Assessing the physical and mechanical properties of poly 3-hydroxybutyrate-chitosan-multi-walled carbon nanotube/silk nano–micro composite scaffold for long-term healing tissue engineering applications

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The new nano–micro hybrid scaffolds were fabricated through electrospinning the poly 3-hydroxibutyrate-chitosan-multi-walled carbon nanotube (MWNT) nanofibres on the silky knitted microfibers, orderly and randomly. The scaffolds were prepared by varying the concentration of MWNT. The samples were compared as to their alignment and MWNT concentration. The morphological and physical properties were assessed through scanning electron microscopy, Fourier transform infra-red (FT-IR) spectroscopy and water contact angle test. Mechanical properties were determined through tensile strength test run on optimal samples chosen according to the results obtained from above-mentioned tests. The morphological view of the scaffolds showed that an increase in the amount of MWNT up to 1 wt% led to a better fibres diameter distribution and alignment in comparison with other samples. The porosity percentage of all scaffolds were >80% which is appropriate for tissue engineering applications. The FT-IR spectra indicated that the nanofibrous coat on knitted silk did not have any effect on crystallinity structures of silk fibroin. There existed a direct relation between hydrophilicity of scaffolds and MWNT concentration according to water contact angle. The presence of nanofibrous coat on knitted silk had no effect on tensile strength increment in comparison with pure knitted silk. The poly 3-hydroxybutyrate-Chitosan-1 wt% MWNT/Silk scaffolds could be an appropriate biomimetic for extracellular matrix of long-term healing tissues in order to their tissue engineering applications.

1. Introduction: Compact connective tissues such as tendon, ligament and articular cartilage consist of collagen I and they are involved in joint movement and stability of tissues. These tissues have a long-term healing process due to lack in blood vessels and neurons existed in them [1]. Tissue engineering is a promising treatment which can introduce scaffolds which intern regenerate and heal the damaged tissue(s) through replacing it with engineered tissue(s) [2]. This process is a combination of engineering and biologic sciences [3]. Designing a biodegradable scaffold which could simulate bioenvironmental conditions is the first step in tissue engineering. The function of scaffold is to simulate extracellular matrix (ECM) of the natural tissue and be a biomimetic of it; consequently, being an appropriate environment for cell survival, regulate cell behaviour and function is mandatory for a scaffold [2]. An ideal scaffold should have biocompatibility, a 3D porous structure with interconnected porosity, biodegradability, harmonic degradation rate together with new tissue growth and must have controllable mechanical properties during degradation process [4]. ECM consists of regular nanostructure which surrounds the cells inside the natural tissues. An implementation of nanofibrous scaffolds provides an appropriate place for cell attachment and proliferation. Moreover, nanofibres have high aspect ratio and appropriate biomimetic of ECM architecture [5]. One of the most efficient technics in fabricating nanofibrous scaffolds is electrospinning; because of its 3D and porous architecture and ease in application. This technic consists of syringe dosage pump, high-voltage power supply and collector. The power supply has two electrodes that provide an electromagnetic field between the solution and the collector. At this phase, the fluid becomes charged and stretched from the tip of the needle to the collector sheet [6, 7].

Bioresorbable polymers are the appropriate choice in producing electrospun scaffolds. Synthesis polymers have good mechanical properties with a weak cell interaction which is due to their hydrophobicity properties [8]. Two of the procedures in enhancing polymers properties are alloying and coating [8]. Poly 3-hydroxybutyrate (P3HB) is a synthesis thermoplastic polyester with microorganisms as their source. P3HB has appropriate mechanical properties, biodegradability, biocompatibility, non-toxicity and is hydrophobic with high crystallinity degree and slow degradation rate [4, 9]. Natural polymers are of high hydrophilicity. Chitosan is a highly biodegradable biopolymer, biocompatible, accessible and cost effective with no adequate mechanical properties; consequently, its application in combination with P3HB is beneficial [9, 10]. The P3HB/chitosan scaffold that was prepared by electrospinning for cartilage tissue engineering applications indicated that the presence of chitosan in the composition led to an increase in degradation rate and hydrophilicity properties in comparison with pure P3HB [11]. Consumption of P3HB plus chitosan polymeric composition provide inadequate mechanical properties for long-term healing tissue engineering [11]; consumption of ceramic reinforcement can enhance the strength of the composition and consuming nanomaterial in preparing scaffolds can mimic the nanostructure of ECM [12]. Functionalised carbon nanotubes (CNTs) in two forms of single walled carbon nanotube (SWNT) and multi-walled carbon nanotube (MWNT) are consumed as biomaterial in order to improve the features of scaffolds [12]. CNTs have specific features such as high mechanical strength, high flexibility, low density, high length to diameter ratio and surface-to-volume ratio, electrical and thermal conductivity [13]. Functionalisation of CNTs leads to an increase in hydrophilicity, biocompatibility, uniform distribution and provides a good connection with polymeric matrix [14, 15]. These properties allow CNTs to be applied as fibers reinforcement in low-density composite materials like polymeric-based composites [12, 15]. As for P3HB-Chitosan/MWNT scaffold in cartilage

