Use of Self-Assembled Monolayers as Substrates for Atomic Force Imaging of Hydroxyapatite Crystals from Mammalian Skeletal Tissues

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Atomic force microscopy (AFM) has recently been successfully used to describe the surface topography of hydroxyapatite crystals from mammalian skeletal tissues. To further characterize the growth mechanisms of skeletal hydroxyapatite crystals and the role of adsorbed proteins in these processes, imaging under biological fluids is essential. However, under aqueous solutions, these crystals do not bind to the usual AFM substrate such as mica and graphite and therefore alternative substrates are necessary. The aim of the present study was to evaluate the use of self-assembled monolayer technology with controllable chemical functionality to provide "designer surfaces" for crystal binding in fluid environments which simulate the normal physiological milieu. We have found that hydroxyapatite crystals from developing enamel are bound most effectively by negatively charged self-assembled monolayer (COO− and SO3−) surfaces, demonstrating an important role for such substrates in AFM imaging of biological samples under aqueous fluids and suggesting that the prevalent charge on enamel crystal surfaces is positive.

Introduction

Atomic force microscopy (AFM) has provided unprecedented opportunities for the imaging of biological specimens, generating quantitative data in three dimensions with molecular resolution.1 AFM is ideally suited for biological imaging as specimens do not need to be dehydrated, fixed, stained, or coated, and most importantly, imaging can be carried out under fluids, maintaining the correct biological conditions.2-5 We have recently used the AFM to provide topological images of hydroxyapatite crystals from the mammalian skeleton,6 revealing putative kink and growth sites which may be important determinants in the mechanisms of crystal growth during mineralized tissue development.

The control of hydroxyapatite crystal deposition and growth is central to morphogenesis, morphostasis, and physical strength in the skeletal tissues. Precise control mechanisms remain obscure, but interaction between crystal domains and specific stereochemical arrays on extracellular matrix proteins have been implicated.7-9 The relationship between hydroxyapatite crystals and their corresponding matrix proteins and the role of the previously described topological features such as kink sites, steps, and grooves in the crystal surface is an area where the application of AFM under fluids could result in a significant step forward. This would not only permit the imaging of crystals under conditions approximating those in vivo but also permit direct observations of crystal growth to be as well as investigations of matrix protein-mineral interactions. In addition, chemical force microscopy (CFM) using AFM tips chemically modified by thiol self-assembly10-12 could also be used to provide more specific information in respect of surface charge properties of skeletal mineral crystals and binding strengths of interacting ligands. However, imaging biological samples under aqueous fluids is often difficult as the samples are frequently unstable and even detach from the surface or are disturbed by the scanning process. Hydroxyapatite crystals from developing enamel and bone are small (approximately 300 nm × 60 nm × 20 nm for enamel crystals and 15 nm × 10 nm × 5 nm for bone crystals) and high-resolution AFM images have so far only been obtained on mica in air or under organic solvents.6,13

Modification of a gold surface using thiol self-assembly14 can provide substrates with controllable chemical properties (hydrophobicity, predisposition for hydrogen bonding, or electrostatic charge) to which biological crystals may bind under aqueous fluids. Selection of an appropriately suitable crystal substrate is essential for high-resolution imaging. In the reported studies on hydroxyapatite crystals from developing bone,7-9 bone apatite crystals from developing tooth,10-12 and bone apatite crystals isolated from bone,13,16 the crystal substrates used were mica, graphite, or silicon oxide, and imaging was carried out in air or under organic solvents.

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terminated functional group would permit a designer substrate to be constructed at the molecular level which, in addition to binding the sample to the substrate, would also provide information in respect of the surface properties of the crystals themselves. The aim of the present study was to investigate the efficacy of a range of SAMs for binding hydroxyapatite crystals from developing enamel under aqueous conditions simulating those found in vivo. Four different thiol compounds, HS-(CH$_2$)$_2$-X (where X = -COOH, -OH, -NH$_2$ and -CH$_2$SO$_3$Na), were used as substrates for AFM imaging under a range of fluids and varying conditions of pH, and the stability of crystals on the surfaces during scanning was determined.

