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Diffusion and Transfer of Antibody Proteins from a Sugar-Based Hydrogel

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ABSTRACT

Diffusion of antibody protein from hydrogel films and hydrogel encapsulated in a microcapillary was studied. Thin hydrogel films were formed by crosslinking 6-acryloyl-B-*O*-methylgalactoside with *N,N'*-methylene-*bis*-acrylamide and the diffusive transport of monoclonal antimouse IgG-FITC into and out of the hydrated films was measured. Diffusion coefficients in 2 and 4% crosslinked hydrogel films were measured. The measured diffusion constants determined for IgG in both the 2 and 4% hydrogel films were comparable to the free diffusion of IgG in bulk water ($D_{mean} \sim 10^{-7}cm^2/s$). In addition, 2% crosslinked hydrogels were prepared in a capillary tube and the transport of antimouse IgG-FITC into and out of the hydrated hydrogel was measured. Kinetic analysis indicated that the protein transport through the capillary hydrogel was faster than would be expected for a simple diffusion process. Finally, by utilizing the diffusion of antibody from the capillary hydrogel, transfer of antibody to a silica surface was demonstrated. A capillary hydrogel loaded with antimouse IgG-FITC was used to transfer the protein to a silica surface forming a 30-μm spot of antibody, which was imaged using fluorescence microscopy. These results may lead to the development of a nonlithographic method of patterning antibodies on surfaces for use in integrated microimmunosensors.

Index Entries: Hydrogel; multianalyte sensor; immunosensor; device miniaturization; diffusion; transfer; capillary; antibody.

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INTRODUCTION

There is considerable interest in the biosensor and medical diagnostic communities in developing multiplexed immunosensors for the simultaneous detection of multiple antigens *(1–4)*. The key feature of immunosensors is the coupling of an antibody or antigen to a solid support that facilitates the binding and separation of antibodies or antigens from a sample for detection. Single analyte immunoassays and sensors have been successfully developed to detect and measure pharmaceuticals, biomolecules, explosives, and environmental toxins *(1,2,5)*. However, the widespread exploitation of this technology has been limited by a number of problems. Prime among these problems are the nonspecific binding of biomolecules to the solid support; reproducible device fabrication; rapid, simultaneous detection of a number of analytes; and miniaturization of the immunosensor for field use *(1,2,4)*. Strategies have been developed to deal with nonspecific binding by blocking the surface regions that do not contain coupled antibody or antigen through coating of those surface areas with proteins, such as BSA, streptavidin, or deglycosylated forms of avidin *(4,6,7)*, or through novel encapsulation and antibody-labeling strategies *(1,8)*. The needs for miniaturization and multiple analyte detection are the driving forces behind efforts to develop array biosensors that have a number of different antibodies coupled to a solid support. Development of such a biosensor requires the immobilization of dense, well-resolved arrays of the antibodies to maximize antigen binding. This will require that novel deposition and immobilization techniques that minimize nonspecific binding will have to be employed. Current efforts to fabricate such a device involve the use of photolithographic techniques to couple multiple proteins to solid supports in high-resolution patterns *(4,9)*.

An alternative method of delivering antibody proteins to surfaces in well-resolved patterns would be to use antibody-loaded polymer sponges as the transfer tool. The sponge could be used to deliver the antibody to any location on the support surface, thereby creating the desired pattern. A somewhat similar approach has been used to pattern gold surfaces with alkanethiols, using an elastomeric poly(dimethylsiloxane) (PDMS) stamp *(10)*.

Hydrogels are insoluble hydrophilic polymers that swell in the presence of water *(11)*. The chemical nature of the polymer and the degree of crosslinking are the primary factors that determine the extent of the swelling and pore size *(12,13)*. Because of their biocompatibility, hydrogels have been used in a variety of biomedical applications (e.g., encapsulation of cells, controlled release of proteins or pharmaceuticals, wound-healing, biosensors, and contact lenses) *(11,14–20)*. Hydrogels are a suitable candidate as a protein transfer material, because of their ability to encapsulate and release proteins. Also, their ductility permits conformal contact with a

support surface. In order to test the viability of this approach, the diffusion of an antibody protein into and out of the hydrogel was studied.

