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ProteoChip: A highly sensitive protein microarray prepared by a novel method of protein immobilization for application of protein-protein interaction studies

We have developed a highly sensitive microarray protein chip, ProteoChip, coated with ProLinker™, novel calixcrown derivatives with a bifunctional coupling property that permits efficient immobilization of capture proteins on solid matrixes and makes high-throughput analysis of protein-protein interactions possible. The analysis of quartz crystal microbalance showed that both monoclonal antibody (mAb) and antigen (Ag) bound to the gold film of the sensor surface coated with ProLinker™ B and that it is useful for studies of Ab-Ag interactions. ProteoChip, aminated glass slide coated with ProLinker™ A, was also demonstrated to be useful for preparation of high-density array spots by using a microarrayer and for analysis of analyte Ags either by direct or sandwich methods of fluorescence immunoassay. The detection sensitivity of ProteoChip was as low as 1–10 femtogram/mL of analyte protein, useful for detection of tumor markers. ProteoChip was also useful for studies of direct protein-protein interactions as demonstrated by analysis of integrin-extracellular matrix protein interaction. These experimental results suggest that ProteoChip is a powerful tool for development of chip-based lead screening microarrays to monitor protein-protein interactions (*i.e.* drug target) as well as for biomarker assays which require high detection sensitivity.

Keywords: ProLinker / Protein immobilization / Protein microarray / Protein-protein interaction / ProteoChip
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1 Introduction

The protein microarray, a solid phase assay method to detect protein-protein interactions, is becoming an attractive tool in proteomics today for diagnostics and therapeutic purposes as well as for basic research [1]. Array-based methods to study proteins allow high-throughput determination of protein functions in parallel [2]. Ekins and coworkers [3, 5] demonstrated, using the ambient analyte assay theory, that a miniaturized binding assay system, like the microarray, can be highly sensitive. Recently, MacBeath and Schreiber [6] reported that a protein microarray chip prepared with aldehyde slides was useful for studying protein-protein interactions with several different protein systems. It was also demonstrated that a high-density antibody (Ab) microarray could be applied for global analysis of expression profiles of proteins [7–9]. Different types of protein or peptide microarrays have been reported to be useful for immunoassays as well as for analyzes of enzymatic activity such as kinase activity [10–12].

The major advantages of protein array technologies include (1) being a highly parallel and miniaturized solid phase assay system, (2) high detection sensitivity, (3) useful for very high-throughput approaches, (4) low consumptions of reagent samples (nL level), (5) potentially attractive manufacturing costs, and (6) they require only a few nanograms of protein. The core technologies for protein microarrays that are currently practiced by protein chip developers are surface chemistry for immobilization of proteins or capture agents, capture molecules which are immobilized onto a solid support and used for capturing target proteins or molecules, and systems to detect protein-protein interactions based on fluorescence, chemiluminescence, MS, electrochemical or surface plasmon resonance (SPR) [9]. Currently, the SPR detection system, which requires immobilization of capture proteins on gold substrate, has been extensively studied and widely applied for analyses of protein-protein interactions [13–15]. Since a SPR sensor system does not require

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Abbreviations: Ab, antibody; **AFM**, atomic force microscopy; **Ag**, antigen; **β-HCG**, beta-human choryonic gonadotropin; **CEA**, carcinoembryonic antigen; **CRP**, C-reactive protein; **ECM**, extracellular matrix; **QCM**, quartz crystal microbalance; **SAM**, self-assembled monolayer; **SPR**, surface plasmon resonance

labeling of analyte proteins, it has been recognized to be a promising tool for proteomics research. Recently, it was reported that a peptide microarray on a gold-coated surface can be coupled with various downstream detection methods, such as SPR [12].

The development of a protein microarray, with improved methods of protein immobilization on solid matrix, is of great interest to the field of biotechnology [16, 17]. Unlike DNA, proteins are chemically and physically heterogeneous, unstable, have a three-dimensional structure that is critical to their function, and can not be amplified [18]. Therefore, development of unique surface chemistry is needed for protein microarrays, a challenge that is not present in DNA microarray applications. For miniaturizing protein microarray devices, surface chemistry plays an increasingly important role for maintaining the conformation and orientation of proteins. An efficient immobilization method is required for attachment of a wide range of proteins to prevent loss of their activity when bound to the surface of solid substrates [19]. Immobilization methods using efficient surface chemistry should be reproducible, applicable to proteins with different properties, amenable to high-throughput automation, and compatible with retention of fully functional protein activity through maintenance of the correct orientation of surface-bound proteins, and minimization of nonspecific protein binding [18, 20].

Several different immobilization methods and tags have been described for construction of protein microarrays. Protein immobilization has been carried out by applying various methods, such as noncovalent absorption [21, 22], covalent linkage by chemical bonding [23, 24], and affinity capture methods such as the streptavidine-biotin interaction [25–27]. There are both advantages and disadvantages to these methods of protein immobilization. Noncovalent binding of proteins has the advantage of low nonspecific binding to glass surface and high capacity and retention of protein function [20]. The method of noncovalent physical adsorption to solid surfaces is simple and allows conformation of immobilized protein to remain intact. In particular, a hydrogen method appears to be useful for enzyme assays. However, these methods suffer from some key limitations such as little control of orientation or quantitative adsorption of biomolecules and, hence, result in lower reproducibility, lower interaction efficiency, and higher error rates of assays.

Covalent bonding has been used for generation of protein microarrays by using a variety of chemically activated surfaces such as aldehyde, epoxy, amine, and active esters [6, 22, 24] as well as attachment by histidine-tag-nickel-chelates [9, 27]. High M_r biopolymers such as various derivatives of collagen, dextran, or cellulose have been commonly used as solid supports for coupling with solid substrates for protein immobilization [29–31]. Covalent coupling methods allow robust protein immobilization with good reproducibility. They can be applied to a wide range of proteins and versatile linkage processes. However, chemical modification of proteins causes loss of activity and low immobilization efficiency. It also requires multiple functional surfaces. Abs immobilized on aldehyde-surfaced solid support had lower binding affinities or reduced specificities of Ab-antigen (Ag) interactions [32].

A specific biomolecular affinity interaction such as the streptavidine-biotin interaction has been widely used as a biological capture method [25, 26]. The major problem with this method is reported to be that biotinylation of Ab molecules causes changes in specificity and affinity of the capture Ab [27]. Biological capture methods employing protein tags provide a stable linkage and bind protein specifically in a reproducible orientation. However, it requires biological reagents to be tagged to a target protein adequately and special handling for the preparation of arrays, and often results in variable stability.

The decreases in activity of proteins captured on solid matrixes may be due to the random orientation of capture proteins, which causes decreased accessibility for interacting proteins, or conformational changes in a protein especially around its active site due to tight binding to the chip surface [33, 34]. Furthermore, the immobilization process of a capture protein should be simple and easy for microarray preparation for high-throughput applications. Improved surface chemistry for immobilization of capture proteins, to preserve their activity and maintain a correct orientation, has been suggested to be necessary for development of high quality protein microarrays.

We present a novel immobilization method to capture proteins which uses calixcrown derivatives (ProLinker™ A and B) on solid substrates. It provides effective high-density protein immobilization without activity loss or incorrect orientation of the capture protein. The ProteoChip is a useful tool for analysis of protein-protein or Ab-Ag interactions as demonstrated by several experimental results with Quartz crystal microbalance (QCM) or fluorescence immunoassay methods. A mechanism of protein binding to solid surface coated with ProLinker™ which results in improved detection sensitivity is provided.

2 Materials and methods

2.1 Reagents

C-reactive protein (CRP) and carcinoembryonic antigen (CEA) were obtained from Fitzgerald Industries International (Concord, MA, USA). All chemicals, solvents,

β-galactosidase (β-Gal), and BSA (96–99%) were from Sigma (St. Louis, MA, USA). To label proteins with fluorescence probes, FluoroLinker Cy3 or Cy5 Mono Reactive Dye (Amersham Biosciences, Uppsala, Sweden) was dissolved in 1 M sodium bicarbonate buffer (pH 9.3) and allowed to react with target proteins for 1 h. Free dye was then separated from labeled proteins by gel filtration in a Sephadex G-50 column (Amersham Biosciences).

2.2 Synthesis of calixcrown-5 derivatives (ProLinker™ A and B)

Two different types of calixcrown-5 derivatives, novel bifunctional molecular linkers, were prepared according to the methods previously described [35]. These are ProLinker™ A which contains CHO groups for amine glass and ProLinker™ B which contains SH groups for gold-coated substrates. The improved processes for the production of calixcrown-5 derivatives described in this work resulted in high product yields without affecting the crown moiety; an important site for protein immobilization. The chemical structure of ProLinker™ A and B are presented in Fig. 1A.

2.3 Preparation of ProteoChips

Two different types of ProteoChips, A and B, were developed by using ProLinker™ A and B, respectively. ProteoChip A was prepared with aminated glass slide, which was produced by treating glass slide with 3-aminopropyltriethoxysilane solution (0.125%) in anhydrous ethanol at 25°C overnight. To prepare ProteoChip A, aminated slide glass was immersed in $CHCl_3$ solution containing 10 mM ProLinker™ A at 25°C for 5 h as previously described [35]. ProteoChip A, coated with ProLinker™ A, can be used for analysis of fluorescence probed proteins with a fluorescence scanner. Patterned glass slide with gold film was used for preparation of ProteoChip B. Gold film was coated on glass slides by thermal evaporation. Prior to coating with ProLinker™ B, the gold-coated glass slides were cleaned in freshly prepared piranha solution (3:1 mixture of concentrated H_2SO_4 and 30% H_2O_2). After washing with deionized water, the glass slides were dried under a stream of N_2 gas. ProteoChip B was prepared by soaking the clean gold-coated ProteoChip base plate in 3 mM ProLinker™ B solution for 1 h and rinsing sequentially with $CHCl_3$, acetone, ethanol, and deionized water. ProteoChip B can be used for protein analyses with SPR or the QCM system without probing for analyte proteins.

