Formation of Gradients of Proteins on Surfaces with Microfluidic Networks

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The capability of microfluidic networks to pattern substrates with proteins is extended to create density gradients of proteins on surfaces. The networks are micromachined in Si, and the substrates are hydrophobic poly(dimethylsiloxane) (PDMS) elastomers. The gradients result from the progressive depletion of proteins in the fluids due to their adsorption onto the PDMS substrate as the solution travels along the microchannel. Forming gradients of rhodamine-tagged antigens on PDMS and binding the antigens with fluorescein-tagged antibodies from solution enable us to study the binding behavior of these partners on a surface: Detection of the fluorescence associated with either partner suggests that recognition of the surface-immobilized antigens by an antibody from solution is more effective for a low density of antigens on the surface.

Introduction

Biological processes rely on molecular interactions between ligands and receptors, including enzymes and their substrates or inhibitors, adhesion factors and cell receptors, and antigens and antibodies, for example, all of which fulfill vital functions in organisms. The specificity and occurrence of ligand−receptor interactions must be exquisitely tuned to the needs of organisms for this reason. A particular and important use of ligand−receptor interactions in vitro is for immunodiagnostics where the interaction between an antigen and its antibody often occurs at a solid−liquid interface.[1]

A binding event at a solid−liquid interface is not comparable to one in solution, however. Proteins tend to alter their native conformation[2,3] as they adsorb onto liquid−solid interfaces and onto hydrophobic surfaces in particular. The binding properties of antibodies to antigens depend for both on their native three-dimensional structure; thus conformational changes of these proteins upon adsorption can affect their binding characteristics.[4,5] Steric access to the binding sites, orientation of the immobilized protein, and the proximity between these proteins are additional factors that affect interactions between ligands and receptors near a surface.[6−8]

Simultaneous control over the position and density of proteins on a surface could constitute a powerful means to investigate the binding properties between two partners on a surface.[9−14] We propose in this Letter that microfluidic networks (μFNs)[15,16] be used to create gradients of immobilized proteins on a surface to investigate how the density of the immobilized proteins on the surface influences their recognition by antibodies from solution. Specifically, we prepared gradients of proteins on PDMS which served as antigens to bind antibodies from solution. The advantage of this method compared to previous studies that investigated the effect of the density on the binding properties of immobilized proteins[6,7] is that (i) forming gradients of proteins with μFNs is simple, fast, and uses small amounts of reagents, (ii) it provides a large ensemble of adjacent zones of a substrate with varying density of deposited proteins, which facilitates screening the results, and (iii) fluorescence tagging can be used to reveal the presence of both binding partners on the surface simultaneously.

Figure 1 summarizes our approach in which a PDMS substrate is placed across the channels of a Si micromachined μFN to pattern lines of proteins onto the substrate. The μFN comprises two macroscopic pads (2×3 mm^2) separated by an array of 5-mm-long microchannels, Figure 1B. Capillary forces between a solution containing proteins and the hydrophilized walls of the μFN induce filling of the channels,[17] leading to deposition of proteins over the hydrophobic regions of the PDMS substrate exposed to the filled channels, Figure 1C.[16] The gradient of proteins on the PDMS substrate forms during this step: by flowing into the channels, the solution of protein is gradually

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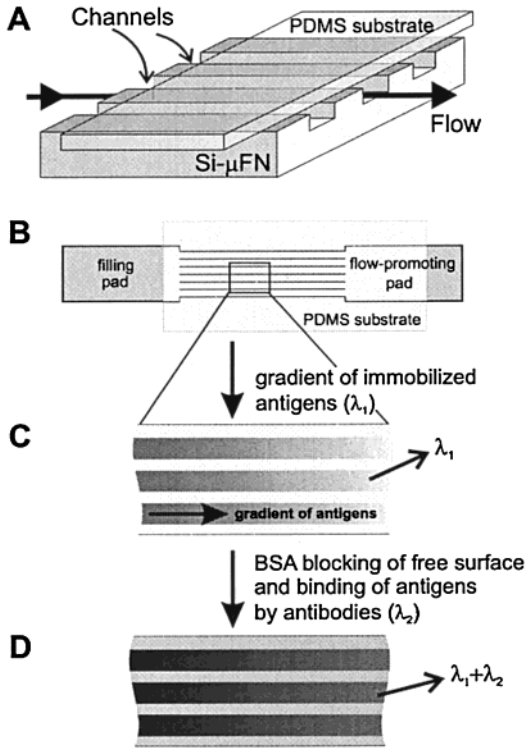

