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Protein Microchips: Use for Immunoassay and Enzymatic Reactions

Pavel Arenkov,* Alexander Kukhtin,* Anne Gemmell,† Sergey Voloshchuk,* Valentina Chupeeva,* and Andrei Mirzabekov*,†,[1]

**Joint Human Genome Program, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 117984 Moscow, Russia; and †Biochip Technology Center, Argonne National Laboratory, Argonne, Illinois 60439*

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Different proteins such as antibodies, antigens, and enzymes were immobilized within the 100 × 100 × 20-μm gel pads of protein microchips. A modified polyacrylamide gel has been developed to accommodate proteins of a size up to 400,000 daltons. Electrophoresis in the microchip reaction chamber speeded up antigen–antibody interactions within the gel. Protein microchips were used in immunoassays for detection of antigens or antibodies, as well as to carry out enzymatic reactions and to measure their kinetics in the absence or presence of an inhibitor. A protein microchip can be used several times in different immunoassays and enzymatic kinetic measurements. © 2000 Academic Press

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The development and application of the arrays of immobilized biological compounds (biological microchips) have become a significant trend in modern biology, biotechnology, and medicine (1). The main advantage of biological microchips over conventional analytical devices is the possibility of massive parallel analysis. Biological microchips are smaller than conventional testing systems and highly economical in the use of specimens and reagents. Major progress has been achieved in the manufacturing and application of DNA microchips (1).

Our group has been developing the microarray of gel-immobilized compounds on a chip (MAGIChip) (2, 3). The main advantage of the use of a three-dimensional gel support for fixation of biological compounds is the large capacity for immobilized compounds. In addition, the gel pads in the array are separated from each other by a hydrophobic surface. Therefore, the gel pad arrays can be used as a large number of individual microtest tubes to carry out specific interactions and chemical and enzymatic procedures with microchip substances.

The technology of manufacturing oligonucleotide microchips either by application and chemical immobilization of oligonucleotides within gel pads (3, 4) or by their copolymerization with acrylamide (5) has been described. Preliminary data on the use of similar techniques for manufacturing protein microchips have been reported (6).

The further development of protein microchip fabrication and its applications is presented in this work. The microchips were used for various types of immunoassays, detection, and recording of activity of immobilized enzymes and inhibition.

MATERIALS AND METHODS

Proteins

Proteins, enzymes, and antibodies were purchased from Sigma (U.S.A) unless otherwise posted.

Monoclonal antibodies were purified from ascite fluid by ammonium persulfate precipitation following gel filtration on a PD-10 column with Sephadex G-25M (Pharmacia Fine Chemicals, Sweden). All proteins for the direct immunoassay were labeled with FITC[2] ac-

[1] To whom correspondence should be addressed at the Biochip Technology Center, Argonne National Laboratory, 9700 S. Cass Ave., Argonne, IL 60439. Fax: (630)-252-3387. E-mail: amir@everest.bim.anl.gov.

[2] Abbreviations used: AU, arbitrary units; DHEBA, *N*,*N*′-(1,2-dihydroxyethylene)bisacrylamide; Bis, *N*,*N*′-methylenebisacrylamide; DATD, *N*,*N*′-diallyltartardiamide; PB, phosphate buffer; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; MAb, monoclonal antibody; AFP, α-fetoprotein; hCG, human chorionic gonadotropin; HRP, horseradish peroxidase;

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cording to (7). The conjugates were purified by gel filtration on PD-10 columns and then concentrated on a Centricon 30 system (Amicon, U.S.A.). The FITC/protein molar ratios for the conjugates were determined spectrophotometrically to be around 2. Protein concentration in the conjugates was adjusted to 1 μg/μl.

Manufacturing of Protein Microchips

Fabrication of gel micromatrices (6). The polymerization chamber consisted of a glass slide (Corning Micro Slides, U.S.A.) treated with Bind-Silane (LKB, Sweden) and a quartz plate mask of a specified topography: transparent 100 $\times$ 100-μm square windows spaced by 200 μm were arranged on a 1-μm-thick chromium nontransparent layer. The slide and the mask were separated by 20-μm-thick Teflon spacers. The chamber was filled with solution of 3% acrylamide (Bio-Rad, U.S.A.), 0.86% *N,N′*-(1,2-dihydroxyethylene)bisacrylamide (DHEBA) (Sigma), 0.14% *N,N′*-methylenebisacrylamide (Bis) (Bio-Rad), 40% glycerol, 2×10^{-4}% methylene blue (Sigma), 1.2% TEMED (Sigma) in 0.1 M sodium phosphate buffer (PB), pH 7.2. The chamber was illuminated with 254-nm uv light for 40 min in a 254-nm UV Stratalinker 1800 (Stratagene, U.S.A.) oven at a distance of 5 cm from the uv lamps. Then the matrix was washed with water for 10 min to remove nonpolymerized monomers, dried, and kept at room temperature. For fabrication of a conventional polyacrylamide gel matrix, solution containing 5% acrylamide [40% acrylamide/Bis stock solution 19:1 (Bio-Rad)], 40% glycerol, 2×10^{-4}% methylene blue, 1.2% TEMED in PB was used. Time of photopolymerization was 15 min in this case. The standard 9 $\times$ 9-mm micromatrix contained six hundred and seventy-six 100 $\times$ 100 $\times$ 20-μm gel pads with center to center distance of 300 μm (6).

