Effect of Surface Structure on Cell Growth Prepared by the Terminal Immobilization Method

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ABSTRACT: Substrate effects of surface morphology and chemical structure for cell cultures prepared by molecular terminal immobilization method were studied. When we focused attention on a phenyl group as a functional moiety, the cell growth on the surface prepared by the immobilization method showed a better proliferation rate than that of a substrate prepared by the casting method. Further, from the results of mouse fibroblast L929 cell (L-cell) growth on poly(amino acid)-immobilized surfaces in Dulbecco's minimal essential medium containing 10% FBS, it was indicated that the amino group was more effective than the phenyl group, and that a slight difference of chemical structure had a substantial influence on cell growth. In addition, mouse bone marrow–derived Sp2/0-Ag14 cell (Sp2/0 cell) culture in ASF-104 serum-free medium, poly(amino acid)-immobilized substrates showed an almost equal proliferation rate to that in a serum-containing medium. These results showed that effective cell growth can occur on immobilized surfaces, and that detection of a weak interaction depends on the functional groups. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 91: 3001–3008, 2004

Key words: biocompatibility; terminal immobilization method; cell growth; poly(amino acid)s; biological activity

INTRODUCTION

Problems for developments of biomedical applications, and biocompatibility of synthetic materials with living tissue such as toxicity, carcinogenicity, and allergic response are first discussed.[1–5] In such cases, the chemical and physical structures of the material interfaces in contact with the living tissue strongly influence the vital functions.[6,7] Clinical applications require various properties; for example, the development of blood-contacting materials has required the designing of antithrombogenic surfaces that reduce protein adhesion,[8–16] but in the case of cell culture there needs to be cell-adhesive surfaces.[17–22] In some cases by using these surfaces, life processes cannot be maintained. For this reason, with tissue culture *in vitro*, it is imperative to construct an interface having biological activity.

Because of its optical property, cost, and sterilization by γ-ray irradiation, polystyrene has been widely used.[23–27] Although these tissue cultures have histocompatibility, a lowering of vital functions cannot be ignored. In addition, expensive serum protein is needed to improve the growth ability of a cell. Because of these problems, various modified methods to retain specific physiology activity were investigated; such methods as tissue-culture ware coated with gelatin,[28–30] poly(amino acid)s,[31–34] and collagen[35–37] were developed and have shown high cell growth promotion actions. In particular, wares coated with poly(amino acid)s were evaluated with respect to the effects of side chain[38] and the secondary structures[39] for cell culture. Recently, it was reported that Arg-Gly-Asp (RGD) peptide sequences progressively inhibit binding of cell-surface receptors, and examinations have proceeded regarding cell growth on polymer films with this sequence introduced into various polymer chains.[40–44] However, many issues regarding use of the cast method, such as solubility, surface bulky characteristics, and membrane holding remain, although here we do not examine in detail the relevance between surface structure and cell growth.

In this study, to construct a different biological activity surface, we report the possibility of cell cultures on modified glass surfaces prepared by the terminal immobilization method. This method has the advantages that a monolayer-like structure on a substrate can be immobilized and that a weak interaction can be detected depending on the functional moiety applied to the medical polymer materials. From this perspective, we focused on participation of phenyl and amino groups in cell culture. Initially, we synthesized polymeric styrene having a trimethoxysilane moiety at the

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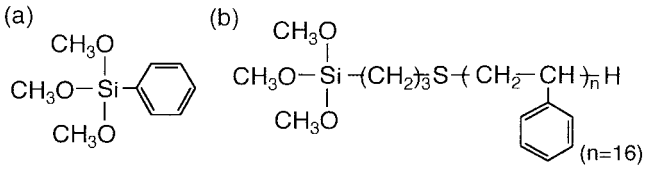


Scheme 1 Chemical structure of (a) monomeric and (b) polymeric styrene having trimethoxy group at the terminal end.

terminal, and immobilized it onto a glass surface to compare with the cell growth on polystyrene cast film. We also focused on poly(amino acid)s as immobilized polymers having many characteristics, not only of biocompatibility but also of side chain modification and secondary structure. We synthesized various types of phenyl groups containing poly(amino acid)s having trimethoxysilane moiety at the terminal to highlight the relevance of surface structure and cell growth. This demonstrated a versatile approach to the manipulation of cellular responses in biomaterials and in tissue engineering applications.

