A simple procedure to improve the surface passivation for single molecule fluorescence studies

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Abstract
The single-molecule fluorescence technique is becoming a general and mature tool to probe interactions and dynamics of biomolecules with ultra high precision and accuracy. However, nonspecific adsorption of biomolecules to the flow cells remains a major experimental riddle for the study of many complex biological systems, especially those exhibiting low binding affinity and presenting with weakly populated intermediates. Many novel surface passivation methods have been introduced to reduce nonspecific interactions. Here, we present an effective and inexpensive method to significantly reduce nonspecific binding of biomolecules in conventional poly (ethylene glycol) (PEG)–based surface passivation protocols, without additional exogenous effects. In particular, we propose a simple 10 min Tween-20 treatment for the PEG passivated surface, which could further increase the hydrophilicity of the surface and thus promote passivation efficacy by about 5 to 10 times. We anticipate that this new procedure will find broad practical applications and extend the current reaches of single-molecule fluorescence studies.

Introduction
The single-molecule fluorescence technique has been greatly advanced and extensively applied in biological research over the decades [1–6]. It allows direct observation of the dynamics and heterogeneity of many biological processes, which are otherwise hidden in ensemble measurements [7–10]. Due to its unprecedented spatial and temporal resolution, weakly populated states or transient intermediates can be revealed unambiguously. In typical single-molecule fluorescence experiments, the concentrations of biomolecules of interest should be kept as low as possible to minimize the background noises and improve the image quality [11]. Such a strategy works well for many strong protein–protein or protein–DNA interactions [12, 13]. However, for weak interactions with dissociation constants at the micromolar range, strong nonspecific interactions between biomolecules of interest and the glass or quartz flow cells typically lead to a strong background, making the single-molecule fluorescent measurements difficult. Moreover, the nonspecific binding of fluorophore-labeled biomolecules to the imaging surface could introduce random errors, thus dramatically diminishing the accuracy and precision of the data statistics [14].

Conventionally, there are two ways to block nonspecific binding of biomolecules of interest to imaging surfaces in single-molecule experiments: [15, 16] covalent poly (ethylene glycol) (PEG)–based surface passivation protocols, without additional exogenous effects. In particular, we propose a simple 10 min Tween-20 treatment for the PEG passivated surface, which could further increase the hydrophilicity of the surface and thus promote passivation efficacy by about 5 to 10 times. We anticipate that this new procedure will find broad practical applications and extend the current reaches of single-molecule fluorescence studies.
coating, directly applying Tween-20 to the hydrophobic silane-treated surface could lead to improved surface passivation [14]. Nonetheless, these new approaches use a hydrophobic surface to facilitate Tween-20 coating. Here we show that even without using hydrophobic modification, a simple 10 min Tween-20 treatment of a PEG passivated surface could lead to a 5- to 10-fold increase in the passivation efficacy. Tween-20 treatment could further increase the hydrophilicity of the surface and thus inhibit the non-specific binding between biomolecules and the surface [9, 21]. We anticipate that this new procedure will find broad applications in single-molecule fluorescence studies, especially for the studies of weak binding and transient intermediates.

Materials and methods

Chemicals
Cy3-labeled ssDNA and Holliday junction (HJ) DNA were purchased from GeneScript, Nanjing, China; Cy3-labeled IgG and streptavidin were from Invitrogen. The Holliday junction sequence is the same as McKinney, et al used [22]. Specifically, we used Junction7, whose sequences are listed below:

- Cy5−b−arm, 5′−Cy5−CCCTAGCAAGCCGCT GCTACGG;
- Cy3−h−arm, 5′−Cy3−CCGTAGCAGCGCGA GCCGTTGGG;
- Biotin−r−arm, 5′−Biotin−CCCACCGCTCG GCTCAACTGGG;
- x−arm, 5′−CCCCAGTTGAGCCGCTTGCTAGGG.

We used Cy3-h-arm DNA for the test of non-specific binding of Cy3-ssDNA on different surfaces. Modified PEG (mPEG)-SVA (MW = 5000 Da) and biotin-PEG-SVA (MW = 5000 Da) were purchased from Laysan Bio. N-(2-Aminoethyl)-3-(trimethoxysilyl)propylamine, glucose, glucose oxidase, catalase, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and Tween-20 were available commercially from Sigma-Aldrich. All other chemicals were of reagent grade, and buffers were filtered with 0.22 μm filters before use.

