

Polyhydroxyalkanoate Chip for the Specific Immobilization of Recombinant Proteins and Its Applications in Immunodiagnosics

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Abstract In this study, a novel strategy was developed for the highly selective immobilization of proteins, using the polyhydroxyalkanoate (PHA) depolymerase substrate binding domain (SBD) as an active binding domain. In order to determine the appropriacy of this method for immunodiagnostic assays, the single-chain antibody (ScFv) against the hepatitis B virus (HBV) preS2 surface protein and the severe acute respiratory syndrome coronavirus (SARS-CoV) envelope protein (SCVe) were fused to the SBD, then directly immobilized on PHA-coated slides via microspotting. The fluorescence-labeled HBV antigen and the antibody against SCVe were then utilized to examine specific interactions on the PHA-coated surfaces. Fluorescence signals were detected only at the spotted positions, thereby indicating a high degree of affinity and selectivity for their corresponding antigens/antibodies. Furthermore, we detected small amounts of ScFv-SBD (2.7 ng/mL) and SCVe-SBD fusion proteins (0.6 ng/mL). Therefore, this microarray platform technology, using PHA and SBD, appears generally appropriate for immunodiagnosis, with no special requirements with regard to synthetic or chemical modification of the biomolecules or the solid surface.

Keywords: Poly(3-hydroxybutyrate), Microarray, HBV preS2 surface protein, SARS-CoV envelope protein, P(3HB) depolymerase substrate binding domain

INTRODUCTION

The immobilization of proteins and peptides on solid surfaces has become the focus of a great deal of attention, most notably in fields concerning the development of biosensors and microarrays [1-5]. Protein immobilization on solid surfaces has been previously conducted via the application of a variety of methods [2,6,7]. However, non-specific binding and incorrect protein orientation have proven to be major problems in this regard, resulting in generally low specificity and selectivity in the application of such methods [5]. Chemical modifications of the solid surface or the protein to be immobilized can sometimes solve these problems, but this tends to be both costly and cumbersome. Therefore, there is continued interest in the development of an efficient method for the immobilization of proteins, which does not require

chemical modifications of the solid surface or the relevant proteins.

With the continuing emergence of new pathogens, the development of a simple, sensitive and rapid diagnostic method is clearly a matter of significance [8]. In the diagnosis of hepatitis B virus (HBV), several detection methods, including RT-PCR, DNA chip, and enzyme-linked immunosorbent assay (ELISA) methods constitute the most commonly-employed techniques [9-11]. A variety of strategies for the diagnosis of severe acute respiratory syndrome coronavirus (SARS-CoV) have been recently developed, including sequence detection, serological assays, and real-time RT-PCR [12,13]. However, a great demand exists for the development of a simpler, more accurate method for the detection of these viruses.

Protein microarray systems, miniaturized and sensitive platforms for the performance of immunoassays, have recently been developed, and have been demonstrated to yield efficient diagnostics [3,14]. However, there remains a need for an efficient method for the immobilization of biomolecules for sensitive and accurate assays. In this

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i> strains		
XL1-Blue	<i>recA1, endA1, gyrA96, thi, hsdR17, suppE44, relA1, I⁻, lac⁻, F['][proAB lacI^q lacZΔM15, Tn10 (tet)^r]</i>	Stratagene ^a
BL21 (DE3)	F ['] <i>ompT hsdS_B (r_B⁻ m_B⁻) gal dcm</i> (DE3)	Novagen ^b
Plasmids		
pET-22b(+)	5.5 kb, Ap ^r , T7 promoter, T7 terminator	Novagen
pTrc99A	4.2 kb, Ap ^r ; <i>trc</i> promoter	Pharmacia ^c
pScFv(S)-ScFv(S2)-Fc	3.0 kb, Ap ^r ; <i>lac</i> promoter, anti-preS ScFv, anti-preS2 ScFv-Fc domain	[18]
pET-ScFv-SBD	6His and ScFv fused-SBD, pET-22b(+) derivative	This study
pET-EGFP-SBD	6His and EGFP fused-SBD, pET-22b(+) derivative	[15,19]
pET-RFP-SBD	6His and RFP fused-SBD, pET-22b(+) derivative	[15,19]
pTrc-SCVe-SBD	6His and SCVe fused-SBD, pTrc99A derivative	[15]

^a Stratagene Cloning Systems, La Jolla, CA, USA.

