Imaging the ordered arrays of water-soluble protein ferritin with the atomic force microscope

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ABSTRACT Individual water-soluble molecules of the protein ferritin have been imaged on a silicon surface in pure water at room temperature with the atomic force microscope (AFM). The ferritin molecules formed an ordered monolayer by binding to a charged polypeptide monolayer of poly-1-benzyl-L-histidine (PBLH) spread at the air–water interface. The film, fully wetted with water, was horizontally transferred onto an alkylated silicon wafer for AFM imagings. The hexagonal arrangement of ferritin molecules was imaged with high reproducibility on the whole surface of the film, since the forces between cantilever and the sample could be kept sufficiently smaller than 10^{-10} N, mainly due to a "self-screening effect" of the surface charges of the ferritin–PBLH layer. This is the first observation of two-dimensional ordered arrays of water-soluble protein molecules directly confirmed by AFM with molecular resolution.

INTRODUCTION

Fabrication and characterization of biological macromolecular systems have been the subject of considerable study as dimensionally controlled molecular assembly, which is important both to fundamental issues in biophysics and biochemistry and to future possibilities such as novel material structures for molecular devices. Especially in protein engineering, it has been recognized that the characterization of three-dimensional structures of protein molecules has a great importance in revealing the detailed nature of their function and associated phenomena.

In the structural analysis fields, electron microscopy has long been successful as a well established imaging technique for microscopic determination having wide applications, and is still improving in terms of the resolution quality for imaging the three-dimensional structures of biological molecules (Henderson et al., 1990). However, observation with an electron microscope must be carried out in a vacuum, resulting in difficulty for imaging the biological specimen under physiological conditions. The three-dimensional structures are observed as two-dimensional projections, often distorted by the drying processes in the electron microscope. In addition, to obtain a sufficient contrast in the electron microscope analysis, the specimen usually must be treated by heavy-metal stain, which easily induces denaturation of the biological molecules.

Currently, the atomic force microscopy (AFM), which was introduced by Binnig, Quate, and Gerber (Binnig et al., 1986), has been increasingly drawing our attention as an entirely new approach in the study of surface topography. This is especially evident in the case of biological macromolecular systems (Butt et al., 1990*a*; Edstrom et al., 1990; Egger et al., 1990; Zasadzinski et al., 1991), where AFM provides an outstanding capability for the structural analysis of nonconductive biological specimens under physiological conditions during imaging, such as ionic strength, pH, and temperature.

In order to elucidate the applicability of AFM to protein molecules, it has been realized that the structurally well characterized proteins should be used for the AFM samples. This is especially true in the case of the two-dimensional crystalline arrays of proteins studied by electron microscopy or x-ray diffraction measurements (Uzgiris and Kornberg, 1983), where such precise structural data allow us to evaluate the utility of AFM on protein samples with clear discrimination against artifacts.

Butt et al. (1990*b*) have studied the two-dimensional crystal of the membrane protein bacteriorhodopsin (purple membrane), as deposited on mica, silanized glass, or lipid bilayers, and reported the two-dimensional Fourier transform of AFM images, which was in agreement with results of electron diffraction measurements. In the case of scanning tunneling microscope (STM) imagings, high quality images of bacterial HPI (hexagonally packed intermediate) layer have been reported by Guckenberger et al. (1989).

While there exist some AFM and/or STM images reported for biological specimens, these high resolution images of protein molecules have so far been restricted to "naturally formed" two-dimensional protein crystals, which are sufficiently stable, or "randomly anchored" protein particles on membrane proteins or lipid bilayers. To demonstrate the first trial observations of "artificially formed" two-dimensional arrays of water-soluble protein molecules, we carried out an AFM study of monolayers of ferritin molecules electrostatically bound to a charged polypeptide layer of poly-1-benzyl-L-histidine (PBLH) on a silicon wafer.

In this paper, we present AFM images of two-dimensional ordered arrays of ferritin molecules arranged in a predesigned manner on a silicon wafer for the first time. The ordered structure of ferritin confirmed by AFM is in good agreement with our previous results obtained by

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high resolution scanning electron microscopy (SEM) (Furuno et al., 1989). In addition, we demonstrate the ability to identify individual protein molecules by introducing smaller forces than 10^{-10} N due to the "self-screening effect" of surface charges.

