Nanoscale variation in surface charge of synthetic hydroxyapatite detected by chemically and spatially specific high-resolution force spectroscopy

Jennifer Vandiver\textsuperscript{a}, Delphine Dean\textsuperscript{b}, Nelesh Patel\textsuperscript{c}, William Bonfield\textsuperscript{c}, Christine Ortiz\textsuperscript{a,*}

\textsuperscript{a}Departments of Materials Science and Engineering, RM13-4022, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA
\textsuperscript{b}Electrical Engineering and Computer Science, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA
\textsuperscript{c}Department of Materials Science and Metallurgy, University of Cambridge, Pembroke Street, Cambridge CB2 3QZ, UK

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Abstract

The normal intersurface forces between nanosized probe tips functionalized with COO\textsuperscript{-} and NH\textsubscript{3}\textsuperscript{+}-terminated alkanethiol self-assembling monolayers and dense polycrystalline phase pure synthetic hydroxyapatite (HA) were measured via a powerful nanomechanical technique called chemically specific high-resolution force spectroscopy. The data taken on approach of the probe tip to the HA surface was compared to the nonlinear Poisson–Boltzmann-based electrostatic double layer theory to predict the surface charge per unit area of the HA, $\sigma_{HA}$ (C/m\textsuperscript{2}), as a function of ionic strength, position within a variety of grains, and across grain boundaries. The average $\sigma_{HA}$ was found to be $\sim-0.02$ C/m\textsuperscript{2} and to vary from $-0.0037$ to $-0.072$ C/m\textsuperscript{2} with nanoscale position in relation to grain boundaries and crystal planes up to $-0.19$ C/m\textsuperscript{2}/m\textsuperscript{m}. Positional measurement of nanoscale surface properties holds great promise in elucidating the molecular origins of physicochemical processes occurring at the biomaterial interface.

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1. Introduction

Currently, there is a significant need for improved synthetic materials, for use as orthopedic implants, to replace human bone lost and damaged due to disease (e.g. osteoporosis) and/or injury. Certain ceramics, like calcium phosphates, have the special property of being bioactive, meaning that an interfacial bond between the implant and the surrounding bone forms, leading to good fixation, and generally no fibrous tissue encapsulation [1–3]. One such ceramic, hydroxyapatite (HA) (Ca\textsubscript{5}(PO\textsubscript{4})\textsubscript{3}OH), the stable phase of calcium phosphate at body temperature and pH $>4.2$ [1], is one of the main constituents of natural bone ($\sim70$ wt\%) [4] and is being investigated in a wide variety of forms for use in different bone implant applications [1,3–5]. The nanoscale surface chemical properties (e.g. surface functional groups, charge distribution), and morphological structure (e.g. grain size, shape, distribution, roughness) will critically influence the implant’s interaction with the biological environment, and how well the implant subsequently performs in vivo [2].

When HA is implanted into a bony site, a cascade of physiochemical interactions takes place with the biological environment upon exposure to extracellular fluid, resulting in the build up of interfacial layers that bond the bone tissue to the implant material [5–7]. The three main processes thought to occur upon implantation are adsorption of ions and biomolecules, formation of calcium phosphate (apatite) layers, and interactions with various cells [6]. Transmission electron microscopy (TEM) suggests the precipitation of microcrystals of CO\textsubscript{3}-apatite directly onto the implant surface [2] from calcium and phosphate ions released from partially dissolving ceramic HA and ions such as CO\textsubscript{3}\textsuperscript{2} from the biological fluid. The precipitation of apatite onto the
HA could be a form of epitaxy [2,5,8] or be controlled by electrostatic interactions [9–12], thus yielding a bioactive apatite layer [2,6,13,14]. It is expected that surface charge will have a strong influence on the processes of inorganic and organic deposition and structural evolution on the implant material, especially in the initial stages of implantation. Reports of increased crystal growth of bone-like HA [15], cell adhesion [16], and osteobonding [17] to negatively charged surfaces of electrically polarized HA support this hypothesis. Si-substituted HA is known to have improved bioactivity both in vitro and in vivo [18–20], which has been suggested to be due to increased negative surface charge from substitution of PO₄³⁻ with SiO₄⁻ groups [18]. Stoichiometric HA (bulk Ca/P ratio 1.67 [3]) has been shown to have a negative charge though zeta potential measurements of powder suspensions with granule sizes 5–50 µm [9,18] which is thought to arise from the preferential concentration of PO₄³⁻ groups to the top few nanometers [18] of the surface [3,21]. It is believed this Ca deficient surface layer (Ca/P ratio ~1.5 [21]) is caused by solid–solution equilibrium during the precipitation used to form stoichiometric HA whereby there is the creation of a vacancy on one of the 10 Ca sites, the creation of a vacancy on one of the two hydroxyl sites, and protonation of one of the six PO₄³⁻ groups [21].

Typically, surface charge is measured via the zeta potential method [22,23] which yields an averaged, bulk value for colloidal dispersions, fibers, films, and other microscopic structures. Here, we employ the complementary and relatively new technique of chemically specific high-resolution force spectroscopy (HRFS) [24] that allows for the direct measurement of piconewton (pN) level forces in fluid between a nanosized probe tip functionalized with molecules of uniform and known structure, chemistry, and charge (e.g. self-assembling monolayers (SAMs)) as a function of separation distance from a sample of interest. By comparing HRFS data on approach of the probe tip to the sample surface to appropriate electrostatic double layer theory [25–27], an estimation of the surface charge per unit area, σ (C/m²), of the sample of interest can be made. In this paper, we measure, for the first time, the normal electrostatic double layer forces between COO⁻ and NH₃⁺-terminated alkanethiol SAM-functionalized probe tips and bioactive dense, polycrystalline, phase pure synthetic HA via chemically specific HRFS with new nanomechanical instrumentation [28] that allows for both high-resolution topographic imaging and HRFS with nanoscale spatial resolution. Nanomechanical data on approach were compared to the nonlinear Poisson–Boltzmann-based electrostatic double layer theory for surfaces of constant surface charge to predict σ_HA (C/m²) as a function of ionic strength (IS), position within a variety of grains, and across grain boundaries. This new methodology allows for precise and positionally sensitive measurement of nanoscale surface properties that control bioactivity.

