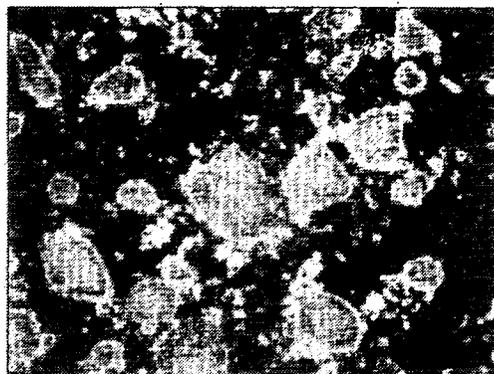100 μm

LIFなしでのマウスES細胞培養

100 μm

固定化LIF (10^4 U/ well)上
でのマウスES細胞培養

図8　LIFを固定化した基板上と固定化しない基板上で6日間マウスES細胞の培養を行い，アルカリフォスファターゼ染色。

たい。

5. 胚性幹（ES）細胞の体外増幅

ES細胞培養法についても実際の医療に用いるためには，倫理的な面以外でも技術的に解決しなければならない問題は多い。その一つは，マウスES細胞では様々な培養法が確立されているが，ヒトを含む霊長類ES細胞については，未分化状態で増幅する培養法は，異種動物由来細胞やヒト中絶胎児由来細胞をナース細胞として用いる以外に一般的には確立されていないことである。[6] そこで，我々はサルES細胞培養のためのヒト由来ナース細胞の探索を行った結果，実用的なナース細胞としてヒト胎盤由来の羊膜上皮細胞及び絨毛膜板細胞が機能することを発見した。[7] 特に希アンモニア水で処理すると，サルES細胞を半年以上，未分化状態で安定に増幅できた（図7）。

また，マウスES細胞のフィーダー細胞を用いない培養法は確立されているものの，いくつかの添加物を必要とする。その中の一つが，Leukemia Inhibitory Factor（LIF）である。そこで，LIFを固定化し，その上でマウスES細胞を培養した（図8）。するとLIF固定化基材上で培養し

たマウスES細胞は，アリカリフォスファターゼ活性を指標に染色でき，このことから未分化を維持したまま増殖できることがわかった。これまでに固定化した様々なサイトカイン同様，LIFは固定化しても活性を維持することがわかった。[8] 今後，分化を制御できる様々のバイオシグナル分子を固定化することにより，ES細胞から様々な分化状態を制御した組織構造化ができると期待できる。

6. 終わりに

体性幹細胞を用いた再生医療は着実に発展してきている。一方で，ES細胞については，2004年のアメリカ大統領選でも争点の一つになった。結局は，ヒトES細胞研究に消極的なブッシュ大統領の勝利となったが，このような連邦政府とは別に，アーノルド・シュワルツネッガー知事のカルフォルニア州では，住民投票による賛成多数の支持を受け，今後10年間で州予算から約30億ドルを，ヒトES細胞研究を中心とした再生医療の基礎研究に出資することになった。カルフォルニア州は，もともと生殖医療の発達した地域の1つであり，治療を受けるための訪問者も多く，ヒト

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ES 細胞樹立のためのヒト胚の入手は容易な地域である。ヒト ES 細胞の研究は，アジア地域での進展も著しいが，カリフォルニア州がこの領域の研究を大きく発展させることは間違いない。日本でも基礎研究に根ざす様々な可能性を生かしながらの臨床応用への展開が望まれる。

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再生医療用ナノファイバー
Nanofiber for Regenerative Medicine

――――――――――――伊藤嘉浩[*1]　Oh Hyeong Kwon[*2]　Inn-Kyu Kang[*3]

近年，エレクトロスピニング法によるナノファイバーの研究が盛んに行われるようになり，様々な素材や形状のものが作られるようになった。そこで，ナノファイバーを用いた再生医療用材料，主に生分解性高分子を用いたティッシュエンジニアリングのためのスキャホールド（足場）として，将来へ期待がかかっている。本稿では，再生医療分野におけるナノファイバー研究についての現状を概説した。

1. はじめに

2004年2月，シーエムシー出版から『ナノファイバーテクノロジーを用いた高度産業発掘戦略』[1]なる書籍が出版された。ナノファイバーテクノロジーは米国と韓国で特に盛んに研究されている。バイオマテリアル分野で特に用いられる方法はエレクトロスピニング法あるいはエレクトロスプレー法と呼ばれる方法で，安価な装置でナノサイズの材料ができることもあり，様々な素材が加工されている。ここでは，エレクトロスピニング法について概説し，その医療分野への応用について述べる。

2. エレクトロスピニング法

エレクロスピニング法の歴史を表1に示す。

1917年に Zeleny により，溶液に高い電場をかけると不安定な cone が形成され，臨界電圧以上ではスプレー現象が生じることが発見され，エレクトロスピニング法の特許は，1934年になる。その後，医用目的の特許なども現れたが，今日のように研究が盛んになったのは，90年代半ばからで，製造装置が安価で，容易にナノ構造体が得られることによる。

図1には製造原理を示す。試料は，微細な先端をもつキャピラーに収められ，キャピラリー先端から導電性基板に数キロボルトの高電圧を印加すると試料が静電気力によって噴射される。

写真1には，実際筆者らが作成した装置の写真を示す。エレクトロスピニング法のメカニズムはまだ十分に解明されていないが，概略は以下のように考えられている[2]。まず，電圧の印加により

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*[3]Inn-Kyu Kang　Department of Polymer Science　Kyungpook National University　Professor

表1　エレクロスピニング法によるナノファイバーの歴史

| 1917	Zeleny	エレクトロスプレー現象の研究
1934	Formhals	電場を使った酢酸セルロースのエレクトロスピニング (USP 1975504 (1934), USP 2160962 (1939))
1971	Baumgarten	アクリルポリマーのエレクトロスピニング（繊維径 500〜1,100nm）
1977	ICI	創傷被覆用ナノファイバーマット（USP 4044404）
1981	Manley *et al.*	ポリエチレンとポリプロピレンのエレクトロスピニング
1989	Reneker *et al.*	エレクトロスピニング装置の開発と様々な高分子のナノファイバー化
1994	Reneker *et al.*	ポリエチレンオキシドのエレクトロスピニング
1995 以降		加工条件による繊維形状の分析 ナノファイバーの用途展開 エレクトロスピニング法の原理と機構の解明
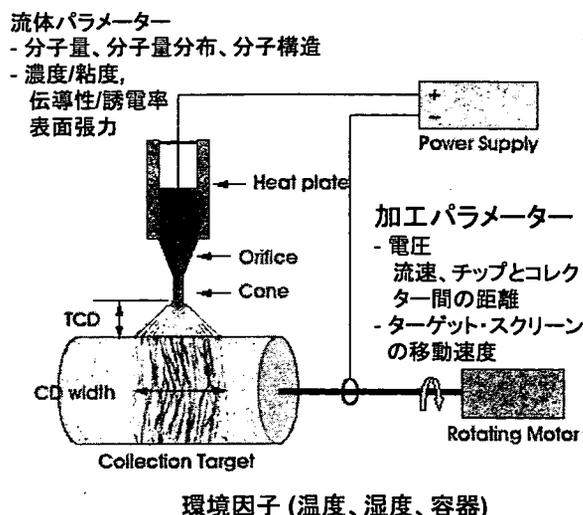
図1