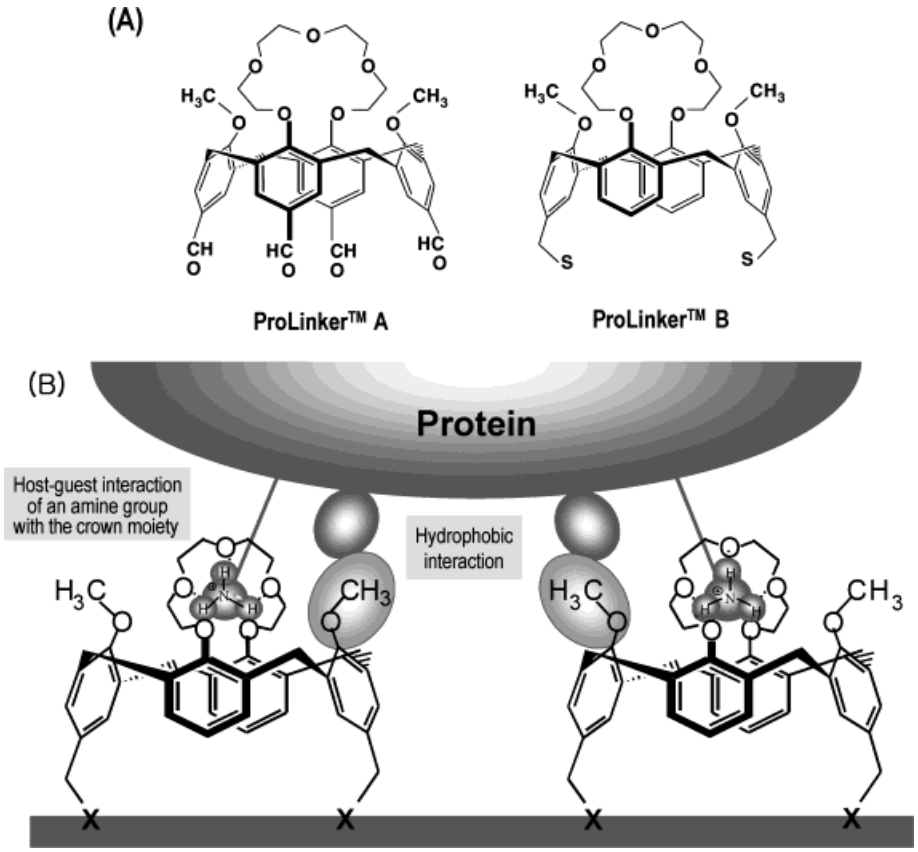

Figure 1. (A) The chemical structures of ProLinker™ A and B, used for preparation of ProteoChip A by coating with amine glass or ProteoChip B by coating with gold substrate. (B) A proposed mechanism of protein binding to molecular linkers (ProLinker™). The major binding force could be attributed to the ionized amine groups of capture proteins, which bind to the crown moiety of the linker molecule *via* host-guest interactions. Hydrophobic interactions between hydrophobic residues of a protein and methoxy groups of the linker molecule may also be involved in protein immobilization.

2.4 Atomic force microscopy analysis

Si (100) wafer (MEMC Electronic Material, Texas, USA) coated with gold film (2000Å thickness) was used as a basic substrate. The coating process of ProLinker™ B on the gold film was the same as described for the preparation of ProteoChip B. Wafer chip coated with ProLinker™ B was immersed in PBS solution (10 mM phosphate buffer with 120 mM saline, pH 7.4) containing Abs or Ags in a concentration of 1 μM for 1 h. The chip coated with immobilized proteins was washed with buffer solution and subjected to analysis by Atomic force microscopy (AFM) images. AFM images were obtained with an Autoprobe CP model (Park Scientific Instrument, Sunnyvale, CA, USA). Experiments were performed in humid conditions using a noncontact mode.

2.5 QCM analysis

QCM sensor chips were purchased from International Crystal Manufactoring (Oklahoma, USA). They were made with unpolished A-T-cut quartz crystals, coated with gold film on both sides (5 mm diameter, 100 μm thickness) and had a fundamental resonance frequency of 10 MHz. ProLinker™ B was coated on the gold film of the QCM sensor. The QCM sensor was horizontally placed to make contact with buffer solution in a flow cell made with Teflon. The flow rate of the input protein solution into the cell was 1 mL/min. At time = 0, the flow was stopped and, when a baseline was stabilized, real time changes of resonant frequency were observed by injecting a protein solution for interaction with the sensor surface. All experiments were performed at room temperature.

2.6 Capture protein immobilization

To fabricate protein microarrays, we applied methods of manual spotting as well as a microarrayer depending on the purpose of the experiments. The microarrayer used for automatic spotting was a high-precision contact-printing robot (Proteogen CM-1000; Proteogen, Seoul, Korea) equipped with either stealth micro-spotting pins (SMP; Telechem, Sunnyvale, CA, USA) SMP10 (335 μm) or SMP3 (100 μm) pins. The SMP (Telechem) were able to deliver nanoliter volumes of protein sample onto the ProteoChip base plate, yielding spots of 300–330 μm or 90–100 μm diameter.

Microarray experiments for immobilization of capture proteins and protein-protein or Ab-Ag interactions were carried out with slight modifications to the method described by MacBeath [6] and Pavlickova *et al.* [36]. A capture protein (Cy5-CEA monoclonal antibody (mAb), 100 μg/mL) in PBS solution, with 30% glycerol to prevent evaporation of nanodroplets, was spotted on a ProteoChip base plate and incubated at 37°C for 3 h. The chip was then washed twice with 30 mL of PBST solution (10 mM PBS with 0.5% Tween 20, pH 7.8) *per* glass slide for 10 min at room temperature and dried under a stream of N_2 gas [36]. Subsequently, the chip was immersed in 30 mL of 3% BSA in PBS solution for 1 h at room temperature to block the chip surface. Blocking and washing processes were performed with the base plate placed in an upside-down position, with gentle shaking on a shaking plate. The immobilized proteins were analyzed by fluorescence scanning. The effects of cations on the immobilization of capture proteins on the ProteoChip base plate were also evaluated using 1 M NaCl or 0.5 M NH_4Cl in PBS solution.

2.7 Protein-protein interactions (manual)

A capture mAb was diluted to a working concentration of 10 μg/mL or 100 μg/mL in PBS solution with 30% glycerol. A ProteoChip, with microspots of a capture protein, was prepared by spotting 1 μL of the protein solution in defined concentrations. It was then washed and dried as described in Section 2.6. In order to carry out further experiments on protein-protein interactions, one μL of an analyte Ag probed with Cy5 (Ag-Cy5) diluted in varying concentrations (from 1 μg to 1 fg/mL) in PBS solution was added onto each spot of mAb on a chip blocked with BSA and incubated for 1 h at 37°C in a humidity chamber. The chip was washed with PBST solution and dried as described in Section 2.6.

2.8 Protein-protein interactions (arrayer)

Protein-protein interactions were also carried out by using automatic spotting of proteins with a microarrayer (Proteogen CM-1000; Proteogen) equipped with a SMP10 stealth pin. Ab-Ag (CEA) and integrin-extracellular matrix (ECM) pairs were employed as models for protein-protein interaction studies. The prefabricated ProteoChip immobilized with a capture protein such as a mAb or an integrin receptor (integrin $\alpha_{IIb}\beta_3$) was prepared by spotting with CM-1000 as described in Section 2.6. Washing and drying steps were performed as described in Section 2.6. The protein microarray thus prepared was also incubated and washed in PBST solution as described in Section 2.6, to remove unbound protein molecules. Subsequently, the base plate with protein microarray spots was blocked with 3% BSA in PBS solution by incubating it for 1 h at room temperature. After blocking, the microarray was washed in PBST solution to remove excess BSA. For protein-protein interaction analysis, an interacting protein probed with Cy5 in various concentrations (100 μg/mL–1 fg/mL for CEA and 1 μg/mL–1 fg/mL for fibrinogen)

was spotted on the array spots of a capture protein and incubated for 1 h at 37°C in a humidity chamber. The microarray chip thus prepared was washed in PBST solution to remove unbound fluorescent material. Fluorescence scanning was performed for analyses of protein-protein interactions.

2.9 Fluorescence scanning analysis

The ProteoChip arrays prepared for protein immobilization and protein-protein interaction studies were scanned in a Scan Array Lite model (GSI Lumonics, Kanata, Ontario, Canada) or GenePix 4000B (Axon Instruments, CA, USA) scanner using 532 nm or 635 nm lasers. The scanners were set to optimize the quality of the microarray images by adjusting the laser power and contrast. The signal intensity was quantified with GenePix Pro 3.0 software purchased from Axon Instruments (California, USA).

2.10 Sandwich fluorescence immunoassay

The present sandwich fluorescence immunoassay was carried out using the manual spotting method. A capture Ab was diluted to 100 μg/mL or 10 μg/mL in PBS solution containing 30% glycerol. Diluted antibody solution (0.5 μL) was subsequently spotted on a ProteoChip base plate. The steps for immobilization of capture Ab on ProteoChip were followed as described in Section 2.6. For the sandwich fluorescence immunoassay, an analytic Ag (0.5 μL) diluted in different concentrations ranging from 100 μg/mL to 1 fg/mL, in PBS solution containing 10% BSA and 30% glycerol, was added onto each spot of mAb on a chip and incubated for 1 h at 37°C in a humidity chamber. The chip was then washed with PBST solution and dried as described in Section 2.6. Polyclonal Ab (0.5 μL of 1 μg/mL or 5 μg/mL solution) was added onto each spot and incubated at 37°C for 30 min in a humidity chamber. It was then washed with PBST solution and dried. The chip was incubated with 0.5 μL of IgG-Cy5 (1.0 μg/mL or 5.0 μg/mL) at 37°C for 30 min in a humidity chamber, washed with PBST solution and dried. The fluorescence intensity of each spot on the chip was determined in a fluorescence scanner and displayed either colored spots or as numerical values in a dose-response curve.

