Journal of Applied Polymer Science

A Novel Honey-Based Nanofibrous Scaffold for Wound Dressing Application

H. Maleki,[1] A. A. Gharehaghaji,[1] P. J. Dijkstra[2]

[1]Department of Textile Engineering, Amirkabir University of Technology, Tehran, Iran

[2]Department of Polymer Chemistry and Biomaterials, Faculty of Science and Technology, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands

Correspondence to: A. A. Gharehaghaji (E-mail: aghaji@aut.ac.ir)

ABSTRACT: In this study, nanofiber meshes were produced from aqueous mixtures of poly(vinyl alcohol) (PVA) and honey via electrospinning. The Electrospinning process was performed at different PVAs to honey ratios (100/0, 90/10, 80/20, 70/30, and 60/40). Dexamethasone sodium phosphate was selected as an anti-inflammatory drug and incorporated in the electrospinning solutions. Its release behavior was determined. Uniform and smooth nanofibers were formed, independent of the honey content. In case honey content increased up to 40%, some spindle-like beads on the fibers were observed. The diameter of electrospun fibers decreased as the ratio of honey increased. The release characteristics of the model drug from both the PVA and PVA/honey (80/20) nanofibrous mats were studied and statistical analysis was performed. All electrospun fibers exhibited a large initial burst release at a short time after incubation. The release profile was similar for both PVA and PVA/honey (80/20) drug-loaded nanofibers. This study shows that an anti-inflammatory drug can be released during the initial stages and honey can be used as a natural antibiotic to improve the wound dressing efficiency and increase the healing rate. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: honey; electrospinning; nanofiber; wound dressing; drug release

Received 14 December 2011; accepted 27 February 2012; published online 00 Month 2012

DOI: 10.1002/app.37601

INTRODUCTION

Since ancient times, honey has been used by many cultures in wound care, owing to its medicinal properties.[1] It is a viscous and saturated sugar liquid which is derived from nectar and gathered and modified by honey bees. Honey generally is composed of glucose (30%), fructose (40%), sucrose (5%), water (20%), and many other substances, such as amino acids, vitamins, minerals, and enzymes.[2,3]

The effects of honey on wound healing have been reported in both acute (burns, lacerations) and chronic wounds (venous leg ulcers, pressure ulcers).[2] There are many articles published, describing the effectiveness of honey in rapidly clearing infections from wounds and protecting wounds from becoming infected.[4] Honey has been shown to have anti-inflammatory, antimicrobial, and antibiotic properties. It inhibits a broad spectrum of bactericidal activities.[5,6] Therefore, it can provide a moist healing environment without the risk of bacterial growth occurring, and with no adverse effects to slow the healing process. The acidic nature of honey provides an optimal environment for activity of fibroblast cells. In addition, it can provide a nonadherent interface between the dressing and the wound bed and prevents the dressing from tearing away the newly formed tissue during removing.[6,7] According to ancient medical writings by Egypt, Greece, Persia, and a part of India, honey is considered to be the oldest wound dressing. Many randomized controlled trials have been carried out comparing honey as a wound dressing with various other wound treatments. Some of these trials and the successful results obtained from them are summarized in Table I. A dressing containing honey can undoubtedly be used on many types of wounds and currently various dressings and gels containing honey are commercially available.[6,18]

Fibers with antimicrobial properties are particularly interesting for the medical area such as wound dressings or textiles for patients.[19] Hamzeh and Miraftab[20] described a process for producing fibers which are able to import antimicrobial effects and use in control and treatment of physiological site infections. They used an extrusion setup to encapsulate honey within fibers. The WO/2008/049251 relates to a method for producing electrospun fibers from polymers and honey with microbicidal properties for textile applications.[19]

Table I. Randomized Controlled Trials That Have Been Carried Out on Honey as a Wound Dressing