Application of proteins on the micromatrix. The proteins were dissolved in a phosphate-buffered saline (PBS) solution containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2, at concentration of 0.1–10 μg/ml. One nanoliter of protein solution was transferred from individual wells of a 384-well plate by a pin onto the gel pads by using a robot (3) or a manual device (6). The pin and the micromatrix glass slide were kept at dew point to avoid evaporation of protein solution.

Immobilization of proteins. Two methods of chemical attachment of proteins to polyacrylamide gels were used. In method A, the gel was activated by glutaraldehyde solution and then conjugated with proteins (8). The gel matrix was treated with 0.1 M $NaIO_4$ in water for 20 min at room temperature, washed with water for 30 min, then dried. The matrix was immersed into 5% glutaraldehyde (Serva) in 0.1 M PB, pH 7.4, for 48 h at room temperature. It then was washed in water three times for 15 min each, dried, treated with Repel-Silane (LKB) for 10 s, rinsed with ethanol and water, and dried in nitrogen atmosphere.

After the application of protein solutions onto the glutaraldehyde-activated gel pads, the microchip was incubated for 16 h at 4°C in a humid chamber. It was then washed for 10 min in PBS with 0.5% Tween 20, in PBS, and in PB. The microchip was treated for 20 min at 4°C in 0.1 M $NaBH_4$ and washed briefly with water then PBS with Tween 20 for 1 h and finally in PB. The microchip was stored in sterile PBS at 4°C.

In method B, the polyacrylamide gel was activated by partial substitution of amide groups with hydrazide groups, and aldehyde groups were introduced into the polysaccharide portion of the antibodies by periodate oxidation. The hydrazide groups of the gel effectively interact with the aldehyde groups on the antibodies under mild condition with the formation of a stable covalent bond between them. The micromatrix was incubated for 45 min at room temperature in 100% hydrazine hydrate (Sigma), washed with water for 5 min, with 1% acetic acid for 5 min, and with water for 15 min, dried, and treated with Repel-Silane for 10 s.

The antibodies to be immobilized were oxidized with 40 mM $NaIO_4$ in PB for 30 min at room temperature, and then PB was substituted with PBS via gel filtration on the PD-10 columns. The solutions of the activated antibodies were applied onto the activated gel pads, and the microchips were incubated for 16 h at 4°C in a humid chamber. Unreacted aldehyde groups of the proteins were either blocked by incubation of the microchips in 1% glycine in PBS for 2 h at 4°C (for enzyme microchips) or reduced by $NaBH_4$ as in method A. Further procedures were carried out as described above.

Immunoassays on the Microchips

Immuno- and enzymatic assays on a microchip were usually carried out in a 30-μl chamber. Thus, usually 30 μl of solution was taken for each step of an assay. Usually 1:100 dilutions were used for immunoassays unless otherwise posted.

The immunoassay on microchips can be significantly accelerated by microelectrophoresis. The microchip was placed on a chamber for horizontal electrophoresis (LKB 2117 Multiphor chamber with LKB 2103 power supply) and connected with filter paper strips with the buffer compartments containing 0.01 M Tris–HCl, pH

AP, alkaline phosphatase; GUS, β-D-glucuronidase; DAB, diaminobenzidine tetrahydrochloride; BCIP/NBT, 4-chloro-3-indolyl phosphate/nitroblue tetrazolium.

7.6–7.8. The sample of the fluorescently labeled specific antibody or antigen in 20 μl of the same buffer was applied to the filter strip connected with the cathode chamber. The application of an electric field (3 V/cm) moved the proteins into the microchip and also removed unbound molecules toward the anode in a matter of 20 min. Following the assay the microchip was washed three times for 1 h at room temperature or at 4°C overnight in PBS, containing 0.1% Tween 20.

The protein microchips can be reused for immunoassays. Dissociation of the antibody–antigen complex was achieved by incubation of the microchip in either 0.05 M HCl–glycine buffer (pH 2.5, 48 h, 4°C) or 4 M urea (24 h, 4°C) or by microelectrophoresis in 4 M urea, 0.05 M Tris–HCl, pH 8.0, for 20 min. After complex dissociation, the microchip was washed three times with PBS containing 0.5% Tween 20 for 20 min at room temperature and three times with PBS for 5 min and was used for the following assays. After washing, the remaining fluorescence background was measured. The microelectrophoresis under denaturing conditions resulted in a smaller background in shorter time and thus seems to be the method of choice for repeated immunoassays.

Up to 10 immunoassays can be carried out successively on the same microchip, with a decrease in the binding signal of less than 3.0% each time.

Enzymatic Assays on the Microchips

Horseradish peroxidase. Enzyme activity on a chip was detected with 5 mM 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.03% H_2O_2 in 50 mM Tris–HCl buffer, pH 7.4, for 30 min.