3 Results

3.1 Structures of ProLinker™ and a proposed mechanism of protein binding

ProLinker™ A and B, calixcrown-5 derivatives, are novel bifunctional molecular linkers for efficient protein immobilization on solid surfaces. ProLinker™ A, which contains aldehyde groups, is used for aminated glass to make ProteoChip A. ProLinker™ B which contains thiol groups is used for gold-coated glass to prepare ProteoChip B (Fig. 1A). Conformers (cone or partial cone) of calixarene derivatives are known to act as excellent host molecules for interaction with organic guests. Interaction between neutral organic molecules and calixarene strongly depend on the extent of distortion in the conformation of the host cavity [37]. A coupling mechanism of proteins to ProLinker™ was proposed to be mediated by the ionized amine group of the capture proteins, which binds to the crown moiety of the linker molecule in a host-guest interaction (Fig. 1B). This mechanism was confirmed by the inhibitory effect of ammonium ion on protein binding to ProteoChip coated with ProLinker™ (Section 3.5).

3.2 Use of AFM for observation of immobilized Ab and Ab-Ag interactions

Figure 2A shows a diagram of the simple steps for immobilization of a capture protein and interaction of an analyte protein on ProteoChip base plate coated with ProLinker™. The self-assembled monolayer (SAM) of ProLinker™ which forms on the surface of the ProteoChip does not allow space for nonspecific binding. This is thought to be the key factor responsible for the high sensitivity and accuracy of analysis of protein-protein interactions obtained with the protein microarray. To observe physical characteristics of bound proteins immobilized on ProLinker™ B, AFM analysis was carried out. As shown in Fig. 2B, AFM images on the nanometer scale revealed the protein monolayer formed either by β-Gal Ag (Fig. 2B, middle panel) or anti-β-Gal mAb (Fig. 2B, right panel) on the surface of the gold film of the wafer coated with ProLinker™ B (Fig. 2B, left panel). Both β-Gal Ag and anti-β-Gal Ab were uniformly and densely assembled on the entire gold surface without leaving room for nonspecific binding of other proteins. This observation suggests that the protein layer formed on the surface of gold substrate coated with ProLinker™ may provide smooth surface characteristics and maintain the biochemical properties of the immobilized protein on the chip surface intact.

3.3 QCM analysis of protein immobilization and Ab-Ag interaction

To confirm the efficiency of protein immobilization on gold substrate coated with ProLinker™ B and of protein-protein interactions, QCM analyses were carried out. The time-dependent changes in frequency caused by binding of proteins to the sensor surface coated with ProLinker™ B were analyzed using the QCM method. As shown in

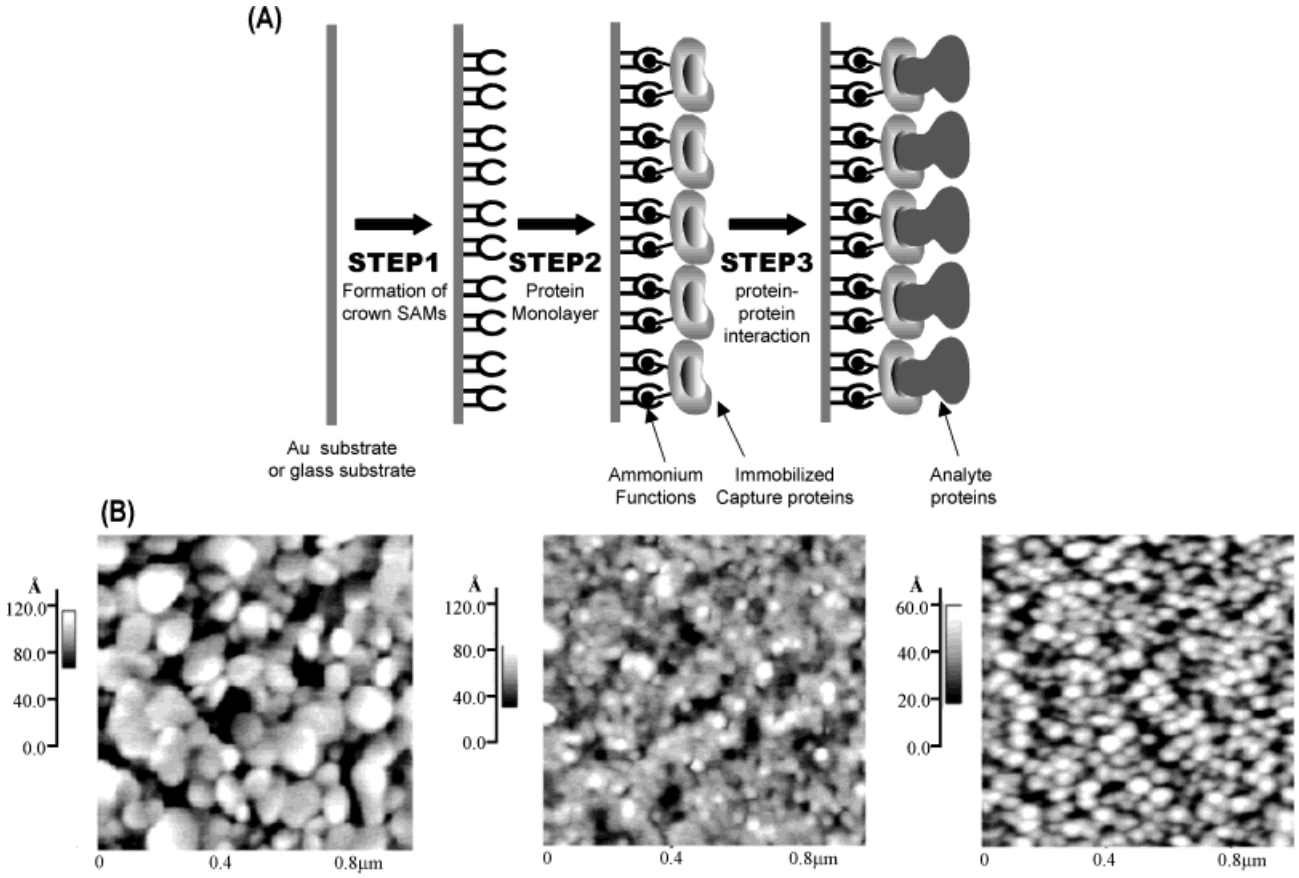

Figure 2. (A) A diagram of the simple steps for immobilization of a capture protein and interaction of an analyte protein on ProteoChip coated with ProLinker™. (B) Noncontact mode AFM images of a gold substrate without an immobilized protein (left panel), with immobilized β-Gal Ag (middle panel), and with immobilized β-Gal mAb (right panel). Ab and Ag can bind uniformly and tightly to the surface of gold substrate coated with ProLinker™ B.

Figs. 3A and B, both CRP mAb and Ag bound well to the QCM sensor surface coated with ProLinker™ as seen by the changes in frequency following addition of the proteins. The frequency changes reached a plateau, when protein binding became saturated. At this point, further addition of an excess amount of the same proteins did not cause frequency changes. This result provides evidence that once a monolayer of protein is formed on the sensor chip surface, there is no space available for additional binding onto the layer of immobilized protein. However, subsequent addition of CRP Ag to the immobilized mAb (Fig. 3A) or CRP mAb to the immobilized Ag (Fig. 3B) resulted in dramatic changes in frequencies suggesting that the Ab-Ag interaction occurred on the QCM sensor. A similar result was also observed with QCM analysis of the ferritin Ab-Ag interaction as shown in Fig. 3C.

To investigate if IgG molecules can bind to the ProLinker™ surface with the correct orientation, the Protein A-IgG interaction was examined by QCM analysis, since its known that Protein A binds to the Fc region of IgG. When Protein A was immobilized first and CRP mAb was subsequently incubated with Protein A-captured QCM sensor, it was demonstrated that CRP mAb bound to Protein A instantaneously (Fig. 3D). However, Protein A did not bind to CRP mAb immobilized to the sensor surface (Fig. 3E). In fluorescence analysis of Ag-Ab and IgG-Protein A interactions, CRP mAb immobilized on the ProteoChip base plate interacted with the CRP Ag. In contrast, Protein A did not interact with immobilized CRP mAb (Fig. 3F). These data clearly show that IgG molecules immobilized on the ProLinker™ surface form a tight molecular monolayer with vertical orientation, in such a way that the Fc domain of IgG binds to the ProLinker™ surface hindering Protein A interaction with the binding site on the Fc domain. In contrast, the Fv domain of IgG is open to the medium allowing for the Ag to interact freely.

The estimated number of protein molecules bound on the surface of the solid matrix was determined by analysis of vibration frequencies. When the weight of a protein bound on the surface is increased, the vibration num-

