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Carboxylate-modified Latex Bead Surface for Analysis of Blood Proteins

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Abstract

We developed a new carboxylate-modified latex bead surface for determining the quantitative level of blood proteins. In this study, antibody arrays are fabricated by immobilizing a target protein-specific antibody on a carboxylate-modified latex bead surface of well-type arrays. C-reactive protein (CRP), carcinoembryonic antigen, and prostate-specific antigen were conjugated with fluorophores for use as internal standards for the analysis of blood proteins. We found that the carboxylate-modified latex bead surface had a higher binding affinity for Alexa 546-CRP than dextran, aldehyde, and amine surfaces. The carboxylate-modified latex bead surface was found to be specific and reproducible. In addition, the antibody arrays were successfully applied to the analysis of CRP, carcinoembryonic antigen, and prostate-specific antigen in normal human serum (r^2=0.997, 0.988 and 0.999, respectively). Thus, an antibody array based on a carboxylate-modified latex bead surface has a strong potential for a rapid serodiagnosis of human diseases.

Keywords: Antibody array, Carboxylate-modified latex bead, C-reactive protein, Carcinoembryonic antigen, Prostate-specific antigen

Introduction

Protein arrays have appeared as a key technology in proteomics and serodiagnosis, as the technology allows a high-throughput and large-scale analysis of protein interactions, drug analysis, and the diagnosis of diseases in a small sample format[1-4]. Multiple techniques are in development for the analysis of protein interactions on arrays using various methods such as surface plasmon resonance, atomic force microscopy, mass spectrometry, and fluorescence detection[1,5-7]. Fluorescence detection methods are quite popular in the analysis of antigen and antibody arrays because they are simple, highly sensitive, and compatible with the standard DNA microarray[1,8]. Recently, antibody arrays are rapidly increasing in importance as prospective tools for the functional analysis of cellular activity and the diagnosis of diseases[9-11].

There have been an accumulating number of reports on the applications of antibody arrays toward the analysis of blood proteins by fluorescence detection methods[12-14]. Two different methods based on the competition principle, termed one-color and two-color approaches, have been proposed to analyze protein expression profiling in human cells and tissues[12,15]. A two-color comparative fluorescence detection method, which is similar to the two-color labeling strategies used for cDNA microarray experiments, has been successfully applied toward identifying protein biomarkers of various cancers in human sera, such as prostate cancer, pancreatic cancer, lung cancer, and bladder cancer[16-19]. However, the two-color approach provided relative, rather than quantitative, protein levels of experimental samples compared to those of the control samples[15,18]. Another fluorescence detection method based on antibody arrays is a single-color-detection, competitive-displacement assay, and this method was proposed to overcome the weak-point of the two-color assay and has been used to determine protein expression profiling in tissues and cells[20,21].

In this paper, we have developed a new carboxylate-modified latex bead (CML) surface for determining the quantitative level of blood proteins based on one-color antibody arrays. In this study, we used antibody arrays to measure the levels of blood proteins; C-reactive protein (CRP), carcinoembryonic antigen (CEA), and prostate-specific antigen (PSA) in normal human serum. The CML surface for antibody arrays was specific, reproducible, and successfully applied to the analysis of blood proteins in normal human sera. Thus, an antibody array using a CML surface has a strong potential for rapid serodiagnosis of human diseases.

