

# Immobilization of Proteins in Immunochemical Microarrays Fabricated by Electrospray Deposition

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**Electrospray (ES) deposition has been applied to fabricate protein microarrays for immunochemical assay. Protein antigens were deposited as arrays of dry spots on a surface of aluminized plastic. Deposition was performed from water solutions containing a 10-fold (w/w of dry protein) excess of sucrose. Upon contact with humid air, the spots turn into microdroplets of sucrose/protein solution from which proteins were either adsorbed or covalently linked to clean or modified aluminum surfaces. It was found that covalent binding of antigens via aldehyde groups of oxidized branched dextran followed by reduction of the Schiff bonds gives the highest sensitivity and the lowest background in microarray-based ELISA, as compared to other tested methods of antigen immobilization. The minimum concentration of a primary mouse antibody detected in indirect ELISA with such antigen microarrays was ~0.3–1.0 ng/mL for ELF-97 or BCIP/NBT substrates of alkaline phosphatase.**

Miniaturization of analytical instrumentation and combining assays in multianalytical test systems are two major trends in modern biochemical analysis. Microarrays containing hundreds of thousands different oligonucleotides are now commercially available and widely used in genetic studies.<sup>1</sup> Originally, the idea of a biochemical microchip as a bioanalytical device combining many assay elements in a single unit was advanced by R. Ekins in connection with arrays of antibodies and antigens in immunoassays.<sup>2–4</sup> Protein microarrays containing 100–200 elements, each 80  $\mu\text{m}$  in diameter, were first fabricated at Pharmacia using ink-jet deposition.<sup>4</sup>

Until recently, three basic techniques for deposition of biomacromolecules have been known: microspotting,<sup>5,6</sup> ink-jetting,<sup>7,8</sup>

and microcontact printing.<sup>9–11</sup> The first two techniques have limitations on size of spots (typically larger than 80  $\mu\text{m}$ ) and on speed of deposition that are imposed by a necessity for mechanical motion following deposition of every spot. Microcontact printing is distinguished by its remarkable nanometer scale resolution and ability for parallel manufacturing of many arrays simultaneously; however, certain problems, such as low efficiency of transfer, limited amount of material transferred from stamp, and difficulties in controlling such transfer to different surfaces, have to be solved before its wide application can be implemented.

Recently, a new electrospray-based technique has been developed to deposit proteins and other substances as ions and charged nanoclusters under control of electrostatic field.<sup>12,13</sup> This technique allows a reduction in spot size to 2–7  $\mu\text{m}$  and a manyfold increase in fabrication rate as a result of its ability to simultaneously manufacture thousands of identical microchips. ES-deposited enzyme molecules retain their catalytic activity, and antigens and DNA keep their ability to bind specific antibodies and complementary strands, respectively.

Fabrication of any biochip involves two stages: deposition itself and immobilization of the deposited molecules on a substrate surface. Adsorption of ES-deposited proteins on wet membrane filters has been tested as one immobilization procedure;<sup>12</sup> however, membrane filters have two major drawbacks as a carrier for immunoassays: a high background, especially in fluorescence detection, and the long times for binding and washing steps. Therefore, many assay protocols now use flat polystyrene surfaces as a carrier. Polystyrene, however, cannot be used directly in ESD because of lack of conductivity. Moreover, preservation of functional activity of protein molecules in ESD requires low humidity (20–30%) where proteins are deposited in a dry state,<sup>13</sup> whereas protein molecules can be adsorbed or covalently bound to surfaces only from a solution.

Here we describe one way to solve these problems by using a metallized plastic as a substrate and by introducing a special procedure that enables immobilization of proteins from dry ES

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deposits. We also compare here different techniques of protein immobilization based on such a procedure to develop microarrays having high sensitivity and low background in ELISA.

## EXPERIMENTAL SECTION

**Reagents.** Dextran,  $M_n = 20\,000$ , was obtained from Loba-Chemie, Austria.  $\text{NaCNBH}_3$  was a product of Jensey Company, Japan. An ELF-97 cytological labeling kit was purchased in the Molecular Probes, Inc., Eugene, OR. Human serums with standard amounts of IgE were taken from the "Diaplus IgE IFA" kit, a product of the Diaplus Company, Germany. Dichlorodimethylsilane (DDS) was an Aldrich product. *p*-Nitrophenyl phosphate,  $\gamma$ -aminopropyl triethoxysilane (APTES), 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate tablets, glutaraldehyde (GA), mouse IgG, AP conjugated anti-mIgG ( $F_c$ -specific, from goat), anti-mIgG (polyclonal, against whole molecule, from goat), AP conjugate of anti-hIgE antibody from goat ( $\epsilon$ -chain specific), monoclonal antibody to human IgE (mouse ascite liquid), BSA, casein and other proteins were products of Sigma. Defatted dry milk was kindly provided by Dr. O. S. Morenkov. All other chemicals were of analytical grade or purer.