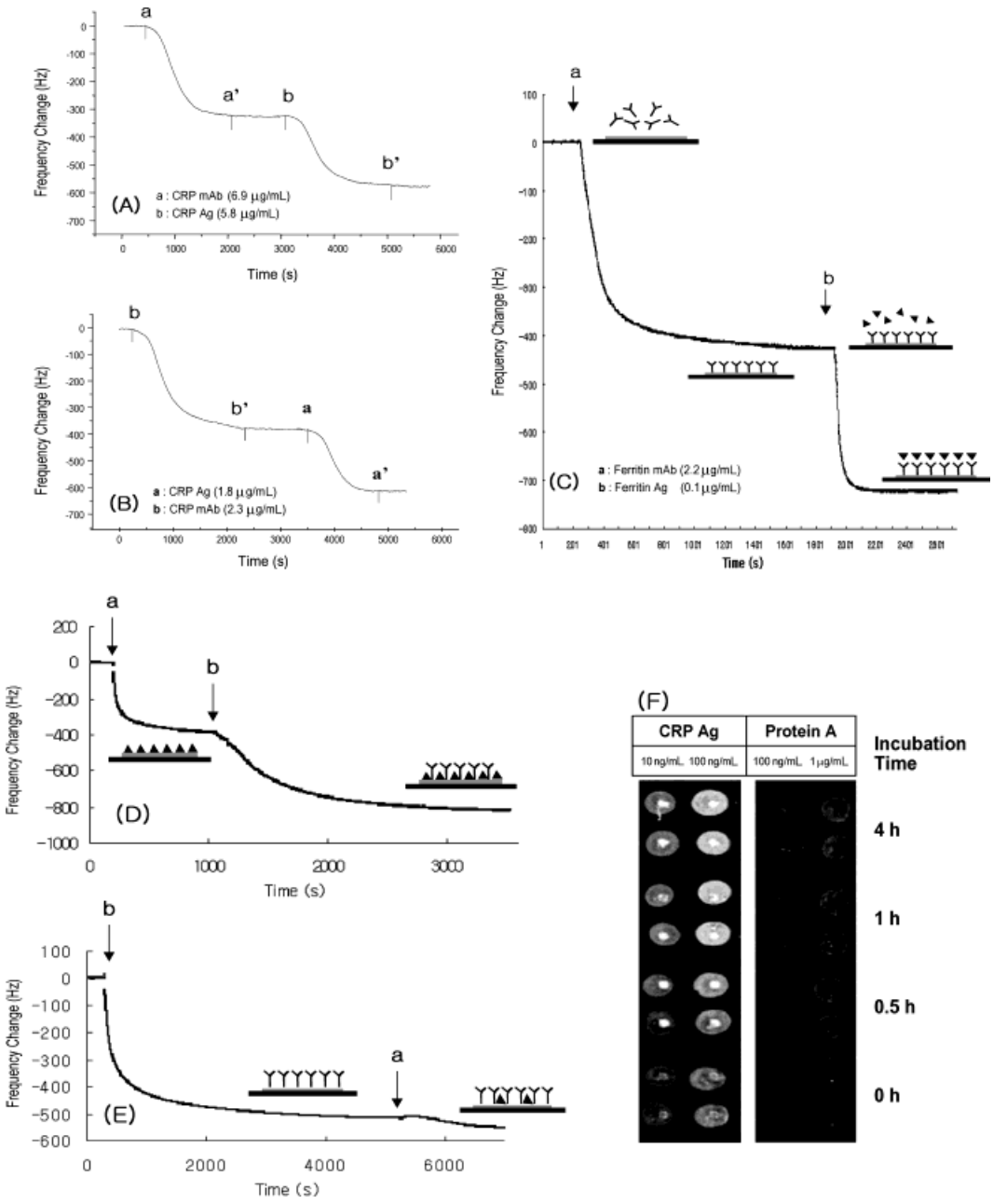

Figure 3. Kinetic analysis of Ag-Ab and IgG-Protein A interactions by QCM. (A) QCM analysis of CRP Ab-Ag interaction, a and a' indicate addition of CRP mAb (6.9 μg/mL) and b and b' addition of CRP Ag (5.8 μg/mL), respectively. The result shows CRP Ag binding to CRP mAb, immobilized on the sensor chip surface. (B) QCM analysis of CRP Ag-Ab interaction CRP mAb, a and a' indicate addition of CRP mAb (2.3 μg/mL) and b and b' addition of CRP Ag (1.8 μg/mL), respectively. The result shows interaction with CRP Ag, immobilized on the sensor chip surface. (C) QCM analysis of the ferritin Ab-Ag interaction. (a) and (b) indicate the time of addition of 100 μL mAb and Ag, respectively. (a') and (b') indicate the time of the second injection of an additional 100 μL mAb and Ag, respectively. These experiments were carried out with low ionic strength buffer (0.83 mM PBS) at room temperature. (D) QCM of Protein A-IgG interaction. Protein A (100 μL of 10 μg/mL) was immobilized (a) and 100 μL of CRP mAb solution (10 μg/ml) was subsequently added (b) to interact with protein A demonstrating that mAb was bound to protein A. (E) Protein A did not bind to IgG immobilized on the QCM sensor surface. (F) Fluorescence analysis of Ag-Ab and IgG-Protein A interactions. The capture CRP mAb immobilized on ProteoChip interacted with the corresponding Ag tagged with Cy3, but did not bind Protein A as detected by fluorescence scanning.

ber of the quartz plate is decreased. According to the Saubery Eq. [38], $\Delta f = -C_f \Delta$ m, where the value of C_f is $2.26 \times 10^2 cm^2 MHz/g$ and the change of 1 Hz represents the weight change of 4.42 ng/cm^2, one can estimate the number of protein molecules bound. Based on the above formula, the weight of anti-β-Gal mAb immobilized on the surface is approximately 4.2 μg/cm^2, suggesting that approximately 26 picomoles of the molecule are bound on the surface.

Table 1 summarizes the numbers of Ab or Ag molecules bound on the chip surface and the stoichiometric ratios of Ag-Ab interaction calculated with data obtained from QCM analysis of Ags with different M_r. It was estimated that the stoichiometric binding ratios for CRP (23 kDa), a-fetoprotein (70 kDa), β-galactosidase (116 kDa), ferritin (440 kDa), and Protein A (45 kDa) with their respective antibodies (150 kDa) are approximately 5, 3, 1.5, 1.0, and 3.2, respectively. These experimental values of stoichiometric ratios are reasonable considering the molecular sizes of the Ags relative to that of the Abs. It was estimated that the number of Ab molecules bound on the solid surface were 5.6×10^{12}, 5.4×10^2, 6.4×10^{12}, 6.8×10^{12}, and 2.3×10^{13} for CRP, α-fetoprotein, β-galactosidase, ferritin, and Protein A, respectively. The average number of Ab molecules bound on the surface was calculated to be 6×10^{12} molecules/cm^2, which is in agreement with the theoretical value (4.06×10^{12} molecules/cm^2) estimated based on the molecular dimension of IgG molecules (7 nm × 3.5 nm) that may cover the solid surface (Fig. 4A). The result suggests that bound Abs form a dense molecular monolayer on the gold film surface coated with ProLinker™.

Table 1. Stoichiometric ratios of Ag-Ab interaction[a)]

Interacting Ag (M_r)	Order of protein interaction	Number of molecules bound[a)] (molecules/cm^2)	Binding ratios for Ab-Ag interaction
CRP (23 kDa)	1. Ab 2. Ag	5.6×10^{12} 2.9×10^{13}	1.0 : 5.0
α-Fetoprotein (70 kDa)	1. Ab 2. Ag	5.4×10^{12} 1.7×10^{13}	1.0 : 3.0
β-Galactosidase (116 kDa)	1. Ab 2. Ag	6.4×10^{12} 9.6×10^{12}	1.0 : 1.5
Ferritin (440 kDa)	1. Ab 2. Ag	6.8×10^{12} 2.0×10^{12}	3.4 : 1.0
Protein A (45 kDa)	1. Protein A 2. Human IgG	2.3×10^{13} 7.2×10^{12}	3.2 : 1.0

a) Ab-Ag interaction and the number of Ag or Ab molecules bound were estimated by the QCM method carried out (see Fig. 3).

From these results, it is postulated that the immobilization of capture Ab on the solid chip surface by ProLinker™ brings about formation of a monolayer of the capture Ab in high density and the correct orientation to capture analyte proteins more efficiently than conventional immobilization processes which allow random binding orientations (Fig. 4C). It was expected that not all single mAbs would be in the right-side-up position in the microarray spots. However, a major part of the immobilized mAb molecules must be attached in the correct orientation on the chip surface. The novel surface property of ProLinker™ is thought to be responsible for the high detection sensitivity of analyte proteins by ProteoChip without loss of activity of capture Ab.

3.4 Immobilization of capture proteins on ProteoChip A

In order to examine the immobilization efficiency of a capture protein on ProteoChip, a series of experiments on spot density and binding capacity, as a function of capture protein concentration, were carried out by using a microarrayer, CM-1000 (Proteogen). Figure 5A shows microarray spots of several different capture proteins (Protein A-Alexa546 (yellow), CRP mAb-Cy5 (green) and mouse IgG-Cy3 (red)) immobilized on the ProteoChip A coated with ProLinker™ A. The array spot densities shown in Fig. 5A are 4900 ea/cm^2 and 460 ea/cm^2 as spotted using ArrayIt (Telechem) SMP3 (100 μm) and SMP10 (335 μm), respectively. The spot sizes of the two different microarrays are estimated to be 0.03 mm^2 (0.6 mm diameter) and 0.28 mm^2 (0.2 mm diameter), respectively. Each microspot was densely and regularly arrayed on ProteoChip without diffusion of reagent materials between spots. These experiments demonstrated that the ProteoChip base plate can be used for preparation of protein microarrays using an automatic spotting machine.

Figures 5B and C show a rainbow color display of array spots and dose-response curves, respectively, tested with spotting pins of two different sizes, SMP3 and SMP10. Gradual changes in fluorescence intensity of the spots as a function of varied concentrations of a capture Ab, CEA mAb-Cy5, spotted on ProteoChip A are seen. The fluorescence intensity increased gradually as the concentration of mAb increases until the spots were saturated with mAb. The saturation concentration of capture mAb was about 100 μg/mL when spots of two different sizes were spotted with SMP10 and SMP3 stealth pins using a 0.5 s dwelling time. It is noteworthy that larger microarray spots spotted with the SMP10 pin (335 μm) can provide better conditions for protein immobilization and better visualization of microspots by delivering a larger volume (about 23 nL)

