Micropatterning Proteins on Polyhydroxyalkanoate Substrates by Using the Substrate Binding Domain as a Fusion Partner

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Abstract: A novel strategy for micropatterning proteins on the surface of polyhydroxyalkanoate (PHA) biopolymer by microcontact printing (μCP) is described. The substrate binding domain (SBD) of the Pseudomonas stutzeri PHA depolymerase was used as a fusion partner for specifically immobilizing proteins on PHA substrate. Enhanced green fluorescent protein (EGFP) and red fluorescent protein (RFP) fused to the SBD could be specifically immobilized on the micropatterns of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). Laser scanning confocal microscopic studies suggested that two fusion proteins were micropatterned in their functionally active forms. Also, antibody binding assay by surface plasmon resonance suggested that protein–protein interaction studies could be carried out using this system. © 2005 Wiley Periodicals, Inc.

Keywords: polyhydroxyalkanoates; PHA; substrate binding domain; microcontact printing; surface plasmon resonance

INTRODUCTION

Immobilization of biomolecules onto the solid surfaces has become increasingly important for the development of biosensors and biomolecular microarrays (Frey et al., 2003; Herbert et al., 1997; Hodneland et al., 2002; Kane et al., 1999; Shirahata et al., 2003; Wilson and Nock, 2002, 2003; Zhu and Snyder, 2003). Several strategies including microcontact printing (μCP) and photolithography have been developed to immobilize proteins and peptides on the glass, polymer, and metal substrates (Lee et al., 2003). μCP has an advantage over conventional methods in that it requires no harsh chemicals, making it suitable for patterning biological molecules (Shirahata et al., 2003; Lee et al., 2004). Some enzymes contain specific domains enabling their binding to biopolymers. For example, the cellulose binding domain allows specific binding of cellulase to the surface of cellulose. Unfortunately, cellulose is a fibrous material and unsuitable for manufacturing solid chips. In this aspect, polyhydroxyalkanoates (PHAs) are attractive.

PHAs are polyesters of various (R)-hydroxycarboxylic acids that are intracellularly accumulated as carbon and energy storage material in numerous microorganisms (Lee, 1996). PHAs are completely biodegradable and can exhibit thermoplastic or elastomeric properties depending on the monomer composition (Jendrossek and Hardrick, 2002; Kasuya et al., 1999; Lee, 1996; Ohura et al., 1999; Yamashita et al., 2001). Even though PHAs are hydrophobic and water-insoluble, they can be degraded by PHA depolymerases secreted by many microorganisms (Jendrossek and Hardrick, 2002). PHA depolymerase has a substrate-binding domain (SBD) at the C-terminus, and uses it to bind to the surface...
of PHAs by recognizing the hydrophobic side chains and chemically interacting with it (Jendrossek and Handrick, 2002; Kasuya et al., 1999; Ohura et al., 1999; Yamashita et al., 2001). Among the four important amino acid residues (serine, histidine, arginine, and cysteine) in the SBD, positively charged histidine and arginine residues have been suggested to electrostatically interact with carbonyl groups of polyesters with high specificity (Jendrossek and Handrick, 2002).

In this study, we describe the first example of specifically patterning proteins on PHA surface by μCP. Our method is based on the site-selective binding of the SBD to PHA, which allows micropatterning of proteins fused to the SBD on PHA thin film as shown in Figure 1.

**MATERIALS AND METHODS**

**Materials**

(Tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane was purchased from Gelest (Morrisville, PA). Rabbit anti-GFP polyclonal antibody was obtained from Molecular Probes (Eugene, OR). Polyvinyl alcohol (PVA, Mw: 22,000, 88% hydrolyzed) was purchased from ACROS Organics (Pittsburgh, PA). Poly(3-hydroxybutyrate), PHB, and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), PHBHH, were produced and purified as previously reported (Choi et al., 1998; Park et al., 2001).

