The FASEB Journal express article 10.1096/fj.04-2098fje. Published online June 6, 2005.

Calixarene derivative as a tool for highly sensitive detection and oriented immobilization of proteins in a microarray format through noncovalent molecular interaction

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ABSTRACT

One important factor in fabricating protein microarray is to immobilize proteins without losing their activity on a solid phase. To keep them functional, it is necessary to immobilize proteins in a way that preserve their folded structural integrity. In a previous study, we developed novel Calixarene derivatives for the immobilization of proteins on the surface of a glass slide (1). In this study, we compared the sensitivity and the specificity of the linker molecules with those of five other protein attachment agents on glass slides using a prostate-specific antigen and its antibodies as a model system. The Calixcrown-coated protein chip showed a superior sensitivity and a much lower detection limit than those chips prepared by other methods. When we tested the capability of Calixcrown to immobilize antibody molecules, it appeared that Calixcrown makes arrangement of antibody be more regular with the vertical orientation than the covalent-bond agent. We also observed that the Calixcrown chip could be used for the diagnostic application with clinical samples from prostate cancer and HIV patients. Finally, we applied the Calixcrown chip using an antibody microarray to identify up- or down-regulated proteins in specific tissue and detected several up- or down-regulated proteins from a rat liver by administering toxin. Thus, the Calixcrown chip can be used as a powerful tool with a wide range of applications, including protein-protein interaction, protein-DNA interaction, and an enzyme activity assay.

Key words: protein microarray • Calixcrown • antibody • orientation • diagnostic application

Protein microarrays have recently had an explosion of interest due to advances in proteomics, robotics, microelectronics, and bioinformatics (2–4). Microfabricated protein chips for an enzyme assay, antibody screening, and protein-protein interaction have been reported (5–7). Low-density protein array based on the 96-well microtiter plate format was

followed by generation of high-density automated microarray system on a small flat surface, such as glass slides or filter membranes (8). High-density protein microarray accommodates extremely low sample volumes and enables a simultaneous processing of thousands of proteins for determining high-throughput function (9, 10). The use of protein microarrays was extended to identify novel proteins in complex cell or tissue mixture. Using the high-throughput screening methods, they have identified novel proteins associated with hepatocellular carcinomas and novel radiation-regulated proteins as well as determined 50 or more CD antigens on leukemia or leukocytes in one assay (11–13).

Over the years, a variety of immobilization strategies, including entrapment in a cross-linked matrix, coupling proteins to polymeric layers, dendrimer-activated solid supports, and covalent attachment on derivatized surfaces, have been investigated for creating selective reaction layers of immunoassays, immobilized enzymes, and biosensors (14–18). Among those methods, covalent linkages are being widely used for protein immobilization. Two major questions exist regarding immobilizing proteins onto a solid phase: 1) How can their native structural integrity be preserved? 2) How can they be attached with high density? When proteins are immobilized with chemical linkage or physical absorption, it usually accompanies modification/denaturation of their three-dimensional structure and a problem of protein orientation (19). Change of native conformation and random orientation of protein greatly affects the specificity and the selectivity of protein-protein interaction. Inefficient immobilization of protein makes it difficult to detect and analyze low-level proteins. This may cause nonspecific reactions and serious problems in interpreting experimental results. Thus, many ongoing studies on protein chips are focusing on development of molecular linkers that make immobilized protein maintain its structural integrity and of detection systems that catch even low-level signals.

Calixarenes are cup-shaped molecules that can form inclusion complexes with a wide range of guest species. Each Calixarene contains a repeating phenolic unit formed into a macrocycle via methylene bridges (20, 21). The Calixarene “cups” have a vaselike structure defined by an upper rim, lower rim, and central annulus. The polar and nonpolar features of cavities enable Calixarenes to interact with a wide range of guest species, depending on the binding groups substituted at each rim and the number of repeating units in the macrocycle. As such, they belong to a broad range of compounds, such as crowns, crypands, and cyclodextrins, which are already well-known for forming host-guest complexes in solution (22). Calixarenes have been used in sensor devices, in nuclear waste treatment, and as a catalyst in synthetic reactions and liquid crystals (23). Compared with other applications, the study on the complex formation between proteins and Calixarene derivatives is less common. A Calixarene carboxylic acid derivative has been found to form a complex with the cationic protein cytochrome *c* (24). Calixarene derivatives can even be used as synthetic receptors that mimic the role of hypervariable loops in antibody combining regions (25).

In a previous study, we synthesized two novel Calixarene derivatives, ProLinker A and ProLinker B, and used them for immobilization of proteins onto the surface of glass slides and gold substrates, respectively (1). We demonstrated that protein microarrays fabricated with Calixcrown could be used as an excellent tool to monitor protein-protein interactions. In this study, we further characterized the properties of the Calixcrown as a biolinker molecule in immobilization of proteins and applied the protein chip to analyze samples collected in clinical practices. We also compared the sensitivity, the limit of detection, and the reproducibility of the

Calixcrown chip with those of other covalent linkage-based chips. We applied the Calixcrown chip to identify up- or down-regulated proteins of a specific tissue after administering toxin into animals.

MATERIALS AND METHODS

Preparation of Calixcrown chip

With minor modifications, we prepared two different types of Calixcrown chip, A and B, by using ProLinker A and B, respectively, as described previously (1). Before glass slides were coated with ProLinker agents, they were precleaned by a series of steps. First, glass slides were soaked in a solution containing MeOH and 35% HCl (1:1) for 30 min, treated in piranha solution (3:1 mixture of H_2SO_4 and H_2O_2) for 10 min, and thoroughly washed in distilled water (DW). The slides were then dried under a stream of N_2 gas. For preparation of Calixcrown chip A, the precleaned glass slides were aminated by immersing them in toluene containing 3% APS (3-amino-propyl-trithoxysilane) for 6 h at room temperature. Then the amine slides were washed once with toluene and absolute EtOH, and incubated at 100°C for 1 h. The slides were immersed in $CHCl_3$ containing 10 mM ProLinker A for 3 h, rinsed once with $CHCl_3$, EtOH, and DW, and stored at room temperature in a vacuum desiccator. For preparation of Calixcrown chip B, the precleaned glass slides were gold-coated by thermal evaporation with Magnetron Sputtering System (Cliotech, Seoul, Korea). Then, the slides were incubated in 3 mM ProLinker B solution for 2 h and rinsed sequentially with $CHCl_3$, EtOH, and DW.

Preparation of other protein chips

We purchased Superaldehyde chips from Telechem (Sunnyvale, CA). All the slides were precleaned as described above before being treated with specific agents for protein chips. For the carboxyl chip, the precleaned slides were coated with 3% MPS (3-mercapto-propyl-triethoxysilane) for 6 h and soaked in toluene containing 3 mM mercaptoundecanoic acid for 3 h at room temperature to form a disulfide bond. After they were rinsed the with EtOH, the slides were treated with a 100 mM NHS:EDC mixture (1:1) to activate the carboxyl group. The aminated glass slides were coated with NHS-LC-biotin (200 μg/ml) for 3 h to form an amide bond between APS and biotin. For biotin-avidin slides, avidin solution (100 μg/ml) was applied on the slides (26). To prepare the Protein A slide, the glass slide was incubated with 3% MPS, followed by sequential rinsing with toluene and EtOH, baked for 1 h in an oven, and incubated in an MBS (*m*-maleimidobenzoyl-*n*-hydroxysuccinimide-ester) cross-linker solution. Protein A (100 μg/ml) was then spotted on the slides.

