ATTACHMENT KINETICS OF ANIMAL CELLS IMMEDIATELY AFTER CONTACT ONTO SPECIFIC AND/OR NON-SPECIFIC SURFACES

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Attachment kinetics of BHK cells in very short contact times (0, 1, 2, 3 and 5 min) under static conditions was investigated on tissue-culture-treated polystyrene (TC-PS) dishes preadsorbed with various proteins. Although cells attached rapidly onto bare TC-PS, they did not attach at all within 1-2 min of contact when the surfaces were preadsorbed with serum albumin (Alb), even at low surface coverage. The specific interaction between adsorbed fibronectin (FN) and cells required a short contact time to accomplish the attachment. Moreover, FN adsorption at low surface coverage retarded the cell attachment up to 2 min as compared to the bare TC-PS. These inhibitory effects of FN were completely overcome by postcoating with polylysine (PL), which did not inhibit the spreading promoting activity of FN. Both Alb and FN adsorption had some contributions in enhancing the surface hydrophobicity, whereas post-treatment of the FN adsorbed surfaces by PL did not change the characteristics at all. Having considered that Alb and FN themselves are negatively charged at physiological pH, cell contact and attachment initiation onto solid surfaces once preadsorbed with proteins were strongly suggested to be predominated by electrostatic interactions simply by evaluating the charge sign of adsorbed proteins.

Introduction

Attachment, spreading and growth of various anchorage-dependent mammalian cells on artificial solid surfaces have not been clarified so far, because a large number of factors are involved. Generally, charge and wettability of surfaces are major global factors[1], which also influence the adsorbability of proteins supplied from culture media. In addition, some reports show that spreading and growth are based on a mechanism different from that at the initial attachment stages[3, 7, 8].

Cell spreading and growth on artificial surfaces are considered to be predominated by the preadsorption of various attachment glycoproteins having biospecific interaction with cells, such as fibronectin and vitronectin[7, 8]. Usually, long-term cell attachment, spreading and growth occur preferably on surfaces having moderate wettability[7, 8, 20, 21], presumably because both cells themselves and specific attachment proteins in sera or derived from the cultured cells are preferentially adhered onto these surfaces.

In contrast to the long-term stability of the cells on solid surfaces, the initial contact and attachment step is postulated to be based on physical interactions rather than on specific ones[3]. Cell contact onto solid surfaces and attachment initiation are the first steps allowing subsequent cell spreading, growth and/or sometimes differentiation. In particular, in the case of cell inoculation in the fluid phase by continuous stirring to obtain rapid confluence and/or maximal cell growth and its yield, which is often carried out in large-scale cell culture, the cells should be trapped and settled down within a very short contact time onto the support surfaces.

To elucidate cell attachment onto solid surfaces under flow conditions, various types of well-defined flow systems have been used[4, 13, 23]. In these systems, cell attachment is the integral result of cell contact, trap, attachment and detachment repeated in very short contact times. However, due to the large number of parameters involved, the analysis of experimental data obtained seems to be difficult. Moreover, the main concern of these studies has been restricted to biospecific bond formations between the ligand in the attachment proteins and its receptor of cell membranes, resulting in poor attention to the physical interactions[2, 13]. Therefore, the actual factor predominating cell attachment within a short contact time, and the contribution of both non-biospecific or physical and biospecific interactions have remained obscure.

In the present study, we set a time period allowing the cell-surface contact to be very short (0, 1, 2, 3 and 5 min) in a simple and conventional attachment assay using culture dishes in static conditions, and tried to elucidate the factors controlling cell behaviors at the initial contact and attachment stages. Tissue-culture-treated polystyrene (TC-PS) dishes are employed as a standard surface. Other surfaces are prepared by pretreatment of the TC-PS with various proteins. The individual and integrated effects of both specific and non-specific interactions on cell attachment are investigated. Also, surface characteristics controlling initial cell attachability onto protein adsorbed surfaces are

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Table 1 Pretreatment of TC-PS

Surface	Pretreatment
bare TC-PS	bare TC-PS without any treatment
TC-PS/FBS	precoated with 10 % non-heat-inactivated FBS
TC-PS/PL	precoated with 100 μg-PL*/ml
TC-PS/Alb	preadsorbed with various Alb** concentrations
TC-PS/FN + Alb	preadsorbed with various FN cncentrations followed by treatment with heat-denatured 10mg-Alb/ml
TC-PS/FN	preadsorbed with various FN*** concentrations
TC-PS/FN + PL	preadsorbed with 80 ng-FN/ml followed by 100 μg-PL/ml postcoating

* : Poly-D-lysine (M. W. = 30,000-70,000, Sigma Chemical Co.)
** : Bovine serum albumin (Sigma Chemical Co. St. Louis, MO)
***: Bovine plasma fibronectin (Biomed. Technol. Inc. Stoughton, MA)

studied in terms of surface wettability and surface charge.

