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Dual surface activation of thermoplastic polyurethane (TPU) guided membranes

Termoplastik poliüretan (TPU) kılavuz membranlarının ikili yüzey aktivasyonu

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Abstract

Thermoplastic polyurethane (TPU), which consisting of alternant hard- and soft segment is a kind of segmented block copolymer. Amazing elasticity, transparency, and strength at break have expanded the application of TPU in automotive, buildings, coatings, sealants, medicine, and rubber industries. Further, TPUs lack active group, they have high crystallinity, low surface energy, and chemical inertness. Therefore, its properties needed to restore. Recently, plasma or alkali treatment have been suggested to modify the surface properties of nanostructures. Especially, alkali treatment is also versatile one, and creates changes in dimensions, and fine structure without change the surface functional groups. The aim of this study was to evaluate the influence of surface treatment method on newly identified TPU membranes containing of phosphatidylcholine (PC) and polyethylene glycol (PEG). Raw TPU surface was modified by alkali treatment with different percentages of NaOH: 1M and 3 M without heating for a constant soaking time of 30 min. Surface morphology, roughness and wettability properties of treated TPU membranes were investigated. The experimental results showed that the all treated TPU membranes showed surface feature morphology with increasing roughness, i.e. Sa (areal average roughness) values of the TPU-PEG or TPU-PC after the submersion in 1 M solution of NaOH became about $2.51x10^{2}$±15.6 and $2.79x10^{2}$±17.3 nm while that of TPU was $6.24x10^{1}$±6.9 nm. Furthermore, while the contact angle values of TPU-PEG after alkalization reduced from 40.6±0.5° to 21±0.2° and patterned TPU-PC showed significantly superior cell attachment to the MC3T3-E1 cells than the pristine TPU. The study's findings indicate NaOH-treated composite TPU membranes could be a possible guided agent, which supported the bone induction, and differentiation.

Keywords: Thermoplastic polyurethane (TPU), Phosphatidylcholine (PC), Polyethylene glycol (PEG), Guided bone regeneration, Alkali treatment.

Öz

Termoplastik poliüretan (TPU), yumuşak ve sert segmentten oluşan blok kopolimer türüdür. İlgi çekici elastisitesi, transparanlığı ve kopmaya karşı direnci, TPU'nun otomotiv, inşaat, kaplama, dolgu, tıp ve kauçuk endüstrisindeki uygulamalarını genişletmiştir. Ancak TPU aktif gruplara sahip değildir ve bu özellik onu yüksek kristalin, düşük yüzey enerjili ve kimyasal olarak inert yapar. Bu nedenle özelliklerinin iyileştirilmesine ihtiyacı vardır. Son zamanlarda plazma ya da alkali muamelesi nanoyapıların yüzey özelliklerinin modifiye edilmesi için önerilmektedir. Özellikle, alkali müdahalesi çok yönlü yöntemlerden bir tanesidir ve yüzeydeki fonksiyonel grupları değiştirmeden küçük yapılar oluşturur ve boyutlarda değişiklik yaratır. Bu çalışmadaki amaç, fosfatidilkolin (PC) ve polietilenglikol (PEG) içeren yeni tanımlanmış TPU membranlar üzerindeki yüzey modifikasyon metodunun etkisini araştırmaktır. Saf TPU, farklı yüzdelerdeki (1 M ve 3 M) NaOH ile ısıtma olmaksızın 30 dakika süreyle, alkali muamelesi ile modifiye edilmiştir. Muamele edilmiş TPU membranların yüzey morfolojisi, pürüzlülüğü ve ıslanabilirlik özellikleri incelenmiştir. Deney sonuçları, tüm muamele edilmiş TPU membranlarında artan yüzey pürüzlüğüne işaret etmektedir; şöyle ki 1 M NaOH solüsyonuna daldırılan TPU-PEG ya da TPU-PC'nin Sa (yüzey alanı pürüzlülüğü) değeri $2.51x10^{2}$±15.6 ve $2.79x10^{2}$±17.3 nm iken TPU'nun $6.24x10^{1}$±6.9 nm'dir. Ayrıca TPU-PEG'in temas açısı alkalizasyon sonrasında 40.6±0.5'den 21±0.2°'e düşmüştür ve yüzeyi modifiye edilmiş TPU-PC, saf TPU'ya kıyasla üstün bir hücre yapışması göstermiştir. Çalışmanın bulguları, NaOH-muamele edilmiş kompozit TPU membranların kemik indüksiyonunu ve farklılaşmasını destekleyen potansiyel bir kılavuz ajanı olacağını göstermektedir.

Anahtar kelimeler: Termoplastik poliüretan (TPU), Fosfatidilkolin (PC), Polietilen glikol (PEG), Kılavuzlu kemik rejenerasyonu, Alkali muamelesi.

