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RESEARCH ARTICLE

Interferon α-2a interactions on Glass Vial Surfaces Measured by Atomic Force Microscopy

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ABSTRACT: Atomic force microscopy was used to study adsorption and adhesion peculiarities of interferon α-2a on glass and mica surfaces. The specific protein adsorption behavior as a function of the pH value was illustrated on mica by single molecule imaging, while adhesion forces between interferon molecules and inner surfaces of borosilicate glass vials were measured directly under aqueous buffer conditions by force microscopy. We found that the adhesion force on Schott FIOLAX Type I plus® was reduced by 40% of the total adhesion force measured on Schott FIOLAX®, a standard type I borosilicate glass quality. These results reflect the anticipated superiority of the special "Type I plus" coating over undesired protein adsorption to glass. In addition, this study gives insight into a new method to predict unintended protein adsorption to glass container walls and to characterize the adsorption process by force measurement.

KEYWORDS: Interferon α-2a, force microscopy, adhesion, borosilicate glass, vials

Introduction

The growing problem of protein adsorption to glass container surfaces in the pharmaceutical field is demanding extensive inquiries of the molecular adsorption and adhesion process. Chemico-physical interactions at the interface protein-substrate have to be understood in detail in order to successfully prevent adsorption. The invention of atomic force microscopy (AFM) (1) has provided new techniques to investigate force interaction on the molecular level. A schematic illustration of the operation of AFM is given in Figure 1. The sample is scanned over a defined area in the x,y-plane underneath the sharp tip of a flexible cantilever (Figure 2) by piezoelectric scanning elements. A laser beam is pointed on the back of the cantilever spring and reflected towards a segmented photodiode. The changes in deflection of the cantilever in the Z axis, caused by varying interaction forces between the apex of the tip and the sample surface, are detected by the photodiode. Current signals from the detector are used to generate data, such as topography and friction force in the constant height mode of contact AFM operation. In the constant deflection mode, topography of the sample is acquired by recording the movement of the z-piezo scanner that is needed to keep the cantilever deflection constant. In this mode, the varying force acting between the tip and the surface is kept small to minimize damage to the surface being imaged. In non-contact mode AFM, the cantilever is vibrated at or near its resonant frequency by applying an additional AC-voltage to the piezo material that is attached to the rigid end of the cantilever. Since the resonant vibration of the cantilever is changing in frequency, amplitude, and

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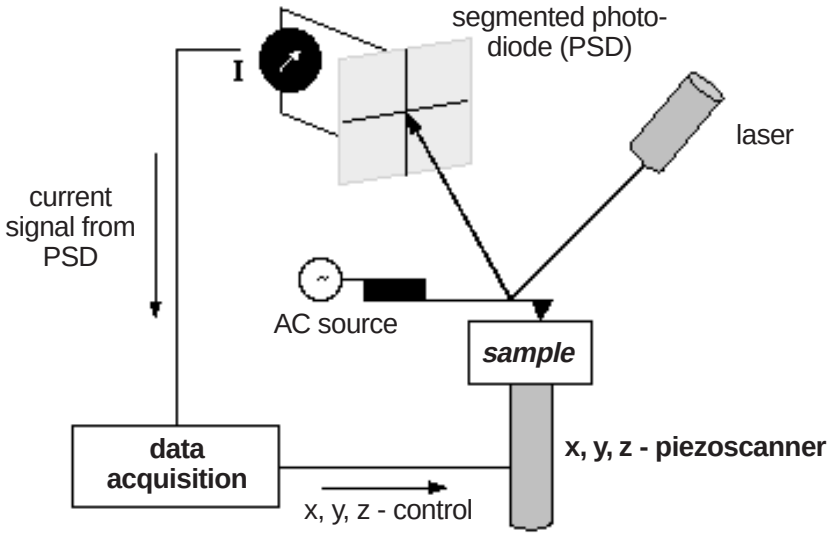
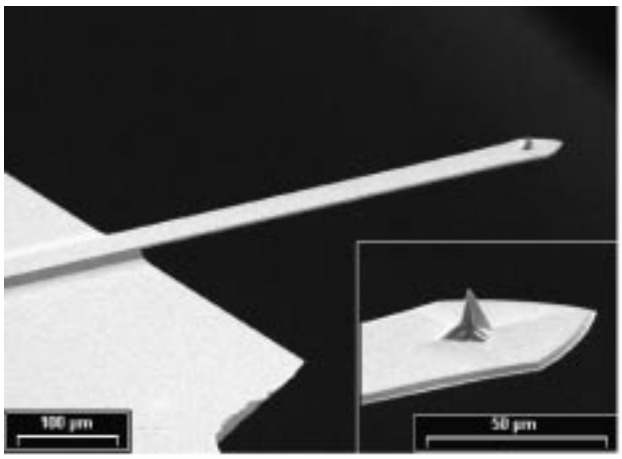
Figure 1: Illustration of the principle and operation of an AFM system.



phase when the apex of the tip is approaching the sample surface, topographical data can be detected in a very sensitive and gentle way. AFM allows forces to be measured in the piconewton range and it enables, at the same time, study of biological specimens *in situ* in a physiological environment (2-4).

