Direct observation of uncoated spectrin with atomic force microscope*

ZHANG Pingcheng (张平城), BAI Chunli (白春礼)**, CHENG Yingjun (成英俊), FANG Ye (方 晔),

(Scanning Tunneling Microscopy Laboratory, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100080, China)

FENG Liming (冯立明) and PAN Huazhen (潘华珍)

(Institute of Basic Sciences, Chinese Academy of Medical Sciences, Beijing 100005, China)

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Abstract Spectrin molecules extracted from human blood cell membrane have been examined by atomic force microscopy (AFM) without using shadowing or staining procedures. A drop of the solution containing spectrin molecules was deposited on the freshly cleaved mica substrate. After about 1 min, the residual solution was removed with a piece of filter paper. Afterwards the sample was imaged with a home-made atomic force microscope (AFM) in air in a constant force mode. The obtained AFM images revealed that the spectrin molecules prepared from the above procedures exhibit several kinds of structures as follows: (i) the compact rod-like spectrin heterodimers with a length of around 100 nm; (ii) bent or curved linear tetramers with a length of around 200 nm; (iii) somewhat curved spectrin hexamers, octomers or decamers with lengths of about 300, 400, or 500 nm; and (iv) high oligomers with a length above 1 000 nm.

Keywords: atomic force microscopy (AFM), spectrin, red blood cell, structure.

Scanning probe microscopes, such as the atomic force microscope (AFM), can be used under near-ambient conditions and can yield even atomic resolution on some surfaces[1-3]. The potential capability of AFM to generate very high resolution images of biological samples and the possibility of obtaining their images under conditions of near native state have motivated immense interest in imaging biological molecules. So far the AFM studies related to biological samples are usually limited to imaging of DNA molecules[4-8].

On the other hand, the investigation on the red blood cell (RBC) membrane skeleton has greatly increased. Spectrin is the most abundant protein of the erythrocyte membrane skeleton. The molecular structures of human erythrocyte spectrin have been visualized with a transmission electron microscope (TEM), but the conventional TEM method usually requires a procedure of shadowing or staining the molecules with heavy metal to increase image contrast. The above procedure in TEM not only is an additional inconvenience for sample

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** To whom correspondence should be addressed.

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preparation, but also impedes the direct observation of biological macromolecules. AFM may be a new method to tackle the above problem. In this paper, we will explore the application of AFM to direct observation of spectrin structures.

1 Materials and methods

1.1 Preparation of spectrin sample

An extraction solution (0.1 mol/L Na_2HP_4, 0.1 mol/L EDTA, 0.1 mol/L thioethanol, pH 8.0) in a volume ratio of 30:1 was added into a solution containing human red cell membrane. After homogeneously mixing, the above solution was centrifuged at 15 000 round per minute for 10 min. The supernatant solution was removed, then another 1.5 volume of the above extraction solution (0.1 mol/L Na_2HPO_4, 0.1 mol/L EDTA, 0.1 mol/L thioethanol, pH 8.0) was added into the residual solution. The obtained solution was completely mixed, then stored at 0℃ overnight. After that, the solution was centrifuged at 15 000 round per minute for 10 min. The collected supernatant solution was the specimens containing spectrin for subsequent AFM experiments. The purity of the spectrin was examined with electrophoresis.

1.2 Preparation for AFM specimens

Three to five μL of the solution containing spectrin was spread on a freshly cleaved mica surface. About 1 min later, the residual solution on the mica was removed by attaching a piece of filter paper to the edge of the liquid drop. After natural air-drying, the obtained specimens were directly imaged with AFM. The procedures for the sample preparation did not include any shadowing or staining treatment.

1.3 Instrumentation

All AFM experiments were performed with a home-made AFM. A detailed description of the AFM instrument has been reported elsewhere[9]. The results presented here were obtained by optical detection of a cantilever deflection, which was sensed by measuring the deflection of a reflected laser beam at a photodiode, which has become common practice in the AFM field. All results were imaged in air at room temperature and the images were recorded in a constant-force mode. A grey scale typically represents the sample height with lighter features being the taller. Commercial cantilevers microfabricated from Si_3N_4 were used in this study (Nanoprobes, Digital Instruments Inc.), having a spring constant of $k=0.12$ N/m (200 μm long) and a pyramidal tip. Piezoelectric scanner was calibrated by taking the images of X-ray diffracting grating. AFM images were stored as 180 × 180 point arrays. Raw (unfiltered) data are presented unless otherwise stated in the study. Freshly cleaved mica surface was used as the substrate for adsorption of spectrin molecules.

