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Development of sub-micron patterned carbon electrodes for immunoassays

Narasaiah Dontha, Wilbur B. Nowall, Werner G. Kuhr *

Department of Chemistry, University of California, Riverside, CA 92521, USA

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Abstract

Sub-micron sized domains of a carbon surface are derivatized with antibodies using biotin/avidin technology. These sites are spatially-segregated from, and directly adjacent to, electron transfer sites on the same electrode surface. The distance between these electron transfer sites and enzyme-loaded domains are kept to a minimum (e.g. less than a micron) to maintain the high sensitivity required for the measurement of enzyme-linked cofactors in an enzyme-linked immunoassay (ELISA). This is accomplished through the use of photolithographic attachment of photobiotin using an interference pattern from a UV laser generated at the electrode surface. This allows the construction of microscopic arrays of active ELISA sites on a carbon substrate while leaving other sites underivatized to facilitate electron transfer reactions of redox mediators; thus maximizing sensitivity and detection of the enzyme mediator. The carbon electrode surface is characterized with respect to its chemical structure and electron transfer properties following each step of the antibody immobilization process. The characterization of specific modifications of micron regions of the carbon surface requires analytical methodology that has both high spatial resolution and sensitivity. We have used fluorescence microscopy with a cooled CCD imaging system to visualize the spatial distribution of enzyme immobilization sites (indicated by fluorescence from Texas-Red labeled antibody) across the carbon surface. The viability of the enzyme attached to the surface in this manner was demonstrated by imaging the distribution of an insoluble, fluorescent product.

Keywords: Photobiotin; Avidin; Maskless photolithography; Alkaline phosphatase; Cyclic voltammetry; Antigen-antibody; Interference pattern

1. Introduction

Photolithography has been used extensively in the formation of electronic circuits. The use of ultraviolet or visible light as a radiation source for the initiation of photochemical reactions forms the present basis for the synthesis of microstructures for electronics on single crystal silicon wafers [1]. Complex interconnections of electronic components can be generated on the micron-length scales by using a variety of photosensitive organic polymers to mask various areas of a silicon chip. Areas of the silicon not coated with

* Corresponding author. Tel.: +1-909-787-3485; fax: +1-909-787-4713; e-mail: werner.kuhr@ucr.edu.

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polymer are thermally reacted to produce silicon oxide coated regions and etched regions providing specific electronic functions. Interfacial photolithography as applied to silicon-based electronic materials is limited to sculpting organic polymers of specific topography due to various degrees of cross-linking and thus solubility.

More recently, it has been used in the construction and design of templates for the parallel synthesis of peptides and oligonucleotides [2]. Spatial control of the derivatization of solid substrates has been achieved through the use of photolabile protecting groups where such groups are removed after application of an appropriate wavelength of light to allow activation of a specific chemical synthetic step. These photolabile protecting groups, which are good chromophores that are very sensitive to specific wavelengths of light must be relatively stable to the other chemical reagents that they will be exposed to as part of the synthetic procedure. The selective removal of these photolabile groups has been directed through conventional masking technology to obtain structures with dimensions of 100 μm on a slide [3].

The purpose of this study is to immobilize antibody and enzyme molecules directly adjacent to electron transfer sites. Several reports on the patterning of biomolecules on different substrates by using photolithography have already appeared. Pirrung and Huang [4], immobilized biotin using caged biotin, which has a photoremovable nitrobenzyl group which is activated by UV light. This method generated 350–500 μm wide structures on glass surfaces. Bhatia et. al. [5,6], attached antibodies on to the glass surface via oxidation of the thiol terminus of an organosilane self assembled monolayer film by deep ultraviolet (DUV) irradiation. The antibodies immobilized in this way retained their antigenic binding activity. The generated patterns of protein were less than 10 μm wide. Several groups have used photobiotin to pattern antibodies and enzymes on gold and modified glass surfaces [7–10]. All these techniques involved the use of conventional mask-based photolithography, where the dimensions of the irradiated structures ranged from ten to several hundreds of microns.

The technique which we have developed utilizes maskless patterning of biomolecules on a carbon substrate. Carbon is an attractive substrate since it can be treated in various manners to yield a variety of surface carbon–oxygen functionalities. Carbon electrodes can be conveniently functionalized to serve as enzyme electrodes [11]. Considerable work has been done in our laboratory on the derivatization of surfaces with a hydrophilic tether which is linked to biotin [12–15], which in turn, allows construction of an enzyme modified carbon-fiber microelectrode through covalent attachment of the biotin to the carbon surface. In recent years, the biotin–avidin–biotin complex has become very useful as an extremely versatile, general mediator in a wide variety of bioanalytical applications. The rapid rise in its appeal and almost universal employment in so many different fields of biological expertise are due largely to the exceptionally high affinity (10^{15} M^{-1}) and consequent stability of this noncovalent interactions.