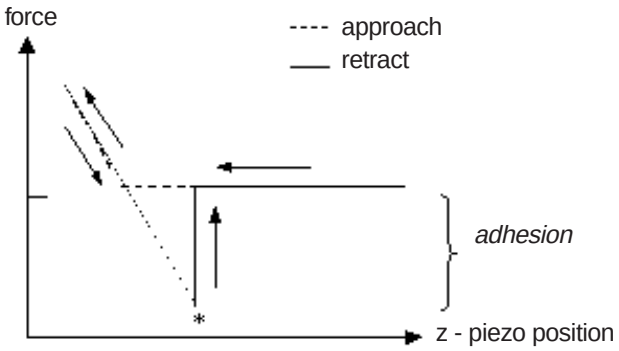
Figure 2: Scanning electron microscopy images of a silicon cantilever with an integrated pyramidal silicon tip (inset).



One of the outstanding examples for specific recognition and measuring interaction forces between individual molecules is the AFM application for antigen/antibody pairs. By immobilizing the two interacting partners, one to the AFM tip and the other to the substrate, the binding force can be determined by so-called force-distance curves or force spectroscopy (5,6). A drawing of a typical approach/retract curve is shown in Figure 3. During approach, the tip moves down and eventually contacts the glass surface. Approaching further causes a deflection of the cantilever due to repulsive forces. During retrace, the cantilever deflection is relaxed following approximately the same linear force slope until the tip looses contact with the surface. However, this point can be shifted relative to the contact point if adhesion forces are present. A characteristic feature of the unspecific adhesion event is the size of the jump-out of contact. At this point, the force acting on the cantilever spring is equal to the adhesion force of the molecules attached to the cantilever tip interacting with those immobilized to the substrate.

Other authors were able to map affinity and adhesion forces of a specific substrate by performing force-distance curves with chemically modified AFM tips

Figure 3: Typical force distance curve with an adhesion event between the tip and the sample. The 'jump out of contact' is indicated by the asterix.



(7,8). Furthermore, when measuring under well-defined aqueous conditions, it is possible to distinguish the forces acting between tip and sample (9). However, working with complex biological molecules, force differentiation can get quite complicated and indefinable.

In this report, we present a method of how to determine, experimentally, the adhesion force between interferon α-2a and glass vial surfaces. In our experiment, interferon was covalently coupled to the AFM tip through a short divalent linker molecule, always ensuring a buffered environment. Then, force spectroscopy was applied to the surfaces of two types of glasses, Schott FIOLAX® and Schott FIOLAX Type I plus®, both suitable for pharmaceutical packaging of parenteral drug formulations (10). Adsorption of protein molecules to borosilicate glass surfaces is a well known phenomenon. FIOLAX Type I plus is the same glass as FIOLAX but with a coating. This coating technology, called Type I plus, has been developed by Schott Germany to produce a highly inert glass container for sensitive drug formulations (11). The quartz-like silicon dioxide coating provides a high purity and dense barrier between the drug solution and the underlying conventional and reactive glass surface. Test results with low dosed formulations of different proteins have shown that protein adsorption to the container wall was significantly reduced in Type I plus modified glass containers. Ion release and hydrolytic resistance of Type I plus glass is improved by orders of magnitudes compared to standard borosilicate glass. Thus, drug formulations containing low dose proteins or complexing agents, as well as products with high sensitivity against pH shifts, are preferentially stored in SiO_2-coated Type I plus containers. Surface roughness of the two glass substrates included in the adhesion force experiments are measured and discussed in the context with the results obtained.

Additional information about the structure of adsorbed interferon α-2a was achieved by non-contact mode AFM imaging. To obtain the highest resolution of single molecules, freshly cleaved and atomically flat mica was used (2,12). Interferon α-2a was allowed to adhere to the surface at two different pH conditions around the isoelectric point of the protein in order to study the adsorption behavior as a function of electrostatic effect.

Experimental

Adsorption of interferon α-2a on mica

Purified interferon α-2a in buffer of pH 5 (0.1 mM citrate buffer, Merck, Switzerland) or pH 7 (0.1 mM phosphate buffer, Merck, Switzerland) was adsorbed to freshly cleaved mica (Plano W. Planet, Germany) at the given concentration. After incubation for two minutes at room temperature, the samples were rinsed with pure water (≤ 18 MΩ/cm, filtered 0.2 µm) to remove non-adsorbed protein molecules and dried in a pure nitrogen stream (purity: 99.9997%). All images were performed in air at room temperature on a commercial AFM (Topometrix Explorer, Thermomicroscopes, USA) by means of non-contact mode atomic force microscopy. The silicon cantilevers (Pointprobe, Nanosensors, Germany) had spring constants of about 42 nN/nm and were driven at their resonance frequencies of about 300 kHz. Feedback was adjusted to 10 – 20% amplitude reduction.

