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Detection Technology for Antibody-Antigen Interaction on ProteoChip using Quantum Dot

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Abstract

Quantum Dot (CdSe-ZnS nanocrystals, QD or Qdot) nanometer-size particles have a number of potential applications in a variety of fields. Qdots have already been put into use as an alternative to organic fluorescent dyes and fluorescent proteins. In this study, the applications of Qdots were explored in protein microarray techniques, using Angiogenin (ANG) and Anti-ANG antibody on a ProteoChip. We compared the signal-to-noise ratios between the Cy5 labeling and Qdot labeling methods. The Qdot method evidenced a higher signal-to-noise ratio than was observed in the Cy5 labeling method. This indicates that Qdots can be used as an enabling detection material for the specific quantification of proteins and profiling in protein chips.

Keywords: Quantum dots, ProteoChip, Protein chip, Cy5

Introduction

Protein microarray technology involves the use of a variety of fluorescent dyes for protein visualization and labeling. Many fluorescent dyes have been reported effective as tools for biomaterial labeling. Cyanine reagent (Cy5, Cy3) is accepted as one of the more useful dyes for protein and biological compound labeling[1]. Recently, quantum dots (Qdot) have been reported as an alternative labeling dye for protein microarray techniques. Qdots are crystalline materials, constructed of a CdSe core of a few nanometers, surrounded by a thin ZnS shell[2]. This CdSe/ZnS core/shell nanostructure has the ability to emit light upon UV excitation. The emission is relatively narrow (~20-25 nm fwhm) and can be tuned by adjusting the size of the CdSe core, in accordance with the quantum confinement effect. Qdot-antibody conjugates have been studied for possible application as a tool for fluoroimmunoassays and in immunosensor devices[3]. This technology has also been studied in conjunction with reverse-phase protein microarray techniques, which are desirable because they allow for the analysis and comparison of multiple samples side by side in a single array[4].

The ProteoChip (Proteogen, Korea), ProLinker™-coated slide, was previously developed as a powerful tool for protein-protein interaction analyses based on stable protein immobilization. Thus, the technology has been utilized for the study of protein-ligand interactions, protein-protein interactions, protein expression profiling, new lead screening, and a variety of proteomics research efforts[5].

In the protein microarray technique, the signal-to-noise ratio is crucial for sensitivity and specificity. This may be related to non-specific binding of the labeled material. In particular, the signal-to-noise ratio plays a critical role in the study of quantitative biomarker analysis, expression profiling, and pathogen detection. In this study, we have attempted to assess the efficacy of Qdots in the quantitative analysis of antibody-antigen interaction. Anti-ANG polyclonal antibody was labeled with Qdot or Cy5, and the signal-to-noise ratios of the Cy5 labeling method and the Qdot labeling method were compared.

Results and Discussion

Angiogenin and Angiogenin antibodies were used for the study. Angiogenin-induced protein expression profiling was also conducted[6]. We purified angiogenin from expressed recombinant *E. coli* via a refolding process[7].

Cy5 labeling efficiency is more efficient than that achievd with Qdot. Different labeling methods were employed. The Cy5 dye molecules bound to Anti-ANG antibody /antibody molecule (D/P) ratio was approximately 10. The Qdot signals were much brighter than those seen with the organic dyes[8]. In order to assess the applicability of Qdots in a protein chip detection system, we conducted antibody-anti-