Preparation of antibodies

The immunization of human α-fetoprotein (AFP), prostate-specific antigen (PSA), and C-reactive protein (CRP), determination of antibody titer, cell fusion, screening of hybridoma by ELISA, and production of ascitic fluids were conducted according to a standard protocol (27, 28). In brief, 6- to 8-wk-old Balb/c mice were immunized with 50 μg of immunogen premixed with an equal volume of complete Freund's adjuvant. The first injection was followed by three or four booster injections with same amount of immunogen mixed with incomplete Freund's adjuvant every other week. We conducted two fusion experiments with the spleens of immunized mice and myeloma cells to select many hybridomas responding to different epitopes of

immunogen. Ascitic fluids were generated for a large production of monoclonal antibody (mAb) and applied onto a Protein G column to purify mAb after clearing by centrifugation. The mAb was eluted with 0.1 M glycine-HCl (pH 2.5), neutralized with 1 M Tris (pH 8.0), dialyzed against PBS, and then stored at –80°C until ready to be used. We measured the protein concentration according to the Bradford method (29). To prepare the antibody microarray, we purchased all of the antibodies from commercial sources. We obtained 15 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), 10 antibodies from Transduction Laboratories (San Jose, CA), 7 antibodies from Sigma (St. Louis, MO), and 5 antibodies from Serotech (Kidlington, UK).

Construction and purification of Gal4 proteins

We constructed two Gal4 clones to learn the effect of the amino group of proteins with which the Calixcrown molecule interacts. The control Gal4 construct encodes residue 1-147 of the Gal4 DNA binding domain, plus six His-tags at the N terminus for purification (30). The 9R-Gal4 construct was generated by introducing 45 oligonucleotide pairs of six His-tags and an extra nine arginines into the immediate 5′-end sequence of Gal4 DNA binding domain. We confirmed positive clones by DNA sequencing and transformed recombinant DNA into BL21 cells. We induced the expression of protein with 1 mM IPTG for 4 h at 30°C and checked the protein induction with SDS-PAGE and immunoblotting. The recombinant proteins were purified using imidazole and Ni-column chromatography system of the pET vector as described by the manufacturer (Novagen, San Diego, CA).

Conjugation of fluorescence dye to protein

To label proteins with fluorescence dye, we mixed 10 μl of a 1 M sodium carbonate buffer (pH 8.3) with 100 μl of the proteins (1 mg/ml in PBS). Then, 1 μl of activated fluorescence dye (10 mg/ml) was added to the mixture and incubated first for 1 h at room temperature before incubating overnight at 4°C. Glycine solution was added to the mixture to quench unreacted sites of activated fluorescence dye. The mixture was applied onto a Sephadex G25 chromatography column to separate the conjugates from free dye. After centrifugation of the column at 2500 rpm for 2 min, the fluorescence conjugates were collected from the column as elutes.

Protein immobilization

To fabricate protein chips, we used manual spotting as well as automatic spotting with an arrayer, depending on the purpose of the experiments. The microarrayer used for automatic spotting was a high-precision, contact-printing robot (Proteogen CM-1000; Proteogen, Chuncheon, Korea) equipped with stealth micro-spotting pins (Telechem, Sunnyvale, CA) SMP10 (335 μm). To attach protein to the Calixcrown slide, 50 nl of anti-PSA-, anti-AFP-, and anti-CRP-mAb (40 μg/ml) in PBS were spotted on the protein array and incubated in a humidity chamber at 37°C for 2 h. The slides were rinsed in PBST three times for 5 min and incubated with 3% BSA containing 0.1% Tween-20 solution at 37°C for blocking a nonspecific binding. After extensive rinses with PBST, the mixture of PSA, AFP, and CRP antigen (stock: 1 μg/ml) labeled with different fluorescence dye in blocking solution was spotted onto the sites of mAb immobilized for direct immunodetection. We incubated the slides for an antigen-antibody reaction in a humidity chamber at 37°C for 1 h. After rinsing with PBST and DW, the slides

were dried in a stream of N_2 gas and scanned for quantification of fluorescence intensity. In an experiment comparing the sensitivity between different protein linker-coated slides, we manually spotted the given concentration of PSA onto the slide on which the anti-PSA-mAb had been previously immobilized. The sandwich pair of anti-PSA-mAb labeled with Cy5 was a probe, and we extensively washed it with PBST before scanning. To coat Gal4 and 9R-Gal4 protein, we manually spotted each protein onto the glass slides coated with ProLinker A or Superaldehyde. After incubation and washing as noted above, we probed the slides with Gal4 binding oligonucleotide (1 μg/ml) labeled with Cy5 in a blocking solution to detect the immobilized proteins.

Fluorescence-scanning analysis

We scanned the protein chips probed with fluorescence conjugates with a GSI Scan Array Lite model (Ontario, Canada) or a GenePix 4000B (Axon Instruments, Union City, CA) scanner. By adjusting the laser power and contrast, we set the scanners to optimize the quality of the microarray images and quantified the signal intensity with GenePix Pro 3.0 software.

Patient serum

After fully explaining the nature and possible consequences of the studies to the patients, we obtained their informed consent. Clinical samples of prostate cancer patients with known PSA concentrations were serially diluted and then applied on Calixcrown chip and Superaldehyde chip that anti-PSA-mAb had been previously immobilized. PSA antigen in serum was detected by probing the Cy5-labeled sandwich pair of anti-PSA-mAb. For clinical samples of HIV patients, gp120 protein (10 ng/ml) was spotted on Calixcrown chip and Superaldehyde chip, and then they were incubated in a humidity chamber at 37°C for 1 h. The serum serially diluted with a blocking solution was applied to antigen-immobilized spots. The HIV antibody in patient serum was detected by probing Cy5-conjugated anti-human IgG.

RESULTS

Fabrication of Calixcrown chip

We fabricated two types of Calixcrown chips to test their selectivity and reproducibility in antigen-antibody interaction. We evaluated the specificity of interaction using a method of highly sensitive detection with fluorescence-labeled probes. The ProLinker A contains aldehyde groups and thus is used for an aminated glass surface (Fig. 1*A*). Meanwhile, the ProLinker B has thiol groups and is used for gold-coated glass to prepare Calixcrown chip. To prepare antibody microarrays on Calixcrown chip, we used a contact-printing arrayer to deliver nanoliter volumes of protein sample to the slides. The antibodies in PBS were printed to yield microspots ~300 μm in diameter, ~400 spots per square centimeter. The volume of protein was ~50 nl for one spot. We included 30% glycerol in the protein sample to prevent evaporation of protein nanodroplets and used a direct method to detect protein antigens. The fluorescence-labeled protein mixture, including AFP, PSA, and CRP, was overlaid on the antibodies-immobilized slide for 1 h in a humidified chamber. To distinguish each antigen, AFP, PSA, and CRP were labeled with Cy5, Cy3, and Cy2, respectively. As expected, fluorescence signal was detected in only a pair of antigen-antibody reactions, and no cross-reactivity was observed. We printed 30 spots for each

antibody and all of the 30 spots showed uniform intensity of fluorescence as shown in Fig. 1*B*. This finding suggests that Calixcrown chip coated with the supramolecular Calixcrown displayed a high selectivity and a high reproducibility. When we compared the Calixcrown chip A with the ProLinker B-coated Calixcrown chip B, the former gave a much better signal than the latter (data not shown). The gold surface of the ProLinker B-coated chip appeared to interfere or reduce the fluorescence intensity during scanning with a commercial fluorescence scanner. Thus, we used the ProLinker A-based chip throughout this study for highly sensitive detection of fluorescence-labeled probe.