Surface structure of glass substrates

Adhesion forces of interferon α-2a were investigated on inner surfaces of glass vials used for pharmaceutical packaging. Vials made of FIOLAX® glass (Schott, Germany) are typical borosilicate glass containers, whereas FIOLAX Type I plus® vials (Schott, Germany) have been subsequently coated with a pure silica layer. In order to prepare glass samples, the vials were carefully broken into small pieces and the glass dust blown off by a stream of dry nitrogen. These samples were then glued onto the sample holder in such a way that the area to be measured at the edge of the inner

surface was easily accessible to the cantilever tip. The same procedure was applied to the force measurements on FIOLAX and FIOLAX Type I plus.

Surface topography and standard roughness were acquired by non-contact mode AFM. Images were taken in air at room temperature with silicon cantilevers (Pointprobe, 42 nN/nm, 300 kHz, Nanosensors, Germany).

Adhesion force measurements

All adhesion force measurements were carried out in buffered solutions (0.1 mM phosphate buffer, pH 7, Merck, Switzerland; or 0.1 mM citrate buffer, pH 5, Merck, Switzerland). The experiments were performed at room temperature on the Topometrix AFM (Explorer, Thermomicroscopes, USA), equipped with a home-built holder for liquids. Interferon α-2a was covalently attached to silicon cantilever tips (Pointprobe, 0.07 – 0.4 nN/nm, Nanosensors, Germany) by using a divalent linker molecule, 11, 11'-Dithio-bis(succinimidy-lundecanoat) (DSU). This protein-coupling method and synthesis of DSU was described by Wagner et al. (13). Gold-coated cantilever tips were achieved by thermal evaporation under vacuum at a pressure of 10^{-6} mbar. Dipping fresh gold surfaces into a solution of 1 mM DSU in 1,4-dioxane puriss. (Fluka, Switzerland) for 15 minutes led to an amino-reactive self-assembled monolayer of DSU on the AFM tips. Before immersing these tips into a solution of 0.95 mg/ml interferon α-2a in phosphate buffer of pH 7, they had to be rinsed carefully with dioxane and phosphate buffer of pH 7. After an incubation time of 50 minutes at room temperature, these modified tips were ready for use for at least three days if kept under buffer at 4°C.

Adhesion forces between tip-bound interferon molecules and glass substrates were monitored by approach/retract cycles at velocities of 1 µm/s and at a maximum applied repulsive force of 500 pN. Each modified tip was used for 50 binding-unbinding cycles on three different spots on each of the two glass substrates investigated.

Glass substrates were prepared in the same way as described in the section above for topography imaging. The cantilevers were calibrated before and after each series of force measurement by a non-destructive method determining thermal fluctuations of the cantilever (14).

Results

Adsorption of interferon α-2a on mica

In Figure 4, we show a series of different concentrations of physically adsorbed interferon α-2a to mica in buffer solution of pH 7. Starting from the bare mica surface, the density of single interferon molecules increases when a higher protein concentration is applied. At 4.75 µg/ml interferon in pH 7, only a few individual molecules were found on the surface. The drifting of molecules in the image was caused by a weak attachment to the surface. However, a nearly full monolayer of interferon α-2a has been formed on mica at a concentration of 14.25 µg/ml.

In conformity with other experimental results (data not shown), a higher adsorption density of interferon to mica was achieved in pH 5 than in pH 7. Corresponding results are given in Figure 5. Protein densities in Figure 5 are compared to those shown in Figure 4 at concentrations of 4.75 and 9.5 µg/ml at pH 7.

Surface structure of glass substrates

In force spectroscopy, knowledge of the surface roughness of the substrates used can help to correctly interpret the results. AFM imaging of inner surfaces of FIOLAX Type I plus vials revealed a significantly higher line roughness (R_a = 0.4 nm) than that of standard FIOLAX glass vials (Figure 6). From the cross-section analysis, the grain size of the special silica coating is estimated to 4 to 8 nm in diameter (after correction for convolution of a 10 nm tip radius, Figure 7), and 1 to 2 nm in height. Untreated FIOLAX surfaces have a typical R_a-roughness of about 0.1 nm and show an amorphous structure.

Adhesion force measurements

The immobilization setup for modified tips is illustrated in Figure 8a, while the covalent binding reaction of Interferon α-2a to DSU is shown schematically in Figure 8b. In a pH-range of 6.5 – 8.5, reactive succinimide groups at the free end of the DSU monolayer on the gold surface are able to bind primary amines of proteins. This configuration is stable enough to perform more than 300 approach/retract cycles with the same protein-modified tip without loss of reproducibility.

Figure 4: Interferon α-2a adsorbed at different concentrations from phosphate buffer (pH 7) onto mica. Topography images (size: 500 x 500 nm, grey scale: 1.6 nm) were taken in air from (a) the bare mica substrate, (b) with interferon at a concentration of 4.75 µg/mL, (c) 9.5 µg/mL and (d) 14.25 µg/mL.

