Nanoscale Analysis of Bone Mineral Crystals

H. Chen†, C. Robinson‡, R. C. Shore‡, S. J. Brookes‡, J. Zhang‡, D. A. Smith†, B. H. Clarkson†,* and J. Kirkham†,*

† School of Dentistry, University of Michigan, Ann Arbor, MI 48109-1078, USA, bricla@umich.edu
‡ Division of Oral Biology, Leeds Dental Institute, Leeds LS2 9LU, UK, J.Kirkham@leeds.ac.uk
‡ Department of Physics and Astronomy, University of Leeds, Leeds LS2 9JT, UK

ABSTRACT

The mineral component of both bones and teeth comprises of crystals of substituted calcium hydroxyapatite. However, there are very few studies which have characterised and compared individual crystals from these mineralised tissues at the nanoscale. In our previous studies, we have utilised atomic force microscopy (AFM) to characterise crystals isolated from rat developing enamel and have described arrays of repeating charge domains on the crystal surfaces. The present aim was to utilise AFM and Self-Assembled Monolayer (SAM) technologies to investigate the topography and surface properties of crystals isolated from rat femoral cortical bone and compare these with the known characteristics of developing enamel crystals. In contrast to enamel crystals, AFM images clearly showed that individual bone crystals are thin, plate-like structures with sub-nm surface “steps” approximating to the unit cell dimensions for hydroxyapatite. These surface steps may be associated with step or spiral growth. Crystal aggregates were also present, suggesting that crystal fusion may have occurred in vivo. Bone crystals were successfully bound to charged SAM surfaces (both negative and positive) permitting imaging in contact mode AFM. No crystals were observed on uncharged SAMs. Preferential binding was seen on negatively-charged SAM surfaces, indicating a predominantly positive charge on the crystal surfaces similar to that seen in developing enamel crystals. The charge on the crystal surface may play a key role in the mechanism of matrix-mineral interactions.

Keywords: bone mineral, atomic force microscopy, self-assembled monolayers, nanoscale

1 INTRODUCTION

Bones and teeth are the principal mineralized tissues of the mammalian skeleton. Both comprise of crystals of a substituted calcium hydroxyapatite within an organic matrix [1]. However, unlike mature tooth enamel that is comprised of 95 % (by volume) nanorod-like crystals which have a cross section of 25-100 nm and lengths of 100 nm to 10 µm or longer along the c-axis, the crystals in bone are much smaller and irregular [1, 2]. The crystals in bone constitute 60-70 % (by volume) of the tissue and are tightly bound to their organic matrix (mainly collagen) [3]. It is widely accepted that these nanometer sized crystals are needle or plate-like in morphology based on studies using conventional ultrastructural techniques, such as TEM, X-ray diffraction line width broadening techniques and infrared spectroscopy [3-6]. However, the extremely small size and difficulties in extracting these crystals from the tissue has precluded any direct characterization of their surface properties. TEM is unable to provide details of crystal surface topography or chemistry and X-ray diffraction and infrared spectroscopy only allow indirect computation of the crystal dimensions [4].

The development of atomic force microscopy has provided a powerful way to explore biological specimens because of its high resolution and ability to provide a range of imaging environments [7]. Specimens can be scanned and images generated quantitatively in all 3 dimensions, providing height data and measurements of surface rugosity. This offers previously unprecedented opportunities to investigate the crystals of mineralised tissues [8, 9]. In our previous studies, we have utilized this technique to characterize the physico-chemical properties of crystals isolated from rat developing enamel. More recently, we have used dendrimers (artificial proteins) to probe enamel crystal surfaces [2, 10]. These studies showed that the hydroxyapatite crystals isolated from rat incisors were able to bind -COOH and -NH2 terminated polyamidoamine (PAMAM) dendrimers. This binding survived rinsing with water whereas the bound acetamide (-NHC(O)CH3) terminated, neutral charge dendrimers could be easily removed [10]. The Tapping mode AFM phase images show clearly that many of the crystal surfaces appeared to have a nanoscale structure of approximately 20 nm. Studies using self-assembled monolayers (SAMs) revealed that developing enamel crystals were able to bind to -COOH and -NH2 terminated SAMs, again implying that they carried surface charge [11]. Chemical force microscopy (CFM) studies disclosed that the surfaces of individual enamel crystals comprised of a series of discrete and alternating charge arrays aligned along and perpendicular to the crystal c-axis [12]. Further atomic force microscopy studies have showed that many proteins including serum albumin, amelogenin, DPP, DSP and ameloblastin, were able to bind to the enamel crystal surfaces under physiological conditions [13]. CFM images revealed a “banded” appearance along the crystal surfaces following protein binding that corresponded closely with the periodicity or the charge arrays described using CFM.
[12-14]. All of these proteins were expected to carry an overall negative charge at physiological pH. The reported charge arrays on the crystal surfaces were therefore assumed to play a key role in the binding to the proteins and charged dendrimers via electrostatic interactions.

