Two-Dimensional Artificial Extracellular Matrix: Bioadhesive Peptide-Immobilized Surface Design

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SYNOPSIS

A novel artificial extracellular matrix derivatized with a cell adhesive peptide, Arg–Gly–Asp–Ser (RGDS) tetrapeptide, which is the central peptidyl sequence of the adhesive site of fibronectin, was designed. RGDS coupling was achieved via isocyanation of surface hydroxyl groups of poly(vinyl alcohol) (PVA) film and subsequent conversion to activated ester. The surface-modified PVA film was quantitatively analyzed by ESCA. The surface density of RGDS was partly controlled by the degree of isocyanation of the PVA film. Bovine endothelial cells (ECs) adhered and grew well on the RGDS-derivatized PVA film, irrespective of the presence or absence of the serum. The adhesion and growth of ECs were enhanced with an increase in the surface density of RGDS. When a sufficient amount of RGDS was added to the medium, the adhered ECs were delaminated. This indicated that the adhesion of ECs on an RGDS-derivatized PVA surface is mediated by the RGD–ligand/receptor interaction. Thus, a peptidyl artificial matrix via surface derivatization was developed. © 1993 John Wiley & Sons, Inc.

INTRODUCTION

Living cells in the tissues adhere to and grow on extracellular matrices (ECMs) that are composed mainly of proteins and glycosaminoglycans. It has been established that the ECMs regulate cell behavior such as adhesion, growth, migration, and differentiation. Of biomolecular components of ECMs, it has been reported that adhesive proteins such as fibronectin, collagen, vitronectin, and laminin serve as a cell-adhesive matrix. It has been established that the minimal amino acid sequence of the adhesive site of fibronectin is Arg–Gly–Asp–Ser (RGDS; one-letter description of amino acid).[1,2] In fact, previous studies showed that peptides containing the RGD sequence dose-dependently inhibited the cell-adhesion promoting effect of fibronectin when mixed with various anchorage-dependent cells including endothelial cells, smooth muscle cells, and platelets.[3] It has also been evidenced that the adhesion receptors in cell membranes molecularly recognize the RGD sequence.[4]

The background, as mentioned above, on the cell-adhesion mechanism via RGD–ligand/receptor interaction prompted us to design an artificial extracellular matrix into which an RGD–ligand is incorporated. Imanishi et al. reported that the RGDS tetrapeptide immobilized on polyamine-grafted silicone films significantly enhanced the adhesion of fibroblasts.[5] We have attempted to prepare RGD-derivatized surfaces and polymers.[6–8] As a part of the series of our RGD–peptidyl-immobilized studies aiming at the development of 2-dimensional and 3-dimensional artificial ECMs, a surface derivatization approach is demonstrated here.

In this paper, we report a surface derivatization of RGDS on a nonadherent hydrophilic surface, poly(vinyl alcohol) (PVA). RGDS derivatization resulted in the conversion of the surface from nonbioactive to bioactive.

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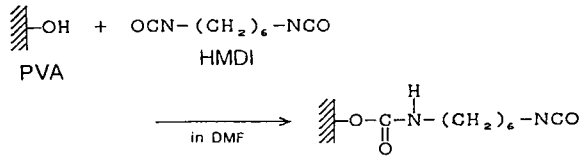
EXPERIMENTAL

Materials

Poly(vinyl alcohol) (PVA) film obtained from Nichigo Film Co. Ltd. (Ogaki, Japan) was thoroughly washed with a Soxhlet extraction with methanol prior to the chemical modification. Arg–Gly–Asp–Ser (RGDS) peptide was obtained from the Peptide Institute, Inc. (Minoo, Japan). Hexamethylene diisocyanate (HMDI) and 4-dimethylaminopyridine (DMAP), both of which were of a special grade, purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and *N*,*N*-dimethylformamide (DMF), a special grade, purchased from Nacalai Tesque, Inc. (Kyoto, Japan), were dried on molecular sieves. Disuccinimidylsuberate (DSS) was purchased from Pierce Chemical Co. (Rockford, IL).