**Preparation of Mouse Serum.** Whole serum was obtained from a mouse immunized intraperitoneally with a mixture of soybean trypsin inhibitor and concanavalin A, 60  $\mu\text{g}$  each. The proteins were dissolved in 0.25 mL of d/d water and mixed with 0.25 mL of the complete Freund's adjuvant. A second injection was given 3 weeks later with the same mixture of proteins (20  $\mu\text{g}$  each) dissolved in 80  $\mu\text{L}$  of d/d water and mixed 1:1 with the incomplete Freund's adjuvant. Plasma was collected 2 weeks after the second injection and stored at  $-20\text{ }^\circ\text{C}$  after the addition of 50% (v/v) glycerol.

**Materials.** Aluminized (0.5–0.6- $\mu\text{m}$  layer of Al) 50- $\mu\text{m}$ -thick Mylar film was used as a substrate for microarray fabrication. Polypropylene mesh obtained from Small Parts Co. (Miami, FL) was used as a mask in ES deposition of arrays of protein spots.

**Preparation of Proteins for ES Deposition.** Proteins were dissolved in d/d water to a final concentration of 100–150  $\mu\text{g}/\text{mL}$ , and the conductivity of the solutions was measured using a homemade 4- $\mu\text{L}$  microcell. If conductivity exceeded 0.05 S/m, the solution was subjected to dialysis against d/d water. Concentration of protein in the dialyzed solutions was determined using a homemade quartz microbalance.<sup>13</sup> Sucrose was added to all of the protein solutions in 10-fold (w/w) excess relative to the protein content.

### Different Modifications of Al Surface for ES Deposition.

**Plasma Cleaning (PAL).** The Aluminized surface of plastic was treated for 20–30 s in a plasma discharge at low air pressure,<sup>14</sup> rinsed with d/d water, and dried. All other modifications started with plasma cleaning.

**Modification of Al Surface with Proteins (PALP).** The PAL surface was exposed for 10–15 min to 0.5% protein (BSA or casein) in the physiological solution (0.15 M NaCl, pH = 7.2–7.5), rinsed with water, and dried.

**Modification of Al Surface with Proteins and Glutaraldehyde (PALP/GA).** PALP was treated for 15–20 min in a 1% solution of GA prepared on PBS (0.14 M NaCl, 2.5 mM KCl, 4.4 mM

$\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , pH = 7.2–7.5). The surface was then rinsed with water and dried.

**Hydrophobization of Al Surface (PALH).** PAL was placed in a 0.5-L flask with a small plastic dish inside. Dichlorodimethylsilane (10–15  $\mu\text{L}$ ) was applied onto the dish, and the flask was kept closed for 1–2 h. The film was then removed from the flask and baked in an oven at  $80\text{ }^\circ\text{C}$  for 2–3 h.

**Covering Al Surface with Poly(methyl methacrylate) Layer (PAL/PMMA).** The PAL surface was covered with a 1% solution of PMMA in ethylenedichloride. Excess solution was drained, and the wet surface was dried in air. The thickness of the PMMA layer determined under a Linnik microinterferometer was about 0.1–0.2  $\mu\text{m}$ .

**Treatment of Substrate Surface with Oxidized Dextran (PAL/Dex).** The PAL surface was aminated for 30 min in APTES solution (0.25 mL of APTES in 5 mL of d/d water, pH 7; the solution was prepared 1 h before use to allow APTES hydrolysis<sup>15</sup>), then it was rinsed with d/d water, dried, and baked at  $90\text{--}100\text{ }^\circ\text{C}$  for 9–12 h in a nitrogen atmosphere. After baking, the pieces were immediately brought into contact with a solution of oxidized dextran (30–50 mg/mL, 4% of oxidized monomers, prepared as described in ref.<sup>16</sup>) for 1 h at room temperature, rinsed with d/d water, dried, and stored at  $-20\text{ }^\circ\text{C}$  until use.

