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ORIGINAL CONTRIBUTION

Effects of denaturation and association of collagen on adsorption behavior: two-dimensional nanostructure and its property

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Abstract Effects of denaturation and association of collagen on adsorption behavior were studied in various pH and temperature T by a quartz crystal microbalance QCM. The surface nanostructure, the adhesion force F_{ad}, and the local frictional coefficient μ of collagen were studied by an atomic force microscope AFM and a lateral force microscope LFM. Adsorptions of collagen were Langmuir type in the regions of pH 3.0–5.8 and T=25–50 °C. With increasing pH and T, adsorption mass Γ increased, and adsorbed fibrils increased in width. At interface, the association of collagen molecules in solution enhanced the formation of fibrils. The results of F_{ad} in the solution of pH 3.0 increased with increasing Γ and T but decreased in pH 5.8. The results of μ increased with increasing Γ and T, and those in pH 3.0, were much greater than those in pH 5.8. From comparing them with the results of bovine serum albumin and sodium hyaluronate monolayer, we concluded that nonelectrostatic interactions and the softness of collagen layer contribute primarily to F_{ad} and μ.

Keywords Collagen · Adsorption · Adhesion force · Frictional force · Atomic force microscope

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Introduction

Numerous applications require controlling the morphology and arrangement of the protein adsorption layers in a precise way. In recent years, template method is used extensively to synthesize nanostuctured and multifunctional materials. By using kinds of templates, various nanostructures with various shapes such as spherical particles; one-dimensional nanorod, nanowire, and nanotube; and two-dimensional ordered nanostructures, etc., could be fabricated. As for template, kinds of material were used, such as alumina [1], silicon dioxide [2], carbon nanotube, surfactant [3, 4], and biomolecule [5]. These techniques are expected to bring significant progress in nanostructure formation of proteins, which have attracted more and more attention in the field of biomaterials science, biosensors design, immunological test systems, immobilized-enzyme bioreactors, and food industry.

Collagen is an important protein component localized in skin, tendon, bone, etc. Collagen molecules compose of three polypeptide chains which are coiled in a left-handed helix being a rod-like structure (300 nm in length and 1.5 nm in diameter) [6]. The collagen molecules are thrown into right-handed helixes stabilized [7, 8] and assembled by intermolecular cross-linking to become thicker and stronger strands to form networks, which might determine their particular properties in the body.

Controlling the arrangement of collagen is an important point in designing various functional nanostructured materials because of its particular properties and functions in the body. In the past years, many studies on morphologies and properties of collagen at interface were reported. Rouxhet's group [9–11] studied collagen adsorption layers on native and oxidized poly(ethylene terephthalate) layers. They discussed the adsorption of collagen on hydrophilic

and hydrophobic substrates. Furthermore, collagen was investigated on other substrates such as mica [12–14], poly(methyl methacrylate) [15], tissue-culture polystyrene, and plasma-oxidized polystyrene [16]. It was found that collagen films formed on different substrate surfaces showed different adsorption behavior and properties.

In our lab, we are working on nanostructures and nanoproperties of biopolymers (protein, acid polysaccharides, and their complexes [17–21]) in the solution and at the interface to discuss how the nanostructures, nanoadhesion force, and frictional properties of those adsorption layers would give strong effect on cell or tissue morphogenesis and self-repair. In this paper, self-assembly layers of collagen on a gold surface of quartz crystal microbalance QCM tip were investigated as a basic study on the arrangement of collagen on various substrate surfaces. QCM is an ideal apparatus for obtaining the adsorption mass to investigate the adsorption behavior at various T and pH. Their surface structure and properties were tried to be studied by an atomic force microscope AFM and a lateral force microscope LFM.

Experimental section

Materials Acid soluble type-I collagen prepared from bovine Achilles dermis was purchased from Koken as an aqueous solution (3.0 g dm^{-3} at pH 3.0). Its molar mass and isoelectric point were 300,000 g mol^{-1} and 9.0, respectively. Other reagents were all of special grades, and water was distilled and deionized.

