

Application of NHS-dextran-based Protein Arrays for Analysis of Blood Proteins with a Spectral SPR Biosensor

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Abstract

In general, antibody arrays were favored to analyze proteins in blood samples or cell extracts because the arrays may provide selective immobilization of target proteins. However, in this study, we proposed protein arrays based on amide-linked (AL) NHS-dextran as an alternative approach to analyze C-reactive protein with a spectral SPR biosensor. The surface modification with AL NHS-dextran was based on amide linkage between amine-modified gold arrays and NHS-modified CM-dextran. The AL NHS-dextran arrays were characterized with FT-IR spectroscopy and atomic force microscopy (AFM). Moreover, AL NHS-dextran arrays were compared with the previously used epoxide-linked carboxymethyl dextran. In order to analyze quantitatively C-reactive protein in human sera on protein arrays, we have investigated the effect of buffer pH on the binding affinity of proteins to the arrays. Then, we have successfully analyzed C-reactive protein in human sera on the dextran arrays. Thus, protein arrays based on the AL NHS-dextran surface can be used as alternative method for rapid analysis of blood proteins.

Keywords: Protein arrays, NHS-dextran, C-reactive protein blood proteins, Spectral SPR biosensor

Introduction

Recently, there have been many reports associated with applications of protein arrays for serodiagnosis and proteomics based on the large-scale and high-throughput analysis^{1,2}. For these applications, various techniques have been demonstrated as detection methods such as fluorescence labeling^{2,3}, mass spec-

trometry⁴, atomic force microscopy (AFM)⁵ and surface plasmon resonance (SPR). Among these techniques, the most appropriate one for label-free and high-throughput analysis is SPR.

There are many reports on analysis of proteins mainly based on antibody arrays in a high-throughput manner because target proteins in sample solutions can be selectively immobilized onto the antibody arrays. It was reported that proteomic analysis of secreted proteins in early rheumatoid arthritis was performed on antibody arrays⁶. In addition, there are many reports on applications of antibody arrays for diagnosis of cancers based on serum-protein profiling^{7,8}. However, there are a few reports on use of protein arrays in the analysis of proteins, since it is not easy to immobilize target proteins selectively on protein arrays. Furthermore, the level of the target proteins in blood is much lower than major blood proteins such as albumin, immunoglobulin G and haptoglobin. Thus, there is no report on the high-throughput analysis of blood proteins based on protein arrays with SPR biosensors.

In this study, we introduced a protein array system based on NHS-dextran and an appropriate pH to analyze blood proteins. It is known that buffer pH has a significant influence on binding affinity of proteins to the arrays. In order to find an optimal buffer pH for selective immobilization of target proteins in human sera, we prepared blood samples in different buffer and applied to the NHS-dextran arrays. The AL NHS-dextran arrays provide several advantages such as short fabrication time, possibility to analyze protein interactions in a high-throughput format on gold arrays and higher binding affinity of protein to the arrays. Then, the protein arrays were probed with anti-CRP antibody and analyzed with a self-developed spectral SPR biosensor (Figure 1).

Results and Discussion

To prepare protein arrays, the surface of gold arrays was modified by two different approaches using new AL NHS-dextran and previously used EL CM-dextran, and various proteins were immobilized onto the dextran arrays. Then, two approaches were compared in the binding affinity of proteins (Table 1). One of major differences between AL CM-dextran and EL



Figure 1. A self-developed spectral SPR biosensor.

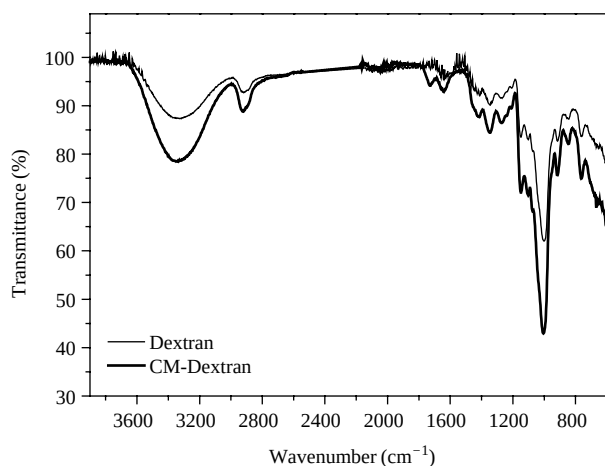


Figure 2. Characterization of synthesized CM-dextran by FT-IR.

