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In-Vitro NaOH Surface-Treated and Fibrin-Coated Electrospun Polyethylene Terephthalate Scaffolds for Small Diameter Vascular Graft

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ABSTRACT

The surface inertness of polyethylene terephthalate (PET) gives disadvantages including noncompliance, thrombogenicity, intimal hyperplasia, aneurysms, calcium deposition and infection also limited growth for small diameter vascular graft application thus limits their efficacy. To overcome this problem, electrospun PET fiber was treated with sodium hydroxide (NaOH) at 65°C for 1-hour. Fibrin gel was then coated onto NaOH treated samples and bare PET for comparison before seeding human umbilical vein endothelial cells (HUVECs) for 1, 7 and 14 days. The NaOH treatment resulted in reduced fibre diameter, enhanced hydrophilicity due to the existence of carboxyl and hydroxyl groups confirmed by FTIR results and contact angle measurements showed that the hydrophilicity of NaOH treated samples improved from 123° to 86°. After fibrin gel was coated on PET treated NaOH, in vitro tests showed increased cell adhesion and proliferation after 14 days of culture also formation of collagen after 21 days of culture. The PET treated with NaOH fibers coated with fibrin was proven attract HUVEC cells and promote endothelialization.

1. Introduction

Tissue engineering (TE) has been seen capable to replace and repair the problem caused by host tissue [1]. However, research in tissue-engineered small-caliber vascular graft has been critically debated until now due to the blood clot formations following intimal hyperplasia where constant and extreme tissue ingrowths in the persistent phase [2]. Several investigators have proven that the protein immobilization on the surface [3-5] of synthetic materials can be done to introduce a monolayer of endothelial cells (EC) onto the graft before implantation to mimic a natural human vascular state [6]. This method leads to functionalize vascular graft that can function very well. In fact, monolayer endothelium not only prevents thrombosis but also mediate the migration and proliferation of the EC [7].

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In order to construct scaffolds with required strength and good biological properties for cardiac TE, electrospinning is widely used compared to other methods such as phase separation and self-assembly. Electrospinning utilizes electrical forces to produce polymer fibers in nanometer scales mimicking native extracellular matrix (ECM) of the heart [8]. Polyethylene terephthalate (PET) has been electrospun to nano/microfibers over the last few years to be applied in different areas [9] including TE due to its promising advantages – giving good mechanical properties, moderate biocompatibility and cost-effectiveness [9]. The appropriate intrinsic properties of PET and the relatively low cost make PET a favorable material among others.

PET is a hydrophobic material and has low surface energy in nature thus makes other material hard to stick well to its surface [10]. Therefore, modification of PET is needed to increase the wettability and inertness in order to attain good interaction of cell-material compatibility and thus increase their patency. Various functionalization methods have been introduced without altered its bulky properties such as graft copolymerization [10], plasma treatment [11], radiation [12], aminolysis [13], and enzymatic modification [14], and hydrolysis [15]. Among these methods, hydrolysis by sodium hydroxide (NaOH) [13,15] is an easy and cheapest method that gives better results for biomedical application. Numerous works describe the use of NaOH surface treatment methods to functionalize PET surface for applications in the field of biomedical (i.e. antibacterial material, tissue engineering and wound healing) [16]. However, the use of NaOH surface treatment to functionalize PET fiber with fibrin has not yet been reported especially in terms of SDVG application.

Fibrin is used as a substance to coat synthetic polymer since synthetic polymers normally do not support the growth of cells [17]. Fibrinogen is capable to generate the adhesion, spreading and microfilament arrangement of HUVEC either in 2D or 3D in-vitro culture system [18].

Most research focuses on short-term cell proliferation, and there is insufficient information about the long-term stability of PET and fibrin coatings treated with NaOH under physiological conditions. Accordingly, the PET fiber was first surface functionalized using NaOH in this investigation, and then its surface was coated using fibrin gel for a 21-day culture. Surface morphology, chemical composition, wettability, cell adhesion, cell proliferation, and cytotoxicity were then used to characterize the resultant changed fibers.

