Chapter 23

Immobilization of Biomolecules onto Silica and Silica-Based Surfaces for Use in Planar Array Biosensors

Lisa C. Shriver-Lake, Paul T. Charles, and Chris R. Taitt

Summary

Several methods are described in which a biological recognition molecule – a critical element in any biosensor – is immobilized onto a silica or silica-based sensing substrate. Although several variations are described, the methods for covalent immobilization share a common theme and are generally composed of three steps: modification of the surface to add specific functional groups (using appropriate silanes or an amine or carboxyl-containing hydrogel), covalent attachment of a crosslinker through one of its reactive moieties, and finally, covalent linking of the biomolecule (recognition element) to the remaining reactive moiety of the crosslinker. One final method is presented in which the surface is modified with a highly hydrophobic silane and a glycolipid recognition element immobilized, essentially irreversibly, by hydrophobic interactions. All of the methods described have been successfully used to immobilize biological recognition molecules onto sensing surfaces, with full functionality in biosensor-binding assays.

Key words: Silanization, Crosslinking, Biomolecules, Immunoassay, Array, Detection, Biosensor.

1. Introduction

A key component of any biosensor is the biological molecules used to recognize and quantify the target of interest. These recognition species can be enzymes, antibodies, artificial and natural receptors, peptides, carbohydrates, or nucleic acids. The recognition molecules are chosen for their specificity and affinity for the analyte, as well as their ability to convert a recognition event into a measurable change in physical properties of the system. As they are immobilized onto the sensing surface in most sensor systems,

Avraham Rasooly and Keith E. Herold (eds.), *Methods in Molecular Biology: Biosensors and Biodetection, Vol. 504*

DOI:10.1007/978-1-60327-569-9_23

the means used to attach these biomolecules to the surface is a critical factor in sensor development and optimization. Retention of antigenicity, binding affinity, and/or enzymatic activity by the recognition molecule not only requires functional integrity and correct orientation of the immobilized species but may also be influenced by steric hindrance, molecular freedom of movement, and diffusional barriers.

This chapter describes several methods to attach different biomolecules to silica or silica-based surfaces, although other surfaces such as indium tin oxide, tin oxide, tantalium pentoxide, and platinum may also be suitable for similar treatment (1–6). **Subheadings 3.2.1** and **3.2.2** describe direct covalent linking of a biomolecule to the surface. The biomolecule may be the recognition species itself (e.g., antibody, peptide, enzymes), or may be an intermediary species, such as avidin or its derivatives, for subsequent *noncovalent* linking to a biotin-labeled molecule. Use of an intermediary species such as avidin enables preparation of a generic surface, with the end user able to change the (biotin-labeled) recognition species at will. An additional immobilization method **(Subheading 3.2.3)** utilizes hydrophobic interactions between a long chain (C_{18}) silane layer on the sensor surface and the hydrophobic moiety of glycolipids. A separate section has been included describing preparation of hydrogel surfaces for biomolecule immobilization **(Subheading 3.2.5)**; these surfaces are capable of binding higher quantities of immobilized biomolecules, providing higher avidity "capture" surfaces in detection assays.

In addition, **Subheading 3.2.4** describes general methods for creating patterned arrays of biomolecules on treated surfaces. These arrays can be used to screen samples for the presence of many targets of interest using any one of a number of multianalyte sensors described in the literature (7–19). The NRL Array Biosensor described in a later chapter is one such system that takes full advantage of the two-dimensional nature of the patterned substrate to enable simultaneous testing of multiple samples for multiple targets.

2. Materials

2.1. Cleaning Procedures (for Subheading 3.1)

1. *Coplin jars:* Eight slots to process batches of 16 slides.
2. Microscope slides were purchased from A.J. Daigger, Vernon Hills, IL.
3. *HCl*: *Methanol solution*: Mix hydrochloric acid (HCl) and methanol 50/50 v:v. For Coplin jars with eight slots, 100 mL is sufficient to cover the microscope slides. Personnel should

wear safety goggles or full face shield, a lab coat, and acid-resistant gloves.

4. *Sulfuric acid*: As this is a strongly acidic material, personnel should wear safety goggles or full face shield, a lab coat, and acid-resistant gloves.
5. *KOH solution*: Dissolve 10 g potassium hydroxide in 100 mL methanol. This is a very basic solution. Personnel should wear safety goggles or full face shield, a lab coat, and base-resistant gloves.

2.2. Mercapto-Modified Surfaces (for Subheading 3.2.1)

1. Glove bag was obtained from the Instruments for Research and Industry (I^2R, Cheltenham, PA). The glove bag should be located in a chemical hood to reduce exposure to silane odors and toluene fumes.
2. High purity toluene and methanol were purchased from Aldrich Chemical Co., St. Louis, MO.
3. *MTS/toluene solution*: This solution should be prepared in a glove bag under nitrogen just prior to use to preserve the reactive thiols. Mix 1 mL mercaptopropyltriethoxysilane (MTS, Fluka Chemical Corp., St Louis, MO) with 49 mL toluene to make a 2% MTS solution (*see* **Notes 1** and **2**).
4. *GMBS/EtOH solution*: Dissolve 12.5 mg N-[γ-maleimidobutyryloxy]succinimide ester (GMBS, Fluka Chemical Corp., St. Louis, MO) in 250 µL dimethyl sulfoxide (DMSO). Absolute ethanol (43 mL, EtOH, Warner-Graham, Cockeysville, MD) is added to this solution. This solution is prepared immediately before use, as the reactive groups degrade (*see* **Note 3**). GMBS is moisture sensitive and must be stored at 4°C in a desiccator.
5. *Phosphate buffered saline, pH 7.4 (PBS, Sigma Chemical Co., St. Louis, MO, P-3813)*: Prepared with 18 mΩ water from Millipore Milli-Q system per manufacturer's directions.
6. *NeutrAvidin (Pierce Chemical Co., Rockland, IL)*: A stock solution of 10 mg/mL NeutraAvidin in PBS is prepared by adding 1 mL PBS to 10 mg NeutrAvidin vial. Just prior to use, 120 µL of the 10 mg/mL stock is added to 40 mL PBS.
7. *Biomolecule solution (thiol–amine linking)*: 10–50 µg/mL (antibodies, enzymes) or 20–250 µg/mL (peptides) in PBS (*see* **Note 4**).

