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# Nanoindentation as a Tool to Clarify the Mechanism Causing Variable Stiffness of a Silane Layer on Diamond

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Additional information is available at the end of the chapter

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## 1. Introduction

For utilizing biological molecules as nanomachines, it is usually necessary to immobilize them on a solid surface of abiological materials. Covalent modification or physical adsorption is used for retaining the target molecules on the solid surface. The following four properties are required for successful immobilization: 1) the surface enables the immobilization with high enough surface density of the target molecules to exert their functions, unless an overly high density prevents interaction as in the case of hybridization; 2) such exerted functions are not significantly perturbed by non-target substances adsorbed on the same surface during use; 3) the surface is suitable for stable immobilization of many kinds of biological materials with high enough mechanical stiffness; 4) the perturbation caused by immobilization does not alter the functions of the immobilized molecules. Immobilization inevitably diminishes the accessibility to the immobilized molecules and changes the dynamic structure of water molecules surrounding the target biomolecules, affecting their structure and conformation as well as their interactions with other substances. Soluble proteins are generally much softer than structured nucleic acids, and thus some are easily flattened in the close vicinity of a hydrophobic surface due to the surface tension. In contrast to proteins, small molecules and structured nucleic acids tend to maintain their native structures, but the limitation of accessibility becomes significant in the close vicinity of the surface. The most problematic consequence of immobilization is the alteration of the function, while the loss of function, to a certain extent, is usually tolerable as long as a significant fraction of homogeneously active molecules is still present on the surface. Therefore, a direct fixing on a solid support often leads to denaturation of proteins due to the surface tension. To avoid these

detrimental perturbations, linkers and porous intervening layers may be introduced onto the solid support to buffer the surface effects.

Here, we present an example of such an intervening layer, namely an aminosilane layer formed on a diamond surface. One of the useful characteristics of this layer is that its stiffness can be controlled by changing the solvent used for the deposition. To clarify the mechanism causing the varying stiffness, we used Atomic Force Microscopy (AFM) nanoindentation. Our method does not require sophisticated equipment and is suitable for a typical wet biochemical lab, which enables the immobilization to be easily performed in the same lab where biological material is purified and used.

## 2.1. Functionalization of diamond surface

The material for immobilization of biomolecules on its surface is selected according to its properties, such as mechanical, chemical, electrical, and optical properties, as well as availability. Diamond is at present drawing attention as a material for biological applications because of its hardness, absence of toxicity, and potential conductivity. It is now used for electrodes and biosensors, DNA and protein chips, and for coating of implants [1-3]. Though it is known to be chemically inert, there are many approaches, which allow modification of its surface [2,4,5]. The effects of immobilizing biomolecules on their activities have also been discussed [6,7].

Diamond has always been an expensive material, but the chemical vapor deposition (CVD) method, developed several decades ago for the synthesis of nano- and polycrystalline diamond films on silicon and other substrates on a large scale and at a reasonable cost, increased the commercial availability of diamond and facilitated its use in research and development [2]. Nonetheless, diamond surface modification often requires sophisticated equipment and facilities.

To introduce any chemical groups onto the diamond surface, at first, the entire surface must be either hydrogenated or oxidized, hereafter H- or O-terminated. The H<sub>2</sub> plasma treatment, used in the CVD method in a diamond film synthesis, automatically results in H-termination [5]. According to our observation, the water contact angle on an H-terminated surface gradually decreased at room temperature, probably because of the local oxidation to generate an inhomogeneous surface, or because of the contamination of the surface. The uniform oxidation of the diamond surface can be achieved by various processes, such as anodic polarization [8], treatment with oxygen plasma [9], treatment with thermally activated oxygen [10], UV irradiation in the presence of ozone [11], or treatment with acids [12-14].

An H-terminated surface can be directly functionalized, either photochemically by UV irradiation in halogen gases or alkenes [15-17], or chemically by thermal decomposition of benzoyl peroxide, activated by argon gas [18], or by electrochemical diazonium salts reduction in an inert atmosphere [19,20]. In contrast, an O-terminated surface can be chemically modified by silanization or by esterification [4], or, as recently reported, aminated by NH<sub>3</sub>-plasma treatment [1].

Biological molecules can be covalently immobilized on a diamond surface either directly, or indirectly through an intervening layer. The former may be suitable for structured nucleic acids, if their lying down in parallel to the surface is prevented. Alternatively, their unstructured part can play as a linker, increasing the accessibility of the rest of the part of the molecule. In contrast, direct attachment is generally unadvisable for proteins, especially enzymes, which tend to lose their activities due to deformation caused by immobilization, though it has been actualized for particular proteins in several studies [1,21]. Diamond may be more biocompatible for direct protein immobilization than, for example, metal surfaces [22]. However, from our experience of single-molecule dynamics [23,24], as well as that of others' [10,25-30], we still recommend the use of an intervening layer for preserving enzymatic activities. Polymer layers have a distinct advantage compared with flat solid surfaces in terms of retaining protein activity. Their porous or mesh-like structure allows an easier access of water to the immobilized biological molecules, maintaining their conformations and accessibilities.

Diamond technology is still immature, and the biologically active interfaces on a diamond have not yet been extensively studied. Several pioneering works, reporting the properties of polymer layers, formed on a diamond surface, describe testing of the layers on H-terminated diamond by scratching with an AFM cantilever [28,29,31,32]. Since different proteins require different treatments for maintaining their activities, an intervening layer needs the adjustment of thickness, roughness, and stiffness. Therefore, control over the properties of the layer is important.

## 2.2. Deposition of aminosilane on the diamond surface

The most common functionalization used for immobilizing biological molecules is the introduction of amine residues on the surface or the use of an amine-rich intervening layer, which is fixed on the surface. The utility of amine residue arises from its activity as a nucleophile in coupling chemistries under moderate aqueous conditions [29,33]. Silanization is commonly used in a wide variety of both industrial and research-oriented applications as a coupling agent for creating intervening silane layers on different kinds of surfaces. The chemical formula of a typical silane molecule is  $X_3Si-R$ , where X is the group leaving during its polymerization, and R is the hydrocarbon-containing functional group, which remains after the reaction. A wide range of various silanes, such as aminosilanes ( $-NH_2$ ), mercaptosilanes ( $-SH$ ), and glycidoxytrimethoxysilanes (epoxide), is commercially available, allowing different chemical functionalities to be incorporated on the surfaces [34]. Formation of aminosilane layers on a diamond surface has been reported in several studies [10,26,27]. In our work, we used 3-aminopropyltriethoxysilane (APTES), which is one of the commonly used aminosilanes.