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2 Results and discussion

2.1 AFM images of spectrin heterodimers

The spectrin molecules isolated from the red blood cell according to the above procedures were adsorbed on the freshly cleaved mica surface, then the specimens were directly imaged with AFM at room temperature in air. The stable AFM image of spectrin molecules can be obtained as shown in fig. 1(a). The spectrin molecules in the image were neither shadowed with any substances such as heavy metal, carbon, and so on, nor stained with any solution such as phosphotungstic acid and uranyl acetate. However, a clear AFM image with high contrast during a long AFM scanning can be observed. The AFM image

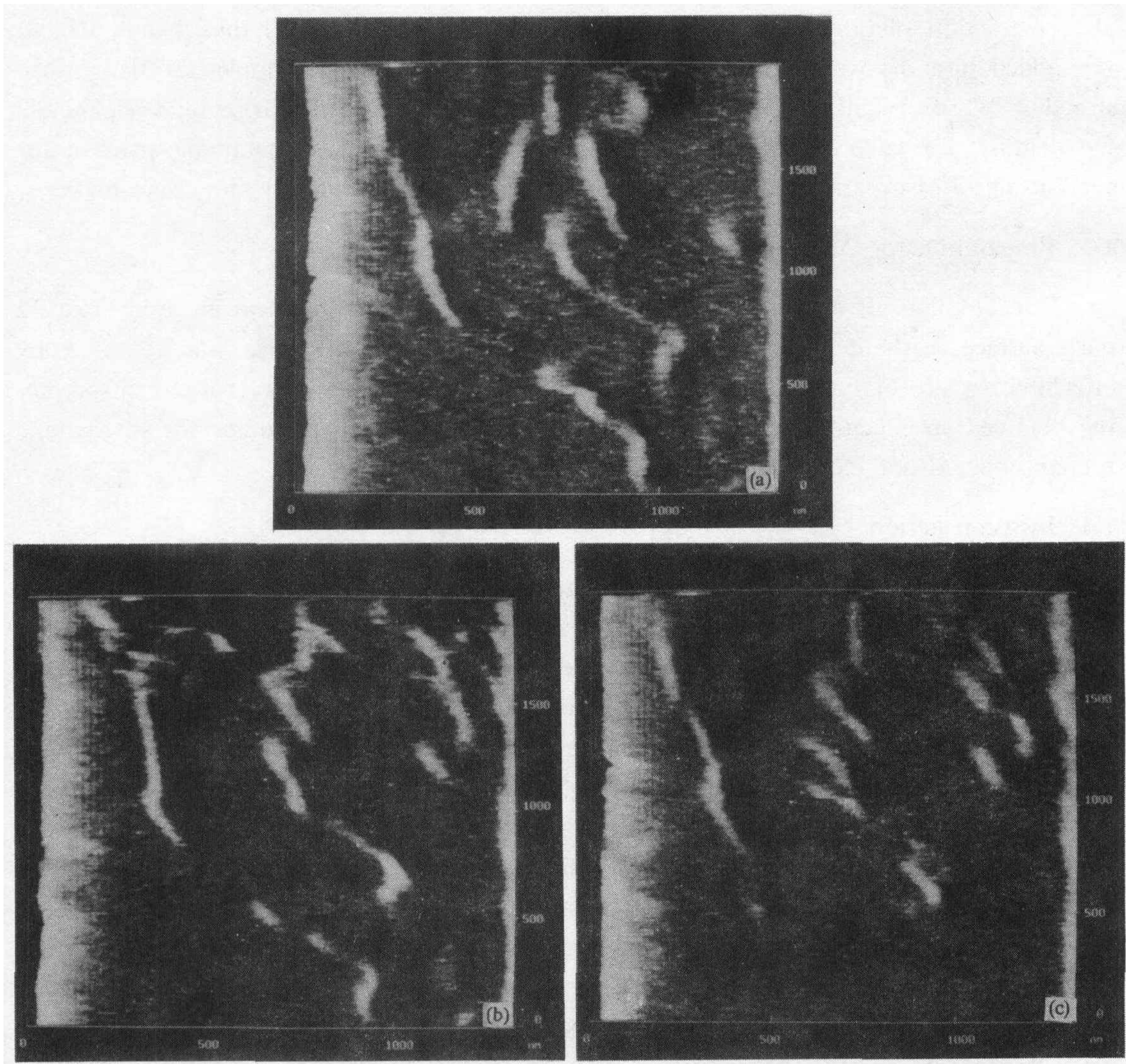

Fig. 1. AFM images of uncoated spectrin heterodimers in air. (a), (b) and (c) were taken from different scan areas. The characteristic appearance of spectrin heterodimers with compact rod-like form can be seen in the above areas.