^b Novagen, San Diego, CA, USA.

^c Amersham Pharmacia Biotech, Uppsala, Sweden.

study, we report a novel strategy for the development of protein microarrays, which exploits the specific interaction occurring between the biopolymer polyhydroxyalkanoate (PHA) and the substrate-binding domain (SBD) of PHA depolymerase. Using HBV and SARS-CoV, we have determined that this strategy should prove generally applicable to the development of protein microarrays for immunodiagnosics.

In the following experiments, enhanced green fluorescent protein (EGFP), red fluorescent protein (RFP), single chain antibody (ScFv) against hepatitis B virus preS2 surface protein, and SARS-CoV surface envelope protein (SCVe) were employed as model proteins, in order to demonstrate the immunodiagnostic feasibility of this technique. Polyclonal antibody against SCVe was synthesized at Peptron (Daejeon, Korea). On the basis of our analysis of the primary structure of the SCVe protein, we predicted the putative antigenic regions of the SCVe protein. The hydrophilicity, surface probability, flexible regions, and antigenicity of the SCVe protein were calculated using the Protean™ program from DNASTAR, Inc. (Madison, WI, USA), using Kyte-Doolittle plots, the Emini prediction, the Karplus-Schulz prediction, and the Jameson-Wolf prediction, respectively [15]. In brief, polyclonal rabbit serum was generated via immunization with a peptide predicated on SCVe residues 58-75 (H₂N-VYSRVKLNLSSEGVPDLL-COOH), harboring cysteine for conjugation [16]. Female rabbits (age 12 to 22 weeks) were injected 3 times with 500 mg of peptide-KLH conjugate in Freund's complete adjuvant (FCA; Pierce Chemical Company, Rockford, IL, USA), in accordance with the manufacturer's instructions. Sera were screened via indirect ELISA. Each of the wells of the ELISA plates were coated with 10% (w/v) peptide-ovalbumin conjugate in 50 mM carbonate buffer (pH 9.0), and the plates were incubated overnight at 4°C. Without blocking, 100 μL of antiserum or hybridoma supernatant was incubated for 45 min at 37°C. Bound antibodies were detected with horseradish-conjugated goat anti-rabbit IgG and *O*-phenylene-diamine dihydrochloride (Sigma, St. Louis,

MO, USA). The titer of the rabbit antiserum after immunization was approximately 1:100,000. This antibody was then purified through a column and concentrated to 1.6 mg/mL. The fluorophore Cy5 was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The anti-rabbit IgG polyclonal antibody was labeled with Cy5, in accordance with the product specifications. Antibody and Cy5 solutions prepared in 0.1 M sodium carbonate buffer (pH 8.0) were combined, and allowed to settle for 45 min in darkness. The reaction samples were brought up to 0.5 mL with phosphate-buffered saline (PBS), then loaded onto microconcentrator spin columns (Millipore Microcon 30) set to a 30,000 Da molecular weight cutoff. After centrifugation, the samples were washed in PBS and re-centrifuged, yielding a sample volume of 10 μL. PBS was then added, to increase the volume to 20 μL. The efficiency of the labeling procedure was analyzed via measurements of the molar ratio of dye to antibody.

All bacterial strains and plasmids used in this study were listed in Table 1. The PCR experiments were conducted with a PCR Thermal Cycler T1 (BioMetra Co., Germany), using the High Fidelity PCR System (Boehringer Mannheim, Mannheim, Germany). Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA, USA). All DNA manipulation procedures, including restriction digestion, ligation, and agarose gel electrophoresis were conducted according to standard protocols [17]. The DNA sequences of all of the clones were confirmed via sequencing with an automatic DNA sequencer (ABI Prism model 377, Perkin Elmer Co., IL, USA). The DNA fragments encoding for SBD and ScFv against HBV preS2 surface protein were generated via PCR amplification, using the genomic DNA of *Alcaligenes faecalis* T1 and pScFv(S)-ScFv(S2)-Fc [18], respectively, as templates, using the primers shown in Table 2. An *Nde*I site was introduced at the 5' end of oligonucleotide primer 1, which was then employed as the forward primer for the amplification of the fusion gene. It harbors 18 nucleotides, encoding a six-histidine tag at the *N*-terminus for

