Nanoarray fabrication is a multidisciplinary endeavor encompassing materials science, chemical engineering, and biology. We formed nanoarrays via a new technique, porphyrin-based photocatalytic nanolithography. The nanoarrays, with controlled features as small as 200 nm, exhibited regularly ordered patterns and may be appropriate for (a) rapid and parallel proteomics screening of immobilized biomolecules, (b) protein-protein interactions, and/or (c) biophysical and molecular biology studies involving spatially dictated ligand placement. We demonstrated protein immobilization utilizing nanoarrays fabricated via photocatalytic nanolithography on silicon substrates where the immobilized proteins are surrounded by a non-fouling polymer background. Molecular & Cellular Proteomics 8:1823–1831, 2009.

Biomolecular arrays facilitate molecular aggregate investigation and high throughput analysis of immobilized biomolecules. Current biomolecular arraying capabilities are limited by relatively large sample volumes (typical spot sizes are on the order of ~100–200 μm) and relatively long incubation times (1). Despite their limitations, protein microarray applications include autoantibody profiling, antibody response profiling, and identification and detection of bacterial and protein analytes as well as disease proteomics (oncoproteomics) (2–5). Typically a specific analyte is bound and detected by fluorescence, resulting in an expression profile or protein atlas.

Nanoarrays are expected to expand the use of biomolecular arrays beyond drug discovery, medical diagnostics, and genetic testing to include point-of-care and in-the-field applications (6). We present a rapid and low cost photocatalytic lithography method for generating biomolecular nanoarrays on a non-fouling background appropriate for analysis of immobilized biomolecules.

A number of publications have reviewed approaches to nanofabrication and bionanopatterning (7–9) of substrates including photolithography, contact printing (9, 10), imprint lithography (11–13), dip-pen lithography (14–17), and block co-polymer formation (18, 19). These approaches exhibit varying degrees of efficiency and success. Photocatalytic lithography (PCL) is a more recently described technique that is capable of generating arrays. Lee and Sung (20) patterned silane layers by activating TiO₂ (a photocatalytic semiconductor) via 2-min exposure to UV light. They produced ~600-nm parallel lines with 400-nm spaces, and they subsequently performed atomic layer deposition of zirconium dioxide (ZrO₂) onto exposed silanol groups (alternating octadecysiloxane regions did not have necessary precursor molecules). The subsequent decomposition of alkylsiloxane monolayers with TiO₂ was reported to be 20 times faster than under UV irradiation in air.

Previously we have described patterning via porphyrin-based PCL (21). Patterning is achieved within 10 s and with extremely low energy sources (an LED flashlight is sufficient). As presented below, we have miniaturized features patterned with this new technique and are now able to pattern on the scale of 200 nm; this is roughly half the reported scale achieved with PCL using photocatalytic semiconductors. We refer to patterning on this scale as porphyrin-based photocatalytic nanolithography (PCNL). We describe the implementation of porphyrin-based PCNL to form large scale nanoarrays appropriate for rapid, parallel, quantitative proteomics screening of immobilized biomolecules and to form spatially dictated ligand arrays for functional proteomics studies.

EXPERIMENTAL PROCEDURES

Mask Masters—Masters for the nanometer scale structures were fabricated by electron beam lithography using a Raith150 system (Raith GmbH, Dortmund, Germany). After cleaning, the silicon masters were coated with a 264-nm-thick ZEP-520 positive tone electron beam resist (ZEON Corp., Tokyo, Japan) and soft baked for 120 s at 200 °C. Wafers were mounted on a leveled electrostatic chuck. The

1 The abbreviations used are: PCL, photocatalytic lithography; PCNL, photocatalytic nanolithography; TiO₂, titanium dioxide; LED, light-emitting diode; MgPC, magnesium phthalocyanine; P(AAm), polyacrylamide; AFM, atomic force microscopy; SEM, scanning electron microscopy; POP, polylefin plasotomer; RIE, reactive ion etching; UPW, ultrapure water; ATC, allytrichlorosilane; Fn, fibronectin.
resist was exposed to an electron beam at an acceleration voltage of 20 kV using an electron dose of 70 microcoulomb cm$^{-2}$ for the grid pattern in Fig. 1 and using an electron dose of 250 microcoulomb cm$^{-2}$ for the grid pattern shown in Fig. 3. After electron beam lithography exposure, the resist was developed in xylene for 40 s at 20 °C with gentle agitation followed by soaking in 1:3 methyl isobutyl ketone/isopropanol alcohol solution for 30 s and then rinsed with pure 2-propanol for 30 s to stop development.

