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Original Article

A multicompartment vascular implant of electrospun wintergreen oil/ polycaprolactone fibers coated with poly(ethylene oxide)

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ABSTRACT

Background: The aim of the present study was to fabricate double layered scaffolds of electrospun polycaprolactone (PCL) and poly(ethylene oxide) (PEO). The electrospun PCL fibers were functionalized with wintergreen oil (WO) as a novel approach to prevent vascular grafts failure due to thrombosis by adjusting biomaterial–blood interactions.
Methods: PCL tubular scaffolds were prepared by electrospinning approach and coated with PEO as a hydrophilic polymer. The single and double layered scaffolds were characterized in terms of their morphological, chemical properties -as well as-hemocompatibility assays (i.e. prothrombin time, hemolysis percentage and platelets adhesion). Moreover, the anti-oxidant potential of WO-PCL samples were measured by 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical assay.
Results: The results demonstrated that incorporation of WO during the electrospinning process decreased the PCL fiber diameter. In addition, the prothrombine time assay shows that WO could be used to lower the electrospun PCL fiber tendency to induce blood clotting. Moreover, SEM observations of platelets adhesion of both single and double layered PCL/ PEO scaffolds fiber shows an increase of platelets number, compared with the scaffolds containing WO.
Conclusions: The antioxidant potential and blood compatibility measurements of WO-PCL/ PEO samples highlight the approach made so far as an ideal synthetic small size vascular grafts to overcome autogenous grafts shortages and drawbacks.

Although autologous vascular grafts can provide an effective solution for surgeons to redirect blood flow around the injured or diseased vascular vessels via bypassing two spots within the vessel using natural veins or arteries [1–3], the bypass surgical operation is restricted under certain circumstances including, limited supply, secondary site injury

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and *anatomical* - as well as physiological mismatches [4,5]. Today, artificial vascular grafts made of biocompatible materials are often applied to replace autologous grafts [6,7]. Unfortunately, the development of synthetic vascular grafts of small diameter (less than 6 mm) - with functional features similar to the native ones - represents a huge obstacle to overcome the common drawback of traditional bypass vascular surgery [8].

Electrospinning has been highlighted as a fruitful approach to simply draw one dimension (1 D) polymeric fibers of nano/microsize thats directly assembled into 2D (or 3D) mats for different biological applications [9–11]. The electrospun nanofiber exhibit a large surface area and a high porous microstructure which are intrinsically essential for gas and nutrient perfusion - as well as various cellular activities [12]. Aliphatic polyesters, i.e. polyglycolic acid (PGA), polylactic acid (PLA), polycaprolactone (PCL) and their copolymers, have received a special attention as a result of their biocompatibility, mechanical strength and biodegradability [13,14]. However, electrospun polyester vascular scaffolds have been limited due to their lower bioactivity and hydrophobic nature which decrease their capability to interact or integrate with biological systems [15,16]. Hence, bioactive molecules have been conjugated or loaded to improve the blood compatibility of polymeric fiber for vascular grafts application i.e. anticoagulant agents as heparin and pharmaceutical drugs-that appear to be valuable components to promote tissue regeneration process via inducing endothelial precursor cells differentiation or reducing the inflammatory response [17,18].

Natural essential oils (NEOs) are hydrophobic volatile liquids that have a strong aroma because of their high concentration of the aromatic compounds. NEOs are usually extracted as secondary metabolites from the flowers, leaves, roots, fruits and other parts of the plant [19,20]. NEOs have garnered the attention of many researchers for medicinal and health care purposes as effective therapeutic agents due to their unique features including antibacterial, antimicrobial, anti-inflammatory and antioxidant [21–24]. Among them, wintergreen oil (WO) is mainly composed of methyl salicylate (up to 99%), which is responsible for its medicinal activity with minor organics ingredients as α-pinene, myrcene, 3,7-guaiadiene, delta-3-carene, limonene, and delta-cadinene, [25,26]. Despite WO is used topically to reduce inflammations and pains, the lower solubility of WO under physiological conditions and the systematic toxicity of methyl salicylate limit its application [27,28].

Herein, electospun PCL fiber were coated with a hydrophilic layer of PEO as a novel approach to develop multifunctional vascular scaffold. The effects of WO on PCL fiber formation and stability were studied at different concentrations (1, 2 and 3 wt%). The fabricated single/double layered scaffolds were thoroughly characterized in terms of their microstructure properties via Scanning Electron Microscopy (SEM) and Attenuated Total Reflectance Fourier transform infrared spectroscopy (ATR-FTIR). The water uptake and antioxidant capabilities were also measured. Moreover, the blood compatibility of the fabricated single and double layered scaffolds was assessed by testing, prothrombin time, hemolysis percentage and platelets adhesion.