2. Methodology

2.1 PET Fabrication

The electrospinning solutions were prepared by dissolving the PET pellets (0.82g/dl) instantaneously into TFA (20 wt%) and stirring at 350 rpm for 2 hours in order to absolutely dissolve PET. The solution was completely dissolved when all PET pellets were dissolved in TFA solvent and thus made the solution become clear and viscous. The PET solution was put into the 5mL plastic syringe along with a needle with an inner diameter size 0.21 mm. The solution was injected out with flow rate 1 ml/hr. Voltage was set-up at the low voltage at 13kV and maximum voltage was 15kV while current and frequency were set at zero. The outcome fibers were collected on a 6cm x 6cm static flat aluminum collector. The electrospinning process was operated for 4 hours.

2.2 PET Surface Treatment

PET electrospun fibers were put in 1M NaOH aqueous solution. Then the samples were put in the oven at 65^{o}C for 1 hour. The samples were rinsed with plenty of distilled water until pH~7. Later the samples were dried at ambient temperature (T~25^{o}C) for 24 hours. This NaOH surface treatment was expected to promote the wettability of PET surfaces by introducing polar functional groups. Enhanced hydrophilicity is particularly important for biomedical applications, as it promotes better cell adhesion and proliferation.

2.3 Fibrin Gel Preparation

Fibrinogen solution was prepared by dissolving the fibrinogen powder in Hanks' Balanced Salt Solution (HBSS) while thrombin was prepared by the concentration of 100U mL^{-1} in HBSS and supplemented with 350 KIU mL^{-1} of aprotinin. Unsterile fibrinogen and thrombin were warm up in the water bath (37^{o}C) about 15 minutes before used. Both solutions were filtered through a 0.22 µm syringe filter. The moderate fibrin gel with concentration of the 2mg/mL was developed by immediate transfer and fast stirring of the fibrinogen-thrombin mixture into the 24-well plate for 3 minutes stirring [18]. The fibrinogen polymerized with sterile PET electrospun fiber embedded in the gel. The samples then were dried on filter paper at room temperature in a sterile condition.

2.4 Characterization

The morphology of all samples was captured using field emission scanning electron microscope (FESEM). Fiber was coated via gold plated for 5 minutes before mounted on the holder and was run at the acceleration voltage of 20 kV under low vacuum. The images were taken at several different magnifications 10K and 25K.

The fourier transform infrared spectroscopy (FTIR) is commonly used to analyze the chemical bonds of the fibers. The FTIR spectra of the fibers were collected using the Nicolet 5700 instrument (Thermo Company, USA) and was used to analyze the chemical structure of fibers over a range of 500-4000 cm^{-1} at a resolution of 4 cm^{-1}.

The wettability of the samples was measured using water contact angle (WCA) sessile drop method using a VCA Optima instrument (AST Product, MC). A 5 µL of distilled water was dropped on a fiber's surface and contact angle images were analyzed using VCA optima software.

2.5 Cell Seeding Procedure

All samples were placed nicely into the bottom of 24 well-plate with the rounded dimension (d=15mm). Next, ~ l x $l0^{6}$ cells/ml of Human Umbilical Vein Endothelial Cells (HUVEC) from passage 3 to 5 was seeded on the surface of the fibers. Fibers were then placed in the incubator for 20 minutes to allow cells make a contact with the surface of the fibers. Fibers were taken out from the incubator after 20 minutes and the remaining medium was added (500µl) to each sample of the well and incubated again in the incubator at 37°C with 5% CO_2.

2.6 Cell Growth Morphology and Collagen Formation Analysis

In order to screen for cell growth morphology, culture was maintained in the incubator for 1, 7, and 14 days while the medium was replaced every 2 days. The adhered cells on the sample were

stained with 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI), Alexa Fluor 488 and phalloidin. First, cell-seeded were rapidly washed with PBS three times and fixed with 3.75 % wt/v paraformaldehyde for 20 minutes. Then the samples were washed with PBS three times for 3 minutes each. The cell adhered on the sample was permeabilized with 0.5 v/v % of Triton diluted in PBS (Sigma Aldrich) for 5 minutes. The samples were washed again with PBS three times. Next, the cytoskeletons of cells on the samples were stained using Alexa Fluor 488 (Molecular) for 30 minutes at room temperature and placed in the dark to avoid bleaching. After that, the samples were washed with PBS three times. For nuclei staining, samples were stained with DAPI (Molecular) for 3 minutes and then washed with PBS. The images were visualized under the fluorescence microscope (Carl Zeiss, Germany) and imaging using ZEISS software.

