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Microcontact Printing of Biotin for Selective Immobilization of Streptavidin-fused Proteins and SPR Analysis

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Abstract In this study, a simple procedure is described for patterning biotin on a glass substrate and then selectively immobilizing proteins of interest onto the biotin-patterned surface. Microcontact printing (μCP) was used to generate the micropattern of biotin and to demonstrate the selective immobilization of proteins by using enhanced green fluorescent protein (EGFP) as a model protein, of which the C-terminus was fused to a core streptavidin (cSA) gene of *Streptomyces avidinii*. Confocal fluorescence microscopy was used to visualize the pattern of the immobilized protein (EGFP-cSA), and surface plasmon resonance was used to characterize biological activity of the immobilized EGFP-cSA. The results suggest that this strategy, which consists of a combination of μμCP and cSA-fused proteins, is an effective way for fabricating biologically active substrates that are suitable for a wide variety of applications, one such being the use in protein-protein assays.

Keywords: microcontact printing (μCP), pattern generation, protein-protein assay, surface plasmon resonance

INTRODUCTION

Immobilization of biomolecules onto solid surfaces has become increasingly important for the development of biosensors and biomolecular microarrays [1-5]. Numerous techniques have been developed to immobilize proteins and peptides onto silicon and glass substrates, but only a few procedures exist for selectively patterning biomolecules at specific sites of the substrates. Among them, microcontact printing (μCP), a soft lithographic technique, is an attractive method for generating two-dimensional patterns of biological molecules on solid substrates because of its simplicity and flexibility [6-7]. In the technique of μCP, an elastomer, which is typically poly(dimethylsiloxane) (PDMS), is cast against a microfabricated silicon or photoresist master, which has micrometer-sized features etched onto the surface. Curing the PDMS prepolymer and peeling the cured PDMS away from the master provides an elastomeric stamp, which presents a negative replica of the features on the master. Then, the stamp is inked with the biomolecule that is to be printed and pressed into contact with the substrate [6]. The inked biomolecule is transferred only from the protruding areas of the stamp that come into contact with the substrate, thereby generating spatially resolved patterns on the substrate. The μCP has intensively been used to generate patterns of self-assembled monolayers (SAMs) and has recently been applied to generate patterns of biomolecules and cells on SAMs, which were usually formed on gold or silicon substrates, through biospecific interactions [7]. Among the biospecific interactions, the streptavidin (SA)-biotin interaction is specifically known to be one of the strongest interactions between proteins and ligands. This model system is a useful tool for studying biomolecular interactions as it has been demonstrated in numerous experiments [8-12].

In this study, we present a simple approach for patterning biotin on modified glass substrates by μCP and also present a subsequent pattern generation of enhanced green fluorescent protein (EGFP) fused to SA. The results of monitoring antibody-EGFP interaction by surface plasmon resonance (SPR) analysis are also reported.

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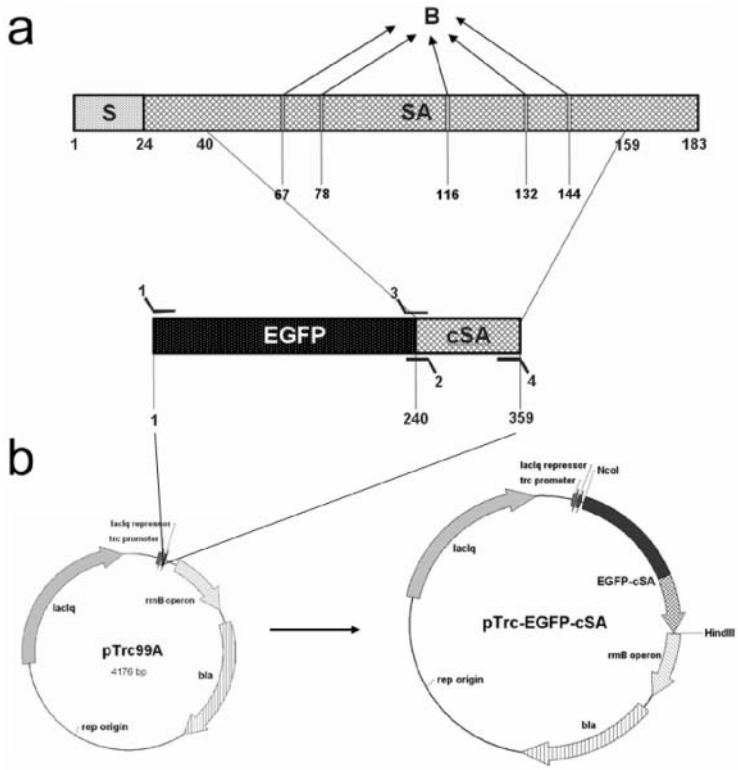

