Phospholipid biotinylation of polydimethylsiloxane (PDMS) for protein immobilization

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Received 8th November 2005, Accepted 13th January 2006
First published as an Advance Article on the web 26th January 2006
DOI: 10.1039/b515840k

Polydimethylsiloxane (PDMS) surfaces can be functionalized with biotin groups by adding biotinylated phospholipids to the PDMS prepolymer before curing. The addition of β-D-dodecyl-*N*-maltoside (DDM) in the solution blocks non-specific protein binding on these functionalized PDMS surfaces. We characterize the surface by measuring fluorescently labeled streptavidin binding. Single molecule tracking shows that the phospholipids are not covalently linked to PDMS polymer chains, but the surface functionalization is not removed by washing. We demonstrate the immobilization of biotinylated antibodies and lectins through biotin–avidin interactions.

Introduction

Polydimethylsiloxane (PDMS) is a popular material for microfabrication aimed for various applications, among which the use of microchips for immunoassay or protein–ligand interaction analysis is highly attractive. Such applications need the immobilization of certain proteins on the PDMS surface, but the relative chemical inertness of Si–C and Si–O–Si bonds makes the derivation of PDMS surfaces much more difficult than silicon or glass. Makamba *et al.*[1] have extensively reviewed various methods for modifying PDMS surfaces. Approaches for immobilizing proteins can be divided into three categories. (1) Non-specific adsorption on the hydrophobic (native) PDMS surface and subsequent protein binding.[2–4] These methods are simple to realize, but they rely on blocking the PDMS surface with the first protein layer to reduce further non-specific protein binding; therefore, the specificity is sometimes limited owing to non-specific protein–protein interactions. (2) Lipid bilayer formation on the hydrophilic (plasma oxidized) PDMS surface.[5,6] This approach can achieve high specificity, but the instability of lipid bilayer limits its storage time to only a few days.[1] (3) Activation of the PDMS surface (plasma, UV light or chemical vapor deposition) and subsequent chemical reaction (polymer grafting or silanization).[7–9] In this way, target molecules are covalently linked to the surface. This approach, however, often requires complicated reactions, and the activation of the PDMS surface makes it more prone to protein adsorption.

Here we present a new method of functionalizing the PDMS surface with biotinyl groups by adding the derivatizing reagent to the liquid PDMS prepolymer prior to curing (Fig. 1). This premixing method has been previously used to create catalytic PDMS films with porphyrin doping.[10] For protein immobilization, such reagents must have a long hydrophobic tail for attachment to the PDMS matrix and a hydrophilic linker for attachment to the biotin group, so that it can reach into the buffer solution for avidin binding. We have found that commercially available biotinylated phospholipids are ideal candidates. After biotin groups have been introduced to the PDMS surface, other proteins can be immobilized through biotin–avidin interactions. Combined with our recent observation that the use of a non-ionic surfactant, β-D-dodecyl-*N*-maltoside (DDM), almost completely eliminates the non-specific adhesion of proteins on native PDMS surfaces,[11] we can achieve highly specific protein immobilization. We then demonstrate the immobilization of anti-FLAG M2 antibody and concanavalin A, which allows the subsequent binding of a transmembrane protein (β_2 adrenergic receptor).

Results and discussion

Biotinyl functionalization of PDMS

We choose 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(biotinyl) (Bio-DOPE, Fig. 1A) to test the functionalization of PDMS polymer. A 10 μL 5 mg mL^{-1} phospholipid–chloroform solution is added to 1 g of PDMS prepolymer/hexane solution, *i.e.*, 150 μg phospholipids per 1 gram of PDMS prepolymer mixture. The addition of more than 50 μL of chloroform solution to 1 g of PDMS prepolymer–hexane solution results in poor spin coating on glass because of the insolubility of chloroform in hexane at this concentration.

