Orientation and conformation of osteocalcin adsorbed onto calcium phosphate and silica surfaces

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(Received 21 February 2017; accepted 2 May 2017; published 18 May 2017)

Adsorption isotherms, circular dichroism (CD) spectroscopy, x-ray photoelectron spectroscopy (XPS), and time-of-flight secondary ion mass spectrometry (ToF-SIMS) were used to investigate the adsorption of human osteocalcin (hOC) and decarboxylated (i.e., Gla converted back to Glu) hOC (dhOC) onto various calcium phosphate surfaces as well as silica surfaces. The adsorption isotherms and XPS nitrogen signals were used to track the amount of adsorbed hOC and dhOC. The intensities of key ToF-SIMS amino acid fragments were used to assess changes in the structure of adsorbed hOC and dhOC. CD spectra were used to investigate the secondary structure of OC. The largest differences were observed when the proteins were adsorbed onto silica versus calcium phosphate surfaces. Similar amounts (3–4 at. % N) of hOC and dhOC were adsorbed onto the silica surface. Higher amounts of hOC and dhOC were adsorbed on all the calcium phosphate surfaces. The ToF-SIMS data showed that the intensity of the Cys amino acid fragment, normalized to intensity of all amino acid fragments, was significantly higher ($\sim \times 10$) when the proteins were adsorbed onto silica. Since in the native OC structure the cysteines are located in the center of three α -helices, this indicates both hOC and dhOC are more denatured on the silica surface. As hOC and dhOC denature upon adsorption to the silica surface, the cysteines become more exposed and are more readily detected by ToF-SIMS. No significant differences were detected between hOC and dhOC adsorbed onto the silica surface, but small differences were observed between hOC and dhOC adsorbed onto the calcium phosphate surfaces. In the OC structure, the α -3 helix is located above the α -1 and α -2 helices. Small differences in the ToF-SIMS intensities from amino acid fragments characteristic of each helical unit (Asn for α -1; His for α -2; and Phe for α -3) suggests either slight changes in the orientation or a slight uncovering of the α -1 and α -2 for adsorbed dhOC. XPS showed that similar amounts of hOC and dhOC were adsorbed onto hydroxyapatite and octacalcium phosphate surfaces, but ToF-SIMS detected some small differences in the amino acid fragment intensities on these surfaces for adsorbed hOC and dhOC. © 2017 American Vacuum Society. [<http://dx.doi.org/10.1116/1.4983407>]

I. INTRODUCTION

Mineralizing proteins control the size, structure, morphology, and functions of bone and other hard tissues by an

intricate crystal engineering process called biomineralization, and thereby provide structural support and protection to the vertebrates.^{1–5} Proteins adsorbed onto the crystal face often undergo structural rearrangements to execute their mineralization role effectively. Any obstruction in such transformations can lead to complications such as osteoporosis,

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osteomalacia chronic kidney disease, dental calculus formation, artificial heart valve calcification, and arthritis.⁶⁻⁸ Extracellular matrix mainly consists of type 1 collagen, non-collagenous proteins, proteoglycans, and the inorganic mineral hydroxyapatite (HAP).⁹ The noncollagenous proteins are present in smaller but significant levels, and undergo numerous posttranslational modifications such as phosphorylation and carboxylation that confers increased negative charge density and helical modulation required for efficacious interaction with bone minerals. Osteocalcin (OC) is one such noncollagenous protein that is secreted by osteoblasts during bone remodeling and serves as an important biological marker of bone formation.^{10,11}

OC belongs to the vitamin K dependent family of proteins and undergoes vitamin K carboxylase assisted posttranslational carboxylation of glutamic acid (Glu) residues at positions 17, 21, and 24, converting them into γ -carboxy glutamic acid (Gla) residues. Under physiological conditions, the negatively charged Gla residues enhance the protein incorporation by strongly chelating with Ca^{2+} ions in the HAP surface.¹²⁻¹⁴ Osteoblast cells treated with the carboxylation inhibitor warfarin produced a lower percentage of OC bound HAP and higher undercarboxylated OC (ucOC) in serum compared to untreated osteoblast cells.¹⁵ Ducy *et al.* reported that OC inhibits bone formation by observing that an OC knockout mouse has increased rate of bone formation and higher bone mass.¹⁶ Recently, OC has also been shown to be involved in a variety of endocrinal regulatory process such as energy metabolism, β -cell proliferation, testosterone, and insulin secretion in addition to its role in mineralization.¹⁷⁻²³ For example, adipocytes exposed to ucOC increased its ability to release adiponectin, a glucose regulating protein.¹⁵ Similar enhancements have also been noticed with pancreatic islet cells in producing insulin and cyclin D.^{22,23} The above findings combined with other investigations suggest that post-translational carboxylation of OC is vital for regulating the mineral density of bone²⁴ while ucOC specifically functions as a hormone like substance that controls glucose metabolism, insulin secretion, and male fertility.^{25,26}

Results gathered from various immunological experiments on animal models have been used to demonstrate the general roles of OC and ucOC. However, there is a remarkable difference between human and other genomes, and how vitamin D regulates OC gene expression.^{27,28} Most of the species studied have higher percentage of γ -carboxylation and bone mineral density compared to human bone, and this variation is due to lower dietary vitamin K intake in humans.²⁹⁻³¹ Although the central portion of the OC is strongly conserved with high sequence similarity among vertebrates, the N terminus vary significantly and accounts for the differences in calcium binding affinity and induced helicity between species.^{10,11} To better understand the functional impact of OC carboxylation on human bone morphology, a molecular level structural analysis needs to be carried out specifically on human OC (hOC) and its decarboxylated version (dhOC). In the present study, we have investigated the

conformational transition and mineralization effect of hOC and dhOC on HAP surfaces in the presence and absence of calcium ions with circular dichroism (CD) and binding isotherm analysis. In addition, x-ray photoelectron spectroscopy (XPS) and time of flight secondary ion mass spectrometry (ToF-SIMS) studies were carried out for the proteins bound on HAP surfaces to investigate the amount, conformation and orientation of hOC and dhOC adsorbed onto HAP surfaces. Complementary, multitechnique analysis of surface bound proteins is required because unlike the situation where detailed structures can be obtained by one technique when proteins are crystalized (x-ray diffraction) or in solution (NMR or cryoelectron microscopy), no single surface analysis technique can be used to obtain detailed structural information.³²

