Electrospinning of Type I Collagen and PCL Nanofibers Using Acetic Acid

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ABSTRACT: Fabrication of nanofibrous biomaterials based on natural materials (collagen, gelatin, etc.) through various techniques is an important research topic. Electrospinning, a well-established technique for nanofiber production has also been extended for producing nanofibrous structures of natural materials. Collagen nanofiber production utilizes hexafluoro isopropanol (HFIP) as a solvent for electrospinning. Research efforts are now focused on replacing HFIP with an environmentally benign solvent. In this study, electrospinning of Type I collagen of bovine skin with polycaprolactone (PCL) as a blend and an environmentally benign solvent, acetic acid, was carried out. The samples produced were subjected to contact angle measurements, porosity estimation, SEM, FTIR, TGA, and DSC. Nanofibers in the range of 100–200 nm were produced with an optimum porosity of 60%. The instrumental analyses confirm the physical interaction between collagen and PCL. Electrospinning of collagen in an environmentally benign solvent has been carried out and its usage in tissue engineering is being investigated by our research group. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 125: 3221–3227, 2012

Key words: electrospinning; biomaterial; biopolymer; nanofibers

INTRODUCTION

Tissue engineering is a fast growing research field, which offers solution for the regeneration of damaged tissue and organ transplantation, etc.[1,2] Wide number of fabrication methods based on synthetic and natural materials has been developed to meet the existing demand. The engineered scaffold should not elicit an immune response while remaining a viable framework for cellular infiltration/proliferation, ultimately mimicking both the fibrillar form and the complex function of the native extra cellular matrix (ECM).[3,4] By nature, human organs are constituted by fibrous structures made up of nanofibers and replacing or mimicking these constructions is a challenging task. Electrospinning is one of the most established process for producing nanofibrous structures that mimic the ECM.[5] It has also been reported that nanofibrous materials obtained upon electrospinning improve tissue regeneration and decrease scar formation.[6]

In electrospinning, nanofibers are obtained as a result of the electrostatic charges overcoming the surface tension of polymer droplet and subsequent deposition of these nanofibers onto a collector to obtain nonwoven matrices. The parameters influencing the process are broadly classified into three groups,[7] namely solution properties (viscosity, conductivity, surface tension, polymer molecular weight, dipole moment, and dielectric constant); controlled variables (material flow rate, electric field strength, distance between tip and collector, needle tip design, collector composition, and geometry); ambient conditions (temperature, humidity, and air velocity).

Even though lots of synthetic materials are being electrospun, the major bottleneck in usage of synthetic materials for biomaterial production lies in their biocompatibility. Hence, the primary focus of research community is to develop biomaterials with biocompatible properties. Materials of natural origin (Collagen, Gelatin, Casein, Zein, Elastin) are likely to overcome the above discussed problem and can be used for the production of highly biocompatible biomaterials.

Collagen, a well-studied natural structural protein has always been a choice as a substrate for tissue engineering, because of its biocompatible and biodegradable properties. Nevertheless, the low melting point of collagen and its quick denaturation is the major drawback encountered. However, cross-linking of collagen with suitable cross-linkers in turn provides stability and mechanical strength to the final material. Electrospinning of collagen has been attempted by number of researchers either, alone[3,8,9] or in the presence of blends like chitosan,[10,11] PCL,[12,13]

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elastin,[14,15] nanohydroxyapatite,[16] silk fibroin,[17] polyurethane,[18] etc. In particular, collagen-PCL composite nanofibers have been shown to be promising candidates for skin[13] and vascular[12] tissue engineering applications. Invariably, electrospinning of collagen has been carried out in the presence of HFIP for most of the blends and with TFP and acetic acid in combination with HFIP for some blends. The nanofibers obtained from the mixture of said blends and collagen apparently mimic the tissue fibers in terms of promoting cell adhesion, adherence, etc. However, the biochemical cross-talk between the tissue and the engineered spun material could not be achieved because of the toxic nature of the HFIP employed during spinning. It has also been reported that electrospinning using HFIP transforms collagen to gelatin.[18] Thus, the major challenge in producing electrospun collagen is to use an appropriate solvent system that does not denature or alter the biologically favorable properties of collagen. In this study, an attempt was made to electrospin collagen with a suitable blend in the absence of HFIP and characterize the resultant material for biomedical applications.

