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The electrospun polyhydroxybutyrate fibers reinforced with cellulose nanocrystals: Morphology and properties

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ABSTRACT: Polyhydroxybutyrate (PHB) has been used in the biomedical field. However, the poor mechanical properties of PHB have limited its application. Here, electrospun fibrous nanocomposite mats reinforced with cellulose nanocrystals (CNCs) were fabricated by using PHB as polymeric matrix. The morphological, thermal, mechanical properties, as well as cytotoxicity were characterized. Increasing the concentration of CNCs caused a decrease in diameter of the electrospun fibers. Moreover, thermal analysis indicated that melting temperature of PHB/CNCs electrospun fibers were improved with the increased CNCs content. The addition of CNCs gradually enhanced the tensile strength till 8 wt % content followed by a gradual decrease at higher CNCs content (12–22 wt %) in tensile strength. The PHB/CNCs electrospun fibers were nontoxic to L-929 and capable of supporting cell proliferation in all conditions. This study demonstrates that fibrous PHB/CNCs electrospun fibers are cytocompatible and potentially useful mechanical properties for biomedical application. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43273.

KEYWORDS: cellulose and other wood products; composites; electrospinning; fibers; nanoparticles; nanowires and nanocrystals

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INTRODUCTION

Polyhydroxybutyrate (PHB) is a linear polyester of D(-)-3-hydroxybutyric acid which is intracellular storage material produced by a large variety of bacteria.[1] PHB offers many advantages over polymers derived from a petrochemical source, e.g. it is formed from renewable resources, is completely biodegradable, and biocompatible making it suitable for environmental and biomedical applications.[2,3] However, the disadvantages of PHB include the fact that its production is more expensive and that it lacks mechanical strength compared with conventional plastic. In addition, its high crystallinity, stiffness, and brittleness leads to limited applications.[4] There are various methods for enhancing the mechanical properties of PHB, for example, by means of creating polymer blends,[5] modifiying polymers,[6] and forming polymer composites[7] and polymer nanocomposites.[8]

Polymer nanocomposites have received considerable attention in recent years. This is because nanocomposites show better mechanical performance compared to that of the microcomposites because of the high surface to volume ratio of the reinforcing phase.[9] The reinforcing material can be made up of particles (carbon black),[10,11] sheets (clays),[11,12] or fibers (carbon nanotube,[13] cellulose nanocrystals).[14]

Cellulose nanocrystals (CNCs) have several advantages over other types of nanofillers which are easily modified, given that they are inexpensive, renewable, and biocompatible. Furthermore, they have a low density, high aspect ratio, and high modulus and strength.[15,16] CNCs are rod-like in appearance with high crystallinity which exhibit relatively high mechanical properties. The tensile strength and Young's modulus of CNCs determined by experimental and theoretical approaches are in the range of 14.3–28.6 and 100–160 GPa, respectively.[17,18] Up to date, CNCs have been successfully used as a reinforcing material for sub-micron polymer fibers fabricated by using the electrospinning technique. The various polymers including poly (methyl methacrylate),[18] polyvinyl alcohol,[19] poly(lactic acid),[20] chitosan–polyethylene oxide,[21] polystyrene,[22] polycaprolactone,[23] and polyethylene oxide[24] can be used as a matrix. However, research on CNCs for reinforcing PHB can hardly be found in the literature.

Electrospinning is a very effective method for producing micron/submicron diameter fiber mats. In the electrospinning process, a high voltage is applied to a polymer droplet, and electrostatic repulsion overcomes the liquid surface tension thus enabling the formation of fibers in nonwoven mats. These mats have a high surface area and are utilized for a variety of applications such as filtration, tissue engineering scaffolds, sensors,

energy generation, drug delivery, and protective clothing.[18] Although electrospinning has developed quickly with the occurrence of coaxial, side-by-side and tri-axial processes,[25–27] the mainstream of this technology is still the single-fluid electrospinning of a working solution containing a guest functional ingredient and a host filament-forming polymer matrix.