Type of wound	Findings	Reference
Pressure ulcers: To compare the effect of a honey dressing versus an ethoxydiaminoacridine plus nitrofurazone dressing in patients with pressure ulcers	Patients treated with honey had a faster healing rate and a smaller ulcer at 5 weeks	Yapucu Günes and Eser [8]
Burns: To assess the efficiency of honey as a dressing in comparison with silver sulfadiazine gauze dressing in superficial burn injury	Patients treated with honey had a faster healing rate; honey gave better relief of pain, less irritation of the wound, less exudation, a lower incidence of hypertrophic scar, and postburn contracture	Subrahmanyam [9]
Burns: To compare honey-impregnated gauze with OpSite® as a cover for fresh partial thickness burns	Patients treated with honey had a faster healing rate; honey gave a soothing effect, and ease of removal of dressings with little pain	Subrahmanyam[10]
Burns: To compare honey-impregnated gauze with amniotic membrane dressing in partial thickness bums	Patients treated with honey had a faster healing rate	Subrahmanyam[11]
Burns: To compare honey dressing with boiled potato peel dressings as a cover for fresh partial-thickness burns	Patients treated with honey had a faster healing rate	Subrahmanyam[12]
Burns: To compare honey dressing with silver sulfadiazine on histological and clinical studies of wound healing in fresh partial thickness burns	Patients treated with honey had a faster healing rate and more histological evidence of reparative activity; honey gave early subsidence of acute inflammatory changes and better control of infection	Subrahmanyam[13]
Surgical wounds: To study the effect of topical crude undiluted honey in the treatment of severe acute postoperative wound infections	Patients treated with honey had a faster healing rate, a shorter hospital stay, a smaller scar formation, and a shorter antibiotic use	Al-Waili and Saloom [14]
Surgical wounds: To evaluate and compare the effectiveness of honey-impregnated gauzes, hydrocolloid dressings, and as a conventional dressing, saline-soaked gauzes for skin graft donor sites	Patients treated with honey had a faster healing time than those who had paraffin gauzes and saline-soaked gauzes	Misirlioglu et al.[15]
Surgical wounds: To clinically compare the healing of abscess wounds dressed with either crude undiluted honey or Edinburgh University solution of lime	Patients treated with honey had a shorter hospital stay and a further healing process at day 21	Okeniyi et al.[16]
Infections: To compare honey versus antimicrobial therapy, broad debridement, exhaustive cleaning, and split thickness skin grafts in Fournier's gangrene	Patients treated with honey had a better cosmetic result	Tahmaz et al.[17]

Electrospinning is a simple and effective method to produce thin fibers with micro to nanoscale diameters[21,22] especially meshes composed of nanofibers show outstanding characteristics, such as very large surface area to volume ratio and high porosity with a very small pore size. Owing to the high surface area to volume ratio, nanofibrous webs exhibit higher water absorption compared to polymer films. These characteristics have extended their potential applications to wound dressings.[23] In case hydrophilic polymers are applied, such nanofibrous webs are expected able to absorb wound exudates much more efficiently than film-type dressings.[24] The porous structure of nanofibrous webs is suitable for the drainage of the wound exudates and allows appropriate permeation of atmospheric oxygen to the wound. Also, they may prevent the microbial attack and protect the wound from infections, which would ultimately result in delayed healing. The porous structure of a nanofiber dressing would be excellent for the creation of an appropriate moist environment for the wound.

Poly(vinyl alcohol) (PVA) is a water-soluble, semi–crystalline, and hydrophilic polymer with good chemical and thermal stability.[23,25] It is produced by polymerization of vinyl acetate followed by hydrolysis.[26] PVA has been proposed for various biomedical applications owing to its good mechanical properties, biocompatibility, nontoxicity, and biodegradability.[26–28]

In this study, we describe a nanofibrous wound dressing prepared by electrospinning. Mixtures of PVA and honey were spun into nanofibers at different weight ratios. Dexamethasone sodium