Comparison of different methods in protein immobilization

We next compared the sensitivity of our Calixcrown method with those of various methods that are being widely used for protein immobilization on glass slides. Other methods included Superaldehyde chip from TeleChem, chemical cross-linking agent, biotin-avidin system, and immobilized Protein A on slide glass. We used the interaction of PSA and its antibodies as a model system and adopted a sandwich immunoassay for detecting specific interaction between PSA and its antibody pairs. Capture and detector PSA antibody were immobilized on the glass surface and labeled with Cy5 according to the methods described in Materials and Methods. In the assay, different concentrations of antigen were manually spotted on glass sides that had been immobilized with capture anti-PSA-mAb. The mold of Tefron membrane was coated on each slide before immobilizing anti-PSA-mAb. Thus, each spot was physically separated from other spots to prevent mixing. In the case of the biotin slides, biotin-conjugated capture anti-PSA-mAb was overlaid onto streptavidin-coated slides for immobilization before applying PSA antigen. After washing unbound PSA, the fluorescence-labeled detector anti-PSA-mAb was probed, and florescence signals were read with a laser-confocal scanner. The Calixcrown chip A showed a superior sensitivity and a much lower detection limit than those of other protein chips (Fig. 2*A*). The detection limit of Calixcrown chip A was 100 pg/ml, and the Superaldehyde and the Carboxyl chip were next, but they were 10–100 times less sensitive. The biotin-avidin and the Protein A chip were the least sensitive with a high detection limit (Fig. 2*A*, 2*B*). Depending on the affinity and specificity of the antigen-antibody interaction, the low limit of detection in the Calixcrown chip corresponded well with our previous results (1).

After learning of the high sensitivity of the Calixcrown chip in the protein-protein interaction with the model system of PSA and its antibodies, we adapted an enzyme-substrate interaction on the chips as a more direct method for comparing sensitivity. For this application, we chose an enzyme activity assay of alkaline phosphatase because of color visualization of on-site deposition of end products. The enzyme was spotted by triplicate onto the slides in a serial-diluted manner. Since the Superaldehyde chip showed the second-highest sensitivity in the protein-protein interaction test (Fig. 2*A*, 2*B*), we compared the enzyme activity between Calixcrown chip A and Superaldehyde chip. The enzyme activity on the ProLinker A-coated slide was much higher than that on the Superaldehyde-coated slide (Fig. 2*C*). One possibility of Calixcrown chip's superior sensitivity is that the proteins immobilized on Calixcrown chip may maintain more intact configuration after their noncovalent attachments to Calixcrown. Other explanations could be that more IgG molecules are bound to the Calixcrown chip or IgG molecules are posed with better exposure pattern to their partners on Calixcrown chip than on other chips.

Ionic interaction of ProLinker A with proteins

In the previous study, we suggested that a coupling mechanism of proteins to the Calixcrown is mediated by the ionized amine group of capture proteins, which bind to the crown moiety of the linker molecule as host-guest interaction (1). The ionic interaction between Calixcrown and protein may explain the superior sensitivity and the lower detection limit of Calixcrown chip A. To further prove that it is not a physical absorption but instead a inclusion of protonated amines that plays a key role in capturing the proteins, we immobilized a fixed amount of protein under various ionic concentrations. According to a model study, Na^+ is smaller than the inclusion size of Calixcrown-5, K^+ is almost same size, and NH_4^+ is between Na^+ and K^+ (31). Figure 3*A* shows the immobilization of a fixed concentration of CRP-Cy5 under various ion concentrations of Na^+, NH_4^+, and K^+. The CRP-Cy5 (5 μg/ml) was manually spotted on a Calixcrown glass slide and immobilized for 30 min in a humidity chamber at 37°C. The slide was then rinsed with PBST thoroughly, dried under a stream of N_2 gas, and scanned to measure fluorescence intensity. Whereas the presence of only 200 mM NH_4^+ ion resulted in a complete inhibition of protein immobilization, even 800 mM of K^+ ionic concentration was not able to inhibit protein immobilization completely, but only partially (Fig. 3*A*). As expected from considering the molecular size of Na^+ and crown moiety, Na^+ ion showed no effect at all on protein immobilization. This result confirmed that the protonated amine of protein is an important factor in the interaction with Calixcrown-5 linker. The scale of fluorescence intensity shown in Fig. 3*B* indicated NH_4^+ and K^+ ions can be recognized 10 times more than Na^+ ion by the Calixcrown-5.

To demonstrate that an ionic interaction between protonated amine group of protein and crown moiety of ProLinker A plays a key role in protein immobilization, we constructed two mutant yeast *GAL4* clones using molecular cloning techniques and compared binding efficiencies of their proteins to the Calixcrown chips. The Gal4 construct, as a control, contained six His-tag for protein purification at the amino-terminal of 1-147 residue of the Gal4 DNA binding domain. Nine arginine residues were added between six His-tag and 1-147 residue of the Gal4 protein in an experimental 9R-Gal4 construct. As a probe to detect the Gal4 DNA binding domain on protein chips, we prepared a fluorescence-labeled oligonucleotide (Fig. 3*C*). After the control Gal4 protein was spotted at a serially diluted concentration, we compared the binding efficiency of control Gal4 protein between Calixcrown chip and Superaldehyde chip by probing the Cy5-labeled Gal4 binding oligonucleotide (Fig. 3*D*). The detectable concentrations of Gal4 protein by fluorescence-labeled DNA on ProLinker- and Superaldehyde-coated glass slides were 100 pg/ml and 1 μg/ml, respectively (Fig. 3*D*, 3*E*). This result again showed a superior sensitivity of the Calixcrown chip over the chemical cross-linking based chip. Under the assumption that the same amount of Cy5-labeled oligonucleotide was applied to the duplicated Calixcrown chips, the higher fluorescence intensity would imply that more Gal4 proteins were immobilized on the slides. While 9R-Gal4 mutant protein became detectable as low as at 312 pg/ml, 2.5 ng/ml was a detectable concentration of the control Gal4 protein, as shown in Fig. 3*D*, 3*F*. This result suggested that 9R-Gal4 mutant protein would have more chances to interact with the ProLinker A monolayer than the control protein so that more Cy5-labeled oligonucleotide could bind to 9R-Gal4 mutant protein. Considering that most of the $–NH_2$ group of protein exists as an $–NH_3^+$ form in a physiological condition (pH 7.4), this result indicated that Calixcrown-5 interacts with the ammonium ion of protein by ionic interaction. The extent of immobilization of protein on Calixcrown chip A tended to be reduced as buffer pH shifted toward the basic condition (unpublished data). A p*K* value of pH 8.5 affects the interaction between Calixarene

and protein, which also suggests that the amino group is to be an interacting group with ProLinker A. In addition, this result suggested that the Calixcrown chip A can be applied as a powerful tool to investigate protein-DNA interaction.

Oriented immobilization of IgG on Calixcrown chip

In our previous study, we investigated how antibody molecules localize onto the Calixcrown chip- based plate, and we demonstrated that IgG molecules form a tight molecular monolayer with a vertical orientation by QCM and fluorescence analysis (1). To further prove that IgG molecules on the Calixcrown chip are placed with the right orientation in a tight molecular monolayer, we designed an experiment using the characteristic of Protein A that specifically recognizes Fc domain of IgG molecule as shown in Fig. 4*A*. If Fc region of immobilized IgG is not exposed on the surface of chip but hidden inside of the surface, Protein A could not bind to the IgG (Fig. 4*Aa*). If some of IgG molecules are arranged on the surface of chip in such a configuration that Fc domain is located up and Fab domain is on the down side, Protein A does bind to the IgG on the surface (Fig. 4*Ab*).