2.3. Amine-Modified Surfaces (for Subheading 3.2.2)

1. *Amino silane solution*: Mix 45 mL methanol and 5 mL deionized water. Add one drop of acetic acid. To this, add 1 mL of 3-aminopropyl triethoxysilane (Gelest, Inc., Tullytown, PA). Prepare immediately before use.
2. *BS^3 solution*: Dissolve 1.43 mg bis(sulfosuccinimidyl) suberate (BS^3, Pierce, Rockland, IL) in 50 mL of 10 mM sodium

phosphate, pH 6.0; the final concentration of this solution is 50 µM (*see* **Note 5**). Prepare immediately before use.

3. *GMBS/EtOH*: Dissolve 12.5 mg *N*-succinimidyl-4-maleimidobutyrate (GMBS, Pierce, Rockland, IL) in 0.25 mL anhydrous dimethyl sulfoxide (DMSO); this solution can be kept for several hours. Add GMBS/DMSO solution to 43 mL absolute ethanol and mix vigorously. Once diluted in ethanol, use the GMBS solution immediately (*see* **Note 3**). GMBS is moisture-sensitive and must be stored in a dessicator at 4°C. Store opened stock bottle of EtOH in glove bag.
4. *Biomolecule solution (amine–amine linking,* **Subheading** "Amine–Amine Homobifunctional Crosslinkers"*)*: 5–25 µg/mL in PBS for proteins. For peptides and carbohydrates where achieving maximal surface density is important, concentrations may be as high as 1 mg/mL (in PBS).
5. *Biomolecule solution (amine–thiol linking,* **Subheading** "Amine–Thiol Crosslinking Heterobifunctional Crosslinkers"*)*: 5–25 µg/mL (proteins) or 10–50 µg/mL (peptides and thiol-derivatized sugars) in 10 mM sodium phosphate/10 mM NaCl, pH 7.5. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Pierce) is added to the protein/peptide/sugar solution at a 0.8 molar equivalent *(11)* (*see* **Note 6**).

2.4. Hydrophobic Silane-Modified Surfaces (for Subheading 3.2.3)

1. *OTS solution*: Add 0.4 mL octadecyltrichlorosilane (OTS, Fluka, Ronkonkoma, NY) to 100 mL anhydrous toluene. Use immediately (*see* **Note 7**).
2. *Glycolipid*: 1 mg/mL ganglioside GM1, GT1b, or other glycolipid in 10 mM bicarbonate, pH 8.0.

2.5. Patterning with PDMS (for Subheading 3.2.4)

1. *Plexiglas molds for PDMS*: Plexiglas plates (»1/2 in. thick) are milled with a numerically controlled milling machine (Techno Isel) to form the molds for the patterning and assay PDMS flow chambers (**Fig. 1**). Specific measurements can be found in Rowe et al. *(12)* Fins approximately 1 mm wide and tall are milled in the Plexiglas to form the channels in the PDMS through which the solutions will flow.
2. *PDMS patterning gasket*: NuSil MED-4011 (Nusil Silicone Technology, Carpinterai, CA). Mix Part A and Part B at a 10:1 w/w ratio in a large plastic disposable beaker. The mixture is degassed in a vacuum oven at room temperature (RT) until all the bubbles are removed (»30 min). The PDMS is poured into the Plexiglas mold (*see* **Note 1**). This mold is placed in the vacuum for further degassing until all the bubbles are at the surface, because bubbles near the fins can cause problems in the final PDMS flow chamber. After degassing, the mold is left at room temperature to cure, or it may be

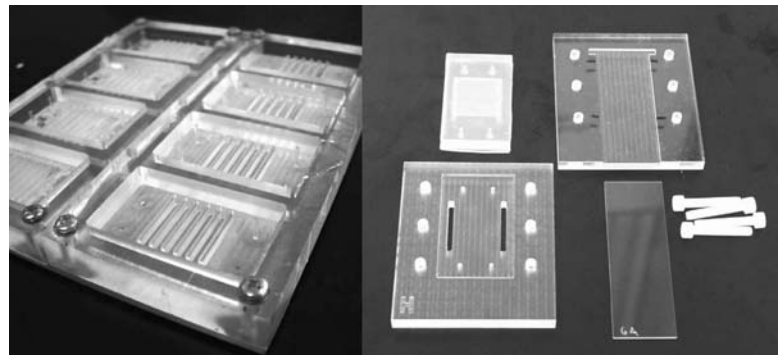

Fig. 1. Plexiglas and PDMS pieces for patterning. **(a)** Plexiglas mold for PDMS patterning gasket. The number of fins shown is 6 but up to 15 patterning channels have been designed in the same region. **(b)** Shown clockwise from the upper left are the PDMS patterning gasket, bottom piece of Plexiglas patterning chuck, screws, microscope slide with number etched, and the top piece of Plexiglas patterning chuck.

placed in a 60°C oven for 30 min. Some caution is advised with the oven method, as the Plexiglas mold can warp at higher temperatures. After curing, the PDMS is removed from the mold, trimmed, and washed with soap to remove any residue. For many applications, it is also useful to incubate the PDMS in 1% BSA prior to use to prevent nonspecific adsorption of molecules to the walls of the channel.

3. *Patterning chucks*: A chuck made of two Plexiglas plates (**Fig. 1**) to apply pressure evenly on the PDMS gasket was designed such that one plate has a recess to fit the slide and six tapped holes around the perimeter. The other plate is the same size and has holes that line up with the tapped holes. This plate also has access holes for the syringe needles, used to pierce the PDMS at both ends of the flow channels, for flowing the reagents.
4. *Phosphate-buffered saline, pH 7.4 (PBS)*: Sigma Chemical Co., St Louis, MO, P-3813. Dissolve 1 packet in 1 L 18 mΩ water.
5. *PBSTB*: Phosphate buffered saline pH 7.4/ 0.05% Tween-20/ 1 mg/mL BSA. Dissolve 1 g bovine serum albumin (# A3912, Sigma Chemical Co, St Louis, MO) and 500 µL Tween 20 (Sigma Chemical Co.) in 1 L PBS to make the correct final concentrations.
6. *Patterning buffer*: 10 mM phosphate buffer, 10 mM sodium chloride, and 0.05% Tween 20, pH 7.4.
7. *Biotin-labeled biomolecule for use with NeutrAvidin-coated slides*: In sandwich immunoassays, 10–20 µg/mL biotinylated antibody in patterning buffer is routinely used for patterning. In competitive immunoassays, the biotinylated analyte concentration varies from 0.2 to 5 µg/mL.

8. *Unlabeled biomolecule for direct immobilization*: 5–50 µg/mL (antibodies and other proteins) or 10–50 µg/mL (peptides and thiol-derivatized sugars) in patterning buffer.
9. Tuberculin syringes (1 mL) with 25 gauge needles.