It is only recently however, that this complex has found wide use as a scientific tool to detect, quantitate and separate minute amounts of biological substances. Radioisotopes and radioimmunoassays used earlier for these purposes were satisfactory, however the hazard of working with, and disposing of, radioactive materials prompted scientists to begin using enzymes as detector labels. By determining the presence of detector enzymes with colorogenic or fluorogenic substrates sensitive, safe and usually less expensive assays could be developed. In an attempt to further improve these immunoassays, the avidin–biotin complex has been useful in amplifying detection methodologies [16,17].

2. Experimental

2.1. Chemicals and apparatus

Photobiotin [*N*-(4-azido-2-nitrophenyl)-*N*-(3-biotinyl amino propyl)-*N*-methyl-1,3-propane diamine) acetate salt, ExtrAvidin, Bovine Serum Albumin (BSA) (Sigma, St. Louis, MO)], Biotinylated goat IgG, Texas Red anti-goat IgG, Alkaline Phosphatase anti-goat IgG (Vector

Laboratories, Burlington, CA) were used as received. ELF^R-97 Endogenous phosphatase detection kit was used according to the manufacturers protocol. (Molecular Probes, Oregon). Phosphate-buffered saline (PBS; 150 mM NaCl and 100 mM Na_2HPO_4, pH 7.4) and Tris (1 mM MgCl and 100 mM Tris) buffers were prepared with DI water (E-Pure, Barnstead, Debuque, IA) before adjusting pH. α-Naphthol and 1-α-Naphthyl phosphate were purchased from Sigma. Nail polish for sealing the substrate and probe tips was Revlon # 61 clear (New York, NY). A hair drier (Conair, East Winsdor, NJ) was used to quickly dry the nail polish. The electrode substrate was a glassy carbon plate 1 mm thick with a 1 mm^2 diameter. Silver epoxy (APS, Peabody, MA) was used to mount the electrodes to locally-constructed aluminum holders (Matt McCormick, Chemistry Machine Shop, UC Riverside). The polishing apparatus was built in-house and 0.05 μm gamma alumina polishing powder was added to deionized water and placed on Microcloth™ polishing cloth (Buehler, Lake Bluff, IL.). Ag/AgCl reference electrode was purchased from Bioanalytical Systems (West Lafayette, IN).

2.2. Spatially-selective modification of carbon surface with photobiotin

Glassy carbon (GC) electrodes (glassy carbon plates, 1 mm thickness; Alfa Aesar; MA) were polished using 0.3 and 0.05 μm alumina powder in deionized water and metacloth on a home built polishing apparatus. After each polishing step, the carbon surface was cleaned thoroughly with deionized water to remove any residual polish. GCs were then air dried in a virtually dust free environment. 10 μl of photobiotin (1 mg ml^{-1}) was placed on the polished GC and left to dry in the dark at room temperature for 2–3 h. Photoactivation of the photobiotin was accomplished with a 10 mW, 325 nm HeCd laser (Omnichrome, Chino, CA).

The laser apparatus used for interference patterning is described elsewhere [18]. Briefly, UV (325 nm) light was sent through a beam splitter and reflected off mirrors to make two parallel beams of approximately similar power. The parallel beams were sent through a 10 cm focal length lens and the substrate (a photobiotin-coated GCE) was placed where the two laser beams merge (near the focal point). The two beams form an interference pattern with less than 2 μm spacing when the angle between the two beams is $\approx 10°$. The interference pattern spacing can be easily changed by modifying the angle between the beams [18].

Photobiotin was exposed to UV light for different time intervals to optimize the patterning process. The laser interference pattern was applied to the photobiotin covered carbon surface for 20–300 s. Once photobiotin is exposed to the UV laser light, a covalent bond is formed between the biotin and the carbon surface. A more detailed account of the chemistry of the photolabels were discussed elsewhere [19]. Afterwards, the electrodes were washed with phosphate buffer to remove any unbound photobiotin. The electrodes were then incubated with 1% Tween-20 for 1 h to minimize non-specific adsorption of proteins. After 1 h, the GCs were washed again with phosphate buffer.