We first immobilized Cy5-labeled IgG molecules on Calixcrown chip A surface and Superaldehyde glass (Fig. 4*B*). We, then, overlaid Cy3-labeled Protein A on the IgG-immobilized chips. Thus, Cy5 signal shows the amount of IgG captured on the Calixcrown chip and Cy3 signal reflects the amount of protein A bound to Fc region of the immobilized IgG molecules. When the IgG layer was compared, Calixcrown chip showed higher overall Cy5 fluorescence intensity (Fig. 4*Ba*, 4*Bb*), suggesting that Calixcrown chip captured or immobilized IgG molecules more efficiently than Superaldehyde slide did at the same concentration of the IgG solution. Next, we compared Cy3 signal to measure the amount of protein A bound to the IgG layer (Fig. 4*Bc*, 4*Bd*). Little signal was detected at 4 μg/ml or higher concentration from both chips. One plausible explanation for this would be that at the high concentration each chip was densely packed with the IgG molecules, so that Protein A could not penetrate into the layer. In the meantime, at low concentration, the IgG layer was loosely packed and thus contained some empty room for the access of protein A. Actually, we observed that at 0.16 and 0.8 μg/ml, the fluorescence intensity of Superaldehyde chip was higher than that of Calixcrown chip, indicating that more Protein A molecules were bound to the IgG layer (Fig. 4*Bc*, 4*Bd*). The graphs of Fig. 4*C* show quantified fluorescence intensities on chips of Fig. 4*B*. This observation could support our assumption that IgG molecules on the Calixcrown chip may be arranged more regularly with a vertical orientation than IgG molecules on the Superaldehyde slide. The right orientation of IgG molecules on Calixcrown chip could lead to not only high-density immobilization in a unit area but also high sensitivity of the chip.

Application of Calixcrown chip to clinical samples

We tested the Calixcrown chip with two clinical samples for checking specificity and background, one for detecting antigen and the other for detecting antibody in blood. For the detection of an antigen, serum from a prostate cancer patient was applied onto Calixcrown chip or Superaldehyde chip where capture anti-PSA-mAb had been immobilized. When Cy5-labeled detector antibody was probed for PSA in serum, the detection limit of the Calixcrown chip was 100 times lower than that of the Superaldehyde chip (Fig. 5*A*). For the detection antibody against HIV virus in serum, we used HIV gp120 recombinant protein as a capture molecule on

Calixcrown chip or Superaldehyde chip. We probed Cy5-labeled anti-human-IgG to detect anti-gp120 antibody in the patient's serum. A strong signal was detected on the Calixcrown chip even after 10^5-fold dilution of the HIV serum, whereas the Superaldehyde chip gave a weak signal at 10^4-fold dilution (Fig. 5*B*). In addition, we observed a much lower level of a background fluorescence signal with undiluted sera than we expected. The crown moiety could capture any kind of serum proteins or peptides because of the high concentration and the unexpected nature of the serum components. This result indicated that the Calixcrown chip can be used for medical testing without an extensive blocking process to reduce the background signal.

Antibody microarray on Calixcrown chip for identifying toxicity-specific proteins

We applied Calixcrown using an antibody microarray chip to identify up- or down-regulated proteins. For the fabrication of an antibody microarray, we applied 36 antibodies against a variety of cellular proteins, including cell cycle-specific proteins, cytoskeletal proteins, cell-signaling proteins, and proteins with other cellular functions, on a ProLinker A base slide as a triplicate. For comparison, we prepared cellular proteins from normal rat liver and dimethylnitrosamine (DMN)-treated rat liver as target proteins. DMN has been known to induce fibrosis or cirrhosis in rat liver (32). The total proteins extracted from normal and DMN-treated rat liver were labeled with Cy5 and applied onto a ProLinker A slide of antibody microarray. The analysis of DMN-treated and normal rat liver produced a comparable spot pattern on the microarray on which 36 different antibodies lay. The DMN-treated rat liver proteins were distinguished from normal liver proteins by strong binding to several antibodies (Fig. 6*A*). While some proteins showed either enhanced or decreased fluorescence intensity, most of the proteins displayed little change in their expression pattern. Caspase-3 was detected as one of the proteins that increased during DMN treatment. Caspase-3, a member of the caspase enzyme family, has a central role in the execution of apoptosis, and thus could be increased by DMN treatment (33). Also of interest, integrin was reported to up-regulate in liver inflammation and fibrosis (34). This proves to be a good coincidence with the observation that integrin is also increased by DMN treatment using the antibody microarray. The points inside the yellow box in Fig. 6*B* show proteins whose expression levels vary significantly depending on DMN treatment.

DISCUSSION

In the previous study, we reported the development of a highly sensitive microarray protein chip with novel Calixcrown derivatives that permit efficient immobilization of capture proteins on solid matrixes (1). Using a ProLinker A slide, in this study, we were able to detect a low level of target proteins in clinical samples and apply the antibody microarray for the expression profile of selected proteins in rat liver. Generally, a detection system with high sensitivity accompanies with high background. The background signal from the clinical sample, including undiluted serum, was much lower than we expected in Calixcrown chip. Another advantage of the Calixcrown chip is that the dispensed material does not spread on the surface of the chip. Microarray smearing is a common artifact found in most glass-based arrays. It is also one of the methods by which cross-talk between the adjacent probes occurs, which leads to false results. Most of the current analyses of protein-based interactions on microarrays are based on the surfaces and formats that were used earlier for DNA arrays. Thus, it is also important that ProLinker A-coated protein chips are compatible with most commercial arrayers and

fluorescence scanners. For instance, other substances such as polystyrene, polyvinylidene fluoride, and nitrocellulose membranes are often not compatible with those instruments. In addition, the directional immobilization of proteins on a solid surface is one of the most powerful advantages of the chip. The oriented immobilization on the Calixcrown chip might lead to superior test performance over other protein chips.

Conventional protein-immobilization methods for diagnostics include simply dispensing a protein solution onto the nitrocellulose membrane or coating the wells of an ELISA plate with protein solution (35). In those cases, the proteins are attached onto the surface by physical absorption, and a relatively high concentration of protein solution is required. To achieve more specific and stronger protein attachment, several groups have created a reactive surface on glass that can covalently cross-link to protein. The chemical binding methods using Schiff's base or the aldehyde functional group of activated carboxylic acid are slowly deactivated during the immobilization step, which makes it difficult to get a full coverage of proteins on a solid phase. On the contrary, Calixcrown is functioning to accommodate the protonated amines without any chemical modification. The molecular size of Calixcrown is far smaller than that of an antibody molecule. And since antibody molecule has many numbers of positively charged amino acids, there might be multiple ionic interactions between an antibody molecule and Calixcrown linker. Calixcrown can also form a self-assembled monolayer on gold-coated or amine-coated slide glass just like other SAM molecules. Researchers have used immobilization by SAM to control density and orientation of capture proteins and to obtain better reproducibility on a solid surface (36, 37). The gold-coated glass surface, in particular, has improved the design of controlled interfacial architectures over physical absorption and conventional techniques. Although we used amine-derivatized glass slides in most cases, we also fabricated an antibody microarray on a gold-coated slide (data not shown). However, we could not test sensitivity and reproducibility of gold-coated slides because the gold surface made the emission wavelength of Cy5 on proteins shift to a far longer wavelength than the original one. Presently, we are designing an experiment for reading the shifted wavelength toward the red zone with the gold-fabricated ProLinker B base chip.

A number of studies reported that Calixcrown derivatives form complexes with amino acids using electrospray ionization (ESI) mass spectrometry (38, 39). Using MALDI mass spectrometry, researchers have also studied the inclusion complexes formed between amino acids and Calixarene derivatives. The amino acid complexes were not observed for most of the 20 amino acids in an ion cyclotron resonance (ICR) detector, but were observed in a time-of-flight (TOF) analyzer. However, the most basic amino acids, arginine, histidine, and lysine, formed stable adducts with high incidence (40). As expected, the ammonium complex was the most strong and abundant signal in the mass spectra due to the high availability of NH_4^+. This result showed that the positively charged amino acids are strong binders to the Calix[6]arene derivative and interact with Calixarene by noncovalent linkage. However, they studied complexes in the gas phase only. Our previous report was the first application of Calixcrown-5 for immobilizing proteins on a solid surface (1). To confirm that amine groups of immobilized protein are primarily responsible for the host-guest interaction, we put extra positively charged amino acids to a protein and showed the enhanced interaction between the model protein and the Calixcrown derivative.

