

LAB UNIT 2: Non-Contact Scanning Force Microscopy in Air and Liquid Environment

Specific Assignment: Protein Adsorption Kinetics

Objective

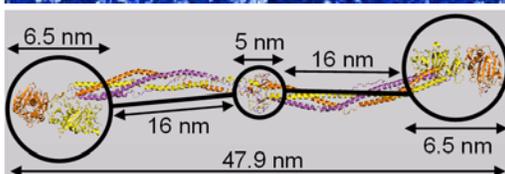
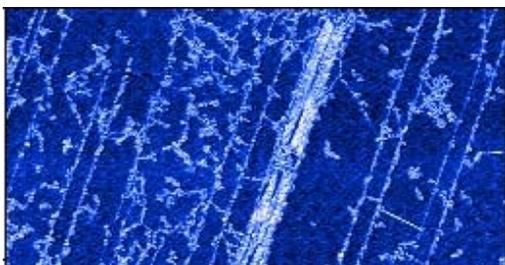
In this lab unit students are characterizing protein-material interactions using intermittent non-contact (NC) scanning force microscopy (SFM) in both fluid medium and in air to quantify complex surface adsorption processes. The material analyzed is graphite adsorbed with a blood clotting protein, fibrinogen (Fb), to mimic a bio-response to prosthetic heart valve devices.

Outcome

Gain insight into macromolecular surface interaction at the molecular level and its role in understanding/improving the field of engineered biomaterials. Learn about proteins and adsorption from a physiological perspective, and to quantify equilibrium adsorption constant K_a as well as the Gibbs Free Energy of adsorption using high resolution SFM and the Langmuir model.

Synopsis

Implant rejection by the body accounts for a large percentage of preventable surgeries occurring in modern medicine today. At the earliest stages of the immune response, foreign bodies are marked by clotting agents such as fibrinogen (Fb) which signals larger platelets and white blood cells to initiate a response pathway and eventually to isolate it from the rest of the body.



Fibrinogen a blood clotting protein, adsorbed on graphite imaged by intermittent NC-SFM

By understanding the initial stage of protein-solid interactions and engineering materials to camouflage them from early protein adsorption, the immune response can be bypassed and long-term complications avoided by the implant patient. This lab seeks to characterize protein-

solid interactions via SFM imaging in order to understand the adsorption behavior of fibrinogen in real time for the purpose of simulating a graphitic carbon modern prosthetic heart valve.

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1. Assignment

The assignment is to use the SFM in both air and a dynamic fluid medium to observe the binding morphology of a common blood clotting factor towards a graphitic surface. Further, we will seek to quantify this adsorption in order to characterize its binding modality based on the observable surface coverage from resulting SFM images. In fluid mode, scanning will begin in buffer solution while the protein is introduced and observed to bind over time. To quantify surface coverage trends, we will scan previously prepared samples which have been exposed to protein solutions, at equilibrium, of increasing concentrations in air for enhanced resolution. The module's emphasis will be on understanding the precedent for such experiments and their application for improving engineered biomaterials. The steps are outlined here:

1. Familiarize yourself with the background information provided in Section 4.
2. Test your background knowledge with the provided Quiz in Section 2.
3. Conduct the fluid-mode and in-air experiments in Section 3. Follow the step-by-step experimental procedure.
4. Analyze your data as described in Section 3.
5. Finally, provide a report with the following information:
 - (i) Results section: In this section you show your data and discuss instrumental details (i.e., limitations) and the quality of your data (error analysis).
 - (ii) Discussion section: In this section you discuss and analyze your data in the light of the provided background information.
It is also appropriate to discuss sections (i) and (ii) together.
 - (iii) Summary: Here you summarize your findings and provide comments on how your results would affect any future SFM work you may do. The report is evaluated based on the quality of the discussion and the integration of your experimental data and the provided theory. You are encouraged to discuss results that are unexpected. It is important to include discussions on the causes for discrepancies and inconsistencies in the data.

2. Quiz – Preparation for the Experiment

Background Questions

Biology Background

1. Explain, briefly, the role(s) of fibrinogen in the context of implant rejection.
2. Why are smooth surfaces preferred over rough ones as implant coatings?
3. Why don't proteins generally aggregate in aqueous solutions? Why do they aggregate on surfaces?
4. What is the difference between the 'intrinsic' bio-response and the 'extrinsic' one?
5. Why is graphite the material of choice for heart valve prostheses?
6. Stoney's formula (Klein, C. A. J. Appl. Phys. 2000, 88, 5487)

$$\sigma = \frac{1}{3} \left(\frac{D}{L} \right)^2 \frac{E}{(1-\nu)} \Delta z$$

relates the tensile surface stress σ to the normal deflection Δz measured at the front of the cantilever. With the cantilever spring constant k_N , given as

$$k_N = \frac{EWD^3}{4L^3},$$

derive the function $\sigma(k_N)$.

Prelab Quiz*Langmuir Isotherm*

- (1) Determine based on Figure 1 the binding constant K of the adsorption process. Hint: Use the Langmuir Model.

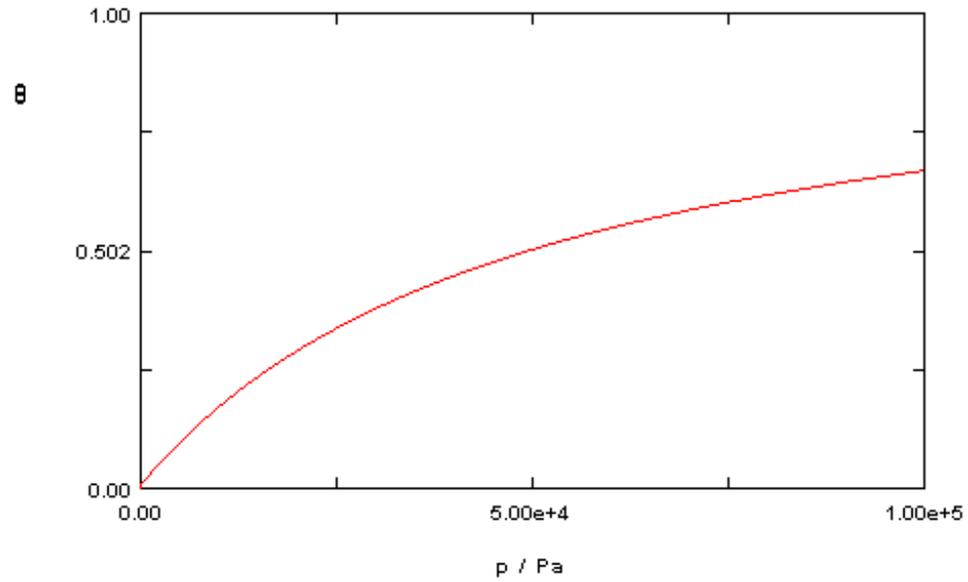


Figure 1

3. Experimental Assignment

Goal

Following the step-by-step instructions below, determine the adsorption kinetics of fibrinogen on highly oriented pyrolytic graphite (HOPG) in phosphate buffer solution. This experiment will be performed in AC-mode (intermittent non contact (NC) mode) both in air using dried samples and in liquid to obtain ‘real time’ data. Analyze and discuss the data with the background information provided in Section 4. Provide a written report of this experiment.

Specifically provide answers to the following questions:

- (1) What morphologies do you notice on the surface? Where does the protein seem to bind first (i.e. at earlier times)? Why is this?
- (2) Is it easier to determine protein surface coverage from a topography image or a phase contrast image? Why?
- (3) According to the analysis, what was (a) K_a and (b) θ_{max} for the measurements taken in air?
- (4) If possible, according to the analysis of measurements taken in liquid what was (a) θ_{inf} and (b) τ ?
- (5) Compare your results the results previously reported[1] how are your results different/similar? Discuss your findings and what experimental aspects may be different between your study and the literature
- (6) Why is the resonance frequency in liquid much different from that in air? If possible, report your values for both liquid and air.

Safety

- Wear safety glasses.
- Refer to the General rules in the SFM lab.
- Wear gloves when handling Fibrinogen in solution.
- Take particular care to avoid using too much liquid in the liquid mode experiment as this could damage the instrument and cause an electrical shock

Instrumental Setup

- Easy Scan 2 SFM system with Force Modulation tip (FMR) 3 N/m spring constant.
- Nanosurf Easyscan 2 Flex AFM system with Nanosensor or Vista FMR cantilevers with ~3 N/m spring constant.

