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Single-Molecule Imaging on Living Bacterial Cell Surface by High-Speed AFM

Hayato Yamashita¹[†], Azuma Taoka^{2,3}[†], Takayuki Uchihashi^{1,3}, Tomoya Asano⁴, Toshio Ando^{1,3}[‡] and Yoshihiro Fukumori^{2,3*}[‡]

¹School of Mathematics and Physics, College of Science and Engineering, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan
²School of Natural System, College of Science and Engineering, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan
³Bio-AFM Frontier Research Center, College of Science and Engineering, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan
⁴Division of Functional Genomics, Advanced Science Research Center, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan

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Keywords:

scanning probe microscopy; live cell imaging; porin; molecular resolution; molecular dynamics Advances in microscopy have contributed to many biologic discoveries. Electron microscopic techniques such as cryo-electron tomography are remarkable tools for imaging the interiors of bacterial cells in the near-native state, whereas optical microscopic techniques such as fluorescence imaging are useful for following the dynamics of specific single molecules in living cells. Neither technique, however, can be used to visualize the structural dynamics of a single molecule at high resolution in living cells.

In the present study, we used high-speed atomic force microscopy (HS-AFM) to image the molecular dynamics of living bacterial cell surfaces. HS-AFM visualizes the dynamic molecular processes of isolated proteins at sub-molecular resolution without the need for complicated sample preparation. In the present study, magnetotactic bacterial cells were anchored in liquid medium on substrate modified by poly-L-lysine and glutaraldehyde. High-resolution HS-AFM images of live cell surfaces showed that the bacterial outer membrane was covered with a net-like structure comprising holes and the hole rims framing them. Furthermore, HS-AFM captured the dynamic movement of the surface ultrastructure, showing that the holes in the net-like structure slowly diffused in the cell surface. Nano-dissection revealed that porin trimers constitute the net-like structure. Here, we report for the first time the direct observation of dynamic molecular architectures on a live cell surface using HS-AFM.

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^{*}Corresponding author. Department of Life Science, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan. E-mail address: fukumor@staff.kanazawa-u.ac.jp.

[†] H.Y. and A.T. contributed equally to this work.

 $[\]ddagger$ T.A. and Y.F. contributed equally to this work.

Abbreviations used: AFM, atomic force microscopy; HS-AFM, high-speed atomic force microscopy; MALDI-TOF/ TOF, matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry; TEM, transmission electron microscopy.

Introduction

Imaging of single molecules within a cell is achieved using various techniques. Conventional transmission electron microscopy (TEM) techniques reveal supramolecular structures at high resolution, but these techniques require sample preparation methods such as fixation, staining, dehydration, embedding, and thin sectioning, all of which potentially damage or alter the native supramolecular structure. Cryo-electron microscopy does not require prior sample preparation and therefore allows for visualization of molecular structures in a near-native, frozen hydrated state.^{1,2} While the cryo-electron microscopy shows the static molecular images, the molecular dynamics of the specimen cannot be visualized because the specimen is in a frozen hydrated state. Optical microscopic methods, such as fluorescent video imaging, show molecular dynamics in single living cells.3,4 The optical microscopic technique has been applied to imaging of bacterial cells and provides insights about molecular dynamics in vivo. For example, Kim et *al.* observed the dynamics of the single cytoskeletal protein MreB in living bacterial cells using a total internal reflection fluorescence microscope.⁵ This method visualizes fluorescence signals of labeled target molecules, and therefore only indirect molecular movement can be detected as a trajectory. Moreover, the spatial resolution of optical microscopy is not sufficient for observing the dynamic structural change of intracellular molecules. Visualization of the structural dynamics in living cells is necessary to gain more insight into the precise function of biologic molecules.

Atomic force microscopy (AFM) is a complementary technique that has been used to visualize labelfree organic samples at sub-nano-order resolution under physiologic conditions. Recent advances in AFM provide new opportunities to study single cells and single molecules in microbiology.^{6,7} In particular, visualization of the architecture of a living cell is a novel powerful approach to unveil the molecular organization *in vivo*. For example, the hexagonal S-layer of *Corynebacterium glutamicum*,⁸ the peptidoglycan organization of *Lactococcus lactis*,⁹ and the germinating spores of *Bacillus atrophaeus*¹⁰ have been directly visualized in living microbial cells using AFM.