2. Materials and methods

2.1. Preparation of hydroxyapatite pellets

Synthetic HA pellets were prepared by an aqueous precipitation reaction between calcium hydroxide (Ca(OH)₂) and phosphoric acid (H₃PO₄) solution as described in detail previously [2,29]. Analytical grade reagents (BDH AnalR, Merck Ltd., Lutterworth, UK) were used. To ensure purity of the samples, the reaction vessels were thoroughly cleaned and rinsed with deionized (DI) water prior to use. The precipitation process proceeded as follows: a calcium hydroxide solution was made up by initially stirring 0.5 mol of Ca(OH)₂ in 1 L of DI water. Similarly, 0.299 mol of H₃PO₄ solution was dissolved in 1 L of DI water. The precipitation reaction occurred when the H₃PO₄ solution was added dropwise to the Ca(OH)₂ solution over a period of 2–3 h at ambient temperature. During the precipitation process the pH of the reaction was maintained at 10.5 by small additions of 25% ammonia solution. The precipitate was filtered under vacuum, thoroughly washed with approximately 100 ml of DI water, and then placed in a glass drying dish to dry at 80°C for 24 h in air. The dried HA filter-cake was then crushed using a pestle and mortar and sieved to a particle size less than 75 µm in diameter. The green powder was then pressed into pellets using an isostatic press and subsequently sintered to 1200°C for 2 h in air. The pellets have an average width of 3.8 mm, an average diameter of 8.64 mm, and are >98% of the theoretical density (3.13 ± 0.015 g/cm³) as measured by water displacement.

2.2. General characterization

Purity of the HA pellets was determined via wide angle X-ray diffraction (WAXD) and X-ray fluorescence spectroscopy (XRF). Surface wettability was assessed via contact angle measurements with DI water on advancing and receding (Video Contact Angle System 2000, AST Inc.). Grain size analysis, via grain diameter measurements, was performed using a FEI/Philips XL30 FEG environmental scanning electron microscopy (ESEM). To prepare the sample for ESEM, the HA pellet was polished to a 3 µm finish using an aluminum oxide film on a uni-polisher (Geoscience Instruments Corp.) and then etched in 10% H₃PO₄ for 10 s. Three SEM images of different sites were then taken at 10,000 X and these images were analyzed using Scion Image (Scion Corporation) to determine grain size and
distribution. Surface topography was imaged using a Digital Instruments Nanoscope IIIA System Controller with multimode atomic force microscope in contact mode in air (CMAFM) with a Thermomicroscopes Si$_3$N$_4$ V-shaped cantilever with a spring constant ($k_c$) $\sim$ 0.01 N/m (Appendix Tables 1 and 2).

2.3. Chemically specific HRFS with SAMs

2.3.1. Functionalization of probe tips with SAMs

HRFS experiments were performed with electroplated Au-coated Si$_3$N$_4$ cantilever probe tips chemically functionalized with alkanethiol SAMs terminated with either COO$^-$ groups (11-mercaptoundecanoic acid, HS-(CH$_2$)$_{10}$-COOH, Aldrich—used as received) or NH$_3$$^+$ groups (11-aminoundecanethiol, HS-(CH$_2$)$_{11}$-NH$_2$ Dojindo Laboratories—used as received). The Au-coated cantilever probe tips were cleaned in O$_2$ plasma for 10 s and immediately placed in 1 mTorr SAM solutions in 100% ethanol for 18 h, after which they were rinsed with 100% ethanol and stored in Milipore water (18 MΩ cm resistivity).

2.3.2. Probe tip end-radius measurements

The probe tip end-radius, $R_{TIP}$, was measured by a JEOL 6320FV field-emission high-resolution SEM. Tip radii were determined by digitally drawing a circle on a 100,000 $\times$ SEM image within the point of the tip and comparing the radius of the circle to the scale bar on the image.

