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The Movement of a Water Droplet on a Gradient Surface Prepared by Photodegradation

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Received August 24, 2006. In Final Form: November 4, 2006

A hydrophobic to hydrophilic gradient surface was prepared using the tuned photodegradation of an alkylsilane self-assembled monolayer (SAM) using irradiation of vacuum ultraviolet light (wavelength = 172 nm). The water contact angle on the photodegraded SAM surface was adjusted using the intensity and time photoirradiation parameters. The formation of a gradient was confirmed by fluorescent labeling. The water drop moved from the hydrophobic to hydrophilic surface with a velocity that depended on the gradient. The higher the gradient, the faster the water moved. For the first time, we have prepared a gradient surface using photodegradation where the movement of a water drop was regulated by the degree of gradation. Considering that the photodegradation technique can be applied to various surfaces and to lithography, this technique will be useful for various material surfaces.

1. Introduction

A gradient surface displays a gradual change in the chemical and physical properties along its length. Gradient surfaces have a wide range of applications in material science.[1−20] A gradient in a surface can induce the net mass transport of liquids, which affords a driving force for operation of microfluidic devices and for biological cell motility in vitro.

Therefore, different preparation methods for gradient surfaces, including thermal,[21] vapor-phase diffusion,[1,4−9,11,12] electrochemical,[22−24] photolithographic,[10] gradual immersion,[14,15] photoinitiator-mediated photopolymerization,[25] corona treatment,[26] contact printing,[27] microfluidic device,[28] and microstructured[29] approaches have been developed. Not only the surface properties but also protein gradients[30−36] have been devised, and these techniques are generally based on a generation process.

Recently, Sugimura and co-workers[37−39] have reported on an effective method that can photopattern alkyl- and fluoroalkyl-

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10.1021/la0624992 CCC: $37.00
Published on Web 12/22/2006

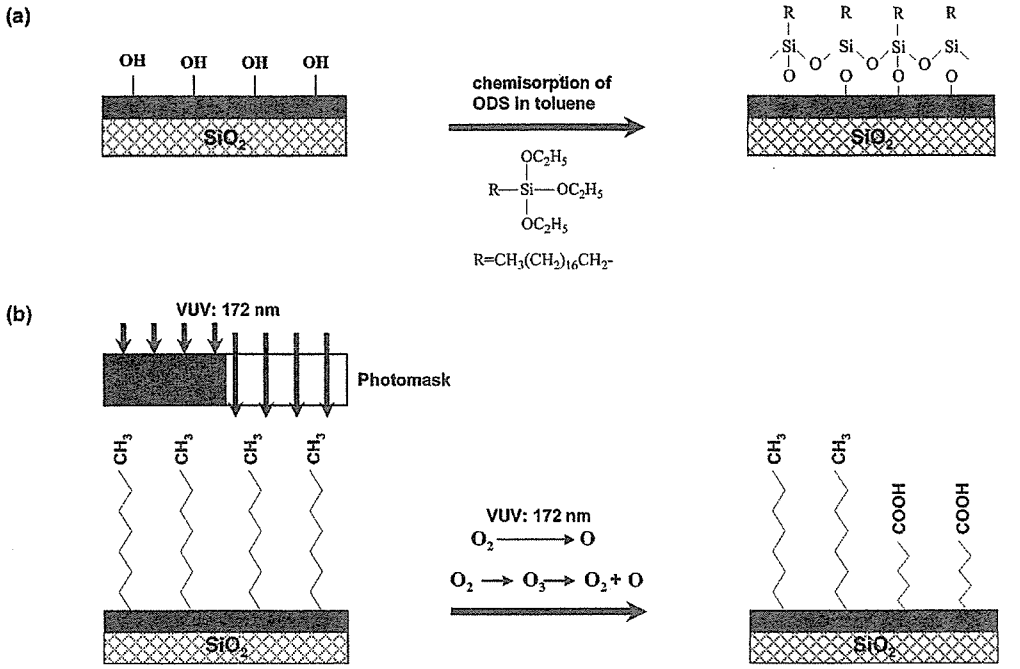

Figure 1. Preparation of surface gradient by photodegradation. (a) Surface treatment of glass plates by a silane coupling reaction. (b) Excitation of the surface molecules by VUV irradiation and oxygen molecules cleaved by the VUV.

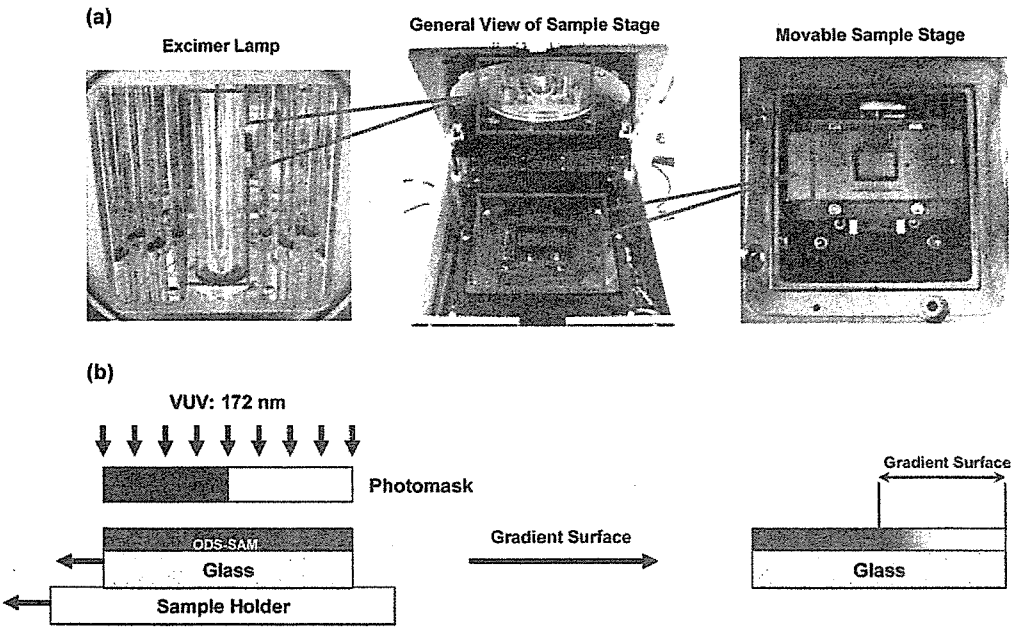

Figure 2. The photoirradiation apparatus used to prepare gradient surface. (a) A general view of the excimer device and (b) preparation of a gradient surface on an ODS-SAM surface using a VUV excimer device. A drop of water moved from the hydrophobic to the hydrophilic surface. The hydrophobicity and hydrophilicity of the SAM surface is shown by the gray and white colors.

silane self-assembled monolayers (SAMs) and collagen using vacuum ultraviolet light (VUV), which has wavelengths shorter than 200 nm. They showed that SAMs were effectively decomposed under VUV irradiation at 172 nm through a photomask.

In this work, for the first time, we have prepared a gradient surface using photodegradation. Considering that the photodegradation technique can be applied to various surfaces, and to lithographic studies, this technique will be useful to various applications. In addition, it was possible to reproducibly prepare well-defined gradient surfaces. Here, the movement of a water drop on different gradient surfaces was investigated.

2. Materials and Methods

2.1. Materials. The *N,N*-dimethylformamide (DMF) and dehydrated toluene used were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan). The dicyclohexylcarbodiimide (DCC) used was purchased from Tokyo Kasei Co. (Tokyo, Japan). The *n*-octade-

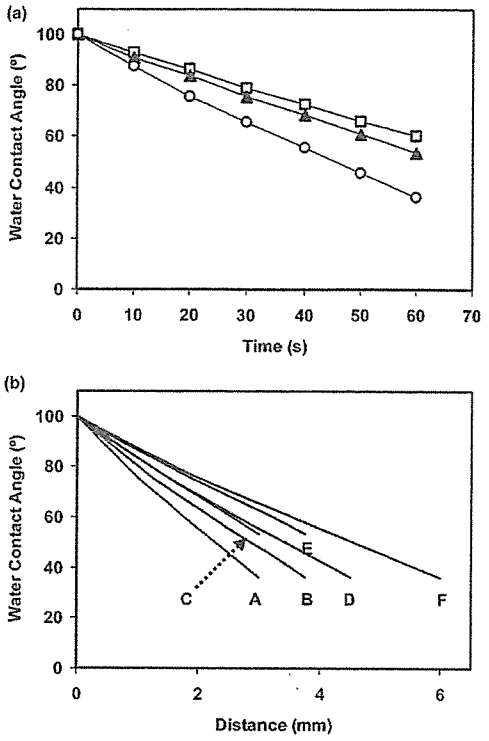

Figure 3. Water contact angle measurements. (a) Static water contact angles under different irradiation light intensities: open circle, 3.3 mW/cm^2; closed triangle, 1.2 mW/cm^2; and open square, 0.6 mW/cm^2. (b) The distance-dependent water contact angle is calculated using data from Figure 3a. The irradiation energy and slide movement rate were (A) 3.3 mW/cm^2 and 0.05 mm/s, (B) 3.3 mW/cm^2 and 0.0625 mm/s, (C) 1.2 mW/cm^2 and 0.05 mm/s, (D) 3.3 mW/cm^2 and 0.075 mm/s, (E) 1.2 mW/cm^2 and 0.0625 mm/s, and (F) 3.3 mW/cm^2 and 0.1 mm/s.

cyltrimethoxysilane [ODS, $CH_3(CH_2)_{17}Si(OCH_3)_3$] used was purchased from the Shin-Etsu Chemical Co. Ltd. (Tokyo, Japan). The fluoresceinamine isomer I (FA) used was purchased from Kanto Kagaku Chemicals (Tokyo, Japan). The microcover glass plates (area = 18 × 18 mm^2, thickness = 0.25−0.35 mm) used were purchased from Matsunami Glass Co. Ltd. (Osaka, Japan).