Materials and methods

Materials

PCL pellets (Mw = 80,000 g/mol), poly(ethylene oxide) powder (PEO, MW = 9,00,000 g/mol) and wintergreen oil (WO, Mw = 152.15 g/mol) were purchased from Sigma–Aldrich. Heparin solution (5000 I.U./1 ML) was purchased from Amoun Pharmaceutical Industries Co., APIC, Cairo, Egypt. Chloroform ($CHCl_3$, 99%) and Acetone was supplied by Carlo Erba Reagents. All reagents and solvents were used without any further purification.

Preparation of PCL/PEO double-layered tubular scaffolds

The PCL (6, 8 and 10 wt.%) solutions were prepared by dissolving PCL granules in a mixed solution of acetone chloroform (1:1 v/v). The optimized electrospinning parameters for the PCL solution were carried out to select the favorable conditions to obtain neat PCL nanofibers. The electrospinning process was performed using a homemade apparatus in which PCL solutions were loaded to a plastic syringe with a metallic needle of 0.9 mm diameter and connected to a high-voltage power supply. The selected processing parameters were as following: an applied voltage of 20 kV, a needle-collector distance of 12 cm, while the polymeric solution was fed in using a syringe pump at a flow rate of 1 mL/h. A rotating stainless steel tube of 5 mm diameter was used as a collector to fabricate tubular scaffolds. The WO-PCL nanofiber were obtained under the above processing parameters by the addition of WO to PCL solution before the electrospinning process. Different amount of WO (100, 200, 300 μl) were added to a 10 ml of PCL solution and stirred for 3 h. The samples were named WO1-PCL, WO2-PCL and WO3-PCL. Finally, PEO layer was added to the WO-PCL tubular scaffolds by dipping the whole scaffold into PEO solution of 5% and 10% conc. 2 ml of Heparin (Hep) - as an anticoagulant agent - was mixed with PEO solution. The obtained PCL/PEO double layered scaffolds in absence and presence of Hep were named as WO-PCL/PEO and WO-PCL/PEO-Hep, respectively. The scaffolds were dipped into PEO three times before air drying. The prepared tubular scaffolds were dried at room temperature for solvent traces removal and then stored in the desiccator for further characterizations.

Structural characterization

The microstructure and morphology of elctrospun fiber PCL and double layered PCL-PEO mats were observed via SEM. The prepared samples were coated with a thin layer of gold by sputtering and then the morphology of electrospun mats was determined. The micrographs were taken using a Hitachi S-4100 electron microscope (Tokyo, Japan) at an accelerating voltage of 5 kV and a working distance of 8–10 mm. While the functional groups of neat PCL, PEO and WO, as well as PCL/PEO double layered mats were characterized by ATR-FTIR spectroscopy using Vertex 70 FTIR spectrometer from BrukerOptik GmbH, Germany, equipped with diamond ATR crystal system

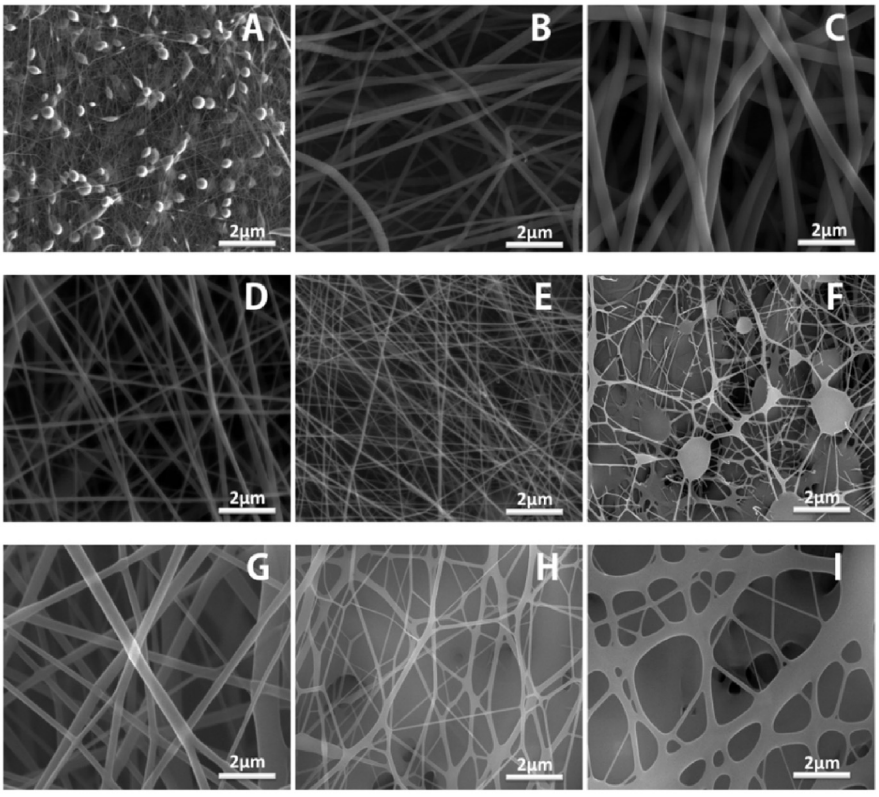

Fig. 1 Optimization of processing parameters (**A, B& C**) obtained fibers at 15 kV and distance of 10 cm for solution conc. of 6, 8 &10%, respectively, (**D& E**) effect of applied voltage 17 and 20 kv, (**F**) WO1-PCL solution conc. of 8%, (**G, H& I**) WO1-PCL, WO2-PCL WO3-PCL solution conc. of 10%, respectively.