キャピラリー先端の液体表面に電極と反対符号の電荷をもつイオンが集まる。液体表面に蓄積された電荷と電場の相互作用によってキャピラリー先端ではメニスカスが半円状に盛り上がる。より高い電場の下では，Taylor-Cone と呼ばれる円錐状のメニスカスが形成される。電場をさらに大きくし，静電気力反発が表面張力を上回ると，液体の一部が Taylor-Cone から飛び出し，液滴あるいはジェットとして噴出を始める。噴出された液滴あるいはジェットは，強く帯電しており，電場により導電性基板へ引き寄せられる。場合によっては，液滴内部での静電気力反発によってさらに分裂して細かい液滴あるいはジェットを形成する。形成された液滴のサイズは極めて小さく，表面積が体積より非常に大きいため，きわめて短時間のうちに溶媒が蒸発する。通常，溶媒は飛行過程中に蒸発するので，基板上には乾燥した溶質分子がデポジットされる。このとき基板上にナノスケールの粒状，紡錘状，繊維状などの構造体が形成される。一般に，分子量の比較的低い試料からは粒状構造（ナノパーティクル）が，高い試料からは繊維構造（ナノファイバー）が形成されやすく，このような方法を最も一般的にはエレクトロスプレー法といい，特に繊維構造体のみを形成させる場合にエレクトロスピニング法と呼ばれている。

図2には電界強度とファイバーの形成の様子を表す。これまでに，表2に示すように様々なナノファイバーが得られてきた。生体高分子としても多くの研究が行われてきている。

ナノファイバーの特徴をまとめると，
①高い比表面積
②高いアスペクト比（長さ/直径）
③不織布の多孔性制御可能
④様々な高分子液体（溶液，溶融体）に適用可能
⑤簡便で安価なプロセス
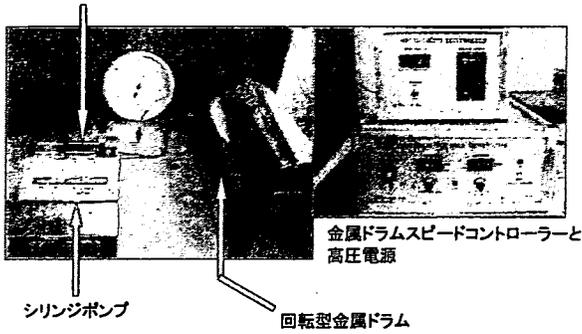となり，現在，医療分野では，創傷被覆材，ティッシュエンジニアリングのスキャホールド（足場），

写真1

ガラス・シリンジ (10ml, 22G 金属ニードル)
金属ドラムスピードコントローラーと高圧電源
シリンジポンプ
回転型金属ドラム

図2

半球形
表面張力
円錐形
安定ジェット
荷電ジェット
不安定領域
短時間露出時のイメージ
電圧
エレクトロスピニング条件：表面張力　電圧

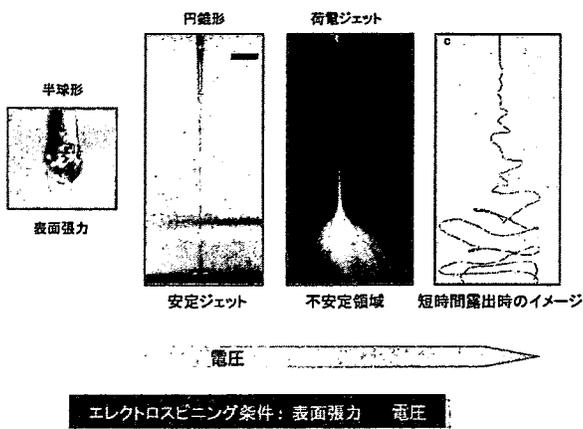| ポリマー分類	ポリマー	溶　媒
高性能ポリマー	ポリイミド ポリアミック酸	フェノール m-クレゾール
液晶ポリマー	アラミド ポリ (γ-ベンジルグルタメート)	硫酸 DMF
繊維ポリマー	ポリアクリロニトリル ナイロン	蟻酸 蟻酸
ポリオレフィン	ポリプロピレン 低密度ポリエチレン	溶融 溶融
伝導性ポリマー	ポリアニリン	硫酸
生体高分子	DNA コラーゲン フィブリノーゲン PLGA ポリ (ヒドロキシブチレート-co-バレレート)	水 HFP HFP/MEM HFP クロロホルム
コポリマー	ナイロン 6-ポリイミド	蟻酸
ブレンド	PET/PEN ブレンド	溶融

表2　ナノファイバーの例

写真2

濃度減少
拡大倍率 (X 10K)
6 wt%　　4 wt%　　2 wt%

人工血管，ドラッグデリバリーシステム (DDS) への応用が進められている。

3. ナノファイバー作成

ここで，どのようなナノファイバーが作られるかを，筆者らの例をもとに説明する[3]。ここでは，バクテリアが産生する生分解性高分子として知られるポリ (β-ヒドロキシアルカノエート) 誘導体 (PHBV) をナノファイバーにする。写真2には，高分子溶液の濃度を変えたときのナノファイバーの形状を示す。低濃度溶液ほど細い直径のナノファイバーが得られる。また，写真3に示すようにスピニング距離を長くするほど，また写真4に示すように電場を強くするほど，直径が細くなる傾向も見られた。

