Immobilization of Soluble Eggshell Membrane Protein on Polyethylene Film Surface: Effect on the Culture of NIH3T3 *in Vitro*

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ABSTRACT: Polyethylene (PE) film surface is modified by combining plasma treatment and soluble eggshell membrane protein (SEP) immobilization. Contact angle measurements, attenuated total reflectance infrared spectroscopy, and X-ray photoelectron spectroscopy confirm that SEP adheres tightly to the PE film surface. Mouse 3T3 fibroblasts are used as model cells to evaluate the biocompatibility of PE film surfaces before and after modification. Plasma pretreatment can incorporate polar groups onto the surface, benefitting tight immobilization of SEP. The hydrophilicity of the PE film surface modified by combining plasma treatment and SEP immobilization is increased as evidenced by contact angle measurements, and the biocompatibility of the surface is greatly improved as shown by cell culture. The surface of the modified material can endure rinsing with 10% acetic acid (a good solvent of SEP), which would be an advantage for further application as a biomaterial. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 99: 1340–1345, 2006

Key words: biomaterial; biocompatibility; plasma modification; polyethylene (PE); soluble eggshell membrane protein

INTRODUCTION

Hen eggshell membrane (ESM) consists mainly of proteins such as collagen (types I, V, and X) and sialoprotein and plays a key role in the formation of eggs and the development of chick embryos.[1,2] Stromal cells can adhere and proliferate well on ESM, proving that ESM has good biocompatibility.[3] Maeda and Sasaaki have used ESM as biological dressing materials for burns and obtained satisfactory results compared with other kinds of biological dressing materials such as human amniotic membrane.[4] ESM has good attachment to the wound and the burn area epithelializes well.[4] Although raw ESM is inexpensive and can be readily obtained as an industry waste product, it also has some drawbacks. Dry ESM is brittle, and its surface is not flat (usually curved). Synthetic polymer, such as polyethylene (PE), usually has good toughness and can easily be processed into the desired shape. Therefore, we want to immobilize ESM on the film surface of a synthetic polymer to overcome the drawbacks of natural ESM. Being the cheapest synthetic polymer with good toughness, PE was chosen as the support film in this work.

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Natural ESM is known to be insoluble in almost any solvent due to the presence of a large number of crosslinks of disulfide bonds. We recently reported a preparation method of soluble eggshell membrane protein (SEP), in which raw ESM was treated with aqueous β-mercaptopropionic acid in the presence of acetic acid followed by neutralization with aqueous NaOH.[5] The biocompatibility of SEP, as demonstrated by cell culture of NIH3T3, is comparable to collagen type I and superior to raw ESM.[6] The availability of SEP makes immobilization possible.

Many biological macromolecules have been immobilized on polymeric substrates to improve the biocompatibility of the surfaces. Those immobilized by simple coating methods are easily removed when exposed to the environment. Plasma treatment is a convenient and powerful method for modifying polymeric materials without altering their bulk properties and can easily introduce functional groups (-COOH, -CHO, -OH, etc.) onto the polymeric surfaces and, consequently, provide the anchorage sites and facilitate the immobilization of biological macromolecules.[7,8] Therefore, plasma pretreatment is often used for obtaining tight immobilization.

In this work, the biocompatible SEP, a novel natural biocompatible material, is immobilized on a PE film surface by combining plasma treatment with SEP immobilization. The modified PE film is characterized by means of contact angle measurements, attenuated to-

tal reflectance infrared spectroscopy (ATR-FTIR), and X-ray photoelectron spectroscopy (XPS). The biocompatibility of PE film surfaces before and after SEP anchorage is evaluated and compared by NIH3T3 fibroblast culture.

EXPERIMENTAL

Material

SEP was prepared by dissolving raw ESM powder in aqueous 3-mercaptopropionic acid and acetic acid followed by neutralizing to pH 5 according to our previous report.[5,6] Polyethylene film of 0.1 mm thickness was used in this study.

Surface modification

Air plasma treatment was carried out on a plasma instrument (13.56 MHz). PE film was placed in the plasma chamber. After the pressure of the chamber had stabilized to 15 Pa, a glow-discharged plasma (30 W) was created by controlling the electrical power at a radiofrequency of 13.56 MHz for 3 min.