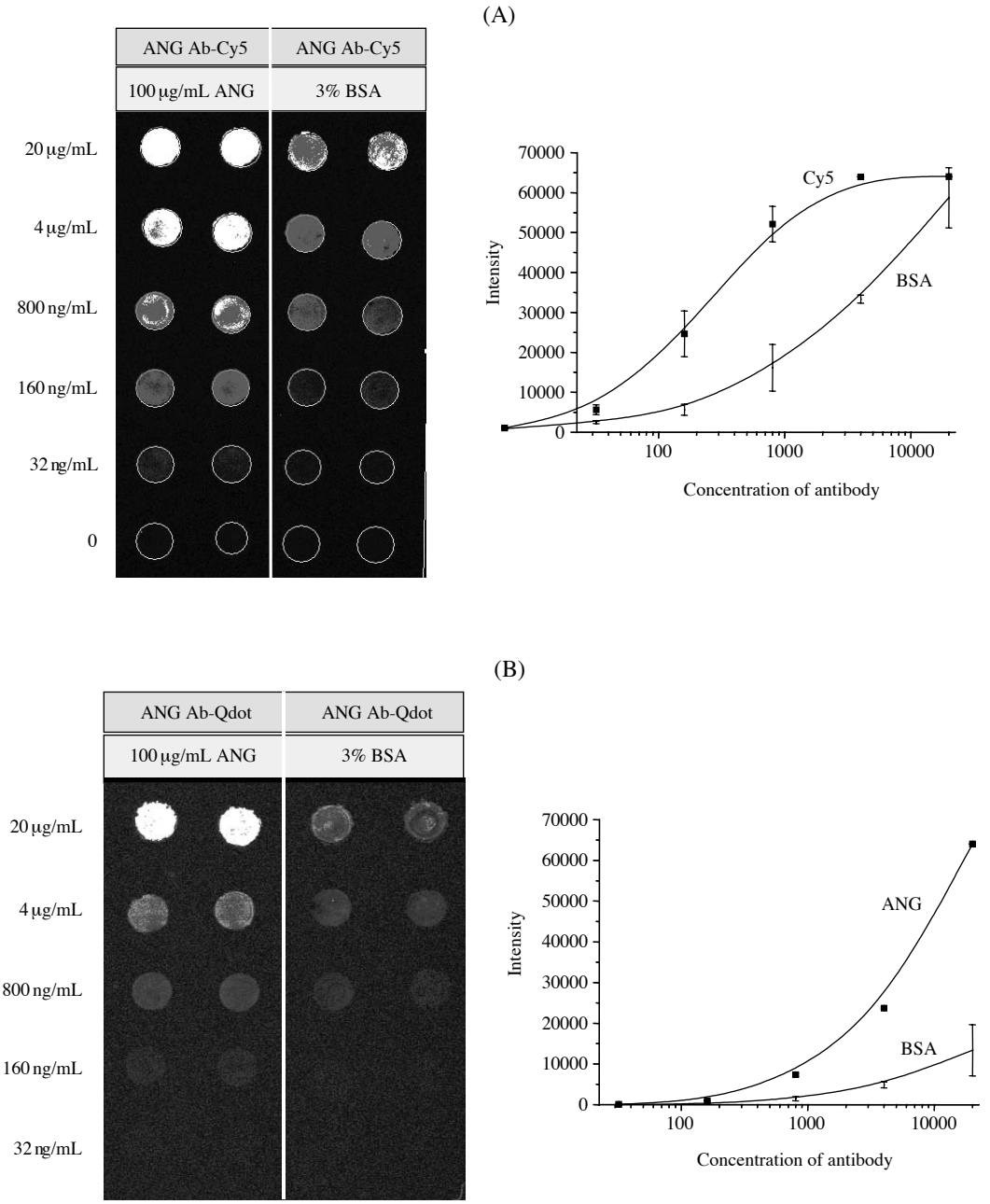

Figure 1. Interaction of ANG with anti-ANG Ab-Qdot on ProteoChip. (A) Cy5-labeled ANG antibody-ANG interaction on ProteoChip; (B) Qdot-labeled ANG antibody-ANG interaction on ProteoChip.

gen interaction assays using the Qdot labeling method on a ProteoChip system. Angiogenin (ANG) protein was initially immobilized in a well-on-a-chip ProteoChip base plate, then incubated with Anti-ANG Ab labeled with Qdot in different concentrations ranging from 20 μg/mL to 32 ng/mL (Figure 1B). The image analysis evidenced good correlation between fluorescence intensities and Qdot-labeled ANG antibody concentrations (Figure 1). It was clear that the Qdot-labeled ANG antibody was able to bind specifically to ANG as compared with BSA, similar to what was observed with Cy5-labeled ANG antibody. Cy5-labeled Ab evidenced a lower Limit-of-Detection (LOD) than was achievable with Qdot-labeled Ab (Figures 1 and 2). This result might be attributable to the size difference between Qdot and