Materials

- Samples: 6 pieces of HOPG that were exposed to Fibrinogen solution in PBS (provided) for 1.5 hrs. Concentrations of protein solution were: 0.05 $\mu\text{g/mL}$, 0.667 $\mu\text{g/mL}$, 3.33 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$.
- A separate HOPG sample for use in liquid mode
- Double sided tape (to cleave graphite and secure graphite samples)
- Pipetter (100 μL) with pipette tips

- 10mL of ethanol to sterilize pipette tips
- 50mL of PBS
- 1mL of a 1mg/mL solution of Fibrinogen in PBS
- Vista FMR probes
- Nanosensors PPP-FMR probes
- Tweezers

Experimental Procedure

Read the instructions below carefully and follow them closely. They will provide you with information about (i) preparation of the experiment, (ii) the procedure for dry dynamic mode imaging, (iii) the procedure for liquid dynamic mode imaging, and (iv) how to conduct the data analysis.

(i) Preparation of the experiment

(1) Dry dynamic mode system set-up: (This part will be performed with a TA).

- a. System set-up
 - i. Remove the scan head from the sample stage.
 - ii. Load a Vista FMR cantilever with a spring constant of 3 N/m on the EasyScan 2.
 - iii. Position one of the provided dry graphite samples with Fibrinogen on the sample stage and electrically ground the sample.
 - iv. Carefully place the scan head onto the sample stage ensuring by watching the video feed that you have sufficient clearance to avoid crashing the tip into the surface. Carefully adjust the height of the scan head such that the tip is ~ 1 mm from the surface.
- b. Software preparation
 - i. Once the cantilever is loaded click on ‘Operating Mode’ icon in the menu list on the left side of the screen in the Easyscan 2 software. The operating mode panel will appear. Select ‘FMR’ as the mounted cantilever and ‘Phase Contrast’ as the operating mode.
 
 - ii. Click on the ‘Imaging’ icon (top right). Select the ‘Topography – Scan Forward’ plot. Add two new plots by clicking the new chart icon twice (middle right). Next, select one of the new charts and change the signal of the first to ‘Amplitude - Scan Forward’ by clicking the select signal icon (bottom right) and choosing amplitude from the drop down menu. Select the second new chart and change the signal to ‘Phase – Scan Forward’. The amplitude plot provides a map of the oscillation amplitude of the lever as a function of position, while the phase plot gives the phase shift of the photodiode signal as a function of position.
 


 - iii. Click on the ‘Topography – Scan Forward’ line graph and create new line graphs for both amplitude and phase in the same manner as above.
- c. Determination of cantilever resonance frequency
 - i. In the operating mode panel set the free vibration amplitude to 200mV and check the ‘Display sweep chart’ box.

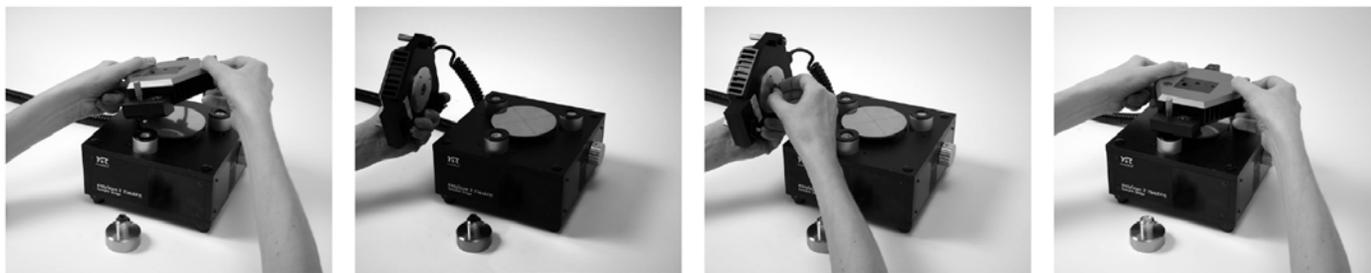
- ii. Click the ‘Set’ button just below the vibration frequency box. The software will automatically perform two sweeps to find the resonance frequency. Two plots will appear. Examine them to ensure that a peak frequency of reasonable height ($>100\text{mV}$) was found.
- iii. The probe status light should be red before this process and yellow afterward.

(2) Liquid mode system set up: (This part will be performed with a TA).

- a. System set-up:
 - i. Using double sided tape, cleave the graphite to be used for liquid mode imaging.
 - ii. Remove the SFM head from the sample stage and set it upside down on a secure surface.
 - iii. Crank the sample stage down until it is fully lowered and load the freshly cleaved graphite onto the sample stage.
 - iv. Remove the detachable liquid cantilever mount from the SFM and clean it with dish soap and water. Rinse with DI water followed by ethanol. Load a Nanosensor FMR cantilever and then place the holding spring on the mount as shown below.



- v. Place back the mount as shown below.



- vi. Take the SFM head and carefully place it onto the sample stage.
- vii. Crank the sample stage up until the sample is $<1\text{mm}$ from the lever (see below). Watch this process using the view port to ensure you do not crash the tip into the sample surface.



- b. Software preparation
 - i. Follow the same steps as listed above for dry dynamic mode.
- c. Determination of cantilever resonance frequency
 - i. Follow the same steps as listed above for dry dynamic mode.
 - ii. Note that the highest peak in the frequency sweep may not be the resonance, but may be the 2nd harmonic. For FMR levers, the resonance frequency should be less than 50kHz.
- d. Oscilloscope set-up:
 - i. Ensure that an oscilloscope is present and is connected by BNC cable to the ‘Deflection’ output on the Nanosurf break-out box and to the ‘Z-axis’ output signal as well. This will allow you to watch the cantilever’s oscillation and z-axis signals when coming into contact.
 - ii. Press the ‘autoscale’ button on the oscilloscope and ensure that you have a sine-wave after finding the resonance frequency.

(ii) Dry Dynamic Mode Imaging

- 1) Coming into contact:
 - a. Once the cantilever is approximately 1mm from the surface, click on the ‘Positioning’ icon  and click ‘Approach’ to bring the lever into contact.
 - b. Close the frequency tuning window that appears.
 - c. Ensure that the probe status light is green when approaching. If not, stop the approach and consult your TA for the cause of this. Note that a blinking red light means that no lever is detected, while a solid red light means that a tip is detected but the frequency is not set properly.
 - d. The program will automatically switch to the imaging window once imaging begins.
- 2) Adjusting slope:
 - a. Once imaging has begun, the slope will most likely need adjustment.
 - b. This can be done automatically by selecting ‘Imaging – adjust slope’ from the ‘Script’ menu.
 - c. The software automatically adjusts slope and begins the scan again.
- 3) Optimize scan quality:
 - a. Open the Z-Controller Panel (right) by clicking the z-controller icon. 
 - b. Set the set point to be 50%. Use the default values for

- the P-Gain and I-Gain.
- c. In the Imaging Panel, change the image width to $2\mu\text{m}$ and ensure that the resolution is set to 256.
 - d. Vary the set point, P-Gain, I-Gain and time/line values to optimize the image quality according to the following guidelines:
 - i. Faster scan speeds (lower time/line values) generally require higher gains.
 - ii. Excessively high gains cause the controller to ring, resulting in very noisy amplitude and topography signals.
 - iii. Very large surface features generally require higher gains or higher amplitude or slower scan speed.
 - iv. Noisy measurements may improve with increased set point, but very high set points may result in coming out of contact. You may also employ the minimum force trick, that is, increase the set point (%) until you are out of contact and then decrease it again until a reasonable image is obtained. This results in using the minimum force.
- 4) Obtain images:
- a. Once scan quality is acceptable complete the scan by clicking on the 'Finish' icon.
 - b. If the completed image looks good, click 'Photo' and save the image.
 - c. Each image should be in a different area of the sample. To move to a different area, simply select random values (from -20 to $20\mu\text{m}$) and input these into the 'Image X-Pos' and 'Image Y-Pos' fields in the imaging panel. Alternatively, you may withdraw the cantilever under the 'Positioning' window and use the translation stage to move to a new area of the sample surface. Be sure to 'Approach' again after moving.
 - d. Click 'Start' to begin a new image. Repeat slope adjustment and image optimization as necessary.
 - e. Obtain at least three images for each sample.
- 5) Change sample:
- a. Once all images have been obtained for that sample, go to the 'Positioning' window and click 'Withdraw'.
 - b. Once the cantilever is well off the surface, click 'Retract' and raise the tip farther ($\sim 3\text{ mm}$ off the surface).
 - c. Remove the scan head from the surface and change the sample, making sure it is appropriately grounded.
 - d. Repeat steps 1)-5) for all samples.
- 6) Conclude imaging:
- a. Come out of contact using 'Withdraw' and 'Retract'.
 - b. Remove the cantilever and store it appropriately.
 - c. Shut down the Easyscan 2 software and turn off the controller.