The time required to record a high-resolution image with conventional AFM is much longer than the timescale of most biologic processes. Therefore, visualization of the molecular dynamics in living cells using AFM has not been achieved. High-speed atomic force microscopy (HS-AFM) was developed to simultaneously capture the structure and dynamics of functioning biomolecules.^{11–13} Large improvements in its performance were recently demonstrated in practical imaging studies of proteins *in vitro*.^{14–16} Moreover, the dynamics of membrane proteins isolated from bacterial cells can be visualized at high resolution with HS-AFM.^{17–19} HS-AFM will become even more useful in biologic science if it can be used to observe the molecular dynamics of living cell membranes. A large number of membrane proteins play important roles in cell function (e.g., transport, signal transduction for extracellular stimuli, and maintenance of cellular structure). Little is known, however, about their dynamic molecular processes on living cells.

Here, we used HS-AFM to visualize the dynamics of molecular complexes on living cell surfaces. In the present study, we used the magnetotactic spirillum *Magnetospirillum magneticum* AMB-1 as a specimen for observing the live cell surface, because previous TEM analyses have provided data about the outer membrane structure of this bacterium.^{20,21} High-resolution HS-AFM images of the live cell surface showed that the bacterial outer membrane is covered by a net-like structure, and the movies captured by HS-AFM revealed dynamic movements of outer membrane proteins.

Results

AFM observations of living cells on mica

M. magneticum AMB-1 cells were chemically immobilized on mica to observe the detailed surface architecture of living cells. First, bare mica was coated with poly-L-lysine and then treated with glutaraldehyde. After the remaining unreacted glutaraldehyde was completely removed by washing at least 10 times, the cell suspension was loaded onto the treated mica and incubated for 1 h. Based on light microscopic observations, the cells were densely attached to the mica substrate (Fig. S1). The viability of the immobilized cells was assessed using a LIVE/DEAD BacLight Bacterial Viability Kit (Fig. 1a and b). Most of the cells remained alive on the mica for at least 1 h after immobilization, which is enough time for HS-AFM observation. HS-AFM images of cells immobilized on mica are shown in Fig. 1c and d. The cell shape revealed by HS-AFM was consistent with that observed by TEM (Fig. 1e). The cells had a spiral shape, which is the characteristic architecture of M. magneticum AMB-1. The height and width of the bacterial cells were measured vertically along the long axis of the cells to estimate the cell diameter (Fig. 1f). Based on AFM, the cell was 542 ± 65 nm wide and 526 ± 78 nm high (n=12), whereas based on TEM, the cell diameter was 653 ± 74 nm (*n*=12). The larger diameter measured on TEM images may be due to observation of the dried sample in the vacuum environment, whereas AFM allows for visualization of the three-



Fig. 1. Imaging of M. magneticum AMB-1 cells using AFM. (a) LIVE/ DEAD BacLight bacterial viability stain of M. magneticum AMB-1 cells in phosphate-buffered saline. (b) Viability of immobilized cells on mica. Live bacteria with intact membranes fluoresce green, while those with damaged membranes fluoresce red. (c) AFM observation of a living M. magneticum AMB-1 cell chemically immobilized on mica in medium. (d) AFM image of M. magneticum AMB-1 cells densely attached on mica. All cells had a spiral shape and a smooth surface. (e) TEM observation of M. magneticum cells. Magnetosome chains were observed in the center of the cell (arrows). (f) Surface profile along the line indicated in (c). AFM images were recorded at imaging rates of 5.0 s/frame with 200×200 pixels (c and d).

dimensional cellular structure in liquid environment. The magnetotactic bacterium *M. magneticum* AMB-1 has unique bacterial organelles—'magnetosomes'—that comprise the membrane vesicle and magnetite crystal. TEM images revealed chain-like arrays of magnetosomes (Fig. 1e). AFM observation, however, revealed no structural signature on the cell surface at the predicted position of the magnetosomes. This finding suggests that intracellular magnetosomes are not affected on the outer surface structure.