tissue engineering, the scaffold with more MWNT had lower degradation rate and higher mechanical strength than pure P3HB and P3HB/Chitosan scaffolds [16]. Regeneration of long-term healing tissues last about 6 months; therefore nanofibrous scaffolds must hold its full mechanical strength in the first 3 months and hold at least half of the rest for the next 3 months [2]. Nanofibres can just mimic tissue surface properties and stimulate cell behaviour [17]. The mechanical behaviours of texture scaffolds are similar with connective tissues like cartilage. Texture scaffolds are developed for long-term healing tissue engineering and are in woven, wire-roped and knitted forms [1]. Texture microfibers can provide adequate mechanical strength until the end of tissue heals period [2]. Silk which is an organic biomaterial with a fibroin core surrounded with a gummy protein named sericin is consumed as microfibers in texture scaffolds. Semi-crystalline structure of fibroin is of high strength and toughness while being flexible. Fibroin due to its biocompatibility, hydrophilicity and slow degradation rate is of major concern. The presence of sericin near the fibroin is toxic with adverse features. A process of separating sericin from silk named degumming is essential in silky texture scaffolds production [1]. Nano–micro hybrid scaffolds are made of a texture of microfibers substrate coated with electrospun nanofibres in a two-step procedure. This hybrid scaffold unlike their single-phase counterpart is of mechanical properties due to microfibers and appropriate cell response due to nanofibres features as one [1]. The starch-based hybrid scaffold with poly ε-caprolactone (PCL) electrospun nanofibres revealed that the nanofibres act as nanobridges between microfibers, similar to ECM architecture [17]. Electrospinning poly (lactic-*co*-glycolic) (PLGA) on knitted PLGA microfibers led to sufficient mechanical strength and integration, moreover the presence of nanofibres enhanced cell behaviour [18]. Electrospinning PLGA nanofibres on silky knitted microfibers revealed that the presence of silk led to higher mechanical strength and lower degradation rate during regeneration of long-term healing tissues [2]. As for electrospinning P3HB or PCL nanofibres on a silky knitted microfibers substrate, the hybrid scaffolds provided adequate mechanical strength [1]. Electrospinning P3HB-Chitosan nanofibres on knitted silk microfibers revealed higher mechanical strength than P3HB/silk hybrid scaffold and pure knitted silk texture. Moreover, cell studies on chondrocytes for the same scaffold revealed the highest growth, attachment and proliferation of cells in comparison with control samples [19].

In this Letter, applying knitted silky texture and the presence of MWNT in P3HB-Chitosan-MWNT/Silk nano–micro hybrid scaffold is a new technic in order to enhance the mechanical properties and improve mechanical support in long-term healing tissue engineering. The physical and mechanical properties of this newly proposed nano–micro hybrid scaffold are assessed by scanning electron microscopy (SEM), Fourier transform infra-red (FT-IR), water contact angle and tensile strength test.

2. Method and materials: The method adapted in this Letter is experimental.

2.1. Materials: P3HB (M_w = 300,000 g/mol) and chitosan (M_w = 1526.454 g/mol, deacetylation degree = 75–85%) were purchased from Sigma-Aldrich (USA). MWNTs (with inner diameter = 3–5 nm, outer diameter = 5–15 nm, length = 50 µm and purity of 95%) functionalised by carboxyl group (COOH) was purchased from US Nano Co. (USA). trifluoroacetic acid (TFA = CF_3COOH, M_w = 169.87 g/mol, density = 1.49 g/ml with purity of 99%) and chloroform ($CHCl_3$, M_w = 119.38 g/mol with purity of 99.5%) were purchased from Merck (Germany). Bombyx mori Silk of three twisted filaments, each with 63 filaments (totally 189 filaments) was purchased from Kiashahr Co. (Iran).

2.2. Fabricating the knitted silk substrate: Knitted ribbon silk (width = 1 cm) was knitted through the knitting machine (Passap duomatic, Isfahan University of Technology, Iran) knitting machine. The degumming process was run on the knitted silk.

2.3. Degumming knitted silk: The knitted silk scaffold was first boiled in the degumming solution of 0.25% sodium carbonate (Na_2CO_3) at 94–98°C for 30 min, next the degumming solution was changed with a fresh degumming solution and the process is repeated for another 60 min, then the knitted silk is rinsed with distilled water [1, 2]. The colour change from yellow to white is the end point of this process, indicating that the sericin is completely removed, according to the literatures [2, 20, 21].

2.4. Preparing the electrospinning solution: First a 9 wt% optimal concentration of P3HB was stirred and dissolved in TFA for 50 min at 45–50°C; next a 20 wt% optimal concentration of chitosan is added to P3HB solution and stirred and dissolved in 30 min [11, 16, 19]; then different concentration of MWNT at 0, 0.5 and 1 wt% was added to P3HB/Chitosan solution and was stirred for 6 min. Sonication is run for 12 s in order to distribute MWNTs uniformly.