in the spectral range of 4000–400 cm^{-1} with the resolution of 4 cm^{-1}.

Water uptake

The obtained single and double layered tubular scaffolds were incubated for 24 h in phosphate buffer solution (PBS) at 37 °C. The degree of water uptake was determined according to the following equation [29]:

$$\text{Water uptake (\%)} = [(Ww - Wd)/ Wd] \times 100.$$

where Wd is the dry sample weight before immersion in PBS and Ww is the wet weight of the sample after soaking 24 h in the PBS.

Antioxidant assay

Radical scavenging activity was measured against stable 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) [30]. The WO1-PCL and WO2-PCL mats were kept in the dark for different time intervals(0.5, 1, 2, 4, 8, 16 and 32 h) at room temperature to react with DPPH solution (0.1 mM in ethanol). The change in colour (from deep violet to light yellow) was measured using UV–visible light spectrophotometer at 517 nm. The experiment was carried out five times for each sample. DPPH free radical scavenging activity was expressed as remaining DPPH % against different time intervals.

Prothrombin test

Prothrombin test (PT), also called International Normalised Ratio test (INR), is a laboratory approach to measure the blood coagulation time [31,32]. In brief, 2.5 ml human blood was taken from a healthy adult and drawn into a sterilized tube containing liquid sodium citrate, as an anticoagulant agent. The samples were spread over a glass slide and then 200 μl of the anticoagulated blood was added on the surface of the samples. The blood coagulation was promoted by adding 20 μl PT liquid (isi = 1.5) and coagulation time was recorded. The data were taken after five measurements were averaged.

$$INR = [(PT_S / PT_b]^{isi}$$

Where PT_s and PT_b represent the blood coagulation time in presence and absence of the samples, respectively.

Hemolysis assay

A fresh blood sample was treated with sodium citrate solution and normal saline, according to a method describe elsewhere

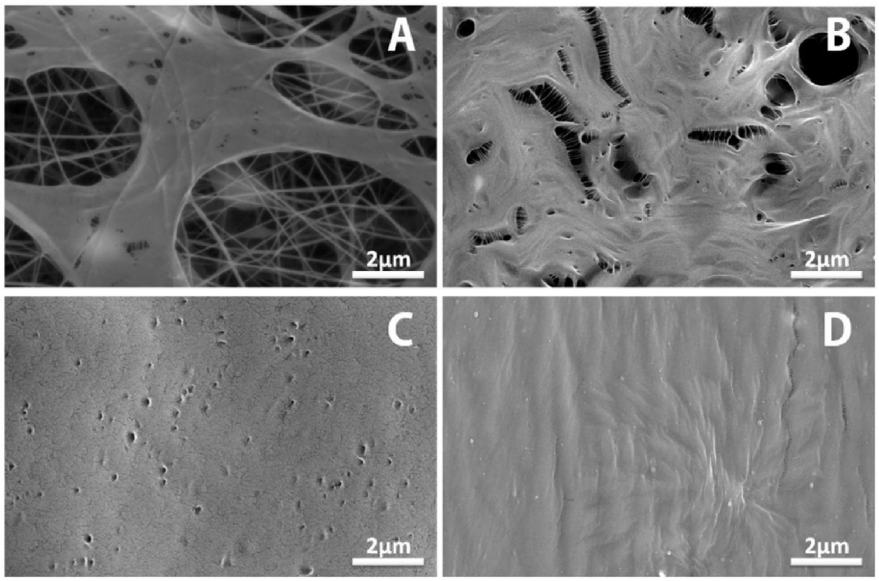

Fig. 2 SEM micrographs of double layered mats (**A**) PCL coated PEO solution of 5%, (**B**) PCL coated PEO solution of 10%, (**C**) PCL/PEO-Hep and (**D**) WO1-PCL/PEO-Hep.

