lift mode

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ABSTRACT

Kelvin probe force microscopy (KPFM) is a powerful technique to visualize the differences of work function in metals and lateral surface potential distribution in thin organic films. Earlier we have shown that frequency modulated-Kelvin probe force microscopy has significant advantages in both sensitivity and resolution when applied to metal and inorganic interfaces in vacuum. High resolution, high sensitivity, and performance in ambient conditions are required in order to study biologically relevant samples. In this work we compared the resolution of frequency modulation (FM-KPFM), amplitude modulation (AM-KPFM), and lift modes KPFM for imaging the local electrical surface potential of complex biomolecular films and demonstrated that FM-KPFM mode has superior resolution for biological applications. The power of the method was illustrated on pulmonary surfactant films, revealing nm spatial resolution and mV potential sensitivity in ambient air. At this level of resolution this method can provide critical insight into the molecular arrangement and function of complex biosystems in a way that other KPFM modes cannot do. Based on the observed changes in the local surface potential we discovered that excess cholesterol produces nm size electrostatic domains and change the electric fields.

1. Introduction

The local electrical surface potential in a heterogeneous biomolecular film is a direct reflection of its molecular-level structure. Mapping the electric potential on the nanometer scale provides a highly localized glimpse of the function of these complex materials in a view that is not possible with other scanning probe or molecular identity techniques. Kelvin probe force microscopy (KPFM) is a scanning probe technique that is capable of measuring the local distribution of contact potential difference (CPD), which is a measure of the electrical surface potential or work function of the sample. KPFM is carried out by applying a voltage to a conducting AFM probe. Scanning the probe across the structure of interest produces a map of the electrostatic interaction at each point of the sample. Commonly used KPFM methods, such as "lift mode", have significant disadvantages such as low resolution, low sensitivity, and inability to perform AFM and KPFM imaging simultaneously. Moreover, the resolution and performance of KPFM methods are highly affected by the environment [1,2]. As a result, existing KPFM methods have limited or no applications in biological research as biological samples require high resolution and high sensitivity at the same time due to very small electrostatic signals, and these samples cannot be studied in vacuum.

All KPFM methods operate by applying an AC voltage (100–500 mV) to a scanning probe at a frequency (f_{mod}). If the CPD ($\Delta\varphi$) between the tip and sample differ, then the AC voltage induces mechanical oscillations proportional to the tip–surface electrostatic interaction strength. A feedback loop applies an additional DC voltage to the probe until the mechanical oscillations induced by electrostatic interactions are compensated. When the oscillations are exactly nullified, this voltage corresponds to the surface potential of the sample. The total voltage applied to the cantilever (as derived by [1]) is given by

$$U = U_{dc} - \frac{\Delta\varphi}{e} + U_{mod}\cos(2\pi f_{mod} t) \quad (1)$$

and the electrostatic force that the tip experiences is given by

$$F = \frac{1}{2}\frac{\partial C}{\partial z}U^2 \quad (2)$$

Abbreviations: AFM, atomic force microscopy; FM-KPFM, frequency modulated-Kelvin probe force microscopy; AM-KPFM, amplitude modulated-Kelvin probe force microscopy; CPD, contact potential difference; PS, pulmonary surfactant

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which can be expressed in three terms as

$$F = \frac{1}{2}\left[\left(U_{dc} - \frac{\Delta\varphi}{e}\right)^2 + \frac{1}{2}U_{mod}^2\right]\frac{\partial C}{\partial z} + \frac{\partial C}{\partial z}\left(U_{dc} - \frac{\Delta\varphi}{e}\right)U_{mod}\cos(2\pi f_{mod}t) + \frac{1}{4}\frac{\partial C}{\partial z}U_{mod}^2\cos(2\times 2\pi f_{mod}t). \quad (3)$$

Note that the electrostatic force is minimized when the DC voltage is equal to the surface potential, or work function $\Delta\varphi/e$. Scanning the probe across the surface of interest produces a map of the electrostatic interaction at each point of the sample.