The plasma-treated sample was immersed in 1% SEP dissolved in 10% acetic acid for 2 h and then dried in a desiccator over silica gel for 24 h. The sample was rinsed in 10% acetic acid, which is a good solvent of SEP, for 3 h under vigorous stirring to remove any weakly adsorbed SEP, followed by drying in a desiccator over silica gel.

Contact angle measurements

The contact angles were measured using the JY-82 type contact angle meter. Four independent determinations at different sites were averaged. Deionized water and ethylene glycol were used for the measurements. The surface free energy was calculated according to the Harmonic mean equations and expressed as[3]

$$(1 + \cos\theta_1)\gamma_1 = 4\left(\frac{\gamma_1^d\gamma_s^d}{\gamma_1^d + \gamma_s^d} + \frac{\gamma_1^p\gamma_s^p}{\gamma_1^p + \gamma_s^p}\right) \quad (1)$$

$$(1 + \cos\theta_2)\gamma_2 = 4\left(\frac{\gamma_2^d\gamma_s^d}{\gamma_2^d + \gamma_s^d} + \frac{\gamma_2^p\gamma_s^p}{\gamma_2^p + \gamma_s^p}\right), \quad (2)$$

where γ_d is the dispersive component; γ_p is the polar component; θ_1 is the contact angle to water, and θ_2 is the contact angle to ethylene glycol. For water, $\gamma_1 = 72.8$ mJ/m^2, $\gamma_1^d = 22.1$ mJ/m^2, and $\gamma_1^p = 51.0$ mJ/m^2. For ethylene glycol, $\gamma_2 = 48.2$ mJ/m^2, $\gamma_2^d = 31.5$ mJ/m^2, and $\gamma_2^p = 16.7$ mJ/m^2.[9]

ATR-FTIR

ATR-FTIR measurements were performed on a Fourier transform infrared spectrometer from a Nicolet 560, coupled with ATR accessory (split-pea). One hundred scans were performed with a resolution of 4 cm^{-1}.

XPS

XPS spectra of the modified samples and the control were acquired on a XSAM800 (UK, Kratos) spectrometer using AlK_α radiation at a power of 120 W. A take-off angle of 90° with respect to the sample surface was used. All measurements were carried out under vacuum (1.3×10^{-6} Pa). The high-resolution spectra C_{1s}, N_{1s}, and S_{2p} were deconvoluted and curve-fit to analyze the chemical bonding state.

Cell culture

NIH3T3 cells were cultured in Dulbecco's minimum essential medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Gibco) in a water-saturated atmosphere of 5% CO_2 at 37 °C. The medium was changed every 2 days. The cell monolayer was washed twice with PBS and incubated with trypsin–EDTA solution (0.25% trypsin, 1 m*M* EDTA; Gibco) for 3 min at 37 °C to detach the cells. The effect of trypsin was then inhibited by adding the complete medium at room temperature and the cells were reseeded and grown in new culture flasks.

NIH3T3 cells were seeded onto virgin, plasma-treated, and SEP-modified PE film surfaces at a density of approx. 10,000 cells/cm^2 in 24-well plates. The cells were allowed to attach to the films undisturbed in a humidified incubator (37 °C and 5% CO_2) for 3 days. The photomicrographs of NIH3T3 cells were obtained with a phase contrast microscope after a definite interval.

A 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay was used to examine the cell viability. MTT reagent is a pale-yellow substrate that produces a dark-blue formazan product when incubated with viable cells. Therefore, the level of the reduction of MTT to form formazan can reflect the level of cell metabolism. After each period of culturing time in 24-well plates, MTT solution (100 μL, Sigma) was added to each well and incubated for 4 h at 37 °C. At the end of the assay, dimethyl sulfoxide (500 μL) was added to dissolve the formazan crystals and cells were transferred to a 96-well plate. The optical density of the formazan solution was measured on an ELISA plate reader (Bio-tek) at 570 nm.[10] Data from six sets of these experiments were averaged.

TABLE I
Effect of Plasma Treatment on Surface Hydrophilicity and Surface Energy

Sample	Contact angle		Surface energy[a] (mJ/m^2)			
	θ (H_2O)	θ (Glycol)	γ_s	γ_s^d	γ_s^p	χ^p
PE	101 ± 2.0	77 ± 1.2	21.30	14.57	6.73	0.316
Plasma-treated PE	85 ± 1.6	61 ± 1.5	29.07	15.64	13.43	0.462
SEP-modified PE	70 ± 2.1	35 ± 1.3	40.79	23.12	17.67	0.433

[a] γ_s, surface energy; γ_s^d, dispersive component; γ_s^p, polar component; $\chi^p = \gamma_s^p/\gamma_s$.