Currently, PHB has found acceptance in biomedical application.[28,29] However, the lack of mechanical properties limits the use of PHB. As a result, nanocomposite would be one good solution for the above problem. Therefore, cellulose nanocrystals (CNCs) were used as reinforcing agent for PHB by adding CNCs into PHB solution prior to electrospinning. The morphology of the resulting nanocomposites was investigated by using the scanning electron microscopy (SEM). Moreover, mechanical properties, water absorption, thermal analysis, and cytotoxicity tests were studied.

EXPERIMENTAL

Materials

PHB was obtained from Tianan Biologic Materials, China, whereby its reported molecular weight was 400,000. The PHB was used to prepare polymer solutions for electrospinning without further purification. The PHB was dissolved in a mixture of chloroform and dimethylformamide solvent with a 90/10 ratio and stirred at 60 °C for 20 h. Cellulose nanocrystals (CNCs) extracted from corn husk by means of sulfuric acid hydrolysis were produced as described in our previous work[30] and used as a nanocomposite material.

Methods

The Electrospinning of PHB Mixed with Cellulose Nanocrystals. The CNCs were prepared by means of acid hydrolysis and dispersed well in water; however, water is not a solvent system for PHB electrospinning. Freeze-dried particles were re-dispersed in an organic solvent by sonication. However, few re-dispersed CNCs were found because of the hydrophilic nature of cellulose and the strong hydrogen bonding interactions between the cellulose nanocrystals.[18] In this experiment, the solvent exchange method was used as reported by Dong *et al.*[18]

The PHB solution was mixed with various amounts of CNCs in a mixed solvent of chloroform (CF) and dimethylformamide (DMF) in the ratio of 90:10. Various amounts of CNCs were added to obtain a final CNCs content of 0, 5, 8, 12, 17, and 22 wt % while the total PHB concentrations were maintained at 4 wt %. The solution was then placed in a 5 mL syringe tube with a 16 G needle connected with a syringe pump (NE-300, New Era Pump Systems) that controlled the flow rate at a rate 2.4 of mL/h. A voltage of 15 kV was applied to the needle. This process resulted in polymers in the form of fine fibers accumulated on the aluminum foil covered collector that was placed at a working distance of 15 cm from the needle tip.

Fiber Characterization. The morphology of fibers was observed under a 4510-Jeol scanning electron microscope, SEM (Jeol, Japan). About 100 measurements of individual fiber diameter were measured by means of the ImageJ software (National Institutes of Health, USA) from the SEM micrographs in their original magnification.

Thermal Analysis. Specimens (10 mg) were randomly cut from the electrospun mats. The thermal analysis of specimens was measured by means of 204 F1 Phoenix differential scanning calorimeter (NETZSCH Instruments North America, LLC., USA). The typical procedure included heating and cooling cycles with a temperature rate of 10 °C/min. Each specimen was heated from −10 °C to 200 °C. The melting temperature was taken as the peak temperature of the melting endotherm.

Mechanical Properties. The tensile behavior of the electrospun mats was tested on a LF Plus testing machine (Lloyed Instruments, USA) with a cross-head speed of 10 mm/min at room temperature. Rectangular tensile specimens of size 2 cm × 20 cm prepared from electrospun mats were used for testing.

Water Absorption. The water absorption of electrospun mats was tested according to ASTM D570.The electrospun mats were cut into pieces with dimensions of 5 cm × 5 cm, dried in the oven at 100 °C for 2 h and placed in a desiccator to cool. Immediately upon cooling, the mats were weighed. After that, the mats were placed into a beaker filled with distilled water for 24 h. The mats were then removed from beaker, patted dry with a lint free cloth and weighed.

$$\text{Water absorption} = \frac{\text{wet weight-dry weight}}{\text{dry weight}} \times 100$$

Cytotoxicity. The *in vitro* cytotoxicity was evaluated using a direct contact test and MTT assay. In the direct contact assay, a sample was placed in direct contact with L-929 fibroblasts cells. After 24 h of culture, the specimens were prepared for examination in the scanning electron microscope. The negative and positive control materials are Thermanox (Nunc) cover slips and polyurethane film containing 0.1% zinc diethyldithiocarbomate (ZDEC), respectively.