Direct visualization of molecules on the living cell surface

We recorded highly magnified AFM images of cells in liquid medium to visualize the molecules on the cell surface. Figure 2a and b show the cell surface morphology at ranges of 150×150 nm² and 100×100 nm², respectively. The cell surface was not flat but had a slightly undulating topography (Fig. 2c: cross section of Fig. 2a) and small holes all over. These holes produced a net-like structure (Fig. 2b) composed of the holes and hole rims (frame). The cell surface was covered by the net-like hole structures, which were irregularly arranged, suggesting that these structures are not packed tightly. The diameter of the holes in the net-like structure was 7.3 ± 1.4 nm (n=210) and the depth was $0.85 \pm$ 0.52 nm (n=210; Fig. 2e). Compactly arranged particles were observed in the frame (Fig. 2b, arrows). Movie S1 shows the entire surface of a whole cell. When the imaging area was moved along the long axis of the cell, these structures were observed on the entire cell surface. All of the cells that were examined under high magnification had



these structures. We also observed the surface structure of cells that were cultivated in low-iron medium (1.8 μ M Fe). The iron concentration, however, did not affect the architecture or the number of hole structures (Fig. S2).

Dynamic molecular movement on living cells

To reveal the molecular dynamics on a living cell surface, we recorded successive AFM images of the outer cell surface in liquid medium. Figure 3a shows still images of a successive AFM movie (Movie S2) with a frame time of 0.5 s. Many particles were observed on the rims of the holes in the net-like structure (Fig. 3a, arrows). Because the particles rapidly diffused in these structures (Movie S2), we could not accurately follow their movements. Representative trajectories of the holes in the netlike structure on the cell surface are shown in Fig. 3b and c. A single hole in the net-like structure exhibits

Fig. 2. Highly magnified AFM observation of the cell surface. (a) AFM image of the cell surface at a range of 150×150 nm². The cell surface appeared undulated. (b) AFM image of cell surface at a range of 100×100 nm². Small holes were observed over the entire cell surface. Compactly arranged particles were observed on the frame (the rims of these holes) in a net-like structure (arrows). (c and d) Surface profile along the lines indicated in (a) and (b), respectively. (e) Histogram for widths and depths of netlike structure observed on the cell surface. Curve represents the fit to a Gaussian distribution (diameter, 7.3 ± 1.4 nm; depth, 0.85 ± 0.52 nm). AFM images were recorded at imaging rates of (a) 2.0 s/frame, (b) 0.7 s/frame, and $200 \times 200 \text{ pixels}$ (a and b).

random movement, indicating that these movements are not the effect of a tip-sample interaction force, but rather molecular diffusion on the living cell surface. The diffusion speed was extracted from each single-structure trajectory (n=23). The diffusion constants for individual holes are shown in Fig. 3d. Wade *et al.* reported that the diffusion constants of molecules in the cell membrane follow a log-normal distribution.^{22,23} In our study, the distribution of diffusion constants was fitted with a lognormal distribution and the mean diffusion constant was 3.2 ± 0.4 nm²/s. This is the first report of the observation of molecular dynamics on a living cell surface using HS-AFM. Because of the slow diffusion speed, the AFM scanning speed (0.5 s/frame)used for the observation seems to have sufficient temporal resolution to detect the movement of the holes in the net-like structure (ca 7.3 nm in diameter). A faster scanning rate (0.2 s/frame) did not affect the diffusion speed or the resolution of the



net-like structure. We also treated the cell surface with glutaraldehyde and observed immobile netlike structures (Movie S3). Although movement of the holes in the net-like structure ceased, more details of the structure could not be observed due to molecular fluctuations and vertical movement in the non-supported membrane.

In vitro imaging of the isolated outer membrane

M. magneticum AMB-1 belongs to alpha-proteobacteria and is enveloped by two membrane layers: the outer and cytoplasmic membranes. To elucidate the composition of the net-like structure, we isolated the outer membrane of *M. magneticum* AMB-1 and observed it using HS-AFM. The purified outer membrane was first negatively stained with uranyl acetate and observed by TEM (Fig. 4a). The outer membrane formed a round-shaped patch that was 40 to 100 nm in diameter. TEM observation revealed no distinct structural features in the patches. On the other hand, HS-AFM showed the net-like structures on the membrane patch (Fig. 4b). This structure was the same as that observed on the living cell surface.