To investigate the collagen formation, scaffolds were stained with sirius red after 21 days of seeding. Samples were fixed in 4% formaldehyde diluted in distilled water for 20 minutes. Samples then were washed with distilled water three times. Since collagen is a major constituent for ECM, this staining technique is relevant to detect tissue formation [19].

3. Results

3.1 Surface Morphology

Figure 1(a) shows the micrographs of bare PET fiber obtained from electrospinning. It was observed that the bare PET fiber showed homogenous morphology with featureless and smoothness. The fibre weight and volume ratio will influence the orientation of the fibre [30]. The average fiber diameter of PET fiber is 565 ± 38 nm. Figure 1(b) shows the fabricated PET coated with fibrin gel. Fibrin constructs looked smooth flat surface and had translucent appearance after coating process. The fibrin gel constructs after coating of fibrin gel on PET fibers could not be seen due to the hydrogels translucent characteristic [20]. However, the existence of fibrin gel can be proven by increase in average fiber diameter 594 ± 55 nm. In that case, surface treatment was conducted to increase the hydrophilicity of PET fiber. This hydrophilic surface has advantages to adsorb organic substance followed by attracting the cells on its surface [16]. After the surface treatment in Figure 1(c), the average fibers diameter was 374 ± 12 nm and PET becomes rough with uneven surface seen along the fibers. It can be concluded that surface treatment of NaOH at 65°C for 1 hour was good enough as this has increased the surface hydrophilicity of PET fiber without affecting much the physical properties. After PET treated with NaOH, fiber was subsequently coated with fibrin gel. The average fiber diameter increased to 409 ± 53 nm and the surface became smooth, indicating a fibrin gel was formed on the surface of the fiber.

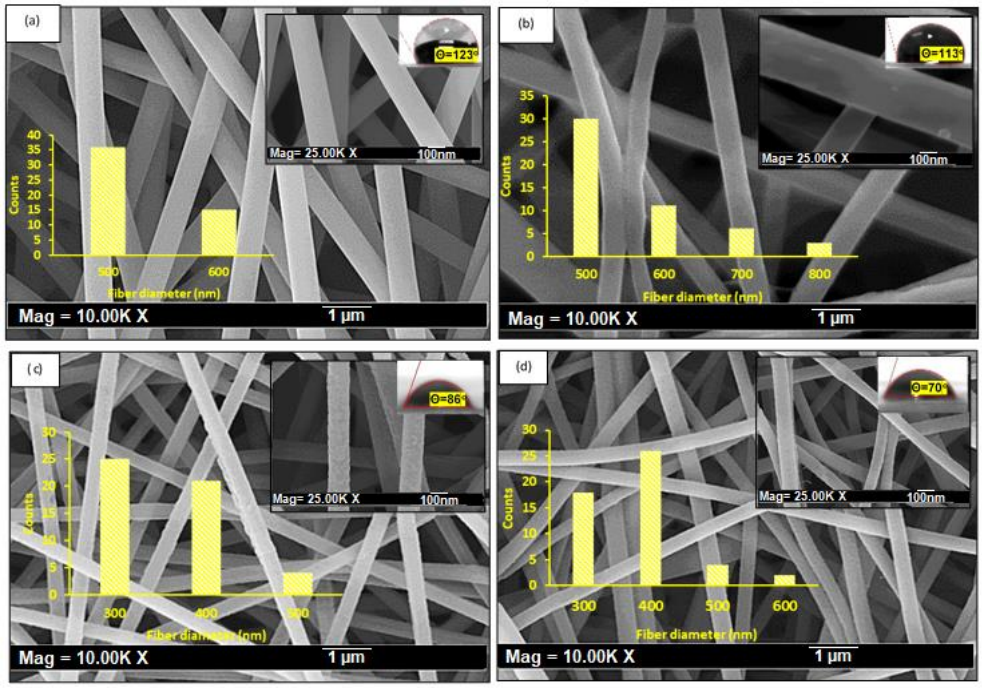

Fig. 1. Surface morphology, fiber diameter counting and water contact angle of (a) bare PET; (b) PET coated fibrin gel (c) PET treated NaOH (d) PET treated NaOH coated fibrin gel