Some silanes form uniform monolayers, while APTES tends to form multilayers on various substrates, and the layer's thickness increases during deposition [35,36]. In several studies, APTES monolayers have been reported, including those on GaN [37], silicon oxide [38], and porous silicon [39]. However, it is difficult to distinguish a monolayer from dispersed

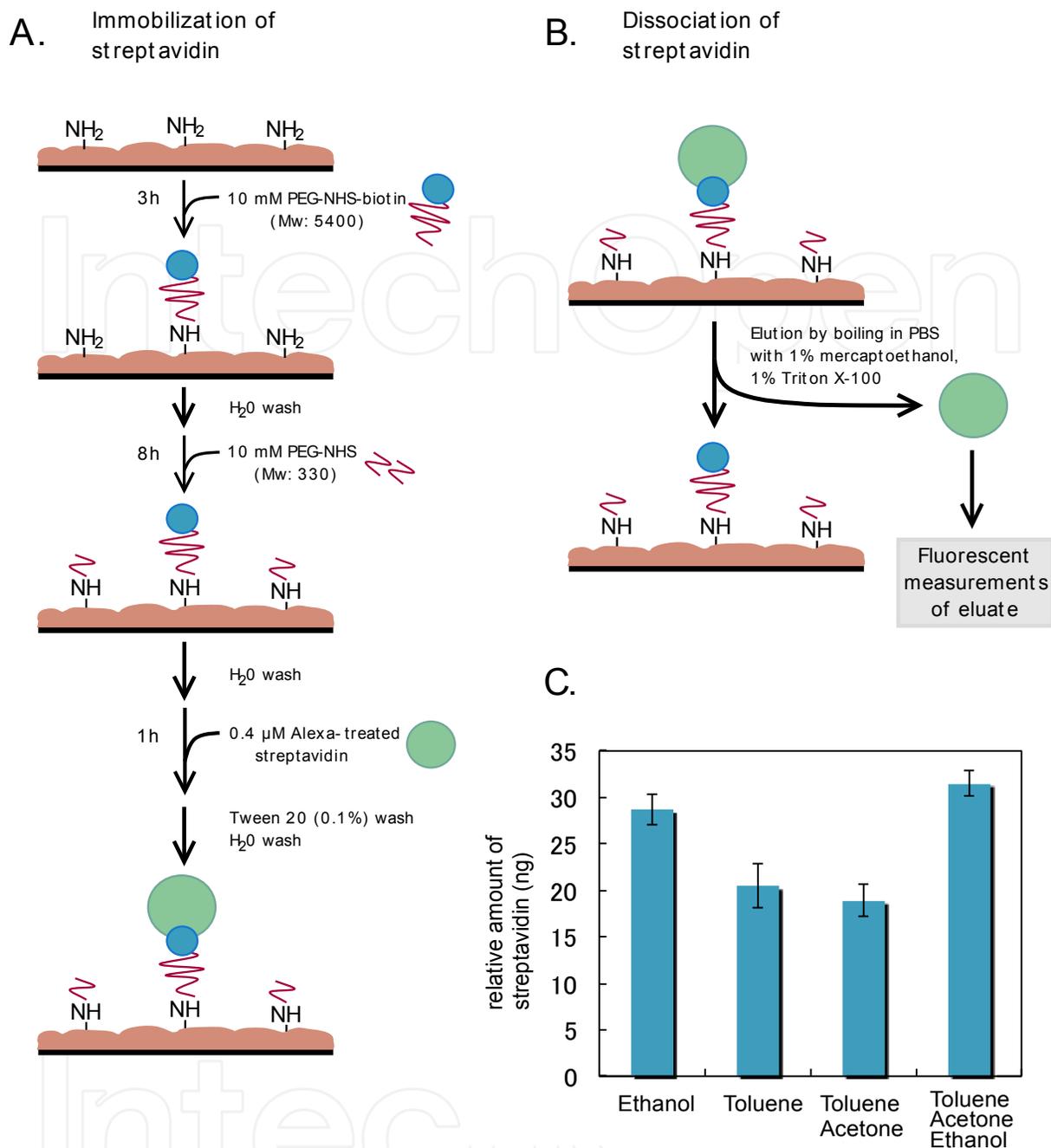
molecules of APTES deposited in a limited time [40,41]. Thus, in a long enough time period, a multilayer is expected to be formed. Such a multilayer is likely to have a disordered mesh-like structure as a consequence of the flexibility of APTES polymer molecules, which is suitable for protein attachment as it is.

It is generally recognized that aqueous conditions and a sufficient accessibility of water molecules to the protein are important for the functions of many soluble proteins which tend to be adsorbed on hydrophobic surfaces [42]. Therefore, the rough and disordered surface of an APTES multilayer is suitable for protein attachment as it creates an environment of high water accessibility for protein molecules. Using APTES for protein immobilization is also often considered to be a means of introducing linkers, intended to reduce steric hindrance, thus providing a greater freedom of movement to immobilized biological molecules [34]. Thus, the introduction of the APTES multilayer to the diamond surface contributes to keeping immobilized molecules active. The disordered mesh-like structure might not be the best choice in all cases. For example, in microfabrication of arrays for DNA attachment, a regular array could not be prepared with the mesh-like structured APTES, whereas another silane gave much better regularity and spatial resolution [35].

