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Short communication

Construction of a more sensitive fluorescence sensing material for the detection of vascular endothelial growth factor, a biomarker for angiogenesis, prepared by combining a fluorescent peptide and a nanopillar substrate

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ABSTRACT

We have established a highly sensitive and selective protein detection technology in combination with the nanofabrication technique. A silica nanopillar chip with a 200-nm pitch and 1000-nm height pillar substrate was fabricated by electron beam lithography and deep reactive ion etching method. Fluorescent peptides, with high affinity towards vascular endothelial growth factor (VEGF), were immobilized on nanopillar chip via a self-assembled monolayer made from 3-aminopropyltrimethoxysilane and glutaraldehyde under optimal conditions. The fluorescence intensity of the fluorescent peptide on the nanopillar substrate increased with increasing VEGF concentrations, as determined by a fluorescence spectrophotometer and fluorescent scanning image analysis. The dissociation constant (K_d value) calculated by the non-linear least square curve fitting method was 6.0×10^{-9} M, which contributed to the highly sensitive detection of VEGF. The fluorescence intensity of the fluorescent reagent on the nanopillar substrate upon binding to VEGF was higher than that obtained using the flat substrate because the dense and tall nanopillar array increased the virtual protein binding area. The reproducibility tests and lifetime measurement indicate the fluorescent reagent to be a useful biosensor for the detection of VEGF in this system. These experimental results clearly showed that the combination of a fluorescent reagent and a nanopillar substrate may be widely applicable as a convenient method for the detection of VEGF.

1. Introduction

Angiogenesis is related to the proliferation and metastasis of cancer cells, and recently, studies have been conducted on the molecular mechanisms underlying angiogenesis (Brahimi-Horn and Pouyssegur, 2007; Ghosh et al., 2008). Vascular endothelial growth factor (VEGF) is an important regulator of angiogenesis, and it promotes the migration and proliferation of endothelial cells and the formation of new blood vessels from preexisting capillaries (Ferrara et al., 2003; Ferrara and Kerbel, 2005). Biological responses to VEGF expression result from the binding of VEGF to 2 membrane-embedded receptors and the subsequent intracellular signaling induced by receptor activation (D'Andrea et al., 2006). Therefore, it is important to develop technology for easy, rapid, and highly sensitive detection of VEGF.

In our previous study, we developed an excellent fluorometric reagent (compound 1) for the detection of VEGF (Suzuki and Yokoyama, 2009). The chemical structure of compound 1 is shown in Fig. 1. This reagent contains a peptide as the binding site for VEGF, and the amino acid sequence of the peptide ensures that it has a high affinity for the tyrosine kinase receptor — kinase insert domain-containing receptor (KDR) — and for the fms-like tyrosine kinase-1 (Flt-1) receptor of VEGF (Fairbrother et al., 1998; Millauer et al., 1993; Terman et al., 1992). On binding to VEGF, the fluorescence quantum yields responded linearly to the amount of VEGF in the solutions and on the gold substrate. The enhancement of fluorescence intensity occurred due to the formation of a complex in which the fluorophore in compound 1 bound to the hydrophobic residues in VEGF. The difference between the ground and excited states is influenced by factors in the external environment of the fluorophore, such as solvent polarity and desolvation. As a result, there was a drastic linear change in the fluorescence intensity in response to the amount of VEGF in the solution.

However, the VEGF sensitivity of compound 1 immobilized on the Au substrate was poor because the amount of reagent immobilized on the substrate was insufficient for VEGF detection.

Recently, studies have been conducted on the possible use of a sensor fabricated using the microelectromechanical system (MEMS) technology in compact analytical systems (Lee et al., 2008;

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Fig. 1. Chemical structure of the fluorescent VEGF probe (compound 1).

Shukla et al., 2008). In MEMS-based industries, substantial research has been conducted to develop nanofabrication techniques; to date, these techniques have mainly used silicon substrates. These well-developed techniques can be directly used in the fabrication of micro- and nano-fluidic devices for chemical and biological applications. A typical nanofabrication process consists of electron beam lithography (EBL) and deep reactive ion etching (DRIE); it is used to create nanofabricated structures such as nanopillar structures for biological applications. As typical and successful examples of bioanalysis using nanopillar structure, DNA separation technologies combined with nanofabrication technologies identified a breakthrough in genotyping and DNA sequencing. The size of the pillars and the spacing between pillars were designed as a DNA sieving matrix for analysis of large DNA fragments over 10 kb pairs (Austin, 2007; Baba et al., 2003; Bakajin et al., 2001; Huang et al., 2002, 2004; Kaji et al., 2004; Volkmuth et al., 1992).