3.2 Surface Chemistry

Figure 2(a) shows a typical FTIR spectrum of the PET polymer [10]. The main absorption bands in the spectrum were assigned as follows, 2958 cm^{-1} to aromatic and aliphatic –C-H bond stretching, 1716 cm^{-1} to the ester carbonyl bond (-C=O) stretching, 1577cm^{-1} benzene normal mode and 1504cm^{-1} *para*-substituted benzene ring, 1470-1350 cm^{-1} to bending and wagging vibrational modes of the ethylene glycol segment, 1243 cm^{-1} to the ester group (-COO-) stretching, 1096cm^{-1} to the esters group, 1017cm^{-1} gauche formation and aromatic bands in-plane/out-plane bending at 725 cm^{-1}. One of the major bands sensitive to configurational changes was the carboxyl (C–O) stretch at 1096 cm^{-1} that correspond to the C–O stretch in the gauche form of the $–O–CH_2CH_2–O–$ moiety. This moiety exists in two forms, trans and gauche. Mancini and Zanin [27] have pointed out that thermodynamic estimates show that the weakest chemical connection in the PET chain is the ester link.

After the bare PET fiber was coated with fibrin gel, amide I ($-NH_2$) and II (-NH) peaks were readily observed. In Figure 2(b), the peak with a broad feature between 1500 and 1600 cm^{-1} was the amide II signal, while the broad peak between 1600 and 1700 cm^{-1} was the amide I band. The amide I signal is dominated by the protein backbone –O stretching modes. The amide II signal has the main combinations from the backbone N-H bending and C-N stretching modes [21].

Considerable changes in the spectra were observed in the PET treated NaOH as compared to bare PET sample (Figure 2(c)). PET ester bond was thought to be hydrolyzed upon the addition of NaOH as evidenced in the wave number shift and the broadening of carbonyl (–C=O) stretch. The increased in the absorption intensity and the broadening of the carbonyl (–C=O) stretch indicate the formation of acid as NaOH-hydrolysis product of PET along with the overlap of carbonyl (–C=O) stretch of the ester and acid bond of NaOH-treated PET. It is noteworthy that –C=O stretch of the ester is around 1705–1720 cm^{-1} for acid – C=O stretch [14]. An increase in the absorption intensity of the –OH stretch in the PET treated NaOH indicates an increase of the ethylene glycol and carboxyl (-COOH) functionalities known hydrolysis products of PET [14]. This transmittance may result from NaOH

bonded to the fiber surface. According to Sellitti *et al.,* [29] the treatment of PET fiber with NaOH solution results in the transformation of carbonyl groups (C=O) to carboxylates (COO-) in the hydrolysis of esters.

A predominant amide I (-NH_2) peak at 1652 cm^{-1} was observed for the samples PET treated NaOH coated with fibrin gel (Figure 2(d)). The peaks at 1651 cm^{-1} for PET treated NaOH coated fibrin gel and 1537 cm^{-1} at PET coated fibrin gel corresponded to the α-helix/random coil structure, and the peak at 1651 cm^{-1} was due to the intermolecular β-sheet structure [22]. The peak near 1407 cm^{-1} was mainly due to the deformation of CH_2 and CH_3. The peaks ranging from 1300 to 1200 cm^{-1} corresponded to amide III(-C=ONCH$_2$) bands and the peak at 1095 cm^{-1} was due to the C-O stretching modes of glycoprotein in fibrinogen structure for both PET fibrin coated fibrin gel and PET NaOH coated with fibrin gel. Amide II (-NH stretch) for PET treated NaOH coated fibrin gel showed strong peak compared with PET coated fibrin gel proved that NaOH surface treatment contributed in adsorbed fibrinogen molecules on PET fiber [22].

