Preparation of Silk Fibroin and Polyallylamine Composites

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ABSTRACT: Composite films made of silk fibroin (SF) and polyallylamine (PAA) are prepared that contain various compositional ratios. These materials are analyzed to elucidate the resulting physical properties and to assess their potential toward advanced applications as industrial materials. The composite films are obtained from a SF and PAA binary system by dry casting from aqueous solution. These composite films exhibit excellent processability such as film forming capabilities, and the elongation at break is increased in the wet state. The differential scanning calorimetry (DSC) curves of the composites suggest that a mutual interaction takes place between the SF and PAA. This interaction is believed to occur because the endothermic peak, corresponding to the individual polymer, shifts with increasing SF content. The random coil conformation of the SF is present, regardless of the PAA blending, as confirmed by FTIR and DSC measurements. Additionally, living cells from *Antheraea pernyi* and *Bombyx mori* insect tissues are shown to grow effectively on the composite films. Maximum growth levels occur when the cultivation flask is coated with the material in SF/PAA ratios of 75:25 to 25:75. © 2002 Wiley Periodicals, Inc. J Appl Polym Sci 84: 1963–1970, 2002; DOI 10.1002/app.10491

Key words: silk fibroin; polyallylamine; blend; differential scanning calorimetry; compatibility; cell attachment

INTRODUCTION

New materials prepared by hybridizing naturally occurring biopolymers and synthetic organic high polymers are attractive for the production of functional polymer composites with varied and custom-tailored properties that neither individual polymer possesses.

The term silk refers to a wide range of filaments spun by several species of *Lepidoptera* and *Arthropoda* to build structures external to the body such as cocoons and webs. The silk protein is formed by the dehydration of various amino acids, giving rise to a polypeptide chain with silk fibroin (SF), referring specifically to silk protein derived from the silkworm. SF has been noted as a suitable medical material because of its high biocompatibility with living tissues, as is suggested by its present use as sutures in the surgical field. Additionally, biotechnological and biomedical applications utilizing silk proteins have been widely reported, not only because of their outstanding

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biocompatibility characteristics with living cell attachment[1,2] but also for their ease of oxygen permeation[3] and enzyme immobilization.[4]

SF has good film-forming capabilities and is a natural product safe for the human body; however, these films are very brittle and stiff in the dry state.[5] Therefore, the physical properties of silk films require improvement by physical or chemical modification. Among the possible modifications, the technique of polymer blending is both an effective and economical option.[5,6]

Polyallylamine (PAA) is a synthetic cationic high polymer made of olefin copolymers, which is soluble and positively charged in aqueous solution. In addition to this, PAA is nontoxic and utilized extensively in chemical and biomedical applications. PAA has also been widely used in the industrial field and has been used as a slow releaser or immobilizing substrate for pharmaceuticals and medicines.[7] It has also been shown that living cells or tissues attach well on culture substrates rinsed with PAA solution.[8,9]

However, because of its highly hydrophilic nature, PAA compounds have drawbacks such as lack of processability and difficulties in film, fiber, and hydrogel preparations. After adding an anionic polymer into a PAA solution, PAA precipitates and coagulates, making production of PAA composite materials with other polymers impossible. Similar to SF films, PAA films are brittle in the dry state and exhibit low processability as described above. Applications for PAA are limited because of these negative aspects.

This article discusses the production of new composite materials made of silk protein and PAA to overcome the above limitations and to improve the individual mechanical and biomedical properties of each polymer. Optimum conditions for preparing the composites and applications for these materials as living cell substrates are also presented.

EXPERIMENTAL

Materials

A 3% (w/v) PAA solution was prepared by diluting a commercially available 10–40% PAA solution from Nitto Boseki Col., Ltd. with water. The solution was successively neutralized using a diluted NH_3 aqueous solution.

Composite films were obtained by dry casting the corresponding binary system containing SF and PAA on a polyethylene film at 20°C for 24 h. The thickness of the blend films was about 80–100 μm.

