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Applied Surface Science 188 (2002) 506–512

applied surface science

www.elsevier.com/locate/apsusc

Intra- and intermolecular mechanics of proteins and polypeptides studied by AFM: with applications

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Received 1 September 2001; accepted 25 September 2001

Abstract

In order to use an atomic force microscope to measure the intramolecular mechanics of a single protein or polymer molecule, the very initial stage of stretching must be recorded without complications from nonspecific adhesions of target molecules to substrate materials. Although polyethylene glycol itself shows a certain level of adhesion to the functionalized surface of crystalline silicon wafer used as a substrate, adhesion of polypeptides and proteins can be minimized by covering the substrate surface with flexible polyethylene glycol chains. Determination of the cantilever spring constant is also important in the quantitative analysis of the result of force measurements. We introduce a hydrodynamic method of cantilever calibration as an effective method of estimating the relative spring constant of the cantilever. Finally, for mechanical manipulation of DNA, we introduce the use of a positively charged glass surface to keep DNA molecules in the stretched conformation for molecular manipulation including mechanical cutting of stretched DNA.

Keywords: Atomic force microscope; Bio-nanotechnology; Protein nano-mechanics; Spring constant calibration; DNA manipulation

1. Introduction

The atomic force microscope (AFM) has been increasingly used to monitor the mechanical events that accompany macromolecular deformations induced by externally applied forces from the AFM cantilever [1–4]. We apply the method to study intramolecular mechanics of proteins and DNA to facilitate artificial manipulations of biological structures such as biomembranes and chromosomes for the promotion of future medical technology. In the mechanical studies of tandemly repeated proteins such as titin and fibronectin, the adsorption of proteins to the gold surface has been usefully exploited to provide secure anchors during mechanical stretching experiments [4]. Some effort has been made to produce tandemly repeated version of normally monomeric proteins such as calmodulin (CaM) or lysozyme to take advantage of the above-mentioned studies [5,6]. In many of the studies using physical adsorption of sample proteins to a gold substrate, the initial parts of the force–extension relationships are obscured by spurious force peaks due primarily to a ''peeling process'' of various parts of the sample from the substrate or possibly to the tip. Information on the force required to destroy tertiary structures of individual domains in the repeated structure can be obtained but the process leading to the breakdown of a single domain is difficult to obtain.

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0169-4332/02/$ – see front matter
PII: S0169-4332(01)00962-X

We have developed a method to study the process leading to the breakdown of the secondary and tertiary structure of monomeric proteins such as carbonic anhydrase II (CAII), CaM, and a model compound polyglutamic acid (PGA), by using various types of bifunctional covalent cross-linkers to anchor sample proteins at well-defined sites on the molecular surfaces. For this purpose we constructed proteins and PGA with cysteine residues at specific sites to be used as reactive sites towards cross-linkers grafted on the surface of a silicon substrate as well as an AFM tip. Construction of modified proteins has been done by inserting cysteine residues at two selected sites, usually at the N- and C-termini, using genetic engineering techniques. PGA was synthesized with a cysteine residue at its C-terminus, thus providing a unique –SH group on one end as well as a unique –NH_2 group on the N-terminus. In the case of PGA and CAII, mechanical extension experiments produced large force peaks in the initial parts of the force curves. The use of the cross-linker with a long and flexible spacer of polyethylene glycol helped to remove most of the spurious force peaks.

Understanding protein-mechanics on the single molecular level helped us to start more practical work in biomedical applications of probe microscopy in extracting constituent proteins and DNA from large biostructures such as plasma membranes and chromosomes. In this review paper, we present a short account of the work published from our laboratory.

2. Experimental outlines

2.1. Adsorption of PGA to the silicon substrate

When PGA was, on one hand, covalently immobilized on the aminosilanized surface of a crystalline silicon wafer by using the amino reactive bifunctional covalent cross-linker, disuccinimidyl suberate (DSS), and on the other, bonded to the tip through *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Pierce, Rockford, IL), there was a strong indication of physical adsorption of PGA to the substrate. Force curves obtained by increasing the distance between the tip and the substrate were characterized by large force peaks in the very initial part, ranging in the peak force value between 100 and 600 pN as shown in Fig. 1.