The MTT assay is another cell viability assay often used to determine cytotoxicity following exposure to toxic substances. In brief, 1×10^5 viable cells/mL were plated into the 96-wells plate. After incubation at 37 °C for 24 h, the media were replaced with fresh complete media containing crude extract. The extract from Thermanox (Nunc) cover slips act as a negative control, while polyurethane film containing 0.1% zinc diethyldithiocarbomate (ZDEC) extraction act as a positive control. After 24 h, the supernatants were removed. Then, the MTT solution (0.5 mg/ml) was added and the specimens were incubated for 2 h. Dimethylsulfoxide (DMSO) was added for solubilization. The solution was homogenized by shaking the plates for 3 min. The homogenized solutions were transferred to another 96-wells plate. The optical density (OD) was read at 570 nm on a multiwall microtiter plate photospectrometer. All assays were performed in triplicate. The results were presented as a reduction of metabolic activity in percentage when compared to control cells cultured in medium only.

$$\%\ \text{viability} = \frac{OD_{570c}}{OD_{570b}} \times 100$$

where OD_{570c} is the mean value of the measured optical density of the 100% extracts of the test sample and OD570b the mean value of the measured optical density of the 100% extracts of the blanks.

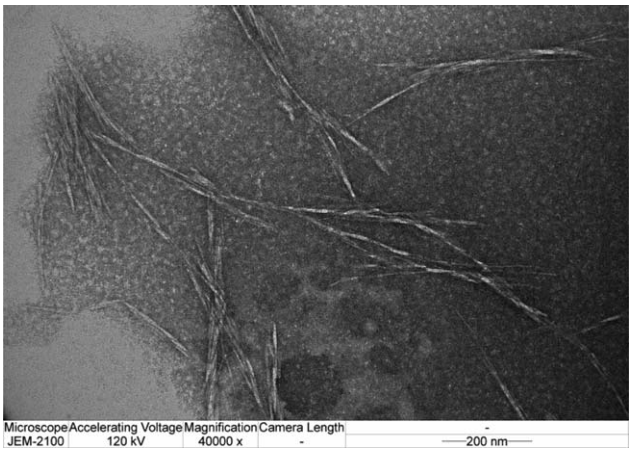

Figure 1. TEM micrograph of CNCs extracted from corn husk.

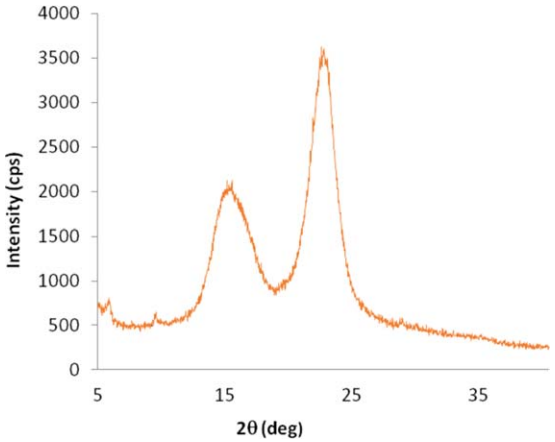

Figure 2. X-ray diffraction pattern of cellulose nanocrystals from corn husk. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS AND DISCUSSION

The TEM micrograph of CNCs extracted from corn husk at 45 °C for 45 min by means of chemical hydrolysis using 64 wt % sulfuric acid are shown in Figure 1. The individual CNCs were rod-like in shape with an average diameter of 7.4 nm and length of 230.3 nm. The calculated aspect (length to width) ratio was 32.4.[30] X-ray diffraction pattern of CNCs showed peaks around $2\theta = 16°$, 22°, and 35° were assigned to the *hkl* (110), (200), and (004) crystallographic planes of cellulose I structure (Figure 2).[30]

Electrospun Fiber Mats

PHB with different weight percentages of CNCs in electrospun fibers were examined using SEM. The morphology and average diameter of PHB/CNCs nanocomposite electrospun fibers are shown in Figures 3 and 4, respectively.