Measurement of thermal denaturation of collagen by circular dichroism A thermal denaturation of collagen was estimated by an ellipticity obtained by using a spectropolarimeter (Model J-725, Jasco). A cell with 10-mm light path was used in this study. The concentration of collagen C was 0.03 g dm^{-3}, and the experimental temperature T was in the range of 20–50 °C, which was controlled by a thermostatic cell holder (PTC-343, Jasco). The solutions were kept at each T for 40 min before the measurement. The ellipticity at 221 nm can be used as a signal of the helix-coil transition from a native helix type to a denatured coil one. The collagen molecule is a native type at 25 °C and a denatured one at 50 °C. The ratio of the native type $[\theta]$ can be expressed by Eq. 1 using the mean residue ellipticity $[\theta]^T$ at 221 nm and T °C [22],

$$[\theta] = \left([\theta]^T - [\theta]^{50\,^\circ\mathrm{C}}\right) \Big/ \left([\theta]^{20\,^\circ C} - [\theta]^{50\,^\circ\mathrm{C}}\right) \quad (1)$$

Measurement of adsorption mass of collagen on Au surface Adsorption mass of collagen Γ was measured by immersing a QCM tip (the area: 0.159 cm^2) in collagen solutions under various T and pH. In addition, three or five samples were prepared under every same condition. The solutions were also kept at each T for 40 min before the measurement as mentioned above. The concentration of collagen solutions was in the region of $0.6–15\times10^{-4}$ g dm^{-3}. The surface of QCM tip was covered by Au and was cleaned by ethanol and distilled water before the measurements. The value of Γ on Au surface of the tip was determined from the deviation of the resonance frequency according to Sauerbrey's equation. The frequency decrease of 1 Hz corresponded to an increase of 0.8 ng [17]. The collagen adsorption layers prepared in this study were used in following experiments.

Observation of collagen adsorption layer by AFM The collagen adsorption layers were observed in air by a tapping mode using AFM (Nanoscope, Digital Instruments). After drying the adsorption layers at room temperature under atmospheric pressure, the surface structures were observed at six to eight spots on each sample. AFM tip (type: NCH-W, Digital Instruments) was composed of silicon monocrystal Si which was used without any surface treatment. Its normal spring constant K_N was 33 $N{\cdot}m^{-1}$.

Measurement of adhesion force F_{ad} using contact mode of AFM The value of F_{ad} between the collagen adsorption layer and the AFM tip was measured in the solution at six to eight spots on each sample and was averaged. To protect the electronics of AFM from the solution, a Teflon skirt was placed above the cantilever. The forces between the collagen adsorption layer and the AFM tip were measured at various vertical positions of the tip. The value of F_{ad} is equal to a pull-off force and can be given directly from a force–distance curve. The AFM tip for a contact mode with no modification was also composed of Si, and the value of K_N was 0.12 N m^{-1}, which was much smaller than that used in the tapping mode.

Measurement of frictional force F_f using LFM The value of F_f of the collagen layers were measured in the solution by LFM using the same AFM tip as mentioned in the measurement of F_{ad}. The deflection of the tip of cantilever in lateral direction ΔX_L was measured by scanning in trace and retrace, and the values of F_f were obtained by Eq. 2:

$$F_f = K_L \times \Delta X_L \quad (2)$$

where K_L is the cantilever spring constant in lateral direction, which can be calculated from normal spring constant K_N [23]. The value of frictional coefficients μ

between adsorption layer and an AFM tip can be available from Eq. 3.

$$F_f = (F_{load} + F_{ad}) \quad (3)$$

F_{load} is a load force exerted by the tip [24].

In this study, F_{ad} was ignored in the calculation of F_f because the values of F_{ad} were very small compared with those of F_f. The values of F_{ad} and μ measured at several points on each sample were averaged.

Results

Thermal denaturation of collagen

After collagen solutions were kept at various T in the range of 15–50 °C for 40 min, the ellipticities were measured. In the solutions, any insoluble collagen was not found even under the conditions of pH 5.8 and 50 °C. This would be due to the low concentration (0.03 g dm^{-3}) of the solution. The spectrum of an ellipticity of the collagen solution showed the same feature in pH 3.0 and 5.8, which possessed a positive peak at 221 nm and decreased with increasing T. The peak can be used as a signal of the helix–coil transition [22] from a native helix type to a denatured coil one. The collagen molecule is a native type at 25 °C and a denatured one at 50 °C. The results of $[\theta]$ are shown in Fig. 1 as a function of T. The ratio of the native type decreased abruptly with increasing T. The transition temperatures T_m defined by the midpoint of the transition were 39.0 and 41.0 °C in the solutions of pH 3.0 and 5.8, respectively. The thermal stability of collagen increased with increasing pH. At 37 °C, collagen did not denature in pH 5.8, but 5% of collagen denatured in pH 3.0. When T>45 °C, collagen denatured completely at both pH 3.0 and 5.8.