To model the reaction of protein immobilization, we attached biotin and then streptavidin onto the APTES layer (Fig. 1), which has formed on the O-terminated diamond surface by a wet method, which is described later. To enhance specificity of binding, we pretreated the surface with a mixture of polyethylene glycol (PEG) with different chain lengths according to a previously established method for preventing non-specific adsorption [43]. The best specificity of attachment was achieved, when APTES was consequently treated first with a long NHS-PEG-biotin and then with a short NHS-PEG.

We then immobilized fluorescently labeled streptavidin on an APTES layer and measured the density of its immobilization. By the measured density, we estimated the average distance between flanking streptavidin molecules. On the two layers with the highest densities this distance was estimated to be 5.6 and 5.3 nm. Since the size of the streptavidin tetramer, derived from the crystallographic data, was  $5.4 \times 5.8 \times 4.8 \text{ nm}^3$  [44], the streptavidin on these layers was immobilized in a state proximate to the hexagonal closest packing, if streptavidin molecules were assumed to distribute in a flat plane.

There is a strategic problem as to whether one should use the most refined methods or the just-satisfactory methods in an experiment. For the combination of two or more lineages of technologies, we chose the latter strategy, because preparing a new pure protein in the lab already requires a number of techniques. In this way, rather than absolute accuracy of the measurements by sophisticated instruments, we preferred familiar and common technologies giving a sufficient accuracy: we formed an APTES layer in a wet process and performed nanoindentation by AFM. Therefore, the developed method can be performed in a standard biological laboratory where target biomolecules are prepared, and does not require any commercially unavailable reagents or sophisticated equipment.



**Figure 1.** Immobilization of streptavidin on an APTES multilayer. A. The steps of the PEG treatment. B. Elution of streptavidin from the multilayer for measuring the immobilization density. C. The immobilization densities observed for the multilayer deposited in one or more steps in different solvents.

There is a consensus on the basic mechanisms of APTES polymerization. The reaction begins with the hydrolysis of silane molecules, resulting in siloxane bonds forming and attaching APTES molecules to the surface. Hydrolysis may occur both at the surface of the substrate, or in solution, depending on the water concentration. If water is predominantly present in solution, the polymerization will occur predominantly in the solution, resulting in formation of aggregates of APTES, which otherwise form covalent bonds with the

substrate [40,41]. Therefore, a distribution of water can determine the size of aggregates and the degree of covalent linking to the surface. In our study, we used several solvents with different isoelectric constants for APTES deposition and found that the layers, formed in different solvents, showed various morphology and nanoscopic hardness, which is discussed below.

### 2.3. Nanoindentation

Nanoindentation is a powerful and useful method for assessing the mechanical properties of a material. In a typical indentation test, a load is applied to the sample examined with a hard indenter, and the analysis of the load-depth curve and the morphological changes in the indented material allow the measurement of such properties as hardness, Young's modulus, and stress relaxation data [45-47].

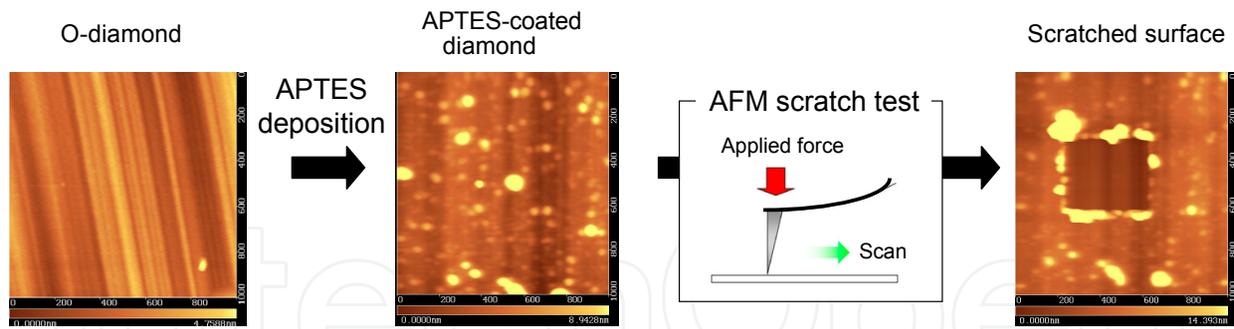
Over the recent few decades, AFM, initially invented as an imaging tool, has been extensively used for nanoindentation, especially on soft materials. The conventional nanoindentation instruments, which utilize the Oliver and Pharr's procedure [48], are preferentially used for hard materials. However, they cannot offer a wide enough range of loads necessary for soft materials, which must be indented with less force [49]. AFM is a very useful tool to study the properties of biologically relevant materials [50,51]. By using AFM cantilevers as indenters, it is possible to measure nanomechanical properties with high force and depth resolutions. Imaging can be performed with the same tool right after the indentation, without resetting the sample. Furthermore, AFM can detect pile-up or sink-in effects, which conventional indenters cannot [52-54].

Several serious reservations have been asserted regarding the problem of whether or not AFM measurements are sufficiently quantitative in indentation [55]. Because the cantilever's apex is deformed in the indentation process, and may not be exactly vertical, the AFM cantilever requires more corrections of measurements compared with conventional indenters [54]. However, we still hold the view that AFM is usable for our purpose, according to our strategy, by taking into account the distortion of the apex.

### 2.4. Scratching manipulation and AFM nanoindentation

In our experiments, single-crystalline synthetic (100) type Ib diamond (Sumimoto Electric Industries) was etched with acids to prepare an O-diamond surface and then APTES was deposited. Then we performed the imaging with scratching manipulation [29] on our APTES layers (Fig. 2), prepared under different deposition conditions. We used AFM (SPI3700, Seiko Instruments) for both topographic imaging and nanoindentation. We calibrated silicon AFM cantilevers (SI-DF-3) with the Cleveland method [56] and selected tips with a spring constant of 1.2-1.4 N/m.

We found that one can control the roughness of the layer by changing the polarity of the solvent. Deposition of APTES in the nonpolar solvents resulted in a rougher surface, and the use of polar solvents resulted in a smoother surface.