Figure 3 shows that all of the polymer compositions generated uniform fibers with no beads could be observed. By adding a low content of CNCs, the diameter of the PHB/CNCs nanocomposite electrospun fibers decreased (Figure 4). A possible explanation for this phenomenon is that the addition of CNCs increased the conductivity of the electrospinning solution as a result of the negative charges from the sulfate groups on the CNCs' surface, which were grafted via sulfuric acid hydrolysis during the CNCs production process.[31] A high content of CNCs was introduced into the same concentration of polymer solution, which resulted in increased viscosity. When the effect of

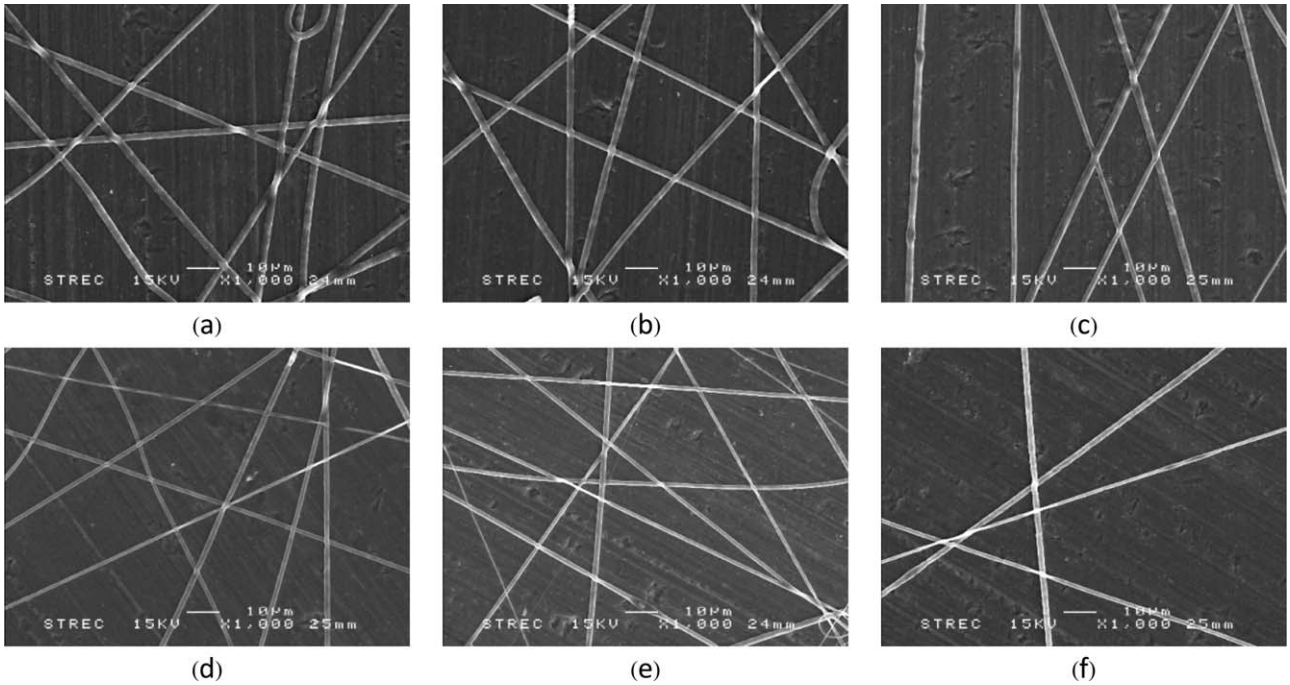

Figure 3. Scanning electron micrographs of (a) PHB electrospun fibers and (b–f) PHB/CNCs electrospun fibers at various w/w percentages of CNCs in fibers: (b) 5% CNCs, (c) 8% CNCs, (d) 12% CNCs, (e) 17% CNCs, and (f) 22% CNCs.