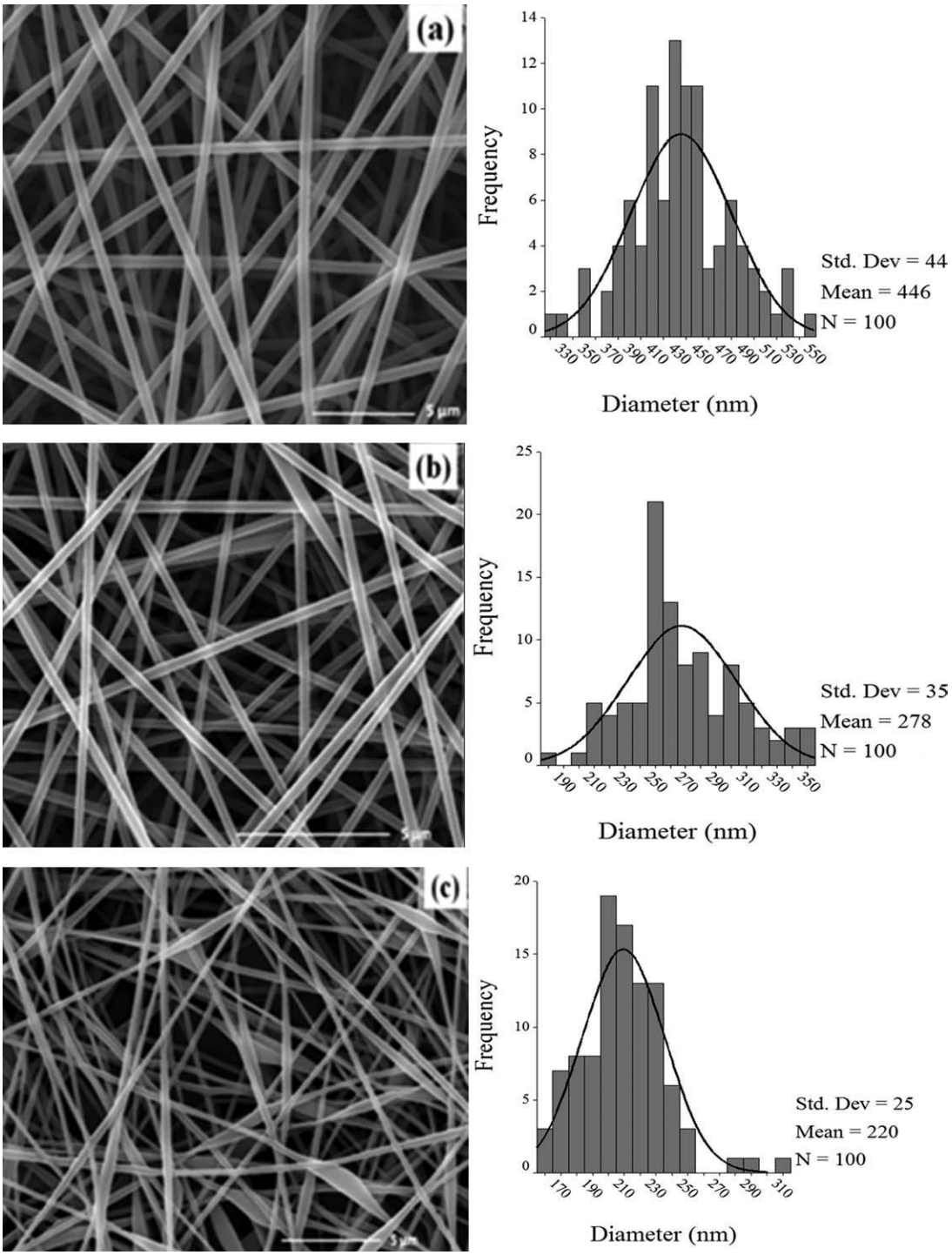

Figure 1. SEM images and diameter distribution of PVA/honey nanofibers at different ratios. (a) PVA, (b) PVA/honey (80/20), and (c) PVA/honey(60/40).

phosphate (Dex-P) was chosen as model drug as it is well known and applied as an inflammatory drug, and its release behavior in a physiological environment was discussed and compared. Dex-P was incorporated into the fibers to provide anti-inflammatory properties in the early stages of treatment[29–31] and the release behavior in a physiological environment was studied.

EXPERIMENTAL

Materials

PVA (Mw = 195,000, degree of polymerization = 4300, and degree of hydrolysis = 98.0–98.8 mol %) and Dex-P was purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Iran-Tabriz honey is used in this study.

Table II. The Diameters of the Individual Fibers Within the Electrospun Fiber Webs ($n = 100$)

Type of electrospun webs	Sample	Fibers diameter (nm)
Neat	PVA	446 ± 44
	PVA/honey (90/10)	336 ± 47
	PVA/honey (80/20)	278 ± 35
	PVA/honey (70/30)	224 ± 35
	PVA/honey (60/40)	220 ± 25
Drug loaded	PVA + 5 wt % Dex-P	327 ± 39
	PVA + 10 wt % Dex-P	264 ± 27
	PVA + 15 wt % Dex-P	250 ± 28
	PVA/honey (80/20) + 5 wt % Dex-P	189 ± 16
	PVA/honey (80/20) + 10 wt % Dex-P	166 ± 22
	PVA/honey (80/20) + 15 wt % Dex-P	163 ± 20

Electrospinning

PVA/honey solutions were prepared by dissolving weighted amounts of PVA and honey at ratios of 100/0, 90/10, 80/20, 70/30, and 60/40 in distilled water to afford 7.5 wt % solutions. The solutions were stirred gently at 80°C for at least 4 h.

To prepare drug-loaded fibers by electrospinning, a weighted amount of Dex-P was added to the PVA/honey 100/0 and 80/20 solutions and the mixture was stirred until a clear and homogeneous solution was obtained. The resulting solutions contained 5, 10, and 15 wt % Dex-P.

Electrospinning was carried out by using a high-voltage DC power supply (RP50-1.25R/230DDPM, Gamma high-voltage Research, USA) and a syringe pump (KD-100, KD Scientific, Holliston, MA) on which a 5-mL syringe was connected to a stainless steel needle (inner diameter, 0.7 mm). In preliminary experiments, electrospinning was conducted at voltages in between 15 and 20 kV, and the flow rates varied from 0.5 to 1.2 mL/h. Based on the results, nonbeaded and smooth fibers were obtained at a fixed voltage of 19 kV, a distance of 20 cm between the tip of the needle and the collector, and at a constant flow rate of 1 mL/h. The electrospinning process was performed at room temperature.

