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First page image caption : The surfaces of malaria infected red blood cells exhibit knob like structures responsible for cytoadherence. This overlayed image of simultaneous topography (red color coded) and CD36 binding site mapping (gold color coded) on the surface of human malaria infected erythrocytes using PeakForce QNM in fluid clearly shows that the specific CD36 binding sites locate precisely on the knobs where they have higher chance to contact and form intermolecular bonds with endothelial receptors. The image is captured at 2um x 2um scan size, taken by Ang Li, from Singapore-MIT Alliance for Research and Technology in Singapore.

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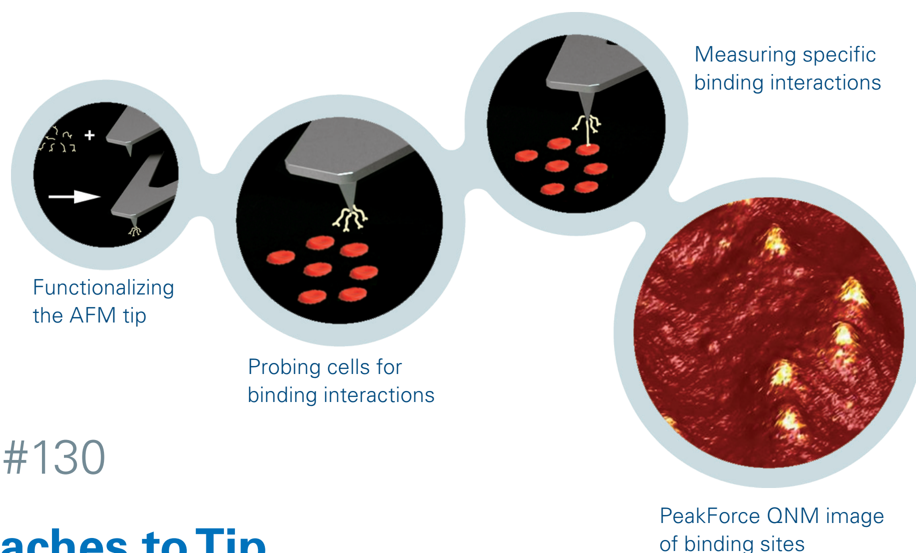

Application Note #130

Common Approaches to Tip Functionalization for AFM-Based Molecular Recognition Measurements

Though most often considered as a nanoscale imaging technique, atomic force microscopy (AFM) is also uniquely well suited to measuring force interactions at the piconewton and nanonewton scale. This capability has been widely used to measure the nanomechanical properties of materials ranging from polymers to living cells and even to characterize single molecules. These measurements are normally made using common, unmodified AFM probes. However, another type of measurement is made possible by the chemical functionalization of AFM probes. This enables the interaction between two specific molecules to be investigated. Most commonly, these molecules are biomolecules and the interactions measured are specific binding interactions, for instance between an antibody and an antigen. Such interactions are critical to life and it's known that many diseases have their cause at the molecular level, sometimes resulting in a malfunctioning of molecular recognition and directly impacting cell behavior. Thus, understanding the specific molecular forces between individual receptors and ligands can be of great relevance in biomedical research.[1-10] AFM force measurements can be used to estimate the specific unbinding forces between such molecules with piconewton accuracy by attaching one of the molecules (referred to as the ligand) to the AFM tip and probing corresponding receptors on surfaces of interest. This technical note reviews common approaches to functionalizing AFM tips for this type of research.

Principles of molecular recognition measurements

AFM molecular recognition measurements are based on the interaction between two molecules: one attached to the AFM tip and the other bound to the surface of interest (groups A and B respectively in figure 1). What we refer to as tip functionalization are all the chemical steps that lead to the fixation of molecules A to the AFM tip. The right half of figure 1 shows a typical approach/retract cycle, also called a force-distance curve. First the tip is brought down to the surface until it contacts and exerts a positive load on the surface (blue trace). Then the tip is retracted and moves back to its original position (red trace). During this retraction, a downward peak may occur in the retraction curve that indicates adhesion between the tip and the sample. If the spring constant (in N/m) and the deflection sensitivity (in nm/V) of the cantilever are known, one can calculate the maximum tip-sample adhesion (in N).