EXPERIMENTAL

Materials

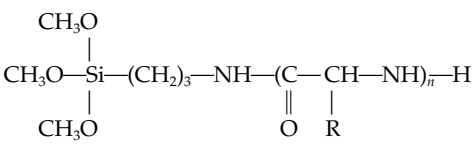
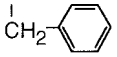
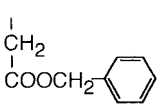
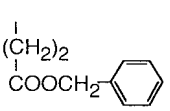
Polymeric styrene [Scheme 1(b)] was prepared by radical telomerization of styrene with 3-mercaptopropyltrimethoxysilane (MPS) as described.[45] The structure and polymerization degree of PS_n was determined by ^{1}H-NMR spectroscopy. ^{1}H-NMR [in $CDCl_3$, δ = 7.0–6.5 ppm (5H, aromatic), 3.6 ppm (9H, SiOCH_3), and 0.8 ppm (2H, SiCH_2)]. The average degree of polymerization (n) was estimated to be 16 from the ratios at 7.0–6.5 and 0.8 ppm.

Poly(amino acid)s with a trimethoxy group at the terminal were obtained by polymerizing *N*-carboxyanhydrides of L-Lys(Z), L-Ala, L-Phe, L-Asp(OBzl), and L-Glu(OBzl) with 3-aminopropyltrimethoxysilane as an initiator.[46] L-Amino acids–NCA were synthesized by the triphosgene method.[47] 3-Aminopropyltrimethoxysilane (APTS) dissolved in tetrahydrofuran (THF) was prepared and L-amino acid–NCA (25 mol equivalent against APTS) dissolved in THF (5.0 mL) was added to the initiator solution at room temperature. The polymerization reaction was monitored by the FTIR spectrum of the C═O stretching vibration of L-amino acid–NCAs exhibited at 1850 cm^{-1}. After the disappearance of the C═O stretching vibration was confirmed, we stopped the reaction by plunging the reaction mixture into methanol. The precipitate was filtered off and washed with methanol and diethyl ether. The structure and polymerization degree were determined by the ^{1}H-NMR spectrum and Ostwald's viscometer measurement.[48] The side-chain structure and polymerization degree are shown in Table I.

Surface preparation

A monomeric styrene–immobilized surface was prepared as follows. A glass dish (ϕ 45 mm) was cleaned by a previously described cleaning protocol,[49] immersed in acetone in an ultrasonic bath for at least 2 min, rinsed with 95% ethanol, and then immersed in 0.5*N* NaOH aqueous solution for 2 h. After ultrasonic cleaning in 95% ethanol for 20 min, it was washed with deionized water. The glass dish was then dried under reduced pressure. Phenyltrimethoxysilane [PMS, Scheme 1(a)] immobilized surface was prepared as follows. A cleaned glass dish was immersed in a 1.0 wt % CCl_4 solution of PMS for 15 h at 60°C and then

TABLE I
Chemical Structure and Polymerization Degree of Poly(amino acid)s Having Trimethoxy Group at Terminal

Main structure	Name	Sidechain structure (R)	Polymerization degree (n)
	L-Ala	CH_3	30[a]
	L-Phe		39[a]
	L-Asp(OBzl)		38[a]
	L-Glu(OBzl)		42[a]
	L-Lys	$(CH_2)_4NH_2$	110[b]

[a] Calculated from ^{1}H-NMR measurement.
[b] Calculated from Ostwald viscometer measurement.

TABLE II
Contact Angles of Water to Cell Culture Substates

Sample	Contact angle (°)
Glass	22.3
Monomeric styrene	64.8
Polymeric styrene	91.8
Polystyrene cast film	93.4
Poly(L-Lys)	71.6
Poly(L-Phe)	64.5
Poly(L-Asp(OBzl))	69.4
Poly(L-Glu(OBzl))	57.0
Poly(L-Ala)	44.3

rinsed with fresh CCl_4. To remove unimmobilized PMS, consecutive 10-min ultrasonic cleanings were performed in CCl_4, then rinsed with 95% ethanol and dried under reduced pressure for 24 h. The polymeric styrene–immobilized surface was prepared in the same way as with PMS.