Figure 1. Si μFNs can pattern a PDMS substrate with a gradient of immobilized biomolecules. (A) The seal between the network and the elastomeric substrate confines the deposition of biomolecules from solution onto the PDMS regions exposed to the microchannels. (B) The PDMS substrate is placed across an array of microchannels and covers only a fraction of the filling- and flow-promoting pads to enable filling of the channels and displacement of air by the liquid, respectively. (C) The geometry of the μFN and the limited amount of proteins (antigens) flowing through the channels lead to a depletion gradient of immobilized antigens on the surface. These antigens can be tagged for example with a fluorophore λ_1. (D) After removal of the μFN and blocking the surface against nonspecific binding with BSA, the immobilized antigens are bound by fluorescently labeled (λ_2) antibodies from bulk solution. The two different fluorophores allow for their simultaneous, yet individual, detection by fluorescence microscopy.

depleted by fast adsorption of the proteins to the PDMS. Blocking the underivatized areas of PDMS with bovine serum albumine (BSA) is done after separating the PDMS layer from the μFN structures. Binding the immobilized antigens with antibodies from solution is the last step of this assay, and "simultaneous" detection of the binding partners proceeds when both proteins are tagged with a different fluorophore, Figure 1D.

The μFNs used here were made in a Si wafer because Si is a convenient material to micromachine: many geometries for the μFN patterns are possible without compromising the stability of the microchannels;[18] the SiO_2 layer present at the surface of the μFN can be readily cleaned and hydrophilized. PDMS was used as a substrate because this elastomer establishes conformal contact[19] with the raised structures of the μFN and effectively seals the channels. The surface of PDMS is hydrophobic and promotes deposition of proteins from solution.[20−22] It is transparent and has little interfering fluorescence at the standard wavelengths of excitation of common fluorescent tags.

Experimental Section

2.1. Fabrication of Microfluidic Networks. Standard photolithography was used to pattern microchannels and pads in Si wafers. The wafers were first spin-coated at 4000 rpm with ~1.2 μm of AZ6612 photoresist (Hoechst), photoexposed through a Cr mask (Photronics, Dresden, Germany) and developed for 30 s in AZ400/H_2O (1:4; Hoechst). The photoresist acted as a mask for an inductively coupled plasma (reactive ion etcher from Surface Technology Systems, Cambridge, U.K.) to pattern the silicon wafer. Three different geometries for the μFNs were used in this work. A first geometry consisted of two 3 × 2 mm^2 pads (a filling and a flow-promoting pad) connected via an array of 30 channels, which were 5 mm long, 15 μm wide, and separated by 20 μm. Both pads and microchannels had a depth of 6.5 μm. The second type of μFNs was 1.5 μm deep. The third one had 16 parallel channels (5 mm long, 20 μm wide, and separated by 20 μm) that were each connected to a filling and flow-promoting pad (1.5 × 1.5 mm^2). For this geometry, all structures were 20 μm deep.

2.2. Preparation of Substrates. PDMS substrates were obtained by curing the prepolymer components of Sylgard 184 (Dow Corning, Midland, MI) at 60 °C for at least 24 h against the bottom of a polystyrene dish; these components were mixed according to the recommendations of the manufacturer, using an automatic dispenser (DOPAG MICROMIX E, Cham, Switzerland).

2.3. Proteins and Reagents. Biomolecules, assay reagents, and immunoconjugates were obtained from Sigma (Sigma Chemie, Buchs, Switzerland). Deionized water or Millipore water (R > 18.2 MΩ cm^{-1}) produced with a Milli-Q purification unit (Millipore) was used in this work. Phosphate-buffer saline (PBS) was prepared with 150 mM NaCl, 4 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4 in deionized water and had a pH of 7.4.