Table I Prominent Characteristics of Polyallylamines

Polyallylamine	Molecular Weight	Characteristics
PAS-A-120L	~ 100,000	Stable in acidic solution
PAS-H-10L	~ 200,000	Stable in all pH ranges, soluble in methanol
PAS-92	~ 5,000	Stable in acidic solution
PAA-HCl-10L	~ 100,000	Stable in all pH ranges
PAA-10C	~ 10,000	Soluble in alcohol

Varying types of PAAs were obtained from Nitto Boseki Co., Ltd.; the prominent characteristics are listed in Table I after slight modification of the technical reports from Nitto Boseki Co., Ltd.

Regenerated silk fiber solutions were obtained by degumming silk fibers obtained from *Bombyx mori* cocoons by treatment with a 0.2% aqueous solution of Marseille soap containing 0.05% Na_2CO_3 at 98°C for 30 min. The fibers were then washed with distilled water and dried at room temperature. The SF fiber was prepared by dissolving the fibers with a 9*M* LiBr solution at 50°C for 15 min. The salt was completely removed by dialysis against distilled water for 3 days at 5°C. The 3% regenerated SF solution was thus obtained and the final concentration of SF was adjusted by slowly drying the solution in air.

A binary system of SF and PAA was prepared by the addition of a 3% regenerated SF solution into an aqueous PAA solution so that the concentration of PAA attained the required amount. The composite material of fibroin and PAA was then prepared to a thickness of approximately 80–100 μm by casting the binary system at 25°C for 12 h on polyethylene film.

Among the PAAs listed in Table I, the PAA-10C (pH 11) became turbid when the SF solution was added and an oily PAA-10C precipitate formed on the bottom of the glass tube after allowing the solution to stand for 30 min. Preparation of the SF and PAA-10C binary system therefore required the addition of an ammonium solution into the 3% regenerated SF solution with a subsequent addition of 6% PAA-10L so that the amount of PAA-10L met the required concentration.

Analytical Measurements

The tensile properties of composite films (3-mm width, 15-mm length, 80 μm thickness) were measured with an automatic tensile testing machine (Tensilon UTM-II, Toyo Baldwin Co., Ltd.) in the above standard conditions. Each value is the average of 20 measurements.

Differential scanning calorimetry (DSC) measurements were performed on a Rigaku Denki instrument (DSC-10A) at a heating rate of 10°C/min. The DSC range and sample weight were 2.5 mcal/s and 2 mg, respectively. The open aluminum cell was swept with N_2 gas during the course of the analysis.

The IR spectra of the composite films were obtained directly from the film with a Perkin–Elmer FTIR 1725 spectrophotometer in the 2000–400 cm^{-1} wavenumber range.

Moisture Regain

The composite films were allowed to stand at standard conditions (25°C, 65% relative humidity) for 1 week to stabilize the absorption amount of humidity. The composite films were then dried at 85°C until the weight became constant. The moisture regain (MR) was measured by the following equation:

$$\mathrm{MR} = (A - B)/A \times 100\ (\%)$$

where A is the weight of the sample before the absorption test and B is the weight of the sample dried at 80°C for 3 h.

State of Cell Growth

Living cells from *Antheraea pernyi* (*Ae*), *Bombyx mori* (*Bm*) and insect strain were utilized for cell growth studies. Cells were grown in Grace medium (commercial product G8142, Sigma) supplemented at 37°C for 65 h with 5% fetal bovine serum (Gibco), 5% *Bm* silkworm hemolymph (Nihon Nosan Kogyou, Ltd.), and 1% penicillin–streptomycin (Life Technologies). Cells were permitted to grow in a water-saturated atmosphere of 5% CO_2 and 95% air at 37°C.

L-929 fibroblast cells derived from mouse connective tissue were purchased from Riken Gene Bank. The cells were grown in MEM medium (Gibco) supplemented with 5% fetal bovine serum, L-glutamine, and 20 mL of HEPES (Sigma) at 37°C for 65 h.

The growth of the living cells on the substrate wall was examined by visual inspection using an inverted microscope. For measuring the number of cells grown in the cultivation, all cells were removed by use of an ultrasonic irradiator for 15 min. Any residual cells left in the container were removed via a pipette. Thus, the attached cells were removed from the wall of the tissue culture flask in the cultivation medium. The number of cells grown was measured by use of a hemocytometer.

