

Effect of ProNectin F derivatives on cell attachment and proliferation

SATOSHI SOMAMOTO^{1,2} and YASUHIKO TABATA¹

Short / Running Title: Cellular effects of ProNectin F derivatives

¹Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University,

53 Kawara-cho Shogoin, Sakyo-ku Kyoto 606-8507, Japan

²Sanyo Chemical Industries Ltd.,

1-40, Goryo Ohara, Nishikyo-ku, Kyoto 615-8245, Japan

TEL +81-75-751-4121

FAX +81-75-751-4646

E-mail: yasuhiko@frontier.kyoto-u.ac.jp

Abstract

ProNectin F (PnF) was chemically modified by introducing some functional groups to prepare various derivatives of primary amino (PnF-N1), tertiary amino (PnF-N3), quaternary ammonium (PnF-N4), carboxyl (PnF-COOH), and sulfonyl groups (PnF-SO₃H) introduction. When C3H10T1/2 cells were cultured on non-treated dishes coated with the derivatives, the number of mesenchymal cells attached onto culture dishes increased for the coating with PnF-COOH and PnF-SO₃H even at their low adsorption amount. The cytotoxicity was high for the coating of PnF-N1 and PnF-N4 compared with that of the PnF-N3, PnF-COOH, and PnF-SO₃H. The treatment with integrin $\alpha 5$ and αV antibodies suppressed the cell attachment onto the dishes coated with PnF-COOH and PnF-SO₃H. The phosphorylation of extracellular signal-regulated kinase (ERK) was upregulated for cells attached onto the dishes coated with PnF-COOH and PnF-SO₃H, indicating their enhanced proliferation. It is concluded that the chemical derivatization of PnF enhanced the ability of cell attachment and proliferation.

Keywords: ProNectin F, artificial protein, cell adhesion, proliferation, integrin

1. Introduction

ProNectin F (PnF) is an artificial protein of 980 amino acids commercially available [1, 2]. It contains a repeated peptide segment of GAGAGS originated from silk fibroin, and GAAVTGRGDSPASAAGY from fibronectin [3]. This segment is repeated 13 times on one molecule of PnF. The protein is resistant to heat, biologically stable for several months at room temperature, highly water-soluble, and adsorbable to the hydrophobic surface of polystyrene even in the presence of water. PnF can be autoclaved at 121 °C without any loss of cell adhesive activity [4].

Interaction of extracellular matrix (ECM) proteins and integrin receptors of cells is required for the survival of anchorage-dependent cells [5]. In anchorage-dependent cells, the interactions activate various intracellular signaling pathways that play an important role in the cell viability, proliferation, and differentiation [6, 7]. Integrins are members of a widely expressed family of heterodimeric transmembrane receptors that bind to cell adhesive motifs present in various ECM proteins including fibronectin, vitronectin, laminin, and collagen [6, 8]. It's known that the ECM interaction through the integrin of cells contribute to their survival and their apoptosis suppression [5, 9-11]. Therefore, ECM proteins have been used to manipulate the cell behavior. PnF with integrin ligand sequences have been developed as a substitute of natural ECM proteins [4, 12-15].

The surface properties of biomaterials play critical roles in cell behaviors. It has been demonstrated that the type, quantity, and activity of proteins adsorbed on cell substrates are influenced by the physicochemical properties, such as the charge and hydrophobicity [16-19]. Various cellular responses were observed for substrates with different surfaces [20]. The surface property of substrates modifies the functional presentation of major integrin binding

domain of fibronectin, and consequently alters the integrin binding to potentiate the extent of cell attachment [21].

Fundamentally, cell-ECM interaction is composed of the interaction between the ligand of ECM and the receptor of cell surface. The interaction is based on various physicochemical interactions of coulombic and hydrophobic forces in the molecular level. PnF contains 13 arginine–glycine–aspartic acid (RGD) sequences of a cell adhesion motif which contains in the ECM [4]. The sequence mainly contributes to the cell interaction of PnF. The objective of this study is to obtain the fundamental knowledge whether or not the chemical derivatization of PnF can modify the manner of cell interaction. PnF was chemically derivatized by the introduction of cationic and anionic functional groups. Cells were cultured on the dishes coated with PnF and the derivatives to evaluate the coating effect on the attachment and proliferation of cells. We examine the conformation change of PnF through the chemical derivatization.

2. Materials and Methods

2.1. Materials

ProNectin F (Lot #:100630) was kindly supplied from Sanyo Chemical Industries, Kyoto, Japan. Other chemicals were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan and used without further purification.

2.2. Chemical modification of ProNectin F and conformational characterization

The chemical modification of ProNectin F was performed according to the method previously reported with partial modification [22]. ProNectin F (0.5 μ mol) was dissolved in 0.5 ml of 4.5 M lithium perchlorate in water. 2-Chloroethyl amino hydrochloride (1.5 mmol), 2-chloroethyl-N,N-dimethyl ammonium chloride (1.5 mmol), (2-chloroethyl) trimethyl ammonium chloride (1.5 mmol), chloroacetic acid (1.5 mmol), or 2-chloroethanesulfonic acid sodium salt (1.5 mmol) was dissolved in 0.5 ml of 4.5M lithium perchlorate solution, and each solution was added into the mixture with stirring at room temperature for the chemical derivatization of primary amino, tertiary amino, quaternary ammonium, carboxyl, and sulfonyl groups, respectively. Aqueous solution of 4.5 M lithium perchlorate (0.4 ml) containing 0.8 mmol of NaOH (2.0 M) was added into the mixture, followed by stirring at room temperature for 24 hr. The reaction was stopped by the neutralization of solution at pH 6.5-6.8 by adding glacial acetic acid. Then, the mixture was dialyzed for 24 hr against double-distilled deionized water (DDW) using a dialysis membrane (the cutoff molecular weight.6000–8000, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) and the content of dialysis bag was frozen and lyophilized.

The percentage of functional groups introduced was determined by the decrease of serine. The percent introduced was calculated based on the percent decrease in the OH groups of serine before and after the reaction according to the amino acid analysis. The samples were solubilized in DDW and measured at 25°C on a Circular dichroism (CD) Jasco J-820 spectropolarimeter (Jasco Ltd., Tokyo, Japan) in a cell with a 0.1 cm path length. The conformational content was calculated by using a reference spectrum according to the procedure reported [23].

2.3. Preparation of polystyrene dishes coated with different amounts of PnF and the derivatives

The solution of PnF and the derivatives (50 μ l) in 10 mM phosphate-buffered saline (PBS, pH 7.4) was added into each well of 96-well multi-well untreated polystyrene plates (7.15 mm in diameter, Cat. No. 1860-096, Asahi Glass Co., Ltd, Tokyo, Japan) and incubated for 2 hr at 37°C. The solution was removed, and each well was washed twice with PBS. The amount of protein adsorbed was measured with a Micro BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA). Next, 0.05 wt% bovine serum albumin (BSA) solution in PBS was added into each well and incubated for 2 hr at 37 °C. The BSA solution was removed, and the well was washed twice with PBS to use as polystyrene dishes coated. Experiments were performed independently three times and the mean value is shown as the amount of adsorption.

2.4. Cells culture

A cell line of mesenchymal cells (C3H10T1/2) was used and cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco Lifetechnologies Co., Carlsbad, CA) supplemented with 10 wt% fetal bovine serum (FBS) (Gibco Lifetechnologies Co., Carlsbad, CA) and 1 wt% of both penicillin and streptomycin (Sigma-Aldrich Co., St. Louis, MO) at a humidified 5 % CO₂-95 % air atmosphere at 37 °C. Cells were detached from dishes by the treatment of trypsin- ethylenediaminetetraacetate (EDTA) solution (Sigma-Aldrich Co., St. Louis, MO) and used for the following cell culture experiments.