2.4. μFN Patterning of PDMS Substrates. PDMS substrates were cut from a PDMS layer cured against the bottom of a Petri dish and placed by hand onto the μFN without applying pressure. Conformal contact between PDMS and the raised structures of the μFN was sufficient to ensure good sealing of the channels. Solutions of protein with a concentration in PBS ranging from 10 to 1000 μg mL^{-1} were applied with an Eppendorf micropipet on the filling pad, from which the liquid entered and filled the hydrophilic channels of the μFN owing to capillary forces. This defined the starting time for the duration of the experiments. The fluid rapidly (1 mm s^{-1}) reached the flow-promoting pad. Removal of the PDMS substrate from the μFN was done quickly under a flow of BSA (1% in PBS). The PDMS substrates were then rinsed for 20 s under deionized water to remove partially bound proteins and were dried under a stream of N_2. Each experiment for a given duration of immobilization and concentration of the protein solution was done in triplicate and with simultaneous use of at least three adjacent μFNs.

2.5. Immunobinding Assays. Sites of adsorption on the PDMS substrates that remained free after derivatization with the antigens were blocked by exposing the entire substrates to a 100-μL solution of 1% BSA in PBS for 30 min at room temperature. The BSA solution was then pipetted back with care, and the PDMS substrates were covered with 100 μL of diluted antispecies IgG solutions (1:200 in PBS). The substrates were not allowed to dry during removal of the BSA solution so as to use the remaining thin film of liquid to help cover the surface homogeneously with the antispecies solution. The recognition step was performed for 30 min and stopped by rinsing the substrate with deionized water for 20 s and then blow-dried with N_2. Fluorescence images of the tagged proteins present on the surface were acquired with a fluorescence microscope (Nikon Labophot-2) equipped with band-pass filters, a 100-W Xe lamp, and a charge-coupled device camera (ST-8, SBIG, Santa Barbara,

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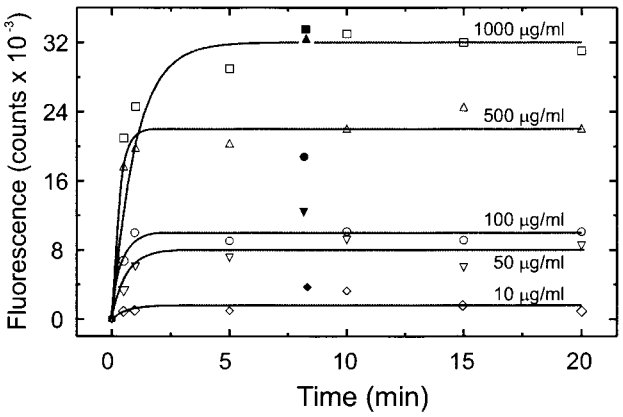

Figure 2. Fluorescence intensity of TRITC-labeled rabbit-IgGs deposited on PDMS as a function of protein concentration and incubation time. The comparison of protein adsorption from solution (filled symbols) and from a μFN (open symbols) having the same geometry as in Figure 1 as a function of the initial concentration revealed that the solutions of IgG were depleted in the channels (see text). A maximal coverage of IgG was reached for the highest protein concentration (1000 μg mL^{-1}), corresponding here to 32 000 counts.

CA) cooled to 0 °C. The fluorescence images were acquired with 765 × 510 pixels (16 bits dynamic range), 4-s integration time, and a magnification of ~25, using SkyPro software (Bisque, Golden, CO). A series of images covering the gradient area was acquired under identical conditions, assembled with the software Photoshop (Adobe Systems, Inc., San Jose, CA), and analyzed with ImagePro (Media Cybernetics, Del Mar, CA).

Results and Discussion

The first set of experiments characterizes the deposition of serum antibodies (rabbit) from solutions in PBS onto PDMS using a Si μFN having a geometry as shown in Figure 1. These antibodies are tagged with a rhodamine (TRITC) derivative so that the fluorescence intensity at the surface indicates how many proteins were immobilized for a given solution concentration and time of deposition, Figure 2. The profile of the curves reveals that initially deposition is fast but saturates at a certain protein coverage, which depends on the initial concentration of protein in the solutions that filled the μFN. For deposition from solution (filled symbols in Figure 2), maximum coverage is obtained using a concentration of antibodies in PBS of at least 500 μg mL^{-1}.[6,8,24] In contrast, for solutions flowing in the μFNs (open symbols in Figure 2), reducing this concentration from 1000 to 500 μg mL^{-1} already decreases the amount of antibodies immobilized on the PDMS surface. The curves in Figure 2 reveal that this observation is general: at equivalent concentrations and durations of reaction, fewer antibodies are deposited onto the substrate using a μFN than when a drop of solution of proteins was placed directly onto the PDMS substrate. We attribute this difference to the depletion of antibodies in the solutions filling the channels.[16] The site for assessing the amount of surface-adsorbed proteins by fluorescence was always the same distance from the filling pad of the μFN. The high surface-to-volume ratio of the microchannels provides a large area for derivatization per unit volume of protein solution. As a consequence, only the beginning of the channels is covered with proteins when the concentration of proteins in the solution is not high enough. Promoting the flow of the solution through the channels is in some cases important for ensuring the renewal of their content and for reducing or preventing this depletion effect when necessary.