Table 2. Oligonucleotides used for PCR in this study

No. Primer	Sequences ^a (5' → 3')
Primer 1 ^b	GGAATTCCATATGCACCATCACCATCACCATTTCGACTGAGGAGTCT
Primer 2	CGCGGGATCCTTAGTTGCTGCAGCGTCC
Primer 3	CTTGCTGCCGTTGCGGTAACGTTTTATTTCAGGTA
Primer 4	TACCTGGAAATAAACGTTACCGCAACGGCAGCAAG

^aThe restriction enzyme sites are indicated by underlines.

^bThe sequence for six histidine is shown in italic.

easy purification. A *Bam*HI site and a stop codon were introduced at the 5' end of oligonucleotide primer 2, which was employed as the reverse primer. The DNA fragments encoding for 6His-ScFv and SBD were amplified via PCR using the primer combinations 1 plus 3 and 2 plus 4, respectively. The PCR products containing 36 overlapping nucleotides were employed as templates for the PCR amplification of the 6His-ScFv-SBD fusion gene, using primers 1 and 2. The amplified product acquired using overlapping PCR was then digested with *Nde*I and *Bam*HI, and ligated into the same restriction sites of pET-22b(+), yielding pET-ScFv-SBD. Plasmids pET-EGFP-SBD, pET-RFP-SBD, and pET-ScFv-SBD, which harbored the fusion genes encoding for 6His-EGFP-SBD, 6His-RFP-SBD, and 6His-SCVe-SBD, respectively, have all been previously described [15,19]. The fusion genes encoding for 6His-EGFP-SBD, 6His-RFP-SBD, and 6His-ScFv-SBD were expressed in *E. coli* BL21 (DE3) using the strong *T7* promoter, whereas the 6His-SCVe-SBD fusion gene was expressed in *E. coli* XL1-Blue, using the *trc* promoter in pTrc99A, to obtain soluble proteins. For the production of 6His-ScFv-SBD fusion protein, *E. coli* BL21 (DE3) harboring pET-ScFv-SBD was cultivated in 100 mL of Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with ampicillin (Ap, 50 µg/mL) in a shaking incubator at 200 rpm, at 37°C. After the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma), the cells were cultivated for an additional 4 h. The cells were harvested and disrupted via sonication (Braun Ultrasonics Co, Danbury, CT, USA) for 1 min at 20% output. After 10 min of centrifugation at 12,000 rpm at 4°C, the supernatant containing the soluble protein fraction was saved, for further use in the purification of fusion proteins. An Ni-NTA column (Qiagen, Valencia, CA, USA) was employed in the purification of the fusion proteins.

Poly(3-hydroxybutyrate), P(3HB), was produced as described previously [20]. The spin-coating of P(3HB) on the surface of a glass slide was conducted as reported previously [19,21]. In brief, the glass substrate was modified via treatment with octadecyltrichlorosilane (OTS). The OTS-modified glass substrate was spin-coated (3,000~4,000 rpm for 40 sec) three times with P(3HB) in chloroform (0.25 wt %). In order to determine whether the SBD-fusion proteins could be immobilized on the P(3HB)-coated substrates, 6His-EGFP-SBD and 6His-RFP-SBD fusion proteins were utilized as readily visible model proteins, and spotted on P(3HB) chips using a robotic microarrayer (BioRobotics, Cambridge, UK).

