MORPHOLOGICAL, MECHANICAL AND BIOLOGICAL PROPERTIES OF NOVEL PCL- Cs/PVA MULTI LAYER NANOFIBROUS SCAFFOLDS

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Poly caprolactone and chitosan were used to fabricate multi-layer scaffolds using electrospinning technique. The effect of layers arrangement on the biological and mechanical properties of the nanofibrous multilayer composites was studied. PCL was dissolved a) in methylene chloride/dimethyl formamide (MC/DMF 4:1) to make 10% solution and, b) in 90% aqueous acetic acid (90AA) to make 15% solution. Chitosan/PVA (2:3) was dissolved in 80% aqueous acetic acid. Nanofibers webs were prepared from each solution under constant conditions. The number of layers were varied from 2-4 to make the final samples. SEM was used to study the morphology of the webs. Cross sectional micrographs show the interface of layers and the layer arrangements. Mechanical properties of the webs were measured by Universal testing machine. The results show that the webs containing PCL dissolved in 90AA had better mechanical properties. The porosity of each nanofibrous layer was calculated and the results showed that the porosity of PCL in MC/DMF is better than the others. MTT assay was carried out on the resulted nanofibrous web to investigate the proliferation rate of fibroblast cells on the scaffolds. SEM micrographs also were used to study the morphology of cells adhering on the nanofibrous scaffolds.

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1. Introduction

Chitosan is a natural polysaccharide derived from de-acetylation of chitin. It has intrinsic biological properties such as biocompatibility, biodegradability and antimicrobial property. Because of this unique nature, chitosan is one the most interesting materials for different application such in biomedical, filtration, tissue engineering, etc [1,2]. On the other hand, nanotechnology as a novel technology in recent century has opened a new door of knowledge for scientific and industrial researches. In the recent years, electrospinning has been found to be a novel technique to produce nanofibers from different polymer solutions. In a typical electrospinning process, fibers are drawn from a solution or melt through a blunt needle by electrostatic forces. Nanofibrous webs regardless of the material used and because of such interesting properties like porosity, high surface to volume ratio, 3-D structure, etc find enormous applications [3-5]. In this regards, chitosan nanofibrous webs are used for biomedical and tissue engineering application [1, 6, 7]. But there are some complicities for converting chitosan or any other natural polymers (e.g., gelatin, alginate, chitin, etc.) to nanofibers using electrospinning. In fact because of their polyionic nature and high viscosity of solution, they do not produce uniform fibers. Usually hydrophilic polymer such as polyvinyl alcohol, polyethylene oxide and etc are used as second component to prepare a blend solution to facilate electrospinning [6-11]. Another drawback is low mechanical properties of the nanofibrous webs made from chitosan for some applications such as skin tissue engineering.

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This problem may be overcome by using another polymer with higher mechanical properties. This may be in the form of fabricating multi-layer webs or producing web from mixed polymer solution or polyblend solutions [12]. Polycaprolactone is a semi-crystalline biocompatible poly (α -hydroxy ester) and its mechanical properties are high enough for biomedical applications