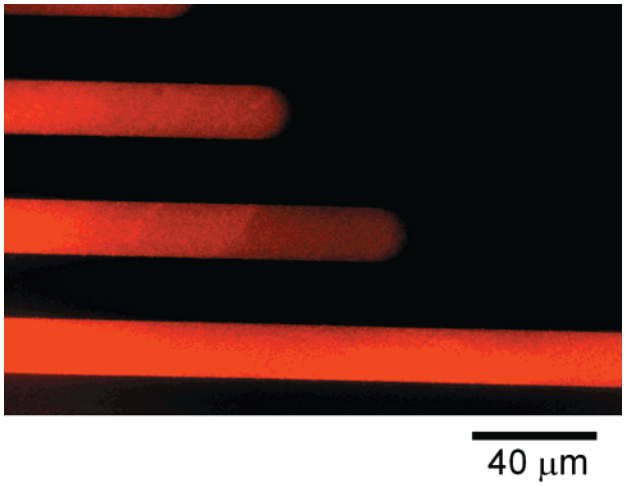

Figure 3. Short-scale gradient of a fluorescently labeled antibody deposited from a μFN onto a PDMS substrate. The protein solution had a concentration of 100 μg mL^{-1} of TRITC-tagged IgGs in PBS, and the Si μFN had 20-μm-wide and 1.5-μm-deep channels. The Poiseuille flow profile and the sharp drop of surface coverage reflect the fast rate of deposition of the IgGs from the fluid, the laminar nature of the flow, and the high speed of filling.

In this work, the depletion of proteins in the fluids displaced along the microchannels enabled the formation of surface-density gradients of immobilized proteins. The small dimensions of the channels are desired and have an important influence on the creation of surface-density gradients. Depletion of reactants flowing in a microchannel is exacerbated when the characteristic diffusion length of the reactants attains the same order of magnitude of or becomes greater than the cross section of the conduits. In this case, deposition of proteins, for example, can occur rapidly because the adsorption process is not limited by the transport from the solution to the substrate. The fluorescence microscopy image in Figure 3 illustrates such a situation. The 15-μm-wide and 1.5-μm-thin channels used here were very efficient in guiding a solution of antibodies dissolved in PBS and favored the loss of antibodies from solution by deposition onto the PDMS substrate. In this example, the length scale of the gradient is only ~30 μm. The shape of this gradient is equally interesting: it reflects the profile of fluid velocity within the channel near the depletion zone. The flow was fastest in the middle of the channel (Poiseuille type of flow profile); hence proteins could be carried by the fluid a little bit farther along the channels before they were deposited.

Even though it is straightforward to create a gradient of proteins on a surface like the one displayed in Figure 3, we suggest that it is important to have the factors affecting the shape and the length scale of the gradient well under control. The gradient should not be too short in order to facilitate the evaluation of the results. The concentration of proteins in the fluid and the geometry of the channels can serve to "shape" the gradient; the wettability of the channels and the reactivity between the proteins and interfaces are not convenient parameters for controlling the formation of the gradient.