1 Introduction

Nanostructures, which have unique characteristics, present a synthetic tissue mimic for cell adhesion and proliferation in regenerative applications [1]. However, sometimes, the surface properties of these nanostructures can be unsuitable for tissue growth. All these features such as roughness, surface charge, wettability, and porosity can be manipulate strongly in terms of the cell adhesion, proliferation, and even, the formation of a new tissue. The scientist shown that the hydrophilic structures are key contributor to regulate the cell activity and cellular behaviors in applications in biomedical field. Furthermore, as is well known, hydrophobic nanostructures cannot also provide suitable support to the cellular interactions. Therefore, the surface properties of these nanomaterials need to be tuned to better cell adhesion and bioactivity. Plasma and sodium hydroxide (NaOH) hydrolysis have been suggested as surface modification technique because these methods can modify effectively the biocompatibility and hydrophobicity of nanostructures [2],[3]. Relative to many other surface modification methods, NaOH hydrolysis has attracted extensive attention because it is a simple, low-cost, and efficient technique [4]. The results of many studies have shown that NaOH treatment enhanced hydrophilic properties of the

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nanostructures and created new active sites including carbonyl and hydroxyl groups on the nanosurface. Namely, the NaOH hydrolysis leads to a significant change on the contact angle and surface morphology of these nanostructures by increasing the surface energy triggered capillary reaction and thus, the water molecules strongly attract to the nanostructures [5],[6].

Preferably, bone substitutes could mimic the micro/nanostructures of natural cancellous bone as much as possible in order to promote the cell attachment, and proliferation, as well as be osteoconductive, biodegradable, osteoinductive to present an excellent bone regeneration ability, and induce new functional bone tissue [7],[8]. It is possible way to improve these substitutes used in bone tissue engineering, which should be simulate natural bone structure at nanoscale, and tuned graft surface feature size into nanometer regime. In the works by research groups, the authors detailed the fabrication of nanofeatured poly-caprolactone (PCL), poly-l-lactic-co-glycoloic acid (PLGA) 2D- and 3D- scaffolds which are treated with NaOH. Especially, Thapa et al. investigated nitric acid- and sodium hydroxide-treated PU and PLGA films *in vitro* bladder cell response [9]. A similar conclusion was reached also by Miller et al., the concentration of smooth muscle cell increased on the nanostructured PLGA [10]. Although there are the positively findings related to nanostructured TPU, there are limited studies about the cell response of nanostructured TPU fibers, and films, which created by alkali treatment.

As well known, polyurethane (PU) is a polymer by approved FDA and has been already manufactured in various shapes for different applications in tissue engineering [11],[12]. Although extensively used in practice, polyurethane-based materials have some drawbacks. One of the major limitations of PU is its hydrophobic character. Hence, PU shows low cell activity although usually exhibits acceptable mechanical properties such as high ductility, high tensile strength, and excellent abrasion resistance [13],[14]. Therefore, the surface architecture of PU is not particularly appropriate for bone tissue response, and eventually bone regeneration, even if it is a flexible and strength polymer, and needs to improve through chemical or physical modifications for cover these specifications. Herewith, these possible tunable properties become TPU more useful guided membrane candidate for bone tissue engineering. TPU in form of nanostructures improved by alkalization has been already investigated, and further did not study the effects of alkalization on the surface texture of TPU for using in TE applications.

In our previous work, we aimed to fabricate hybrid TPU vascular graft enriched with natural (phosphatidylcholine, PC), and synthetic molecules (poly(ethylene glycol, PEG), and the incorporation of these additives in PU scaffolds enhanced wettability and hemocompatibility of the graft. Because, it is well known that PEG is a hydrophilic versatile polyether being used in biomedical applications and the addition of PEG as an additive to the structure can hinder the protein adsorption besides reduced the bacterial adhesion [15],[17]. Other hand, PC namely lecithin, which is a phospholipid found in nervous tissue and brain substance has been frequently used in many applications in order to improve the hydrophilicity and biocompatibility, especially in tissue engineering [18],[19]. Our preliminary results have shown also that this composite polyurethane nanofiber mat showed remarkable hemocompatibility, and hydrophilicity compared to the raw TPU fiber. In addition to that, Yan et al. only treated thermoplastic polyurethane elastomer (TPU)/polyester woven fabric with NaOH, and investigated their mechanical properties [20]. Agour et al. also alkali-treated titanium surface coated a polyurethane, containing hydroxyapatite (HAp) nanoparticles (NPs) and magnesium (Mg) particles, and the cell response to the variably coated Ti substrates was examined using MC3T3-E1 cell line [21]. Consequently, in consideration of the following reasons; (1) there is no alkali-based modification of TPU/PEG or TPU/PC nanofiber membranes, the presented method provides bioactive, developable, and low cost modification option, (2) the surface and biological properties of alkali-treated TPU nanofiber membrane form consisting of PEG or PC did not investigated in the literature before, therefore this study is unique. On this basis, the objective of this study was also to achieve a dual-functionalization through NaOH etching besides chemical group coupling on the surface via PEG and PC, and investigate also the effects of a double modification of the surface. Herein, the surface wettability properties of TPU fibrillar membrane was characterized by static contact angle. The changes of dual chemical treatment created on the surface topography were evaluated by scanning electron microscopy (SEM), and atomic force microscopy (AFM). Moreover, the effects of these modified membranes on bone cell adhesion and viability were respectively evaluated ***in vitro*.**

