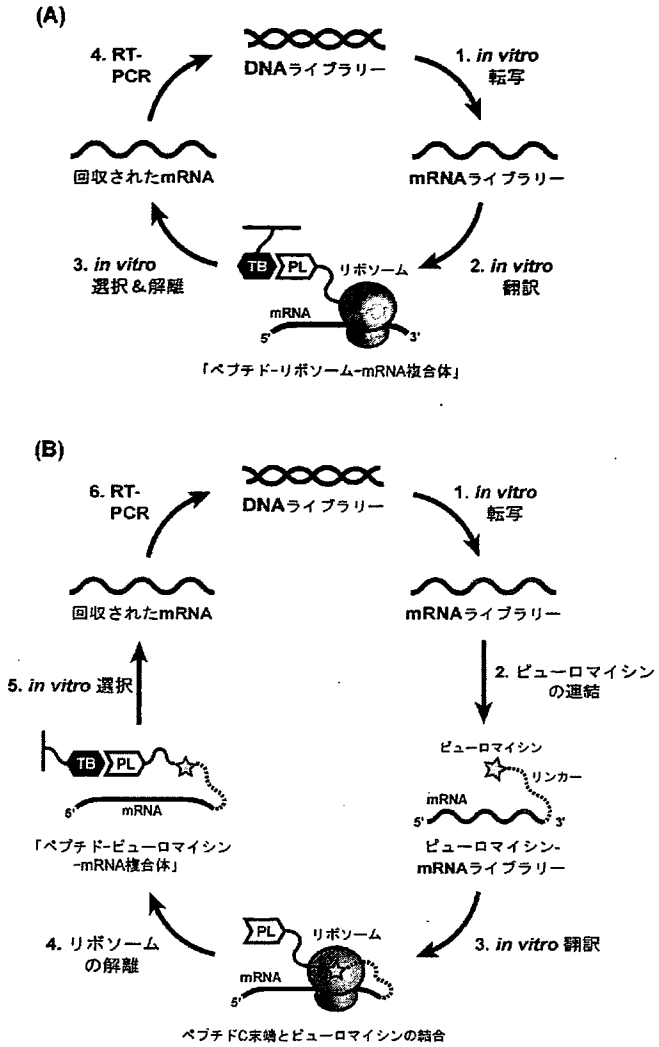

図4　新しいペプチドアプタマー獲得法

(A)リボソームディスプレイ法，(B)mRNAディスプレイ（*in vitro* virus）法

TB : Target Biomolecule（例．タンパク質／酵素／生理活性低分子），PL : Peptide Library

4　アプタマーの機能化

4.1　安定性の向上

オリゴ核酸を医薬品として用いる際，その生体内安定性が低いことが問題点となる。通常，RNAやDNAの血中での半減期は，およそ数秒から数分である。それゆえ，核酸アプタマーの生体内安定性を高めるため，非天然核酸の導入が試みられている。これまでにも，多様な核酸誘導体が数多く開発されている（図5）。現在，非天然核酸をアプタマーに組み込む方法は主に2つある。一つは，すでに結合活性のある天然核酸アプタマーの一部を非天然核酸に置換する方法（ポスト修飾法）である。Schmidらは，抗tenasin-Cアプタマーの核酸配列の一部を，LNA

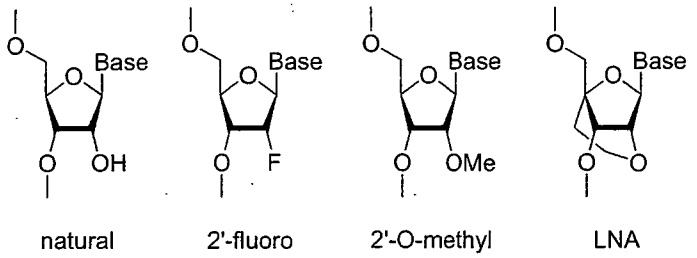

図5　核酸アプタマーへ導入された非天然核酸の例

(Locked Nucleic Acid) で置換することにより，その熱的安定性と酵素分解耐性の向上に成功した[37]。

もう一つは，非天然核酸アナログをポリメラーゼの基質として，SELEX法の過程に組み込む方法である。例えば，アプタマー医薬品であるMacugen (抗VEGFアプタマー)の開発過程では，2'-位フッ素化ピリミジンアナログ及び天然プリンを用いたSELEX法が行われ，第一世代のアプタマーを獲得している。そして，ポスト修飾法により，2'位メトキシヌクレオチドを導入することで，高い生体内安定性をもつアプタマーを完成させた[5]。また，松田らは，4'位硫黄化ピリミジンアナログを用いて，抗トロンビンアプタマーの創製を報告している[38]。

現在，ポリメラーゼを用いて非天然核酸アナログを導入する場合，必ず起こりえる問題として，オリゴ核酸鎖への導入効率が低いことが挙げられる。そこでEllingtonらは，2'位メトキシヌクレオチドアナログを基質として効率よく鎖伸長できる変異型T7RNAポリメラーゼを開発した[39]。一方，Keefeらは，変異型T7RNAポリメラーゼを用いて，すべて2'位メトキシヌクレオチドアナログ骨格からなるアプタマーをSELEX法により創出した[40]。このアプタマーは，VEGFを標的としており，2'位メトキシヌクレオチドの高い生体内安定性から，Macugenに勝る有望なアプタマー医薬品となり得る。近い将来，様々な非天然核酸を含むオリゴ核酸を導入したアプタマー創製法が確立すれば，これまでに創造し得なかった新たなアプタマー医薬の誕生が期待できる。

4.2　デリバリー機能付与

一般的にウイルスが細胞に感染するとき，10から20個のアミノ酸から構成される特徴的なペプチド機能を利用することが知られている。例えば，HIV-1 (Human immunodeficiency virus type-1) のTAT[41]やHSV (Herpes-simplex-virus) のVP-22[42]のタンパク質配列中には，カチオン性アミノ酸（アルギニン・リジン）に富んだペプチド配列（PTD : Protein Transduction Domain）が存在し，哺乳類細胞の細胞膜に対して特異的に結合するだけでなく，細胞内へ能動的に侵入する機能を発現する。つまり，これらのペプチドは，ウイルスが利用する天然型の機能

性ペプチドアプタマーといえる。このPTDの細胞透過性と核移行性は，目的の組織・臓器に薬物としての低分子化合物・核酸を送達するドラッグデリバリーシステムのキャリアーとして極めて魅力的な性質であり，近年，数多くの大学・研究機関・企業が早期実用化を目指して研究に取り組んでいる[43,44]。それゆえ，薬物送達のキャリアーとしてのペプチドアプタマーの開発・改良[45]も，ペプチドを利用した新たな医療を開拓する重要な研究として位置づけられる。

4.3　機能の複合化

オリゴ核酸の特徴である配列特異性とアプタマーとしての機能を巧みに利用した新しい試みもある。それは，核酸アプタマーに対するアンチセンス核酸を用いて活性を制御するという方法である[46]。例えば，トロンビンに対するアプタマーの抗血液凝固作用が強すぎる場合，生体において有害となる。このとき，アプタマーに対するアンチセンス核酸を投与すれば，アプタマーとアンチセンス核酸は二本鎖を形成し，トロンビンへの結合は阻害される。この結果，アプタマーの作用は緩和される。このように，解毒（antidote）機能を付与･制御する分子レベルの設計戦略は，核酸配列を有するアプタマーであるからこそ可能であったといえる。

5　おわりに

現在の抗体医薬は，疾病原因分子に対して特異的に作用する優れた分子標的薬として確固たる地位を築きつつある。しかし，ある頻度で治療効果の低い患者が存在することを始め，中和抗体による影響，製造に関する特許権の錯綜，高額な生産コスト等，解決すべき問題点も多く残している。一方，アプタマー医薬は，細胞内外のいずれの標的分子に対しても作用するマルチ医薬として開発することが可能であり，上記の抗体医薬に関わる諸問題を解決・補完するものとして活躍できるであろう。それゆえ，アプタマーの新規開発を支える進化分子工学的手法の更なる改善，オリゴ核酸やペプチドの生体内動態・薬効・安全性を評価するシステムの確立により，将来のアプタマー医薬によるテイラーメイド医療の実現を大いに期待したい。

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ORIGINAL ARTICLE

A Photo-immobilized Allergen Microarray for Screening of Allergen-specific IgE

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ABSTRACT

Background: We developed an *in vitro* system to diagnose allergy using an allergen microarray and photo-immobilization technique. Photo-immobilization is useful for preparing the allergen microarray because it does not require specific functional groups of the allergen and because any organic material can be immobilized by a radical reaction induced by photo-irradiation.

Methods: To prepare the plates, allergen solutions were mixed with polymer and a bis-azidophenyl derivative, a photo-reactive cross-linker, the mixtures were micro-spotted on the plate, and the droplets were dried. The plate was irradiated with an ultraviolet lamp for immobilization. For the assay, human serum was added to the microarray plate.

Results: Allergen-specific immunoglobulin E (IgE) adsorbed on the micro-spotted allergen was detected by peroxidase-conjugated anti-IgE antibody. The chemiluminescence intensities of the substrate decomposed by the peroxidase were detected with a sensitive CCD camera.

