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Improved Glass Surface Passivation for Single-Molecule Nanoarrays

Haogang Cai¹ and Shalom J. Wind^{2,*}

¹Dept. of Mechanical Engineering, Columbia University, New York 10027, USA

²Dept. of Applied Physics and Applied Mathematics, Columbia University, New York 10027, USA

Abstract

Single-molecule fluorescence techniques provide a critical tool for probing biomolecular and cellular interactions with unprecedented resolution and precision. Unfortunately, many of these techniques are hindered by a common problem, namely the nonspecific adsorption of target biomolecules. This issue is mostly addressed by passivating the glass surfaces with a poly(ethylene glycol) (PEG) brush. This is effective only at low concentrations of the probe molecule, because there are defects inherent to polymer brushes formed on glass coverslips, due to the presence of surface impurities. Tween-20, a detergent, is a promising alternative that can improve surface passivation, but it is incompatible with living cells, and it also possesses limited selectivity for glass background over metallic nanoparticles, which are frequently used as anchors for the probe molecules. To address these issues, we have developed a more versatile method to improve the PEG passivation. A thin film of hydrogen silsesquioxane (HSQ) is spin-coated and thermally cured on glass coverslips in order to cover the surface impurities. This minimizes the formation of PEG defects and reduces nonspecific adsorption, resulting in an improvement comparable to Tween-20 treatment. This approach was applied to single-molecule nanoarrays of streptavidin bound to AuPd nanodots patterned by e-beam lithography (EBL). The fluorescence signal to background ratio (SBR) on HSQ coated glass was improved by ~ 4-fold as compared to PEG directly on glass. This improvement enables direct imaging of ordered arrays of single molecules anchored to lithographically patterned arrays of metallic nanodots.

Graphical Abstract

*Corresponding Author: sw2128@columbia.edu.

Supporting Information. Chemical composition of glass coverslips. Nanoarray fabrication: EBL vs. NIL. SiO₂ deposition: PECVD vs. HSQ. PSF and optical resolution. Intensity threshold of particle detection. Molecular occupancy in defective regions of PEG. Nonspecific adsorption on three types of surfaces. Cell assays on HSQ + PEG surfaces. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

Glass surface passivation; nonspecific adsorption; single-molecule; PEG; HSQ; SBR

Introduction

The past two decades have seen the development of a new class of tools for controlling, manipulating and probing individual molecules, and such single-molecule techniques have become a mainstay in investigations of biophysical and biomolecular systems.^{1, 2} The result has been an explosion of new information regarding structure, function and the nature of biomolecular interactions that is simply impossible to access with ensemble averaged measurements. A particular class of such studies, in which single molecules are presented on a surface (primarily glass), has been seeing increasing use in biological studies with specific advantages over solution-based techniques, particularly for imaging by microscopy, where many individual interactions can be monitored in parallel.^{3–6}

For applications that use fluorescence to probe molecular interactions, single molecules from a dilute solution can be randomly distributed on a surface by various approaches including physical adsorption,^{7–10} vesicle encapsulation,^{11–13} and covalent binding on either bovine serum albumin (BSA)^{14, 15}, or poly(ethylene glycol) (PEG).^{16–18} Alternatively, they can be selectively bound to nanoparticles or nanodots formed into arrays on the surface. This is advantageous in that the use of such nano-anchors provides control over the number or density of the molecules as well as a high degree of spatial order. Metallic nanodot arrays have been used to investigate the effect of molecular tension^{19, 20} and geometric organization^{21–24} involved in critical cellular functions, from adhesion^{19, 24} to immune recognition,^{20–23} with the single-molecule resolution. A sparsely spaced nanoarray enables high-throughput, parallel monitoring of biological activity in real time with conventional epi-fluorescence microscopy.²⁵ These various single-molecule platforms suffer a common problem of nonspecific adsorption, which not only results in a high background for fluorescence measurement, but can also alter cellular response.