2.6. Hydrogels (for Subheading 3.2.5)

1. *MTPTS solution*: Add 8.0 mL of 3-(trimethoxysilyl) propylmethacrylate (MTPTS) to a mixture of methanol (186 mL), water (5.4 mL), and acetic acid (0.6 mL).
2. *DCDM solution*: Add 4.0 mL of dichlorodimethylsilane (DCDM) to 196.0 mL of hexane.
3. *Carboxy-hydrogel solution*: Dissolve 60 mg galactose monomer, 6-acryloyl-β-*O*-methyl galactopyranoside *(20)*, in 0.5 mL 18 mΩ Milli-Q water at a final concentration of 12% (w/v). Add this monomer solution to 30 mg of 2-acrylamidoglycolic acid monohydrate at a final concentration of 50% (w/w) of the galactose monomer concentration. Dissolve *N*,*N*-methylene bis-acrylamide (Bis) cross-linker at 13% (w/w) of the monomer concentration in 100 µL of 18 mΩ H_2O. Heat briefly with heat gun to dissolve. Add carboxy - amine-hydrogel monomer solution to Bis crosslinker. Mix gently. Add 0.55 mg sodium persulfate and 2 µL of TEMED. Purge solution briefly with nitrogen. Use immediately.
4. *Amine-hydrogel solution*: Dissolve 110 mg of the galactose monomer, 6-acryloyl-β-*O*-methyl galactopyranoside in 0.5 mL of 18 mΩ H_2O at a concentration of 22% (w/v). Add the monomer solution to 5.5 mg of *N*-(3-aminopropyl)methacrylamide at a 5% (w/w) of the sugar monomer concentration. Add 0.55 mg of *N*,*N* methylene bis-acrylamide cross-linker for a final 0.5% (w/w) of the monomer concentration. Add sodium persulfate (5.5 µL) from a 10 mg/mL solution and TEMED (2.0 µL) to the hydrogel solution. Each monomer solution is purged with a stream on nitrogen. Use immediately.
5. *EDC/NHS solution*: Weigh out 11 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl (EDC) and 28 mg *N*-hydroxysuccinimide (NHS) in separate vials. Keep dry. Immediately before use, pipette 0.75 mL 50 mM MES buffer, pH 6.0 into each tube and mix well. Combine the two aliquots into a single EDC/NHS solution and *use without delay*. Final concentrations of EDC and NHS are 40 mM and 80 mM, respectively.
6. *BS^3 solution*: Dissolve 2.1 mg BS^3 (Pierce, Rockland, IL) in 1.5 mL 10 mM sodium phosphate, pH 6.0; the final concentration is 2.5 mM. Prepare immediately before use.
7. PDMS patterning gasket (*see* **Subheading 2.5** for preparation).
8. Phosphate-buffered saline, pH 7.4 (PBS).
9. *Antibody solution*: 100–300 µg/mL antibody in PBS.

10. *Blocking solution*: Add 2 g bovine serum albumin (Sigma, # A3912) and 2 g casein (BDH Laboratory Supplies) to 80 mL PBS. Add 10 M NaOH, with stirring, until the pH is approximately 12 (*see* **Note 8**). Once solution is clarified, bring the pH back to neutral by adding (with stirring) first 6 M HCl, then 1 M HCl, dropwise to the solution. Once the pH is approximately 7.4, add PBS to bring the volume to 100 mL (*see* **Note 9**).
11. 1 mL tuberculin syringes and 1 mL tuberculin syringe barrels (without plunger), fitted with 1.5-in., 25-gauge needles.
12. Multichannel peristaltic pump, set to flow at a rate of 0.8 mL/min. Inlet tubing is fitted with 25-gauge syringe needles, which will be used to connect the pump to the PDMS patterning gasket.

3. Methods

For all the procedures described in this section, microscope slides are used as the silica-based support and are labeled with a diamond scribe away from the biomolecule immobilization region. Other silica-based surfaces have also been used. Two slides are placed back-to-back into a Coplin jar with the etched numbers to the outside.

3.1. Cleaning

No matter which silanization method is employed, and cleaning of the solid support to generate reactive hydroxyl groups is critical for effective immobilization of biomaterials. There are several types of Si–OH groups that can form on silica surfaces. The geminal and isolated silanols are reactive, whereas the vicinal silanol and the siloxane groups are not (**Fig. 2**). If the surface is not properly cleaned of oils, dirt, detergents, etc., the reactive hydroxyl groups will not be formed and the silane will not be deposited in a uniform manner. Many comments against silanization methods concern the nonuniformity of the silane monolayer. The major contributors to such nonuniformity are inadequate surface cleaning and decomposure or

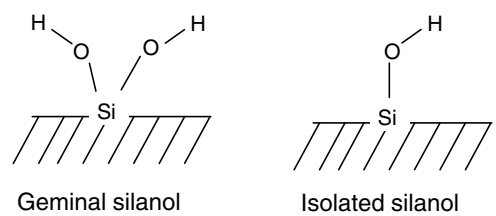

Fig. 2. Types of reactive surface hydroxyls.

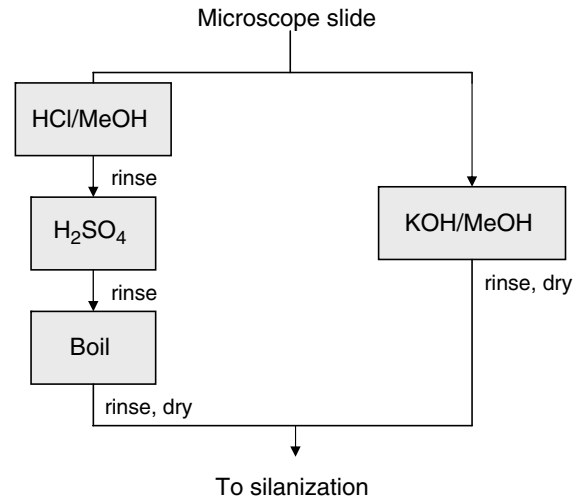

Fig. 3. Flow chart for cleaning methods.

polymerization of the silane. Two methods for cleaning silica-based supports are described; a flow chart for each cleaning method is shown in **Fig. 3** *(21, 22)*.