After lithography, the wafers were soft baked at 90 °C for 2 min immediately before loading into an Applied Materials P-5000 MERIE system for reactive ion etching. RIE was performed in a two-step process. The initial breakthrough step removed the intrinsic native silicon dioxide layer over the silicon substrate using CF$_4$ for 5 s (100 millitorrs, 250 watts, 40 gauss magnetic field). Target depth was 250–300 nm. The ZEP-520 thin film was stripped using Piranha etchant (9:1 H$_2$SO$_4$:H$_2$O$_2$, 120 °C).

**Photomask Fabrication**—Affinity polyolefin plas stereomers (POPs) (22) were used to construct nanoscale masks. These materials are co-polymers of ethylene and R-olefin (butane or octane) and undergo metallocene polymerization, which selectively polymerizes the ethylene and co-monomer sequences. POP pellets (Affinity EGB150 (stiffer) or EGB200 (softer), Dow Chemical Co., Midland, MI) were melted into 40 × 20 × 5-mm blocks at 190 °C under a pressure of 4 bars using an appropriate metal template. Thin polyimide foils were placed between metal and polymer foil to avoid sticking. After cooling to room temperature, the solid polymer bars were removed from the template, rinsed with ethanol, and dried under a stream of nitrogen. In the next step, the bars were placed over the electron-beamed masters (10 × 10 mm) prior to placement between two silicon wafers. This “sandwich” was placed on a heatable plate (heated to 130 °C from both sides). A weight of 200 g was put on top of the sandwich for 5 min followed by a weight of 700 g for 4 min. After cooling, the master was peeled off from the POP bar, which was then cut to its proper size with a razor blade. Prior to usage, the POP masks were cleaned with acetone for 5 min in an ultrasonic bath.

**Substrate Preparation**—Silicon (cut to −1 cm$^2$, Micralyne, Edmonton, Alberta, Canada) substrates were placed in Fluoroware$^\text{TM}$ (Chaska, MN) baskets and sonicated first in UPW, then in 2-propanol, and finally in UPW (each step, 10 min). The substrates were immersed in a Piranha etch bath comprising concentrated H$_2$SO$_4$, 30% H$_2$O$_2$ (5:1 v/v) for 20 min followed by thorough rinsing in UPW. Substrates were individually blown dry under a filtered nitrogen stream and exposed to oxygen plasma (Structure Probe, Inc., West Chester, PA) at 50 mA, 300-millitorr vacuum. As previously described, we drafted allyltrimethoxysilane (ATC; United Chemicals, Bristol, PA), prepared in anhydrous toluene (1.25% by volume) in a glove box purged with nitrogen, to the substrates (21, 23). Briefly immobilization was performed in toluene for 1 min followed by rinsing and a 5-min bake at 120 °C to accelerate bond formation.

**Photocatalytic Nanolithography**—We dissolved 1 mg/ml magnesium phthalocyanine (MgPC; Frontier Scientific, Logan, UT) in ethanol with sonication. A drop of solvated porphyrin was applied to the photomask and blown dry with nitrogen. Masks held by tweezers were carefully placed by hand on top of the ATC-coated silicon chips.