1st Stage



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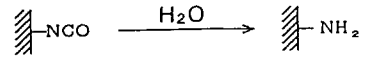

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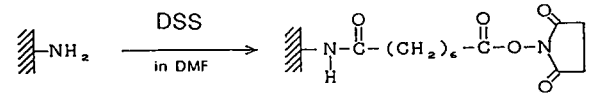
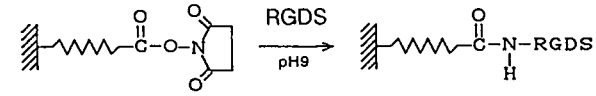


4th Stage



Scheme 1 Schematics of RGDS derivatization on the surface of the PVA film. The first stage: isocyanation of the surface hydroxy group. The second stage: alkaline hydrolysis of the surface isocyanate group. The third stage: generation of the activated ester. The fourth stage: derivatization of RGDS tetrapeptide in an aqueous solution. The reaction time in the first stage was the only variable controlling the surface density of RGDS. The reaction conditions in the second stage to the fourth stage were fixed.

The preparation of RGDS-derivatized PVA films is briefly described below.

Surface Modification

The first stage, isocyanation of the hydroxyl group, was performed using HMDI (10% v/v) in DMF in the presence of DMAP (0.5% w/v). At the second stage, the surface isocyanate group was hydrolyzed in 1*N* NaOH aqueous solution for 1 h. At the third stage, the activation reaction of the surface amino group was performed for 1 h in DMF containing 2.5 mg/mL DSS. At the fourth stage, an RGDS coupling reaction was performed for 1 h in 10 mg/mL RGDS aqueous solution that was adjusted to pH 9. All the reactions were carried out at room temperature. After a thorough wash with distilled water, RGDS-derivatized PVA film was desiccated and stored in a desiccator until surface analysis and culturing of cells.

Surface Analysis by ESCA

The surface-modified PVA films were analyzed by electron spectroscopy for chemical analysis (ESCA). ESCA measurements were performed with the ESCA 750 spectrometer (Shimadzu Co., Kyoto, Japan) with the aid of the grading-angle technique. The X-ray source was MgKα radiation. The spectra data were collected using ESCAPAC 760 (Shimadzu Co.) and were analyzed by a computer-aided curve-deconvolution method.

Endothelial Cell Culture

Bovine endothelial cells (ECs) harvested from bovine aortae were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (FCS) at 37°C in 95% air and 5% CO_2. RGDS-derivatized PVA films, sterilized by brief irradiation with UV light, were subjected to the cell-adhesion experiments. The adhered cells were observed by phase-contrast microscopy and scanning electron microscopy.

RESULTS AND DISCUSSION

Surface Modification

RGDS tetrapeptide is not soluble in organic solvents such as DMF, but is very soluble in water. Therefore, the coupling reaction must be performed in an aqueous phase. RGDS tetrapeptide was coupled on

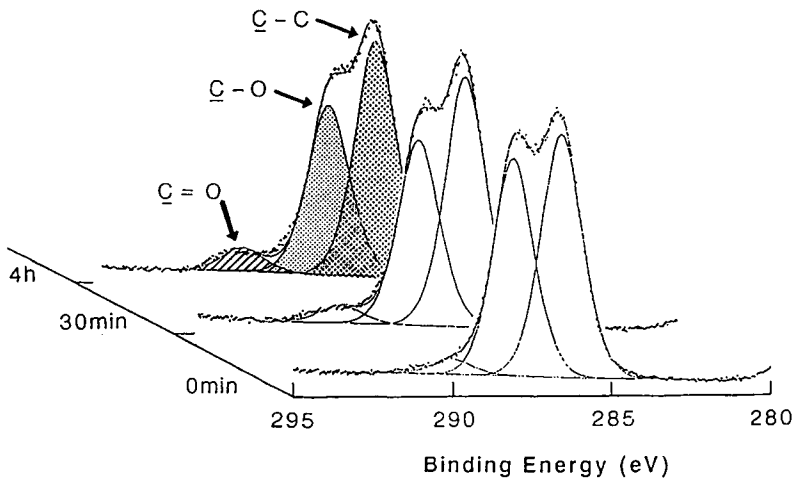


Figure 1 C1s spectra of the surface-isocyanated PVA film. C1s spectra were separated into three subpeaks, assigned as indicated in the figure, by a computer-aided curve-deconvolution method.

the surface-modified PVA film according to Scheme 1. This surface coupling method consists of a sequence of four reactions: The first reaction is isocyanation of surface hydroxyl groups using HMDI in DMF. The second reaction is alkaline hydrolysis of the surface isocyanate group to generate the amino group. The third reaction is the coupling reaction of DSS with surface amino groups in DMF to generate the activated ester group: succinimide ester. The last reaction is the derivatization of RGDS with the activated ester in an aqueous solution. The reaction conditions, including reagent concentrations and reaction times, in the second to the fourth reaction were fixed. The reaction time of the first reaction was the only variable controlling the surface density of derivatized RGDS in this experiment.