### Fabrication of Microarrays. Electrospray Deposition of Protein.

Deposition was performed on the modified Al surfaces through a polystyrene mesh<sup>12</sup> under the following conditions: humidity, 20–30%; distance between the capillary tip and the substrate surface, 60–70 mm; o.d. of capillary tip, 20–30  $\mu\text{m}$ ; voltage, +(3.0–6.5) kV; current, 20–25 nA. Typically, 10–40 pg of sucrose and 1–4 pg of protein were deposited in each spot with a lateral diameter of 7–15  $\mu\text{m}$ . The amount of solid within the spots was estimated from the spot volumes measured under a Linnik microinterferometer, assuming that the density of the deposit is 1000  $\text{kg}/\text{m}^3$ . If not used immediately, such microarrays could be stored at  $-20\text{ }^\circ\text{C}$  over silica gel for up to 8 months without a notable deterioration of the ELISA response.

**Immobilization of Proteins.** The film with antigen microarray was cut into pieces, 3  $\times$  3 mm in size. The pieces were glued to special floating holders and placed in a Petri dish with 100% humidity for about 30 min. Microdroplets of sucrose/protein solution are formed quickly under these conditions, and proteins are either physically adsorbed or chemically bound to the surface.

The arrays were then quickly rinsed with the blocking solution (0.5% BSA or 1% dry milk in 100 mM TRIS/HCl, 0.15 M NaCl, pH = 7.2–7.5) to remove any excess protein, kept for 45 min in a fresh portion of the blocking solution, rinsed 2–3 times with washing solution (0.05% Tween-20 dissolved in the physiological solution), and used in ELISA. Such a blocking procedure was used in all cases except for the one described below when blocking was combined with the reduction of Schiff bonds formed between amino groups of APTES and proteins and aldehyde groups of the oxidized dextran.

**Reduction of Schiff Bonds.** To prevent release of proteins from arrays as a result of hydrolysis of Schiff bonds, the latter were reduced for 15 min in 5% cyanoborohydride solution freshly

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prepared using the physiological solution with an addition of 0.5% of BSA to block the free surface and avoid blurring the spots. The arrays were then transferred into fresh cyanoborohydride solution without BSA and held there for 10 min, rinsed with the physiological solution, and additionally treated for 45 min in the blocking solution. Then the arrays were rinsed 2–3 times with the washing solution. If not used immediately, the samples were soaked in 10% sucrose solution, dried, and stored at  $-20^{\circ}\text{C}$ . We will further refer to these samples as PAL/DexR to distinguish them from PAL/Dex samples for which the reduction procedure was omitted.

**Immunoassays with Protein Microarrays.** Microarray-based ELISA (MB-ELISA) was performed in wells of the standard 96-welled microtiter plate. Before filling with antibody solutions, the wells were pretreated for 45 min in the blocking solution and rinsed with d/d water. All of the dilutions were made using the blocking solution. Pieces of the microarrays glued to the floating holders were placed face down into the wells filled with 100  $\mu\text{L}$  of antibody solutions. The solutions inside the wells were stirred with magnetic stir bars.

*Indirect ELISA of Mouse IgG.* Microarrays were fabricated from anti-mIgG using all of the immobilization techniques described above. After incubation in a solution of the primary antibody for 1.5–2 h, the microarrays were triply rinsed with washing solution and reacted for 3 h or overnight in fresh wells filled with anti-mIgG-AP conjugate diluted 40 000 times.

*Indirect ELISA of Human IgE.* Arrays of monoclonal anti-hIgE were prepared on PAL/DexR substrate, incubated for 2 h in standard human serum (diluted 1:6) with known amounts of IgE, rinsed 4–5 times with washing solution, and incubated overnight with a solution of anti-hIgE conjugated with AP (diluted 1:4000).

*Direct ELISA.* Microarrays of mIgG were fabricated on PAL/DexR substrate and incubated overnight in solutions of anti-mIgG-AP-conjugate of different dilutions.

*Detection of Immunobinding.* After the final 3–4 rinses with the washing solution, the microarrays were detached from the holders and placed face down for 1.5–2 h onto 10–20- $\mu\text{L}$  droplets of cytological ELF-97 or for 0.5–1 h onto droplets of BCIP/NBT solutions prepared as recommended by the manufacturers. The pH of the BCIP/NBT solution was reduced from 9.5 to 8.2–8.3 to avoid destruction of the Al layer at alkaline pH. The microarrays were then rinsed with water and dried.

