Acceleration of coupling by pre-adsorption

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Acceleration of covalent ligand coupling to the tip by pre-adsorption

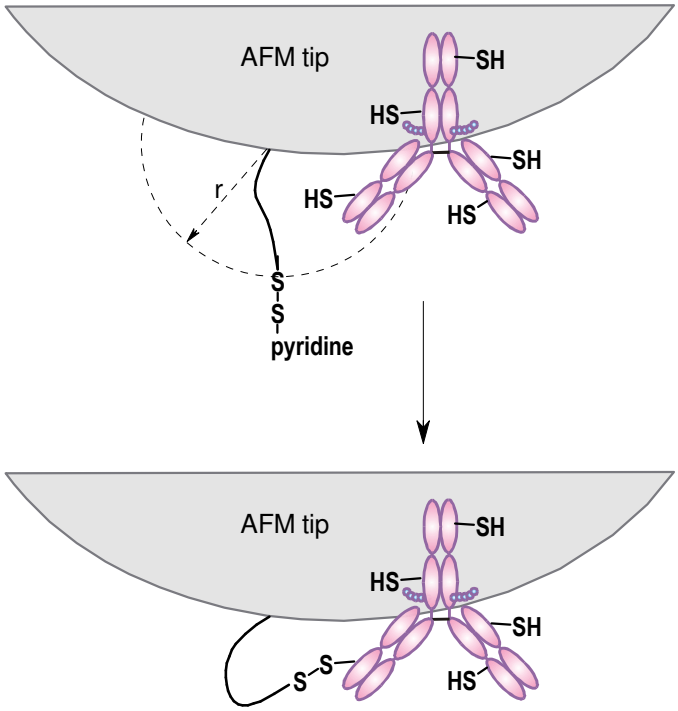

Figure 1: Model for acceleration of antibody linking to tip-bound pyridyl-S-S-PEG tentacles, due to pre-adsorption of protein next to the PEG linker. The free end of a 6 nm long, surface bound PEG linker can reach all locations in a hemisphere with r = 6 nm. One particle per hemisphere (450 nm^3) corresponds to a formal concentration of 3 mM [Kamruzzahan et al., 2006]. Tip radius, antibody dimensions, and PEG length were roughly drawn to scale.

Figure 1 shows our original version of AFM tip functionalization with antibody molecules [Hinterdorfer et al., 1996]. The tip was equipped with polyethylene glycol chains (PEG) which carried a reactive disulfide (S-S-pyridine) at its free end. In parallel, about 5-7 SH groups were generated on the antibody surface. When the tip was incubated with 1 µM of this antibody, coupling was achieved on the time scale of 1 h.

In the coupling step, the S-pyridine group at the free end of the PEG linker is replaced by HS-antibody. The released HS-pyridine isomerizes into 2-thiopyridone which has a UV absorption maximum at 343 nm (see Figure 2). We wanted to estimate the speed of the coupling reaction under various conditions which mimicked the situation on the AFM tip more or less closely [Kamruzzahan et al., 2006].

In the spectrophotometer, we had to use high concentrations of the reactants, for two reasons: First, the molar absorptivity of thiopyridone is not very high (ε_{343} = 8080 M^{-1} cm^{-1}), therefore 10-100 µM thiopyridone must be released to reach absorbance values between 0.1 and 1.0. Second, the kinetics turned out to be relatively slow if the concentrations of the reactants were in the low micromolar range. In the desired concentration range (10-100 µM) the experiments would have been very costly when using antibody molecules. We, therefore, replaced the

antibody by bovine serum albumine (BSA) which had also been decorated with several SH groups.

In the cuvette, the AFM tip had to be omitted for obvious reasons. However, the PEG linker (symbolized by the dashed line - - - - - - in Figure 2) was included in the reaction, and the "immobilization" of the PEG linker on the AFM tip (Figure 2A) was additionally simulated by attaching the PEG linker to BSA (Figure 2B). Like the AFM tip, the PEG-bound BSA molecule also slowed the thermal motion of the PEG, as compared to a free PEG chain.