3.1.1. HCl/Methanol

1. Mark all slides using a diamond-tipped pencil.
2. Load slides into upright Coplin jar. Two slides can be placed back to back for each slot in the Coplin jar.
3. Mix 50 mL concentrated HCl with 50 mL MeOH in a large flask (250 mL). Pour HCl into premeasured MeOH in flask to avoid spattering of concentrated acid. Swirl flask until both components are thoroughly mixed and no schlieren lines are observed.
4. Pour HCl/MeOH mix over slides and incubate 30 min without mixing.
5. Remove HCl/MeOH mix from the Coplin jar and pour into a labeled glass bottle for waste disposal. Do not pour down the drain.
6. Rinse slides exhaustively with deionized water (no schlieren lines observed).
7. Dry slides individually under stream of nitrogen and place back into clean, dry Coplin jar.
8. Pour concentrated sulfuric acid over slides until slides are covered. Incubate for 30 min without mixing. CAUTION: Highly acidic. Use appropriate personal protective equipment.
9. Remove concentrated sulfuric acid from Coplin jar and pour into a labeled glass bottle for waste disposal. Do not pour down drain.
10. Rinse slides exhaustively with deionized water until no schlieren lines are observed. Rinse each slide individually with deionized water.

11. Add hot 18 mΩ water (100°C) to the slides. Incubate for 15 min.
12. Dry each slide individually under a stream of nitrogen and place back into a clean dry Coplin jar. Each slide should look pristine. There should be no spots or cloudiness to the slide. If spots or cloudiness are present repeat from **step 8**. Slides should be cleaned no more than 3 days before use.

3.1.2. KOH/Methanol

1. Dissolve 10 g KOH in 100 mL methanol (MeOH) in a 250 mL flask.
2. While KOH is dissolving, etch appropriate number in upper right corner of each slide.
3. Place slides into upright Coplin jar. Two slides can be placed back to back for each slot in the Coplin jar. For metal coated slides the front is where the metal coating is located.
4. Pour KOH/MeOH over slides until slides are completely immersed. Incubate for 30 min without mixing.
5. Remove KOH/MeOH mix from Coplin jar and discard into an appropriate waste container.
6. Wash slides exhaustively with deionized water. Rinse each slide individually until no schlieren lines are visible.
7. Dry each slide individually under a stream of nitrogen and place back into clean dry Coplin jar. Each slide should look pristine. There should be no spots or cloudiness on the slide. If spots or cloudiness are present, repeat from **step 3**. Slides should be cleaned no more than 3 days before use.

3.2. Covalent Immobilization Procedures

Biomolecules such as antibodies, enzymes, and cells can be attached to silane-treated surfaces using the free thiols and/or amines on the surface and on the biomolecules. After treatment of clean silica surfaces with a thiol or amine-terminated silane, a homo or hetero-bifunctional crosslinker that possesses appropriate reactive groups can be used to attach a biomolecule to the surface. **Figure 4** shows several illustrations of the general schemes for immobilization, described below.

3.2.1. Attachment of Biomolecules to Thiol-Modified Surfaces

In this section, only the heterobifunctional crosslinker that contains a maleimide for reacting to the thiols from the mercaptosilane and *N*-hydroxysuccinimidyl ester (NHS) to react with primary amines on the biomolecule will be described. A generalized flow chart for this procedure is shown in **Fig. 5**.

Thiol Silanization

In this step, the surface hydroxyl groups on the slide react with the ethoxy groups on the mercaptopropyltriethoxy silane resulting in a thiol-derivatized slide **(22, 23)**

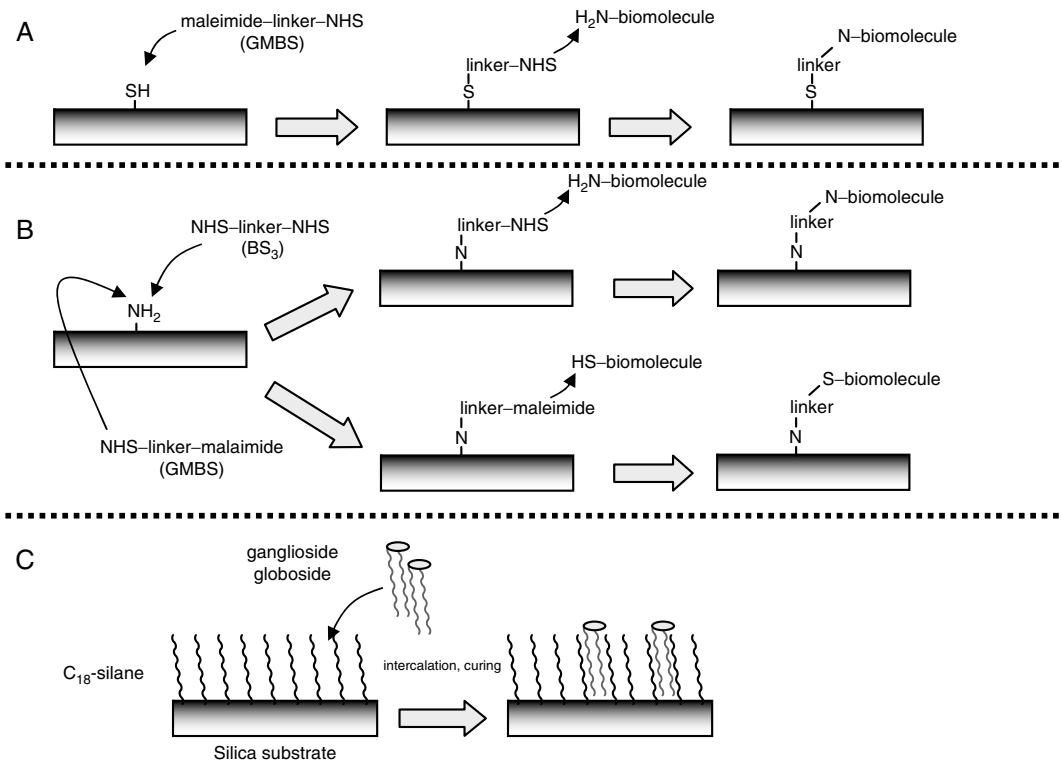

Fig. 4. Immobilization methods. **(A)** Attachment of biomolecules onto thiol-derivatized surfaces (*see* **Subheading 3.2.1** for experimental details). **(B)** Attachment of biomolecules onto amine-derivatized surfaces **(Subheading 3.2.2)** using amine–amine linking (*upper pathway*, **Subheading "Amine–Amine (Homobifunctional Crosslinkers)"**) and amine–thiol linking (*lower pathway*, **Subheading "Amine–Thiol Crosslinking (Heterobifunctional Crosslinkers)"). (C)** Noncovalent attachment of gangliosides/globosides onto hydrophobic surfaces **(Subheading 3.2.3)**.

