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Identification and characterization of peptide fragments for the direct and site-specific immobilization of functional proteins onto the surface of silicon nitride

Yoichi Kumada[a,*], Takeru Ootsuka[b], Masashi Asada[a], Saori Yoshizuka[c], Masateru Chiyama[c], Masayasu Sakane[c], Hasan M.D. Fida[d], Kazuaki Sawada[d], Koichi Okumura[d], Michimasa Kishimoto[a]

[a] Department of Biomolecular Engineering, Kyoto Institute of Technology, Kyoto, Japan
[b] Department of Chemistry and Materials Technology, Kyoto Institute of Technology, Kyoto, Japan
[c] Nissha Printing Co. Ltd., Kyoto, Japan
[d] Department of Electrical and Electronic Information Engineering, Toyohashi University of Technology, Aichi, Japan

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ABSTRACT

In this study, we successfully identified peptide fragments that have a strong affinity toward the surface of a silicon nitride (SiN) substrate. An *E. coli* soluble protein, which was preferentially adsorbed onto the surface of a SiN substrate was isolated by 2D electrophoresis, and it was identified as "elongation factor Tu (ELN)" via the peptide MS fingerprinting method. A recombinant ELN that was originally cloned and produced, also maintained its adsorptive ability to a SiN substrate, by comparison with BSA that was used as a control protein. The peptide fragments derived from the recombinant ELN were prepared via 3 types of proteases with different recognition properties (trypsin, chymotrypsin and V8 protease). The peptide mixture was applied to the surface of a SiN substrate, and then, the SiN-binding peptide candidates were isolated and identified. The amino acid sequences of the peptide candidates were genetically fused with the C-terminal region of glutathione S-transferase as a model protein, and the adsorption properties of mutant-type GSTs on the surface of a SiN substrate were directly monitored using a reflectometric interference spectroscopy (RIfS) sensor system. Consequently, among the 8 candidates identified, the genetic fusion of TP14, V821 and CT22 peptides resulted in a significant enhancement of GST adsorption to the surface of the SiN substrate, while the adsorption of a wild-type GST was hardly detectable by RIfS sensor. These peptide fragments were located at the C-terminal region in the aminoacid sequence of recombinant ELN. Interestingly, the sequence with the shortest and strongest SiN-binding peptide, TP14 (GYRPQFYFR), was also found in that of V821 (GGRHTPFFKGYRPQFYFRTTDVTGTIE). The TP14 peptide might be the smallest unit of SiN-binding peptide, and a clarification of the amino acid contribution in TP14 peptide will be the next subject. Three-fold higher enzymatic activities were detected from the SiN substrate immobilized with GST-TP14 and GST-V821 due to a higher density of enzyme through the SiN-binding peptides.

Thus, the SiN-binding peptides identified in this study will be considerably useful for the immobilization of target proteins with high density and biological activity onto the surfaces of SiN substrates, and these will be applicable to the task of coating proteins onto the surface of SiN-based RIfS sensors and semiconductors.

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1. Introduction

Silicon nitride (SiN or Si_3N_4), which has high mechanical strength and chemical resistance, is a basic material used in the manufacture of semiconductors. Also, the surface of SiN is well known as a pH-responsive semiconductor, and thus, in recent years, SiN-based ion sensors have been developed for the ultra-sensitive

* Corresponding author. Tel.: +81 75 724 7542; fax: +81 75 724 7542.
E-mail addresses: kumada@kit.ac.jp, kumada@chem.kit.ac.jp (Y. Kumada).

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detection of ion changes in biomolecular interactions such as DNA–DNA and antigen-antibody (Bergveld, 1970; Besselink et al., 2003; Maruyama et al., 2009; Selvanayagam et al., 2002; Tsuruta et al., 1994). Especially, development of antibody-immobilized semi-conductor sensors such as ISFET for label-free detection of serum biomarkers is one of important subjects in the biochemical and electrical research fields (Hideshima et al., 2011, 2012). Therefore, technologies for denser and site-directed immobilization of functional proteins such as antibodies and enzymes onto the surface of SiN substrate have been requisite.

On the other hand, reflectometric interference spectroscopy (RIfS) is based on the spectral distribution of white-light reflectance from transparent thin layers wherein distinct alternating maxima and minima reflectance patterns result from the interference of beams that are partially reflected from each interface of the interference layer. (Birkert et al., 2000; Jung et al., 2001; Piehler et al., 1996, 2000) SiN-coated silicon (Si) substrate has also been utilized as a sensor chip of RIfS sensors. (Choi et al., 2012) In RIfS, wavelength shift ($\Delta\lambda$) can be detected label-free and with high sensitivity according to the direct adsorption of biomolecules onto the SiN surface as well as to the indirect association of a biomolecule with a ligand immobilized on the surface of a sensor chip.

Therefore, the development of technologies for the site-specific immobilization of biomolecules, particularly proteins, onto the surface of a SiN substrate may be a considerably important subject for the enhancement of sensitivity in these biosensor systems. Choi et al. prepared a grafted carboxymethyl (CM) dextran layer for the indirect immobilization of anti-CRP antibodies via protein A (Choi et al., 2012). However, it is often difficult to control the uniform thickness of a dextran layer, as well as the dissociation of a ligand antibody from the protein A-coupled CM layer, because the dissociation of the antibody from a protein A-coupled layer is too great when a RIfS sensor must be used long-term. Therefore, methodologies that will allow both direct and site-specific immobilization of target proteins are necessary. They may enhance detection sensitivity on above label-free sensors significantly, and make it possible to simplify the immobilization steps of ligand proteins.