Preparation of the surface
Coverslips were PEG-treated following reported protocols with minor adjustments [15, 23, 24]. First, the coverslips were rinsed with Milli-Q H2O 3 times and sonicated in Milli-Q H2O for 1030 min. Then, the H2O was replaced by KOH solution (1 M), and the coverslips were sonicated for additional 30 min. After sonication, the coverslips were taken out, rinsed by Milli-Q H2O at least 3 times, and dried by blowing with N2 gas. Next, the dried coverslips were put in the piranha solution (H2SO4:H2O2 = 3:1) and incubated for 3030 min. Finally, the coverslips were rinsed with Milli-Q H2O at least 3 times, acetic acid at least 3 times, and stored in aceton. After cleaning procedures, coverslips were incubated in the mixture of 3 mL of aminosilane and 30 mL of aceton for 30 min. Then 2 mg of biotin-PEG and 40 mg of mPEG were dissolved in 140 μL of PEG buffer (100 mM of sodium bicarbonate buffer, pH 8.0) for 2 sandwiches of coverslips, incubated for typically more than 3 hours. Additionally, T50 buffer (10 mM of Tris-HCl, 50 mM of NaCl, pH 8.0) containing 1% (v/v) Tween-20 was injected after assembling the flow cell. After incubation for about 1030 min, the flow cell was washed using T50 buffer. The concentration of Tween-20 does not affect the passivation too much. We found that 1–5% (v/v) of Tween-20 showed similar passivation effects.

Contact angle measurement
Contact angles were measured using an optical contact angle meter (CAM200 of KSV Instruments Ltd, Finland) [25]. The volume of the water droplet was about 5 μL. Images of a drop were acquired by a charge-couple device camera. The contact angle was an average of 5 replications, calculated using image analysis software with integrated Laplace equation of capillarity and experimental drop profile fitting. All experiments were carried out at room temperature of 21 ± 2 °C.

Objective-type total internal reflection fluorescence imaging
An Olympus IX-71 with an oil immersion UAPON 100XOTIRF objective lens (N.A. = 1.49, Olympus) was used for objective-type total internal reflection fluorescence (TIRF) [15, 23]. A 532 nm laser was used for excitation of Cy3. The emission fluorescence was collected by an electron-multiplying charge-coupled device camera (IXon 897, Andor Technology) with temporal resolution of 40 ms. The imaging area was typically 2500 μm² (50 × 50 μm) and averaged over 10 frames. We counted dots with an intensity at least 6 times higher than that of the background.

Single-molecule fluorescence resonance energy transfer (smFRET) detection of conformational dynamics of HJ
HJ was annealed by mixing 4 single-stranded DNA arms in a Tris buffer (20 mM, pH 8.0) and slowly cooled from 80 °C to 20 °C using a polymerase chain reaction machine. The final concentration of the annealed HJ was 2 μM. The emission fluorescence was split into two channels by a dichroic mirror (FF640-D01, Semrock), and two band-pass filters (FF01-585/40 and FF01-675/67, Semrock) were used for green and red emission channels, respectively. All smFRET experiments for HJ were carried out under the buffer condition of 10 mM of Tris-HCl, 50 mM of NaCl, 30 mM of MgCl2, and pH 8.0, with an
oxygen scavenger system containing 0.8% (w/v) glucose, 1 mg mL\(^{-1}\) glucose oxidase, 0.04 mg mL\(^{-1}\) catalase and 2 mM Trolox. Since the images for the PEG-only surface under the sample concentration of 10 nM was difficult to map and analyze in the experiments, we used a regular sample concentration (about 50–100 pM) to obtain the HJ conformational switching rates between two states. The temporal resolution was 40 ms.

**Results and discussion**

We first developed the new surface passivation procedure using PEG in combination with Tween-20. In a typical PEG passivation procedure, glass or quartz coverslips for single-molecule studies are typically aminosilanized and reacted with the N-hydroxysuccinimide ester modified PEG (mPEG) as the blocking reagent and biotin-PEG as the anchoring points to immobilize fluorophore-labeled molecules through biotin-streptavidin (or neutravidin) interaction [15]. However, PEG typically adopts coiled structures in solution and different PEG molecules are mutually excluded due to steric hindrance, which leads to incomplete coating of the surface. The gaps between individually packed PEG molecules are exposed to the biomolecules and could potentially cause nonspecific adsorption. This scenario is depicted in figure 1. However, Tween-20 is a surfactant and has a much smaller molecular weight. Therefore, it can form ordered membrane-like structures in between neighboring PEG molecules to further cover the empty space (figure 1). We propose that because the exposed area between individual PEG molecules is typically small, Tween-20 molecules could stably cover the gaps without the need of a hydrophobically treated supporting surface. Therefore, using Tween-20 as a complementary reagent to block the exposed area that PEG is incompetent to cover, we should be able to further reduce the hydrophobicity of the imaging surface and improve the passivation quality. The new passivation procedure is as follows: first, the coverslips for objective-type TIRF imaging were prepared following the same procedure as the regular PEG passivation [15]. Then, T50 buffer containing 1% (v/v) Tween-20 was injected to the flow cell and incubated for 10 min to allow the formation of Tween-20 clusters in between PEG molecules. The flow cell was subsequently washed with T50 buffer and was ready for single-molecule fluorescent measurements.