Since we suspected it as resulting from a ''peeling process'' of the physically adsorbed PGA to the substrate, next we used a cross-linker having a long polyethylene glycol spacer (PEG3400-MAL, Shearwater Polymer, Clearwater, USA. This will be abbreviated as PEG cross-linker) of an average molecular weight of 3400, corresponding to the extended length of 28 nm. The histogram of extension length of the PEG cross-linker gave a major peak around 25–30 nm

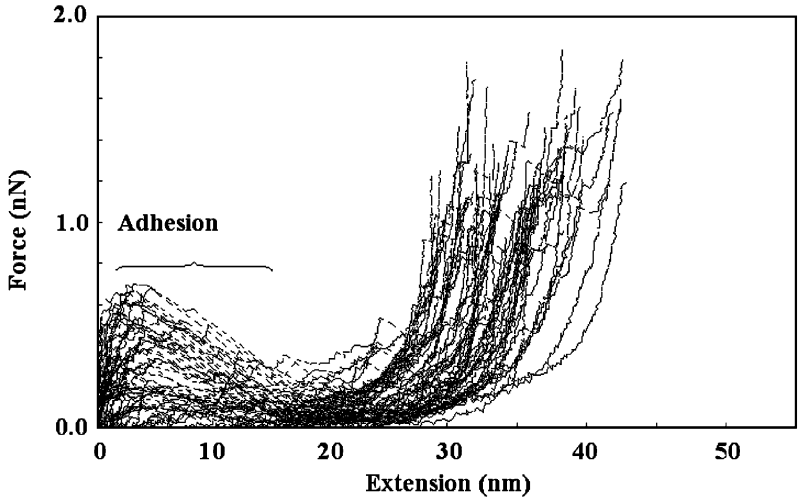

Fig. 1. Force–extension curves representing physical adsorption of PGA to modified silicon surface (Ref. [6] with permission).