Tip-sample adhesion is often observed when using "bare" (non-functionalized) tips, though in this case it corresponds to a non-specific interaction. When the tip is functionalized for molecular recognition measurements one challenge becomes the ability to distinguish between these non-specific interactions and the desired specific interaction. For this reason it is common to use intermediate molecules called spacers or linkers between the tip and molecule A, which have a key role in helping to enable and recognize

specific interactions. The greater flexibility of the linker provides the ligand molecule some mobility to best access the binding receptor. It also introduces a characteristic curved adhesion peak (see inset force curve in figure 1) due to entropic stretching of the linker molecule, which helps distinguish the desired specific interactions from non-specific interactions.

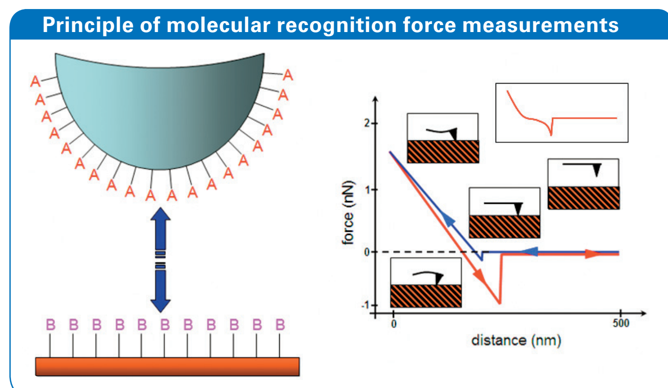

Figure 1: The AFM tip is extended toward and then retracted from the surface as the deflection of the cantilever is monitored as a function of distance. The retract part of the curve (in red) will show any adhesion force between the tip and the sample. In molecular recognition force measurements, ligand molecules (A) are attached to the AFM tip, whereas receptor molecules (B) are present on the sample surface. Use of a linker molecule (e.g. PEG) results in a characteristic curved unbinding peak as the linker stretches, enabling easier identification of specific unbinding interactions between A and B (see representative curve in inset).

Overview of tip functionalization strategies

Though many different approaches have been used to attach molecules to AFM probes, several basic issues must always be considered:

1) One must choose an appropriate AFM probe. Usually the most important consideration is the spring constant of the cantilever, but sometimes tip sharpness is also important. Molecular binding interactions are usually quite small, so typically very soft cantilevers are used ($k < 0.1$ N/m). If the interaction is to be measured quantitatively, the spring constant must be calibrated using an appropriate method.[11]

2) One must select the tip functionalization chemistry, keeping in mind that the ligand molecule must be attached to the tip such that the binding strength between it and the tip is greater than the interaction between the ligand and the surface receptor. Covalent binding or strong chemisorption (e.g. gold-thiol) approaches are typically used to ensure that this condition is met.

3) In cases where it is desired to measure single binding events, techniques should be considered that limit the surface density of the ligand on the tip. These approaches can reduce the occurrence of multiple molecular interactions during a single tip-sample interaction.

4) One should consider the use of a linker or spacer molecule for the benefits it can provide in promoting and recognizing specific binding events.

5) One must ensure that environmental factors (buffer composition, pH, temperature) during tip functionalization and the measurement itself are appropriate, such that the binding molecules maintain their binding activity.

This technical note will focus on the options available for the tip functionalization itself (i.e., issues 2–4).