[13]. There are some reports available dealing with chitosan-PCL blend products [14-20]. However, we could not find any report regarding the effect of multilayer chitosan/PCL on the biological properties of the nanofibrous webs. Sarasam et al., [14] had prepared homogenous blends of chitosan (Mw>310KDa) and PCL (80KDa) in a common solvent of ~77% aqueous acetic acid. They found that, 50:50 blends when processed at 55°C in an oven showed significant improvement in mechanical properties as well as support for cellular activity relative to chitosan. Malheiro et al., [16] reported production of poly (e-caprolactone)/chitosan blend fibers via wet spinning for the first time. Fibers of chitosan and PCL were prepared from blend solutions, using a formic acid/acetone 70:30 v% mixture as common solvent and methanol as coagulant by wet spinning process. The SEM micrographs showed the existence of porous and rough surface which could be potentially advantageous for cell attachment. Shalumon et al., [19] fabricated a fibrous scaffold comprising of chitosan (Cs) (0.5% to 2%) and poly (E-caprolactone) (PCL) (6%) using electrospinning from a novel solvent mixture consisting of formic acid and acetone. The results showed that lower concentrations of PCL lead to beaded fibers where as 8% and 10% of PCL in lower compositions of chitosan resulted in fine nanofibers. In our previous work, the blend nanofibrous webs of PCL/Cs/PVA (2:1:1.33 and 2:1:1.5) were fabricated successfully. The results show that the blend webs of PCL/CS/PVA have higher tensile strength than Cs/PVA webs [20]. Hong and Kim [21] fabricated biocomposites composed of poly (ε-caprolactone) (PCL) fibers and chitosan by two different fabrication methods: a two-step layer-by-layer technique (normal electrospinning of PCL and air-spraying of chitosan solution) and a co-electrospinning process (electrospinning of mixture of PCL solution and chitosan powder). The authors concluded that resulting composites exhibited improved mechanical properties in compared with the pure PCL fiber mats. In addition the biological response of mesenchymal stem cells (MSCs) i.e.; cell attachment, higher proliferation, and morphological homogeneity, on the biocomposites was superior to that on pure PCL.

In the present work, 2, 3, 4-layer webs were produced from layer-by-layer electrospinning of Cs:PVA (2:3) layer(s) and PCL layer(s). With respect to solvent selected, there are two different group of multi-layer. In first group, 90% acetic acid was the solvent for PCL and in second group, Methylene Chloride/Dimethyl formamide (4/1) was PCL solvent. SEM micrographs were used to investigate the cross section of multi-layer webs. Tensile strength tests were carried on the webs using Universal testing machine. Bioactivity and cell-compatibility of the multi-layer nanofibrous scaffold was evaluated by in vitro cell culture and contact angle measurements. MTT assay was carried on the resulted nanofibrous web to investigate the proliferation rate of fibroblast cells on the scaffolds. SEM micrographs also were used to study the morphology of cells adhering on the nanofibrous scaffolds.

2. Material and methods

Chitosan (Mw 1000 KDa, 85% DD), Poly (vinyl alcohol) 88% hydrolyzed (Mw 120 KDa) were purchased from Merck, Co. PCL (Mw 80 KDa) were purchase from Sigma-Aldrich Glacial acetic acid, methylene chloride and dimethyl formamide (DMF) were purchased from Merck Co. and used without any purification.

Solutions preparation

5% solutions of Chitosan in 80% acetic acid were mixed with 10% concentration of PVA to make 2/3 (w/w) ratio. Polycaprolactone (PCL) was dissolved in two different solvents i.e.; MC/DMF and 90% acetic acid (90AA). 10% concentration of PCL solution in MC/DMF and 15% concentration of PCL solution in 90% acetic acid were prepared according to our previous report [22].

Electrospinning procedure and webs preparation

Briefly in this technique, polymer solution is placed in a 20ml syringe and a high voltage is applied using a high voltage power supply (Gamma high voltage research) to overcome the surface tension of polymer solution, and then a charged jet is ejected. The jet extends in a straight line for a certain distance and then bends and follows a looping and spiraling path. These jets get dried to form nanofibers which are collected on a target (aluminum foil) as nanofibers. Cs/PVA solution was electrospun at the following conditions: voltage: 15 KV, extrusion rate: 1ml/hr and the distance between nozzle and collector: 15 cm. PCL layers were electrospun at 20 KV applied voltage, 20 cm needle to collector distance and 1ml/hr extrusion rate. All electrospinning were carried out at room temperature.

2-layer (Cs/PVA + PCL), 3-layer (CS/PVA + PCL + CS/PVA) and 4-layer (CS/PVA + PCL + CS/PVA + PCL) webs were prepared with Cs/PVA and PCL electrospun on top of each other.

