Interaction of Biological Molecules with Clay Minerals: A Combined Spectroscopic and Sorption Study of Lysozyme on Saponite

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ABSTRACT: The interaction of hen egg white lysozyme (HEWL) with Na- and Cs-exchanged saponite was investigated using sorption, structural, and spectroscopic methods as a model system to study clay–protein interactions. HEWL sorption to Na- and Cs-saponite was determined using the bicinchoninic acid (BCA) assay, thermogravimetric analysis, and C and N analysis. For Na-saponite, the TGA and elemental analysis-derived sorption maximum was 600 mg/g corresponding to a surface coverage of 0.85 ng/mm² with HEWL occupying 526 m²/g based on a cross-sectional area of 13.5 nm²/molecule. HEWL sorption on Na-saponite was accompanied by the release of 9.5 Na⁺ ions for every molecule of HEWL sorbed consistent with an ion exchange mechanism between the positively charged HEWL (IEP 11) and the negatively charged saponite surface. The d-spacing of the HEWL–Na-saponite complex increased to a value of 4.4 nm consistent with the crystallographic dimensions of HEWL of 3 × 3 × 4.5 nm. In the case of Cs-saponite, there was no evidence of interlayer sorption; however, sorption of HEWL to the “external” surface of Cs-saponite showed a high affinity isotherm. FTIR and Raman analysis of the amide I region of the HEWL–saponite films prepared from water and D₂O showed little perturbation to the secondary structure of the protein. The overall hydrophilic nature of the HEWL–Na-saponite complex was determined by water vapor sorption measurements. The clay retained its hydrophilic character with a water content of 18% at high humidity corresponding to 240 H₂O molecules per molecule of HEWL.

INTRODUCTION

Interest in the interaction of biomolecules with inorganic surfaces has significantly increased in recent years. Hybrid organic–inorganic materials that combine biological concepts with inorganic material chemistry are emerging at a rapid pace in a wide range of disciplines that span from biotechnology, materials science, and nanomedicine.¹–⁵ One class of inorganic materials that continues to attract attention is the group of 2:1 phyllosilicates known as smectites. Smectites are among the most abundant naturally occurring particles. Smectites are among the most abundant naturally occurring materials on earth on a surface area basis.⁶ Fundamental particles of smectite are sheet or lathe-like with a thickness of 1 nm and aspect ratios that range from 20 to 1000 and surface areas that approach 750 m²/g.⁷ These surfaces are also reactive resulting from the release of 9.5 Na⁺ ions for every molecule of HEWL sorbed consistent with an ion exchange mechanism between the positively charged HEWL and the negatively charged saponite surface. The d-spacing of the HEWL–Na-saponite complex increased to a value of 4.4 nm consistent with the crystallographic dimensions of HEWL of 3 × 3 × 4.5 nm. In the case of Cs-saponite, there was no evidence of interlayer sorption; however, sorption of HEWL to the “external” surface of Cs-saponite showed a high affinity isotherm. FTIR and Raman analysis of the amide I region of the HEWL–saponite films prepared from water and D₂O showed little perturbation to the secondary structure of the protein. The overall hydrophilic nature of the HEWL–Na-saponite complex was determined by water vapor sorption measurements. The clay retained its hydrophilic character with a water content of 18% at high humidity corresponding to 240 H₂O molecules per molecule of HEWL.

hydrophobic compounds such as nitroaromatics, pesticides, and dibenzo-p-dioxin.¹¹,¹² This dual hydrophobic–hydrophilic character²⁹,³⁰ has been attributed to account for the high affinity for certain classes of organic compounds with smectites, which include metal–ligand complexes,¹⁴ cationic dyes,¹⁶ and cationic surfactants.¹⁷ Although not fully understood or recognized, this dual hydrophobic/hydrophilic nature is thought to influence the interaction of proteins with smectites.