In addition to structural imaging, HS-AFM can be used as both a manipulator for the dissection of individual biologic samples and a recorder of the dissection process.^{24,25} HS-AFM images of the dissection process of the net-like structure observed

Fig. 3. HS-AFM observation of the molecular dynamics on a living cell surface. (a) Highly magnified AFM image of cell surface at a range of 80×80 nm², showing a still image of a successive AFM movie (Movie S2). (b) AFM image with trajectories of four net-like structures. These trajectories were drawn by tracing the movement of each hole. (c) Total trajectories of the two-dimensional diffusion for four net-like structures. The analyzed time spans were 82 s for holes 1, 2, and 4, and 47.5 s for hole 3. (d) Distribution of diffusion constants for individual trajectories. The data follow a lognormal distribution characterized by a mean $D = (3.2 \pm 0.4 \text{ nm}^2/\text{s})$ (continuous line). Inset shows mean square displacements of individual trajectories. The mean square displacement increased linearly with time, yielding diffusion constants. AFM images were recorded at imaging rates of 0.5 s/frame with 200×200 pixels (a and b).

on the isolated outer membrane are shown in Fig. 4c and Movie S4. While the net-like structures were being imaged, an additional tapping force was applied (3–7 frames). The net-like structures were disassembled by the scanning stylus, which resulted in the observation of several triangular molecules in the membrane patch that were diffusing (13 frames in Fig. 4c). A cross section of membrane patches before and after dissection is shown in Fig. 4d and e, respectively. The height of the membrane remained unchanged before and after dissecting, indicating that the structural alteration was not due to the detachment of any component from the outer membrane. Subsequently, we recorded highly magnified AFM images on the membrane patch after dissecting. Figure 4f shows that most of the molecules in the membrane patch formed a triangle shape very similar to the porin trimer structure.²⁶ Together, the findings from the dissection experiment provided direct evidence that the triangular molecule is one of the components constituting the net-like structure.

Identification of the molecular components in the net-like structure

An SDS-PAGE profile of the outer membrane fraction isolated from *M. magneticum* is shown in Fig. 4g. Approximately 15 bands were observed.



Fig. 4. In vitro imaging and nanodissection of isolated outer membrane. (a) TEM image of isolated outer membrane from M. magneticum AMB-1. (b) AFM image of isolated outer membrane adsorbed on mica. Net-like structure was observed in a membrane patch. (c) HS-AFM observation of dissociation process of a net-like structure in an outer membrane patch. Numbers indicate frame number. Note that triangular molecules appeared after dissection of the net-like structure. (d and e) Surface profile along the lines indicated in (c). (d) and (e)show the surface profile before (frame number 0) and after (frame number 13) the dissection process, respectively. (f) Highly magnified AFM image of molecules observed after dissociation. (g) SDS-PAGE gel profile of extracted proteins from the isolated outer membrane (lane 2). The protein band indicated by the arrow was identified as porin. The molecular masses of the standards (Precision Plus protein standards; Bio-Rad) are indicated on the left sides of the lanes. AFM images were recorded at imaging rates of 1.0 s/frame with 100×100 pixels (b and c) and 160×160 pixels (f).

The trypsin-digested gel slices containing these bands were analyzed by MALDI-TOF/TOF (matrix-assisted laser desorption/ionization time-offlight tandem mass spectrometry). The mass spectra from the tandem mass spectrometry were analyzed against the M. magneticum AMB-1 whole genome database²⁷ (Fig. S3). The result corresponded to the previous proteomic analysis of M. magneticum AMB-¹ membrane fractions.²⁸ The most abundant protein in the outer membrane fraction is a 40-kDa protein (Fig. 4g) that was identified as a porin homolog (amb0025: Msp1). The amino acid sequence of amb0025 was similar to porin 41 of Rhodospirillum rubrum (30% identity) and the major outer membrane protein OmaA of Azospirillum brasilense Sp245 (26% identity). Tanaka et al. reported the high expression level of this outer membrane protein, which was used as an anchoring molecule for a cell surface display system.²⁹

The crystal structures of a number of bacterial porins have been deposited in the Protein Data Bank database. In the present study, AFM images were constructed from the crystal structural data by simulation software SPM simulator (Fig. S4). Extracellular and periplasmic faces of simulated porins were determined by referring to general bacterial porin structures reviewed in Refs. 30 and 31. The simulated AFM images of the extracellular sides are well reproduced in the observed image (Fig. S4). In the construct, the three ring structures corresponding to the periplasmic side were never observed in the HS-AFM images of the outer membrane. The periplasmic side of the outer membrane might be attached selectively on the mica. The simulated porin image is well fitted to the observed triangular structure. These results suggest that the net-like structure on the outer cell surface comprises porin molecules.