**Cloning of the 6HEGFP-SBD and 6HRFP-SBD Fusion Genes**

DNA fragments encoding the SBD plus linker domain, enhanced green fluorescent protein (EGFP), and red fluorescent protein (RFP) were obtained by polymerase chain reaction (PCR) amplification using the genomic DNA of *Pseudomonas stutzeri* JCM 10168, and plasmids pEGFP and pDsxRed2-N1 (BD Biosciences Clontech, Palo Alto, CA) carrying the EGFP gene and the RFP gene, respectively, as templates. For the cloning and expression of the 6HEGFP-SBD and the 6HRFP-SBD fusion genes in *Escherichia coli*, PCR experiments were carried out using the primers listed in Tables I and II, respectively. PCR experiments were performed with a PCR Thermal Cycler T1 (BioMetra Co., Goettingen, 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). All DNA manipulations including restriction digestion, ligation, and agarose gel electrophoresis were carried out following the standard procedures (Sambrook and Russell, 2001). The DNA sequences of all clones were confirmed by sequencing with the automatic DNA sequencer (ABI Prism model 377, Perkin Elmer Co., Downer’s Grove, IL).

An NdeI restriction site, start codon and six histidine codon were introduced at the 5’ end of oligonucleotide primer 1 and used as the forward primer for amplifying the 6HEGFP-SBD

![Figure 1. Schematic illustration of specific immobilization of fusion proteins on the polyhydroxyalkanoate (PHA) surface by microcontact printing (μCP).](image-url)
and 6HRFP-SBD fusion genes. A BamHI restriction site and stop codon were introduced at the 5’ end of oligonucleotide primer 2, which was used as the reverse primer for amplifying the fusion genes. The 6HEGFP and SBD fragments were prepared by PCR using the primer combinations 1 plus 3 and 2 plus 4, respectively. The 6HEGFP-SBD gene was obtained by PCR using the above PCR products as templates and primers 1 and 2. The 6HRFP-SBD gene was obtained by PCR using P. stutzeri genomic DNA and pDsRed2-N1 as templates and primers listed in Table II. The 6HRFP and SBD fragments were obtained by PCR using primers 1 plus 4 and 2 plus 3, respectively. The 6HRFP-SBD gene was obtained by PCR using the above PCR products as templates and primers 1 and 2. The 6HEGFP-SBD and 6HRFP-SBD fusion gene PCR products were digested with NdeI and BamHI and ligated into the same sites of pET-22b(+) (Novagen, Darmstadt, Germany) to make pET-EGFP-SBD and pET-RFP-SBD, respectively. The SBD fragment corresponds to the C-terminal 218 amino acids of P. stutzeri PHB depolymerase.

Production of 6HEGFP-SBD and 6HRFP-SBD

The 6HEGFP-SBD and 6HRFP-SBD fusion proteins were produced by cultivation of E. coli BL21 (DE3) harboring pET-EGFP-SBD and pET-RFP-SBD, respectively. For the flask cultures, Luria-Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L) was used. Cells were cultivated in 250 mL flask containing 100 mL LB medium supplemented with ampicillin (Ap, 50 μg/mL) in a shaking incubator at 37°C and 200 rpm. Batch culture was carried out in a 5 L bioreactor under the same condition. At the OD600 of 0.4–0.6, isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma, St. Louis, MO) was added to a final concentration of 1 mM. Cells were further cultivated for 4 h and were harvested by centrifugation at 6,000 rpm for 10 min at 4°C. Cells were disrupted by sonication (Braun Ultrasonics Co., Danbury, CT) for 1 min at 40% output. After centrifugation at 12,000 rpm for 10 min at 4°C, the supernatant fluid (soluble protein fraction) was saved for the purification of fusion proteins. Ni-NTA column (Qiagen, Valencia, CA) was used for the purification of the fusion proteins.

Spin-Coating of Polymers

For the spin coating of PHB and PHBHH, glass substrate was modified by treating with octadecyltrichlorosilane (OTS). The OTS-modified glass substrate was spin-coated (3,000 ~ 4,000 rpm for 40 s) with PHAs in chloroform (0.1–0.5 wt%).

