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Lithographic techniques and surface chemistries for the fabrication of PEG-passivated protein microarrays

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Abstract

This article presents a new technique to fabricate patterns of functional molecules surrounded by a coating of the inert poly(ethylene glycol) (PEG) on glass slides for applications in protein microarray technology. The chief advantages of this technique are that it is based entirely on standard lithography processes, makes use of glass slides employing surface chemistries that are standard in the microarray community, and has the potential to massively scale up the density of microarray spots. It is shown that proteins and antibodies can be made to self-assemble on the functional patterns in a microarray format, with the PEG coating acting as an effective passivating agent to prevent non-specific protein adsorption. Various standard surface chemistries such as aldehyde, epoxy and amine are explored for the functional layer, and it is conclusively demonstrated that only an amine-terminated surface satisfies all the process constraints imposed by the lithography process sequence. The effectiveness of this microarray technology is demonstrated by patterning fluorescent streptavidin and a fluorescent secondary antibody using the well-known and highly specific interaction between biotin and streptavidin.

Keywords

Protein microarrays; Poly(ethylene glycol); Lithography; Non-specific adsorption

1. Introduction

The invention of microarray techniques for the analysis of DNA (Schena et al., 1995) in the past decade has opened up enormous possibilities for the highly parallel or multiplexed analysis of genomic DNA. Such microarray analysis has been used to simultaneously profile multiple genes and mRNAs in cells through the hybridization of fluorescently-labeled DNA strands to DNA of known sequences immobilized on a glass slide, thereby providing extensive information about the nature of genetic material and its role in cellular processes. An important application of DNA microarray technology is the investigation of the nature and abundance of expression of various proteins in cells. Despite a certain degree of success, genetic and mRNA information alone are often not enough to understand cellular reaction pathways and their relation to various diseases and conditions. This is because there is often no one-to-one correlation between mRNAs and the proteins they encode for, and many proteins often undergo

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post-translational modifications that are not coded for by their mRNAs. The consensus today is that only direct protein profiling and quantitation through protein microarrays can provide the kind of information and insight necessary to understand cellular cycles, pathways, and diseases (Zhu and Snyder, 2001). In addition to analyzing cellular proteins, protein microarrays can be employed to assay antibodies and antigens by adapting traditional sandwich ELISA protein assays (Wiese et al., 2001) and for drug discovery by determining protein–protein and protein–ligand interactions (MacBeath and Schreiber, 2000). Microarrays of proteins thus serve as a highly versatile platform to perform quantitative and multiplexed analysis of a wide range of proteins, antibodies and antigens.

Macbeath and Schreiber (2000) were the first to demonstrate the highly reliable and parallel patterning of proteins in a microarray format using a modified robotic system used originally for DNA microarrays. Following up on this, Haab et al. (2001) demonstrated both antibody and antigen microarrays for the simultaneous detection of more than 100 pairs of antibody–antigen interactions, using a calibrated, two-color fluorescence based detection. Around the same time, Wiese et al. (2001) demonstrated that protein microarrays can be employed in a classic sandwich assay to detect antigens such as prostate specific antigen (PSA) and interleukin in protein mixtures with very little cross-reactivity across antigens. In a pioneering experiment, Zhu et al. (2001) printed 5800 purified proteins from the yeast proteome onto glass slides and used this microarray to identify proteins interacting with biologically significant markers such as calmodulin and phospholipids. Such an extensive microarray technique opens up the arena to the analysis and screening of entire eukaryotic proteomes for various biological activities. In an interesting variation of microarray technology, Angenendt et al. (2003) spotted capture antibodies as well as subsequent layers of proteins onto the microarray slide using the robotic spotter on the original spots to perform an ELISA test. While we have so far discussed the most representative of protein microarray techniques and experiments, comprehensive surveys of a multitude of protein microarray array technologies and their applications can found in Templin et al. (2004) and Cutler (2003). Zhu and Snyder (2003) and Kusnezow and Hoheisel (2003) review the different substrates and chips currently being employed for different assays.

Despite rapid advances significant challenges remain before protein microarrays can achieve the same status for proteomics that DNA microarrays have attained for genomics. As discussed by Haab et al. (2001) and Pawlak et al. (2002), the three most important issues confronting protein microarray technology today are: (a) preserving the active conformation of proteins upon printing them, (b) minimizing non-specific adsorption of proteins on the surface of the microarray slide, (c) cross-reactivity of target proteins with probe proteins. The focus of this article is on the second of these challenges, namely, the minimization of non-specific adsorption of proteins onto the bare microarray surface surrounding protein spots. Currently, the most widely used method to reduce non-specific protein adsorption is blocking the regions surrounding the microarrays with a solution of bovine serum albumin (BSA) (MacBeath and Schreiber, 2000) or non-fat milk (Haab et al., 2001). Though this method works quite well for purified solutions of proteins at higher concentrations, the background noise due to proteins adhering to this coating limits detection at lower concentrations. This method is, therefore, not the most effective method to minimize or eliminate non-specific adsorption. In addition, the enormous size of the BSA molecule can sometimes obscure the printed protein molecules, thereby preventing their interaction with proteins in subsequent steps of the assay (MacBeath and Schreiber, 2000). It is, therefore, of great interest to investigate into alternative coatings to reduce non-specific protein binding onto microarray slides. Conventional microarray technology employs robotic spotters to print proteins on glass slides, and is limited in terms of surface chemistry available to prevent non-specific protein adsorption. Lithography-based patterning techniques, on the other hand, are capable of patterning thin films of various materials as well as molecular monolayers such as aminosilanes, mercaptosilanes and poly (ethylene glycol) silanes, and allow the exploration of various surface chemistries for protein

microarrays to increase signal-to-noise ratios. Various research groups have explored the use of different types of lithography for protein microarrays, the most important of which are reviewed here.