Discussion

Recent dramatic improvements in AFM have opened up new avenues for analyzing cellular structures, such as isolated membranes²⁶ and cell envelopes,^{8,9,32} and for probing their spatial organization, interaction, and elasticity at a molecular resolution under physiologic conditions. The temporal resolution of conventional AFM, however, is not sufficient to analyze the molecular dynamics on living cell membranes. Direct observation of the movement of single molecules on living single cells is one of the most challenging techniques in cell biology. Our study presents, for the first time, direct observation of dynamic movements of outer membrane proteins on living cells at single molecular resolution using a laboratory-built HS-AFM. We improved the spatial resolution to capture the dynamics of membrane proteins at a high resolution

and succeeded in visualizing lateral movement and structural changes of proteins in isolated biologic membranes.^{17–19} HS-AFM is, at present, capable of imaging soft biologic molecules non-invasively without damage, suggesting that it has the potential to capture the molecular dynamics of the surface of a living cell. The bacterial cell, however, has a substantially higher topography than isolated molecules. In the present study, we used a longer AFM stylus, which seemed suitable for cell imaging, because a normal stylus disturbs imaging when the cells come into contact with the cantilever. We were able to discern the movement of surface structures at a lateral resolution of about 2 nm. Although this resolution is insufficient to determine the precise molecular organization, the lateral resolution obtained in this study is the best achieved so far for the dynamics of living cell surface molecules. The low resolution may be due to the spiral and undulating topography of the specimen or to the softness of the cell surface.

Our AFM observations revealed that the cell surface is completely covered with a net-like structure in the liquid medium. The AFM movies showed many small particles in the net-like structure. Moreover, according to the nano-dissection experiment and proteomics analysis, we suggest that outer membrane porin constitutes the net-like structure. The small particles visualized in the netlike structure may correspond to porin molecules. The prokaryotic surface layer (S-layer) resembles a net-like structure.^{8,33} While a variety of S-layers have been observed in archaea and Gram-positive bacteria, only a few Gram-negative bacteria possess the S-layer.^{34,35} We detected no homologs of the Slayer subunit in our proteomic analysis of the M. magneticum AMB-1 outer membrane fraction. Also, there is no typical S-layer protein subunit in the annotated genome sequence of M. magneticum AMB-1. Additionally, after dissociation of the netlike structure by dissection with the AFM stylus, the height of the outer membrane patch was not changed. If the single layer of the S-layer subunit array is bound to the surface of the outer membrane, the height of the outer membrane patch should be decreased. Moreover, the holes and small particles in the net-like structure randomly moved in the membrane. These findings are not consistent with a crystalline monomolecular lattice, which could not have such dynamics.

In this study, we successfully produced highresolution images of cell surface structures on bacterial cells using HS-AFM. Surface structures on various types of cells were visualized using conventional AFM. High-resolution images were achieved mainly on microbial cells,^{8–10} while high-resolution images of animal cells remain challenging.³⁶ This difference may be due to the softness of animal cells. Actually, Young's modulus values (indicating the softness of a sample surface) of living microbial cells (in the 0.1 MPa-to-0.1 GPa range)^{37–39} are significantly larger than those obtained on animal cells (in the 100 Pa to 0.1 MPa range),^{40–42} which was estimated by nano-indentation measurements using AFM. In the next step, further improvements of our HS-AFM will lead to elucidate the structures on soft cell surfaces in high resolution.

Our live cell AFM images provide novel visualization of the bacterial cell outer membrane. The netlike arrayed structure might contribute to the high permeability of the outer membrane. Our observations are the first to show the advantage of HS-AFM for recording dynamic changes on the surfaces of living single cells. This report demonstrates the enormous potential of HS-AFM imaging for microbiology and cellular biology. The HS-AFM technique can be applied to other cells such as plant, animal, protozoan, and yeast cells, and even to eukaryotic cell organelles such as mitochondria and chloroplasts.