EXPERIMENTAL

Materials

Reconstituted Type I Collagen of bovine skin was obtained as a gift from Dr. A. Gnanamani, Central Leather Research Institute, Adyar, Chennai. Polycaprolactone (PCL) (M_n of 80,000) was obtained from Sigma Aldrich, USA. Glacial Acetic acid, Analytical Grade was obtained from SRL, Mumbai.

Apparatus

An electrospinning setup consisting of a programmable infusion pump (KD scientific), high voltage apparatus (Gamma High voltage Research, USA), a 5-mL syringe with a 21-G needle and a stationary aluminum plate as collector was used for the production of nanofibers. The diameter of nanofibers was measured using image analysis software, Digimizer®.

Nanofiber production

Table I displays the test combinations chosen for this study. An additional combination of 85/15 (collagen/PCL) was also electrospun and characterized only for Fourier transform infrared (FTIR) and thermal properties since a peelable matrix was not obtained. The prepared solutions were loaded with care into a 5-mL syringe fitted with a 21-G needle. The needle tip was connected to the high voltage source. Electrospinning was carried out at a constant electric field of 20 kV with a solution feed rate of 0.70 mL/h and a tip to collector distance of 10.5 cm. The fibers were collected on the aluminium plate in the form of nonwoven matrices. These matrices after sufficient drying were peeled off from the collector with the aid of a surgical knife and stored in clean polythene bags at 4°C until subjected to characterization.

Characterization

Surface contact angle measurement

Biomaterials inadvertently come into contact with water, blood, and other body fluids. Thus, while intending to produce materials for biomedical applications such as scaffolds for wound healing or skin regeneration, cellular proliferation or tissue engineering, it is essential to characterize them for their wettability. This is usually done by assessing the contact angle made by a liquid on the surface of the electrospun matrix. Surface contact angle measurements for the electrospun materials obtained from the said composition of PCL and collagen in the presence of acetic acid were made according to the methods summarized[20] using contact angle meter (Holmarc Optomechatronics).

Porosity

Both porosity and scaffold density of the electrospun collagen/PCL material were measured according to the equation reported elsewhere.[21]

$$\text{Porosity} = \left(1 - \frac{\text{scaffold density}}{\text{bulk material density}}\right) \times 100 \quad (1)$$

$$\text{Scaffold density} = \left(\frac{\text{mass of scaffold}}{\text{area of scaffold} \times \text{thickness of scaffold}}\right) \quad (2)$$

Tensile testing

To assess the mechanical properties of the electrospun sample, tensile testing was carried out using universal tensile tester (INSTRON 1408) with a 5-kN load cell. Dog-bone shaped samples with width 5 mm and gauge length 20 mm were prepared and tested at a speed of 5 mm/min. The thickness of the samples was around 0.2 mm.

Scanning electron microscopy analysis

Surface morphology of the electrospun fibers and membrane was observed under a scanning electron microscope (SEM) (S3400NSEM, HITACHI) at an accelerating voltage of 15 kV. Prior to scanning under the SEM, the samples were sputter coated with gold using a fine coater (E 1010, HITACHI). On the basis of the SEM photographs, the diameters of fibers were analyzed using analysis software Digimizer®.

TABLE I
Composition Details on Collagen and PCL Used for Electrospinning of Collagen Nanofibers

S. No.	Collagen (%)	PCL (%)
1.	100	0
2.	75	25
3.	50	50
4.	25	75
5.	0	100

FTIR spectroscopy analysis

An ATR-FTIR spectroscopic analysis of electrospun material was made using Spectrum One (Perkin-Elmer, USA model).