Although lift mode is the simplest implementation of KPFM, it involves several disadvantages when imaging biological samples. It takes two passes to make topography and potential scans. The first pass determines the surface topography, and the return trace measures the electrostatic interactions by raising the tip (~50 nm) above the measured topography of the first trace while applying KPFM feedback. This method ensures that only the long-range electrostatic interactions are measured, and at equal heights above all areas of the surface. Raising the tip away from the surface eliminates cross talk between topography and surface potential measurements, at the expense of decreasing lateral resolution with increasing tip–sample separation. Furthermore, the topography measurement does not decouple the electrostatic information, which can drastically affect topography measurements. Additionally, the entire cantilever contributes to the measured electrostatic forces, which greatly reduces the sensitivity of the measurement [2,3].

Amplitude modulation (AM-) KPFM improves upon lift mode by simultaneously measuring surface topography and KPFM feedback on orthogonal frequencies. An off-resonant KPFM frequency reduces cross talk with topography, and unlike lift mode, the topography and surface potential are completely decoupled since they are simultaneously measured. The largest advantage that AM-KPFM provides over lift mode is the ability to measure the surface potential very close to the surface, which greatly improves the lateral resolution of electrostatic forces. However, AM-KPFM requires a soft cantilever (1–5 N/m) and a low resonant frequency (~70 kHz) as it is not very sensitive to the electrostatic potential [1]. In UHV experiments, this difficulty is overcome by using the first overtone ($f_1 = 6.28 * f_0$) frequency. Since the quality factor (Q) of a cantilever in vacuum is very high (~10^5), this greatly enhances the sensitivity of the measurement. In contrast, the Q factor of a cantilever in air is an order of magnitude smaller.

FM-KPFM overcomes these deficiencies of other Kelvin methods by measuring the force gradient of the electrostatic force [2,4]. The cantilever is mechanically oscillated at f_0 and electrically oscillated at f_{mod} with non-contact feedback. When the tip experiences electrostatic interactions, frequencies f_0 and f_{mod} mix to produce side bands at $f_0 \pm f_{mod}$ (see Fig. 1). FM-KPFM overcomes all the disadvantages of other Kelvin set-ups by tracking the electrostatic signal of interest at these side band frequencies. These side bands in FM-KPFM are very sensitive to the electrostatic forces and can be separated from the main frequency f_0 and any noise bands or other disturbing resonances. Moreover, as FM-KPFM records the force gradient rather than the electrostatic force [1], the largest sensitivity stems from the tip apex, avoiding any contribution from the tip shaft and cantilever beam (which reflect DC components in Kelvin signal). FM-KPFM allows simultaneous recording of topographic and local surface potential images with very high resolution and sensitivity, reaching quantitative values even below 1 nm resolution [1]. In FM-KPFM operation, it is possible to use cantilevers with high spring constants (~42 N/m), causing it to be less affected by humidity factor and allows high spatial resolution.

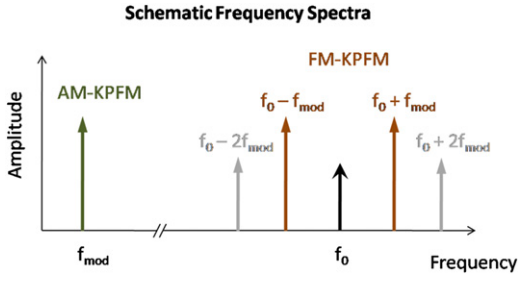

Fig. 1. Schematic frequency spectra used in FM-KPFM.