RESULTS AND DISCUSSION

Surface hydrophilicity and surface free energy

Surface contact angles and free energy of virgin PE, plasma-treated PE, and PE modified by a combination of plasma treatment with SEP immobilization (referred to as SEP-modified PE) are shown in Table I. After air plasma treatment, the water contact angle of the PE film surface obviously decreases from 101 to 85°, and both the surface energy and the contribution of polar components increase, indicating an enhancement of surface hydrophilicity. Polar groups (such as -OH, -CHO, and -COOH, etc.) can be incorporated into the surface of the PE film by air plasma treatment, thus resulting in an improvement of surface hydropilicity. However, the hydrophilicity of the surface would decline with preserving time owing to the surface mobility.[11] In the present research, SEP immobilization is used in combination with air plasma treatment to give a stable surface and also to introduce biocompatible macromolecules to the surface. After SEP immobilization, the contact angle to water is 70°, which is less than that of virgin PE film, and the surface energy is higher than that of virgin PE film. Therefore, combining air plasma treatment with SEP immobilization can improve the hydrophilicity and surface energy of PE film.

Analysis of ATR-FTIR

ATR-FTIR spectra of SEP-modified PE (dipping for 2 h and washing for 3 h), plasma-treated PE, and virgin PE film surfaces are shown in Figure 1. Compared to those of virgin and plasma-treated PE, the spectrum of the SEP-modified sample shows additional peaks in the region of 1500–1700 cm^{-1}, which can be assigned to the characteristic peaks of the amide group, indicating the formation of a thin layer of biocompatible macromolecules (SEP) on the surface of the PE film. The strong adhesion of SEP may be attributed to hydrogen bonding and polar interaction between SEP, which has amino and carboxylic groups, and plasma-treated PE film, which has polar functional groups (such as –COOH, -CHO, -OH, etc.) introduced by

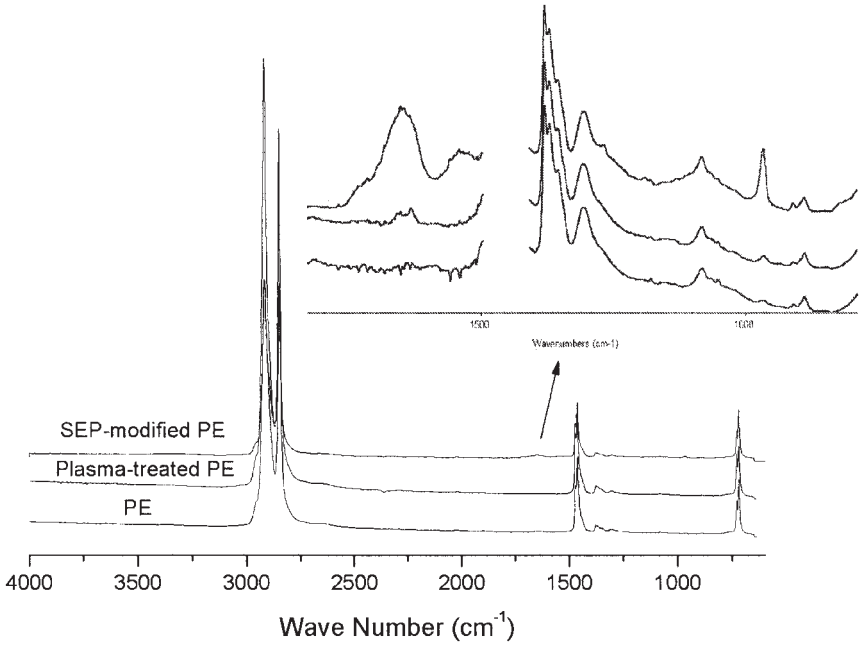

Figure 1 ATR-FTIR spectra of SEP-modified (after washing for 3 h), plasma-treated, and virgin PE film surfaces.