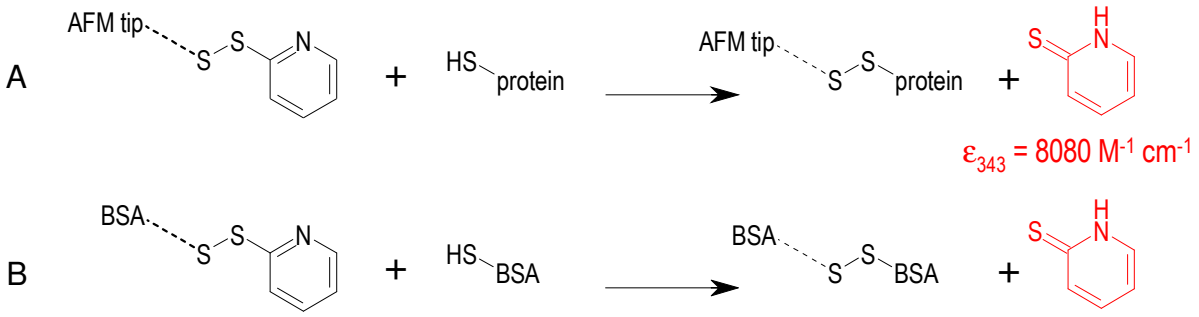

Figure 2: Mimicking of AFM tip functionalization (A) by a similar reaction in homogeneous solution (B), and monitoring of the coupling kinetics in the cuvette of a UV-vis spectrometer [Kamruzzahan et al., 2006]. In the coupling step, the S-pyridine part in tip-PEG-S-S-pyridine is replaced HS-protein. The released HS-pyridine isomerizes into 2-pyridone which has an absorption maximum at 343 nm. Consequently, the progress of the reaction can be monitored by the UV absorbance at 343 nm.

The kinetic data gave a bimolecular kinetic constant of ~1.6 M^{-1} s^{-1}. We then used this number to predict the kinetics of antibody coupling to the AFM tip (Figure 2A) in a situation where the antibody concentration is 1 µM. We calculated the half time for the derivatization of the PEG linker on the AFM tip with antibody. The surprising result was that the half time was 5 days (see Table 1). This means that after 5 days the probability for antibody coupling was only 50%. If this were true, then five half times (25 days) would be required to reach 97% coupling efficiency (see Table 1).

Table 1

[HS-protein]	$t_{1/2}$	$5 \times t_{1/2}$ (97% coupling)
1 µM = 150 µg/ml, antibody in bulk solution	5 days	25 days
3 mM = 1 antibody per 6 nm hemisphere	2 min	10 min

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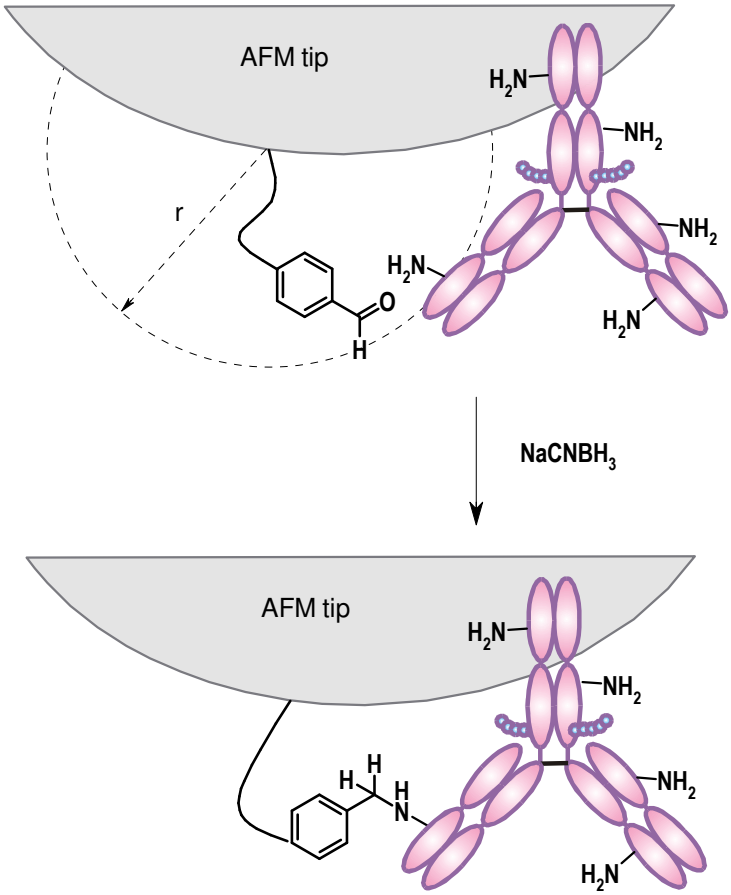

