Thiol-based, site-specific and covalent immobilization of biomolecules for single-molecule experiments

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The success of single-molecule (SM) experiments critically depends on the functional immobilization of the biomolecule(s) to be studied. With the continuing trend of combining SM fluorescence with SM force experiments, methods are required that are suitable for both types of measurements. We describe a general protocol for the site-specific and covalent coupling of any type of biomolecule that can be prepared with a free thiol group. The protocol uses a poly(ethylene glycol) (PEG) spacer, which carries an *N*-hydroxy succinimide (NHS) group on one end and a maleimide group on the other. After reacting the NHS group with an amino-functionalized surface, the relatively stable but highly reactive maleimide group allows the coupling of the biomolecule. This protocol provides surfaces with low fluorescence background, low nonspecific binding and a large number of reactive sites. Surfaces containing immobilized biomolecules can be obtained within 6 h.

INTRODUCTION

Single-molecule (SM) experiments are continuously becoming more important for the study of biological systems. In contrast to classical ensemble measurements, which average over the whole population of individual molecules, SM experiments are able to detect temporal or spatial heterogeneities, identify transient or rare events, follow the time series of events and reveal parallel reaction pathways. Because of this unique potential, attempts are being made to combine SM fluorescence with SM force experiments[1–6]. These integrated measurements greatly increase the amount of information that can be obtained from one experiment. For example, it has been shown to be possible to manipulate molecules with force and read out the result optically[2,3,5,7–9].

A number of methods have been developed to immobilize proteins, DNA and RNA molecules to various types of surfaces for either SM force[10] or SM fluorescence experiments[11]. In most cases, these methods are tailored for one specific experiment and are not always useful for other types of applications. This is mainly because of the fact that SM force and fluorescence experiments have other requirements. Atomic force microscope (AFM)-based SM force measurements demand covalent immobilization through a sufficiently long spacer molecule. Although other strategies such as site-specific immobilization through noncovalent, specific interactions (e.g., His tag) seem attractive, they might lead to a misinterpretation of data as there is a possibility that this bond might rupture as well[12]. In contrast, for SM fluorescence measurements, a covalent immobilization is not required and the most critical point is a low fluorescence background allowing the specific detection of the molecule of interest. Besides these specific requirements, the immobilization protocol should fulfill several other criteria. Most importantly, immobilization should not lead to additional heterogeneities in the population of molecules, a problem that can occur with procedures that are not site specific, such as coupling through the amino groups of lysine residues. Furthermore, the immobilization procedure should allow for the possibility to adjust the density of biomolecules to ensure that single molecules can be addressed. Finally, nonspecific binding needs to be low to avoid either fluorescent background or nonspecific interactions in the force measurements.

We describe a protocol that fulfills the above criteria using the Michael addition between a maleimide group and a thiol group[13] to site specifically immobilize different types of thiol-containing biomolecules to maleimide-activated surfaces. During our experiments, we have found that coupling biomolecules through thiol groups is the most reproducible method. This probably originates from the following facts: first, the thiol group itself is very reactive compared with other groups (e.g., amino groups). Second, maleimide groups for thiol coupling have a relatively good stability compared with other reactive groups used for bioconjugation (e.g., *N*-hydroxy succinimide (NHS)). Furthermore, especially when the site-specific immobilization of a protein is desired, coupling through thiol groups (i.e., cysteines) has the additional advantage that cysteines occur only rarely on the surface of many proteins. In fact, many proteins do not contain any exposed cysteine residues at all, but they can be introduced by mutagenesis at a desired position (see below).

The process of immobilization

An overview of the chemistry of the immobilization procedure can be found in **Figure 1**. To obtain a surface containing maleimide groups, the surface (e.g., glass slide or cantilever) is first amino-functionalized with a monoreactive aminosilane to prevent crosslinking and multilayer formation, which might cause problems in force measurements. Thereafter, a hetero-bifunctional poly(ethylene glycol) (PEG) carrying an amino-reactive (NHS) group on one end and a thiol-reactive maleimide group on the other end is coupled to the surface. As PEG is a linear polymer, all PEG molecules that are coupled to the surface contain only one reactive maleimide group. The use of PEG as the spacer molecule has additional advantages. PEG can be synthesized with a relatively low polydispersity and, therefore, has a relatively defined spacer