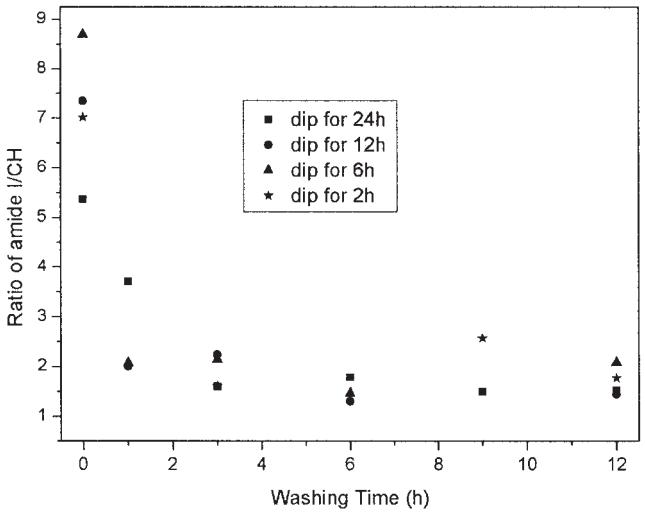

Figure 2 IR peak ratio (amide I/CH) as a function of washing time (effects of dipping times and washing times on SEP immobilization amount).

plasma treatment. Even covalent bonding between amino groups of SEP and the aldehydes of plasma-treated PE through formation of imino bonds is likely to give some contribution for the strong adhesion of SEP on plasma-treated PE.

To investigate the immobilized amount and stability of SEP anchored on the PE film surface, the amounts of SEP immobilized on the PE film surface under different dipping and washing times (washed by 10% acetic acid) were estimated by ATR-FTIR. The characteristic peaks of the virgin PE at 690–750 cm^{-1} (CH) were used as the reference. The relative ratio of the peak area of SEP at 1589–1735 cm^{-1} (amide I) to that of the reference peak is used to express the relative amount of SEP. As shown in Figure 2, washing with 10% acetic acid (a good solvent of SEP) can obviously reduce the immobilized amount of SEP. The immobilized amount of SEP becomes unchanged after washing for 3 h, indicating that a washing time of 3 h is sufficient to remove any weakly adsorbed SEP. The dipping time has no obvious influence on the tightly immobilized amount of SEP, suggesting that a dipping time of 2 h is sufficient to get maximum immobilized amount of SEP. Therefore, in the latter experiments, a dipping time of 2 h and a washing time of 3 h were used for all the samples.

XPS analysis

Figure 3 shows XPS spectra of virgin PE, plasma-treated PE, SEP, and SEP-modified PE film surfaces. Compared to virgin PE and plasma-treated PE, signals for N and S appear at 400 and 164 eV, respectively, for SEP-modified PE, which are also present in the spectrum of SEP and can be assigned to nitrogen-containing groups and sulfur-containing groups, respectively, indicating the presence of SEP on the surface of the PE film. The deconvoluted C_{1s} spectra provide more information. The virgin PE has only one single C_{1s} peak at 284.6 eV assigned to -CH. Both plasma-treated PE and SEP have three C_{1s} peaks at 284.6, 286.4, and 288.3 eV, but their relative peak areas are notably different. For plasma-treated PE, the peaks at 286.4 (-C-O-) and 288.3 eV (-C = O) have almost equal areas. As to SEP, the peak at 286.4 eV has much larger area than that at 288.3 eV, attributed to the presence of -C-O-, -C-N-, and -C-S-. The SEP-modified PE also has the above-mentioned three C_{1s} peaks; the peak at 286.4 eV has larger area than that at 288.3 eV, proving the success of SEP immobilization on the PE surface.

Table II lists the atomic concentration of pure SEP, SEP-modified PE, plasma-treated PE, and virgin PE film surfaces. The surface components of virgin PE film are C and O. After plasma treatment, the surface carbon concentration decreases from 98.4 to 87.5%, and that of oxygen increases accordingly. After immobilization of SEP, the surface carbon concentration further decreases to 79.2%; nitrogen and sulfur appear with contents up to 7.5 and 2.0%, respectively, indicating the formation of a thin layer of SEP on the PE film.