Many researchers have created a reactive surface on a solid matrix that immobilizes antibodies on protein chips for the specific detection and enrichment of an antigen from complex biological fluids. However, the process of antibody immobilization has not been quite optimal (19). The principal reason for this is that antibody molecule can bind to the surface in various orientations, many of which block antigen recognition. In the covalent cross-linking approaches, the immobilized proteins attach to the surface in a random fashion because the reactive groups also exist in the side chains of proteins. The physical absorption approach can also make the protein on the surface orientate in random mode, which may alter the native conformation of proteins, reduce the activity of proteins, or make them inaccessible to probes. When the orientation of Y-shaped antibodies appears on a charged surface by Monte Carlo simulations, the molecules prefer lying flat on the surface (41). The absorption of proteins on a solid surface appears to depend mainly on the nature of the proteins themselves. Some striking differences between the absorption behaviors of antibody and albumin were observed (42). By comparing other immobilization methods in this study, we found that Calixcrown-coated slide showed a high efficiency of detection while maintaining a low background level. The oriented attachment of proteins on the ProLinker A-coated chip explains the overall improved test performance. The superiority of Calixarene chip over Superaldehyde slide could be explained by the observation that protein immobilization via ionic interaction may maintain a better folding conformation and keeps the protein more functional, although the electrostatic charges present on the glass may lead to the denaturation of the proteins.

The advantages of the oriented immobilization of proteins are a good steric accessibility of the active binding site and an increased stability (19). The Protein A binding experiment suggests more oriented immobilization of antibodies on Calixcrown slide than on Superaldehyde slide (Fig. 4). In addition, the experiments with clinical samples indicate that the antigen protein may be immobilized in a regular format (Fig. 5). Since the amine group of proteins is the primary guest moiety for complex formation in the ProLinker A slide, the introduction of extra positive amino acids into a protein helps a more efficient site-specific attachment of proteins to the surface of sold supports. Adding positive amino acids, including arginine and lysine in the N or C terminus will give the protein orientation. Inserting a stretch of basic amino acids into a protein by molecular-cloning techniques is expected to become an easy and convenient method for the oriented immobilization of protein on a solid surface.

In conclusion, with a Calixcrown-5 derivative molecule as a fabrication agent for protein microarray, interesting proteins were immobilized on a solid surface via noncovalent ionic interaction without a chemical-linkage reaction. This type of immobilization provides several advantages. First, the binding reaction between Calixcrown-5 and protein occurs spontaneously without any energy input so that protein is immobilized more easily than in other methods. Second, changes of affinity or specificity in the protein-protein interaction can be minimized since there is no chemical reaction involved during the protein immobilization process. Third, high-density immobilization can be achieved by guided interaction between a guest antibody and a host Calixcrown bifunctional linker.

ACKNOWLEDGMENTS

We thank Prof. J. S. Park and K. H. Han at Hallym University for the HIV sera and the preparation of Gal4 protein, respectively. This work was funded by the National Research Laboratory Program (M1-0104-00-0164) of the Korean Ministry of Science and Technology.

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Received May 10, 2004; accepted April 13, 2005.

Fig. 1

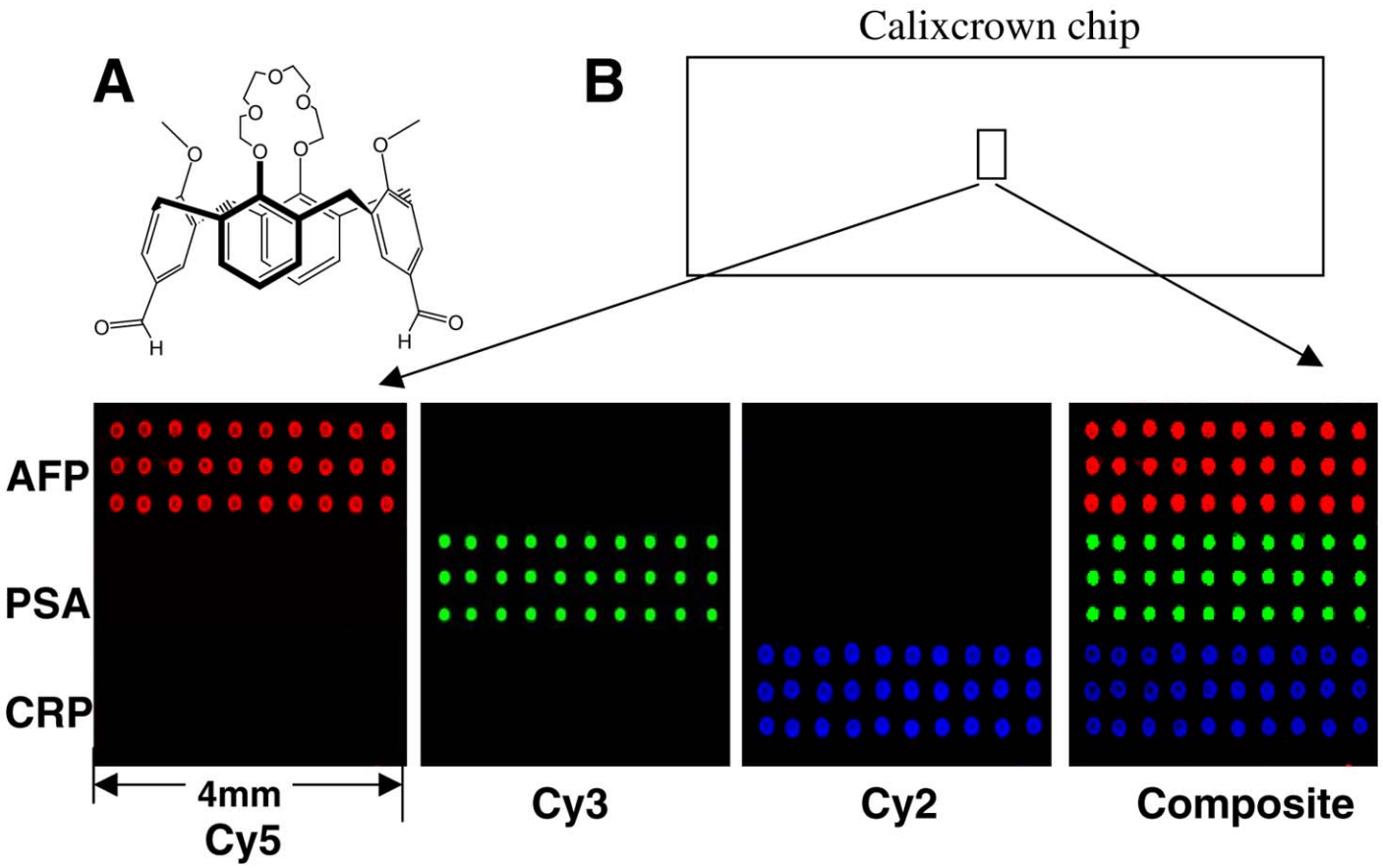

Figure 1. Molecular structure of Calix[4]crown-5 with aldehyde groups (ProLinker A) (***A***) and fabrication of a Calixcrown-coated protein microarray (***B***). Nanoliter volume of anti-AFP, anti-PSA, and anti-CRP-mAb was delivered onto Calixcrown-coated slide by a contact-printing robot and subsequently probed with fluorescence-labeled antigen. The fluorescence signal shows the uniformity of intensity in each spot of the antigen-antibody interaction. ***B*)** Image shown is one representative of five independent experiments.

