Stabilization of Silver Metal in Citrate Buffer: Barcoded Nanowires and Their Bioconjugates

Rebecca L. Stoermer,† James A. Sioss,† and Christine D. Keating*

Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

Received April 29, 2005. Revised Manuscript Received June 20, 2005

Nanobarcodes (NBCs) are striped metallic nanowires typically several micrometers in length and ∼300 nm in diameter, having segments of Au and Ag along their length. NBCs are promising for multiplexed bioanalysis due to the large number of striping patterns that can be synthesized and the ease of optical read-out using simple reflectance microscopy. Since Ag metal is susceptible to oxidation, we were interested in determining the long-term stability of NBCs in aqueous buffers. We report the effect of storage in high-salt hybridization buffer for both undisturbed and continuously vortexed NBC samples. We find that wires stored in hybridization buffer for longer than 2 weeks begin to show significant degradation of Ag segments. When agitated with continuous vortexing, the Ag oxidation progressed more rapidly, rendering the NBCs stored in hybridization buffer unidentifiable in less than 1 week. Addition of 40 mM citrate as a mild reducing agent increased Ag stability by 17 weeks over those stored in hybridization buffer. NBCs subjected to continuous vortexing in 40 mM citrate buffer retained Ag segment stability for longer than 2 weeks. Derivatization of the wires with biomolecules, such as are used in bioassays, affords some additional protection against Ag degradation. We find that wires coated with rhodamine-tagged DNA oligonucleotides attached via Neutravidin−biotin chemistry are stable for 12 days in hybridization buffer and for at least 63 days when 40 mM citrate is added. Silver deterioration in these experiments was coupled to loss of fluorescence from the labeled DNA, as well as wire breakage.

Introduction

Nanobarcodes (NBC) are striped metal nanowires generally several micrometers in length and ∼300 nm in diameter, prepared by sequential electrodeposition of metals such as Au, Ag, Pd, Ni, or Pt in alumina template membranes.[1] These particles are attractive as identification tags for applications ranging from brand protection in the retail market to multiplexed bioanalysis.[2] They can be fabricated in a multitude of striping patterns and are identifiable by simple optical reflectance microscopy.[2,3] Of the metals used as segments in these particles, Au and Ag are particularly attractive due to the large difference in reflectivity between these metals under blue illumination, which provides excellent contrast for readout of the striping pattern, or barcode.[3] Ag metal is less noble than Au and prone to oxidation. Indeed, we had previously noted some degradation of Ag nanowire segments when stored in air or H_2O and have found that storage in EtOH protects against oxidation.[3] It is, however, of interest to preserve protein and DNA conjugated NBCs, which require storage in aqueous buffer.

Silver metal surfaces and particles are important in a wide variety of biosensing applications.[4] Ag is attractive due to its favorable optical properties in surface plasmon resonance (SPR),[5,6] localized surface plasmon resonance (LSPR),[7,8] and surface-enhanced spectroscopies including fluorescence,[9−11] absorbance,[11] and Raman scattering.[12] In addition, Ag films have been used on quartz crystal microbalance[13,14] and electrochemical sensors.[15] Biosensor construction generally requires attachment of DNA, proteins, or other biomolecules to the Ag substrate.[1−3,16−21] Unfortunately, the coupling of biomolecules onto Ag surfaces has often proven difficult,

* To whom correspondence should be addressed: 104 Chemistry Building, The Pennsylvania State University, University Park, PA 16802. E-mail: keating@chem.psu.edu. Phone: 814-863-7832.

† These authors contributed equally to this work.