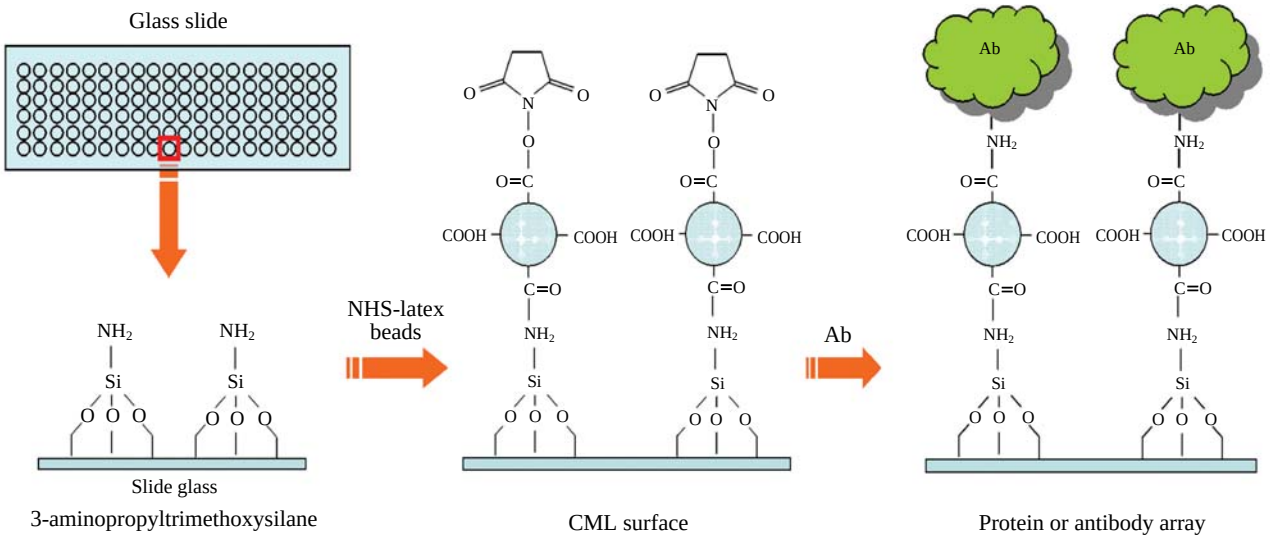

Figure 1. Schematic diagram of antibody array preparation. Antibody arrays were fabricated by immobilizing anti-CRP on the carboxylate-modified latex bead surface of well-type arrays, as described under Materials and Methods.

