

Poly-Electrolyte Surface-Chemistry Platform for Fluorescence Studies of DNA on Glass

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Title running head: Poly-Electrolytes for Single-Molecule Studies

Abstract:

We report on the use of Poly-Electrolyte Multilayers (PEM) in a stable, robust surface chemistry recipe for specific anchoring of DNA to glass. DNA polymerase remains active on the anchored DNA template. The non-specific binding of fluorescently-tagged nucleotides is suppressed down to single-molecule level. Thus, we have developed a surface-chemistry platform for single-molecule studies of DNA and DNA polymerase.

Keywords: Poly-Electrolytes, layers, surface chemistry, biotin, streptavidin, DNA polymerase, tagged nucleotides, fluorescence, single-molecule detection

Brief: Poly-Electrolytes in surface chemistry for single-molecule fluorescence studies of DNA and DNA polymerase.

Introduction

Poly-Electrolytes are polymers whose chains contain charged functional groups. Some examples are Poly(Acrylic Acid) (PAcr), Poly Ethyleneimine (PEI), and Poly Allylamine. Decher et al. built poly-electrolyte multilayers (PEM) and extensively studied their properties (1). Others used PEM to deposit DNA (2), a charged virus (3), streptavidin (4), and more recently, various proteins (5).

We use similar PEM deposition, but we anchor DNA through covalent and biotin-streptavidin specific bonding, instead of non-specific deposition in a dense layer. This allows us to anchor and observe DNA on the single-molecule level and to ensure that DNA polymerase remains active on the anchored template. Additionally, the negative charge of the final PEM layer repels the negatively-charged fluorophore-tagged nucleotide. Non-specific binding is thus suppressed enough to enable fluorescence studies of single DNA molecules. A number of experiments both in bulk and with single molecules demonstrate the high reproducibility of the results as well as the quality of the electrostatic shielding. Thus we have developed a platform, which is well suited for single-molecule studies of DNA and DNA polymerase.

Materials and Methods

We use Corning microscope slides (3x1 inch) and VWR Micro Cover Glasses #1 coverslips (25x25 mm or 22x22 mm). The glass is cleaned using our version of the RCA protocol (6) and stored in HP water.

PAcr and PEI are dissolved at 2 mg/ml in HP water. The solutions are adjusted to pH 8 using NaOH and HCl. This pH ensures that both components have their functional groups charged (dissociated carboxyl and protonated amino groups respectively). Finally, the polyelectrolyte solutions are passed through a 0.22-micron filter to remove dust residue from the solid phase.

RCA glass is loaded into machined plastic holders and then immersed in solutions of PAcr (-) and PEI(+) according to the scheme +/wash/-/wash+/wash/-/wash. Each polyelectrolyte step is 10 min of immersion, whereas a wash step is thorough rinsing with HP water. Ready PEM glass is stored in HP water. The same results are obtained if we use Poly Allylamine instead of PEI.

PEM glass is biotinylated according to the Biotin-EZ-Link kit from Pierce. We freshly dissolve EDC (1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride) at 50mM in MES (2-[N-Morpholino]ethanesulfonic acid) 10 mM buffer pH5.5 (MESb). The EDC solution is filtered to remove any dust residues from the solid phase. BLCPA (Biotin-LC-PEO-Amine from the kit) 50mM in MESb is mixed with the EDC solution at 1:1 and the result is diluted to 5mM final concentration of each in MESb. The PEM glass is treated with this solution to be biotinylated. The chemical linking is a reaction between the carboxyl group of the PAcr and the amino group of the BLCPA, activated by EDC. After incubation of 30 min, the unreacted excess is washed away with MESb and then with Trisb (10 mM Tris, 10mM NaCl, pH 8).