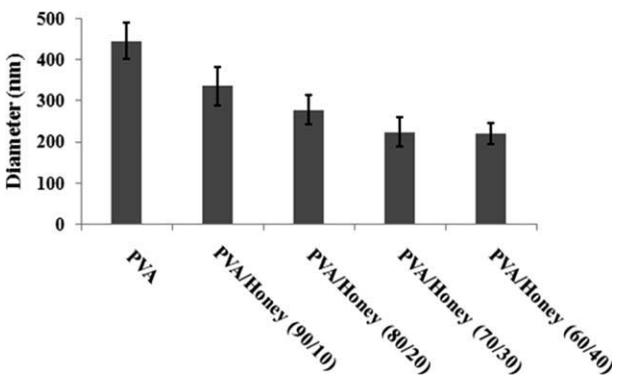

Figure 2. Fiber diameter changes at different ratios of PVA/honey.

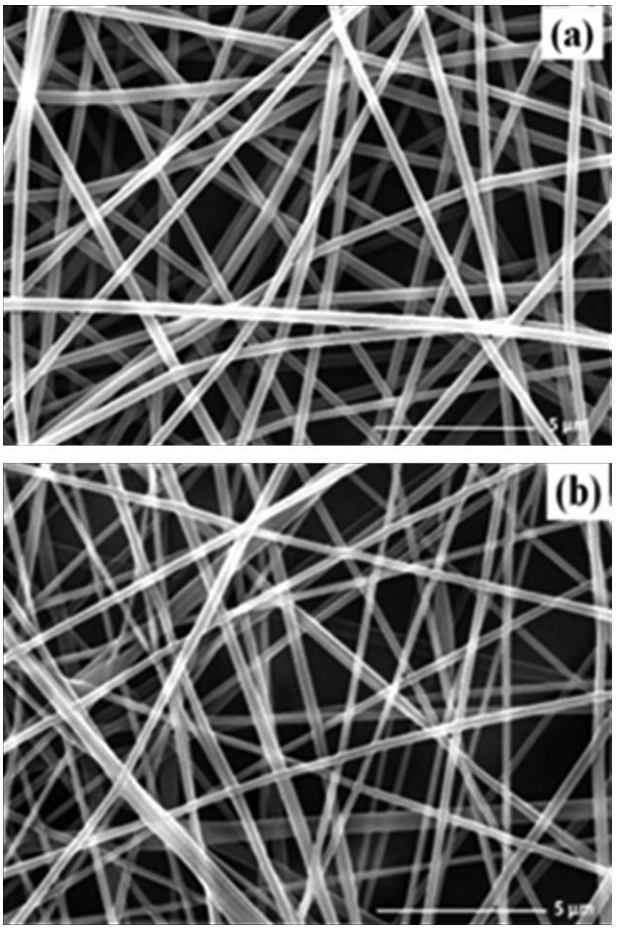

Figure 3. SEM images of drug loaded nanofibers of (a) PVA and (b) PVA/honey (80/20) at 10 wt % Dex-P.

Characterization

Morphology. The morphology of the electrospun fibers was examined by scanning electron microscopy (SEM) (XL30 ESEM FEG). At least five different positions on the fibrous web were analyzed to determine the diameter of the electrospun fibers.

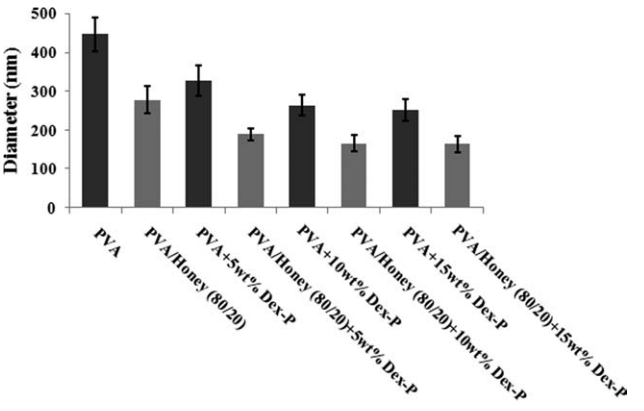

Figure 4. Diameter changes for drug-loaded PVA and PVA/honey (80/20) nanofibrous webs at different drug loadings.