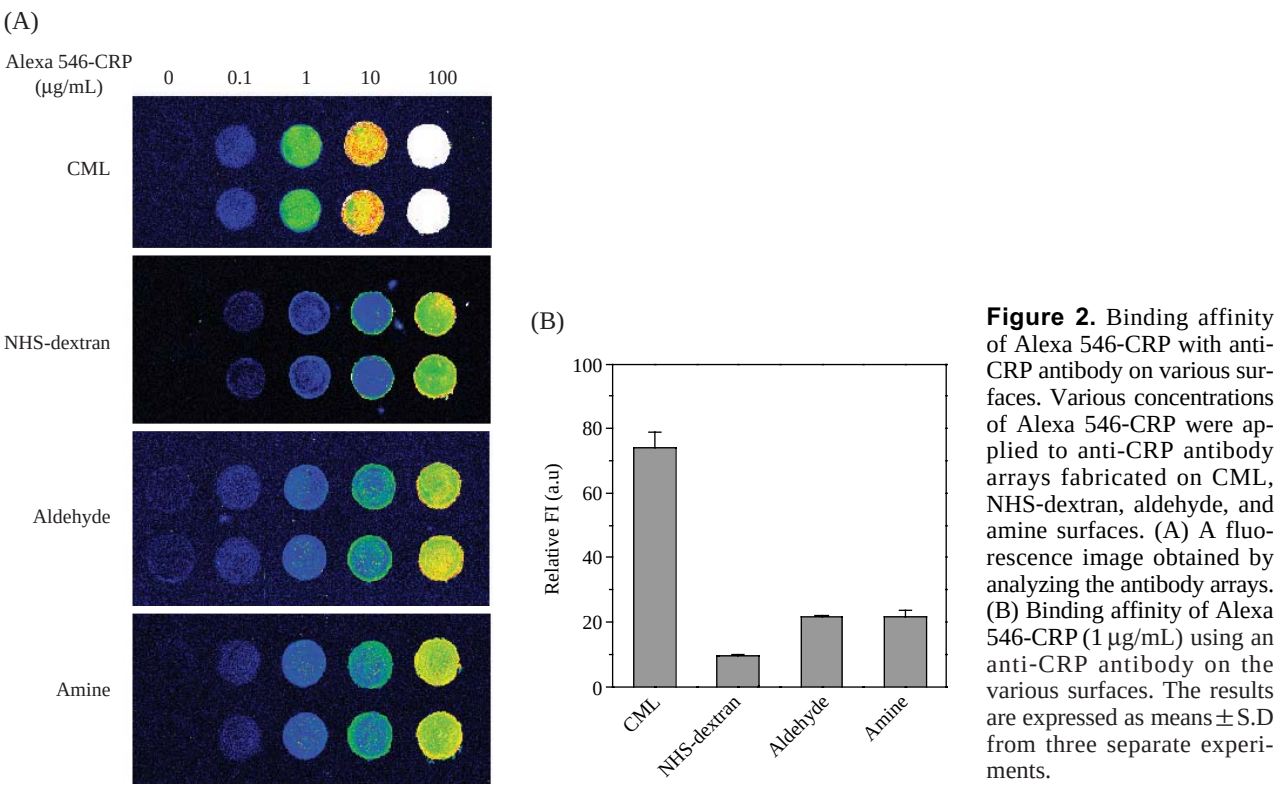

Figure 2. Binding affinity of Alexa 546-CRP with anti-CRP antibody on various surfaces. Various concentrations of Alexa 546-CRP were applied to anti-CRP antibody arrays fabricated on CML, NHS-dextran, aldehyde, and amine surfaces. (A) A fluorescence image obtained by analyzing the antibody arrays. (B) Binding affinity of Alexa 546-CRP (1 μg/mL) using an anti-CRP antibody on the various surfaces. The results are expressed as means ± S.D from three separate experiments.

Results and Discussion

Characterization of Antibody Arrays Using the CML Surface

In order to prepare well-type antibody arrays, Teflon tapes with 200 holes were mounted onto glass slides modified with 3-aminopropyltrimethoxysilane, and the surface of the resulting well-type arrays were then further modified with carboxylate-modified latex beads (Figure 1). Well-type antibody arrays were fabricated using immobilizing anti-CRP on the latex bead

surface of the array wells.

First, we tested various surfaces for the binding of Alexa 546-CRP to the well-type antibody arrays. Various concentrations of Alexa 546-CRP were applied to anti-CRP antibody arrays fabricated on CML, NHS-dextran, aldehyde, and amine surfaces, and the arrays were then analyzed using a fluorescence scanner. As shown in Figure 2A, the CML surface had a higher binding affinity for Alexa 546-CRP than the NHS-dextan, aldehyde and amine surfaces. At 1 μg/mL of Alexa 546-CRP, the fluorescence intensity of the CML surface was about 74, whereas those of the other surfaces ranged from 9 to 21 (Figure 2B), indicating that the CML surface was better than the other surfaces in the analysis of blood proteins using antibody arrays. Thus, we investigated whether the well-type antibody arrays prepared on latex beads were appropriate for an analysis of blood proteins. When we prepared Alexa 546-CRP in a phosphate buffer, the Alexa 546-CRP interacted with the antibody arrays in a dose-dependent manner, showing an apparent relative fluorescence intensity at 100 ng/mL, and an almost maximal intensity at 10 μg/mL. However, when the Alexa 546-CRP samples were prepared in bovine serum albumin or normal human serum, the relative fluorescence intensity exponentially increased, starting at 1 ng/mL, becoming almost saturated at 1 μg/mL of Alexa 546-CRP (Figure 3A). These results indicate that the interaction of Alexa 546-CRP with the antibody arrays is enhanced by human serum, and that this effect was caused by the serum albumin used in this experiment.

Next, we investigated the specificity of Alexa 546-CRP interactions with anti-CRP antibody arrays. To test this, we applied Alexa 546-CRP to the antibody arrays of CRP, PSA, CEA, alpha-fetoprotein, haptoglobin, and hemoglobin, and then analyzed their interactions. As shown in Figure 3B, the relative fluorescence intensity (arbitrary unit) of Alexa 546-CRP on anti-CRP arrays was approximately 200, whereas the intensities on the other antibody arrays ranged from16 to 27, indicating that Alexa 546-CRP had a strong affinity for only the anti-CRP array, with negligible interactions with the other antibody arrays. Thus, the interaction between Alexa 546-CRP and anti-CRP antibody arrays based on the CML surface was specific.

