Functionalization of Surfaces with Short Acetal Linker

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Surface with Short Acetal Linker

short version for experts

for risks and details see full length procedure further below

1. Aminofunctionalization of the substrate (see *AFM_tip_aminofunctionalization*).
2. Transfer the glass vial with SDMB from the freezer into a box with blue gel (or orange silica gel). Let warm to room temperature (30 min). Weigh 2-3 mg SDMB into a small beaker which is large enough for your substrate. Store the original SDMB vial in the freezer in a box with blue gel (or orange silica gel).
3. Dissolve the 2-3 mg portion of SDMB (= Acetal-NHS) in chloroform (2 mL), add triethylamine (100 µL), and mix. This SDMB concentration will give a lateral density of aldehyde functions which is fully sufficient for protein coupling. Higher SDMB concentrations may be necessary for extremely high aldehyde densities on the aminosilanized surface.
4. Immediately place substrate in the beaker, cover the beaker and incubate for 2 h. Several layers of aluminum foil are best suited for tight coverage of a glass beaker.
5. Wash with chloroform (3 × 10 min), dry with nitrogen gas.
6. Store the substrate for up to several months under argon, or continue with next step.
7. Immerse the substrate for 20 min in 2 % citric acid (in water).
8. Wash in water (3 × 5 min), dry with nitrogen gas. Continue with the next step!
9. Freshly prepare a 1 M solution of sodium cyanoborohydride (with 20 mM NaOH) from the following components.
   - 13 mg $NaCNBH_3$ (or 32 mg) – toxic!
   - 20 µL 100 mM NaOH (or 50 µL)
   - 180 µL water (or 450 µL)
10. Place substrate on Parafilm in a polystyrene Petri dish.
11. Pipet 100 µL protein solution (1-2 µM) onto the substrate (or n × 100 µL).
12. Add 2 µL (or n × 2 µL) of the 1 M sodium cyanoborohydride stock solution, mix carefully, cover with lid, incubate for 1 h.
13. Meanwhile dissolve 3 pellets NaOH in 500 mL water, add the unused $NaCNBH_3$ solution, mix, pour into the drain and flush with tap water.
14. Add 5 µL (or n × 5 µL) of ethanolamine (1 M, pH 8.0) to the drop on the cantilever(s), mix cautiously, cover with lid, incubate for 10 min.
15. Wash in PBS or any other buffer of choice (3 × 5 min).
16. Mount the support in AFM setup (or store in a 24 well plate under PBS at 4°C for 1-2 weeks).

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Functionalization of Surfaces with short Acetal Linker

This introduction starts with a short recapitulation of AFM tip functionalization which begins with aminosilanization (**Fig. 1**) and proceeds to protein coupling via long crosslinker molecules (**Fig. 2**). Fortunately, ultra-flat supports like mica and silicon (nitride) chips can also be aminosilanized, therefore the same methods can also be used for flexible tethering of target molecules to these supports.

Fig. 1 shows our standard method for generation of primary amino groups (NH_2) on AFM tips. Aminosilane (APTES) is applied via the gas phase, using triethylamine vapors as catalyst (see manual *AFM_tip_aminofunctionalization*).

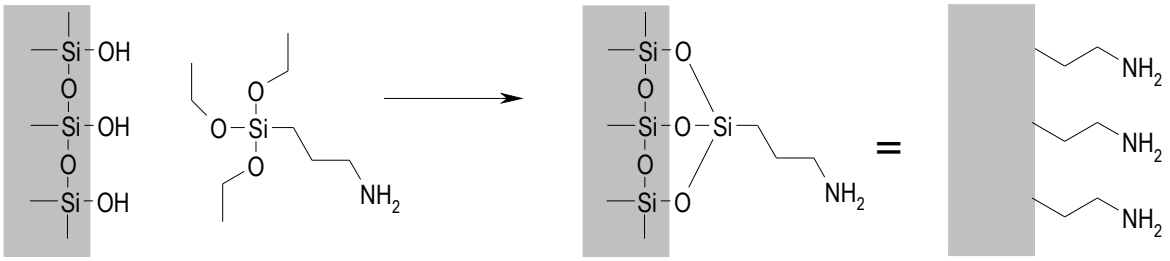

Fig. 1: Covalent grafting of AFM tip surfaces with aminosilane. The silicon or silicon nitride surface of the AFM tip spontaneously oxidizes in air, and thus contains silanol groups (Si-OH) which replace the ethoxy groups of APTES (aminopropyltriethoxysilane), forming covalent bonds [Riener et al., 2003].

For flexible attachment of proteins to the AFM tip, polyethylene glycol chains (PEG) are coupled to the amino groups with one end, resulting in surface-linked PEG chains which carry a benzaldehyde group at their free end (stage **A** in **Fig. 2**). The benzaldehyde group provides for stable binding of proteins in presence of $NaCNBH_3$ (stage **B** in **Fig. 2**) The procedure is described in the manuals "AFM_tip_with_aldehyde" and "AFM_tip_with_acetal".

The procedure shown in **Fig. 2** can also be used for covalent linkage of proteins to ultra-flat supports, such as mica or silicon nitride. In this way two complementary proteins can flexibly be linked to the tip and to the support, allowing for force spectroscopy studies with minimal perturbation by steric hindrance [Rankl et al., 2008].