Fluorescent products of the AP reaction with ELF-97 were imaged using a conventional fluorescence microscope equipped with a digital camera (Pixera Corp.). Products of the BCIP/NBT reaction were registered using the same microscope in a reflection mode, that is, with a cutoff filter removed. The optical density of the spots (gray level) in the images was measured using the SCION/NIH program freely available on the Internet.

**ELISA on Disks. Comparison of Different Techniques of Antigens' Immobilization.** Disks 7 mm in diameter were cut out of aluminized Mylar film with the Al surface modified in different ways as described above. The disks were glued to the bottoms of the wells in the standard 96-welled plate using a silicon glue (Sylgard 184 from Dow Corning Corp., Midland, Michigan) in such a way that only one side of the disks (plastic or Al) was exposed to the solution. Trypsin inhibitor was used as the antigen in these experiments. The disks were covered with 15  $\mu\text{L}$  of a

solution containing 0.5% antigen and 5% sucrose. After incubation for 30 min in the antigen/sucrose solution, the disks were rinsed twice with the washing buffer, and the wells were kept with 200  $\mu\text{L}$  of the blocking solution in them for 45 min. For part of the disks (PAL/Dex), the blocking procedure was combined with the reduction of Schiff bonds as described above. After blocking, the disks were incubated for 2 h with 100  $\mu\text{L}$  of plasma from the mouse immunized with trypsin inhibitor (diluted 500 times). The wells were rinsed 4–5 times and then incubated overnight with 100  $\mu\text{L}$  of anti-mIgG-AP that was diluted 1:40 000. The disks were again thoroughly washed with washing solution and transferred into fresh microtiter wells filled with 100  $\mu\text{L}$  of pNPP solution (10 mM pNPP in 0.2 M TRIS/HCl, 5 mM  $\text{MgCl}_2$ , pH = 8.0). After incubation in the substrate solution for 4 h at room temperature, the reaction was stopped by an addition of 100  $\mu\text{L}$  of 2 N NaOH, and the optical density was read at 405 nm.

Two types of controls were used for each type of surface tested in these experiments. In the first one, BSA was immobilized onto the disk surface instead of the antigen. In the second control, the disks with the immobilized trypsin inhibitor were incubated with plasma from a nonimmunized mouse.

## RESULTS AND DISCUSSION

We found here that commercially available aluminized plastic film is a good conductive substrate for protein microchips because of several useful properties: (i) the metal protected with a layer of oxide is relatively inert in neutral and slightly acidic or basic solutions, (ii) the mirror surface of the Al layer makes it easy to control the deposit visually, (iii) the surface has a low background and allows rapid washing, (iv) aluminized plastic is mechanically robust, and (v) microchips formed on such a plastic can be easily cut off to be tested separately.

To preserve functional activity of proteins upon ESD, the humidity should be kept relatively low<sup>13</sup> (20–30%). AFM analysis (our unpublished data) and theoretical estimations<sup>17</sup> show that under these conditions, proteins are mostly deposited as conglomerates of dry nanoclusters, thus posing a problem for protein immobilization. The problem is solved here by deposition of protein in a mixture with a large excess of a highly hydrophilic substance that readily adsorbs water in humid air, turning the array of solids into array of microdroplets from which solubilized proteins can be bound to a substrate surface. Sucrose has been chosen as such a substance, because it is highly hydrophilic and protects proteins upon drying<sup>18</sup> and upon ES deposition.<sup>13</sup>

**Formation and Composition of Microdroplets.** The inset in Figure 1 illustrates how the size of droplets changes in a humid atmosphere. Microinterferometric measurements of volumes of solid deposits and microscopic measurements of droplet volumes allowed the estimation of sucrose and protein concentration in microdroplets as a function of time. Figure 1 shows that the sucrose concentration rapidly decreases to 5–7% and then changes rather slowly. Thus, when sucrose/protein ratio is 10:1 upon ESD, immobilization of proteins proceeds from microdroplets containing 0.5–0.7% of protein and 5–7% sucrose during 90% of the total time

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Table 1. Optical Density in Standard ELISA on Different Surfaces and Sensitivity Limit in Microarray-Based Indirect ELISA on the Same Surfaces

	surface type <sup>a</sup>				
	PALP/GA	PAL/Dex	PAL/DexR	polymer surfaces	PAL
immobilization	covalent linking	covalent, via Schiff bases	covalent linking	physical adsorption	physical adsorption
optical density in ELISA <sup>b</sup>	0.036 ± 0.01	0.040 ± 0.013	0.072 ± 0.015	0.12 ± 0.025 <sup>d</sup>	0.037 ± 0.009
sensitivity limit <sup>c</sup> , ng/mL	10	10	1	10–30 <sup>e</sup> , 10–100 <sup>f</sup>	100–1,000