1. Experimental

1.1 Surface preparation

TC-PS dishes (Falcon 35 mm, Becton Dickinson and Comp., Lincoln Park, NJ) were pretreated with fetal bovine serum (FBS) or various protein solutions in phosphate buffered saline (PBS) at 37°C for 1 h with gentle shaking on a rotatory shaker (**Table 1**).

Albumin (Alb) and fibronectin (FN) were selected as representatives because their adsorption shows non-specific attachment-preventing and specific attachment-improving effects, respectively. The concentration ranges in this study referred to those in 10%-FBS containing media, 4.0 mg-Alb/mL and 20 μg-FN/mL, which were calculated based on the concentrations in FBS (40 mg-Alb/mL and 200 μg-FN/ml). Polylysine (PL) often serves as a readily attachable surface for various cells.

To study the effect of specific interactions of FN, after FN precoating, the residual attachment sites of TC-PS dishes were completely blocked with 10 mg/ml heat-denatured Alb at 37°C for 1 h (TC-PS/FN+Alb)[5]. FN preadsorbed TC-PS was postcoated with PL in some experiments. All the dishes thereby prepared were rinsed three times with plenty of PBS, and 0.9 ml of MEM with Hank's BSS (DIFCO Lab., Detroit, MI) was added to each dish to equilibrate at 37°C under humidified air.

1.2 Measurements of the amount adsorbed of FN and Alb

The adsorbed amount of FN on the TC-PS dish was determined from the decrease in the concentration in 1.0 ml solution exposed to a 35 mm TC-PS dish for 1 hr. The concentration was measured by sandwich ELISA using gelatin instead of the first antibody according to the method of Ruoslahiti *et al*[14]. The anti-bovine FN rabbit IgG and peroxidase-conjugated anti-rabbit IgG were purchased from Cosmo Bio Co. (Tokyo) and E-Y Lab. (San Mateo, CA), respectively. The amount of FN adsorbed was determined by using the adsorption isotherm and a simple equation derived from the FN mass balance in the adsorption system as follows.

$$Q_{FN} = (V / S) \cdot (C_{FN,0} - C_{FN}) \quad (1)$$

Where, Q_{FN} is the amount adsorbed (μg/cm^2), V is the volume of the solution (1.0 ml), S is the surface area contacting with the solution (13.45 cm^2), C_{FN} and $C_{FN,0}$ are the concentration (mg/ml) after 1 hr of exposure and time zero, respectively. The amount adsorbed onto TC-PS agreed well with a previous report[9].

Alb adsorption onto TC-PS was indirectly estimated by the adsorption measurement onto TC-PS microcarriers (TC-PS MCs, Hazelton Biologics Inc., St. Lenexa, KS, mean diameter of 120 μm), because the ratio of amount adsorbed to the added concentration was very low. The MCs were washed with PBS and air dried prior to use, 250 mg of each of them (total surface area of 120 cm^2) was added to 1.0 ml of Alb solutions in a test tube and completely shaken on a water bath shaker at 37°C. After 1 hr, the decrease in concentration was measured by sandwich ELISA as previously described[15].

1.3 Cell preparation and inoculation

Baby hamster kidney cell line BHK21 c13[12] was obtained from the Japanese Cancer Research Bank (JCRB). The growth medium was Eagle's minimum essential medium (MEM with Earle's BSS; Nissui Pham. Co., Japan) supplemented with 10%-FBS (Filtron Pty. Ltd., Altona, Australia). To obtain actively growing cells, the confluent monolayers were trypsinized and inoculated at 5.0×10^4 cells/cm^2 on the day before the assay[14].