Fluorescence imaging with Alexa Fluor 647 labeled streptavidin (AX647-SA) is performed to visualize the surface. Four different buffers are used in the experiments: HEPES-buffered saline (HBS, containing 20 mM HEPES, pH 7.5, 100 mM NaCl), TRIS-buffered saline (TBS, containing 20 mM TRIS, pH 7.4, 100 mM NaCl), DHBS and DTBS (HBS and TBS with 0.1% DDM). 50 μL of 1 μM AX647-SA in DHBS or DTBS is injected into the channel using a pipet or a syringe, allowed to stand for 5 min. and then washed away with 50 μL of the corresponding buffer. The presence of 0.1% DDM in the buffer eliminates the non-specific adsorption of

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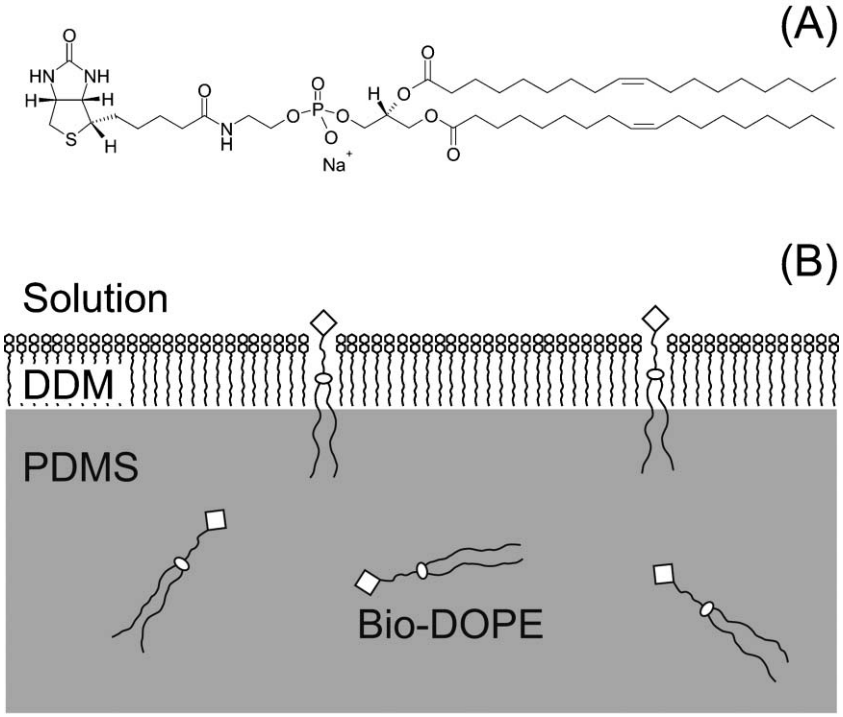

Fig. 1 (A) Structure of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(biotinyl) (Bio-DOPE); (B) schematic illustration of biotinyl functionalization of PDMS using phospholipids.

streptavidin on hydrophobic PDMS surfaces,[11] including the non-functionalized top and side walls of the channel. As can be seen from Fig. 2A, homogeneous fluorescence can be observed from modified PDMS surfaces. On the contrary, with the presence of 0.1% DDM, only scattered molecules can be observed from 1 μM AX647-SA on the native PDMS surface[11] (Fig. 2D), and we are unable to detect the modified PDMS surface after incubating with 200 nM tetramethylrhodamine labeled β_2 adrenergic receptor because of the lack of adsorbed fluorescent molecules. These results indicate that biotin groups are present on the PDMS surface after phospholipid modification, and their presence allows streptavidin binding. It also shows that DDM is effective in preventing protein adsorption to the Bio-DOPE-modified PDMS surface, which suggests that the surface might still be hydrophobic.

When DDM is absent in the buffer, streptavidin adsorbs strongly to native PDMS, resulting in almost full surface coverage. With 1 μM AX647-SA, the surface fluorescence is about 6 times stronger on a native PDMS surface without DDM in the buffer (Fig. 2(C)) than on a Bio-DOPE-modified surface with DDM (Fig. 2(A)), suggesting that the streptavidin coverage is about 17% in the latter case. Actually, in some applications a lower surface coverage might be preferable because it reduces the possibility of non-specific interactions between the target molecules and avidin (or avidin variants). In these cases, the surface density of biotin groups can be reduced by decreasing the amount of phospholipids added to the PDMS prepolymer. No effort was made to determine the extent of surface coverage by phospholipids, and it is possible that some phospholipids do not attach to streptavidin.