Coating of Tissue Culture Flask

The walls of the tissue culture flasks were coated with the binary system after ensuring a good state of mixing and compatible condition sets. The binary system was prepared by adding 3% SF solution and 6% PAA solution so that the content of SF attained blending ratios of 100, 90, 75, 50, 25, and 0% (w/w). Pure SF and pure PAA were used for 100 and 0% (w/w) polymer ratios, respectively. Tissue culture slides were then coated with the SF and PAA binary solution system. A small amount of the SF and PAA system was poured into the tissue culture flask in order to coat the sides of the flask with the composite films. This solution was then stirred slowly so that the solution covered all surfaces of the slides (24-chamber slides, Falcon Laboratories, Inc.). After decantation the binary solution was allowed to dry on the surfaces of the flask at room temperature. This resulted in a superthin composite film coat on the substrate walls. In order to desolublize this film, a mixture solution containing chloroxirane (Kanto Kagaku, Co.), water, and ethanol, (5:10:85% v/v) was poured in the tissue culture flask and allowed to dry on the surfaces of the flask at room temperature. The *Ae* and *Bm* silkworm cells were subsequently incubated in the culture slides that had been coated with the thin composite film.

Compatibility of Binary Mixture

A preliminary evaluation of the extent of compatibility exhibited by SF and PAA (pH not adjusted) was performed in aqueous solution according to the following three-stage criteria:

1. (+) good compatibility: SF and PAA mixed well, resulting in a transparent solution in which no precipitate formed;
2. (±) weak compatibility: there was partial formation of the precipitate, and the result-

Table II Compatibility of Silk Fibroin Solution and Various PAA Solutions

	Silk Fibroin Content (w/w %)						
Polyallylamine	95	90	80	75	50	25	Solubility
PAS-A-120L	−	−	+	+	+	+	±
PAS-H-10L	±	±	+	+	+	+	+
PAS-92	+	+	+	+	±	±	+
PAA-HCl-10L	−	+	+	+	+	±	±
PAA-10C	−	−	−	±	+	+	+

ing solution became turbid after mixing; and
3. (−) no compatibility: there is no mixing of solutions and two separate visible phases can be easily discerned.

Solubility of Composite Films

The film solubility was then evaluated for the composite films produced from the SF and PAA binary mixtures in aqueous solution according to the following three-stage criteria:

1. (+) soluble in water;
2. (±) partially soluble in water; and
3. (−) insoluble in water.

RESULTS AND DISCUSSION

Compatibility of SF Solution and PAA Solution

The most important consideration in the described process is the compatibility or state of mixing of SF and various PAA polymers in aqueous solution. For the preliminary studies the compatibility in the aqueous state and the water solubility were evaluated by mixing 3% SF solution into 6% PAA solutions of differing types (Table II).

From these rough estimations, the PAA-HCL-10L and PAS-92 solution (pH not adjusted) show excellent compatibility with the SF solution in a wide composition range, especially when the SF content is less than 80%. PAA-10C does not show good compatibility or state of mixing with SF specifically when the content of SF is above 80%. The mixing for PAA-10C can be improved by adjusting its pH (pH 11) using diluted ammonium solution. The extent of water solubility was adequate for composite films prepared just after dry casting.

These preliminary studies give important information for obtaining good composite films. In this way, composite films can be obtained by dry casting specific solution compositions that show high compatibility and a good state of mixing for the SF and PAA polymers.

Moisture Regain

In order to elucidate the dependence of the moisture regain on the polymer compositional ratios, the moisture regain of the blended films is plotted as a function of the SF weight fraction. Membranes were allowed to stand at normal conditions of 65% relative humidity at 25°C for 7 days to achieve swelling equilibrium. Figure 1 shows the moisture regain of the composite blend films made of SF and different kinds of PAAs including PAS-A, PAS-92, PAS-H-10L, and PAS-HCl-10L. The moisture regain of SF/PAA-HCL-10L remained unchanged below 10% PAA content, then the value was slightly increased at concentrations above 10% PAA. The moisture regain of SF/PAS-H-10L and SF/PAS-A displayed similar behavior, while the moisture regain of SF/PAS 92 did not show significant moisture regain under the conditions examined.