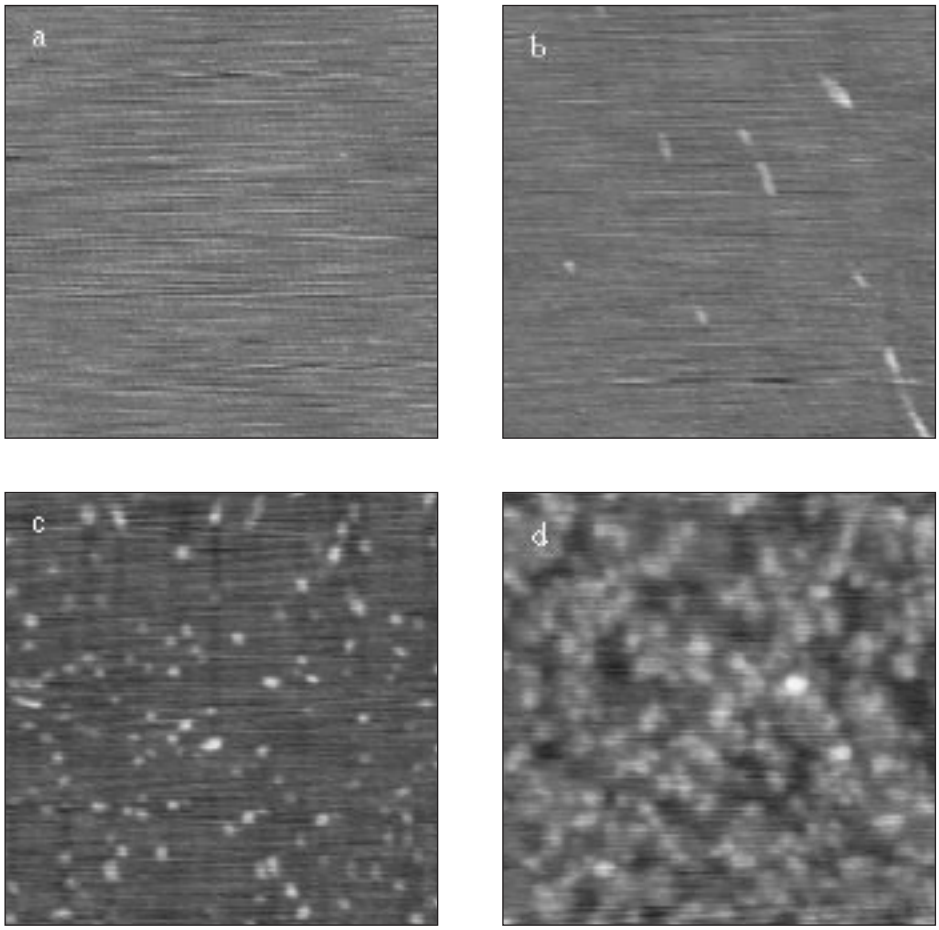


Figure 5: Interferon α-2a adsorbed from citrate buffer (pH 5) onto mica at a concentration of (a) 4.75 µg/mL and (b) 9.5 µg/mL. Topography images (size: 500 x 500 nm, grey scale: 1.6 nm) were taken in air.

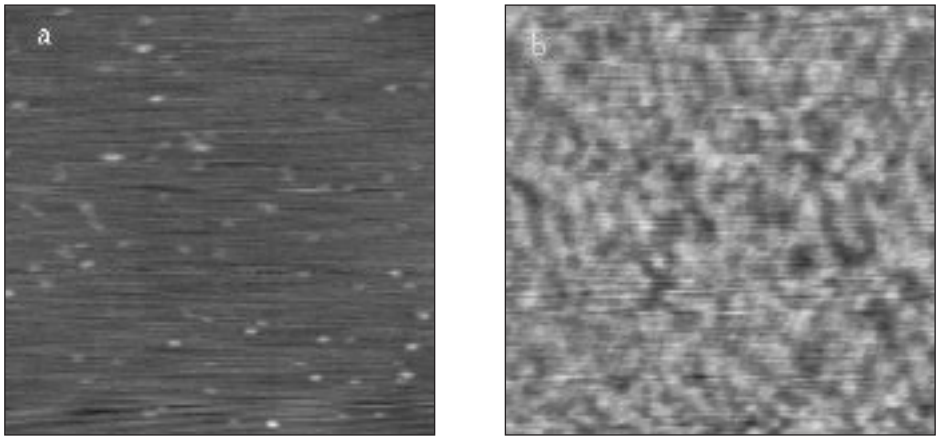


Figure 6: Topography of the inner side of (a) a FIOLAX Type I plus vial, and (b) a FIOLAX vial imaged in air (size: 500 x 500 nm, grey scale 4.3 nm). From the profile shown in (a) we estimate the average grain size of Type I plus to about 1 - 2 nm in height and about 4 - 8 nm in diameter regarding convolution with the tip. The R_a-value of Type I plus is with 0.4 nm significantly higher than for FIOLAX with a line roughness of about 0.1 nm. The average roughness R_a is the arithmetic average of the absolute values of the measured profile height deviations.