Characterization and measurement

The cross sections of multi-layer webs were observed with a scanning electron microscope (SEM) (XL30-SFEG, FEI/Philips) with gold coating. Mechanical properties were measured using INSTRON (model 5566 made in U.K.). The hydrophilicity of the resulted webs was qualitatively determined by measuring the contact angle of distilled water drop on the web, using Kruss drop shape analyzer (DSA 100). The webs were cut into 12mm^2 and fixed into the custom made sample holder of the drop shape analyzer. The distilled water was taken in a 2 ml syringe fitted with blunt edge needle. A single drop of volume~2µl was dropped on the webs. The drop shapes on the webs surfaces were recorded using the camera attached to the drop shape analyzer after 3 seconds. Initial contact angle values on the webs were measured from recorded images. The contact angle was measured by the sessile drop approximation of the inbuilt software of the instrument.

Nanofibrous web sterilization

All nanofibrous webs on coverslips were kept in 24 well culture plates. The nanofiber scaffolds on coverslips were sterilized using UV-ray. Thereafter, the scaffolds were soaked in PBS and in cell culture medium overnight prior to cell seeding, in order to facilitate protein absorption and cell attachment on nanofibers.

Cell culture

Rat fibroblast cells (L929) according to ASTM-F813 were cultured in containing 5% FBS and growth factors in 75 cm² flasks. The cell culture was maintained at 37 °C in a humidified CO_2 incubator for 3 days and Fibroblast cells were harvested from 3rd passage cultures by trypsine EDTA treatment and replated. Populations of cell lines used in this experiment were between passages 6 and 7.

MTT assay

For MTT test, fibroblasts were cultured on the scaffolds for 72 hr (scaffolds plus fibroblasts samples) and their proliferation rate was compared with the fibroblasts cultured on standard plastic culture surfaces (fibroblasts only samples). Six samples were prepared in each group for this purpose. MTT test was performed according to the manufacturer's (Sigma) instructions and the colorimetry was performed at 530 nm. To correct the possible absorption of MTT or formazan by scaffolds, the same weight of scaffolds used for scaffolds plus fibroblasts samples were added to the fibroblasts only samples at the time of adding MTT solution. A blank OD value was reduced from each sample's reading.

Fibroblast study by SEM

Fibroblast cells were seeded $(9 \times 10^4$ cells/well) on control TCP (Tissue Culture Plate), nanofibrous webs from PCL solutions in 90AA and MC/DMF, CS:PVA web and the resulted 2-layer and 3-layer webs, on 24 well plates. After 72 h of the experiment, fibroblast cells grown on scaffolds were washed with PBS to remove non-adherent cells and then fixed in 4% glutaraldehyde for 1 h at room temperature, dehydrated through a series of graded alcohol solutions and finally dried into hexamethyldisilazan overnight. Dried cellular constructs were

sputter-coated with gold and observed under a scanning electron microscope (SEM) at an accelerating voltage of 15 kV.

Statistical analysis

All quantitative results were obtained from triplicate samples. Data were expressed as the mean GSD. Statistical analysis was carried out using unpaired Student's t-test. A value of p<0.05 was considered to be statistically significant.

3. Results and discussion

Morphological study on resulted nanofibrous webs

Cross sectional SEM micrographs of 2, 3 and 4-layer nanofibrous webs are shown in Fig.1 and Fig.2 (PCL layer(s) was/were made of PCL dissolved in MC/DMF and 90% acetic acid respectively). As it is clear from Fig.1, due to dissimilarity of the solvents of different layers (CS:PVA in 80% acetic acid and PCL in MC/DMF), there is (are) distinct gap(s) between layers. This phenomenon is clearly observed in 3-layer web (Fig1.b). It could be due to lower boiling point of MC/DMF in compared with acetic acid. In this case dried PCL nanofibers were collected on semi-dried CS:PVA nanofibers and a distinct boundary is obtained.



Fig. 1: SEM cross sectional micrographs of multi-layer webs with different number of layer: a) 2-layer, b) 3-layer web and c)4-layer web; PCL layer is electrospun from 10% PCL in MC/DMF (4/1).



Fig. 2: SEM cross sectional micrographs of multi-layer webs with different number of layer: a) 2-layer, b) 3-layer web and c)4-layer web; PCL layer is electrospun from 15% PCL in 90% acetic acid.