2.2. Surface Treatment with ODS. The surfaces of the glass plates were treated as shown in Figure 1. First, the surfaces were photochemically cleaned using an excimer VUV lamp (λ = 172 nm, power = 10 mW/cm^2, Ushio Electric, Model UER20-172V, Tokyo, Japan) for a period of 10 min before forming an alkylsilane SAM on the surface. The VUV treatment was applied to decompose any organic molecules that may have contaminated the surface. The complete removal of any organic material was confirmed by the observed decrease in the water contact angle, with the value of $\theta \approx 0°$ being indicative of a fully hydrophilic surface.

The cleaned plates were placed in a flask containing 40 mL of *n*-octadecyltrimethoxysilane (10 mM ODS in dehydrated toluene). The coupling reaction was carried out at 80 °C for a period of 5 h. The hydrophobilized plates were then rinsed in fresh toluene followed by drying in a clean vacuum oven at 80 °C for a period of 4 h.

2.3. Gradient Surface by the Photoirradiation Method. The ODS-SAM covered glass plate was irradiated using VUV light with wavelength = 172 nm through a photomask using an apparatus that was specifically developed for this study, as shown in Figure 2a. The operational mechanism is illustrated in Figure 2b. An excimer lamp (power = 10 mW/cm^2, Ushio Electric, UER20-172V, Tokyo, Japan) was used as the light source. The photomask consisted of a 2-mm-thick quartz glass plate with 93% transparency at 172 nm and a 0.1-μm thick chromium. The distance between the lamp and the photomask was 1 mm. The distance between the mask and sample was 1.5 mm. The light irradiation was conducted in air. The total light intensity was controlled using mesh filters, and the intensity at the surface was estimated using a photometer (Ushio Electric, Unimeter UIT-150). The gradient surface was prepared by moving a sample stage at a controlled velocity of 50−100 μm/s.

2.4. FA Labeling Method. The pattern and gradient surfaces were labeled with FA. To enhance the nucleophilic attack by amino group FA, DCC was used for the coupling reaction of amines with carboxyl groups by converting the carboxylate groups into activated esters. If the aldehyde groups were on the surface, they were considered to react with FA without such activation. First, DCC (95.04 mg) was dissolved in DMF (4 mL) and the sample plates were then soaked in this solution and were incubated for a period of 2 h at room temperature. Subsequently, a freshly prepared solution of FA (79.99 mg) in DMF (4 mL) was added. The reaction was carried out in darkness at room temperature for a period of 12 h. Then, the FA-labeled plates were washed thoroughly with DMF and were observed using a fluorescence microscope (Model T 01359, Carl Zeiss, Tokyo, Japan).

2.5. Water Contact Angle Measurements. The static water contact angles of the sample surfaces were measured at 25 °C in air using a contact angle meter (Kyowa Interface Science Co, Tokyo, Japan) on the basis of the sessile drop method. All of the contact angles were determined by averaging values measured at nine different points on each sample surface. The water contact angle error was about ±1°. For the contact angle measurements, a nonpatterned quartz glass plate was placed on the sample to ensure full irradiation of the sample and to investigate the surface states of the irradiated sample.

2.6. Measurement of the Movement of a Water Drop. The movement of a drop of water was measured using the same technique as reported in a previous paper.[40] A 2-μL water droplet was carefully placed on a sample plate. Sequential photographs of the sliding action of the water droplet on the surface were taken at 4−5 ms intervals using a high-speed digital camera system (Model 512 PCI, Photon Ltd., Tokyo, Japan). The sliding acceleration was estimated by measuring the sliding distance of the front or rear edge of the contact line between the droplet and sample surface from its initial starting point (Dipp Macro, Direct Co. Ltd., Tokyo, Japan).

3. Results and Discussion

3.1. Water Contact Angle Measurements. The surface hydrophilicity of ODS-SAM covered glass was estimated from water contact angle measurements (Figure 3). The water contact angle decreased with VUV irradiation time and also with VUV light intensity. Figure 3a shows that the water contact angle decreased monotonically with increasing irradiation time. This result also indicates that the gradient can be controlled by regulation of the irradiation time and light intensity.

Figure 3b shows the variation in contact angle versus distance, which was calculated from the data in Figure 3a by assuming that the irradiation time corresponds to the velocity of the moving stage. The surface exhibited a gradient in hydrophobicity as the water contact angle changed from 100° to about 25° over a distance of 6 mm.

3.2. Fluorescence Microscopy of the Pattern and Gradient Surface. The formation of a gradient on the ODS-SAM surface was confirmed using the FA labeling method. There have been a few reports on photochemical reactions involving chloromethylphenylsilane SAMs and COOH-terminated SAMs.[37,38] In these cases, the $-CH_2Cl$ groups absorbed UV light and subsequently oxidized on reacting with oxygen. On the other hand, in the case of VUV degradation of ODS-SAMs, the VUV light dissociatively excites chemical bonds, for example, C−C, C−H, and C−Si bonds, to form radicals,[41] as in the case where

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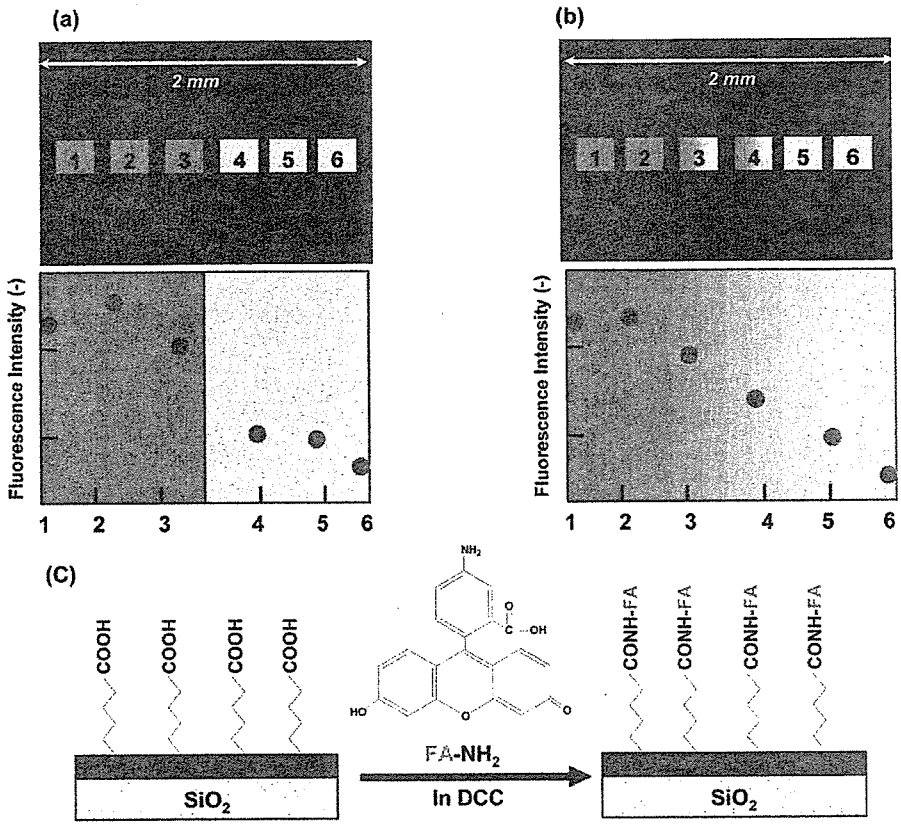

Figure 4. (a) Fluorescence microscopy of a pattern surface and (b) a gradient surface. The surface was labeled with 5-aminofluorescein ($FA\text{-}NH_2$), which targeted the VUV irradiated regions on the surface. Luminance = 1 mW/cm^2 and exposure time = 20 s.

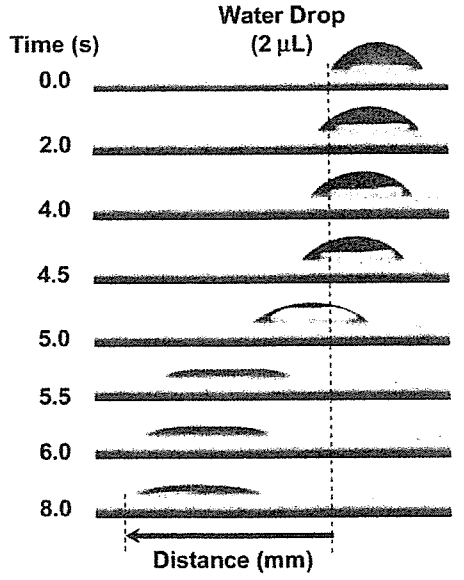

Figure 5. The movement of a water drop and its related equation. Water drop size = 2 μL.

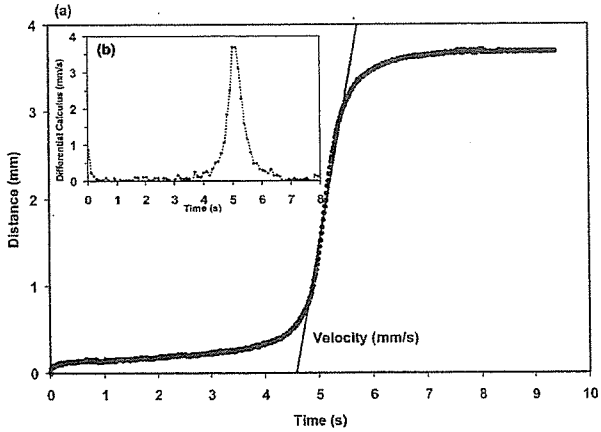

Figure 6. (a) Position of a droplet with nominal volume = 2 μL plotted against the transverse time, on a surface prepared such that irradiation energy and slide movement rate were 1.2 mW/cm^2 and 0.05 mm/s, respectively. The velocity of the water droplet was determined by the sharpest slope. (b) Time dependence of the differential of a.

soft X-ray irradiation is used.[42] These radicals can react further with oxygen and water molecules in the atmosphere. Furthermore, the VUV light is simultaneously absorbed with oxygen molecules and generates atomic oxygen species.[43] Since these activated oxygen atoms have a strong oxidative reactivity, the organic radicals formed from the direct VUV excitation of ODS-SAMs can react with the activated oxygen atoms to form −COOH groups. When the VUV irradiation is prolonged further, the ODS-

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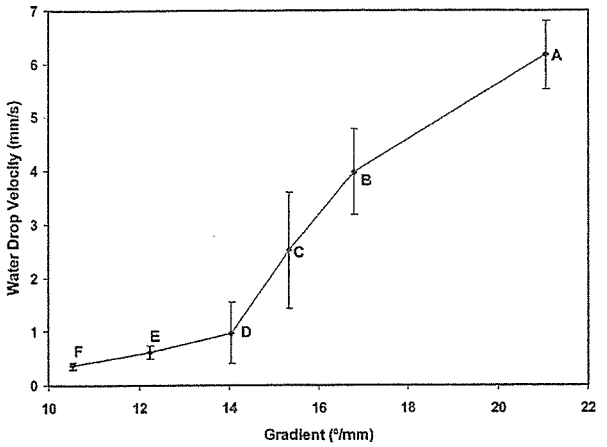

Figure 7. Relationship between the movement of a water droplet and the gradient of the contact angle with distance. A–F correspond to the data obtained from Figure 3b.