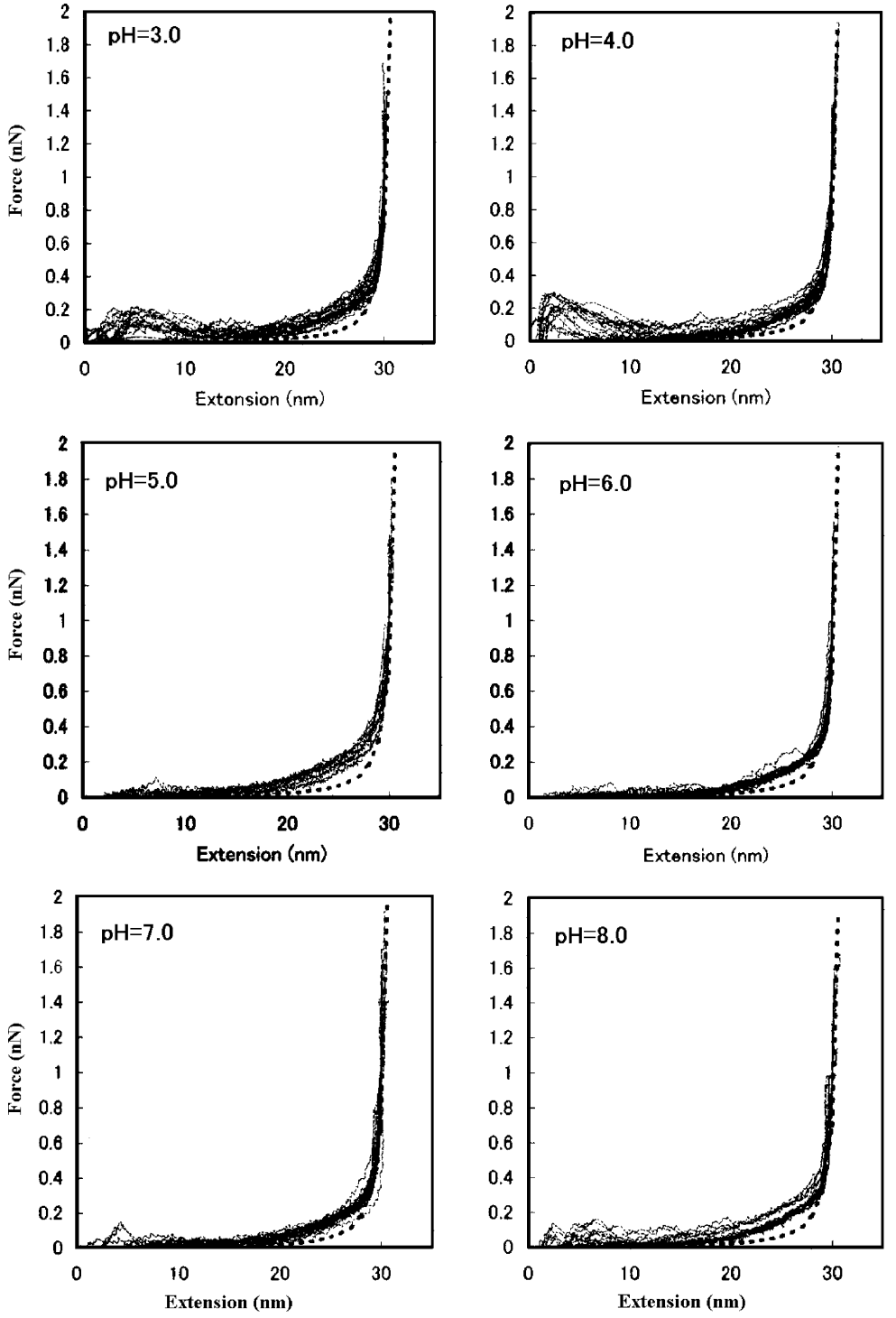

Fig. 2. Force–extension curves of PEG3400-MAL cross-linker at six different pHs. Some spurious force peaks are observed in the initial parts. The overall shape of the curves are almost independent of solution pH. Vertical and horizontal scales are common for all six graphs.

and a second small peak around 40–45 nm, indicating the possible presence of longer spacers in commercial samples. Fig. 2 shows a collection of force–extension curves of PEG cross-linker at different pHs.

There was a small degree of adsorption, but the shape of the force–extension curves was constant between pH 3 and 8. This nature of PEG cross-linker was effective when we studied the force–extension relationship of PGA between pH 3 and 8. Force peaks that appeared in the initial part of the curves in Fig. 1 subsequently disappeared completely.

The polymer has been known to assume helical and random coil conformations in acidic and neutral solutions, respectively. The net extension curves of PGA were obtained by subtracting the PEG extension from the averaged extension curves of the type given in Fig. 3 [5].