Stability of the surface

When the streptavidin concentration is lower than 100 pM, discrete fluorescent spots can be observed using total internal reflection (TIR) excitation. As is evident by stepwise photobleaching (data not shown), these spots represent individual AX647-SA molecules. By tracking the behavior of these molecules, we can monitor the motion of biotinylated phospholipid molecules on the surface. We have found that more than 80% of AX647-SA molecules show random walk behavior in the presence of DDM (Fig. 3(A)). Replacing the buffer in the flow channel with HBS immediately stops the motion, possibly because of the non-specific adsorption of streptavidin to the surface. Such movement returns when changing back the buffer in the channel to DHBS. We have also measured the behavior of AX647-SA molecules on a saturated phospholipid (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(biotinyl), Bio-DPPE) doped PDMS surface. Except for some rare cases, almost all the observed streptavidin molecules are mobile.

The movement of streptavidin molecules suggests that the phospholipid molecules are mostly embedded in the PDMS polymer matrix. It also indicates that the majority of the unsaturated phospholipids do not participate in the cross-linking reaction when the PDMS prepolymer is cured, which involves the reaction between Si–H groups and vinyl groups.[12,13] Although the biotin groups are not covalently linked to PDMS polymer chains, we have observed that the surface functionalization is essentially permanent, that is, not removed by buffer (including surfactant) washing. In one experiment, after 1 μM AX647-SA is bound to Bio-DOPE-modified PDMS, the channel is washed and incubated with

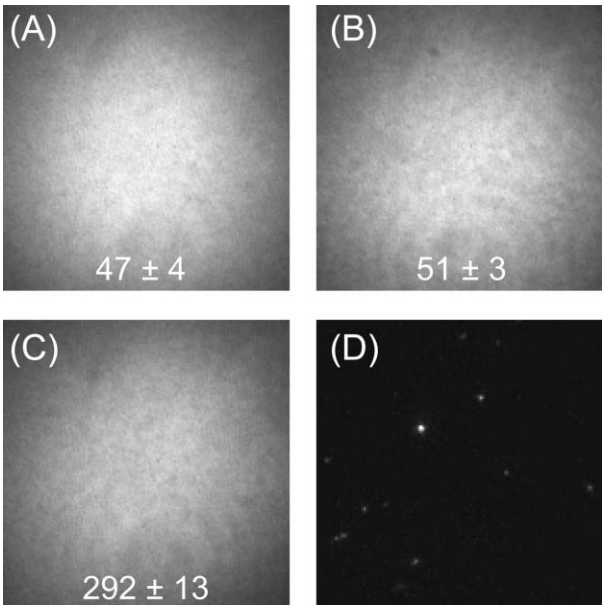

Fig. 2 Fluorescent images of 1 μM AX647-SA in different buffers on PDMS surfaces. (A): DTBS solution on a Bio-DOPE surface; (B): same as (A) but after incubation in DTBS for 1 h; (C): HBS solution on an unmodified surface; (D): DHBS solution on an unmodified surface. The image sizes are 34 μm × 34 μm. Images (A), (B) and (C) are adjusted to have the same contrast. They are acquired using epifluorescence with an integration time of 200 ms and a laser power of 67 μW, 67 μW and 9.2 μW, respectively. Numbers in each image show the average fluorescence (normalized by excitation power) and the standard deviation from eight different locations in the channel. The difference of fluorescence between the center and the edges reflects the laser profile. (D) is acquired using TIR excitation with an integration time of 100 ms and a laser power of 823 μW so that fluorescence from individual molecules can be observed.

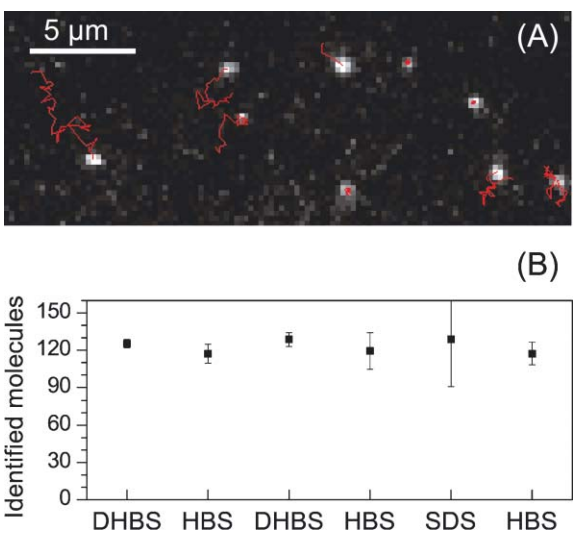

Fig. 3 (A) The first frame of a stack of images from 1 pM AX647-SA on a Bio-DOPE-modified PDMS surface. Trajectories of the molecules are marked in red. The image stack is recorded at 100 ms per frame for 100 frames using TIR excitation with 1.43 mW excitation laser power. (B) Average number of identified molecules in 45 μm × 45 μm squares (2–5 observations) from 10 pM AX647-SA on Bio-DOPE modified PDMS surface after a series of solution washings.