2 Materials and methods

2.1 Materials

A polyether Elastollan® 1185A (polyether type polyurethane elastomer) was supplied from BASF GmbH (Lemförde, Germany). For preparation of TPU membranes, poly(ethylene glycol (PEG, Mw=1000 Da), phosphatidylcholine (PC, lyophilized powder), N,N'-Methylenebisacrylamide (MBAm), and benzophenone (BP) were supplied from Sigma Aldrich (USA). For *in vitro* cell culture studies, MC3T3-E1 culture, alpha minimum essential medium (α-MEM), fetal bovine serum (FBS), penicillin-streptomycin, L-glutamine and trypsin-EDTA were supplied from Biological Industries (Israel). TACS Annexin V-Fluorescein Apoptosis Detection Kit was supplied from R&D Systems, Inc (MN, USA).

2.2 Cell culture

Pre-osteoblastic, MCT3T3-E1 cells (ATCC, CRL-2593™) were cultured in α-MEM (Minimum Essential Medium) containing 10% (v/v) FBS, 1% penicillin-streptomycin, and L-glutamine. The conventional culture flasks were maintained in an incubator supplied with 5% CO_2 at 37°C for optimal growth. When the cells reached to approximately 80% confluence, were detached using trypsin/EDTA, continuously were subcultured.

2.3 Preparation of TPU-PEG and TPU-PC membranes

As described in our previous work, TPU membranes were fabricated by electrospinning equipment (Spellman CZE1000R) [22]. First, raw TPU of 0.5 g was dissolved in a solvent mixture of THF (tetrahydrofuran) and DMF (dimethylformamide) of 5 mL [1:1 (v/v)]. The raw TPU was electrospun onto a collector from a TPU solution (10% w/v). Raw random fibers were collected on a drum wrapped with aluminum foil at a feeding rate of 0.2 mL/h, a distance of 15 cm, and an applied voltage of 20 kV. For composite membranes, TPU solution (10% w/v in THF:DMF) containing weight ratio of 25 wt% PC was prepared and the optimum conditions for this solution were obtained at a feeding rate of 0.01 mL/h, a high voltage of 22 kV and a distance of collector from the needle tip of 15 cm. Another

composite formulation, a 1:4 weight ratio of PU-PEG solution was prepared following by mixing of PEG, MBAm and BP in a weight ratio PEG:MBAm:BP [97.5:2.0:0.5 (wt%)]. Then, the polymer solution was transferred to a 10-mL syringe, and the distance from the needle tip to the grounded collector was adjusted to 15 cm, and a 23 kV charge was applied to the needle under UV light.

2.4 Modification of TPU membranes by alkalization and characterization

To determine optimal treatment concentration, the membranes were hydrolyzed with NaOH solution at two different concentrations (1 and 3 M NaOH), and at a constant submersion time, 30 min. For that, the samples were immersed in NaOH solution at room temperature, and subsequently were washed with distilled water until the pH was 7. Herewith all NaOH residues on the surface of membranes were removed, and before being tested for characterization, all samples were dried at room temperature overnight.

Changes in the morphological structure of the TPU membranes modified due to alkali treatment were determined using scanning electron microscopy (SEM, JEOL JSM700F), atomic force microscopy (AFM, Nanomagnetics Instruments, Ankara, Turkey), respectively. For SEM examinations, the square-shaped membranes (1x1 cm in size) having the average thickness of 0.035±0.05 mm were coated a thin layer of gold-platinum using sputter coating machine for 5 min before taking SEM images, and scanned at 5 kV accelerating voltage. Furthermore, the average fiber diameter was determined by measuring the diameter of 250 different fibers from the randomly selected five SEM images using ImageJ software. AFM was employed to analyze three-dimensional topographic structure and surface roughness. The areal average roughness (Sa) data were attained using silicon cantilever (noncontact mode) with a scan size of 10 µm and a scan rate of 0.5 Hz at ambient temperature. From all pictures with a pixel resolution of 512×512, the average surface roughness with standard deviations was calculated, and presented. To analyze the contact angle, hydrophilicity of the TPU membranes, static contact angle measurements at ambient temperature were carried out by DSA100, KRUSS, Germany. A droplet of deionized water was dropped on the sample surface carefully with the help of microsyringe. The contact angle between the solid surface and droplet was determined from the images taken with a digital camera, and an average of six measurements on three different samples for each category was considered.