Fig. 2

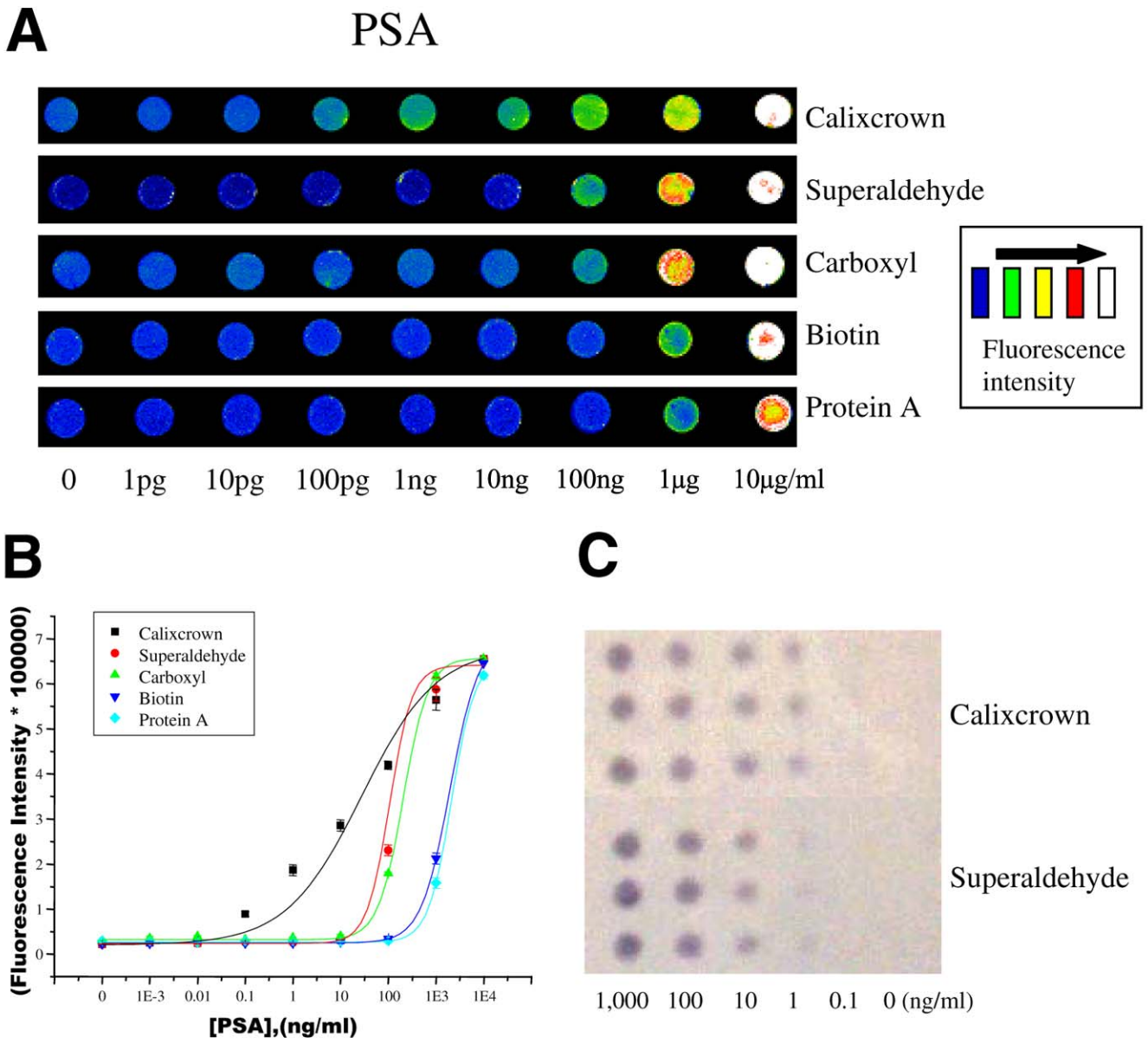

Figure 2. Comparison of detection limits of PSA on different matrices (***A***) and corresponding fluorescence intensity as a function of PSA concentration (***B***). The numerical values of fluorescence intensity were calculated by software of a GSI scanner. The same amount of anti-PSA-mAb was spotted onto different matrix-coated slide glasses, and an indicated amount of PSA antigen was mounted on each spot. Then fluorescence-labeled sandwich pair of anti-PSA-mAb was probed to detect PSA antigen. Fluorescence intensity represents the relative amount of antibody immobilized on different matrix-coated glass slide. ***B*)** Bars represent standard deviation of three independent experiments. ***C*)** Comparison of enzymatic activity of alkaline phosphatase between an Calixcrown-coated glass slide and an Superaldehyde-coated glass slide is shown. The assay was evaluated with a color reaction by adding enzyme substrates BCIP and NBT to the enzyme spot on each glass slide.