Alkaline phosphatase. Enzyme activity on a chip was detected with either 5 mM 5-bromo-4-chloro-3-indolyl phosphate and 5 mM nitroblue tetrazolium (Sigma) upon incubation at room temperature for 20 min or 5 mM ELF-97 phosphate (Molecular Probes, U.S.A.) after 1 h of incubation.

β-D-Glucuronidase. Enzyme activity on a chip was detected with either 10 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide in 0.01 M PB, pH 7.6, for 6 h at 37°C or 20 mM ELF-97 β-D-glucuronide (Molecular Probes) for 2 h at room temperature.

Monitoring of Fluorescence and Color Assays on Microchips

Quantitative fluorescence measurements were carried out simultaneously for all 676 gel pads in the 9 × 9-mm microchip area with a fluorescence microscope equipped with a cooled CCD camera (Princeton Instruments Inc., U.S.A.), a computer, and respective software (3, 6, 9). Accumulation of color precipitate during enzymatic reactions on microchips was also recorded with a Polaroid SLR camera and Polaroid color instant film.

RESULTS

Gel Support

Polyacrylamide gel provides a stable, low-fluorescence background, low nonspecific absorption, high-capacity support for immobilization of different compounds on microchips. However, the rather low porosity of conventional polyacrylamide gels retards diffusion into the gel of the macromolecules that interact with the immobilized compounds. More porous gels can be prepared by increasing the ratio of the crosslinker Bis to acrylamide from 1:19 to 1:3 (10). The use of *N,N′*-diallyltartardiamide (DATD) as a crosslinker also improves the porosity of the microchip gels (6); however, DATD inhibits polymerization of acrylamide-based gels (10). Here, a further increase in porosity was achieved by the use of *N,N′*-(1,2-dihydroxyethylene)bisacrylamide (DHEBA)–Bis mixture (10, 11) instead of DATD as a crosslinker and partial splitting of the crosslinking DHEBA molecules by oxidation of their vicinal dihydroxy groups with $NaIO_4$. The high porosity of this gel and easy access of macromolecules inside the gel pads enabled us to regulate the amount of immobilized compounds and thus the sensitivity of measurements by variations in the size of the microchip elements. The change in thickness of the 100 × 100-μm gel pads from 20 to 40 μm increased the amount of immobilized antibodies and enhanced the fluorescence signals for antigen detection by about twofold.

Two methods of protein immobilization were found to be satisfactory for manufacturing protein microchips. In the first method, activation of polyacrylamide gel with glutaraldehyde (8) provides a convenient procedure to immobilize different antibodies and enzymes with preservation of their biological activity. Our estimations show that the immobilization yield was about 40% at protein concentrations of 0.1–1.0 μg/μl and decreased to 20–25% at higher concentrations (not shown). The second procedure, used previously for manufacturing the oligonucleotide microchips (2), is based on the activation of the gel by partial substitution of the amide groups on the polyacrylamide with hydrazide groups and site-specific generation of reactive aldehyde groups in the polysaccharide component of the antibodies by their oxidation with $NaIO_4$. The reaction between the hydrazide and aldehyde groups efficiently crosslinks the proteins to the gel. Hydrazine-activated microchips demonstrated higher capacity than glutaraldehyde-activated microchips and gave signals 1.8-fold higher after immobilization of the same antibodies. Both methods produce protein microchips that sustained many cycles of immunoassays, but the