The cells were washed with PBS, exposed to 0.25% trypsin/0.02% EDTA at room temperature, and the digestion was terminated by adding 10% FBS-containing media. The cells were centrifuged and resuspended in 2.1 ml of serum-free MEM with Hank's BSS, and 0.1 ml was used to measure the cell concentration and viability by trypan blue dye exclusion. 93-97% of the cells were viable on average. The cells were finally resuspended with serum-free MEM at a density of 2.0×10^6 cells/ml and preincubated at 37°C to equilibrate cell surfaces in the medium 37°C prior to inoculation. An aliquot (0.1 ml) was added to each equilibrated dish containing 0.9 ml-medium, and the attachment assay was started in a 37°C incubator immediately after the cells were completely distributed over the bottom surface of the dish.

1.4 Measurements of attachment kinetics

After 0-5, 20, and 60 min incubation , the dishes were shaken by a reciprocal shaker (2.2 cm amplitude) at 90 rpm for 15 sec. The 1.0 ml suspension was transferred to a 20 ml plastic beaker. An additional 0.5 ml of MEM was added to the dish and collected into the same beaker.

The cell concentrations were measured by a particle counter (PC-602A, ERMA Inc., Tokyo, Japan). The cell attachment at t (min), A_t (-), was defined as follows.

$$A_t = (N_i - N_s) / N_i \quad (2)$$

Where N_i is the inoculated cell number and N_s is the number of cells recovered into the beaker, which was defined to be non-attached cells.

From the A_t values, the attachment rate constant for 0-1 min of contact, k_{A01}, was calculated by Equation (2)

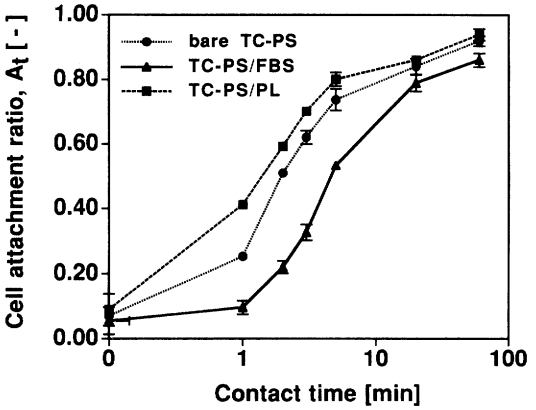

Fig. 1 Attachment kinetics on three surfaces. Each point represents mean ± maximal errors in two runs

and its integral form, Equation (3), assuming the cell attachment is approximated by the first order reaction within the short contact time.

$$dA_t / dt = k_A(1 - A_t) \quad (3)$$

$$K_{A01} = \ln (A_1 / A_0) / \Delta t \quad (4)$$

Where Δt is the net contact time for 0-1 min. Since the inoculated cells settle down through the medium (0.1 cm depth) until contact with the bottom surface, all the cells do not necessarily contact simultaneously. The time at which a half number of the cells initiate the contact with the bottom surface was measured and determined to be 20 sec after settling. Thus, Δt was determined to be 40 sec, and the k_{A01}s were calculated by Eq. (3).

1.5 Measurement of cell spreading

Cell spreading was described by the ratio of spreading cells to attached cells at 60 min contact, S_{60}, according to the methods of Grinnel and Feld[5].

1.6 Measurement of water contact angles

Surface wettability was determined in terms of the water contact angle. The TC-PS surfaces preadsorbed by proteins were washed with plenty of distilled water, air-dried, and the angles were measured with a contact angle meter (CA-A, Kyowa-Kagaku Co., Tokyo) at 25°C.

2. Results

2.1 Attachment kinetics on bare TC-PS, TC-PS/FBS, TC-PS/PL

In **Figure. 1**, the attachment kinetics of BHK cells betweer 0-5 min and during the subsequent incubation (20, 60 min) are compared for three kinds of surfaces, *i. e.*, bare TC-PS, TC-PS/FBS and TC-PS/PL. Although no significant differences in A_t values were found after 20 min contact among the three surfaces, the k_{A01} values, calculated by Eq. (2) and listed in **Table 2**, were markedly varied. The value on TC-PS/PL was two times larger than that on the bare TC-PS. Despite the fact that the TC-PS/FBS had adsorbed specific-attachment proteins, the k_{A01} on it was below half that on bare TC-PS.