The rise of the equilibrium moisture regain induced by a small amount of PAA is attributable to the higher hygroscopic nature of PAA. A PAA content between 10 and 30% increased the moisture regain of the SF/PAA-HCL-10L composite films significantly, suggesting that this film becomes strongly hygroscopic because of the hydrophilic nature of PAAs. In addition to this, PAA-HCL-10L is stable in a wide range of pH values (Table I), which is convenient for preparing such composite films.

This presents an advantage in using PAA-HCl-10L as a partner of SF in preparing new and functional composite materials. As a result, the

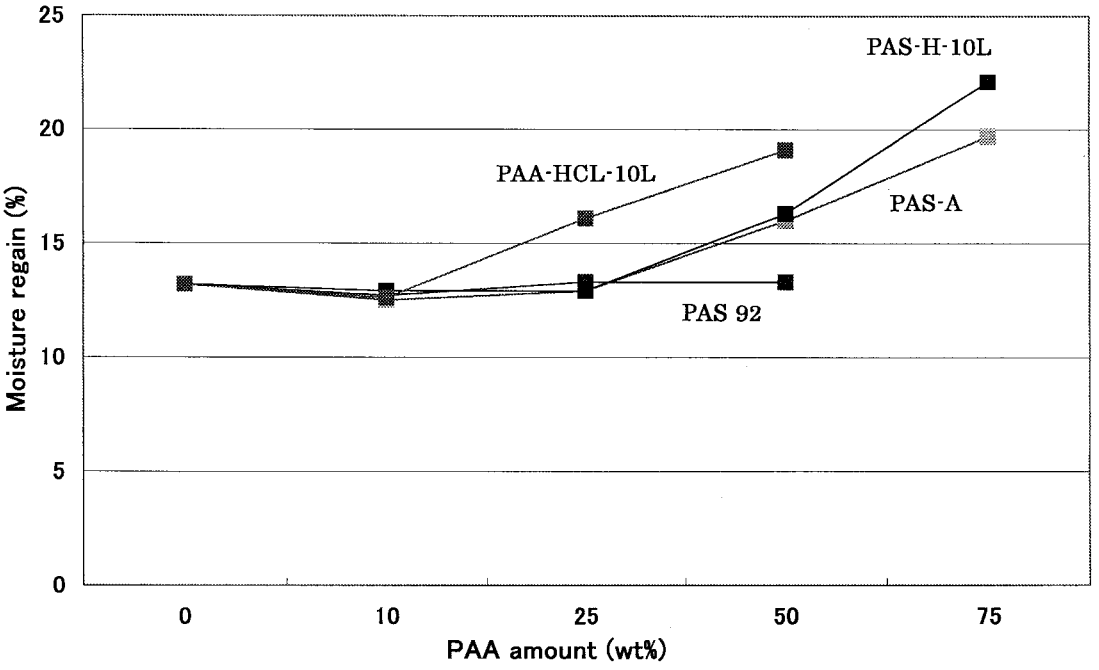

Figure 1 The moisture regain of composite blend films made of silk fibroin and polyallylamines containing various compositional ratios as a function of the polyallylamine content (wt %).

physical properties of composite films made with PAA-HCl-10L and SF were chiefly evaluated in this study.

FTIR Spectroscopy

The conformation of the composite films made of SF and PAA-HCl-10L after blending was examined by means of IR spectroscopy. The FTIR spectra of the composite films are shown in Figure 2.