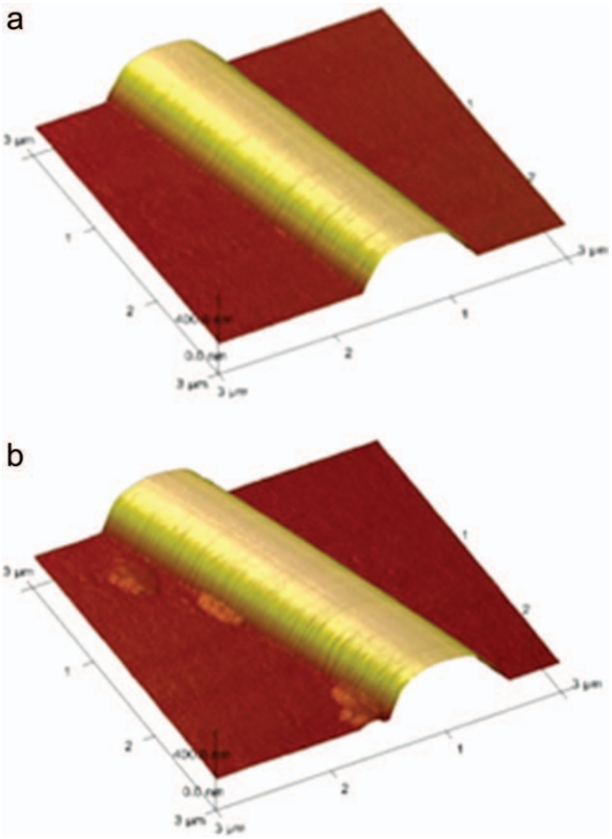

Figure 5. AFM images of nanofibers without honey (a) and with honey (b). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The samples were sputtered with a thin layer of gold prior to SEM analysis. Accelerating voltage of 15 kV was applied. Based on these SEM images, the average diameter of electrospun fibers was determined by means of Image J 1.44p software. In the case of fibers spun from PVA/honey (30/70) and (40/60) mixtures, the diameter of the free beaded fibers was measured. The results reported as average values of 100 measurements.

The surface morphology of electrospun nanofibers was evaluated by using a Bioscope Catalyst atomic force microscopy (AFM) (Bruker AXS). All samples were scanned at room temperature in normal atmosphere and scan rate of 1 Hz.

In Vitro Release

To study the release behavior of dexamethasone from drug-loaded PVA and PVA/honey (80/20) nanofibrous webs, dried sections of webs were weighted and immersed in 5 mL of a phosphate buffer solution (pH = 7.4) in 15-mL plastic tubes which were placed in a shaking incubator at 37°C. At scheduled time points, 1-mL samples of the buffer solution were taken and replaced by an equal volume of fresh phosphate buffered saline (PBS). The amount of dexamethasone in the buffer samples was determined using a UV–Visible spectrophotometer (UV-2401PC SHIMADZU) at a wave length of 241 nm. The amount of dexamethasone released was related to a calibration curve of dexamethasone in PBS prepared from solutions containing 25, 50, 75, and 100 mg/mL. Three samples were tested

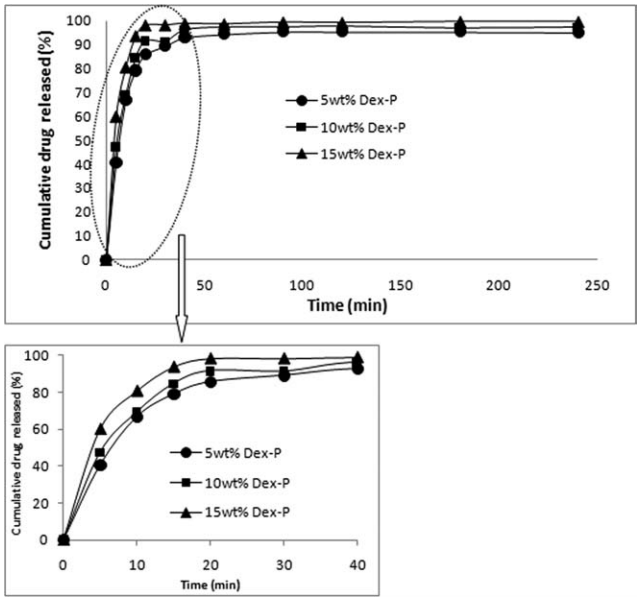

Figure 6. Release profile of dexamethasone from PVA nanofibrous mat in PBS at pH 7.4 and 37°C.

for each nanofibrous membrane and the results were averaged. To compare the release behavior between drug-loaded PVA and PVA/honey (80/20) nanofibrous webs at different amounts of dexamethasone, the statistical analysis was performed. The ANOVA tests were arranged in SPSS software for this purpose. All statistical tests were carried out within 95% significance level ($\alpha = 5\%$).