PEG is the most commonly used material for glass surface passivation, *i.e.*, to prevent nonspecific adsorption, due to its protein resistance and low cytotoxicity²⁶. Thin films of PEG can be covalently bound to glass through silane chemistry²⁷ using either a two-step process using silane reagents with reactive organic moieties (*e.g.*, amines^{4,5}, epoxides²⁸), or

a one-step formation of a self-assembled monolayer (SAM) of PEG-silane^{22–25, 28–31}. Unfortunately, regardless of the recipe used, the PEG film is usually not a uniform, defect-free monolayer. There are two primary reasons for this: Firstly, silanation depends critically on the glass chemical composition and surface characteristics (cation concentration, roughness, *etc.*).³² Common glass (particularly the kind found in standard borosilicate microscope slides and coverslips) contains impurities (modifiers), which are introduced to adjust its physical and chemical properties (Supporting Information Table S1).^{33, 34} Some of the impurities (*e.g.*, oxides of boron, iron) form less stable bonds with Si-O,^{35, 36} and some of them (*e.g.*, alkali metal oxides, carbonate) do not form stable bonds,³⁶ leaving defective regions in the PEG film (Figure 1a). Molecular dynamic simulation have found that silane binding is weaker on glass with a high concentration of sodium due to the presence of sodium-rich regions on the surface.³² Secondly, silane molecules could hydrolyze in solution and cross-link with one another before being immobilized on the hydroxylated surface, thereby leaving gaps between the patchy multilayer and the underlying surface. As a result, conventional PEG coating is typically most effective at low (nanomolar) concentrations of the molecule of interest¹⁵.

Efforts have been made to improve PEG passivation. The silanol cross-linking could be eliminated by using monoreactive silanes,³⁷ chlorine-terminated surfaces,³⁸ although these involve complicated chemistry and specific reagents. Alternatively, the defectivity can be minimized by enhancing the anhydrous conditions.^{39, 40} The passivation can also be improved by filling the defective regions with blocking reagents, such as BSA,⁴¹ Pluronic F-127⁴² or polysiloxane.⁴³ Recently, Tween-20, a detergent, was found to significantly improve surface passivation either in combination with PEG + polysiloxane,⁴³ PEG,⁴⁴ or even alone on hydrophobic silane-treated surfaces¹⁵. Tween-20 reduces nonspecific adsorption by 5 to 10 fold, typically,^{15, 44} and it is the most promising method so far for randomly distributed single molecules.

In the context of orderly arranged single molecules tethered to metallic nanoarrays, a generally higher concentration (micromolar^{22–25, 31}) is required to ensure functionalization efficiency of the nanodots, making the passivation more challenging. Tween-20 is not suitable for these surfaces, as it is adsorbed by hydrophobic interactions rather than covalent binding, so its selectivity for the PEG background over the metallic nanodots (which are typically already partly functionalized with a linker, *e.g.*, alkylthiol on gold) is limited and could severely reduce the functionalization efficiency (Figure 1b). Moreover, Tween-20 permeabilizes cell membranes⁴⁵, which makes it inappropriate for cellular studies. Thus, a more versatile method is needed in order to improve PEG passivation, particularly for single-molecule nanoarrays.

A simple solution for improving passivation is to replace the borosilicate glass with quartz or fused silica. But these materials are relatively costly, and they are fragile, so glass is preferable, particularly for micro- or nanofabricated surfaces. For the same reason, glass is also more often used in objective-based total internal reflection fluorescence (TIRF) microscopy,⁶ an important tool in surface-based single-molecule studies. Further, quartz or fused silica might require additional adjustments, as the refractive index is significantly lower than that of glass.⁴⁶ In this work, we present a more general approach to glass surface

passivation, in which a thin film of SiO₂ is applied to glass coverslips prior to the nanoarray fabrication. This film covers the impurities affecting the selective binding on nanodots (Figure 1c). Other approaches mentioned above can be used in combination, including anhydrous conditions and blocking BSA, in order to achieve the optimized background passivation. We demonstrate the efficacy of this approach by monitoring the binding of streptavidin on the nanoarrays (Figure 1), since streptavidin is widely used to immobilize a variety of other biomolecules on metallic nanodots through the biotin-streptavidin-biotin bridge.^{22–25, 47} Streptavidin has a two-dimensional size of $\sim 5.4 \text{ nm} \times 6.5 \text{ nm}$,⁴⁸ so the metallic nanodots in this work were made to be smaller than 5 nm in diameter, in order to ensure that each nanodot presents, on average, a single streptavidin molecule.³⁰