**Materials and Methods**

**Source of Material.** Individual crystals from the maturation stage of enamel development were obtained from mandibular incisors of 6 week old male Wistar rats following dissection from the mandibles as described previously by Hiller et al.$^{15}$

**Preparation of Crystals.** Particles of enamel were dissected from the maturation stage of developing rodent enamel as described above. All detectable traces of matrix protein were removed from the particles using a sequential extraction procedure as described by Robinson et al.$^{16}$ Briefly, enamel particles were first extracted with 0.1 M phosphate buffer (pH 7.4) to desorb mineral-bound and freely mobile components. The insoluble residue was pelleted by centrifugation and the supernatant removed. The pellet was re-extracted by resuspending in fresh phosphate buffer followed by further centrifugation. The pellet was extracted six times in total using the same procedure. The pelleted material was then further extracted but using 50 mM Tris containing 4 M urea at pH 7.4 to dissolve aggregated protein. The insoluble residue was then re-extracted for a further six times with 0.1 M phosphate buffer, pH 7.4, to ensure final desorption of any mineral-bound components. The final residue was washed with distilled water with the pH adjusted to 7.0 in order to remove all traces of buffer and urea. Removal of protein from the crystals was monitored by spectrophotometric determination of absorbance of the extract solutions at 216 nm and by polyacrylamide gel electrophoresis (SDS PAGE) of the extraction solutions. In addition, aliquots of the final crystal preparation were dissolved using 10% acetic acid to release any potentially residual proteins and the solutions subjected to SDS PAGE.

**Preparation of SAM-Modified Substrates.** Gold substrates were prepared by vacuum evaporation of gold (Goodfellow, 99.99% pure) onto glass slides to a thickness of 150 nm at an evaporation rate below 0.1 nm/s. To improve the adhesion of the gold to the glass, the glass substrates were prepared by vacuum evaporation of gold (99.99% pure) onto glass slides to a thickness of 150 nm at an evaporation rate below 0.1 nm/s. The gold substrates were then cleaned in hot "piranha" solution (30% H$_2$O$_2$/70% concentrated H$_2$SO$_4$ by volume) at 90 °C followed by rinsing with polishing water and then removed from the solution, washed carefully with HPLC-grade ethanol, blown dry with nitrogen, and imaged under the AFM immediately. Figure 1 shows a diagrammatic representation of the relationship between enamel crystals and SAM substrates. The illustration is not intended to imply the degree of order of the monolayer, which would be expected to be low given the short length of the alkyl spacers of the thiol compounds used in the study.

**Atomic Force Microscopy of Crystals.** AFM images of crystals were obtained both in air and under fluid using the Molecular Imaging picoSPM controlled Nanoscope IIIa electronics (Digital Instruments, USA). All images were obtained with a 40 μm x 40 μm scanner in contact mode using 200 μm Si$_3$N$_4$ cantilevers (Park Scientific Instruments, U.K.). All measurements were carried out at room temperature. Imaging under fluid was carried out using either neutral water (pH 7.4) or a physiological salt solution based upon the reported composition of the developing enamel fluid, pH 7.4$^{17}$ (see Table 1).

**Results**

**Imaging in Air.** Figure 2 shows typical AFM images of developing enamel crystals adsorbed on to the surfaces of the different SAM modified substrates obtained in air. All four SAM surfaces (-COOH, -OH, -NH$_2$, and -CH$_2$SO$_3$Na) showed adsorption of hydroxyapatite crystals which were stable under contact mode AFM imaging in air but the amount of crystals present on each of the substrates was very different. The carboxyl- and sulfate-terminated SAMs appeared to adsorb larger amounts of the mineral crystals compared with the amino and hydroxylated surfaces. For comparison, the AFM image
of developing enamel crystals adsorbed onto mica following previous preparation procedures is also shown in Figure 2.

**Enamel Crystal Stability under Fluid.** Figures 3 and 4 show typical AFM images of developing enamel crystals under neutral water and enamel fluid solutions, respectively. When compared with the results obtained in air (Figure 2), it is clear that the addition of water at
neutral pH (pH = 7.4) resulted in loss of crystals from the hydroxyl-terminated SAMs and that this loss became complete under enamel fluid. For the amino-terminated SAMs, no crystals were present under both neutral water (pH = 7.4) and enamel fluid (pH = 7.4). In contrast, the crystals present on the surface of both the carboxylated

Figure 3. AFM images obtained in neutral water of developing enamel crystals adsorbed on to different self-assembled monolayer-modified substrates and mica: A, COOH-terminated SAM; B, OH-terminated SAM; C, NH2-terminated SAM; D, CH3SO3Na-terminated SAM; E, mica. All images show 10 μm x 10 μm areas. Crystals were completely lost from the −NH2 terminated SAM (C) under water (* height scale = 50 nm).
and sulfated SAMs were stable for contact mode AFM imaging in both fluid environments. The AFM images obtained under fluids using mica as substrate indicated a significant loss of crystals under neutral water ($\text{pH} = 7.4$) and a complete loss of crystals under enamel fluid solution ($\text{pH} = 7.4$).

