COMMUNICATION TO THE EDITOR

Adsorption of Avidin on Microfabricated Surfaces for Protein Biochip Applications

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Abstract: The adsorption of the protein avidin from hen egg white on patterns of silicon dioxide and platinum surfaces on a microchip and the use of fluorescent microscopy to detect binding of biotin are described. A silicon dioxide microchip was formed using plasma-enhanced chemical vapor deposition while platinum was deposited using radiofrequency sputtering. After cleaning using a plasma arc, the chips were placed into solutions containing avidin or bovine serum albumin. The avidin was adsorbed onto the microchips from phosphate-buffered saline (PBS) or from PBS to which ammonium sulfate had been added. Avidin was also adsorbed onto bovine serum albumin (BSA)-coated surfaces of oxide and platinum. Fluorescence microscopy was used to confirm adsorption of labeled protein, or the binding of fluorescently labeled biotin onto previously adsorbed, unlabeled avidin. When labeled biotin in PBS was presented to avidin adsorbed onto a BSA-coated microchip, the fluorescence signal was significantly higher than for avidin adsorbed onto the biochip alone. The results show that a simple, low-cost adsorption process can deposit active protein onto a chip in an approach that has potential application in the development of protein biochips for the detection of biological species. *Biotechnol Bioeng* **73:** 324–328, 2001.
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INTRODUCTION

In the recent years, there has been a merger of microelectronics and the biological sciences for the purpose of developing microscale biosensors, or biochips. The principle of microscale detection of a specific target cell, protein, or biomolecule is based on binding of the target to its antibody or other receptor fixed to the surface of the chip, once it is fabricated. The receptor must not only be fixed to the chip's surface which might consist of an insulator (e.g., oxide) and a metal (e.g., platinum), but its ability to selectively bind the target from a mixture of other species must be retained. Binding of the target to the receptor must occur in a manner so that fluorescence microscopy or other means can detect the binding event. The chip itself is designed and fabricated to contain micron-scale channels through which the fluid containing the target molecules can be introduced to micron-sized wells containing the receptors. These devices are called biochips because they resemble a computer chip and consist of micron-scale structures to which biological molecules are fixed. The term biochip has been used in various other contexts, but can be defined as "microelectronic-inspired construction of devices that are used for processing (delivery, analysis, or detection) of biological molecules and species." Such devices have been used for the interrogation of electronic properties of cells (Ayliffe et al., 1997; Borkholder et al., 1996), microscale capillary electrophoresis (Harrison et al., 1996; Woolley et al., 1995), and optical detection of hybridization of DNA using fluorescence signals in commercially available "DNA-chips" (Fodor et al., 1991; Heller, 1996). As the human genome decoding is completed, studies on proteomics and the functionality of proteins will accelerate and protein biochips could play a critical role.

The attachment of proteins on microfabricated surfaces will be vital to the success of protein biochips. Clearly, much needs to be learned from prior work involving proteins at metal surfaces and electrodes (Fukuzaki et al., 1996; Roscoe and Fuller, 1992). Although the attachment of antibodies and proteins has been demonstrated on microfabricated surfaces using functional groups such as silane (Brit-

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land et al., 1992; Mooney et al., 1996), amine (Nicolau et al., 1998), carboxyl (Williams et al., 1994), and thiols (Lahiri et al., 1999), and for 12-mer peptides with respect to GaAs (Whaley et al., 2000), it is still desirable to devise techniques to attach the proteins on chip surfaces while minimizing the number of chemical processing steps and hence the total cost of the system. In this study, we report on the preliminary experimental results of attachment of avidin on microfabricated oxide and platinum surfaces using direct adsorption. The adsorption process maintains the activity of the protein, as demonstrated by subsequent biotin binding. Fluorescence microscopy is used to validate the attachment of avidin and other proteins onto the chip surfaces. Histograms of the emission intensity are used to provide qualitative insights into the attachment processes.