2.3. Antibody attachment

After the carbon surface was patterned with biotin, the modified GCE was incubated with 3% BSA in PBS and left for 1 h at room temperature. After it was washed thoroughly to remove excess protein on the surface, the modified GC electrode was incubated in 1 mg ml^{-1} ExtrAvidin in PBS solution for 1 h. This forms the strong avidin–biotin bond due to high affinity of avidin to biotin [17]. The avidin-modified electrode surface is washed thoroughly with PBS to remove unbound avidin; then it is placed in a solution containing biotin-labeled goat IgG solution (b-goat IgG; 50 μg ml^{-1}) and left for 1 h. This attaches b-goat IgG to the avidin-modified surface; this goat IgG patterned surface was washed thoroughly. Finally, either Texas Red-labeled anti-goat IgG (TR-anti-goat-IgG; 100 μg ml^{-1}), or Alkaline phosphatase-labeled anti-goat IgG (AP-anti-goat-IgG; 100 μg ml^{-1}), was added to form the antigen–antibody complex with the goat IgG patterned surface. This forms a nice sandwich of Ag/Ab on the carbon

surface. A schematic diagram of immobilization of the biomolecules is shown in Fig. 1.

2.4. Fluorescence imaging microscopy

The Texas Red-labeled anti-goat IgG was visualized using fluorescence imaging microscopy (Excitation wavelength = 595 nm and emission wavelength = 615 nm). The activity of Alkaline phosphatase-labeled anti-goat IgG was also imaged with fluorescence microscopy by using a fluorogenic substrate known as ELF-97. The ELF-97 substrate is prepared according to the manufacturers protocol, and the modified electrode surface was incubated with ELF-97 for 30 min, after which the electrode was washed thoroughly and imaged for fluorescence due to the presence of the product of ELF-97 hydrolysis (with DAPI filter set; Excitation wavelength = 358 nm and emission wavelength = 461 nm).

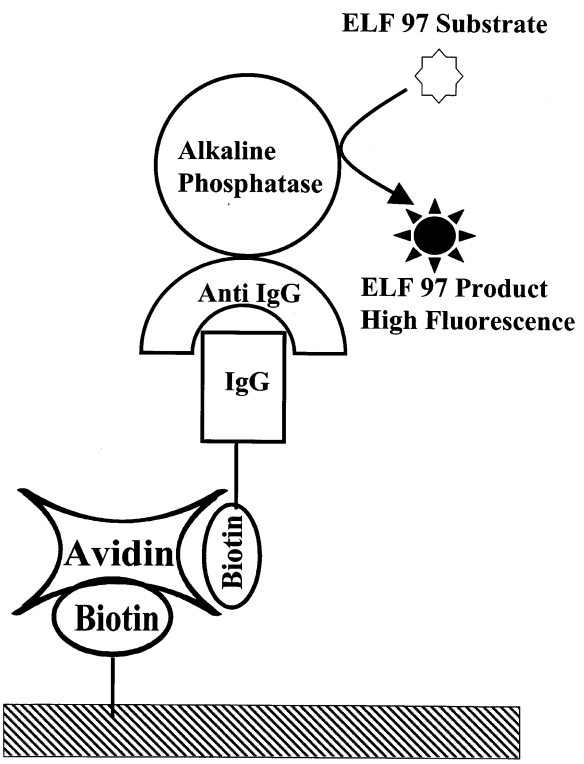

Fig. 1. Schematic diagram of photoimmobilization of biomolecules via photobiotin on the carbon surface (see text for details).

ELF-97 itself is only weakly fluorescent under these conditions. Once the phosphate group is enzymatically removed from ELF-97, the resulting product is an intensely fluorescent yellow–green precipitate that remains at the site of hydrolysis, where this product has an emission wavelength that is more than 100 nm longer than its excitation wavelength.

2.5. Optical instrumentation

The surfaces of Texas Red and Alkaline phosphatase-ELF-97 modified carbon electrodes were positioned face-up toward either an Epiplan™ Neofluar 20x (air lens), a 40x (water immersion), or a 100x (oil immersion) objective. Electrode surfaces were imaged with an epi-fluorescence microscope (Zeiss Axioskop™. Thornwood, NY) equipped with a 100 W Hg arc lamp for epi-illumination and a 50 W halogen lamp for transmitted illumination. All images were collected in a darkened room with a cooled Thompson 7895B CCD (class 2, 512 × 512, 200 × 200, Metachrome II UV coated MPP mode, Photometrics, Tucson, AZ) that was operated at −45°C. Images were collected through a Photometrics NU-200 controller (16 bit, 40 khz A/D, Macintosh IIci configuration) and saved on a Macintosh IIci. Subsequent data processing was performed with IP-LAB 2.1.1c (Signal Analytics, Vienna, VA) and/or NIH Image 1.40 (Public domain, Wayne Rasband) imaging software.