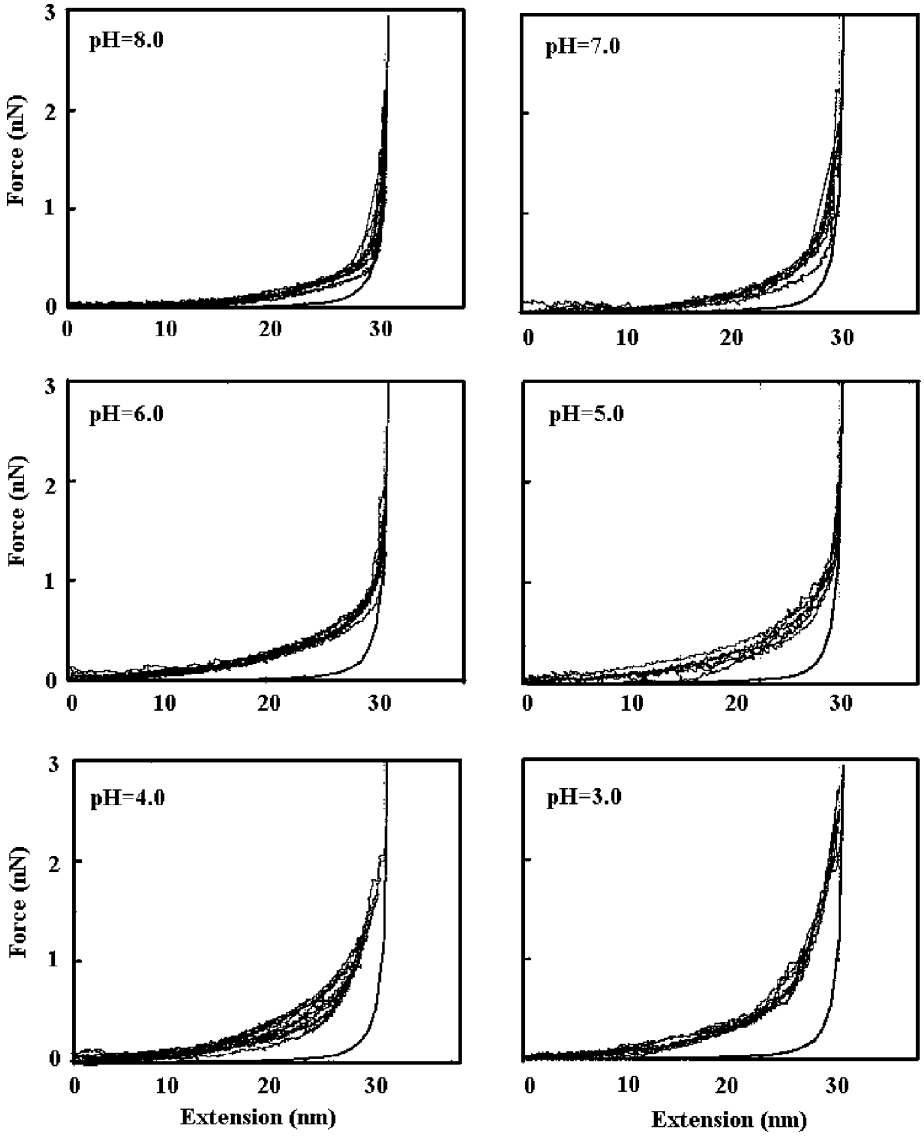

Fig. 3. Force–extension curves of PGA tethered to the substrate with PEG cross-linker after subtraction of PEG extension. Force curves having no initial force peaks were processed to obtain PGA extensions. Vertical and horizontal scales are common for all six graphs.

2.2. Calibration of cantilever spring constant by hydrodynamic method

Calibration of the cantilever spring constant has been realized as a crucial step in force measurement experiments using the AFM [6]. Various methods have been proposed to measure the value assuming the Hookean behavior of the cantilever. The cantilever spring constant is usually calibrated by the thermal vibration method [7]. In many of single molecular extending experiments, cantilevers are chemically modified and measurements are done in liquid. To confirm whether the spring constant is not changed by chemical treatments or during the measurement, a simple in situ method can be performed during, and/or just after the measurement. We found that the difference of the cantilever deflection during its movement in opposite directions can be correlated with the relative spring constant of the cantilever.