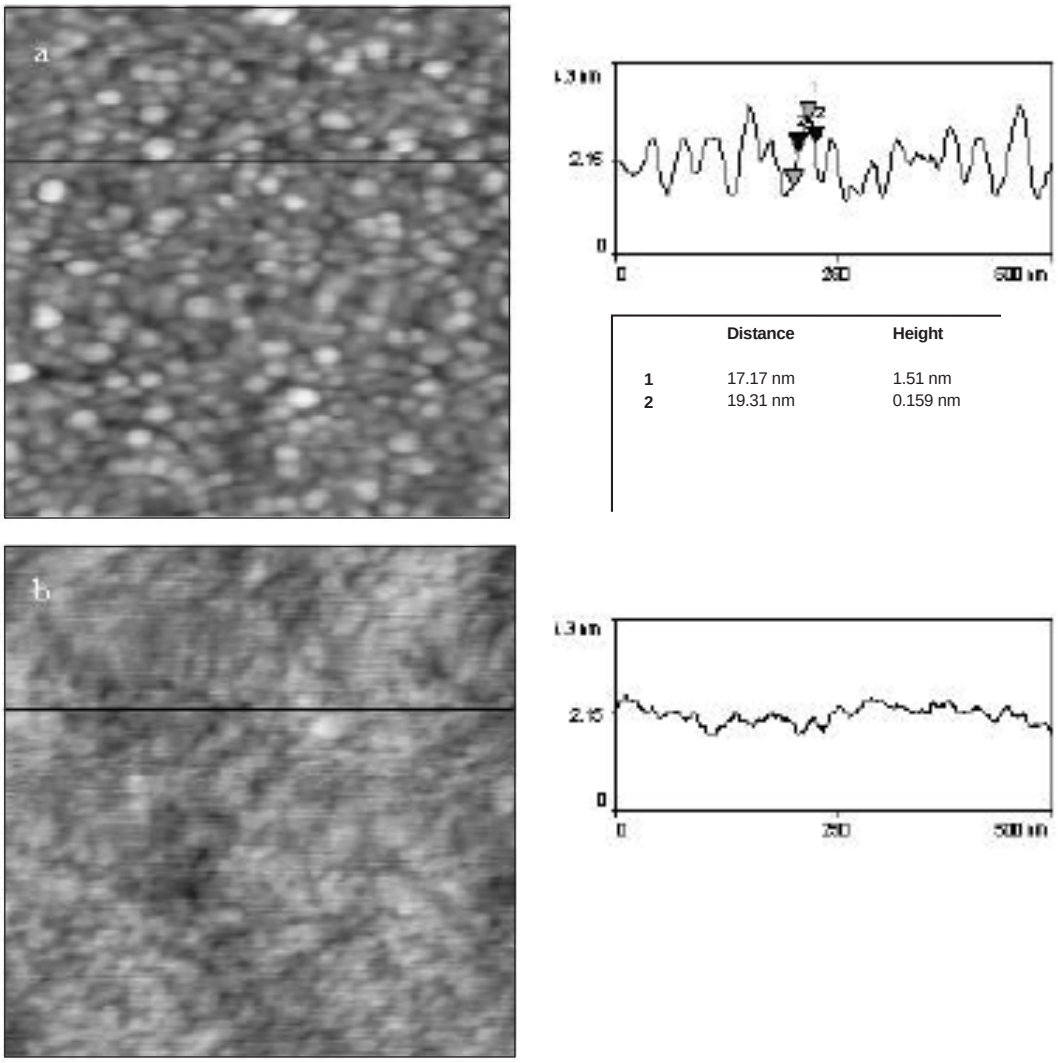


Figure 7: Enlargement of the size of a topographic feature with radius r when imaged with an AFM-tip with radius R. The observed width W of the molecule can be calculated by using the following equation:

$$W = 2\sqrt{(R+r)^2 - (R-r)^2}$$

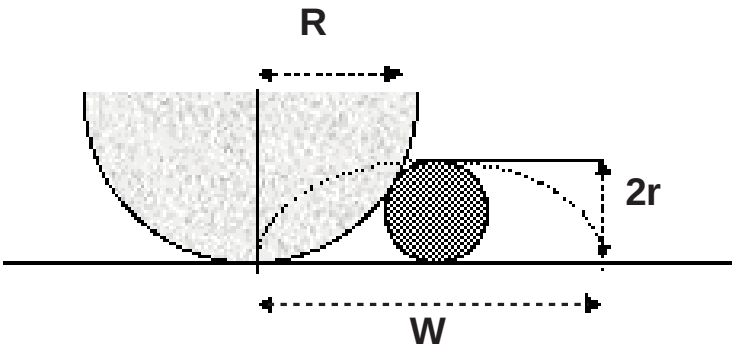
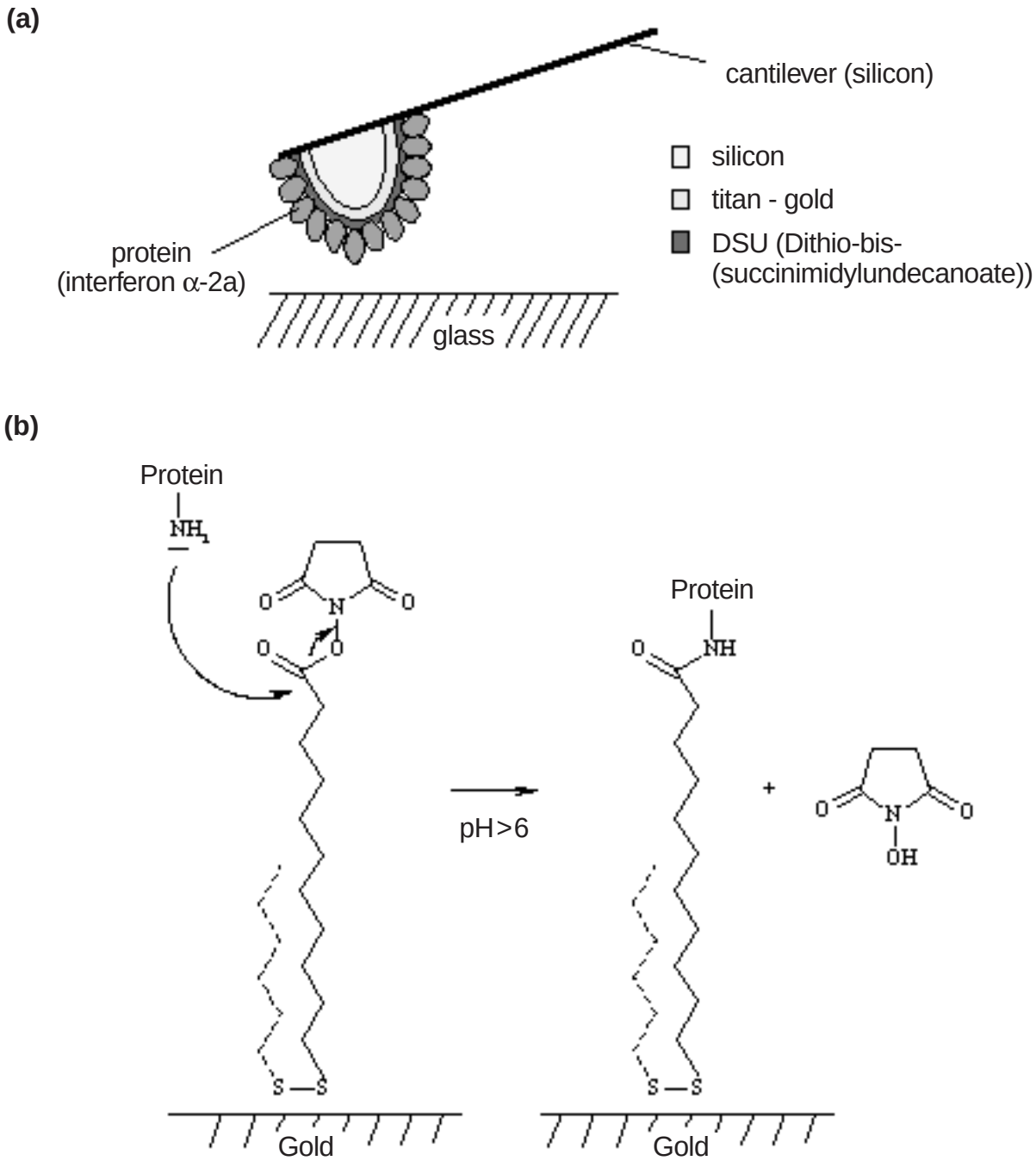


Figure 8: (a) Scheme of the immobilization setup. Interferon α-2a is covalently linked to the cantilever tip by the chemical reaction shown in (b). The cantilever tip has been coated with titan and gold before the immobilization step. The scale of the drawings is set randomly. The size of the protein molecules is about 3 nm in diameter, the thickness of the crosslinker monolayer is 1.7 - 1.9 nm (15), the titan-gold coating is about 20 nm.



A typical retract cycle on a Type I plus surface is shown in Figure 9. The adhesion force can be determined by measuring the distance on the force scale between the jump out of contact and the free cantilever oscillation. The most probable adhesion force of a total of 150 force distance curves monitored with one protein-modified tip is determined by a Gaussian fit through the force distribution, as seen in the example given in Figure 10. The mean adhesion force on a FIOLAX glass surface extracted from the plot is about twice as high as on a Type I plus surface.

Figure 9: Retract curve on a FIOLAX Type I plus surface. The adhesion force measurement was performed with an interferon α-2a coupled cantilever tip in citrate buffer (pH 5).