Sorribas et al. (2002) developed a relatively straightforward process in which, proteins are first coated on a silicon surface, protected by a layer of sucrose, patterned with photoresist and finally subjected to microarray reactions with other proteins. The main problems with this method are that proteins are exposed to the high-temperature processing steps, which can destroy the activity of the patterned proteins. Furthermore, protein spots are surrounded by bare silicon with no chemical coating to reduce non-specific proteins adsorption during subsequent binding steps of a multi-step assay. Lee et al. (2004) have demonstrated an improved lithography-based process in which fluorocarbon thin films are patterned on silicon using lift-off and simple proteins such as BSA are subsequently attached to the interstitial microarray spots using standard covalent chemistry. While fluorocarbon films help in reduction of non-specific protein binding compared to bare silicon, there is still measurable protein adsorption, and hence, they are not the best coatings available.

It has been known for a long time that poly(ethylene glycol) (PEG) forms highly inert coatings that can resist adsorption of a variety of proteins (Zalipsky and Harris, 1997). PEG is a water-soluble polymer capable of extensive hydrogen bonding that swells in aqueous solutions and satisfies all the properties for surface coatings that greatly resist non-specific protein adsorption, as outlined in Ostuni et al. (2001a,b). Table 1 below summarizes the results of protein microarray experiments performed by different research groups that employ PEG and other surface chemistry techniques to minimize non-specific protein adsorption. These experiments provide a definitive demonstration that PEG coatings are far superior to bare silicon and other molecular coatings such as BSA/non-fat milk and fluorocarbon coatings in preventing non-specific protein adsorption, and are therefore highly desirable as passivation agents in protein microarrays. During the past few years, researchers have used standard lithographic techniques to fabricate microscale patterns of a functional thin film surrounded by a PEG such that proteins attach covalently to the functional microarray spots, while being repelled by the PEG coating effectively. Veiseh et al. (2002) fabricated patterns of thin gold films on a silicon wafer, coated the surrounding silicon with a PEG silane layer, and assembled a layer of the functional NHS ester on the gold patterns to demonstrate that the proteins assembled almost exclusively onto the gold with little protein adsorption on the PEG-coated silicon. Falconnet et al. (2004) lithographically fabricated patterns of biotin-terminated PEG surrounded by PEG regions on niobia-coated silicon wafers and assembled streptavidin molecules exclusively onto the biotin patterns, with no visible protein being bound to the surrounded PEG-coated regions. It should, however, be noted that while the above two approaches successfully employ PEG to eliminate non-specific adsorption, they are incompatible with industry-standard protein microarray technology, since they make use of non-standard thin-films such as gold and niobia-based materials for protein immobilization.

To our knowledge, no research group has thus far successfully fabricated protein microarrays in standard format on conventional glass slides in which PEG coatings are used to eliminate non-specific binding of proteins, antibodies, and antigens. Therefore, in this article, we discuss a protein microarray technology we have developed based on standard lithography, which incorporates the two important features lacking in the techniques discussed above: (a) PEG coatings to minimize non-specific protein adsorption, (b) complete compatibility with current microarray technology and methods of protein detection. To achieve these objectives, we perform lithographic patterning on standard glass slides to create patterns of the functional molecule aminosilane that are surrounded by an inert PEG coating, and subsequently assemble proteins exclusively onto the functional regions to demonstrate protein patterning using biotin, streptavidin and biotin-conjugated antibodies. The process developed in this article has the

potential to serve as a platform for a new generation of protein microarrays that can provide the sensitivity necessary to detect ultra-low amounts of proteins for highly critical biological assays.

2. Experimental

2.1. Materials and methods

Three types of glass slides modified with amine groups (SuperAmine® slides), aldehyde groups (SuperAldehyde® slides) and epoxy groups (SuperEpoxy® slides) were purchased from Telechem International (Sunnyvale, CA). 2-[Methoxy(polyethyleneoxy) propyl] trimethoxysilane, henceforth referred to as PEG silane (average molecular weight 360 Da), and 3-aminopropyltrimethoxysilane, referred to as APS, were purchased from Gelest, Inc. (Morrisville, PA). NHS-LC-biotin was purchased from Pierce Biotechnology, Inc. (Rockford, IL), AlexaFluor dye, AlexaFluor-conjugated streptavidin and protein-labeling kits were purchased from Molecular Probes, Inc. (Eugene, OR) and biotin-conjugated rat anti-mouse IgG was purchased from Zymed Laboratories (South San Francisco, CA). Microfabrication equipment for lithographic patterning was provided by the Berkeley Microfabrication Laboratory.

