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Improved Morphology and Biocompatibility of Chitosan/PEO Casting Films Adding Hyaluronic Acid

Hyaluronik Asit Ekleyerek Kitosan/PEO Döküm Filmlerinin Morfolojisinin ve Biyouyumluluğunun İyileştirilmesi

Research Article

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ABSTRACT

Biopolymer-based blends can be used within the form of cast film in various proportions with polyethylene oxide (PEO) in order to obtain more durable and biocompatible surfaces. Chitosan and hyaluronic acid (HA) are the most used biocompatible biopolymers for the production of films. On the other hand, as a synthetic polymer, PEO has been mostly used to improve durability of the surfaces by using in various molecular weights. This study was carried out to determine the effect of HA on the chitosan and PEO biopolymer blends different from reported Chitosan/PEO film surfaces in the literature. FTIR studies presented no any polycationic amine groups belong to chitosan through the surface of four films in different compositions after casting. Surface morphology were determined by SEM, EDS and polarized microscopy analyses. Surface morphology was observed as well-oriented spherulitic crystallization by the increasing amount of PEO in the films. Astonishingly, it was proven that HA improved the biocompatibility feature of PEO-rich surfaces which were tested by using healthy mouse fibroblast cell lines, L929 (ATCC CCL-1).

Key Words
Biopolymer blends, chitosan, PEO, hyaluronic acid, film casting.

ÖZ

Biyopolimer esaslı harmanlar, polietilen oksit (PEO) ile daha dayanıklı ve biyouyumlu yüzeyler elde etmek için çeşitli oranlarda dökme film formunda kullanılabilir. Kitosan ve hiyaluronik asit (HA), film üretimi için en çok kullanılan biyouyumlu biyopolimerlerdir. Öte yandan, sentetik bir polimer olarak, PEO çeşitli molekül ağırlığında yüzeylerin dayanıklılığını arttırmak için çoğunlukla kullanılmaktadır. Bu çalışma, literatürde bildirilen Kitosan/PEO film yüzeylerinden farklı olarak, kitosan ve PEO biyopolimer karışımları üzerine, HA'nın etkisini belirlemek amacıyla yapılmıştır. FTIR sonuçları, farklı oranlarda dört filmin, yüzeyi boyunca kitosana ait herhangi bir polikatyonik amin grubunun dökümden sonra sunmamıştır. Yüzey morfolojisi SEM, EDS ve polarize mikroskop analizleri ile belirlenmiştir. Filmlerde artan PEO miktarı ile birlikte iyi yönlendirilmiş küresel kristalleşme yüzey morfolojisi gözlenmiştir. Şaşırtıcı olarak, HA'nın sağlıklı fare fibroblast hücre hattı, L929 (ATCC CCL-1) kullanılarak test edilen PEO bakımından zengin yüzeylerin biyouyumluluk özelliğini geliştirdiği kanıtlanmıştır.

Anahtar Kelimeler
Biyopolimer karışımlar, kitosan, PEO, hyaluronik asit, film döküm.

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INTRODUCTION

Biopolymer blend films have started to be used in many industrial areas by dint of expanding environmental awareness. These bio-based resources are aimed to be alternative to the conventional synthetic polymer blends for the many different industrial products [1,2]. Especially, the sugar-based polymers have many advantages for the replacement of synthetic polymers due to their low cost, non-toxicity, biodegradability and availability [3]. The one of them is cationic chitosan which is generally derived by a full or partial N-deacetylation of chitin which is obtained from exoskeleton of crustaceans as the one of the most abundant biopolymer in the nature [4]. Its copolymer structure composed of glucosamine and N-acetyl-glucosamine [5]. Chitosan has antibacterial, metal binding, mucoadhesive superior properties [6-8]. It is easily soluble in acid environment and can be used in gels, films, nanoparticle, patches, sutures, and fiber applications in various blend forms with other polymers [3,9].