また，同じ高分子でも溶解させる溶媒によってもナノファイバーの形状はかなり異なる。その例を写真5に示す。図3には直径の分布を示す。
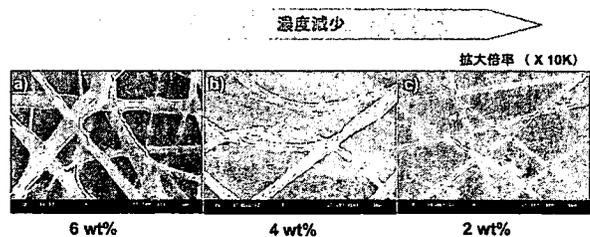
松田らは，このエレクロスピニング法を発展させて多層法や混合法を編み出している[4]。例えば，I型コラーゲン，スチレン化ゼラチン，セグメント化ポリウレタン，ポリオキシエチレンの4種類の高分子を連続あるいは同時に噴出させることで様々な複合組織体を創出できることを報告している。

スピニング距離増加

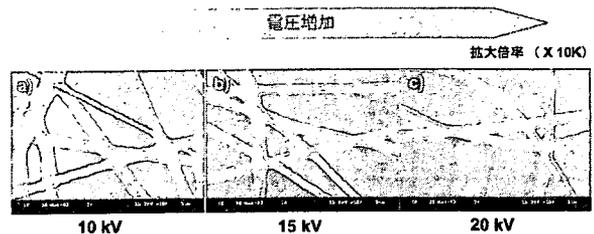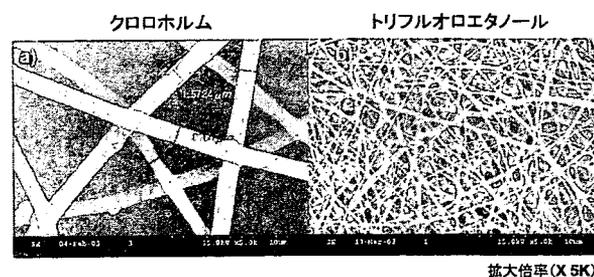拡大倍率 (X 10K)
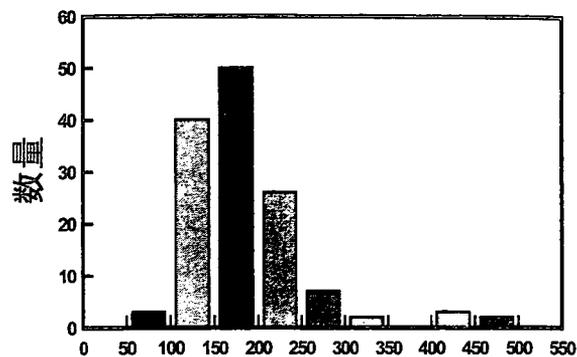
9 cm 15 cm 21 cm

写真 3

電圧増加

拡大倍率 (X 10K)

10 kV 15 kV 20 kV

写真 4

クロロホルム トリフルオロエタノール

拡大倍率(X 5K)

最小径形成条件下でナノファイバー化

写真 5

繊維径 (nm)

最小	56.569
最大	466.905
幅	410.336
平均	184.715
標準偏差	69.352
サンプル数	130

図 3

さらなる展開として繊維を交差させたり，平行に並べたりもできるようになっている。また，ゾルーゲル法と組み合わせてセラミックのナノファイバーを作成したり，中空のナノファイバー化も可能になってきており，これらの製造技術の発展により，新しい可能性が広がってきている[5]。

4. 医療への応用研究

医療分野への応用を目指した研究としては，生分解性高分子のナノファイバーを用いたものが最も多く報告されている。例えば，Kim らは，ポリ乳酸ナノファイバーを作成し，比表面の増加により，これらの分解速度が顕著に速くなることを報告している[6]。Li らは，ポリ(ε-カプロラクトン)ナノファイバーから3次元構造物を調製し，

軟骨細胞の培養を行っている[7]。細胞培養用のポリスチレンプレートに単層で存在するナノファイバーより，軟骨細胞の発現型を保持し，細胞成長も促進したことから，軟骨ティッシュエンジニアリングのスキャホールド（足場）として有望であると報告している。Boland らは，ポリグリコール酸でナノファイバーを作成し，さらに酸処理を行うことで，生体適合性が向上することを報告している[8]。ポリ乳酸共重合体として，Ramakrishna のグループは，ポリ(L-乳酸-co-カプロラクトン)共重合体からナノファイバーを作成し，溶媒キャスト法で作成した平膜に比べて，平滑筋細胞や内皮細胞が接着しやすく成長しやすいこと[9, 10]を，Bhattarai らは，ポリ(p-ジオキサノン-co-L-乳酸)-ブロック-ポリエチレングリコール共重合体からナノファイバーを作成し，NIH3T3 繊維芽細胞がナノファイバーの配向に沿って並び成長することを報告している[11]。

Katti らは，生分解性ナノファイバーを利用した抗生物質徐放システムを報告している[12]。ポリ(乳酸-co-グリコリド)（PLAGA）をエレクロス

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BIO INDUSTRY

ピニング法でナノファイバーにし，抗生物質としてセファゾリンを導入した。すなわち，PLAGAとセファゾリンをジメチルホルムアミドとテトラヒドロフランの混合溶媒に溶解して，紡糸することにより調製された。筆者らは，生分解性のポリ(3-ヒドロキシブチル酸-co-3-ヒドロキシバレリック酸)からエレクトロスピニング法でナノファイバーを調製し，ヒドロキシアパタイト（HAp）との複合化を行っている[13]。ナノファイバー化によって生分解性が顕著に促進され，HApとの複合化によりさらに促進されることが見出された。しかし現在のところ，HApとの複合化による顕著な細胞応答の差は見出されていない。複合化の方法を変えることにより，HApの効果が現れるかを検討中である。

　天然高分子のナノファイバーとしては，絹フィブロインの報告が最近2つある。Minら[14]は，絹フィブロインの蟻酸溶液からの再生ナノファイバーを作成し，50%メタノール水溶液で化学処理した。これは，ヒト・ケラチノサイトや繊維芽細胞の接着を促進したと報告されている。さらに，Jinら[15]は，カイコの絹水溶液からのナノファイバー（直径約700nm）を作成し，通常の絹ファイバー（直径約15μm）との比較を行ったところ，前者が，ヒト骨髄ストローマ細胞の接着，伸展，成長において高い支持能があったと報告しており，ナノファイバーのティッシュエンジニアリングへの有用性を明らかにしている。

　非分解性高分子としては，ポリウレタンのナノファイバー膜を創傷被覆材として用いた例も報告されている[16]。これは，水分蒸発を制御し，高い酸素透過性をもち，浸出液の排出を促進するが，その孔が超微細であるために微生物の侵入を阻止できる。組織学的にもで創傷被覆材として有用であると結論している。