In the other group because of similarity of the solvents of different layers, there were good attachments between the layers (Fig.2). On the other hands, welding is occurred between the layers which could lead to higher strength in this group of multi-layer webs (PCL in 90AA) in compared with other multi-layer webs (PCL in MC/DMF).

1440

Mechanical results

The mechanical properties of the webs are shown in table 1. The mechanical results shows that the tenacity of PCL in 90AA web is better than PCL web in MC/DMF, which causes better tenacity for the multi-layer webs including PCL in 90AA compared to the webs consisting PCL in MC/DMF. On the other hand, existence of gaps between chitosan layer(s) and PCL layer(s) in the multi-layer webs in which the PCL layers were made from PCL dissolved in MC/DMF, which leads to interruption of layers, may be another reason of lower tenacity of this group. Another reason could be the nature of the solvents used. 90AA is a single and homogenous solvent whereas; MC/DMF is a solvent system which can turn to inhomogeneous solvent system by changing the ratio of MC to DMF. In 90AA it seems fibers get more oriented and this molecular orientation leads to faster solidification and better mechanical properties.

Table 1: Mechanical properties of electrospun webs from PCL, CS/PVA and the multi-layers webs.

Sample	Max. Load (CN)	Tensile strain (%)	Thickness (micron)	Tenacity (Max.load/area) CN/cm ²
PCL 90AA	86.42	16.11	50	17284
PCL MC/DMF	66.66	74.44	48	13604
Cs/PVA (2:3)	81.25	15	45	18055
2-layer (PCL in MC/DMF)	51.6	113.89	50	10320
3-layer (PCL in MC/DMF)	114.02	21.11	80	14252
4-layer (PCL in MC/DMF)	157.28	32.33	110	14298
2-layer (PCL in 90AA)	66.6	5.56	50	13320
3-layer (PCL in 90AA)	124.62	28.89	80	14661
4-layer (PCL in 90AA)	164.6	10	110	14963

As it can be seen from the fiber diameters, the average diameter of fibers electrospun from 90AA is much lesser than ones electrospun from MC/DMF. In another word a constant volume of polymers solution is ejected from the tip of needle but due to higher conductivity of 90AA, electrical field has more severe effect on the jet and the path travelled by the jet is longer, resulting in finer narrow fibers (Table 2).

Sample	Average diameter (nm)		
PCL in 90AA	117±30 nm		
PCL in MC/DMF	767±307 nm		
Cs/PVA (2:3)	97±12 nm		

Porosity of scaffolds

The porosity of the electrospun nanofibrous webs were calculated using Eq.(1) and Eq.(2).

$$\rho_{\text{web}=\frac{W_{\text{web}}}{D\times S}}$$
(1)
$$P(\%) = \left[1 - \frac{\rho_{\text{web}}}{\rho_m}\right] \times 100$$
(2)

Which ρ_{web} is the web density (g/cm³), W_{web} is the web mass (g), D and S are web thickness (cm) and web area (cm²) respectively P is the web Porosity (%) and ρ_m is density of the raw materials (g/cm³).

The density of PCL, Chitosan and PVA which were used in this work were 1.14 g/cm³, 0.5 g/cm³ and 0.5 g/cm³, respectively. The calculated porosity of each layers of the webs are showed in Table 3. According to table 3, the porosity of PCL layers made of PCL solution dissolved in 90AA and MC/DMF were around 85% and 88%, respectively.

Sample	Weight (g)	Thickness (µ)	Web density (g/cm ³)	Web porosity (%)
PCL in 90AA	0.0069	400	0.1725	84.8%
PCL in MC/DMF	0.0004	30	0.134	88.3%
Cs/PVA (2:3)	0.0004	40	0.125	75%

Table 3: The porosity of each layer of the multi-layers electrospun webs.

In the same number of layers the porosity of multi-layer web which its PCL layer(s) was/were from PCL in MC/DMF, was higher. It may lead to better cell spreading, attaching and proliferation for the multi-layer webs with PCL layer(s) made of PCL dissolved in MC/DMF solvent.