Thermo gravimetric analysis and differential scanning colorimetric analysis

Thermo gravimetric analysis (TGA) was carried out under nitrogen (40–60 mL/min) using TGA Q 50(V20.6 build 31) instrument. Differential scanning colorimetric (DSC) analysis was carried out using DSC Q 200(V 23.10 Build 79) with standard mode at nitrogen (50 mL/min) atmosphere.

RESULTS AND DISCUSSION

Collagen, a well-known structural protein has fascinated and continues to fascinate researchers around the globe. The intricate triple helical conformation of collagen, the abundance of collagen in the ECM and its ability to mimic the ECM when electrospun, are the predominant reasons for it being preferred as a raw material for biomaterial fabrication. Electrospinning of collagen for producing scaffold like materials, biocompatible cellular structures, tissue engineering materials etc. is thus having an exponential growth in the scientific research domain. It is important to note that when preparing materials for applications such as wound healing and tissue engineering, prevention of any residual toxicity that can evoke adverse immunogenic reactions in the body becomes a necessity. Electrospinning of collagen predominantly uses HFIP, a fluroalcohol, which is a natural pollutant of the environment and is not cost effective. The denaturing effects of HFIP on collagen, when it is used as a solvent for electrospinning has also been reported previously[19] on the basis of low denaturation temperatures of gelatin and collagen and circular dichroism spectral properties. During biomaterial fabrication a change in the inherent properties of the raw material may ultimately affect the application. Similarly, in electrospinning of collagen using HFIP, the denaturation of collagen may affect the regeneration of the damaged tissues. REACH (registration, evaluation, authorization, and restriction of chemicals), an European community regulation on chemicals and their safe use, calls for the progressive substitution of the most dangerous chemicals when suitable alternatives have been identified. It will not be farfetched to consider the possibility of HFIP being restricted in the near future by regulatory organizations. Thus, the need to identify and standardize an environmentally benign solvent system for the electrospinning of collagen in such a way that its inherent structure and biocompatible properties are not affected becomes essential.

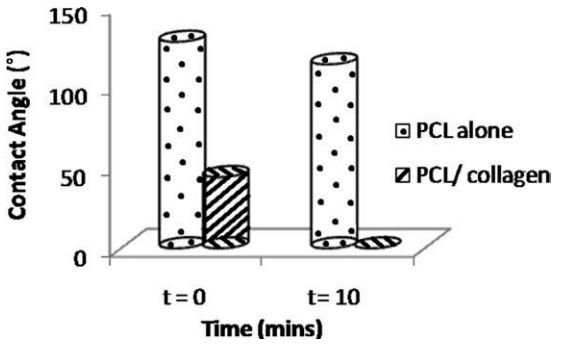

Figure 1 Water contact angle measurements for collagen/PCL and PCL electrospun matrices.

It is well known that extraction of collagen without any denaturation is usually carried out using dilute acetic acid[22] and the stability is provided through crosslinking. The above statement gives us an indication that acetic acid may be a better solvent for electrospinning of collagen. Hence, collagen was electrospun using acetic acid as a solvent. Subsequently, it was found that collagen alone in acetic acid did not result in fiber formation when electrospun. Thus, in order to stabilize the solution, PCL was added as a blend. Subsequently, electrospinning was carried out with different concentrations of PCL and collagen and the matrix obtained from each combination was subjected to characterization and compared.

Contact angle measurement

Figure 1 demonstrates the contact angle measurements made for PCL alone and for the collagen-PCL blended material. An average contact angle of 128° was

TABLE II
Details on Thickness, Density, and Porosity of Collagen/PCL Electrospun Materials at Different Ratios

Collagen/PCL blend	Thickness[a] (mm)	Apparent density of scaffold (g/cc)[a]	Porosity* (%)
100/0	NA	NA	NA
75/25	0.042 ± 0.002	0.4782 ± 0.02	59 ± 2
50/50	0.042 ± 0.001	0.4948 ± 0.04	57 ± 3
25/75	0.042 ± 0.001	0.5896 ± 0.02	57 ± 2
0/100	0.042 ± 0.003	0.4419 ± 0.02	61 ± 2

[a] mean ± SD.