2.3.3. Averaged (blind) high-resolution force spectroscopy

HRFS experiments were conducted using a 1-D molecular force probe (1DMFP) [28], to measure force, $F$ (nN), vs. tip–sample separation distance, $D$ (nm), on approach and retract ($F$–$D$ curves). A full description of this instrument, its limits, procedures for spring constant calibration and raw data conversion, and details of measurement errors are given in previous work [30]. In all HRFS experiments described in this paper, the spring constant of the cantilever is much less than the stiffness of the substrate such that little or no deformation occurs, leading to the $D = 0$ vertical region of apparent infinite slope in the high-force constant compliance regime. HRFS experiments were performed using two cantilevers (Thermomicroscopes, V-shaped, $k_c = 0.01$ N/m); one with a COO$^-$-terminated SAM probe tip ($R_{TIP} = 64$ nm) and the other with NH$_3$$^+$-terminated SAM probe tip ($R_{TIP} = 37.5$ nm). HRFS experiments for the COO$^-$-terminated SAM probe tip ($pK_a$(COO$^-$) $\sim$ 4.75) were carried out in aqueous electrolyte solutions having ISs of 0.001–1 m NaCl and constant pH $= 5.98 \pm 0.043$ (henceforth, referred to as pH $\sim$ 6), which were prepared by dissolving NaCl crystals in DI water. An equilibration time of 20 min was allowed during solution changes and the order of experiments was from low to high IS. HRFS experiments for the NH$_3$$^+$-terminated SAM probe tip ($pK_a$(NH$_3$) $\sim$ 5.5) were carried out in aqueous electrolyte solutions having IS of 0.01–1 m formate buffer, pH $= 4.02 \pm 0.005$ (henceforth referred to as pH $\sim$ 4), prepared by mixing formic acid (HCHO$_2$) with DI water and adding NaCl crystals to get desired IS. Both NaCl and formate buffer create only monovalent electrolyte solutions. The probe tip–surface force as a function of tip–sample separation distance on approach and retract was measured at a $z$-piezo rate of 2 μm/s. HRFS experiments using each of these probe tips were first conducted on Au-coated Si substrates [30] functionalized with the same SAM as the probe tip in varied IS solutions of constant pH (pH $\sim$ 6 for COO$^-$-terminated SAM and pH $\sim$ 4 for NH$_3$$^+$-terminated SAM as described above) to determine the surface charge per unit area of the SAM (described in detail in a later section) and then used to probe the HA pellet. Approximately 30 individual HRFS probes were performed and averaged at each of three random locations on the samples of interest.

2.3.4. Positionally specific high-resolution force spectroscopy

HRFS was additionally performed using a 3-D molecular force probe (3DMFP) [28] which has all the same features as the 1DMFP but additional capabilities to image the surface and perform positionally sensitive HRFS in the plane of the sample surface. An Au-coated Si$_3$N$_4$ cantilever probe tip (Thermomicroscopes, V-shaped, $k_c = 0.03$ N/m, $R_{TIP} = 89$ nm) chemically functionalized with COO$^-$-terminated SAM was used to image and probe an HA sample simultaneously within a variety of grains and across grain boundaries in order to compare forces to observed topographical surface features. The experiment was carried out in 0.01 m NaCl solution (pH $= 5.94$). The probe tip–surface forces as a function of tip–sample separation distance were measured on approach and retract at a $z$-piezo rate of 2 μm/s.

2.3.5. Electrostatic double layer theory

HRFS data on approach were compared to the numerical solutions of the full nonlinear electrostatic double layer theory based on a Poisson–Boltzmann formulation for a surface of constant charge per unit area [31]. The setup and parameters for this analysis are shown in Fig. 1. The Poisson–Boltzmann equation gives an expression for the electrical potential, $\Phi$ (V), between two charged planar surfaces in an electrolyte solution. For a monovalent 1:1 electrolyte, the solution has the form:

$$\nabla^2 \Phi = \frac{2fC_0}{\epsilon_w} \sinh \left( \frac{f\Phi}{RT} \right),$$

(1)

where $f$ is the Faraday constant (96,500 C/mol), $C_0$ is the bulk concentration of ions (mol/m$^3$), $\epsilon_w$ is the dielectric permittivity of water (6.9 $\times$ 10$^{-10}$ C/Nm$^2$), $R$ is the
universal gas constant (8.314 J/mol K), and $T$ is the absolute temperature (K) [25–27]. Constant charge boundary conditions were used such that the electric field at the substrate and probe tip surfaces was related to the surface charge per unit area (i.e., $\nabla \phi_{\text{surface}} = \sigma_{\text{surface}}/\varepsilon_w$ and $\nabla \phi_{\text{tip}} = -\sigma_{\text{tip}}/\varepsilon_w$). In this study, constant charge boundary conditions on both bounding surfaces are employed because in the experiments, neither the probe tip nor the substrate is electrically connected to any source that would maintain them at a constant potential [32]. A numerical method, known as the Newton method on finite differences, was used to solve the full nonlinear Poisson–Boltzmann equation [31]. For the 1DMFP experiments, control HRFS experimental data of the COO\(^{-}\)-terminated SAM probe tip vs. a COO\(^{-}\)-terminated SAM planar substrate were compared to the theory (data not shown) and $\sigma_{\text{COO}^-}$ was estimated to be $-0.0084 \text{C/m}^2$ where the fixed parameters in the analysis were $R_{\text{TIP}}$ and the IS. Analogous NH\(^3\)+-terminated SAM HRFS experiments and theoretical fits (data not shown) yielded $\sigma_{\text{NH}^3^+} = +0.0207 \text{C/m}^2$. For the 3DMFP experiment on HA $\sigma_{\text{COO}^-}$ of $-0.0178 \text{C/m}^2$ was used. These probe tips of known $\sigma$ were then used to test HA in varied IS solutions and this experimental data were fit to the theoretical solution using $\sigma_{\text{HA}}$ as the only free fitting variable.

3. Results

3.1. General characterization: WAXD, contact angle, ESEM, and AFM

The XRD experiments demonstrated the HA pellets to be phase pure while XRF analysis confirmed that the pellets had a Ca/P molar ratio of approximately 1.67 and did not reveal any unexpected elements. Contact angle measurements with DI water gave an averaged ($n = 6$ different positions on sample) advancing contact angle of $65.2^\circ \pm 0.85^\circ$ and an averaged receding contact angle of $18.1^\circ \pm 2.9^\circ$, demonstrating that the HA surface is slightly hydrophilic. The large variation between the advancing vs. receding contact angles (hysteresis) observed is likely due to water absorption through interaction with hydroxyl vacancies near the surface of HA [21]. Grain size analysis was performed on the ESEM images (e.g., Fig. 2a) where the diameter of every distinct grain on three separate digital images was measured, for a total of 93 grains. The grain size distribution (Fig. 2b) was found to have a mean value of $1.48 \pm 0.68 \mu\text{m}$. Five $5 \mu\text{m} \times 5 \mu\text{m}$ AFM scans were analyzed, and each scan encompassed several grains. The grain sizes determined via CMAFM images agreed well with the ESEM images where 28 separate grains were measured giving an average size of $1.17 \pm 0.76 \mu\text{m}$. The average root mean squared (RMS) surface roughness of the five $5 \mu\text{m}$ scans was $113.6 \pm 21.0 \text{nm}$, which takes into account several grains and grain boundaries.