Hydrophilicity

Hydrophilicity of the single and multi-layer webs were determined by contact angle measurements to investigate the effect of layers arrangement using distilled water on hydrophilicity of webs. Generally, hydrophilic surfaces would give a contact angle between 0° and 30° and less hydrophilic surfaces show a contact angle up to 90°. Materials which show a contact angle more than 90° is usually termed as hydrophobic. The contact angle images of CS: PVA blend webs, PCL webs and multi-layer webs are shown in Fig. 2. CS: PVA web shows a contact angle of 43.8±2 which indicates the hydrophilic nature of the blend nanofibrous web. However, due to hydrophobic nature of poly(caprolactone), PCL in 90AA and MC/DMF webs show 107.7±3 and 100.9±2 contact angles (Fig. 2b,f). PCL in MC/DMF shows lower contact angle compare with PCL in 90AA due to its higher porosity (88.3%). Generally, when CS: PVA layer(s) was/were incorporated with PCL layers a decrease in the contact angle values was observed. 2-layer webs (CS: PVA + PCL (in 90AA or in MC/DMF)) show contact angle 64.3±6 and 60.9±3.



Fig. 2: The contact angle images on a) CS: PVA; b, c, d, e) PCL in 90AA and 2-layer, 3-layer, 4-layer; f, g, h, i) PCL in MC/DMF and 2-layer, 3-layer, 4-layer scaffolds using distilled water.

1442

SEM study of scaffolds with cells

 9×10^4 cells/well Fibroblast cells were seeded on control TCP (Tissue Culture Plate), PCL nanofibrous layers made of PCL solution dissolved in 90AA and MC/DMF, 2-layer, 3-layer and 4-layer webs of CS/PVA and PCL (in 90AA and in MC/DMF) on 24 well plates.

Fig. 3 shows the SEM micrographs of cells morphology and interaction between cells and nanofibrous webs (PCL in 90AA (Fig.3a), PCL in MC/DMF (Fig. 3b), CS: PVA (Fig. 3c), 2-layer, 3-layer and 4-layer nano fibrous webs (Fig. 3 d, e and f) which the PCL layer(s) was/ were from PCL in MC/DMF) after 72 h. AS it can be seen fibroblast cells attached and spread on the surface of all scaffolds showed normal morphology (Fig.3). Due to random orientation of nanofibers in webs, the cells grew in all direction on the webs surface. On the other hand, forming a three-dimensional and multicellular network need to cell proliferation to fill the pores of webs.



Fig. 3: SEM micrographs of Fibroblast cells seeded on different nanofibrous scaffold: a) PCL in 90AA (2500x); b)PCL in MC/DMF (5000x); c) CS/PVA (1000x); d) 2-layer Cs/PVA+PCL in MC/DMF (1000x); e)3-layer CS/PVA+PCL (MC/DMF)+ CS/PVA (2500x); f) 4-layer CS/PVA+PCL (MC/DMF)+ CS/PVA+PCL (MC/DMF) (2500x).

Fibroblast cells show a proper morphology and well spreading and attaching on CS/PVA nanofibrous webs (Fig. 3c), whereas PCL nanofibers (Fig. 3a,b) showed that the cells were not attached and spread firmly. For 3-layer and 4-layer nanofibrous webs (Fig. 3e and 3f) it can be seen that cells attached to the nanofibrous webs and normal cell morphology leading to tissue-like architecture after72 h. But for 2-layer nanofibrous web (Fig 3d), cells attaching and spreading were not as well as the others webs. Existence of another CS: PVA layer as bellower layer in 3-layer and 4-layer webs could be considerable response to better biological behavior of these layers.

The observations show that in multi-layer nanofibrous webs, chitosan biological properties are added to PCL mechanical properties and lead to a nanofibrous webs that can definitely support cell adhesion, infiltration and proliferation to form a new tissue.