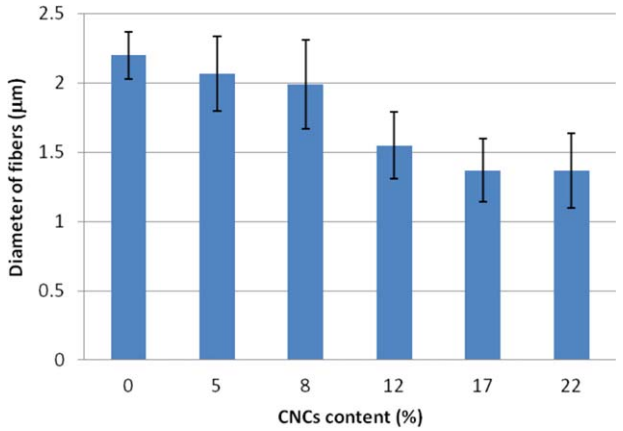

Figure 4. Diameter of electrospun fibers. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the increased viscosity was greater than the enhanced electrical conductivity, this rendered the weakened elongation of the jet between the needle tip and collector, hence the generation of thicker fibers.

Thermal Analysis

The melting temperature (Tm) of the PHB pallet and PHB electrospun fiber mats loaded with different CNCs content were investigated by using a differential scanning calorimeter, DSC, as shown in Table I.

Table I demonstrates the measured melting temperature of used materials. It was observed that the melting temperature of PHB electrospun fiber mats was considerably higher than that of the PHB pallet (Table I). This is because of the high shear stress present during the electrospinning process, leading to the enhanced crystallization of the polymer chains within the individual PHB fibers.[32] When CNCs are added into the system, the melting temperature of electrospun mats was slightly increased. This phenomenon implied that CNCs act as nucleating agents for PHB during the crystallization process, inducing significant increases in the nucleation rate.[33]

Mechanical Properties

To evaluate the mechanical properties of electrospun mats, the mats were electrospun for a longer period of time (~6 h) so as to produce thicker mats. The results are presented in Table II.

Table I. Thermal Properties of the PHB Pallet and PHB/CNCs Electrospun Fibers

Sample	CNCs content (%)	Tm (°C)
PHB pallet	0	178.2
PHB electrospun mats	0	182.6
	5	183.2
	8	184.7
	12	185.2
	17	185.9
	22	186.6

Table II. Mechanical Properties of PHB/CNCs Electrospun Fibers

CNCs contents (wt %)	Tensile strength (N/mm^2)	Young's modulus (MPa)	Elongation (%)
0	3.9 ± 0.3	4,837.5 ± 134.1	25.9 ± 1.4
5	4.2 ± 0.3	4,931.3 ± 245.4	19.4 ± 1.7
8	4.4 ± 0.2	5,120.1 ± 215.3	18.3 ± 1.2
12	3.0 ± 0.3	3,062.2 ± 216.7	19.5 ± 1.1
17	3.0 ± 0.2	2,991.7 ± 187.4	19.4 ± 2.1
22	2.9 ± 0.2	2,925.4 ± 156.1	19.4 ± 1.8

Tensile strength first increased reaching a maximum of 8 wt % of CNCs contents and then decreased with the increased CNCs content. According to the morphology results presented previously (Table II), the smaller-diameter fibers were obtained by adding CNCs to the PHB solution. This induced higher overall relative bonding between the fibers because of the increased surface area, bonding density, and better distribution of bonds.[31] An increase in CNCs content up to 8 wt % lead to an increase in the tensile strength of electrospun mats. This was despite the fact that at the beginning, CNCs were well dispersed in the PHB matrix, resulting in the increase of the electrospun mats strength. However, with a further increase in CNCs content, the reunion of CNCs would weaken intermolecular interaction of PHB.