Fig. 1. Schematic of chemically specific HRFS experiments showing parameters used in Poisson–Boltzmann electrostatic double layer model: $\sigma_{\text{tip}}$ is the charge per unit area of a hemispherical probe tip, $\sigma_{\text{surface}}$ is the charge per unit area of the surface, $\varepsilon_w$ is the dielectric permittivity of water, $R_{\text{TIP}}$ is the probe tip end-radius measure by SEM, $F$ is the measured probe tip–surface interaction force, and $D$ is the probe tip–sample separation distance.

Fig. 2. (a) ESEM image of acid etched HA pellet (10,000 x) and (b) grain size distribution of acid etched HA pellet ($1.48 \pm 0.68 \mu\text{m}$).
Additional scans inside five different grains were performed in order to quantify the surface roughness inside the grains and the average RMS surface roughness was found to be 17.9 ± 3.2 nm. Typical AFM images are shown in Fig. 3.

3.2. Chemically specific HRFS: charged SAM probe tips vs. HA

3.2.1. Averaged (blind) HRFS with 1DMFP

3.2.1.1. Approach. The averaged $F - D$ curves with standard deviations for the COO$^-$-terminated SAM probe tip vs. an HA pellet surface at pH ~ 6 and varied IS (Fig. 4a) all show a purely repulsive, nonlinear forces on approach of the probe tip to the HA surface. The repulsive forces observed in the 0.1 and 1 M IS solutions are observed to be significantly less in both magnitude and range than the lower IS solutions of 0.01 and 0.001 M. A small attractive “jump-to-contact” was observed ~20% of these 1DMFP data and the distance, $D_{jump-to-contact} = 4.39$ nm at 0.01 M IS was used to estimate the Hamaker constant ($\kappa$) of the surface and found to be $4.33 \times 10^{-20}$ J. The averaged $F - D$ curves with standard deviations for the NH$_3^+$-terminated SAM probe tip vs. HA at pH ~ 4 and varied IS (Fig. 4b) all show an attractive, nonlinear force that begins at increasingly smaller distances from the sample surface with increasing IS, i.e. 10.8, 9.2, and 6.9 nm at 0.01, 0.1, and 1 M IS, respectively, and exhibit minimum values of $F/R_{tip} \approx 0.20, \sim 0.17$, and $\sim 0.25$ mN/m at $D \sim 3.4, D \sim 3.7$, and $D \sim 1.9$ nm, respectively. These results clearly suggest an electrostatic double layer origin for the surface interaction force with the HA possessing a net negative surface charge. One observation of note was that the variance in the HRFS data for the COO$^-$-terminated SAM probe tip with position on the HA surface (Fig. 5a) was significantly greater than the variance of HRFS curves with position on the COO$^-$-terminated SAM (Fig. 5b). Hence, the effect of position was investigated further using the 3DMFP and discussed in the following section.

In order to estimate $\sigma_{HA}$, the averaged HRFS curves shown in Fig. 4 were fit to the numerical solution of the Poisson–Boltzmann theory for surfaces of constant charge per unit area using the following fixed parameters: surface charge of probe tips ($\kappa_{COO^-} = -0.0084$ C/m$^2$ or $\kappa_{NH_3^+} = +0.0207$ C/m$^2$), IS = 0.01 M, $R_{tip}$ ($R_{tip}$(COO$^-$) = 64 nm or $R_{tip}$(NH$_3^+$) = 37 nm), and $\sigma_{HA}$ (C/m$^2$) as the only free variable fitting parameter [33]. HRFS data of
Fig. 5. 1DMFP HRFS data. Averaged data for COO\textsuperscript{−}-terminated SAM probe tip at three randomly selected positions (pH=5.94, IS=0.01 m) vs. (a) HA and (b) COO\textsuperscript{−}-terminated SAM planar surface.

Fig. 6. 1DMFP HRFS data. (a) Averaged data for COO\textsuperscript{−}-terminated SAM probe tip vs. HA (pH=5.94) and Poisson–Boltzmann theoretical fit (fixed parameters: $\sigma_{\text{COO}}=-0.0084 \text{ C/m}^2$, IS=0.01 m, $R_{\text{TIP}}=64 \text{ nm} \rightarrow \sigma_{\text{TIP}}=-0.005 \text{ C/m}^2$). (b) Averaged data for NH\textsubscript{3}\textsuperscript{+}-terminated SAM probe tip vs. HA (pH=4.01) and Poisson–Boltzmann theoretical fit (fitting parameters: $\sigma_{\text{NH}}=+0.0207 \text{ C/m}^2$, IS=0.01 m, $R_{\text{TIP}}=37 \text{ nm} \rightarrow \sigma_{\text{TIP}}=-0.0048 \text{ C/m}^2$).