RESULTS AND DISCUSSION

Morphology of Electrospun Nanofibers

In Figure 1, the SEM images of electrospun PVA/honey nanofibers and corresponding diameter distributions at different ratios of 100/0, 80/20, and 60/40 are shown.

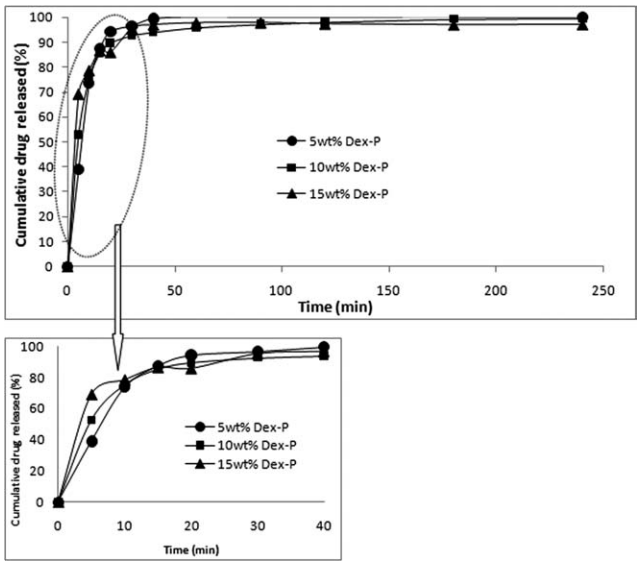

Figure 7. Release profile of dexamethasone from PVA/honey (80/20) nanofibrous mat in PBS at pH 7.4 and 37°C.

Table III. ANOVA Results to Analyze the Effect of Drug Amount (wt %) on Cumulative Release (%) of Nanofibrous Webs

Kind	Source	Type III sum of squares	df	Mean square	*F*	Sig. (*P*)
PVA	Drug amount (wt %)	59.801	2	29.900	5.532	0.043
	Error	32.428	6	5.405		
	Total	725.249	9			
PVA/honey (80/20)	Drug amount (wt %)	65.989	2	32.994	17.061	0.003
	Error	11.604	6	1.934		
	Total	1104.864	9			

As can be seen from the SEM images, uniform nanofibers were formed at all weight ratios. When the content of honey in the blend solution was 40%, spindle-like beads in webs were observed [Figure 1(c)]. At an increasing wt % of honey, the viscosity of the solution decreases having a significant effect on the electrospinning process and resulting fiber morphology. At lower viscosities, the degree of chain entanglements is not high enough to withstand the Coulombic stretching force acting on a charged jet, and thus the possibility of bead formation increases.[32]

From the SEM images, the diameters of the nanofibers were determined by image J analysis software and are listed in Table II. The diameters of fibers ranged from 220 to 446 nm depending on the composition of the spinning solution. The diameter of electrospun fibers decreased as the weight percentage of honey in the spinning solution increased (Figure 2). Up to a concentration of honey of 40 wt %, the average diameter of nanofibers decreased from 446 to 220 nm. This trend is likely owing to the increased conductivity of solution with increasing honey content. Interestingly, the electrical conductivity is one of the main characteristics of honey and is a parameter allowing distinguishing different kinds of honey. The electrical conductivity of honey is closely related to the concentration of minerals, organic acids, proteins, some complex sugars, and polyols.[33–35] As a result, the higher charge density at higher honey concentrations leads to higher stretching forces in the jet, and thus decreased fiber diameters.[32]

To study the release of an inflammatory drug-like Dex-P from electrospun fibers, solutions of PVA and PVA/honey (80/20) containing 5–15 wt % of the drug were prepared and electrospun. The morphology of the obtained drug-loaded fibers is shown in Figure 3 and the calculated fiber diameters are listed in Table II. All electrospun drug-loaded fibers exhibited a smooth surface. The average fiber diameter depended on the amount of loaded drug and appeared highly influenced by the addition of honey. As shown in Table II and Figure 4, it is found that the average diameter of the fibers decreased as the Dex-P concentration increased from 5 to 15 wt % for both PVA and PVA/honey (80/20) fibers. The average diameters of the PVA and PVA/honey (80/20) nanofibers containing no Dex-P were 446 and 278 nm, respectively, whereas the average diameters of the fibers when loaded with 5 wt % Dex-P decreased to 328 and 190 nm.

SEM analysis was used to characterize the structure and diameter of nanofibers. AFM was used for further characterizations and surface analysis. Figure 5 shows the three-dimensional surface images of PVA/honey nanofibers by AFM imaging. It can be seen from the AFM images, that the electrospun fibers have uniform smooth surfaces with cylindrical structure.