Fig. 1. (a) Domain structure scheme of SA and EGFP-cSA. The numbers refer to the positions of the amino acid sequence of the peptides. (S, signal peptide; B, biotin-binding region; SA, streptavidin; EGFP, enhanced green fluorescent protein; cSA, core streptavidin). (b) Construction of plasmid of pTrc-EGFP-cSA.

Table 1. Oligonucleotides that were used in this study

No.	Sequences (5' → 3')
Primer 1	CGAATT*CCATGG*TGAGCAAGGGC
Primer 2	CAGGTGCCGGTGATGCCCTTGTACAGCTCGTCCATG
Primer 3	CATGGACGAGCTGTACAAGGGCATCACCGGCACCTG
Primer 4	TAG*AAGCTT*GCTCAGCTTACGGCTTCACCTTGGTGA

[a] Restriction sites (italic) and/or the start/stop codon (underlined) were introduced in the oligonucleotides.

MATERIALS AND METHODS

Chemicals and Reagents

Rabbit anti-GFP polyclonal antibody was obtained from Molecular Probes, Inc. (Eugene, OR, USA). Pentafluorophenol (PFP), 11-mercaptoundecanoic acid, 1- ethyl-3-(dimethylamino) propylcarbodiimide (EDC), and 2-(2-aminoethoxy)ethanol (EG_2-amine) were purchased from Aldrich (St. Louis, MO, USA). 6-Hex-1-enyltrichlorosilane (HTS) and (+)-biotinyl-3,6,9-trioxaundecanediamine (biotin-amine) were purchased from Gelest, Inc. (Morrisville, PA, USA) and Pierce, Inc. (Rockford, IL, USA), respectively.

Cloning and Expression of EGFP-cSA Fusion Protein

A recombinant protein that was examined in this study was the enhanced green fluorescent protein (EGFP) from the jellyfish, *Aequorea victoria*. As shown in Fig. 1, DNA fragments encoding core streptavidin (cSA) and EGFP were obtained by polymerase chain reaction (PCR) amplification using the plasmid pEGFP (BD Biosciences Clontech, Palo Alto, CA, USA) that carried the EGFP gene and genomic DNA of *Streptomyces avidinii* as templates, respectively. For the cloning and expression of EGFP-cSA-fused genes in *Escherichia coli*, several PCR experiments were carried out using the primers listed in Table 1. A *Nco*I restriction site and start codon were introduced at the 5' end of oligonucleotide primer 1, while a *Hin*dIII restriction site and stop codon were introduced at the 5' end of oligonucleotide primer 4. The EGFP-cSA PCR product that was prepared using the primers 1 and 2, 3 and 4 were used as the template DNA. Finally, PCR was performed to obtain the EGFP-cSA fused gene using primers 1 and 4. In this way, the 120 amino acids from *S.*