TABLE III
Tensile Test

Ratio (collagen/PCL)	Tensile strength (MPa) $n = 5$	Elongation at break (%)
75/25	0.84 ± 0.3	54 ± 0.4
50/50	1.2 ± 0.4	56 ± 0.2
25/75	1.7 ± 0.8	60 ± 0.3
0/100	1.88 ± 1.3	61 ± 0.8

reduced to 114° after 10 min of exposure to water droplet for the electrospun material of PCL alone. However, it was only 44° at initial period and 0° within a minute for matrices made up of collagen and PCL, irrespective of the combinations. The observed contact angle for PCL may be attributed to its hydrophobic nature, and upon blending with collagen, an increase in hydrophilicity and the porous structures trap the water molecules and reduce the contact angle abruptly. A similar finding has been reported while blending PCL with collagen in the presence of trifluoroethanol (TFE).[21]

Porosity measurement

Porosity is a crucial factor that is to be characterized for a tissue engineered scaffold. The porous nature being essential for facilitating cell infiltration and proliferation should also continue to aid hemostasis, as well as ensure sufficient gas and nutrient exchange for wound healing. The preferred porosity of scaffolds used for cellular penetration should generally be within the range of 60–90%.[5]

TABLE IV
Measurements of Average Fiber Diameter of Collagen/PCL Electrospun Nanofibers for Different Ratios of Collagen and PCL

Collagen/PCL blend ratio (%)	Average fiber diameter (nm)
100/0	NA
75/25	115 ± 39
50/50	130 ± 38
25/75	148 ± 30
0/100	160 ± 35

With reference to the porosity measurements, a total of five samples for each combination were tested for porosity and the average reported. Table II demonstrates the thickness, density, and porosity obtained for the electrospun matrices of collagen and PCL blend. The density of collagen was taken as 1.16 g/cc as reported elsewhere.[16] Porosity was calculated using eqs. (1) and (2) and was observed to be in the range of 59 ± 2%.