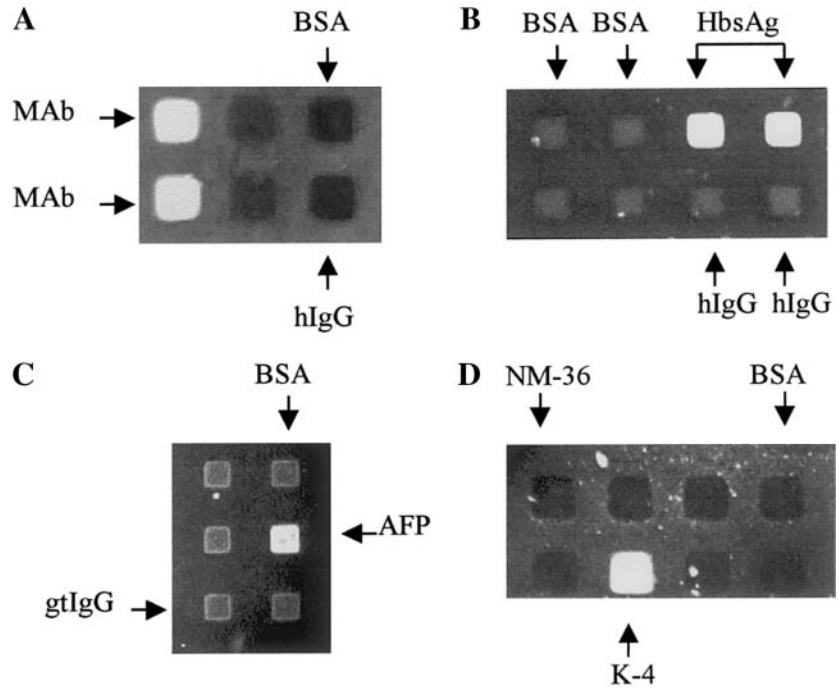

FIG. 1. Immunoassays on protein microchips. (A) Direct antibody detection. Monoclonal antibodies (MAb) to laminin were immobilized on the microchip and were developed with laminin–FITC. MAb to human IgG (hIgG) and bovine serum albumin (BSA) were immobilized as controls. (B) Direct antigen determination. Recombinant hepatitis B virus antigen was immobilized on the microchip and was developed with FITC-labeled antibodies purified from positive to HBV human serum. Human IgG and BSA were immobilized as controls. (C) Indirect antigen detection. α-Fetoprotein (AFP) was immobilized on the microchip which was reacted consequently with mouse ascites fluid MAb to AFP and with second goat anti-mouse IgG antibodies labeled with FITC. Goat IgG (gtIgG) and BSA were immobilized as controls. (D) Indirect antigen detection. The microchip with immobilized peptide K-4 (Institute of Immunology, Moscow, Russia), epitope of glycoprotein gp-41 of human immunodeficiency virus (HIV) was covered consequently with rabbit anti-gp-41 serum and goat anti-rabbit IgG antibodies labeled with FITC. Peptide NM-36, epitope of the core antigen of hepatitis B virus (HBV), and BSA were immobilized as controls. Note the presence of empty wells.

hydrazine-activated microchips were more stable (results not shown).

Immunoassays on the Microchip

Several types of immunoassays were performed on protein microchips in which either the antigen or the antibody was immobilized within the gel pads and detected directly with labeled antibodies or antigens, respectively. In indirect methods, the immobilized antigen was reacted with specific antibodies and the reaction was detected by second antibodies. To detect antigen–antibody interactions by using a fluorescence microscope, the target molecules were conjugated to fluorescent dyes or to enzymes catalyzing a color precipitate reaction.

Direct antibody detection by antigens (Fig. 1A). Monoclonal antibodies to laminin, human IgG, and bovine serum albumin (BSA) were immobilized within separate gel pads. The microchip was stained with laminin–FITC conjugate. One can see that the labeled laminin stained only the anti-laminin antibody gel pad and the detection was highly specific.

Direct antigen detection by antibodies (Fig. 1B). Affinity-purified recombinant hepatitis B surface antigen (Institute of Immunology, Moscow, Russia), human IgG, and BSA were immobilized on the microchip and stained with human IgG antibodies purified from the serum of a hepatitis B virus (HBV)-positive patient and labeled with FITC. No significant reaction was observed for background and nonspecific immunostaining compared with the specific interaction of hepatitis B surface antigen and the fluorescently labeled antibody.

Indirect antigen detection (Fig. 1C). A microchip with immobilized α-fetoprotein (AFP), goat IgG, and BSA was covered with a solution of MAb to AFP (ascites fluid, specific antibody concentration 3 mg/ml, dilution 1:100) and, after washing, with solution of secondary goat anti-mouse IgG antibodies, labeled with FITC. Only specific interaction was observed.

In another experiment (Fig. 1D), peptide K-4 (1 mg/ml), epitope of glycoprotein gp-41 of human immunodeficiency virus (HIV), as well as the control peptide NM-36, epitope of the core antigen hepatitis B virus,

and BSA were immobilized on the microchip. The microchip was developed consequently with solution of rabbit anti-K-4 serum and then with solution of secondary goat anti-rabbit IgG antibodies labeled with FITC. Again, only the specific interaction was detected by this assay.

Kinetics of immunobinding on different microchips. To study the time course of immunobinding and to compare microchips prepared using different gel compositions, the kinetics of binding of fluorescently labeled goat anti-rabbit IgG with rabbit IgG immobilized on different microchips was studied. Rabbit IgG (1.0, 0.2, 0.04 μg/μl) was immobilized on a microchip prepared using the DHEBA-containing gel composition (DHEBA microchip) and on a conventional polyacrylamide gel microchip (see Materials and Methods). The microchips were covered with 30 μl of solution containing goat anti-rabbit IgG labeled with FITC and placed in airtight chambers with quartz glass windows, and the kinetics of the interaction was recorded in the same way as the kinetics of hybridization on oligonucleotide microchips (12). Figure 2 shows the time dependence of the intensity of the immunostaining signal on the two different microchips. The process of immunobinding proceeds slower on the conventional microchip (Fig. 2A) than on the DHEBA microchip (Fig. 2B), and the resulting signals on the conventional microchip are 7–10 times lower than on the DHEBA microchip. Though the fluorescent signals on the DHEBA microchip reached a plateau within 7–25 h, the times required for 50% equilibrium signals were 0.2, 1, and 3 h for antibodies immobilized at 0.04, 0.2, and 1.0 μg/μl, respectively.

Sandwich immunoassay (Fig. 3). Two microchips with immobilized MAb_1 to human chorionic gonadotropin (hCG) (2A3 clone, α + β subunits) were covered with solution of hCG with concentrations of 5 and 50 μg/ml, respectively, while another microchip was covered with BSA solution (1 μg/μl). After washing, the microchips were developed with FITC-labeled MAb_2 to hCG (7C8 clone, β subunits). Specific interaction was observed only on the microchips covered with the hCG solution, and the intensity of the immunostaining signals increased upon increasing the hCG concentrations.