2.5. Cell attachment assay

Cell attachment was evaluated according to the method reported previously [24]. Cells proliferated were washed twice with FBS-free DMEM to remove cell adhesive factors from the serum. The cell suspension in FBS-free DMEM (2.0×10^4 cells, 100 μ l) was added to PnF-, PnF derivatives-, fibronectin- or collagen-coated dishes, and cultured for 2 hr at 37 °C. Non-adhesive cells were removed by washing with FBS-free DMEM, and a cell-counting reagent (Cell count Reagent SF, Nacalai Tesque Co., Ltd, Kyoto, Japan) was added to each well. After incubation with the reagent for 2 hr, the absorbance of each well was measured using a UV-microplate reader (VERSA max, Molecular Devices Inc, Sunnyvale, CA) at 562 nm. The number of cells attached was calculated with a standard curve prepared by titrating the known number of cells.

2.6. Cytotoxicity assay

The cytotoxicity of PnF and PnF derivatives was examined by measuring the leakage of cell membrane with lactate dehydrogenase (LDH) [25]. After incubation of cells for 1 hr in the presence of PnF and PnF derivatives, the amount of LDH leaked from cells was measured with LDH-cytotoxic test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The present cytotoxicity was calculated by dividing the amount of LDH leakage in PnF and PnF derivatives by that of the positive control group of 0.2 wt% Tween20 treatment and the negative control PBS group, while the cell number was fixed at 1.0×10^4 cells.

2.7. Proliferation assay

Cell proliferation was evaluated according to the method reported previously [24]. Cells proliferated were washed twice with FBS-free DMEM to remove cell adhesive factors from the serum. The cell suspension in FBS-free DMEM (2.0×10^3 cells, 100 μ l) was added to PnF-, PnF derivatives-, fibronectin- or collagen-coated dishes, and cultured for 1 day. Then, the medium was changed to the DMEM containing 0.1 vol% FBS. Cells cultured for 5, 12, and 15 days. The cell-counting reagent was added to each well. After 2 hr of incubation with the reagent, the absorbance of each well measured using a UV-microplate reader at 562 nm. The number of cells was calculated with a standard curve prepared by titrating the known number of cells with the same cell counting method. The number of cells was defined as that of cells proliferated for 5, 12, and 15 days.

2.8. Blocking assay by integrin antibodies

To evaluate the effect of integrin receptors on the attachment of cells, the blocking assay was performed. Cells proliferated were washed twice with FBS-free DMEM, and then cells washed were treated with 5 μ g/ml anti-integrin- α 2 and - α V antibodies (Millipore Inc., Billerica, MA), 5 μ g/ml anti-integrin- α 3 antibodies (Chemicon Inc., Temecula, CA), 5 μ g/ml anti-integrin- α 4 and - α 6 antibodies (R&D Inc., Minneapolis, MN), or 5 μ g/ml anti-integrin- α 5 antibodies (Abcam Plc., Cambridge, UK) for 30 min at 37 $^{\circ}$ C. Next, the cells were centrifuged at 1,000 rpm for 5 min at room temperature, and the cell pellet was resuspended in FBS-free DMEM. Then, the cell attachment assay was performed as described above.

2.9. Estimation of ERK phosphorylation

Cells were cultured on PnF-, PnF derivatives-, fibronectin- or collagen-coated dishes for 2 hr. The cells were treated with a lysis buffer (50 mmole/l Tris-HCl, 150 mmole/l NaCl, 1 mmole/l ethylenediaminetetraacetate (EDTA), 0.1wt% Nonidet P-40 ; pH 7.4) containing a protease inhibitor mixture (Cat. No. P8340, Sigma-Aldrich Co., St. Louis, MO) for cell lysis. The protein content of cell lysates was measured with a Micro BCA protein assay kit. The cell lysates prepared were applied to sodium dodecylsulfate polyacrylamide electrophoresis (SDS-PAGE) and transferred to an Immobilon-P membrane (Millipore Inc., Milford, MA). 15 % SDS-PAGE gel was used and 50 µg of proteins were applied to each lane. The membrane was immunoblotted with an antibody against phospho-ERK or ERK (Lot No. 23 and 16, Cell Signaling Technology Inc., Beverly, MA) and β -actin (Sigma-Aldrich Co., St. Louis, MO) as an internal control as the diluted ratios of 1:1000, 1:1000, and 1:5000, respectively. After incubation with a peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (Lot. No. FK928983 or LB142282, Sigma-Aldrich Co., St. Louis, MO), the target proteins were immunologically visualized with SuperSignal West Pico Chemiluminescent Substrate (Lot. No. LF144837, Pierce, Thermo Fisher Scientific, Rockford, IL). The western blot gel image was analyzed by image J (National Institutes of Health, Bethesda, MD). The band intensity was normalized by cell culture dish (96 well cell culture cluster, Cat. No. 3595, Corning Inc., One Riverfront Plaza, NY).

2.10. Statistical analysis

All the results were statistically analyzed by Tukey-Kramer test and $p < 0.05$ was considered to be

statistically significant. Data were expressed as the mean \pm the standard deviation. Experiments were replicated independently three times unless otherwise mentioned.

3. Results

3.1. Chemical modification of PnF

Primary amino, tertiary amino, and quaternary ammonium groups were introduced to PnF as cationic functional groups. Carboxyl, and sulfonyl groups were also introduced to PnF as anionic functional groups. **Figure 1** shows the percent introduction of functional groups as a function of reaction time. The percent introduction increased with time of reaction, irrespective of the type of functional groups.

Figure 2 shows the CD spectra of PnF derivatives. **Table 1** shows the percentage of functional groups introduced and the conformation of PnF derivatives. The conformation of PnF derivatives changed with an increase in the amount of chemical residues in a similar fashion, irrespective of the residues type (data not shown). When the functional groups were introduced around 15 mol% of PnF OH, the β -sheet structure was collapsed, and then converted to the randomcoil one.

3.2. Cell attachment onto PnF or the derivatives-coated dishes

Figures 3A and **3B** show cells attachment to PnF or the dishes coated with different adsorption concentrations of PnF and the derivatives. **Figure 3A** shows cells attachment as a function of the amount of PnF

derivatives adsorbed on surface. **Figure 3B** shows cells attachment to PnF derivatives at low protein adsorption amount. To evaluate the initial cell attachment to the PnF, non-adhesive cells were excluded 2 hr after incubation. At a high amount of PnF adsorbed, a constant number of cells attached was observed for the PnF-N3-coated dishes. At a low amount of adsorption, significantly high cell number was observed for PnF-N3, PnF-COOH, and the PnF-SO₃H-coated dishes. On the contrary, the coating of PnF-N1 and PnF-N4 decreased the cell attachment at a high amount of protein adsorbed. **Figures 3C** shows the morphology of cells attached on the dishes coated with PnF and the derivatives. The shape of cells cultured on the PnF-, PnF derivatives-, fibronectin-, and collagen-coated dishes was similarly spread while adhered.

3.3. Effect of PnF modification rate on cell attachment

Figure 4 shows the cell attachment onto dishes coated with PnF derivatives with different introduction percentages. The introduction percentage was determined by the decrease of serine hydroxyl groups before and after the reaction according to the amino acid analysis. The number of attached cells decreased with an increase in the introduction percentages. However, the PnF-N3, PnF-COOH, and PnF-SO₃H coating did not decrease the cell attachment.