2.2. Lithographic patterning of glass slides

All three types of functionalized glass slides (amine, aldehyde and epoxy) were patterned using the same lithographic process sequence outlined in Fig. 1. In addition to patterning these three standard glass slides, a fourth type of patterning process was also employed involving a biotin layer. In this process, a SuperAmine® glass slide was first coated with the molecule NHS-LC-biotin by immersing the glass slide in a 5 mM solution of NHS-LC-biotin in 1 × phosphate buffered saline (PBS) for 1 h, followed by washing in water to remove excess NHS-LC-biotin. After the coating with biotin the glass slide was subjected to the same lithographic process sequence as the rest of the slides. The lithographic mask was fabricated to replicate a microarray format, where square regions of various sizes (500, 300, 200, 100 and 50 μm) were printed in successive rows with each row consisting of microarray spots of the same size. The fabrication process starts with coating the pre-functionalized glass slide (Fig. 1(a)) with a layer of photoresist of approximately 1.6 μm thickness using a Headway Photoresist Spinner at 3000 RPM for 30 s, followed by a baking the glass slide a 90 °C for 1 min (Fig. 1(b)). The glass slide is then exposed through the microarray mask in a Karl Suss MA6 Mask Aligner system, following which it is baked at 120 °C for 1 min for a post-exposure bake. The glass slide is then washed in developer solution to remove the photoresist from the exposed regions, thereby patterning the glasss slide (Fig. 1(c)). After baking for 10 min at 120 °C, the slide is placed in a Technics® plasma etching system, where it is exposed to O_2 plasma to remove the organic functional coating around the photoresist patterns (740 mTorr, 300 W for 2 min) and treated with 10:1 buffered hydrofluoric acid (BHF) for approximately 1 min to completely remove any organic material around the resist patterns (Fig. 1(d)). The glass slide is then dried at 120 °C for 5 min after which it is again subjected to O_2 plasma under the same conditions as before, in order to make the glass surface surrounding the resist patterns hydrophilic and prepare for coating it with PEG silane.

2.3. Coating the glass slide with PEG silane

The protocol developed by Papra et al. (2001) was adopted to coat the glass slides with PEG silane. PEG silane and concentrated HCl were mixed in toluene in a freshly cleaned glass beaker with 100 μl PEG and 40 μl HCl present in 50 ml of toluene. The beaker was then placed in a sonicator for 10 min after which, it contents were transferred to another clean glass beaker. The glass slide was placed in the PEG toluene solution for approximately 1 h followed by washing once in toluene for 2 min, once in acetone for 5 min, once in ethanol and finally in

de-ionized water for 2 min each time. It should be noted that the acetone wash strips the photoresist off the glass surface, thereby exposing the functional molecular layer. Thus, at this stage, the glass slide consisted of patterns of functional molecules surrounded by a coating of PEG molecules (Fig. 1(e and f)).

2.4. Conjugation of fluorescent dye to antibody

AlexaFluor 488 fluorescent dye (absorption peak = 495 nm, emission peak = 519 nm) was conjugated to the biotinylated secondary antibody using a standard protocol outlined in the Molecular Probes protein labeling kit. The final antibody concentration was measured as 7 μM. The antibodies were stored in the dark in a −20 °C freezer for further use in a 1 × PBS buffer.

2.5. Assembly of fluorescent streptavidin on different types of glass slides

A stock solution of fluorescent streptavidin conjugated AlexaFluor 488 dye was prepared at a concentration of 25 μM and suspended in a 1 × PBS buffer and experiments were performed to assemble the protein on PEG-patterned glass slides with various functional microarray patterns. In all the following experiments, streptavidin suspended in 1 × PBS was used at a dilution of 1:100 of the stock solution. The first experiment consisted of incubating a solution of streptavidin on microarray slides with aldehyde patterns for a period of 2 h, followed by washing of glass slides with a 0.1% solution of the detergent Tween-20 in 1 × PBS for 3 min to remove excess streptavidin. The glass slides were subsequently washed in 1 × PBS twice and dried under a continuous flow of N_2 before imaging. The second experiment consisted of the same sequence of steps as before with epoxy-patterned microarray slides. It should be noted that in these two experiments, the protein is expected to covalently attach to the aldehyde and epoxy functional groups. In the third experiment, amine-patterned glass slides were first treated with NHS-LC-biotin by immersing them in a 5 mM solution of NHS-LC-biotin in 1 × PBS for a period of 1 h, followed by washing in water for 2 min to remove excess biotin. Fluorescent streptavidin was subsequently immobilized onto these biotin-functionalized glass slides using the same procedure used with aldehyde and epoxy slides. In the final experiment a SuperAmine® glass slide was *first* treated with a solution of NHS-LC-biotin (same procedure as before) and subsequently subjected to the lithography and PEG functionalization process as outlined in Fig. 1, after which the slides was then treated with streptavidin as in the previous three experiments.

2.6. Patterning of fluorescent antibody molecule

Fluorescent, biotin-conjugated secondary antibody was assembled onto microarray slides as follows. Microarray slides patterned with biotin and PEG molecules were incubated with non-fluorescent streptavidin to create a surface containing streptavidin molecules in the microarray spots, surrounded by PEG coating. This microarray was subsequently functionalized with secondary biotin-conjugated antibody by placing a solution of the antibody at a concentration of 70 nM for a period of 2 h. After the reaction, the glass slides were washed in a 0.1% solution of the detergent Tween-20 in 1 × PBS for 3 min to remove excess streptavidin and then in 1 × PBS twice before being dried under a continuous flow of N_2.

2.7. Imaging of microarrays

Fluorescence signals from the microarrays were measured with a commercial objective-scanning confocal Axon 3000i scanner (Axon Instruments), with two excitation laser diodes (532 and 635 nm). Though the laser wavelength of 532 nm is not at the absorption peak of AlexFluor 488, it is still within its excitation spectrum, and hence, this channel was used for all measurements.

3. Results and discussion

Fig. 2 presents fluorescent images of microarray slides on which AlexFluor 488-labeled streptavidin was immobilized using aldehyde, epoxy, and amine surface chemistries, with PEG silane coating the regions surrounding the patterns. Fig. 3 shows the result of performing antibody patterning using glass slides with biotin patterns surrounded by PEG. Fig. 4 consists of intensity line plots for the microarray images shown in Figs. 2(c and d) and 3, which are obtained by measuring the intensity at every pixel along a horizontal line drawn across a single row of microarray spots.