2.5. Fabrication of nano–micro scaffold: The knitted silk of 1 × 20 cm was saturated with 5 wt% P3HB dissolved in chloroform as mediate solution in order to enhance the interface connection between electrospun nanofibres and silk microfibres. The knitted silk was placed on the collector. After the silk saturated with mediate solution was dried, P3HB-Chitosan/MWNT solution was electrospinned on knitted silk in both the aligned and random manners. The optimal electrospun parameters are tabulated in Table 1. In all the analyses run on P3HB-Chitosan-x% MWNT/Silk nano–micro scaffolds, the aligned samples were compared with random samples where the control sample was without MWNT.

2.6. Physical properties: In order to assess surface, nano/micro interface morphology and porosity of scaffolds, SEM (SEM-AIS 2300 C, SEI; Japan) was applied. Surface of the scaffolds was coated with gold by sputter-coating machine (SC7620). Sectional imaging was made for determining the nano/micro interface of the samples. Average and distribution of fibre diameters was calculated by measuring 100 segments of fibers of each sample image obtained through SEM using the Imagej software (Imagej 1.51, Java 1.6.0_24 (64-bit)) National Institutes of Health, USA) [11, 15, 16, 19]. Porosity percentage is assessed in three layers through SEM and analysed by MATLAB (MATLAB R2013a (8.1.0.604) 64-bit, The MathWorks Inc., USA) [15, 22]. In order to assess the complete sericin removal in degumming process, FT-IR-attenuated total reflectance (FTIR-ATR,JASCO, 6300, Japan) was applied on the knitted silk; for assessing chemical structure of scaffolds in the presence and the absence of MWNT the same analysis was run in transition mode. For determining the availability of peaks in MWNT-included scaffolds, FT-IR was applied on pure MWNTs. All of the IR spectroscopies were obtained in 4000–400 cm^{-1} wavenumber range [19]. In order to determine the wettability and hydrophilicity of scaffolds, water

Table 1 Optimised parameters of electrospinning process for aligned and random scaffolds

Electrospinning parameters	Aligned	Random
voltage, kV	21	20
needle-collector distance, cm	20	20
flow rate, ml/h	0/25	0/50
rundle rotation speed, rpm	1000	0

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contact angle analysis was run according to ASTM D7334 [16, 23]. In this Letter, a distilled water droplet was dropped on the surface of samples and a photo was taken after 10 s by video contact angle system (CA-ES10, Fars EOR Tech.; Iran). By analysing the obtained image by the ImageJ, the angle of the water droplet was measured ($n = 5$) [16, 23].

2.7. Mechanical properties: The mechanical properties of the nano–micro structure were tested trough uniaxial testing machine (Zwick Roell Materials Testing Systems, model 1 M/H; Germany). The test was run until the sample was ruptured in 5 mm/min strain rate and the load cell applied in this test was 2 kN ($n = 3$) [19].

2.8. Statistical analysis: All of the tests are run by using the statistical software (IBM SPSS statistics version 24 release 24.0.0.0 2016 64-bit edition). One-way analysis of variance (ANOVA) was chosen for this purpose. All data presented are reported as mean ± standard deviation (SD). A value of $p \leq 0.05$ was considered statistically significant [11, 15, 16, 19, 24, 25].

3. Results and discussion

3.1. Morphology of nano–micro scaffolds: As observed in Fig. 1*c*, the aligned scaffold of 482.94 ± 107.52 nm average diameter had the best fibre diameter distribution and alignment. An increase in MWNT amounts up to 1 wt% in aligned scaffolds decreased the average diameter. Fiber diameter distribution and average together with alignment in the samples containing 1 wt% MWNT did not have significant difference in comparison with the sample containing 0.5 wt% MWNT and control sample ($p > 0.05$). In random scaffolds, an increase in MWNT amount reduced the average fibre diameter while the scaffold containing 1 wt% MWNT had 544 ± 73.21 nm, the least average fibre diameter ($p \leq 0.05$). There was no significant difference between aligned and random scaffolds ($p > 0.05$).

Fig. 2 shows that the scaffolds containing 1 wt% MWNT have presented better connection in interface of nano/micro in comparison with other samples (Figs. 2*c* and *f*). An increase in MWNT with amount of 1 wt% led to an increase in electrical conduction due to an increase in the amount of MWNT in polymeric solution and this caused the most alignment in aligned scaffolds, a good connection between nano/micro interface and a decrease in fibre diameter in both the aligned and random scaffolds. An increase in MWNT up to optimal content overcomes the MWNT electrical conduction effect on increasing the viscosity related to MWNT. This phenomenon lead to a reduction in fibre diameter, that is more fibers stretch in electrical field in electrospinning process. The presence of COOH group in MWNT and carbonyl group (C=O) in P3HB-mediated solution could be a justification for better connection between nano/micro phase interfaces. High aspect ratio and high reactivity of MWNT could provide a better connection with the silk substrate. Sonication of MWNT causes appropriate distribution of MWNT among the polymeric chains and prevention of agglomeration; which could be effective in enhancing diameter distribution and morphology, and causing more reduction in fibre diameter average. Moreover high length-to-diameter ratio of CNTs leads to their placement and orientation along the polymeric chains in a sense that here the electrical conductivity increases and fibre diameter decreases. The SEM image from the final morphology of the nano–micro scaffolds is shown in Fig. 3.