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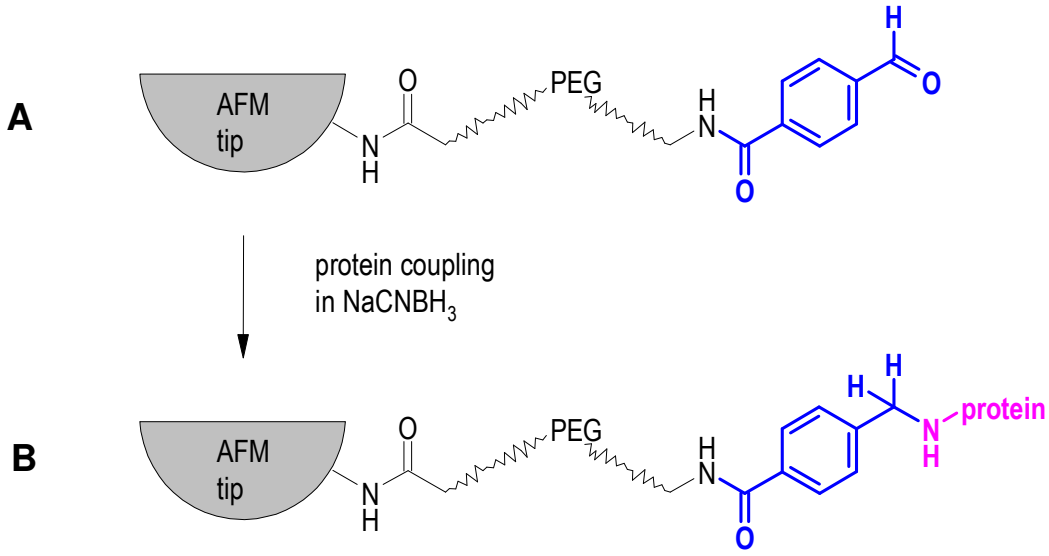

Fig. 2: Scheme of protein coupling to tip-linked PEG chains which carry a benzaldehyde group at the free end. In the last step, the benzaldehyde reacts with a lysine residue (NH_2) of the protein, resulting in a semi-stable C=N bond which is usually converted into a stable CH–NH bond by reaction with $NaCNBH_3$ [Ebner et al., 2007; Wildling et al., 2011].

Often it is desirable to use a much shorter linker for the covalent immobilization of a biomolecule to the ultra-flat support. One reason for this strategy is that immobilization occurs at much higher densities when using short crosslinkers. For example, different mutants of avidin were covalently bound to mica by the procedure depicted in **Fig. 3** [Rangl et al., 2014]. First, amino groups were introduced with aminosilane (APTES) as depicted in **Fig. 1**. The amino groups were then reacted with a short homo-bifunctional crosslinker called EGS. The term "homo-bifunctional" means that the linker has two equal reactive groups. In EGS, the two reactive groups are so-called NHS esters (= activated COOH groups) which react with the amino groups on the surface under formation of stable amide bond.

The procedure shown in **Fig. 3** has two critical aspects:

(1) The short crosslinker has two amino-reactive end groups. At high lateral density of NH_2 groups a large fraction of the short crosslinkers will react with two adjacent NH_2 groups under formation of a loop which cannot undergo any further chemical reaction.

(2) The reaction of the short crosslinker is performed in organic solvent where the NHS ester groups can only react with the NH_2 groups on the surface but not with the solvent. The coupling of protein, however, is performed in aqueous solution at neutral pH. In this situation, the NHS ester groups can undergo two competing reactions, (a) coupling of protein, and (b) hydrolysis into a non-reactive COOH group (see **Fig. 3**). For this reason, it is essential that the surface is not exposed to water (or moisture) before the protein solution is applied to the surface.

The procedure shown in **Fig. 3** will usually work well for coupling of proteins because the surface with the short linkers ensures rapid preadsorption of protein, even from dilute solution (~1 µM), as explained in the manual "acceleration of coupling by preadsorption". In case of other amine-containing molecules, however, it may be necessary to use very high concentrations of this molecule (~10 mM), in order to ensure that amine coupling is faster than hydrolysis.