　この他にも，ナノファイバー化ではないが，エレクトロスプレー法でリン酸化カルシウム（CaP）をデポジットしたバイオマテリアルが報告されている[17]。この技術では，カルシウムとリン酸を含んだ有機溶液がノズルから噴出される。チタン合金（TiAl6V4）の上にCaPをエレクトロスプレーし，その上でラットから採取した骨髄細胞を播種して培養すると，他の方法でCaP被覆した場合より，骨芽様細胞の増殖促進が観測されたとしている。

5. おわりに

　通常の紡糸では2マイクロメートル以下のサイズのものを作るのは難しい。また，フラーレンやカーボンナノチューブとは違い，組織化の制御がしやすい。そのようなことから，エレクトロスピニング法を用いたナノファイバーについては指数関数的に発表論文が増加しており，医療以外に様々な応用展開が報告されている[1,5]。繊維は日本が得意とする技術分野であるが，ナノファイバーに関しては遅れをとっているようである。今後の大いなる発展が期待されるだけに，日本における一層の展開が望まれる。

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特集にあたって

————————————————————————————————横山昌幸[*1]　伊藤嘉浩[*2]

ナノテクノロジーが大々的に展開されるようになり，バイオテクノロジーと融合したナノバイオテクノロジーへ，そして近年，ナノメディシンとして応用を志向する研究領域が注目されるようになってきた。しかし，ナノメディシンといっても，医学分野では，すでにナノレベルのデバイスは使われている。それは，ドラッグデリバリーシステムの分野である。

血液を介して薬物を疾病組織・臓器に運搬するためには，毛細血管の直径約5μmよりも薬物キャリヤーが小さいことが前提となるが，実際にはこの上限よりも遙かに小さな100nm以下のものが用いられている。その理由は，400nm位より大きいと肝臓・脾臓などで異物と判断されてしまい，血液中をうまく循環できないためによる。それに加えて，固形がん組織などに血液から移行する経路を通過するためにも400nm以下が必要とされる。このようにナノレベルの大きさが最も直接的に医療で活用されるのが薬物ターゲティングである。

これと同様にナノサイズのシステムで診断用の放射性同位元素や造影剤のキャリヤーシステムは，

「分子イメージング」の概念と半導体のナノ粒子の出現により，これからの大きな発展が期待されている。再生医療材料の分野でも，精緻な構造を求める動きがある。それは，細胞のようなマイクロメートルレベルの領域でも，細胞骨格系タンパク質の制御に基材のナノメートル・サイズの構造が影響を与え，細胞接着に影響することが報告されるようになってきているからである。ナノメートル・レベルの構造制御は，細胞機能を積極的に制御する因子になる可能性がある。

ただ，最近気にかかることとして，ナノ物質の生体や環境への悪影響を指摘する研究が多く発表されるようになってきたことがある。2004年英国では，フラーレンやナノチューブはフード内で使うようにと指示するようなマニュアルが公的機関から出されるようになってきている。サイズにより，体内での集積場所や作用効果も異なるという研究が報告されている。このような欧米でのナノテク材料の危険視に，日本のナノテク産業隆盛への牽制球ではないかという多少穿った見方もあるものの，日本でも，安全性に関して研究や議論が行われ始めている。カーボン系ナノマテリアル

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を医療にすぐに応用しようとするものはないもの
の，ナノメディシンの更なる発展のためには，慎
重に研究を進めてゆく必要がある。

　世界でナノメディシンの研究が活発化する中，
2004年に，横山は㈶神奈川科学技術アカデミー
で横山「高分子ナノメディカル」プロジェクトを，
伊藤は㈱理化学研究所で伊藤ナノ医工学研究室を
立ち上げるなど，「ナノ」で医療応用分野を切り
拓こうと志を新たにしている。

　このようなことを背景に，ナノメディシンの現
状の今後の展望についての特集を企画し，第一線
でご活躍の研究者の方々に研究を紹介していただ
いた。

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Technical note

Photoimmobilized array of panel cells for assay of antibodies

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Abstract

Antibodies in blood are checked with panel blood cells before blood transfusion. In this investigation, for the first time, a panel cell-microarray was prepared by using a photoimmobilization method. Different types of red blood cells were microarrayed on a plate. A water-soluble photoreactive polymer as a matrix was synthesized by the coupling reaction of azidoaniline with poly(2-methacryloyloxyethylphosphorylcholine-*co*-methacrylic acid). The polymer was mixed with cells and the mixtures were microspotted on substrate and photoirradiated after drying in air. For the antibody assay, monoclonal antibodies or human serum was added to the cell-arrayed plate and adsorbed antibodies were detected by horseradish peroxidase-labeled secondary antibody, which recognized the adsorbed antibodies. Antibodies specifically adsorbed on the immobilized cells as expected. The aggregation method has been available for this type of assay, but extensive experience was needed to apply it correctly. The method using a cell array will be useful for antibody detection.
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Keywords: Adsorption; Cross-linking; Phosphorylcholine; Protein adsorption

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 exactly the same conditions [1–3]. DNA microarray approaches have demonstrated a rapid and economic way to interpret gene functions [4,5]. In recent years, there have been considerable achievements in preparing small-molecule arrays [6,7], peptide arrays [8–11], protein arrays [12–15], polysaccharide arrays [16–18], antigen arrays [19–21], antibody arrays [22–27], and tissue arrays [28,29].

Although the preparation of the microarray is important, no universal immobilization method has been developed. Noncovalent immobilization (physical adsorp-

tion) or standard 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, Ito et al. developed a photoimmobilization method for the preparation of a microarray chip [30]. Using this method, any material can be immobilized on a substrate by the use of radical reactions. Ito et al. microarrayed proteins using photoreactive polymers as the matrix on a tissue culture polystyrene plate [30,31]. In the present study, cells were microarrayed using this method. Although a microarray of cultured cell lines for antibody detection of cell surface proteins has been reported by Schwenk et al. [32], the use of a cell microarray for clinical analysis has not been reported.

Here, the assay of antibodies in blood was chosen as the target of the cell array. Usually, A-type blood does not have anti-A antibody. However, irregular antibodies are sometimes produced by previous transfusion or pregnancy. Blood that contains irregular antibodies cannot be used for

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transfusion, because they aggregate with the patients' cells. Therefore, the presence of irregular antibodies is usually checked before transfusion. However, as the check is performed by an aggregation assay of blood cells, it is a time-consuming process that requires some skill to judge a positive result. If the cell array method is realized, washing and staining processes can be automated and the assay system will be conveniently performed. Recently, an automatic analysis using an allergen-microarray was reported [21].