Extending and retracting traces of a force distance curve do not overlap perfectly in many cases according to several causes including hysteresis of the piezoelectric. The deflection of the cantilever in baseline where the probe is not touching the substrate or the sample has shown significant differences in liquid at high scan rates as shown in Fig. 4. The differences are likely due to the hydrodynamic force acting on the cantilever. The hydrodynamic force is expected to be applied uniformly on the flat surface of the cantilever. If the force per unit area is q, the deflection of the cantilever, d, with length, l,

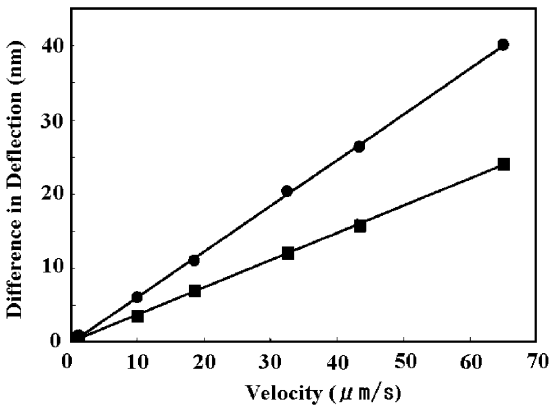

Fig. 4. Dependence of the difference of cantilever deflections between the approach and retraction period of the cantilever in aqueous medium.

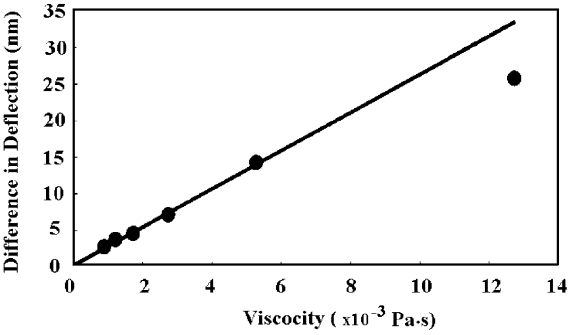

Fig. 5. Dependence of the difference of cantilever deflections between the approach and retraction period of the cantilever on the viscosity of the solvent.

Young's modulus, E, and moment of inertia, I, will be:

$$d = \frac{ql^4}{8EI} \tag{1}$$

The value of q is expected to be proportional to the relative velocity and the viscosity coefficient of the liquid. We, therefore, measured the dependency of the difference on the rate of sample stage movement and on the viscosity of the solution. The difference of deflection in the base line of the force distance curve was plotted against the rate of piezoelectric movement (Fig. 5). The viscosity of the liquid was controlled by adding sucrose to water. Fig. 5 shows how the deflection difference changed when the sucrose concentration varied from 0 to 50%. The difference increased linearly with viscosity while the viscosity was less than 5×10^{-3} Pa s. The deflection was found to be proportional to both the rate and the viscosity within a limited range, which proved that the force bending the cantilever is likely caused from the hydrodynamic effect.

We can use this difference to calibrate the spring constant of a cantilever relative to a standard cantilever whose spring constant is determined by an absolute method [7]. One important assumption is that the two-dimensional projection of the cantilever and its holding angle to the cantilever holder be constant. The variation in the projected shape of cantilevers of same types were found constant to a satisfactory degree by using optical and scanning electron microscopes. Based on this assumption, we measured the spring constant of some cantilevers by the thermal vibration method and compared them with

the results of the hydrodynamic method. Correlation constants c were calculated to be 2.71×10^{-5} and 6.25×10^{-5} for NP-S narrow long cantilevers and TR400 long cantilevers respectively, whose spring constant can be calculated as:

$$k = \frac{cv}{\Delta} \quad (2)$$

where k is the spring constant (N/m), c the correlation constant, v is the rate of piezoelectric movement (m/s) and Δ the difference of the deflection between extension and retractions (m). Details of application of this method will be published elsewhere.

2.3. Electrostatic adsorption of DNA to the substrate for the cutting manipulation

Cutting a selected DNA strand at a desired locus is becoming one of the important operations of DNA manipulation for the development of precise methods in future gene therapy. DNA can be immobilized on a solid surface in extended conformations with various methods and we devised a way to use a slide glass having a modified surface with amino groups, being positively charged at a neutral pH. In a neutral buffer, DNA is negatively charged and exhibits strong electrostatic interactions with a positively charged surface. Fig. 6 shows fluorescently labeled DNA stretched out on an aminosilanized glass surface. The figure shows the tip of a nano-manipulator approaching the DNA from the back side to cut the DNA with a mechanical force. It was observed that DNA after cutting, kept a stretched conformation. This is a very preliminary result of our ongoing work on the mechanical scission of DNA and chromosomes [8].