The interaction of proteins with clay minerals has been studied over the past 70 years²⁸ with emphasis in the past 10 years on the development of novel hybrid structural and functional biomaterials applied to biosensing, biocatalysis, and drug delivery.²⁸ Hen egg white lysozyme (HEWL) has been used as a model protein to study clay–protein interactions because of its high affinity for smectites and ability to intercalate into the clay interlayer.²¹–²³,²⁸ Sorption of HEWL can exceed values of 2000 mg lysozyme/g clay and is accompanied by interlayer expansion to d-spacings of 4.4 nm.²¹,²²,²⁸ HEWL is a “hard”, globular protein with a MW of 14 300 Da. The high affinity of HEWL
for smectites is attributed to its isoelectric point (IEP) of 11.4 that is attracted to the negatively charged clay surface at essentially all pH ranges of interest.\textsuperscript{31}

Recent studies have exploited HEWL as a type of molecular probe to study clay surfaces. Layer-by-layer (LbL) assembly methods were used recently to produce smectite–protein complexes using protamine, papain, and HEWL.\textsuperscript{32} All three of these positively charged proteins showed a high affinity for the Na-smectite. In related work, HEWL has been used in conjunction with surface force apparatus (SFA) studies and with AFM to investigate the adsorption of positively charged lysozyme onto negatively charged mica surfaces.\textsuperscript{33–36} In the SFA experiments, the surface forces between parallel sheets of mica have been examined in the presence of HEWL under a variety of conditions. Mica is a 2:1 phyllosilicate, similar to smectites, but characterized by a higher layer charge and by nonexpanding interlayers. SFA studies of HEWL–mica complexes reveal a hard wall repulsion at 3 nm, and another “force wall” at 6 nm, corresponding to 1 and 2 layers of HEWL between the mica sheets.\textsuperscript{33}

Although the general behavior of HEWL interactions with clay minerals is known including its use as a model probe in SFA and LbL studies, a detailed molecular understanding of HEWL–smectite complexes has not been reported. For example, clay-induced changes in protein secondary and tertiary structure and orientation of sorbed HEWL were made by Pall Life Sciences) on a Millipore holder under vacuum. The unapodized

**MATERIALS AND METHODS**

Lysozyme, a protein made of chicken-egg white purchased from Sigma-Aldrich (MW 14 600 with an isoelectric point of 11.4), was stored at 4 °C and used for this study. Saponite was obtained from the Source Clays Repository of the Clay Minerals Society. It was saturated with Na\textsuperscript{+} by repeated exchange with 1 M NaCl solutions followed by dialysis with water until the water tested Cl\textsuperscript{−}–free using AgNO\textsubscript{3}. The particle size fraction of 0.5–2.0 μm was obtained by centrifugation. The resulting 0.5–2.0 μm saponite dispersion was freeze-dried and stored as a powder at room temperature. After the 0.5–2.0 μm fraction of the Na-exchanged saponite was obtained by centrifugation, a portion of the clay was washed with a 0.1 M CsCl solution, followed by washing and freeze-drying to obtain Cs\textsuperscript{−}-exchanged saponite.

Sorption experiments were conducted in 25 mL glass (Correx) screw cap centrifuge tubes with two replications. Aliquots of a lysozyme stock solution and deionized distilled water were added to 12.5 mg of Na-saponite in the tubes to reach a final volume of 15 mL with lysozyme concentrations ranging from 0 to 2000 μg mL\textsuperscript{−1}. The tubes were shaken overnight (16 h) in a reciprocating shaker. The tubes were centrifuged at 6000 rpm for 20 min, and 7 mL of the supernatant was removed for analysis of pH, lysozyme, and Na content. The saponite–lysozyme complexes were redispersed with the remaining supernatant to make self-supported clay films.

The aqueous clay–protein dispersion was passed through a 0.45 μm Supor-450 hydrophilic polyethersulfone membrane (47 mm diameter, made by Pall Life Sciences) on a Millipore holder under vacuum. The resulting saponite–lysozyme deposits on the filter were allowed to air-dry and were removed from the filter by running the filter and clay deposit over a knife edge.

Lysozyme concentration in the supernatant was determined using the BCA Protein Assay Kit (Pierce). An adsorption isotherm was developed by plotting lysozyme sorbed (mg g\textsuperscript{−1} saponite) against the equilibrium concentration in the supernatant. Na\textsuperscript{+} concentration in the supernatant was measured using an atomic adsorption spectrophotometer-6800 (Shimadzu). Acetylene/air flame and a Na lamp were used with a Shimadzu auto sampler (model ASC-1600).