Microscopic surface features were brought into focus with light from the Hg arc lamp after it passed through two neutral density filters with a 1 ms CCD acquisition time and a camera gain setting of 4. Fluorescent images of modified carbon surfaces were obtained by passing the light from the Hg arc lamp through an excitation filter specific for the Texas red absorption band (595nm) and collecting all fluorescence at 615nm with a 1 s camera collection time. All images were acquired in the central zone of the CCD.

2.6. Electrochemical measurements

Cyclic voltammetry was used to examine the electron transfer properties of α-naphthol and

α-naphthyl phosphate at carbon electrodes after polishing, after global derivatization with the protocol described above, and after patterning the Alkaline Phosphatase-labeled antibody. The latter represents an enzyme-linked immunoassay (ELISA) format for the detection of anti-goat IgG using electrochemical detection of the product, α-naphthol. A three-electrode potentiostat was used for electrochemical measurements. Staircase cyclic voltammetry was run at a scan rate of 50 mV s^{-1}. The experiments were run in either 100 mM phosphate buffer with 150 mM NaCl added at pH 9.0 or 100 mM Tris buffer with 150 mM NaCl added at pH 9.0 with 1 mM MgCl added. All potentials reported are versus Ag/AgCl via a salt bridge filled with 3 M NaCl. Potentials were controlled via computer through an EI-400 potentiostat (Cypress Systems) and all data was collected via the same system.

3. Results and discussion

Recently Flounders et al. described the patterning of antibodies on silicon surfaces using 15 and 0.4 μm masks [20]. Mouse monoclonal antibodies were covalently linked through free amino groups to aminosilanized silicon dioxide films using glutaraldehyde. Immobilized antibody layers were stabilized with sucrose, dehydrated and stored refrigerated with desiccant. The photolithographic pattern was obtained with a positive photoresist with modified bake temperatures and timed, selective UV exposure with a contact mask. This was followed by an aqueous alkaline solubilization of exposed resist. Exposed regions of immobilized antibody were then removed by exposure to a low power, radio frequency oxygen discharge. Finally, residual resist was stripped with acetone. Successful patterning was demonstrated by challenging surfaces with anti-IgG labeled with TRIC [20].

Several workers have used photoactivatable biomolecules to pattern surfaces with photolithography. Cooper et al. used photobiotin to pattern antibodies on silicon and gold surfaces (mask dimensions $\approx$10, 4, 1.5 μm) [9]. Photobiotin has a terminal aryl azide group, which is stable in the dark but forms a reactive aryl nitrene group upon exposure to light (340–370 nm). This will insert into any organic surface and covalently bind biotin to the surface. Other workers have used photobiotin, caged biotin or other types of photoreactive molecules but they have always used contact masks to determine the exposed area. The use of a mask in photolithography has its own inherent problems. The mask should be tightly bound on the substrate before exposing the substrate to light, otherwise the light will diffuse and random immobilization occurs.

In this work, we have immobilized photobiotin onto an electrode surface with the objective to provide regions of antibody-modified surfaces directly adjacent to unmodified areas, which have facile electron transfer kinetics for electroactive products of enzyme-lined tags. An array of micron-sized wide lines can be formed by using the interference pattern produced when two parallel coherent light sources are combined at a surface at an angle [18]. Basically, the laser beam is passed through a 50% beam splitter, and each beam is directed to recombine at the sample surface at approximately the same angle of incidence. The placement of the beams is fine-tuned to allow complete overlap of the mode structure of the laser spot. This produces a well-defined pattern of lines across the electrode surface, where the spacing between points of positive interference (D) can be approximated by the equation: $n\lambda = 2D \sin(\theta/2)$, where $\lambda =$ wavelength, $\theta =$ angle between beams, and n is order.

With a HeCd laser at 325 nm, an incidence angle of $\approx 10°$ is needed to generate an interference pattern with lines spaced at 2 μm. The biotin-coated GCE was exposed to the diffraction pattern formed with 325 nm light ($\approx$1 W cm^{-2}) for various times (20–300 s) to induce photolysis of the nitroaryl moiety to generate a nitrene, which inserts readily into the glassy carbon lattice. An exposure time of 60 s was found to reproducibly form a linear array of biotin on the GCE with a line width of $\approx$0.85 μm. A very regular pattern of lines, closely resembling the intensity distribution expected for the laser diffraction pattern, could be visualized by optical microscopy as shown in Fig. 2 (upper left). The distance between adjacent lines of bound photobiotin was examined