Conclusions: All allergens were immobilized by this method and used to screen allergen-specific IgE.

KEY WORDS

allergen microarray, allergen-specific IgE, allergy diagnosis, chemiluminescence, photo-immobilization

INTRODUCTION

To study allergic reactions, it is important to develop test systems to measure immunoglobulin E (IgE) concentration in serum samples. The first radioallergosorbent test (RAST) to detect allergen-specific IgE in serum was described in 1967.[1] Subsequent tests replaced the radioactive labels used in the RAST with various procedures such as the chromogenic-enzyme immunoassay (EIA) or fluorescence-enzyme immunoassay (FEIA). However, few of these have become routine methods in the diagnosis of allergy in research and clinical practice.[2-7] The most common *in vitro* technique used in the clinical settings is the Pharmacia CAP System (PCS) to measure total and allergen-specific IgE (specific IgE FEIA, Pharmacia, Uppsala, Sweden). Other methods such as the FAST FEIA (MAST Diagnostica, Reinfeld, Germany) and HYTEC EIA (Hycor Biomedicals, Kassel, Germany) are available commercially.[8] Some methods are based on liquid-phase inhibitor assays (e.g., AlaSTAT, DPC Biermann, Los Angeles, CA, USA) or multiallergen-coated nitrocellulose strips (e.g., IgEquick, Teomed AG, Greifensee, Switzerland; CMG Immunodot, Trimedal AG, Brüttisellen, Switzerland).[9,10] The CAP System contains a cellulose polymer densely conjugated with allergen extracts or recombinant allergens.

Although it is possible to measure a multitude of allergen-specific IgEs by immunoassays in the patient's blood, these tests are expensive, time consuming, and some need a high volume of reagents and serum. An increasing number of patients are experiencing immediate-type allergic diseases, such as allergic rhinoconjunctivitis, atopic eczema, and food and drug allergies.[11] It is desirable to develop a fast and economic screening technology to detect allergen-specific IgE in serum samples that allows the simultaneous analysis of hundreds of allergens in a single run. Multiallergen dipstick tests were a first step in

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Table 1 UniCap data of sera (IU/ml)

Allergen	M-14	SIC311276	AHP9580	AHP9549	SIC31181
Japanese cedar	8.79	< 0.34	< 0.34	9.37	30.9
Dermatophagoides pteronyssinus	0.88	36.4	0.63	4.50	12.8
Orchard grass	< 0.34	< 0.34	65.1	99.9	> 100
Cow milk	< 0.34	< 0.34	< 0.34	14.7	37.9
Egg white	< 0.34	0.52	< 0.34	11.4	33.1

The values corresponding to the chemiluminescent spots of Figure 4 are underlined.

the miniaturization and cost savings of such techniques,[10,12] but most could not be run automatically. Microarrays produced with spotting devices are another strategy to miniaturize such tests, which allow proteins to be immobilized in the lower nanoliter range on defined positions on a surface. The first experimental microarray system for allergy diagnosis was reported in 2000,[13] and an allergen microarray based on fluorescence detection was published in 2002.[14] Fall *et al.* reported recently on an application of the parallel affinity sensor array (PASA) technology that automatically performs allergy diagnosis.[2] Purified recombinant and natural allergens and allergen extracts were immobilized on glass slides to detect allergen-specific IgE. However, not all allergens were immobilized by the technique because specific functional groups are needed by the allergens.

We have developed a photo-immobilization method to apply the microarray to various materials including proteins and cells.[15,16] We used this photo-immobilization technique to prepare a microarray of allergens. The advantages of the photo-immobilization method are that it is not limited by functional groups and that it can immobilize any organic material in any organic substrate.

METHODS

REAGENTS AND CHEMICALS

Plates for microarray (polystyrene slides, 2.5 cm × 7.6 cm × 0.5 mm) were cleaned using ethanol with sonication for 15 minutes at room temperature. The washed polystyrene slides were dried and stored. The raw allergen materials, Japanese cedar, orchard grass, *Dermatophagoides pteronyssinus*, cow milk and egg white, were purchased from Allergon (Ängelholm, Sweden). The polyclonal affinity-purified horseradish peroxidase (HRP) -labelled goat anti-human IgE antibody was purchased from Serotec Ltd (Oxford, UK). The ECL Advance Kit for HRP was purchased from Amersham Biosciences UK Ltd (Buckinghamshire, UK). 4,4'-diazido-stylbene-2,2'disulfonic acid, disodium salt (BIS), polyethylene glycol methacrylate (molecular weight, 526 Da), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co (Milwaukee, WI, USA). Sera containing allergen-specific IgE for Japanese cedar (M-14), *Dermatophagoides pteronyssinus* (SIC311276), orchard grass (AHP9580), cow milk (AHP9549), and egg white (SIC31181) were purchased from Uniglove Research Corp. (Rivera, CA, USA). The reference measurements were performed with the UniCap System (CAP specific IgE-FEIA, Pharmacia, Uppsala, Sweden) and the data are shown in Table 1.

SYNTHESIS OF PEG-350

The polymer matrix carrying polyethylene glycol in the side chains (PEG-350) was prepared as follows. Polyethylene glycol methacrylate (molecular weight 350 Da, 7.0 *g*) was dissolved in ethyl acetate (80 mL) and bubbled with nitrogen gas for 30 seconds. Azobisisobutyronitrile (46.0 mg) was added to the solution, which was then allowed to stand for 6 hours at 60℃. The solution was concentrated and added to diethyl ether. A viscous solid was obtained after stirring. The precipitation procedure was repeated four times and the final precipitate was dried *in vacuo*. The yield was 1.57 *g* (22.4%).

PREPARATION OF ALLERGENS

To prepare the allergen extracts, 5% raw allergen material (w/v) was suspended in 0.05 M phosphate buffer (pH7.4) for 2 hours at 4℃. The supernatant was collected and filtered through a 0.45 μm cellulose acetate membrane (Sartorius, Göttingen, Germany). The supernatant was dialyzed against water for 24 hours and then lyophilized.

PHOTO-IMMOBILIZATION OF ALLERGEN

The principle of immobilization is illustrated in Figure 1. We propose that BIS works as a photo-reactive cross-linker to immobilize the allergen with PEG-350 and that photo-irradiation causes the cross-linking reaction to occur between allergen and allergen, allergen and PEG-350, allergen and the plate surface, and PEG-350 and the plate surface.

The extracted allergens were dissolved in deionized water at various concentrations (0.625–40 mg/mL). The allergen solutions were mixed with an aqueous solution of BIS (0–0.5 mg/mL), and PEG-350 (0.25 mg/mL) at a 2:1:1 volume ratio. The mixtures were micro-spotted (50 nL) with the microarray spotter (PixSis-4500, Cartesian, Irvine, CA, USA) on

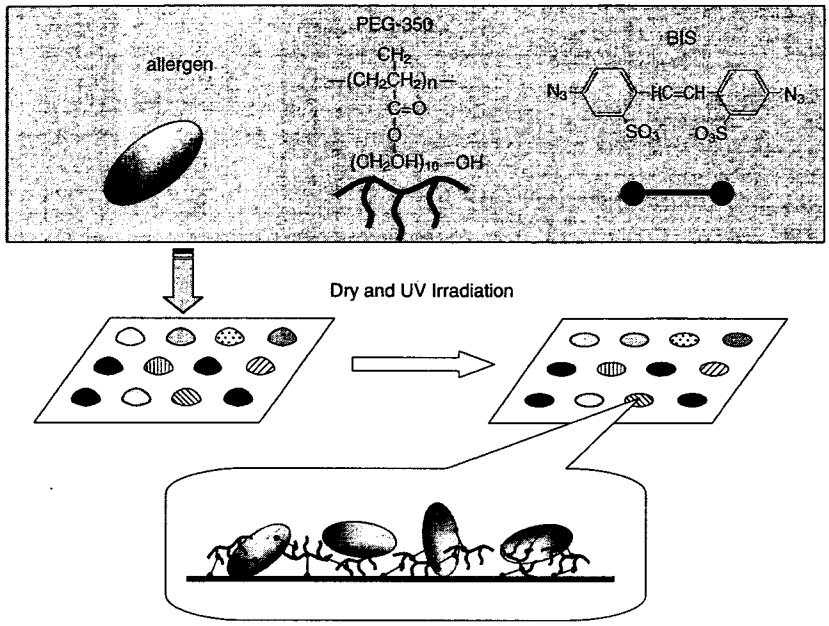

Fig. 1 Illustration of the photo-immobilization method. Abbreviations: PEG-350, polymer carrying polyethylene glycol in the side chains; BIS, 4,4'-diazido-stilbene-2,2'-disulfonic acid disodium salt.

the plate and the droplets were dried. The microarrayed plate was irradiated with an ultraviolet lamp (300–400 nm, Nippo Electric Co. Ltd. FL15BLB) for 7 minutes. Finally, the allergen-immobilized plates were rinsed with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (the washing buffer), and stored until use in a refrigerator.