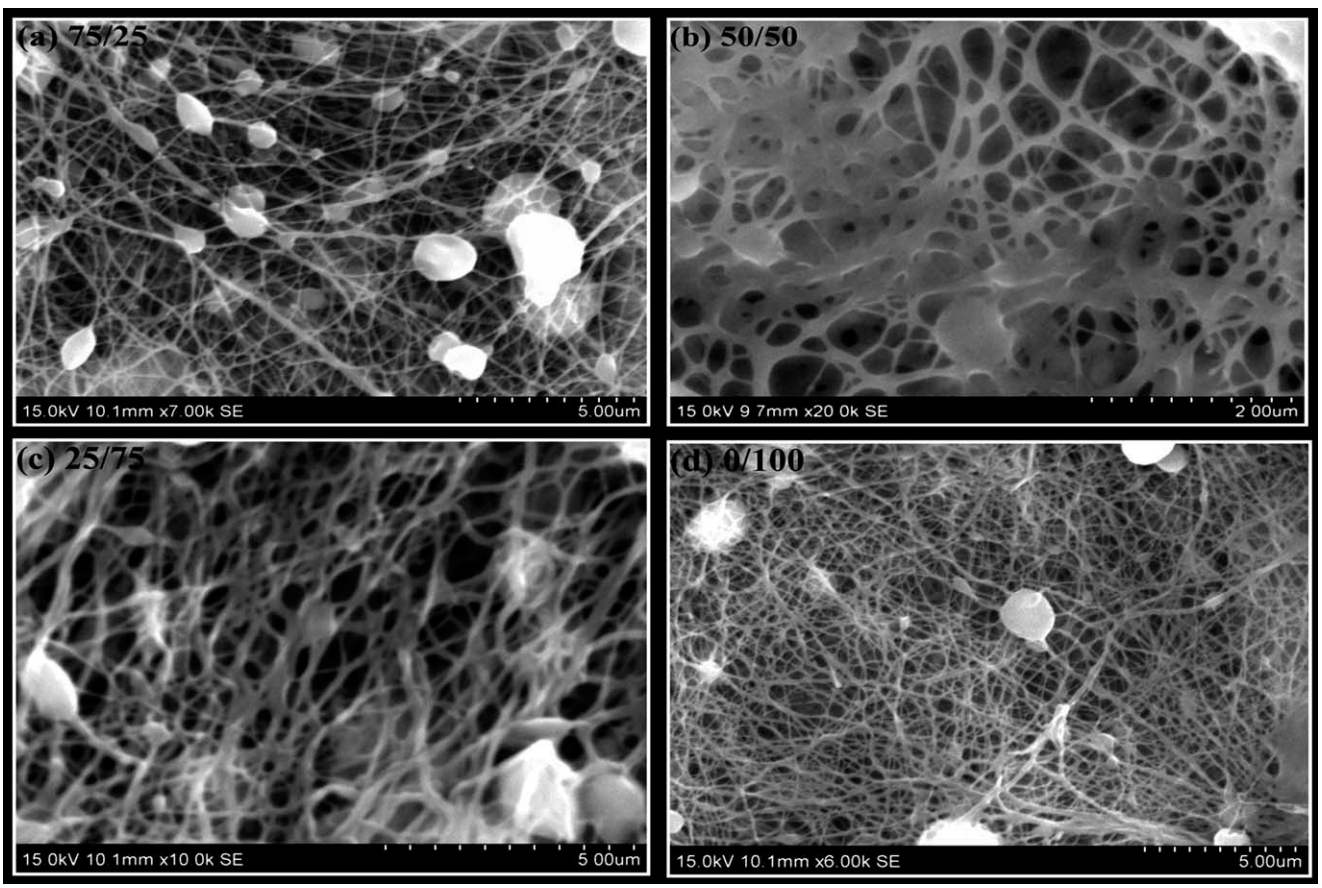

Figure 2 SEM images of collagen/PCL blended matrices made at different ratios; (a) 75/25 (b) 50/50 (c) 25/75 (d) 0/100.

Tensile testing

Table III displays the results of the tensile testing of the electrospun scaffolds. For each ratio, five samples were tested and the average tensile strength is shown. Samples containing more collagen displayed an average tensile strength of 0.84 ± 0.3 MPa. There is a gradual increase in the tensile strength as the PCL content is increased with considerable variations in the readings.

SEM analysis

Figure 2 displays the SEM micrographs of electrospun matrices obtained from the collagen/PCL blends. Presence of fibers is clearly evidenced along with globule structures. The diameters of the fibers were calculated using image analysis software, Digimizer®. From each SEM image 50 measurements were made and the average reported. Table III displays the diameters of the fibers obtained. The addition of PCL is found to increase the diameter from below 100 nm to around 200 nm. The increase in diameter might be due to the increase in viscosity of the solution due to addition of PCL.

FTIR spectra analysis

In order to confirm the interaction of PCL with collagen, the electrospun matrices obtained from various combinations of collagen and PCL were subjected to FTIR analysis. Figure 3 demonstrates the IR spectrum of the matrices along with the IR spectrum of PCL and collagen. With respect to collagen alone, the NH stretching vibrations, CH_2 vibrations, C=O vibrations of amide linkages, —NH bending vibrations, CH_2 bending vibrations and C—N vibrations are observed respectively at 3326, 2928, 1658, 1553, 1375, 1455, and 1239 cm^{-1}. Whereas the peaks observed for PCL alone at 2946, 2870, and 2821 cm^{-1} are due to CH_2 vibrations, the intense sharp peak at 1728 cm^{-1} due to C=O vibrations, CH_2 bending vibrations at 1465, 1407, and 1362 cm^{-1}, and COO vibrations at 1238 and 1181 cm^{-1} and C—O vibrations at 1047 and 1099 cm^{-1}. With regard to the FTIR spectrum of the combinations made, the broad peak observed at 3304 cm^{-1} might be due to the hydrogen bonding interaction of collagen with PCL. The low intense peak observed at 1725 cm^{-1} is attributed to the reduced availability of C=O of PCL upon interaction with collagen. The C=O vibrations of the blend observed at 1633 cm^{-1} was about 20 cm^{-1} lower than collagen alone, and suggests that the collagen fibers are well dispersed and as a result, nitrogen lone pair of amide link might be highly delocalized over the adjacent C=O group. This delocalization might be the cause for the shift of C=O vibrations to low value. This observation also suggested that formation of newer hydrogen bonds between the amide groups of collagen and the carboxyl groups of PCL. This observation was also supported by the broadening of NH vibrations and shift of C=O vibration of PCL to a slightly low value. The slight shift in all the characteristic peaks is evidence of the interactions occurring between collagen and PCL.