Fig. 3

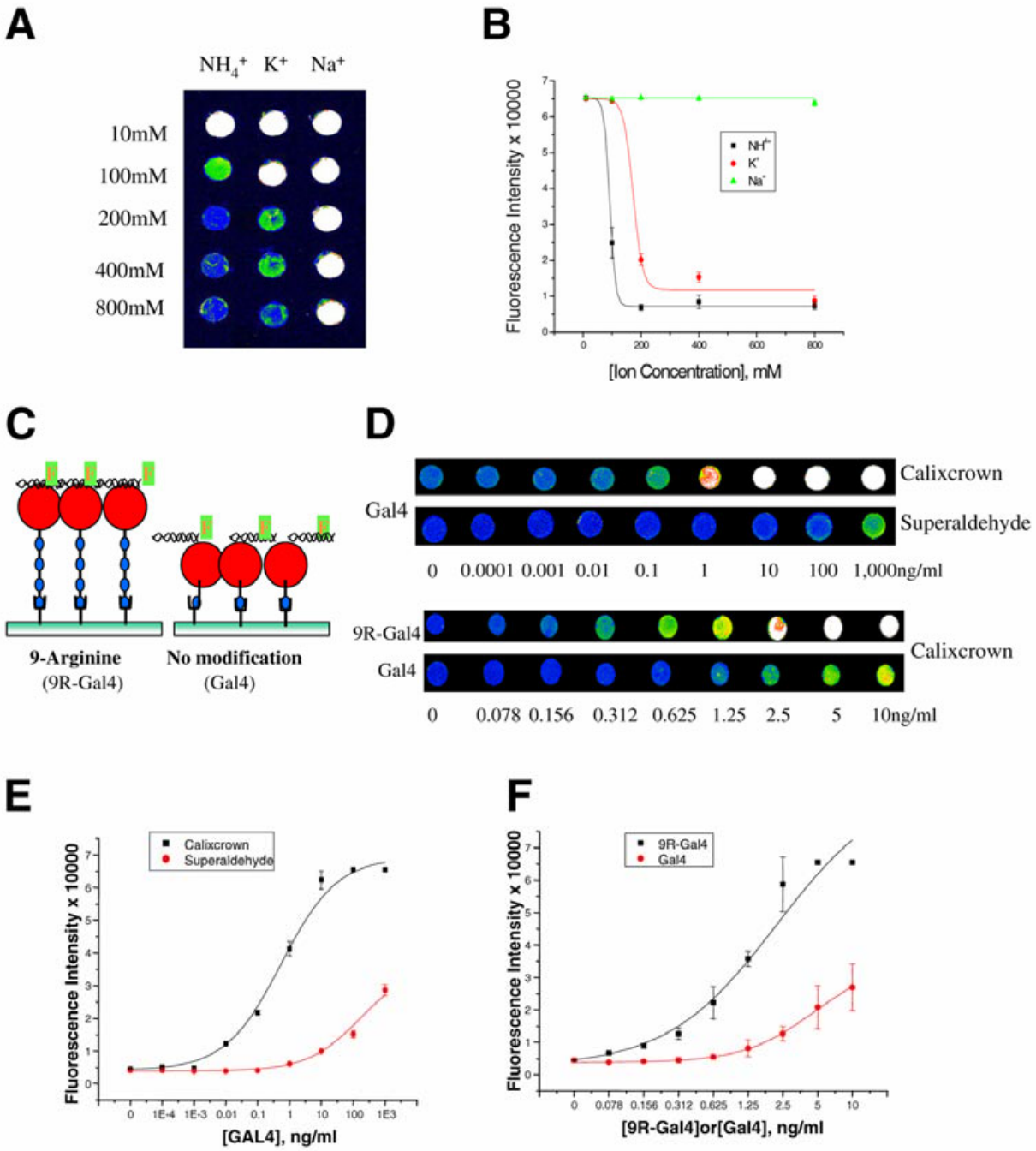

Figure 3. Ionic interaction of amino group with Calixcrown in protein immobilization. Effect of ammonium ion (***A***) and corresponding fluorescence intensity as a function of ion concentration (***B***). Cy5-labeled CRP (CRP-Cy5, 5 μg/ml) solution containing indicated concentration of Na^+, NH_4^+, or K^+ was spotted on Calixcrown glass slides (***A***) and incubated at room temperature for 10 min. The relative fluorescence intensity of Calixcrown chip on which CRP-Cy5 had been immobilized was plotted against the ion concentration (***B***). Bars represent standard deviation of three independent experiments. ***C)*** Schematic diagram to detect a wild and a mutant Gal4 protein with extra basic nine arginines in testing Calixcrown's ability to interact with ammonium ion. 9R represents a mutant Gal4 protein containing consecutive nine arginine residues at the N-terminus. ***D)*** Efficient protein immobilization of Calixarene with Gal4 (***upper panel***) and the effect of extra arginines on the immobilization of mutant Gal4 (***lower panel***) are shown. Protein immobilization was evaluated by application of fluorescence-labeled Gal4 binding oligonucleotide. ***E***) Relative fluorescence intensity of the ***upper panel*** of ***D***. ***F)*** Relative fluorescence intensity of the ***lower panel*** of ***D***. Bars represent standard deviation of three independent experiments.

Fig. 4

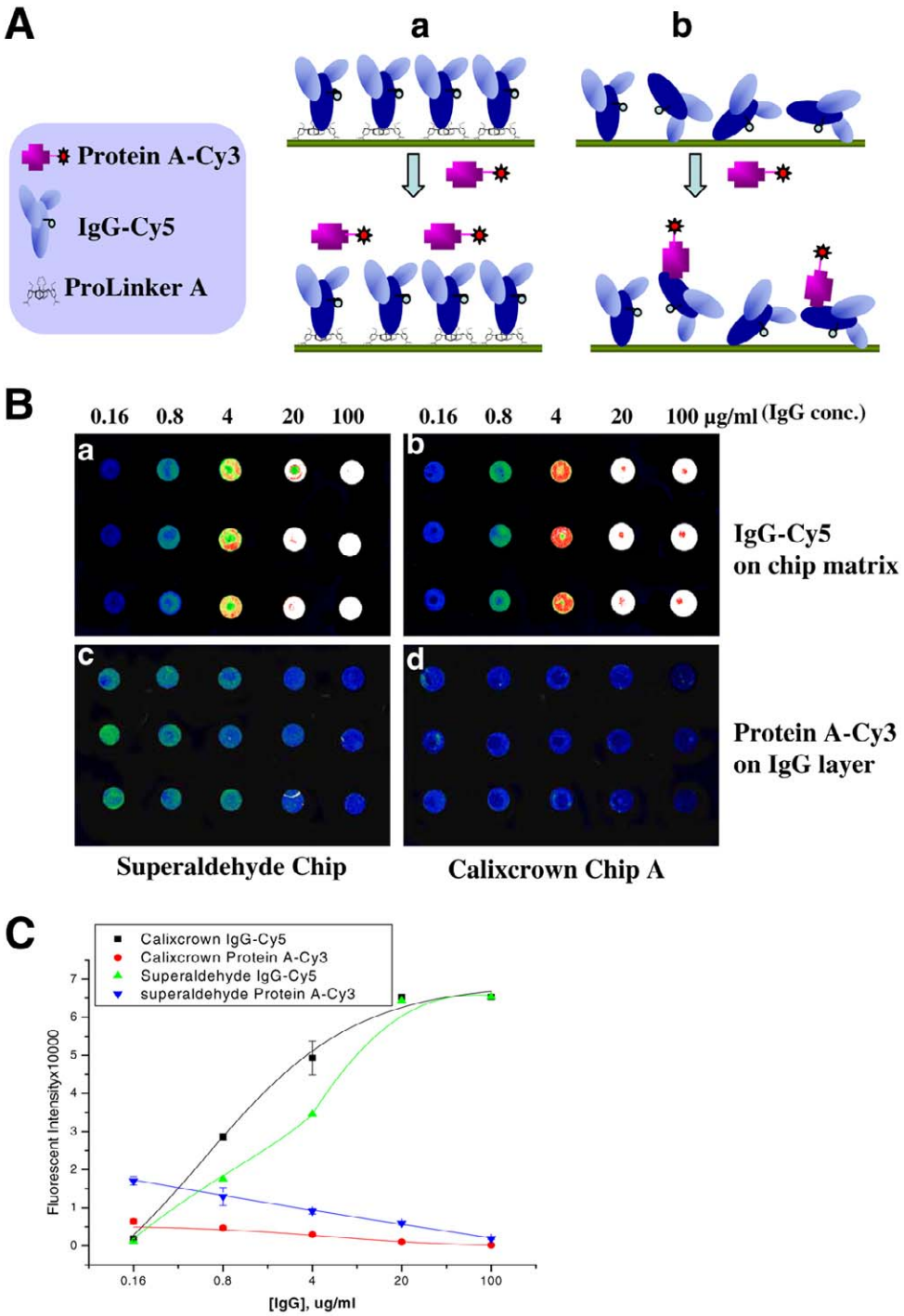

Figure 4. Oriented immobilization of IgG molecules on Calixcrown chip. ***A*)** Schematic diagram showing that IgG molecules' orientation could affect the accessibility of protein A to IgG layer. ***Aa)*** If Fc region of the antibody molecule is captured on the chip as a regular mode, protein A has little chance to bind to the IgG. ***Ab*)** If IgG molecule is immobilized on the chip as a random mode, more numbers of protein A can bind to the IgG. ***B*)** Scanning images of an immobilized IgG molecule and protein A on Superaldehyde slide (***Ba***, ***Bc***) and on Calixcrown slide (***Bb***, ***Bd***) are shown. ***Ba, Bb*)** The higher fluorescence intensity of IgG on Calixcrown chip indicates the more efficient immobilization. Overlay of Cy3-labeled protein A on IgG-immobilized Superaldehyde slide (***Bc***) and on Calixcrown slide (***Bd***) is shown. The lower fluorescence intensity on the Calixcrown slide suggests the possible oriented arrangement of IgG molecules on the chip. ***C*)** Graph shows quantified fluorescence intensities of the chips, and bars represent standard deviation of three independent experiments.