MATERIALS AND METHODS

Chip Fabrication

The chips used in this study were fabricated in silicon wafers using standard microfabrication techniques. Silicon wafers were thermally oxidized in oxygen and water vapor ambient to form a 1-μm-thick silicon dioxide (SiO_2) surface. Platinum metal was deposited, using a radio-frequency sputtering process in a Perkin Elmer (Wellesley, MA) sputtering system, to a thickness of 1000 Å. A plasma-enhanced chemical vapor deposition process using silane (SiH_4) and nitrous oxide (N_2O) sources at 250°C was used to deposit an 8000-Å-thick hydrogen-rich silicon oxide layer on top of the platinum. Optical photolithography was used to open contact pads on top of the platinum using liquid hydrofluoric acid to etch the oxide. The silicon wafer was diced into 1.0 cm × 0.5 cm chips, which were used in the adsorption experiments. The chips were cleaned by rinsing in acetone and methanol and in an oxygen plasma to remove any carbon contamination. Figure 1a shows a schematic representation of a cross-section of the chip, indicating the various regions where the proteins were adsorbed. Figure 1b is a SEM of a portion of the chip. The channels are 20 μm wide. The length scale is as indicated by the bar in the lower right-hand corner. The total volume of the channels in a typical cross-section of the chip is 0.1 μL or less. Because the samples are introduced in fluid (liquid) form, this type of architecture is referred to as a microfluidic chip.

Adsorption Protocols

Fluorescein-labeled immunopure avidin (Cat. #21221, Lot #AI612511, Pierce, Rockford, IL) was purchased at a concentration of 5 mg/mL in 10 m*M* HEPES, 0.15 *M* NaCl, and 0.08% sodium azide buffer. Unlabeled avidin was also purchased from Pierce (Cat. #21228, Lot #AI41673). The specified pH of the stock solution, as provided, was 8.5. Phosphate-buffered saline (PBS) was prepared by mixing 8 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4, and 1.15 g of

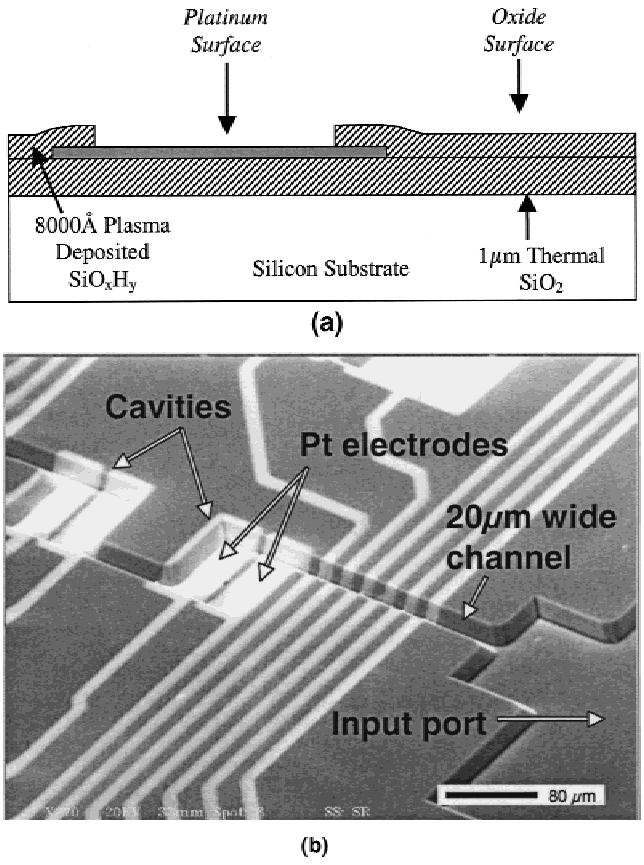

Figure 1. (a) Cross-section of the surfaces in the microchip where the proteins were adsorbed. (b) SEM of typical section of microchip showing channels and wells.

Na_2HPO_4 in 1000 mL of distilled water, resulting in pH 7.4. A volume of 0.2 mL of the avidin solution was mixed with 0.8 mL of PBS buffer to get a final, avidin concentration of 1 mg/mL. A final pH of 7.4 was maintained due to the buffering capacity of the PBS. For each of the experiments described in what follows, the chips were incubated in clean vials by immersion in 200 μL of the avidin solution at the specified temperature for a period of 15 h. Labeled biotin was purchased (Cat. #22030, Lot #AK42310, Pierce) in powder form and then dissolved in the aforementioned PBS to a concentration of 4 mg/mL. The biotin incubation was done for 5 min at room temperature in 200 μL of the dissolved solution. Crystallized unlabeled BSA (Cat. #77110, Lot #AD40111, Pierce) and fluorescein-labeled BSA (Cat. #A-9771, Lot #89H7613, Pierce) were purchased and dissolved in PBS to a concentration of 10 mg/mL. The chips were incubated in BSA at 40°C for 2 h and stored at 4°C for 15 h. After incubation, the chips were rinsed three times for 5 min each in PBS to allow excess unbound proteins to be removed. BSA, with and without the fluorescein label, was purchased and used as appropriate in the various experiments to be described.