(iii) Procedure for Liquid Dynamic Mode Imaging

(1) Prepare for Liquid Mode

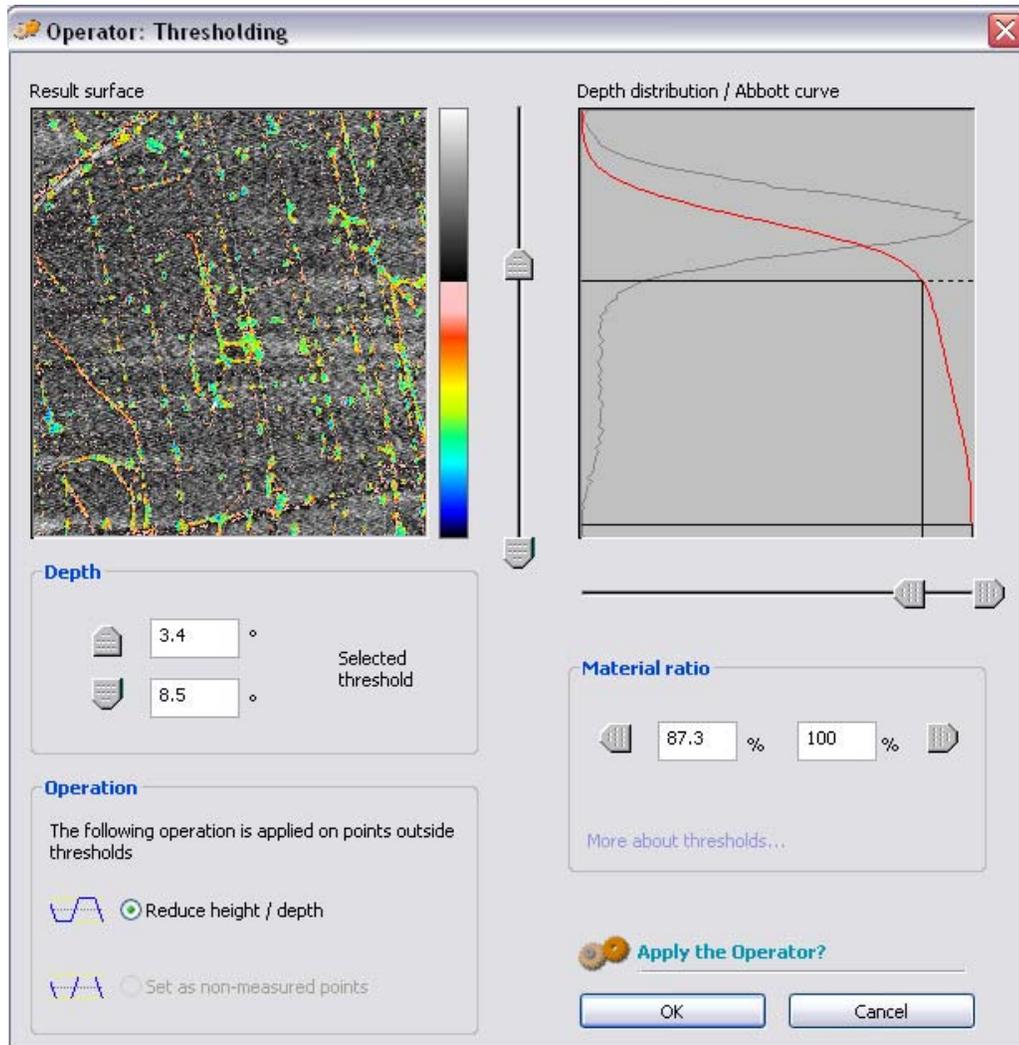
- a. Remove the SFM head from the sample stage.
- b. Obtain a new pipette tip and sterilize the tip by filling it with ethanol ($100\mu\text{L}$) three times, followed by DI water three times.

- c. Using the sterilized tip, place 200 μL of PBS onto the graphite surface.
- (2) Find the cantilever resonance frequency:
- a. Replace the SFM head onto the sample stage, ensuring that the tip does not crash into the surface by monitoring the lever through the view port.
 - b. Look at the set up to ensure that the lever is entirely submerged in liquid. The cantilever status light should be red at this point. If it is flashing red, the cantilever is not detected and you must remount the lever.
 - c. In the Operating Mode Panel set the free vibration amplitude to 100mV.
 - d. Under 'Freq. Peak Search' in the Operating Mode Panel uncheck Auto set and set the start frequency to 0Hz and the end frequency to 100,000Hz.
 - e. Click the 'Set' button just below the vibration frequency box. The software will automatically perform two sweeps to find the resonance frequency. Two plots will appear. Ensure that the resonance frequency is found and that the software successfully set the value. If not, reduce the free vibration amplitude to as low as 60mV and try again.
 - f. The probe status light should be yellow after this process.
- (3) Come into contact:
- a. Under the 'Imaging' window ensure that a 20 μm area is selected.
 - b. When you are ready, click 'Withdraw' and then 'Stop' to reset the system. Watch the oscilloscope while slowly lowering the cantilever by turning the front leg of the SFM counterclockwise (down).
 - c. The oscilloscope signal will 'shudder' and then the amplitude of the signal will rapidly decrease as you approach. Once it has decreased to about half of its original value it is in contact. Also watch the z-position signal to check the contact as it will move substantially when you come in contact, continue lowering the front leg until the z-position signal is approximately zero.
 - d. Once in contact go to the 'Imaging' window and level the image by going to Script \rightarrow Imaging Adjust Slope.
 - e. In the imaging panel change the rotation angle to 90 $^\circ$ and again select Script \rightarrow Imaging Adjust Slope.
- (4) Find flat area:
- a. While imaging a 20 μm area, determine the stability of the system by changing parameters such as P-Gain, I-Gain and scan speed.
 - b. During the scan look for an area on the surface that is free of topography changes.
 - c. When you have found such a region, zoom in by selecting a 2 μm by 2 μm region using the zoom feature in the Imaging window.
 - d. Optimize image quality in this region (see method in dry imaging section above) and ensure that the system is continuously scanning by not selecting 'Finish' but by selecting 'Photo' each time a new image starts.
 - e. Obtain 2-3 images of the bare surface.
- (5) Inject protein:
- a. Using the pipetter, carefully inject 7 μL of a 1mg/mL solution of Fibrinogen in PBS into the existing 200 μL of buffer.
 - b. Continue to obtain images in the same area as they are generated for 1 hour after injection.

- (6) Close out experiment:
- After 1 hour, come out of contact by turning the front leg screw clockwise, while watching the oscilloscope signal to ensure that it is becoming larger.
 - Carefully remove the SFM head.
 - Remove the detachable liquid cantilever mount and remove the lever, placing it in a designated area for used levers.
 - Clean the mount with soap and water, dry it and replace it on the SFM head.
 - Lower the sample stage all the way and remove the graphite.
 - Place the SFM head back on the sample stage.

(iv) Instructions for data analysis

- All images obtained in dry dynamic mode will be processed using Nanosurf Report software, which is installed on the same computer used to control the instrument.
- Open the Nanosurf Report software and go to Options→General Preferences→File formats and ensure that ‘Load all layers as individual studiables’ is selected.
- Go to File→ Open a Studiable and choose the desired image.
- Once the image is loaded, click on the ‘Scan Forward—Phase’ image.
- Correct the lines in the image by going to Operators→line correction. Settings should be:
 - Direction of correction: Line by line
 - Include/Exclude area: Use whole surface
 - Method of correction: Subtract the LS line
- Level the resulting image by selecting Operators→Leveling
 - Leveling method: Least Square Plane
 - Leveling operation: by subtraction
- Find the area occupied by the protein by selecting Operators→Thresholding
- The thresholding box (below) pops up with the image on the left and the depth distribution/Abbott curve on the right.
- The proteins should form a shoulder on the main distribution curve, as shown below. By moving the slides in the middle of the box, the colored area may be highlighted, while the area can be found by the difference in the material ratio numbers, in this case $100\% - 87.3\% = 12.7\%$.