Experimental methods

Surface preparation

Glass coverslips (22 × 22 mm, no. 1.5, Thermo Scientific) were immersed in a diluted 7× cleaning solution (1:4 with deionized (DI) water), which was heated to boiling temperature for 30 min on a hot plate. After cooling down, the samples were rinsed in DI water, followed by IPA, and were then blown dry with inert gas (Ar or N₂). A portion of the samples were coated with 15 nm SiO₂ by plasma-enhanced chemical vapor deposition (PECVD, Oxford PlasmaPro NPG80). Another portion of the samples were spin-coated with 15 nm hydrogen silsesquioxane (HSQ, Dow Corning XR-1541, diluted 1:5 in methyl-isobutyl ketone) and then cured at 540 °C for 2 h in ambient air.

Nanoarray fabrication and functionalization

Arrays of sub-20 nm spots were patterned in a 60 nm thick PMMA film, either by e-beam lithography (EBL, NanoBeam nB4) or by nanoimprint lithography (NIL, Nanonex BX-200) (Supporting Information Figure S1).^{22, 23, 49} A 12 nm-thick Ti hard mask was deposited at an angle of 30° by e-beam evaporation (Semicore SC2000); the angled deposition reduces the opening size to sub-15 nm, and creates a negative resist profile suitable for lift-off. The hard mask also protects the unexposed resist in the descumming by oxygen plasma (Oxford PlasmaLab 80). A 0.5 nm Ti adhesion layer and a 2 nm AuPd (60/40 alloy, Kurt J. Lesker) film were evaporated (Semicore SC2000) and then lifted off in boiling acetone, forming ultra-thin metal platelets at the sites of the patterned openings. These platelets agglomerated into spherical particles with a diameter $\lesssim 5 \text{ nm}$ after annealing at 540 °C for 2 h, as measured by both scanning electron microscopy (SEM, Hitachi 4700) and atomic force microscopy (AFM, Park XE-100). The NIL molds were HSQ nanoarrays patterned by EBL, cured at 540 °C for 1 h, and then treated by anti-adhesion coating (Nanonex Ultra-100). The two approaches of EBL and NIL are compared and discussed in the Supporting Information (Figure S2).

Following the protocols developed in our previous work,^{22–25, 31} the nanoarray samples were first cleaned in a 1.5 h-aged piranha solution (3:1 H₂SO₄:H₂O₂) for 3 min, and then in an oxygen plasma for 5 min. After thiolation in a solution of 1 mM HS-C₁₁-EG₆-Biotin (ProChimia Surfaces) in ethanol for 18 h, and PEGylation in a solution of 2 mg mPEG-Silane (MW 5000, Laysan Bio) in 25 mL anhydrous toluene with 30 μL acetic acid for 48 h,

the samples were incubated for 30 min in a solution of 200 nM streptavidin (Streptavidin, Alexa Fluor 555 Conjugate, F/P 3:1, Life Technologies) in 1.5 mL phosphate-buffered saline (PBS, Gibco DPBS 1 ×) with 1 mg/mL BSA. This protocol (with specified streptavidin concentration and incubation time) was used for both bare glass and HSQ coated glass surfaces, which are referred to as "PEG" and "HSQ + PEG" surfaces, respectively. The "PEG + Tween-20" surface was prepared by an additional incubation of the "PEG" surface in PBS buffer containing 1% (v/v) Tween-20 for 10 min before adding streptavidin.⁴⁴

Microscopy

The functionalized samples were rinsed and incubated in PBS for at least 15 min, in order to wash out free molecules. The samples were mounted in a chamber for replaceable coverslips (CSC-22 × 22, Bioscience Tools) and covered with PBS. An Olympus IX81 with an oil-immersion objective (100 ×, 1.49NA, Olympus) and a DSRed filter was used for epifluorescence microscopy. The fluorescence was collected by an electron-multiplying charge-coupled device (EM-CCD) camera (Cascade II 512, Photometrics) at 25 frame/s for 40 s. A typical image (40 × 40 μm²) was averaged over the first 25 frames (which is different from a constant exposure of 1 s). For the nonspecific adsorption measurement, at least five random locations were imaged on each sample, and the results were averaged.