2.5 Flow cytometry analysis

The effect of alkali treatment on the cellular viability and apoptotic behavior of TPU membranes in compliance the manufacturer's guidance were assessed by Annexin V-FITC/propidium iodide (PI) apoptosis detection kit. Initially, all TPU membranes were treated with 5 mL of cell culture medium for 24 h. MC3T3-E1 cells were similtaneously seeded in a 25 cm^2 conventional cell culture flask at a density of 2 x 10^5 cells/mL, and cultivated at 37 °C with 5% of CO_2. The flasks were replaced with a 50% extract medium when the cells reached 80% confluence. After 24 h to allow the cells to treat, the cells were detached by trypsinization, pelleted at 3500 rpm for 5 min. The cells were rinsed twice in 1xPBS buffer (pH 7.4). Then, according to the manufacturer's instructions, the cell pellets were suspended in 500 µL of binding buffer, and stained using double staining mix containing of 5 µL of Annexin V-FITC and 5 µL of PI in the dark for 15 min. Finally, the cell suspensions were immediately passed through the counting chamber, and analyzed the data.

2.6 Confocal microscopy

Cell adhesion and morphology on coumarin-labeled TPU nanofibers were examined by staining with DAPI on Day 3 after seeding. In brief, the cells (1x10^2 cells/mL) were grown on the TPU specimens (1x 1 cm in size) for 3 days, and the culture medium was replenished with fresh media every day. For staining, the specimens were washed several times with PBS and the fixation of the cells was accomplished using 4% (w/v) paraformaldehyde (PFA) solution for at least 15 min. Then, the fibers were stained with coumarin, the cell nuclei was counterstained with DAPI to visualize the cell morphology following cell washing step. The observation of the cells was done with a confocal laser-scanning microscope (Zeiss LSM 800).

2.7 SEM examinations

MC3T3-seeded TPU membranes were also investigated through a scanning electron microscopy (SEM). For that, on Day 3, at a density of 2x10^2 cells/mL cell seeded membranes were fixed with 4% (w/v) PFA as mentioned confocal microscopy section, and were washed thrice in 1x PBS for desalination. Thereafter, the fixed specimens were dehydrated in increasing concentration of ethanol solutions (with 10 min in each 60%, 70%, 80%, 90%, and 100%). Finally, the specimens were coated with Au/Pd for 30 sec at 20 mA, and imaged using a scanning electron microscope (SEM, JEOL JSM700F K) at an accelerating voltage of 5 kV.

3 Results and discussions

3.1 Characterization of TPU membranes

To assess the morphology of the TPU membranes, the SEM images before and after alkalization procedure were obtained, and the average fiber diameter was determined using ImageJ from SEM images. As can be seen from Table 1, the diameter of the untreated TPU, TPU/PC and TPU/PEG composite fibers was found to be larger than that of the alkali-treated. For instance, TPU was about 0.591±0.148 µm while the fiber diameter obtained after alkalization reduced to 0.385±0.071 µm as such in PEG or PC compounding TPU samples. Consequently, the fiber diameter decreased significantly with the increasing concentration of NaOH solution as reported by Hashim et al. They investigated the effect of the modification method on the morphological and physical properties of kenaf fiber, and found an intensive decline in kenaf fiber diameter after alkali treatment [23].

Table 1. The mean fiber diameters of bare and composite TPU samples.

Samples	Fiber Diameter (µm)	Samples	Fiber Diameter (µm)	Samples	Fiber Diameter (µm)
TPU	0.591±0.148	TPU-PEG	0.732±0.150	TPU-PC	0.502±0.163
1 M/TPU	0.325±0.099	1M/TPU-PEG	0.595±0.127	1 M/TPU-PC	0.403±0.09
3 M/ TPU	0.385±0.071	3 M/TPU-PEG	0.479±0.110	3 M/TPU-PC	under 100 nm

In our previous study, the mean fiber diameters for the bare PU, PU-PEG, PU-PC were found to be 573±146 nm, 680±126 nm and 648±160 nm, respectively [22]. A similar trend was also observed, the diameter of the electrospun fibers increased with compounding of PEG or PC, which the fiber diameter of the pure TPU was about 0.591±0.148 μm while that of TPU/PEG is 0.732±0.150 μm. It supports that the increasing solution viscosity after compounding leads to increase in fiber diameter, and is an expected result [24]. SEM images (Figure 1) display the surface properties of the untreated and alkali-treated TPU fibers at two different concentration of alkali solution, and show a morphology structure with bead-free, smooth, uniform, and randomly-oriented fibers in the range of approximately 100-800 nm. Furthermore, the surface of nanofibers after alkalization became pitted without fracture, especially on the surface of TPU-PEG samples. Consequently, when NaOH concentration was increased, the morphology change was noticed.

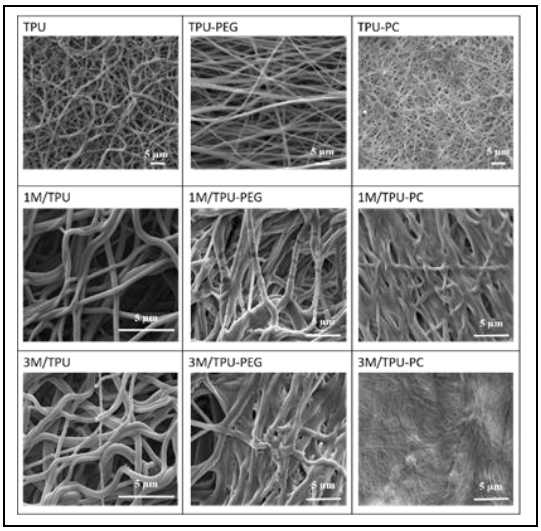

Figure 1. SEM images of untreated and treated electrospun TPU samples. Scale bar 5 μm.