Controlled patterning and removal of the ATC were achieved by local oxidation of the photocatalyst on the topographically patterned POP masks through 10 s illumination of the photocatalyst with an LED flashlight (Restoration Hardware, San Francisco, CA) exhibiting intensity peaks at 455 and 550 nm. Control experiments exposed ATC-coated substrates to photomasks without porphyrin in the presence of excitation energy (light). Selective patterning was not observed. Illumination with 660 nm red LED light (LUMEX, Glenview, IL or Superbright LEDs, St. Louis, MO) also effectively achieved patterning.

Localized patterning and removal of the ATC occurred at locations in close contact to the excited porphyrin on the photomasks, i.e. occurred at elevated areas of the masks that were selectively created from the silicon masters. ATC areas positioned under recessed mask regions remained intact. After patterning, surfaces were sonicated in solvent for 1 min and blown dry with nitrogen. A thin (~15 nm) polyacrylamide film was then grafted to remaining silane.

**Optical Microscopy**—Surface patterning was monitored at each step of the patterning process by exposing the patterned substrates to water vapor (24) and acquiring images with a Nikon D100 camera mounted on a reflectance-based Nikon Labphot 2 microscope. A few images were acquired in quick succession after introduction of water vapor to view differences in surface energy between patterned and background substrate regions.

**Scanning Electron Microscopy (SEM)**—An FEI Co. Quanta 200 environmental scanning electron microscope (Hillsboro, OR) was used in high vacuum mode. Figs. 1 and 2 include micrographs obtained using the secondary electron detector at 1 kV and a spot size of ~100 nm. No staining was necessary to image patterned polymer films; however, contrast was enhanced for the micrographs presented.

**Atomic Force Microscopy (AFM)**—Features on patterned silicon substrates were visualized using a Digital Instruments Dimension 3100 atomic force microscope (Digital Instruments/Veeco Metrology Group, Inc., Santa Barbara, CA) with Si$_3$N$_4$ (DNP-S) probes. Contact mode was used for friction and topography imaging. Image processing was performed with WSXM 2.1 Scanning Probe Microscopy Software (Nanotec Electronica).

**Protein Adsorption**—Fluorescently labeled protein experiments were conducted by first immersing photocatalytically patterned substrates in a 25 μg/ml solution of recombinant protein A (Pierce) in PBS for ~1 h at room temperature. After thoroughly rinsing in PBS, the substrates were immersed in 50 μg/ml fluorescein isothiocyanate-labeled IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in PBS for 30 min. Photocatalytically patterned samples exposed to FITC-IgG were stored in polystyrene dishes sealed with Parafilm and wrapped in aluminum foil to keep out light. Substrates subsequently were rinsed three times in PBS, then rinsed in distilled water, and dried before imaging. Detection of FITC-labeled IgG on surfaces exposed to fluorescently labeled proteins was achieved using a Roper Scientific Photometrics CoolSnap charge-coupled device camera (Tucson, AZ) and Universal Imaging’s Metamorph software (v6.1). Images were acquired at 100× magnification for 10,000 ms and binned 3 × 3. The detector had a well depth of 16 bits. Postacquisition images were analyzed using Image J 1.40g, and contrast was enhanced.

**RESULTS**

**Creation of Nanoscale Arrays**—Micrometer scale PCL results have demonstrated the retained functionality, shape, and spacing of patterns, proteins, and prokaryotic and eukaryotic cells (21, 25). PCL using poly(propylene sulfide)-b-poly(ethylene glycol) on gold substrates controls protein adsorption (26). Our present efforts show that PCL can dictate nanoscale protein patterning, thereby enabling biophysical and molecular biological studies involving spatially dictated ligand placement.

We used the organic semiconductor MgPC to accomplish robust, reproducible, and low cost substrate patterning down to line widths of 200 nm. We created transparent, three-dimensional polymeric photomasks by melting the polyolefin
elastomer POP pellets into silicon masters created with an electron beam. MgPC solvated in ethanol was applied to the patterned side of the mask and blown dry with nitrogen. The masks were placed by hand onto silane-coated silicon substrates.