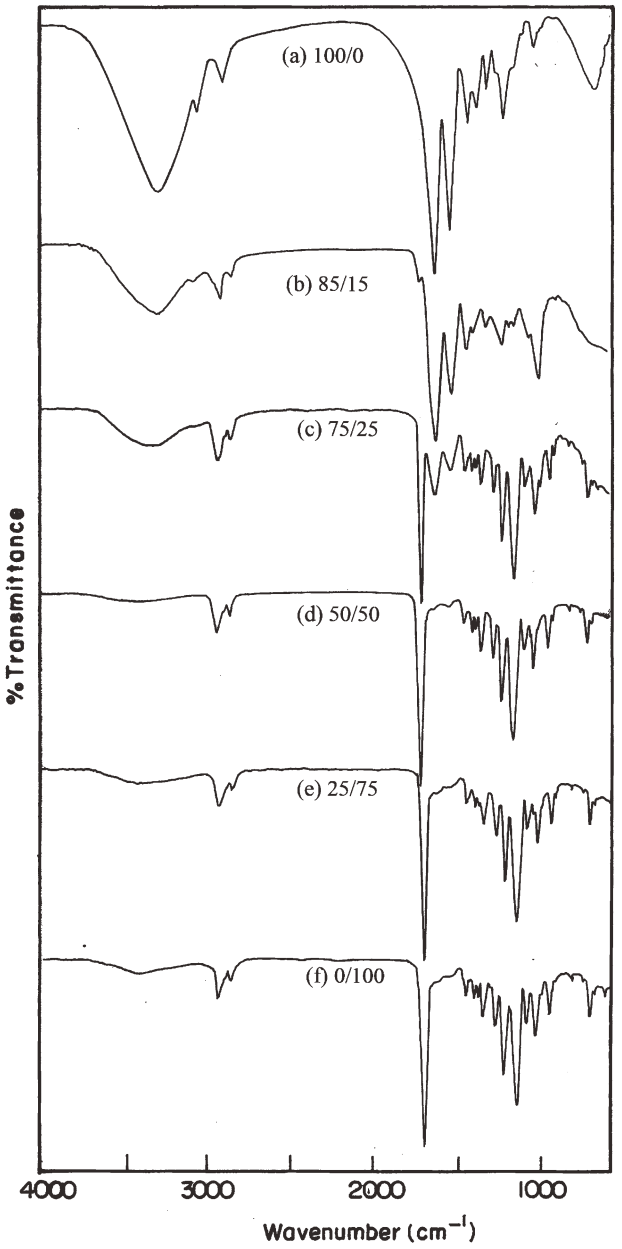

Figure 3 FTIR spectra of collagen/PCL blended matrices made at different ratios; (a) 100/0 (b) 85/15 (c) 75/25 (d) 50/50 (e) 25/75 (f) 100/0.

