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Kelvin-probe force microscopy of the pH-dependent charge of functional groups

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Kelvin-probe Force Microscopy (KFM) is an established method to map surface potentials or surface charges at high, spatial resolution. However, KFM does not work in water, which restricts its applicability considerably, especially when considering common, functional chemical groups in biophysics such as amine or carboxy groups, whose charge depends on pH. Here, we demonstrate that the KFM signal of such groups taken in air after exposure to water correlates qualitatively with their expected charge in water for a wide range of pH values. The correlation was tested with microcontact-printed thiols exposing amine and carboxy groups. Furthermore, it was shown that collagen fibrils, as an example of a biological material, exhibit a particular, pH-sensitive surface charge pattern, which could be caused by the particular arrangement of ionizable residues on the collagen fibril surface. © 2016 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

The ionization state of functional, chemical groups is of paramount importance in a vast range of natural processes and technical applications, ranging from molecular biology1–2 over surface treatments3,4 to nanoparticle technology.5,6 It is usually determined by zeta-potential measurements, titration, or similar methods.5,7 However, such methods do not provide spatial resolution. A possible approach to determine the ionization state could be Kelvin-probe Force Microscopy (KFM),8 a variant of Atomic Force Microscopy (AFM). However, KFM, while well-established in areas such as semiconductor physics, cannot be applied in water because a bias voltage is applied to a conductive AFM tip in KFM.9 The measurement principle is, usually, to perform two consecutive scans of the same scan line, the first scan providing the sample topography and the second scan a profile of the local surface potential.9 The DC-component of the bias voltage is then readjusted by a controller so that it matches the local surface potential. Because of this DC-voltage, conventional KFM measurements can only be performed in a non-conductive, non-polar medium (air, vacuum, non-conductive liquids),10 which is not the “natural” environment of, for example, biomolecules.

A promising variant which omits the DC-voltage, Open-Loop KFM, was introduced a few years ago and used to determine the surface potential of molecule layers and nanoparticles in water.11,12 However, these experiments were conducted in simple electrolyte solutions of relatively low molarity (up to 10 mM) and have yet to be applied to biomolecular systems under physiological-like conditions at higher molarity.

Despite this restriction, an increasing number of studies has been published, in which KFM is used in air to determine the surface potential of biomolecules deposited on a solid surface.13–20 However, it is by no means obvious that the KFM signal measured in air, that is, after drying the sample, reflects the surface charge in water. For example, effects such as counterions deposited on the sample or the ambient air humidity during the KFM measurement could influence the signal.21 Nevertheless, it is conceivable that, in carefully controlled, simple cases, there is indeed a correlation between the charge of a surface in water at given pH and the KFM signal in air.

To test this hypothesis, we prepared test samples, which expose amine (NH2) and carboxy (COOH) groups at the surface, immersed them in water at various, defined pH values, and then dried and measured their KFM signal in air at relative humidity of ca. 30% (Figs. 1(a)–1(d)).33 NH2- and COOH-groups stand for the most important ionizable groups in biomolecules and also various applications in surface technology.

As KFM can only determine relative variations of the surface potential, we used microcontact-printing with thiol-coated polydimethylsiloxane (PDMS) stamps to produce micrometer-sized patterns of surface-exposed NH2- and COOH-groups (Figs. 1(a) and 1(b)),22 which are ionized into NH3+- and COO−-groups to a varying degree, dependent on pH and their respective acid dissociation constants (pKas).23 The pKas of a chemical group vary depending on its position in a molecule and its immediate, molecular environment. A pK of 6.5 was reported for NH2-(CH2)11-SH monolayers on Au and a pK of 7.4 for COOH-(CH2)11-SH monolayers on Au by surface plasmon resonance spectroscopy.24 For the shorter chain length we used in the present work, Marmisolle et al. recently reported a pK of NH2-thiol (6-amino-1-hexanethiol) of approximately 7 for monolayers on Au measured by titration and impedance spectroscopy.25 Thus, we can reasonably assume that our patterns have similar pKas, which means that, in terms of their ionization state, we would expect predominantly positively charged NH3+-groups at low (acidic) pH and predominantly neutral NH2-groups at high (basic) pH. Likewise, we would expect predominantly negatively charged COO−-groups at high pH and predominantly neutral COOH-groups at low pH.
Figure 2 shows an example of a printed NH₂-thiol pattern backfilled with CH₃-thiols (a)–(c) and typical examples of printed CH₃-thiol patterns backfilled with NH₂-thiols (d)–(f) and COOH-thiols (g)–(i), respectively. All images were taken in air, and the pH-values indicated are those of the solution the samples have been exposed to before imaging. The expected pH-dependent behavior of NH₂- and COOH-groups can be seen: At acidic pH, a more positive KFM signal was observed for NH₂-thiols compared to neutral pH (Fig. 2(e) vs Fig. 2(f)), which reflects the fact that NH₂-groups are more likely to be ionized into NH₃⁺-groups under acid conditions. Similarly, for COOH-thiols, the KFM signal at acidic pH is less negative compared to neutral pH (Fig. 2(h) vs Fig. 2(i)), which indicates that COOH-groups are more likely to be protonated at acidic pH.