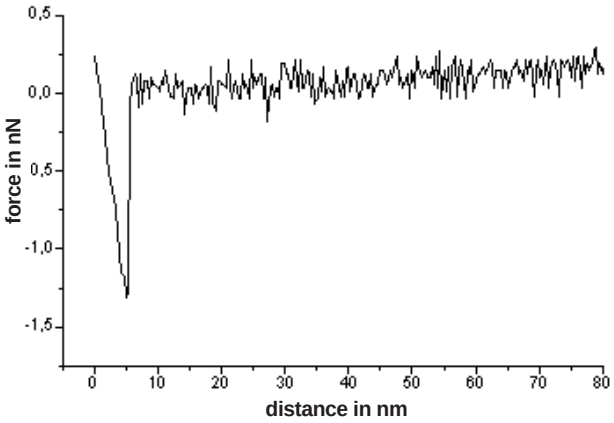
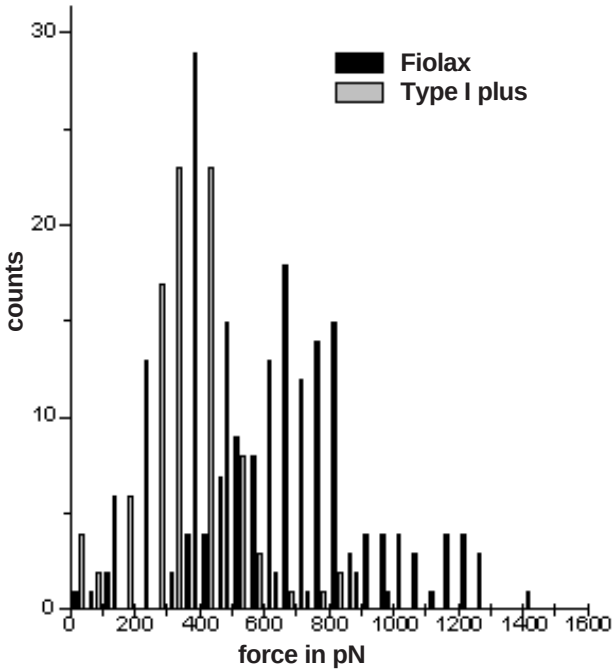


Figure 10: Histogram of absolute adhesion forces measured between interferon α-2a and a FIOLAX Type I plus surface or a FIOLAX surface, respectively, in buffer, pH 5. The mean adhesion force on FIOLAX is about twice as high (~700 pN) as on Type I plus (~360 pN) measured with the same cantilever.



The results obtained from six experiments performed with individually modified tips in pH 5 or pH 7 are summarized in Figure 11. Independent from pH conditions, we found that the total adhesion on Type I plus is reduced by about 40% compared to the values obtained on FIOLAX surfaces. Control experiments were carried out with non-modified tips in pH 7 in order to confirm that we are indeed measuring interactions both between protein and surface, and bare tip and surface. Statistical errors are estimated by the width of the distribution σ from N rupture events by $2\sigma/\sqrt{N}$ (95% confidence level). To convert the deflection signal into forces, the spring constants of the cantilevers had to be experimentally determined for each series of measurements (see experimental section). The spring constants of all cantilevers tested ranged from 170 to 400 pN/nm. Maximum forces applied to the surface during approach averaged 320 ± 150 pN.

Discussion

AFM-imaging of biological material can only be successful if the molecules are firmly attached to the substrate, and if the tip-molecule and tip-substrate interactions are small (2,15). Regarding our results, we assume that physical adsorption of interferon α-2a to mica in aqueous solutions is mainly driven by electrostatic interactions. The isoelectric point of interferon α-2a is around 6 (16), which results in a slightly positive net surface charge at pH 5. Due to dissociation of potassium ions from the surface in aqueous solution, mica is negatively charged (15), and thus, the positively charged interferon α-2a molecules are attracted towards the surface. Moreover, above pH 6, all free carboxylic groups of the protein are deprotonated and the surface charge of the protein switches to negative values. Therefore, at pH 7, the

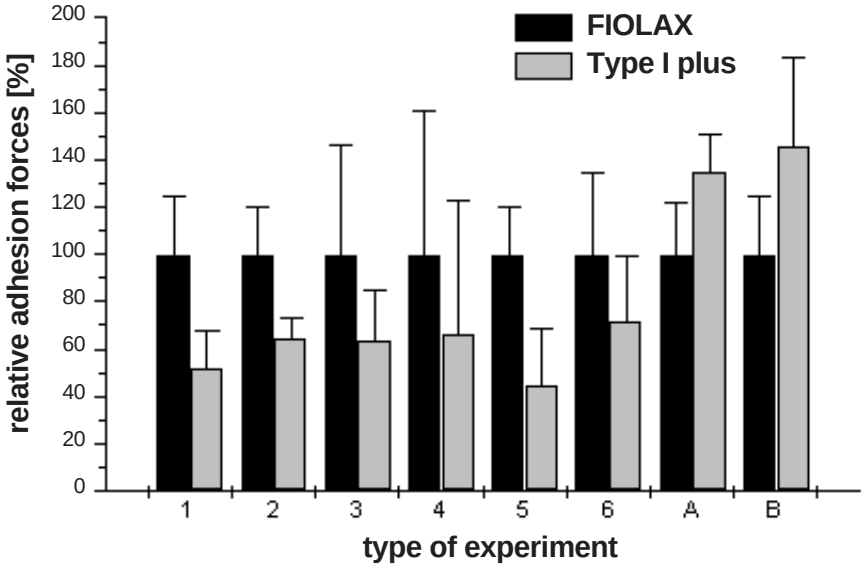
protein molecules are slightly repelled by the negatively charged mica surface and adhere less firmly to the mica substrate than at pH 5.