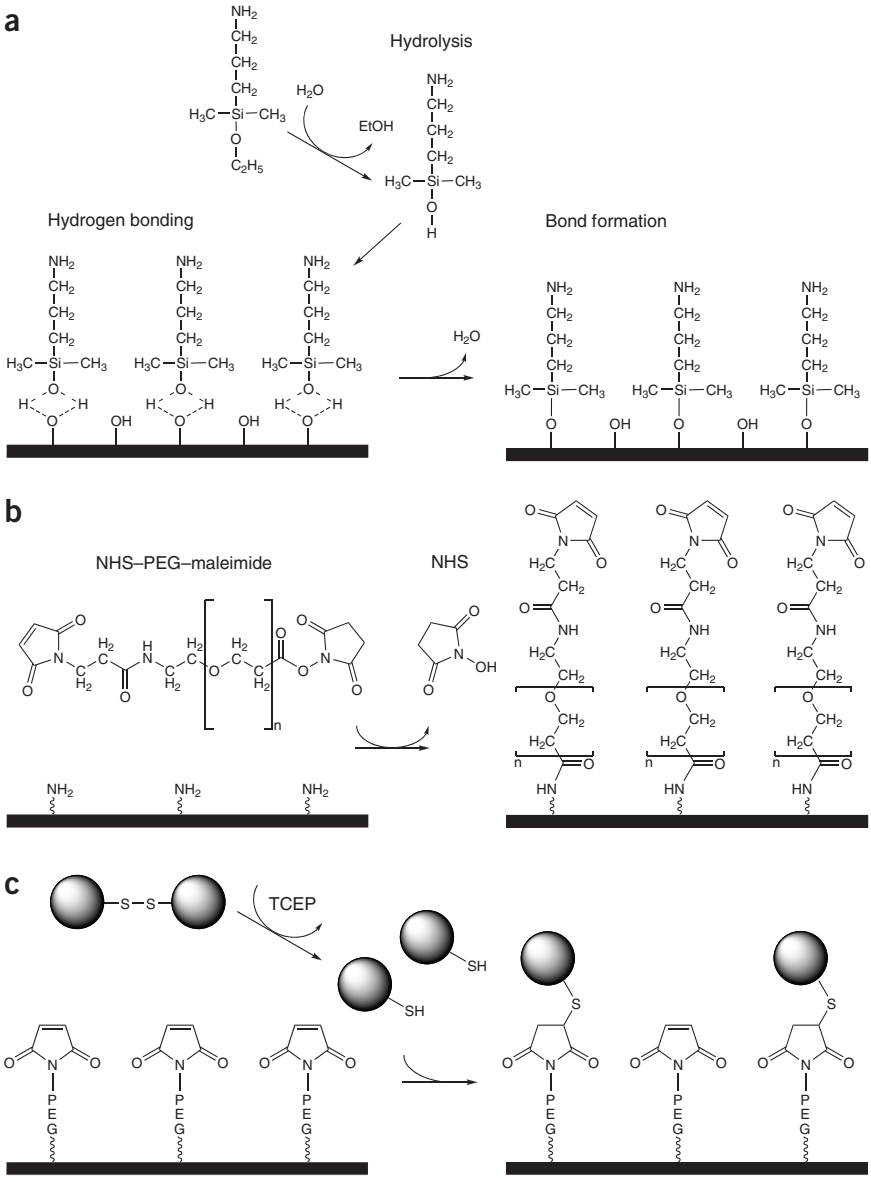

Figure 1 | An overview of the immobilization procedure. The protocol can be used for every kind of surface that reacts with silanes, and consists of three steps. (**a**) Cleaned surfaces are incubated in a solution of pure monoalkoxy amino silane to prevent the formation of crosslinks between silane molecules that might lead to the formation of multilayers. After this step, silane is not yet covalently bound. To obtain covalent bonds, the surface needs to be baked to remove the layer of water, which stabilizes the interaction of silane with the surface by hydrogen bonds. (**b**) After obtaining amino-functionalized surfaces, the hetero-bifunctional NHS–PEG–maleimide spacer is coupled to the surface, yielding a covalent amide bond between PEG and the silane. (**c**) Finally, the reduced biomolecules are coupled to the PEG surface displaying thiol reactive maleimide groups. The surface density of biomolecules is adjusted in this last step and needs to be optimized for the desired application.

length. Because of its defined length and well-characterized elastic behavior, PEG leads to very defined force-extension curves and, as a result, provides a kind of 'internal control' for force measurements[14,15]. Furthermore, PEG is highly biocompatible, and surfaces with a sufficiently high density of PEG become 'resistant' to the adsorption of biomolecules[16–18]. In this context, the crucial point of this protocol is that both silane and PEG are applied in high concentrations to achieve a dense layer of PEG, which is essential to obtain a well-passivated surface with low nonspecific binding. Having obtained a high density of spacers carrying reactive groups, the density of the biomolecule can be adjusted by using an appropriate concentration of the biomolecule in the respective coupling step. The remaining, nonreactive maleimide groups may be blocked with a thiol-containing compound (e.g., cysteine) if desired. This strategy is in contrast to other protocols that control the number of coupling sites in the silanization step to adjust the density of biomolecules[10,19]. This strategy might result in a low density of spacer molecules. As mentioned above, a large number of PEG spacers are, however, required for the passivation of the surface toward nonspecific binding.

Although the protocol was developed mainly for AFM and combined AFM-fluorescence measurements, it will also be useful for other experiments that rely on the immobilization or conjugation of biomolecules, such as the preparation of polymer conjugates[20], the coupling to nanomaterials (e.g., quantum dots[21]), protein or DNA microarrays[22] and biosensors[23,24].

Possibilities and limitations relating to the introduction of thiol groups into biomolecules

Thiol groups can be incorporated into peptides and into DNA or RNA oligonucleotides during solid-phase synthesis. For proteins, either the thiol of a free cysteine can be used for the coupling or a reactive thiol group can be generated using a number of other methods depending on the protein of interest. Before the possibilities are described in detail, it is important to note that only reduced cysteines carrying the free –SH group are able to react with maleimide. Therefore, measures need to be taken to either prevent the formation of disulfide bonds or reduce them directly before the coupling reaction. In this context, it should also be mentioned that many reducing agents carry a free thiol group themselves (e.g., dithiothreitol or 2-mercaptoethylamine) and that their presence interferes with the coupling reaction. Considering these difficulties, we also describe two methods for reducing disulfide bonds in proteins/peptides and in DNA/RNA oligonucleotides, neither of which require time-consuming dialysis or sometimes inefficient gel-filtration steps. For RNA and DNA oligonucleotides, the most efficient reduction procedure uses the reducing agent tris(2-carboxyethyl) phosphine (TCEP)[25], which is removed from the mixture by ethanol precipitation. For proteins and peptides, the use of TCEP beads has been shown to provide a convenient and fast way for disulfide bond reduction: the protein or peptide is incubated

with the beads for 1–2 h and the beads are separated from the solution by centrifugation directly before the coupling step[15,26,27]. In addition, the use of TCEP beads has the additional advantage that only the surface of the protein is accessible for the reducing agent. In none of our examples were problems with the reduction of internal disulfide bonds observed. It should be noted that this procedure (i.e., with beads) did not lead to reproducible results with oligonucleotides, and reduction efficiency seemed to depend on the method used to introduce the thiol group into the oligonucleotide.