The other valuable biopolymer, hyaluronic acid, of which biological functions have been pointed more and used in blends very recently [10-14]. It structurally consists of linear, N-acetyl-d-glucosamine and glucuronic acid chain [15]. Hyaluronic acid is the one of anionic glycosaminoglycans (GAGs) [10]. It has mostly found within extracellular matrix (ECM) and its astonishing viscoelastic property determines its polyelectrolyte characteristics [11,16,17]. Moreover, it can form polyelectrolyte complexes or multilayer structures with chitosan [17,18]. Hyaluronic acid has many functions in embryogenesis; such as, signal transduction, cell motility, cancer invasiveness, protecting delicate tissues and injured tissue surfaces [19].

Polyethylene oxide (PEO) is a colorless, semicrystalline, water-soluble, low toxic synthetic polymer. It is used with other biopolymers during the production of biological materials such as nano- or micro- films, spins, sprays and capsules [20-23]. It has flexible chain depending on the molecular weights that provide high viscosity and durability to the films [24]. It has flexible chain conformation due to molecular weight and this provides high durability within the films. When they are used with biopolymers, especially chitosan, it can improve mechanical properties of cast film by the aid of chain conformation. Since, PEO has glassy physical characteristics, it can be generally used in making blend with other low durable biopolymers providing more flexibility to PEO. [21,25,26].

In this study, it was investigated that the effect of hyaluronic acid within blends of Chitosan/PEO for four different proportions. As the investigating material, hyaluronic acid was set as 5% vv^{-1} in each blend to form ternary blend. Changes in cast film surfaces were evaluated and its reflection to cell culture studies were carried out by using healthy mouse fibroblast cells, L929 cells and interpreted.

MATERIALS and METHODS

Blend Preparation

Blend composition of polymer films, Chitosan 120K and degree of deacetylation 85%; PEO 20K and Hyaluronic Acid 800K were used. Four different polymer blend mixture (Film 1, Film 2, Film 3, Film 4) were prepared in 2% acetic acid vv^{-1} in dd water according to Chitosan/PEO ratio setting as 90:10%, 80:20%, 70:30%, 60:40%, respectively. Hyaluronic acid was added 5% in each blend composition. Total polymer amount was set 26.25 mg/mL. Prepared films were stirred during 18 h at room temperature. Clear polymer solutions were poured onto 60-mm-diameter polystyrene Petri dishes immediately after degassing. Solvent was slowly evaporated at room temperature. Finally, the blends were kept in a vacuum oven for 4 days at 40°C to remove traces of water and acetic acid. Thickness of films was found to be in the range of 75 ± 5 μm.

FTIR Studies

FT-IR spectra of the blends were recorded on a Shimadzu 8400S FT-IR spectrophotometer. Measurements were conducted between 600-4000 cm^{-1} in transmission mode. FT-IR images were taken by using directly or by embedding the films in KBr pellets.

SEM and EDS Analysis

The prepared blend films were cut as 2 cm^2. Then, they are put on metal grids with double sided adhesive carbon tape, coated with gold to ~500×10^{-8} cm in thickness using sputter coater under high vacuum, 0.1 Torr, 1.2 kV, and 50mA at 27◦C ± 1◦C. The surface morphology of coated samples was evaluated by scanning electron microscopy 20.000X magnification of SEM; and EDS (JEOL JSM Tokyo, Japan) at 20 kV.

Polarized Light Microscopy Analysis

Four different blends were observed under light microscopy with using polarized attachment of Olympus BX53. All images were recorded by 40X magnification at room temperature.

In vitro Cell Culture Studies

Sterilization of all blend films was performed under UV light during 6 hour. The mouse fibroblast cell lines, L929 (ATCC number CCL-1) were obtained from Marmara University Department of Bioengineering and cultured in DMEM supplemented with 10% FCS, 1% Glutamax, 50 U. mL^{-1} penicillin, and 50 mg mL^{-1} streptomycin (Gibco). Casting films were fixed into each well as 5 mm by selfadhesion to bottom. Immediately after, fixed films were subjugated UV light within sterile laminar flow hood approximately 1h. Cultured cells during 3 days in a 5% CO_2 humid atmosphere at 37°C were used for cell culture studies by seeding into each sample in 96-well plates at a density of 2.1×10^4 cells in the per well. After incubated for 72 h, MTT Assay (CellTiter 96 aqueous one solution cell proliferation assay kit from Promega) were tested to measure cell proliferation of film samples. Plates were applied to measured at the optical density at 570 nm of cells at 37°C by using ELISA Reader. Samples were tested in triplicate, and results were showed as the average of the measured values. The absorbance values are proportional to the number of metabolically active cells on the surface of biomaterials. Control cells were considered according to their viability. Statistical analyses were performed by one way ANOVA.

