Characterization of Patterned Self-Assembled Monolayers and Protein Arrays Generated by the Ink-Jet Method†

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Commercial ink-jet printers were used with little modification to deposit alkanethiols onto gold substrata and several proteins onto silica supports. The resulting patterns of alkanethiols form self-assembled layers comparable to those obtained by microcontact printing or solution adsorption. The method has been used successfully to create binary chemical gradients and patterns of tertiary functionality. The proteins form dense patterns on the substrates and seem to maintain their configuration as measured by their ability to bind their specific ligands. Four different proteins were printed simultaneously, allowing for positive and negative controls. This "drop-on-demand" printing method is an inexpensive, flexible alternative to current binary technologies of chemically functionalizing surfaces.

Introduction

Ink-jet technology has been used for over 20 years to control delivery of nanoliter volumes of liquid to defined locations onto surfaces. Examples of patterning of thin films include dip-pen nanolithography,[1] capillary gel stamping,[2] and direct ink deposition.[3] Ink-jet devices for the consumer market combine a static pressure ink reservoir, a small diameter orifice, and a voltage-gated orifice that is positioned using a two-dimensional translation mechanism. While the orifice determines the volume of liquid dispensed, the relative position of the nozzle is sensed by an encoder, which also determines the lateral resolution of the device. Drop-on-demand (DOD) devices have recently been used for creation of ceramic pillar arrays,[4] deposition of gold conductive tracks,[5] deposition of optical microlens arrays,[6] and deposition of polymers for microelectronics applications.[7] They have recently been used for manufacture of DNA microarrays, be it by a step-by-step synthesis of DNA on a chip[8] or the subsequent synthesis of DNA and immobilization on a surface.[9]

The ink-jet technology has the advantages of being inexpensive, flexible, simple, desktop computer controlled, and fast. DOD printers may prove advantageous over competing technologies such as pin arrayers or microcontact printing because of their speed and high-throughput nature. Furthermore, DOD printers deliver a liquid drop that in the case of aqueous solutions may not dry until after the printing process is finished. The ability to work with hydrated or solvated molecules makes this technique extremely versatile, allowing for example the deposition of easily denaturing proteins or living cells. However, this comes at the expense of larger drop feature sizes as compared to pin arrayers or microcontact printing.

Additional advantages over microcontact printing or the more expensive photolithography are that multifunctional surfaces can be created easily, by using a multitude of nozzles. Commercial printers use 4 or 6 nozzles, but designs of up to 200 nozzles have been investigated. Furthermore, mixing of the feed molecules is possible by simultaneously actuating several nozzles, thus allowing chemical gradients to be created with relative ease.

Although the technology has been investigated for synthesis of DNA, proteins, and other chemicals, relatively few studies addressed the surface chemistry effects of jet printing of proteins. In this article, we report the characterization of patterned films on surfaces. The initial experiments focused on well-understood model systems, that of the self-assembled monolayers (SAMs) of alkanethiols on gold and of biotin−streptavidin patterns. The self-assembly of alkanethiol monolayers onto gold has been studied extensively due to its utilization in a myriad of applications ranging from electrically conducting molecular wires[13] to corrosion protection[14,15] to molecular recognition[16,17] and protein adsorption studies.[18]

Experimental Section

Printer Design. Two printers were designed to print the SAMs and protein arrays. The first printer was designed to print protein solution to solid surfaces of any thickness less than 2 in. The body of the printer is made from poly(methyl methacrylate)

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with printing components (print head, logic board, encoder, etc.) stemming from a HP 660C printer (Hewlett-Packard, Palo Alto, CA). Based upon the technical drawings for the original printer, the new printer was designed using new gear mount pillars with closer tolerances by adding a horizontal support, changing the transistor in the circuit to one with higher amplification, and reentering the horizontal position encoder.

The second printer, based on a Cannon BJC 2100 (Cannon USA Inc., Lake Success, NY) was designed to accommodate samples with sizes up to those of typical microscope slides (2.5 × 1 in.). The modifications were limited to removing the rubber rolls and removing the center springs while tightening the remaining springs, which are used to advance the paper. For both printers, the samples are fixed to a piece of paper using double-sided tape.

The software drivers were rewritten to allow different viscosities of protein solutions to be printed. The new driver software constantly adjusts the voltages to the nozzles to account for different impedances of the solutions, thus allowing the appropriate amount to be dispensed. Other software modifications include lowering the resistive voltages to avoid heating of the solutions above 37 °C. The modified drivers for the HP 660C and Cannon BJC-2100 are in the public domain and can be downloaded from the manufacturers' or authors' Web sites.