Polystyrene (Wako Pure Chemicals, Osaka, Japan; $n = 1600–1800$) casting film was prepared from 5.0 wt % toluene solution cast onto a glass dish at 60°C for 5 h and then dried at 30°C for 12 h to remove the solvent.

The immobilization of poly(amino acid)s was performed on the immersed glass dish in 1.0 wt % DMSO solution for 15 h at 80°C and then rinsed with fresh DMSO. For the poly(L-Lys(Z))-immobilized surface, we treated the surface with HBr/CH_3COOH solution to deblock the carbobenzoxy (Z) group.

Measurements

The static contact angle of distilled water on the immobilized surface was used to evaluate hydrophobicity using a contact angle meter (CA-D; Kyowa Interface Science Co., Saitama, Japan). Static contact angles were measured at 25°C on profiles of sessile drops using a microscope with a fixed goniometer eyepiece (magnification ×20). The average drop size was about 0.05 mL and angles were measured in 10 different regions of each surface; averages are shown in Table II.

To evaluate the secondary structure of poly(amino acid)s on a glass surface, poly(amino acid)-immobilized quartz glass was prepared following the same conditions and measured by circular dichroism (CD) spectropolarimeter (J-550; Jasco International Co., Tokyo, Japan).

Evaluation of cell spreading and growth

Mouse fibroblast L929 cells (L-cells) were obtained from the skin of an 8-week-old male mouse, and after 5 days of primary culture on a tissue culture polystyrene (TCPS), the cells were seeded onto surface-modified glass dishes at a cell density of 1.0×10^5 cells/dish. They were then incubated in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS) under a humidified atmosphere of 95% air and 5% CO_2 at 37°C. On days 2 and 4 of incubation, the medium was replaced. At each time point, after DMEM was removed, the dish surfaces were rinsed twice in Dulbecco's phosphate-buffered saline (PBS). Then L-cells were collected by enzymatic dissociation with a PBS solution of 0.25% trypsin and

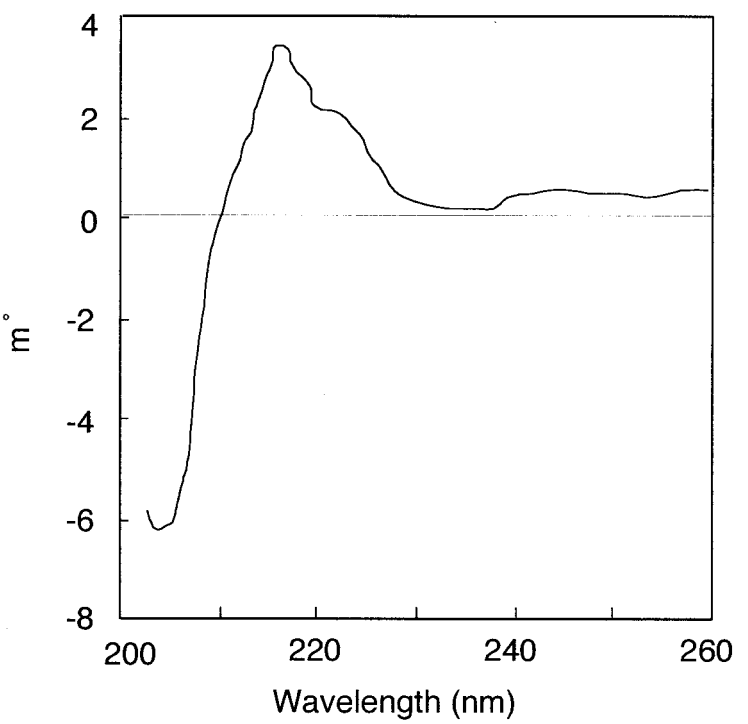


Figure 1 CD spectrum of poly(L-Lys) immobilized onto quartz glass surface.