The IR spectrum of the SF film shows the characteristic absorption band at 1656 cm^{-1} (amide I) that is attributed to random coil conformation.[10] The SF/PAA blend films show absorption bands at 2975 (not shown in Fig. 2), 1625, 1522, 1418, 1396, 1313, 1175, 1109, 1069, 1002, and 862 cm^{-1}, which are attributed to the pure PAA component of the blends. The SF/PAA blend films also exhibited typical absorption bands at 1648 (amide I) and 1242 cm^{-1} (amide III), which are also attributed to a random coil conformation.[10] The IR spectrum of pure PAA film was eliminated because of its ready availability in previously published technical reports of Nitto Boseki Co. Ltd. The IR spectra of the SF-PAA blend films overlap with the pure PAA and the random coiled conformation of SF. These results indicate that the random coil conformation of SF remains even after blending with PAA. The absorption intensity of the bands contributed by SF is gradually lowered with increasing amounts of PAA.

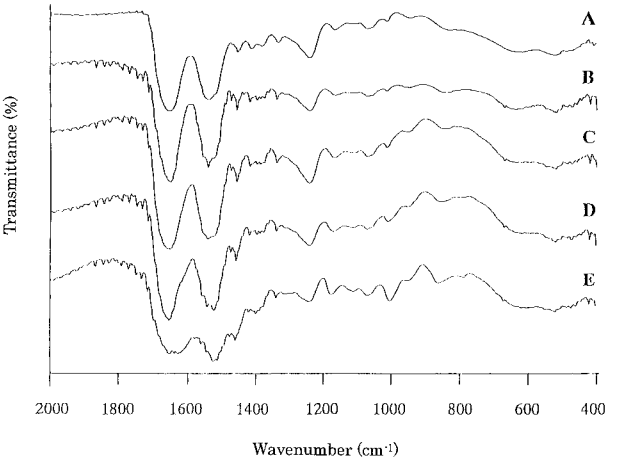

Figure 2 FTIR spectra of the composite films made of silk fibroin and polyallylamines containing various compositional ratios. The compositional ratios of silk fibroin at 100 (spectrum A), 90 (spectrum B), 75 (spectrum C), 50 (spectrum D), and 25 (spectrum E) are shown.

Table III Mechanical Properties of Composite Films

PAA	Strength (gf)		Elongation (%)		Water Content (%)
	D	W	D	W	
SF/PAA-HCl-10L (75 : 25)	76	20	2.1	65	539
SF/PAA-10C (75 : 25)	41	22	0.9	25	686
SF/PAS-92 (75 : 25)	35	—	1.9	—	267
SF/PAS-A-120L (75 : 25)	56	27	11.5	63	494
SF/PAS-H-10L (75 : 25)	38	28	30	68	391
SF	47	37	0.6	19	179

SF, Silk fibroin without PAA; SF/PAA-10C (75 : 25), a composite film made of silk fibroin and PAA-10C with a ratio of 75 : 25 (% w/w); D, W, the tensile properties in the dry state (25°C, 65% RH) and in water.

Mechanical Properties

The tensile properties of the composite films containing SF and various PAA polymer types were also measured (Table III). Composite films were obtained by dry casting a binary system consisting of 6 mL of 3% SF solution and 4 mL of 6% PAA polymer solution such that the SF and PAA composite films were at a 75:25 (w/w) ratio in composition.

Regardless of the polymer composition, the tensile strength of the composite films in the dry state does not show large variations, and the elongation at break of the composite films was increased significantly by polymer blending. Additionally, the elongation at break was considerably improved when the materials were allowed to stand in water and the tensile strength was lowered with swelling. From Table III the combination of SF and SF/PAA-HCl-10L seems advantageous for increasing the tensile strength in the dry state. The tensile strength and elongation at break of a pure SF film are 2.0 kg/cm^2 and 0.6%, respectively, suggesting that SF is very brittle. However, after the addition of PAA into the SF, the mechanical properties of the blend were much improved as evidenced by a significant increase in both the tensile strength and elongation at break.