Surface Preparation. Surfaces for alkanethiol printing were freshly evaporated gold samples, made by e-beam evaporation of 100 Å of chromium followed by 2000 Å of gold in a 10^{-7} Torr ion pumped deposition chamber (Varian Inc., Palo Alto, CA). Silicon and glass surfaces were cleaned by immersion into piranha solution (60% H_2SO_4/40% H_2O_2 [30%]) for about 20 min. The surfaces were analyzed by fixed wavelength ellipsometry (Autoel III, Rudolph Technologies Inc., Flanders, NJ) immediately after preparation.

Printing Solutions. The printer cartridges were emptied and thoroughly rinsed with deionized water (Barnstead Nanopure II, Dubuque, IA) between 50 and 100 times to ensure no ink was left in the reservoir. The cartridges were then rinsed alternatively with ethanol and water several times. For SAMs, three cartridges were filled with recrystallized ω-substituted alkanethiols, namely, $HS(CH_2)_{15}X$, with $X = CH_3$, CO_2H (Aldrich Chemical Inc., Milwaukee, WI), and CH_2OH (gift from David L. Allara) each dissolved in pure ethanol (Aaper Alcohol and Chemical Co., Shelbyville, KY) at millimolar concentrations. The alkanethiols will hereafter be referred to as hexadecanethiol (HDT), mercaptohexadecanoic acid (MHA), and mercaptohexadecanol. For the protein experiments, the four cartridges of the BCJ-2100 were filled with 1 mg/mL solutions of bovine serum albumin (BSA) (Sigma Chemical Inc., St. Louis, MO), biotinylated BSA (biotin−BSA) (Sigma Chemical Inc.), streptavidin (Sigma Chemical Inc.), and Lucifer yellow labeled biotin (Y-biotin) (Molecular Probes, Eugene, OR). Each of the solutions was prepared in pH 7.4 phosphate buffered saline solution (PBS) (Sigma Chemical Inc.).

Patterns were designed using Microsoft PowerPoint software (Microsoft Inc., Redmond, WA). The color scheme was adjusted to print the desired compounds from their respective cartridges. After printing, the samples were dried, rinsed, and analyzed. For comparison, patterned surfaces were also prepared by microcontact printing (μ-cp). A poly(dimethylsiloxane) (PDMS) stamp with a smooth surface was prepared by pouring the siloxane Sylgard 184 (Dow Corning, Midland, MI) and curing agent according to the manufacturer's instructions into a Petri dish and allowing the polymer to form within a period of 24 h. Excessive rinsing in acetone, chloroform, ethanol, and Nanopure water followed this. A drop of the hexadecanethiol solution was placed on the stamp and allowed to dry under nitrogen. The stamp was then handpressed onto a freshly prepared gold substrate for 1 min and then removed. Both stamp and surface were copiously rinsed with ethanol immediately. For comparison with SAMs obtained through solution adsorption, fresh gold was immersed in 1 mmol ethanolic solutions of the SAM of interest for a 24 h period, removed, and rinsed copiously with ethanol.

Surface Analysis. The resulting surfaces were analyzed by FTIR with a glancing angle attachment, ellipsometry, contact angle, condensation figure inspection, and optical microscopy. All spectral measurements were performed on a Nicolet 550 FTIR spectrometer equipped with an external reflection accessory (Harrick Scientific, Ossining, NY). Spectral measurements were acquired at 2 cm^{-1} resolution with 500 scans per sample conducted on bare gold and SAM surfaces, in p-polarization and in a nitrogen-purged environment. Freshly evaporated gold samples cleaned in an UV ozone cleaner (UV clean, Boekel Industries Inc., Feasterville, PA) were used to obtain reference spectra. Condensation figures were obtained by blowing water-saturated air through a glass pipet onto the sample and recording the resulting images with a digital camera. Contact angles were measured using an automated analyzer with a CCD camera (Cam 200, KSV Instruments Ltd., Helsinki, Finland), fitted with drop shape analysis software, which was used to determine the angle between the surface and the 8−15 μL drops of Nanopure water. Thickness measurements were carried out by a Rudolph Research AutoEL III ellipsometer equipped with a He−Ne laser ($\lambda = 632.8$ nm) as the light source. The light was incident at an angle of 70° relative to the surface normal. The film thickness calculations were based on a three-phase ambient/film/substrate model and assigned a scalar refractive index of $n = 1.50$ and $k = 0$. The thickness measurements were converted into the mass of adsorbed protein assuming a density of 1.3 g/cm^3. The beam of the ellipsometer, which averages over several hundred microns, was placed onto a printed pattern with millimeter or larger dimensions. The reported values are thus estimates of the average protein deposited using the ink-jet method. Contact angle and ellipsometry measurements were recorded from three different locations on the sample, and data was analyzed for significance using the two-tailed paired Student's *t*-test.