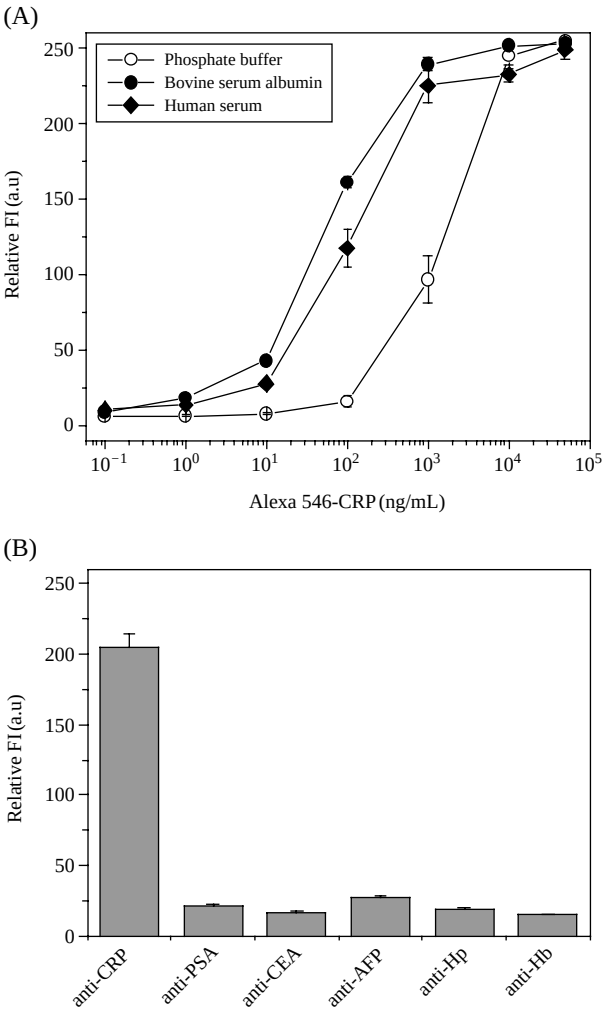

Figure 3. Enhanced affinity and specificity of Alexa 546-CRP interaction with anti-CRP antibody arrays. (A) Enhanced affinity. The indicated concentrations of Alexa 546-conjugated CRP were prepared in a 9.3 mM phosphate buffer (pH 7.4), 1% BSA in a buffer, or normal human serum, and then applied to the anti-CRP antibody arrays. (B) The antibody arrays were prepared by immobilizing six antibodies (200 μg/mL), and then incubated with Alexa 546-CRP. The results are expressed as the mean value of relative fluorescence intensity (FI) ± S.D from three separate experiments.

Reproducibility of Anti-CRP Antibody Arrays on the CML Surface

We evaluated the inter-array and inter-labeling/-array reproducibility of antibody arrays on the CML surface. The reproducibility was determined by analyzing 20 human sera using the same batch of Alexa 546-CRP, or those with different batches of Alexa 546-CRP, on different anti-CRP antibody arrays. As shown in Figure 4A, we found the inter-array reproducibility to be high, with a correlation coefficient of 0.991. The inter-labeling/-array reproducibility was also high, with a correlation coefficient of 0.973 (Figure 4B). Taken together, antibody arrays based on a CML surface is reproducible and appropriate for a parallel analysis of CRP in human sera.