- (10) Save the report by going to File→Save the document *As*
- (11) Enter the resulting areas into the provided Excel spreadsheet entitled 'Langmuir Fit program' as fractions rather than % values.
- (12) Repeat steps 3)-11) for the remaining images.
- (13) Once all data has been obtained follow the instructions on the Excel spreadsheet to determine θ_{inf} and τ from the data.

4. Background: Fibrinogen's Role in Biomaterial Response and Protein-Solid Interactions

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Brief Overview on Blood Clotting

Medical implants used today can incur thousands of dollars in cost to the patient and often require invasive methods of maintenance and eventual replacement (see Fig. 1) to correct unintended physiological responses by the body (bio-response). While many of these complications stem from the implant design, broad limitations exist in designing proper material interfaces that can coexist the dynamic environment of the body and its complex biochemical response to foreign surfaces. Therefore, the design of proper biomaterials requires a fundamental understanding of the bio-response mechanism from the body and its ultimate effects at the interface of the material surface.



Figure 1. Prosthetic carbon-based mechanical heart valve, (*left*) before implantation, (*right*) after implantation rejected by the body.

Courtesy of T. Horbert (University of Washington)

To understand the body's response to implanted materials, it is insightful to first study how the body responds to normal internal and external wounds (lacerations), as well as imperfections in everyday functional tissues via blood clotting. The formation of a blood clot is the result of a

concerted interplay between various blood components, such as the platelets, or thrombocytes, The platelets are cells in the blood that are involved in the cellular mechanisms of the primary blood clotting process, the hemostasis.

Initial response to a laceration begins when platelets from the blood plasma aggregate at the wound site, to create a clot that impedes blood loss. Blood platelet aggregation is assisted at the wound site by a protein known as the *von Willebrand factor* (vWF). The von Willebrand factor found in both tissue cells as well as the blood stream supports the clotting factor VIII. When people show a deficiency in the von Willebrand factor, factor VIII can weaken and cease to perform its function in blood clotting leading to excessive bleeding upon injury. With von Willebrand factor, platelet cells are biochemically stimulated to bridge exposed tissue cells that further initiates clotting factors to jumpstart the coagulation phase of the clotting response. Generally, the *intrinsic response* is contained within the blood plasma itself and responsible for a larger part of clot formation while the *extrinsic response* is by the surrounding tissue cells. This is meant to supplement the intrinsic pathway to accelerate clot formation. These two pathways ultimately convene to arrive at the *common response*. This common pathway, as highlighted in Fig. 2a, activates crucial proteins involved in forming an adhesive matrix to bind and solidify the existing platelets, forming what is known as a “hard clot.” As with all biochemical processes, the formation, usage and ultimate degradation of the numerous clotting factors is self-regulated via feedback mechanisms recognized by the factors themselves at each individual reaction stage.

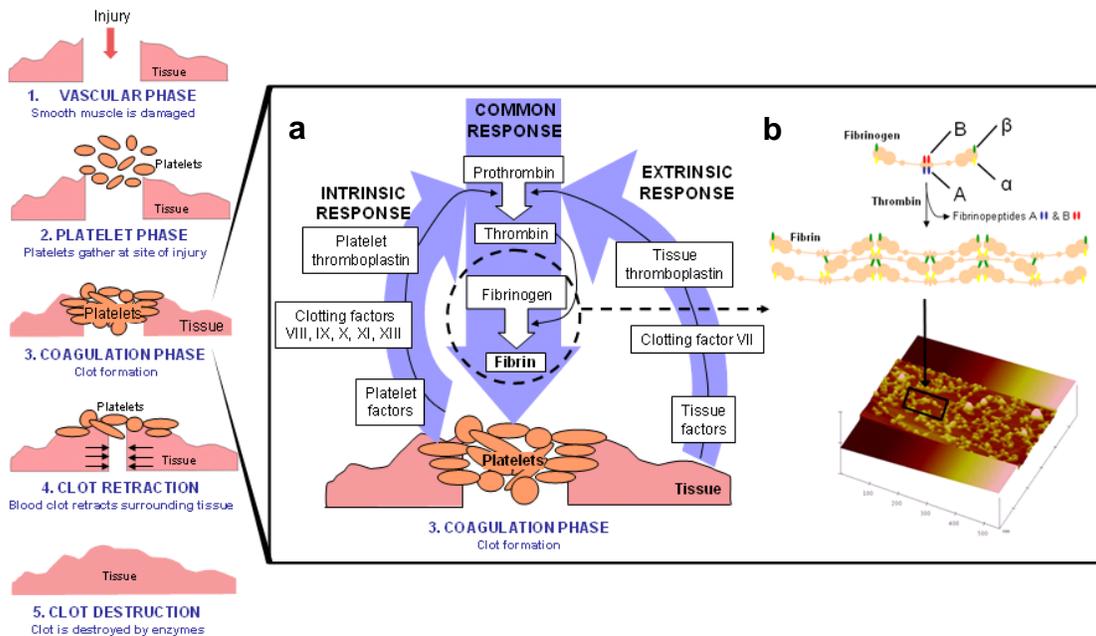


Figure 2a (left) Blood clotting pathway for tissue injuries with emphasis on fibrinogen activation and regulation, circled. **2b (right)** Specific mechanism of the activation of fibrinogen by thrombin, and SFM image of a fibrin clot on highly oriented graphite.

The common pathway is responsible for the formation of an adhesive protein based gel that interacts with and further coordinates the final stages of clotting. Here, a common product from both intrinsic and extrinsic pathways, clotting factor X_a , transforms an existing factor

‘prothrombin’ to its active form ‘thrombin’. This active form then proceeds to activate another factor, fibrinogen, by breaking specific intramolecular connections in a process known as cleavage. Active fibrinogen, referred to as ‘fibrin monomer’, is responsible for polymerizing with itself at the previously cleaved sites to rapidly form an adhesive gel to support the surrounding platelet aggregation in what is known as a ‘soft clot’. Thus, the common pathway and resulting fibrin polymer is the product of both the intrinsic and extrinsic pathways and the main driving force in the clotting cascade’s coagulation phase. The mechanism of fibrin polymer formation is shown in Figure 2b, which also provides a visualization of the fibrin matrix on a model implant surface (graphitic carbon) by scanning force microscopy (SFM).

Fibrinogen Structure and Functioning Mechanism

Fibrinogen in its inactive form is 340 kD (~47.5 nm) in size and exists as a covalently bound two part molecule (known as a “dimer”) associated through three disulfide bridges, as shown in Fig. 3. It is comprised of three intertwined strands of amino acids shown in Fig. 3b as the A, B, and C strands. These strands associate with each other to form several functional domains, including the terminal sticky α , β , and γ domains (shown as tangled lines in Fig. 3b) of the protein as well as the rigid spacer linking the two portions of the dimer together. From the center, strands A and B contain short sequences of amino acids which together form the thrombin cleavage site, shown as stemmed circles in Fig. 3b. After the short sequences (known as ‘fibrinopeptides’) are cleaved off, the newly vacant sites (pathway shown in Fig. 2b) are now free to interact specifically with the sticky α and β domains from adjacent fibrin monomers for polymerization and the formation of a ‘soft clot’. Further, polysaccharides contained within the terminal sticky ends of fibrin (shown as black hexagons in Fig. 3b) help provide an even stronger fibrin polymer through a process called cross-linking (off-axis bonding) to ultimately form a ‘hard clot’ via a factor known as XIIIa. These domains of fibrin are spaced ~16 nm from the center domain via a structured triple-helix spacer domain, where the three strands are intertwined to give fibrinogen and the resulting clot a rigid structure.

The last domain, the γ -sticky end also plays a crucial role (as seen in Fig. 4a) in interacting with platelet cell surface receptors to ultimately incorporate the existing platelets into the fibrin clot. Typically, both inactive and active forms of fibrinogen can mediate adhesion via γ -domain interaction with platelet surface-bound factors known as GPIIb/IIIa. When these surface receptors are bound, platelets switch from inactive to active form and begin to secrete cofactors (a factor designed to work with another factor) and signaling proteins (including fibrinogen and vWF) which act as positive feedback agents to further promote clot formation. As covered in the next section, this interaction plays a crucial role in implant rejection due to the lack of need for an active form of fibrinogen to initiate a clotting cascade.