Figure 5 shows the LDH activity of cells cultured in the medium containing PnF derivatives. The coating concentration is 10 µg/ml. The LDH activity increased with an increase in the concentration. The LDH activity increased for cells cultured on the dishes coated with PnF-N1 and PnF-N4. On the contrary, the LDH activity was

suppressed for cells cultured on the PnF-N3, PnF-COOH, and PnF-SO₃H-coated dishes.

3.4. Effect of functional groups on the cell adhesion manner

Table 2 shows the percent decrease in the number of cells attached after the treatment with antibodies against different integrin α subunits. **Figure 6** shows the effect of integrin antibody treatment on the cell attachment onto PnF derivatives-coated dishes. The primary amino, the tertiary amino, and the quaternary ammonium groups tended to increase the association with $\alpha 2$ and $\alpha 6$ integrins when compared the association of PnF. On the contrary, the association with the $\alpha 4$ integrin decreased. The association with the integrin αV increased by the derivatization of carboxyl and sulfonyl groups.

Figure 7 A shows the ERK phosphorylation of cells cultured on dishes coated with PnF and the derivatives 2 hr after incubation. Figure 7 B shows the westernblotting image of ERK phosphorylation of cells cultured. The band density was normalized by that of cells cultured on the original culture dish. The significant increase of ERK phosphorylation was observed for cells cultured on PnF-COOH- and PnF-SO₃H-coated dishes compared with that of PnF-coated one. On the contrary, the PnF-N1, PnF-N3, and PnF-N4 coating had no influence.

3.5. Proliferation of cells cultured on dishes coated with PnF and the derivatives

Figure 8 shows the number of cells after culturing on dishes coated with PnF and the derivatives. The number of cells was counted after 5, 12, and 15 days after incubation. After 15 days culture, cells cultured on the dish

coated with the original PnF reached at the 80~90 % confluence. The number of cells cultured on fibronectin- and collagen-coated dishes slightly increased in a low serum condition. On the other hand, the number of cells cultured on PnF derivatives-coated dishes increased to a significantly higher extent. The significant increase in the cell number was observed on PnF-COOH- and PnF-SO₃H-coated dishes compared with that of PnF-coated one. On the contrary, the PnF-N1 coating decreased the cell number although the PnF-N3 and PnF-N4 coating had no influence.

4. Discussion

The present study demonstrated that the effect of chemical modification of PnF affects the cell attachment and proliferation. The serine content of PnF decreased with time when determined by the amino acid analysis (**Figure 1**). This indicates that PnF was chemically modified by introducing different functional groups. The PnF derivatization lost the β -sheet structure of original PnF and brought about a random coil structure (**Figure 2**). It is reported that the chemical modification by the amino, carboxyl, and sulfonyl groups allows silk fibroin to have a random coil conformation [27]. It is highly conceivable that this conformational change causes change in the affinity of RGD sequences to bind to the integrin of cells.

It has been reported that some types of cells (fibroblast and endothelial cells) attached onto PnF- and PnF-N3-coated dishes in FBS-free condition. The cells attached onto the PnF- and PnF-N3-coated dishes to a greater extent than fibronectin-coated dishes [30, 31]. The similar result was obtained in this study. In addition, cells attached much more onto PnF-COOH- and PnF-SO₃H-coated dishes. The number of cells attached on dishes coated with

PnF-N3, PnF-COOH, and PnF-SO₃H increased even at the low amount of protein adsorbed (**Figure 3**). There are various plausible reasons to change the interaction manner of PnF derivatives and integrins. Among them, the phenomenon can be explained in terms of change in the interaction manner. It is possible that the chemical modification with positive and negative residues causes the changes in overall conformation of PnF as well as changing the receptor-ligand affinity between PnF and integrins. For the dishes coated with PnF-N1 and PnF-N4, however, the number of cells attached did not increase and decreased with an increase in the percent introduction (**Figures 3 and 4**). It is recognized that the water wettability of substrate surface linearly increased with the increasing carboxyl group content of collagen and no cytotoxicity was detected [32]. A good correlation between the number of positive charges on the protein surface and the cytotoxic activity is demonstrated [33, 34]. The basicity of amino groups in water is in the order of the secondary > the primary > the quaternary > the tertiary, because of the hydration extent [35]. In the case of cationized PnF, the cytotoxicity of positive charges from the primary and quaternary amino groups resulted in the decrease of cells attachment while the tertiary amino group showed no cytotoxicity (**Figure 5**). It is conceivable that the lowest basicity of the tertiary group resulted in the low cytotoxicity. As a result, the number of cell attachment onto PnF-N3-coated dishes was larger than PnF-N1- and PnF-N4-coated dishes.

It is known that the integrin α V has a wide association with various ECMs with the positive amino block of RGD and GRKRR [36, 37]. Therefore, it is possible that the carboxyl and sulfate groups of negative charge are likely to adsorb positive charge substances, resulting in enhanced association with the α V integrin. **Figure 6** showed that the

treatment of integrin $\alpha 5$ and αV antibodies suppressed the cell attachment on the dishes coated anionic groups-introduced PnF. It is well recognized that the α -subunit of integrins associate with particular trans membrane and cytoplasmic proteins to specifically activate the intracellular signaling pathways [38]. Caveolin-1 links the integrin $\alpha 1$, $\alpha 5$, and αV binding to the Src pathway that plays a key role in the promotion of cell proliferation through the phosphorylation of ERK [39]. Integrins $\alpha 5$ and αV which modulate the cell proliferation, interacted with the PnF-COOH and PnF-SO₃H (**Figure 6**). It is known that fibronectin has an affinity for many types of integrins [26]. In this study, fibronectin mainly bound to integrins $\alpha 4$ and $\alpha 6$, but the reason is unclear at present. In addition, the phosphorylation of ERK was increased (**Figure 7**). It is possible that for the carboxyl and sulfonyl groups-coated dishes, the enhanced ERK phosphorylation resulted in the promoted cell proliferation (**Figure 8**). It is concluded that the chemical modification of PnF with the tertiary amino, carboxyl, and sulfonyl groups was promising to enhance the cell attachment and the subsequent proliferation. The design of cell culture substrate is important to manipulate the proliferation and differentiation of cells in terms of regenerative medicine where the cellular potential can be artificially enhanced for cell-based therapy. Based on the results obtained, the ProNectin and the derivatives may be promising as a coating material which makes the substrate surface cytocompatible to modify the cell behavior. The cell line of C3H10T1/2 was used in this study as a stem cell with high potential of proliferation and differentiation. The effect of ProNectin derivatives on the cell differentiation will be reported in the future.

5. Conclusions

PnF was chemically modified by the primary, tertiary amino, quaternary ammonium, carboxyl, and sulfonyl groups. The cytotoxicity was low for PnF-N3, PnF-COOH, and PnF-SO₃H. The number of cells attached onto the dishes coated with PnF-N3, PnF-COOH, and PnF-SO₃H was higher than that of original PnF. In addition, the phosphorylation of ERK was upregulated for cells attached onto the dishes coated with PnF-COOH and PnF-SO₃H. Actually, integrin subunits attached PnF-derivatives are changed each derivatives. The derivatization of the tertiary amino, and carboxyl, and sulfonyl groups increase cell attachment and proliferation.

