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Optimized straight forward procedure for covalent surface immobilization of different biomolecules for single molecule applications

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ABSTRACT

Covalent chemisorption of biomolecules to surfaces with high density and low unspecific background is prerequisite for most optical and mechanical single molecule experiments and accordingly, many recipes have been developed. However, new establishment of the surface functionalization process in the lab usually is still difficult and time consuming due to the complex procedures containing many pitfalls. Therefore, based on the known recipes, we developed and optimized a simple straight forward protocol. We demonstrated it resulting in a high density of the coupled biomolecules, homogeneous surfaces and a low unspecific background when binding nucleic acids, peptides and proteins.

The protocol was optimized for borosilicate cover glasses and silicon nitride atomic force microscope cantilevers commonly used in single molecule experiments and takes advantage of commonly used chemicals. It consists of only four steps, silanol group generation, amination, grafting of poly(ethylene glycol) to the surface and biomolecule coupling. All individual steps were optimized comparing different variations partially described in the literature. Finally, a detailed description is provided which allows avoiding most sources of contamination, often being a main hurdle on the way to single molecule experiments.

1. Introduction

Due to the exceptional sensitivity of single molecule detection and its ability to detect unique events in an ensemble of molecules it presents a huge potential for the study of biorecognition processes. The development of biosensors and supports with conjugated biomolecules has important applications in areas such as environmental science and biomedical research.

However, due to problems associated with the sensitivity, reproducibility, long-term stability, and non-specific binding of biomolecules to surfaces, biosensors for DNA, peptides and proteins are not widely available from commercial sources. In order to circumvent these problems new approaches have to be developed to create biosensors with high chemical stability, high reproducibility and fewer non-specific surface adsorption artefacts.

Attachment of DNA, peptides and proteins to a solid support is of great interest for biotechnological, molecular biological and sensitive diagnostic applications. Different solid supports, such as glass, silicon, silicon nitride, magnetic beads and polymers are used for single molecule application studies. Different immobilization methods, including entrapment, adsorption and chemical binding are applied to couple different biomolecules both covalently and non-covalently to surfaces. Due to the high background resulting from non-specific adsorption with non-covalent immobilization methods, covalent immobilization which selectively links biomolecule probes to solid supports is the method of choice for single molecule applications.

To allow easy access to bio-functionalized surfaces we developed a simple straight forward protocol for coupling different biomolecules (DNA, peptides and proteins) to silicon nitride AFM tips and borosilicate glasses with different biomolecules (DNA, peptides and proteins) in only three steps (Fig. 1): (i) generation of amino groups as grafting sites on the surface, (ii) attachment of a heterobifunctional PEG linker with its one end using a pre-activated carboxylic group, and (iii) coupling of biomolecules via a peptide bond formation. These three steps and the pre-activation of glass and silicon nitride surfaces to generate silanol groups before amination have been analyzed and optimized to gain a reproducible and high coupling efficiency and homogenous surface functionalization.

A comparison of surface amination through amino silane (APTES) and silanol group esterification with ethanolamine hydrochloride is described in the literature [1] and both methods

Abbreviations: AFM, atomic force microscope (or microscopy); BSA, bovine serum albumin; DMSO, dimethylsulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; H_2O_2, hydrogen peroxide; H_2SO_4, sulphuric acid; HCl, hydrogen chloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MeOH, methanol; NHS, N-succinimidyll; PBS, phosphate buffered saline; PEG, poly(ethylene glycol); SSC, saline-sodium citrate.

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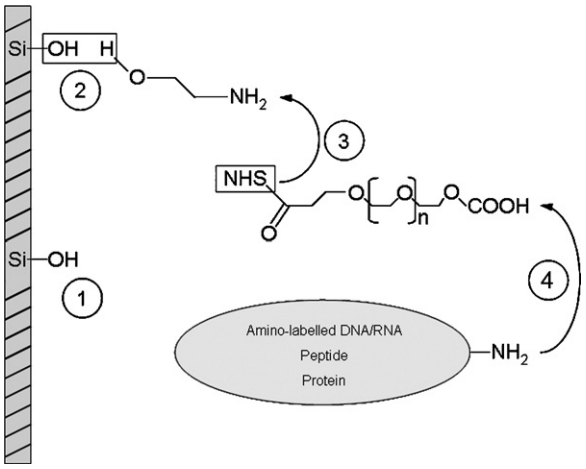


Fig. 1. Scheme of the immobilization procedure. Freshly cleaned and pre-activated support was incubated for 24 h in solution of 5 M ethanolamine hydrochloride in water-free DMSO at room temperature (1). After the esterification reaction of ethanolamine hydrochloride to the surface silanol groups, the supports were washed in DMSO, pure ethanol and water and then dried in a nitrogen stream (2). Aminated supports were immersed for 1 h in a dry DMSO solution containing 2 mM of heterobifunctional poly(ethylene glycol)-linker (NHS-PEG-$COOH_{MW3400}$) and 0.5% (v/v) triethylamine. The supports were washed afterwards in chloroform and water (3). Covalent immobilization of different biomolecules with free accessible amino groups was achieved via peptide bond formation. Here one-step direct coupling with EDC or a two-step indirect coupling with pre-activated carboxylic groups through EDC/NHS-activation was performed, which depends on the desired biomolecule for immobilization. After immobilization, the surfaces were washed stringent with an adequate washing procedure, dependent on the attached biomolecule (4).

were proven to be equally suitable to create an acceptable number of amino grafting sites for poly(ethylene glycol) attachment. However, ethanolamine hydrochloride serves better as amination substance in respect to the overall binding rate and has shown homogeneous coating compared to silanization in our observations. Generally, alkoxysilane molecules hydrolyze rapidly in water forming isolated monomers, cyclic oligomers and large intramolecular cycles [2,3], whereas the dominating species is dependent on the silane type, concentration, pH, temperature and reaction time as well as storage conditions. It has been shown, that silanizing with amino ethoxy silane on solid surfaces produces no homogeneous surfaces, but "islands" with large dimensions [4]. The polysiloxane structure inside such islands is also non-uniform and is composed of a 3D network of polysilane [5,6]. However, most single molecule applications and ligand–receptor investigations with AFM, optical tweezers and magnetic tweezers require a well-defined linker length, which is difficult to achieve using silanes because of their high tendency of self-polymerization. Instead we used ethanolamine hydrochloride, which creates homogeneous monolayers, an important prerequisite for homogeneous functionalization procedures.

To allow the immobilized biomolecules to interact with other molecules in their native way a distance of several nanometres between the biomolecule and the surface through flexible linkers is needed. Linear PEG chains have been commonly used to attach single biomolecules to glass surfaces, beads and AFM tips [7–12]. In comparison to other linker molecules, such as carboxyamylose [13] or dextran [14], poly(ethylene glycol) is simpler in handling, well characterized and chemically and physically inert. Additionally, it allows a rapid and free re-orientation of the attached biomolecule and also shields the surface due to non-specific interactions between biomolecules and surface [15,16]. The carboxylic group of the custom-synthesized heterobifunctional NHS-PEG-COOH applied here can be used for biomolecule immobilization and reacts less often non-specifically compared to the more commonly used amino and aldehyde end groups [11,17–19]. In this study, the binding capacity of three different PEG linkers (NHS-PEG-$COOH_{MW3400}$, NHS-PEG-$Aldehyde_{MW800}$ and NHS-PEG-$Maleimide_{MW3400}$) was compared with different immobilization strategies.