Figure 4. AFM images obtained in simulated enamel fluid ($\text{pH} = 7.4$) of developing enamel crystals adsorbed on to different self-assembled monolayer-modified substrates and mica: A, COOH-terminated SAM; B, OH-terminated SAM; C, NH$_2$-terminated SAM; D, CH$_2$SO$_3$Na-terminated SAM; E, mica. All images show $10 \mu \text{m} \times 10 \mu \text{m}$ areas. Crystals were most stable on –COOH- and –CH$_2$SO$_3$-terminated SAMs and were completely lost from the –OH- and NH$_2$-terminated SAMs and mica (* height scale = 20 nm).
Discussion

The results shown here clearly demonstrate that under conditions of neutral pH, developing enamel crystals remain stable for AFM imaging on carboxyl and sulfate functionalized SAM surfaces but are unstable on hydroxyl- and amino-terminated SAMs. The results of our force titration (a detailed discussion of these and other data is in preparation) agree with previous published data which show that the pKₐ values for charged groups at surfaces, such as we have with the SAMs here can be very different from those for the free groups titrated in solution. At the pH used in the present experiments (7.4) the conditions are above the surface pKₐ of the carboxyl (5.2) and sulfate (lower than carboxyl) groups and we may therefore assume that more than 50% of these moieties will be present in their dissociated forms providing a negatively charged surface. The hydroxyl-terminated SAM would be expected to provide a polar but uncharged surface and the amino-terminated SAM partially protonated (pKₐ near 10.0) and hence slightly positively charged surface.

Enamel crystals were not stable on the positively charged surface provided by the amino-terminated SAM when imaged under either water or "enamel fluid" but were stable on the negative surfaces of the carboxylated and sulfated SAMs under both fluid environments. This strongly suggests that there is a net positive surface charge on the developing enamel hydroxyapatite crystals. There are conflicting reports in the literature which claim both overall positive and negative surface charge for hydroxyapatite crystals, highlighting the need for further work in this area. The very nature of hydroxyapatite itself makes such determinations difficult in view of the frequency of heteroionic substitutions into the crystal lattice and its capacity to take up excess calcium and phosphate ions. The presence of a calcium-rich surface layer would support the findings reported here.

It is of interest to note that developing enamel crystals were not stable on cleaved mica surfaces when imaged in the AFM under fluid, despite the fact that mica is purported to be negatively charged. It is possible therefore that the charge density provided by the SAM surfaces may be an important consideration in crystal binding and may be a reflection of charge domains on the crystal surfaces. On the other hand, mica has a composite layer structure with the typical formula KAl₂Si₃AlO₁₀(OH)₂. freshly cleaved mica has a negative charge due to the presence of hydroxyl groups recessed slightly below the surface with spacing of 0.5 nm. Potassium ions bridge between the hydroxyl groups, or alkoxide ions, in adjacent layers. When immersed into an aqueous solution containing cations such as Mg²⁺ or Ca²⁺ (see Table 1), mica undergoes an ion exchange process in which the potassium atoms on the surface are replaced by the cations in solution. In this case, the Mg ions in solution which have a valence of 2+ can easily replace the potassium ions in the mica surface which have a valence of 1+. This would clearly affect the overall surface charge on the mica substrate.

The results obtained using the hydroxylated SAM-modified substrate presented a slightly more complex picture since the crystals were stable under water at neutral pH (albeit adsorbed at much lower concentrations compared with the negative SAM substrates) but were lost when the fluid environment was changed to enamel fluid at the same pH. We tentatively suggest that a weaker polar interaction and hydrogen bonding are responsible for the binding in water but that at higher ionic strengths in enamel fluid (ionic strength I ≈ 0.2 M) this weaker interaction may be destroyed or is reduced sufficiently to cause loss of the sample from the substrate when the AFM probe is applied during scanning.

In summary, we have shown that small hydroxyapatite crystals which cannot be imaged under aqueous fluids on common substrates such as mica or graphite can be effectively immobilized by using negatively charged self-assembled monolayers as modified substrates. In addition, this immobilization has no discernible effect on the AFM images of the samples and the surface of the mineral appears to carry a net positive charge surface. We have also demonstrated that the use of the "designer surfaces" at the molecular level can be employed for the successful imaging of biological samples under fluids simulating those in vivo. Using this technology, it will now be possible to further explore the phenomena of crystal growth and crystal surface charge characteristics using AFM and CFM.

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