MTT results

In order to monitor cell adhesion and proliferation on different substrates, the number of cells was determined using the colorimetric MTT assay. The mechanism behind this assay is that metabolically active cells react with a tetrozolium salt in the MTT reagent to produce a soluble formazan dye. The cellular constructs were rinsed with PBS followed by incubation with 20% MTT reagent in serum free medium for 3 h. Thereafter, aliquots were pipetted into 96 well plates and the samples were read in a spectrophotometric plate reader. Table 4 shows the MTT results for the resulted nanofibrous webs. If P-value^{*} is more than 0.05 it indicates that there is no significant difference between the scaffold and its positive control.

Fibroblasts on		Colorimetric Reading		
	Number of samples	Average OD (± SD)	P value*	
PCL in 90AA	6	0.42967 (± 0.019775)	< 0.001 [†]	
plastic surface	6	$0.69033 (\pm 0.083546)$	< 0.001	
PCL in MC/DMF	6	0.83517 (± 0.029424)	< 0.001 [†]	
plastic surface	6	0.3195 (±0.035331)	< 0.001	
CS:PVA 2:3	6	0.94633 (± 0.037495)	> 0.05	
plastic surface	6	0.94983 (± 0.063528)	> 0.05	
2-layer web (PCL in MC/DMF)	6	0.88083 (± 0.010907)	$< 0.001^{\dagger}$	
plastic surface	6	1.1105 (± 0.038172)		
3-layer web (PCL in MC/DMF)	6	0.81633 (± 0.02225)	> 0.05	
plastic surface	6	0.83333 (± 0.06413)		
2-layer web (PCL in 90AA)	6	0.709 (± 0.087038)	< 0.01 [†]	
plastic surface	6	0.86833 (± 0.03176)		
3-layer web (PCL in 90AA)	6	0.484 (± 0.01352)	< 0.001 [†]	
plastic surface	6	$0.76783 (\pm 0.028266)$		

Table 4: MTT results for electrospun nanofibrous webs.

* Independent samples t test, SPSS 16.0 for Windows (Release 16.0.1)

† Statistically significant difference with assumption of equality of variances based on Levene's test

As it can be seen from table 3 for CS: PVA and 3-layer web (PCL dissolved in MC/DMF) scaffolds P-value^{*} is more than 0.05. So these scaffolds enhance the infiltration amount and are proper to select for other biological investigation like in vivo tests.

Cell proliferation

Fibroblast cells proliferation assay indicated that the living cells in a scaffold and the cells proliferated in culture to fill the void space present between the polymer fibers under all conditions, resulting in the generation of new tissue. The proliferation of cells was observed after 72 h (Fig. 3). PCL nanofibers, 2-layer and 4-layer web showed significant (P-value^{*} < 0.01) decrease in cell proliferation rate compared to control TCP, whereas CS: PVA nanofibrous web and 3-layer web (PCL in MC/DMF) showed significant levels of increase in proliferation compared to TCP (P-value^{*} > 0.05), after 72 h.

4. Conclusion

In this study nanofibrous multi-layer scaffolds from Chitosan (CS) / Polyvinyl alcohol (PVA) and polycaprolactone (PCL) were electrospun successfully. All multi-layer webs showed proper morphology for utilizing as nanofibrous scaffolds. The tenacity of 4-layer webs without considering the PCL solvent was more than other 3-layer and 2-layer webs, but generally the

multi-layer webs from PCL in 90AA showed better mechanical strength in compared with the multi-layer webs from PCL in MC/DMF. The contact angle measurements showed that the hydrophobic character of poly(caprolactone) has been reduced by fabricating multi-layer webs with embedding CS: PVA layer(s) among PCL layer(s) and this could enhancing the bioactivity of the resulted webs. The results obtained from the porosity of the webs show that the porosity of PCL layers made of PCL solution dissolved in 90AA and MC/DMF were around 85% and 88%, respectively. And it may lead to better cell spreading, attaching and proliferation for the multi-layer webs with MTT results. It was observed from SEM micrographs that in multi-layer nanofibrous webs that can definitely support cell adhesion, infiltration and proliferation to form a new tissue. From MTT assay, CS: PVA and 3-layer webs (PCL in MC/DMF) enhance the infiltration amount and are proper to select for other biological investigation like in vivo tests.

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