Figure 3: Model for acceleration of antibody linking to tip-bound PEG-aldehyde tentacles, due to pre-adsorption of protein next to the PEG linker. The free end of a 6 nm long, surface-bound PEG linker can reach all locations in a hemisphere with r = 6 nm. One protein per hemisphere (450 nm^3) corresponds to a formal concentration of 3 mM. Tip radius, antibody dimensions, and PEG length were roughly drawn to scale. The number of NH_2 groups per antibody is about 80-90 [Dorner et al., 1967]. At least one antibody (3 mM protein) and at least 10 amino groups per antibody (10 × 3 mM = 30 mM amino groups) are expected to be within the reach of one PEG linker. This explains the fast effective kinetics of antibody coupling, in spite of the slow intrinsic kinetics of amino group coupling to benzaldehyde groups. (*Please, note that the initially formed –CH=NH– group is reduced to a –CH_2–NH– group by $NaCNBH_3$ which is included in the reaction.*)

How can we explain that, in reality, the antibody molecules are efficiently coupled to the AFM tip within 1 hour, while theoretically it should take one month? – The clue to the explanation was found in comparable experiments on gold surfaces. We checked the kinetics of antibody coupling to aldehyde-functionalized gold surfaces. In one study [Hahn et al., 2007], the gold surfaces were sticky and here it took few seconds to cover the whole surface

with covalently bound antibody molecules (at 7 µM antibody concentration). In the other study [Hölzl et al., 2007] the surfaces were protein resistant and here no significant amounts of antibody were coupled on the time scale of one hour (also at 7 µM antibody concentration).

By analogy to the above cited experiments on gold surfaces, we concluded that the unexpected acceleration of antibody coupling to the AFM tip is most likely due to transient pre-adsorption of antibody to the AFM tip [Kamruzzahan et al., 2006]. Figures 1 and 3 show PEG linkers that are coupled to the AFM tip surface with one end. The extended length of a PEG_{18} linker is about 6 nm, therefore its outer end can diffuse within a hemisphere of 6 nm radius. If one adsorbed antibody molecule is within reach of the PEG linker, then the formal antibody concentration within the hemisphere is 3 mM. This is 3000 times higher than the typical bulk concentration of the antibody (1 µM), therefore we predict a half time of 2 min (rather than 5 days). Fife half times (10 min) are sufficient to ensure 97% coupling efficiency.

We are aware that the numbers in our model calculations are crude estimates. Nevertheless, the message is clear: In free solution, the kinetics of disulfide formation (Figure 1) is very slow [Kamruzzahan et al., 2006]. The same applies to the kinetics of antibody coupling to aldehydes in absence of pre-adsorption [Hölzl et al., 2007]. In absence of pre-adsorption, antibody concentrations of up to 1 mM (= 150 mg/mL !!!) would theoretically be required to ensure coupling within 1 h. In reality, however, we see efficient coupling within 1 h at antibody concentrations of 0.1 – 1 µM. Obviously, some "magic factor" accelerates coupling by at least 3 orders of magnitude. The "magic factor" can only be pre-adsorption of antibody the AFM tip, enriching the local concentration next to the PEG linker from micromolar to millimolar.

It is essential to pay attention to the pre-adsorption hypothesis in the daily practice of AFM tip functionalization:

- If detergent is present, then pre-adsorption will be abolished and the time scale of antibody coupling is indeed expected to shift from minutes to weeks (Table 1).
- If the disulfide coupling scheme in **Figure 1** is performed with a thiol protein (HS-protein) and a second protein without a thiol group is present in large excess, then the second protein will preferentially adsorb to the AFM tip and the first protein will not be enriched next to the PEG linker by pre-adsorption. No coupling will be seen in this case.
- The same problem will arise if a thiol protein is to be coupled to a maleimide-functionalized AFM tip (see manual "AFM_tip_with_maleimide"). Here the efficiency of tip coupling will also be greatly reduced by the presence of a second protein, even if the second protein has no free SH group and cannot react with maleimide!
- Another example would be coupling of periodate-oxidized antibody to hydrazide tips (see manual "AFM tip with hydrazide"). Commercial antibodies are usually stabilized with a large excess of BSA. The BSA is not oxidized by periodate, only the carbohydrate on the antibody is converted into an aldehyde by periodate. Theoretically, it should be possible to selectively couple the oxidized antibody to the hydrazide tip. In practice, this will not work because the surface next to the PEG

linker will preferentially be occupied by adsorbed BSA molecules, thereby suppressing both pre-adsorption and covalent coupling of antibodies.