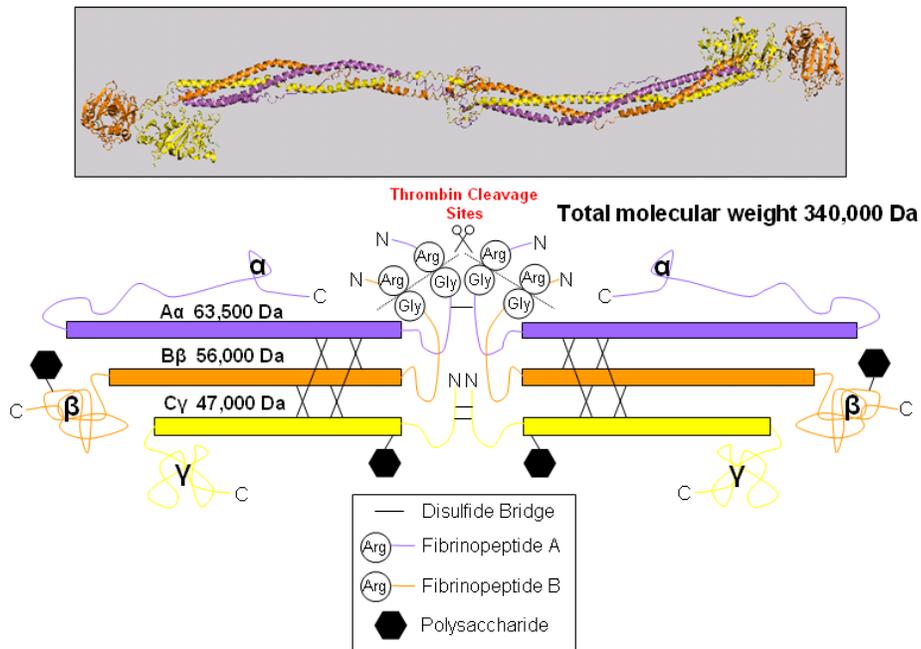
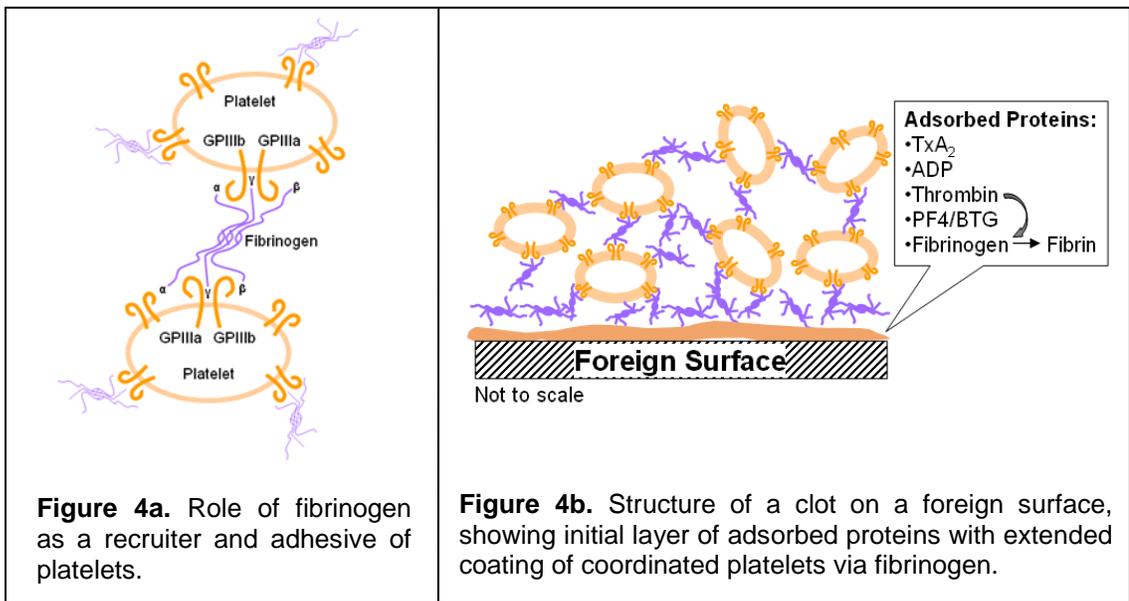


Figure 3. a (top) Crystal structure of fibrinogen, showing the triple helix structure of linker regions and **b (bottom)** color corresponding diagram of three separate protein strands A,B,C and their association with each other via disulfide bridges.



Bio-Response toward Implant Devices and Foreign Bodies

Many of the same factors play a role in the identification and isolation of foreign material surfaces in the body, which leads to ‘rejections’. One major difference, however, is the lack of vWF or other existing extrinsic pathways to supplement or jumpstart the bio-response cascade as

in normal blood clotting. The initial response to foreign surfaces is predominately intrinsic and stems from the material surface's abilities to adsorb and aggregate various factors and proteins, which then, jumpstart the clotting mechanism and ultimately the formation of a fibrous capsule that walls off the implant from the rest of the body. One example of this is by fibrinogen, which can recruit platelet cells in its inactive form (from Fig. 4b) via its γ domain and GPIIIa/IIb platelet-surface protein receptor interactions. Thus, if the implant surface displays affinity towards aggregation of fibrinogen, it will also display affinity towards platelet cells in the blood plasma. As shown in Fig. 4a, this protein aggregation phase is one of the primary factors in initiating the implant bio-response cascade.

Another differentiating factor between foreign body response and blood clotting is the involvement of certain immune system elements in the cascade. Shown in Fig. 5, the lack of normal extrinsic pathway signals can persuade the body to identify a material as foreign and attack it with immune cells, such as 'neutrophils,' 'macrophages' and others. This attack typically ends with the formation of an encapsulating cell caused by fusion of macrophages, and known as a 'foreign body giant cell'. The foreign body giant cell engulfs the entire surface and recruits connective tissue cells known as fibroblasts to the implant site. The fibroblasts then form a dense fibrous capsule to wall off the implant from the rest of the body in a stage termed 'fibrosis'. This stage of bio-response is also termed 'thrombosis' and can occur within 3 weeks of the initial response. It is useful here to note that the non-specific adsorption of various bodily factors towards any material surface will likely initiate a bio-response cascade by the body and ultimately complications in the lifetime of the implant device. This has been one of the main challenges fueling the development of novel engineered biomaterial systems.

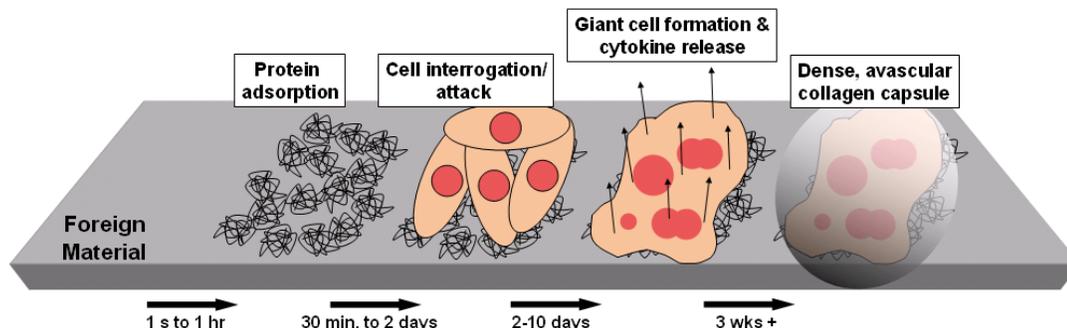


Figure 5. Stages of implant rejection over time, beginning with protein aggregation and ending with capsule formation

Implant Material Design

It is widely accepted that the prevention of non-specific protein adsorption can be highly correlated with improved implant lifetime and viability. To understand strategies in camouflaging material surfaces chemically, it is useful to understand the general properties of physiological proteins. The body, comprised of ~70% water, is a highly aqueous environment in which proteins have evolved in to function. In the course of evolution, the majority of proteins found in humans are folded to exhibit hydrophilic exteriors and hydrophobic cores, as illustrated in Fig. 6, much like lipid micelles found in soaps. Without this phase segregated property,

proteins would tend to precipitate out of solution and lose the ability to travel in the blood stream or any aqueous environment as needed for bodily functions.