Reference

- [1] J c. microbial production of structural protein polymers, in: plastics from microbes. carl hanser verlag 1994:34-92.
- [2] j c. genetically engineered protein polymers. handbook of biodegradabel polymers 1997:1-32.
- [3] DeSimone DW, Stepp MA, Patel RS, Hynes RO. The integrin family of cell surface receptors. *Biochem Soc Trans* 1987;15:789-91.
- [4] Heslot H. Artificial fibrous proteins: a review. *Biochimie* 1998;80:19-31.
- [5] Meredith JE, Jr., Fazeli B, Schwartz MA. The extracellular matrix as a cell survival factor. *Mol Biol Cell* 1993;4:953-61.
- [6] Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell* 2002;110:673-87.
- [7] Giancotti FG, Ruoslahti E. Integrin signaling. *Science* 1999;285:1028-32.
- [8] Ruoslahti E, Pierschbacher MD. Arg-Gly-Asp: a versatile cell recognition signal. *Cell* 1986;44:517-8.
- [9] Frisch SM, Screaton RA. Anoikis mechanisms. *Curr Opin Cell Biol* 2001;13:555-62.
- [10] Liddington RC, Ginsberg MH. Integrin activation takes shape. *J Cell Biol* 2002;158:833-9.
- [11] Vuori K. Integrin signaling: tyrosine phosphorylation events in focal adhesions. *J Membr Biol* 1998;165:191-9.
- [12] Hosseinkhani H, Tabata Y. PEGylation enhances tumor targeting of plasmid DNA by an artificial cationized protein with repeated RGD sequences, Pronectin. *J Control Release* 2004;97:157-71.
- [13] Ohyama T, Ko IK, Miura A, Iwata H, Taki W. ProNectin F-grafted-ethylene vinyl alcohol copolymer (EVAL) as a liquid type material for treating cerebral aneurysm--an in vivo and in vitro study. *Biomaterials* 2004;25:3845-52.
- [14] Kurokawa M, Sato S. Growth and poliovirus production of Vero cells on a novel microcarrier with artificial cell adhesive protein under serum-free conditions. *J Biosci Bioeng*;111:600-4.
- [15] Okazaki A, Jo J, Tabata Y. A reverse transfection technology to genetically engineer adult stem cells. *Tissue Eng* 2007;13:245-51.
- [16] McClary KB, Grainger DW. RhoA-induced changes in fibroblasts cultured on organic monolayers. *Biomaterials* 1999;20:2435-46.
- [17] McClary KB, Ugarova T, Grainger DW. Modulating fibroblast adhesion, spreading, and proliferation using self-assembled monolayer films of alkylthiolates on gold. *J Biomed Mater Res* 2000;50:428-39.
- [18] Scotchford CA, Gilmore CP, Cooper E, Leggett GJ, Downes S. Protein adsorption and human osteoblast-like cell attachment and growth on alkylthiol on gold self-assembled monolayers. *J Biomed Mater Res* 2002;59:84-99.

- [19] Mrksich M, Whitesides GM. Using self-assembled monolayers to understand the interactions of man-made surfaces with proteins and cells. *Annu Rev Biophys Biomol Struct* 1996;25:55-78.
- [20] Allen LT, Fox EJ, Blute I, Kelly ZD, Rochev Y, Keenan AK, et al. Interaction of soft condensed materials with living cells: phenotype/transcriptome correlations for the hydrophobic effect. *Proc Natl Acad Sci U S A* 2003;100:6331-6.
- [21] Keselowsky BG, Collard DM, Garcia AJ. Surface chemistry modulates fibronectin conformation and directs integrin binding and specificity to control cell adhesion. *J Biomed Mater Res A* 2003;66:247-59.
- [22] chemical modification of repetitive polymers to enhance water solubility. United states patent 5,760,004 1994.
- [23] Yang JT, Wu CS, Martinez HM. Calculation of protein conformation from circular dichroism. *Methods Enzymol* 1986;130:208-69.
- [24] Kurihara H, Nagamune T. Cell adhesion ability of artificial extracellular matrix proteins containing a long repetitive Arg-Gly-Asp sequence. *J Biosci Bioeng* 2005;100:82-7.
- [25] Yoshida N, Koizumi M, Adachi I, Kawakami J. Inhibition of P-glycoprotein-mediated transport by terpenoids contained in herbal medicines and natural products. *Food Chem Toxicol* 2006;44:2033-9.
- [26] Johansson S, Svineng G, Wennerberg K, Armulik A, Lohikangas L. Fibronectin-integrin interactions. *Front Biosci* 1997;2:d126-46.
- [27] Murphy AR, St John P, Kaplan DL. Modification of silk fibroin using diazonium coupling chemistry and the effects on hMSC proliferation and differentiation. *Biomaterials* 2008;29:2829-38.
- [28] Keselowsky BG, Collard DM, Garcia AJ. Surface chemistry modulates focal adhesion composition and signaling through changes in integrin binding. *Biomaterials* 2004;25:5947-54.
- [29] Lan MA, Gersbach CA, Michael KE, Keselowsky BG, Garcia AJ. Myoblast proliferation and differentiation on fibronectin-coated self assembled monolayers presenting different surface chemistries. *Biomaterials* 2005;26:4523-31.
- [30] Esty A. Receptor-specific serum-free cell attachment using a highly stable engineered protein polymer. *Am Biotechnol Lab* 1991;9:44.
- [31] Ishii-Watabe A, Kanayasu-Toyoda T, Suzuki T, Kobayashi T, Yamaguchi T, Kawanishi T. Influences of the recombinant artificial cell adhesive proteins on the behavior of human umbilical vein endothelial cells in serum-free culture. *Biologicals* 2007;35:247-57.
- [32] Bet MR, Goissis G, Vargas S, Selistre-de-Araujo HS. Cell adhesion and cytotoxicity studies over polyanionic collagen surfaces with variable negative charge and wettability. *Biomaterials* 2003;24:131-7.
- [33] D'Errico G, Ercole C, Lista M, Pizzo E, Falanga A, Galdiero S, et al. Enforcing the positive charge of N-termini enhances membrane interaction and antitumor activity of bovine seminal ribonuclease. *Biochim Biophys Acta*;1808:3007-15.

- [34] Fischer D, Li Y, Ahlemeyer B, Krieglstein J, Kissel T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* 2003;24:1121-31.
- [35] Nagy P. Theoretical calculations on the basicity of amines. *journal of molecular structure* 1989;201:271-86.
- [36] Akhtar K, Broekelmann TJ, Song H, Turk J, Brett TJ, Mecham RP, et al. Oxidative modifications of the C-terminal domain of tropoelastin prevent cell binding. *J Biol Chem*;286:13574-82.
- [37] Bax DV, Rodgers UR, Bilek MM, Weiss AS. Cell adhesion to tropoelastin is mediated via the C-terminal GRKRR motif and integrin α V β 3. *J Biol Chem* 2009;284:28616-23.
- [38] Giancotti FG. Complexity and specificity of integrin signalling. *Nat Cell Biol* 2000;2:E13-4.
- [39] Wary KK, Mariotti A, Zurzolo C, Giancotti FG. A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. *Cell* 1998;94:625-34.

Figure captions

Figure 1. The profile of PnF chemical modification by introduction of different functional groups: PnF-N1 (\triangle), PnF-N3 (\square), PnF-N4 (\bullet), PnF-COOH (\blacktriangle), and PnF-SO₃H (\blacksquare).

Figure 2. Conformational analysis of PnF and the derivatives. CD spectra of PnF (\circ), PnF-N1 (\triangle), PnF-N3 (\square), PnF-N4 (\bullet), PnF-COOH (\blacktriangle), and PnF-SO₃H (\blacksquare).

Figure 3. Initial attachment of cells onto culture dishes coated with PnF (\circ), PnF-N1 (\triangle), PnF-N3 (\square), PnF-N4 (\bullet), PnF-COOH (\blacktriangle), PnF-SO₃H (\blacksquare), fibronectin (∇), and collagen (\blacktriangledown). **A** Effect of protein adsorption amount on the cell attachment. **B** Cell attachment at low Protein adsorption amount. The cell attachment was examined 2 hr after incubation. **C** The morphology of cells attached on the dishes coated with PnF and the derivatives 2 hr after incubated.