Diffusion studies have been reported for hydrogels synthesized from a variety of polymeric materials (e.g., polyethylene glycol, polyvinyl alcohol, polyurethane, polyethylene oxide, polyhydroxyethylmethacrylate, polymethylmethacrylate, alginate, and agarose); *(21–27)*. Recently, sugar-based polyacrylate hydrogels have been prepared by crosslinking 6-acryloyl-B-*O*-methylgalactoside with *N,N'*-methylene-*bis*-acrylamide or ethylene glycol dimethacrylate *(28,29)*. For our study, we chose to work with the acrylamide crosslinked hydrogel, because of its high water content (90–99%), homogeneity, stability, expected nontoxicity, and ease of synthesis. In addition, we decided to use preswollen gels for our experiments to enable removal of any contaminants, such as unreacted regents and reaction byproducts, from the gels prior to antibody uptake *(25)*. Using this approach, the uptake and release of monoclonal antimouse IgG-FITC into and from the hydrogel films was examined in 2 and 4% crosslinked hydrogels. The results were used to determine the proper level of crosslinking required for the use of the capillary hydrogel as an antibody transfer tool. The transport properties of the capillary hydrogel were studied by examining the loading and release of the antibody and the transfer of the protein to a silica surface.

MATERIALS AND METHODS

N,N'-methylene-*bis*-acrylamide, *Pseudomonas fluorescens* lipase, bovine serum albumin (BSA), and monoclonal antimouse IgG (whole molecule) fluorescein (FITC) conjugate (1:128 dilution, ca. 1 mg/mL in 0.01 *M* phosphate-buffered saline, pH 7.4, containing 1% BSA with 0.1% sodium azide, purified by immunospecific affinity chromatography) were obtained from Sigma (St. Louis, MO); 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (AIPD) was obtained from Wako Pure Chemical Industries (Osaka, Japan); and trimethoxy-silylpropyl diethylenetriamine, methacryloxypropyl-trimethoxy silane, pyridine, and acetone were obtained from Aldrich (Milwaukee, WI). The monomer 6-acryloyl-B-*O*-methylgalactoside and the hydrogel polymer were prepared according to literature procedures *(28)*. Fused silica slides were obtained from NSG Precision Cells (Farmington, New York). Deionized water was obtained using a Milli-Q (Millipore, Bedford, MA) filtration system.

Preparation of Hydrogel Films and Capillary Hydrogel

Fused silica slides (2.5 × 2.5 cm) were cleaned by immersing the plates in 1:1 HCl:CH_3OH for 30 min, rinsing with deionized water, immersing in conc. H_2SO_4 for 30 min, rinsing with water, and, finally, heating them under

water at 100°C for 15 min. The slides were dried under a stream of N_2. The dried slides were then coated with methacryloxypropyltrimethoxy silane. The slides were silanized by adding 1 mL of the silane to 94 mL of a 1 m*M* solution of acetic acid in methanol in a N_2-filled dry box. This solution was removed from the dry box and 5 mL of water was added. The resulting mixture was poured into a Coplin jar containing the fused silica slides. After 15 min, the silane mixture was decanted and the slides were washed three times with methanol and then dried under a stream of N_2. The films were cured by drying overnight in the dry box at room temperature.

A 2.5 × 2.5 cm piece of parafilm (100 μm thickness) with a 1 × 1 cm center cut-out was placed on the silanized fused-silica slides. One hundred μL of the hydrogel reaction mixture (67.5 mg 6-acryloyl-B-*O*-methylgalactoside, 1.35 mg [2% crosslinking] or 2.70 mg [4% crosslinking] *N,N'*-methylene-*bis*-acrylamide, and 3 μL of 5% aq solution of AIPD in 382.5 μL water, degassed under vacuum for 8 min) was added to the 1 × 1 cm × 100 μm mold. The slide was then placed in a glass reactor and kept under N_2. The reactor was then lowered into an oil bath. The reactor was kept at 50°C under N_2 for 30 min, and then the slide was removed and allowed to cool to room temperature. The parafilm mold was then peeled away to reveal the hydrogel film. In a similar manner, the capillary hydrogel was prepared in flame-drawn glass capillary tubes with narrow-end IDs of 30–100 μm.

