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CHEMICAL MODIFICATION OF PROTEINS FOR BIOCOMPATIBLE POLYMERS

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ABSTRACT

Chemical modification of biosignal proteins for design and synthesis of biocompatible polymers is described. Biosignal proteins were immobilized onto polymer film to obtain hybrid film for regulating cellular functions. Immobilization of adhesion or growth protein enhanced cell adhesion or growth, respectively. Coimmobilization of these proteins markedly enhanced the growth of anchorage-dependent cells. This concept of materials design was applied for endothelialization of artificial blood vessel.

INTRODUCTION

Many attempts have been made to impart biocompatibility, in particular, nonthrombogenicity, to synthetic polymers which can be used for artificial organ materials [1]. These attempts began with controlling surface properties of polymer film, e.g., roughness or smoothness, morphology, hydrophilicity or hydrophobicity, electric charge, etc. Hybridization of polymer film with antithrombogenic substances such as heparin, prostacyclin, argatroban (a hydrophobic derivative of arginine), thrombin substrate peptides, urokinase, and heparinoid polymers—e.g., poly(vinyl sulfonate)—has been investigated, too. However, these attempts were only successful in obtaining a temporary (short-term) or an imperfect nonthrombogenicity.

Recently, there has been interest in polymer materials for promotion of tissue construction or materials hybridized with living tissues for long-term nonthrombo-

genicity or biocompatibility. The control of cellular functions—e.g., adhesion, movement, growth, and differentiation—with polymeric compounds is considered to be essential in the investigation of artificial organ materials.

ENHANCEMENT OF CELL ADHESION ON POLYMER FILM BY IMMOBILIZATION OF ADHESION PROTEIN

Cell-adhesion proteins such as collagen, fibronectin, and vitronectin; or a tetrapeptide, Arg-Gly-Asp-Ser (RGDS), which is involved in the active site of cell-adhesion proteins, were immobilized on various polymer films to enhance cell adhesion. We immobilized fibronectin on poly(acrylic acid)-grafted polystyrene film in the following way. First, polystyrene film was glow discharged and acrylic acid was graft polymerized on the surface. The proteins were coupled to the poly(acrylic acid) grafts with water-soluble carbodiimide. The immobilized adhesion proteins were found to enhance markedly cell adhesion.

Similarly, the RGDS-immobilized polystyrene film increased cell adhesion, and the activity was comparable to the fibronectin-immobilized polystyrene film as shown in Fig. 1 [2]. Although the unit activity of RGDS was lower than that of fibronectin, the total activity of immobilized RGDS was increased with increasing density on the surface. In addition, RGDS was more resistant against pH change, thermal treatment, and treatment with ethanol than fibronectin.

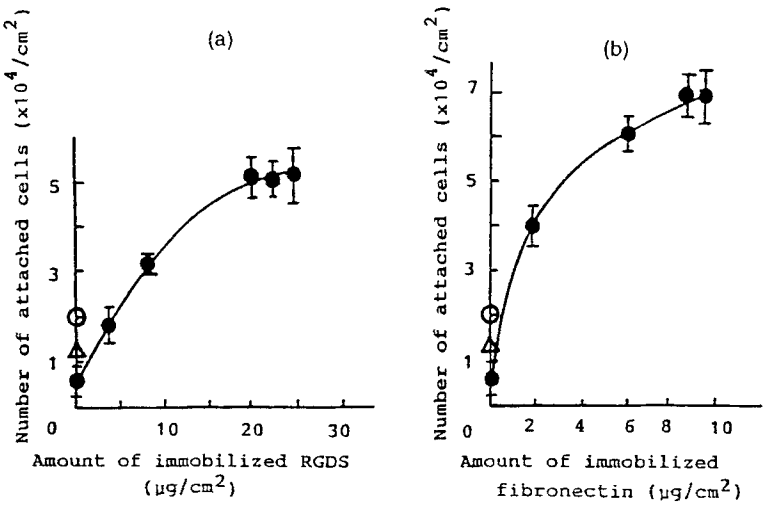

FIG. 1. Adhesion of mouse fibroblast cell STO on modified polystyrene films: △, unmodified polystyrene; ○, glow-discharged polystyrene. (a) RGDS-immobilized poly-(acrylic acid)-grafted polystyrene; (b) fibronectin-immobilized poly(acrylic acid)-grafted polystyrene. n = 10. Bars represent standard deviations.