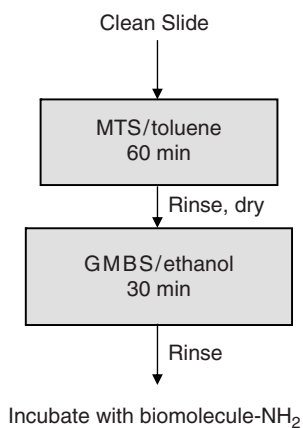

Fig. 5. Protocol for attaching amine-containing biomolecules to thiol-coated surfaces.

1. Place 16 cleaned, dried slides back-to-back in a clean, dry Coplin jar, with etched labels facing outward.
2. Place Coplin jar in glove bag.
3. For each batch of 16 slides, prepare 50 mL MTS/toluene solution immediately prior to use in the glove bag. Pour the silane/toluene mix over the slides and incubate for 1 h under nitrogen in the glove bag.
4. Remove the Coplin jar from the glove bag and place elsewhere in the chemical fume hood.
5. With forceps, remove each slide from MTS/toluene solution and rinse three times in toluene by swishing the slides 3–5 times sequentially in three 150 mL beakers filled with toluene. Caution needs to be taken when working with toluene. Proper protective gear needs to worn, and the work needs to be carried out in a chemical fume hood (*see* **Note 10**).
6. Dry each slide completely under a stream of nitrogen and place slides back-to-back in a clean, dry Coplin jar. The slides should still appear pristine. If not, the slides should not continue on to the next step as either the cleaning was insufficient or the silane has degraded. Since the thiols will rapidly oxidize within 24 h, the crosslinking step must be performed immediately after silanization.

Heterobifunctional Crosslinking

During this step, the surface thiols react with the maleimide group of the crosslinker. The following step uses the linker's NHS reactive group to bind to primary amines on the biomolecule. Crosslinking and all subsequent steps may be performed on the bench top, outside of the chemical hood.

1. For each batch of 16 slides, prepare 43 mL of GMBS/ethanol immediately prior to use.
2. Pour GMBS solution over slides and cover jar. Incubate for 30 min at room temperature. Time is critical at this step as the reactive groups on the GMBS degrade over time in the ethanol and air atmosphere.
3. Rinse the slides three times in deionized water by swishing the slides 3–5 times sequentially in three 150 mL beakers filled with water.
4. If immobilizing a single biomolecule (e.g., avidin, NeutrAvidin) over the entire slide surface, place slides back-to-back in a Coplin jar and add the 40 mL patterning buffer containing the biomolecule of interest. Incubate the slides overnight at 4°C. If using metal-coated slides, the NeutrAvidin or other biomolecule should be prepared in 10 mM phosphate buffer pH 7.4 and immobilized on the same side of the slide as the metal coating. Rinse the slides three times with PBS. They are stored in PBS (or in 10 mM phosphate pH 7.4 if metal-coated) at 4°C until patterning.

5. If immobilizing multiple biomolecules in a patterned array, dry each slide under a stream of nitrogen and proceed immediately with patterning (**Subheading 3.2.4**).

3.2.2. Attachment of Biomolecules to Amine-Modified Surfaces

Biomolecules such as peptides, modified carbohydrates, F_{ab} fragments, and other proteins can be attached to amine-modified surfaces using free thiols and amines (*11, 24, 25*). The cleaned surface is first treated with an amino silane, followed by a homo or heterobifunctional crosslinker possessing one or two amine-specific reactive groups and/or a thiol-specific reactive group (amine–thiol linking). After the *N*-hydroxysuccinimidyl ester (NHS) terminus of the crosslinker has been reacted with the surface-immobilized amines, the remaining reactive group of the crosslinker (maleimide or additional NHS) is reacted with thiol or amine moieties on the biomolecule. A generalized flow chart showing procedures for attaching the thiol and amine-containing biomolecules to amine-modified surfaces is shown in **Fig. 6**.

Amine Silanization

During this process, the hydroxyl groups on the surface of the slide react with the ethoxy groups of aminopropyl triethoxy silane resulting in an amine-derivatized slide *(24)*.

1. Place 16 cleaned, dried slides back-to-back in a Coplin jar, with etched labels facing outward.
2. For each batch of 16 slides, prepare 50 mL silane/methanol/HOAc mix immediately prior to use. Pour the silane/methanol/HOAc mix over the slides and incubate for 1 h.
3. With forceps, remove each slide from silane/methanol/HOAc solution and rinse three times in methanol by swishing the slides 3–5 times sequentially in three 150-mL beakers filled with methanol.
4. Wash three times in deionized water (as above for methanol wash).
5. Dry each slide completely under a stream of nitrogen.

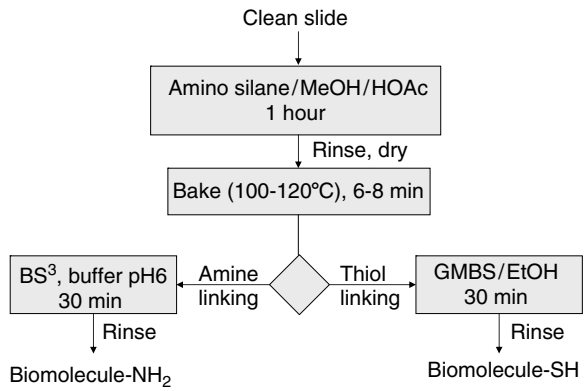

Fig. 6. Protocol for attaching amine-containing biomolecules (*left pathway*) and thiol-containing biomolecules (*right pathway*) to amine-coated surfaces.

6. Place slides in a drying oven at 100–120°C for 6–8 min. Allow slides to cool to room temperature.
7. Place slides back-to-back in a clean, dry Coplin jar. Slides treated with amino silane may be kept under nitrogen for several weeks. These slides may be used for amine–amine crosslinking **(Subheading Amine–Amine (Homobifunctional Crosslinkers))** or amine–thiol crosslinking **(Subheading Amine–Thiol Crosslinking (Heterobifunctional Crosslinkers))** of biomolecules to the surface.

Amine–Amine (Homobifunctional Crosslinkers)

During this process, the surface amine groups first react with one NHS reactive group of the crosslinker; this initial step is performed at pH 6 to minimize reaction of both NHS groups with surface amines *(24)*. The second NHS group is later reacted with amines on the biomolecule to be attached at pH 7.5. Crosslinking and all subsequent steps may be performed outside of the chemical hood, on the bench top.

