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Silk fibroin immobilization on poly(ethylene terephthalate) films: Comparison of two surface modification methods and their effect on mesenchymal stem cells culture

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ABSTRACT

Silk fibroin (SF) has played a curial role for the surface modification of conventional materials to improve the biocompatibility, and SF modified poly(ethylene terephthalate) (PET) materials have potential applications on tissue engineering such as artificial ligament, artificial vessel, artificial heart valve sewing cuffs dacron and surgical mesh engineering. In this work, SF was immobilized onto PET film via two different methods: 1) plasma pretreatment followed by SF dip coating (PET-SF) and 2) plasma-induce acrylic acid graft polymerization and subsequent covalent immobilization of SF on PET film (PET-PAA-SF). It could be found that plasma treatment provided higher surface roughness which was suitable for further SF dip coating, while grafted poly(acrylic acid) (PAA) promised the covalent bonding between SF and PAA. ATR-FTIR adsorption band at 3284 cm^{-1}, 1623 cm^{-1} and 1520 cm^{-1} suggested the successful introduction of SF onto PET surface, while the amount of immobilized SF of PET-SF was higher than PET-PAA-SF according to XPS investigation (0.29 vs 0.23 for N/C ratio). Surface modified PET film was used as substrate for mesenchymal stem cells (MSCs) culture, the cells on PET-SF surface exhibited optimum density compared to PET-PAA-SF according to CCK-8 assays, which indicated that plasma pretreatment followed by SF dip coating was a simple and effective way to prepare biocompatible PET surface.

1. Introduction

Poly(ethylene terephthalate) (PET) is a commercial material for industrial and domestic applications because of its desirable bulk properties, such as excellent mechanical strength, good stability against body fluids, and high radiation resistance for sterilization [1,2]. PET is generally bio-inert, it has to be modified by biocompatible matter to meet the requirements of biological and medicine applications, especially as substrate for cell culture. Therefore, biomolecules immobilization onto PET surface has been widely employed to provide the material surface biocompatibility and functionality while maintaining the substrate bulk properties [3–5]. For this purpose, PET surface needs to be activated, and a number of methods such as UV irradiation [6,7], chemical hydrolysis [8,9], enzymatic hydrolysis [10–12], plasma treatment [13,14], and plasma-induce graft copolymerization [3,5,15,16], etc. have been applied. Among these methods, plasma treatment, which can be carried out in the presence of specific gases such as oxygen, argon, nitrogen or hydrogen is an extremely attractive way to modify the surface chemistry and morphology of polymeric material. Meanwhile, in the process of plasma-induced graft polymerization, a desired monomer may be graft-polymerized onto a plasma-activated polymer surface, the grafted surface may then provide active sites for the binding of biomacromolecules [3,5,16]. Plasma-induced graft copolymerization provides the covalent linkages between biomacromolecule and grafted brush layer on top of material surface, except that the process is complicated and time-consuming. In contrast, as plasma treatment results in roughness, hydrophilicity and the generation of active species on polymer surface, plasma treatment followed by biomacromolecules immobilization may be a simple way for the surface modification of a material.

On the other side, silk fibroin (SF), a protein produced from silkworms, has attracted considerable attention and has been utilized in biotechnological and biomedical applications recently. SF is a natural material with remarkable biocompatibility [17] and biodegradability [18], good oxygen and water vapor permeability [19,20], relatively low inflammatory response [21] while it is cheap in terms of commercial availability. Comprehensive studies on SF applications in biological and biomedical fields have emerged such as enzyme immobilization [22], drug carrier and delivery [23,24], scaffolds for tissue engineering including infarcted cardiac tissues [25], wound-dressing [26,27], ligament [28,29], bone tissues [30,31], etc. In these studies, SF matrices have been employed to culture various cells like fibroblasts, chondrocytes and osteoblasts, while in ligament and bone tissues engineering, SF matrices have been shown to be non-immunogenic, biocompatible and capable of supporting the attachment, spreading, growth and differentiation of mesenchymal stem cells (MSCs) as well as eliciting a negligible response [28,29,32].

In the present work, PET film surface was modified by SF immobilization for its potential application in ligament tissue engineering. Two

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different methods were used for comparison: 1) plasma pretreatment followed by SF dip coating (PET–SF), and 2) plasma-induced acrylic acid graft polymerization and subsequently covalent immobilization of SF on PET films (PET–PAA–SF). Various techniques such as ATR-FTIR, XPS, AFM and water contact angle (WCA) were performed to characterize the modified surfaces, the capabilities of MSCs attachment and growth onto these surfaces were studied as well to evaluate the biocompatibility of SF modified PET films.

2. Materials and methods

2.1. Materials

PET films of about 100 μm in thickness were purchased from Redsun Inc. of Shanghai, China. The samples were cut into 15×30 mm^2 pieces and ultrasonically cleaned with acetone and ethanol for 20 min, respectively. The films were then dried under vacuum at ambient temperature. Acrylic acid obtained from Dahe Chemical Co. of Shanghai, China, was purified by distillation under vacuum. All other chemicals (AR) were used as received without further purification.

2.2. Plasma treatment

A quartz cylindrical-type glow discharge cell was used for the plasma treatment. The plasma power applied was kept at 100 W. PET films were placed between the two parallel plate electrodes and subjected to the glow discharge for 120 s at an argon flow rate of 160 mL/min [5,15,16,33]. The Ar-plasma pretreated PET films were divided into two groups, one for subsequent dip coating of SF, the other was used for the following acrylic acid graft polymerization.