RESULTS and DISCUSSION

FTIR Studies

Four polymer blends were prepared in different compositions of chitosan/PEO/HA and characterized by FT-IR analyses (Figure 1). In IR spectra of four blend films, OH stretchings of chitosan and of HA (3473-3381 cm^{-1}) appeared as mixed broad absorption peaks in 80/20 and 70/30 blend compositions but corresponding OH stretchings of 90/10 and 60/40 blend films were observed as less broader peaks at lower wavenumbers (3452, 3414 cm^{-1}). These broad absorptions and slight shifts can be the evidences for the presence of hydrogen-bonding interactions in corresponding blends. C=O stretching band of pure chitosan at 1660 cm^{-1} shifted slightly to lower wavenumbers (1653-1641 cm^{-1}) in all blends. Similarly, NH bending absorption peak of pure HA (1616-1620 and 1458 cm^{-1}) shifted to lower wavenumbers (1570-1558 cm^{-1}) in all blend compositions. Increases in CH_2 bending band intensities of PEO (1469-1456, 1280, 1242 cm^{-1}) were also observed by increasing amounts of PEO in 80:20, 70:30 and 60:40 blends. However, CH_2 bending band intensity of chitosan (1379,1321 cm^{-1}) was dominant in only 90:10 blend. Besides, C-O-C stretching bands of chitosan appeared at 1095 cm^{-1} as dominant band in 90:10 blend composition, but C-O-C stretching bands of PEO appeared at 1041 cm^{-1} as dominant band in only 60:40 blend composition by increasing amount of PEO. In all blends, C-O-C absorption bands of HA mostly coincided with the C-O-C bands of chitosan and of PEO at around 1095-1022 cm^{-1}.

Our results are in accordance with Zivanovic and coworkers' results [25]. Furthermore, in another study, Eroğlu and coworkers evaluated FT-IR spectra ternary blend film chitosan/PEO/Levan. This study indicated the chitosan and levan suppressed the PEO crystallinity that changed physicochemical properties of blend films [21]. Also, our results confirmed the same tendency of chitosan and hyaluronic acid within Film 4 which move to the top side of the surface.

SEM and EDS Analysis

SEM images were obtained for four different blend films (Figure 2). SEM images clearly indicated that the Film1 has the smoothest surface depending on the increased amount of Chitosan (A). By increasing the ratio of PEO in blend film, surface seemed more indented as indicated in SEM image of Film2 (B). However, the SEM image (C) proved the crystalline surface of Film3 (Ch/PEO: 70/30). Kit and coworkers showed ultra-thin film blends even in low molecular weight chitosan (✥150 kDa)