1. For each batch of 16 slides, prepare 100 mL of BS^3 solution immediately prior to use.
2. Pour BS^3 solution over slides and cover jar. Incubate for 30 min at room temperature.
3. Rinse three times in deionized water by swishing the slides 3–5 times sequentially in three 150-mL beakers filled with water.
4. If immobilizing a single biomolecule (e.g., avidin, NeutrAvidin) over the entire slide surface, place slides back-to-back in a Coplin jar containing 33 mL solution of the biomolecule of interest.
5. If immobilizing multiple biomolecules in a patterned array, dry each slide under a stream of nitrogen and proceed immediately with patterning **(Subheading 3.2.4)**.

Amine–Thiol Crosslinking (Heterobifunctional Crosslinkers)

During this process, the surface amines first react with the NHS reactive group of the crosslinker **(11, 25)**. A subsequent step uses the pendant maleimide to attach the biomolecule to the surface via its thiols. Crosslinking and all subsequent steps may be performed outside of the chemical hood, on the bench top.

1. For each batch of 16 slides, prepare 43 mL of GMBS solution immediately prior to use.
2. Pour GMBS solution over slides and cover jar. Incubate for 30 min at room temperature.
3. Rinse three times in deionized water by swishing the slides 3–5 times sequentially in three 150-mL beakers filled with water.
4. If immobilizing a single biomolecule (e.g., avidin, NeutrAvidin) over the entire slide surface, place slides back-to-back in a Coplin jar containing 33 mL solution of the biomolecule

of interest. Incubate overnight at 4°C (proteins, peptides) or room temperature (thiol-derivatized sugars). Rinse three times with PBS and store at 4°C in PBS.

5. If immobilizing multiple biomolecules in a patterned array, dry each slide under a stream of nitrogen and proceed immediately with patterning (**Subheading 3.2.4**).

3.2.3. Attachment of Lipophilic Biomolecules to Hydrophobic Silane-Treated Surfaces

Gangliosides, globosides, and other lipid-linked biomolecules can be immobilized onto silica or silica-based substrates using hydrophobic interactions. A long-chain hydrophobic silane is used to coat the slide surface, in essence, creating a monolayer-type structure into which the lipid moieties can intercalate. After incubation of the glyolipid with the silanized surface, the slides are cured, essentially locking the intercalated lipid chains in place. Ganglioside and globoside-patterned surfaces produced in this manner have been used to detect toxins *(26, unpublished)*. A flowchart illustrating the procedure is shown in **Fig. 7**.

Hydrophobic Silanization

During this process, the hydroxyl groups on the surface of the slide react with the trichloro groups of the OTS, resulting in formation of a monolayer on the slide surface.

1. Place 16 cleaned, dried slides back-to-back in a Coplin jar, with etched labels facing outward.
2. For each batch of 16 slides, prepare 100 mL OTS solution and pour the silane/toluene mix over the slides. Incubate for 1 h under nitrogen in a glove bag.
3. Remove the Coplin jar from the glovebag and place elsewhere in chemical fume hood.
4. With forceps, remove each slide from the OTS solution and rinse three times in toluene by swishing the slides 3–5 times sequentially in three 150-mL beakers filled with toluene.

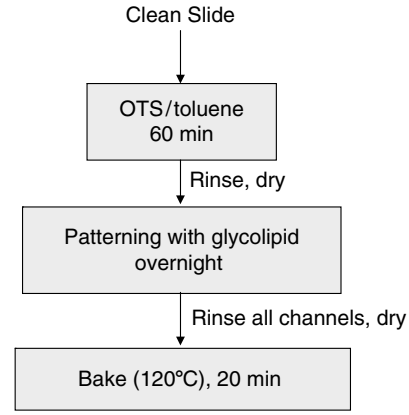

Fig. 7. Procedure for immobilizing lipid-linked biomolecules to glass slides treated with hydrophobic silane.

5. Dry each slide completely under a stream of nitrogen.
6. Place slides back-to-back in a clean, dry Coplin jar. Slides treated with OTS can be stored in a sealed jar (in air) at room temperature for up to a week without measurable changes in surface characteristics.

Glycolipid Immobilization

During this process, glycolipid solution is incubated with the OTS-treated slide and the lipid moiety allowed to intercalate into the hydrophobic layer.

1. Rinse each slide three times in water. Place into patterning templates and pattern with glycolipid as described in **Subheading 3.2.4**. Let incubate overnight in the presence of glycolipid solution.
2. Rinse each channel with 1 mL deionized water under flow.
3. Remove slides from PDMS patterning template.
4. Rinse each slide three times by swishing 3–5 times sequentially in three 150 mL beakers filled with water.
5. Dry under a stream of nitrogen.
6. Bake each slide for 20 min in a drying oven at 120°C (*see* **Note 11**). Allow to cool to room temperature. Store dry at 4°C for up to one week.

3.2.4. Creation of PDMS Patterned Arrays

For multianalyte sensing, different biotinylated recognition molecules can be immobilized in each of the patterning channels. **Figure 8** shows a fully assembled unit for patterning a single slide. For many of our studies, a positive control using an irrelevant biomolecule (usually anti-chicken IgY) is patterned in the outermost patterning channels and a negative control (buffer) is injected into one of the middle channels. Place slide on bottom piece of patterning chuck with etched labeled side up.

1. Place PDMS patterning gasket over the distal end (away from etched label) of the slide with channels being formed by slide and PDMS.
2. Place top piece of patterning chuck over PDMS. Finger tighten four screws (each corner) evenly. The screws should be finger tight; over-tightening will result in slide breakage; too loose and the channels leak into each other.
3. Syringes with needles and without plunger are placed on one end of each channel as an effluent reservoir.
4. Using a 1 mL syringe, inject 60–100 µL solution containing the biotinylated biomolecule (NeutrAvidin-coated surfaces) or unlabeled biomolecule (activated surface) in the patterning buffer into the appropriate channel.
5. Incubate the biomolecule with the slide overnight at 4°C.