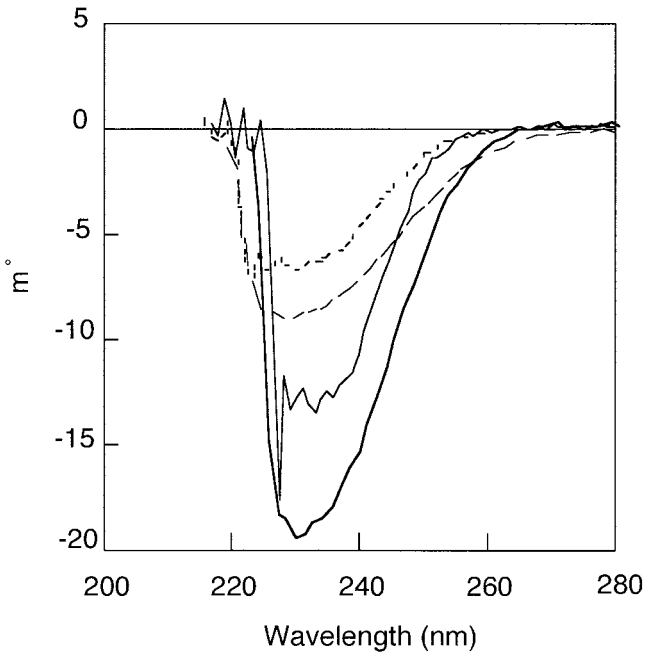


Figure 2 CD spectra of poly(amino acid)s immobilized onto quartz glass surface. (—): poly(L-phenylalanine); (▬): poly(L-Asp(OBzl)); (· · ·): poly(L-Glu(OBzl)); (– – –): poly(L-alanine).

the number of cell were counted using a hemocytometer.

The mouse bone marrow–derived Sp2/0-Ag14 cells (Sp2/0 cells) were obtained from the marrow of an 8-week-old male mouse, and then 4 days of primary culture on a TCPS incubated in ASF-104 medium (nonserum medium) under a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cell culture was evaluated in the same way as were the L929 cells, and Sp2/0-Ag14 cells were collected by pipet and the number of cell counted using a hemocytometer.

Cell spreading on the substrate was observed using an inverted laboratory microscope (CK2; Olympus Optical Co., Tokyo, Japan) at ×100 magnification. The cell growth activity on the substrate was estimated by total cell counts after incubating for a number of days (S_d) per initial cell count (S_0).

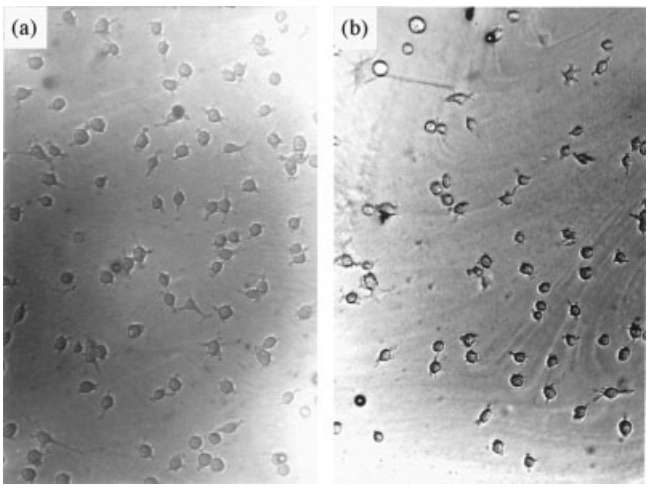


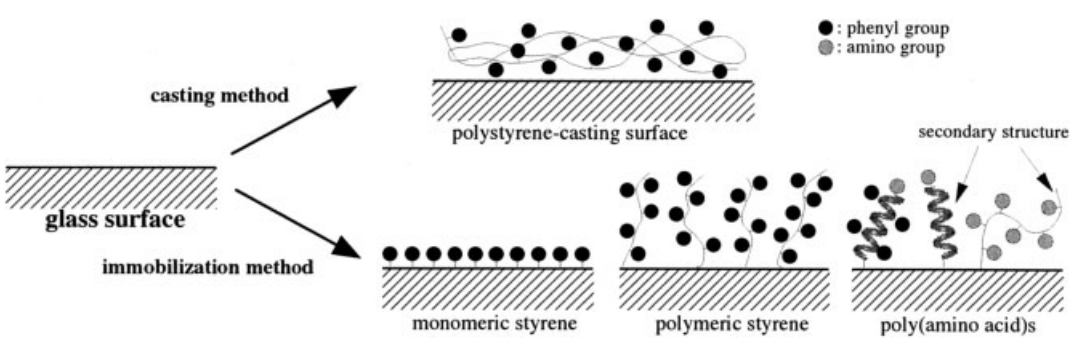
Figure 3 Optical micrographs of L-cell extended onto (a) PS_{16}-immobiized surface and (b) polystyrene casting film 1 day after cell culture started.