TGA and DSC analysis

Figure 4 depicts the results of thermo gravimetric analysis of electrospun matrices of collagen and PCL

blends. Two decomposition stages were visualized at 330 and 410°C in TG analysis. It has been observed that the peak maxima for PCL and collagen was not constant for all the blends but, vary by a small degree and suggests the interaction between both collagen and PCL, by which thermal property of one might be influenced by the other. The interaction of amide groups of collagen with carboxyl groups of PCL through hydrogen bonding as evidenced from FTIR analysis might be the reason for change in thermal properties. With regard to DSC analysis of the blends (Fig. 5), the peaks around 56°C correspond to the melting point of PCL and peaks around 155°C correspond to the melting (denaturing) of collagen. In the curves corresponding to the ratios 50/50 and 25/75 of collagen/PCL blend, masking of denaturation of collagen has been observed. This might be due to the presence of larger amount of PCL. The observations clearly support the results of TGA, in that there was no weight loss till 155°C, as the denaturing of collagen does not produce any volatile substances, which might affect the mass of the residue. The slight shift in the peaks and the masking of the collagen denaturing peaks in samples with lesser amount of collagen might indicate the presence of interaction between the components of the matrix.

CONCLUSION

Biomaterials made from natural sources such as collagen are preferred over those made from synthetic materials. Electrospinning has gained worldwide acceptance as a process for producing nanofibrous structures. Since last decade, electrospinning of collagen has come into practice for producing materials that mimic the human ECM and act as scaffolds and cellular constructs. At present hexafluoro isopropanol (HFIP), despite its harmful nature, ranks as the prime solvent for collagen electrospinning. In the present study, electrospinning of collagen has been attempted without the usage of HFIP, in order to avoid the denaturation of collagen during electrospinning. Electrospinning of collagen was carried out using acetic acid in the presence of a blend, PCL, which provided a nano fabric network with water absorption capacity, optimum porosity of 60% and fiber diameter in the range of 100–200 nm. The diameter seems to be in a finer range when compared to other reported literatures.[20] FTIR analysis confirms the presence of interaction between PCL and collagen through hydrogen bonding which is also substantiated by thermal analyses. Thus, electrospinning of collagen in an environmentally benign solvent has been carried out and its usage in skin tissue engineering (wound healing applications) is being investigated by our research group.

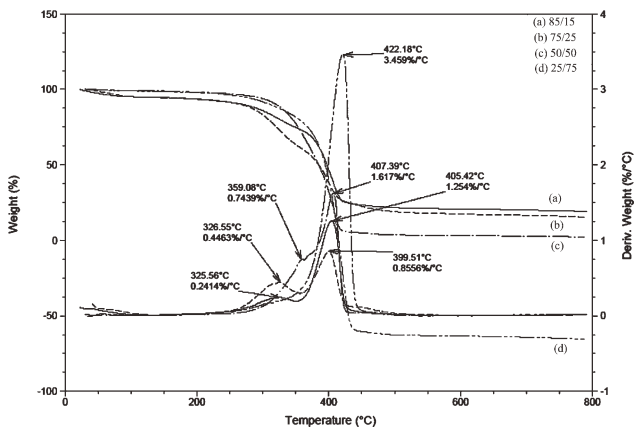

Figure 4 TGA curves of collagen/PCL blended matrices made at different ratios; (a) 85/15 (b) 75/25 (c) 50/50 (d) 25/75.

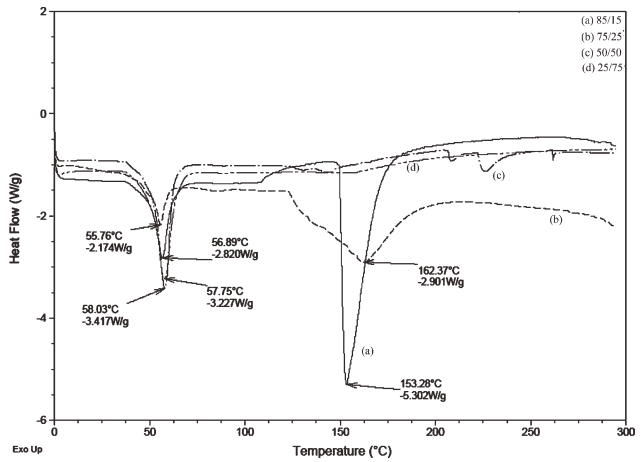

Figure 5 DSC curves of collagen/PCL blended matrices made at different ratios; (a) 85/15 (b) 75/25 (c) 50/50 (d) 25/75.