[33]. 200 μl of the diluted blood were added to a standard falcon tube containing normal saline and the prepared samples which were cut into equal squares of 4 mm diameter and kept for 1 h at 37 °C. The positive and negative controls were distilled water and saline, respectively. All the samples and the controls were measured in triplicate and mean values were noted. The supernatant was slowly collected after centrifugation at 3000 rpm for 5 min and used to measure the absorbance at 541 nm by a spectrophotometer. The hemolysis percentage was calculated as according to the following relationship.

$$\text{Hemolysis (\%)} = [(\text{O.D.}_{\text{Sample}} - \text{O.D.}_{\text{Negative}})/ (\text{O.D.}_{\text{Positive}} - \text{O.D.}_{\text{Negative}})] \times 100.$$

where O. D_{Sample} is the measured absorbance of the tested sample as well as $\text{O.D.}_{\text{Negative}}$ and $\text{O.D.}_{\text{Positive}}$ represents the absorbance of negative and the positive control, respectively.

Platelets adhesion

To investigate the effect of prepared samples on platelets adhesion, a human blood was taken from a donor and then centrifuged at 3000 rpm for 5 min to collect the platelet-rich plasma [34]. Equal volume of platelets rich plasma were seeded on the top of electrospun PCL fiber and double layered tublar scaffolds and incubated at 37 °C for 1 h. The platelet were fixed with 4% glutaraldehyde for 10 min and washed several times with PBS. The samples were coated with gold and visualized using SEM.

Statistical analysis

The results are expressed as the means standard deviations. Statistical analysis was performed using Origin 8.0 (OriginLab, Northampton, MA, 01060 USA). The statistical analysis of the obtained results was evaluated using paired Student's *t*-test. The differences between groups were considered significant at a significance level of $p<0.01$.

Results

Morphology of the obtained PCL nanofibers and PCL/PEO double layered scaffolds

Electrospinning is a highly sensitive method due to the numerous processing parameters that influence the morphology and diameter of obtained fibers. In the current study, PCL solutions concentration. and applied voltage were controlled to select the optimal processing parameters for the fabrication of PCL nanofiber. Fig.(1A–C) shows the obtained PCL fiber under different concentrations. (6, 8 and 10 wt. %) of PCL solution and fixed voltage at 15 kv. The SEM observations indicate that PCL solution with solution conc. lower than 8% result in beads formation. The increase of polymer conc. from 8 to 10 wt. % leads to a significant increase in fiber diameter from 812 ± 103 to 1400 ± 300 nm, respectively. While, the increasing of applied voltage from 17 kv and 20 kv results in a clear decrease in fiber diameter from 244 ± 75 to 127 ± 58 nm, respectively. For WO-PCL fibers, PCL solution conc. was kept at 10% as the addition of WO to 8% PCL solution leads to beads formation, as shown in Fig. (1F). The obtained fiber diameter of 10% PCL solution containing different amounts of 100 and 200 μl of WO were 900 ± 175 nm, 225 ± 40 nm, while the ones containing 300 μl appeared to be fused together, as shown in Fig.(1G–I).

To adapt the coating of PCL electrospun layer, the dip casting was performed at different PEO concentrations. Fig.(2A, B) shows the PCL fiber coated with PEO of 5 and 10 wt%., respectively. The homogenous layers were also

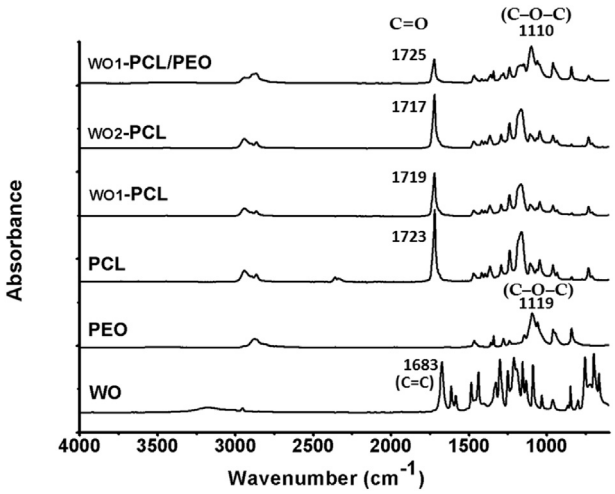

Fig. 3 FTIR spectra of pure WO, PEO and PCL - as well as fabricated WO1-PCL electrospun mats and double layered mats of WO1-PCL/PEO.

observed for PCL/PEO-Hep and WO1-PCL/PEO-Hep, as shown in Fig. (2C, D).

