Surface Modification of Silicon Pillar Arrays To Enhance Fluorescence Detection of Uranium and DNA

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ABSTRACT: There is an ever-growing need for detection methods that are both sensitive and efficient, such that reagent and sample consumption is minimized. Nanopillar arrays offer an attractive option to fill this need by virtue of their small scale in conjunction with their field enhancement intensity gains. This work investigates the use of nanopillar substrates for the detection of the uranyl ion and DNA, two analytes unalike but for their low quantum efficiencies combined with the need for high-throughput analyses. Herein, the adaptability of these platforms was explored, as methods for the successful surface immobilization of both analytes were developed and compared, resulting in a limit of detection for the uranyl ion of less than 1 ppm with a 0.2 μL sample volume. Moreover, differentiation between single-stranded and double-stranded DNA was possible, including qualitative identification between double-stranded DNA and DNA of the same sequence, but with a 10-base-pair mismatch.

INTRODUCTION

Nanostructures have been the subject of a great deal of recent work owing to the complex and unique nature of their optical properties.1–4 Recently, work has been done investigating the ability of nanopillars to act as field enhancers for fluorescence analysis.5–7 While nanopillars may be too large for quantum confinement effects, silicon pillars at or near 100 nm in diameter have been shown to exhibit optical resonances within the visible spectrum, making them valuable for fluorescence research.8–10 Not only does the nanoscale platform of pillar arrays minimize the reagent consumption and sample volumes, but the vertical geometry of the pillars and the capability of coating them with an interfacial, porous surface also increases the number of sites available to bind fluorophores within the same field of view. Additionally, it is possible to functionalize the surface with a wide variety of functionalities via siloxane and other chemistries to facilitate the binding of analytes. In this work, we utilize the versatility and fluorescent-enhancing capabilities of nanopillars in conjunction with different interfacial chemistries to immobilize and quantify two analytes different except that they both have low quantum yields and are frequently present in only trace amounts: uranium and DNA.

Events such as the nuclear disaster at the Fukushima Nuclear Power Plant in 2011 have and will continue to pose significant hurdles to traditional uranium detection and analysis, mostly because of the sheer number and variety of samples that need to be evaluated. Current approaches typically consist of extraction chromatography followed by radiometric counting or ionization and mass analysis.8–11 While these methods are ideal for trace level samples, method complexity as well as instrumentation investment, field portability, and footprint can be limiting and have driven the investigation into alternate methodologies. Herein, we examine using the coordinating compound N,N-diisobutyl-2-(octyl(phenyl)-phosphoryl)-acetamide (CMPO), used in many actinide recovery resins, to capture uranyl (UO₂²⁺) at the porous layer surface of silicon nanopillars for fluorescence detection. The waveguide enhancement of the nanopillars will work to combat the notoriously low quantum yield associated with the uranyl ion, and the nature of the arrays implies that very small sample sizes may be used and analyzed quickly, creating an ideal screening method.

Supporting Information

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Similar advantages must be taken into consideration when entering the realm of bioanalytics. Like the uranyl ion, nucleic acids exhibit low quantum yields, and only very small sample sizes are frequently available. DNA quantitation has been accomplished in the literature by a variety of methods including UV absorbance spectroscopy and spectrofluorimetry. There has also been recent progress using microfluidic chips in nucleic acid research as all-in-one platforms for processes such as ligation and digestion. Denaturation and reassociation are important physiochemical processes of DNA, the study of which can provide valuable insight into not only the growth of cells and viruses but also the taxonomic and evolutionary relationships between organisms. Reassociation of DNA was first measured by binding small fragments of labeled DNA to long strands immobilized in an agar-based supporting medium, a method later replaced with the use of a hypoxypatite column, on which double-stranded DNA is retained but single-stranded DNA is not. However, both of these methods are time- and labor-intensive, and while work has been done in an effort to reduce these time constraints, such as using size-selective capillary electrophoresis to separate double-stranded DNA samples, to our knowledge a high-throughput method such as presented here has not been developed for the purpose of reassociation evaluation. Herein, we investigate the use of nanoscale pillar arrays for the detection of double- and single-stranded DNA via sequestration of the strands on the pillar surface and fluorescence measurement of added intercalating dyes.