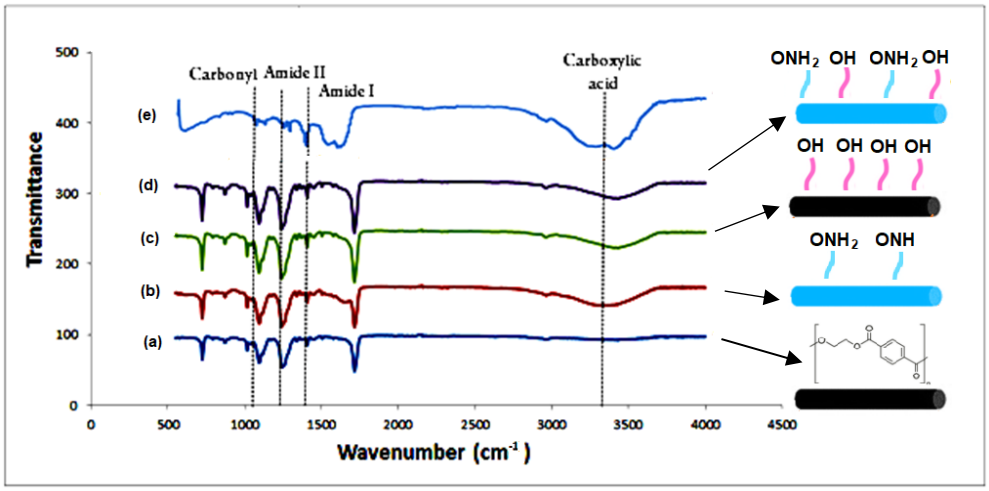

Fig. 2. FTIR results of (a) bare PET; (b) PET coated fibrin gel (c) PET treated NaOH (d) PET treated NaOH coated fibrin gel (e) fibrin

3.3 Wettability

Bare PET fiber as expected displayed an intrinsically hydrophobic surface with a relatively stable water contact angle (WCA) at ~123° (Figure 1(a)), while for the PET coated fibrin gel the contact angle was reduced to ~113° (Figure 1(b)), indicating improved wetting of the fiber. In the case of PET treated with NaOH, the WCA decreased sharply from 123° to 65° after the treatment (Figure 1(c)). The fibers' surface after NaOH treatment had a very low contact angle implying that it is highly hydrophilic than other samples. These results are attributed to the rapid wetting due to intramolecular hydrogen bond formation between the C=O and N-H groups in the PET chain and water molecules [14]. The decrease in contact angle is probably caused by an increase of negatively charged ions (OH−) on the surface after the treatment. When NaOH treatment was suitably enlarged, the polar groups increased and formed hydrophilic property, at the same time contact angle decreased [23]. In this research, the transformation of hydrophilic molecules could be the result of reactions between NaOH radicals and polymer surface radicals caused by NaOH dissociation [24]. Moreover, the results indicated that the surface hydrophilicity was obviously improved due to the cleavage of hydrophobic groups and the formation of the hydrophilic groups on the PET treated NaOH surface as confirmed by FTIR analysis, shown in Figure 2(c). NaOH treatment lowered the water absorption of the specimens because the parts of PET fiber volatile extractives were removed after

NaOH surface treatment [15]. The WCA for the PET surface decreased after treated with NaOH may be attributed to the formation of various kinds of the oxidized group such as carboxylic acids and hydroxyl due to chain scission and followed by air exposure [25].

PET treated NaOH coated fibrin gel surfaces shows contact angle at 70^{o} (Figure 1(d)). The contact angle for the PET treated NaOH coated fibrin gel showed relatively improved hydrophilicity compared to bare PET, confirming the presence of fibrin gel on the fiber layer. This finding is in agreement with the FTIR data as shown in Figure 2 where relatively high oxygen content was observed on the surface of the fiber.