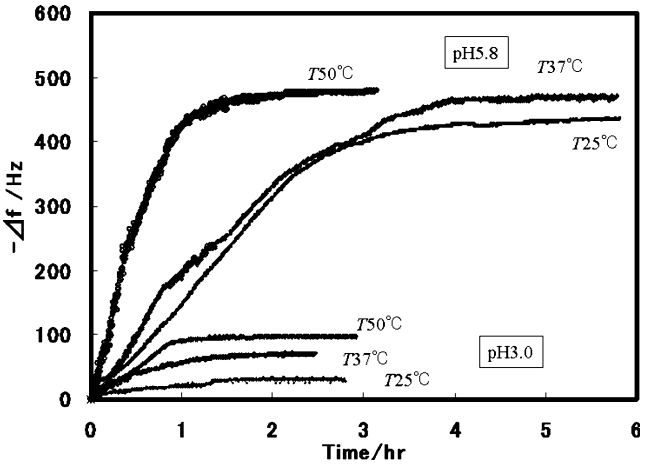


Fig. 2 Time courses of resonance frequency of quartz crystal microbalance $-\Delta f$ with collagen adsorption on the Au surface at different temperatures T and pH. The concentration of collagen was 7.5×10^{-4} g dm^{-3}. The three data at the top were those of pH 5.8, and the three at the bottom were those of pH 3.0

Effect of denaturation of collagen on its adsorption layer The QCM tip was immersed in collagen solution, and the decrease of the resonance frequency $-\Delta f$ was measured. The concentration of collagen solution was in the region of $0.6–15\times10^{-4}$ g dm^{-3}. Typical time courses of $-\Delta f$ are shown in Fig. 2. Time reaching the equilibrium

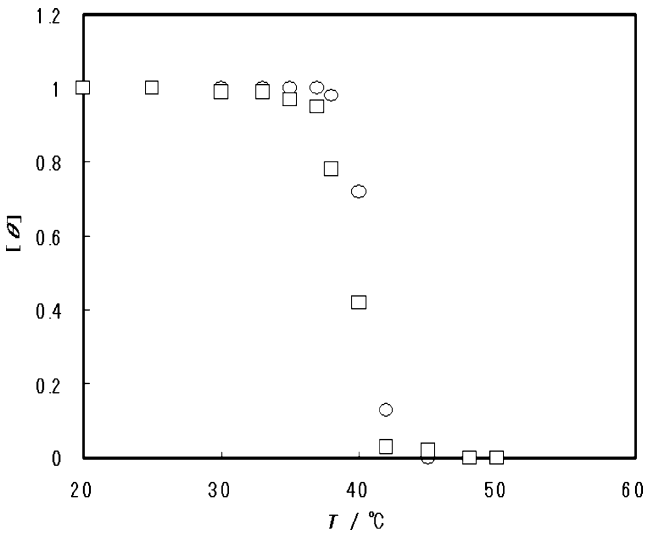


Fig. 1 Effect of pH on thermal denaturation of collagen. The ratios of the thermal denaturation were estimated from $([\theta]^T-[\theta]^{50\ °C})$ / $([\theta]^{20\ °C}-[\theta]^{50\ °C})$ in which $[\theta]^T$ is the mean residue ellipticity at T (°C) and at 221 nm. The concentration of collagen was 0.03 g dm^{-3}. *Empty squares* pH 3.0, *empty circles* pH 5.8

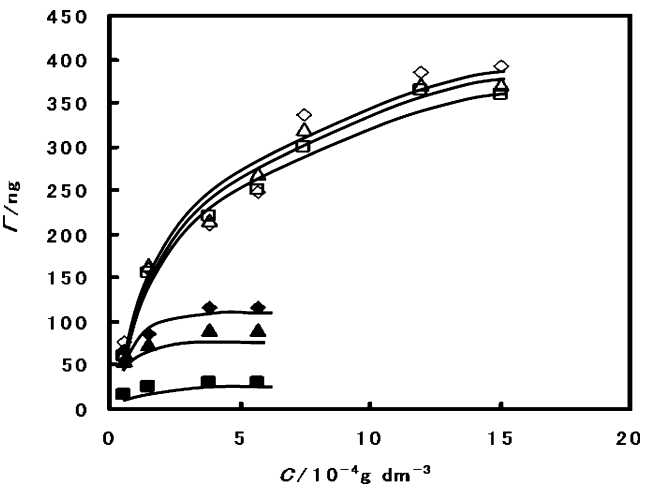


Fig. 3 Effect of concentration of collagen C on adsorption mass Γ on Au surface at different temperatures T and pH. *Filled squares* pH 3.0, T=5 °C; *filled triangles* pH 3.0, T=37 °C; *filled diamonds* pH 3.0, T=50 °C; *empty squares* pH 5.8, T=25 °C; *empty triangles* pH 5.8, T=37 °C; *empty diamonds* pH 5.8, T=50 °C