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quality of spectrin molecules is as good as that of TEM images of shadowed or stained biological macromolecules. Similarly, several high-contrast images of spectrin molecules can also be found in other scan areas as shown in figure 1(b), (c).

Spectrin is a heterodimer, composed of nonidentical α and β subunits with calculated molecular weights (M_r) of 280 and 246 Kd, respectively. The spectrin molecule heterodimers were often observed in the AFM experiments on the above spectrin specimens. The AFM images in fig. 1 (a), (b), (c) show that the spectrin heterodimers exhibit a compact rod-like form and have a tendency to condense to ellipsoid granules. The lengths of the spectrin heterodimers measured from the AFM images are in the range from 100 to 120 nm. These lengths are much shorter than one would expect if the two α-helical polypeptides with molecular weight of 240 000 and 220 000 formed supercoiled α-helical rods. This result shows that the spectrin monomers must possess more folded tertiary conformations. In fact, highly folded tertiary models have been raised in the previous work on spectrin structures[10]. The α and β subunits of spectrin are supposed to be a form with the triple helical structure. The α subunit and β subunit contain 22 and 17 segments, respectively. Most of them are typical 106 amino-acid homologous spectrin repeats. On theoretical grounds, most investigators believe that each repeating segment folds into three α helices[10–13].

2.2 AFM images of spectrin tetramers

The TEM images of spectrin tetramers after unidirectional and rotary shadowing have been observed by Shotton *et al.*[13] Measurements of the shadowed lengths gave an uncorrected value of 197 nm. The distribution of molecular lengths is in the range from 160 to 240 nm. The AFM images of spectrin molecules observed in several scan areas are indicated in fig. 2(a), (b). A number of extended straight molecules can be seen in fig. 2(a). Most of the spectrin molecules have a length of around 200 nm. The length data of the uncoated spectrin molecules measured with AFM under ambient conditions are consistent with the values of shadowed spectrin tetramers observed in TEM[14]. As a result, it can be deduced that most of the spectrin molecules shown in fig. 2(a) may be spectrin tetramers. In fig. 2(b), the AFM images of spectrin molecules having a similar straight form are also observed, some of them are probably spectrin tetramers. The tetramers may be formed by end-to-end association of two spectrin heterodimers in two binding ways. One is the head-to-head association, the other is the head-to-tail association. In addition, in fig. 2(b), several spectrin molecules longer than the tetramers can also be found. Therefore, two ways of interactions between the ends of spectrin molecules are likely to occur during the association process.

2.3 AFM images of the hexamers, octomers and decamers

Besides tetramers, the AFM images of spectrin oligomers with relatively long lengths

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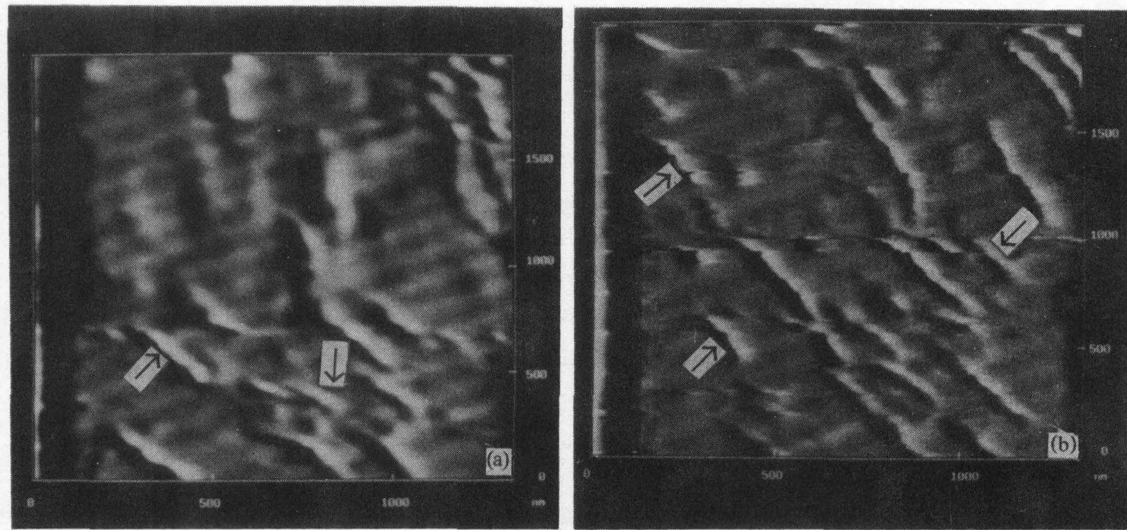