2. Materials and methods

2.1. Preparation of photoreactive polymer

Photoreactive polymer was prepared as previously reported [33]. A copolymer consisting of 2-methacryloyloxyethyl phosphorylcholine (90 mol%) and methacrylic acid (10 mol%) was obtained from NOF Co. (Tokyo, Japan); it is referred to as PMAc. The molecular weight of PMAc, as measured by gel permeation chromatography, was 2.2×10^5. Modification of PMAc was performed as follows. 4-Azidoaniline (12.44 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (17.47 mg), purchased from Dojindo Lab. (Kumamoto, Japan), were dissolved in 2 mL of PMAc solution (5 wt%); 98 mL of water was added to the solution. The solution was left to stand for 24 h. After reaction, the product was dialyzed with a dialysis cassette (PIERCE, Rockford, IL, USA) until ultraviolet absorption confirmed no further release of azidoaniline through the cassette. The resultant solution was freeze-dried. The azidophenyl-derivatized PMAc is referred to as Az-PMAc. Elemental analysis indicated that the amount of azidophenyl group in Az-PMAc was 6%.

2.2. Preparation of cell array

Cell array was carried out as follows. Cells carrying M, N, A, B, AB, O, or Rh (D) antigen were chosen and nine kinds of panel cells, M+, N+, A; D+, B; D+, AB; D+, O; D+, O; D−, D+, and D− were employed. The symbols + or − mean the presence or absence of antigen on the cells, respectively. The cells were washed twice with phosphate buffered saline (PBS). After the washed cells were incubated in 2 vol% glutaraldehyde solution for 15 min or left untreated, they were mixed with 1 wt% of photoreactive polymer. The suspension (0.5 μL) was spotted on a tissue culture polystyrene dish (diameter 35 mm, 3000-035X) purchased from Iwaki Glass Co., Ltd. (Chiba, Japan). After drying in air, the spot was immobilized by UV irradiation. The immobilized cell spot was washed three times with PBS containing 0.1 wt% Tween 20.

2.3. Binding assay of antibodies

Mouse antibodies or human sera were prepared as follows. In the case of anti-M, -A, -B, or -GPA, mouse (BALB/c) was immunized with each human erythrocyte and the immunized spleen cell was fused with a myeloma cell (NS-1). The fused cell was repeatedly cloned, each antibody-producing cell was established, and each antibody was collected from the cell. In the case of anti-D antibody, B lymphocyte isolated from humans carrying anti-D antibody was transformed with Epstein-Barr virus, and the transformed cell was cultured and fused with the myeloma cell. The fused cell was repeatedly cloned, an antibody-producing cell was established, and the antibody was collected. In the case of human sera, they were collected from healthy volunteers.

Binding assay was performed as follows. PBS containing anti-M, -A, -B, -D, or -glycophorin A (GPA) mouse monoclonal antibody (53, 62, 57, 58, and 86 mg/L, respectively), or human serum of A, B, O, or AB was added to the cell array plate and incubated at 22 °C for 2 h. After incubation, the plate was washed with PBS containing 0.1 wt% Tween 20. Subsequently, the plate was incubated with a PBS containing anti- or -human whole antibodies IgG, linked with horseradish peroxidase (Amersham Bioscience Co., Piscataway, NJ, USA) at 22 °C for 1 h. After washing three times with PBS containing 0.1 wt% Tween 20, ECL reagents (Amersham Bioscience Co.) were added. The plate was analyzed using a chemiluminescence image analyzer, $n = 4$.

3. Results and discussion

First, the M+ cells were fixed with glutaraldehyde and mixed with PMAc or Az-PMAc, and the mixture was arrayed on a tissue culture polystyrene plate for the binding assay of monoclonal anti-M antibody. It was found that anti-M antibody bound to the spot of M+ cells immobilized with Az-PMAc, but not with PMAc. Because the cells were immobilized by the photocrosslinking reaction, the cells were not considered to be washed out during the binding assay. This result indicates that photoimmobilization was important for the binding assay.

Second, the binding of anti-M and -GPA antibody to the glutaraldehyde-fixed and photoimmobilized M+ or N+ cells was investigated. Anti-GPA was used as a control and it recognized both immobilized M+ and N+ cells. On the other hand, anti-M antibody bound to immobilized M+ cells, but not to N+. These results demonstrate that the recognition of antibody with the cells was specific. Dilution of the cells for the immobilization reduced the amount of bound antibodies.

Third, the effect of glutaraldehyde treatment on antigens locating on panel cells was investigated. Fig. 1 shows that anti-A and -B antibodies bound to A and B cells, respectively. Anti-D antibody bound to D+-type cells, but not to D−-type cells. In this case, because all the A, B, and AB cells have D antigen, anti-D antibody bound to all the D+ panel cells. However, a comparison of Figs. 1a and b shows that the amount of anti-D antibody bound to cells treated with glutaraldehyde (Fig. 1a) was lower than for those that were not treated (Fig. 1b). D antigen is a membrane protein with a complex structure and anti-D antibody recognizes the three-dimensional structure of D antigen. For recognition of D antigen, glutaraldehyde treatment was not appropriate. Considering that glutaraldehyde fixation affects the molecular recognition, the fixation was not carried out before photoimmobilization for further experiments.

Fig. 2 shows photographs indicating monoclonal antibodies bound to spotted panel cells that were photoimmobilized with Az-PMAc. Anti-A and -B antibodies bound to the immobilized A; D+ and B; D+ cells, respectively. In addition, the antibodies bound to the AB cells. On the other hand, in this case, because O; D− cells had no D antigen, no reaction of anti-D antibody was found. These phenomena correspond to the result of an aggregation test for conventional blood types.