Infrared spectra of the self-supported clay films were obtained on a Perkin-Elmer GX2000 Fourier transform infrared (FTIR) spectrometer equipped with deuterated triglycine (DTGS) and mercury–cadmium–telluride (MCT) detectors, an internal wire grid IR polarizer, a KBr beam splitter, and a sample bench purged with dry air. The unpolarized resolution for the FTIR spectra was 2.0 cm\textsuperscript{−1}, and a total of 64 scans were collected for each spectrum. The FTIR spectrum of lysozyme crystals was obtained in KBr pellets corresponding to 2 mg sample in approximately 248 mg of spectral grade KBr. Postprocessing of the FTIR spectra was restricted to baseline correction, curve fitting, and integration using GRAMS/32 program (Galactic Software).

A set of self-supported clay films were used in thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) analysis after taking the FTIR spectra. The clay films were lightly ground into a powder and weighed in 70 μL ceramic crucibles. The crucibles were placed in the thermogravimetric analyzer (model — TGA/SDTAA851e, Mettler Toledo, OH) and ran in the temperature range of 25–1000 °C at an increment rate of 20 °C per minute in a N\textsubscript{2} atmosphere. For DSC analysis, powder samples were weighed into 40 μL aluminum sample pans. Sample pans were sealed and placed in the DSC (model — DSC 30
equipped with a TC15-TA controller, Mettler Toledo, OH) and run with two temperature segments in a N2 atmosphere. The first was from 25 to −150 °C at a cooling rate of 20 °C per minute followed by the heating portion from −150 to 600 °C at a heating rate of 20 °C per minute. Both TGA and DSC results were analyzed using STARRe software Version 9.0X (Mettler Toledo), which included normalization using the sample size, integration, and analysis of the first derivative.

X-ray diffraction patterns of self-supporting clay films of the lysozyme–saponite bionanocomposites were obtained from a PANalytical X-ray diffractometer (model X’Pert PRO; Natick, MA) using Co radiation. The self-supporting films were mounted horizontally on aluminum sample holders. Data were collected from 2° to 80° 2θ, counting for 1 s every 0.02° 2θ step using a 1° slit between 2θ and 12° 2θ. The d-spacing of all of the treatments of lysozyme sorbed Na-saponite was determined using Bragg’s Law \( n\lambda = 2d \sin \theta \), where \( n \) is an integer, lambda \( \lambda \) is the wavelength (1.78901 Å) of the incident X-ray beam, \( d \) is the distance between atomic layers of a crystal, and \( \theta \) is the angle between the incident rays and the scattering planes. Given the fixed variables \( n \) and \( \lambda \) with the angle at each position, we calculated the d-spacing (\( d \)) of the characteristic protein—clay peaks.

Raman spectra were collected on lysozyme crystals and self-supported clay films on an Action Research Corp. SpectroPro500 spectrograph. A Spectra-Physics (model 127) helium—neon laser with 632 nm wavelength was used as the excitation source. The spectrograph used a holographic grating with 1200 grooves per millimeter with a blaze wavelength of 532 nm. The detector was a Princeton Instruments liquid N2 cooled CCD detector with an active array of 1100 (H) x 330 (V) pixels. Lysozyme crystals and self-supported clay films were placed on a flat 23 x 23 mm SpecTRIM (hydrophobic stainless steel substrate; Tenta Sciences) slide. Spectra were collected using 60 s of acquisition on the CCD array through an Olympus BX 60 microscope with a 50× objective. Winspec (Princeton Instruments, Inc.) and Grams software were used to collect and analyze spectra, respectively. Carbon and nitrogen content of clay—protein complexes was also analyzed using a LECO CHN-1000 analyzer.