FTIR analysis

The ATR-FTIR spectroscopy was used to observe the changes in the characteristic absorption bands of PCL due to the incorporation of WO and PEO, as shown in Fig. 3. The spectrum of pure PCL electrospun fiber exhibits two bands at 2940 cm^{-1} and 2868 cm^{-1} which are typically due to the symmetric and asymmetric C–H stretching. The sharp band at 1723 cm^{-1} belongs to stretching vibrations of the ester-carbonyl groups (C=O). The two bands at 1293 cm^{-1} and 1165 cm^{-1} are attributed to the C–C and C–O stretching vibrations, respectively. The band at 1238 cm^{-1} is related to the asymmetric (C–O–C) stretching mode [35,36]. The main component of WO is the methyl ester of salicylic acid which has two functional groups carboxylic acid and phenol [37]. Within the spectrum of WO, the broad band with a high intensity at 3194 cm^{-1} represents the stretching vibration of the hydroxyl groups which reflect the strong intra and intermolecular H-bonding of phenolic and carboxylic hydroxyl groups. The band at 2916 cm^{-1} is due to the C–H stretching vibration. The carbonyl stretching (C=O) due to aromatic carboxylic acid was found at 1683 cm^{-1}. The main bands that remain are the aryl ν(C=C) stretches at 1612 cm^{-1}, the asymmetric and symmetric ν(C–O) stretches of the ester groups at 1328 and 1165 cm^{-1} and the asymmetric stretch for acetates around 1213 cm^{-1}. However, the characteristics bands of PCL were also observed for WO-PCL, the WO carbonyl band at 1683 cm^{-1} is not observed which could be due to the small amount of WO. Notably, with increasing amount of WO, the intensity of the absorption band assigned to ester band at 1723 cm^{-1} was changed and shifted to 1719 cm^{-1} for WO1-PCL and 1717 cm^{-1} for WO2-PCL that indicate the interaction between PCL and WO. Moreover, the spectra of pure PEO and WO1-PCL/PEO

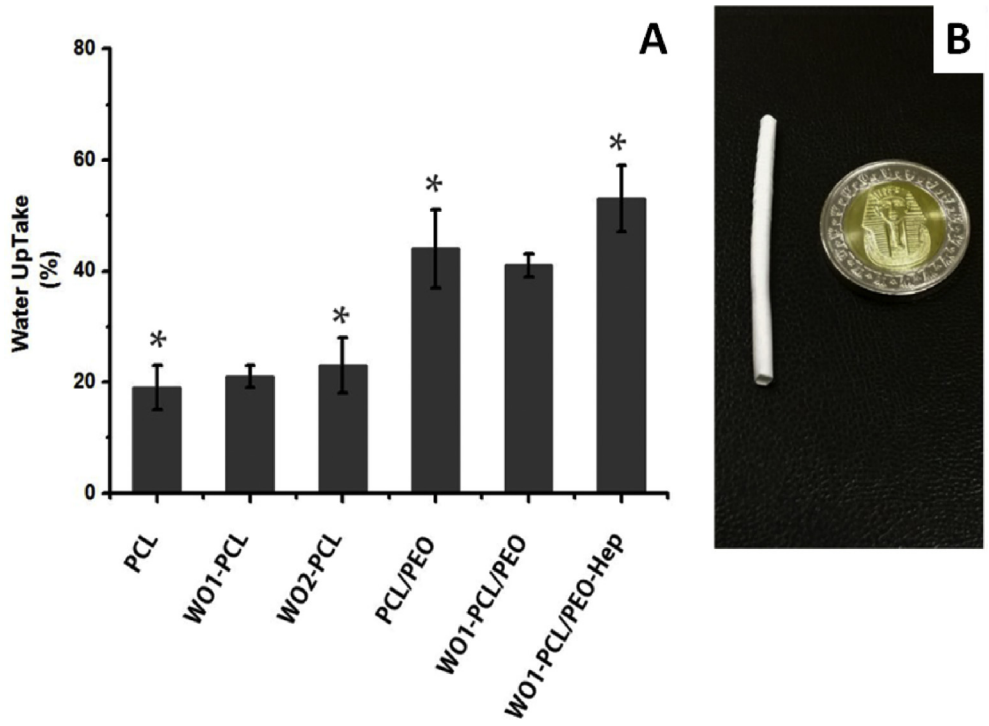

Fig. 4 Represents water uptake percentage of fabricated PCL and PCL/PEO double layered fibers. The inserted photograph of fabricated tubular scaffolds which was obtained using a rotating mandrel to develop a vessel like structure of electrospun PCL fibers. Data are evaluated as mean values ± SD for three experiments. Differences between the samples were determined using the unpaired *t*-test (*$p \leq 0.01$).