We then compared the performance of the flow cell prepared using this new passivation method with that prepared using the traditional PEG method. First, we quantified the nonspecific adsorption of Cy3-labeled ssDNA at different ssDNA concentrations. The

![Figure 1. Schematic of the surface modification using the PEG and Tween-20. (A) aminosilane-modified glass coverslip surface, (B) PEG-coated surface, and (C) PEG + Tween-20-coated surface.](image-url)
number of fluorescent dots on TIRF images with an imaging area of 2500 μm² at a wide range of ssDNA concentrations was shown in figure 3(A). Using this new method, the number of fluorescent spots corresponding to nonspecific binding was reduced by ~80% at the concentration ssDNA of 800 nM.
To quantify the nonspecific binding of proteins to the surface, we used Cy3-labeled IgG (figure 3(C)). The improvement was 10-fold at the protein concentration of 200 nM. Proteins showed much stronger adsorption to the surface than ssDNA. Due to the occurrences of massive nonspecific binding events for the PEG-coated surface, we did not test the Cy3-IgG nonspecific binding capacity at protein concentrations above 200 nM (figure 3(D)). However, even at such high protein concentrations, it is still possible to obtain single-molecule fluorescence imaging using our new surface passivation procedure. It is worth mentioning that even for a commercially available PEG-coated glass coverslip (PEG_01GC, Microsurfaces) with better initial PEG passivation, our approach could also further reduce the nonspecific adsorption of biomacromolecules (figure S1).

Subsequently, we tested whether other nonionic detergents could also improve the surface passivation similar to Tween-20. We used the same passivation procedure but replaced Tween-20 by Tween-80, which has an oleic acid hydrophobic chain instead of a lauric acid one. The nonspecific adsorption of Cy3-labeled IgG and ssDNA at a concentration of 200 nM for the three different imaging surfaces was shown in figure 4. Clearly, both Tween-80 and Tween-20 could lead to improved surface passivation. However, the performance of Tween-80 was not as good as Tween-20. This is probably because the hydrophobic side chain of Tween-80 is longer and kinked, making the formation of the uniformed surface layer difficult. We have also tested other detergents, such as Triton X-100 and SDS. They also did not show better improvement on the surface passivation as compared with Tween-20.

In order to make sure that the addition of Tween-20 does not affect the intrinsic dynamics of biomolecules, we further tested the biocompatibility of the surface prepared by this new method with HJs using smFRET (figure 5). HJs are DNA molecules that can switch conformations between two states (figure 5(A)) [22, 26, 27]. We compared the performance of two surfaces, PEG only and PEG + Tween-20, at a DNA concentration of 10 nM, which is 100 times higher than the concentrations used in regular smFRET experiments. The surface modified with PEG only was packed with a tremendous number of labeled HJ molecules, making the mapping of the images from the two channels impossible (figure 5(B)). However, on the Tween-20 treated surface, the DNA molecules were well scattered, allowing easy mapping of the...
corresponding points (figure 5(C)). The Cy3–Cy5 intensity trajectory extracted from the Tween-20 surface data demonstrates how HJ molecules switched between two conformational states (figure 5(D)), based on which the FRET efficiency ($E_{\text{FRET}}$) could be calculated. By analyzing the dwell time at the low $E_{\text{FRET}}$ state ($\tau_{\text{low}}$) and the high $E_{\text{FRET}}$ state ($\tau_{\text{high}}$), we compared the dynamics of HJs on both surfaces. Our results clearly indicated that introducing Tween-20 into the system had negligible effects on the intrinsic dynamics of HJs (figures 5(F) and (G)). Since Tween-20 is a widely used detergent for protein purification and quantification, we anticipate that it will also show good compatibility to protein systems.

**Conclusion**

In summary, we have presented a simple 10 min procedure to substantially reduce nonspecific adsorption of biomolecules in traditional PEG-based surface passivation methods without affecting the activity and stability of biomolecules of interest. It is also possible to combine Tween-20 coating with protein-based passivation methods. Recently Hua *et al* have introduced a similar approach to directly passivate the surface using Tween-20 and anchor biomolecules using BSA-biotin, which has been proven to be very efficient, robust and timesaving [14]. In our approach, Tween-20 is filled in the PEG unprotected gaps of the surface. Although in our approach PEGylation of the surface is still time consuming, combining PEGylation and Tween-20 coating could potentially make the surface more inert and stable, thus suitable for almost all kinds of single-molecule experiments. We anticipate that due to its simplicity and high efficacy, our approach will find broad applications in single-molecule fluorescence studies, especially the studies that require high protein concentrations.

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