Transport Experiments

All transport experiments were performed at 23°C. The hydrogel films or capillary hydrogel were hydrated in water overnight, and then immersed in the monoclonal antimouse IgG-FITC solution (4 mL, eightfold dilution of stock solution with water) to begin the filling process. Immersion time before beginning release measurements for both the films and the capillary hydrogels was 15 min. In the case of the films, the slides were placed in a 3.5 × 0.5 cm polystyrene dish, and the dish was covered to minimize any effects from evaporation. None of the outer reservoir solutions were stirred, because the solutions used to fill the capillary hydrogel, and through which the protein was to be transferred to the silica surface, would not be stirred. The solution was agitated during loading and removal of the slide into and from the dish. After a predetermined time, the slides were removed from the antibody solution, rinsed with water and the hydrogel fluorescence was measured at 524 nm using a SLM 8000 fluorimeter. The fluorescence beam was set orthogonal to the plane of the hydrogel films and capillary hydrogel. The wavelength of 524 nm was chosen for monitoring the fluorescence, based on the emission spectrum of antimouse IgG-FITC on a fused-silica slide. The hydrogels were then reimmersed into the antibody solution until the time for the next measurement. Care was taken to minimize the time

that the hydrogels were out of the antibody solution, in order to prevent significant drying. Measurements were made until the fluorescent readings reached a plateau. Then the hydrogel films were immersed in 4 mL water and the release of the antibody was monitored in a similar manner. After each experiment with the hydrogel films, each film was carefully removed from the silanized fused-silica slide and the residual fluorescence on the slide was measured. A minimum of three isotherms for both uptake and release was acquired in this manner. Release data for capillary hydrogel were gathered separately from the uptake data. The capillary was immersed in the antibody solution described above in a capped glass vial for 15 min, and then immersed in 4 mL water. Data were then collected as described for the films. Great care was taken to position the capillary in the fluorimeter so that the beam passed through the same portion of the capillary for each measurement. Six isotherms for both uptake and release of antibody were acquired.

Calculations

Fluorescence uptake and release data for the hydrogel film and the capillary encapsulated hydrogel was fit to an exponential of the form

$$f = f_0\left(1 - e^{-t/\tau}\right)(\text{uptake}) \text{ and } f = f_0 e^{-t/\tau}(\text{release}), \tag{1}$$

where f was the measured fluorescence intensity, f_0 was the starting fluorescence for the release data or the asymptotic fluorescence for the uptake data, and τ is the characteristic decay time.

Diffusion constants (D) for hydrogel release were calculated using the one-dimensional diffusion equation with the solution,

$$C(t)/C_0 = 8/\pi^2 \sum_{n=0}^{\infty} 1/(2n+1)^{2^{e^{-(2n+1)^2\pi^2 Dt/4L^2}}}, \tag{2}$$

where $C(t)$ is the concentration of protein as a function of time, C_0 is the concentration in the gel at time $t = 0$, and L is the thickness of the gel *(30)*. For the hydrogel films, $L = 100$ μm and for the capillary hydrogel, $L = 1.2$ cm. For sufficiently large t, the higher order terms can be neglected. So, at time $t_{1/2}$ when $C(t)/C_0 = 1/2$, we have:

$$D = 4/\pi^2 \ln\left(16/\pi^2\right) L^2/t_{1/2} = (0.1958) L^2/t_{1/2} \tag{3}$$

Hydrogel uptake data were calculated using a trivial modification of equation *(2)*, which also yields equation *(3)* for the determination of D.

Diffusion constants for the capillary release and fill data were also calculated using equations *(2)* and *(3)*, when in this case L is the length of the hydrogel region in the capillary. The one-dimensional diffusion equation was used, because diffusion of material was limited to flow along the length of the capillary to the end. To calculate D, $t_{1/2}$ was taken from the experimental fits in Eq. 1 ($t_{1/2} = \tau \ln 2$), and was substituted into Eq. 3.