- If a small molecule is to be coupled, then the chances are high that it is not pre-adsorbed to the AFM tip. In this case, millimolar concentrations have to be used to ensure fast coupling within 1 h. A published example is shown in **Figure 4**. Here the ethylenediamine derivative of ATP (EDA-ATP) was coupled to the aldehyde tip, using 10 mM EDA-ATP [Wildling et al., 2011].

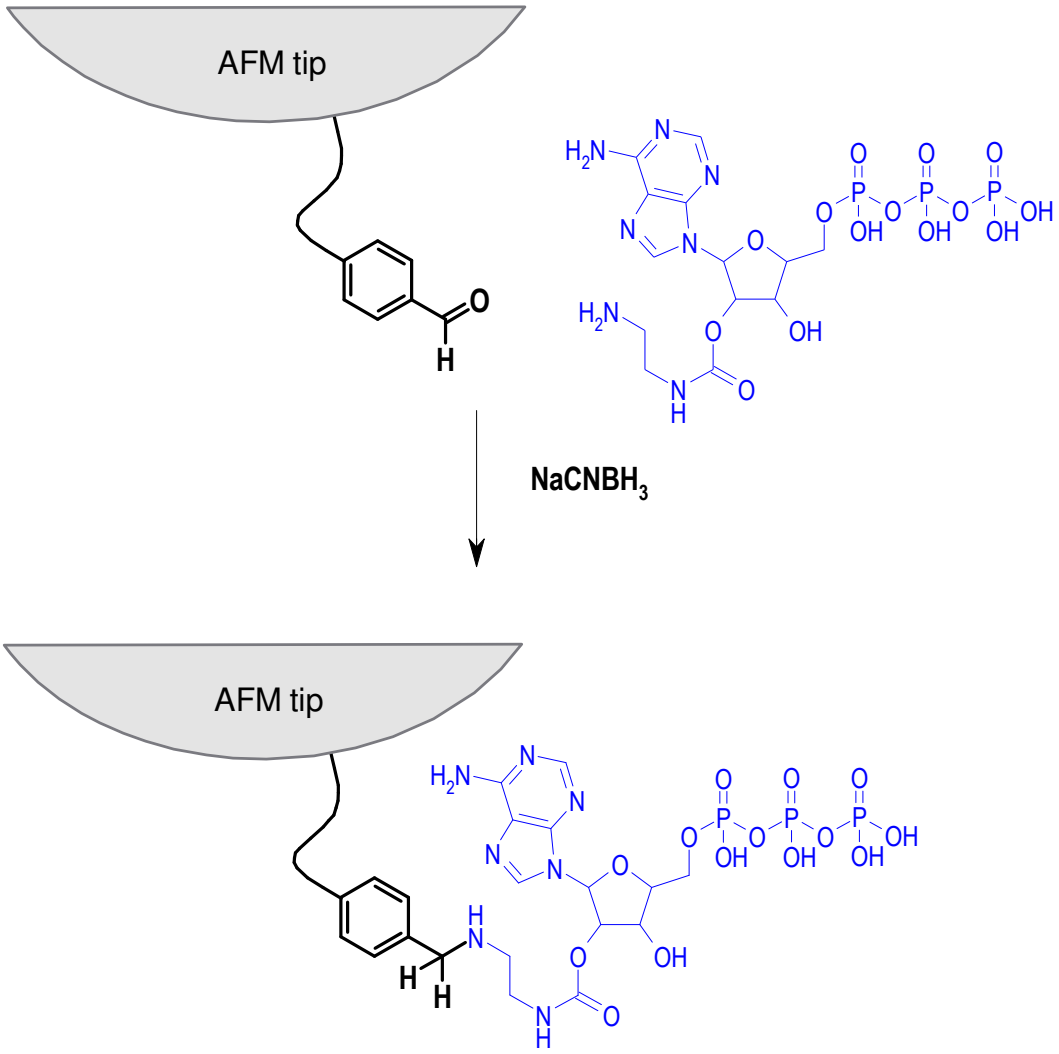

Figure 4: Reaction of tip-PEG-aldehyde with an ethylene diamine derivative of ATP (EDA-ATP) [Wildling et al., 2011]. The concentration of the ATP derivative was 10 mM, in order to ensure fast coupling kinetics without the help of pre-adsorption. (*Please, note that the initially formed –CH=N– group is reduced to a –CH₂–NH– group by NaCNBH₃ which is included in the coupling reaction.*)

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