### RESULTS

The sorption isotherms of HEWL on Na- and Cs-saponite determined using the bicinchoninic acid (BCA) assay are shown in Figure 1. The BCA-derived sorption isotherm for Na-saponite shown in Figure 1 (left side) represents the average of four different sorption experiments, and the x- and y-error bars represent the standard deviation of error in the equilibrium solution concentration determinations and amount of protein sorbed. At low protein concentrations of <50 μg/mL, sorption was quantitative with no HEWL detected in solution after sorption, the amount being 150 mg/g. As the amount of lysozyme offered increased, a gradually increasing amount of lysozyme was sorbed. No maximum sorption level was attained. Similar observations were made for Cs+-saponite (Figure 1, right). However, the amount of quantitatively adsorbed lysozyme was 230 mg/g, and with increasing amounts of lysozyme offered in solution the amount adsorbed increased in a concave fashion without reaching a maximum. In the experimental conditions of the present research, the maximum amount of lysozyme sorbed was 500 mg/g, 2.5 times lower than on Na+-saponite.

The amount of HEWL sorbed on Na-saponite was also determined by C and N analyses of the HEWL—saponite complexes based on the chemica formula of HEWL of C_{613}H_{995}N_{193}O_{188}S_{10} with a mass of 14313.3 and a C/N ratio of 2.73.\(^{43}\) These data are shown in Figure 1 (left side) and presented in Figure 1 (right). The carbon-to-nitrogen ratios are given in Table 1. Good agreement was found between the protein content obtained from the BCA-derived sorption isotherm and the C- and N-derived protein concentrations with the exception of the highest protein concentration (Figure 1 and Table 1). The carbon-to-nitrogen ratios are given in Table 1 and converge on a value of 2.76, which compares to a value of 2.72 based on the composition of HEWL.\(^{43}\) We note that saponite contains small amounts of C and N in the absence of lysozyme, indicating the presence of organic residues.

The amount of HEWL sorbed by the Na-saponite was also determined using thermogravimetric analysis (TGA). The TGA mass loss curves under N2 for Na- and Cs-saponite and HEWL—saponite bionanocomposites are shown in Figure 2, and the first derivative analysis of these curves is shown in Figure 3. Mass loss occurs in three distinct regions: loss of water from 25 to ~200 °C, loss of protein or residual carbon from 200 to 550 °C, and dehydroxylation of the clay from 800 to 900 °C. The TGA-derived protein content values for Na- and Cs-saponite, expressed as mg of protein/g of clay, are included in Figure 1. In the case of Na-saponite, these results agree well with those of the C and N analysis and with the BCA-assay sorption isotherm for all but the highest concentration. In the case of Cs-saponite, however, significantly greater sorption of lysozyme was observed from the BCA assay as compared to the TGA results. The observed minima in the first derivative curves at 86, 342−359, and the doublet at 835 and 878 °C correspond to the temperatures of maximum mass loss for, respectively, water, lysozyme, and dehydroxylation (Figure 3). At the lowest protein concentration, the temperature of maximum loss of protein is 359 °C, and this value decreases with increasing protein concentration to 342 °C. These values compare to a temperature of decomposition HEWL at 325 °C in N2.

In the case of Cs-saponite, the amount of sorbed lysozyme obtained from the thermogravimetric data gives a rectangular adsorption isotherm (Figure 1, right). The maximum amount sorbed is 150 mg/g. It does not change significantly with increasing amounts of lysozyme in the equilibrium solutions. The first derivative curves of lysozyme loaded Cs-saponite show minima around 86, 348, and 865 °C, due to, respectively, release of water, decomposition of lysozyme, and dehydroxylation of the saponite structure (Figure 3). This dehydroxylation feature is not influenced by the presence of adsorbed lysozyme. In contrast, the two-step dehydroxylation of Na-saponite disappears with increasing amounts of adsorbed lysozyme and is replaced by a continuous weight loss, stretching over a much broader temperature range.
The amount of Na⁺ ions released to the supernatant as a function of the amount of HEWL sorbed (determined using BCA assay) is shown in Figure 4. The slope of this curve indicates that, on average, 9.5 Na⁺ ions were released for every molecule of HEWL sorbed. At lower ionic strength, the slope is slightly higher, indicating a greater release of Na⁺ initially similar to what was observed in prior work for protamine.40 The positive y-intercept shown in Figure 4 is due to the excess release of Na⁺ ions in the solutions at low ionic strength. In addition, the pH of the clay/C₀ HEWL dispersion decreased with increasing protein concentration (data not shown). A slight decrease from 7 to ∼6.5 occurred for initial protein concentrations of 500 mg/L with a more pronounced decrease to pH ≈ 4 at the higher protein concentration of 2000 mg/L.

