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Enhanced Single-Molecule Detection using Porous Silver Membrane

Yi Fu and Joseph R. Lakowicz

Center for Fluorescence Spectroscopy, University of Maryland School of Medicine, 725 W. Lombard Street, Baltimore, MD 21221, Phone: 410-706-8409, Fax: 410-706-8408

Yi Fu: yifu@cfs.umbi.umd.edu

Abstract

We evaluated a commercial porous silver membrane as a support substrate for direct localization and visualization of single molecule events. We characterized the fluorescence behaviors of fluorescently labeled DNA oligonucleotides on the silver membranes. The fluorescence from the fluorescent probes that were immobilized on the porous silver is greatly enhanced. Additionally, correlated to reflectance contour image, it appears that enhanced fluorescence came from location close to the “valley” of the pore channels (or in the voids). These results are of great interest to increase the effectiveness of fluorescence-based single molecule DNA analysis.

Keywords

nanoporous metal; single molecule detection; metal-enhanced fluorescence; plasmons

Introduction

The development of high density oligonucleotide arrays with fluorescence detection have resulted in increased understanding gene expression, single nucleotide polymorphisms (SNPs), medical diagnostics and personalized medicine^{1–4}. There is a continuous need to increase the sensitivity of DNA detection to avoid the need for amplification steps, reduced costs, and to move towards detection of single DNA molecules or single nucleotides during sequencing⁵. Recent advances in optical detection have allowed the detection and dynamics studies of single biomolecules by fluorescence microscopy^{5–11}. Current single molecules studies however are based mainly on the use of organic fluorophores that often suffer from poor photostability, low signal intensities and on/off blinking^{11–15}. We have studied the effects of metallic particles and metal surfaces on the fluorescent probes^{16–19}. These studies implicate the opportunity to increase the sensitivity of fluorescence detection and to increase the brightness of single labeled oligonucleotides. These effects occur because of fluorophores coupling with surface plasmons on the metal surface which results in increased rates of both excitation and emission²⁰.

In recent years, the creation of nanopores in thin membranes has attracted much interest due to the potential to isolate and detect single DNA molecules while they translocate through the defined channels^{21–25}. But little attention has been paid for nanoporous metal materials in DNA analysis. Nanoporous metals are sponge-like structures which are about 50% metal and 50% open space. Unlike bulk metal surfaces, nanoporous metals coupled with a rich surface chemistry have great potential for applications in heterogeneous catalysis, electrocatalysis and biosensing^{26–28}. The relatively rigid and flat surface, and the high porosity and flow rate make nanoporous metals attractive materials for sample filtration and nucleic acid visualization. The surface properties of the noble metals allow for chemical modification to the membrane that can increase their use as a substrate for single molecule sequencing application. Additionally,

porous metals possess outstanding optical properties in plasmonics and surface-enhanced Raman scattering (SERS)^{29–31}. They hold promise to act as highly active, stable, and economically affordable substrates in high-performance instrumentation applications for chemical inspection and biomolecular diagnostics.

We are interested in nanoporous metals because their potential use in high throughput sequencing without PCR amplification. In this work, we characterized the effects of porous silver membranes on fluorescently-labeled DNA oligomers. We observed up to 100-fold enhancement in fluorescence emission on the porous silver substrate. These results indicate the advantages of these structures for use in DNA detection and sequencing. We expect nanoporous metals have high potential for DNA analysis as nanopores have been successfully employed as a new tool to rapidly detect single biopolymers. The porous silver layer would provide metal-enhanced fluorescence (MEF) of the released nucleotides for single nucleotide detection. The increased sensitivity would result from both the MEF effect and the increased surface area. Additionally, these substrates provide opportunities for both single molecules and many molecule DNA assays in a flow-through format. We can imagine a new generation of devices which provide high-throughput data by imaging the fluorophores as they exit nanoporous metal substrates.

Experimental

Sample Preparation

All oligonucleotides were obtained from the Biopolymer Core Facility at the University of Maryland, School of Medicine. Nanopure water purified using Millipore Milli-Q gradient system, was used for all experiments. All other compounds were purchased from Sigma-Aldrich and used as received. The coverslips used in the experiments were first soaked in a 10:1 (v:v) mixture of concentrated H₂SO₄ and 30% H₂O₂ overnight, extensively rinsed with water, sonicated in absolute ethanol for 2 min and dried with air stream.

The porous silver membranes were purchased from SPI Inc. with 200nm pore sizes and thickness of 50 μ m. The films were immersed in a 10% concentration of hydrochloric acid for ten minutes, followed by ultrasonic cleaning in ethanol. After cleaning, the films were thoroughly rinsed with water and air-dried prior to DNA immobilization.