The final step in the immobilization process was also analyzed and optimized depending on the desired biomolecule to be attached. In this study an NH_2-labelled ssDNA oligonucleotide, a His_{10} containing peptide as His-tag and the prion–protein antibody IgG_{saf32} were tested to establish a general functionalization method for all kinds of biomolecules. For the final biomolecule attachment, the known method of peptide binding [20] including the use of EDC and sulfo-NHS with a high covalent binding rate [7] was applied reproducibly, without damaging the biomolecules or affecting their native behavior.

In this study a general "easy-to-use-protocol" for reliable and improved surface functionalization with ssDNA oligonucleotides was developed and evaluated by quantitative comparison to existing protocols. Finally, the applicability of the optimized method was demonstrated also for the immobilization of peptides and proteins.

2. Experimental procedures

2.1. Materials

Super pure-grade materials were used, when commercially available. Si_3N_4 AFM tips (MLCT series) were bought from Veeco Instruments (Dourdan, France). NHS-PEG-$COOH_{MW3400}$ was custom-synthesized by LaysanBio (AL, USA). Epoxy-PEG-$COOH_{MW3400}$ was purchased from Iris Biotech (Marktredwitz, Germany). NHS-PEG-$Aldehyde_{MW800}$ was synthesized by Hermann Gruber (Keppler-University Linz, Austria). NHS-PEG-$Maleimide_{MW3400}$ was purchased from Rapp Polymere (Tübingern, Germany). Borosilicate glass slides were purchased from Schott (Jena, Germany). Used DNA oligonucleotides (sequence of binding-ssDNA: 5′-NH_2-CCACTCGTGACGCATTCACCTCAGC-AGCACTCCTCCTCGG-3′; complementary fluorophor labelled ssDNA: 5′-CCGAGGAGGAGTGCTGCTGAGGTGAATGCGTCACGAGTGG-Atto647N-3′) were synthesized by PURIMEX (Grebenstein, Germany). Antibody IgG_{saf32} were purchased from SpiBio (Montigny-le-Bretonneux, France). His_{10} containing peptide (sequence: N-GGSGSGHHHHHHHHHH-C) was synthesized at the BMFZ (Heinrich-Heine University, Germany). SybrGreenII was bought from Sigma–Aldrich (Hamburg, Germany). Hellmanex cleaning detergent was purchased from Hellma (Mühlheim, Germany). Labelled Penta-His antibody labelled with Alexa488 was bought from Qiagen (Hilden, Germany). Alexa Fluor 633 F(ab′)-fragment of goat anti-mouse IgG (H + L) was purchased from Invitrogen (Karlsruhe, Germany). Ultra pure water was gained from the water processing apparatus Arium 611 from Sartorius (Goettingen, Germany).

2.2. Glass supports

The experiments in this study were done with glass slides due to their simpler handling compared to cover slips commonly used in single molecule experiments. Since cover glasses are generally made of borosilicate, the experiments were consequently done on borosilicate cover slides. Furthermore, an approximately 15% higher immobilization density on borosilicate glass slides was achieved compared to common used soda-lime glass slides (data not shown).

2.3. General cleaning procedure of borosilicate glass supports and silicon nitride AFM tips

Cleaning before pre-activation and amination with ethanolamine hydrochloride is important for glass and silicon

nitride surfaces. The glass cleaning procedure was developed in the Institute of Physical Chemistry (Heinrich-Heine University) for both, cleaning and reducing fluorescence background and controlled via TIRF measurements. Glass surfaces were covered in 5% (v/v) Hellmanex solution and sonicated for 20 min at 37 °C. After washing 10 times with water, the surfaces were covered in pure acetone and sonicated for further 20 min. After an additional washing step in water a 20 min ultrasonication in pure ethanol was performed. The plate was washed again with water before a final 20 min ultrasonication step in pure water. After cleaning, the surfaces were dried in a nitrogen flow. The silicon nitride AFM tips were just washed twice in dry chloroform and dried in a nitrogen stream before further functionalization.

2.4. Working protocol for surface functionalization and immobilization of ssDNA oligonucleotides

This easy to use surface functionalization and amino-labelled oligonucleotide immobilization protocol is based on different published protocols and is used in the following as a starting point in the detailed protocol optimization process (Fig. 1).

Pre-activation of a cleaned surface is important in order to generate a high density of silanol groups on the surfaces, which allows surface amination through an esterification reaction. The borosilicate glass and silicon nitride supports were pre-activated in piranha solution (3:1 (v/v), H_2SO_4:H_2O_2) for 30 min at room temperature. After washing shortly in pure water and drying in a nitrogen flow, the supports were immersed in 5 M ethanolamine hydrochloride in water-free DMSO for 24 h at room temperature for homogeneous amination. This procedure was based on studies where ethanolamine amination was tested with different molarities and temperatures [1]. After amination, the supports were washed in dry DMSO, in pure ethanol and finally 5 times with ultra pure water and dried again in a nitrogen flow. In the second step of functionalization, the amino-reactive heterobifunctional poly(ethylene glycol) NHS-PEG-$COOH_{MW3400}$ was immobilized on the supports which serves as a linker between the surface and the amino-labelled ssDNA oligonucleotide. 2 mM of the PEG linker was dissolved in water-free chloroform with 0.5% (v/v) triethylamine and the supports were incubated for 1 h at room temperature in this PEGylation solution. The supports were washed several times in ultra pure water after the PEGylation process. As our standard process, amino-labelled ssDNA oligonucleotides were immobilized to the free accessible carboxylic groups of the PEG linker via peptide binding. The peptide binding method is based on the one-step method with EDC in acidic buffer as carboxyl activation substance [7] to achieve the highest possible ssDNA immobilization rate. 10 μM amino-labelled ssDNA oligonucleotide was dissolved in 100 mM MES buffer (pH 4.75) with 50 mM EDC and added to the PEGylated supports. After 1 h reaction time at room temperature, the supports were washed for 5 min with ultra pure water, 10 min in a 100 mM KCl solution and finally again 5 min with ultra pure water.

2.5. Sample preparation and specific ligand binding

For the sample preparation and the optimization of the protocol in this study, the following sample configuration was used.

The 40b ssDNA oligonucleotide was covalently bound to the surface according to the working protocol (Fig. 2A and C) as a volume defined droplet of 0.5 μl. To test non-specific binding of ssDNA to the PEGylated surface, complementary 40b fluorophor labelled ssDNA was given on the support with a drop of about 20 μl volume in a wet atmosphere which covered a wider area around the previously immobilized ssDNA to allow simultaneous control of unspecific background interactions. The hybridization was done with a concentration of about 10 μM on the prepared surface in

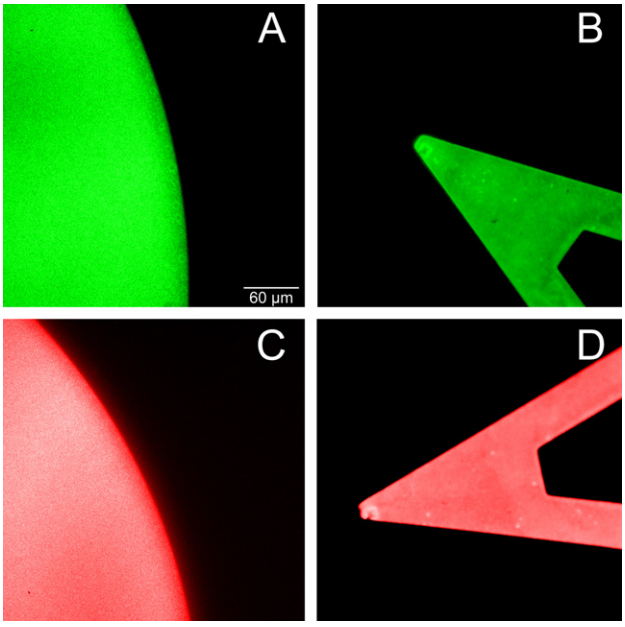


Fig. 2. Immobilization of 40b ssDNA oligonucleotide on borosilicate glass slides and silicon nitride AFM tips. On glass slides (A and C), 40b amino-labelled oligonucleotide was covalently bound to the PEG-modified surface in a round spot (approximately 200 μm). On silicon nitride supports (B and D), 40b amino-labelled ssDNA was covalently bound to the entire PEG-modified cantilever. (A and B) Fluorescence control was done via 0.02× single strand DNA staining SybrGreen. (C and D) A complementary 40b ssDNA oligonucleotide, labelled with Atto647N fluorophor was given to the supports.