Figure 4. Initial attachment of cells onto dishes coated with PnF-N1 (\triangle), PnF-N3 (\square), PnF-N4 (\bullet), PnF-COOH (\blacktriangle), and PnF-SO₃H (\blacksquare).

Figure 5. LDH activity of cells cultured in medium containing PnF-N1 (\triangle), PnF-N3 (\square), PnF-N4 (\bullet), PnF-COOH (\blacktriangle), and PnF-SO₃H (\blacksquare). The coating concentration is 10 μ g/ml. The percent LDH activity for unmodified PnF is that at the percent OH introduced of 0.

Figure 6. Inhibition patterns of cells attachment onto the dishes coated with 10 μ g/ml of PnF, PnF-derivatives, fibronectin, and collagen 2 hr after incubation in the presence of anti-integrin α 2 (\square), α 3 (\square), α 4 (\blacksquare), α 5 (\boxtimes), α 6 (\boxminus), and α V (\square) antibodies.

Figure 7. **A** ERK phosphorylation of cells cultured on dishes coated with PnF and the derivatives. *, $p < 0.05$; significant against PnF. **B** Westernblot image of ERK phosphorylation of cells. Lane # 1 PnF, # 2 PnF-N1, # 3 PnF-N3, # 4 PnF-N4, #5 PnF-COOH, # 6 PnF-SO₃H, # 7collagen, # 8 fibronectin

Figure 8. Proliferation of cells on dishes coated with PnF, PnF-derivatives, fibronectin, and collagen for 5 (\square), 12 (\square), and 15 (\blacksquare) days incubation. The coating concentration is 10 μ g/ml. *, $p < 0.05$; significant difference between the two groups.

Table 1. Conformational information of PnF and the derivatives.

| | Percentage introduced | Conformation (%) | | | |
|-----------------------|-----------------------|--------------------|----------------|---------------|-----------|
| | | α -helix | β -sheet | β -turn | radomcoil |
| PnF | 0 | 0 | 30 | 20 | 50 |
| PnF-N1 | 15 | 0 | 1 | 32 | 67 |
| PnF-N3 | 26 | 0 | 0 | 29 | 71 |
| PnF-N4 | 20 | 0 | 0 | 19 | 81 |
| PnF-COOH | 22 | 0 | 7 | 33 | 61 |
| PnF-SO ₃ H | 28 | 0 | 6 | 33 | 61 |

Table 2. The affinity of PnF and the derivatives for integrins.

| | Percent decrease in the number of cells attached by the blocking with antibody against | | | | | |
|-----------------------|--|---------------|----------------|---------------|---------------|---------------|
| | $\alpha 2$ | $\alpha 3$ | $\alpha 4$ | $\alpha 5$ | $\alpha 6$ | αV |
| PnF | 9.3 ± 2.7^a | 0 ± 5.0 | 42 ± 8.5 | 17 ± 6.9 | 8.7 ± 2.3 | 0 ± 0.75 |
| PnF-N1 | 14 ± 9.4 | 0 ± 4.3 | 18 ± 4.2 | 32 ± 8.5 | 39 ± 4.8 | 0 ± 5.1 |
| PnF-N3 | 23 ± 15 | 3.1 ± 1.7 | 11 ± 5.8 | 19 ± 5.4 | 44 ± 7.7 | 0 ± 3.4 |
| PnF-N4 | 13 ± 3.8 | 0 ± 2.5 | 2.0 ± 4.7 | 6.0 ± 3.4 | 24 ± 3.6 | 4.0 ± 2.6 |
| PnF-COOH | 13 ± 9.3 | 2.6 ± 1.5 | 38 ± 8.1 | 33 ± 8.7 | 13 ± 5.2 | 14 ± 1.5 |
| PnF-SO ₃ H | 2.5 ± 0.72 | 0 ± 2.2 | 0 ± 4.5 | 18 ± 4.5 | 0 ± 1.3 | 14 ± 2.1 |
| Fibronectin | 4.7 ± 3.8 | 0 ± 3.2 | 34 ± 8.0 | 0 ± 4.2 | 35 ± 8.5 | 0 ± 3.2 |
| Collagen | 46 ± 6.3 | 2.2 ± 5.7 | 0.14 ± 9.7 | 0 ± 6.6 | 9.8 ± 4.1 | 2.2 ± 5.7 |

a) The percentage is calculated when 100 % is defined for the number of cells attached without the antibody treatment.

Figure 1.

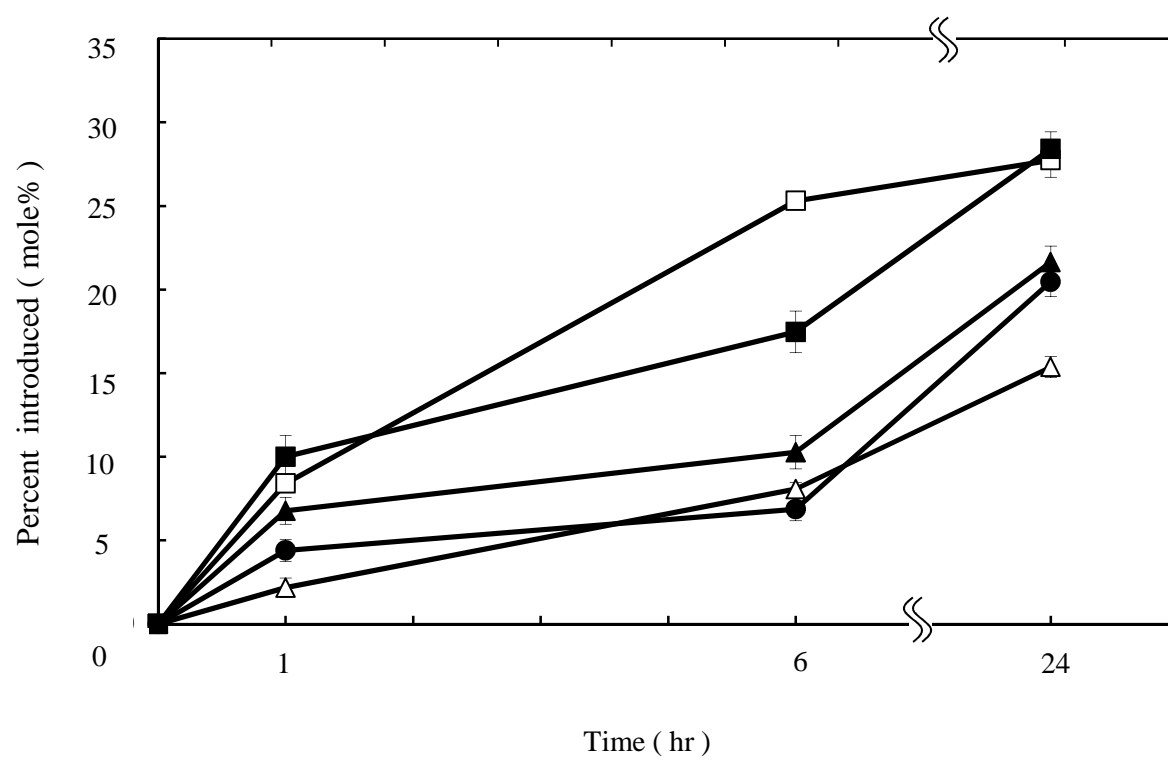


Figure 2.