X-ray diffraction (XRD) patterns in the 2θ range are shown in Figure 5. The XRD pattern of Na-saponite (pattern C) is characterized by a mixed 1- and 2-layer hydrate of the Na-saponite with d-spacings of, respectively, 1.28 and 1.45 nm. Cs-saponite has a d-spacing of 1.16 nm (pattern A). It is indicative for a mixture of 1- and 0-layer hydrates. This d-spacing slightly decreases to 1.12 nm upon adsorption of lysozyme. In contrast, adsorption of progressively higher amounts of HEWL on Na-saponite reveals the intercalation of HEWL into the interlamellar region, resulting in an expansion of the d₀₀₁ spacing from 1.28 to 1.45 nm to a maximum value of 4.4 nm (patterns D and E).

Water sorption isotherms were obtained from self-supporting clay films of HEWL/C₀ saponite using a gravimetric/FTIR apparatus described in prior work44,46 and compared to those of

### Table 1. Initial Lysozyme Concentration, Equilibrium Lysozyme Concentration, and Amount of Lysozyme Sorbed by Na-Saponite and Cs-Saponite Sorption Experiments

<table>
<thead>
<tr>
<th>initial lysozyme conc. (mg/L)</th>
<th>equilibrium lysozyme conc. (mg/L)</th>
<th>amount of lysozyme sorbed (mg/g clays)</th>
<th>TGA — amount of lysozyme sorbed (mg/gclays)</th>
<th>carbon content (%)</th>
<th>nitrogen content (%)</th>
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Amount of protein sorbed calculated using thermogravimetric analysis data, carbon content, nitrogen content, and C/N ratio of lysozyme sorbed self-supported clay films is also included.

Figure 2. Thermogravimetric analysis conducted on lysozyme sorbed Na-saponite and Cs-saponite self-supported clay films. Graph depicts the loss of water, protein, and structural OH from clay. Lysozyme treatments were: 0 µg mL⁻¹ (a); 50 µg mL⁻¹ (b); 100 µg mL⁻¹ (c); 200 µg mL⁻¹ (d); 300 µg mL⁻¹ (e); 500 µg mL⁻¹ (f); and 1000 µg mL⁻¹ (g).
Na- and Cs-saponite. The sorption isotherms (Figure 6) show hysteresis between the adsorption and desorption legs, which is more pronounced for Na-saponite than for Cs-saponite with HEWL—saponite falling in between. The data points (green squares) of the adsorption branch of HEWL—saponite follow the Cs-saponite curve up to a relative humidity (RH) of 60%. At higher RH’s, the amount of water sorbed on HEWL—saponite falls between the amounts of Na-saponite and Cs-saponite. The desorption branch (△) of HEWL—saponite lies above the adsorption branch until a RH of 20%. Below this 20% RH, the desorption branch of HEWL—saponite follows the Cs-saponite desorption branch.

FTIR spectra were obtained from self-supporting HEWL—saponite films corresponding to known points along the sorption isotherm shown in Figure 1. Survey FTIR spectra of the regions of interest (3700—2800 and 1750—1200 cm⁻¹) are shown in Figure 7A and B. The FTIR spectra clearly reveal the structural hydroxyl groups of saponite at 3676 cm⁻¹ and the presence of the sorbed HEWL by the characteristic protein absorption bands. These features increase linearly with increasing HEWL sorption. Bands around 3317, 3205, and 2976 cm⁻¹ are indicative for N—H and O—H stretching vibrations of both lysozyme and water. The bands at 2962, 2936, and 2876 cm⁻¹ are the C—H stretch modes of the adsorbed lysozyme molecules. In the 1750—1200 cm⁻¹ region, the two most important bands are the amide I band at 1657 cm⁻¹, superimposed on the 1620 cm⁻¹ band of adsorbed water, and the amide II band with two maxima at 1534 and 1517 cm⁻¹.