Fig. 2(a) shows that the signal from the aldehyde-functionalized patterns surrounded by PEG silane. The aldehyde-functionalized surface is used extensively in DNA and protein microarray analysis because a variety of proteins can be directly conjugated to the aldehyde moiety using the Schiff's base reaction. Such a direct chemistry has the advantages of covalent attachment and high protein surface density, and would be preferable for any microarray technique that aims to achieve high signal to noise ratio. It is seen, however, that the signal from this slide is extremely weak, and hence, this chemistry cannot be used to pattern proteins using our microfabrication process sequence. Epoxy surfaces are also highly reactive towards primary amines, and hence, can be used for direct, covalent attachment of protein on glass surfaces with high surface density. Fig. 2(b) shows a magnified image of a single microarray pattern on an epoxy-coated glass slide that was patterned to have a PEG-coated surface around the microarray patterns. The center region is the epoxy pattern, while the surrounding region is the PEG coating. This image was taken using a Nikon TE2000-U inverted epi-fluorescence microscope because no signal was observed using the Axon microarray scanner. It is seen that the signal from the epoxy slide is actually slightly lower than the background due to the PEG, thereby making SuperEpoxy® slides also unsuitable for protein assembly. Fig. 2(c) shows the result of using amine surface chemistry and NHS-LC-biotin for streptavidin assembly, corresponding to the third type of experiment described in Section 2.5. In this case, a glass slide with amine patterns surrounded by a PEG coating was first coated with biotin on the amine surface, using the reaction between the NHS ester and primary amines. This process resulted in a surface terminated with biotin molecules, and fluorescent streptavidin was subsequently assembled on the microarray surface. It is observed that the signal is quite high and the noise from the PEG-coated regions, while observable, is quite low. This may be better visualized in the intensity line plot shown in Fig. 4(a), where the noise from the regions between microarray spots is seen to be around an order of magnitude lower than the signal from the spots themselves. The average signal-to-noise ratio calculated from many such plots was approximately 15:1. Fig. 2(d) shows the results of the last type of protein patterning experiment mentioned in Section 2.5, in which NHS-LC-biotin is initially coated on the SuperAmine® slides to obtain a biotin-terminated glass slide. Assuming that biotin groups survive the various processing steps during lithography, photoresist patterning and PEG functionalization were performed on the biotin-coated surface, resulting in a glass slide in which biotin patterns should be surrounded by a PEG coating. It is seen that the signal is still very high, while the noise from the PEG background is below the detection limits of the flatbed scanner, resulting in extremely high signal-to-noise ratios as can be see in Fig. 4(b) (signal-to-noise for this case was not calculated since the noise is below detection threshold of the scanner).

In the antibody experiments, biotin/PEG patterned microarray glass slides were first incubated with non-fluorescent streptavidin to immobilize the latter onto the biotin patterns. The results are presented in Fig. 3. For reasons not clear at this point, the smallest microarray patterns measuring 50 μm square were not visible in the case of antibody patterning. The streptavidin molecule has four binding pockets that can bind with biotin with very high specificity. Hence, the streptavidin immobilized on biotin patterns will have additional binding sites to bind with a biotin-conjugated antibody, which is the basis for the antibody experiments outlined Section

2.6. It is seen that in this instance too, the signal is quite high and that the noise is very low, the signal-to-noise ratios usually ranging between 150:1 and 250:1, as may be calculated from the intensity line plots such as presented in Fig. 4(c). It should be noted that the signal to noise ratios in all our experiments are quite high, especially in Figs. 2(d) and 3. It is worth mentioning at this point that these high signal-to-noise ratios were obtained despite the fact that the AlexaFluor 488 dye, whose absorption peak is at 495 nm, is actually excited by the scanner at a wavelength of 532 nm, which lies at the tail end of its excitation spectrum. We believe that even higher ratios can be obtained with an optimized dye/laser combination such as AlexaFlour 488/Ar^+ ion laser.

Of all the standard surface chemistries available, it is seen that only amine-coated glass slides are able to provide the necessary reactive surface for protein assembly. This is most likely due to the fact that aldehyde and epoxy groups are highly reactive, and hence, lose their functionality during any of the various steps in the patterning process such as photoresist coating and baking at high temperatures that are described in detail in Section 2.2. The epoxy group is very susceptible to hydrolysis, while the aldehyde group is susceptible to redox reactions that covert it to carboxylic acids or alcohols, thereby destroying its functionality. While the exact step responsible for the degradation is not obvious, it is clear that the lithography processing sequence is responsible for these groups' losing their capability to conjugate proteins. In contrast, it is seen that the amine-coated glass slides still retain their functionality despite the harsh conditions that the glass slide is subjected to during the patterning process. In addition, it is important to note that both the amine-terminated surface and the biotin-functionalized surfaces survive the lithography process intact as observed in Fig. 2(d).