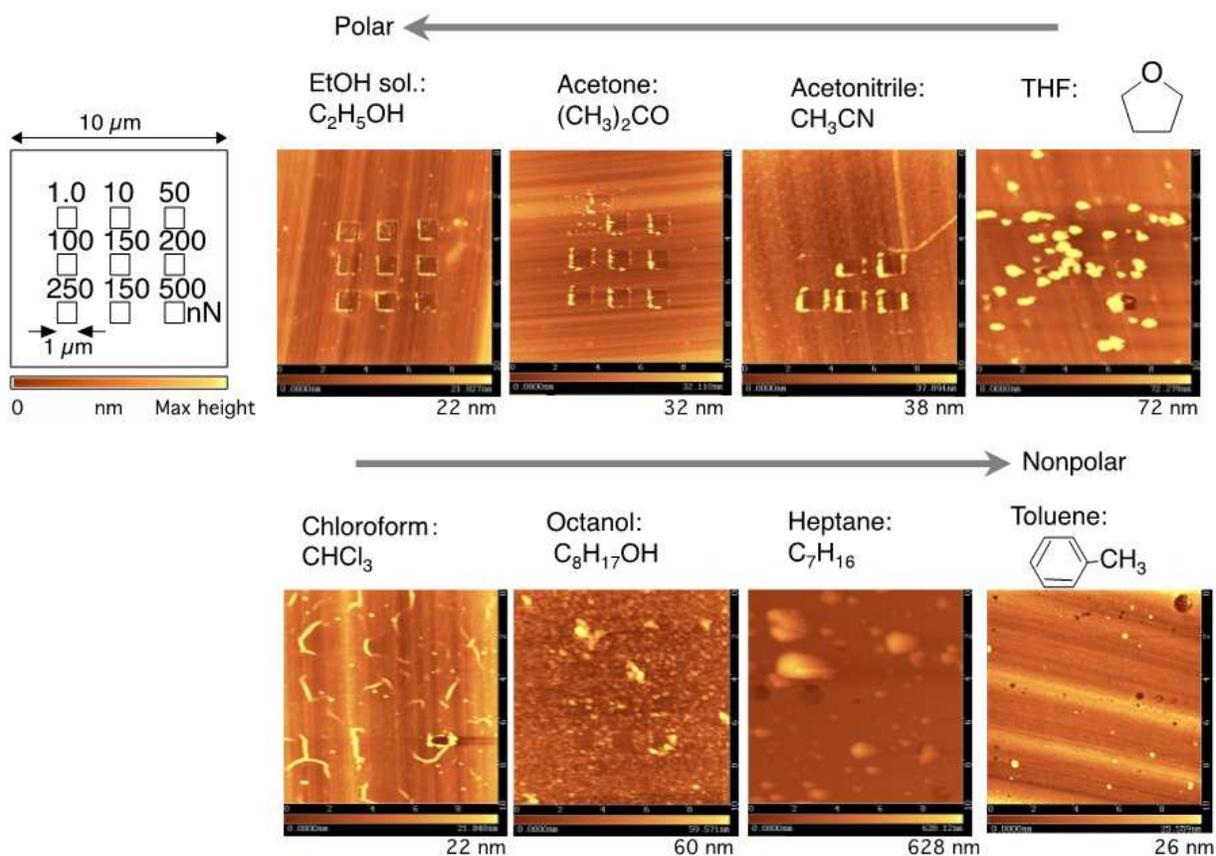


**Figure 2.** Scratching manipulation (Modified from [57]).

In the scratching manipulation, the stiffness was indexed by the minimum force essential to disrupt the layer and to remove it from the diamond support. The lower the dielectric constant of a solvent, the greater the force had to be applied to remove the layer: only 1 nN for the layers prepared in ethanol mixture or acetone, 100 nN for octanol, and even greater forces for more nonpolar solvents. In this way, we could draw arbitrary nanopatterns composed of lines and squares on softer layers, though we could not scratch the harder layers even at forces greater than 500 nN (Fig. 3). The composed patterns remained stably on the diamond surface. Therefore, both roughness and stiffness increased relative to the decreasing polarity.

It is important to clarify the relationship between scratching, which is terminologically a macroscopic examination, and microscopic nanoindentation. In the macroscopic world, scratching means scraping or removing a part of a substance by disrupting it with a sharp edge, which is a macroscopic process and is on a different level from molecular events. Therefore, the force, essential to break the APTES layer with the edge, is dependent on the sharpness and the shape of the edge, as well as its velocity and moving direction. However, the scratching manipulation in our experiment includes breaking molecular bonds between APTES and the diamond by vertical pressing with the cantilever, which we consider to be close to nanoindentation and a microscopic process. In the microscopic model, the break should be independent of the shape of the apex and dependent solely on the work of the pressing force. In other words, the macroscopic model predicts that the force breaking the layer depends on the sharpness of the edge and, thus, the horizontal movements in case of a pyramidal cantilever. Alternatively, the microscopic model asserts the independence of the cantilever used, its shape, and the horizontal direction of its movement (Fig. 4).

The experimental results demonstrated that an APTES layer could be scratched and removed from the diamond surface with a common pressuring force within one order of magnitude in different examinations and was independent of cantilevers used (Seiko SI-DF-3, SI-DF-20S, and Olympus OMCL-AC240TS-C3), the velocity of scratching, and the horizontal direction of scratching. These results indicate that the microscopic model is more accurate in describing the mechanism of scratching of the APTES layer.



**Figure 3.** The results of the scratching manipulation of APTES multilayers formed in various solvents (Modified from [57]).