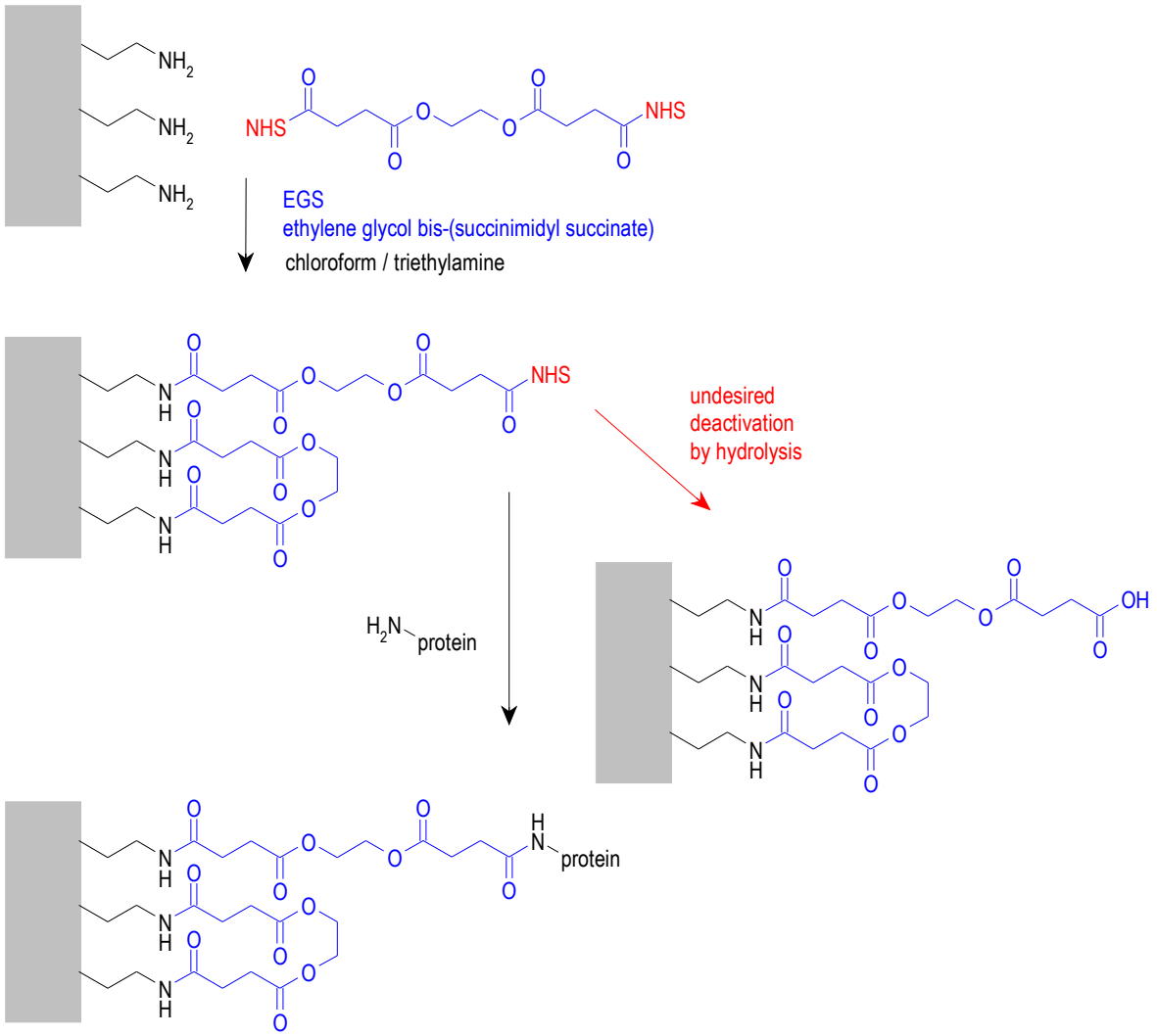

Fig. 3: Three-step protocol for covalent attachment of a protein to mica (or some inorganic oxide surface). First, amino groups are introduced as shown in **Fig. 1**. Second, the amino groups are reacted with a short linker that has to NHS ester functions. Third, the free ends of the linkers react with lysine groups of proteins, resulting in stable amide bonds.

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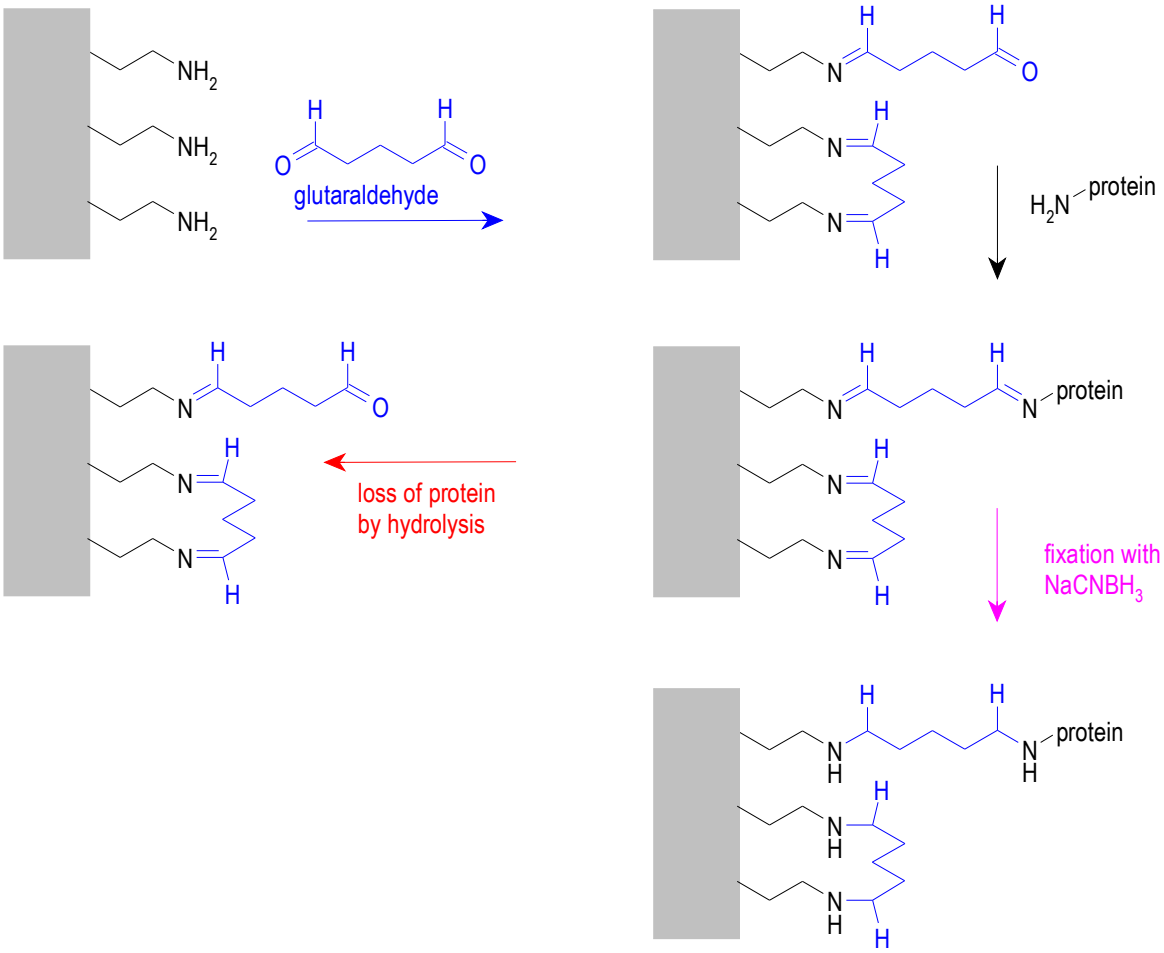

Fig. 4: Glutaraldehyde method for coupling of proteins (or other amines) to aminofunctionalized surfaces.

The problem of ring formation is also encountered when using glutaraldehyde (**Fig. 4**) in place of a bis-NHS ester (**Fig. 3**). The hydrolysis problem, however, is quite different. The aldehyde group itself cannot hydrolyse, therefore the free aldehyde ends of surface-linked glutaraldehyde molecules retain their reactivity for protein even during prolonged exposure to water. The coupling product of aldehyde and amine, however, is not permanently stable and can be cleaved by hydrolysis – unless the initially formed C=N bond is converted into a CH–NH bond by reaction with $NaCNBH_3$.

The above considerations about the EGS-coupling (**Fig. 3**) and glutaraldehyde coupling (**Fig. 4**) should not be misunderstood in the sense that these methods would not work well or that it is complicated to use them in practice. What we want to say is this: Due to the many side reactions, these procedures are prone to give unexpected and/or irreproducible results when used without care – or for other purposes than coupling of proteins.