Microcontact Printing (μCP) of Fusion Proteins

Polydimethylsiloxane (PDMS) stamps were prepared according to the protocol reported previously (Bowden et al., 1999; Park et al., 2004) using Sylgard 184 silicone elastomer (Dow Corning Corp. Midland, MI). To cast the PDMS stamp, the master was covered in a Petri dish with PDMS oligomers. After curing for 6 h at 60°C, the PDMS stamp was peeled off from the master.

For the μCP of fusion proteins, PDMS stamp was sonicated in 50% ethanol for 10 min, rinsed with deionized water three times, and dried under argon stream as previously reported (Bowden et al., 1999; Park et al., 2004). After inking with fusion proteins (0.4 mg/mL in PBS buffer), the PDMS stamp was manually brought into contact with the PHA-coated glass substrate. The stamp was carefully removed after 10 min, and the glass substrate was washed three times with PBS buffer and deionized water under shaking condition. Fluorescence images were acquired using an LMS 510 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY). Fluorescence intensities of the images obtained by confocal microscopy were estimated by using Adobe Photoshop program (San Jose, CA).

Surface Plasmon Resonance (SPR) Analysis

All SPR measurements were performed with Biacore-X instrument (BIAcore, Uppsala, Sweden) and a commercial sensor chip (Pioneer J1, BIAcore) having an evaporated gold surface. The chip surface was first cleaned with 0.1 N NaOH containing 1% (v/v) Triton X-100 for 5 min. Fifty microliters of HEPES buffer was injected to the activated surface at 5 μL/min to saturate the gold chip. After washing, the flow rate was reduced to 1 μL/min and 50 μL of the 6HEGFP-SBD fusion proteins (0.4 mg/mL) were flown over the surface of the chip. Antibody solution (0.5 μg/mL) was injected at the indicated time. SPR response was measured in resonance units (RU).
RESULTS AND DISCUSSION

The domain structure of the *Pseudomonas stutzeri* (Ps) PHA depolymerase was analyzed (Fig. 1a). Then, two plasmid vectors were constructed for the production of two fluorescent proteins fused to the Ps SBD (Fig. 1b). A conventionally fabricated master was replicated by pouring liquid prepolymer of PDMS onto the master followed by curing. After releasing, the stamp serves as a vehicle to transfer the fusion proteins containing the SBD to PHA substrates upon contact (Fig. 1c). In detail, we constructed recombinant plasmids pET-EGFP-SBD and pET-RFP-SBD, which were pET-22b(+) harboring the EGFP-SBD fusion gene and the RFP-SBD fusion gene, respectively (Fig. 1b). For the efficient purification of the fusion proteins to be patterned, the six histidine (6His)-tag was introduced at the N-termini of the fusion genes (Fig. 1b). These plasmids were introduced into *E. coli* BL21 (DE3) for the production of two fusion proteins, 6HEGFP-SBD and 6HRFP-SBD. These fusion proteins could be simply purified using Ni-chelating resin with high purity.

For spin-casting of PHAs on the glass substrate, the glass substrate was modified with octadecyltrichlorosilane (OTS, CH$_3$(CH$_2$)$_{17}$SiCl$_3$) to improve the adhesion of PHA to the glass by hydrophobic interaction. This allowed formation of uniform PHA films on the glass substrate. Plasma-cleaned

![Figure 2. Confocal fluorescence images of 6HRFP-SBD (a and c) and 6HEGFP-SBD (b and d) fusion proteins patterned by microcontact printing on PHB-coated surface. The images are those obtained by the microcontact printing followed by washing with PBS buffer three times (a and b) and ten times (c and d). The graphs show the fluorescence intensities estimated. Bar represents 50 μm. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]](image-url)
glass substrate was immersed in toluene containing 10 mM OTS. After the formation of self-assembled monolayers (SAMs), the samples were rinsed with toluene and dried under the stream of argon. The OTS-modified glass substrate was coated with PHA by spin-casting of chloroform containing 0.1–0.5 wt% PHA. It was found that the best spin-coating of PHA could be achieved with 0.25 wt% PHA solution. Above 0.25 wt%, the formation of inhomogeneous multilayers with aggregates was observed. As a result, a clean and thin film of PHA was fabricated on the glass substrate, which was confirmed by FT-IR spectroscopy (data not shown).