Light was shone through the transparent, patterned, MgPC-coated photomasks for \( \frac{1}{10} \) s to locally oxidize substrate silane chemistry in close contact with the photomask while leaving substrate silane chemistry disposed more distant from, and along recessed regions of, the patterned photomask intact. Our chosen silane chemistry was unsaturated so that we could graft polyacrylamide (P(AAm)) to retained matrix silane in a subsequent step to create a passivating background for protein nanoarray fabrication (Scheme 1). We used a suite of techniques to characterize masters and photomasks, pattern resolution, and homogeneity as well as the ability to pattern protein on the nanoscale.

Porphyrin-based PCNL utilizes materials with interesting physical properties and low material costs. The porphyrin MgPC is known in biotechnology as a photodynamic therapy molecule (27) and in nanotechnology as a gas-sensing material (28); it is also used in solar cells (29–31). Advantageously, MgPC absorbs light in the visible spectrum and can be solvated, making it preferable to other semiconducting photocatalysts used for lithography, such as TiO\(_2\) (20, 32, 33).

**Corroborative Feature Characterization**—With reference to Fig. 1, we provide an assembly of AFM, optical microscopy, and low voltage SEM images that reveal the integrity and reproducibility of 600-nm line width silicon lines surrounded by a matrix of P(AAm) chemistry using PCNL. AFM of the POP mask is shown as a contact mode surface plot in Fig 1A. Fig. 1B shows the friction mode image of the photocatalytically patterned P(AAm)/silicon substrate. We found that friction mode provided more contrast than height mode in our contact mode force microscopy experiments.

An optical image acquired at 500× is depicted in Fig. 1C. This image was acquired after lightly hydrating the P(AAm)/silicon patterned surface. As described by López et al. (24), condensation of a vapor to a liquid correlates with the molecular structure of the surface. Thus, light hydration is a fast, inexpensive, and non-destructive technique that provides a valuable method for characterizing chemically patterned surfaces. In our case, the P(AAm) hydrogel absorbed water preferentially over the silicon lines and thus resulted in strong (time-dependent) optical contrast. Low voltage SEM was performed at 1 kV to convey the secondary electron contrast.
between the P(AAm) matrix and the semiconducting silicon lines (Fig. 1D, "L" boxes).

We also patterned features across a range of line widths and 160:1 two-dimensional aspect ratios (Figs. 2 and 3). The silicon slits surrounded by a P(AAm) matrix depicted in the low voltage SEM in Fig. 2 ranged from 2.4 μm to 320 nm in line width. Squares surrounded by a similar matrix ranged from 1.7 μm to 340 nm in diameter. Because we performed microscopy at 1 kV, we did not need to coat our substrate. The P(AAm) matrix provides contrast against the silicon features because of greater interaction of carbon, nitrogen, and oxygen atoms with the secondary electrons from the beam as
compared with the interaction of the silicon atoms with the secondary electrons from the beam.

Nanoscale Protein Arrays—Fig. 3 depicts AFM (A), computer-aided design drawing (B), and fluorescent imaging (C) of both a field of 500 nm-diameter spots and of various features, including a modified version of the Paul Klee work “Lady Apart.” The fluorescent protein spots demonstrate feasibility of a direct immunoassay architecture, immobilizing protein and then probing it with labeled antibody (commonly used in proteomics arrays). This construct has been used by Pawel et al. (34) and Madoz-Gülpide and co-workers (35–37) for microarray-based cancer research and has been documented by Pollard et al. (38) for clinical proteomics. A signal to noise ratio of 1.94 was calculated using the histogram tool in Image J. Signal from $n = 12$ spots from 16 pixel regions of interest was equal to 160.6 ± 26.4. Background signal from $n = 12$ spots from 16 pixel areas between regions of interest was equal to 82.8 ± 22.7. Scale was from 0 to 255.

The modified Lady Apart figure demonstrates the ability to traverse the micrometer to submicrometer scale within the length and line width aspects of the figure. The figure is almost 30 μm tall, but the lines are only 200 nm wide. The broad, dynamic range of pattern size and the spanning of such length scales have been problematic in the past because of mask collapse (39). As noted, this work was done by placing masks onto substrates by hand; yet line widths of magnitude similar to those found in state-of-the-art integrated circuit chips were achieved.