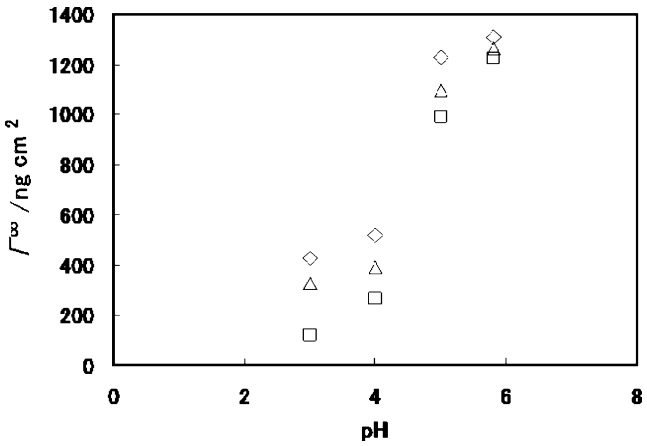


Fig. 4 Effect of pH on saturated adsorption mass Γ^{∞} of collagen. The results of Γ^{∞} showed the constant adsorption masses in sufficiently high concentrations. The results of pH 3.0 and 5.8 agreed well with the values obtained from the Langmuir's reciprocal plots. *Empty squares* T=25 °C, *empty triangles* T=37 °C, *empty diamonds* T=50 °C

decreased with increasing T in both pH 3.0 and 5.8. The value of Γ obtained from the values of $-\Delta f$ are shown in Fig. 3 as a function of C. They showed Langmuir-type adsorptions. As shown in Fig. 4, the saturated adsorption mass Γ^{∞} increased abruptly with increasing pH. From the slope and intercept of the reciprocal plot of Γ and C, the adsorption constant K and Γ^{∞} were obtained. They are shown in Table 1. Although the values of Γ^{∞} in pH 5.8 were much more than those in pH 3.0, the values of K in pH 5.8 were smaller than those in pH 3.0. The values of Γ^{∞} and K increased with increasing T in both pH.

Effects of pH and T on the structure of collagen adsorption layers The adsorbed collagen layers on Au surface of QCM tip were observed using a tapping mode of AFM in air. The surface structure of Au-evaporated layer of QCM tip is shown in Fig. 5a. Au particles were arranged closely and were 100–140 nm in diameter and about 10 nm in height. Fig. 5b–d shows the topographic images of the saturated adsorption layers of collagen at 25, 37, and 50 °C in pH 3.0. As shown in Fig. 5b, the image of the adsorbed layer at 25 °C and pH 3.0, which was abbreviated to layer 3.0–25, was almost the same as that of the Au surface. Although the adsorption mass was measured to be 119 ng, collagen molecules could hardly be found. This is due to the low adsorbed mass and the small dimension of the adsorbed collagen molecules compared with other adsorption layers. The monomeric collagen molecule (diameter: 1.5 nm, length: 300 nm) in the solution is considered to adsorb as it is. With increasing T, the images of the layer became obvious and showed a patch-like structure in which collagen fibrils took a parallel arrangement as shown in Fig. 5c,d. Regarding the directions of the patches as those of the collagen fibrils, the directions of the patches were more random at 50 °C than at 37 °C. The widths of the fibrils increased with increasing T. They were 20 nm at 37 °C and 30 nm at 50 °C on average.

Figure 5e–g shows the topographic images of the saturated layers of collagen adsorbed in pH 5.8. As shown in Fig. 5e, the surface of the layer 5.8–25, which stands for the collagen adsorption layer prepared in pH 5.8 at 25 °C, showed obvious fibrils arranged in the state of the parallel. The fibrils were 17 nm in width on average. However, the layer 5.8–37 and layer 5.8–50 did not show the parallel arrangement but the entangled structures. With increasing T, the fibrils became wider and thicker. Their entangled fibrils were 25 nm at 37 °C and 45 nm at 50 °C in width.

Adhesion force F_{ad} between collagen adsorption layer and AFM tip F_{ad} between the collagen layer and the AFM tip was measured by a contact mode of AFM in the solution. Figure 6 showed the results of F_{ad} as a function of the value of Γ. The result at Γ=0 shows the adhesion force of the Au surface of the QCM tip. The values of F_{ad} in pH 3.0 increased with increasing Γ and T. On the other hand, those in pH 5.8 decreased with increasing Γ and became 0 in the region of Γ>250 ng irrespective of T.