It is interesting compare the protein patterning processes that resulted in Fig. 2(c and d). In the case of Fig. 2(c), the microarray slide initially consisted of patterns of amine regions surrounded by PEG silane, on which NHS-LC-biotin was subsequently assembled, following which, streptavidin was functionalized. Thus the PEG coating is exposed to two patterning steps, the first step involving the small molecule NHS-biotin. In the case of Fig. 2(d), streptavidin is directly assembled onto the biotin/PEG patterned microarray surface and the PEG is exposed only to streptavidin. It should be mentioned that this difference in noise levels between the two types of patterning was very repeatable, and hence, not an artifact of the different experimental conditions employed. We hypothesize that there could be two reasons for the higher noise observed in Fig. 2(c). First, it is possible that the PEG chain, which is essentially a polyether, degrades upon repeated exposure to atmosphere and aqueous solutions resulting in loss of its inertness (Ostuni et al., 2001a,b). Secondly, it is possible that exposing the PEG to the small molecule NHS-LC-biotin before the streptavidin step results in patches of non-specifically adsorbed NHS-LC-biotin resulting in higher noise when streptavidin is subsequently reacted. The above set of experiments demonstrate that the most robust and successful patterning sequence involves creating biotin/PEG patterns first and then assembling proteins on top of these patterns. This is the rationale for employing biotin-terminated glass slides lithographic patterning for antibody experiments.

In the context of background noise, it is useful to briefly discuss the reasons for the high protein rejection capabilities PEG molecules. Various research groups have theoretically investigated the physical basis for rejection of proteins by PEG coatings (Halperin, 1999; Jeon and Andrade, 1991; Jeon et al., 1991; Ostuni et al., 2001a,b; Sharma et al., 2002; Xia et al., 2002). Currently, it is accepted that the PEG molecule consists of very dynamic and heavily hydrated chains of poly(ethylene oxide) that are hydrogen bonded to the surrounding water. Hermans (1982) was the first to suggest that purely physical steric interactions could result in large exclusion volumes for the case of PEG polymer brushes, thereby resulting in highly reduced protein adsorption on PEG-coated surfaces. Following up on these arguments, Jeon et al. (Jeon and

Andrade, 1991; Jeon et al., 1991) performed extensive theoretical modeling to understand more fully the various attractive and repulsive forces at play when protein molecules approach a PEG-coated surface. These models suggested that the PEG's rejection capacity is a balance between attractive van der Waals forces between the protein and the surface and repulsive steric forces and osmotic pressure on the protein as it is pressed into the heavily hydrated PEG layer.

A few improvements to the technique presented here may be envisioned in order to make it more robust and to standardize it for applications in industry-standard protein microarray technology. One of the most important concerns is that the PEG layer tends to deteriorate upon repeated and prolonged exposure to ambient conditions. It should be possible to mitigate this problem by synthesizing more robust variants of the PEG molecule, while retaining inertness towards proteins. Halperin's generalized theory for polymer brushes (Halperin, 1999), including PEG coatings, suggests that both the PEG molecule's surface grafting density as well as its length play an important role in determining its rejection capabilities. If the protein is very small compared to the radius of gyration of the PEG molecules, then it may be able to penetrate the surface coating easily, and hence, a high enough surface density is necessary to generate the osmotic repulsive forces necessary to repel it. On the other hand, if the proteins are large enough compared to radius of gyration approach the PEG-coated surface, they will not be able to penetrate the surface coating, and hence, the length of the PEG chain is the determinant of the rejection capabilities. Therefore, the PEG coating's resistance to protein adsorption may be tailored and optimized through synthetic chemistry techniques to significantly suppress noise due to non-specific protein adsorption. Another aspect of the current technique is that the protein assembly process involves a biotin–streptavidin–biotinylated protein sequence, which is probably not the most efficient method to assemble proteins, antibodies, and antigens on surfaces. This is due to the fact that streptavidin is a large molecule, and therefore, its surface density is bound to be much lower than small molecules such as functional silanes. This can lead to a significant reduction in the surface density of the assembled antibody, and hence, a lower signal. It should be possible to obtain higher surface density of proteins using a different surface chemistry compatible with the lithography process. In addition, unlike conventional microarrays printed using robotic spotters, it is currently not possible to functionalize each spot with a different protein. This can be resolved through the development of a microfluidic system to address each array spot individually, resulting in a multiplexed microarray. With these and other enhancements, the lithography-based microarray that we have presented here has genuine potential to scale down the size of microarray spots using standard technology and chemistry, thereby greatly increasing the number of patterns per unit area and allowing the analysis of a very large number of proteins simultaneously.

4. Conclusions

Our experiments have demonstrated that it is possible to use lithography in conjunction with standard surface chemistry and PEG-based passivation techniques to assemble proteins and antibodies in a microarray format with very high selectivity. From among the various surface chemistries considered, it has been shown conclusively that only amine-functionalized glass slides are robust enough to survive the lithographic patterning process. In addition these experiments show that patterning of biotin surfaces provides better results than direct patterning of amine surfaces when streptavidin-based protein assembly is performed. Our lithography-based protein microarray technique is the first known instance in which standard functionalized glass slides, standard lithographic tools and off-the-shelf chemicals such as PEG silane have been used to fabricate microarrays in which a PEG coating provides resistance to non-specific protein adsorption. While others researchers have demonstrated selective protein assembly using PEG molecules, such experiments have involved processes such as gold film patterning on silicon surfaces (Veiseh et al., 2002), exotic surfaces such as niobia-based thin films for

PEG self-assembly (Falconnet et al., 2004) and other non-standard techniques not accessible to a broad cross-section of the protein microarray community. The present process, when used in conjunction with protein assay techniques such as ELISA, can therefore serve as a simple, viable and a general platform for the highly multiplexed detection of a broad class of proteins, antibodies and antigens.