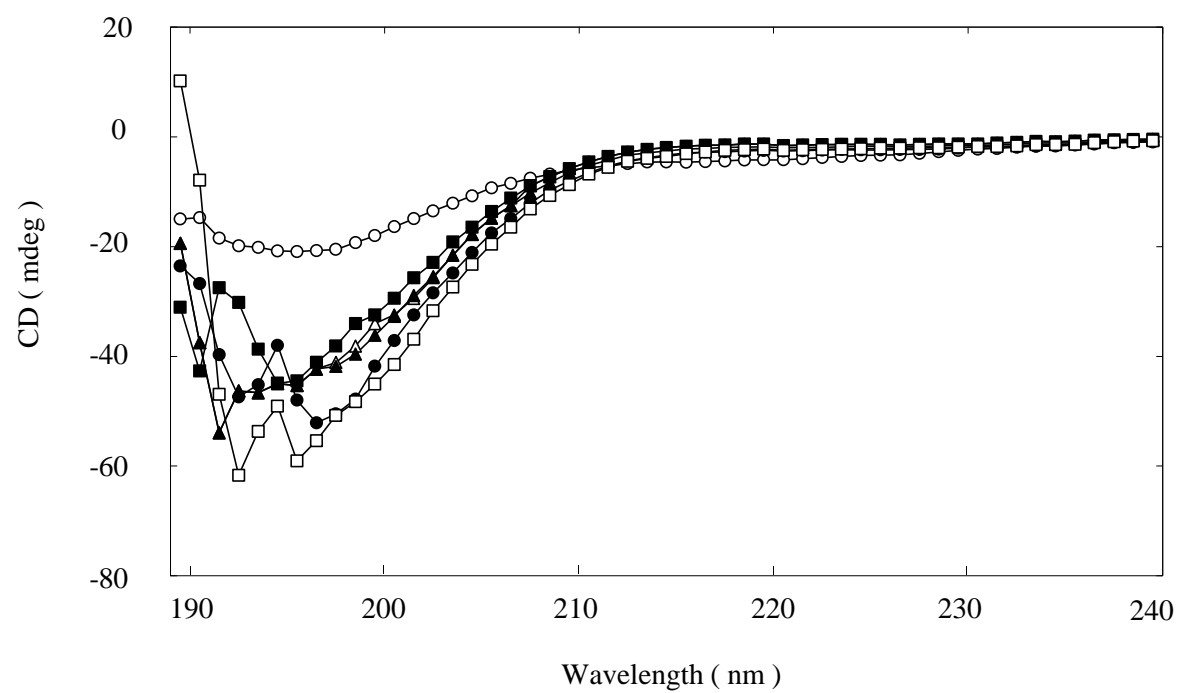
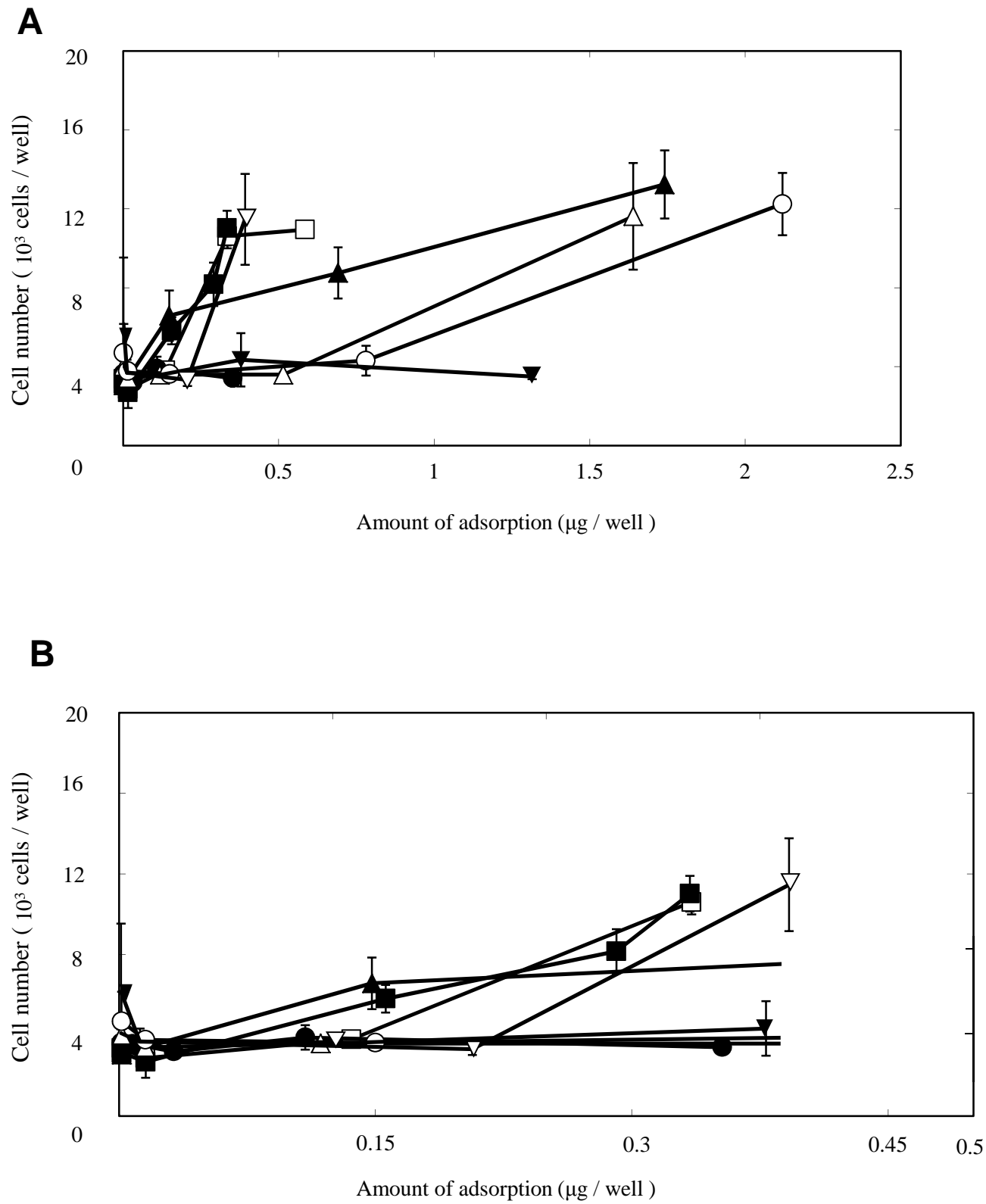
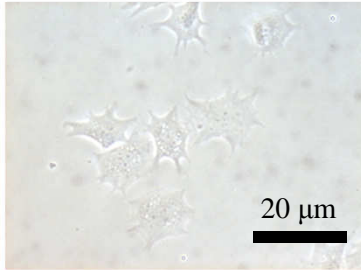


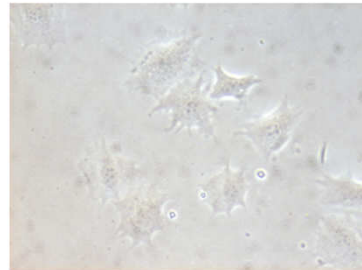
Figure 3.