**RESULTS AND DISCUSSION**

**Determination of the Optimal Pillar Width and Comparison To Flat Silicon.** Figure 1 shows arrays with pillar diameters ranging from 60 to 100 nm and with either 5, 10, or 25 nm of porous silicon oxide (PSO), after being soaked in fluoresceinisothiocyanate (FITC), rinsed, and imaged. Plot profiles of the presented images may be found in the Supporting Information. It was determined that 80–90 nm diameter pillars with 5 nm of PSO showed the greatest fluorescence with the least amount of background, and so fabrication parameters that would create approximately this size were used for the fabrication of the dewetted (DW) pillars for uranyl and DNA analysis. By using this electron beam lithography (EBL) wafer layout, we were also able to demonstrate the large on-to-off-piller ratio of the fluorescence signal. This ratio decreases as the pillar width deviates from the optimum and as the PSO thickness increases. With pillar thicknesses greater than 100 nm and PSO thicknesses of 25 nm or greater, the effect is nullified and the fluorescence signal on flat silicon (with a functionalized PSO coating) can even become greater than that on pillars. The thickness of the PSO determined the measured on/off pillar ratio. For instance, we measured an off-pillar signal of zero for 5 nm PSO on 60–100 nm pillar diameters, which results in an on/off pillar ratio of infinity, but for 10 nm PSO on 80 nm pillars, we were able to calculate an on/off pillar ratio of 3.92.

**Detection of Uranyl.** Figure 2 shows the range of uranyl concentrations when spotted on CMPO arrays with their respective intensity profiles, and the corresponding calibration...
curve, which plots the background-corrected average integrated density of each spot versus its concentration. Regression analysis indicated that the experimental data closely fit the linear statistical model between 0 and 200 ppm, with a coefficient of determination ($R^2$) of 0.9842. Over 200 ppm, oversaturation of the signal resulted in not only a leveling out of the calibration curve but also an increase in error of 400%. The noise of the blank was used to determine figures of merit. The limit of detection (LOD) was calculated as 0.97 ppm with a 0.2 μL sample size, resulting in a detection platform capable of subnanogram uranium detection. This LOD is approximately 3 orders of magnitude less sensitive than previously reported using Uraplex, but correspondingly, the sample volume here is minimized over 3 orders of magnitude, creating a platform to screen for low ppm levels of the uranyl ion.\footnote{31} Furthermore, the more sensitive techniques using Uraplex were developed using time-resolved pulsed laser phosphorescence detection, not simple fluorescence microscopy, and it stands to reason that exploration into that high excitation intensity method of detection combined with these nanopillar array systems could achieve substantially lower detection limits.

Detection of DNA. It was quickly determined that spotting was unfeasible as an application method. The arrays did not exhibit enough of a hydrophobic nature to take advantage of the sample concentrating within a small area. When the droplet made contact with the array surface, it immediately wicked through the pillars and spread to an area of ≥2 mm in diameter, greater than the field of view on the fluorescence microscope, and dried with a significant coffee-ring effect. Additionally, while fluorescence was visible immediately after spotting, it was not resistant to rinsing, resulting in a negligible signal postrinse.

Application by soaking, however, resulted in even coverage across the array and quantifiable fluorescence even after rinsing. Indeed, rinsing appeared to be necessary, as prerinse, the different solutions all exhibited the same fluorescence, indicating that there were large amounts of excess dye. The differences between pre- and postrinse, as well as the differences between the different solutions, are illustrated in Figure 3. The average integrated densities of the arrays pre- and postrinse are also shown in the figure. The error in Figure 3b was calculated as the standard deviation of the fluorescence intensity of five 400 px square areas within one array, to account for irregularities in functionalization, and therefore analyze retention on the surface. For ethidium bromide (EtBr), the matched solution of the ssDNA and its exact complement exhibited approximately 175% the fluorescence of the alone solution postrinse, whereas the mismatched solution of the ssDNA and its 10-base-pair-mismatched complement exhibited 105% the fluorescence of the alone solution postrinse (and 60% the magnitude of the matched signal postrinse).

It is possible that the fluorescence of the alone solution is artificially high because of self-hybridization of the single strand or possible electrostatic interaction of EtBr with ssDNA,\footnote{32} as the signal of the alone solution was roughly 4 times that of the blank. If no hybridization was present, we would expect the two signals to be more similar. We also hypothesize that the fluorescence of the mismatched solution is affected by the way that EtBr is able to intercalate with the mismatched strands. The mismatches between the strands were designed such that no portions of the strands were mismatched by more than three base pairs, so we do not expect that the signal is artificially high because of self-hybridization. However, it could be that the mismatches are insufficient to prevent EtBr from binding, resulting in a signal which is greater than anticipated.