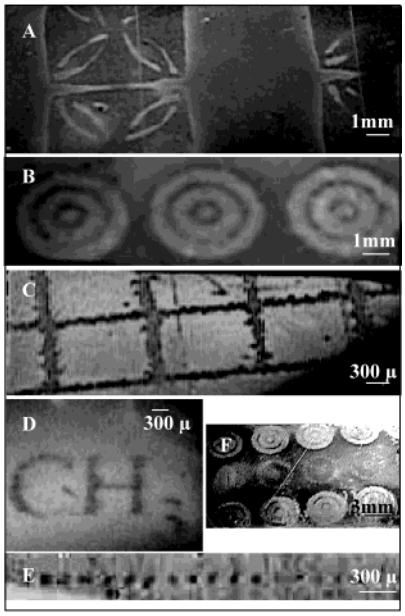

Figure 1. Schematic diagram of the DOD printer and condensation images of alkanethiol patterns deposited onto gold. The patterns include arcs and concentric circles of HDT (images A and B; the scale bar is 1 mm), a grid pattern, a "CH_3" pattern, and a single drop pattern of HDT (images C, D, and E; the scale bar is 300 μ), and a pattern of concentric circles of HDT and mercaptohexadecanol (image F; the scale bar is 3 mm). All samples were backfilled with MHA.

Results and Discussion

SAMs. A schematic of the experiments is shown in Figure 1. Many different patterns were transferred onto clean gold using the HDT solution. After rinsing, the samples were immersed into the MHA solution. Figure 1 shows a series of representative patterns visualized by the condensation method. The HDT SAM is visualized by the dark areas since the water does not as easily condense on these hydrophobic areas. As indicated by the scale bar, drops as small as 100 microns were transferred onto the gold (Figure 1E). Resolutions in the order of 100−300

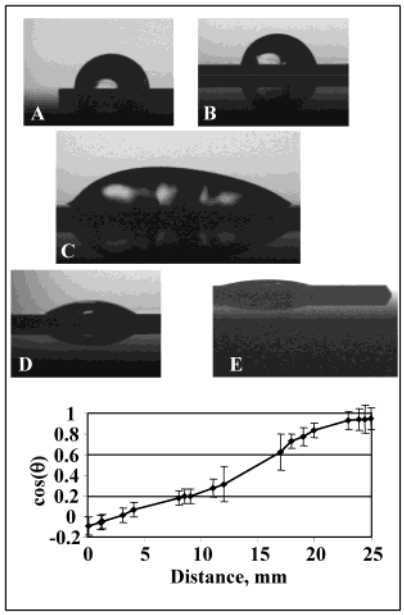

Figure 2. Images of advancing water drops (15 μL, image C; 8 μL, all others) deposited along a gradient of HDT and mercaptohexadecanol (A−E). The graph below is representative of the surface energy along the gradient plotting cos(θ) as a function of distance from the sample edge.

microns are also observed for the circular or square patterns. The sample shown in Figure 1F depicts three rows of concentric circles, created by depositing HDT, mercaptohexadecanol, and HDT, respectively, and backfilling with MHA, thus creating a tertiary surface chemistry. The resolution of ~100 microns falls in the range expected from the nozzle diameter and the resolution of the original printer. As discussed later, the resolution depends on the drop size and liquid/solid surface interfacial tension and may therefore be improved by choosing smaller nozzles. Print feature reproducibility is very good, as the constant spacing between the grid pattern of Figure 1C suggests. The spacing and alignment of the single drops in Figure 1E vary somewhat, possibly due to slight sample movement during printing. Concerning the repeatability of the pattern, we observed that the orifice nozzles did not lose their quality due to clogging by the alkanethiol solutions. We estimate the number of repeated orifice firings before the print quality is compromised to be in the thousands, since we routinely produce SAMs on letter size films or foils. The number of different printing cycles, however, varies with each cartridge; we have received best results when the nozzles were not allowed to dry out between cycles by keeping them in solution.