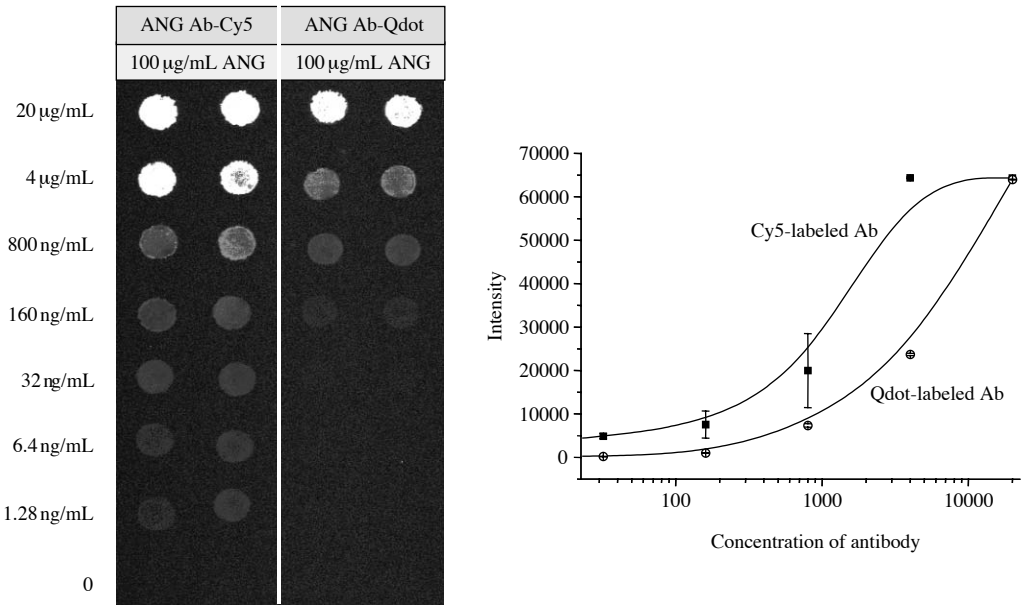

Figure 2. Comparison of detection efficiency between Cy5 and Qdot on ProteoChip. Cy5-labeled ANG antibody or Qdot-labeled ANG antibody was utilized for analyses of antibody-antigen interactions on a ProteoChip immobilized with ANG.

Cy5. The diameter of the antibody conjugated with Qdot is at least 20 nm, whereas the Cy5-labeled antibody was much smaller than the Qdot-labeled one. The Well-on-a-Chip surface has a limited area, allowing for less binding of Qdot-labeled antibody than is the case with Cy5-labeled antibody. This means that the Qdot-labeled system was less sensitive than the Cy5-labeled system; however, it does not mean that the fluorescence intensity of Qdot was less sensitive than that of Cy5 when compared on a molecule-to-molecule basis. In addition to this result, we assumed that our results might be attributable, in part, to the differences in the signal-to-noise ratios of the Qdot and Cy5 labeling methods. In order to evaluate the non-specific binding ability of the Qdot material, we assessed signal-to-noise ratio of Qdot and compared it with that of Cy5, a conventional fluorescent dye. The saturated signal intensity of the scanned images was 65535. The signal-to-noise ratio was calculated from 65000 to 5000. The S/N ratio of the Qdot-labeled antibody was higher than that of the Cy5-labeled one and was invariable, regardless of the antibody concentration. However, the S/N ratio of the Cy5-labeled antibody fluctuated in a concentration-dependent manner (Figure 3). These data indicate that the Qdot labeling system was significantly less non-specific than the Cy5 labeling system. This result strongly indicates that the Qdot technique is applicable to solid-phase biomarker assays on protein microarrays, as well as protein expression profiling

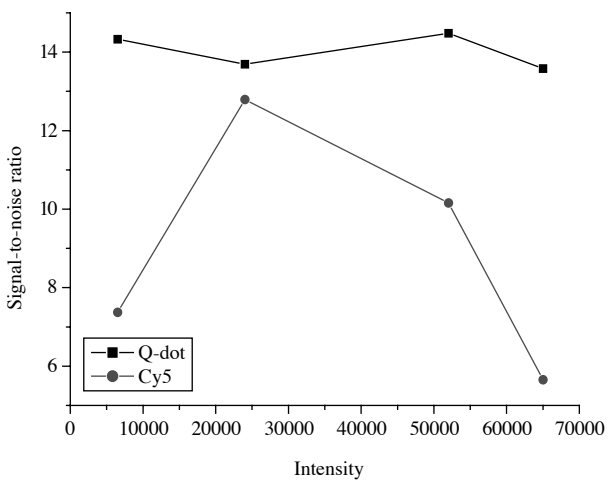

Figure 3. Comparison of signal-to-noise ratio between Cy5 and Qdot on ProteoChip. Signal-to-noise ratios (S/N or SNR) were calculated via the following equation: signal=Vs, noise =Vn, S/N=20 log10 (Vs/Vn).

on an antibody microarray. We demonstrated protein differential expression using an antibody-arrayed ProteoChip (Proteogen)[6]. In this study, the ratios of two Cyanine dyes, Cy3 and Cy5, were employed for protein profile analyses. Cyanine dyes are limited with regard to long-term observation due to photobleaching effects[1]. Therefore, it seems clearly necessary that progress be made in the development of

new detection technology for protein chips.