3.4 HUVEC Growth Morphology and Collagen Formation

As shown in Figure 3(a), cells grown on the bare PET scaffold demonstrated a small spherical or cobblestone shape which is typical non-spreading morphology for HUVEC after day 1 of culture. HUVEC cells had a rounded morphology and no focal adhesion points were observed. As the bare PET is a hydrophobic scaffold thus, surface tension prevents liquid media and cells from entering 3D scaffolds. Consequently, cells grew only on the scaffold surface and were not able to penetrate into the scaffold. This resulted non-uniform cell distribution limits available surface areas for cell growth [26].

After 1-day culture, the cytoskeletons of HUVEC seeded on PET coated fibrin gel began to organize along fibrin on fibers (Figure 3(b)). They remained primarily as a monolayer with the spread filopodia attached on the scaffold as shown in Figure 3(e) on day 7. After 14 days, it shows cells on the PET coated fibrin gel started to follow the bare PET cells morphology (Figure 3(h)). This is because the dissolution of the fibrin on the surface of PET coated fibrin gel on day 14.

Similarly, HUVEC were well spread and oriented along the PET fibers treated NaOH coated fibrin gel on day 1. The cells also showed stretched cytoskeleton with spindle shape when grown on PET treated NaOH coated fibrin gel. The increase in cell adhesion on the scaffold might be attributed to the adsorption of fibrin gel coating on the surface of PET treated NaOH and the enhancement in the hydrophilicity of the scaffolds (Figure 3(c)). The cells increased significantly in numbers, reaching an estimated confluence of about 70% for PET treated NaOH coated fibrin gel scaffold on day 7 (Figure 3(f)). HUVEC cells were rapidly grown on the coated surface on day 14 of incubation than bare PET surfaces. They exhibited filopodia that protrudes on top of the surface of the fibers (Figure 3(i)) which improves the interaction and adhesion of the cells. This is an indication of initial strong cell attachment and adequate signalling for cell growth survival [27]. After day 14, HUVEC-structured and -oriented on the PET treated NaOH coated fibrin gel allow the designing of the tube-like structure [3,26] as shown yellow arrow in Figure 3(i). When compared to the bare PET, there is no tube-like structure formed nor cell-cell interactions detected (See Figure 3(g)). This result shows that the original material, PET was successfully improved using the coatings giving good inherent biocompatibility.

After 21 days of culture, results showed a small area of collagen content on bare PET with no formation of tube-like structure (Figure 3(j)). However, the PET treated NaOH coated with fibrin gel (Figure 3(l)) shows the highest percentage of collagen content area with 60% after 21 days of culture while the average tube-like structure diameter is 48.63 ± 0.39 µm. The deposition of collagen indicated a similar distribution of HUVEC [28]. Fibrin receptor activation induces phosphorylation of specific proteins and promotes HUVEC migration and tube formation [29]. Compared to other techniques [10-14], NaOH immersion ensures uniform surface modification, which is critical for ensuring consistent biological performance across the scaffold. Thus, this modified PET exhibited

improved HUVEC adhesion and proliferation. This outcome would open the door for clinical studies by offering thorough insights into the long-term behavior of treated PET scaffolds.

4. Conclusions

PET hydrophobic nature may lead to problems, particularly protein absorption, when used for biomedical application. As a result of NaOH surface treatment of electrospun PET fiber, the hydrophilicity of fiber was improved. This NaOH surface treatment introduced hydroxyl groups on the fiber surface which led to higher wettability of PET treated NaOH fiber than bare PET. It was also observed that wettability of fibrin gel coating on the PET treated NaOH fiber surface was increased due to enhanced of hydrophilicity of the fiber. From the results, PET treated NaOH coated fibrin gel enhanced endothelialization, as observed in spreading cell morphology and increasing cell proliferation until day 14 of culture. It is also showed that HUVEC adhere to the surface of the scaffold and lead the collagen formation after 21 days of culture. Therefore, this study suggests that PET treated NaOH coated fibrin gel has the potential to be used in SDVG application.