<sup>a</sup> See Experimental Section for description of surface types. <sup>b</sup> Trypsin inhibitor was immobilized on the disks, as described in the Experimental Section. Optical densities of ELISA readings at 405 nm are presented as average of 6 independent measurements with averaged control intensities subtracted (O.D. obtained for both control types was very close to the blank pNPP solution). RMS deviations from the average are presented. <sup>c</sup> Microarray (spots of 10–20 μm in diameter spaced 100 μm apart) of anti-mIgG was ES deposited on the surfaces and tested using different concentrations of mIgG used as the primary antibody in indirect ELISA. The lowest concentration of mIgG at which an array of fluorescent spots was observed is presented. See Experimental Section for more details. <sup>d</sup> Surface of Mylar film. <sup>e</sup> Al surface coated with dichlorodimethylsilane from gas phase (PALH). <sup>f</sup> Layer of PMMA on Al surface (PAL/PMMA).

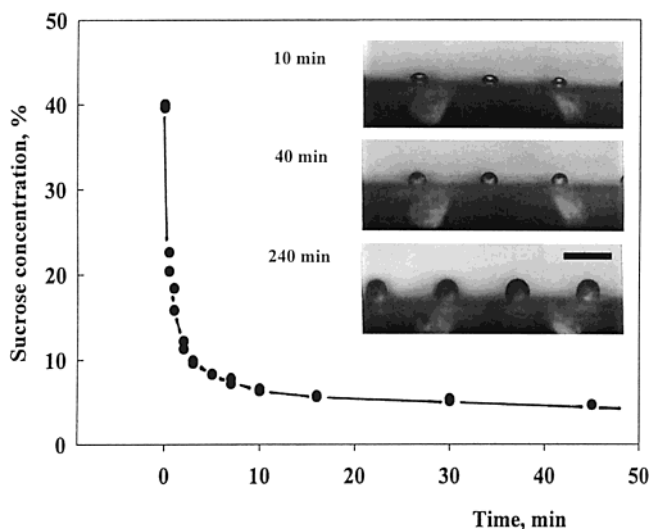


Figure 1. Changes of sucrose concentration in microdroplets exposed to 100% humid air. The inset presents microphotos of the droplets after different times of exposure to humid atmosphere. Bar denotes 100 μm.

(30 min) of exposure to humid air. The sugar concentration in these droplets is high enough to protect proteins<sup>19</sup> without a notable interference with protein solubility.

Protein concentration in microdroplets ( $C = 5$  mg/mL) seems excessively high as compared to that used upon protein adsorption in the standard ELISA ( $\sim 20$  μg/mL), but the ratio of protein amount,  $VC$ , ( $V$  is droplet volume) to surface area,  $S$ , is comparable in both cases. For a microdroplet approximated as a hemisphere with a radius  $R = 5$  μm this ratio is  $VC/S = C(1/2)(4/3)\pi R^3/\pi R^2 = (2/3)CR = 1.7$  μg/cm<sup>2</sup>, very close to 2–3 μg/cm<sup>2</sup>, used upon protein adsorption in the standard ELISA. Only a small fraction of this protein needs to be adsorbed to give a maximum signal in ELISA. As Balcells et al.<sup>20</sup> found for a number of polymer surfaces, the amount of adsorbed protein accessible for antigen–antibody interactions did not exceed 50 ng/cm<sup>2</sup>. Taking this into account, one can estimate that at least 2% of all of the protein should be bound from the droplet with  $R = 5$  μm and  $C = 5$  mg/mL. A 10-fold decrease of  $R$  would increase the amount of bound protein

to 20%. These estimates show that the concentration of protein in our microdroplets is not excessively high.

#### Comparison of Different Immobilization Procedures.

Standard indirect ELISA on disks was used to quantitatively characterize the amount of immobilized antigen available to react with the antibody after different types of immobilization. To model a real situation upon immobilization of proteins on microarrays, a composition of the antigen solution similar to that found in the microdroplets (0.5% protein and 5% sucrose) was chosen.