Immobilization of single stranded DNA samples on silver membranes was accomplished by the method reported. The membrane was placed in a low concentration solution of thiol-derivatized ssDNA (HS-5' TCC-ACA-CAC-CAC-TGG-CCA-TCT-TC-3') for 48 hrs at 5 °C. A final washing step largely removed the unbound DNA probes from the substrate. The DNA hybridization were performed by immersing the tethered silver membrane in a 5nM complementary oligonucleotide (3'-AGG-TGT-GTG-GTG-ACC-GGT-AGA-AG-5'-Cy5) solution containing 5 mM Hepes (pH 7.5), 0.1 M KCl and 0.25 mM EDTA. The membrane was slowly cooled down after incubation at 70 °C for 2 min. After hybridization, the sample was rinsed with copious of deionized water and air dried before the experiments.

Single-molecule Imaging

The single molecule studies were performed with a time-resolved confocal microscopy (MicroTime 200, PicoQuant). A single mode pulsed laser diode (635 nm, 100ps, 40 MHz) (PDL800, PicoQuant) was used as the excitation light. The collimated laser beam was spectrally filtered by an excitation filter (D637/10, Chroma) before directing into an inverted microscope (Olympus, IX 71). An air objective (Olympus, 100 $\times$, 0.95NA) was used both for focusing laser light onto sample and collecting the reflected fluorescence emission from the sample. The fluorescence that passed a dichroic mirror (Q655LP, Chroma) was focused onto a 75 μ m pinhole for spatial filtering to reject out-of-focus signals and then reached the single

photon avalanche diode (SPAD). To further isolate single-molecule fluorescence emission and reduce background, the desired spectral detection range was selected by placing a long-pass filter (HQ685/70, Chroma) in front of the single-photon avalanche diode (SPAD). Images were recorded by raster scanning (in a bidirectional fashion) the sample over the focused spot of the incident laser with a pixel integration of 0.6 ms. The excitation power into the microscope was maintained less than 200 nW. Time-dependent fluorescence data were collected with a dwell time of 50 ms. The fluorescence lifetime of single molecules was measured by time-correlated single photon counting with time-tagged-time-resolved (TTCR) mode (TimeHarp 200, PicoQuant). The reflectance images were recorded using the same optics setup after removing the emission band-pass filters. All measurements were performed in a dark compartment at room temperature.

Results and Discussion

The silver membranes used in this study are commercially available (SPI Inc.) and are composed of pure silver with a mean pore size of 200 nm and a thickness of 50 μm ³². The SEM image of porous silver membranes is present in Figure 1. The film itself is porous with many voids around several microns across. The filters were manufactured by consolidating silver particles of carefully controlled size. These filters are used to trap particles in, rather than on, the membrane. All of the different pore sizes are produced from particles of the same sizes, so the surface appearance of the filters is the same for all of the filters, no matter what their pore size.

The sample configuration is sketched in Figure 1. The thiolated single stranded DNA was initially tethered to the silver membrane via thiol-silver linkage. The density of thiolated single stranded DNA was controlled to be low enough so that clustering could be excluded. As a control experiment, a small portion of Cy5-labeled double stranded DNA (dsDNA) molecules with a nanomolar concentration was spin-cast on glass substrate. Typical 10 $\times$ 10 μm confocal fluorescence images are illustrated in Figure 2. The apparent emission intensities from the dye-labeled dsDNA on glass (Figure 2a) and unlabeled single stranded DNA on silver porous structure (Figure 2b) are generally less than 50 arbitrary units (a.u.) per pixel. With application of complementary single stranded DNA molecules hybridized to the tethered single stranded DNA on silver membranes, the brightness of round spots greatly increases as shown in Figure 2c. The fluorescence signals from dye-labeled dsDNA on glass substrate and unlabeled ssDNA on porous silver membrane are hardly observable under the contrast scale as shown in Figure 2c (Data not shown).

The density of spots observed on membrane surface increased with higher incubation dye concentrations, confirming the hybridization of single complementary ssDNA molecules. Basically all fluorescent molecules suffered from irreversible photobleaching after a certain number of excitation cycles³³. To be certain that the studied bright spots arise from single dye molecules and not from silver nanostructure scattering or other optical process, we have investigated time transients for these emission spots. Figure 3 shows typical photobleaching transitions from the bright spots. The on-off blinking and abrupt photobleaching from the spots are characteristic of single molecule behavior³⁴. Most of bright spots in the images correspond to the fluorescent signal from one Cy5 probe or one DNA hybrid. However, we found that different bright spots had different survival times before photobleaching.