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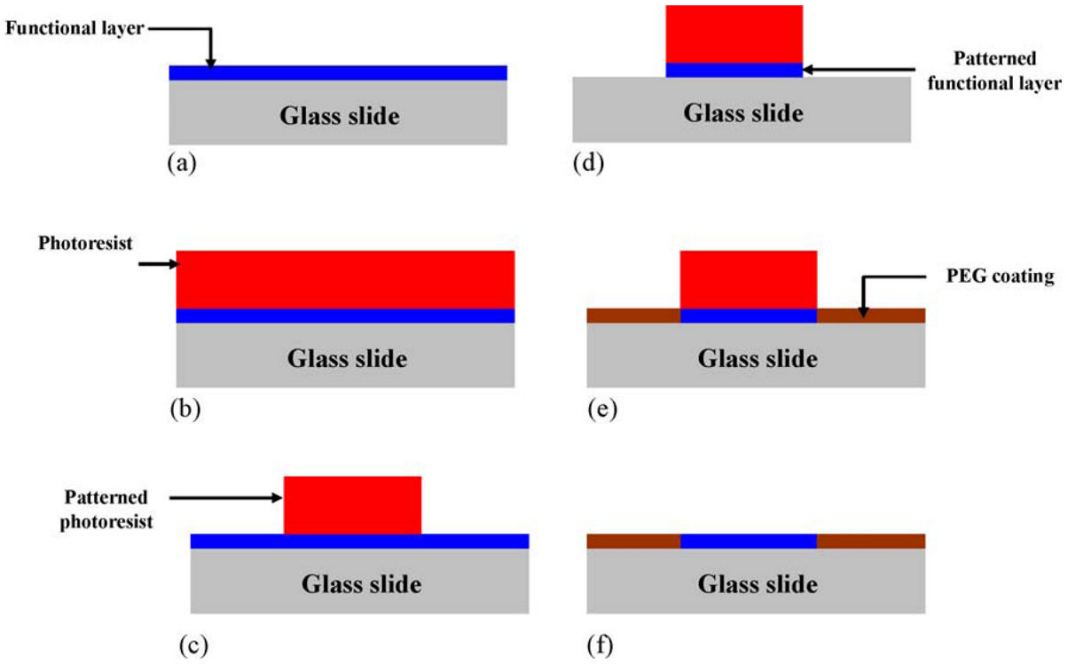

Fig. 1.

The general PEG-based patterning process. (a) Glass slide coated with a functional layer (aldehyde, epoxy, amine or biotin), (b) functional glass slide coated with photoresist for patterning, (c) glass slide after lithographic patterning of resist, (d) etching of functional layer surrounding patterned resist and cleaning through a HF dip, (e) functionalization of surrounding glass with PEG silane, (f) removal of photoresist to create glass slides with patterns of functional molecule/PEG.