CM-dextran is how dextran layers are introduced onto the gold surface. The formation of CM-dextran layer using EL CM-dextran was processed on solid arrays, whereas fabrication process using AL NHS-dextran was mainly performed in a liquid phase except the final immobilization. Thereby, AL NHS-dextran surface has several advantages such as short fabrication time, high-throughput analysis on gold arrays and higher binding affinity of proteins. To fabricate AL NHS-dextran arrays, initially, we functionalized dextran through the addition of carboxyl groups to hydroxyl functionalities by ether linkage, and the addition of carboxyl groups to dextran backbone was verified by the presence of a carboxylic FT-IR band from the carbonyl stretching of CM-dextran at ca. 1,734 and 1,580 cm^{-1} (Figure 2). Surfaces of AL NHS-dextran and EL CM-dextran were characterized with AFM, focused on surface topology and film thickness

Table 1. Differences between EL CM-dextran and AL NHS-dextran surfaces.

	EL CM-dextran	AL NHS-dextran
Time for surface modification	2 day	2 hr
Synthesis of modified dextran	on chip	in solution
Thickness of layer (nm)	3.2	4.2
Binding capacity of proteins	1	2.2 ± 0.8
High-throughput analysis on gold arrays	Inappropriate	Appropriate

(Table 1).

Subsequently, we investigated whether AL NHS-dextran arrays were appropriate for the analysis of blood proteins with the spectral SPR biosensor. It is difficult to analyze proteins in blood samples on protein arrays since the level of the target proteins in human blood is generally lower than major blood proteins such as albumin, immunoglobulin G and haptoglobin. It is known that albumin, immunoglobulin G and haptoglobin occupy 54, 17 and 3%, respectively, of total human blood proteins. In general, antibody arrays were favored to analyze proteins in blood samples or cell extracts because the arrays may provide selective immobilization of target proteins. However, in this study, we used protein arrays as an alternative method to analyze CRP in human sera since antibody arrays were not so sensitive to analyze CRP at low concentrations with the SPR biosensor (data not shown). In addition, when antibody arrays are analyzed by SPR biosensors, it is necessary to check whether the SPR signals obtained from the binding of proteins are specific.

In preparation of protein arrays, selective protein immobilization onto the array is important. It is known that buffer pH has a significant effect on binding affinity of proteins to the arrays. Thus, we investigated the effect of buffer pH on the binding affinity of proteins to the NHS-dextran arrays with a spectral SPR biosensor (Figure 3). Protein solutions of BSA, hemoglobin, haptoglobin, immunoglobulin G, CRP and GST were prepared in acetate buffer (pH 4.5) or phosphate buffer (pH 7.4), and then applied onto the dextran arrays. Most proteins showed strong binding affinity to the dextran surface at pH 4.5. However, when the proteins were prepared in the phosphate buffer, there was a dramatic decrease in the binding affinity of BSA, haptoglobin and GST with a slight decrease in that of hemoglobin and CRP. These results showed that selective immobilization of proteins can be achieved by choosing a proper buffer pH. Thus,