In order to determine how common sample treatments and pH affect KFM measurements, we investigated the repeatability upon cycles of immersion and drying (Figs. 3(a) and 3(b)), the pH-dependence of the KFM signal (Figs. 3(c) and 3(d)), and the effect of immersion in solutions with large pH-variations (Figs. 3(e) and 3(f)). We made sure that, by using topographical landmarks, approximately the same sample area was chosen for repeated immersion-measurement cycles in order to minimize variations of the KFM signal due to a possible, spatially inhomogeneous thiol distribution.

Figures 3(a) and 3(b) show that, for both thiols, repeated immersion in solutions of the same pH does not significantly alter the measured KFM signal. The data also show that the KFM signal remains distinguishable between acidic and neutral pH. That is, the mere act of immersion and drying does not alter the ionization state of the groups significantly, which indicates that possible counterion deposition from the buffers during the drying-step has only negligible effect.

To confirm our hypothesis that the KFM signal reflects the charge of the ionizable groups specifically at the pH of the solution to which they have been exposed immediately prior to KFM measurements, we varied the pH from 2 to 10. As predicted, the KFM signal shows the typical charge-vs-pH behavior of NH₂- and COOH-groups (Figs. 3(c) and 3(d)). NH₂-thiols always appear positively charged on average (the only possible states being NH₂⁺ ↔ NH₃⁺ + H⁺ to the right). COOH-thiols always appear negatively charged on average (the only possible states being COOH ↔ COO⁻ + H⁺ to the right).
The charge-vs-pH relation of a monoprotic acid-base dissociation in an ideal solution would normally be expected to follow a sigmoidal (i.e., S-shaped) pattern as described by the Henderson-Hasselbalch equation, which essentially reflects the chemical equilibrium between dissociated (here NH₂ or COO⁻) and non-dissociated groups (here NH₃ or COO⁻), and relates it to pH and pKₐ. However, the data in Figures 3(c) and 3(d) does not appear to follow such a pattern.

There could be many reasons for this: For example, the actual pH at the surface might not necessarily be identical to the measured one in bulk, or the measured surface potential might not be directly proportional to the number concentration of ionized groups on the surface. These observations would have to be investigated in more detail in future studies.

Figures 3(e) and 3(f), however, show that, when changing the pH widely, a “saturation” effect occurs. That is, for example, when going from pH4 to pH10 and back again, the KFM signal does not return to the original value measured at pH4.26 Fibrils were prepared from rodent tail tendon and KFM was performed, first after immersion of the sample in deionized water (Figs. 4(a)–4(c)) and then after re-immersion in a diluted HCl solution adjusted to pH2 (Figs. 4(d)–4(f)). It can be clearly seen in the KFM maps (Figs. 4(b) and 4(e)) and the KFM profiles (Figs. 4(c) and 4(f)) that, at neutral pH, the surface potential of the gaps is about 10 mV higher than that of the overlaps, whereas at acidic pH, the difference becomes negligible. The KFM signal is unlikely to be a mere artifact of cross-talk with the topography as, first, the topographic profile does not significantly change between pH7 and pH2, whereas the KFM signal does (Figs. 4(c) and 4(f)), and, second, the topography is very “shallow”, that is, the overlaps have a height of only a few nm, whereas their horizontal extent is approximately 30–40 nm. KFM-topography edge effect artifacts usually occur when there are “steep” features such as sidewalls on a sample, which is not the case here. These very preliminary results indicate a possible, inhomogeneous distribution of ionizable and accessible residues on the surface of collagen fibrils. This interpretation is corroborated by the fact that characteristic banding-patterns of charge-sensitive, heavy-metal fibrils. This interpretation is corroborated by the fact that characteristic banding-patterns of charge-sensitive, heavy-metal salts were observed on collagen fibrils.28

In conclusion, we have shown that KFM performed with dry samples can be used to qualitatively determine the ionization state of amine- and carboxy-groups on surfaces and that the KFM signal in air is related to the charge they have in solution. Obviously, in the present study, we only
investigated very well-defined and simple chemical structures as test samples. Future work would need to assess the influence of the earlier mentioned effects such as uncontrolled counterion adsorption, the exact charge-potential relationship, or the effect of ambient humidity in more detail. Especially, the time a sample was exposed to humid air between drying and the actual KFM measurement could cause a significant, unwanted alteration/dissipation of the charge of ionizable groups, for example, through adsorption/desorption of $\text{H}^+$-ions. Furthermore, the surface charge measured by KFM of dielectric solids such as oxides, sulfates, or phosphates has been shown to be clearly dependent on ambient humidity, which was attributed to the acid or base character of the surfaces and corresponding adsorption/desorption of $\text{H}^+$ or $\text{OH}^-$ ions from ambient water. These observations underpin our hypothesis and show that also the surface charge of other chemical groups (e.g., $\text{SiO}_2$) measured by KFM in the dry state is strongly dependent on the environmental conditions of the measurement.

Although the spatial resolution of KFM is not as high as that of AFM topography measurements, what makes KFM appealing is its extremely high sensitivity. This is because of the high force sensitivity of AFM cantilevers and the relatively high strength of electrostatic forces at nm distances. Employing KFM to detect and follow subtle charge alterations on surfaces could open up entirely new routes for functional imaging, especially in biophysics, a few examples being enzymatic reactions, glycation, histone-DNA interactions, or the influence of charged peptides on membranes.

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