In Vitro Drug Release

The release behavior of dexamethasone as a model anti-inflammatory drug from the electrospun fiber webs was investigated by immersing the webs in PBS at pH 7.4 and 37°C. The cumulative release of the drugs from the PVA and PVA/honey (80/20) drug-loaded nanofibrous webs as a function of immersion time is shown in Figures 6 and 7. For both kinds of PVA and PVA/honey (80/20) nanofibrous webs, the release profile is very similar at three concentrations. A large initial burst release at short times after incubation was observed owing to high solubility of Dex-P in PBS. It appeared that the release of a drug is mainly controlled by the swelling behavior of the fibers.[28] As soon as the PVA fibers started to swell, molecules of drug were dissolved and were released from the fibers. Another important factor is the dissolution of the PVA fibers in the PBS solution. Visual observation revealed a fast dissolution of the web in PBS solution. Despite the short-time frame up to almost complete the release, the rate of the drug released from the samples appears in first order. At similar time points, the amount of released drug was slightly increased with increasing initial amounts of Dex-P. When the amount of drug was increased from 5 to 15 wt %, this leads to a larger initial burst release of the drug. This trend was observed for both PVA and PVA/honey (80/20) drug-loaded nanofibers (Figures 6 and 7). Statistical analysis confirms this behavior (Table III). The ANOVA results are summarized in Table IV which provides a comparison between the release behavior of drug-loaded PVA and PVA/honey (80/20) webs. It was found that honey does not have significant effect on the release behavior of nanofibrous webs ($P > 0.05$). Hence, honey can be used as a kind of natural antibiotic besides drugs to improve the wound dressing efficiency and increase the healing rate.

Table IV. ANOVA Results to Compare the Release Behavior of PVA and PVA/Honey (80/20) Nanofibrous Webs

	Sum of squares	df	Mean square	*F*	Sig. (*P*)
Between-groups	23.744	1	23.744	2.237	0.154
Within-groups	169.821	16	10.614		
Total	193.565	17			

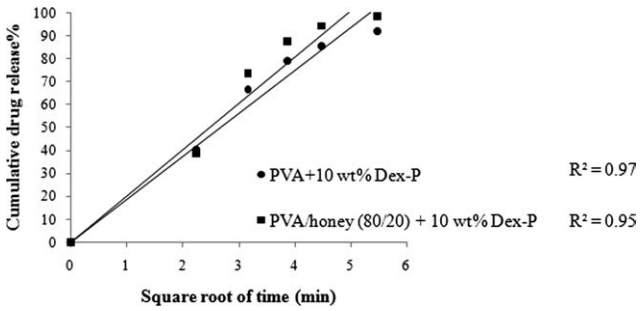

Figure 8. Release kinetics of PVA and PVA/honey (80/20) represented as cumulative dexamethasone percentage.

The release of dexamethasone from the webs shows a diffusion-controlled first-order release profile. A plot of the cumulative release as the square root of time affords a straight line and complies with the Higuchi model (Figure 8).[36]

CONCLUSIONS

According to ancient medical writings, honey was the first wound dressing and it has been used for wound care since many years ago owing to its medical properties. In this study, the novel PVA/honey nanofibers webs were successfully fabricated by electrospinning. According to SEM and AFM images, uniform and smooth nanofibers were formed at PVA/honey ratios up to almost 40 wt % of honey. When the content of honey in the solution increased up to 40%, spindle-like beads in the fibers were observed. The diameter of electrospun fibers decreased as the ratio of honey increased. The nanofibers could be loaded with dexamethasone at different concentrations by electrospinning. These drug-loaded nanofibers have a smooth and uniform surface. It was found that the diameter of the fibers decreased as the Dex-P amount increased from 5 to 15 wt % for both PVA and PVA/honey (80/20) fibers. The release characteristics of the model drug from both the PVA and the PVA/honey (80/20) nanofibrous mats exhibited a large initial burst release and release was completed within 1 h. Statistical analysis confirmed that honey does not have significant effect on the release behavior of nanofibrous webs. Electrospun nanofibrous mats based on PVA and honey in combination with an anti-inflammatory drug are interesting materials to be applied as dressing for many type of wounds.