ENHANCEMENT OF CELL GROWTH ON POLYMER FILM BY IMMOBILIZATION OF GROWTH PROTEIN

It has been considered that the control of cell growth with polymer materials should be more difficult than the control of cell adhesion, because the mechanism of signal transmission for cell growth has not been clear. As illustrated in Fig. 2, growth proteins interact with their specific receptors on the cell surface and the protein/receptor complexes are internalized into the cell to be dissociated in the lysosome, and only a part of the liberated receptors is transported back to the cell surface, resulting in a downregulation phenomenon. However, it is expected that the protein/receptor complexes might not be internalized if the biosignal proteins are immobilized on nonbiodegradable polymer film, resulting in unattenuated transmission of strong biosignals to the cell nucleus.

Insulin is a typical growth factor and is often added to serum-free culture media [3–6]. Mouse fibroblast cell line STO was cultured in the presence of poly(methyl methacrylate) (PMMA) film on which insulin was immobilized. As a result,

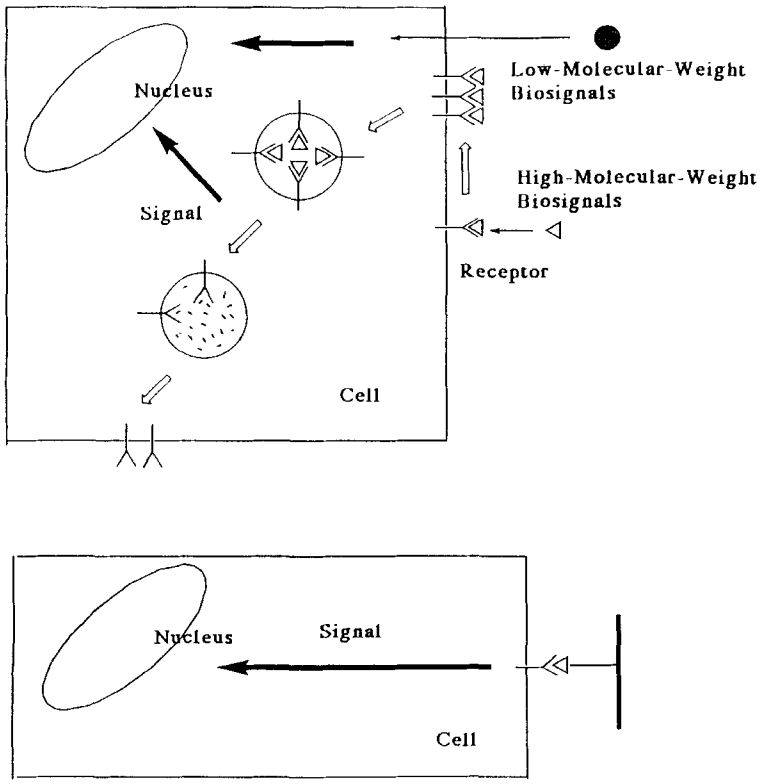

FIG. 2. Schematic illustration of biosignal molecule/cell interaction. Low molecular weight molecules (e.g., steroid hormones) permeate cell membrane without intervention of receptors. On the other hand, high molecular weight molecules (e.g., growth proteins) permeate cell membrane after interaction with receptors. In the case of immobilized biosignals, the complex with receptor is not endocytosed.

the cell growth was more accelerated on the insulin-immobilized film than in the presence of free (soluble) insulin, as shown in Fig. 3. This result suggests a possibility to control complicated cellular functions such as movement, growth, and differentiation by immobilization of biosignal proteins onto polymer films.