Frictional force F_f between collagen adsorption layer and AFM tip F_f between the collagen layer and the AFM tip was also measured in the solution using LFM. The values of F_f were obtained from Eq. 3 under several F_{load}. Figure 7a,b showed the results of the saturated adsorption collagen layers in pH 3.0 and 5.8 as a function of F_{load}. The values of F_f increased linearly with increasing F_{load}. The value of μ can be obtained from Eq. 3. As

Table 1 Adsorption characteristics of collagen layers adsorbed on Au surface

	Values		
pH 3.0			
T (°C)	25	37	50
K (10^3 dm^3 g^{-1})	5.0	6.3	6.5
Γ^{∞} (ng cm^{-2})	119	330	428
pH 5.8			
T (°C)	25	37	50
K (10^3 dm^3 g^{-1})	3.2	3.4	3.6
Γ^{∞} (ng cm^{-2})	1,226	1,264	1,308

K Adsorption constant, Γ^{∞} saturated adsorption mass

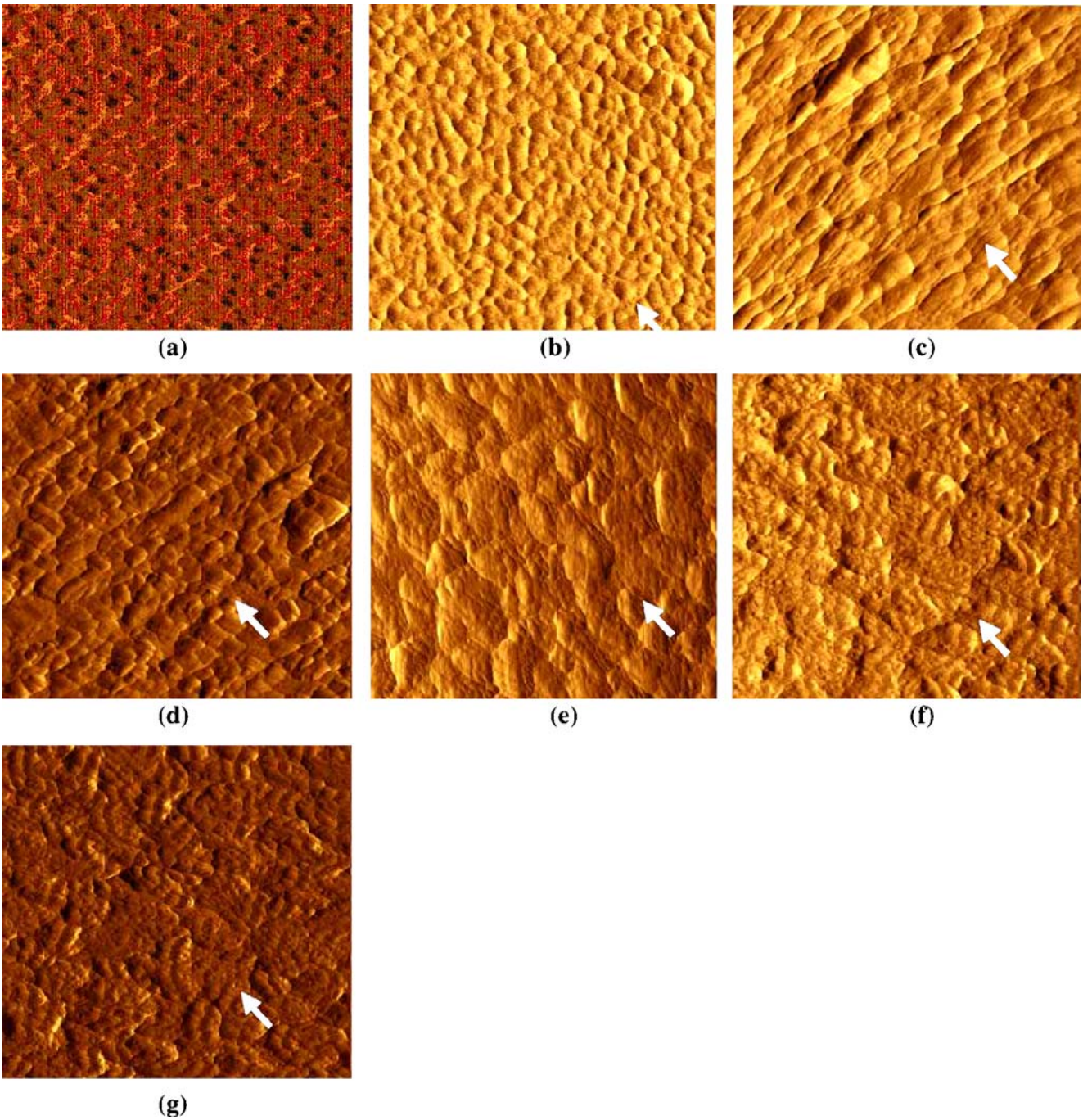


Fig. 5 Topographic images (2×2 μm) of saturated adsorption collagen layers on Au surface observed by atomic force microscope in air. **a** Au surface, **b** layer(pH 3.0, T=25 °C), **c** layer(pH 3.0, T=37 °C), **d** layer(pH 3.0, T=50 °C), **e** layer(pH 5.8, T=25 °C), **f** layer(pH 5.8, T=37 °C), **g** layer(pH 5.8, T=50 °C). A collagen fibril is indicated by an *arrow*