3.2. Porosity: Porosity is one of important parameters for choosing a scaffold because of its significant effect on cell behaviour in cell culturing. The result of porosity percentage is shown in Fig. 4. There was no significant difference in porosity percentage in first layer between nano–micro scaffolds with different amounts of MWNTs in both the aligned and random groups ($p > 0.05$). The differences among interconnected porosity percentage average in second and third layers were not significant ($p > 0.05$). The presence of nanoparticle often leads to reduction in porosity percentage in polymeric composite scaffolds [16, 26], while in this Letter there existed no adverse effect on porosity percentage. By increasing the MWNT amount from 0.5 to 1 wt% the porosity percentage was still >80% in both the aligned and random groups and they are appropriate for tissue engineering applications [22].

3.3. FT-IR analyses: The FT-IR-ATR spectra of degummed knitted silk, P3HB-Chitosan-0% MWNT/Silk, P3HB-Chitosan-1% MWNT/Silk and pure MWNTs are shown in Fig. 5. Degummed knitted silk spectrum included N–H stretching bond in 3274 cm^{-1} and C–H stretching bond in 2922 cm^{-1}; and the presence of amide I bonds in 1620 cm^{-1} (C=O stretching), amide II in

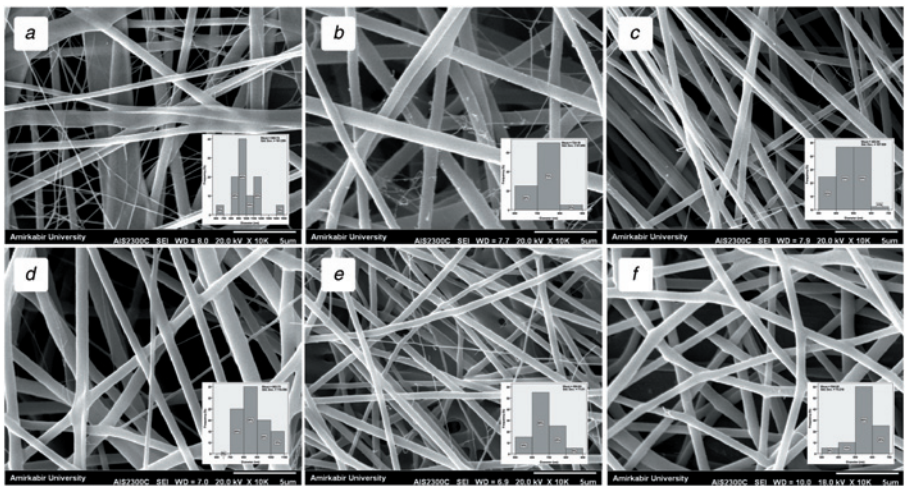

Fig. 1 *SEM images from surface of aligned and random nano–micro scaffolds*
a P3HB-Chitosan-0% MWNT/Silk-aligned
b P3HB-Chitosan-0.5% MWNT/Silk-aligned
c P3HB-Chitosan-1% MWNT/Silk-aligned
d P3HB-Chitosan-0% MWNT/Silk-random
e P3HB-Chitosan-0.5% MWNT/Silk-random
f P3HB-Chitosan-1% MWNT/Silk-random