MATERIALS AND METHODS

Ordered arrays of ferritin monolayers

Horse spleen ferritin (Sigma Chemical Co., St. Louis, MO) was dissolved in pure water and fractionated by ultracentrifugation several times at 200,000 *g* for 40 min. The heavier fractions were diluted with a solution of sodium chloride (10 mM) to a final concentration of 100 mg/ml. The ferritin solution was diluted with phosphate buffer (5 mM, pH 4.8–7.0) and prepared to a concentration of 30 μg/ml. The micro Langmuir trough (20 mm in width, 35 mm in length, and 2 mm in depth) was filled with the ferritin solution until the air–water interface became a slightly convex shape. PBLH (Sigma Chemical Co.) with an average degree of polymerization of 100 was dissolved in chloroform containing dichloroacetic acid (0.68 μl to 1 mg of PBLH) to a concentration of 0.54 mg/ml. The desired amount (3 μl) of PBLH was spread over the ferritin solution and allowed to incubate condensed ferritin monolayers at a ferritin–PBLH interface for 3 h at room temperature.

We used a silicon wafer (n-type, [100]) as the substrate because the two-dimensional ordered arrays of ferritin molecules have been observed previously with high resolution SEM on this substrate prepared without staining or metal shadowing (Furuno et al., 1989). Before the film transfer, the silicon wafers were irradiated with UV light of a low pressure mercury lamp to obtain hydrophilic surfaces, placed in a glass desiccator filled with hexamethyldisilazane vapor, and then heated to 60°C for 1 h to bind hexamethyldisilazanes covalently to the surface, thus forming the alkylated hydrophobic surface. The interfacial film (hetero-bilayer of ferritin–PBLH) on the trough was transferred onto such an alkylated silicon wafer by a horizontal transfer method. The ferritin–PBLH film transferred on a silicon wafer was rinsed with pure water. The wafer glued to a steel disc was placed on an electrically grounded magnetic disc on top of a piezoelectric translator in an AFM system without drying. The film was imaged using a fluid cell in pure water.

AFM imagings

The AFM system used in this study was a commercially available NanoScope II (Digital Instruments, Inc., Santa Barbara, CA). After slowly circulating pure water around the sample in a fluid cell, a Si_3N_4 cantilever with a spring constant of 0.12 N/m (reported by Digital Instruments, Inc.), which is V-shaped and 200 μm long, was positioned and scanned over the sample in pure water at room temperature. AFM images (400 × 400 pixels) were obtained using the "height mode," which kept the force constant. Typical AFM parameters were as follows: integral gain = 3; proportional gain = 5; two-dimensional gain = 0.3; scan rate = 8.68–19.6 Hz; scan width = 40–700 nm. In order to obtain the best imagings, the applied force was minimized and stabilized by adjusting the height of cantilever (set point voltage) during scanning the sample surface.

RESULTS AND DISCUSSION

Ferritin monolayers on a silicon wafer imaged in water

Ferritin is composed of 24 polypeptide subunits, which are arranged to form a nearly spherical hollow shell with outside and inside diameters of ~12 and 8 nm, respectively. The polypeptide shell can store up to 4,500 iron atoms forming an iron oxide core (Harrison 1963). Currently, the structure and function of the outer shell itself are of considerable interest (Meldrum et al., 1991), since the presence of six channels (about 1 nm in diameter) passing through the shell and providing access routes for iron atoms, has been reported (Harrison et al., 1980). Even in the field of electron microscopy, however, it is well known that the crystallographic determination of protein structures, for example, has to overcome great difficulties such as single-crystal sample preparation, three-dimensional analysis of electron density maps, and computer processing. From this point of view, only the AFM imaging is a method that has so far been expected to provide the remarkable potential to directly map topological features of protein molecules under physiological conditions.

In Fig. 1 *A*, we show the typical unfiltered AFM image of the molecular packing of ferritin on a silicon surface taken in pure water at room temperature. Individually distinguishable spherical patterns and regular alignment are observed. The imaging was carried out for the ferritin monolayers prepared with phosphate buffer at pH 5.3. We will return to this suitable pH condition in the discussion of the next section.

Fig. 1 *B* shows an AFM image in small scanning area. It is evident that seven spheres form a hexagonally packed arrangement. The hexagonally packed structure is in good agreement with the two-dimensional array of ferritin molecules observed by high resolution SEM, as we reported previously (Furuno et al., 1989). In addition, the spacing between these spheres in this pattern is about 14 nm with a diameter of about 12 nm, which agrees well with that of a ferritin crystal deduced from x-ray analysis (Harrison 1963). From these results, each spherical image obtained with AFM is identified with the individual ferritin molecule.

In the case of SEM imagings, a ferritin molecule has been observed as a 7–8-nm particle, corresponding to the inner iron oxide core, which has high electron density. AFM imagings, on the other hand, provide a direct image of the surface topography of ferritin molecules, so that the diameter observed with AFM agreed well with that of the outer shell of ferritin molecule.