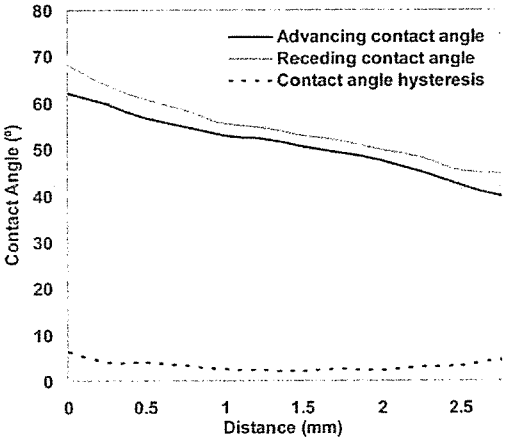

Figure 8. The time course of the movement of a water droplet on the surface (line A in Figure 3b).

SAM finally converts to form volatile species, such as H_2O, CO, and CO_2, and accordingly are removed from the substrate. In the VUV irradiation in our work, oxygen molecules were easily photolyzed to generate highly reactive oxygen radicals. COOH groups were considered to appear by the reaction of oxygen and carbon radicals with the formation of peroxy-radicals that react further with water.

Therefore, the photodegraded surfaces were considered to be labeled with FA-NH_2, while the masked areas (the alkyl chain of the ODS) did not react with the FA. Figure 4 shows fluorescence micrographs of the surfaces with, and without, moving the photomask. In Figure 4a, the bright and dark regions in the images correspond to the photoirradiated and masked areas, respectively. On the other hand, with the photomask moving, a gradient surface was generated, as shown in Figure 4b.

3.3. Observations of the Movement of Water Drops. When a 2-μL water droplet was placed in the gradient region (i.e., at the hydrophobic region), it moved from the hydrophobic to the hydrophilic region by spreading out. To evaluate the movement of a water drop under different conditions, the speed of the water drop was measured as illustrated in Figure 5. The movement of the water droplet as a function of its position is shown in Figure 6. The velocity of the water droplet varied across the gradient

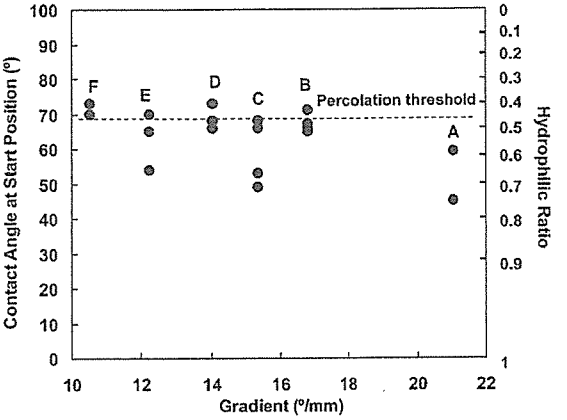

Figure 9. The relationship between the advancing contact angle of a water droplet at the position where it began to move (the hydrophilic ratio deduced from Cassie and Baxtor's equation) and the surface gradient. A–F correspond to the data in Figure 3b.

surface under different conditions, with an average speed of 0.5–6 mm/s, as shown in Figure 7.

The movement of the water droplet is reported to be caused by a contact angle hysteresis resulting from the surface gradient. Figure 8 shows the difference in the advancing and receding contact angles of a moving droplet. Recently, Moumen et al.[11] have analyzed the velocity using "sharp" and "gentle" gradient surfaces, according to a model they developed.[9]

In our work, we added a new factor, which is based on percolation theory. The possibility of water spreading by percolation of the hydrophilic region was proposed after a study on the photoinduced wettability conversion of TiO_2 polycrystalline thin films.[44] The polycrystalline TiO_2 thin film was composed of grains without specific orientation. Therefore, it was reasonable to assume that the hydrophilic conversion rate of each grain in the film was different. A proper UV illumination time induced the surface composed of hydrophilic grains where wettability conversion was fast and of hydrophobic grains where wettability conversion was slow.[45] When both hydrophilic and hydrophobic regions were within a certain ratio, the amphiphilicity of anatase polycrystalline films was attained, probably because of the connection of each region (between hydrophilic and hydrophilic regions or hydrophobic and hydrophobic regions) and the resultant two-dimensional capillary phenomena by these regions.[44]

Study of the effect of percolation between the hydrophilic region and the hydrophobic one on dynamic wettability is quite limited. Semal et al.[46] investigated spreading kinetics of an organic liquid (squalane) on the surface of mixed alkanethiol monolayers. They changed the ratio of hydrophilic and hydrophobic thiols and reported that a wetting transition occurred for squalane when the degree of hydroxylation of the surface was more than 70%. However, their surface was not a gradient surface, and this kind of research on a water droplet has not been reported so far. Therefore, here we applied the theory for the movement of a droplet on a gradient surface. The percolation threshold of a two-dimensional surface is approximately 0.45.[47] According to Cassie and Baxtor's equation,[48] the contact angle of a composite

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solid surface is expressed as

$$\cos\theta = f_1 \cos\theta_1 + f_2 \cos\theta_2$$

where f_1 and f_2 are the contributions of the hydrophilic and hydrophobic ratios, respectively. In our case, this was from the most hydrophilic VUV-irradiated surface, $\theta_1 = 0°$, and from the most hydrophobic ODS-modified surface, $\theta_2 = 100°$. When $f_1 = 0.45$, the contact angle, θ, of the percolation threshold of the contact angle is calculated to be 69°. Figure 9 shows the contact angle of positions where the water droplets began to move. As is clear from the figure, once the front edge of the droplet made contact with the position where the advancing contact angle was 69°, it spread and moved rapidly on the surface. Thus, in this study, it is deduced that the water droplet moved dominantly by the capillary force because of the formation of the percolation structure of the hydrophilic region. The driving force on the movement of a water droplet as far as the position where hydrophilic percolation was formed might be the surface energy difference.

In this experiment, the hydrophilic/hydrophobic ratio was continuously changing on the solid surface. Moreover, the change contains both increasing capillary width and the practical amount of capillary itself. For the modeling of the motion behavior of a water droplet on this surface, precise analysis of the change of hydrophilic region and the estimation of the resultant capillary effect are required. Considering that the velocity is not constant during the movement of a water droplet, it is difficult to explain the phenomenon by a single mechanism. In our investigation, the movement of the water droplet should be explained by both the contact angle hysteresis and the percolation, although it is difficult to predict the velocity of the movement from the latter factor.

Concluding Remarks. We prepared a gradient surface using photodegradation. The photodegradation method using ultraviolet light is useful for the reproducible preparation of a gradient surface, and these gradient surfaces can be used to quantitatively determine the movement of a water droplet. The sharper the surface gradient is, then the faster the water droplets move.

LA0624992

Biotechnol. Prog. **2006**, *22*, 924–932

Combinatorial Bioengineering: Review

Photoimmobilization for Microarrays

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A photoimmobilization method has been developed for the preparation of microarray biochips. This photoimmobilization method makes it possible to easily covalently immobilize various types of organic molecules and cells on a chip. In addition, by using hydrophilic polymers as matrixes, it is possible to reduce nonspecific interactions with biological components. Various proteins, antibodies, and cells have been microarrayed using this technique, and interactions between these proteins, antibodies, and cells have been investigated. This type of microarray biochip will be important for academic applications such as genomics, proteomics, and cellomics, and clinical analyses.

Contents

Introduction

Recent progress in the life sciences has been significantly enhanced by microfabrication technology. DNA microarray technology is a typical result of the combination of biotechnology and microfabrication technology. Now, targets that can be analyzed are expanding, with applications in genomics, proteomics, glycomics, cellomics, and metabolomics, and along with progress in bioinformatics, the importance of microarray technology is increasing in clinical analysis. The integrity of microarrays has increased in comparison with that of the integrated circuit (IC) of computer technology, in which the number of transistors per square inch has doubled every year since the IC was invented, in accordance with Moore's Law. The clinical use of DNA microarrays began in 2004, and some antibody and protein microarray chips have recently become commercially available.

Microarray technology is a crucial tool for large-scale and high-throughput biological science and technology (*1*). It allows fast, easy, parallel detection of thousands of addressable elements in a single experiment under identical conditions. The technology has provided scientists with various types of multiple analyte assay systems, which are likely to transform medical diagnosis in the future. Various types of protein microarrays and a variety of other methodologies have been developed to immobilize biological molecules, as shown in Table 1 (*1*–*11*). Snyder's group synthesized proteins carrying oligohistidine, immobilized the proteins on nickel-coated glass slides, and then investigated protein interactions using protein microarrays (*3*). Espejo et al. (*12*) fused protein domains with glutathione S-transferase for the preparation of microarrays, and Newman and Keating (*13*) have identified new protein–protein interactions using protein microarrays. Recently, Kiyonaka et al.