II. MATERIALS AND METHODS

A. Protein synthesis and purification

hOC and dhOC were synthesized by Fmoc solid-phase peptide synthesis. Fmoc-protected amino acids and the pre-loaded resin Fmoc-Val-NovaSyn-TGA (0.25 mmol/g substitution) were purchased from Novabiochem. Fmoc- γ -carboxy-Glu(OtBu)₂-OH was purchased from Bachem. All other chemicals were obtained from Sigma Aldrich and used without further purification. The synthesis was carried out on an automated Rainin PS3 peptide synthesizer. All amino acids were coupled with a fourfold excess of protected amino acids. Coupling was performed using the activator 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate in 0.4 M *N,N*-diisopropylethylamine in dimethylformamide. For Fmoc deprotection, 20% piperidine in *N*-methyl-2-pyrrolidone was used. Resin cleavage and deprotection of side chain protecting groups were simultaneously carried out with a cleavage cocktail composed of 94% trifluoroacetic acid (TFA), 2.5% 1,2-ethanedithiol, 1% triisopropylsilane, and 2.5% water. After 3.5 h stirring at room temperature, the solution was filtered and concentrated to approximately 5 ml. The TFA/protein solution was then precipitated into a cold hexane/diethyl ether (3:7) mixture. The disulfide bond between Cys 23 and Cys 29 was obtained by passing air bubbles into the solution of protein containing 3 M guanidine. The solution was concentrated using pressure ultrafiltration with regenerated cellulose membrane. All protein samples were purified by reverse-phase high performance liquid chromatography (HPLC) using a 0.1% TFA containing acetonitrile/water mobile phase and characterized by electrospray ionization mass spectrometry (ESI-MS).

B. HAP preparation

1. HAP

All the reagents required for HAP synthesis were prepared using carbon dioxide free, nanopure water. A two neck round bottom flask fitted with a reflux condenser was charged with 1 l of 0.02 M $\text{Ca}(\text{OH})_2$ solution and heated to 50 °C. An aqueous solution of 0.24 M H_3PO_4 was added

slowly at a rate of 150 ml/min using a syringe pump. The addition was stopped when the reaction reached a *pH* of 10. The reaction mixture was continuously stirred for additional 36 h at 50 °C. The resulting HAP crystals were filtered, washed thoroughly with water. The wet HAP was dispersed in 60 ml of water and the *pH* of the suspension was brought down to 7.4 using H₃PO₄. The resulting HAP suspension was aged at 37 °C for 4 days. The surface area was determined using N₂ Brunauer-Emmett-Teller (BET) measurements and the crystal structure was confirmed by x-ray powder diffraction.

2. HAP90

Crystalline HAP90 powder sample was prepared by a dropwise addition of 0.12 M (NH₄)₂HPO₄ solution to an aqueous solution of 0.2 M Ca(NO₃)₂ · 4H₂O, at 90 °C. The *pH* was held constant at 11 by adding NH₄OH. After the (NH₄)₂HPO₄ addition was completed, the mixture was allowed to digest for 2 h and then filtered. The powder was rinsed, dried by lyophilization, and then sieved using an opening of 74 μm. The crystal structure was confirmed by x-ray powder diffraction.

3. HAP5

A HAP powder with a lower degree of crystallinity was made with the same reagents used to synthesize HAP90, but changing a few parameters. The synthesis temperature was lowered to 5 °C, the *pH* was raised to 12 by using KOH, and the digesting time was increased to 3 h. The crystal structure was confirmed by X-ray powder diffraction.

4. OCP

Crystalline octacalcium phosphate (OCP) was made following the LeGeros route³³ where 250 ml of a 0.04 M phosphate solution was added to 250 ml of a 0.04 M calcium solution. The *pH* was held at 6, and the temperature was 60 °C. After 4 h, the OCP precipitate was collected by filtration. The crystal structure was confirmed by x-ray powder diffraction.

C. Circular dichroism spectroscopy

CD spectra were recorded on a Jasco J-720 spectropolarimeter equipped with an electronic temperature controller using 1.0 mm path length quartz cuvette at 22 °C. A total of eight scans were averaged to obtain each spectrum. All the samples were prepared in 20 mM Tris buffer containing 150 mM NaCl at *pH* 7.4. OC solutions of 20 μM were incubated at 22 °C with Ca²⁺ concentrations ranging from 0.1 to 5 mM. CDs were recorded for calcium free OC and then as the Ca²⁺ solution concentration was increased stepwise with a 5 min incubation period at each Ca²⁺ concentration. All spectra were collected using a bandwidth of 2 nm and were corrected for solvent effects. CD spectra are presented as the mean residue ellipticity versus wavelength. The raw CD data

obtained in millidegrees was converted into mean residue ellipticity using the following equation:

$$[\theta] = (\theta \times 10^{-3} \times 100) / C \times n \times l,$$

where $[\theta]$ is the mean residue ellipticity in deg cm² dmol⁻¹, θ the raw output-ellipticity in millidegrees, *C*, the protein concentration in molar, *n*, the number of amino acid residues, and *l* is the cell path length in centimeter.