(1) Nicewarner-Pena, S. R.; Freeman, R. G.; Reiss, B. D.; He, L.; Pena D. J.; Walton, I. D.; Cromer, R.; Keating, C. D.; Natan, M. J. *Science* **2001**, *294*, 137.
(2) Keating, C. D.; Natan, M. J. *Adv. Mater.* **2003**, *15*, 451.
(3) Nicewarner-Pena, S. R.; Carado, A. J.; Shale, K. E.; Keating C. D. *J. Phys. Chem. B* **2003**, *107*, 7360.
(4) Rosi, N. L.; Mirkin, C. A. *Chem. Rev.* **2005**, *105*, 1547.
(5) Raether, H. *Surface Plasmons on Smooth and Rough Surfaces and on Gratings*; Springer-Verlag: Berlin, 1988.
(6) Neumann, T.; Johansson, M.-L.; Kambhampati, D.; Knoll, W. *Adv. Funct. Mater.* **2002**, *12*, 575.
(7) Haes, A. J.; Van Duyne, R. P. *Expert Rev. Mol. Diagn.* **2004**, *4*, 527.
(8) Haes, A. J.; Van Duyne, R. P. *J. Am. Chem. Soc.* **2002**, *124*, 10596.
(9) Lakowicz, J. R.; Geddes, C. D.; Gryczynski, I.; Malicka, J.; Gryczynski, Z.; Aslan, K.; Lukomska, J.; Matveeva, E.; Zhang, J.; Budugu, R.; Huang, J. *J. Fluoresc.* **2004**, *14*, 425.
(10) Lakowicz, J. R. *Anal. Biochem.* **2001**, *298*, 1.
(11) Moskovits, M. *Rev. Mod. Phys.* **1985**, *57*, 783.
(12) Vo-Dinh, T. *Sens. Actuators, B* **1995**, *29*, 183.
(13) Su, X. *Biochem. Biophy. Res. Commun.* **2002**, *290*, 962.
(14) Baily, L. E.; Kambhampanti, D.; Kananzawa, K. K.; Knoll, W.; Frank, C. W. *Langmuir* **2002**, *18*, 479.
(15) Matsuura, H.; Sato, Y.; Sawaguchi, T.; Mizutani, F. *Sens. Actuators, B* **2003**, *91*, 148.
(16) Sokolov, K.; Chumanov, G.; Cotton, T. M. *Anal. Chem.* **1998**, *70*, 3898.
(17) Malicka, J.; Gryczynski, I.; Lankowicz, J. R. *Anal. Chem.* **2003**, *75*, 4408.
(18) Wang, J.; Song, F.; Zhou, F. *Langmuir* **2002**, *18*, 6653.
(19) Yokota, H.; Saito, K.; Yanagida, T. *Phys. Rev. Lett.* **1997**, *80*, 4606.
(20) Malicka, J.; Gryczynski, I.; Gryczynski, Z.; Lankowicz, J. R. *Anal. Biochem.* **2003**, *315*, 57.
(21) Walton, I. D.; Norton, S. M.; Balasingham, A.; He, L.; Oviso, D. F.; Gupta, D.; Raju, P. A.; Natan, M. J.; Freeman, R. G. *Anal. Chem.* **2002**, *74*, 2240.

10.1021/cm050910l CCC: $30.25
Published on Web 07/27/2005

due to the instability of Ag surfaces and nanoparticles.[22,23] While many of the optical investigations of Ag have been carried out under vacuum, biosensing is generally done in (oxygenated) aqueous buffers.[3,21,22]

Dry Ag films in oxygen-rich environments undergo an alteration in interference color and appear rough within a few days.[24] During oxidation, cracking and flaking of the oxide takes place which facilitates diffusion of the oxygen to the surface beneath, thus causing continual oxidation damage to the film.[24−26] It has been proposed that the silver oxide formation on the surface during oxidation acts as a catalyst for further oxidation of underlying silver.[24] Although this oxidation process has not been characterized to the same degree in oxygenated aqueous solutions, it is known to occur. The instability of Ag films has caused difficulties for biosensing applications due to oxidation.[13,27]

When Ag-containing NBCs oxidize in air, water, or salt buffer, the initial deterioration appears as pits in the surface of the Ag segments. Eventually, larger portions of the surface disintegrate, leaving behind large voids in the Ag segments. When agitated, the wires degrade faster, presumably due to increased O_2 diffusion and improved access to underlying Ag.[24] The degradation of NBCs and their bioconjugates is of interest because it may impact the long-term storage of these materials for use in biological analysis. Potential consequences of Ag oxidation in NBCs include (1) the loss of attached biomolecules as the surface deteriorates, (2) alteration of particle optical properties as the surface pits and becomes less uniform, which could lead to difficulties in barcode pattern identification, and (3) wire breakage as Ag segments disappear, which could also lead to misidentification of barcode patterns.

Silver metal surfaces can be protected from oxidation by coating with alkanethiol monolayers or polymeric or inorganic thin films.[13,23,27−32] For example, Su and co-workers have demonstrated that polystyrene or carboxy-poly(vinyl chloride) films protected Ag-coated quartz crystal microbalance biosensors from Ag oxidation and the associated degradation in sensor response.[13,27] Silver nanospheres have been coated with Au or SiO_2 shells for added stability and biofunctionalization.[22,29] Takenouti and co-workers have used hexadecanethiol self assembled monolayers to prevent Ag tarnishing and corrosion.[31,32] These methods have been successful in protecting Ag; however, they all require surface modification and may not be readily adopted by the molecular biology community. We were interested in preventing Ag oxidation in barcoded nanowires without introducing surface coatings.