Table 1. Attachment Strategies for Microarray Production

functional groups	attachment: non-covalent	attachment: covalent	references
nitrocellulose	physical adsorption		15, 27, 68, 29, 69, 82, 83
polystyrene, silanized glass	physical adsorption		81, 84
self-assembled hydrogel	encapsulation		14
poly(L-lysine), poly(ethyl ene imine), amino	electrostatic adsorption		18–20, 86
cyano groups		amino groups	2, 17
aldehyde groups		amino groups	16
amino/bifunctional *N*-hydroxysuccinimide		amino groups	
mercapto groups		amino, thiol groups	
epoxy groups		amino, thiol, hydroxy	26, 30
Au		thiol groups	23, 24, 28
solid synthesis			25
nickel coating	His-tag		3
neutravidin	biotinylated		
avidin	biotinylated		
phenyl nitrene		organic groups	32–38, 44–46, 51–61
phenyl carbene		organic groups	39–43, 48–50

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10.1021/bp060143a CCC: $33.50
Published on Web 06/17/2006

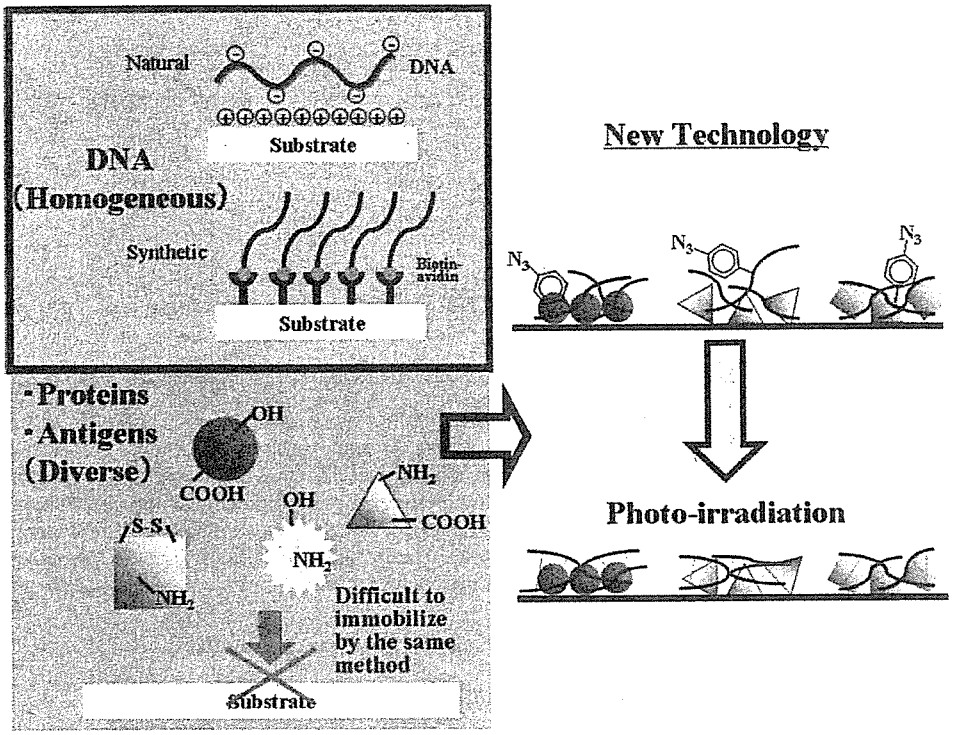

Figure 1. Comparison of DNA- and protein-immobilization and photoimmobilization. Because DNA is chemically homogeneous and can be specifically modified by organic synthesis it is easy to immobilize on a chip (top left). However, because proteins are chemically different, it is difficult to immobilize them with the same methods used to immobilize DNA (bottom left). The photoimmobilization method was invented to covalently immobilize various biomolecules, including proteins (right).

invented a microencapsulation method for microarrays using hydrogels (*14*). Various immobilization methods, including protein microarrays (*15*–*17*), membrane–protein microarrays (*18*–*21*), antibody microarrays (*22*), peptide microarrays (*23*–*25*), small-molecule microarrays (*26*), and sugar microarrays (*27*–*29*), have been developed by many research groups (Table 1).

Although many types of biopolymer microarray have been investigated, the practical technology has not yet been developed. One of the reasons why biopolymer microarray technology has not been developed as rapidly as DNA microarray technology is the difficulty in immobilizing proteins. Although DNA contains varied genetic information, its structure is composed of four chemically similar bases. Therefore, DNA is considered to be a homogeneous biopolymer. In addition, the DNA biopolymer is conveniently synthesized by the solid phase method. In principle, different DNAs and peptides can be immobilized or synthesized on one plate by the same method (Figure 1, top left panel). In contrast, the chemical structure of individual proteins is different. Proteins have different functional groups, and they also differ in quantity and location. Therefore, it is very difficult to covalently immobilize different proteins, polysaccharides, antigens, and cells on the one chip using the same method (Figure 1, bottom left panel). Fall et al. (*30*) reported some difficulties in screening of some allergen-specific IgEs, although they did not attribute it to difficulties in the immobilization method. It is easy to understand why covalent immobilization is difficult, because biological molecules may not have enough functional groups or their recognition sites may be used in the immobilization process. To overcome the difficulties of covalent immobilization of various types of biocomponents by the same method, we have invented a new photoimmobilization technique (Figure 1, right panel) for the preparation of microarrays (*31*).

The photoimmobilization method uses photogenerated radical cross-reactions. Because radical reactions occur on every organic material, including biological molecules, the polymer matrix, and the chip surface, the method does not require any special functional groups, such as amino, carboxyl, hydroxyl, or thiol groups, unlike other conventional immobilization methods. Therefore, different biocomponents can be immobilized by the same method. In addition, as shown in Figure 2, it is possible to immobilize biological materials without molecular orientation. Some orientation usually occurs when the conventional immobilization method is used because of the uneven distribution of functional groups on biological molecules. In contrast, there is no particular orientation of immobilized molecules when the photoimmobilization method is used. This method is suitable for multiple interactions of immobilized molecules with analytes because recognition sites on the immobilized molecules are not as limited.

To date, we have used this technique to immobilize growth factor proteins on a solid matrix to show the effect of immobilized protein on cell function (*32*–*38*). Using micropattern-immobilization, the effect of the immobilized growth factor proteins was visualized by observing cellular behavior on immobilized and nonimmobilized regions of the matrix (*32*–*38*). Because the immobilized proteins retain their biological activity, even after immobilization, we considered that photoirradiation does not significantly destroy the 3D structure of the immobilized biomolecules and applied this immobilization technology to the preparation of microarray biochips. In the present article, the photoimmobilization method is introduced and its applications in cell and antibody analysis are described.

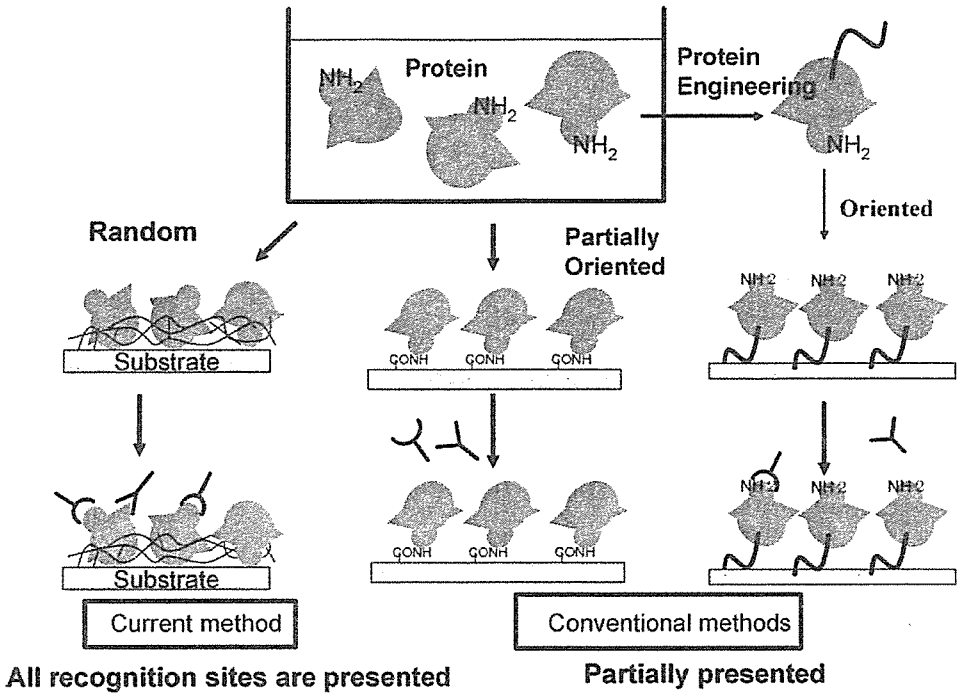

Figure 2. Comparison of immobilization methods. Photoimmobilization leads to the random orientation of immobilized molecules, whereas other covalent immobilization methods lead to some orientation of immobilized molecules because of the uneven distribution of functional groups on the molecules being immobilized. In genetically engineered proteins, which have adhesive peptide sequences on the end chain, the recognition site is limited to the remaining part of the molecule.

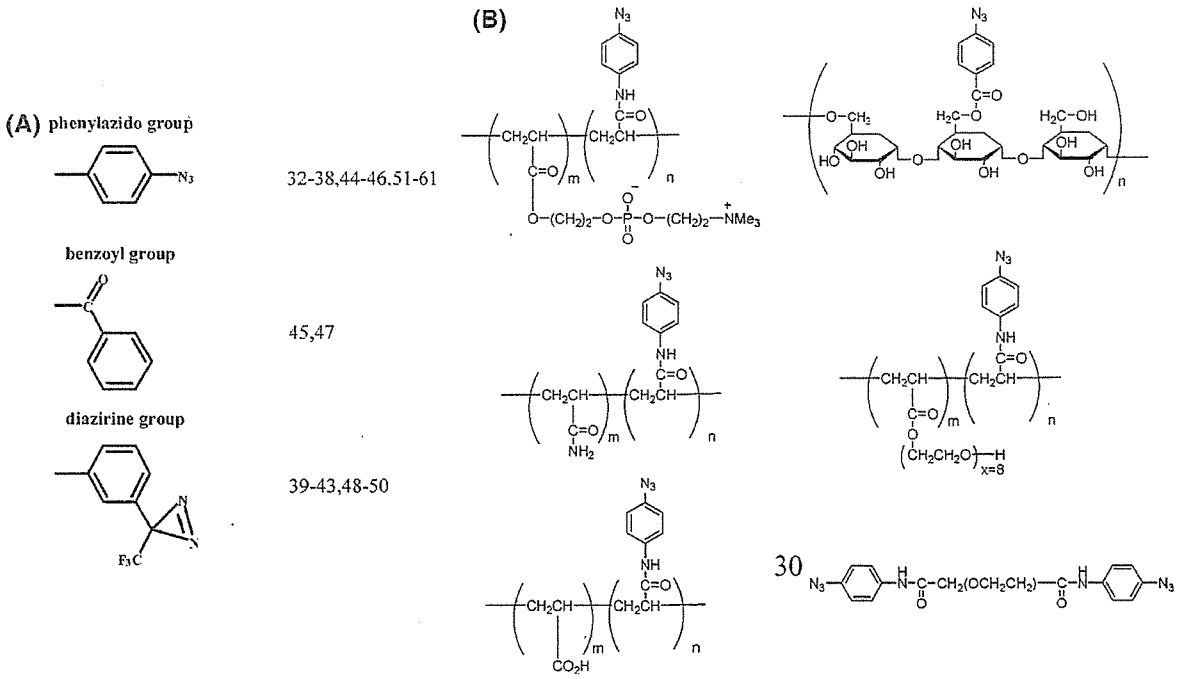

Figure 3. (A) Examples of photoreactive functional groups used in photoimmobilization. The numbers on the right indicate the references for photoimmobilization studies that have been conducted on each photoreactive functional group. (B) Photoreactive polymers synthesized by our group's current work.