To choose the best strategy for the coupling of a protein, one should first check for the amount and positions of the cysteines of the protein. If the protein has surface-exposed disulfide bonds, there is a possibility that they can potentially be reduced[28]. If there is only one disulfide bond, the position of coupling is relatively well defined. However, one should consider the fact that this method generates two reactive thiols for coupling. They are located at the same site of the protein but, depending on the desired application, it might make a difference if one or the other is coupled. In addition, as the reduced thiols are in close proximity, there is a high chance of reoxidation before coupling to the surface can take place. If the coupling site needs to be better defined than with the above procedure, it might be a better strategy to mutate one cysteine of the disulfide bond to alanine[26]. With this strategy, the disulfide bond cannot form anymore and the remaining cysteine can be used for site-specific coupling. If the protein of interest does not contain any surface-accessible cysteines, they can then be introduced by site-directed mutagenesis at the position of interest. The most general strategy is the attachment of an additional cysteine at the C-terminus of the protein[15,26,27]. This strategy further allows the addition of a tag for purification (e.g., His tag), together with the cysteine yielding a universal expression, purification and immobilization system. In our experience, the attachment of the cysteine after the His tag also yielded a higher coupling efficiency than the mutation of disulfide bonds, because of better accessibility of the C-terminal cysteine[26]. Finally, if none of the above strategies can be applied but one still wants to use the thiol–maleimide reaction, it is possible to convert amino groups on the surface of the protein into thiols. Several reagents exist, which introduce protected thiol groups into a protein[29] (e.g., *N*-succinimidyl-*S*-acetylthioacetate (SATA)). The protecting group is removed directly before coupling the protein to the surface. With this strategy, however, no site-specific coupling can be achieved and, depending on the degree of modification of the protein with SATA, even multipoint attachment might occur.

In summary, there are several methods that allow for the introduction of thiols into proteins and the described protocol can be used for a broad range of different proteins. However, it should be noted that not all of them are site specific and rare cases might exist in which a protein carries multiple free cysteines on the surface or needs a free cysteine for its function, such as certain enzymes that have a free cysteine in their active site. In these cases, it needs to be evaluated on a case-by-case basis if the protocol described here can still be used for the desired application. In this context, possible site-specific coupling through the *N*-terminal amino group or tyrosine residues might provide an alternative[20,29].

Experimental design

An overview of the protocol is shown in **Figure 2**. Before starting the SM experiments, it is generally recommended to test the immobilization procedure at least once using a fluorescently labeled binding partner for the immobilized molecule and appropriate negative controls (see TROUBLESHOOTING for details). Buying or preparing the respective controls and fluorescently labeled detection molecules might be cheaper and less time consuming than carrying out many unsuccessful SM experiments. As the basic chemistry used for cantilevers and glass surfaces is identical, the immobilization of the molecule, which should be coupled to the cantilever, can also be tested earlier on a glass surface. An example of such a control experiment including positive and negative controls is explained in more detail in ANTICIPATED RESULTS and in **Figure 3**.

In general, the preparation of surfaces needs to be carried out in parallel to the reduction of the biomolecule, so that the freshly reduced biomolecule can be coupled to the maleimide-functionalized surface immediately. The steps of the different procedures for different types of surfaces and biomolecules are summarized in **Figure 2** to give an overview of the duration of the individual steps. For control and AFM experiments, it is possible to use commercially available amino-functionalized glass slides. In contrast, SM fluorescence experiments normally require the

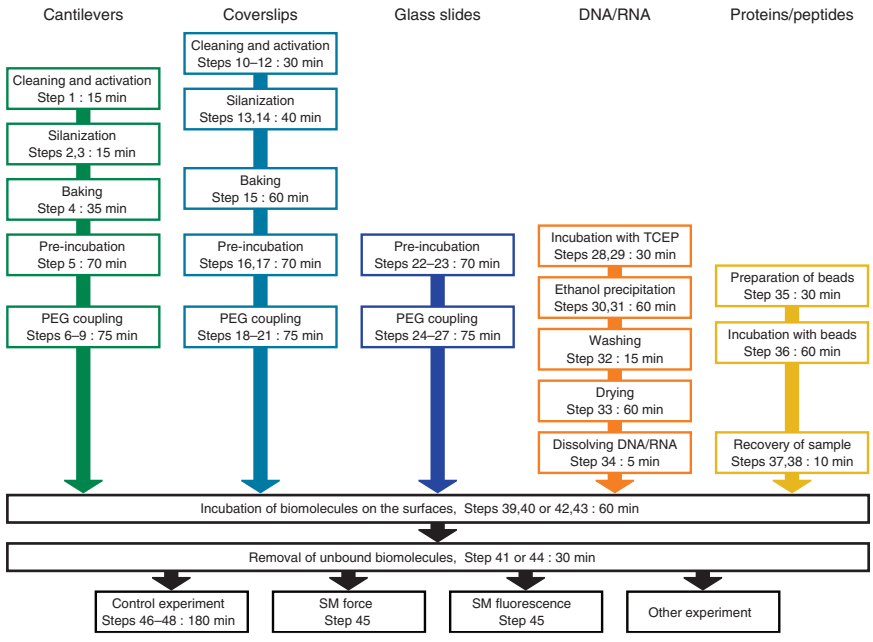

Figure 2 | An overview of the protocol. Depending on the intended experiments, various preparative steps need to be carried out in parallel before the biomolecules are coupled to the surface. For example, for an AFM experiment, the surfaces of the cantilever and of a glass support need to be functionalized while, in parallel, reduction of biomolecules needs to be carried out. The figure gives an overview about the time sequence of the necessary steps.