Finally, the array assay was used for human serum, as shown in Fig. 3. O-type serum reacted with A, B, and AB type cells. A- and B-type serum reacted with B and A cells,
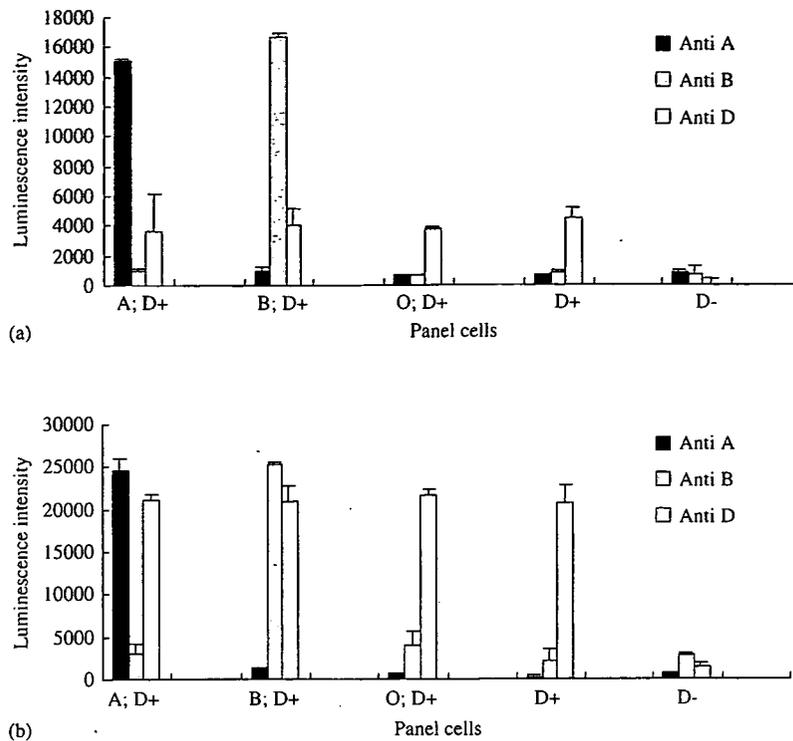
Fig. 1. Effect of glutaraldehyde fixation of panel cells on binding of antibody. (a) Glutaraldehyde-fixed panel cells were arrayed and (b) nonfixed panel cells were arrayed. Binding of anti-A (black), anti-B (gray), and anti-D (white) antibodies onto the cells.
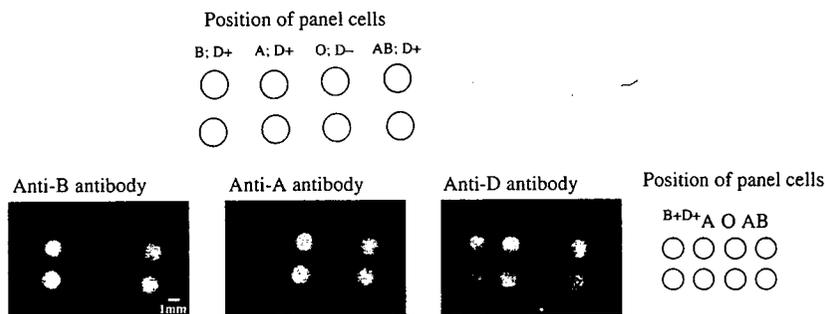

Fig. 2. Detection of chemiluminescence of antibodies bound on photoimmobilized panel cells array.

respectively, about 2-fold as high as with other cells. This is because A- and B-type serum have anti-B and -A antibody, respectively. AB-type serum did not react with either type of cell, because it has no antibody. Because O-type serum has both anti-A and -B antibodies, the panel cells except for O; D+ cells were stained. These phenomena correspond to the result of an aggregation test for conventional blood types.

Recently, Zhang et al. reported the capture of red blood cells by blood group antibodies (anti-A and -B) [34]. Conversely, this study has demonstrated that cells can be arrayed and immobilized on a plate and that the immobilized array was successfully employed for antibody detection, although some treatment of cells, for example

glutaraldehyde fixation, depends on the characteristics of the antigen. The photoimmobilization method will be useful in the array technique as an "everything array method", even for cells.

4. Conclusions

A panel cell-microarray was prepared by using a photoimmobilization method. A photoreactive polymer was mixed with different types of red blood cells and the mixture was microspotted on substrate and photoirradiated. By this method the cells were immobilized on the susbstrate. Human serum was added to the cell-arrayed plate and adsorbed antibodies in the serum were detected
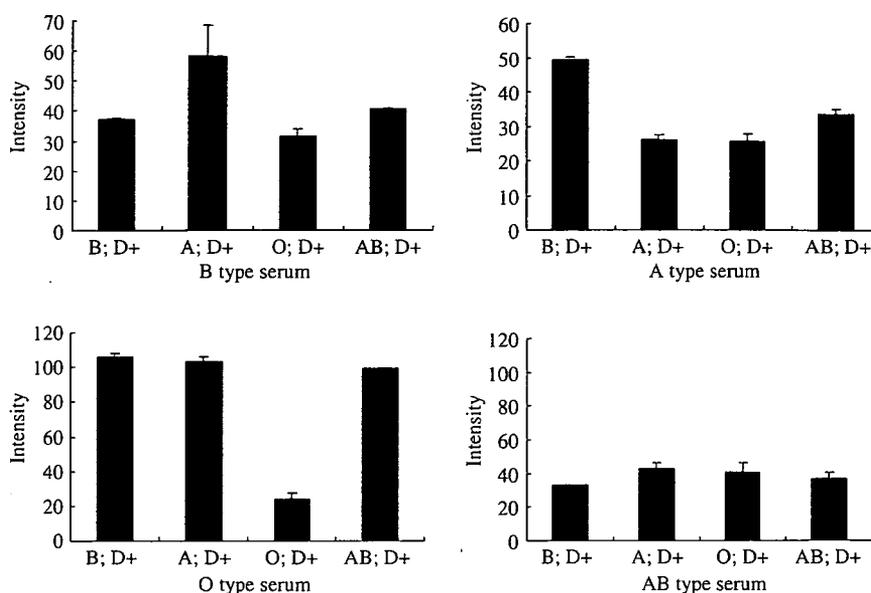
Fig. 3. Detection of chemiluminescence of antibodies in the serum of some blood types bound on photoimmobilized panel cells array.

as expected. The method using a cell array is useful for antibody detection because of its convenient multiple analysis of antibodies in human serum.