In order to clarify if there are similar surface and charge properties on crystals isolated from bone, we have applied AFM and SAMs technologies to study the crystals isolated from rat femoral cortical bone. AFM was used to probe the surface topology and determine the size distribution of individual apatite crystals in order to generate quantitative three dimensional images at sub-nm resolution. SAMs were used to investigate the overall surface charge characteristics of the crystals. Each SAM has a hydrocarbon chain of finite length with a terminal head group to generate charged, hydrophilic or hydrophobic substrates. By binding the isolated bone crystals to these chemically well characterized SAMs substrates, we were able to probe bone crystal surface properties.

2 MATERIALS AND METHODS

2.1 Source of materials

Rat bone was obtained from dissected femoral bone of 3-6 week old male Wistar rats.

2.2 Isolation of individual bone crystals

Isolation of individual bone crystals was carried out based upon a modification of the method described by Weiner and Price [3]. Briefly, bone was first defatted with acetone and frozen in liquid nitrogen. The sample was then ground and placed in a 15 mL conical polypropylene tube. Ten milliliter of 3 % sodium hypochlorite was added. After sonicating in ice for 2 hours, the sample was centrifuged. The supernatant was discarded and another 10 mL of 3 % sodium hypochlorite was added. After a further hour of sonication the sample was again centrifuged and fresh reagents added. After a total of 4 hours of treatment with sodium hypochlorite, 10 mL of double distilled water was added and the sample was sonicated briefly, centrifuged for 5 min and the precipitate resuspended. After washing twice more in double distilled water, the sample was rinsed twice with 10 ml 95 % ethanol. The pellet was resuspended in 2ml of 100 % ethanol and sonicated in a water bath for 15 minutes. The pellet was then dried at 37 °C in a vacuum dessicator over phosphorus pentoxide. After drying for 24 hours, a 15 mg sample was placed in a glass test tube, and 3 mL 95 % ethanol was added. After 5 minutes sonication, the sample was vortexed and allowed to settle for 5 minutes. The procedure was repeated and allowed to settle for 30 minutes, 90 minutes and 48 hours. The final supernatant was removed and each pellet was dried in vacuum dessicator. The samples were resuspended in 100% HPLC-grade methanol. The 48 hour-pellet was used in this work as the others were not completely disaggregated.

2.3 Preparation of SAMs-modified substrates

Flat gold films were achieved by vacuum evaporation (Advent Research Materials Ltd, with purity of 99.99 %) onto silicon wafers (thickness of 120 nm, evaporation rate of 0.1-0.2 nm/s and vacuum pressure of 1.0 × 10⁻⁶). Glass plates were then bonded to the exposed gold surface with EpoTek 377 (Promatech Ltd.) and the wafer peeled away exposing a flat gold substrate. Carboxyl, amino, methyl and hydroxyl terminated SAMs were then covalently assembled on to the gold film via thiol linkages by immersing the slide into 0.1-0.5 mM ethanol solutions containing each thiol compound (11-mercaptoundecanoic acid (Aldrich, U.K.), 11-amino-1-undecanethiol hydrochloride (Dojindo, Japan), 10-mercaptopdecanol, 1-dodecylmercaptan (Aldrich, U.K.)) for 40 minutes at room temperature. Upon removal from the solution, the substrates were rinsed extensively with absolute ethanol and finally with distilled water (pH = 7.4) before use [11].

![AFM Scanner](image)

Figure 1: Crystals on the SAMs modified substrate for viewing under AFM [11].

2.4 Adsorption of crystals onto SAMs-Modified Substrates

The bone crystals, dispersed in methanol solution, were sonicated for 5 minutes to promote disaggregation. The SAMs-modified substrates were then immersed into the crystal suspension. After 20 minutes the slides were rinsed with methanol to remove loosely bound crystals. The sample was then dried in air and imaged under AFM immediately. Figure 1 shows the schematic relationship of the crystals on the SAMs for viewing by AFM.

2.5 AFM Analysis

Contact mode images were obtained using a Molecular Imaging pico SPM (Molecular Imaging) controlled by Nanoscope IIIa (Veeco, CA) electronics equipped with a 10 µm × 10 µm scanner and oxide-sharpened Si₃N₄ probe. All imaging was carried out in ethanol at room temperature.

The size of particles was determined using Digital Instruments’ off-line section routines.

![Image](image1)

**Figure 2**: AFM images of bone crystals adsorbed on to carboxyl-, amino-, hydroxyl- and methyl-terminated SAMs modified substrates, imaged under ethanol. The load force of tip to sample was approximately 2 nN. (a) on carboxyl-terminated SAMs modified substrate, (b) on amino-terminated SAMs modified substrate, (c) on hydroxyl-terminated SAMs modified substrate, (d) on methyl-terminated SAMs modified substrate. The left images in (a) and (b) show the height topography, while the right images are the lateral force image. The lateral force images provide higher resolution. The images in (c) and (d) are both height images. All images shown are 2 µm × 2 µm in area. The scale bar for all height images is 50 nm. The arrow in (a) shows the spiral-like structures and/or several crystals stacked on top of each other.