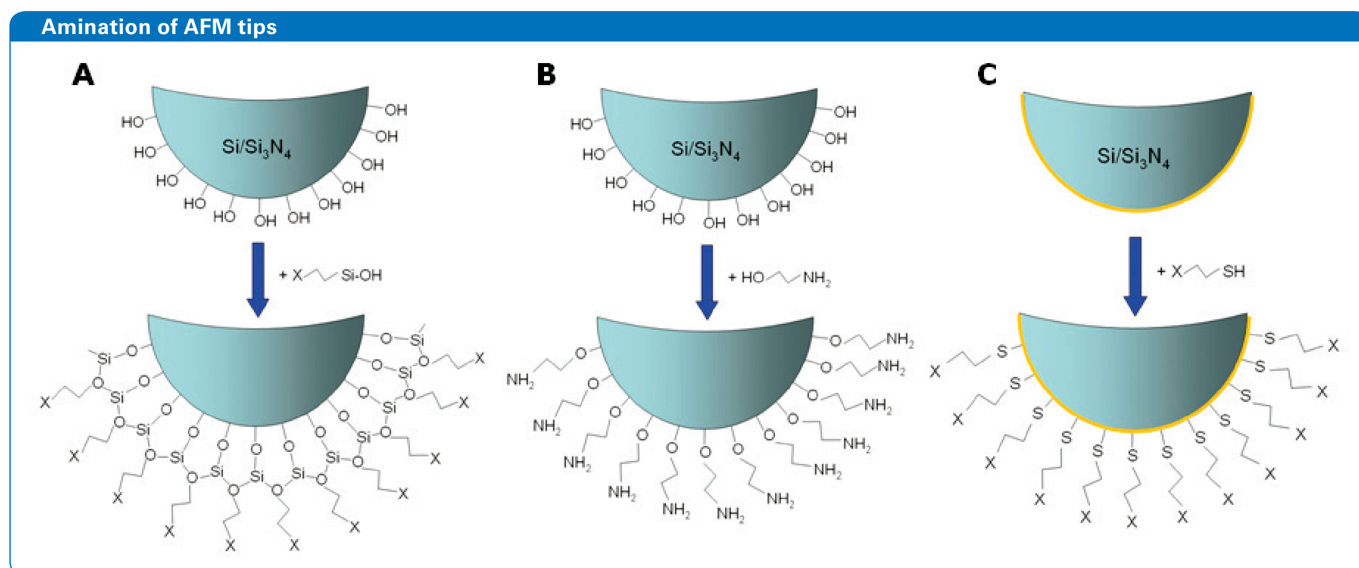

Figure 2: The first step of tip functionalization is generally to introduce amine groups (shown here as "X") to the tip surface. Three methods are widely used: A) treatment with silanes; B) esterification with ethanolamine; and C) formation of a SAM using thiol-gold chemistry.

Choosing a starting point for tip functionalization

Tip functionalization is almost always a multi-step process, but fundamentally it always starts with a silicon or silicon nitride tip on an AFM probe. From this point two general approaches appear most commonly in the literature: 1) direct amination of the tip by silanization or esterification, or 2) amination via a thiol-based self-assembled monolayer (SAM) on a gold-coated tip. These approaches are depicted schematically in figure 2.

The silanization and esterification approaches offer the advantage that the probe can be functionalized directly, or at least after simple cleaning of the tip surface, without other prior surface preparation. The silanization reaction occurs between silanol groups on the AFM tip and a trichlorosilane group in the silane reagent, which lead to the formation of an organosilane layer.[12] This results in Si-O-Si covalent bonds between the tip and silane molecules and hydrogen bonding interactions between the aliphatic chains of the silane molecules (figure 2A). In this figure "X" represents a reactive group (e.g., most commonly amino, NH_2) that will be used for the next step of the functionalization. Note that it is also possible to modify this terminal group after the formation of the SAM, for example, converting a vinyl groups to hydroxyl groups.[13] Alternatively, amination may be performed via esterification by reaction of surface silanol groups with ethanolamine [$HO-(CH_2)_2-NH_2$] (figure 2B).[14]

Though one of the most commonly used reagents for amination of the tip is APTES (3-aminopropyl triethoxysilane)[15], some work suggests that esterification with ethanolamine or an alternative silane, aminophenyl-trimethoxysilane (APhS), may lead a slightly higher density of amino groups than APTES treatment.[16] Though used effectively in many examples, the silanization reactions can be difficult to control reproducibly, in part because the silane reagents themselves are sensitive to contamination, easily crosslink, and can have a short shelf life. This can lead to wide variations in the surface density of the reactive functionalization groups, which is particularly undesirable when the experimental strategy calls for close control of this parameter in order to favor single-molecule interactions. In this regard the ethanolamine esterification approach is preferred.