Table 2 Cell attachmnent rates immediately after the contact on the three surfaces*

	Bare TC-PS	TC-PS/FBS	TC-PS/PL
k_{A01} [min^{-1}]	0.33 ± 0.02	0.13 ± 0.03	0.65 ± 0.11

*: Data represents mean ± maximal errors in two runs

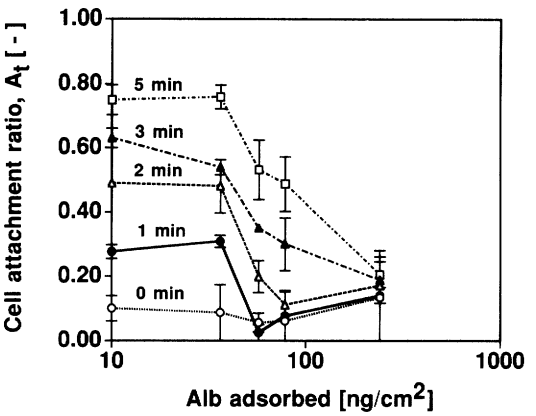

Fig. 2 Dependence of initial cell attachment upon non-specific interactions (TC-PS/Alb). Each point represents mean ± maximal errors in two runs

2.2 Attachment kinetics on TC-PS/Alb, TC-PS/FN+Alb and TC-PS/FN

Figure. 2 shows the dependence of attachment kinetics upon the non-specific interactions between the surface and the cells (TC-PS/Alb). The attachment was inhibited markedly by Alb preadsorption, particularly over 80 ng-Alb/cm^2, where the cells initiated attachment after 1-2 min contact. The initial Alb concentration giving the 80 ng-Alb/cm^2 is 62.5 μg/ml, corresponding to one-sixtieth that in 10% FBS-containing medium.

The dependence of attachment kinetics upon the specific interactions is presented in **Figure. 3A** (TC-PS/FN+Alb). By the specific interaction with FN only (TC-PS/FN+Alb), the cells attached after 5 min contact at 80, 287, 869 ng/cm^2 of adsorbed FN were almost the same (A_5 = 0.80). The attachment rate was maximized at 869 ng-FN/cm^2, even immediately after the contact, whereas they were attached after a short time lag(1-2 min) in the region below 80 ng-FN/cm^2.

Integral effects of specific and non-specific interactions are presented in **Figure. 3B** (TC-PS/FN). In the region over 869 ng-FN/cm^2, the specific interactions by FN were dominant, because almost the same kinetics were observed over 869 ng-FN/cm^2 in Fig. 3A and B. In contrast, in the region below 15 ng-FN/cm^2 in Fig. 3B, non-specific and direct cell attachment onto bare TC-PS is dominant due to the low surface coverage of FN molecules. However, in the intermediate region (80-287 ng-FN/cm^2), cells started to attach after 1-2 min of contact time. FN adsorption in this region exhibited an influence of inhibition rather than improvement on the cell contact and attachment initiation, in spite of its specific attachment activity, even though the non-specific interaction by bare TC-PS coexists.

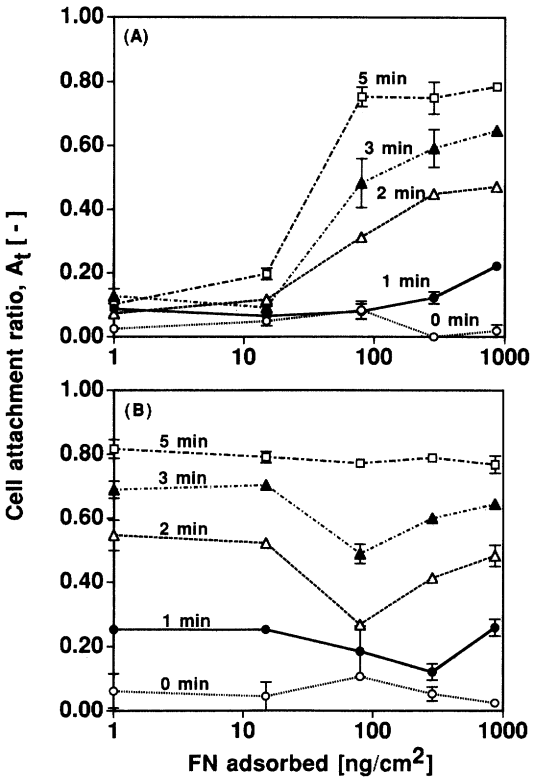

Fig. 3 Dependence of initial cell attachment upon specific interactions (A) (TC-PS/FN+Alb), and upon integrated effects of both specific and non-specific interactions (B) (TC-PS/FN). Each point represents mean ± maximal errors in two runs