Therefore, the observed strength of PHB/CNCs electrospun mats declined.[34] In the case of percentage elongation of electrospun mats, this percentage was lower than that of neat PHB. Notwithstanding the foregoing, the percentage elongation is not much affected by the addition of CNCs. The decreased percentage elongation of electrospun mats with the addition of CNCs as reinforcing agents is generally known to be an expected phenomenon given that they act as stress concentrating components.[35]

Water Absorption

PHB material is rarely used as scaffold in tissue engineering application because it is hydrophobic. The water absorption on the composites of PHB electrospun fibers was shown in Figure 5.

The introduction of CNCs to PHB electrospun fiber mats induced a significant improvement in water absorption qualities. The percentage of water absorption of PHB/CNCs electrospun fibers increased from 0.78 to 3.21 with an increase of CNCs content from 0 to 22 wt %. Owing to the abundance of surface hydroxyl groups, CNCs, a hydrophilic, were able to form hydrogen bonds with water molecules. The results indicated that PHB, a hydrophobic polymer, decreased with increased hydroxyl density, resulting in hydrophilicity and increased water absorption.[34]

Cytotoxicity

Cell viability and cytotoxicity of PHB/CNCs electrospun fibers were investigated and the results are shown in Table III and Figures 6 and 7.

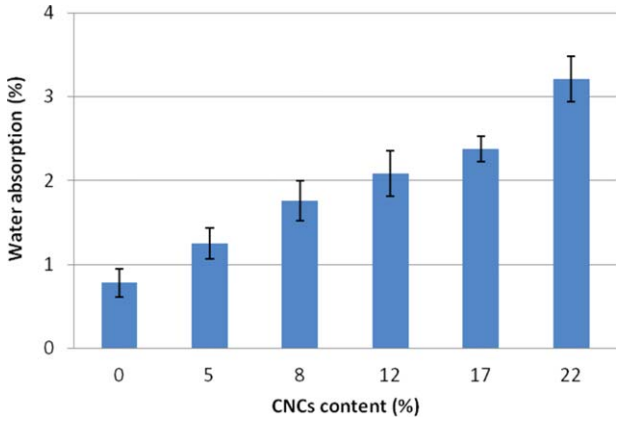

Figure 5. Water absorption of PHB/CNCs electrospun fibers. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table III. Cell Viability

Sample	Average	
	OD 570 nm	% Viability
Blank	1.089	100
Negative control	1.015	93
Positive control	0.000	0
PHB electrospun mat	0.788	72
PHB/CNCs electrospun mats (CNCs 22 wt %)	0.764	70

MTT Cytotoxicity Test. To determine cell viability, an enzyme-based method using MTT was employed in this experiment. This method relied on a reductive coloring reagent. Viable cells convert yellow MTT to purple formazan, thus color formation serves as cell-viability indicator. According to the ISO 10993-5,