MICROARRAY ASSAY PROCEDURE

The allergen-immobilized plates were incubated with serum (30 μL) for 1 hour at room temperature with shaking in a chamber. The plate was washed with 30 mL of the washing buffer for 3 minutes in a chamber. HRP-conjugated anti-human-IgE antibody (diluted 1: 100 with PBS-10% BSA) was loaded on the microarray plates and the plates were incubated for 1 hour at room temperature with shaking in a chamber. Finally, the substrate solutions (ECL Advance Kit) were added to the plates and the plates were incubated for 3 minutes at room temperature. The chemiluminescence intensities of each micro-spot were measured for 30 seconds with a cooled CCD camera system (AE-6960 Light Capture, ATTO Corp., Tokyo, Japan).

RESULTS

PHOTO-IMMOBILIZATION

To examine the photo-immobilization method, we immobilized orchard grass under various conditions. No chemiluminescence was observed when allergen was not contained in the micro-spot (data not shown). This result indicates that neither BIS nor PEG-350 induces non-specific adsorption of antibodies. Although neither BIS nor PEG-350 immobilized allergen, some intensity of chemiluminescence was observed (Fig. 2). Non-specific (physical) adsorption of allergen was considered to occur on the plate surface. In contrast, micro-spotting with both BIS and PEG-350 produced maximum intensity (Fig. 2). This result indicates that the allergen was stably immobilized on the plate surface; BIS caused cross-linking between allergen and allergen, and between allergen and the plate surface, and thus immobilized allergen on the plate and PEG-350 was considered to increase the amount of immobilized allergen by entrapping in the crosslinked matrix

CALIBRATION CURVE

Using the condition producing the highest chemical luminescence intensity, we produced calibration curves for orchard grass, Japanese cedar, *Dermatophagoides pteronyssinus*, cow milk, and egg white, which are shown in Figure 3. The coefficients of variation (CV) were <10.0%, although the pattern of the calibration curve differed between the allergens.

ASSAY USING MULTIPLE MICRO-SPOTTED PLATES

Figure 4 shows the chemiluminescence image produced by IgE adsorbed on micro-spotted orchard grass, Japanese cedar, *Dermatophagoides pteronyssinus*, cow milk, and egg white. Although Fall *et al.*[2] could not immobilize all the antigens they studied,

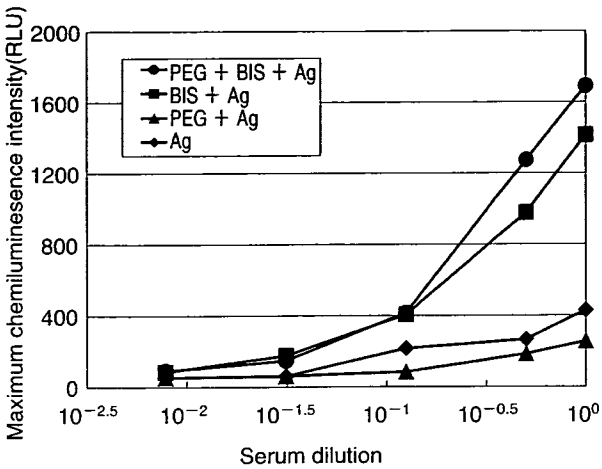

Fig. 2 Adsorption of orchard grass-specific IgE on the surface micro-spotted with ◆, allergen only; ▲, allergen with PEG-350; ■, allergen with BIS; and ●, allergen with PEG-350 and BIS. The concentrations of micro-spotted allergens were 2.5 mg/mL for orchard grass allergen, 2.5 mg/mL for PEG-350, and 0.125 mg/mL for BIS. Serum was diluted 1-, 2-, 8-, 32- and 128-fold with PBS-4% BSA. $N = 4$.

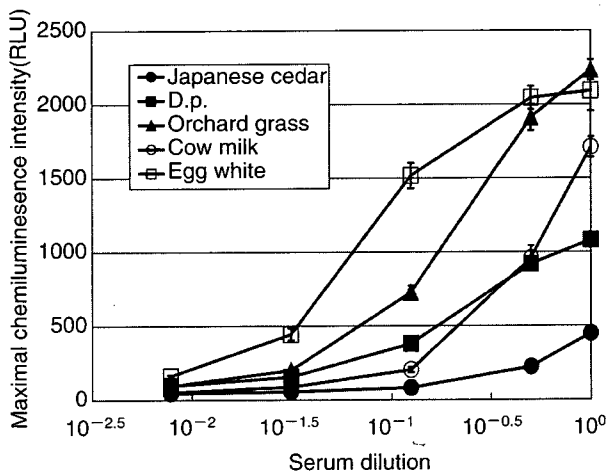

Fig. 3 Calibration curve of allergen-specific IgE on micro-spotted allergens. Orchard grass (2.5 mg/mL), Japanese cedar (10.0 mg/ml), *Dermatophagoides pteronyssinus* (20.0 mg/ml), cow milk (10.0 mg/ml), and egg white (10.0 mg/ml) were micro-spotted and photo-immobilized with BIS (0.125 mg/mL) and PEG-350 (2.5 mg/mL). Serum was diluted 1-, 2-, 8-, 32- and 128-fold with PBS-4% BSA. Data are presented as means ± SD ($N = 4$).

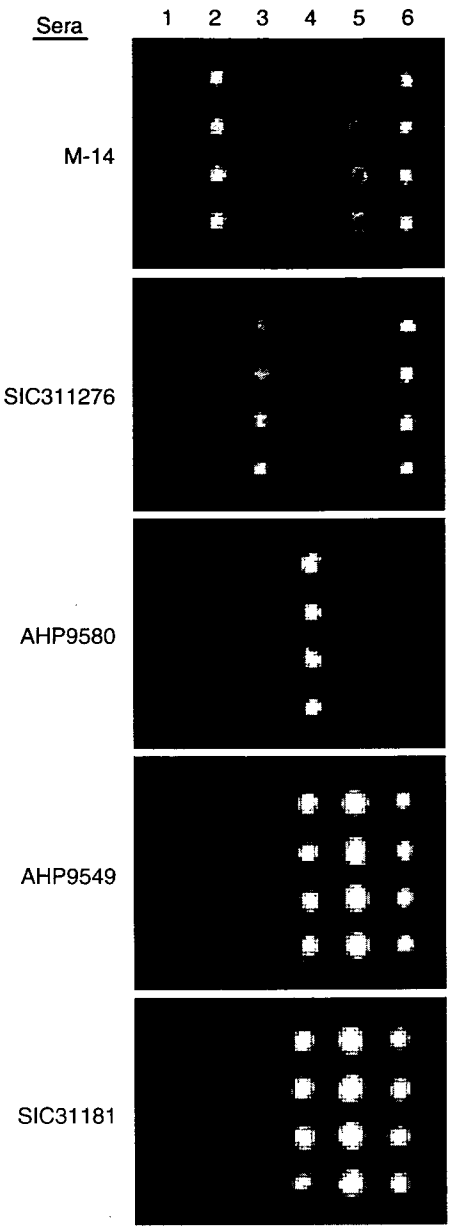

Fig. 4 Chemiluminescence image of IgE adsorbed on micro-spotted Japanese cedar, *Dermatophagoides pteronyssinus*, orchard grass, cow milk, and egg white.

our results show that all antigens were immobilized and that we observed chemiluminescence of each allergen spot by adsorption of IgE from the corresponding serum. In addition to the IgE, which should be contained in serum according to the supplier, Figure 4 shows the allergic reactions between the various allergens and serum. M-14 and SIC311276 each produced an allergic response to cow milk and egg white. AHP9549 produced an allergic response to or-

chard grass and egg white, and SIC31181 produced an allergic response to orchard grass and cow milk.

DISCUSSION

The photo-immobilization method uses the radical reaction for cross-linking, making it possible to immobilize any organic materials independently of functional groups of chemicals or proteins.[15-17] It is easy and convenient to prepare multiple micro-spots by the same method. We have previously immobilized various types of proteins to analyze cell adhesion and panel cells to detect antibody in blood.[15,16,18]

One additional characteristic of the assay is that the orientation of immobilized molecules is random. This property is suitable for the allergen microarray because various sites of immobilized molecules are exposed from the surface, enhancing recognition by polyclonal IgE (Fig. 1). The ability to measure the amount of IgE in the diluted serum, shown in Figure 3, indicates that this property enhances the assay's sensitivity.

Since standardization was not completely performed, the chemiluminescent spots were not completely in accord with the data in Table 1. However, in each sample higher values of UniCap measurement corresponded to the chemiluminescent spots. This indicates the adequacy of the present measurement.

Another characteristic is the small amount of serum required for the microarray assay. Usually 300 μL of serum is required to analyze five different allergens in the conventional one-to-one assay. However, this system required one-tenth that amount (30 μL) to analyze five allergens. Integrating the microarray density will increase the number of allergens that can be analyzed.

Our study demonstrates that this photo-immobilization technique is useful for screening of allergen-specific IgE to diagnose allergy. The ability to adapt this technique to immobilize any organic material has potential applications in developing assays to analyze auto-immunity and infection.