F_{load},F_{ad}, F_{ad} was ignored, and the values of μ of the adsorption layer were obtained from the slope. As shown in Fig. 8, the results of μ increased with increasing Γ, and the values in pH 3.0 were more than those in pH 5.8. The value of Γ=0 is that of the Au surface. As shown in Fig. 9, the results of μ of the saturated adsorption layers increased with increasing T.

Discussion

Denaturation of collagen in the solution The thermal stability of collagen increased slightly with increasing pH of the solution as shown in Fig. 1. At pH 3.0, the acid-soluble collagen molecules are soluble and exist in a monomer state possessing positive charges. The monomeric

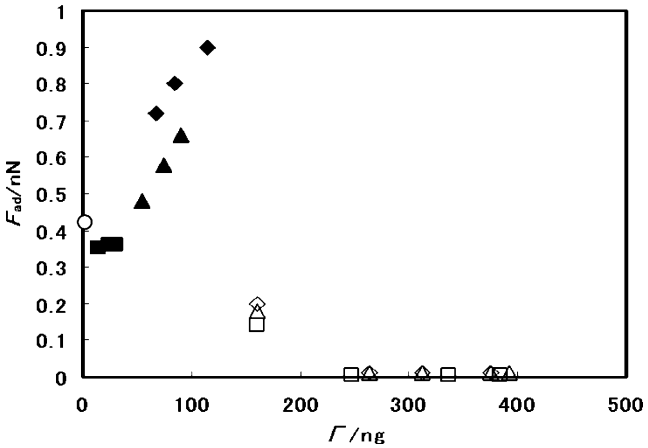


Fig. 6 Adhesion force F_{ad} between AFM tip and collagen layer adsorbed at different temperatures T and pH. *Empty circles* Au surface (F_{ad}=0.4 nN), *filled squares* layer(pH 3.0, T=25 °C), *filled triangles* layer(pH 3.0, T=37 °C), *filled diamonds* layer(pH 3.0, T=50 °C), *empty squares* layer(pH 5.8, T=25 °C), *empty triangles* layer(pH 5.8, T=37 °C), *empty diamonds* layer(pH 5.8, T=50 °C)

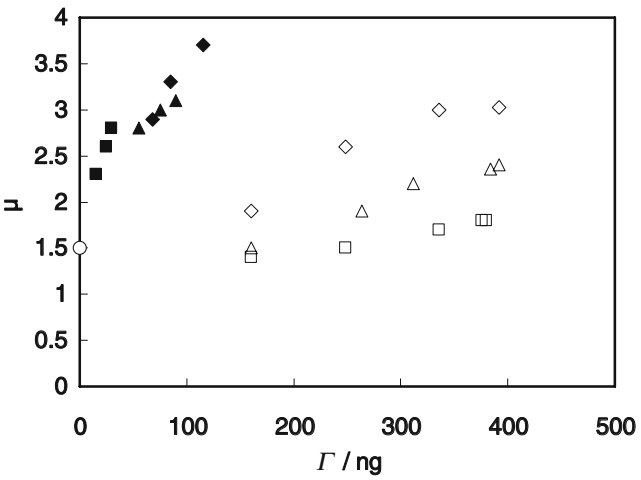
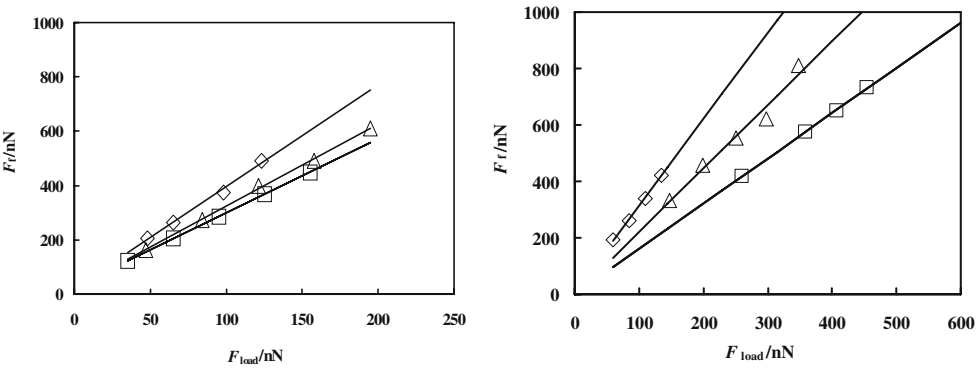