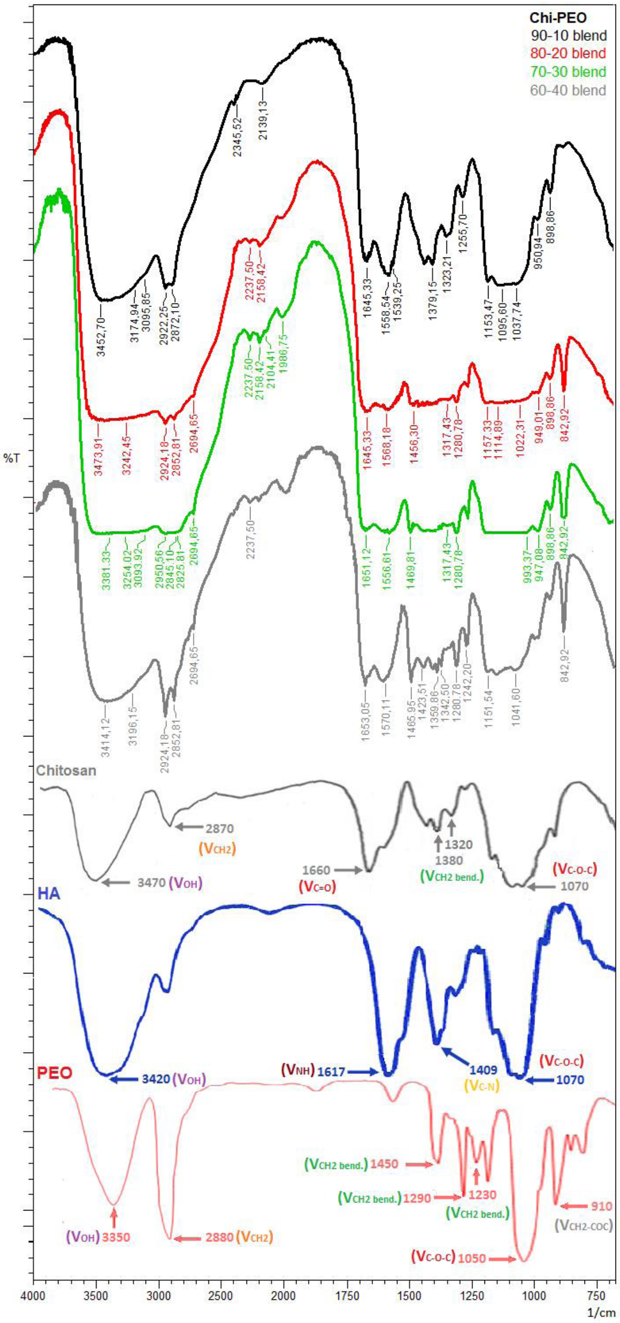

Figure 1. Characterization of FTIR Spectra of Chitosan/PEO/HA Blends (4000-600 cm^{-1}). Chitosan/PEO ratio: Film1 (90/10); Film2 (80/20); Film3 (70/30); Film4 (60/40).

and high molecular weight PEO (900 kDa), 10% chitosan in blend increased surface roughness [27]. In our study, it was achieved the formation of smoother and regular indented surface as regular layers by the composition of Film4, even if the amount of PEO has been increased. This evidence proved that hyaluronic acid provides more homogenous environment in Film4 between chitosan and PEO.

Energy-dispersive X-ray photoelectron spectroscopy (EDS) results of polymer blends were analyzed to ensure the elemental composition of blends. Surface of films were essentially consisted of carbon and oxygen. It wasn't observed any other sharp electron diffraction ring that implies other group on the surface for each film. The results verified the similar amount of components

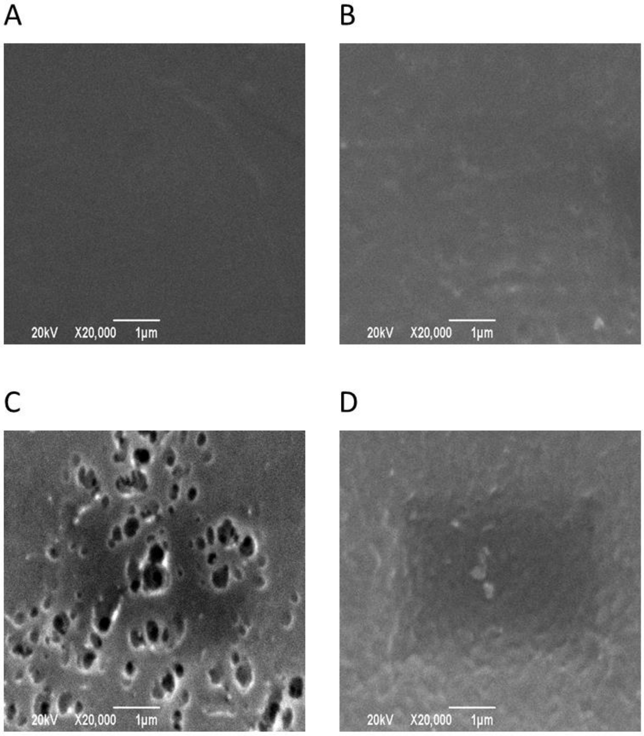

Figure 2. SEM image of (A) Film 1: smooth chitosan surface, (B) Film2: intended chitosan surface, (C) Film3: crystalize surface, (D) Film4: smooth intended miscible chitosan/PEO/HA surface under 20.000X magnification.

on the selected area of blend films (Film1-4). However, Film4 has the highest amount of carbon groups and the least amount of oxygen groups (Table 1). When the amount of PEO increased from Film 1 to Film 4, the number of oxygen groups in blends decreased. On the contrary, the number of carbon groups in blends increased on the surface of films**.** These results supported FT-IR spectra and surface analysis of film blends.