The now biotinylated PEM glass is treated with Streptavidin Plus from Prozyme at 0.1 mg/ml in Trisb. Streptavidin has four binding sites for biotin, so, as it deposits on the surface, it is probable that at least some of the sites remain unoccupied. After a 30-min incubation, the unreacted excess is washed off with Trisb. Then a solution of DNA is put down in TrisMg (10 mM Tris, 10mM NaCl, 100 mM MgCl₂, pH 8). The biotinylated DNA anchors to the surface at the previously unoccupied sites of Streptavidin. After 30 min of DNA deposition, the unreacted excess is washed off with Trisb. This completes the procedure.

Optical observations were conducted using an inverted microscope (Olympus IX50) with a mercury lamp, a cooled CCD camera (SBIG ST-7I), and fluorescence filter sets appropriate for the particular dyes.

Results and Discussion

The RCA procedure leaves hydroxyl groups on the glass surface, which imparts a negative charge. Thus the positively charged PEI sticks to it electrostatically. Same-charge poly-electrolytes build up a layer because it is energetically favorable for them to hide their hydrophobic carbon chains from the ambient water; some degree of mechanical entwining may also be present. PAcr sticks to PEI electrostatically. The final fourth layer of the PEM is PAcr, which is negatively charged and thus repels the negatively charged fluorescently tagged nucleotides.

The rest of the chemistry is built to anchor biotinylated DNA to the PEM. The high concentration of Mg^{2+} in the DNA deposition buffer helps to deactivate the electrostatic shielding so that the DNA can defuse onto the surface and anchor by its biotin tag. In subsequent feeds, the salt concentration is reduced to standard levels, so the repulsive shielding is reactivated. The reactivated shielding probably repels the unanchored end of the DNA off the surface, which would explain why the polymerase remains active on the DNA.

To demonstrate the benefits of this surface chemistry, we enclose the results of several experiments:

A. PEM Shielding

Four PEM coverslips were prepared according to the general procedure up to the biotinylation step. Then two of the slips were put through an extra treatment of PEI/wash. These slips are called PEM(+) to indicate that they are positively terminated. For symmetry, the standard slips are called PEM(-). Then one slip from each group was incubated for 15 min with $1\mu M$ dATP-TMR (A-nucleotide tagged with tetramethylrhodamine) in Trisb, and the other with only Trisb, as a control. All slips were then washed with Trisb and observed on the microscope. The Trisb controls establish the background signal from the PEM and the glass, whereas the dATP-TMR cases give information about the charges on the surface.

A series of several pictures was taken at different locations on each coverslip, using a dichroic filter set appropriate for TMR. The camera signal was integrated over a large area for each picture to produce an average value for the fluorescence signal in counts per pixel. An average of these values was calculated for each series of pictures. Uncertainty was assigned based on the spread among the values in each series. These averages and the associated uncertainties are plotted on Figure 1.

The results confirm that dATP-TMR sticks with much higher probability (~ 18 times) to PEM(+), compared to PEM(-). Some non-specific binding to PEM(-) still occurs, probably at imperfections on the surface, establishing the noise floor.

B. Biotinylation and Streptavidination

A series of experiments was conducted to test the biotinylation by leaving out some of the critical components and then testing the presence of anchored biotin by attachment of Streptavidin-TRITC and fluorescence detection. The SA-TRITC massively attached to the surface only if all the biotinylation components were present. Similar tests were conducted for streptavidination by leaving out streptavidin and then trying to attach Biotin-Fluorescein or biotinylated fluorescently-tagged DNA. In both cases, strong attachment was observed only if the surface was treated with streptavidin. We do not present the respective data for purposes of brevity.

C. Bulk On-Surface Incorporations in Anchored DNA

Sixteen coverslips were prepared with the complete chemistry including the anchoring of Biotin-Mu50, which is a biotinylated single-strand 50-mer annealed to the corresponding 15-mer primer. The sequence of Mu50 had been taken out of Lambda DNA. The DNA was deposited at 0.1 μM in TrisMg. The coverslips were organized in 8 pairs, so that the level of consistency could be measured by the difference between the results in each pair. Next, the 8 pairs were organized in 4 groups of 2 pairs. Group 1 Pair 1 was treated with a solution of dATP-TMR, dCTP, dGTP, and dTTP, each at 1 μM in EcoPol buffer, in which there was also Klenow Exo- DNA polymerase. Group 1 Pair 2 was treated with the same solution but without polymerase. The same procedure was done to the other groups, except the mixture of nucleotides was different: Group 2 (A-Texas Red, C, T, G), Group 3 (A-Lissamine, C, T, G), Group 4 (C-Cy3, A, T, G). After 40 min of simultaneous incubation, all slips were washed with Trisb. Fluorescence measurements were conducted using dichroic filter sets appropriate for the particular dyes.