Table 3 Effect of PL postcoating on the attachment rates and the subsequent cell spreading*

	TC-PS/FN	TC-PS/PL	TC-PS/FN + PL
k_{A01}[min^{-1}]	0.17 ± 0.07	0.61 ± 0.07	0.76 ± 0.04
A_{60} [-]	0.95 ± 0.06	0.92 ± 0.05	0.96 ± 0.02
S_{60} [-]	0.9 ± 0.0	0.1 ± 0.0	0.9 ± 0.0

*: Data represents mean ± maximal errors in two runs

2.3 Effect of PL postcoating on attachment kinetics

To improve the low initial attachability as observed onto TC-PS/FN at a low FN coverage (Fig. 3B, 80 ng-FN/cm^2), PL postcoating (TC-PS/FN+PL) was tried and the results are listed in **Table 3**. The k_{A01} was enhanced 4.5-fold by the postcoating. No significant differences were observed in A_{60} and S_{60} values between the surfaces with and without the postcoating, suggesting that the PL postcoating has no inhibitory effects on cell spreading activity.

2.4 Effects of surface wettability

To elucidate the effects of surface wettability on initial cell attachment rates, particularly for the low attachability on TC-PS/FN of low FN coverage (Fig. 3B, 80 ng-FN/cm^2), water contact angles were measured on the surfaces where the attachment assay was performed. The results are shown in **Figure. 4** The contact angles increased with the increase in the amount of adsorbed protein for both TC-PS/Alb and TC-PC/FN up to 60° (1,000 ng-Alb/cm^2) and 70° (869 ng-FN/cm^2), respectively. The angle remained unchanged, irrespective of the PL postcoating over the

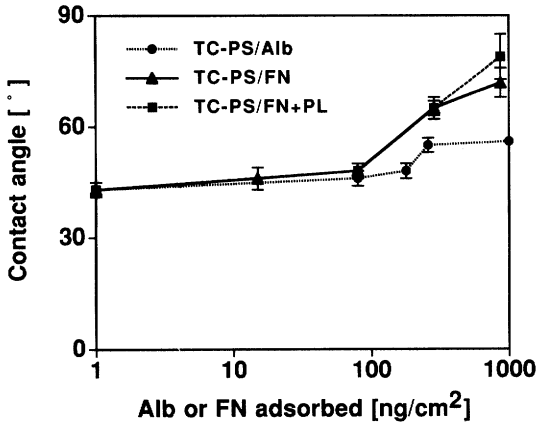

Fig. 4 Surface wettability of TC-PS/FN, TC-PS/Alb and TC-PS/FN+PL. Each point represents mean ± S. D. in twenty readings

entire range. Since surface pretreatment with 100 mg-PL/ml in itself did not alter the contact angle of bare TC-PS (data not shown), on TC-PS/FN+PL, the increase in the wettability seems to be attributable mainly to the adsorbed FN.

3. Discussion

Himes *et al.* reported[6] that FBS retarded CHO cell attachment onto MCs under continuous stirred conditions, despite the fact that the supplementation of FBS with culture medium was indispensable for cell spreading and growth. The markedly low attachment rate, k_{A01}, in our study (Table 2) is in good agreement with their results.

From Fig. 3A, the specific interaction between adsorbed FN and the FN receptor was concluded to need a certain contact time to accomplish cell attachment. Schakenraad *et al.* observed[16] existence of a small time lag (about 20 min) prior to the spreading initiation of human skin fibroblast, and suspected cells formed their binding to the surface within the lag time. Worthen *et al.*[23], for neutrophil leukocytes and endothelial cells, showed that the binding strength of the cell-surface obeyed a time-dependent reaction. The kinetics of biospecific receptor-ligand bond formation has been incorporated in some theoretical models describing cell attachment under a flow[2, 13]. Our observation is considered to have revealed the intermediate step.

On TC-PS/FN, high attachment rates were attained in two different regions of the FN coverage, that is, over 869 ng-FN/cm^2 and below 15 ng-FN/cm^2 (Fig. 3B). To obtain the same attachment rate as on bare TC-PS by FN alone, more than 869 ng-FN/cm^2 was required. The attachment rates in a short contact time such as under flow conditions may be drastically reduced in the region of 15-869 ng-FN/cm^2. In fact, FN adsorption onto a glass surface reduced markedly the attachment of neutrophil leukocytes under flow condition despite their having FN receptors as reported by Forrester *et al.*[4].