The results on mica are in good agreement with other experimental and theoretical considerations (9,17). However, within our adhesion force measurements on glass, no correlation could be found between the adhesion of interferon to glass and different pH conditions. Yet, this was not completely unexpected for two reasons. First, the surface charge density of borosilicate glass might be smaller than that of mica and second, we were not able to differentiate between the various forces. In our experiments we measured a total of unspecific interactions that include both van der Waals and electrostatic interactions.

In this work, we measured the strength of unspecific adhesion interactions between interferon α-2a and glass substrates by means of AFM. Absolute force values of the adhesion experiments differ in a wide range due to variation of tip radius, monolayer uniformity, and protein coverage of individual cantilevers. Nevertheless, estimating the mean contact area at the tip to approximately 900 nm^2, there are about 150 interferon molecules contributing to the average adhesion force of about 600 pN measured on FIOLAX glass. For this calculation, tip radius was estimated to 25 nm (including the titan-gold coating), diameter of single interferon molecules to 3 nm, and a hexagonal protein coverage of the cantilever tip. Consequently, disregarding the different types of interactions involved, each protein molecule is experiencing an average adhesion force of about 12 pN to the glass substrate, which is in the range of unspecific single molecule binding strengths to surfaces found by other authors (6,18,19).

Another reason for comparing the force values obtained relatively to each other is based on the experimental setup of this study. In force spectroscopy, the proteins covalently bound to the tip surface are intentionally pushed against the glass sample while approaching the surface. In liquid, the force applied is kept to a minimum. Nevertheless, the protein can be compressed under the load in such a way that possibilities of interactions increase relatively to the unintentional adsorption/diffusion process. In addition, the surface roughness of Type I plus is about three to four times

Figure 11: Relative adhesion forces measured on FIOLAX (=100%) and FIOLAX Type I plus. Experiments were performed with interferon a-2a coupled cantilever tips in pH 5 (no. 1 - 3) and in pH 7 (no. 4 - 6). Protein-free cantilever tips were used in both control experiments. Experiment A was performed in pH 7 with an untreated fresh silicon cantilever tip, while experiment B was carried out with a gold-coated tip. In comparison to protein-modified tips, realtive adhesion forces achieved with non-modified tips on FIOLAX Type I plus were always higher than on FIOLAX.



higher than that of standard FIOLAX surfaces. Thus, Type I plus coating exhibits, again, a larger contact area with the interacting cantilever tip. Since the number of protein molecules contributing to the adhesion experiment on Type I plus is greater than on FIOLAX, the relative distinction between the actual interaction force of an individual protein on Type I plus and FIOLAX would be even greater than 40%.

Conclusions

In our study, force spectroscopy was used to characterize adhesion properties for one specific protein to glass surfaces. With this method, we were able to distinguish between glass containers that show more protein adsorption and those with less protein adsorption. We were comparing non-coated with coated borosilicate glass surfaces. The latter was specifically developed for low protein adsorption. However, in future experiments we will further enhance the sensitivity to differentiate between similar glass types by improving the accuracy of the electronics and the detection system of the AFM instrumentation.

The detailed molecular process of interferon α-2a adsorption to borosilicate glass is still unknown. From the adhesion study presented in this article, we learn that topography in terms of surface roughness is not directly responsible for protein adhesion, but that the occurrence of protein adhesion is rather affected by chemical properties of the substrate surface. A surface exhibiting a variety of functional groups provides a great deal of interaction possibilities with complex biological molecules like proteins. It is generally acknowledged that protein adsorption is always subject to a multitude of different interactions (20-22). Besides electrostatic interaction with charged groups, hydrophobic and hydrophilic domains can be responsible for adherence and adsorption. Therefore, it is easily understood that proteins are less likely to adhere to a chemically homogeneous surface, like the pure SiO_2-layer on Type I plus, than to a standard borosilicate glass surface endowed with alkali oxides, e.g. FIOLAX.

A perspective for the future is certainly to develop a standardized method that can be applied to every kind of protein in order to decide which container material and/or storage buffer is appropriate for the corresponding pharmaceutical product.

Acknowledgements

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