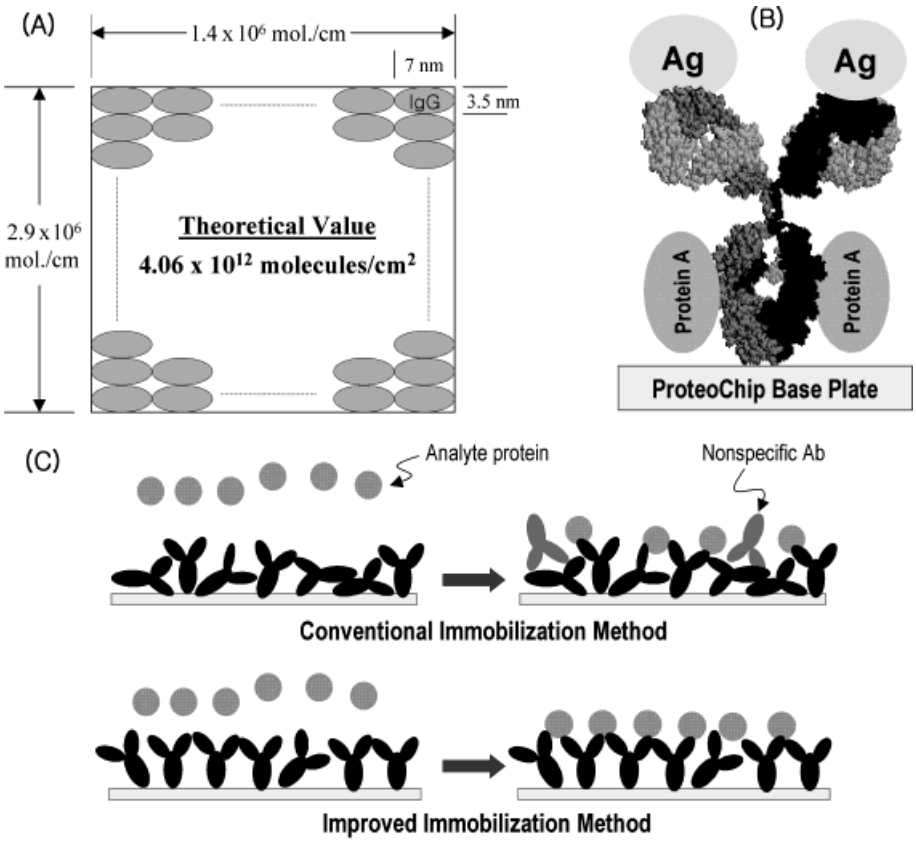

Figure 4. (A) Theoretical estimation of the number of IgG molecules immobilized on an unit surface. The theoretical value of IgG molecules bound on a 1 cm^2 surface is 4.06×10^{12} molecules (mol.) as calculated by assuming that the dimension of an IgG molecule (top view) is 7.0 nm × 3.5 nm and forms a monolayer. The estimated experimental values from QCM analysis are 6.8–5.4×10^{12} molecules/cm^2 (see Table 1). (B) A model of IgG bound on solid surface demonstrating that the Fc domain of IgG is the binding site fixing it onto solid surface. It has been proven by experimental facts that the Fv sites are always open for Ag binding, while the Protein A binding site is hindered by Ab attachment on the chip surface. (C) A model for increased sensitivity based on improved surface properties of capture Ab immobilized on the microarray spot surface. (Top panel) Nonspecific Ab binding due to irregular orientation of capture mAb and reduced Ag binding to mAb may occur with conventional immobilization methods. (Bottom panel) Increased detection sensitivity can be attained by minimized nonspecific Ab binding and increased Ag binding to Ab due to the correct orientation of capture mAb immobilized in high density on the surface of ProteoChip.

of protein solution than that obtained with the smaller pin, SMP3. Therefore, all experiments on protein-protein interaction described in this paper were carried out with a SMP10 pin.

3.5 Effect of ammonium ion and pH on protein immobilization with ProLinker™ and a mechanism of protein binding to the linker molecule

We examined the effect of two different salt ions, ammonium and sodium, on immobilization of capture mAb onto the surface of ProteoChip. As shown in Figs. 6A and B, the fluorescence intensity of CEA mAb immobilized on the ProteoChip base plate in the presence of 0.5 M NH_4Cl was almost the same as that of the negative control even when the concentration of CEA mAb increased. In contrast, the fluorescence intensity of CEA mAb in the presence of 1 M NaCl increased steadily as the concentration of CEA mAb increased. These results demonstrate that ammonium ion inhibited binding of CEA mAb to the ProLinker™ surface of the ProteoChip, suggesting that the amine groups of a protein could be involved in the immobilization of a capture protein to the ProLinker™ surface by way of host-guest interaction with the crown moiety of ProLinker™.

To confirm the effect of ammonium ion on the immobilization of CEA mAb onto the surface of the ProteoChip, the effect of pH on this process was investigated (Figs. 6C and D). These studies demonstrated that the binding affinity or the number of bound molecules increased at acidic pH, whereas alkaline pH decreased the binding affinity of the capture Ab. This suggests that the ionizing residue is an amine group, since the interaction of a capture Ab with the ProLinker™ appears to be dependent on a pK group of 8.5. This result confirms

the result of ammonium ion inhibition of the protein binding on the surface of ProteoChip suggesting that amine groups of a capture protein bind to the crown moiety of ProLinker™. In our proposed mechanism of capture protein immobilization (Fig. 1B), hydrophobic interactions are also considered as a binding force.

3.6 The detection sensitivity of ProteoChip

To evaluate whether the detection sensitivity of ProteoChip for analyte proteins improved, we conducted experiments on direct Ag-Ab interactions as a function of analyte protein concentration using the fluorescence assay method with either manual (Figs. 7A and B) or arrayer spotting (Fig. 7C). Figures 7A and B show the effect of the concentration of capture mAb (CRP) on the detection sensitivity of the Ab-Ag interaction. In experiments of Ab-Ag interaction, the array spots of CRP mAb immobilized at two different concentrations (10 μg/mL and 100 μg/mL) were treated with varied concentrations of CRP-Cy5 as an interacting Ag. It was observed that the fluorescence intensity of the spots increased gradually with increasing amounts of applied Ag. It is of interest to note that the lowest detection limit of interacting Ag was 1 fg/mL when capture mAb was prepared with CRP-Cy5 at a concentration of 100 μg/mL, whereas a detection limit of 1 pg/ml was obtained for arrays spotted with 10 μg/mL CRP-Cy5 solution. The fluorescence intensities of the array spots were saturated at a concentration of 1 ng/mL and 1 μg/mL CRP-CY5 for CRP mAb microarrays spotted with 100 μg/mL and 10 μg/mL mAb solution, respectively. This observation suggests that a low concentration of capture mAb results in less mAb immobilized to ProteoChip with decreased efficiency of Ag interaction (Figs. 7A and B). These data imply that the detection sensitivity of the Ab-Ag interaction is dependent on the saturation state of capture Ab immobilized on ProteoChip.

The detection sensitivity of ProteoChip was confirmed by monitoring a second Ab-Ag interaction. CEA was spotted with the CM1000 microarrayer (Figs. 7C and D). To investigate the detection limit of interacting Ag (CEA-Cy5) on ProteoChip, 100 μg/mL of CEA mAb as a capture Ab was spotted on the ProteoChip with a CM-1000 microarrayer equipped with a SMP3 (300 μm in diameter) and the fluorescence intensities were determined after subsequent incubation with CEA-Cy5 in different concentrations ranging from 100 μg/mL to 1 fg/mL (Figs. 7C and D). The fluorescence intensity of the array spots were saturated at a concentration level of 100 ng/mL CEA-Cy5. The detection limit of CEA Ag was found to be 1 fg/mL confirming the previous result with the CRP system. These results strongly support the premise that ProteoChip is an extremely sensitive protein microarray with the lowest detection limit of analyte Ag (1 fg/mL) ever reported. The data also support our postulation that the high detection sensitivity of analyte Ag by ProteoChip may be due to monolayer formation of capture mAb immobilized on the ProteoChip surface in high density and in the correct orientation (Fig. 4C). The experimental results reported in this paper strongly suggested that ProteoChip will be a promising protein microarray applicable for immunoassays of low abundant proteins and for studies of various protein-protein or Ag-Ab interactions with a high detection sensitivity.

3.7 Applications of ProteoChip for sandwich immunoassay of biomarkers and protein-protein interaction

To explore possible applications of ProteoChip in biomarker assays, we carried out experiments of sandwich immunoassays for the analysis of biomarkers such as β-human choryonic gonadotrophin (β-hCG), CEA, and CA19-9, well-known tumor markers, as model targets (Figs. 8A and B). As shown in Fig. 8A, the relative fluorescence intensity increased with increased concentrations of analyte marker proteins. The detection limits of the three marker proteins were found to be as low as 1 fg/mL. The dose-response curves, estimated from the data in Fig. 8A, are shown in Fig. 8B. Sigmoid curves with a broad dynamic range for all of three biomarker assay systems were obtained. These preliminary experimental results indicate that ProteoChip is applicable for biomarker assays in biological fluids for the method of sandwich immunoassay.

It was also expected that ProteoChip could be applied effectively for new lead screening by using a protein-protein interaction system as drug targets. In order to prove this concept, we have developed an experimental system to detect a protein-protein interaction on ProteoChip. In this experiment, an intergin-ECM system was used as a model to demonstrate a protein-protein interaction on ProteoChip. As shown in Figs. 8C and D, integrin $\alpha_{IIb}\beta_3$ receptor immobilized on ProteoChip can directly interact with its ligand, fibrinogen labeled with Cy-5, in a concentration-dependent manner. Fluorescence intensities increased as the concentration of fibrinogen-Cy-5 increased. Direct interaction of integrin with fibrinogen does occurr on the ProteoChip base plate. It is evident that this system for analysis of protein-protein interactions with ProteoChip will be useful for further development of a chip-based lead screening system for new drug discovery.