3.2.1.2. Retract. The retract $F-D$ curves were also examined and the average adhesion forces and distances with standard deviations for the COO\textsuperscript{−}-terminated SAM probe tip probing HA vs. solution IS were calculated (Fig. 7). The adhesion force magnitudes at 0.1 and 1 m are statistically lower ($p<0.01$) than the adhesion force magnitudes at 0.001 and 0.01 m although the forces between 0.1 and 1 m were not statistically different, nor were the forces between 0.001 and 0.01 m. The adhesion pull-off distances ranged up to 60 nm and were also statistically lower ($p<0.01$) at higher IS except for 0.001 compared to 0.01. The adhesion distances are similar to the surface roughness within individual grains (17.9 ± 3.2 nm).

The Derjaguin–Muller–Toporov elastic contact mechanics theory [34,35] can provide an upper limit estimate for the number of molecular contacts that exist at the maximum compressive force and contribute to the adhesive interaction. The elastic contact area between the probe tip and a planar surface, $A_{\text{contact}} = \pi a^2$, can be calculated from $a$, the elastic contact area radius, as follows:

$$a = \left( \frac{F + F_{\text{adhesion}}}{K} \right)^{1/3},$$

$$K = 4 \frac{1 - \nu^2}{E_1} \frac{1 - \nu^2}{E_2},$$

where $K$ is the reduced elastic modulus, $\nu$ the Poisson’s ratio, $E$ the Young’s (elastic) modulus, $E_1(\text{Au})=64 \text{ GPa}$, $E_2(\text{HA})=95 \text{ GPa}$, and $E_2(\text{HA}) = 0.28$. Taking a maximum compressive force of ~1 nN at $D = 0$ and a maximum observed adhesive force of ~1 nN, $A_{\text{contact}}$ was found to be ~5.2 nm\textsuperscript{2} for the 64 nm radius COO\textsuperscript{−}-terminated SAM probe tip used for the 1DMFP experiments. Since the area per SAM molecule is approximately 0.216 nm\textsuperscript{2} [36], the number of molecules within the maximum elastic contact area corresponds to 24. Hence, the maximum adhesive force per SAM molecule within this elastic contact area is...
Fig. 7. Analysis of 1DMFP HRFS retract data ($R_{\text{TIP}} = 64 \text{ nm}$, pH~6) vs. IS: (a) averaged adhesion forces and (b) averaged adhesion distances on retract between COO$^-$/terminated SAM probe tip and HA vs. solution IS.

~42 pN, an upper limit since the contact radius will decrease from the maximum value before piezo reversal and upon retract, as well as the fact that the adhesion force taken was the maximum observed.

3.2.2. Positionally specific HRFS with the 3DMFP

3.2.2.1. Approach. As mentioned previously, when performing HRFS experiments with the 1DMFP, a variance in the experimental data with position on the HA surface was noticed which was significantly greater than the variance of HRFS curves with position on the COO$^-$/terminated SAM (Fig. 5). Hence, further investigation was carried out with the 3DMFP where AFM imaging and HRFS could both be performed together, making it possible to correlate intersurface forces with topographical features. The first set of HRFS experiments (labeled Scan 1) traversed three different grains on the HA surface corresponding to the positions shown as “x’s” in the CMAFM deflection (topographical) image given in Fig. 8a. Underneath the AFM image in Fig. 8a, a topographical profile of the surface height vs. horizontal distance across the image (corresponding to the horizontal black line in the top image of Fig. 8a) is given and shows the surface geometry of the probed area. A second smaller scan (labeled Scan 2) was made at the grain boundary between probe positions 5 and 6 of Scan 1, and the surface locations of the HRFS experiments taken across the grain boundary are shown as “x’s” in Fig. 8b. Fig. 8c shows the averaged $F-D$ curves including maximum standard deviation for the probe positions of Scan 1. Fig. 8d shows the averaged $F-D$ curves including maximum standard deviation for the probe positions of Scan 2. Position 5 on Scan 1 and position 1 on Scan 2 probe the same topographical face. The probe at point 4 on Scan 2 showed unusual nanomechanical behavior, most likely due to geometric and interlocking effects between the probe tip and the grain boundary. A positional variance of the surface forces was observed in both of these data sets and so the data were analyzed in this context. In particular, the positions were grouped by facet as shown in Fig. 9, where a facet was defined as a distinct topographical face whose area had a relatively constant slope.

Generally, the 3DMFP HRFS data and the 1DMFP HRFS data taken at 0.01 m IS were consistent in magnitude and range, even using different probe tips where variations always arise due to geometry and other nanoscale factors such as variations in surface roughness, SAM density, etc. The 3DMFP data show slightly larger $F/R_{\text{TIP}}$ correlating with a higher probe tip surface charge density than that used for the 1DMFP.

Using the same process as for the 1DMFP data, the 3DMFP HRFS data on approach were compared to electrostatic double layer theory and $\sigma_{HA}$ for each position was calculated by averaging the fitted $\sigma_{HA}$ for three individual curves at that position. Figs. 10a and b show the averaged $\sigma_{HA}$ for each probe position for Scans 1 and 2, respectively, along with standard deviations. The $\sigma_{HA}$ of each facet was examined since facets likely have different exposed crystallographic planes with differing numbers of exposed charged groups causing $\sigma_{HA}$ variations. Statistical analysis shows the average $\sigma_{HA}$ for each facet to be significantly different from the others in nine out of 10 comparisons ($p<0.05$). The average $\sigma_{HA}$ over all positions in both scans was calculated in this experiment to be $-0.0188 \pm 0.0198 \text{ C/m}^2$. The magnitude of the force and hence, the fitted value of $\sigma_{HA}$, showed variations within individual grains, between differing grains, and across the grain boundary. Fig. 10c shows $\sigma_{HA}$ vs. the distance of the probe position from the grain boundary (left side of grain boundary, including data from both scans). A linear regression of these data points gives a correlation factor of $>98\%$ which is statistically significant ($p<0.05$) and a slope of $-0.19 \text{ C/m}^2/\mu\text{m}$. This trend is consistent with surface charge being due to ion arrangements in crystallographic planes which become disordered and shifts from one plane to another at grain boundaries.