DSC Measurements

The thermal behavior of the composite film was investigated utilizing DSC (Fig. 3). The SF film (Fig. 3, spectrum A) showed a prominent endotherm at 280°C, which is attributed to thermal decomposition of unoriented SF chains.[12] The endothermic shift at about 180°C and the exotherm at 228°C correspond to the glass transition[11] and crystallization of amorphous SF chains, respectively.[12] The existence of a random coil conformation from SF was also confirmed by DSC as previously suggested from FTIR measurements of the same specimen (Fig. 2, spectrum A). The composite film (SF/PAA = 25/75, Fig. 2, spectrum E) showed a major endothermic peak at about 351°C, which is attributed to the melting of PAA. The composite films (Fig. 2, spectra B, C) showed

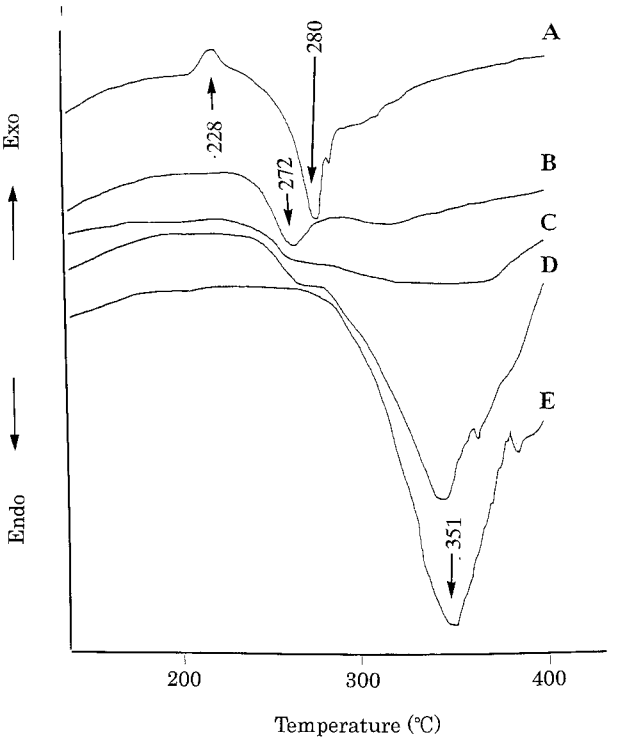

Figure 3 DSC curves of the composite films made of silk fibroin and polyallylamines containing various compositional ratios. The compositional ratios of fibroin at 100 (spectrum A), 90 (spectrum B), 75 (spectrum C), 50 (spectrum D), and 25 (spectrum E) are shown.