Image Analysis

Images were observed in a Nikon Labophot fluorescence microscope with an FITC filter and an Optronics 470T CCD

camera. The images were acquired with METAMORPH software (Universal Imaging Corp.). Using the image-processing toolbox available in MATLAB (The Mathworks, Inc., Natick, MA), pixel sections of 1600 × 1600 were analyzed to extract histograms of the fluorescence intensity from the surface of the chip. These histograms indicate the number of pixels for each level of brightness. Because the fluorescent label used (fluorescein) has an emission peak in a frequency corresponding to green color, only the green component of the image was analyzed (using a red/green/blue [RGB] decomposition of the color space as produced by the image acquisition software). These histograms were used to compare the degree of adsorption on the various surfaces within each particular experiment. A comparison was also made with histograms from images of untreated surfaces. It is assumed that the intensity of the emission, as indicated by the position of the peaks in the histograms, was directly proportional to the amount of adsorbed protein on the surface. It should be pointed out that the emission from the platinum metal surface may have been altered due to local and surface quenching processes, and hence should not be directly compared with the emission from the oxide surface. Only like surfaces were compared under the various experimental conditions within a given experiment and are the basis of the results summarized.

RESULTS AND DISCUSSION

Avidin is a glycoprotein (molecular mass = 68 kDa) found in egg white; it has an isoelectric point of about 10 (Wilchek et al., 1990), and hence will carry a net positive surface charge at the pH used in these experiments. In the first experiment, direct adsorption of avidin on the chip surfaces was established by incubating the chips in fluorescein-labeled avidin according to the procedure described earlier. Two different chips were incubated at room temperature (about 25°C) and at 37°C for 15 h. After washing away the excess unbound proteins, the chips were imaged using the fluorescence microscope. Figure 2 shows the emission intensity histograms on the surfaces (oxide and Pt) from this first experiment. Clearly, there was adsorption of avidin on the surfaces as evidenced by the emission profiles shown in Figure 2, which graphs normalized counts as a function of intensity. A stronger signal (which reflects a greater presence of labeled protein) is indicated by a larger intensity and the position of the peak to the right. The intensity was higher on chips where the adsorption was done at 37°C (open diamonds in Fig. 2). Hence, this procedure was used for avidin adsorption in subsequent experiments. It is also of interest to note that when the chips (both incubated at room temperature and at 37°C) were rinsed in de-ionized (DI) water for 30 seconds and dried with compressed gas (100% chlorodifluoromethane) for 15 seconds, the emission was reduced to background level (indicated by open triangles in Fig. 2). The chip that was washed and dried in this way was subsequently rewetted in PBS. An absence of fluorescence confirmed that the protein had been removed from the surfaces. The compressed air (delivered at about 120 psig), when blown across the 0.5-cm^2 surface of the chip, was sufficient to dry the protein and literally blow it off of the surface of the chip. However, if the protein was kept in a wet state it was retained on the surface, as shown by the other experiments.

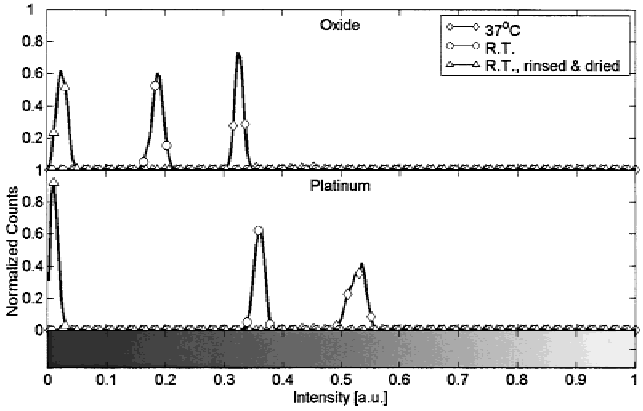

Figure 2. Histograms of fluorescence emission of fluorescein-labeled avidin from the chip surfaces under incubation at room temperature (RT) for 15 h, incubated at 37°C for 15 h, and when the chips were rinsed in DI and dried. Highest emission coincides with peaks on the right of the intensity scale.