Fig. 5

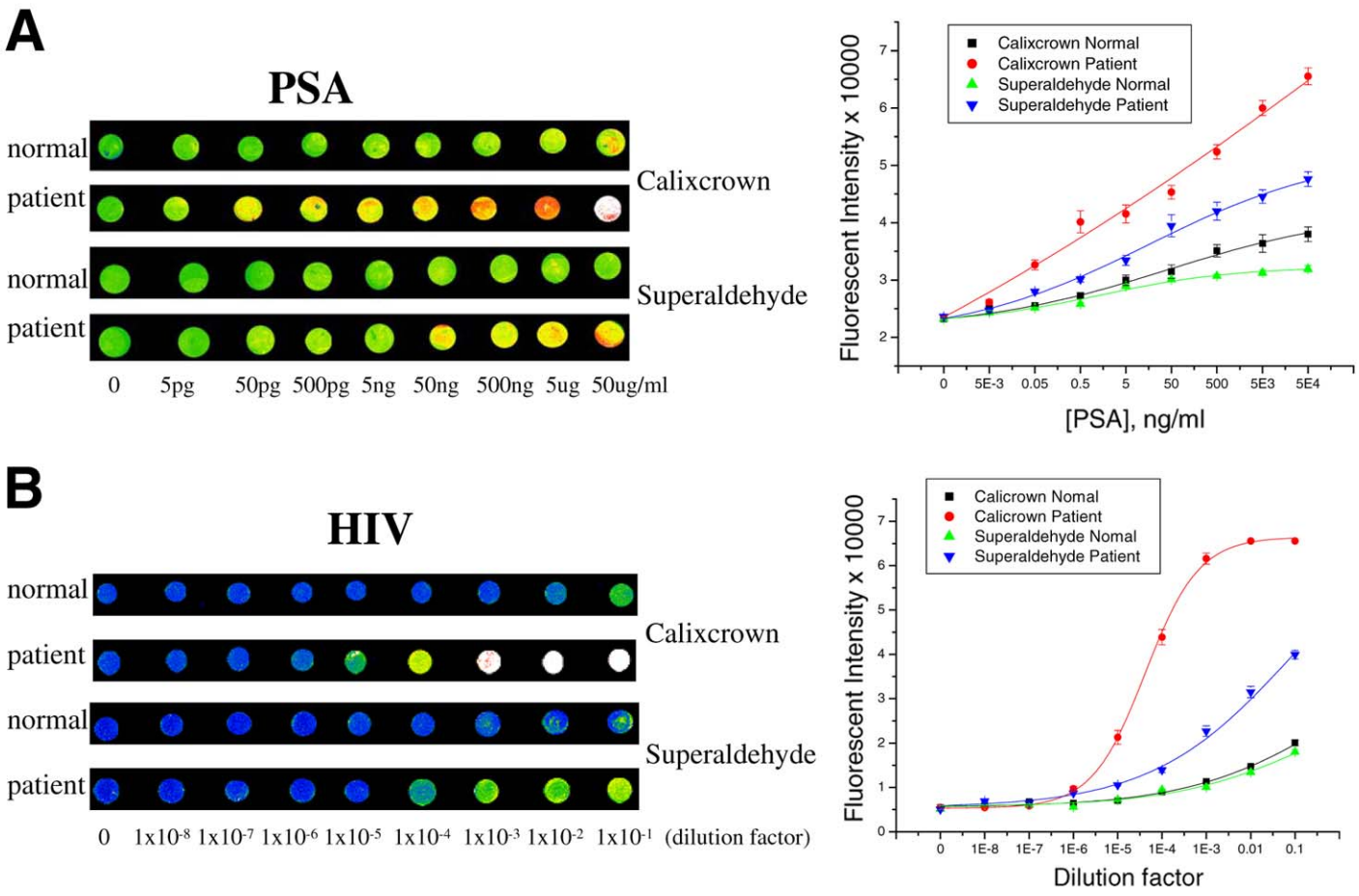

Figure 5. Application of Calixcrown chip for analysis of clinical samples. ***A*)** To detect an antigen in serum, anti-PSA-mAb was immobilized onto Calixcrown- or Superaldehyde-coated glass slides before applying normal and patient serum with an indicated PSA concentration. Anti-PSA-mAb sandwich pair was used to detect PSA antigen in serum. ***B*)** To detect an antibody in serum, HIV gp 120 protein was immobilized onto Calixcrown- or Superaldehyde-coated glass slides. Normal or patient serum was subsequently applied on the chips. The slide was probed with anti-human IgG to detect HIV antibody in the serum. ***A, B*)** Graphs show quantified fluorescence intensities of the chips, and bars represent standard deviation of three independent experiments.

Fig. 6

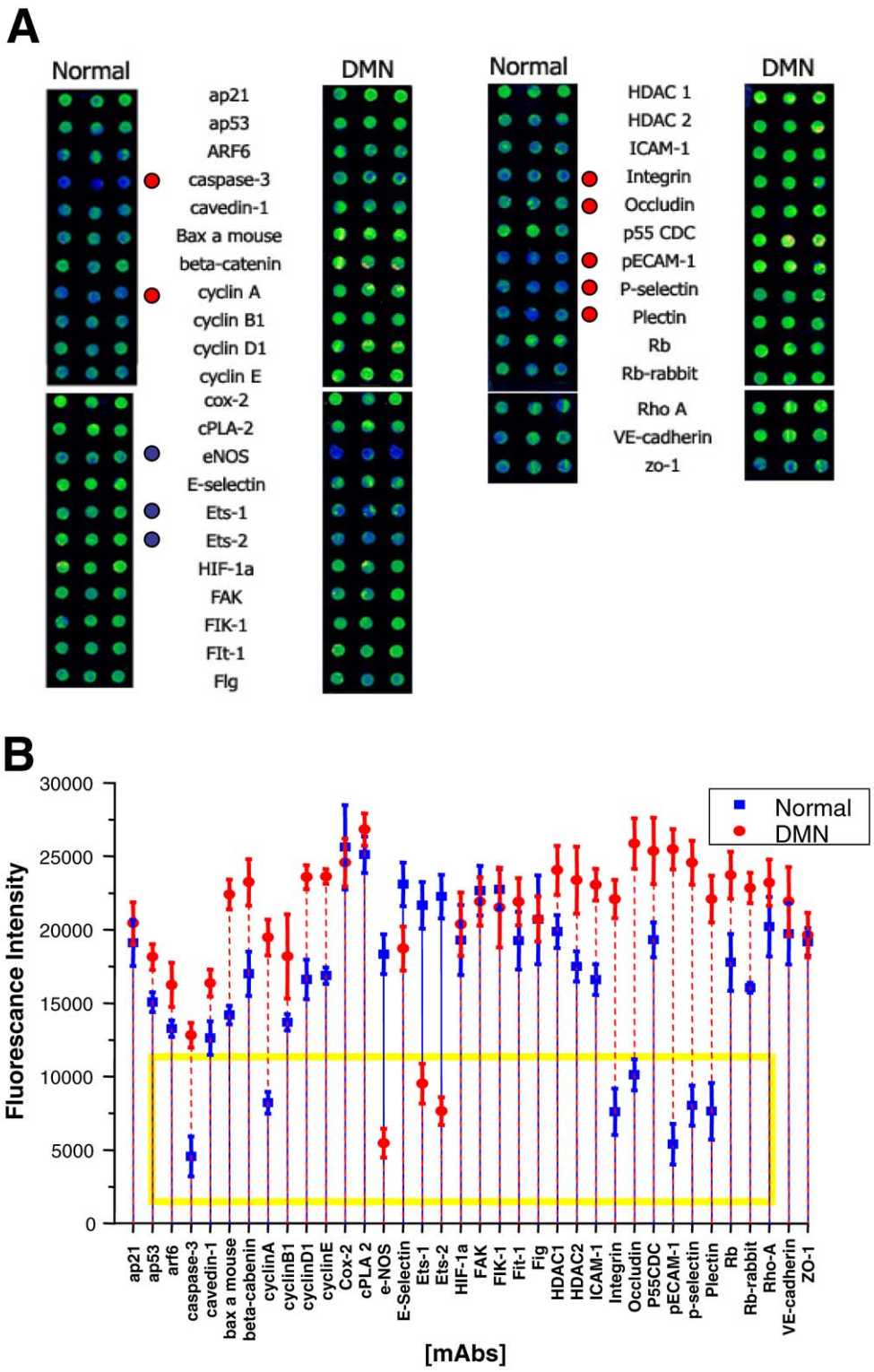

Figure 6. Application of Calixcrown chip for the identification of toxicity-specific proteins in rat tissue. ***A*)** Thirty-six mAbs to various cellular proteins were immobilized on Calixcrown slide. Total liver proteins were extracted from normal and DMN-treated rat, labeled with Cy3, and applied onto the mAbs-immobilized Calixcrown chip. Red and blue circles indicate that the proteins increased and decreased, respectively, in the DMN-treated rat. ***B*)** For the statistical anaysis of the scanned image, the fluorescence intensities were scored from three independent experiments. The points inside the yellow box shows proteins whose expression levels vary significantly depending on DMN treatment.