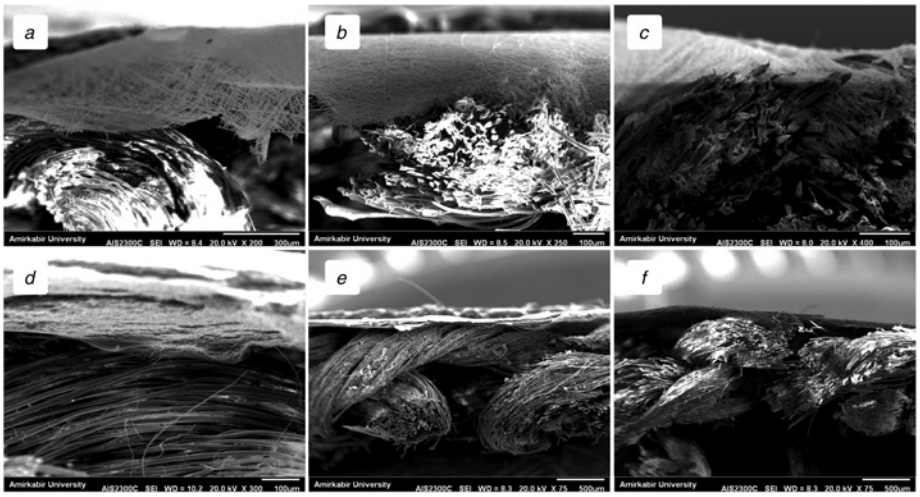

Fig. 2 *SEM images from interface between nano and micro phase of aligned and random nano–micro scaffolds*
a P3HB-Chitosan-0% MWNT/Silk-aligned
b P3HB-Chitosan-0.5% MWNT/Silk-aligned
c P3HB-Chitosan-1% MWNT/Silk-aligned
d P3HB-Chitosan-0% MWNT/Silk-random
e P3HB-Chitosan-0.5% MWNT/Silk-random
f P3HB-Chitosan-1% MWNT/Silk-random

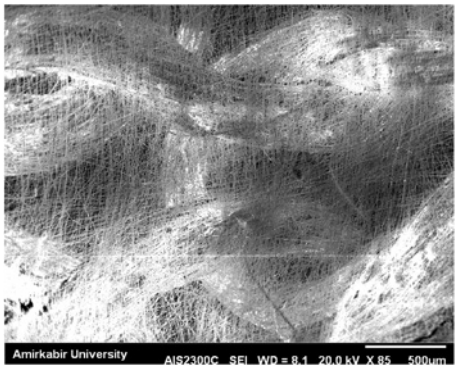

Fig. 3 *SEM image of P3HB-Chitosan-MWNT/Silk hybrid nano–micro scaffold*

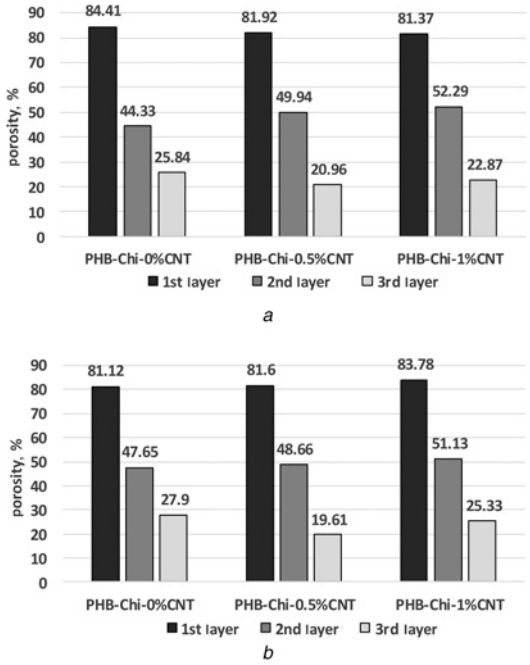

Fig. 4 *Porosity percentage charts of*
a Aligned
b Random nano–micro scaffolds in three layers

1508 cm^{-1} (C–N stretching plus N–H in-plane bending) and amide III in 1223 cm^{-1} assure the main fibroin structure in degummed silk [27]. The presence of amide I bond in 1620 cm^{-1} indicated the change from random α-helix to β-sheets conformation in silk structure [27, 28]. P3HB stretching bonds included 1725 cm^{-1} (C=O), 1181 and 1284 cm^{-1} (C–O–C) and 1455 cm^{-1} (CH_2). In chitosan structure spectrum stretching bonds including 1055 cm^{-1} (C–O), amide I in 1682 cm^{-1} (C=O) and amide II in 1540 cm^{-1} (N–H) were observed; likewise, the presence of the peak in 1104 cm^{-1} related to ester formation from free carboxylic functional group inside P3HB along with free alcoholic group from chitosan [19]. Vibrating state of CNTs functionalised by COOH was observed in 1430 and 1622 cm^{-1} (C–C), 3437 cm^{-1} (O–H), 807 cm^{-1} and 1151 cm^{-1} (C–O), and COOH functional group of MWNT in 2965 cm^{-1}. The presence of peak in 3437 cm^{-1} indicated the presence of MWNTs in 1 wt% MWNT scaffold and the peak in 2929 cm^{-1} in the scaffold containing MWNT was related to COOH bond in CNT structure. The peak in 1720 cm^{-1} could indicate RCONHR' connection between free chitosan amine group and MWNT carboxylic group or be related to amide bond inside the chitosan structure [16]. The silk FT-IR results indicated the stability of β-sheets in silk fibroin structure and electrospinning solution of P3HB-Chitosan/1% MWNT did not change the chemical structure of silk. The presence of chitosan in the scaffolds caused disorder in P3HB crystalline phase; consequently, the peaks move towards higher wavelength (lower wave number); this movement was due to intermolecular bonds formation between amino acid group of chitosan and carbonyl group of P3HB, thus, a reduction in P3HB crystallinity [19].