D. Equilibrium adsorption binding isotherm

Stock solutions of protein and HAP were prepared in 20 mM tris buffer containing 150 mM NaCl at *pH* 7.4 with or without 3 mM CaCl₂. Protein samples of eight different concentrations (5 to 80 mM) were equilibrated with the HAP suspension (1 mg/ml) for 2 h at 22 °C under constant, gentle vortexing. The samples were then centrifuged, and the protein concentration in the supernatant was determined using a Micro BCA protein assay reagent kit (Pierce), recording the absorbance at 562 nm on a Safire TECAN plate reader. The difference in protein concentration [*C*] (moles/l) between before and after adsorption onto the HAP particles was divided by the HAP surface area per gram (m²/g) and the concentration of HAP suspension (g/l) to get the number of moles adsorbed per square meter (*N*) onto the HAP surface. This value was plotted against the equilibrium concentration [*C*] of protein in solution to obtain the adsorption isotherms. The *K_d* values were obtained by nonlinear curve fitting in the origin software program using a single-site binding model.

E. Protein adsorption for XPS and ToF-SIMS

A 0.3 mg/ml protein solution in 20 mM tris buffer containing 150 mM NaCl at *pH* 7.4 was mixed with 0.2 mg/mL solution of calcium phosphate particles (HAP, HAP90, HAP5, and OCP) for 3 h at room temperature using a constant gentle vortexing. The silicon wafer substrates used for OC adsorption were 1 × 1 cm in size and covered by a native oxide layer. This native oxide layer is typically ~2 nm thick with a stoichiometry of SiO₂. At a *pH* of 7.4, the oxide surface should be negatively charged and contain hydroxyl groups. For adsorption onto the silicon wafer surface, the wafer was placed into 500 μl of the protein solution for 3 h. After adsorption, the powder and the Si wafer samples were rinsed 2 × with buffer and 3 × with purified water. After rinsing, the Si wafer was dried with flowing N₂, and the powder was dropped onto a Si wafer and let dry overnight.

F. X-ray photoelectron spectroscopy

XPS is a quantitative, surface sensitive (up to 10 nm sampling depth) analytical technique used to measure surface element composition.³⁴ The composition is calculated from the peak areas detected from each element, corrected for the instrument parameters (transmission function, etc.) and sensitivity factors.

The XPS system used was an S-Probe from Surface Science Instruments equipped with a monochromatic Al K α

TABLE I. Amino acid sequences and ESI-MS determined masses of hOC and dhOC (see Fig. S1 for mass spectra).

Protein	Amino acid sequence ^a	ESI-MS
hOC	YLYQWLGA VPYPDP LY EP RR γ EVC γ ELNPDCDELADHIGFQEAYRRFYGPV	5930.0
dhOC	YLYQWLGA VPYPDP LE PR REVCE LN PDCDELADHIGFQEAYRRFYGPV	5797.6

^aGla (γE) residues are highlighted bold.

X-ray source and a hemispherical analyzer at a 55° take-off angle (angle between the sample surface normal and the axis of the XPS analyzer lens). The x-ray source was operated at 10 kV and 20 mA (200 W anode power) with an 800 μm spot size on the sample. The spectra used to determine the surface elemental compositions were determined from scans acquired with an 150 eV analyzer pass energy using a step size of 1.0 eV. A low-energy electron flood gun was used for charge neutralization. Three spots on each of the three replicate samples were analyzed. The hydrocarbon peak in the C (1s) spectrum at 284.6 eV was used to reference the binding energy scale for each spectrum. The C (1s) spectra were acquired using 50 eV pass energy and 0.065 eV step size.

G. Time of flight secondary ion mass spectrometry

ToF-SIMS in the static mode provides a mass spectrum of the species in the outer ~2 mm of the sample.³⁵ ToF-SIMS data were acquired with an ION-TOF 5–100 (ION-TOF, Münster, Germany) instrument with a pulsed 25 keV Bi₃⁺ primary ion source. The total ion dose used to acquire each spectrum was $<9 \times 10^{11}$ ions/cm². The area analyzed in each spectrum was 100 × 100 μm, and spectra were acquired from five spots on each of three replicate samples. Only positive secondary ion spectra were acquired since it has been shown previously that for adsorbed proteins they are most informative.³⁶ Positive ion spectra were mass calibrated using the CH₃⁺, C₂H₃⁺, C₃H₅⁺, C₂H₆N⁺, and C₃H₃O⁺ fragments.

H. Principal component analysis

Due to the complexity ToF-SIMS data, where each spectrum can contain hundreds of peaks, it is beneficial to use methods such as principle component analysis (PCA) to process the data.^{37,38} PCA transforms the data into two cross-product matrices: scores and loadings. The scores plot provides the relationship between the samples and the loadings plot shows which peaks are responsible for any differences among samples. Before performing PCA, the diagnostic amino acids peaks (see supplementary material Table I)^{36,47} were selected and normalized by sum of selected peaks and then mean-centered. Also peaks ratios, selected based on the location of unique amino acids in the protein structure, were calculated to provide information about the orientation of the adsorbed protein. The NESAC/BIO Toolbox software was used for all PCA processing.³⁸ Confidence limits of 95% are shown in all PCA scores figures.³⁶

III. RESULTS AND DISCUSSION

A. Osteocalcin characterization

The hOC and dhOC sequences with their corresponding ESI-MS values are given in Table I. Both sequences were synthesized by automated solid phase peptide synthesis using standard Fmoc strategy and purified by HPLC. The purity of the sequence was analyzed by ESI-MS (see supplementary material Fig. S1). The OC 3D structure (RSCB Protein Data Base structure 1Q8H) is shown in Fig. 1. Although the amino acid sequence in the three helical regions is conserved among vertebrates, the unstructured N terminus region does vary among vertebrates.