After incubation in a humid chamber at 30°C for immobilization, the slides were washed three times in PBS buffer, and visualized with a ScanArray 5000 (GSI-Lumonics, Ottawa, Canada). Each of the fluorescence signals was analyzed using Scion image analysis software (Scion Corp., Frederick, MD, USA). As expected, each of the fluorescence intensities was detected only at the spotted positions with native fluorescence signals, either green or red. As can be seen in Figs. 1A and B, the specific and strong fluorescence signals were detected in the positions at which the fusion proteins had been spotted. However, in the negative control experiment, no fluorescence signals were detected at the spots of the *E. coli* cell lysates. The native EGFP and RFP proteins were determined not to bind to the P(3HB) chip, which suggests that the binding of SBD is site-specific to the P(3HB) chip. More remarkably, the fluorescence intensities changed in a linear fashion, in accordance with the concentrations of fusion proteins (Fig. 1C). Furthermore, small amounts of 6His-EGFP-SBD (10.7 ng/mL) and 6His-RFP-SBD (6.3 ng/mL) were also detected (Fig. 1D). It is worthy of noting that this system evidenced a good correlation between fluorescence intensity and fusion protein concentration, thereby suggesting that both qualitative and quantitative immunodiagnostic assays might be run on the P(3HB) chips. After the successful immobilization and detection of SBD-fluorescent fusion proteins had been confirmed, we examined the possible efficacy of this strategy in immunodiagnostic assays.

In order to visualize the specific interactions occurring between the ScFv and HBV preS2 surface antigen, and between the SCVe antigen and its polyclonal antibody, the ScFv-SBD and SCVe-SBD fusion proteins were diluted to 1 mg/mL in PBS buffer (pH 7.5), and were spotted on P(3HB)-coated glass slides, using a robotic microarrayer (BioRobotics). The spot diameter and spot-to-spot distance were measured to be 100 and 225 µm, respectively. After spotting, the glass slides were air-dried for 1 h at room temperature. In order to determine the concentration-dependent detection limit, the fusion proteins were serially diluted in PBS to the desired concentrations prior to spotting. After incubation in a humid chamber at 30°C for immobilization, the slides were washed three times in PBS, and incubated with fluorescein-5-isothiocyanate (FITC)-conjugated HBV preS2 surface antigen, which was acquired from LG Life Sciences (Daejeon, Korea), or with Cy5-labeled anti-SCVe antibody, for 30 min at 37°C. After reaction, the slides were washed three times in PBS. Finally, the slides were scanned using a ScanArray 5000. Signal intensity was

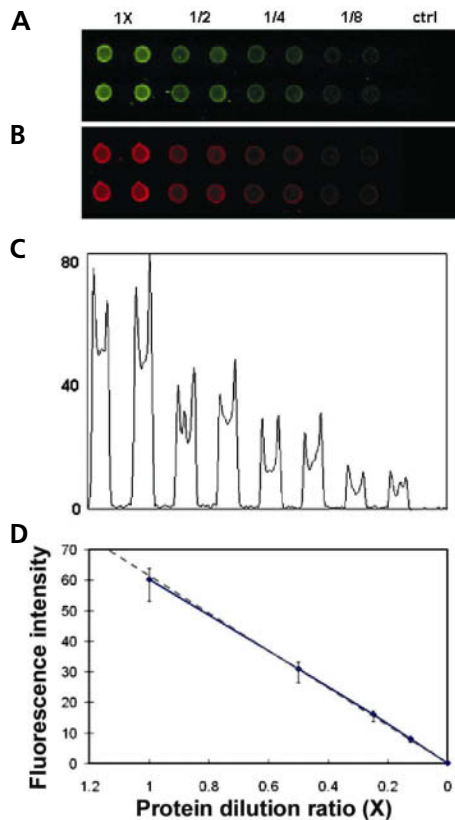


Fig. 1. Protein microarray of two fluorescent fusion proteins on P(3HB)-coated substrate. (A) Microspottings of 6His-EGFP-SBD fusion proteins at varying concentrations (1X, 86 ng/mL; 1/2, 43 ng/mL; 1/4, 21.5 ng/mL; 1/8, 10.75 ng/mL). (B) Microspottings of 6His-RFP-SBD fusion proteins at varying concentrations (1X, 50 ng/mL; 1/2, 25 ng/mL; 1/4, 12.5 ng/mL; 1/8, 6.25 ng/mL). The negative control (ctrl) contains *E. coli* cell lysates in PBS buffer. (C) Fluorescence intensity profile of the spots shown in (A). (D) Relationship between fluorescence intensity and proteins at varying concentrations in the case of (A). The error bar represents standard deviation.

quantified using Image-Quant and Scion image analysis software.