Two members of PHAs were examined in this study: poly(3-hydroxybutyrate), PHB, which is the most frequently found member of PHAs, and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), PHBHH, which has more flexible material characteristics than PHB (Choi et al., 1998; Ohura et al., 1999; Park et al., 2001) as another substrate. The 6HEGFP-SBD fusion protein was patterned on the surface of PHB coated glass by μCP using the PDMS stamps that have relief features of 50 μm stripes and circles as shown in Figure 3. The patterns generated on PHBHH were much better than those generated on PHB, which suggests that this copolymer was a better substrate for protein micropatterning than PHB homopolymer. Taken together, the SBD of PHA depolymerase can be used as a fusion partner for specifically immobilizing and micropatterning proteins on PHA substrates.

Surface plasmon resonance (SPR) spectroscopy was then employed to further study specific immobilization of fusion protein and its interaction with antibody on the PHA surface. The 6HEGFP-SBD fusion protein was flown over PHBHH spin-coated SPR sensor chip to monitor its binding affinity. The immobilization of 6HEGFP-SBD fusion proteins resulted in an increase of SPR signal, indicating the strong binding of the fusion proteins on to the PHBHH film (Fig. 4, solid line). When 6HEGFP without the SBD was used as a control, it did not bind much to the PHBHH film (Fig. 4,

![Figure 3. Confocal fluorescence images of 6HEGFP-SBD fusion protein (0.4 mg/mL) micropatterned in stripes (a) and circles (b) on PHBHH-coated surface after washing with PBS buffer three times. Bar represents 50 μm. (Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.)](image)

![Figure 4. SPR data showing the binding of fusion proteins on the PHA-coated SPR sensor chips and subsequent washing followed by antibody binding. The flow rate in all experiments was 1 μL/min. The HEPES buffer (10 mM HEPES, pH 7.4, 150 mM NaCl) containing 0.005% (v/v) Tween 20 was used. Solid and dotted lines represent 6HEGFP-SBD fusion protein and 6HEGFP without SBD, respectively. The rabbit anti-GFP antibody was introduced as indicated by arrow.](image)
dotted line). To examine if protein–protein interaction studies can be carried out using this system, the rabbit anti-GFP antibody was flown over the sensor chip that has bound 6HEGFP-SBD. As can be seen from Figure 4 (solid line), anti-GFP antibody binding to 6HEGFP-SBD could be successfully detected. Again, anti-GFP antibody binding to the surface flown with 6HEGFP was negligible. Furthermore, washing was intentionally carried out for a long time (1 h) to demonstrate the good stability of fusion protein binding. These results suggest that the system developed in this study can be successfully used for examining protein–protein interactions.

We finally examined the long term stability of the micro-patterned fusion protein chip. Two 6HEGFP-SBD printed chips were manufactured as described earlier. One chip was examined by confocal fluorescence microscopy on the day of manufacturing, while the other chip was stored at 4°C for 1 week before examination. As can be seen from Figure 5, the 6HEGFP-SBD fusion proteins were stably bound at least for a week, thus proving the long term stability of the chip.

In summary, a novel strategy for protein micropatterning was developed by employing the SBD of PHA depolymerase as an anchoring motif for specific binding on to PHA coated glass substrate. The micropatterned SBD fusion proteins retained their biological activity (fluorescence in this case) and ability to interact with the antibodies. Since any proteins (or peptides) can be fused to the SBD motif, the strategy reported here should be generally useful in micropatterning proteins and peptides on PHA substrate, generating protein chips and peptide chips, and also in studying protein–protein and protein–peptide interactions.

References