Effect of modification on biocompatibility

To examine the biocompatibility of modified PE film, NIH3T3 cells were cultured on virgin PE, plasma-treated PE, and SEP-modified PE film surfaces, the last having been thoroughly washed with 10% acetic acid to remove any weakly adsorbed SEP. The morphology of NIH3T3 fibroblasts cultured on the control and modified samples was observed by inverted phase contrast microscope. Figure 4 shows the photomicrographs of the cells attached to the surfaces of the three substrates, which were cultured for 24 h. The cells stretched very well on the SEP-modified PE film and their filopodia can be seen, and their shape is spindle-like, which takes advantage of the good metabolizability and functionality of the cells. However, based on a counting exercise, all cells on the virgin PE and about 70% of the cells on plasma-treated PE film surfaces were round and not well stretched. This suggested that the SEP-modified PE film surface is most beneficial for the attachment and proliferation of NIH3T3 fibroblasts.

The cell viabilities on the three substrates were measured at day 3. As shown in Figure 5, the cell viability on the SEP-modified PE film surface is obviously greater than that on the virgin PE and plasma-treated PE film ($P < 0.01$). Since the initial seeding density of the cells was the same on the three substrates, the difference in cell viability indicates different cell affinities; the SEP-modified PE film surface has improved

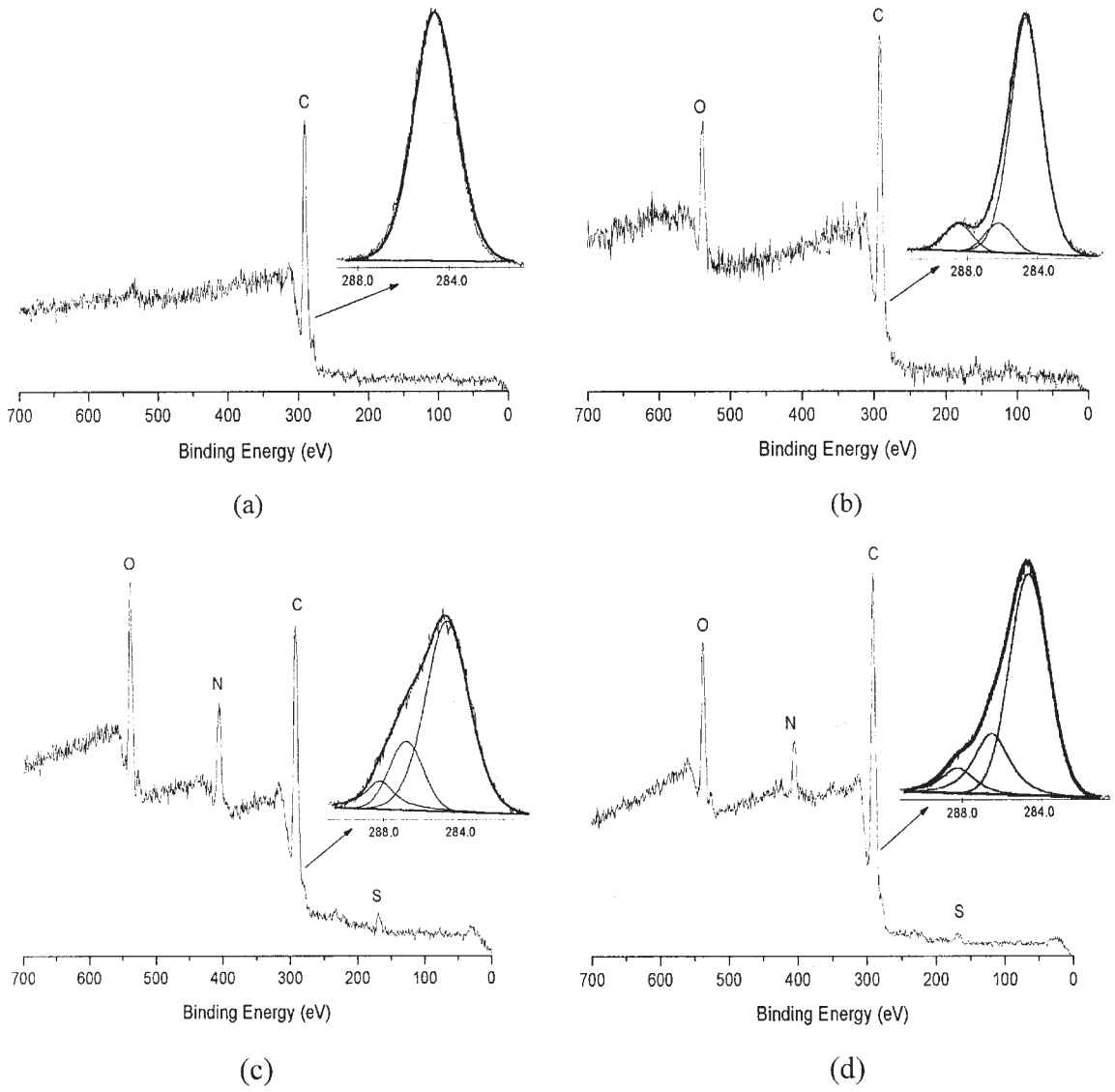

Figure 3 XPS spectra and deconvoluted C_{1s} curves of (a) virgin PE, (b) plasma-treated PE, (c) SEP, and (d) SEP-modified PE film surfaces.