**EXPERIMENTAL SECTION**

Fabrication of EBL Arrays and Deposition of PSO. EBL pillar arrays were created using a previously reported process.\footnote{5,24,25} This process involved writing of the circular patterns in a positive tone electron beam resist (300 nm of ZEP 520A), followed by vacuum deposition of a 20 nm chromium layer (VE-240, Thermonics Laboratory, Inc.) and metal lift-off in acetone to form a mask, and finally anisotropic reactive ion etching (RIE) of the silicon substrate not masked by chromium. The pattern of arrays on the wafer is shown in the Supporting Information. The arrays used for this study were 10 × 10 pillar arrays with diameters ranging from 60 to 160 nm varying along a horizontal axis and etched to create pillars with a height of 1 ± 0.2 μm. Each array was separated from those adjacent by distances greater than 20 μm to avoid any possibility of optical coupling or crosstalk.

The structure of the pillars was confirmed with scanning electron microscope (SEM) images obtained from a MERLIN (Carl Zeiss) microscope with a field-emission gun operating at approximately 3 kV. Wafers were then annealed in 10% H2 in Ar at atmospheric pressure at 800 °C for 15 min in a cold wall furnace (FirstNano) to remove any residual organics from the pillar arrays.

The PSO coating was accomplished via room temperature plasma enhanced chemical vapor deposition (PECVD, Oxford Instruments) with the chamber temperature at 27 °C and pressure at 600 mTorr.\footnote{24,26,27} Deposited thicknesses included 5, 10, or 25 nm of PSO.

**Determination of Ideal Pillar Widths.** While using the precise control of pillar geometries afforded by the EBL fabrication would create a system whereby the enhancement of
the analyte could be evaluated and optimized, the EBL approach requires expensive equipment and is a slow serial process, creating practical limits as to the size and quantity of fabricated arrays. Therefore, pillar arrays created via the EBL fabrication method are unsuitable as platforms because of the time and monetary demands of a high throughput analytical method. To meet these high demands, we decided to use a platform that may compromise some of the optimized enhancement for quickly fabricated vast pillar array substrates, namely, stochastic platinum DW pillar arrays, whose usefulness has been previously expounded upon.\textsuperscript{6,24,25} To create these arrays, we first needed to determine the optimum pillar diameter range.

To this end, EBL arrays with a range of pillar diameters were first functionalized with APTES by placing the pieces in a 10 v/v % APTES/toluene solution and soaking at room temperature for 1 h. The pieces were then sequentially rinsed with toluene, methanol, and deionized water and then soaked in 1 mM FITC (in ethanol) for 45 min. After a triplicate rinse in ethanol to remove any excess FITC, fluorescence imaging of the pieces was performed with a Nikon Eclipse E600 microscope and QCapture software. The blue-light excitation (450–490 nm excitation) was used with the same collection time (20 s), gain (2), and offset (−500).

**Fabrication of DW Pillars and Deposition of PSO.** To create high-throughput U platforms, we created silicon nanopillar arrays using the thermal DW of a Pt-film process previously developed.\textsuperscript{5,24,28} This method begins with physical vapor deposition of a thin, 8 nm Pt film (Thermonics Laboratory, VE-240) on a p-type silicon wafer with 100 nm of thermally grown SiO\(_2\). During the Pt deposition, the deposition rate and the average (mass-based) thickness of the deposited metal were monitored with a quartz-crystal microbalance. The platinum film was then thermally annealed in a 10:1 mixture of argon and hydrogen at 735 Torr in a cold wall furnace (EasyTube 3000, First Nano, Ronkonkoma, NY) equipped with a radiative heat source. During the annealing step, the heat source was set to its maximum power (22 kW) for 8 s, yielding an estimated maximum substrate temperature of 900 °C.

The thermally induced metal-film dewetting created circular masking patterns, which, while nonuniform, exhibited the average diameters and spacing desired. These platinum islands were subsequently used as a selective mask for RIE (Oxford Instruments) was diluted 1:20 in deionized water and then placed under vacuum overnight with a reservoir of 1:10 N-butyldimethylchlorosilane (C4)/toluene, after which the array was taken to ensure that the stirring did not cause any direct impact with the pillar array surface. Subsequently, 5 mL of 1 mM APTES was added to the solution and stirred for an additional 120 min under ambient nitrogen. Then the substrate was removed from the solution and dried under nitrogen.