The time traces were collected using nearly the same excitation power throughout the experiment. The time profiles presented in Figure 3 is representative of more than half of those emission spots in the scanned images and illustrates the overall trend observed from more than 30 single molecules in each environment. The various time-dependent behaviors indicate the heterogeneity of the surface properties. Using the same incident excitation power, we observed

significantly more fluorescence from the molecules on the silver substrates as compared to the control samples on glass substrate. Most of observed time traces recorded from DNA molecules tethered to the silver porous membranes (black lines) show characteristic one-step photobleaching and discrete blinking behaviors as observed from those on glass surface (gray lines). However, much higher and fairly constant emission rates are observed from these time profiles, which are generally more than 20-fold from those observed in the absence of silver nanostructures. In fact, different sites on the silver surface in which the probe molecules were tethered are moderately varied. In effect, the single molecules randomly experienced the possible enhancements that can occur for various positions and orientations on the silver surface. In the case presented in Figure 3c, the brightness of the molecule tethered to silver surface is about 100-fold higher than that on glass substrate. The intensity is fairly constant until it drops abruptly to a lower level in a single step. The eventual photobleaching of 88% of the total signal can be attributed to a single fluorophore's dynamics, revealing that most of the fluorescence from this spot is due to a single molecule.

Once we identified single-molecules, we implemented the time-correlated single photon counting (TCSPC) measurement on a single fluorescent spot. Single-molecule lifetime was determined by recording the arrival time of each photon with respect to laser pulse. Figure 4 shows fluorescence decay curves of two probe molecules immobilized on glass (Figure 3b) and tethered to silver membrane (Figure 3d). The curves decay exponentially with averaged time constants of $\tau_{av} = 2.19$ ns for the free labeled DNA molecule and $\tau_{av} = 0.86$ ns for the DNA molecule tethered to silver porous membrane, respectively. The average lifetimes τ_{av} present here were obtained from the intensity weighted average lifetime calculated from the result of the fittings.

$$\tau_{av} = \frac{\sum_i Ampl_i \cdot Lifet_i^2}{\sum_i Ampl_i Lifet_i}$$

The nearly single exponential decay of fluorescence observed for different molecules in the absence of silver nanostructure suggests that most of the molecules adsorbed on glass are in a relatively homogeneous environment. Much faster intensity decay was found in the case of silver porous structure. Double exponential decay analysis yields two decay components of about 0.19 ns and 1.81 ns. We observed a predominant intensity decay contribution (94%) from the short lifetime component below 200ps for the investigated single molecules. The increase in local field results in a higher excitation rate but barely modify the fluorescence lifetime of the molecules. An increase in the radiative decay rate yields shorter lifetimes. The radiative decay rate alteration of the fluorophore in close proximity to the silver nanostructures is consistent with the previous reports on the Metal-enhanced fluorescence (MEF) phenomenon. When the fluorophore is placed close to the metal nanostructure, the lifetime becomes $\tau_m = (\Gamma + \Gamma_m + k_{nr})^{-1}$ (the radiative rate is given by $\tau + \tau_m$, where τ_m is the part due to the metal)³⁵. An increase in the radiative decay rate of the fluorophore can dramatically shorten the lifetime of the excited state, leading to a fast de-excitation. This is in good agreement with our observations that, as shown in Figure 3, a dramatic increase in the brightness was observed from a single fluorophore residing on silver porous films.

We note that the interactions of fluorophores with metallic nanostructures have been studied intensively^{36–38}. The enhancement of fluorescence intensity generally depends on the surface nano-features and the optimal distance of fluorophores from the surface. The major concern was the dependence of the emission rate on the distance between the dye layer and the metallic