2. Materials and methods

2.1. Samples preparation

Pulmonary surfactant Bovine Lipid Extract Surfactant (BLES) is a hydrophobic extract of bovine lung lavage that differs from natural surfactant in the lack of surfactant specific proteins SP-A and SP-D and cholesterol. Pulmonary surfactant BLES is a lipid–protein mixture where phosphatidylcholines (PC) represent 80% of its mass with half of the PC being the disaturated dipalmitoyl-phosphatidylcholine (DPPC). 5–10 mass% is the negatively charged phosphatidylglycerol (PG), and two hydrophobic surfactant-associated proteins (SP-B, SP-C). BLES in non-buffered normal saline (pH 5–6) with a phospholipid concentration of 27 mg/ml was a kind gift by the manufacturer (BLES Biochemical Inc. of London, Ontario). Cholesterol was purchased from Sigma Chemicals, St. Louis, MO. In order to add desired amount of cholesterol a solution of 1:1:1 ratio of methanol, chloroform, and BLES by volume was first vortexed and then spun in a centrifuge at the speed 100g for 5 min. The methanol/water phase was discarded and the BLES in chloroform was retained and either 0% or 20% of cholesterol (by mass) with respect to phospholipids in chloroform was added. Each solution was then dried under N_2 and re-suspended with Goerke's buffer (140 mM NaCl, 10 mM Hepes, and 2.5 mM $CaCl_2$; pH 6.9) or chloroform to obtain solution of BLES at a concentration of 27 mg/ml phospholipids, containing 0% or 20% of cholesterol. BLES was spread on a NIMA Langmuir–Blodgett trough, compressed at 47 mN/m, and deposited on freshly cleaved mica substrates by Langmuir–Blodgett deposition for imaging.

2.2. KPFM imaging

KPFM images were collected in air using NanoWorld cantilevers with spring constant 42 N/m with the samples grounded. All lift mode KPFM images were recorded on the JPK Nanowizard II. AM-KPFM data was recorded from using Agilent Technologies SPM/AFM-5500. FM-KPFM mode images were collected using a home-built setup, developed at Technical University Dresden [1]. It comprises a home-made AFM, operated in true non-contact mode using a Nanosurf easy PLL, with addition of Scientific Instruments SR830 and SR844 lock-in models, and conductive Chromium/Platinum tips. Constant tip–sample separation is controlled by the phase-locked loop (PLL) by maintaining a constant frequency shift during scanning (and constant phase difference between tip excitation and detected resonance). The internal reference of lock-in amplifier 2 is the applied AC voltage to the surface (3 kHz). The deflection signal is supplied to lock-in amplifier 1, which down-mixes the signal using the resonant