In numerous related AFM studies, we find no evidence of deformation of the packing structures or denaturation of ferritin molecules. This periodic structure comprises a large homogeneous domain over an area of $\sim 700 \times 700$ nm^2, approaching the limiting area that the AFM can scan. Such nondestructive AFM imagings could be attained with high reproducibility, when the operating force condition was stabilized as shown in Fig. 1 *C*. The interpretation of the force curve in terms of cantilever deflection is given in the previous paper (Butt, 1991*b*). The applied force operated in Fig. 1 *A* was estimated at 10^{-10}–10^{-11} N from the force curve in Fig. 1 *C*. This is the first AFM observation of artificially formed two-dimensional arrays of water-soluble protein monolayers without any filtration.

"Self-screening effect" of surface charges

In an aqueous medium, electrostatic force is one of the most crucial components (Israelachvili et al., 1988) between the cantilever and the sample surface, and is strongly affected by the surface charges. From this viewpoint, to achieve the stabilized low force and high resolution AFM imagings, a screening of the surface charges should be considered as one of the most effective factors.

Horse spleen ferritin has an isoelectric point of about 4.5 and is negatively charged in a solution of phosphate buffer of pH 5 or above (Arbuthnott et al., 1975). The imidazole ring of PBLH, on the other hand, should be positively charged, when PBLH is spread on a subphase at the lower pH than the pKa of the histidyl residue (in the range of 6–7). Therefore, it is expected that ferritin in the subphase would bind electrostatically to a PBLH film at slightly acidic pH 4.5–6.5 and form only a monolayer, even if there exist excess ferritin molecules near the interface. In this case, electrostatic charges at a ferritin–PBLH interface are canceled, thereby forming the more efficient screening of the surface charges of ferritin monolayers.

To elucidate the effect of pH of the buffer on the stability of the sample and the AFM imaging, the relationship between the pH condition during the sample preparation and the AFM imagings has been investigated. Ferritin molecules were bound to PBLH in phosphate buffer at each pH condition, 7.0, 6.0, 5.7, 5.5, 5.3, and 4.8, transferred onto the silicon wafer, and then AFM imagings were carried out in pure water.

Before discussing the pH dependence, it is of interest to briefly compare the AFM image of ferritin monolayers with that of the PBLH layer itself without ferritin molecules. (We later on show the force curve from the PBLH layer in Fig. 3 *A*.) While large attraction could be seen in the order of 10^{-7} N, we could not get any unambiguous images of the PBLH layers. The large attractive force can be attributed mainly to hydrophobic attractive interaction, since the PBLH film on the silicon substrate has a hydrophobic surface. Moreover, the surface of the cantilever is negatively charged, because silicon nitride bears a slight negative surface charge in water (Butt 1991*b*). The interaction between negatively charged cantilever surface and positively charged PBLH may also enhance the electrostatic attraction.

pH 7.0. The ferritin layer prepared at pH 7.0 could not be imaged with AFM, since the stylus of the cantilever often comes off from the sample surface during scanning.

pH 6.0. In Fig. 2 *A*, we show the AFM image of the sample prepared at pH 6.0. It is clear that ferritin molecules scattered on the sample surface and distributed randomly in two dimensions. This is mainly due to the