REFERENCES

1. Gorzalski, P. *Am. Bee J.* **2009,** *149*, 518.
2. Jull, A. B.; Rodgers, A.; Walker, N. *Cochrane Database Syst. Rev.* **2009,** *4*, 48.
3. Robson, V.; Dodd, S.; Thomas, S. *J. Adv. Nurs.* **2009,** *65*, 565.
4. Khoo, Y. T.; Halim, A.; Singh, K. K.; Mohamad, N. A. *Biomed. Chromatogr. Complement. Altern. Med.* **2010,** *10*, 48.
5. Chang, J.; Cuellar, N. G. *Home Healthcare Nurse* **2009,** *27*, 308.
6. Pieper, B. J. *Wound Ostomy Continence Nurs.* **2009,** *36*, 60.
7. Malik, K. I.; Malik, M. A. N.; Aslam, A. *Int. Wound J.* **2010,** *7*, 413.
8. Yapucu Günes, Ü.; Eser, I. J. *Wound Ostomy Continence Nurs.* **2007,** *34*, 184.
9. Subrahmanyam, M. *Br. J. Surg.* **1991,** *78*, 497.
10. Subrahmanyam, M. *Br. J. Plast. Surg.* **1993,** *46*, 322.
11. Subrahmanyam, M. *Burns* **1994,** *20*, 331.
12. Subrahmanyam, M. *Burns* **1996,** *22*, 491.
13. Subrahmanyam, M. *Burns* **1998,** *24*, 157.
14. Al-Waili, N. S.; Saloom, K. Y. *Eur. J. Med. Res.* **1999,** *4*, 126.
15. Misirlioglu, A.; Eroglu, S.; Karacaoglan, N.; Akan, M.; Akoz, T.; Yildirim, S. *Dermatologic. Surg.* **2003,** *29*, 168.
16. Okeniyi, J. A. O.; Olubanjo, O. O.; Ogunlesi, T. A.; Oyelami, O. A. *J. Altern. Complement. Med.* **2005,** *11*, 511.
17. Tahmaz, L.; Erdemir, F.; Kibar, Y.; Cosar, A.; YalcÝN, O. *Int. J. Urol.* **2006,** *13*, 960.
18. Iftikhar, F.; Arshad, M.; Rasheed, F.; Amraiz, D.; Anwar, P.; Gulfraz, M. *Phytother. Res.* **2010,** *24*, 583.
19. Greiner, A.; Hehl, J. Schoeller Textil Greiner Andreas Hehl Judith **2008,** p PCT/CH2007/000510.
20. Hamzeh, S.; Miraftab, M. UNIV BOLTON, United Kingdom, UK Patent, 2,474,851, **2009.**
21. Subbiah, T.; Bhat, G. S.; Tock, R. W.; Parameswaran, S.; Ramkumar, S. S. *J. Appl. Polym. Sci.* **2005,** *96*, 557.
22. Yacoob, C.; Liu, W.; Adanur, S. *J. Ind. Text.* **2010,** *40*, 33.
23. Kang, Y. O.; Yoon, I. S.; Lee, S. Y.; Kim, D. D.; Lee, S. J.; Park, W. H.; Hudson, S. M. *J. Biomed. Mater. Res. B Appl. Biomater.* **2010,** *92B*, 568.
24. Zahedi, P.; Rezaeian, I.; Ranaei-Siadat, S. O.; Jafari, S. H.; Supaphol, P. *Polym. Adv. Technol.* **2010,** *21*, 77.
25. Linh, N. T. B.; Min, Y. K.; Song, H.-Y.; Lee, B.-T. *J. Biomed. Mater. Res. B Appl. Biomater.* **2010,** *95B*, 184.
26. Puppi, D.; Piras, A. M.; Detta, N.; Ylikauppila, H.; Nikkola, L.; Ashammakhi, N.; Chiellini, F.; Chiellini, E. *J. Bioact. Compat. Polym.* **2011,** *26*, 20.
27. Lee, S. J.; Lee, S. G.; Kim, H.; Lyoo, W. S. *J. Appl. Polym. Sci.* **2007,** *106*, 3430.
28. Taepaiboon, P. *Nanotechnology* **2006,** *17*, 2317.
29. Hao, H. X.; Wang, J. K.; Wang, Y. L. *J. Chem. Eng. Data* **2004,** *49*, 1697.
30. Gurney, A. B.; Wascher, D. C. *Am. J. Sports Med.* **2008,** *36*, 753.
31. Hochhaus, G.; Barth, J.; al-Fayoumi, S.; Suarez, S.; Derendorf, H.; Hochhaus, R.; Mollmann, H. *J. Clin. Pharmacol.* **2001,** *41*, 425.
32. Ramakrishna, S.; Fujihara, K.; Teo, W. E.; Lim, T. C.; Ma, Z. An Introduction to Electrospinning and Nanofibers; World Scientific Pub Co. Inc.: Singapore, **2005.**
33. Akbulut, M.; Özcan, M. M.; Çoklar, H. *Int. J. Food Sci. Nutr.* **2009,** *60*, 577.
34. Teresa Szczêsna, H. R. C. *J. Apicult. Sci.* **2004,** *48*, 97.
35. PŘIDAL, A. *Czech. J. Anim. Sci. UZPI* **2002,** *47*, 439.
36. Higuchi, T. *J. Pharm. Sci.* **1961,** *50*, 874.