Animal cells attach and grow preferably on moderate wettable solid surfaces[7, 8, 20, 21]. Tamada and Ikada[20] observed no significant differences in L cell attachment after 60 min of contact in a serum free system when the

wettability of solid surfaces was varied within 60-80° of water contact angles, although the cell attachment was markedly enhanced when the contact angle was raised from 40° to 60°. In contrast, in this study, the increase by 20-30° in bare TC-PS wettability (42°) caused by Alb and FN preadsorption (Fig. 4) brought elimination to cell attachment rates in a reversed manner (Figs. 2 and 3A). Moreover, by postcoating with PL, the initial attachment on a FN-preadsorbed surface of low coverage (80 ng-FN/cm^2) was remarkably enhanced (Table 2), whereas the surface wettability was not at all altered, irrespective of the PL treatment (Fig. 4).

Therefore, different from in long-term attachment, spreading, and growth stages, the surface wettability never concluded to be a governing factor in the initial cell contact and attachment onto protein preadsorbed surfaces. The study of Van Wachem *et al.*[21] examining human endothelial cell attachment onto methacrylate polymers while varying their wettability showed that moderate wettability did not govern the attachability at 30 min of contact time, whereas the moderate wettable polymers showed maximal cell adhesion after 1 hr of contact, suggesting that other parameters control the cell attachment within short contact times.

One of the other possible factors strongly influencing cell-surface phenomena is the surface electrostatic conditions. In physiological pH, the overall net charge of animal cells is negative[17]. Alb and FN covered surfaces may also have overall net negative charge due to the their low isoelectric points (pI) under that condition.

Takeichi and Okada[19] investigated attachment of chick embryonic fibroblasts on TC-PS with and without Alb precoating under varying medium pH. Without Alb preadsorption, cells attached on TC-PS over a wide pH range (pH 3.4-9.0). In contrast, the cell attachment onto Alb-preadsorbed TC-PS was gradually enhanced when lowering the medium pH, whereas the cells could not attach at all over pH 5.8. Although the time period permitting the cell to attach was 3 hr in that study, it indicates that electrostatic interaction is the dominant factor when the surface was once preadsorbed with proteins.

Forestell *et al.* reported[3] that lowering the medium pH value lead to enhancement in MRC-5 cell attachability onto MCs under gently-stirred conditions in the presence of 4%-FBS. It was presumably due to the net negative charge decrease both for the cell membranes and MC surfaces adsorbed with serum proteins. In that study, since cell-surface contact time should be very short, it supports the mechanism of cell contact and attachment onto protein adsorbed surfaces derived from our experiments.

On the basis of the above discussion, although long-term cell-surface phenomena were recognized to be dependent not upon charge sign but upon charge density of the surfaces[11], once being preadsorbed with proteins, electrostatic interaction between cells and adsorbed protein as simply accounted by charge sign seems to be a controlling factor in the initial contact and attachment stages. As postulated by Van Wachem *et al.*[21], the cells coming in contact with the surfaces should overcome the high electrostatic energy barrier between the surface-preadsorbed serum proteins and the cell surface before accomplishing their attachment.

Rat hepatocytes[10], human endothelial cells[2], and human diploid fibrobrasts[18] were reported to attach solid surfaces more slowly than BHK cells, even when the surface was preadsorbed with biospecific attachment proteins at an amount sufficent for their cell spreading and growth. Initial contact and attachment support of biospecific interactions by positively-charged proteins such as PL is more advantageous over utilizing biospecific interactions only, even when, as recently reported by Varani *et al.*[22], synthetic peptides such as ProNectin^{TM} F (Protein Polym. Technol. Inc.) containing a plentiful amount of RGD sequences were used as coating materials of TC-PS MCs.

Conclusions

Specific interaction of adsorbed FN with the cells needs a certain contact time to accomplish cell attachment. Direct and non-biospecific attachment onto bare TC-PS was very rapid. Once being preadsorbed with proteins, however, even though they have biospecific interactions with the cells, electrostatic interactions between the cells and adsorbed protein layers were suggested to be predominant.

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