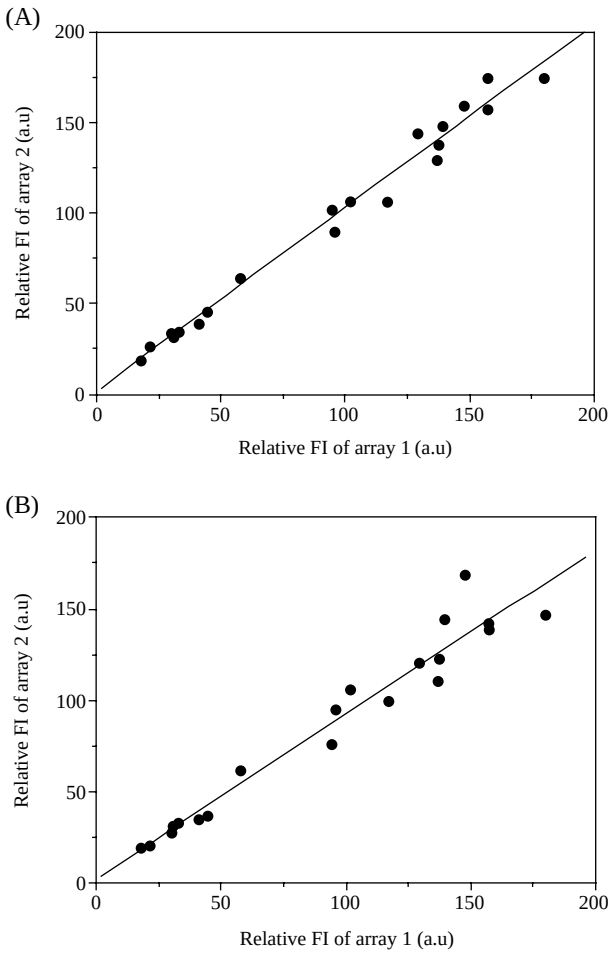

Figure 4. Reproducibility of anti-CRP antibody arrays. Twenty unlabeled human sera, mixed with Alexa 546-CRP, were applied to anti-CRP antibody arrays and analyzed using a fluorescence scanner. The correlation between array 1 and array 2 was analyzed using the fit Linear function of the Origin program. (A) Inter-array reproducibility ($r=0.991$). (B) Inter-labeling/-array reproducibility ($r=0.973$).

Analysis of CRP, CEA and PSA in Normal Human Serum

Next, we applied antibody arrays using the CML surface to a rapid analysis of CRP, CEA, and PSA in normal human serum. First, various concentrations of CRP were prepared in normal human serum containing Alexa 546-CRP (a final concentration of 1 μg/mL), and the resulting mixtures were applied in duplicate toward anti-CRP antibody arrays prepared on the CML surface. The arrays were analyzed using a fluorescence scanner, and normalized reverse fluorescence intensities were obtained using Equation (1). As shown in Figure 5A, the normalized fluorescence intensity increased in a CRP concentration-dependent manner, showing an apparent elevation at 10 ng/mL,