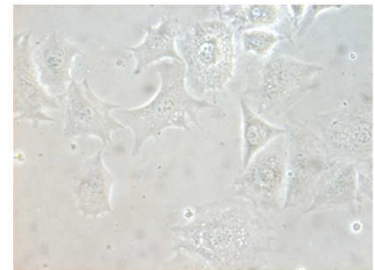
C



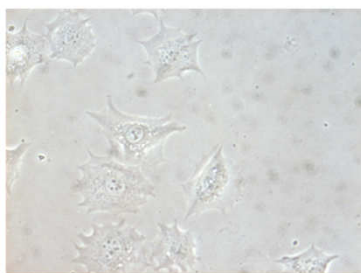
PnF



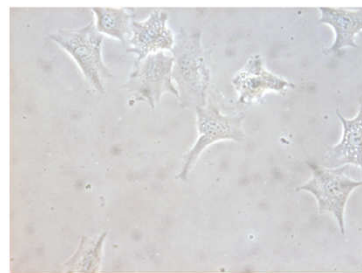
PnF-N1



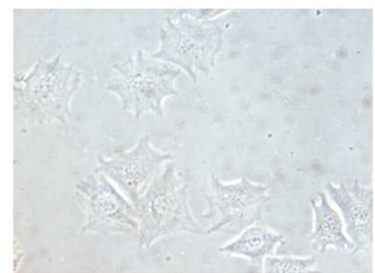
PnF-N3



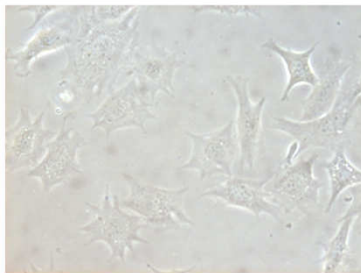
PnF-N4



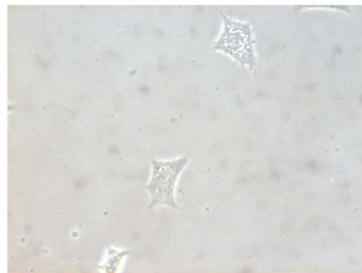
PnF-COOH



PnF-SO₃H



Fibronectin



Collagen

Figure 4.

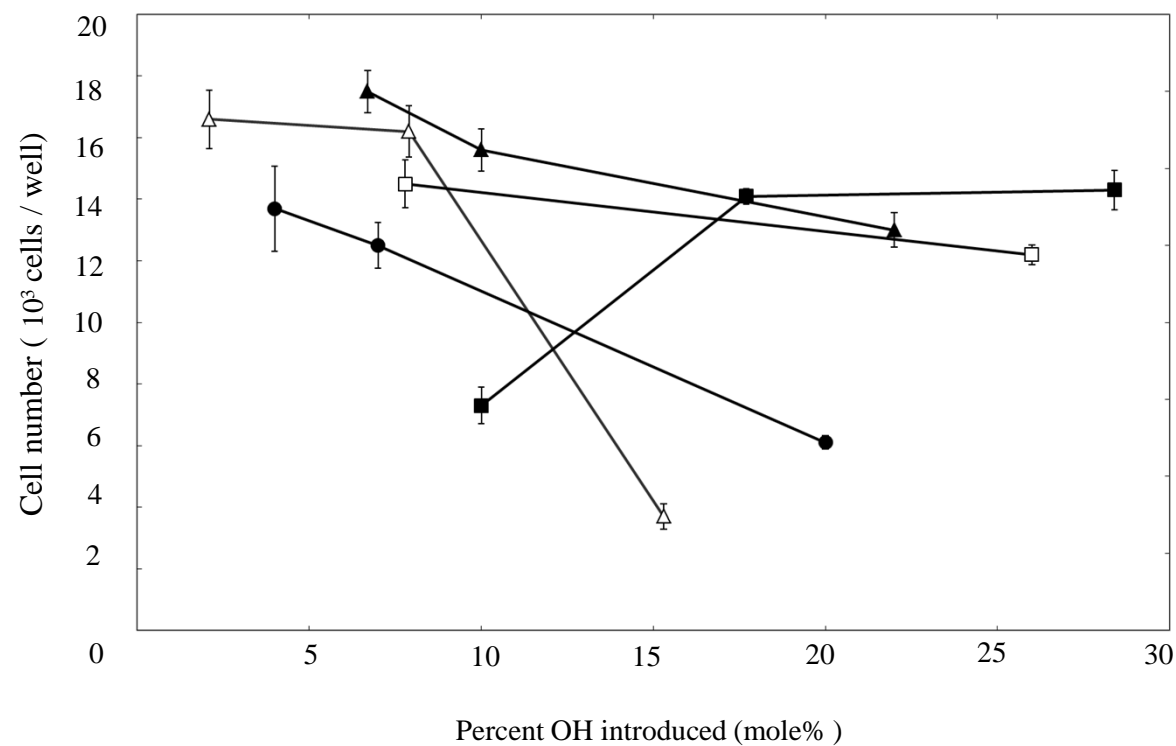


Figure 5.

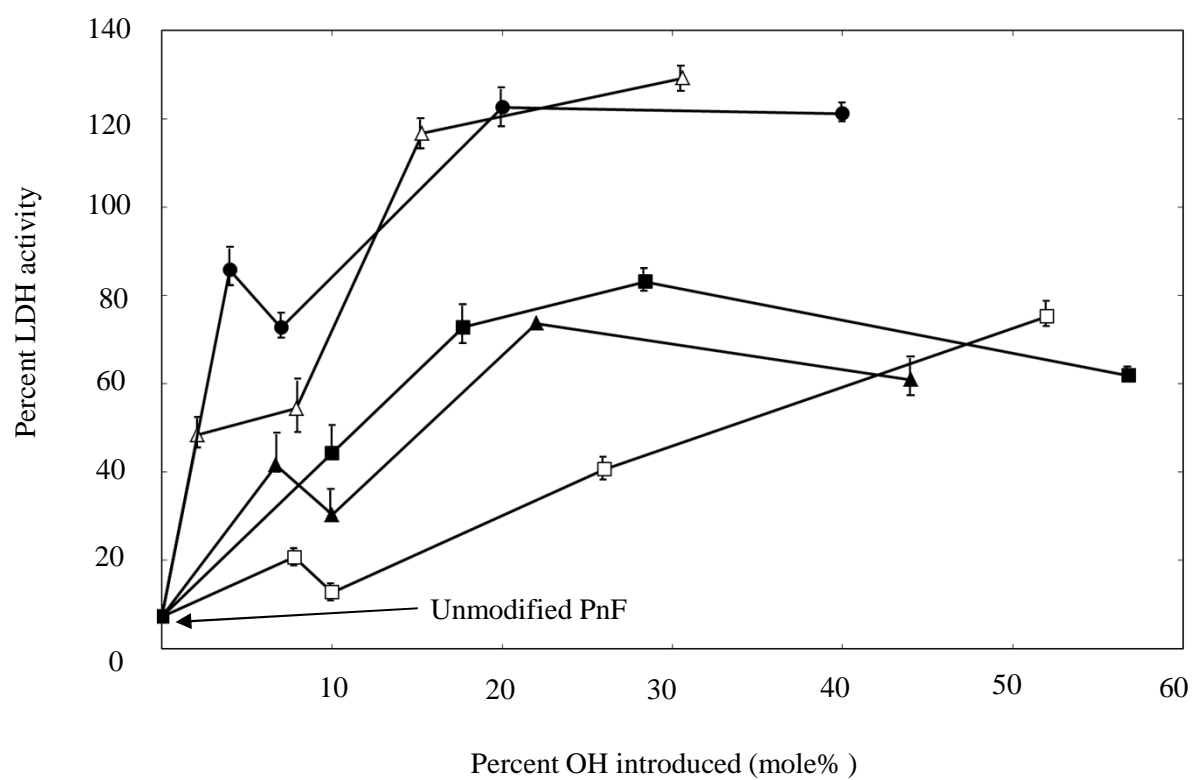


Figure 6.