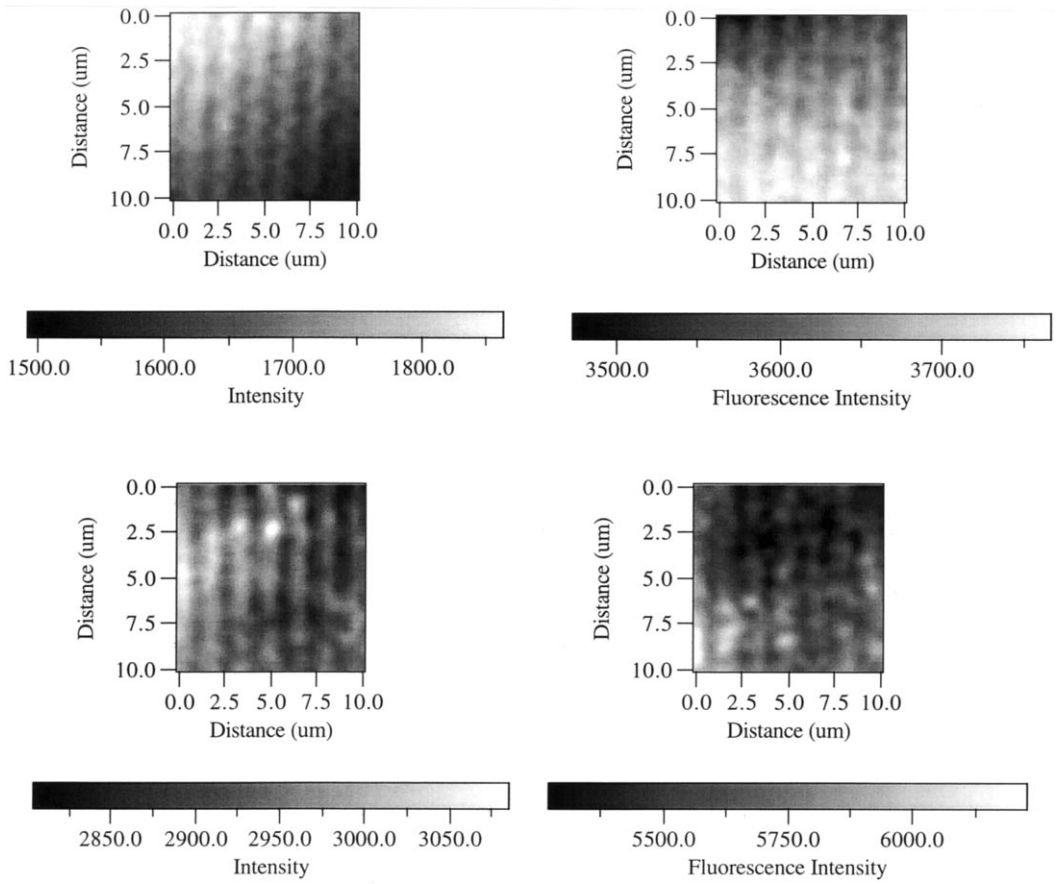

Fig. 2. GC electrodes photolithographically derivatized with photobiotin using laser interference pattern. Photobiotin was irradiated for 60 s with 325 nm light in a interference pattern produced with an angle of 3.6° between the beams. The white light reflectance image of the patterned photobiotin/avidin bound surface (upper left) and the fluorescence image of Texas Red-labeled antigoat antibody immobilized on the same biotin–avidin modified surface (lower left). The white light reflected image of a biotin/avidin/alkaline phosphatase-modified surface is shown in the upper right, and the fluorescence image of the product of ELF-97 generated by alkaline phosphatase-labeled antigoat antibody bound on the modified carbon surface is shown in the lower right. The fluorescence images were integrated for 1 s using appropriate excitation and emission filters, all white light images were integrated for 0.01 s without excitation or emission filters. All the images were processed with one moving average smooth to improve image quality.

by plotting the average intensity as a function of lateral distance (Fig. 3, upper left). The average width of each photopatterned line (taken at half-maximum intensity) was 0.84 ± 0.10 μm ($n = 5$). This corresponds to a standard deviation less than ± 0.5 pixel.

ExtrAvidin was allowed to bind to the immobilized biotin after photopatterning, then the avidin-patterned surface was exposed to b-goat IgG to form the basis for the antigen-antibody reaction. TR-anti-goat IgG, the antibody labeled with a fluorescent dye (Texas Red), was used to image the microscopic spatial distribution of the completed antigen/antibody reaction, as shown in Fig. 2 (lower left). Virtually the same linear array was observed, but each individual line was not continuously labeled by TR-avidin. This is similar to what was observed with TR-avidin directly [18], where the density of surface oxides on the carbon surface influence the availability of biotin for binding. We have observed similar heterogeneity when carbon fiber surfaces were derivatized using