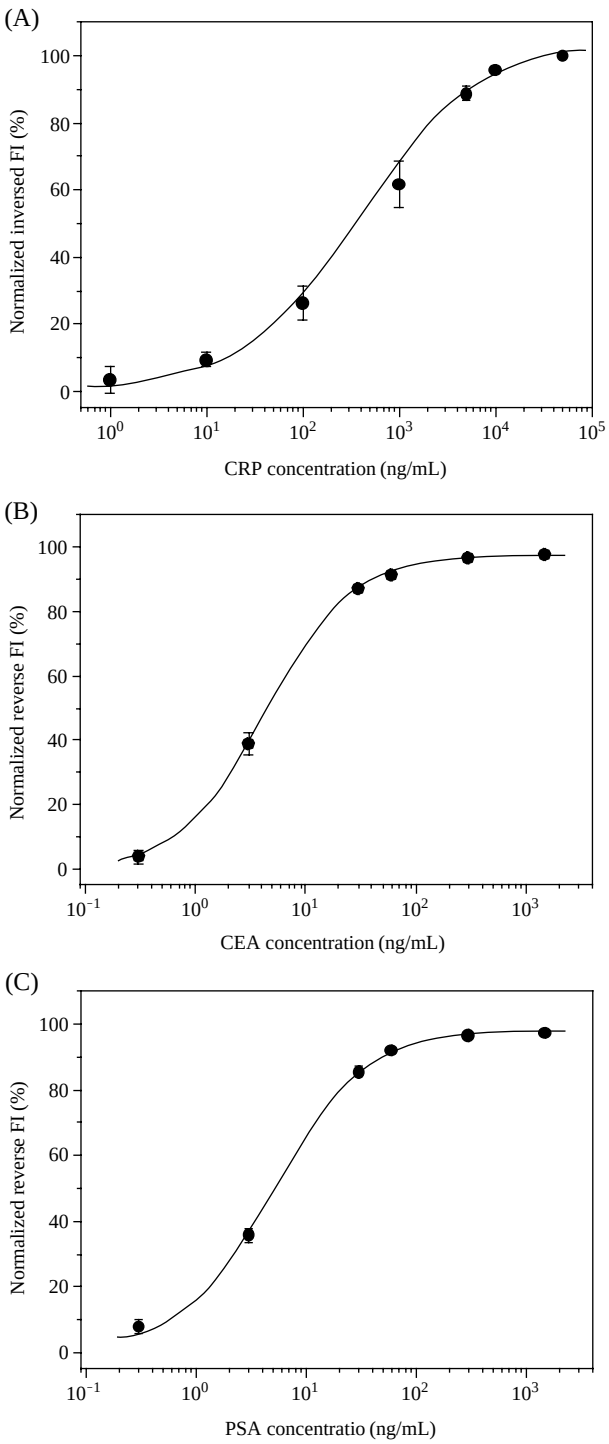

Figure 5. Analysis of CRP, CEA and PSA in normal human serum. The indicated concentrations of CRP, CEA, and PSA were prepared in normal human serum, 10-times diluted with a 9.3 mM phosphate buffer (pH 7.4) containing 1% BSA, and mixed (1 : 1, v/v) with Alexa 546-CRP, Cy3-CEA, and Cy3-PSA, respectively. Normalized reverse fluorescence intensities were obtained using Equation (1). (A) CRP ($r^2=0.997$), (B) CEA ($r^2=0.988$), (C) PSA ($r^2=0.999$).

becoming almost saturated at 50 μg/mL of CRP (r^2= 0.997). Considering the reference range (0 to 3 μg/mL) of CRP for serodiagnosis, the CML surface-based antibody arrays are appropriate for a sensitive analysis of CRP in human blood. We then analyzed CEA and PSA in normal human serum using the same approach. The two blood proteins showed a concentration-dependent increase of normalized fluorescence intensity (Figures 5B and 5C). The normalized fluorescence intensity of CEA was detectable at 0.3 ng/mL and was saturated at 300 ng/mL (r^2=0.988), demonstrating that the antibody arrays were sensitive to an analysis of CEA in human blood samples. Similar results were obtained using PSA (r^2=0.999). Thus, an antibody array based on the carboxylate-modified latex bead surface has a strong potential for a quantitative analysis of blood proteins.

Conclusion

In this report, we presented a new carboxylate-modified latex bead surface for determining the quantitative level of blood proteins. The carboxylate-modified latex bead surface for antibody arrays was specific, reproducible, and successfully applied toward the analysis of serum proteins in human sera. Thus, the carboxylate-modified latex bead surface for an antibody array has a strong potential for a rapid serodiagnosis of human diseases.

Materials and Methods

Chemicals and Reagents

3-Aminopropyltrimethoxysilane, ammonium hydroxide, hydrogen peroxide, bovine serum albumin (BSA), and Cy3-conjugated streptavidin were purchased from Sigma (St. Louis, MO). Monoclonal anti-CRP antibody was purchased from Bethyl Lab (Montgomery, TX). C-reactive protein was purchased from Scripps Lab (San Diego, CA). Alexa Fluor 546 carboxylic acid-succinimidyl ester, succinimidyl D-biotin, and carboxylate-modified latex beads were purchased from Molecular Probes™ (Eugene, OR). Cy3 mono NHS ester was purchased from Amersham Biosciences (Piscataway, NJ).

Serum Samples

Human sera were obtained from Kangwon National University Hospital, divided into aliquots and stored at −20°C until used. Experiments using the human samples were performed under the approval of the local institute's Ethics Committee for human subject research. In preparation for the experiments, the sera were diluted 10-fold with a 9.3 mM phosphate buffer (pH 7.4) containing 1% BSA and protease inhibitors (one tablet in a 10 mL buffer).

Surface Modification of Glass Slides

Glass slides (75 × 25 mm) were cleaned using a solution of $H_2O_2/NH_4OH/H_2O$ (1 : 1 : 5, v/v) at 70°C for 10 min, incubated with a 1.5% 3-aminopropyltrimethoxysilane solution (v/v) in 95% ethanol for 2 h, and sequentially washed with ethanol and water. The amine-modified glass slides were dried under an air gas and baked at 110°C for 1 h. Teflon tapes (75 × 25 mm) with 200 holes (25 × 8) 1.5 mm in diameter each were attached to the modified glass slides to prepare the well-type arrays.