20 mM TRIS/HCl hybridization buffer (pH 8.4) containing 100 mM NaCl and washed afterwards to remove non-specific ssDNA surface adsorptions in 2× SSC buffer at 65 °C (10°C below the melting temperature) for 5 min, in 0.01× SSC buffer at room temperature for another 5 min and finally stored in the hybridization buffer for further measurement.

The silicon nitride AFM tips were completely covered in the ssDNA binding and hybridization solutions because of their small dimensions (Fig. 2B and D).

To analyse the peptide immobilization quality, the immobilized His_{10} containing peptide was detected via the fluorophor labelled His-tag specific Penta-His-antibody labelled with an Alexa488 fluorophor.

The IgG_{saf32} antibody immobilization was verified through an Alexa633-labelled second antibody of goat anti-mouse IgG. The experimental procedure is described in detail in the respective description.

2.6. Experimental evaluation procedure

To evaluate the successful immobilization, the overall binding efficiency and the homogeneity of the functionalization procedure, the surfaces and AFM tips were analyzed via fluorescence detection of fluorophor labelled specific ligands binding to the immobilized biomolecules.

The samples were measured using an inverted microscope (Olympus IX71, Hamburg, Germany) with a peltier-cooled ECCD camera (Andor IXON, 512 × 512 pixels, Belfast, Ireland). Fluorophor excitation was achieved by a 150 W Xenon-lamp with the specific filter sets (AHF, Tübingen, Germany) due to the different fluorophores of the counter molecules (F41-054 for Alexa488, F41-008 for Alexa633 and Atto647N). On each sample the fluorescence intensity (in counts/s) was measured by taking the average over five areas of 10 × 10 pixels. Each experiment was performed five times to test the reproducibility.

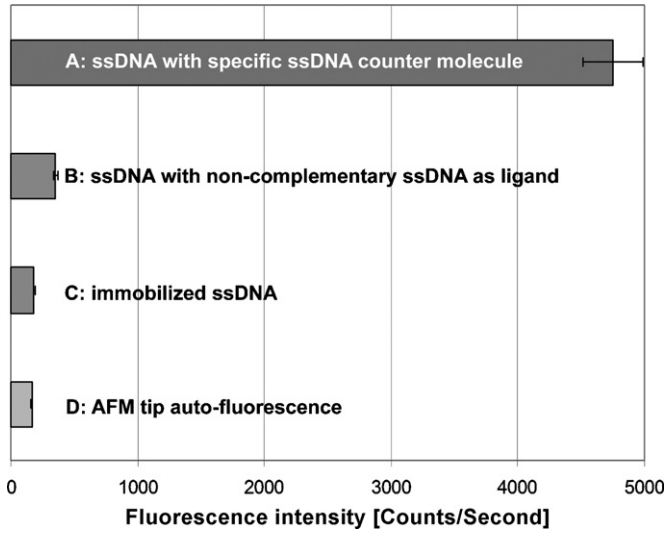


Fig. 3. DNA immobilization on silicon nitride AFM tips. (A) Immobilized 40b amino-labelled ssDNA, hybridized with complementary Atto647N-labelled oligonucleotide. (B) Same functionalization as in (A), but hybridized with non-complementary Atto647N-labelled ssDNA. (C) Immobilized ssDNA as in (A) and (B), but without further treatment with fluorescent counter molecule. (D) Auto-fluorescence of cleaned silicon nitride AFM tips.

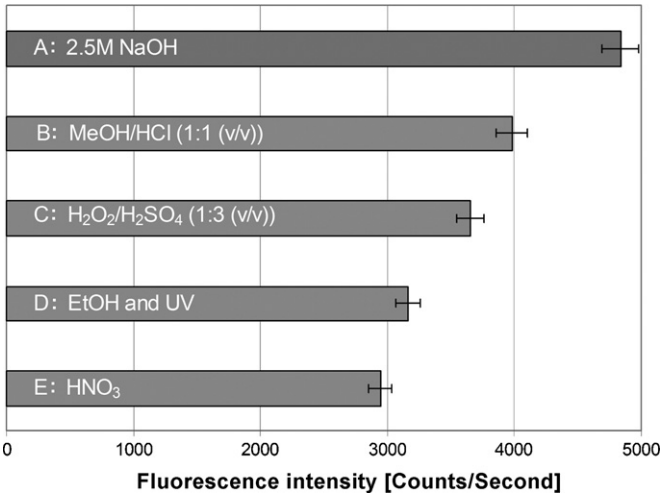


Fig. 4. Comparison of different methods to generate silanol groups on borosilicate glass surface. Solutions and treatment prior to the amination step were varied. (A) 24 h in 2.5 M NaOH at room temperature. (B) 1 h in 1:1 (v/v) MeOH:HCl at room temperature. (C) 30 min in piranha solution (1:3 (v/v) H_2O_2/H_2SO_4). (D) 30 min in ethanol, dried with N_2, and 1 h exposed to UV. (E) 1 h in nitric acid in boiling water bath. All surfaces were washed afterwards shortly in ultra pure water and dried with N_2 before further functionalization.

2.7. Verification via atomic force microscopy measurements

We used combined TIR (total internal reflection) fluorescence microscopy with an atomic force microscope (MFP3D, Asylum Research, USA) to verify the immobilization of biomolecules on surface and the topography change of glass supports due to different pre-activation substances.

2.8. Supplemental information

A brief flowchart with the functionalization and biomolecule immobilization strategy and a commented protocol for each step of the process can be found in the supplemental information.

3. Results and discussions

3.1. Covalent immobilization of ssDNA oligonucleotides to glass and silicon nitride supports

The amino-labelled 40b ssDNA oligonucleotide was successfully immobilized covalently to PEG-functionalized borosilicate glasses and silicon nitride supports with the described working protocol. The results from this preparation will be taken as reference data when varying the protocol in the further described optimization process. The immobilization on borosilicate glass and silicon nitride AFM cantilevers, which was analyzed by SybrGreenII ssDNA staining and by hybridization of complementary Atto647N labelled ssDNA as ligand, is shown in Fig. 2. The immobilization is homogeneous with low fluorescence background of about 3% on borosilicate glass by analysis via complementary fluorophor labelled ssDNA oligonucleotide. This low background ratio demonstrates that the used NHS-PEG-COOH linker also passivates the surface in respect to non-specific binding. The non-specific adsorption could occur from the complementary oligonucleotide itself or due to the attached Atto647N fluorophor, adsorbing to the PEG chain or the carboxylic end groups of the PEG linker.