Herein we describe an experimental study conducted in an effort to characterize and prevent the deterioration of Ag in bare and bioconjugated NBCs stored in aqueous buffer. We have found that buffer solutions containing sodium citrate as a reducing agent greatly reduce the rate of nanowire degradation. Our results indicate that bare, unfunctionalized NBCs survive in hybridization buffer ∼2 weeks before extensive deterioration. This is comparable to the length of time that the wires survived in both air and water (14 days before extensive pitting).[3] When sodium citrate is added to the buffer, NBCs last on average three times longer than those in hybridization buffer alone. Agitation of NBC suspensions in any buffer greatly increases the rate of Ag deterioration. However, addition of citrate still offers significant preservation. We also compared hybridization buffer with and without citrate as a storage solution to prevent the oxidation of Ag in fluorescent DNA:NBC bioconjugates. After 12 days of storage in hybridization buffer, fluorescent DNA detached from the DNA-conjugated NBCs as they deteriorated, while the NBCs stored in citrate-containing hybridization buffer did not show significant deterioration at 9 weeks.

Experimental Section

Materials. Monobasic and dibasic sodium phosphate, trisodium citrate, and PBS buffer (0.01 M phosphatebuffered saline; 0.138 M NaCl; 0.0027 M KCl; pH 7.4) were purchased from Sigma. Sodium chloride was purchased from Aldrich. All water used was distilled and purified to 18.2 MΩ through a Barnstead Nanopure system. NeutrAvidin was obtained from Pierce and was reconstituted to appropriate concentrations in nanopure water. The DNA sequences used are as follows: (A) 5′biotin-AAA AAA ACG TTG TCT GAT GCG TCA, (B) 5′-ACA CAG ACG TAC TAT CAT TGA CGC ATC AGA CAA CGT, and (C) 5′-ATG ATA GTA CGT CTG TGT-ROX, where ROX is a rhodamine-based dye sold by Integrated DNA Technologies. Two of the DNA sequences (A and B) were synthesized on an Expedite 8909 DNA synthesizer using reagents purchased from Glen Research, and the third sequence (C) was purchased from IDT, Inc.

Striped metal nanowires (NBCs) with striping patterns encoded 011110, 000111, and 001100, where 0 and 1 represent 0.75 μm length segments of Au and Ag, respectively, were purchased from Nanoplex Technologies (Menlo Park, CA). These particles were "bare" (i.e., no molecules had been intentionally added postsynthesis) unless otherwise noted. NBCs were rinsed three times in water before use, as they were previously stored in ethanol. Typically, a 1000 μL batch of NBCs contains 1×10^9 nanowires. NBSee Analysis Software (version 1.0.26, from Nanoplex Technologies) was used for NBC identification of nanowire patterns and to investigate the degree of degradation that caused the software to no longer recognize the wire patterns.

Buffer Preparation. Buffers were made using a standard high-salt hybridization buffer (HB), consisting of 0.3 M NaCl and 10 mM sodium phosphate at pH 7.2. The citrate-containing buffers (CB) were prepared to concentrations of 40, 100, or 300 mM of sodium citrate in HB.

(22) Charles, S. A.; Endericks, T.; Evans, A. G.; Garnham, S. E.; Irlam, J. C.; Pollard-Knight, D.; Downes, M.; Heaney, P. J.; Finlan, M. F.; Garland, P. B. In *UCLA Symposia on Molecular and Cellular Biology*; Cantor, C. R., Caskey, C. T., Hood, L. E., Kamely, D., Omenn, G. S., Eds.; Biotechnology and Human Genetic Predisposition to Disease, Vol. 126; John Wiley & Sons: New York, 1990; p 291.
(23) Cao, Y. W.; Jin, R.; Mirkin, C. *J. Am. Chem. Soc.* **2001**, *123*, 7961.
(24) Pettersson, L. A. A.; Snyder, P. G. *Thin Solid Films* **1995**, *270*, 69.
(25) Zheludkevich, M. L.; Gusakov, A. G.; Voropaev, A. G.; Vecher, A. A.; Kozyrski, E. N.; Raspopov, S. A. *Oxid. Met.* **2004**, *61*, 39.
(26) Czanderna, A. W. *J. Phys. Chem.* **1964**, *68*, 2765.
(27) Su, X.; Ng, H. T.; Dai, C.-C.; O'Shea, S. J.; Li, S. F. Y. *Analyst* **2000**, *125*, 2268.
(28) Li, W.; Virtanen, J. A.; Penner, R. M. *Langmuir* **1995**, *11*, 4361.
(29) Morales, A.; Duran, A. J. *Sol−Gel Sci. Technol.* **1997**, *8*, 451.
(30) Liu, S.; Zhang, Z.; Han, M. *Anal. Chem.* **2005**, *77*, 2595−2600.
(31) Bernard, M. C.; Dauvergne, E.; Evesque, M.; Keddam, M.; Takenouti, H. *Corros. Sci.* **2005**, *47*, 663.
(32) Evesque, M.; Keddam, M.; Takenouti, H. *Electrochim. Acta* **2002**, *49*, 2937.