Transfer of Antibody to Silica Surface

Fused-silica plates were chemically modified with an aminosilane (trimethoxy-silylpropyl diethylenetriamine), as described above for the preparation of methacryloxypropyltrimethoxy silane-coated slides to enhance reception of the antibody. The capillary hydrogel was immersed in monoclonal mouse anti-FITC (eightfold dilution of stock solution with water, approx 1 mg/mL) for 30 min. The hydrogel swelled slightly from solution uptake, causing the tip to extrude from the end of the capillary. The extruded hydrogel tip was then brought into short contact (approx 1 s) with the DETA-coated silica plate through a small water droplet. The capillary was withdrawn, positioned over a second droplet, and the process repeated. The water droplets were used to ensure that the deposited antibody did not dry on the surface and denature. Following the deposition of the antibody spots, the plate was immersed in a bovine serum albumin (BSA) solution (1:1000 w:w) to block nonspecific binding on the remaining areas of the surface. The plate was washed and immersed in a solution containing FITC-conjugated BSA (antigen). The plate was washed a final time and observed using fluorescence microscopy.

RESULTS AND DISCUSSION

The formation of the hydrogel films was straightforward. Typically, the water content of the hydrogels immediately following their preparation was approx 85% *(28)*. Upon hydrating the 2 and 4% crosslinked gels overnight, water content is known to increase to 97.3 and 90%, respectively *(28,29)*. Representative uptake and release profiles obtained for the transport of monoclonal antimouse IgG-FITC into and out of the thin 2 and 4% crosslinked hydrogel films are presented in Fig. 1. The uptake and release of antibody from the hydrogels was modelled on diffusion processes to give the average diffusion coefficients listed in Table 1. The values of D are similar to those obtained for the diffusion of the protein through water (6.29×10^{-7} cm^2/s) *(31)*, as well as the release of similarly sized proteins through hydrogels prepared from other materials *(22)*, indicating that protein transport through the 2 and 4% crosslinked hydrogel films is a largely diffusion-controlled process. However, the release rates are consistently faster than the

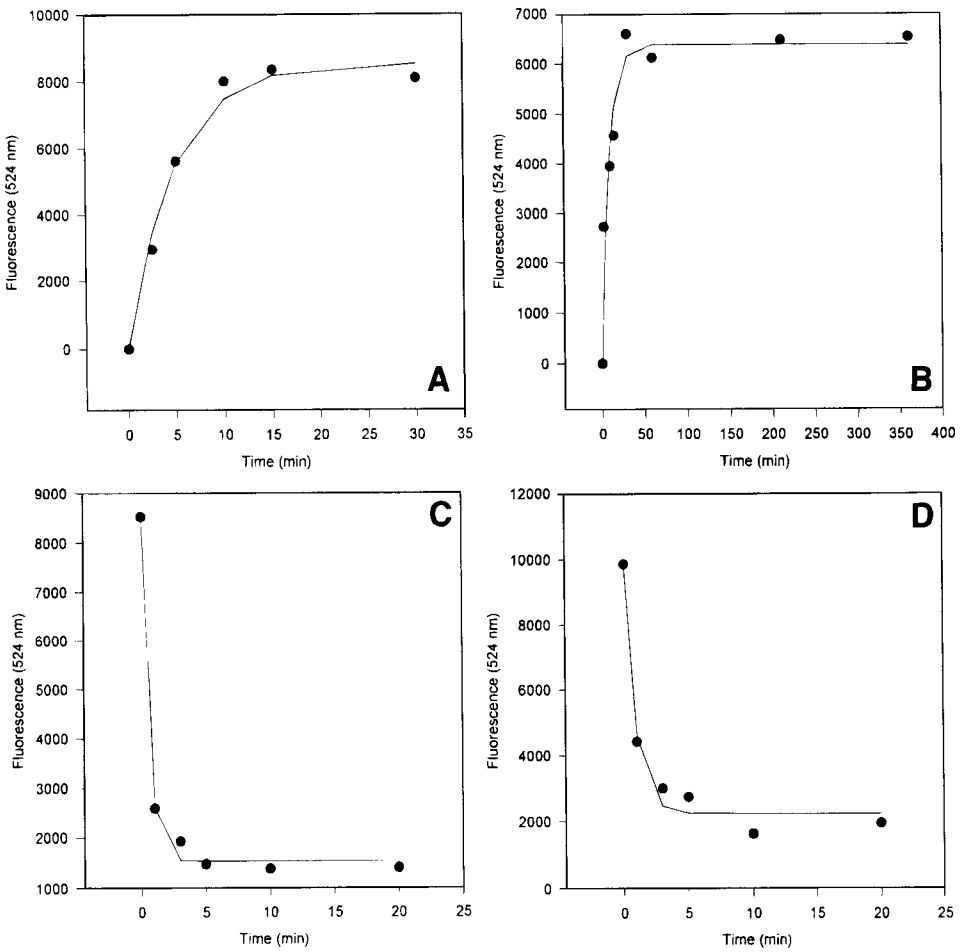

Fig. 1. Representative isotherms for fill of antimouse IgG-FITC into 2% **(A)** and 4% **(B)** crosslinked hydrogel films, and release of antimouse IgG-FITC from 2% **(C)** and 4% **(D)** crosslinked hydrogel films.