Photoimmobilization

Photoreactive functional groups that have been used in photoimmobilization are shown in Figure 3A. Many researchers have performed the photoimmobilization of biomolecules to material surfaces, and some types of photogenerated nitrenes, carbenes, and ketyl radicals have been used to form covalent links with target molecules and materials. Sigrist's group (*39*–*43*), for example, used diazirine derivatives to generate carbene groups by photoirradiation. Matsuda's group (*44*, *45*) used azidophenyl, benzophenone, dithiocarbamate, and camphorquinone as UV-reactive groups, and fluorescein, eosin, and Rose Bengal as visible light-sensitive groups. In addition, they used

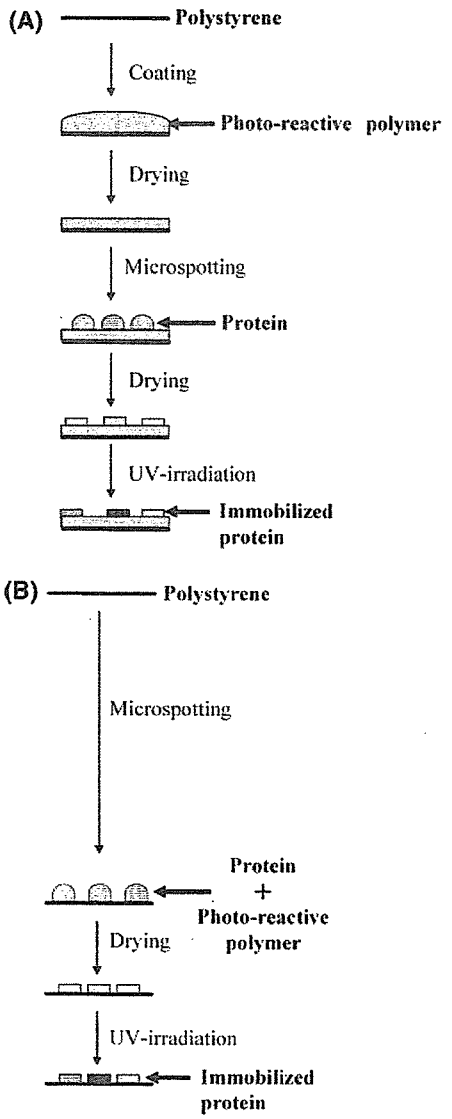

Figure 4. Synthesis of microarray chips by photoimmobilization. (A) After coating with a photoreactive polymer, proteins were microspotted and immobilized by photoirradiation. (B) Proteins were mixed with photoreactive polymer, and the mixture was microspotted and immobilized by photoirradiation.

photocross-linking and photopolymerization methods for the photoimmobilization of target molecules. Recently, Miller et al. (*46*) used azidophenyl groups for the preparation of an antibody microarray. Prucker et al. (*47*) incorporated benzophenone on a plate and immobilized organic molecules and macromolecules to investigate binding strength. Recently, Sigrist's group synthesized diazirine-containing dextran to reduce nonspecific interactions and immobilize proteins on a plate (*43*). Kanoh et al. (*48*–*50*) prepared photoaffinity glass slides containing aromatic diazirine through a poly(ethylene glycol) anchor and immobilized low molecular weight molecules on the slide by irradiation with 360 nm light.

We developed some photoreactive polymers in previous work (*32*–*38*, *51*–*56*). Figure 3B shows the polymer that was employed in the current work (*57*–*61*). Azidoaniline or azidobenzoic acid has been incorporated into water-soluble synthetic polymers, including poly(acrylic acid) (*57*), poly(vinyl alcohol) (*58*), poly(phosphatidylcholine methacrylate) (*59*, *60*) and poly-

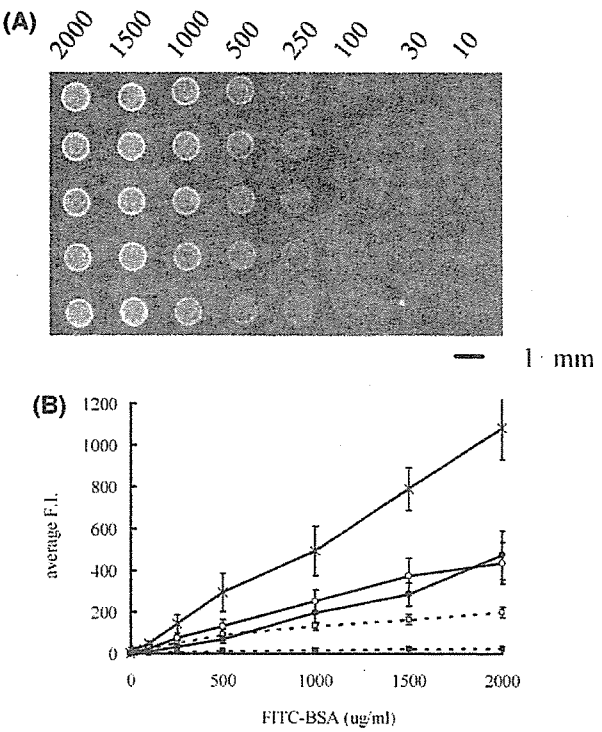

Figure 5. (A) Micrograph of a microarray of fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) with a photoreactive polymer after washing with phosphate buffered saline (PBS). The concentrations of FITC-BSA used in spotting were 2000, 1500, 1000, 500, 250, 100, 30, and 10 μg/mL. Bars represent 1000 μm. (B) Fluorescence intensity of cast FITC-BSA (crisscross), FITC-BSA immobilized with photoreactive polymer and washed with 0.1% Tween-20 (●) or PBS (○), and FITC-BSA without photoreactive polymer washed with 0.1% Tween-20 (■) or PBS (□).

(ethylene glycol) (*61*). Because these water-soluble polymers do not nonspecifically interact with biological components, they have been used to form an immobilization matrix to reduce nonspecific interactions (N) and to enhance specific interactions (S), resulting in a high S/N ratio (*57*–*61*).

Two schemata for the synthesis of a microarray chip are shown in Figure 4. Some researchers have adopted the method shown in Figure 4A, and the commercially available "Photochip" is made by this method (*43*). A high amount of protein is considered to be immobilized when using the method shown in Figure 4B because the photoreactive polymers, shown in Figure 3B, are soluble in water, thus allowing proteins or cells to be mixed with them in water (*60*, *61*). The aqueous solution containing the photoreactive polymer and biomolecules of interest is then microarrayed, dried, and photoirradiated.

The photoimmobilization method can be applied to various biological components including proteins, antibodies, and cells. Here, we demonstrate some applications of photoimmobilized microarrays.

Cell Analysis

The modulation of cellular activity through substrate interactions can have a significant effect on biomaterial-based therapies. Material-based control of cellular function is a potentially powerful tool for controlling stem cells, which have the potential to differentiate into many different tissue types. The development of new biomaterials has been an iterative process: polymers are rationally designed and then individually tested for their properties. More recently, attention has focused on the

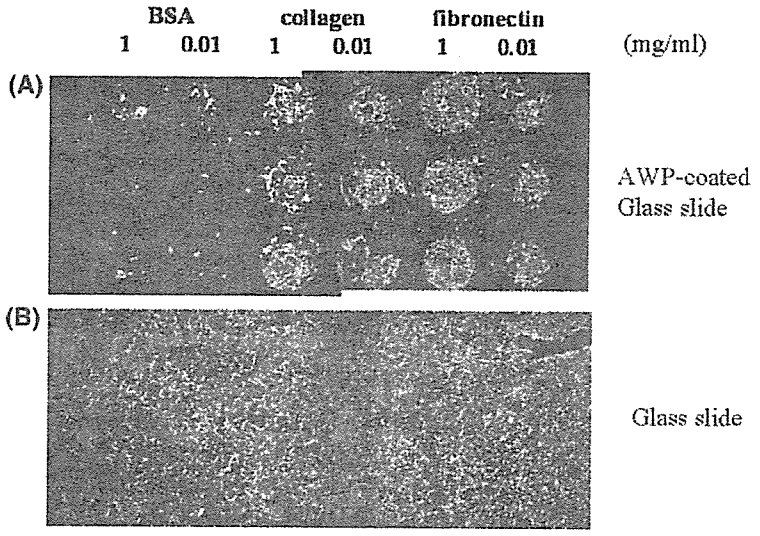

Figure 6. Micrograph of mouse fibroblast STO cells adhered to bovine serum albumin (BSA), collagen, and fibronectin-spotted AWP-coated glass slides (A) or glass slides only (B) over 2 h. The AWP-coated surface reduced nonspecific adhesion of cells to the slide.

development of parallel, combinatorial approaches, and the development of diverse libraries of polymeric biomaterials. Langer's group (*62*, *63*) described a platform that enables the nanoliter-scale synthesis and cell-based screening of thousands of microarrayed biomaterials using human embryonic stem cells. In addition, they prepared an array comprised of blends of well-characterized biodegradable polymers and tested these to examine the ability of this system to efficiently screen biomaterials in a range of cell types.