Fig. 8 Effect of adsorption mass Γ on frictional coefficient μ of collagen layer at different temperatures T and pH. *Empty circles* Au surface (μ=1.4), *filled squares* layer(pH 3.0, T=25 °C), *filled triangles* layer(pH 3.0, T=37 °C), *filled diamonds* layer(pH 3.0, T=50 °C), *empty squares* layer(pH 5.8, T=25 °C), *empty triangles* layer (pH 5.8, T=37 °C), *empty diamonds* layer(pH 5.8, T=50 °C)

molecules denatured while increasing T. At 50 °C, they denatured completely and turned into a random coil structure. At 37 °C, they denatured partly, and their structures are considered to be intermediate between a rod and a random coil. With increasing pH, the monomeric molecules associate spontaneously each other to form subfibrils. Under the condition of pH 5.8, the native collagen molecule is reported to form a subfibril being 5 nm in diameter even at 25 °C [25]. From the difference in transition temperature shown in the result, it can be considered that the formation of the collagen subfibril in a higher pH solution enhanced its thermal stability.

Effects of pH and T on adsorption behavior and surface structure of collagen Collagen is reported to adsorb on both surfaces of hydrophobic and hydrophilic films [14]. As collagen contains more hydrophobic amino acid groups, such as methionine, alanine, proline, etc. than other proteins [26], it would prefer a hydrophobic surface than a hydrophilic one. In addition, Au surface is hydrophobic, and collagen would adsorb on it primarily by a hydrophobic interaction.

As shown in Fig. 4, the results of Γ^{∞} increased significantly with increasing pH but increased slightly with increasing T. The charge amount, the structure, and the state of the association of collagen molecules in solution would affect the adsorption. When increasing pH, the hydrophobicity increases due to the decreases of the charge density, and they associate to form a subfibril in the solution as mentioned above. The hydrophobic amino acid residues facing outside are considered to enhance its insoluble property and the adsorption on Au surface.



(a) pH3.0 (b) pH5.8

Fig. 7 Frictional force F_f plotted against load force F_{load} between AFM tip and saturated adsorption collagen layer at various temperatures T and pH. **a** pH 3.0, **b** pH 5.8. *empty squares* T=25 °C, *empty triangles* T=37 °C, *empty diamonds* T=50 °C

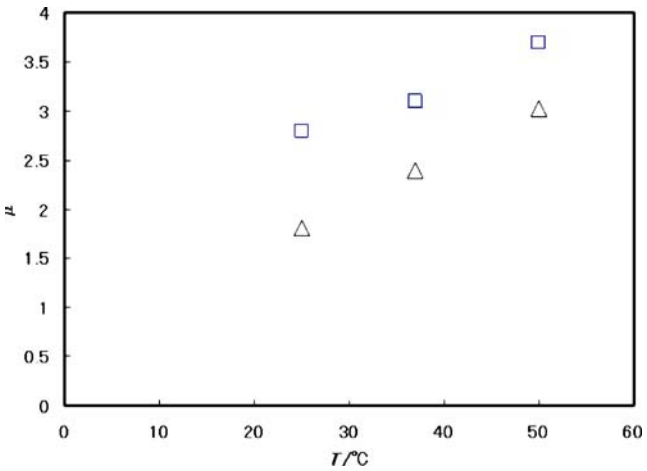


Fig. 9 Effect of temperature T on frictional coefficient μ of saturated adsorption collagen layer at different pH. *Empty squares* pH 3.0, *empty triangles* pH 5.8

The image of collagen molecule in layer 3.0–25 was not obvious as shown in Fig. 5b. However, its value of Γ^{∞} was 119 ng cm^{-2} as shown in Table 1. Assuming that the collagen molecules (diameter: 1.5 nm, length: 300 nm) adsorb in the state of the saturated monolayer, the adsorption mass was calculated to be 110 ng/cm^2, which was almost equal to the experimental result. This indicates that collagen molecules adsorb on the Au surface in a monomer state in pH 3.0.