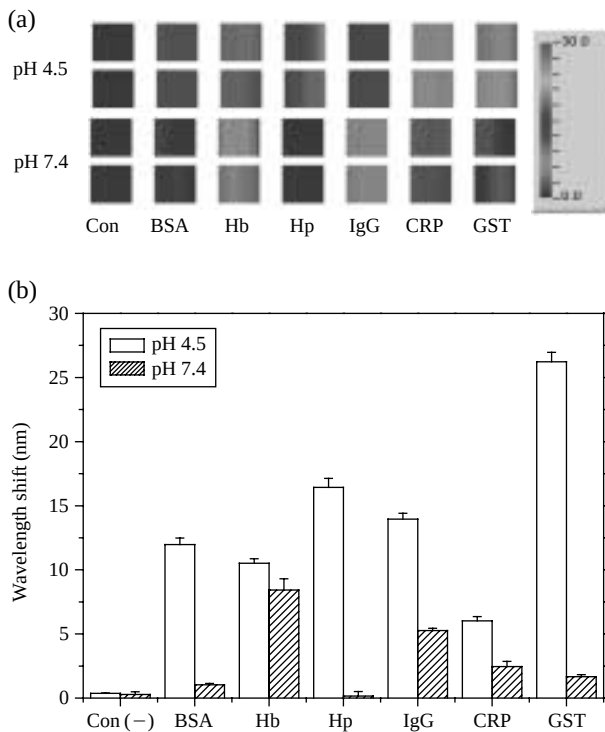


Figure 3. Differential binding affinity of proteins according to buffer pH. 100 $\mu\text{g/mL}$ BSA, hemoglobin (Hb), haptoglobin (Hp), immunoglobulin G (IgG), CRP and GST were prepared in acetate buffer (pH 4.5) and phosphate buffer (pH 7.4), and applied onto NHS-dextran arrays. Then, the arrays were analyzed in the line-scanning mode of the spectral SPR biosensor as described in Materials and Methods (a). The results are expressed as means $\pm$ S.D from three separate experiments (b).

the phosphate buffer (pH 7.4) was selected to immobilize human sera onto the dextran arrays to analyze CRP in human blood samples.

In order to analyze CRP in human sera with the spectral SPR biosensor, initially, various concentrations of CRP ranging from 0.1 to 50 $\text{ng}/\mu\text{L}$ were prepared in the normal human serum, diluted ten times with the phosphate buffer (pH 7.4), and applied to the AL NHS-dextran arrays. Then, the arrays were probed with anti-goat CRP, incubated with the secondary antibody, and then analyzed with the spectral SPR biosensor. CRP caused a dose-dependent increase of SPR signals (Figure 4) and a standard curve was obtained by the Sigmoid fit of the Origin program. Then, human sera were analyzed with a spectral SPR biosensor after diluting the sera ten times with the phosphate buffer (Figure 4). The level of CRP was determined by using the standard curve. The level of CRP in the human sera was also determined by a commercialized method, the latex-enhanced turbidi-

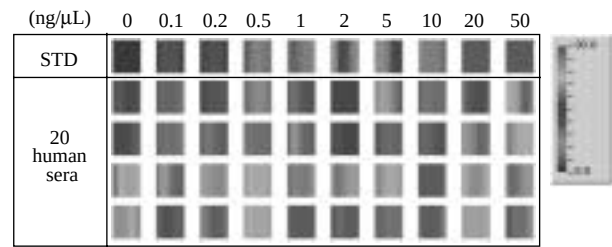


Figure 4. Analysis of CRP in human sera. Standard samples ranging from 0.1 to 50 $\text{ng}/\mu\text{L}$ and 20 human sera were applied onto NHS-dextran arrays. The protein arrays were incubated with anti-CRP antibody and anti-goat IgG, and analyzed in the line-scanning mode of the spectral SPR biosensor as described in Materials and Methods.

metry immunoassay using an automated chemical analyzer. Then, the relationship between the results obtained by two methods was investigated with the Fit Linear of the Origin program, and the results showed a good correlation with those determined by the latex-enhanced turbidimetry immunoassay. Thus, protein arrays are an alternative approach to analyze blood proteins in an appropriate pH environment.

Materials and Methods

Protein and Serum Samples

GST was prepared by expressing the genes in *E. coli* (BL21) according to the procedures specified in a previous report⁹. C-reactive protein was obtained from Scripps Lab (San Diego, CA). Human sera were obtained from the Kangwon National University Hospital, divided into aliquots and stored at -20°C until use. Experiments using the human samples were performed under the approval by the local Institute Ethics Committee for human subject research.

Hydrophobic Modification of Gold Arrays

Gold arrays were fabricated and modified according to the previous report⁹. Briefly, gold arrays with fifty spots each of 2 mm diameter were fabricated by depositing Ti/Au (50/450 $\text{\AA}$) films on pyrex glass, and were cleaned with a cleaning solution of $\text{NH}_4\text{OH} : \text{H}_2\text{O}_2 : \text{H}_2\text{O}$ (1 : 1 : 5, v/v) at 70°C for 10 min. Then, the glass slides were incubated with a mixture of hexadecane/carbone tetrachloride/octadecyltrichlorosilane (20 : 5 : 0.04, v/v) to generate hydrophobic glass surface at 50°C for 30 min. And the gold arrays were washed with a mixture of hexadecane/carbone tetrachloride (20 : 5, v/v), carbon tetrachloride and ethanol in order. The gold spots were hydrophilized with the cleaning solution just before using.