Multi-immunoassay on a microchip (Fig. 4). Monoclonal antibodies to laminin, fibronectin, α-fetoprotein, c-reactive protein, and human IgG were immobilized on different gel pads of a microchip. Bovine IgG, BSA, and MAb to BSA were immobilized as controls. The same microchip was developed with different mixtures of corresponding antigens labeled with FITC. The antigen–antibody complexes were dissociated and washed off before the next reaction step; the microchip was washed and tested with other antigens. It appears that seven rounds of different assays can be carried out on one microchip without any loss in specificity of interactions.

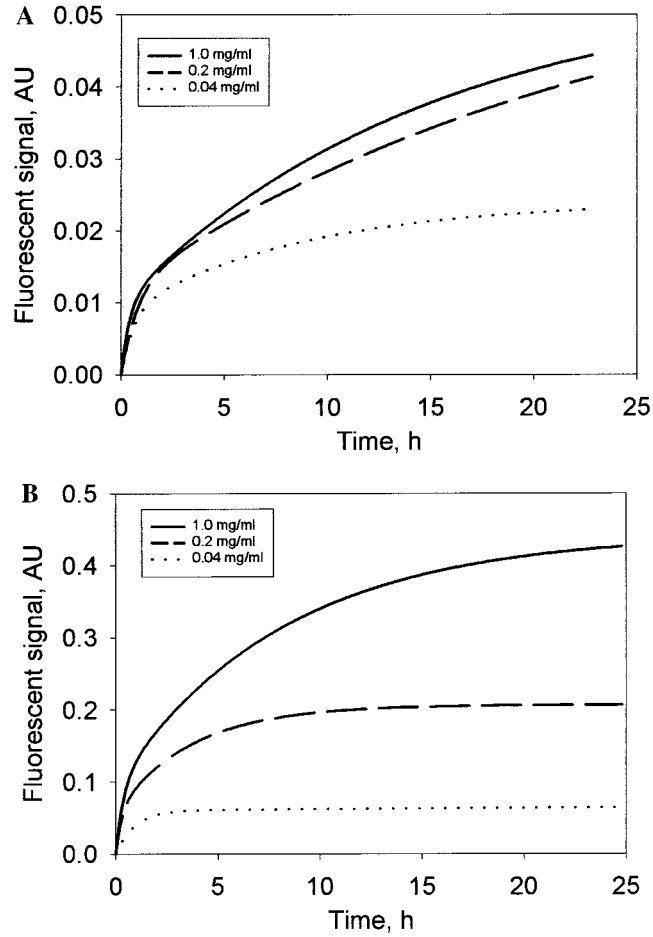

FIG. 2. Kinetics of binding of goat anti-rabbit IgG to rabbit IgG on conventional acrylamide–Bis and acrylamide–DHEBA gel microchips. Rabbit IgG (1.0, 0.2, 0.04 μg/μl) was immobilized on conventional (A) and DHEBA (B) microchips, and the kinetics of the interaction with FITC-labeled goat anti-rabbit IgG (dilution 1:100) was recorded.

Enzyme immunoassay on a microchip (Fig. 5). AFP, BSA, and goat IgG were immobilized on a microchip. The microchip was treated first with a solution of MAb to AFP and then with the secondary goat anti-mouse IgG antibodies labeled with horseradish peroxidase (HRP). After washing, the microchip was developed with a solution of 3,3′-diaminobenzidine tetrahydrochloride. A positive signal was observed only in the proper place.

Enzyme Microchips

Three enzymes of various size, which are widely used for different applications, were tested for immobilization on microchips by glutaraldehyde:HRP (M_r 44,000),

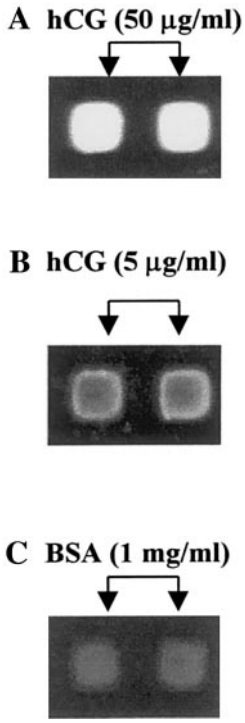

FIG. 3. Sandwich immunoassay. One of the microchips with immobilized MAb_1 to human chorionic gonadotropin (hCG) (2A3 clone, $\alpha + \beta$ subunits) was covered with hCG (A, B) while another microchip was covered with BSA (C). The microchips were developed with FITC-labeled MAb_2 to hCG (7C8 clone, β subunits).