The surface isocyanation reaction was quantitatively analyzed by ESCA, which enabled us to determine the chemical composition up to several tenths of angstroms in depth. C1s spectra were separated into subpeaks by a curve-deconvolution method (Fig. 1). The C1s fraction assigned to the carbonyl carbon (C=O), which is derived from the isocyanate group and urethane linkage formed, increased with an increase in the reaction time. The

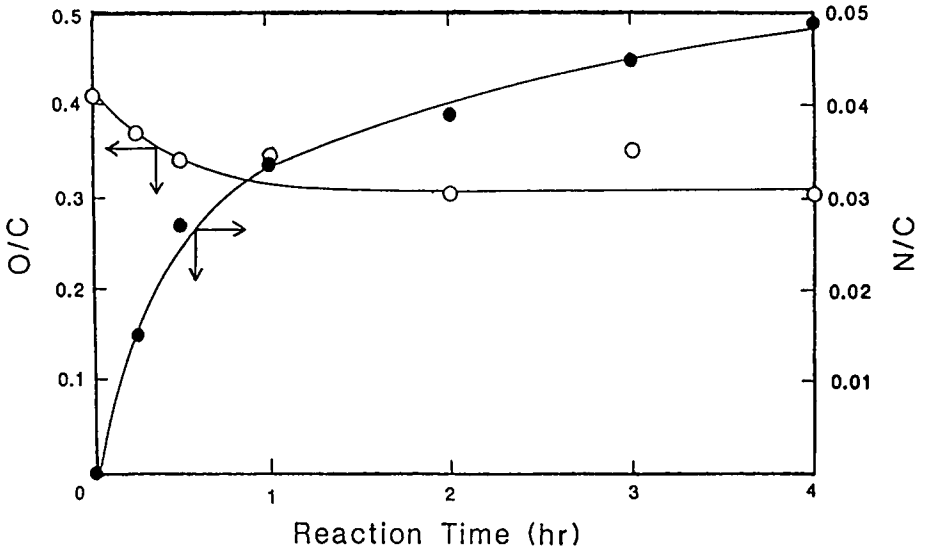


Figure 2 The time-dependent elemental ratio of O/C and N/C on the surface-isocyanated PVA film. Open circle: O/C; closed circle: N/C.

elemental ratios of O/C and N/C were plotted against the isocyanation time (Fig. 2). The O/C ratio gradually decreased as the reaction proceeded. On the other hand, the N/C ratio markedly increased with reaction time. These findings indicate that the degree of isocyanation increased with the reaction time, which may determine the surface density of derivatized RGDS because reaction conditions of the second to the fourth reaction were fixed.

Little appreciable change in the ESCA spectra was observed upon the hydrolysis of surface isocyanation groups. The increase in the C1s fraction ascribed to C=O was observed as the third reaction was completed. The RGDS-derivatizing density of the fourth reaction was qualitatively measured by ESCA measurement coupled with a grading-angle technique. Figure 3 shows the isocyanation time dependence of the N/C ratio of RGDS-derivatized surfaces. The N/C ratio increased with an increase in the isocyanation time. This was greatly enhanced with the spectra recorded at the takeoff angle of 15° than at 90°. If it is considered that the effective measuring depth of the ESCA spectrum is proportional to the sine of the takeoff angle, the spectra recorded at 15° determines the surface chemical composition closer to the outermost layer than do those recorded at 90°. These results indicate that derivatized RGDS peptide is localized in the outermost layer and that its surface density increases with an increase in the isocyanation time.

Adhesion and Growth of Endothelial Cells

On RGDS-derivatized PVA films, the surface densities of which were controlled by the isocyanation time at the first-stage reaction as shown in Figure 3, bovine aortic endothelial cells (ECs) were seeded and cultured in DMEM with or without fetal calf serum (FCS). Neither adhesion nor growth of ECs was seen for nontreated PVA film (Figs. 4 and 5). Upon further culturing, ECs floated in the medium to form aggregates. This is due to the hydrophilic nature of the surface, which causes generally minimal adsorption of proteins and facilitates desorption. To the contrary, ECs adhered well on RGDS-derivatized surfaces, irrespective of the presence or absence of FCS. Figure 6 shows that adhered ECs spread well on RGDS-derivatized PVA film, whereas ECs, which merely settled down on nontreated PVA film, remained round. The number of adhered ECs increased with an increase in the isocyanation time, which reflects the surface density of derivatized RGDS (Fig. 4). This adhesion dependence on the surface density of RGDS found here is in good agreement with previous observation by Imanishi et al., who showed that the adhesion of fibroblast cells is proportional to the amount of immobilized RGDS on aminated silicone films.[5] Further culturing in the medium supplemented with FCS resulted in the formation of a confluent monolayer that exhibited the cobblestone morphology characteristic of ECs, irrespective of the isocyanation time (Fig. 5).