Since the surface charge calculation is based on an approximated fit to the HRFS data, specific data from...
each individual curve was also examined. The force between the probe tip and HA at one Debye length away from the surface \((\kappa^{-1} = 3 \text{ nm})\) was recorded for each curve \((n = 5)\) at each position in each scan (data not shown). An increase in this force would indicate a larger surface charge per unit area if the force is caused mainly by electrostatics. The trends for surface charge and force between the probe tip and HA at one Debye length away from the surface were consistent.

3.2.2. Retract. Similar to the 1DMFP data, the retract \(F-D\) curves were also examined and the average adhesion forces and distances for the COO\(^-\)-terminated SAM probe tip probing HA were recorded. The average adhesion force magnitudes and the adhesion pull-off distances were similar to the values obtained from the 1DMFP experiment and although they differed per facet

![Fig. 8](image)

Fig. 8. (a) Scan 1 CMAFM deflection image taken using 3DMFP with COO\(^-\)-terminated SAM probe tip in fluid \((R_{\text{TIP}} = 89 \text{ nm}, IS = 0.01 \text{ m}, \text{pH} = 5.94)\) showing specific positions \((X's)\) in three grains probed via HRFS. Below is a plot showing the height profile along the solid black line in the image. (b) Scan 2 CMAFM deflection image taken using 3DMFP with COO\(^-\)-terminated SAM probe tip in fluid \((R_{\text{TIP}} = 89 \text{ nm}, IS = 0.01 \text{ m}, \text{pH} = 5.94)\) showing specific positions \((X's)\) in two grains probed via HRFS. Below is a plot showing the height profile along the solid black line in the image. (c) Averaged HRFS data of seven probe locations in Scan 1 labeled in (a) (each position, \(n = 5\)). (d) Averaged HRFS data of six probe locations in Scan 2 labeled in (b) (each position, \(n = 5\)).

![Fig. 9](image)

Fig. 9. Labeling for different facets among the grains imaged in Scan 1.
there were no obvious correlations with surface charge. Similar to the 1DMFP experiments, $A_{\text{contact}}$ was calculated to be $\sim 6.5 \text{ nm}^2$ for the 89 nm radius COO- terminated SAM probe tip used for the 3DMFP experiments, corresponding to 30 SAM molecules and a maximum adhesive force per SAM molecule of 33 pN.

4. Discussion

In this paper, we have shown how the sensitive and powerful nanomechanical technique of chemically and spatially specific HRFS can measure the nanoscale forces that exist at the interface between a biomaterial surface and physiological fluids. Nanosized probe tips of known chemistry and geometry were used to test a promising bone implant material, i.e. phase pure, dense, polycrystalline synthetic HA, and fits of these data on approach to electrostatic double layer theory [25–27] enabled approximation of the HA surface charge per unit area, $\sigma_{\text{HA}}$. On retract of the probe tip away from the surface, nanoscale adhesive interaction forces, $F_{\text{adhesion}}$, were measured. One of this most unique aspects of the methodology presented in this paper is that it allows for the determination of local nanoscale variations in $\sigma_{\text{HA}}$ and $F_{\text{adhesion}}$ within grains and across grain boundaries, important information unable to be obtained by other standard techniques such as zeta potential measurements. Nanoscale variations in the local interface potential are certain to affect the adsorption process of ions and biomolecules, formation of calcium phosphate (apatite) layers, and interactions with various cells which determine the build up of interfacial layers that bond the bone tissue to the implant material. With the new capabilities presented in this paper, the relation of such local nanoscale parameters to bioactivity will be able to be explored, for example by correlating $\sigma_{\text{HA}}$ and $F_{\text{adhesion}}$ measured by HRFS measurements with the kinetics of apatite-layer growth when the same samples are incubated in simulated body fluid (SBF) or implanted in vivo. In addition, chemically specific HRFS will be able to detect at extremely high resolutions the differences in nanoscale surface properties between the original HA surface and the precipitated bone-like apatite layer for example, in SBF. Lastly, the same experimental and theoretical methodology can be used for studying the nanoscale interactions between biomaterial surfaces and proteins and cells since the nanosized probe tips can be functionalized accordingly. Overall, it is clear that this method holds great potential for fundamental research on the physicochemical processes occurring at biomaterial interfaces and elucidating the molecular origins of bioactivity. Following is a discussion of specific aspects of the data presented in this paper.

4.1. Approach HRFS data

All trends in HRFS experimental data with IS and theoretical fits support the fact that on approach the intersurface interaction is dominated by electrostatic double layer forces and that HA has a net negative surface charge per unit area, $\sigma_{\text{HA}}$, ranging from $-0.0037$ to $-0.072 \text{ C/m}^2$ with an average value of $-0.019 \text{ C/m}^2$, which is similar to that found for the COO- terminated SAM probe tip ($\sigma_{\text{COO}} \sim 0.018 \text{ C/m}^2$).
and as mentioned in Section 1 has been suggested to be due to preferential surface migration of PO₄³⁻ groups [3,21]. Considering the maximum magnitude of σ̄₂⁺ = (−0.072 C/m²) obtained by HRFS, for comparison, if one was to consider an alkanethiol SAM of Ca²⁺ and PO₄³⁻ terminal groups with roughly 1 group/0.22 nm² [36] and a Ca/P ratio of ~1.5, equivalent to the surface Ca/P ratio in HA [21], this SAM would have a fully ionized charge density of ~0.74 C/m². Therefore, the HA surfaces measured have a ~10× smaller charge density than would be expected from a densely packed collection of phosphate and calcium ions with a Ca/P ratio of 1.5.