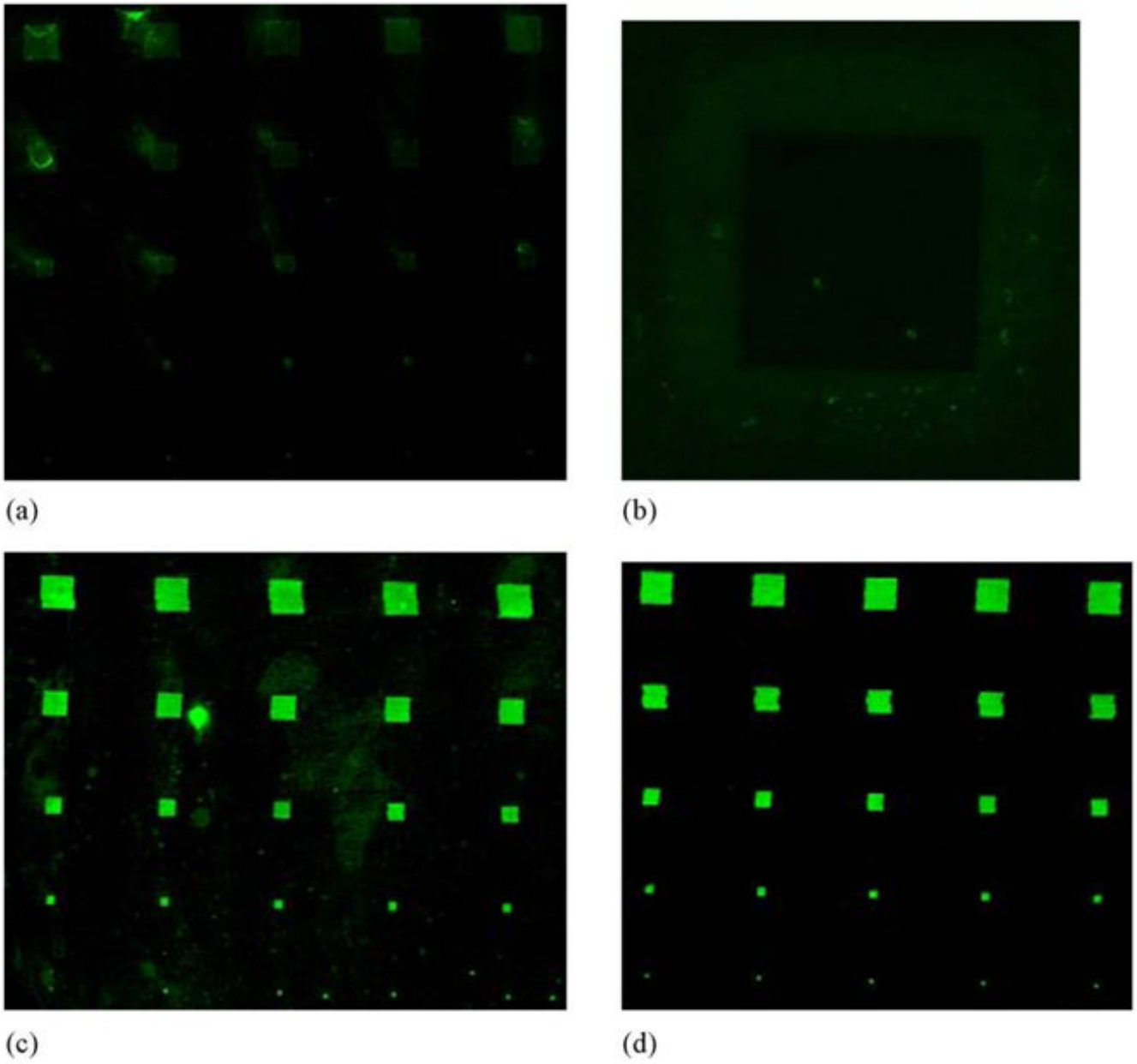

Fig. 2.

Microarray images of fluorescent streptavidin patterning experiments using four different surface chemistries in conjunction with PEG coating for passivation. (a) Streptavidin on aldehyde patterns, (b) streptavidin on epoxy pattern, (c) streptavidin on NHS-LC-biotin patterns, with NHS-LC-biotin deposited *after* lithographic patterning of amine slides, (d) streptavidin on NHS-LC-biotin patterns, with NHS-LC-biotin deposited *before* lithographic patterning of amine slides. The sizes of the squares in different rows are 500, 300, 200, 100 and 50 μm from top to bottom.

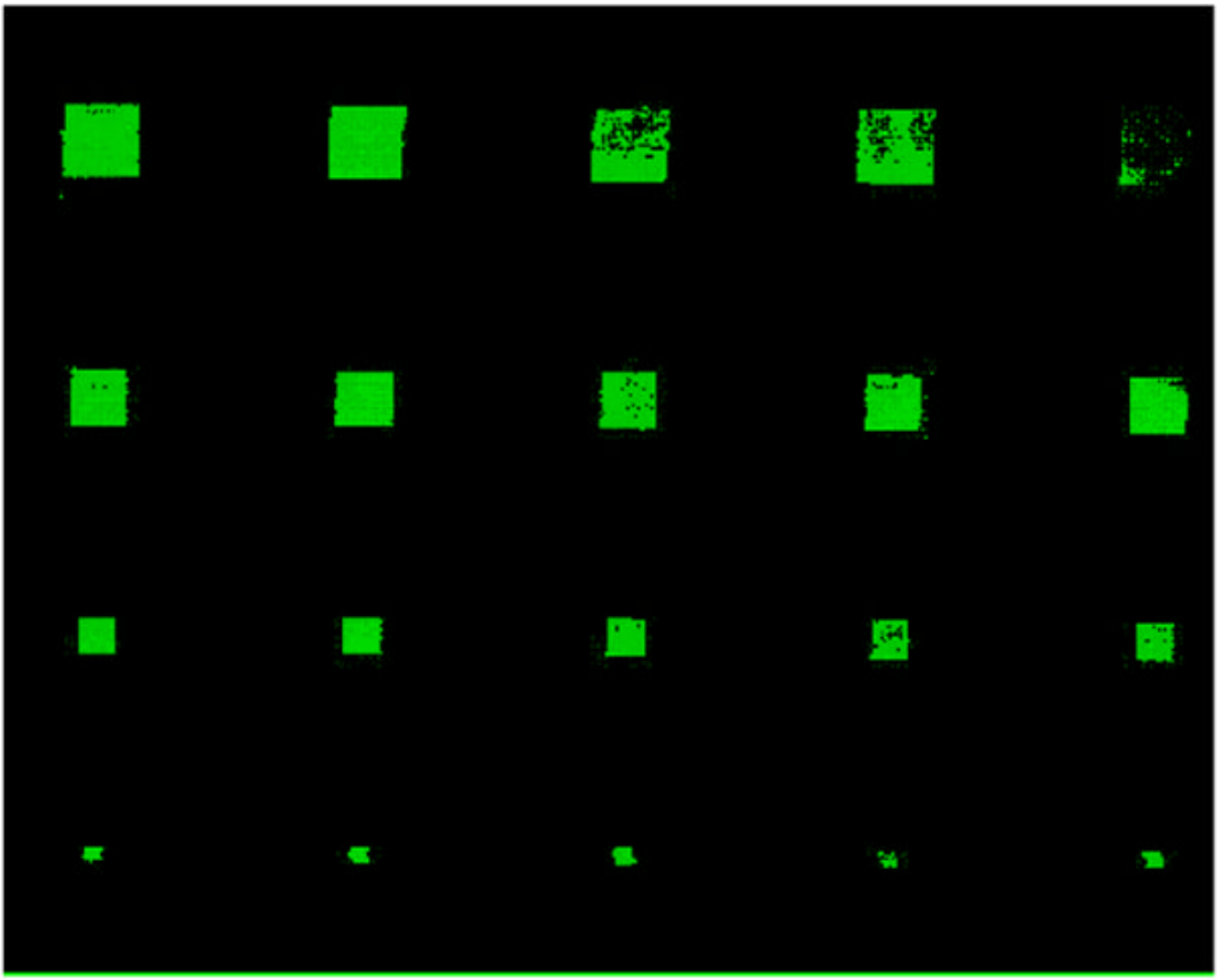

Fig. 3.