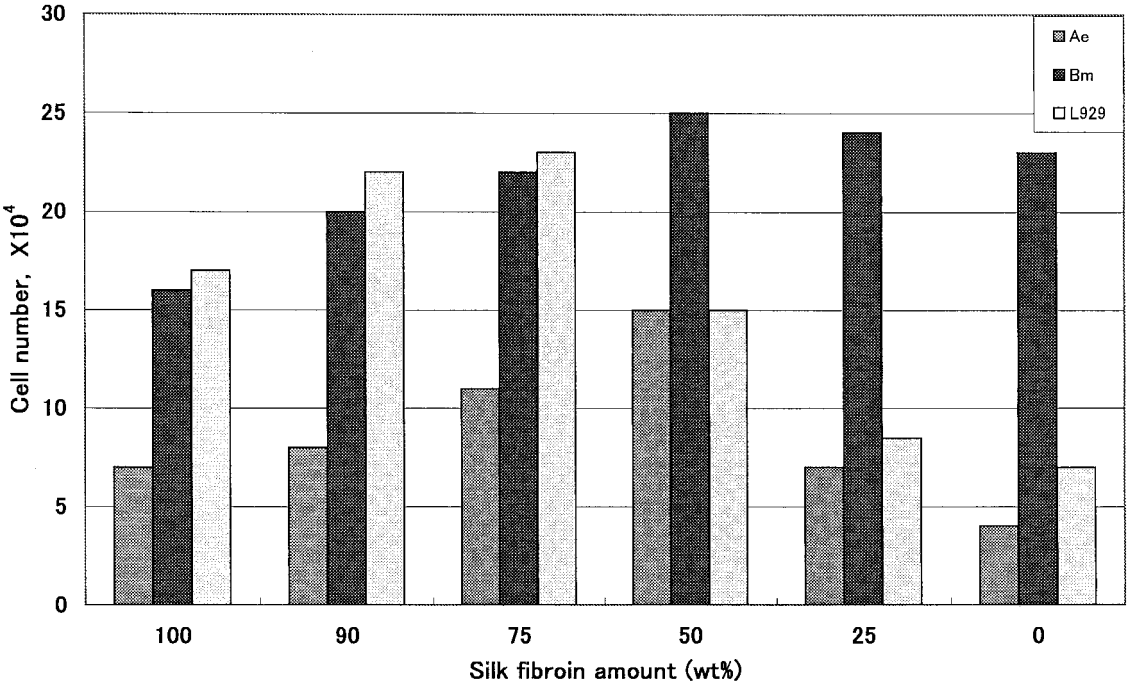

Figure 4 The numbers of *Antheraea pernyi* cells, *Bombyx mori* cells, and L-929 fibroblast cells proliferated by use of tissue culture flasks, whose walls are coated with the binary system containing silk fibroin and polyallylamines. The 75 wt % silk fibroin content refers to 75 wt % silk fibroin and 25 wt % polyallylamine. The initial number of cells in the cultivation medium is 2×10^4/mL.

two endothermic peaks corresponding to the thermal degradation of SF and PAA. In addition to the endotherm at 351°C, the blended film (Fig. 2, spectrum E) showed broad endothermic peaks from about 275°C. Although the DSC curve of pure PAA films is not shown in Figure 3, PAA-HCL-10L shows a prominent single endothermic peak on the DSC curve in the temperature region of 266–360°C. Its DSC curve is shifted toward the endothermic region and exhibited a prominent endothermic peak at around 348°C, which was probably due to thermal decomposition in the polymer. The endothermic peak, which was attributed to the thermal degradation of PAA, shifted to a lower position with increasing SF content. These DSC data suggest that a mutual interaction, possibly due to hydrogen bonding, occurs between the SF and PAA.

Living Cell Growth

The cell growth of *Ae*, *Bm*, and L-929 fibroblast cells was evaluated after studying a number of living cells, which were permitted to grow in the cultivation process described (Fig. 4). Because we are mainly concerned with the effect of the composite materials on cell growth and proliferation rather than cell morphology, we focused only on the increased numbers of living cells in the cultivation process rather than their visual observation.

The composite SF/PAA-HCL-10L film was coated on the wall of the tissue culture flask. Among the living cells examined, the *Bm* and L929 cells proliferated considerably as compared to the *Ae* cells. It was determined that the number of cells grew most effectively when the flask was coated with composite films in ratios of 75:25 and 50:50. It was also shown that the cells multiply freely on the substrate with high mobility. However, the above results suggest cell growth and proliferation occurs best on the binary composite film rather than on pure SF or pure PAA-HCl-10L alone.

CONCLUSION

Composite films can be obtained by casting a binary system made of SF and PAA solutions that

display good compatibility and state of mixing on polyethylene film. Among the various PAAs examined, the use of PAA-HCl-10L presents an advantage in that a transparent composite film can be obtained from the binary system without adjusting the PAA concentration.

According to FTIR it seems that the molecular interactions between SF and PAA are very weak or even absent in the blend films, and new absorption bands do not appear after blending the two components of SF and PAA. Analysis of the FTIR data suggests that the component bands overlap without forming any new chemical interaction. However, the slight change of thermal movement evaluated after DSC indicates the development of a weak interaction between SF and PAA in the composite film, as might be expected from hydrogen bond formation, but not of chemical coordination/covalent bonding.

The most interesting aspect of the present article is that of *Ae*, *Bm*, and L-929 fibroblast cell attachment to the composite films. Cell growth is excellent when compared to cell growth and attachment to pure SF or PAA films. It appears that the cells grow well on a substrate less hydrophilic in nature than pure PAA-HCl-10C, which is strongly hydrophilic. Nevertheless, the cationic properties, water content, hydrophobicity, and hydrophilicity are all major factors that determine the cell growth rate and proliferation in a culture medium; a more precise analysis should be conducted to include all of these aspects.

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