surface. The emission can be quenched due to radiation energy transfer to the metal as molecules adsorbed directly on the surface. Another consideration is the enhanced fluorescence due to increased electromagnetic field of surface plasmon and/or enhanced quantum yield. Thus it is interesting to investigate the variation in measured fluorescence and surface morphology from these structures. We performed simultaneous reflectance and fluorescence imaging of immobilized probes on the silver membrane with the diffraction limited spatial resolution. Scanning confocal fluorescence imaging of the sample slide was first performed to locate the fluorescent probe. Then, the same sample area was recorded by reflected scattering light to reveal its rough surface features. Figure 5 shows fluorescence emission of single DNA molecules over silver surface on a two-dimensional space. The single molecules on a silver membrane appear as bright spots at a spatial resolution at about the diffraction limit (400 nm). Correlated to its reflectance contour image, it appears that enhanced fluorescence came from location close to the “valley” of the pores (or in the voids). Despite the strong enhancement near the surface nanostructures, we can find little evidence that strong emission arises from the outer or relatively continuous surfaces of highly porous samples. Highly localized surface plasmons can arise at some locations in or on certain exceedingly disordered nanoporous structures, resulting in high fluorescence enhancement. The mechanism of the surface plasmons on porous metal films is rather complex and has been discussed in detail elsewhere^{39–41}. It has been proved that the plasmons in the voids are very strongly localized and the enormous resonant absorption of light occurs at the plasmon resonance of the void network. We assume that intensively localized void plasmons in a thin layer of porous metal would strongly interacting with surface plasmons, which can greatly enhance excitation light and alter radiative and nonradiative decay rates of nearby fluorophores. The observed maxima in the fluorescence enhancement at specific locations illustrate the tradeoff between the high local field around the porous surface and their ability to scatter light into the far field. The interaction of excited fluorophores with plasmons dominating at very close distances can be observed as fluorescence quenching. In our case, we intent to minimize such an effect as the distance between the fluorophore and the tethered location was controlled by the DNA oligomer length, which is estimated as about 8 nm. However, due to the complex surface feature of the porous structure, there still exist the possibilities of fluorophores interacting with silver nano-objectives in a very close proximity.

In conclusion, single molecules of labeled DNA were used as probes immobilized on the silver porous membrane. Using the prevailing emission that arises from highly enhanced molecules, fluorescence brightness enhancements of more than 20-fold (up to 100-fold) were observed. Lifetime measurements support the coupling mechanism between the fluorophore and metal particle. The porous silver membranes possess the advantage of large specific surface area available for the tethering of fluorescent probes with enormous emission amplification. Currently there are extensive ongoing efforts to develop highthroughput low-cost DNA sequencing. One of these methods is the use of an exonuclease to sequentially remove DNA bases from a single strand of DNA. Intrinsic emission from DNA bases is extremely weak. It is possible to increase the intrinsic base emission from DNA by the use of metallic nanostructure. Thus, nanoporous metallic substrates offer new opportunities to detect and identify the bases with high accuracy using the enhanced intrinsic fluorescence from the DNA bases.

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References

1. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ. Science 2001;291:1304. [PubMed: 11181995]