The ssDNA immobilization with NHS-PEG-$COOH_{MW3400}$ as linker molecule was applied to silicon nitride AFM cantilevers in the same way (Fig. 3) as to borosilicate glass slides. For detailed analysis of the immobilization and detection quality, the ssDNA immobilization experiments were repeated five times. To determine the amount of non-specific ssDNA and linked fluorophor interaction with the functionalized silicon nitride surface, three negative tests were simultaneously performed. In the first negative test ssDNA functionalized silicon nitride AFM tips were incubated in a solution with non-complementary Atto647N-labelled ssDNA, in a second, ssDNA functionalized cantilevers were measured without further treatment and finally, the auto-fluorescence of cleaned cantilevers was determined.

The auto-fluorescence of the silicon nitride support and the immobilization without fluorescent ligand molecule shows an equal non-specific adsorption amount of 3% in respect to the immobilization on borosilicate glass slides. The successful and reproducible immobilization on silicon nitride AFM cantilevers agrees with the binding characteristics observed on borosilicate glass surfaces (Fig. 4C).

3.2. Optimizing the single functionalization steps to achieve higher immobilization efficiency

For optimizing the surface functionalization protocol, all individual steps in the entire functionalization process were varied systematically with respect to the working protocol, detecting the density and homogeneity via the complementary fluorophor labelled DNA oligonucleotides hybridized to the immobilized ssDNA. The images were taken with the IXON EMDDC camera and results are given in camera counts/s.

3.3. Pre-activation of supports

Already the pre-activation, which aims for silanol group generation on the substrate, is a very important step with respect to the amount of biomolecules finally bound to the surface. Thus, we compared four different protocols described in the literature to pre-activate borosilicate glass supports (Fig. 4) with our approach of using sodium hydroxide as pre-activation substance. Freshly cleaned borosilicate glass slides (see methodical part) were (i) covered in nitric acid (65%) in a boiled water bath for 1 h [7]; (ii) treated in pure ethanol for 10 min, dried in nitrogen stream and exposed to UV-light (UVO-Cleaner 42-220, Jelight Company Inc.,

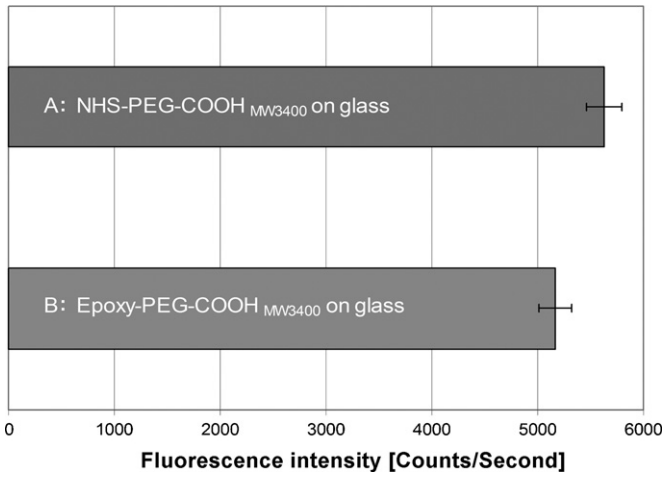


Fig. 5. Comparison between direct coupling of Epoxy-PEG-$COOH_{MW3400}$ to clean, pre-activated glass surface and the use of NHS-PEG-$COOH_{MW3400}$ with ethanolamine hydrochloride aminated surface. (A) Indirect coupling of NHS-PEG-$COOH_{MW3400}$ to prior aminated surface in dry DMSO solvent with 0.5% (v/v) triethylamine. (B) Direct coupling of Epoxy-PEG-$COOH_{MW3400}$ in dry $CHCl_3$ solvent with 0.5% (v/v) triethylamine.

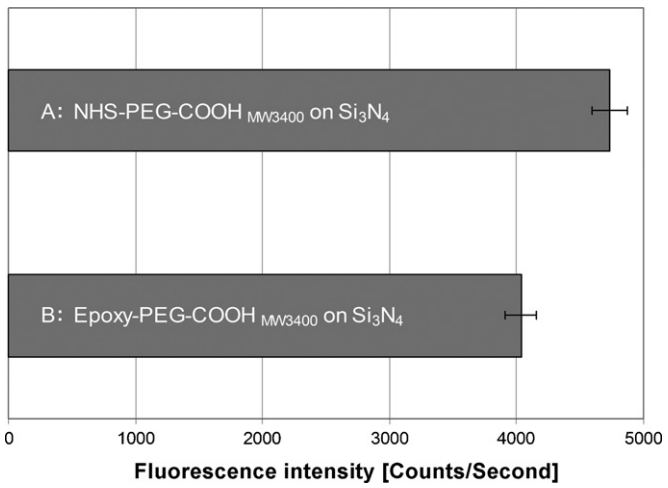


Fig. 6. Comparison between direct coupling of Epoxy-PEG-$COOH_{MW3400}$ to clean, pre-activated silicon nitride AFM tips and the use of NHS-PEG-$COOH_{MW3400}$ with ethanolamine aminated surface. (A) Indirect coupling of NHS-PEG-$COOH_{MW3400}$ to prior aminated AFM tip in dry DMSO solvent with 0.5% (v/v) triethylamine. (B) Direct coupling of Epoxy-PEG-$COOH_{MW3400}$ in dry $CHCl_3$ solvent with 0.5% (v/v) triethylamine.

Irvine, USA) for 1 h [11]; (iii) deposited in fresh piranha solution for 30 min at room temperature [8,17]; (iv) incubated for 1 h in 1:1 (v/v) MeOH:HCl [14] and (v) stored for 24 h in 2.5 M NaOH at room temperature. After treatment, the pre-activated glass supports were rinsed quickly with ultra pure water and dried in a nitrogen flow. Further treatment was performed as described in Section 2.4.

The comparison showed that the use of 2.5 M NaOH over 24 h was the best method to generate a high number of silanol groups on borosilicate glass supports, which allows a high density of amination and further covalent biomolecule immobilization. The commonly used piranha solution, which was also applied in our standard functionalization procedure, results in 24% less fluorescence intensity compared to the NaOH pre-activation.

Additionally, the surface roughness of cleaned and NaOH-activated glass was investigated with an AFM to determine, if the strong alkalise treatment significantly changes the surface topography, which would be a disadvantage of this pre-activation method. However, the AFM measurements showed no difference in the surface topography and the roughness of about 1.0 ± 0.2 nm in average was equal to non-treated cleaned glass.

3.4. Direct coupling of silanol-reactive PEG linker to glass and silicon nitride supports

We took advantage of the general property of epoxy rings to bind covalently to amino groups and hydroxyl groups, and also tested the direct coupling of a heterobifunctional PEG linker which bears an active epoxy ring to the silanol groups of the pre-activated glass surfaces and silicon nitride AFM tips. This alternative functionalization procedure saves preparation time and possible contamination in respect to the used substances and solvents for the additional functionalization and washing steps.

2 mM Epoxy-PEG-$COOH_{MW3400}$ was dissolved in dry $CHCl_3$ with 0.5% (v/v) triethylamine and was let to react with the pre-activated glass (Fig. 5) and silicon nitride supports (Fig. 6) for 1 h at room temperature. Here, all supports were pre-cleaned as described before, pre-activated with 2.5 M NaOH for 24 h, washed shortly with ultra pure water and were then either directly used for PEGylation with Epoxy-PEG-$COOH_{MW3400}$ or functionalized with ethanolamine hydrochloride and NHS-PEG-$COOH_{MW3400}$. Further treatment was performed as described in Section 2.4.