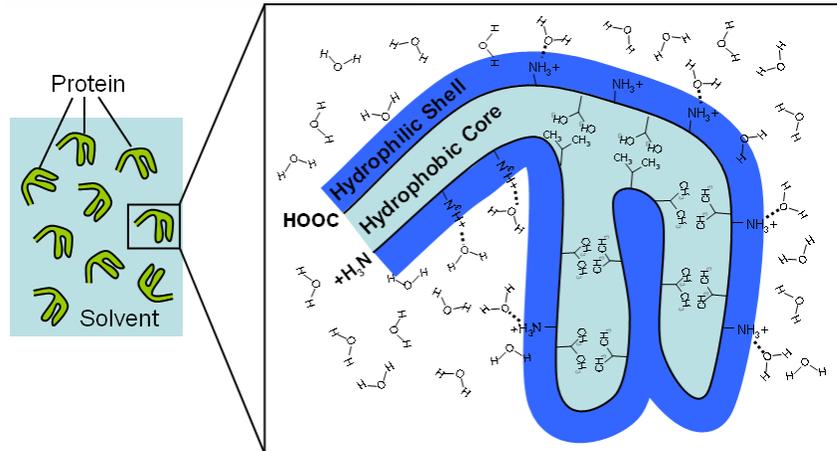


Figure 6. Schematic representing a protein backbone (black outline) with corresponding charged hydrophilic side-chains facing outward (dark blue region) and water-fearing aliphatic groups facing inward (light blue region).

Many foreign surfaces act as condensers of proteins due to their insolubility with aqueous environments, causing proteins to denature at the interface and aggregate. Thus, for specific properties of aggregation prevention, solvability is a key factor in surface engineering for improved biomaterials. In particular, an effective strategy for implant surface chemistry design has been to maximize the hydrophilicity of implant surfaces to tightly bind layers of water in place of potential proteins. This also orients the tightly bound water molecules towards the biological environment so blood proteins see no significant difference between the blood and the implant surface, effectively camouflaging the implant device.

For this reason but also because of their mechanical strength, common biomaterials used for today's implants include hydrophilic metal-oxides such as titanium, cobalt, chromium, and some stainless steels. Where softer plastics and gels are used, hydrophilic polymers such as polyethylene glycol (where oxygen in the carbon backbone enhances polarity and water affinity) and even protein coatings such as heparin are used to prevent aggregation.

Lastly, a major factor in implant design is concerned about the biomaterial surface topography and total surface area of the exposed material. As seen in Fig. 7, roughness and porosity play key roles in bioactivity. In general, smooth surfaces present less surface area of the material chemistry to the environment and also maintain a lower surface energy. Metallic grain boundaries and porosity often introduce increased densities of unsatisfied bonding which increase the overall surface energy and interaction with the environment. Rough surfaces also tend to obstruct flow patterns in the bloodstream, increasing the likelihood of biological agents in contacting the foreign surface due to the generation of flow turbulences. These properties, particularly the surface energy and resulting roughness, play significant roles in the performance when considering a bio-interface.

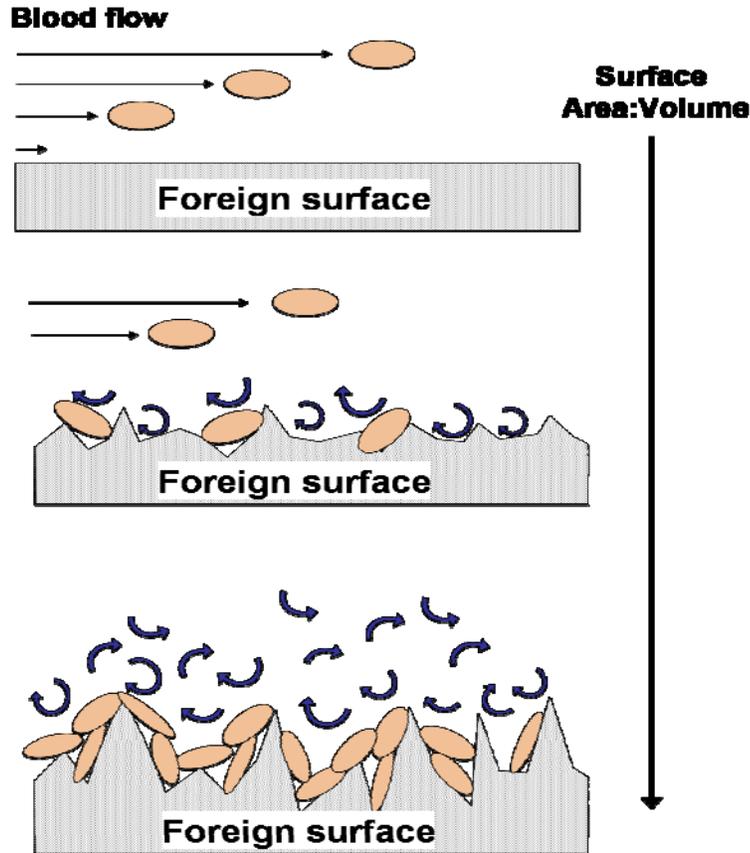


Figure 7. Effects of surface roughness on blood flow as well as surface area to volume ratio, both enhancing cell adhesion probabilities.

A material that is often used for prosthetic heart valves is pyrolytic carbon because of its enhanced properties of toughness and apparent bio-inertness to high amounts of blood flow. Due to its large and atomically flat surface, graphite remains inert both because of its relatively low surface energy and also little disruption to the bloodstream flow. From previous clinical study[2], an as-deposited layer of pyrolite brand carbon is significantly rough and is observed to elicit a decrease in thromboresistance (the tendency to resist blood clotting). The polished version, on the other hand, is commonly used in implants today with low levels of inflammation and bio-response. These observations confirm the previously discussed principles of implant material design, where topography and surface energy play key roles in bio-inertness. While the failure rate is moderately low for these carbon prostheses, many cases of patient rejection still occur. This is because proteins and cofactors still have some affinity towards a graphitic surface, as illustrated in Figure 8.

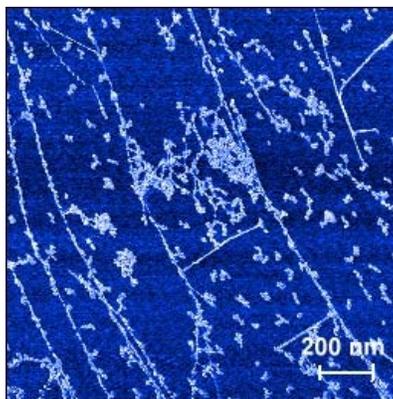


Figure 8. Fibrinogen adsorbed on highly oriented pyrolytic graphite (HOPG) visualized by intermittent non-contact SFM.

Characterization of Adsorption Processes

Surface adsorption processes are manifold depending on variables such as solute concentrations, pressures, temperatures, and deposition environments. Furthermore, the bonding mechanism is influenced by the level of molecular and surface interactions, surface diffusion, and local and integral substrate properties and morphologies.

A straightforward molecular model for adsorption is the Langmuir model, developed by Irving Langmuir in 1916. It describes the dependence of the surface coverage of an adsorbed inert gas on the pressure (or partial pressure) of the gas above the surface at a fixed temperature (isothermal state). While this so-called *Langmuir Isotherm* provides one of the simplest models, it offers a good starting point towards a molecular understanding of adsorption processes. Although developed for non-interacting simple gases, it is extensively employed to analyze macromolecular adsorption processes in biology.

With the Langmuir model we assume the following:

1. All surface sites have the same activity for adsorption.
2. There is no interaction between adsorbed molecules.
3. All of the adsorption occurs by the same mechanism (e.g., physisorption or chemisorption), and each adsorbent complex has the same structure.
4. The extent of adsorption is no more than one monolayer.