Additionally, the increase in alkali concentration resulted in generation of bonding points as can be seen from Figure 1, and thus, the bonding force among the fibers enhanced. According to the previous studies, interfacial bonding properties of the fibers are also typical for treated fibers [25],[26].

As is well known, surface roughness has been regarded as a critical parameter for the adherence and growth of different cell lines [27]. Therefore, in addition to SEM analysis, AFM was used to determine the changed surface features of the TPU samples after alkalization. According to the data shown in Table 2 and Figure 2, it can be noted that the alkalization has a significant impact on the surface properties of the material. In fact, the Sa values for pristine TPU, 1 M/TPU, and 3 M/TPU were 3.36x101±1.2, 6.24x101±6.9, and 1.16x102±11.7 nm, respectively. The Sa values of the alkali treated nanofibers were greater than that of the without the alkali treatment, even the alkali concentration was increased, the Sa values of TPU membranes were increased proportionally. Similarly, Shu et al. fabricated chitosan (Chi) and heparin (Hep) multilayer coated titanium surface using a layer-by-layer (LbL) self-assembly technique. They concluded that Ra for Ti-OH, and Ti/PLL/(Hep/Chi were about 65.7, and 47.5 nm, respectively when the surface roughness of the multilayer-coated samples (Ti/PLL/(Hep/Chi) and only NaOH-treated Ti (Ti-OH) were compared via AFM analysis [28]. This result clearly indicated the impact of modification on the roughness of the surface, and the enhanced surface characteristics provide more growth sites for cells and promote cell attachment, these findings are in accordance with the presented results.

In consideration of the impact of alkalization on the surface pattern, the texture of the composite membranes was varied considerably from the alone TPU membrane's in nanoscale, and TPU-PEG after alkali treatment was the highest Sa values. Especially, the topographies of the TPU-PEG or TPU-PC formed after alkalization were like valleys, and Sa values of the TPU-PEG or TPU-PC after the submersion in 1 M solution of NaOH became about 2.51x102±15.6, and 2.79x102±17.3 nm, respectively.

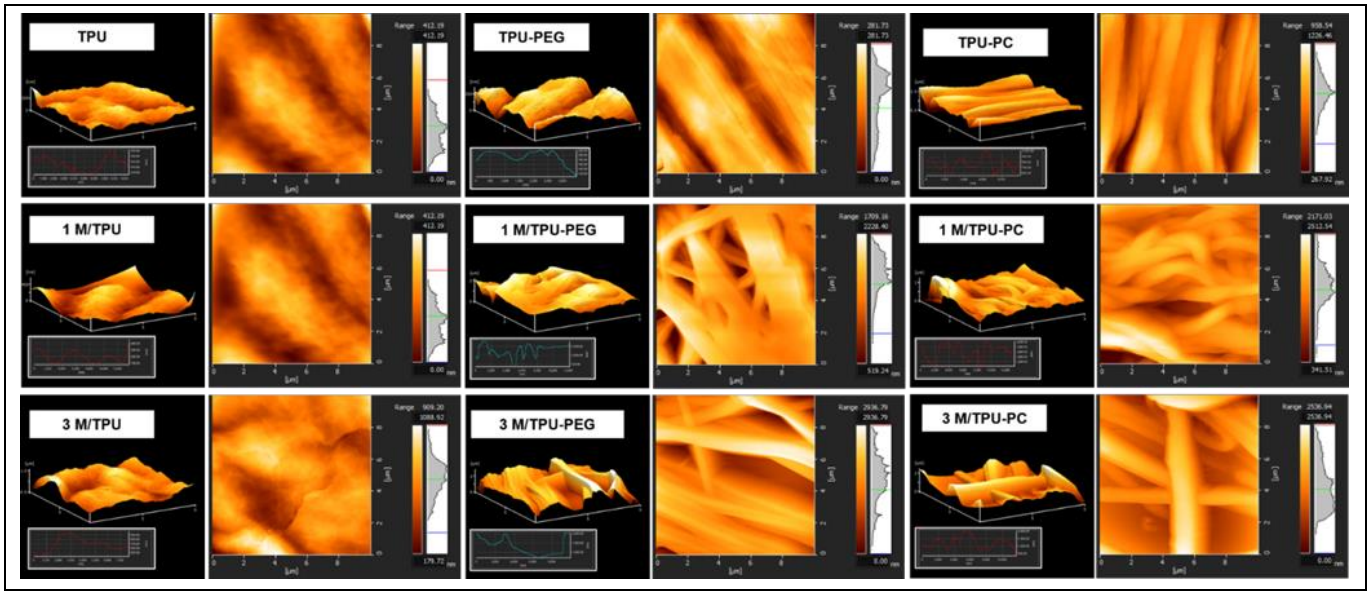

Figure 2. AFM images of untreated and treated electrospun TPU samples. 3D images, 2D images, and the surface roughness values obtained by AFM were presented.