On the other hand, the AFM image of layer 5.8–25 showed obvious fibrils whose mean diameters were 17 nm as shown in Fig. 5e. This indicates that the subfibrils in the solution aggregated on the Au surface in the state of the fibril, i.e., when pH increased, the collagen molecules associated to become the subfibrillar structure in solution and then aggregated further on the Au surface in the state of the fibril. From the ratio of the sectional area of the fibril (diameter: 17 nm) to the subfibril (diameter: 5 nm), one adsorbed fibril is estimated to be composed of 12 subfibrils. As shown in Table 1, the value of Γ^{∞} of the layer 5.8–25 increased to be 1,226 ng/cm^2, which was much more than that in pH 3.0. Assuming that the mean length of the fibrils is equal to that of the monomer in the solution (300 nm) and the adsorption is in the state of the monolayer, the calculated value of Γ^{∞} is 1,329 ng cm^{-2} and agrees well with the experimental result. Then, the adsorbed fibrils would be also in the state of the monolayer.

At pH 3.0, native collagen denatured when increasing T and resolved into three chains (α-component), two chains joined by covalent cross-linkages (β-component), and three chains joined by covalent cross-linkages (γ-component) [27]. These denatured collagen molecules formed the fibrils on the adsorption as shown in Fig. 5c,d. Their orientation in the patches should result from the presence of the β- and γ-components in the solution. At pH 5.8 and 25 °C, the native collagen associated and formed the subfibrils in the solution. With increasing T, the subfibrils denatured, and thick entangled fibrils were formed on adsorption as shown in Fig. 5e–g.

Effects of pH and T on adhesion force F_{ad} of collagen adsorption layers As shown in Fig. 6, the value of F_{ad} increased with increasing Γ at pH 3.0, but at pH 5.8, it decreased to the contrary. F_{ad} is related closely to surface chemical composition, especially terminal groups [28] such as carboxylic and amino groups. The big differences between pH 3.0 and 5.8 should result from the pH dependencies of the charge density of collagen. As the isoelectric point of collagen is 9.0, the charge density is positive at pH 3.0 and is near 0 at pH 5.8. The surface of AFM probe composed of a silicon monocrystal is non-charged –Si–OH– at pH 3.0 and negatively charged –Si–O– slightly at pH 5.8 [29]. These indicate that the big difference of F_{ad} between the solution of pH 3.0 and of pH 5.8 should not result from electrostatic interaction but primarily from nonelectrostatic one.

We reported F_{ad} of the saturated adsorption monolayers of BSA and NaHA [18, 20]. Comparing them with the results of collagen gives important information. The result of F_{ad} of the saturated adsorption monolayers of BSA was 0.5 nN and that of NaHA was 3.0 nN at pH 5.8, which are greater than those of collagen. As the isoelectric point of BSA is 5.2, the charge density at pH 5.8 is negative that is near to 0 in contrary to collagen. Predicting the value of F_{ad} due to electrostatic interaction, the value of collagen should be greater than that of BSA in contrary to the experimental results. Furthermore, the result of F_{ad} of the negatively charged NaHA was much more than that of the collagen at pH 5.8, which suggests that nonelectrostatic interactions primarily hydrogen binding participate in adhesion force. From these results, we can conclude that the interaction between the collagen adsorption layer and the Si probe would result from nonelectrostatic ones.

Effects of pH and T on frictional coefficient μ of collagen adsorption layers As shown in Fig. 8, the results of μ increased with increasing Γ, and those of pH 3.0 were much greater than pH 5.8. Friction of a polymer surface is affected by the surface morphology, the charge density, the surface energy, the softness, and the viscoelasticity [30]. The values of μ obtained by LFM are local ones, and the effects of the local morphology are cancelled out. The value of μ of the saturated adsorption monolayer of BSA was 0.115 and that of NaHA was 0.19 at 25 °C and pH 5.8 [18, 20]. The results of collagen were much greater than those results. Because the charge densities of collagen and BSA in pH 5.8 are almost zero, the big difference of μ

between collagen and BSA should result from their softness and viscoelasticity, i.e., collagen fibrils would be much softer than the BSA adsorbed layer. The greater value of μ at pH 3.0 indicates that the softness of the collagen fibrils decreases with increasing pH due to the electrostatic repulsion. It is worth to notice that the result of NaHA was much smaller than that of collagen although the result of F_{ad} of the NaHA was much more than that of collagen. This indicates that the collagen fibrils are softer than NaHA adsorbed layer. These properties might relate to living functions in body tissues such as skin, cartilage, etc.

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