Here, we offer an alternative method which closely resembles our method of protein coupling to the AFM tip (Fig. 2), except that the long crosslinker "Acetal-PEG-NHS" (**Fig. 5A**) is replaced by the short crosslinker "Acetal-NHS" which lacks the long PEG chain (**Fig. 5B**).

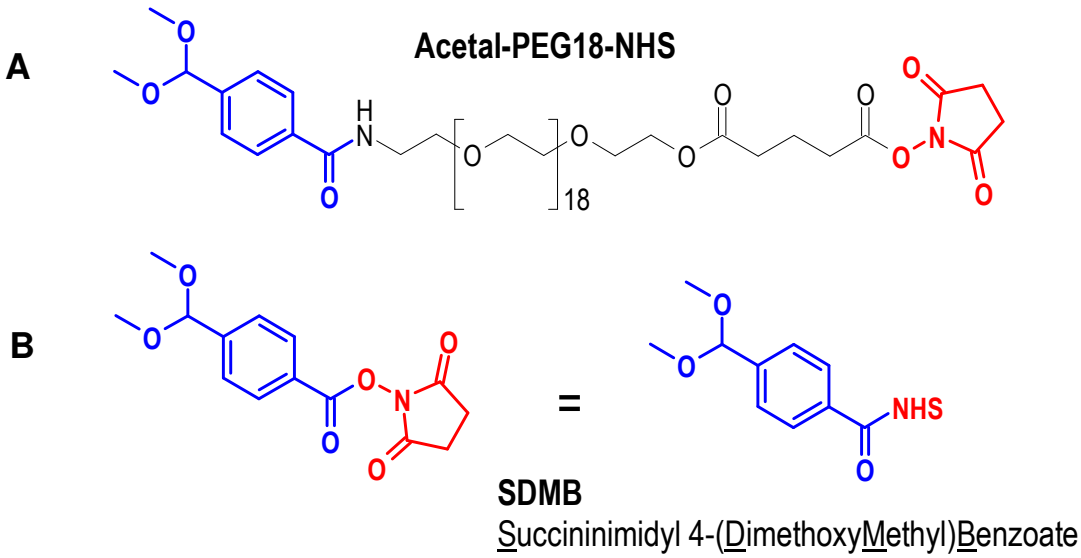

Fig. 5: Comparison of the long crosslinker Acetal-PEG18-NHS (**A**) with SDMB (**B**) which does not contain a long PEG chain in the center. The correct chemical name of the short linker is **S**uccinimidyl 4-(**D**imethoxy**M**ethyl)-**B**enzoate, and the official abbreviation is **SDMB** [Wildling et al., 2011].