Hydroxyapatite crystals were prepared by a wet chemical synthesis using calcium hydroxide and orthophosphoric acid in carbon dioxide free nanopure water. Transmission electron microscopy images showed the HAP morphology was needlelike crystals [see supplementary material Fig. S2(a)] and BET measurements gave a surface area of 60.6 m²/g. The crystal structure of the HAP crystals was confirmed by x-ray diffraction [see supplementary material Fig. S2(b)].

A comparison of calcium induced conformational changes between hOC and dhOC was investigated using CD spectroscopy. Figure 2 shows the CD spectra of hOC and dhOC in the absence and presence of 5 mM of Ca²⁺. The negative CD band displayed by Ca²⁺ free hOC at 205 represents a random coil structure, and the enhanced CD signals at 208 and 222 nm in the presence of Ca²⁺ correspond to the formation of a highly α-helical structure. Although dhOC displayed somewhat higher helicity compared to hOC in the absence of Ca²⁺ due to decreased net negative charge and charge repulsive

FIG. 1. 3D structure of OC (RSCB Protein Data Base structure 1Q8H, <http://www.rcsb.org/pdb/explore.do?structureId=1q8h>) showing the arrangement of the three helical secondary units and the location of the Cys, Asn, His, and Phe amino acids.

FIG. 2. Circular dichroism spectra of hOC (solid lines) and dhOC (dotted lines) in 20 mM Tris with 150 mM NaCl at pH 7.4 before and after treatment with 5 mM Ca^{2+} at 22 °C.

interactions, it did not undergo pronounced conformational transition when Ca^{2+} was added to the solution. This is in agreement with the earlier studies that has been done on other species such as bovine, chicken, and fish using spectroscopy and x-ray crystallography.^{12–14,39–41} OC in metal free solution is relatively unstructured and, in the presence of Ca^{2+} ions, exhibits multiple helical regions. This induced α -helical conformation brings the highly negatively charged Gla residues to the highly conserved first helical region at regular spacing intervals, which provide a desirable spatial arrangement for HAP surface coordination. The distance between the dicarboxylic acid groups of Gla in OC (0.45–0.6 nm) aligns with the interatomic lattice spacing of calcium ions (0.55 nm) in the x-y planes of the HAP crystal and facilitates the binding of OC with metal ions and HAP.^{10,11} The higher α -helical change obtained for calcium bound hOC compared to dhOC indicates the important role of Gla dicarboxylic acid groups in calcium binding and subsequent conformational transition.

CD titration curves and the corresponding one-site binding fit obtained for hOC with increasing amounts of Ca^{2+} are

shown in Fig. 3. hOC (20 mM) was titrated with incremental additions of Ca^{2+} ranging from 0 to 5 mM. Induced conformational change reached a maximum at 3 mM of Ca^{2+} concentration. Molar ellipticity difference at 222 nm relative to Ca^{2+} free hOC was plotted versus increasing Ca^{2+} concentration, and a K_d value of 0.33 ± 0.04 mM was obtained for the Ca^{2+} binding.

B. Osteocalcin binding efficiency and equilibrium isotherms

OC-HAP binding efficiency was examined by treating a 40 mM protein solution with 1 to 4 mg/ml of HAP, both in the presence and absence of 3 mM Ca^{2+} . As can be seen from Fig. 4(a), HAP bound more hOC in the presence of Ca^{2+} (almost 80% for 1 mg of HAP with Ca^{2+} compared to 55% in the absence of Ca^{2+}). A similar trend was observed for dhOC; however, the percent bound was lower than that exhibited by hOC under same conditions. Although dhOC did not undergo a prominent helical shift in the presence of Ca^{2+} , its binding affinity toward HAP was significantly increased in the presence of Ca^{2+} . This indicates that calcium plays other mechanistic roles than just inducing conformational changes.

Surface coverage and binding affinity in the presence and absence of Ca^{2+} were measured by equilibrium adsorption isotherm analysis. The best-fit values for the equilibrium constant K and maximal coverage N_{max} determined with a single site Langmuir adsorption model are reported in Table II. Binding isotherms in Fig. 4(b) indicate that hOC has the highest surface coverage of 0.61 mmol/m² in the presence of Ca^{2+} . Relative to hOC the surface coverage of dhOC was not influenced as much by Ca^{2+} , likely due to its inability to undergo the helical transformation needed for HAP adsorption. However, the binding affinity was higher in the presence of Ca^{2+} than in the absence of Ca^{2+} .

C. Osteocalcin adsorption onto silicon wafer and HAP powder surfaces

Since the position of the Gla residues in OC are optimal for binding to calcium ions on HAP surfaces, we first

FIG. 3. (a) CD titration of hOC; (b) change in molar ellipticity at 222 nm relative to Ca^{2+} free hOC with the Ca^{2+} concentration increasing from 0 to 5 mM at 22 °C. All measurements were made in 20 mM Tris with 150 mM NaCl at pH 7.4.

FIG. 4. (a) Percentage of hOC (green) and dhOC (red) bound to HAP at 22 °C in the absence and presence of 3 mM Ca²⁺ in 20 mM Tris with 150 mM NaCl at pH 7.4; (b) Equilibrium adsorption isotherms of hOC (green) and dhOC (red) at 22 °C in the absence (•) and presence (■) of 3 mM Ca²⁺ in 20 mM Tris with 150 mM NaCl at pH 7.4.

compared OC adsorption onto a HAP surface with OC adsorption onto a surface that did not contain calcium (silicon wafer). XPS survey scans (supplementary material Fig. S3) were used to measure the surface concentration of each element present in the samples (Table III) as well as the binding energy of each peak (see supplementary material Table S2). Also, since the substrates do not contain N and the protein does, the N was used as a marker to indicate the presence of adsorbed protein.