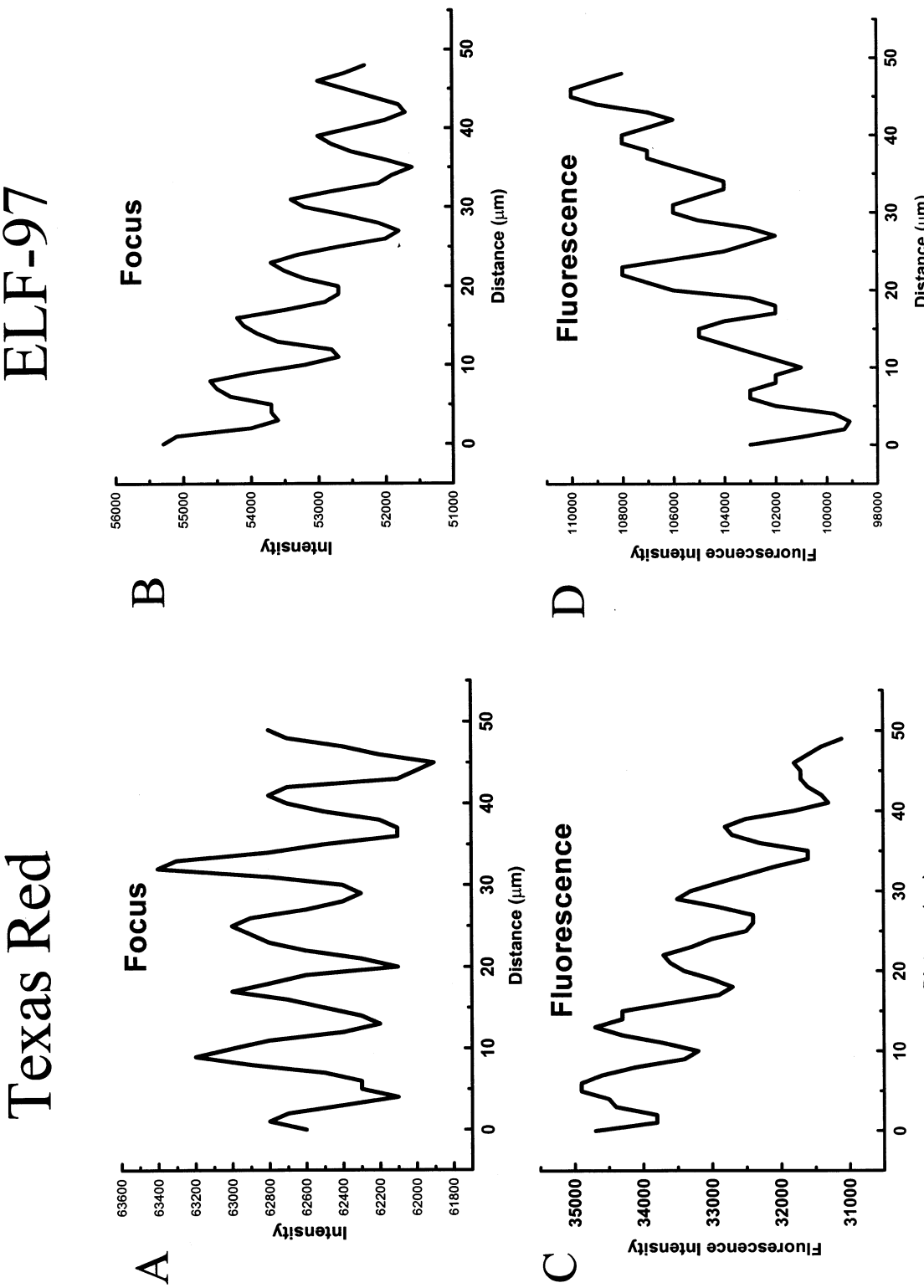

Fig. 3. Lateral profile of the interference pattern derivatized biomolecules on the carbon surface. The intensities of reflected light (upper figures) and fluorescence (lower figures) were plotted as a function of lateral distance across the electrode. The lateral profiles were collected from four different electrodes corresponding to the images in Fig. 2.

biotin/avidin chemistry [15,21]. The size of the TR-anti-goat IgG features were examined by plotting fluorescence intensity as a function of lateral distance (Fig. 3, lower left). The average width of each photopatterned structure (taken at half-maximum intensity) was 0.83 ± 0.10 μm ($n = 5$). This is not statistically different from that observed for photobiotin only, indicating that the antibody is binding specifically to the antigen photolithographically immobilized to the electrode surface. This also implies that there is no degradation of resolution in the formation of these nanostructures produced in any of the affinity interactions.