In the next experiment, the precipitation of avidin using $(NH_4)_2SO_4$ was investigated. Ammonium sulfate $(NH_4)_2SO_4$, a kosmotrope (promoter of an ordered arrangement of water molecules), attracts water molecules to itself and away from the hydration layer around the protein, which is necessary to keep the protein in solution. This in turn promotes hydrophobic interactions between protein molecules and leads to their aggregation and precipitation. This rationale led to the experiment in which ammonium sulfate was added to the PBS to promote precipitation of the protein on a surface (the microchip in this case) to produce a concentrated protein layer. Such a layer was expected to enhance the adsorption of the protein onto the surface. Fluorescently labeled avidin solution was added to a vial containing the chip, along with ammonium sulfate at a final concentration of 50% (Bollag et al., 1996), mixed gently, and incubated on ice for 30 min. The vial with the fluorescently labeled avidin and ammonium sulfate solution was then stored at 37°C for 15 h, followed by washing of the chip in PBS. Figure 3 shows the histograms of the emission intensity showing that the precipitation process induced by ammonium sulfate enhanced the deposition of avidin on platinum. The ammonium sulfate promoted greater adsorption on the platinum (Fig. 3, lower graph, triangles) than the oxide (Fig. 4, upper graph, triangles). In both cases, adsorption (intensity) was greater than the background (control, diamonds). This procedure could be useful in ensuring that a concentrated source of avidin is present over the chip surfaces, even when using a solution with low concentration, in order to allow for a more efficient precipitation.

Once the presence of the protein is confirmed on the

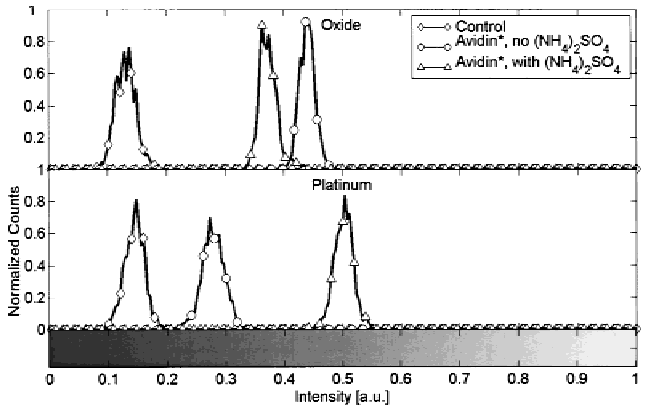

Figure 3. Histograms of fluorescence emission from the chip surfaces of a reference unprocessed chip, avidin adsorption at 37°C for 15 h, and avidin adsorption using ammonium sulfate precipitation. The histograms labeled "control" correspond to untreated surfaces. An asterisk next to a particular species indicates that there is a fluorescent label attached to it.

surface of the chip, it is important to establish that its ability to bind is still present. This was verified using fluorescein-labeled biotin (a vitamin with a molecular mass of 244 Da), which binds to avidin with very high affinity ($K_a = 10^{15}$ M^{-1}). After avidin adsorption on the chip surface using an unlabeled form of this protein, as per experiment 1, the chip was incubated in a biotin solution for 5 min. As shown in Figure 4, the emission intensity from the chip with both avidin and biotin was about the same or less than the surfaces with avidin only. This result indicates that biotin did not strongly bind to avidin, perhaps because the avidin was not in an active conformation when directly adsorbed on the chip surface. When biotin alone in PBS was incubated with the chip, it did not bind as strongly to the oxide as on the platinum surface. These results are consistent with earlier studies (Mooney et al., 1996), which reported that bacterial streptavidin alone does not adsorb to bare oxide surfaces directly.

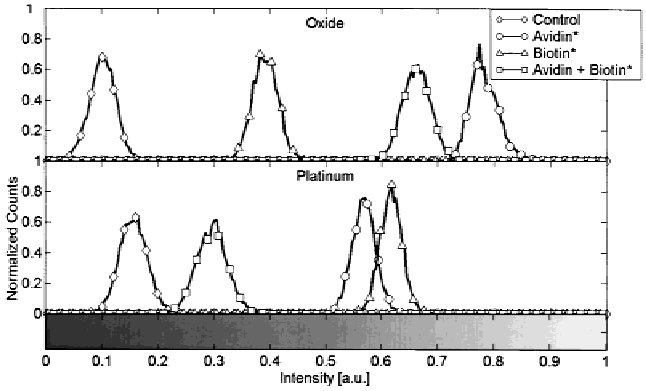

Figure 4. Histograms of fluorescence emission from the chip surfaces of a reference unprocessed chip, avidin adsorption, biotin adsorption, and avidin biotin reaction. Histograms labeled "control" correspond to untreated surfaces. An asterisk next to a particular species indicates that there is a fluorescent label attached to it.