fill rates. One possible explanation for this behavior involves the effect of the differences in solute concentration between the reservoir and hydrated gel. The protocol for the transport experiments was designed to mimic the conditions under which a capillary hydrogel would be loaded with protein and used in transferring the protein to a silica surface. Because the protein would be loaded into capillary hydrogels hydrated in water, all of the hydrogels used in the transport experiments were hydrated in water and not buffer. In addition, the protein was to be transferred to the silica surface through a water droplet, and therefore the outer reservoir solution for all release experiments was water. The swelling of the hydrogel would be affected by any resulting differences in solute concentration between the outer reservoir and the hydrogel film (*32*). As solute concentration in the medium is increased,

Table 1
Average Diffusion Coefficients for Transport of Antimouse IgG-FITC Into and Out of Hydrogel Films

Crosslinking	Transport	D cm^2/s
2%	Fill	$1.1 \times 10^{-7} \pm 4.2 \times 10^{-9}$
4%	Fill	$5.5 \times 10^{-8} \pm 1.6 \times 10^{-8}$
2%	Release	$5.8 \times 10^{-7} \pm 3.0 \times 10^{-7}$
4%	Release	$3.3 \times 10^{-7} \pm 1.5 \times 10^{-7}$

gel swelling decreases slightly. In the protein-filling process, the hydrogel film initially contains deionized water when it is immersed in a large reservoir of protein/buffer. The resulting solute concentration differences set up an osmotic potential that draws water from the hydrogel to the buffer phase, causing gel contraction and a pore-size decrease that impedes entry of protein. Conversely, in the protein release process the hydrogel film initially contains protein/buffer when it is immersed in a large reservoir of deionized water. Water is osmotically drawn into the gel, swelling occurs, pore sizes increase, and protein release is facilitated.

The swelling of similar neutral sugar-based hydrogels has been demonstrated to be essentially independent of pH from 1.5 to 8.0 *(32)*. Therefore, effects resulting from differences in the pH of the reservoir (pH 7.4 during filling process, and pH 5.5 during release process) and the film (pH 5.5 during the filling process, and pH 7.4 during the release process) and probably negligible. It should also be noted that the fluorescent label of the antibody is hydrophobic, which may cause the antimouse IgG-FITC conjugate to diffuse more rapidly through the hydrophilic environment of the hydrogel than would be the case for the nonlabeled antibody.

The residual fluorescence observed for the protein-release isotherms reflect the amount of nonspecific binding of the protein to the silica plates, as determined by measuring the fluorescence of the plates minus the hydrogel film at the completion of each release experiment. In each case, the plate fluorescence equaled the residual fluorescence. Therefore, under the conditions of these experiments, no protein remains entrapped in the hydrogel after release is complete. This is a very important point, because it demonstrates that protein is not adhering to the gel or becoming entangled in any sidechains within the gel. Also, the results for each 2 and 4% crosslinked hydrogel film indicate that the pores produced during hydrogel crosslinking are consistently large and probably homogeneous from preparation to preparation. Thus, the results demonstrate that the pore structure and chemical nature of the hydrogel do not hinder the rapid, free flow of the protein through the 2 and 4% crosslinked gels.