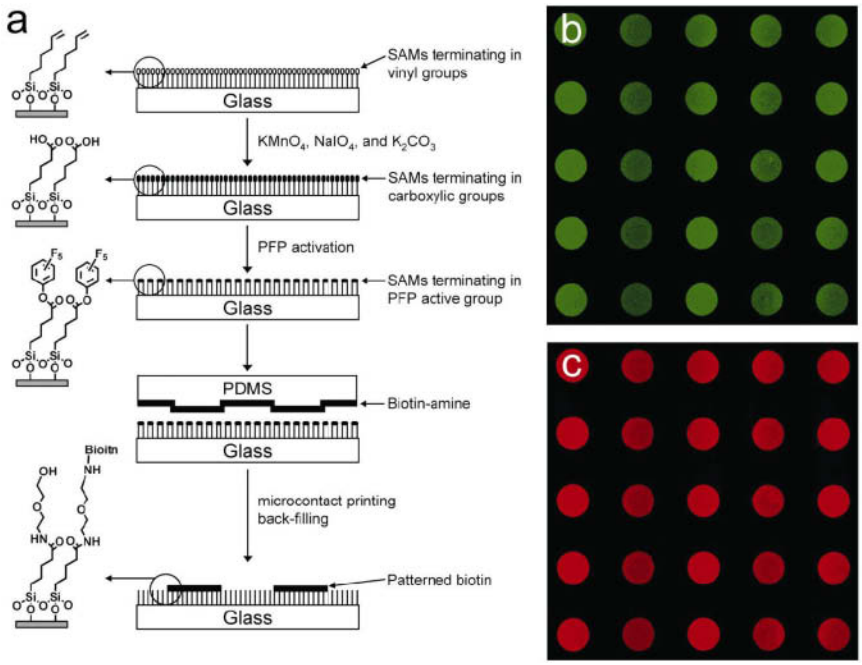

Fig. 2. (a) Schematic representation for patterning biological ligands. Confocal micrographs of EGFP-cSA (b) and Cy5-labeled anti-GFP antibody (c) bound to 50-μm micropatterns of biotin.

avidinii that contained 5 biotin-binding regions [13] were fused to the C-terminus of EGFP. The PCR product was digested with *Nco*I and *Hin*dIII, and it was ligated into the same sites in pTrc99A in order to construct the plasmid, pTrc-EGFP-cSA (Fig. 1b). PCR experiments were performed with the PCR Thermal MP TP 3000 (Takara Shuzo Co., Japan) using a high fidelity PCR System (Boehringer Mannheim, Mannheim, Germany). Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs (Berverly, MA, USA). All DNA manipulations were carried out according to the general molecular biological procedures [14]. The sequences of cloned genes were analyzed by an automatic DNA sequencer (ABI Prism model 377, Perkin Elmer Co., Connecticut, IL, USA).

Total Protein Assay

Total proteins were assayed in a 50 mM Tris-HCl buffer (pH 7.5) using the Bradford's method. Bovine serum albumin (BSA, Sigma) was used to generate a standard curve. All measurements were carried in microplates in triplicates. The absorbance was measured at 595 nm by using a microplate reader (Molecular Devices Co., Sunnyvale, CA, USA).

Surface Plasmon Resonance (SPR) Spectroscopy

A bare gold chip for the SPR biosensor (2 nm of chromium as an adhesion layer and 45 nm of gold deposited on an 18 × 18 × 0.3 mM glass) was obtained from K-MAC (Daejeon, Korea). SPR measurements were performed through the use of a cuvette-based AutoLab SPR instrument (Eco Chemie, KM Utrecht, Netherlands). The bare gold surface was cleaned with H_2SO_4/H_2O_2 (3/1, v/v) solution at 50°C for 30 min. For the biotin functionalization of the gold surface, the gold substrates were washed with absolute ethanol before the adsorption of alkanethiol. SAMs of 11-mercaptoundecanoic acid [15] were formed overnight in the 2 mM solution of ethanol/water/acetic acid (85/10/5, v/v/v), and the resulting gold substrate was rinsed with ethanol and was dried under a stream of argon. After the formation of the SAMs, the terminal carboxylic acid group was activated by immersing the substrate for 30 min in an ethanol solution that consisted of 0.1 M EDC and 0.2 M PFP [15]. Then, the PFP-activated gold substrate was immersed in a 0.1 M sodium bicarbonate solution containing 10 mM biotin-amine for 30 min and was rinsed with distilled water. The biotin-modified gold chip was attached to a half-cylinder prism with refractive-index matching oil (n_D=1.517) and was inserted into the AutoLab SPR instrument. A 50 μL sample was loaded onto the chip using a micropipette. After each step, the gold chip was washed and equilibrated with a 10 mM HEPES buffer that contained 0.15 M NaCl and 0.05% Tween 20 (pH 7.4). A mixing rate of 16.7 μL/sec with a mixing volume of 10 μL and a temperature of 25 °C were chosen for conditions of operation of the SPR system.