As shown in Table III, nitrogen was only detected after exposure to hOC and dhOC solutions, indicating that hOC and dhOC were adsorbed onto both Si wafer and HAP surfaces. The decrease in the concentration of substrate species (e.g., Ca and P for HAP) along with the increase of the C concentration after hOC and dhOC adsorption is also consistent with the presence of adsorbed protein on both surfaces. The XPS determined Ca/P ratio of the HAP surface prior to adsorption was close to the expected stoichiometric value of 1.67 and did not change significantly after OC adsorption. However, the standard deviation of the Ca/P ratio did increase noticeably, suggesting the HAP surfaces with adsorbed proteins had larger concentration variations than the surfaces without adsorbed proteins. A small amount of adventitious carbon (~6 at. %) was detected on the HAP surface prior to protein adsorption. Protein adsorption did not result in any significant changes in the substrate peak binding energies (see Table S2).

The XPS results in Table III show that the amount of protein adsorbed onto the Si wafer surface was similar for hOC

and dhOC (~3.5 at. %N). For the adsorption onto the HAP surface, the nitrogen concentration was higher for both proteins compared with the adsorption onto the Si wafer surface. This can be explained by the affinity of OC with the Ca ions at the HAP surface. For the HAP surface, adsorbed hOC resulted in slightly higher nitrogen concentrations than adsorbed dhOC (~7 vs ~5 at. %). However, the large standard deviation of the nitrogen concentration for hOC adsorbed onto HAP (7.0 ± 1.6 at. %) reduces the significance of the small difference between hOC and dhOC adsorbed onto HAP. These adsorption experiments were done without Ca²⁺ ions in solution, and it should be noted that without the presence of Ca²⁺ ions in the adsorption solution the equilibrium isotherm results in Table II show the amount of hOC and dhOC adsorbed onto the HAP surfaces is the same within experimental error.

The ToF-SIMS/PCA results for OC adsorption onto HAP and Si wafer surfaces are shown in Fig. 5. PC1 captures 91% of the total variance in the data set and the PC1 scores plot in Fig. 5(a) clearly distinguishes the adsorption onto Si wafer substrates (positive scores) from adsorption onto HAP substrates (negative scores). The dotted lines in the scores plot represent the 95% confidence limit for each sample. ToF-SIMS/PCA does not show a significant difference between hOC and dhOC adsorbed onto Si wafer surfaces, but does show a small, but significant at the 95% confidence level, difference between hOC and dhOC adsorbed onto HAP surfaces. The PC1 loading plot shown in Fig. 5(b) can be used to provide some insight for the differences observed in the PC1 scores. The PC1 loadings indicate that the intensity of the Cys amino acid fragment is higher for hOC and dhOC adsorbed onto the Si wafer surfaces compared to hOC and dhOC adsorbed onto the HAP surfaces. The PC1 loadings also indicate that the intensity of the Arg and Pro amino acid fragments are higher for hOC and dhOC adsorbed onto the HAP surfaces compared to hOC and dhOC adsorbed onto the Si wafer surfaces.

To investigate in further detail the differences between hOC and dhOC adsorption onto HAP, PCA was done using

TABLE II. Surface coverage and binding affinity of hOC and dhOC to HAP at 22 °C in the presence and absence of 3 mM Ca²⁺ in 20 mM Tris with 150 mM NaCl at pH 7.4.

Protein	Ca ²⁺ (mM)	N _{max} (10 ⁻⁷ mol/m ²)	K (10 ⁵ /M)
hOC	0	3.73 ± 0.38	3.90 ± 0.92
hOC	3	6.13 ± 0.56	3.53 ± 1.05
dhOC	0	3.38 ± 0.33	0.55 ± 0.12
dhOC	3	4.28 ± 0.10	3.45 ± 0.31

TABLE III. XPS determined surface element composition (atomic percent) for HAP and Si wafer surfaces, before and after protein adsorption. The Ca/P atomic ratio is also shown for the HAP surfaces. nd = not detected.

Sample	O at. %	Ca at. %	P at. %	C at. %	N at. %	Si at. %	Ca/P atom ratio
HAP	59.8 ± 0.9	21.0 ± 0.5	12.8 ± 0.5	6.3 ± 0.5			1.64 ± 0.01
HAP-dhOC	47.3 ± 1.5	15.8 ± 0.6	10.0 ± 0.4	21.2 ± 2.9	5.0 ± 0.4		1.58 ± 0.09
HAP-hOC	42.2 ± 4.0	13.2 ± 1.9	8.3 ± 1.1	29.3 ± 5.4	7.0 ± 1.6		1.59 ± 0.60
Si-dhOC	35.2 ± 1.2	nd	nd	28.9 ± 2.3	3.7 ± 0.5	32.3 ± 1.5	—
Si-hOC	37.1 ± 1.5	nd	nd	24.5 ± 2.2	3.1 ± 0.2	35.1 ± 1.3	—

only the ToF-SIMS data from these two samples (see Fig. 6). PC1 captures 85% of the variance between these two samples. The PC1 scores plot in Fig. 6(a) shows the two samples are separated at the 95% confidence level. The PC1 loading plots in Fig. 6(b) show which amino acid fragments are responsible for this separation. The Arg and Pro amino acid load positively, suggesting they have higher intensity when dhOC is adsorbed onto HAP. Conversely, the Gln/Glu, Ile/Leu, His, Phe, Trp, and Tyr fragments load negatively, suggesting they have higher intensities when hOC is adsorbed onto HAP.