Insulin was coimmobilized with other kinds of growth factor, such as transferrin, on various polymer matrices, including PMMA, poly(ethylene terephthalate), polyurethane, and poly(hydroxyethyl methacrylate-*co*-ethyl methacrylate) films, and polyacrylamide, glass, and collagen beads as summarized in Fig. 4 [7–14]. Anchorage-dependent cells were cultured on these hybrid materials and the growth was accelerated. On the other hand, the growth of anchorage-independent cells was not accelerated on the insulin/transferrin coimmobilized matrices. The latter phenomenon might have been caused by the absence of contact of the anchorage-independent cells with the immobilized proteins.

FURTHER ENHANCEMENT OF CELL GROWTH ON POLYMER FILMS BY COIMMOBILIZATION OF GROWTH AND ADHESION PROTEINS

Several attempts to enhance the activity of the growth protein immobilized polymer film were made, as illustrated in Fig. 5. On one hand, a spacer chain was inserted between the growth proteins and polymer matrix [Fig. 5(a)] to obtain a flexible immobilization which might facilitate encounter with receptor proteins [9].

In other attempts [Fig. 5(b)], an enhanced contact of cell with the immobilized proteins was aimed at. Positively charged polymers were coimmobilized with insulin

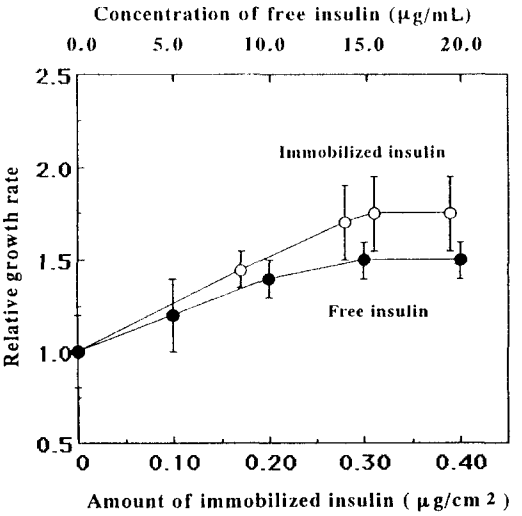

FIG. 3. The relative growth rate of mouse fibroblast cell STO on insulin-immobilized poly(methyl methacrylate) film (○) or on surface-hydrolyzed poly(methyl methacrylate) film in the presence of free insulin (●). The relative cell growth rate was calculated by counting cells on surface-hydrolyzed poly(methyl methacrylate) film in the absence of insulin after 48-h culture.