The data acquisition and analysis was conducted similarly to Expt A. The results are plotted in Figure 2. It is clear that in all cases, the surface signal is much stronger where polymerase was present. This supports the conclusion of on-surface incorporation in anchored DNA.

D. Single-Molecule On-Surface Incorporation in Anchored DNA:

Some single-fluorophore studies (6, 7) were conducted in parallel with these bulk incorporation measurements. Thus, it was natural to ask if the two techniques could be united to observe incorporation on single-molecule level.

Three coverslips were prepared with the complete chemistry. The DNA used was Biotin-Mu50 at 1 nM in TrisMg. Then coverslip #1 was treated with a mixture of dATP-Lissamine, dCTP, dGTP, and dTTP each at 0.1 μM in EcoPol buffer also containing Klenow Exo- DNA Polymerase. The coverslip #2 was prepared as #1 but without polymerase. The #3 was prepared as #2 except for using untagged dATP instead of

dATP-Lissamine. After 30 min of incubation, all slips were washed with Trisb. Observations were made with a dichroic filter set appropriate for Lissamine.

Samples of the resulting pictures are shown on Figure 3a (#1), Figure 3b (#2), and Figure 3c (#3). Comparison between 3a and 3b shows the on-surface incorporation on single-molecule level. Comparison between 3b and 3c shows that the several objects in 3b are not contamination but Lissamine possibly attached to local imperfections on the surface.

Conclusions

We have developed a PEM-based optimized robust surface-chemistry recipe for anchoring DNA to glass surfaces, while ensuring very low background for fluorescence studies and keeping the DNA polymerase active on the surface. Thus we have developed a PEM-based surface-chemistry platform for single-molecule fluorescence studies of DNA and DNA polymerase.

Acknowledgements

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Figures and Captions:

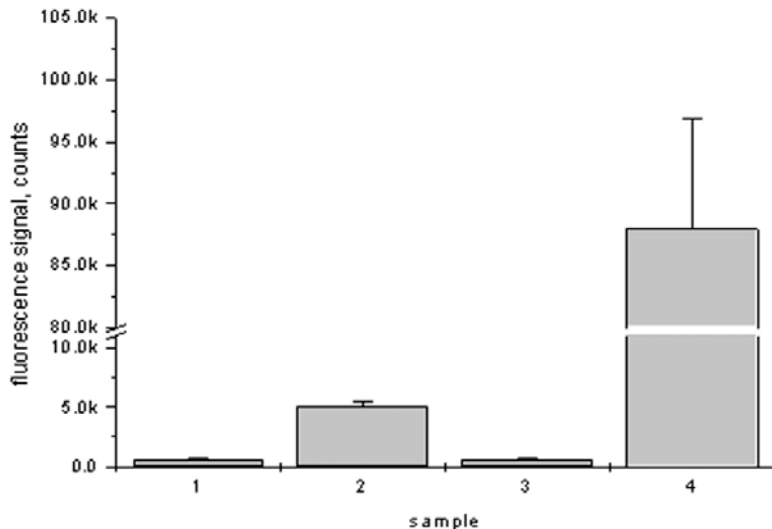


Figure 1. PEM electrostatic shielding: 1: {PEM(-) + Trisb (control)}, 2: {PEM(-) + dATP-TMR}, 3: {PEM(+) + Trisb (control)}, 4: {PEM(+) + dATP-TMR}. Negative probes bind far more strongly to a positive surface than to a negative surface.