The presence of the sorbed HEWL is clearly evident in the ν(NH), ν(OH), and ν(CH) regions from 3400 to 2800 cm⁻¹ as well as from 1700 to 1200 cm⁻¹. The strong ν(Si—O) bands below 1200 cm⁻¹ obfuscate IR-active bands of the protein from 1200 to ~800 cm⁻¹ as well as bands <500 cm⁻¹. Expanded spectral regions in the 3750—2800 cm⁻¹ region and in the 1750—1200 cm⁻¹ region are plotted in Figure 7A,B. Raman and FTIR spectra of HEWL and of the HEWL—saponite complex were obtained in the 1800—500 cm⁻¹ region and are included as Supporting Information.

Analysis of the amide I region can provide information about the secondary structure of the sorbed protein. The amide I region was analyzed in two ways to provide insight about the secondary structure of sorbed HEWL. In the first method, the spectrum of water sorbed on saponite (spectrum a in Figure 8A) was subtracted from the spectrum of HEWL sorbed on Na-saponite, which contained some water (spectrum b in Figure 8A), and the resulting spectrum is shown in spectrum c in Figure 8A. In addition, the ATR-FTIR spectrum of a thin deposit of Na-saponite in the presence of H$_2$O is shown by the dashed line in Figure 8B. The water was replaced by D$_2$O, and the corresponding D$_2$O—HEWL—saponite complex is shown by the solid trace in Figure 8B. In this case, the corresponding D—O—D bending band (νd mode) occurs at ~1200 cm⁻¹ and does not interfere with the amide I band. Deuteration of the HEWL results in a slight shift of the amide I band by about 10 cm⁻¹ to lower energies; the shift of the amide II band is about 80 cm⁻¹. Thus,
adsorbed lysozyme is deuterated by D2O. The amide II band consists of N–H bending vibrations, which are converted to N–D bendings. The amide I band is comprised of coupled vibrations, consisting of approximately 60% C=O stretch with 40% from the N–H bending vibration. The weak vibration at 1590 cm$^{-1}$ in the deuterated spectrum is likely due to COOH or COO$^{-1}$ and has been observed in prior work.

### DISCUSSION

Good agreement was found between the three independent measures of HEWL sorption on Na- and Cs-exchanged saponite at low to medium surface coverage based on the BCA assay, thermogravimetric analysis (TGA), and C/N analysis (Figure 1; Table 1). HEWL with an isoelectric point (IEP) of 11.431 has a thermodynamic driving force for the negatively charged surface of Na-saponite in the presence of HEWL, indicating interlayer expansion in agreement with earlier studies. On the basis of the crystallographic dimensions for HEWL of 3 × 3 × 4.5 nm and the thickness of a fundamental particle of saponite of 0.96 nm, the observed d-spacing of 4.4 nm indicates that the sorbed HEWL molecule is oriented with the long axis of the molecule oriented parallel or slightly tilted with respect to the basal surface of the clay. If HEWL was laying “flat” on the surface, a d-spacing of 3.96 nm would be expected (3.0 nm of HEWL + 0.96 nm saponite). Interestingly, recent molecular dynamics simulations of HEWL sorbed to a model clay surface show a slightly tilted orientation of HEWL in good agreement with the observed d-spacing of 4.4 nm found in this study. In an earlier LbL study of HEWL sorption to Na-saponite, we observed a d-spacing of 3.6 nm. As noted earlier, the LbL construction process is fundamentally different from the batch equilibration sorption used in this study. The presence of excess water in the batch sorption method used here may contribute to the increased d-spacing consistent with the high water content of these complexes corresponding to 146 H2O molecules per molecule of HEWL at high relative humidity.