In our previous work, we demonstrated that some immobilized growth factor proteins enhanced cell growth more than soluble growth factor proteins and that other immobilized growth factors induced effects that were different from the effects induced by soluble growth factors (*64*−*67*). In current work, we microarray-immobilized proteins to examine the effect of immobilized proteins on cellular functions, especially cell adhesion.

Protein Microarray. ***Preparation.*** One of the photoreactive polymer, poly(acrylic acid) containing a phenylazido group in the side chains, was synthesized by a coupling reaction of poly(acrylic acid) with azidoaniline (*57*). An aqueous solution containing the photoreactive polymer was microarrayed onto on the surface of a polystyrene dish (chip). After drying, protein of various concentrations was spotted onto the polystyrene chip. Finally the chip was irradiated with UV light.

Fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) of different concentrations was then microspotted by a microarrayer onto the polymer cast and photoimmobilized (Figure 5A). Figure 5B demonstrates the binding strength of the immobilized proteins. When BSA alone was microspotted onto the chip and the chip was washed with water or the detergent Tween, the BSA immobilized by the photoreactive polymer was not washed out. This result demonstrated that the proteins were covalently immobilized on the chip.

Using the method shown in Figure 4B, a microarray chip was fabricated using another type of photoreactive polymer, the azide-unit pendant water-soluble photopolymer (AWP; *58*). AWP is a poly(vinyl alcohol) with 0.7% phenylazido groups on its side chains and has an affinity to glass surfaces. AWP solution was spin-coated onto a glass slide and pattern-immobilized in the presence of a photomask. When adsorption onto the glass slide was measured, the pattern formed was stable in organic solvents, including methanol, acetone, and tetrahydrofuran. BSA, collagen, and fibronectin were photoimmobilized on AWP-precoated glass slides.

Assay of Cell Adhesion. Different amounts of BSA or fibronectin were microarrayed, and adhesion of some cell types, including mouse leukemia monocytes RAW264, African green monkey COS-7 kidney cells, and rat pheochromocytoma PC12 cells, was investigated (*57*). The number of cells that adhered to the photoreactive polymer-immobilized region was the same as the number of cells that adhered to nontreated polystyrene. In contrast, the adhesion of COS-7 and PC12 cells to BSA-immobilized regions was strongly suppressed. Although immobilized fibronectin did not enhance cell adhesion, it did enhance cell spreading for a short time. These cellular behaviors are similar to those observed on protein-adsorbed surfaces. This indicates that the photoimmobilized proteins have the same activity as the adsorbed proteins, which are usually employed in the biological sciences.

Proteins were microarrayed onto glass slides precoated with AWP, and cell adhesion was investigated (*58*). Figure 6 shows the results of the adhesion of mouse fibroblast STO cells. The STO cells adhered not only to collagen- and fibronectin-spotted AWP-coated glass slides, they also adhered to the glass surface. Thus, when proteins are microspotted onto glass slides, it is difficult to assay for cell adhesion. However, no adhesion was observed on the AWP-coated surface. Therefore, the AWP-coated surface allows proteins to be assayed for cell adhesion. In addition to the STO cells, human hepatocyte HepG2 cells, COS-7 cells, and RAW264 cells did not adhere to the AWP-coated surface. Thus, the AWP-coated surface provided good contrast micrographs for the cell adhesion assay and was useful for profiling cell properties.

Antibody Microarray. Antibody microarray for the detection of cell-surface antigens has also been reported (*68*−*72*). Analysis of cell-surface antigens is usually performed by flow cytometry. The principle of this technique is the adsorption of fluorescent-labeled antibody onto a cell-surface antigen and the detection of fluorescence by a laser. It is now possible to detect six different types of fluorescence simultaneously. However, it is difficult to simultaneously detect more than six types of fluorescence. Therefore, to replace and match flow cytometry, 50 different antibodies to anti-cluster of differentiation (CD)

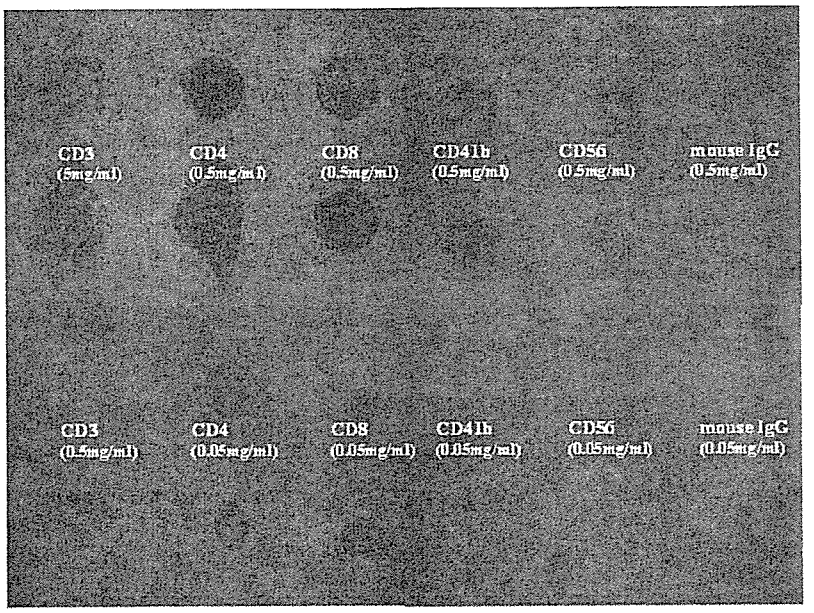

Figure 7. Human leukocyte adhesion to anti-cluster of differentiation (CD) antibody-immobilized surface in the presence of serum. Because the leukocytes have CD4, CD8, and CD41b antigens on their surface, they adhered to the corresponding antibodies immobilized on the chip. The concentrations indicate the antibodies spotted onto the chip.

antigens were microarrayed onto a nitrocellulose membrane to detect CD antigens in human lymphocytes (*68*, *69*). The lymphocytes of healthy individuals were then compared with the lymphocytes of patients who had different levels of expression of CD19, CD20, CD21, CD22, CD23, CD24, CD25, and CD37. These results were confirmed by flow cytometry.

Figure 7 shows the immobilization of anti-CD antibodies by our photoimmobilization method for use in an assay of CD antigens in human monocytes. Glass slides were treated with octadecyltriethoxysilane, and the antibodies were microarrayed onto the surface with a photoreactive polymer. Human monocytes were then placed on the microarray glass for 90 min. After staining with Giemsa dye, it was demonstrated that human monocytes did not adhere to mouse IgG; however, they did adhere to CD4 and CD8 antibodies. Therefore, the microarray method is very useful for detecting multiple CD antigens.

Antibody Analysis

Allergen Microarray. The photoimmobilization method not only has the ability to covalently immobilize organic materials on a chip, it also orientates the immobilized materials more randomly than conventional methods, as illustrated in Figure 2. Therefore, the most suitable application for this technique is basic research that investigates various interactions between biological molecules and clinical diagnosis for the detection of polyclonal antibodies in blood. Because a polyclonal antibody has many recognition sites, the immobilized antigen also provides many recognition sites. Oriented immobilization must cover some of these recognition sites. Therefore, the photoimmobilization method is suitable for allergy diagnosis.

Allergy diagnosis using a microarray biochip was performed as follows (*31*). Various allergens were immobilized on a polymeric chip using the photoreactive polymer, and human serum was added to the chip. After 10 min, the chip was washed, and the bound IgE was detected by anti-IgE-antibody-containing peroxidase. Finally, a substrate for chemiluminescence was added to the chip. Chemiluminescent detection is useful for the construction of a relatively cheap measurement machine. Figure 8 shows the results of chemiluminescent detection of an allergy test using microarrays (*31*). Five allergens were microarrayed and the adsorption of IgE was investigated. Serum from five different patients had different reactivities to the five allergens.

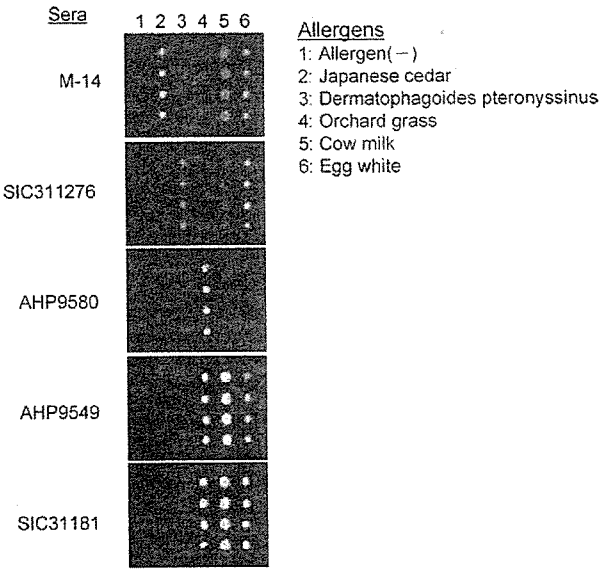

Figure 8. Microarray showing detection of adsorbed IgE from the sera of five different patients (M-14, SIC311276, AHP9580, AHP9549, and SIC31181) to five different allergens (Japanese cedar, *Dermatophagoides pteronyssinus*, orchard grass, cow's milk, and egg white).

To date, various types of allergen microarrays have been prepared (*61*, *73*−83). However, as described above, there are some difficulties with some antigen arrays, as reported by Fall et al. (*30*). The photoimmobilization method provides a solution for this problem.