3.4. Contact angle measurement: The results obtained from contact angle measurement on aligned and random P3HB-Chitosan-x% MWNT/Silk nano–micro scaffolds are tabulated in Tables 2 and 3, respectively. Water contact angle in all scaffolds was

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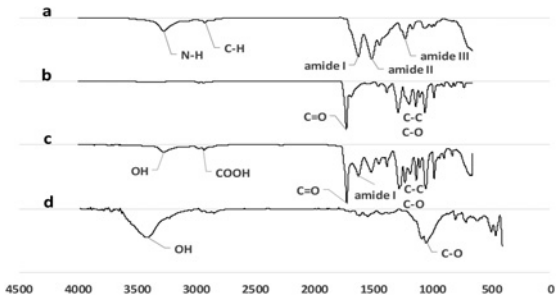

Fig. 5 *FT-IR spectrum of*
a Pure degummed silk
b P3HB-Chitosan-0%MWNT/Silk
c P3HB-Chitosan-1%MWNT/Silk
d Pure MWNTs

Table 2 Water contact angles of aligned scaffolds

Aligned scaffolds	Mean ± SD, °
P3HB-Chitosan-0% MWNT/Silk	59.47 ± 7.36
P3HB-Chitosan-0.5% MWNT/Silk	44.53 ± 3.44
P3HB-Chitosan-1% MWNT/Silk	37.24 ± 0.67

Table 3 Water contact angles of random scaffolds

Random scaffolds	Mean ± SD, °
P3HB-Chitosan-0% MWNT/Silk	55.90 ± 4.97
P3HB-Chitosan-0.5% MWNT/Silk	39.65 ± 5.99
P3HB-Chitosan-1% MWNT/Silk	31.41 ± 5.68

lower than 90°. Hydrophilicity of the scaffolds without MWNT is because of amine group (NH_2) due to the presence of chitosan biopolymer. An increase in carboxylic group (COOH) due to an increase in MWNT amount up to 1 wt% in P3HB-Chitosan hydrophilic polymeric solution led to a decrease in water contact angle. An increase in the MWNT amount led to a decrease in water contact angle in all aligned and random samples. Amount of 1 wt% MWNT in scaffolds increased hydrophilicity in comparison with 0.5 wt% MWNT and control samples significantly ($p < 0.05$). As presented in Tables 2 and 3, aligned scaffolds revealed more hydrophilicity than random scaffolds, with no significant difference ($p > 0.05$). According to morphology, fibre diameter distribution, porosity, hydrophilicity and alignment (in aligned scaffolds) in scaffolds with different amounts of MWNT, the aligned and random scaffolds containing 1 wt% MWNT were chosen as optimum samples. The tensile strength test was run on these scaffolds and the results were compared with the control samples (without MWNT).

3.5. Tensile strength test: The results of this test are tabulated in Tables 4 and 5, respectively. Scaffolds containing 1 wt% MWNT have the highest tensile strength in comparison with control samples and pure knitted silk. The presence of P3HB-Chitosan/1 wt% MWNT electrospun nanofibres coated on knitted silk led to an increase in tensile strength 1.69 and 1.64 times in comparison with pure knitted silk, respectively. No significant difference was observed between 1 wt% MWNT and control samples (without MWNT) ($p > 0.05$), while this difference was significant between nanofibres coated samples and pure knitted silk without nanofibres ($p < 0.05$). An increase in tensile strength in samples containing MWNT could be justified due to contribution of high intrinsic tensile strength in carbon nanotubes

Table 4 Average tensile strength of aligned scaffolds and degummed knitted silk

Aligned scaffolds	Mean ± SD, MPa
P3HB-Chitosan-1% MWNT/Silk	48.13 ± 2.2
P3HB-Chitosan-0% MWNT/Silk	45.64 ± 2.81
pure knitted silk	28.32 ± 7.46

Table 5 Average tensile strength of random scaffolds and degummed knitted silk

Random scaffolds	Mean ± SD, MPa
P3HB-Chitosan-1% MWNT/Silk	46.68 ± 5.95
P3HB-Chitosan-0% MWNT/Silk	41.18 ± 2.75
pure knitted silk	28.32 ± 7.46

and improvement of nano/micro interface connection in these scaffolds. Coating of nanofibres on knitted silk had a significant effect on increasing tensile strength in comparison with pure knitted silk. The average tensile strength in aligned samples was higher than that of random samples, and this fact is due to nanofibres' alignment and tensile stress along P3HB-Chitosan/MWNT nanofibres coating on knitted silk that is an inherent property of fibrous composites, while their difference has no statistical significance ($p > 0.05$). The CNTs tensile strength is high at its length and its best orientation is along the fibre length [29]; thus, an increase in tensile strength in aligned scaffolds. As to PCL/CNT it was found that an increase in CNT amount, initially increased the mechanical strength and then decreased due to agglomeration [30], while according to this Letter, adding 1 wt % MWNT to P3HB-Chitosan composition did not have significant effect on mechanical strength and this amount did not lead to agglomeration. Adding nanomaterial to polymeric matrix as reinforcement leads to filling the pores and reducing the porosity, thus, an increase in mechanical strength [31], while according to this Letter adding 0.5 and 1 wt% MWNT to P3HB-Chitosan solution stabilise the appropriate porosity limit for tissue engineering application (>80%) and did not fill the pores; moreover, the tensile strength of samples containing 1 wt% MWNT increased. These findings reveal that the P3HB-Chitosan-1% MWNT/Silk can be appropriate for long-term healing tissue engineering applications in their mechanical context.