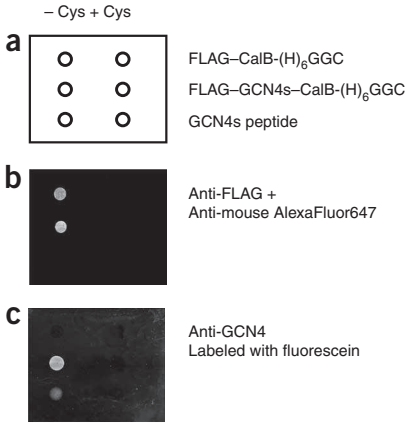

Figure 3 | An example of an experiment carried out to verify the site-specific immobilization of an enzyme. (**a**) Schematic of the position of different spots on the slide. The left column contained the protein/peptide samples dissolved in coupling buffer (−Cys). The right column contains samples that have been spotted with an excess of free cysteine (10,000×) in the coupling buffer (+Cys). (**b**) Fluorescence scan showing detection with the anti-FLAG antibody. Only enzyme variants spotted in cysteine-free buffer are detected clearly, showing that cysteine specifically blocks the coupling of enzymes to the surface. (**c**) Fluorescence scan showing detection with the antibody fragment directed against the GCN4s peptide. The anti-GCN4 antibody fragment specifically detects the enzyme fusion protein containing the GCN4s sequence spotted in the cysteine-free buffer, thereby confirming the results obtained with the anti-FLAG antibody. Furthermore, the anti-GCN4 antibody detects the immobilized peptide, which was used as a positive control.

use of coverslips, which need to be amino-functionalized with the procedure described here. To provide one example for a typical procedure, AFM experiments carried out to determine the rupture forces between an antibody and an antigen, e.g., as in Morfill *et al.*[15,27], require the reduction of both antibody and antigen while the cantilevers and the glass surface are being prepared.

MATERIALS

REAGENTS

- Cantilevers (Bio-lever without gold coating, Olympus; Park-lever, Veeco microscopes or other cantilevers consisting of either silicon or silicon nitride)
- Coverslips (Menzel Gläser)
- Amino-functionalized slides type A+ (Schott Nexterion, cat. no. 1064875)
- 3-Aminopropyl dimethyl ethoxysilane (ABCR GmbH, cat. no. AB110423)
- NHS–PEG–maleimide (MW according to the desired application; Rapp Polymere, Thermo Scientific, cat. no. 22114)
- Bond-breaker TCEP solution (Thermo Scientific, cat. no. 77720)
- Immobilized TCEP disulfide reducing gel (Thermo Scientific, cat. no. 77712)
- Ethanol abs (Roth)
- Isopropanol p.a. (Roth)
- Toluol p.a. (Roth)
- DNA- or RNA-oligonucleotides with thiol group (IBA)
- Peptide with cysteine at the desired position (Jerini Peptide Technologies GmbH)
- Recombinant protein with a single cysteine
- BSA fraction V, protease free (Roth, cat. no. T844.2)
- Borate buffer (BB, 50 mM sodium borate pH 8.5)
- Coupling buffer (CB, 50 mM sodium phosphate pH 7.2 at 4 °C, 50 mM NaCl, 10 mM EDTA)
- Sodium acetate solution (NaAc 3 M, pH 6.0)
- 10× PBS (Roche)
- Ethanol, 85% (vol/vol)

EQUIPMENT

- UV–ozone cleaner (FHR)
- Refrigerated centrifuge for Eppendorf tubes (Heraeus)
- Thermomixer (Eppendorf)
- Microarray scanner (Tecan)
- Orbitrary shaker (IKA)
- Ultrasonic bath (Sonorex)
- Vortex (Scientific Industries)
- Cold room
- Filter paper (Macherey–Nagel)
- Teflon holder for coverslips
- Several glass petri dishes (Roth)
- Disposable plastic petri dishes (Roth)
- Quadriperm petri dishes for slides (Greiner bio-one, cat. no. 96077307)

REAGENT SETUP

Silanes and PEG are moisture and/or oxidation sensitive. It is recommended to aliquot them on arrival and store them under argon or N_2 at the temperature stated by the manufacturer. Storage under argon or N_2 is also required for the amino-functionalized glass slides. In general, solvents need to be of 'p.a.' quality. For single-molecule fluorescence experiments, solvents 'for spectroscopy' should be used. Buffer salts and EDTA should be of the highest available purity. Prepared buffers BB and CB need to be filtered through a 0.22-µm membrane.

EQUIPMENT SETUP

It is recommended to use gloves in all the steps and to use only tweezers to touch cantilevers and glass surfaces. The tweezers should be cleaned with ethanol and water before using them. In several steps of the protocol, glass petri dishes, beakers, slides and coverslips are used. These should be cleaned with ethanol and then with ultrapure water for 10 min in an ultrasonic bath, and finally dried in a clean oven at 80 °C. In general, glass petri dishes or beakers should be used when working with organic solvents, whereas disposable plastic petri dishes should be used for aqueous solutions containing biomolecules to avoid nonspecific adsorption and cross-contamination between experiments.

PROCEDURE

▲ CRITICAL STEP As already mentioned in Experimental design, the preparation of surfaces needs to be carried out in parallel to the reduction of the biomolecule so that the freshly reduced biomolecule can be coupled to the maleimide-functionalized surface immediately (see **Fig. 2** for ● TIMING the individual steps).

Preparation of cantilevers ● TIMING ~3.5 h

1| Deposit the cantilevers with the tips facing up on a cleaned glass slide in a UV–ozone cleaner for 10 min.

▲ CRITICAL STEP The generated ozone does not only oxidize all organic dirt on the surface, which might interfere with the coupling reaction, but also oxidizes the surface of the cantilevers to provide a higher density of Si–OH groups for coupling.

2| For silanization, completely submerge the cantilevers in concentrated 3-aminopropyl dimethyl ethoxysilane in a small cleaned glass petri dish for 60 s at room temperature (18–25 °C).