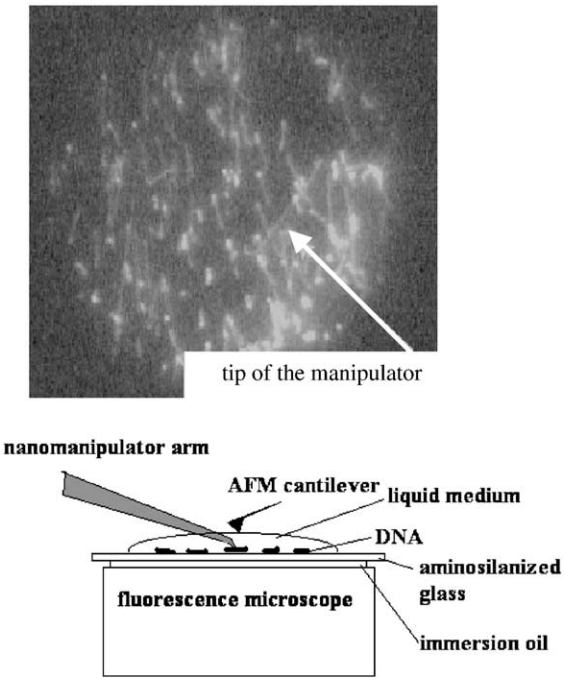

Fig. 6. Top: fluorescence microscopic image of DNA fragments stretched out on an aminosilanized glass surface with the tip of the nano-manipulator approaching from the other side of the glass to cut the stretched DNA. Bottom: schematic diagram of combined AFM, nano-manipulator and total internal reflection fluorescence microscope. The nano-manipulator arm with a thin needle is manipulating on a piece of DNA stretched on an aminosilanized glass in liquid medium.

3. Conclusions

The use of AFM in the mechanical study of proteins and polypeptides can be done by using appropriate methods to avoid physical adsorption between the sample and the substrate which gives rise to spurious force peaks in the force–extension curves, especially in their very initial parts. The internal mechanics of proteins can only be studied when appropriate precautions to avoid adsorptions are taken as indicated. When AFM is combined with optical microscopes and micro- or nano-manipulators, more versatile uses leading to direct cellular and chromosome manipulations are possible. In such operations, it will be desirable to replace a contact mode AFM which is currently in use in our laboratory with a noncontact mode AFM, especially when functionalized AFM tips for special chemical groups are used. When the tip is reactive towards specific chemical groups on the sample, it will be required to image the sample without touching it, then make limited contacts on specifically chosen areas of the sample with the functionalized surface tip.

References

[1] U. Dammer, W. Hegner, D. Anselmetti, P. Wagner, M. Dreier, W. Huber, H.J. Guntherodt, Biophys. J. 70 (1996) 2437.

[2] P. Hinterdorfer, W. Baumgartner, H.J. Gruber, K. Schilcher, H. Schindler, Proc. Nat. Acad. Sci. 93 (1996) 3477.
[3] G.U. Lee, L.A. Chrisey, R.J. Colton, Science 266 (1994) 771.
[4] M. Rief, M. Gautel, A. Schemmel, H.E. Gaub, Biophys. J. 75 (1998) 3008.
[5] A. Idiris, M.T. Alam, A. Ikai, Protein Eng. 13 (2000) 763.
[6] A. Ikai, Surf. Sci. Rep. 26 (1996) 261.
[7] J.L. Hutter, J. Bechhoefer, Rev. Sci. Instrum. 64 (1993) 1868.
[8] S. Nishida, A. Ikai, Ultramicroscopy, in print.