4.2. Spatial heterogeneity of surface charge of hydroxyapatite

Geometrical calculations have ruled out the variance in apparent surface charge being due to the slope of the sample surface as shown in Appendix A. Unlike SAM layers, the HA pellets do not present a uniform charge over its surface but is locally (at the nanoscale level) heterogeneous. The degree to which these nanoscale heterogeneities are relevant to Ca²⁺ binding, apatite precipitation, protein adsorption, and ultimately biocompatibility is an area of great interest and is currently being investigated further.

4.3. Relation of HRFS data to zeta potential measurement

Until recently, the majority of surface charge measurements have been accomplished through zeta potential measurements. Zeta potential is dependant on surface charge density and is defined as the electrostatic potential at the hydrodynamic shear plane which is located approximately at the Stern surface. The Stern surface is the boundary between a layer of more rigidly bound counterions and the diffuse electrostatic double layer of highly mobile, hydrated counterions, and is typically a few molecular diameters from the surface [22]. Zeta potential measurements are averaged, bulk measurements most often performed via electrophoresis on dilute colloidal suspensions or by the streaming potential method on fibers, films, and other macroscopic structures [39], and have the variability of particle size [9,40] and IS dependence [22].

The negative sign for surface charge of phase pure HA measured in this paper is in agreement with zeta potential measurements of HA in the literature [9,37,38,41]. In previously reported work [18], the zeta potentials of pure, synthetic, dense polycrystalline HA particles were measured at varied pH. At pH 6, similar to the pH at which the experiments in this paper were carried out, and IS 0.0001 M, the zeta potential for these HA particles was measured to be approximately −35 mV [18]. When the surface potential is calculated via the nonlinear Poisson–Boltzmann equation from the average surface charge measured by 3DMFP in this paper, σ̄ = −0.0188 C/m², a value of ~64 mV is obtained. Previous zeta potential measurements are ~50% of the potential calculated from the HRFS data, which is consistent with previously published data [42,43] that found zeta potential measurements to be 30–50% of HRFS derived surface potentials for zirconia surfaces in poly(acrylic acid) solutions probed with spherical zirconia colloidal probe tips and poly(ether ether ketone) probed with Si₃N₄ probe tips, respectively. It has been hypothesized that the disagreement between zeta potential measurements and HRFS derived surface potentials is due to the potential drop in the immobilized liquid layer close to the surface which moves with the particle in zeta potential measurements, but is not included in Poisson–Boltzmann theory [42]. The surface charge calculated in this paper via HRFS data compared to Poisson–Boltzmann theory is the effective charge at the Stern surface since electrostatic double layer theory is valid only within the diffuse double layer that begins at the Stern surface [22]. The HRFS surface charge model used in this paper should more closely resemble the electrostatic interaction of a biomacromolecule or cell approaching a biomaterial surface, where the effective charge at the Stern surface is what the biomacromolecule or cell feels inside the diffuse double layer.

4.4. Retract HRFS data

Both the 1DMFP and 3DMFP data produced similar adhesion forces and distances, with a maximum adhesive force per SAM molecule of 42 pN, clearly typical of a noncovalent interaction (e.g. hydrogen bonding, van der Waals, or ionic). The adhesion forces measured are likely not hydrophobic interactions since HA is relatively hydrophilic as seen in the contact angle measurements. Scarcity of jump-to-adhesion occurrences indicates van der Waals forces to be minimally important and the lack of consistent trend in adhesion forces and distances with surface charge indicates the interactions is unlikely to have a significant component that is electrostatic in nature. Adhesion interactions most likely have contributions from both surface forces and surface topology which can be difficult to deconvolute.

5. Conclusions

The average surface charge for the phase pure, polycrystalline hydroxyapatite studied here was found to be ~−0.02 C/m² which correlates reasonably well with zeta potential measurements reported in the literature [9,37,38,41]. The surface charge varies with nanoscale position on the surface and across grain
boundaries and is most likely associated with exposed crystal plane since different facets in the same grain have statistically different surface charges. Extra $\text{PO}_4^{3-}$ groups at the surfaces cause all surfaces to have a negative charge and variance is most likely due to different arrangements on each crystal plane of the additional charged ions making up the HA lattice. It is expected that surface charge has a strong influence on the processes of inorganic and organic deposition and structural evolution on the implant material, especially in the initial stages of implantation. New HRFS methodologies can give positionally sensitive measurement of nanoscale surface charge variation, which is an initial step in elucidating electrostatic effects on the bioactivity of HA.

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**Appendix A**

**Geometrical effects**

Since the HA sample probed is topographically heterogeneous, the variance in HRFS curves due to geometrical effects was evaluated. HRFS was approximated as the interaction between a spherical probe tip and surface $\text{PO}_4^{3-}$ groups at the surfaces cause all surfaces to have a negative charge and variance is most likely due to different arrangements on each crystal plane of the additional charged ions making up the HA lattice. It is expected that surface charge has a strong influence on the processes of inorganic and organic deposition and structural evolution on the implant material, especially in the initial stages of implantation. New HRFS methodologies can give positionally sensitive measurement of nanoscale surface charge variation, which is an initial step in elucidating electrostatic effects on the bioactivity of HA.