To demonstrate the feasibility of ELISA-based assays, the antibody-patterned carbon surface was prepared as described above. An alkaline phosphatase conjugate of anti-goat IgG (AP-anti-goat-IgG) was attached to the patterned antigen, then the electrode was washed and the enzyme-derivatized carbon surface was imaged with light microscopy. As shown in Fig. 2 (upper right), a pattern similar to that of biotin-only is observed. Additionally, the dimensions of the AP-anti-goat-IgG pattern was examined by plotting intensity as a function of lateral distance (Fig. 3, upper right). The average width of each photopatterned line (taken at half-maximum intensity) was 0.77 ± 0.10 μm ($n = 5$) indicating that the enzyme–antibody complex is binding specifically to the biotin photolithographically immobilized to the electrode surface.

The viability of the APase attached to the antigen–antibody complex in this manner was demonstrated by imaging the distribution of the product of the alkaline hydrolysis of ELF-97™ (Fig. 3, lower right). An insoluble, fluorescent product is formed when ELF-97™ is hydrolyzed by the enzyme. The distribution of the precipitated product shows the activity of the patterned enzyme because the fluorescent product is formed only where active enzyme is present. As shown in Fig. 2 (lower right), the spatial distribution of the product of APase hydrolysis of ELF-97™ is similar to that of biotin-only (Fig. 2, upper right) and to that of the bound TR-anti-goat IgG (Fig. 2, lower left). Additionally, the product distribution was examined by plotting fluorescence intensity as a function of lateral distance (Fig. 3, lower right) and the average width of each photopatterned line (taken at half-maximum intensity) was 0.76 ± 0.10 μm ($n = 5$). This is not statistically different from that observed for photobiotin only, indicating that the spatial distribution of the activity of the enzyme–avidin complex is virtually identical to that of the enzyme–avidin complex (as well as the biotinylated surface).

3.1. Electrochemical studies

Alkaline phosphatase is a versatile enzyme. This enzyme has been used in many electrochemical studies [22–27], because it is robust and has several sensitive electroactive substrates. We have used α-naphthol as a electroactive substrate, since it is commonly used in similar applications for the electrochemical detection in ELISA [28]. AP-anti-goat-IgG was patterned on the carbon surface as described above, and the electrode was incubated with α-naphthyl phosphate for 2 h in Tris buffer as described in the literature [29]. As shown in Fig. 4(A), cyclic voltammogram of the dephosphorylated product shows a peak around 300 mV, identical to that observed for α-naphthol [29]. In contrast, the cyclic voltammogram of the substrate, α-naphthyl phosphate, shows very irreversible voltammetry (Fig. 4B).

The importance of photopatterning was demonstrated by examining the voltammetry of naphthol at carbon surfaces when the surface is globally derivatized. When a carbon electrode is completely covered with the biotin–avidin–antigen–antibody complex, electron transfer is greatly inhibited. This loss of electron transfer facility is readily apparent by examining the cyclic voltammetry of 1 mM α-naphthol phosphate (dotted line, Fig. 4C). This effect is due to the presence of the complex only, regardless of whether the enzyme is biotinylated, fluorescently labeled or used in its native form. The use of photopatterned immobilzation sites on the surface permits the retention of facile electron transfer for α-naphthol, presumably at the underivatized part of the surface (dashed line, Fig. 4D). This shows that the antibody-APase is active and can be used in detecting various environmental pollutants by patterning various antigens to the surface to create multianalyte sensors [30].

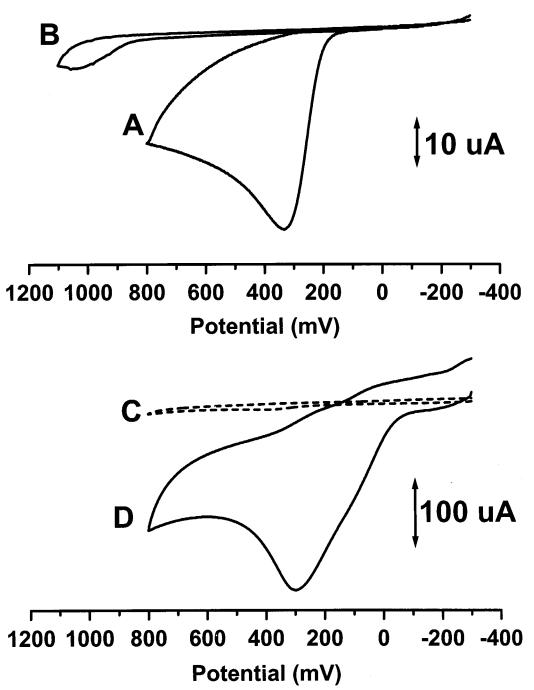

Fig. 4. Cyclic voltammograms (50 mV s^{-1}) of 10 μM α-naphthol (A) and 6 mM α-naphthyl phosphate (B) at carbon electrodes immediately after polishing. Cyclic voltammograms (50 mV s^{-1}) were recorded after 6 mM α-naphthyl phosphate was applied to carbon surfaces for 2 h after (C) completely global derivatization with the biotin/avidin/antigen/APase-labeled antibody described above, and (D) at a patterned Alkaline Phosphatase-labeled antibody surface. This demonstrates the dramatic increase in sensitivity in the voltammetry of α-napthol at the patterned surface compared to the globally-derivatized surface.