Our results in Fig. 3C demonstrate nanoscale protein arraying. Subsequent to PCNL, P(AAm) was grafted and cross-linked to the remaining silane, resulting in a patterned substrate of a non-fouling P(AAm) matrix and adhesive silicon/SiO₂ features. Protein nanoarrays then were formed by first bathing the substrate in a single concentration of protein A and then a single concentration of FITC-IgG solution in buffer. As our present goal is to demonstrate protein immobilization utilizing nanoarrays fabricated via PCNL, we intentionally conducted experiments with a single protein concentration and labeling molecule concentration where fluorescence saturation makes it easy to see the strikingly small features.

Although we did not attempt to multiplex our initial demonstration, we note that others have used Teflon barriers to subdivide regions, allowing numerous interactions to be tested (40). Teflon barriers would be difficult to fabricate and assemble with submillimeter accuracy. Although the PCNL technique does not easily allow for side by side (less than 1-μm spacing) placement of different proteins, we envision etching silicon or glass substrates before any chemistry has been performed to set up micrometer scale physical boundaries. Within such boundaries, large nanoarrays or small microarrays then may be chemically fabricated in conjunction with PCNL to allow for reproducibility testing per protein as well as protein multiplexing.

DISCUSSION

Formation of patterned substrates via PCNL provides a platform for protein nanoscale arrays. Such nanoarrays could significantly advance the capabilities of, for example, quantitative proteomics wherein agents capture target proteins from complex mixtures for protein detection and quantification. Quantitative proteomics typically utilizes protein-detecting microarrays with spot sizes on the order of 100 μm (1, 41). Protein nanoarrays cover less than 0.1% of the surface area of today’s microarray spots while maintaining enough antibodies to provide a useful dynamic range. PCNL is well positioned to assist in the transition from microarray to nanoarray research and may be used to obtain global proteome analysis or even small scale, on-chip bioreactors.

Lynch et al. (42) describe the useful limit of an individual antibody capture domain to be about 250 nm, which is on the scale of our protein arraying results described below. Lynch et al. (42) point out that although even smaller, single molecule...
detection systems are seductive they are subject to statistical time because of the necessity for repeated sampling to obtain a diagnostic readout (below a threshold size, array spots lack an adequate number of active capture molecules and may, therefore, suffer from inaccurate quantitation and poor dynamic range (42)).

Using fluorescence microscopy, we were able to detect the presence of protein down to 200 nm line widths (Fig. 3C). Signal from the FITC-IgG, bound to proteins on silicon, was easily detectable. Regular periodicity of 500 nm spots also was readily detectable. Although the emission region on the sample is not spatially resolved (emission is resolved as a larger recorded line width with high contrast over background), the presence of 200 nm line width features is clearly ascertained in the AFM images in Fig. 3A. Although such an approach of using a single concentration of protein may be relevant to protein-protein interactions or nanoscale ligand placement, we point out that concentration range and linear detection range analyses will be necessary to enable proteomic chip studies with the PCNL technique and proteins of interest. However, conventional microscopy cameras and detectors are not sufficient to complete such a study with spot sizes primarily between 200 and 500 nm, and therefore these experiments are not straightforward to conduct. Fluorescence microscopy performed on 200–500 nm spot sizes can present a detection problem because of limited signal if the recorded detector counts are not well above background. With spot sizes this small, cryogenically cooled detectors are required to image fluorescence in a quantifiable way. We plan a future series of studies that include a range of protein concentration and analysis of the linear detection range. For this set of experiments, we will appropriately design masks with spot sizes of ~600–800 nm so that the results are relevant to researchers with conventional biomedical imaging equipment while still providing significantly higher density arrays than are available today.