RESULTS AND DISCUSSION

Surface characterization

From the static contact angle measurements shown in Table II, all of the immobilized surfaces were higher than those of a bare glass surface, indicating that the compounds were immobilized onto the glass dishes. The higher the contact angle, the lower the tendency of water not to spread over the surface, given that hydrophilic surfaces are expected to have lower contact angles than those of hydrophobic ones.

The CD spectra of poly(L-Lys) immobilized on a quartz surface showed a single maximum at 218 nm, characteristic of a random-coil structure (Fig. 1). The CD spectra of the poly(amino acid)-immobilized surfaces were qualitatively that of a polypeptide in a α-helical conformation, very different from the typical spectra of pure helices (Fig. 2). The negative bands at 222 and 208 nm are features characteristic of a polypeptide in the α-helical conformation, although these poly(amino acid)s showed only a negative peak around 226 nm. This band shift depends on the aggregation form of the α-helices,[45] which thus indicated



Scheme 2 Preparation of modified surfaces by casting and molecular terminal immobilizing methods.

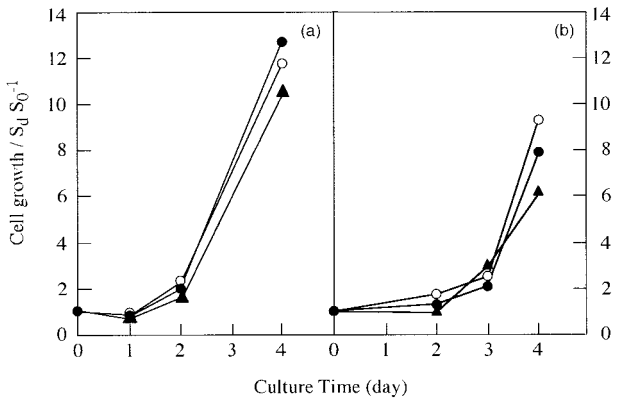


Figure 4 Effect of culture time on cell growth of (a) L-cell and (b) Sp2/0 cell. The cell growth was determined by stationary culture method (a) with or (b) without serum protein. St, total cell count on substrate after incubating for each day; So, initial cell count. (●): monomeric styrene; (○): polymeric styrene; (▲): polystyrene cast film.

that the poly(amino acid)-immobilized surfaces formed monolayer-like structures on glass surfaces with closely packed α-helical molecules. The surface structures of the immobilized substrates are schematically represented in Scheme 2.

Cell morphology and growth according to the modification methods

Substrate effects of the phenyl group–modified surface morphology were apparent in the cell growth. Monomeric and polymeric styrene–immobilized and polystyrene cast film surfaces induced adhesion of all cell types attributed to the surface hydrophobicity caused by the surface modification and the promotion of hydrophobic interaction between the cells and the modified surfaces. In optical microscopic observations, the L-cell morphology after 24 h under condi-