To examine the OC orientation on the Si wafer and HAP surfaces, the intensities of following amino acid fragments were used: (1) Asn fragments at $m/z = 70, 87, 88$, and 98 u; (2) His fragments at $m/z = 81, 82$, and 110 u; and (3) Phe fragments at $m/z = 120$ and 130 u. These three amino acids are each located in a different α helix unit of the OC structure (Asn in $\alpha 1$, His in $\alpha 2$, and Phe in $\alpha 3$). Previously, ToF-SIMS amino acid fragments ratios have been used to provide insight regarding the orientation and conformation of surface immobilized proteins.^{42–46} Since the secondary ions emitted in a ToF-SIMS experiment are preferentially emitted from the outermost surface of a sample, the amino acid fragment intensities will vary with changes in protein orientation and conformation. Therefore, examining how the intensity ratios of these peaks vary with type of protein (hOC versus dhOC) and surface (silica versus HAP) can provide insight to the structure of hOC and dhOC on these surfaces. Figure 7

shows the amino acid intensity ratios for Asn/His ($\alpha 1/\alpha 2$), Asn/Phe ($\alpha 1/\alpha 3$) and His/Phe ($\alpha 2/\alpha 3$) as well as the ratio of Cys to the total amino acid intensity for hOC and dhOC adsorbed onto both HAP and Si wafer surfaces.

For hOC and dhOC adsorbed onto the silicon wafer surfaces, no significant differences were observed between the two proteins in the ToF-SIMS Asn/His (0.8 and 0.8), Asn/Phe (2.4 and 2.3), and His/Phe (3.2 and 3.0) intensities ratios, suggesting that whether or not the Glu residues are carboxylated does not have an effect on the adsorbed protein structure. For hOC and dhOC adsorbed onto the HAP surfaces, the ToF-SIMS Asn/His (0.8 and 1.1), Asn/Phe (1.7 and 2.2), and His/Phe (2.0 and 2.1) intensities ratios were similar, with the Asn/His and Asn/Phe intensity ratios being slightly higher for adsorbed dhOC. The largest difference between the proteins adsorbed onto Si wafer and HAP surfaces was the ToF-SIMS Cys intensity, normalized to the total amino acid fragment intensities, which was $>10\times$ higher for hOC and dhOC adsorbed onto silica (1.0 and 0.8) compared to HAP (0.02 and 0.05) surfaces [Fig. 7(c)]. Since in the native OC structure the cysteines are in the center of the three α -helices,¹⁸ the high Cys intensity from adsorbed hOC and dhOC onto the Si wafer surfaces indicates both proteins are denatured upon adsorption, exposing the Cys. On the other hand, the low Cys intensity from adsorbed hOC and dhOC onto the HAP surfaces indicates both proteins likely retain their native 3D structure. It is not surprising that there are significant differences between OC adsorption onto the Si

FIG. 5. PC1 (a) scores and (b) loadings plots for the adsorption of hOC and dhOC onto wafer and HAP surfaces. See Fig. 6 for the PCA scores and loadings for the adsorption of hOC and dhOC adsorption onto just the HAP surface.

FIG. 6. PC1 (a) scores and (b) loadings plots for the adsorption of hOC and dhOC onto HAP surfaces.

wafer and HAP surfaces. The HAP surface has positively charged Ca^{2+} ions located in an arrangement that are well-suited to strongly interact with the negatively charged Glu residues in hOC. In contrast, the silica oxide overlayer on the Si wafer not only lacks the positive Ca^{2+} ions, but it is negatively charged at the protein adsorption conditions (pH 7.4). Thus, the interactions of the Glu residues will be significantly different on silica surfaces.

Some additional differences were observed for the ToF-SIMS intensity ratios from the three helices. Comparison of the amino acid ToF-SIMS peak intensity ratios for hOC

adsorbed onto HAP and Si wafer surfaces shows that Asn($\alpha 1$)/His($\alpha 2$) is the same (0.8 and 0.8), Asn($\alpha 1$)/Phe($\alpha 3$) increases (1.7–2.4), and His($\alpha 2$)/Phe($\alpha 3$) increases (2.0–3.2), which suggests the peaks from the $\alpha 1$ and $\alpha 2$ helices are most intense, and therefore less covered by the $\alpha 3$ helix when hOC is adsorbed onto the Si wafer surface. This is consistent with the ToF-SIMS Cys results that show hOC is more denatured on the Si surface compared to the HAP surface.

For dhOC adsorbed onto HAP and Si wafer surfaces Asn($\alpha 1$)/His($\alpha 2$) decreased (1.1–0.8), Asn($\alpha 1$)/Phe($\alpha 3$) was

FIG. 7. ToF-SIMS Asn/His ($\alpha 1/\alpha 2$), Asn/Phe ($\alpha 1/\alpha 3$) and His/Phe ($\alpha 2/\alpha 3$) peak ratios for hOC and dhOC adsorbed onto (a) HAP surfaces and (b) Si wafer surfaces. (c) ToF-SIMS Cys/(total amino acid) peaks ratios for hOC and dhOC adsorbed onto HAP and Si wafer surfaces.

TABLE IV. Table with the XPS determined atomic percentage of each element detected in HAP5, HAP90, and OCP surface along with the Ca/P atomic ratio, before and after protein adsorption. nd = not detected.

Sample	O at. %	Ca at. %	P at. %	C at. %	N at. %	Ca/P atom ratio
HAP5	46.8 ± 0.9	24.7 ± 0.3	19.4 ± 0.6	9.2 ± 0.5	nd	1.27 ± 0.06
HAP5-dhOC	46.2 ± 1.2	13.2 ± 0.6	8.6 ± 0.5	24.0 ± 1.7	5.2 ± 0.3	1.55 ± 0.10
HAP5-hOC	47.4 ± 1.7	13.5 ± 0.7	8.7 ± 0.5	22.7 ± 2.2	5.4 ± 0.6	1.55 ± 0.11
HAP90	48.6 ± 0.6	23.8 ± 0.1	18.3 ± 0.3	9.3 ± 0.2	nd	1.30 ± 0.01
HAP90-dhOC	47.2 ± 1.7	14.2 ± 0.7	9.3 ± 0.5	21.6 ± 2.2	4.7 ± 0.4	1.53 ± 0.10
HAP90-hOC	47.4 ± 2.1	15.5 ± 0.8	9.9 ± 0.5	22.5 ± 3.4	4.8 ± 0.3	1.56 ± 0.10
OCP	48.7 ± 0.1	24.2 ± 0.1	21.0 ± 0.3	6.2 ± 0.3	nd	1.15 ± 0.01
OCP-dhOC	48.0 ± 1.2	14.9 ± 0.5	10.7 ± 0.2	21.5 ± 1.2	4.9 ± 0.5	1.40 ± 0.05
OCP-hOC	49.2 ± 0.8	15.6 ± 0.3	11.0 ± 0.3	20.0 ± 0.6	4.2 ± 0.2	1.43 ± 0.05