ACKNOWLEDGEMENTS

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Culture of human umbilical vein endothelial cells on immobilized vascular endothelial growth factor

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Abstract: Vascular endothelial growth factor (VEGF) was immobilized on substrata in photoreactive gelatin to control the adhesion and growth of vascular endothelial cells. The gelatin and VEGF were mixed in water and cast on a polystyrene dish or a silane-coated glass plate. The surface was then photoirradiated in the presence or absence of a photomask and washed. Toughness of the immobilized material was confirmed by ethanol treatment. Human umbilical vein endothelial cells (HUVECs) grew on the immobilized VEGF but not on a nontreated surface. Growth of HUVEC increased significantly with an increase in the amount of immobilized VEGF, and the effects were inhibited by treatment with anti-VEGF antibody. Thus, immobilized VEGF specifically interacted with HUVECs to permit growth in culture. Micropatterning of HUVEC cultures was also achieved using micropattern-immobilized VEGF. This patterning technique may be useful for the formation of blood vessel networks *in vitro*. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 74A: 659–665, 2005

Key words: immobilization; vascular endothelial growth factor; human umbilical vein endothelial cells; micropatterning

INTRODUCTION

The construction of blood vessels is a fundamental challenge for regenerative medicine. To construct or regenerate organs, formation of a capillary network is essential to transport nutrients and gas into organs, and control of vascular endothelial cell growth is necessary to achieve this *in vitro*. Recently, Koike and colleagues[1] showed that a network of long-lasting blood vessels can be formed in mice by implantation of vascular endothelial cells and mesenchymal precursor cells cocultured in a gel matrix, thus bypassing the need for risky genetic manipulation. Vascular endothelial growth factor (VEGF) is a mitogen primarily acting on vascular endothelial cells. It was purified by Gospodarowicz and colleagues[2] and Ferrara and Henzel[3] from a conditioned medium of bovine pituitary follicular stellate cells, utilizing an endothelial cell proliferation assay to monitor the biological activity. Purified VEGF is a protein of approximately 46 kDa, which dissociates upon reduction into two apparently identical 23 kDa subunits.

Ito and colleagues[4,5] found that biosignal molecules (growth factors and cytokines) immobilized on a solid matrix enhanced cell growth. Since this discovery, various groups have confirmed the biological activity of biosignal molecules immobilized on various matrices.[6–18] Immobilized growth factors transduce a signal for a longer time than do soluble growth factors, and this enhances growth to a greater extent. On the other hand, Ishikawa and colleagues[19] and Hayashi and coworkers[20] synthesized chimeric proteins containing adhesion and growth factors and showed that these enhanced adhesion and growth of cells. Recently, Zisch and colleagues[21] reported that fibrin-bound ephrin-B2 acts as multivalent ligand for endothelial cells. Here, VEGF was photoimmobilized in gelatin, and human umbilical vein endothelial cells (HUVECs) were successfully cultured on the resulting surface. Micropattern-immobilization was employed not only to investigate the effect of immobilized growth factor, whether the cell behavior was affected on the growth factor-immobilized surfaces or not, but also to induce network formation.

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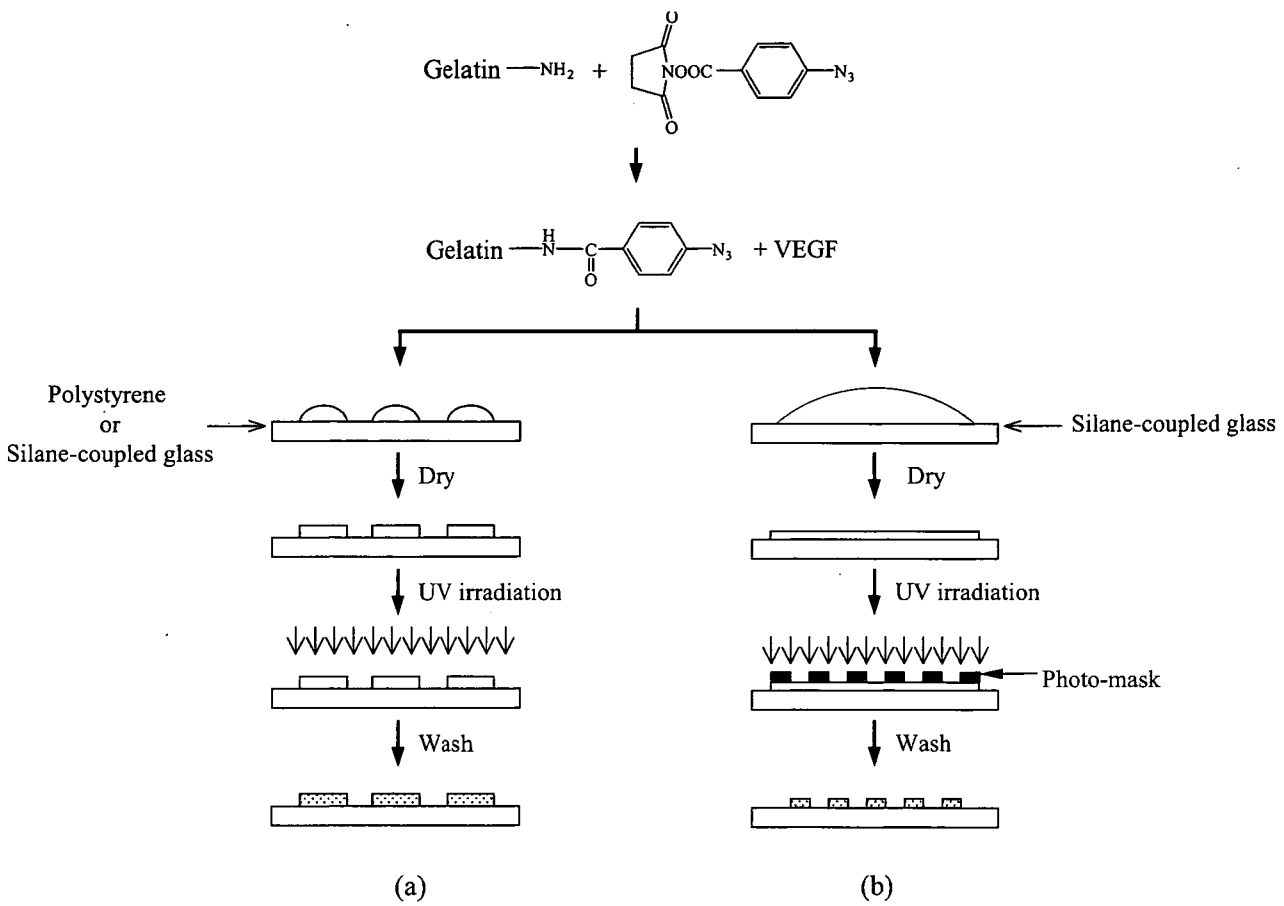

Figure 1. Immobilization of VEGF with photoreactive gelatin. (a) Microspot formation; (b) micropattern formation.

MATERIALS AND METHODS

Materials

Human recombinant VEGF 121, recombinant human VEGF receptor 2 (KDR/Fc) chimera (357-KD/CF), and antihuman VEGF antibody (MBA293) were purchased from R & D Systems, Inc. (Minneapolis, MN). Antihuman Fc antibody (rabbit), antirabbit IgG antibody conjugated with biotin, and streptavidin-bound horseradish peroxidase (HRP) were purchased from Dako Cytomation A/S (Copenhagen, Denmark). DAB was purchased from Dojin Chem, Inc. (Kumamoto, Japan). Gelatin (porcine) was purchased from BD Diagnostic Systems (Sparks, MD). Dicyclohexylcarbodiimide (DCC), dioxane, *N*-hydroxysuccimide, and dimethylformamide (DMF) were purchased from Wako Pure Chem Ind. (Osaka, Japan) and used without further purification. 4-Azidobenzoic acid was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and used without further purification. Polystyrene dishes were purchased from Iwaki & Co., Ltd. (Tokyo, Japan). Octadodecylethoxysilane for surface treatment of glass plate was purchased from Shinetsu Chem. Ind. Co., Ltd. (Tokyo, Japan). HUVECs were purchased from Cambrex (Wakersville, MD).

Synthesis of photoreactive gelatin

Immobilization was performed as reported.[22] Photoreactive gelatin was synthesized as follows. First *N*-(4-azidobenzoyloxy) succinimide was prepared. A solution of DCC (380 mg) was added dropwise to a solution of *N*-hydroxysuccinimide (210 mg) and 4-azidobenzoic acid (300 mg) in dioxane (20 mL), and then cooled in an ice bath while stirring for 12 h. The white solid that formed was filtered off and the solvent was removed under reduced pressure. The yellow residue obtained was crystallized twice from dioxane/diethyl ether.

Gelatin, dissolved in 10 mL phosphate-buffered solution (pH 7.0), was added to a solution of *N*-(4-azidobenzoyloxy) succinimide (25.8 mg) in DMF (20 mL) while being stirred on ice. After a stirred incubation at 4°C for 24 h, the solution was filtered using Millipore ultrafiltration membranes (10,000 molecular weight cutoff). The dialyzed sample was freeze dried and the content of azidophenyl groups in the modified gelatin was calculated from the light absorbance at 270 nm.