Materials and Methods

Microorganism and culture

M. magneticum AMB-1 (ATCC 700264) was cultured in MS-1 liquid media under an O₂ (1%)–N₂ (99%) atmosphere at 25 °C in the dark.⁴³ For AFM analyses, bacterial cells were collected by centrifugation at 8000 g for 10 min at room temperature from 45 ml of late exponentially growing cultures (optical density at 600 nm of ca 0.1). The bacterial pellet was resuspended in 100 μ l of MS-1 liquid medium.

LIVE/DEAD staining

The viability of immobilized *M. magneticum* AMB-1 on mica was assessed using the BacLight LIVE/DEAD staining system according to the manufacturer's protocol (Molecular Probes, Carlsbad, CA). Cells that retained the green fluorescence color were live, whereas red fluorescent cells were considered dead. Fluorescence microscopy images were observed using Eclipse E600 (Nikon, Tokyo, Japan). Images were acquired using a DS-Qi1Mc monochrome quantitative digital camera (Nikon) and processed with NIS-Elements software (Nikon) and/or Adobe Photoshop CS2 (Adobe, San Jose, CA).

HS-AFM measurements

Imaging was performed with a laboratory-built HS-AFM, an extensively improved version of the previously reported AFM.^{11,12} The HS-AFM was equipped with small cantilevers (k=0.1–0.2 N/m, f=800–1200 kHz in water) and operated in tapping mode. The AFM styli were grown on each cantilever by electron beam deposition.⁴⁴ The previous deposition time was 90 s, whereas the deposition time in this study was increased to 180 s to

generate a longer stylus, which was adjusted for cell imaging in this study. The substrate for observation of the cell surface was prepared as follows. Freshly prepared mica was treated by depositing 0.1% poly-L-lysine solution and left to dry at room temperature. The resulting mica was then treated with 0.1% glutaraldehyde for 15 min. The *M. magneticum* cell suspension was loaded on the treated mica in liquid medium. After 60 min, the sample was rinsed with fresh medium and measured in liquid medium. The purified outer membrane was adsorbed on freshly cleaved mica and observed in 300 mM KCl and 10 mM phosphate buffer (pH 7).

Simulation of AFM image

We used software (SPM simulator, Advanced Algorithm Systems Co., Tokyo, Japan) to simulate AFM images of the porin attached to a substrate surface at either the extracellular or the periplasmic side. The simulation was performed with a simple hard-sphere model. The cantilever tip was modeled as a circular cone (apex angle, 5°) with a small sphere (radius, 0.5 nm) at the apex. The crystal structures of the porin trimer were used as the samples. Each atom in the protein was modeled as a hard sphere with a corresponding van der Waals radius. We simulated AFM images using various radii for the tip-apex sphere and found that a radius of 0.5 nm produced the images most similar to the actual AFM images. The simulated images were processed using a low-pass filter with a cutoff wavelength of 2 nm, because the spatial resolution of the AFM image was approximately 1 nm based on 2D Fourier transformation of the actual AFM images.

Tandem mass spectrometry analyses and protein identification

In-gel digestion of protein bands and MALDI-TOF/ TOF analysis were performed as described previously.⁴⁵ To identify proteins, we used the 4800Plus MALDI-TOF/ TOF[™] Analyzer (Applied Bioscience, Carlsbad, CA), and results were analyzed using Protein Pilot[™] software.

Preparation of cellular component

Outer membrane fractions were prepared using the method reported by Mizushima and Yamada.⁴⁶

Specimen preparation for TEM

Formvar- and carbon-coated grids were put on a drop of the cell suspension or purified outer membrane for about 1 min to prepare the specimen for TEM observation. Some of the grids were negatively stained with 4% uranyl acetate or 2% sodium tungstate for several seconds. The specimens were studied with a JEOL JEM 2000EX TEM operating at 120 kV or JEOL JEM 2010FEF TEM operating at 200 kV in bright-field mode.

Physical and chemical measurements

The protein content was determined using the bicinchoninic acid method (BCA Protein Assay Kit, Pierce Chemical) with bovine serum albumin as the standard. SDS-PAGE was performed according to the method of Laemmli.⁴⁷ Immunoblotting analysis was performed as described previously.⁴⁸

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Supplementary Data

Supplementary data related to this article can be foundonline at doi:10.1016/j.jmb.2012.05.018.

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