The direct binding of the Epoxy-PEG-$COOH_{MW3400}$ linker to the pre-activated silanol groups showed successful oligonucleotide immobilization. Compared to the three-step procedure immobilization yielded approximately 91% on the borosilicate glass supports (Fig. 5) and 85% on silicone nitride AFM tips (Fig. 6). Thus, this two-step functionalization procedure serves as a suitable alternative depending on the desired density of immobilized biomolecules for both supports. The loss in binding yield may be explained by hydroxylation of the epoxy group during binding and the use of non-modified or non-reactive PEG linkers during synthesis. These unmodified or abreacted PEG molecules could adsorb to the surface during the binding process and hinder other PEG linkers to bind.

3.5. Effect of solvent for NHS-PEG-COOH linker attachment

The use of the polymeric linkers plays also an important role with respect to the passivation against non-specific interactions of the biomolecules with the surface. At this point, the density of the used linkers is a crucial factor. Thus, beside the coupling chemistry also the reaction condition, especially the solvent is important for covalent surface coupling of the used polymeric linkers. Therefore, after cleaning, pre-activation and amination of the borosilicate glasses according to the working protocol given above, the NHS-PEG-$COOH_{MW3400}$ linker was dissolved in either water-free chloroform or water-free DMSO containing 0.5% (v/v) triethylamine and was given to the fresh, aminated supports for 1 h at room temperature for covalent attachment (Fig. 7). Further treatment was performed as described in Section 2.4.

Generally, using dry solvents is crucial for obtaining high binding yields of the PEG linker. However, the comparison of dry chloroform and dry DMSO (Fig. 7) shows, that DMSO as reaction solvent leads to an approximately 42% higher number of successfully bound heterobifunctional PEG linkers on aminated borosilicate glass. The PEG itself is less soluble in DMSO as in chloroform and accordingly for larger NHS-PEG-COOH chains (MW 35,000) it was observed, that DMSO was not a suitable solvent any more. Short PEG linkers lead to a less extended coil of the solvated polymer and thus enable a more tight packing on the surface.

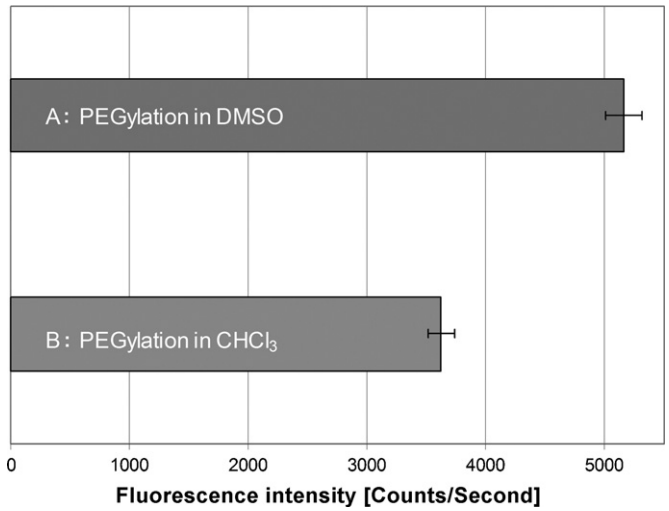


Fig. 7. Comparison of two solvents for the covalent PEG linker coupling on aminated surfaces. The surfaces were functionalized with amino-labelled 40b ssDNA and hybridized with complementary fluorescent Atto647N-labelled ssDNA as counter molecule. (A) NHS-PEG-$COOH_{MW3400}$ coupled in dry DMSO with 0.5% (v/v) triethylamine. (B) NHS-PEG-$COOH_{MW3400}$ bound in dry chloroform with 0.5% (v/v) triethylamine.

3.6. Biomolecular coupling chemistry

For the immobilization of biomolecules most commonly aldehyde and maleimide groups [11,17–19] are used. Thus, the use of the NHS-PEG-$COOH_{MW3400}$ linker was compared quantitatively with the two commonly used poly(ethylene glycol) linkers, NHS-PEG-$Aldehyde_{MW800}$ and NHS-PEG-$Maleimide_{MW3400}$. The cleaning, pre-activation, amination and PEGylation steps were done on borosilicate glass according to the working protocol given above.

For amino-labelled ssDNA coupling to the aldehyde end group of the NHS-PEG-$Aldehyde_{MW800}$ linker, the pegylated supports were treated with 10 μM of the same amino-labelled oligonucleotide which was used for the standard immobilization procedure in PBS buffer (pH 7.4). Additionally, 1 μl of chemical elimination solution, which contains 450 μl H_2O, 50 μl 100 mM NaOH and 32 mg $NaCNBH_3$, was added to the ssDNA coupling solution. After 1 h reaction time at room temperature, the washing of the surfaces was done by immersing in ultra pure water for 5 min, 10 min in a 100 mM KCl solution and finally again 5 min in ultra pure water.

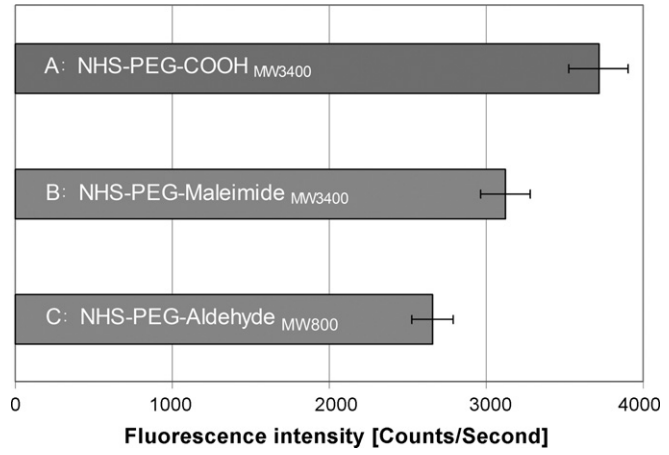


Fig. 8. Comparison between three immobilization strategies with different PEG linker molecules. (A) NHS-PEG-$COOH_{MW3400}$ as PEG linker with immobilized amino-labelled ssDNA oligonucleotide. (B) NHS-PEG-$Maleimide_{MW3400}$ as linker molecule with coupled thiol-labelled oligonucleotide with the same sequence as in (A). (C) NHS-PEG-$Aldehyde_{MW800}$ linker with same bound amino-labelled ssDNA as in (A). Fluorescence emission data results due to hybridized complementary ssDNA labelled with Atto647N as fluorophor.

For immobilization of ssDNA to the NHS-PEG-$Maleimide_{MW3400}$ linker, thiol-modified ssDNA oligonucleotides were given to the supports in PBS buffer at pH 7.4 at room temperature for 1 h reaction time. The surfaces were washed afterwards with the same procedure as described for aldehyde coupling.

Further hybridization with the complementary fluorescent ssDNA was done for all surfaces equally and measured as described in the methodical part.

Using a carboxyl end group for coupling the amino-labelled ssDNA oligonucleotides, an approximately 28% higher amount of coupled molecules compared to the binding via NHS-PEG-$Aldehyde_{MW800}$ (Fig. 8) was detected in spite of the fact, that the smaller molecular weight of the aldehyde linker in principle might favor a more dense packing on the surface. Compared to the use of NHS-PEG-$Maleimide_{MW3400}$, still a 16% higher amount was observed. Thus, the use of NHS-PEG-$COOH_{MW3400}$ appears as the best choice as reactive group for immobilization of biomolecules to the PEGylated surface with respect to a high immobilization efficiency and high reproducibility.