Another example of the versatility of the drop-on-demand method is the creation of chemical gradients. To achieve such a gradient, the spatial density of HDT drops was continuously changed along 2.5 cm of a gold surface using the Microsoft PowerPoint graphical interface. Upon drying, the sample was backfilled with mercaptohexadecanol and then rinsed with pure ethanol and dried. Figure 2 shows a series of contact angle pictures obtained along the modified gold surface. As can be seen from the graphical representation of the surface energy with respect to the distance from the sample edge, there is a gradual change in the surface energy along the gradient. DOD may be particularly useful for creation of surface gradients because of its simplicity compared to the gradient methods using a polysaccharide matrix deposited on top of a gold substrate,[19] a scanning tunneling microscope for replacement lithography,[20] or photoimmobilization techniques.[21] Furthermore, the ability to create patterns and gradients of controlled chemistry using the simple and reproducible DOD method may be very useful for directing cell adhesion, spreading, and proliferation.[22]

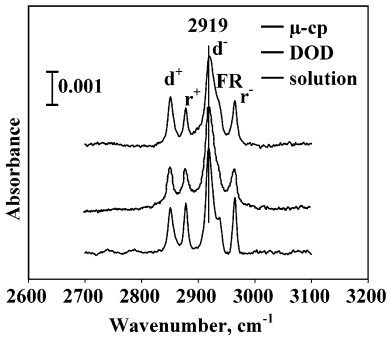

Figure 3. Infrared spectra (2 cm^{-1} resolution) showing the spectral features of the SAMs. The C−H stretching mode spectra of the $H_3C(CH_2)_{15}S$−/Au SAM are shown. The d^+ (CH_2 symmetric), r^+ (CH_3 symmetric), d^- (CH_2 antisymmetric), FR (Fermi resonance), and r^- (CH_3 asymmetric) mode peaks are indicated along with the mode of fabrication.

An FTIR comparison study between the SAMs prepared by DOD, μ-cp, and solution adsorption is shown in Figure 3. For this study, drops of the HDT solution were uniformly deposited on the entire sample using the printer. Similarly, the entire PDMS stamp was inked with HDT and employed to transfer the SAM onto a gold surface. The figure shows C−H stretching mode spectra of the $H_3C(CH_2)_{15}S$−/Au SAM along with the d^+ (CH_2 symmetric), r^+ (CH_3 symmetric), d^- (CH_2 antisymmetric), FR (Fermi resonance), and r^- (CH_3 asymmetric) mode peaks. Although small differences between the spectra are observed as discussed later, the SAMs obtained by the DOD method are at least of comparable quality to the μ-cp SAMs.

SAM Feature Size and Structure. The SAM feature size using the DOD method depends on the drop size and the interfacial tension between the drop and substrate. The choice of ethanolic alkanethiol solutions on gold surfaces was primarily motivated by their use as a well-studied model system, rather than the size of the drops or their spreading on the gold surface. Furthermore, the choice of the 37 °C temperature restriction was based upon the possible use of the device for biological applications, although it comes at the expense of larger drops. Nevertheless, the feature size in these experiments of about 100 microns may suffice for many biological applications such as microarrays or cell arrays, which have typical spot sizes of several hundred microns. Employing different solvents, other noble metals, smaller gate times, or higher temperatures may potentially reduce the feature size.

The structure of the HDT SAMs obtained by the DOD method compares favorably with those obtained by microcontact printing in our laboratory but somewhat less favorably with those of solution-based SAMs. In a comparison of the d^- peak position (2919 vs 2020 cm^{-1}) and width (22 vs 27 cm^{-1}) of the DOD and μ-cp SAMs shown in Figure 3, the DOD SAM is somewhat more uniform and crystalline. Although these differences are

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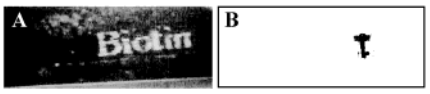

Figure 4. Images of the protein printing experiment. Three successive passes will print first BSA and biotin−BSA according to the yellow and red pattern, then streptavidin, and finally Y-biotin. Each passage spells out the word "biotin" on identical positions on the glass slide. (A) Optical image after the initial deposition. (B) Epifluorescent image after the final deposition and rinse of yellow-labeled biotin.

small, the most striking difference between the spectra is the lower d^- intensity coupled with an increased d^+ intensity of the μ-cp sample when compared to the DOD sample or the solution-based SAM. This has been attributed to a higher twist in the alkanethiol chain.[23] The μ-cp sample also shows significantly reduced d^- intensity, pointing toward an increased overall tilting of the alkane chains as compared to the other two samples.