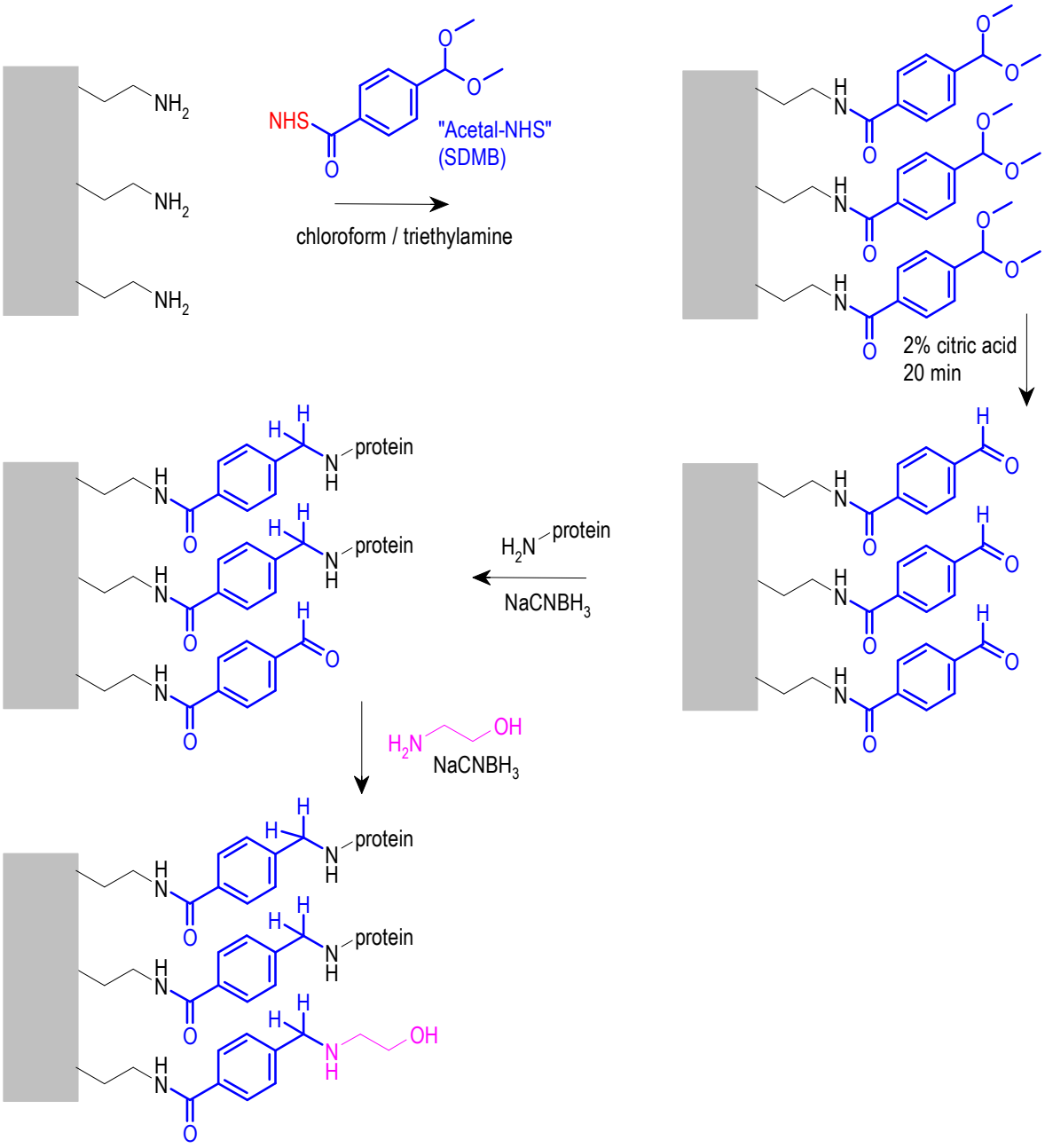

Fig. 6: Five-step scheme for immobilization of proteins (or DNA etc.) via the short crosslinker "Acetal-NHS" [Posch et al., manuscript in preparation]. First, amino groups are introduced with aminosilane (see **Fig. 1**). Second, the amino groups react with the NHS ester group of "Acetal-NHS", yielding a stable amide bond. Third, the acetal function is hydrolyzed into a benzaldehyde group by use of aqueous citric acid. Fourth, the benzaldehyde groups react with the amino groups of proteins or other ligand molecules, yielding a semi-permanent C=N bond. The latter is instantaneously converted into a stable CH–NH bond by NaCNBH3 which is present at the same time. Fifth, ethanolamine is added after protein coupling, providing for block of unused aldehyde groups.

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The full scheme for surface immobilization of proteins (or other amine-containing molecules) is shown in **Fig. 6**. The details of the reaction are explained in the legend to **Fig. 6**. – Please, read this legend because the details are essential and they are not explained in the regular text!

Immobilization with SDMB (**Fig. 6**) has significant advantages over the conventional methods with EGS (**Fig. 3**) or glutaraldehyde (**Fig. 4**):

- The surface-bound benzaldehyde groups do not lose their reactivity upon prolonged exposure to water – in contrast to surface-linked NHS ester functions (compare **Fig. 3**).
- No loops can be formed on the surface because SDMB has only one amino-reactive group while it is attached to the surface. Consequently, the lateral density of benzaldehyde groups will be directly proportional to the lateral density of the NH_2 groups on the aminosilanized surface. – The opposite is true with EGS and glutaraldehyde, where loop formation will be absent at low NH_2 group densities and predominant at high NH_2 group densities.
- Higher lateral densities of reactive aldehydes can be reached with SDMB where every accessible NH_2 group on the surface can be derivatized with one SDMB molecule – provides that the reaction is performed at high SDMB concentration and over a sufficient time period.
- If desired, much lower lateral densities of reactive benzaldehyde functions can be adjusted in a reproducible way. The trick is to mix SDMB with NHS acetate before the mixture is reacted with the aminosilanized surface (see **Fig. 7**). NHS acetate will cause acetylation of a large number of NH_2 groups on the solid surface, thereby lowering the number of sites where SDMB can couple. – Please, note that the ratio of SDMB over NHS acetate in the solution does not allow prediction of the fraction of NH_2 groups on the surface will react with each of the two reagents. The reason is that the reactivity of the two reagents with the NH_2 groups can be quite different. The small NHS acetate may be much faster in the reaction with NH_2 groups. We have not tested this option so far. Those interested in applying this method are advised to prepare different mixtures and to test for the effective outcome of the different mixtures.