Oxidation of Silver NBCs Segments. To a 25 μL aliquot of rinsed nanowires (000111), 200 μL of 40 mM citrate buffer was added. To a separate aliquot, hybridization buffer was added in the same quantities. The nanowire samples were allowed to sit undisturbed on the benchtop over the course of the experiment, except when an aliquot was removed every third day for imaging. When imaged, both reflectance optical microscopy and FE-SEM data were obtained.

NBSee Software Analysis. Nanowire samples consisting of 80 μL wires, rinsed in water with the supernatant removed, were resuspended in 80 μL of either hybridization buffer, 40 mM citrate buffer, or ethanol. Two samples were made containing each solution such that one sample could be left to rest on the benchtop and the other could be continuously agitated on a vortex genie. The samples were sonicated briefly before imaging to reduce clumping of wires, as the NBSee program cannot identify wires that are touching or clumped. These samples were more concentrated with nanowires than previous experiments, which allowed for a greater number of nanowires per image and more statistical data when analyzed by NBSee.

Optical Microscopy. Brightfield reflectance images were acquired using a Nikon TE-300 inverted microscope equipped with a 12 bit high-resolution Coolsnap HQ camera (Photometrics). A CFI plan fluor 100× oil immersion lens (N. A. = 1.3) was used in conjunction with Image-Pro Plus software (version 4.5) to image the samples. The light source was a 175 W ozone-free Xe lamp, and a Sutter Instruments filter wheel (Lambda 10-2) allowed for wavelength selection. Samples were prepared by either drying 10 μL aliquots of nanowires onto glass coverslips (Fisher 12-542-C) and then adding a 10 μL drop of water to the sample to adhere the coverslip to a glass slide or by sandwiching an 8 μL sample between two coverslips. All reflectance images were taken at 430 nm, which is the wavelength that gives the biggest reflectance contrast between Au and Ag.[3]

Electron Microscopy. Field emission-scanning electron microscopy images (FE-SEM) were obtained using a JEOL 6700F FE-SEM located at the PSU Materials Characterization Lab. Secondary electron imaging (SEI) mode was used with an accelerating voltage of 15 KV. To prepare the samples, an Al stud covered in copper tape (EM Sciences) was used to support 8 μL of nanowires, which were dried in a vacuum desiccator for 2 h prior to imaging.

Fluorescent-DNA-Coated Nanowires. A 40 μL sample of NBCs (patterned 011110) was washed three times in water prior to the addition of 200 μL of 0.25 mg/mL NeutrAvidin. The wires were then vortexed for 2 h and rinsed three times in water. They were then resuspended in 10 μM DNA (A) in PBS buffer (0.01 M phosphate, 0.138 M NaCl, 0.0027 M KCl, pH = 7.4) and vortexed for 4 h at room temperature. The NBCs were then rinsed three times in PBS buffer and resuspended to a final concentration of 10 μM DNA (B) in HB and were vortexed for 4 h at room temperature. Following three rinsings in HB, the final fluorescent DNA strand (C) was hybridized at a concentration of 10 μM in HB for 4 h at room temperature. The excess DNA was rinsed from the system in three washings using HB. The DNA conjugated wires were divided equally into two tubes of which 100 μL of HB was added to one tube and 100 μL of CB was added to the other. The tubes were then allowed to sit on the benchtop at room temperature, undisturbed with the exception of the occasional removal of an aliquot for imaging.

Results and Discussion

NBCs are routinely stored for long periods in ethanol after synthesis, and Ag segments stored in this way retain their integrity indefinitely.[3] However, once biomolecules such as antibodies or DNA are attached to NBCs, it is necessary to store the bioconjugates in an aqueous buffer solution. Although oxidation could be prevented by substitution of Pd or Pt in place of Ag segments, Ag provides the greatest contrast with Au, and therefore is generally preferred.[3] Therefore, it is of interest to identify conditions under which Ag-containing NBCs can be stored in aqueous buffer. The mechanistic details of Ag degradation in aqueous buffers are not generally discussed in the literature; however, it is widely appreciated that oxidation of Ag by dissolved O_2 is responsible. The presence of solutes such as Cl^-, which alter the reduction potential of Ag^0, may also play a significant role.[33] Our previous observations indicate that Cl^- is not necessary for Ag dissolution, which also occurred after storage in deionized H_2O.[3]