As one can see in Table 1, the highest ELISA response is provided by the antigen physically adsorbed on the plastic side of the aluminized Mylar film. The PAL surface produces an ELISA response three times lower than that on plastic. Binding on a PAL/Dex surface without reduction of Schiff base is unstable and results in an ELISA response as low as on PAL. Reduction of the Schiff links increases the response considerably, reaching 60% of the response on the plastic surface. The PALP/GA substrate was not effective: the ELISA response did not differ from that on a clean Al surface (PAL). Probably binding via short glutaraldehyde links results in more steric hindrance for interaction with antibodies than does binding via much longer dextran spacers. Thus, of all the methods tested, physical adsorption on a Mylar surface and covalent binding via dextran with reduced Schiff bonds (PAL/DexR) resulted in immobilization of the largest amounts of antigen accessible to reaction with antibodies.

#### Sensitivity of MB-ELISA with Different Types of Immobilizations.

The same types of surface modification were tested in the indirect MB-ELISA to find out which immobilization technique provides the highest sensitivity. Defined as the minimum concentration of antibody in solution capable of generating a reliable signal on microarrays, the sensitivities on different surfaces are listed in Table 1. It is seen that the highest sensitivity is obtained on PAL/Dex substrate with the reduction of Schiff bases. Omission of the reduction procedure resulted in a decrease in the sensitivity by an order of magnitude, which indicates that (i) antigens are really bound on PAL/Dex via Schiff bases and (ii) the Schiff bases are not stable enough to keep antigen molecules bound throughout the ELISA.

We failed to fabricate a microarray on the plastic side of aluminized Mylar film, which yielded the highest response in the ST-ELISA. Instead, we prepared PAL/PMMA and PALH surfaces in which a thin plastic layer formed on the Al surface is conductive

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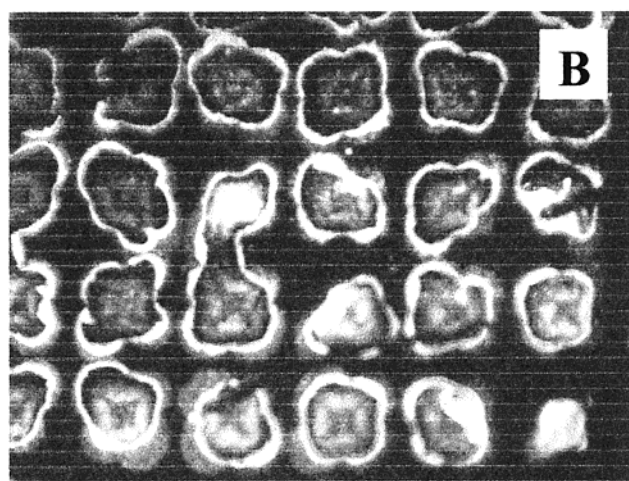
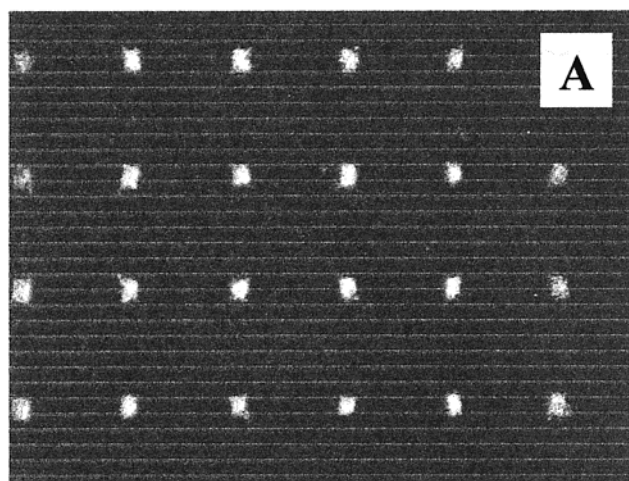


Figure 2. Illustration of quality of ELISA responses obtained using microarrays deposited onto different substrates. White areas show deposition pattern in distribution of fluorescent product of ELF-97 substrate. (A) Microarray of anti-mIgG deposited onto PAL/DexR; Al surface covered by oxidized dextran with Schiff bases reduced by NaCNBH<sub>3</sub>. (B) Similar microarray deposited on plasma-cleaned Al surface (PAL). Distance between the spots is 140  $\mu\text{m}$ .

enough to support ESD. As seen from Table 1, sensitivity of MB-ELISA on these surfaces is higher than that on PAL, but notably lower than those on PAL/DexR.

It was a surprise to find that the ability of a PAL surface to bind antigens greatly increases (instead of the expected decrease due to surface blocking) after the adsorption of proteins, providing a sensitivity of  $\sim 10$  ng/mL. This enhanced binding capacity of PALP is not connected with the electrospray procedure, because a direct spotting and drying of antibody solution on such surfaces shows a similar high ELISA response. Contrary to our expectations, GA treatment of the PALP did not increase the sensitivity of the microarray-based ELISA.