3| Wash the cantilevers in 100-ml toluene for 1–2 min. The toluene, which remains on the cantilevers, is removed by drying the cantilever on a filter paper. Now, wash the cantilevers in 100-ml ultrapure water for 1–2 min. Again, the remaining liquid film on the cantilever surface has to be removed with a filter paper. Deposit the cantilevers with the tips facing up in a cleaned small glass petri dish.

4| Place the glass petri dish, containing the cantilevers, in an oven heated to 80 °C for 30 min. After this procedure, the cantilevers have to cool down to room temperature for 1–2 min.
▲ **CRITICAL STEP** Without baking the cantilevers, the silane layer is not stable in aqueous solutions. This step is essential for the formation of the covalent bond between the surface and the silane. When using cantilevers that are gold coated on their reverse side, irreversible bending might occur because of different thermal expansion coefficients of the gold and silicon material. In this case, the baking step might be omitted, although this will result in a lower stability of the silane layer. Ideally, cantilevers that are not gold coated should be used.

5| For every cantilever, pipette one droplet of 50-µl BB into a disposable plastic petri dish. Place the cantilevers in the droplets of BB and place the petri dish containing the cantilevers in a box with a water-saturated atmosphere to prevent evaporation. Incubate for 1 h at room temperature.
▲ **CRITICAL STEP** The reaction between the amino groups on the surface and the NHS groups of the PEG requires deprotonated amino groups. The slightly alkaline pH of the borate buffer ensures that most amino groups are deprotonated. In principle, any other buffer with a similar pH can be used as well. However, buffers containing free amino groups (such as Tris) have to be avoided, as they block the reaction of the NHS ester with the surface.

6| Ensure that the powder of NHS–PEG–maleimide is warmed up to room temperature before weighing the required amount (for one cantilever, at least 25 µl of a 50-mM solution is required). Dissolve the NHS–PEG–maleimide in BB. Centrifuge for 1 min at maximum speed at room temperature to remove potential insoluble impurities. Use the solution immediately.
▲ **CRITICAL STEP** The reactive groups on the PEG (especially the NHS ester) are sensitive to hydrolysis. Once dissolved in an aqueous solution, the reaction of the NHS ester with amino groups competes with autohydrolysis. Both the desired reaction and the autohydrolysis proceed faster at higher pH values. Therefore, the used pH is a compromise to balance stability and reactivity. Nevertheless, it is essential to bring the PEG on the surface as soon as it is dissolved. Therefore, PEG solutions cannot be reused. However, in our hands, it has been possible to reuse the same vial of PEG powder several times. Storage under argon or N_2 and warming up the vial to room temperature before opening prevents the contact of water in the air with the PEG.

7| For every cantilever, pipette droplets of at least 25 µl of PEG solution in a disposable plastic petri dish. Remove the cantilevers from the BB droplets and place them directly into the PEG solution droplets. Place the petri dish containing the cantilevers in a box with a water-saturated atmosphere to prevent evaporation. Incubate for 1 h at room temperature.

8| Remove the cantilevers from the PEG solution droplets and wash them in 100-ml ultrapure water. Store the cantilevers in a clean petri dish containing ultrapure water.

9| Proceed to 'Coupling of biomolecules to cantilevers' as quickly as possible.

Preparation of coverslips ● TIMING 4.5–5 h
10| Place the coverslips in an appropriate Teflon holder. Place the holder with the coverslips in a beaker containing 50% (vol/vol) isopropanol. Sonicate for 10 min.

11| Wash the coverslips with ultrapure water and dry them under a stream of nitrogen.

12| Deposit the coverslips in a UV–ozone cleaner for 10 min.

13| Silanize the coverslips with pure 3-aminopropyl dimethyl ethoxysilane for 30 min.

14| Wash the coverslips carefully with isopropanol. Wash with ultrapure water and dry under a stream of nitrogen.

15| Incubate the coverslips at 80 °C for 1 h.
■ **PAUSE POINT** Coverslips can be stored under argon or N_2 for a few days.

16| Pipette 5-ml BB into each of the required number of wells in a Quadriperm petri dish. Place one amino-functionalized coverslip in each well and shake for 1 h.
▲ **CRITICAL STEP** The reaction between the amino groups on the surface and the NHS groups of the PEG requires deprotonated amino groups. The slightly alkaline pH of the borate buffer ensures that most amino groups are deprotonated.

17| Take the coverslip out of the BB and dry it under a stream of nitrogen. Place it into a disposable plastic petri dish.

18| Ensure that the powder of the NHS–PEG–maleimide is warmed up to room temperature before weighing the required amount (for one coverslip, ~15 µl cm^{-2} of a 50-mM solution is required). Dissolve the NHS–PEG–maleimide in BB. Centrifuge for 1 min at maximum speed at room temperature to remove potential insoluble impurities. Use the solution immediately.
▲ **CRITICAL STEP** NHS–PEG–maleimide is sensitive to autohydrolysis (see Step 6).

19| Pipette the PEG solution on one amino-functionalized coverslip and cover it with a second coverslip to form a sandwich. For the second coverslip, either another amino-functionalized coverslip or a clean nonfunctionalized coverslip can be used. Place the coverslips in a box with a water-saturated atmosphere to prevent evaporation. Incubate for 1 h at room temperature.

20| Separate the two coverslips carefully and wash them under flowing ultrapure water. Dry the coverslip(s) under a stream of nitrogen and put them in a clean plastic petri dish.

21| Proceed to 'Coupling of biomolecules to glass surfaces' as quickly as possible.

Preparation of glass slides ● TIMING 2–2.5 h
22| Pipette 5-ml BB into each of the required number of wells in a Quadriperm petri dish (one slide fits in there exactly). Place one amino-functionalized slide in each well and shake for 1 h.
▲ **CRITICAL STEP** The reaction between the amino groups on the surface and the NHS groups of the PEG requires deprotonated amino groups. The slightly alkaline pH of the borate buffer ensures that most amino groups are deprotonated.