Fig. 2. AFM images of spectrin tetramers in air. Freshly cleaved mica surfaces were used as substrates. (a) and (b) were taken from different scan areas. A number of extended straight linear molecules can be seen in the above areas. Some of them (←) are around 200 nm in length, which may be spectrin tetramers.

were also found. Multiple spectrin dimers can be associated with each other in turn to form hexamers, octomers, and decamers via interaction between their ends. A number of spectrin molecules with lengths of about 300, 400, or 500 nm were seen in fig. 3(a) and (b). They may

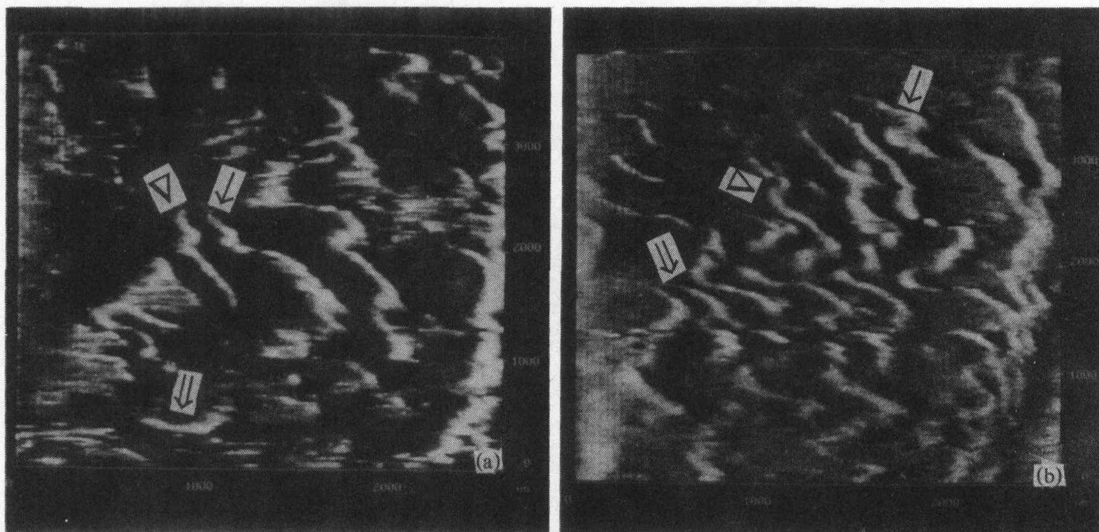

Fig. 3. AFM images of spectrin hexamers, octomers and decamers in air. Freshly cleaved mica surfaces were used as the substrates. (a) and (b) were taken from different scan areas. Several spectrin molecules with lengths of about 300 (←), 400 (⇐), and 500 nm (▷) can be seen. They may correspond to spectrin hexamers, octomers and decamers, respectively. These spectrin molecules appear as linear structures without branch.

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correspond to spectrin hexamers, octomers or decamers. These spectrin molecules exhibit straight, bent or curved linear structures, but no branch structures were found in their AFM images. These structures are different from the multiple-armed branched form observed in spectrin-actin band 4.1 complex[15]. The branch structure can be ascribed to the presence of actin band 4.1 including several sites which can bind to multiple spectrin molecules. For the specimens containing only one kind of molecules such as spectrin, the multiple-armed structure was not found in their AFM images. Therefore, it can be deduced that each end of the single spectrin subunit may include only one binding site. In this case, spectrin heterodimers can only form linear molecules without branch structures via interaction between their ends of molecules.

2.4 AFM images of high oligomers

A few spectrin molecules of above 1 000 nm in length can be visualized in an AFM image (fig. 4). These spectrin molecules may be high oligomers. These extended long spectrin molecules can be seen more often in fig. 5 (a), (b). In a few areas, the compact molecular aggregates formed by side interaction are also visible. Some of molecules appeared somewhat unfolded or curved. The curved molecules can be obviously found in fig. 5(a) and (b). The flexible spectrin molecules were randomly curved into different forms in L-, C-, or S-shape. These curved conformation forms of spectrin molecules reflect the flexibility of spectrin molecules in some sense.