One of the goals of proteomics research is to understand how proteins interact with each other and with other biomolecules to control processes at the cellular, tissue, and whole organism level. Biomolecular arraying allows for molecular aggregate investigation (focal adhesions serve as one example of hierarchically organized cooperation among proteins) as well as for high throughput protein analysis. The effect of spatially positioning signaling cues at different length scales on cell response is a key question in the other branch of proteomics known as functional proteomics. As with quantitative proteomics, PCNL is positioned to advance functional proteomics studies.

Synergistic interaction studies by Elbert and Hubbell (43), Irvine et al. (44, 45), Koo et al. (46), Maheshwari et al. (47), Lussi et al. (48), and Arnold et al. (49) provide initial insight into cell response to spatially positioned signals and suggest research avenues for directing cell adhesion through placement of nanoscale peptide and protein ligands. Cell adhesion and motility may be governed by growth factor receptors and integrin adhesion receptors interacting with the extracellular environment followed by collaborative intracellular signaling (50). There is clearly a complex synergy between receptors and integrins, and modeling shows that spatial ligand presentation affects cell regulation behavior (51).

We believe that PCL/PCNL is well suited for functional proteomics studies as it can traverse the micrometer to nanoscale patterning requirements incumbent in the design of substrates for such studies. We have previous experience creating cell arrays on the micrometer scale with PCL (21). Now that we have patterned on the nanoscale, we are positioned to control cell arrays on a much smaller scale by placing small molecule ligands or ligand clusters beneath patterned cells.

As seen in Scheme 2, we envision a three-step process. First, porphyrin-based PCL exposes silicon substrate regions ranging from 20 μm (for single cell attachment) to 100 μm (for cell cluster attachment) on substrates homogenously coated with non-fouling chemistry, such as P(AM), poly(acrylamido-co-ethylene glycol) (23), or poly(L-lysine)-g-poly(ethylene glycol) (52, 53). Second, silicon regions are coated with fibronectin (Fn). Third, PCNL, performed with a secondary mask and a mask aligner, re-exposes a majority of the silicon, leaving nanoscale regions of fibronectin on cell-adhesive silicon patches surrounded by non-fouling matrix. In such a way, PCL/PCNL may facilitate multidimensional parameter testing on the same substrate to examine cooperativity not just among proteins but among cell contacts as well.

PCNL allows for patterning across multiple orders of magnitude, for a range of substrates, and in an ambient environment with little energy input. However, we have found that, similar to contact printing techniques, lateral diffusion of materials not strongly bound to substrates can deleteriously influence pattern resolution. Smaller, tightly bound materials and covalently bound materials perform better than larger, more loosely bound materials, which may result in pattern blurring.

Also similar to contact printing techniques, mask modulus influences pattern resolution. Although the polyolefin elastomer POP proved sufficient for line widths down to a few hundred nanometers, future work should investigate new materials, such as polyanides or low viscosity monomers that may be UV light-cured to fabricate higher modulus, transparent masks that should enable patterning of spotted sizes 50 nm or smaller.

We note that all work done in our laboratory to date has utilized physical placement of mask materials on substrates by hand. We expect that the use of mask aligners to interface the mask and substrate would facilitate the higher resolution (estimated at less than 50 nm) that we believe is attainable with PCNL. Furthermore all work done to date has relied on a thin layer of photosensitizer applied to masks via a volatile solvent film. We have performed preliminary experiments embedding photosensitizer within the masks and have found that
(on the micrometer scale) they retain their function. We believe this may present a way of minimizing potential substrate contamination.

Biological applications for nanoarrays are increasing in both academia and industry, and biotechnology now plays a notable role in the chip fabrication market. Ariga et al. (54) and Truskett and Watts (13) suggest that biotechnology is the future of nanoscale methods and applications. We have demonstrated that porphyrin-based PCNL is a rapid, low cost lithographic technique for nanoscale protein arraying on a non-fouling background, and we have reproducibly patterned protein on 200 nm diameter features. Porphyrin-based PCNL expands present nanoarray fabrication and proteomics study capabilities.

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This paper is dedicated to one of the fathers of biomaterials, Professor Eugene Lautenschlager.

§ The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

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