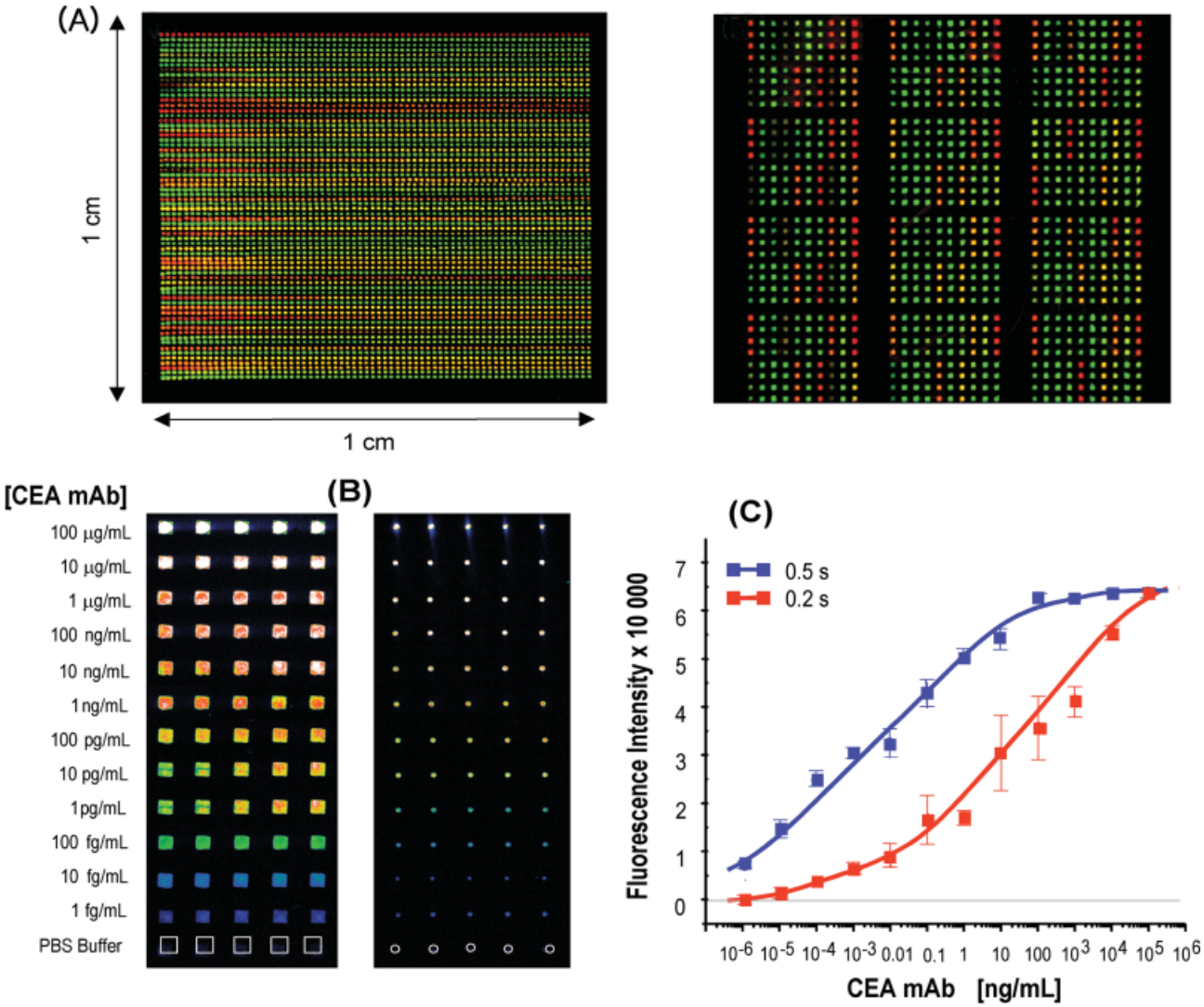

Figure 5. (A) Microarray images spotted with a Proteogen CM 1000 microarrayer. (Left panel) Spotting tests for ProteoChip base plate prepared with an ArrayIt SMP3 (100 μm). The array spot density is 4900 ea/cm^2. (Right panel) Spotting tests for ProteoChip base plate prepared with an ArrayIt SMP10 (335 μm). The array spot density is approximately 460 ea/cm^2 in this test. The proteins used for the spotting tests are protein A-Alexa546 (yellow), CRP mAb-Cy5 (green) and mouse IgG-Cy3 (red). (B), Rainbow color displays spotted using two different pin sizes, 300 μm (left panel) and 100 μm (right panel), for immobilization of a capture mAb (CEA) in varied concentrations from 1 fg/mL to 100 μg/mL on ProteoChip. The microarrayer and fluorescence scanner used for this experiment were the Proteogen CM 1000 and GSI scanner, respectively. (C) Dose-response curves for immobilized capture mAb represented by fluorescence intensity as a function of mAb concentration. The numerical values of fluorescence intensity were calculated using software from the GSI scanner. The immobilized capture mAb was detectable at a concentration level of 1 fg/mL and appeared saturated at a mAb concentration of 100 μg/mL.

4 Discussion

We have developed novel bifunctional molecular linkers, ProLinker™, for immobilization of proteins *via* interaction between protein on the surface of glass substrate or gold substrate. ProLinker™ has a unique surface chemistry which forms a self-assembled monolayer on solid substrates and allows tight binding of capture proteins to the crown moiety of the linker molecules. The properties of ProLinker™-coated surface of ProteoChip are summarized in Table 2.

Immobilization by organic thin films such as self-assembled alkanethiol and silane monolayer on noble metals and glass substrates have been used to control biomolecular density and orientation of capture proteins

Table 2. Surface properties of ProteoChip BP

– Format:	Flat surface or patterned microarray	
– Support:	Amine glass or patterned glass slide coated with gold film	
	ProteoChip Type A	– Amine glass coated with ProLinker™ A for fluorescence scanning systems
	ProteoChip Type B	– Gold substrate coated with ProLinker™ B for SPR, QCM and AFM systems
– Number of spots:	25–5000 spots/cm^2	–
– Application volume:	For manual spotting	– 0.5–1.0 μL
	With a microarrayer	– 10–50 nL
– Spot Diameter:	For manual spotting	– 1.5–2.0 mm
	With a microarrayer	– 100–300 μm
– Capture Ab density:	10^5 sites/μm^2	
– The LOD:	1–10 femtogram/mL	

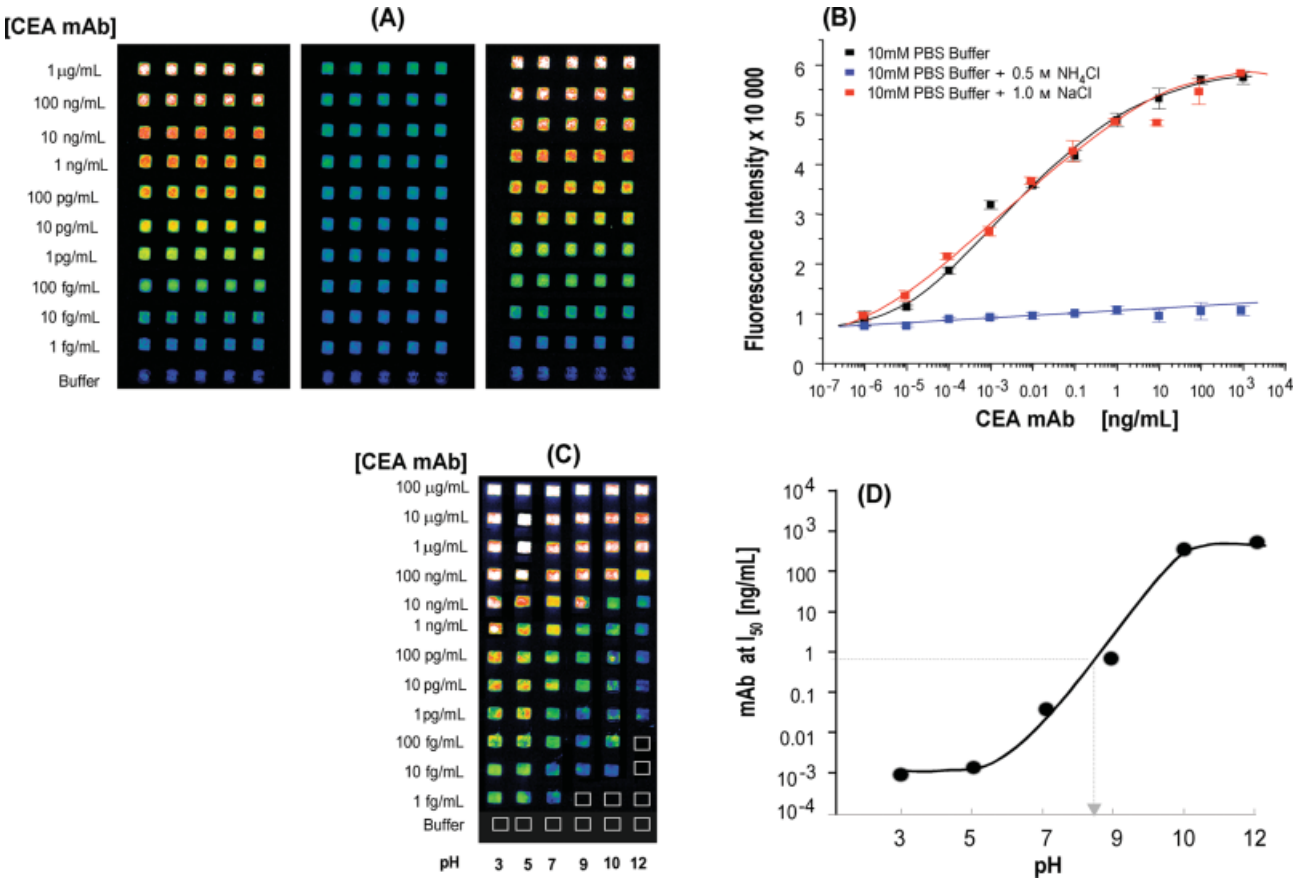

Figure 6. The effect of ammonium and sodium ions on binding activity of a capture mAb (CEA) and of pH on its immobilization activity. (A) Rainbow displays of fluorescence intensity of capture mAb immobilized on ProteoChip BP; (Left panel) in 10 mM phosphate buffer (pH 7.4), (middle panel) in the presence of 0.5 M NH_4Cl or (right panel) 1 M NaCl in 10 mM phosphate buffer. (B) Dose-response curves of fluorescence intensity as a function of CEA mAb concentration in the presence and absence of NH_4Cl and NaCl. Numerical data were obtained from (A). (C) A rainbow display of fluorescence intensity of immobilized mAb. (D) A plot of I_{50} (50% of the maximum intensity) values of fluorescence intensity of immobilized mAb as a function of pH showing that the pK is approximately 8.5. The result suggests that an amine group of a capture mAb is the ionizing group that determines the binding affinity of the capture protein to the crown moiety of ProLinker™ coated on ProteoChip BP.