Results and discussion

SiO₂ deposition and nanoarray fabrication

PECVD is the most common way to deposit thin films of SiO₂, due to its simple process and low cost relative to other techniques such as e-beam or sputter deposition⁵⁰, however PECVD SiO₂ films usually have a high surface roughness that is not easily controlled.⁵¹ HSQ, on the other hand, is a spin-on glass originally developed as a low dielectric constant material with excellent planarization and gap-filling properties⁵² (it was later found to be an excellent high-resolution negative e-beam resist⁵³). It is a silicon-based polymer whose molecular structure changes from cage form to network form under either thermal curing or electron irradiation. The thermally induced transformation is affected by the film thickness, temperature, and ambient gas⁵². A thinner film (~ a few tens of nm) cured at high temperature and O₂ concentration tends to cause the Si-H bonds to disassociate completely, resulting in a chemical composition close to that of SiO₂, which serves our purpose quite well.

Glass coverslips with a 15 nm coating of either PECVD SiO₂ or cured HSQ, were characterized by AFM (Supporting Information Figure S3). In both cases, the thickness was sufficient to form a continuous film that was resistant (*i.e.*, no change in the surface roughness or continuity was observed) to the harsh physical or chemical processes involved in the nanoarray fabrication or functionalization (*e.g.*, thermal annealing, immersion in piranha solution). Overall, the PECVD SiO₂ had the highest surface roughness R_{RMS} (root mean squared), making it unsuitable as a substrate for small nanodots, while the cured HSQ had the smallest R_{RMS} - even smoother than the original bare glass surface (Figure 2a), effectively planarizing the surface. The HSQ coating was therefore chosen for this work over

PECVD SiO₂. Nanoarrays were fabricated on both bare glass and HSQ coated glass surfaces (Figure 2b, c).

Nonspecific adsorption measurement

Nonspecifically bound streptavidin molecules on PEG surfaces were counted by fluorescence microscopy^{7, 15, 44}. In order to improve the accuracy, multiple fluorophore labels (F/P ratio 3:1), instead of a single one, were used. This minimizes lost information caused by initial bleaching. The fluorescence spots were identified and counted based on both spatial and intensity criteria. Firstly, the point-spread function (PSF) was measured and approximated by a Gaussian function, in order to determine the proper radius to use for particle detection (Supporting Information Figure S4). A larger radius would mistakenly identify nearby spots as a single particle, while a smaller radius would increase the noise. Secondly, the particle detection threshold was optimized by comparing the intensity histogram of detected spots with the intensity of a single fluorophore, rather than arbitrarily picking a predetermined value as in Ref^{15, 44}.

In single-molecule experiments, there are usually a small number of fluorophores in each spot sufficiently far apart, so that the intensity-time curve shows discrete steps, which represent individual events of fluorophore bleaching, blinking or disassociation. Step changes of intensity have been used to determine the molecular occupancy of lipid vesicles¹³, monitor single QD blinking⁴⁷, or DNA cleavage by a restriction enzyme²⁵. Here the average bleaching step size was simply used to determine the single fluorophore intensity (see, *e.g.*, Figure 3a). The initial intensity histogram was fitted by a mixed Gaussian distribution using an expectation maximization algorithm, with peaks around integer multiples of the single fluorophore intensity (Figure 3b). Therefore, each single Gaussian represents the intensity distribution of streptavidins with a given number of labeling fluorophores.

The first peak shows the intensity distribution of a single fluorophore, whose average should be the same as measured by the bleaching step size. This was used as a criterion when choosing the proper threshold in particle detection, since the peak is shifted by adjusting the threshold (Supporting Information Figure S5). The bleaching curves with two and three steps (Figure 3a) correspond to the second and third peaks, representing streptavidins with two and three fluorophores, which comprise the majority of the histogram (Figure 3b). The overlapping of adjacent Gaussians is due to the variation of single fluorophore intensity, commonly observed in bleaching experiments,¹³ which makes identification of particles by a predetermined intensity threshold problematic. Finally, the probability of each Gaussian curve reveals the distribution of fluorophore number per streptavidin molecule, with an average of 2.86, close to the given F/P ratio of 3 (Figure 3c). (This analysis could serve as a precise measurement to determine the F/P ratio, whose average is loosely controlled by the concentrations of fluorophores and target biomolecules.⁵⁴)