The alternative approach is formation of a SAM via adsorption of alkanethiol molecules to a gold coated tip (figure 2C). This strategy presents three main advantages. First, gold is a rather inert metal and gold-coated probes can be easily recycled by removing all attached molecules (e.g., with detergents and UV-ozone treatments). Second, thiol groups have a high affinity with gold, forming strong bonds that help ensure that the tip-ligand interaction is stronger than the ligand-receptor interaction (i.e. issue #2 above). Third, the acyl chains of the SAM form a very dense, close-packed structure that rigidifies it and increases the robustness of the tip functionalization. A notable disadvantage of this technique is that it requires tip-side gold coated AFM probes, which are not universally available commercially and have larger tip radii due to the gold layer.

Certain approaches have been found to assist in the formation of reproducible, robust alkanethiol SAMs. Though both sputtering and thermal evaporation can produce thin layers of gold (generally less than 100nm), it is known that the roughness of the gold layer will directly impact formation and quality of the thiol SAM.[17] Thermal evaporation followed by carefully controlled annealing is known to produce atomically flat gold crystals (terraces) whereas sputtering or similar techniques will produce larger, rougher aggregates. Note that another thin metal layer between the gold layer and the silicon or silicon nitride tip is also required for the gold to adhere, for which chromium is most often used.[18] The choice of alkanethiol molecule is also important, where it is recommended to use chain lengths between 10 and 18 carbon atoms to enhance hydrogen bonding and thus stability.[19] The speed at which SAMs form is highly dependent on the solvent used, the temperature, and the length of the acyl chains (where longer chains assemble faster than short ones).

It should be noted that both of these approaches have been used successfully in many molecular recognition studies. In addition to the considerations already presented, one path or the other may be preferred due to your expertise or the availability of the necessary reagents and equipment. Whichever approach is chosen, these initial steps are crucial for preparing the tip for the following steps that complete the functionalization a linker molecule and the desired ligand.

Attaching flexible linker molecules to the tip

Though it is possible to attach proteins directly to the tip at this point using the reactions discussed in the next section, it is generally recommended to first introduce linker molecules. In addition to the advantages already discussed regarding promotion and recognition of specific binding events, this stage of the functionalization process also presents the opportunity to more systematically control the eventual surface density of the ligand molecules. This can be accomplished by using a mixed SAM containing two different molecules with different terminal groups. For instance, a SAM containing 99% of molecule HS-R-X' and 1% of molecule HS-R-X, where X represents a reactive chemical group able to bind to the ligand and X' represents an inert terminal group. This approach is commonly used, for instance by Vansco et al. to study the interaction between ferrocene and cyclodextrin molecules by using a mixed SAM made primarily of 2-mercaptoethanol with a very low percentage of thiol-modified ferrocene.[20]

Options at this stage depend somewhat on the initial approach taken to treating the tip. If the gold-thiol SAM approach is chosen, then it is possible with the appropriate reagents to directly incorporate linker molecules at the same time as formation of the SAM. One possibility is to use a PEG (Polyethylene glycol) / NTA (N-nitrilotriacetic acid) strategy.[21] This strategy can be combined with use of mixed SAMs to control ligand surface density, for instance in an example by Dufrene et al.[22] In this example, the majority (typically 90 to 95%) of the SAM was comprised of a triethylene-glycol-alkyl-thiol [PEG-thiol] and the remainder was a NTA-triethylene-glycol-alkyl-thiol [NTA-PEG-thiol] (see figure 3). The tetradentate NTA can form a hexagonal complex with metal cations like nickel. Four chelation bonds are established with Ni^{2+} whereas the two remaining ones can be used to target histidine groups (see figure 3) belonging to poly-His-modified proteins (i.e., the ligand). Thus only this low percentage of NTA-PEG-thiol binds the ligand whereas the PEG-thiol remains inert, thereby limiting the density of proteins on the tip surface. In the Dufrene et al. example this approach was used successfully to study the specificity of interaction between an Fv fragment of anti-lysozyme antibodies and lysozyme molecules attached to a plane support.[22]