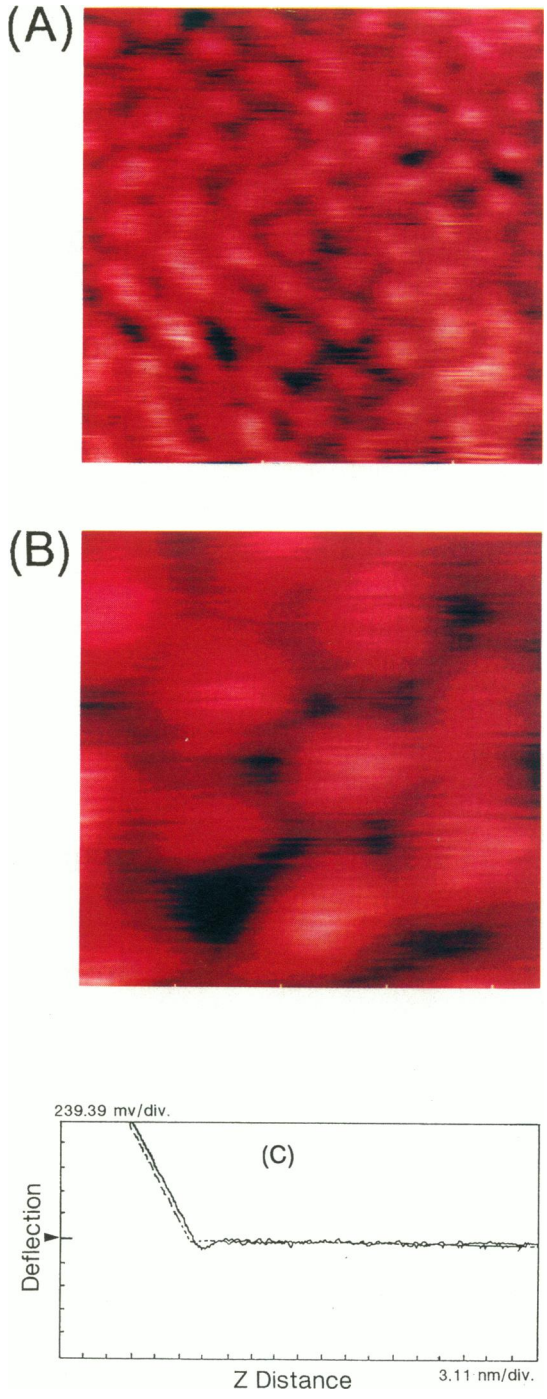

FIGURE 1 (*A*) Unfiltered AFM image of a regular array of ferritin molecules bound to a PBLH layer on a silicon wafer. The imaging was carried out in pure water using a fluid cell. Image area is 125 × 125 nm^2. (*B*) Higher magnification AFM image showing a hexagonal packing of ferritin molecules. Image area is 45 × 45 nm^2. (*C*) Force curve for Fig. 1 *A*. Cantilever deflection in millivolts (*ordinate*) versus distance between stylus and sample in nanometers (*abscissa*). The two curves are for approach (*dashed line*) and for withdrawal (*solid line*) of the stylus from the sample. Arrowhead (▶) indicates the operation point. It was estimated from this curve that the force applied was 10^{-10} N ~ 10^{-11} N.