Photoimmobilization of VEGF

Immobilization is illustrated in Figure 1. VEGF was mixed with photoreactive gelatin in water (180 μL) and aliquots of

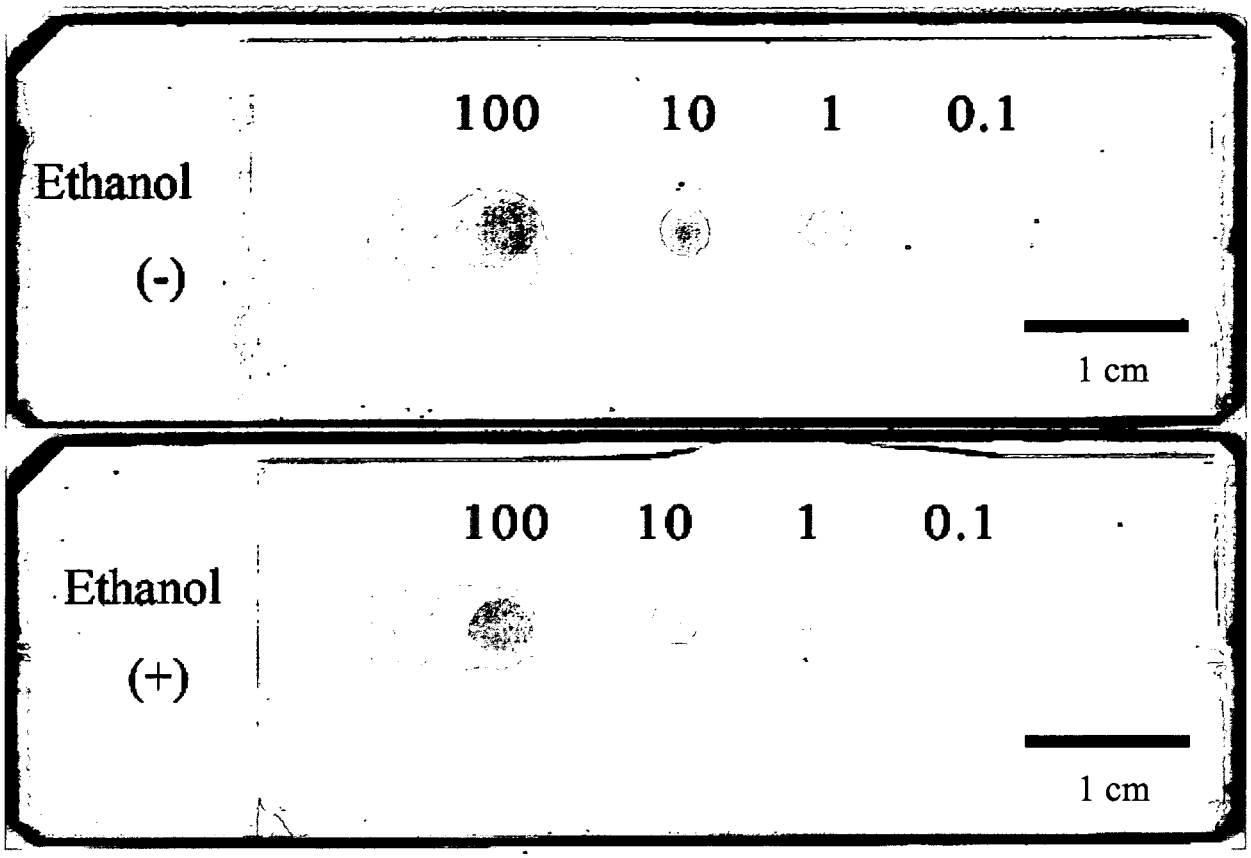

Figure 2. Staining of immobilized VEGF. The immobilized slide glass plate was incubated with recombinant VEGF receptor 2 (KDR/Fc), with anti Fc antibody, with the secondary antibody coupled with horseradish peroxidase, and finally with DAB solution. The plate is shown before (−) and after (+) 70% ethanol treatment. The numbers show the concentrations of VEGF in micrograms per milliliter. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the aqueous solution were dropped onto tissue culture polystyrene dishes or silane-coated glass plates. The silane-coated glass plates were prepared as follows. Plates were incubated in Piranha solution (sulfuric acid: 30% aqueous peroxide solution, 7:3) under sonication for 10 min and left in the solution for 1 h. The glass plate was rinsed with MilliQ water five times and dried *in vacuo* at 50°C for 2 h. The pretreated glass plate was incubated in a solution of octadecylethoxysilane (10 m*M*) in toluene at room temperature overnight. The plate was then washed with toluene and dried *in vacuo* for 1 h.

Each dish or plate coated with photoreactive gelatin plus VEGF was covered with or without a photomask (Nippon Filcon Co. Ltd., Tokyo, Japan) and irradiated using a UV spot Light Source L5662 (Hamamatsu Photonics, Hamamatsu, Japan) from a distance of 5 cm for 10 s (16 mW/cm^2). The dish or plate then was washed repeatedly with distilled water.

Staining of immobilized VEGF

The glass plate with immobilized VEGF was treated with or without 70% ethanol for 10 min at room temperature. Subsequently the plate was washed with phosphate-buffered saline (PBS) three times and incubated in a PBS solution containing 1 μg/mL of KDR/Fc and 0.1% bovine serum albumin (BSA) for 2 h at 37°C. The plate was washed with PBS three times and incubated in a PBS solution containing antihuman Fc antibody (diluted 1:1000) and 0.1% BSA for 1 h at room temperature. The plate was washed with PBS three more times and incubated in a PBS solution containing biotinylated antirabbit IgG antibody (diluted 1:500) and 0.1% BSA for 30 min at room temperature. The plate was washed with PBS three more times and then incubated in PBS containing streptavidin-HRP (diluted 1:700) and 0.1% BSA for 30 min at room temperature. Finally the plate was washed with PBS five times and incubated in DAB solution for 3 min at room temperature.

Cell culture

HUVECs were cultured in EBM-2-MV bullet kits (Cambrex); basal endothelial medium (EBM-2) supplemented with 5% fetal calf serum (FCS), VEGF, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), R3-insulinlike growth factor (R3-IGF-1), hydrocortisone, ascorbic acid, and antibiotics (SingleQuots kit, Cambrex). For

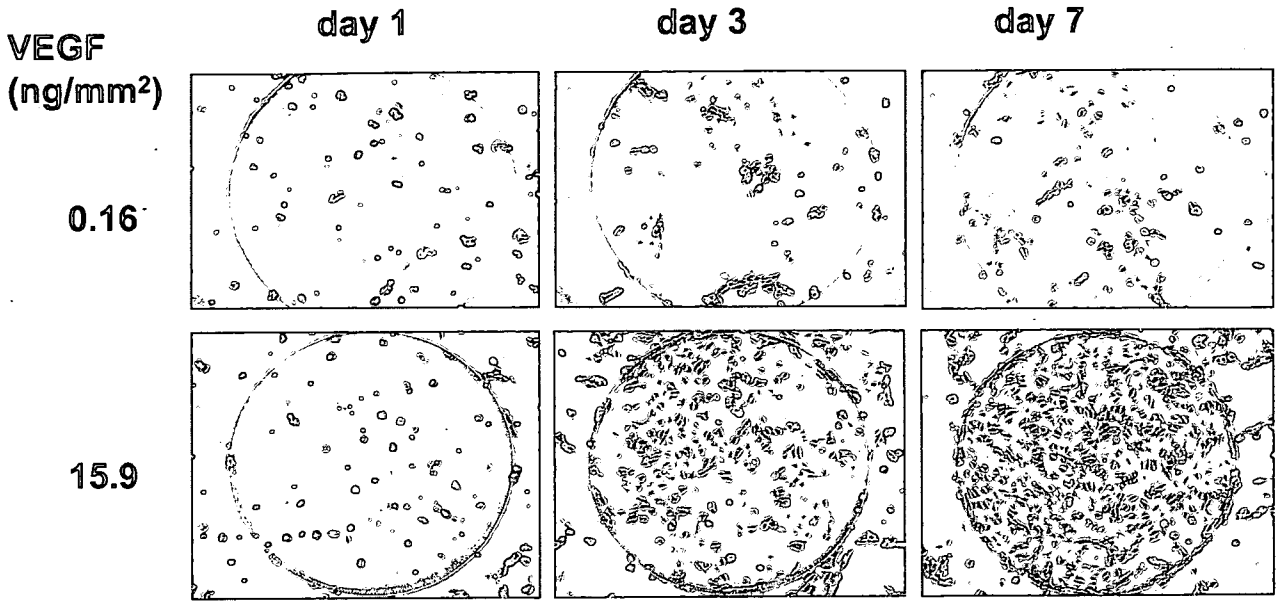

Figure 3. Phase-contrast micrographs of HUVECs cultured on tissue culture polystyrene dishes with different concentrations of immobilized VEGF. The immobilized region is in the center of each photo. The surface concentration of gelatin was 15.9 ng/mm^2.

experiments, they were harvested after trypsinization and washed with EBM-2 medium supplemented with 0.5% FCS and SingleQuots components except for VEGF, bFGF, and EGF. Subsequently, the harvested cells were resuspended in the same medium (1×10^3 cells/mL) and were cultured under an atmosphere containing 5% CO_2 at 37°C for the prescribed number of days.