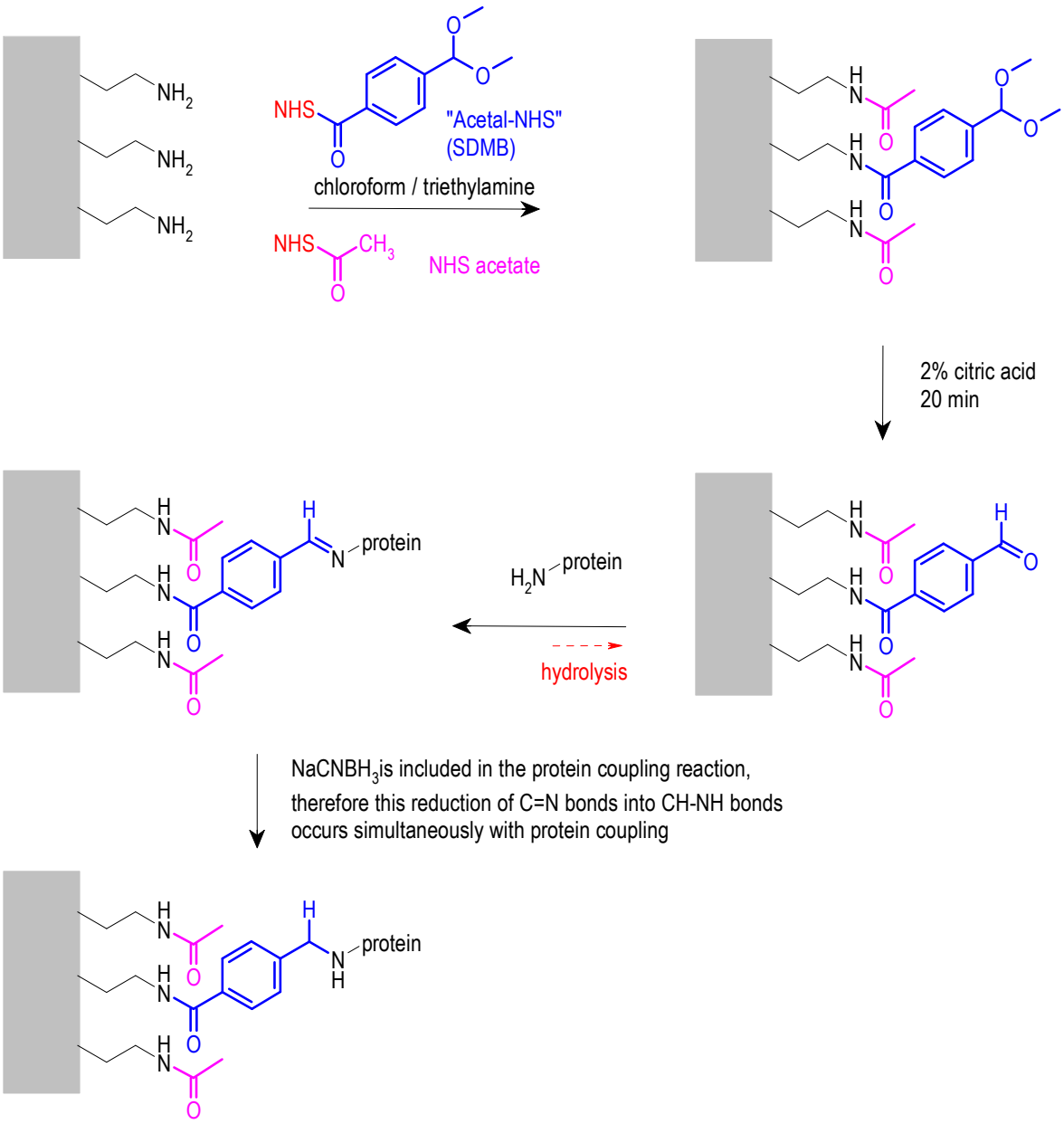

Fig. 7: Systematic strategy for reproducible adjustment of a low lateral density of reactive benzaldehyde groups on aminosilanized surfaces. The details of the procedure are the same as in **Fig. 6**, except that SDMB is pre-mixed with NHS acetate at a defined ratio, resulting in block of a major fraction of NH_2 groups by acetylation. Please, note that the reactivity of NHS acetate for the NH_2 groups on the aminosilanized surface is possibly higher than that of SDMB, therefore the fraction of acetylated groups on the chip surface may be quite different from the fraction of NHS acetate (as compared to SDMB) in the reagent solution.

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The experimental details for the **first step** are as described in the manual *AFM_tip_aminofunctionalization*. It makes no difference whether AFM tips or larger substrates are placed into the argon-filled desiccator, next to a tray with APTES and a tray with triethylamine.

For the second step, the glass vial with SDMB is transferred from the freezer into a box with blue gel (or orange silica gel). Let warm to room temperature (30 min). SDMB (2-3 mg) is weighed into a small beaker which is large enough for for the substrate which is to be derivatized. The original SDMB vial is placed back in the freezer in a box with blue gel (or orange silica gel). Chloroform (2 mL) is added to the beaker and SDMB is dissolved by swirling. Triethylamine (100 µL) is added and mixed into the solution. The amino-functionalized substrate is immersed in this solution and the beaker is covered with a aluminum foil (4 layers) to minimize evaporation of chloroform and triethylamine. After two hours the substrate is washed in chloroform (3 × 10 min incubation) and dried with nitrogen gas. At this stage, the acetal-functionalized substrate can be stored in an argon-filled desiccator for up to several months!

For the third step, the substrate is immersed in aqueous 2% citric acid for 20 min at room temperature. Subsequently, the substrate is washed in water (3 × 5 min) and dried in a stream of nitrogen gas. At this stage, the substrate must not be stored but used immediately for the subsequent coupling steps.

Before the fourth step, a 1 M solution of sodium cyanoborohydride is freshly prepared by one of the following methods.

- 13 mg sodium cyanoborohydride ($NaCNBH_3$ = $NaBH_3CN$) is weighed into a screw-cap glass vial (2 mL). Exert due caution because of the high toxicity of this compound! See last Note for the proper procedure! Then, 20 µL of 100 mM NaOH (4 mg/mL NaOH in water, see list of materials at the end of the manual) is added which ensures that no toxic HCN is being released. Subsequently, 180 µL of water is added and the solution is carefully mixed with the pipette tip. Only 2 µL of this solution is required in the fourth step, the rest should be diluted with water (e.g., 500 mL) containing >3 pellets of NaOH (or ~5 g of sodium carbonate) and subsequently flushed down the drain with copious amounts of water.
- Alternatively, if the crystals of sodium cyanoborohydride are large, it may be easier to work with 32 mg, adding 50 µL of 100 mM NaOH and 450 µL of water. Only 2 µL of this solution is required in the fourth step, the rest should be diluted with water (e.g., 500 mL) containing >3 pellets of NaOH (or ~5 g of sodium carbonate) and subsequently flushed down the drain with copious amounts of water.
- We do not recommend using a stock solution of sodium cyanoborohydride (e.g., 5 M sodium cyanoborohydride, Sigma-Aldrich No. 296945) because its high sodium hydroxide content (1 M) makes proper pH adjustment during the coupling reaction extremely tricky. Moreover, the large quantity of this highly toxic solution (50 mL) poses a high safety risk.

For the fourth step, a piece of Parafilm is pressed onto the inner surface of a polystyrene Petri dish and the substrate is placed onto the Parafilm. Then, 100 µL of the protein solution (or n × 100 µL) is pipetted onto the surface of the substrate. The preferred buffer is PBS. Hepes buffer can also be used. TRIS or glycine are strictly forbidden because they would

react with the aldehyde functions on the substrate. The typical concentration of proteins in this reaction is 1-2 µM but successful coupling has also been observed at 0.2 µM. Subsequently, 2 µL of the 1 M sodium cyanoborohydride solution (or n × 2 µL) is added and cautiously mixed into the droplet with the pipette. The Petri dish is covered with the lid and the reaction is allowed to proceed for one hour at room temperature. Subsequently, a 5 µL aliquot (or n × 5 µL) of ethanolamine (1 M, pH 8.0, see list of materials at the end of the manual) is added and cautiously mixed into the droplet with the pipette. After 10 min, the cantilevers are washed in PBS (3 × 5 min) and stored in a 24 well plate under PBS at 4°C for 1-2 weeks.