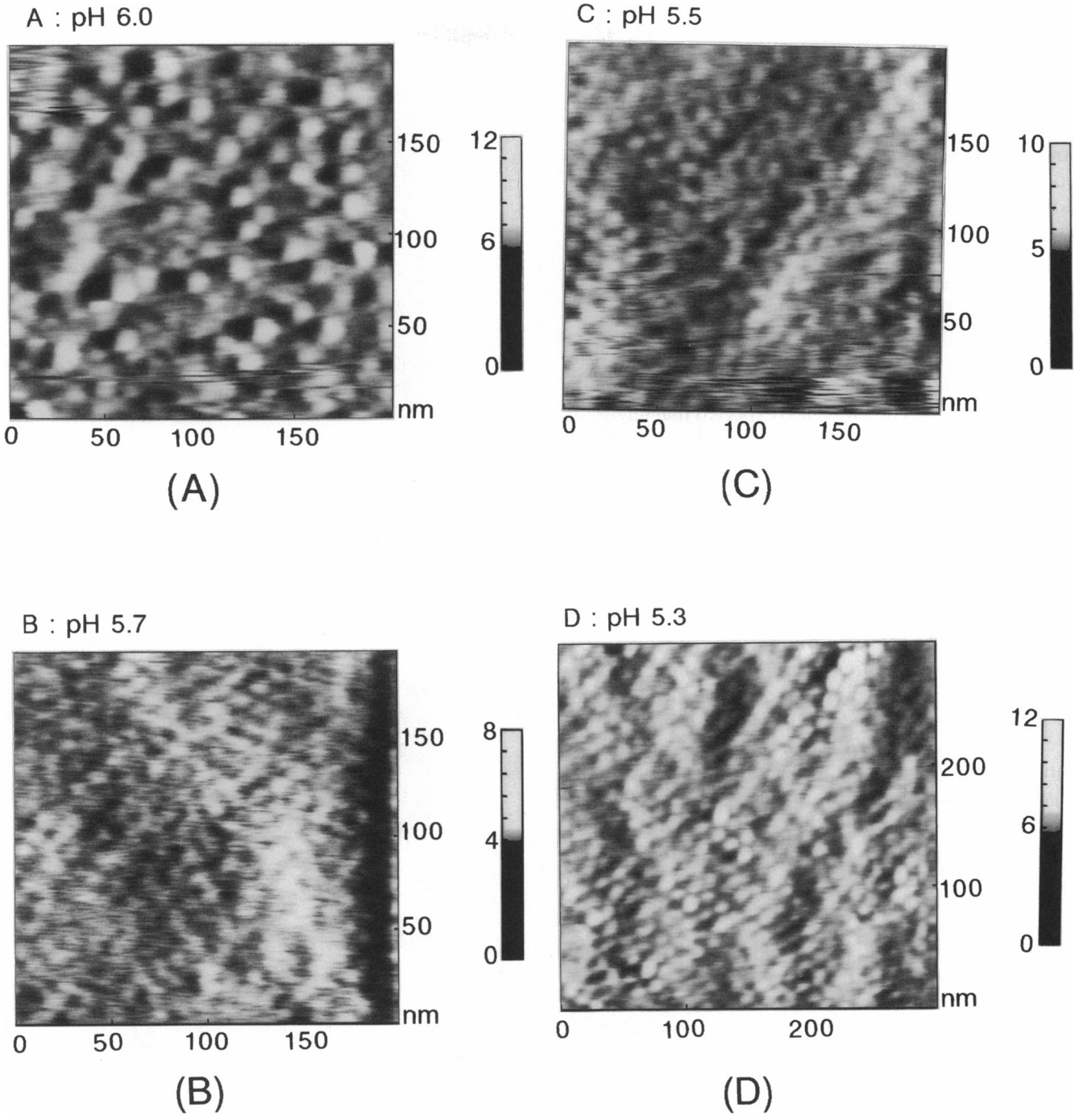

FIGURE 2 Unfiltered AFM images of ferritin samples prepared at pH: (*A*) 6.0; (*B*) 5.7; (*C*) 5.5; and (*D*) 5.3. The image area is: (*A–C*) 200 × 200 nm^2; and (*D*) 300 × 300 nm^2. The imaging was carried out in pure water.

low density of the positive charge coming from the protonated imidazole of PBLH at pH 6.0, where the surface coverage of ferritin molecules should be inevitably low. In such images, the averaged diameter of the spheres can be observed as larger than that of the actual size of ferritin molecule (Harrison 1963). In the case of scanning over an isolated molecule, the shoulder of the stylus still has contacts with the molecule, even if the top of the stylus comes off the molecule. It follows that the size of molecules imaged is slightly larger than the actual size of ferritin molecule. Because of the nonuniform charge distribution on the sample surface, the reproducible force curve could not be obtained. The unstable attractive force was in the range of 10^{-8}–10^{-9} N.

pH 5.7. In Fig. 2 *B*, we show the AFM image of the sample at pH 5.7. While the ordered array of ferritin molecules could be observed, individual ferritin mole-

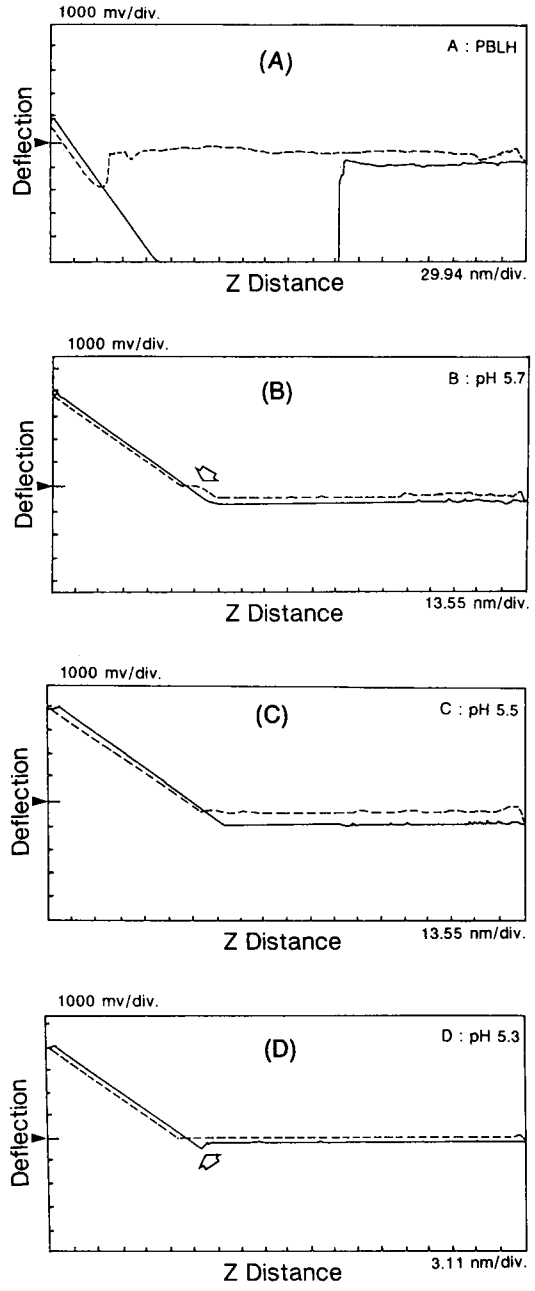

FIGURE 3 AFM force curves in pure water. The dashed and solid curves are for approach and withdrawal of the stylus, respectively. Operating point is indicated with arrowhead (▶). (*A*) The force curve for pure PBLH layer. A large attractive force of the order of 10^{-7} N is observed despite the measurement in pure water. (*B*) pH 5.7: Weak repulsion is pointed out by an arrow (◊). (*C*) pH 5.5. (*D*) pH 5.3: Weak attraction is pointed out by an arrow (◊).