23| Take the slide out of the BB and dry it under a stream of nitrogen. Place it into a disposable plastic petri dish.

24| Ensure that the powder of the NHS–PEG–maleimide is warmed up to room temperature before weighing the required amount (for one coverslip, ~15 µl cm^{-2} of a 50-mM solution is required). Dissolve the NHS–PEG–maleimide in BB. Centrifuge for 1 min at maximum speed. Use the solution immediately.
▲ **CRITICAL STEP** NHS–PEG–maleimide is sensitive to autohydrolysis (see Step 6).

25| Pipette the PEG solution on one amino-functionalized glass slide and cover it with a coverslip to form a sandwich. Place the slide in a box with a water-saturated atmosphere to prevent evaporation. Incubate for 1 h at room temperature.

26| Take out the slide and wash it carefully under flowing ultrapure water. The coverslip will be removed by the flowing water. Dry the slide under a stream of nitrogen and place it in a clean container.

27| Proceed to 'Coupling of biomolecules to glass surfaces' as quickly as possible.

Preparation of DNA and RNA oligonucleotides ● TIMING 2.5–3 h
28| Dilute the TCEP stock solution (500 mM) to a concentration of 10 mM in ultrapure water.

29| For one slide/coverslip or two cantilevers, 18 µl of the respective DNA (100 µM) or RNA is necessary to carry out force spectroscopy measurements. Mix 18 µl of the respective DNA or RNA with 18 µl of 10-mM TCEP in an Eppendorf tube and store at 4 °C for 30 min.
▲ **CRITICAL STEP** On storage of thiol-containing oligonucleotides in an aqueous solution, thiols can oxidize and form disulfide bonds that are not reactive toward maleimide. Therefore, reduction of disulfide bonds is essential to ensure a high fraction of free thiols for the coupling reaction.

30| To remove TCEP from the solution, add 4-µl NaAc and vortex for 60 s. Thereafter, add 160-µl ethanol abs, mix carefully using a pipette and store the solution at −20 °C for at least 30 min.
■ **PAUSE POINT** The ethanol precipitation can be carried out at any time and kept at −20 °C for at least 1 month.

31| Take the sample(s) out of the freezer and centrifuge at maximum speed for 10 min at 4 °C. Remove the supernatant. Be careful not to disturb the pellet.

32| Wash the pellet with 800-µl 85 % (vol/vol) ethanol. Centrifuge the solution at maximum speed for 10 min at 4 °C. Remove the supernatant carefully.

33| Air-dry the pellet at room temperature until the ethanol has evaporated.

34| Dissolve the pellet in 50 µl (solution for one slide/coverslip or two cantilevers) of CB and use as quickly as possible (Step 39 or 42).
▲ **CRITICAL STEP** The composition of the buffer CB is critical for the performance of the protocol. The EDTA in the buffer slows down the rate of reoxidation of the generated free thiols.

Preparation of proteins and peptides ● TIMING 1.5–2 h

35| The starting concentration of the protein should be between 0.2 and 1.0 mg ml^{-1}. For peptides, a concentration of 2 mM is recommended. Always keep the molecules on ice. Mix TCEP beads well. Take 10 µl of beads (corresponds to 20-µl suspension as supplied by the manufacturer) and add them to 1 ml of cooled CB in an Eppendorf tube. Vortex and centrifuge at 2,500*g* for 2 min at 4 °C. Remove the supernatant and wash the beads two more times with 1 ml of CB. After the final washing step, remove the supernatant carefully.
▲ **CRITICAL STEP** The composition of the buffer CB is critical for the performance of the protocol. The pH value ensures that the reaction of maleimides is specific for thiol groups. For higher pH values, the probability increases that the reaction of maleimides also occurs with amino groups, which are present on the surface of proteins. Furthermore, the EDTA in buffer slows down the rate of reoxidation of the generated free thiols.

36| Add 10-µl protein or peptide solution and shake the mixture in a Thermomixer (set to 4 °C) at maximum speed for at least 1 h and for a maximum of 2 h.

37| Take the sample(s) out of the Thermomixer and centrifuge at maximum speed for 2 min at 4 °C. Remove the supernatant containing the reduced proteins or peptides without sucking any beads into the pipette.

38| Dilute the reduced proteins and peptides to an appropriate concentration (depending on the type of experiment) using CB. Use as quickly as possible (Step 39 or 42). In the meantime, keep the samples on ice.

Coupling of biomolecules to cantilevers ● TIMING 1.5 h

39| For every cantilever, pipette droplets of at least 25 µl of the desired protein, peptide or oligonucleotide solution in a clean plastic petri dish. Remove the cantilevers from the ultrapure water (see Step 8) and place them directly into the prepared protein, peptide or DNA solution droplets. Ensure that only a small amount of water remains on the cantilever to avoid significant dilution of the solution of the biomolecule.

40| Incubate for at least 1 h at 4 °C in a water-saturated atmosphere.
■ **PAUSE POINT** This reaction can also be performed over night.

41| Wash the cantilevers in 100-ml PBS (or any other buffer used for the final experiment) for 1–2 min and repeat this procedure one more time with fresh PBS. Store the cantilevers in PBS in a clean container at room temperature until use.
▲ **CRITICAL STEP** This washing step is crucial for the following experiments. If noncoupled biomolecules remain on the surface, they might interfere with the experiment, e.g., by reducing the amount of available binding sites in AFM experiments.

Coupling of biomolecules to glass surfaces ● TIMING 1.5 h

42| Pipette the desired amount of reduced sample on the surface at the desired position(s). Spots with a volume starting from 1 µl can be prepared this way depending on the application. For AFM or single-molecule fluorescence measurements, use at least 20 µl per spot. For initial tests to check the performance of the protocol, 1–2 µl of every sample is sufficient.