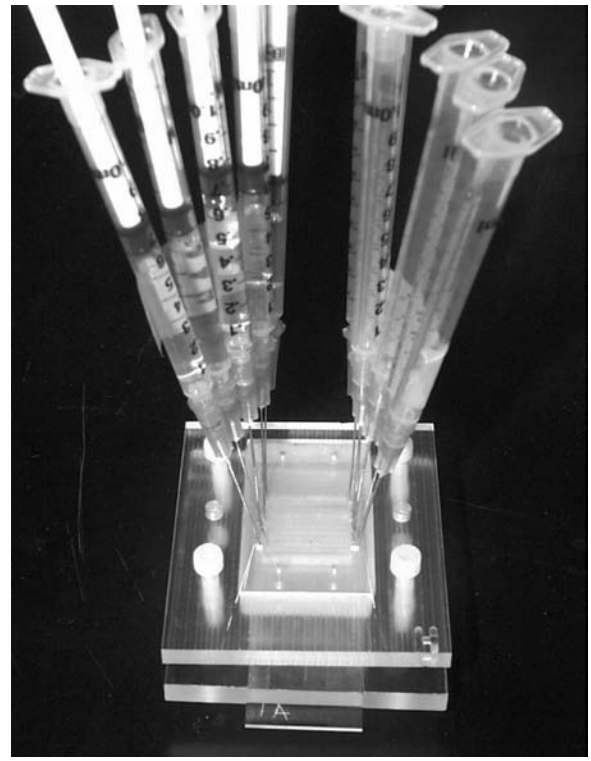

Fig. 8 Fully assembled unit for patterning. Solutions containing capture antibodies are in the closed syringes on *the left* side. Syringes on *right side* are inserted to allow flow through the chambers and are used as effluent reservoirs. Reprinted from ref. 10, with permission from Elsevier

6. Rinsed each channel with 1 mL PBSTB followed by air using the syringe format in **step 5**.
7. Remove slide from the patterning PDMS and chuck and insert into a 50 mL centrifuge tube containing 10 mg/mL bovine serum albumin in phosphate buffer, pH 7.4, for 20 min to block the slide.
8. Rinse slide with 18 mΩ water and dry with nitrogen. The dried patterned slide is stored at 4°C until use.

3.2.5. Preparation and Patterning of Hydrogel Thin Films

Recently, hydrogels have been used as an alternative substrate for the immobilization of biomolecules, because of their potential advantages over standard two-dimensional surfaces: increased capacity for immobilized species, low background in fluorescence-based assays, and a network conducive for affinity experiments. The following sections describe methods to prepare slides for casting of hydrogels, preparation of two types of hydrogels (amine and carboxylate-terminated), and direct covalent immobilization of biomolecules within the hydrogel matrix. Hydrogels prepared in this manner are amenable to use in biosensor assays. **Figure 9**

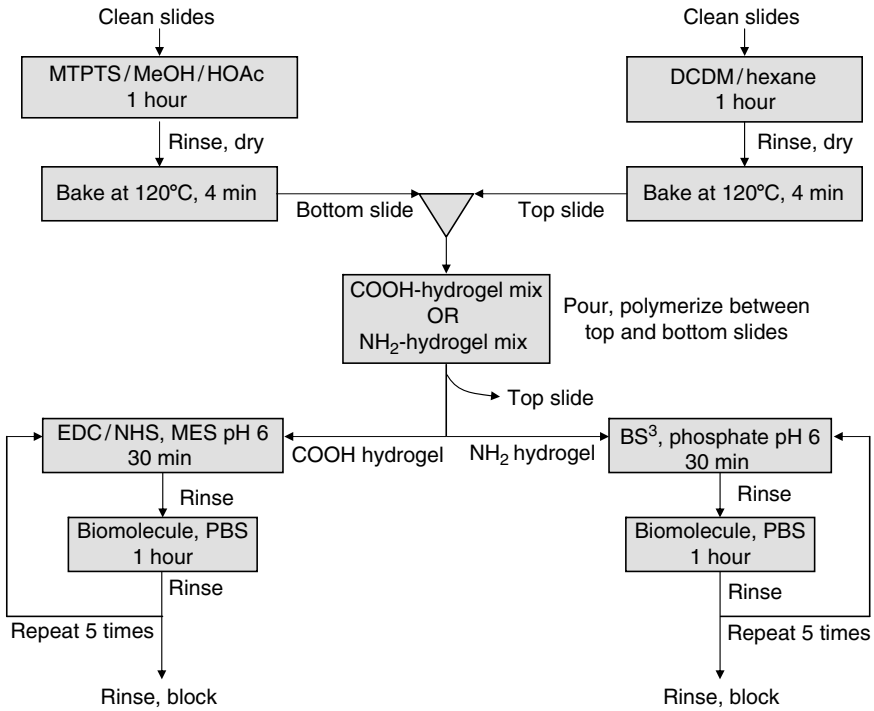

Fig. 9. Procedure for immobilizing biomolecules inside COOH-hydrogels (*lower left pathway*) or amine-hydrogels (*lower right pathway*). Procedures for preparation of the top and bottom slides for casting the hydrogel slabs are shown in the upper right and left pathways, respectively.

shows a flow chart with the entire process of coating the top and bottom slides, preparing the hydrogel slab between the top and bottom slides, and patterning each type of gel.

Treatment of Slides with Hydrophobic and Methacrylate Silanes

For each hydrogel prepared, two glass slides are modified using different silanes to impart differential chemical and physical properties. The slide onto which the hydrogel is cast is treated with MTPTS. The second slide, which forms the top layer of the hydrogel casting assembly, is functionalized with a silane with nonreactive pendant moieties (DCDM silane). This latter slide will eventually be removed from the assembly, leaving a hydrogel covalently attached to the MTPTS-treated slide.

1. Divide 32 acid cleaned glass slides into two groups for respective silane treatment.
2. Inscribe 16 slides with number 1–16 in top right corner of slide.
3. Inscribe the remaining 16 slides A–P in top right corner of slide.
4. Treat slides 1–16 with a MTPTS solution and incubated for1 h at 60°C.
5. Rinse MTPTS-treated slides three times with methanol and dry with a stream of nitrogen.

6. Bake MTPTS-treated slides for 4 min at 120°C (scribed number side up).
7. Treat slides A–P with a DCDM solution for 1 h at room temperature.
8. Rinse DCDM-treated slides with hexane then dried with a stream of nitrogen.
9. Bake DCDM-treated slides for 4 min at 120°C (scribed number side up).
10. Store desiccated until further use.

Preparation of Hydrogel Slabs

Hydrogel formation is accomplished via a free radical polymerization process using the initiator sodium persulfate and catalyst TEMED. The hydrogel slab is formed by creating a sandwich-type assembly composed of the MTPTS-treated slide on the bottom, the hydrogel matrix in the middle, and the DCDM-treated slide on the top. The hydrophobic nature of the DCDM-treated slide (prepared above) helps reduce the stress and shear forces upon removal from the hydrogel film, minimizing tearing or delamination of the hydrogel from the MTPTS-treated surface. Attachment of the poly(acrylate)-acrylamide film to the MTPTS-treated surface is accomplished though free radical polymerization of the polymer network and the pendant vinyl group of the MTPTS-surface. Teflon tape, which serves as the spacer between the two slides, provides control of the hydrogel polymer thickness.