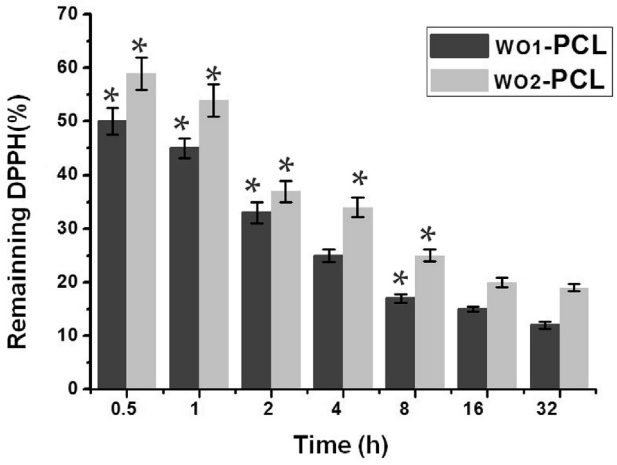

Fig. 5 Shows the remaining DPPH % for WO1-PCL and WO2-PCLsamples after different time intervals. The antioxidant potential was calculated and expressed as mean values ± SD for five experiments ($p \leq 0.01$).

were also investigated for comparison of WO1-PCL and PCL. The absorption bands of PEO were found at 3436 cm^{-1} (O–H stretch), 2906 cm^{-1} (C–H stretch), 1464 cm^{-1} and 1356 cm^{-1} (CH_2 groups in PEO), 1119 cm^{-1} (C–O–C stretch) [37–39]. For WO1-PCL/PEO, the PEO adsorption on the surface was confirmed by the changes of peak intensity at 1725 and around 1110, corresponding to the carbonyl group of PCL and C–O–C linkage of PEO, respectively [37,38].

Water uptake

Since, vascular grafts are in direct contact with blood, the water uptake capability is an important aspect for protein absorption and cellular attachment and nutrient diffusion, but high water uptake could result in morphological changes and destruction of the implant. Therefore, water uptake is a debatable issue that should be investigated. Fig.(4A) shows the water uptake of the obtained fiber mats electrospun mats after soaking in PBS for 24 h at 37 °C. As expected, the overall water uptake was increased for PCL/PEO double layered mats due to hydrophilic nature of PEO. The water uptake of PCL fiber was 19% ± 4 and obviously increase to 44% ± 7 for PCL/PEO, 41% ± 2 for WO1-PCL/PEO and 53% ± 6 for WO1-PCL/PEO-Hep. Meanwhile, the water uptake was slightly increased to 21% ± 2 for WO1-PCL and 23% ± 5 for WO2-PCL. According to the above result, both single and double layered PCL fiber scaffolds are not under huge changes upon exposure to water. Moreover, the double layered scaffolds show higher water uptake that would induce diffusion of WO and Hep to the surroundings as well as biodegradation of PCL electrospun fibers.

Antioxidant assay

The antioxidant activity is well known to depend on the type and polarity of the extracting solvent, the isolation procedures, and the purity of the active compounds, as well as the assay techniques and substrate used [40,41]. The antioxidant activity of WO-PCL fiber mats were measured by DPPH assay after 0.5, 1, 2, 4, 8, 16 and 32 h, as shown in Fig. 5. The results indicate that the difference between WO1-PCL and WO2-PCL samples was not much. Both of the samples exhibit a stable antioxidant capability after 8 h in which the remaining DPPH % were not slightly changed after 16 and 32 h. This could be attributed to the controlled release of WO form PCL occurs after 8 h.

Hemocompatibility: prothrombin time, hemolysis and platelets adhesion

Fig. 6 shows the INR that was applied to measure blood clotting tendency of fabricated samples. The INR for PCL and PCL-

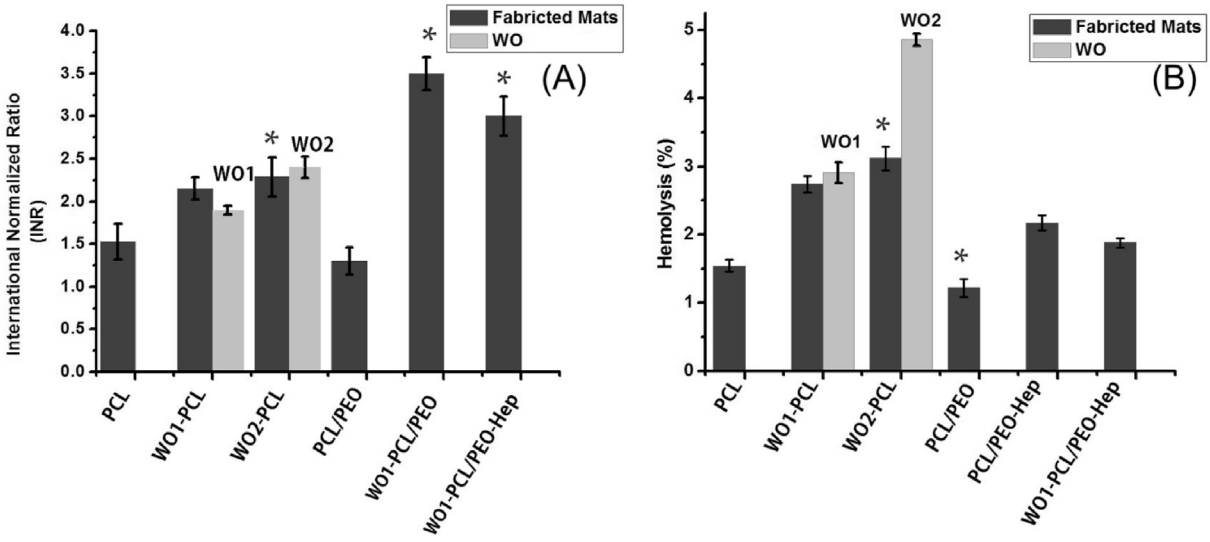

Fig. 6 Represents blood compatibility assay of fabricated single and double layered mats (**A**) Prothrombin assay and (**B**) Hemolysis assay. Data were evaluated as mean values ± SD for five measurements (*$p \leq 0.01$).