Finally, the adsorption of avidin through BSA was examined. The incubation of a chip in fluorescein-labeled BSA demonstrated its binding to oxide and platinum, as shown by the histograms in Figure 5a. Binding of BSA on platinum was stronger than BSA onto oxide (compare peaks and squares, on lower and upper scales, respectively). This is indicated by the brighter emission. Avidin alone and BSA with avidin showed similar emission and intensity peaks to one another (circles and triangles in lower scale of Fig. 5a), indicating that avidin bound to the surfaces through BSA. Even more noteworthy are the results in Figure 5b, which show that a chip with BSA and avidin exhibits very bright fluorescence upon subsequent binding with biotin. The emission is intense, which is consistent with the biotin binding to the avidin, and is in contrast to Figure 4, where the intensity suggests that the extent of biotin binding is less. BSA thus serves to promote greater adsorption of the avidin to the surface and/or interacts with the avidin in a manner

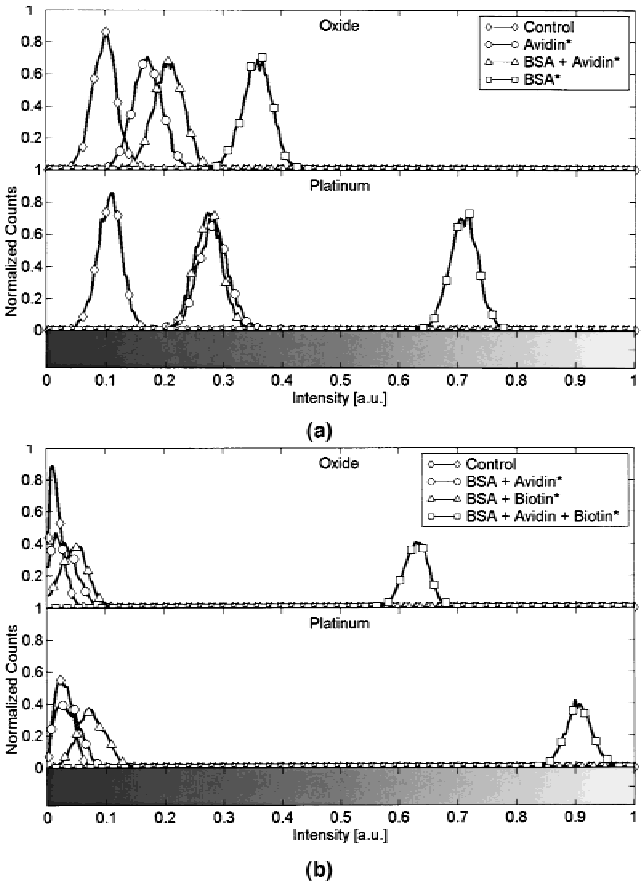

Figure 5. (a) Histograms of fluorescence emission from the chip surfaces of a reference unprocessed chip, avidin adsorption, BSA adsorption, and avidin + BSA binding. Histograms labeled "control" correspond to untreated surfaces. An asterisk next to a particular species indicates that there is a fluorescent label attached to it. (b) Histograms of fluorescence emission from the chip surfaces of a reference unprocessed chip, BSA + avidin binding, BSA + biotin binding, and BSA + avidin + biotin binding. Histograms labeled "control" correspond to untreated surfaces. An asterisk next to a particular species indicates that there is a fluorescent label attached to it.

that increases the intensity of its complexing with biotin. The time elapsed between the completion of the adsorption experiment to the inspection of emission ranged up to 6 h, suggesting that the proteins remained stable for this period of time.

CONCLUSIONS

Adsorption protocols for the protein avidin on oxide and platinum surfaces of a biochip show that avidin adsorption is promoted by ammonium sulfate and biotin binding is strongest for avidin adsorbed onto BSA. Avidin adsorbs on the chip surfaces at 37°C better than at room temperature and can be removed when a chip previously rinsed with DI water is dried. Washing alone is insufficient to remove the protein, as long as the chip's surface is kept wet. The enhancement obtained with the BSA/avidin sequential procedure was surprising, because BSA is often used to block adsorption or binding to antibodies. The sequence with which adsorption is carried out and the environment of the protein molecules (platinum and oxide surfaces) may be a factor. Developing an understanding of such attachment schemes will be very important for linking of proteins and antibodies to surfaces of platinum and oxide surfaces for biochip applications.

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