In this paper, we propose a protein detection technique that combines fluorescent molecular probes with MEMS fabrication technologies. We have effectively immobilized fluorescent reagents on a nanopillar substrate to overcome the limitation of poor VEGF detection sensitivity of the fluorescent peptide immobilized on the substrate while maintaining excellent photophysical and chemical properties. We successfully employed our method to recognize VEGF in rat serum with high sensitivity and high reproducibility, thus enabling us to measure trace levels of a disease marker protein owing to signal amplification by fluorescence enhancement with a low noise level.

2. Materials and methods

2.1. Reagents

All the chemicals used were of analytical reagent grade and were purchased from TCI (Tokyo, Japan), Wako (Osaka, Japan), Dojindo (Kumamoto, Japan), Pierce (USA), and Aldrich (USA). Recombinant human VEGF121 was purchased from Peprotech (UK), whose purity was greater than 98% by SDS–PAGE gel and high performance liquid chromatography (HPLC) analyses.

2.2. Fluorescent peptide synthesis

The fluorescent peptides designed by our group were synthesized by Scrum Inc. (Tokyo, Japan). The molecular weight and purity of the peptides were 2965.49 and >95%, respectively, which were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and HPLC. The data from the above analyses are shown in the supplementary material.

2.3. Nanopillar substrate fabrication

The silica nanopillar structure was fabricated with a silicon wafer using nanolithographical techniques (Kan et al., 2009). EBL was conducted on the silicon wafer, and the silicon surface was etched by inductively coupled plasma reactive ion etching (ICP-RIE). The silicon nanopillar array was thermally oxidized in a furnace for 2 h at 1000 °C so that the silicon in the pillars was completely converted to silica.

2.4. Fluorescent peptide immobilization on a nanopillar substrate

The nanopillar substrate was immersed in a diluted alkaline detergent overnight, washed well with distilled water, and dried. The cleaned nanopillar substrate was immersed in a 5% toluene solution of 3-aminopropyltrimethoxysilane for 5 min. After the silane coupling reaction, the substrate was rinsed 5 times in EtOH with sonication for 5 min, and 2 times in distilled water for 5 min. The silanized nanopillar substrate was immersed in a 5% glutaraldehyde solution and stirred for 1 h. After washing with distilled water, the nanopillar plate coated with formyl groups was immersed in a 10% fluorescent peptide solution for 2 h and then washed with distilled water.

2.5. Miscellaneous

The fluorescence spectra were recorded at 25 °C with a JASCO FP-6500 spectrofluorophotometer. The image acquisition and analysis of fluorescent images were performed before and after washing the gels by using an LS400 scanner (Tecan, USA).

3. Results and discussion

A surface electron microscopy (SEM) photograph of a nanopillar is shown in Fig. 2(a). The silicon-wafer-based nanopillar was prepared by EBL and ICP-RIE. The silicon nanopillar array was thermally oxidized in a furnace for 2 h at 1000 °C to convert the silicon in the pillars to silica. The pitch and height of the nanopillar array were 200 and 1000 nm, respectively. The surface area of this structure was calculated on the basis of SEM data and was found to be approximately 6-fold greater than that of the flat substrate.

Compound 1 was immobilized onto a nanopillar substrate coated with self-assembled monolayers (SAMs). The SAMs on the nanopillar substrate were derived by the silane coupling reaction between 3-aminopropyltrimethoxysilane and the surface of the nanopillar substrate. Amino groups on the SAMs reacted with glutaraldehyde. Compound 1 was immobilized onto the SAMs by bond formation between the lysine residue in compound 1 and the formyl group in the SAM, and this was mediated by a Schiff base reaction. To identify the optimum experimental conditions for immobilization of compound 1 onto the nanopillar substrate via the SAMs, the response was investigated by changing the concentration of compound 1, 3-aminopropyltrimethoxy silane, glutaraldehyde, and the reaction time. The processing solutions, which were toluene solution, containing concentrations of 3-aminopropyltrimethoxysilane ranging from 0% to 2.0% and glutaraldehyde (0–20%), were prepared, and their reactions were carried out by changing the reaction time (silane coupling reac-