*Compatibility of Different Surfaces with the Technology of Protein Immobilization from Microdroplets.* Factors other than the ability of surfaces to bind proteins and to generate a high ELISA signal are also important in the choice of substrates for microarray fabrication. Thus, the size of the immobilized protein spots depends both on the size of the solid deposit and on the spreading of microdroplets over the surface during immobilization. Moderately hydrophobic surfaces such as PALP and PAL/Dex, where

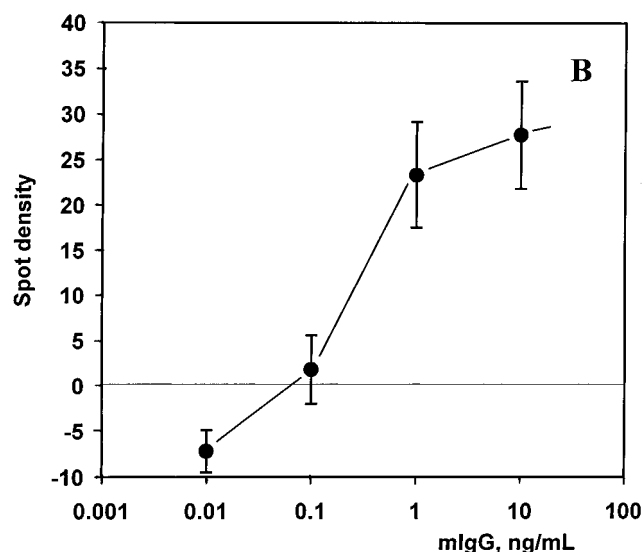
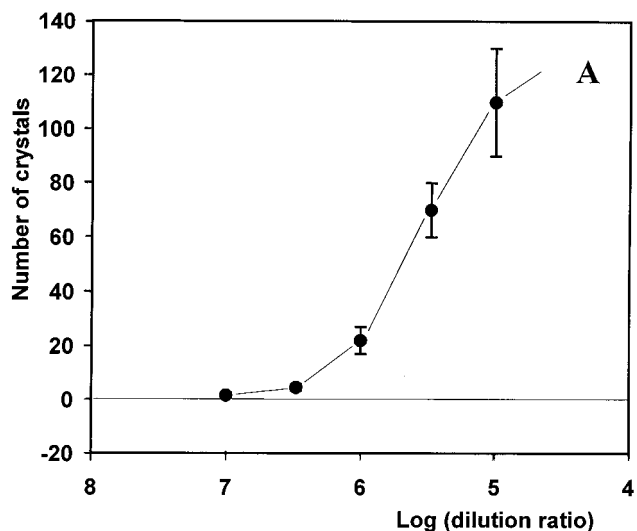


Figure 3. Dependence of ELISA response from protein microarrays on dilution of antibody conjugate, A, and on concentration of a primary antibody in solution, B. (A) Average number of ELF-97 product crystals in spots is shown as a function of anti-mIgG-AP conjugate dilution in direct immunoassay protocol using dextran-covered Al substrate (PAL/DexR). Crystals were directly counted for 10 different spots and averaged. An average of  $1.6 \pm 1$  and  $4.5 \pm 1.3$  crystals were found in spots at dilutions  $1:10^7$  and  $1:3 \times 10^6$ , respectively. (B) Intensity of spots of BCIP/NBT products measured as the difference between the gray level of the spots and the background in the vicinity of each spot related to 256 (maximum difference between gray levels). Indirect ELISA protocol was used with anti-mIgG deposited onto the dextran-covered Al surface (PAL/DexR), incubated with different concentrations of mouse IgG and anti-mIgG-AP conjugate diluted 40 000 times. Intensities of 10 different spots were averaged in each image.

spreading of sugar/protein droplets is restricted, allow obtaining microdroplets as small as the deposits themselves. As illustrated in Figure 2A, an array of identical fluorescent dots 10  $\mu\text{m}$  in diameter is observed by ELISA after ES deposition and the immobilization of antibodies on PAL/DexR. Highly hydrophilic surfaces, such as PAL, on which droplets are readily spread, are not suitable for microarray fabrication, as seen in Figure 2B.