In the case of sensitive proteins, it is recommended to carry out this step in a cold room. This also prevents the evaporation of spotted sample(s) while preparing other surfaces in parallel.

43| Incubate for at least 1 h at 4 °C in a water-saturated atmosphere.
■ **PAUSE POINT** This reaction can also be carried out overnight.

44| Pipette 5 ml of PBS (or any other buffer used for the final experiment) into the required amount of wells of a Quadriperm petri dish. Remove the spotted liquid from the surface of the slide or coverslip by suction, e.g., with a pipette or with a pipette tip attached to a pump. Ensure that the solution is removed as completely as possible without touching the surface. Place the slide in the buffer immediately. Place on a shaker at 25 r.p.m. for 5 min. Exchange the buffer and shake again for 5 min. Depending on the experiment, either store the slide in fresh buffer until use or dry the slide under a stream of nitrogen.
▲ **CRITICAL STEP** This washing step is crucial for the following experiments. If noncoupled biomolecules remain on the surface, they might interfere with the experiment, e.g., by reducing the amount of available binding sites in AFM experiments.

AFM or fluorescence experiment
45| Proceed with the planned experiments.
■ **PAUSE POINT** In principle, it should be possible to store cantilevers and surfaces dry either under argon or N_2, or in an appropriate buffer. However, this has never been tested in detail, as storage might not only lead to inactivation or degradation of biomolecules but also to the accumulation of dirt from the environment on the surfaces.
? TROUBLESHOOTING

Control experiment ● TIMING 1.5–3 h
46| Prepare a PBS solution containing 0.4 % (wt/vol) BSA. Dilute the fluorescently labeled detection molecule(s) to the appropriate concentration in 5 ml of the prepared buffer. For labeled oligonucleotides, a concentration of 0.1 μM is recommended. For proteins (e.g., antibodies), the final concentration can vary between 0.1 and 10 μg ml^{-1} depending on the affinity of the respective interaction. Pipette the mixture into a Quadriperm petri dish and shake the slide or coverslip for 1 h at 25 r.p.m. in dark surroundings to prevent bleaching of the fluorescent dye.

47| Wash the slide or coverslip at least 2× in 5-ml PBS+BSA in the Quadriperm petri dish in dark surroundings. Take out the slide or coverslip and dry it under a stream of nitrogen. (The dried buffer on the surface does not interfere with the fluorescence measurements. Never wash the slide or coverslip with water, as this might disrupt the biological interaction.)

48| Scan the fluorescence on the surface using a microarray scanner or a confocal microscope.

● TIMING
Steps 1–9, Preparation of cantilevers: ~3.5 h
Steps 10–21, Preparation of coverslips: 4.5–5 h (silanization procedure 2–2.5 h)
Steps 22–27, Preparation of glass slides: 2–2.5 h
Steps 28–34, Preparation of DNA and RNA oligonucleotides: 2.5–3 h
Steps 35–38, Preparation of proteins and peptides: 1.5–2 h
Steps 39–41, Coupling of biomolecules to cantilevers: 1.5 h
Steps 42–44, Coupling of biomolecules to glass surfaces: 1.5 h
Step 45, AFM and/or flourescence experiment: several hours
Steps 46–48, Control experiment: 1.5–3 h

? TROUBLESHOOTING
SM experiments do not work
This is most likely the result of either a low fraction of functionally coupled molecules or a large number of nonspecific events. In many cases, it is extremely difficult to determine the reason for this with SM experiments. Therefore, a control experiment, as described below, is highly recommended to identify the step in the procedure that causes the problems. The control experiment requires a fluorescently labeled detection molecule that can form a specific interaction with the immobilized molecule. The use of a biomolecule that does not carry a thiol group and should therefore not couple to the surface provides a meaningful negative control reporting on the degree of nonspecific binding to the surface. A list of possible detection molecules is provided in the following and an example of such a control experiment is shown in ANTICIPATED RESULTS.

Suggested detection molecules for different types of biomolecules

An immobilized DNA oligonucleotide can be detected most efficiently with a complementary oligonucleotide carrying a fluorescent label. Many different labels are available to suit the specific needs for detection. However, we recommend the use of Cy3.

RNA oligonucleotides can be detected using the same strategy, provided that they do not form a secondary structure. In that case, the use of a fluorescently labeled ligand that binds to the RNA structure might be an alternative. If there are no other possibilities, the RNA oligonucleotide might be fluorescently labeled itself.

In the case of proteins, one can use fluorescently labeled antibodies in a similar way as in western blots. If a recombinant protein was prepared with a tag for purification (e.g., His tag as suggested previously), it is a good strategy to use an antibody against the tag. Alternatively, any other ligand (antigen, covalent inhibitor and so on) can be fluorescently labeled and used for the detection.

For peptides, it might be more difficult to identify and prepare a fluorescently labeled binding partner. It might be an interesting option to include the amino-acid sequence of one of the 'tags' (His tag, FLAG tag, Strep tag, HA tag and so on) into the peptide sequence at either terminus, as fluorescently labeled antibodies are commercially available for these tags.

When the final goal is to analyze the interaction between two biomolecules with force spectroscopy, it is needless to say that the most useful results will be obtained if the second binding partner used in force spectroscopy experiments is used as the detection molecule.