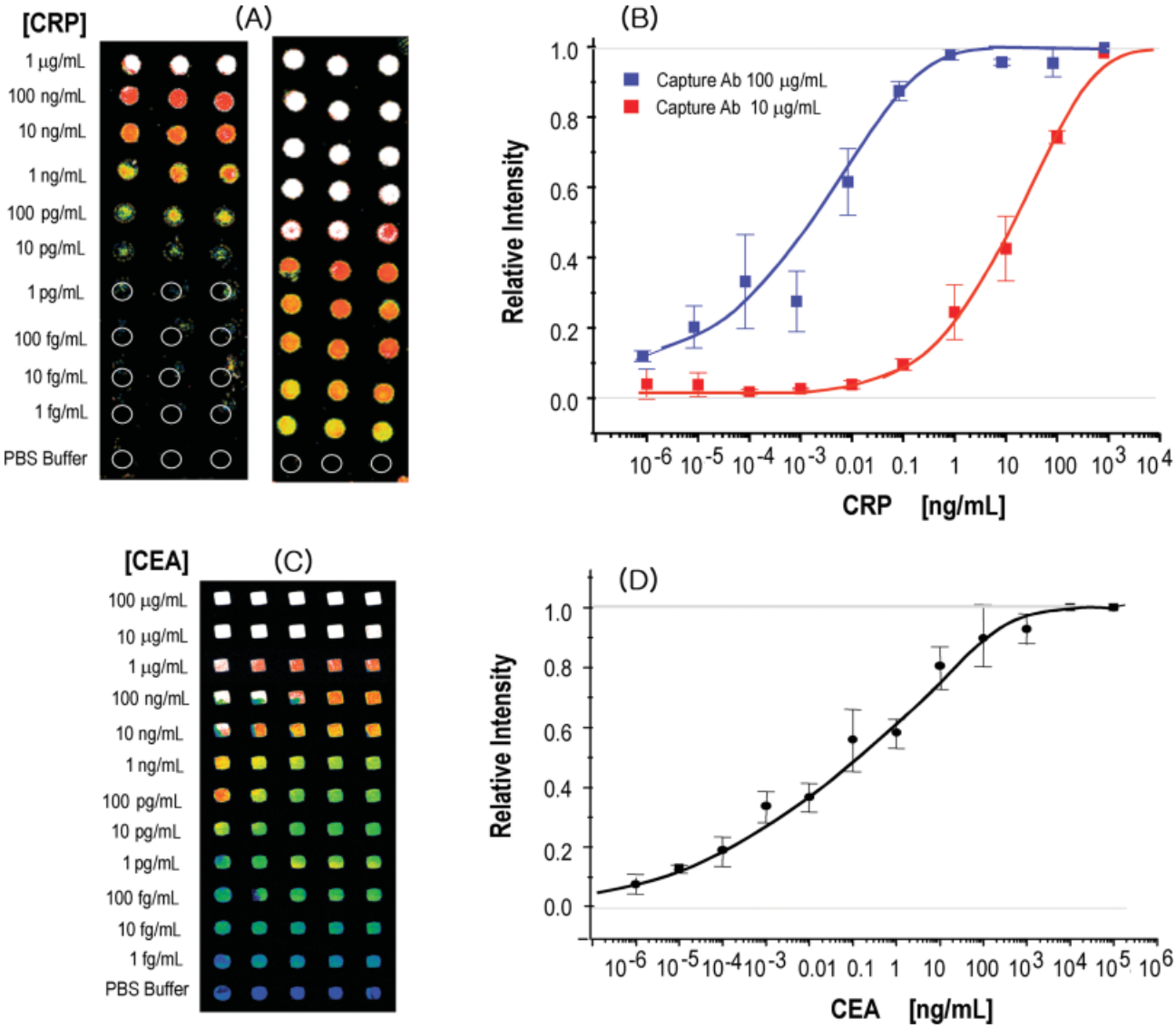

Figure 7. Effect of concentration of applied capture mAb (CRP) on detection sensitivity of the Ab-Ag interaction and sensitivity of Ab-Ag interaction with CEA. (A) Fluorescence intensity of Ag bound to capture mAb (CRP) immobilized on ProteoChip BP. The capture mAb and interacting Ag were applied to microarray spots with a 2 mm diameter manually. The application volume and concentrations of the capture mAb used were 1 μL and 10 μg/mL (left panel) and 100 μg/mL (right panel), respectively. (B) Dose-response curves of Ab-Ag interactions with two different concentrations of capture mAb (10 μg/mL and 100 μg/mL) applied on microarray spots. (C) Microarray spots of Ag (CEA) in varying concentrations interacted with capture mAb (CEA) immobilized on ProteoChip. (D) Dose-response curve of relative fluorescence intensity of bound Ag as a function of Ag concentrations from 1 fg/mL to 100 μg/mL. CEA mAb and Ag were spotted on a ProteoChip with CM 1000 containing an ArrayIt SMP10 pin. The detection sensitivity of the bound Ag to the capture mAb was as low as 1 fg/mL.

on solid surface and to obtain better reproducibility [39]. These approaches of protein immobilization on solid surfaces have improved controlled interfacial architectures compared to that obtained with physisorption techniques. These SAM forming linker molecules, however, require further activation processes to be able to react with nucleophilic groups of proteins and to be captured on the surface of SAM for immobilization since they only have simple functionalities such as *C*- or *N*-terminals. ProLinker™ is a different type of SAM forming linker molecule, which provides an efficient method for immobilization of capture proteins on glass or gold substrates noncovalently without requiring additional modification processes for coupling capture proteins to linker molecules (Fig. 1B).

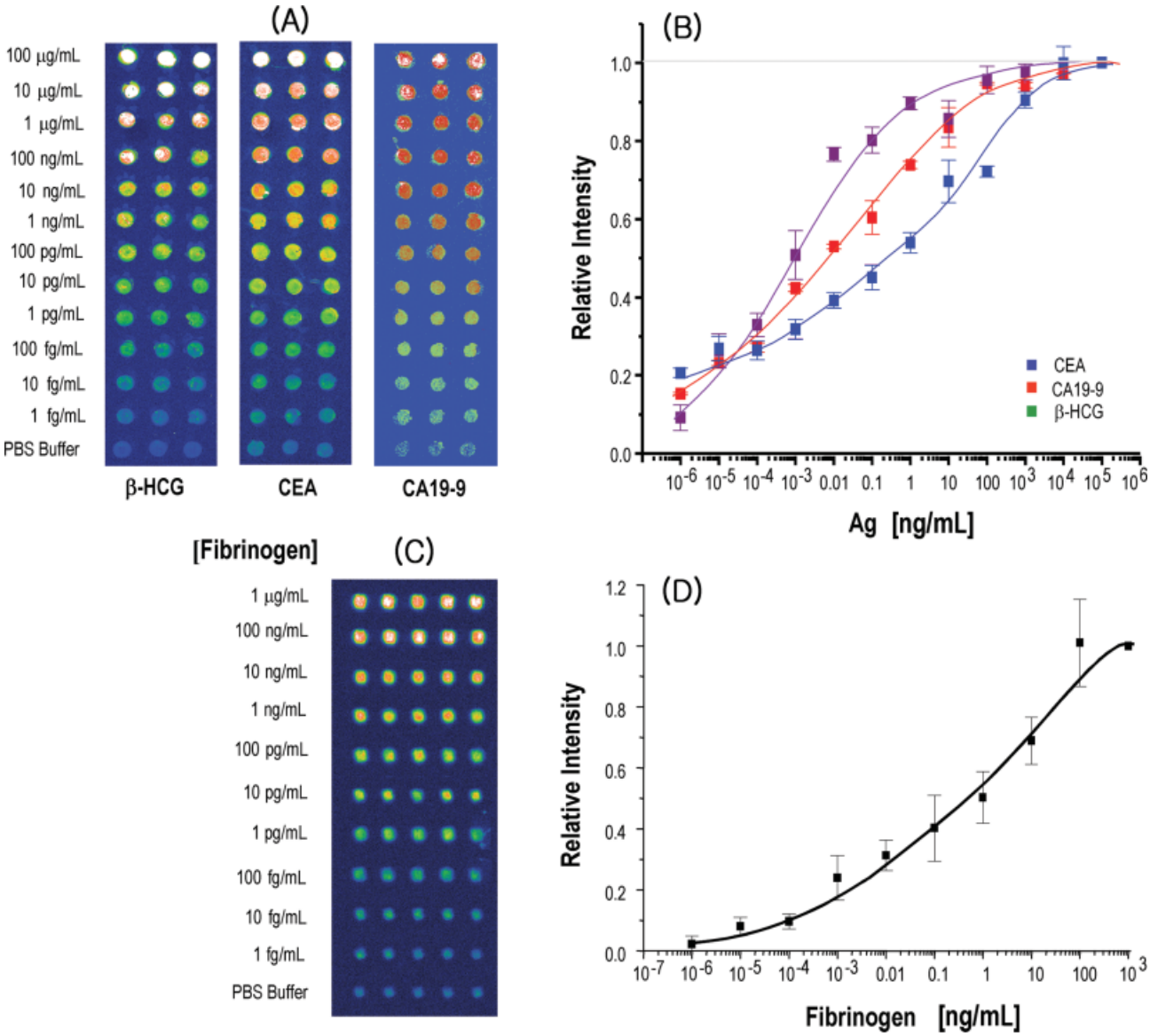

Figure 8. Results of sandwich fluorescence immunoassay for several tumor markers and of the integrin-fibrinogen interaction. The LOD was as low as 1 fg/mL. (A) Microarray spots showing fluorescence intensity scanned with a GSI scanner for β-HCG and an Axon scanner for CEA and CA19-9. (B) Dose-response curves of relative intensity as a function of Ag concentration. The numerical values of the fluorescence intensities were estimated from scanning data in (A). (C) Integrin $\alpha_{IIb}\beta_3$ was immobilized on ProteoChip BP and fibrinogen-Cy5 (1 g/mL–1 fg/mL) was applied on the integrin spots to observe direct interaction between integrin and ECM as shown by increased fluorescence intensity with increased concentration of ECM and the corresponding dose-response curve (D).