A layer of tetraethyl orthosilicate (TEOS) was then added. The patent\textsuperscript{29} indicated that the addition of TEOS was to be done in solution, but some heavy polymerization of the siloxane compounds was observed via SEM analysis. This polymerization both caused damage to the pillar arrays and created a nonuniform pillar array surface. To combat this polymerization, the TEOS layer was deposited via a vapor phase by placing the array under vacuum overnight with a reservoir of TEOS. The substrate was then rinsed in triplicate by each toluene, tetrahydrofuran (THF), ethanol, and deionized water. To create a hydrophobic surface for spotting purposes,\textsuperscript{30} the array was placed under vacuum overnight with a reservoir of 1:10 N-butyldimethylchlorosilane (C4)/toluene, after which the array was rinsed for a final time in duplicate with toluene, THF, 90:10 (v/v) water/THF, and deionized water.

**Detection of Uranyl.** A 1000 μg/mL (1000 ppm) stock solution of uranyl nitrate hexahydrate (SPI Supplies) in 5% HNO\(_3\) (High Purity Standards, Lot 604605) was created and diluted to form sample solutions containing 1, 25, 50, 75, 100, 200, 300, 400, and 500 μg/mL uranyl in 5% HNO\(_3\). They were then spotted onto the DW arrays functionalized according to the modified patent procedure, hereafter referred to as “CMPO arrays”, in volumes of 0.2 μL and in triplicate.

The fluorescence images were taken with a Nikon Eclipse LV150 microscope using the 10× objective. The microscope was equipped with a halogen-lamp light source, a multicolor fluorescence cube (DAPI–FITC–TRITC), and a color charge-coupled device camera (DS-2M, Nikon, Inc.) controlled by NIS-Elements software. Fluorescence color (RGB) images with 16-bit color depth per channel were acquired by integrating a sequence of 16 8-bit color images. The blue-light excitation was used with the same collection time (20 s), gain (2), and offset (−500) for both background and sample measurements.

Images were analyzed using ImageJ software. The signal was integrated over a circular area with a diameter equal to the largest spot. This was done by centering the area on each spot and integrating the intensity via ImageJ software. All data were background corrected by subtracting the intensity produced by the surface without uranyl surrounding the spots. The standard deviations and averages of the intensities for each concentration were then calculated and plotted against concentration to obtain a linear relationship.

Upon first evaluation, there was a lack of luminescence and we surmised that the native fluorescence of the \((\text{UO}_2)^{2+}\) was either too weak or being quenched by the system. However, recent work has been done in aqueous samples with Uraplex, a uranium complexant which extends the lifetime and minimizes quenching of \((\text{UO}_2)^{2+}\).\textsuperscript{31} As such, it was decided to use this complexant with our samples. Uraplex concentrate (Chemchek Instruments) was diluted 1:20 in deionized water and then incorporated as 50% of the sample mixtures by volume.

**Functionalization of the Surface for DNA Detection.** Our procedure for functionalization of the pillar surface for the immobilization of DNA is based upon work previously done by First, the arrays were acid-etched in 50:50 sulfuric/nitric acid for 30 min and dried overnight at 80 °C. Then, 0.05 g of CMPO was dissolved in 25 mL of ethanol, at which point pillar arrays were added and the solution was stirred for 90 min. Care was taken to ensure that the stirring did not cause any direct impact with the pillar array surface. Subsequently, 5 mL of 1 mM APTES was added to the solution and stirred for an additional 120 min under ambient nitrogen. Then the substrate was removed from the solution and dried under nitrogen.

A layer of tetraethyl orthosilicate (TEOS) was then added. The patent\textsuperscript{29} indicated that the addition of TEOS was to be done in solution, but some heavy polymerization of the siloxane compounds was observed via SEM analysis. This polymerization both caused damage to the pillar arrays and created a nonuniform pillar array surface. To combat this polymerization, the TEOS layer was deposited via a vapor phase by placing the array under vacuum overnight with a reservoir of TEOS. The substrate was then rinsed in triplicate by each toluene, tetrahydrofuran (THF), ethanol, and deionized water. To create a hydrophobic surface for spotting purposes,\textsuperscript{30} the array was placed under vacuum overnight with a reservoir of 1:10 N-butyldimethylchlorosilane (C4)/toluene, after which the array was rinsed for a final time in duplicate with toluene, THF, 90:10 (v/v) water/THF, and deionized water.