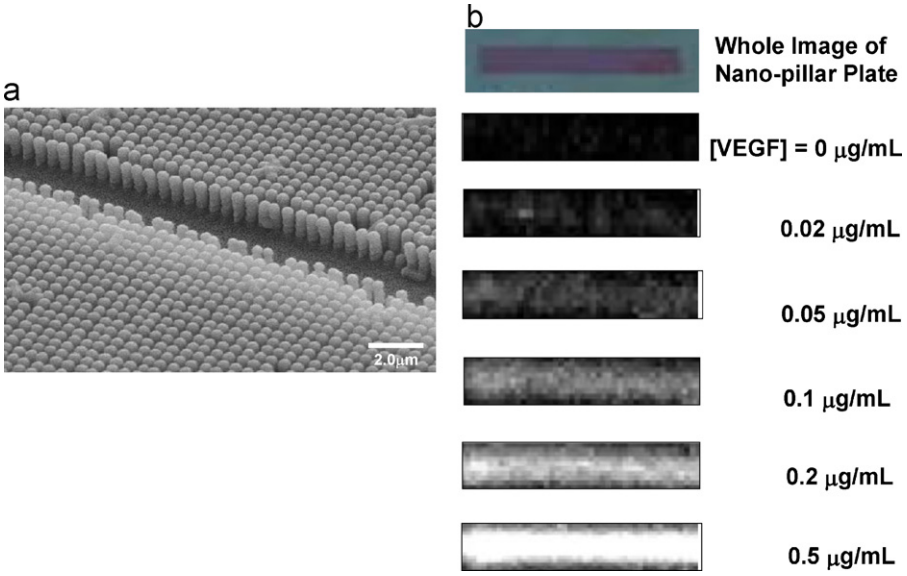

Fig. 2. SEM image of nanopillar structures (a) and fluorescent images of the nanopillar substrate before and after addition of VEGF (b).

tion: 0, 5, 10, and 20 min; glutaraldehyde reaction: 0, 30, 60, 120, and 300 min). The response of the immobilized nanopillar substrate for 1.0 μg/mL of VEGF was measured after the immobilization of compound 1, whose fixed concentration and reaction time were 10% and 2 h, respectively. As a result, the response linearly increased as a function of reagent concentration and reaction time, with saturation at 5.0% 3-aminopropyltrimethoxysilane (5 min) and 5.0% glutaraldehyde (60 min). Therefore, the silane coupling and glutaraldehyde reactions were carried out under the above experimental conditions.

To examine the dependence of the concentration of compound 1 and its immobilization reaction time, nanopillar substrates made by immersion in processing solution (3-aminopropyltrimethoxysilane 5.0%, glutaraldehyde 5.0%) were prepared and then immersed in various concentrations (0–50%) of compound 1 with the change of the reaction time from 0 to 300 min. After reaction with 1.0 μg/mL of VEGF, fluorescence intensity of compound 1 was monitored, and we observed that the response for VEGF slightly increased with the increase in concentration and reaction time. With over 10% of compound 1 and a reaction time of 120 min, fluorescence intensity was saturated. To take advantage of this phenomenon, immobilization of compound 1 on a nanopillar plate was carried out under the above optimum experimental conditions.

To study the *in vitro* photophysical properties of compound 1 immobilized on the nanopillar substrate, we recorded its fluorescence intensity in rat serum at 25 °C by using an image analysis system. Various concentrations of VEGF solution in rat serum (200 μL) were added to the fluorescent peptides immobilized on the nanopillar substrate, and were then left standing for 5 min. After briefly washing the substrate, it was scanned using image analysis systems. The typical fluorescent images of the nanopillar substrate before and after the reaction between the immobilized fluorescent peptides and VEGF in rat serum are shown in Fig. 2(b). Compound 1 itself exhibited very weak emission, whereas the compound 1-VEGF complex exhibited a strong green fluorescence emission, and there was a drastic increase in the fluorescence intensity centered at approximately 525 nm.

On staining with compound 1, it was observed that the integrated volume of the densitometry units for the scanned bands of the proteins in the gel showed a good linear relationship with the VEGF concentration from 0 to 0.05 μg/mL ($r^2 > 0.996$), as shown in Fig. 3. The K_d value was 6.0×10^{-9} M, calculated by the non-linear least square curve fitting method. This numerical value contributes to the highly sensitive detection of VEGF and allows for the search of angiogenesis biomarkers. To examine the reproducibility of the calibration graph, 3 reproducibility tests were performed, and the coefficient of variation (CV) value was within 3.8%.