Latex beads were washed with a PBS (8.1 mM Na_2HPO_4, 1.2 mM KH_2PO_4, 2.7 mM KCl, 138 mM NaCl, pH 7.4) and suspended in PBS (1 : 10, v/v). To prepare the CML bead surface, the latex beads were mixed (1 : 1, v/v) with a solution of 200 mM EDC and 50 mM NHS, and immediately applied to the wells of the arrays for 20 min. The CML bead-modified arrays were washed with 0.1% Tween-20 in PBS for 1 h, milliQ water for 5 min, and air dried. An N-hydroxysuccinimide-dextran surface was prepared by applying an N-hydroxysuccinimide-dextran solution to the amine-modified glass slides[22]. An Aldehyde surface was fabricated using a 1% glutaraldehyde solution applied to the amine-modified slides[23].

Preparation of Antibody Arrays

CML bead-modified arrays were incubated using a solution of 200 mM EDC and 50 mM NHS for 10 min and rinsed with milliQ water. Following incubation with 500 μg/mL protein G in a 10 mM sodium acetate buffer (pH 4.5) for 2 h at 37°C, the arrays were washed with 0.1% Tween-20 in PBS for 10 min and milliQ water for 5 min, and then air dried. One microliter of antibody solution (200 μg/mL), prepared in a 9.3 mM phosphate buffer (pH 7.4), was applied to each well of the arrays for 2 h at 37°C. After incubation, the well-type antibody arrays were washed with 0.1% Tween-20 in PBS for 10 min and milliQ water for 5 min.

Labeling of CRP, CEA and PSA with Fluorophores

The CRP was labeled with Alexa Fluor 546 as described in a previous report[17]. Briefly, 100 μL of 1 mg/mL CRP in a 100 mM sodium bicarbonate buffer (pH 8.3) was mixed with 1 μL of 10 mg/mL Alexa Fluor 546 carboxylic acid-succinimidyl ester in dimethyl sulfoxide. CEA and PSA were also labeled with Cy3

mono NHS ester using the same procedure for labeling the CRP. The reaction mixtures were incubated for 1 h at room temperature and loaded onto a 2.5 mL column of Sephadex G-25, and the unbound fluorophore was removed using centrifugation for 2 min at 2,500 rpm.

Analysis of CRP, CEA and PSA

Alexa 546-CRP (2 μg/mL, tagged-internal standard) in a 9.3 mM phosphate buffer (pH 7.4) containing 1% BSA was mixed (1 : 1, v/v) with normal human serum containing various concentrations of CRP, or with 10-fold diluted human sera with a 9.3 mM phosphate buffer (pH 7.4) containing 1% BSA and protease inhibitors (one tablet in 10 mL buffer). Cy3-PSA and Cy3-CEA (3 μg/mL) in a 9.3 mM phosphate buffer (pH 7.4) containing 1% BSA were mixed (1 : 1, v/v) with human serum containing various concentrations of the two unlabeled proteins. Then, 1 μL of the mixtures was applied to each well of the antibody arrays, which were then incubated for 1 h at 37°C. The arrays were washed and scanned using a fluorescence scanner with a 532 nm laser (Perkin Elmer, MA, USA).

Data Analysis

The average fluorescence intensities of the array spots were measured using a Fluoview program (Olympus, Japan). The normalized inverse fluorescence intensity (y) was expressed as follows:

$$y = 100(F_{max} - F_x)/(F_{max} - F_{min}), \quad (1)$$

where F_{max} is the maximum fluorescence intensity obtained from normal human serum containing no unlabeled CRP, F_x is the fluorescence intensity obtained from normal human serum containing an x-amount of unlabeled CRP or sera, F_{min} is the minimal fluorescence intensity obtained from a normal human serum containing the maximal concentration of unlabeled CRP, and x is the CRP concentration. The level of CRP in each serum was determined by

$$x = x_0[(y_1 - y_2)/(y - y_2) - 1]^{1/p}, \quad (2)$$

where x is the CRP concentration of the sample, x_0 is the CRP concentration at half the maximal normalized inverse fluorescence intensity, y_1 is the minimal normalized inverse fluorescence intensity, y_2 is the maximal normalized inverse fluorescence intensity, y is the normalized inverse fluorescence intensity, and p is the power.