To illustrate the model, we shall assume a surface with a fixed number of active adsorption sites for molecule A that is exposed to a gas containing A . If we define θ as the fraction of surface sites covered by adsorbed molecules then $(1 - \theta)$ is the fraction of surface sites that are still active; i.e., not bound to A . Depending on the gas, we will use either the gas pressure P for a monomolecular gas, or the partial pressure p_A for a multicomponent gas mixture. We can expect that with increasing gas pressure, the rate with which the surface is covered will increase linearly, according to:

$$r_a = k_a p_A (1 - \theta). \quad (1)$$

Thereby, we assumed dealing with a gas mixture and implied that the rate of adsorption can be expressed in the same manner as any kinetic process with a kinetic order of one. In other words, the adsorption rate is linearly expressed with the applied partial pressure, according to $r_a = k_a p_A$ via the adsorption rate constant k_a . Analogous, we express the desorption rate constant as

$$r_d = k_d \theta, \quad (2)$$

where r_a is the desorption rate and k_d is the rate constant for desorption. k_a and k_d are determined from kinetic experiments

At equilibrium, we can equate Eqs (1) and (2), which yields

$$\frac{\theta^*}{1-\theta^*} = \frac{k_a}{k_d} p_A. \quad (3)$$

Thereby, θ^* defines the equilibrium fractional surface coverage. Substituting the rate constant ratio k_a/k_d with the binding equilibrium constant K_a (also referred to as equilibrium association constant), the fractional surface coverage can be expressed as

$$\theta^* = \frac{K_a p_A}{1 + K_a p_A}, \quad (4)$$

which is the analytical expression for the *Langmuir Isotherm*. For a typical equilibrium experiment, adsorption data are gathered over a range of partial pressures, and final coverages are plotted with respect to concentration as illustrated in Fig. 9. Equation (4) yields for the equilibrium association constant

$$K_a = \frac{\theta^*}{(1-\theta^*) p_A}. \quad (5)$$

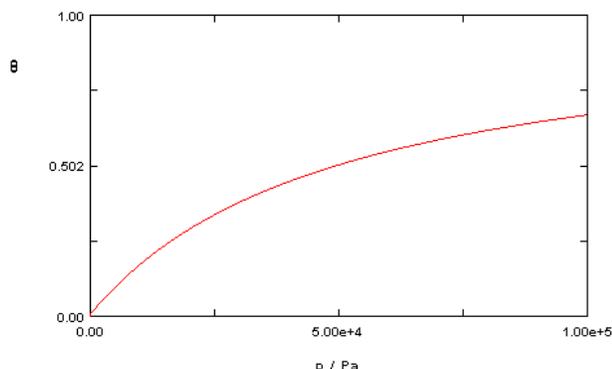


Figure 9. *Langmuir Isotherm* yielding an equilibrium binding constant K_a of $2 \times 10^5 \text{ Pa}^{-1}$.

Accordingly, it is common to analyze adsorptions from solutions with the Langmuir model, expressing θ^* as:

$$\theta^* = \frac{K_a \times [X] \times [S]}{[S] + K_a \times [X] \times [S]} = \frac{K_a \times [X]}{1 + K_a \times [X]}. \quad (6)$$

Thereby, we considered the reaction



$[X]$ and $[S]$ representing the solute (e.g., protein) concentration and the substrate immobilized active site concentration, respectively. $[XS]$ is the compound concentration at the surface. The equilibrium constant for association K_a and dissociation K_d are related via $K_a = [XS]/[X][S] = 1/K_d$. Note, the equilibrium constants results from both (i) the protein-surface interaction, and (ii) the protein-surface interaction with the buffer solution (solvent). Thus, instead of the partial pressure, it is the free adsorbate concentration $[X]$, which we assume to be constant that is the variable parameter in the *Langmuir Isotherm*. The *Langmuir Isotherm*

provides the equilibrium constant K_a , from which the standard free energy of adsorption $\Delta G = -RT \ln(K_a)$ can be determined.

Under transient (non-equilibrium) conditions, where the coverage is changing over time, we can express the change in the fractional surface coverage in terms of the reaction and desorption coefficient as

$$\frac{d\theta}{dt} = r_A - r_d = k_a p_A (1 - \theta) - k_d \theta. \quad (8)$$

After integration, the transient fractional surface coverage is given as

$$\theta(t) = \theta^* [1 - e^{-t/\tau}], \quad (9)$$

where θ^* is the equilibrium fractional surface coverage provided in Eq. (4), and

$$\tau \equiv \frac{1}{k_a p_A + k_d} \quad (10)$$

is the process relaxation time. $k_a p_A + k_d$ reflects the observed rate constant. Equation (10) is used to fit the data collected in time-varied experiments. A time-dependant illustration of a Langmuir adsorption process is illustrated in Figure 10. Here, the surface is initially unoccupied and undergoes a rapid population until sites are screened (sterically) from the free particles above, resulting in a slow saturation towards the equilibrium surface coverage asymptote.

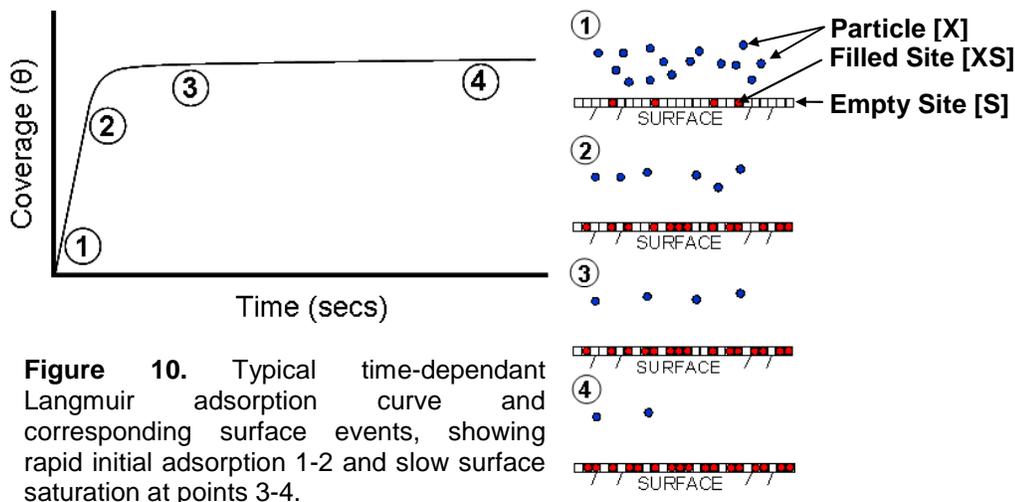


Figure 10. Typical time-dependant Langmuir adsorption curve and corresponding surface events, showing rapid initial adsorption 1-2 and slow surface saturation at points 3-4.

Although the Langmuir equation and its derivatives provide a method to quantify the interaction strengths of inert molecular adsorption processes, it holds certain limitations that restrict its applicability. Proteins, in particular, have been shown to possess unique aggregation mechanisms at solid interfaces exhibiting adsorption curves that largely deviate from Langmuir. Also surface imperfections, with preferential sites for adsorption as on stepped graphite surfaces (see Fig. 8) modify the adsorption kinetics. Thus, the *Langmuir Isotherm* method is to be understood as a primer to building a foundation for further exploration of peculiar adsorption mechanisms, or as an initial assessment of general affinity without regard for specific interaction mechanisms.

Artificial Nose or Biosensor

The Langmuir adsorption isotherm provides a useful foundation for understanding a variety of applications. One such application is a novel scanning force microscopy (SFM) tool known as the “artificial nose” that has also found applications as a biosensor.⁴ With this tool, molecular concentrations on the picomolar scale can be “rapidly” sensed. *Briefly*, the working principle is as follows: An array of free-standing cantilevers that are coated or “functionalized” for sensitivity to adsorption of molecules (see Figure 10a) is exposed to either a gas or a buffer solution, respectively. While cantilever material coatings, typically polymers, serve as adsorption (more precisely absorption) membranes for gaseous solutes, chemical functional materials act as adsorption sites (receptors) for liquid buffer dissolved solutes, Fig. 10a. Due to the single side coating and adsorption process of the cantilever probes, the cantilevers will be asymmetrically strained, which leads them to bend, Fig. 10b. This degree of bending is captured by the laser beam deflection scheme of the SFM, as illustrated in Figure 10.

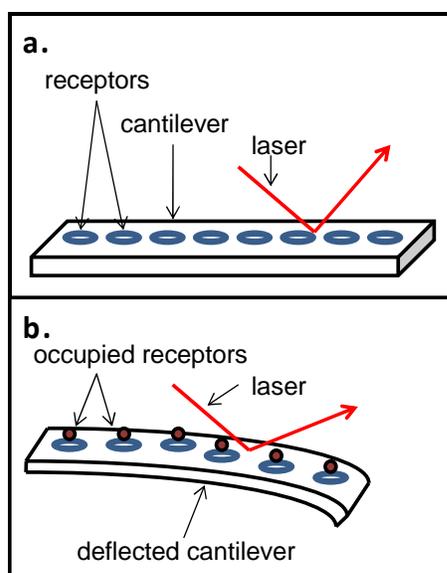


Figure 10. Working principle of a functionalized SPM biosensor; (a) before and (b) after adsorption of bio-molecules.