Another limitation for the choice of substrate is connected with keeping a low background. Some modifications of the Al surface

(PALH, PALP/GA) resulted in a heavy background, making these surfaces also unsuitable for microarraying. The reduced ability of these surfaces to be blocked by BSA or dry milk or reversibility of the blocking process<sup>21</sup> may be responsible for the heavy background. Protein adsorption is greatly inhibited on PAL/DexR, yielding a very small background of MB-ELISA on this surface, as seen in Figure 2A.

**MB-ELISA on PAL/DexR.** Considering all the factors determining the quality of microarrays, we came to the conclusion that covalent binding via dextran with reduced Schiff bonds (PAL/DexR) is the most suitable technique for protein immobilization. It combines a high level of signal, high sensitivity, and a low background. That is why microarrays prepared with such an immobilization technique were studied in more detail using different ELISA protocols.

Direct immunoassay with an array of mouse-IgG was performed using ELF-97 as the AP substrate. The dependence of the average number of fluorescent crystals in spots as a function of the dilution is presented in Figure 3A. One can see that MB-ELISA is highly sensitive. An array of fluorescent ELF-97 product crystals was seen up to a 10 000 000-fold dilution of anti-mIgG-AP. This dilution is 300 times higher than that indicated by the manufacturer for a direct assay in dot blot protocol.

Indirect ELISA on an anti-mIgG array with ELF-97 substrate displayed fluorescent crystals at mIgG concentrations up to 1 ng/mL. Omission of the array treatment with the mIgG resulted in no visible array of AP products. It is interesting that the same sensitivity (1 ng/mL) was obtained with BCIP/NBT substrate, because a mirrorlike perfect Al surface allows observation of nonfluorescent insoluble product as easily as the fluorescent one. The dependence of the spot density of the BCIP/NBT product on the mIgG concentration is presented in Figure 3B. The optical density was measured as a difference between the gray level of the spot and the background in the vicinity of each spot. As seen in Figure 3B, a characteristic feature of the BCIP/NBT substrate system is a negative contrast at low mIgG concentration and in controls when the density of the spots is lower than the density of the surrounding background. Such a negative contrast may reflect catalysis of BCIP/NBT hydrolysis by the Al surface and an inhibition of this catalysis by the layer of immobilized protein. We have observed formation of a blue deposit on a clean Al surface when the latter was kept in contact with BCIP/NBT solution for more than 2 h.

Indirect ELISA with a microarray of anti-hIgE showed that the lowest detectable concentration of hIgE is  $\sim 1$  ng/mL or 0.5 I.U. No crystals were observed in the control experiments with a standard serum depleted of hIgE and when human serum was replaced with BSA solution.

Thus, antigen microarrays on PAL/DexR work well in different ELISA protocols, with different types of antibodies, and with different insoluble AP substrates. This method has been recently used in the fabrication of immunoarrays containing 28 different antigens. Their antigenic properties were tested in assays with mouse and human serums, and it was shown that they are well-retained (to be published elsewhere). We suggest, therefore, that the microfabrication technique described here can be applied for many different proteins.

## CONCLUDING REMARKS

Fabrication of protein microarrays by a technique combining dry ES deposition and immobilization of proteins from microdroplets offers several advantages. First, the deposited protein is kept dry and protected by carbohydrate during the entire fabrication procedure, which is favorable for preserving its structure and functional properties. Second, the immobilization procedure can be started and stopped simultaneously for all of the spots on the microchip. Third, the deposit can be stored for a prolonged time without immobilization.

**Abbreviations.** ES, electrospray; ESD, electrospray deposition; d/d, double-distillate; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; ELISA, enzyme-linked immunosorbent assay; MB-ELISA, microarray-based ELISA; ST-ELISA, standard ELISA; GA, glutaraldehyde; APTES,  $\gamma$ -aminopropyl triethoxysilane; DDS, dichlorodimethylsilane; PAL, plasma cleaned Al; PALH, PAL treated with DDS; PALP, PAL treated with protein; PALP/GA, PALP treated with GA; PAL/Dex, PAL treated with APTES and oxidized dextran; PAL/DexR, sample prepared on PAL/Dex with reduced Schiff bonds; PBS, phosphate-buffered saline; BSA, bovine serum albumin; AP, alkaline phosphatase; mIgG, mouse IgG; anti-mIgG, anti-mouse IgG; anti-mIgG-AP, anti-mouse IgG conjugated with alkaline phosphatase; hIgE, human IgE; anti-hIgE, anti-human IgE; anti-hIgE-AP, anti-human IgE conjugated with alkaline phosphatase; pNPP, *p*-nitrophenyl phosphate.

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