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References

[1] A.B. Bocarsly, C.C. Chang, Y. Wu, E.P. Vicenzi, J. Chem. Educ. 74 (1997) 663–667.
[2] J.W. Jacobs, S.P. Fodor, Trends Biotechnol. 12 (1994) 19–26.
[3] S.P. Fodor, J.L. Read, M.C. Pirrung, L. Stryer, A.T. Lu, D. Solas, Science 251 (1991) 767–773.
[4] M.C. Pirrung, C.Y. Huang, Bioconj. Chem. 7 (1996) 19–26.
[5] S.K. Bhatia, L.C. Shriver-Lake, K.J. Prior, J.H. Georger, J.M. Calvert, R. Bredehorst, F.S. Ligler, Anal. Biochem. 208 (1989) 408–413.
[6] S.K. Bhatia, J.L. Teixeira, M. Anderson, et al., Anal. Biochem 208 (1993) 91–93.
[7] M. Hengsakul, A.G. Cass, Bioconj. Chem. 7 (1996) 249–254.
[8] D.J. Pritchard, H. Morgan, J.M. Cooper, Anal. Chem. 67 (1995) 3605–3607.
[9] D.J. Pritchard, H. Morgan, J.M. Cooper, Angew. Chem. Int. Ed. Engl. 34 (1995) 91–93.
[10] H. Morgan, D.J. Pritchard, J.H. Cooper, Biosens. Bioelectron. 10 (1995) 841–846.
[11] R.M. Ianniello, T.J. Lindsay, A.M. Yacynych, Anal. Chem. 54 (1982) 1098–1101.
[12] W.G. Kuhr, V.L. Barrett, M.R. Gagnon, P. Hopper, P. Pantano, Anal. Chem. 65 (1993) 617–622.
[13] P. Pantano, T.M. Morton, W.G. Kuhr, J. Am. Chem. Soc. 113 (1992) 1832–1833.
[14] P. Pantano, W.G. Kuhr, Proc. Electrochem. Soc. 92 (1992) 829–830.
[15] P. Pantano, W.G. Kuhr, Anal. Chem. 65 (1993) 617–622.
[16] D. Fucillo, Biotechniques 3 (1985) 494–501.
[17] M. Wilchek, E.A. Bayer, Anal. Chem. 171 (1988) 1–32.
[18] N. Dontha, W.B. Nowall, W.G. Kuhr, Anal. Chem. 69 (1997) 2619–2625.
[19] J. Staros, TIBS 5 (1980) 320–322.
[20] A.W. Flounders, D.L. Brandon, A.H. Bates, Biosens. Bioelectron. 12 (1997) 447–456.
[21] P. Hopper, W.G. Kuhr, Anal. Chem. 66 (1994) 1996–2000.
[22] K.R. Wehmeyer, H.B. Halsall, W.R. Heineman, Clin. Chem. 31 (1985) 1546–1549.
[23] O. Niwa, Y. Xu, H.B. Halsall, W.R. Heineman, Anal. Chem. 65 (1993) 1559–1563.
[24] P.H. Treolar, A.T. Nkohkwo, J.W. Kane, D. Barber, P.M. Vadgama, Electroanalysis 6 (1994) 561–566.
[25] L.M. Christie, P.H. Treolar, Z.B. Koochaki, P.M. Vadgama, G.N. Smith, Anal. Chim. Acta 257 (1992) 21–28.
[26] M. Santandreu, F. Cespedes, S. Alegret, E. Martinez-Fabregas, Anal. Chem. 69 (1997) 2080–2085.
[27] O. Bagel, B. Limoges, B. Schollhorn, C. Degrand, Anal. Chem. 69 (1997) 4688–4694.
[28] C.G. Bauer, A.V. Eremenko, E. Ehrentreich-Forster, et al., Anal. Chem. 68 (1996) 2453–2458.
[29] C. Fernandez-Sanchez, A. Costa-Garcia, Biosens. Bioelectron. 12 (1997) 403–413.
[30] R. Pande, S. Kamtekar, M.S. Ayyagari, M. Kamath, K.A. Matrix, J. Kumar, S.K. Tripathy, D.L. Kaplan, Bioconj. Chem. 7 (1996) 159–164.