Panel Cell Microarray. Cells were also microarrayed using the photoimmobilization method. In addition to molecular immobilization, some pathogens or cells were microarrayed for multiple analyses (*84*−*86*). Schwenk et al. (*86*) prepared a

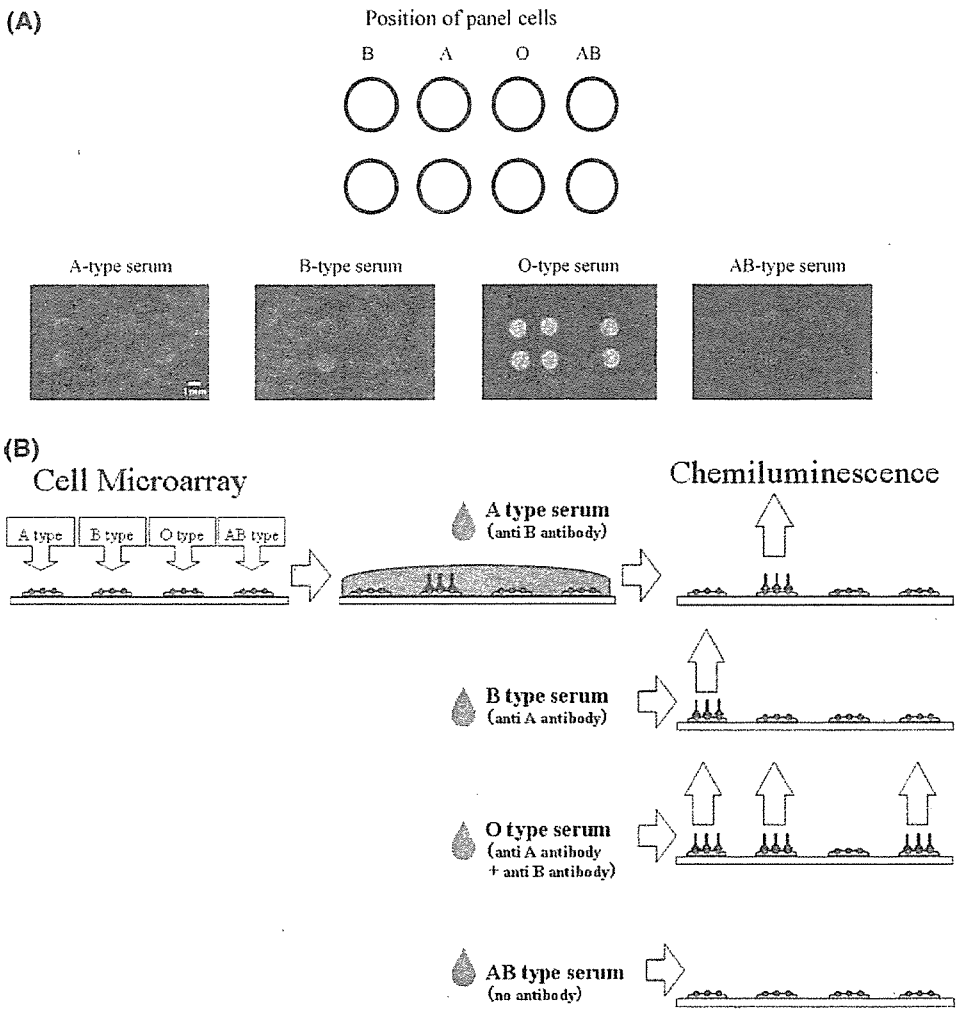

Figure 9. (A) Image of antibodies adsorbed onto blood cells microarrayed on a chip with photoreactive poly(phosphatidylcholine methacrylate). (B) Diagram of the image shown in (A). A-Type serum contained anti-B antibodies that adsorbed onto B-type blood cells, whereas B-type serum contained anti-A antibodies that adsorbed onto A-type cells. O-Type serum contained anti-A and anti-B antibodies that adsorbed onto A, B, and AB cells, and AB-type serum contained no antibodies to any of the cells.

microarray of cultured cell lines for antibody detection. Recently, a cell microarray for clinical analysis was performed (*60*); the target was the assay of antibodies in blood.

Usually, A-type blood does not have anti-A-type antibodies present; however, irregular antibodies are sometimes produced by previous transfusion or pregnancy. Blood containing irregular antibodies cannot be used for transfusion because the irregular antibodies aggregate with cells of the patient receiving the transfusion. Therefore, the presence of irregular antibodies is usually checked before transfusion. However, because the check is performed by an aggregation assay of blood cells, it is a time-consuming process and skill is required to interpret the results. If a cell array method was realized for detecting irregular antibodies, the assay system could be automated, reducing the time required to obtain results; therefore, we microarrayed a panel of cells that expressed the A, B, O, and AB antigens (*60*). The cells were mixed with photoreactive polymers and microspotted onto a polystyrene dish that was irradiated with UV light.

Antibody solution or serum was added to the microarray plate and incubated for some time. After washing, the array assay was used for human serum. Figure 9 shows the result of interactions of the cell microarray with antibodies. The O-type human serum reacted with A-, B-, and AB-type cells, and the A- and B-type sera reacted with B- and A-type cells, respectively, although the reaction intensity was relatively low. This is because A- and B-type sera contain anti-B and anti-A antibodies, respectively. AB-type serum did not react with either type of cell, because it contains no antibodies to these antigens. Because O-type serum contains anti-A and anti-B antibodies, this serum reacted with the panel of cells, except for OD+ cells. These phenomena correspond to the results of an aggregation test for conventional blood types.

Conclusion

The possibility of miniaturizing and carrying out biological assays in parallel has had a great impact on the development of biomedical technologies. Here, we discuss how the photoimmobilization method can be employed for the microarray immobilization of various biological components such as proteins, antibodies, antigens, and cells. Microarray technology will increase in importance with the progress of bioinformatics and clinical informatics, and this progress will produce new applications for these technologies. Further efforts to combine chemistry, biotechnology, and nanotechnology are required for additional progress in this interdisciplinary field.

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Accepted for publication May 17, 2006.

BP060143A

新機能性核酸・タンパク質を生み出すコンビナトリアル・バイオエンジニアリング

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コンビナトリアル・バイオエンジニアリングは，化学とバイオテクノジーを融合した新しい手法として，これからの「ものづくり」になくてはならない．ダーウィンの進化論の「突然変異」，「自然淘汰」，「増殖」のサイクルをそのまま対応させたプロセスを分子レベルで試験管内で行うことで，さまざまな新しい機能性核酸やタンパク質・ペプチドの合成が可能であることがわかってきた．無生物で増殖させることができるDNAを有効に使うことで，図1に示すような進化分子工学が可能となる．試験管内で行えるため，有機合成した非天然ヌクレオチドや非天然アミノ酸を組み込むことも可能になる．ここでは，これら手法を用いた機能性高分子創製の最前線を解説する．

核酸のインビトロセレクション

核酸の試験管内進化法（*in vitro* selection あるいは systematic evolution of ligands by exponential enrichment; SELEX）は，ランダム配列の核酸ライブラリー（「突然変異」）から出発し，アフィニティーカラムによる選別（「淘汰」）をへ，「増殖」にあたるPCRによる核酸の増幅を行うことにより，1サイクルが完了する．このサイクルを繰り返すことにより，標的分子に高い結合能を有する配列のみが生き残り，増殖することになる．本過程で得られる結合性の核酸分子はaptamer（アプタマー）と呼ばれる．現在までに，さまざまなタンパク質や低分子化合物に結合するアプタマーが得られている（http://aptamer.icbm.utexas.edu/）．

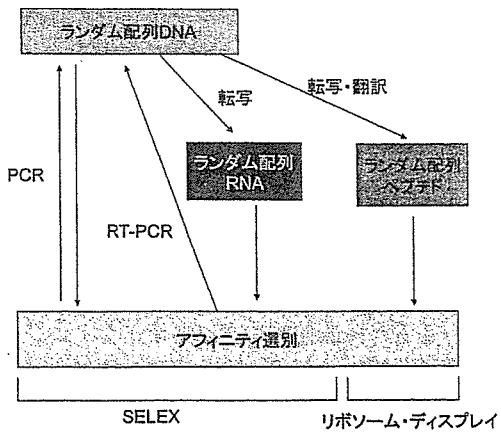

図1．核酸とペプチドの進化分子工学

この試験管内進化法の選別のステップを工夫することにより，現在までに単に結合するだけに限らずさまざまな機能性分子が獲得されている[1,2]．その中でもとくに興味深いものが触媒機能をもつ核酸分子である．

触媒機能を有するオリゴ核酸を探索する方法には二つの方法がある．一つは間接法と呼ばれ，抗体触媒と同じように遷移状態アナログをターゲット分子として，これに結合する分子を得る方法である．たとえば，抗体触媒でも試された，平面状のポルフィリンが少し歪んだ構造をとるメチルメソポルフィリンを遷移状態アナログとして，これと結合するRNAやDNAが得られた．これらのメチルポルフィリン結合性核酸は，ポルフィリンに対するメタレーション反応を加速した[1]．また，コレステロールとパラニトロベンゼンアルコールのリン酸ジエステルを遷移状態アナログとして，獲得されたRNAは，同基質の炭酸エステルの加水分解反応を触媒した[3]．

もう一つの方法は直接法と呼ばれる．たとえば，基質をビオチンでラベル化して，ランダム配列の核酸分子ライブラリーと反応させる．ライブラリーの中で基質と自己触媒反応により結合する核酸配列はビオチンラベル化され，アビジン結合担体で回収される．この方法では，アミド結合やエステル結合形成反応，さらにはDiels-Alder反応を触媒するオリゴ核酸などが得られている．我々の研究室では，この直接法を用いて，中性環境下でのみ活性のあるリボザイムを酸性条件下で活性を持つように調整することに成功している[1]．

非天然核酸組み込み

天然ヌクレオチドは4種類に限られているが，新たに合成した非天然ヌクレオチドを用いて，進化分子工学により新しい機能性オリゴ核酸を合成することができる[1]．

非天然核酸を機能性高分子に組み込むには，二つの方法がある．一つは，すでに機能がある天然オリゴ核酸の一部を非天然核酸に置換する方法で，ポスト修飾法とい

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える．Vesterらは，DNAzymeに彼らが開発したLNA（LOCKed nucleic acid）構造に置換し，安定性の高いLNAzymeを考案している[4]．