cules were not visualized clearly. In the force curve from this sample (Fig. 3 *B*), the small repulsive component (indicated by an arrow) can be seen, when the stylus of the cantilever approaches the surface of the sample. Below pH 5.7, the surface density of the positive charge in the PBLH film should be larger than that at higher pH. This would enable a binding of negatively charged ferritin, resulting in a formation of dense packing or ordered arrays, which we observed in the present work. The negative charge density of ferritin molecules, on the other hand, becomes larger as the pH increases. Therefore, the repulsion is probably due to electrostatic repulsive force between the negatively charged surface of the stylus and the ferritin monolayer. Under such a force condition, the stylus cannot trace the exact surface profile of ferritin molecules, and consequently the contrast of the image was low, despite the small z range.

pH 5.5. In Fig. 2 *C*, we show the AFM image of the sample at pH 5.5. Hexagonally packed ferritin molecules could be observed, while any repulsion or attraction could not be seen in the force curve, as shown in Fig. 3 *C*. The force curve suggests that the surface charge density at pH 5.5 is lower than that at pH 5.7 due to cancellation of the positive charges of PBLH by binding of negatively charged ferritin molecules.

pH 5.3. In the case of the sample prepared at pH 5.3, clear images of hexagonally close-packed ferritin molecules could be obtained, as shown in Fig. 2 *D*. The individual image of the ferritin molecule has a 12-nm diameter. Figure 3 *D* shows the force curve of the sample at pH 5.3. When the stylus leaves the sample surface, a tiny attractive force (indicated by an arrow) in the range of 10^{-10}–10^{-11} N was observed. Such attractive forces can be attributed to the slightly charged positive surface of the sample, since the positive charge of the PBLH layer is not canceled completely by the slightly lower negative charge of the ferritin molecules at pH 5.3. For this small attraction, however, the stylus could contact and scan the close-packed ferritin surface properly, and the shape of the ferritin surface was traced and imaged very clearly.

pH 4.8. In the case of a lower pH condition, the imagings were too distorted to obtain stable images (Data are not shown). The attractive force was in the order of 10^{-9} N.

From these results, it is clear that the samples prepared at pH 5.3 give the best AFM images of ferritin molecules with a suitable attractive condition. The binding condition of ferritin molecules to the PBLH layer determines the surface charge density of the sample itself and hence affects the AFM imagings, especially for soft biological macromolecules.

In the case of the negatively charged membrane protein bacteriorhodopsin (purple membrane), it has been reported that the high salt concentration was effective in reducing the electrostatic force due to the screening of surface charges (Butt, 1991*a*). In our AFM studies, on the other hand, the sample itself has intrinsic properties of the screening by canceling the charges at the interface, so that the imagings can be carried out on the ideal surface without controlling the environmental conditions. Actually, as shown in Fig. 1 *C*, the applied forces can be stabilized and weakened to the order of 10^{-11} N due to the "self-screening effect" of the surface charges, result-

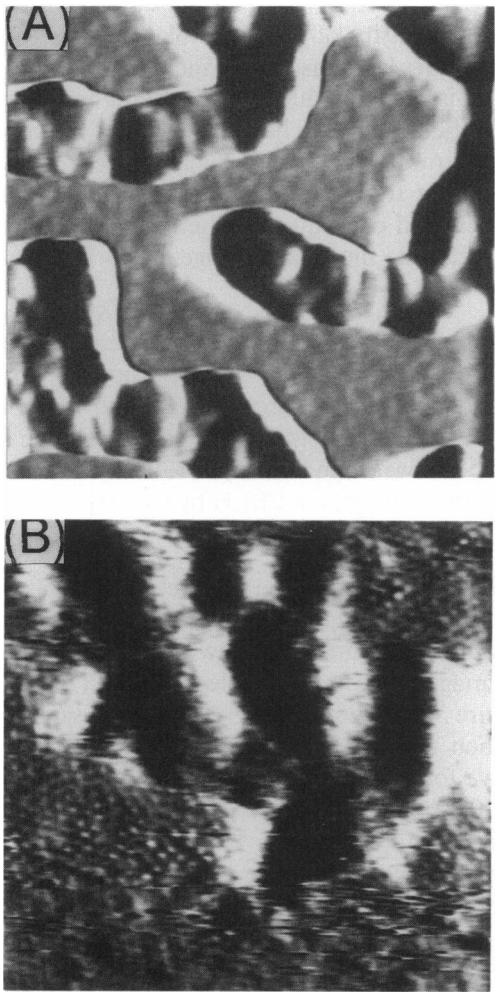

FIGURE 4 (*A*) Unfiltered AFM image of an island structure of the ferritin–PBLH film, which was observed when the specimen was scanned in air at forces higher than 10^{-8} N. Image area is 700 × 700 nm^2. (*B*) Unfiltered AFM image of large holes formed by scratching with the stylus. It was imaged in pure water after scanning several small areas at forces higher than 10^{-10} N. Image area is 500 × 500 nm^2. Hexagonally packed ferritin molecules are observed in the unscratched region.