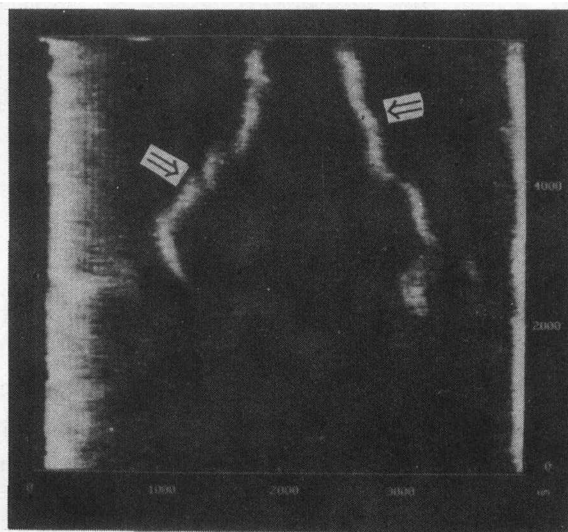

Fig. 4. The AFM image of high spectrin oligomers in air. Freshly cleaved mica surfaces were used as the substrate. Two high spectrin oligomers of above 1 000 nm in length can be observed (⇐).

3 Conclusions

With AFM, several kinds of uncoated spectrin molecules, such as the heterodimers, tetramers, hexamers, octomers, decamers and high oligomers, have been successfully observed. The formation of the various lengths of oligomers suggests that there are two connecting ways, in which spectrin molecules are associated head-to-head or head-to-tail. In the absence of actin band 4.1, no branch structures were found in AFM images of spectrin molecules. Those results show that the end of a spectrin molecule contains only one binding site.

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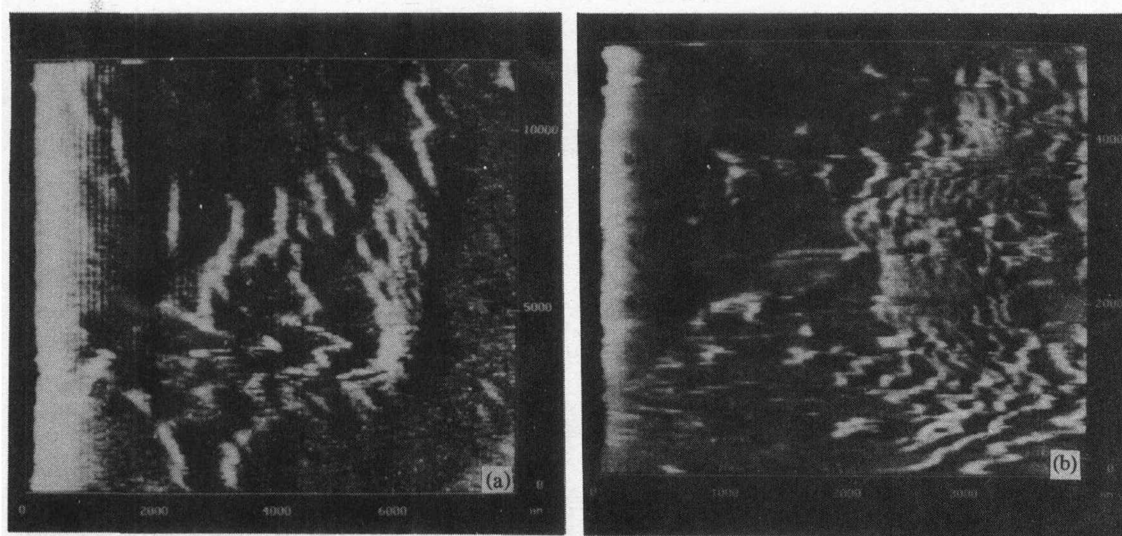

Fig. 5. AFM images of high spectrin oligomers in air. Freshly cleaved mica surfaces were used as the substrates. (a) and (b) were taken from different scan areas. Relatively long spectrin oligomers can be visualized in the picture. In a few areas, these spectrin molecules tend to compact together via side interaction.

As seen from the above, AFM provides a convenient method for observing structures of spectrin molecules. AFM not only has a similar or even higher resolution in comparison with the conventional electron microscope, but also can image spectrin molecules under ambient conditions, under which it is impossible to carry out observation by electron microscope. AFM will possibly become a challenging tool to image structures of biological macromolecules such as spectrin.

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