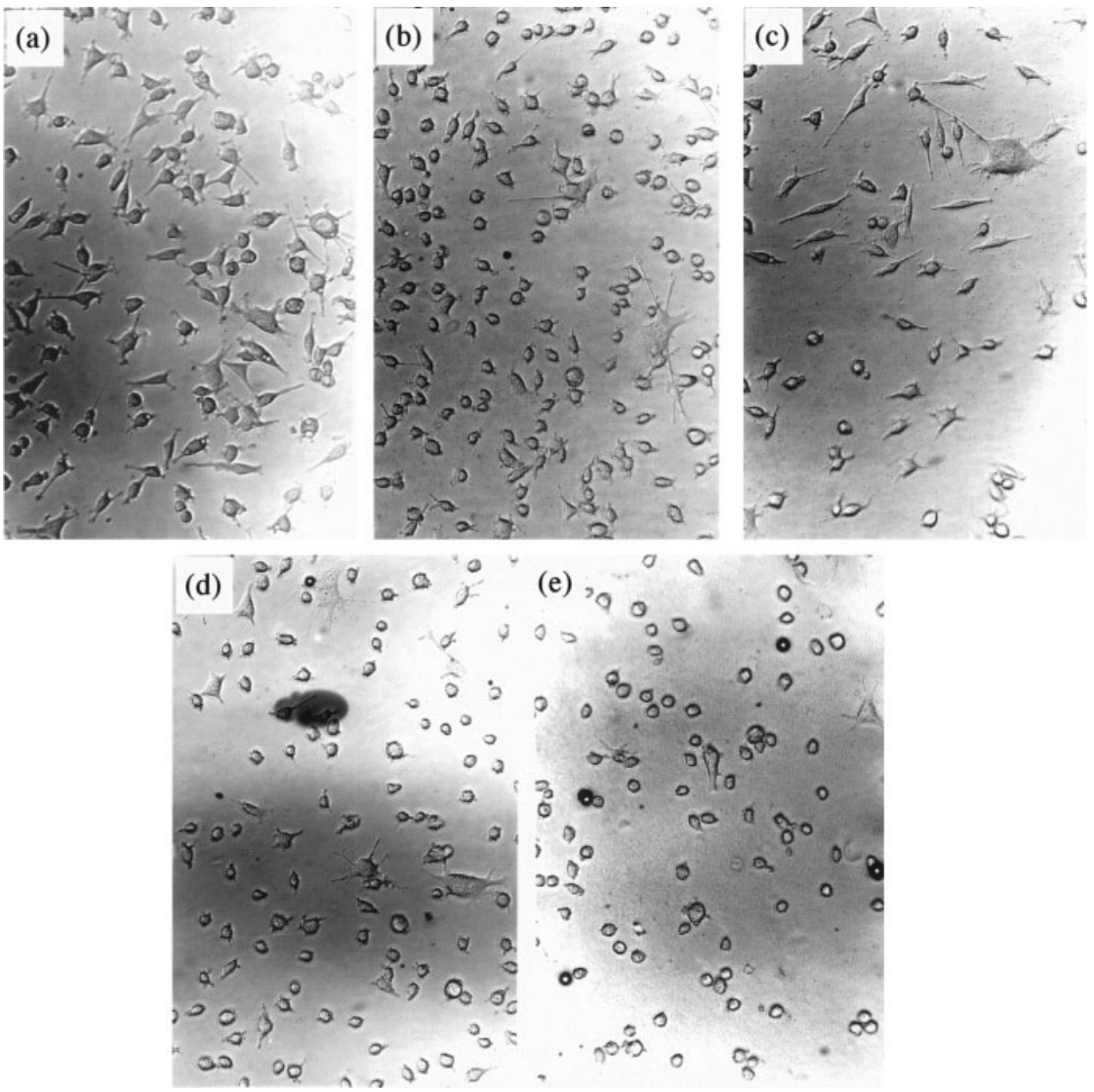


Figure 5 Optical micrographs of L-cell extended onto (a) poly(L-Lys), (b) poly(L-Phe), (c) poly(L-Asp(OBzl)), (d) poly(L-Glu(OBzl)), and (e) poly(L-Ala)-immobilized surface 1 day after cell culture started.

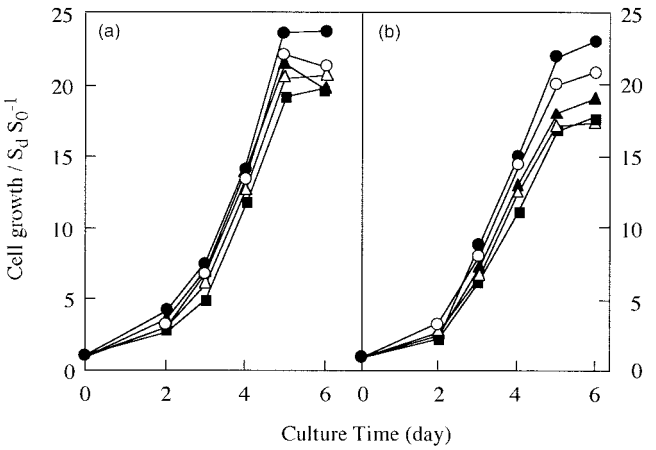


Figure 6 Effect of culture time on cell growth of (a) L-cell and (b) Sp2/0 cell. The cell growth was determined by stationary culture method (a) with or (b) without serum protein. St, total cell count on substrate after incubating for each day; So, initial cell count. (●): poly(L-Lys), (○): poly(L-Phe), (▲): poly(L-Asp(OBzl)), (△): poly(L-Glu(OBzl)), (□): poly(L-Ala).