**Table 2**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition of abbreviations</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>CMAFM</td>
<td>Contact mode atomic force microscopy</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>$F-D$ curves</td>
<td>Force vs. tip-sample separation distance curves</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>HRFS</td>
<td>High-resolution force spectroscopy</td>
</tr>
<tr>
<td>IS</td>
<td>Ionic strength</td>
</tr>
<tr>
<td>MFP</td>
<td>Molecular force probe</td>
</tr>
<tr>
<td>RMS</td>
<td>Root mean squared</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-assembled monolayer</td>
</tr>
<tr>
<td>SBF</td>
<td>Simulated body fluid</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>VDW</td>
<td>Van der Waals</td>
</tr>
<tr>
<td>WAXD</td>
<td>Wide angle X-ray diffraction</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
</tr>
<tr>
<td>XRF</td>
<td>X-ray fluorescence spectroscopy</td>
</tr>
</tbody>
</table>

**Table 1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Name</th>
<th>Value/units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a$</td>
<td>Elastic contact radius between probe tip and surface</td>
<td>nm</td>
</tr>
<tr>
<td>$A_{\text{contact}}$</td>
<td>Elastic contact area between probe tip and surface</td>
<td>nm$^2$</td>
</tr>
<tr>
<td>$A$</td>
<td>Hamaker constant</td>
<td>J</td>
</tr>
<tr>
<td>$C_0$</td>
<td>Bulk concentration of ions</td>
<td>mol/m$^3$</td>
</tr>
<tr>
<td>$D$</td>
<td>Probe tip–surface separation distance</td>
<td>nm</td>
</tr>
<tr>
<td>$D_{\text{jump-to-contact}}$</td>
<td>Separation distance at which the cantilever exhibits an attractive jump to the surface</td>
<td>nm</td>
</tr>
<tr>
<td>$E$</td>
<td>Young’s (elastic) modulus</td>
<td>Pa</td>
</tr>
<tr>
<td>$\varepsilon_w$</td>
<td>Dielectric permittivity of water</td>
<td>6.9 $\times 10^{-10}$ C/Nm$^2$</td>
</tr>
<tr>
<td>$f$</td>
<td>Faraday constant</td>
<td>96,500 C/mol</td>
</tr>
<tr>
<td>$F$</td>
<td>Probe tip–surface force</td>
<td>nN</td>
</tr>
<tr>
<td>$F_{\text{adhesion}}$</td>
<td>Maximum attractive force measured on retraction</td>
<td>nN</td>
</tr>
<tr>
<td>$F/R_{\text{TIP}}$</td>
<td>Force per probe tip end radius</td>
<td>mN/m</td>
</tr>
<tr>
<td>$K$</td>
<td>Reduced modulus for probe tip and surface</td>
<td>Pa</td>
</tr>
<tr>
<td>$k_B$</td>
<td>Boltzmann’s constant</td>
<td>$1.38 \times 10^{-23}$ J/K</td>
</tr>
<tr>
<td>$k_c$</td>
<td>Cantilever spring constant</td>
<td>N/m</td>
</tr>
<tr>
<td>$n$</td>
<td>Number of force vs. distance curves</td>
<td></td>
</tr>
<tr>
<td>$pK_a$</td>
<td>The pH at which the ionizable compound is 50% protonated and 50% deprotonated</td>
<td></td>
</tr>
<tr>
<td>$R$</td>
<td>Universal gas constant</td>
<td>8.314 J/mol K</td>
</tr>
<tr>
<td>$R_{\text{TIP}}$</td>
<td>Probe tip end radius measured experimentally by SEM</td>
<td>nm</td>
</tr>
<tr>
<td>$T$</td>
<td>Absolute temperature</td>
<td>K ($RT = 298$)</td>
</tr>
<tr>
<td>$Z$</td>
<td>Direction normal to sample surface</td>
<td>Unitless</td>
</tr>
<tr>
<td>$\phi$</td>
<td>Electrostatic potential</td>
<td>V</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Surface charge per unit area</td>
<td>C/m$^2$</td>
</tr>
<tr>
<td>$\kappa^{-1}$</td>
<td>Electrical interaction Debye length</td>
<td>nm</td>
</tr>
<tr>
<td>$\nu$</td>
<td>Poisson’s ratio</td>
<td>Unitless</td>
</tr>
</tbody>
</table>

Units: C = Coulombs, J = Joules, K = Kelvins, m = meters, N = Newtons, nN = nanoNewtons, nm = nanometers, Pa = Pascals, and V = Volt.
and a sloped surface (Fig. 11a). Variable $D$ is the tip-surface distance measured by the MFP. However, due to the possible slope of the surface there are portions of the probe tip that could be interacting with the surface at distance $D'$, which would change how the tip–surface interaction area varied with distance. The change in tip interaction distance vs. $D$ at various possible surface slopes is shown in Fig. 11b. Height profiles via the 3DMFP software were taken across the probe locations as demonstrated in Figs. 8a and 9a, and the slope of the profile corresponding to probe location was calculated. Absolute values of slopes ranged from 0.34°/C14 to 22.64°/C14.

The plot of averaged ($n = 3$) Poisson–Boltzmann fitted surface charge vs. slope as shown in Fig. 12 demonstrates no significant trend. The $R^2$ value was found to be 0.0292 while the $R^2$ value needed for statistical significance ($p = 0.05$) is at least 0.532. In conclusion, the range of sample surface slope is not large enough to significantly change tip interaction area or force data. This is consistent with geometrical calculations since a sample surface slope of 22.64°, the maximum slope probed in this experiment, would change the tip interaction area by less than 8% at 15nm (5k−1 at 0.01 m), the approximate maximum range of electrostatic interactions at 0.01 IS. Because the geometrical constraints do not significantly alter the force curves, trends seen in force curves are likely due to electrostatic differences.

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