cell affinity due to SEP anchorage. Generally speaking, higher hydrophilicity and higher free energy of the surfaces favor the attachment and proliferation of cells.[12] Although plasma-treated PE has higher hydrophilicity and higher free energy compared to virgin PE, the cell affinity is practically not improved. Only after SEP immobilization is the cell affinity greatly improved, indicating the importance of SEP immobilization. The enhancement of cell affinity by SEP immobilization may mainly be attributed to the good cell affinity of SEP itself. Therefore, it can be concluded

TABLE II
Elemental Composition of the PE Film Modified with SEP

Sample	C (%)	N (%)	O (%)	S (%)
PE	98.4	0	1.5	0
Plasma-treated PE	87.5	0	12.4	0
SEP	67.8	14.4	14.1	3.5
SEP-modified PE	79.2	7.5	11.3	2.0

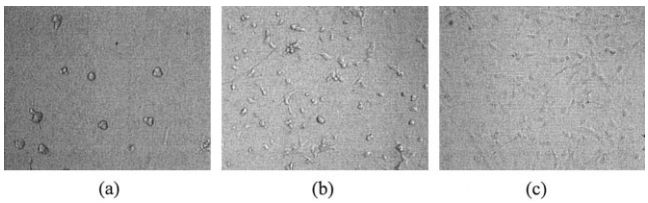

Figure 4 Photomicrographs (×200) of NIH3T3 fibroblasts cultured on (a) virgin PE, (b) plasma-treated PE, and (c) SEP-modified PE films for 24 h.

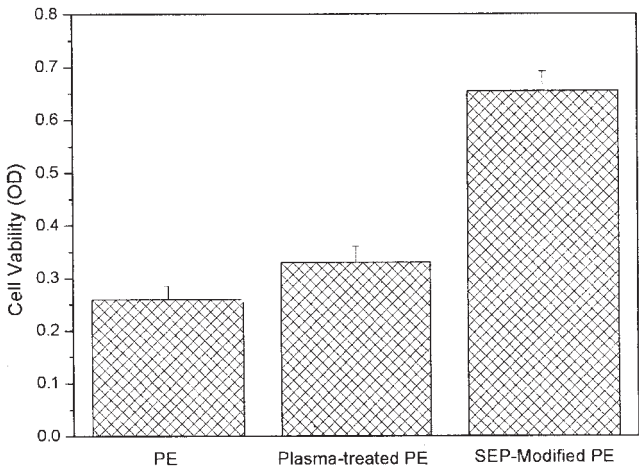

Figure 5 MTT assay; formazan absorbance is expressed as a measure of cell viability from NIH3T3 seeded onto virgin PE, plasma-treated PE, and SEP-modified PE films for 3 days.

that the PE film modified by combining plasma treatment with SEP anchorage has good biocompatibility.

CONCLUSIONS

In the present study, SEP is immobilized on a PE film surface by combining plasma treatment with SEP immobilization. The appearance of amide groups at 1500–1700 cm^{-1} in ATR-FTIR spectrum and that of N and S signals in XPS spectrum confirm the immobilization of SEP on the PE film surface. Plasma pretreatment facilitates immobilization of SEP, and a certain amount of SEP is retained on the PE film surface even after drastic rinsing with 10% acetic acid, which is a good solvent of SEP. Contact angle measurements have shown that the surface hydrophilicity is improved. The biocompatibility of the film surface is greatly enhanced compared to that of the virgin PE film surface, as demonstrated by cell culture of mouse 3T3 fibroblasts. Therefore, SEP-modified PE film can be potentially used as a biomaterial, for example, as a dressing material for burns.

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