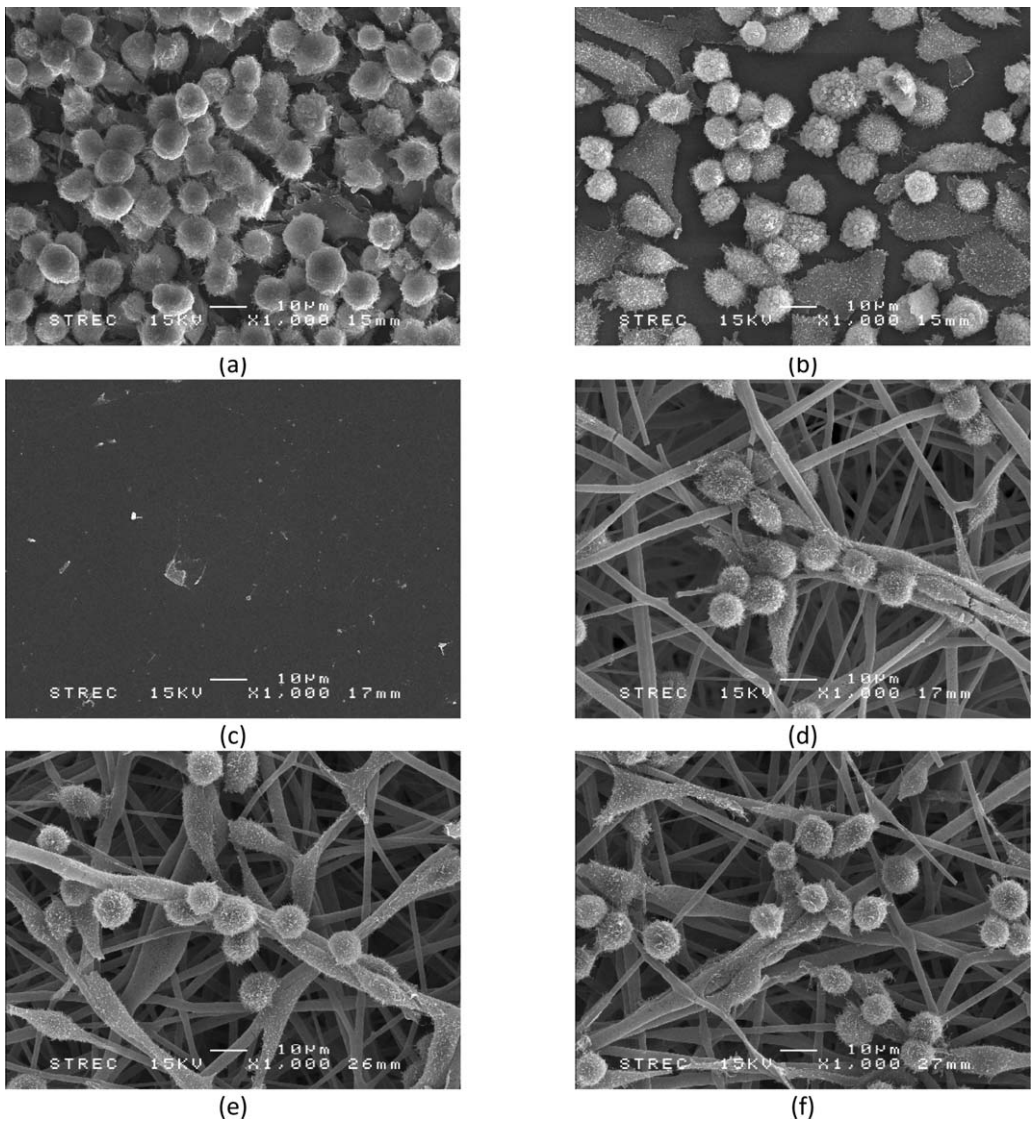

Figure 6. SEM images of fibroblasts (L929) cultured (a) on blank, (b) on negative control, (c) on positive control, (d) on PHB electrospun mat, (e) on PHB electrospun mat containing 8 wt % CNCs, and (f) on PHB electrospun mat containing 22 wt % CNCs.