Surface coverage with cells was estimated by digital counting of phase-contrast micrographs of cultured cells as an indicator of cell growth. When the immobilized VEGF was blocked with anti-hVEGF antibody, the antibody was first added to the culture medium at 1 μg/mL. The cells were stained with Giemsa at room temperature for 10 min after fixation of cells with methanol at 4°C for 10 min.

RESULTS AND DISCUSSION

Photoimmobilization of VEGF

VEGF was mixed with the photoreactive gelatin, and the mixture solution was dropped onto a polystyrene plate and the plate was dried and photoirradiated. When the immobilized spot was stained using VEGF receptor, concentration-dependent binding of the receptor was observed, as shown in Figure 2. Thus the immobilized VEGF could actively interact with the cognate receptor. The active site of VEGF was therefore considered exposed to the medium. In addition, washing with ethanol did not significantly reduce the amount of bound receptors, so the immobilized VEGF was stable against ethanol.

Previously, we used azidophenyl-derivatized gelatin to immobilize erythropoietin and no release of immobilized erythropoietin was confirmed.[22] Therefore, in the present study, VEGF was considered to be quantitatively immobilized because the same method was employed for immobilization.

Cell culture

In the following experiments, HUVECs were cultured under starved conditions. The culture medium

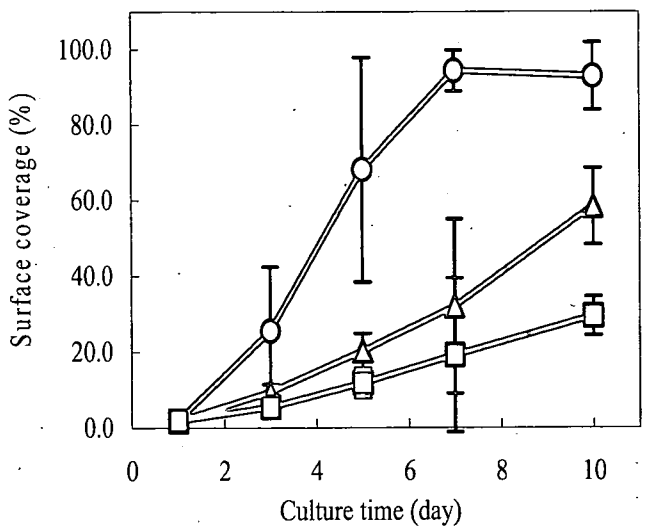

Figure 4. Cell growth of HUVECs on tissue culture polystyrene dishes immobilized with VEGF (□; 0.32, △; 3.2, and ○; 15.9 ng/mm^2). The surface concentration of gelatin was 15.9 ng/mm^2. Surface coverage by HUVEC was determined by phase contrast microscopy (n = 3).

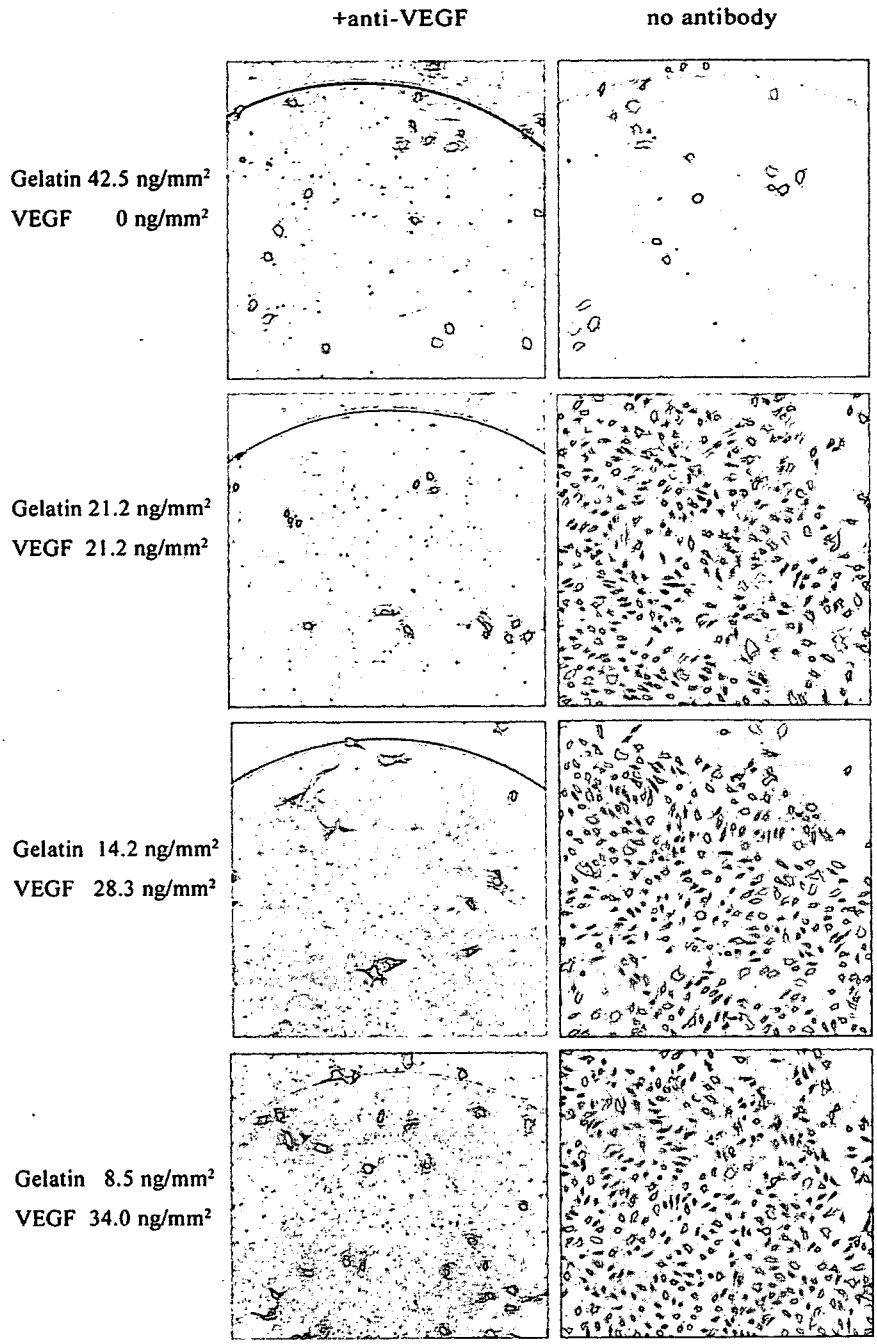

Figure 5. HUVEC growth on a tissue culture polystyrene dish immobilized with VEGF in the absence and the presence of anti-VEGF antibody. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

contained only 0.5% FCS and no angiogenic growth factors (bFGF, VEGF, or EGF). When HUVECs were placed on the microspot-immobilized VEGF surface and cultured, the cells adhered to the VEGF-immobilized regions as well as to nontreated regions (polystyrene surface). There was no significant increase in cell numbers after 1 day of culture. However, after 3 days, the numbers of cells on the VEGF-immobilized regions were significantly higher than on nontreated regions, as shown in Figure 3. Neither significant adhesion nor growth was observed on gelatin-immobilized regions lacking VEGF. These results indicated that no significant release of VEGF occurred to affect the HUVECs' behaviors.

Immobilized VEGF enhanced cell growth in a concentration-dependent manner as shown in Figure 4. Cell growth was linear until 7 days and the surface was completely covered with cells at a concentration of 15.9 ng/mm^2 immobilized VEGF. After 1 day, the cell concentration was 17.1 ± 2.9 cells/mm^2, which