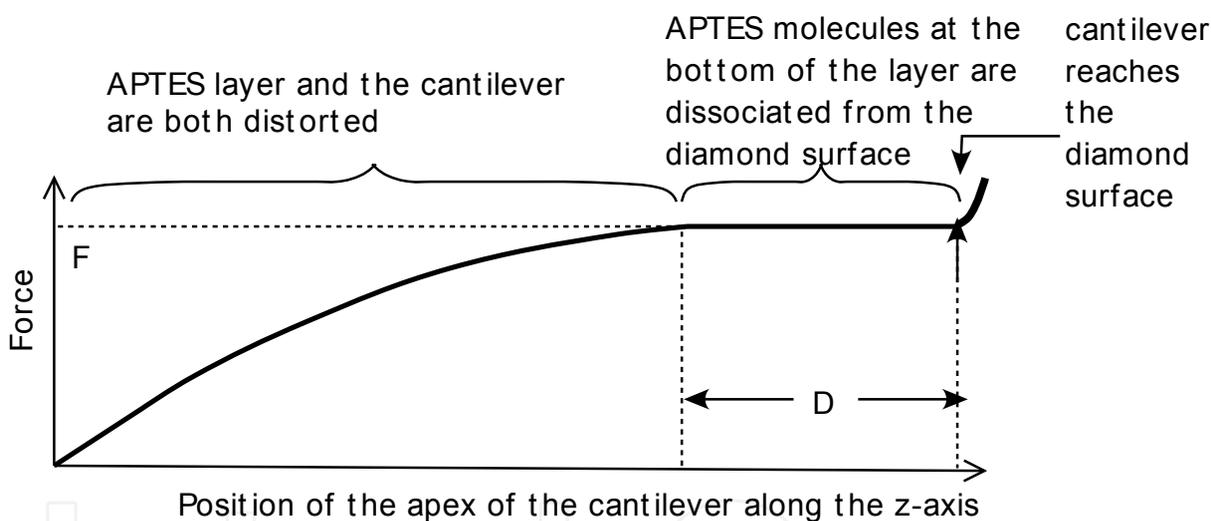
As a control for checking whether or not the result is independent of AFM instruments, we performed a scratching manipulation with our instrument on a layer of  $\omega$ -unsaturated 10-trifluoroacetic amide-dec-1-ene (TFAAD), photo-chemically attached to the diamond surface, which had been inspected with the same manipulation [29]. The measured force was well aligned with the value ( $\sim 100$  nN) previously obtained. When toluene was used for the APTES deposition, the forces obtained in the scratching manipulation were greater than 500 nN, the upper limit of our instrument. The attachment of APTES to the diamond, therefore, is stronger than that of TFAAD, which is generally recognized to be covalently bound to diamond [29], suggesting that APTES is also covalently bound to the diamond. This circumstantial evidence for the microscopic model led us to compare the results of the scratching manipulation and the AFM indentation. The nanoindentation with no horizontal movements was performed by pressing the APTES layer in the contact mode. The image of the contact area was then obtained in the noncontact mode.

The results of nanoindentation showed an agreement of forces with the scratching manipulation within the same order of magnitude. The layer could not be scratched even at 500 nN, showing no trace after the indentation at 500 nN. Indentation of the softest and smoothest layer, which had been formed in the ethanol mixture, showed a force  $< 1$  nN in the scratching manipulation. This semi-quantitative agreement again suggests that the microscopic model is more appropriate than the macroscopic one. Therefore, the force

measured in the indentation test can be used for scratching and nanopatterning of the surface of a material. The indentation test sometimes required pressing for up to 10 min to yield reproducible results. This hysteresis indicates that the APTES layer is elastic in nanoscale when a force is rapidly removed.

## 2.5. Interpretation of the results

As discussed above, the result of the scratching manipulation suggests the existence of covalent bonds between APTES and diamond, at least for the multilayers deposited in nonpolar solvents. If APTES were physically adsorbed on the surface, the changes of the breaking force of more than 100-fold could not be explained, since more than a 100-fold change of the contacting area is unlikely for such a soft material as APTES polymer which is easily flattened. The likely explanation is the change in the surface density of the covalent bonds between APTES and diamond, although its evidence is required. Since the shape of the apex of the silicone cantilever used in the indentation should be deformed significantly at forces in the order of magnitude of 100 nN as shown in Fig. 4, a detailed analysis of the force curve of AFM indentation is not very informative.



**Figure 4.** Schematic illustration of a force-position curve during AFM indentation.

However, the following consideration of energetics is still possible. The force giving the plateau (Fig. 4) is about 500 nN for chloroform and greater than 500 nN for heptane and toluene. The distance  $D$  is the thickness of the compressed multilayer and is close to 1 nm. This value was obtained from the AFM imaging of the surface with uncoated holes with exposed diamond. During this period when the force is constant, the energy is used for breaking the covalent bonds connecting between APTES and diamond for squeezing the layer out of its original position. Since a Si-O bond is stronger than a C-O bond, the energy is used for breaking the C-O bonds, which costs  $6 \times 10^{-19}$  J/bond [58]. A part of the energy may be used for frictional dissipation and eventual bond breakage within the APTES multilayer, too. Therefore, the work done by the force  $F$  is  $FD$ , and  $FD$  must satisfy Eq. 1, where  $S$  is the area of the place where APTES was removed.

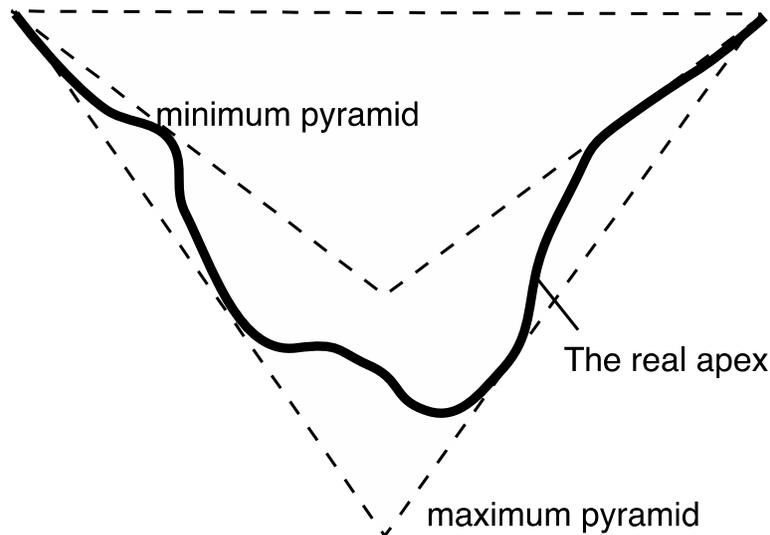
$$FD \geq (\text{density of the anchoring } C-O \text{ bond}) \times S \times (\text{energy of a } C-O \text{ bond}) \tag{1}$$

Since  $F = (\text{strain}) \times ES$

$$(\text{density of the anchoring } C-O \text{ bond}) \leq \frac{(\text{strain}) \times ED}{(\text{energy of a } C-O \text{ bond})} \tag{2}$$

where  $E$  is Young’s modulus of silicone, 150 Gpa. It is noted that Eq. 2 is independent of  $F$  and  $S$ , which indirectly contribute through the strain.