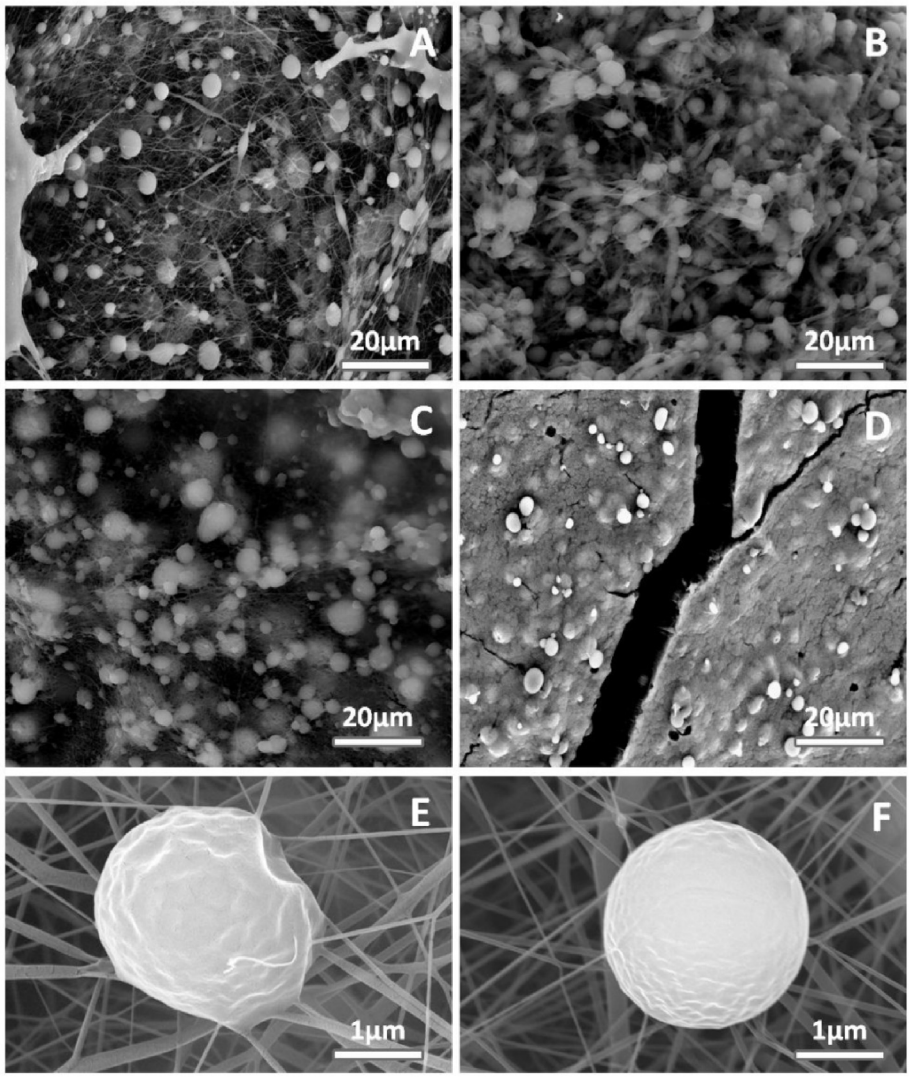

Fig. 7 SEM micrograph of adhered plated on the surface of (**A**) Electrospun PCL mats,(**B**) PCL/PEO mats,(**C**) WO1-PCL/PEO mats, (**D**) WO1-PCL/PEO-Hep, (**E**) Higher magnification of adhered platelet on the surface of PCL/PEO and (**F**) adhered platelet on WO1-PCL/PEO.