tions of 10% FBS attached effectively and spread on each of the surfaces. In the case of PS_{16}-immobilized surface, the cell extension was slightly better than that immobilized on polystyrene casting film (Fig. 3). The proliferation rates of cells are shown in Figure 4. The proliferation rate of L-cell growth on each surface indicated logarithmic growth curves, whereas the rate on the polystyrene cast film surface was less than that of the polymeric and monomeric styrene–immobilized surfaces [Fig. 4(a)]. In the Sp2/0 cell culture, the same tendency was observed, with the proliferation rates on polymeric and monomeric styrene–immobilized surfaces having been reversed [Fig. 4(b)]. These results indicated that immobilized surfaces show higher proliferation rates than those prepared by the casting method. Further, compared with the proliferation rate of L-cells (in serum protein conditions) and Sp2/0 cells (without serum protein conditions) after 4 days, a high proliferation rate was shown under serum-containing conditions. From these results, we concluded that the existence of proteins encouraged cell growth on these surfaces because protein adsorption to the surface involved multiple electrostatic, hydrophobic, and hydrogene bonding, and van der Waals interaction.

Cell growth on poly(amino acid)-immobilized substrate

Effects of poly(amino acid)-immobilized surface chemistry were apparent in the morphology of spreading cells and their growth in each medium. Figure 5 shows the optical micrographs of L-cell attached on the poly(amino acid)-immobilized surface. From the observation, a cell having extended the most is on the poly(L-Lys)-immobilized surfaces, and became lower in the order of poly(L-Phe), poly(L-Asp(OBzl)), poly(L-Glu(OBzl)), with the poly(L-Ala)-immobilized surface being the lowest. In addition, the results of the proliferation rates of L-cells and Sp2/0

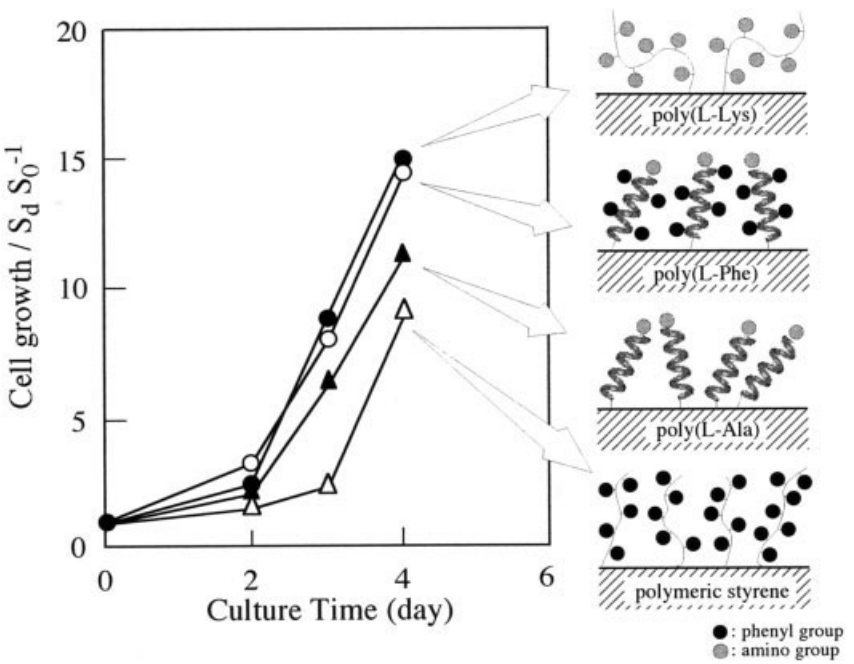
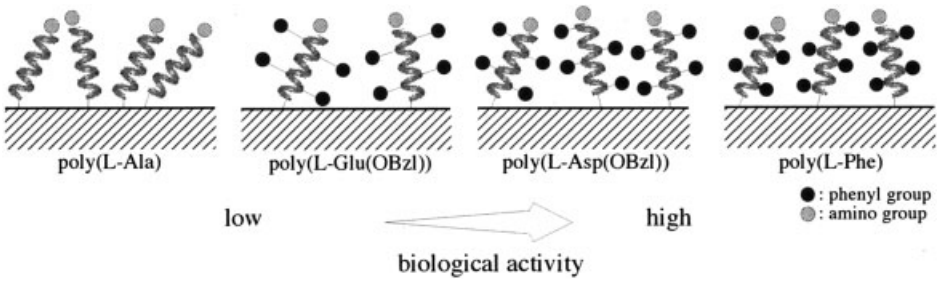