もう一つは，非天然ヌクレオチドアナログをポリメラーゼの基質として，直接試験管内分子進化の過程に組み込む方法である．これまでにも，さまざまなヌクレオチドアナログを用いて本手法が適用されてきた．たとえば，Ellingtonらは，蛍光基を導入したヌクレオチドを用いて類似の選別を行い，分析対象物を分子認識すると蛍光強度が変化するセンサーアプタマーを獲得した[5]．非天然ヌクレオチドを導入した核酸触媒の最初の例としては，イミダゾール基を導入したヌクレオチドを用いた試験管内進化法で，Diels-Alder反応を触媒するリボザイムが調製された[6]．これは，非天然ヌクレオチドを用いた進化分子工学の最初の例で，大きなインパクトを与えた．我々は，2'位に水酸基の代わりにアミノ基をもつポルフィリン・メタレーションを触媒するリボザイムをRNAの代わりに得て，過酸化水素の還元反応を触媒するペルオキシダーゼとして働くことを示した[1]．

Ellingtonらは，最近，2'OMeリボ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[7]．さらに，Keefeらは，変異型T7RNAポリメラーゼを用いて，すべて2'-OMeRNA骨格からなるアプタマーを創出した[8]．このアプタマーはVGEF（vascular endothelial growth factor）を標的としており，2'-OMeRNAの高い生体内安定性から有望な核酸医薬となり得る．今後，さまざまな非天然ヌクレオシドを含むオリゴ核酸ライブラリーが調製され，そこからの選別が行われるようになれば，これまで考えられなかった新しい機能性触媒も種々生まれてくる可能性がある．

タンパク質・ペプチドのインビトロセレクション

核酸のインビトロセレクションは，PCRなどによる複製と増幅が可能であることから飛躍的に発展してきた．しかし，機能性タンパク質を選択する場合，この増幅法が適応できない．それゆえ，微量のタンパク質をいかにして回収し，どのようにアミノ酸配列を決定するかが大きな問題であった．そこで，表現型としてのタンパク質とその情報を記録している遺伝子型としてのmRNA/DNAを連結した複合体（対応付け分子）を作製し，それらを選択実験に利用するという概念が生まれた．つまり，対応付け分子のタンパク質部位の機能により選択した後，その核酸部位を回収・解析することで，目的の機能性生体分子を同定するという手法である．これにより，対応付け分子の作製と選択操作以外は，核酸の試験管内進化サイクルの操作をそのまま適応することができるようになった．

現在，この原理を基礎として開発された試験管内分子選択法[9]として，リボソームディスプレイ法，mRNAディスプレイ / *in vitro* virus（IVV）法，*in vitro* compartmentalization（IVC）法が報告されている．これらの技術では，無細胞転写・翻訳系を採用しており，すべての実験操作が試験管内レベルで迅速に遂行できるように設計されている．

リボソームディスプレイ法　一般にリボソームは，タンパク質をコードするmRNA上を移動しながら翻訳を行い，終止コドンに出会うことで遊離し，翻訳は終了する．しかし，リボソームディスプレイ法では，mRNAの終止コドンを取り除くことでリボソームの遊離を抑制し，表現型と遺伝子型を結合させたタンパク質 / ペプチド-リボソーム-mRNA対応付け分子を形成させる（図2-1）．そして，この対応付け分子に提示するタンパク質 / ペプチドにランダム配列を導入したライブラリーを構築し，標的分子に対する結合性を条件にしてスクリーニングを行えば，目的の生体分子を選択することが可能となる．たとえば，Plückthum[10]らは，超可変領域にラン

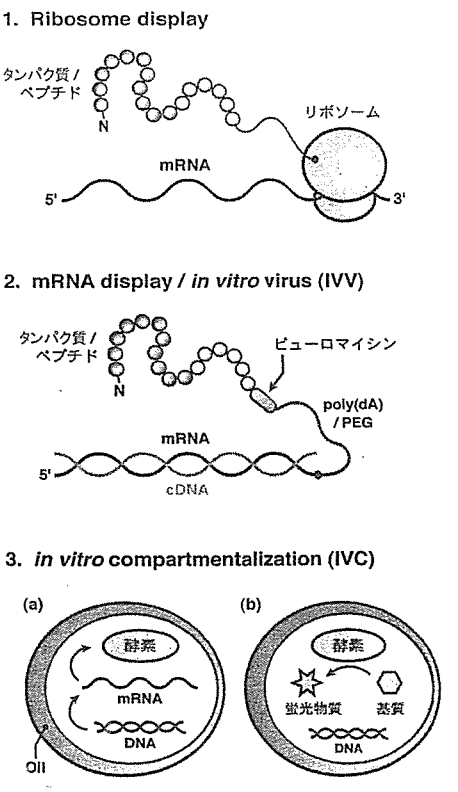

図2．試験管内分子選択法における対応付け分子

ダム配列を挿入した一本鎖抗体scFvライブラリー(～10^{12}種類）を作製し，各種抗原（インスリン/フルオレセイン/GCN4ペプチド)に対する親和性を指標にしたスクリーニング実験を行った．その結果，いずれの抗原においてもpMオーダーの解離定数を有する高親和性scFvの選択に成功した．

mRNAディスプレイ/*in vitro* virus（IVV）法　これらの方法では，ピューロマイシンをmRNAの3'末端にpoly（dA）もしくはPEGリンカーを介して結合させた後，翻訳したタンパク質のC末端とピューロマイシンをリボソーム内にて反応させる．その結果，リボソームの解離により，表現型と遺伝子型が共有結合により連結したタンパク質-mRNA対応付け分子が形成される（図2-2)．さらに，高次構造を取りやすい一本鎖RNA部分を逆転写することにより，cDNA-mRNAの二本鎖核酸へと変換する．これにより，構造的剛直性の獲得と共にRNAアプタマーの選択が抑制され，より信頼度の高い選択実験を行うことが可能となった．たとえば，Szostak[11]らは，80アミノ酸の完全なランダムペプチド配列（6×10^{12}種類）のmRNAライブラリーから，新たなATP結合性タンパク質の選択に成功している．一方，柳川[12]らは，IVV法をゲノムネットワーク解析に応用しており，マウスcDNAを元に作製したmRNAライブラリーから，Fosタンパク質と相互作用する既知および候補タンパク質を同定している．

***In vitro* compartmentalization（IVC）法**　この方法では，無細胞転写・翻訳に必要な因子と酵素をコードするDNAライブラリーをエマルジョン（w/o型）液滴内に閉じこめることで，表現型と遺伝子型を対応付けている（図2-3a)．つまり，1個のエマルジョン内（直径～2.6 μm）に1分子のDNAのみが存在するように調整されており，翻訳された酵素を完全に区画化された環境で機能させることができる．これにより，液滴内の基質に対する反応性を条件にして，より高い触媒機能を発現する酵素を選択することが可能となった．Griffiths[9,13]らは，基質から蛍光性物質を生成する反応とその反応を触媒する酵素（DNAメチルトランスフェラーゼ，β-ガラクトシダーゼなど）のライブラリーを組み合わせたIVC選択システムを数多く構築している．そこでは，ライブラリーとしてのエマルジョンの調整→転写→翻訳→酵素反応(図2-3b)を行った後，FACS（fluorescence activated cell sorting）によりエマルジョン（w/o/w型）を単離する．このとき，触媒機能を反映した蛍光量を基準にしてエマルジョンを分取するため，触媒活性が向上・進化した酵素のみを選択することが可能となる．

非天然タンパク質・ペプチドへ

最近では，非天然アミノ酸をランダムな位置に導入したタンパク質/ペプチドライブラリーの開発と選択実験への応用も検討されている．たとえば，宍戸・芳坂ら[14]は，4塩基コドン法とmRNAディスプレイ法（図2-2）を組み合わせることにより，非天然アミノ酸導入ペプチドを提示したmRNAライブラリー（1×10^{12}種類）を作製した．そして，ストレプトアビジンに対する結合性を指標にしてスクリーニング実験を行ったところ，非天然アミノ酸としてベンゾイルフェニルアラニンを含む親和性ペプチドを選択することに成功した．

今後，新たな機能性非天然アミノ酸の合成と試験管内分子選択システムへの応用により，天然アミノ酸では獲得し得なかった性質・機能（蛍光性，光感受性，化学反応性など)を有する人工タンパク質の創出が可能となる．そして，上記の天然・非天然核酸/アミノ酸を利用したコンビナトリアル・バイオエンジニアリングの更なる進展は，高感度バイオセンサー・ナノ抗体・人工核酸医薬などをテーラーメイドに創製する新たなバイオ技術の開発において，重要な役割を果たすであろう．

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アレルギー診断用抗原マイクロアレイ・チップ

Allergen Microarray Chip for Allergy Diagnosis

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Key words：Allergy Diagnosis, Allergen, Microarray, Photo-immobilization, IgE

Abstract

アレルギー治療の第一歩は，起因アレルゲンの特定である。我々は，これまで様々なアレルゲンを同一の方法で一枚のチップ上に固定化できる光固定化法を開発してきた。アレルゲンとなる様々な生体分子，有機分子を固定化できるため，簡単な自動化装置で抗体検査が可能となった。この成果を，社会還元するためにベンチャー企業が設立され，通常は抗体検査できないアレルギー反応も受託できるようになっている。エピトープ分析も含め，きめ細かな診断法への応用に向け，臨床家，臨床研究者との連携が期待される。

はじめに

アレルギー検査は，皮膚試験と試験管内試験に大別される。皮膚試験として，即時型アレルギーの検査にはプリックテスト，遅延型アレルギーの検査としてはパッチテストが最もよく行われる。しかし，皮膚試験は患者への負担が大きいため，試験管内試験が現在のアレルギー検査の主流となっている。中でも，測定が簡便なうえ，結果が客観的な数値として表れることから，血中の特異IgE抗体の測定が最も多く用いられている。アレルギー治療の第一歩は起因アレルゲンの特定であるため，特異IgE抗体のスクリーニング検査が注目されている。

表1には，現在主に市販あるいは開発されている検査法をまとめる。著者らは，わずかな血液量で，様々な抗原に対するアレルギーを，一括して一度に検査できる比較的安価なシステムを，新しいタイプの抗原マイクロアレイ・チップ（ヒラソルチップ）を用いて可能にしたので，これについて解説する。

1. マイクロアレイ技術

最近のライフサイエンスの研究は，材料の微細加工やナノテクノロジーの発展により新しい展開をみせつつある。DNAマイクロアレイ（DNAチップ）の出現は，バイオテクノロジーと微細加工技術の融合の賜物であ