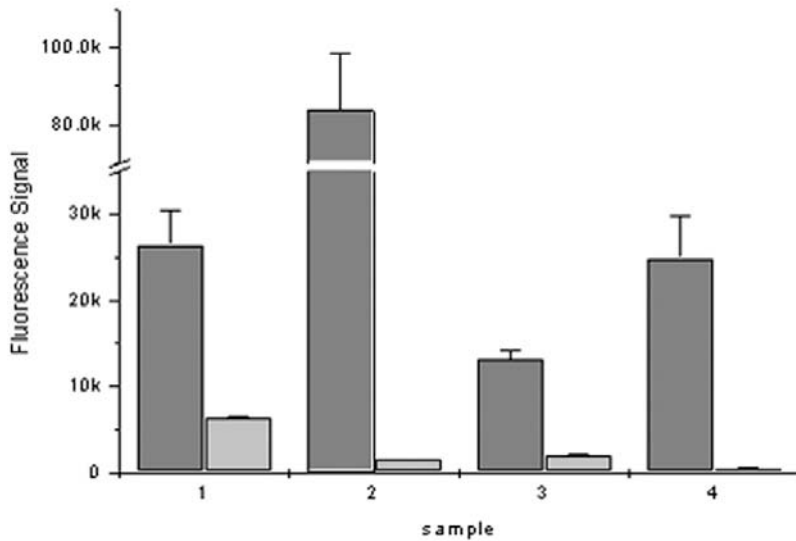


Figure 2. Bulk on-surface incorporation in anchored DNA: 1: {PEM-Mu50 + [C,T,G, A-TMR]}, 2: {PEM-Mu50 + [C,T,G, A-Lis]}, 3: {PEM-Mu50 + [C,T,G, A-TeX]}, 4: {PEM-Mu50 + [A,T,G, C-Cy3]}. In each pair, left/dark had polymerase and right/light had no polymerase. Much stronger signal detected in polymerase cases due to incorporation.

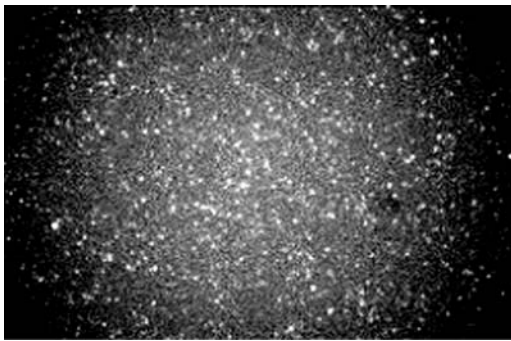


fig3a

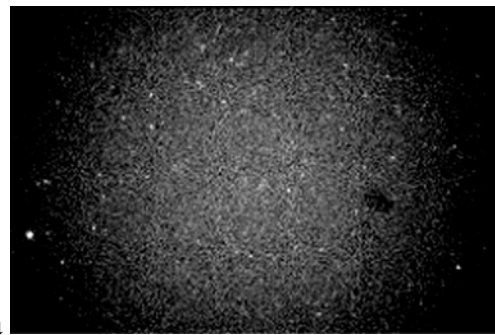


fig3b

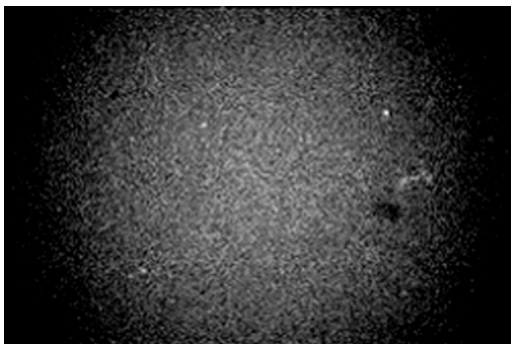


fig3c

Figure 3. On-Surface incorporation in anchored DNA on single-molecule level. Fig 3a {A-Lis,C,G,T, polymerase}, Fig 3b {A-Lis,C,G,T}, Fig 3c {A,C,G,T}.

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