Table 2. Roughness analysis results of the bare and composite TPU samples. (Sa=areal average roughness).

Samples	Sa (nm)	Samples	Sa (nm)	Samples	Sa (nm)
TPU	$3.36x10^{1}$±1.2	TPU-PEG	$5.01x10^{1}$±4.7	TPU-PC	$1.16x10^{2}$±12.3
1 M/TPU	$6.24x10^{1}$±6.9	1M/TPU-PEG	$2.51x10^{2}$±15.6	1 M/TPU-PC	$2.79x10^{2}$±17.3
3 M/ TPU	$1.16x10^{2}$±11.7	3 M/TPU-PEG	$4.29x10^{2}$±47.2	3 M/TPU-PC	$3.66x10^{2}$±64.6

The improved roughness in composite structures confirmed by Rajeshkumar et al. They reported cellulosic fiber reinforced epoxy composite, and confirmed that the mercerization both the surface of the fiber makes rougher and more number of wrinkles on the surface constitues [29].

Contact angle measurement is an effective method to examine the surface wettability properties. Figure 3 presents the corresponding contact angle images of both the untreated and treated TPU samples with various NaOH concentrations. From the observation, the treatment of the TPU membranes with the alkali solution reduced the contact angle by ~ 20°. For instance, the contact angle of the bare TPU-PC nanofiber membrane was 40.6±0.5°, whereas that of the TPU-PC treated with alkali solution of 1 M was 27±0.3°. Similarly, after NaOH treatment, a significant decrease in the contact angle in the TPU-PEG was observed compared to the bare TPU samples (from 40.6±0.5° to 21±0.2°). Furthermore, the surface hydrophilicity was more improved following the submersion in 3 M solution of NaOH. This demonstrated that the higher alkali concentration resulted in significant reductions in contact angle of the membranes. A similar conclusion was reached by Bosworth et al. They reported electrospun polycaprolactone (PCL) in various NaOH concentration and submersion time and investigated theirs surface hydrophilicity, biocompatibility, and material properties. According to contact angle measurement results, the contact angle value of random fibers at 0.1 M NaOH and 4 h submersion time was ~ 70° , whereas at 1 M 4 h reduced to ~ 10° [30]. Both the PEG-or PC compounding and NaOH treatments significantly improved the hydrophilicity of the TPU membrane surface. These results implied that the treated PEG or PC compounding TPU nanofiber membranes are suitable for cell adhesion, and could be a perfect barrier membrane for guided bone regeneration since an ideal membrane should allow cell colonization and thus guide bone regeneration.

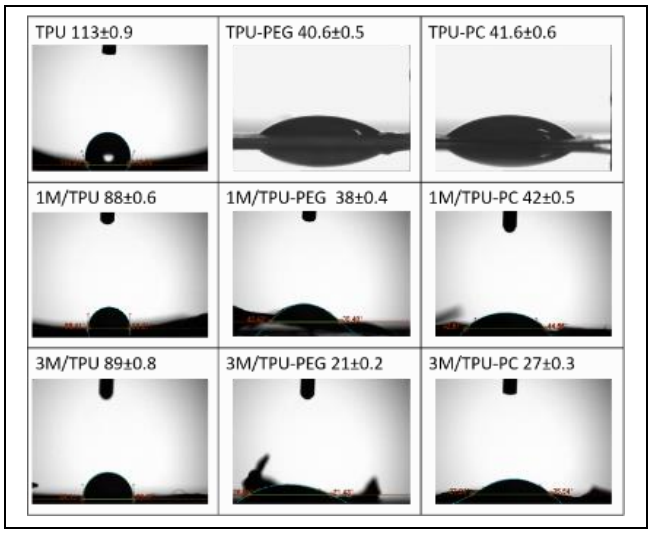

Figure 3. Water contact angle measurements of both the untreated and treated TPU membranes with various NaOH concentrations.

The effect of TPU fibers on the MC3T3-cell growth was assessed by flow cytometry analysis. As can be seen from inset table in Figure 4, the pre-osteoblast cells treated with all TPU membranes induced no prominent change in apoptosis/necrosis distribution. Furthermore, with increasing alkali concentration, the apoptotic/necrotic cell amounts in cell cycle did not significantly changed. Additionally, the live cell percent for all groups was above 95%, and there is no statistically difference among the treated groups. Further, the results were in agreement with our previous study [31], which showed that nanofeatured PLGA membranes created by alkali treatment method as a skin graft material reduced bacterial growth, fibroblast cell growth was not, which may be responsible for the prevention of focal contacts by surface topographies upon alkali treatment, and thus, the growth rate of fibroblasts may be decreased.