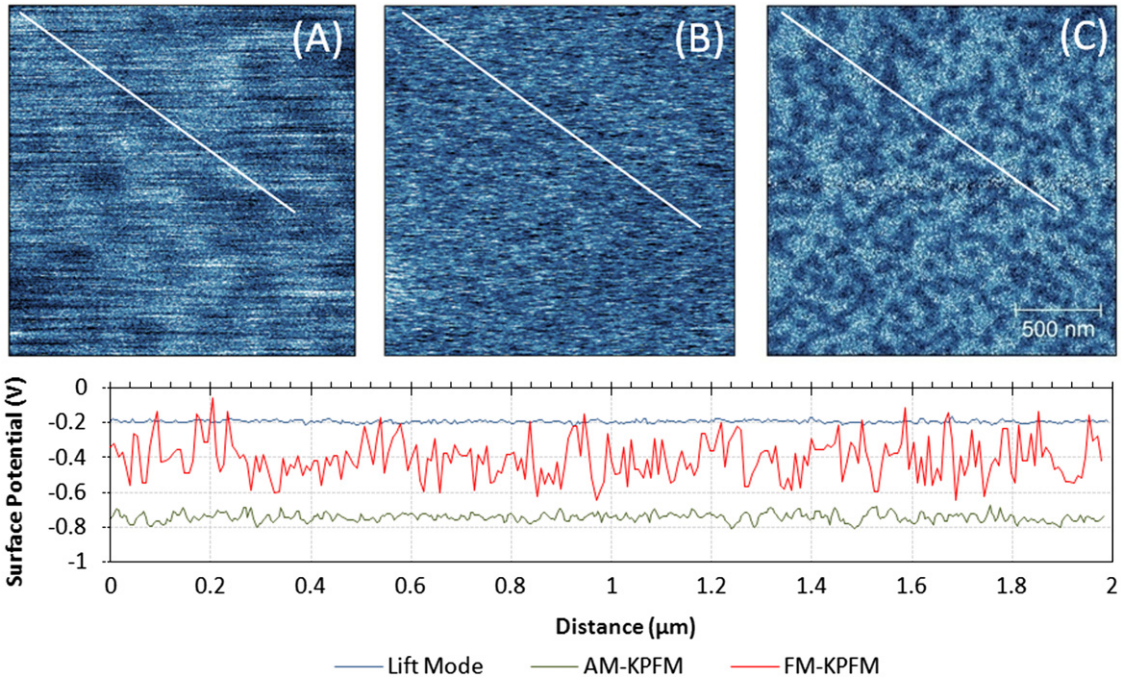

Fig. 2. Kelvin probe force microscopy images of BLES with 20% cholesterol in (A) lift mode, (B) amplitude modulation AM-KPFM, and (C) frequency modulation FM-KPFM. All images are 2×2 μm in scan size.

excitation frequency (Fig. 1). The mixed signal is supplied to lock-in amplifier 2, which uses the internal reference to demodulate the side bands at frequencies $f_0 \pm f_{mod}$.

3. Results and discussion

We demonstrated earlier the superior resolution of FM-KPFM over other KPFM methods by deducing the work function in metallic and inorganic surfaces in vacuum [1]. To demonstrate its great advantages for imaging biological samples, we adapted a FM-KPFM method for mapping the local electric surface potential of pulmonary surfactant (PS) films in ambient air.

PS forms a lipid–protein molecular film at the interface of the lung's epithelia, providing stability to the alveolar structure, reducing the work of breathing, and serving as the first barrier to inhaled nano-sized particles (NSPs). NSPs are found in the ambient polluted air and pose a major health threat [5], on the other hand NSPs are prospective systems for aerosolized drug delivery to the lung. Both man-made and natural particles are invariably electrically charged [6], and may be affected strongly by electrostatic forces at the air–lung interface. Functional PS films show a laterally highly structured surface morphology (multilayered structures) [7,8], suppressed by cholesterol excess (20% w/w), which has been associated with surfactant inhibition in adult respiratory distress syndrome [8,9].

Fig. 2 compares the three KPFM modes with images of pulmonary surfactant BLES with excess of cholesterol (20% by weight). Lift mode (Fig. 2A) and AM-KPFM (Fig. 2B) show low resolution and low surface potential sensitivity, as the signal to noise ratio is insufficient to observe electrostatic domains. FM-KPFM , however, offers superior signal to noise ratio, and provides lateral resolution capable of localizing nm scale electrostatic domains within the surfactant. FM-KPFM allowed us to achieve high resolution and revealed clearly small 10–50 nm electrostatic domains in the surfactant monolayer, produced by cholesterol, which cannot be well resolved by other KPFM modes. BLES monolayer containing no cholesterol shows mostly unstructured local surface potential (image not shown). Excess of cholesterol is related to surfactant inhibition and has been shown to reduce adhesion, and elasticity of the film. FM-KPFM showed that cholesterol also alters the electrical potential in the surfactant film, which is intimately related to its function, and impacts immediately on the manner in which charged species interact with pulmonary surfactant film. This example application of FM-KPFM method to pulmonary surfactant illustrates a key point of central importance: that a previously unknown phenomenon (that cholesterol excess changes surface potential distribution in PS films) can be visualized with this high resolution FM-KPFM technique, which may lead to a new understanding of the mechanism of surfactant structure and function, as well as how the inhalation of charged airborne particles interact synergistically with these cholesterol-laden lung surfactants providing a new explanation for the way pollution causes respiratory disease.

4. Conclusions

In summary, electrostatic interactions are the driving forces in many biological processes. Knowing the nano-scale electrostatic potential in bio-films is a crucial function-related characteristic without which the important link between the structure and function of the biomolecular film in vivo might be missed. High resolution imaging techniques such as FM-KPFM, which sense electrostatic forces at high resolution and high sensitivity, are seminal to studying electrostatic properties providing these crucial data.

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