On the basis of the plateau observed in the HEWL sorption isotherm for Na-saponite of 600 mg/g and a cross-sectional area of 3 × 4.5 nm, the surface area occupied by one HEWL molecule is estimated to be 27 nm$^2$ given that HEWL contact both the “upper” and the “lower” surface of Na-saponite in the interlayer, the specific surface area occupied by HEWL is 682 m$^2$/g. This value is remarkably close to measured specific surface areas that range from 710 to 760 m$^2$/g for Na-saponite obtained with different methods. As noted earlier, HEWL sorption on clay mineral surfaces has also been studied using layer-by-layer assembly and in surface force and AFM studies. In earlier work, we observed a surface area per molecule value of...
17 nm² (one side only) per molecule in LbL films of HEWL–Na-saponite, slightly greater than the 13.5 nm² value assumed here. Because the d-spacing did not indicate a bilayer of protein in the interlayer, HEWL sorption in excess of 600 mg/g is indicative of excess sorption on the edges and external surface of Na-saponite.

HEWL sorption resulted in the release of 9.5 Na⁺ ions for every molecule of HEWL sorbed (Figure 4), which agrees with the charge density of HEWL from titration and synchrotron-based structural studies of HEWL exchanged with NaBr. In addition, the ATR-FTIR spectra of HEWL–Na-saponite in H₂O and D₂O reveal minimal perturbation to the HEWL structure upon deuteration (Figure 8), indicating the overall HEWL structure of HEWL sorbed to saponite is preserved and consistent with the LbL results. Together, these observations point to the nearly “ideal” behavior of the HEWL–saponite complex. The amount of HEWL sorbed and the Na-release data indicate that electrostatic attraction sorbed, and the Na-release data indicate that sorption is driven mainly by electrostatic attraction resulting in monolayer surface coverage of HEWL in the clay interlayer. The XRD results indicate no partial collapse of HEWL–saponite complex. In addition, the well-defined second order reflection at 2.2 nm indicates a high degree of order, indicating complete intercalation and lack of interstratification of expanded and collapsed layers. The XRD results are further supported by the FTIR results, which showed no evidence of protein unfolding or denature.

In the case on Cs-saponite, the d-spacing did not increase, and there was no evidence of interlayer sorption of HEWL (Figure 5). The amount of HEWL sorbed by Cs-saponite was also much less as compared to Na-saponite with a TGA-derived sorption capacity of ~140 mg/g (Table 1). On the basis of the cross-sectional area of HEWL and assuming that HEWL can only contact the external surface of Cs-saponite, the specific surface area values range from 53 to 80 m² g⁻¹, depending on the orientation of the sorbed HEWL. The increased surface coverage observed using the BCA assay is assigned to protein aggregation on the external surface of Cs-saponite resulting in multilayer sorption. Additional support for this mechanism comes from AFM studies of HEWL sorption on flat mica surfaces where no additional clay layers are present, which show surface diffusion of lysozyme monomers occurs forming clusters consisting of approximately 5 molecules.

A common feature of the HEWL adsorption isotherms for both Na- and Cs-saponite is the vertical adsorption branch of 100 mg/g for Na-saponite and 140 mg/g for Cs-saponite. This means that lysozyme is quantitatively taken up from solution; that is, the amount of lysozyme in the equilibrium solution is below the detection limit of our analytical methods. Such a vertical branch in the adsorption isotherm has also been observed for adsorption of protamine and papaain. It is indicative for adsorption at the edges of the saponite layers and reveals that HEWL has a high affinity for edge and external surface sites on both Na- and Cs-saponite based on the shape of the isotherm.

The BCA assay resulted in systematically higher amounts of HEWL sorbed for both Cs- and Na-saponite at the higher surface coverage. The BCA assay is determined by measuring the loss of HEWL from solution. In contrast, the TGA and C/N analyses are based on high temperature removal of sorbed HEWL. The difference between the sorbed amounts obtained by BCA analysis and those obtained by C, N analysis or thermogravimetry is the sorbed fraction, which can be washed off the biocomposite. It is weakly adsorbed HEWL. In addition, it is likely that some pyrolysis of the sorbed protein occurred in the TGA measurements because they were run in a N₂ atmosphere resulting in some residual carbonaceous material (e.g., black carbon), which remained on the surface of the clay at high temperature. The C/N ratio of sorbed HEWL did not change significantly with protein surface loading and is in agreement with the chemical composition of pure HEWL, indicating that the clay surface does not retain a nitrogenous or carbonaceous component of HEWL.