4. Conclusion: The results obtained from SEM indicated that in aligned and random scaffolds containing MWNT, the fibre diameter average decreased due to an increase in solution conductivity and fibre stretched in electrical field due to MWNT presence. This reduction in samples containing 1 wt% MWNT was significant. These scaffolds had better diameter distribution and nano–micro interface connection in comparison with control sample and the sample containing 0.5 wt% MWNT. Porosity percentages of aligned and random scaffolds did not reduce, while by adding MWNT the porosity percentage still remained above 80%. Water contact angle test revealed that the scaffolds containing 1 wt% MWNT were more hydrophilic due to presence of many COOH groups of functional MWNT. Tensile strength of the samples containing 1 wt% MWNT in aligned and random scaffolds were more than their counterparts without MWNT and pure knitted silk. This result was indicated a significant effect of electrospun nanofibres on knitted silk in its mechanical sense. High intrinsic tensile strength along CNTs length and improvement of nano–micro interface connection led to an increase in tensile strength in scaffolds containing 1 wt% MWNT,

thus, an increase of tensile strength in aligned samples containing 1 wt% MWNT in comparison with random samples.

According to the obtained results here, P3HB-Chitosan-1% MWNT/Silk aligned and random scaffolds consist of appropriate physical and mechanical properties.

The aligned and random nano–micro scaffolds of P3HB-chitosan-1 wt% MWNT/Silk could constitute an appropriate biomimetic of ECM of long-term healing tissues in order to their tissue engineering applications.

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6 References

[1] Naghashzargar E., Farè S., Catto V., *ET AL.*: 'Nano/micro hybrid scaffold of PCL or P3HB nanofibers combined with silk fibroin for tendon and ligament tissue engineering', *J. Appl. Biomater. Funct. Mater.*, 2015, **13**, (2), p. 156

[2] Sahoo S., Lok Toh S., Goh H., *ET AL.*: 'PLGA nanofiber-coated silk microfibrous scaffold for connective tissue engineering', *J. Biomed. Mater. Res. B, Appl. Biomater.*, 2010, **95**, (1), p. 19

[3] Kasoju N., Bhonde R.R., Bora U.: 'Fabrication of a novel micro–nano fibrous nonwoven scaffold with *Antheraea assama* silk fibroin for use in tissue engineering', *Mater. Lett.*, 2009, **63**, (28), p. 2466

[4] Güven E.Ö., Demirbilek M., Saglam N., *ET AL.*: 'Preparation and characterization of polyhydroxybutyrate scaffolds to be used in tissue engineering applications', *Hacet. J. Biol. Chem.*, 2008, **36**, p. 305

[5] Chen S.-H., Chang Y., Lee K.-R., *ET AL.*: 'A three-dimensional dual-layer nano/microfibrous structure of electrospun chitosan/poly (D, L-lactide) membrane for the improvement of cytocompatibility', *J. Membr. Sci.*, 2014, **450**, p. 224

[6] Zheng Y., Monty J., Linhardt R.J.: 'Polysaccharide-based nanocomposites and their applications', *Carbohydr. Res.*, 2015, **405**, p. 23

[7] Liu X., Ma P.X.: 'Phase separation, pore structure, and properties of nanofibrous gelatin scaffolds', *Biomater.*, 2009, **30**, (25), p. 4094

[8] Jiankang H., Dichen L., Yaxiong L., *ET AL.*: 'Preparation of chitosan–gelatin hybrid scaffolds with well-organized microstructures for hepatic tissue engineering', *Acta Biomater.*, 2009, **5**, (1), p. 453

[9] Cao W., Wang A., Jing D., *ET AL.*: 'Novel biodegradable films and scaffolds of chitosan blended with poly (3-hydroxybutyrate)', *J. Biomater. Sci. Polym. Ed.*, 2005, **16**, (11), p. 1379

[10] Giretova M., Medvecky L., Stulajterova R., *ET AL.*: 'Effect of enzymatic degradation of chitosan in polyhydroxybutyrate/chitosan/calcium phosphate composites on in vitro osteoblast response', *J. Mater. Sci. Mater. Med.*, 2016, **27**, (12), p. 181

[11] Sadeghi D., Karbasi S., Razavi S., *ET AL.*: 'Electrospun poly (hydroxybutyrate)/chitosan blend fibrous scaffolds for cartilage tissue engineering', *J. Appl. Polym. Sci.*, 2016, **133**, (47), p. 44171

[12] Ma Y., Zheng Y., Wei G., *ET AL.*: 'Processing, structure, and properties of multiwalled carbon nanotube/poly (hydroxybutyrate-co-valerate) biopolymer nanocomposites', *J. Appl. Polym. Sci.*, 2012, **125**, (S1), p. 620