In an effort to slow the oxidation of Ag segments, the addition of the reducing agents citrate and dithiothreitol (DTT) to buffer solutions was investigated. These were selected because they are frequent additives in buffers for biological molecules. Trisodium citrate is also commonly used in the preparation of Ag sols in aqueous solution, where it reduces Ag^+ to Ag^0 to form nanoparticles.[34] We found that DTT-containing buffers hastened the rate of Ag degradation in nanowires over those stored in HB alone, perhaps because this molecule can serve as a good ligand for Ag(I). We have observed similar effects from other short-chain thiols such as mercaptoethanol and mercaptopropanol. Therefore, we focused on citrate, and DTT was eliminated from the study.

To determine the length of time Ag-containing nanowires would survive in citrate buffer solution as compared to those stored in a noncitrate buffer solution, two separate aliquots of NBCs patterned 000111 (half Au and half Ag) were stored in either hybridization buffer (HB) or in HB containing 40 mM citrate buffer (CB) on the benchtop for up to 53 days. Reflectance optical microscopy and FE-SEM data were obtained daily over the length of this study. The optical microscopy provides information on changes in reflectance, which are important for barcode pattern identification and would impact the homogeneity of a fluorescence assay carried out on the NBC surface. FE-SEM enables visualization of the nanostructure as Ag oxidation proceeds. Figure 1 shows representative reflectance and FE-SEM images of wires imaged after 0, 7, and 17 days. The first signs of degradation are observed in the form of Ag segment pitting in FE-SEM and darker spots in the reflectivity images for the nanowires stored in HB as early as 2 days after addition of buffer. Samples stored in CB do not show this initial degradation until day 7 in both FE-SEM and reflectivity images. By day 17, the wires in HB displayed substantial degradation, while the corresponding wires in CB showed very little damage. It was not until day 53 that the NBCs stored in CB had similar degradation as those stored in HB for 17 days (see the Supporting Information, Figure 1). In

(33) (a) Burstein, G. T.; Liu, C.; Souto, R. M.; Vines, S. P. *Corros. Eng. Sci. Technol.* **2004**, *39*, 25−30. (b) *J. Appl. Electrochem.* **2002**, *32*, 157−164.
(34) Lee, P. C.; Meisel, D. *J. Phys. Chem. B* **1982**, *86*, 3391−3395.

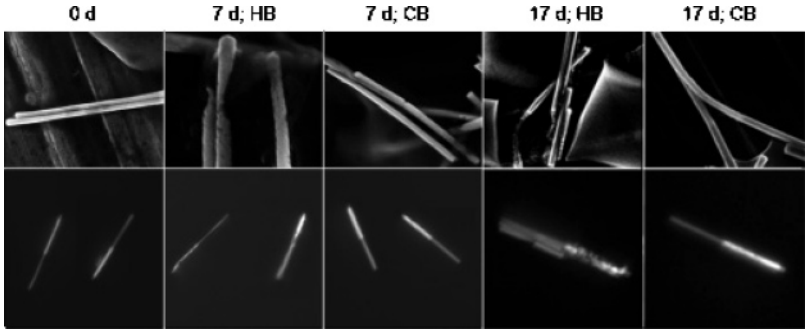

Figure 1. Nanowires (patterned 000111) after storage in hybridization buffer (HB) or citrate buffer (CB) for the number of days indicated. Top images are FE-SEM and bottom images are optical reflectance.

general, NBCs stored in CB extended the life of the Ag nanowires by nearly three times over those stored in HB.

Beyond the fact that this reductant prevents oxidative dissolution of the Ag metal, our experiments do not elucidate a detailed mechanism of citrate protection against Ag degradation. Citrate ions can adsorb to the Ag surface via their carboxylate moieties;[35] however, we do not expect that this weakly adsorbed citrate layer is sufficient to prevent O_2 from reaching the Ag metal. Citrate may compete effectively for binding sites with various oxygen species or may itself react with oxygen species adsorbed to the Ag. Any Ag^+ that does form will be re-reduced by the excess citrate.

During a typical bioassay experiment, NBCs are agitated on a rotary shaker, tumbler, or vortex mixer during both derivatization with capture probes and reaction with target biomolecules. Since agitation will help oxygenate the solution, it was of interest to determine to what extent it accelerated the rate of damage to Ag segments. We selected the most vigorous of the mixing methods, vortexing. To determine whether vortexing the samples increased the rate of degradation, NBCs (pattern 001100) were prepared in HB, 40 mM CB, or 95% ethanol. Ethanol is our standard NBC storage solvent when no biomolecules are attached and is very effective at preventing Ag oxidation; unstirred samples are undamaged after one year.[3] Each sample was divided in half; one-half was vortexed continuously and the other half was stored in the respective solutions on the benchtop. The wires were imaged daily over a 12 day period. Optical reflectance images for these samples after 7 and 12 days are shown in Figures 2 and 3, respectively.