3.7. Optimized protocol for surface functionalization and DNA immobilization

On summary, the optimization process resulted in the following optimized protocol: The pre-activation has to be done with both, the borosilicate glass and silicon nitride supports in 2.5 M NaOH for 24 h at room temperature. After rinsing quickly with ultra pure water, the supports have to be incubated in 5 M ethanolamine hydrochloride solution with dry DMSO as solvent and containing 0.5% (v/v) triethylamine for another 24 h at room temperature. PEGylation with 2 mM NHS-PEG-$COOH_{MW3400}$ was performed in dry DMSO as solvent and 0.5% (v/v) triethylamine for 1 h reaction time at room temperature. Alternatively, the supports can be directly coated with 2 mM Epoxy-PEG-$COOH_{MW3400}$ linker in dry chloroform with 0.5% (v/v) triethylamine for 1 h at room temperature. The PEG coated surfaces should be washed several times with ultra pure water afterwards. Amino-labelled ssDNA, dissolved in 100 mM MES buffer (pH 4.75) with 50 mM EDC, should be given to the PEGylated supports for 1 h at room temperature. Finally, the supports have to be washed 5 min with ultra pure water, 10 min with 100 mM KCl and again 5 min with ultra pure water. The supports can be stored at this point in appropriate buffer or in dried state.

3.8. Surface functionalization and DNA immobilization validation via AFM imaging

Finally we applied an additional quality control via AFM imaging and manipulation. Therefore we functionalized a standard borosilicate cover slide commonly used in single molecule fluorescence and AFM experiments according to our optimized functionalization protocol. Subsequently, we immobilized 40b ssDNA oligonucleotides onto a spot with approximately 3 mm of diameter. AFM imaging of the edge of the spot revealed the height difference between the only PEG coated glass slide and the spot, originating from the additional layer thickness due to the immobilized ssDNA oligonucleotides (Fig. 9A).

In our total internal reflection fluorescence setup routinely used for single fluorophor detection and AFM based nano-manipulation experiments we imaged the spot after hybridizing the fluorophor labelled complementary 40b ssDNA oligonucleotides. Before imaging, we used the nano-manipulation setup to scratch out a rectangular area of about 5 μm × 3.5 μm with a force of about 7 nN. Fig. 9B clearly shows the removal of the fluorophor labelled

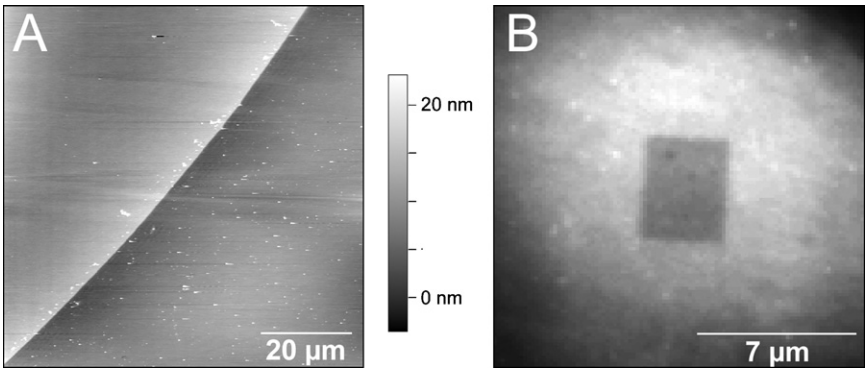


Fig. 9. (A) Topography image of dense immobilized 40b amino-labelled ssDNA oligonucleotides on a prepared PEGylated surface. (B) Fluorescence TIRF image of the same prepared surface as in (A), after hybridization of complementary fluorophor (Atto647N) labelled DNA oligonucleotide. A rectangular area with 5 μm × 3.5 μm in dimensions was scrapped free of DNA via the AFM tip with a contact force of 7 nN.

oligonucleotides as a dark rectangular area within the fluorescent surface.

3.9. Covalent peptide immobilization on PEG-functionalized supports

For immobilization of biomolecules via the peptide binding method, the carboxyl groups of the PEG linker on the surface were activated prior to immobilization. If the biomolecules to be immobilized bears no accessible carboxyl groups at the C-terminus, the activation can be done in one step with the immobilization by adding just EDC to the buffer. Otherwise carboxyl groups have to be activated in an additional previous step to avoid polymerization of the biomolecules. To demonstrate that the immobilization protocol developed here also serves for peptide coupling and to compare the two step pre-activation procedure with the one-step binding method, a 16 amino acids peptide containing 10 histidine (N-GGSGSGHHHHHHHHHH-C) was immobilized according to the optimized protocol. The C-terminus of the peptide was protected by an amide modification to prevent polymerization of the peptides during surface coupling. For comparison, the same peptide was immobilized with pre-activation of the surface bound carboxyl groups of the PEG linker, which may serve as an example for coupling peptides without inactivated C-terminus. Here, the carboxylic end group of the PEG linker was activated with NHS in presence of EDC in 100 mM MES buffer (pH 4.75) prior to the peptide binding in PBS buffer [7].

In the one-step method, analogue to the ssDNA immobilization, 10 mM of the peptide with protected C-terminus was let to react in 100 mM MES buffer (pH 4.75) with 50 mM EDC to the surface for 1 h at room temperature. In the two-step method, the carboxylic groups of the PEG linker were pre-activated with 50 mM EDC and 50 mM NHS in 100 mM MES buffer (pH 4.75) for 1 h. After washing shortly in ultra pure water, 10 μM of the peptide was given to the pre-activated surface in slight basic HEPES buffer (pH 8) at room temperature for 1 h reaction time.

The peptide immobilization was detected via binding of a histidine specific antibody (Penta-anti-HIS, Qiagen) labelled with Alexa488 and fluorescence imaging (Fig. 10).

The one-step immobilization procedure led to an approximately 29% higher number of immobilized peptides compared to the two-step procedure. This may happen due to hydrolyzation of NHS during the activation of the carboxylic groups and the washing procedure prior to peptide immobilization. While studying peptide immobilization, we also tried to immobilize a His_6 peptide without the additional N-GGSGS-C linker sequence. However, in that case the N-terminus of the His_6 was not accessible for coupling to the surface bound carboxyl groups. Only by adding the linker sequence N-GGSGS-C to the N-terminus the peptide could be bound covalently to the carboxylic groups of the NHS-PEG-$COOH_{MW3400}$ linker.

3.10. Covalent protein immobilization on PEG-functionalized supports

The optimized protocol for surface functionalization was also tested for the coupling of proteins by immobilizing the prion–protein antibody IgG_{saf32} (Fig. 11) analogue to the two-step immobilization procedure of peptides described before.

After pre-activation of the carboxyl groups of the PEG-functionalized glass surface through 50 mM EDC and 50 mM NHS in 100 mM MES buffer (pH 4.75) for 45 min, 1 mM IgG_{saf32} was given to the surface in PBS buffer with a moderate lower pH of about 6 for 1 h at room temperature and washed afterwards four times in PBS buffer (pH 7.4). For fluorescent analysis, 125 mM Alexa633-labelled second antibody of goat anti-mouse IgG fragment (H + L) in PBS buffer (pH 7.4) with 1.5% (v/v) BSA was given to the surface at room temperature for 1 h and washed again four times with PBSTT buffer (PBS (pH 7.4), 0.05% Tween 20, 0.2% Triton X100) and finally three times in PBS buffer (pH 7.4).