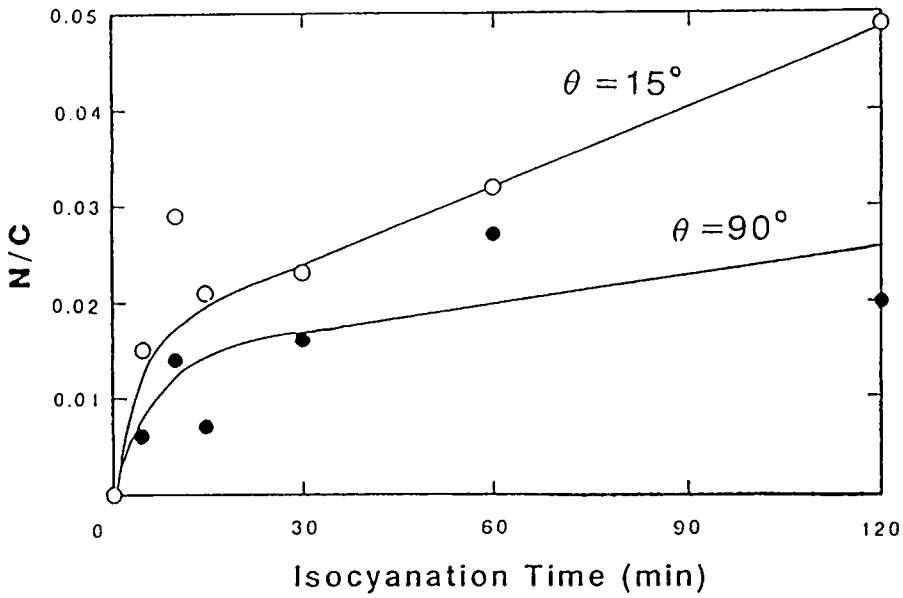


Figure 3 The time-dependent elemental ratio of N/C on the RGDS-derivatized PVA film, measured at the takeoff angle (θ) of 15° (open circles) and 90° (closed circles).