As illustrated in Fig. 5, the strain is overestimated if the shape of the apex is substituted by an inscribing pyramid with the same base, while the strain is underestimated if it is substituted by another circumscribing pyramid with the same base. Therefore, the two pyramids give the maximum and the minimum values of the strain, respectively. If their values are within the same magnitude, the actual one should also be within it. Therefore, this approximation is sufficient in estimating the order of the strain and the density. Therefore, we consider the distortion of a pyramidal cantilever (Fig. 6).



**Figure 5.** The relationship among the actual shape of the apex, the maximum pyramid, and the minimum pyramid. If the heights of two pyramids agree within the factor of 3.2, the actual value of the strain also agrees within the factor. Because a force is parallel to the square of the height, the factor 3.2 maintains the value of the force or the density within a factor of 10. This condition is likely to be established because of the use of different cantilevers and because of the loss of sharpness of the apexes used repeatedly.

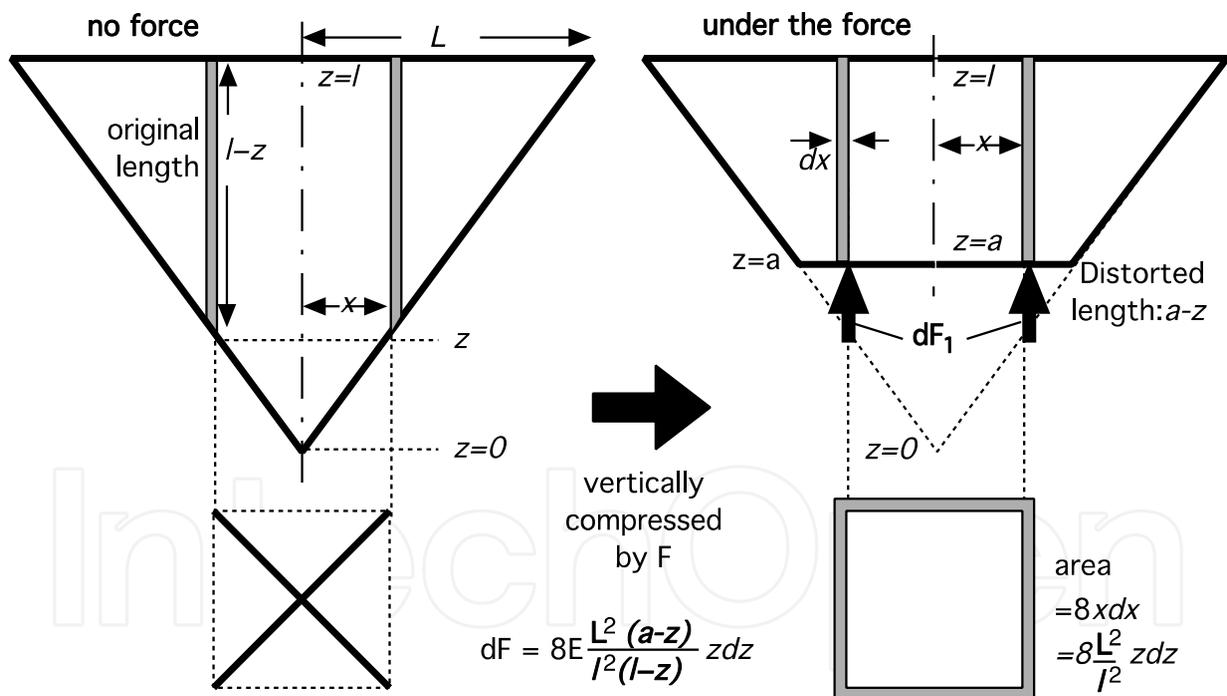
If  $dF$  shown in Fig. 6 is integrated from 0 to  $a$ ,

$$F = 8E \frac{L^2}{l^2} \int_0^a \frac{a-z}{l-z} z dz = 8EL^2 \left( \frac{a}{l} \right)^2 = 8EL^2 (\text{strain})^2 \tag{3}$$

Because  $a \ll l$ , and  $\int_0^a \frac{a-z}{l-z} z dz = \frac{a^2}{2} + (l-a-b)a + (l-a)(l-b) \ln \left( 1 - \frac{a}{l} \right)$

The strain obtained for the present parameters ( $L=4.5 \mu\text{m}$ ,  $l=10 \mu\text{m}$ , and  $F=500 \text{ nN}$ ) is the order of magnitude of  $10^{-4}$ , leading to the order-estimated maximum value of the density to be 3 C-O bonds in a square of  $10 \text{ nm} \times 10 \text{ nm}$ . Since there are about 10 carbon atoms per  $(\text{nm})^2$  of (100) plane, APTES is covalently linked to a maximum 0.3 % of the surface carbon. This value is for the APTES multilayer deposited in chloroform, while the one in toluene is more densely bound to the diamond surface.