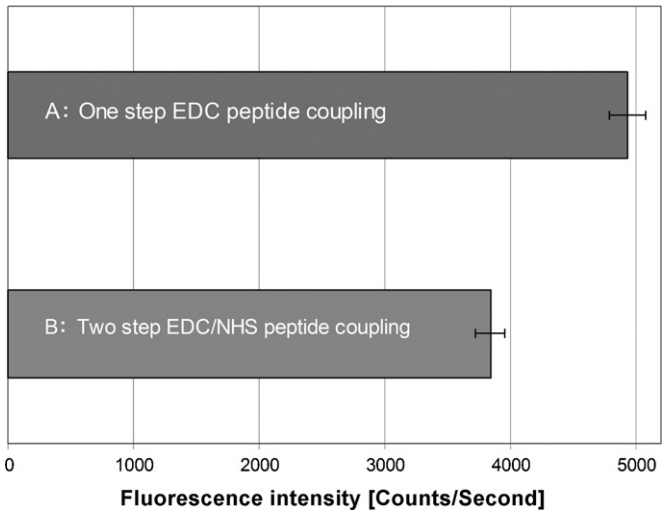
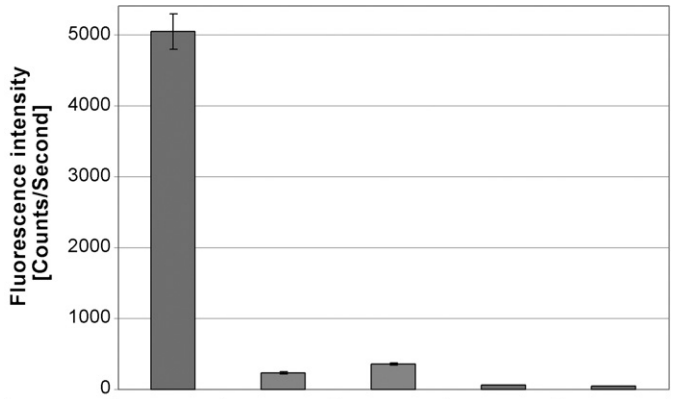


Fig. 10. Covalent immobilization of a 16 amino acid peptide containing 10 histidine and fluorescence analysis via a Penta-anti-His antibody labelled with Alexa488 fluorophor. Comparison between the direct and indirect coupling method. (A) Peptide with protected carboxylic group (amide modification) was immobilized directly in 100 mM MES buffer (pH 4.75) and 50 mM EDC for 1 h. (B) Carboxylic group of PEG linker was pre-activated with 50 mM EDC and 50 mM NHS in 100 mM MES buffer (pH 4.75). Peptide was immobilized in HEPES buffer (pH 8) for 1 h reaction time.



Sample	A	B	C	D	E
Ethanol amine	X	X	X	X	X
PEG linker	X	X	X		
First Antibody	X				
BSA Blocking	X	X			
Second Antibody	X	X	X	X	

Fig. 11. Prion-antibody IgG_{saf32} was immobilized through the two-step EDC/NHS-coupling method to PEGylated surface with NHS-PEG-$COOH_{MW3400}$. Fluorescence measurements were performed using fluorescent second antibody Alexa Fluor 633 F(ab′) fragment of goat anti-mouse IgG (H + L). (A–F) Different sample compositions with positive and negative controls. Sample conditions specified in table below.

To investigate the amount of non-specific interactions, the passivation characteristics of the NHS-PEG-$COOH_{MW3400}$ linker and the influence of additional BSA (3% in H_2O) in respect to surface blocking after protein immobilization, several additional negative tests were performed and compared (Fig. 11).

Similar to the two-step EDC/NHS binding method of peptides, the antibody IgG_{saf32} was successfully immobilized on the NHS-PEG-$COOH_{MW3400}$ modified glass support (Fig. 11A), where the different experimental compositions are also shown.

The negative tests show an overall low background signal (Fig. 11E) of ethanolamine hydrochloride as amination substance and low non-specific physisorbtion of the fluorescent second antibody (Fig. 11D) on the amine surface layer. A low, but still significant adsorption about 5.9% (Fig. 11C) of the fluorescent second antibody was observed on the PEGylated surface which can be explained through spontaneous low-rate peptide binding and non-specific adsorption to the carboxylic end groups of the PEG linker. However, it can be reduced down to approximately 3.4% by additional BSA (3%) blocking (Fig. 11B).

Additionally, previous experiments have shown (data not shown), that directly bound proteins using epoxy silane without a PEG linker in-between, were not accessible for second antibody counter molecules, which shows that a linker is important for the immobilized protein accessibility for applications in sensoring and interaction detection.

4. Conclusions

In this study, a ubiquitous, simple and straight forward coupling procedure of biomolecules (DNA, peptides and proteins) with a heterobifunctional poly(ethylene glycol) linker to borosilicate glass and silicon nitride supports was developed. In addition, the passivation character of the PEG linker which prevents the non-specific adsorption of the different biomolecules to the functionalized surfaces was proven. In respect to the commonly used functionalization methods which were compared in this study for each single step in the entire functionalization process, we yield an increase up to 3-fold in binding efficiency by optimizing each functionalization step and using a NaOH solution of high molarity for pre-activation and heterobifunctional NHS-PEG-$COOH_{MW3400}$ as biomolecule linker molecule. By applying a silanol-reactive PEG linker, which offers a faster and easier preparation effort, we still achieve a significant higher coupling density up to 173%, compared to the common methods, although the binding efficiency is slightly lower than our optimized three-step procedure with ethanolamine as surface linker molecule.

The reliable functionalization procedure is highly reproducible and leads to homogeneously modified surfaces, which is important for single molecule applications, biosensors and other sensitive ligand–receptor interaction studies. Especially for this kind of research, the demonstrated modification method does not influence or harm the native structure and behavior of the individual biomolecules. Also, the use of the PEG linker to depart the biomolecules from the surface is essential as it allows steric freedom and thus fully operating molecules and also leads to low non-specific adsorption. Taken together, the insights from this study provide a great approach to bio-functionalization of any probes with single molecules.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.colsurfb.2009.02.011.