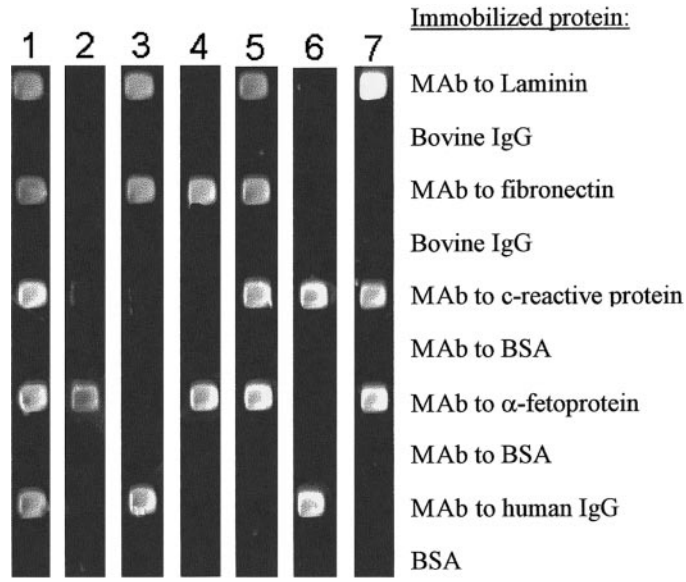

FIG. 4. Multianalyte immunoassay on one microchip. The microchip with immobilized monoclonal antibodies (MAb) to laminin, fibronectin, c-reactive protein, α-fetoprotein (AFP), and human IgG (hIgG) was successively covered with samples of FITC-labeled analytes in different combinations: 1, laminin, fibronectin, c-reactive protein, AFP, hIgG; 2, AFP; 3, laminin, fibronectin, hIgG; 4, fibronectin, AFP; 5, laminin, fibronectin, c-reactive protein, AFP; 6, c-reactive protein, hIgG; 7, laminin, c-reactive protein, AFP. Bovine IgG, MAb to bovine serum albumin (BSA), and BSA were immobilized as controls.

calf intestinal alkaline phosphatase (AP) (M_r 140,000), and β-D-glucuronidase (GUS) (M_r 290,000). The activities of the immobilized enzymes were detected with substrates yielding color or fluorescent precipitates during enzymatic reactions.

Horseradish peroxidase (Fig. 6A). The enzyme at a concentration of 1 μg/μl was immobilized on a gel pad. The microchip was covered with diaminobenzidine tetrahydrochloride solution. Insoluble brown precipitate was formed only in the enzyme gel pad.

Alkaline phosphatase (Figs. 6B and 6C). Two different substrates were used for enzyme detection. The solution of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma) developed upon incubation an insoluble dark gray precipitate in the corresponding gel pad of the microchip (Fig. 6B). Alternatively, the solution of ELF-97 phosphate developed an insoluble fluorescent precipitate (E_{ex} = 365 nm, E_{em} = 540 nm) in a gel pad containing alkaline phosphatase (Fig. 6C). In the latter chip, the enzyme was immobilized at concentrations of 10, 1, and 0.1 μg/μl per gel pad for 10 h at 4°C.

β-D-Glucuronidase (Figs. 6D and 6E). The enzyme was immobilized for 20 h at 4°C at concentrations of 10, 1, and 0.1 μg/μl. The immobilized enzyme was covered with 5-bromo-4-chloro-3-indolyl β-D-glucuronide solution and developed a green precipitate (Fig. 6D). Incubation of the enzyme microchip with a solution of ELF-97 and β-D-glucuronide resulted in a fluorescent precipitate (Fig. 6E) (E_{ex} = 365 nm, E_{em} = 540 nm). Even for the largest immobilized protein, β-D-

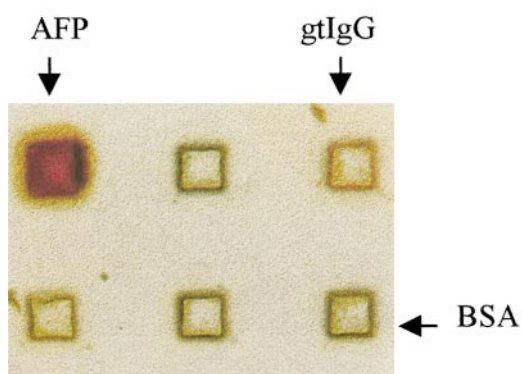

FIG. 5. Enzyme-linked immunoassay on a microchip. The microchip with immobilized AFP was covered consequently with mouse liquid ascites MAb to AFP and then with goat anti-AFP antibodies (gtIgG) labeled with horseradish peroxidase (HRP). The 3,3′-diaminobenzidine tetrahydrochloride liquid substrate system was used for the development of this microchip.