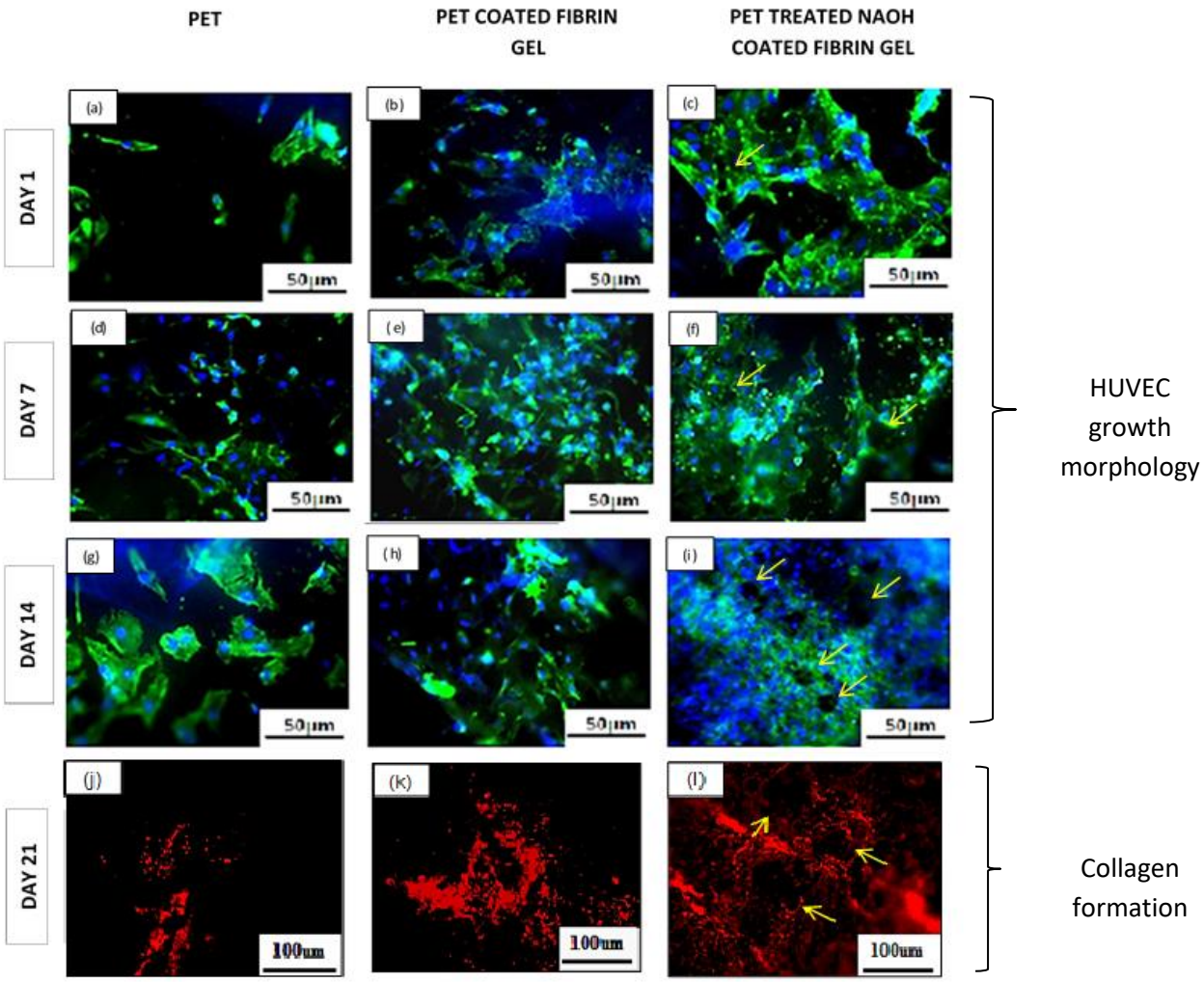

Fig. 3. (a)-(i) HUVEC cell growth morphology on bare PET, PET coated fibrin gel and PET treated NaOH coated fibrin gel for day 1, 7 and 14 days of culture; (j-k) Collagen formation of bare PET, PET coated fibrin gel and PET treated NaOH coated fibrin gel for 21 days of culture (yellow arrow indicate tube-like structure).

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