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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

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Table 1. Attachment Strategies for Microarray Production

functional groups	attachment		references
	non-covalent	covalent	
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

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.
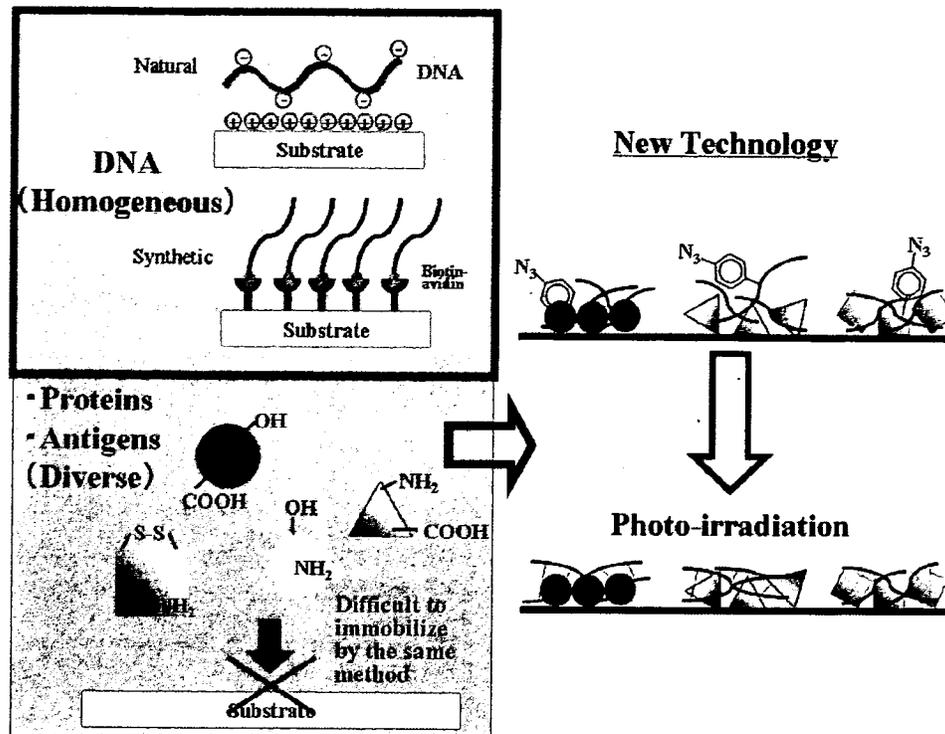
Figure 1. Comparison of DNA- and protein-immobilization and photoimmobilization. Because DNA is chemically homogeneous and can be specifically modified by organic synthesis it is easy to immobilize on a chip (top left). However, because proteins are chemically different, it is difficult to immobilize 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 micro-pattern-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 photoir-radiation 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.
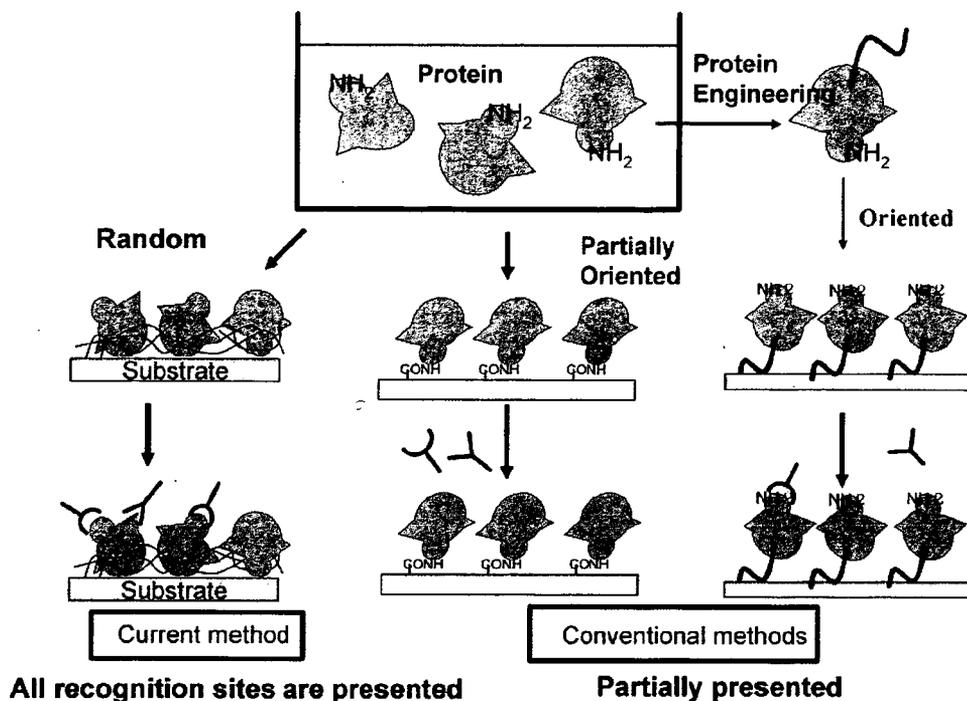
Figure 2. Comparison of immobilization methods. Photoimmobilization leads to the random orientation of immobilized molecules, whereas other covalent immobilization methods lead to some orientation of immobilized molecules because of the uneven distribution of functional groups on the molecules being 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.
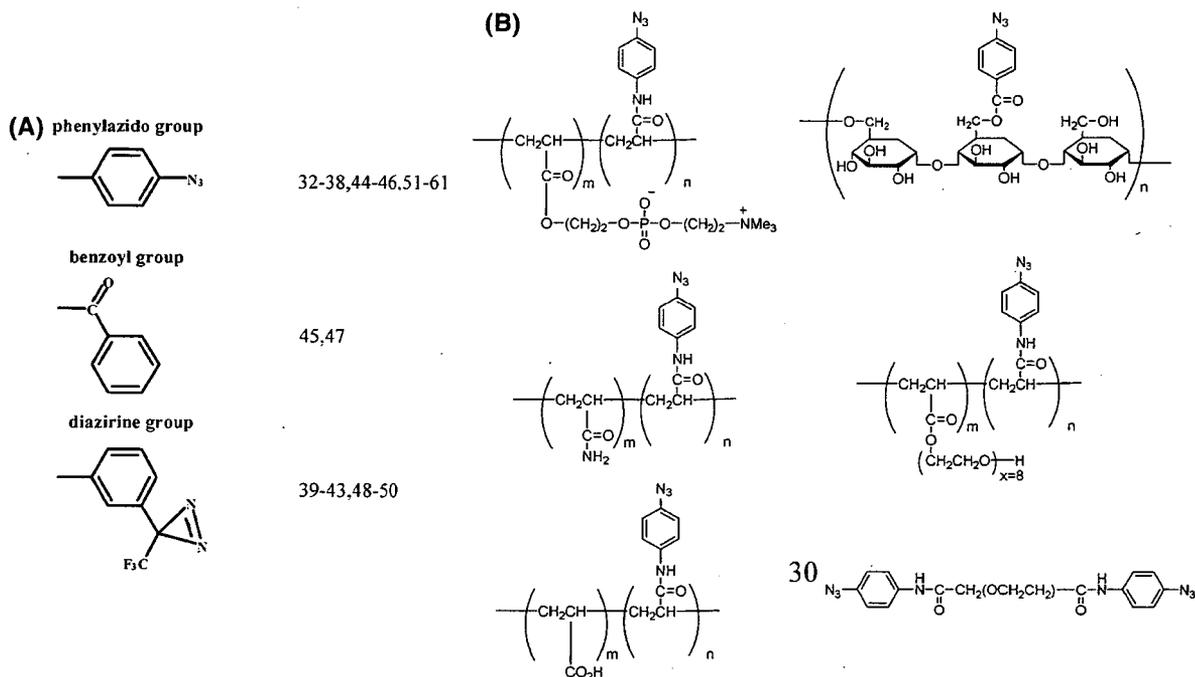