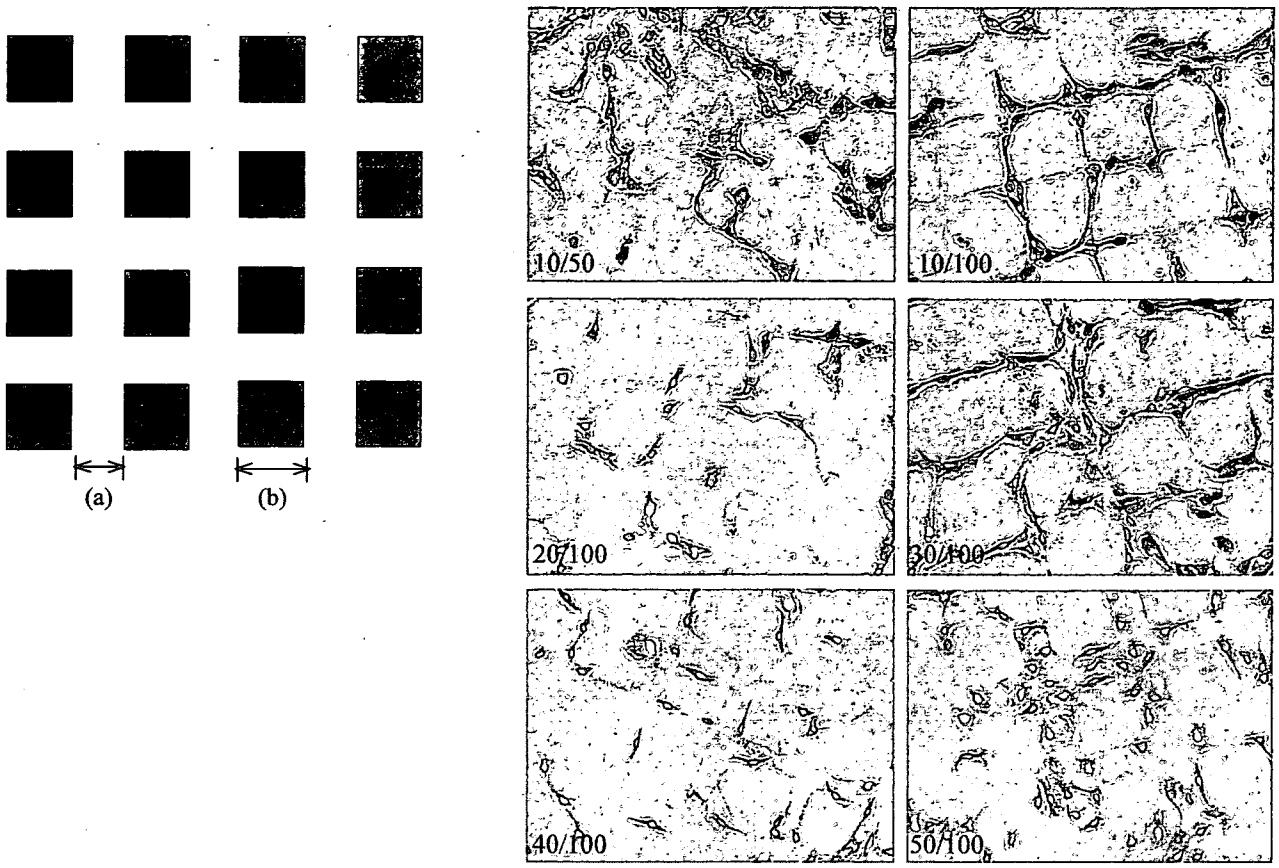

Figure 6. Photomasked and micropattern-cultured HUVECs on glass plates micropattern-immobilized with VEGF. The numbers in the photos indicate the width of immobilized regions (a) and the gap width of the lattice (b). The surface densities of gelatin and VEGF were both 21.2 ng/mm^2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

meant that 2% of surface immobilized with VEGF was covered with cells. Thus, the cells increased 50-fold over the number seeded. Such a dramatic increase has not been observed on other VEGF-immobilized surfaces.[13] In addition, approximately half of the cells on these surfaces survived for 7 to 10 days after reaching confluence. Thus, the immobilized VEGF appears to have maintained its activity for 14 to 17 days, including the time needed for cells to reach confluence.

When HUVECs were cultured in the presence of anti-VEGF antibody, the numbers of cells were significantly lesser than in the absence of antibody after 9 days (Fig. 5). Inhibition by the antibody was reduced with increased concentrations of immobilized VEGF. No such effect was observed on the gelatin-immobilized surface not treated with antibody. Thus, the immobilized VEGF specifically interacted with the HUVEC.

Figure 6 shows the micropatterning of HUVEC growth. Cells grew only on the VEGF-immobilized regions. When the lattice size was small, a clear form was not observed because of cell aggregation. However, when the lattice size was relatively large, networks of HUVECs formed on the surface.

VEGF is a key physiological regulator of angiogenesis during embryogenesis, skeletal growth, and reproductive functions.[23] Taguchi and colleagues[13] immobilized VEGF 165 on poly(acrylic acid)–grafted polyethylene film via a reaction between the amino group of VEGF and the carboxyl group, using water-soluble carbodiimide. They found that coimmobilization of fibronectin and VEGF enhanced the growth of HUVECs. Stone and colleagues[24] synthesized a VEGF–BSA covalent complex and this retained its chemotactic and proliferative properties. They suggested that bare prosthetic surfaces lined with VEGF might support endothelial cell proliferation and migration, and thereby offer new strategies to improve graft reconstruction and patency.

Here we showed that VEGF actively supported HUVEC growth in the immobilized state by specific interaction and by blocking with anti-VEGF antibody. As this photoimmobilization technique is applicable for any organic material, it will be possible to immobilize VEGF on various substrates. Immobilized VEGF significantly enhanced the surface coverage with endothelial cells. This enhancement is very important, considering the scarcity of endothelial progenitor cells

in the body. In addition, using this photoimmobilization technique it should be possible not only to promote endothelialization but also to regulate the formation of a network of vascular capillaries *in vitro*.

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Photo-reactive polyvinylalcohol for photo-immobilized microarray

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Abstract

A new photo-reactive polymer, polyvinylalcohol modified with phenylazido groups, was synthesized as a microarray matrix. The polymer is soluble in water and spin-coated onto glass plate. Aqueous solutions of proteins were micro-spotted onto the coated glass and were fixed by ultraviolet light irradiation. Subsequently, cell adhesion on the photo-immobilized protein microarray was investigated. Non-specific adhesion of cells onto non-protein-spotted regions was reduced in comparison with the previously prepared microarray chip (Biomaterials 24 (2003) 3021). The adhesion behavior of cells depended on the kind of immobilized proteins and the type of cells. The microarray will be useful for cell diagnosis and for the selection of biomaterials to regulate cell behavior.

Keywords: Biochip; Microarray; Photo-immobilization; Cell adhesion; Cell chip

1. Introduction

Microarray technology has become a crucial tool for large-scale and high-throughput biological science and technology. It allows fast, easy and parallel detection of thousands of addressable elements in a single experiment under the same conditions [1,2]. DNA microarray approaches have demonstrated a rapid and economic way to interpret gene function [3,4]. In recent years, there have been considerable advancements in the preparation of small-molecule arrays [5–7], peptide arrays [8–11], protein arrays [12,13], polysaccharide arrays [14–16], antigen arrays [17–19], antibody arrays [20–22], and tissue arrays [23,24]. The microarray is important not only for genomics or proteomics but also for cellomics, where very few studies have been carried out. Recently, Ziauddin and Sabatini [25] prepared transfected cell microarrays from cDNA microarrays after addition of a lipid transfection reagent and adherent mammalian cells. In addition, microarrays of different kinds of antibody and proteins have been used for the assay of lymphocytes and cells, respectively [26,27]. Such microarrays of proteins are useful for cell surface profiling. By using the chip it is possible to observe cell behavior on a single surface under the same condition.

Although microarraying proteins is important, no universal immobilization method for the preparation of arrays has been developed. Non-covalent immobilization (physical adsorption) or chemical coupling reactions (using amino or carboxyl groups in the materials) were usually used. However, the former is not suitable for stable immobilization and the latter is limited by the structure of the immobilized materials. Therefore, a photo-immobilization method was devised for the preparation of microarray chips for immunological [28] and cell adhesion assays [27]. Although the immobilization method does not regulate the molecular orientation of the immobilized molecules, any materials can be immobilized on a substrate because of the radical reactions. Because all molecules are randomly immobilized, the activity was considered to be averaged on each immobilized molecule.

Previously, we have microarrayed proteins using photo-reactive poly(acrylic acid) as a matrix on a polystyrene tissue culture plate [27]. However, since cells significantly adhered to areas without immobilized proteins (glow-discharged polystyrene surface), it was

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difficult to differentiate between immobilized and non-immobilized areas. In other words, the S/N ratio was low. Therefore, in the present study, a new photo-reactive polymer inhibiting non-specific cell adhesion was developed.

2. Materials and methods

2.1. Preparation of photo-reactive polyvinylalcohol

The synthetic scheme of photo-active polyvinylalcohol is shown in Fig. 1. First 2-(2-(4-azidophenyl)vinyl)-4-(3-pyridylmethylene)-1,3-oxazolin-5-on (Az-8) was synthesized as follows. 2-(3-(4-Azidophenyl) prop-2-enoylamino)acetic acid (9 g), 3-pyridylaldehyde (4 g), acetic anhydride (12 g), sodium acetate (0.6 g), and cyclohexane (35 g) were mixed and allowed to stand for 6 h at 70°C. After heating, the mixture was cooled and isopropyl alcohol (30 g) was added to the mixture, which was then again allowed to stand for 15 h at room temperature. The resulting precipitate was recovered by filtration at room temperature and the solid material washed with cooled methanol (30 g). Finally, the product was dried under vacuum. Approximately 7 g of Az-8 was obtained. The product had an absorbance at 390 nm.