[13] O'connell M.J.: 'Carbon nanotubes: properties and applications' (CRC Press, USA, 2006)

[14] Li Q.-H., Zhou Q.-H., Dan D., *ET AL.*: 'Enhanced thermal and electrical properties of poly (D, L-lactide)/multi-walled carbon nanotubes composites by in-situ polymerization', *Trans. Nonferrous Met. Soc. China*, 2013, **23**, (5), p. 1421

[15] Zarei M., Karbasi S.: 'Evaluation of the effects of multiwalled carbon nanotubes on electrospun poly (3-hydroxybutirate) scaffold for tissue engineering applications', *J. Porous Mater.*, 2018, **25**, (1), p. 259

[16] Karbasi S., Mohammad Alizadeh Z.: 'Effects of multi-wall carbon nanotubes on structural and mechanical properties of poly (3-hydroxybutyrate)/chitosan electrospun scaffolds for cartilage tissue engineering', *Bull. Mater. Sci.*, 2017, **6**, (40), p. 1247

[17] Tuzlakoglu K., Bolgen N., Salgado A., *ET AL.*: 'Nano-and micro-fiber combined scaffolds: a new architecture for bone tissue engineering', *J. Mater. Sci. Mater. Med.*, 2005, **16**, (12), p. 1099

[18] Sahoo S., Cho-Hong J.G., Siew-Lok T.: 'Development of hybrid polymer scaffolds for potential applications in ligament and tendon tissue engineering', *Biomed. Mater.*, 2007, **2**, (3), p. 169

[19] Karbasi S., Fekrat F., Semnani D., *ET AL.*: 'Evaluation of structural and mechanical properties of electrospun nano-micro hybrid of poly hydroxybutyrate-chitosan/silk scaffold for cartilage tissue engineering', *Adv. Biomed. Res.*, 2016, **5**, (1), p. 180

[20] Teh T.K., Toh S.-L., Goh J.C.: 'Optimization of the silk scaffold sericin removal process for retention of silk fibroin protein structure and mechanical properties', *Biomed. Mater.*, 2010, **20**, (3), p. 035008

[21] Farè S., Torricelli P., Giavaresi G., *ET AL.*: 'In vitro study on silk fibroin textile structure for anterior cruciate ligament regeneration', *Mater. Sci. Eng. C*, 2013, **33**, (7), p. 3601

[22] Ghasemi-Mobarakeh L., Semnani D., Morshed M.: 'A novel method for porosity measurement of various surface layers of nanofibers mat using image analysis for tissue engineering applications', *J. Appl. Polym. Sci.*, 2007, **106**, (4), p. 2536

[23] ASTM International, W.C., PA. ASTM D-7334-08: standard practice for surface wettability of coatings, substrates and pigments by advancing contact angle measurement. 2008

[24] Ghasemi-Mobarakeh L., Prabhakaran M.P., Morshed M., *ET AL.*: 'Electrospun poly (ε-caprolactone)/gelatin nanofibrous scaffolds for nerve tissue engineering', *Biomaterials*, 2008, **29**, (34), p. 4532

[25] Karbasi S., Zarei M., Foroughi M.: 'Effects of multi-wall carbon nano-tubes (MWNTs) on structural and mechanical properties of electrospun poly (3-hydroxybutyrate) scaffold for tissue engineering applications', *Sci. IR Trans. F Nanotechnol.*, 2016, **23**, (6), p. 3145

[26] Mohammadian M., Haghi A.: 'Systematic parameter study for nanofiber fabrication via electrospinning process', *Bulg. Chem. Commun.*, 2014, **46**, (3), p. 545

[27] Mobini S., Hoyer B., Solati-Hashjin M., *ET AL.*: 'Fabrication and characterization of regenerated silk scaffolds reinforced with natural silk fibers for bone tissue engineering', *J. Biomed. Mater. Res. A*, 2013, **101**, (8), p. 2392

[28] Vepari C., Kaplan D.L.: 'Silk as a biomaterial', *Prog. Polym. Sci.*, 2007, **32**, (8), p. 991

[29] Spinks G.M., Shin S.R., Wallace G.G., *ET AL.*: 'Mechanical properties of chitosan/CNT microfibers obtained with improved dispersion', *Sens. Actu. B Chem.*, 2006, **115**, (2), p. 678

[30] Mattioli-Belmonte M., Vozzi G., Whulanza Y., *ET AL.*: 'Tuning polycaprolactone–carbon nanotube composites for bone tissue engineering scaffolds', *Mater. Sci. Eng. C*, 2012, **32**, (2), p. 152

[31] Jalal M., Fathi M., Farzad M.: 'Effects of fly ash and TiO_2 nanoparticles on rheological, mechanical, microstructural and thermal properties of high strength self compacting concrete', *J. Mech. Mater. Struct.*, 2013, **61**, p. 11

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