similar (2.2 and 2.3) and His($\alpha 2$)/Phe($\alpha 3$) increased (2.1–3.0), which suggests the peaks from $\alpha 2$ helix are more intense, and therefore, the $\alpha 2$ helix is less covered by the $\alpha 3$ helix when dhOC is adsorbed onto the Si wafer surface. This is consistent with the ToF-SIMS Cys results that show hOC is more denatured on the Si wafer surface compared to the HAP surface. However, the differences between hOC and dhOC adsorbed onto the HAP surface for the ToF-SIMS peak intensity ratios involving the $\alpha 1$ helix [Asn($\alpha 1$)/His($\alpha 2$) increasing from 0.8 to 1.1 and Asn($\alpha 1$)/Phe($\alpha 3$) increasing from 1.7 to 2.2] suggest hOC and dhOC have slightly different orientations on the HAP surface with the $\alpha 1$ helix more surface exposed in adsorbed dhOC. This is not surprising since the Glu residues are in the $\alpha 1$ helix and once they have been decarboxylated back to Glu residues the $\alpha 1$ helix interactions with the HAP surface are expected to decrease.

D. Osteocalcin adsorption onto OCP, HAP90, and HAP5 powders

The effect of the calcium phosphate composition and crystallinity on OC adsorption was also examined. For these studies, a crystalline hydroxyapatite (HAP90), a less crystalline hydroxyapatite (HAP5), and another type of calcium phosphate (OCP) were used. The x-ray powder diffractions for HAP90, HAP5, and OCP are shown in Figs. S2(c) and S2(d). The XPS determined surface compositions in Table IV and Fig. S4 show for all substrates that the nitrogen peak only appeared after protein adsorption, while Ca and P concentrations decreased in all samples after protein adsorption. This is consistent with an overlayer of protein attenuating the peaks from the calcium phosphate substrates. The peak positions of all elements detected by XPS did not vary significantly with either the type of calcium phosphate or protein adsorption (see Table S3). Several similarities and differences were observed when comparing the XPS results for the HAP5, HAP90, and OCP samples to the XPS results for the HAP sample. The amount of hOC and dhOC adsorbed onto all calcium phosphate surfaces was similar (~5 at. % N). The Ca/P atomic ratio for all protein covered hydroxyapatite surfaces (HAP, HAP5, and HAP90) was similar (~1.55) and close to the expected stoichiometric value of 1.67. The protein covered OCP surface exhibited a lower

Ca/P (~1.40), as expected since the stoichiometry Ca/P atomic ratio in OCP is 1.33. Unlike HAP, where the Ca/P atomic ratio did not change significantly upon protein adsorption, on the HAP5, HAP90, and OCP samples, there was a significant increase in the Ca/P atomic ratio upon protein adsorption. Since the Ca/P atomic ratios for protein covered HAP5, HAP90, and OCP were all close to the expected stoichiometry values, this suggests the preparation method for these three samples resulted in a slight surface excess of P on the starting surfaces, which was removed by the protein adsorption process. It is also possible that these calcium phosphate surfaces could be restructured by the protein adsorption.

The ToF-SIMS/PCA results from hOC and dhOC adsorbed onto HAP5, HAP90, and OCP surfaces show that all dhOC samples except one spot on one of the HAP5 replicates have positive PC1 scores, while all hOC samples except one spot on one of the OCP replicates have negative PC1 scores [Fig. 8(a)]. Thus, for these three calcium phosphate surfaces, the largest difference is between the type of adsorbed protein (i.e., hOC versus dhOC). PC2 did not show any significant separation among the samples, largely because of the larger spread of data points for both hOC and dhOC adsorbed onto the HAP5 sample (data not shown). Thus, to see if any differences between the type of calcium phosphate surfaces could be observed, a direct comparison of hOC and dhOC adsorption onto HAP90 and OCP was done. Again PC1 separates the hOC samples from the dhOC samples (see supplementary material Fig. S5). dhOC on both surfaces have positive PC1 scores while hOC on both surfaces have negative PC1 scores (PC1 captures 93% of variance in the data). PC2 captures 5% of the variance in the data and separates the samples by substrate with hOC and dhOC on OCP having positive PC2 scores and the majority of the hOC and dhOC on HAP90 having negative PC2 scores (see supplementary material Fig. S5). Thus, ToF-SIMS/PCA shows largest difference among the HAP5, HAP90, and OCP samples was between the type of adsorbed protein (hOC separated from dhOC) with the difference between the type of calcium phosphate substrate having a much smaller effect.

ToF-SIMS/PCA results for hOC and dhOC adsorbed onto HAP and HAP90 surfaces in the PC1 versus PC2 scores plot

FIG. 8. (a) PC1 scores plot and (b) PC1 loading plot for the adsorption of both proteins onto HAP5, HAP90, and OCP.