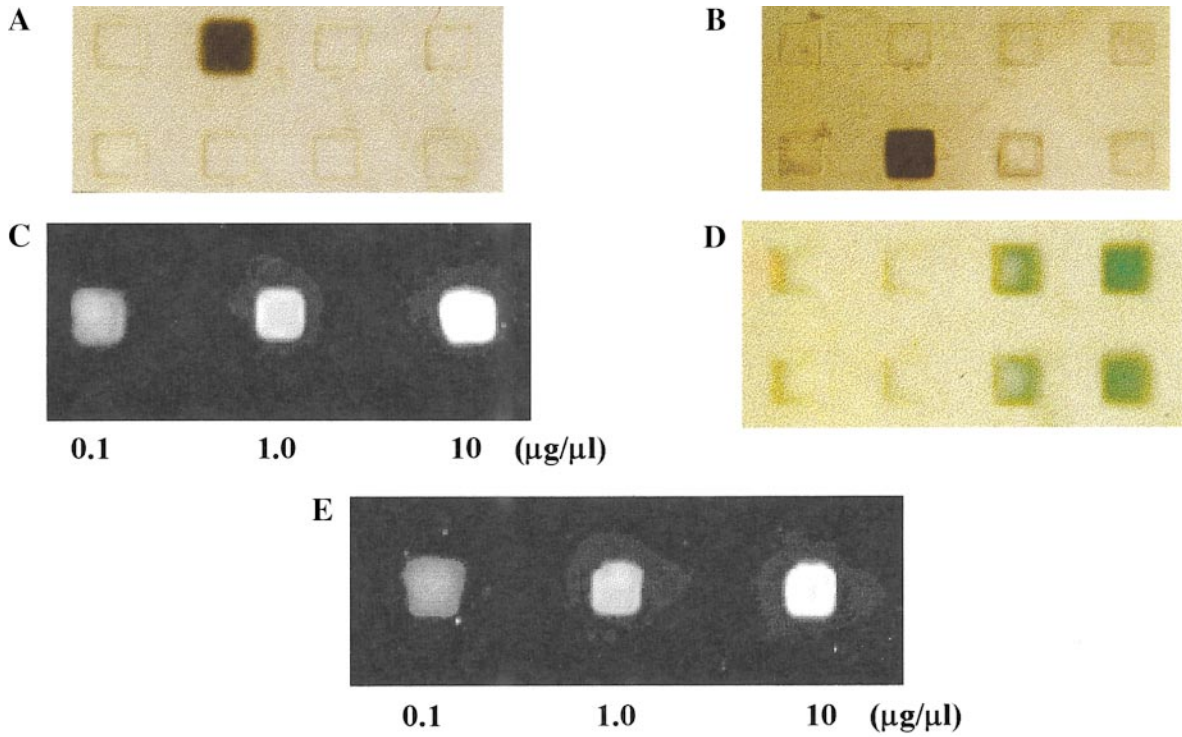

FIG. 6. Detection of activity on enzyme microchips. (A) A microchip with immobilized horseradish peroxidase (HRP) was covered with the 3,3′-diaminobenzidine tetrahydrochloride liquid substrate system. (B) A microchip with immobilized alkaline phosphatase (AP) was covered with the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium liquid substrate system. (C) A microchip with AP immobilized at concentrations 0.1, 1.0, and 10.0 μg/μl was covered with ELF-97 phosphate substrate. (D) A microchip with immobilized β-D-glucuronidase was covered with 5-bromo-4-chloro-3-indolyl β-D-glucuronide substrate solution. (E) A microchip with β-D-glucuronidase immobilized at concentrations 0.1, 1.0, and 10.0 μg/μl was covered with ELF-97 β-D-glucuronide substrate solution.

glucuronidase, the gel pad was stained rather smoothly indicating that the protein was immobilized within the whole volume of the gel pad.

Measurements of enzyme activity and inhibition on a microchip (Figs. 7A and 7B). To confirm a possible application of the enzyme microchips for purposes of combinatorial analysis and drug discovery, we studied kinetics of enzymatic reaction and inhibition on a microchip. Figure 7 shows the dependence of the initial rate of ELF-97 dephosphorylation on enzyme concentration used for immobilization. It should be emphasized that activities from different gel pads containing the immobilized enzyme were measured simultaneously during a single experiment (Fig. 7A). The dependence of observed activity upon enzyme concentration is linear below the concentration of 3 mg/ml, and the rate of enzymatic reaction tends to zero with decreasing enzyme concentrations (Fig. 7B). However, enzyme concentrations above 3 mg/ml fall outside the linear portion of the curve, which probably indicates saturation of the gel matrix by the protein.

Inhibition of alkaline phosphatase immobilized on a chip was studied with the stereospecific noncompetitive inhibitor L-phenylalanine (13). The dependence of the dephosphorylation rate on inhibitor concentration (Fig. 8) gives $[I]_{50}$ of 0.5 mM; this value corresponds to literature data (13). Thus, enzyme microchips could be used for quantitative analysis of inhibitor–enzyme interactions.

DISCUSSION

Microarrays of gel pads containing immobilized proteins were manufactured with the same technique developed earlier for making oligonucleotide and DNA microchips (2, 3). The procedure consists of three steps: manufacturing the micromatrix of polyacrylamide gel pads by photopolymerization, application of a protein solution onto each gel pad by a pin (3), and, finally, chemical fixation of the proteins within the gel pads (4). The standard size and the volume of the gel pads are 100 × 100 × 20 μm and 0.2 nl, respectively. Recently, a two-step procedure was suggested for manufacturing oligonucleotide and protein gel-based microchips involving a copolymerization of acrylamide with these compounds (5). The minimal size and volume of these microchip gel pads are 10 × 10 × 5 μm and 0.5 pl, respectively.