The fluorophores were bound to primary amines on lysine residues in the streptavidin, meaning that there are 28 (average of 24–32 lysines) possible binding sites per molecule. Theoretically, the fluorophore occupancy should follow a binomial distribution $B(28, 0.1071)$. But the measured distribution was narrower, close to $B(6, 0.4767)$, suggesting that

there may be fewer binding sites available, possibly due to the molecular structure and/or interactions between fluorophores. This distribution also suggests that each spot accommodated only a single streptavidin molecule, which was achieved by the optimized PEGylation and BSA blocking (Multiple molecules per spot were observed on a glass surface with poor PEG passivation, as shown in Supporting Information Figure S6).

Surface passivation comparison

Based on the measurements with improved accuracy, the nonspecific adsorption was investigated under a variety of conditions. There are many factors that can affect the PEG integrity (*e.g.*, the PEG length and PEGylation time in the experimental methods section were optimized in Ref.⁵⁵). In addition, the density of nonspecific adsorption apparently increases with the molecule concentration and incubation time (Figure 4a). As mentioned above, a low concentration of 20 – 100 pM⁶ is typically used for randomly distributed single molecules. On the other hand, a high concentration from 50 nM³⁰ to 2 – 10 μ M²⁴ is needed in nanoarrays, in order to achieve a high functionalization efficiency. Here, 200 nM streptavidin in PBS solution for 15 min was used for a comparison between the three types of surfaces (Figure 4b, with corresponding fluorescence images in Supporting Information Figure S7). The PEG + Tween-20 surface reduced the nonspecific adsorption by approximately 8-fold, as compared to conventional PEG, similar to previous work^{15, 44}. The improvement of the HSQ + PEG surface was comparable, with a density of nonspecific adsorption down to 0.2/ μ m². It provides sufficient background inertness for cellular studies, which is critical in applications involving highly sensitive cellular functions (*e.g.*, T cells react to extremely low densities of agonist peptides in immune recognition⁵⁶). A high density of nonspecific adsorption could alter the cellular response and blur the investigation of geometric effect and/or stoichiometry. The HSQ + PEG combination also improves the fluorescence signal to background ratio (SBR), which is defined as

$$\text{SBR} = \frac{\mu_{\text{sig}}}{\sigma_{\text{bg}}} \quad (1)$$

where μ_{sig} is the average signal of nanoarrays, σ_{bg} is the standard deviation of the background. As shown in Figure 4c, σ_{bg} of the HSQ + PEG surface was reduced by approximately 4-fold, which is a very low noise level considering the system noise (the standard deviation of a surface without fluorophores). According to Eq. 1, the SBR of nanoarrays on HSQ + PEG surfaces could be improved by 4-fold relative to PEG surfaces, given the same signal level.

Single-molecule nanoarrays with improved passivation

Sparsely spaced nanodot/cluster arrays were fabricated on both bare glass and HSQ coated glass surfaces. In order to resolve single molecules and minimize the intensity profile overlap, the spacing was set to 1 μ m, larger than twice the optical resolution, which was determined by the measured PSF and the Rayleigh criterion (Supporting Information Figure S4). To compare the performance at different signal levels, heptamer arrays (Figure 5a, inter-dot spacing 60 nm) were made in addition to single dot arrays (Figure 5b, with a typical

height profile). The insets of SEM images show the lateral size of the nanodots, which, together with AFM height measurements, confirms their spherical shape. After functionalization with streptavidin on the nanodots and PEG-silane on the background, as in Figure 1a, c, fluorescence images were taken on both PEG and HSQ + PEG surfaces for both heptamer and single dots, respectively (Figure 5c–f). The reduced nonspecific adsorption and improved SBR on HSQ coated surfaces are clearly demonstrated.

Needless to say, the reduction of background noise is more important when the signal per spot is weak. For example, a heptamer array on PEG was resolved, albeit with a higher background noise level than HSQ + PEG (Figure 5c, d). On the other hand, the signal of a single dot is only 1/7 of a heptamer, and this was buried in the background noise on PEG, as shown by the intensity profile (Figure 5e). The locations of single dots were determined based on that of registers (an 11×11 dot array with 10 μm inter-register spacing). In contrast, with the improved (~ 4 -fold) SBR on HSQ + PEG, a single dot/molecule array was resolved. A typical intensity profile shows peaks with 1 μm spacing, which matches the nanoarray geometric arrangement (Figure 5f).