### 3 RESULTS

After disaggregating the ground cortical bone into particles, we were able to obtain individual units. The EDS analysis of these small units gave a calcium and phosphorus ratio of 1.73 ± 0.05 which approximates to the theoretical ratio of hydroxyapatite crystals (1.66). This result indicates that these isolated units are individual bone mineral crystals.

These separated crystals were then adsorbed onto the surfaces of different SAMs substrates. Figure 2 shows typical contact AFM images of the bone crystals adsorbed on to these surfaces. Both carboxyl- and amino-terminated SAMs were able to bind the crystals and allowed images (including lateral force images) to be obtained under ethanol but carboxyl-terminated SAMs (Figure 2a) appeared to adsorb larger amounts of the bone crystals compared with the amino-terminated SAMs (Figure 2b). No crystals were observed on hydroxyl- and methyl-terminated SAMs (Figure 2c and 2d). In Figure 2a and 2b, we can see that most of the crystals were well separated. However, the images also showed many larger units. The individual small particles were thin and plate-like in shape. Measurement of the bone crystal dimensions confirmed this observation, providing quantitative data. The average maximum length, width and height were 84.0 ± 36.3 nm, 63.6 ± 28.4 nm and 6.3 ± 5.0 nm, respectively. The lateral force images are of higher resolution and depict more clearly the sub-nm surface features of the crystal. Surface features such as this were observed on all of the particles and surface irregularities were more apparent on the large particles than the small ones. Some of the large particles appear to be individual crystals stacked face to face with step dimensions of 1-2 nm (Figure 3).

![Image](image2)

**Figure 3**: The AFM image of (presumably) two crystals stacked one upon the other. Line scan taken through the steps indicated a step height of approximately 1-2 nm.

### 4 DISCUSSION

The topographic features of individual bone crystals described here are of interest in that they may be related to special aspects, possible defects, in the crystallographic structure. The general observation of rugosity and steps in the crystallographic structure may reflect possible growth sites of the crystals. These steps approximate to the
hydroxyapatite unit cell (c = 0.68 nm) dimensions and may be the potential point or edge-defects, which provide a low-energy site for preferential attachment of ions or ion clusters enabling incremental growth in thickness and width [7]. Figure 3 shows two crystal plates stacked on top of each other. From the section analysis, each plate is only 2 nm in thickness. The results indicate that the crystals here are only 2-3 cell units in thickness and most of the organic matrix had been removed during development. It is even possible that these two plates became fused during the removal of the organic matrix either artefactually in our extraction procedure or during development within the bone itself. The images also clearly show that the bone crystals are thin and plate-like in shape. Some of the crystals even seem to have a spiral-like appearance, suggesting a possible growth mechanism of the crystal along a screw-dislocation.

The results showing that the bone crystals have a wide distribution of size and shape may also reflect different origins for the crystals within the tissue. It is assumed that the distribution of mineral crystals between intra- and inter-collagen fibrils results in crystals of different shape and size [8].

The results that crystals are bound and stable to scanning on carboxyl- and amino-terminated SAMs but are unstable on hydroxyl- and methyl-terminated substrate surfaces may indicate overall positive or negative charge on the crystal surfaces. Carboxyl groups on functionalized SAMs surfaces are likely to dissociate with the help of water, providing a negatively charged substrate surface [11]. The amino-terminated SAMs are partially protonated and hence provide an overall positively charged surface with lower charge density. The hydroxyl-terminated SAM would be expected to provide a polar but uncharged substrate surface, while the methyl-terminated SAM would provide a hydrophobic and uncharged surface. It appears that the bone crystals prefer to adsorb onto to carboxyl-terminated SAMs rather than amino-terminated SAMs. This may indicate a predominantly positive charge on the bone crystal surfaces. Surface charge on mineral crystals may arise from the presence of excess calcium (positive charge) or phosphate ions (negative charge). The results here are similar to those reported previously describing charges arrays observed on the surface of crystals isolated from enamel crystals [10-12]. This charged surface may play an important role in matrix-mineral binding.

5 CONCLUSION

In summary, we have utilised AFM to examine the crystals isolated from rat cortical bone. The AFM images show that the individual crystals are in the form of thin, plate-like structures with sub-nm surface “steps” approximating to the dimensions of the unit cell for hydroxyapatite. The surface steps may be associated with step or spiral growth of crystals in bone. We have also observed that the crystals are stacked one on another. We have found that bone crystals bind to carboxyl-terminated or amino-terminated SAMs, permitting imaging in contact mode AFM. Their binding to the SAM substrate suggests a net positive or negative charge on the crystal surfaces. The charge on the crystal surface may play the key role in their protein binding capacity.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Special Trustees of the Leeds Hospitals Trust for their support of this work. The author (H. Chen and B. H. Clarkson) thank the support of USPHS Research Grant DE015599 from the National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892.

REFERENCES