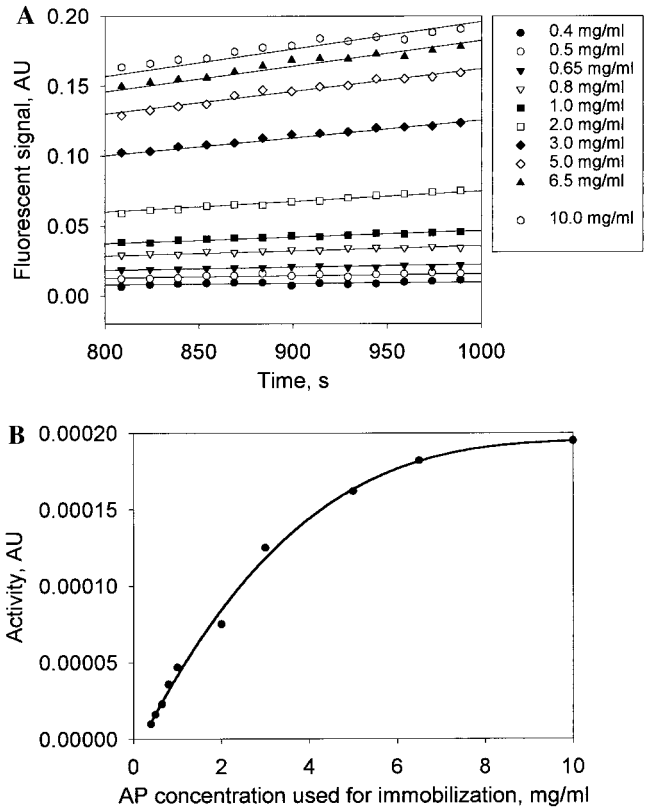

FIG. 7. The effect of alkaline phosphatase concentration used for the immobilization on its activity. (A) Initial rates of ELF-97 dephosphorylation catalyzed by different amounts of alkaline phosphatase immobilized on a single chip. (B) Dependence of alkaline phosphatase activity on enzyme concentration used for immobilization.

Gel pads are fixed on a glass slide and separated from each other by a hydrophobic glass surface preventing communication of water solutions between adjacent microchip elements. This allows one to study specific macromolecule interactions (12) and to carry out chemical and enzymatic reactions (14) in individual gel pads as microtest tubes or microtiter plate wells of a volume from pico- to nanoliters.

Alternative technologies for protein microchip manufacturing include photolithography of silane monolayers (15), inkjetting onto polystyrene film (16), and spotting onto polyvinylidene fluoride filters (17). They are focused on fabrication of miniaturized formats for single and multianalyte immunoassays (15, 16) and high-throughput for protein expression (17) and could be considered as two-dimensional patterns of functional antigens or antibodies on a surface.

In our approach, the use of three-dimensional gel support provides a much higher capacity for immobilization over a two-dimensional glass or plastic surface, homogeneous water environment for immobilized proteins, and some other essential advantages (6). The immobilized proteins are well spaced and do not interact with each other or with the glass surface. This prevents the aggregation of immobilized proteins or their interphase-induced denaturation on a solid surface.

However, the polyacrylamide gel used previously for microchip manufacturing is of rather low porosity (10), causing a retarded diffusion of macromolecules into the gel pads (18). We used here high concentrations of crosslinkers during gel polymerization, including DHEBA (10) and DHEBA cleavage by periodate oxidation; these modifications increased significantly the gel porosity. This gel is able to accommodate the rather large sandwich complex of antibody–antigen–antibody (M_r = 300 kDa) and a large enzyme such as GUS (M_r = 290 kDa). Diffusion of macromolecules into the gel pads of the microchip can be accelerated by using electrophoresis (see Results) or mixing the solutions within the microchip chamber with a micropump (not shown).

The protein microchips can be used at least 7–10 times. The high density of gel-immobilized proteins increases the sensitivity of the procedure. At the present level one can detect about several attomoles (unpublished results) of Texas Red as a fluorescent label within a standard element of the microchip.

Different monoclonal and polyclonal antibodies, antigens, and enzymes were used for manufacturing the gel-based protein microchips. Several applications of the protein microchips were demonstrated. Antibody or antigen microchips were applied to detect complementary molecules in the fluorescence or enzymatic direct or indirect immunoassay. Detection of enzymatic activity and measurements of the kinetics of their reaction in the absence or presence of an inhibitor were carried out on enzyme microchips.

Our experience with microchips containing all possible 4096 hexadeoxynucleotide sequences (19) allows us to conclude that it is possible to carry out massive parallel immuno- and enzyme assays on protein microchips containing hundreds and thousands of different immobilized proteins.

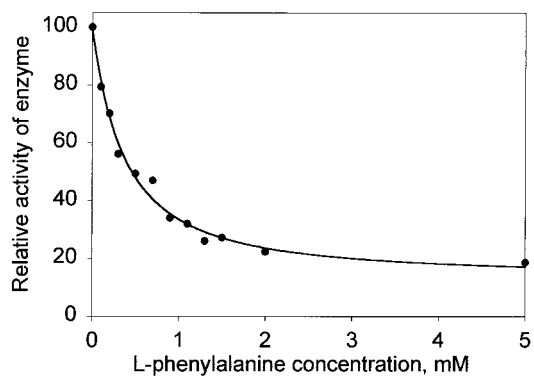

FIG. 8. Inhibition of alkaline phosphatase immobilized within microchip gel pads with L-phenylalanine.

There are many other possible applications for protein microchips that depend on availability of proteins, their stability within the gel pads, and cost efficiency of microchip production.

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