Microcontact Printing (μμCP) of Biotin-Amine

Fig. 2a shows a schematic representation of the formation of reactive SAMs of PFP ester groups on a glass substrate and the μCP-based pattern generation [16-17]. A freshly cleaned glass substrate was immersed in a solution of 2 mM HTS in hexane. After the formation of

SAMs, the substrate was rinsed with hexane and was dried with a stream of argon. The terminal vinyl groups were then oxidized to carboxylic acid groups by immersing the substrate for 24 h in an aqueous solution that consisted of 0.5 mM $KMnO_4$, 19.5 mM $NaIO_4$, and 1.8 mM K_2CO_3. The COOH-terminated substrate was placed in a freshly prepared solution of 0.1 M EDC and 0.2 M PFP for 30 min at room temperature, was rinsed with ethanol, and was dried with a stream of argon. The substrates that presented the PFP groups were used immediately. PDMS stamps were prepared according to the method previously reported [18] using Sylgard 184 silicone elastomer (Dow Corning, Midland, MI, USA). Before being used, the PDMS stamp was oxidized by an oxygen plasma cleaner (Harrick PDC-002, Broadway Ossining, NY, USA) at the medium setting for 1 min. After inking with 10 mM biotin-amine in ethanol, the PDMS stamp was made to be in contact with the PFP-activated glass substrate for 60 sec. Then, the substrate was immediately immersed for 30 min in a 0.1 M sodium bicarbonate solution containing 10 mM EG_2-amine [19-20]. The substrate was taken and rinsed with distilled water. For the protein immobilization studies, the biotin-patterned substrate was incubated for 1 h with the 0.1 mg/mL EGFP-cSA solution in PBS (pH 7.4) containing 0.1 % (w/v) BSA and 0.02 % (v/v) Tween 20. The substrate was rinsed several times with phosphate buffered saline (PBS). Fluorescence confocal microscopy was used to examine the EGFP-cSA-bound glass substrate (λ_{ex} = 488 nm, λ_{em} = 520 nm).

Image Analysis

Fluorescence images were obtained using an LMS 510 laser scanning confocal microscope (Carl Zeiss, Jena, Germany). Green fluorescence images were obtained through excitation with a 488-nm argon laser, and the images were filtered by a longpass 505-nm filter. A red fluorescence sample was excited by a 543-nm HeNe laser, and the images were filtered by a longpass 575-nm filter.

RESULTS AND DISCUSSION

EGFP-cSA Fusion Protein Production

E. coli XL1-Blue strain (Stratagene Cloning Systems, La Jolla, CA, USA) was used as the host strain for EGFP-cSA fusion protein expression. 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 a 250-mL flask containing 100 mL of LB medium that was supplemented with ampicillin (50 μg/mL) in a shaking incubator at 37°C and 200 rpm. At the OD_{600} of 0.4-0.6, isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma, St. Louis, MO, USA) was added to the final concentration of 1 mM. The cells were further cultivated for 4 hrs and harvested by centrifugation at 6,000 rpm for 10 min at 4°C. The cells were disrupted by sonication (Braun Ultrasonics Co, Danbury, CT, USA), and the supernatant fraction containing soluble EGFP-cSA fusion proteins was used in other processes.

Microcontact Printing

To achieve the covalent attachment of amine-terminated ligands (*e.g.*, biotin-amine) onto glass substrates, we used a method of common reactive intermediates, called PFP esters [15] (Fig. 2a). The PFP ester groups were introduced on the surface, and the activated surface was allowed to react with the amine group of the biotin ligand by μCP because the PFP ester groups were easily coupled with amines, leading to the formation of amide bonds. The PFP-activated surfaces were brought into conformal contact with an oxidized PDMS stamp that presented micrometer-sized relief features, which had been inked with biotin-amine.