Polarized Microscopy Measurements

Film blends were evaluated by using polarized microscope with a 40X magnification in order to observe crystalline regions, thereof semi-crystalline PEO chains (Figure 3). All the films were transparent and slightly yellowish in color and PEO changed the physicochemical structure of blend mixtures. Film1 had no crystalline region in 90/10 blend ratio due to higher amount of chitosan which suppresses the spherulitic growth of PEO crystals (Figure 3-A). With the small increase in amount of PEO in Film2 (80/20), some of the monoclinic crystals of PEO chains has started to form (Figure 3-B). By increasing the amount of PEO in 70/30 blend ratio, the spherulitic formation of monoclinic PEO crystals has increased further on the surface of Film3 (Figure 3-C). However, the greatest extent of spherulitic PEO crystal formation were obviously seen on the film4 surface (60/40) (Figure 3-D). Besides, the similar investigations with the increased ratio of PEO more than 50% in the films indicated the formation of colorless films and increased amount of chitosan in PEO blend films reduce the PEO spherulitic crystallization of PEO chains [25,27]. Thereby, our findings on PEO crystal formation are coherent with the results of biopolymer blend film studies in the literature.

In vitro Cell Culture Studies

Cell viability tests were performed to investigate the effect of hyaluronic acid on the cell proliferation of mouse fibroblast L929 on the cell surface for 72 hour. The cells in direct interaction with Film1, Film2, Film3 and Film4 were subjugated to MTT assay. Control cells were 100% viable. All films were highly biocompatible even after 72 hour. All the cell viabilities increased (Film1: 473%, Film 2: 1204%, Film 3: 1297% and Film 4: 1603%) by the decreasing amount of PEO and increasing amount of chitosan. However, Film4 has induced the maximum cell proliferation. This evidence

Table 1. EDS results of surface components (Carbon, Oxygen and Hydrogen) of films; the Chitosan/PEO ratio Film1 (90/10); Film2 (80/20); Film3 (70/30); Film4 (60/40).

Surface Component	C wt.%	O wt.%	H wt.%
Film 1	50.76	49.23	0.00
Film 2	52.27	47.73	0.00
Film 3	52.78	47.22	0.00
Film 4	56.77	43.23	0.00

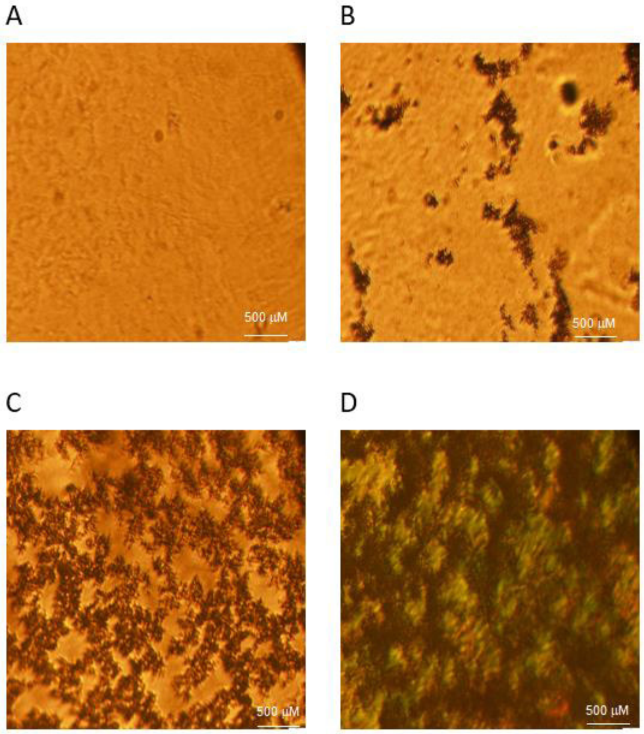

Figure 3. Polarized optical microscope images under 40X of (A) Film1; (B) Film2; (C) Film3; (D) Film4.

showed that the hyaluronic acid maintain miscibility of chitosan and PEO in 60/40 ratio. Film4 displayed good adherent morphology as in the SEM images (Figure 2D) for the attachment and proliferation of L929 cells. In fact, it is the other evidence as Zhao and coworkers indicated that good miscibility is closely related to good biocompatibility. While the amount of chitosan is nearly 50% or less, the miscibility and biocompatibility increase [28].