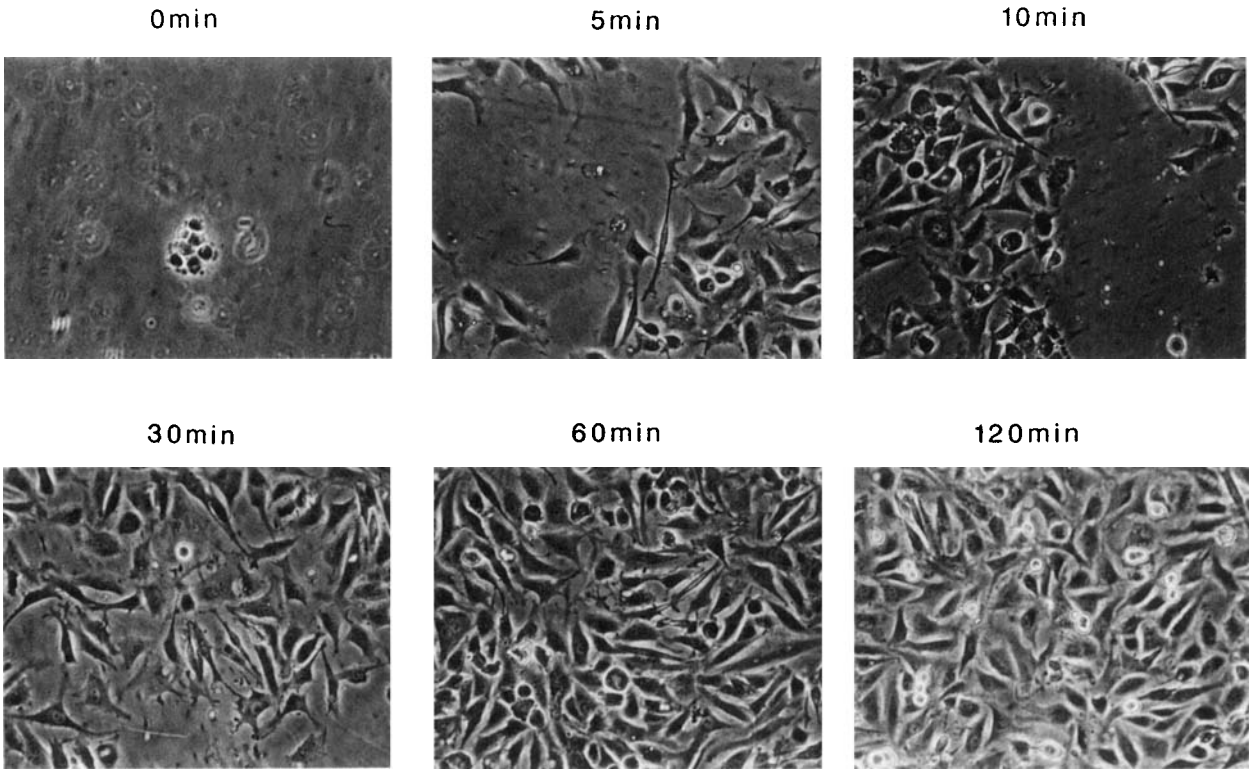


Figure 4 Initial adhesion of ECs on the RGDS-derivatized PVA films at 3 h after seeding (phase-contrast microscopy). The numbers shown denote isocyanation time.

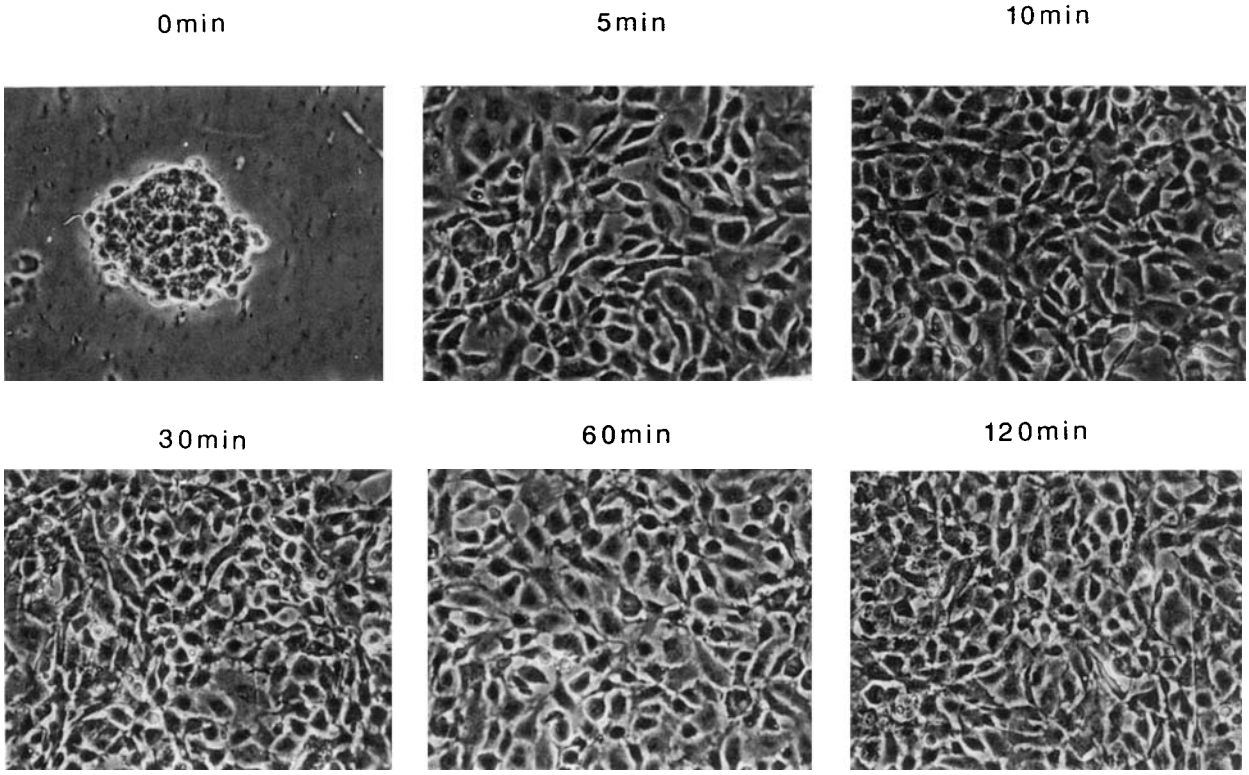


Figure 5 The growth of ECs on the RGDS-derivatized PVA films at 24 h after seeding (phase-contrast microscopy). The numbers shown denote isocyanation time.