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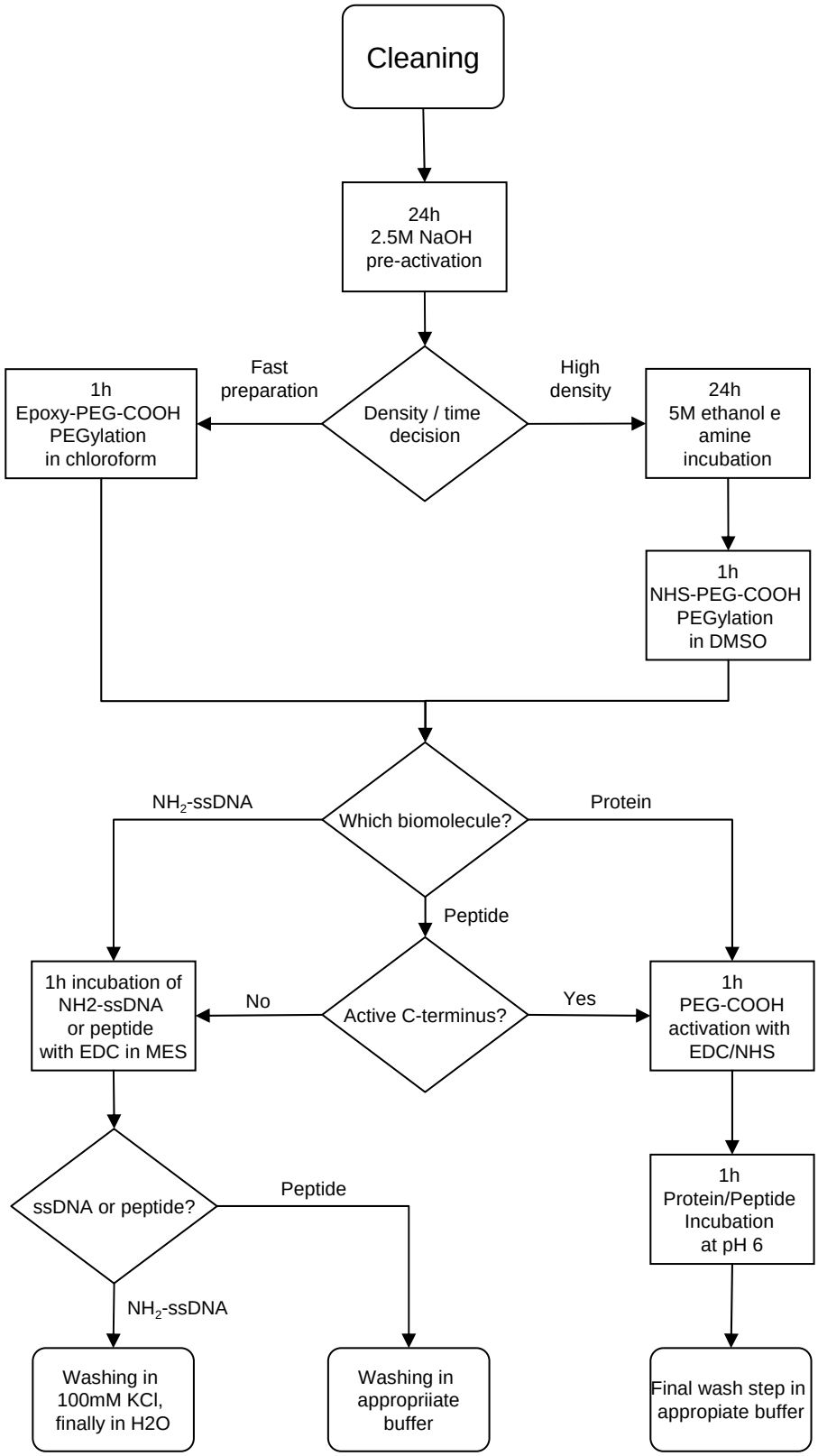
Functionalization and immobilization of biomolecules to glass and silicon nitride supports



Flowchart: Strategy for borosilicate glass and silicon nitride functionalization and covalent coupling of biomolecules

Optimized protocol for borosilicate glass and silicon nitride AFM Tip functionalization and immobilization of biomolecules

0. General advices

The preparation has been done with cleaned pincers, pre-cleaned glass repositories (see step 2), cleaned gloves (with water) and all steps were performed in a clean bench to avaoid contamination.

1. Materials

The materials used for the optimized functionalization and biomolecule immobilization were tested with the TIR single molecule fluorescence method. Following substances were tested and finally used:

Substance	Company	Product number
Chloroform	Sigma-Aldrich	650471
DMSO	Merck	1.02950.0500
MES	Sigma	M3671
Triethylamine	Fluka	65897
N-Hydroxysuccinimid	Aldrich	13,067
Ethanolamine hydrochloride	Sigma	E6133
Ethanol	Merck	1.00980.2500
Aceton	Merck	1.00022.2500
Sodium chloride	Merck	1.06400.0500
Tri-sodium citrate	Normapur	27 833.294
EDC	Fluka	03450
Potassium chloride	Merck	1.04938.0500
Sodium hydroxide	J.T. Baker	0402

1. Cleaning of supports (glass, glass beads, silicon, silicon nitride)

This cleaning method is for cleaning the surfaces from inorganic and organic dirt and also to reduce fluorescent background:

Attention: Glass repositories and the glass holding tool (we use custom made teflon blocks) have to be cleaned prior to use with the same procedure to reduce contamination. Also, for the

different washing steps, the glass repositories have to be filled and emptied completely, if possible.

- in 5% **HELLMANEX II** (in H2O) 20 minutes ultrasonic
- 10 times washing with H2O
- **70% Ethanol / 30% Acetone** (v/v) 20 minutes ultrasonic
- 10 times washing with H2O
- In **H2O** 20 minutes ultrasonic
- 10 times washing in H2O
- Dry in nitrogen or argon flow
- Store in excicator

2. Pre-activation of supports

This is important to generate silanole groups on surface to get enough grafting sites for ethanol amine esterification. Here it is recommended to use glass repositories for incubation.

- store clean supports in **2,5M NaOH** for **24 hours** in appropriate, clean glass storage
- washing shortly with **H2O** in appropriate, clean glass storage (fill and empty completely)
- dry in argon or nitrogen flow
- go **FAST** to next functionalization step

3. Surface amination with ethanol amine hydrochloride

- wash supports in water-free **DMSO** in appropriate, clean glass storage
- store supports in 5M **ethanol amine hydrochloride** in **DMSO** (water-free) solution for 24 hours (ethanol amine hydrochloride can be well dissolved before at 60°C) in appropriate, clean glass storage
- wash with **DMSO** in appropriate, clean glass storage
- washing several times with **water** (fill and empty completely)
- dry in nitrogen or argon flow

4. Coupling heterobifunctional PEG linker to freshly aminated surface

- dilute 2mM **NHS-PEG-COOH** (MW3400) (LaysanBio, USA) with **0,5% triethylamine** (v/v) in dry chloroform
- store supports in PEG solution (to save material put a drop between two glass slides as sandwich) for 1 hour at room temperature

- wash several times with **H2O** in appropriate, clean glass storage (fill and empty completely)
- dry in nitrogen or argon flow

5. A. Immobilization of NH_2-labelled ssDNA and c-terminus-inactivated peptide to PEG linker

- dilute 1-10 µM **NH_2-labelled ssDNA or peptide** in 100mM **MES buffer** (pH 4,75) with 50mM **EDC**(1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide)
- store supports in solution for 1 hour (or put drops of solution to glass slides in wet atmosphere)
- wash 5 minutes in water (**ssDNA**) (fill and empty completely)
- wash 10 minutes with **100mM KCl (for ssDNA) or appropriate buffer (e.g. HEPES for peptide)** in appropriate, clean glass storage (fill and empty completely)
- wash 5 minutes with **water (for ssDNA) or appropriate buffer**

5. B. Immobilization of c-terminus-active peptides and proteins to PEG linker

- dilute peptides or proteins in appropriate buffer (PBS, HEPES, etc…) - But not amino groups containing buffer!
- pre-activate supports with 50mM **EDC**, 50mM **NHS** in 100mM **MES buffer** (pH 4.75) for 1 hour
- wash shortly with **water** (fill and empty completely)
- store supports (or drop at glass slides in wet atmosphere) for 1 hour in prior preparated **peptide/protein solution**
- wash several times with appropriate buffer and going further with adequate procedures (e.g. BSA blocking, aply second antibody, apply interaction partner, etc.)