This research group has developed protein immobilization technologies utilizing material-binding peptides with the ability to recognize the surfaces of target materials with high-binding affinity. So far, affinity peptide tags binding to hydrophilic polystyrene (phi-PS), as well as bare polycarbonate (PC) and poly (methyl methacrylate) (PMMA) have been identified. In particular, polystyrene-binding peptides (PS-tag) have been identified via biopanning utilizing a random peptide-displayed *E. coli* library, while PC- and PMMA-binding peptides were identified from the amino acid sequences of *E. coli* soluble proteins that were preferentially adsorbed onto the surface of target substances (Kumada et al., 2006, 2012a) Genetic fusion of these binding peptides to target proteins such as glutathione S-transferase, single-chain variable fragments (scFvs) etc., have improved the immobilization density and the maintenance of high biological activity in the adsorption state, due to the site-specific attachment of material-binding peptides, and an avoidance of the direct adsorption of target proteins (Kogot et al., 2012; Kumada et al., 2009b, 2010a; Tang et al., 2013). In particular, the genetic fusion of a PS-tag to scFvs resulted in an increase in sensitivity for the detection of biomarkers in a sandwich ELISA (Kumada et al., 2009a, 2010b, 2011, 2012b, 2013).

The aim of the present study was the isolation and characterization of SiN-binding peptides (SiN-tags) for the site-specific immobilization of recombinant proteins onto the surface of a SiN substrate. We investigated the isolation of SiN-binding peptide fragments from an *E. coli* soluble protein that was preferentially adsorbed onto the surface of a SiN substrate. Candidates of SiN-binding peptides (SiN-tag) were genetically fused with the C-terminus of glutathione S-transferase (GST), and the adsorption characteristics of GSTs to the surface of a SiN substrate were directly evaluated using a RIfS sensor.

2. Materials and methods

2.1. Materials

Silicon nitride was coated onto the surface of a silicon substrate (sized 4) using a low-pressure thermal chemical vapor deposition (LPCVD) method (Riley, 2000). Then the substrate was cut finely (approximately 0.5 cm square pieces) to use in the screening of SiN-binding peptide candidates. The Reflective Interference Spectroscopy (RIfS) sensor that was used was equipped with SiN-coated Si sensor chip and was originally manufactured by NISSHA printing co. Ltd. Bugbuster™ was purchased from Merck for the lysis of an *E. coli* cell membrane to extract protein from the cells. Modified trypsin (Promega), chymotrypsin (Promega) and V8 protease (Roche) were used for the preparation of peptide fragments from SiN-binding proteins. A GSTrap HP column was purchased from GE Healthcare Bioscience (USA) and was used to purify the glutathione S-transferase (GST), as well as its fusion protein with SiN-tag candidates. TSKgel ODS-100V was purchased from TOSOH and used for the separation of a peptide mixture digested from SiN-binding protein. Other chemicals were of reagent grade, unless otherwise specified.

2.2. Preparation of E. coli soluble protein mixture

E. coli soluble protein mixture was prepared by the following method and was used as a sample for the isolation and identification of SiN-binding protein. Briefly, *E. coli* W3110 was inoculated to 10 ml of LB medium in a 200 ml shake flask. The flask was shaken at 200 rpm and 37 °C overnight. Then, an aliquot of the culture was transferred to 50 ml of LB medium in 500 ml shake flask for a final DO_{600} of 0.1. The flask was shaken at 200 rpm and 37 °C for 7 h. The cells were harvested by centrifugation at 4500 rpm for 15 min. After removal of the supernatant, 5 ml of bugbuster™ containing benzonase nuclease (Merck) and protease inhibitor cocktail (Nacalai Tesque) was added to disrupt the *E. coli* cell membrane and extract protein from the cells. The mixture was incubated at 37 °C for 1 h followed by centrifugation at 10000 rpm and 4 °C for 20 min. The supernatant was collected as a protein sample for the screening of SiN-binding protein.

2.3. Screening and identification of SiN-binding protein

SiN substrate chips with a total surface area of 118 cm^2 were mixed with 5 ml of *E. coli* soluble protein mixture and were incubated with gentle shaking at 25 °C for 1 h. The SiN chips were washed 3 times with 40 ml of PBS, and further washed 3 times with 40 ml of milli-Q water. After removal of the milli-Q water by aspiration, the adsorbed proteins were eluted by the addition of 4 ml of solubilization buffer containing 8 M urea, 4% CHAPS, 2 (v/v)% Pharmalyte and 1% DTT. The mixture was shaken at 130 rpm and 25 °C for 1 h. The supernatant was collected as a SiN-binding protein fraction and passed through a syringe filter with a pore size of 0.45 μm. The adsorption and extraction procedures described above were performed 3 times and all extracted solutions were mixed. Then, the solution was dialyzed against milli-Q water overnight followed by lyophilization. A protein pellet was dissolved with 150 μl of solubilization buffer and was used as a sample for two-dimensional electrophoresis (2DE). Peptide-mass fingerprinting and 2DE were performed by the same method reported previously for the separation and identification of proteins that had preferentially adsorbed onto the surface of SiN substrate (Kumada et al., 2012a).

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