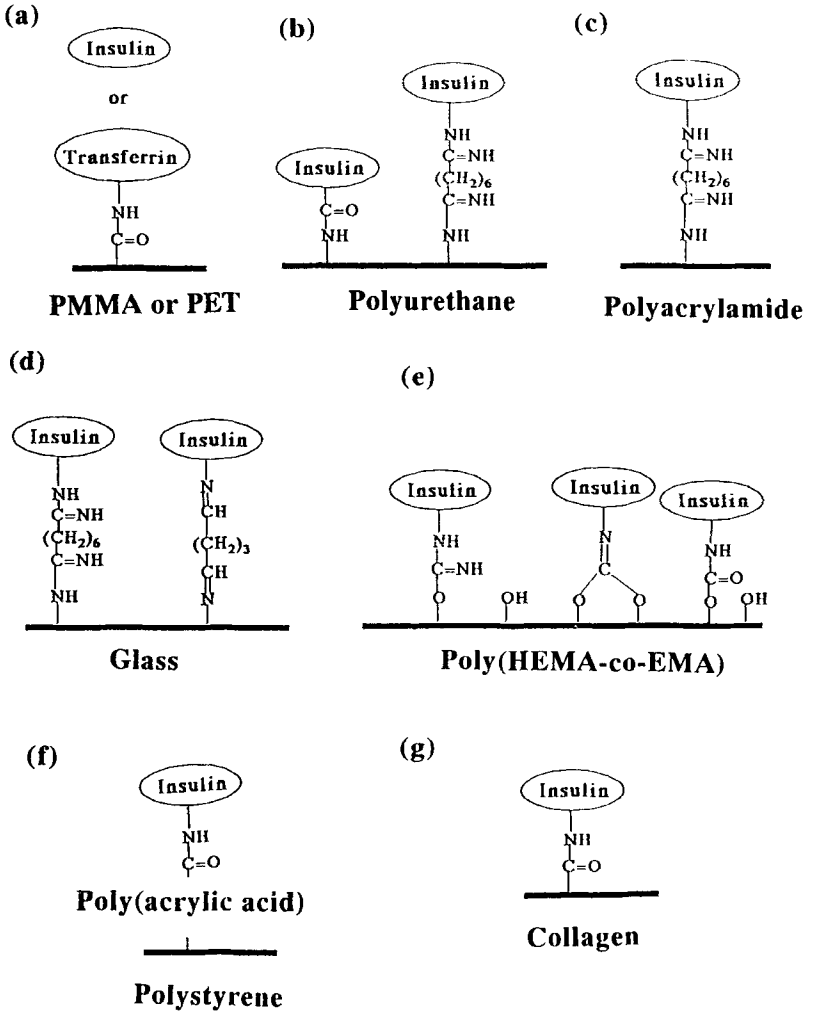

FIG. 4. The growth factors immobilized upon various polymer matrices. (a) Insulin or transferrin immobilized on surface-hydrolyzed poly(methyl methacrylate) or poly(ethylene terephthalate) film with water-soluble carbodiimide. (b) Insulin immobilized on aminated polyurethane tube or film with water-soluble carbodiimide or dimethyl suberimidate. (c) Insulin immobilized on NaOCl-treated polyacrylamide beads with dimethyl suberimidate. (d) Insulin immobilized on silane-coupled glass beads with dimethyl suberimidate or glutaraldehyde. (e) Insulin immobilized on poly(hydroxyethyl methacrylate-*co*-ethyl methacrylate) film with CNBr. (f) Insulin immobilized on poly(acrylic acid)-grafted polystyrene dish. (g) Insulin immobilized on macroporous collagen beads with water-soluble carbodiimide.

to enhance electrostatic interactions with negatively charged cells. Adhesion proteins were coimmobilized with growth proteins, too [9]. In the latter approach, insulin and fibronectin were coimmobilized on the surface-hydrolyzed PMMA film, and the mouse fibroblast cell line STO was cultured in the presence of the coimmobilized film. As shown in Table 1, an acceleration of cell growth to the maximum of