1. Place the methacrylate-treated slide onto a horizontal surface and form "spacers" at each end of the slide using Teflon tape or commercial spacers.
2. Pipette 110 μL of the carboxy or amine-hydrogel solution onto the methacrylate-treated slide. Place the dichlorodimethyl (DCDM) silane-treated slide on top of the liquid, forming a sandwich-type assembly.
3. Place the assembly into an inert atmosphere (nitrogen). Polymerize overnight at room temperature.
4. Remove the DCDM-treated slide (top slide) from the MTPTS-treated slide. This will reveal a thin film of highly crosslinked sugar polyacrylate hydrogel containing a carboxyl or amine-terminated moiety covalently attached to the MTPTS-treated surface.
5. Immerse the slides briefly (1.0 min) in Milli-Q water and air-dry at room temperature. Store hydrogel slides semihydrated at 4°C until further use.

Immobilization of Antibodies Within Hydrogel Slabs

Biomolecules are covalently immobilized onto amine and carboxylic acid-modified hydrogel films in a method similar to that described for two-dimensional surfaces above (**Subheadings 3.2.1** and **3.2.2**). The key difference in the method used for

hydrogels is the requirement for *multiple* cycles of cross-linking and attachment of biomolecules. For amine-terminated hydrogels, crosslinking occurs via the homobifunctional crosslinker BS^3, whereas for carboxylate gels, EDC/NHS coupling chemistry is used. However, the procedure is identical for both types of hydrogels – i.e., 30 min crosslinker, wash, 60 min antibody, wash, repeat. Only the crosslinker solutions are changed: BS^3 for amine hydrogels, EDC/NHS for carboxylate gels (**step 5**, below).

1. Place hydrogel-coated slide on bottom piece of patterning chuck with number side up.
2. Place PDMS patterning gasket over the distal end of the slide, ensuring that all channels of the patterning gasket are within the boundaries of the hydrogel slab.
3. Place top piece of patterning chuck over PDMS. Finger-tighten four screws (each corner) evenly. The screws should be finger tight; over-tightening will result in slide breakage or damage to the hydrogel, whereas undertightening will result in leakage.
4. Place a syringe barrel (without plunger, fitted with a 25-gauge needle) at one end of every channel for use as a reservoir. These reservoirs will remain in place throughout **steps 5–15**.
5. Using a 1 mL syringe, inject 60–100 μL freshly prepared BS^3 solution (amine-terminated gels) or EDC/NHS solution (carboxylate gels) into each channel.
6. Incubate for 30 min.
7. Hook each slide up to the peristaltic pump. One end of each channel will have a syringe barrel reservoir (*see* **step 4**, above), while the other end is hooked to the pump's inlet tubing via a syringe needle.
8. Start the pump (0.8 mL/min flow rate), pulling the BS^3 or EDC/NHS solution from each channel. Evacuate all channels.
9. Pipette 1 mL PBS into the syringe-barrel reservoir of each channel and continue pump flow until the entire volume has flushed through the channel and each channel is filled with air.
10. Unhook the slide from the pump.
11. Using a 1 mL syringe, inject 60–100 μL Antibody Solution into each channel.
12. Incubate for 60 min.
13. Using the same syringe, remove Antibody Solution from each channel and store on ice for subsequent rounds of patterning.

14. Hook the slide up to the peristaltic pump, and rinse each channel with PBS, as in **steps 7–10**, above. This completes 1 round of patterning.
15. Repeat **steps 5–14**, for a total of six rounds of patterning. The final (sixth) incubation with Antibody Solution can be performed overnight at 4°C.
16. After final antibody incubation and rinsing of each channel with PBS, disassemble the slide from the patterning assembly.
17. Place the patterned slides into a Coplin jar filled with Blocking Solution.
18. Incubate with Blocking Solution for 1 h at room temperature, or overnight at 4°C.
19. Replace Blocking Solution with PBS and incubate for 30 min, with agitation. Repeat twice more for a total of three post-blocking washes.
20. Allow slides to air dry. Store slides at 4°C in a moist chamber until use.

4. Notes

1. Silanization steps are performed in a N_2-filled glove bag in a ventilated hood. This is to prevent air-oxidation of the thiol-silane, as well as to protect laboratory personnel from inhaling silane fumes. Triethoxy silanes are used in preference to trimethoxy silanes, due to decreased toxicity.
2. The MTS silane solution should be prepared just before use. It is very important that the silane be kept as dry as possible and that its exposure to air/oxygen is minimized to avoid decomposition/polymerization of the silane and oxidation of the thiols. Aliquoting of the silane stock with storage in dark glass vials under nitrogen is highly recommended.
3. Other heterobifunctional crosslinkers with NHS and maleimide reactive groups (e.g., BMPS, MBS, SMCC) can be used in place of GMBS. The final concentration of these other linkers in the ethanolic mix should be 1 mM.
4. The biomolecule solution should not contain any amine-containing materials such as Tris and BSA. If present in initial protein solution, they should be removed by dialysis or another appropriate method.
5. Other homo-bifunctional NHS-based crosslinkers (e.g., DSS and EGS) may be used in place of BS^3, provided the final diluted molar concentration is 50 μM.

6. Addition of 0.8 molar equivalents of TCEP to the solution of peptide, protein, carbohydrate, etc., is crucial for linking of oxidized thiols to amine-modified surfaces. TCEP will reduce disulfide bonds in proteins, peptides, and derivatized sugars without introducing new thiols, as occurs with thiol-containing reducing agents such as dithiothreitol (DTT) and 2-mercaptoethanol. In general, care must be exercised with this reagent, as its redox potential is significantly higher than that of DTT, and only half the concentration of TCEP is required when compared with DTT for the same application.
7. As with all halogenated silanes, trichlorosilane is highly reactive and freshly opened stocks should be used for optimal results.
8. An alternate method for dissolving casein in the Blocking Solution is slow heating to near-boiling, followed by cooling to room temperature.
9. This solution can be stored at 4°C for extended time if azide is added (final concentration 0.03 wt%).
10. Used (waste) silane and toluene are poured into labeled brown glass bottles. Glassware contaminated by silane and/or toluene are rinsed several times with acetone; rinse acetone is discarded in same waste container as silane and toluene.
11. High temperature curing of the slides *after* patterning is essential for sensitive detection of ganglioside-binding toxins *(26)*. Omission of this step has resulted in great diminished binding of cholera toxin to immobilized GM1. Curing of the OTS-treated slides *before* patterning will prevent immobilization of glycolipids onto the surface.

Acknowledgments

The development of these methods was in part funded by the Office of Naval Research. The views expressed here are those of the authors and do not represent those of the U.S. Navy, the U.S. Department of Defense or the U.S. Government.

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