At day 7, the wire samples vortexed in HB showed clear signs of Ag degradation, with dark regions visible in the reflectance images (Figure 2E). All other samples, including wires vortexed in CB, showed essentially no sign of damage. After 12 days, the HB sample showed dark regions in the reflectance from Ag segments, much like what was observed after 7 days of vortexing. The HB sample that had been continuously vortexed for 12 days showed mainly broken wires (i.e., the Ag segments had failed entirely). In contrast, the ethanol and CB samples show no change. It should be noted that vortexing continually for 7 days goes far beyond the demands of any potential bioassay application. Nonetheless, this study showed that the degradation of agitated wires stored in HB was accelerated greatly over those allowed to rest undisturbed on the benchtop and that protection by addition of 40 mM citrate to the HB of a sample continuously vortexed for 12 days was as effective as undisturbed storage in ethanol.

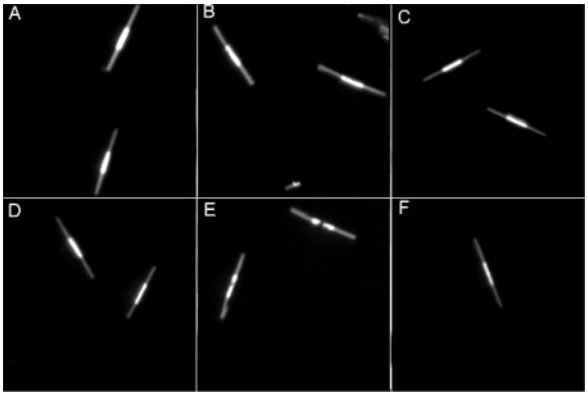

Figure 2. Reflectivity images of nanowires (patterned 001100) at 430 nm illumination after 7 days of benchtop storage in ethanol (A), hybridization buffer (B), and citrate buffer (C). Images D−F represent wires vortexed in ethanol (D), hybridization buffer (E), and citrate buffer (F).

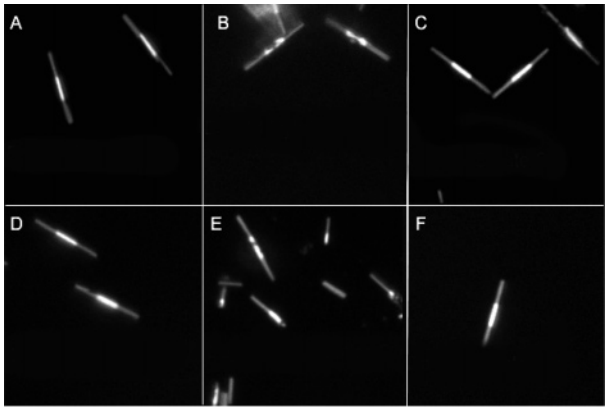

Figure 3. Reflectivity images of nanowires (patterned 001100) using 430 nm illumination. Top: images taken after 12 days of undisturbed benchtop storage in ethanol (A), hybridization buffer (B), and citrate buffer (C). Bottom: images taken after 14 days of continuous vortexing in ethanol (D), hybridization buffer (E), and citrate buffer (F).

The use of NBCs in multiplexed bioanalysis requires accurate pattern recognition. Since Ag degradation leads to areas of reduced reflectance, it could hinder NBC identification. To investigate this possibility, we tested the impact of NBCs Ag segment degradation on the ability of the NBSee software to correctly identify the nanowires. This software was designed by Nanoplex Technologies for the purpose of identification and analysis of NBCs.[36] Image sets were analyzed using NBSee software programmed to discard wires

(35) Rospendowski, B. N.; Kelly, K.; Wolf, C. R.; Smith, W. E. *J. Am. Chem. Soc.* **1991**, *113*, 1217−1225.

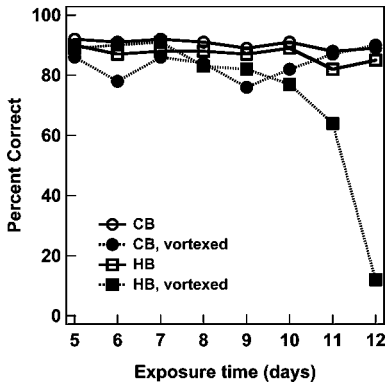

Figure 4. Graph of percentage of nanowires correctly identified by NBSee software from days 5−12.

not at least 4.3 μm in length; unbroken wires should be 4.5 μm long. This range was used to identify only wires that were not broken. Typically during bioanalysis it is desirable to identify which biomolecules are attached to which NBCs. Therefore, it is important to identify only the NBCs that are not broken and are fully identifiable.