2. Shendure J, Mitra RD, Varma C, Church GM. *Nature Rev* 2004;5:335.
3. Brown PO, Botstein D. *Nat Genet Suppl* 1999;21:33.
4. Deyholos MK, Galbraith DW. *Cytometry* 2001;43:229. [PubMed: 11260590]
5. Tiemann-Boege ICC, Shinde DN, Goodman DB, Tavare S, Arnheim N. *Anal Chem* 2009;81:5770. [PubMed: 19601653]
6. Trabesinger W, Schutz GJ, Gruber HJ, Schindler H, Schidt T. *Anal Chem* 1999;71:279. [PubMed: 9921136]
7. Singh-Zocchi M, Dixit S, Ivanov V, Zocchi G. *PANS* 2003;100:7605.
8. Taylor JR, Fang MM, Nie S. *Anal Chem* 2000;72:1979. [PubMed: 10815954]
9. Zhang CY, Chao SY, Wang TH. *Analyst* 2005;130:483. [PubMed: 15776157]
10. Garcia-Parajo MF, Veerman J-A, Bouwhuis R, Vallee R, Hulst NFv. *CHEMPHYSICHEM* 2001;2:347.
11. Weiss S. *Science* 1999;283:1676. [PubMed: 10073925]
12. Heilemann M, Margeat E, Kasper R, Sauer M, Tinnefeld P. *J Am Chem Soc* 2005;127:3801. [PubMed: 15771514]
13. Kohn F, Hofkens J, Gronheid R, Van der Auweraer M, De Schryver FC. *J Phy Chem A* 2002;106:4808.
14. Panzer O, Gohde W, Fischer UC, Fuchs H, Mullen K. *Adv Mater* 1998;17:1469.
15. Peterman EJG, Brasselet S, Moerner WE. *J Phy Chem A* 1999;103:10553.
16. Fu Y, Lakowicz JR. *J Phys Chem B* 2006;110:22557. [PubMed: 17092001]
17. Fu Y, Lakowicz JR. *Anal Chem* 2006;78:6238. [PubMed: 16944907]
18. Zhang J, Lakowicz JR. *Optics Express* 2007;15:2598. [PubMed: 19532498]
19. Zhang J, Fu Y, Chowdhury MH, Lakowicz JR. *Nano Letters* 2007;7:2101. [PubMed: 17580926]
20. Lakowicz JR. *Plasmonics* 2006;1:5. [PubMed: 19890454]
21. Branton D, Deamer DW, Marziali A, Bayley H, Benner SA, Butler T, Di Ventra M, Garaj S, Hibbs A, Huang XH, Jovanovich SB, Krstic PS, Lindsay S, Ling XSS, Mastrangelo CH, Meller A, Oliver JS, Pershin YV, Ramsey JM, Riehn R, Soni GV, Tabard-Cossa V, Wanunu M, Wiggan M, Schloss JA. *Nature Biotechnology* 2008;26:1146.
22. Healy K. *Nanomedicine* 2007;2:459. [PubMed: 17716132]
23. Milos PM. *Expert Review of Molecular Diagnostics* 2009;9:659. [PubMed: 19817551]
24. Ryan D, Rahimi M, Lund J, Mehta R, Parviz BA. *Trends in Biotechnology* 2007;25:385. [PubMed: 17658190]
25. Voelkerding KV, Dames SA, Durtschi JD. *Clinical Chemistry* 2009;55:641. [PubMed: 19246620]
26. Asefa T, Duncan CT, Sharma KK. *Analyst* 2009;134:1980. [PubMed: 19768203]
27. Sanchez C, Boissiere C, Grosso D, Laberty C, Nicole L. *Chemistry of Materials* 2008;20:682.
28. Sharma KK, Anan A, Buckley RP, Ouellette W, Asefa T. *Journal of the American Chemical Society* 2008;130:218. [PubMed: 18076164]
29. Ko H, Chang S, Tsukruk VV. *Acs Nano* 2009;3:181. [PubMed: 19206265]
30. Lang XY, Chen LY, Guan PF, Fujita T, Chen MW. *Applied Physics Letters* 2009;94.
31. Qian LH, Inoue A, Chen MW. *Applied Physics Letters* 2008;92.
32. http://www.2spi.com/catalog/spec_prep/silver-membrane-filtration-media.html
33. Xie XS, Trautman JK. *Annu Rev Phys Chem* 1998;49.
34. Yip W, Hu D, Yu J, Vanden Bout DA, Barbara PF. *J Phy Chem A* 1998;102:7564.
35. Lakowicz JR. *Anal Biochem* 2001;298:1. [PubMed: 11673890]
36. Aussenegg FR, Leitner A, Lippotich ME, Reinisch H, Reigler M. *Surface Sci* 1987;139:935.
37. Enderlein J. *Chemical Physics* 1999;247:1.
38. Enderlein J. *Biophysical Journal* 2000;78:2151. [PubMed: 10733992]
39. Teperik TV, Popov VV, Abajo FJGd. *Phys Stat Sol* 2005;202:362.
40. Teperik TV, Popov VV, Abajo FJGd, Kelf TA, Sugawara Y, Baumberg JJ, Abdelsalem M, Philip NB. *Optics Express* 2006;14:75620.

41. Maarooof AI, Gentle A, Smith GB, Cortie MB. J Phys D: Appl Phys 2007;40:5675.

Figure 1. Schematic experimental setup used to monitor single DNA molecules on silver membranes. The left panel shows a SEM image of a porous silver membrane, the image was recorded using a Scanning Electron Microscope Quanta 200 (FEI Inc.).

Figure 2. Confocal fluorescence images ($10 \times 10 \mu\text{m}$) of samples: (a) dsDNA molecules spin-cast on glass coverslips; (b) thiol derivatized ss-DNA (unlabeled) molecules tethered to silver porous membranes; (c) hybridized dsDNA molecules immobilized on silver porous membranes. Excitation wavelength: 633nm

Figure 3.

Typical fluorescence time traces from single fluorescently-labeled DNA molecules: (a) & (b) ds DNA molecules on glass coverslips (gray lines); (c)&(d) dsDNA molecules immobilized on silver porous structures (black lines).

Figure 4.

Fluorescence decay curves of single fluorescently labeled DNA molecules. Gray line: a single molecule deposit on glass coverslip; black line: a single molecule immobilized on silver porous membrane.

Figure 5.

An overlaid image of single DNA molecules fluorescence emission over a surface contour on a silver porous membrane. Different colors are used to represent different fluorescence intensity levels. The shaded contour represents surface feature derived from the reflectance image recorded from the same region. Excitation wavelength: 633nm.