The fabrication processes of ProteoChip coated with ProLinker™ and immobilization of a capture protein were demonstrated to be simple, easy, and reproducible without requiring extensive chemistry. It was noted that the robustness of immobilized capture protein on the surface of ProteoChip would be useful for protein-protein interaction studies, which may require a series of washing steps in a solid phase assay system.

In addition to the handling simplicity of ProteoChip, its detection sensitivity was shown to be as low as 1–10 fg/mL of analyte protein. This is the lowest detection sensitivity ever reported for a protein microarray chip. It is noteworthy that Wagner has reported a low detection limit (LOD) of 200 fg/mL for IL10 with the Zyomix microarray system [40], while MacBeath has shown the LOD of FKBP12 to be approximately 150 pg/mL with aldehyde

glass [6]. The detection sensitivity of ProteoChip is at least 1000 times lower than that of aldehyde glass under the same experimental conditions.

It should be pointed out that the detection limit demonstrated with ProteoChip is basically at the intrinsic limit of analyte protein concentration. In the case of IgG (M_r 150 kDa), 25 nL of 10 fg/mL solution would contain approximately one IgG molecule, which can be considered the lowest limit of detection. The LOD for IgG is not a problem in practice for protein-protein interaction studies, since a saturated level of a capture protein has been applied for its immobilization in microarray spots. In the case of assay studies of analyte proteins, however, it is important to determine the lowest limit of a test concentration and to consider the intrinsic detection limit of the molecule when preparing the application volume of analyte protein. For example, when an analyte protein with M_r 20 kDa is subjected to the assay, 100 nL of a 1 fg/mL protein solution may contain approximately three molecules of the analyte protein in the test volume, which is considered the intrinsic detection limit.

The high detection sensitivity of ProteoChip can be attributed to the improved surface chemistry of protein immobilization by the bifunctional affinity linker. Specifically, a capture protein, such as a mAb, can be immobilized to the chip surface (1) without conformational change and thus no loss of binding activity and (2) by forming a high density monolayer with the correct orientation. We have presented indirect evidence that the Fc region of Ab molecules can bind to the chip surface and that the Fv domains are open to the solution phase for Ag interaction. In fact, it was demonstrated by both QCM and fluorescence scanning that Protein A, which has binding activity to the Fc domain of IgG, did not bind to the site properly after Ab was immobilized on the chip surface (Figs. 3D–F). It is thus proposed that most Ab molecules immobilized on the ProteoChip are in the correct orientation resulting in increased detection sensitivity.

Given that ammonium ion strongly inhibited protein binding to the linker molecules and that the pK of the responsible ionizing group is 8.5, secondary amine groups of basic amino acid residues may be responsible for protein binding to the crown moiety of ProLinker™. It is known that a crown moiety binds an $-NH_3^+$ group *via* host-guest interactions [41]. It was suggested that the host-guest interaction could be attributed to ion-dipole interaction and hydrogen bonding between the $-NH_3^+$ group and the ethyl oxygen of the crown structure. However, additional binding forces such as hydrophobic interactions between hydrophobic side chains of proteins and methoxyl groups of ProLinker™ may also be involved in the strengthened binding of capture proteins to ProteoChip base plate coated with linker molecules.

Proteins immobilized with ProLinker™ appear to be more stable than those immobilized by chemical binding, because the host-guest interaction mediated by the molecular linker may occur spontaneously without drastic chemical modification of capture Ab. In stability tests of immobilized Ab, it was found that the Ab microarray constructed on ProteoChip was stable without activity loss for more than 3 months at 4°C.

ProteoChip A was successfully used for the preparation of an integrin chip to study direct protein-protein interactions as demonstrated by an experiment of integrin-ECM (fibrinogen) interactions. The integrin-ECM systems are considered as potential drug targets for screening new lead molecules for various integrin-associated diseases such as blood clotting, tumor-induced angiogenesis, and rheumatoid arthritis. The application of high-throughput new lead screening with the integrin chip has been successfully demonstrated with ProteoChip in our laboratory. The results will be reported elsewhere. It is expected that the ProteoChip platform can be extended to development of microarray chips for other membrane receptor proteins.

5 Conclusion

In conclusion, various experimental results presented in this report suggest that ProteoChip is a highly sensitive protein microarray containing an improved surface chemistry with ProLinker™, a novel bifunctional linker molecule for efficient protein immobilization. It is a useful tool for development of chip-based lead screening by using a protein-protein interaction system as a drug target as well as for biomarker assays which require high detection sensitivity.

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6 References

[1] Abbott, A., *Nature* 1999, *402*, 715–720.
[2] Albala, J. S., *Expert Rev. Mol. Diagn.* 2001, *1(2)*, 145–152.
[3] Ekins, R. P., *J. Pharm. Biomed. Anal.* 1989, *7*, 155–168.
[4] Ekins, R. P., Chu, F., *Ann. Biol. Clin. (Paris)* 1992, *50*, 337–353.
[5] Finckh, P., Berger, H., Karl, J., Eichenlaub, U. *et al*., in: *Proc. UK NEQAS Meeting*, 1998, *3*, 155–165.

[6] MacBeath, G., Schreiber, S. L., *Science* 2000, *289*, 1760–1763.

[7] Haab, B. B., Dunham, M. J., Brown, P. O., *Genome Biol.* 2001*, 2: Research 4*, 1–13.

[8] Zhu, H., Bilgin, M., Bangham, R., Hall, D. *et al.*, *Science* 2001, *293*, 2101–2105.

[9] Templin, M. F., Stoll, D., Schrenk, M., Traub, P. C. *et al*., *Trends Biotechnol.* 2002, *20(4)*,160–166.

[10] Arenkov, P., Kukhtin, A., Gemmell, A., Voloshchuk, S. *et al*., *Anal. Biochem.* 2000, *278*, 123–131.

[11] Zhu, H., Klemic, J. F., Chang, S., Bertone, P. *et al*., *Nat. Genet.* 2000, *26*, 283–289.

[12] Houseman, B. T., Huh, J. H., Kron, S. J., Mrkisch, M., *Nat. Biotechnol.* 2002, *20*, 270–274.

[13] O'Shannessy, D. J., Brigham-Burke, M., Peck, P., *Anal. Biochem*. 1992, *205,* 132–136.

[14] Nelson, R. W., Krone, J. R., *J. Mol. Recognit*. 1999, *12*, 77–93.

[15] Gomes, P., Giralt, E., Andreu, D., *J. Immunol. Methods* 2000, *235*, 101–111.

[16] Eisenberg, D., Marcotte, E. M., Xenarios, I., Yeates, T. O., *Nature* 2000, *405*, 823–826.

[17] Mann, M., *Anal. Chem*. 2000, *72*, 565A.

[18] Metzger, S. W., Lochhead, M. J., Grainger, D. W., *IVD Technol.* 2002, June, 39–46.

[19] www.sric-bi.com/BIP/summaries/2360. shtml

[20] www.functionalgenomics.org.uk/sections/resources/protein arrays.html

[21] Muller, W., Ringsdorf, H., Rump, E., Wildburg, G. *et al*., *Science* 1993, *62(5140),* 1706–1708.

[22] Ziauddin, M., Sabatini, D. M., *Nature* 2001, *411*, 107–110.

[23] Sanders, G. H. S., Manz, A., *Trends Anal. Chem.* 2000, *19*, 364–378.

[24] Blawas, A. S., Reichert, W. M., *Biomaterials* 1998, *19*, 595–609.

[25] Nakanishi, K. H., Muguruma, H., Karube, I., *Anal. Chem*. 1996, *68*, 1695–1700.

[26] Mendoza, L. G., McQuary, P., Mongan, A., Gangadharan, R. *et al*., *Biotechniques* 1999, *27*, 778–788.

[27] Hoyer-Hansen, G., Hamers, M. J., Pedersen, A. N., Nielsen, H. J. *et al., J. Immunol. Methods* 2000, *235*, 91–99.

[28] Pritchard, D. J., Morgan, H., Cooper, W. G., *Anal. Chem*. 1997, *69*, 1619–2625.

[29] Howard, J. C., Heinemann, C., Thatcher, B. J., Martin, B. *et al*., *App. Environ. Microbiol.* 2000, *66*, 4396–4400.

[30] Delgado, A. D., Leonard, M., Dellacherie, E., *Langmuir* 2000, *17*, 4386–4391.

[31] Muchova, L., Jirsa, M., Kuroki, M., Dudkova, L. *et al*., *Biomed. Chromatogr.* 2001, *15,* 418–422.

[32] Lueking, A., Horn, M., Eickhoff, H., Bussow, K., *Anal. Biochem*. 1999, *270*, 103–111.

[33] Büssow, K., Nordhoff, E., Lubbert, C., Leharch, H., Walter, G., *Genomics* 2000, *65*, 1–8.

[34] Spitznagel, T. M., Clark, D. S., *Biotechnology* 1993, *11*, 825–829.

[35] Kim, T. S., Proteogen, US Patent no. 6,485,984 B1, Nov 26, 2002.

[36] Pavlickova, P., Jensen, N. M., Paul, H., Schaeferling, M. et al., *J. Proteome Res*. 2002, *1(3)*, 227–231.

[37] Kunsagi-Mate, S., Bitter, I., Grun, A., Nagy, G., Kollar, L., *J. Biochem. Biophys. Methods* 2002, *53(1–3)*, 101–108.

[38] Rickert, J., Brecht, A., Gopal, W., *Anal. Chem*. 1997, *69*, 1263–1272.

[39] Tsai, M. Y., Lin, J. C., *J. Biomed. Mater. Res.* 2001, *55(4)*, 554–565.

[40] Wagner, P., Protein Microarray Technology, IBC Microtechnology Series, March 21–21, 2001, San Diego, USA.

[41] Kubo, Y., Maeda, S., Tokita, S., Kubo, M., *Nature* 1996, *382*, 522–524.