With the use of NBSee, all samples were identified with greater than 80% accuracy through day 12, with the exception of the vortexed HB sample (Figure 4). Less than 100% identification accuracy indicates that some of the wires were identified as having patterns other than 001100. Misidentification can arise from polydispersity in the initial NBC sample or postsynthesis degradation; decreases over time indicate that pitting of the Ag segments and/or wire breakage caused incorrect wire assignment. Nanowires in ethanol were imaged as a control, as no Ag oxidation is observed for these samples over an entire year.[3] NBCs left undisturbed in ethanol remained relatively constant at 94% for day 5 and 92% for day 12 (see the Supporting Information, Figure 2). The percentage of correctly identified wires stored in CB decreased from 92% to 89% (Figure 4). Those stored in HB decreased from 90% to 85%. These very small changes reflect the fact that the wires do not undergo much degradation in either CB or HB after only 12 days when sitting undisturbed. However, the percent correctly identified dropped precipitously to just 12% for the vortexed HB sample by day 12. This substantial decrease tracks the increase in Ag segment degradation and wire breakage observed in the reflectance images of these samples. In contrast, NBCs vortexed for 12 days in CB showed no significant decrease in pattern identification accuracy, underscoring the protective effect of the citrate. The fact that nanowire identification by NBSee did not decline significantly for the 12 day undisturbed HB and 7 day vortexed HB samples, despite noticeable changes in their reflectance images (Figures 2E and 3B), indicates the robustness of the pattern identification by the NBSee software. The program anticipates that each segment will be 750 nm in length, which reduces the impact of small dark regions within a Ag stripe, and the user interface enables selection of identification criteria to discard broken wires from analysis by dictating the expected length. From the standpoint of multiplexed analysis, this means that small amounts of Ag degradation will not impact particle identification.

We initially selected citrate based on its use in Ag(I) reduction for preparation of colloidal Ag sols.[34] These recipes call for a 1% (38.8 mM) citrate solution.[34] To determine whether additional reducing agent would provide further increases in Ag stability, we compared hybridization buffer with 40 mM, 100 mM, and 300 mM citrate. NBCs (patterned 000111) were left undisturbed on the benchtop in each of these buffers. Reflectance images of these wires after 7, 12, and 19 weeks show no additional improvement for the higher citrate concentrations (see the Supporting Information, Figure 3). Whole, slightly pitted, and broken wires can be found in all three samples after 19 weeks. These data suggest that 40 mM citrate is sufficient for preserving the wires over several months.

NBC-based multiplexing is generally performed using fluorescence to report the presence and amount of target biomolecules. Fluorescence intensity for NBC-based assays is sensitive to both the underlying metal identity (i.e., Au vs Ag) and to inhomogeneities such as can result from Ag degradation. We performed a DNA sandwich hybridization assay on nanowires composed almost entirely of Ag, with short Au caps on each end. Capture probes biotinylated on the 5′ end were attached to the wire surface via adsorption to a NeutrAvidin protein, after which the particles were exposed to the target strand, followed by a fluorescently tagged detection strand with a 3′ rhodamine dye. After this DNA sandwich was assembled on the nanowire surface, the sample was divided into two aliquots; half of the wires were stored in HB, and the other half in CB. Both nanowire bioconjugate samples showed greater longevity as compared with bare Ag segments, due to the protective effect of the protein and DNA layer. Eventually, however, the wires in HB began to show signs of Ag oxidation. By day 18 pitting is evident in the wires stored in HB. After 63 days of storage in their respective buffers, the optical reflectance and fluorescence images of the wires show that the samples stored in HB have degraded substantially, while those in CB show little evidence of oxidation (Figure 5). The sample stored in HB contained many broken wires, and very low fluorescence intensity remained on the wires. Note that, although increased surface roughness can lead to enhanced fluorescence due to increased electromagnetic fields,[11] we observe only a decrease in fluorescence intensity upon Ag degradation. Unlike in surface-enhanced fluorescence experiments, however, the adsorbed molecules in this case were present prior to the surface roughening and have presumably been removed with the Ag as it dissolved. Once in solution, these molecules could readsorb to the Ag surface or to the container walls, or they could remain in solution, perhaps bound to Ag^+. If they do readsorb to the Ag, they may do so via the DNA rather than the thiol, leading to lower surface coverages and likely quenching of rhodamine emission by the metal surface. It is also possible that the thiol moieties are oxidized, resulting in lower affinity for the Ag surface. In contrast, samples stored in CB showed minimal degradation, and fluorescent DNA was still present even after 63 days, the

(36) Walton, I. D.; Norton, S. M.; Balasingham, A.; He, L.; Oviso, D. F., Jr.; Gupta, D.; Raju, P. A.; Natan, M. J.; Freeman, R. G. *Anal. Chem.* **2002**, *74*, 2240.