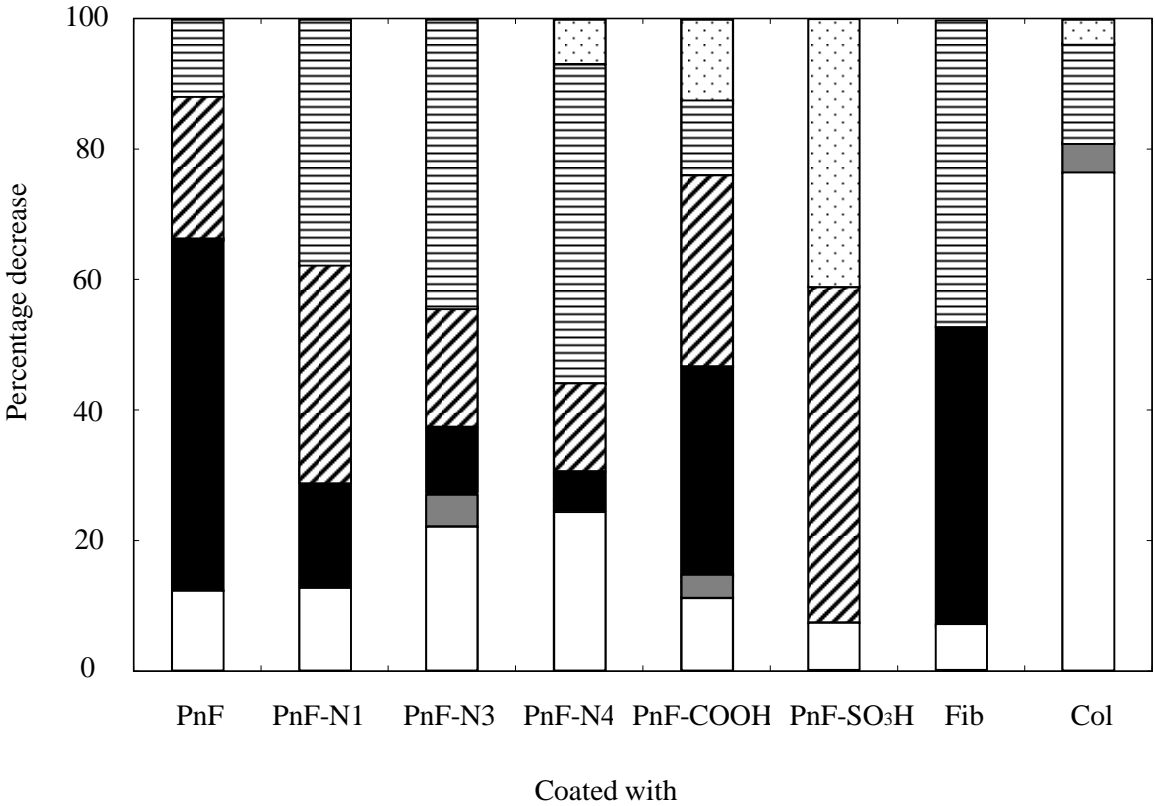


Figure 7.

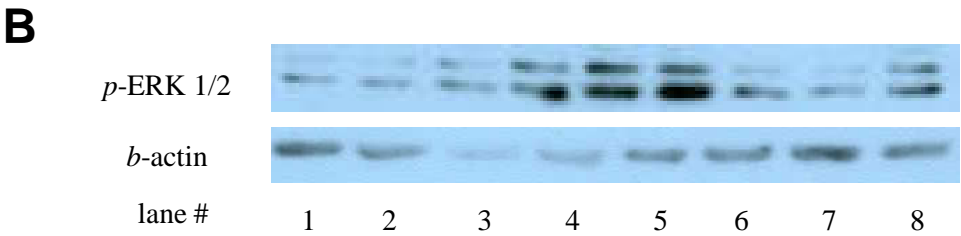
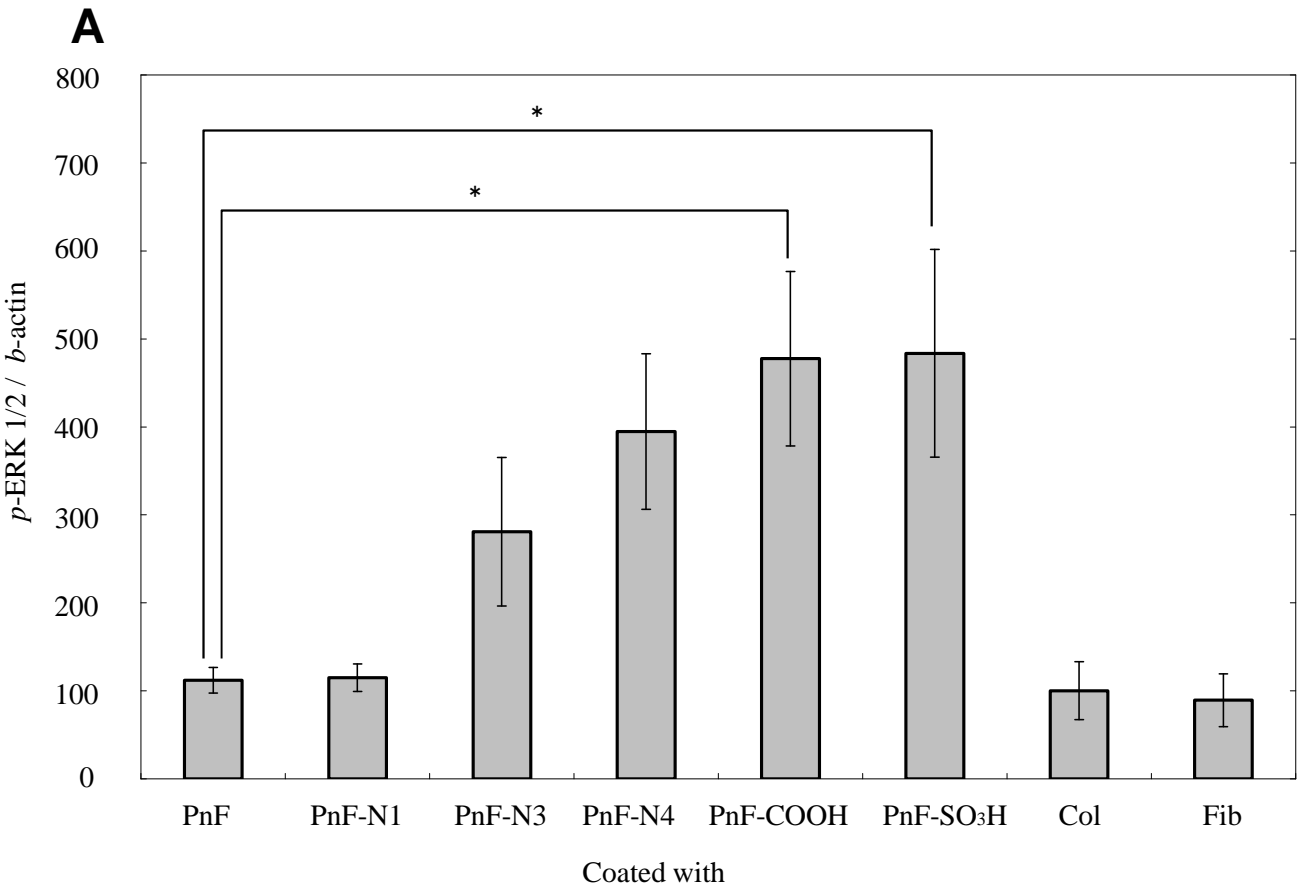


Figure 8.