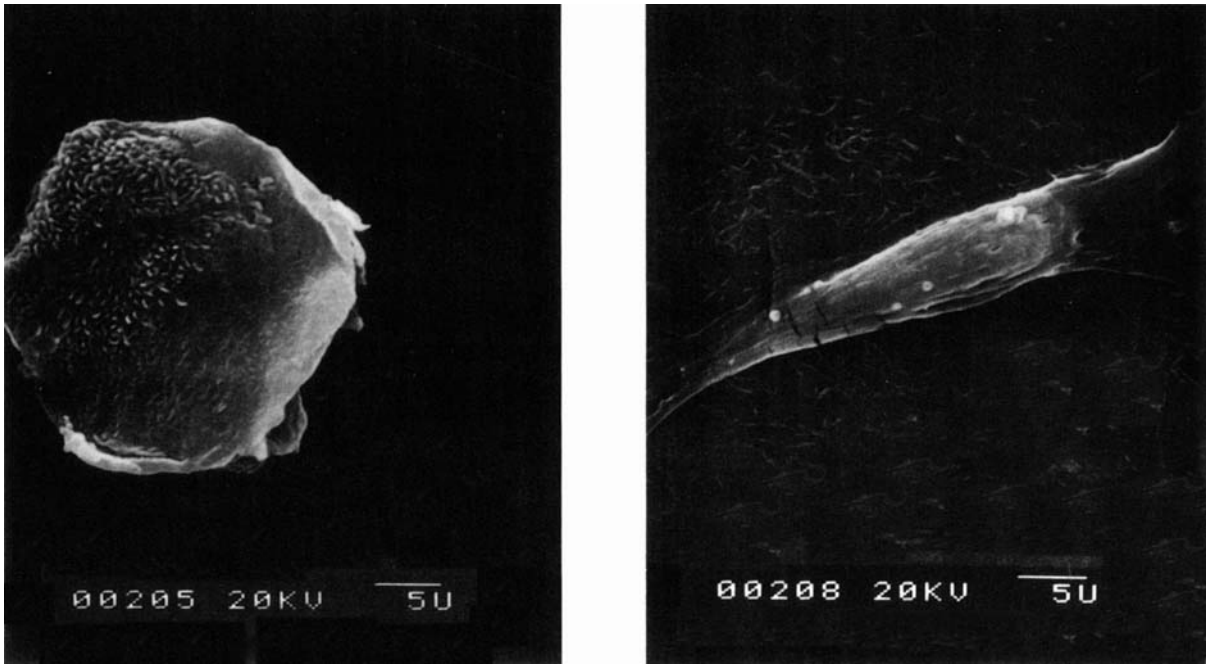


Figure 6 Scanning electron microscopic observation of EC on the nontreated PVA film (left) and the RGDS-derivatized PVA film (right) after 3 h of incubation.

It is of interest to see whether the adhesion of ECs on RGDS-derivatized PVA film is mediated via RGD–ligand/receptor interaction. If such a mechanism operates for this case, the addition of a sufficient amount of RGDS into the medium may result in the delamination of adhered ECs, due to the antagonistic action. This is based on the fact that ECs have receptors that can molecularly recognize the adhesive sites containing the RGD tripeptidyl sequence common to adhesive proteins. In fact, the addition of 1 mg/mL RGDS into the medium resulted in delamination of most adhered ECs, indicating that RGD–ligand receptor interaction is responsible for the adhesion of ECs on the artificial substrate.

In conclusion, we demonstrated that RGDS peptide-derivatized surfaces enhanced the adhesion and growth of ECs. The derivatization of RGDS tetrapeptide onto a PVA surface converted it from a nonadhesive to a bioadhesive matrix even in the absence of adhesive proteins. Since the present method of derivatization of RGDS on a PVA surface is rather complex, only qualitative information on the relationship between RGDS surface density and adhesion capability was obtained. A simpler and more reliable method of surface derivatization and detailed surface characterization as well as adhesion kinetics will be reported in the near future.

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