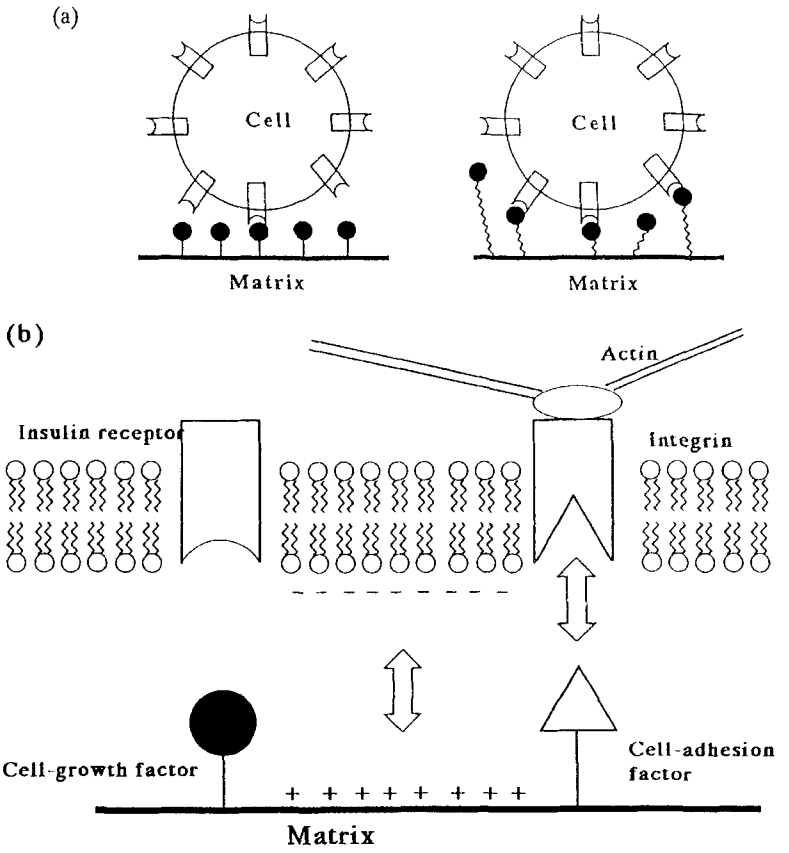

FIG. 5. Schematic explanation of several attempts to enhance the effect of immobilized biosignal proteins. (a) Introduction of a spacer chain enhances formation of biosignal/receptor complex. (b) Enhancement of contact of cell with the immobilized growth proteins either by immobilization of positively charged polymers or by coimmobilization of growth proteins with adhesion proteins.

2.7- to 2.9-fold was obtained in the presence of immobilized insulin plus soluble fibronectin or immobilized fibronectin plus soluble insulin. The extent of acceleration was less than that (3.13-fold) attained by a simultaneous addition of two kinds of soluble proteins. The highest acceleration of cell growth (3.32-fold) was obtained in the culture in the presence of insulin/fibronectin-coimmobilized film. The strong acceleration of cell growth by insulin/fibronectin coimmobilization might be due to increased susceptibility toward immobilized insulins of the receptors of adhered cells.

APPLICATION OF BIOSIGNAL-IMMOBILIZED MATERIALS

Endothelialization of synthetic polymers seems promising for acquisition of perfect nonthrombogenicity or blood compatibility. We tested the effectiveness of growth factor/adhesion factor coimmobilization on a polyurethane tube for endo-

TABLE 1. Cell Growth with Free and/or Immobilized Proteins

Immobilized insulin/fibronectin ($\mu g/cm^2$)	Free insulin ($\mu g/mL$)	Free fibronectin ($\mu g/mL$)	Relative growth rate
0.38/0			1.80 ± 0.07
0.27/0.12			2.56 ± 0.04
0.22/0.13			3.32 ± 0.07
0.15/0.13			3.12 ± 0.06
0.15/0.15			2.74 ± 0.11
0/0.59			1.75 ± 0.08
0/0.59	15	5	3.13 ± 0.04
0/0.59	10		2.90 ± 0.05
0.38/0		10	2.70 ± 0.05

thelialization [10]. A polyurethane tube, on which insulin and collagen were coimmobilized, was rapidly covered with endothelial cells. The endothelial cell layer was stable for more than 9 months, as shown in Table 2. In addition, the grown endothelial cells efficiently secreted prostacyclin. In this bioproduction experiment, coimmobilization of transferrin and collagen was found most effective.

Our concept of immobilized biosignal proteins provided an innovative culture technique and attained a rapid and stable endothelialization of synthetic polymers.

TABLE 2. Coverage of Interior of Polyurethane Tubes with Endothelial Cells and Prostacyclin Secretion

Polymer tube	Time required to cover the interior of polymer tube (days)	Time lapse until the beginning of cell detachment (days)	Rate of prostacyclin secretion ($pg/10^5$ cell day)
Without immobilization	18 ± 1	19 ± 1	274 ± 20
Insulin immobilized	13 ± 1	20 ± 1	336 ± 25
Transferrin immobilized	15 ± 1	19 ± 1	310 ± 21
Collagen immobilized	17 ± 1	>270	435 ± 26
Insulin/collagen coimmobilized	10 ± 1	>270	360 ± 25
Transferrin/collagen coimmobilized	13 ± 1	>270	522 ± 30

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