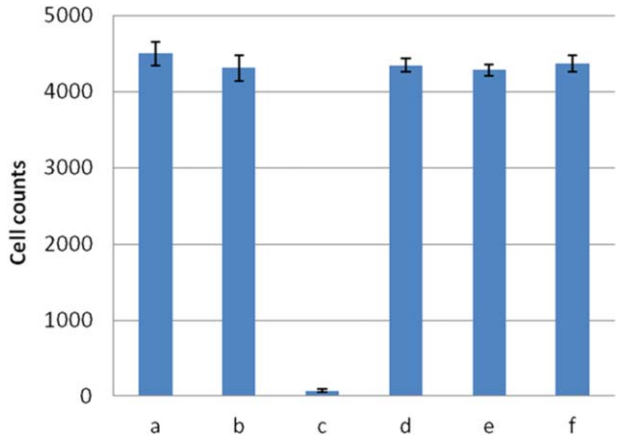

Figure 7. Cell counts of L929 fibroblasts grown (a) on blank, (b) on negative control, (c) on positive control, (d) on PHB electrospun mat, (e) on PHB electrospun mat containing 8 wt % CNCs, and (f) on PHB electrospun mat containing 22 wt % CNCs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the average cell viability of less than 70% is considered as cytotoxic. The observed percentage viability of both the PHB electrospun fibersand PHB electrospun fibers with 22 wt % CNCs was between 70 and 72 (Table III). Therefore, the cells are deemed to be viable and the electrospun fibers are regarded as having low toxicity.

Direct Test. The PHB/CNCs electrospun mats were exposed to mammalian cell cultures (L-929 cells) by direct contact. After the appropriate incubation time, the cytotoxic effect is analyzed by using the scanning electron microscope (Figures 6 and 7).

In the cytotoxicity assay, the controls should be included as a positive control, a negative control, and a blank control. The positive control should be based on materials known to induce a cytotoxic response, while the negative control should be a known as a noncytotoxic material. The L-929 cells grown in direct contact on PHB electrospun fibers with different amounts of CNCs exhibited no significant differences when compared with the negative control group (Figures 6 and 7). However, cell death was observed in the positive control group. It can be concluded that PHB/CNCs electrospun fibers did not reduce cell numbers in comparison to the negative control group. In addition, the results of morphological analysis obtained from direct contact were similar to the results from the MTT assay (Table III), i.e. the PHB/CNCs electrospun fibers are nontoxic and biocompatible.

Despite PHB is biodegradable and biocompatible polymer, it is extremely brittle and relatively hydrophobic causing its application in biomaterials is limited. Reinforcing CNCs in polymer is one of the most common methods for providing scaffolds with improved mechanical properties. In addition, the porous structures of electrospun fibers have a high specific surface area that is beneficial for biomedical application.

By adding a low content of CNCs (5–8 wt %), PHB/CNCs electrospun fibers with improved melting temperature, water absorption, and tensile strength were produced. However, tensile strength of PHB/CNCs electrospun fibers decreased with an increase in CNCs content (12–22 wt %), while both melting temperature and water absorption of PHB/CNCs electrospun fibers gradually increased from 185.2 °C to 186.6 °C and from 2.08% to 3.21%, respectively. Moreover, in the case of cytotoxic effect, all conditions of PHB/CNCs electrospun fibers were nontoxic to L-929 fibroblast cells.

To identify the optimal CNCs contents reinforced in PHB fibers, both mechanical properties and toxicity of PHB/CNCs electrospun fibers should be considered. The electrospun fibers made of PHB containing 8 wt % CNCs showed the best tensile strength and strongly supported the growth of L-929 fibroblasts cells. The results provide further evidence that PHB/CNCs electrospun fibers have a promising application as biomedical materials.

CONCLUSIONS

Electrospun composite mats based on PHB and CNCs were successfully produced via the electrospinning technique. The concentration of CNCs and the fiber diameter affected the mechanical properties of the nanocomposite mats. Thermal analysis proved that CNCs promote the crystallization of the PHB matrix, thus resulting in a higher melting temperature. Furthermore, the water absorptivity of electrospun mats was improved by adding CNCs to the PHB electrospun mats. All of the developed materials used in medical applications must be subjected to a cytotoxicity test in order to evaluate their biological properties and associated risk factors. Overall, the PHB electrospun mats with different CNCs loadings proved to be nontoxic and biocompatible. Therefore, the PHB/CNCs electrospun fibers have potential to be biomaterials.

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