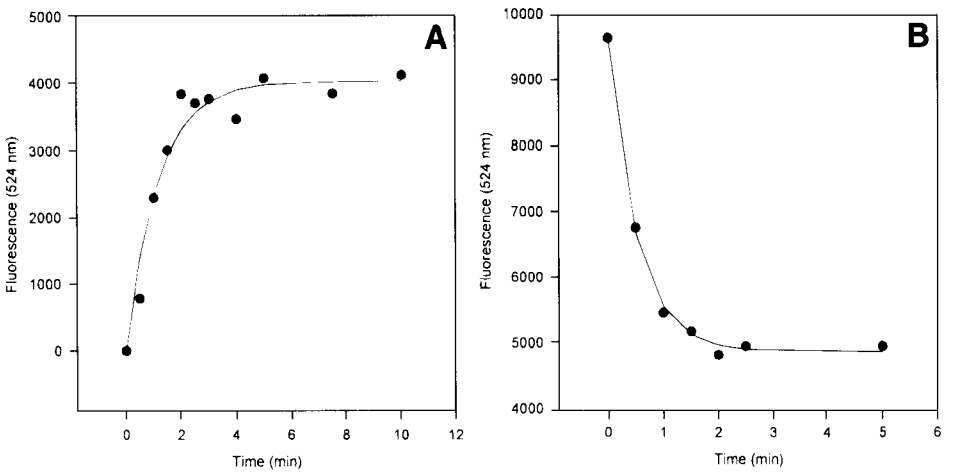

Fig. 2. Representative isotherms for fill **(A)** and release **(B)** of antimouse IgG-FITC into and from capillary hydrogel.

Based solely on the filling and release profiles and the calculated diffusion coefficients, either the 2 or 4% crosslinked hydrogel would be a suitable choice for forming the capillary hydrogel that will be used as the protein transfer sponge. The 2% hydrogel, however, was found to be the most appropriate for several reasons. The 2% crosslinked hydrogel was repeatedly handled and manipulated without any resulting damage to the gel. Increasing the crosslinking from 2 to 4% resulted in increased fragility of the hydrogel. In addition, in some cases the hydrogel may have to be extruded through the capillary in order to have a small piece of the gel protrude from one end of the capillary to form a contact point for protein transfer to a surface. Because the lower crosslinked hydrogel is more elastomeric and more stable to handling, the 2% hydrogel would be a better choice to form the capillary hydrogel.

Representative filling and release profiles obtained for monoclonal antimouse IgG-FITC transport into and out of the capillary hydrogel are shown in Fig. 2. The capillary hydrogel was formed directly inside the capillary. Differences in the maximum fluorescence observed between the uptake and release profiles result from differences in the positioning of the capillary in the fluorimeter. Care was taken to maintain capillary positioning throughout each individual experiment. The results demonstrated the stability of the capillary hydrogel to repeated operations. The results also demonstrate that, as with the hydrogel films, a rapid free flow of antibody through the hydrogel was achieved. In fact, diffusion coefficients calculated for the transport of the antibody through the hydrogel ($D = 7.4 \times 10^{-3} \pm 2.3 \times 10^{-3}$ cm^2/s for filling, and $1.3 \times 10^{-2} \pm 2.0 \times 10^{-3}$ cm^2/s for release) were several orders of magnitude greater than would be anticipated for a process involving a simple