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References

1. Zhu, H. & Snyder, M. Protein chip technology. *Curr. Opin. Chem. Biol.* **7**, 55-63 (2003).
2. Janzi, M. *et al*. Serum microarrays for large scale screening of protein levels. *Mol. Cell. Proteomics* **4**, 1942-1947 (2005).
3. Huang, R.P. *et al*. The promise of cytokine antibody arrays in the drug discovery process. *Expert Opin. Ther. Targets* **9**, 601-615 (2005).
4. He, M., Stoevesandt, O. & Taussig, M.J. In situ synthesis of protein arrays. *Curr. Opin. Biotechnol.* **19**, 4-9 (2008).
5. Yuk, J.S. & Ha, K.-S. Proteomic applications of surface plasmon resonance biosensors: analysis of protein arrays. *Exp. Mol. Med.* **37**, 1-10 (2005).
6. Jung, J.-W. *et al*. High-throughput analysis of GST-fusion protein expression and activity-dependent protein interactions on GST-fusion protein arrays with a spectral surface plasmon resonance biosensor. *Proteomics* **6**, 1110-1120 (2006).
7. Jung, S.-H. *et al*. Analysis of C-reactive protein on amide-linked N-hydroxysuccinimide-dextran arrays with a spectral surface plasmon resonance biosensor for serodiagnosis. *Anal. Chem.* **79**, 5703-5710 (2007).
8. MacBeath, G. & Schreiber, S.L. Printing proteins as microarrays for high-throughput function determination. *Science* **289**, 1760-1763 (2000).
9. Borrebaeck, C.A. Antibody microarray-based oncoproteomics. *Expert Opin. Biol. Ther.* **6**, 833-838 (2006).
10. Kingsmore, S.F. Multiplexed protein measurement: Technologies and applications of protein and antibody arrays. *Nat. Rev. Drug Discov.* **5**, 310-320 (2006).
11. Lv, L.-L. & Liu, B.-C. High-throughput antibody microarrays for quantitative proteomic analysis. *Expert Rev. Proteomics* **4**, 505-513 (2007).
12. Haab, B.B. Antibody arrays in cancer research. *Mol. Cell. Proteomics* **4**, 377-383 (2005).
13. Sanchez-Carbayo, M., Socci, N.D., Lozano, J.J., Haab, B.B. & Cordon-Cardo, C. Profiling bladder cancer using targeted antibody arrays. *Am. J. Pathol.* **168**, 93-103 (2006).
14. Ingvarsson, J. *et al*. Detection of pancreatic cancer using antibody microarray-based serum protein profiling. *Proteomics* **8**, 2211-2219 (2008).
15. Barry, R. & Soloviev, M. Quantitative protein profiling using antibody arrays. *Proteomics* **4**, 3717-3726 (2004).
16. Orchekowski, R. *et al*. Antibody microarray profiling reveals individual and combined serum proteins associated with pancreatic cancer. *Cancer Res.* **65**, 11193-11202 (2005).
17. Miller, J.C. *et al*. Antibody microarray profiling of

human prostate cancer sera: antibody screening and identification of potential biomarkers. *Proteomics* **3**, 56-63 (2003).
18. Hamelinck, D. *et al*. Optimized normalization for antibody microarrays and application to serum-protein profiling. *Mol. Cell. Proteomics* **4**, 773-784 (2005).
19. Gao, W.-M. *et al*. Distinctive serum protein profiles involving abundant proteins in lung cancer patients based upon antibody microarray analysis. *BMC Cancer* **5**, 110 (2005).
20. Barry, R., Diggle, T., Terrett, J. & Soloviev, M. Competitive assay formats for high-throughput affinity arrays. *J. Biomol. Screen*. **8**, 257-263 (2003).
21. Yeretssian, G., Lecocq, M., Lebon, G., Hurst, H.C. & Sakanyan, V. Competition on nitrocellulose-immobilized antibody arrays: from bacterial protein binding assay to protein profiling in breast cancer cells. *Mol. Cell. Proteomics* **4**, 605-617 (2005).
22. Jung, S.-H., Jung, J.-W. & Ha, K.-S. Application of NHS-dextran-based prtein assays for analysis of blood proteins with a spectral SPR biosensor. *Biochip J.* **1**, 213-217 (2007).
23. Feng, J., Gao, C., Wang, B. & Shen, J. A novel process for inking the stamp with biomarcromolecule solution used in reactive microcontact printing. *Colloids and Surfaces B: Biointerfaces* **36**, 177-180 (2004).