Following the other approach, where the native silicon or silicon nitride tip is amino-functionalized with silanes or ethanoloamine, the strategy is slightly different. In this case one end of the PEG linker molecule must react with the surface amino groups, leaving the other end free to bind to the protein of interest. These PEG molecules are commonly referred to as heterobifunctional PEG linkers.[23,24]

Many companies offer a wide range of heterobifunctional PEG linkers.[25] Detailing all the possible reactive group functionalities and their binding targets is not possible here, but some of the most commonly encountered ones are summarized in Table 1 and shown schematically in figure 4.

Because the tip is amino-functionalized, one end of the heterobifunctional PEG linkers should generally have an NHS-ester reactive group. The reactive group on the other end of the PEG linker should be chosen based on the desired ligand binding chemistry options, described in the next section.

The optimal length of PEG linkers is still debated. Though it's generally accepted that the PEG linker should not be less than 12 carbons, it has been demonstrated very long chains (>35nm) can cause the binding probability to significantly decrease.[26] The chosen length may be a compromise forced by the limited selection of commercially available PEG linkers

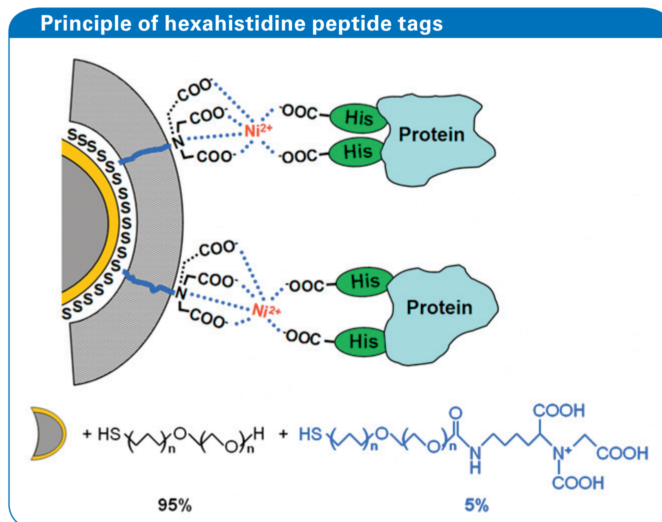

Figure 3: Mixed SAMs are formed on a gold-coated tip. Only a very low percentage of so-called NTA-terminated alkanethiols will establish a chelation with cations, which will also interact with polyhistidine groups belonging to peptides or proteins.

Attaching proteins and other ligands

The final step of the tip functionalization is completed by reaction of the reactive terminal group (X) with amino acids within the desired protein or other ligand. Care should be taken, where possible, to avoid targeting known points within the functional binding site of the ligand in order to prevent changes in its activity or function. The availability of reactive amino acids within the protein can be modified by site directed mutagenesis or direct modification of the protein with various reagents, but again, none of them should be involved in the binding site.