Figure 7 Effect of culture time on cell growth of Sp2/0 cell without serum protein. The well-defined surface structure promotes biospecific interaction with cell in spite of serum protein existence. St, total cell count on substrate after incubating for each day; So, initial cell count. (●): poly(L-Lys); (○): poly(L-Phe); (▲): poly(L-Ala); (△): polymeric styrene.



Scheme 3 Schematic illustration of surface morphology on immobilized surfaces. The side-chain phenyl group and terminal amino group, arranged spatially by α-helix structure, promote the surface biological activity.

cells on each of the poly(amino acid)-immobilized surfaces showed the same tendency as the results of the cell extension (Fig. 6). Both the proliferation profiles indicated logarithmic growth phases for 5 days and thereafter shifted to a stationary phase. From these proliferation curves, poly(L-Lys)-immobilized surfaces showed the highest ability for initial cell attachment and proliferation, with or without serum proteins, attributed to the electrostatic interaction between positive amino group and negative cell surface. Proliferation rates became lower in the order of poly(L-Phe), poly(L-Asp(OBzl)), poly(L-Glu(OBzl)), with the poly(L-Ala)-immobilized surface being the lowest. This indicated that the amino group was more effective than the phenyl group in hydrophobic interaction for cell growth. It was considered that the amino group acts on cell adhesion at the initial stage and also during the ongoing culture. The cell culture on poly(L-Phe), poly(L-Asp(OBzl)), poly(L-Glu(OBzl)), and poly(L-Ala)-immobilized surfaces indicated that a slight difference of side-chain structure affected cell growth. For example, the proliferation rate of poly(L-Ala)-immobilized surface was the lowest in spite of having a hydrophobic surface similar to that of other phenyl groups containing poly(amino acid)s. Furthermore, on this kind of surface containing a phenyl group, cell growth showed a tendency to deteriorate when the length of the side chain increased.

To study the substrate effects of surface morphology and chemical structure on cell culture, we prepared two kinds of surfaces using the molecular terminal immobilization method. This method provides monolayer-like surfaces and constructs functional interfaces having a physiological activity that uses the slightly differing effects of the functional moieties. From cell growth on polymeric and monomeric styrene–immobilized surfaces and on polystyrene cast film, the terminal immobilization method was more useful for cell culture than the casting method, when focused on the phenyl group. In such cases, high cell adhesion was observed on both modified surfaces, but a conspicuous difference was seen in cell growths. That is, the tendencies observed in these styrene series depended on the existence of serum proteins, meaning that hydrophobic interaction was mainly by the accumulation of phenyl moiety–enhanced cells and protein absorption. It was thought that the increase of the proliferation rate was from this adhesion effect.

On the other hand, having examined the relevance of proliferation rates and side-chain structures containing the phenyl group, cell growth on poly(amino acid)-immobilized surfaces showed that the amino group better promotes cell growth. Growth was lower in the phenyl group than in the alkyl group. In addition, poly(L-Phe)-immobilized surfaces showed overwhelmingly high proliferation rates after 4 days compared with polymeric styrene–immobilized surfaces (Fig. 7). This was caused by the side-chain phenyl group and the terminal amino group being arranged spatially by the secondary structure of the α-helix formed by the main chain. It was thought that these alternative functions influenced cell growth. In particular, promotion of such cell growth is amplified in cell cultures under serum-free conditions. Thus a poly(amino acid)-immobilized surface could lead to the construction of a well-defined surface that allows the promotion of biospecific interactions with cells at minimal levels.

CONCLUSIONS

Surfaces prepared by the molecular terminal immobilization method improved cell adhesion and growth. Furthermore, poly(amino acid)-immobilized surfaces promoted cell growth without serum proteins (Scheme 3). These results indicate that a bioactive surface can be constructed through the design of the molecular structure and immobilized morphology.

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