Figure 3. (A) Examples of photoreactive functional groups used in photoimmobilization. The numbers on the right indicate the references for photoimmobilization studies that have been conducted on each photoreactive functional group. (B) Photoreactive polymers synthesized by our group's current work.

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
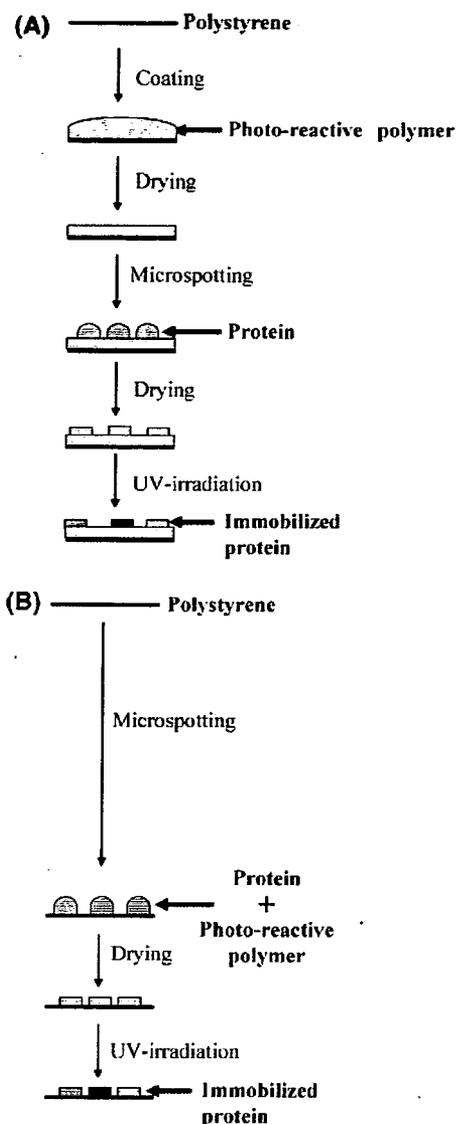
(A) ———— Polystyrene

↓ Coating

◁—— Photo-reactive polymer

↓ Drying

↓ Microspotting

◁—— Protein

↓ Drying

↓ UV-irradiation

◁—— Immobilized protein

(B) ———— Polystyrene

Microspotting

↓

Protein
+
Photo-reactive
polymer

↓ Drying

↓

↓ UV-irradiation

◁—— Immobilized protein

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.

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 (□).

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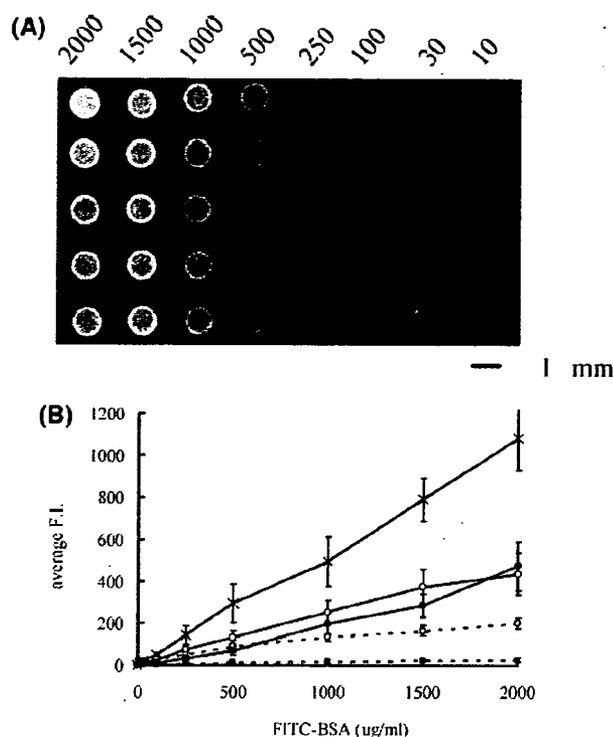
(ethylene glycol) (61). Because these water-soluble polymers do not nonspecifically interact with biological components, they have been used to form an immobilization matrix to reduce nonspecific interactions (N) and to enhance specific interactions (S), resulting in a high S/N ratio (57—61).

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
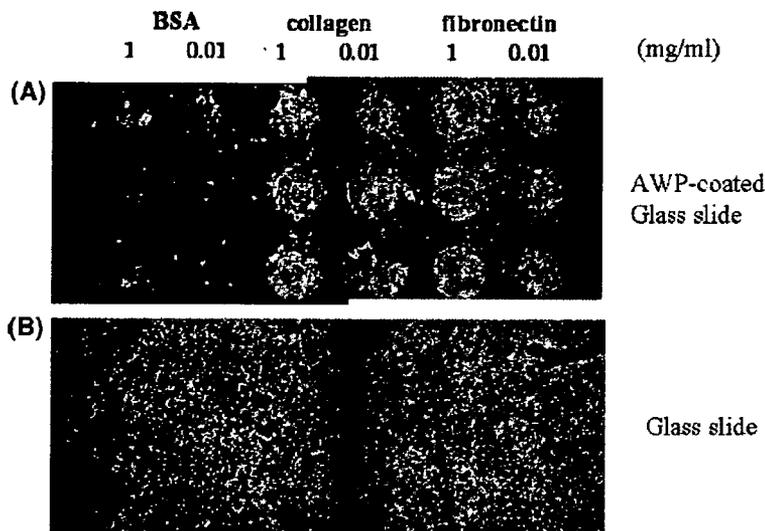
BSA		collagen		fibronectin		
1	0.01	1	0.01	1	0.01	(mg/ml)

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)