Notes:

- The solid SDMB should be stored at -20°C over a desiccant like blue gel (or orange silica gel). Silica gel must not be breathed because it causes damages to the lung, similar to asbestos. In addition, blue gel contains cobalt chloride which acts as a carcinogen in the lung. Orange gel contains ferric ammonium sulfate in place of cobalt chloride, and it consists of aluminium silicate which releases much less fines than blue gel, therefore its hazard potential is much lower. For storage of SDMB over desiccant it is convenient to fill a plastic box (the same kind as used to store food in a refrigerator) with a layer of desiccant (~5 mm in height), to cover the desiccant with Kleenex or a folded sheet of kitchen roll, to place the SDMB vial on top and to press the lid firmly onto the box. For thawing, the vial with SDMB should be transferred into a second plastic box ("thawing box") which also contains desiccant covered with Kleenex but is always kept at room temperature. Please, keep an eye on the color of the desiccant: If the blue color turns white or pink (or if the orange gel gets colorless), then the old desiccant must be replaced by fresh desiccant. The moist desiccant can be regenerated by heating to 150-180°C in a metal pan in a well ventilated hood. The pan must be covered with the proper metal lid, with a thin wire (e.g., a properly bent paper clip) between pan and lid to allow for escape of the water vapors. When the color indicates that the desiccant has been regenerated, then the heater is turned off, the wire is removed, the lid is put in place, and the pan is allowed to come to room temperature before the desiccant is transferred into a tightly sealing jar for storage.
- A gas mask with particle filter (type 3) must be used during all manipulations of blue gel, and preferably also with orange silica gel.
- Phosphate, borate, and Hepes (or other Good buffers) are fully compatible with the aldehyde linker and need not be dialyzed.
- The acetal/aldehyde linking method is not applicable if the protein sample contains buffer components with primary amines (e.g., Tris or glycine) or ammonium bicarbonate or ammonium acetate. In this case, the protein sample (50-100 µL) must be dialyzed, preferably with a "Side-A-Lyzer MINI Dialysis Device" from Thermo Scientific, with a molecular weight cut-off (MWCO) of 10K or 20K. Dialysis should be against 500 mL buffer for ~36 h, with several buffer changes!!!
- The acetal/aldehyde linking method does not work for proteins in presence of detergent. The reason is that detergent eliminates pre-adsorption of protein to the surface which is indispensable in case of slow coupling reactions such as disulfide or

aldehyde coupling [Kamruzzahan et al. (2007) Bioconjugate Chem. 17, 1473-1481], as explained in the manual "acceleration_of_coupling_by_preadsorption".

- In contrast, detergent is allowed if the concentration of the amine-containing ligand is ≥5 mM because then no pre-adsorption is required for fast coupling to the aldehyde functions.
- By analogy to the cyanhydrine reaction of cyanide (NaCN, KCN), **sodium azide (NaN_3)** could theoretically react with the benzaldehyde function on the tip. The chances are very low that it actually happens because this reaction has never been reported in the literature. Moreover, even cyanide has a low tendency towards the cyanhydrine reaction under the conditions of AFM tip functionalization. In fact, low concentrations of cyanide are formed by hydrolysis of sodium cyanoborohydride, without any adverse effect on protein coupling. If you want to be on the safe side, you can remove azide by dialysis with a Slide-A-Lyzer (see first note).
- If antibodies are stabilized with an excess bovine serum albumin (BSA) or gelatin, then coupling to the aldehyde on the AFM tip is impossible because BSA has a much higher chance to get coupled. BSA can be removed from the sample by gel filtration on a Superdex 200 column (GE Healthcare). Alternatively, you can use a Melon gel kit from Thermo Scientific for removal of BSA or gelatin (http://www.piercenet.com/files/TR0055-Melon-Gel-for-BSA.pdf).
- By the same rationale, crude ascites fluid or serum cannot be used because only a minor fraction of the total protein content consists of the desired antibody.
- Pipetting of chloroform and triethylamine should be performed with glass pipettes and a pipette bulb (or with a graduated glass capillary in case of triethylamine) and not with the plastic tips of digital pipettes because the plastic tips may release polymeric material in chloroform and triethylamine. – Never use your mouth for pipetting, especially not with such toxic liquids as chloroform and triethylamine.
- The ideal tools for accurate and easy pipetting of chloroform and triethylamine are gas-tight Hamilton syringes. A 500 µL syringe is suggested for chloroform (part no. 81230, RN-type) and a 50 µL syringe for triethylamine (part no. 80230, RN-type). Two removable needles with a blunt ends (part no. 7780-02) need to be purchased in addition.
- Immediately after pipetting, these syringes should be washed free of chloroform or triethylamine. Use nitrile or rubber gloves (not latex) for the cleaning procedure. First, isopropanol (same as 2-propanol) is pulled into the syringe and disposed into the solvent waste (3 ×). Then, the piston is removed and carefully rinsed with isopropanol. The barrel filled with isopropanol several times from a squeeze bottle and the contents poured into the solvent waste. Finally, the barrel is again filled with isopropanol and the piston is inserted to push the contents into the solvent waste bottle. This step is repeated twice. Subsequently, the syringe must be dried by one of two alternative methods:
    - Diethyl ether can be pulled into the syringe and pushed into the solvent waste. Then the piston is moved up and down (15-20 ×).

- The outsides of the barrel and the piston are dried with soft paper tissue or with a stream of gas. The barrel interior is dried with nitrogen gas from a Pasteur pipette which is inserted into the barrel. Then, the needle is inserted into the tip of the Pasteur pipette until the glass base of the syringe needle softly touches the tip of the Pasteur pipette. Now the gas flow is forced through the needle which causes evaporation of isopropanol from the interior of the needle (takes about 1 min).

- Be careful when using Pasteur pipettes which are connected to a nitrogen gas tank via silicon tubing. Make sure that the needle valve is closed when you open the main valve of the gas tank! Slowly open the needle valve! Always hold (or fix) the Pasteur pipette and not the silicon tubing. In this way, only the soft tubing may jump off the pipette if the nitrogen flow is opened too quickly. In the opposite case, the Pasteur pipette may become a dangerous weapon hurting yourself or your colleague.
- In spite of the small quantities of chloroform and triethylamine, the reactions should be performed in a well ventilated hood.
- If your institution forbids the use of chloroform, then you can use DMSO for the reaction of the tip with the linker and isopropanol (or ethanol) for the subsequent washing steps. Test experiments have shown that this gives good results.
- Unfortunately we have no experience whether chloroform can be replaced by some other reagent in the initial washings, before aminosilanization. Chlorinated solvents are much more efficient in the removal of impurities. You can probably use dichloromethane if that is allowed at your institution. If not, then it may be better to use ozone cleaning or piranha, followed by washing with water and then with isopropanol or ethanol.
- Strictly avoid contact of the organic solvents and of triethylamine with your skin. Latex gloves cannot protect you for more than 1-2 seconds. Nitrile gloves provide slightly longer protection. In case of splashing the gloves must be immediately removed from the hands.
- Extreme caution is necessary when weighing the aliquots of sodium cyanoborohydride. The highest risk is breathing the dust and contact with the skin. Use a gas mask, a lab coat, and robust gloves for weighing. All material transfers must be performed in a well ventilated hood. Place the empty vial (2 mL) with the screw cap on the balance, press the re-zero button, carry the vial to the hood, add a tiny amount of $NaCNBH_3$, close the screw cap and put the closed vial onto the balance. Go back and forth between hood and balance and add/remove until the net weight of cyanoborohydride in the vial is 13 mg. Alternatively, you can place the balance into the hood and prepare many aliquots which are then stored in a desiccator in a poison cupboard. Clean the area in the hood with an alkaline solution (e.g., 1% Na_2CO_3).