The sorption data of HEWL on Na- and Cs-saponite can be interpreted as follows. The overall sorption process is driven by ion exchange of the positively charged HEWL species to the negatively charged clay surface. In the case on Na-saponite, HEWL sorption occurs on both the internal and the external surfaces of saponite. Interlayer sorption is nearly complete, forming a monolayer of HEWL with little perturbation to the structure of HEWL. HEWL sorption on the external surface of Na-saponite also occurs resulting from protein aggregation or a related process resulting in multilayer sorption. For Cs-saponite, HEWL sorption is restricted to the external surface of the clay particles with evidence for the multilayer sorption here as well. It is as if sorption on the edges of saponite creates a biocomposite with the favorable environment (hydrophilic–lipophilic balance) for physisorption of lysozyme. One could also see this in the frame of double layer theory. Lysozyme forms a double layer: a strongly adsorbed monolayer and a weakly adsorbed phase on top of this monolayer; the concentration of this weakly adsorbed phase decreases with the distance from the surface. For both Na- and Cs-exchanged clays, ion exchange appears to be the overall driving force. However, given that both HEWL and saponite have a distribution of hydrophobic and hydrophilic regions, it is likely that additional hydrophilic and hydrophobic interactions contribute to the overall sorption process. Although protein desorption was not measured in this study, prior studies have shown the HEWL is strongly retained by smectite. Similarly, AFM “washout” studies have shown that HEWL is irreversibly adsorbed on mica and it is expected that sorbed HEWL would not be easily removed. The experimental results and the proposed ion exchange mechanism proposed in this study are in good agreement with recent molecular dynamics results.

The role of water is central to many applications of hybrid materials in biosensing, biocatalysis, and drug delivery applications involving supported enzymes. The interaction of H₂O with a clay–protein complex has not been reported previously. As noted earlier, we did not detect the presence of H₂O in HEWL–saponite.
films produced using the LbL construction method.\textsuperscript{22} Interestingly, the \(d\)-spacing of the HEWL–saponite LbL films was 3.6 nm, considerably less than the 4.4 nm spacing observed here. From the XRD data, the proposed structure of HEWL–saponite complex is shown in Figure 9. The presence of the 3 nm globular spheres of HEWL in the clay interlayers impacts water sorption in two ways. First, depending on the packing of protein molecules into the interlamellar region, the protein spheres occupy space and serve to block some potential water sorption sites. On the other hand, the 3 nm sphere in the clay interlayer functions as a type of “molecular prop”, which holds the clay layers apart. Even though protein molecules occupy between 50\% and 70\% of the available volume, a considerable amount of “free” volume is available to hold water. From the water sorption isotherms, the HEWL–saponite complex is found to be hydrophilic. In comparison to Cs-saponite, water sorption on HEWL–saponite is exceeded by about 85 mg of H\(_2\)O/g at high relative humidity, or 135 H\(_2\)O molecules per sorbed HEWL. It is likely that most of this water is located within the clay interlayer, indicating that the protein is hydrated in the clay interlayer and that the overall HEWL–saponite complex is hydrophilic.

\textbf{CONCLUSION}

Lysozyme is adsorbed on saponite in a three-step process: (1) adsorption at the edges and external surfaces of the saponite layers; (2) adsorption of a monomolecular layer of lysozyme molecules in the interlamellar space; and (3) weak adsorption of lysozyme molecules, which can be washed away. The first two processes are ion exchange reactions. The three processes occur on Na-saponite. Processes (1) and (3) occur on Cs-saponite. These three adsorption processes/steps have also been observed for other positively charged protein molecules, which can be washed away. The intercalated clay layers are open to adsorb water molecules, thus allowing the intercalated protein molecules to create the aqueous environment, necessary for enzymatic reactions.

\textbf{ASSOCIATED CONTENT}

\textbf{Supporting Information.} Spectroscopic details about the HEWL–saponite complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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