In the presented study, the surface pattern generated after alkali treatment may be trigger for the inhibition of the cell proliferation, but not for adhesion. Furthermore, especially TPU-PEG and TPU-PC samples showed more less necrotic cell amounts compared to their naked formulations. For instance, the necrotic cell count of naked TPU-PEG was 0.7%, whereas at 3 M NaOH reduced to 0.2% or the necrotic cell percent of TPU-PC after alkalization in dot-plot analysis was 0.4%. In general, flow cytometric analysis confirmed that alkali-treated TPU samples exhibited no considerable deformation in cell cycle of MC3T3-E1. The attachment of MC3T3-E1 cells on the TPU membranes was observed by SEM. Figure 5 shows the cellular behavior on the membranes after 3 days of *in vitro* cultivation. The cells are flattened, so that well-spread with cell-cell contacts on TPU nanofiber membranes. Further, the better cell spreading, and more filopodial extensions were obviously observed in the NaOH-treated groups compared to bare membranes. Clearly, at the end of three days of cell culture, the cells begin to make connections, and well-integrate with TPU surface. In case of the alkali treated TPU-PC, the cells on nanofibers were also nicely spread, and begin to cover the all membrane surface. Furthermore, as is well known, contact guidance of the cells is key factor in regulating cell migration, and focal adhesion contacts via filopodia formation facilitated the osteoblast adhesion and migration [32],[33]. Therefore, alkali modification of surface besides PEG- and PC-coupling is more efficient method, and could be promote cell viability and proliferation, and thus osteogenesis-related genes expression level. In order to assess the biological effects of the alkali modification on the TPU membranes, a confocal microscopy analysis was conducted. From the confocal microscopy images (Figure 6), the MC3T3-E1 cells were successfully adhered on the TPU membranes after culturing for 3 d. Obviously, the pre-osteoblast cells on the PC-or PEG-compounding membranes treated with the highest concentration NaOH were densely attached compared to those attached on the untreated TPU or composite membranes, and exhibited polygonal, elongated shape. It was found that the alkali treatment greatly increased the cell adhesion onto the membranes, which is closely related to increased surface wettability.

Samples	Q1 (%)	Q2 (%)	Q3 (%)	Q4 (%)
Control	2.9	0	97	0.1
TPU	0.2	0	99.7	0.1
1 M/TPU	0.3	0	99.5	0.2
3 M/TPU	0.4	0	98.9	0.7
TPU-PEG	0.7	0	99.1	0.2
1 M/TPU-PEG	0.3	0	99.4	0.2
3 M/TPU-PEG	0.2	0	99.3	0.5
TPU-PC	0.5	0	99.3	0.1
1 M/TPU-PC	0.4	0.1	99.2	0.3
3 M/TPU-PC	0.4	0	99.1	0.4

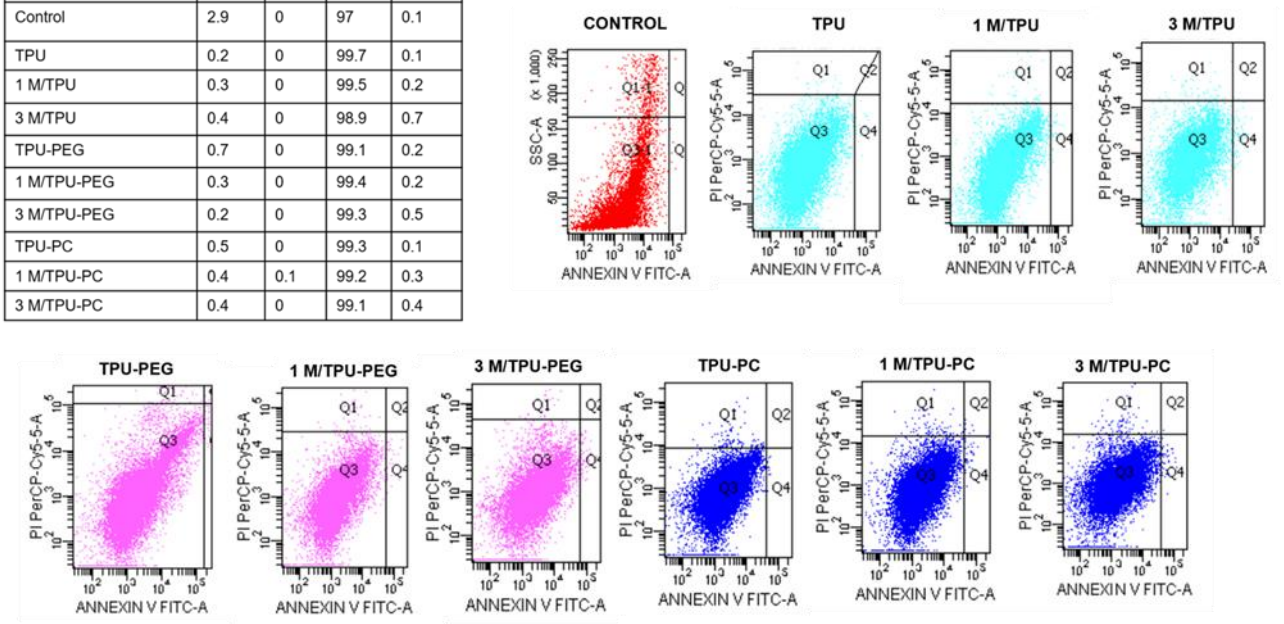

Figure 4. Cell apoptosis analysis determined by the flow cytometry of MC3T3-E1. The results were assessed after incubation with untreated and treated TPU samples for 24 h (Quadrant Q1, Quadrant Q2, Quadrant Q3, and Quadrant Q4 correspond to necrotic cells, late apoptotic cells, living cells, and early apoptotic cells, respectively).