After patterning the biotin ligand on the glass surface and passivating the other area with EG_2-amime, the substrate was incubated with the EGFP-cSA fusion protein in PBS (pH 7.4) for 1 h at room temperature. Confocal fluorescence microscopy was used to examine the pattern formation of EGFP-cSA that was immobilized onto the biotin micropatterns (Fig. 2b). Fig. 2c shows the formation of spatially resolved patterns of fluorescent Cy5-labeled anti-GFP antibody, which was bound to the EGFP-cSA. In the control experiment, where an unmodified glass substrate was printed with biotin-amine, was washed with ethanol and buffer, and was successively incubated with EGFP-cSA fusion protein and Cy5-labeled anti-GFP antibody, no micropatterns could be observed (result not shown). These results clearly showed that the interaction between the EGFP-cSA fusion protein and the micro-patterned surface was mediated by the biotin. This strategy of immobilizing the cSA fusion protein onto the biotin-patterned solid surface can be applied to immobilizing any pair of capture protein and ligand.

SPR Study on Binding Property of the Immobilized EGFP-cSA Fusion Protein

In order to demonstrate the selective immobilization of EGFP-cSA onto the biotin- functionalized chip, both the specific binding of EGFP-cSA onto the sensor chip and its subsequent interaction with the antibody were directly monitored by SPR. EGFP-cSA was flown over the biotin-functionalized sensor chip. Specific binding led to an increased SPR-signal over time as shown in Fig. 3 (curve 1).

To examine whether the immobilized EGFP-cSA fusion protein could interact with the antibody, the anti-GFP antibody was flown over the EGFP-cSA derivatized sensor chip. Upon introducing 0.4 mg/mL of the antibody, the SPR signal increased over time (Fig. 3, curve 1), while no binding of the antibody was observed on the biotin-functionalized sensor chips that lacked the attached EGFP-cSA. These results showed that the immobilized EGFP-cSA was able to maintain biological functionality and was also able to interact with the antibody. No signal was observed when the extracts of *E. coli* expressing only EGFP without the fusion of cSA were

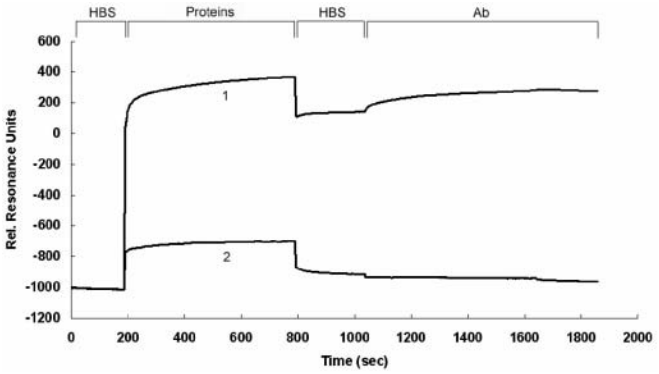

Fig. 3. SPR data for immobilized EGFP-cSA and its subsequent interaction with anti-GFP antibody. (curve 1) EGFP-cSA (0.4 mg/mL) was immobilized onto the biotin-functionalized sensor chip. (curve 2) EGFP (0.4 mg/mL) was directly immobilized onto the same chip as a negative control. The flow rate in all experiments was 1 μL/min. Abbreviations are: HBS, [10 mM HEPES (pH 7.4), 150 mM NaCl, 0.005% Tween 20]; Proteins, EGFP-cSA or EGFP; Ab, anti-GFP antibody (0.4 mg/mL).

tested (Fig. 3, curve 2). All together, the strategy described in this paper should be useful in studying protein-protein interactions in general. This examples establishes an efficient strategy for protein immobilization that shares the advantages that binding of capture protein to the immobilized capture ligand is selective, reaction of the capture ligand gives a covalent immobilization, and the recombinant expression of the capture protein avoid the need for synthetic modification and other purification of proteins.

CONCLUSION

The methods described in this study can be considered as a general process for fabricating biotin-functionalized substrates for the selective immobilization of cSA-fused proteins and its possible application for studying protein-protein interactions through the SPR method. The binding of cSA-fused proteins to the surface is very selective, and the fusion protein remains functional. Since many different proteins (or peptides) can be fused to the cSA moiety, the strategy should be useful in various applications that involve biomolecule-protein (peptide) interactions.

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