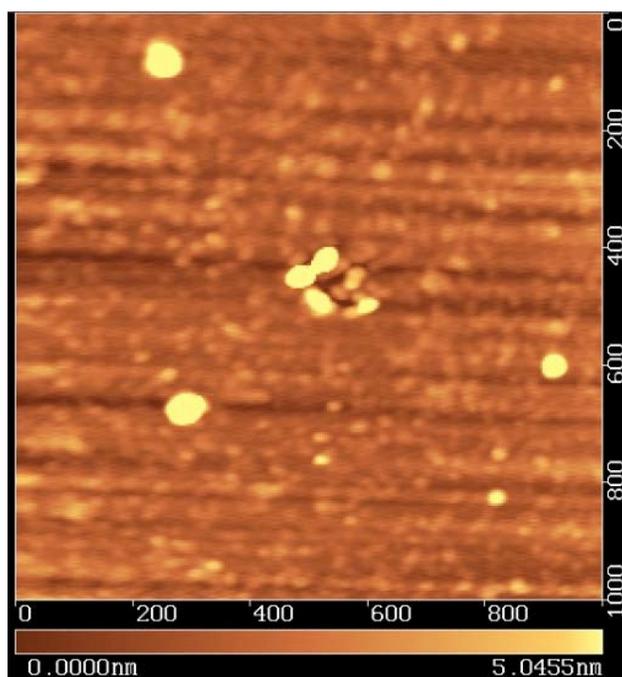
These sparse covalent bonds on diamond can be explained by the sparseness of the reactive carbon of the diamond surface. The diamond surface used in this experiment is (100) crystal plane, and the carbon atoms on (100) plane have two dangling bonds. On the O-terminated surface, they form bridge-bonded ether bonds with their nearest surface carbon atoms, or become carbonyl carbon atoms [10]. The reactive carbon atom must have a single dangling bond, which is converted into the hydroxyl form during the O-termination [27,59]. Such carbon atoms will exist at defects of (100) plane, the edge of steps between two (100) planes, for example. The steps can be seen in Fig. 2 as stripes on the surface of uncoated O-diamond. Since APTES is known to react with a hydroxyl residue [40], a covalent bond between APTES and the O-diamond surface is limited to the carbon atoms with single dangling bonds.



**Figure 6.** Pyramidal approximation of the apex of the cantilever.

In the above consideration, we assumed that the effect of different shapes of apices is smaller than an order of magnitude. Such a difference was reported to be 2-3 fold in the scratching manipulation using dull apices at 10-40 nN [62]. Since the effect becomes smaller at a larger force, the assumption is likely to be reasonable at a force larger than 100 nN. The estimation by an order of magnitude, therefore, is a productive criterion almost independent of the shape of the apex.

In our consideration, we took into account only C-O bond between diamond and APTES, but not the Si-O bonds crosslinking between APTES molecules. The contribution of the Si-O bonds could be significant in the scratching manipulation but not in the nanoindentation, because scratching may rip the APTES layer. However, the work consumed in ripping the layer must be done by a horizontal force, which is the driving force of the AFM stage, and would not be included in the vertical force required. Actually, the results of the scratching manipulation and the nanoindentation agreed with each other at the accuracy of an order of magnitude. Furthermore, we observed that a similar ripping happens also after the measurement of nanoindentation, namely when the force applied to the cantilever is removed. As Fig. 7 shows, a hole much larger than the apex of the cantilever was formed on the APTES layer deposited in ethanol. The APTES layer, which had been covering the hole, was ripped from the surface and remained around the hole. In conclusion, these results indicate that the observed variation of the stiffness is due to the various densities of the covalent bonds between APTES and diamond.

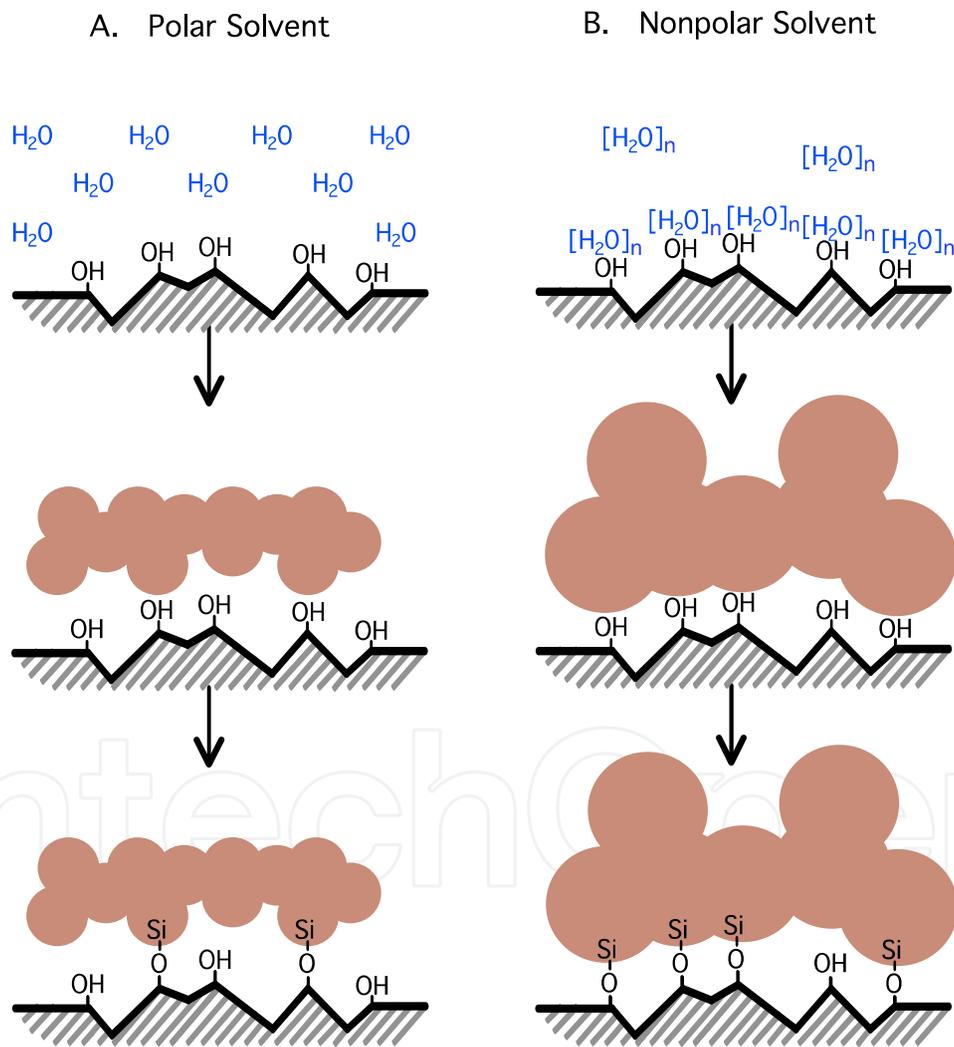


**Figure 7.** The trace after AFM nanoindentation of a multilayer deposited in ethanol at 500 nM. The trace is as great as 50 nm, which is much greater than the apex of the cantilever, ~ 10 nm.