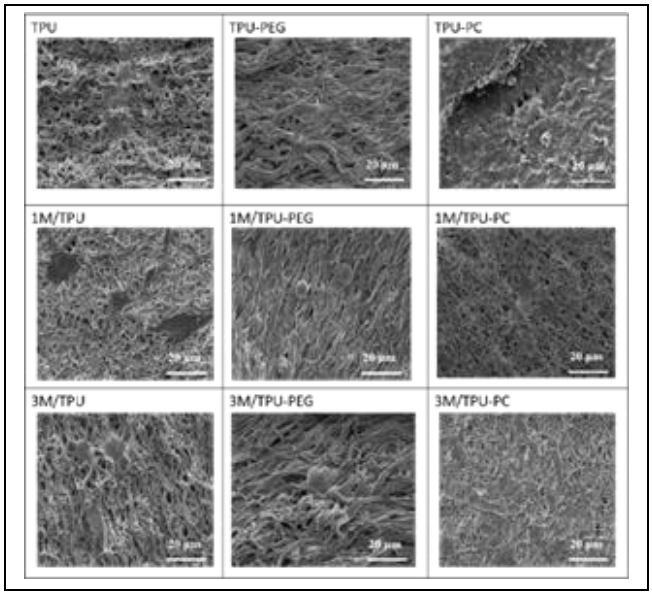

Figure 5. SEM images of the TPU membranes before and after surface modification. Scale bar 20 µm.

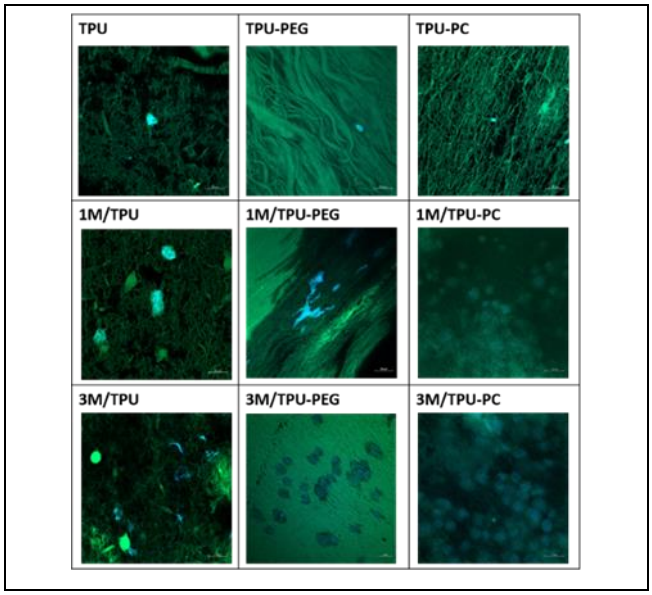

Figure 6. Fluoresent micrographs of the MC3T3-E1 attached on the TPU membranes on day 3. Green fluorescence indicates coumarin-stained membranes while blue fluorescence for nucleus. Scale bar 20 µm.

4 Conclusions

In this study, it was aimed to investigate the effect of the alkali post treatment on a TPU membrane as a guided tissue membrane because there is no study related to the guided bone regeneration application of alkali treated-TPU membranes. This study is first clearly to evidence how MC3T3-E1 cells respond to increased surface roughness and hydrophilicity by alkali treatment of TPU nanofibers doped with PEG or PC. The characterization of the TPU membranes showed that the fiber diameter was drastically reduced upon alkali contact, and further, obtained a significant change in the surface roughness

values and surface wettability of alkali-treated membranes against to non-treated. Furthermore, flow cytometry and confocal microscopy analysis exhibited that post-alkali treatment promoted the initial attachment of preosteoblasts on the membrane. Based on the findings as well as of supporting arguments of other authors, it was interpreted the alkaline post-treatment of TPU composite membrane is a promising technique to produce a an ideal barrier membrane for GBR applications, and to improve the osteogenic potential of the membranes.

5 Author contribution statement

Zeynep KARAHALİLOĞLU conceived of the presented idea, and carried out the all experiments. Then, Zeynep KARAHALİLOĞLU analysed the data, and designed the figures. Finally, discussed the results, and drafted the manuscript.

6 Ethics committee approval and conflict of interest statement

There is no need to obtain permission from the ethics committee for the article prepared.

There is no conflict of interest with any person / institution in the article prepared.

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