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References

1. Sia, S. K.; Gillette, B. M.; Yang, G. J. Birth Defects Rese (Part C) 2007, 81, 354.
2. Grodzinski, J. J. Polym Adv Technol 2006, 17, 395.
3. Matthews, J. A.; Wnek, G. E.; Simpson, D. G.; Bowlin, G. L. Biomacromolecules 2002, 3, 232.
4. Sell, S. A.; Francis, M. P.; Garg, K.; McClure, M. J.; Simpson, D. G.; Bowlin, G. L. Biomed Mater 2008, 3, 045001 (11 pp).
5. Chong, E. J.; Phan, T. T.; Lim, I. J.; Zhang, Y. Z.; Bay, B. H.; Ramakrishna, S.; Lim, C. T. Acta Biomater 2007, 3, 321.
6. Webster, T. J.; Waid, M. C.; McKenzie, J. L.; Price, R. L.; Ejiofor, J. U. Nanotechnology 2004, 15, 48.
7. Pham, Q. P.; Sharma, U.; Mikos, A. G. Tissue Eng 2006, 12, 1197.
8. Dong, B.; Arnoult, O.; Smith, M. E.; Wnek, G. E. Macromol Rapid Commun 2009, 30, 539.
9. Shih, Y. R. V.; Chen, C. N; Tsai, S. W.; Wang, Y. J.; Lee, O. K. Stem Cells 2006, 24, 2391.
10. Chen, R.; Huang, C.; Ke, Q.; He, C.; Wang, H.; Xiumei, M. Colloids Surfaces B: Biointerfaces 2010, 79, 315.

11. Xiumei, M.; Zonggang, C.; Weber, H. J. Front Mater Sci China 2007, 1, 20.
12. Venugopal, J.; Zhang, Y. Z.; Ramakrishna, S. Nanotechnology 2005, 16, 2138.
13. Zhang, Y. Z.; Venugopal, J.; Huang, Z. M.; Lim, C. T.; Ramakrishna, S. Biomacromolecules 2005, 6, 2583.
14. Boland, E. D.; Matthews, J. A.; Pawlowski, K. J.; Simpson, D. G.; Wnek, G. E.; Bowlin, G. L. Frontiers Bioscience 2004, 9, 1422.
15. Buttafoco, L.; Kolkman, N. G.; Buijtenhuijs, P. E.; Poota, A. A.; Dijkstra, P. J.; Vermes, I.; Feijen, J. Biomaterials 2006, 27, 724.
16. Thomas, V.; Dean, D. R.; Jose, M. V.; Mathew, B.; Chowdhury, S.; Vohra, Y. K. Biomacromolecules, 2008, 8, 631.
17. Zhou, J.; Cao, C.; Ma, X.; Lin, J. Int J Biol Macromol, 2010, 4, 514.
18. Chen, Z. G.; Wang, P. W.; Wei, B.; Mo, X. M.; Cui, F. Z. Acta Biomaterialia 2010, 6, 372.
19. Zeugolis, D. I.; Khew, S. T.; Yew, E. S. Y.; Ekaputra, A. K.; Tong, Y. W.; Yung, L. Y. L.; Hutmacher, D. W.; Sheppard, C.; Raghunath, M. Biomaterials, 2008, 29, 2293.
20. Lee, J. J.; Yu, H. S.; Hong, S. J.; Jeong, I.; Jang, J. H.; Kim. H. W. J Mater Sci: Mater Med 2009, 20, 1927.
21. Szentivanyi, A.; Chakradeo, T.; Zernetsch, H.; Glasmacher, B. Adv Drug Deliv Rev 2011, 63, 209.
22. Cliche, S.; Amiot, J.; Avezard, C.; Gariepy, C. Poultry Sci 2003, 82, 503.