ing in the nondestructive imagings of soft biological macromolecules. In addition, the PBLH film also plays an important role as an electrostatically charged "flexible two-dimensional mesh" to stabilize the packing arrangement of ferritin molecules during transferring from the air–water interface to the solid substrate and scanning the cantilever on the ferritin surface. From this point of view, the sample preparation with electrostatic binding is one of the most suitable methods for biological macromolecules to achieve the low force, high resolution AFM imagings.

Ferritin molecules embedded in polyvinyl alcohol imaged in air

It is of interest to briefly compare such nondestructive AFM images with other images that have been obtained under different conditions. Figs. 4 *A* and 4 *B* show the typical AFM images of the same ferritin–PBLH samples on a silicon wafer obtained in air and in pure water at higher force than 10^{-10} N, respectively. In the case of the AFM imagings in air, only some island structures were observed (Fig. 4 *A*). Individual ferritin molecules were invisible in the islands, while the existence of ferritin was recognized by SEM after the AFM imagings. Since the forces higher than 10^{-8} N were applied to sample surfaces during scanning in air, the stylus on the cantilever would scratch the surface and make an aggregation of molecules in islands (Weisenhorn et al., 1989). Furthermore, from the viewpoint of drying processes, polypeptide shells would have been flattened by dehydration, while the structures of the iron core would remain the same. Even in the case of imagings in pure water, large holes (~100 nm) scratched by the stylus were often observed (Fig. 4 *B*) after scanning the cantilever at higher forces than 10^{-10} N. These images demonstrate that it is essential for AFM imagings of soft biological macromolecules to operate at sufficiently small forces in pure water or a suitable solution.

To investigate the possibilities of the biological AFM imagings in air, ferritin molecules were embedded and immobilized in a film of polyvinyl alcohol (PVA) by casting and drying the ferritin–PVA solution on a silicon wafer. Fig. 5 *A* shows the AFM image of the ferritin–PVA composite film taken in air at a force acting on the stylus of 4×10^{-8} N. Although the force curve, as shown in Fig. 5 *B*, represented the typical operating condition in air, in which nondestructive images could hardly be obtained (Weisenhorn et al., 1989), individual ball-like ferritin patterns (~15 nm in diameter) randomly distributed in the entangled PVA host could be clearly observed. This "resin embedding" would be one of the simple fixation methods of obtaining the images of biological macromolecules by AFM in air.

CONCLUSIONS

AFM imagings of artificially formed two-dimensional arrays of water-soluble molecules of the protein ferritin have been demonstrated on a silicon surface for the first time. The hexagonal arrangement and individual ferritin molecules were imaged with high reproducibility at sufficiently small forces in the order of 10^{-11} N. Due to the newly introduced "self-screening effect" of the surface charges, the forces acting on the stylus can be successfully reduced without controlling the environmental conditions.

Since the ferritin is an iron-storage protein, there is currently great interest in utilizing ferritin itself as a reaction cavity for the preparation of inorganic nanometer particles (Meldrum et al., 1991). To investigate the structure and function of the channels on the shell surface is one of the most basic approaches to demonstrating nanoscopic materials with ferritin molecules. From

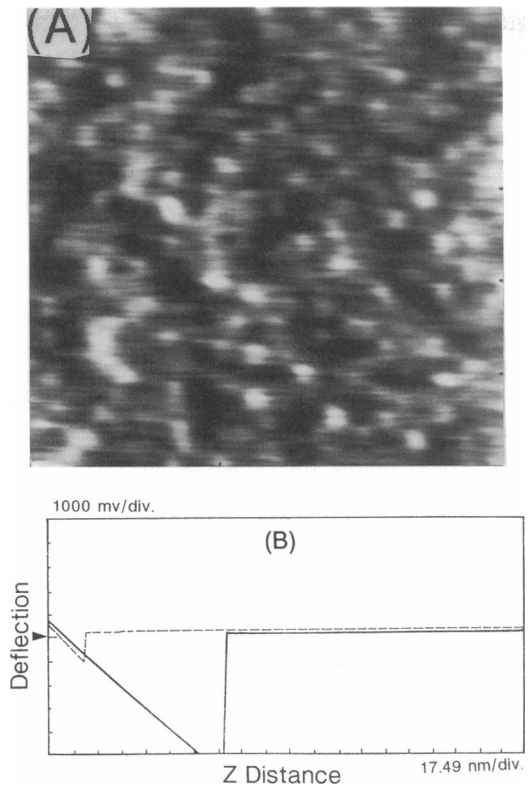

FIGURE 5 (*A*) Unfiltered AFM image of ferritin molecules embedded in polyvinyl alcohol (imaged in air). Image area is 480 × 480 nm^2. Isolated individual ferritin molecules are imaged apparently as larger particles than those in pure water. (*B*) Force curve for Fig. 5 *A*. Operating point is indicated with arrowhead (▶).