Conclusions

We have developed a method to improve the conventional PEG passivation on glass surfaces, by simply applying a thin film of HSQ, which has a chemical composition similar to SiO_2 after thermal curing. The HSQ layer minimizes PEG defects by covering surface impurities which interfere with silanol condensation. The defects caused by silane hydrolysis and cross-linking was also minimized by enhancing the anhydrous conditions. Therefore, the PEG integrity was improved, resulting in a reduction of nonspecific adsorption of biomolecules. The density of nonspecifically bound streptavidins (200 nM, 15 min) was reduced by nearly 8-fold to $0.2/\mu\text{m}^2$, comparable with the Tween-20 treatment. Meanwhile, the standard deviation of the background σ_{bg} was reduced by approximately 4-fold.

The underlying HSQ layer has no influence on the molecular or cellular interactions above the PEG passivation, making it more versatile than the Tween-20 treatment. For example, Tween-20 is adsorbed via hydrophobic interactions, rather than covalent binding; this limits selectivity for glass background over metallic nanodot arrays. Tween-20 also lyses living cells by permeabilizing the cell membrane. Therefore, our method is more suitable for metallic nanodot arrays, particularly in cellular studies (Supporting Information Figure S8). The HSQ layer is not only sufficiently smooth (compared with PECVD SiO_2), but also sufficiently robust to withstand processes involved in the nanoarray fabrication and functionalization.

1 μm -spaced AuPd nanodot/cluster arrays with sub 5 nm nanodot size were fabricated by EBL, and then functionalized with a single streptavidin per dot, on average, which can be resolved by conventional epi-fluorescence microscopy. At the same signal level, the SBR was improved by approximately 4-fold on HSQ coated glass. This is sufficient for the direct imaging of orderly arranged single molecules, which is generally impossible on bare glass. (Randomly distributed single molecules are feasible on bare glass only at low concentrations, which is insufficient for the functionalization of nanodots). Overall,

conventional PEG passivation on glass is significantly improved by the HSQ coating, providing not only enhanced SBR for single-molecule fluorescence, but also sufficient biological inertness for cellular studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic diagrams of metallic nanodot arrays with background passivation. (a) PEG: Conventional PEG coating does not cover all the impurities on glass surface, leaving defects for nonspecific adsorption; (b) PEG + Tween-20: Tween-20 improves the PEG passivation by blocking the defects, but may also block the specific binding on nanodots; (c) SiO₂ (HSQ) + PEG: A thin film of SiO₂ (cured HSQ) covers the impurities, which reduces the PEG defects without affecting the specific binding.

Figure 2.

(a) Comparison of surface roughness on bare glass, glass coated with 15 nm PECVD SiO_2 , and glass coated with 15 nm cured HSQ. AFM images and typical height profiles of AuPd nanodot arrays fabricated on the HSQ coated glass: (b) an extended hexagonal array, (c) a cluster (heptamer) array.

Figure 3. (a) Typical bleaching curves of nonspecifically bound streptavidins. (b) An intensity histogram with multiple peaks, corresponding to different fluorophore numbers per molecule. (c) The probability distribution of fluorophore number per molecule.

Figure 4.

(a) Counts of nonspecifically bound streptavidins on PEG surface with different concentration and incubation time. Comparison between the three types of surfaces at 200 nM, 15 min: (b) Passivation efficiency on PEG, PEG + Tween-20 and HSQ + PEG surfaces; (c) The standard deviation of the background σ_{bg} on PEG, HSQ + PEG surfaces, and a surface without fluorophores (system noise).

Figure 5.

AFM images and SEM images (insets) of 1 μm -spaced (a) heptamer array, and (b) single dot array with a typical height profile. Fluorescence images of the heptamer array on (c) PEG, and (d) HSQ + PEG surfaces. Fluorescence images and intensity profiles of the single dot array on (e) PEG (possible locations of single dots are circled in green), and (f) HSQ + PEG (nonspecifically bound streptavidins are circled in orange).