CONCLUSION

This study proved that hyaluronic acid has changed physicochemical and biological characterization of chitosan/PEO/hyaluronic acid blend films. The well-oriented spherulitic PEO crystallization was clearly observed on the surface of Film4 through the 40X polarized microscopy images. Similarly, Kit and coworkers showed that the spherulitic crystallization was dominant in each film by an increase only in the amount of PEO in Chitosan/PEO blend films (over 80/20) in 100X polarized microscopy studies [25]. SEM and EDS analyses showed that the PEO enrichment provided a reliable direction of radial crystallization on the surface depending on the ratio of chitosan/PEO/hyaluronic acid 57.1/38.1/4.8 (v/v%) in Film4. Cell viability and proliferation studies were performed by using mouse fibroblast L929 cells on four

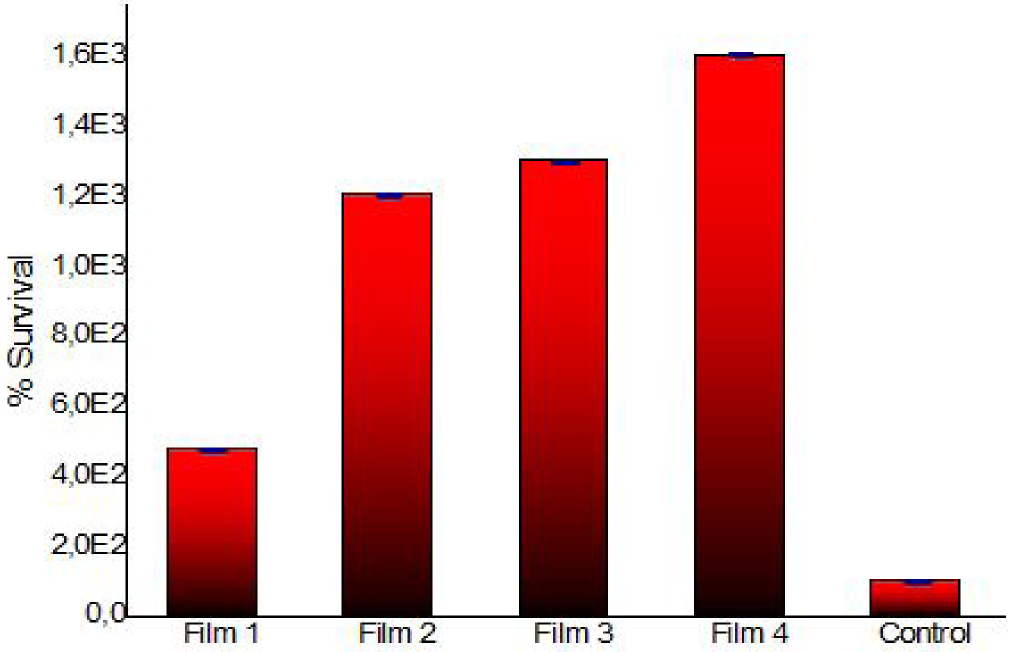

Figure 4. Cell viability of L929 cells after being cultured with the blend films; Film1, Film2, Film3, Film4 after 72 h.

different content of polymer film surfaces. Even after 72hour, no toxicity was observed for each film. L929 cells clearly achieved attachment and proliferation onto all films via performing their normal adherent morphology; in which, all were higher than control group. However, by increased amount of PEO and decreased amount of chitosan from Film 1 to 4, the biocompatibility has increased. On the contrary, in the another study, pure chitosan /PEO films in 70/30 and 60/40 ratios affected the viability of mouse fibroblast cells, L929 (CCL-1) after 24 h, the viabilities were below 50% depending on increasing amount of PEO [21]. It is evident from the results that hyaluronic acid chains provide not only good entanglement between chitosan and PEO; but also, serve as the best three dimensional vicinity with high molecular weight chitosan for cells to adhere for proliferation.

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