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Yang et al. A schematic of the process is shown in Scheme 1. First, the arrays were acid-bathed and dried as above. Then, the arrays were functionalized with APTES and rinsed as above. The arrays were then soaked in a 10 μg/mL solution of sulfo-N-hydroxysuccinimide-biotin in phosphate-buffered saline (PBS, 1×, pH 7.4) for 1 h at room temperature. After rinsing in triplicate with additional 1× PBS, the arrays were dried under nitrogen and then soaked in 1 μg/mL streptavidin in PBS (1×, pH 7.4) for 1 h at room temperature. Finally, the arrays were rinsed in triplicate with 1× PBS and then dried under nitrogen. To discourage denaturing, the arrays were stored at 4 °C until used.

Detection of DNA. Three single-stranded DNA (ssDNA) 100-base-pair oligomers were designed and obtained from Invitrogen. The first was biotin-terminated so that it could be bound to the pillar array surface via the strong biotin–avidin affinity (K_{affinity} ≈ 10^{15}). The other two oligomers were the exact complement of the first and the complement with a 10-base pair mismatch scattered throughout the strand, respectively. The oligomers were reconstituted in 1 mL of Tris–EDTA buffer (TE, 1×, pH 8). Four different solutions were then created to produce samples with a 5:1 base pair/dye molecule ratio and ∼60 nM (or ∼2 ng/μL) DNA concentrations in TE buffer (a concentration of similar magnitude to that used in quantitative analyses). These solutions consisted of the biotinylated ssDNA (“alone”), the biotinylated ssDNA with its complement (“matched”), the biotinylated ssDNA with the complement containing 10-base pair mismatches (“mismatched”), and a dye blank.

The intercalating dye used for fluorescence imaging was EtBr (Sigma-Aldrich, 1.15 × 10^{-5} M). These solutions were allowed to incubate for 1 h at room temperature before being used or being stored at 4 °C for later use.

DNA solutions were then applied to avidin-functionalized arrays in one of two ways, either by spotting or soaking. For application by spotting, 0.1 μL of sample was applied to the array surface and allowed to dry. The spots were then imaged, rinsed with ∼1 mL 1× PBS buffer, and imaged again. For application by soaking, ∼100 μL of sample was applied to the surface of the array, with surface tension keeping it in a droplet form over the entire array surface, and allowed to soak for 1 h at room temperature. Also, the area of the array was then imaged, rinsed with ∼1 mL 1× PBS buffer, and imaged again. The same Nikon microscope as was used for the uranyl detection was used here but with the green-light excitation (510–560 nm) for EtBr detection. The same collection time (5 s), gain (2), and offset (−500) were used for both background and sample measurements.

Images were analyzed using ImageJ software. The signal was integrated over a circular area with a diameter equal to the largest sample spot. This is done by centering the area on each spot and integrating the intensity via ImageJ software. In spotted arrays, the signal was background-corrected by subtracting the integrated signal within the same-sized circular area where there was no sample applied.
CONCLUSIONS

In this work, we have introduced two surface chemistry approaches for the detection of the uranyl ion and DNA, which are ideal to use as high-throughput screening methods. Both methods consisted of creating a multilayer extraction resin on silicon nanopillar systems. In the case of uranyl, this allowed for the fast analysis of many samples at subppm concentrations using subμL volumes, which, while not as sensitive as other methods using Uraplex, could still be used for screening purposes. In the case of DNA, this allowed for the qualitative differentiation between picomole amounts of single-stranded and double-stranded DNA, including identification of base-pair mismatches. The retention of sensitivity while using minimal sample and reagent volumes and the lack of complex and highly specific instrumentation combine to create a platform, which is both accessible and economically advantageous. Future avenues of research include investigating the pH dependency of the uranyl fluorescence for additional optimization.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00912.

Wafer and array layout with SEM images, functionalization optimization for uranyl studies, and plot profiles of FITC images (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CMPO, N,N-diisobutyl-2-(octyl(phenyl)-phosphoryl)-acetamide; EBL, electron beam lithography; RIE, reactive ion etching; SEM, scanning electron microscope; PSEO, porous silicon oxide; PECVD, plasma enhanced chemical vapor deposition; APTES, 3-aminopropyltriethoxysilane; FITC, fluoresceinisothiocyanate; DW, dewetted; TEOs, tetraethyorthosilicate; THF, tetrahydrofuran; C4, N-butyldimethylchlorosilane; NHS, N-hydroxysuccinimide; PBS, phosphate-buffered saline; ssDNA, single-stranded DNA; TE, Tris–EDTA buffer; EtBr, ethidium bromide

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