Figure 4 illustrates how it is possible to take advantage of an array of independent channels to pattern lines with different densities of proteins onto PDMS.[23] Solutions with decreasing concentration of rabbit antigens in PBS were drawn in each channel where their deposition was homogeneous and resulted in a decreasing amount of

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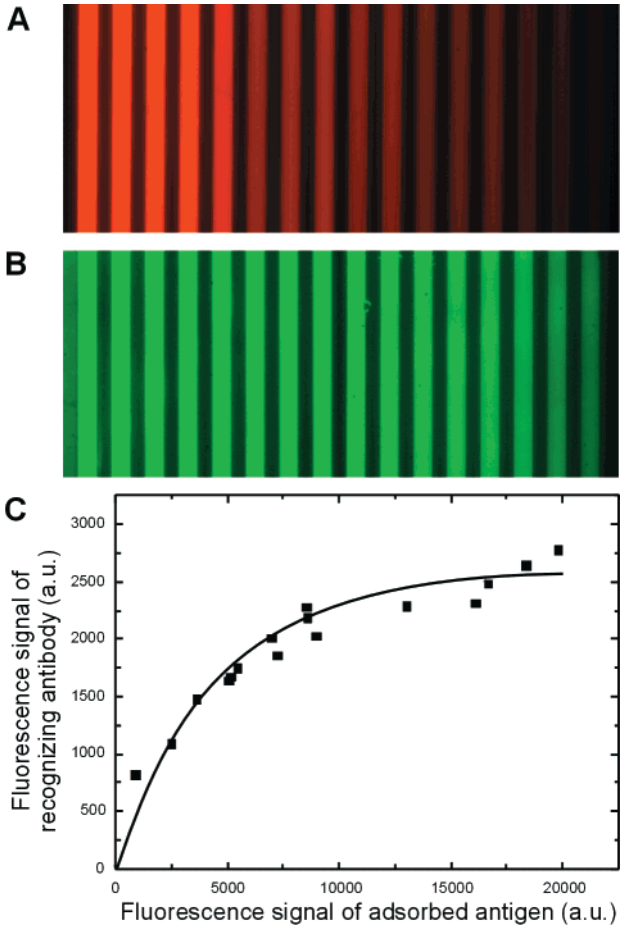

Figure 4. Detecting the antigens on a surface and their bound antibodies reveals the nonlinear binding behavior of these partners on the surface. (A) A 16-channel μFN was used to pattern 20-μm lines of decreasing antigen coverage onto a PDMS substrate (left line to right line) using solutions ranging from 2 mg mL^{-1} to 5 μg mL^{-1} in PBS. The μFN was 20 μm high, and the antigen was labeled with rhodamine. (B) The deposited antigens were recognized by a specific antibody carrying a fluorescein label. Although the former pattern was reproduced accurately, the fluorescence of the recognizing species did not scale proportionally to the one of the immobilized antigen. (C) A plot of the amount of bound antibody versus the amount of surface-immobilized antigen corroborates the observation from (B): the line fitting the data deviate from a linear binding correlation.

proteins on the surface from the left line to the right line. No gradient has formed in the direction of the channels because the μFN used here had 20-μm-deep channels (and thus had a less critical surface-to-volume ratio) that were connected to a "large" flow-promoting pad. Filling of each pad ensured at least an ~50-fold renewal of the content of the associated channel. In some sense, the substrate here is decorated with a "discontinuous" gradient in the direction across the lines of proteins. Such a pattern is useful to investigate the binding behavior of the immobilized antigens by antibodies from solution as a function of the antigen density on the surface in a discrete manner. Detection and quantitation of both partners on the surface are done simultaneously using fluorescence. This has the advantage that all the necessary information is present in a small region of the surface: the proteins forming the pattern are rigorously exposed to the same conditions for rinsing, blocking, and recognition, which provides a high level of self-consistency in this experiment.

The left line in Figure 4A has the highest coverage of antigens on the PDMS substrate and the right line the lowest coverage. Immunocomplexation of the TRITC-tagged rabbit antigens was done, after a BSA blocking step, by exposing the patterned PDMS substrate to a solution containing FITC-labeled anti-rabbit antibodies (diluted 1:200 from the commercial solution). Detection of the second antibodies using fluorescent labels, Figure 4B, reveals that their presence on the surface matches the pattern of the rabbit antibodies immobilized in the regions corresponding to the channels. The microfluidic networks used here were optimized for manipulating minute amounts of protein and having a micrometer-scale pattern yet well within the reach of conventional fluorescence microscopes. The red and green fluorescences are proportional to the density of antigens and antibodies on the surface, respectively, but it is not possible to compare these signals directly because several factors contribute to their magnitude. A slightly different number of fluorophores per molecule, distinct spectral properties between TRITC and FITC labels, and the sensitivity of the detector for FITC and TRITC are the principal factors. This is the reason we calibrated the fluorescence signals with the amount of protein on the surface (see below). Plotting the fluorescence from the antigens versus the fluorescence associated with the antibodies, Figure 4C, reveals a nonlinear correlation between these two quantities. Apparently, proportionally more antibodies are present on the surface when their respective antigens have a lower coverage on the surface.[6−8,24]