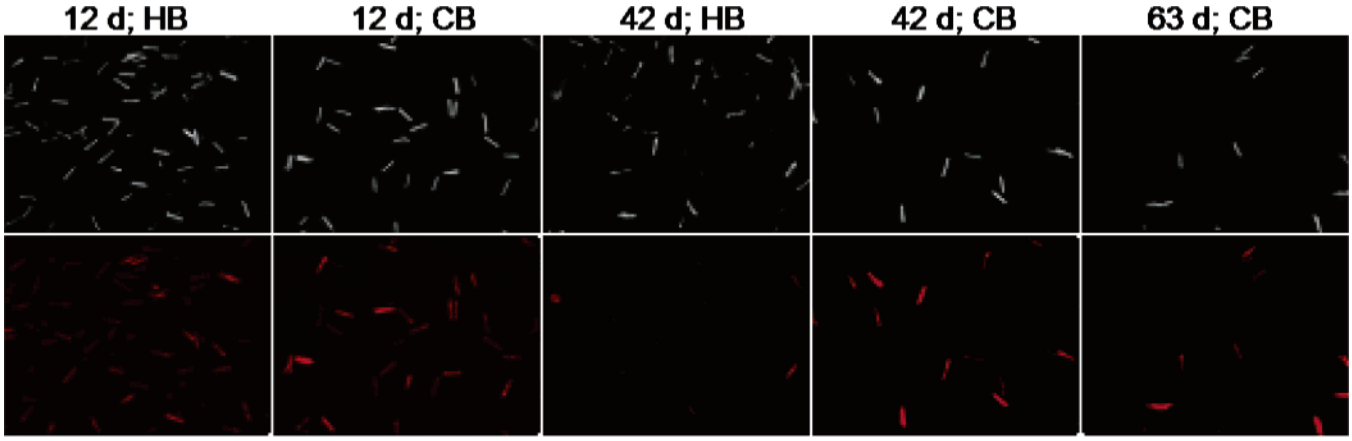

Figure 5. Reflectance (top) and corresponding fluorescence images (bottom) of nanowires, patterned 011110, after storage in hybridization buffer (HB) or citrate buffer (CB) for the number of days indicated.

longest time evaluated (Figure 5). Thus, we recommend that when Ag-containing nanowire bioconjugates are stored prior to use, citrate or another mild reducing agent be added to maintain sample integrity.

Conclusions

Barcoded nanowires offer a promising route for multiplexed bioanalysis.[2] Silver and Au segmented nanobarcodes (NBCs) are of particular interest, as these metals provide the greatest contrast with blue illumination and can provide uniform intensity for fluorescent assays performed in the red.[3] Unfortunately, Ag is less stable than other possible NBC metals (Au, Pd, Pt) and will degrade over a time scale of weeks in aqueous buffers. Although this does not present any problems for uses in which the NBCs are conjugated and used within a few days, slow air oxidation of stored bioconjugated wires could lead to degradation of Ag segments and eventual wire breakage. We have shown that adding 40 mM citrate to the buffer markedly slows Ag oxidation. No surface modification was necessary. Bioconjugated NBCs were stable for more than two months in citrate-containing 0.3 M NaCl, 50 mM phosphate buffer (pH 7). Presumably, similar results would be achieved by storing the NBCs in deoxygenated buffers, under Ar(g); however, addition of citrate is much more convenient. Addition of citrate will enable longer storage of biolabeled nanobarcodes prior to use and may aid in the prevention of Ag surface degradation of biotagged Ag thin films such as those used in surface plasmon resonance and surface-enhanced Raman scattering studies.

Acknowledgment. This work was supported by the National Institutes of Health (Grant R01 EB00268) and The Pennsylvania State University. C.D.K. also acknowledges support from a Beckman Foundation Young Investigator Award and a Sloan Fellowship.

Supporting Information Available: FE-SEM and optical reflectance images for 000111 nanowires after 53 days in CB, optical reflectance images for 000111 nanowires after storage in various concentrations of CB for 7 to 19 weeks, graph of percentage of nanowires correctly identified by NBSee software after storage for 5−12 days in CB and ethanol. This material is available free of charge via the Internet at http://pubs.acs.org.

CM050910L