## 2.6. Mechanism yielding the dependence of the stiffness on solvent polarity

The last problem to be addressed is why the density of the covalent bonds varies by 100-fold depending on the solvent used in the deposition. As already mentioned above, the polymerization of the APTES layer proceeds by reacting with water, indicating that there are two places for the polymerization as shown in Fig. 8: in the solvent and on the surface where water molecules are trapped. In a polar solvent like ethanol, water is homogeneously dispersed in the solvent but there is less water distributed on the surface. Therefore, homogeneous polymerization occurs in the bulk, while infrequent polymerization occurs on

the surface. Thus, the size of aggregates is uniform, which makes a smooth surface of the multilayer, but it is sparsely bound to the diamond surface, making the stiffness less. In contrast, in a nonpolar solvent, water content is small and with water tending to exist as water clusters or small droplets. Since the C-OH is more hydrophilic than the solvent, it holds a water droplet. Therefore, APTES aggregates are much fewer, and their size is inhomogeneous, making a rough surface of the multilayer. Instead, more C-OH carbons are covalently bound to APTES, making the stiffness greater. In this case, a longer period would be required for deposition. In fact, the deposition in ethanol-water mixture lasts for 1 h, while the deposition in toluene requires longer than 10 h to cover the surface. This proposed mechanism [57] explains not only the variation of the stiffness but also the roughness and the required time period for deposition.



**Figure 8.** Mechanism yielding the dependence of stiffness on the solvent polarity.

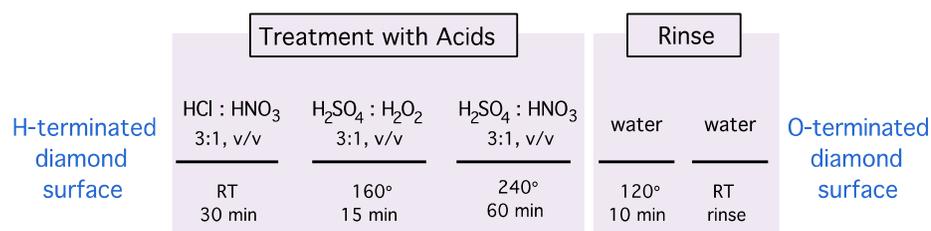
Thus, when a stiff multilayer is required, we must deposit APTES in a nonpolar solvent, a time-consuming process. The proposed mechanism suggests that the time required will be shortened if we increase the number of water droplets in a nonpolar solvent. Therefore, we used water-saturated toluene, which may be a rare treatment of a nonpolar solvent in

chemistry. In this way, we accelerated the deposition in toluene from overnight to an hour. This success supports our proposed model for APTES deposition. If the second deposition is made in ethanol-water, a smoother multilayer is obtained.

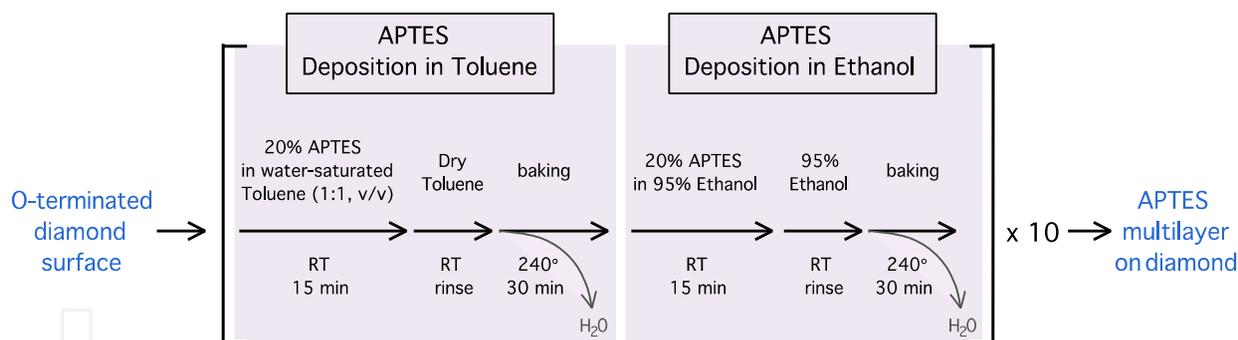
## 2.7. Efficient deposition of APTES multilayer with a great stiffness and on diamond

According to the described mechanism, a method for preparing a stiff APTES multilayer on a diamond surface has been developed. The 2-step deposition in toluene and ethanol has been repeated in quick steps, followed by baking to remove the water remaining inside the layer after each step. This procedure has yielded the best results so far of any tried in our lab in terms of stiffness, which is essential for long-term stability in practical use (Fig. 9).

### A. Oxidation of Diamond Surface



### B. 2-step APTES Deposition



**Figure 9.** An efficient procedure forming a stiff APTES multilayer on diamond.

## 3. Conclusions

We have developed a new method for forming a bioactive APTES multilayer on an O-diamond surface with a controlled stiffness, roughness, and capacity for immobilizing streptavidin. This method does not require sophisticated machinery or expensive materials, and thus, it can be adopted for use in many biological laboratories where proteins and nucleic acids are prepared and immobilized.

Using AFM nanoindentation, the scratching manipulation with AFM, and AFM imaging, we examined the stiffness and morphology of the APTES multilayers formed on a CVD diamond. The results of nanoindentation and the scratching manipulation were interpreted semi-

quantitatively rather than fully quantitatively. This interpretation enables us to propose a model which explains the various levels of stiffness of APTES multilayers, its correlation with polarity of the solvent, and the time required for covering the surface with an APTES multilayer. The model also allows us to improve the method to make a stiff and smooth APTES surface.

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