Forming a continuous gradient of immobilized IgGs and recognizing them with their binding partners from solution should complement the previous experiment: such a gradient can offer a continuum of decreasing densities of immobilized proteins and should prove experimentally simpler than using a μFN with a series of independent channels. Figure 5 illustrates this situation. The fluorescence microscope image in Figure 5A corresponds to a gradient of rabbit IgGs deposited onto PDMS from a μFN having an array of channels connected to the same filling zone. The image covers the beginning of the channels from where the depletion phenomenon already started. We again evaluated binding between the immobilized antigens and their partners, after a BSA blocking step, with fluorescence microscopy, Figure 5B. The intensities of the fluorescence associated with either partner on the surface along the gradient are reported in Figure 5C. The red fluorescence from the immobilized antigens decreases along the channels due the depletion phenomenon. The green fluorescence does not evolve in a similar manner, however. As anticipated from Figure 4, the fluorescence from the antibodies (green curve in Figure 5C) decreases less quickly than that associated with the antigens (red curve).

It is possible to calculate the surface coverage for both proteins for a given fluorescence intensity measured on the surface. We assume that up to one layer of proteins can be deposited on the substrate. As a reference for full coverage we use the maximal fluorescence intensity measured after a long deposition time (1 h) from a high concentration of proteins (1 mg mL^{-1}, see Figure 2). Another two important assumptions are that the simultaneous presence of the two fluorophores on the surface does not affect their emission (no fluorescence resonance energy transfer) and that their fluorescence is independent of their density on the surface (no self-quenching). Control experiments suggested that these latter assumptions are

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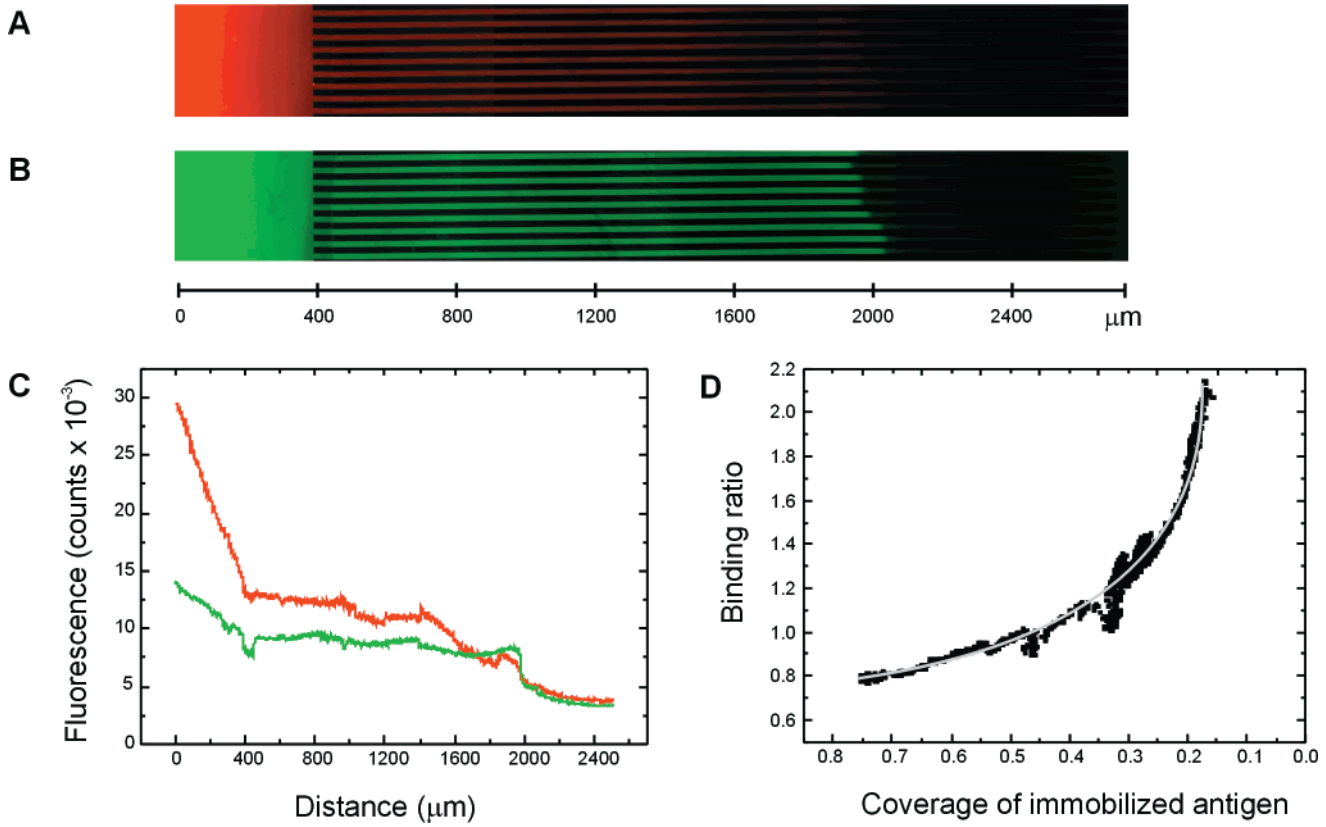