Synthesis of CM-dextran Hydrogel

CM-dextran was synthesized according to the procedures of McArthur *et al.*¹⁰ Six grams dextran (M.W. 500 K) was dissolved in 20 mL of 100 mM bromoacetic acid in 2 M NaOH and stirred overnight. Then, the solution was dialyzed against milliQ water for 24 h, against 0.1 M HCl for 24 h and finally against water for 24 h. The dialyzed CM-dextran solution was lyophilized, and the lyophilized CM-dextran was stored until use.

Surface Modification of Gold Arrays with AL NHS-dextran

The surfaces of gold arrays were modified with AL NHS-dextran according to the procedures of Jung *et al.*¹¹ First, the gold arrays were incubated with a mixed thiol solution of 0.1 mM MUA and 0.9 mM 6-mercaptohexanol in ethanol for 16 h, and then washed with ethanol to remove the excess thiols¹². For amination of carboxyl groups, the arrays were incubated with 500 mM ethylenediamine for 1 h after preincubation with a mixture of 50 mM NHS and 200 mM N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride for 10 min. The CM-dextran was functionalized with NHS groups by incubating 0.3 g/mL CM-dextran in the mixed solution of 50 mM NHS and 200 mM N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride at room temperature for 10 min. Then, AL-NHS dextran layer on gold arrays was formed by applying the NHS-dextran solution to the monolayer of N-(2-aminoethyl)-11-mercaptoundecanamide and 6-mercaptohexanol.

Preparation of Protein Arrays on the AL NHS-dextran Layer of Gold Arrays

In order to investigate the effect of buffer pH on the binding affinity of proteins onto the AL NHS-dextran layer of arrays, various proteins including BSA, hemoglobin, haptoglobin, immunoglobulin G, CRP and GST prepared in 10 mM sodium acetate buffer (pH 4.5) or 9.3 mM phosphate buffer (pH 7.4) were applied to the AL NHS-dextran surface of gold arrays for 1 h. Then, the arrays were washed twice with 0.1% Tween 20 in PBS for 5 min, rinsed with milliQ water, flushed with N₂ gas to remove the water and immediately analyzed with a self-developed spectral SPR biosensor.

High-throughput Analysis of CRP in Human Sera

The high-throughput analysis of CRP in human sera on protein arrays was performed in the line-scanning mode of the spectral SPR biosensor according to a previous report¹³. Briefly, the spectral SPR sensor

was configured using the Kretschmann geometry of the attenuated total reflection method. The arrays were coupled with a fused silica prism via an index matching fluid and mounted on an x-y linear stage. Then, the array spots were automatically scanned every 100 μm along the central line by the line-scanning mode.

For the quantitative analysis of CRP in human sera by the spectral SPR biosensor, first, it was necessary to obtain the standard curve through analysis of standard samples. Thus, pure CRP was serially mixed, ranging from 0.1 to 50 ng/ μL , with normal human serum diluted with the phosphate buffer (pH 7.4) and applied to AL NHS-dextran surface. Collected SPR signals were used to obtain a standard curve by the Sigmoidal Fit of the Origin program. Then, human sera were diluted ten times with the phosphate buffer and immobilized onto the AL NHS-dextran layer of arrays. The protein arrays were blocked with 1% BSA containing 0.1% Tween 20 in PBS for 30 min and probed for 1 h with 200 $\mu\text{g/mL}$ monoclonal anti-CRP antibody (2 $\mu\text{L/spot}$) in 1% BSA containing 0.1% Tween 20 in PBS. After washing with 0.1% Tween 20 in PBS, the arrays were incubated with anti-goat IgG and immediately analyzed with the spectral SPR biosensor. The level of CRP in human sera determined by a spectral SPR biosensor was compared with that determined by latex-enhanced turbidimetry immunoassay, another method commercially used. Then, correlation between two methods was analyzed with the Linear Fit of the Origin program.

Conclusions

In this paper, we proposed protein arrays based on AL NHS-dextran as an alternative method to analyze blood proteins. AL NHS-dextran surface showed a higher binding affinity of proteins than EL CM-dextran surface. Proteins were selectively immobilized on the NHS-dextran surface in an appropriate pH environment. CRP in human sera was successfully analyzed on the NHS-dextran arrays with a spectral SPR biosensor. Thus, protein arrays based on the AL NHS-dextran surface has a potential for rapid analysis of blood proteins.

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