The d^- intensity of the DOD sample is comparable to that of the solution-based SAM, but the r^- and r^+ intensities are reduced, indicating a larger overall tilt of the molecules compared to that in the solution-based SAM. Since this larger tilt is accompanied by a dense crystalline packing, it is reasonable to conclude that DOD SAM packing on the gold surface is somewhat less than the ideal $\sqrt{3} \times 11$ overlayer but that the lower surface coverage is compensated for by a slight tilting of the alkane chains toward the surface. One possible explanation for the lower coverage is that the DOD SAMs are rinsed immediately after the printing is completed, allowing at best several minutes for the formation of the SAM. Previous studies have found that the kinetics of SAM formation is complicated and although a majority of the SAM is formed after seconds, complete coverage approaching the ideal overlayer may take several days depending among other factors on the temperature and crystallinity of the gold.[24]

Proteins. The pattern "Biotin" (size 12 font) was selected for the protein printing. The pattern was designed so that the letter "t" was printed with the cartridge containing biotin−BSA while the remainder of the pattern was printed with the cartridge containing pure BSA. For the next subsequent pass, the cartridge containing streptavidin was addressed, thus transferring it to the entire pattern, and finally for the last pass, the Y-biotin cartridge was addressed, thus transferring the fluorescent agent to the entire pattern. Between passes, the slides were rinsed with PBS buffer solution. Figure 4 shows an optical image of the pattern after the initial transfer and after the final rinse. The fluorescently labeled biotin is clearly visible. Ellipsometry measurements indicate that about 1890 ± 20 ng/cm^2 of protein is initially deposited, which was reduced to about 190 ± 10 ng/cm^2 after rinsing. These data suggest that most of the proteins are physisorbed on top of each other and easily rinsed away, while the proteins with surface contacts most likely remain after rinsing. The figure is somewhat lower than that of a monolayer coverage which is expected to remain on the surfaces after rinsing[25] and may possibly be increased by allowing more time before rinsing as longer adsorption times are often correlated with tenacity of the protein binding.[25]

Protein printing is another application of the DOD method. As the images in Figure 4 show, different proteins may easily be immobilized in patterns using this technique. If the amount of protein deposited is not crucial, such as in the example presented here, the DOD technique is simple and effective. However, for deposition of a known quantity of protein, careful calibration may be necessary. In the example shown in Figure 4, the 1 mg/mL BSA solution resulted in 1800 ng/cm^2 of protein being deposited. For monolayer deposition ($\sim$400 ng/cm^2),[25] one may need to reduce the solution concentration by a factor of 4−5. Nonspecific binding is negligible in the experiments, as there is no fluorescence observed where nonbiotinylated albumin is printed. This is attributed to the rinsing step, during which all nonspecifically bound dye is removed.

The DOD method may have applications in the area of protein microarrays. The most common use of the microarrays is for microimmunoassays, in which different antibodies are arrayed on a surface and subsequently exposed to an unknown sample. Binding of proteins to the immobilized antibodies is then detected by a variety of optical techniques such as fluorescence, luminescence, and so forth. The DOD method may also be employed for other protein-binding compounds such as synthetic proteins,[26] RNA or DNA,[27] allosteric ribozymes,[28] peptides, and other small biological molecules.[29] DOD may provide an alternative to pin arrayers[30] which have been used as a platform for cDNA arrays as well as protein arrays.[31,32]

The DOD delivery mechanism has several advantages over μ-cp or spotting techniques. These include the ability to work with hydrated samples, the simplicity of the technique, the possibility of using many types of organic molecules or proteins, and its high-throughput nature. The ability to work with hydrated samples is important when using proteins that are highly susceptible to denature upon drying. The technique may be used for membrane-bound proteins by using liposomes, thus arraying membrane proteins that are otherwise difficult to immobilize in patterns. Furthermore, a striking difference between DOD and μ-cp or spotting techniques is that the molecules are delivered without contact between the substrate and the device. This may be important in cases where contamination is to be minimized[33] or sterile conditions are called for. On the other hand, the microfluidic channels of the DOD cartridges need to be maintained properly by excessive cleaning to prevent clogging and contamination problems. Since proteins have been shown to adsorb to the walls of microfluidic channels, cartridges may be replaced regularly. Finally, DOD is fully computer controlled, allowing for repeated high-throughput patterning.

Conclusions

With little modification, commercial ink-jet printers have been used for DOD patterning of alkanethiols on gold. The resulting SAM structures compare favorably with those of microcontact-printed samples. The feature size is above 100 microns, which may be sufficient for

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many biological applications of patterned SAMs. The DOD technique allows for deposition of up to four different solutions simultaneously, and thus multifunctional surfaces, which may include binary or higher gradients, can be created with relative ease.

The ability to print proteins and other hydrated samples opens the possibility of generating inexpensive and dense bioarrays such as phage libraries, bacterial artificial chromosome libraries, or cell-based sensors. Systematic protein or cellular assemblies may become possible using the DOD approach.

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