Materials

Phosphate-buffered saline (PBS) is prepared by dissolving the components listed below in about 800 mL water, transferring the solution into a 1 L volumetric flask and adding water to give exactly 1 L volume. Mix carefully. The pH will automatically be 7.3 if

all steps are performed correctly. No pH adjustment is required. Aliquots can be stored at -20°C.

- 140 mmol NaCl (58,44 g/mol × 0,140 mol = **8.182 g NaCl**)
- 2.7 mmol KCl (74.56 g/mol × 0.0027 mol = **0.201 g KCl**)
- 10 mmol Na_2HPO_4 (in case of anhydrous dibasic sodium phosphate: 141.96 g/mol × 0.010 mol = **1.420 g anhydrous Na_2HPO_4**; in case of the heptahydrate: 268.07 g/mol × 0.010 mol = **2.681 g $Na_2HPO_4.7H_2O$**)
- 1.8 mmol KH_2PO_4 (136.09 g/mol × 0.0018 mol = **0.245 g anhydrous KH_2PO_4**).

2% citric acid is prepared by dissolving 1 g citric acid in 50 mL water. The resulting pH is 2.0 and it must not be adjusted to another value. Small aliquots (e.g., 1 mL) are frozen and stored at -20°C for up to several years.

100 mM NaOH is prepared by weighing 1 pellet of solid NaOH (~ 300 mg) into a beaker (100-250 mL) and adding water to give a final concentration of 4 mg/mL. As an example, 320 mg would be dissolved in 320/4 = 80 mL of water to give a 100 mM concentration. Aliquots (100 µL or larger) are frozen and stored at -20°C.

1 M ethanolamine (pH 8.0) is prepared by dissolving 975 mg ethanolamine hydrochloride in water at a final volume of 10 mL and adjusting the pH to 8.0 with 20% NaOH (towards the end with 2% NaOH). Aliquots (20 µL and larger ones) are frozen and stored at -20°C for up to several years.

Literature focusing on aldehyde- and acetal-functionalized AFM tips:

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Kamruzzahan, A. S. M., Ebner, A., Wildling, L., Kienberger, F., Riener, C. K., Hahn, C. D., Pollheimer, P. D., Winklehner, P., Hölzl, M., Lackner, B., Schörkl, D. M., Hinterdorfer, P., and Gruber, H. J. (2006) Antibody linking to atomic force microscope tips via disulfide bond formation. *Bioconjugate Chem. 17*, 1473-1481.

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Risk and Safety

	**Chloroform** ($CHCl_3$): toxic, co-carcinogenic, H302-H315-H351-H373, P281, R22-38-40-48/20/22; S36/37
	**Diethyl ether,** extremely flammable, narcotic, forms explosive peroxides when allowed to evaporate, H224-H302-H336, P210-P261, R12-19-22-66-67 (Europe), S9-16-29-33 (Europe)
	**Ethanolamine hydrochloride,** H315-H319-H335, P261-P305 + P351 + P338, R36/37/38, S26
	**Isopropanol = 2-propanol:** flammable, H225-H319-H336, P210-P261-P305 + P351 + P338, R11-36-67 (Europe), S7-16-24/25-26 (Europe)
	**SDMB,** CAS No. 120465-57-8, no hazard information available. At weakly acidic pH, this compound will be hydrolyzed into 4-formylbenzoic acid N-hydroxysuccinimide ester, CAS No. 60444-78-2. The latter compound is not a dangerous material according to EU regulations No. 1272/2008 and 67/548/EWG. Further hydrolysis gives 4-formylbenzoic acid, CAS No. 619-66-9 which is a strong irritant, hazard codes H315, H319, H335, precautionary measures P261, P305+P351+P338. The GHS symbol on the left refers to 4-formylbenzoic acid.
	**Silica gel blue**, carcinogenic, teratogenic, ingredients silica gel CAS No. 7631-86-9 and cobalt chloride CAS No. 7646-79-9, H350, H360, H400, H410
-	**Silica gel orange**, no hazard information available, ingredients aluminium silicate CAS No. 1327-36-2 and ferric ammonium sulfate CAS No. 10138-04-2
	**Sodium cyanoborohydride, $NaCNBH_3$ = $NaBH_3CN$,** contact with acids liberates very toxic gas (hydrogen cyanide, HCN), H228-H260-H300-H310-H314-H330-H410, P210-P223-P231 + P232-P260-P370 + P378-P422, R11-15-26/27/28-32-34-50/53 (Europe), S26-28-36/37/39-43-45-61

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	**Triethylamine** (TEA): flammable, irritant, caustic, H225-H302-H312-H314-H332, P210-P280-P305 + P351 + P338-P310, R20/21/22-35, S3-6-26-29-36/37/39-45

Be careful when using Pasteur pipettes which are connected to a nitrogen gas tank via silicon tubing. Make sure that the needle valve is closed when you open the main valve of the gas tank! Slowly open the needle valve! Always hold (or fix) the Pasteur pipette and not the silicon tubing. In this way, only the soft tubing may jump off the pipette if the nitrogen flow is opened too quickly. In the opposite case, the Pasteur pipette may become a dangerous weapon hurting yourself or your colleague.

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