[Fig. 9(a)] does not show as clear of separation among the four different samples compared to the results in Figs. 8(a) and S5(a). There is a significant overlap of the PC1 and PC2 scores for dhOC adsorbed onto both HAP and HAP90, both residing primarily in the positive PC1 and positive PC2 quadrant. hOC adsorbed onto both HAP and HAP90 have negative PC2 scores, but adsorbed hOC is separated by substrate type on PC1. The amino acid fragments that

consistently show high loadings for all comparisons of hOC and dhOC adsorbed on the calcium phosphate surfaces [Figs. 6(b), 8(b), 9(b), 9(c), S5(b), and S5(c)] are Pro, Arg, and Tyr. Both Pro (6 of 7) and Tyr (4 of 6) are predominately located in the unstructured regions near the C and N termini. Thus, it is not surprising that the ToF-SIMS intensity of these fragments vary with protein type and surface type as the unstructured terminal regions should have sufficient

FIG. 9. (a) PC1 vs PC2 scores plot, (b) PC1 loading plot and PC2 loading plot for the adsorption of hOC and dhOC onto HAP and HAP90. PC1 and PC2 capture 91% and 5% of the total variance in the data set, respectively.

flexibility to explore, for example, small differences in surface structure of the substrates. Two Arg residues are located in the $\alpha 1$ helix with another two Arg residues located in the $\alpha 3$ helix. Although the Arg residues are located in α helical regions, they have long side chains that also could allow them sufficient flexibility to rearrange in response to small differences in the surface structure. The fragment CNH_4^+ also consistently shows high loadings for these samples. Although this fragment is typically associated with Gly residues, it can also be formed many other amino acids, and so, it is difficult to uniquely interpret the reason for its high loading.

For further insights into the structural differences of the adsorbed protein the α -helix peak ratio intensities were examined. For hOC and dhOC adsorbed onto HAP90 the Asn($\alpha 1$)/His($\alpha 2$) ratio was 1.1 and 1.2, the Asn($\alpha 1$)/Phe($\alpha 3$) ratio was 1.9 and 1.8, and the His($\alpha 2$)/Phe($\alpha 3$) ratio was 1.8–1.5, respectively [see Fig. S6(a)]. For hOC and dhOC adsorbed onto OCP, the Asn($\alpha 1$)/His($\alpha 2$) ratio was 1.5 and 1.3, the Asn($\alpha 1$)/Phe($\alpha 3$) ratio was 2.1 and 1.6 and the His($\alpha 2$)/Phe($\alpha 3$) ratio was 1.4 and 1.3, respectively [see Fig. S6(b)]. In addition, the ratio of the Cys fragment intensity to the total amino acid fragment intensity from hOC and dhOC adsorbed onto HAP5, HAP90, and OCP was <0.05 , consistent with low Cys fragment intensity observed from hOC and dhOC adsorbed onto HAP.

When comparing the three α -helical and the Cys peak ratios for both proteins on all surfaces (Si, HAP, HAP5, HAP90, and OCP), it is apparent that the largest difference is between the proteins adsorbed on the Si wafer surface and the proteins adsorbed onto the calcium phosphate surfaces. There are some small differences observed for the two proteins on the different calcium phosphate surfaces, which suggest only small changes in the protein conformation and orientation among those surfaces. However, when comparing the $\alpha 1/\alpha 3$, $\alpha 2/\alpha 3$, and Cys ratios for the proteins adsorbed onto the Si wafer surface, those ratios are all always smaller for the proteins adsorbed onto the calcium phosphate surfaces. In addition, the $\alpha 1/\alpha 2$ ratio is always higher for the proteins adsorbed on the calcium phosphate surfaces compared to the proteins adsorbed onto the Si wafer surfaces. The observations are all consistent with both proteins being more denatured and the $\alpha 3$ helix covering less of the $\alpha 1$ and $\alpha 2$ helices on the Si wafer surface.

IV. CONCLUSIONS

A multitechnique approach (equilibrium isotherms, CD, XPS, and ToF-SIMS) was used to characterize hOC and dhOC adsorbed onto various calcium phosphate surfaces as well as silica surfaces. An increased conformational transition of solution from random coil to α -helical structure in the presence of Ca^{2+} ions was observed for hOC, which in turn led to higher surface coverage of hOC on the HAP surface. Both hOC and dhOC were more denatured on the Si wafer surfaces compared to the calcium phosphate surfaces. Similar amounts of the proteins were adsorbed onto all

calcium phosphate surfaces, but the ToF-SIMS intensity patterns of the amino acids suggested that there were small differences in their conformation and orientation on the different calcium phosphate surfaces. The ToF-SIMS data showed that the intensity of the Cys amino acid fragment was a key marker for the extent of OC denaturation. Since in the native OC structure the cysteines are located in the center of three α -helices, as OC denatures, the cysteines will become more surface exposed and the ToF-SIMS intensity of the Cys fragment will increase. The ToF-SIMS Cys fragment intensity, normalized to the total amino acid fragment intensity, was $\sim 10\times$ higher from both hOC and dhOC adsorbed onto the Si wafer surfaces. No significant differences were detected between hOC and dhOC adsorbed onto the silica surface, but small differences were observed between hOC and dhOC adsorbed onto the calcium phosphate surfaces. ToF-SIMS intensities from amino acid fragments characteristic of each helical unit (Asn for $\alpha-1$; His for $\alpha-2$; and Phe for $\alpha-3$) were used to follow these differences.

ACKNOWLEDGMENTS

The authors gratefully acknowledge support from NIH Grant Nos. EB-002027 and DE-012554 for this study. L.A.S. gratefully acknowledges a scholarship from FAPERJ (support from Science, Technology and Innovation in the State of Rio de Janeiro). Elisa Harrison is thanked for discussions about the osteocalcin structure and preparing Fig. 1. Dan Graham, Mike Robinson, and Gerry Hammer are thanked for assistance with and discussions about XPS, ToF-SIMS, and PCA.

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