DHBS. No decrease in surface fluorescence intensity is observed after one hour (Fig. 2(B)). In another experiment, 10 pM AX647-SA on a Bio-DOPE-modified PDMS is repeatedly washed with DHBS, HBS and 1% SDS solution; the surface densities of AX647-SA molecules do not show significant change (Fig. 3(B)).

We routinely store Bio-DOPE-modified PDMS channels in air at 6 °C for up to one week without affecting the AX647-SA binding. Nevertheless, there is still concern that phospholipids are not stable in an oxygen environment. A possible solution is to attach biotin groups to 1-alkenes. In this way, the vinyl group can participate in the crosslinking reaction when curing the PDMS prepolymer, which covalently links biotin to PDMS. Such reagents can be obtained by reacting an amine-reactive protein biotinylation reagent (*e.g.*, sulfo-NHS-biotin) with amino alkenes (*e.g.*, 1-amino-10-undecene).

Immobilization of antibody and lectin on PDMS

After the PDMS surface is functionalized with biotin groups and bound with avidin, other biotinylated proteins can be immobilized. We chose biotinylated anti-FLAG mouse monoclonal M2 antibody (BioM2) and biotinylated concanavalin A (Con A) as a demonstration for immunoassay and glycoprotein analysis. Their activities are confirmed by monitoring the binding of recombinant human β_2 adrenergic receptor (β_2AR), which can be recognized by BioM2 through a genetically engineered FLAG peptide sequence at the N terminus[14,15] and by Con A through its glycosylation. In the experiment, a Bio-DOPE-modified channel is sequentially incubated with 1 μM neutravidin for 10 min, 100 nM BioM2 or 100 nM Con A for 15 min and finally 200 nM tetramethylrhodamine-labeled β_2AR for 15 min. Buffer washings are performed between protein incubations and after β_2AR binding. As the control, we add β_2AR directly to the neutravidin-bound surface. DHBS is used as the buffer for BioM2 experiments and DHBS with 1 mM $CaCl_2$ and 1 mM $MnCl_2$ is used for Con A experiments.

As can be seen in Fig. 4, the fluorescence from β_2AR on PDMS surfaces attached with BioM2 and Con A is 18 times and 61 times higher than from the control, which indicates that β_2AR is bound to the modified PDMS surface specifically through the interaction with BioM2 or Con A. As was

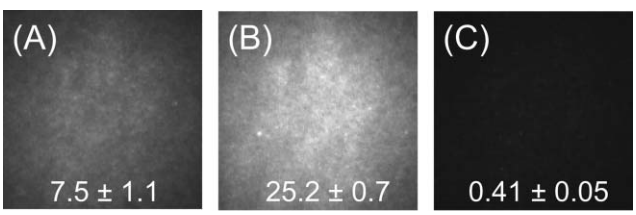

Fig. 4 Fluorescent images of 200 nM β_2AR on Bio-DOPE-modified PDMS surfaces that has: (A) neutravidin and BioM2; (B) neutravidin and Con A; and (C) neutravidin. Each image is that of a 34 μm × 34 μm square field of view. The images are acquired by epifluorescence with an integration time of 500 ms (300 ms for (B)) and an excitation laser power of 162 μW. All three images are displayed with the same contrast. Numbers in each image mark the average fluorescence (normalized by laser power and integration time) and its standard deviation from eight locations in the PDMS channel.

mentioned before, we have shown that β_2AR does not interact with the modified PDMS surface. Therefore, the residual binding in the control is mainly caused by the interaction between β_2AR and neutravidin.

Conclusions

We have demonstrated that by adding biotinylated phospholipids to the PDMS prepolymer before curing, the PDMS surfaces can be functionalized with biotin groups. Most of these phospholipids are embedded in the PDMS polymer matrix, but remain stable during storage in air and during surfactant rinsing. DDM on the modified PDMS surface blocks non-specific protein adsorption. Using this method, biotinylated antibodies and lectins can be immobilized on the PDMS surface through a linker layer of avidin or avidin variants. This approach of functionalizing PDMS surface is very easy to implement. It can also be generalized to other functional groups such as carboxylic acid, amine, and nitrilotriacetic acid (NTA) because phospholipids bearing these modifications are commercially available.