A simple strategy for quick tip functionalization

It is possible to avoid these more complicated functionalization steps by using the so-called "glutaraldehyde strategy." First, the AFM probes must be NH_2 activated as previously explained. Then they are treated with glutaraldehyde, which reacts with the amine groups. The tips are then exposed to the desired ligand, which binds to the remaining free end of the glutaraldehyde molecule (figure 5). The main drawback of this strategy is that there is no way to control the density of proteins on the tip surface and that the protein attachment is done randomly. Thus only a random percentage of ligands will be oriented in such a way that they can interact with their corresponding receptors. The main benefits are the simplicity and generality of the method. AFM probes can be functionalized in half an hour and have been used to generate excellent results.[27-29]

Table 1. Common binding targets and matching reactive groups

Binding target	Reactive group on PEG	Bond formed
–COOH (carbonxyl) *found in:* aspartate glutamate	amine *(reaction requires activation with EDC)* *or* hydroxyl	amide *or* ester
–NH$_2$ (amine) *found in:* lysine silane treated tip ethanolamine treated tip	NHS –ester *or* carboxyl	amide *or* ester
–SH (sulfhydryl) *found in:* cysteine	maleimide *or* carboxyl	thio –ether *or* thio –ester
–CHO (carbonyl) *found in:* oxidized carbohydrates	hydrazide	hydrazone
–OH (hydroxyl) *found in:* serine threonine	carboxyl	ester
Avidin *found in:* avidin modified proteins	biotin	avitin-biotin bond

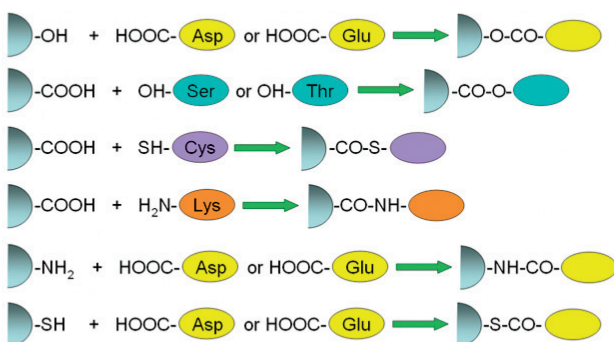

Figure 4: Typical reactions between chemically modified AFM tips and some amino acids (Asp = Aspartate, Glu = Glutamate, Ser = Serine, Thr = Threonine, Cys = Cysteine and Lys = Lysine).

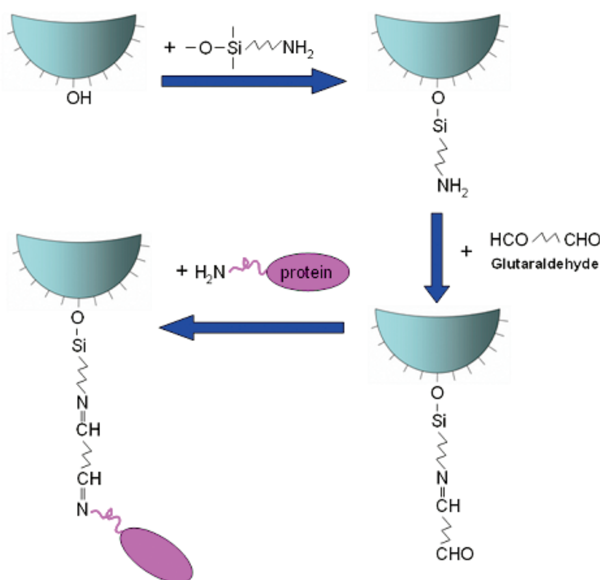

Figure 5: After silanization of the AFM tips, glutaraldehyde is used as a binding agent to attach proteins of interest to the tip. This strategy doesn't guarantee any specificity of interaction since any amino moiety of the protein can randomly be used for the covalent bond, but it does offer the advantage of being extremely easy to apply, with the entire functionalization occurring in no more than 30 minutes.

Conclusions

This technical note reviewed the major strategies and applications of tip functionalization. Though the discussion of existing techniques here is not exhaustive, it should provide a good start for those getting started with chemically modified AFM probes and molecular recognition experiments. The methods described have been applied widely for single point force measurements and force-volume molecular recognition mapping. Preliminary results suggest that tip functionalization will also be useful when used with the PeakForce QNM imaging mode, which should enable faster, higher resolution, and more quantitative mapping of molecular interactions.