Figure 5. (A) Continuous gradients of antigens (rabbit IgGs) were produced by a μFN on a PDMS surface and (B) recognized by their antibodies (anti-rabbit IgGs) from solution. The gradient profiles in (C) were measured using the fluorescence associated with either protein and those in (D) were converted into monolayer quantities of immobilized proteins to give the binding behavior between the antigens and antibodies. The latter graph illustrates that binding of the immobilized antigen by its antibody from solution is more favorable for antigens dilute on the surface.

correct, at least for the density of fluorophores reported in the experiments. Exciting FITC while simultaneously monitoring light emission in the red wavelengths did not result in an observable signal. These results indicate that color conversion was not a path for loss of signal from FITC. In contrast, the loss of fluorescence by self-quenching is more difficult to estimate.[25] The limited amount of fluorophores per IgG (3.7 on average per antigen and ~3.9 per antibody), the relatively large size of IgGs compared to the fluorophores, and the strong dependence of fluorescence quenching with the distance between fluorophores suggest that fluorescence quenching should not be a problem, at least for intermediate and low-density coverage of the IgGs on the surface.

In consequence, the fluorescence signals indicate how much of a monolayer of antigens and antibodies is present on the surface and hence leads to the binding ratio between the partners. This binding ratio is reported in Figure 5D and, importantly, confirms the nonlinear binding behavior of the partners on the surface. For the conditions of recognition used here, one antibody from solution binds on average one antigen on the surface for an antigen coverage of up to ~0.5 of a monolayer. The binding efficiency then gradually increases to ~2 as progressively fewer antigens are present on the surface. This observation was expected because there are many causes that can affect the recognition of an antigen on a surface by its antibody from solution. Among these causes, steric hindrance seems to be a particularly important factor: proteins that form a dense monolayer on a surface are likely to be less accessible for binding partners than if they are free in solution or adsorbed at a lower density on the surface. Orientation of the immobilized antigens, their conformation with respect to their density on the surface, the specificity of the immunocomplexation (monoclonal system vs polyclonal), and the relative sizes of the partners are also likely to influence their binding behavior at the solid−liquid interface.

Conclusion

It has long been recognized that the binding between a partner from solution and another on a surface can be affected by the density of the immobilized partner. Several additional factors may also be of importance, such as the orientation of the partners on the surface, their relative size, and the distribution of their sites of interaction. In our work, we create gradients of biomolecules on surfaces using μFNs on length scales from micrometers up to millimeters with minute amounts of protein solutions. Our methodology uses fluorescence labeling with which the amount of deposited antigens is readily compared to the amount of bound antibodies from solution. The binding ratio of such partners is not linear with the density of the antigen on the surface but improves with decreasing density of antigens on the surface. We suggest that gradients of biomolecules on surfaces created by μFNs are an excellent means to study the binding behavior between ligands and receptors on surfaces in general.

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