Experimental

Chemicals and materials

PDMS prepolymer (RTV-615 A and RTV-615 B) is purchased from R. S. Hughes (Santa Clara, CA). negative photoresist (SU 8-50) is from MicroChem (Newton, MA), perfluoro-1,1,2,2-tetrahydrooctyltrichlorosilane is from United Chemical Technologies (Bristol, PA). Phospholipids are purchased from Avanti Polar Lipids (Alabaster, AL) as chloroform solutions. Alexa Fluor 647 labeled streptavidin is from Invitrogen (Carlsbad, CA). 10% sodium dodecyl sulfate (SDS) solution, biotinylated anti-FLAG monoclonal mouse M2 antibody, and biotinylated concanavalin A from Jack bean (Type VI) are obtained from Sigma–Aldrich (St. Louis, MO). Recombinant human beta-2 adrenergic receptors are expressed in SF9 cells, purified, and labeled with tetramethylrhodamine-5′-maleimide (Invitrogen).[16] β-D-Dodecyl-*N*-maltoside is purchased from AnaTrace (Maumee , OH). Neutravidin is obtained from Pierce Biotechnology (Rockford, IL).

Microchannel fabrication

A silicon master is created by patterning SU-8 photoresist into 25 mm long × 1 mm wide × 100 μm high strips on a silicon wafer with a standard photolithography procedure.[17] The surface of the master is then treated with perfluoro-1,1,2,2-tetrahydrooctyltrichlorosilane vapor in a vacuum desiccator to prevent the adhesion of PDMS during the curing step. PDMS prepolymer (RTV 615 A and B mixed with a 10:1 mass ratio) is then cured against the master at 70 °C for 30 min before being cut into rectangular slabs. Inlet and outlet holes are punched into these slabs.

Biotinyl functionalization of PDMS

PDMS prepolymer (10:1 of RTV 615 A and B) is dissolved in hexane (as the thinner for spin coating) with a mass ratio of 1:2. A certain volume of 5 mg ml^{-1} phospholipid–chloroform solution is then added to the mixture. The mixture is spin coated on a micro coverglass (900 rpm for 9 s and then 2500 rpm for 30 s) to form a PDMS film that is about 10 μm thick. Such a small thickness is essential for total internal reflection (TIR) fluorescence imaging. The top slab of the flow channel is then put on the PDMS prepolymer coated coverglass before the latter is cured at three different temperatures: (1) 70 °C for 30 min; (2) 40 °C overnight; or (3) room temperature for two days. These three curing methods result in similar surface properties; nevertheless, 40 °C curing is preferred because it is relatively fast, and it does not cause the curvature of the coverglass, which arises from the difference in the thermal expansion between PDMS and glass. After curing, the top piece is tightly bound to the coverglass.

Fluorescence imaging

Fluorescence imaging is performed on a Nikon TE2000-U inverted microscope (Nikon, Melville, NY) with a 100× NA 1.4 oil immersion objective. A 532-nm diode-pumped frequency-doubled Nd:YAG laser (Compass 215M, Coherent, Santa Clara, CA) is used for tetramethylrhodamine (TMR) excitation and a 636-nm diode laser (RCL638-025, Crystalaser, Reno, NV) is used for the excitation of Alexa Fluor 647 (AX647). A translation stage enables the switching between epifluorescence, wide-field excitation (for ensemble imaging), and TIR excitation (for single molecule imaging).[18] The emitted fluorescence is filtered with a dichroic mirror (565 DRLPXR for TMR and 400-535-635 TBDR for AX647, Omega Optical, Brattleboro, VT) and a bandpass filter (HQ595/50 m for TMR and HQ675/50 m for AX647, Chroma Technology, Rockingham, VT) before being detected by a intensified CCD camera (I-PentaMax, Roper Scientific, Trenton, NJ). The intensifier gain is set to 70 in all experiments. Images are analyzed by home-coded programs.

Acknowledgements

S.K. is grateful for a Stanford Bio-X Graduate Fellowship. We thank Gayathri Swaminath and for purifying and labeling β_2AR. This work is supported by National Science Foundation NSF BES-0508531.

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