Image of fluorescent and biotin-conjugated secondary antibody patterned as a microarray using amine surface chemistry. A Superamine® slide was first coated with NHS-LC-biotin, lithographically patterned and coated with PEG on surrounding silicon. Streptavidin was first assembled on the biotin patterns, followed by assembly of the antibody. The sizes of the squares in different rows are 500, 300, 200 and 100 μm from top to bottom. The 50 μm squares were not visible in this experiment.

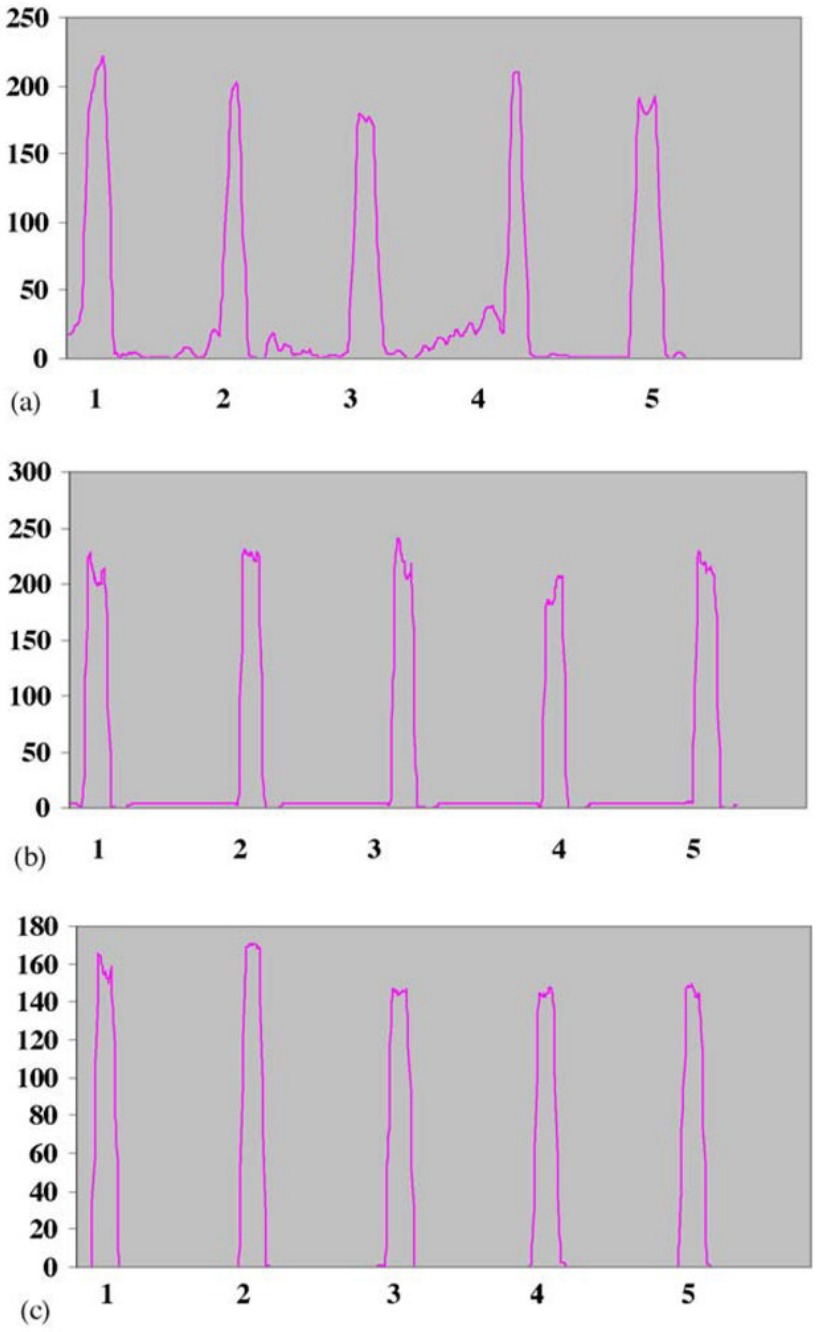

Fig. 4.

Intensity line plots of microarray images, with intensity in arbitrary units on the vertical axis and the index of the spot on the horizontal axis. (a, b and c) correspond to Figs. 2(c and d) and 3, respectively. In all three images, the third row is used for line intensity analysis.

Table 1

Comparison of non-specific adsorption of proteins on PEG and other coatings employed by different researchers

Reference	Proteins studied	Measurement technique(s) used	Non-specific binding on PEG	Other surfaces/ coating(s) used for passivation
Sorribas et al.	IgG	Radiolabeling	28 ng/cm^2	500 ng/cm^2 on fluorinated silane
Sharma et al.	Albumin, fibrinogen	Ellipsometry	~50–100 ng/cm^2	~600–700 ng/cm^2 on bare silicon
Lee et al.[a]	BSA, streptavidin	AFM, fluorescence microscopy	–	Substantial non-specific adsorption on fluorocarbon-coated surfaces
Veiseh et al.[a]	IgG, BSA	AFM, fluorescence microscopy	Very low	Much higher protein adsorption on bare silicon
Falconnet et al.[a]	Streptavidin	Fluorescence and mass spectroscopy	Below detection threshold	–
Haab et al.[a]	Various antibodies and antigens	Fluorescence using flatbed scanners	–	Substantial non-specific adsorption on non-fat milk-coated regions

Both qualitatively and quantitatively, it is seen that PEG coatings are far superior to any other surface chemistry for minimizing non-specific protein adsorption.

[a]These authors do not provide quantitative data pertaining to non-specific adsorption either on PEG or other coatings.