Several lines of our experimental data indicate that the Qdot-labeling system may facilitate the advancement of technology in a variety of biological fields, including the quantification of protein biomarkers, protein-protein interaction assays, protein expression profiling, and the detection of pathogens.

Materials and Methods

Preparation of Bovine-angiogenin and Angiogenin Antibody

The bovine-Angiogenin (bAng) recombinant was expressed in *E. coli* in the form of inclusion bodies[7]. Angiogenin inclusion bodies were dissolved in 20 mL of denaturing buffer (6 M Gdn-Cl, 100 mM Tris-Cl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 10 mM DTT) and diluted for refolding to 0.2-0.3 mg/mL in refolding buffer (0.3 mM GSSG, 1.5 mM GSH, 1 mM EDTA, 100 mM Tris-Cl, pH 8.0), then incubated for 24 h at 4˚C. The solution was then concentrated via ultrafiltration, loaded onto a SP-Sepharose Fast Flow column, and purified via FPLC with a linear gradient of 0-1 M NaCl. The collected fractions were then dialyzed against water and lyophilized. The purity of the purified angiogenin was determined via SDS-PAGE. The anti-ANG polyclonal Ab was obtained from Dr. Soo-Ik Chang at the Chungbuk National University.

Angiogenin Antibody Labeling

300 μg of angiogenin antibody was labeled with a Cy5 fluorescent mono reactive dye pack (Amersham Pharmacia Biotech, Uppsala, Sweden). Antibody containing 0.1 M sodium carbonate buffer was added to the dye vial, and incubated for 1 hr. The labeled proteins were separated from excess free dye using SigmaSpin post-reaction Clean-Up Columns (Sigma). 300 μg of angiogenin antibody was then conjugated using a Qdot 655 Antibody Conjugation Kit (Invitrogen, Netherlands). The kit contains all of the reagents and components required for conjugation reaction. The conjugation utilizes a well-known chemistry, which is predicated on the rapid and efficient coupling of thiols to maleimide groups. The starting materials provided in the kit are chemically derivatized Qdot 655 nanocrystals harboring a number of amine groups on their surfaces. The initial step in the conjugation process involves the conversion of these amines to thiol-reactive maleimide groups. This can be readily achieved using the hetero-bifunctional crosslinker, 4-(maleimidomethyl)-1-cyclohexanecarboxylic acid N-hydroxysuccinimide ester (SMCC). After 60 minutes of reaction, the excess crosslinker is removed from the activated quantum dots using a desalting column. Antibody reduced by DTT was then desalted with a desalting column. Then, the antibody and Qdots were allowed to conjugate for 1 hr and were quenched with β2-mercaptoethanol. The conjugated antibody was separated via gel filtration chromatography.

Protein Microarray Assay

Purified angiogenin was diluted to a concentration of 100 μg/mL in PBS solution with 30% glycerol, and 3% BSA in PBS was prepared for parallel control or noise. Diluted angiogenin and 3% BSA were spotted onto ProLinker A coated ProteoChip well chips (Proteogen, Korea) in duplicate and incubated overnight at 4˚C. The chips were washed in PBST buffer (0.5% Tween-20 in PBS) for 30 min and blocked for 1 hour with blocking buffer (1% BSA in PBS). After blocking, the chip was washed in PBST buffer to remove excess BSA and dried under N_2 gas. Cy5-Angiogenin antibody and Qdot-Angiogenin antibody were diluted five-fold from the initial dilution to 20 μg/mL in PBS solution with 30% glycerol, and loaded onto well chips, then incubated for 1 hour at 37˚C. The chip was washed with PBST buffer and dried under N_2 gas.

Image Analysis

In order to detect Qdot signals and Cy5 signals, a slide stained with Qdot-anti Ang and Cy5-anti Ang was scanned with a Genetix aQuire™ scanner (Genetix, UK) and saved as a TIFF file. The scanned images were analyzed with GenePix Pro 6.0 (Axon Instruments, CA, USA). Data were analyzed using Excel (Microsoft). The signal-to-noise ratios (S/N) were calculated using the following formula:

Signal-to-noise ratio (S/N or SNR),
signal=Vs, noise=Vn,
S/N=20 log10 (Vs/Vn)

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