A commonly used technique for the detection of HBV in blood samples involves the measurement of surface antigen levels in hepatitis patients [22]. Other serological assays for the diagnosis of viral infection are predicated on the detection of specific antibodies against viral antigens [23]. Thus, we decided to demonstrate both antigen and antibody detection. The strategies for the detection of HBV antigen and the antibody against SARS-CoV are shown in Fig. 2A. Two fusion proteins, 6His-ScFv-SBD and 6His-SCVe-SBD, were spotted onto a P(3HB) chip at varying concentrations, then incubated with FITC-conjugated HBV preS2 surface antigen (2.2 μ g/mL in PBS) and Cy5-labeled polyclonal antibody against SARS-CoVe (1.7 μ g/mL in PBS), respectively. The slides were then washed three times in PBS buffer and distilled water. After drying the slides, the fluorescence intensities of the

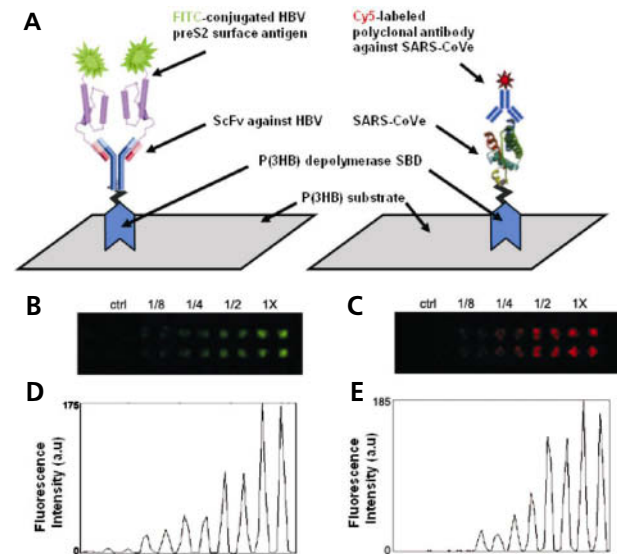


Fig. 2. Specific detection of the HBV antigen and SARS antibody on P(3HB) coated substrate. (A) Schematic diagram of immunodiagnostics on the P(3HB) chip. (B) Detection of FITC-conjugated HBV preS2 surface antigen at varying concentrations (1/8, 1.35 ng/mL; 1/4, 2.7 ng/mL; 1/2, 5.4 ng/mL; 1X, 10.8 ng/mL). (C) Detection of Cy5-labeled polyclonal antibody against SARS-CoVe at varying concentrations (1/8, 0.3 ng/mL; 1/4, 0.6 ng/mL; 1/2, 1.2 ng/mL; 1X, 2.4 ng/mL). The negative control (ctrl) contains *E. coli* cell lysates in PBS buffer. (D) and (E) Fluorescence intensity profiles of the spots shown in (B) and (C), respectively.

spots were determined with the ScanArray 5000 system (GSI-Lumonics). As is shown in Figs. 2B and C, the 6His-ScFv-SBD and 6His-SCVe-SBD fusion proteins immobilized onto the P(3HB) chips were specifically recognized by its specific antigen and antibody, respectively. No fluorescent signals were detected in the negative control spots containing the *E. coli* cell lysates. The fluorescence intensities, measured with Scion image software, are shown in Figs. 2D and E. It is, again, worthy of note that the 6His-ScFv-SBD and 6His-SCVe-SBD fusion proteins were detectable and measurable even at concentrations as low as 2.7 and 0.6 ng/mL, which indicates the high degree of sensitivity inherent to this system.

In this work, we have developed and described a novel strategy for protein immobilization on a P(3HB) microarray, a highly selective and sensitive substrate. Our system is predicated on the selective binding occurring between SBD and the P(3HB) surface, which allows for the specific immobilization of proteins fused to the SBD on the surfaces of the P(3HB) chips. The fusion proteins can be readily generated via the cultivation of the recombinant bacteria harboring the SBD fusion gene-expressing plasmids. Additionally, P(3HB) can simply be spin-coated on slide glasses, and require no special chemical modifications for the protein immobilization. In summary, this new protein microarray system, which employs SBD-fusion proteins immobilized onto P(3HB)-coated surfaces,

should prove effective in a variety of immunodiagnostic assay protocols, and may also possibly be used in the study of other protein-protein and protein-peptide interactions.

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