While carrying out control experiments, one might observe two problems. In one case, the ratio of signal intensities for the biomolecule of interest and the negative control is low (low fraction of functionally and site-specifically coupled molecules). In the other case, the background is high (fluorescent impurities or high nonspecific binding). In the following section, possible reasons and potential solutions will be summarized on the basis of our experience:

Little or no fluorescent signal at the positions wherein the biomolecule of interest was immobilized or high signal for the negative control

1. The coupling of PEG to the surface did not work. This is very likely if the fluorescent background is very high in addition. Check the reactivity of the NHS group on the PEG with other methods.
2. The coupling of the biomolecule did not work. Although the maleimide group is relatively stable and a deactivation under the conditions of the experiment is unlikely, it might become deactivated if the PEG is stored for long times or under inappropriate conditions (e.g., UV light). Check the reactivity of the maleimide group on the PEG with other methods. Alternatively, the thiol group on the biomolecule could be the problem. Check thiol accessibility with other methods. If the biomolecule solution is relatively old or has not been stored properly and the reaction has worked before, consider replacing it.
3. The concentration of the immobilized biomolecule was too low. The concentrations of the biomolecules described in this protocol are those that worked best in our experiments. Perform a dilution series of the biomolecule and spot these different dilutions on the surface.
4. The concentration of the fluorescently labeled detection molecule is too low. Perform a dilution series to find the optimal concentration.
5. No specific interaction with the labeled detection molecule takes place. Check the interaction between the biomolecules with other methods. If the solution of the detection molecule is relatively old or has not been stored properly and the reaction has worked before, consider replacing it.

High fluorescence background

1. One of the used reagents contains fluorescent impurities. This can be analyzed by analyzing the surface after every step.
2. The coupling of PEG did not work and the fluorescently labeled detection molecule adsorbs nonspecifically to the surface. This is very likely if no specific spots appear on the expected positions. Check the reactivity of the NHS group on the PEG with other methods.
3. The coupling of biomolecules did not work. We have observed that the detection molecules adsorb nonspecifically if no specific interaction can take place. Although the maleimide group is relatively stable and a deactivation under the conditions of the experiment is unlikely, it might become deactivated if the PEG is stored for long times or under inappropriate conditions (e.g., UV light). Check the reactivity of the maleimide group on the PEG using other methods. Alternatively, the thiol group on the biomolecule could be the problem. Check thiol accessibility with other methods. If the biomolecule solution is relatively old or has not been stored properly and the reaction has worked before, consider replacing it.
4. The concentration of the fluorescently labeled detection molecule is too high. Perform a dilution series to find the optimal concentration.

5. No specific interaction with the labeled detection molecule takes place. Check the interaction between molecules using other methods. If the solution of the detection molecule is relatively old or has not been stored properly and the reaction has worked before, consider replacing it.
6. The number of washing steps before scanning the surface was not sufficient. Extend the washing procedure.
7. Depending on the detection molecule used, it might become necessary to block the remaining maleimide groups on the surface (e.g., with cysteine).

ANTICIPATED RESULTS

Here we first describe a typical control experiment to confirm the specific immobilization of an enzyme to a glass surface. This example will be followed by a short summary of single-molecule experiments that used this protocol. The intention of the control experiment was to investigate whether the enzyme *Candida antarctica* lipase B (CalB) is immobilized site specifically through a C-terminal cysteine. CalB was prepared as a fusion protein carrying an N-terminal FLAG tag[30] and a C-terminal His tag, followed by two glycines and a cysteine. In addition, one CalB variant contained an additional 12-amino-acid-long peptide sequence (GCN4s) between the N-terminal FLAG tag and the *calB* gene. Both the FLAG tag and the His tag, as well as the GCN4s peptide, can be detected with corresponding antibodies[15,27,31]. In the experiment described here, detection was based on anti-FLAG/anti-mouse AlexaFluor647 antibodies or alternatively on a fluorescein-labeled antibody fragment directed against the GCN4s sequence. The experiment included the GCN4s peptide containing a C-terminal cysteine as a positive control. Samples containing an excess of cysteine were used as the negative control. Results (**Fig. 3**) show that coupling can be blocked completely with an excess of free cysteine, proving specific immobilization by the cysteine residue. Similar results have been obtained for a range of biomolecules (oligonucleotides[5,7,8,32,33], peptides[15,27], recombinant antibody fragments[15,27] and enzymes[9,26]) and surfaces (glass slides and coverslips[5,7–9,15,26,27,32,33], Si_3N_4 cantilevers[5,7,8,15,27,32–34], agarose beads[9] and even PDMS).

The described method has been used for two series of SM experiments in which AFM was used to manipulate the biological system and a TIRF microscope was used to read out the result of this manipulation. In the first series of experiments, fluorescently labeled DNA oligonucleotides were picked up with the AFM cantilever at one position and subsequently deposited at another position with nanometer precision ('single molecule cut-and-paste'[5,7,8]). Because of the possibility of adjusting the functionalization density easily, single molecules could be picked up with high efficiency and two-dimensional nanostructures could be assembled and imaged using the TIRF microscope.

In the second series of experiments, an enzyme molecule was stretched periodically using an AFM while its activity was monitored with the TIRF microscope[6]. To carry this out, the enzyme was coupled to a coverslip through its C-terminal cysteine. At the N-terminus, the GCN4s peptide was fused, which allowed picking up the enzyme with an anti-GCN4 antibody fragment coupled to the cantilever. Once the antibody–antigen interaction was established, the enzyme was stretched until this interaction ruptured and the cycle was repeated. While performing these stretching and relaxation cycles, the conversion of a fluorogenic substrate into a fluorescent product was detected with the TIRF microscope, revealing that the applied force had an influence on the activity of the enzyme.

In addition to these combined AFM-fluorescence experiments, SM force measurements have been carried out to analyze the interactions between DNA oligonucleotides[32,33] and the interaction between peptides and recombinant antibody fragments[15,27]. All experiments showed very low nonspecific binding and very high interaction frequencies (up to 50%). As a result, very good statistics have been obtained, allowing the discrimination of only small differences in the binding strength of these interactions[15,27]. For several examples, the whole range of loading rates, which is accessible using the AFM, could be measured on one day with one cantilever only[27,33].

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