With Stoney’s formula applied to a cantilever beam,⁵ the tensile surface stress⁶ σ acting on the lever can be related to the cantilever properties and normal deflection Δz , as:

$$\sigma = \frac{4}{3} \left(\frac{L}{W} \right) \left(\frac{\Delta z}{D} \right) \frac{k_N}{(1-\nu)} = \frac{4}{3} \left(\frac{L}{W} \right) \frac{\Delta F_N}{D(1-\nu)} = \theta \sigma^* \quad (11)$$

where L , W and D are the lever size dimensions (length, width and thickness), k_N and ν are the cantilever normal spring constant and Poisson’s ratio, respectively, and $\Delta F_N = k_N \Delta z$ is the normal force acting on the lever. Thereby, changes in σ can be assumed to be directly proportional to changes in the fractional surface coverage θ with $\theta = \sigma / \sigma^*$, where σ^* is the equilibrium stress imposed by the adsorbed film for infinite exposure time. Surface stresses imposed by a monolayer adsorption of macromolecules such as proteins are on the order of tens of dyne/cm (10^{-3} N/m). This translates according to Eq. (11) to ~ 10 nN normal cantilever deflection forces for a Poisson’s ratio of 0.23 (silicon lever), and cantilever dimensions (L , W , D) of 100 10 and 0.1 μm , respectively.

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5. *Nanoscience – Friction and Rheology on the Nanometer Scale*, Meyer, E., Overney, R.M., Dransfeld, K., Gyalog, T., World Scientific (New Jersey) 1998, p. 186.
6. For an adsorbed film of thickness t_F , the surface tensile stress σ [N/m] is related to the stress in the adsorbed film along the cantilever beam σ_F [Pa = N/m²] via $\sigma = \sigma_F / t_F$.

5. Appendix

Simple Harmonic Motion

Having covered the fundamentals of the materials in question in this lab we now move to the operation of the scanning probe microscope itself. For this lab we will be using ‘dynamic’ or ‘AC’ mode imaging, to prevent the tip from damaging the protein as it scans. We will use this method both in air and in liquid (phosphate buffer solution, PBS) to measure protein adsorption behavior in situ (PBS) and ex situ (air).

The SPM tip sits at the end of a long, flexible cantilever. This cantilever is flexible and behaves like a spring: if the tip is pushed in one direction the cantilever exerts a force in the opposite direction in an attempt to restore the tip to its original position. Since we are going to be examining the motion of the tip in more detail, we first define some parameters:

$z(t)$	position of the tip as a function of time
F	force exerted on the tip
m	mass of the tip
k	effective spring constant of the cantilever

Remember that the velocity $v(t)$ and acceleration $a(t)$ of the tip are related to its position $z(t)$ through the following derivatives:

$$v(t) = \frac{dz}{dt} \quad a(t) = \frac{dv}{dt} = \frac{d^2z}{dt^2} \quad (1a,b)$$

Newton’s third law ($F = ma$) relates the forces on the tip to its motion. We already mentioned that the cantilever behaves much like a spring, and will we approximate the restoring force using Hooke’s law relating spring constants and restoring forces ($F = -kz$). Combining these two equations gives us the following equation of motion:

$$F = ma = -kz$$

$$m \frac{d^2z}{dt^2} = -kz \quad (2a, b)$$

The tip and cantilever are real materials moving through air, so the motion is also damped by both air friction (or water friction in fluid mode) and by losses in the spring. These losses are both approximately proportional to the velocity and so we modify our equation of motion with a “drag force” or damping term $-bv$:

$$F = ma = -kz - bv$$

$$m \frac{d^2z}{dt^2} = -kz - b \frac{dz}{dt} \quad (3a, b)$$

Rearranging a bit, we can write this as a differential equation describing basic motion of the tip far away from any substrate (and still without any driving force yet either)

$$m \frac{d^2 z}{dt^2} + b \frac{dz}{dt} + kz = 0 \quad (4a,b)$$

$$\frac{d^2 z}{dt^2} + \beta \frac{dz}{dt} + \omega_0^2 z = 0$$

where $\omega_0^2 \equiv k/m$ and $\beta \equiv b/m$. ω_0 is the natural frequency of the cantilever.

Most non-contact/intermittent contact SPM is performed while applying a sinusoidally oscillating drive force on the tip, so we must also add the driving force to our equation of motion:

$$\frac{d^2 z}{dt^2} + \beta \frac{dz}{dt} + \omega_0^2 z = D \cos \omega t \quad (5)$$

This equation may look familiar. It is the classic equation for the damped and driven simple harmonic oscillator. The solution to this equation is a steady-state motion of the system should oscillate with the driving force, plus a potential phase shift δ . You can check that the solution has the form:

$$z(t) = A \cos(\omega t - \delta) \quad (6)$$

where:

$$\delta = \tan^{-1} \left(\frac{\omega \beta}{\omega_0^2 - \omega^2} \right) \quad (7)$$

The amplitude, A , of oscillation is:

$$A = \frac{D}{\sqrt{(\omega_0^2 - \omega^2)^2 + \omega^2 \beta^2}} \quad (8)$$

The “resonance frequency” of the oscillator is defined as the driving frequency at which A is maximized. If the damping is small, the amplitude is at a maximum when the driving frequency equals the natural frequency ω_0 . The amount of damping in a simple harmonic oscillator is commonly characterized by the quality factor, Q . Q is defined as the resonance frequency divided by β , and is a measure of the total energy stored in the oscillator divided by the energy lost per period of oscillation. For systems that are only weakly damped (like an SFM tip vibrating in air), a practical method of determining Q is to divide the resonance frequency by the width of the resonance peak (where the width is taken at the points where the amplitude is equal to $1/\sqrt{2} \approx 0.707$ of the maximum). In the case of liquid imaging, the resonance frequency changes substantially as the system is not only further damped, but also must move a larger mass (including liquid) during the oscillation process.

AC-Mode Imaging

In a common form of topography imaging, called intermittent-contact mode (or a variation called “Tapping Mode™”, or “dynamic mode” by some manufacturers), the SFM is driven at a

frequency close to the resonant frequency of the tip. When the tip comes close to the surface, it interacts with the surface through short range forces such as van der Waals forces. These additional interactions change the resonance frequency of the tip, thereby changing the amplitude of oscillation and its phase lag. Typically, an image is formed using a feedback loop to keep the oscillation amplitude constant by varying the tip sample distance with the z-piezo (see Fig. 10 below). By plotting the z-piezo signal as a function of position it is then possible to generate an image of the height of features on the surface. It is possible to image in both the attractive, and the repulsive regions of the van der Waals potential, and strictly speaking this divides the classification of AC-Mode imaging techniques into “non-contact” and “intermittent contact” SFM respectively). However, intermittent contact AC-mode imaging is more common for routine imaging.

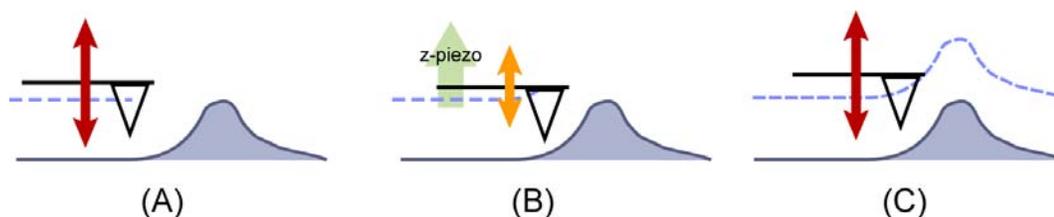


Figure 10. Schematic for intermittent-contact mode SFM. (A) The SFM tip is driven near its natural resonance frequency to obtain a target amplitude of oscillation. (B) As the tip approaches topography changes, the increasing van der Waals forces shift the resonance frequency, which causes the tip's oscillation amplitude to decrease. In response, the Z-piezo lifts the tip away from the surface so (C) the original oscillation amplitude is reestablished. By tracking the Z-motion of the tip, we obtain the measured topography of the surface (dashed line).

Of course, the material properties of the sample affect tip-surface interactions, so the topography image obtained by AC-mode imaging isn't perfectly free of artifacts (some imaging modes even exploit differences in elastic properties of the surface to differentiate materials). Nevertheless, AC-mode imaging is much gentler and can be used to image a wider variety of soft samples than contact mode imaging.