PEO were 1.53 ± 0.21 and 1.30 ± 0.16, respectively, which reflect the tendency of electrospun PCL and PEO to induce blood coagulation. While the value for WO1-PCL was 2.15 ± 0.13 and 2.29 ± 0.23 for WO2-PCL. The INR ratio was further increased for PCL/PEO-Hep to 3.5 ± 0.19 and 3 ± 0.23 for WO1-PCL/PEO-Hep. It is important to mention that the INR values of WO were also measured to compare those with WO-PCL fibers as shown in Fig.(6A). The measured ratio for WO1 and WO2 were 1.9 ± 0.05 and 2.4 ± 0.13. The high values of INR (more than 2) highlight the anticoagulation action of heparin and WO. In addition, the results of the H% of fabricated samples are given in Fig. 6. From the data obtained, the H% of neat elctrospun PCL was 1.54 ± 0.9%. However the pure WO samples show a higher H% (2.91 ± 0.15% for WO1 and 4.86 ± 0.09% for WO2), the electrospun WO1-PCL and WO2-PCL exhibit lower values 2.74 ± 0.12% and 3.12 ± 0.17%, respectively. While the hemolytic values for the double layered scaffolds were 1.22 ± 0.13 for PCL/PEO, 2.17 ± 0.11% for PCL/PEO-Hep and 1.88 ± 0.7% for WO1-PCL/PEO-Hep.

The platelets adhesion after incubation with platelet-rich plasma for 2 h at 37 °C was observed by using SEM images, as shown in Fig. 7. In comparison to neat PCL, the double layered PCL/PEO mats resulted in an increase of platelets deposition which could be attributed to the hydrophobic nature of the PCL surface and hydrophilic nature of PEO, as shown in Fig.(7A, B). Remarkably, the number of adhered platelets were reduced with the addition WO (WO1-PCL/PEO) and further decreased by the WO1-PCL/PEO-Hep surface. Fig.(7E, F) show the discoid morphology of platelets on the PCL and (WO1-PCL/PEO-Hep) surfaces at higher magnification.

Discussion

The ideal vascular scaffolds should be biocompatible and biodegradable as well as able to integrate within the host vascular tissue. Moreover, vascular grafts should allow blood flow without any physical or chemical interactions with blood

components (i.e., blood cells or platelets) to avoid thrombosis that is commonly associated with currently used natural and synthetic grafts, especially for small-size blood vessel implants [4,7,42]. Electrospinning has been introduced to simply draw polymeric fibers of ultrafine diameter and extremely large surface area, which mimic the natural extracellular matrix for biomedical application due to their capability to promoted cell adhesion, migration and proliferation [9,11,15]. Over the last decade, electrospun polymeric fibers of aliphatic polyesters have received special attention, because of their biocompatibility, mechanical strength and biodegradability such as poly(caprolactone) (PCL) etc [16]. Although, electrospun mats have been successfully used in tissue engineering, wound dressing and drug delivery systems. The applicability of electrospun fibers as a vascular grafts is often restricted because of their hydrophobic nature and their tendency to induce blood coagulation. To overcome the above description, we introduced a facial approach to improve performance of PCL fibers via coating with PEO and the addition of WO as a natural oil to offer a range of biological benefits. The result within this study demonstrates the capability of WO to improve the blood clotting tendency and hemolytic abilities of electrospun PCL vascular scaffolds. For instance, the prothrombin time test shows that the INR value of PCL and PCL-PEO were lower than 2, which reflect the tendency of electrospun PCL and PEO to induce blood clotting. On the other hand, the values for the sample containing WO were higher than 2 which indicates capability of WO to delay blood clotting process [42]. These result were supported with H% test which indicate that the fabricated PCL and PCL/PEO with and without WO or Hep are classified as non-hemolyic materials and meet the essential requirements for vascular grafts due to their hemolytic percentages which are lower than 5% [43]. Moreover, it is well known that platelet adhesion and aggregation are one of the essential key parameters to activate the coagulation pathway. The release of platelet factor from the active platelets can activate pro-thrombin to result in coagulation. Therefore, the adhesion of platelets on the vascular scaffolds can be used to determine the anticoagulant potential and blood compatibility of the vascular scaffolds [44]. Remarkably, the number of adhered platelets were reduced with the addition WO (WO1-PCL/PEO) and further decreased by the WO1-PCL/PEO-Hep surface, as shown in Fig. 7 which reflect the ability of WO to decrease the tendency of electrospun PCL to induce blood clotting via delaying platelets adhesion on the surface of vascular grafts.

Conclusions

Tubular vascular grafts of multiple functions were prepared by combining electrospinning and dip coating approaches. The obtained electrospun PCL fibers functionalized by WO and Hep were added to PEO as an anticoagulant agent. The hydrophicity of the double layered PCL/PEO mats was improved upon comparing with single layered PCL mats. The double layered grafts exhibited a good blood compatibility in terms of their non hemolytic features and tendency to delay blood clotting processes - as well as antioxidant activity. However, it is important to highlight that the amount of WO should be controlled to avoid side effects. In summary, the fabricated PCL/PEO mats may be considered as a multifunctional conduit for small-diameter (less than 6 mm) and are valuable for further assessment - such as *in-vitro* cell culture and *in-vivo* studies.

Conflicts of Interest

The authors have no conflicts of interest to disclose.

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