Subsequently, Az-8 (7 g) was dissolved in tetrahydrofuran (70 ml) and 3 g of aminobutylaldehyde dimethylacetal was added dropwise to the solution at 5–10°C. After 2 h, the absorbance at 390 nm in the reactant suspension disappeared and a new absorbance at 316 nm appeared. Water (200 ml) and aqueous ammonium solution (2 ml) were added to the solution and the mixture was stirred for 2 h. The resulting precipitate was filtered from the solution. Approximately 3.5 g of 2-(3-(4-azidophenyl)prop-2-enoylamino)-*N*-(4,4-dimethoxybutyl)-3-(3-pyridyl)prop-2-enamide (Az-8-γAB) was obtained. ^{1}HNMR, 8.97 (s, 1H), 8.55 (s, 1H), 8.42 (d, 1H, *J=4*), 7.73 (d, 1H, *J=8*), 7.51 (d, 1H, *J=15.6*), 7.39 (d, 2H, *J=8.8*), 7.24 (dd, 1H, *J=4,8*, 8.0), 7.08 (t, 1H, *J=5.2*), 6.96 (d, 2H, *J=8.4*). 6.60 (s, 1H), 6.51 (d, 1H, *J=15.6*), 4.36 (t, 1H, *J=4.8*), 3.31 (s, 6H), 3.31–3.28 (m, 2H), 1.66–1.59 (m, 4H). Elemental analysis showed; C, 61.05; H, 5.78; N, 18.56, Calculated; C, 61.32; H, 5.82; N, 18.66.

Finally, the photo-reactive polyvinylalcohol (Azido-unit pendant Water-soluble Photopolymer, AWP) was synthesized as follows. Fifty grams of polyvinylalcohol (degree of polymerization was 1700 and 88% of hydroxyl groups were converted to acetal, EG-30, Nippon Gosei Co., Ltd) was dissolved in 300 ml of water at 80°C for 1 h and Az-8-γAB (3.2 g) was added to the solution with 1.5 g of phosphate acid and 100 ml of water at 60°C. The mixture was allowed to stand for 24 h at 60°C. After the reaction, 30 g of ion-exchange resin (WA-20, Mitsubishi Chem. Co., Ltd.) was added to the solution and allowed to stand for 3 h to remove the phosphoric acid until the pH of the solution reached 6.5. The resin was removed by polyester filter (400 mesh). The solution contained 10% (w/v) AWP and had a viscosity of 2825 cP/25°C.

GPC measurement was performed using GS 320HQ (Shodex Asahipak; column size, 7.6 × 500 mm^2; eluent, mixture of 1.428% NaCl water/acetonitrile (7/3); flow rate, 0.5 ml/min; temperature, 50°C; detection absorbance, 290 nm).

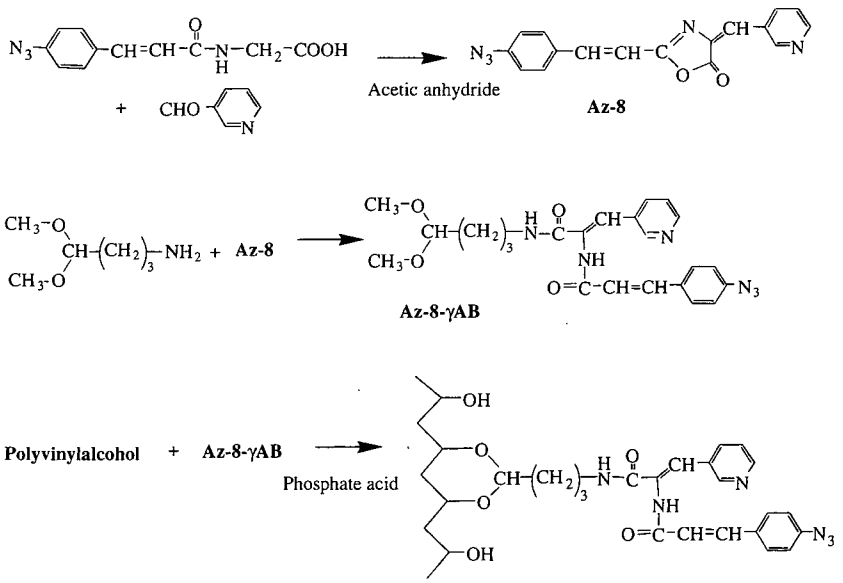

Fig. 1. Synthetic scheme of photo-reactive polyvinylalcohol.

2.2. AWP coating and characterization

An aqueous solution of AWP (1 wt%) was spin-coated onto a glass plate at 350 rpm for 2 s, followed by 1400 rpm for 30 s. The coated plate was air-dried at 40°C for 5 min. The thickness of coated AWP was measured by surface roughness measurement (Toyo Seimitsu Co., Ltd., Surfcom920B) after scratching the surface to a 0.1 μm width. Percentage of the remaining resin layer was determined by the thickness before and after UV-irradiation.

2.3. Protein microarray

A micro-spotter (Stampman®) produced for DNA microarraying by the Nippon Laser Electric Co. (Nagoya, Japan) was used for the microarray experiment as previously reported [27]. The arrayer dip pen was incubated in an aqueous solution located in one of the wells of the plate, lifted from the well, and placed on a cell culture dish for a prescribed time. Subsequently, the pen was moved to a washing dish and washed as described below. The dip pen was incubated in a 0.1% sodium dodecylsulfate solution for 5 s, water for 5 s, and ethanol for 5 s. Finally, it was dried in vacuo for 14 s. The dried pen was then incubated in the next aqueous solution. These processes were controlled by personal computer and were repeated for the complete microarray construction.

A protein microarraying was carried out as shown in Fig. 2. First, the glass plate was spin coated with the prescribed concentrations of AWP. After AWP coating, an aqueous solution of protein at various concentrations was cast onto the coated plate. Subsequently, the plate was irradiated with ultraviolet light (153 mW/cm^2) using an UV Spot Light Source L5662 (Hamamatsu Photonics Co., Hamamatsu, Japan). The plate was then washed five times with water. Except for the light-irradiation, all processes were carried out at 4°C.

2.4. Cell adhesion assay

COS-7 cells were cultured in Dulbecco modified Eagle's medium (DMEM, Sigma) with 10% fetal bovine serum (FBS) and RAW264, HepG2 and STO cells were cultured in a minimum essential medium (MEM, Sigma) with 10% FBS and 1% non-essential amino acids (Invitrogen Life Technologies). The cells were harvested with a 0.25% trypsin solution containing 0.5 mM EDTA. The recovered cells were washed with culture medium and suspended in each medium (4.0×10^4 cells per 12 well culture plate, Iwaki). The cell suspension was added to the protein microarrayed dishes, and allowed to stand for 2 h or 3 d at 37°C. After incubation, the dishes were washed three times with phosphate-buffered saline, and cells were directly observed by phase-contrast microscopy (× 40, Olympus).

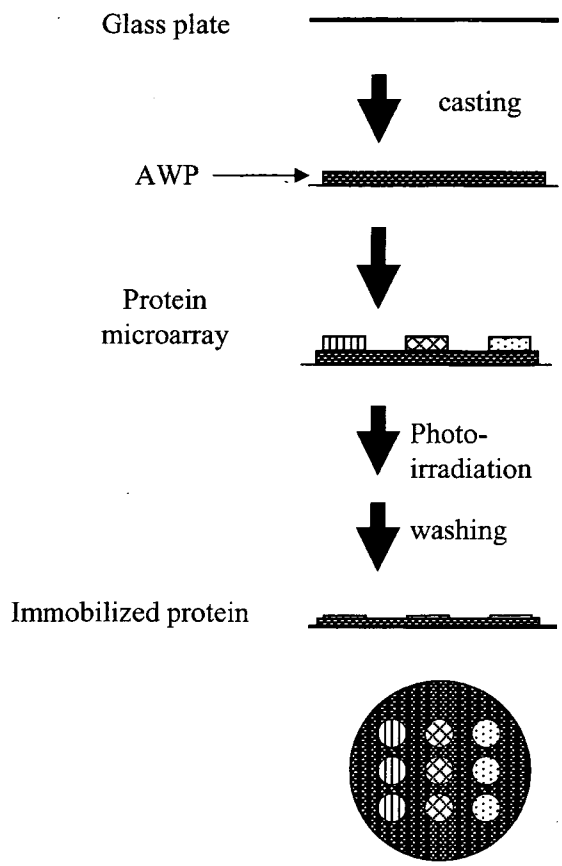

Fig. 2. Preparative scheme of protein microarray.

3. Results and discussion

3.1. Preparation of AWP

GPC charts of Az-8-γAB and AWP are shown in Fig. 3. The retention time of Az-8-γAB was 38.4 min. On the other hand, the retention time of AWP was 11.2 min. Aldehyde of Az-8-γAB, which was produced at acidic conditions, was observed at the retention time of 44.2 min. The GPC chart indicated that almost 100% of the Az-8-γAB reacted with the polyvinylalcohol.

Fig. 4 shows the ultraviolet spectrum of Az-8-γAB and AWP. The absorbance peak slightly shifted. However, there is no liquid dissolving both compounds, therefore a precise comparison based on the chemical structure is difficult. The content of incorporated azidophenyl groups in the polymer was the same as the feed content 0.7 mol%. The value was the same as that calculated from the calibration curve using Az-8-γAB.