this point of view, our AFM results are indeed encouraging. Further measurements to identify specific sites on individual protein surfaces are in progress and an extended discussion will be presented separately.

Received for publication 13 January 1992 and in final form 9 July 1992.

REFERENCES

Arbuthnott, J. P., and J. A. Beeley. 1975. Isoelectric Focusing. Butterworths, London. 125 pp.

Binnig, G., C. F. Quate, and Ch. Gerber. 1986. Atomic force microscope. *Phys. Rev. Lett.* 56:930–933.

Butt, H.-J., E. K. Wolff, S. A. C. Gould, B. D. Northern, C. M. Peterson, and P. K. Hansma. 1990*a*. Imaging cells with the atomic force microscope. *J. Struct. Biol.* 105:54–61.

Butt, H.-J., K. H. Downing, and P. K. Hansma. 1990*b*. Imaging the membrane protein bacteriorhodopsin with the atomic force microscope. *Biophys. J.* 58:1473–1480.

Butt, H.-J. 1991*a*. Electrostatic interaction in atomic force microscopy. *Biophys. J.* 60:777–785.

Butt, H.-J. 1991*b*. Measuring electrostatic, van der Waals, and hydration forces in electrolyte solutions with an atomic force microscope. *Biophys. J.* 60:1438–1444.

Edstrom, R. D., M. H. Meinke, X. Yang, R. Yang, V. Elings, and D. F. Evans. 1990. Direct visualization of phosphorylase–phosphorylase kinase complexes by scanning tunneling and atomic force microscopy. *Biophys. J.* 58:1437–1448.

Egger, M., F. Ohnesorge, A. L. Weisenhorn, S. P. Heyn, B. Drake, C. B. Prater, S. A. C. Gould, P. K. Hansma, and H. E. Gaub. 1990. Wet lipid-protein membranes imaged at submolecular resolution by atomic force microscopy. *J. Struct. Biol.* 103:89–94.

Furuno, T., H. Sasabe, and K. M. Ulmer. 1989. Binding of ferritin molecules to a charged polypeptide layer of poly-l-benzyl-L-histidine. *Thin Solid Films.* 180:23–30.

Guckenberger, R., W. Wiegräbe, A. Hillebrand, T. Hartmann, Z. Wang, and W. Baumeister. 1989. Scanning tunneling microscopy of a hydrated bacterial surface protein. *Ultramicroscopy.* 31:327–332.

Harrison, P. M. 1963. The structure of apoferritin: molecular size, shape and symmetry from x-ray data. *J. Mol. Biol.* 6:404–422.

Harrison, P. M., G. A. Clegg, and K. May. 1980. *In* Iron in Biochemistry and Medicine. M. Worwood and A. Jacobs, editors. Academic Press, New York. 160 pp.

Henderson, R., J. M. Baldwin, T. A. Ceska, F. Zemlin, E. Beckmann, and K. H. Downing. 1990. Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *J. Mol. Biol.* 213:899–929.

Israelachvili, J. N., and P. M. McGuiggan. 1988. Forces between surfaces in liquids. *Science (Wash. DC).* 241:795–800.

Meldrum, F. C., V. J. Wade, D. L. Nimmo, B. R. Heywood, and S. Mann. 1991. Synthesis of inorganic nanophase materials in supramolecular protein cages. *Nature. (Lond.).* 349:684–687.

Uzgiris, E. E., and R. D. Kornberg. 1983. Two-dimensional crystallization technique for imaging macromolecules, with application to antigen-antibody-complement complexes. *Nature. (Lond.).* 301:125–129.

Weisenhorn, A. L., P. K. Hansma, T. R. Albrecht, and C. F. Quate. 1989. Forces in atomic force microscopy in air and water. *Appl. Phys. Lett.* 54:2651–2653.

Zasadzinski, J. A. N., C. A. Helm, M. L. Longo, A. L. Weisenhorn, S. A. C. Gould, and P. K. Hansma. 1991. Atomic force microscopy of hydrated phosphatidylethanolamine bilayers. *Biophys. J.* 59:755–760.