2.3. Acrylic acid graft polymerization

Ar-plasma pretreated PET films were exposed to the air for about 30 min to promote the formation of surface peroxides and hydroperoxides [5,33], then were immersed in the reaction bottle filled with a 20% (v/v) acrylic acid aqueous solution for reaction. The acrylic acid graft polymerization was carried out at 80 °C under nitrogen protection with magnetic stirring for 4 h. Samples were then washed thoroughly with deionized water in an ultrasonic cleaner for 10 min and rinsed for three times at ambient temperature to remove the monomer and possible homopolymer on the surface. The films referred to as the PET-PAA were then dried under vacuum at ambient temperature for 24 h and stored in a desiccator or used for the following SF immobilization directly [15,16,33]. Grafted carboxylic acid groups on the PET film surfaces were determined using the colorimetric method with Toluidine Blue O (TBO) staining as previously reported, assuming the molar ratio between carboxyl groups and the dye to be unity [5,15,16]. A calibration curve was initially generated from the optical density of TBO solutions of known concentrations at 631 nm measured by a UV-visible spectrophometer (Hitachi U-2910, Japan). An aqueous solution containing 0.5 mmol/L TBO and adjusted to pH 10 with 0.1 mmol/L NaOH was prepared, and the grafted films were placed in this solution for 6 h at 37 °C. The PET films were then rinsed with an excess amount of 0.1 mmol/L NaOH solution to remove the noncomplexed TBO molecules. The complexed TBO on PET films were then desorbed from the surfaces in 50% (v/v) acetic acid solution overnight, and the final dye content was obtained by measuring the optical density of the solution at 631 nm.

2.4. Silk fibroin immobilization

2.4.1. Methods

Silk fibroin was prepared by the degumming and dissolving of *Bombyx mori* silkworm silk according to established procedures [34], during which the silk cocoon was treated with 5% (w/w) Na_2CO_3 boiling solution for 45 min to remove sericin and the degummed silk was then dissolved in 9.3 mol/L LiBr aqueous solution. After being filtered, the SF solution was dialyzed against deionized water for 72 h at room temperature with a 12000–14000 molecular weight cutoff dialysis membrane to remove the salt. The dialyzed solution was then clarified by spinning in a centrifuge at 6000 rpm for about 5 min. The supernatant which was an aqueous regenerated SF solution with concentration of around 4% (w/w) was collected and carefully stored at 4 °C.

Immobilization of SF on the PET films was carried out by dip coating and covalent immobilization [35–37], respectively. For the dip coating, plasma-treated PET film was exposed to the air for about 10 min and then immersed in a 2% (w/w) SF solution (pH 7.5) for 12 h at 4 °C. The film referred to as the PET-SF film was then taken out, rinsed with phosphate buffer of pH 7.5, and dried for 1 h in air and under vacuum overnight. For the covalent immobilization, PET-PAA film was first reacted with 0.03 mol/L of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/N-Hydroxysulfosuccinimide sodium salt (EDC-HCl/Sulfo-NHS) solution in 0.05 mol/L phosphate buffer of pH 4.8 at 4 °C for 2 h to activate the carboxylic acid groups on its surface. The film was then washed with 0.05 mol/L phosphate buffer of pH 7.5, and immersed in a 2% (w/w) SF solution (pH 7.5) for 12 h at 4 °C. The film named PET-PAA-SF was then taken out and rinsed with phosphate buffer of pH 7.5, and dried in the same manner as described above. Both PET-SF and PET-PAA-SF films were treated with 80% (v/v) ethanol solution for 2 h after drying process to enable the conformation transition [38] for the stability of the silk fibroin on PET film surfaces and vacuum-dried again.

2.4.2. Characterization of the surface-modified PET films

All the samples, if not noted further, were used for characterization after stored in a desiccator at ambient temperature for over 20 days.

ATR-FTIR measurement was carried out using a Nicolet Nexus 6700 spectrometer. Each spectrum was recorded with 64 scans and 4.0 cm^{-1} resolution. The spectra of virgin and surface modified PET films were analyzed in the range of 650–4000 cm^{-1}.

XPS experiment was performed on a PHI 5000C ESCA system (Perkin Elmer, USA). The X-ray anode was run at 300 W and the high voltage was kept at 14.0 kV. The samples were detected by using Mg (Ka) radiation (1253.6 eV) operating at a working pressure of about 10^{-6} Pa with a photoelectron take-off angle of 90°. Overlapping peaks were deconvoluted into their individual components by XPSPEAK 4.1 software, the line width (full width at half-maximum, FWHM) of the Gaussian peaks was maintained constant for all components in a particular spectrum [5,39], surface elemental stoichiometries were determined from peak-area ratios. All binding energies (BEs) were referenced to the C 1 s hydrocarbon peak at 284.6 eV.

Virgin and surface-modified PET films (5×5 mm^2) were attached to a magnetic steel disc (serving as sample holder) for AFM measurements. Imaging was recorded on a Nanoscope IV Digital Instruments atomic force microscope (Veeco Metrology Group, USA) in tapping mode. In each case, an area of 5×5 μm^2 was scanned and all images were fitted to a plane using the 1st degrees flatten procedure enclosed in NanoScope software version 6.12r1. An arithmetic mean of the surface roughness (Ra) was calculated from the roughness profile determined by AFM.

Surface wettability of the films before and after modification was characterized by static water contact angle measurement. The image of the droplet on the film was visualized through the image analyzer (OCA40, Dataphysics, German) and the angle between the water droplet and the surface was measured. All of the surface-modified samples were dried overnight under vacuum and stored in a desiccator at ambient temperature for over 20 days before the measurements, except those of fresh plasma treated and fresh-made PET-PAA films. An ultrapure water droplet of 3 μL was placed on the film and the recording was taken 30 s after the application of the