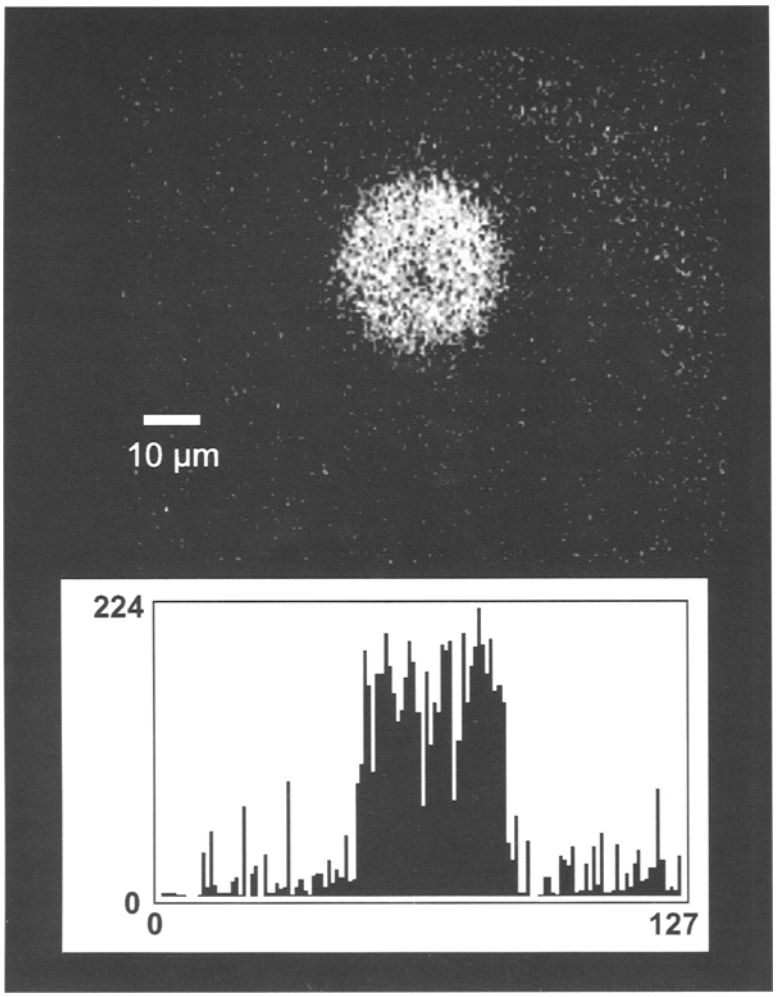

Fig. 3. Fluorescence microscope image of a representative micropattern of antimouse IgG-FITC transferred to aminosilane-coated silica surface using the capillary.

diffusion mechanism. One possible explanation for the accelerated transport behavior is the influence of tip hydration. In contrast to the 2% crosslinked hydrogel film (1 × 1 cm × 100 μm), only a small piece of the gel protruding from one end of the capillary (approx 30 μm diameter × 1.2 cm length) was directly exposed to aqueous filling or release reservoirs. The extent of the protrusion from the capillary depends on the state of hydration of the gel. As with the films, data was obtained using a noncontinuous assay, which involved removing the capillary from the reservoir for fluorescence measurements, and then reimmersing the capillary back into the reservoir until it was time for the next measurement. Because of the differential in size

between the capillary hydrogel and the hydrogel films, it is reasonable to assume that the relative degree of drying in the extruded portion of the capillary hydrogel would be much greater than that for the hydrogel films. Consequently, the capillary hydrogel will be subjected to the effects of dehydration to a much greater degree than the hydrogel films. This dehydration and subsequent rehydration with water and/or protein molecules cause a rapid swelling of the hydrogel, which draws in water and protein molecules at superdiffusional rates. Thus, antibody uptake/release will be significantly greater than for simple diffusion of the protein into a capillary hydrogel with a constant level of hydration. In fact, a fully dehydrated capillary hydrogel draws in protein and water rapidly, and fills the gel in a matter of seconds. The rate of filling for the dehydrated hydrogel was much too fast to obtain sufficient data points to measure a diffusion constant, given the limitations of our experimental protocol.

In order to test whether or not the capillary hydrogel would transfer the antibody to a surface, an antibody-loaded capillary hydrogel (30-μm diameter tip) was brought into contact with a sticky aminosilane (DETA: trimethoxy-silylpropyl diethylenetriamine)-coated, fused-silica plate within a water droplet on the surface of the plate. After removal of the capillary, the remaining plate surface area was blocked with BSA, and then the antigen, FITC-conjugated BSA was added. A representative circular micropattern obtained via fluorescence microscopy is shown in Fig. 3. The pattern arises from FITC-BSA bound to the transferred monoclonal anti-mouse IgG-FITC, clearly indicating that the antibody remains active after the transfer. The average and peak fluorescence intensities of the circular region are 14.1 and 19.6 times greater than background, indicating good transfer of active antibody to the surface at reasonably high spatial resolution (approx 5 μm). No transfer of the hydrogel to the surface was observed. The same hydrogel tip can be used at least three times without reloading for significant transfer of protein to the silica surface.

CONCLUSION

Transport of antibody protein through sugar-containing poly(acrylate) hydrogels has been demonstrated to be rapid and free flowing. Protein transport through hydrogel films could be described by a diffusion mechanism, but protein transport through a capillary hydrogel was several orders of magnitude faster, because of capillary tip dehydration occurring in addition to diffusion. Finally, transfer of an antibody to a silica surface was accomplished using the capillary hydrogel. These experiments represent an essential first step in the development of a nonlithographic protein-patterning technology for the fabrication of multianalyte sensor arrays.

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