To investigate whether the nanopillar substrate contributes to the sensitivity of VEGF detection, a flat substrate prepared using the same materials and methods as those used for the nanopillar substrate were used as reference, and fluorescent peptides were immobilized on it in the same manner. The fluorescent images of both nanopillar and flat substrates in the presence and absence of VEGF were monitored and the data are shown in Fig. 3(a). The flat substrate exhibited only a small change in fluorescence intensity in the presence of VEGF. In contrast, a remarkable change was observed in the fluorescence intensity of the nanopillar plate after the addition of the same concentration of VEGF. For example, the fluorescence intensity at 0.5 μg/mL of VEGF for the nanopillar sub-

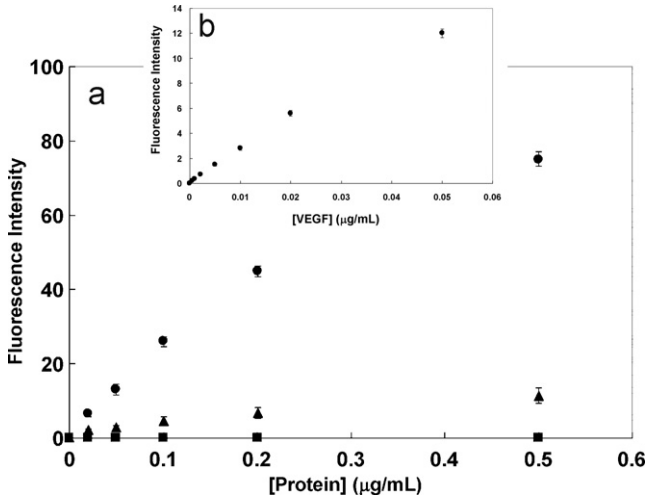

Fig. 3. (a) Fluorescence intensity of compound 1 immobilized on various plates after addition of VEGF (●: nanopillar, ▲: flat plate), and compound 1 immobilized on nanopillar substrate after addition of BSA (■). (b) The relationship between fluorescence intensity of compound 1 on nanopillar substrate and VEGF concentrations ranging from 0 to 50 ng/mL.

strate was about 5 times higher than that for the flat substrate. Moreover, compound 1 on the nanopillar substrate was able to detect VEGF below 1.0 ng/mL, whereas the fluorescent sensor based on the flat substrate did not detect VEGF at a concentration of 5.0 ng/mL. This was caused by the fact that the calculations based on SEM data indicated that the surface area of the nanopillar substrate was approximately 6-fold greater than that of the flat substrate. From these results, the amount of compound 1 immobilized on the nanopillar substrate played an important role in the increase in the fluorescence intensity and in the highly sensitive detection of VEGF.

In order to investigate the selectivity of compound 1 against VEGF, fluorescence intensity of compound 1 on the nanopillar substrate was monitored as a function of bovine serum albumin (BSA). The data are shown in Fig. 3(a). Fluorescence enhancement was not observed in the presence of BSA at concentrations from 0 to 0.5 μg/mL. Moreover, other famous proteins such as transferrin and IgG did not enhance fluorescence intensity. From these results, it was clear that this fluorescent biosensor has a high selectivity for VEGF.

The lifetime of the nanopillar substrate containing compound 1 is important for monitoring the VEGF. The substrates were stored in a freezer at 4 °C. After storage for about 1 month, the response to the VEGF was about 95% of the original value. The data are shown in supporting information. This result indicates that compound 1 is a very stable compound and that this substrate produces a reliable quantitative response to VEGF after a long period of nonexposure to the solution.

4. Conclusions

The present study assesses the immobilization of a VEGF-binding fluorescent peptide on a silica-based nanopillar substrate. Immobilized fluorescent peptides interacted noncovalently with VEGF in the rat serum sample and exhibited a large increase in fluorescence intensity in response to VEGF binding. The detection limit of VEGF detection obtained using the nanopillar substrate was better than that obtained using the flat plate because a larger amount of peptide had been immobilized on the nanopillar plate than on the flat substrate. Recently, early detection and treatment of cancer cells has been emphasized, and rapid progress has been made in the study of angiogenesis. This monitoring system with the VEGF indicator allows for the specific, easy, and rapid detection of VEGF; therefore, it is a convenient method that can be widely applied for VEGF monitoring in hospitals, homes, and other fields.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.02.007.

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